

Ana Isabel Azevedo Serralheiro

Intranasal Delivery of Antiepileptic Drugs: Non-clinical Evaluation of Pharmacokinetics and Brain Biodistribution

Tese de Doutoramento em Ciências Farmacêuticas, especialidade de Farmacologia e Farmacoterapia, orientada pelo Professor Doutor Amílcar Celta Falcão Ramos Ferreira e pelo Professor Doutor Gilberto Lourenço Alves e apresentada à Faculdade de Farmácia da Universidade de Coimbra.

Setembro 2015



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FFUC FACULDADE DE FARMÁCIA
UNIVERSIDADE DE COIMBRA

The experimental work presented in this thesis was carried out at the Laboratory of Pharmacology, Faculty of Pharmacy, University of Coimbra and at the Health Sciences Research Centre, Faculty of Health Sciences, University of Beira Interior, under the supervision of Professor Amílcar Celta Falcão Ramos Ferreira and Professor Gilberto Lourenço Alves.

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FCT Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA EDUCAÇÃO E CIÊNCIA



UNIÃO EUROPEIA
Fundo Social Europeu

Ao Miguel

Aos meus Pais

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ABBREVIATIONS

A

AED	Antiepileptic Drug
AUC	Area Under the Concentration-time Curve
AUC _{inf}	AUC from Time Zero to Infinity
AUC _{ext} (%)	Percentage of AUC Extrapolated from t _{last} to Infinity
AUC _t	AUC from Time Zero to the Time of the Last Quantifiable Drug Concentration

B

BBB	Blood-Brain Barrier
BCSFB	Blood-Cerebrospinal Fluid Barrier
Bias	Deviation from Nominal Value

C

C-974P	Carbopol 974P
CBZ	Carbamazepine
CBZ-E	Carbamazepine-10,11-epoxide
C _{last}	Last Quantifiable Drug Concentration
CL/F	Apparent Clearance
C _{max}	Maximum Peak Concentration
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CV	Coefficient of Variation
CYP	Cytochrome P450

D

DGAV	Food and Veterinary General Divison
DMSO	Dimethyl Sulfoxide
DTE	Drug Targeting Efficiency
DTP	Direct Transport Percentage

E

EMA	European Medicines Agency
-----	---------------------------

F

F	Absolute Bioavailability
FDA	US Food and Drug Administration

G

GABA	γ-Aminobutyric Acid
------	---------------------

H

HLB	Hydrophilic-Lipophilic-Balanced Sorbent
HPLC	High Performance Liquid Chromatography
HPMC	Hydroxypropyl Methylcellulose

I

ILAE	International League Against Epilepsy
IN	Intranasal
IS	Internal Standard
IV	Intravenous

K

k	Tissue Elimination Rate Constant
k_{el}	Apparent Elimination Rate Constant

L

Lic	Licarbazepine
LLE	Liquid-Liquid Extraction
LLOQ	Lower Limit of Quantification
LOD	Limit of Detection
LTG	Lamotrigine
LogP	Octanol/Water Partition Coefficient

M

MCC	Mucociliary Clearance
MRT	Mean Residence Time
MS	Mass Spectrometry

O

OXC	Oxcarbazepine
-----	---------------

P

PB	Phenobarbital
PF-127	Pluronic F-127
PHT	Phenytoin
pK_a	Negative Decadic Logarithm of the Acid Dissociation Constant
PP	Protein Precipitation
PRM	Primidone

Q

QC	Quality Control
----	-----------------

R

r^2	Coefficient of Determination
rpm	Rotations per Minute

S

SEM Standard Error of the Mean
SPE Solid-Phase Extraction

T

TDM Therapeutic Drug Monitoring
TEA Triethylamine
 $t_{1/2}$ Tissue Elimination Half-Life
 $t_{1/2el}$ Apparent Elimination Half-Life
 t_{last} Time of the Last Quantifiable Drug Concentration
 t_{max} Time to Achieve Maximum Peak Concentration
trans-diol 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine

U

UGT Uridine 5'-diphosphate-glucuronosyltransferase
ULOQ Upper Limit of Quantification
UV Ultraviolet

V

VIP Vasoactive Intestinal Peptide

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ABSTRACT / RESUMO

ABSTRACT

Even though remarkable advances in the treatment of epilepsy have been made over the last years, the currently available anticonvulsant pharmacotherapy is unsatisfactory as it provides only the management of epileptic seizures, is not effective in a significant percentage of patients, and is often associated to several adverse effects. Therefore, the development of novel and alternative therapeutic approaches towards a safer and more effective seizure control is urgently needed. The search for an ideal antiepileptic drug (AED) that will be capable of preventing, delaying or modifying epilepsy is still ongoing. However, since the process of discovery and development of new chemical entities is very expensive and commonly accompanied by reduced rates of success, the use of already existing AEDs by improving some of their formulation properties, delivery systems or routes of administration aiming at allowing their efficient and prompt delivery to the brain could also be an attractive strategy.

Owing to the unique anatomical connection between the nasal cavity and the central nervous system, a great deal of interest has recently been focused on the exploitation of the intranasal (IN) route for the delivery of therapeutics directly to the brain by circumventing the blood-brain barrier (BBB). Indeed, the olfactory region is the only site in the human body where the nervous system is in direct contact with the surrounding environment, providing a great opportunity for drugs intranasally administered to gain a quick and easy access to the brain, minimising their systemic exposure.

The purpose of the present thesis was to assess and compare the pharmacokinetic behaviour of phenytoin (PHT), carbamazepine (CBZ), oxcarbazepine (OXC) and lamotrigine (LTG) administered via the IN and intravenous (IV) routes to mice and to investigate whether a direct transport of the referred compounds from nose to brain could be involved.

This project started with the development and validation of appropriate and reliable bioanalytical techniques to support the execution of the intended pharmacokinetic and brain biodistribution studies. In essence, two high performance liquid chromatographic methods coupled with ultraviolet detection were properly validated for the quantification of drugs and some of their respective main metabolites in mouse plasma, brain (whole brain, olfactory bulb and frontal cortex) and liver matrices. An additional technique developed in human plasma has also shown to be a useful tool to be applied in clinical practice.

Due to unexpected difficulties encountered during the definition and optimisation of the *in vivo* experimental setup, two of the four initially proposed test molecules were

excluded from further investigation, which was performed only with CBZ and LTG. The fairly comparable concentration-time profiles of both of these drugs obtained in plasma, brain and liver following IN and IV administrations to mice, together with a high IN absolute bioavailability, underscored the fact that a substantial absorption of the drugs from the nasal vasculature into the systemic circulation has occurred. Conversely, the uneven biodistribution profile after IN delivery of either CBZ or LTG, with the highest drug concentration levels attained in the olfactory bulb contrasted with the homogeneous biodistribution pattern typically observed following IV injection, strongly suggesting the involvement of a pathway to directly transport these drugs from nose-to-brain, bypassing the BBB.

According to our results, it seems that the IN route can be assumed as a suitable and a valuable drug delivery strategy for the chronic treatment of epilepsy and, in the specific case of CBZ, it gathers favourable conditions to be also applied in acute convulsive emergencies. Considering all its inherent potential and indisputable advantages, the IN administration may likewise emerge as a promising and a non-invasive alternative approach for a prospective management of pharmacoresistance.

RESUMO

Apesar de, ao longo dos últimos anos, terem sido alcançados avanços notáveis no que ao tratamento da epilepsia diz respeito, a terapêutica farmacológica anticonvulsivante atualmente disponível não é satisfatória, uma vez que possibilita apenas o controlo das crises epiléticas, não é eficaz numa percentagem muito significativa de doentes e é frequentemente associada a vários efeitos adversos. Deste modo, afigura-se urgente o desenvolvimento de abordagens terapêuticas novas e alternativas com vista a um controlo mais seguro e eficaz das crises. A procura pelo fármaco antiepilético (AED) ideal que permita prevenir, retardar ou modificar a doença ainda permanece uma realidade. Porém, tendo em conta que o processo de descoberta e desenvolvimento de novas entidades químicas é geralmente muito dispendioso e acompanhado de uma reduzida taxa de sucesso, a otimização das propriedades de formulação, sistemas de entrega ou vias de administração de AEDs já estabelecidos e disponíveis na clínica também poderá constituir uma estratégia muito atrativa.

A ligação anatómica ímpar entre a cavidade nasal e o sistema nervoso central tem recentemente suscitado um interesse particular na exploração da via intranasal (IN) para a entrega de agentes terapêuticos diretamente para o cérebro, circunscrevendo a barreira hemato-encefálica (BBB). De facto, a região olfativa representa o único local do corpo humano onde o sistema nervoso se encontra em contacto direto com o meio ambiente, oferecendo assim uma grande oportunidade aos fármacos administrados por via IN de atingirem o cérebro de uma forma rápida e facilitada, minimizando a sua exposição sistémica.

Com o trabalho de investigação subjacente à presente dissertação pretendeu-se avaliar e comparar o comportamento farmacocinético da fenitoína (PHT), carbamazepina (CBZ), oxcarbazepina (OXC) e lamotrigina (LTG) administrados por via IN e intravenosa (IV) a murganhos, e investigar o eventual envolvimento de um transporte direto para estas moléculas desde a cavidade nasal até ao cérebro.

Este projeto iniciou-se com o desenvolvimento e validação de técnicas bioanalíticas adequadas e confiáveis para suportar a execução dos estudos farmacocinéticos e de biodistribuição cerebral pretendidos. Para tal, foram validados dois métodos de cromatografia líquida de alta eficiência acoplada a deteção ultravioleta para a quantificação dos referidos fármacos e alguns dos seus principais metabolitos em matrizes de plasma, cérebro (cérebro total, bolbo olfativo e córtex frontal) e fígado de murganho. Uma técnica adicional em plasma humano foi ainda desenvolvida demonstrando aplicabilidade na prática clínica.

Face a dificuldades inesperadamente encontradas durante a definição e otimização do protocolo experimental *in vivo*, apenas a CBZ e a LTG, de entre as quatro moléculas teste inicialmente propostas, foram amplamente avaliadas neste trabalho. A semelhança observada entre os perfis de concentração-tempo em plasma, cérebro e fígado após a administração IN e IV a murganhos, juntamente com a elevada biodisponibilidade absoluta IN indicaram a ocorrência de uma absorção substancial de ambos os fármacos a partir da vasculatura nasal para a circulação sanguínea. Por outro lado, em oposição a um padrão de biodistribuição cerebral homogêneo tipicamente observado mediante administração IV, após administração IN foi obtido um perfil muito heterogêneo com níveis de concentração de CBZ e LTG mais elevados no bolbo olfativo, sugerindo assim o provável envolvimento de uma via de transporte direto desde a cavidade nasal até ao cérebro contornando a BBB.

De acordo com os nossos resultados, pressupõe-se que a via IN seja assumida como uma estratégia válida e apropriada para a administração continuada de ambos os fármacos visando o tratamento crónico da epilepsia, reunindo no caso específico da CBZ condições favoráveis para ser aplicada também em situações de emergência convulsiva. Considerando todas as suas vantagens e potencial inerente, a administração IN poderá igualmente representar uma abordagem alternativa, não-invasiva e promissora para um controlo prospetivo da epilepsia farmacorresistente.



CHAPTER I

GENERAL INTRODUCTION

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I. 1. EPILEPSY

Disease and Pharmacological Therapy

I. 1.1. HISTORICAL BACKGROUND

Epilepsy is perhaps one of the oldest recorded medical illnesses in history (WAHAB, 2010). The earliest detailed account of epilepsy dates back to the Babylonian ancient times, over 3000 years ago, when it was described as a condition representing an evil state of mind or possession (GOLDENBERG, 2010; WAHAB, 2010). Epileptic attacks were once thought to be the result of invasion and possession of the body by supernatural forces, usually malign or evil influences, requiring spiritual treatment based on exorcism, incantations or other religious or social approaches (WORLD HEALTH ORGANIZATION, 2005). At about 500 B.C., this supernatural view was firstly challenged by Hippocrates who suggested that the brain was the origin of epileptic seizure activity (WEIERGRÄBER *et al.*, 2010; WORLD HEALTH ORGANIZATION, 2005). Unfortunately, this concept was not widely accepted and the first modern definition of epilepsy was only given in the 19th century when it was recognised that seizures derived from disordered electrical discharges in the brain, with consequences at the level of consciousness, sensation and behaviour (WAHAB, 2010). Since then, the discovery of human electroencephalography and the remarkable advances in molecular biology, neurophysiology, genetics, functional imaging and numerous neurochemical techniques have contributed to deep the understanding of the basic mechanisms underlying seizures and epilepsy, allowing the exploitation of the concepts behind excitation, inhibition, modulation, neurotransmission and synchronisation (WORLD HEALTH ORGANIZATION, 2005). Despite these tremendous developments, a complete characterisation of the pathophysiological mechanisms involved in the initiation and propagation of seizures (ictogenesis), as well as those involved in transforming the normal brain into a seizure-prone brain (epileptogenesis) still remains to unveil.

I. 1.2. EPIDEMIOLOGY

Today, epilepsy is regarded as one of the most common serious neurological disorders worldwide which is associated with an increased risk of morbidity and mortality that significantly affects patients' quality of life. With an estimated prevalence of 0.7% (SCHMIDT and SCHACHTER, 2014), overall annual incidence of epilepsy ranges from 40 to 70 cases per 100,000 people in industrialised countries and up to 190 per 100,000 people in developing countries (SANDER, 2003). Without any racial, geographical or social boundaries, epilepsy affects individuals at all ages, regardless gender or social-economic status; however, high incidence levels are observed in childhood and in the elderly since in about 50% of the cases, the onset of epilepsy arise in these two extremes of life (BELL and SANDER, 2001). Around 50 million people in the world have epilepsy (BANERJEE *et al.*,

2009; NELIGAN *et al.*, 2012). It is believed that approximately 10% of the general population will experience at least one seizure at some point in their lives, and about a third of them will go on to develop epilepsy (PERUCCA and TOMSON, 2011; WORLD HEALTH ORGANIZATION, 2006).

I. 1.3. EPILEPSY AND EPILEPTIC SEIZURES

According to the International League Against Epilepsy (ILAE), an **epileptic seizure** is defined as “*a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain*”; whereas **epilepsy** is assumed as “*a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures and by the neurobiological, cognitive, psychological, and social consequences of this condition*”, requiring the occurrence of at least one epileptic seizure (FISHER *et al.*, 2005).

The above definitions were created in a document generated by an ILAE Task Force in 2005 but currently, they are being considered conceptual (theoretical) and not sufficiently detailed to provide guidance on how *enduring predisposition* should be defined, particularly for those individuals presenting a single unprovoked seizure. In this regard, the ILAE commissioned a second Task Force to formulate a more practical (operational) definition of epilepsy. Several years of deliberations on this issue resulted in new recommendations that have recently been published and adopted as a position of the ILAE. According to the revised definition, “*epilepsy is a disease of the brain defined by any of the following conditions: (1) At least two unprovoked (or reflex) seizures occurring greater than 24 h apart; (2) one unprovoked (or reflex) seizure and a probability of further seizures similar to the general recurrence risk (at least 60%) after two unprovoked seizures, occurring over the next 10 years; (3) diagnosis of an epilepsy syndrome*” (FISHER *et al.*, 2014).

Generically, epilepsy is considered a chronic and often progressive brain disorder as a result of recurrent, periodic, unpredictable and unprovoked seizures which can be generated in response to a loss of balance between excitatory and inhibitory influences within the central nervous system (CNS) (LASOŃ *et al.*, 2013; LÖSCHER and POTSCSKA, 2002).

The underlying causes and the precise pathophysiological mechanisms behind the incidence of epileptic seizures and the development of epilepsy are so far only partially understood. Notwithstanding, the abnormal and sustained neuronal discharges may be commonly associated with a variety of causative factors such as trauma, hypoxia, infection, stroke, tumours, metabolic derangements or even genetic mutations (BIALER and WHITE, 2010; ENGELBORGHES *et al.*, 2000). It is due to these multifactorial aetiologies that epilepsy is characterised as a complex and heterogeneous neurological disorder with

highly diversified symptomatology and manifestations. In fact, the term epilepsy is a misnomer concept (DUNCAN, 2002). Epilepsy is not just one single pathological condition but, otherwise, it is a varied family of brain disorders comprising many seizure types and syndromes (FISHER *et al.*, 2005; SCHMIDT and SCHACHTER, 2014). Each distinct form of epilepsy has its own natural history and response to treatment. In this context, the determination and identification of the different seizure types and syndromes is fundamental in order to focus the diagnostic approach on a particular aetiology, select the appropriate therapeutic option and provide vital information regarding the prognosis. With the aim of providing an universal vocabulary that not only facilitates communication among clinicians but also establishes a taxonomic foundation for performing quantitative clinical and basic research on epilepsy, the ILAE afforded standardised classifications and terminology for both epileptic seizures and syndromes (ENGEL JR., 2001).

Official ILAE classifications were firstly published in 1981 for epileptic seizures and in 1989 for epileptic syndromes (COMMISSION ON CLASSIFICATION AND TERMINOLOGY OF THE ILAE, 1989, 1981). Over the past three decades, this international classification has been extremely useful for both clinicians and researchers; nevertheless, new data from modern neuroimaging techniques, molecular biology studies and genetics research have prompted the continuous review of the adopted system, and new updated concepts and terminology have been proposed in the last few years (BERG and SCHEFFER, 2011; BERG *et al.*, 2010; RUDZINSKI and SHIH, 2011). The most recent document on this matter was published in 2010, emphasising the reduction of the existing dichotomy between focal and generalised seizures and epilepsies (BERG and SCHEFFER, 2011; BERG *et al.*, 2010). Accordingly, a new network concept for the definition of focal (partial) and generalised seizures is adopted, in which focal seizures are conceptualised as originating within networks limited to one brain hemisphere, while generalised seizures are generated at some point within, and rapidly engaging, bilaterally distributed networks (LEE, 2013). The terms “generalised” and “focal” were also recommended to be abandoned as overall classification categories for epilepsies (BERG and SCHEFFER, 2011). The aetiological designations of idiopathic, symptomatic and cryptogenic were advocated to be replaced by genetic, structural-metabolic and unknown aetiologies. A new database scheme of epilepsies was suggested by separating the multiple diseases and syndromes into four different groups: electroclinical syndromes, constellations, epilepsies associated with structural or metabolic conditions and finally, epilepsies of unknown causes (LEE, 2013). The list of the currently recognised seizure types and epilepsies are presented in Tables I.1.1 and I.1.2, respectively.

Table I.1.1 Classification of epileptic seizures (BERG *et al.*, 2010; COMMISSION ON CLASSIFICATION AND TERMINOLOGY OF THE ILAE, 1981; MATTSON, 2003; RUDZINSKI and SHIH, 2011).

Seizures types	Characterisation
Focal Seizures	Origin within networks limited to one hemisphere; only a certain area of the body is usually involved.
Generalised Seizures	Simultaneous arising from both cerebral hemispheres with symptom manifestation bilaterally in the body.
Tonic-clonic (grand mal)	<p>Abrupt loss of consciousness followed by tonic contraction of the muscles which then evolves to clonic convulsive movements.</p> <ul style="list-style-type: none"> - <u>Tonic phase</u>: rigid, violent and sustained contraction of whole body musculature. The upper extremities often symmetrically abduct and flex at the elbows while the lower extremities may briefly flex and adduct with the toes pointed. Momentary cessation of breathing and tongue biting. - <u>Clonic phase</u>: repetitive spasms and rhythmic jerking of the extremities. It is characterised by a progressive muscle relaxation until the end of the ictal phase.
Absence (petit mal) Typical Atypical Absence with special features	Sudden, brief lapses of consciousness without loss of postural control.
Myoclonic	Sudden, brief and arrhythmic muscle contractions that may involve the whole body or certain focal areas.
Clonic	Repetitive rhythmic clonic jerks with impairment of consciousness and a short post-ictal phase.
Tonic	Tonic contraction of the face, neck, axial, or appendicular musculature lasting from 10 to 60 seconds. Usual upward deviation of the eyes.
Atonic	Sudden loss of postural muscle tone and consciousness that usually cause abrupt falls.
Unknown	Seizures that cannot be clearly diagnosed into one of the preceding categories due to incomplete data.

Table I.1.2 Classification of epilepsies and epileptic syndromes (BERG *et al.*, 2010).

Electroclinical Syndromes (arranged by typical age at onset)
<i>Neonatal Period</i>
Benign familial neonatal epilepsy
Early myoclonic encephalopathy
Ohtahara syndrome
<i>Infancy</i>
Benign infantile epilepsy
Benign familial infantile epilepsy
Dravet syndrome
Epilepsy of infancy with migrating focal seizure
Myoclonic encephalopathy in nonprogressive disorders
Myoclonic epilepsy in infancy
West syndrome
<i>Childhood</i>
Autosomal-dominant nocturnal frontal lobe epilepsy
Benign epilepsy with centrotemporal spikes
Childhood absence epilepsy
Early onset childhood occipital epilepsy (Panayiotopoulos syndrome)
Epilepsy with myoclonic atonic (previously astatic) seizures
Epilepsy with myoclonic absences
Epileptic encephalopathy with continuous spike-and-wave during sleep
Landau-Kleffner syndrome
Late onset childhood occipital epilepsy (Gastaut type)
Lennox-Gastaut syndrome
<i>Adolescence – Adult</i>
Autosomal dominant epilepsy with auditory features
Epilepsy with generalised tonic-clonic seizures alone
Juvenile absence epilepsy
Juvenile myoclonic epilepsy
Progressive myoclonus epilepsies
Other familial temporal lobe epilepsies
<i>Less Specific Age Relationship</i>
Familial focal epilepsy with variable foci
Reflex epilepsies
Distinctive Constellations
Gelastic seizures with hypothalamic hamartoma
Hemiconvulsion-hemiplegia-epilepsy
Mesial temporal lobe epilepsy with hippocampal sclerosis
Ramussen syndrome
Epilepsies Attributed to and Organised by Structural-Metabolic Causes
Malformations of cortical development (hemimegalencephaly, heterotopias, etc.)
Neurocutaneous syndromes (tuberous sclerosis complex, Sturge-Weber, etc.)
Tumour, infection, trauma, angioma, perinatal insults, stroke, etc.
Epilepsies of Unknown Cause

I. 1.4. THERAPEUTIC APPROACH

The primary focus of care for patients with epilepsy is the prevention of further seizures which may lead to additional morbidity or even mortality. The goal of treatment should rely on the maintenance of a normal lifestyle, preferably by complete seizure control without or with minimal side-effects, ultimately aiming to restore the functional capacity of patients in what concerns to their usual psychosocial and vocational activities.

Currently, antiepileptic drug (AED) therapy is the mainstay of treatment for the majority of patients with epilepsy. Non-pharmacological options include surgery, vagus nerve stimulation and ketogenic diet which are primarily reserved for a few selected cases, usually upon AED failure (SHETH *et al.*, 2005). Overall, clinically available AEDs lead to satisfactory control of seizures and a favourable risk-benefit balance for 60-70% of newly diagnosed patients, albeit with considerable differences in response depending on the type of seizure, epilepsy syndrome and rare serious adverse events (SCHMIDT, 2009). Unfortunately, in about 30-40% of the patients, seizures persist regardless the choice of an adequate AED and carefully monitored treatment (LÖSCHER and POTSCHKA, 2002).

The modern pharmacological treatment era of epilepsy began in 1857, over than 150 years ago, with the introduction of potassium bromide to provide seizure control (BRODIE, 2010; SCHMIDT, 2009). Despite their limited efficacy and severe side-effects, bromides were the only drugs available for the treatment of epilepsy at that time and thus, they were regularly used for the following 50 years.

A major turning point in the management of epilepsy occurred in 1912 when phenobarbital (PB) emerged. Its anticonvulsant properties were accidentally discovered by Alfred Hauptmann, who originally used the drug as an hypnotic and found that it also attenuated epileptic attacks (BRODIE, 2010). Since then, PB has been worldwide employed, still being the most widely prescribed AED in the developing world and remaining one of the first popular choices in many industrialised countries partly due to its undeniable efficacy, but especially because of its modest cost (BRODIE, 2010; PERUCCA, 2001). In the ensuing 60 years, new molecules including **phenytoin (PHT)**, primidone (PRM), ethosuximide, **carbamazepine (CBZ)**, valproic acid and a range of benzodiazepines became available on the pharmaceutical market and nowadays, they are regarded as “established” AEDs in clinical practice (BRODIE and DICHTER, 1996). Most of these represented an important improvement over barbiturates since they displayed better tolerability and, particularly in the case of valproate, also a broader spectrum of efficacy against different seizure types (PERUCCA, 2001). While these agents remain the mainstay of the modern treatment of epilepsy, they also have important shortcomings such as a narrow therapeutic index, a highly variable and often non-linear pharmacokinetics, suboptimal response rates, and a large propensity to cause significant adverse effects and

drug-drug interactions (PERUCCA, 2001). In an attempt to overcome at least in part the above limitations, several efforts have been made in order to widen the CNS activity and improve the efficacy, safety and/or tolerability of the existing old AEDs. Thus, focusing on enhancing brain penetration, eliminating toxic metabolites and optimising pharmacokinetic properties, a second generation of AEDs was developed and licensed by the end of the 1990s, which it was mainly represented by zonisamide, **lamotrigine (LTG)**, **oxcarbazepine (OXC)**, felbamate, gabapentin, topiramate and levetiracetam. Although these new AEDs do not offer significant advantages in terms of efficacy compared to the older ones, some benefits were ascertained concerning better tolerability, fewer drug interactions and simpler pharmacokinetics. In general, modern AEDs have fewer and less severe side-effects being also associated in a smaller extent to hypersensitivity reactions, weight problems and teratogenic phenonema. Moreover, the lower plasma protein binding and the absence or minimal hepatic enzyme-inducing properties reduce the likelihood occurrence of undesirable drug interactions. Higher rates of compliance can be achieved with new AEDs since most of them have long half-lives and therefore can be administered only once or twice daily (WAHAB, 2010).

In spite of the encouraging and welcome advantages that new AEDs have brought for the management of epilepsy, there is a growing concern that the efficacy of pharmacological treatment of epilepsy has not substantially improved (LÖSCHER and SCHMIDT, 2011). In fact, second generation of AEDs has often been promoted as having advantages over the older ones; however no evidence that these new drugs are more effective really exists. Altogether, their use leads to seizure freedom in no more than 15-20% of patients with epilepsy who are refractory to old AEDs (PERUCCA *et al.*, 2007). Therefore, a novel and more effective third generation of AEDs was needed and since the beginning of the 21st century, about seven new molecules (stiripentol, pregabalin, rufinamide, lacosamide, eslicarbazepine acetate, retigabine and perampanel) have been approved and are currently available on the market. Unlike previous generations in which the most clinically effective AEDs have been empirically found by serendipitous screening programmes or structural variation of already known drugs, third generation of AEDs has been mainly characterised by a “rational” process of discovery and development (LÖSCHER and SCHMIDT, 2011, 2002). Indeed, thanks to a better insight in the pathophysiology of epilepsy and improved understanding on AED mechanisms of action, the designing of new compounds is nowadays directed to novel therapeutic targets and mechanisms critically involved in the occurrence of epileptic seizures.

A summary of the main clinically approved AEDs is presented in Table I.1.3.

Table I.1.3 The main clinically approved AEDs.

First Generation	Second Generation	Third Generation
Phenobarbital (Oral, IV, IM)	Zonisamide (Oral)	Stiripentol (Oral)
Phenytoin (Oral, IV)	Vigabatrin (Oral)	Pregabalin (Oral)
Primidone (Oral)	Lamotrigine (Oral)	Rufinamide (Oral)
Ethosuximide (Oral)	Oxcarbazepine (Oral)	Lacosamide (Oral, IV)
Benzodiazepines	Felbamate (Oral)	Eslicarbazepine acetate (Oral)
Carbamazepine (Oral)	Gabapentin (Oral)	Retigabine (Oral)
Valproic Acid (Oral, IV)	Topiramate (Oral)	Perampanel (Oral)
	Tiagabine (Oral)	
	Levetiracetam (Oral, IV)	

IV, intravenous; IM, intramuscular.

The last decades have witnessed unprecedented progress in the pharmacotherapy of epilepsy with the introduction of more than 20 anticonvulsant drugs and a considerable advance in the understanding of how AEDs exert their effects at the cellular level. This undoubtedly beneficial expansion of the pharmacological armamentarium does, however, hamper the selection of the most suitable AED (or combination of AEDs) for each patient individually (DECKERS *et al.*, 2003). Because AED therapy is typically maintained for several years and often for life, a decision to initiate treatment has far-reaching consequences and needs to be based on thorough risk-benefit analyses (PERUCCA and TOMSON, 2011).

The ultimate goal of AED treatment is the lasting freedom from seizures with minimal adverse effects. AEDs differ in many important aspects ranging from efficacy against different seizure types, side-effect profiles, potential for pharmacokinetic interactions and ease of use. Moreover, there is evidence that each type of seizure has a specific drug that usually proves to be the most effective and adequate (SHAJU and ABRAHAM, 2013). Accurate seizure classification with judicious choice of pharmacological treatment is imperative since some drugs that are effective in the treatment of one seizure type may exacerbate another. In this context, several treatment guidelines were established (FRENCH *et al.*, 2004; GLAUSER *et al.*, 2006; NATIONAL INSTITUTE FOR HEALTH AND CARE EXCELLENCE, 2013), recommending a range of drugs as potential first-line treatments for the variety of seizure types and epilepsy syndromes (Table I.1.4). Notwithstanding, the choice of the AED among first-line agents always needs to be carefully individualised, mainly on the basis of the patient profile regarding type of seizure, age, gender, co-existing illnesses and concomitant medication. Tolerability, safety, ease of use, pharmacokinetics and cost are fundamental elements that should also be taken into account (SCHMIDT, 2009). Ideally, therapy should be initiated using a single appropriate AED (GARNETT *et al.*, 2009). If after gradual increasing of the dosage to the maximum

tolerated level the seizures remain or become uncontrolled, a progressive switching of the first prescribed drug should be performed by an alternative AED that can also be used as monotherapy (GARNETT *et al.*, 2009; SHAJU and ABRAHAM, 2013). Combination therapy may be considered when two or three AEDs given as monotherapy regimens fail. Nevertheless, this therapeutic strategy is usually driven by concerns of excessive drug load and increased toxicity by exposing the patient to a greater risk of drug interactions and side-effects, compromising both the prognosis and compliance (KAMINSKI *et al.*, 2009; SHAJU and ABRAHAM, 2013). Even though there is still controversy over when and how polytherapy approach could be used, the selection of AED combinations should invariably be cautious and rational (FRENCH and FAUGHT, 2009; LEE and DWORETZKY, 2010; ST LOUIS, 2009). The concept of “rational polytherapy” presupposes that the association of AEDs with different mechanisms of action is more effective and may provide better seizure control than those which share similar mechanistic (ST LOUIS, 2009; STAFSTROM, 2010). Intentionally, AED combinations should yield synergistic or, at least, additive clinical effectiveness along with antagonistic adverse effects (STAFSTROM, 2010). Unfortunately, the adherence to the above principles is not always fulfilled, often making the process of selecting optimal drug combinations complex and challenging.

Table I.1.4 AEDs used in different seizure types and epilepsy syndromes (DUNCAN *et al.*, 2006; NATIONAL INSTITUTE FOR HEALTH AND CARE EXCELLENCE, 2013; SCHMIDT and SCHACHTER, 2014; SHAJU and ABRAHAM, 2013).

	First-line AEDs	Alternative AEDs
Seizure Type		
Focal	CBZ, LTG, LEV, OXC, VPA, TPM	GBP*, PGB, TGB, ZNS, CLB, CLZ, ESL, LCM, PB, PHT, VGB
Generalised		
Tonic-clonic	CBZ, LTG, OXC, VPA, TPM	CLB*, LEV*, ZNS, CLZ, PB, PHT
Absence	ESM, LTG, VPA	CLB, CLZ, TPM, ZNS
Myoclonic	LEV, VPA, TPM	CLB, CLZ, LTG, ZNS
Tonic	LTG, VPA	CLB, CLZ, TPM, ZNS, FLB, LEV, PB, PHT
Atonic	LTG, VPA	CLB, CLZ, TPM, ZNS, FLB, LEV, PB
Epileptic Syndrome		
Childhood absence epilepsy	ESM, LTG, VPA	CLB, CLZ, LEV, TPM, ZNS
Dravet syndrome	VPA, TPM	CLB*, STP*
Benign epilepsy with centrotemporal spikes	CBZ, LTG, LEV, OXC, VPA	CLB*, GBP*, OXC*, TPM*, ESL, LCM, PB, PHT, PGB, TGB, VGB, ZNS
Panayiotopoulos syndrome	CBZ, LTG, LEV, OXC, VPA	CLB*, GBP*, OXC*, TPM*, ESL, LCM, PB, PHT, PGB, TGB, VGB, ZNS
Lennox-Gastaut syndrome	VPA	LTG*, FBM, RFM, TPM
Juvenile absence epilepsy	ESM, LTG, VPA	CLB, CLZ, LEV, TPM, ZNS
Juvenile myoclonic epilepsy	LTG, LEV, VPA, TPM	CLB, CLZ, ZNS
Epilepsy with generalised tonic-clonic seizures alone	CBZ, LTG, OXC, VPA	CLB*, LEV*, TPM*, CLZ, PB, PHT

* As adjunctive therapy. CBZ, carbamazepine; CLB, clobazam; CLZ, clonazepam; ESL, eslicarbazepine acetate; ESM, ethosuximide; FLB, felbamate; GBP, gabapentin; LCM, lacosamide; LEV, levetiracetam; LTG, lamotrigine; OXC, oxcarbazepine; PB, phenobarbital; PGB, pregabalin; PHT, phenytoin; RFM, rufinamide; STP, stiripentol; TGB, tiagabine; TPM, topiramate; VGB, vigabatrin, VPA, valproic acid; ZNS, zonisamide.

Unlike other therapeutic classes, AEDs are not usually classified into categories according to their respective modes of action (PERUCCA, 2005). While many AEDs have been categorised in line with a single and principal mechanism of action, it is increasingly recognised that several agents have multiple primary effects at therapeutic concentrations, possessing more than one mechanism of action (CZAPIŃSKI *et al.*, 2005; DECKERS *et al.*, 2003). Furthermore, most of them have less well-characterised additional mechanisms and the fundamental pharmacology of others remains to be determined (DECKERS *et al.*, 2003).

Pharmacological treatment of epilepsy has traditionally relied on the control of the symptoms, i.e. suppression of seizures, rather than correcting the cause or preventing the development of the disease. Seizures can be generated either from excessively hyperexcitatory processes or from hypoactivity of neuronal inhibition in a specific neuronal cell population within the brain (LASON *et al.*, 2013). Although the mechanisms of action of the currently marketed AEDs are still not completely understood, these agents essentially redress the balance between neuronal excitation and inhibition. Therefore, three major mechanisms are recognised at the cellular level: modulation of voltage-dependent ion channels (Na^+ , Ca^{2+} , K^+), enhancement of γ -aminobutyric acid (GABA)-mediated inhibitory neurotransmission, and attenuation of glutamate-mediated excitatory neurotransmission (KWAN *et al.*, 2001) (Figure I.1.1).

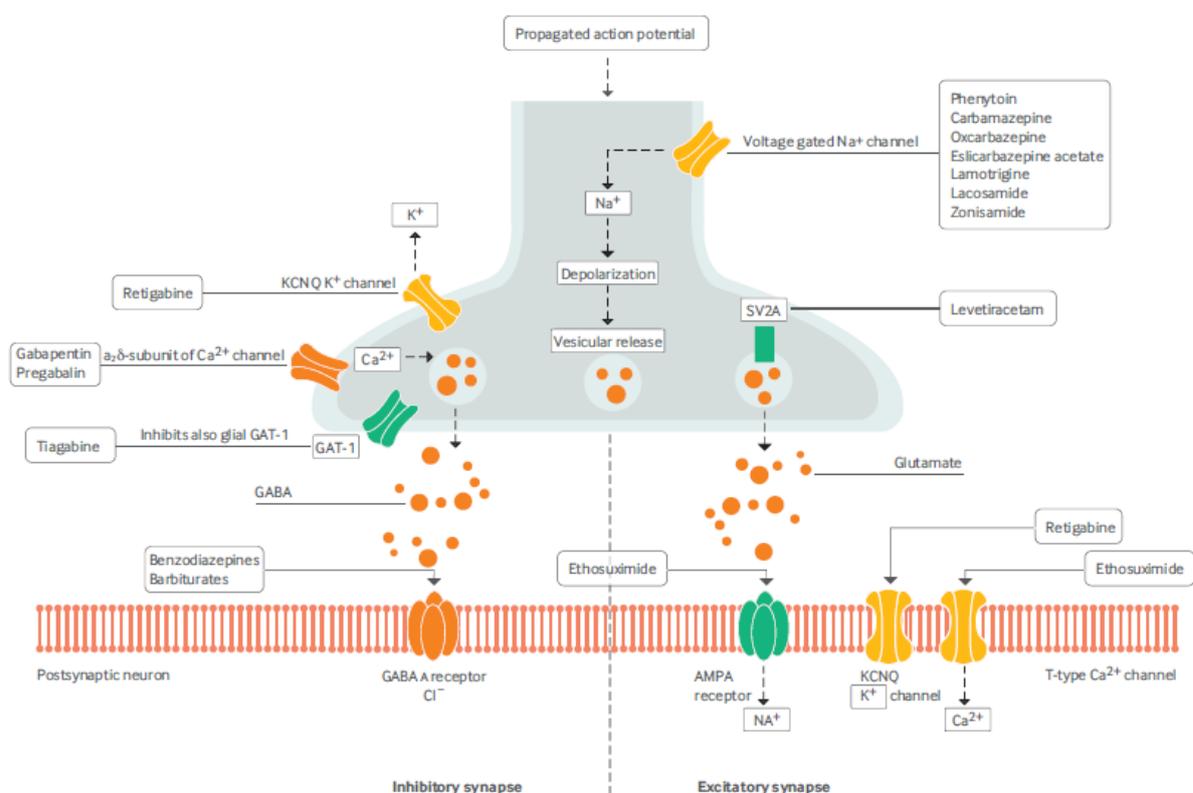


Figure I.1.1 Mechanisms of action of the main AEDs currently available on the market. AMPA, α -amino-3-hydroxy-5 methyl-4-isoxazole propionic acid; GABA, γ -aminobutyric acid; GAT-1, sodium dependent and chloride dependent GABA transporter 1; SV2A, synaptic vesicle glycoprotein 2A (Adapted from SCHMIDT and SCHACHTER, 2014).

Despite its undeniable importance, at the current state of knowledge, the information upon how the various AEDs work has demonstrated limited value in predicting their therapeutic efficacy and adverse effects. Inclusively, it has even been a matter of debate whether the AEDs' mechanism of action is relevant for their use as epilepsy therapy (BRODIE *et al.*, 2011; PERUCCA, 2011; SCHMIDT, 2011). Indeed, due to incomplete understanding of the precise pathophysiological mechanisms behind epilepsy and seizure activity, most of the AEDs have been identified by screening compounds in seizure models without targeting the specific mechanisms involved in ictogenesis or epileptogenesis (LÖSCHER *et al.*, 2013). As a result, the mechanism of action was only determined after the discovery of anticonvulsive effects, hampering a mechanistically-driven approach to drug therapy. So far, the AED selection has primarily relied on clinically acquired evidence of efficacy spectrum and side-effect profile (PERUCCA, 2001).

Notwithstanding, evidence is starting to emerge whereby a clear relationship can be ascertained between certain mechanisms of action and efficacy against specific types of seizures and epileptic syndromes. Accordingly, it seems that focal seizures and primary generalised tonic-clonic seizures respond well to sodium channel blockers (e.g. CBZ, PHT). In contrast, T-type calcium channel blockers (e.g. ethosuximide) may be effective against absence seizures, and GABAergic agents (e.g. PB, benzodiazepines) and AEDs with multiple mechanisms of action (e.g. valproic acid) are more likely to exert broad-spectrum protection over both focal and generalised seizure disorders (DECKERS *et al.*, 2003; PERUCCA, 2011). Likewise, there are mechanisms of action apparently associated with potential seizure aggravation. For example, some AEDs that block selectively sodium channels or enhance the synaptic availability of GABA are commonly ineffective and may even exacerbate myoclonic and absence seizures, respectively (PERUCCA, 2011). Apart from the abovementioned, clinical activity of several AEDs cannot always be explained based exclusively on their main mode of action. In fact, potentially subtle differences may exist in the intimate molecular mechanisms by which AEDs interact with ion channels or neurotransmission function, thus resulting in distinct therapeutic profiles.

In spite of the diversity of AED modes of action, it is interesting to note that the antiepileptic efficacy of these drugs in initial add-on trials does not seem to differ substantially, which indicates that seemingly similar anticonvulsant activity can be obtained by different therapeutic targets (LÖSCHER *et al.*, 2013). Moreover, the fact that many new AEDs share similar mechanisms of action with older agents may account, to some extent, why the vast majority of the patients who do not respond to older generation AEDs also fail to achieve seizure freedom on the newer drugs (PERUCCA, 2001). A deeper understanding of the AED pharmacology and the pathophysiological processes

underlying epileptic disorders may contribute to the identification of novel promising pathways and potential new drug targets.

I. 1.5. PHARMACORESISTANT EPILEPSY

Drug resistant epilepsy is one of the most important unmet needs in the daily management of epilepsy. Although the concept of drug resistant, also called as “medically refractory/intractable” or “pharmacoresistant” epilepsy may appear self-explanatory and intuitive, a precise definition has remained elusive (KWAN *et al.*, 2010). Indeed, there is no true or ideal definition of pharmacoresistant epilepsy. Several studies have defined it according to the number of AEDs the patient has tried without success, the frequency of seizures, the duration of illness, and the period of remission (WEAVER and POHLMANN-EDEN, 2013). Recently, an ILAE Task Force commission proposed a global consensus definition: “*failure of adequate trials of two tolerated and appropriately chosen and used AED schedules (whether as monotherapies or in combination) to achieve sustained seizure freedom*” (KWAN *et al.*, 2010). The above description is consistent with the clinical observation that if complete seizure control is not accomplished with trials of two appropriate AEDs, the likelihood of success with subsequent regimens is much reduced (KWAN *et al.*, 2011).

Pharmacoresistant epilepsy is a major health concern associated with increased morbidity and mortality. Recurrent uncontrolled seizures and the exposure to high doses of multiple ineffective medications result in an augmented risk of premature death, physical injury, cognitive impairments, psychosocial dysfunctions and a reduced quality of life (ALEXOPOULOS, 2013; KWAN *et al.*, 2011; WEAVER and POHLMANN-EDEN, 2013). Effectively, the recent introduction of various new generation AEDs in the market has not changed the problem of intractable seizures to any significant extent. As a consequence, approximately 20-40% of patients with primary generalised epilepsy, and up to 60% of patients with focal epilepsies continue to manifest pharmacoresistance (ALEXOPOULOS, 2013). A striking obstacle in developing new strategies for treatment of refractory epilepsy is that the regulatory events underlying drug resistance are only poorly understood. Pharmacoresistance occurs besides the use of medications with distinct pharmacologic properties and modes of action, suggesting that a non-specific mechanism is involved in limiting the effectiveness of AEDs. This therapeutic failure is likely to be regarded as a variable multifactorial process with many possible causes (KWAN *et al.*, 2011; LÖSCHER and POTSCHKA, 2002). In this context, three major pathomechanisms have been advocated to explain medical refractoriness of epilepsy: disease-related, drug-related and genetic-related mechanisms (SCHMIDT and LÖSCHER, 2005). Among all, the two

key theories based on the target hypothesis and the multidrug-transporter hypothesis have gained an increasing support (SCHMIDT and LÖSCHER, 2005). While the target hypothesis postulates that acquired alterations in the structure and/or functionality of target ion channels and neurotransmitter receptors result in loss of AED affinity and efficacy; multidrug-transporter hypothesis suggests that an overexpression of efflux proteins at the blood-brain barrier (BBB) restricts drug uptake into the brain leading to insufficient AED levels at their target sites (LÖSCHER and SCHMIDT, 2011; REMY and BECK, 2006). Beyond that, other disease-related factors may also contribute to the different clinical patterns of drug resistance, including the aetiology of seizures, progression of the disease and structural brain alterations and/or network changes such as seizure induced synaptic reorganisation (SCHMIDT and LÖSCHER, 2005). The development of pharmacologic tolerance, i.e. the loss of anticonvulsant efficacy during treatment after initial control, is likewise presented as a possible mechanism involved in inadequate seizure regulation (SCHMIDT and LÖSCHER, 2005; WAHAB, 2010). Finally, genetic alterations like for example, polymorphisms in genes encoding for drug targets, multidrug transporters or proteins involved in pharmacokinetics and pharmacodynamics of AED action may be important to explain why two patients with the same type of epilepsy or seizures may differ in their initial responses to AEDs (LÖSCHER and SCHMIDT, 2011; SCHMIDT and LÖSCHER, 2005).

However, none of the current hypotheses is able to convincingly explain how drug resistance arises in human epilepsy as a stand-alone theory (SCHMIDT and LÖSCHER, 2009). Clinical evidence demonstrated that patients who are resistant on monotherapy with the first AED have a chance of only about 10% or lower to be controlled by other AEDs, even when they operate by diverse mechanisms of action (LÖSCHER and POTSCHEKA, 2002). Hence, it is now recognised that despite their better tolerability and safety profiles, the therapeutic efficacy of modern AEDs has not substantially improved comparatively to the older generation compounds, and the proportion of patients with drug resistant epilepsy does not seem to have changed over the last decades (SCHMIDT, 2011). The multifaceted nature of pharmacoresistant epilepsy dictates a multidisciplinary approach to future research and therapeutic interventions. Regarding this, next generation of therapies must necessarily evolve to include targets that specifically contribute to epileptogenesis and drug resistance in relevant epilepsy models (LÖSCHER and SCHMIDT, 2011; WEAVER, 2013). A major breakthrough in the pharmacological treatment of refractory epilepsy will only be achieved when the development of drugs is directed towards the underlying cause of the disease.

I. 1.6. NEED FOR NEW THERAPEUTIC STRATEGIES

Over the last years, remarkable advances in the treatment of epilepsy have been made; however, the currently available antiepileptic therapies are unsatisfactory as they provide only symptomatic relief, are not effective in a significant percentage of epileptic patients, and are often accompanied by persistent adverse effects (LÖSCHER and SCHMIDT, 2002; SCHMIDT, 2009; WAHAB, 2010). Therefore, new concepts and alternative approaches for developing safer and more effective therapeutic options are urgently needed.

In this regard, several strategies have been explored to improve the pharmacological therapy of epilepsy: high throughput screening of potential new agents, modification of existing compounds, optimisation of drug formulations, enhancing delivery to the brain of current existing drugs, serendipitous discovery and rational drug development as a result of better insight of the mechanisms underlying epilepsies and epileptic seizures (DUNCAN, 2002; LÖSCHER *et al.*, 2013; VENTOLA, 2014).

I. 1.6.1. DISCOVERY AND DEVELOPMENT OF NOVEL AEDs

Despite considerable progress in understanding the pathogenesis of seizures and epilepsy, the cellular basis of human epilepsy remains a mystery. In the absence of a specific aetiological comprehension, approaches to pharmacological therapy of epilepsy must necessarily be directed at the control of symptoms. Nowadays, none of the old or new AEDs appears to represent a “cure” for epilepsy or an efficacious mean for preventing epilepsy or its progression (LÖSCHER and SCHMIDT, 2002). Among the various therapeutic options, resective surgery is often considered as the only curative treatment of the disease (LÖSCHER and SCHMIDT, 2002). Nevertheless, surgical procedures are restricted to patients with focal epilepsy in which the epileptogenic focus can be adequately identified and, in the majority of the cases, AED therapy has to be continued even after surgery in order to avoid relapse and achieve complete seizure freedom. Thus, the search for new drugs that are capable of preventing, delaying or modifying epilepsy is clearly warranted.

Traditionally, the pharmacological treatment of epilepsy has been essentially empirical often based on trial and error, aiming at suppressing initiation or propagation of seizures rather than correcting the processes involved in the natural history of the disease (LÖSCHER and SCHMIDT, 2002; SCHMIDT and SCHACHTER, 2014). There is no single drug of choice in treating all types of seizures and no drug has solved the problem of intractable epilepsy yet. Even though first-generation AEDs are potent anticonvulsants, most of them have dose-limiting toxicity and/or unacceptable side-effects that usually prevent the

achievement of adequate brain levels to attain a complete seizure control (GERLACH and KRAJEWSKI, 2010). In contrast, second generation AEDs present an improved side-effect and/or pharmacokinetic profile, but due to their close analogy to the original compounds and the corresponding mechanisms of action, it is not expectable that their therapeutic potential could be broadened (GERLACH and KRAJEWSKI, 2010).

From a clinical perspective, the ideal AED should meet the following criteria: great efficacy in refractory epilepsy; ability to prevent, delay or modify the progression of the disease; few adverse effects; acceptable tolerability; improved ease of use and favourable pharmacokinetic properties (BIALER and WHITE, 2010; PERUCCA *et al.*, 2007). Ultimately, the most desirable pharmacokinetic profile of an AED is the one that has high bioavailability, rapid brain penetration, a volume distribution with a single compartment, low and non-saturable protein binding, no active or toxic metabolites, no autoinduction of enzymatic biotransformation, an elimination half-life suitable for once or twice daily dosing, linear elimination kinetics, and finally, lack of pharmacological interactions (PODELL, 2013). In view of the numerous limitations and challenges that typify the current epilepsy pharmacotherapy, it is mandatory to revisit conventional AED discovery and development. There is an imperative need for the development of new strategies that can address both the remaining unmet medical demands in epilepsy and also simultaneously provide a favourable business case to be successfully executed by the pharmaceutical industry (LÖSCHER *et al.*, 2013).

In the 1990s, epilepsy conferred an opportunity to enter a therapeutic space in which there was a good chance for investment return; however, these prior incentives on AED development are now negatively balanced by the drug discovery and development challenges that face industry overall (LÖSCHER *et al.*, 2013). Indeed, the development of new AEDs is costly and risky since the chances for a successful conclusion of the development and the subsequent approval by the regulatory authorities are less than 10%, even for those drugs that are already at phase Ia of clinical research (PERUCCA *et al.*, 2007). The refund of the investment requires that future AEDs bring additional value or differentiation to an already crowded and highly generic AED field. In essence, evidence of better tolerability and safety profiles over the existing AEDs is not enough; thereby an improvement in efficacy has to be necessarily demonstrated. In this way, efforts must be conducted in a further research into the pathophysiologic mechanisms underlying both epilepsy and drug resistance. Given the heterogeneity and complexity of seizures in humans, it is unlikely that a single anticonvulsant animal model can be used to predict the full therapeutic potential of an AED candidate (BIALER and WHITE, 2010). Moreover, once the conventional preclinical models have been originally validated by old AEDs, there is a growing conviction that they are likely to identify only more of the same new AEDs

without possessing significant advances concerning efficacy and toxicity (LÖSCHER and SCHMIDT, 2002; PERUCCA *et al.*, 2007). Through a greater understanding of the molecular and genetic basis of different epilepsies and seizure types, new pharmacological targets and more representative animal models are likely to arise and lead to the discovery of novel and more effective therapies.

I. 1.6.2. NEW METHODS FOR AED DELIVERY

Since the process of discovery and development of new chemical entities is very expensive and generally associated to high attrition rates, some possible strategies to improve the performance of the pharmacological treatment of epilepsy may involve the use of already existing AEDs by modifying and optimising some of their formulation properties, delivery systems and/or routes of administration. Taking into account that a considerable percentage of the epileptic patients are drug resistant to a wide range of AEDs with different mechanistic, it may be profitable to consider other avenues beyond the new mechanisms of drug action to improve seizure control (SCHMIDT and HOLMES, 2009). In this context, more effective drug delivery approaches towards the epileptogenic brain tissue may enhance efficacy and decrease toxicity of AEDs (FISHER and CHEN, 2006; FISHER and HO, 2002; SCHMIDT and HOLMES, 2009).

It seems that betting in new alternative methods to efficiently deliver AEDs to the CNS could be an attractive business opportunity to the pharmaceutical industry. The investment necessary for discovery and development would be markedly reduced and the technical hurdles and regulatory data requirements would also be potentially minimised, thereby upgrading the premises for a very positive business case. Thus, the topic of drug delivery has become an intense point of interest not only for academy but also for industry and clinicians. Biopharmaceutical scientists and drug delivery industries have been searching for new ways to deliver drugs and explore innovative strategies aside from the traditional oral or injectable routes of administration aiming at creating new product opportunities and treatment paradigms (MATHIAS and HUSSAIN, 2010).

Conventional and Unconventional Routes of Drug Delivery

Oral administration remains the mainstay of AED therapy (FISHER and HO, 2002). At present, numerous AED oral formulations are commercially available, ranging from tablets and capsules to solutions, syrups and suspensions, and extended release products. Although it is easier and more practical for daily chronic use, oral administration is commonly associated to a variety of limiting factors, such as gastrointestinal absorption

and hepatic first-pass metabolism, which may compromise drug bioavailability and increase the possibility of high inter-subject variability regarding the relationship between dose and plasma concentration (ANDERSON and SANETO, 2012). The passage of the drug from the oral site of administration into the bloodstream is significantly influenced by the physicochemical properties of the drug, the type of dosage formulation, the rate of gastric emptying and the gastrointestinal motility. Therefore, oral administration of medicines does not assure a quick and immediate onset of action which is invariably demanded on acute seizure situations.

Unlike the slow and unreliable pharmacokinetics of oral administration, intravenous (IV) injection provides a precise dosing through a direct delivery of the drug to the systemic circulation, creating appropriate conditions to allow a better, faster and less variable systemic incorporation and biodistribution. Typically, IV administration is only applicable for drugs that are aqueous soluble. Due to poor water solubility, most of the AEDs are only available in oral dosage forms, with the exception of PB, PHT and its pro-drug fosphenytoin, valproic acid, levetiracetam, lacosamide and some benzodiazepines (clonazepam, diazepam, lorazepam and midazolam) that can be intravenously administered as parenteral formulations (ANDERSON and SANETO, 2012). IV injection is frequently used when the oral administration is unavailable or a rapid clinical response is required, being the route of choice for emergencies (e.g. *status epilepticus*) (ANDERSON and SANETO, 2012; FISHER and HO, 2002). However, the establishment of an IV access may entail delays and necessarily demands sterile equipment and skilled personnel, often making it impractical and inconvenient to be used outside the hospital setting.

The chronic management of epilepsy has commonly been performed using oral administration of AEDs while in case of *status epilepticus*, IV benzodiazepines are the first-line treatment option (ANDERSON and SANETO, 2012; MANNO, 2011; MEIERKORD *et al.*, 2010). Considering the above limitations, systemic delivery of drugs via alternative and unconventional routes of administration appears to be a valuable strategy to circumvent these inherent problems. Therapeutic-toxic ratio can be potentially improved and novel opportunities to efficiently and safely treat out-of-hospital acute seizures can thus be given to both patients and caregivers. In accordance, alternative routes of AED delivery may include buccal/sublingual, intranasal, intramuscular, pulmonary, rectal and subcutaneous/transdermal administrations. Each route has its own potential of application which is determined by the corresponding advantages and limitations (Table I.1.5). A recent consensus document from ILEA Task Force on Status Epilepticus recommended the use of rectal diazepam, buccal midazolam or intranasal midazolam as alternative emergency therapies for the early treatment of seizures outside the hospital environment (SHORVON *et al.*, 2008).

Transmucosal administration via buccal cavity, nasal passage and rectum generally provides an efficient absorption of drugs by offering large areas of potentially accessible mucosal surfaces that are rich in blood supply, yielding the means for rapid drug transport to the systemic circulation and avoiding, in most cases, degradation by gastric and hepatic first-pass metabolism (AMERICAN ACADEMY OF PEDIATRICS. COMMITTEE ON DRUGS, 1997). Although no AED is specifically approved for sublingual or buccal administration, such use has been occasionally performed among patients in clinical practice. Off-label use attests to the effectiveness of buccal/sublingual administration of benzodiazepines, but formal controlled studies are actually few (FISHER and HO, 2002). Hence, lorazepam sublingual tablet can be placed under the tongue at the start of a seizure in an attempt to interrupt seizure clusters, and liquid formulation of midazolam can be squirted by using a syringe between the gum and cheek for seizure prophylaxis. Recently, an oromucosal solution of midazolam (Buccolam®) has been licensed by the European Medicines Agency (EMA) and it is available in the European market for the paediatric treatment of prolonged acute convulsive seizures (ANDERSON, 2013). For a significant period of time, rectal diazepam has been the treatment of choice to attain the cessation of prolonged or serial seizures, whereby, several studies have been conducted in order to compare buccal administration of midazolam with the already established IV injection and rectal delivery of diazepam. Clinical evidence shows that comparatively to rectal diazepam, buccal midazolam has equal or greater efficacy in achieving seizure control without being associated to an increased incidence of respiratory depression (MCINTYRE *et al.*, 2005; SCOTT *et al.*, 1999). The comparison between buccal midazolam and IV diazepam revealed no remarkable differences in terms of efficacy, notwithstanding the mean time to initiating treatment was significantly shorter for the first one which makes it a viable alternative to IV diazepam, especially when getting an IV line is problematic (TALUKDAR and CHAKRABARTY, 2009).

Table I.1.5 Summary of the main advantages and limitations of conventional (oral and intravenous) and alternative routes for AED administration (ANDERSON and SANETO, 2012; FISHER and HO, 2002; MATHIAS and HUSSAIN, 2010).

Delivery Route	Advantages	Limitations
Oral	<ul style="list-style-type: none"> - Convenient, practical, painless - Well accepted by patients - Variety of dosage forms available - Non-invasive 	<ul style="list-style-type: none"> - Gastrointestinal degradation - Hepatic first-pass metabolism - Unpredictable absorption - Fluctuant bioavailability - Delayed onset of action - Pharmacokinetic variability - Patient cooperation/consciousness is required - Not suitable for emergencies
Intravenous	<ul style="list-style-type: none"> - Fast onset of action - Precise dosing - High bioavailability - Avoidance of absorption and hepatic first-pass metabolism - Useful in case of emergency 	<ul style="list-style-type: none"> - Requirement of an IV access - Sterile equipment and skilled personnel are essential - Impractical to be used in out-of-hospital acute seizure situations - Painful, invasive
Buccal/ Sublingual	<ul style="list-style-type: none"> - Avoidance of first-pass metabolism - Rapid absorption (due to the highly vascularised area) 	<ul style="list-style-type: none"> - A portion of the drug can be swallowed - Incomplete bioavailability - Patient cooperation is required - Not feasible during an acute seizure - The increased secretion of saliva during a seizure can cause loss of the drug
Intranasal	<ul style="list-style-type: none"> - Fast onset of action - Avoidance of first-pass metabolism - Convenient, practical, non-invasive - Useful for treatment of prolonged and acute seizures - Self administration 	<ul style="list-style-type: none"> - Limited dose, low dose volume - AEDs must present excellent aqueous solubility or could be formulated using solubilising agents - Potential irritation of nasal mucosa - Mucociliary clearance - Nasal congestion due to cold or allergy
Intramuscular	<ul style="list-style-type: none"> - Useful when ready IV access is lacking - Rapid administration - Fast onset of therapeutic effect 	<ul style="list-style-type: none"> - Irregular absorption - Absorption is influenced by the degree of vascularisation of the injection site - Local irritation - Generally inappropriate for the majority of AEDs due to their poor solubility and non-physiological pH of the formulation - Painful, invasive
Pulmonary	<ul style="list-style-type: none"> - Large surface area for absorption - Thin epithelial barrier featuring moderate permeability characteristics - High blood perfusion 	<ul style="list-style-type: none"> - Limited dose, low dose volume - Drug deposition in mouth and gastrointestinal tract - Safety and lung function
Rectal	<ul style="list-style-type: none"> - Partial avoidance of first-pass metabolism - Useful in children and when the oral route is unavailable 	<ul style="list-style-type: none"> - Erratic absorption - Inconvenient, not socially accepted
Subcutaneous/ Transdermal	<ul style="list-style-type: none"> - Convenient, good patient compliance - Large surface area - Prolonged drug delivery - Systemic effect 	<ul style="list-style-type: none"> - Absorption is slow and incomplete - Skin irritation

Despite its undeniable potential, some reservations have been put on the feasibility and safety to use the buccal/sublingual administration of drugs during an acute convulsive event. In fact, lowering the jaw to open the oral cavity might be difficult and the increased salivary secretion that can occur may also cause a loss of the drug by diverting it from the targeted absorption site (ANDERSON and SANETO, 2012; MCINTYRE *et al.*, 2005). In addition, as a portion of the drug can often be swallowed, the bioavailability can be incomplete and the time to peak concentration delayed (ANDERSON and SANETO, 2012).

In patients receiving AED treatment, maintaining the therapeutic concentrations of the drug is essential for optimal seizure control which must be guaranteed even when the patient is unable to take medication orally or an IV access is lacking. Under these circumstances, rectal administration of AEDs may represent an attractive alternative. The rectal route has been used for transitory or long term administration of anticonvulsants such as diazepam, valproic acid, PB and CBZ; however, the only medication approved by the US Food and Drug Administration (FDA) for non-intravenous treatment of prolonged seizures is the rectal gel of diazepam (Diastat®) (ANDERSON and SANETO, 2012; ANDERSON, 2013). In general, rectal absorption is relatively complete for many drugs, but it is usually erratic and associated to a significant patient-to-patient variability. This irregular drug uptake is reflected in a prolonged or delayed absorption in some cases, but in other patients, a rapid drug uptake occurs, as an IV bolus had been administered. Therefore, due to the prolonged rate of absorption, the achievement of CBZ therapeutic levels after rectal administration is only attained within 5-10 h of initial dosing, which indicates that rectally administered CBZ is effective as long-acting AED therapy for the maintenance of seizure freedom rather than in the treatment of acute convulsive episodes (PATEL, V. *et al.*, 2014). In contrast, rectal administration of diazepam results in rapid and complete drug absorption in which the time needed to accomplish seizure cessation ranges from 2 to 15 min (HOLSTI *et al.*, 2010). Currently, and even though the social stigma, rectal diazepam is still often one of the preferred choices among the first-line treatment options to control cluster of seizures and *status epilepticus* (MCMULLAN *et al.*, 2010; NATIONAL INSTITUTE FOR HEALTH AND CARE EXCELLENCE, 2013).

Intranasal (IN) delivery is conceivably the most attractive option when first considering a non-invasive and non-oral route of administration (MATHIAS and HUSSAIN, 2010). Traditionally, the nasal route has been exploited for the treatment of local diseases like nasal allergy, rhinitis, sinusitis, nasal infections and nasal congestion; however, the high degree of vascularisation, the high permeability due to the porous endothelial membrane and the large surface area of the nasal mucosa gather the conditions necessary for a rapid and extensive drug absorption to the systemic circulation (GRASSIN-DELYLE *et al.*, 2012). Indeed, systemic delivery of drugs via the IN route has already proven to be a valuable approach since often similar concentration-time profiles of drugs are

observed in plasma following both IN and IV administrations, which leads to a rapid onset of pharmacological action (GRASSIN-DELYLE *et al.*, 2012; SINGH *et al.*, 2013; UGWOKE *et al.*, 2001). Apart from being practical and convenient, one of the major advantages of the IN route is the avoidance of both gastrointestinal and hepatic first-pass effects, constituting a very profitable option for the administration of active principles with low oral bioavailability (SINGH *et al.*, 2013). Accordingly, given its characteristics, IN administration seems to be potentially advantageous in the home treatment of prolonged seizures, in the treatment of pre-hospital seizures by emergency medical technicians, and in the treatment of severely behaviourally challenged and cognitively impaired patients whose cooperation may be compromised (ANDERSON and SANETO, 2012). Conversely, some limitations arise concerning the limited volume of the nasal cavity that restricts the total amount of the formulation that can be administered, and the physicochemical properties of the molecules which must allow a highly concentrated incorporation of the drug in the formulation and, simultaneously, an easy and efficient permeation across the nasal mucosa (GRASSIN-DELYLE *et al.*, 2012). In theory, a drug candidate for IN administration should possess among other features, high aqueous solubility, efficiency at low doses (generally less than 25 mg), minimal nasal irritation and good nasal absorption characteristics in order to provide adequate bioavailability and achieve the desired therapeutic effects in terms of both intensity and onset of action (BEHL *et al.*, 1998; GRASSIN-DELYLE *et al.*, 2012; SINGH *et al.*, 2013; UGWOKE *et al.*, 2001). The most often nasally applied anticonvulsant drug has been the benzodiazepine midazolam. Several clinical studies have been performed in order to evaluate its pharmacokinetics, demonstrating the efficacy and safety of IN midazolam in the emergency treatment of prolonged convulsive seizures (DE HAAN *et al.*, 2010; GOLDENBERG, 2010; HASCHKE *et al.*, 2010; HOLSTI *et al.*, 2010; IVATURI *et al.*, 2009; THAKKER and SHANBAG, 2013). Overall, effective midazolam serum concentrations are reached within less than 10 min after nasal application, enabling a prompt control of the acute seizures either in medical centres or at home (HASCHKE *et al.*, 2010; HOLSTI *et al.*, 2010).

Intramuscular administration is another alternative route that can be considered for individuals who lack a ready IV access. Appropriate intramuscular formulations are merely available for some benzodiazepines (diazepam, lorazepam and midazolam), fosphenytoin and PB once the poor solubility and the non-physiological pH of the majority of AEDs formulations render them unsuitable to be administered by this route (ANDERSON and SANETO, 2012; FISHER and HO, 2002). Intramuscular injection usually delivers the exact quantity of drug desired; nevertheless, it is painful and the rate and extent of absorption is variable and not always complete being dependent on the degree of vascularisation at the injection site (ANDERSON and SANETO, 2012).

AEDs are rarely administered subcutaneously. In exceptional circumstances such as end-of-life palliative care, the continuous subcutaneous infusion of midazolam, clonazepam and PB has been advocated (FISHER and HO, 2002). The idea of a subcutaneous AED implant for the chronic management of epilepsy is attractive. However, the low solubility, highly alkaline pH in solution and low potency of most AEDs make such implants bulky and impractical (FISHER and HO, 2002). Moreover, most of the AEDs have a relatively narrow therapeutic index which demands for little variability in release rates from an implant.

Delivery of AEDs through transdermal or pulmonary routes has not been conveniently exploited yet. Notwithstanding, the concept of a hypothetical AED administration by means of skin patches or inhalators appears to be very interesting.

Alternative Strategies for Direct CNS Drug Targeting

As the brain is an organ structurally supplied with a large blood vessel network, direct systemic delivery of drugs by avoiding first-pass metabolism has a great potential to get their access to the brain by crossing the vascular barrier (Figure I.1.2). Unfortunately, both the abovementioned conventional and unconventional drug administration methods frequently fall short in delivering a number of therapeutic agents efficiently to the brain. Brain availability of systemic drugs is largely affected by the half-life of the drug in the plasma, rapid metabolism, level of non-specific binding to plasma proteins and the permeability of the compound across the BBB and into peripheral tissues (ALAM *et al.*, 2010). Although the main function of the BBB is to protect the CNS against a variety of pathogens and toxicants, it also plays a significant role on restricting the transport of drugs/xenobiotics from the systemic circulation into the brain. It is remarkable that approximately 98% of small-molecule and essentially 100% of large-molecule drugs do not cross the BBB (PARDRIDGE, 2007). Hence, regardless of their high potential, many neurotherapeutic agents may have been abandoned because of their inability to achieve sufficient levels in the brain through systemic circulation, and the BBB often turns out to be the sole reason for this clinical failure (PARDESHI and BELGAMWAR, 2013; PATHAN *et al.*, 2009). Inevitably, a number of new alternative strategies have been investigated and developed to circumvent the BBB and increase the CNS targeting of drugs (Figure I.1.2). Such novel delivery approaches may, on one hand, enhance the therapeutic properties of the existing AEDs, and on the other hand, enlarge the spectrum of molecules with antiepileptic potential that were previously excluded from experimental and clinical evaluation simply because they could not cross the BBB.

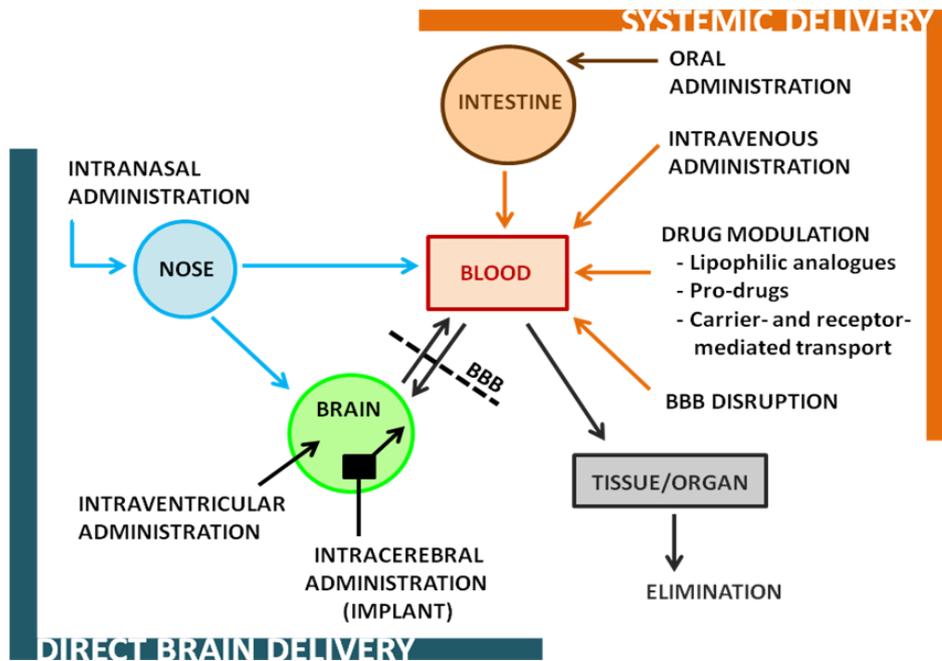


Figure I.1.2 Different methods of drug administration to the CNS.

The brain is a delicate organ that plays a set of vital functions to maintain convenient body homeostasis. Hence, its integrity is ensured by physiological barriers and mechanisms of defence which efficiently protect and isolate the CNS from harmful endogenous substances and external insults (e.g. xenobiotics and virus), while still preserving the appropriate nutrient supply. Overall, there are two main physiological barriers that separate the CNS environment from the systemic circulation, thereby controlling the influx and efflux of endogenous and exogenous compounds: the epithelial blood-cerebrospinal fluid barrier (BCSFB) and the endothelial BBB (ABBOTT, 2014).

The BCSFB separates the blood from the cerebrospinal fluid (CSF) that runs within the subarachnoid space surrounding the brain (PAVAN *et al.*, 2008). This barrier results from the combined action of the arachnoid membrane with the choroid plexus which is responsible for almost all the production of the CSF and is distributed throughout the third, fourth and lateral ventricles of the brain (ABBOTT, 2014; ILLUM, 2000; MISRA *et al.*, 2003). Each choroid plexus comprises a secretory epithelium that is perfused by blood at a local high perfusion rate (ILLUM, 2000). Although the capillaries of the choroid plexus are non-continuous, fenestrated and have gaps between the endothelial cells allowing the free-movement of small molecules, the adjacent choroid epithelial cells are closely held together by tight junctions which carefully regulate the passage of substances from blood into the CSF (DEEKEN and LÖSCHER, 2007).

The BBB represents one of the strictest structural and functional barriers in segregating the brain interstitial fluid from the circulating blood. Its protective character is due, among other features, to very specific morphological properties of the capillary endothelial cells located in the brain. In contrast to the peripheral organs, brain endothelial cells are absent of fenestrations, linked by continuous and strong tight junctions, and exhibit very low pinocytotic activity. Moreover, they are also surrounded by a basal membrane and pericytes as well as astrocyte end-foot processes that encapsulate the capillaries, restraining its permeability (ABBOTT, 2014; DEEKEN and LÖSCHER, 2007). Apart from being a selective structural diffusion barrier, the BBB also displays an efficient functional regulation. Owing to the presence of tight junctions between endothelial cells, a very high transendothelial electric resistance of about 1500-2000 $\Omega \cdot \text{cm}^2$ is found in the brain capillaries compared to 3-33 $\Omega \cdot \text{cm}^2$ of other tissues (e.g. skin, bladder, colon and lung), which significantly hinders the entry of polar and ionic substances to the brain (MISRA *et al.*, 2003; VYAS *et al.*, 2005). Furthermore, the high metabolic activity as well as the expression of numerous carrier-mediated efflux transporters in the brain endothelial cells, also contributes to the restrictive nature of the BBB (ANDERSON, 1996; RAUTIO *et al.*, 2008). Therefore, solutes crossing BBB cell membranes may be susceptible to enzymatic degradation, or can be actively removed from brain endothelial cell cytoplasm returning back to the bloodstream by means of a variety of efflux proteins, such as P-glycoprotein or multidrug-resistance proteins.

It is undeniable the importance of the BBB role in the preservation of brain integrity by creating a unique extracellular fluid environment within the CNS. Strikingly, despite the extensive human brain capillary network with approximately 650 km in length, BBB provides an efficient barrier against the diffusion of most substrates, making the brain practically inaccessible for high molecular weight and hydrophilic compounds such as polar molecules and small ions (MISRA *et al.*, 2003). Unfortunately, the mechanisms involved in the protection of the brain against intrusive chemicals are also responsible for preventing the CNS delivery of beneficial therapeutic agents (TACHIKAWA *et al.*, 2014).

The stringent permeability of the BBB by restricting the entry of various substances into the brain remains the key hurdle for a successful development of many CNS drugs. To overcome the multitude of obstacles that commonly hampers the CNS permeation of potential therapeutic agents, intense research efforts have recently focused on the design and exploitation of new strategies to more effectively deliver drug molecules into the brain. Thus, different approaches for enhanced CNS drug targeting have been investigated in the last years and may be divided into three main categories: (1) optimisation of systemic CNS acting drugs administration based on chemical or

physiological non-invasive methods, (2) transient disruption of BBB and (3) direct delivery of therapeutics into the brain by employing invasive administration techniques (DWIBHASHYAM and NAGAPPA, 2008; MISRA *et al.*, 2003).

(1) Optimisation of Systemic CNS Acting Drugs Administration Based on Chemical or Physiological Non-Invasive Methods

A variety of non-invasive CNS drug delivery methods have been proposed making use of the large brain blood vessel network to gain widespread drug distribution. Such methods usually rely upon chemical manipulation of the molecules which may include alterations as lipophilic analogues and pro-drugs, carrier-mediated and vector-mediated drug delivery systems (PATHAN *et al.*, 2009).

Delivery of therapeutic agents to the CNS is arguably a huge challenge. The endothelial cells that line the BBB microvasculature are joined together by highly resistant tight junctions thus preventing the paracellular passage of polar solutes; and BBB capillaries allow minimum pinocytosis which makes the transcellular diffusion across the cell membranes the only feasible passive route for entering the brain (RAUTIO *et al.*, 2008). Experimental observations dictate that CNS penetration is clearly favoured by low molecular weight, lack of ionisation at the physiological pH and high lipophilicity (MISRA *et al.*, 2003). In point of fact, the majority of neurotherapeutics has molecular weight between 150 and 500 Daltons and Log octanol/water partition coefficient values ranging from -0.5 to 6.0 (BEGLEY, 2004). A direct correlation exists between BBB permeability and the ability of a drug to passively diffuse through the cell membrane lipid bilayer, whereby, chemical manipulation by modifying drug physicochemical properties in order to increase its lipophilicity is a possible strategy to improve CNS uptake. The design of more lipophilic analogues has however lead frequently to very disappointing results. In practice, simply increasing the lipid solubility of a drug molecule may have undesirable effects as the solubility and bioavailability in plasma decreases and the extent of plasma protein binding increases, resulting in lower concentrations of drug available for diffusion into the CNS (BEGLEY, 2004; MISRA *et al.*, 2003). In addition, it has been recognised that highly lipophilic molecules present a greater predisposition to be substrates of the ATP-binding cassette group of efflux transporters (e.g. P-glycoprotein, multidrug-resistance proteins and breast cancer resistance protein), which are responsible for the extrusion of the drugs from the cerebral capillary endothelial cells and the CNS back into the blood against a concentration gradient (BEGLEY, 2004; GABATHULER, 2010). Hence, when a systemic administration of a CNS acting drug is considered, a delicate balance between cerebrovascular permeability and plasma solubility is required. Specifically, an optimum octanol-water partition coefficient with a LogP value of approximately 1.5 to 2.5 is recommended

for a drug to be effective following the delivery via the circulatory system (DWIBHASHYAM and NAGAPPA, 2008; MISRA *et al.*, 2003).

An attractive lipidization strategy that has been successfully employed to improve the CNS transport of poorly penetrating therapeutic agents is their transient chemical modification by using the pro-drug approach. Pro-drugs are bioreversible derivatives of drug molecules that must undergo a chemical or enzymatic biotransformation to the active forms within the body, prior to exerting a pharmacological effect (RAUTIO *et al.*, 2008). The major goal in pro-drug design is to overcome the various physicochemical, pharmaceutical, biopharmaceutical, and/or pharmacokinetic limitations of the parent drug, which otherwise would hinder its clinical use (RAUTIO *et al.*, 2008). Among several problems, pro-drug technology may provide an effective tool in solving drug formulation and delivery issues, such as poor water solubility or inadequate brain penetration. To enhance drug permeation through the BBB, pro-drugs are often formulated by attaching chemical moieties that increase the lipophilicity of the parent compound (DWIBHASHYAM and NAGAPPA, 2008). Fairly lipid soluble pro-drugs can thus enter the CNS more readily, and then be converted into the pharmacologically active form solely within the CNS. Nevertheless, as it was mentioned before, while high lipophilicity may facilitate drug movement across the BBB, it also tends to increase the efflux processes and the uptake into other peripheral tissues, causing an augmented tissue burden. Furthermore, distinct metabolic conversions can occur beyond the transition of the pro-drug into the parent compound which may contribute to the formation of potential toxic products (MISRA *et al.*, 2003). Therefore, the low selectivity, the poor biophase retention and the possibility of reactive metabolites generation often compromise the therapeutic index of pharmacological agents masked as pro-drugs (MISRA *et al.*, 2003).

CNS targeting of either drugs or pro-drugs can also be improved by taking advantage of the physiologic nature of the BBB. Many polar molecules such as glucose, amino acids, insulin, growth hormone, etc. are fundamental substances for the maintenance of brain homeostasis which, unlike their low lipid solubility might suggest, present high permeability through the BBB (BEGLEY, 2004; GABATHULER, 2010). In fact, these molecules are recognised by specific endogenous receptors or transport mechanisms expressed in the membranes of brain capillary endothelial cells that allow a carrier-mediated entry of the compounds either by facilitated diffusion or energy-dependent transport (BEGLEY, 2004; GABATHULER, 2010). Conjugation of drugs to ligands like endogenous transporter substrates, modified proteins or anti-receptor specific monoclonal antibodies results in a higher affinity of the compounds to the BBB influx transporter systems, thus enabling an easier CNS penetration of molecules that would otherwise have minimal access to the brain (BEGLEY, 2004; GABATHULER, 2010; RAUTIO *et al.*,

2008). In general, these physiological strategies are promising, but need careful attention to capacity and rates of transport *versus* plasma clearance (Lo *et al.*, 2001).

(2) Transient Disruption of BBB

Modulating the strength of the tight junctions between cerebral endothelial cells, so that the paracellular route of access to the brain is either partially or completely opened, is an approach that has been used to enhance BBB permeation of drugs. Theoretically, the weakening of BBB intercellular connections provides an enhanced endothelium extravasation of systemically administered drugs, leading to increased brain parenchymal concentrations (MISRA *et al.*, 2003; STENEHJEM *et al.*, 2009). A variety of different techniques that transiently disrupt the BBB integrity have been explored. An often tested approach, primarily used for the treatment of brain tumours, is the osmotic opening of the barrier involving the infusion via carotid artery of hyperosmolar substances like mannitol or arabinose (LO *et al.*, 2001; PARDRIDGE, 2007, 2005). The high osmotic pressure imposed by the injection of hypertonic solutions into the cerebral circulation leads to passive diffusion of water out of the cells causing brain endothelial cell shrinkage. As a result, the disarrangement of extracellular domains of the proteins forming and regulating the endothelium tight junctions occurs, which transiently facilitates the entry of drugs paracellularly (ALAM *et al.*, 2010). Pharmacological opening of the BBB comprising the administration of bradykinin analogues and alkylglycerols has also been proposed (ALAM *et al.*, 2010; BEGLEY, 2004). More recently, the application of ultrasound and electromagnetic radiation emerged as a very attractive BBB disruption technique since it can be focused with some precision to a particular region of the brain, allowing a selective modulation of the BBB at a preferred site, rather than globally throughout the entire brain (BEGLEY, 2004; STENEHJEM *et al.*, 2009).

Regardless the methodology employed, BBB disruption, even for brief periods of time, is usually associated to vascular pathology and chronic neuropathological changes in the brain (PARDRIDGE, 2007). Undermine BBB integrity by breaking down the interendothelial tight junctions is, of course, a non-selective process and all serum constituents, including albumin, viruses, toxicants and other potentially circulating damaging substances may gain entry from blood to brain (ALAM *et al.*, 2010; STENEHJEM *et al.*, 2009). Astrogliosis, as a result of astroglial reactions produced by plasma proteins leakage like albumin to parenchymal compartment, is the most serious complication (ALAM *et al.*, 2010; PARDRIDGE, 2007). Owing to the induced brain vulnerability, BBB disruption techniques always require patient hospitalisation in order to ensure an expert administration and close medical monitoring (STENEHJEM *et al.*, 2009).

(3) Direct Delivery of Therapeutics into the Brain by Employing Invasive Administration Techniques

Despite advances in rational CNS drug design and BBB disruption techniques, many potentially efficacious drug molecules still cannot penetrate into the brain parenchyma at therapeutic concentrations. Therefore, a third class of strategies aiming at enhancing CNS targeting has been explored based on direct delivery methodologies that do not rely on the cardiovascular system. These approaches have the advantage of delivering much higher concentrations of neurotherapeutics by directly administering the drug into the CSF or parenchymal space, subsequently reducing the drug distribution throughout peripheral tissues and, as a consequence, the unwanted systemic side-effects (ALAM *et al.*, 2010). Intracranial techniques comprising intracerebral, intraventricular and intrathecal administration routes are invasive delivery methods extensively studied both in laboratory and clinical trials that afford a direct neurosurgical introduction of drugs into the brain or CSF, bypassing both the BBB and BCSFB (ALAM *et al.*, 2010; LO *et al.*, 2001; PARDRIDGE, 2005). In general, drug instillation can be achieved by first drilling a hole in the skull and then, inserting an intrathecal catheter or a locally controlled release polymeric implant (also called as wafer). Intracranial drug administration results in immediate high drug concentrations and long drug half-life in the CSF due to the minimised protein binding and decreased enzymatic activity comparatively to blood. Since CSF freely exchanges molecules with the extracellular fluid of the brain parenchyma, direct delivering of drugs into the CSF could theoretically be converted in therapeutic CNS drug concentrations (MISRA *et al.*, 2003). Regrettably, apart from the required surgical intervention, transcranial delivery approaches are commonly associated to a number of drawbacks that compromise their potential. The success of these methodologies is restrained by the slow rate of drug distribution within the CSF, the limited diffusion coefficient of the compounds from the depot site into the brain parenchyma, and the brain tissue damage induced by the insertion of solid implants and rapid injection of fluids into small ventricular volumes, increasing intracranial pressure and thereby, the incidence of haemorrhage, CSF leaks, neurotoxicity and CNS infections (BEGLEY, 2004; LO *et al.*, 2001; MISRA *et al.*, 2003; PARDRIDGE, 2007, 2005; PATHAN *et al.*, 2009). The high turnover rate of the CSF, with the entire volume being produced and completely absorbed into the venous circulation every four to five hours, implies that, after intraventricular drug injection, the compound is rapidly drained back into the blood, becoming essentially equivalent to a slow IV infusion (LO *et al.*, 2001; MOHANACHANDRAN *et al.*, 2012; PARDRIDGE, 2007). Therefore, intra-CSF drug injection is a suitable strategy to deliver therapeutic agents to the surface of the brain but fail to result in drug accumulation in parenchymal structures of the deep

brain which is highly essential for sustained drug release (ALAM *et al.*, 2010; MOHANACHANDRAN *et al.*, 2012).

Although regarded as promising strategies, the aforementioned CNS direct drug delivery techniques are not frequently applied in the clinic primarily because of their complexity, invasiveness and associated risks. A great deal of interest has recently been focused on the exploitation of the IN route for a non-invasive delivery of therapeutic agents directly to the brain. Until not so long ago, due to the high vascularisation and permeability of the nasal epithelium, IN route was assumed as a suitable and reliable approach for systemic delivery of drugs, allowing a rapid and extensive absorption of the compounds with plasma drug profiles sometimes almost identical to those obtained after IV injection (GRASSIN-DELYLE *et al.*, 2012). Nonetheless, the unique anatomical connection between the nasal cavity and the CNS has been widely investigated during the last decades in what concerns to its feasibility to directly transport therapeutics into the CSF and brain by circumventing the BBB (MERKUS and VAN DEN BERG, 2007). Indeed, the olfactory region is the only site in the human body where the nervous system is in direct contact with the surrounding environment, providing a great opportunity for drugs administered by the IN route to gain a quick and easy access to the brain, minimising their systemic exposure (GRAFF and POLLACK, 2005; JOGANI *et al.*, 2008).

IN administration has thus come to the forefront as an alternative to invasive delivery methods like intracerebral or intraventricular injections to bypass the BBB and rapidly target therapeutics directly to the CNS by using pathways along the olfactory and trigeminal nerves innervating the nasal passages (SINGH *et al.*, 2013). IN delivery not only circumvents the BBB but also avoids hepatic first-pass and systemic dilution effects, reduces drug delivery to non-targeted tissues, facilitates administration of low doses and in turn minimises toxicity (JOGANI *et al.*, 2008). Taking into account these merits allied to the simple, practical, non-invasive and convenient way of drug delivery, IN administration has been assigned as a relevant and promising route for CNS targeting of drugs.

The recognition of the nose-to-brain transport and the therapeutic viability of IN route arouse a wide interest by the scientific community and currently, it is an area of intense research. Physiological and histological data from animals and man have already demonstrated that mucosa in the upper part of the nose is connected with the cerebral perivascular and subarachnoid spaces of the brain olfactory lobes, which would make this pathway for drug delivery feasible. Several experimental studies have been conducted to establish the qualitative and quantitative transport of various low molecular weight drugs as well as therapeutic peptides and proteins through the nasal mucosa to the brain. To date, numerous evidences supporting the target-specific delivery of molecules from nasal cavity to the CNS have been reported.

In many CNS disorders like Alzheimer's disease, Parkinson's disease, migraine, pain and epilepsy, a rapid and/or specific targeting of drugs to the brain would definitely be beneficial; whereby, a major therapeutic advantage from IN drug delivery could be taken if the nose-to-brain pathway is confirmed. The question is whether this new route of drug delivery is a real treatment option or merely a scientific claim. Unfortunately, the nose-to-brain transport hypothesis is still controversial. While improved delivery to the brain via the IN route has been accounted for some therapeutic molecules, there are other cases for which no evidence of preferential delivery to the brain following IN dosing was found.

In this context, aware of the high potential that IN delivery has to offer and the limitations of the current pharmacological therapy of epilepsy, we intend with the work underlying the present thesis to investigate the feasibility of the IN delivery as an alternative route for AED administration. Thus, in order to get a better understanding of this possible transport route, a comprehensive characterisation of the nose and its particular features will be thoroughly addressed in the next section.



I. 2. INTRANASAL DRUG DELIVERY

I. 2. INTRANASAL DRUG DELIVERY

Oral delivery is undeniably the most desirable, preferred and convenient method of drug administration whenever systemic effects are intended. However, low oral bioavailability of some compounds due to extensive hepatic metabolism and gastrointestinal degradation has prompted the search of more effective routes, apart from the IV injection, to improve their systemic delivery (BEHL *et al.*, 1998; PIRES *et al.*, 2009). Different transmucosal routes involving nasal, buccal, rectal and vaginal cavities have demonstrated distinct advantages over peroral administration. Along with their convenience, the avoidance of hepatic first-pass metabolism and pre-systemic elimination in the gastrointestinal tract is the key attribute offered by these unconventional routes. While rectal and vaginal routes are commonly less preferred as they usually cause irritation and are less patient compliant, buccal administration frequently deals with the problem of acceptability when an unpleasant taste of drug formulation is present (SINGH *et al.*, 2013). Thus, nasal cavity has been put forward as a highly promising site for drug delivery, being now recognised as a useful and reliable alternative to both oral and parenteral administrations.

The administration of therapeutic compounds via nose is actually not a totally new approach for drug delivery (BHISE *et al.*, 2008). Smelling salts, tobacco, and hallucinogens have been used by inhalation for centuries by different cultures worldwide. Historically, the delivery of drugs via the nasal route has received attention of mankind since ancient time. This administration route was considered by various traditional systems of medicine such as Unani, Ayurvedic as well as Persian (ZARSHENAS *et al.*, 2013). Nasal therapy, also called “Nasya Karma”, has been an established therapeutic procedure in the Ayurvedic system of Indian medicine wherein herbal medicinal oils, powders, liquids or fumes are administered through the nostrils for treating diseases affecting the head area (APPASAHEB *et al.*, 2013).

Conventionally, drugs have been administered intranasally for their local effect on the mucosa, whereby the prevention and treatment of topical nasal conditions like allergy, sinusitis, congestion and infections via the nose is a common routine practice (BHISE *et al.*, 2008; CHOUDHARY and GOSWAMI, 2013). Nevertheless, in the last few decades, IN drug delivery has been given a new lease of life. As the market for the delivery of topical drugs matures, the potential for IN administration of systemically-acting drugs is developing at a remarkably fast pace (ILLUM, 2012; SOUTHALL and ELLIS, 2000). In fact, the early 1980s saw the introduction of nasal route as a promising systemic delivery alternative to other conventional drug delivery methods (ALSARRA *et al.*, 2010). Many drugs have been shown to achieve better systemic bioavailability through nasal route as compared to oral administration. Inclusively, it has been reported that lipophilic drugs are

generally well absorbed from the nasal cavity with pharmacokinetic profiles often identical to those obtained after an IV injection and bioavailability approaching 100% (ILLUM, 2003). The recognition of nasal mucosa as a therapeutically viable route for systemic administration of drugs came from its high permeability and large surface area providing a fast and extensive absorption of the compounds into the systemic circulation, also avoiding the hepatic first-pass elimination. A special interest has been laid on those drugs that are orally ineffective and must be administered by injection, as for instance small polar molecules, peptides and proteins. Hence, the pharmaceutical world has seen an increasing number of systemically acting drugs being marketed as nasal formulations and many more are at various stages of development. Currently, IN delivery is being employed in treatments for migraine, smoking cessation, acute pain relief, nocturnal enuresis, osteoporosis and menopausal hormone replacement (ALAGUSUNDARAM *et al.*, 2010; ILLUM, 2012). Other examples of therapeutic areas under development or with potential for IN delivery include cancer, epilepsy, nausea, rheumatoid arthritis and insulin-dependent diabetes (ALAGUSUNDARAM *et al.*, 2010).

Recent developments have highlighted the possibility of exploiting the nasal route for direct transport of drugs from nose to brain by circumventing the BBB (CHAPMAN *et al.*, 2013; ILLUM, 2004). Although the ability of IN delivery to enhance systemic bioavailability of drugs is remarkably important for some applications, the potential of bypassing the systemic circulation and delivering drugs directly into the brain represents a particularly novel, attractive but yet little understood feature (DJUPESLAND *et al.*, 2014). Targeting the brain via the olfactory epithelial region comes to offer a new favourable approach for treating CNS disorders, in view of the unique anatomical connection between the brain and nasal mucosa which may constitute a promising non-invasive pathway to deliver therapeutic agents directly into the biophase (DJUPESLAND *et al.*, 2014). Therefore, many CNS-acting compounds that do not normally cross and even those that actually can permeate the BBB upon systemic administration may likely find in the IN route a viable option to be efficiently delivered to the brain (HANSON and FREY II, 2008). As a result, a preferential drug uptake into the brain can be achieved and the peripheral exposure minimised, improving not only the therapeutic efficacy but also tolerability.

IN administration is steadily gaining momentum since, in addition to local application, its positive attributes also offer an important avenue to deliver drugs for systemic and CNS effects. To comprehend the potential of the nasal cavity as a route for drug delivery it is essential to understand the morphological structures and physiological features affecting the uptake of drugs across the nasal mucosa. Thus, in the beginning of this section, a brief description of the general anatomical, histological and physiological characteristics of the human nose will be given. As the purpose of the present work

mainly relies on the evaluation of the IN route potential to administer certain AEDs, a special focus will be placed on nose-to-brain drug delivery. The theoretical concepts underlying the hypothesis of CNS delivering of drugs via the nasal route will be presented. At the end, several relevant and pertinent aspects concerning the experimental procedures commonly employed in the assessment of nose to CNS drug transfer will also be appropriately discussed.

I. 2.1. ANATOMY AND PHYSIOLOGY OF THE NASAL CAVITY

The nose as a drug delivery site has a number of unique features related to its anatomy and physiology. In order to fully comprehend the intricacies of nasal drug delivery and to make any rational assessment about the usefulness of the IN route either for systemic or CNS applications, it is important to have an understanding of the most relevant nasal cavity morphological and physiological factors affecting these functions.

I. 2.1.1. NASAL ANATOMY AND HISTOLOGY

The nose is a protruding structure in the face supported by a framework of bone and cartilage which gives it a pyramidal conformation that can greatly differ in size and shape depending on race. The human nasal cavity comprises the space between the base of the skull and the roof of the mouth consisting of a chamber with about 12-14 cm long and approximately 5 cm high, superiorly enclosed by the ethmoid and sphenoid, and laterally, by the ethmoid, maxillary and inferior conchae bones (GIZURARSON, 2012; PIRES *et al.*, 2009). The total surface area of the human nasal cavity is about 150-160 cm² and the corresponding total volume is near 15-20 mL (GIZURARSON, 2012; LOCHHEAD and THORNE, 2012). The nose is longitudinally divided by a cartilaginous nasal septum in two symmetrical and non-connected passages, each one opening at the face through nostrils and extending posteriorly to the upper part of the throat at the level of the nasopharynx, where the two halves of the airway join together.

According to its anatomical and histological characteristics, the nasal cavity is mainly composed of three distinct functional areas which include the nasal vestibule, the respiratory region and the **olfactory region** (Figure I.2.1).

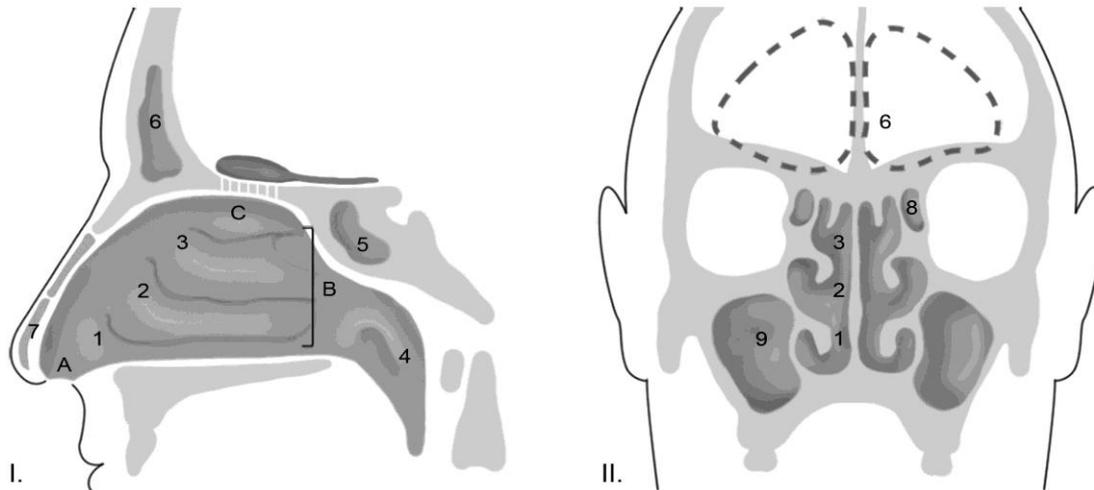


Figure I.2.1 Schematic representation of the human nasal cavity. (I) Longitudinal cross-section; (II) Transversal cross-section; A. Nasal vestibule; B. Respiratory region; C. Olfactory region; 1. Inferior turbinate; 2. Middle turbinate; 3. Superior turbinate; 4. Nasopharynx; 5. Sphenoid sinus; 6. Frontal sinus; 7. Septal cartilage; 8. Ethmoid sinus; 9. Maxillary sinus (Adapted from SERRALHEIRO *et al.*, 2012).

The nasal vestibule is the most anterior part of the nasal cavity and corresponds to the region just inside the nostrils with an area of about 0.6 cm^2 (ILLUM, 2003). The luminal surface is lined by a stratified squamous and keratinised epithelium containing nasal hairs in addition to sebaceous and sweat glands, which is gradually converted into a posterior pseudostratified ciliated columnar epithelium that covers the respiratory region (ILLUM, 2004; LOCHHEAD and THORNE, 2012). Being very similar to the external skin, the vestibular area serves as a first barrier against airborne particles with poor vascularisation, affording on one hand a desirable protection of the underlying tissues from atmospheric toxic agents while limiting the absorption of drugs on the other (BITTER *et al.*, 2011; PIRES *et al.*, 2009).

The respiratory region is the largest area of the nasal cavity comprising three nasal turbinates (also called conchae): the inferior, the middle and the superior that are projected from the nasal lateral walls into the airway lumen and play an important role in filtering, humidification and warming of the inspired air (MYGIND and DAHL, 1998; PIRES *et al.*, 2009). These turbinates are part of a convoluted and folded structure which, by increasing the resistance to the airflow through the nasal passages creates turbulence, thus ensuring an intimate contact between the inhaled air and the mucosal surface (GIZURARSON, 1990; ILLUM, 2003). Due to its large surface area and rich vascularisation, the

respiratory mucosa provides the essential conditions for a significant drug absorption into the systemic circulation (ALSARRA *et al.*, 2010; ARORA *et al.*, 2002).

The **olfactory region** is located in the roof of the nasal cavity, just below the cribriform plate of the ethmoid bone, lying partly on the nasal septum and partly on the superior turbinate (JONES, 2001; MISTRY *et al.*, 2009). The cribriform plate is a horizontal bone that separates the nasal chamber from the brain. Nevertheless, its perforated structure by small vascular apertures, also called foramina, enables access for the nerve endings to enter the outer surface at the olfactory mucosa (GIZURARSON, 2012). Similarly to the respiratory epithelium, the olfactory one is highly vascularised and pseudostratified but contains specialised olfactory receptor cells that are responsible for smell perception (PIRES *et al.*, 2009; WATELET and CAUWENBERGE, 1999). While humans have relatively simple noses with breathing as the primary function, other mammals have more complex nasal structures mainly targeted to olfaction (HARKEMA *et al.*, 2006; REZNIK, 1990). Hence, the area of the nasal cavity covered by olfactory mucosa in humans is restricted and corresponds to less than 10% which is much smaller in comparison to dogs (~77%) and rodents (~50%), whose sense of smell is extremely developed (ILLUM, 2004; LOCHHEAD and THORNE, 2012). Owing to the unique direct connection between the nose and the brain, absorption of drugs across the olfactory neuroepithelium appears to be a valuable option to target therapeutics from the nasal cavity to the CNS.

Surrounding the nasal cavity there are paired air-filled spaces – the paranasal sinuses – that are located inside the bones of the face and head. According to their location and, therefore, the bone involved, paranasal sinuses can be divided in maxillary, frontal, sphenoid and ethmoid sinus (JONES, 2001). Even though their intrinsic functions have not been yet clarified, it is suggested that they form a collapsible framework to protect the brain from trauma, act as auxiliary chambers to heat and humidify the inspired air, also providing resonance to the voice (GIZURARSON, 2012).

Respiratory Mucosa

The nasal respiratory mucosa covers approximately 80-90% of the total nasal cavity area in humans (LOCHHEAD and THORNE, 2012). It is constituted by a pseudostratified columnar secretory epithelium resting on a collagen basement membrane and a *lamina propria* which is a connective tissue richly supplied with blood vessels, nerves, glands, lymphatic tissue and immune cells (PIRES *et al.*, 2009).

The human respiratory epithelium is comprised of four major and dominant types of cells which include ciliated and non-ciliated columnar cells, goblet cells and basal cells (Figure I.2.2). The latter lie on the basement membrane and, unlike the remaining

epithelial cells, do not extend sufficiently to the apical surface in order to reach the airway lumen. Carrying the essential information on each of the other cell types, the basal cells function as a reserve population by maintaining individual cell replacement in the epithelium (GIZURARSON, 2012). Moreover, it is also believed that they help in the adhesion of the other epithelial cells, specifically the columnar cells, to the basement membrane (MYGIND and DAHL, 1998).

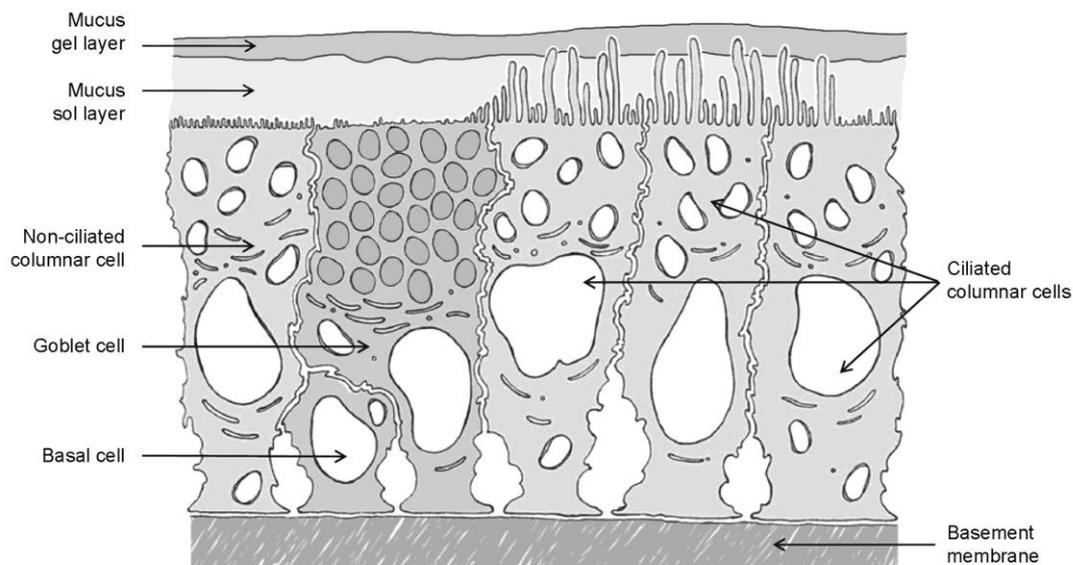


Figure 1.2.2 Schematic representation of the different cell types constitutive of the nasal respiratory epithelium (Adapted from GRASSIN-DELYLE *et al.*, 2012).

Another cell type characteristic of the nasal respiratory epithelium is the goblet cell, whose primary function is the secretion of the mucus that covers the epithelial cell layer. These glandular cells are interspersed among the columnar cells throughout the epithelium and differentiate by the presence of multiple granules filled with mucin on their inside. Their contribution to the volume of nasal secretions is probably small compared to that of the submucosal glands. Although little is known about their release mechanisms, it seems that physical and chemical irritants of the environment as well as biochemical mediators may stimulate goblet cell secretion (GIZURARSON, 2012; MYGIND and DAHL, 1998).

The columnar cells correspond to the majority of the epithelium cells. Ciliated or not, all of them are covered by numerous microvilli that are uniformly distributed over the entire apical surface. These short and slender finger-like cytoplasmic expansions of

the cell membrane considerably increase the surface area of the respiratory epithelium available for systemic drug absorption, also preventing the surface from drying by retaining the moisture essential for ciliary function (FORTUNA *et al.*, 2014a; MYGIND and DAHL, 1998). About 15-20% of the total number of respiratory cells display long cytoplasmic mobile projections called cilia (ILLUM, 2000; MISTRY *et al.*, 2009). Under physiologic conditions, the cilia move in a coordinated way to propel mucus across the epithelial surface towards the nasopharynx where it is either swallowed or expectorated. This phenomenon, known as mucociliary clearance, is one of the main mechanisms of defence of the nose and will be further discussed.

Owing to its vast surface area and richly supplied vascular nature, the respiratory mucosa is considered the major site for drug absorption into the systemic circulation. The blood supply of nasal mucosa comes mainly from ophthalmic, sphenopalatine and facial arteries. The arterial blood flow irrigates a dense bed of capillaries and large venous sinusoids that exist near the turbinate respiratory zone. The venous return involves the ophthalmic, sphenopalatine and facial veins that convey into the internal jugular vein, which in turn drains into the right heart chambers via the subclavian vein and the superior vena cava (FORTUNA *et al.*, 2014a; GRASSIN-DELYLE *et al.*, 2012). These anatomical features explain the absence of hepatic first-pass effect of drugs administered by the IN route.

Olfactory Mucosa

The olfactory organ is the only externally exposed portion of the CNS, being generally recognised as a “window to the brain” (ESCADA *et al.*, 2009). The human olfactory mucosa consists of a pseudostratified columnar epithelium resting on a basement membrane and a highly cellular *lamina propria* (ESCADA *et al.*, 2009). This epithelium is a modified form of the respiratory epithelium and is predominantly composed of three different cell types: ciliated olfactory receptor cells, sustentacular (or supporting) cells and basal cells (Figure 1.2.3).

The olfactory receptor cells are bipolar neurons primarily responsible for the mediation of the sense of smell by conveying sensory information from the peripheral environment to the CNS. They are appropriately interspersed among the sustentacular cells, projecting a single dendrite to the surface of the olfactory neuroepithelium and a single unmyelinated axon to the olfactory bulb (GIZURARSON, 2012; ILLUM, 2000). The dendritic process, which extends to the free apical surface, terminates in a small knob containing numerous non-motile cilia with specialised membrane chemical receptors for odour molecules binding (ESCADA *et al.*, 2009; MISTRY *et al.*, 2009). The axon is gathered together with other olfactory axons in small nerve bundles to pass through the *lamina*

propria and the cribriform plate of the ethmoid bone towards the olfactory bulb, where it establishes a synapse with a second order neuron by connecting to a mitral or a tufted cell at the level of the olfactory glomerulus (NAGAYAMA *et al.*, 2004) (Figure I.2.3). These nerve bundles, also called *fila olfactoria*, are conveniently ensheathed by Schwann cells (or glial cells) which, in addition to supporting the transition of the axons from the peripheral olfactory epithelium to the CNS, also create continuous fluid-filled perineural channels that act as ionic reservoirs for action potential propagation (ESCADA *et al.*, 2009; MISTRY *et al.*, 2009).

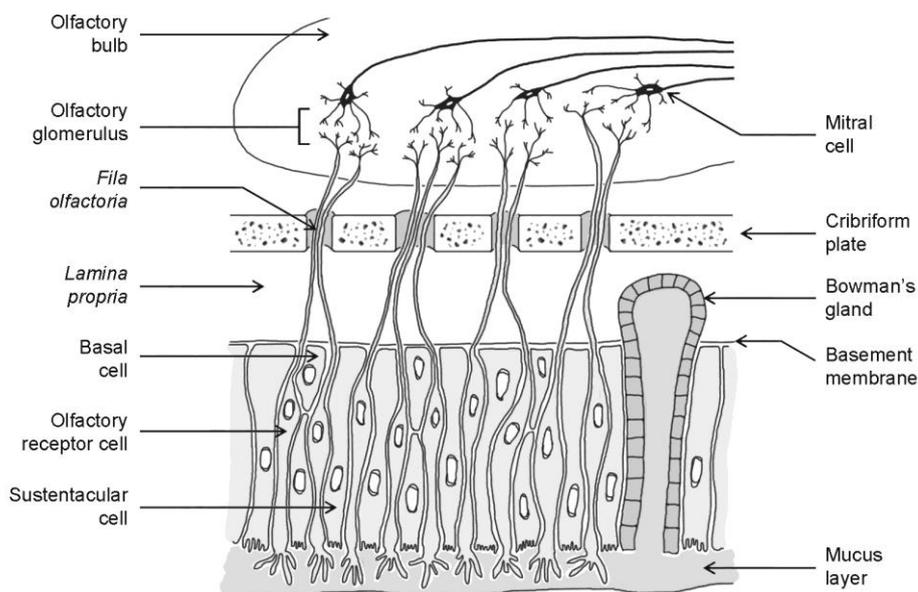


Figure I.2.3 Schematic illustration of the various cell types representative of the nasal olfactory epithelium.

The sustentacular cells are elongated columnar cells with their tapered bases resting on the basement membrane and many long microvilli extending from their luminal surface. They surround olfactory receptor neurons providing metabolic and physical support and contributing to regulate and maintain the appropriate extracellular potassium levels required for neuronal activity (GIZURARSON, 2012; JONES, 2001). Basal cells are the only type of cells that do not project to the epithelial surface, being merely in contact with the underlying basement membrane. They are a well-recognised distinct stem cell population of the olfactory epithelium once they are capable to continuously regenerate olfactory receptor neurons along the life span (ESCADA *et al.*, 2009). Upon

differentiation, these cells can replace the neuroepithelium approximately every 40 days (JONES, 2001).

Beneath the olfactory epithelium there is the *lamina propria* which contains blood and lymphatic vessels, olfactory axon bundles, autonomic and trigeminal nerve fibres and mucus-secreting tubuloalveolar Bowman's glands (ILLUM, 2000; MISTRY *et al.*, 2009). These exocrine acinar-type glands are under the control of the parasympathetic nervous system to produce and secrete mucus that is essential for the olfactory transduction by acting as a solvent for odour molecules.

I. 2.1.2. NASAL PHYSIOLOGY

The nose is a structurally and functionally complex organ of the upper respiratory tract whose primary functions are breathing and olfaction. In addition to heating, moistening and filtering the particles in suspension of the inhaled air, the nose also serves as a first-line of defence against external insults, once it presents several physiological mechanisms of protection that avoid the access of pathogenic agents, toxicants and airborne pollutants to the lungs. Therefore, the absorption and delivery of drugs across the nasal passages are likewise conditioned by such physiological barriers.

Nasal Mucus and Mucociliary Clearance

Under physiological conditions, the nasal respiratory epithelium is covered with a thin layer of a watery and sticky fluid, the mucus, which is produced and secreted by submucosal glands and goblet cells (SAHIN-YILMAZ and NACLERIO, 2011). Approximately 1.5-2 L of mucus is produced daily (UGWOKE *et al.*, 2005). This nasal secretion consists of a complex mixture of several materials mainly composed of water (95%), mucin (2%), other proteins such as albumin, immunoglobulins, lysozyme and lactoferrin (1%), inorganic salts (1%) and lipids (<1%), presenting a pH value that ranges from 5.5 to 6.5 (JADHAV *et al.*, 2007; MYGIND and DAHL, 1998). Due to its adhesive characteristics, the nasal mucus acts as a physical barrier of protection onto which particles, pathogens and xenobiotics in the turbulent inhaled airstream can impact and be entrapped.

The continuous blanket of mucus lining the respiratory epithelium is a double layer with about 10-15 μm thick consisting of an aqueous lower sol phase surrounding the cilia and a superficial layer of viscous gel that covers the tips of the cilia (MYGIND and DAHL, 1998; SAHIN-YILMAZ and NACLERIO, 2011; UGWOKE *et al.*, 2001). Through the action of the coordinated cilia beating, the more viscous gel layer, together with deposited noxious substances, is propelled posteriorly along the epithelial surface towards the nasopharynx from where it can be swallowed or expectorated. The mucus flow rate has been

measured in humans to be in the order of 5 mm/min, so that the renewal of the nasal mucus layer is accomplished every 15-20 min (ILLUM, 1996).

The combination of the viscoelastic nasal mucus properties with the ciliary activity of the respiratory epithelium results in an important and efficient physiologic mechanism of defence that, by removing toxic materials from the nasal cavity, prevents them from reaching the lower airways. This mechanism is designated as mucociliary clearance (MCC) and it is fundamental to maintain the integrity of the respiratory tract. Unfortunately, it also exerts a very significant impact on IN drug delivery by limiting the time available for absorption to occur (UGWOKE *et al.*, 2001).

MCC has been described as a “conveyer belt” wherein ciliated cells provide the driving force and mucus functions as a tacky fluid that collects and disposes foreign particles (MARTTIN *et al.*, 1998). The efficiency of this cleansing system is therefore dependent on the length, density and beat frequency of cilia as well as the viscoelastic character and amount of mucus (JONES, 2001; MARTTIN *et al.*, 1998; PIRES *et al.*, 2009). Any changes concerning mucus production, mucus composition or ciliary activity, whether by environmental or pathological conditions, may influence the normal behaviour of the MCC system. While in view of nasal drug delivery, a decrease in MCC rate would be beneficial to increase the residence time of drugs within the nasal cavity thus enhancing their permeation, a longer contact of the airway mucosa with irritant substances and pathogenic microorganisms could eventually lead to local tissue damage and respiratory infections (ARORA *et al.*, 2002; MARTTIN *et al.*, 1998).

The rapid MCC of drug formulations that are deposited in the nasal cavity is inevitably considered an important factor underlying the low bioavailability of intranasally administered compounds. In particular, polar drugs are the most affected since they are highly soluble in mucus and their permeation across the nasal epithelium is very slow. In order to attain a prolonged residence time of therapeutic agents within the nose, several strategies have been performed; among them, the incorporation of bioadhesive excipients into the pharmaceutical formulations seems to be a suitable approach (UGWOKE *et al.*, 2001).

Nasal Metabolism

It is generally accepted that the IN route circumvents the gastrointestinal elimination and the hepatic first-pass metabolism associated with oral drug delivery. However, the nasal mucosa has a defensive enzymatic barrier against the entry of xenobiotics, and the original concept of nasal drug delivery without first-pass effect is no longer applicable (SARKAR, 1992). In fact, the metabolic activity in nasal cavity plays an important role in bioactivation and deactivation of inhaled or systemically applied

toxicants, and therefore it can also be responsible for a possible reduction of the intranasally administered drugs bioavailability, especially those containing peptides or proteins (WONG and ZUO, 2010).

Drug-metabolising enzymes are present in nasal secretions, in nasal epithelial cells (where they may be free or membrane bound), and in the *lamina propria* (HINCHCLIFFE and ILLUM, 1999). Several oxidative phase I enzymes such as cytochrome P450 (CYP) isoenzymes, carboxylesterases and epoxide hydrolases, and conjugative phase II enzymes like glucuronyltransferases and glutathione S-transferases have been identified in human nasal tissue (MINN *et al.*, 2002; SARKAR, 1992). Particularly important is the high levels of various CYP isoenzymes detected in both respiratory and olfactory mucosa, since, as it is known, this catalytic system is largely implicated on the metabolism of a huge number of drugs. Interestingly, it has been found that the specific content of CYP in nasal mucosa was the second most abundant after the liver when it is expressed in relation to gram of tissue, and its metabolic activity is actually more pronounced than in any other tissue (ILLUM, 1996; SARKAR, 1992). Notwithstanding, due to the short period of contact time between the drug and enzyme within the nasal cavity, as well as the higher drug-to-enzyme ratio compared to the gastrointestinal tract and liver, no significant effect is generally accounted on the extent of absorption of small molecule drugs administered by the IN route (HUSSAIN, 1998). In contrast, the same aspect is no longer observed for therapeutic peptides and proteins once enzymatic degradation, either by nasal peptidases or proteases, has been suggested as one of the main barriers against the absorption of many biocompounds (SARKAR, 1992; WONG and ZUO, 2010).

Regardless of its impact on drug disposition, the extensive enzymatic machinery within the nose contributes to a “nasal first-pass effect” that affects the pharmacokinetic and pharmacodynamic profiles of nasally applied drugs, and thus it should not be neglected.

Nasal Permeability

For effective systemic or CNS effects, intranasally administered therapeutic molecules have to primarily cross the mucus layer and nasal epithelial membranes before reaching the blood circulation or the brain. The first step in nasal drug permeation is the passage through the mucus. In general, small and uncharged particles can easily permeate through this layer, whereas large or charged particles may find it more difficult to cross (FORTUNA *et al.*, 2014a). Indeed, a greater drug permeation is usually achieved at a nasal pH that is lower than the drug's pK_a because under such conditions, the molecules exist as unionised species (ARORA *et al.*, 2002). On the other hand, the establishment of intermolecular interactions between the drugs and the glycoprotein chains of the mucus

may hamper their diffusion across the layer, becoming more difficult as the molecule size increases.

After transposing the mucus, drugs have to transverse the nasal epithelial barrier. Therapeutic agents are able to pass through the nasal mucosa by two different pathways which include the *transcellular route* (across the cell) and the *paracellular route* (between the cells) (Figure I.2.4).

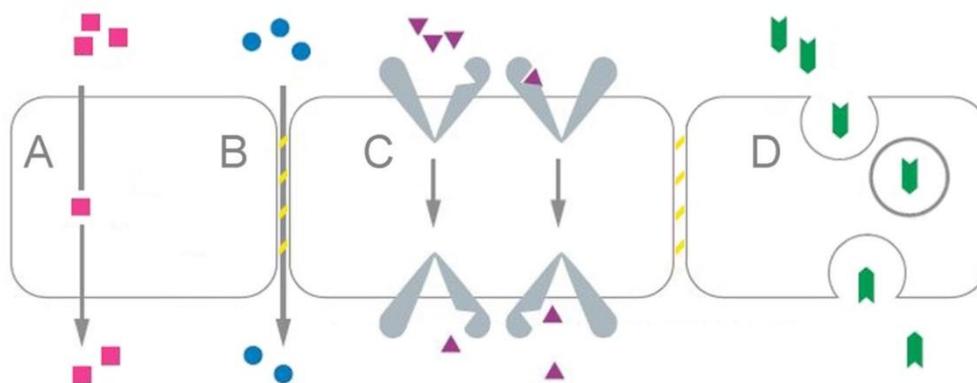


Figure I.2.4 Mechanisms of drug transport across nasal mucosa. A. Transcellular passive diffusion; B. Paracellular passive diffusion; C. Carrier-mediated transcellular transport; D. Vesicular-mediated transcellular transport (Transcytosis).

The *transcellular route* mainly encompasses transport via a lipoidal pathway in which drugs can cross the epithelial cell by a simple concentration-dependent passive diffusion process, by receptor/carrier mediation or by vesicular transport mechanisms (ILLUM, 2002; JADHAV *et al.*, 2007) (Figure I.2.4). Therefore, lipophilic drugs may incorporate into the lipid bilayer of the cell membrane, diffuse through it and transverse the cell cytoplasm by a process of passive diffusion. The passive transcellular transport across the nasal mucosa is mainly a function of the lipophilic nature of a drug compound and it shows rate dependency on its lipophilicity, whereby, highly lipophilic drugs are expected to have rapid and complete transnasal uptake (JADHAV *et al.*, 2007; VYAS *et al.*, 2005). An active internalisation of the drugs can also be accomplished after recognition by carrier transporters expressed at the membrane of epithelial cells. These carriers comprise organic cation and amino acid transporters that mediate the influx of a wide variety of drugs across the membrane (FORTUNA *et al.*, 2014a; SINGH *et al.*, 2013).

The *paracellular route* involves a slow and passive aqueous mode of transport via gaps or pores between adjacent epithelial cells. The nasal epithelial cells are closely

packed by intercellular connections consisting of tight junctions and desmosomes that create a dynamic, adjustable and semi-permeable diffusion barrier between the cells, hindering the paracellular passage of various hydrophilic compounds (ILLUM, 2004). In general, tight junctions are observed in both respiratory and olfactory epithelium. They are domains of occluded intercellular clefts composed by multiprotein complexes, whose opening and closing is regulated by multiple cell signalling mechanisms (BALDA and MATTER, 1998; WOLBURG and LIPPOLDT, 2002). As their normal diameters in nasal mucosa vary between 3.9 and 8.4 Å (ILLUM, 2007), the transport of drugs through the paracellular space is strictly controlled, being highly dependent of their molecular weight. Hence, there is an inverse relationship between nasal permeation and the molecular weight of water-soluble compounds (ARORA *et al.*, 2002). A poor bioavailability has been reported for drugs presenting a molecular weight greater than 1000 Daltons (JADHAV *et al.*, 2007; VYAS *et al.*, 2005).

The nose is a complex organ from a kinetic point of view since three different processes – deposition, clearance and absorption of drugs occur concurrently within the nasal cavity (JOGANI *et al.*, 2008). Indeed, apart from the physicochemical properties of the compound and formulation, drug transport across the nasal epithelium is largely affected by several nasal physiological factors. The foremost obstacles to drug permeation relate to the distribution and deposition site of drugs within the nasal cavity, the possible nasal first-pass metabolism, and the rapid elimination of the drug from the absorption site as a result of the MCC mechanism.

The permeability at the site where the formulation is deposited and the area of the nasal cavity exposed are determinant factors that affect the efficiency of nasal absorption (ARORA *et al.*, 2002). Thus, while an anterior deposition at the vestibule may prolong the nasal residence time, the absorption of drugs is relatively low. In opposition, an enhanced permeability could be achieved if the drug was deposited in the respiratory area, however, the rapid elimination of the dosage form due to the MCC process may limit the time available for absorption to occur which significantly compromises its rate and extension. A posterior distribution targeting the olfactory epithelium may facilitate direct transport of the drug into the brain, but in case of susceptible compounds, it could increase enzymatic degradation (UGWOKE *et al.*, 2001). Therefore, according to the intended therapeutic effect, deposition of nasally instilled substances in a specific region should be regular and predictable in order to attain desirable results. Nonetheless, nasal drug distribution and deposition are influenced by a myriad of determinants which may include the type of formulation, administration device, particle size and pathological condition of the nasal cavity (CHUGH *et al.*, 2009; TALEGAONKAR and MISHRA, 2004).

Another contributing, but often less considered factor to the low transport of drugs across the nasal membrane is the possibility of an enzymatic degradation of the molecules either within the lumen of the nasal cavity or during the passage through the nasal epithelial barrier (UPADHYAY *et al.*, 2011). In fact, as aforementioned, many compounds, such as cocaine, nicotine, alcohols, decongestants and anaesthetics are known to be metabolised by the nasal P450-dependent monooxygenase system (BEHL *et al.*, 1998). However, owing to the high expression of proteolytic enzymes in nasal tissues, the effect on the stability of nasally administered peptides and proteins is generally much more pronounced.

The general fast nasal removal of the administered drug formulations as a result of the MCC mechanism is considered one of the most significant constraints underlying the poor bioavailability of compounds administered by the IN route. This natural process of defence severely limits the retention and the intimacy of the contact between the drug and the mucosa, being especially critical for drugs that are not easily permeable across the nasal membrane. Effectively, whenever a substance is nasally applied, it is rapidly cleared from the nasal cavity with a clearance half-life of approximately 15-20 min (MARTIN *et al.*, 1998). The consequences are not only restricted to the limited time allowed for absorption; MCC also rules out the possibility for a controlled IN drug delivery (UGWOKE *et al.*, 2001).

Taking into account that absorption is a prelude to pharmacological effect, the permeability of a drug across the nasal mucosa should be high enough in order to achieve the therapeutic purpose. In this view and accordingly to the above considerations, several strategies have been suggested to improve the nasal permeability of drugs which include, among others, the use of pro-drugs, enzymatic inhibitors, absorption enhancers and mucoadhesive delivery systems.

I. 2.2. THE NOSE AS A SITE FOR DRUG DELIVERY – THERAPEUTIC APPLICATIONS

IN delivery represents an attractive and promising option for local, systemic and CNS administration of a wide variety of therapeutic agents including not only small molecules but also biocompounds. Clearly, the specific nature of the nasal mucosa provides a series of unique attributes which render the nose both a therapeutic target and a portal of entry for drug delivery.

The nose as a route for drug delivery offers a number of advantages (Table I.2.1) that afford the maximisation of patient convenience, comfort and compliance (COSTANTINO *et al.*, 2007). Compared to other mucous membranes, the nasal mucosa is readily accessible, highly vascularised and highly permeable, conferring a prompt and practical doorway for small and large molecules. Its vast surface area allows a rapid onset of pharmacological effect, an extensive systemic drug bioavailability and a potential pathway for the transport of therapeutics directly into the CNS. By avoiding gastrointestinal degradation and hepatic first-pass metabolism, the IN route is particularly effective for drugs that are poorly orally absorbed or have to be given by injection (BAHADUR and PATHAK, 2012). In addition, being non-invasive, essentially painless and easily administered by patients or physicians, IN administration can be applied in both chronic and emergency treatments and be potentially performed either inside or outside the hospital environment (COSTANTINO *et al.*, 2007).

Nevertheless, despite its multiple key benefits, the IN route also presents several intrinsic limitations (Table I.2.1) that should be taken into account during the discovery and development of new chemical entities/drug formulations intended for nasal application. Such drawbacks relate to the specific anatomical, physiological and pathological conditions of the nasal cavity that, together with physicochemical properties of drugs and their final formulations, may compromise the extent of nasal drug absorption and ultimately, the therapeutic efficacy (COSTANTINO *et al.*, 2007; PIRES *et al.*, 2009).

Table I.2.1 The major advantages and limitations of IN drug delivery (APPASAHEB *et al.*, 2013; ARORA *et al.*, 2002; PARDESHI and BELGAMWAR, 2013; PATEL *et al.*, 2012; SINGH *et al.*, 2012; UPADHYAY *et al.*, 2011).

Advantages	Limitations
<ul style="list-style-type: none"> • Non-invasive, simple, safe and comfortable; • Ease of administration and self-medication, improving patient convenience and compliance; • Rapid absorption and quick onset of action due to the large surface area and high vascularisation; • Avoidance of gastrointestinal and hepatic first-pass metabolism; • High permeability and improved bioavailability; • Potential for direct delivery of drugs to CNS, bypassing the BBB; • Lower enzymatic activity compared to the liver and gastrointestinal tract; • Lower doses and reduced side-effects; • Reduced risk of overdose and infection; • Effective in long-term and emergency therapies as an alternative to oral and parenteral routes of administration; • No complex formulation requirement. 	<ul style="list-style-type: none"> • Delivery volume in nasal cavity is restricted to 25-200 μL; • Poor permeability for hydrophilic drugs or compounds with molecular weight > 1 kDa; • Rapid elimination of drug substances from nasal cavity due to MCC mechanism; • Possible enzymatic degradation, especially for peptides and proteins; • Nasal congestion due to cold or allergy; • Local irritation; • Frequent use may result in mucosal damage; • Possible mechanical loss of the dosage form due to improper technique of administration.

Faced with surging development costs and fierce generic competition, the overall pharmaceutical companies are under increasing pressure to find ways to enhance and prolong the profitability of existing and new products (DJUPESLAND, 2003). Furthermore, many of the advanced therapeutic molecules being developed require more efficient delivery than that offered by conventional administration routes and delivery systems (DJUPESLAND, 2003). Therefore, in view of its inherent virtues, it is legitimate to consider IN administration as a novel delivery route when developing new therapeutics, or when improving the profile of an existing drug including life cycle management (BITTER *et al.*, 2011; COSTANTINO *et al.*, 2007). In general, among the primary targets for IN administration are pharmacologically active compounds with poor stability in gastrointestinal fluids, poor intestinal absorption and/or extensive hepatic first-pass elimination, such as peptides, proteins and polar drugs (DHAKAR *et al.*, 2010). Based upon the advantages and limitations of IN drug delivery, the selection of drug candidates for transmucosal nasal application may become challenging. According to the biopharmaceutical drug classification system, therapeutic molecules that fit into class I (high permeability, high solubility) have the

highest potential for nasal administration (BITTER *et al.*, 2011; MATHIAS and HUSSAIN, 2010). Ideally, the profile of a nasal drug candidate should fulfil the following criteria (ARORA *et al.*, 2002; BEHL *et al.*, 1998; KUMAR *et al.*, 2014; LO *et al.*, 2001; SINGH *et al.*, 2013):

- Appropriate aqueous solubility to provide the desired dose in a 25-150 μL volume formulation administered per nostril;
- Appropriate nasal absorption properties – molecular weight < 500 Da, LogP < 5;
- Suitable drug formulation characteristics – pH within 4.5-6.5, isotonic;
- Low dose (generally less than 25 mg);
- No nasal irritation;
- No toxic nasal metabolites;
- No offensive odours/aroma associated with the drug;
- Convenient stability features.

I. 2.2.1. LOCAL DELIVERY

IN route is the logical natural delivery choice for the treatment of local (or topical) nasal disorders. Typical examples for locally acting administered drugs are decongestants for nasal cold symptoms relief, antihistamines and corticosteroids for allergic rhinitis, and antimicrobials for nasal infections (BITTER *et al.*, 2011; COSTANTINO *et al.*, 2007). Since nasal topical therapy enables direct delivery of drugs to the target organ (biophase), a quick relief of the symptoms can be easily achieved with a very favourable adverse-event profile (SALIB and HOWARTH, 2003). In fact, unlike systemic therapies, relatively low doses are needed to be effective when administered locally which minimises the potential for systemic toxic effects that are commonly associated to oral and parenteral administrations (PIRES *et al.*, 2009; SERRALHEIRO *et al.*, 2013a). Currently, topical drug formulations continue to represent the most significant portion of the total nasal products available in the market (Table I.2.2).

Table I.2.2 Examples of commercially available nasal drug formulations for local (topical) delivery (BEHL *et al.*, 1998; KUMAR *et al.*, 2014; PIRES *et al.*, 2009).

Drug	Formulation	Product Name	Manufacturer	Therapeutic Indication
Oxymethazoline	Nasal drops	Nasorhinathiol® Nasex®	Sanofi Aventis Johnson & Johnson	
Phenylephrine	Nasal drops	Neo-Sinefrina®	GlaxoSmithKline	Nasal congestion
Tramazoline	Nasal spray	Rhinospray®	Unilfarma	
Xylometazoline	Nasal drops Nasal spray	Otrivina®	Novartis	
Azelastine	Nasal spray	Allergodil®	Meda Pharma	
Beclometasone	Nasal spray	Beconase®	GlaxoSmithKline	
Budesonide	Nasal spray	Pulmicort®	AstraZeneca	
Fluticasone furoate	Nasal spray	Avamys®	GlaxoSmithKline	
Levocabastine	Nasal spray	Livostin®	Johnson & Johnson	Management of seasonal and perennial (allergic) rhinitis
Mometasone	Nasal spray	Nasomet®	Schering-Plough	
Olapatadine	Nasal spray	Patanase®	Alcon Laboratories	
Sodium cromoglicate	Nasal spray	Fenolip®	Angelini	
Triamcinolone acetonide	Nasal spray	Nasacort®	Sanofi Aventis	
Mupirocin	Nasal ointment	Bactroban®	GlaxoSmithKline	Eradication of nasal staphylococci

I. 2.2.2. SYSTEMIC DELIVERY

The nasal respiratory region is considered to be the major site for nasal drug absorption into the systemic circulation. Underlying the great potential of the IN route for systemic drug delivery is the rapid and direct systemic absorption of compounds that, by circumventing gastrointestinal and hepatic first-pass metabolism, enables a reduction of the administered dose; a quick achievement of relevant therapeutic levels; a fast onset of pharmacological action and fewer side-effects compared to conventional administration routes (FORTUNA *et al.*, 2014a). Consequently, the awareness that drugs may reach widespread circulation in few minutes after nasal administration remarkably expanded the number of systemically acting drugs marketed as nasal formulations (Table I.2.3) and many more are in the pipeline. Examining the commercially available nasal products for systemic delivery, it is evident that the majority of the approved therapies are for acute and sub-chronic treatment regimens (MATHIAS and HUSSAIN, 2010). Accordingly, the rapid

absorption and prompt onset of action of nasally administered drugs may suit the unmet medical needs in crisis treatment such as acute pain, migraine, cardiovascular attacks, erectile dysfunction, seizures, nausea and vomiting. On the other hand, the high nasal bioavailability coupled with the ease of administration has provided another IN treatment paradigm in the field of long-term illnesses that has gained significant success in the management of several disorders like endometriosis, osteoporosis, fertility treatment, prostate cancer and diabetes (MATHIAS and HUSSAIN, 2010).

Table I.2.3 Examples of commercially available nasal drug formulations for systemic delivery (BITTER *et al.*, 2011; FORTUNA *et al.*, 2014a; GRASSIN-DELYLE *et al.*, 2012; KUMAR *et al.*, 2014; PIRES *et al.*, 2009; SINGH *et al.*, 2013).

Drug	Product Name	Manufacturer	Therapeutic Indication
Buserelin	Suprefact®	Sanofi Aventis	Prostate cancer
Butorphanol	Stadol NS®	Bristol Myers Squibb	Pain management; Migraine
Cyanocobalamin	Nascobal®	Par Pharmaceutical	Vitamin B12 deficiency
Desmopressin	Desmospray®	Ferring	Nocturnal enuresis; Diabetes <i>insipidus</i>
Dihydroergotamine	Migranal®	Novartis	Migraine and cluster headaches
Estradiol	Aerodiol®*	Servier Laboratories	Hormone replacement therapy
Fentanyl	Instanyl®	Takeda Pharma	Breakthrough pain in patients with cancer
Nafarelin	Synarel®	Pfizer	Endometriosis
Nicotine	Nicotrol NS®	Pfizer	Smoking cessation
Oxytocin	Syntocinon®	Novartis	Labour induction; Lactation stimulation
Salmon calcitonin	Miacalcin®	Novartis	Osteoporosis
Sumatriptan	Imigran®	GlaxoSmithKline	Migraine and cluster headaches
Zolmitriptan	Zomig®	AstraZeneca	Migraine and cluster headaches

* Discontinued in 2006 due to commercial reasons.

I. 2.2.3. VACCINE DELIVERY

The vast majority of disease-causing bacteria, viruses and parasites reach the body through mucosal surfaces by which it is natural that most of the immune system is either located in or in direct contact with mucosal membranes, thus providing a “first-line of defence” system against harmful microorganisms (BITTER *et al.*, 2011). Hence, preventing infection at these sites via mucosal immunisation seems to be a promising and rational approach for vaccine development. In addition to being a rapid and non-invasive needle-free option for antigen delivery, mucosal vaccination also provides an efficient immunisation method by inducing both local and systemic immune responses, thus emerging as an interesting alternative to the classic injection (SHARMA *et al.*, 2009; ZAMAN *et al.*, 2013).

Among other mucosal routes, nasal delivery is becoming particularly attractive for immunisation as the nasal epithelium is characterised by easy access, reasonable permeability, low enzymatic activity and the presence of an important number of immunocompetent cells (SLÜTTER *et al.*, 2008). In upper airways, the local and systemic immunological responses are mainly mediated by the nasal associated lymphoid tissue which is an organised structure of cells located underneath the nasal epithelium and composed of agglomerates of dendritic cells, T-cells and B-cells (SLÜTTER *et al.*, 2008; ZAMAN *et al.*, 2013). As opposed to what could be expected, mucosal immunity after nasal vaccination is, however, not only restricted to the upper airways affections. The detection of significant immunoglobulin A levels in other mucosal secretions, following IN vaccine administration, may account for a possible cross immune protection against non-respiratory infections in distant mucosal organs, such as urogenital and gastrointestinal tracts (PIRES *et al.*, 2009; SLÜTTER *et al.*, 2008).

Despite the promise of nasal vaccine delivery, only very few products are currently approved for human use, indicating that advances towards new effective nasal vaccines are still progressing slowly. An example of a fully developed and commercially available option is the FluMist® by AstraZeneca, the first nasal trivalent vaccine for seasonal influenza. Compared to the corresponding injection form, FluMist® conferred several advantages in terms of ease of use, longer duration of protection, enhanced efficacy and both mucosal and systemic immunity (ZAMAN *et al.*, 2013).

I. 2.2.4. CNS DELIVERY

According to a recent World Health Organization report (WORLD HEALTH ORGANIZATION, 2006), neurological disorders like epilepsy, migraine, stroke, multiple sclerosis, Alzheimer's disease and Parkinson's disease, are currently estimated to affect as many as a billion people worldwide, being one of the leading causes of disability. Their intricate pathophysiology and the difficulty of systemically administered therapeutics in accessing the brain represent a great challenge in the development of efficacious agents for central targets (DJUPESLAND *et al.*, 2014). In spite of the intense research efforts, many drugs are still not being effectively and efficiently delivered to the CNS, mainly due to the impenetrable nature of BBB that restricts substances from entering the brain depending on their lipophilicity, molecular size and charge (GRAFF and POLLACK, 2005; HANSON and FREY II, 2008).

In the last decades, several different strategies have been attempted in order to circumvent the BBB and improve the delivery of drugs to the brain for therapeutic and diagnostic applications (ILLUM, 2000). Intranasal administration, especially to the olfactory region located in the upper portion of the nasal passages, has been shown to noninvasively deliver a wide variety of compounds from the nasal cavity directly to the CNS, bypassing the BBB within few minutes. Increasing evidence of nose-to-brain transport has been reported by numerous research studies wherein the levels of certain drug molecules determined in CSF and olfactory bulb, after IN administration to rats, were considerably higher than those observed following IV injection (VYAS *et al.*, 2005). The rapid and preferential distribution of drugs to the brain via the IN route may lead to the reduction of systemic exposure and peripheral side-effects. Similarly, the dose and frequency of dosing could be decreased, the toxicity minimised and the therapeutic efficacy improved by achieving desired drug concentrations at the biophase (KUMAR *et al.*, 2008; SEJU *et al.*, 2011).

Up to date, the investigations have attracted researchers to place the IN delivery option for CNS drug targeting under the microscope. Even though the clinical potential of this drug administration avenue still remains controversial, there is substantial curiosity in exploring the nasal route for the treatment of common intra-cerebral diseases.

Having these premises in mind, the work underlying the present thesis was structured and developed. In order to deepen the comprehension of the phenomena inherent to the ability of the IN route to directly deliver therapeutic compounds to the CNS, the following section (*section I. 2.3.*) will provide thorough information with emphasis on this specific topic.

I. 2.3. NOSE-TO-BRAIN DRUG DELIVERY

With the enlargement of life expectancy and ageing of populations, the incidence of many chronic and progressive conditions including neurological disorders is expected to increase significantly in the 21st century. Many CNS disorders affect individual's functioning resulting in disabilities that limit activities and restrict participation. Currently, they are an important cause of mortality, constituting about 12% of total deaths globally (WORLD HEALTH ORGANIZATION, 2006).

Recent advances in the fields of pharmacology and molecular neurobiology have led to a better understanding of the CNS disease processes, allowing the development of new therapeutic approaches. Nevertheless, drug discovery and development programs have for too long been centred on selecting molecules with activity at a particular site or receptor in the brain, disregarding the extent of the delivery of the compounds to the biophase. For this reason, many promising molecules have been failing in the early stages of clinical trials, simply because they are unable to cross the BBB in sufficient quantity to be therapeutically effective (BEGLEY, 2004; GABATHULER, 2010; WAGER *et al.*, 2011).

Traditionally, the management of neurologic disorders have been predominantly performed through peripheral administration of medicines via oral or IV routes. However, there are a variety of troublesome issues concerning the use of systemic drug delivery to treat CNS diseases. Many drugs, and in particular macromolecules, are generally degraded in the gastrointestinal tract and/or metabolised in the liver, severely reducing their bioavailability to the point where only a small fraction of the active compound actually reaches the circulatory system and ultimately the brain (CHAPMAN *et al.*, 2013; DJUPESLAND *et al.*, 2014). Increasing the oral dose to compensate drug loss and direct delivery of therapeutics into the blood via IV injection seem to be obvious alternatives to overcome these limitations. Notwithstanding, the former option may cause unacceptable peripheral adverse events, and the latter remains rather impractical and unattractive, especially for treatments requiring frequent dosing or home administration (DJUPESLAND *et al.*, 2014). Beyond the above inconveniences, systemic administration of drug towards the CNS invariably predicates the passage of the compounds from the bloodstream to the brain by crossing the BBB which is not always an easy task, mainly for large and/or charged molecules. In addition, plasma protein binding, another consequence of systemic delivery, can also affect both the duration and intensity of a drug's action, diminishing its ability to efficiently permeate the BBB (CHAPMAN *et al.*, 2013).

Although invasive strategies, such as intracerebroventricular injection, have been stated as a viable solution to defeat BBB through the introduction of therapeutics directly into the brain, these methods are therefore not realistic for clinical applications. Besides being risky, they involve expensive techniques, demand surgical expertise and are not

somehow appropriate for multiple dosing regimens (DHURIA *et al.*, 2010; HANSON and FREY II, 2008). In view of this fact, IN administration has been proposed as a promising non-invasive, practical, safe and convenient delivery method which may allow an increased CNS penetration of compounds that otherwise display limited brain uptake, by providing a potential and efficient way to circumvent the BBB while minimising systemic exposure (GRAFF *et al.*, 2005; PARDESHI and BELGAMWAR, 2013).

The exploitation of the nasal cavity as a means of delivering therapeutic agents preferentially to the brain has gained significant recent interest. Detailed pharmacokinetic and pharmacodynamic studies, following IN delivery in both animals and humans, have shown that a broad spectrum of compounds not only reach specific areas of the brain, but also have effects on CNS-mediated behaviours within a short period of time (DHURIA *et al.*, 2010). Curiously, this route of administration has long been adopted by individuals who consume cocaine illicitly. In fact, it is well known that after sniffing, cocaine is rapidly absorbed from the nasal mucosa allowing the achievement of a state of euphoria within only 3-5 min (ILLUM, 2002). Apart from a quick nasal absorption of the narcotic to the systemic circulation, it was hypothesised that such rapid cocaine effects on CNS were probably due to the presence of a direct pathway from the nasal cavity to the brain. Chow and collaborators (1999) performed a study to determine whether cocaine could be directly transported from nose to brain by comparing drug concentration-time profiles in plasma and different brain regions, following both IN and IV administrations to rats. This work revealed that, unlike what it was found with IV route, cocaine concentration in samples collected within 60 min after IN administration, was considerably different among distinct brain regions, showing the highest level in the olfactory bulb. Moreover, it was also observed that brain-to-plasma ratios of cocaine in the olfactory bulb following IN administration were significantly higher than those in other brain areas up to 60 min post-dose. Inclusively, at 1 min after IN instillation, this parameter exhibited an order of magnitude about 3 times greater comparatively to the IV injection. Thus, based on these results, the authors concluded that a direct pathway from the nasal cavity to the brain was probably involved in the delivery of cocaine when it was administered nasally; and the transport of the referred abuse substance from nose to brain may have presumably been carried out via the olfactory system.

I. 2.3.1. PATHWAYS AND MECHANISMS OF TRANSPORT FROM NOSE TO BRAIN

While the precise mechanisms governing direct IN drug delivery to the CNS are still not completely understood, an accumulating body of evidence has demonstrated that pathways involving nerves connecting the nasal passages to the brain play an important role. In general, there are mainly three possible pathways along which a drug administered into the nasal cavity may travel and attain the CNS (Figure I.2.5). One of these routes includes the systemic pathway by which part of the drug is absorbed to the blood circulation and subsequently reaches the brain by crossing the BBB. The other two enclose direct transfer of the compounds from nose to the CSF or brain tissue via neuronal transport, which can be accomplished either through olfactory or trigeminal nerves (MITTAL *et al.*, 2014).

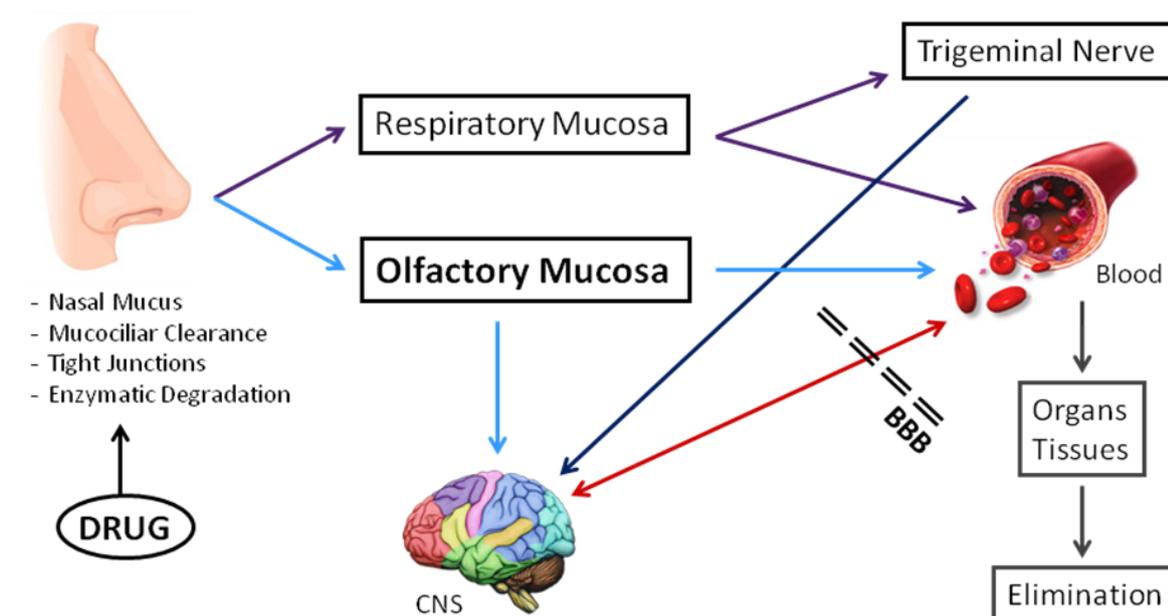


Figure I.2.5 Schematic representation of the possible pathways involved in the transport of drugs from nose to brain (Adapted from SERRALHEIRO *et al.*, 2013a).

When a drug formulation is nasally applied, it can be deposited in the respiratory region from where, after escaping enzymatic degradation and the normal rapid clearance by the MCC system, it may be absorbed to the bloodstream by successfully traversing the mucus and nasal epithelial layers. Once in the systemic circulation, therapeutic compounds can freely diffuse through the BBB and enter the CNS. Nevertheless, as mentioned hereinbefore, the systemic delivery of drugs to the brain is highly conditioned

by the permeability of the molecules across the BBB, which is subsequently dependent on their lipid solubility, molecular size and charge. Hence, the absorption of substrates to the systemic circulation after IN administration constitutes an indirect pathway for delivering nasally administered drugs to the brain, and therefore it does not afford any preferential advantage on CNS targeting (GRAFF and POLLACK, 2005; WONG and ZUO, 2010). Moreover, some limiting factors such as plasma protein binding, plasma protease degradation, systemic dilution and potential peripheral side-effects, also contribute to make it not ideal for nose-to-brain delivery (DHURIA *ET AL.*, 2010).

Taking into account that the olfactory region is a well-recognised direct portal of entry for drug substances into the brain, several investigations have been made in order to describe the mechanisms involved in the transport of molecules across the olfactory epithelium. In theory, therapeutic agents can be transported from the nasal cavity directly to CSF or brain parenchyma via two possible routes along the olfactory neurons: the *olfactory nerve pathway* (intracellular axonal transport) and the *olfactory epithelial pathway* (extracellular perineural transport) (LOCHHEAD and THORNE, 2012; MERKUS and VAN DEN BERG, 2007).

The *olfactory nerve pathway* has been proposed as a feasible route to transfer drugs directly to the brain via intracellular axonal transport along the olfactory sensory neurons. Consistent evidences have been described by several authors for the uptake of different substances into the olfactory neurons with subsequent distribution to the olfactory bulb and other brain areas by the anterograde axoplasmic flow. Axonal internalisation of numerous metal compounds, such as gold (DE LORENZO, 1970), nickel (HENRIKSSON *et al.*, 1997), mercury (HENRIKSSON and TJÄLVE, 1998), aluminium (PERL and GOOD, 1987), manganese and cadmium (TJÄLVE *et al.*, 1996) were reported to occur by passive diffusion or different mechanisms of endocytosis. A study carried out in rats to assess the possibility of transneuronal transport of wheat germ agglutinin-horseradish peroxidase, after IN application, revealed that such substance was intra-axonally delivered to the olfactory bulb following neuronal uptake through surface receptors binding and subsequent adsorptive endocytosis (BAKER and SPENCER, 1986; THORNE *et al.*, 1995). Notwithstanding, despite the ability of the intracellular axonal route to deliver agents to the olfactory bulb, distribution to other CNS regions beyond the olfactory system is unclear. Furthermore, it is believed that such transport is slow, taking hours or even days for drugs to reach the brain parenchymal tissue which, therefore, cannot account for the rapid delivery of some therapeutics to the CNS that exhibit considerably high concentrations in both CSF and brain almost immediately after or within an hour post IN dosing (CHAPMAN *et al.*, 2013; DHURIA *et al.*, 2010; DJUPESLAND *et al.*, 2014; THORNE and FREY II, 2001). Hence, this pathway is not likely to explain the observed sudden

appearance of certain drugs in the CNS like for example cocaine (CHOW *et al.*, 1999), CBZ (BARAKAT *et al.*, 2006), PB (CZAPP *et al.*, 2008), propiomazine (BJERRE *et al.*, 1996), hydroxyzine (CHOU and DONOVAN, 1997) and testosterone (BANKS *et al.*, 2009) after nasal administration. Accordingly, as an alternative to intracellular axonal uptake, it was suggested that drugs, after traversing the olfactory epithelium, either by transcellular or paracellular mechanisms, could make their way by entering into the *lamina propria* and then diffusing into the perineural channels, which appear to be the result of continuous subarachnoid extensions that surround the olfactory nerves from the base of the epithelium to the brain by crossing the cribriform plate (Figure I.2.6).

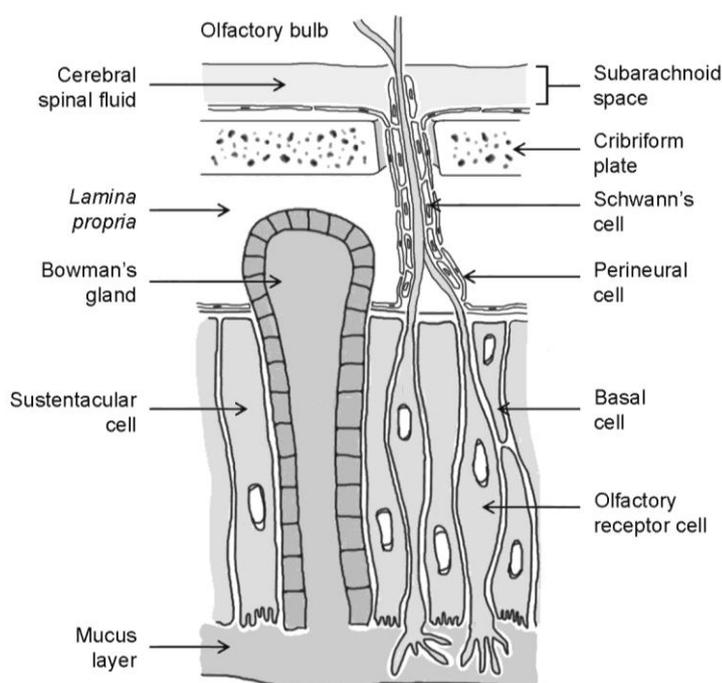


Figure I.2.6 Schematic illustration of the anatomical neuronal connection between the olfactory epithelium and the subarachnoid space of the brain.

This extracellular mechanism, also known as *olfactory epithelial pathway*, is considered a faster route of nose-to-brain transfer as compounds are able to paracellularly cross the perineural epithelium into the fluid-filled perineuronal space, requiring only few minutes (<30 min) for drugs to travel, most likely by a bulk flow transport phenomenon, along the olfactory axon up to the subarachnoid space filled with CSF (DHURIA *et al.*, 2010). Nevertheless, some controversial facts have been questioning

the viability of this pathway since distinct directions and rates are ascertained between the desired drug diffusion and the CSF flow, which creates a functional barrier against the free movement of molecules from the subarachnoid space to the brain tissue (GRAFF and POLLACK, 2005; ILLUM, 2004; PARDRIDGE, 2011). Furthermore, diffusion decreases exponentially with the distance (PARDRIDGE, 2011), and in humans, CSF is continuously renewed approximately every 5 hours, being totally replaced more than four times a day (ILLUM, 2000). Thus, after reaching CSF, drugs can be rapidly cleared by drainage into the lymphatic vessels and thereafter into the systemic circulation, hindering its penetration into the brain parenchyma. Upon these facts, despite the intimate contact of the CSF and the brain, one cannot assure that a thorough dynamic balance exists between both compartments, and therefore the drug concentrations will not be equivalent (GRAFF and POLLACK, 2005).

More recently, *trigeminal nerve pathway* has been suggested as another valid route for the transport of molecules directly from the nasal cavity to the brain (ROSS *et al.*, 2004). The trigeminal nerve is the largest cranial nerve with both sensory and motor functions, and its name derives from the fact that it has three major branches: the mandibular, the maxillary and the ophthalmic nerve divisions. The last two are particularly involved in nose-to-brain drug delivery, since their branches are directly connected to the nasal mucosa, in contrast with the mandibular division which extends to the lower jaw and teeth, away from the nasal cavity (DHURIA *et al.*, 2010; PARDESHI and BELGAMWAR, 2013). The maxillary ramification allows the innervation of the lateral walls of the nasal mucosa, whereas the ophthalmic branch innervates both dorsal segment of nasal mucosa and the anterior portion of the nose (DHURIA *et al.*, 2010). The three peripheral trigeminal branches meet at the trigeminal ganglion, yielding in a single incoming nerve that ends in the brainstem, at the level of the pons (MISTRY *et al.*, 2009).

The transport of nasally administered therapeutic agents via the *trigeminal nerve pathway* was firstly demonstrated by Thorne and collaborators (2004) for insulin-like growth factor I. Since then, similar mode of transport was also evidenced for other proteins and peptides, including interferon- β 1b (ROSS *et al.*, 2004; THORNE *et al.*, 2008) and hypocretin-1 (DHURIA *et al.*, 2009). Following IN instillation of the referred radiolabeled compounds to animal models, higher levels of radioactivity were observed in the trigeminal branches, trigeminal ganglion and pons, strongly indicating the involvement of the trigeminal nerve in the delivery of drugs from the nasal passages to the CNS. Even though no factual data have been reported on the ensheathing cells and channels associated with the trigeminal nerve comparable to those observed with the olfactory ones, there is a substantial conviction that these anatomical features may likewise be present along the trigeminal pathway (PARDESHI and BELGAMWAR, 2013). Interestingly,

unlike the *fila olfactoria*, trigeminal nerve extends from the respiratory epithelium and enters the brain at two different sites: (1) the anterior lacerated foramen near the pons and (2) the cribriform plate near the olfactory bulb, which allows for drug delivery to both caudal and rostral areas of the brain (CHAPMAN *et al.*, 2013; PARDESHI and BELGAMWAR, 2013).

Although several hypothetic mechanisms were described for the direct delivery of drugs from nasal cavity to the CNS, the contributions underlying each one have not been yet enlightened. Generally, the rapid appearance of a drug in the brain and CSF indicates preferential involvement of the extracellular transport pathway rather than the olfactory intraneural route. However, the possibility of occurring later axonal drug internalisation cannot be entirely ruled out. In fact, the vast majority of the studies performed in animal models was often conducted for limited periods of time (<240 min) (ILLUM, 2004), therefore preventing the opportunity to assess if, beyond the *olfactory epithelial pathway*, the molecules would also explore the axonal transport to travel within the neurons and reach the brain. Upon the above considerations, it is likely that drugs may use one or a combination of various pathways in the transport from nose to CNS; notwithstanding, so far, the extent of each mechanism still remains experimentally hard to define.

I. 2.3.2. NOSE-TO-BRAIN ASSESSMENT – EXPERIMENTAL CONSIDERATIONS

The neuronal connection between the nasal cavity and the brain has been extensively investigated on the hypothesis for CNS targeting of drugs. Albeit the numerous examples that support the success and potential of the IN administration to rapidly deliver a great diversity of neurotherapeutics into the brain, direct transport following IN dosing is not always consistently demonstrated. Beyond that, some contradictory results are occasionally encountered for the same tested molecule and using equal experimental models. For instance, while van den Berg *et al.* (2004a) concluded that IN estradiol held no advantage in drug targeting to the CSF over IV injection, other research group has shown that, after IN application, the same compound was transported into the CSF via olfactory neurons, confirming, for this particular case, the existence of a direct transport route from the nasal cavity to the CSF (WANG *et al.*, 2006). Opposing results concerning the involvement of a direct nose-to-brain pathway were likewise accounted for melatonin following IN administration to rats (BABU *et al.*, 2011; VAN DEN BERG *et al.*, 2004b). These contrasting conclusions for similar drugs may be due to differences in the methodologies employed which raise important issues in what concerns to experimental and formulation factors that can significantly influence the outcome of the studies and the interpretation of the obtained results. Therefore, the

distinct methodologies used to study drug transport via the nasal route to the CNS will be discussed below. Some relevant considerations will be given regarding the choice of the experimental models, the definition of the study design and the application of the assessment parameters.

Experimental Models

Many different experimental models are used in nasal drug delivery research which include *in vitro*, *in situ* and *in vivo* techniques (AGU and UGWOKE, 2008; KIM, 2008). Each model has its advantages and limitations that affect how the results obtained can be interpreted and more importantly, how they can be extrapolated to the human situation. In effect, it must not be forgotten that the ultimate goal of exploration the nose-to-brain pathway is to be able to apply this administration route to humans, promoting an efficient delivery of drugs to the CNS and, as a consequence, improving their therapeutic efficacy.

With the intent of formulating a successful novel nasal drug delivery system, testing with reliably *in vitro*, *in situ* and *in vivo* models is crucial, being their application dependent on the aim of the study and the phase of the drug discovery and development program (KIM, 2008). *In vitro* techniques present the ability to control a lot of variables, are cheap, use low amounts of drug and animals, and allow high throughput screening of a large number of compounds. These approaches comprise the use of nasal cell cultures or excised tissues from different animal species and are extremely helpful in evaluating the mechanistic aspects of nasal drug delivery, such as absorption, metabolism and toxicity of drugs at the level of nasal epithelium (UGWOKE *et al.*, 2001). Aware of the major importance that the determination of drugs' permeability across the nasal mucosal membrane has to address their systemic exposure, and thereby, their therapeutic potential, the usefulness of predictive nasal *in vitro* models at the early stages of research and development process is unquestionable, contributing to optimise the selection of drug candidates and formulations prior to further pre-clinical and clinical phases (AGU and UGWOKE, 2008). However, owing to the simplistic environmental conditions under which these models are frequently performed and their limitation with regard to the absence of several physiologic factors, like for example the MCC mechanism, led to the need of complementing the obtained experimental *in vitro* data with other information generated from *in situ* models. By perfusing a drug solution through the nasal cavity of an anaesthetised animal and monitoring the changes in drug concentration levels remaining in the perfusate at specified time intervals, it is possible to estimate drug absorption through nasal mucosa (HUSSAIN, 1998). Even though *in situ* techniques provide detailed information about nasal drug absorption kinetics, they also have some drawbacks related

to significant differences between experimental and real physiologic conditions. Among other factors, the mucus layer is gradually eroded by the circulating solution, exposing the epithelium and thus increasing the drug absorption rate. Additionally, comparing to the *in vivo* situation, the nasal drug residence time is much more prolonged which would give a false impression of improved absorption and/or increased toxicity of the drug (UGWOKE *et al.*, 2001).

Granted that the complete pharmacokinetics and pharmacodynamics of a drug can only be evaluated in whole animal models, *in vivo* approaches have been employed in more advanced stages of drug discovery and development programmes. Although they are more expensive and labour-intensive, also involving a bigger number of animals, *in vivo* techniques provide the most comprehensive pharmacological characterisation of the fate and activity of drug molecules within the body, which is not possible to achieve simply resorting to *in vitro* or *in situ* methods.

As with any biomedical area of research, the majority of the nasal delivery studies has been preferably accomplished in animal models, such as rats, mice, rabbits, sheep, dogs and monkeys (ILLUM, 1996; KOZLOVSKAYA *et al.*, 2014). Due to methodological and ethical limitations, much less studies have been conducted in humans and therefore, to date, very few investigations providing evidence for nose-to-brain drug delivery in man have been reported. Since sampling of human CSF and brain tissue specimens is difficult and risky, the indirect evaluation of pharmacodynamic effects [e.g. event-related brain potentials (KERN *et al.*, 1999; PIETROWSKY *et al.*, 1996), attention (SMOLNIK *et al.*, 1999) and working memory function (SMOLNIK *et al.*, 2000)] have been commonly described rather than absolute measurements of CNS drug concentrations (ILLUM, 2004). Specialised techniques like positron emission tomography (WALL *et al.*, 2005; YATES *et al.*, 2005), neurosurgical CSF drainage (MERKUS *et al.*, 2003), and intraspinal catheterisation (BORN *et al.*, 2002) have recently allowed CNS quantitative determinations after nasal administration of drugs in human volunteers, but convincing unequivocal proof of the nose-to-brain pathway via demonstration of mass transport is still lacking (DJUPESLAND *et al.*, 2014).

Taking into account the large amount of data obtained from nasal delivery studies carried out in laboratory animals, the key question is lifted on whether these experimental results can be applied or not to the human clinical situation (PADOWSKI and POLLACK, 2010). As a matter of fact, there are valid reasons to be sceptical of the potential for nose-to-brain transport in man based on evidence from animal research whereby, the *in vivo* model used to study the transfer of drugs from nasal mucosa to the brain must be properly selected in order to allow a feasible extrapolation of the results (DJUPESLAND *et al.*, 2014). Indeed, substantial differences exist between the nasal anatomy and

physiology of humans and animals, which should be carefully considered when the results are interpreted and compared, avoiding misleading conclusions (Table I.2.4).

Table I.2.4 Comparison of the anatomical and physiological characteristics of the nasal cavity and cerebrospinal fluid (CSF) among different animal species (GIZURARSON, 2012, 1990; GROSS *et al.*, 1982; ILLUM, 2004, 2000; KAUR and KIM, 2008; LOCHHEAD and THORNE, 2012; UGWOKE *et al.*, 2001).

Species	Nasal Cavity Characteristics					CSF Characteristics	
	Length (cm)	Volume (mL)	Surface Area (cm ²)	Olfactory Area (cm ²)	Clearance Half-life (min)	Volume (mL)	Turnover Rate (mL/h)
Human	7.5	20.0	160.0	10.0	15.0	160.0	21.0
Monkey	5.3	8.0	62.0	9.0	10.0	NF	2.5
Sheep	18.0	114.0	327.0	NF	42.0	14.2	7.1
Dog	10.0	20.0	221.0	150.0	20.0	NF	NF
Rabbit	5.2	6.0	61.0	6.0	10.0	2.3	0.6
Rat	2.3	0.4	14.0	7.0	5.0	0.15	0.18
Mouse	0.5	0.03	2.8	1.3	1.0	0.035	0.018

NF, not found.

In general, rodents are the most widely used animals for IN drug delivery experiments (ILLUM, 1996; KAUR and KIM, 2008; KOZLOVSKAYA *et al.*, 2014; MERKUS and VAN DEN BERG, 2007). As mentioned in *section I. 2.1.1.*, the amount of the nasal epithelium that corresponds to the olfactory mucosa is proportionally smaller in humans (3-8%) comparing to mice or rats (45-50%). In addition, the olfactory region in such animals covers a large part of the nasal mucosa, whereas in man the olfactory epithelium is restricted to a small area in the roof of the nasal cavity (HARKEMA *et al.*, 2012). Therefore, it is expected that the predisposition of rodents to olfactory deposition and transport of compounds to the brain via the olfactory route may be significantly more pronounced. On the other hand, the volume and renovation rate of the CSF are likewise distinct between the two species once the CSF turnover in rodents is about five to six times higher relatively to man (DJUPESLAND *et al.*, 2014). These differences may have considerable impact on nose-to-brain drug delivery, especially when the diffusion of compounds from the CSF into the brain tissue is accounted.

Bearing in mind the above considerations, conclusions regarding the bioavailability of a compound and the extent of its delivery to the brain in humans should not be straightforwardly drawn from the results solely obtained in animal models. In order to verify the actual feasibility of the IN route for CNS drug delivery, it is imperative to compare data derived from both animal and human research studies. A realistic comparison between humans and rats was performed using resembling experimental

setups (MERKUS *et al.*, 2003; VAN DEN BERG *et al.*, 2004b, 2003). By administering identical IN and IV drug formulations of melatonin and hydroxocobalamin and employing similar sampling times, analogous results were obtained for both evaluated species. Even though no evidence of direct transport of the compounds from the nasal cavity to the CSF has been found, the comparison of these three studies demonstrated that, in this particular case, rat experiments can be predictive for nose-to-CSF transport in humans, strengthening the basis for extrapolating the results from animals to man.

Study Design

It is important to consider the different methodologies used in IN studies since several experimental aspects such as delivery volume, administration technique, drug formulation and anaesthesia, can all influence the distribution and deposition of drugs in the nasal cavity and consequently, the pathways by which a drug may travel to the CNS after IN administration. As discussed in *section 1. 2.1.2. – “Nasal Permeability”*, following IN delivery, the deposition of the drug within the nasal chamber should be appropriately optimised for the intended therapeutic goal, whether it may be highly concentrated at the olfactory mucosa for a CNS effect or low deposited in that area when a systemic outcome is desired. Actually, the distance from the nostril to the olfactory epithelium is very short; however its location in the slit-like olfactory cleft behind the narrow nasal vestibule and at the end of a complex labyrinth of respiratory turbinates severely complicates the access (DJUPESLAND *et al.*, 2014). Expectably, deposition in adequate innervated regions of the nasal cavity can therefore be a critical component of the overall approach towards realising the full potential of nose-to-brain drug delivery (DJUPESLAND *et al.*, 2014).

Typically, the administration of drugs into the nose is most often performed by using either a pipettor for delivering drops to alternating nostrils, a piece of tubing attached to a microsyringe/micropipette for inserting within the nasal cavity, or a nasal spray device (DHURIA *et al.*, 2010; DJUPESLAND, 2013; MERKUS and VAN DEN BERG, 2007). Placing the nasal drops at the opening of the nostrils allows the animal to sniff them into the nasal cavity but the tendency to deposit on the nasal floor and be subjected to a rapid MCC is elevated (DHURIA *et al.*, 2010). Nasal sprays usually provide high reproducibility in terms of the emitted dose and a widespread distribution of the drug within the nasal cavity; nevertheless, the probability of deposition either in oesophagus and/or lungs is much more augmented (DHURIA *et al.*, 2010; DJUPESLAND, 2013). Conversely, the use of flexible tubing, by giving the possibility of being inserted inside the nose, enables a precise delivery of the drug formulation on a localised mucosal area, inasmuch the

deposition in the respiratory and olfactory epithelia essentially depends on the length of the tube (CHARLTON *et al.*, 2007a; DHURIA *et al.*, 2010).

The selection of the drug formulation is also a fundamental issue that should be gingerly taken into account. A big part of the compounds intranasally applied is in the form of simple solutions (KOZLOVSKAYA *et al.*, 2014). Nasal drops are the simplest and the most convenient pharmaceutical dosage form, but the precise dosing of drug delivered is somewhat difficult to control in addition to be also associated to a rapid nasal drainage (KUMAR *et al.*, 2014). When using liquid formulations, a special concern must be considered regarding the administration volume. Differences in delivery volumes are closely related to the amount of the nasal surface area covered by the formulation, affecting the deposition within the nasal cavity and the distribution to the CNS. Depending on the experimental model used, the administration technique applied and the therapeutic effect intended, the delivery nasal volume should be properly adjusted and defined. Hence, delivery volumes that do not overload the total capacity of the nasal cavity must be used, and should not exceed 5-10 μL /nostril in mice, 10-25 μL /nostril in rats and 75-100 μL /nostril in humans (MERKUS and VAN DEN BERG, 2007). Although small volumes may not be sufficient to reach and cover the desired area of the nasal mucosa, an excessive dosage volume may cause loss of the drug formulation by resulting in deposition at the nasopharynx with subsequent swallowing or drainage to the lower respiratory tract, which in the latter case, it may give rise to respiratory distress and eventually death (DHURIA *et al.*, 2010; SOUTHAM *et al.*, 2002). Aiming at preventing drainage of nasally dosed drug solution, some researchers have employed the nasal cavity isolation technique by applying an established surgical procedure in rodents (CHOW *et al.*, 1999; HUSSAIN *et al.*, 2000; ISHIKAWA *et al.*, 2002; RAGHAVAN and ABIMON, 2001; SINSWAT and TENGAMNUAY, 2003; WANG *et al.*, 2007; YOKOGAWA *et al.*, 2006). Briefly, after an incision has been made in the neck, trachea is cannulated to allow animal free breathing, the upper part of the oesophagus is tied off to restrain swallowing, and the nasopalatine duct is sealed to avoid any drug formulation from being cleared into the mouth. Since this method involves a closed and confined system, the data obtained is very reproducible and reliable providing more concrete information about the transport of the molecules from nose to brain (DEY *et al.*, 2011). Nonetheless, the use of such treatment would be unrealistic, even unthinkable in humans and therefore, one should exercise caution in the interpretation and extrapolation of these animal results to man (MERKUS and VAN DEN BERG, 2007).

Given the limitations described for liquid formulations, alternate semi-solid dosage forms, such as gels, have been developed for a more precise drug delivery through the nose. They reduce post-nasal drip and anterior leakage by maintaining the drug formulation in nasal mucosa. This enhances the drug residence time inside the nasal

cavity and diminishes MCC, thereby providing a better chance for the drug to be absorbed and transported to the CNS (BEHL *et al.*, 1998; PIRES *et al.*, 2009). Recently, *in situ* nasal gelling systems have emerged as a promising approach that benefit dual advantages from both sol and gel and present themselves for more accurate dosing (SINGH *et al.*, 2013; SWAMY and ABBAS, 2012). Displaying low viscosity properties at the time of administration, *in situ* gels are easily handled and precisely delivered. Following IN instillation, they should readily undergo phase transition to form a gel that is strong enough to reside within the nasal cavity for a prolonged period of time, thus preventing MCC and enhancing drug bioavailability (SINGH *et al.*, 2013).

Albeit most often depreciated, the influence of the anaesthesia on the rate and extent of drug transport from nose to brain is another matter of debate. In effect, the majority of the preclinical studies has been carried out in anaesthetised mice and rats in order to facilitate nasal drug administration, so it seems possible that both the category of anaesthetic and its delivery route could affect the drug pharmacokinetic profile (WONG and ZUO, 2013; WU *et al.*, 2008). Generally, compared with the conscious condition, the anaesthetic state may result in a more effective contact time of the formulation with the nasal mucosa, as well as higher absorption in the olfactory epithelium, which may further increase brain drug uptake (WU *et al.*, 2008). Even though few published records exist regarding the influence of different types of anaesthesia on IN brain drug delivery, some authors claim a probable decrease on nasal mucosa cilia clearance rate with the consequent reduction of mucociliary activity (KAUR and KIM, 2008; MAYOR and ILLUM, 1997). Ideally, to avoid any interference caused by the anaesthesia, the use of conscious animals is clearly preferred; however, rodents commonly do not tolerate IN delivery without being sedated and therefore, this experimental variable should also be appropriately considered.

The design of the experimental setup to investigate nose-to-brain drug delivery also involves the definition of the biological samples collected, the sampling times analysed, the analytical methodologies used to measure the concentration of drugs in different biocompartments and finally, the type of control or reference employed to make comparisons. Overall, IN studies should include frequent sampling of, at least, both plasma and brain tissue or CSF to enable calculation of the global drug exposure in those specimens. In practice, the brain tissue is sampled by dissection; the organ is usually used as a whole or divided in different anatomical regions after slicing. The analysis of whole brain concentrations by treating the tissue as an entire piece may disguise the contribution of direct neurological pathways to transport the drug from nose to brain; instead, measuring the levels of drug distribution in specific target areas of the brain may afford to more accurate determination of the routes by which the molecules have

travelled (WONG and ZUO, 2013). When measurement of drug concentrations in brain tissue is intended, one sample per animal per time point has to be taken which means that several animals are needed to delineate a complete pharmacokinetic profile, probably increasing the variability in the obtained data. On the contrary, similarly to blood, serial sampling of CSF is also feasible provided by the replacement of the withdrawn volume with artificial CSF via infusion into the lateral ventricle (CHOU and DONOVAN, 1997). Serial CSF sampling is usually performed in rats by microdialysis (BAGGER and BECHGAARD, 2004a, 2004b; CHOU and DONOVAN, 1998) or cisternal puncture (DAHLIN and BJÖRK, 2001; KAO *et al.*, 2000; ZHAO *et al.*, 2008) and maximal care should be taken to avoid contamination with blood. Mice are unsuitable for CSF sampling due to its extremely low total volume (approximately 35 μL), but the relatively small amount of CSF samples (<50 μL) that can be collected from rats generally imposes a major problem with respect to drug analysis, requiring highly sensitive analytical methods (such as mass spectrometry) for drug quantification (MERKUS and VAN DEN BERG, 2007). Nevertheless, the main advantage of this procedure is the possibility of obtaining a drug concentration-time profile in CSF from one single subject, which saves animals and reduces variability in data.

The importance of frequent and early sampling is highlighted by a study performed by Chow *et al.* (1999) in which cocaine concentrations in rat brain tissue were evaluated after IN and IV administrations. Interestingly, the area under the concentration-time curve (AUC) of cocaine in the olfactory bulb following 60 min post-dose was lower for IN comparatively to IV delivery; however, when calculated for times up to 4 min, the AUC values determined after IN drug administration exceeded those obtained after IV injection (CHOW *et al.*, 1999). Hence, this indication of direct nose-to-brain transfer could easily have been overlooked if samples had not been taken as soon as 1, 2 and 4 min. A particular attention should be posed when the sampling times are selected.

By tracking the analytical methodologies commonly utilised in the assessment of IN delivery it was found that the investigation of the potential for direct nose-to-brain transport was accomplished either in a qualitative or a quantitative way. Several imaging tools such as microscopy, radioactivity, fluorescence and gamma scintigraphy have been frequently employed. Although microscopic analysis of brain tissue provides a qualitative answer whether or not there is direct drug transfer, it does not give an indication about the proportion of the administered drug that reaches the brain from the nasal cavity. More sophisticated imaging techniques like autoradiographic and gamma scintigraphy analysis, can otherwise enable a direct visualisation and quantification of where the drug formulation has been delivered, what is doing, and whether or not it is behaving according to its proposed rationale (NEWMAN and WILDING, 1998). However, it should be noted that these imaging methods alone do not confirm the presence of intact, free or biologically active drug present in the CNS tissues, besides their inability to distinguish

between parent drug and active/inert metabolites (WONG and ZUO, 2013). Furthermore, attaching radioligands to the drug might also alter the physicochemical and pharmacological properties of the therapeutic agent, which may interfere with the pharmacokinetic and pharmacodynamic outcomes of the experiments (WONG and ZUO, 2013). In view of these limitations, more accurate and specific assays such as liquid chromatography coupled with different types of detection could be used, providing more complete and definitive descriptions of the pharmacokinetic profiles of the intranasally administered drug and the corresponding active metabolites formed *in vivo*. Currently, high performance liquid chromatography (HPLC)-based techniques are the most commonly applied analytical methods for drug separation and subsequent quantification in a wide variety of biological matrices (KOZLOVSKAYA *et al.*, 2014).

Taking into account that IN delivery is, in the majority of cases, considered as a new route of drug administration, the experimental protocol should be designed to allow the comparison of the obtained results with other alternative routes. In fact, some compounds, even when administered non-nasally, can be preferentially distributed to the olfactory mucosa and the olfactory bulbs (BERGSTRÖM *et al.*, 2002), highlighting the need for appropriate controls when designing studies in this field. In order to discriminate the exact transport pathway by which the drug is transferred from nose to brain (either via the BBB or the direct olfactory route), an adequate IV pharmacokinetic control has commonly been included in the investigations as an experimental comparator. IV delivery is definitively the most widely used alternative route to make comparisons with the IN administration (DHURIA *et al.*, 2010; MERKUS and VAN DEN BERG, 2007). Other parenteral delivery routes like for instance, intramuscular or intraperitoneal, and even oral administration, may also serve as comparators as long as the pharmacokinetic profiles are properly measured and matched (DJUPESLAND *et al.*, 2014).

Assessment Parameters

Investigating nose-to-brain drug targeting presupposes the assessment of the relative distribution of the drug by determining and comparing its concentrations attained on therapeutic sites of action (i.e. brain and/or CSF) with other non-targeted biocompartments such as blood and other peripheral tissues. Bearing in mind that the concentrations observed in the CNS after IN administration of drugs could be due not only to the amount of drug directly transported to the brain via neuronal routes, but also to the fraction that could be absorbed into the systemic circulation and reach the CNS by crossing the BBB and BCSFB, IV delivery controls are required to determine the contribution of this indirect vascular pathway on blood-mediated brain drug deposition (DHURIA *et al.*, 2010; MERKUS and VAN DEN BERG, 2007). Therefore, the measurement of drug

exposure (given by AUC) in both CNS (brain and/or CSF) and blood (plasma or serum) as well as the comparison of CNS-to-blood concentrations ratio after IN and IV administrations provide an evaluation of the route of drug transport to the brain (CHOW *et al.*, 1999; MERKUS and VAN DEN BERG, 2007; PADOWSKI and POLLACK, 2010) (Equation 1). Hence, when CNS-to-blood ratios following IN delivery are greater than that after IV administration indicate that direct transport pathways either via epithelial and/or nerve olfactory routes are primarily involved in the transfer of the compounds from the nasal cavity to the brain. Conversely, when IN ratio is equal or lower than the IV one, it is assumed that the concentrations observed in CNS derive mainly from the absorption into the nasal vasculature with further systemic distribution.

$$CNS \text{ to blood ratio} = \frac{AUC_{CNS}}{AUC_{blood}} \quad \text{Equation 1}$$

With the aim of surveying the extent of drug delivery to the brain, specific indexes have been suggested and broadly adopted by several researchers. In accordance, the *drug targeting efficiency* (DTE) quantifies the overall tendency of the drug to accumulate in the brain subsequently to IN vs. systemic administration (KOZLOVSKAYA *et al.*, 2014). It can be described as the ratio of the AUC_{CNS}/AUC_{blood} following IN administration compared to that after IV injection (WANG *et al.*, 2003) and it is calculated conforming to Equation 2.

$$DTE = \frac{(AUC_{CNS}/AUC_{blood})_{IN}}{(AUC_{CNS}/AUC_{blood})_{IV}} \quad \text{Equation 2}$$

The value of DTE can range from zero to $+\infty$, exhibiting a proportional relationship to the efficiency of drug targeting to the brain. Thus, it can be assured that there is direct transport of drugs from nose to the CNS whenever the DTE value is higher than 1, as it was demonstrated by Wang and co-workers (2003) in their experiments with IN administration of methotrexate to rats. Based on their results, the authors suggested that this antineoplastic agent could be directly delivered from the nasal cavity to the CSF, since the value of DTE achieved was 21.7, far greater than 1. By contrast, van den Berg *et al.* (2004b) found a DTE value of only 0.76, demonstrating that there was no additional transport of melatonin via the nose-CSF pathway in rats.

Another valuable used index is the nose-to-brain *direct transport percentage* (DTP), which can be derived from the DTE expression and estimates the relative amount of the drug that achieves the brain via direct neuronal routes (KOZLOVSKAYA *et al.*, 2014). DTP represents the percentage of drug directly transported via olfactory and/or trigeminal pathways to the brain, subtracting from the total AUC_{CNS} the fraction (represented by B_x) which corresponds to the amount of drug administered intranasally

that reaches the brain through the BBB by means of the systemic circulation (DHURIA *et al.*, 2010) (Equation 3). In fact, since the extent of drug in blood is proportional to the AUC, it can be assumed that the AUC_{CNS} fraction contributed by systemic circulation (B_x), divided by AUC_{blood} after nasal administration is equal to the same ratio following the IV route (JOGANI *et al.*, 2007) (Equation 4).

$$DTP = \frac{(AUC_{CNS})_{IN} - B_x}{(AUC_{CNS})_{IN}} \times 100 \quad \text{Equation 3}$$

$$B_x = \frac{(AUC_{CNS})_{IV}}{(AUC_{blood})_{IV}} \times (AUC_{blood})_{IN} \quad \text{Equation 4}$$

Overall, the DTP can range from $-\infty$ to 100% with the values below zero indicating more efficient drug delivery to the brain following IV injection as compared to the IN administration (KOZLOVSKAYA *et al.*, 2014).

The above described indexes are inter-dependent, so that, more efficient drug uptake to the brain via direct routes will lead to higher values of both DTE and DTP. The calculation of the referred parameters is based on several assumptions that should not be entirely ignored. Specifically, the drug pharmacokinetics is assumed to be linear and the analysed AUC values are pretended to reflect the pharmacologically relevant drug concentrations in the brain and blood, despite the fact that the compound can exist in several forms at these biocompartments (e.g. free, protein bound or encapsulated) and different brain drug disposition patterns can result from distinct transport pathways from nose to brain (KOZLOVSKAYA *et al.*, 2014). Notwithstanding, it seems that these measures are helpful in comparing findings across various studies conducted in different labs, also being useful for assessing the effects of diverse formulations on enhancing IN delivery of drugs to the CNS (DHURIA *et al.*, 2010).

The determination of brain distribution profiles after IN and IV drug administration also contributes to elucidate whether direct nose-to-brain transport pathways are involved. The assessment of drug distribution is usually performed in animal models and consists on analysing the drug exposure in different regions of the brain. Following drug administration, animals are sacrificed and the whole brain is removed and sectioned in sequential slices from rostral to caudal direction. Depending on drug physicochemical properties and route of administration, distinct brain distribution patterns are observed. In general, nasal delivery is characterised by preferential exposure in the rostral portions of the brain (i.e. olfactory bulb and frontal cortex), whereas systemic administration results in a more homogenous distribution of the substrate throughout the CNS structures (GRAFF *et al.*, 2005; WONG and ZUO, 2013). Interestingly, higher drug concentrations can also be encountered in more distant and specific caudal brain areas.

The quantitative analysis of lidocaine levels in multiple brain regions upon IN administration to rats demonstrated that brain tissue drug content decreased in a rostral-to-caudal direction from the cortex to the cerebellum; however, a considerable augment was observed at the level of the olfactory bulb and brainstem, suggesting a direct delivery of lidocaine to the CNS along the olfactory and trigeminal nerve pathways (Johnson *et al.*, 2010). Similar heterogeneous brain distribution patterns were likewise observed by quantitative autoradiographic analysis after IN perfusion of the vasoactive intestinal peptide (VIP) to rats (DUFES *et al.*, 2003). Unlike the uniform brain distribution profile of VIP when intravenously administered, an irregular deposition of the peptide in specific areas of the brain was observed following IN delivery. Higher drug concentrations were attained in sections corresponding to the cerebellum, midbrain, olfactory bulb and frontal cortex. While elevated VIP levels in the olfactory bulb were due to effective delivery of the peptide via the olfactory pathway, accumulation of the drug in distant brain areas remains to be clarified. Apparently, preferential localisations of the peptide corresponded to the brain structures particularly rich in VIP receptors. Authors hypothesised that intranasally administered VIP could perhaps be redistributed from the nasal absorption site into the brain blood microcirculation thus reaching these specific regions of the brain. On the contrary, Kaur and Kim (2008) concluded that there was no evidence of direct nose-to-brain transport of diazepam since apart from the lower DTE values, homogenous distribution patterns were observed throughout the various regions of rat and rabbit brain after both IN and IV administrations. The high lipophilicity value is probably the cause for this rapid and extensive absorption of the substrate into the nasal vasculature.

I. 2.4. INTRANASAL ADMINISTRATION AND EPILEPSY

Whether manifested in the form of an acute isolated seizure, repetitive or recurrent seizures and *status epilepticus*, all of them are deemed as medical emergencies. Clearly, since mortality and neurological morbidity are directly associated with the duration of seizure activity, patients experiencing epileptic seizures have a medical emergency and require immediate medical care (WERMELING, 2009). A rapid medication is essential to prevent the spread of the electrical discharge and subsequent brain damage, but while epilepsy requires a long-term prophylactic treatment, *status epilepticus* demands a prompt and quick management of seizures. In this context, several consensus statements have been published describing pharmacologic treatment protocols for a diversity of epileptic disorders. Oral administration of anticonvulsant drugs has generally been associated with high systemic distribution into non-targeted tissues, peripheral adverse effects and limited brain uptake. Moreover, patient's physical condition immediately after a convulsive episode is incompatible with the oral ingestion of a tablet

dosage form. Although IV administration is probably the most rapid way to suppress epileptic convulsions, an alternative route of drug delivery is needed when IV injection is impractical and inconvenient since it depends upon sterile equipment and skilled personnel which are only available in hospital setting (Li *et al.*, 2000). In this respect, the IN route of administration potentially offers a simple and non-invasive method for a rapid, selective and efficient delivery of anticonvulsant drugs to the brain. Apart from its advantages on the clinical emergencies in acute seizure situations, nasally administered AEDs may represent a valuable approach for the long-term treatment of epilepsy by allowing the decrease of the dose, frequency of dosing and related side-effects, improving therapeutic efficacy and tolerability. Inclusively, it is hypothesised that the possible direct nose-to-brain delivery might provide a valuable alternative means for reversing AED resistance. By circumventing the BBB and reinforcing the delivery of substrates to the brain, IN anticonvulsive treatment may yield a prospective management of intractable epilepsy (TAN *et al.*, 2008).

IV benzodiazepines, such as diazepam, clonazepam, lorazepam and midazolam have been widely used as the first-line therapy for acute seizure activity termination (MANNO, 2011; MEIERKORD *et al.*, 2010). They act by enhancing the effectiveness of the GABA inhibitory neurotransmitter within the CNS. However, IV dosing generally leads to excessive systemic drug exposure which, in case of benzodiazepines, may unleash hypotension, cardiac dysrhythmia and respiratory failure (Li *et al.*, 2000). Aiming to minimise the disadvantages and potentiate the therapeutic index of such drugs, numerous studies have been carried out on the subject of IN benzodiazepine delivery. Specifically, IN midazolam has been extensively investigated in epileptic patients and, currently, it is already recommended in some consensus guidelines as a viable alternative drug delivery technique for prompt abortive treatment of convulsive seizures (MAZURKIEWICZ-BEŁDZIŃSKA *et al.*, 2014; SHORVON *et al.*, 2008; WOLFE and MACFARLANE, 2006). Several preclinical studies involving various IN preparations of other benzodiazepines like for example, clonazepam (ABDEL-BAR *et al.*, 2013; VYAS *et al.*, 2006a), clobazam (FLORENCE *ET AL.*, 2011), diazepam (KAUR AND KIM, 2008) and lorazepam (YAO *et al.*, 2009; ZHAO *et al.*, 2012) have been broadly reported in literature presenting very promising results. Likewise, the potential of the IN route for the administration of distinct antiepileptic agents such as CBZ (BARAKAT *et al.*, 2006; PATEL, R.B. *et al.*, 2014), PB (CZAPP *et al.*, 2008), valproic acid (ESKANDARI *et al.*, 2011) and even leptin (XU *et al.*, 2008) has also been tested in different animal models exhibiting similar encouraging outcome.

During the last years, scientific community has been striving to improve the care of epileptic patients by developing and optimising novel and more efficient AED delivery approaches to reduce the time of treatment and cessation of seizures. Up to now,

increasing evidence from both experimental and clinical research has demonstrated the enormous potential that the IN route has to offer in the field of pharmacological therapy of epilepsy and epileptic disorders. Moreover, the nose-to-brain transport theory is gradually gaining support. Despite the extraordinary progress that has been made since the introduction of the IN administration to directly deliver therapeutics into the brain, much more remains to be explored in this area.



I. 3. AIMS

I. 3. AIMS OF THIS THESIS

The pharmacological therapy has been, and is likely to remain, the mainstay of treatment for the majority of epileptic patients. Regardless of the large number of currently available AEDs, and albeit their broad spectrum of efficacy, these drugs have revealed to be relatively insufficient to successfully attain the most basic assumptions of epilepsy treatment, which are to provide a complete seizure control while maintaining acceptable tolerability and safety profiles. Since the process of discovery and development of new and more efficient AEDs is complex, laborious and expensive, it seems that the exploitation of alternative strategies of drug delivery in order to improve both the efficacy and toxicity of the existing pharmacological armamentarium could be a very attractive approach. In this context, the main objective of the present work was to evaluate the potential of the IN route for the administration of four AEDs, selected among the various anticonvulsant agents that comprise the current therapeutic options of the first- and second-drug generations: CBZ, PHT, OXC and LTG.

In essence, all the AEDs herein chosen are widely used in the clinic as first- or second-line options for the management of a variety of different seizure types and epileptic syndromes. Notwithstanding their high anticonvulsant activity, both CBZ and PHT exhibit narrow therapeutic index, pronounced pharmacokinetic variability and large propensity to cause relevant adverse effects and drug-drug interactions, hindering their application in polytherapy regimens. Although neither OXC nor LTG offer significant advantages in terms of efficacy compared to CBZ or PHT, some benefits were ascertained in what concerns to tolerability and safety. Therefore it seems possible that, for any of the considered AEDs, favourable pharmacokinetic profiles following IN administration could potentially result in an improved therapeutic outcome.

A comparative study of the pharmacokinetics as well as the brain biodistribution pattern of CBZ, PHT, OXC, and LTG after IN and IV administrations in mice may allow to infer about the feasibility of the IN route to deliver the referred compounds and to investigate the involvement of an hypothetical direct transport pathway of the drugs from the nose to the brain. To make these goals achievable, appropriate analytical methods must be conveniently developed and validated in different sample matrices in order to obtain accurate and reliable quantitative data.

The specific aims outlined for the implementation of this work were as follows:

- Development and validation of an analytical high performance liquid chromatographic method with ultraviolet detection (HPLC-UV) to simultaneously quantify six AEDs [PRM, PB, CBZ, PHT, OXC and LTG] and three of their main metabolites [carbamazepine-10,11-epoxide (CBZ-E), 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine (*trans*-diol) and licarbazepine (Lic)] in human plasma.
- Laboratorial implementation of two analytical HPLC-UV methods to quantify CBZ, CBZ-E, OXC, Lic, PHT and LTG in plasma, brain (whole brain, olfactory bulb and frontal cortex) and liver tissue homogenates of mice.
- Pharmacokinetic characterisation of CBZ, PHT, OXC and LTG following single and individual IN and IV administrations to mice.
- Assessment and comparison of the brain biodistribution patterns following IN and IV administrations of CBZ, PHT, OXC and LTG to mice.



CHAPTER II

QUANTITATIVE DRUG ANALYSIS

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II. 1. BIOANALYSIS

General Concepts

II. 1.1. INTRODUCTION

Bioanalysis includes the set of analytical techniques and procedures applied in the characterisation and quantitative determination of drugs and their metabolites in various biological matrices such as blood, plasma, serum, urine, other fluids (e.g. saliva, CSF) or tissues. By generating reproducible and reliable data, bioanalytical methods are the key determinants in the acquisition, evaluation and interpretation of the results derived from non-clinical, clinical and biopharmaceutical studies, playing a critical role not only during the process of drug discovery and development but also in implementing and monitoring of therapy after drug approval (NOVÁKOVÁ and VLČKOVÁ, 2009; SHAH *et al.*, 2000; WAL *et al.*, 2010). In effect, the quality of these studies, which are often used to support regulatory filings or diagnosis, establish appropriate dosage schemes and monitor therapeutic efficacy or safety, is directly related to the reliability of the underlying bioanalytical outcome (NOVÁKOVÁ and VLČKOVÁ, 2009; VISWANATHAN *et al.*, 2007). Thus, the employment of well-characterised and fully validated analytical methods is essential to ensure that any decision based on the obtained bioanalytical results can be taken with trust and confidence (CHANDRAN and SINGH, 2007; SHAH *et al.*, 2000).

Aiming at implementing a bioanalytical method for routine use, it must be first conveniently developed, optimised and validated. Each analytical method has its own characteristics, which in turn vary from analyte to analyte, and the appropriateness of the technique may also be influenced by the ultimate goal of the study (SHAH *et al.*, 2000). The development phase is an inherently laborious and complex process that, depending on its specific requirements, the stage of drug discovery and development process and the analyst's experience, can be conducted in multiple ways (ALVES *et al.*, 2010; FORTUNA *et al.*, 2014b). Whereas at one extreme, it may simply involve the adaptation of an existing method by making minor changes so that it is suitable for a new application, at the other extreme, the analyst may start out with a few sketchy ideas and apply expertise to develop a proper method (EURACHEM, 2014). During the optimisation stage, the initial sets of conditions that were evolved along the method development proceedings are improved and maximised in terms of the overall ability to quantify the specific analytes of interest (DEVANSHU *et al.*, 2010).

After developing and optimising analytical conditions, the method must be properly validated in order to guarantee that it provides accurate, precise and reproducible data throughout study-sample analysis. Bioanalytical method validation is the systematic process of defining the analytical requirements and confirming that the method under consideration has performance capabilities consistent with the demands of its intended application (CHANDRAN and SINGH, 2007). Therefore, it includes all the procedures required to demonstrate that a particular analytical method developed and

employed for the quantitative determination of the analytes in a given biological matrix is suitable and reliable (SHAH *et al.*, 2000). In essence, each methodological step should be investigated to determine the extent to which environment, matrix or procedural variables can affect the estimation of the analyte from the time of sample collection up to the analysis and interpretation of the obtained results. The success of validation is a reflection of a well performed bioanalytical method development and optimisation. In fact, the development and validation of any bioanalytical method is an iterative process in which there can be no demarcation between these two phases (CHANDRAN and SINGH, 2007). By carrying out preliminary validation studies during the phase of method development it is possible to ascertain whether the established analytical conditions are satisfactory or require additional alterations to successfully meet the standards of the acceptance criteria.

This chapter sets up the beginning of the experimental work underlying the present thesis. With the intent of evaluating the potential of CBZ, PHT, OXC and LTG to be administered by the IN route to mice, the availability of appropriate and reliable analytical tools that support the execution of comprehensive pharmacokinetic studies was fundamental to enable the quantification of the drugs and their respective main metabolites in different biological matrices. Accordingly, a bioanalytical method that allows the simultaneous quantification of all the compounds of interest was initially developed and validated in human plasma, which can be potentially applied in the clinic for the therapeutic monitoring of the referred AEDs. Since the human plasma is a relatively complex matrix, easily accessible, available in large quantities and associated with few ethical constraints, its characteristics make it ideal for the optimisation of the various analytical conditions that will form the basis for the subsequent development and validation of the bioanalytical methods used for the quantitative drug determination in animal matrices. Simple, rapid, sensitive, accurate and reproducible HPLC techniques were developed in order to measure the concentration of the selected drugs and some of their metabolites in plasma, brain and liver tissues of mice, allowing the analysis of the samples derived from the *in vivo* pharmacokinetic experiments.

Prior to a further detailed description of the techniques herein employed, a brief theoretical approach will be provided concerning the main concepts inherent to the general process of development and validation of a chromatographic method for the quantification of small drugs and/or metabolites in biological matrices.

II. 1.2. BIOANALYTICAL METHOD DEVELOPMENT

A given compound can often be measured by several methods. Nowadays, HPLC remains the methodology of choice as it is able to separate and quantify quite complicated mixtures of compounds with low and high molecular weight, as well as different polarities and acid-base properties in a variety of matrices (NOVÁKOVÁ and VLČKOVÁ, 2009).

The development of a bioanalytical method is unique and specific for each drug candidate and it depends, among various factors, on the analyte physicochemical features (e.g. molecular weight, pK_a and lipophilicity), nature of the matrix, sensitivity required and the available instrumentation. Generally, it involves considerable trial and error procedures to test and evaluate as much variables as possible in order to establish the most suitable analytical conditions for the intended application. In essence, HPLC method development comprises the consideration of all the parameters pertaining to both the chromatographic conditions and sample preparation.

Chromatographic Conditions

Chromatographic analysis pursues the separation of all the compounds of interest in a sample with good resolution, without interferences at the retention times of the analytes and within a reasonable running time. Sharp, symmetrical and well resolved peaks are crucial in quantitative analysis to attain low quantification limits, low relative standard deviation between injections and reproducible retention times (WAL *et al.*, 2010). In general, the HPLC separation of the analytes is accomplished based on the differences in affinity of the compounds between two phases: one mobile and the other stationary. Since the majority of the drugs and metabolites are polar in nature, the reversed-phase chromatography is the most commonly applied method in which the mobile phase is polar, composed of a mixture of water/buffer and organic solvents (acetonitrile or methanol); and the stationary phase is non-polar, consisting of hydrocarbon chains chemically attached to the silica base packing of the column (e.g. C_{18} and C_8). Hence, while polar compounds get eluted firstly, the non-polar ones are retained in the column for a longer period of time and therefore, the separation of the analytes is sorted according to their increasing molecular hydrophobicity.

There are many factors that should be taken into account during the process of defining the most appropriate chromatographic conditions for a specific bioanalytical method. Depending on the number and polarity of the compounds to be resolved or separated, the mode of elution can be stipulated as isocratic or gradient. In opposition to the gradient mode, isocratic elution is characterised by keeping the mobile phase

composition constant over time, being simpler and more reproducible (KUPIEC, 2004). The main criterion in selection and optimisation of the mobile phase composition is to achieve appropriate separation of all the analytes of interest from each other, as well as from matrix endogenous substances or impurities (DEVANSHU *et al.*, 2010). In most cases, changing the polarity or pH of the mobile phase will alter the elution of the compounds and, as a consequence, the resolution and the efficiency of separation can also be affected. Buffers are needed when an analyte is ionic or ionisable under reversed-phase conditions (pH values within the range 2-8) (WAL *et al.*, 2010). In fact, pH changes as little as 0.1 pH units might have a significant impact on peak symmetry and separation, affecting the reproducibility of the results (WAL *et al.*, 2010). The flow rate and temperature of analysis may likewise have quite significant effects on the analytes' resolution and retention time, whereby these are parameters that should not be neglected during the chromatographic method development.

The choice of the most convenient system for the detection of the eluting components essentially depends on the analytes' properties. Different types of detectors, such as ultraviolet (UV), fluorescence and mass spectrometry, are available to be used in HPLC analysis. Even though, in general terms, either fluorescence or mass spectrometry provide better sensitivity and selectivity than UV detection, the latter is cheaper, more easily accessible and presents a broad spectrum of application; for these reasons, it still being one of the most frequently employed detection techniques. The wavelength of the UV incident light must be selected in accordance with the absorption characteristics of the analyte. Theoretically, it should correspond to the value at which the maximum absorbance occurs aiming to enhance sensitivity. Nonetheless, care should be taken to minimise the absorption of potential interfering endogenous substances of the matrix, providing a more specific detection of the compounds. UV detection often requires the search for an optimal compromise between specificity/selectivity and sensitivity.

Sample Preparation

Samples obtained from biological materials are usually not directly compatible with HPLC analyses due to their complexity and protein content (NOVÁKOVÁ and VLČKOVÁ, 2009). As a matter of fact, the overall biological matrices present a variable biochemical nature comprised of numerous components like salts, acids, bases, proteins, cells and lipids that can be problematic if not properly removed, potentially leading not only to an undesirable interference with the analysis, but also to clogging and deterioration of the stationary phase with the consequent reduction of the chromatographic efficiency (KOLE *et al.*, 2011; NOVÁKOVÁ and VLČKOVÁ, 2009).

Sample preparation is one of the most important stages of the bioanalytical method development since it includes all the procedures involved in the selective isolation and/or pre-concentration of the target compounds from various matrices, making them more suitable for separation, identification, detection and quantification in chromatographic systems (ASHRI and ABDEL-REHIM, 2011; NOVÁKOVÁ and VLČKOVÁ, 2009). It is the most labour-intensive and error-prone process in the overall bioanalytical methodology, accounting for up to 80% of the total analysis time (KOLE *et al.*, 2011). Different strategies can be applied to clean up a sample before chromatographic analysis, ranging from a simple dilution to more complex sample pre-treatment procedures, such as protein precipitation (PP), liquid-liquid extraction (LLE) and solid-phase extraction (SPE). Recently, several innovative sample preparation techniques have been developed which include, among others, liquid-liquid micro-extraction, salting-out liquid-liquid extraction, solid-phase micro-extraction, restricted access material, stir bar sorptive extraction and micro-extraction by packed sorbent (KOLE *et al.*, 2011; NOVÁKOVÁ and VLČKOVÁ, 2009). The selection of the sample preparation technique relies essentially on the chemical properties of the analyte, the nature of the biological matrix and the detection mode employed (ASHRI and ABDEL-REHIM, 2011).

PP is the simplest and the least time-consuming sample pre-treatment technique being the method of choice when fast and high throughput analysis of plasma or serum specimens is desired (HENDRIKS, 2009; NOVÁKOVÁ and VLČKOVÁ, 2009). It consists in denaturing the proteins present in the matrix by submitting the samples to an external stress like the addition of an appropriate miscible organic solvent (e.g. acetonitrile, methanol), strong acid (e.g. perchloric acid, trichloroacetic acid) or some salts of heavy metals (e.g. zinc sulphate) (ASHRI and ABDEL-REHIM, 2011; KOLE *et al.*, 2011). As denaturation leads to the active change of protein structure, the drugs or analytes formerly bound to proteins become freely soluble in the medium. After centrifugation, a pellet of denatured proteins is formed while a clear supernatant containing the target compounds is separated and used for chromatographic analysis, which can be performed either directly or after dilution/concentration (HENDRIKS, 2009). Since PP is one of the most crude and non-selective sample preparation techniques, analyte concentrations should be high enough to achieve a signal that dominates the outcome of the endogenous material for accurate determination, particularly if a concentration step is not performed (HENDRIKS, 2009). Precipitation efficiency and analyte recovery are remarkably influenced by the type and amount of the precipitating agent used. Indeed, strong acids are considered the most potent precipitating agents and among organic solvents, the acetonitrile appears to be more effective in removing sample proteins comparatively to methanol (WAL *et al.*, 2010). Regardless of the precipitating agent employed, the risk of analyte co-precipitation should always be considered. Complete deproteinisation is commonly achieved in a

volume acid:sample ratio of about (0.2-0.5):1.0 (v/v) whereas in case of organic solvents, the required proportion of solvent to sample volumes frequently ranges within (1.0-4.0):1.0 (v/v) (ASHRI and ABDEL-REHIM, 2011). For some analytes, PP may be the first pre-treatment step which is frequently followed by purification and concentration by LLE or SPE in order to achieve higher sensitivity (ASHRI and ABDEL-REHIM, 2011).

LLE is one of the most commonly used sample preparation techniques in bioanalysis, affording clear samples with satisfactory recovery of the target compounds. Separation of the analytes from interferences is based on the principle of their differential solubility and partitioning equilibrium between two immiscible liquid phases: one aqueous (the sample to be extracted) and the other organic (ASHRI and ABDEL-REHIM, 2011; DEVANSHU *et al.*, 2010). While hydrophilic compounds are retained in the polar aqueous phase, the more hydrophobic ones are transferred to the organic component, being easily recovered by evaporation of the extract and reconstitution of the residue with a small volume of mobile phase. The efficiency of the extraction is highly influenced by the polarity of the organic solvent and the pH of the sample (ASHRI and ABDEL-REHIM, 2011; HENDRIKS, 2009). This technique is simple, rapid and has a relatively small cost factor per sample when compared to SPE. Nevertheless, several shortcomings have been associated to LLE such as the consumption of large amounts of toxic organic solvents, emulsion formation, variable recovery, unsuitability for hydrophilic compounds and difficulty in extracting a wide variety of analytes with different lipophilicities (KOLE *et al.*, 2011; NOVÁKOVÁ and VLČKOVÁ, 2009).

The precept of SPE is similar to the affinity-based separation mechanism of liquid chromatography in which the analytes to be extracted are partitioned between a solid phase (sorbent) and a liquid phase (sample) (KOLE *et al.*, 2011; WAL *et al.*, 2010). In general, SPE technique involves a set of procedures comprising the conditioning of the sorbent to allow efficient solvent compatibility with the incoming sample matrix; sample loading to ensure the retention of the analytes in the sorbent; washing to eliminate impurities; and finally, elution to extract the desired analytes (DEVANSHU *et al.*, 2010). There is a wide variety of currently available SPE sorbents whereby the retention of the compounds can be established either through non-polar, polar or ionic interactions. The choice of the ideal sorbent strongly depends on the physicochemical properties of the target analytes and matrix components, thus having a significant impact on the selectivity and capacity of the extraction (NOVÁKOVÁ and VLČKOVÁ, 2009). However, other parameters such as the composition and volume of the washing and elution solutions also play a determinant role for the extractive efficiency. Nowadays, SPE is unequivocally considered the leading sample preparation methodology used in routine bioanalytical laboratories since it provides an efficient isolation and extraction of the analytes with the highest recovery values. In spite of all these advantages, SPE is time-consuming and relatively expensive, as

the SPE cartridges are manufactured for a single use only. Moreover, it sometimes has been associated with poor batch-to-batch reproducibility and still requires a significant amount of harmful organic solvents (NOVÁKOVÁ and VLČKOVÁ, 2009).

As stated above, sample preparation is the most critical and error-prone process in the overall bioanalytical methodology. Depending on the complexity of the extraction technique employed, it may be necessary to use an internal standard (IS) to compensate for any loss of the analytes during sample preparation, thereby minimising the error and improving both the accuracy and precision of the analysis (ALVES *et al.*, 2010; DADGAR *et al.*, 1995). Hence, a known and constant amount of IS must be added to the sample at the earliest stage prior to the initiation of the pre-treatment procedures. The choice of the appropriate IS is guided by its chromatographic behaviour, which should be similar to that of the interest compounds, extraction recovery value, and potential interference with analytes or endogenous substances of the biological matrix. Ideally, it must display similar physicochemical properties to the analytes, be stable and unreactive with any of the sample components. More importantly, it must not be present in the original sample (ALVES *et al.*, 2010; KUPIEC, 2004).

II. 1.3. BIOANALYTICAL METHOD VALIDATION

Before a bioanalytical method can be employed for routine use, it is widely recognised that it must first be validated to demonstrate and document, through the implementation of specific laboratory investigations, that the performance characteristics of a particular method used for quantitative determination of analytes in a given biological matrix are suitable, reliable and reproducible for the intended analytical applications (PANDEY *et al.*, 2010; US FOOD AND DRUG ADMINISTRATION, 2001). In essence, it is crucial to assure that the analytical methods implemented generate accurate, precise and consistent data in order to permit valid interpretation of the studies they support.

Owing to the undeniable importance of method validation in whole field of bioanalysis, it was felt the need to standardise the overall validation parameters and acceptance criteria by globally establishing appropriate guiding principles. In this context, since 1990, a number of conferences have been held concerning this issue, resulting in the publication of relevant reports (SHAH *et al.*, 2000, 1992) which, in turn, served as a base template for the definition of the current United States Food and Drug Administration (US FDA) general recommendations on bioanalytical method validation (US FOOD AND DRUG ADMINISTRATION, 2001). Originally issued in 2001, the US FDA guidance has been revised to reflect the advances in science and technology and recently (in September 2013), an updated draft (US FOOD AND DRUG ADMINISTRATION, 2013) of this

document has been made available for public review and comment before its implementation. Although US FDA guidance is considered one of the most influential guiding documents, other international guidelines have also come into force from various regulatory entities, such as EMA, to facilitate the regulatory submissions in their countries. The EMA guideline for validation of bioanalytical methods applied in the quantitative analysis of samples from animal and human studies was released in July of 2011 (EUROPEAN MEDICINES AGENCY, 2011).

The validation of the analytical methodologies employed during the course of the present thesis was performed in accordance to the general recommendations described in both US FDA and EMA guidelines, which will be briefly discussed.

II. 1.3.1. TYPES OF METHOD VALIDATION

As aforementioned, method validation is the process through which the reliability of a method to quantify a specific analyte in a certain matrix is demonstrated. There are three different levels/types of validation whose application depends on the circumstances in which the method was developed and how it is going to be used: full validation, partial validation and cross validation (CHANDRAN and SINGH, 2007; EURACHEM, 2014; GONZÁLEZ *et al.*, 2014).

When a bioanalytical method is developed and implemented for the first time or a new drug/metabolite is included, a *full validation* is mandatory in which all the fundamental parameters required to ensure the acceptability of the performance and the reliability of the analytical results must be evaluated. In situations where minor alterations are made to a previously validated method, such as transfer between laboratories or analysts, changes in equipment, in species within matrix (e.g. rat plasma to mouse plasma), in matrix within species (e.g. mice plasma to mice brain homogenate), in sample processing procedures, in storage conditions, etc., a full validation may not be necessary and therefore, a *partial validation* can be performed ranging from as little as one intra-assay accuracy or precision determination to an almost full validation (GONZÁLEZ *et al.*, 2014; TIWARI and TIWARI, 2010). Finally, a *cross validation* consists in a comparison of the validation parameters when two or more bioanalytical methods are used to generate data within the same or across different studies conducted in distinct laboratories. In fact, differences in sample preparation or the application of another analytical method may result in variable outcomes among separate analytical sites (EUROPEAN MEDICINES AGENCY, 2011).

II. 1.3.2. VALIDATION PARAMETERS

A full method validation demands rather high workload and, for that reason, should only start when promising results are obtained for the explorative validation performed during the early method development phase, i.e. when the preliminary experiments indicate that the required quality will be achieved (HARTMANN *et al.*, 1998). There is a general agreement that at least the following validation parameters should be evaluated for quantitative procedures: selectivity, calibration curve and linearity, precision, accuracy, limit of quantification and limit of detection, recovery and stability. Each parameter will be described below in detail. At the end, a summary of the main validation procedures and requirements as well as the respective acceptance criteria according to the US FDA and EMA recommendations will be provided in the Table II.1.1.

Selectivity

Selectivity is defined as the ability of an analytical method to unambiguously quantify and differentiate the analyte(s) of interest from other interfering components that may be expected to be present in the sample, typically, endogenous biological matrix substances, metabolites, degradation products or co-administered drugs (BANSAL and DE STEFANO, 2007; TIWARI and TIWARI, 2010). The general procedure to assess selectivity consists in the comparison of the response of the analytes in biological sample at the lower limit of quantification level (LLOQ) with that of blank matrix specimens, which should not produce any significant interference at the retention time of the target compounds and IS (BANSAL and DE STEFANO, 2007; KOLLIPARA *et al.*, 2011).

Even though US FDA guidance does not provide specific recommendations for selectivity, EMA guidelines states that the absence of interfering components is accepted when the peak response in blank matrices at the retention time of the analyte(s) is less than 20% of the response for the LLOQ samples, and lower than 5% for the IS (KOLLIPARA *et al.*, 2011).

Calibration Curve and Linearity

The selection of an appropriate calibration model that yields a well defined and reproducible relationship between instrument response and known concentrations of the analyte is fundamental for reliable quantification (HARTMANN *et al.*, 1998; TIWARI and TIWARI, 2010). Therefore, a calibration curve should be prepared in the same biological matrix as the test samples by using six to eight standards, and should be plotted for each

analyte, covering the expected range of concentrations in the study (EUROPEAN MEDICINES AGENCY, 2011; US FOOD AND DRUG ADMINISTRATION, 2001).

Analyte concentrations in unknown samples are typically interpolated using the regression results obtained from calibration curves, being the linear regression the most commonly adopted model (ALMEIDA *et al.*, 2002). The linearity refers to the ability of a method to produce a signal which is, either directly or by mathematical transformation, proportional to the concentration of the analyte present in the sample over the entire range of interest (CHANDRAN and SINGH, 2007; KOLLIPARA *et al.*, 2011). Ideally, it should be employed the simplest mathematical model that can properly explain the relationship between concentration and detector response. The ordinary least square model is one of the simplest linear regression models most commonly used; however it only can be applied when the condition of homoscedasticity, i.e. constant variance over the whole calibration range (assessed by plotting the residuals *versus* concentration values), is confirmed for the analytical data (Hartmann *et al.*, 1998; TIWARI and TIWARI, 2010). Indeed, unlike pharmaceutical analysis, the concentration range in bioanalytical methods is usually dynamic and broad in order to monitor drug concentrations effectively (ALMEIDA *et al.*, 2002). Inevitably, in broad calibration ranges, even relatively small deviations from an assumed linear model can lead to substantial errors in the predicted concentrations at the extremes of the calibration range (HARTMANN *et al.*, 1998). As a result, larger deviations at higher concentrations tend to influence the regression line more than slight deviations at smaller concentrations, and thus the accuracy in the lower end of the calibration range is impaired (ALMEIDA *et al.*, 2002; GONZÁLEZ *et al.*, 2014). When heteroscedasticity (significant difference among variances at lowest and highest concentration levels) is observed and the calibration range cannot be reduced, the use of a weighted least squares linear regression is preferable and justified (ALMEIDA *et al.*, 2002; HARTMANN *et al.*, 1998; KOLLIPARA *et al.*, 2011).

Whether the applied calibration model is direct or whether it includes the use of weighting factors, the linearity of the method should always be demonstrated. Accordingly, a coefficient of determination (r^2) greater than 0.990 is generally considered as evidence of acceptable fit of the data to the regression line (CHANDRAN and SINGH, 2007; KOLLIPARA *et al.*, 2011). The quality of the calibration curve is guaranteed if the back calculated concentrations of all the calibration standards do not deviate more than $\pm 15\%$ of the nominal value with the exception of the LLOQ, whose deviation can be up to $\pm 20\%$ (EUROPEAN MEDICINES AGENCY, 2011; US FOOD AND DRUG ADMINISTRATION, 2001).

Precision and Accuracy

The precision and accuracy with which known concentrations of an analyte can be determined in a certain biological matrix must be demonstrated, constituting one of the most important factors in assessing method quality (SHAH *et al.*, 2000). The precision of a bioanalytical method is the ability to produce reproducible results between series of determinations, i.e., describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of the same homogeneous sample, being generally expressed as % coefficient of variation (% CV) (KOLLIPARA *et al.*, 2011; US FOOD AND DRUG ADMINISTRATION, 2001). The accuracy, also termed as trueness, is defined as the closeness of the experimental value obtained by the method to the true or nominal concentration of the analyte, and it is expressed as % Bias, deviation of the test results from the accepted reference value (TIWARI and TIWARI, 2010).

The precision and accuracy should be determined both inter- and intra-daily by analysing replicate sets of quality control (QC) samples prepared at the LLOQ and three different concentration levels covering the whole dynamic range of the method: low (equivalent to three times of LLOQ concentration), medium (somewhere within the middle concentration value of the calibration range) and high (closer to the upper end of the standard curve and ideally, it should not be greater than 80-90% of the upper limit of quantification [ULOQ]) (BANSAL and DE STEFANO, 2007; KOLLIPARA *et al.*, 2011). The acceptance criteria for both the parameters establish that, at each QC level, either % CV or % Bias should not exceed the absolute value of 15% with the exception of LLOQ, whose deviations can reach values up to 20% (EUROPEAN MEDICINES AGENCY, 2011; US FOOD AND DRUG ADMINISTRATION, 2001).

Limit of Quantification and Limit of Detection

The limit of quantification is the lowest concentration of the analyte in a sample that can be quantitatively determined with acceptable precision and accuracy (% CV and % Bias < 20%). In practice, it corresponds to the lowest point of the calibration curve and should be determined by analysing at least five replicates of the sample at the LLOQ concentration, independently of those used for construction of the calibration curve (BANSAL and DE STEFANO, 2007). Quantification below LLOQ is, by definition, considered not acceptable and thereby, for determinations accomplished under this minimum value, the method can only produce semi-quantitative or qualitative data (TIWARI and TIWARI, 2010). The limit of quantification is frequently identified directly with sensitivity when these terms are actually conceptually different. Indeed, sensitivity refers to the capability of the method to discriminate small differences in concentration of the analyte, reflecting the

slope of the calibration curve obtained when the chromatographic response is represented *versus* concentration (GONZÁLEZ *et al.*, 2014).

The limit of detection (LOD) is the lowest concentration of an analyte which can be detected and reliably differentiated from background noise but not necessarily quantified as an exact value. The LOD can be estimated using various procedures. Hence, LOD is commonly determined for an analyte concentration at which the response signal-to-noise ratio is equal or greater than three; however, simple visual examination after successive dilutions of the LLOQ standard is also an approach very often performed (CHANDRAN and SINGH, 2007; KOLLIPARA *et al.*, 2011).

Recovery

Recovery is the parameter that reflects the extraction efficiency, in other words, it corresponds to the ability of a bioanalytical method to extract the analyte(s) from the biological matrix. According to US FDA guidelines, the extraction efficiency should be determined by comparing the analytical response of replicate sets of extracted samples at three concentration levels representative of the calibration range (low, medium and high) with non-extracted standards containing the same amount of the analyte(s), which represent 100% recovery (US FOOD AND DRUG ADMINISTRATION, 2001). It is important to point out that there is not a minimum established recovery value either for the analyte or IS, nevertheless, it should be precise, consistent and reproducible over the dynamic range of the method (GONZÁLEZ *et al.*, 2014; KOLLIPARA *et al.*, 2011). Even though high recovery of analyte(s) from the matrix is always desirable, sometimes, it may be necessary to intentionally sacrifice the extraction efficiency in order to achieve better selectivity (DADGAR *et al.*, 1995). Factors such as organic solvent composition, buffer ionic strength and pH, acid or base concentration and temperature are just some of the variables that can be investigated and optimised to improve the extent of recovery (WAL *et al.*, 2010).

Stability

The chemical stability of a drug/metabolite is a function of the physicochemical properties of the compound, the storage conditions, the matrix components and the container system (US FOOD AND DRUG ADMINISTRATION, 2001). In effect, the stability of the analyte in biological matrices is often critical, and therefore, it is imperative to verify that there is no relevant degradation between the time of samples collection and their analysis that would compromise the reliability of the obtained results (HARTMANN *et al.*, 1998).

Evaluation of stability should be carried out to ensure that every step taken during sample preparation and analysis, as well as the storage conditions used, do not affect the final concentration of the analyte (EUROPEAN MEDICINES AGENCY, 2011). Stability can be tested by comparing the results of replicate sets of both low and high QC samples analysed before (reference samples) and after (stability samples) being exposed to the conditions for stability assessment. Although US FDA guidance does not specify acceptance criteria for the stability exercises, EMA recommendations indicate that the deviation between experimental and nominal values should be within $\pm 15\%$ (EUROPEAN MEDICINES AGENCY, 2011). The conditions employed in stability experiments should cover all the situations likely to be encountered during the whole analytical procedure which include sample handling, storage and analysis:

- *Short-term (bench top) stability* – Evaluates the stability of the analyte before processing a sample, which is done at room temperature and/or 4°C for 4-24 h based on the expected duration for which samples will be maintained at these conditions from collection until sample preparation;
- *Long-term stability* – Ensures the integrity of the drug in the biological matrix stored in the freezer (-70°C and/or -20°C) since the date of the first sample collection up to the date of the last sample analysis. It is advised to analyse the samples at least on three different occasions to ensure enough stability data before starting the study;
- *Freeze-thaw stability* – It simulates situations of transport or re-analysis in which samples are commonly frozen and thawed several times. Analyte stability is determined for at least three freeze and thaw cycles wherein QC samples are stored in the freezer for 24 h, thawed unassisted at room temperature and then once again refrozen for 12-24 h under the same conditions. After completing three freeze-thaw cycles, the samples are finally analysed;
- *Post-preparative stability* – Assess the stability of the analyte in the final extract during the anticipated maximum analysis time under the conditions of analysis (e.g. residence time in the autosampler).

Table II.1.1 Summary of the main validation parameter requirements according to both US FDA and EMA guidelines on bioanalytical method validation.

Validation Parameter	Requirement <i>- Acceptance Criteria</i>
Selectivity	Analysis of blank samples of the appropriate biological matrix should be obtained from at least six different sources. <i>- Absence of interfering components is accepted when the response is < 20% of the LLOQ for the analyte and < 5% for the IS.</i>
Calibration Curve/ Linearity	Should be prepared in the same biological matrix as the test samples by using six to eight calibration standards, covering the whole expected concentration range. <i>- At least 3 calibration curves (generated in 3 different days) should be plotted.</i> <i>- $r^2 > 0.990$; % Bias within $\pm 15\%$ (all concentrations) or $\pm 20\%$ (LLOQ).</i>
Precision	Should be evaluated inter- and intra-daily. A minimum of five replicates per concentration (LLOQ, low QC, medium QC and high QC) should be analysed. <i>- % CV (QC samples) < 15%; % CV (LLOQ) < 20%.</i>
Accuracy	Should be evaluated inter- and intra-daily. A minimum of five replicates per concentration (LLOQ, low QC, medium QC and high QC) should be analysed. <i>- % Bias (QC samples) within $\pm 15\%$; % Bias (LLOQ) within $\pm 20\%$.</i>
LLOQ	Should be determined by analysing at least five replicates. <i>- % CV < 20%; % Bias within $\pm 20\%$.</i>
Recovery	Should be performed at three concentration levels (low QC, medium QC and high QC) by comparing the analytical results of extracted samples with non-extracted standards, which represent 100% recovery. <i>- Recovery should be precise, consistent and reproducible.</i>
Stability	Should be performed for at least three replicates of low and high QC samples. Stability evaluations should comprise short-term, long-term, freeze/thaw and post-preparative experiments. <i>- % Deviation within $\pm 15\%$.</i>

Bias, deviation from nominal value; CV, coefficient of variation; IS, internal standard; LLOQ, lower limit of quantification; QC, quality control; r^2 , coefficient of determination.



II. 2. EXPERIMENTAL

Development and Validation of an HPLC-UV Method for the Simultaneous Quantification of PB, PRM, PHT, CBZ, OXC, LTG and their Main Metabolites in Human Plasma

II. 2.1. INTRODUCTION

Epilepsy is a chronic neurological disorder that affects approximately 50 million people worldwide (BIALER and WHITE, 2010). Among several therapeutic approaches, pharmacotherapy based on a wide variety of AEDs is the first-line treatment option to achieve seizure control (GERLACH and KRAJEWSKI, 2010). Indeed, since the introduction of PB, several molecules with anticonvulsant properties were developed and, presently, more than twenty AEDs are patented in the world market, including older drugs such as PHT, PRM, ethosuximide, CBZ and valproic acid; and newer AEDs like LTG, levetiracetam, OXC, zonisamide and eslicarbazepine acetate.

Dealing with such a wide arsenal of AEDs does not make the clinical selection of the best pharmacotherapy an easy task and, therefore, several guidelines have been established (GLAUSER *et al.*, 2006; PATSALOS *et al.*, 2008). Although monotherapy is still the preferred therapeutic approach in newly diagnosed epilepsy, it has failed in many patients even when two or more different AEDs are attempted and gradually titrated to the maximum tolerated dose (GARNETT *et al.*, 2009). Thus, if an epileptic condition is refractory to an initial monotherapy regimen, patients should be switched to monotherapy with another AED or to polytherapy (ST LOUIS, 2010, 2009). However, to ensure that therapeutic failure is due to the inadequate seizure control of the selected AED monotherapy regimen rather than the inability to achieve the proper drug plasma concentrations in the patient, therapeutic drug monitoring (TDM) of plasma/serum levels remains necessary. Considering the variable nature of epilepsy, patient's pathophysiological features, comorbidities and co-medications, AED therapy must be carefully optimised for each individual patient concerning both therapeutic and toxicological profiles. Hence, the TDM of AEDs has become a routine practice in the management of epilepsy (KRASOWSKI, 2010) not only when AED polytherapy combinations are implemented, but also during the course of pharmacological conversion between different AED monotherapies.

The present work aimed at developing a sensitive and fast bioanalytical method able to simultaneously determine the plasma concentrations of PB, PRM, PHT, CBZ, LTG and OXC as they represent the main frontline of old and new AEDs in the current clinic (LANDMARK *et al.*, 2007), but also some of their main metabolites, CBZ-E, *trans*-diol and Lic (Figure II.2.1). Regarding the metabolic and pharmacotoxicological properties of such metabolites, it is clear that they should also be quantified in several circumstances. Specifically, PRM is mainly metabolised in the liver to PB, its active metabolite, and both exhibit anticonvulsant activity. In humans, CBZ is mainly oxidised to CBZ-E which is the main responsible for the toxic effects of the parent compound; then, the epoxide undergoes microsomal hydrolysis and is converted to the inactive *trans*-diol (PATSALOS *et*

al., 2008). Although structurally similar to CBZ, OXC is extensively reduced to the pharmacologically active metabolite Lic.

Bearing in mind that PB, PRM, PHT and CBZ are mainly metabolised by the hepatic cytochrome P450 system, and that LTG and OXC are second-generation AEDs frequently used when switching those first-generation AEDs, it is expected that during these conversion processes drug-drug interactions may occur. Therefore, TDM is crucial because abruptly stopping an existing baseline AED increases the risk of breakthrough seizures, while introducing a new adjunctive AED too quickly can cause an exacerbation of adverse effects (ST LOUIS, 2010). Hence, the availability of rapid, simple, sensitive, accurate and reliable analytical techniques is primordial to support TDM of these drugs in clinical routine.

During the last years, several HPLC methods coupled to UV detection or mass spectrometry detection (HPLC-MS and HPLC-MS/MS) for the simultaneous determination of AEDs and some of their metabolites have been reported in literature. Notwithstanding, to the best of our knowledge, an HPLC-UV assay that provides a simultaneous quantification of PB, PRM, PHT, CBZ, OXC, LTG and three of their main metabolites (CBZ-E, *trans*-diol and Lic) in human plasma has not been published yet.

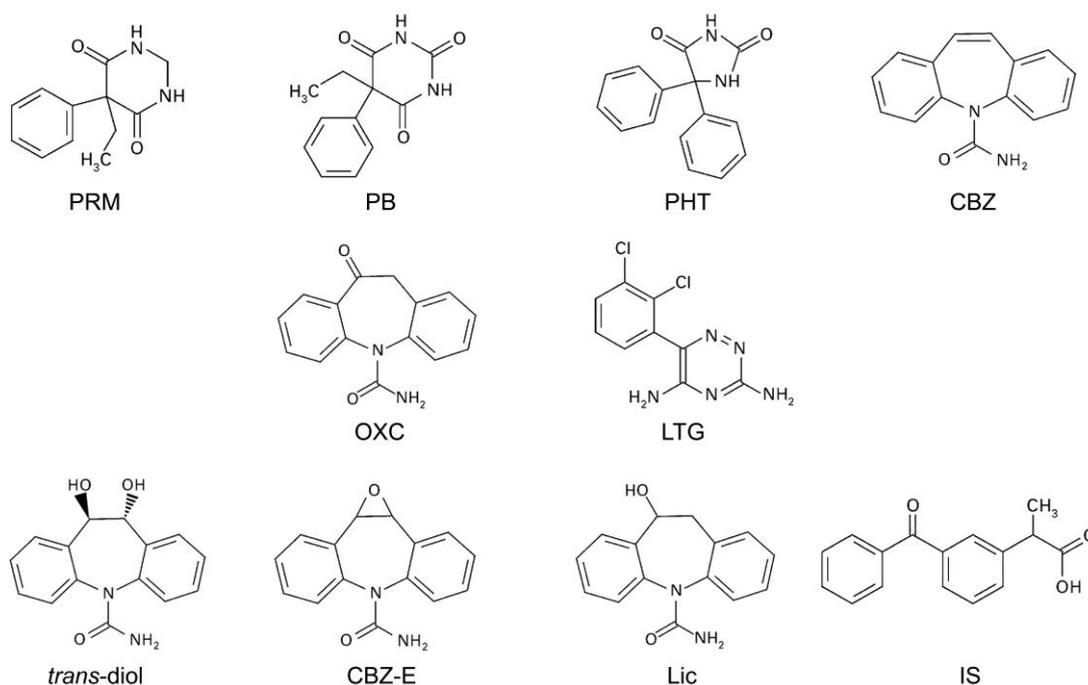


Figure II.2.1 Chemical structures of primidone (PRM), phenobarbital (PB), phenytoin (PHT), carbamazepine (CBZ), oxcarbazepine (OXC), lamotrigine (LTG), 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine (*trans*-diol), carbamazepine-10,11-epoxide (CBZ-E), licarbazepine (Lic), and ketoprofen which was used as internal standard (IS).

II. 2.2. MATERIALS & METHODS

II. 2.2.1. Chemicals and Reagents

PRM, PHT, CBZ, CBZ-E, OXC and ketoprofen, used as IS, were purchased from Sigma-Aldrich (St Louis, MO, USA). Lic and *trans*-diol were kindly supplied by BIAL-Portela & C^a S.A. (S. Mamede do Coronado, Portugal). LTG was kindly provided by Bluepharma (Coimbra, Portugal). PB was commercially obtained from Labesfal (Campo de Besteiros, Portugal). Methanol (HPLC gradient grade) was purchased from Fisher Scientific (Leicestershire, UK), acetonitrile (HPLC gradient grade) from Lab-Scan (Sowinskięo, Poland) and ultrapure water (HPLC grade, 18.2 M Ω .cm) was prepared by means of a Milli-Q water apparatus from Millipore (Milford, MA, USA). Ethyl acetate and triethylamine (TEA) were acquired from Fisher Scientific (Leicestershire, UK) and Merck KGaA (Darmstadt, Germany) respectively, while *ortho*-phosphoric acid (85%) was purchased from Panreac Química SA (Barcelona, Spain).

Blank human plasma samples from healthy donors were gently provided by the Portuguese Blood Institute after written consent of each subject.

II. 2.2.2. Preparation of Standard Solutions

Stock solutions of PB (20 mg/mL), PRM (10 mg/mL), PHT (10 mg/mL), CBZ (10 mg/mL), LTG (10 mg/mL), OXC (5 mg/mL), CBZ-E (10 mg/mL), *trans*-diol (10 mg/mL), Lic (20 mg/mL) and IS (1 mg/mL) were individually prepared by dissolving appropriate amounts of each compound in methanol. These solutions were then adequately diluted with methanol to obtain the corresponding intermediate solutions. Afterwards, stock and intermediate solutions of drugs and metabolites were properly mixed to afford six combined spiking solutions with final concentrations of 6.25, 12.5, 50, 250, 100 and 2500 μ g/mL for PB; 10, 20, 50, 125, 500 and 1250 μ g/mL for PRM; 12.5, 25, 50, 125, 500 and 1250 μ g/mL for PHT; 2.5, 5, 12.5, 50, 250 and 1250 μ g/mL for CBZ, LTG and CBZ-E; 2.5, 5, 12.5, 25, 125 and 625 μ g/mL for OXC; 6.25, 12.5, 25, 50, 100 and 250 μ g/mL for *trans*-diol and 3.75, 7.5, 20, 100, 500 and 2000 μ g/mL for Lic. Each combined solution was daily used for spiking aliquots of blank human plasma in order to prepare six calibration standards in the concentration ranges of 0.25-100 μ g/mL for PB, 0.4-50 μ g/mL for PRM, 0.5-50 μ g/mL for PHT, 0.1-50 μ g/mL for CBZ, LTG and CBZ-E, 0.1-25 μ g/mL for OXC, 0.25-10 μ g/mL for *trans*-diol and 0.15-80 μ g/mL for Lic.

QC samples at three representative concentration levels (low, medium and high) of the calibration curves were independently prepared in the same biological matrix. Thus, aliquots of blank human plasma were spiked to attain final concentrations of 0.75, 50 and 90 μ g/mL for PB; 1.2, 25 and 45 μ g/mL for PRM; 1.5, 25 and 45 μ g/mL for PHT; 0.3,

25, 45 $\mu\text{g}/\text{mL}$ for CBZ, LTG and CBZ-E; 0.3, 12.5 and 22.5 $\mu\text{g}/\text{mL}$ for OXC; 0.75, 5 and 9 $\mu\text{g}/\text{mL}$ for *trans*-diol and 0.45, 40 and 72 $\mu\text{g}/\text{mL}$ for Lic.

A methanolic IS working solution at 250 $\mu\text{g}/\text{mL}$ was also prepared by dilution of an appropriate volume of the respective stock solution.

All stock, intermediate and combined solutions were stored at 4°C and protected from light, with the exception of the IS working solution which was daily prepared.

II. 2.2.3. Sample Preparation

An aliquot of human plasma (500 μL) added of 20 μL of the IS working solution was mixed with 1 mL of methanol in order to precipitate plasma proteins. After centrifuging at 13400 rpm for 10 min, the resulting supernatant was evaporated under a gentle nitrogen stream at 80°C for 10 min; then, the residual volume of supernatant was diluted with 1.5 mL of ultrapure water and vortex-mixed for 30 s. Afterwards, the pre-treated sample was subjected to a SPE on the Waters Oasis[®] HLB cartridge (30 mg of hydrophilic-lipophilic-balanced sorbent; 1 mL of capacity; from Milford, MA, USA), which was previously conditioned with 1 mL of methanol, 1 mL of acetonitrile and 1 mL of water-acetonitrile (95:5, v/v). The loaded cartridge was subsequently submitted to -60 kPa and washed four times with 1 mL of water. After drying the sorbent under airflow for 5 min, analytes were eluted with 1 mL of ethyl acetate using gentle vacuum. The eluate was evaporated to dryness at 45°C under a gentle stream of nitrogen gas and reconstituted with 500 μL of mobile phase by vortexing and ultrasonication. At last, 20 μL of the final mixture were injected into the chromatographic system.

II. 2.2.4. HPLC-UV Instrumentation and Chromatographic Conditions

The chromatographic analysis was performed on a BAS-480 liquid chromatograph equipped with a PM-80 pump, a Rheodyne manual injector with a 20 μL loop, a BAS UV-116 UV-Vis detector, a BAS DA-5 chromatography control and a data system interface (Bioanalytical Systems, West Lafayette, IN, USA). Data acquisition was achieved by means of BAS Chromgraph Control and Chromgraph Report software version 2.30.

The chromatographic separation of all the six drugs, metabolites and IS was carried out at room temperature on a reversed-phase LiChroCART[®] Purospher Star[®] C₁₈ column (55 mm x 4 mm; 3 μm particle size) purchased from Merck KGaA (Darmstadt, Germany). An isocratic elution was applied at a flow rate of 1.0 mL/min with a mobile phase composed of water-methanol-acetonitrile-TEA (68.7:25:6:0.3, v/v/v/v) adjusted at pH 6.5 with *ortho*-phosphoric acid 85%. The wavelength detection was set at 237 nm.

II. 2.2.5. Method Validation

The method was validated according to the international recommendations for bioanalytical method validation regarding selectivity, linearity, precision, accuracy, limits of quantification and detection, recovery and stability (EUROPEAN MEDICINES AGENCY, 2011; US FOOD AND DRUG ADMINISTRATION, 2001).

Blank human plasma samples from six different subjects were used in order to assess method selectivity, analysing the eventual interference of matrix endogenous substances at the retention times of PB, PRM, PHT, CBZ, LTG, OXC, CBZ-E, *trans*-diol, Lic and IS. Interferences from several commonly co-administered drugs were also tested and included amitriptyline, chlorpromazine, clonazepam, dexamethasone, diazepam, digoxin, fluoxetine, haloperidol, hydrochlorothiazide, ibuprofen, levetiracetam, lorazepam, mirtazapine, naproxen, omeprazole, paracetamol, ranitidine, salicylic acid, sertraline, theophylline, and venlafaxine.

The linearity of the method was evaluated within the defined plasma concentration ranges, using calibration curves prepared on five different days ($n = 5$), constructed with six spiked plasma calibration standards and plotting analyte-IS peak height ratio *versus* the corresponding plasma nominal concentrations. The data were subjected to a weighted linear regression analysis using $1/x^2$ as weighting factor, taking the plots and the sums of absolute percentage of relative error into account (ALMEIDA *et al.*, 2002).

Inter-day precision and accuracy were investigated analysing each QC sample on five consecutive days of the assay ($n = 5$), while the intra-day data were obtained by analysing five sets of QC samples in a single day ($n = 5$). According to bioanalytical method validation guidelines, the acceptance criterion for precision, which is expressed as % CV, must be equal to or lower than 15%; whereas accuracy, which is expressed as % Bias, must be within $\pm 15\%$.

The LLOQ was defined as the lowest concentration of the calibration curve that can be measured with adequate precision and accuracy. The LLOQ was stipulated analysing plasma samples intra- and inter-daily ($n = 5$) and absolute deviations lower than 20% for both CV and Bias values were accepted. The LOD was determined for all the compounds by analysing spiked plasma samples with known concentrations after successive dilutions.

The absolute recovery of the compounds from human plasma samples submitted to the treatment previously described was investigated at the three concentration levels

(low, medium and high) representative of the calibration range. It was calculated comparing the analytes peak heights from extracted QC plasma samples with those obtained after direct injection of non-extracted aqueous solutions at the same nominal concentrations ($n = 5$). The absolute recovery of the IS was also determined by calculating its peak height ratio between extracted samples and non-extracted aqueous solutions.

Human plasma stability of the nine analytes was investigated at low and high QC concentration levels comparing the data of samples analysed before (reference samples) and after being exposed to the conditions for stability assessment (stability samples). Stability/reference samples ratio between 85-115% was defined as stability criterion ($n = 5$) according to the guidelines. Short-term and long-term stability were evaluated at room temperature for 4 h, at 4°C for 24 h and at -30°C for up to 30 days in order to simulate sample handling and storage time in the refrigerator and freezer before analysis ($n = 5$). The effect of three freeze-thaw cycles on the stability of the compounds in human plasma samples was additionally studied. For that, aliquots of spiked plasma samples were stored at -30°C for 24 h, thawed unassisted at room temperature and then refrozen for 24 h under the same conditions until completing the three cycles. In order to assess the post-preparative stability of processed samples in usual autosampler conditions, the stability of the analytes on the reconstituted extracts was also assessed at 4°C for 24 h.

II. 2.2.6. Clinical Application

After development and validation of the method, it was applied for the quantification of parent compounds and main metabolites in real plasma samples of epileptic patients treated with PB, PHT or CBZ who were being monitored at the Coimbra University Hospital Centre (CHUC).

II. 2.3. RESULTS & DISCUSSION

II. 2.3.1. Development and Optimisation of Chromatographic Conditions

During the development and optimisation phase of the assay, several chromatographic conditions were tested in order to achieve the best separation of the compounds within the shortest running time. In the early stages, a mobile phase composed of a mixture of water-methanol-acetonitrile in the proportion of 64:30:6 (v/v/v) was tested. However, the poor peak resolution of LTG concerning both shape and symmetry demanded the incorporation of TEA to the mixture. Indeed, TEA saturates the free silanol groups of the stationary phase, allowing the decrease of the asymmetry and peak tailing phenomenon (Sousa *et al.*, 2012). The influence of mobile phase pH in the separation and retention time of the analytes was also evaluated in the range of 3-10. Hence, a mixture of water-methanol-acetonitrile-TEA in the proportion of 68.7:25:6:0.3 (v/v/v/v) and pH at 6.5 was chosen, as it provided a good separation and peak shape for all compounds of interest (including AEDs, metabolites and IS). Under these analytical conditions, the last-eluting analyte was CBZ, with a retention time of approximately 15 min, and the order of elution of the compounds was PRM, LTG, *trans*-diol, PB, Lic, CBZ-E, OXC, IS, PHT and CBZ (Figure II.2.2).

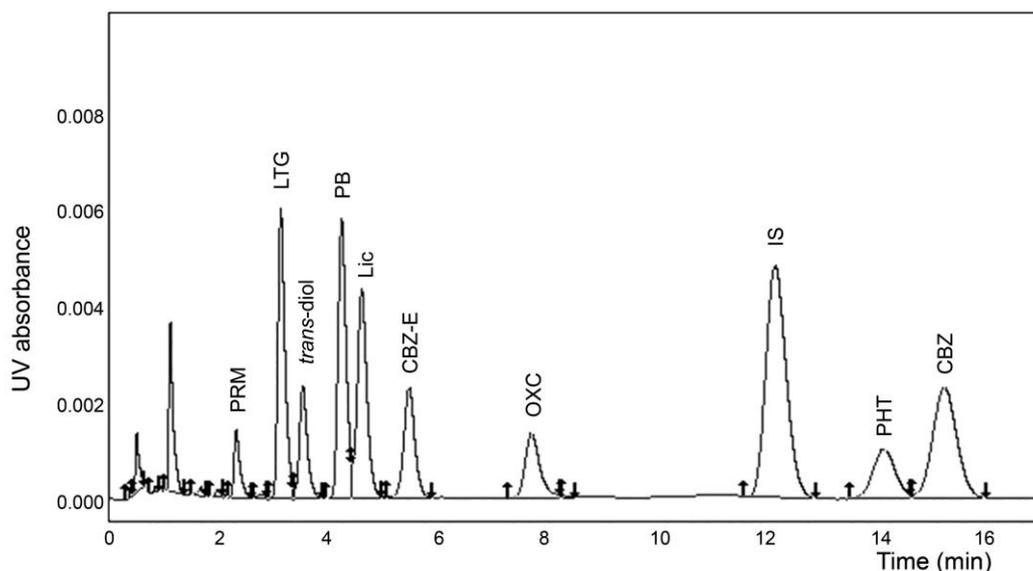


Figure II.2.2 Typical chromatogram of an extracted human sample plasma spiked with internal standard (IS) and analytes at intermediate concentrations of the calibration ranges. CBZ, carbamazepine; CBZ-E, carbamazepine-10,11-epoxide; Lic, licarbazepine; LTG, lamotrigine; OXC, oxcarbazepine; PB, phenobarbital; PHT, phenytoin; PRM, primidone; *trans*-diol, 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine.

The development of a practical and accessible HPLC method that could be broadly applied in hospitals and clinical pharmacokinetic departments was our main concern. Therefore, the use of UV detection and isocratic elution were preferred to mass spectrometry (KIM *et al.*, 2011; PAGLIA *et al.*, 2007; SHIBATA *et al.*, 2012; SUBRAMANIAN *et al.*, 2008) and gradient elution (JUERGENS, 1984; MAURER *et al.*, 2002; SHIBATA *et al.*, 2012), which involve more complex and expensive equipments, and thus cannot be easily adopted by the majority of clinical laboratories. Several wavelength values ranging from 210 nm to 250 nm were assessed and the best compromise in terms of sensitivity and selectivity was achieved at 237 nm.

In order to select the appropriate IS, some compounds including amitriptyline, chloramphenicol, naproxen, 10,11-dihydrocarbamazepine and ketoprofen were tested. The latter was selected as it presented the most adequate retention time and it also displayed a chromatographic behaviour and absolute recovery values similar to those exhibited by the AEDs under investigation.

II. 2.3.2. Optimisation of the Sample Preparation Procedure

Sample pre-treatment methodology was initially investigated by plasma precipitation with acetonitrile and methanol (BHATTI *et al.*, 1998; CONTIN *et al.*, 2010; PATIL and BODHANKAR, 2005); however, no profitable results were achieved because the resulting processed samples were relatively unclean, still presenting matrix endogenous substances that interfered with the retention times of the analytes. As previous studies (BUGAMELLI *et al.*, 2002; FORTUNA *et al.*, 2010) revealed that CBZ-E is easily degraded in acid environments, the use of strong precipitation agents like perchloric and trichloroacetic acids was herein excluded. Then, since SPE procedures are usually associated to high and reliable extraction of AEDs from human plasma (ALVES *et al.*, 2007; FORTUNA *et al.*, 2010; FRANCESCHI and FURLANUT, 2005; VERMEIJ and EDELBROEK, 2007), several SPE conditions were tested, including washing steps and eluting solvents, but also without success. Thus, the combination of a plasma deproteinisation with methanol followed by a SPE procedure was tested and demonstrated to be the best option as it allowed a more effective elimination of interfering substances and avoided sample dilution, improving the selectivity and sensitivity of the analytical method. Furthermore, the sample preparation methodology herein developed is faster, less cumbersome, less pollutant and affords greater recovery values than LLE procedures reported in literature (BUDAKOVA *et al.*, 2008; KIMISKIDIS *et al.*, 2007; OH *et al.*, 2006).

II. 2.3.3. Method Validation

II. 2.3.3.1. Selectivity

Representative chromatograms of blank plasma and spiked human plasma samples with IS and analytes at concentrations of the LLOQ are depicted in Figure II.2.3. The analysis of blank plasma samples from six healthy volunteers confirmed the absence of interfering peaks from matrix endogenous substances at the retention times of the studied compounds.

In addition, none of the tested drugs potentially co-administered with the considered AEDs were found to interfere with peaks of the analytes or IS.

II. 2.3.3.2. Linearity, LLOQ and LOD

The calibration curves prepared in human plasma for all the compounds at the concentration ranges defined in *section II. 2.2.2.* were linear ($r^2 \geq 0.992$) and showed a consistent correlation between analyte-IS peak height ratios and corresponding plasma concentrations. The regression equations of the calibration curves and the corresponding coefficients of determination attained for each AED and metabolites are summarised in Table II.2.1. At this point, it is important to highlight that the concentration range for each compound is much wider than its respective therapeutic window (PATSALOS *et al.*, 2008), allowing the applicability of this assay to both pharmacokinetic and toxicological determinations.

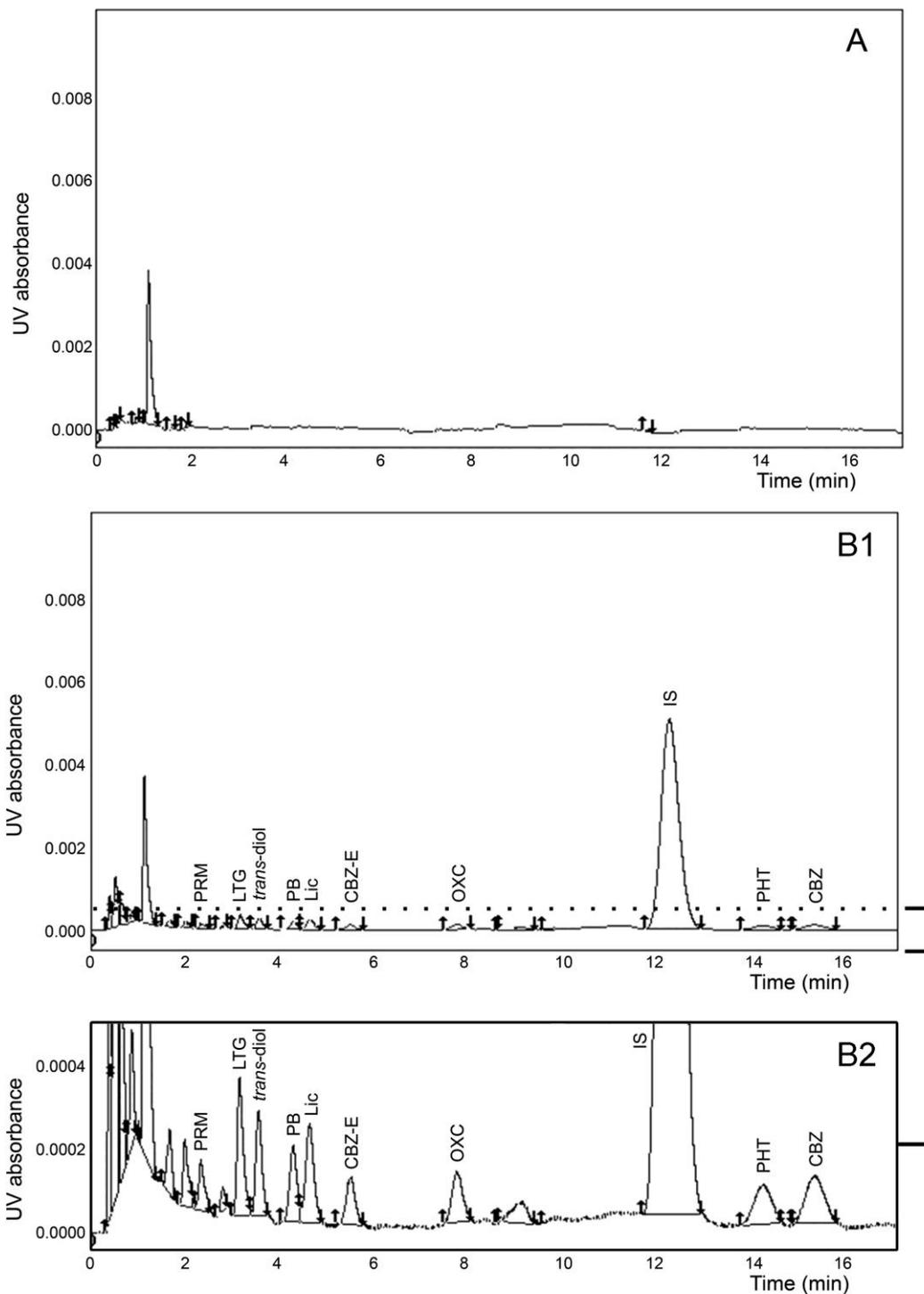


Figure II.2.3 Typical chromatograms of extracted human plasma: (A) blank plasma; (B1) plasma spiked with the internal standard (IS) and analytes at concentrations of the LLOQ; (B2) expanded partial representation of the chromatogram depicted in (B1). CBZ, carbamazepine; CBZ-E, carbamazepine-10,11-epoxide; Lic, licarbazepine; LTG, lamotrigine; OXC, oxcarbazepine; PB, phenobarbital; PHT, phenytoin; PRM, primidone; *trans-diol*, 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine.

Table II.2.1 Mean calibration parameters ($n = 5$) of primidone (PRM), lamotrigine (LTG), 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine (*trans*-diol), phenobarbital (PB), licarbazepine (Lic), carbamazepine-10,11-epoxide (CBZ-E), oxcarbazepine (OXC), phenytoin (PHT) and carbamazepine (CBZ) in human plasma.

Analyte	Calibration Parameters		
	Concentration Range ($\mu\text{g/mL}$)	Regression Equation ^a	r^2
PRM	0.40 - 50	$Y = 0.0485x - 0.0012$	0.992
LTG	0.10 - 50	$Y = 0.5412x + 0.0031$	0.994
<i>trans</i> -diol	0.25 - 10	$Y = 0.2163x - 0.0073$	0.994
PB	0.25 - 100	$Y = 0.0995x + 0.0095$	0.996
Lic	0.15 - 80	$Y = 0.1898x + 0.0108$	0.994
CBZ-E	0.10 - 50	$Y = 0.2088x - 0.0005$	0.996
OXC	0.10 - 25	$Y = 0.2410x - 0.0032$	0.993
PHT	0.50 - 50	$Y = 0.0384x - 0.0030$	0.992
CBZ	0.10 - 50	$Y = 0.2132x - 0.0021$	0.994

^a Y represents analyte-IS peak height ratio (expressed in arbitrary height units); x represents analyte concentration (expressed in $\mu\text{g/mL}$).
 r^2 , coefficient of determination.

The LLOQ was experimentally defined as 0.50 $\mu\text{g/mL}$ for PHT, 0.40 $\mu\text{g/mL}$ for PRM, 0.25 $\mu\text{g/mL}$ for PB and *trans*-diol, 0.15 $\mu\text{g/mL}$ for Lic and 0.10 $\mu\text{g/mL}$ for OXC, CBZ, CBZ-E and LTG with acceptable precision ($\text{CV} \leq 18.27\%$) and accuracy (Bias varied from -13.93% to 16.43%) as depicted in Table II.2.2. Interestingly, the LLOQs obtained with this method are often lower than those achieved with other HPLC-UV techniques reported in literature (BUDAKOVA *et al.*, 2008; BUGAMELLI *et al.*, 2002; FRANCESCHI and FURLANUT, 2005; GREINER-SOSANKO *et al.*, 2007; HEIDELOFF *et al.*, 2010; KHOSHSORUR *et al.*, 2001; LEVERT *et al.*, 2002; MA *et al.*, 2007).

After successive dilutions of the lowest calibration standard, the LOD was reliably established at 0.050 $\mu\text{g/mL}$ for PHT, 0.040 $\mu\text{g/mL}$ for PRM, 0.025 $\mu\text{g/mL}$ for PB and *trans*-diol, 0.015 $\mu\text{g/mL}$ for Lic and 0.010 $\mu\text{g/mL}$ for OXC, CBZ, CBZ-E and LTG.

Table II.2.2 Inter- and intra-day precision (% CV) and accuracy (% Bias) of the analytes in human plasma samples at the lower limit of quantification (LLOQ) ($n = 5$).

Analyte	LLOQ						
	Nominal Concentration	Inter-day			Intra-day		
		Experimental Concentration ^a	% CV	% Bias	Experimental Concentration ^a	% CV	% Bias
PRM	0.40	0.43 ± 0.055	13.48	7.38	0.41 ± 0.014	3.31	1.94
LTG	0.10	0.11 ± 0.017	14.95	5.78	0.12 ± 0.019	14.29	16.43
<i>trans</i> -diol	0.25	0.26 ± 0.022	9.60	5.46	0.27 ± 0.006	2.42	6.09
PB	0.25	0.25 ± 0.062	18.27	-1.58	0.22 ± 0.026	8.09	-13.93
Lic	0.15	0.14 ± 0.036	17.93	-5.28	0.16 ± 0.019	7.62	5.47
CBZ-E	0.10	0.11 ± 0.011	10.22	9.24	0.11 ± 0.011	10.41	10.87
OXC	0.10	0.11 ± 0.012	12.40	6.26	0.10 ± 0.007	7.04	0.43
PHT	0.50	0.51 ± 0.052	12.05	1.22	0.50 ± 0.021	4.66	-0.17
CBZ	0.10	0.11 ± 0.012	12.31	10.88	0.11 ± 0.017	14.85	14.74

^a Mean ± Standard deviation, $n = 5$. Nominal and experimental concentrations are expressed in µg/mL. CBZ, carbamazepine; CBZ-E, carbamazepine-10,11-epoxide; Lic, licarbazepine; LTG, lamotrigine; OXC, oxcarbazepine; PB, phenobarbital; PHT, phenytoin; PRM, primidone; *trans*-diol, 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine.

II. 2.3.3.3. Precision and Accuracy

The results of inter- and intra-day precision and accuracy analyses are reported in Table II.2.3. The acceptance criteria were fulfilled for all the compounds at the three concentration levels assessed, since the overall inter- and intra-day CV values were below 12.15% and Bias varied between -14.91% and 13.63%. These data clearly demonstrate that the HPLC-UV method herein developed is reliable, accurate and reproducible.

Table II.2.3 Inter- and intra-day precision (% CV) and accuracy (% Bias) of the analytes in human plasma samples at the low, medium and high concentrations of the calibration ranges ($n = 5$).

Nominal Concentration	Inter-day			Intra-day		
	Experimental Concentration ^a	% CV	% Bias	Experimental Concentration ^a	% CV	% Bias
PRM						
1.2	1.14 ± 0.110	9.90	-4.90	1.22 ± 0.133	10.76	1.43
25	25.54 ± 3.002	11.76	2.18	26.22 ± 1.167	4.45	4.88
45	42.49 ± 2.600	6.12	-5.57	43.95 ± 2.126	4.84	-2.34
LTG						
0.3	0.28 ± 0.028	9.92	-6.42	0.27 ± 0.031	10.67	-8.96
25	24.83 ± 2.565	10.33	-0.66	24.46 ± 0.699	2.86	-2.16
45	45.96 ± 3.601	7.83	2.13	48.49 ± 2.890	5.96	7.76
trans-diol						
0.75	0.70 ± 0.067	9.99	-6.56	0.69 ± 0.032	4.80	-8.62
5	4.95 ± 0.443	9.00	-0.94	4.89 ± 0.229	4.70	-2.11
9	9.40 ± 0.821	8.76	4.49	10.13 ± 0.431	4.27	12.55
PB						
0.75	0.70 ± 0.096	12.08	-6.41	0.66 ± 0.051	6.67	-11.80
50	48.74 ± 4.300	8.80	-2.52	47.52 ± 1.496	3.14	-4.95
90	87.45 ± 7.313	8.35	-2.83	94.91 ± 6.040	6.36	5.46
Lic						
0.45	0.45 ± 0.047	9.38	-0.33	0.46 ± 0.039	7.06	1.91
40	40.91 ± 4.061	9.91	2.28	40.54 ± 1.669	4.11	1.36
72	73.53 ± 5.700	7.75	2.12	76.63 ± 3.847	5.01	6.44
CBZ-E						
0.3	0.27 ± 0.023	8.55	-8.97	0.27 ± 0.016	6.16	-10.92
25	25.28 ± 2.151	8.51	1.11	24.60 ± 1.004	4.08	-1.61
45	44.98 ± 2.833	6.30	-0.04	45.65 ± 2.469	5.41	1.45
OXC						
0.3	0.27 ± 0.022	8.43	-9.63	0.27 ± 0.015	6.62	-10.57
12.5	13.61 ± 1.204	8.86	8.85	13.30 ± 0.523	3.93	6.43
22.5	25.57 ± 1.481	5.80	13.63	24.50 ± 1.471	6.01	8.90
PHT						
1.5	1.34 ± 0.086	6.81	-10.79	1.32 ± 0.102	8.11	-12.22
25	24.46 ± 2.765	11.34	-2.15	23.72 ± 0.730	3.08	-5.14
45	46.99 ± 5.700	12.15	4.43	51.08 ± 4.370	9.13	13.50
CBZ						
0.3	0.27 ± 0.027	10.57	-10.39	0.26 ± 0.025	9.76	-14.91
25	25.12 ± 2.590	10.31	0.47	24.94 ± 0.781	3.13	-0.25
45	46.16 ± 3.957	8.57	2.58	48.82 ± 3.266	9.69	8.48

^a Mean ± Standard deviation, $n = 5$. Nominal and experimental concentrations are expressed in $\mu\text{g}/\text{mL}$. CBZ, carbamazepine; CBZ-E, carbamazepine-10,11-epoxide; Lic, licarbazepine; LTG, lamotrigine; OXC, oxcarbazepine; PB, phenobarbital; PHT, phenytoin; PRM, primidone; *trans*-diol, 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine.

II. 2.3.3.4. Recovery

The overall recovery values obtained using the sample preparation methodology herein developed are presented in Table II.2.4. The absolute mean recoveries determined for AEDs and metabolites ranged from 78.49% to 101.04% and showed CV values lower than 10.54%; while the mean recovery of IS was of 69.07% and CV was lower than 7.57%. These results undoubtedly demonstrate that the sample preparation methodology employed is consistent, precise and reproducible.

Table II.2.4 Absolute recovery of primidone (PRM), lamotrigine (LTG), 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine (*trans*-diol), phenobarbital (PB), licarbazepine (Lic), carbamazepine-10,11-epoxide (CBZ-E), oxcarbazepine (OXC), phenytoin (PHT) and carbamazepine (CBZ) from human plasma ($n = 5$).

Analyte	Nominal Concentration ($\mu\text{g/mL}$)	Absolute Recovery (%) ^a	Precision (% CV)
PRM	1.2	92.24 \pm 3.99	4.33
	25	80.17 \pm 2.84	3.54
	45	94.47 \pm 2.80	2.96
LTG	0.3	84.87 \pm 5.89	6.94
	25	84.16 \pm 3.20	3.81
	45	85.96 \pm 2.41	2.80
<i>trans</i> -diol	0.75	83.57 \pm 3.94	4.71
	5	83.13 \pm 2.57	3.06
	9	88.68 \pm 2.48	2.79
PB	0.75	89.77 \pm 4.77	5.32
	50	81.35 \pm 2.60	3.19
	90	83.80 \pm 2.60	3.10
Lic	0.45	101.04 \pm 3.83	3.79
	40	87.45 \pm 3.05	3.49
	72	91.42 \pm 2.67	2.92
CBZ-E	0.3	89.43 \pm 6.02	6.73
	25	85.27 \pm 3.05	3.57
	45	90.46 \pm 2.67	2.95
OXC	0.3	82.88 \pm 1.95	2.36
	12.5	86.96 \pm 2.98	3.43
	22.5	89.01 \pm 2.47	2.77
PHT	1.5	84.19 \pm 7.42	8.81
	25	82.81 \pm 3.63	4.39
	45	78.49 \pm 2.60	3.31
CBZ	0.3	88.89 \pm 9.37	10.54
	25	88.46 \pm 3.35	3.78
	45	86.37 \pm 2.57	2.97

^a Mean \pm Standard deviation, $n = 5$. CV, coefficient of variation.

II. 2.3.3.5. Stability

The stability of the six AEDs and metabolites was assessed under the conditions previously stated in *section II. 2.2.5.* and the results are reported in Table II.2.5. Accordingly, all the analytes demonstrated to be stable in unprocessed plasma samples for up to 4 h at room temperature, for 24 h at 4°C, for 1 month at -30°C and after three freeze-thaw cycles. Stability of processed plasma samples was also ensured for at least 24 h at 4°C for all the compounds.

Table II.2.5 Stability (values in percentage) of primidone (PRM), lamotrigine (LTG), 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine (*trans*-diol), phenobarbital (PB), licarbazepine (Lic), carbamazepine-10,11-epoxide (CBZ-E), oxcarbazepine (OXC), phenytoin (PHT) and carbamazepine (CBZ) under different conditions of sample handling and storage.

Analyte Concentration (µg/mL)	Stability Conditions				
	Human Plasma				Mobile Phase
	RT 4h	4°C 24 h	-30°C 30 days	Freeze/thaw (three cycles)	4°C 24 h
PRM					
1.2	100.23	98.18	101.45	97.18	103.68
45	103.28	103.82	94.89	91.47	106.33
LTG					
0.3	98.02	96.01	104.30	102.30	101.06
45	103.07	103.31	95.50	90.12	104.73
<i>trans</i>-diol					
0.75	103.47	102.98	101.64	100.49	100.65
9	105.86	97.19	95.15	89.84	105.28
PB					
0.75	105.78	100.78	102.05	104.53	99.87
90	103.88	105.08	95.30	94.97	105.31
Lic					
0.45	107.49	98.14	104.20	93.70	101.39
72	104.60	101.15	95.06	89.62	105.30
CBZ-E					
0.3	96.52	94.32	103.51	97.27	99.86
45	105.50	100.96	95.10	88.98	105.01
OXC					
0.3	106.22	100.53	94.14	107.41	98.32
22.5	106.06	99.21	95.87	88.26	104.64
PHT					
1.5	96.47	100.47	104.14	95.91	100.32
45	105.53	101.03	95.38	87.16	103.15
CBZ					
0.3	99.69	100.39	104.73	98.52	100.64
45	106.56	99.82	95.64	87.18	103.73

RT, room temperature.

II. 2.3.4. Clinical Application

The method herein developed and fully validated was employed in the analysis of some plasma samples from patients treated with PB, PHT or CBZ. Representative chromatograms are shown in Figure II.2.4. Analytical results revealed good peak shape and resolution and no interferences from endogenous matrix constituents were observed. Drug plasma concentrations were calculated by interpolation on the corresponding calibration curves and the experimental levels obtained were within the concentration range established in the chromatographic technique (Table II.2.6). Furthermore, even though the patients were co-treated with other drugs, no interfering peaks were detected (Table II.2.6, Figure II.2.4). Therefore, the method showed to be highly reliable and selective and thus it seems to be suitable for monitoring plasma concentrations of the selected AEDs and metabolites.

Table II.2.6 Characteristics of drug therapy instituted to the epileptic patients whose plasma samples were analysed by the HPLC-UV method herein developed and the respective measured concentrations obtained for phenobarbital (PB), phenytoin (PHT), carbamazepine (CBZ) and metabolites.

Patient	AED Therapy	Concentration (µg/mL)	Concomitant Administered Drugs
A	PB	PB – 32.34	azitromycin; acetylcysteine; enoxaparin sodium; ceftriaxone; furosemide; ipatropium bromide; pantoprazole; paracetamol; theophylline; salbutamol.
B	PHT	PHT – 8.24	amoxicillin; azitromycin; bromazepam; clonazepam; chlorpromazine; ipatropium bromide; lysine acetylsalicylate; olanzapine; omeprazole; metilprednisolone; paracetamol; risperidone; salbutamol; theophylline; valproic acid.
C	CBZ	<i>trans</i> -diol – 7.22 CBZ-E – 2.97 CBZ – 10.18	amoxicillin; azitromycin; acetylcysteine; diazepam; enoxaparin sodium; lactulose; pantoprazole; paracetamol; piracetam; valproic acid.

CBZ-E, carbamazepine-10,11-epoxide; *trans*-diol, 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine.

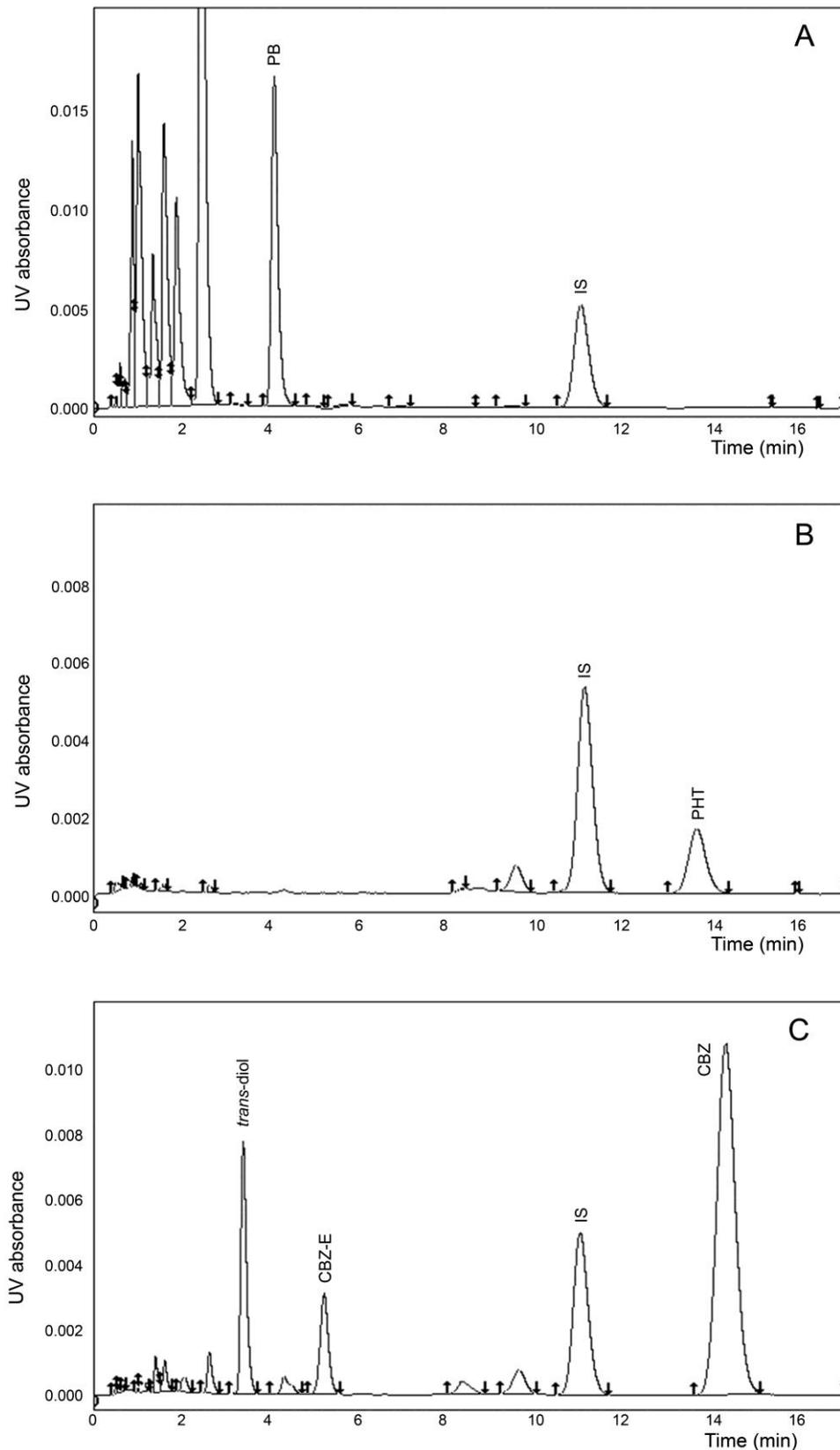


Figure II.2.4 Representative chromatograms of plasma samples obtained from epileptic patients treated with: (A) Phenobarbital (PB); (B) phenytoin (PHT) and (C) carbamazepine (CBZ). IS, internal standard.

A novel isocratic reversed-phase HPLC-UV method associated to a sample pre-treatment methodology based on protein precipitation followed by SPE was herein developed and fully validated for the simultaneous determination of PB, PRM, PHT, CBZ, LTG, OXC and some of their main metabolites CBZ-E, *trans*-diol and Lic in human plasma. The experimental results demonstrated that the reported bioanalytical method is selective, precise, accurate and sensitive, yielding also an efficient and reproducible sample extraction. Therefore, it can be applied in clinical settings as a useful tool for routine TDM of patients treated with such AEDs either in monotherapy, transitional or chronic polytherapy regimens, as well as in other pharmacokinetic-based studies involving these drugs.



II. 3. EXPERIMENTAL

HLPC-UV Methods for the Quantitative
Determination of PHT, CBZ, OXC, LTG
and their Main Metabolites in
Mouse Biological Matrices

II. 3.1. GENERAL INTRODUCTION

To achieve the utmost objective of the work underlying the present thesis, it was of paramount importance to have appropriate analytical techniques that enable the quantification of PHT, CBZ, OXC, LTG and their main metabolites in various biological matrices derived from the pharmacokinetic studies carried out in mice.

The chromatographic method previously described in *section II. 2.* for the simultaneous quantitative determination of the referred compounds in human plasma (SERRALHEIRO *et al.*, 2013b) was developed and validated with the intent of providing not only a suitable and useful tool for TDM of epileptic patients, but also to test and optimise the analytical conditions that would serve as a basis for the development of the bioanalytical methods applied in the quantification of drugs from the required animal samples. In fact, taking into account that several mouse biological matrices, and specifically mouse plasma, are commonly available in very limited amounts, the use of a surrogate matrix like the human plasma, with all its inherent features, may be assumed as a preferential approach during the first stages of any bioanalytical method optimisation. As a result, the process of method development is considerably faster, the number of animals needed is substantially lower and both the economical and ethical issues are more advantageous.

Therefore, taking as a starting point the formerly optimised conditions of the quantitative analytical method in human plasma, it was initially attempted the development and validation of an HPLC-UV technique for the simultaneous quantification of PHT, CBZ, OXC, LTG and some of their main metabolites (CBZ-E and Lic) in mouse plasma, brain and liver homogenates. Indeed, to obtain reliable experimental results it is essential that they are generated by properly developed and validated methods in the same and each separate biological matrix (of the same animal species) of the real samples to be analysed (SHAH *et al.*, 2000). Unfortunately, during the preliminary validation process, multiple and time-consuming difficulties have emerged which prevented the achievement of consistent, precise and accurate data. Exploratory results indicated that the transposition of the bioanalytical methodology from human plasma to mouse specimens did not gather the appropriate performance characteristics essential to assure a valid interpretation of the analytical outcome derived from the subsequent pharmacokinetic studies and therefore, it could not be implemented with confidence into our routine laboratory practice. On account of that, we decided to take advantage of techniques already developed and validated in our laboratory (CASTEL-BRANCO *et al.*, 2001; FORTUNA *et al.*, 2010) by introducing few adjustments to make them suitable for the intended purposes. As referred in *section II. 1.3.1.*, the inclusion of minor alterations in the original methods commonly implies the accomplishment of a *partial validation*

procedure to ensure the reliability of the obtained results; for this reason, two basilar analytical techniques were herein developed and partially validated enabling:

- Quantification of CBZ, CBZ-E, OXC, Lic and PHT in mouse plasma, brain (whole brain, olfactory bulb and frontal cortex) and liver tissues;
- Quantification of LTG in mouse plasma, brain (whole brain, olfactory bulb and frontal cortex) and liver tissues.

The next sections will provide a description of all the analytical procedures and results concerning the quantitative HPLC-UV methods developed and validated to support the analysis of the samples originated from the pharmacokinetic studies that will be presented in the following chapters of this thesis.

II. 3.2. HPLC-UV METHOD FOR THE QUANTITATIVE DETERMINATION OF CBZ, OXC, CBZ-E, LIC AND PHT IN MOUSE PLASMA, BRAIN AND LIVER HOMOGENATES

II. 3.2.1. INTRODUCTION

The unfruitful and longstanding efforts of developing and validating a reliable analytical technique that would enable the simultaneous quantification of PHT, CBZ, OXC, LTG and some of their metabolites in mouse matrices drove us to take advantage of an HPLC method, already implemented in our laboratory (FORTUNA *et al.*, 2010), by introducing the alterations required to make it adequate to be applied in the course of the current work. As mentioned before, when minor modifications are imposed to a previously validated method, the overall impact on bioanalytical performance should always be evaluated and therefore, a partial validation must be performed. In this section, a description of the HPLC-UV technique employed for the simultaneous determination of CBZ, CBZ-E, OXC and Lic and the individual quantification of PHT (Figure II.3.1) in mouse plasma, brain (whole brain, olfactory bulb and frontal cortex) and liver tissues will be provided. The validation parameters analysed in order to ensure the reliability of the experimental results obtained will be thoroughly discriminated as well.

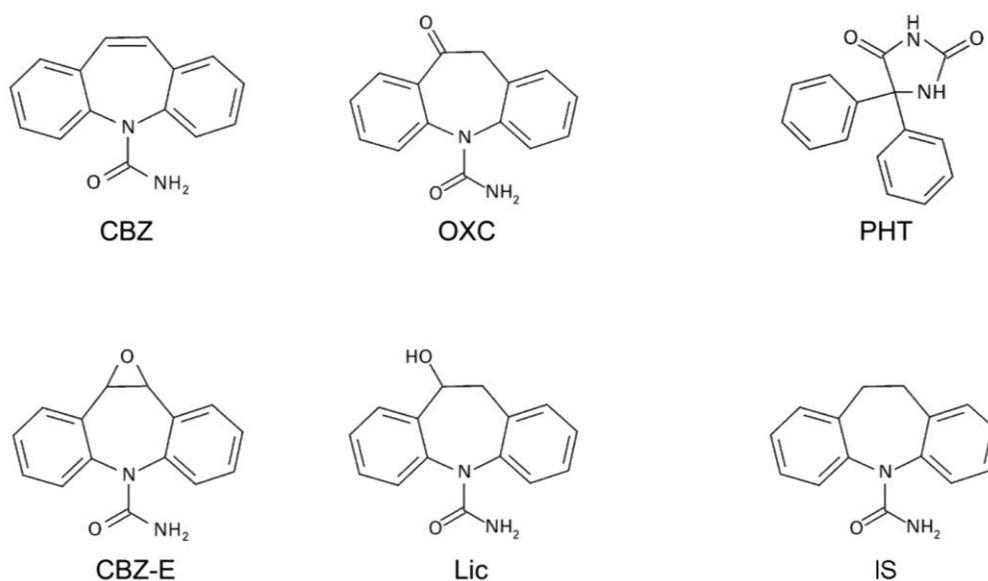


Figure II.3.1 Chemical structures of carbamazepine (CBZ), oxcarbazepine (OXC), phenytoin (PHT), carbamazepine-10,11-epoxide (CBZ-E), licarbazepine (Lic) and 10,11-dihydro-carbamazepine which was used as internal standard (IS).

II. 3.2.2. MATERIALS & METHODS

II. 3.2.2.1. Chemicals and Reagents

CBZ, CBZ-E, OXC and PHT were purchased from Sigma-Aldrich (St Louis, MO, USA). Lic and 10,11-dihydro-carbamazepine, used as IS, were kindly supplied by BIAL-Portela & C^a S.A. (S. Mamede do Coronado, Portugal). Methanol (HPLC gradient grade) and ethyl acetate were purchased from Fisher Scientific (Leicestershire, UK) while acetonitrile (HPLC gradient grade) was acquired from Lab-Scan (Sowinskięo, Poland) and ultrapure water (HPLC grade, 18.2 M Ω .cm) was obtained by means of a Milli-Q water apparatus from Millipore (Milford, MA, USA). Sodium dihydrogen phosphate dihydrate, di-sodium hydrogen phosphate dihydrate and hydrochloric acid fuming 37%, all used to prepare 0.1 M sodium phosphate buffer pH 5.0, were provided from Merck KGaA (Darmstadt, Germany).

II. 3.2.2.2. Animals and Blank Biological Matrices

Adult male CD-1 mice weighting 30-40 g were obtained from local certified animal facilities (Faculty of Healthy Sciences of the University of Beira Interior, Covilhã, Portugal) and maintained under controlled environmental conditions (12 h light/dark cycle, at 20 \pm 2 $^{\circ}$ C and relative humidity 50 \pm 5%) with free access to tap water and standard rodent diet (4RF21, Mucedola, Italy). Mice not subjected to any pharmacological treatment were used as the source of drug-free plasma, brain and liver tissues required for the method optimisation and validation studies. Blank matrices were gathered after sacrificing the animals by cervical dislocation followed by decapitation. Blood samples were collected into heparinised tubes and plasma was separated by centrifugation at 4000 rpm for 10 min (4 $^{\circ}$ C) and stored at -30 $^{\circ}$ C until use. After exsanguination, mice brain and liver tissues were quickly removed, weighed and homogenised with 0.1 M sodium phosphate buffer pH 5.0 (4 mL per gram of tissue) using a THOMAS[®] Teflon pestle tissue homogeniser. Tissue homogenates were then centrifuged at 4800 rpm for 15 min (4 $^{\circ}$ C) and the resultant supernatants were likewise frozen at -30 $^{\circ}$ C until being used. In order to mimic the sample processing of olfactory bulb and frontal cortex specimens, mice brains were dissected in small tissue amounts weighing approximately 0.02 g and 0.1 g, respectively. Each brain fragment was separately transferred into a 2.0 mL Eppendorf tube, homogenised with 1 mL of 0.1 M sodium phosphate buffer pH 5.0 using an ULTRA-TURRAX[®] device, and finally centrifuged for 15 min at 13400 rpm (4 $^{\circ}$ C). The obtained homogenate supernatants were conveniently packaged and stored according to the conditions described above.

All the experiments involving the collection of mice blank matrices and their care were conducted in conformity with the international regulations of the European Directive (2010) regarding the protection of laboratory animals used for scientific purposes (2010/63/EU) (EUROPEAN PARLIAMENT AND COUNCIL OF THE EUROPEAN UNION, 2010), and the experimental procedures employed were reviewed and approved by the Portuguese Food and Veterinary General Division (DGAV).

II. 3.2.2.3. Preparation of Standard Solutions

Stock solutions of CBZ, OXC, CBZ-E, Lic and PHT were individually prepared by dissolving appropriate amounts of each compound in methanol and then adequately diluted with the same solvent to obtain the corresponding intermediate solutions. Afterwards, different volumes of drug/metabolite stock and intermediate solutions were properly mixed and further diluted with methanol to afford six combined spiking solutions of all the analytes at distinct concentrations in order to spike the biological matrices always with the same volume of the appropriate combined spiking solution (10 μ L). Thus, each combined solution was daily employed for spiking aliquots of mice blank plasma and tissue (whole brain, olfactory bulb, frontal cortex and liver) homogenates allowing the preparation of the calibration standards required to construct six-point calibration curves in all the matrices considered. QC samples were likewise independently prepared in the same biological matrices at the LLOQ and at three representative concentration levels [low (QC₁), medium (QC₂) and high (QC₃)] of the calibration ranges.

Two methanolic IS working solutions at the concentration of 200 μ g/mL and 100 μ g/mL were prepared by dilution of an appropriate volume of the respective stock solution (1 mg/mL). All the stock, intermediate and combined solutions were stored at 4°C and protected from light, with exception of the IS working solutions which were prepared in every day of analysis.

A summary of all the solutions, calibration standards and QC samples prepared in the five biological matrices considered (plasma, brain, olfactory bulb, frontal cortex and liver of mice) were presented in *Appendix A1*. (Tables A1.1 and A1.2).

II. 3.2.2.4. Sample Preparation

Sample preparation was performed by submitting mice plasma and tissue homogenates to a SPE procedure according to the methodology formerly developed and optimised by Fortuna *et al.* (2010) with slight modifications.

Aliquots of 200 μL of mice plasma spiked with 10 μL of IS methanolic working solution (200 $\mu\text{g}/\text{mL}$) were added to 800 μL of 0.1 M sodium phosphate buffer (pH 5.0). The samples were briefly vortex mixed and subsequently loaded into Waters Oasis[®] HLB (30 mg, 1 mL) cartridges (Milford, MA, USA), previously conditioned with 1 mL of methanol, 1 mL of acetonitrile and 1 mL of water-acetonitrile (95:5, v/v). Upon sample elution, the SPE loaded cartridges were submitted to -30 kPa and washed four times with 1 mL of water followed by four more times with 1 mL of water-methanol (90:10, v/v); then the sorbents were dried under airflow for about 5 min. Thereafter, analytes were eluted with 1 mL of ethyl acetate applying a gentle vacuum and the eluates were evaporated to dryness at 45°C under a moderate nitrogen stream. The resultant residues were reconstituted with 100 μL of mobile phase, vortexed and placed in an ultrasonic bath at room temperature for approximately 1 min each. At the end, 20 μL of the final reconstituted extracts were injected into the HPLC system for analysis.

Posterior to thawing, tissue homogenates were centrifuged a second time at 13400 rpm for 30 min to obtain clearer samples, and 500 μL of brain and 250 μL of liver supernatants were used after being added to an appropriate volume of 0.1 M sodium phosphate buffer (pH 5.0) in order to make a total of 1 mL sample amount. All the brain and liver tissue samples, including the olfactory bulb and frontal cortex homogenate supernatants (1 mL) were spiked with 10 μL of IS working solution (100 $\mu\text{g}/\text{mL}$ for the olfactory bulb and 200 $\mu\text{g}/\text{mL}$ for the remaining matrices), and then subjected to the same SPE procedure already described for plasma. Finally, aliquots of 20 μL (brain, frontal cortex and liver) or 40 μL (olfactory bulb) of the reconstituted extracts were likewise injected into the chromatographic system to be analysed.

II. 3.2.2.5. HPLC-UV Instrumentation and Chromatographic Conditions

The HPLC analysis was carried out on a Shimadzu liquid chromatographic system equipped with a GDU-20A₅ degasser, a SIL-20A_{HT} autosampler, a CTO-10AS_{VP} column oven and a SPD-M20A diode array detector, all from Shimadzu Corporation (Kyoto, Japan). Data acquisition and instrumentation control were achieved by means of LCsolution software (Shimadzu Corporation, Kyoto, Japan).

Chromatographic separation of drugs/metabolites and IS was accomplished at 40°C on a reversed-phase LiChroCART[®] Purospher Star[®] C₁₈ column (55 mm x 4 mm, 3 μm ; Merck KGaD), using an isocratic elution with a mobile phase consisting of water-methanol-acetonitrile (64:30:6, v/v/v) pumped at a flow rate of 1 mL/min. The analytes' detection was performed at the wavelength of 235 nm and the total running time was set at 10 min.

II. 3.2.2.6. Method Validation

Even though the present HPLC-UV technique stemmed from an existing and already implemented bioanalytical method that has previously been fully validated (FORTUNA *et al.*, 2010), the reliability of this modified assay had to be necessarily demonstrated through a partial validation process. Indeed, partial validations are usually conducted to assess the validity of minor changes in a method with the intent of assuring comparable performances (BRIGGS *et al.*, 2014; DESILVA *et al.*, 2003). Generally, a partial validation can range from a single intra- or inter-assay precision and accuracy experiment to nearly a full validation, being the extent essentially determined by the nature of the modifications performed (BRIGGS *et al.*, 2014; EUROPEAN MEDICINES AGENCY, 2011; US FOOD AND DRUG ADMINISTRATION, 2001). In the absence of an established protocol, the partial validation procedure inevitably relies on the criterion of the analyst (GONZÁLEZ *et al.*, 2014). Therefore, the selection of the parameters to be evaluated should be made using a risk-based approach considering the potential impacts of any alterations in the method. The decision process of whether to carry out particular validation experiments will materially depend on the nature of changes to the method and their degree of significance (BRIGGS *et al.*, 2014). Taking all these considerations into account, the set of validation parameters analysed were selectivity, linearity, inter-day precision, inter-day accuracy and limit of quantification.

Selectivity was determined by evaluating the potential interference of mouse plasma, brain and liver endogenous substances at the same retention times of the analytes and IS. For that, all the considered blank matrices derived from six different mice were analysed under the described chromatographic conditions, and the resultant chromatograms were compared with those from spiked samples. In addition, possible interferences from ketamine and xylazine, the specific anaesthetics that will be employed during the pharmacokinetic studies conducted in mice, were also ascertained by injecting standard solutions of these compounds.

The linearity was assessed in each biological matrix within the defined concentration ranges, using six-point calibration curves prepared on three separate days ($n = 3$) by plotting analyte-IS peak height ratio against the corresponding nominal concentrations. The data were subjected to a weighted linear regression analysis using $1/x^2$ as weighting factor, which was selected taking the plots and the sums of absolute percentage of relative error into account (ALMEIDA *et al.*, 2002).

Inter-day precision and accuracy were investigated analysing each QC sample (QC₁, QC₂ and QC₃) on three consecutive days of the assay ($n = 3$). According to

bioanalytical method validation guidelines (EUROPEAN MEDICINES AGENCY, 2011; US FOOD AND DRUG ADMINISTRATION, 2001), the acceptance criterion for precision, which is expressed as % CV, must be equal to or lower than 15%; whereas accuracy, which is expressed as % Bias, must be within $\pm 15\%$.

The LLOQ was defined as the lowest concentration of the calibration curve that can be measured with adequate precision and accuracy. It was stipulated by analysing three replicates of each matrix spiked samples inter-daily ($n = 3$), wherein the absolute deviations lower than 20% for both CV and Bias values were accepted.

II. 3.2.3. RESULTS & DISCUSSION

II. 3.2.3.1. Optimisation of Chromatographic Conditions

The analytical conditions herein applied to achieve chromatographic separation and detection of CBZ, CBZ-E, OXC, Lic and PHT were the same to those employed in the method developed by Fortuna and collaborators (2010). In a preliminary phase, the potential for simultaneous quantification of CBZ, CBZ-E, OXC, Lic, PHT and IS was investigated by injecting individual solutions of all the analytes in HPLC system and subsequent comparison of their retention times with those obtained in a combined solution. Chromatographic separation of all the compounds was attained in less than 9 min. The IS was the last-eluting analyte with a retention time of approximately 8 min, and the order of elution of the remaining compounds was the following: Lic, CBZ-E, OXC, PHT and CBZ. Unfortunately, a partial co-elution between CBZ and PHT was observed which demanded the quantification of the latter separately.

Comparatively to the HPLC technique described before in *section II. 2.*, this method displays several important advantages in what concerns to the chromatographic conditions used. The absence of buffer and salts in the mobile phase allows the reduction of the back-pressure and prolong the column lifetime by avoiding the consequent formation of precipitates due to the limited solubility of salts in organic modifiers. The chromatographic separation was accomplished at 40°C, providing not only a good shape and resolution of the peaks but also a decrease of the analytes' overall retention times. As a result, the total running time was reduced, diminishing the amount of expensive and polluting solvents consumed.

II. 3.2.3.2. Optimisation of the Sample Preparation Procedure

The SPE methodology employed to isolate and extract the analytes of interest was the same for all the biological matrices considered (plasma, brain, olfactory bulb, frontal cortex and liver) and very similar to that developed by Fortuna *et al.* (2010), presenting only one additional step in the washing phase. Since no significant changes to the sample preparation procedure have been made, it was assumed that the extraction efficiency remained unaffected thus not requiring the assessment of the recovery parameter (BRIGGS *et al.*, 2014).

Given that this assay is based on a single SPE procedure, minimal sample manipulation is required which, in essence, reduces the time of handling and the source of errors.

II. 3.2.3.3. Method Validation

II. 3.2.3.3.1. Selectivity

The analysis of drug-free mouse samples derived from six different animals confirmed the absence of endogenous interferences at the retention times of the analytes and IS for all the biological matrices under investigation (plasma, brain and liver tissues). Moreover, none of the tested anaesthetics (ketamine and xylazine) were found to interfere with the intended chromatographic analysis. Representative chromatograms of blank and spiked mouse plasma and tissue homogenate samples are depicted in Figures II.3.2, II.3.3 and II.3.4.

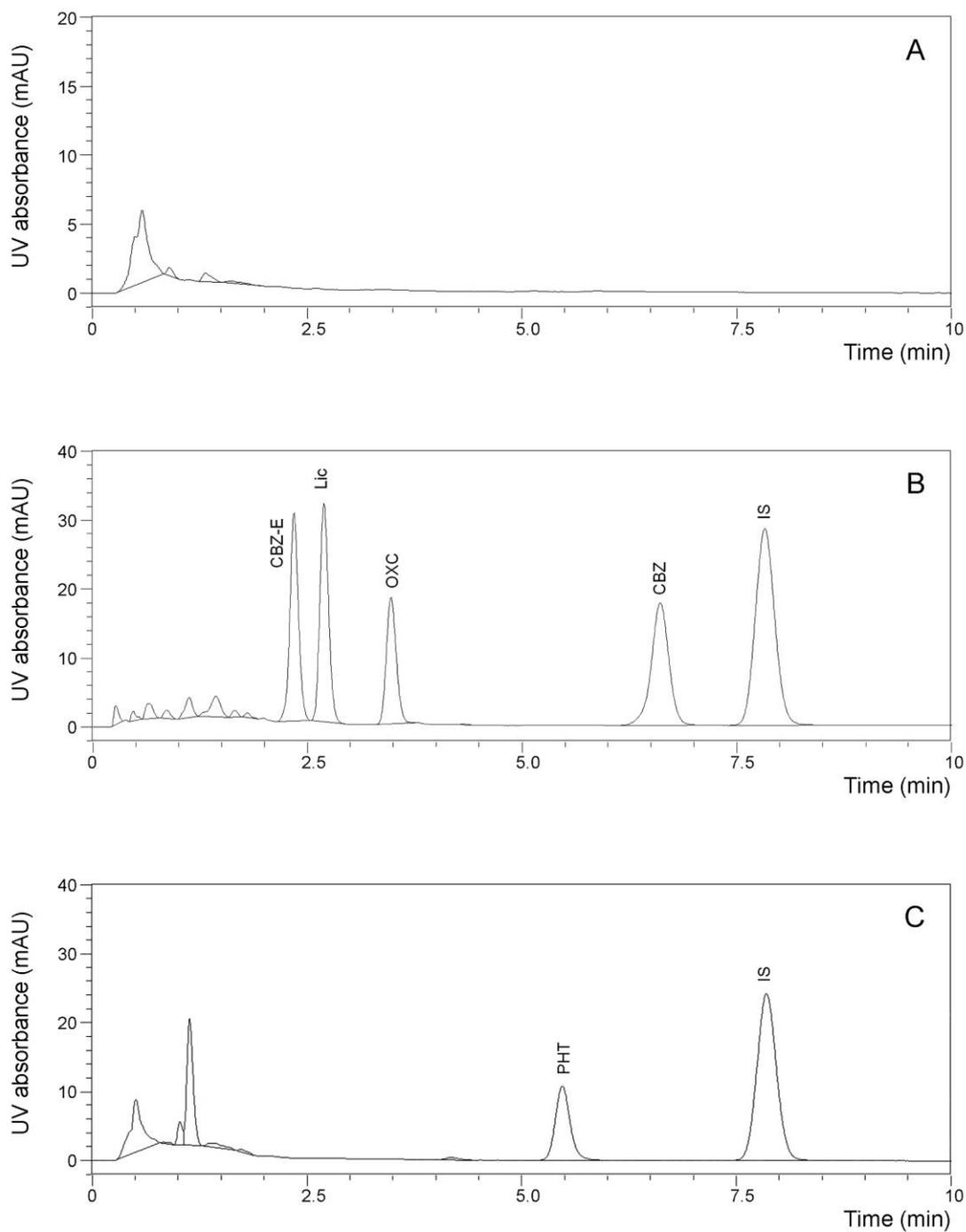


Figure II.3.2 Representative chromatograms of extracted mouse plasma: (A) blank plasma; (B) plasma spiked with licarbazepine (Lic), carbamazepine-10,11-epoxide (CBZ-E), oxcarbazepine (OXC), carbamazepine (CBZ) and internal standard (IS) at intermediate concentrations of the calibration ranges; (C) plasma spiked with phenytoin (PHT) and IS at the intermediate concentration of the calibration range.

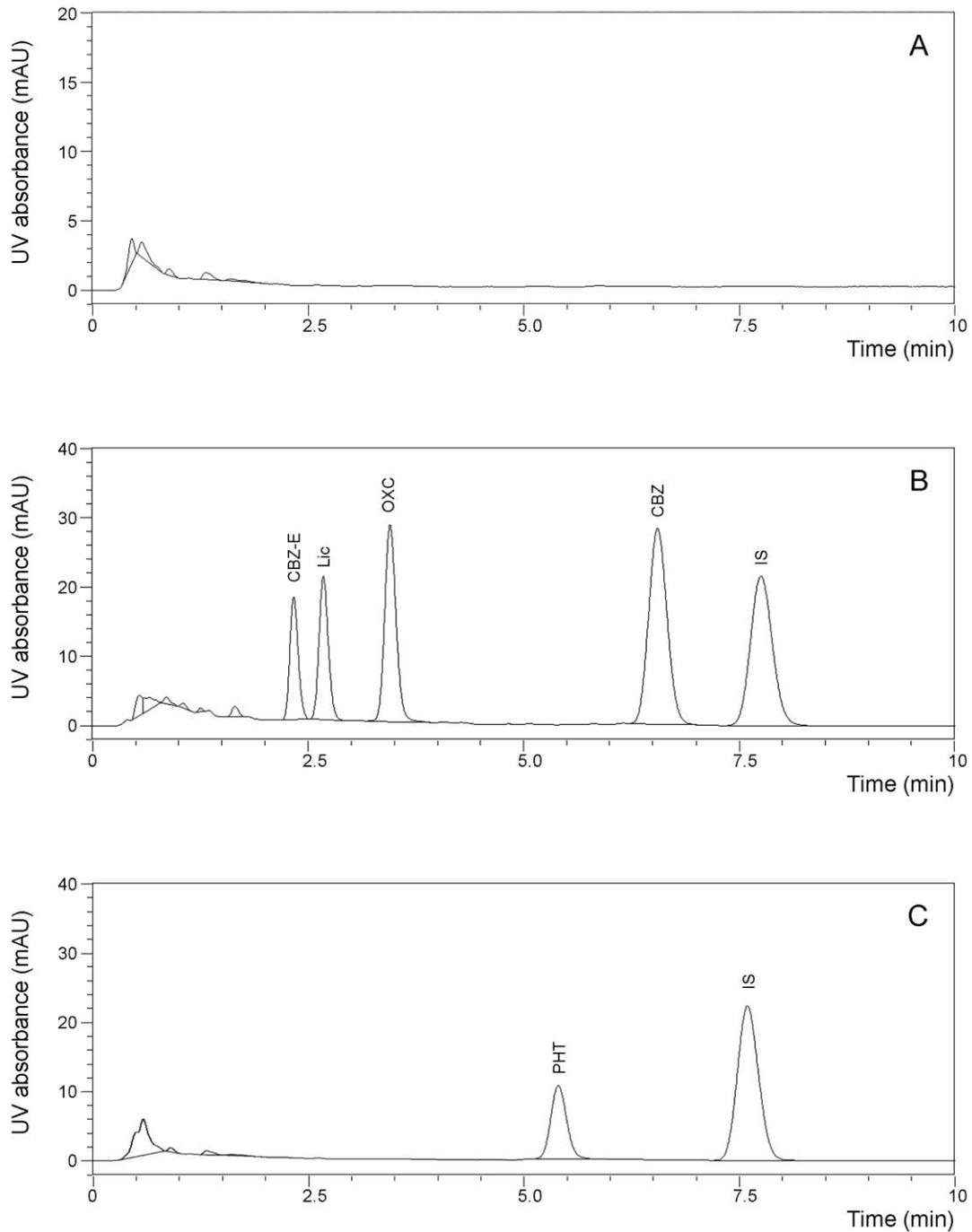


Figure II.3.3 Representative chromatograms of extracted mouse brain homogenate supernatant: (A) supernatant of blank brain homogenate; (B) supernatant of brain homogenate spiked with licarbazepine (Lic), carbamazepine-10,11-epoxide (CBZ-E), oxcarbazepine (OXC), carbamazepine (CBZ) and internal standard (IS) at intermediate concentrations of the calibration ranges; (C) supernatant of brain homogenate spiked with phenytoin (PHT) and IS at the intermediate concentration of the calibration range.

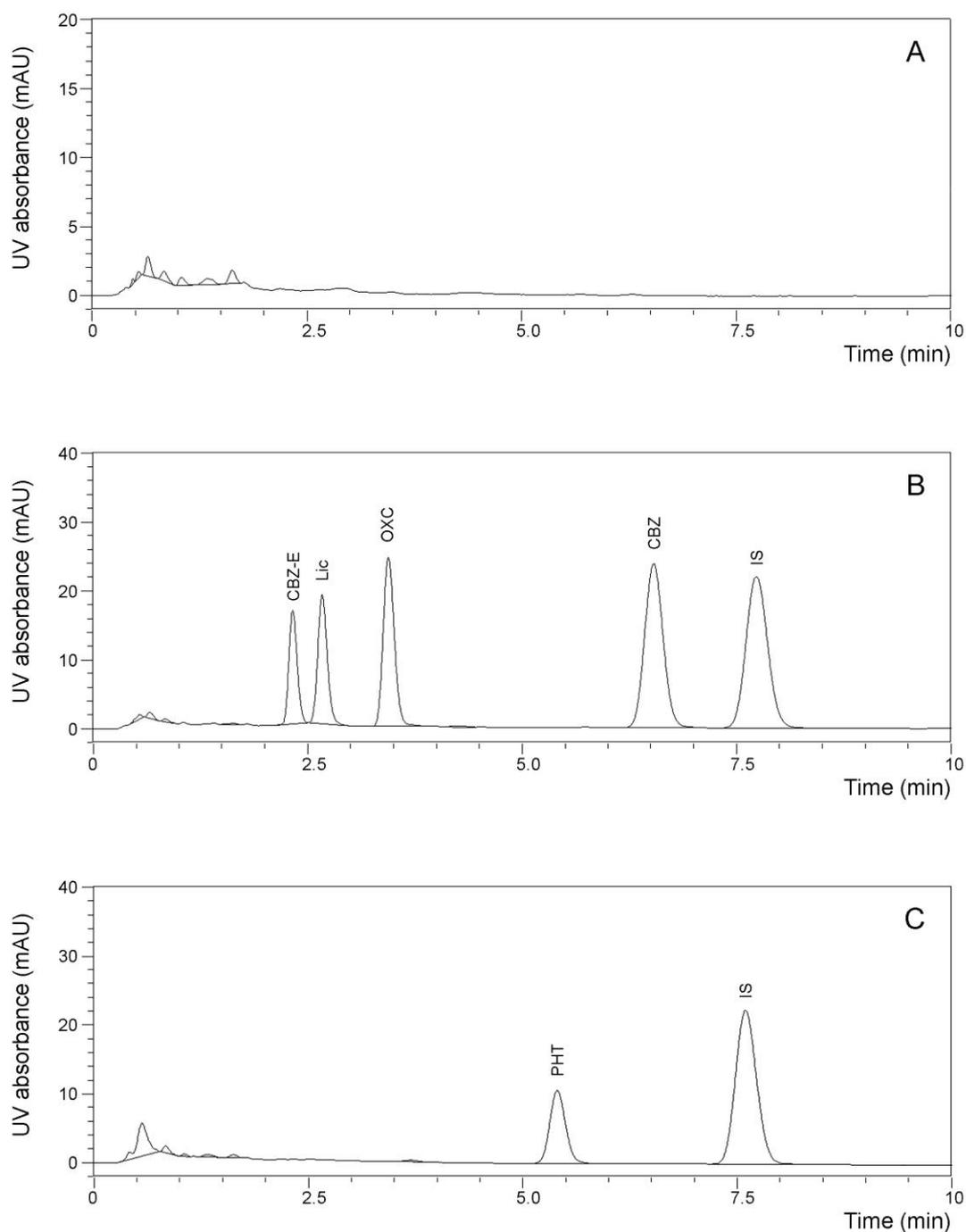


Figure II.3.4 Representative chromatograms of extracted mouse liver homogenate supernatant: (A) supernatant of blank liver homogenate; (B) supernatant of liver homogenate spiked with licarbazepine (Lic), carbamazepine-10,11-epoxide (CBZ-E), oxcarbazepine (OXC), carbamazepine (CBZ) and internal standard (IS) at intermediate concentrations of the calibration ranges; (C) supernatant of liver homogenate spiked with phenytoin (PHT) and IS at the intermediate concentration of the calibration range.

II. 3.2.3.3.2. Linearity and LLOQ

Six-point calibration curves were constructed for each compound in all matrices, plotting peak-height of analyte/IS ratios *versus* nominal concentrations and applying the best-fit weighting factor of $1/x^2$ in order to compensate for heteroscedasticity. The mean regression equations of the calibration curves and their determination coefficients are summarised in Table II.3.1, demonstrating the good linearity ($r^2 \geq 0.991$) of the method within the specified concentration ranges. As shown in Table II.3.1, the experimental values of LLOQ defined for all the compounds in all the considered mouse matrices were appropriately measured with acceptable inter-day precision and accuracy (% CV and % Bias $\leq 20\%$).

II. 3.2.3.3.3. Precision and Accuracy

The data depicted in Tables II.3.2, II.3.3 and II.3.4 correspond to the values of inter-day precision and accuracy determined at three different concentration levels (QC₁, QC₂ and QC₃) representative of the calibration range for all the compounds in mouse plasma and tissue homogenate supernatants (brain, olfactory bulb, frontal cortex and liver). The results revealed that the acceptance criteria of the international guidelines were fulfilled since the overall CV values did not exceed 10.77% and Bias ranged from -8.31% to 9.14%. These data indicate that, under the current analytical conditions, excellent precision and accuracy can be achieved for the quantitative determination of the referred drugs/metabolites in mouse biological matrices, being this HPLC-UV method reliable, accurate and reproducible.

Table II.3.1 Mean calibration parameters ($n = 3$) and inter-day precision (% CV) and accuracy (% Bias) values at the lower limit of quantification (LLOQ) ($n = 3$) of licarbazepine (Lic), carbamazepine-10,11-epoxide (CBZ-E), oxcarbazepine (OXC), carbamazepine (CBZ) and phenytoin (PHT) in mouse plasma, brain, olfactory bulb, frontal cortex and liver homogenate supernatants.

Analyte	Calibration Parameters			LLOQ			
	[] Range	Regression Equation ^a	r^2	Nominal Concentration	Experimental Concentration ^b	% CV	% Bias
Plasma							
Lic	0.2 - 60	$Y = 0.2421x - 0.0035$	0.996	0.2	0.210 ± 0.004	1.85	4.80
CBZ-E	0.4 - 30	$Y = 0.3027x - 0.0191$	0.999	0.4	0.421 ± 0.014	3.82	5.24
OXC	0.1 - 60	$Y = 0.4109x - 0.0071$	0.996	0.1	0.105 ± 0.003	2.84	5.26
CBZ	0.1 - 30	$Y = 0.3296x - 0.0037$	0.999	0.1	0.099 ± 0.006	6.67	-0.66
PHT	1.0 - 40	$Y = 0.0512x - 0.0032$	0.996	1.0	0.993 ± 0.024	2.55	-0.66
Brain							
Lic	0.05 - 15	$Y = 0.4601x - 0.0014$	0.991	0.05	0.048 ± 0.003	7.17	-3.89
CBZ-E	0.05 - 15	$Y = 0.5944x - 0.0058$	0.996	0.05	0.052 ± 0.002	4.48	3.90
OXC	0.05 - 15	$Y = 0.7826x - 0.0037$	0.993	0.05	0.055 ± 0.007	14.49	9.74
CBZ	0.1 - 15	$Y = 0.7101x - 0.0178$	0.997	0.1	0.102 ± 0.006	7.89	2.25
PHT	0.2 - 15	$Y = 0.1094x - 0.0058$	0.996	0.2	0.219 ± 0.020	12.06	9.43
Olfactory Bulb							
Lic	-	-	-	-	-	-	-
CBZ-E	-	-	-	-	-	-	-
OXC	0.01 - 4	$Y = 3.5558x - 0.0011$	0.992	0.01	0.009 ± 0.001	5.75	-5.14
CBZ	0.02 - 4	$Y = 3.6064x - 0.0089$	0.998	0.02	0.020 ± 0.001	3.63	-1.28
PHT	0.05 - 4	$Y = 0.5923x - 0.0037$	0.995	0.05	0.049 ± 0.001	1.76	-1.30
Frontal Cortex							
Lic	-	-	-	-	-	-	-
CBZ-E	-	-	-	-	-	-	-
OXC	0.025 - 7.5	$Y = 1.5652x - 0.0037$	0.993	0.025	0.027 ± 0.004	14.49	9.74
CBZ	0.05 - 7.5	$Y = 1.4202x - 0.0178$	0.997	0.05	0.051 ± 0.003	7.89	2.25
PHT	0.1 - 7.5	$Y = 0.2187x - 0.0058$	0.996	0.1	0.109 ± 0.010	12.06	9.48
Liver							
Lic	0.2 - 20	$Y = 0.2785x - 0.0105$	0.999	0.2	0.204 ± 0.011	6.31	2.13
CBZ-E	0.2 - 20	$Y = 0.2976x - 0.0019$	0.999	0.2	0.218 ± 0.012	5.82	9.18
OXC	0.2 - 20	$Y = 0.4049x - 0.0154$	0.997	0.2	0.208 ± 0.002	1.08	4.05
CBZ	0.2 - 20	$Y = 0.3820x - 0.0079$	0.999	0.2	0.203 ± 0.001	0.28	1.55
PHT	0.5 - 20	$Y = 0.0524x - 0.0026$	0.999	0.5	0.503 ± 0.015	3.36	0.51

^a Y represents analyte-IS peak height ratio (expressed in arbitrary height units); x represents analyte concentration (expressed in $\mu\text{g}/\text{mL}$ for plasma, brain and liver or μg for the olfactory bulb and frontal cortex samples). ^b Mean \pm Standard deviation, $n = 3$.

r^2 , coefficient of determination. Calibration range, nominal and experimental concentrations are expressed in $\mu\text{g}/\text{mL}$ for all matrices with the exception of olfactory bulb and frontal cortex, whose values are presented in μg .

Table II.3.2 Inter-day precision (% CV) and accuracy (% Bias) of licarbazepine (Lic), carbamazepine-10,11-epoxide (CBZ-E), oxcarbazepine (OXC), carbamazepine (CBZ) and phenytoin (PHT) in mouse plasma and brain samples at the low, medium and high concentrations of the calibration ranges ($n = 3$).

Analyte	Plasma				Brain			
	Nominal Value	Experimental Concentration ^a	% CV	% Bias	Nominal Value	Experimental Concentration ^a	% CV	% Bias
Lic	0.6	0.579 ± 0.015	2.69	-3.54	0.15	0.146 ± 0.004	2.82	-2.95
	30	30.675 ± 1.612	5.26	2.25	7.5	7.657 ± 0.186	2.43	2.10
	54	57.910 ± 2.160	3.73	7.24	13.5	14.504 ± 0.391	2.70	7.43
CBZ-E	1.2	1.206 ± 0.046	4.06	0.54	0.15	0.141 ± 0.003	2.23	-6.04
	15	15.335 ± 0.776	5.08	2.23	7.5	7.356 ± 0.221	3.02	-1.92
	27	27.223 ± 0.920	3.39	0.83	13.5	14.086 ± 0.398	2.82	4.34
OXC	0.3	0.291 ± 0.009	3.16	-2.88	0.15	0.161 ± 0.014	9.10	7.55
	30	30.589 ± 1.105	3.62	1.96	7.5	7.312 ± 0.215	2.94	-2.50
	54	57.707 ± 1.465	2.54	6.87	13.5	13.482 ± 0.840	6.24	-0.13
CBZ	0.3	0.290 ± 0.003	1.10	-3.24	0.3	0.284 ± 0.010	3.90	-5.41
	15	15.189 ± 0.401	2.64	1.26	7.5	7.263 ± 0.211	2.91	-3.17
	27	27.607 ± 0.482	1.75	2.25	13.5	14.007 ± 0.222	1.59	3.75
PHT	3	3.037 ± 0.079	2.64	1.22	0.6	0.605 ± 0.010	1.75	0.79
	20	20.107 ± 0.447	2.23	0.54	7.5	7.526 ± 0.390	5.22	0.35
	36	34.829 ± 2.603	7.49	-3.25	13.5	12.843 ± 1.38	10.77	-4.87

^a Mean ± Standard deviation, $n = 3$. Nominal and experimental concentrations are expressed in µg/mL.

Table II.3.3 Inter-day precision (% CV) and accuracy (% Bias) of oxcarbazepine (OXC), carbamazepine (CBZ) and phenytoin (PHT) in mouse olfactory bulb and frontal cortex samples at the low, medium and high concentrations of the calibration ranges ($n = 3$).

Analyte	Olfactory Bulb				Frontal Cortex			
	Nominal Value	Experimental Value ^a	% CV	% Bias	Nominal Value	Experimental Value ^a	% CV	% Bias
OXC	0.03	0.029 ± 0.001	3.74	-3.79	0.075	0.081 ± 0.007	9.10	7.55
	2	2.126 ± 0.091	4.30	6.30	3.75	3.656 ± 0.107	2.94	-2.50
	3.6	3.929 ± 0.177	4.50	9.14	6.75	6.741 ± 0.420	6.24	-0.13
CBZ	0.06	0.060 ± 0.003	5.39	0.45	0.15	0.142 ± 0.005	3.90	-5.41
	2	1.984 ± 0.054	2.75	-0.82	3.75	3.631 ± 0.105	2.91	-3.17
	3.6	3.815 ± 0.103	2.69	5.98	6.75	7.003 ± 0.111	1.59	3.75
PHT	0.15	0.138 ± 0.003	2.02	-8.31	0.3	0.302 ± 0.005	1.75	0.83
	2	1.931 ± 0.083	4.33	-3.45	3.75	3.765 ± 0.195	5.22	0.39
	3.6	3.625 ± 0.384	10.62	0.71	6.75	6.425 ± 0.689	10.77	-4.82

^a Mean ± Standard deviation, $n = 3$. Nominal and experimental values are expressed in µg.

Table II.3.4 Inter-day precision (% CV) and accuracy (% Bias) of licarbazepine (Lic), carbamazepine-10,11-epoxide (CBZ-E), oxcarbazepine (OXC), carbamazepine (CBZ) and phenytoin (PHT) in mouse liver samples at the low, medium and high concentrations of the calibration ranges ($n = 3$).

Analyte	Liver			
	Nominal Value	Experimental Concentration ^a	% CV	% Bias
Lic	0.6	0.577 ± 0.008	1.57	-3.83
	10	10.101 ± 0.422	4.20	1.01
	18	17.992 ± 0.245	1.37	-0.05
CBZ-E	0.6	0.580 ± 0.019	3.27	-3.41
	10	9.974 ± 0.449	4.50	-0.26
	18	18.190 ± 0.174	0.96	1.06
OXC	0.6	0.571 ± 0.014	2.57	-4.81
	10	10.534 ± 0.324	3.08	5.34
	18	18.903 ± 0.138	0.73	5.02
CBZ	0.6	0.602 ± 0.016	2.77	0.28
	10	10.136 ± 0.397	3.92	1.36
	18	18.213 ± 0.154	0.84	1.18
PHT	1.5	1.455 ± 0.098	6.98	-3.00
	10	10.426 ± 0.324	3.12	4.26
	18	18.274 ± 0.214	1.17	1.52

^a Mean ± Standard deviation, $n = 3$.

Nominal and experimental concentrations are expressed in µg/mL.

The reversed-phase HPLC-UV technique associated to the SPE sample pre-treatment procedure adapted from the method previously developed and fully validated by Fortuna *et al.* (2010) was herein successfully employed for the quantification of CBZ, CBZ-E, OXC, Lic and PHT in plasma and tissue (brain, olfactory bulb, frontal cortex and liver) homogenates of mice. Partial validation results have demonstrated that this assay is linear over the wide concentration ranges of each analyte in all the matrices analysed and fulfils the fundamental international requirements, being selective, precise, accurate and reproducible. Accordingly, it has been proven that the present method is suitable to support and can be applied with confidence in the analysis of samples from the subsequent pharmacokinetic studies underlying the work of the current thesis.

II. 3.3. HPLC-UV METHOD FOR THE QUANTIFICATION OF LTG IN MOUSE PLASMA, BRAIN AND LIVER HOMOGENATES

II. 3.3.1. INTRODUCTION

The analytical methodology used for the quantitative determination of LTG in biological matrices of mice was initially based on the assay already developed and validated by Castel-Branco *et al.* (2001). The introduction of some changes to the original method have been made with the intent of achieving the optimal experimental and analytical conditions for the reliable quantification of LTG (Figure II.3.5) in mouse plasma, brain (whole brain, olfactory bulb and frontal cortex) and liver tissues. Similarly to the preceding section (*section II. 3.2.*), a thorough description of the HPLC-UV technique and sample preparation procedure employed along with the validation parameters analysed will also be herein supplied.

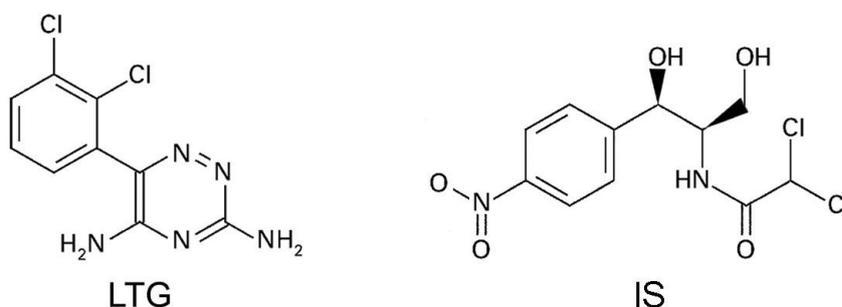


Figure II.3.5 Chemical structures of lamotrigine (LTG) and chloramphenicol which was used as internal standard (IS).

II. 3.3.2. MATERIALS & METHODS

II. 3.3.2.1 Chemicals and Reagents

LTG was kindly supplied by Bluepharma (Coimbra, Portugal) whereas chloramphenicol, used as IS, was commercially acquired from Sigma-Aldrich (St Louis, MO, USA). Methanol (HPLC gradient grade) was purchased from Fisher Scientific (Leicestershire, UK), acetonitrile (HPLC gradient grade) from Lab-Scan (Sowinskięgo, Poland) and ultrapure water (HPLC grade, 18.2 M Ω .cm) was prepared by means of a Milli-Q water apparatus from Millipore (Milford, MA, USA). Ethyl acetate was acquired from

Fisher Scientific (Leicestershire, UK). TEA and potassium dihydrogen phosphate, as well as sodium dihydrogen phosphate dihydrate, di-sodium hydrogen phosphate dihydrate and hydrochloric acid fuming 37%, used to prepare 0.1 M sodium phosphate buffer pH 5.0, were all obtained from Merck KGaA (Darmstadt, Germany).

II. 3.3.2.2. Animals and Blank Biological Matrices

Adult male CD-1 mice weighting 30-40 g were obtained from local certified animal facilities (Faculty of Healthy Sciences of the University of Beira Interior, Covilhã, Portugal) and maintained under controlled environmental conditions (12 h light/dark cycle, at $20\pm 2^\circ\text{C}$ and relative humidity $50\pm 5\%$) with free access to tap water and standard rodent diet (4RF21, Mucedola, Italy). Mice not subjected to any pharmacological treatment were used as the source of drug-free plasma, brain and liver tissues required for the method optimisation and validation studies. Blank matrices were gathered after sacrificing the animals by cervical dislocation followed by decapitation. Blood samples were collected into heparinised tubes and plasma was separated by centrifugation at 4000 rpm for 10 min (4°C) and stored at -30°C until use. After exsanguination, mice brain and liver tissues were quickly removed, weighed and homogenised with 0.1 M sodium phosphate buffer pH 5.0 (4 mL per gram of tissue) using a THOMAS® Teflon pestle tissue homogeniser. Tissue homogenates were then centrifuged at 4800 rpm for 15 min (4°C) and the resultant supernatants were likewise frozen at -30°C until being used. In order to mimic the sample processing of olfactory bulb and frontal cortex specimens, mice brains were dissected in small tissue amounts weighing approximately 0.02 g and 0.1 g, respectively. Each brain fragment was separately transferred into a 2.0 mL Eppendorf tube, homogenised with 0.5 mL of 0.1 M sodium phosphate buffer pH 5.0 using an ULTRA-TURRAX® device, and finally centrifuged for 15 min at 13400 rpm (4°C). The obtained homogenate supernatants were conveniently packaged and stored according to the conditions described above.

All the experiments involving animals and their care were conducted in conformity with the international regulations of the European Directive (2010) regarding the protection of laboratory animals used for scientific purposes (2010/63/EU) (EUROPEAN PARLIAMENT AND COUNCIL OF THE EUROPEAN UNION, 2010), and the experimental procedures employed were reviewed and approved by the Portuguese DGAV.

II. 3.3.2.3. Preparation of Standard Solutions

Stock solutions of LTG were prepared by dissolving appropriate amounts of compound powder in methanol, and then adequately diluted with the same solvent to obtain the two corresponding intermediate solutions (intermediate solutions A and B). Thereafter, different volumes of LTG stock and intermediate solutions were once again properly diluted with methanol to afford six spiking solutions at distinct concentrations. Calibration curves were daily constructed using the resultant calibration standards to spike aliquots of all the mice matrices under investigation: plasma, brain (whole brain, olfactory bulb and frontal cortex) and liver homogenates. QC samples were likewise independently prepared in the same biological matrices at the LLOQ and at three representative concentration levels [low (QC₁), medium (QC₂) and high (QC₃)] of the entire calibration range.

A methanolic stock solution of IS (2 mg/mL) was daily diluted in the same solvent to afford a working solution at the concentration of 400 µg/mL. All the stock, intermediate and spiking solutions were stored at 4°C and protected from light, with exception of the IS working solution which was prepared in every day of analysis.

A summary of all the solutions, calibration standards and QC samples prepared in the five biological matrices considered (plasma, brain, olfactory bulb, frontal cortex and liver of mice) were presented in *Appendix A2*. (Tables A2.1 and A2.2).

II. 3.3.2.4. Sample Preparation

The pre-treatment of samples was performed by submitting mice plasma and tissue homogenate supernatants to a LLE procedure optimised from the methodology presented by Castel-Branco *et al.* (2001).

Each aliquot of 200 µL of mice plasma spiked with 10 µL of IS methanolic working solution (400 µg/mL) was added to 500 µL of ethyl acetate (extraction solvent), vortex-mixed for 1 min and then centrifuged during 5 min at 13400 rpm. The upper organic layer was transferred to a clean glass tube while the residual aqueous phase was re-extracted once again, applying the same experimental proceeding. The obtained organic extracts were combined, evaporated to dryness under a gentle nitrogen stream at 45°C, and the residue was further reconstituted with 100 µL of mobile phase. The reconstituted extracts were transferred to a 1.5 mL Eppendorf tube, centrifuged (2 min, 13400 rpm) and subjected to an additional 0.22 µm Spin-X filtration before injection. At the end, 20 µL of the final filtrates were injected into the chromatographic system for analysis.

To extract LTG from tissue samples, 250 μL of liver and 500 μL of brain, olfactory bulb and frontal cortex homogenate supernatants were used after being spiked with 10 μL of the IS working solution. While brain specimens (whole brain, olfactory bulb and frontal cortex) were immediately submitted to the LLE procedure described above, liver samples were firstly added to 250 μL of acetonitrile, vortex-mixed for 1 min and centrifuged over 10 min at 13400 rpm in order to precipitate protein content. Then, the resultant purified supernatants were likewise extracted according to the same pre-treatment conditions aforementioned. In contrast with plasma, wherein a supplementary filtration step was required prior to injection, following centrifugation (2 min, 13400 rpm), 20 μL of the final reconstituted extracts from all tissue samples were directly injected into the HPLC apparatus to be analysed.

II. 3.3.2.5. HPLC-UV Instrumentation and Chromatographic Conditions

The HPLC analysis was carried out on a Shimadzu liquid chromatographic system equipped with a GDU-20A₅ degasser, a SIL-20A_{HT} autosampler, a CTO-10AS_{VP} column oven and a SPD-M20A diode array detector, all from Shimadzu Corporation (Kyoto, Japan). Data acquisition and instrumentation control were achieved by means of LCsolution software (Shimadzu Corporation, Kyoto, Japan).

Chromatographic separation was performed at 25°C on a reversed-phase LiChroCART® Purospher Star® C₁₈ column (55 mm x 4 mm, 3 μm ; Merck KGaD), using an isocratic elution with a mobile phase composed of 0.05 M potassium dihydrogen phosphate solution-methanol-acetonitrile-TEA (79.7:10:10:0.3, v/v/v/v) pumped at a flow rate of 1 mL/min. LTG and IS were detected at the wavelength of 306 nm and the total running time was set at 12 min.

II. 3.3.2.6. Method Validation

As previously mentioned in *section II. 3.2.2.6.*, the scarcity of established guidelines that would help to standardise the procedures of a partial validation protocol, dictates that the selection of the parameters to be evaluated should essentially be made using a risk-based approach in which the nature of all the changes imposed to the original method, their potential impacts and degree of significance are taken into account. Considering that a major change to the mobile phase in terms of composition and proportion of constituents has been herein performed, the overall performance of the assay should be critically assessed and therefore, the linearity, limit of quantification, inter- and intra-day precision and accuracy have been the fundamental validation

parameters analysed. Given that a different IS has been used and few alterations were introduced into the initial sample preparation methodology, the assessment of the analytes' recovery as well as the selectivity of the method also proved to be required.

Selectivity was determined by evaluating the potential interference of mouse plasma, brain and liver endogenous substances at the same retention times of LTG and IS. For that, all the considered blank matrices derived from six different mice were analysed under the described chromatographic conditions, and the resultant chromatograms were compared with those from spiked samples. In addition, possible interferences from ketamine and xylazine, the specific anaesthetics that will be employed during the pharmacokinetic studies conducted in mice, were also ascertained by injecting standard solutions of these compounds.

The linearity was assessed in each biological matrix within the defined concentration ranges, using six-point calibration curves prepared on three separate days ($n = 3$) by plotting LTG-IS peak height ratio against the corresponding nominal concentrations. The data were subjected to a weighted linear regression analysis using $1/x^2$ as weighting factor, which was selected taking the plots and the sums of absolute percentage of relative error into account (ALMEIDA *et al.*, 2002).

Precision and accuracy were investigated both inter-daily, by testing each QC sample (QC₁, QC₂ and QC₃) on three consecutive days of the assay ($n = 3$) and intra-daily, through the analysis of three replicates of the each QC sample (QC₁, QC₂ and QC₃) within a single day ($n = 3$). According to bioanalytical method validation guidelines (EUROPEAN MEDICINES AGENCY, 2011; US FOOD AND DRUG ADMINISTRATION, 2001), the acceptance criterion for precision, which is expressed as % CV, must be equal to or lower than 15%; whereas accuracy, which is expressed as % Bias, must be within $\pm 15\%$.

The LLOQ was defined as the lowest concentration of the calibration curve that can be measured with adequate precision and accuracy. It was stipulated by analysing three replicates of each matrix spiked samples inter-daily ($n = 3$), wherein the absolute deviations lower than 20% for both CV and Bias values were accepted.

The extraction efficiency of LTG from the overall biological matrices considered was investigated at the three independent concentration levels (QC₁, QC₂ and QC₃) representative of the calibration range. The absolute recovery was calculated comparing the analytes peak heights from extracted QC samples with those obtained after direct injection of non-extracted aqueous solutions at the same nominal concentrations ($n = 3$). The recovery of the IS was also determined at the concentration used in sample analysis.

II. 3.3.3. RESULTS & DISCUSSION

II. 3.3.3.1. Optimisation of Chromatographic Conditions

As a starting point, the chromatographic separation of LTG and IS was firstly attempted applying analytical conditions similar to those originally employed by Castel-Branco *et al.* (2001). A mobile phase consisting of 69.7% 0.1 M potassium dihydrogen phosphate buffer, 30% of methanol and 0.3% of TEA pumped at a flow rate of 1 mL/min provided a good shape and symmetry of the peaks and the elution of the compounds was accomplished in a short period of time (< 5 min); however it was associated to a poor peak resolution. Indeed, under these conditions, LTG and chloramphenicol did not separate adequately whereby the adjustment of both the composition and proportion of the mobile phase constituents was found to be necessary. Thus, a mixture composed of 0.05 M potassium dihydrogen phosphate solution-methanol-acetonitrile-TEA in the proportion of 79.7:10:10:0.3 (v/v/v/v) demonstrated to give the best correlation in terms of separation, resolution and retention time of the peaks in which LTG and IS eluted at approximately 5.1 and 7.8 min, respectively. In fact, no significant differences concerning the chromatographic response were ascertained between the use of 0.1 and 0.05 M potassium dihydrogen phosphate buffer and the inclusion of acetonitrile, as a component of the mobile phase, allowed the complete separation of the analytes within a convenient running time.

The unfeasibility of using the structural analogue [3,5-diamino-6-(2-methoxyphenyl)-1,2,4-triazine] of LTG as the IS led to the search for a suitable alternative compound. Chloramphenicol was herein selected since it has already been successfully used by Mattar *et al.* (1998) for the quantification of LTG in plasma samples and exhibited an appropriate extractive and chromatographic behaviour in the course of our preliminary experiments.

II. 3.3.3.2. Optimisation of the Sample Preparation Procedure

The sample preparation methodology implemented for the extraction of LTG and IS from plasma and tissue homogenates of mice was essentially based on the experimental procedures reported by Castel-Branco *et al.* (2001) with slight modifications. In an early phase, a variety of LLE solvents, including chloroform, diethyl ether, *tert*-butyl methyl ether, *n*-hexane and ethyl acetate were tested to extract the analytes from mouse biological matrices but it was the latter that enabled the best outcome in regard to selectivity (absence of endogenous interfering substances) and recovery. The influence of sodium hydroxide addition onto the overall extractive

efficiency was investigated and no notable differences were observed between those samples that were or were not previously basified. In opposition to a one-step LLE, the multiple sample preparation procedure consisting of two partial extractions, using small volumes of the organic solvent at a time, yielded higher recovery values.

Due to the more complex nature of liver tissue specimens, a combined sample preparation methodology comprising a first PP followed by the LLE was required. Among the distinct precipitating agents (20% trichloroacetic acid, methanol and acetonitrile) investigated, the acetonitrile, in the proportion of 1:1 proved to be the most appropriate one since it allowed an efficient sample deproteinisation by providing cleaner chromatograms with minimal compound loss.

II. 3.3.3.3. Method Validation

II. 3.3.3.3.1. Selectivity

The selectivity of the method was established by analysing six independent sources of the drug-free mouse samples. The tested blanks from all the biological matrices under investigation (plasma, brain and liver tissues) confirmed the absence of endogenous interferences at the retention times of the drug and IS. Furthermore, none of the anaesthetics standard solutions tested (ketamine and xylazine) were found to interfere with the intended chromatographic analysis. Representative chromatograms of blank and spiked mouse plasma and tissue homogenate samples are depicted in Figures II.3.6, II.3.7 and II.3.8.

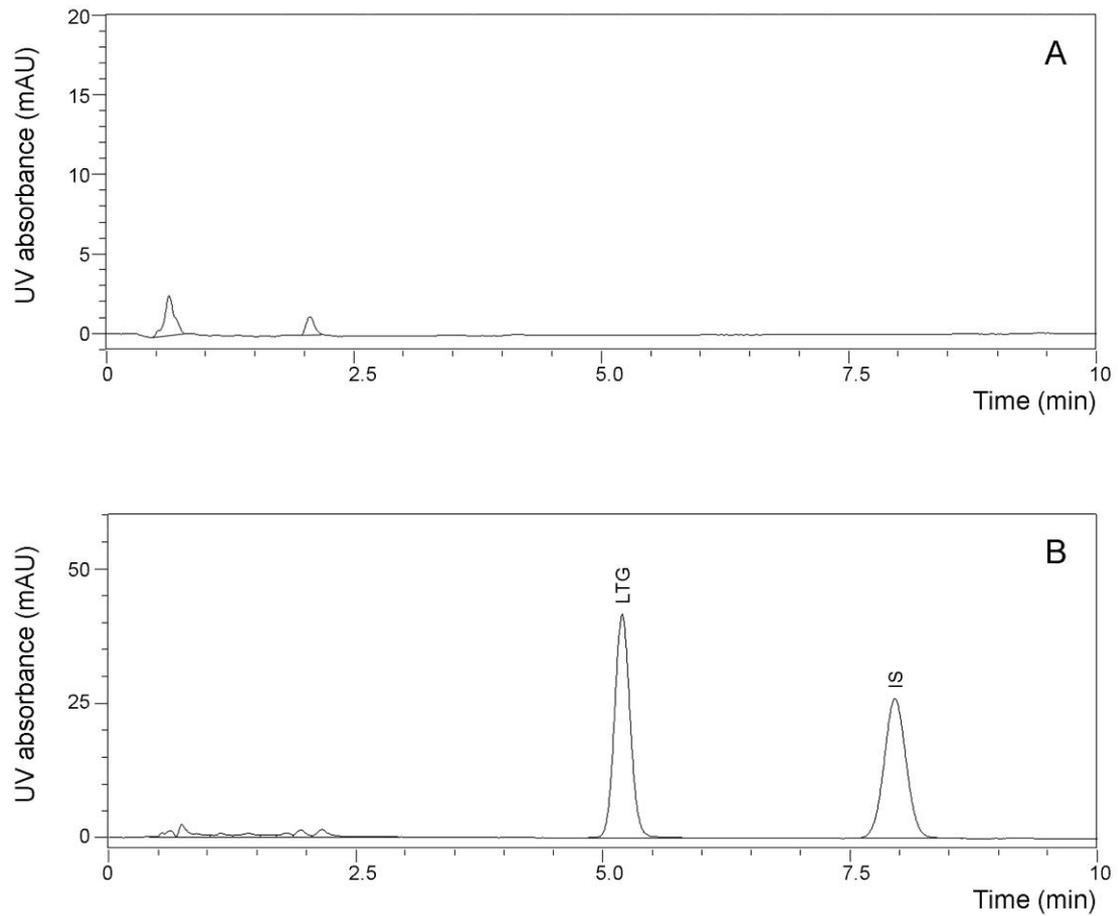


Figure II.3.6 Representative chromatograms of extracted mouse plasma: (A) blank plasma; (B) plasma spiked with lamotrigine (LTG) and internal standard (IS) at the intermediate concentration of the calibration range.

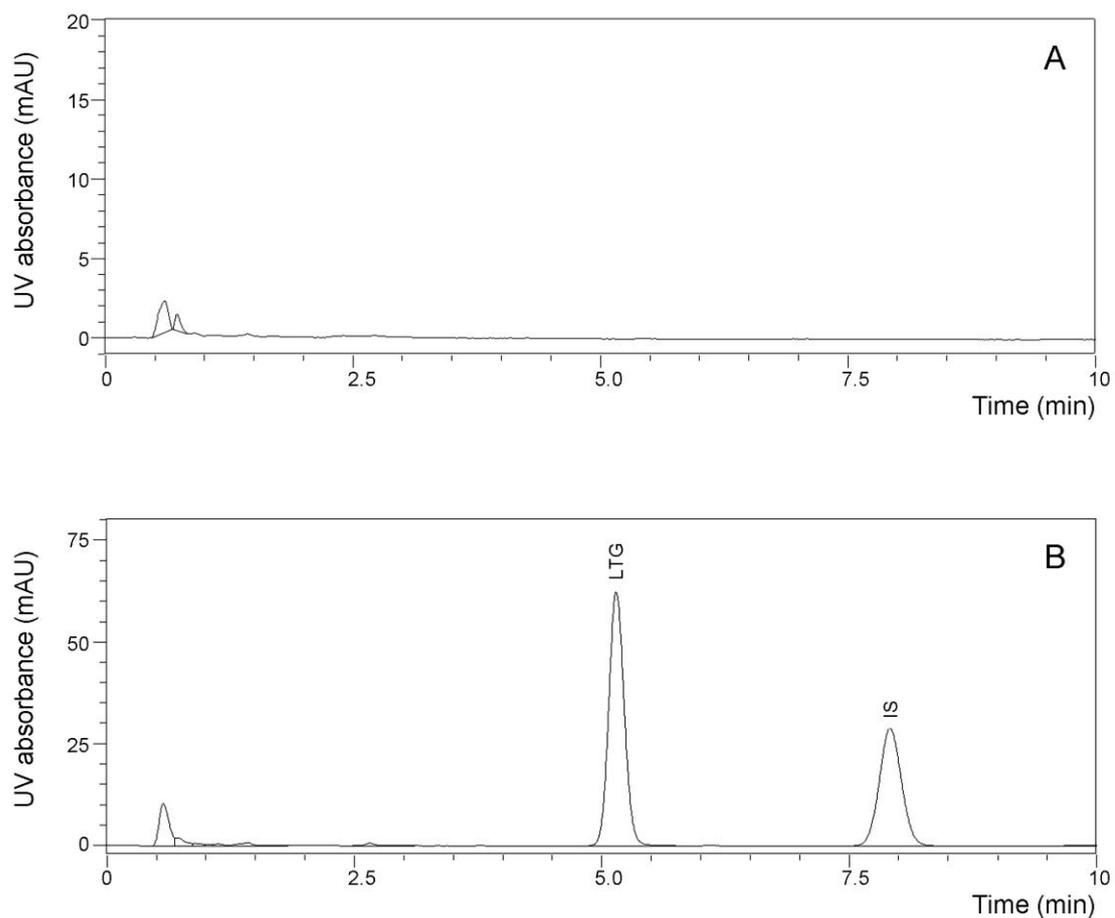


Figure II.3.7 Representative chromatograms of extracted mouse brain homogenate supernatant: (A) supernatant of blank brain homogenate; (B) supernatant of brain homogenate spiked with lamotrigine (LTG) and internal standard (IS) at the intermediate concentration of the calibration range.

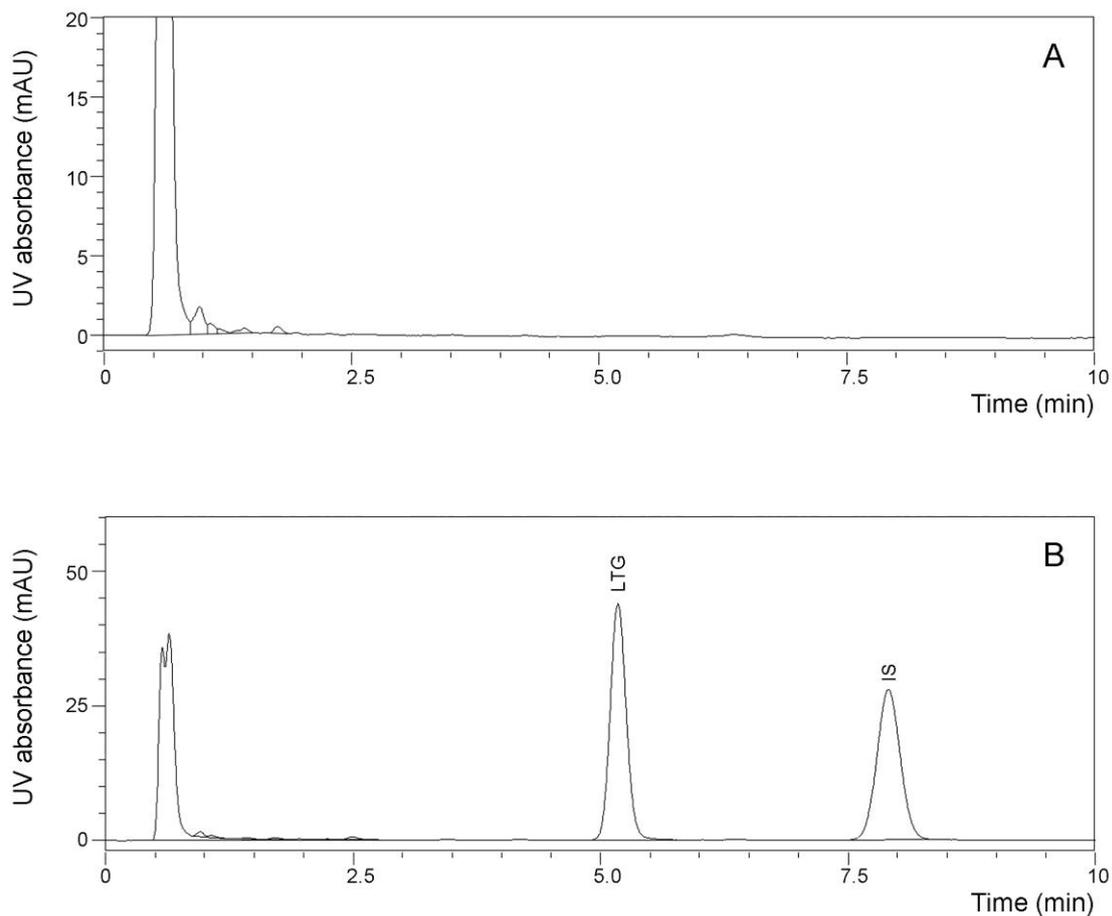


Figure II.3.8 Representative chromatograms of extracted mouse liver homogenate supernatant: (A) supernatant of blank liver homogenate; (B) supernatant of liver homogenate spiked with lamotrigine (LTG) and internal standard (IS) at the intermediate concentration of the calibration range.

II. 3.3.3.3.2. Linearity and LLOQ

Calibration curves of LTG in all the mouse biological matrices considered were constructed on three consecutive days by plotting LTG-IS peak height ratios against the corresponding nominal concentrations. The slope, intercept and determination coefficient (r^2) were calculated through a weighted least squares linear regression using $1/x^2$ as the best-fit weighting factor. Mean calibration parameters are summarised in Table II.3.5, demonstrating the linearity ($0.989 \leq r^2 \leq 0.997$) of the method over the LTG concentration ranges studied. The LLOQ of the assay was experimentally set with acceptable precision (% CV ≤ 6.97) and accuracy ($3.94 \leq \% \text{ Bias} \leq 9.06$) in all the matrices under investigation, as shown in Table II.3.5.

Table II.3.5 Mean calibration parameters ($n = 3$) and inter-day precision (% CV) and accuracy (% Bias) values at the lower limit of quantification (LLOQ) ($n = 3$) of lamotrigine (LTG) in mouse plasma, brain, olfactory bulb, frontal cortex and liver homogenate supernatants.

	Matrix				
	Plasma	Brain	Olfactory Bulb	Frontal Cortex	Liver
Calibration Parameters					
Concentration Range	0.20 - 30	0.05 - 15	0.025 - 7.5	0.025 - 7.5	0.20 - 20
Regression Equation ^a					
Slope	0.1254	0.2670	0.5536	0.5341	0.1677
Intercept	-0.0026	-0.0007	-0.0035	-0.0007	-0.0027
r^2	0.997	0.992	0.993	0.992	0.989
LLOQ					
Nominal Value	0.20	0.05	0.025	0.025	0.20
Experimental Value ^b	0.218 \pm 0.014	0.052 \pm 0.002	0.0266 \pm 0.0004	0.0260 \pm 0.0010	0.217 \pm 0.006
% CV	6.97	4.25	1.96	4.25	3.02
% Bias	9.06	3.95	6.36	3.94	8.58

^a $Y = bx + a$, where Y represents LTG-IS peak height ratio (expressed in arbitrary height units); b represents the slope of the calibration curve; x represents LTG concentration (expressed in $\mu\text{g}/\text{mL}$ for plasma, brain and liver or μg for the olfactory bulb and frontal cortex samples); a represents the intercept with the origin.

^b Mean \pm Standard deviation, $n = 3$.

r^2 , coefficient of determination. Calibration range, nominal and experimental concentrations are expressed in $\mu\text{g}/\text{mL}$ for all matrices with the exception of olfactory bulb and frontal cortex, whose values are presented in μg .

II. 3.3.3.3. Precision and Accuracy

The inter- and intra-day precision and accuracy were determined from three replicate analysis ($n = 3$) of mouse plasma and tissue homogenate samples containing LTG at three different concentrations covering the low, medium and high levels of the calibration curves. According to the results depicted in Table II.3.6, the overall CV values did not exceed 12.38% and Bias varied from -9.71% to 12.12%, fulfilling the acceptance limits recommended by the international guidelines. Thus, these data demonstrate that this HPLC-UV method is precise, accurate and reproducible, enabling the reliable quantification of LTG in all the mouse matrices analysed.

Table II.3.6 Inter- and intra-day precision (% CV) and accuracy (% Bias) of lamotrigine (LTG) in mouse plasma, brain, olfactory bulb, frontal cortex and liver samples at the low, medium and high concentrations of the calibration range ($n = 3$).

Matrix	Nominal Value	Inter-day			Intra-day		
		Experimental Value ^a	% CV	% Bias	Experimental Value ^a	% CV	% Bias
Plasma							
	0.6	0.582 ± 0.005	0.89	-3.06	0.585 ± 0.007	1.15	-2.56
	15	13.976 ± 0.533	3.82	-6.83	14.279 ± 0.189	1.33	-4.80
	27	27.736 ± 0.086	0.31	2.72	27.525 ± 0.505	1.84	1.94
Brain							
	0.15	0.145 ± 0.002	1.46	-3.36	0.143 ± 0.002	9.10	-4.50
	7.5	7.341 ± 0.351	4.78	-2.12	7.425 ± 0.153	2.06	-0.99
	13.5	14.227 ± 0.300	2.11	5.38	14.781 ± 0.337	2.28	9.49
Olfactory Bulb							
	0.075	0.072 ± 0.008	12.38	-3.49	-	-	-
	3.75	3.868 ± 0.373	9.66	3.16	-	-	-
	6.75	7.234 ± 0.238	3.30	7.16	-	-	-
Frontal Cortex							
	0.075	0.072 ± 0.001	1.46	-3.37	-	-	-
	3.75	3.670 ± 0.175	4.78	-2.14	-	-	-
	6.75	7.112 ± 0.150	2.11	5.36	-	-	-
Liver							
	0.6	0.543 ± 0.013	2.44	-9.56	0.573 ± 0.010	1.78	-4.51
	10	9.029 ± 0.617	6.84	-9.71	9.769 ± 0.160	1.64	-2.31
	18	20.089 ± 0.569	2.83	11.60	20.181 ± 0.551	2.73	12.12

^a Mean ± Standard deviation, $n = 3$. Nominal and experimental values are expressed in $\mu\text{g/mL}$ for all matrices with the exception of the olfactory bulb and frontal cortex samples in which the values are expressed in μg .

II. 3.3.3.3.4. Recovery

Absolute recoveries of LTG from plasma, brain and liver homogenates were estimated by comparing the peak height of extracted samples with those obtained after direct injection of standards at the same concentration levels in mobile phase. The results are presented in Table II.3.7, indicating that the sample preparation methodology employed is consistent precise and reproducible. Indeed, the overall absolute mean recoveries of LTG ranged from 76.97% to 97.99% and CV values were lower than 8.53%. Concerning the IS, an absolute mean recovery of 77.17% in plasma, 86.61% in brain and 90.33% in liver were found, exhibiting CV values below 7.01%.

Table II.3.7 Absolute recovery of lamotrigine (LTG) from mouse plasma, brain and liver homogenate supernatants ($n = 3$).

Matrix	Nominal Concentration ($\mu\text{g/mL}$)	Absolute Recovery (%) ^a	Precision (% CV)
Plasma			
	0.6	86.28 \pm 1.69	1.96
	15	84.94 \pm 1.84	2.17
	27	83.81 \pm 0.24	0.29
Brain			
	0.15	77.71 \pm 6.63	8.53
	7.5	76.97 \pm 1.79	2.33
	13.5	84.81 \pm 2.47	2.92
Liver			
	0.6	97.99 \pm 4.00	4.08
	10	84.71 \pm 2.72	3.21
	18	85.62 \pm 0.56	0.66

^a Mean \pm Standard deviation, $n = 3$.

The LLE-HPLC technique optimised from the method previously developed and validated by Castel-Branco *et al.* (2001) was herein successfully employed for the quantification of LTG in plasma and tissue (brain, olfactory bulb, frontal cortex and liver) homogenates of mice. Partial validation results have demonstrated that this assay is linear over the entire concentration range of LTG in all the mouse matrices analysed and fulfils the fundamental international requirements, being selective, precise, accurate and reproducible, also yielding satisfactory and consistent recovery values. Accordingly, it has been proven that the present method is suitable to support and can be applied with confidence in the analysis of samples from the subsequent pharmacokinetic studies underlying the work of the current thesis.



CHAPTER III

INTRANASAL ADMINISTRATION OF CARBAMAZEPINE

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III. 1. CARBAMAZEPINE

Pharmacological Characterisation

III. 1.1. HISTORICAL BACKGROUND

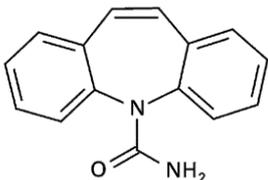
CBZ was firstly synthesised by the chemist Walter Schindler at the Swiss pharmaceutical firm Geigy in 1953 as part of a programme to investigate analogues of imipramine and chlorpromazine for depression and psychosis (BRODIE, 2010; SHORVON, 2009a; TOLOU-GHAMARI *ET AL.*, 2013). Notwithstanding, clinical evidence of its positive effects on relieving neuropathic pain symptoms led it to be licensed and marketed in the early sixties (1962) for the treatment of trigeminal neuralgia (SHORVON, 2009a). Its antiepileptic properties were then later reported in 1964, following successful seizure control had been achieved in 70 patients treated with CBZ who were refractory to conventional drugs (JONGMANS, 1964). CBZ rapidly gained a reputation in Europe as a very promising new AED and was approved for management of seizures in the United Kingdom in 1965 (SHORVON, 2009a; TOLOU-GHAMARI *et al.*, 2013). Its use as an anticonvulsant drug was authorised in Portugal in 1966, but approval in the United States of America was delayed until 1974 (TOLOU-GHAMARI *et al.*, 2013). Shortly after being licensed, CBZ became very widely used and, together with PHT, had evolved into the first choice drug to manage psychomotor and grand mal epilepsy.

By the mid-1980s, CBZ was the most frequently prescribed AED in Europe and it remains so up to the present day. After 50 years since it has been introduced in clinical practice, CBZ is assigned as one of the first-line AEDs for simple or complex focal and generalised tonic-clonic seizures, still being the gold standard for comparative studies of antiepileptics and the drug to beat for any new compound (SHORVON, 2009a; VUCIĆEVIĆ *et al.*, 2007).

III. 1.2. PHARMACEUTICAL INFORMATION

CBZ (a first-generation AED) is an iminostilbene, a dibenzazepine derivative that is chemically and pharmacologically related to tricyclic antidepressant agents (BAZIL and PEDLEY, 2003). Structurally, the molecule consists of a tricyclic nucleus composed by two benzene rings fused to an azepine group, bearing a carboxamide substituent at 5th position which is responsible for its potent anticonvulsant activity (WANG and KETTER, 2005). CBZ is a white or almost white crystalline powder, practically insoluble in water. This characteristic has hindered the development of a CBZ parenteral drug formulation, and therefore, it is only commercially available in oral dosage forms. Detailed pharmaceutical information of CBZ is summarised in Table III.1.1.

Table III.1.1 Pharmaceutical information of carbamazepine (DRUGBANK; NOVARTIS, 2014a; PUBCHEM).

Drug Identification		Physicochemical Properties	
Generic Name	Carbamazepine	Phase State	Solid / White powder
Chemical Name	5H-dibenz[<i>b,f</i>]azepine-5-carboxamide	Solubility in Water	0.018 mg/mL (at 25°C)
Chemical Formula	C ₁₅ H ₁₂ N ₂ O	Solubility in Ethanol	20.8 mg/mL (at 25°C)
Molecular Weight	236.27 g/mol	LogP	2.45
Molecular Structure		pK_a	13.9
Drug Information			
Brand Name	Tegretol®	Manufacturer	Novartis
Dosage Forms	Immediate-release tablet Extended-release tablet Chewable tablet Suspension	Route of Administration	Oral

LogP, octanol/water partition coefficient; pK_a, negative decadic logarithm of the acid dissociation constant.

III. 1.3. PHARMACOKINETICS

The pharmacokinetics of CBZ is complex and challenging from a clinical point of view. It is influenced by the limited hydrosolubility of the drug and the autoinduction phenomenon of its own metabolism by the hepatic microsomal enzymes (BAZIL and PEDLEY, 2003). CBZ exhibits a non-linear kinetics and is commonly associated to high pharmacological variability between and within patients (PERUCCA and JOHANNESSEN, 2003; TOLOU-GHAMARI *et al.*, 2013). This section and Table III.1.2 will provide a brief overview of the CBZ pharmacokinetic profile.

Table III.1.2 Summary of the main pharmacokinetic parameters of carbamazepine in epileptic adult patients (BERTILSSON, 1978; LANDMARK *et al.*, 2012; NEELS *et al.*, 2004; PATSALOS *et al.*, 2008; TOLOU-GHAMARI *et al.*, 2013).

Pharmacokinetic Parameter	Mean Value
t_{\max} (h)	4-8, possible delay up to 24 h
F (%)	75-85
Plasma Protein Binding (%)	70-80
Vd/F (L/kg)	0.9-1.4
$t_{1/2el}$ (h)	8-20
CL (L/h)	1.5

CL, plasma clearance; F, absolute bioavailability; $t_{1/2el}$, apparent elimination half-life; t_{\max} , time to achieve maximum peak concentration; Vd/F, apparent volume of distribution.

Absorption

The rate of CBZ absorption from the gastrointestinal tract is often relatively slow and erratic probably due to its poor solubility in water (NEELS *et al.*, 2004; VUCIĆEVIĆ *et al.*, 2007). Maximum concentration in plasma (C_{\max}) is usually attained within 4-8 h after oral ingestion, but it may be delayed by as much as 24 h, especially following the administration of a large dose (NEELS *et al.*, 2004). Generally, the time required to reach peak plasma concentrations is formulation-dependent since the suspension is absorbed more quickly and the controlled-release tablet more slowly and less completely than the conventional tablet (NOVARTIS, 2014a; PATSALOS *et al.*, 2008). Upon long-term administration, CBZ peak plasma levels occur at 1.5 h with the oral suspension, at 4-5 h with immediate-release or chewable tablets, and at 3-12 h with the extended-release formulation (BAZIL and PEDLEY, 2003; NOVARTIS, 2014a). Overall, the oral bioavailability of CBZ ranges from 75-85% and it can be increased when the drug is taken together with meals (BERTILSSON, 1978; LANDMARK *et al.*, 2012; PATSALOS *et al.*, 2008).

Distribution

CBZ is a neutral and highly lipophilic compound that easily diffuses through biological membranes. Once absorbed, the drug is rapidly and fairly uniformly distributed to all body tissues without any preferential affinity for particular organs (BAZIL and PEDLEY, 2003; BERTILSSON and TOMSON, 1986). The apparent volume of distribution (Vd/F) after peroral administration varies from 0.9-1.4 L/kg and the degree of plasma protein binding is of about 70-80% with very little inter-individual variation (PATSALOS *et al.*, 2008; TOMSON, 1987).

Metabolism

CBZ undergoes extensive hepatic metabolism (Figure III.1.1), with only approximately 2-3% of the dose orally given being excreted unchanged in the urine (KETTER *et al.*, 1999; NEELS *et al.*, 2004). More than 30 different metabolites of CBZ have been identified but the major metabolic pathway involves its oxidative conversion to CBZ-E which appears to be mediated primarily by the hepatic CYP3A4, with a minor contribution by CYP2C8 (KETTER *et al.*, 1999). This metabolite accounts for about 40% of CBZ disposition, being pharmacologically active and responsible for neurotoxic and idiosyncratic adverse effects (KECK and McELROY, 2002; KETTER *et al.*, 1999; PELLOCK, 2002). CBZ-E, in turn, undergoes almost complete hydrolysis via microsomal epoxide hydrolase to 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine, a *trans*-diol compound which is inactive and the major metabolite of CBZ found in human urine, corresponding to near 20% of the administered dose (EADIE, 1991; LANDMARK *et al.*, 2012). Other minor pathways of CBZ metabolism include aromatic hydroxylation to 2- and 3-hydroxycarbamazepine (25%) apparently mediated by CYP1A2, and glucuronide conjugation of the carbamoyl side chain (15%) by uridine 5'-diphosphate-glucuronosyltransferase (UGT) (KETTER *et al.*, 1999; STONER *et al.*, 2007).

CBZ induces many enzyme systems such as CYP1A, CYP3A, CYP2C and UGT resulting in the increase of the metabolism of many other co-administered drugs, including its own (VUCIĆEVIĆ *et al.*, 2007). Autoinduction of CBZ metabolism is observed during the first 3 to 5 weeks after starting therapy, significantly reducing CBZ plasma concentrations and elimination half-life, often demanding an upward dosage adjustment (BERTILSSON and TOMSON, 1986; STONER *et al.*, 2007; VUCIĆEVIĆ *et al.*, 2007). The clinical effect of autoinduction means that the process of introducing the drug can be tricky. Therapy should be initiated with low doses and progressively increased the days afterwards. Once autoinduction is complete, the relatively short and variable elimination half-life requires

frequent daily dosing to maintain adequate blood levels and therapeutic outcome (BAZIL and PEDLEY, 2003).

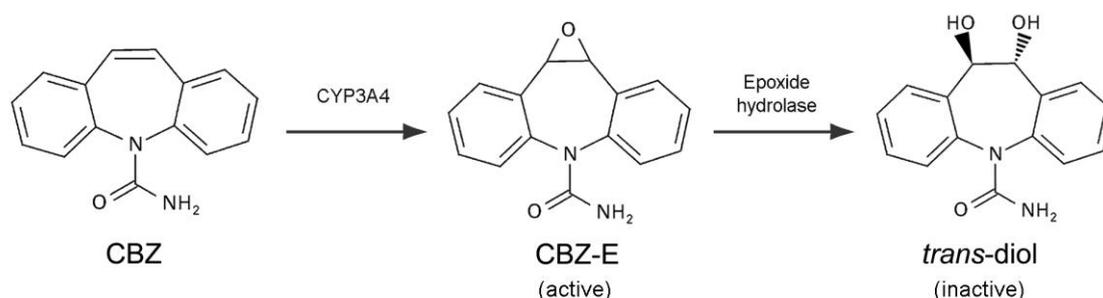


Figure III.1.1 The major metabolic pathways of carbamazepine (CBZ). CBZ-E, carbamazepine-10,11-epoxide; *trans*-diol, 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine.

Elimination

The elimination of CBZ follows a non-linear kinetics probably due to dose-dependent autoinduction phenomenon since increments in CBZ dose can result in less than proportional increases in drug concentrations (BERTILSSON, 1978; CLOYD and REMMEL, 2000; PERUCCA, 2005; STONER *et al.*, 2007). As a consequence, whole plasma concentrations of CBZ-E correlate more closely with CBZ dose than that of the parent compound (EADIE, 1991). Following a single oral dose, plasma elimination half-life in adults is approximately 30 h and the clearance is of about 1.5 L/kg. With multiple dosing, during maintenance CBZ monotherapy, half-life values shorten by two-thirds to approximately 10 h and clearance rises 3-fold to about 4.5 L/kg as a result of metabolism autoinduction, leading to a progressive decrease in CBZ plasma levels (BAZIL and PEDLEY, 2003; WANG and KETTER, 2005).

CBZ elimination depends almost entirely on hepatic biotransformation (BAZIL and PEDLEY, 2003; TOLOU-GHAMARI *et al.*, 2013). The urinary excretion is the primarily route of CBZ elimination with 72% of the administered dose being excreted in the urine mainly in the form of epoxidated, hydroxylated and conjugated metabolites. The remaining 28% are excreted in faeces, via biliary secretion (BERTILSSON, 1978; NOVARTIS, 2014a).

III. 1.4. PHARMACODYNAMICS

Mechanism of Action

A large body of evidence indicates that CBZ may interact with different types of ion channels, receptors and signalling pathways, bearing clear that it does not act by a single mechanism (AMBRÓSIO *et al.*, 2002). Nonetheless, it is conceivable that prevention or reduction of sustained repetitive firing of action potentials in depolarised neurons by stabilising the inactive form of sodium channels in a use-, voltage- and frequency-dependent manner may be its main mechanism of action (AMBRÓSIO *et al.*, 2002; KWAN *et al.*, 2001). In fact, CBZ blocks voltage-sensitive sodium channels in a similar fashion to PHT, promoting fast inactivation and increasing the number of channels in the inactivated state (PORTER *et al.*, 2012; POTSCSKA, 2013). However, while CBZ has a greater binding rate constant, but lower affinity for the inactivated sodium channels, PHT produces a more pronounced slowing of recovery from the fast inactivated state (KWAN *et al.*, 2001).

Inhibition of glutamatergic neurotransmission and indirect facilitation of GABA activity have also been ascribed to CBZ, albeit they are likely to be relatively minor physiologic effects at therapeutic dosage levels (AMBRÓSIO *et al.*, 2002; STONER *et al.*, 2007). Modulation of calcium currents by blocking voltage-sensitive calcium channels, particularly those of the L-type, has likewise been indicated as a possible mechanism implicated in the anticonvulsive activity of CBZ, even though this effect is apparently less often observed at concentrations that are therapeutically relevant (AMBRÓSIO *et al.*, 2002; PORTER *et al.*, 2012). Despite inconclusive experimental data, an interference of CBZ with potassium conductance through modulation of potassium channels has also been hypothesised (AMBRÓSIO *et al.*, 2002; KECK and McELROY, 2002). Such an action on potassium currents is expected to oppose or diminish hyperexcitability, thus being potentially relevant to seizure protection (PORTER *et al.*, 2012).

III. 1.5. CLINICAL USE

Therapeutic Indications

CBZ is an anticonvulsant and mood-stabilizing drug that is commonly used in the management of epilepsy, neuropathic pain (trigeminal neuralgia), mania and bipolar affective disorders (NOVARTIS, 2014a; WANG and KETTER, 2005). It remains the first drug of choice for the treatment of simple or complex focal (with or without secondary generalisation) and generalised tonic-clonic seizures, but it is not effective against absence, atonic and myoclonic seizures, which may actually be aggravated or exacerbated (NOVARTIS, 2014a; PELLOCK, 2002; TOLEDANO and GIL-NAGEL, 2008).

It can be given either as monotherapy or in combination with other anticonvulsant agents. To date, any AED has demonstrated to be more effective than CBZ against focal-onset seizures. Notwithstanding, besides being equally efficacious, several new AEDs are often better tolerated and present more favourable pharmacokinetic properties (ABOU-KHALIL and SCHMIDT, 2012).

Tolerability

CBZ therapy is associated with several noteworthy idiosyncratic adverse events that range from commonly occurring but benign to rarely occurring but serious, often requiring discontinuation of the drug. During long-term CBZ treatment, the most frequent undesirable adverse effects are typically neurotoxic, and are dose- and titration-dependent (PELLOCK, 2002; WANG and KETTER, 2005). Dose-related CNS side-effects includes sedation, ataxia, diplopia, blurred vision, nystagmus, headache, dizziness and motor incoordination that may appear early in the course of treatment during dose adjustment (BAZIL and PEDLEY, 2003). Neurotoxicity can however be minimised and better tolerated if the treatment is initiated at low doses with further progressive slow titration (NEELS *et al.*, 2004; TOLEDANO and GIL-NAGEL, 2008).

Mild to severe dermatological, haematological and hepatic dysfunctions are likewise commonly associated to CBZ. Skin rash reactions of the morbilliform type are frequently developed in around 10% of patients but life-threatening conditions like Stevens-Johnson syndrome are less common or even very rare (PELLOCK, 2002; TOLEDANO and GIL-NAGEL, 2008). Reversible modest leucopenia can usually be detected in first two months of treatment. This suppression of leukocyte count does not require discontinuation of therapy unless there is evidence of infection or the decline continues to values <3000 leucocytes/mm³ (WANG and KETTER, 2005; WARNER *et al.*, 1998). Serious cases of agranulocytosis or aplastic anaemia have occasionally been reported (TOLEDANO and GIL-NAGEL, 2008; WARNER *et al.*, 1998). Transient elevations of hepatic enzymes occur at a frequency of 5-10% and are of no clinical significance; fulminant hepatitis is exceedingly rare (BAZIL and PEDLEY, 2003; WANG and KETTER, 2005).

Since CBZ has anti-diuretic effects, the induction of hyponatremia and water retention frequently occurs as a late complication (NEELS *et al.*, 2004). This mild to moderate reduction of plasma sodium concentrations is generally unimportant, although it sometimes be related to patient complaints of dizziness, headache and nausea. Severe hyponatremia can exacerbate seizures or contribute to congestive heart failure, being particularly problematic in patients with compromised cardiac function (BAZIL and PEDLEY, 2003).

Therapeutic Range

The high inter-individual variation in plasma elimination half-lives, as well as the differences concerning the extent to which CBZ induces its own metabolism among distinct subjects hamper the prediction of drug concentrations achieved in plasma based on the administered dose (BERTILSSON, 1978). In fact, plasma levels of CBZ may range from 0.5 to 25 µg/mL without an apparent relationship to the daily intake of the drug (NOVARTIS, 2014a). Therefore, establishing a suitable dosing regimen of CBZ is generally complicated not only due to its wide pharmacokinetic variability but also to a weak relationship between plasma concentration and therapeutic effects (VUCIĆEVIĆ *et al.*, 2007). Generally, in order to attain an optimal management of seizures, the recommended therapeutic concentrations for CBZ may be settled within the range of 4-12 µg/mL (NEELS *et al.*, 2004; NOVARTIS, 2014a; PATSALOS *et al.*, 2008). Nevertheless, achieving a specific therapeutic level is not an absolute goal of the therapy since some patients respond differently to concentrations below or above this interval. At this point, similar CBZ concentrations have inclusively been reported to provide seizure control in certain individuals and adverse toxic effects in others (PATSALOS *et al.*, 2008).

The unpredictable relationship between dose and CBZ plasma concentration, the large inter- and intra-individual CBZ pharmacokinetic variability, its narrow therapeutic index and its high predisposition to establish numerous clinically significant pharmacological interactions support the need to monitor and adjust therapy individually in order to optimise CBZ therapeutic/toxic ratio, thus improving patient's clinical outcome.

Drug Interactions

CBZ drug-drug interactions are predominantly pharmacokinetic. Since it is a powerful inducer of catabolic enzymes, including various CYPs and UGTs, CBZ decreases the serum concentrations of multiple co-administered medications, potentially attenuating their clinical effects (JOHANNESSEN and LANDMARK, 2010; WANG and KETTER, 2005). Therefore, CBZ commonly accelerates the metabolic rate of numerous therapeutic compounds such as valproic acid, LTG, topiramate, benzodiazepines, warfarin, theophylline, haloperidol, ethinyl estradiol among others, often demanding appropriate adjustments of their corresponding dose (JOHANNESSEN and LANDMARK, 2010; KETTER *et al.*, 1999; PATSALOS and PERUCCA, 2003). As a result, particular caution must be imposed when CBZ is discontinued, since the effect of enzymatic stimulation is reduced, leading to an unpredictable increase of plasma concentrations to levels that can be eventually toxic for

other drugs whose catabolism is no longer induced (JOHANNESSEN and LANDMARK, 2010; WANG and KETTER, 2005).

Even though CBZ mediates a large number of clinically significant pharmacological interactions, its own metabolism may likewise be affected by the action of many therapeutic compounds. Several drugs, including known inhibitors of CYP3A4, can precipitate signs of CBZ toxicity by increasing its serum concentrations (JOHANNESSEN and LANDMARK, 2010). Conversely, CBZ therapeutic efficacy can be potentially compromised when potent enzyme-inducers, like for example PHT, PB or PRM are concomitantly administered (BERTILSSON and TOMSON, 1986; KETTER *et al.*, 1999; NEELS *et al.*, 2004; WANG and KETTER, 2005).



III. 2. EXPERIMENTAL

Intranasal Administration of CBZ to Mice:
A Direct Delivery Pathway
for Brain Targeting

III. 2.1. INTRODUCTION

Epilepsy is one of the most common and devastating neurological disorders which is estimated to have a worldwide prevalence of about 0.7% (SCHMIDT and SCHACHTER, 2014). There are several antiepileptic drugs currently available to control and suppress seizures. However, despite the ongoing development of new pharmacological therapies, more than 30% of the patients do not become seizure free mainly due to the pharmacoresistance phenomena (WEAVER and POHLMANN-EDEN, 2013). Moreover, conventional antiepileptic drug administration via either oral or IV routes commonly exhibits high systemic drug distribution into CNS and non-targeted tissues which can potentiate the occurrence of drug-drug interactions and undesirable side effects that range from a CNS impairment (e.g. somnolence, dizziness and ataxia) to more severe peripheral pathological conditions such as skin reactions and hematologic, hepatic and renal dysfunctions (TOLEDANO and GIL-NAGEL, 2008).

Arguably, the delivery of drugs to the CNS remains a great challenge owing to the strict structural and functional BBB (GABATHULER, 2010). Thus, over the last decades, different strategies have been attempted in order to circumvent the BBB and to deliver drugs efficiently into the brain for therapeutic and diagnostic applications (GABATHULER, 2010; ILLUM, 2000). In fact, the development of new alternative drug delivery methods could enhance the efficacy and minimise the toxicity of antiepileptic drugs, thereby improving their therapeutic index (FISHER and Ho, 2002). The IN administration has long been widely used for the symptomatic relief and treatment of local nasal dysfunctions, but recently, it has received a great attention as a convenient and reliable route for the systemic administration of drugs (FORTUNA *et al.*, 2014a; GRASSIN-DELYLE *et al.*, 2012). Nevertheless, assuming the olfactory region as a unique direct connection between the nose and the brain, an increasing interest has been posed on the potential of the IN route for the delivery of therapeutic agents directly to the CNS bypassing the BBB (ILLUM, 2004; VYAS *et al.*, 2005). Indeed, IN administration represents an attractive alternative to parenteral and oral routes since, in addition to be non-invasive, it also avoids gastrointestinal and hepatic first-pass metabolism. The rapid-onset of action and the preferential delivery of drugs to the brain also enable the IN route to be successfully applied in the management of emergency situations (LI *et al.*, 2000; WOLFE and BERNSTONE, 2004).

CBZ is one of the first-line AEDs most commonly prescribed despite its narrow therapeutic window, complex pharmacokinetic profile, potential for drug interactions and severe side effects (GERLACH and KRAJEWSKI, 2010; NEELS *et al.*, 2004; PATSALOS *et al.*, 2008). Currently, CBZ is only available in tablet or suspension oral dosage forms due to its poor water solubility that prevents its incorporation in therapeutic dosages in aqueous

solutions for IV injection. Following oral administration, the absorption of CBZ is relatively slow, erratic and formulation-dependent (LANDMARK *et al.*, 2012); its oral bioavailability is within the range 75-85% (LANDMARK *et al.*, 2012) and the time to reach peak concentration in plasma is approximately 4-8 h post-dosing but it may be delayed by as much as 24 h with high doses (NEELS *et al.*, 2004). Furthermore, CBZ undergoes extensive hepatic metabolism and considerable enzymatic induction that result in unpredictable plasmatic fluctuations and unexpected clearance increments which demand successive dose adjustments (PATSALOS *et al.*, 2008; TOMSON, 1987). Taking all those pharmacokinetic limitations of CBZ oral administration into account, we do believe that this AED is a promising candidate to be administered by the IN route. A prompt and efficient IN drug delivery to the brain may decrease the systemic exposure, improving both efficacy and tolerability profiles. The opportunity to control seizures by reducing the dose makes IN administration of CBZ a valuable approach for long-term treatment of epilepsy. Likewise, it could also give an attractive advantage in the management of acute and severe convulsive seizure episodes. In fact, IV administration of benzodiazepines is the first-line option for the treatment of *status epilepticus* (LOCKEY, 2002; MANNO, 2011); however, it is generally associated with hypotension, cardiac dysrhythmia and respiratory failure. Furthermore, IV injection requires sterile equipment and skilled personnel which often makes it impractical and inconvenient to be used outside the hospital setting. Bearing in mind that quick cessation of the seizures is essential to prevent serious neurological damages, a rapid access and a high brain bioavailability of CBZ administered via IN route may probably contribute to its recognition as a viable alternative to IV administration of the drugs used in emergency conditions.

Interestingly, IN administration of CBZ has already been studied in rats by Barakat and collaborators (2006), reporting high levels of drug penetration in the brain solely based on the analysis of plasma and whole brain homogenates. Therefore, a comprehensive pharmacokinetic characterisation of IN CBZ and its active metabolite mainly responsible for the toxic effects (CBZ-E) is lacking. In this context, plasma, brain and liver levels of both CBZ and CBZ-E, were, in this study, determined following IN and IV administrations to mice, and the corresponding pharmacokinetic profiles were assessed and compared. Additionally, in order to establish a more sustained basis for an hypothetic direct transport of the drug from nose to brain via the olfactory pathway, CBZ concentrations were also determined in different brain regions and the rostral-caudal brain distribution of the drug was studied following the two routes of administration considered.

III. 2.2. MATERIALS & METHODS

III. 2.2.1. Chemicals and Reagents

CBZ and 10,11-dihydrocarbamazepine, used as IS, as well as Pluronic F-127 (PF-127) and propylene glycol were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Carbopol 974P (C-974P) was kindly supplied from Lubrizol (Wickliffe, OH, USA). Methanol and acetonitrile of HPLC gradient grade were acquired from Fisher Scientific (Leicestershire, UK) and Lab-Scan (Sowinskięo, Poland) respectively. Ultrapure water (HPLC grade, 18.2 M Ω .cm) was prepared by means of a Milli-Q water apparatus from Millipore (Milford, MA, USA). Ethyl acetate was obtained from Fisher Scientific (Leicestershire, UK). Sodium dihydrogen phosphate dihydrate, di-sodium hydrogen phosphate dihydrate and hydrochloric acid fuming 37%, all used to prepare 0.1 M sodium phosphate buffer pH 5.0, were purchased from Merck KGaA (Darmstadt, Germany). Ketamine (Imalgene 1000[®], 100 mg/ml) and xylazine (Vetaxilaze 20[®], 20 mg/ml) were commercially acquired.

III. 2.2.2. Animals

Adult male CD-1 mice aged between 6 and 7 weeks and weighing 30-40 g were obtained from local certified animal facilities (Faculty of Health Sciences of the University of Beira Interior, Covilhã, Portugal). Mice were housed under controlled environmental conditions (12 h light/dark cycle, at 20 \pm 2 $^{\circ}$ C and relative humidity 50 \pm 5%) with free access to tap water and standard rodent diet (4RF21, Mucedola, Italy). All the experiments involving animals and their care were conducted in conformity with the international regulations of the European Directive (2010) regarding the protection of laboratory animals used for scientific purposes (2010/63/EU) (EUROPEAN PARLIAMENT AND COUNCIL OF THE EUROPEAN UNION, 2010), and the experimental procedures employed were reviewed by the Portuguese DGAV.

III. 2.2.3. Preparation of Carbamazepine Formulations

For IN administration, CBZ was previously dissolved in ethanol at the concentration of 20 mg/mL. Then 50 μ L of this ethanolic solution was incorporated in 950 μ L of a thermoreversible nasal gel so that the final drug concentration was 1 mg/mL and the total percentage of ethanol in the formulation was equivalent to 5%. Thermoreversible gel was prepared using the cold method described by Schmolka (1972). Briefly, 1.8 g of PF-127 was slowly added to 10 mL of distilled cold water (5-10 $^{\circ}$ C), under

gentle magnetic stirring, to achieve an efficient hydration of the flakes and then, the mixture was left at 4°C overnight to attain a complete dissolution of the polymer (18% PF-127, w/v). Afterwards, according to the technique employed by Badgujar and co-workers (2010), the mucoadhesive polymer C-974P was gradually dispersed in the prepared PF-127 solution with continuous agitation, until a final concentration of 0.2% w/v was reached. At this point, a nasal hydrogel formulation composed of 18% PF-127 and 0.2% C-974P was obtained, exhibiting thermo-sensible properties. In fact, PF-127 is a triblock copolymer of poly(ethylene oxide) and poly(propylene oxide) units that is fluid at or below room temperature, but turns to a semisolid gel when the temperature increases, as a consequence of the micelle packing disorder-order transition phenomenon (SWAMY and ABBAS, 2012). This thermo-sensible behaviour makes the final formulation suitable for gelation within the nasal cavity, providing a sustained residence of the drug at the absorption site.

For the IV administration, a CBZ parenteral solution was prepared as a mixture of propylene glycol-physiological saline solution (0.9% NaCl)-ethanol (5:3:2, v/v/v) at a final drug concentration of 0.1 mg/mL.

III. 2.2.4. IN and IV Administrations

Before CBZ dosing, mice were always anaesthetised with an intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and kept in a heated environment to maintain the body temperature.

CBZ was intranasally and intravenously administered at the dose of 0.4 mg/kg. For IN administration, mice were placed on one side and 12 µL of the nasal gel per 30 g of mice body weight were instilled using a polyurethane tube (24G x 19 mm) attached to a microliter syringe. The tube was inserted about 10 mm deep into one of the nares, enabling the delivery of the formulation towards the roof of the nasal cavity. IV administration of CBZ (0.4 mg/kg) was performed by means of a short and slow infusion of the formulation for over 30 s into the lateral tail vein (120 µL per 30 g body weight) using an appropriate syringe.

III. 2.2.5. Pharmacokinetic Study

Mice were randomly divided into two experimental groups of 40 animals each. One of the groups received IN formulation whereas the other group was treated with the IV dosage form. At predetermined time points (5, 10, 15, 30, 45, 60, 90, 120, 180 and 240 min) after CBZ dosing (4 animals per time point, $n = 4$), the mice were sacrificed by

cervical dislocation followed by decapitation and the blood was immediately collected into heparinised tubes, while brain and liver tissues were quickly removed and weighed. Blood samples were centrifuged at 4°C and 4000 rpm for 10 min to obtain plasma supernatants that were stored at -30°C until analysis. Mice brain and liver tissues were homogenised with 0.1 M sodium phosphate buffer pH 5.0 (4 mL per gram of tissue) using a THOMAS® Teflon pestle tissue homogeniser. Tissue homogenates were centrifuged at 4800 rpm for 15 min (4°C) and the resultant supernatants were also frozen at -30°C until analysis.

III. 2.2.6. Brain Biodistribution Study

Mice were divided at random into two experimental groups (20 animals each). The animals were treated with CBZ (0.4 mg/kg) using the IN or IV formulations. After administration, mice were sacrificed at 5, 10, 15, 30 and 60 min post-dosing ($n = 4$). Blood samples were taken and plasma was separated as described above. Brains were removed and carefully dissected with the help of a scalpel into three different regions: olfactory bulb, frontal cortex and the remaining portion of the brain. The remaining portion of the brain was homogenised and centrifuged in accordance to the procedure used for brain and liver tissues, while olfactory bulb and frontal cortex specimens, regardless of the weight, were homogenised with 1 mL of phosphate buffer using an ULTRA-TURRAX® device and centrifuged at 4°C for 15 min at 13.400 rpm. The resultant homogenate supernatants were conveniently packaged and stored at -30°C until analysis.

III. 2.2.7. Drug Analysis

Plasma and tissue (brain and liver) concentrations of CBZ and CBZ-E were determined by using a SPE procedure followed by a reversed-phase HPLC analysis, according to the method previously developed and fully validated by Fortuna *et al.* (2010) with slight modifications (*Chapter II, section II. 3.2.*).

Briefly, aliquots of plasma (200 µL), brain (500 µL) and liver (250 µL) homogenate supernatants were added to an appropriate volume of 0.1 M sodium phosphate buffer (pH 5.0) to make a total of 1 mL sample amount. Regarding the matrices of brain specified regions, 1 mL of both olfactory bulb and frontal cortex homogenate supernatants were used. All the samples were spiked with 10 µL of the methanolic IS working solution (200 µg/mL for all matrices excluding for the olfactory bulb, which was 100 µg/mL). After vortex mixed, samples were loaded into Waters Oasis® HLB (30 mg, 1 mL) cartridges (Milford, MA, USA), which were previously conditioned with 1 mL of methanol, 1 mL of

acetonitrile and 1 mL of water-acetonitrile (95:5, v/v). Upon sample elution, the loaded cartridges were submitted to -30 kPa and washed four times with 1 mL of water followed by four more times with 1 mL of water-methanol (90:10, v/v). After drying the cartridge under airflow for 5 min, the drugs were eluted with 1 mL of ethyl acetate applying a gentle vacuum. The eluates were then evaporated to dryness at 45°C under moderate nitrogen stream and reconstituted with 100 µL of mobile phase by vortexing and ultrasonication. Finally, an aliquot of 20 µL (plasma, brain, liver and frontal cortex) or 40 µL (olfactory bulb) of each reconstituted extracts was injected into the chromatographic system for analysis.

The HPLC analysis was carried out on a Shimadzu liquid chromatographic system equipped with a GDU-20A₅ degasser, a SIL-20A_{HT} autosampler, a CTO-10AS_{VP} column oven and a SPD-M20A diode array detector, all from Shimadzu Corporation (Kyoto, Japan). Data acquisition and instrumentation control were achieved by means of LCsolution software (Shimadzu Corporation, Kyoto, Japan). Chromatographic separation was performed at 40°C on a reversed-phase LiChroCART® Purospher Star® C₁₈ column (55 mm x 4 mm, 3 µm; Merck KGaD), using an isocratic elution with a mobile phase consisting of water-methanol-acetonitrile (64:30:6, v/v/v) pumped at a flow rate of 1 mL/min. CBZ and CBZ-E were detected at the wavelength of 235 nm and the total running time was set at 10 min. The main partial validation parameters of the analytical method employed were in agreement with the international guidelines (EUROPEAN MEDICINES AGENCY, 2011; US FOOD AND DRUG ADMINISTRATION, 2001) and are summarised in Table III.2.1.

Table III.2.1 Validation parameters of the HPLC method employed for the quantification of carbamazepine (CBZ) and carbamazepine-10, 11-epoxide (CBZ-E) in plasma, brain, liver, olfactory bulb and frontal cortex homogenate supernatants ($n = 3$).

Drug	Validation Parameters	Plasma	Brain	Liver	Olfactory Bulb	Frontal Cortex
CBZ	Calibration Range (µg/mL)	0.10 - 30	0.10 - 15	0.20 - 20	0.02 - 4 ^b	0.05 - 7.5 ^b
	Coefficient of Determination (r^2)	0.999	0.997	0.999	0.998	0.997
	LLOQ (µg/mL)	0.10	0.10	0.20	0.02 ^b	0.05 ^b
	Precision (% CV) ^a	≤ 6.67	≤ 7.89	≤ 3.92	≤ 5.39	≤ 7.89
	Accuracy (% Bias) ^a	-0.66 - 2.25	-5.41 - 3.75	0.28 - 1.55	-1.28 - 5.98	-5.41 - 3.75
CBZ-E	Calibration Range (µg/mL)	0.40 - 30	0.05 - 15	0.20 - 20	-	-
	Coefficient of Determination (r^2)	0.999	0.996	0.999	-	-
	LLOQ (µg/mL)	0.40	0.05	0.20	-	-
	Precision (% CV) ^a	≤ 5.08	≤ 4.48	≤ 5.82	-	-
	Accuracy (% Bias) ^a	0.54 - 5.24	-6.04 - 4.34	-3.41 - 9.18	-	-

^a Inter-day values, $n = 3$; ^b Values expressed in µg. Bias, deviation from nominal value; CV, coefficient of variation; LLOQ, lower limit of quantification.

III. 2.2.8. Pharmacokinetic Analysis

The maximum peak concentration (C_{max}) in plasma and tissues of CBZ and its main metabolite (CBZ-E) and the corresponding time to reach C_{max} (t_{max}) were directly derived from the experimental data obtained. The remaining pharmacokinetic parameters were estimated based on the mean concentration values ($n = 4$) determined at each time point by a non-compartmental pharmacokinetic analysis employing the WinNonlin® version 5.2 (Pharsight Co, Mountain View, CA, USA). The pharmacokinetic parameters evaluated were the area under the drug concentration time-curve (AUC) from time zero to the time of the last quantifiable drug concentration (AUC_t) which was calculated by the linear trapezoidal rule; the AUC from time zero to infinity (AUC_{inf}) that was calculated from $AUC_t + (C_{last}/k_{el})$, where C_{last} is the last quantifiable concentration and k_{el} (or k) is the apparent (or tissue) elimination rate constant estimated by log-linear regression of the terminal segment of the concentration-time profile; the percentage of AUC extrapolated from t_{last} to infinity [$AUC_{extrap}(\%)$], where t_{last} is the time of the C_{last} ; the apparent terminal (or tissue) elimination half-life ($t_{1/2el}$ or $t_{1/2}$), and the mean residence time (MRT).

The absolute bioavailability [F (%)] of CBZ after IN administration was calculated as follows (Eq. 1):

$$F (\%) = \frac{(AUC_{inf IN} \times Dose_{IV})}{(AUC_{inf IV} \times Dose_{IN})} \times 100 \quad (\text{Eq. 1})$$

where $AUC_{inf IN}$ and $AUC_{inf IV}$ are the areas under the drug concentration-time curves from time zero to infinity following IN and IV administration, respectively; $Dose_{IV}$ and $Dose_{IN}$ are the values of the CBZ dosage (mg/kg) given by IV and IN route to mice.

In order to assess brain targeting efficiency of nasally delivered CBZ, the DTE index was calculated (WANG *et al.*, 2003). DTE index represents the brain-to-plasma partitioning ratio of the drug administered by IN route compared to that after IV injection and can be calculated according to the following equation (Eq. 2):

$$DTE = \frac{(AUC_{brain}/AUC_{plasma})_{IN}}{(AUC_{brain}/AUC_{plasma})_{IV}} \quad (\text{Eq. 2})$$

where AUC_{brain} and AUC_{plasma} are the areas under the drug concentration-time curves for brain and plasma after both IN and IV administration to mice. It is assured that preferential transport of drug to the brain occurs when DTE index is greater than 1 (WANG *et al.*, 2003).

With the aim of evaluating the distribution of CBZ to specific brain regions (olfactory bulb, frontal cortex and the remaining portion of the brain) after its IN and IV

administration, the drug concentrations in each specimen were determined at predefined time points ($n = 4$). The corresponding tissue-to-plasma and tissue-to-remaining portion of the brain CBZ concentration ratios were calculated and compared.

III. 2.2.9. Statistical Analysis

The data were expressed as mean \pm standard error of the mean (SEM). Statistical comparisons between IN and IV administration groups were performed using unpaired two-tailed Student's *t*-test. Differences were considered statistically significant for a *p*-value lower than 0.05 ($p < 0.05$).

III. 2.3. RESULTS

III. 2.3.1. Pharmacokinetics of Carbamazepine after IN and IV Administration

The mean plasma, brain and liver concentration-time profiles of CBZ and CBZ-E obtained in mice after a single dose of CBZ (0.4 mg/kg) administered as nasal gel and IV solution are depicted in Figure III.2.1. The corresponding main pharmacokinetic parameters estimated by non-compartmental analysis are summarised in Table III.2.2. It is noteworthy that, in all the three biological matrices, the pharmacokinetic profiles obtained after IN and IV administration are fairly comparable. As expected, the C_{\max} of the parent drug (CBZ) was attained almost instantaneously (5 min) after IV administration, and it occurred not only in plasma but also in brain and liver tissues. In comparison to IV delivery, only a slight delay in the time to reach the C_{\max} of CBZ ($t_{\max} = 10$ min) was observed for IN administration. Particularly interesting is the resemblance found in the magnitude of the peak concentrations of CBZ achieved in brain and plasma via IN and IV delivery. After reaching the C_{\max} , CBZ concentrations in plasma, brain and liver decreased similarly following the two administration routes. As shown in Table III.2.2, the extent of systemic and brain exposure to CBZ was also comparable after either IN or IV administration (as assessed by AUC_t and AUC_{inf}), whereas the extent of hepatic exposure to CBZ was 1.4-fold greater after IV injection (as assessed by AUC_t). Thus, the absolute bioavailability estimated for CBZ delivered via the IN route was found to be very high (107.6%), indicating that a comparable amount of the drug was easily and rapidly accessible in the systemic circulation following both IN and IV administrations. Regarding the MRT parameter presented in Table III.2.2, it can be noted that higher values were attained for plasma and brain after IN administration comparatively to IV administration, in contrast with the liver, where the highest MRT value was assigned to the IV route. The DTE index calculated for IN delivery of CBZ was 0.98 which did not provide any

discriminative information of the potential for direct nose-to-brain transport of the drug via IN route. In opposition, the estimated DTE value appears to suggest that the uptake of CBZ into the CNS through the nasal cavity is predominantly achieved by crossing the BBB after a quick nasal absorption of the drug to the systemic blood. Therefore, taking into account these pharmacokinetic data, the impact of the direct nose-to-brain delivery of CBZ after IN instillation was not evident when considering only the analysis of whole brain homogenate concentrations.

The concentrations of CBZ-E were also simultaneously determined in the referred matrices. Overall, the CBZ-E levels were near or below the LLOQ of the analytical method, thus the estimation of the corresponding pharmacokinetic parameters was limited and therefore their values are not very informative (Table III.2.2).

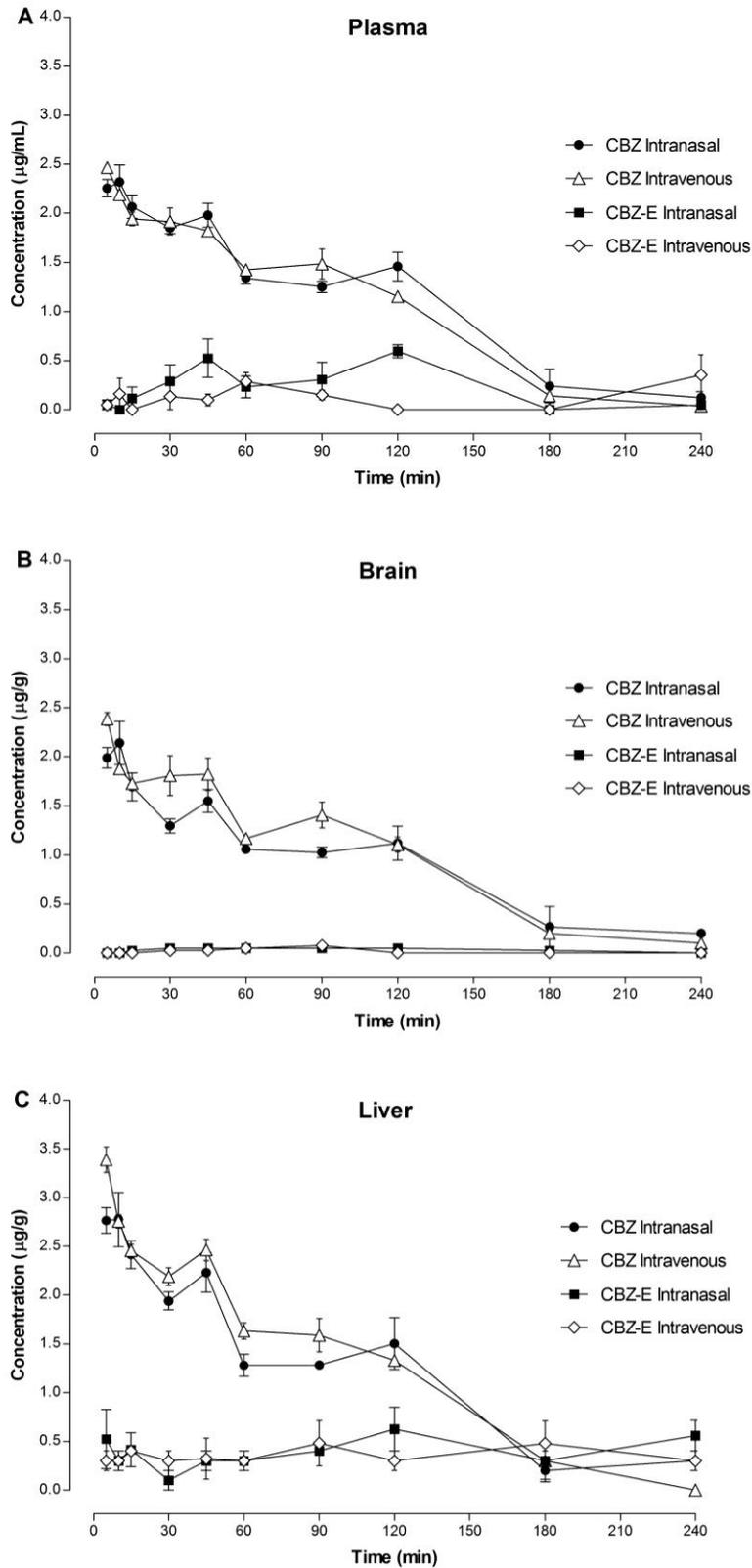


Figure III.2.1 Concentration-time profiles of carbamazepine (CBZ) and carbamazepine-10,11-epoxide (CBZ-E) in (A) plasma, (B) brain and (C) liver tissues following IN thermoreversible gel and IV solution administration of CBZ (0.4 mg/kg) to mice. Symbols represent the mean values \pm SEM of four determinations per time point ($n = 4$).

Table III.2.2 Pharmacokinetic parameters of carbamazepine (CBZ) and carbamazepine-10,11-epoxide (CBZ-E) in plasma, brain and liver tissues following the administration of CBZ (0.4 mg/kg) to mice through intranasal (IN) and intravenous (IV) routes.

Drug	Pharmacokinetic Parameters ^a	Plasma		Brain		Liver	
		IN	IV	IN	IV	IN	IV
CBZ	t _{max} (min)	10.0	5.0 ^c	10.0	5.0	10.0	5.0
	C _{max} (µg/mL)	2.32	2.47 ^d	2.14 ^e	2.39 ^e	2.78 ^e	3.39 ^e
	AUC _t (µg.min/mL)	252.58	238.65	193.19 ^f	185.71 ^f	204.15 ^f	288.88 ^f
	AUC _{inf} (µg.min/mL)	262.46	243.84	220.58 ^f	NC	NC	304.33 ^f
	AUC _{extrap} (%)	3.76	2.13	12.4	NC	NC	5.07
	k _{el} (min ⁻¹)	0.013	0.027	-	-	-	-
	k (min ⁻¹)	-	-	0.010	0.006	0.006	0.019
	t _{1/2el} (min)	55.2	25.3	-	-	-	-
	t _{1/2} (min)	-	-	70.8	127.1	112.1	35.7
	MRT (min)	76.1	64.9	71.0	52.9	53.1	64.2
F (%) ^b	107.6	-	-	-	-	-	
CBZ-E	t _{max} (min)	120.0	NA	NA	NA	120.0	90.0
	C _{max} (µg/mL)	0.60	NA	NA	NA	0.63 ^e	0.48 ^e
	AUC _t (µg.min/mL)	45.99	NA	NA	NA	122.87 ^f	50.40 ^f
	AUC _{inf} (µg.min/mL)	NC	NC	NC	NC	NC	NC
	AUC _{extrap} (%)	NC	NC	NC	NC	NC	NC
	k _{el} (min ⁻¹)	NC	NC	-	-	-	-
	k (min ⁻¹)	-	-	NC	NC	NC	NC
	t _{1/2el} (min)	NC	NC	-	-	-	-
	t _{1/2} (min)	-	-	NC	NC	NC	NC
	MRT (min)	NC	NC	NC	NC	NC	NC

^a Parameters were estimated using the mean concentration-time profiles obtained from four different animals per time point ($n = 4$). ^b Absolute intranasal bioavailability (F) was calculated based on AUC_{inf} values. ^c t_{max} in plasma after IV administration corresponds to the first sampling time point. ^d C_{max} in plasma after IV administration is equivalent to the maximum concentration value determined at the first sampling time point (5 min). ^e Values expressed in µg/g. ^f Values expressed in µg.min/g. AUC_{extrap}, extrapolated area under the drug concentration time-curve; AUC_{inf}, area under the concentration time-curve from time zero to infinity; AUC_t, area under the concentration time-curve from time zero to the last quantifiable drug concentration; C_{max}, maximum peak concentration; k, tissue elimination rate constant; k_{el}, apparent elimination rate constant; MRT, mean residence time; NA, not available; NC, not calculated; t_{1/2}, tissue elimination half-life; t_{1/2el}, apparent terminal elimination half-life; t_{max}, time to achieve the maximum peak concentration.

III. 2.3.2. Brain Biodistribution of Carbamazepine after IN and IV Administration

To achieve more specific and informative data on the rostral-caudal brain biodistribution of CBZ following its IN and IV administration (0.4 mg/kg) to mice, some particular brain regions (olfactory bulb, frontal cortex and the remaining portion of the brain) were analysed as well as the plasma samples taken at the corresponding sampling time points. The mean concentrations of CBZ in plasma, olfactory bulb, frontal cortex and

the remaining portion of the brain up to 60 min post-dosing are presented in Figure III.2.2. Accordingly, CBZ concentrations attained in plasma and in the different brain regions after IV administration of CBZ solution were very similar, assuming a homogenous brain distribution pattern. In contrast, following IN administration of CBZ nasal gel, different drug concentrations were observed throughout the specific brain regions analysed. Indeed, at 10 min post-dosing, higher CBZ concentrations were determined in the olfactory bulb ($3.16 \pm 0.09 \mu\text{g/g}$) and frontal cortex ($3.05 \pm 0.09 \mu\text{g/g}$) homogenates comparatively to the remaining portion of the brain ($2.58 \pm 0.09 \mu\text{g/g}$), showing an uneven distribution of the drug from rostral to more caudal brain areas (Figure III.2.2). Interestingly, this heterogeneous brain distribution of CBZ is more evident during the first three time points (5, 10 and 15 min) after the IN instillation, whereas a more uniform diffusion was accomplished from 30 min onwards. In fact, it is noteworthy that, up to 15 min, the highest concentrations of CBZ after IN administration were always found in the olfactory bulb in comparison to plasma, frontal cortex and remaining portion of the brain, sustaining a direct passage of the drug from nose to the brain.

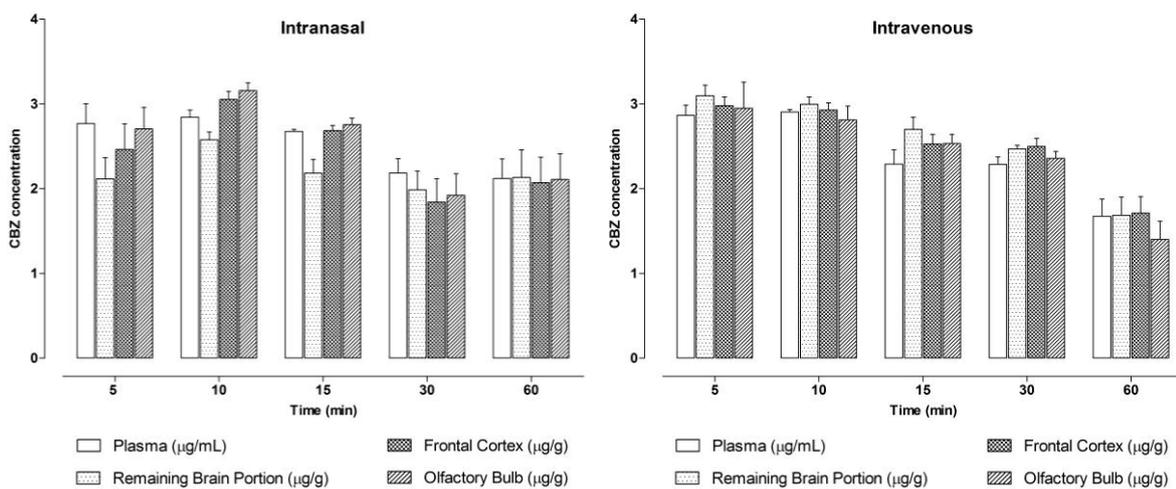


Figure III.2.2 Carbamazepine (CBZ) concentrations (mean \pm SEM) up to 60 min post-dosing in plasma and different brain regions (olfactory bulb, frontal cortex and the remaining portion of the brain) after IN and IV administration of CBZ (0.4 mg/kg) to mice ($n = 4$, at each time point). Statistical significant differences ($p < 0.05$) between both delivery routes (IN vs IV) are marked with (*).

The tissue-to-plasma and tissue-to-remaining portion of the brain concentration ratios were calculated for the olfactory bulb and frontal cortex specimens following both routes of administration (Table III.2.3). After IV injection, similar ratios were observed at all sampling time points within the first hour post-dosing, while after IN administration, discrepant values were ascertained, mainly up to 15 min. These results support the hypothesis that a direct transfer of CBZ from nose to the brain may be involved. Focusing particularly on the olfactory bulb-to-remaining portion of the brain ratios, it can be inferred that a direct nose-to-brain transport of CBZ occurs and probably via the olfactory pathway since the value of 1.29 ± 0.05 found at 5 min after IN delivery is significantly higher ($p < 0.05$) than that achieved after IV injection (0.95 ± 0.07) (Table III.2.3).

Table III.2.3 Tissue-to-plasma and tissue-to-remaining portion of the brain concentration ratios of carbamazepine (CBZ) in different brain regions following intranasal (IN) and intravenous (IV) administration to mice (0.4 mg/kg).

Route	Post-dosing Time (min)	CBZ Concentration Ratios				
		B/P	FC/P	OB/P	FC/B	OB/B
IN	5	$0.76 \pm 0.04^{\S}$	$0.88 \pm 0.05^*$	0.98 ± 0.04	$1.17 \pm 0.05^{\#}$	$1.29 \pm 0.05^{\#}$
	10	0.91 ± 0.06	1.08 ± 0.04	1.11 ± 0.03	$1.19 \pm 0.05^{\#}$	$1.23 \pm 0.07^*$
	15	$0.82 \pm 0.07^{\#}$	1.01 ± 0.03	1.03 ± 0.04	$1.25 \pm 0.09^*$	$1.28 \pm 0.06^{\#}$
	30	$0.90 \pm 0.03^{\#}$	$0.83 \pm 0.05^{\#}$	$0.87 \pm 0.04^*$	0.92 ± 0.03	0.96 ± 0.02
	60	0.99 ± 0.07	0.97 ± 0.06	0.99 ± 0.06	0.98 ± 0.02	1.00 ± 0.03
IV	5	1.08 ± 0.02	1.04 ± 0.02	1.03 ± 0.09	0.96 ± 0.02	0.95 ± 0.07
	10	1.03 ± 0.03	1.01 ± 0.03	0.97 ± 0.06	0.98 ± 0.02	0.94 ± 0.04
	15	1.19 ± 0.07	1.11 ± 0.08	1.12 ± 0.11	0.94 ± 0.05	0.94 ± 0.07
	30	1.08 ± 0.02	1.09 ± 0.02	1.04 ± 0.05	1.01 ± 0.03	0.96 ± 0.03
	60	1.01 ± 0.02	1.03 ± 0.02	0.84 ± 0.08	1.02 ± 0.02	0.83 ± 0.08

Data are expressed as the mean values \pm SEM of four animal determinations ($n = 4$). Statistically significant differences between the two routes of administration (IN versus IV) are marked with an asterisk (*) for $p < 0.05$, (#) for $p < 0.01$ and (§) for $p < 0.001$. B/P, remaining portion of the brain-to-plasma ratio; FC/B, frontal cortex-to-remaining portion of the brain ratio; FC/P, frontal cortex-to-plasma ratio; OB/B, olfactory bulb-to-remaining portion of the brain ratio; OB/P, olfactory bulb-to-plasma ratio.

III. 2.4. DISCUSSION

It is estimated that more than 98% of all small molecules and nearly 100% of large molecular weight drugs systemically delivered to the CNS, either by oral or IV routes, do not readily cross the BBB and reach the brain parenchyma at pharmacologically active concentrations (PARDRIDGE, 2005). As a consequence, many promising therapeutic agents may have been discarded due to its inability to effectively permeate BBB and others are given at high systemic doses to attain therapeutic levels at the biophase, which commonly lead to undesirable peripheral adverse effects and drug interactions.

In the light of the current knowledge, drug transport across the nasal mucosa into the CNS depends on a variety of factors that can range from the physicochemical properties of the drug to the formulation design and physiological conditions at the absorption site (PIRES *et al.*, 2009; VYAS *et al.*, 2006b). Aware that nasal MCC is one of the major limitations for nasal drug delivery (MARTIN *et al.*, 1998), the choice of a convenient nasal dosage form that avoids the rapid nasal drainage and promotes the increase of drug residence time within the nasal cavity is fundamental (MAJITHIYA *et al.*, 2006). Therefore, in order to avoid a fast MCC of the drug but to simultaneously keep an easy administration form, a thermoreversible mucoadhesive gel composed of 18% PF-127 and 0.2% C-974P was herein selected to incorporate and deliver CBZ by the IN route since, according to the results reported by Badgujar *et al.* (2010), the viscous properties of this formulation offer an appropriate and promising compromise between *in situ* gelling and ease of administration. Being a liquid-like solution at room temperature but changing to a firm gel at the physiological temperature within the nasal cavity (32-35°C) (BADGUJAR *et al.*, 2010), *in situ* thermoreversible mucoadhesive gel displays a huge advantage over the conventional and more viscous hydrogels (BARAKAT *et al.*, 2006; CZAPP *et al.*, 2008) concerning not only the ease of handling but also the accuracy of dosing (BASU and BANDYOPADHYAY, 2010).

Although CBZ is only currently available in oral dosage forms, it seems that the use of the IV route as a control is the most appropriate for this study. Indeed, due to the direct delivery of the drugs to the systemic circulation, IV administration will be responsible for the highest systemic exposure by comparison with any other route, creating appropriate conditions to allow a less variable drug incorporation and biodistribution. Moreover, considering that after IN administration drugs reach the CNS either via systemic circulation or olfactory epithelium, the contribution of the blood-mediated drug delivery to the brain can be inferred by employing IV injection and, consequently, the fraction of the drug directly transported from nose to brain could be more accurately discriminated.

The pharmacokinetic results herein described revealed that, similarly to what happens following IV injection, the IN administration of CBZ nasal gel brought a rapid and extensive systemic absorption of the drug (assessed by C_{max} , t_{max} , AUC_t and AUC_{inf}). The high CBZ concentrations attained in plasma after IN instillation, as well as the almost parallel time course of plasma and brain concentrations (Figure III.2.3), clearly indicate that a substantial fraction of the drug has effectively been absorbed to the systemic circulation and reached the brain parenchymal tissue by crossing the BBB. In addition, comparable parent drug plasma concentration-time profiles following IN and IV administrations were also observed (Figure III.2.1), supporting a similar bioavailability value (107.6%) achieved for the IN delivery of CBZ. These findings could be explained on the basis of the high lipophilic nature of the drug which LogP value is 2.45. Indeed, small lipophilic molecules nasally administered can be rapidly absorbed to the blood stream by easily crossing the nasal membrane via transcellular diffusion and then enter into the brain after traversing the BBB. Experimental data reported in other research studies using both low molecular weight and lipophilic compounds such as diazepam (LogP = 2.8) (KAUR and KIM, 2008), PB (LogP = 1.47) (CZAPP *et al.*, 2008), NXX-066 (LogP = 4.35) (DAHLIN and BJÖRK, 2001), progesterone (LogP = 4.03) and estradiol (LogP = 3.51) (VAN DEN BERG *et al.*, 2004a) underscored the fact that IN drug delivery occurred predominantly via the systemic pathway. The higher MRT values observed for plasma and brain on one hand and the lower MRT value attained in liver after IN administration comparatively to IV injection on the other hand, could also underlie the high bioavailability achieved for CBZ delivered by the IN route (Table III.2.2). In fact, according to these results, CBZ molecules stayed for a longer time in plasma and brain after IN instillation in comparison with the IV injection, which in turn led to a greater retention of the drug in the liver.

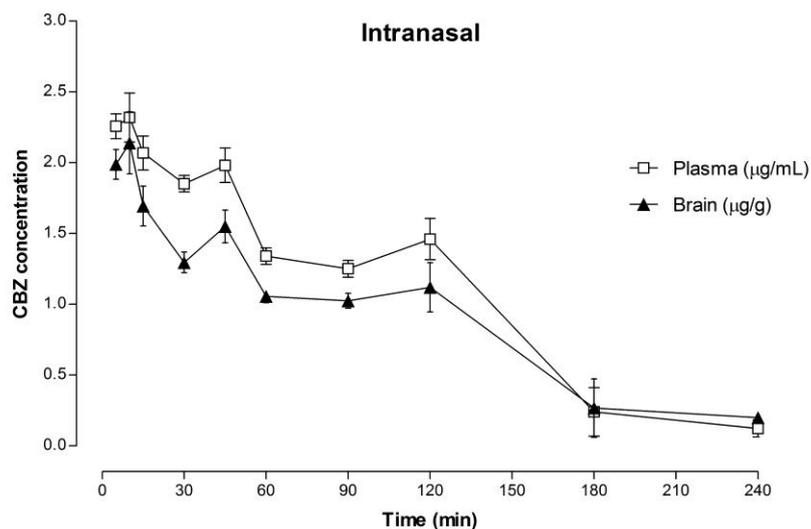


Figure III.2.3 Concentration-time profiles of carbamazepine (CBZ) in plasma and brain following IN administration (0.4 mg/kg) to mice. Symbols represent the mean values \pm SEM of four determinations per time point ($n = 4$).

Apart from the indirect pathway via the systemic circulation, it is believed that, there are two other different pathways by which a drug administered through the IN route may reach the CNS: the olfactory and the trigeminal neuronal routes (DHURIA *et al.*, 2010). Although both of them provide a direct nose-to-brain delivery of the drug, the uptake via the olfactory neurons affords a preferential drug delivery to the olfactory bulb and rostral portion of the brain while the transference via the trigeminal nerve generally yields a more distant drug distribution to caudal brain areas. Thus, aiming at evaluating whether a direct transport of CBZ was occurring from nose to brain, the drug distribution in different brain regions was characterised after IN and IV administration. Interestingly, distinct distribution of CBZ through plasma, olfactory bulb, frontal cortex and remaining portion of the brain following IN and IV administration was herein reported for the first time. While a homogeneous brain distribution was observed for CBZ after IV injection, in the case of IN administration, the CBZ concentrations were different according to the respective brain area, presenting higher values in the rostral portion comparatively to the cerebral caudal region. Given that the CBZ brain concentration ratios determined at 5 min were 1.36-fold higher in the olfactory bulb and 1.22-fold higher in the frontal cortex employing the nasal delivery route than those obtained for IV injection (Table III.2.3), it seems probable that a direct transport of the drug from nose to brain may be involved and that it occurred preferentially via the olfactory neuronal pathway. These findings assume particular interest in the field of the pharmaco-resistant epilepsy. Indeed, it is nowadays scientifically accepted that the overexpression and/or upregulation of

multidrug efflux transporters in the BBB is one of the main mechanisms responsible for the development of resistance to the AEDs (KWAN *et al.*, 2011; LÖSCHER and POTSCSKA, 2002; REMY and BECK, 2006). Overall, these transmembrane proteins pump the AEDs back to the systemic circulation, restricting their access to the brain (LÖSCHER and POTSCSKA, 2002; LUNA-TORTÓS *et al.*, 2008). In this context, the results herein obtained, demonstrate that the IN route may be considered as a novel approach to overcome the pharmacoresistance phenomena since a direct delivery of CBZ from nose to brain was clearly evidenced and it occurred in a considerable extent.

Pooling the data derived from both the pharmacokinetic and brain biodistribution studies following IN administration, it seems that with the high plasma concentrations on one hand and the superior delivery to the rostral regions of the brain on the other hand, CBZ reached the CNS through a combination of routes. Even though it is not possible to accurately quantify the contribution of each of these routes, we presume that a small fraction of the drug is in fact delivered to the brain via the olfactory pathway, while the most representative amount is still attributable to the systemic circulation. The 0.98 value obtained for the DTE index also strengthens this hypothesis. Notwithstanding, a further optimisation of the CBZ nasal formulation will probably contribute to a better exploitation of the maximum potential that the IN route has to offer.

In summary, IN delivery seems to represent a suitable and promising alternative route for the CBZ administration regarding not only its use on the chronic treatment of epilepsy but also in the case of more severe and acute emergency situations, such as *status epilepticus*. Indeed, the IN administration of CBZ allowed extensive plasma and brain exposures to the drug as well as a fast and pronounced drug uptake in the brain. Apart from being very practical and adequate to be used outside the hospital setting, the uneven biodistribution pattern with the highest CBZ concentration levels attained in the rostral areas of the brain, reinforces the potential of IN delivery to be employed in acute convulsive emergencies.

From the pharmacokinetic point of view, IN and IV administration of CBZ exhibited similar concentration-time profiles which probably point out to very similar pharmacological responses. In order to foresee whether IN delivery of CBZ could become clinically relevant, technological optimisation of the nasal drug formulation, as well as further pre-clinical investigations are needed to evaluate the therapeutic efficacy attained via this route.



CHAPTER IV

INTRANASAL ADMINISTRATION OF LAMOTRIGINE

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IV. 1. LAMOTRIGINE

Pharmacological Characterisation

IV. 1.1. HISTORICAL BACKGROUND

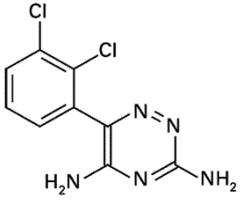
LTG was firstly synthesised in the 1970s as part of a rational effort to develop and test folic acid antagonists as possible anticonvulsants (MESSENHEIMER, 1995). In fact, since the beginning of the 1960s, it had been hypothesised that the disturbances of folate metabolism caused by some AEDs, such as PHT and PB, could be related to their antiepileptic properties, and that folate could act as a pro-convulsing agent leading to an exacerbation of seizures (BRODIE, 1992; RAMBECK and WOLF, 1993; SHORVON, 2009a). Although this hypothesis was later proven to be incorrect, with the antifolate effects of the two AEDs actually demonstrating no relevance to the corresponding anticonvulsive action, it resulted in the development of numerous antifolate agents and their further testing in various conventional animal models of epilepsy. Among a whole series of compounds studied by the Wellcome Research Laboratories, a group of phenyltriazine derivatives was deepened investigated, and of these, BW430C, later known as *lamotrigine*, proved to be one of the most promising (SHORVON, 2009a). Ironically, structure-activity studies subsequently revealed that little or no folic acid antagonism was indeed associated to LTG, and therefore, the very weak antifolate activity could not be responsible for its potent antiepileptic effects (GOA *et al.*, 1993; MESSENHEIMER, 1995).

After its therapeutic effectiveness had been successfully demonstrated in a number of studies performed on different laboratory animals and humans, LTG was introduced into clinical practice in Europe in 1990 (LTG was licensed in Portugal in December of 1994), and in the United States of America in 1994, as adjunctive treatment for focal seizures with or without secondary generalisation in adults with refractory epilepsy (SHORVON, 2009a). Several years after its launch, it became apparent that, in addition to the therapeutic indication for which it had been originally licensed (focal-onset seizures), the drug had also value in the treatment of generalised seizures, both primary and secondary, and soon the drug was being hailed as an anticonvulsant with a broad spectrum of activity (SHORVON, 2009a). Subsequent large scale controlled comparative trials of LTG monotherapy with CBZ (BRODIE *et al.*, 1995) and PHT (STEINER *et al.*, 1999) indicated that the former offered similar efficacy but better tolerability; since then, LTG has been increasingly used as first-line monotherapy, particularly for generalised epilepsy as an alternative to valproate (FITTON and GOA, 1995; SHORVON, 2009a).

IV. 1.2. PHARMACEUTICAL INFORMATION

LTG, a second-generation AED, is a phenyltriazine derivative which is structurally, chemically and pharmacologically unrelated to any established antiepileptic medication (GARNETT, 1997; GOA *et al.*, 1993). The molecule consists of a phenyl group with two chloride substituents joined to a triazine structure with two amino substitutions (WALKER and SANDER, 1999). It is a white to pale cream-colored powder with poor solubility in water or ethanol, and similarly to CBZ, it is only available in formulations for oral administration. LTG has lipophilic properties and is a weak base that in the presence of strong acids or bases is hydrolysed and converted into a mono- or di-hydroxotriazine. Detailed pharmaceutical information of LTG is summarised in Table IV.1.1.

Table IV.1.1 Pharmaceutical information of lamotrigine (DRUGBANK; GLAXOSMITHKLINE, 2013; PUBCHEM).

Drug Identification		Physicochemical Properties	
Generic Name	Lamotrigine	Phase State	Solid / White powder
Chemical Name	6-(2,3-dichlorophenyl)-1,2,4-triazine-3,5-diamine	Solubility in Water	0.17 mg/mL (at 25°C)
Chemical Formula	C ₉ H ₇ Cl ₂ N ₅	Solubility in Ethanol	4.1 mg/mL (at 25°C)
Molecular Weight	256.09 g/mol	LogP	2.5
Molecular Structure		pK_a	5.7
Drug Information			
Brand Name	Lamictal®	Manufacturer	GlaxoSmithKline
Dosage Forms	Compressed tablet Chewable/dispersible tablet	Route of Administration	Oral

LogP, octanol/water partition coefficient; pK_a, negative decadic logarithm of the acid dissociation constant.

IV. 1.3. PHARMACOKINETICS

The pharmacokinetic properties of LTG have been studied after its administration as single and multiple oral dosing regimens to animals, healthy volunteers and patients with epilepsy receiving concurrent antiepileptic medications. LTG exhibits a first-order linear kinetics which varies considerably among different individuals and species (GARNETT,

1997; RAMSAY *et al.*, 1991). This section and Table IV.1.2 will provide a brief overview of the LTG pharmacokinetic profile.

Table VI.1.2 Summary of the main pharmacokinetic parameters of lamotrigine in both adult healthy volunteers and patients with epilepsy (FITTON and GOA, 1995; GLAXOSMITHKLINE, 2013; GOLDSMITH *et al.*, 2003).

Pharmacokinetic Parameter	Mean Value
t_{max} (h)	1-3
F (%)	98
Plasma Protein Binding (%)	55
Vd/F (L/kg)	0.9-1.4
$t_{1/2el}$ (h)	25-35
CL/F (L/h/kg)	0.021-0.035

CL/F, apparent plasma clearance; F, absolute bioavailability; $t_{1/2el}$, apparent elimination half-life; t_{max} , time to achieve maximum peak concentration; Vd/F, apparent volume of distribution.

Absorption

Following oral administration, LTG is rapidly and almost completely absorbed with negligible first-pass metabolism, displaying an absolute bioavailability of about 98% (GARNETT, 1997; RAMBECK and WOLF, 1993). In the presence of food, the rate of absorption is slightly reduced but the extent remains unchanged (GLAXOSMITHKLINE, 2013). C_{max} is typically achieved within 1 to 3 hours post-dosing, and a second peak or plateau may occur at 4-6 hours, suggesting a possible enterohepatic recycling phenomenon (FITTON and GOA, 1995; GARNETT, 1997). Over the range of 50-400 mg given as a single dose, a linear relationship is ascertained between the dose administered, the C_{max} and the AUC in plasma (GLAXOSMITHKLINE, 2013; GOA *et al.*, 1993). Linearity has also been reported for doses up to 700 mg, indicating that the absorption of LTG is not saturable in clinical doses (GARNETT, 1997).

Distribution

Due to its high lipophilicity, LTG is widely and uniformly distributed throughout all organs and tissues. The Vd/F ranges from 0.9 to 1.4 L/kg and it is independent of dose, dosage regimen and the presence or absence of epilepsy (BITON, 2006). The degree of plasma protein binding is estimated to be constant at approximately 55% over LTG plasma concentrations within 1-10 $\mu\text{g/mL}$, and therefore, clinically significant interactions

with other drugs through competition for protein-binding sites are unlikely to occur (BITON, 2006; GARNETT, 1997; GLAXOSMITHKLINE, 2013).

Metabolism

LTG is a weak base that is extensively metabolised in the liver predominantly by glucuronic acid conjugation which appears to be the rate-limiting step in the elimination of the drug (COHEN *et al.*, 1987) (Figure IV.1.1). During the first 6-7 days after a single oral dose of LTG, about 75% to 90% of the total dose administered is recovered in urine, of which, 80-90% is found in the form of 2-N-glucuronide while the rest is eliminated as the 5-N-glucuronide (10%), 2-N-methyl (0.1%), unidentified metabolites and finally, the unchanged parent drug (10%) (BITON, 2006; GOA *et al.*, 1993; GOLDSMITH *et al.*, 2003; WERZ, 2008). Glucuronidation reactions are catalysed by the UGTs. None of the metabolites are pharmacologically active or associated to appreciably toxic effects (GARNETT, 1997; GOLDSMITH *et al.*, 2003; LU and UETRECHT, 2007).

Similarly to CBZ, LTG also exhibits an induction of its own metabolism, but in a smaller extent. For most patients, autoinduction is complete within the first two weeks of therapy and can be associated to a reduction of approximately 20% in LTG plasma concentrations (JOHANNESSEN *et al.*, 2003; KRASOWSKI, 2010). Nevertheless, since this phenomenon occurs mainly in the early stages of treatment, when plasma concentrations are rising to steady-state, and in view of the wide therapeutic margin of LTG, it is commonly considered that such effect is devoid of clinical relevance (RAMBECK and WOLF, 1993; WALKER and SANDER, 1999).

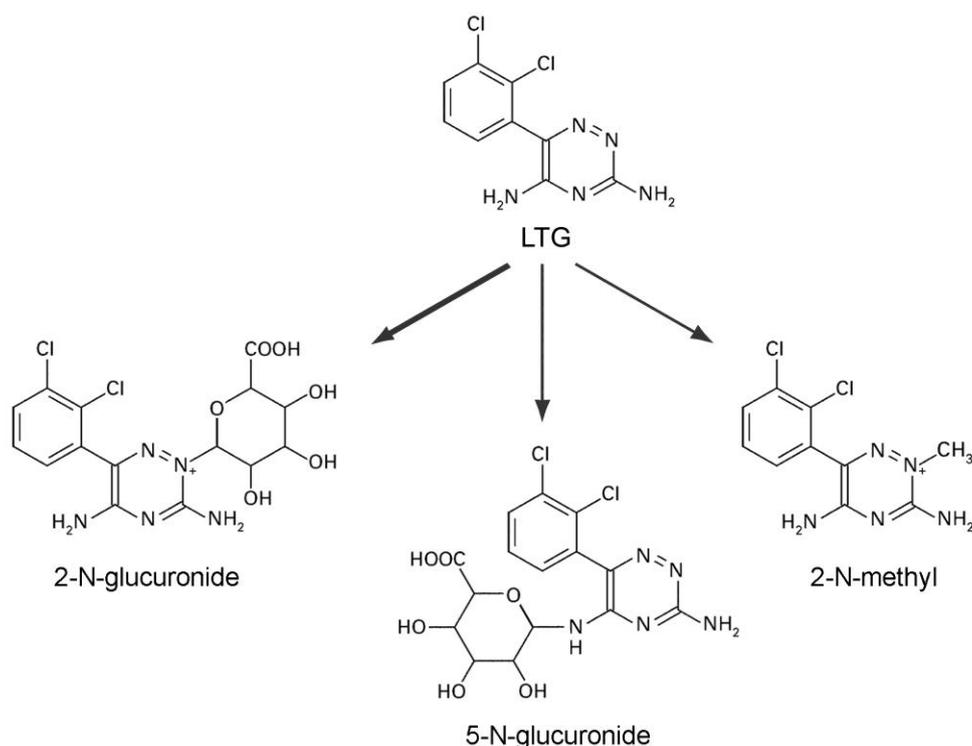


Figure IV.1.1 The major metabolites of lamotrigine (LTG).

Elimination

As aforementioned, the elimination of LTG follows a first-order linear kinetics. The plasma elimination half-life ($t_{1/2el}$) of LTG in healthy adults is reported to be in the range of 25-35 h, whereas the values of total clearance varies between 0.021-0.035 L/h/kg (GOA *et al.*, 1993; GOLDSMITH *et al.*, 2003).

The urinary excretion is the primarily route of LTG elimination with approximately 90% of total oral dose being recovered in urine. A very small amount of the drug (around 2%) could also be encountered in faeces, suggesting the involvement, although insignificant, of a biliary excretion (GARNETT, 1997).

IV. 1.4. PHARMACODYNAMICS

Mechanism of Action

LTG shares a very similar mechanism of action with conventional AEDs such as CBZ and PHT. Hence, it is suggested that LTG inhibits sustained repetitive firing of action potentials by stabilising pre-synaptic neuronal membranes through the blockade of voltage-sensitive sodium channels in a time- and use-dependent manner, preventing the

release of excitatory neurotransmitters, particularly glutamate and aspartate (FITTON and GOA, 1995; POTSCSKA, 2013). Identically to PHT, there is evidence that at therapeutic concentrations, LTG binds slowly to the fast inactivated state of the sodium channels, playing a key role on its selective effect against the sustained depolarisations and high frequency discharges characteristic of epileptic foci without disturbing most of the normal neuronal activities (FITTON and GOA, 1995; KUO and LU, 1997).

The LTG broader clinical spectrum of activity comparatively to other sodium channel-blocking antiepileptic agents points out that the interaction with sodium voltage-dependent channels is unlikely to be its sole antiseizure mechanism (PORTER *et al.*, 2012). Therefore, in addition to its inhibitory effect on sodium channels, LTG may also modify voltage-gated calcium currents by blocking pre-synaptic N-type and P-type calcium channels, resulting in decreased glutamate release (KWAN *et al.*, 2001; PORTER *et al.*, 2012). Notwithstanding, it remains possible that supplementary unidentified mechanisms of action for LTG may still exist.

LTG is also believed to act on serotonin reuptake, which may contribute to its antidepressant effects (NG *et al.*, 2007).

IV. 1.5. CLINICAL USE

Therapeutic Indications

LTG was initially approved for the add-on treatment of focal seizures in adults and later in children above the age of two (WERZ, 2008). Clinically, the drug shows a broad spectrum of antiepileptic activity proving to be highly effective against focal seizures with or without generalisation and primarily generalised seizures, including tonic-clonic, absence, atonic and drop attacks (FITTON and GOA, 1995; NEELS *et al.*, 2004).

Similarly to other novel AEDs, LTG possesses comparable anticonvulsant efficacy but exerts much less adverse effects when compared to conventional antiseizure drugs (CZAPIŃSKI *et al.*, 2005). It is indicated for use as adjunctive therapy for the management of adult and paediatric patients with epilepsy who are not satisfactorily controlled; as monotherapy following withdrawal of other AEDs (CBZ, PHT, PB, PRM and valproic acid) and as adjunctive therapy for the management of generalised seizures associated with Lennox-Gastaut syndrome (GLAXOSMITHKLINE, 2013). Recently, LTG was also licensed for the maintenance treatment of bipolar disorder to delay the time to occurrence of mood episodes (GOLDSMITH *et al.*, 2003).

Tolerability

In comparison to older AEDs, LTG is generally associated to less clinically significant neurological, cognitive, metabolic, hepatic or reproductive endocrine toxicity in the majority of patients. Commonly reported adverse experiences of LTG administered as mono- or adjunctive therapy are primarily CNS-related, including dizziness, diplopia, ataxia, somnolence, blurred vision, headache and asthenia (10-14%) (GOA *et al.*, 1993; RAMBECK and WOLF, 1993). Likewise CBZ or PHT, LTG has also been associated with serious hypersensitivity reactions such as skin rash, which although very rare, is the most common adverse event leading to discontinuation of therapy (BITON, 2006; GOA *et al.*, 1993). Skin rashes have been reported in about 10% of patients receiving LTG and they are usually of the simple morbiliform type, benign and self-limited. Occasionally, more severe forms of rash like Stevens-Johnson syndrome and toxic epidermal necrolysis have also been described (BITON, 2006; FITTON and GOA, 1995; WERZ, 2008). Skin rash manifests almost always within the first few weeks after initiation of LTG therapy but resolves quickly upon treatment withdrawal (FITTON and GOA, 1995). It appeared to be associated with the use of high initial doses, rapid dose-escalation and concomitant administration with valproic acid (BITON, 2006; RAMBECK and WOLF, 1993). Accordingly, in order to reduce the risk of rash development, it is recommended a gradual dose-titration from a low starting dose and appropriate dose adjustments when LTG is administered to patients already receiving valproate.

Therapeutic Range

There is not a clear-cut relationship between plasma concentrations of LTG and clinical or toxic responses mainly due to a large pharmacokinetic interindividual variability (PATSALOS *et al.*, 2008). A tentative target range of 1-4 µg/mL was initially proposed, however it later appeared not to correlate well with pharmacological effects (MORRIS *et al.*, 1998). Indeed, subsequent studies suggested that these former concentrations may have been too low since some patients have tolerated concentrations above 10 µg/mL with additional therapeutic benefits and without significant clinical toxicity (BRODIE, 1992). Moreover, the incidence of toxic effects have considerably increased only when plasma LTG levels exceed 15 µg/mL (KRASOWSKI, 2010; PATSALOS *et al.*, 2008). Therefore, Morris *et al.* (1998) suggested that an appropriate reference range of plasma concentrations for LTG would be 3-14 µg/mL which is the therapeutic window currently adopted in clinical practice.

Drug Interactions

Since LTG does not appear to interfere with the hepatic mixed function oxidase system and is not highly protein bound, it is therefore expected that the number and extent of drug interactions will be reduced compared with classical AEDs (MESSENHEIMER, 1995). Nevertheless, although LTG itself generally does not affect the metabolism/elimination of other AEDs, important alterations in LTG plasma concentrations are ascertained in patients receiving other antiseizure medications. Thus, in the presence of hepatic enzyme-inducing agents like for instance CBZ, PHT, PB and PRM, the rate of LTG elimination is increased, resulting in a $t_{1/2el}$ reduction of approximately one-half. Conversely, concomitant administration with valproic acid provides a marked prolongation of LTG $t_{1/2el}$ for the double essentially due to competition for hepatic glucuronidation sites (FITTON and GOA, 1995; JOHANNESSEN and LANDMARK, 2010). Co-medication with newer AEDs such as felbamate, gabapentin, levetiracetam, topiramate, vigabatrin and zonisamide does not have a significant impact on LTG pharmacokinetics (WEINTRAUB *et al.*, 2005).

Some relevant interactions between LTG and drugs other than antiepileptic agents have also been described. While sertraline has shown to increase LTG concentrations probably by inhibition of LTG glucuronidation, acetaminophen and rifampicin have been reported to accelerate its metabolism (JOHANNESSEN *et al.*, 2003). Plasma concentrations of LTG can also be reduced by more than 50% on concomitant use with oral contraceptives. This interaction is likely to be caused by glucuronidation induction by the steroids, yielding a diminished seizure control in some women (JOHANNESSEN and LANDMARK, 2010).



IV. 2. EXPERIMENTAL

Direct Nose-to-Brain Delivery
of LTG Following Intranasal
Administration to Mice

IV. 2.1. INTRODUCTION

The lifelong seizure freedom with tolerable adverse effects is considered the ultimate goal of any therapeutic intervention for epilepsy. Although the pharmacological treatment of epilepsy has made remarkable breakthroughs with the introduction of more than 20 different AEDs, it has fallen short of expectations, with up to one-third of the patients continuing to experience seizures or unacceptable medication-related side-effects (CANEVINI *et al.*, 2010; FRENCH, 2007). Indeed, pharmacoresistance has been assumed as one of the major causes underlying the failure of the anticonvulsant therapy and it is commonly associated with an increased risk of morbidity and mortality (ALEXOPOULOS, 2013; WEAVER and POHLMANN-EDEN, 2013). Even though the regulatory events that determine AED resistance have not been completely understood yet, important insights regarding the pathophysiologic mechanisms of intractable epilepsy are emerging. Therefore, considering that the biologic basis of refractoriness is likely to be multifactorial and variable, several putative neurobiological processes have been identified and suggested over recent years (KWAN and BRODIE, 2002; LÖSCHER and SCHMIDT, 2011). Among distinct theories, the multidrug-transporter hypothesis has been gaining an increasing support. Accumulating evidence demonstrate that various efflux transporters such as P-glycoprotein and members of the multidrug resistant-associated protein family, expressed in capillary endothelial cells of the BBB, are particularly upregulated in epileptogenic brain tissue of refractory epileptic patients (LÖSCHER, 2007; POTSCHKA, 2010a; SCHMIDT and LÖSCHER, 2005). This overexpression hampers the penetration of several AEDs into the brain, limiting the amount of the drug that efficiently reaches the therapeutic target, and consequently, it may lead to the reduction or even complete loss of pharmacosensitivity at the biophase (LÖSCHER, 2007; POTSCHKA, 2010b).

LTG is a second-generation AED which exhibits a broad spectrum of efficacy and tolerability, being effective against focal and generalised tonic-clonic seizures, either alone or as adjunctive therapy (GOA *et al.*, 1993; SCHILLER and KRIVOY, 2009). Although it has been initially approved for the add-on treatment of patients with refractory focal-onset epilepsy, only a modest or no significant improvement has been effectively demonstrated on seizure control (SHORVON, 2009a). Inclusively, several recent reports have identified LTG as a P-glycoprotein substrate, which may explain its limited access to the brain throughout the treatment (LUNA-TORTÓS *et al.*, 2008; POTSCHKA *et al.*, 2002; ZHANG *et al.* 2012). Currently, LTG is only commercially available in tablet dosage forms for oral delivery (BITON, 2006), requiring an extensive systemic absorption with further BBB crossing to exert its therapeutic effect. Increasing the oral dose in an attempt to attain therapeutic levels of LTG in the brain and appropriately manage the occurrence of uncontrolled seizures is invariably associated to an augmentation of drug concentrations

in the blood, thus enhancing the predisposition to develop undesirable peripheral adverse events like severe skin rashes (BITON, 2006; SCHILLER and KRIVOY, 2009).

In this context, alternative administration strategies that provide a more efficient delivery of LTG to the brain are undoubtedly required. A number of different invasive approaches like intraparenchymal, intraventricular and intrathecal injections have successfully been used to increase the CNS targeting of drugs. Through the introduction of therapeutics directly into the brain, these techniques have revealed to be very effective on drug delivery; however, they are considered impractical and unrealistic for clinical application because they are complex, risky, expensive and not convenient for multiple dosing regimens (DHURIA *et al.*, 2010). On the other hand, the IN administration as a means of delivering therapeutic agents preferentially to the brain has recently gained significant interest (SERRALHEIRO *et al.*, 2013a). In fact, the unique anatomical connection between the nasal cavity and the CNS provides a great opportunity for drugs administered by the IN route to reach a quick and easy access to the brain through pathways along the olfactory and trigeminal nerves innervating the nasal passages (LOCHHEAD and THORNE, 2014). In accordance, considering its undeniable potential, IN delivery could be a favourable and promising alternative route to improve CNS targeting of LTG. Besides circumventing the BBB, IN administration also avoids hepatic first-pass and systemic dilution effects, facilitates administration of low doses and in turn reduces peripheral drug exposure, minimising toxicity (JOGANI *et al.*, 2008). Moreover, as demonstrated in the previous chapter (*Chapter III, section III. 2.*), the IN administration of CBZ allowed a rapid and extensive cerebral uptake, as well as an uneven biodistribution pattern with the highest drug concentration levels achieved in the rostral areas of the brain, strengthening the potential of this delivery route to be employed in acute convulsive episodes (SERRALHEIRO *et al.*, 2014). Thus, in the absence of a marketed LTG formulation for IV administration, IN delivery may also be regarded as a useful and valuable option in cases of emergency.

Curiously, some formulations for IN delivery of LTG have already been developed and investigated (ALAM *et al.*, 2015; DAVE and PUROHIT, 2013); notwithstanding, little or no information really exists on its pharmacokinetics following administration through this route. To the best of our knowledge, only one study has recently tested the efficacy of the experimental IN formulation of LTG *in vivo* (ALAM *et al.*, 2015), however the drug concentrations determined in plasma and brain were not sufficient to set up a complete pharmacokinetic profile. Aiming at evaluating the potential of LTG to be administered via the IN route, a comprehensive characterisation of the pharmacokinetic behaviour of the drug was herein performed. Hence, following both IN and IV administrations, concentration levels of LTG in plasma, brain and liver tissues were determined and the corresponding pharmacokinetic profiles were assessed and compared. The feasibility of

the drug to be directly transported from nose to the brain, bypassing the BBB, was also investigated. For this purpose, LTG concentrations were analysed in different dissected brain regions and the rostral-caudal brain distribution patterns of the drug were established for the two delivery routes considered.

IV. 2.2. MATERIALS & METHODS

IV. 2.2.1. Chemicals and Reagents

LTG was gently provided by Bluepharma (Coimbra, Portugal). Chloramphenicol, used as IS, PF-127 and propylene glycol were all purchased from Sigma-Aldrich (St. Louis, MO, USA). C-974P was kindly supplied from Lubrizol (Wickliffe, OH, USA). Methanol and acetonitrile of HPLC gradient grade were acquired from Fisher Scientific (Leicestershire, UK) and Lab-Scan (Sowinskięgo, Poland), respectively. Ultrapure water (HPLC grade, 18.2 M Ω .cm) was prepared by means of a Milli-Q water apparatus from Millipore (Milford, MA, USA). Ethyl acetate was obtained from Fisher Scientific (Leicestershire, UK). TEA and potassium dihydrogen phosphate, as well as sodium dihydrogen phosphate dihydrate, disodium hydrogen phosphate dihydrate and hydrochloric acid fuming 37%, used to prepare 0.1 M sodium phosphate buffer pH 5.0, were all purchased from Merck KGaA (Darmstadt, Germany). Ketamine (Imalgene 1000[®], 100 mg/ml) and xylazine (Vetaxilaze 20[®], 20 mg/ml) were commercially acquired.

IV. 2.2.2. Animals

A total of 160 adult male CD-1 mice, with approximately 6-7 weeks old and weighting 30-40 g, were obtained from local certified animal facilities (Faculty of Health Sciences of the University of Beira Interior, Covilhã, Portugal) to be used in this study. Mice were housed under controlled environmental conditions (12 h light/dark cycle, at 20 \pm 2 $^{\circ}$ C and relative humidity 50 \pm 5%) with free access to tap water and standard rodent diet (4RF21, Mucedola, Italy). All the experiments involving animals and their care were conducted in conformity with the international regulations of the European Directive (2010) regarding the protection of laboratory animals used for scientific purposes (2010/63/EU) (EUROPEAN PARLIAMENT AND COUNCIL OF THE EUROPEAN UNION, 2010), and the experimental procedures employed were reviewed by the Portuguese DGAV.

IV. 2.2.3. Preparation of Lamotrigine Formulations

For IN administration, LTG was previously dissolved in 100 μL of propylene glycol and further incorporated in 900 μL of a thermoreversible nasal gel so that the final drug concentration in the formulation was 10 mg/mL. The thermoreversible gel was prepared using the cold method described by Schmolka (1972). Briefly, 1.8 g of PF-127 was slowly added to 10 mL of distilled cold water (5-10°C), under gentle magnetic stirring, to achieve an efficient hydration of the flakes and then, the mixture was left at 4°C overnight to attain a complete dissolution of the polymer (18% PF-127, w/v). Afterwards, according to the technique employed by Badgujar and co-workers (2010), the mucoadhesive polymer C-974P was gradually dispersed in the prepared PF-127 solution with continuous agitation, until a final concentration of 0.2% w/v has been reached. At this point, a nasal hydrogel formulation composed of 18% PF-127 and 0.2% C-974P was obtained, exhibiting thermo-sensible properties. In fact, PF-127 is a triblock copolymer of poly(ethylene oxide) and poly(propylene oxide) units that is fluid at or below room temperature, but turns to a semisolid gel when the temperature increases, as a consequence of the micelle packing disorder-order transition phenomenon (SWAMY and ABBAS, 2012). This thermo-sensible behaviour makes the final formulation suitable for gelation within the nasal cavity, providing a sustained residence of the drug at the absorption site.

For the IV administration, LTG parenteral formulation was prepared as a mixture of propylene glycol-physiological saline solution (NaCl 0.9%)-ethanol (5:3:2, v/v/v) at a final drug concentration of 1 mg/mL.

IV. 2.2.4. IN and IV Administrations

Before LTG dosing, mice were anaesthetised with an intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and kept in a heated environment to maintain the body temperature.

LTG was intranasally and intravenously administered at the dose of 4 mg/kg. For IN administration, mice were placed on one side and 12 μL of the nasal gel per 30 g of mice body weight were instilled using a polyurethane tube (24G x 19 mm) attached to a microliter syringe. The tube was inserted about 10 mm deep into one of the nares, enabling the delivery of the formulation towards the roof of the nasal cavity. IV administration of LTG (4 mg/kg) was performed by means of a short and slow infusion of the formulation for over 30 s into the lateral tail vein (120 μL per 30 g body weight) using an appropriate syringe.

IV. 2.2.5. Pharmacokinetic Study

Mice were randomly divided into two experimental groups of 52 animals each. One of the groups received IN formulation whereas the other group was treated with the IV dosage form. At predetermined time points (0.083, 0.167, 0.25, 0.5, 0.75, 1, 2, 4, 8, 12, 24, 36 and 48 h) after LTG dosing (4 animals per time point, $n = 4$), the mice were sacrificed by cervical dislocation followed by decapitation and the blood was immediately collected into heparinised tubes, while brain and liver tissues were quickly removed and weighed. Blood samples were centrifuged at 4°C and 4000 rpm for 10 min to obtain plasma supernatants that were stored at -30°C until analysis. Mice brain and liver tissues were homogenised with 0.1 M sodium phosphate buffer pH 5.0 (4 mL per gram of tissue) using a THOMAS® Teflon pestle tissue homogeniser. Tissue homogenates were centrifuged at 4800 rpm for 15 min (4°C) and the resultant supernatants were also frozen at -30°C until analysis.

IV. 2.2.6. Brain Biodistribution Study

Mice were divided at random into two experimental groups (28 animals each). The animals were treated with LTG (4 mg/kg) by IN or IV routes. After administration, mice were sacrificed at 0.083, 0.167, 0.25, 0.5, 1, 2 and 4 h post-dosing ($n = 4$). Blood samples were taken and plasma was separated as previously described. Brains were removed and carefully dissected with the help of a scalpel into three different regions: olfactory bulb, frontal cortex and the remaining portion of the brain. The remaining portion of the brain was homogenised and centrifuged in accordance to the procedure used for brain and liver tissues, while olfactory bulb and frontal cortex specimens, regardless of the weight, were homogenised with 0.5 mL of phosphate buffer using an ULTRA-TURRAX® device and centrifuged at 4°C for 15 min at 13400 rpm. The resultant homogenate supernatants were conveniently packaged and stored at -30°C until analysis.

IV. 2.2.7. Drug Analysis

The concentrations of LTG in plasma, brain and liver tissues were determined by using a LLE procedure followed by a reversed-phase HPLC analysis as detailed in *Chapter II, section II. 3.3*.

Aliquots of plasma (200 µL), brain (500 µL), olfactory bulb (500 µL), frontal cortex (500 µL) and liver (250 µL) homogenate supernatants were all spiked with 10 µL of the methanolic IS working solution (chloramphenicol, 400 µg/mL). With the exception of liver samples, which were previously added of 250 µL of acetonitrile, vortexed (1 min) and

centrifuged at 13400 rpm for 10 min in order to precipitate protein content, the remaining specimens were immediately submitted to the LLE process. Therefore, 500 μL of ethyl acetate (extraction solvent) were added to samples and liver protein precipitation resulting supernatant, vortex-mixed for 1 min and then centrifuged during 5 min at 13400 rpm. The upper organic layer was transferred to a clean glass tube while the residual aqueous phase was re-extracted once again, applying the same experimental proceeding. The whole organic extracts obtained were evaporated to dryness under a gentle nitrogen stream at 45°C, and the residues were further reconstituted with 100 μL of mobile phase. At the end, all the reconstituted extracts were transferred to a 1.5 mL Eppendorf tube, centrifuged (2 min, 13400 rpm) and a 20 μL aliquot of each resultant supernatant was injected into the chromatographic system. Particularly for plasma, the final supernatants were subjected to an additional 0.22 μm Spin-X filtration before injection.

The HPLC analysis was carried out on a Shimadzu liquid chromatographic system equipped with a GDU-20A₅ degasser, a SIL-20A_{HT} autosampler, a CTO-10AS_{VP} column oven and a SPD-M20A diode array detector, all from Shimadzu Corporation (Kyoto, Japan). Data acquisition and instrumentation control were achieved by means of LCsolution software (Shimadzu Corporation, Kyoto, Japan). Chromatographic separation was performed at 25°C on a reversed-phase LiChroCART® Purospher Star® C₁₈ column (55 mm x 4 mm, 3 μm ; Merck KGaD), using an isocratic elution with a mobile phase consisting of 0.05 M potassium dihydrogen phosphate solution-methanol-acetonitrile-TEA (79.7:10:10:0.3, v/v/v/v) pumped at a flow rate of 1 mL/min. LTG and IS were detected at the wavelength of 306 nm and the total running time was set at 12 min. The main partial validation parameters of the analytical method employed were in agreement with the international guidelines (EUROPEAN MEDICINES AGENCY, 2011; US FOOD AND DRUG ADMINISTRATION, 2001) and are summarised in Table IV.2.1.

Table IV.2.1 Validation parameters of the HPLC method employed for the quantification of lamotrigine (LTG) in plasma, brain, liver, olfactory bulb and frontal cortex homogenate supernatants ($n = 3$).

Validation Parameters	Plasma	Brain	Liver	Olfactory Bulb	Frontal Cortex
Calibration Range ($\mu\text{g}/\text{mL}$)	0.20 - 30	0.05 - 15	0.20 - 20	0.025 - 7.5 ^b	0.025 - 7.5 ^b
Coefficient of Determination (r^2)	0.997	0.992	0.989	0.993	0.992
LLOQ ($\mu\text{g}/\text{mL}$)	0.20	0.05	0.20	0.025 ^b	0.025 ^b
Precision (% CV) ^a	≤ 7.0	≤ 4.8	≤ 6.8	≤ 12.4	≤ 4.8
Accuracy (% Bias) ^a	-6.8 - 9.1	-3.4 - 5.4	-9.7 - 11.6	-3.5 - 7.2	-3.4 - 5.4
Absolute recovery (%)	83.6 - 88.0	73.5 - 87.2	82.5 - 100.8	-	-

^a Inter-day values, $n = 3$. ^b Values expressed in μg . Bias, deviation from nominal value; CV, coefficient of variation; LLOQ, lower limit of quantification.

IV. 2.2.8. Pharmacokinetic Analysis

The maximum concentration (C_{max}) of LTG in plasma and tissues and the corresponding time to reach C_{max} (t_{max}) were directly derived from the experimental data obtained. The remaining pharmacokinetic parameters were estimated based on the mean concentration values ($n = 4$) determined at each time point by non-compartmental pharmacokinetic analysis employing the WinNonlin® version 5.2 (Pharsight Co, Mountain View, CA, USA). The pharmacokinetic parameters evaluated were the area under the drug concentration time-curve (AUC) from time zero to the time of the last quantifiable drug concentration (AUC_t) which was calculated by the linear trapezoidal rule; the AUC from time zero to infinity (AUC_{inf}) that was calculated from $AUC_t + (C_{last}/k_{el})$, where C_{last} is the last quantifiable concentration and k_{el} (or k) is the apparent (or tissue) elimination rate constant estimated by log-linear regression of the terminal segment of the concentration-time profile; the percentage of AUC extrapolated from t_{last} to infinity [$AUC_{extrap}(\%)$], where t_{last} is the time of the C_{last} ; the apparent terminal (or tissue) elimination half-life ($t_{1/2el}$ or $t_{1/2}$), and the mean residence time (MRT).

The absolute bioavailability [F (%)] of LTG after IN administration was calculated as follows (Eq. 1):

$$F (\%) = \frac{(AUC_{inf IN} \times Dose_{IV})}{(AUC_{inf IV} \times Dose_{IN})} \times 100 \quad (\text{Eq. 1})$$

where $AUC_{inf IN}$ and $AUC_{inf IV}$ are the areas under the drug concentration-time curves from time zero to infinity following IN and IV administration, respectively; $Dose_{IV}$ and $Dose_{IN}$ are the values of the LTG dosage (mg/kg) given by IV and IN route to mice.

In order to assess the overall tendency of the nasally administered LTG to achieve the brain, the DTE index was calculated (KOZLOVSKAYA *et al.*, 2014). DTE index represents the brain-to-plasma partitioning ratio of the drug administered by IN route compared to that after IV injection and can be calculated according to the following equation (Eq. 2):

$$DTE = \frac{(AUC_{brain}/AUC_{plasma})_{IN}}{(AUC_{brain}/AUC_{plasma})_{IV}} \quad (\text{Eq. 2})$$

where AUC_{brain} and AUC_{plasma} are the areas under the drug concentration-time curves for brain and plasma after both IN and IV administration to mice. It is assured that preferential transport of the drug to the brain occurs when DTE index is greater than 1 (WANG *et al.*, 2003).

With the aim of evaluating the biodistribution of LTG to specific brain regions (olfactory bulb, frontal cortex and the remaining portion of the brain) after IN and IV

administrations, the drug concentrations in each specimen were determined at predefined time points ($n = 4$). The corresponding tissue-to-plasma and tissue-to-remaining portion of the brain concentration ratios of LTG were calculated and compared.

IV. 2.2.9. Statistical Analysis

The data were expressed as mean \pm standard error of the mean (SEM). Statistical comparisons were performed between IN and IV administration groups using unpaired two-tailed Student's *t*-test. Differences were considered statistically significant for a *p*-value lower than 0.05 ($p < 0.05$).

IV. 2.3. RESULTS

IV. 2.3.1. Pharmacokinetics of Lamotrigine after IN and IV Administration

The mean concentration-time profiles of LTG obtained in plasma, brain and liver tissues of mice after a single dose administration of the drug (4 mg/kg) as nasal gel or IV solution are depicted in Figure IV.2.1. The corresponding main pharmacokinetic parameters estimated by non-compartmental analysis are summarised in Table IV.2.2. Considering the pharmacokinetic profiles of LTG in the three biological matrices analysed, a clear delay is observed between the t_{\max} following IN *versus* IV administration. Accordingly, despite the similarity in terms of magnitude, the mean C_{\max} of LTG in plasma was attained significantly later after IN delivery comparatively to IV injection (0.75 h *versus* 0.083 h), indicating the occurrence of an extensive and sustained absorption of the drug from the nasal cavity. An even more remarkable delay was determined in liver (t_{\max} after IV and IN administrations was 0.167 h and 8 h, respectively) even though the extent of hepatic exposure to LTG had been equivalent after IN or IV administration (as assessed by AUC_t and AUC_{inf}). Importantly, the extent of systemic and brain exposures to the drug was slightly greater after IN delivery (as assessed by AUC_{inf}) and the absolute bioavailability estimated for LTG delivered via the IN route was found to be very high (116.5%), suggesting a complete absorption of the drug.

The mean C_{\max} of LTG achieved in the brain after IN instillation was significantly lower ($p < 0.001$) in comparison to IV injection; however, regarding the total brain exposure to the drug assessed by the AUC_{inf} parameter, it should be noted that a higher value was assigned to the IN route. Particularly interesting were the differences detected between the values estimated for the pharmacokinetic parameters of elimination. In fact,

contrasting with the other two matrices, the brain was the only one whose $t_{1/2}$ and MRT were higher following IN delivery than after IV injection (Table IV.2.2).

As shown in Table IV.2.2, the DTE index calculated for the intranasally administered LTG amounted to 0.96 which does not provide any discriminative information of the potential for direct nose-to-brain transport of the drug via the IN route. Hence, the estimated DTE value appears to suggest that the uptake of LTG into the CNS through the nasal cavity is predominantly accomplished by crossing the BBB after an extensive nasal absorption of the drug to the bloodstream. Taking solely these pharmacokinetic data into account, the impact of the direct nose-to-brain delivery of LTG after IN instillation is not evident, which may probably be related to the fact that only the analysis of whole brain homogenate concentrations has been considered.

Table IV.2.2 Pharmacokinetic parameters and tissues-to-plasma partitioning ratios of lamotrigine (LTG) following its intranasal (IN) and intravenous (IV) administration (4 mg/kg) to mice.

Pharmacokinetic Parameters ^a	Plasma		Brain		Liver	
	IN	IV	IN	IV	IN	IV
t_{max} (h)	0.750	0.083 ^c	2.000	0.500	8.000	0.167
C_{max} ($\mu\text{g}/\text{mL}$)	2.903	3.019 ^d	1.290 ^e	1.756 ^e	2.145 ^e	3.137 ^e
AUC_t ($\mu\text{g}\cdot\text{h}/\text{mL}$)	52.633	44.794	25.410 ^f	25.411 ^f	54.798 ^f	54.808 ^f
AUC_{inf} ($\mu\text{g}\cdot\text{h}/\text{mL}$)	54.835	47.053	30.149 ^f	26.881 ^f	63.308 ^f	63.845 ^f
AUC_{extrap} (%)	4.02	4.80	15.7	5.47	13.4	14.2
k_{el} (h^{-1})	0.077	0.059	-	-	-	-
k (h^{-1})	-	-	0.051	0.068	0.047	0.044
$t_{1/2el}$ (h)	9.0	11.8	-	-	-	-
$t_{1/2}$ (h)	-	-	13.6	10.2	14.7	15.7
MRT (h)	17.7	17.1	20.1	14.5	23.6	23.4
F (%) ^b	116.5	-	-	-	-	-

AUC_{inf} Ratios	IN	IV	IN/IV
AUC_{brain}/AUC_{plasma}	0.55	0.57	0.96 ^g
AUC_{liver}/AUC_{plasma}	1.15	1.36	0.85

^a Parameters were estimated using the mean concentration-time profiles obtained from four different animals per time point ($n = 4$). ^b Absolute intranasal bioavailability (F) was calculated based on AUC_{inf} values. ^c t_{max} in plasma after IV administration corresponds to the first sampling time point. ^d C_{max} in plasma after IV administration is equivalent to the maximum concentration value determined at the first sampling time point (0.083 h). ^e Values expressed in $\mu\text{g}/\text{g}$. ^f Values expressed in $\mu\text{g}\cdot\text{h}/\text{g}$. ^g Drug targeting efficiency index. AUC_{extrap} , extrapolated area under the drug concentration time-curve; AUC_{inf} , area under the concentration time-curve from time zero to infinity; AUC_t , area under the concentration time-curve from time zero to the last quantifiable drug concentration; C_{max} , maximum peak concentration; k , tissue elimination rate constant; k_{el} , apparent elimination rate constant; MRT, mean residence time; $t_{1/2}$, tissue elimination half-life; $t_{1/2el}$, apparent terminal elimination half-life; t_{max} , time to achieve the maximum peak concentration.

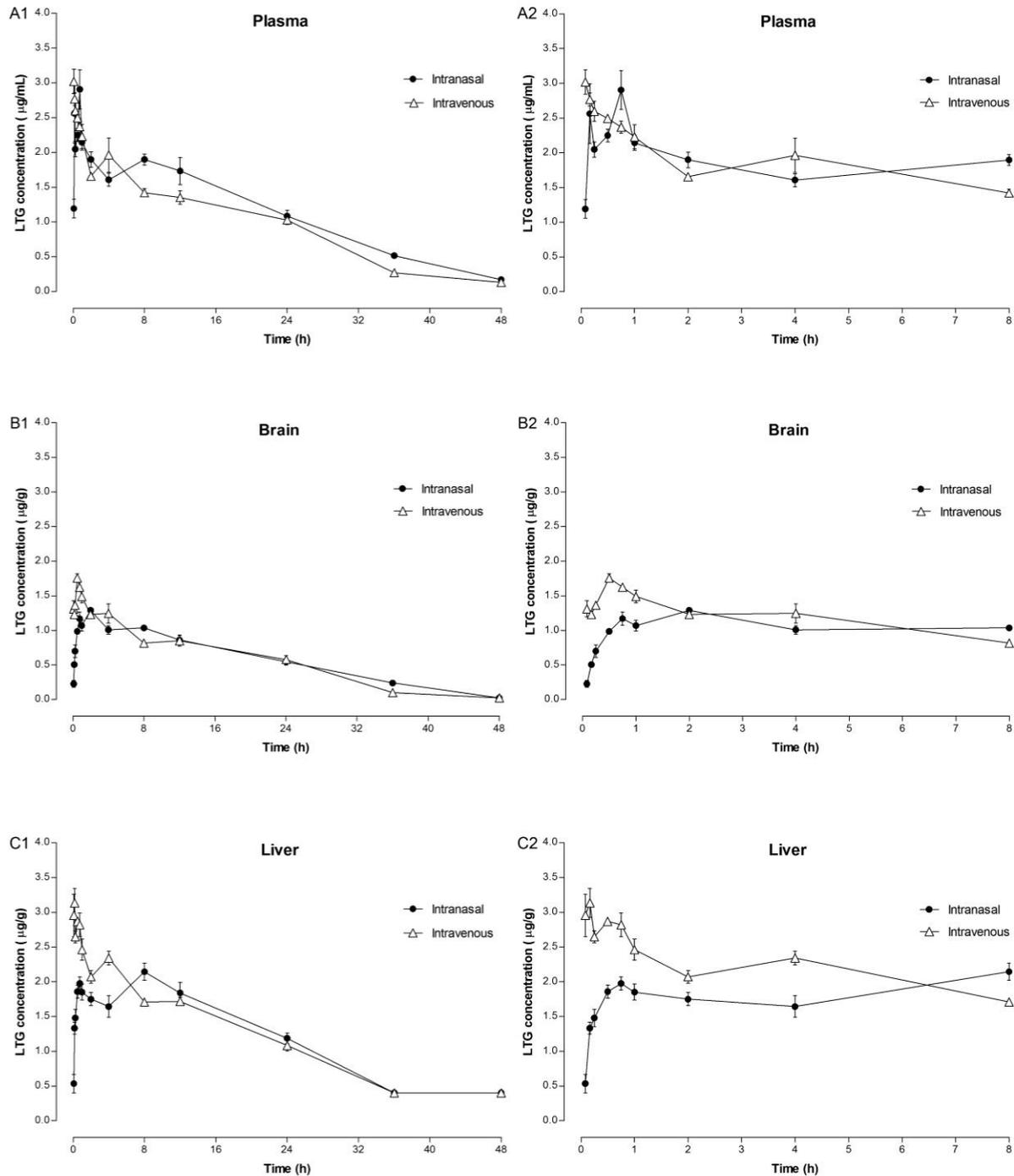


Figure IV.2.1 Concentration-time profiles of lamotrigine (LTG) up to 48 h post-dosing in plasma (A1), brain (B1) and liver (C1) tissues following IN and IV administration (4 mg/kg) to mice. The expanded partial representation of the corresponding concentration-time profiles of LTG up to 8 h post-dosing are also plotted for plasma (A2), brain (B2) and liver (C2), respectively. Symbols represent the mean values \pm SEM of four determinations per time point ($n = 4$).

IV. 2.3.2. Brain Biodistribution of Lamotrigine after IN and IV Administration

In order to investigate the potential involvement of a direct nose-to-brain transport pathway for LTG, more specific and informative data on the rostral-caudal brain biodistribution pattern of the drug following its IN and IV administrations (4 mg/kg) were evaluated in mice. Clearly, the mean LTG concentrations in plasma and two particular brain regions, namely the olfactory bulb and frontal cortex, along with the remaining portion of the brain were analysed at predefined time points up to 4 h post-dosing. The results are illustrated in Figure IV.2.2 as well as the statistical significant differences between both delivery routes (IN *versus* IV). In general, it becomes clear from Figure IV.2.2 that the concentrations of LTG achieved in brain specimens after each administration route are significantly different while those obtained in plasma are very similar.

Particularly regarding the IV administration of LTG, even though higher drug concentrations were achieved in the olfactory bulb comparatively to the frontal cortex and the remaining portion of the brain, all the cerebral specimens evidenced similar drug contents over time, suggesting a homogeneous pattern of brain biodistribution. Conversely, a very heterogeneous and variable drug distribution in the brain was clearly observed for LTG following its administration by the IN route (Figure IV.2.2). Indeed, after IN instillation, higher LTG concentrations were always found in the olfactory bulb relatively to frontal cortex or remaining portion of the brain. Specifically at 0.083 h post-dosing, a mean concentration value of about 25-fold and 67-fold greater was determined in the olfactory bulb (6.70 $\mu\text{g/g}$) in comparison to the frontal cortex (0.27 $\mu\text{g/g}$) and the remaining portion of the brain (0.1 $\mu\text{g/g}$), showing a markedly uneven distribution of the drug from rostral to more caudal cerebral areas (Figure IV.2.2). Interestingly, by analysing the temporal evolution of LTG biodistribution following IN delivery it is possible to notice a decrease of the mean drug concentrations in both plasma and olfactory bulb while a progressive and gradual augment was observed in the frontal cortex and the remaining portion of the brain from 0.083 h onwards.

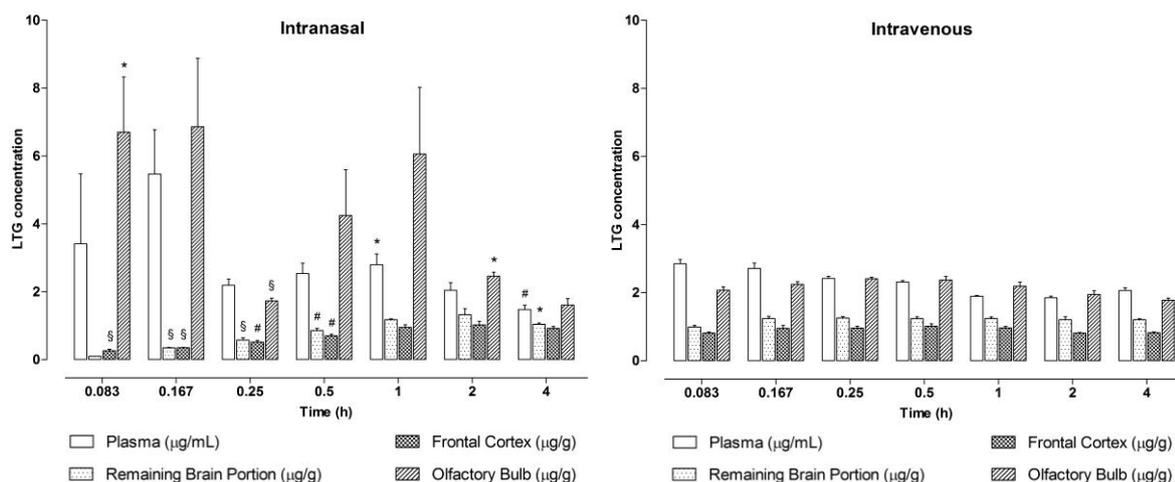


Figure IV.2.2 Lamotrigine (LTG) concentrations (mean \pm SEM) up to 4 h post-dosing in plasma and different brain regions (olfactory bulb, frontal cortex and the remaining portion of the brain) after IN and IV administration (4 mg/kg) to mice ($n = 4$, at each time point). Statistical significant differences between both delivery routes (IN vs IV) are marked with (*) for $p < 0.05$, (#) for $p < 0.01$ and (§) for $p < 0.001$.

The tissue-to-plasma and tissue-to-remaining portion of the brain concentration ratios were calculated for the olfactory bulb and frontal cortex specimens following both routes of administration. As depicted in Table IV.2.3, the concentration ratios of intravenously administered LTG were roughly comparable at all sampling time points, whereas following IN instillation, some discrepant values were ascertained mainly up to 0.167 h post-dosing. Statistically significant differences were found between IN and IV delivery routes for the olfactory bulb-remaining portion of the brain concentration ratios calculated at 0.083 h ($p < 0.01$) and 0.167 h ($p < 0.05$), supporting that a direct transfer of LTG from nose to brain may be involved. Hence, taking into consideration the information derived from Figure IV.2.3, it is evident that, particularly at the first sampling time point (0.083 h), the ratio between LTG concentrations achieved in the olfactory bulb and the remaining portion of the brain is about 32-fold higher after IN administration than IV injection. Accordingly, it can be inferred that a direct nose-to-brain transport of LTG occurs probably via the olfactory pathway.

Table IV.2.3 Tissue-to-plasma and tissue-to-remaining portion of the brain concentration ratios of lamotrigine (LTG) in different brain regions following intranasal (IN) and intravenous (IV) administration to mice (4 mg/kg).

Route	Post-dosing Time (h)	LTG Concentration Ratios				
		B/P	FC/P	OB/P	FC/B	OB/B
IN	0.083	0.07 ± 0.03 [§]	0.20 ± 0.09	3.62 ± 1.40	2.65 ± 0.46 [#]	67.04 ± 16.25 [#]
	0.167	0.08 ± 0.02 [§]	0.08 ± 0.03 [#]	1.90 ± 1.15	1.01 ± 0.06	19.39 ± 5.79 [*]
	0.25	0.26 ± 0.03 [§]	0.24 ± 0.03 [#]	0.80 ± 0.07 [*]	0.92 ± 0.07	3.20 ± 0.55
	0.5	0.35 ± 0.04 [#]	0.28 ± 0.02 [#]	1.81 ± 0.65	0.83 ± 0.08	4.87 ± 1.40
	1	0.44 ± 0.05 [#]	0.35 ± 0.01 [#]	2.09 ± 0.49	0.81 ± 0.05	5.08 ± 1.53
	2	0.64 ± 0.03	0.50 ± 0.02 [*]	1.23 ± 0.11	0.79 ± 0.07	1.93 ± 0.19
	4	0.72 ± 0.04 [*]	0.64 ± 0.06 [#]	1.14 ± 0.19	0.89 ± 0.05 [*]	1.56 ± 0.19
IV	0.083	0.35 ± 0.01	0.29 ± 0.02	0.73 ± 0.04	0.82 ± 0.06	2.09 ± 0.14
	0.167	0.46 ± 0.04	0.35 ± 0.04	0.84 ± 0.07	0.77 ± 0.08	1.82 ± 0.13
	0.25	0.52 ± 0.01	0.40 ± 0.01	1.00 ± 0.010	0.77 ± 0.04	1.93 ± 0.05
	0.5	0.54 ± 0.02	0.44 ± 0.03	1.03 ± 0.06	0.82 ± 0.04	1.92 ± 0.09
	1	0.66 ± 0.03	0.51 ± 0.03	1.16 ± 0.07	0.78 ± 0.06	1.78 ± 0.15
	2	0.65 ± 0.04	0.44 ± 0.01	1.05 ± 0.05	0.68 ± 0.05	1.64 ± 0.16
	4	0.59 ± 0.02	0.40 ± 0.01	0.86 ± 0.02	0.69 ± 0.02	1.47 ± 0.03

Data are expressed as the mean values ± SEM of four animal determinations ($n = 4$). Statistically significant differences between both routes of administration (IN versus IV) are marked with (*) for $p < 0.05$, (#) for $p < 0.01$ and (§) for $p < 0.001$. B/P, remaining portion of the brain-to-plasma ratio; FC/B, frontal cortex-to-remaining portion of the brain ratio; FC/P, frontal cortex-to-plasma ratio; OB/B, olfactory bulb-to-remaining portion of the brain ratio; OB/P, olfactory bulb-to-plasma ratio.

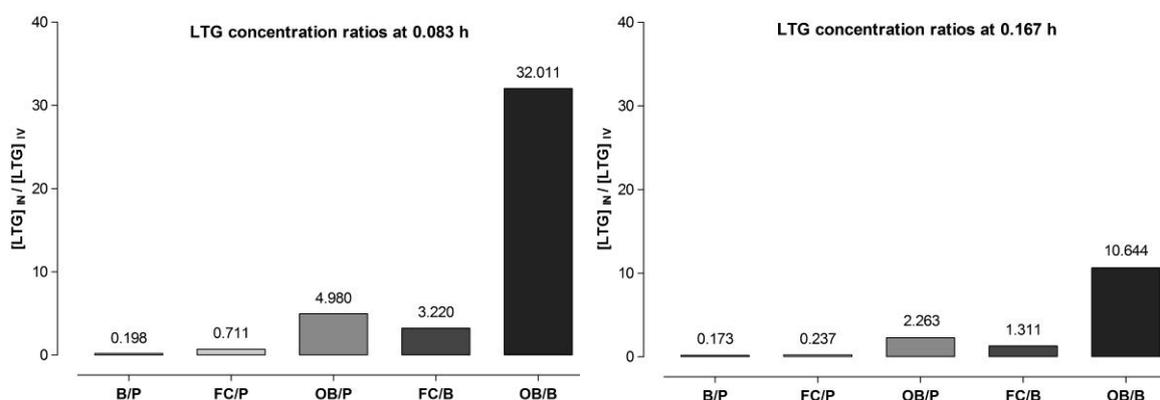


Figure IV.2.3 Relationship between tissue-to-plasma and tissue-to-remaining portion of the brain concentration ratios obtained for lamotrigine (LTG) at 0.083 h and 0.167 h following its intranasal (IN) and intravenous (IV) administration (4 mg/kg) to mice. B/P, remaining portion of the brain-to-plasma ratio; FC/B, frontal cortex-to-remaining portion of the brain; FC/P, frontal cortex-to-plasma ratio; OB/B, olfactory bulb-to-remaining portion of the brain ratio; OB/P, olfactory bulb-to-plasma ratio.

IV. 2.4. DISCUSSION

The past two decades have witnessed the global licensing of several new AEDs with different pharmacological properties and mechanisms of action. Despite the encouraging and welcome advantages that these modern therapies have brought in terms of tolerability and safety, it is however generally recognised that, concerning therapeutic efficacy, no substantial improvement has effectively been introduced (POTSCHKA and BRODIE, 2012). As a result, more than 30% of the epileptic patients continue to experience uncontrolled seizures, and pharmacoresistant epilepsy is nowadays considered a medical problem that urgently demands the development of more effective therapeutic approaches (WEAVER and POHLMANN-EDEN, 2013; WEAVER, 2013). In this regard, the present study was designed with the intent of assessing the potential of LTG to be intranasally administered and investigating whether or not the drug was transferred along the olfactory neuronal pathway directly into the brain.

The pharmacokinetic profiles of LTG herein characterised following both routes of administration revealed that IN delivery provided an extensive but longstanding absorption of the drug, reaching equivalent plasma C_{max} values although later than IV administration. The high LTG systemic exposure ($AUC_{inf} = 54.835 \mu\text{g}\cdot\text{h}/\text{mL}$) and the existence of a clear delay between t_{max} in plasma and brain seems to indicate that, likewise to what happens after IV administration, IN instillation enables the delivery of drug to the brain predominantly through the vascular system (Figure IV.2.4). The absolute bioavailability value achieved for intranasally administered LTG greater than 100% ($F = 116.5\%$) is presumably related to the inter-individual variability among distinct experimental animals since each mouse was assigned not only for one of the two administration groups (IN or IV) but also to a unique sampling time point; though, it underscores the fact that, a substantial fraction of the drug has effectively been absorbed from the nasal cavity into the systemic circulation and gained access to the CNS by crossing the BBB. These findings are in agreement with our previous study conducted with CBZ, an AED that similarly to LTG, is characterised by a low molecular weight and high lipophilicity (SERRALHEIRO *et al.*, 2014). Indeed, the DTE index values calculated for both CBZ (0.98) (SERRALHEIRO *et al.*, 2014) and LTG (0.96) were almost equal to the unity, suggesting no relevant contribution of the IN route to direct brain delivery of these drugs from the nasal cavity. However, the investigations performed with CBZ (SERRALHEIRO *et al.*, 2014) revealed that the analysis of total brain concentrations by treating the organ as a whole may dissipate the contribution of direct neuronal pathways to transport the drug from nose to brain, and therefore, drug distribution levels in specific cerebral areas should be measured in order to afford a more rigorous evaluation of the routes by which molecules have travelled.

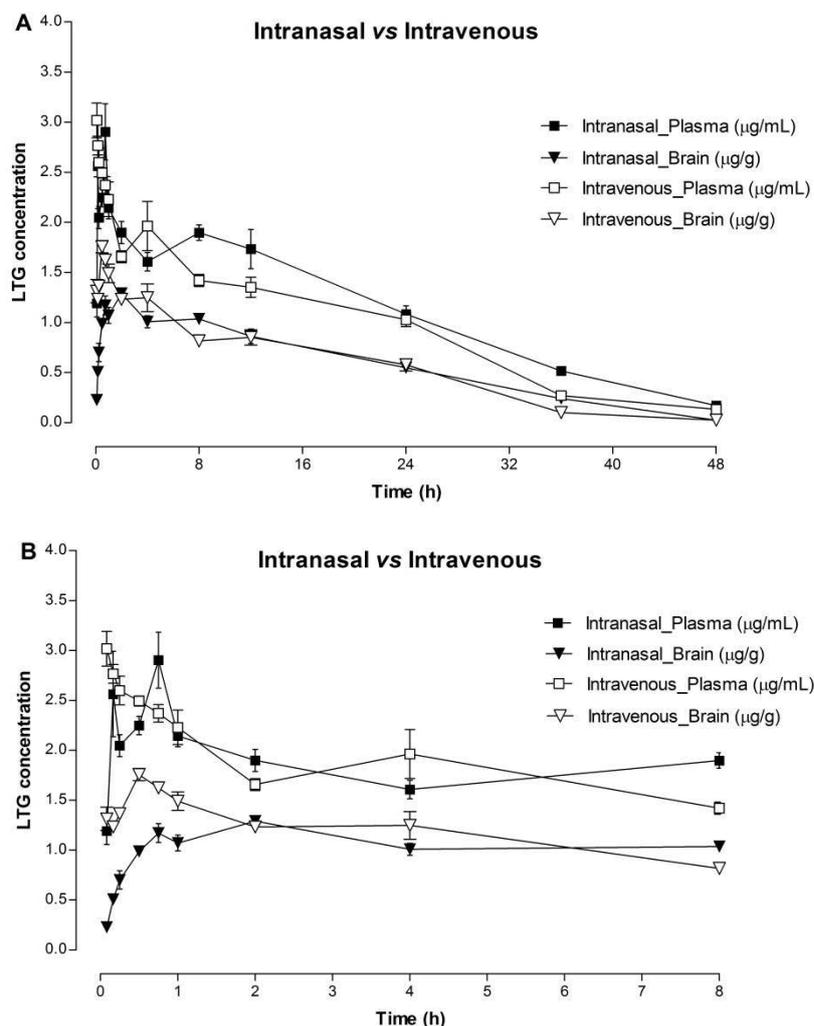


Figure IV.2.4 Concentration-time profiles of lamotrigine (LTG) in plasma and brain following IN and IV administration (4 mg/kg) to mice: (A) from time zero to 48 h post-dosing; (B) from time zero to 8 h post-dosing. Symbols represent the mean values \pm SEM of four determinations per time point ($n = 4$).

Regarding the pharmacokinetic parameters of elimination, it deserves to be mentioned that the brain was the single matrix in which the values estimated for $t_{1/2}$ and MRT were higher following IN delivery than IV injection. In fact, after IN instillation a greater retention of LTG was accomplished in the brain, allowing the maintenance of relevant levels of the drug for a longer period of time. Taking into account that t_{max} was only achieved in the brain at 2 h post-IN dosing and that the respective rate of drug elimination (assessed as k) is lower compared to the IV route, it appears that IN delivery may establish itself as a valid approach for the chronic administration of LTG, more so than for acute convulsive emergencies.

With the purpose of evaluating whether a direct transport of LTG was occurring from nose to the brain, drug concentration levels were determined in different cerebral regions and the corresponding rostral-to-caudal biodistribution patterns were typified following both routes of administration. According to the results herein found, after IV injection, LTG was homogeneously distributed throughout the various parts of the brain, in consonance with the pharmacokinetic data obtained for LTG intraperitoneally administered to rats (CASTEL-BRANCO, 2002; WALKER *et al.*, 2000). Despite the CNS uptake of LTG delivered by the IV route has remained constant over time and differed little among distinct brain regions, a particular intriguing accumulation of the drug was, howbeit, ascertained in the olfactory bulb (Figure IV.2.2). Curiously, a preferential retention of some other therapeutic compounds, such as testosterone (BANKS *et al.*, 2009) and picolinic acid (BERGSTRÖM *et al.*, 2002), has also been reported in the olfactory bulb even when systemically administered, a phenomenon that is possibly related to regional differences in receptor binding or efflux transporters expression.

Indeed, the LTG concentration levels attained in the olfactory bulb relatively to other parts of the brain after IV administration were remarkably lower than those found after IN administration (Figures IV.2.2 and IV.2.3), clearly suggesting the occurrence of a direct nose-to-brain transfer of the drug by means of the olfactory neuroepithelium. Effectively, the quick and striking appearance of LTG in the olfactory bulb immediately at 0.083-0.167 h post-IN dosing, with maximum concentration values of about 3-fold higher than those achieved after IV injection seem to support the existence of a pathway that enables the delivery of the drug to the brain from the olfactory nasal mucosa. Furthermore, specifically at 0.083 h post-dosing, the calculated LTG concentration ratios between the olfactory bulb and the remaining portion of the brain have shown a significant discrepancy among IN and IV routes of administration (67.04 ± 16.25 vs 2.09 ± 0.14 , respectively), thereby strengthening the potential of the IN delivery to improve LTG CNS targeting.

Gathering the overall generated results, it can be assured that due to the high plasma drug concentrations on one hand, and the superior drug distribution to the olfactory bulb on the other hand, intranasally administered LTG reached the CNS through a combination of both systemic and olfactory pathways. Although it is not possible to accurately quantify the contribution of each pathway, we presume that a small fraction of the drug is in fact delivered to the brain via the olfactory route, whereas the most representative amount is most likely to be attributable to the systemic circulation. Following IN delivery of LTG, the high plasma bioavailability value achieved and the sustainment of relevant drug concentration levels in the brain for an extended period of time seem to indicate an advantageous application of this alternative route in chronic

treatment of epilepsy. In addition, strong evidence accounting for a direct transport of LTG from nose to brain, by circumventing the BBB, may likewise render the IN route as a promising and valuable drug delivery strategy for a prospective management of pharmacoresistance.



CHAPTER V

GENERAL DISCUSSION

Currently, millions of people worldwide are affected by serious and debilitating brain disorders, foreseeing that the discovery and development of novel CNS-acting drugs will continue at a significant pace. Despite the advent of numerous new AEDs and the more widespread use of non-pharmacological options like for example surgery, a considerable percentage of epileptic patients continue to manifest seizures, thus providing the impetus for the development of innovative and more efficient therapeutic approaches. In fact, bearing in mind that about 30% of the patients are medically refractory to a wide range of AEDs with different mechanisms of action, it may be profitable to consider other avenues beyond the drug pharmacodynamics to improve seizure control and tolerability. Therefore, it is imperative that novel therapeutic regimens are able to attain relevant pharmacological targets within the CNS, whereby more effective drug delivery strategies towards the epileptogenic brain tissue may enhance efficacy and minimise toxicity of AEDs. The exploitation of the IN route as a means of preferentially delivering therapeutic agents to the brain has steadily been gaining momentum, notwithstanding, the mechanisms governing this uptake are not completely elucidated yet. The work underlying this dissertation intended to assess the feasibility of using the IN delivery as a promising alternative route for the administration of CBZ, PHT, OXC, and LTG in mice and to investigate whether a direct transport of the referred compounds from nose to brain is effectively involved.

In this section, a more integrative discussion of the various topics dealt within the previous chapters will be addressed. A critical overview of the key subjects covering the overall research work carried out to achieve the main objectives initially proposed at the beginning of this thesis will be herein thoroughly provided. Thus, relevant considerations about the HPLC-UV methods developed and validated to support the quantitative analysis of the samples derived from the pharmacokinetic studies performed will be firstly given. The paramount experimental issues concerning the optimisation of the study design will be also discussed. Final remarks are reserved for the examination of the results obtained from the pharmacokinetic and brain biodistribution studies conducted with IN administration of both CBZ and LTG to mice.

Quantitative Drug Analysis

This project started with the development and validation of appropriate and reliable bioanalytical techniques to support the execution of the intended pharmacokinetic and brain biodistribution studies, ensuring an accurate, precise and sensitive quantification of CBZ, PHT, OXC, LTG and their respective main metabolites in different biological samples of mice. Among the analytical methodologies most commonly

employed in the quantitative assessment of IN drug delivery, HPLC remains the method of choice since it enables the separation and quantification of quite complicated mixtures of several compounds within the same sample, and it is also able to determine very small amounts of the analytes that are frequently attained during the pharmacokinetic studies performed in animals. Hence, more extensive and comprehensive descriptions of the pharmacokinetic profiles obtained for drugs intranasally administered can be achieved, easily discriminating the presence of the parent drug from its correspondent metabolites formed *in vivo*.

The beginning of this work was settled by the development and fully validation of an HPLC-UV method for the simultaneous quantitative determination of PRM, PB, CBZ, PHT, OXC, LTG and three of their main metabolites, CBZ-E, *trans*-diol and Lic in human plasma. In point of fact, it is commonly argued that TDM is of particular value for the pharmacological treatment optimisation of patients with epilepsy since the majority of AEDs exhibit narrow therapeutic ranges, pronounced inter- and intra-individual pharmacokinetic variability and high propensity to establish drug-drug interactions (JOHANNESSEN and LANDMARK, 2008; NEELS *et al.*, 2004; PATSALOS *et al.*, 2008). Assuming that drug concentration correlates better with therapeutic and/or toxic effects than the dose, patients' clinical outcome can be considerably improved by managing their medication regimen with the assistance of measured drug concentrations in plasma (JOHANNESSEN *et al.*, 2003; PATSALOS *et al.*, 2008). Being simple, rapid, sensitive, accurate and reliable, this HPLC technique demonstrated suitability to be easily adopted by hospitals in order to support routine TDM of patients treated with such AEDs either in mono-, transitional or polytherapy regimens, also contributing as a useful tool to be applied in pharmacokinetic-based studies involving these drugs. The use of an UV detection coupled with an isocratic elution and a mobile phase essentially composed of water allowed to accomplish a chromatographic separation of all the nine compounds in less than 16 min. Moreover, the combination of PP with SPE resulted in a fast and reproducible sample preparation, affording high values of recovery.

Apart from the undeniable applicability of this analytical assay in clinical TDM, the major intent of developing a methodology that enabled the simultaneous quantification of the referred AEDs in human plasma was to test and optimise the best analytical conditions that could be subsequently transposed into the analysis of animal matrices. As mentioned in *Chapter II (sections II. 1.1 and II. 3.1.)*, due to its relative complexity, high availability and easy access, the use of human plasma as a surrogate matrix enables to expedite the process of method development, also minimising the number of animals that would be necessarily sacrificed to obtain blank matrices.

Therefore, based on the background knowledge acquired during the course of optimising the first analytical technique, the development and validation of a new HPLC-UV method for the simultaneous quantification of PHT, CBZ, OXC, LTG and some of their main metabolites (CBZ-E and Lic) in mouse plasma, brain and liver homogenates was initially attempted. In fact, rodents are the most widely used laboratory animals for IN drug delivery experiments (KOZLOVSKAYA *et al.*, 2014). Mice were herein preferred since, in addition to be more convenient and ethically more appropriate for the purposes of the current work (complete pharmacokinetic characterisation in plasma, brain and liver which implies the sacrifice of the animals at each time point), they are commonly accessible, less expensive, readily available and very easy to handle. Furthermore, the assessment of drug concentrations in plasma, brain and liver tissues was chosen once these are considered to be the matrices with the utmost relevance under the pharmacokinetic point of view. The brain has a particular importance by corresponding on one hand to the biophase and, on the other, to the hypothetical first portal of entry for drugs administered via the IN route. In line with this, it was further recognised that the measurement of drug concentrations in the anterior parts of the brain, such as olfactory bulb and frontal cortex was pivotal to infer about the existence of a pathway to transport drugs from the nasal cavity directly to the CNS.

Unfortunately, in spite of all the efforts carried out aiming the implementation of a unique and reliable HPLC assay that would enable the simultaneous quantification of the four AEDs and their respective metabolites in mouse matrices, this was not possible. Indeed, the basic criteria internationally recommended for the preliminary validation parameters evaluated were not entirely fulfilled, indicating that this method by not guaranteeing the generation of accurate, precise and reproducible data did not gather the appropriate performance characteristics to assure a valid interpretation of the obtained analytical results. Facing such limitation and due to the need to comply with the initially scheduled project tasks, it was decided to take advantage of techniques already developed and fully validated in our laboratory by introducing the alterations required to make them adequate for the purposes of the current work. Thereby, two distinct methodologies were then successfully adjusted and partially validated in mouse plasma, brain (whole brain, olfactory bulb and frontal cortex) and liver, constituting the basilar bioanalytical tools to support the overall *in vivo* studies planned. One allowed the quantification of CBZ, CBZ-E, OXC, Lic and PHT (*Chapter II, section II. 3.2.*), while the other enabled the determination of LTG (*Chapter II, section II. 3.3.*). In essence, both techniques display various favourable attributes, particularly the use of an aqueous mobile phase pumped isocratically, an UV detection and a highly reproducible sample preparation procedure (SPE or LLE) that provide a fast, sensitive and inexpensive analysis of the large number of samples derived from the pharmacokinetic experiments performed. According

to partial validation assessments, analytical results were proven to be confidently accomplished, demonstrating accuracy, precision and reliability.

Study Design

Prior to initiating *in vivo* studies, several preliminary experiments were conducted in order to test and define the optimum experimental conditions to be applied throughout this investigation.

First of all and taking into account the objectives of our work, the most appropriate experimental animal model had to be selected. As with any biomedical research area, many of the studies performed to date on IN delivery of drugs have been undertaken in rodents, being the rat and mouse the most frequently used species (GIZURARSON, 1990; GRAFF and POLLACK, 2005; ILLUM, 1996; KOZLOVSKAYA *et al.*, 2014). The mouse was herein chosen because, as mentioned above, it seemed to be the most adequate for the purpose of the current research. In addition to be cheap, promptly accessible, easy to maintain and handle, mice are normally characterised by a quick breeding and growth, also requiring only few amounts of drug to be administered. Beyond that, it seems that the mouse, comparatively to rat, is the species more related to humans regarding the metabolism of both CBZ and OXC (HAINZL *et al.*, 2001; MYLLYNEEN *et al.*, 1998). Therefore, adult male CD-1 mice with approximately 6-7 weeks old and weighting between 30-40 g were used, ensuring that all the animals' biological systems were completely matured and that possible pharmacokinetic interferences from the female hormonal cycle were avoided.

The search for the most suitable drug formulation, the ideal delivery volume and the most favourable IN administration technique proved to be the critical stage of the overall definition of the *in vivo* experimental setup. Indeed, the mouse nasal cavity presents several intrinsic anatomical and physiological features that impose multiple practical obstacles for an efficient and reproducible IN drug delivery. Since the mouse nasal cavity constitutes a very small drug administration site (approximately 0.03 mL average volume) (GIZURARSON, 1990; GROSS *et al.*, 1982), the total amount of drug which can be delivered by the nasal route is extremely limited. As a result, only a small volume of a nasal dosage form can be administered without compromising the respiratory function of mouse, an obligate nose-breather, and the magnitude of the dose is largely affected by the physicochemical properties of the drugs. Hence, a delivery volume of 12 μ L per 30 g of mouse body weight was established once it appeared to cover a considerable extent of the nasal mucosa surface without evident signs of either anterior leakage or animal respiratory distress. In opposition to the majority of the IN drug

delivery studies reported in literature wherein fixed volumes of nasal formulation were usually administered (DAHLIN *et al.*, 2000; FLORENCE *et al.*, 2011; JAIN *et al.*, 2010; PATHAN *et al.*, 2009; VYAS *et al.*, 2006A; WESTIN *et al.*, 2006), it was decided to standardise the experimental outcome as much as possible by settling down the delivery volume on the basis of each animal body weight, thus minimising a factor that would easily contribute to increase inter-individual variability. Bearing in mind that the reduced volume of IN administration has invariably a significant impact on the dose that can be instilled, higher doses demand the preparation of highly concentrated drug formulations. On top of that, the low aqueous solubility inherent to a great number of therapeutic compounds has generally been pointed out as the main limiting issue (BAHADUR and PATHAK, 2012; BEHL *et al.*, 1998; COSTANTINO *et al.*, 2007). Being the AEDs under evaluation (CBZ, OXC, LTG and PHT) highly lipophilic molecules, remarkable difficulties were encountered concerning the incorporation of a sufficient amount of drugs into the desired nasal formulation. Even if in the initial phases of the work higher doses were expected to be attained, the poor solubility in water and/or ethanol of the referred test compounds stood up as the major technological limitation, determining an administration of much lower values than those expected. Thus, the doses had to be selected essentially based upon drug solubility, corresponding to the maximum quantity of drug that was possible to be incorporated into the nasal dosage form. Just as important as achieving therapeutic concentrations at the biophase is guaranteeing that quantifiable levels of the drug are reached in all the key biological samples analysed. While CBZ and LTG fulfilled this latter assumption, in the case of OXC and PHT such requirement was not afforded (*Appendix B.*).

With the intent of assuring an accurate, precise and reproducible IN dosing, a concerted relationship between the type of the pharmaceutical formulation used as a delivery vehicle and the methodology employed for the IN drug administration was herein successfully found. Aware of the notable influence that MCC mechanism has on the bioavailability of intranasally administered compounds (MARTIN *et al.*, 1998; SCHIPPER *et al.*, 1991), the choice of an appropriate nasal formulation that could avoid the rapid post-nasal drainage and promote the increase of drug residence time within the nasal cavity was one of our foremost concerns. Accordingly, in order to prevent a quick elimination of the drug from the nasal absorption site and to simultaneously keep a practical administration dosage form, a thermoreversible mucoadhesive gel composed of 18% PF-127 and 0.2% C-974P (w/v) was selected to incorporate and deliver the four AEDs by the IN route. Being a low viscosity solution at room temperature, this *in situ* nasal gel was easily and accurately instilled as liquid drops into the nose where it has readily undergone a phase transition to form a semi-solid gel, as a consequence of the increase in temperature up to physiological levels (BADGUJAR *et al.*, 2010; SINGH *et al.*, 2013). Among several advantages, this delivery system has demonstrated to minimise the anterior

leakage and post-nasal drip and offer a favourable compromise between *in situ* gelling and ease of syringeability, undeniably contributing not only for an improved precision and accuracy of dosing but also to sustain the drug formulation within the nasal cavity for a prolonged period of time. After evaluating the performance of distinct IN drug administration techniques, the procedure of inserting a flexible polyurethane tube attached to a microliter syringe inside the nasal cavity of previously anaesthetised mice was adopted. Without causing visible signs of damage to the nasal mucosa, this technique likewise allowed a more accurate and reproducible dosing by avoiding the anterior loss of the administered formulation and providing a more efficient and localised deposition of the drugs onto the desired olfactory area.

Upon the definition and optimisation of the experimental conditions, two different *in vivo* protocols were designed in accordance with the main objectives outlined for this thesis. Specifically, our work was divided into two research studies aiming to assess if CBZ and LTG were promising drug candidates to be administered by the IN route. Thus, the first study consisted in the evaluation of drug concentration-time profiles achieved in plasma, brain (biophase) and liver (the major metabolic organ), with the intent of characterising the pharmacokinetic behaviour of the referred test compounds following IN delivery to mice. In the second one, the brain was sectioned in three different regions (olfactory bulb, frontal cortex and remaining portion of the brain) and the cerebral biodistribution pattern of the intranasally administered drugs was determined in order to infer about the involvement of a direct transport pathway from nose-to-brain. In fact, whole brain measurements of drug concentration generally underestimate the extent of distribution because of dilution effects and do not provide any information concerning the specific pathways and mechanisms underlying the CNS uptake following IN administration (DHURIA *et al.*, 2010). Theoretically, a heterogeneous distribution depicted by a preferential drug exposure in the rostral areas of the brain may suggest the existence of a close connection between the nose and the CNS through which molecules can travel.

Differing not only on the biological matrices collected, those studies also presented some disparity in what concerns to the sampling times. In practice, regardless of the study, the sampling times were always chosen in view of achieving the best compromise between the sufficient number of points to attain a thorough characterisation of the drug concentration-time profiles and the rational use of laboratory animals, thereby reducing to a minimum the number of animals required for a proper interpretation of the results. In the pharmacokinetic study, a broad sampling period involving the use of a large number of time points and mice was needed to obtain a complete and definitive description of the pharmacokinetic profiles, whereas in the brain

biodistribution study this period was shorter on account of a presumably quick transfer of drugs from nose-to-brain.

When investigating the potential of a new route for drug administration, such as the IN route, it is imperative to compare the obtained experimental results with those derived from other conventional delivery alternatives. Even though either CBZ or LTG are only commercially available in oral dosage forms, the use of the IV injection as a control seemed to be the most appropriate for the particular goals of our work. Due to the direct delivery of drugs into the bloodstream, IV administration is responsible for the highest systemic exposure by comparison with any other route, creating optimal conditions to allow a less variable drug incorporation and biodistribution, thus being the reference for absolute bioavailability calculations. On the other hand, bearing in mind that after IN instillation, drugs can reach the CNS either via the systemic circulation, upon nasal vasculature absorption and subsequent BBB crossing, or via the olfactory neuronal epithelium (*Chapter I, section I. 2.3.1.*), the exact contribution of the blood-mediated drug delivery to the brain can only be inferred by using IV injection, enabling a more accurate discrimination of the fraction that could be directly transported from nose-to-brain. Preliminary experiments comparing the pharmacokinetics of CBZ and LTG administered by the IN and oral routes were actually initially attempted (*Appendix C.*). Nevertheless, possibly owing to gastrointestinal degradation and hepatic first-pass metabolism, the values of CBZ oral bioavailability determined were considerably lower than those assumed as a reference for humans. Moreover, relatively to the IN administration, higher doses of orally administered CBZ and LTG were necessarily required to attain quantifiable drug levels in the relevant biosamples. Delivery of similar doses via both IN and IV routes allowed a more direct comparison of the drug concentration-time profiles, attenuating sources of error. The evidence of an equal or superior pharmacokinetic behaviour of drugs administered by the IN route comparatively to the IV injection clearly reflects its potential as a promising alternative approach, implicitly confirming its supremacy also over the conventional oral delivery.

Pharmacokinetics and Brain Biodistribution

Pharmacokinetics is a branch of pharmacology that describes “what the body does to the drug” by contrast with pharmacodynamics which corresponds to “what the drug does to the body”. Therefore, the apprehension of the basic pharmacokinetic parameters of a drug is fundamental to understand its disposition in the body, thereby enabling the interpretation of the relationship between absorption, distribution, metabolism and excretion with the onset, duration and intensity of the therapeutic effect. When analysing

pharmacokinetic data, several approaches can be employed in order to determine pharmacokinetic parameters. In this study, it was used the non-compartmental analysis once it allows the estimation of the main pharmacokinetic parameters without fitting the experimental results to a deterministic model. Indeed, if the primary requirement is to determine the degree of exposure following the administration of a drug (such as AUC), and the associated pharmacokinetic parameters like for instance t_{max} , C_{max} , $t_{1/2el}$, k_{el} and MRT, the non-compartmental pharmacokinetic analysis is generally the preferred methodology to be used since it requires fewer assumptions than the model-based approaches (GABRIELSSON and WEINER, 2012). In practice, the application of a certain deterministic model could eventually hamper or disguise the real perception of possible differences between the pharmacokinetic behaviour of the drug administered by the IN and the IV routes, whereby processing the raw data seems to be the most appropriate strategy.

Regarding the pharmacokinetic results obtained for CBZ and LTG following IN and IV administrations to mice it was notorious that, in all the three biological matrices analysed (plasma, brain and liver), the concentration-time profiles determined were fairly comparable, even though in the case of LTG, a significant delay between the time to reach the maximum peak concentration after IN comparatively to IV injection has been observed. The high values of C_{max} and AUC_{inf} attained in plasma indicate that, following IN instillation, both drugs have been efficiently and extensively absorbed to the systemic circulation, exhibiting a very high absolute bioavailability, easily justifiable by the large vascularisation of the nasal mucosa along with the physicochemical properties of the two molecules considered: high lipophilicity and low molecular weight. Despite the fact that a substantial fraction of either CBZ or LTG has effectively been absorbed from the nasal cavity into the bloodstream, the IN absolute bioavailability values estimated, greater than 100%, revealed to be particularly intriguing. A possible explanation may be related to the existence of an inherent inter-individual variability among animals. In effect, since a complete pharmacokinetic characterisation of the drugs in plasma, brain and liver tissues was intended, each mouse could only be assigned for a single time point of one of the experimental groups investigated (IN or IV), whereby in this situation, it was necessary to pool the data from multiple animals to delineate a thorough concentration-time curve. As a result, there is intrinsically an error associated to the determination of the AUCs which is inevitably reflected in the absolute bioavailability calculations. In general, the measurement of drug concentrations following IN delivery was accompanied by higher SEMs when compared to IV injection (*Appendix D. and E.*). In point of fact, the small size of the nasal cavity may substantially limit the volume of dosing so that slight variations concerning the amount of drug instilled as well as the site of deposition can contribute to

increase the associated relative variability. Nonetheless, the overall SEM values determined in this study are considered reasonably satisfactory on account of an assay performed *in vivo*, allowing the interpretation of the experimental results with acceptable confidence.

Investigating nose-to-brain drug targeting required the assessment of the relative distribution of the drug by determining and comparing its concentration or total exposure (given by AUC) in plasma and brain after both IN and IV administrations. Aiming at surveying the overall tendency of the drugs to accumulate in the brain following IN CBZ and LTG instillation, the DTE index was individually calculated. The estimated DTE indexes amounted to values almost equal to the unity ($DTE_{CBZ} = 0.98$; $DTE_{LTG} = 0.96$), suggesting no relevant contribution of the IN route to directly deliver any of these drugs from the nasal cavity to the brain. Conversely, it seems that the CNS uptake of either intranasally administered CBZ or LTG has been predominantly achieved by a pronounced nasal absorption of the drugs into the systemic circulation with subsequent BBB crossing. Interestingly, in opposition to what would be initially expected, the results derived from the brain biodistribution study demonstrated that a direct pathway other than the vasculature was actually involved in the transport of such compounds from the nasal cavity to the brain. These findings come to illustrate the limitations associated to the analysis of the total brain concentrations by treating the organ as a whole. As already mentioned in this discussion, measurement of drug distribution levels in specific target areas of the brain may afford to a more accurate discrimination of the pathways by which the molecules have travelled, providing a more sustained proof-of-concept of the IN route ability to improve CNS drug targeting.

Contrasting with a homogeneous brain distribution pattern typically attained after IV drug administration, an uneven biodistribution profile was ascertained for both CBZ and LTG following IN delivery, displaying a decreasing exposure to the drug from rostral to caudal areas of the brain. The quick and striking appearance of the drugs in the olfactory bulb immediately at 5-10 min post-IN dosing along with their considerably higher concentrations comparatively to plasma and the remaining parts of the brain seem to support the existence of a direct transport of the drugs from nose to brain, also indicating that it has probably occurred via the olfactory neuronal pathway. The drug concentration ratios between the olfactory bulb and the remaining portion of the brain calculated for the first sampling time points have shown to be, in case of the IN delivery, all greater than 1.0 presenting a significant discrepancy in terms of magnitude relatively to IV injection. While for CBZ these differences between the two administration routes have demonstrated to be more discreet, for LTG they were clearly overwhelming. Notwithstanding, one has to be aware that once distinct doses have been delivered,

direct comparisons among the two test compounds should be made with caution. Apparently, the direct transfer of LTG from nose-to-brain occurred at a higher extent than that of CBZ; however, further investigation would be needed in order to confirm such conjecture.

Gathering the results generated from both pharmacokinetic and brain biodistribution studies it can be stressed that due to the high drug concentrations achieved in plasma, and the heterogeneous brain biodistribution pattern with superior drug exposure in the olfactory bulb, the CNS access of intranasally administered CBZ and LTG was gained through a combination of both systemic and olfactory neuronal pathways.

The extensive plasma and brain drug exposures as well as the fast and pronounced CNS uptake suggest that the delivery of CBZ by the IN route may represent a valuable alternative regarding not only its use on the chronic treatment of epilepsy but also in the case of emergencies. In fact, following IN administration of CBZ, the C_{max} in the brain was attained very quickly at 10 min post-dosing and the evidence of a direct transport of the drug from nose-to-CNS has been demonstrated by the preferential drug distribution to the rostral cerebral areas. Such data together with the practicality and non-invasiveness of the IN route may assemble optimal conditions to promptly and efficiently manage acute convulsive episodes.

In contrast, taking into account the high plasma bioavailability afforded and the relevant drug concentration levels sustained for an extended period of time within the brain, it appears that IN delivery may establish itself as a convenient approach for the chronic administration of LTG, more so than for emergency situations. Accordingly, after LTG IN instillation, among all the matrices analysed, the brain was the single one in which the values estimated for $t_{1/2}$ and MRT were higher relatively to IV injection. Furthermore, t_{max} in the brain was only achieved at 2 h post-IN dosing and the respective rate of LTG elimination (assessed as k) was lower when compared to the IV route. The heterogeneous brain biodistribution profile observed following a single IN administration of LTG could eventually be dissipated under a multi-dose therapeutic regimen, particularly when the *steady-state* is accomplished.

Even though no pharmacodynamic evaluation has been performed in order to assess the therapeutic efficacy of either CBZ or LTG administered by the IN route, the resemblance encountered between the concentration-time profiles obtained in plasma and brain after IN and IV drug delivery probably points out to very similar pharmacological responses. Inclusively, it should be highlighted that considering the magnitude of doses herein intranasally instilled, the maximum drug plasma concentrations attained in mice were almost equal to or near the lower limit of the

clinically defined therapeutic range of LTG (3-14 $\mu\text{g}/\text{mL}$) and CBZ (4-12 $\mu\text{g}/\text{mL}$), respectively. Strong evidence accounting for the existence of a pathway that enables the direct transport of both CBZ and LTG from nose-to-brain by circumventing the BBB, come to offer new perspectives on a future development of more powerful therapeutic strategies to improve the pharmacological management of refractory epilepsy.



CHAPTER VI

CONCLUSIONS & FUTURE PERSPECTIVES

In the last few years, nose-to-brain drug delivery has been widely investigated by the scientific community from all over the world, exploiting the unique advantages that the IN route has to offer. This thesis was designed with the aim of evaluating the potential of the IN administration as a promising alternative strategy to improve CNS targeting of some of the first-line AEDs, soundly established in the current clinical practice. Therefore, a comparative study of the pharmacokinetic behaviour as well as the brain biodistribution pattern of both CBZ and LTG after IN and IV administrations to mice was herein performed to foresee whether a preferential delivery of the referred compounds from the nasal cavity to the brain might be involved.

In summary, the most relevant key findings brought from all the experimental work carried out under the scope of the present dissertation will be succinctly provided below:

- An HPLC-UV method to simultaneously quantify PB, PRM, PHT, CBZ, LTG, OXC and some of their main metabolites, CBZ-E, *trans*-diol and Lic in human plasma was successfully developed and fully validated. Being simple, rapid, sensitive, reliable, accurate and precise within a wide range of concentrations, this assay demonstrated suitability to be easily adopted by hospitals and laboratories to support routine TDM and other pharmacokinetic-based studies.
- Owing to the unfeasibility of directly transpose the best analytical conditions previously optimised in human plasma for the simultaneous quantification of PHT, CBZ, OXC, LTG and some of their main metabolites in mouse matrices, other chromatographic techniques already developed and validated in our laboratory were employed, making the necessary adjustments to render them appropriate for the intended purposes. Thus, after few alterations have been introduced, two distinct HPLC methodologies have been developed and partially validated enabling the quantitative determination of: (1) CBZ, CBZ-E, OXC, Lic and PHT and (2) LTG in plasma, brain (whole brain, olfactory bulb and frontal cortex) and liver of mouse. Consisting on the basilar analytical tools of this entire work, these assays were proven to be properly and confidently applied in the analysis of samples derived from the pharmacokinetic and brain biodistribution studies.
- During the definition and optimisation of the *in vivo* experimental protocol, multiple practical difficulties were faced, precluding the execution of the

studies with all the four molecules (CBZ, PHT, OXC and LTG) initially proposed. The incorporation of the drugs into a thermoreversible *in situ* gel along with the IN administration technique of inserting a flexible tubing inside the mice nasal cavity guaranteed an accurate, precise and reproducible IN dosing. IV injection was used as a control and proved to be the most appropriate for the specific goals of this work, since it enables not only the calculation of the IN absolute bioavailability but also the determination of the exact contribution of the blood-mediated drug delivery to the brain.

- The concentration-time profiles obtained in plasma, brain and liver following IN and IV administrations of CBZ and LTG to mice revealed to be fairly comparable. After IN instillation, the high values of C_{max} and AUC_{inf} observed in plasma indicated that both drugs were efficiently and extensively absorbed from the nasal vasculature into the systemic circulation, displaying a very high absolute bioavailability. The cerebral exposure determined after IN delivery of the referred drugs was rather similar to that estimated following IV injection; nevertheless, while the C_{max} of CBZ in the brain was achieved very quickly at 10 min post IN-dosing, in the case of LTG it was only attained at 2 h.
- Following IN administration of CBZ and LTG, the uneven brain biodistribution profile with the highest drug concentration levels determined at the olfactory bulb contrasted with the homogeneous biodistribution pattern obtained after IV injection, strongly suggesting the involvement of a pathway to directly transport the drugs from nose-to-brain by circumventing the BBB.
- Pooling the data derived from the pharmacokinetic and brain biodistribution studies it can be inferred that following IN administration, the CNS delivery of either CBZ or LTG was accomplished through a combination of both systemic and olfactory neuronal pathways.
- Taking into account all the results found during this work, it seems that IN delivery represents a valuable and promising alternative route to administer CBZ not only for the chronic treatment of epilepsy but also in acute convulsive emergencies. On the other hand, intranasally administered LTG gathers favourable conditions to be mainly applied in a

continuous therapeutic regimen. Strong evidences point out that this route may be considered as an attractive drug delivery strategy for a prospective management of pharmaco-resistance.

Considering all its inherent and indisputable advantages, the non-invasive IN route has been heralded as one of the most promising means to improve the delivery of drugs to the CNS; therefore, it is not surprising that its potential will be exploited for the development of novel and more efficient drug delivery systems in a near future, for which this thesis represents a small but an important contribution. Despite the extraordinary scientific breakthroughs achieved in the field of nose-to-brain drug delivery, there is still a long road to travel before they can be translated into clinical setting. Further development of the work herein presented could possibly focus on a deeper characterisation of the brain biodistribution pattern of drugs including the areas specifically involved in the initiation and propagation of seizures, as well as a pharmacodynamic and a toxicological analysis in order to infer about the impact of the IN route to the efficacy and safety of the antiepileptic pharmacological therapy.



APPENDIX

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APPENDIX A.

A1. HPLC-UV Method for the Quantitative Determination of CBZ, OXC, CBZ-E, Lic and PHT in Mouse Plasma, Brain and Liver Homogenates – Preparation of Standard Solutions

Table A1.1 Concentration of all the solutions used to prepare the corresponding calibration standards (CS) and quality control (QC) samples in plasma, brain, olfactory bulb, frontal cortex and liver tissues of mice.

Matrix/ Analyte	Solutions												
	Stock ($\mu\text{g/mL}$)	Intermediate ($\mu\text{g/mL}$)		Combined ($\mu\text{g/mL}$)									
		A	B	CS1	CS2	CS3	CS4	CS5	CS6	LLOQ	QC1	QC2	QC3
Plasma													
Lic	4000.0	1000.0	100.0	4.0	8.0	20.0	100.0	400.0	1200.0	4.0	12.0	600.0	1080.0
CBZ-E	4000.0	1000.0	100.0	8.0	16.0	40.0	100.0	200.0	600.0	8.0	24.0	300.0	540.0
OXC	4000.0	1000.0	100.0	2.0	4.0	12.0	40.0	400.0	1200.0	2.0	6.0	600.0	1080.0
CBZ	4000.0	1000.0	100.0	2.0	4.0	12.0	40.0	200.0	600.0	2.0	6.0	300.0	540.0
PHT ^a	4000.0	1000.0	250.0	20.0	40.0	80.0	160.0	360.0	800.0	20.0	60.0	400.0	720.0
Brain													
Lic	5000.0	1000.0	100.0	2.5	5.0	20.0	80.0	350.0	750.0	2.5	7.5	375.0	675.0
CBZ-E	5000.0	1000.0	100.0	2.5	5.0	20.0	80.0	350.0	750.0	2.5	7.5	375.0	675.0
OXC	5000.0	1000.0	100.0	2.5	5.0	20.0	80.0	350.0	750.0	2.5	7.5	375.0	675.0
CBZ	5000.0	1000.0	100.0	5.0	10.0	30.0	90.0	350.0	750.0	5.0	15.0	375.0	675.0
PHT ^a	5000.0	1000.0	100.0	10.0	20.0	40.0	100.0	350.0	750.0	10.0	30.0	375.0	675.0
Olfactory Bulb													
Lic	-	-	-	-	-	-	-	-	-	-	-	-	-
CBZ-E	-	-	-	-	-	-	-	-	-	-	-	-	-
OXC	2000.0	1000.0	100.0	1.0	2.0	6.0	30.0	120.0	400.0	1.0	3.0	200.0	360.0
CBZ	2000.0	1000.0	100.0	2.0	4.0	12.0	40.0	160.0	400.0	2.0	6.0	200.0	360.0
PHT ^a	2000.0	1000.0	100.0	5.0	10.0	20.0	60.0	160.0	400.0	5.0	15.0	200.0	360.0
Frontal Cortex													
Lic	-	-	-	-	-	-	-	-	-	-	-	-	-
CBZ-E	-	-	-	-	-	-	-	-	-	-	-	-	-
OXC	5000.0	1000.0	100.0	2.5	5.0	20.0	80.0	350.0	750.0	2.5	7.5	375.0	675.0
CBZ	5000.0	1000.0	100.0	5.0	10.0	30.0	90.0	350.0	750.0	5.0	15.0	375.0	675.0
PHT ^a	5000.0	1000.0	100.0	10.0	20.0	40.0	100.0	350.0	750.0	10.0	30.0	375.0	675.0
Liver													
Lic	5000.0	1000.0	100.0	5.0	10.0	25.0	75.0	225.0	500.0	5.0	15.0	250.0	450.0
CBZ-E	5000.0	1000.0	100.0	5.0	10.0	25.0	75.0	225.0	500.0	5.0	15.0	250.0	450.0
OXC	4000.0	1000.0	100.0	5.0	10.0	25.0	75.0	225.0	500.0	5.0	15.0	250.0	450.0
CBZ	5000.0	1000.0	100.0	5.0	10.0	25.0	75.0	225.0	500.0	5.0	15.0	250.0	450.0
PHT ^a	5000.0	1000.0	100.0	12.5	25.0	50.0	100.0	225.0	500.0	12.5	37.5	250.0	450.0

^a Phenytoin (PHT) combined solutions were prepared independently from the remaining analytes. CBZ, carbamazepine; CBZ-E, carbamazepine-10,11-epoxide; Lic, licarbazepine; LLOQ, lower limit of quantification; OXC, oxcarbazepine.

Table A1.2 Calibration standards and quality control samples prepared in mice plasma, brain, olfactory bulb, frontal cortex and liver homogenate supernatants.

Matrix/Analyte	Calibration Standards (µg/mL)						Quality Controls (µg/mL)			
	1	2	3	4	5	6	LLOQ	1	2	3
Plasma										
Lic	0.2	0.4	1.0	5.0	20.0	60.0	0.2	0.6	30.0	54.0
CBZ-E	0.4	0.8	2.0	5.0	10.0	30.0	0.4	1.2	15.0	27.0
OXC	0.1	0.2	0.6	2.0	20.0	60.0	0.1	0.3	30.0	54.0
CBZ	0.1	0.2	0.6	2.0	10.0	30.0	0.1	0.3	15.0	27.0
PHT ^a	1.0	2.0	4.0	8.0	18.0	40.0	1.0	3.0	20.0	36.0
Brain										
Lic	0.05	0.1	0.4	1.6	7.0	15.0	0.05	0.15	7.5	13.5
CBZ-E	0.05	0.1	0.4	1.6	7.0	15.0	0.05	0.15	7.5	13.5
OXC	0.05	0.1	0.4	1.6	7.0	15.0	0.05	0.15	7.5	13.5
CBZ	0.1	0.2	0.6	1.8	7.0	15.0	0.1	0.3	7.5	13.5
PHT ^a	0.2	0.4	0.8	2.0	7.0	15.0	0.2	0.6	7.5	13.5
Olfactory Bulb^b										
Lic	-	-	-	-	-	-	-	-	-	-
CBZ-E	-	-	-	-	-	-	-	-	-	-
OXC	0.01	0.02	0.06	0.3	1.2	4.0	0.01	0.03	2.0	3.6
CBZ	0.02	0.04	0.12	0.4	1.6	4.0	0.02	0.06	2.0	3.6
PHT ^a	0.05	0.1	0.2	0.6	1.6	4.0	0.05	0.15	2.0	3.6
Frontal Cortex^b										
Lic	-	-	-	-	-	-	-	-	-	-
CBZ-E	-	-	-	-	-	-	-	-	-	-
OXC	0.025	0.05	0.2	0.8	3.5	7.5	0.025	0.075	3.75	6.75
CBZ	0.05	0.1	0.3	0.9	3.5	7.5	0.05	0.15	3.75	6.75
PHT ^a	0.1	0.2	0.4	1.0	3.5	7.5	0.1	0.3	3.75	6.75
Liver										
Lic	0.2	0.4	1.0	3.0	9.0	20.0	0.2	0.6	10.0	18.0
CBZ-E	0.2	0.4	1.0	3.0	9.0	20.0	0.2	0.6	10.0	18.0
OXC	0.2	0.4	1.0	3.0	9.0	20.0	0.2	0.6	10.0	18.0
CBZ	0.2	0.4	1.0	3.0	9.0	20.0	0.2	0.6	10.0	18.0
PHT ^a	0.5	1.0	2.0	4.0	9.0	20.0	0.5	1.5	10.0	18.0

^a Phenytoin (PHT) calibration standards and quality control samples were prepared independently from the remaining analytes. ^b Values are expressed in µg. CBZ, carbamazepine; CBZ-E, carbamazepine-10,11-epoxide; Lic, licarbazepine; OXC, oxcarbazepine.

A2. HPLC-UV Method for the Quantification of LTG in Mouse Plasma, Brain and Liver Homogenates – Preparation of Standard Solutions

Table A2.1 Concentration of all the solutions used to prepare the corresponding calibration standards (CS) and quality control (QC) samples of lamotrigine (LTG) in plasma, brain, olfactory bulb, frontal cortex and liver tissues of mice.

Solution ^a	Matrix				
	Plasma	Brain	Olfactory Bulb	Frontal Cortex	Liver
Stock	2000.0	3000.0	3000.0	3000.0	2500.0
Intermediate					
A	1000.0	1000.0	1000.0	1000.0	1000.0
B	100.0	100.0	100.0	100.0	100.0
Spiking					
CS1	4.0	2.5	2.5	2.5	5.0
CS2	8.0	5.0	5.0	5.0	10.0
CS3	20.0	20.0	20.0	20.0	25.0
CS4	40.0	80.0	80.0	80.0	75.0
CS5	200.0	400.0	400.0	400.0	225.0
CS6	600.0	1000.0	1000.0	1000.0	500.0
LLOQ	4.0	2.5	2.5	2.5	5.0
QC1	12.0	7.5	7.5	7.5	15.0
QC2	300.0	500.0	500.0	500.0	250.0
QC3	540.0	900.0	900.0	900.0	450.0

^a Concentration values are expressed in µg/mL. LLOQ, lower limit of quantification.

Table A2.2 Calibration standards and quality control samples of lamotrigine (LTG) prepared in mice plasma, brain, olfactory bulb, frontal cortex and liver homogenate supernatants.

	Matrix				
	Plasma	Brain	Olfactory Bulb ^b	Frontal Cortex ^b	Liver
Calibration Standards^a					
1	0.2	0.05	0.025	0.025	0.2
2	0.4	0.1	0.05	0.05	0.4
3	1.0	0.4	0.2	0.2	1.0
4	2.0	1.6	0.8	0.8	3.0
5	10.0	7.0	3.5	3.5	9.0
6	30.0	15.0	7.5	7.5	20.0
Quality Controls^a					
LLOQ	0.2	0.05	0.025	0.025	0.2
1	0.6	0.15	0.75	0.75	0.6
2	15.0	7.5	3.75	3.75	10.0
3	27.0	13.5	6.75	6.75	18.0

^a Concentration values of calibration standards and quality controls are expressed in µg/mL for all the matrices with the exception of the olfactory bulb and frontal cortex. ^b Values expressed in µg. LLOQ, lower limit of quantification.



APPENDIX B.

B. Intranasal Administration of Phenytoin and Oxcarbazepine

B1. Phenytoin / Oxcarbazepine – Pharmacological Characterisation

B1. 1. Historical Background

PHT was synthesised for the first time in 1908 by Biltz at the University of Kiel in Germany, and later again in 1923 by the organic chemists Dox and Thomas, at the Parke-Davis laboratories, who were searching for hypnotic drugs (SHORVON, 2009b). Unlike to what would be expected, despite the structural similarity with barbituric acid, PHT did not display any hypnotic properties and therefore it was ignored and shelved until 1936, when it was luckily enrolled in the Putman's and Merrit's experiments with the intent of screening various phenyl compounds to protect laboratory animals from electrically induced convulsions (ANDERSON, 2009; SHORVON, 2009b). The impressive anticonvulsant activity observed for PHT in this early investigation led to subsequent pre-clinical and clinical studies, confirming its undeniable potential on managing patients with different types of epileptic seizures, especially of "grand mal" and psychomotor seizures. In 1938, PHT was brought to the market by Parke Davis company and, in 1939, the American Medical Association Council on Pharmacy and Chemistry accepted it into its pharmacological compendium (ANDERSON, 2009; SHORVON, 2009b). Since then, PHT's popularity grew very quickly and it becomes the predominant medication for the treatment of epilepsy, remaining the first-choice AED until the 1980's, when it was slowly displaced by CBZ and valproic acid. Currently, besides all its faults in what concerns to pharmacokinetic properties, drug interactions and adverse effects, PHT is still one of the drugs most widely used in the world. Even though it has been now largely relegated to second-line therapy, PHT persists as the drug of choice in case of emergency and in *status epilepticus*.

OXC was first synthesised in 1966 (SHORVON, 2009a). The rationale behind its original development was to use the parent molecule CBZ as a template with the intent of producing a drug equally effective, but with an improved tolerability profile (SHORVON, 2000). The key difference between the two drugs is that OXC, due to the addition of an extra oxygen atom on the dibenzazepine ring, is not metabolised to the epoxide derivative mainly responsible for the toxic effects of CBZ (SHORVON, 2000, 2009a). Although the antiepileptic action, spectrum of activity and efficacy of OXC are comparable to that of CBZ, the adverse events associated with it tend to be less severe or frequent and the propensity to establish drug interactions is significantly lower.

OXC was initially subjected to clinical trials in the 1980s, and it was firstly approved for use as an anticonvulsant agent in Denmark in 1990 (SCHACHTER, 1999; SHORVON, 2000). The introduction of OXC in Portugal was held in 1997, and in the United States only in

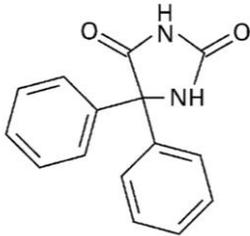
2000 (SHORVON, 2009a). Up to now, the drug is already registered and widely used in more than 50 different countries worldwide; surprisingly, unlike to what was expected, it has not generally superseded CBZ (MAY *et al.*, 2003; SHORVON, 2009a).

B1. 2. Pharmaceutical Information

B1. 2.1. Phenytoin

PHT is the oldest non-sedative AED. It is structurally related to barbiturates, consisting of two phenyl groups attached to a hydantoin ring which are responsible for its anticonvulsive action (ANDERSON, 2009; BAUER, 2008). PHT is usually administered either as the sodium salt or as the free acid (BAUER, 2008; RICHENS, 1979), being available in several oral (tablet, capsule, suspension) and parenteral (IV injection) formulations (GALLOP, 2010). Due to its poor hydrosolubility, the dissolution and, consequently, the absorption rate of PHT oral forms essentially depends on drug particle size rather than whether it is in the acid or salt form (RICHENS, 1979). In order to concentrate the drug in a convenient volume for parenteral administration, propylene glycol and ethanol are added to the vehicle as well as sodium hydroxide to adjust the solution to a pH value between 10 and 12 (BAUER, 2008). Comparatively to an oral loading dose, PHT IV injection yields therapeutic serum levels much faster; nevertheless, it may be associated to local reactions at the infusion site along with serious cardiovascular complications such as hypotension and bradycardia (GALLOP, 2010; PELLOCK, 2002). Detailed pharmaceutical information of PHT is summarised in Table B1.1.

Table B1.1 Pharmaceutical information of phenytoin (DRUGBANK; PUBCHEM).

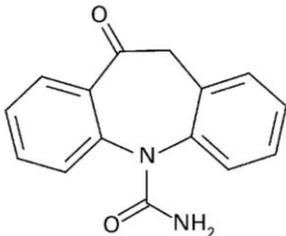
Drug Identification		Physicochemical Properties	
Generic Name	Phenytoin	Phase State	Solid / White powder
Chemical Name	5,5-diphenylimidazolidine-2,4-dione	Solubility in Water	0.032 mg/mL (at 22°C)
Chemical Formula	C ₁₅ H ₁₂ N ₂ O ₂	Solubility in Ethanol	16.67 mg/mL
Molecular Weight	252.27 g/mol	LogP	2.47
Molecular Structure		pK_a	8.33
Drug Information			
Brand Name	Dilantin®	Manufacturer	Pfizer
Dosage Forms	Immediate-release capsule Extended-release capsule Chewable tablet Suspension IV injection	Route of Administration	Oral and Intravenous

LogP, octanol/water partition coefficient; pK_a, negative decadic logarithm of the acid dissociation constant.

B1. 2.2. Oxcarbazepine

OXC is a 10-keto analogue of CBZ with very similar chemical structure. Hence, in comparison to CBZ, OXC has the same 5-carboxamide substituent, but it is structurally different in the 10,11-position where an extra atom of oxygen is added (MAY *et al.*, 2003). It is a white to faintly orange powder, practically insoluble in ethanol and water (FLESCH, 2004; NOVARTIS, 2014b). Currently, OXC is available in the market only in the form of film-coated tablets or suspension for oral administration. Detailed pharmaceutical information of OXC is summarised in Table B1.2.

Table B1.2 Pharmaceutical information of oxcarbazepine (DRUGBANK; FLESCH, 2004; PUBCHEM).

Drug Identification		Physicochemical Properties	
Generic Name	Oxcarbazepine	Phase State	Solid / White powder
Chemical Name	10,11-dihydro-10-oxo-5H-dibenz[b,f]azepine-5-carboxamide	Solubility in Water	0.30 mg/mL (at 25°C)
Chemical Formula	C ₁₅ H ₁₂ N ₂ O ₂	Solubility in Ethanol	< 1 mg/mL (at 25°C)
Molecular Weight	252.27 g/mol	LogP	1.5
Molecular Structure		pK_a	10.7
Drug Information			
Brand Name	Trileptal®	Manufacturer	Novartis
Dosage Forms	Tablet Suspension	Route of Administration	Oral

LogP, octanol/water partition coefficient; pK_a, negative decadic logarithm of the acid dissociation constant.

B1. 3. Pharmacokinetics

B1. 3.1. Phenytoin

The pharmacokinetic characteristics of PHT are markedly influenced by its limited aqueous solubility, variable absorption, high protein binding, saturable metabolism and dose-dependent elimination (NEELS *et al.*, 2004; PATSALOS *et al.*, 2008). The complex and non-linear kinetics of PHT makes the drug administration a real challenge to many clinicians, having contributed to the implementation of TDM in epilepsy (PELLOCK, 2002; SHORVON, 2009b). This section and the following Table B1.3 will provide a brief overview of PHT pharmacokinetic profile.

Table B1.3 Summary of the main pharmacokinetic parameters of phenytoin after oral administration (BAUER, 2008; KUTT and HARDEN, 1999; NEELS *et al.*, 2004; PATSALOS *et al.*, 2008; RICHENS, 1979).

Pharmacokinetic Parameter	Mean Value
t_{\max} (h)	3-12
F (%)	70-100
Plasma Protein Binding (%)	90
Vd/F (L/kg)	0.7
$t_{1/2el}$ (h)	12-36
CL/F (L/kg/h)	0.015-0.065

CL/F, apparent plasma clearance; F, absolute bioavailability; $t_{1/2el}$, apparent elimination half-life; t_{\max} , time to achieve maximum peak concentration; Vd/F, apparent volume of distribution.

Absorption

PHT absorption is highly dependent on drug formulation, particle size and pharmaceutical additives/excipients (PORTER and MELDRUM, 2015; RICHENS, 1979). In general, PHT is slowly but almost completely absorbed when given orally, presenting t_{\max} values in plasma ranging from 3 to 12 h but a bioavailability of about 100% (BAUER, 2008; GALLOP, 2010). At higher doses (single oral doses of 800 mg or more), the rate and extent of absorption are affected, since longer times are needed for maximal drug concentrations to be achieved and decreased bioavailability is frequently observed (BAUER, 2008; PATSALOS *et al.*, 2008).

Distribution

In adults, approximately 90% of plasma PHT is bound to albumin and the degree of binding decreases in the presence of hypoalbuminemia, renal and liver insufficiency (PATSALOS *et al.*, 2008; RICHENS, 1979). PHT exhibits a Vd/F between 0.5 and 1.2 L/kg, quickly diffusing into the tissues and accumulating in the brain, liver, muscle and fat (NEELS *et al.*, 2004; PORTER and MELDRUM, 2015). Since the penetration into the brain is rapid, it has been commonly used in *status epilepticus* conditions. The drug binds to brain tissue at about the same extent as it binds to plasma proteins whereby the brain and serum concentrations are almost identical (RICHENS, 1979). On the other hand, drug concentration in CSF mirrors the free plasma level (PORTER and MELDRUM, 2015).

Metabolism

Less than 5% of a PHT dose is excreted unchanged (BAUER, 2008; PATSALOS *et al.*, 2008). PHT is largely (>95%) metabolised in the liver primarily by CYP2C9 and in a less extent by CYP2C19 to four oxidative metabolites with little or no anticonvulsant activity (Figure B1.1) (BAUER, 2008; YAMAZAKI *et al.*, 2001). Its major metabolite is 5-(4'-hydroxyphenyl)-5-phenylhydantoin which is greatly conjugated with glucuronic acid and subsequently excreted in the urine as a glucuronide (NEELS *et al.*, 2004; RICHENS, 1979). The metabolism of PHT is capacity limited within the therapeutic range, i.e. the drug molecules overwhelms or saturates the enzymes' ability to metabolise the drug (BAUER, 2008). Indeed, the rate of metabolism directly correlates with the amount of available drug in plasma when the concentration is low, but a saturation and, consequently, a decrease of the metabolic process is ascertained for plasmatic levels above 10 µg/mL (BRODIE and DICHTER, 1996; NEELS *et al.*, 2004). Therefore, increments in dosage, even relatively small, may result in unpredictable and disproportionate large augments of PHT plasma concentration, possibly leading to a quick development of toxicity symptoms (NEELS *et al.*, 2004; PATSALOS *et al.*, 2008; PORTER and MELDRUM, 2015). In accordance, the dose required to produce a concentration in the accepted therapeutic range is very close to that which will cause intoxication, and thereby, this issue has important clinical implications (RICHENS, 1979).

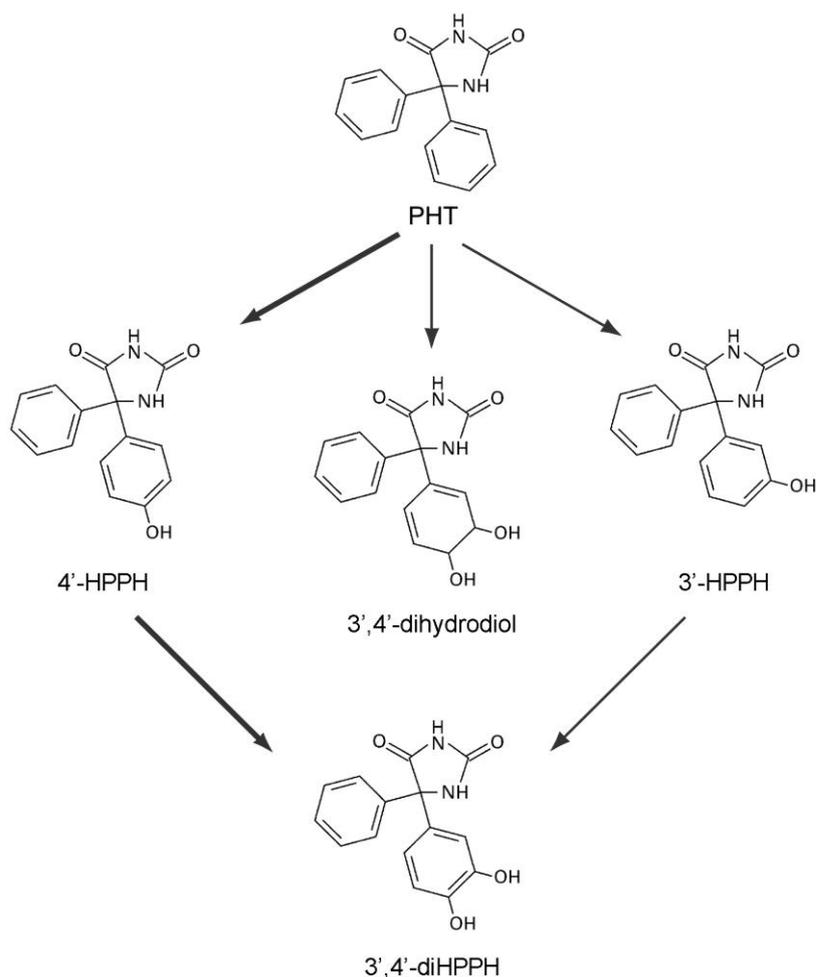


Figure B1.1 The major metabolic pathways of phenytoin (PHT). 4'-HPPH, 5-(4'-hydroxyphenyl)-5-phenylhydantoin; 3',4'-dihydrodiol, 5-(3',4'-dihydroxy-1',5'-cyclohexadien-1-yl)-5-phenylhydantoin; 3'-HPPH, 5-(3'-hydroxyphenyl)-5-phenylhydantoin; 3',4'-diHPPH, 5-(3',4'-dihydroxyphenyl)-5-phenylhydantoin.

Elimination

PHT follows a Michaelis-Menten, saturable or non-linear pharmacokinetics which means that elimination is dose-dependent. Accordingly, as the dose or concentration of PHT increases, the enzymatic system becomes saturated, decreasing the rate of clearance and markedly extending the drug half-life (BAUER, 2008). On average, the effective half-life at very low PHT plasma levels is about 12 h, but it lengthens up to 46 h when the steady-state concentration reaches the top of the therapeutic range (20 µg/mL) (RICHENS, 1979). The time to achieve steady-state therapeutic levels depends upon the dose and the final concentration attained, ranging from 5-7 days after initiation of therapy at a low dose to 4-6 weeks, if a higher dose is administered (PORTER and MELDRUM, 2015).

Most of the drug is excreted in the bile as inactive metabolites which are then reabsorbed from the intestinal tract and subsequently eliminated via urinary excretion (PFIZER, 2015).

B1. 3.2. Oxcarbazepine

Even though OXC is closely related to CBZ, its slight modification in the molecular structure results in major differences regarding to biotransformation and pharmacokinetics (BEYDOUN AND KUTLUAY, 2002). Unlike CBZ, which is oxidised by the CYP450 oxidase system, OXC is rapidly and extensively reduced by cytosolic enzymes to the non-toxic monohydroxy derivative, also known as licarbazepine that is responsible for most of its anticonvulsant effect (BEYDOUN and KUTLUAY, 2002). Compared with Lic, the concentrations of OXC in plasma are low and the metabolite is assumed to be the clinically relevant compound (MAY *et al.*, 2003). In essence, the pharmacological action of the drug is primarily exerted by Lic, and OXC actually acts as a pro-drug (SHORVON, 2000). This section and the following Table B1.4 will provide a brief overview of OXC pharmacokinetic profile.

Table B1.4 Summary of the main pharmacokinetic parameters of oxcarbazepine (OXC) and licarbazepine (Lic) (FLESCH, 2004; MAY *et al.*, 2003; SHORVON, 2000; WELLINGTON and GOA, 2001).

Pharmacokinetic Parameter	Mean Value	
	OXC	Lic
t_{max} (h)	1-2	4-6
F (%)	-	>90
Plasma Protein Binding (%)	60-67	37-43
Vd/F (L/kg)	-	0.75
$t_{1/2el}$ (h)	1-2.5	8-10
CL/F (L/h)	-	0.71-1.26

CL/F, apparent plasma clearance; F, absolute bioavailability; $t_{1/2el}$, apparent elimination half-life; t_{max} , time to achieve maximum peak concentration; Vd/F, apparent volume of distribution.

Absorption

Following oral administration to humans, OXC is almost completely absorbed (>95%), being rapidly and extensively metabolised to the pharmacological active metabolite Lic (FLESCH, 2004). Peak plasma concentration (C_{max}) of OXC after a single dose is reached within 1-2 h, whereas the maximal concentration of Lic is attained somewhat

later at approximately 4-6 h post-dosing (BEYDOUN and KUTLUAY, 2002; FLESCH, 2004). Since OXC works as a pro-drug, only about 2% of the total systemic exposure is due to unchanged OXC, while nearly 70% corresponds to Lic and the remainder is attributable to minor secondary metabolites that are quickly eliminated (FLESCH, 2004). The rate and extent of absorption seem to be not significantly affected by the presence of food (WELLINGTON and GOA, 2001).

At steady-state, OXC pharmacokinetics is linear and shows dose proportionality with plasma levels of the drug and its metabolite over the usual clinical dosage range 300-2400 mg/day (NOVARTIS, 2014b; WELLINGTON and GOA, 2001).

Distribution

OXC and Lic are both neutral lipophilic compounds whereby they are able to easily and reversibly diffuse across many biologic membranes, including the BBB (MAY *et al.*, 2003). The Lic Vd/F varies between 0.7-0.8 L/kg, and the plasma protein binding is around 40% (BEYDOUN and KUTLUAY, 2002; MAY *et al.*, 2003). The binding of Lic to plasma proteins, predominantly albumin, seems to be independent of serum concentrations within the therapeutically relevant range (FLESCH, 2004). Because only a relatively small proportion of Lic is protein bound, it is unlikely that clinically significant displacement by other AEDs might occur (BEYDOUN and KUTLUAY, 2002; WELLINGTON and GOA, 2001).

Metabolism

In humans, once absorbed from the gastrointestinal tract, OXC almost immediately undergoes a presystemic hepatic reduction mediated by non-inducible cytosolic arylketone reductases to form the major active metabolite Lic (MAY *et al.*, 2003; SCHACHTER, 1999). The metabolic reduction of the 10-keto group of OXC is enantioselective, resulting in the formation of an asymmetric carbon and, therefore, a chiral molecule (WELLINGTON and GOA, 2001). From this highly stereoselective conversion, two enantiomers of Lic in a ratio of 80% (*S*-Lic) to 20% (*R*-Lic) are produced, exhibiting equivalent antiepileptic efficacy and tolerability, but different pharmacokinetic properties (FLESCH, 2004; WELLINGTON and GOA, 2001). Both Lic enantiomers are further metabolised by conjugation with glucuronic acid, or to a lesser extent, by oxidation, yielding to a glucuronide conjugate and the inactive metabolite *trans*-diol, respectively (Figure B1.2) (SCHACHTER, 1999; WELLINGTON and GOA, 2001).

Since the metabolism of OXC is mostly independent of CYP450 enzymes, the induction or inhibition of this system by the presence of other AEDs will have little effect

on the conversion of OXC to Lic (SCHACHTER, 1999). In opposition to CBZ, OXC does not undergo autoinduction (BEYDOUN and KUTLUAY, 2002; MAY *et al.*, 2003).

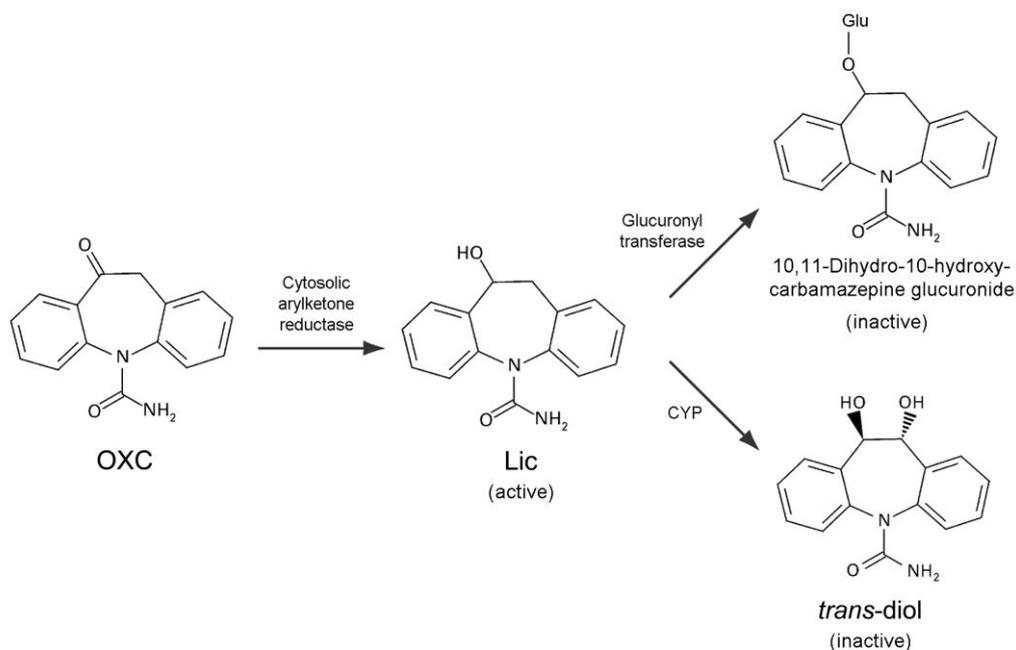


Figure B1.2 The major metabolic pathways of oxcarbazepine (OXC) in humans. Lic, licarbazepine; *trans*-diol, 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine.

Elimination

OXC exhibits first-order linear kinetics during long-term administration (MAY *et al.*, 2003). The plasma elimination half-lives ($t_{1/2el}$) range from about 1-2.5 h for OXC and from 8-10 h for Lic, illustrating the rapid and virtually complete conversion of the parent drug to its active metabolite (WELLINGTON and GOA, 2001). More than 95% of the OXC's administered dose is excreted by the kidneys, the large majority in the form of inactive metabolites and less than 1% as unchanged drug (FLESCH, 2004). Approximately 80% of the dose appears in the urine as glucuronides of Lic (51%) or as unchanged Lic (28%), whereas the inactive metabolite *trans*-diol accounts for nearly 3% and minor conjugates of Lic and OXC amount for 13% of the dose (FLESCH, 2004; NOVARTIS, 2014b). Less than 4% of the administered dose is cleared from the body via faecal excretion (NOVARTIS, 2014b).

B1. 4. Pharmacodynamics

Mechanism of Action

PHT is believed to exert its anticonvulsant effect primarily by the inhibition of voltage-dependent sodium channels, binding to the fast inactivated state of the channels after depolarisation and modifying their sodium permeability, thus reducing the frequency of sustained repetitive firing of action potentials by prolonging the depolarisation of neurons (COOK and BENSALEM-OWEN, 2011; KWAN *et al.*, 2001). The inhibitory potency of PHT is strongly use- and frequency-dependent, being the sodium channel blockade more effective with prolonged or repetitive activation and at higher frequencies of neuronal stimulation (KWAN *et al.*, 2001; PORTER *et al.*, 2012). Another important feature of this blocking is its slow onset without affecting the time course of fast sodium currents which enables that the action potentials evoked by synaptic depolarisations of ordinary duration remain intact (PORTER *et al.*, 2012; ROGAWSKI and LÖSCHER, 2004).

Besides blocking the sodium channel during its peak current phases, an additional impact on persistent late sodium currents has been suggested for PHT. The persistent currents may critically regulate the firing properties of some neurons and could also play a crucial role in epileptic activity (PORTER *et al.*, 2012). Modulation of these currents might delay repolarisation and affect the time span until the threshold potential is reached again triggering the next action potential (POTSCHKA, 2013).

A blockade of high voltage-gated calcium channels and ionotropic glutamate receptors has also been reported for PHT; however, it seems that this occurs only at supratherapeutic drug concentration levels (KWAN *et al.*, 2001; PORTER *et al.*, 2012).

Like PHT and CBZ, OXC and its active metabolite Lic block the voltage-sensitive sodium channels, thereby stabilising hyper-excited neuronal membranes, suppressing high-frequency repetitive firing of action potentials and reducing the propagation of synaptic impulses (FLESCH, 2004; PORTER *et al.*, 2012). In addition, increased potassium conductance as well as modulation of high-voltage activated calcium channels may also contribute to their anticonvulsant effects (NOVARTIS, 2014b). In contrast to CBZ, which modulates L-type calcium channels, OXC has been shown to inhibit the high-voltage N-type, resulting in a dose-dependent inhibition of post-synaptic excitatory glutamatergic transmission at striatal and cortical neurones (BEYDOUN and KUTLUAY, 2002; WELLINGTON and GOA, 2001).

B1. 5. Clinical Use

B1. 5.1. Phenytoin

Therapeutic Indications

PHT is an effective second-line AED for the chronic management of focal and generalised tonic-clonic seizures and it remains the drug of choice for the acute treatment of repetitive seizures and *status epilepticus* (BAUER, 2008). Typical primary generalised seizures such as absence or myoclonic seizures have commonly been reported to be aggravated or precipitated (NEELS *et al.*, 2004; TOLEDANO and GIL-NAGEL, 2008).

Other therapeutic applications of PHT include the treatment of cardiac arrhythmias and trigeminal neuralgia, even though in the latter the use of CBZ is often preferred (BAUER, 2008).

Tolerability

PHT is a drug with many potential side-effects and due to its non-linear or saturable pharmacokinetics it is fairly easy to attain toxic concentrations with modest changes in drug dosage. PHT toxic effects essentially depend on the administration route, the duration of treatment as well as the dose/plasma concentration (ARONSON *et al.*, 1992).

The incidence of adverse effects with PHT intake is similar to that of CBZ, but the cosmetic side-effects of gingival hyperplasia, hirsutism and coarsening of facial features seem to be more prevalent after chronic administration, especially if high doses of PHT are used (KUTT and HARDEN, 1999; PELLOCK, 2002). Diplopia, ataxia, nystagmus, incoordination, confusion, lethargy and coma are the most common dose-related adverse effects (BAUER, 2008; PELLOCK, 2002). Long-term use may also result in abnormalities of vitamin D metabolism contributing to the development of osteomalacia. A modest decline of folate levels as well as megaloblastic anemia have likewise been reported, but clinical importance of these observations is unknown (PORTER and MELDRUM, 2015). Rare idiosyncratic reactions include skin rash, Stevens-Johnson syndrome, lymphadenopathy and blood dyscrasias (BAUER, 2008; PELLOCK, 2002; PORTER and MELDRUM, 2015).

Therapeutic Range

It is generally accepted that, in the majority of patients, optimal suppression of seizures is usually achieved without clinical signs of toxicity when the total (bound + unbound) plasma PHT concentration is within the range 10-20 µg/mL (ARONSON *et al.*, 1992; BAUER, 2008; PATSALOS *et al.*, 2008). Notwithstanding, some patients attain complete seizure control with PHT concentrations below this range, while others obtain benefit from concentrations greater than 20 µg/mL without adverse effects or may be intolerant for concentrations under this level (PATSALOS *et al.*, 2008; RICHENS, 1979). This variability appears to be related to the seizure type and severity of the underlying disorder (KUTT and HARDEN, 1999; PATSALOS *et al.*, 2008), the reason why drug concentrations must be interpreted in the light of each patient's clinical state, and the therapy should be individually defined and monitored (PATSALOS *et al.*, 2008; RICHENS, 1979).

There is a clear relationship between PHT concentration and clinical response since seizure control improves with higher drug levels, unfortunately, at the cost of also increasing the risk of concentration-dependent adverse effects such as ataxia, nystagmus, diplopia and reduced cognition (BAUER, 2008; PATSALOS *et al.*, 2008).

Drug Interactions

Drug interactions involving PHT are primarily related to metabolism or protein binding. In fact, because PHT is so extensively metabolised in the liver and substantially bound to plasma proteins, it is prone to drug interactions in which occurs the inhibition of hepatic microsomal enzymes and the competitive displacement of the drug from its plasma protein-binding sites (BAUER, 2008; PATSALOS *et al.*, 2002). The unpredictable relationship between dose and PHT concentration, as well as its narrow therapeutic index make drug interactions with PHT important, both at pharmacokinetic and clinical levels (PATSALOS *et al.*, 2008; RICHENS, 1979).

An increase of plasma PHT concentration and the consequent risk of toxicity has been reported when many CYP2C9 or CYP2C19 inhibitors such as fluoxetine, isoniazid, cimetidine, amiodarone and omeprazole are concomitantly administered with PHT (ARONSON *et al.*, 1992; BAUER, 2008; PATSALOS *et al.*, 2008). PHT can induce the oxidative metabolism of several CYP450 metabolised drugs, including CBZ, warfarin, ciclosporin and ethinyl estradiol (BAUER, 2008; BRODIE and DICHTER, 1996; RICHENS, 1979). Interactions comprising protein-binding displacement are not clinically important unless it is simultaneously accompanied by enzyme inhibition, producing a significant rise in plasma PHT concentrations, as it occurs with valproic acid (NEELS *et al.*, 2004; PATSALOS *et al.*, 2002).

B1. 5.2. Oxcarbazepine

Therapeutic Indications

Extensive clinical use and a series of clinical trials have proved the effectiveness of OXC in the management of focal-onset seizures, with or without secondary generalisation, and primary generalised tonic-clonic seizures, in adults and children, as initial monotherapy, for conversion to monotherapy and as adjunctive therapy (MAY *et al.*, 2003; NOVARTIS, 2014b; SHORVON, 2000). Presenting equivalent anticonvulsant efficacy when compared to standard AEDs such as CBZ, PHT and valproic acid, OXC undeniably offers clinically relevant advantages both in terms of better tolerability and improved pharmacokinetic profile (BEYDOUN and KUTLUAY, 2002; SHORVON, 2000).

Similarly to CBZ, strong data support the therapeutic efficacy of OXC also in the treatment of patients with trigeminal neuralgia (BEYDOUN and KUTLUAY, 2002).

Tolerability

The type and frequency of adverse effects observed during therapy with OXC depends on the total drug daily dose, whether it is used as monotherapy or adjunctive therapy and on the age of the patient population being treated (BEYDOUN and KUTLUAY, 2002).

Overall, the tolerability profile of OXC is similar in nature to that of CBZ; however the frequency and severity of side-effects have been shown to be less pronounced (SHORVON, 2000). The most common dose-related adverse events associated with OXC are somnolence, headache, dizziness, ataxia, nausea and vomiting (SHORVON, 2000; WELLINGTON and GOA, 2001). Allergy in the form of skin rash appears to occur in less than 10% of patients exposed to OXC, being the main reason for discontinuation of the drug in the comparative monotherapy studies (SHORVON, 2000). The risk of hyponatraemia (serum sodium concentration level below 135 mmol/L), presumably due to an anti-diuretic hormone-like effect, is greater with OXC (20%) than with CBZ (SCHACHTER, 1999; SHORVON, 2000) and is dose- and age-dependent, since it is extremely rare in childhood, increasing to about 7% in the elderly population (BEYDOUN and KUTLUAY, 2002). Nevertheless, the degree of hyponatraemia is usually mild and asymptomatic, having little or no clinical importance (SHORVON, 2000; WELLINGTON and GOA, 2001).

Therapeutic Range

OXC exhibits a linear relationship between dosage and its active metabolite plasma concentrations. The appropriate therapeutic range for Lic has not yet been well defined since similar concentrations have been noted both in groups of patients with optimal seizure suppression as well as in non-responders (JOHANNESSEN and TOMSON, 2006). Even though the exact relation of Lic serum concentrations with efficacy and toxicity has not been determined yet, it is known that adverse effects are particularly likely to start occurring at levels within 35-40 µg/mL (MAY *et al.*, 2003; PATSALOS *et al.*, 2008). Therefore, a tentative target index of 12-35 µg/mL has been suggested for Lic plasma concentrations when patients are treated with therapeutic doses of OXC (JOHANNESSEN and TOMSON, 2006; JOHANNESSEN *et al.*, 2003).

Drug Interactions

Because the biotransformation of OXC to its active metabolite Lic is mediated by non-inducible enzymes, the pharmacokinetic properties of the drug are largely unaffected by induction or inhibition of the CYP system (WELLINGTON and GOA, 2001). Thus, the potential for interactions with other AEDs that interfere with CYP450 isoenzymes is reduced (NEELS *et al.*, 2004). Despite its minimised propensity to establish pharmacological interactions, it was shown that OXC does effectively interact with the hepatic monooxygenase enzymes when used at higher dosages, being a weak but selective metabolic modulator (BEYDOUN and KUTLUAY, 2002; JOHANNESSEN *et al.*, 2003). Hence, inhibition of CYP2C19 may result in increased PHT plasma concentrations by up to 40% (WELLINGTON and GOA, 2001); and the induction of CYP3A4/5 is responsible for the efficacy loss of dihydropyridine calcium antagonists and oral contraceptives (FLESCH, 2004; MAY *et al.*, 2003). A slight induction of the UGT has also been reported, explaining the moderate decrease of LTG concentrations observed during the period of co-medication with OXC comparatively to monotherapy (FLESCH, 2004; MAY *et al.*, 2003).

B2. Preliminary Studies on Phenytoin and Oxcarbazepine Intranasal Administration to Mice

B2. 1. General Introduction

In accordance with the objectives initially outlined at the beginning of this dissertation, the assessment of the IN route potential for the administration of both PHT and OXC was also part of the current work. Indeed, similarly to CBZ and LTG, the oral administration exclusively remains the mainstay of OXC therapy. PHT can be given either orally or intravenously, even though the latter is commonly associated to numerous drawbacks. Effectively, due to poor aqueous solubility of PHT, the preparation of drug parenteral formulation mandatorily requires the use of a hydroalcoholic vehicle, composed of 40% propylene glycol and 10% of ethanol, adjusted to a pH of 12 with sodium hydroxide in order to facilitate dissolution (BIALER, 2009; WANG and PATSALOS, 2003). However, despite enabling a prompt and effective seizure control, the presence of such additives in this formulation is thought to be responsible for the veno-irritant effects, hypotension and cardiac dysrhythmias that are frequently observed after PHT injection (KUTT and HARDEN, 1999; WANG and PATSALOS, 2003). As a result, with the intent of attenuating these complications, IV delivery of PHT has invariably to be infused at a slow rate underneath a close and careful monitoring (BAUER, 2008; WANG and PATSALOS, 2003).

Regardless of being administered either via oral or IV route, PHT displays some unique and peculiar pharmacokinetic characteristics that markedly influence its therapeutic and toxic outcome. Thus, the high protein binding (around 90%), the saturable hepatic metabolism, the non-linear kinetics of elimination and the narrow therapeutic index (10-20 µg/mL) make the use of PHT extremely susceptible to undesirable drug-drug interactions and serious adverse effects, as a consequence of unpredictable and disproportionate increments of drug concentration in plasma (LANDMARK *et al.*, 2012; PATSALOS *et al.*, 2008). Conversely, OXC exhibits an improved pharmacokinetic and tolerability profile when compared to CBZ and the majority of old and established AEDs. However, no significant advantages in terms of anticonvulsant efficacy have however been demonstrated, since it is often needed an OXC dosage of about 1.5-times higher than that of CBZ to achieve equivalent seizure control (PORTER and MELDRUM, 2015). Moreover, several recent reports have identified OXC as a substrate of multidrug efflux transporters, such as P-glycoprotein, which may probably explain the pharmacological failure of OXC therapy in the management of pharmacoresistant epilepsy (CLINCKERS *et al.*, 2005; FORTUNA *et al.*, 2012; MARCHI *et al.*, 2005; ZHANG *et al.*, 2012, 2011).

Therefore, taking all those therapeutic limitations into account and considering the theoretical potential of the IN route to efficiently and directly deliver drugs into the CNS, it was also pertinent to evaluate the feasibility of both PHT and OXC to be intranasally administered. In line with this, several preliminary experiments have been carried out to study the pharmacokinetic behaviour of the two referred compounds following IN delivery to mice. Unfortunately, the overall obtained results were found to be unpromising, and consequently, the investigations did not proceed to a more advanced stage. Some possible reasons for that failure may be related to a number of experimental difficulties, especially concerning the IN dosage form preparation, that hindered the accomplishment of all the studies initially planned.

In true, the first exploratory *in vivo* experiments involving the IN route of drug administration were performed using OXC as the test compound, whereby the long and sinuous process of optimising the methodological conditions, that later allowed the definition of the most appropriate experimental protocol to be applied in both CBZ and LTG IN investigations, was essentially conducted based on OXC preliminary outcomes.

This section is intended to provide an overview of the main results obtained during the preliminary studies on PHT and OXC IN administration to mice. The major experimental problems encountered, the sequential course of experiments tested and the reasons that led us to decline these two molecules will be herein concisely described and discussed.

B2. 2. *In Vivo* Preliminary Studies on Intranasal Drug Delivery

B2. 2.1. Oxcarbazepine

As previously mentioned, the beginning of the studies involving the assessment of the IN route potential to improve CNS targeting of the four AEDs considered (CBZ, PHT, OXC and LTG) was settled by the exploratory trials conducted *in vivo* using OXC as the first test compound.

Aiming at keeping the study design as simple as possible and avoiding the inclusion of a large number of methodological variables that somehow could have a marked impact and a considerable interference with the experimental outcome, the incorporation of the drug in a simple solution with subsequent IN administration by means of a micropipette was chosen as a starting point. Hence, a perfectly clean OXC solution at the concentration of 100 mg/mL was initially prepared in dimethyl sulfoxide (DMSO) enabling the IN administration of a dose of 40 mg/kg. Taking advantage of the fact that mice are obligatory nose breathers (DHURIA *et al.*, 2010; LOCHHEAD and THORNE, 2012), IN delivery was performed by placing the nasal drops formulation at the opening of the mouse nostrils, allowing the animal to sniff them into the nasal cavity. The volume of administration was established at 12 μ L per 30 g of mouse body weight and all the experiments were carried out in immobilised conscious animals. Following IN administration, the animals were sacrificed at 5, 10, 15, 20, 25, 30, 40, 50 and 60 min post-dosing ($n = 1$); blood and brain samples were collected and treated for further chromatographic analysis. The OXC preliminary concentration-time profile achieved in plasma and brain after IN administration was then compared with that obtained, in parallel, after an oral delivery (300 μ L/30 g body weight) of the drug at the same dose (40 mg/kg, $n = 1$) (Figure B2.1). A summary of the main experimental conditions applied in this preliminary study with OXC is presented in Table B2.1.

Table B2.1 Summary of the main experimental conditions applied in the first *in vivo* preliminary study on OXC IN administration to mice.

Dose	40 mg/kg
Formulation	Solution
Administration Volume	12 μ L/30 g body weight
Administration Technique	Nasal drops delivered by means of a micropipette
Anaesthesia	Not used
Sampling Times	5, 10, 15, 20, 25, 30, 40, 50 and 60 min
No. Animals/Time Point	$n = 1$

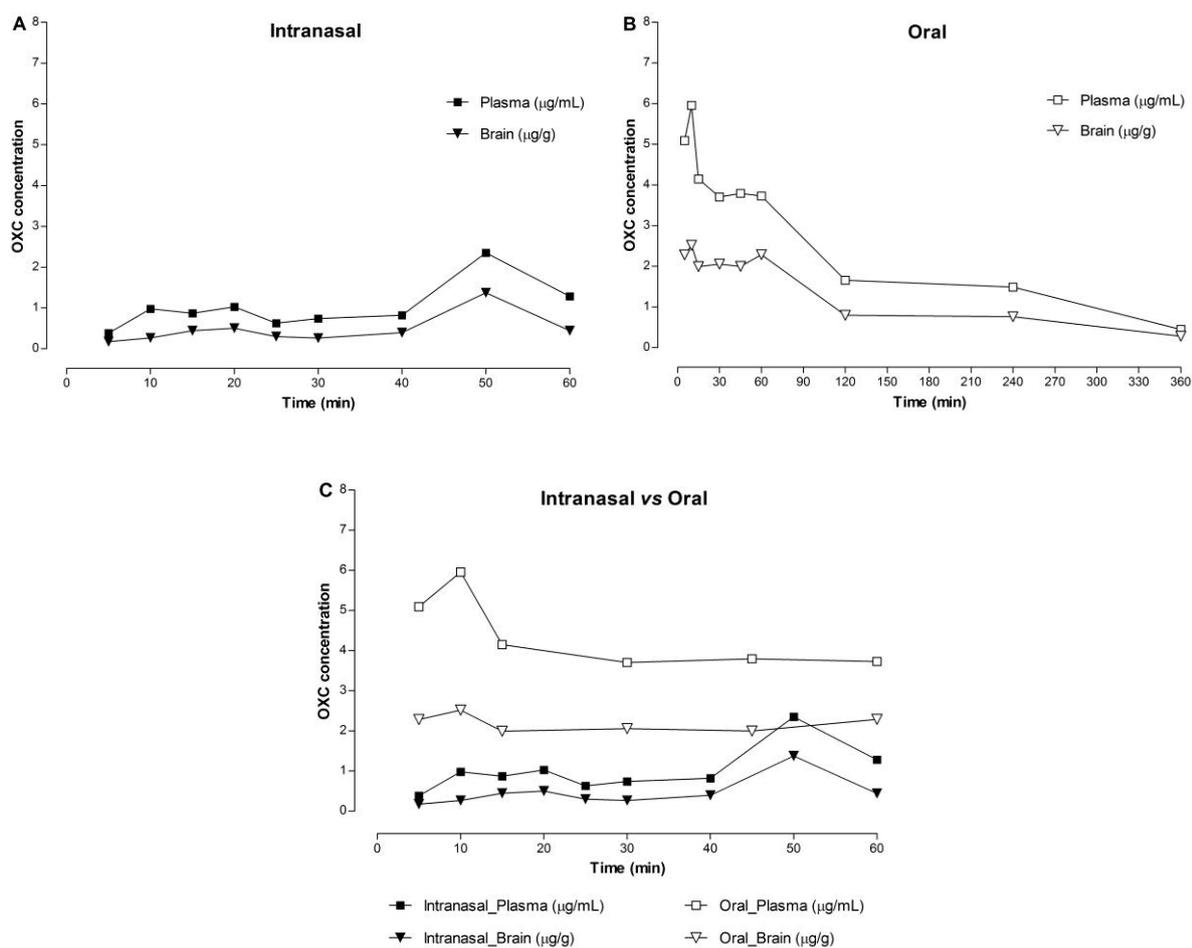


Figure B2.1 Preliminary concentration-time profiles of oxcarbazepine (OXC) in plasma and brain following IN and oral administration (40 mg/kg) to mice: (A) Intranasal; (B) Oral; (C) Intranasal *versus* oral. OXC solution was intranasally delivered in the form of drops at the opening of mice nostrils using a micropipette.

The concentration-time profiles of OXC obtained in plasma and brain after IN administration to mice revealed to be fairly comparable between each other, without exhibiting any advantage, under the pharmacokinetic point of view, over the oral delivery (Figure B2.1). In effect, it is noteworthy that by administering the same dose (40 mg/kg), the extent of systemic and brain exposures to the drug were considerably higher after oral than IN administration. Furthermore, the highest OXC concentration level achieved in plasma and brain following IN instillation was only attained at about 50 min post-dosing which clearly indicate that no direct transport of the drug into the CNS was involved. Instead, a slow absorption of OXC might have been occurred from the nasal cavity to the systemic circulation with subsequent distribution to the brain upon BBB crossing. Bearing in mind that the sampling size is very small ($n = 1$), special care should be imposed when the results are interpreted, since these may not be sufficiently conclusive. Nonetheless,

we strongly believed that this outcome was greatly and primarily influenced by factors related to practical issues. Accordingly, the technique of instillation drops by using a micropipette in awake mice demonstrated to be difficult and inappropriate due to moving and escaping of animals during drug administration. In fact, mice generally do not tolerate IN delivery in the non-anaesthetised state (DHURIA *et al.*, 2010; WONG *et al.*, 2014). Our attempts to intranasally administer OXC in the form of nasal drops resulted in loss of a very significant amount of the formulation at the opening of the nostrils, thus preventing an accurate, precise and reproducible dosing. On top of that, after IN delivery, an appreciable number of animals displayed important signs of respiratory distress and some of them have inclusively died, probably denoting the instillation of an excessive volume of the liquid formulation that ultimately might have been drained to the lower respiratory tract.

Taking all those shortcomings into consideration, we decided to make some alterations in the initial experimental protocol. The OXC nasal formulation administered was maintained, however, the IN delivery technique as well as the volume of administration were adjusted in order to improve the accuracy of dosing and to avoid both the anterior and post-nasal drip drainage (Table B2.2). Thus, the OXC IN administration was accomplished in mice previously anaesthetised with an intraperitoneal injection of ketamine (100 mg/kg)/xylazine (10 mg/kg), by inserting a flexible tube attached to a microsyringe inside the nasal cavity, allowing a more efficient and localised delivery of the drug solution onto the olfactory area. The administration volume was reduced by half (6 μ L/30g body weight) with the intent of eliminating issues associated to respiratory distress (SOUTHAM *et al.*, 2002).

Table B2.2 Summary of the main experimental conditions applied in the second *in vivo* preliminary study on OXC IN administration to mice.

Dose	20 mg/kg
Formulation	Solution
Administration Volume	6 μ L/30 g body weight
Administration Technique	Insertion of a flexible tube attached to a microsyringe
Anaesthesia	Ketamine (100 mg/kg)/xylazine (10 mg/kg), IP
Sampling Times	2, 5, 10, 15, 20, 25, 30, 40, 50 and 60 min
No. Animals/Time Point	$n = 1$

IP, intraperitoneal.

Even though the results obtained were far from being exceptional, considerable improvements were made concerning the methodology previously employed to perform

IN administration. Indeed, the direct delivery of the formulation inside the nasal cavity through the insertion of a flexible tubing enabled a more accurate and reproducible dosing without any evidences of dosage form anterior leakage, and more importantly, without signs of damage to the nasal mucosa. The supremacy of this alternative IN administration technique seemed to be corroborated by the concentration-time profile determined, since the instillation of a dose of 20 mg/kg yielded OXC concentration levels in plasma and brain roughly equivalent to those formerly reached with a dose two-times higher (40 mg/kg) (Figure B2.2). Notwithstanding, once again, the almost parallel time course of plasma and brain OXC levels observed along with the highest peak concentrations attained only at 60 min post-dosing did not evidence the occurrence of a direct drug transport from nose to brain. As a matter of fact, based on these results, we supposed that because of the low nasal solution viscosity allied to the IN delivery technique used, the formulation was easily and rapidly removed from the nasal cavity towards the nasopharynx, where it probably has been swallowed and subsequently absorbed to the systemic circulation via the gastrointestinal tract.

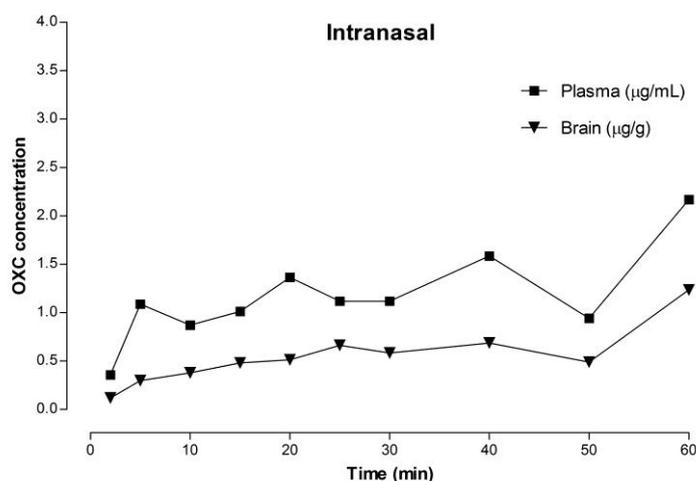


Figure B2.2 Preliminary concentration-time profile of oxcarbazepine (OXC) in plasma and brain following IN administration (20 mg/kg) to mice. OXC solution was intranasally delivered by inserting a flexible tube attached to a microsyringe inside the nasal cavity of previously anaesthetised mice.

Aiming at preventing posterior drainage of nasally dosed solution in mice we tried to isolate the nasal cavity from both the respiratory and gastrointestinal tracts by applying an established surgical procedure firstly described by Hussain *et al.* (1980), with slight modifications (Figure B2.3).

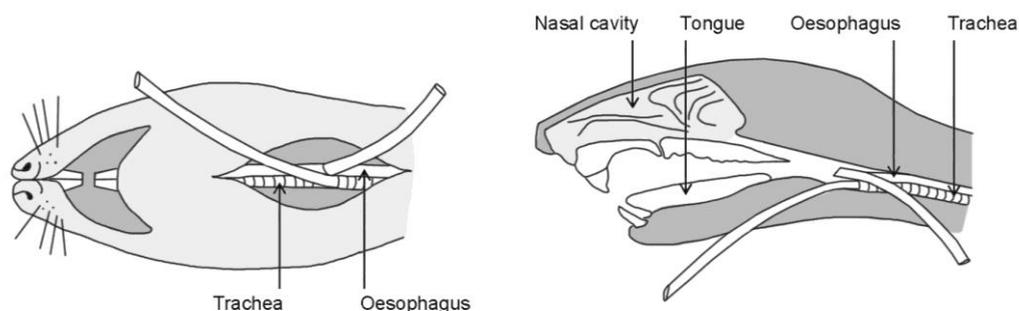


Figure B2.3 Schematic representation of the surgical procedure carried out in mice to isolate the nasal cavity.

Briefly, following an intraperitoneal injection of ketamine (100 mg/kg)/xylazine (10 mg/kg), the anaesthetised mice were placed on their back and an incision was made at the neck to expose and sever the trachea. The upper tracheal segment was tied off with a suture while the lower one was cannulated with a polyurethane tube (22 G) towards the lungs in order to maintain free breathing. The oesophagus was occluded by tying it with a suture thereby ensuring that no loss of the administered dose to the stomach might be involved. Finally, the OXC IN administration was performed by implementing the same experimental conditions employed in the second *in vivo* preliminary assay (Table B2.2).

The main asset of this methodology lies on its ability to provide a clear evaluation of the real and exclusive contribution of the IN delivery to the pharmacokinetic behaviour of drugs administered via this route, a reason why several authors have opted to use this surgical technique in their research work (CHOU and DONOVAN, 1997; CHOW *et al.*, 1999; HUSSAIN *et al.*, 2000; ISHIKAWA *et al.*, 2002; RAGHAVAN and ABIMON, 2001; SINSWAT and TENGAMNUAY, 2003; WANG *et al.*, 2007; YOKOGAWA *et al.*, 2006). Taking advantage of the system tightness, by confining the formulation only to the isolated nasal cavity, it was expected that a direct transport of the drug from nose to brain could be reliably assessed without the interference of other physiological variables. Nevertheless, there are important drawbacks that should be pointed out, namely the invasiveness and complexity of the procedure, the need to keep the animals under the anaesthetised state throughout the whole period of experiment and, most importantly, the complete mismatch between the organism normal function and the unrealistic conditions created. Considering the aforementioned limitations and the inconspicuous results obtained (Figure B2.4), it was taken the decision of disregarding the possibility of applying this procedure in further and more comprehensive studies.

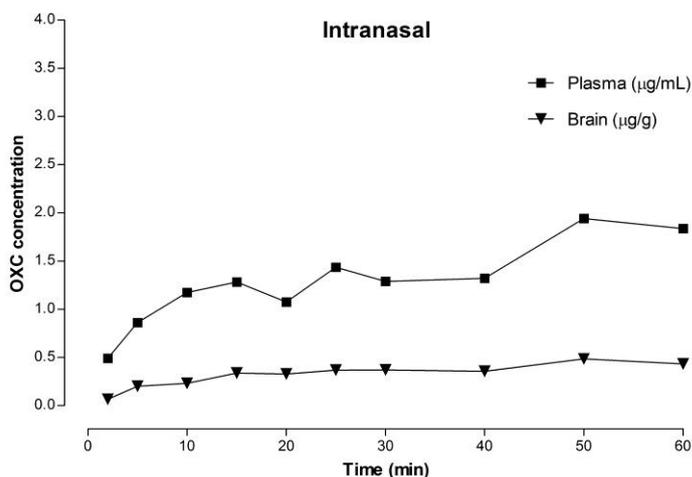


Figure B2.4 Preliminary concentration-time profile of oxcarbazepine (OXC) in plasma and brain following IN administration (20 mg/kg) to mice. The IN administration of OXC solution was performed in mice previously subjected to a surgical procedure to isolate the nasal cavity by using the technique of flexible tubing insertion.

Indeed, examining both plasma and brain concentration-time profiles achieved after IN administration of OXC to mice submitted to the nasal cavity isolation technique, it was still suggested that a direct transport of the drug from nose to brain was absent. Based on these results, we strongly believed that the drug might have been absorbed into the bloodstream through the nasal mucosa and gained access to the brain after traversing the BBB (Figure B2.4).

With the concern of avoiding the early clearance of drug solution from the mice nasal cavity, and at the same time, overcoming the overall limitations associated to the surgical methodology previously described, our efforts were posed onto a new alternative strategy which consisted on the incorporation of the drug into a vehicle with bioadhesive properties. In theory, using such approach could solve a number of problems encountered with IN delivery of simple liquid formulations by providing a localised deposition and an increased contact time between the drug and the olfactory mucosa, thus improving permeability and eventually promoting the preferential transport of drugs from the olfactory region to the CNS (CHARLTON *et al.*, 2007b; UGWOKÉ *et al.*, 2001). In this context, among several mucoadhesive drug delivery systems available, the selection of a temperature-triggered *in situ* gel appeared to be the most appropriate. In essence, this kind of formulation is characterised by a low viscosity at or below room temperature, undergoing a phase transition into a semi-solid state within the nasal cavity, as a result of the increase in temperature (SINGH *et al.*, 2013). Accordingly, the main advantages rely in the reduction of anterior leakage and post-nasal drainage, the ease and precision of

dosage form manipulation allowing for an accurate dosing, and the enhancement of the residence time of the drug in contact with the nasal mucosa (NIRMAL *et al.*, 2010; SINGH *et al.*, 2013).

Therefore, OXC was dissolved in 100 μL of ethanol and further incorporated in 900 μL of a thermoreversible gel, composed of 18% PF-127 and 0.2% C-974P (w/v), so that the final concentration of the drug in the formulation was 0.4 mg/mL. IN administration was performed employing the flexible tubing insertion technique and the volume delivered was settled at 12 μL /30 g of mice body weight (Table B2.3).

Table B2.3 Summary of the main experimental conditions applied in the fourth *in vivo* preliminary study on OXC IN administration to mice.

Dose	0.16 mg/kg
Formulation	Thermoreversible <i>in situ</i> gel
Administration Volume	12 μL /30 g body weight
Administration Technique	Insertion of a flexible tube attached to a microsyringe
Anaesthesia	Ketamine (100 mg/kg)/xylazine (10 mg/kg), IP
Sampling Times	15 and 120 min
No. Animals/Time Point	$n = 1$

IP, intraperitoneal.

The results revealed that in both sampling time points analysed (15 and 120 min), the concentrations determined for OXC in plasma and brain were near or below the limit of quantification established by the analytical method applied. Unfortunately, owing to solubility issues, the maximum amount of drug that was possible to incorporate in the nasal gel only enabled the administration of a dose of 0.16 mg/kg, which proved to be clearly insufficient to attain quantifiable OXC levels in all the relevant biological matrices.

Although the preliminary data obtained for OXC were far from being encouraging, the IN drug administration by means of a thermoreversible gel has shown potential and seemed to be the most promising among all the experimental procedures attempted. Notwithstanding, a more complex galenic development together with a deeper optimisation of the nasal formulation in order to incorporate a greater amount of the drug was definitely required, which revealed to be beyond the scope of the present work. Since the essential conditions to carry out further studies with OXC were not successfully met, it was decided to proceed the investigation with the remaining AEDs. Therefore, the subsequent implementation of these experimental conditions to CBZ provided a more positive outcome, as thoroughly documented in *Chapter III*.

B2. 2.2. Phenytoin

PHT was the third compound to be tested intranasally, subsequently to OXC and CBZ. The preliminary *in vivo* trials of PHT began with the implementation of the same experimental conditions successfully employed in the study carried out with CBZ. Thus, PHT was dissolved in 50 μL of ethanol and further incorporated in 950 μL of the thermoreversible nasal gel (18% PF-127 and 0.2% C-974P, w/v) so that the final drug concentration was 1 mg/mL, affording the administration of a dose of 0.4 mg/kg. IN delivery was performed in anaesthetised mice [intraperitoneal injection of ketamine (100 mg/kg)/xylazine (10 mg/kg)] by using the flexible tubing insertion technique, and the volume of administration was fixed in 12 μL /30g of mouse body weight. Three representative sampling time points (10, 30 and 240 min) were chosen to sacrifice the animals ($n = 1$) and collect the corresponding relevant biological samples for analysis. A summary of the main experimental conditions applied in this preliminary study with PHT is presented in Table B2.4.

Table B2.4 Summary of the main experimental conditions applied in the first *in vivo* preliminary study on PHT IN administration to mice.

Dose	0.4 mg/kg
Formulation	Thermoreversible <i>in situ</i> nasal gel
Administration Volume	12 μL /30 g body weight
Administration Technique	Insertion of a flexible tube attached to a microsyringe
Anaesthesia	Ketamine (100 mg/kg)/xylazine (10 mg/kg), IP
Sampling Times	10, 30 and 240 min
No. Animals/Time Point	$n = 1$

IP, intraperitoneal.

The first exploratory data on PHT IN delivery to mice denoted extremely low concentration levels of the drug in plasma, wherein all the values determined were below the limit of quantification. In addition, it was not even possible to detect any sign of PHT in the brain samples analysed, thereby indicating that the dose administered (0.4 mg/kg) was not enough to provide a desirable drug uptake into the biophase. Regrettably, similarly to what was found with OXC, the poor solubility of PHT in ethanol remarkably precluded the incorporation of a larger amount of the drug into the nasal gel, leading to PHT concentration levels in blood and brain that were not quantifiable.

In line with this, an alternative delivery vehicle that would allow the administration of a significantly higher dose of PHT was definitely needed. On account of the unprofitable initial results obtained with IN administration of OXC in the form of a simple solution, and aware of the importance of bioadhesiveness to avoid both the anterior and posterior leakage of the formulation from the nasal cavity, the use of a slightly viscous aqueous solution consisting of 0.8% hydroxypropyl methylcellulose (HPMC) to incorporate and intranasally deliver PHT to mice was attempted. The administration of a greater dose (12 mg/kg) was primarily achieved by dissolving the drug firstly in DMSO. Hence, an appropriate amount of PHT was dissolved in 300 μ L of DMSO and further incorporated in 700 μ L of 0.8% HPMC solution so that the final drug concentration was 60 mg/mL and the total percentage of DMSO in the formulation was equivalent to 30%. IN delivery was then accomplished applying the experimental conditions outlined in Table B2.5. An IV administration of PHT [propylene glycol-NaCl 0.9%-ethanol (5:3:2, v/v/v); 120 μ L/30g body weight; lateral tail vein injection] at the same dose (12 mg/kg) was also undertaken to be used as a control.

Table B2.5 Summary of the main experimental conditions applied in the second *in vivo* preliminary study on PHT IN administration to mice.

Dose	12 mg/kg
Formulation	Slightly viscous aqueous solution
Administration Volume	6 μ L/30 g body weight
Administration Technique	Insertion of a flexible tube attached to a microsyringe
Anaesthesia	Ketamine (100 mg/kg)/xylazine (10 mg/kg), IP
Sampling Times	10, 30 and 240 min
No. Animals/Time Point	<i>n</i> = 2

IP, intraperitoneal.

PHT concentration values determined in plasma and brain at the three representative sampling time points considered (10, 30 and 240 min) post-IN and IV dosing were systematised in Table B2.6. Following IN administration, higher drug concentrations were achieved in both plasma and brain enabling an accurate and reliable quantitative analysis of the drug in these biological samples. Although the maximal concentration levels of PHT observed in plasma and brain after IN delivery have been fairly comparable to those obtained after IV injection, they were only attained at 240 min post-dosing, suggesting that no preferential transport of the drug from nose to brain was involved. Surprisingly, by analysing the temporal evolution of PHT concentrations

measured either in plasma or brain after IV administration, it was possible to notice that consistent levels of drug were maintained throughout the whole period of sampling time since no decrease in concentration was ascertained from 10 up to 240 min (Table B2.6). A possible explanation for this fact may be related to the saturation of the enzymatic system implied in PHT hepatic metabolism. In fact, at high PHT doses, the enzymes' ability to metabolise the drug can be easily overwhelmed, decreasing the rate of clearance and markedly extending the drug half-life (BAUER, 2008).

Table B2.6 Preliminary PHT concentrations determined in plasma ($\mu\text{g/mL}$) and brain ($\mu\text{g/g}$) following IN and IV administrations (12 mg/kg) to mice.

Post-dosing Time	Mouse	Intranasal		Intravenous	
		Plasma	Brain	Plasma	Brain
10 min	0	2.242	BLQ	11.187	4.074
	I	3.615	BLQ	11.047	3.408
30 min	0	3.165	0.979	-	-
	I	3.579	0.939	11.368	4.110
240 min	0	11.673	3.965	8.315	2.996
	I	7.654	2.588	11.756	4.756

BLQ, below the limit of quantification.

In view of the paramount objectives underlying the present thesis, the use of the IV route as a control to assess the potential of the IN administration to improve CNS targeting of AEDs is undeniably the most appropriate. Additionally to providing the determination of the IN absolute drug bioavailability, it is also crucial to figure out the exact contribution of blood-mediated drug delivery to the CNS, allowing a more accurate discrimination of the fraction that is directly transported into the brain from the nasal cavity. In the particular case of PHT, the suitability of such approach is reinforced by the fact that, among the four AEDs considered in our work, PHT is the only one that is currently commercially available in both oral and parenteral dosage forms.

Given that it was necessary to administer very high doses of PHT via the IN route to achieve measurable drug levels in all the relevant biological matrices analysed, keeping the same order of magnitude for the IV administered dose would compromise a direct comparison of the two pharmacokinetic behaviours. Indeed, probably due to the enzymatic saturation phenomenon, a difference in the doses administered via each route inevitably has to be adopted, introducing an undesirable associated margin of error that ultimately interferes with the reliability of the assumptions. For this reason, the

progression of the research involving this molecule was considered unfeasible, turning our efforts towards the exploitation of LTG IN delivery.



APPENDIX C.

C1. Preliminary Comparative Study of the Pharmacokinetics of CBZ Administered via both IN (0.4 mg/kg) and Oral (4 mg/kg) Routes to Mice

In an early stage, the feasibility of using the oral route as a control was investigated. A summary of the main experimental conditions applied as well as the pharmacokinetic results obtained are herein summarised.

Table C1.1 Summary of the main experimental conditions employed in the preliminary comparative study on CBZ IN and oral administrations to mice.

Experimental Conditions	Intranasal	Oral
Dose	0.4 mg/kg	4 mg/kg
Formulation	Thermoreversible <i>in situ</i> gel	0.5% CMC:DMSO (95:5, v/v)
Administration Volume	12 μ L/30 g body weight	300 μ L/30 g body weight
No. Animals/Time Point	$n = 4$	$n = 4$

CMC, carboxymethyl cellulose; DMSO, dimethyl sulfoxide.

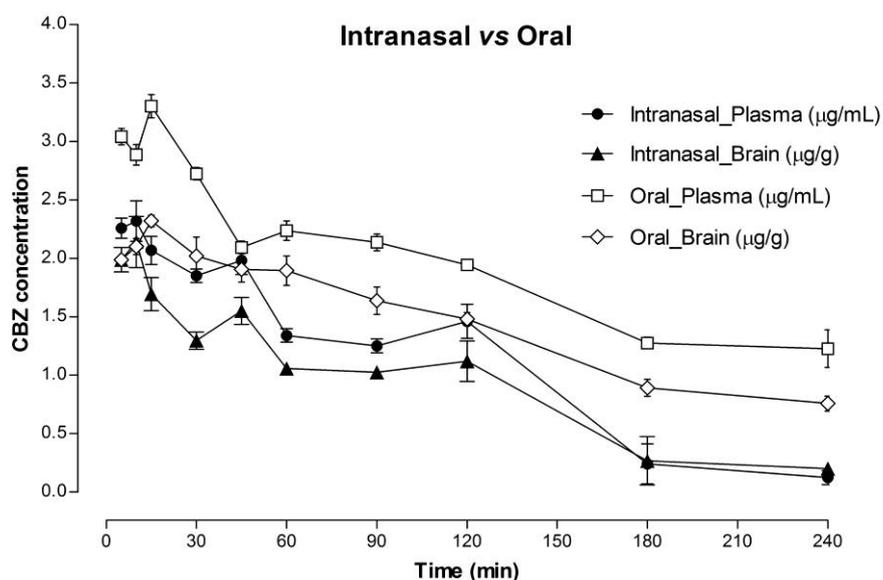


Figure C1.1 Concentration-time profiles of carbamazepine (CBZ) in plasma and brain following IN (0.4 mg/kg) and oral (4 mg/kg) administration to mice. Symbols represent the mean values \pm SEM ($n = 4$).

Table C1.2 The main pharmacokinetic parameters of CBZ following its intranasal (IN) (0.4 mg/kg) and oral (4 mg/kg) administration to mice.

Pharmacokinetic Parameters	Plasma		Brain		Liver	
	IN	Oral	IN	Oral	IN	Oral
t_{max} (min)	10.0	15.0	10.0	15.0	10.0	5.0
C_{max} ($\mu\text{g/mL}$)	2.32	3.30	2.14 ^b	2.32 ^b	2.78 ^b	3.63 ^b
AUC_t ($\mu\text{g}\cdot\text{min/mL}$)	252.58	450.09	193.19 ^c	337.10 ^c	204.15 ^c	499.14 ^c
F (%) ^a	105.8	18.9	-	-	-	-

^a Absolute bioavailability was calculated based on AUC_t values. ^b Values expressed in $\mu\text{g/g}$. ^c Values expressed in $\mu\text{g}\cdot\text{min/g}$. AUC_t , area under the concentration-time curve from time zero to the last quantifiable drug concentration; C_{max} , maximum peak concentration; F, absolute bioavailability; t_{max} , time to achieve C_{max} .

C2. Preliminary Comparative Study of the Pharmacokinetics of LTG Administered via both IN and Oral Routes (4 mg/kg) to Mice

In an early stage, the feasibility of using the oral route as a control was investigated. A summary of the main experimental conditions applied as well as the pharmacokinetic results obtained will be herein provided.

Table C2.1 Summary of the main experimental conditions employed in the preliminary comparative study on LTG IN and oral administrations to mice.

Experimental Conditions	Intranasal	Oral
Dose	4 mg/kg	4 mg/kg
Formulation	Thermoreversible <i>in situ</i> gel	0.5% CMC:Propylene glycol (95:5, v/v)
Administration Volume	12 μ L/30 g body weight	300 μ L/30 g body weight
No. Animals/Time Point	$n = 4$	$n = 1$

CMC, carboxymethyl cellulose.

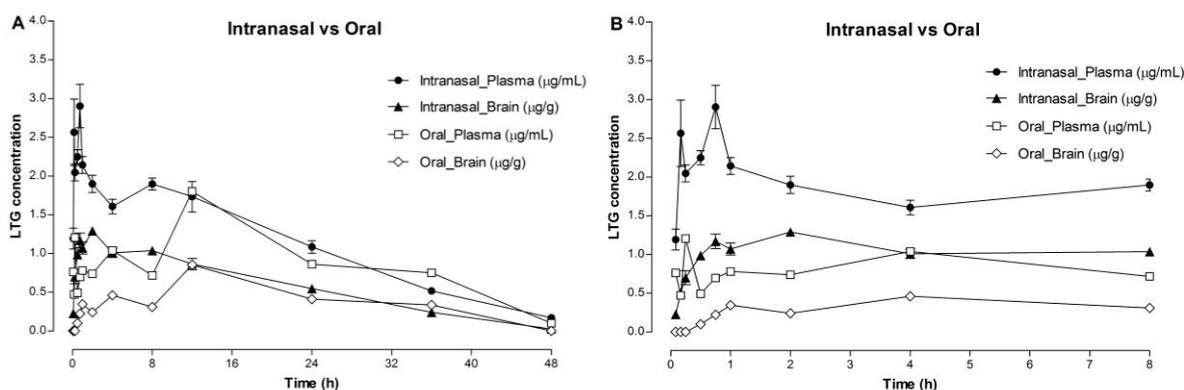


Figure C2.1 Concentration-time profiles of lamotrigine (LTG) in plasma and brain following IN and oral administration (4 mg/kg) to mice: (A) from time zero to 48 h post-dosing; (B) from time zero to 8 h post-dosing.

Table C2.2 The main pharmacokinetic parameters of LTG following its intranasal (IN) and oral administration (4 mg/kg) to mice.

Pharmacokinetic Parameters	Plasma		Brain		Liver	
	IN	Oral	IN	Oral	IN	Oral
t_{max} (h)	0.75	12.0	2.00	12.0	8.00	12.0
C_{max} ($\mu\text{g}/\text{mL}$)	2.90	1.80	1.29 ^b	0.85 ^b	2.15 ^b	1.67 ^b
AUC_t ($\mu\text{g}\cdot\text{h}/\text{mL}$)	52.63	42.50	25.41 ^c	19.04 ^c	54.80 ^c	47.88 ^c
F (%) ^a	117.5	94.9	-	-	-	-

^a Absolute bioavailability was calculated based on AUC_t values. ^b Values expressed in $\mu\text{g}/\text{g}$. ^c Values expressed in $\mu\text{g}\cdot\text{h}/\text{g}$. AUC_t , area under the concentration-time curve from time zero to the last quantifiable drug concentration; C_{max} , maximum peak concentration; F, absolute bioavailability; t_{max} , time to achieve C_{max} .



APPENDIX D.

D1. Pharmacokinetics of CBZ after IN and IV Administration to Mice

Table D1.1 CBZ concentrations in plasma ($\mu\text{g}/\text{mL}$) at predefined time points following both intranasal (IN) and intravenous (IV) administrations (0.4 mg/kg) to mice.

Route	Post-dosing Time (min)	Mouse				Mean ^a	SD	SEM	% CV
		0	I	II	III				
IN	5	2.411	2.376	2.029	2.215	2.258	0.174	0.087	7.728
	10	2.515	2.651	1.865	2.245	2.319	0.347	0.173	14.944
	15	2.346	1.826	2.185	1.916	2.068	0.240	0.120	11.590
	30	1.790	1.789	1.800	2.026	1.852	0.117	0.058	6.298
	45	1.664	2.084	2.234	1.945	1.982	0.243	0.121	12.257
	60	1.363	1.467	1.186	1.343	1.340	0.116	0.058	8.647
	90	1.395	1.111	1.218	1.279	1.251	0.119	0.059	9.485
	120	1.884	1.393	1.224	1.341	1.461	0.291	0.146	19.927
	180	0.050*	0.753	0.050*	0.110	0.241	0.342	0.171	142.341
	240	0.183	0.050*	0.261	-	0.124	0.120	0.060	97.071
IV	5	2.471	2.407	2.477	2.513	2.467	0.044	0.022	1.800
	10	2.203	2.203	2.230	2.135	2.193	0.041	0.020	1.855
	15	1.988	2.116	1.843	1.831	1.944	0.135	0.067	6.933
	30	2.157	1.552	1.869	2.091	1.917	0.273	0.136	14.227
	45	1.970	1.713	1.818	1.785	1.822	0.108	0.054	5.948
	60	1.477	1.559	1.334	1.333	1.426	0.112	0.056	7.829
	90	1.896	1.488	1.398	1.160	1.485	0.307	0.153	20.634
	120	1.153	1.183	1.194	1.083	1.153	0.050	0.025	4.358
	180	0.050*	0.050*	0.170	0.298	0.142	0.118	0.059	83.277
	240	0.050*	0.050*	-	0.050*	0.038	0.025	0.013	66.667

^a CBZ concentration mean values ($n = 4$). * Concentration below the limit of quantification (BLOQ) - these values were assumed as $0.5 \times \text{LLOQ} = 0.050 \mu\text{g}/\text{mL}$. SD, standard deviation; SEM, standard error of the mean; CV, coefficient of variation.

Table D1.2 CBZ concentrations in brain ($\mu\text{g/g}$) at predefined time points following both intranasal (IN) and intravenous (IV) administrations (0.4 mg/kg) to mice.

Route	Post-dosing Time (min)	Mouse				Mean ^a	SD	SEM	% CV
		0	I	II	III				
IN	5	2.133	2.164	1.708	1.948	1.988	0.210	0.105	10.567
	10	2.337	2.549	1.530	2.145	2.140	0.439	0.220	20.516
	15	2.036	1.412	1.805	1.523	1.694	0.282	0.141	16.637
	30	1.105	1.256	1.403	1.420	1.296	0.147	0.074	11.351
	45	1.250	1.669	1.780	1.500	1.550	0.231	0.115	14.881
	60	1.142	1.046	0.934	1.102	1.056	0.090	0.045	8.539
	90	1.134	0.880	1.029	1.056	1.025	0.106	0.053	10.386
	120	1.629	1.034	0.860	0.955	1.119	0.347	0.174	31.020
	180	0.200*	0.871	-	-	0.268	0.413	0.206	154.264
	240	0.200*	0.200*	0.200*	0.200*	0.200	-	-	-
IV	5	2.514	2.394	2.425	2.205	2.385	0.130	0.065	5.450
	10	1.997	1.785	1.932	1.801	1.879	0.103	0.051	5.469
	15	1.831	1.776	1.672	1.641	1.730	0.088	0.044	5.113
	30	1.722	1.467	1.647	2.392	1.807	0.405	0.202	22.399
	45	2.242	1.566	1.923	1.555	1.821	0.328	0.164	18.024
	60	1.288	1.243	1.055	1.082	1.167	0.116	0.058	9.938
	90	1.684	1.151	1.584	1.208	1.407	0.267	0.133	18.950
	120	1.317	1.022	1.106	0.974	1.105	0.152	0.076	13.723
	180	0.200*	0.200*	0.200*	0.200*	0.200	-	-	-
	240	0.200*	-	0.200*	-	0.100	0.115	0.058	115.470

^a CBZ concentration mean values ($n = 4$). * Concentration below the limit of quantification (BLOQ) - these values were assumed as $0.5 \times \text{LLOQ} = 0.200 \mu\text{g/g}$. SD, standard deviation; SEM, standard error of the mean; CV, coefficient of variation.

Table D1.3 CBZ concentrations in liver ($\mu\text{g/g}$) at predefined time points following both intranasal (IN) and intravenous (IV) administrations (0.4 mg/kg) to mice.

Route	Post-dosing Time (min)	Mouse				Mean ^a	SD	SEM	% CV
		0	I	II	III				
IN	5	2.985	2.974	2.451	2.645	2.764	0.261	0.131	9.454
	10	2.951	3.301	1.986	2.864	2.775	0.559	0.279	20.138
	15	2.771	2.199	2.518	2.176	2.416	0.284	0.142	11.741
	30	1.774	1.847	1.944	2.192	1.939	0.182	0.091	9.409
	45	1.716	2.417	2.658	2.134	2.231	0.405	0.202	18.145
	60	1.616	1.167	1.187	1.150	1.280	0.225	0.112	17.555
	90	1.261	1.175	1.374	1.315	1.281	0.084	0.042	6.592
	120	2.255	1.484	1.196	1.070	1.501	0.531	0.266	35.400
	180	-	0.400*	-	0.400*	0.200	0.231	0.115	115.470
	240	0.400*	0.400*	0.400*	-	0.300	0.200	0.100	66.667
IV	5	3.352	3.766	3.226	3.213	3.389	0.259	0.130	7.643
	10	2.867	2.599	2.872	2.684	2.756	0.136	0.068	4.938
	15	2.472	2.620	2.419	2.313	2.456	0.128	0.064	5.205
	30	2.288	1.922	2.215	2.335	2.190	0.185	0.093	8.458
	45	2.725	2.255	2.560	2.320	2.465	0.217	0.109	8.823
	60	1.744	1.807	1.466	1.510	1.632	0.169	0.085	10.373
	90	2.055	1.258	1.594	1.449	1.589	0.340	0.170	21.367
	120	1.372	1.406	1.356	1.185	1.330	0.099	0.049	7.419
	180	-	0.400*	0.400*	0.400*	0.300	0.200	0.100	66.667
	240	-	-	-	-	-	-	-	-

^a CBZ concentration mean values ($n = 4$). * Concentration below the limit of quantification (BLOQ) - these values were assumed as $0.5 \times \text{LLOQ} = 0.400 \mu\text{g/g}$. SD, standard deviation; SEM, standard error of the mean; CV, coefficient of variation.

D2. Brain Biodistribution of CBZ after IN and IV Administration to Mice

Table D2.1 CBZ concentrations in plasma ($\mu\text{g}/\text{mL}$), remaining portion of the brain ($\mu\text{g}/\text{g}$), frontal cortex ($\mu\text{g}/\text{g}$) and olfactory bulb ($\mu\text{g}/\text{g}$) at predefined time points following IN administration (0.4 mg/kg) to mice.

Matrix	Post-dosing Time (min)	Mouse				Mean ^a	SD	SEM	% CV
		0	I	II	III				
Plasma	5	2.938	2.082	3.074	2.989	2.771	0.463	0.231	16.700
	10	2.705	3.079	2.753	2.842	2.845	0.166	0.083	5.848
	15	2.684	2.604	2.704	2.709	2.675	0.049	0.024	1.826
	30	2.689	2.095	2.005	1.952	2.185	0.341	0.170	15.597
	60	2.515	2.268	2.252	1.443	2.120	0.467	0.233	22.016
Remaining Brain Portion	5	2.535	1.439	2.452	2.035	2.115	0.501	0.251	23.697
	10	2.720	2.311	2.625	2.656	2.578	0.182	0.091	7.065
	15	1.805	2.577	2.299	2.047	2.182	0.331	0.166	15.191
	30	2.647	1.864	1.763	1.675	1.987	0.447	0.223	22.486
	60	2.714	2.565	1.968	1.282	2.132	0.652	0.326	30.596
Frontal Cortex	5	2.634	1.679	3.144	2.392	2.462	0.609	0.305	24.741
	10	2.839	2.963	3.167	3.246	3.054	0.186	0.093	6.094
	15	2.665	2.847	2.560	2.674	2.687	0.119	0.059	4.413
	30	2.665	1.589	1.628	1.487	1.842	0.552	0.276	29.950
	60	2.658	2.451	1.843	1.327	2.070	0.604	0.302	29.188
Olfactory Bulb	5	3.004	2.026	3.183	2.608	2.705	0.512	0.256	18.940
	10	3.283	3.327	2.940	3.080	3.158	0.181	0.090	5.727
	15	2.568	2.937	2.767	2.758	2.758	0.151	0.075	5.469
	30	2.673	1.809	1.663	1.544	1.922	0.512	0.256	26.640
	60	2.699	2.493	1.876	1.374	2.110	0.603	0.301	28.569

^a CBZ concentration mean values ($n = 4$). SD, standard deviation; SEM, standard error of the mean; CV, coefficient of variation.

Table D2.2 CBZ concentrations in plasma ($\mu\text{g/mL}$), remaining portion of the brain ($\mu\text{g/g}$), frontal cortex ($\mu\text{g/g}$) and olfactory bulb ($\mu\text{g/g}$) at predefined time points following IV administration (0.4 mg/kg) to mice.

Matrix	Post-dosing Time (min)	Mouse				Mean ^a	SD	SEM	% CV
		0	I	II	III				
Plasma	5	3.084	2.703	3.056	2.624	2.867	0.237	0.119	8.276
	10	2.893	2.936	2.964	2.826	2.905	0.060	0.030	2.064
	15	1.859	2.231	2.409	2.664	2.291	0.338	0.169	14.769
	30	2.332	2.291	2.475	2.051	2.287	0.176	0.088	7.693
	60	1.491	2.063	1.955	1.188	1.674	0.408	0.204	24.373
Remaining Brain Portion	5	3.429	2.939	3.149	2.866	3.096	0.252	0.126	8.154
	10	3.209	3.040	2.807	2.928	2.996	0.171	0.086	5.711
	15	2.375	2.639	2.715	3.071	2.700	0.287	0.144	10.637
	30	2.517	2.476	2.535	2.352	2.470	0.082	0.041	3.339
	60	1.478	2.018	2.065	1.187	1.687	0.427	0.213	25.289
Frontal Cortex	5	3.176	2.743	3.130	2.866	2.979	0.208	0.104	6.985
	10	3.027	3.116	2.800	2.769	2.928	0.170	0.085	5.809
	15	2.197	2.642	2.549	2.717	2.526	0.230	0.115	9.114
	30	2.432	2.658	2.650	2.256	2.499	0.193	0.096	7.714
	60	1.581	2.013	2.035	1.229	1.714	0.385	0.193	22.469
Olfactory Bulb	5	3.848	2.547	2.559	2.847	2.950	0.615	0.307	20.830
	10	3.095	2.902	2.342	2.910	2.812	0.326	0.163	11.589
	15	2.232	2.722	2.522	2.653	2.532	0.217	0.108	8.559
	30	2.580	2.371	2.235	2.255	2.360	0.158	0.079	6.712
	60	0.909 [#]	1.713	1.797	1.190	1.402	0.425	0.212	30.286

^a CBZ concentration mean values ($n = 4$). [#] Concentration below the limit of quantification (BLOQ) - these values were assumed as $0.5 \times \text{LLOQ} = 0.010 \mu\text{g}$. SD, standard deviation; SEM, standard error of the mean; CV, coefficient of variation.



APPENDIX E.

E1. Pharmacokinetics of LTG after IN and IV Administration to Mice

Table E1.1 LTG concentrations in plasma ($\mu\text{g/mL}$) at predefined time points following both intranasal (IN) and intravenous (IV) administrations (4 mg/kg) to mice.

Route	Post-dosing Time (h)	Mouse				Mean ^a	SD	SEM	% CV
		0	I	II	III				
IN	0.083	1.410	1.436	1.002	0.919	1.192	0.270	0.135	22.627
	0.167	1.981	3.825	2.081	2.366	2.563	0.857	0.428	33.425
	0.25	2.131	2.013	1.762	2.280	2.046	0.219	0.109	10.697
	0.5	2.365	2.430	2.033	2.166	2.249	0.183	0.091	8.121
	0.75	3.121	2.510	3.595	2.386	2.903	0.562	0.281	19.356
	1	2.030	2.129	1.961	2.449	2.142	0.216	0.108	10.085
	2	2.089	2.086	1.654	1.764	1.898	0.223	0.112	11.767
	4	1.654	1.466	1.455	1.852	1.607	0.187	0.093	11.638
	8	1.849	2.049	1.699	1.989	1.897	0.156	0.078	8.238
	12	1.813	2.257	1.426	1.437	1.733	0.393	0.196	22.665
	24	1.095	1.022	1.303	0.916	1.084	0.164	0.082	15.079
	36	0.476	0.548	0.618	0.428	0.517	0.083	0.042	16.072
	48	0.216	0.100*	0.264	0.100*	0.170	0.083	0.042	48.935
IV	0.083	3.041	2.846	2.693	3.494	3.019	0.348	0.174	11.523
	0.167	2.986	2.582	2.859	2.638	2.766	0.189	0.094	6.832
	0.25	2.370	2.424	3.005	2.596	2.599	0.288	0.144	11.069
	0.5	2.633	2.389	2.490	2.463	2.494	0.102	0.051	4.105
	0.75	2.415	2.559	2.365	2.141	2.370	0.174	0.087	7.325
	1	2.267	1.762	2.293	2.597	2.230	0.346	0.173	15.508
	2	1.581	1.596	1.617	1.833	1.657	0.119	0.059	7.154
	4	2.338	1.616	2.433	1.466	1.963	0.493	0.246	25.094
	8	1.344	1.597	1.350	1.389	1.420	0.120	0.060	8.424
	12	1.370	1.095	1.360	1.583	1.352	0.200	0.100	14.777
	24	1.142	0.858	0.981	1.134	1.029	0.136	0.068	13.222
	36	0.303	0.236	0.303	0.244	0.271	0.036	0.018	13.329
	48	0.232	0.100*	0.100*	0.100*	0.133	0.066	0.033	49.654

^a LTG concentration mean values ($n = 4$). * Concentration below the limit of quantification (BLOQ) - these values were assumed as $0.5 \times \text{LLOQ} = 0.100 \mu\text{g/mL}$. SD, standard deviation; SEM, standard error of the mean; CV, coefficient of variation.

Table E1.2 LTG concentrations in brain ($\mu\text{g/g}$) at predefined time points following both intranasal (IN) and intravenous (IV) administrations (4 mg/kg) to mice.

Route	Post-dosing Time (h)	Mouse				Mean ^a	SD	SEM	% CV
		0	I	II	III				
IN	0.083	0.313	0.208	0.286	0.100*	0.227	0.095	0.048	42.085
	0.167	0.423	0.559	0.598	0.446	0.507	0.086	0.043	16.882
	0.25	0.699	0.572	0.958	0.573	0.701	0.182	0.091	25.944
	0.5	0.952	1.091	0.930	0.971	0.986	0.072	0.036	7.303
	0.75	0.920	1.287	1.127	1.346	1.170	0.191	0.095	16.282
	1	1.144	0.880	1.016	1.242	1.070	0.157	0.079	14.694
	2	1.281	1.335	1.253	1.293	1.290	0.034	0.017	2.659
	4	1.149	0.882	0.942	1.063	1.009	0.120	0.060	11.904
	8	0.982	1.051	1.054	1.057	1.036	0.036	0.018	3.504
	12	0.913	1.042	0.786	0.710	0.862	0.146	0.073	16.890
	24	0.536	0.523	0.691	0.442	0.548	0.104	0.052	18.979
	36	0.239	0.245	0.263	0.217	0.241	0.019	0.010	7.934
	48	-	-	0.100*	-	0.025	0.050	0.025	200.000
	IV	0.083	1.169	1.270	1.156	1.653	1.312	0.233	0.117
0.167		1.280	1.155	1.269	1.224	1.232	0.057	0.028	4.605
0.25		1.253	1.414	1.362	1.423	1.363	0.078	0.039	5.727
0.5		1.672	1.873	1.632	1.846	1.756	0.122	0.061	6.922
0.75		1.580	1.753	1.614	1.541	1.622	0.092	0.046	5.687
1		1.516	1.255	1.706	1.483	1.490	0.185	0.093	12.426
2		1.302	1.134	1.230	1.257	1.231	0.071	0.035	5.756
4		1.626	1.038	1.281	1.044	1.247	0.277	0.138	22.206
8		0.784	0.873	0.808	0.798	0.816	0.039	0.020	4.823
12		0.811	0.701	0.826	1.067	0.851	0.154	0.077	18.077
24		0.743	0.451	0.537	0.580	0.578	0.123	0.061	21.211
36		0.100*	0.100*	0.100*	0.100*	0.100	-	-	-
48		0.100*	-	-	-	0.025	0.050	0.025	200.000

^a LTG concentration mean values ($n = 4$). * Concentration below the limit of quantification (BLOQ) - these values were assumed as $0.5 \times \text{LLOQ} = 0.100 \mu\text{g/g}$. SD, standard deviation; SEM, standard error of the mean; CV, coefficient of variation.

Table E1.3 LTG concentrations in liver ($\mu\text{g/g}$) at predefined time points following both intranasal (IN) and intravenous (IV) administrations (4 mg/kg) to mice.

Route	Post-dosing Time (h)	Mouse				Mean ^a	SD	SEM	% CV
		0	I	II	III				
IN	0.083	0.937	0.400*	0.400*	0.400*	0.534	0.268	0.134	50.238
	0.167	1.152	1.501	1.449	1.220	1.330	0.170	0.085	12.793
	0.25	1.674	1.465	1.645	1.127	1.477	0.252	0.126	17.028
	0.5	1.730	2.143	1.771	1.788	1.858	0.192	0.096	10.313
	0.75	1.692	2.042	2.066	2.101	1.975	0.191	0.095	9.647
	1	1.935	1.635	1.698	2.140	1.852	0.231	0.116	12.490
	2	1.657	1.779	1.563	2.000	1.750	0.189	0.094	10.789
	4	1.885	1.581	1.224	1.887	1.644	0.315	0.158	19.159
	8	2.030	1.902	2.485	2.163	2.145	0.250	0.125	11.675
	12	2.004	2.180	1.558	1.612	1.839	0.302	0.151	16.428
	24	1.208	1.208	1.346	0.983	1.186	0.150	0.075	12.664
	36	0.400*	0.400*	0.400*	0.400*	0.400	-	-	-
	48	0.400*	0.400*	0.400*	0.400*	0.400	-	-	-
	IV	0.083	2.636	2.570	2.749	3.867	2.956	0.612	0.306
0.167		3.689	2.682	3.056	3.120	3.137	0.416	0.208	13.262
0.25		2.511	2.690	2.875	2.517	2.648	0.173	0.086	6.524
0.5		2.764	2.963	2.803	2.950	2.870	0.101	0.051	3.529
0.75		2.822	3.271	2.746	2.440	2.820	0.343	0.171	12.160
1		2.465	2.088	2.834	2.463	2.463	0.305	0.152	12.368
2		2.072	1.878	2.006	2.327	2.071	0.189	0.094	9.119
4		2.493	2.142	2.534	2.200	2.342	0.200	0.100	8.538
8		1.785	1.772	1.698	1.579	1.709	0.094	0.048	5.523
12		1.723	1.590	1.672	1.886	1.718	0.125	0.062	7.258
24		1.215	0.871	1.063	1.178	1.082	0.155	0.077	14.306
36		0.400*	0.400*	0.400*	0.400*	0.400	-	-	-
48		0.400*	0.400*	0.400*	0.400*	0.400	-	-	-

^a LTG concentration mean values ($n = 4$). * Concentration below the limit of quantification (BLOQ) - these values were assumed as $0.5 \times \text{LLOQ} = 0.400 \mu\text{g/g}$. SD, standard deviation; SEM, standard error of the mean; CV, coefficient of variation.

E2. Brain Biodistribution of LTG after IN and IV Administration to Mice

Table E2.1 LTG concentrations in plasma ($\mu\text{g}/\text{mL}$), remaining portion of the brain ($\mu\text{g}/\text{g}$), frontal cortex ($\mu\text{g}/\text{g}$) and olfactory bulb ($\mu\text{g}/\text{g}$) at predefined time points following IN administration (4 mg/kg) to mice.

Matrix	Post-dosing Time (h)	Mouse				Mean ^a	SD	SEM	% CV
		0	I	II	III				
Plasma	0.083	0.757	9.517	2.274	1.109	3.414	4.120	2.060	120.672
	0.167	6.872	2.229	4.650	8.136	5.472	2.598	1.299	47.477
	0.25	2.694	1.933	1.912	2.233	2.193	0.365	0.182	16.633
	0.5	3.421	2.240	2.431	2.057	2.537	0.609	0.304	23.992
	1	3.504	3.085	2.056	2.558	2.801	0.630	0.315	22.479
	2	2.136	1.708	1.697	2.636	2.044	0.445	0.222	21.752
	4	1.277	1.520	1.291	1.827	1.479	0.258	0.129	17.433
Remaining Brain Portion	0.083	0.100*	0.100*	0.100*	0.100*	0.100	-	-	-
	0.167	0.369	0.342	0.313	0.377	0.350	0.029	0.014	8.209
	0.25	0.698	0.366	0.648	0.593	0.576	0.147	0.073	25.436
	0.5	0.776	0.917	1.006	0.737	0.859	0.125	0.062	14.524
	1	1.242	1.134	1.147	1.185	1.177	0.048	0.024	4.093
	2	1.316	1.172	0.974	1.830	1.323	0.366	0.183	27.664
	4	1.031	1.120	0.934	1.085	1.042	0.081	0.040	7.748
Frontal Cortex	0.083	0.327	0.148	0.346	0.240	0.265	0.091	0.046	34.328
	0.167	0.363	0.371 [#]	0.353	0.324	0.353	0.021	0.010	5.814
	0.25	0.545	0.398	0.643	0.490	0.519	0.102	0.051	19.726
	0.5	0.828	0.712	0.672	0.610	0.705	0.092	0.046	13.018
	1	1.129	0.945	0.772	0.966	0.953	0.146	0.073	15.341
	2	1.158	0.781	0.922	1.238	1.025	0.211	0.105	20.546
	4	1.037	0.836	0.820	0.996	0.922	0.110	0.055	11.950
Olfactory Bulb	0.083	1.836	8.140	8.594	8.244	6.704	3.251	1.625	48.495
	0.167	5.704	11.896	2.232	7.616	6.862	4.029	2.014	58.707
	0.25	1.884	1.779	1.766	1.502	1.733	0.163	0.081	9.381
	0.5	2.169	8.218	3.445	3.165	4.249	2.702	1.351	63.592
	1	11.714	5.057	4.768	2.704	6.061	3.912	1.956	64.546
	2	2.265	2.538	2.278	2.758	2.460	0.235	0.118	9.557
	4	1.888	1.831	1.655	1.078 [#]	1.613	0.370	0.185	22.964

^a LTG concentration mean values ($n = 4$). * Concentration below the limit of quantification (BLOQ) - these values were assumed as $0.5 \times \text{LLOQ} = 0.100 \mu\text{g}/\text{g}$. [#] Concentration below the limit of quantification (BLOQ) - these values were assumed as $0.5 \times \text{LLOQ} = 0.0125 \mu\text{g}$. SD, standard deviation; SEM, standard error of the mean; CV, coefficient of variation.

Table E2.2 LTG concentrations in plasma ($\mu\text{g/mL}$), remaining portion of the brain ($\mu\text{g/g}$), frontal cortex ($\mu\text{g/g}$) and olfactory bulb ($\mu\text{g/g}$) at predefined time points following IV administration (4 mg/kg) to mice.

Matrix	Post-dosing Time (h)	Mouse				Mean ^a	SD	SEM	% CV
		0	I	II	III				
Plasma	0.083	2.604	3.038	2.684	3.092	2.854	0.246	0.123	8.623
	0.167	3.162	2.697	2.442	2.555	2.714	0.316	0.158	11.653
	0.25	2.344	2.301	2.442	2.591	2.419	0.129	0.064	5.313
	0.5	2.227	2.271	2.309	2.438	2.311	0.091	0.046	3.949
	1	1.907	1.844	1.941	1.890	1.896	0.041	0.020	2.147
	2	1.824	1.768	1.993	1.808	1.848	0.099	0.050	5.383
	4	2.037	1.844	2.207	2.172	2.065	0.164	0.082	7.962
Remaining Brain Portion	0.083	0.907	0.976	0.990	1.104	0.994	0.082	0.041	8.203
	0.167	1.261	1.073	1.282	1.355	1.243	0.120	0.060	9.653
	0.25	1.297	1.173	1.186	1.359	1.254	0.089	0.045	7.131
	0.5	1.154	1.218	1.393	1.195	1.240	0.105	0.053	8.501
	1	1.163	1.193	1.234	1.376	1.242	0.094	0.047	7.584
	2	1.266	1.174	1.392	0.987	1.205	0.171	0.085	14.166
	4	1.261	1.132	1.243	1.189	1.206	0.058	0.029	4.815
Frontal Cortex	0.083	0.901	0.778	0.747	0.824	0.813	0.067	0.034	8.248
	0.167	1.024	0.933	1.128	0.720	0.951	0.173	0.087	18.202
	0.25	0.883	0.848	1.029	1.079	0.960	0.112	0.056	11.650
	0.5	0.889	1.155	1.116	0.906	1.017	0.138	0.069	13.620
	1	0.962	1.087	0.923	0.888	0.965	0.086	0.043	8.960
	2	0.840	0.754	0.845	0.811	0.812	0.042	0.021	5.170
	4	0.797	0.781	0.893	0.835	0.826	0.050	0.025	6.021
Olfactory Bulb	0.083	1.907	2.359	2.085	1.931	2.071	0.208	0.104	10.043
	0.167	2.031	2.371	2.192	2.381	2.244	0.166	0.083	7.409
	0.25	2.396	2.293	2.425	2.521	2.409	0.094	0.047	3.904
	0.5	2.380	2.573	2.448	2.108	2.377	0.196	0.098	8.262
	1	2.261	2.371	2.302	1.832	2.192	0.244	0.122	11.126
	2	1.777	1.724	2.195	2.085	1.945	0.230	0.115	11.835
	4	1.862	1.601	1.808	1.847	1.779	0.121	0.061	6.816

^a LTG concentration mean values ($n = 4$). SD, standard deviation; SEM, standard error of the mean; CV, coefficient of variation.



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