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Development and validation of an analytical method for quantification of psychotropic drugs in biological samples by LC-MS/MS

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RESUMO

De acordo com a organização mundial de saúde, muitas pessoas em todo mundo sofrem de doenças mentais e psicológicas, incluindo depressão e esquizofrenia. O tratamento destas doenças muitas vezes é auxiliado pelo uso de antidepressivos e antipsicóticos. Em 2009, houve um aumento de 52% no uso deste tipo de fármacos psicotrópicos. Devido ao aumento do seu uso e o seu envolvimento em situações de intoxicações e suicídio, a sua detecção em amostras biológicas torna-se cada vez mais uma necessidade. Assim o desenvolvimento de uma metodologia analítica para a identificação e quantificação de fármacos psicotrópicos (citalopram, clozapina e haloperidol) em plasma e pêlo foi o principal objectivo deste trabalho.

As amostras de plasma foram processadas com metanol por precipitação de proteínas e as amostras de pêlo foram incubadas com metanol a 45 °C, seguindo-se um passo de limpeza com extração fase sólida. Todas as amostras foram analisadas por cromatografia líquida acoplada a espectrometria de massa sequencial (LC-MS/MS) em modo MRM[1] com um tempo analitico de 9 minutos.

Para garantir a fiabilidade dos resultados analíticos foram definidos parâmetros de validação para este método: selectividade, limites de detecção e quantificação, linearidade, arrastamento[2], precisão intermédia, repetibilidade, exactidão, recuperação e efeito matriz.

O método provou ser selectivo para todos os compostos, com um limite de quantificação de 0,012; 0,014 e 0,015 pmol/µL para o citalopram, clozapine e haloperidol, respectivamente.

Também a linearidade do método foi demonstrada para os intervalos: 0,05 a 5 pmol/µl para o haloperidol; 0,05 a 3 pmol/µl para o citalopram e 0,05 a 2 pmol/µl para a clozapina, com coeficientes determinação (R^2) maiores do que 0,998 para todos os compostos. Os limites de quantificação foram: 0,037; 0,044 e 0,045 pmol/µL para o citalopram, clozapina e haloperidol, respectivamente.

A precisão intermédia do método apresentou alguns valores altos, 13,7-31,3 %. Contudo na exactidão e repetibilidade, os resultados obtidos encontram-se dentro dos critérios estabelecidos.

[1] Do inglês, Multiple Reaction Monitoring
[2] Do inglês, carry-over

Na recuperação do método, a diferentes níveis de concentração, os valores obtidos para o plasma foram de 68,9 a 115,5% e para o pêlo de 8,9 a 45,5%.

Em relação ao efeito de matriz foram obtidos valores negativos para o plasma, indicando supressão iónica. E para o pêlo foram obtidos valores positivos, indicando um aumento do sinal da substância analisada.

O método desenvolvido foi aplicado em amostras de plasma e pêlo que foram recolhidas de ratinhos (5 réplicas independentes por dia) que foram submetidos a um tratamento com fármacos psicotrópicos (citalopram, clozapina e haloperidol) por diferentes períodos de tempo: 1, 2, 4, 8, 15 e 30 dias. A análise da evolução temporal da quantificação dos três compostos no pêlo revela um aumento na concentração ao longo dos dias com um aumento acentuado no dia 15. A evolução temporal da quantificação do citalopram, clozapina e haloperidol para o plasma foi diferente para cada composto, com um pico máximo no dia 8, dias 1 e 2 e dias 4 e 8 para o citalopram, clozapina e haloperidol, respectivamente.

Palavras-chave: fármacos psicotrópicos; plasma; pêlo; LC-MS/MS; quantificação; validação.

ABSTRACT

According to World Health Organization, many people worldwide have mental and psychosocial disabilities, including depression and schizophrenia. The treatment of these diseases is performed with the use of antidepressants and antipsychotics. In 2009 there was a 52% increase in the use of these types of psychotropic drugs. Due to their increased use and their involvement in intoxications and suicide, the ability to reliably detect this class of drugs in biological specimens is a necessity. Thus, the development of an analytical methodology for the identification and quantification of psychotropic drugs (citalopram, clozapine and haloperidol) in biological fluids (plasma and hair) was the main goal of this project.

The plasma samples were processed with methanol, by protein precipitation and the hair samples were incubated overnight with methanol at 45 °C followed by solid phase extraction. All samples were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in MRM mode and with a running time of 9min.

To guarantee reliability in the analytical results validation different parameters were defined for this method: were define for this method: selectivity, limits of detection and quantification, linearity, carry-over, intermediate precision, repeatability, accuracy, recovery and matrix effects.

The method proved to be selective for all compounds with limit of detection of 0.012, 0.014 and 0.015 pmol/µL for citalopram, clozapine and haloperidol, respectively.

Linearity was demonstrated for the intervals: 0.05 to 5 pmol/µl for haloperidol, 0.05 to 3 pmol/µl for citalopram and 0.05 to 2 pmol/µl for clozapine, with determination coefficients (R^2) higher than 0.998 for all compounds. The limits of quantification were: 0.037, 0.044 and 0.045 pmol/µL for citalopram, clozapine and haloperidol, respectively.

The intermediate precision of the method presented some high values (13.7 to 31.3 %). However, for the accuracy and repeatability the results obtained are within the established criteria.

In the recovery of the method, at different concentration levels, the values obtained for plasma were 68.9 to 115.5% and for hair were 8.9 to 45.5%.

In the matrix effects were obtained negatives values for plasma, indicating ion suppression. And for hair were obtained positive values, indicating enhancement of the analyte.

The developed method was applied in plasma and hair samples that were collected from mice (5 independent replicates per day) that were submitted to a treatment with psychotropic drugs (citalopram, clozapine and haloperidol) for different periods of time: 1, 2, 4, 8, 15 and 30 days. The analysis of the time evolution of the quantification for the three compounds in hair reveals an increase in the concentration over the days with a marked increase on day 15. In the time evolution of the quantification in plasma for the citalopram, clozapine and haloperidol, it was different for each compound, with a maximum peak on days 8, 1-2 and 4-8, for citalopram, clozapine and haloperidol, respectively.

Keywords: psychotropic drugs; plasma; hair; LC-MS/MS; quantification; validation.

CONTENTS

ABBREVIATIONS

ACN	Acetonitrile
ANOVA	Analysis of variance
CE	Collision Energy
C_{exp}	Estimated concentration in the sample
CID	Collision Induced Dissociation
CNS	Central Nervous System
CUR	Curtain Gas
CV	Coefficient of Variation
CXP	Collision Cell Exit Potential
DC	Direct Current
DP	Declustering Potential
EMA	European Medicines Agency
EP	Entrance Potential
ESI	Electrospray Ionization
eV	Electronvolt
FA	Formic Acid
F_{crit}	tabulated F value
F_{cal}	calculated F value
FDA	Food and Drug Administration
FGAs	First-generation antipsychotics
HPLC	High-performance liquid chromatography
IS	Internal standard
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
LC	Liquid Chromatography
LC-MS	Liquid Chromatography - Mass Spectrometry
LC-MS/MS	Liquid Chromatography - Tandem Mass Spectrometry
LLE	Liquid–liquid extraction
LOD	Limit Of Detection
LOQ	Limit Of Quantification
m/z	Mass-to-Charge ratio

MAOIs	Monoamine oxidase inhibitors
MeOH	Methanol
ME	Matrix Effects
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
PD	Psychotropic drugs
PP	Protein precipitation
Q1	First Quadrupole
q2	Second Quadrupole (Collision Cell)
Q3	Third Quadrupole
QCs	Quality Controls
R	Coefficient of correlation
R^2	Coefficient of determination
RF	Radiofrequency
ΔRT_{ratio}	Retention time ratio
RT	Retention Time
SGAs	Second-generation antipsychotics
S/N	Signal to noise
SPE	Solid-phase extraction
SSRIs	Selective serotonin reuptake inhibitors
S^2	Variance
$S_{y/x}$	Standard error of the linear regression
S_b	Standard deviation the y-intercept
TCAs	Tricyclic antidepressants
w_i	Weighting Factor
WADA	World Anti-Doping Agency
$\sum \lvert\%\ \mathrm{RE}\rvert$	Sum of the relative errors

1. JUSTIFICATION OF THE THEME

According to World Health Organization, many people worldwide have mental and psychosocial disabilities, including depression and schizophrenia [1]. Mental disorders are on the rise in the European Union. It is estimated that, about 11% of the population has experienced mental disorders [2]. Portugal is the European country with the highest prevalence of mental illness in population, according to the first National Epidemiological Study of Mental Health [3]. Last year, one in five Portuguese suffered from a psychiatric illness (23%) and almost half (43%) already had one of these disorders during life [3].

Depression is already the most prevalent health problem in many European Union-Member States [2] and suicide is very associated with mental illness. Almost one million people die due to suicide every year [1]. Also Schizophrenia is placed among the top causes of disability due to health-related conditions in all countries [1,2].

The treatment of these diseases is performed with the use of antidepressants and antipsychotics. In 2009 there was a 52% increase in the use of psychotropic drugs (PD), especially antidepressants and antipsychotics. An analysis of the total market for medicines, made by the National Epidemiological Study of Mental Health, revealed that drugs belonging to the therapeutic group central nervous system (CNS) therapeutic constitute the second group with more weight on spending, with particularly weight of PD, including antipsychotics and antidepressants [3].

The increased use of antidepressants and antipsychotics may be due to many reasons, such as prevalence of psychotic disorders, increased duration of treatment, drug accessibility, expansion of approved indications for second-generation antipsychotics [3]. The relation between substance abuse and psychiatric disorders is a matter of great concern, both conditions are reportedly linked to increased suicide risk [4].

The pharmacological and toxicological information obtained from the drug distribution in tissues and cells is important for understanding and predicting both drug reaction and toxicity [5]. Analysis of these drugs could be also necessary in forensic cases such as driving under the influence of drugs, cases of violent crimes, cases of drug-facilitated sexual assault and cases of unknown cause of death [6].

Determining the presence of various drugs in samples is an important facet of forensic science [7]. Liquid Chromatography - Mass Spectrometry (LC–MS) has established itself as the clear leader in the quantification of psychotropic drugs in biological samples, such as plasma and hair [5]. The pharmacologic effects of most drugs have a direct correlation with their concentrations in plasma, fact that serves as a basis for therapeutic drug monitoring [8,9]. Therefore the plasma is preferred for quantitative analysis when interpretation of concentrations and effects are required [8]. However, hair has become an important matrix for drug analysis, complementing other specimens like blood [10], since it provides evidence of longer term exposure of drugs and can provide important information as to the time course of drug use [8,11]. Also, it can be used in cases of extreme putrefaction of the body and when there is no longer any matrix available [11].

Whereby stated above, intervention is necessary in terms of therapeutic drug monitoring. Only this way unnecessary consumption associated with therapy can be reduced. Moreover, detection of these drugs in these biological samples is necessary to establish their use and possible contribution to the cause of death. So its detection may be relevant in different situations, presenting indisputable forensic interest.

2. INTRODUCTION

2.1. PSYCHOTROPIC DRUG

Many prescription drugs are widely available today, more developed and marketed every year. Each has the potential for adverse effects and many of them may cause death by overdose [12]. A drug must act on a site of action that is physiologically relevant to the effect. The activation or inhibition of that specific site is termed the drug's mechanism of action. A given drug may affect one or more sites over its clinically relevant dosing range and may produce multiple and different clinical effects [13].

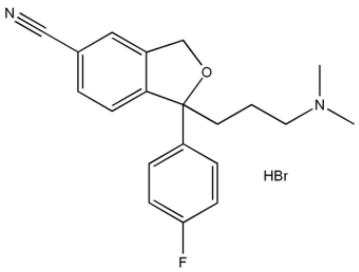
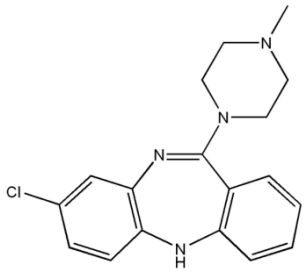
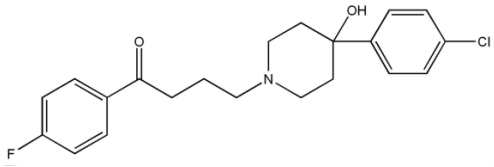
The PD are compounds that affect the functioning of the mind through pharmacological action on the CNS, by other words they have effects on psychological function [7,12]. This category of drugs is widespread in today's society and include both prescribed psychiatric medications and illegal narcotics [7]. It can be divided in four main psychotropic drug categories: antidepressants, antipsychotics, anxiolytics, antiepileptics [7,14].

Due to their abuse potential and their involvement in intoxications and suicides [15], the PD have rapidly gained importance in both clinical and forensic setting [16,17], which makes the ability to reliably detect this class in human biological specimens a necessity [16]. In a clinical environment, the analysis of PD in blood (and/or plasma) is necessary in order to monitor patient compliance and to maintain drug concentrations within the recommended therapeutic range of the respective drug [16]. On the other hand, in a forensic setting, the detection of PD is crucial in determining whether these drugs played a role in the cause of death [16].

The pharmacological treatment of schizophrenia is often performed with the simultaneous use of two or more psychotropic agents to achieve the desired control of psychotic symptoms [5,17]. They are also prescribed to use in the treatment of depression [17].

Most PD are similar in chemical properties such as high lipophilicity, relative molecular weight between 200 and 500 and basicity [18]. In the following pages the main physicochemical characteristics of the compounds that will be studied in this project will be presented (Table 2.1) along with some aspects associated to the mechanism of action, adverse effects and pharmacokinetics properties.

Table 2.1 - Physico-chemical characteristics of psychotropic drugs.

	Citalopram	Clozapine	Haloperidol
Chemical structure			
Chemical name	1-[3-(Dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydro-5-isso-benzofuran carbonitrile hydrobromide	8-Chloro-11-(4-methyl-1-piperazinyl)-5H-dibenzeno[b,e][1,4]diazepine	4-[4-(4-Chlorophenyl)-4-hydroxy-1-piperidinyl]-1-(4-fluorophenyl)-1-butanone
CAS	59729-32-7	5786-21-0	52-86-8
Molecular formula	$C_{20}H_{21}FN_2O$	$C_{18}H_{19}ClN_4$	$C_{21}H_{23}ClFNO_2$
Molecular Weight (g/mol)	405.30	326.82	375.86
Physical Appearance	White Solid	Yellow Crystalline Powder	White Crystalline Powder

2.1.1. ANTIDEPRESSANTS

The antidepressants are currently among the most frequently prescribed therapeutic agents in medicine [12-14], mainly because of their efficacy and good profile of side effects [14]. This type of drug is a therapeutic indicator for the treatment of depression, anxiety disorders, including obsessive-compulsive disorder, bulimia nervosa and psychosomatic disorders [14].

Despite advances in research there is no full explanation of the proper functioning of antidepressants [19]. But it is known that these drugs act on depressive illness through the action on various neurotransmitter systems: serotonine, noradrenaline and dopamine. They produce an increase in the concentration of neurotransmitters in the synaptic gap by inhibiting the metabolism, blocking reuptake of neuronal activity or in the action on presynaptic receptors [19].

The newer antidepressants are much less toxic, safer and more tolerable than the tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs) [12,20], but may still be involved in overdose deaths, particularly when combined with other drugs [12].

Antidepressants can be classified according to the chemical structure or pharmacological action. Because the new-generation antidepressants do not share common structures, the pharmacological action is currently more used [19]. In past years, TCAs and MAOIs were the most commonly used antidepressants [12,14], however the selective serotonin reuptake inhibitors (SSRIs) have emerged as a major therapeutic advance in psychopharmacology [13].

2.1.1.1. SELECTIVE SEROTONIN REUPTAKE INHIBITORS

The SSRIs were all developed to have a similar mechanism of action: block the serotonin reuptake. The inhibition of the serotonin reuptake carrier raises the level of this neurotransmitter in the synapse [13,14,21,22], enhancing serotonin neurotransmission, which results in their antidepressant effects [21].

Serotonin binds to serotonin receptors located in the central and peripheral nervous system and affects various functions such as: sleep, pain perception, blood vessel regulation, anxiety, mood, and depression. The SSRIs have lower binding affinities for other neurotransmitter receptors (for example dopaminergic receptors), providing another reason to be considered better tolerated than TCAs and MAOIs [22].

Although they have a common mechanism of action in this class, each SSRI has a slightly different pharmacologic profile that leads to its distinct clinical activity, side effects and drug interactions [19-21].

After oral administration, SSRIs are well-absorbed, suffer less effect of first-pass metabolism and bind strongly to plasma proteins. SSRIs are metabolized primarily by the liver and their metabolites are mainly eliminated in the urine [19,22].

Overall, the most frequently reported side effects are gastrointestinal (nausea, vomiting, abdominal pain, diarrhea), psychiatric (agitation, anxiety, insomnia, mania, nervousness), sleep disturbances, fatigue, loss or weight gain, sexual dysfunction and skin reactions [19,21,22]. In general, SSRIs have milder adverse effects than older antidepressants, and their adverse effects are often dose related [22].

CITALOPRAM

Citalopram (consult Table 2.1) is a selective and potent serotonin reuptake inhibitor that is used for the treatment of depression [23] and offers an efficient alternative treatment of depression to the TCAs [24].

The main metabolite of citalopram, measurable in plasma, is N-desmethylcitalopram, which is also an SSRI [13,25]. However, the pharmacological activity of the metabolite is weaker when compared to the parent drug [13,25].

As for other lipophilic drugs, the absorption of citalopram from the gastrointestinal tract is almost complete [13,24,25]. The first pass effect of citalopram seems to be of minor importance, since it has an absolute bioavailability of about 80%. [13,25]. As only 50% of the dose is excreted in urine, a significant fecal elimination is suggested [25], with the peak plasma concentrations being reached after 2–4 hours [24].

A linear relationship between citalopram dosage and plasma concentration has been reported, but the interindividual variability increases with dose, which might be due to saturation of an elimination pathway [13].

Citalopram, has been associated with low rates of insomnia, anxiety, and other activating side effects. Nausea is the most common early side effect [21] and it was also associated with weight gain [19].

2.1.2. ANTIPSYCHOTICS

Since their introduction, antipsychotic drugs are medications that have been extensively prescribed and are the primary intervention for the stabilization of acute psychotic episodes, including schizophrenia, bipolar disorder, mania and depression [14,26-28]. Some of these drugs are also used for the palliative treatment of some movement disorders [14].

Dopamine has a central role in excitement, motivation, attention, the extrapyramidal motor system and other pathways. Although the exact etiology is uncertain, dopamine dysregulation plays a role in a number of symptoms. In psychosis, its overactivity leads to excessive information throughput, resulting in hallucinations and delusions [29]. Since the antipsychotic block dopamine D2 receptors [30], they help to correct this overactivity and improve the symptoms [29].

Two primary classes referred in the literature are the typical and atypical antipsychotics. The term atypical originated from the idea that these medications reduce the risk of extrapyramidal side effects. Most recently the classification of these medications has been changed based on pharmacology. The terms first-generation antipsychotics (FGAs) and second-generation antipsychotics (SGAs) have replaced the terms “typical” and “atypical” [18].

The different groups of antipsychotic drugs have slightly different pharmacological profiles, however all of them block the dopamine D2 receptor with different degrees [18,31].

2.1.2.1. FIRST-GENERATION ANTIPSYCHOTICS

The first-generation antipsychotics, include the commonly used butyrophenones (such as droperidol and haloperidol) and phenothiazines (such as chlorpromazine, promethazine and thioridazine) [30].

Their clinical efficacy is strongly correlated with their binding affinities for the receptor subtype [32]. These FGAs were categorized based on their affinity for the dopamine D2 receptor as low potency, such as chlorpromazine, or high potency, such as haloperidol [30]. So, the blocking of dopamine is responsible for the therapeutic effect, with reduction of delusions and hallucinations [14,32-35].

The FGAs are well absorbed orally, have great solubility, easily cross the blood-brain barrier and also the placental barrier. They show a great affinity for plasma proteins (85-90%), which involves risk of toxicity when other drugs that also bind to proteins are available in the plasma simultaneously [14]. Due to their lipophilic properties, antipsychotic are stored in the peripheral fat. FGAs drugs are metabolized in the liver, being removed primarily by urine and feces, through bile, but also by the saliva, tears, sweat, and breast milk. The elimination half-life varies between 18-40 hours [14].

HALOPERIDOL

Haloperidol is a butyrophenone (consult Table 2.1)and is the most used drug for the symptomatic management of psychotic disorders [36].

After oral administration, haloperidol has significant first-pass metabolism in the liver, decreasing oral bioavailability. There is wide inter individual variation in plasma concentrations, so no strong correlation between plasma concentration and therapeutic effect has been found. It was reported to have half-lives between 12 and 38 hours after oral administration. Also 92% of this drug is bound to plasma proteins and is widely distributed throughout the body, including breast milk [37].

2.1.2.2. SECOND-GENERATION ANTIPSYCHOTICS

The SGAs drugs introduced in recent years, besides being dopamine receptor antagonists [27,32], comprise a more heterogeneous pharmacological profile involving actions on multiple neurotransmitter systems [32,33]. The difference between these and the FGA medications is the specificity of the dopamine antagonism at recommended dosages and also the serotonin activity [35]. These agents functionally antagonize dopamine (D2) receptors and antagonize serotonin (5-HT2A) receptors [35]. When compared to phenothiazines and butyrophenones, SGAs have a greater binding affinity for the 5-HT2 receptors than for D2 receptors [38].

They are defined clinically as having minimal or no extrapyramidal symptoms at clinically appropriate doses [30]. Moreover, over the past decade, they have become the treatment of choice for schizophrenia in many countries, due to the perception of a more favourable

tolerability profile, thus raising major hopes of superior effects in a number of areas such as compliance, cognitive functioning, movement disorders, and quality of life [27].

In the case of an overdose, these drugs produce a range of manifestations that affect multiple organ systems. The most serious toxicity involves the cardiovascular system and the CNS, with the most common cardiovascular effects being tachycardia and mild hypotension. [30].

CLOZAPINE

Clozapine is a dibenzodiazepine derivative (consult Table 2.1) that was the first of the atypical antipsychotics to be developed [24,30].

Properties of clozapine are due to the combination of a low affinity for the D2 receptors along with strong affinity to serotonergic, adrenergic and cholinergic receptors. This property is present in many SGAs, so these drugs cause fewer movement disorders as side effects [14].

This drug is efficient in treating the delusions, hallucinations, and disorganization of schizophrenic patients. It has also been demonstrated that clozapine can improve the negative symptoms of schizophrenia, such as lack of emotion [30,39]. It has been shown to have other clinical advantages over FGAs drugs, most notably the ability to improve some aspects of the cognitive dysfunction of schizophrenia, such as attention, verbal fluency (semantic memory) and recall [39].

Clozapine is rapidly and completely absorbed with the peak serum concentration occurring 1h30–2h30 after a single dose [38] and its main metabolite is desmethylclozapine [40].

The most common adverse effects include orthostatic hypotension, gastrointestinal symptoms, and sedation. Seizures may be observed in higher therapeutic doses and in overdose [30].

2.2. DRUGS INTOXICATION

In many countries, intoxication with therapeutic substances exceeds deaths from other types of toxic agents, especially in suicidal and accidental intoxications. Overprescribing or the supply of large a quantities of drugs at one time allows excessive stocks of drugs to be easily available to the public [41].

Deliberate and accidental cases of self-poisoning with psychiatric drugs have become a major medical problem because of their widespread use and the severity of their toxic actions. This fact has led to the development of reliable analytical methods for their analysis [17]. The analysis of these drugs could be also necessary in forensic cases such as driving under the influence of drugs, cases of violent crime, cases of drug-facilitated sexual assault and cases of unknown cause of death [6].

Since forensic science is often concerned with determining the basis of death, investigations are frequently concerned with the influence and effects of toxins. Results of the laboratory procedures must be interpreted and are often used as evidence in legal cases [7]. Thus, the detection of drugs in biological samples is very important and useful in ante-mortem and post-mortem toxicology. Ante-mortem forensic toxicology implies an understanding of drug before the death and the interpretation of result [42]. While, post-mortem forensic toxicology involves analyzing body fluids and organs from death cases and interpreting that information [43].

These toxicological studies are now recognized as an integral part of the proper investigation and evaluation of most medical examiner cases [44]. In sudden unexpected and/or unexplained deaths toxicology studies are useful and necessary for the final decision regarding the cause and manner of death [43]. For example, in many cases, the drug or toxic agent is the direct cause of death, such as by overdose, or may explain the actions of the deceased leading to his death [44]. Also, it is important to investigate a crime scene in order to provide to the toxicology laboratory an idea of what substances might be present in the body, guiding the research and adapting the methodology used for their detection[12].

Intoxication can be understood as the set of disorders that derive from the presence of a substance in the body. There are two forms of intoxication according to the early-onset, severity and symptomatology, which is usually related to the absorption speed of compounds [45]. Acute intoxication is due to short-term exposures, with rapid absorption of compound. Concerns a single dose or multiple doses, but for a brief period, which may be at approximately 24 hours

and the clinical manifestations of poisoning are fast [45]. On the other hand, chronic intoxication is due to a repeated exposure to the compound, during a long period of time. The mechanism may occur due to the following causes: first, the compound accumulates in the body as the elimination rate is less than the absorption. Secondly, effects generated by exposures are added [45].

It is important to clarify that there is a difference between drug, poison and toxic substance. A drug is a substance or composition, characterized by having properties, that is used to treat or prevent a disease or to treat symptoms of a disease or injury [45,46]. Poison is a substance that has an inherent ability to produce adverse effects on the body, whether it is an illness, injury, or death [45,46]. While toxic is any chemical agent that after entering the body, change biochemical elements essential to life [45]. However, it is important to keep in mind that any substance can be harmful and produce balance disorders in the biological cell. Only the amount and time over which the substance is administered will allow to determine how harmful it will be [7,45,46].

Thus, the main issue of the toxicity of a compound is its dose [7,45], which makes it necessary to introduce some concepts related to levels of drug ingested. Therapeutic levels are the steady state concentrations that need to be reached for the drug to exert a significant clinical benefit without causing unacceptable side effects [47]. So the use of the word therapeutic implies a concentration at which a useful response is obtained free from any toxicity [48]. And toxic levels are concentrations above which unacceptable (concentration dependent) side or toxic effects might appear. [47]. However, if a concentration is in the fatal range then it is capable of causing death [48].

In the Table 2.2 are the Therapeutic, Toxic and Lethal Concentrations for each drug that is subject of the study in this project. The drugs that are used for the treatment of psychiatric illness can be misused (for suicide attempts, as an example), so it is important that the physician balances the benefit of pharmacotherapy against the risk of drug overdose.

So, it can be said that another common applications of post-mortem toxicology data is its use to define therapeutic, toxic and fatal doses for drugs. This is useful when establishing a possible role of a drug in a case [48].

Table 2.2- Toxic concentrations, Lethal concentrations and recommended therapeutic range for psychotropic drugs [47].

Psychotropic drug	Therapeutic Concentration (mg/L)	Toxic Concentration (mg/L)	Lethal Concentration (mg/L)
Citalopram [1]	0.02-0.2	_	0.5
Clozapine [2]	0.1-0.6	0.8-1.3	3
Haloperidol [2]	0.005- 0.015	0.05-0.1	0.5

[1] Matrice: plasma
[2] Matrice: serum

Control of dosage is the basis of almost all safety assessment in the use of chemicals [49]. There are drugs that are difficult to define safe and toxic concentrations in the post-mortem setting [48]. Because, although dosage and time are the main factors determining whether or not a particular substance will produce a given effect, there are other factors that influence response, including route of exposure, species and individual differences, sex, age, nutrition, and disease [49].

2.2.1. MANNER OF DEATH BY THE USE OF DRUGS

The manner of death is the category that describes the circumstances that led to infliction of the cause of death [7]. Cases related to therapeutic drugs can involve a variety of special considerations [12]. It can be divided in natural or violent, where the first one involves disease or can be due to the effects of a drug, considered also natural deaths [7,12]. The second one is subdivided in accidental, homicidal, or suicidal. In other words involve some form of physical trauma [7].

Accidental intoxications is the most common type of intoxications and can result from several circumstances [45]. It can occur in a casual way despite the right indication, dose and route of administration. In other situations, it results from self-medication, lack of knowledge about side effects of drugs and possible actions resulting from simultaneous administration with other drugs [45]. It can also be caused by mistakes in the administration of the drug, like medication error or dosing error [12,45]. On the other hand, it may result from an automated action, which occurs in chronic treatments. For example, when an individual, by negligence, takes an amount

of the drug prescribed higher than the normal, leading to overdosing [45]. Drug intoxication occurring in children, are mostly accidental, depending on circumstances such as the attractive appearance and accessibility of drug [12,45]. Another example is the recreational use of drugs, where in most cases a death intoxication due to acute toxic effects of the drug is considered as an accident [12].

The voluntary drug intoxication is the most common method of attempted suicide in developed countries [45]. In fact, according to several studies, suicide by drug intoxication is more common in women than in men [45]. To be considered that a death by intoxication is a suicide, it requires evidence which indicates that the individual purposefully ended his/her life [12]. In these cases elevated levels of drugs (that are not typically used for recreational abuse purpose), or the presence of massive amounts of drugs are found within the stomach [12].

Homicidal poisonings are relatively rare, mainly due to the difficulties in their administration without the victim's knowledge [12,45]. However, in cases where the main goal is to diminish the strength of the victim in order to commit the crime itself, like sexual assault on liberty or property crimes, this usage turns to be very common [45].

2.2.1.1. INTOXICATIONS BY PSYCHOTROPIC DRUGS

One of the most commonly used methods of self-injury worldwide is drug overdose [50]. A study was made to compare the Suicidal overdoses of PD between younger and adults in New York City, whose suicide was determined by the Office of the Medical Examiner to be the result of intentional poisoning or overdose from 1990 through 2006. It was demonstrated that victims of suicidal overdose aged 18–59 had significantly higher rates of death contributed by antidepressants, where female overdose victims had significantly higher rates. Here it can be concluded that antidepressants do not always prevent suicide and that some classes of antidepressants can cause or contribute to overdose death [51].

Fatalities have also been reported at therapeutic concentrations of the second-generation antipsychotic drugs, and as they are increasingly prescribed even among adolescents, they may play a significant role in many intoxications even though they are considered relatively safe [15].

According to the Annual Report of the American Association of Poison Control Centers, the antidepressants and antipsychotics are referred as the substances most frequently involved in

human exposures. The AP are the second and the antidepressants are the third Categories with largest numbers of deaths [52].

Also, in Portugal, the national institute of medical emergency (INEM) did a statistical analysis with incoming calls due to intoxications cases from 2011. It was concluded that in Portugal, the antidepressants and antipsychotics drugs are the second and third groups of drugs responsible for intoxication in adults (Table 2.3) [53].

Table 2.3- Intoxication in adults by drug groups in Portugal [53].

Drug group	Number of cases	Observed cases (%)
Anxiolytics	2542	38.3
Antidepressants	1092	16.5
Antipsychotics	763	11.5
NSAIDs[1]	552	8.3
Antiepileptic	528	7.8
Paracetamol	493	7.4
ACE inhibitor[2]	436	6.6
Beta blockers	239	3.6

[1] Non-Steroidal Anti-Inflammatory;
[2] Agents inhibitor - angiotensin-converting-enzyme inhibitor;

Another conclusion that can be drawn from the data, available on the website, is that intoxications are most frequent in females and the age ranges that have more prevalence is 40-49 years.

Finally, the leading cause of death in adults is intentional (corresponding to 43% of the intoxications) and accidental in children (corresponding to 83% of the intoxication), for the reasons which have been previously discussed. And the second cause is accidental in adults and therapeutic error in children (Table 2.4).

Table 2.4- Characterization of poisoning in adult and child [53].

Manner of death	Adult (%)	Child (%)
Accidental	37.2	83.3
Unknown	0.44	0.16
Therapeutic error [1]	16.3	11.5
Intentional	42.7	4.47
Professional	2.22	0.1
Adverse reaction	1.11	0.46

[1] Therapeutic error: refers to error (the dose or time of administration) in taking the prescribed medication

2.3. BIOLOGICAL MATRICES

A drug may be detected in any body fluid or tissue with which it has been in contact. The primary choice of biological specimen for drug analysis depends on several issues: purpose of the sampling, time interval to study, ease of sampling, cost of sample preparation and analysis, drug concentrations in the sample, and drug stability [54].

Specimens available in post-mortem toxicology investigations can be numerous and variable. Generally, the specimens routinely collected at autopsy include fluids such as blood from peripheral sites and heart blood, urine, bile, cerebrospinal fluid, vitreous humor, gastric contents and organ tissues, particularly liver [55]. In addition to these, a variety of biological specimens are analyzed in forensic science [10]. Each biological matrix has advantages and disadvantages (Table 2.5) and may also be selected based on requests, legal aspects and availability in a given case [9,55].

Table 2.5- Some advantages and disadvantages for different biological matrices. Adapted from [54].

	Blood	**Oral fluid**	**Urine**	**Hair**
Maximum drug detection period [1]	1-2 days	1-2 days	2-4 days	3-6 months
Intrusive sampling	yes	No	Yes	No
Adulteration potential	None	Low	High	Medium [3]
Possibility for environmental contamination	No	No	No	Yes
Potential for negative results after drug use	Low	Medium	Low	Medium to high
On-site screening possible	No	Yes	Yes	No
Analytical costs [2]	Medium	Medium	Medium	High

[1] Approximate detection times after intake of a single dose varying from one substance to another;
[2] Including confirmation testing;
[3] Hair colouring and bleaching.

Of course one of the most important points to choose the biological matrix is the knowledge about the stability of analytes in biological material. This is crucial to ensure the reliability of analytical results, since there are gaps between sample collection, transport to the laboratory and the time of analysis [9].

Blood and urine are the most common specimens used for the analysis of drugs of abuse in post-mortem cases [10,11]. But if a probable death by overdose or intoxication is identified, additional questions may arise such as the route of administration, a long-term or an exclusively recent use/exposure to a drug or poison. In these cases, additional and alternative specimens such as hair, nails or skin samples may be needed [55].

2.3.1. PLASMA

Urine is the sample of choice for non-target comprehensive screening and for identification of unknown drugs. However, blood, plasma, or serum sometimes must be used for at least a limited screening especially for target analytes within multi-analyte procedures [56].

When plasma samples are used, care must be taken, for example, with anticoagulants because it can cause interference with some drugs and assay systems. However, in the absence of such effects, there are no clinically significant differences between serum and plasma, and either may be used [57]. Pharmacokinetic factors such as dose, volumes of distribution, and elimination half-life are also important when laboratory aspects of clinical toxicology are being considered. For example, if the volume of distribution is lower, the amount of drug available in the peripheral plasma for testing purposes is larger [57].

Plasma is traditionally used in clinical settings because blood affords advanced handling in the laboratory procedures [55]. In vivo, the physiological effects of most drugs are directly correlated with their concentrations in blood, plasma and serum, a fact that serves as the basis for therapeutic drug monitoring [8,9].

Drug concentrations provided in literature are usually determined from these fluids. And this is important since analytical results obtained from post-mortem blood are compared valuably with levels previously reported in therapeutic and toxic conditions [55]. Therefore, these matrices are preferred for quantitative analysis when interpretation of concentrations and effects are required [8,9].

Multi-analyte procedures for screening and quantification of drugs in blood, plasma, or serum are relevant tools in these fields because they allow the analysis of several important compounds with a single sample extract injection, thus saving time and resources [56].

2.3.2. HAIR

In the last decade, alternative or unconventional matrices have becoming more important in the field of toxicology, mainly because the advantages when compared with 'conventional' samples used in laboratorial routine analysis [58]. Urine and saliva sampling is non-invasive and has been widely used because of ease of collection, however the main disadvantage of these specimens is the short window of detection [10].

In recent years, remarkable advances in sensitive analytical techniques have enabled the analysis of drugs in unconventional samples such as hair [59]. This alternative matrix offers more several advantages like non-invasive collection, and good stability during storage at room temperature and transport conditions [54,58,60]. However, the most important advantage of hair analysis compared with other human matrices is the much larger detection window [58,61] (weeks to months, depending on the length of the hair shaft, against 2–4 days for most matrices), which allows the retrospective detection of chronic exposure to drugs up to years back [58,60,61].

In fact, hair grows at approximately 1 cm per month, and it is possible to associate the drug distribution pattern in the analyzed segments with a period in the past [58,62]. Segments of single hair may be downsized to 1 mm length if hair concentration and detection limits provide so [62].

The precise mechanisms involved in the incorporation of drugs into hair remain unclear requiring further investigation [63]. However, there are various incorporation models that are suggested for the incorporation of drugs (Figure 2.1).

Beside the physiological characteristics of the individual, dose and time of intake, hair melanine, lipophilicity, chemical structure of drug content plays an important role for the interindividual variation of drug incorporation into the hair matrix [55,63-65].

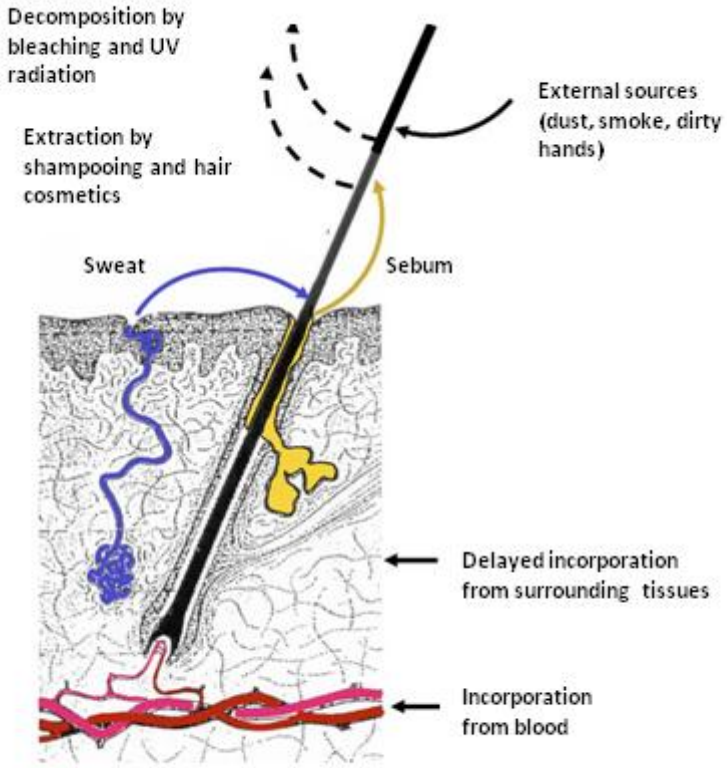


Figure 2.1- Incorporation and elimination mechanisms of drugs in hair. The main mechanism that is typically assumed is the one where the drug enters in the hair by passive diffusion from blood capillaries. Besides incorporation from blood, drugs can be incorporated, with some time delay, from deep skin compartments during hair shaft formation. The other mechanism that is suggested and widely accepted is deposition by diffusion from sweat or sebum secretions into the completed hair shaft. The substances can also be deposited from the external environment, like pollution, smoke and dirty hands. However some losses may occur due to the use of hair cosmetics and ultraviolet radiation. Adapted from [63].

However, the hair analysis has some disadvantages, like low concentrations of some compounds and metabolites, and limited amount of sample supplied for testing [58]. And the most important disadvantage is the fact that substances can be deposited from the external contamination (Figure 2.1) [58,63]. So, if adequate measures are not taken, the risk of reporting false positive results increases, which is unacceptable, especially when there are legal implications of drug consumption. Therefore, to minimize this effect it is strongly recommended that hair analysis procedures include a washing step [58].

Hair analysis is becoming a routine practice in forensic toxicology laboratories [58,61]. Until now, most applications of hair testing have focused on forensic considerations [66], mainly for the detection of illicit drugs owing back to long-term drug consumption [61,64]. However, another exciting application of hair analysis is in the clinical cases, because hair analysis

complements blood and urine analyses by providing long-term information on an individual's drug use [61,64-66].

In addition to the above applications, the hair drug analysis is employed in a wide range of situations, such as workplace drug testing, driving ability probation, doping control, chronic drug abuse intoxication, clarify cause of death, or discover drug use (violence, rape, prison cases, and social cases)[60,67]. In these cases, rapid and secure screening methods are therefore very important [67]. A drug screening in hair can also contribute to the identification of unknown corpses. In such cases, a systematic toxicological analysis, that means the general search for toxic substances in the hair sample, should be performed [61].

3. ANALYTICAL METHODOLOGIES FOR PSYCHOTROPIC DRUG ANALYSIS

3.1. ANALYTICAL METHODOLOGIES FOR SAMPLE PREPARATION

The presence of interfering compounds in complex matrices is a challenge to accomplish reliable results [42,68]. Therefore, the main objective of sample preparation is to convert a real biological matrix into a form suitable for analysis by the desired analytical technique [69].

The pre-treatment of samples to separate drugs and metabolites from the matrices prior the analysis aims to reduce matrix effects via removal of potential interferences [70,71]. This is a fundamental part of the quantitative bioanalysis and is usually the most critical and time-consuming step when using chromatography or affinity techniques for drug analysis in biological matrices [42,68,72].

The first aim of sample preparation is the removal of potential interferences such as proteins and lipids [69,71,72]. In chromatography, proteins increase the baseline, cause noise, and may even ruin chromatographic columns [69,71,72]. The presence of interferences can also influence the ionization efficiency in the mass spectrometers, may block the ion source and contribute to ion suppression for mass spectrometry (MS) assays [69,71-73].

The second aim of sample preparation is to increase the concentration of analytes to achieve adequate signal intensities. The simplest form of enrichment is drying the sample and reconstituting it in a smaller solvent volume [69]. The enrichment is usually performed by extraction methods, protein precipitation (PP), liquid–liquid extraction (LLE), and solid-phase extraction (SPE) [68-70]. Each of these methods has advantages as well as disadvantages [68]. The optimum choice of one is specific for the respective analyte but also for the individual MS/MS system [71].

3.1.1. SAMPLE PREPARATION OF PLASMA

3.1.1.1. PROTEIN PRECIPITATION

A simple work-up method for blood, plasma or serum samples is PP [80]. Probably this is the simplest way to separate proteins from small molecules in biological fluids [69] and it is clearly an attractive sample preparation technique because it's fast and has a good recovery of polar analytes compared with some SPE and LLE procedures [70]. This technique may be used for 'cleaner' matrices such as serum or plasma [16]. However, the precipitated proteins may bind various small molecules and remove them from the solution. This may influence quantification, which has to be taken into account [69].

The theoretical basis of PP is the interaction between the reagent and the protein (directly or indirectly) [73]. Either an organic solvent (typically acetonitrile, methanol or ethanol) or an acid (typically trichloroacetic acid or perchloric acid) is added to a sample to denature the proteins [68,69,71]. However, precipitation with miscible organic solvents is the most commonly used for plasma sample preparation method because of its low cost and minimal method development requirements [74].

The procedure generally begins with the addition of an internal standard (IS). Then a volume of protein precipitation reagent equal to three or four times the sample volume is added to each biological sample [68,73]. The mixture is agitated to increase the aggregation speed of the proteins. The supernatant, which contains the analyte, is then separated from the protein aggregate after centrifugation [69,71,73]. Protein precipitation may be performed alone or in conjunction with another extraction technique [68]. However, this technique does not allow concentration of the analytes; instead, typically a dilution of at least 1:2 is obtained [71].

This method is applicable to a range of LC-MS methods relevant to toxicology [70,73] because it provides sufficient clean-up for most LC–MS analyses [74].

3.1.2. SAMPLE PREPARATION OF HAIR

There is always the possibility that a drug in hair does not originate from consumption but has been incorporated from external sources [66]. And of course, contaminants of hair would be a problem if they were drugs of abuse and if they interfered with the analysis and interpretation of the test results [75].

Thus basically, cleaning the hair sample of external contamination is necessary for two reasons. First, residues of hair products (wax, shampoo, hair sprays) as well as sweat, sebum and dust typically present on hair lead to increased analytical noise/background. Second, drugs could adhere from the environment of the individual and potentially contribute to incorrect test results [63]. To minimize this effect it is strongly recommended that hair analysis procedures include a washing step [54,58]. This can, however, affect the extraction efficiencies of incorporated drugs and must be considered when interpreting quantitative results [54]. Although, basic and lipophilic drugs are well incorporated into hair and less susceptible to wash-out effects [62].

One of the prerequisites of solvents used for hair decontamination is that this should remove external impurities as completely as possible, but not extract drugs from the hair matrix [63].There is no general consensus with respect to the hair washing procedure. For example, one washing sequence for post-mortem hair samples is composed of 0.1% sodium dodecylsulfate in water, distilled water and acetone. Another procedure that is very used includes one or two washes with dichloromethane [63,75]. Non-protic solvents such as dichloromethane or acetone are advantageous because they do not swell the hair thereby extracting materials from the hair [63].

Another step prior to extraction is the cut of the hair typically between 1–3 mm lengths. Alternatively, hair may be processed by grinding. However, this latter approach generally results in loss of sample material and does not improve the extraction process [63].

There are currently no direct methods for the detection of drugs in the hair matrix. Extractions with methanol, extraction by aqueous acids or buffer solutions, digestion of the hair with aqueous Sodium hydroxide are examples of extraction procedures that are more used [54,63,75].

The extraction yield depends on the drug's structure, the state of the hair matrix, polarity of the solvent, duration and manner of extraction [66]. More precisely, in order to make the appropriate choice, the chemical structure of the drug and its sensitivity to agents used for sample preparation must be considered [63].

Clean-up methods used for this purpose are similar to those used in drug isolation from plasma or urine. Although procedures for liquid–liquid as well as solid phase extraction have been described, the latter method is normally used [63]. In this project the clean up of the hair samples was performed using OMIX Tip C18. This pipette tip contains a small bed of

functionalized monolithic sorbent (C18) and it can work as a miniaturized solid phase extraction bed for hydrophobic compounds clean up prior to MS.

3.2. LIQUID CHROMATOGRAPHY COUPLED TO MASS SPECTROMETRY (LC-MS)

Medical examiners determine the cause and manner of death, frequently requiring analysis of fluids and tissues for the presence of drugs and metabolites. In current practice, most medical examiner laboratories screen fluids using immunoassays. Despite being sensitive and capable of high specimen throughput, the matrix effects and substances similar in structure to analytes of interest often cause false positive results [76]. For this reason it is important that specimens with positive immunoassay results are confirmed by chromatographic methods [7,76].

Over the last twenty years there has been a growing interest in the development of methodologies for qualitative and quantitative analysis of several drugs in post-mortem matrices [42]. Liquid chromatography coupled to mass spectrometry is increasingly being used in clinical and forensic toxicology for the identification and quantification of a wide range of compounds in biological samples [60,67] and is often the method of choice because the sensitivity, selectivity and the relatively high throughput that can be achieved [77]. With this technique, the determination of multiple groups of compounds can be performed in a single method [60]. Usually it is used for compounds that are not volatile and are not suitable for gas chromatography [78]. Some of the advantages of this technique include easier sample preparation, derivatization procedures are avoided, and short analysis time [60,67].

Because, the chromatography separation is not sufficient to allow unequivocal identification, further information is usually required from an auxiliary technique [79]. This can be achieve with the combination of high performance liquid chromatography (HPLC) with mass spectrometry that allows more definitive identification and the quantitative determination of compounds, that have similar retention characteristics but a different mass spectra [79]. Mass spectrometer provides not only structural information from the molecule under investigation but it may also provide the molecular weight of the analyte [79].

Tandem mass spectrometry (MS/MS) in combination with Liquid chromatography (LC) now dominates the analytical field, providing a particularly convenient tool in the analysis of PD [16].

Recent advances in this technology enabled the detection and quantification of these drugs present in biological matrices in exceptionally low concentrations [16,72]. The basic information of the procedures normally used for quantification of PD drugs by LC-MS in plasma and hair is summarized in Table 3.1 and Table 3.2, respectively.

3.2.1. LIQUID CHROMATOGRAPHY

In liquid chromatography, the substances are separated based on differential solubilities in the mobile liquid and stationary solid phases, with identification based on retention times within a column [76]. The time required for an analyte to elute from a chromatographic column with a particular mobile phase is termed its retention time (RT) [79]. This interaction may be due to different physical properties and one that is considered is the relative polarities of the species involved [79-81].

High-performance liquid chromatography (HPLC) is the term used to describe LC in which the liquid mobile phase is mechanically pumped through a column that contains the stationary phase [82]. Thus, in an HPLC system, a liquid sample (or a solid sample dissolved in a suitable solvent) is introduced into a column which contains two immiscible phases: the stationary phase (contained in a column) and the liquid mobile phase (which flows through the column) [80,81,83].

The interaction of the analyte with the stationary phase may be adsorption, partition, size exclusion, affinity, and ion exchange [82]. In adsorption chromatography the solute molecules are in contact with both the stationary phase and the mobile phase, simultaneously [79-81,84]. When the analytes interact with the stationary phase, the polar solutes will be retained longest by polar stationary phases, and nonpolar solutes will be retained best by nonpolar stationary phases [82,84].

Table 3.1- Bioanalytical methods based on liquid chromatography for the analysis of plasma.

Compound	Sample	Pretreatment/Extraction	Clean-Up	Analysis	Detection mode	LOD/LOQ	Reference
Citalopram	RP	-Add 2 mL of ter-butyl methyl ether, vortex for 2 min, followed by centrifugation for 10 min at 3200 rpm on Multifuge 3SR (Heraus, Germany).	LLE	LC-MS/MS	ESI, positive mode, MRM	LLOQ: 0.5 ng/mL.	[85]
Citalopram	HP	-Add 4ml of a mixture of hexane–heptane–isopropanol (88:10:2, v/v/v), mix for 10 min and centrifuge at 800 × g for 10 min .	–	LC-MS	ESI, positive mode, SIM	LLOQ: 0.50 ng/mL	[86]
Haloperidol	HP	- Add 3 ml of a mixture of hexane and dichloromethane (70:30, v/v), vortex for 3 min.	LLE	LC-MS	ESI, positive mode, SIM	LLOQ: 70.0 pg/ml	[87]
Haloperidol, Citalopram and Clozapine	HP	- Add 1ml cold acetonitrile and centrifuge for 10 min at 14000 rpm/min	PP	LC-MS	ESI, positive mode, SIM	LOD: HA - 0.5 ng/mL CI - 10 ng/mL CL - 0.5 ng/mL	[88]
Clozapine	HP	- Add 5 mL ethyl acetate and vortex-mixed for 3 min.	LLE	LC-MS/MS	ESI, positive mode, MRM	LLOQ: 0.1 ng/mL	[89]
Citalopram	HP	- Add 600µL methanol, vortex-mixed for 5min and centrifuge at 15.493×g for 10 min.	PP	LC-MS/MS	ESI, positive mode, MRM	LOQ: 0.25–0.5 ng/mL	[90]

Abbreviations: RP: rat plasma, HP: human plasma, PP: protein precipitation, LLE: liquid-liquid extraction, LC: liquid chromatography, MS/MS: tandem mass spectrometry ESI: electrospray ionization, MRM: multiple reaction monitoring, LLOQ: lower limit of quantification, LOQ: limit of quantification, LOD: limit of detection, HA: Haloperidol, CL: Clozapine, CI: Citalopram.

Table 3.2- Bioanalytical methods based on liquid chromatography for the analysis of psychotropic drugs in hair.

Compound	Decontamination	Pretreatment/Extraction	Analysis	Detection mode	LOD	Reference
Citalopram	Three solvents	-Ultrasonication with 4 ml of metanol (2h). -SPE: Chromabond and automated SPE device	LC-MS/MS	ESI, MRM	< 0.1 mg/L	[64]
Citalopram	2 mL dichloromethane (three times, 2 min each).	-Incubated with 2 mL acetonitrile (12 h at 50 ^{o}C in a bath). -LLE followed by a SPE.	LC-MS/MS	ESI, positive mode, MRM	2 pg/mg	[60]
Clozapine	5 mL petroleum benzene (Agitation for 5min in a gas-tight tube)	-Ultrasonification with 3 mL of methanol (55 ^{o}C, 3h).	LC-MS	ESI, positive mode, SRM	–	[62]
Clozapine and Haloperidol	Three solvents	-Ultrasonication with 4 ml of metanol (2h). -SPE: Chromabond and automated SPE device (55 ^{o}C, 3h).	LC-MS/MS	ESI, MRM	CL - 0.017/0.051 ng/mg HA - 0.013/0.039 ng/mg	[65]
Citalopram and Clozapine	–	-Incubation with methanol: acetonitrile: ammonium formate at 37 ^{o}C for 18 h.	UPLC–TOF-MS	ESI, positive mode	CI - 0.01 ng/mg CL - 0.02 ng/mg	[91]

Abbreviations: SPE: solid phase extraction, LLE: liquid-liquid extraction, LC: liquid chromatography, MS/MS: tandem mass spectrometry ESI: electrospray ionization, MRM: multiple reaction monitoring, SRM: single reaction monitoring, LOD: limit of detection, ACN: acetonitrile, HA: Haloperidol, CL: Clozapine, CI: Citalopram.

The widely used chromatographic mode to separate several classes of compounds, based on their hydrophobicity, is reversed-phase chromatography [79,80,84]. Here, the stationary phase is less polar than the mobile phase [69,79,80], the interaction between analyte and the stationary phase has a predominantly hydrophobic (apolar) character [69]. Thus, the more polar analytes elute more rapidly than the less polar ones [79,80] and a decrease in the polarity of the mobile phase results in a decrease in solute retention [82]. Reversed-phase chromatography typically refers to the use of chemically bonded stationary phases, such silica-based alkyl (C4, C8, C18), [69,79,80,84].

The mobile phases used in reversed-phase chromatography are mostly polar solvents such as water, acetonitrile, methanol, and isopropanol [69,82,84]. In LC-MS certain chemicals (ionic modifier) are often added to the mobile phase to influence analyte ionization. Small organic acids like formic and acetic acid are among the most commonly used additives. They improve ionization and resolution of a wide range of molecules [80].

The RT can be controlled by changing polarity of the mobile phase. For example, increasing the polarity of the mobile phase leads to longer retention times, whereas shorter retention times require a mobile phase of lower polarity [80].

With the developed of an elution gradient, the RT also can be controlled. In this mode of elution the initial mobile-phase composition is relatively polar and as the separation progresses, the mobile phase's composition is made less polar [80,84]. In the case of the isocratic, the solvent composition remains constant throughout the analysis [82,84].

3.2.2. MASS SPECTROMETRY

The mass spectrometer provides the most definitive identification of all of the HPLC detectors. The molecular weight of the analyte together with the structural information that may be generated, allows an unequivocal identification [79].

The first step in the mass spectrometric analysis of compounds is the production of gas phase ions of the compound [78]. Thereby, the effluent from the HPLC column is directed to the ionization source of the mass spectrometer [81]. These ions are then introduced in several stages to the high vacuum region of the mass analyzer, where the ions are separated by mass to charge ratio and measured by the detector [76,78,81].

The inlet system for LC, often termed the 'interface' between the two component techniques, must therefore remove as much of the unwanted mobile phase as possible while still passing the maximum amount of analyte into the mass spectrometer [79]. The essential components of a mass spectrometer are represented in the Figure 3.1.

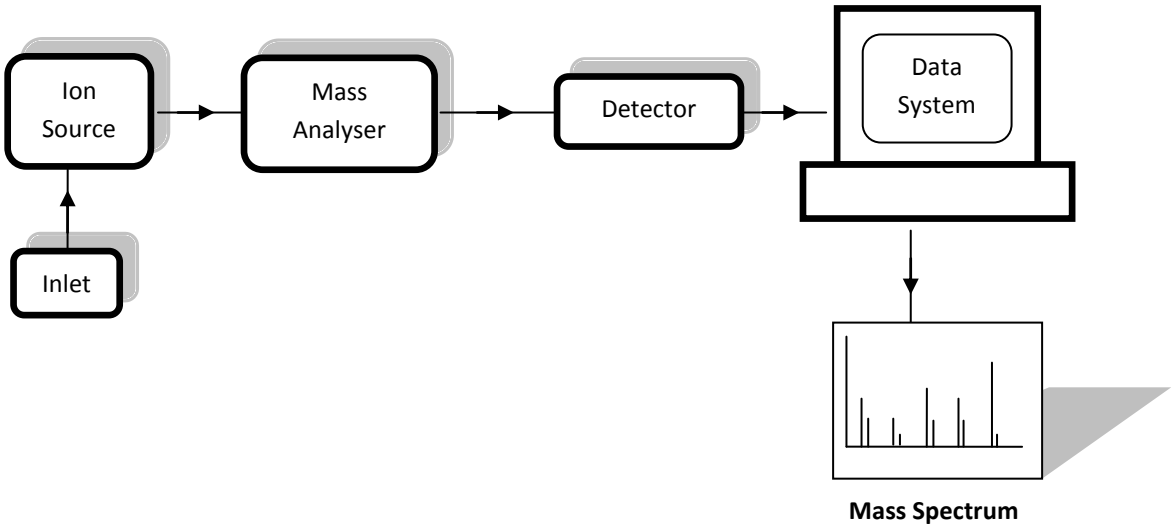


Figure 3.1- The essential components of a mass spectrometer. An inlet system transfers a sample into the ion source. There the neutral sample molecules are converted into gas-phase ions. The mass analyzer separates and analyzes the ionic species and the detector measures and amplifies the ion current of mass-resolved ions. For last, the data system records, processes, stores, and displays data. The mass analyser and detector are operated under high vacuum, which allows ions to move freely in space without colliding or interacting with other species. Adapted from [84].

3.2.2.1. MODES OF IONIZATION

Liquid solutions are difficult to handle by the MS vacuum system and require some novel introduction and ionization systems [84]. Ionization of the analyte is the first step in the analysis of any class of compounds by MS [78,84].

The choice of a particular method is dictated largely by the nature of the sample under investigation and the type of information desired [84]. In the field of toxicology, for analysis of smaller molecules and highly polar compounds associated with the higher sensitivity achieved makes ESI the most widely applied ionization technique [16,70]. The type of ionization that was used in this project was Electrospray Ionization (ESI). This ionization technique has become the most successful interface for LC/MS applications [84].

Electrospray analysis can be performed in positive and negative ionization modes [84], which typically result in protonated molecular ions, $[M+H]^+$, or deprotonated molecular ions, $[M-H]^-$, respectively [78,81,92]. Because most toxicologically relevant compounds have basic properties, positive ionization mode is generally applied (Figure 3.2) [8].

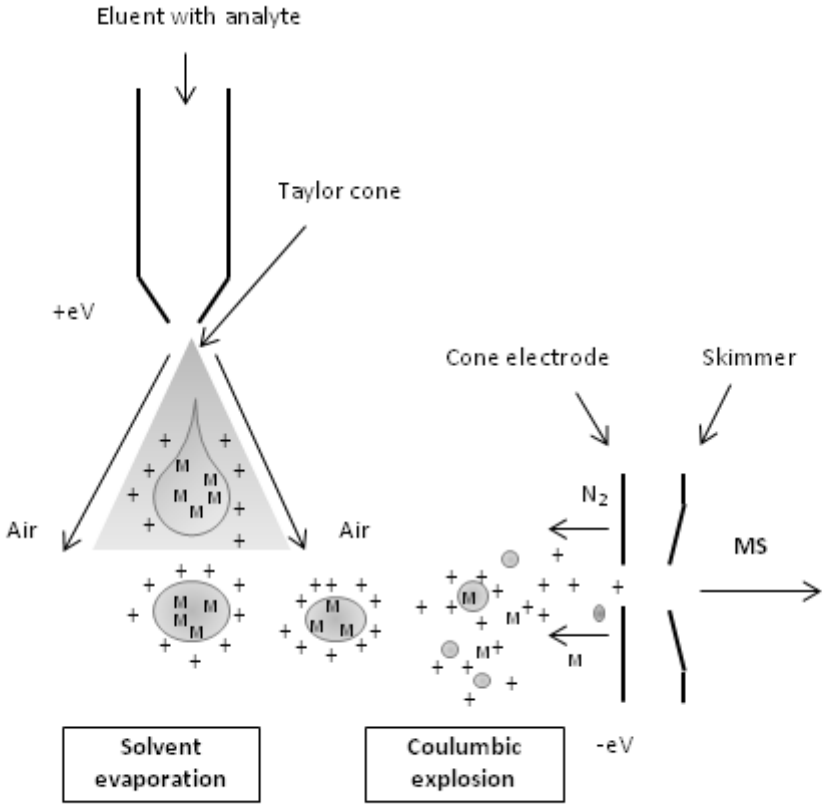


Figure 3.2- A simplified mechanism of ion formation in the positive ESI mode. The analyte is introduced into the ESI source via a needle as an eluent flow from an LC chromatograph. The electrospray itself is formed as a result of a large electrostatic potential difference between the capillary and a cone electrode. Cations concentrate at the tip of the capillary and tend to migrate toward the cone electrode. The migration of the accumulated positive ions toward the cone electrode is counter balanced by the surface tension of the liquid, giving rise to a Taylor cone at the tip of the capillary. The air, which is passed continuously in the region spraying helps the evaporation of the solvent. As the size of the droplet reduces, the repulsive forces between charges on the surface of the droplets overcome the cohesive forces of surface tension and leads to the Coulomb explosion. The skimmer is used to retain these droplets and guide the ion to the analyzer region of the mass spectrometer [84].

In the positive-ion mode, the solution at the end of the needle is polarized and torn away from the needle [69]. The field accumulation of charge on the surface of the liquid emerging from the capillary produces a fine spray of highly charged droplet that are desolvated as they pass

through the atmospheric-pressure region of the source [78,81,92,93]. Because solvent contained in the droplets evaporates, this causes them to shrink, increasing their charge per unit volume [69,78,79,84,92,93]. At some point, the competing force of surface tension causes the droplets to disintegrate (Coulombic explosion) and gas phase ions of the analyte(s) are produced [79,81,92,93].

3.2.2.2. QUADRUPOLE ANALYZER

Mass analyzers are used for ion separation, maximizing the transmission of all ions that enter from the ion source [69,84]. Once the gas-phase ions have been produced, they need to be separated according to their masses, which must be determined. The physical property of ions that is measured by a mass analyser is their mass-to-charge ratio (m/z)[78].

As there are a great variety of sources, several types of mass analysers have been developed [78,81]. All mass analysers use static or dynamic electric and magnetic fields that can be used alone or combined [78]. A quadrupole analyser was the type of mass analyser used in this project and is probably the most used type of mass analyser [84]. It is an ideal detector for chromatography as it is capable of fast scanning and uses low voltages which make it tolerant to relatively high operating pressures, such as those encountered in LC–MS [79].

This device uses the stability of the trajectories in oscillating electric fields to separate ions according to their m/z ratios [78].The field is achieved by using four parallel rods (Figure 3.3) [69,78,92,94] that are arranged symmetrically around a central axis that is the path of ion movement from ion source to ion detector [92].

Two opposite rods have the same voltage, while the perpendicular ones have a voltage with opposite sign (+ and -, respectively) [69,79,92]. The oscillating field applied to the rods alternately attracts and repels ions passing through the mass filter, inducing an ion motion that is exploited to differentiate ions on the basis of their mass [84,92]. Thus a mass spectrum is produced by changing both RF and DC voltages in a systematic way to bring ions of increasing or decreasing m/z ratios to the detector [79,84]. At a specific value of these voltages, only ions of a particular m/z follow a stable trajectory through the rods and reach the detector [69,79,92].

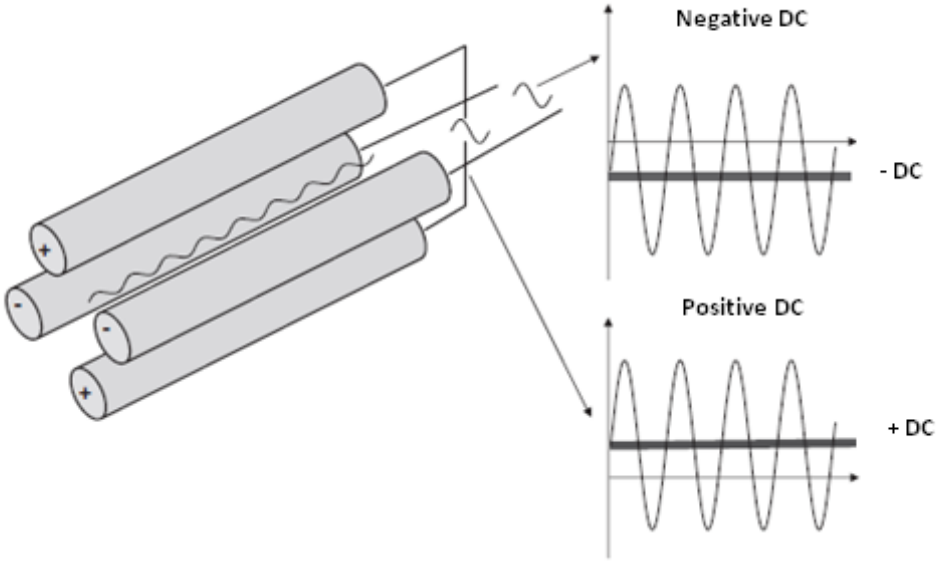


Figure 3.3- Schematic representation of a quadrupole mass analyser and a voltage profile on the rods. One pair of rods receives a superimposed positive direct corrent (DC) potential and a radio-frequency potential. The other adjacent pair of rods receives a negative DC potential and an radio-frequency potential of the same magnitude. Adapted from [69].

The introduction of soft ionization techniques, like ESI, has trigged the rapid development of tandem mass spectrometry (MS/MS) techniques [69]. There are differences between MS mode and MS/MS mode (Figure 3.4).

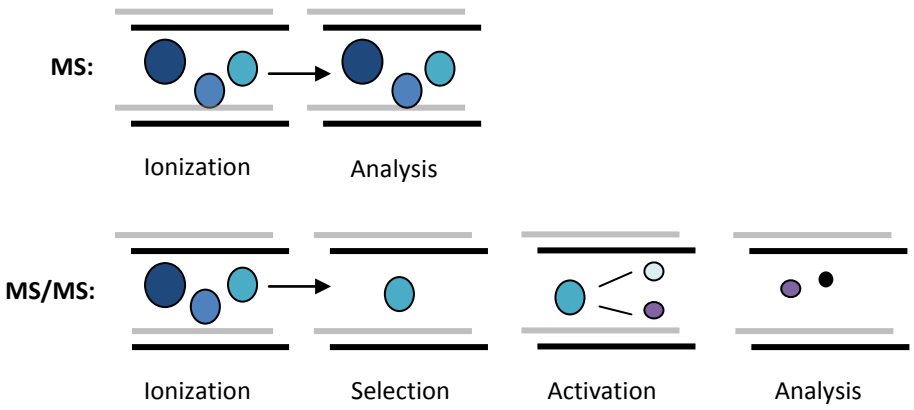


Figure 3.4- Diffrerences between single-stage MS and tandem MS/MS. In the MS mode, ions formed in the ionization source are separated by a single-stage mass analyzer. In tandem in space MS/MS there are three main steps in tandem mass spectrometry: (i) ion selection, (ii) ion activation (fragmentation), and (iii) analysis of the fragments of the selected ion. Adapted from [69].

In MS/MS, any individual ion can be selected and then activated to generate fragments of the selected ion. These fragments are characteristic for the precursor ion structure. The fragments originating exclusively from the precursor ion can then be analyzed separately with another mass analyzer [69]. In the Figure 3.5, the QqQ configuration indicates an instrument with three quadrupoles [78], called a triple quadrupole used for MS/MS experiments.

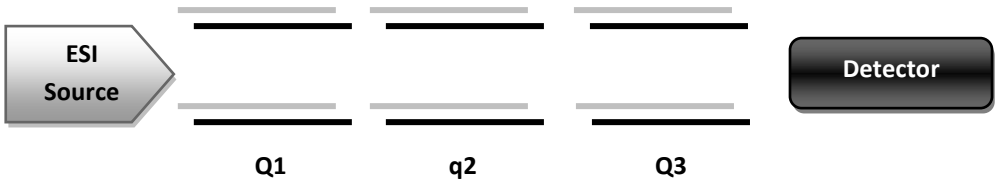


Figure 3.5- General diagram of a triple quadrupole instrument. Q1 and Q3 are mass analyzers, where the Q1 acts as a mass filter and Q3 allows the passage of the fragment ions of the desired mass to charge ratio. The centre quadrupole, q2, is a collision cell made up of a quadrupole using RF only. Adapted from [78].

The first quadrupole (Q1), selects a 'precursor' ion with the desired mass to charge ratio from the ESI source [81,94]. The second quadrupole (q2) is the collision cell, [94] where collisions with a neutral gas such as N_2 or Ar causes the ions to fragment through a process known as collision induced dissociation (CID) [81,94]. Ions are confined to the collision cell by a quadrupole, operated with only a radiofrequency voltage between the poles. The resulting fragment ions are transmitted to third quadrupole(Q3) [78,81,94], where only the fragment ions of the desired mass to charge ratio are allowed to pass and reach the detector [81].

The mass spectrometer used in this project is a Hybrid Triple Quadrupole –Linear Ion Trap. With this mass spectrometer it is also possible to trap ions in between the quadrupole rods for a certain amount of time. In this instrument, the Q3 region can be operated as a normal triple quadrupole with all its scan modes or as a trap in various combinations with the use of the other quadrupoles [78]. This mass spectrometer has an additional quadrupole, Q0 which is a cell that works in high-pressure. Here the ions can be accumulated in the Q0 region of the system while the Q3 trap is scanning ions during MS/MS and scans MS^3.

Tandem mass spectrometry is used to determine ion structure and to detect and quantify targeted compounds in complex mixtures [94]. This improve the selectivity and sensitivity for quantitative assays, and greatly expand the capabilities for gaining qualitative information of unknown metabolites [81].

3.2.2.3. MULTIPLE REACTION MONITORING (MRM)

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a triple quadrupole mass spectrometer operated in multiple reaction monitoring mode (MRM) is the method of choice for quantitative bioanalysis of small molecules (Figure 3.6) [81,84]. And this one was the mode that was used in this project.

The two levels of selectivity in the MRM experiment, combined with the chromatographic separation, provided a very high level of selectivity [81]. Depending on the resolving power of the first mass analyzer, ions can be selected either monoisotopically or with multiple isotopes [69].

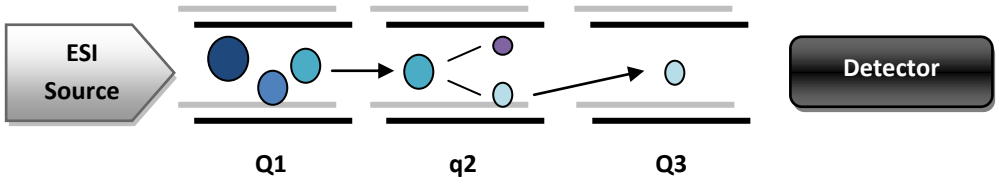


Figure 3.6- Schemating representation of Multiple reaction monitoring. Here a specific m/z value is selected in Q1 and is fragmented in q2. Q3 is set to transmit only ions of a selected m/z rather than scanning the entire fragment ion spectrum . Adapted from [78].

The Q1 (on QqQ scheme) is set to the m/z value of the precursor (first mass analysis step, MS1). Then is induced to dissociate (fragment) via CID in a collision region of the mass spectrometer (q2 on QqQ). Finally, a specific, structurally distinct fragment ion (product ion) is mass selected in Q3 (second mass analysis step, MS2) and detected [69,84,94,95]. Since higher sensitivity is desired, the Q3 is not scanned over a wide mass range of the fragments but, instead, it is set up to monitor only a selected fragment, or fragments [69,84,94].

The term MRM refers to the monitoring of more than one reaction, either from the same precursor or from more than one precursor [84]. This technique is very useful for quantitation [69] and provides enhanced selectivity in quantitative analysis, leading to increased confidence of the analyte of interest being monitored [95].

3.2.2.4. ISOTOPIC ABUNDANCES

Most elements appear in nature as isotope mixtures, each with fixed relative abundances [78,84]. Atoms with nuclei of the same atomic number differing in the number of neutrons are termed isotopes [96]. These isotopes are responsible for the peaks in the mass spectrum appearing as isotopic pattern that are characteristic of the elemental composition [78,84]. With a bar graph representations, it can be visualized the isotopic compositions and show how such a distribution would appear in a mass spectrum (Figure 3.7).

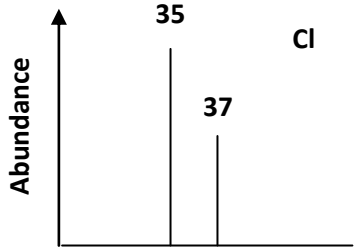


Figure 3.7- Isotopic patterns of chlorine. The bar graph representations of the isotopic distributions have the same optical appearance as mass spectra. Adapted from [96].

Those peaks are isotopically shifted lines that appear at masses one or more units higher than the main peak M; the mass of M is calculated using the atomic masses of the most abundant isotopic species (i.e., the primary isotope). The pattern peaks, designated as M+1, M+2, and so on, reflect the differences in the natural abundances of the isotopes. [84].

Several elements exist naturally in two isotopes and within the context of MS it is useful to deal with them as a class of their own [96], because even without exact mass measurement, the possibilities for elemental composition determination can often be restricted by using isotopic abundance data [78].

So, it is essential to evaluate the compound structure to determine whether it contains any elements such as chlorine, bromine or boron, which would result in a unique isotope pattern [78].

3.3. ANALYTICAL METHOD VALIDATION

Due to the increasing interdependence among the countries during the last years, it has become necessary for the results of many analytical methods to be acceptable internationally. Consequently, the need for and use of validated methods has increased [97]. The international scientific community needs published research results that are valid, reproducible and comparable. In addition, the 'client of the laboratory' will have the implicit expectation of correct results [98].

To ensure that an analytical method generates reliable information, it must be validated. The International Organization for Standardization (ISO) defines validation as the confirmation by examination and provision of objective evidence that the particular requirements for a specified intended use are fulfilled. By another words, method validation is the process of defining an analytical requirement, and confirming that the method under consideration has performance capabilities consistent with what the application requires [99].

Method validation is usually considered to be very closely tied to method development, indeed it is often not possible to determine exactly where method development finishes and validation begins [99].

Reliable analytical data are a prerequisite for correct interpretation of toxicological findings in the evaluation of scientific studies, as well as in daily routine work [100,101]. There are legal reasons, technical and commercial for the need of implementation of validation methods. The cost of carrying out the analyzes is high and additional costs arise from decisions made on the basis of the results [99]. The results are used by others for case report interpretation, by judicial authorities for implementation of legal measures and by medical doctors for patient treatment [98]. So, unreliable results might not only be contested in court, but could also lead to unjustified legal consequences for the defendant or to wrong treatment of the patient [101].

In several countries, judicial authorities impose proficiency testing and/or accreditation according to the ISO standards on laboratories performing analysis of certain samples in a forensic or clinical setting [98]. Therefore, quality management and accreditation have become matters of increasing importance in analytical toxicology in recent years [100,101].

However, all these guidelines not often provide a practical approach to how validation should occur in a particular laboratory setting [98].

Owing to the importance of method validation in the whole field of analytical chemistry, a number of guidance documents about bioanalytical methods in which are definitions, procedures and parameters of validation, were published. This subject has been issued by papers, reviews and conferences [97,100-103]. However, there is no consensus on the extent of validation experiments and on acceptance criteria for validation parameters of bioanalytical methods in forensic and clinical toxicology [101].

International organizations such as ISO, IUPAC (International Union of Pure and Applied Chemistry), EMA (European Medicines Agency), FDA (Food and Drug Administration) and EUROCHEM group [99,104-106], also publish parameters and methodologies for analytical method validation. Guidance documents for analytical method validation differ between them, which creates confusion about the nomenclature and concepts. A single set of global guidelines and use of the same terminology still remains the ultimate goal for full method comparison [91].

Method validation includes all of the procedures required to demonstrate that a method to quantify the concentration of an analyte (or series of analytes) in a particular biological matrix is reliable for the intended application [107]. Any modification of an analytical method would require revalidation of the procedures [108].

It is essential to employ well-characterized and fully validated analytical methods to yield reliable results which can be satisfactorily interpreted [108]. In this way, all of the variables of the method should be considered, including sampling procedure, sample preparation, chromatographic separation, detection and data evaluation [107].

There is a general agreement that at least the following validation parameters should be evaluated for quantitative procedures: selectivity, calibration model (linearity), stability, accuracy, precision (repeatability, intermediate precision), limit of detection and limit of quantification. Additional parameters which might have to be evaluated include recovery, reproducibility and robustness [101,109].

4. MATERIALS AND METHODS

4.1. EQUIPMENTS

- System of Liquid Chromatography coupled to a mass spectrometer, with the following components:
  - Liquid Chromatography, Ultimate™3000 (LC Packings, Dionex);
  - ESI source, turbo V™;
  - Hybrid triple quadrupole/linear ion trap mass spectrometer, 4000 QTRAP® (ABSciex);
  - Software Chromeleon® 6.80 (Dionex) for the LC system;
  - Software Analyst® 1.5.1 (ABSciex) for MS system.
- Analytical balance CP 224S (Sartorius);
- Bench-top Centrifuge (Minispin-Eppendorf®);
- Concentrator Plus (Eppendorf®) - "speedvac";
- Quick spin, model QS 7000 (Edward Instrument Co);
- Sonicator, model VibraCell - Sonics™ 75041 (Bioblock Scientific);
- Thermomixer comfort (Eppendorf®);
- Vortex, model MS3 basic (IKA®).

4.2. MATERIAL

- C18 OMIX Tip - 100µL (Agilent Technologies);
- Eppendorf® CombipTip (w/ pre-cutted end);
- Micropipettes® Research Plus (Eppendorf®);
- Multipipette® Plus (Eppendorf®);
- PS – Microplate 384 well, 128.0/85 mm (Greiner bio-one);
- Microcentrifuge tubes (500µL, 1.5mL, 2mL)
- Vials 500µL (VWR®).

4.3. STANDARDS AND REAGENTS

Standards:

- Citalopram hydrobromide, BIOTREND Chemicals AG (purity 99.8%);
- Clozapine, BIOTREND Chemicals AG (purity 99.0%);
- Haloperidol, BIOTREND Chemicals AG (purity 99.8%);
- Desipramine hydrochloride, Sigma-Aldrich (purity ≥ 98%);
- Haloperidol - D4, Cirilliant® (purity 99.2%);
- Sulfamethazine-D4.

Reagents:

- Acetonitrile (LC Grade, Biosolve) - ACN;
- Dichloromethano (Sigma ≥ 99.9%);
- Formic Acid (LC Grade, Sigma Aldrich) - FA;
- Methanol (LC Grade, Biosolve) – MeOH;
- Water (LC Grade, VWR®).

4.4. ANIMAL PROTOCOL

4.4.1. ANIMALS AND DRUG ADMINISTRATION

Young black male C57BL/6J mice were purchased from Charles River, Laboratories International, Inc (Spain). They were divided into four groups, 5 per group and each animal weighed around 20 - 25 g with access to food and water *ad libitum*.

After a 1 week habituation period to needle punctuation, the animals were injected, via intraperitoneal with clozapine, citalopram and haloperidol at a dose of 1 mg/kg, 20 mg/kg and 10 mg/kg, respectively. An additional group was treated with vehicle (control group).

The mice were injected daily for different periods: 1, 2, 4, 8, 15 and 30 days. It was also added a saline solution control (0.13% HCl at 5 M). Then, the animals were weighed and anesthetised with a mixture of ketamine and xylazine 24 hours after the final injection.

Animals samples were kindly prepared in Dra. Graça Baltazar's lab with the help of Sandra Rocha (University of Beira Interior, Covilhã).

4.4.2. PLASMA AND HAIR COLLECTION

The blood was collected by cardiac puncture and placed in EDTA-coated tubes. Then the samples were centrifuged at 12000x*g* for 2 minutes and the plasma was recovered to another tubes. To each tube was added protease and phosphatase inhibitors and stored at −80°C.

The hair samples were pulled out with tweezers and placed into a centrifuge tube and were also stored at -80°C.

Drug-free plasma and hair samples was courtesy of Professor Carlos Duarte's group of Center for Neuroscience and Cell Biology (Coimbra, Portugal). The collection procedure of plasma and hair was similar.

4.5. METHOD DEVELOPMENT

The development of a LC-MS method requires the optimization of several parameters. This optimization refers to an adjustment of instrumental parameters in order to optimize performance characteristics of each compound.

To set the conditions a direct injection into the mass spectrometer was performed by infusing a standard solution of each analyte with a syringe pump of 1mL, with a concentration of 0.453 µM for haloperidol, 1 µM for clozapine, 0.125 µM for citalopram, 1.14 µM for desipramine and 0.1 µM for haloperidol-d4. Each solution was injected one by one with a flow rate of 9 µL/min.

With this procedure, the conditions to the ESI source to apply to substances and the ideal collision energy (CE) for the fragmentation of each compound were optimized. It was also optimized the best value for declustering potential (DP) to minimize solvent cluster ions.

Each compound fragmentation spectra was analysed with software Peak View™ 1.1.1.2 (ABSciex).

4.6. INSTRUMENTAL CONDITIONS

4.6.1. LIQUID CHROMATOGRAPHY

The separation in the chromatography system was performed with a Gemini® C18 (3 µm, 110 Å, 50 x 2 mm) column. Security Guard™ cartridges Gemini® C18 (4 x 2 mm) was also used.

To have an efficient separation an elution gradient was developed (Table 4.1) with a flow rate of 250 µL/min and 9 min of running time for each sample.

Between samples a blank was injected with a gradient that is also represented in the Table 4.1. Also between batchs three blanks were introduced (solution of 0.1%FA in ACN), with the same program and the same volume of injection that was used for the samples. The volume that was injected for hair samples was 1µL and for plasma samples was 20 µL. For the blanks between samples the injection volume was 10 µL.

Table 4.1- Elution gradient used for chromatographic analysis.

Running program	Time (min)	Mobile phase (% v/v)	
		0.1% FA in H_2O	0.1% FA in ACN
Sample	0	90	10
	0	90	10
	0.3	80	20
	6	70	30
	7	1	99
	9	1	99
Blank	0	100	0
	0	100	0
	1.9	100	0
	2	10	90
	8	10	90

4.6.2. MASS SPECTROMETRY

The equipment and data acquisition were performed by the software with the Analyst® 1.5.1 (ABSciex).

The mass spectrometer is equipped with an ESI source, which was operated in positive ion mode. All values of the source dependent parameters were optimazed: curtain gas (CUR), 30psi; ion source gas 1 (GS1), 30 psi; ion spray voltage, 5500V; source temperature, 450 ^{o}C.

To monitor the precursor ions of each analyte and IS (haloperiodl-D4 and desipramine), the mass spectrometer was operated in MRM mode and the transitions monitored are in the Table 4.2. The haloperidol-D4 was used as IS of haloperidol and the desipramine was the IS used for citalopram and clozapine.

All compound's parameters were determined: Dwell time was 30 ms, entrance potential (EP) was 10eV and collision gas (CAD) was 8psi. The different values of declustering potential (DP), collision energy (CE), collision exit potential (CXP) for each transition are also represented in the Table 4.2. The analytical data were processed by the Multiquant™ 2.1.1 (ABSciex) software.

Table 4.2- Mass spectrometer acquisition parameters: MRM transitions, collision energy (CE), collision exit potential (CXP) and declustering potential (DP), for each transition of all analytes and internal standards.

	Compound	Transitions (m/z)		CE (eV)	CXP (eV)	DP (eV)
		Q1	Q3			
	Citalopram	325.3	109	39	8	66
			261.9	27	24	
			83.1	91	4	
	Clozapine	327.2	269.9	35	18	71
			191.9	57	16	
			163.8	95	10	
	Haloperidol	376.0	164.7	33	10	61
			122.9	55	8	
			94.8	107	16	
Internal standards	Desipramine	267.3	72.2	27	4	56
			208	33	16	
			190.8	83	14	
	Haloperidol – D4	380.2	127.1	61	8	51
			168.4	33	10	
			98	99	6	

4.7. EXTRACTION PROCEDURE FOR PLASMA SAMPLES

4.7.1. PROTEIN PRECIPITATION

To each microcentrifuge tube containing 70 µL of plasma, was added three volumes of methanol (210 µL). The samples were agitated by vortex and after by continuous agitation for 5 minutes at 1000rpm's in the thermomixer. To help the proteins to agregate, they were centrifuged at 14,000×*g* for 10 minutes.

The supernatant was collected to a new microcentrifuge tube and was placed on an evaporator at 60 °C, during approximately 1 hour. Subsequently, the sample was resuspended in 50µL of 2% ACN:0.1% FA.

4.8. EXTRACTION PROCEDURE FOR HAIR SAMPLES

4.8.1. DECONTAMINATION AND SEPARATION OF DRUGS FROM THE HAIR MATRIX

Depending on the length, some hair samples were cut in the middle. Then the samples were weighed in amounts between 0.7 – 9.7 mg (see Appendix 8.1).

First, the hair samples were decontaminated by adding 1mL of dichloromethane for 2 minutes at room temperature in the thermomixer. Then dichloromethane was removed, and this procedure was repeated twice.

The hair was incubated overnight (17 hours) in 1 ml of methanol at 45 °C. Then methanol was evaporated to dryness in the speedvach at 60 °C, during approximately 1 hour.

4.8.1.1. OMIX TIP C18 CLEAN UP

OMIX Tip C18 contains a small bed of functionalized monolithic sorbent (C18) inserted inside a pipette tip, it can work as a miniaturized solid phase extraction bed to remove salts prior to mass spectrometry. This procedure was used as a clean up of the hair samples.

To performe the OMIX C18 SPE, three solution were prepared: 50% ACN, 2% ACN: 1% FA and 70% ACN: 0.1% FA.

To the evaporated sample 100µL of 2% ACN: 1% FA were added and were immediatly sonicated for 2min in the cuphorn (20% amplitude 1s on 1s off cycle). Then, to wet the tip, it was added, from the top, 200µL of 50% ACN and the pre-cutted CombipTip was used to push the solutions and sample through the tip. To equilibrate the tip, 300µL of 2% ACN: 1% FA were added. After, the sample was passed through the tip 5 times. To rinse the tip 100µL of 2% ACN: 1% FA were added and at last the analytes were eluted with 400µL of 70% ACN 0.1% FA.

All samples were evaporated to dryness in the speedvac at 60 °C and then resuspended in 50µL of 2% ACN:0.1% FA and sonicated before being placed in the vials.

4.9. ANALYTICAL METHOD VALIDATION

Only validation can objectively demonstrate the inherent quality of an analytical method by fulfillment of minimum acceptance criteria and thus prove its applicability for a certain purpose [100]. Therefore, the validation of the method was performed and the parameters that were used for validation of analytical method for the identification and quantification of psychotropic drugs in plasma and hair samples by LC-MS evaluated are summarized in Table 4.3.

Table 4.3- Parameters used for the analytical method validation for identification and quantification of psychotropic drugs in plasma and hair by LC-MS/MS. Adapted from [102,109].

Parameter	Qualitative method	Quantitative method
Selectivity	✓	✓
Limit of detection	✓	✓
Limit of quantification		✓
Linearity		✓
Working range		✓
Precision		✓
Accuracy		✓
Extraction efficiency		✓
Carry over	✓	✓
Matrix effects	✓	✓

All the statistical tests and the acceptance criteria applied for each parameter will be explained exposed in the next pages. They were performed with the help of a Microsoft Excel® spreadsheet developed for the validation method, kindly provided by Margarida Coelho.

4.9.1. SELECTIVITY

Selectivity is the ability of an analytical method to differentiate the analyte(s) of interest in the presence of other components in the sample [105,106].

To evaluate the selectivity, for plasma samples, six individual sources of blank plasma were selected and were divided in two aliquots with 70µL of plasma in each:

- The first aliquot was fortified with 20µL of a solution (0.025 µM) containing the three compounds (citalopram, clozapine and haloperidol) and the two IS (desipramine and haloperidol-D4) - positive samples;
- The second aliquot was not fortified with any compound and only 20µL of 2% ACN: 0.1% FA were added, in order to have the same final volume - negative samples.

Then, all samples were subjected to the analytical procedure developed for the extraction of drugs from plasma (see section 4.7). It was injected 20 µL of the sample into the LC–MS/MS system.

In an analogous way, to evaluate the selectivity of hair samples, six individual sources of blank hair were selected. The samples were cut and were placed in two different aliquots, each one with 3-4 mg:

- The first aliquot was fortified with 20µL of a solution (0.5 µM) containing the three compounds (citalopram, clozapine and haloperidol) and the two IS (desipramine and haloperidol-D4) - positive samples;
- The second aliquot was not fortified with any compound and only 20µL of 2% ACN: 0.1% FA were added, in order to have the same final volume - negative samples.

All hair samples were subjected to the analytical procedure developed for the extraction of drugs (see section 4.8). It was injected 1 µL of the sample into the LC–MS/MS system.

In both cases the results obtained from positive samples were compared with the results obtained from negative samples. The criteria used were proposed by the World Anti-Doping

Agency (WADA) which required at least two precursor-product ion transitions should be monitored. The second criterion is monitoring the relative abundance of a diagnostic ion. This is expressed as a percentage of the intensity of the most intense fragment (base peak) and was calculated by dividing the area of the each ion trace by the area obtained from the peak corresponding to the m/z of the base peak ion (corresponding to 100%) [110]. The maximum tolerance range for relative ion intensities used for the identification of compounds are presented in the Table 4.4. The criteria used to calculate the ranges in this parameter are defined by data from the first positive sample.

Table 4.4- Maximum Tolerance ranges for Relative Ion Intensities to Ensure Appropriate Confidence in Identification [110].

Relative Abundance (% of base peak)	Maximum Tolerance Ranges (%)
> 50	± 10 (absolute range)
25 to 50	± 20 (relative range)
5 to < 25	± 5 (absolute range)
< 5	± 50 (relative range)

It was also used as acceptance criterion the relative retention time (RT_{ratio}), which is expressed by the ratio between the RT of the interest compound and RT of the internal standard. Here, the ΔRT_{Ratio} should not differ by more than ±1% (or ± 0.1%, if stable-isotope-labeled internal standard is used), when compared with the ΔRT_{Ratio} of the control sample.

At last, the ratio between the signal of the least intense diagnostic ion and the signal of the noise of the baseline (S/N) shall be greater than 3:1. The determination of S/N was performed by the Multiquant™ 2.1.1. software.

4.9.2. LINEARITY

It is necessary to use a sufficient number of calibrators to define adequately the relationship between concentration and response [107]. Recommendations on how many concentration levels and how many replicates per concentration level should be studied, differ significantly. Most guidelines require a minimum of five to eight concentration levels [101,109,111].

To study the linearity, it was prepared one calibration curve with a solution containing the three analytes (citalopram, clozapine ad haloperidol) with eleven calibrators, uniformly distributed in the working range: 0.05, 0.1, 0.15, 0.2, 0.25, 0.5, 1, 1.5, 2, 3 and 5 µM. At each calibrator a solution (0.5µM) containing the two IS (desipramine and haloperidol-D4) was added.

The choice of an appropriate calibration model is necessary for reliable quantification. Then, if it is assumed that there is a linear relationship, the simplest regression model that is usually applied is the ordinary least squares model [97,109]. This model represents the relationship between two variables by a straight line, mathematically expressed by the equation (4.1), where y is the instrumental response and x is the concentration of the compound [112].

$$y = m.x + b \qquad (4.1)$$

Where, y and x are the independent and dependent variable, b and m are the calibration parameters, the y-intercept and the slope, respectively.

The linearity of the calibration process was first investigated by means coefficient of correlation (R) and coefficient of determination (R^2), that should be above 0.99 in both cases. However, the evaluation of linearity should not rely only on those parameters. Therefore, the zero-value should be included within the confidence interval of 95% [113,114].

Additionaly, the standard error of the linear regression ($S_{y/x}$) was used as a measure of the goodness of fit in order to exclude the residual values (response observed in relation with the response predicted) higher than 2 × $|S_{y/x}|$. In addition, visual inspection of plots for residuals versus concentration was performed [101].

4.9.2.1. MANDEL TEST

Spite of widespread practice of evaluating a calibration model via its coefficients of correlation or determination, this is not acceptable from a statistical point of view [101,115]. For example, calibration models with points not uniformly distributed along the calibration range may provide a good correlation coefficient [115]. Nevertheless, several researchers focused on the fact that R might not be a useful indicator of linearity and other statistical tests or quality parameters have been suggested to ascertain the goodness of fit of the calibration curve [116].

So, the Mandel's fitting test was applied to ascertain which model (linear or quadratic) fitted better in calibration data [116,117].

First, it was calculated the differences between the variance of linear correlation and quadratic correlation (equation (4.2)) [115,117,118].

$$DS^2 = (N-2) \times S^2_{y/x} - (N-3) \times S^2_y \quad (4.2)$$

$$S^2_{y/x} = \frac{\sum(y_i - \hat{x}_i)^2}{N-2} \quad (4.3)$$

$$S^2_y = \frac{\sum(y_i - \hat{y}_i)^2}{N-3} \quad (4.4)$$

Where, $S^2_{y/x}$ is the variance of linear correlation calculated for the linear fit; S^2_y is the variance of quadratic correlation calculated for the quadratic fit and N is the number of calibration standards used to construct the curve.

From this it is possible to calculate the significance of this difference (F_{cal}) through the equation (4.5) [115,118].

$$F_{cal} = \frac{DS^2}{S^2_y} \quad (4.5)$$

Then, it was compared the value obtained for the calculated F value (F_{cal}) with the tabulated value (F_{crit}) of the F distribution of Snedecor $(N-1; N-1)$ at the confidence level of 95% (α = 5%). The criteria for these results were:

- If $F_{cal} \leq F_{crit}$ - the differences between the variances are not statistically significant and therefore the linear adjustment is more appropriate;
- If $F_{cal} > F_{crit}$ - the differences between the variances are statistically significant and therefore the quadratic adjustment is more appropriate.

4.9.3. WORKING RANGE

The working range of an analytical procedure can be defined as the interval between the upper and lower concentration of analyte for which suitable precision, accuracy and linearity have been demonstrated [119,120].

In addition, the concentration range in bioanalytical methods is usually broad and therefore it might be expected that the variance of each standard point of the calibration curve might be different (heterocedastic data) [121]. Therefore, it is mandatory to evaluate the homoscedasticity assumption through the test of homogeneity of variances (or F test) and also by visual evaluation of residuals versus concentration plots [112,121]. Therefore, the study of the working range was performed simultaneous with the study of linearity and ten replicates of the lowest and the highest concentrations levels were performed, 0.1 and 5 µM respectively. To each calibrator it was added a solution (0.5µM) of the IS.

It was calculated the variances of the first (S_1^2) and the last calibrator (S_{10}^2), according to the following equation (4.6) [118,121].

$$S_i^2 = \frac{\sum_{j=1}^{n}(y_{ij} - \bar{y}_i)^2}{n_i - 1} \quad (4.6)$$

Where, S_i^2 represents the variance; i is the calibration sample (i =1 and i=10); j is the number of replicates for each calibration sample (j=1 to 10 for each i); n is the number of results; y_i is the result obtained and $\bar{y}_i$ is the mean of results obtained.

Then, it was obtained the calculated F value (F_{cal}) by the F-test, that uses the ratio between the variances obtained at the lowest (S_1^2) and at the highest (S_{10}^2) concentration level of the working range (equations (4.7) and (4.8)) [112,121,122].

$$F_{cal} = \frac{S_{10}^2}{S_1^2}, \text{ if } S_{10}^2 > S_1^2 \quad (4.7)$$

$$F_{cal} = \frac{S_1^2}{S_{10}^2}, \text{ if } S_1^2 > S_{10}^2 \quad (4.8)$$

The value of $\mathrm{F_{cal}}$ is compared with tabled value (F_{crit}) of F distribution of Snedecor $(\mathrm{N}-1;\mathrm{N}-1)$ at the confidence level of 95% (α = 5%) [112]. The criteria used for these results were:

- If $F_{cal} \leq F_{crit}$ – the difference in variances is not statistically significant and thus the working range is adjusted;
- If $F_{cal} > F_{crit}$ – the difference in variances is statistically significant and so the working range is not adjusted.

It was also performed a visual inspections of residuals versus concentration plots in order to check if residuals are randomly distribuited around the x-axis. If variance is constant over the working range, this condition is verified and also F_{cal} will be lower than F_{crit}.

On other hand, in the presence of heteroscedastic data different approaches could be followed, such as the reduction of the working range and repeat the verification for the homogeneity of variances by F-test, until obtain the $\mathrm{F_{cal}} \leq \mathrm{F_{crit}}$ [118,121]. Another procedure which is used is the inverse of variance ($1/S^2$) in each point of the calibration curve, which is impraticable in routine analysis since several replicates are nedeed to calculate the variance (S^2). Therefore, the Weighted Least Squares Regression [112,120], choosing the approprieted weighting factor (w_i) will overcome this problem [112].

4.9.3.1. WEIGHTED LEAST SQUARES LINEAR REGRESSION

The constant variance over the whole range is not always observed. Larger deviations present at larger concentrations tend to influence (weight) the regression line. Thus if the data are heteroscedastic, the use of WLSLR is the simplest and the most effective way to harmonise the differences of variances of the line points [98,112,123].

For this study calibration curves for all the analytes were prepared with eleven calibrators each. The calibrators were uniformly distributed in the working range: 0.05, 0.1, 0.15, 0.2, 0.25, 0.5, 1, 1.5, 2, 3 and 5 µM. To each calibrator it was added the IS (0.5µM). This procedure was repeated in five different days.

Taking into account the objective of WLSLR, appropriate weighting factors ($\mathrm{w_i}$) can be calculated from the inverse of the variances at the given concentration level [97,123]. However, as was mentioned before, it is not suitable to calculate the inverse of variance in laboratory routine, mainly because it requires several determinations for each calibration point and a fresh calibration line each time the method is used, so other empirical weights based on x-variable

(concentration) or y-variable (response) may provide a simplistic approximation of variance [112,124]. The empirical weights that should be study are: $\frac{1}{x}$; $\frac{1}{x^2}$; $\frac{1}{\sqrt{x}}$; $\frac{1}{y}$; $\frac{1}{y^2}$ and $\frac{1}{\sqrt{y}}$ [112,125]. Therefore, the w_i can be obtained by the equation (4.9) [124].

$$w_i = \frac{1/S_i^2}{(\sum_i^N 1/S_i^2)/N} \tag{4.9}$$

Where, w_i is the weighting factor; S_i^2 is the appropriate empirical weight for the data ($\frac{1}{x}$; $\frac{1}{x^2}$; $\frac{1}{\sqrt{x}}$; $\frac{1}{y}$; $\frac{1}{y^2}$ or $\frac{1}{\sqrt{y}}$) and N is the number of calibration standards.

The effectiveness of the weighted regression can be assessed by calculating the percentage of the relative error (% RE), which compares the estimated concentration of the sample, from the regression equation obtained for each w_i, with theoretical or nominal standard concentration of the sample (equation (4.10)) [112].

$$\%\, RE = \frac{C_{exp} - C_{nom}}{C_{nom}} \times 100 \tag{4.10}$$

Where, C_{exp} is the estimated concentration in the sample and C_{nom} is the nominal standard concentration in the sample.

Plots of %RE versus concentration were performed for the analytes in order to choose the best weighing factor, along with the sum of %RE ($\sum \%RE$). The w_i more adequate will be the one which gives rise to a slight horizontal band of randomly distributed %RE around the x-axis and presents the smallest value of $\sum$ %RE across the whole concentration range [112,123].

In the simple linear regression model, the relationship between variables is established by a straight line, mathematically expressed by the equation (4.6), that is used to calculate the C_{exp} [112]. But, since it is used a WLSLR, the model parameters (m and b) of the weighted straight line equation need to be estimated using the term w_i according to the following equations, before being calculate the C_{exp} [112,123].

$$m = \frac{\sum w_i \times \sum w_i x_i y_i - \sum w_i x_i \times \sum w_i y_i}{\sum w_i \times \sum w_i x_i^2 - (\sum w_i x_i)^2} \tag{4.11}$$

$$b = \frac{\sum w_i x_i^2 \times \sum w_i y_i - \sum w_i x_i \times \sum w_i x_i y_i}{\sum w_i \times \sum w_i x_i^2 - (\sum w_i x_i)^2} \quad (4.12)$$

Where, x_i and y_i is the i[th] data pair of n total data pairs and w_i is the weighting factor chosen.

Finally, the correlation coefficient (R) of the weighted straight line equation, can be obtained by the following modified formula [112].

$$r = \frac{\sum w_i \times \sum w_i x_i y_i - \sum w_i x_i \times \sum w_i y_i}{\sqrt{\sum w_i \times \sum w_i x_i^2 - (\sum w_i x_i)^2} \times \sqrt{\sum w_i \times \sum w_i y_i^2 - (\sum w_i y_i)^2}} \quad (4.13)$$

4.9.4. LIMITS: LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION

The limit of detection (LOD) is the lowest amount of analyte in a sample which can be detected but not necessarily quantified. And the limit of quantification (LOQ) is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy [126].

The evaluation of LOD and LOQ was performed simultaneously with the study of linearity. For this study it was prepared one calibration curve with the three analytes with six calibrators, distributed at a lower range of the calibration curve: 0.01, 0.015, 0.025, 0.05, 0.1 and 0.15 µM. To each calibrator it was added IS solution (0.5µM).

Several approaches for determining the detection limits are possible. The approache used to calculate LOD and LOQ was based on the standard error of the response ($Sy_{/x}$) and the slope, expressed by the following equations [109]:

$$LOD = \frac{3.3 \times Sy_{/x}}{m} \quad (4.14)$$

$$LOQ = \frac{10 \times Sy_{/x}}{m} \quad (4.15)$$

Where, $S_{y/x}$ is the standard error of the response and m is the slope of the calibartion curve.

The above mentioned equations use $S_{y/x}$ for homocedastic data. In the presence of heterosceastic data some alterations must be performed and therefore the standard deviation of a predicted concentration is given by the following equation [122-124].

$$S_{(y/x)w} = \sqrt{\frac{\sum_i w_i (y_i - \hat{y}_i)^2}{n - 2}} \quad (4.16)$$

Where, $S_{(y/x)w}$ is the standard deviation of y-residuals of weighted regression line; w_i is the weight factor used in this study; y_i is the analytical signal measured and $\hat{y}_i$ is the analytical signal predicted.

4.9.5. PRECISION

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from the same sample under the stipulated conditions [105,109,118]. Precision can be expressed as the variance (S^2), standard deviation (S) or coefficient of variation (CV) of a series of measurements [109,119] and is considered at three levels: repeatability, intermediate precision and reproducibility [109].

Repeatability, also termed within-run or intra-day precision, expresses the precision of a determined sample by keeping constant the global factors (human, preparation, instrumental and geographical) over a short period of time [106,109,119].

The intermediate precision, also termed between-run or inter-day precision, expresses the precision of a determined sample by using the same procedure, despite the small changes

introduced that might occur during routine analysis (different analysts, lots of reagents, equipment among others) over an extended period of time (different days) [119].

The term reproducibility refers to reproduce the results by changing one or more of the global factors over a short or an extended period of time [119]. Usually it expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology) [109]. In this project, the reproducibility of the method was not study.

The study of intermediate precision and the repeatability consisted of analysing a test sample in *p* different runs. Within each run, the sample was analysed *n* times under repeatability conditions. All the important sources of variation were varied between each run [127]. Thus, it was prepared one calibration curve with the analytes. Eleven calibrators, uniformly distributed in the working range, were prepared: 0.05, 0.1, 0.15, 0.2, 0.25, 0.5, 1, 1.5, 2, 3 and 5 µM.

Quality controls (QCs) were also prepared at three concentration levels: low, medium and high, respectively 0.075, 0.75 and 2.75 µM for citalopram and haloperidol. For clozapine, the concentrations levels were 0.075, 0.75 and 1.25 µM. Each QC was prepared in triplicate and at each sample it was added 50 µL of IS solution at 0.5µM. The procedure was repeated along five days.

After choosing the best calibration model for each analyte, the calibration curves were obtained by the use of linear regression and the concentration of the QCs were calculated. The results obtained for the different levels of concentration for each compound were analysed by a one-way analysis of variance (ANOVA) (Table 8.7 in Appendix 8.6) [127,128].

Then, it were calculated the intermediate precision and the repeatability, expressed in terms of % CV [129], through the following equations [98,120]:

$$\%\ CV_I = \frac{S_I}{\overline{X}} \times 100 \qquad (4.17)$$

$$\%\ CV_r = \frac{S_r}{\overline{X}} \times 100 \qquad (4.18)$$

Where, $\mathrm{CV_r}$ is the coefficient of variation of repeatability; $\mathrm{S_r}$ is the standard deviation of repeatability; $\mathrm{CV_I}$ is the coefficient of variation of intermediate precision; $\mathrm{S_I}$ is the standard deviation and $\overline{\mathrm{X}}$ is the mean value of concentrations.

The limits of acceptable variability were set at 15% for all the concentrations, except at the LOQ, for which 20% was accepted [105,106].

4.9.6. ACCURACY

The accuracy of an analytical procedure expresses the degree of agreement between the value measured by the procedure to the value accepted as reference value [126].

The experimental procedure to evaluate the accuracy was similar to the procedure used for the precision. Therefore, accuracy was evaluated in terms of percentage of mean relative error $(\%\ MRE)$ [109,112,120,130].

$$\%\ MRE = \frac{\bar{C}_{exp} - C_{nom}}{C_{nom}} \times 100 \qquad (4.19)$$

Where, $\bar{C}_{exp}$ is the mean of the estimated concentration in the sample and C_{nom} is the nominal standard concentration in the sample.

As acceptance criteria, the accuracy for each level of concentration should be within ±15% of the nominal concentration, except for the LOQ for which it should be within ±20% [105,106].

4.9.7. CARRY-OVER

It is important to know how much analyte is retained or "carried over" from a preceding sample into the following sample, since it can affect the accuracy and precision of the method, especially when a low concentrated sample is injected after injections of samples at high levels of concentration. Thus, to evaluate the carry-over phenomena, five blank samples (2% ACN: 0.1% FA) were injected after the injection of the highest level of concentration standard (5 µM).

The procedure was repeated for the calibrators with 0.5 µM and 0.05 µM of concentration. This procedure was repeated in three different days.

Therefore, the carry-over in the blank sample following the highest calibrator should not be greater than 20% of LOQ (equation (4.20))and 5% for the internal standard [105].

$$\bar{A}_{blank\ sample} \leq 0.20 \times \bar{A}_{LOQ} \quad (4.20)$$

Where, $\bar{A}_{blank\ sample}$ is the mean of peak areas of the blanks samples; $\bar{A}_{LOQ}$ is the mean of peak area for LOQ.

4.9.8. RECOVERY

The recovery of a method can be measured comparing the response of analyte spiked in the sample before being processed with the response of the same quantity of analyte, spiked into matrix after the extraction procedure. This measure indicates if the method provides a response for the entire amount of analyte that is present in the sample [120,130,131].

To evaluate the recovery three levels of concentration were selected (low, medium and high), corresponding to 0.1, 0.5 and 3 µM, with plasma from 6 different sources. Each concentration was performed in triplicate. For each level two aliquots were prepared with 70µL each one of them:

- One was spiked with 20 µL of a solution containing citalopram, clozapine, haloperidol, desipramine and haloperidol-D4 and then was subjected to the extraction procedure (see section 4.7);
- The other was first subjected to the extraction procedure (see section 4.7) and at the end of the procedure (when the sample is reconstituted) was spiked with 20 µL of a solution containing citalopram, clozapine, haloperidol, desipramine and haloperidol-D4.

In an analogous way, to evaluate the recovery in the hair three levels of concentration were selected (low, medium and high), corresponding to 0.1, 0.5 and 3 µM, with hair from 6 different sources. Each concentration was performed in triplicate. For each level two aliquots were prepared each one with hair weighing between 1.9 – 3.2 mg:

- One was spiked with 20 µL of a solution containing citalopram, clozapine, haloperidol, desipramine and haloperidol-D4 and then was subjected to the extraction procedure (see section 4.8);
- The other was first subjected to the extraction procedure (see section 4.8) and at the end of the procedure (when the sample is reconstituted) was spiked with 20 µL of a

solution containing citalopram, clozapine, haloperidol, desipramine and haloperidol-D4 and then was subjected to the extraction procedure.

To each sample, for plasma and hair, 10 µL of IS at 0.5µM were added, at the end of the procedure. The IS used in the study of recovery was Sulfamethazine-D4, since it was also evaluated the recovery of the two IS (desipramine and haloperidol-D4) used for the method that was being developed.

The samples that were fortified after the extraction represent 100% recovery [120]. And it was calculated the recovery, in percentage, by the next equation (4.21) [120,131].

$$\%\, Recovery = \frac{A_{ab}/A_{ISb}}{A_a/A_{IS}} \times 100 \qquad (4.21)$$

Where, $\mathrm{A_{ab}}$ is the absolute area of the analyte fortified in the matrix before the extraction; $\mathrm{A_{ISb}}$ is the absolute area of the internal standard fortified in the matrix before the extraction; $\mathrm{A_a}$ is the absolute area of the analyte fortified in the matrix after the extraction at the same level of concentration and $\mathrm{A_{IS}}$ is the absolute area of the internal standard fortified in the matrix after the extraction at the same level of concentration.

The acceptance value for the recovery of the analyte does not need to be 100%, but the extent of recovery of an analyte and of the IS should be consistent, precise, and reproducible [106]. The recovery of the IS should be within 15% of that determined for the analyte [120].

4.9.9. MATRIX EFFECTS

The matrix effects (ME) can be defined as the difference between the mass spectrometric response for an analyte in standard solution and the response for the same analyte in a biological matrix [132].

To evaluate the matrix effect three levels of concentration were selected (low, medium and high), corresponding to 0.1, 0.5 and 3 µM. For plasma, 70µL of blank plasma was subjected to the extraction procedure (see section 4.7). At the end of the procedure (when the sample is reconstituted) the sample was spiked with 20 µL of a solution containing citalopram, clozapine,

haloperidol, desipramine and haloperidol-D4. Each concentration level was prepared in triplicate. In addition it was prepared a standard solution with the five compounds with equivalent levels of concentration.

The procedure to evaluate the matrix effect in hair samples was similar, with the exception to the amount of sample used (3-4 mg) and the extractive procedure (see section 4.8).

To each sample type, plasma and hair, 10 µL of IS at 0.5µM were added, at the end of the procedure. And to prepare a standard solution (citalopram, clozapine, haloperidol, desipramine and haloperidol-D4) with equivalent levels of concentration, to 50 µL of this solution, it was added 50 µL of IS (Sulfamethazine-D4) at 0.5µM.

The difference in response between the post-extraction sample and the standard solution divided by the $\mathrm{standard\ solution}$ response (4.22) determines the degree of matrix effect occurring to the analyte [131,133].

$$ME = \frac{A_{\mathrm{post-extracted\ spiked\ sample}} - A_{\mathrm{standard\ solution}}}{A_{\mathrm{standard\ solution}}} \qquad (4.22)$$

Where, ME is the matrix effect; $\mathrm{A_{post-extracted\ spiked\ sample}}$ is the peak area of the analyte spiked in the sample after the extraction procedure and $\mathrm{A_{standard\ solution}}$ is the peak area of the analyte for the same concentration in standard solution

For this equation, a negative result indicates suppression and a positive result indicates enhancement of the analyte signal. Thus, a calculated value of zero would represent no ME [131,133].

The assessment of the presence of a relative matrix effect, expressed as $\%\ \mathrm{CV}$, can be made based on direct comparison of the peak areas of an analyte spiked into extracts originating from different sources of a Blank matrix (equation (4.23)) [132].

$$\%\ CV_{ME} = \frac{S}{\overline{X}} \times 100 \qquad (4.23)$$

Where, $\mathrm{CV_{ME}}$ is the coefficient of variation of relative ME; S is the sandard deviation and $\overline{\mathrm{X}}$ is the mean value.

When the values of $\%\,\mathrm{CV}$ are high this might indicate that the response originating from the same amount of an analyte is different in different sources of a Blank matrix [132].

4.10. APPLICATION OF THE ANALYTICAL METHOD DEVELOPED IN REAL SAMPLES

The method that was developed was applied for the analysis in samples of hair and plasma collected from mice (five replicates per day) treated with different drugs: citalopram, clozapine, haloperidol, and saline solution (control samples) for different periods of time: 1, 2, 4, 8, 15 and 30 days.

In order to see if any differences statistically significant between the days a non-parametric test, Kruskal–Wallis test (or H test), was performed.

The test proposed by Kruskal and Wallis evaluates whether two or more samples are from the same distribution [134,135]. The null hypothesis is that all the samples come from identical population distributions [134-136].

Given multiple samples (k) with n_i observations in the ith sample, the H statistic tests the null hypothesis that the samples come from identical population distributions [136].

This hypothesis is tested by ranking the observations from 1 to N (giving each observation in a group of ties the mean of the ranks tied), finding the k sum of ranks, and computing an H statistic [134,136]. If there is no tie in all the values, the test statistic is:

$$H = \frac{12}{N(N+1)} \sum_{i=1}^{k} \frac{R_i^2}{n_i} - 3(N+1) \qquad (4.24)$$

Where, N is the total number of values in all samples; n_i is the number of values contained in the i^{th} sample, and R_i is the sum of ranks in i^{th} sample.

For ties in the scores, the tied observations are assigned the average of the ranks that would be assigned if there were no ties [136] and thus the calculation of the test statistic should be changed slightly [134].The correction factor for ties is:

$$C = 1 - \frac{\sum_{i=1}^{g}(t_i^3 - t_i)}{N^3 - N} \quad (4.25)$$

Where, g is the number of groups of tied values, and t_i is the number of tied values in the i^{th} group.

Then it can compute *H* with these new ranks but first the H is divided by this correction [134,136]:

$$H_c = \frac{H}{C} \quad (4.26)$$

Actually the equation (4.26) is the general solution that holds no matter there are ties or not. If there is no tie, C = 1 and thus, $\mathrm{H_c} = \mathrm{H}$ [134].

This statistic is then compared with a tabled value for the H statistic. This comparison will determine whether the null hypothesis is accepted or rejected [136].

If there are more than five observations in each sample, the H statistic has been shown to be distributed approximately as a chi-square distribution (with degrees of freedom = C-1) and therefore chi-square tables are used for the comparison. If the samples have fewer than five observations special approximations through exact tables, called the "critical values" for the H statistic [136].

If the computed value of the H statistic is larger than the tabled value of the H statistic, the results are significant and the null hypothesis is rejected and the probability that the null hypothesis is true is less than 0.05 [136].

When the obtained value of the H statistic is statistically significant, it indicates that at least one of the groups is different from the others. It does not indicate, however, which groups are different or whether the difference is meaningful, nor does it specify how many of the groups are different from each other [136].

In order to see where the differences are presented is used a procedure, called "multiple comparisons methods" that constructs pair-wise multiple comparisons to locate the source of significance [136]. They are also called post hoc or posteriori tests as they are only carried out after the fact, i.e., after a significant effect.

An effective way of doing pairwise simultaneous inference was introduced by Dunn [137]. When sample sizes are unequal, or in the presence of tied ranks, it is recommended the Dunn's test. Because it takes into account tied ranks, when group samples sizes are equal [135].

First the data is combined and ranked. Second it is found the group mean ranks and then is calculated the standardized absolute differences of these average ranks by the next equations, which is the standard error that has a correction term for tied ranks[137,138].

$\frac{|\bar{R}_i - \bar{R}_j|}{S}$, j=1,.., k and j≠i (4.27)

$\bar{R}_i = \frac{R_i}{n_i}$, i=1,....,k (4.28)

$$s = \sqrt{\left(\frac{N(N+1)}{12}\right) \times \left(\frac{1}{n_i} + \frac{1}{n_j}\right)} \quad (4.29)$$

Where, R_i is the sum of the ranks for the i^{th} treatment (i=1,....,k and j=1,.., k); k is the number of samples (k > 2); n_i is the number of observations for the i^{th} treatment.

A new α is computed for each multiple comparisons test based on the overall α level for the study and the number of comparison to be made. The new α is equal to α/C, where C is the number of post hoc tests to be performed [139].

5. RESULTS AND DISCUSSION

5.1. METHOD DEVELOPMENT

The results obtained during method development, used to determine which parameters to use in the spectrometric conditions are presented in this section.

The optimization of some parameters is necessary, as was mentioned in the section 4.6.2, to establish optimal conditions for each of the molecules.

One of the important parameters that is optimized is the CE, which generate product ion scans at different collision energies (gradual increasing) to select the product ion candidate to monitor. This way it can be obtained the optimum CE for each product ion. As an example, the CE values corresponding to three different fragments of haloperidol are represented in the Figure 5.1, where for instance to obtain the lowest fragment (with m/z of 95) will need a higher CE than to obtain the largest fragment (with m/z of 164). They represent the amount of energy that the precursor ions receive as they accelerated into the collision cell, where they collide with gas molecules and fragments.

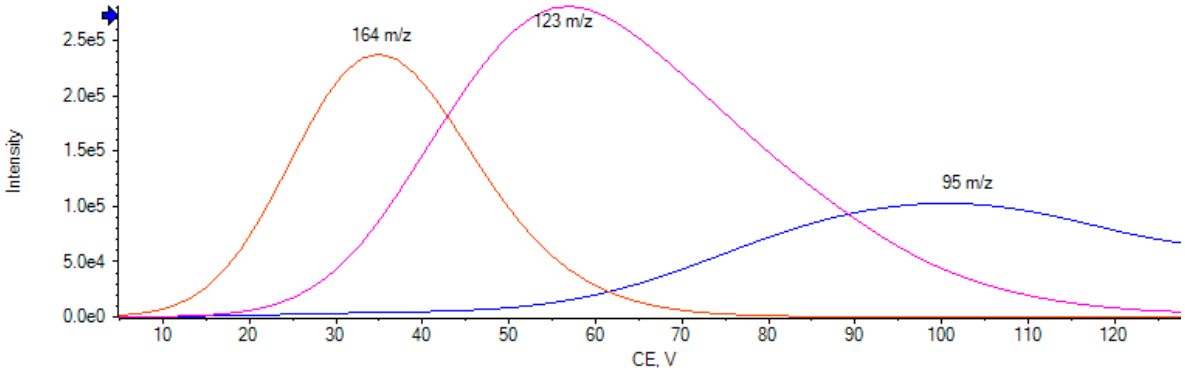


Figure 5.1- Collision energy ramping values for three fragments of haloperidol. The fragments that each peak corresponds are indicated above the peak.

Another parameter that is optimized in the development of the method is the DP. This parameter is used to minimize solvent cluster ions, which may attach to the analyte. If DP is too high, the analyte ion itself may fragment, so an appropriate value for each molecule is necessary. An example of optimization of this parameter is shown in Figure 5.2 for the haloperidol, where the maximum height of the peak corresponds to the best value of DP.

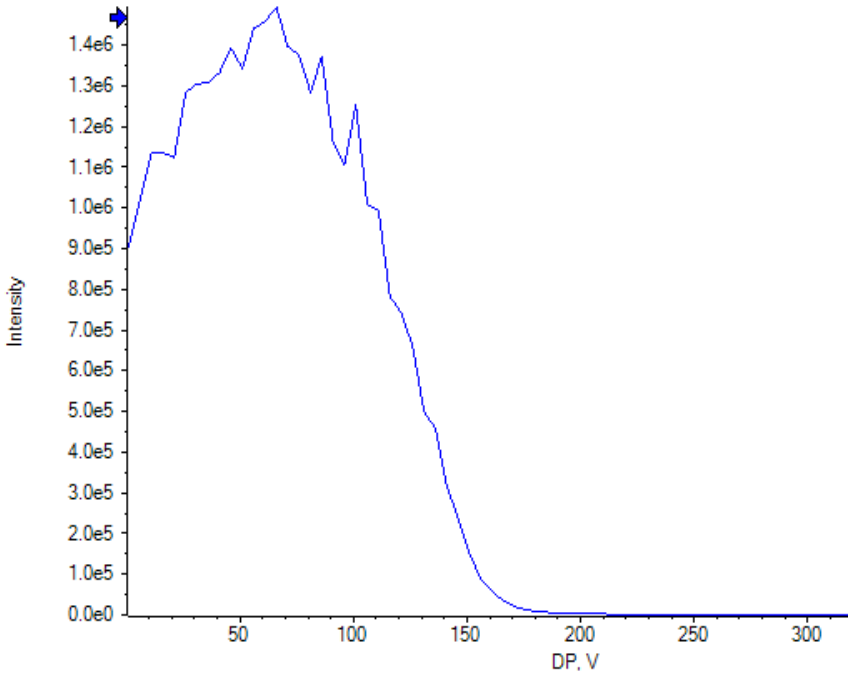


Figure 5.2- Declustering potential ramping for haloperidol. Results show a maximum intensity at 61V for haloperidol.

After these parameters are optimized, fragmentation mass spectra for each analyte of interest can be observed with all fragments of the analyte that is obtained in CID conditions. This parameter controls the pressure of collision gas in the collision cell during Q3 scan, helping to focus the ions as they pass through the collision cell (q2). With the fragmentation spectra it is possible to choose what transitions to monitor. The choice of product ions that are close in m/z to the precursor implies that the neutral loss fragment is of low molecular mass. For many reasons product ions of 'low mass' can be problematic for MRM detection. One of these reasons is due to the observation that 'chemical noise' (background) is considerably more intense at lower m/z values. The ideal product ion to use in MRM method would be the one that can be observed at good relative abundance in the spectrum.

The fragmentation spectrum of haloperidol (Figure 5.3) shows a peak with m/z of 376 corresponding to the intact molecule, and three most intense fragments, with m/z 123, with m/z 165 and with m/z 95, which were chosen to monitor the haloperidol, for the reasons that were stated before. It can also be seen other peaks less intense with m/z of 113 and 75.

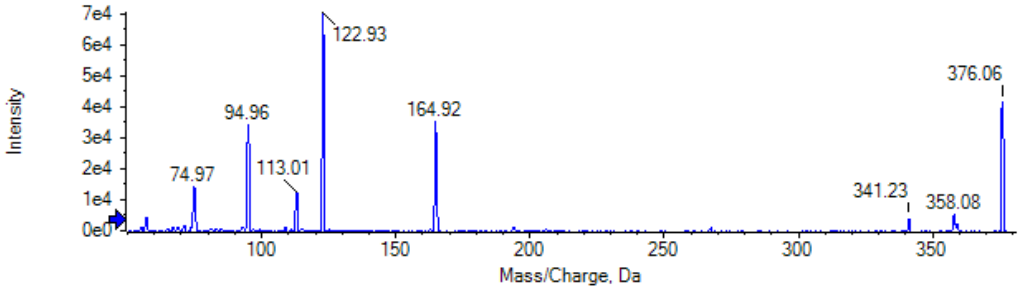


Figure 5.3- Averaged fragmentation mass spectra of haloperidol. A solution of 0.453 µM of haloperidol was infused at a flow rate of 9 µL/min and CE was ramped between 5 to 130 eV.

These fragments are characteristic for haloperidol and they are due to loss of parts of the molecule that are illustrated in the Figure 5.4. The fragment that has the highest intensity, with m/z of 123, corresponds to F-C_6H_4-C≡O^+. The second more intense, with the m/z of 165, corresponds to F-C_6H_4-C(=O)-$CH_2CH_2CH_2^+$. And lastly, the fragment with m/z of 95 corresponds to F-$C_6H_4^+$.

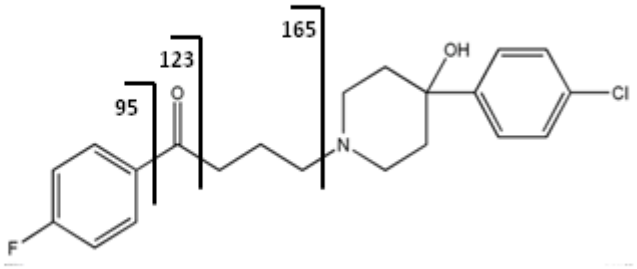


Figure 5.4- Characteristic fragments observed in mass spectrometry for the molecule of haloperidol $[M+H]^+$, with m/z of 376 [140].

The same approach was used for clozapine (Figure 5.5). Once again, the peak with m/z of 327 corresponds to the m/z value of the intact molecule because here the value of CE applied was low. It can be observed in the spectra of clozapine, three more intense peaks. The fragments with m/z of 192, 270 and 164, were consequently chosen to monitor this molecule.

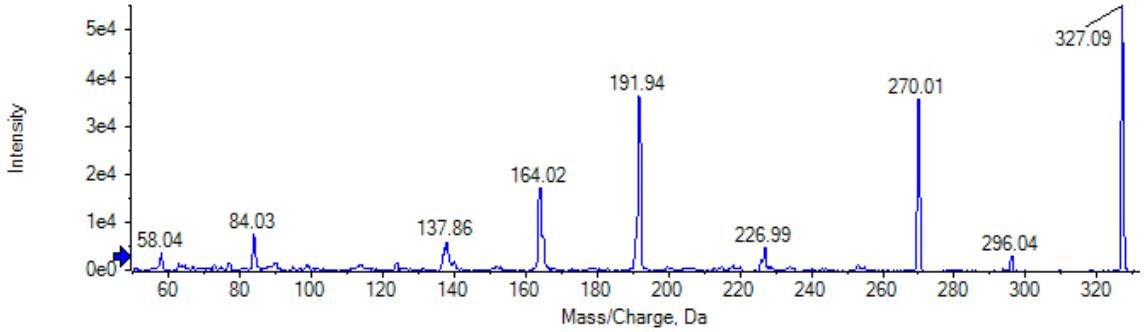


Figure 5.5- Averaged fragmentation mass spectra of clozapine. A solution of 1 µM of clozapine was infused at a flow rate of 9 µL/min and CE was ramped between 5 to 130 eV.

The fragments characteristic for clozapine are illustrated in the Figure 5.6. The most intense peak, with m/z of 192, corresponds to $C_{13}H_8N_2$. The second peak more intense, with m/z of 270, corresponds to $C_{15}H_{12}ClN_3$.

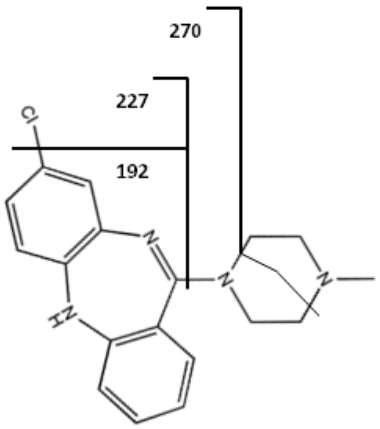


Figure 5.6- Characteristic fragments observed in mass spectrometry for the molecule of clozapine $[M+H]^+$, with m/z of 327 [140].

The same approach was used for citalopram (Figure 5.7). The peak with m/z of 325 corresponds to the m/z value of the intact molecule. As it can be seen in the spectrum, the fragments with m/z of 262 and 109 are the most intense fragments so consequently these were chosen to monitor this molecule.

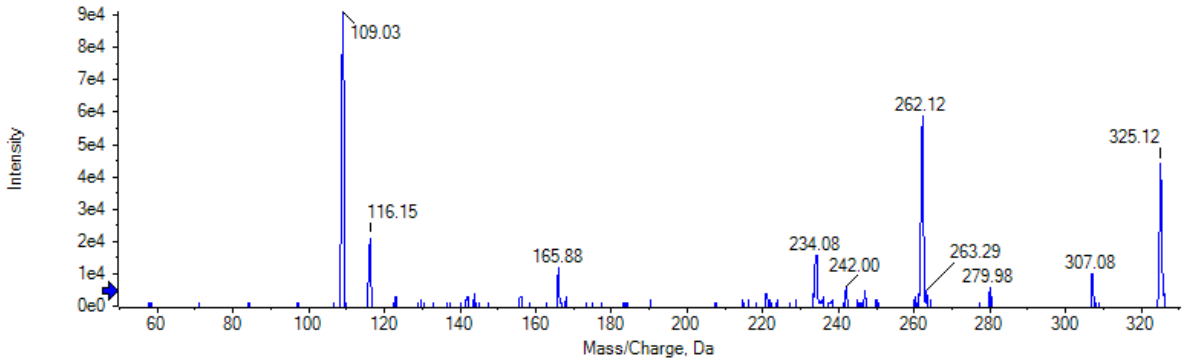


Figure 5.7- Averaged fragmentation mass spectra of citalopram. A solution of 0.125 µM of citalopram was infused at a flow rate of 9 µL/min and CE was ramped between 5 to 130 eV.

The fragment 262 m/z corresponds to C18H13NF, probably is due to the loss of dimethylamine that corresponds to the fragment with m/z of 280. Another intense fragment with m/z of 116 corresponds to the molecular structure $N{\equiv}C\text{-}C_6H_4\text{-}CH_2^+$. And the most intense fragment, with m/z of 109, corresponds to $F\text{-}C_6H_4\text{-}CH_2^+$ (Figure 5.8.)

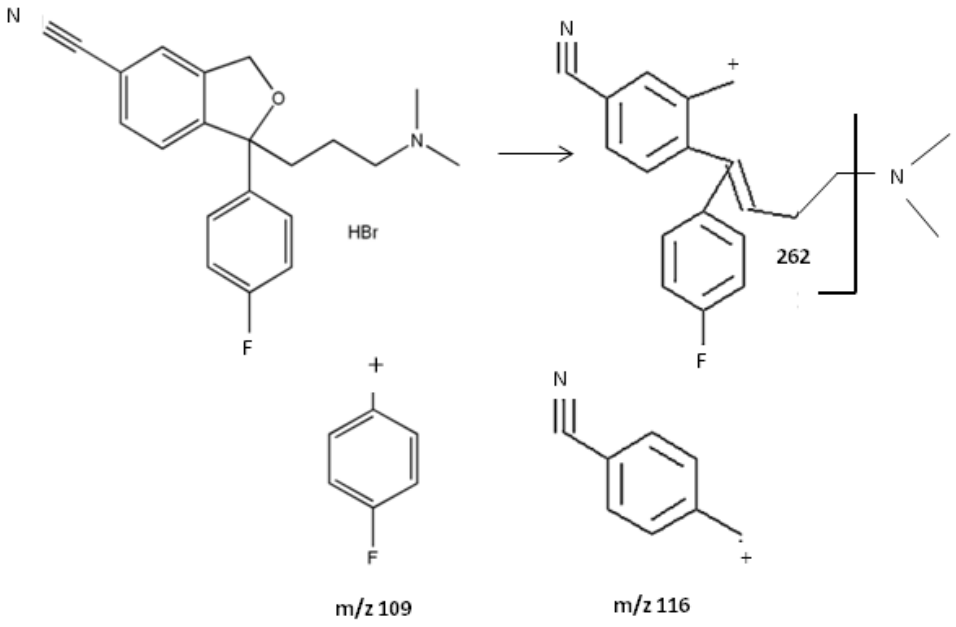
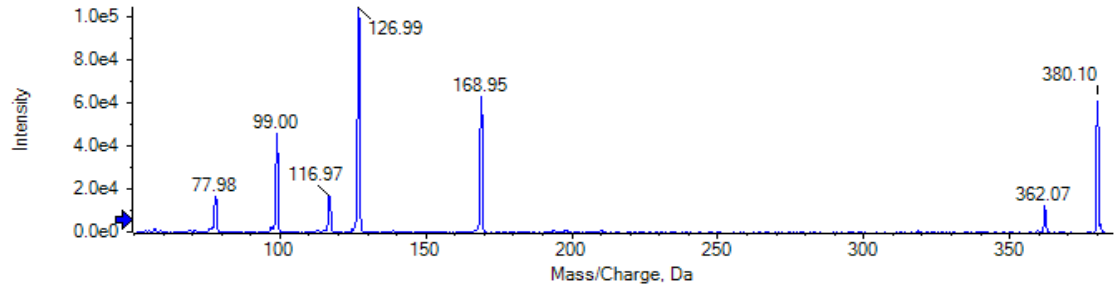


Figure 5.8- Characteristic fragments observed in mass spectrometry for the molecule of citalopram $[M+H]^+$, with m/z of 325 [140].

The fragmentation mass spectra for the two internal standards (Haloperidol-D4 and desipramine) are represented in the Figure 5.9.

a)



b)

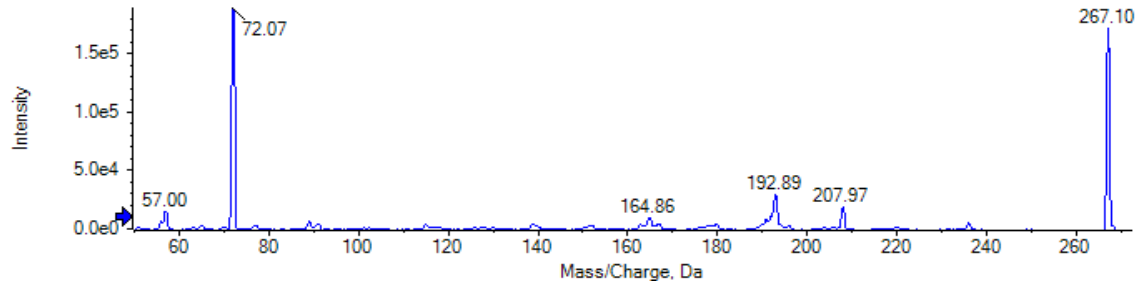
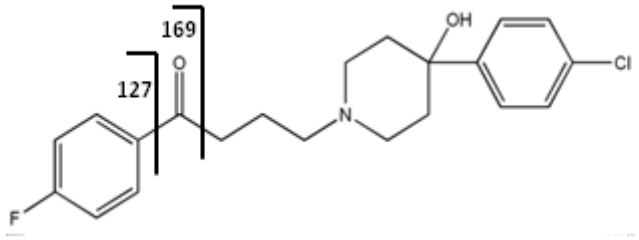


Figure 5.9- Averaged fragmentation mass spectra of haloperidol-D4 a) and of desipramine b). A solution of 0.100 µM for haloperidol-d4 and 1.14 µM for desipramine were infused at a flow rate of 9 µL/min and CE was ramped between 5 to 130 eV.

The fragmentation of the molecule of haloperidol-D4, used here as internal standard is very similar to the fragmentation spectrum of haloperidol, since the difference between them is the substitution of four hydrogens by four deuteriums, with m/z of 380. The characteristic fragments are represents in Figure 5.9.a.

The peak with m/z of 267 corresponds to intact molecule of desipramine. The most intense fragment, with m/z of 72, corresponds to CH_2=CH–CH_2–$NH_2CH_3^+$ and is represented in the Figure 5.10.

a)



b)

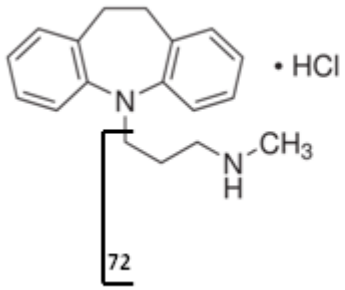


Figure 5.10- Characteristic fragments observed in a) the molecule of haloperidol-D4 $[M+H]^+$, with m/z of 380 and b) the molecule of desipramine $[M+H]^+$, with m/z of 267 [140].

5.1.1. ISOTOPIC IDENTIFICATION

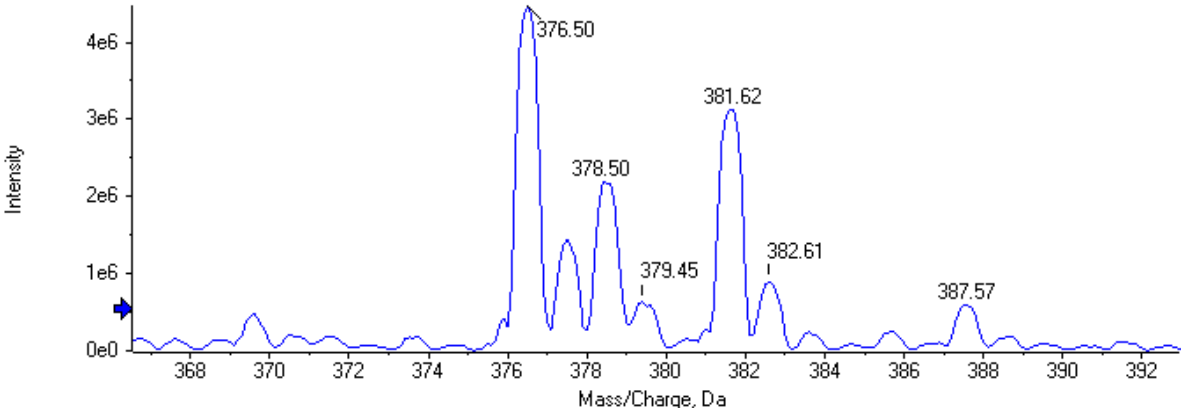
As it was demonstrated before, the compound structure was evaluated and this is important because it can be confirmed the molecule structured and if it contains any elements such as chlorine, bromine or boron, which would result in a unique isotope pattern.

In this project two of the three compounds (haloperidol and clozapine) in their structures, besides carbon and others, have an element that appears as natural isotope, the chlorine. The natural chlorine is a mixture of 75.77% of isotope ^{35}Cl and 24.23% of isotope ^{37}Cl [78,96] .

In the Figure 5.11, it can be observed the isotopic distribution for haloperidol and also for clozapine. For the haloperidol, the peak with m/z of 376 corresponds to the molecule with the isotope ^{35}Cl and the peak with m/z of 378 corresponds to the molecule with the isotope ^{37}Cl (Figure 5.11.a.).

A similar analysis can be performed with the molecule of clozapine, where the peak with m/z of 327 corresponds to the molecule with the isotope ^{35}Cl and the peak with m/z of 329 corresponds to the molecule with the isotope ^{37}Cl (Figure 5.11.b.)

a)



b)

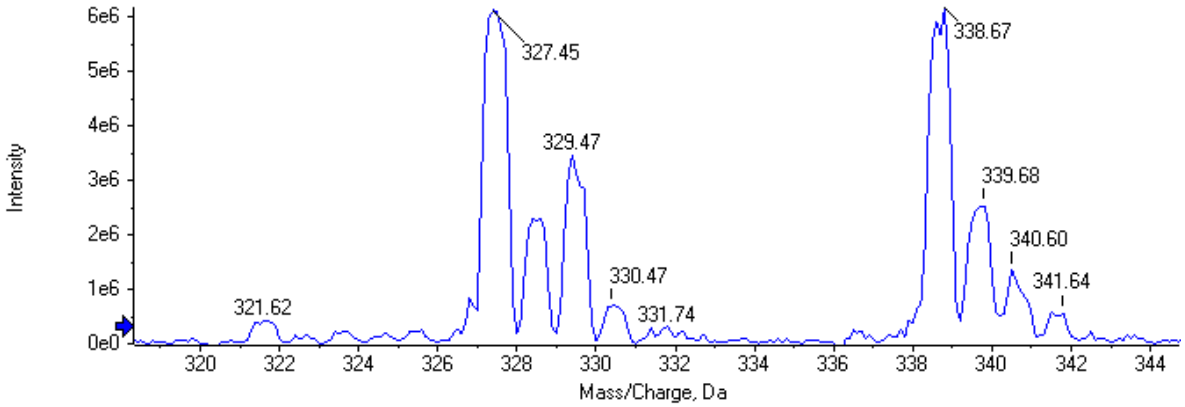


Figure 5.11- Isotopic distribution for haloperidol a) and clozapine b). A solution of 0.08 µM for haloperidol and 1 µM for clozapine were infused at a flow rate of 9 µL/min and CE was ramped between 5 to 130 eV.

Next, the chromatography was evaluated with the RT and the transitions of the isotopes being shown in Table 5.1. These fragments of chlorine 37 were monitorized only for confirmation that the present molecule is the molecule of interest because these transitions do not work so well, for the quantification, as the others transitions. However, this is one more way of confirming that we are in the presence of the molecule of interest.

Table 5.1- Mass/charge ratio (m/z) of the ionic fragments of the isotopic transition of clozapine and haloperidol monitorized in MRM mode and the retention time (RT) used for confirmation of the compounds.

Compound	Transitions (m/z)		RT (min)
	Q1	Q3	
Clozapine (chloride 37)	329.2	84.1	4.05
		192.2	4.05
Haloperidol (chloride 37)	378.2	164.8	6.43
		122.8	6.43
		95.2	6.43

5.2. COMPOUND IDENTIFICATION

The previous results allow the selection of the ionic transitions (m/z) to be monitored in MRM mode (Table 5.2) to each PD and also the IS used.

Table 5.2- MRM transitions and retention time (RT) used for the identification of compounds.

Compound	Transitions (m/z)		RT (min)
	Q1	Q3	
Citalopram	325.3	109	6.06
		261.9	6.07
		83.1	6.06
Clozapine	327.2	269.9	4.07
		191.9	4.06
		163.8	4.05
Haloperidol	376.0	164.7	6.42
		122.9	6.43
		94.8	6.43
Desipramine	267.3	72.2	7.14
		208	7.16
		190.8	7.13
Haloperidol – D4	380.2	127.1	6.39
		168.4	6.41
		98	6.42

The criteria of WADA (section 5.9.) were applied and the results are presented and explained in the section related to the Selectivity (section 5.3.1). To apply the criteria for unequivocal identification of PD, mandatory the use of an IS for all analytes.

Often, the choice of an appropriate internal standard lies on the following criteria: the IS should not be present in the samples to be analyzed and the IS also should be chemically and physically similar to the analyte (ideally, a stable-isotope-labeled IS), and should elute in a similar time to the analyte [79].

An isotope that is used extensively is deuterium, here the molecular weight of the compound is higher than the unlabelled precursor and this is often sufficient to ensure that the ions in the molecular ion region of the unlabelled compound do not occur at the same m/z ratios as those from the labelled molecule [79]. For these reasons, for the haloperidol it was used as IS the haloperidol-D4.

Desipramine was the IS used for the citalopram and the clozapine, as already mentioned in literature [90,141].

5.3. ANALYTICAL METHOD VALIDATION

5.3.1. SELECTIVITY

Potential interfering substances in a biological matrix include endogenous matrix components, metabolites, decomposition products, concomitant medication and other exogenous compounds [106]. So it is very important to obtain a signal free from the influence of other species contained in the sample and this signal should be unequivocally assigned to the analyte of interest [142].

One simple way to establish method selectivity in biological fluids, which is becoming state of the art in the procedures used in the majority in the published work related to the method validation, is to prove the lack of response in blank matrix. Normally is used at least six independent sources of the blank matrix [101,143]. However, interferences, present in small quantities, may adversely affect the quantification of unknown samples at concentrations approaching the limit of quantification [143]. Therefore, it is recommended that the selectivity of the method should be established with respect to endogenous substances, metabolite(s) and

known degradation products [143], spiking pure substance of interest with appropriate levels in a blank matrix and process the sample [144]. These results are compared with blank matrix processed without the analyte [119,120,145].

Thus, as was mentioned in the methodology (section 4.9.1) six individual sources of blank matrix (plasma and hair) were divided in two aliquots, each. One aliquot was spiked with the analytes and another aliquot was not spiked. It was considered that the negative samples were the aliquots without the analytes and the IS (citalopram, clozapine, haloperidol, desipramine and haloperidol-D4) and the aliquots that were spiked with the analytes were considered the positive samples.

In the results for the hair, for instance, the selectivity of the method for haloperidol can be evaluated visually in the chromatogram, where one transition was selected, 376.0→164.7 (Figure 5.12).

It can be observed in the negative samples (Figure 5.12 a-f) that only in sample 5 a small peak appears with the same RT of haloperidol. However, the peak was not integrated because the S/N was lower than 3. Still, in the positive samples (Figure 5.12 g-l), haloperidol was found in the expected retention time, with a good intensity signal and apparently with no more interferents.

Selectivity results for plasma are presented in the Figure 5.13 also for the transition 376.0→165 of the haloperidol.

In the negative samples (Figure 5.13. a-f)) some small peaks are observed in all samples at the same RT of the haloperidol. However, the S/N was lower than 3 for all samples. In the positive samples (Figure 5.13. g-l)) only the haloperidol appears with a higher intensity than the intensity of the interference which appeared in negative samples in the same RT.

To confirm the selectivity, i.e., unequivocal identification of the analytes, was also used the WADA criteria [110] pre-establish in the section 4.9.1. by analyzing the data in the excel spreadsheet created for the analysis of analytical method validation data. Both positive and negative samples were also analyzed in the same excel spreadsheet and the criteria for the negatives were the reverse of the positive ones.

In the WADA technical report, the RT and the RT_{Ratio} are presented as criterion for LC part and also it is stated that between RT and RT_{Ratio} it can be chosen whichever is smaller [110]. In this case it was chosen the RT_{Ratio} because better results were obtained with this one.

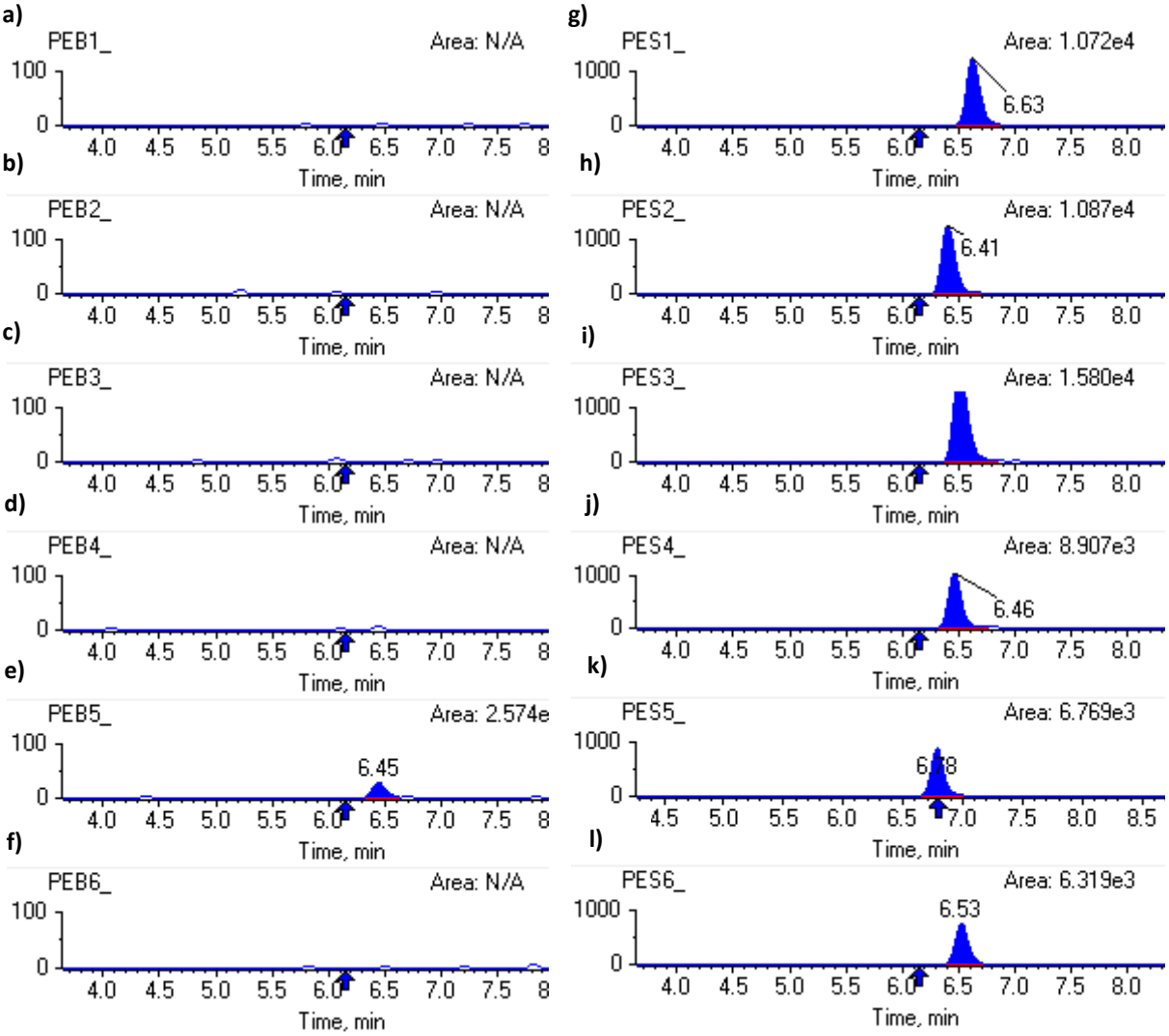


Figure 5.12- Chromatographic spectra of the haloperidol (376→164.7) for the selectivity in hair. a-f) Six different sources of blank samples that were not fortified with the citalopram, clozapine, haloperidol, desipramine and haloperidol-D4 (negative samples). **g-l)** The same six blank samples that were fortified with citalopram, clozapine, haloperidol, desipramine and haloperidol-D4 (positive samples).

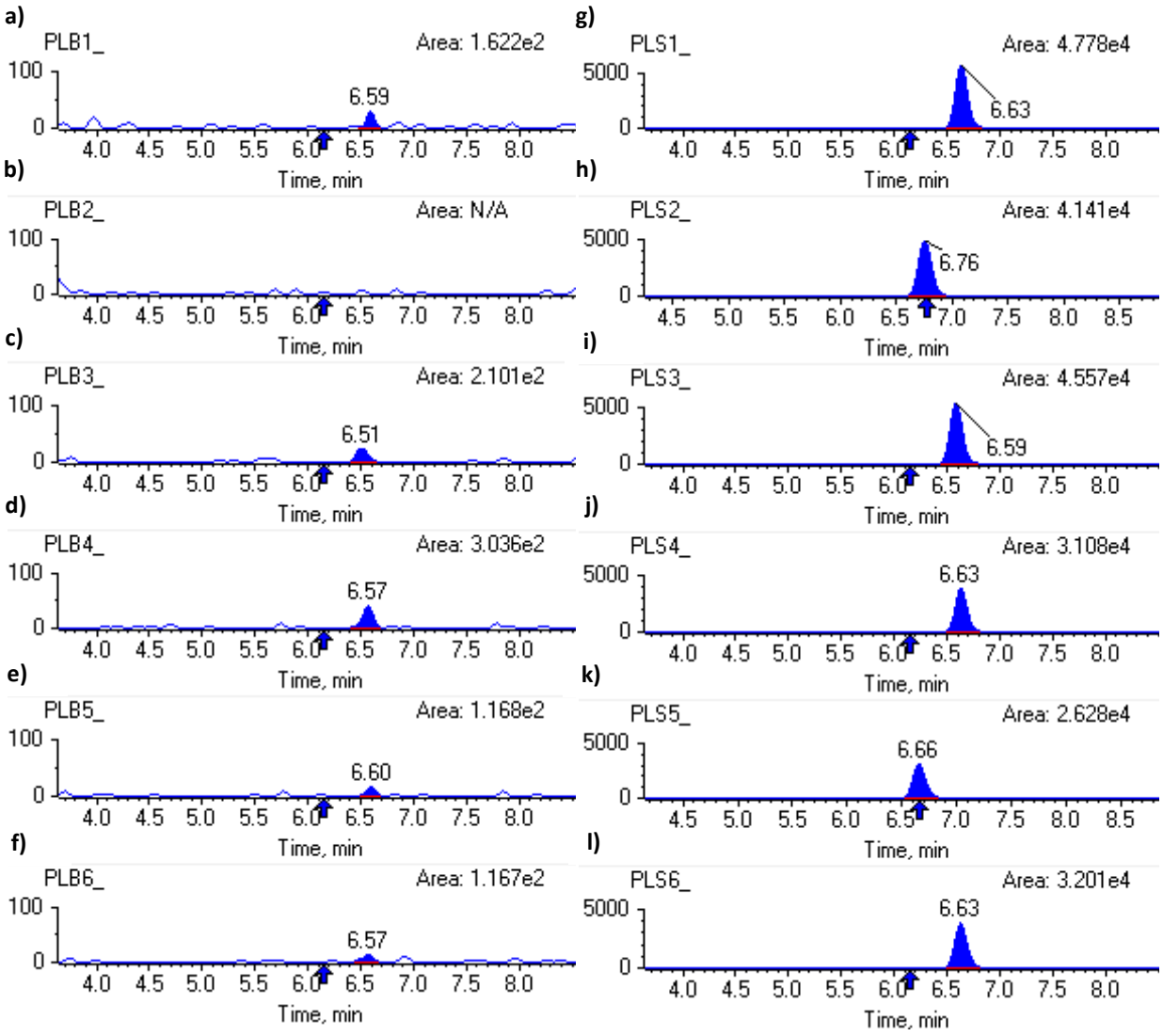


Figure 5.13- Chromatographic spectra of the haloperidol (376→164.7) for the selectivity in plasma. a-f) Six different sources of blank samples that were not fortified with the citalopram, clozapine, haloperidol, desipramine and haloperidol-D4 (negative samples). **g-l)** The same six blank samples that were fortified with citalopram, clozapine, haloperidol, desipramine and haloperidol-D4 (positive samples).

An example for one of the six hair samples that were used to study this parameter for haloperidol is presented in the Table 5.3. Through the analyses of the table, it can be seen that in the negative samples no chromatographic signal was detected and in the positives are within the values calculated according to WADA.

Table 5.3- Application of acceptance WADA criteria for unequivocal identification of the haloperidol in hair.

	Transition	Absolute area	Relative area	S/N	RT_A	RT_{IS}	RT_{Ratio}
Positive 1	376.0→164.7	10716.280	55.225	37.544	6.625	6.591	1.005
	376.0→122.9	19404.737	100.000	67.472	6.622	6.591	1.005
	376.0→94.8	9173.733	47.276	32.296	6.622	6.591	1.005
	Transition	**Absolute area**	**Relative area**	**S/N**	**RT_A**	**RT_{IS}**	**RT_{Ratio}**
Negative 1	376.0→164.7	-	-	-	-	-	-
	376.0→122.9	-	-	-	-	-	-
	376.0→94.8	-	-	-	-	-	-
	Transition	**Relative Abundance range**		**S/N**	**ΔRT_{Ratio}**		
Criteria	376.0→164.7	45.225	65.225		0.995	1.015	
	376.0→122.9	90.000	110.000	>3	0.995	1.015	
	376.0→94.8	37.821	56.731		0.995	1.015	

In relation to the other five positive samples (see Appendix 8.2 in the Table 8.6) only one transition for two positives (positive 4, transition 376.0→94.8 and positive 5, transition 376.0→164.7) is outside the interval for the relative abundance. However the S/N was higher than three and the RT_{Ratio} was inside the interval pre-established. Therefore, it is expectable to unequivocally identify haloperidol. For the other 5 negatives, with the exception of negative 5, nothing was detected that interfere with the detection of haloperidol.

In the case of citalopram (see Appendix 8.2 in the Table 8.4), with the exception of one transition (325.3→261.9) for positive 3, all values for the positives samples are within the criteria. All the negative samples, in the first transition (325.3→109.0), presented a chromatographic signal, but the S/N was lower than three. Therefore the method is selective for this analyte.

In the case of clozapine (see Appendix 8.2 in the Table 8.5), some values of RT_{Ratio} and also of relative abundance do not fit the criteria that was pre-establish for this analyte. Although in negatives no interference peaks were detected.

The same approach was used for another matrix, plasma, and the results of one of the six blank samples that were used for haloperidol are represented in Table 5.4, where all values for all transitions in the positive sample are within the criteria and also for the negative sample. In relation to the other 5 positive samples all transitions are within the criteria proposed by WADA.

Also in negative samples no interferences were observed and therefore, the method is selective for haloperidol in plasma samples (see Appendix 8.2 in the Table 8.3).

Table 5.4- Application of acceptance WADA criteria for unequivocal identification of the haloperidol in plasma.

	Transition	Absolute area	Relative area	S/N	RT_A	RT_{IS}	RT_{Ratio}
Positive 2	376.0→164.7	41407.481	59.955	147.232	6.755	6.719	1.005
	376.0→122.9	69064.705	100.000	256.026	6.754	6.719	1.005
	376.0→94.8	26602.638	38.518	97.336	6.754	6.719	1.005
	Transition	**Absolute area**	**Relative area**	**S/N**	**RT_A**	**RT_{IS}**	**RT_{Ratio}**
Negative 2	376.0→164.7	-	-	-	-	6.497	-
	376.0→122.9	77.749	100.000	0.472	6.302	6.497	0.970
	376.0→94.8	-	-	-	-	6.497	-
	Transition	**Relative Abundance**		**S/N**		**ΔRT_{Ratio}**	
Criteria	376.0→164.7	47.874	67.874		1.004		1.006
	376.0→122.9	90.000	110.000	>3	1.005		1.007
	376.0→94.8	29.723	44.585		1.004		1.006

In the case of citalopram, the second transition (325.3→261.9) of four positives (see Appendix 8.2 in the Table 8.1), the value of relative area is outside the interval of criteria established but the S/N is greater than three. The RT_{Ratio}, only for one transition of one of positive (positive 2, transition 325→83.1) is outside the criteria. In the negative samples, despite of have being detected chromatographic peaks, those were considered irrelevant since fulfilled the criteria for negative samples. The method has proved to be selective for this molecule.

As in the case of hair, some values of RT_{Ratio} and also of relative abundance do not fit the criteria that was pre establish for clozapine (see Appendix 8.2 in the Table 8.2). Although in samples were not detected any interferences at the same retention time of clozapine.

These results reinforce the importance to use multiple sources of blank matrix, since increases the heterogeneity of samples, allowing to check the variability between samples, which can give rise to different results. In addition, the biological samples (hair and plasma) used for the method validation are from different species of mice from those used in the quantification. Furthermore, the WADA state that in ultimate analysis, the laboratory should establish its own criteria for identification of a compound [110].

In this case, a value outside the criteria for more than two positive samples for the same transition was not observed for the citalopram and the haloperidol. So it can be said that no peaks were detected with significance enough that might interfere in the analysis of haloperidol and citalopram. For clozapine the selectivity only was proven to one transition, with m/z 327→192, that was used in the quantification.

5.3.2. LINEARITY

It is important to know the response of the instrument with regard to the concentration of analyte over a specified concentration range [101,105]. Thus, the linearity of an analytical procedure is its ability to obtain test results which are directly proportional to the concentration of the analyte in the sample [126]. This can be performed by the analysis of spiked calibration samples and plotting the resulting responses versus the corresponding concentrations [101].
To conduct this study, it was assumed that the results obtained obeyed a simple linear regression model. Then, to evaluate the linearity it was used a spreadsheet in excel developed in the laboratory. All data were subjected to this statistical analysis (see Appendix 8.3).

The calibration curves of haloperidol, clozapine and citalopram are represented in Figure 5.14, Figure 5.15 and Figure 5.16, respectively.

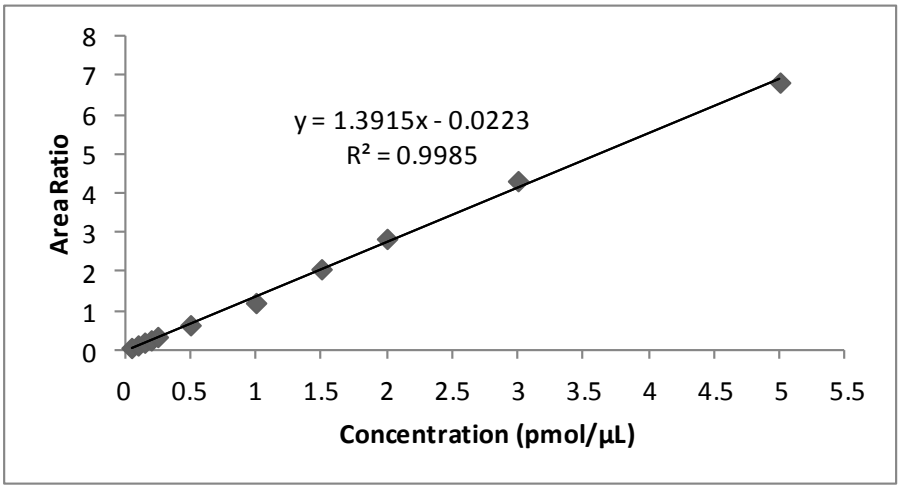


Figure 5.14- Calibration curve for the transition 376.0→164.7 of haloperidol.

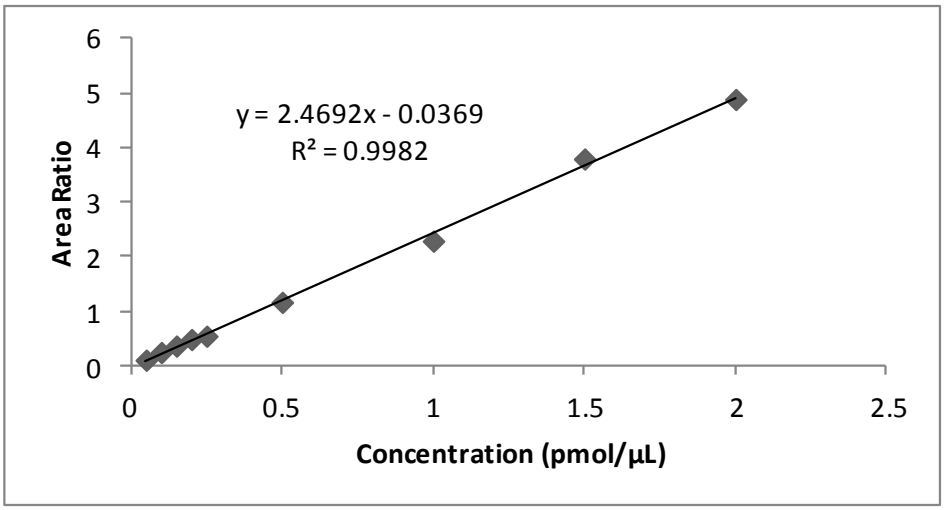


Figure 5.15- Calibration curve for the transition 327.2→191.9 for clozapine.

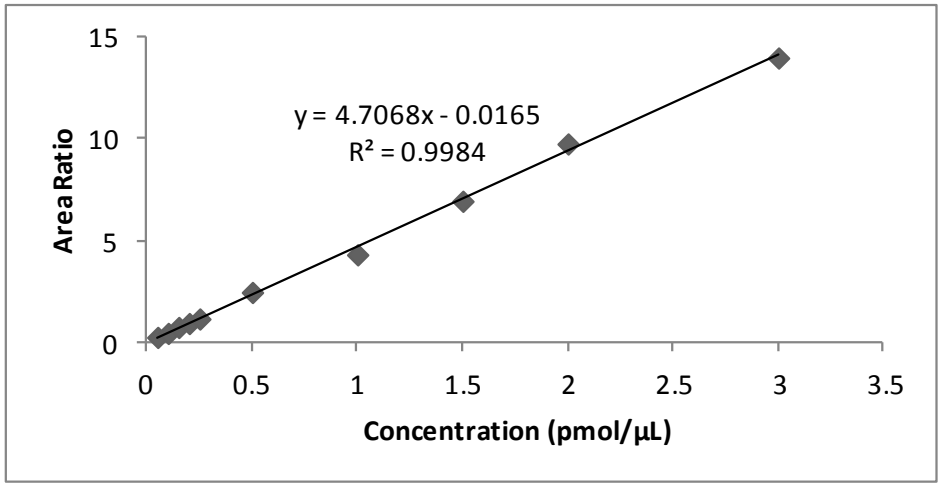


Figure 5.16- Calibration curve for the transition 325.3→109.0 for citalopram.

According to the results, the model appears to be linear for all the compounds, in the working range, through the analysis of the coefficient of correlation and determination (R>0.99 and R^2 >0.99).

However, these parameters are not sufficient to prove the linearity of the calibration model. Indeed, a significant proportion of errors at the lower end of the calibration curve can coexist with acceptable R and R^2 values. Therefore, other parameters, such as standard errors of the regression ($S_{y/x}$), confidence limits for the intercept at 95% confidence level with a zero-value included (Table 5.5) and also visual inspection of plots of residuals versus concentration (appendix 8.3) are needed to perform a complete evaluation of the linearity.

Therefore, the standard error of the linear regression allowed the exclusion of outliers (residual values > 2 × $|S_{y/x}|$), which was necessary in citalopram and clozapine analysis. After outliers have been removed from the data, the linear regression model has been re-evaluated. The value of R and R^2 were indeed higher than 0.99; for the intercept, the zero-value was included within the confidence interval of 95%, however the plots of residuals versus concentration (see Appendix 8.3, Appendix 8.4 and Appendix 8.5) appear to show residuals not randomly distributed around the x-axis. In fact, the variances tend to increase as the concentration increases, which usually points to the hypothesis of others models of calibration beyond the simple linear regression. Therefore, the calibration curve was tested by the Mandel's fitting test.

Table 5.5- Results for the simple linear regression model.

Compound	Transition[1]	Working range (pmol/µL)	Calibration Curve	R^2	Interval of confidence	
					Lower limit 95%	Upper limit 95%
Citalopram	325.3→109.0	0.05 - 3	y = 4.7068x - 0.0165	0.998	-0.215	0.182
Clozapine	327.2→191.9	0.05 - 2	y = 2.4692x - 0.0369	0.998	-0.122	0.049
Haloperidol	376.0→164.7	0.05 - 5	y = 1.3915x - 0.0223	0.998	-0.102	0.058

[1] Transition used in the quantification

5.3.2.1. MANDEL TEST

To evaluate if the simple linear regression adequately fits in the data, visual evaluation, regression statistics and residuals evaluation are not enough [101,115,116]. Thus, it was performed a Mandel's fitting test (see section 4.9.2.1) to determine if it is the linear or the quadratic regression model that better fits the data [116,117].

It was demonstrated that linear adjustment is more appropriate for the calibration curves for all the compounds, since the F_{cal} was lower than the F_{crit} (Table 5.6).

Table 5.6- Results obtained in the Mandel test.

Compound	Transition[1]	Mandel test (F_{cal})	Criteria $F_{cal} \leq F_{crit}$ (N-1; N-1; 0.95)
Citalopram	325.3→109.0	0.004	$F_{cal} \leq 3.179\ F$ (9; 9; 0.95)
Clozapine	327.2→191.9	0.79	$F_{cal} \leq 3.438\ F$ (8; 8; 0.95)
Haloperidol	376.0→164.7	1.75	$F_{cal} \leq 2.978\ F$ (10; 10; 0.95)

[1] Transition used in the quantification.

5.3.3. WORKING RANGE

Linear regression assume that a constant variance of measured values occurs over the range (homoscedasticity) [97,101,121] and that the residuals are randomly distributed along the x-axis [97,112,113,116].

However, this situation is rare, especially in bioanalytical methods, so it is important to confirm if statically there is significant difference between variances within the limits of the working range [112,121]. Plots of residuals versus concentration were obtained and an example for haloperidol is show in the Figure 5.17. These plots clearly show an increase of variance as a function of concentration, which lead us to the hypothesis of heteroscedastic data. Therefore, it was performed a test of homogeneity of variances (F-test).

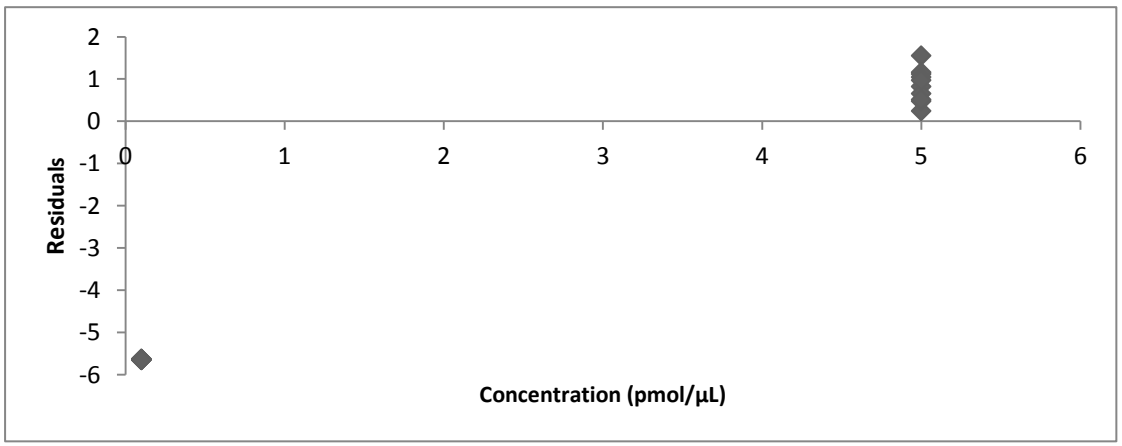


Figure 5.17- Plot of residulals versus concentration in the limits of the working range.

With the test performed to verify the variances (equations (4.7) and (4.8) in section 1.5.3.), differences between the values of the variances within the limits of the working range (0.05 - 5µM) were observed.

Results of the homogeneity test (Table 5.7) show that values for F_{cal} were above the tabled value of F_{crit}., consequently there is a significant difference between the variances, which mean heteroscedastic data, for all compounds.

Table 5.7- Results obtained in the test of homogeneity of variances.

Compound	Transition[1]	Test of homogeneity of variances (F_{cal})	Criteria $F_{cal} \leq F_{crit}$ (N-1; N-1; 0.95)
Citalopram	325.3→109.0	3373.9	$F_{cal} \leq 3.8\ F$ (9; 9; 0,95)
Clozapine	327.2→191.9	1311.6	
Haloperidol	376.0→164.7	2104.0	

[1] Transition used in the quantification.

5.3.3.1. WEIGHTED LEAST SQUARES LINEAR REGRESSION

In presence of heteroscedastic data different approaches could be followed, as already mentioned. Therefore, it is possible to reduce the working range until homoscedasticity is observed ($F_{cal} \leq F_{crit}$) [118,121], to use the inverse of variance ($1/S^2$) in each point of the calibration curve, or even to use the WLSLR [113,122], choosing the approprieted weighting factor, w_i [112]. In this project, it was used WLSLR and therefore it was necessary to choose an appropriate regression model for each compound, with the application of empirical weighting factors: $\frac{1}{x}$; $\frac{1}{x^2}$; $\frac{1}{\sqrt{x}}$; $\frac{1}{y}$; $\frac{1}{y^2}$ or $\frac{1}{\sqrt{y}}$ [112,125].

Plots of %RE versus concentration for unweighted (model 1) and weighted (models 2 – 7) of haloperidol obtained in intermediate precision study are shown in Figure 5.18. As it can be seen, the unweighted model overestimates the concentration, especially in lower ranges of the calibration curves, near LOQ.

As a measure to choose the weighting factor, it was used the percentage of the relative error ($\%\ \mathrm{RE}$), which compares the estimated concentration, from the regression equation obtained for each $\mathrm{w_i}$, with nominal standard concentration in the sample [112]. The best weighting factor is the one which presents the lowest value of the sum of the relative errors ($\sum|\%\ \mathrm{RE}|$) in the working range [112,123].

So, according to what was stated before, the $\sum|\%\ \mathrm{RE}|$ of the different weighting factors were compared and for haloperidol it was chosen the model 3 ($w_i = 1/\mathrm{x}^2$), since the error associated to this model was lower compared to the others models. Additionally, the chosen model presents a better %RE distribution scatter (Figure 5.18.).

For clozapine the model 4 ($w_i = 1/\mathrm{y}$) was chosen and for citalopram was the model 5 ($w_i = 1/\mathrm{y}^2$) (Table 8.9 and Table 8.10 in the Appendix 8.7) for the same reasons presented for haloperidol.

Table 5.8- Relative errors (%RE) and the respective sum of the relative errors ($\sum|\%\ \mathbf{RE}|$) generated by the use of simple linear regression and weighted linear regression for each weighting factor ($\mathbf{w_i}$) for haloperidol.

Nominal concentration (µM)	Model 1 Unweighted ($\mathbf{w_i}$=1)	Model 2 $\frac{1}{\mathbf{x}}$	Model 3 $\frac{1}{\mathbf{x^2}}$	Model 4 $\frac{1}{\mathbf{y}}$	Model 5 $\frac{1}{\mathbf{y^2}}$	Model 6 $\frac{1}{\sqrt{\mathbf{x}}}$	Model 7 $\frac{1}{\sqrt{\mathbf{y}}}$
0.05	511.16	51.89	13.20	58.78	16.43	171.52	176.19
0.1	259.97	47.08	38.70	47.68	38.71	100.68	102.06
0.15	141.77	26.26	26.07	26.34	23.55	37.83	38.94
0.2	104.25	30.07	28.64	30.86	29.40	46.35	47.41
0.25	64.72	25.22	23.26	26.19	25.24	14.28	14.48
0.5	20.75	21.62	15.05	22.01	15.17	18.65	18.60
1	14.85	18.63	9.45	20.02	11.89	20.98	21.63
1.5	33.67	30.20	24.13	31.29	26.49	33.12	33.55
2	40.21	32.11	18.37	33.74	20.13	37.83	38.65
3	29.47	20.10	21.46	22.05	22.02	25.19	26.14
5	16.61	29.19	42.54	27.85	41.36	22.73	22.02
$\sum\|\%\ RE\|$	1237.43	332.36	260.87	346.81	270.39	529.16	539.67

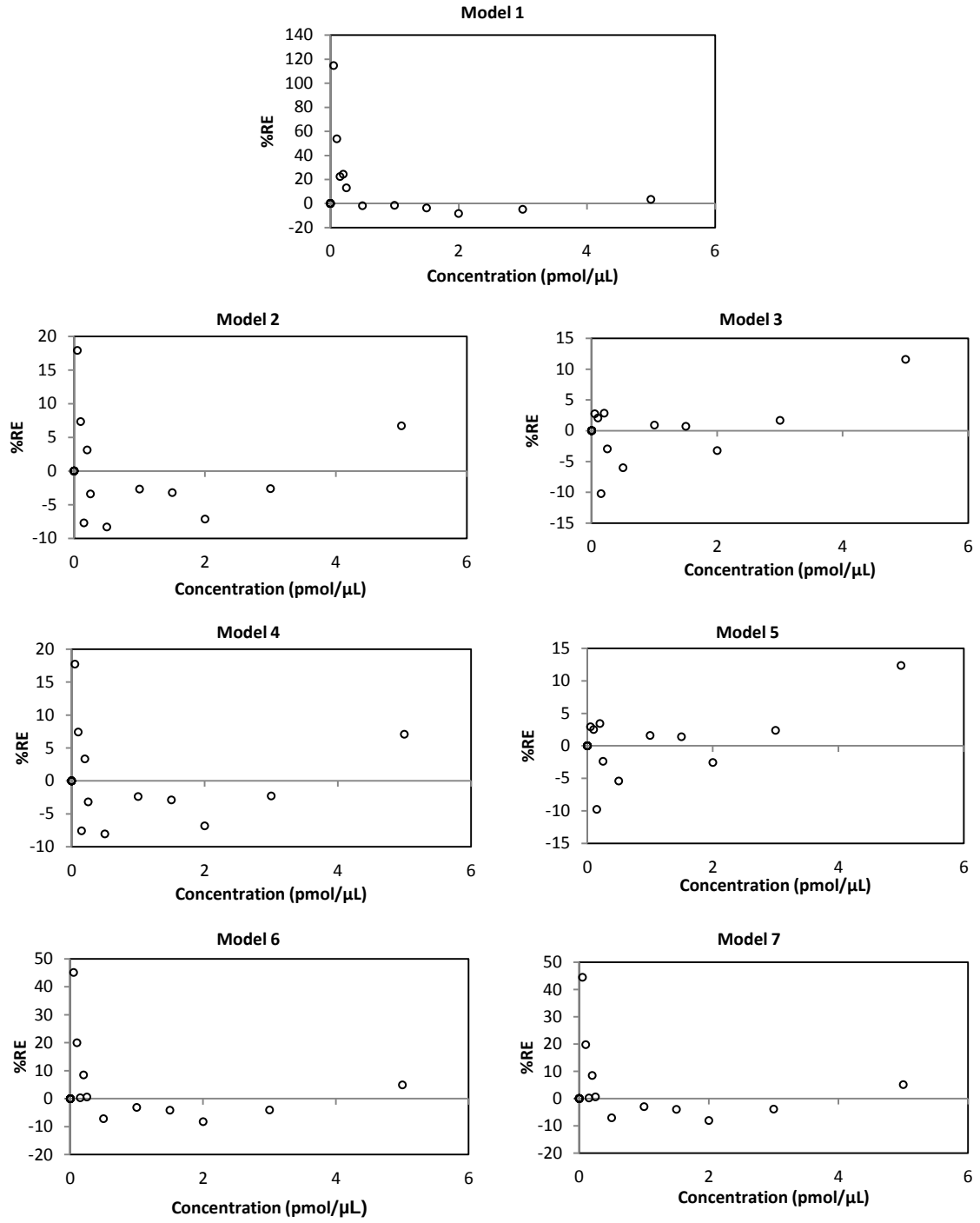


Figure 5.18- Distribution of the %RE versus concentration for haloperidol (transition 376.0→164.7) obtained for model 1 (w_i=1); model 2 ($w_i = 1/x$); model 3 ($w_i = 1/x^2$); model 4 ($w_i = 1/y$); model 5 ($w_i = 1/y^2$); model 6 ($w_i = 1/\sqrt{x}$) and model 7 ($w_i = 1/\sqrt{y}$).

5.3.4. LIMITS: LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION

As was mentioned before, there are different approaches for the determination of LOD and LOQ such as: the precision and accuracy of the data, the signal to noise ratio (S/N), and the parameters of the analytical curve [115].

The first two methods are widely used due to their speed, but the first might give rise to higher values for LOQ and the second has the disadvantage of relying on qualitative parameters.

The estimation method based on parameters of the analytical curve shows greater statistical reliability because it takes into account the confidence interval of the regression. Limit of detection in this case is defined as the minimum concentration of a substance that can be measured and reported with 95% confidence [115]. Therefore, using the equations (4.15) and (4.16) (section 4.10.4), with the appropriate transformation due to the weigh factor, can be calculated the LOD and LOQ.

The results for LOD and LOQ, in the working range previously selected, of each molecule are present in the Table 5.9.

Table 5.9- Results for LOD and LOQ of each molecule

Compound	Transition[1]	LOD (pmol/µL)	LOQ (pmol/µL)
Citalopram	325.3→109.0	0.012	0.037
Clozapine	327.2→191.9	0.014	0.044
Haloperidol	376.0→164.7	0.015	0.045

[1] Transition used in the quantification.

The values achieved for LOD and LOQ were determined using the calibration curve parameters (slope and standard error) and therefore these values are dynamic, since daily calibration curves originate different parameters.

5.3.5. PRECISION AND ACCURACY

Results obtained during the study of precision and accuracy are show in (Table 5.10) and they were estimated through one-way ANOVA analysis and the equations (4.17) and (4.18) (section 4.9.5).

Precision (both repeatibility and intermediate precision) were characterized in terms of the percentage of coeficcient of variation (%CV). The limits of acceptable variability were set at 15% for all concentrations, except at the LOQ, for which 20% were accepted [105,106].

The results obtained for repeatability were within the acceptance criteria, except for the lowest concentration level of citalopram (%CV = 35.6). In the case of the intermediate precision the results obtained were not among the criteria pre-establish,presenting values between 17.69-31.25 %.

Accuracy was evaluated in terms of mean relative error ($\%\ \mathrm{MRE}$) between the measured and the nominal concentrations for the calibrators. The limits of acceptable variability were set to be ± 15% of the accepted true value, except at the LOQ, where ±20% was accepted [130]. As it can be observed in Table 5.10, the results obtained show that the method is accurate for quantification of all the analytes.

Table 5.10- Results of the precision and accuracy at three levels of concentration (n=15).

Compound	Nominal concentration (µM)	Repeatability (% CV_R)	Intermediate precision (% CV_I)	$\%\ MRE$	Estimated concentration (µM)
Citalopram	0.075	35.69	29.72	0.55	0.075
	0.75	5.50	26.88	8.39	0.81
	2.75	5.71	31.25	9.27	3.0
Clozapine	0.075	12.56	17.69	-0.32	0.073
	0.75	7.62	24.92	7.87	0.81
	1.25	7.84	19.16	8.94	1.36
Haloperidol	0.075	14.05	22.35	13.51	0.08
	0.75	7.11	19.03	-2.42	0.73
	2.75	8.40	13.70	-2.00	2.69

5.3.6. CARRY-OVER

As mentioned above, carry-over is the amount of the analyte retained in an LC system from a preceding sample that carries over into the next injected sample [146]. This phenomena can be measured by the response of the blank sample after the injection of a preceding sample at high concentration [146].

Usually, the procedure to evaluate carry-over is performed with the injection of blank samples after samples with an expected high concentration [105], but in this project it was also injected other levels of concentration, 0.5 µM and 0.05 µM (see section 4.9.8).

So, a criterion that is accepted to evaluate if the phenomenon is relevant is proposed by EMA, where the peak area of the analyte in a blank sample that follows the high concentration standard must be less than 20% of the peak area of the limit of quantification and 5% for the IS [105].

Results for carry-over of citalopram and clozapine show that the criterion establish was fulfilled, as for the IS used, desipramine (Table 8.12 in the Appendix 8.8), thus no significant carry over was observed. In the clozapine, same occurs (Table 8.14 in the Appendix 8.8), so it can be said that with this molecule does not occur carry-over.

For haloperidol, the signal detected in the first blank was intense and it was higher than 20% of the LOQ. However, the next blanks that were injected are in accordance to the criteria (Table 8.16).

These peaks that appear in the blank samples may be caused by analyte retention in previous injections [147], which can be absorbed in the autosampler or can be residues on columns [148]. Another cause may be the possibility of the inadvertent addition of the analyte in the sample blank (contamination), or non-analyte related peaks which can arise either from a previous injection (late eluters) or the current injection (interfering endogenous peaks) [147].

Sample carry-over is a major problem that can influence the accuracy and precision of the method, with the consequences being more pronounced at lower concentrations [148]. Therefore, results that were obtained show that carry-over should be investigated and minimized, so it is important to inject blanks with the mobile phase, between samples, to reduce this phenomenon. In this project this phenomenon was minimized with a blank injected between samples and three blanks were introduced between batches. In this way, it was

ensured that the carry-over did not affect the analysis of samples, in particular in the quantification of real samples.

5.3.7. RECOVERY

Recovery is the amount of the substance of interest added to the blank matrix which is capable of being extracted and quantified [145]. This parameter basically evaluates the extraction efficiency of an analytical process of the biological samples [102,129].

Thus, recovery is best tested by comparing the response of blank matrix spiked before extraction with the response of extracted blank matrix to which analyte has been added at the same nominal concentration, just before injection [120,143]. This was the procedure that was performed in this project to study the recovery (see section 4.9.8) and additionally, it was also studied the recovery of the two IS (desipramine and haloperidol-D4), which is recommended to be determined independently [120].

Despite the recommendations related to values close to 100% recovery, Peters et al. [100,130], stated that the value for recovery is not important as long as precision, accuracy, LOQ and LOD are satisfactory.

According to *Ribani et al* [145]*.,* values between 70 to 120%, with a precision of ± 20%, are acceptable intervals for recovery. Also, depending on the analytical complexity and complexity of the sample, these values can be 50 to 120%, with a precision of ± 15%.

Other authors state that it is unlikely that recoveries of 50% or less will compromise the integrity of the method [120] and it is not needed to provide good accuracy and precision if adequate detection can be attained [107].

Values of recovery for hair were between 8.9 to 45.5 % (Table 5.11). The dispersion of the results, measured by % CV, was higher for lower concentrations. Here, it is also important to take in count that the efficiency of the method varies depending on the concentration of the substance and in most cases the dispersion of results increases with the decrease in the concentration, so the recovery can differ substantially from the high and low concentrations [145].

Table 5.11- Recovery, in percentage, of the extraction of the hair for each compound at three concentration levels of concentration.

	Recovery ± % CV		
Compound	**0.1 µM**	**0.5 µM**	**3 µM**
Citalopram	34.0± 50.3	34.0± 27.8	38.9± 10.6
Clozapine	10.7 ± 68.5	8.9 ± 14.4	12.4 ± 9.4
Haloperidol	39.6 ± 36.4	36.5 ± 31.3	39.9 ± 20.2
Desipramine	45.5 ± 36.3	36.6 ± 30.4	36.3 ± 18.6
Haloperidol-D4	43.4 ± 39.7	34.6 ± 27.4	45.2 ± 14.4

One explanation of the low values of recovery and high %CV can be compound degradation during extraction protocol due to the used high temperature and/or incubation time.

Recovery results for plasma were between 68.9 to 115.5 % and the % CV were within the criteria with the exception of haloperidol-D4 and desipramine (Table 5.12).

Table 5.12- Recovery, in percentage, of the extraction of the plasma for each compound at three concentration levels of concentration

	Recovery ± % CV		
Compound	**0.1 µM**	**0.5 µM**	**3 µM**
Citalopram	87.7 ± 7.4	90.9 ± 15.0	101.5 ± 17.4
Clozapine	95.8± 23.0	116.1± 12.0	100.4± 15.5
Haloperidol	89.1 ± 17.9	93.1 ± 19.4	115.5 ± 19.9
Desipramine	69.9 ± 22.5	68.9 ± 10.0	92.2 ± 22.8
Haloperidol-D4	82.9 ± 25.9	136.2 ± 52.1	102.1 ± 19.8

The procedure of the plasma is simpler and with fewer steps where possible losses of the analyte may occur, which may explain better results for the recovery in plasma compared to the recovery of hair.

5.3.8. MATRIX EFFECTS

One of the most important aspects is competition between an analyte and a co-eluting matrix component during ionization. As a result, depending on the environment in which the ionization and ion evaporation processes takes place, there is a decrease in analyte ionization (ion suppression) or an increase in this ionization (ion enhancement) [132,149]. Residual matrix components, endogenous phospholipids in particular, are a significant source of imprecision in quantitative analyses commonly conducted by LC-MS/MS [131,132].

Matrix effects cause a compound's response to differ when analyzed in a biological matrix compared to a standard solution [150], therefore a methodology to compare these differences was developed (see section 4.9.9). Then, it was calculated the ME using the equation (4.22) where a negative result indicates ion supression. Otherwise, if a positive result is obtained this indicates analyte signal enhancement. Thus, a value of zero would represent no ME [131,133].

The ME calculated in this manner may be referred as an absolute matrix effect, since the signal response of the compound present in the sample extract is compared to the response of a compound made directly in a pure mobile phase [132].

Results show that in hair matrix mostly of the analytes had positive results for the absolute ME, which indicates an enhancement of the signal response (Table 5.13).

Table 5.13- Matrix effects for each compound at three levels of concentration in hair matrix

	Matrix Effects		
Compound	**0.1 µM**	**0.5 µM**	**3 µM**
Citalopram	0.182	-0.103	0.889
Clozapine	0.126	-0.004	0.991
Haloperidol	-0.082	-0.383	0.464
Desipramine	0.019	-0.362	0.803
Haloperidol-D4	0.023	-0.307	0.618

The variability in these responses, expressed as % CV can be used as a measure of the relative matrix effect for a given analyte (Table 5.14). The values were high and through the analysis of

the table it can be seen an additional variability of the peak areas for the analytes that were spiked after the extraction procedure than those observed in standard solution. This is an indicative of matrix effects since analytes at the same concentrations were spiked into plasma extracts.

Table 5.14- Relative matrix effects (expressed as %CV) in hair for the standard solution and blank matrix samples spiked after extraction.

	Relative Matrix Effects (% CV)					
	Spike after			Standard solution		
Compound	0.1 µM	0.5 µM	3 µM	0.1 µM	0.5 µM	3 µM
Citalopram	125.6	57.5	35.3	28.1	26.3	26.4
Clozapine	56.1	50.1	26.4	15.6	26.0	23.9
Haloperidol	75.9	53.5	25.7	11.3	16.0	18.3
Desipramine	76.5	56.0	37.6	8.5	35.9	27.7
Haloperidol-D4	72.5	58.9	34.4	3.3	16.3	25.8

The absolute ME were also calculated for plasma and the values obtained were all negatives (Table 5.15) which indicates ion suppression.

Table 5.15- Matrix effects for each compound at three levels of concentration in plasma matrix

	Matrix Effects		
Compound	0.1 µM	0.5 µM	3 µM
Citalopram	-0.855	-0.875	-0.896
Clozapine	-0.951	-0.954	-0.951
Haloperidol	-0.863	-0.878	-0.904
Desipramine	-0.865	-0.856	-0.896
Haloperidol-D4	-0.875	-0.916	-0.887

It was also calculated the relative matrix effect and the results are in the Table 5.16. The values obtained were lower than those presented for the hair, but the variability of the peak areas for

the analytes that were spiked after the extraction procedure was higher in comparison to standard solution, indicating that there is matrix effect.

Table 5.16- Relative matrix effects (expressed as %CV) in plasma for the standard solution and blank matrix samples spiked after extraction.

	Matrix Effects (% CV)					
	Spike after			Standard solution		
Compound	0.1 µM	0.5 µM	3 µM	0.1 µM	0.5 µM	3 µM
Citalopram	30.4	8.7	21.9	17.5	6.7	4.1
Clozapine	23.7	18.0	11.9	0.7	1.8	2.5
Haloperidol	32.7	10.6	15.7	0.7	1.8	2.5
Desipramine	23.3	7.3	24.4	11.9	5.1	3.8
Haloperidol-D4	21.3	86.7	20.9	8.0	1.1	3.3

5.4. APPLICATION OF THE DEVELOPED ANALYTICAL METHOD IN REAL SAMPLES

In the previous section it was developed an analytical method for the determination of citalopram, haloperidol and clozapine in plasma and hair samples by LC-MS/MS.

After this study, it is important the application of the method developed in samples which reflect reality, and where these drugs are indeed present. This application was made in samples that were collected from mice (five replicates per day) treated with different drugs, citalopram, clozapine, haloperidol, and saline solution (control samples) for different periods of time: 1, 2, 4, 8, 15 and 30 days.

Samples were processed according to the protocol defined for plasma and hair samples (see section 4.7 and 4.8) being analyzed on the LC-MS/MS system by a specific order: control samples, citalopram samples, clozapine samples and haloperidol samples. First, all samples of hair were analyzed and then plasma samples.

All compounds were detected in the control samples of plasma. The same was observed for samples which were supposed to contain only one of the compounds with the other two being present. This cross-contamination was more pronounced in plasma samples than hair samples. This fact might indicate an external contamination, for example in the collection procedure (See Appendix 8.9).

The results for the quantification of citalopram, haloperidol and clozapine in hair samples are presented in Table 5.18. In some of the samples no signal was detected, especially in samples from days 1, 2 and 4. Few samples were quantified but below its limit of quantification, so it cannot be said with certainty that this value corresponds to reality. The higher values of quantification were obtained for citalopram. Clozapine was present only in few samples, and on the other hand, haloperidol was detected in almost samples.

To visualize the data it was used a scatter plot, where each point represents a single data point (each replicate for each day). With this type of graph, depending on how tightly the points cluster together, it can be seen a clear trend in the data. Samples used in the graphical representation were samples where quantification of the drug was performed with reliably.

The quantification of citalopram in hair which was collected from mice (5 independent replicates per day) that were administered with a solution of citalopram for different periods of time (1, 2, 4, 8, 15 and 30 days) is summarized in the Table 5.17.

Table 5.17- Summary of the citalopram quantification in hair samples.

Different periods of treatment (days)	Mean concentration (ng/mg)	N
1	0.8	3
2	1.0	1
4	1.2	3
8	3.5	4
15	4.6	3
30	15.2	2

N – sample size

Table 5.18- Quantification of citalopram, haloperidol and clozapine in hair samples.

Label	Concentration (ng/mg)	Label	Concentration (ng/mg)	Label	Concentration (ng/mg)
01PECI1	ND	01PECL1	0.02*	01PEHA1	ND
01PECI2	0.30	01PECL2	ND	01PEHA2	0.41
01PECI3	0.00*	01PECL3	ND	01PEHA3	0.18
01PECI4	1.82	01PECL4	ND	01PEHA4	0.17
01PECI5	0.42	01PECL5	ND	01PEHA5	0.34
02PECI1	1.03	02PECL1	0.58*	02PEHA1	1.48
02PECI2	0.00*	02PECL2	ND	02PEHA2	0.20
02PECI3	0.02*	02PECL3	ND	02PEHA3	0.61
02PECI4	ND	02PECL4	0.19	02PEHA4	0.05*
02PECI5	0.13*	02PECL5	x	02PEHA5	0.73
04PECI1	2.16	04PECL1	ND	04PEHA1	0.20*
04PECI2	0.17*	04PECL2	x	04PEHA2	0.13*
04PECI3	0.87	04PECL3	ND	04PEHA3	0.20*
04PECI4	0.06*	04PECL4	1.51	04PEHA4	0.10*
04PECI5	0.70	04PECL5	ND	04PEHA5	0.18*
08PECI1	5.76	08PECL1	ND	08PEHA1	0.61
08PECI2	0.57*	08PECL2	1.26	08PEHA2	5.55
08PECI3	2.13	08PECL3	0.75	08PEHA3	0.14*
08PECI4	4.06	08PECL4	0.52	08PEHA4	2.78
08PECI5	2.14	08PECL5	2.13	08PEHA5	ND
15PECI1	0.37	15PECL1	x	15PEHA1	0.82
15PECI2	7.97	15PECL2	0.07*	15PEHA2	7.56
15PECI3	15.63*	15PECL3	5.05	15PEHA3	5.51
15PECI4	15.41*	15PECL4	3.89	15PEHA4	0.13
15PECI5	5.43	15PECL5	3.70	15PEHA5	5.96
30PECI1	x	30PECL1	x	30PEHA1	x
30PECI2	26.16*	30PECL2	4.65	30PEHA2	5.88
30PECI3	21.59*	30PECL3	8.00	30PEHA3	3.23
30PECI4	12.63	30PECL4	6.61	30PEHA4	5.26
30PECI5	17.68	30PECL5	x	30PEHA5	4.19

ND - Not detected; x – Insufficient sample or non-existent; *- out of calibration curve.

Label:

- the two first numbers correspond to the different days: 1, 2, 4, 8, 15,30;
- the two first letters correspond to the matrix: PE - hair;
- the two second letters correspond to the drug: CI - Citalopram, CL - Clozapine and HA – Haloperidol;
- the last number correspond to the animal replicate: 1, 2, 3, 4 and 5.

Through the analysis of the scatter plot for the quantification of citalopram, it can be seen an increase in the concentration over the days (Figure 5.19). This suggests that higher amounts of drug can be quantified with increased hair growth. On days 1, 2 and 4, there is no significant increase, so the drug which was incorporate into the hair shaft can be minimal.

To compare the days 4, 8 and 15 it was performed a nonparametric Kruskal-Wallis test. This method compares groups as a whole. In this case, the null hypothesis was accepted, so the difference in the overall data is not statistically significant.

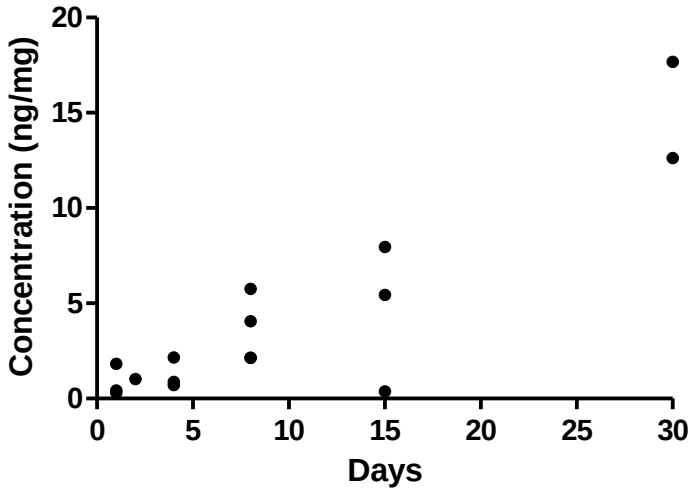


Figure 5.19- Scatter plot for the quantification of citalopram in hair. Quantification of citalopram in hair which was collected from mice (5 independent replicates per day) that were administered with a solution of citalopram for different periods of time: 1, 2, 4, 8, 15 and 30 days. Each point corresponds to a replicate.

The same approach was performed for clozapine. Thus, the quantification of clozapine in hair which was collected from mice (5 independent replicates per day) that were administered with a solution of clozapine for different periods of time (1, 2, 4, 8, 15 and 30 days) is summarized in Table 5.19.

Table 5.19- Summary of the clozapine quantification in hair samples.

Different periods of treatment (days)	Mean concentration (ng/mg)	N
1	-	-
2	0.4	2
4	1.5	1
8	1.2	4
15	4.2	3
30	6.4	3

N – sample size

Also, for clozapine, the analysis of the scatter plot for the quantification reveals an increase in the concentration over the days (Figure 5.20). Again, this suggests that higher amounts of drug can be quantified with increased hair growth.

To compare the days 8, 15 and 30 it was performed a nonparametric Kruskal-Wallis test. This method compares groups as a whole. The difference in the overall data was proved to be statistically significant. In this sense it was performed a Dunn's post test to compare each pair of groups. It was revealed that only between day 8 and day 30 the differences are statistically significant (p value <0.05 - probability of accept the null hypothesis is less than 5 %).

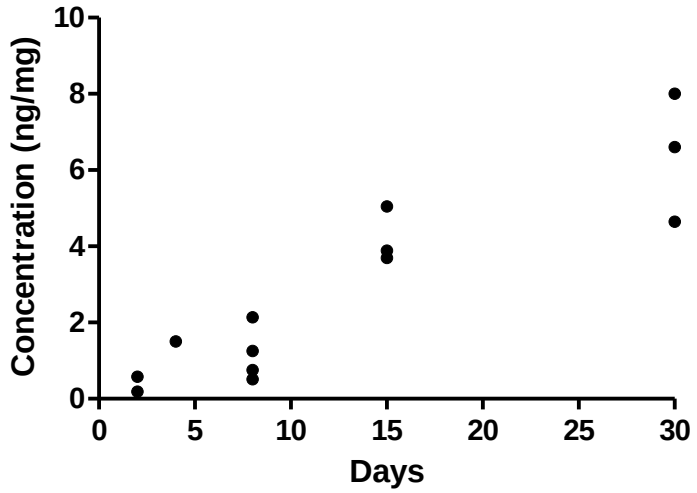


Figure 5.20- Scatter plot for the quantification of clozapine in hair. Quantification of clozapine in hair which was collected from mice (5 independent replicates per day) that was administered with a solution of clozapine for different periods of time: 1, 2, 4, 8, 15 and 30 days. Each point corresponds to a replicate.

The same approach that was used for citalopram and clozapine was performed for haloperidol. The quantification of this compound in hair which was collected from mice (5 independent replicates per day) that were administered with a solution of haloperidol for different periods of time (1, 2, 4, 8, 15 and 30 days) is summarized in the Table 5.20.

Table 5.20- Summary of the haloperidol quantification in hair samples.

Different periods of treatment (days)	Mean concentration (ng/mg)	N
1	0.3	4
2	0.8	4
4	-	-
8	3.0	3
15	4.0	5
30	4.6	4

N - sample size

Through the analysis of the scatter plot it can be seen a large dispersion of data (Figure 5.21). However, the analysis of the Table 5.20 for the quantification profile of haloperidol reveals an increase in the mean concentration over the days

It was performed a nonparametric Kruskal-Wallis test between the days 8, 15 and 30. It was found no differences that were statistically significant in the overall data, so the Dunn's post test wasn't performed. However, there is a large dispersion of the data, as can be seen in Figure 5.21. Two explanations for a result that is not statistically significant can be provided. All data are identical, so there is no difference between them, or data really may be different, but no differences were found due to some combination of small sample size and high variability.

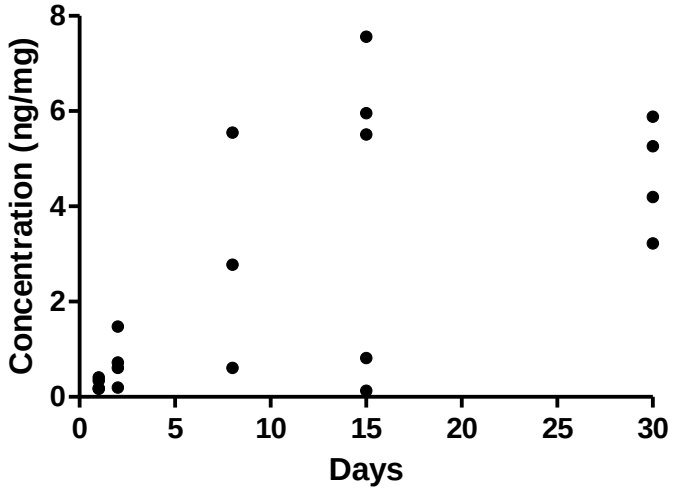


Figure 5.21- Scatter plot for the quantification of haloperidol in hair. Quantification of haloperidol in hair which was collected from mice (5 independent replicates per day) that were administered with a solution of haloperidol for different periods of time: 1, 2, 4, 8, 15 and 30 days. Each point corresponds to a replicate.

Results for the quantification of citalopram, haloperidol and clozapine in plasma samples are presented in Table 5.22. The higher values of quantification were obtained for citalopram.

The same procedure that was used to visualize the data in hair samples was performed in plasma samples. Again, samples used in the graphical representation were samples where quantification of the drug was performed with reliably.

The quantification of citalopram in plasma which was collected from mice (5 independent replicates per day) that were administered with a solution of citalopram for different periods of time (1, 2, 4, 8, 15 and 30 days) is summarized in the Table 5.21.

Table 5.21- Summary of the citalopram quantification in plasma samples.

Different periods of treatment (days)	Mean concentration (pg/µL)	N
1	3.9	3
2	3.0	5
4	5.2	5
8	23.0	4
15	7.0	5
30	2.2	5

N - sample size

Table 5.22- Quantification of citalopram, haloperidol and clozapine in plasma samples.

Label	Concentration (pg/µL)	Label	Concentration (pg/µL)	Label	Concentration (pg/µL)
01PLCI1	x	01PLCL1	12.2	01PLHA1	0.6*
01PLCI2	x	01PLCL2	11.6	01PLHA2	0.8
01PLCI3	3.0	01PLCL3	9.1	01PLHA3	0.4*
01PLCI4	5.0	01PLCL4	4.2	01PLHA4	x
01PLCI5	3.8	01PLCL5	x	01PLHA5	0.3*
02PLCI1	4.4	02PLCL1	x	02PLHA1	0.6*
02PLCI2	2.2	02PLCL2	x	02PLHA2	0.6*
02PLCI3	3.2	02PLCL3	11.3	02PLHA3	0.4*
02PLCI4	2.8	02PLCL4	19.7	02PLHA4	0.7
02PLCI5	2.3	02PLCL5	ND	02PLHA5	0.6*
04PLCI1	4.8	04PLCL1	x	04PLHA1	0.6*
04PLCI2	5.9	04PLCL2	1.9	04PLHA2	0.9
04PLCI3	4.9	04PLCL3	1.5	04PLHA3	1.0
04PLCI4	4.4	04PLCL4	ND	04PLHA4	1.1
04PLCI5	6.0	04PLCL5	0.9	04PLHA5	x
08PLCI1	21.0	08PLCL1	x	08PLHA1	x
08PLCI2	x	08PLCL2	2.4	08PLHA2	1.1
08PLCI3	22.4	08PLCL3	x	08PLHA3	x
08PLCI4	20.8	08PLCL4	4.9	08PLHA4	0.8
08PLCI5	28.0	08PLCL5	2.0	08PLHA5	1.0
15PLCI1	7.5	15PLCL1	x	15PLHA1	0.5*
15PLCI2	5.3	15PLCL2	ND	15PLHA2	0.5*
15PLCI3	4.8	15PLCL3	1.8	15PLHA3	0.5*
15PLCI4	6.7	15PLCL4	0.4*	15PLHA4	0.5*
15PLCI5	10.6	15PLCL5	x	15PLHA5	x
30PLCI1	1.1	30PLCL1	1.4	30PLHA1	0.9
30PLCI2	1.5	30PLCL2	1.8	30PLHA2	0.6*
30PLCI3	4.0	30PLCL3	6.0	30PLHA3	0.4*
30PLCI4	2.2	30PLCL4	1.2	30PLHA4	0.8
30PLCI5	2.1	30PLCL5	3.1	30PLHA5	0.7

ND - Not detected; x - Sample insufficient or non-existent; *- out of calibration curve.

Label:

- the two first numbers correspond to the different days: 1, 2, 4, 8, 15,30;
- the two first letters correspond to the matrix: PL - plasma;
- the two second letters correspond to the drug: CI - Citalopram, CL - Clozapine and HA – Haloperidol;
- the last number correspond to the animal replicate: 1, 2, 3, 4 and 5.

Visualization of the scatter plot for the quantification of citalopram shows a maximum peak in concentration on Day 8 (Figure 5.22).

In order to compare days 2, 4, 8, 15 and 30, it was performed a Kruskal-Wallis test. The difference in the overall data was proved to be statistically significant. In this sense it was performed a Dunn's post test to compare each pair of groups. It was revealed that were differences statistically significant between days 2 and 8 (p value <0.05 - probability of accept the null hypothesis is less than 5 %), days 8 and 30 (p value <0.001 - probability of accept the null hypothesis is less than 0.1%) and days 15 and 30 (p value <0.05 - probability of accept the null hypothesis is less than 5 %).

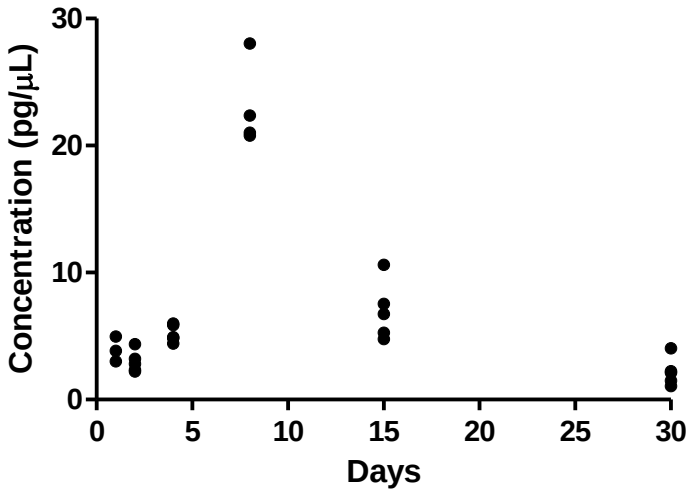


Figure 5.22- Scatter plot for the quantification of citalopram in plasma. Quantification of citalopram in plasma which was collected from mice (5 independent replicates per day) that were administered with a solution of citalopram for different periods of time: 1, 2, 4, 8, 15 and 30 days. Each point corresponds to a replicate.

The same approach was performed to evaluate the quantification of clozapine in plasma which was collected from mice (5 independent replicates per day) that were administered with a solution of clozapine for different periods of time (1, 2, 4, 8, 15 and 30 days) is summarized in the Table 5.23.

Table 5.23- Summary of the clozapine quantification in plasma samples.

Different periods of treatment (days)	Mean concentration (pg/μL)	N
1	9.3	4
2	15.5	2
4	1.4	3
8	3.1	3
15	1.8	1
30	2.7	5

N – sample size

In the scatter plot for the quantification of clozapine, it can be seen that higher concentrations are reached on day 1 and day 2 (Figure 5.23). A nonparametric Kruskal-Wallis test was performed in order to compare days 1, 4, 8 and 30. Day 2 was not analyzed since sample sizes were too small. It was found that there are differences statistically significant between groups as a whole. Then, it was performed a Dunn's post test to compare each pair of groups and it was found that only between day 1 and day 4, the difference was statistically significant (p value <0.05 - probability of accept the null hypothesis is less than 5 %)..

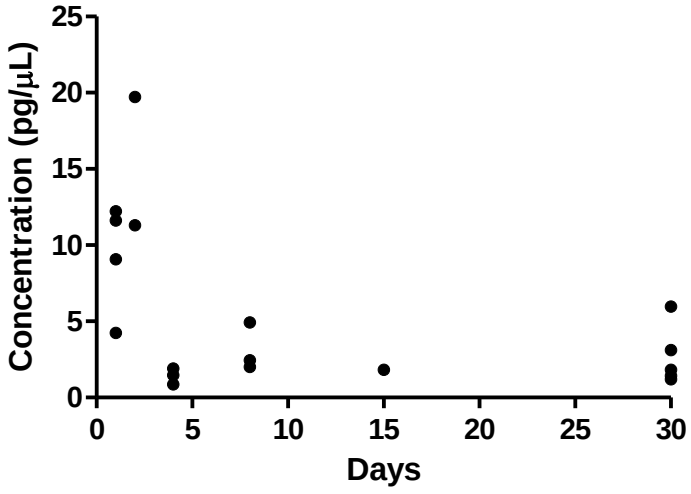


Figure 5.23- Scatter plot for the quantification of clozapine in plasma. Quantification of clozapine in plasma which was collected from mice (5 independent replicates per day) that were administered with a solution of clozapine for different periods of time: 1, 2, 4, 8, 15 and 30 days. Each point corresponds to a replicate.

The same approach of the citalopram and clozapine was performed for haloperidol. The quantification of haloperidol in plasma which was collected from mice (5 independent replicates per day) that were administered with a solution of haloperidol for different periods of time (1, 2, 4, 8, 15 and 30 days) is represented in Table 5.24.

Table 5.24- Summary of the haloperidol quantification in plasma samples.

Different periods of treatment (days)	Mean concentration (pg/µL)	N
1	0.8	1
2	0.7	1
4	1.0	3
8	1.0	3
15	-	-
30	0.8	2

N – sample size

In the scatter plot for the quantification of haloperidol, it can be seen that higher concentrations are reached on day 4 and day 8. However no conclusion can be drawn since there is a large dispersion of data and also a small sample size.

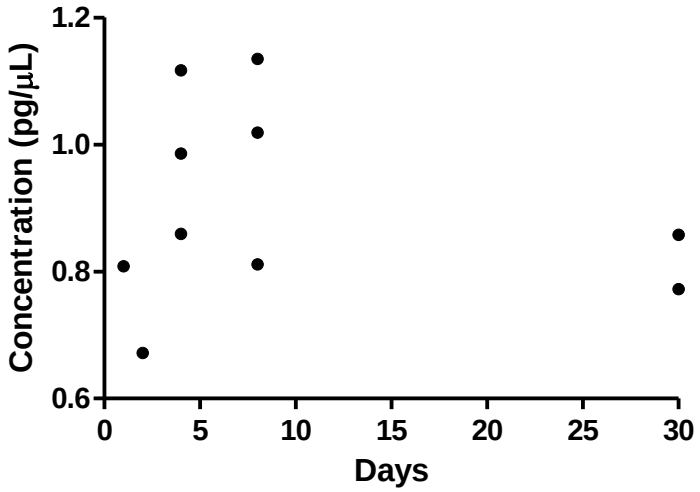


Figure 5.24- Scatter plot for the quantification of haloperidol in plasma. Quantification of haloperidol in plasma which was collected from mice (5 independent replicates per day) that were administered with a solution of haloperidol for different periods of time: 1, 2, 4, 8, 15 and 30 days. Each point corresponds to a replicate.

6. CONCLUSION AND FUTURE PERSPECTIVES

In this project a method was developed for the identification and quantification of psychotropic drugs (citalopram, clozapine and haloperidol) in plasma and hair from mice by LC-MS/MS.

The chromatographic conditions were good, with an efficient separation and a running time for each sample of 9 min. The mass spectrometer was operated in MRM mode, allowing the selection of the transitions to be monitored for each PD and also the internal standards used. The application of the criteria proposed by WADA, allowed the unequivocal identification of these transitions.

In order to obtain reliable analytical data, the method was validated by evaluation of various analytical parameters: selectivity, limits of detection and quantification, linearity, carry over, precision, accuracy, recovery and matrix effects.

The method proved to be selective for citalopram, haloperidol and clozapine.

This methodology was proved to be linear for all compounds over the concentration range studied, with R2 >0.99 and the residuals values were also evaluated (Residuals < 2x|$Sy_{/x}$|). The linearity of the method was demonstrated for the intervals: 0.05 to 5 pmol/µl for haloperidol, 0.05 to 3 pmol/µl for citalopram and 0.05 to 2 pmol/µl for clozapine. However, it was observed a heteroscedastic distribution of the residuals, so it was used a weighted linear regression, with empirical weighting factors of $1/x^2$, $1/y$ and $1/y^2$ for haloperidol, clozapine and citalopram, respectively.

The limits of detection were 0.012, 0.014 and 0.015 pmol/µL for citalopram, clozapine and haloperidol, respectively. These limits were good, since reduced amounts of samples were used for the extraction (70 µl of plasma and 1.2 mg for citalopram, 2.1mg for clozapina, 0.7mg for haloperidol of hair).

The limits of quantification obtained were: 0.037, 0.044 and 0.045 pmol/µL for citalopram, clozapine and haloperidol, respectively.

The precision was studied by analyzing the repeatability and intermediate precision. The results were good for the conditions of reapeatability (5.50 to 35.69 %), with high value in lowest concentration. For intermediate precision the results were slightly higher than the criteria (13.70

to 31.25 %). In future it is proposed to re-study this parameter to be able to get more precise values. The method was proved to be accurate with values within the criteria (-2.42 to 13.51 %).

It was also evaluated the phenomenon of carry over and it was observed that citalopram, haloperidol and clozapine do not have significant values for this parameter. Nonetheless, in the analytical method developed, where multiple samples are analyzed, it was introduced wash injections (blanks with ACN: 0.1%FA) in order to avoid possible contamination between samples.

The study of the recovery at different concentration levels, showed recoveries between 68.9 and 115.5% for plasma, values quite acceptable and within the limits pre-establish. The recovery for hair showed lower values between 8.9 to 45.5%. For future it is proposed the study of different times of incubation for the hair and also different temperatures, in order to achieve the best recoveries for this matrix.

The matrix effects were also studied, where negatives values were obtained for plasma, indicating ion suppression. In the matrix effects for hair were obtained positive values, indicating enhancement of the analyte signal and also the relative ME have high values.

The developed method was applied in biological samples (plasma and hair) that were collected from mice (5 independent replicates per day) that were submitted to a treatment with psychotropic drugs (citalopram, clozapine and haloperidol) for different periods of time: 1, 2, 4, 8, 15 and 30 days.

The time evolution of quantification for the three compounds in hair reveals an increase in the concentration over the days with a marked increase on day 15. This could mean that higher amounts of drug can be quantified with increased hair growth. In the first days the amount of drug which was quantified is very low, so the drug that actually is incorporated into the hair shaft is also low. In these samples, the haloperidol was the compound more difficult to detect and quantified with more disperse values. Contrary to what was expected, since the validation results were in general very good for this compound.

In plasma the time evolution of quantification for the citalopram, clozapine and haloperidol was different for each compound. For citalopram it can be seen a maximum peak in concentration on Day 8. For clozapine it can be seen an increase in the concentration reached on day 1 and day 2 followed by a decrease to a steady state. And for haloperidol it can be seen that higher concentrations are reached on day 4 and day 8. Again, less satisfactory results were obtained with the haloperidol. Based on these data, it is suggested studies on the metabolism of this drug to address the absorption and elimination profiles.

All compounds were detected in the control samples and also for samples which were supposed to contain only one of the compounds with the other two being present. This was observed for plasma samples but not for the hair samples which might indicate a contamination in the plasma collection procedure.

In the future it would be important to continue the study of clozapine with a purpose of creating a method more appropriated, since the validation parameters for this compound were less satisfactory.

Due to the increased use of antidepressants and antipsychotics and their involvement in intoxications and suicide, the ability to reliably detect this class in biological specimens is very important. The biological specimens that were used are both relevant, where plasma is preferred in cases of therapeutic drug monitoring. And hair has become an important matrix, since it provides evidence of longer term exposure of drugs, complementing other specimens and also, it can be used in forensic cases, for example when there is decomposition of the body. Thus, by the foregoing, the methodology developed proves to be of great importance in clinical and forensic approaches.

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8. APPENDIX

Appendix 8.1: Weighing of the hair samples.

Label	Weight (mg)	Label	Weight (mg)	Label	Weight (mg)	Label	Weight (mg)
01PECI1	3.4	01PECL1	6.4	01PEHA1	5.0	01PECT1	5.6
01PECI2	4.1	01PECL2	5.4	01PEHA2	4.2	01PECT2	4.4
01PECI3	4.8	01PECL3	5.9	01PEHA3	5.5	01PECT3	6.3
01PECI4	7.1	01PECL4	3.7	01PEHA4	5.8	01PECT4	8.9
01PECI5	6.6	01PECL5	4.4	01PEHA5	3.9	01PECT5	3.8
02PECI1	8.7	02PECL1	7.7	02PEHA1	7.8	02PECT1	7.0
02PECI2	6.8	02PECL2	4.6	02PEHA2	7.5	02PECT2	4.2
02PECI3	9.7	02PECL3	4.9	02PEHA3	8.0	02PECT3	6.0
02PECI4	7.7	02PECL4	5.5	02PEHA4	6.7	02PECT4	3.1
02PECI5	5.2	02PECL5	x	02PEHA5	6.4	02PECT5	5.8
04PECI1	1.8	04PECL1	1.0	04PEHA1	1.2	04PECT1	0.8
04PECI2	2.1	04PECL2	x	04PEHA2	0.7	04PECT2	0.8
04PECI3	2.4	04PECL3	1.0	04PEHA3	1.3	04PECT3	x
04PECI4	2.0	04PECL4	6.2	04PEHA4	2.2	04PECT4	1.5
04PECI5	2.7	04PECL5	1.1	04PEHA5	2.6	04PECT5	1.2
08PECI1	2.5	08PECL1	1.7	08PEHA1	4	08PECT1	2.8
08PECI2	1.2	08PECL2	2.3	08PEHA2	5.4	08PECT2	3.2
08PECI3	2.8	08PECL3	2.1	08PEHA3	2.8	08PECT3	2.0
08PECI4	3.2	08PECL4	2.9	08PEHA4	3.8	08PECT4	2.9
08PECI5	2.6	08PECL5	3.0	08PEHA5	2.4	08PECT5	3.2
15PECI1	5.9	15PECL1	x	15PEHA1	5.2	15PECT1	3.0
15PECI2	4.5	15PECL2	2.9	15PEHA2	7.8	15PECT2	3.7
15PECI3	7.7	15PECL3	4.4	15PEHA3	4.5	15PECT3	3.6
15PECI4	6.8	15PECL4	3.4	15PEHA4	7.4	15PECT4	4.4
15PECI5	4.7	15PECL5	4.1	15PEHA5	7.6	15PECT5	4.7
30PECI1	x	30PECL1	x	30PEHA1	x	30PECT1	x
30PECI2	2.9	30PECL2	4.6	30PEHA2	4.7	30PECT2	4.5
30PECI3	3.4	30PECL3	5.0	30PEHA3	6.8	30PECT3	2.7
30PECI4	3.3	30PECL4	5.7	30PEHA4	5.9	30PECT4	4.4
30PECI5	2.9	30PECL5	x	30PEHA5	7.2	30PECT5	4.0

x - sample insufficient or non-existent.

Label:

- the two first numbers correspond to the different days: 1, 2, 4, 8, 15,30;
- the two first letters correspond to the matrix: PE - hair;
- the two second letters correspond to the drug: CI - Citalopram, Cl - Clozapine and HA – Haloperidol;
- the last number correspond to the animal replicate: 1, 2, 3, 4 and 5.

Appendix 8.2: Data related to the study of selectivity

Table 8.1- Application of acceptance WADA criteria for unequivocal identification of the citalopram in plasma.

	Transition (m/z)	Absolute area	Relative area	S/N	RT_A	RT_{IS}	RT_{Ratio}
Positive 1	325.3→109.0	8742.177	100.000	32.077	6.271	7.359	0.852
	325.3→261.9	2450.138	28.027	8.611	6.266	7.359	0.851
	325.3→83.1	1800.696	20.598	7.230	6.266	7.359	0.851
Negative 1	325.3→109.0	249.582	100.000	0.850	6.294	6.809	0.924
	325.3→261.9	-	-	-	-	6.809	-
	325.3→83.1	-	-	-	-	6.809	-
Positive 2	325.3→109.0	7587.675	100.000	25.649	6.404	7.445	0.860
	325.3→261.9	1424.155	18.769	5.352	6.402	7.445	0.860
	325.3→83.1	1937.774	25.538	7.039	6.405	7.445	0.860
Negative 2	325.3→109.0	93.434	100.000	0.315	6.055	6.633	0.913
	325.3→261.9	-	-	-	-	6.633	-
	325.3→83.1	-	-	-	-	6.633	-
Positive 3	325.3→109.0	7213.969	100.000	25.362	6.212	7.308	0.850
	325.3→261.9	2101.156	29.126	7.286	6.207	7.308	0.849
	325.3→83.1	1657.643	22.978	6.026	6.209	7.308	0.850
Negative 3	325.3→109.0	-	-	-	-	6.844	-
	325.3→261.9	-	-	-	-	6.844	-
	325.3→83.1	-	-	-	-	6.844	-
Positive 4	325.3→109.0	7213.969	100.000	25.362	6.212	7.308	0.850
	325.3→261.9	2101.156	29.126	7.286	6.207	7.308	0.849
	325.3→83.1	1657.643	22.978	6.026	6.209	7.308	0.850
Negative 4	325.3→109.0	94.289	100.000	0.387	6.186	-	-
	325.3→261.9	-	-	-	-	-	-
	325.3→83.1	-	-	-	-	-	-
Positive 5	325.3→109.0	7549.764	100.000	28.255	6.273	7.372	0.851
	325.3→261.9	1354.053	17.935	4.516	6.283	7.372	0.852
	325.3→83.1	1680.863	22.264	6.620	6.262	7.372	0.849
Negative 5	325.3→109.0	70.030	74.992	0.320	6.266	6.663	0.940
	325.3→261.9	-	-	-	-	6.663	-
	325.3→83.1	93.383	100.000	0.439	6.243	6.663	0.937
Positive 6	325.3→109.0	9586.817	100.000	35.250	6.254	7.340	0.852
	325.3→261.9	1937.547	20.211	6.108	6.258	7.340	0.853
	325.3→83.1	1657.569	17.290	6.078	6.256	7.340	0.852
Negative 6	325.3→109.0	-	-	-	-	6.758	-
	325.3→261.9	-	-	-	-	6.758	-
	325.3→83.1	-	-	-	-	6.758	-
	Transition (m/z)	**Relative Abundance**		**S/N**			**ΔRT_{Ratio}**
Criteria	325.3→109	90	110		0.844		0.861
	325.3→261.9	22.421	33.632	>3	0.843		0.860
	325.3→83.1	15.598	25.598		0.843		0.860

Table 8.2- Application of acceptance WADA criteria for unequivocal identification of the clozapine in plasma.

	Transition (m/z)	Absolute area	Relative area	S/N	RT_A	RT_{IS}	RT_{Ratio}
Positive 1	327.2→269.9	1073.920	80.706	4.146	4.094	7.359	0.556
	327.2→191.9	1330.658	100.000	5.117	4.099	7.359	0.557
	327.2→163.8	536.958	40.353	2.081	4.097	7.359	0.557
Negative 1	327.2→269.9	656.442	84.205	2.922	4.066	6.809	0.597
	327.2→191.9	779.572	100.000	3.662	4.056	6.809	0.596
	327.2→163.8	93.381	11.979	0.419	4.084	6.809	0.600
Positive 2	327.2→269.9	1005.915	68.590	3.684	4.366	7.445	0.586
	327.2→191.9	1466.569	100.000	5.774	4.362	7.445	0.586
	327.2→163.8	373.533	25.470	1.515	4.371	7.445	0.587
Negative 2	327.2→269.9	-	-	-	-	6.633	-
	327.2→191.9	69.999	100.000	0.377	4.408	6.633	0.665
	327.2→163.8	-	-	-	-	6.633	-
Positive 3	327.2→269.9	963.809	76.393	4.105	4.074	7.308	0.557
	327.2→191.9	1261.645	100.000	4.997	4.073	7.308	0.557
	327.2→163.8	326.841	25.906	1.384	4.096	7.308	0.560
Negative 3	327.2→269.9	-	-	-	-	6.844	-
	327.2→191.9	-	-	-	-	6.844	-
	327.2→163.8	-	-	-	-	6.844	-
Positive 4	327.2→269.9	1587.527	73.916	6.761	4.091	7.356	0.556
	327.2→191.9	2147.746	100.000	8.114	4.094	7.356	0.557
	327.2→163.8	747.076	34.784	2.780	4.093	7.356	0.556
Negative 4	327.2→269.9	-	-	-	-	-	-
	327.2→191.9	-	-	-	-	-	-
	327.2→163.8	-	-	-	-	-	-
Positive 5	327.2→269.9	1144.408	71.732	4.847	4.099	7.372	0.556
	327.2→191.9	1595.394	100.000	6.181	4.098	7.372	0.556
	327.2→163.8	723.736	45.364	3.212	4.102	7.372	0.556
Negative 5	327.2→269.9	-	-	-	-	6.663	-
	327.2→191.9	-	-	-	-	6.663	-
	327.2→163.8	-	-	-	-	6.663	-
Positive 6	327.2→269.9	1143.923	62.848	4.575	4.093	7.340	0.558
	327.2→191.9	1820.141	100.000	7.020	4.083	7.340	0.556
	327.2→163.8	542.398	29.800	2.154	4.090	7.340	0.557
Negative 6	327.2→269.9	140.078	100.000	0.724	4.076	6.758	0.602
	327.2→191.9	140.076	99.999	0.525	4.068	6.758	0.602
	327.2→163.8	-	-	-	-	6.758	-
	Transition (m/z)	**Relative Abundance**		**S/N**			**ΔRT_{Ratio}**
Criteria	327.2→269.9	70.706	90.706		0.551		0.562
	327.2→191.9	90.000	110.000	>3	0.551		0.563
	327.2→163.8	32.282	48.423		0.551		0.562

Table 8.3- Application of acceptance WADA criteria for unequivocal identification of the haloperidol in plasma.

	Transition (m/z)	Absolute area	Relative area	S/N	RT_A	RT_{IS}	RT_{Ratio}
Positive 1	376.0→164.7	47778.286	57.874	172.756	6.635	6.600	1.005
	376.0→122.9	82555.313	100.000	300.495	6.639	6.600	1.006
	376.0→94.8	30672.706	37.154	112.539	6.634	6.600	1.005
Negative 1	376.0→164.7	162.229	100.000	0.872	6.592	6.230	1.058
	376.0→122.9	138.565	85.414	0.436	6.254	6.230	1.004
	376.0→94.8	157.300	96.962	0.446	6.575	6.230	1.055
Positive 2	376.0→164.7	41407.481	59.955	147.232	6.755	6.719	1.005
	376.0→122.9	69064.705	100.000	256.026	6.754	6.719	1.005
	376.0→94.8	26602.638	38.518	97.336	6.754	6.719	1.005
Negative 2	376.0→164.7	-	-	-	-	6.497	-
	376.0→122.9	77.749	100.000	0.472	6.302	6.497	0.970
	376.0→94.8	-	-	-	-	6.497	-
Positive 3	376.0→164.7	45566.235	63.522	163.777	6.585	6.548	1.006
	376.0→122.9	71732.851	100.000	254.610	6.586	6.548	1.006
	376.0→94.8	31190.058	43.481	110.149	6.584	6.548	1.005
Negative 3	376.0→164.7	210.114	100.000	0.746	6.508	6.140	1.060
	376.0→122.9	73.158	34.818	0.341	6.274	6.140	1.022
	376.0→94.8	94.546	44.997	0.480	6.470	6.140	1.054
Positive 4	376.0→164.7	31075.385	60.973	117.356	6.632	6.596	1.005
	376.0→122.9	50966.167	100.000	182.164	6.631	6.596	1.005
	376.0→94.8	21352.538	41.896	75.102	6.630	6.596	1.005
Negative 4	376.0→164.7	303.622	100.000	1.215	6.569	6.506	1.010
	376.0→122.9	64.671	21.300	0.325	6.086	6.506	0.935
	376.0→94.8	69.928	23.031	0.313	5.822	6.506	0.895
Positive 5	376.0→164.7	26276.164	57.210	94.598	6.657	6.620	1.006
	376.0→122.9	45929.205	100.000	164.020	6.657	6.620	1.006
	376.0→94.8	19796.612	43.102	71.140	6.652	6.620	1.005
Negative 5	376.0→164.7	116.779	87.326	0.483	6.599	6.581	1.003
	376.0→122.9	133.727	100.000	0.430	5.971	6.581	0.907
	376.0→94.8	-	-	-	-	6.581	-
Positive 6	376.0→164.7	32008.587	67.184	115.245	6.630	6.594	1.006
	376.0→122.9	47643.335	100.000	167.278	6.631	6.594	1.006
	376.0→94.8	18607.559	39.056	66.137	6.633	6.594	1.006
Negative 6	376.0→164.7	116.736	47.689	0.423	6.573	-	-
	376.0→122.9	244.786	100.000	0.946	6.592	-	-
	376.0→94.8	-	-	-	-	-	-

	Transition (m/z)	Relative Abundance		S/N	ΔRT_{Ratio}	
Criteria	376.0→164.7	47.874	67.874		0.995	1.015
	376.0→122.9	90.000	110.000	>3	0.996	1.016
	376.0→94.8	29.723	44.585		0.995	1.015

Table 8.4- Application of acceptance WADA criteria for unequivocal identification of the citalopram in hair.

	Transition (m/z)	Absolute area	Relative area	S/N	RT_A	RT_{IS}	RT_{Ratio}
Positive 1	325.3→109.0	10831.351	100.000	37.960	6.290	7.330	0.858
	325.3→261.9	2894.899	26.727	9.657	6.292	7.330	0.858
	325.3→83.1	1984.660	18.323	6.505	6.290	7.330	0.858
Negative 1	325.3→109.0	132.599	100.000	0.656	6.126	-	-
	325.3→261.9	-	-	-	-	-	-
	325.3→83.1	-	-	-	-	-	-
Positive 2	325.3→109.0	8469.233	100.000	28.450	6.091	7.133	0.854
	325.3→261.9	2288.104	27.017	9.044	6.088	7.133	0.853
	325.3→83.1	1922.074	22.695	7.009	6.089	7.133	0.854
Negative 2	325.3→109.0	296.179	100.000	0.773	6.345	-	-
	325.3→261.9	-	-	-	-	-	-
	325.3→83.1	-	-	-	-	-	-
Positive 3	325.3→109.0	12350.847	100.000	42.149	6.181	7.261	0.851
	325.3→261.9	2162.002	17.505	8.391	6.178	7.261	0.851
	325.3→83.1	2392.538	19.371	8.262	6.179	7.261	0.851
Negative 3	325.3→109.0	116.730	100.000	0.500	6.255	-	-
	325.3→261.9	-	-	-	-	-	-
	325.3→83.1	-	-	-	-	-	-
Positive 4	325.3→109.0	10781.131	100.000	38.682	6.136	7.192	0.853
	325.3→261.9	2545.479	23.611	9.151	6.133	7.192	0.853
	325.3→83.1	1585.524	14.706	5.641	6.131	7.192	0.852
Negative 4	325.3→109.0	273.367	100.000	0.907	6.113	-	-
	325.3→261.9	-	-	-	-	-	-
	325.3→83.1	-	-	-	-	-	-
Positive 5	325.3→109.0	7037.525	100.000	25.665	6.473	7.477	0.866
	325.3→261.9	1610.854	22.889	6.083	6.456	7.477	0.863
	325.3→83.1	1212.095	17.223	4.302	6.462	7.477	0.864
Negative 5	325.3→109.0	439.462	100.000	1.607	6.112	-	-
	325.3→261.9	-	-	-	-	-	-
	325.3→83.1	-	-	-	-	-	-
Positive 6	325.3→109.0	7162.747	100.000	23.736	6.200	7.268	0.853
	325.3→261.9	2264.654	31.617	8.623	6.184	7.268	0.851
	325.3→83.1	1587.975	22.170	5.432	6.181	7.268	0.850
Negative 6	325.3→109.0	184.775	100.000	0.869	6.215	-	-
	325.3→261.9	-	-	-	-	-	-
	325.3→83.1	-	-	-	-	-	-

	Transition (m/z)	Relative Abundance		S/N	ΔRT_{Ratio}	
Criteria	325.3→109.0	90.000	110.000		0.850	0.867
	325.3→261.9	21.382	32.072	>3	0.850	0.867
	325.3→83.1	13.323	23.323		0.850	0.867

Table 8.5- Application of acceptance WADA criteria for unequivocal identification of the clozapine in hair.

	Transition (m/z)	Absolute area	Relative area	S/N	RT_A	RT_{IS}	RT_{Ratio}
Positive 1	327.2→269.9	1639.011	95.043	5.847	4.047	7.330	0.552
	327.2→191.9	1724.491	100.000	6.015	4.038	7.330	0.551
	327.2→163.8	585.835	33.971	2.081	4.040	7.330	0.551
Negative 1	327.2→269.9	-	-	-	-	-	-
	327.2→191.9	-	-	-	-	-	-
	327.2→163.8	70.038	100.000	0.419	3.986	-	-
Positive 2	327.2→269.9	817.094	92.113	2.975	4.005	7.133	0.561
	327.2→191.9	887.052	100.000	3.565	4.007	7.133	0.562
	327.2→163.8	210.112	23.687	1.515	4.004	7.133	0.561
Negative 2	327.2→269.9	-	-	-	-	-	-
	327.2→191.9	-	-	-	-	-	-
	327.2→163.8	-	-	-	-	-	-
Positive 3	327.2→269.9	770.384	63.390	3.235	4.019	7.261	0.554
	327.2→191.9	1215.315	100.000	4.237	4.023	7.261	0.554
	327.2→163.8	513.608	42.261	1.384	4.038	7.261	0.556
Negative 3	327.2→269.9	-	-	-	-	-	-
	327.2→191.9	-	-	-	-	-	-
	327.2→163.8	-	-	-	-	-	-
Positive 4	327.2→269.9	1773.217	61.268	6.650	4.053	7.192	0.563
	327.2→191.9	2894.188	100.000	9.519	4.050	7.192	0.563
	327.2→163.8	910.486	31.459	2.780	4.054	7.192	0.564
Negative 4	327.2→269.9	-	-	-	-	-	-
	327.2→191.9	-	-	-	-	-	-
	327.2→163.8	-	-	-	-	-	-
Positive 5	327.2→269.9	653.69	100.00	2.48	4.07	7.48	0.54
	327.2→191.9	443.58	67.86	1.68	4.09	7.48	0.55
	327.2→163.8	305.74	46.77	3.21	4.09	7.48	0.55
Negative 5	327.2→269.9	-	-	-	-	-	-
	327.2→191.9	93.385	100.000	0.398	3.611	-	-
	327.2→163.8	-	-	-	-	-	-
Positive 6	327.2→269.9	327.884	70.224	1.537	4.026	7.268	0.554
	327.2→191.9	466.913	100.000	1.422	4.056	7.268	0.558
	327.2→163.8	210.117	45.001	2.154	4.037	7.268	0.556
Negative 6	327.2→269.9	-	-	-	-	-	-
	327.2→191.9	-	-	-	-	-	-
	327.2→163.8	-	-	-	-	-	-

	Transition (m/z)	Relative Abundance		S/N	ΔRT_{Ratio}	
Criteria	327.2→269.9	85.043	105.043		0.547	0.558
	327.2→191.9	90.000	110.000	>3	0.545	0.556
	327.2→163.8	27.177	40.766		0.546	0.557

Table 8.6- Application of acceptance WADA criteria for unequivocal identification of the haloperidol in hair.

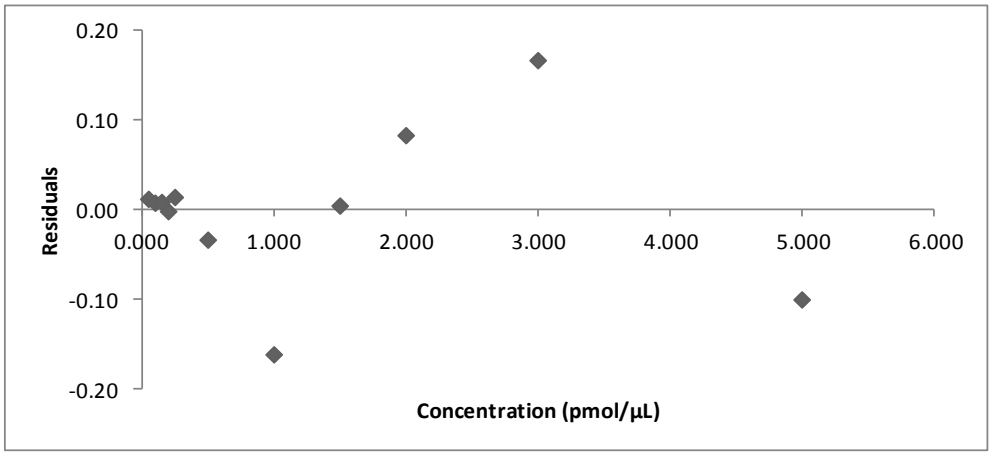
	Transition (m/z)	Absolute area	Relative area	S/N	RT_A	RT_{IS}	RT_{Ratio}
Positive 1	376.0→164.7	10716.280	55.225	37.544	6.625	6.591	1.005
	376.0→122.9	19404.737	100.000	67.472	6.622	6.591	1.005
	376.0→94.8	9173.733	47.276	32.296	6.622	6.591	1.005
Negative 1	376.0→164.7	-	-	-	-	-	-
	376.0→122.9	-	-	-	-	-	-
	376.0→94.8	-	-	-	-	-	-
Positive 2	376.0→164.7	10873.386	63.211	37.926	6.405	6.376	1.005
	376.0→122.9	17201.832	100.000	62.091	6.409	6.376	1.005
	376.0→94.8	6957.090	40.444	24.717	6.411	6.376	1.005
Negative 2	376.0→164.7	-	-	-	-	-	-
	376.0→122.9	-	-	-	-	-	-
	376.0→94.8	-	-	-	-	-	-
Positive 3	376.0→164.7	15799.554	61.363	53.009	6.512	6.487	1.004
	376.0→122.9	25747.751	100.000	96.379	6.518	6.487	1.005
	376.0→94.8	10482.686	40.713	39.603	6.515	6.487	1.004
Negative 3	376.0→164.7	-	-	-	-	-	-
	376.0→122.9	-	-	-	-	-	-
	376.0→94.8	-	-	-	-	-	-
Positive 4	376.0→164.7	8907.074	46.722	31.581	6.464	6.428	1.006
	376.0→122.9	19064.111	100.000	73.133	6.455	6.428	1.004
	376.0→94.8	6496.192	34.076	22.174	6.462	6.428	1.005
Negative 4	376.0→164.7	-	-	-	-	-	-
	376.0→122.9	-	-	-	-	-	-
	376.0→94.8	-	-	-	-	-	-
Positive 5	376.0→164.7	6769.220	72.631	26.670	6.784	6.749	1.005
	376.0→122.9	9319.994	100.000	33.391	6.784	6.749	1.005
	376.0→94.8	4943.972	53.047	17.638	6.788	6.749	1.006
Negative 5	376.0→164.7	257.399	52.430	0.871	6.445	-	-
	376.0→122.9	490.939	100.000	2.019	6.449	-	-
	376.0→94.8	233.460	47.554	0.866	6.440	-	-
Positive 6	376.0→164.7	6319.393	54.834	23.209	6.527	6.493	1.005
	376.0→122.9	11524.690	100.000	43.110	6.524	6.493	1.005
	376.0→94.8	4668.362	40.507	17.019	6.526	6.493	1.005
Negative 6	376.0→164.7	-	-	-	-	-	-
	376.0→122.9	-	-	-	-	-	-
	376.0→94.8	-	-	-	-	-	-
	Transition (m/z)	**Relative Abundance**		**S/N**			**ΔRT_{Ratio}**
Criteria	376.0→164.7	45.225	65.225		0.995		1.015
	376.0→122.9	90.000	110.000	>3	0.995		1.015
	376.0→94.8	37.821	56.731		0.995		1.015

Appendix 8.3: Example of the linearity analysis for the transition 376.0→164.7 of haloperidol.

Concentration (pmol/µL)	Peak Area Ratio	S/N
0.05	0.06	15.05
0.1	0.13	22.52
0.15	0.20	25.93
0.2	0.25	37.13
0.25	0.34	60.53
0.5	0.64	120.58
1.0	1.21	213.96
1.5	2.07	302.20
2.0	2.84	446.62
3.0	4.32	663.66
5.0	6.84	990.64

Regression Statistic	
m	1.39
b	-0.02
R	0.999
R^2	0.999
$S_{y/x}$	0.09
$2xS_{y/x}$	0.18
S_b	0.04
T-Student value	2.26
Upper limit 95%	0.06
Lower limit 95%	-0.10

Concentration (pmol/µL)	Residuals
0.05	0.012
0.1	0.008
0.15	0.009
0.2	-0.002
0.25	0.014
0.5	-0.034
1.0	-0.162
1.5	0.005
2.0	0.083
3.0	0.167
5.0	-0.101

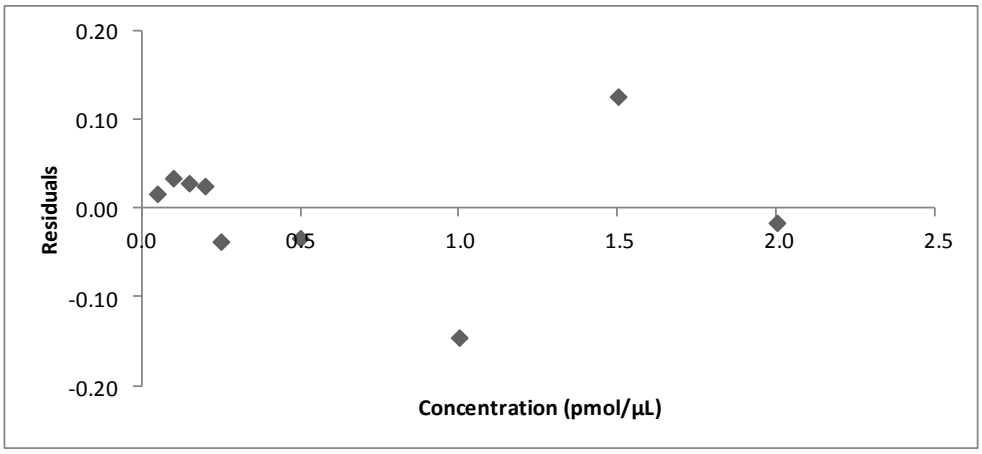


Appendix 8.4: Example of the linearity analysis for the transition 327.2→163.8 of clozapine.

Concentration (pmol/µL)	Peak Area Ratio	S/N
0.05	0.10	38.11
0.1	0.24	79.27
0.15	0.36	135.07
0.2	0.48	178.84
0.25	0.54	209.07
0.5	1.16	439.08
1.0	2.29	940.73
1.5	3.79	1422.99
2.0	4.89	1759.49

Regression Statistic	
m	2.469
b	-0.037
R	0.999
R^2	0.998
$S_{y/x}$	0.078
2x $S_{y/x}$	0.157
S_b	0.036
T-Student value	2.360
Upper limit 95%	0.049
Lower limit 95%	-0.122

Concentration (pmol/µL)	Residuals
0.05	0.017
0.1	0.034
0.15	0.029
0.2	0.026
0.25	-0.037
0.5	-0.033
1.0	-0.146
1.5	0.127
2.0	-0.016

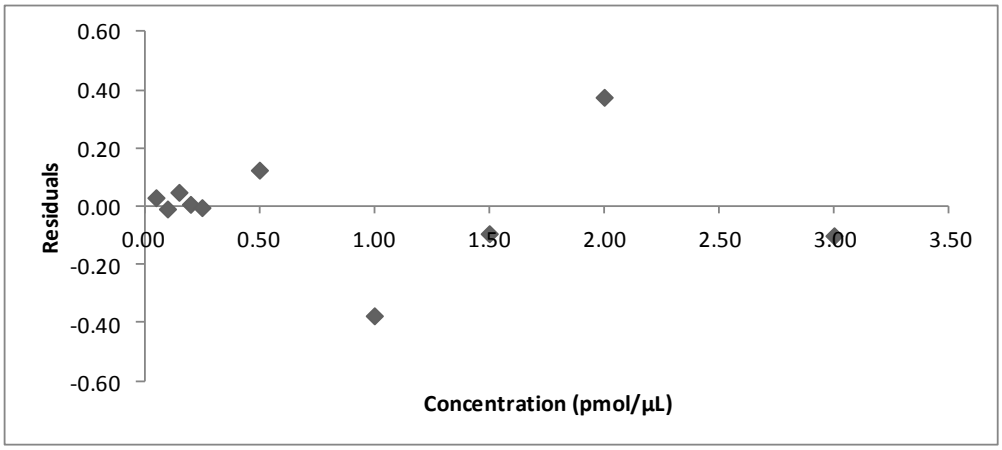


Appendix 8.5: Example of the linearity analysis for the transition 325.3→109.0 of citalopram.

Concentration (pmol/µL)	Peak Area Ratio	S/N
0.05	0.25	80.13
0.1	0.45	146.77
0.15	0.74	246.29
0.2	0.93	302.58
0.25	1.16	377.00
0.5	2.46	783.59
1	4.31	1617.46
1.5	6.95	2312.23
2	9.77	2851.80
3	14.00	4541.72

Regression Statistic	
m	4.707
b	-0.017
R	0.9992
R^2	0.9984
$S_{y/x}$	0.200
2x $S_{y/x}$	0.400
S_b	0.086
T-Student value	2.310
Upper limit 95%	0.182
Lower limit 95%	-0.215

Concentration	Residuals
0.1	0.030
0.1	-0.009
0.2	0.048
0.2	0.007
0.3	-0.004
0.5	0.124
1.0	-0.376
1.5	-0.094
2.0	0.375
3.0	-0.101



Appendix 8.6: ANOVA analysis, repeatability and intermediate precision.

Table 8.7- ANOVA table used for the study of repeatability and intermediate precision.

Source	Sums of Squares (SS)	Degrees of freedom	Mean Squares (MS)
Between Groups (Run)	$SS_{run} = n \sum_{i=1}^{p} (\bar{X}_i - \bar{\bar{X}})^2$	$p - 1$	$MS_{run} = \frac{n \sum_{i=1}^{p} (\bar{X}_i - \bar{\bar{X}})^2}{p - 1}$
Within Groups (Repeatability)	$SS_r = \sum_{i=1}^{p} \sum_{j=i}^{n} (X_{ij} - \bar{X}_i)^2$	$p.(n-1)$	$MS_r = \frac{\sum_{i=1}^{p} \sum_{j=i}^{n} (X_{ij} - \bar{X}_i)^2}{p\,(p-1)}$
Total	$SS_r = SS_{run} + SS_r$	$p.n - 1$	$\mathrm{MS_T} = \frac{\mathrm{SS_T}}{\mathrm{n} - 1}$

p - Number of runs on which the sample is analysed;

n - Number of replicates performed at every run;

X_{ij} - Instrumental response of the sample analysed in the jth replicate and the ith run;

$\bar{X}_i$ – Mean of the j replicates responses perform on run i;

$\bar{\bar{X}}$ - Mean of the mean values obtained in the p different runs.

Table 8.8- Calculation of variances used for the study of repeatability and intermediate precision.

Variance	Expression	Degrees of freedom
Repeatability variance (S_r^2)	$S_r^2 = MS_r$	$p.(n-1)$
Between – run variance (S_{run}^2)	$S_{run}^2 = \frac{MS_{run} - MS_r}{n}$	
Intermediate variance (S_I^2)	$S_I^2 = S_r^2 + S_{run}^2$	
Mean variance ($\mathrm{S_x^2}$)	$\mathrm{S_x^2} = \frac{\mathrm{MS_{run}}}{\mathrm{n}}$	$p - 1$

Appendix 8.7: Data of the study of weighted least squares linear regression.

Table 8.9- Relative errors (RE%) and the respective sum of the relative errors ($\sum|\%\ \mathbf{RE}|$) generated by the use of simple linear regression and weighted linear regression for each weighting factor ($\mathbf{w_i}$) for citalopram

Nominal concentration (µM)	Model 1 Unweighted (w_i=1)	Model 2 $\frac{1}{x}$	Model 3 $\frac{1}{x^2}$	Model 4 $\frac{1}{y}$	Model 5 $\frac{1}{y^2}$	Model 6 $\frac{1}{\sqrt{x}}$	Model 7 $\frac{1}{\sqrt{y}}$
0.05	308.80	124.01	42.11	136.53	64.18	191.91	198.73
0.10	121.78	34.23	39.68	24.02	27.06	51.13	53.59
0.15	78.03	66.48	76.34	63.83	73.06	47.68	46.14
0.20	37.52	37.92	38.28	37.35	39.45	25.03	24.52
0.25	23.70	19.13	16.82	16.82	14.55	15.08	12.54
0.50	15.55	23.27	11.24	23.87	13.31	21.78	21.95
1.00	13.19	11.87	13.81	13.11	11.69	14.01	14.50
1.50	47.87	41.77	43.58	43.25	42.54	45.31	46.02
2.00	26.81	18.61	28.34	20.18	26.58	23.04	23.81
3.00	20.70	29.75	46.08	28.35	38.26	25.18	24.51
$\sum\|\%\ \boldsymbol{RE}\|$	693.95	407.04	356.28	407.32	350.68	460.17	466.30

Table 8.10- Relative errors (RE%) and the respective sum of the relative errors ($\sum|\%\ \mathbf{RE}|$) generated by the use of simple linear regression and weighted linear regression for each weighting factor ($\mathbf{w_i}$) for clozapine.

Nominal concentration (µM)	Model 1 Unweighted (w_i=1)	Model 2 $\frac{1}{x}$	Model 3 $\frac{1}{x^2}$	Model 4 $\frac{1}{y}$	Model 5 $\frac{1}{y^2}$	Model 6 $\frac{1}{\sqrt{x}}$	Model 7 $\frac{1}{\sqrt{y}}$
0.05	198.15	115.11	51.78	138.13	99.85	156.26	165.49
0.10	9.76	35.73	60.92	22.77	39.04	20.86	13.70
0.15	77.85	82.34	90.74	74.21	77.39	77.67	73.70
0.20	40.64	42.93	44.46	38.53	39.61	38.99	37.11
0.25	32.49	38.40	34.30	35.87	34.22	34.81	33.78
0.50	26.42	27.54	27.17	26.70	27.25	27.45	26.57
1.00	28.21	28.22	47.54	26.96	40.84	27.84	27.46
1.50	23.55	20.81	28.16	20.32	20.38	22.58	22.09
2.00	14.18	18.72	40.76	19.43	36.71	16.17	16.68
$\sum\|\%\ \boldsymbol{RE}\|$	451.25	409.78	425.83	402.91	415.29	422.64	416.59

Appendix 8.8: Data of the study of Carry-Over.

Table 8.11- Area of LOQ of citalopram and desipramine used to calculate the criteria for carry-over.

Control LOQ			
Citalopram		**Desipramine**	
Concentration (µM)	**Area**	**Concentration (µM)**	**Area**
0.05	11465.98	0.5	99834.11

Table 8.12- Results of carry-over for citalopram and desipramine. Data obtained of the injections of blanks after sample that was fortified with 5, 0.5 and 0.05 µM with the compounds of the study.

		Citalopram	**Desipramine**
Concentration (µM)	**Injection**	**Area[1]**	**Area[1]**
5	Sample	856213.45	48079.04
	Blank 1	1037.29	340.70
	Blank 2	540.03	69.02
	Blank 3	200.27	112.77
	Blank 4	188.09	205.41
	Blank 5	98.69	103.75
0.5	Sample	100896.00	68323.04
	Blank 1	274.44	108.36
	Blank 2	148.42	129.21
	Blank 3	100.75	222.56
	Blank 4	144.79	114.22
	Blank 5	103.01	140.25
0.05	Sample	9022.31	38356.54
	Blank 1	76.15	296.67
	Blank 2	133.71	235.05
	Blank 3	101.30	157.02
	Blank 4	128.20	96.34
	Blank 5	104.27	125.82

[1] The value of the area presented is the average of the data obtained in the 3 days that the procedure was repeated

Table 8.13- Area of LOQ of clozapine and desipramine used to calculate the criteria for carry-over.

Control LOQ			
Clozapine		**Desipramine**	
Concentration (µM)	**Area**	**Concentration (µM)**	**Area**
0.05	1480.90	0.5	99834.11

Table 8.14- Results of carry-over for clozapine and desipramine. Data obtained of the injections of blanks after sample that was fortified with 5, 0.5 and 0.05 µM with the compounds of the study.

		Clozapine	**Desipramine**
Concentration (µM)	**Injection**	**Area[1]**	**Area[1]**
5	Sample	177879.25	48079.04
	Blank 1	353.18	340.70
	Blank 2	97.63	69.02
	Blank 3	86.01	112.77
	Blank 4	ND	205.41
	Blank 5	45.82	103.75
0.5	Sample	22015.99	68323.04
	Blank 1	127.91	108.36
	Blank 2	70.58	129.21
	Blank 3	58.11	222.56
	Blank 4	ND	114.22
	Blank 5	46.44	140.25
0.05	Sample	2120.56	38356.54
	Blank 1	111.32	296.67
	Blank 2	116.72	235.05
	Blank 3	70.03	157.02
	Blank 4	ND	96.34
	Blank 5	46.11	125.82

[1] The value of the area presented is the average of the data obtained in the 3 days that the procedure was repeated;
ND – Not Detected.

Table 8.15- Area of LOQ of haloperidol and haloperidol-D4 used to calculate the criteria for carry-over.

Control LOQ			
Haloperidol		**Haloperidol-D4**	
Concentration (μM)	**Area**	**Concentration (μM)**	**Area**
0.05	9691.26	0.5	210345.35

Table 8.16- Results of carry-over for haloperidol and haloperidol-D4. Data obtained of the injections of blanks after sample that was fortified with 5, 0.5 and 0.05 μM with the compounds of the study.

		Haloperidol	**Haloperidol-D4**
Concentration (μM)	**Injection**	**Area[1]**	**Area[1]**
5	Sample	887658.99	166663.54
	Blank 1	5123.66	1416.41
	Blank 2	1594.62	542.83
	Blank 3	910.34	214.88
	Blank 4	467.47	163.70
	Blank 5	426.84	122.07
0.5	Sample	108201.71	199866.04
	Blank 1	940.34	1664.06
	Blank 2	296.29	376.27
	Blank 3	307.68	197.21
	Blank 4	244.01	157.89
	Blank 5	129.08	79.69
0.05	Sample	10226.17	174195.86
	Blank 1	214.52	1240.46
	Blank 2	77.35	414.63
	Blank 3	131.49	223.01
	Blank 4	103.58	144.09
	Blank 5	83.58	125.42

[1] The value of the area presented is the average of the data obtained in the 3 days that the procedure was repeated

Appendix 8.9: Data related of the contamination of plasma samples.

Table 8.17- Contamination of citalopram in plasma samples. Citalopram quantification in plasma samples of clozapine, haloperidol and control**.**

Label	Concentration (pg/µL)	Label	Concentration (pg/µL)	Label	Concentration (pg/µL)
01PLCL1	0.58	01PLHA1	1.87	01PLCT1	0.54*
01PLCL2	0.79	01PLHA2	1.82	01PLCT2	0.34*
01PLCL3	0.83	01PLHA3	1.38	01PLCT3	0.58
01PLCL4	0.40*	01PLHA4	x	01PLCT4	0.43*
01PLCL5	x	01PLHA5	1.98	01PLCT5	0.58
02PLCL1	x	02PLHA1	0.77*	02PLCT1	1.00
02PLCL2	x	02PLHA2	0.48*	02PLCT2	0.78
02PLCL3	1.55	02PLHA3	1.00	02PLCT3	0.73
02PLCL4	1.32	02PLHA4	0.68	02PLCT4	1.10
02PLCL5	0.70*	02PLHA5	0.49*	02PLCT5	x
04PLCL1	x	04PLHA1	4.16	04PLCT1	x
04PLCL2	1.26	04PLHA2	3.25	04PLCT2	0.18*
04PLCL3	1.36	04PLHA3	4.29	04PLCT3	0.05*
04PLCL4	0.76	04PLHA4	3.88	04PLCT4	0.05*
04PLCL5	0.88	04PLHA5	x	04PLCT5	0.06*
08PLCL1	x	08PLHA1	x	08PLCT1	17.69
08PLCL2	23.77	08PLHA2	34.83	08PLCT2	7.80
08PLCL3	x	08PLHA3	x	08PLCT3	22.38
08PLCL4	31.30	08PLHA4	35.76*	08PLCT4	14.49
08PLCL5	21.20	08PLHA5	27.78	08PLCT5	16.48
15PLCL1	x	15PLHA1	4.39	15PLCT1	3.73
15PLCL2	3.31	15PLHA2	5.72	15PLCT2	4.12
15PLCL3	6.07	15PLHA3	6.95	15PLCT3	4.58
15PLCL4	4.96	15PLHA4	7.00	15PLCT4	3.84
15PLCL5	x	15PLHA5	x	15PLCT5	3.60
30PLCL1	0.30*	30PLHA1	0.51*	30PLCT1	0.23*
30PLCL2	0.52*	30PLHA2	0.52*	30PLCT2	0.25*
30PLCL3	0.28*	30PLHA3	0.28*	30PLCT3	0.17*
30PLCL4	0.38*	30PLHA4	0.27*	30PLCT4	0.20*
30PLCL5	0.51*	30PLHA5	0.44*	30PLCT5	0.20*

x - Sample insufficient or non-existent; *- out of calibration curve.

Label:

- the two first numbers correspond to the different days: 1, 2, 4, 8, 15,30;
- the two first letters correspond to the matrix: PL - plasma;
- the two second letters correspond to the drug: CL – Clozapine, HA – Haloperidol and CT - Control;
- the last number correspond to the animal replicate: 1, 2, 3, 4 and 5.

Table 8.18- Contamination of clozapine in plasma samples. Clozapine quantification in plasma samples of clozapine, haloperidol and control.

Label	Concentration (pg/µL)	Label	Concentration (pg/µL)	Label	Concentration (pg/µL)
01PLCI1	x	01PLHA1	*	01PLCT1	*
01PLCI2	x	01PLHA2	*	01PLCT2	*
01PLCI3	*	01PLHA3	*	01PLCT3	*
01PLCI4	*	01PLHA4	x	01PLCT4	*
01PLCI5	*	01PLHA5	0.50*	01PLCT5	*
02PLCI1	*	02PLHA1	0.21*	02PLCT1	*
02PLCI2	*	02PLHA2	*	02PLCT2	*
02PLCI3	*	02PLHA3	0.32*	02PLCT3	*
02PLCI4	*	02PLHA4	0.22*	02PLCT4	*
02PLCI5	*	02PLHA5	*	02PLCT5	x
04PLCI1	*	04PLHA1	0.01*	04PLCT1	x
04PLCI2	*	04PLHA2	*	04PLCT2	*
04PLCI3	*	04PLHA3	*	04PLCT3	*
04PLCI4	*	04PLHA4	*	04PLCT4	*
04PLCI5	*	04PLHA5	x	04PLCT5	*
08PLCI1	0.95	08PLHA1	x	08PLCT1	0.77*
08PLCI2	x	08PLHA2	1.96	08PLCT2	*
08PLCI3	1.46	08PLHA3	x	08PLCT3	0.72*
08PLCI4	0.66*	08PLHA4	2.27	08PLCT4	0.38*
08PLCI5	1.34	08PLHA5	1.59	08PLCT5	0.21*
15PLCI1	0.20	15PLHA1	0.10*	15PLCT1	*
15PLCI2	0.12	15PLHA2	*	15PLCT2	*
15PLCI3	*	15PLHA3	0.48*	15PLCT3	*
15PLCI4	*	15PLHA4	0.89	15PLCT4	*
15PLCI5	*	15PLHA5	x	15PLCT5	*
30PLCI1	*	30PLHA1	1.34	30PLCT1	0.82
30PLCI2	*	30PLHA2	0.45*	30PLCT2	0.08*
30PLCI3	0.20*	30PLHA3	1.08	30PLCT3	*
30PLCI4	0.02*	30PLHA4	0.03*	30PLCT4	0.25*
30PLCI5	0.24*	30PLHA5	0.29*	30PLCT5	0.08*

x - Sample insufficient or non-existent; *- out of calibration curve.

Label:

- the two first numbers correspond to the different days: 1, 2, 4, 8, 15,30;
- the two first letters correspond to the matrix: PL - plasma;
- the two second letters correspond to the drug: CI – Citalopram, HA – Haloperidol and CT - Control;
- the last number correspond to the animal replicate: 1, 2, 3, 4 and 5.

Table 8.19- Contamination of haloperidol in plasma samples. Haloperidol quantification in plasma samples of citalopram, clozapine and control.

Label	Concentration (pg/µL)	Label	Concentration (pg/µL)	Label	Concentration (pg/µL)
01PLCI1	x	01PLCL1	0.07*	01PLCT1	0.13*
01PLCI2	x	01PLCL2	0.04*	01PLCT2	0.04*
01PLCI3	0.04*	01PLCL3	ND	01PLCT3	0.06*
01PLCI4	ND	01PLCL4	ND	01PLCT4	0.05*
01PLCI5	0.05*	01PLCL5	x	01PLCT5	0.06*
02PLCI1	ND	02PLCL1	x	02PLCT1	0.05*
02PLCI2	ND	02PLCL2	x	02PLCT2	0.04*
02PLCI3	0.04*	02PLCL3	0.05*	02PLCT3	0.02*
02PLCI4	ND	02PLCL4	ND	02PLCT4	0.06*
02PLCI5	ND	02PLCL5	ND	02PLCT5	x
04PLCI1	0.05*	04PLCL1	x	04PLCT1	x
04PLCI2	0.06*	04PLCL2	ND	04PLCT2	0.04*
04PLCI3	ND	04PLCL3	ND	04PLCT3	0.03*
04PLCI4	0.04*	04PLCL4	ND	04PLCT4	0.09*
04PLCI5	0.05*	04PLCL5	ND	04PLCT5	0.02*
08PLCI1	0.55*	08PLCL1	x	08PLCT1	0.37*
08PLCI2	x	08PLCL2	0.40*	08PLCT2	0.16*
08PLCI3	0.58*	08PLCL3	x	08PLCT3	0.57*
08PLCI4	0.52*	08PLCL4	0.24*	08PLCT4	0.31*
08PLCI5	0.68	08PLCL5	0.34*	08PLCT5	0.34*
15PLCI1	0.09*	15PLCL1	x	15PLCT1	0.10*
15PLCI2	0.12*	15PLCL2	0.14*	15PLCT2	0.07*
15PLCI3	0.17*	15PLCL3	0.07*	15PLCT3	0.14*
15PLCI4	0.10*	15PLCL4	ND	15PLCT4	0.08*
15PLCI5	0.13*	15PLCL5	x	15PLCT5	0.11*
30PLCI1	ND	30PLCL1	ND	30PLCT1	ND
30PLCI2	ND	30PLCL2	ND	30PLCT2	ND
30PLCI3	ND	30PLCL3	ND	30PLCT3	0.03*
30PLCI4	ND	30PLCL4	ND	30PLCT4	ND
30PLCI5	ND	30PLCL5	ND	30PLCT5	ND

ND - Not detected; x - Sample insufficient or non-existent; *- out of calibration curve.
Label:
- the two first numbers correspond to the different days: 1, 2, 4, 8, 15,30;
- the two first letters correspond to the matrix: PL - plasma;
- the two second letters correspond to the drug: CI - Citalopram, CL – Clozapine and CT - Control;
- the last number correspond to the animal replicate: 1, 2, 3, 4 and 5.

Appendix 8.10: Data related of the contamination of hair samples.

Table 8.20- Contamination of citalopram in hair samples. Citalopram quantification in hair samples of clozapine, haloperidol and control.

Label	Concentration (ng/mg)	Label	Concentration (ng/mg)	Label	Concentration (ng/mg)
01PECL1	ND	01PEHA1	ND	01PECT1	ND
01PECL2	ND	01PEHA2	ND	01PECT2	ND
01PECL3	ND	01PEHA3	ND	01PECT3	ND
01PECL4	ND	01PEHA4	ND	01PECT4	ND
01PECL5	ND	01PEHA5	ND	01PECT5	ND
02PECL1	ND	02PEHA1	ND	02PECT1	ND
02PECL2	ND	02PEHA2	ND	02PECT2	ND
02PECL3	ND	02PEHA3	ND	02PECT3	ND
02PECL4	ND	02PEHA4	ND	02PECT4	ND
02PECL5	x	02PEHA5	ND	02PECT5	ND
04PECL1	*	04PEHA1	ND	04PECT1	ND
04PECL2	x	04PEHA2	ND	04PECT2	0.27*
04PECL3	0.01*	04PEHA3	ND	04PECT3	x
04PECL4	ND	04PEHA4	ND	04PECT4	ND
04PECL5	ND	04PEHA5	ND	04PECT5	ND
08PECL1	0.01*	08PEHA1	ND	08PECT1	ND
08PECL2	#VALOR!	08PEHA2	ND	08PECT2	*
08PECL3	0.01*	08PEHA3	ND	08PECT3	*
08PECL4	0.04*	08PEHA4	ND	08PECT4	ND
08PECL5	0.12*	08PEHA5	ND	08PECT5	0.01*
15PECL1	x	15PEHA1	NQ	15PECT1	0.02*
15PECL2	ND	15PEHA2	NQ	15PECT2	ND
15PECL3	0.016*	15PEHA3	ND	15PECT3	ND
15PECL4	ND	15PEHA4	ND	15PECT4	ND
15PECL5	ND	15PEHA5	0.016[#]	15PECT5	ND
30PECL1	x	30PEHA1	x	30PECT1	x
30PECL2	0.011*	30PEHA2	NQ	30PECT2	ND
30PECL3	0.036*	30PEHA3	ND	30PECT3	ND
30PECL4	0.024*	30PEHA4	ND	30PECT4	ND
30PECL5	x	30PEHA5	ND	30PECT5	ND

ND - Not detected; x - Sample insufficient or non-existent; *- out of calibration curve.
Label:
- the two first numbers correspond to the different days: 1, 2, 4, 8, 15,30;
- the two first letters correspond to the matrix: PE - hair;
- the two second letters correspond to the drug: CL – Clozapine, HA – Haloperidol and CT - Control;
- the last number correspond to the animal replicate: 1, 2, 3, 4 and 5.

Table 8.21- Contamination of clozapine in hair samples. Clozapine quantification in hair samples of citalopram, haloperidol and control.

Label	Concentration (ng/mg)	Label	Concentration (ng/mg)	Label	Concentration (ng/mg)
01PECI1	ND	01PEHA1	ND	01PECT1	ND
01PECI2	ND	01PEHA2	ND	01PECT2	ND
01PECI3	ND	01PEHA3	ND	01PECT3	ND
01PECI4	ND	01PEHA4	ND	01PECT4	ND
01PECI5	ND	01PEHA5	ND	01PECT5	ND
02PECI1	ND	02PEHA1	ND	02PECT1	ND
02PECI2	ND	02PEHA2	ND	02PECT2	ND
02PECI3	ND	02PEHA3	ND	02PECT3	ND
02PECI4	ND	02PEHA4	ND	02PECT4	ND
02PECI5	ND	02PEHA5	ND	02PECT5	ND
04PECI1	ND	04PEHA1	ND	04PECT1	ND
04PECI2	ND	04PEHA2	ND	04PECT2	NQ
04PECI3	ND	04PEHA3	ND	04PECT3	x
04PECI4	ND	04PEHA4	ND	04PECT4	ND
04PECI5	ND	04PEHA5	ND	04PECT5	ND
08PECI1	ND	08PEHA1	ND	08PECT1	ND
08PECI2	ND	08PEHA2	ND	08PECT2	ND
08PECI3	ND	08PEHA3	ND	08PECT3	ND
08PECI4	ND	08PEHA4	ND	08PECT4	ND
08PECI5	ND	08PEHA5	ND	08PECT5	ND
15PECI1	ND	15PEHA1	ND	15PECT1	ND
15PECI2	ND	15PEHA2	ND	15PECT2	ND
15PECI3	ND	15PEHA3	ND	15PECT3	ND
15PECI4	ND	15PEHA4	ND	15PECT4	ND
15PECI5	ND	15PEHA5	ND	15PECT5	ND
30PECI1	x	30PEHA1	x	30PECT1	x
30PECI2	ND	30PEHA2	NQ	30PECT2	ND
30PECI3	ND	30PEHA3	NQ	30PECT3	ND
30PECI4	ND	30PEHA4	ND	30PECT4	ND
30PECI5	ND	30PEHA5	ND	30PECT5	ND

ND - Not detected; NQ - Detected but not quantified; x - Sample insufficient or non-existent.

Label:

- the two first numbers correspond to the different days: 1, 2, 4, 8, 15,30;
- the two first letters correspond to the matrix: PE - hair;
- the two second letters correspond to the drug: CI – Citalopram, HA – Haloperidol and CT - Control;
- the last number correspond to the animal replicate: 1, 2, 3, 4 and 5.

Table 8.22- Contamination of haloperidol in hair samples. Haloperidol quantification in hair samples of citalopram, clozapine and control.

Label	Concentration (ng/mg)	Label	Concentration (ng/mg)	Label	Concentration (ng/mg)
01PECI1	ND	01PECL1	ND	01PECT1	ND
01PECI2	ND	01PECL2	ND	01PECT2	ND
01PECI3	ND	01PECL3	ND	01PECT3	ND
01PECI4	ND	01PECL4	ND	01PECT4	ND
01PECI5	ND	01PECL5	ND	01PECT5	ND
02PECI1	ND	02PECL1	ND	02PECT1	ND
02PECI2	ND	02PECL2	ND	02PECT2	0.01*
02PECI3	ND	02PECL3	ND	02PECT3	ND
02PECI4	ND	02PECL4	ND	02PECT4	ND
02PECI5	ND	02PECL5	x	02PECT5	ND
04PECI1	ND	04PECL1	ND	04PECT1	ND
04PECI2	ND	04PECL2	x	04PECT2	0.18*
04PECI3	ND	04PECL3	ND	04PECT3	x
04PECI4	ND	04PECL4	ND	04PECT4	0.06*
04PECI5	ND	04PECL5	ND	04PECT5	0.02*
08PECI1	ND	08PECL1	ND	08PECT1	ND
08PECI2	ND	08PECL2	ND	08PECT2	0.02*
08PECI3	ND	08PECL3	ND	08PECT3	0.03*
08PECI4	ND	08PECL4	ND	08PECT4	0.41
08PECI5	ND	08PECL5	ND	08PECT5	0.03*
15PECI1	ND	15PECL1	x	15PECT1	ND
15PECI2	ND	15PECL2	ND	15PECT2	ND
15PECI3	ND	15PECL3	ND	15PECT3	ND
15PECI4	ND	15PECL4	ND	15PECT4	ND
15PECI5	ND	15PECL5	ND	15PECT5	ND
30PECI1	x	30PECL1	x	30PECT1	x
30PECI2	0.03*	30PECL2	ND	30PECT2	ND
30PECI3	0.04*	30PECL3	ND	30PECT3	ND
30PECI4	0.04*	30PECL4	ND	30PECT4	ND
30PECI5	ND	30PECL5	x	30PECT5	ND

ND - Not detected; NQ - Detected but not quantified; x - Sample insufficient or non-existent.
Label:
- the two first numbers correspond to the different days: 1, 2, 4, 8, 15,30;
- the two first letters correspond to the matrix: PE - hair;
- the two second letters correspond to the drug: CI – Citalopram, CL – Clozapine and CT – Control;
- the last number correspond to the animal replicate: 1, 2, 3, 4 and 5.