QUANTIFICATION OF TRAMADOL AND M1 IN *POST MORTEM* SAMPLES BY GAS CHROMATOGRAPHY–ION TRAP MASS SPECTROMETRY AND PRELIMINARY PHARMACOGENOMIC STUDIES

Master Thesis

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Master Degree in Legal Medicine and Forensic Sciences

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ABSTRACT

Tramadol is a centrally acting opioid analgesic, widely used for moderate to severe pain due to its efficacy and safety. Although tramadol induce less adverse effects compared to other opioids, serious complications can occur in case of intoxication. The intoxication by this drug it is also common in individuals with an abuse history of opioids, despite its low potential in developing dependence. Moreover, the administration of toxic doses of tramadol concomitantly with other central nervous system depressants is one of the most common causes of severe or lethal acute intoxication.

Currently, several analytical techniques are being used to quantify tramadol and its main pharmacologically active metabolite, O-desmethyltramadol (M1) in *ante mortem* specimens, namely in plasma, blood, saliva, urine and hair. However, in *post mortem* samples, there are few studies that evaluate the importance of M1 in the cause of death and the importance of polymorphism in the pharmacokinetics of tramadol.

This work aimed the optimization and validation of an analytical method for the detection and quantification of tramadol and M1 in *post mortem* blood samples by gas chromatography-ion trap mass spectrometry (GC-IT/MS). The same blood specimens were addressed to study cytochrome P450 2D6 (CYP2D6), multidrug resistance protein 1 (MDR1), μ-opioid receptor gene (OPRM1) and catechol-O-methyltransferase (COMT) single nucleotide polymorphisms (SNP) by real-time polymerase chain reaction (PCR).

The Part I of this dissertation describes the use and therapeutic applicability of tramadol as well as considerations about pharmacokinetic and pharmacodynamic of tramadol and M1. A review of the literature was also made on tramadol intoxications and validated analytical methods to quantify tramadol and metabolites in several biological matrices. Special consideration is devoted to the importance of pharmacogenomics in the interpretation of forensic and clinical toxicological results. The Part II is reserved for the general and specific objectives of this work. In Part III the experimental work is described according with the proposed objectives, as well as the method validation, its application to real cases of *post mortem* blood samples and genotyping and phenotyping analysis. In Part IV all references consulted for the present work are presented.

The GC-IT/MS method described in this study exhibited a good selectivity, lower limit of detection (LOD) and lower limit of quantification (LLOQ) (0.74 and 0.56 ng/mL for tramadol and 2.24 and 1.70 ng/mL for M1 respectively) in a matrix with

relevant importance in forensic toxicological analysis. The regression analysis for both analytes showed linearity in the range 5-1000 ng/mL with determination coefficients (r^2) ranging from 0.9991 to 0.9999. The coefficients of variation (%CV) oscillated between 0.70 and 12.45%. This method was then successfully applied for the quantification of tramadol and M1 in real *post mortem* blood samples from five cases of suspected tramadol fatal intoxications.

Regarding polymorphism studies, four subjects with at least one functional allele were categorized as extensive metabolizers (EMs). Nevertheless it was not possible to establish any comparison between polymorphic results and concentrations of tramadol and M1, due to the much reduced number of samples. In addition, high tramadol concentrations could not be completely explained only based on polymorphisms studied. Besides pharmacogenomics, the pharmacokinetics of the drug can be affected by many other factors namely, age, disease, concomitant medication, metabolic interactions and kidney or liver function. One of the five cases was not possible to determine the genotype. In this case, it is important to consider the possibility of being a poor metabolizer (PM) subject for rare mutations that are extremely uncommon in Caucasians, which could not be identified with the assay used.

In conclusion it was develop and validate a GC-IT/MS method to quantify tramadol and M1 in *post mortem* blood samples. Moreover, 4 polymorphisms were applied in blood samples. If further samples were available, interesting correlations were expected to be obtained. Nevertheless, the methods were validated and are ready to be routinely used in forensic aplications.

Keywords: Tramadol; O-desmethyltramadol; Gas Chromatography; Mass Spectrometry; Intoxication; Polymorphisms; CYP2D6; MDR1; OPRM1; COMT.

RESUMO

O tramadol é um analgésico opióide de ação central, muito utilizado para a dor moderada a severa devido à sua eficácia e segurança. Apesar do tramadol induzir menores efeitos adversos comparativamente com outros opióides, podem ocorrer complicações graves em caso de intoxicação. A intoxicação por este fármaco é também comum em indivíduos com um historial de abuso de opióides, apesar do seu baixo potencial de desenvolvimento de dependência. No entanto, a administração de doses tóxicas de tramadol, concomitantemente com outros depressores do sistema nervoso central, é uma das causas mais comuns de intoxicação aguda grave ou letal.

Atualmente, várias técnicas analíticas estão disponíveis para quantificar o tramadol e o seu principal metabolito O-desmetiltramadol (M1), farmacologicamente activo, em amostras *ante mortem*, nomeadamente em plasma, sangue, saliva, urina e cabelo. Contudo, em amostras *post mortem,* são escassos os estudos que avaliaram a importância do M1 na causa de morte e a importância do polimorfismo na farmacocinética do tramadol.

Este trabalho teve como objetivo a otimização e a validação de um método analítico por cromatografia gasosa acoplada a espetrometria de massa com “ion-trap” (GC-IT/MS), para a deteção e quantificação de tramadol e M1 em amostras de sangue *post mortem*. As mesmas amostras de sangue foram utilizadas para estudar os polimorfismos de nucleótido único (SNP) nos genes do citocromo P450 2D6 (CYP2D6), proteína de resistência a múltiplas drogas (MDR1), gene do recetor μ-opióide (OPRM1) e catecol O-metiltransferase (COMT) pela reacção em cadeia da polimerase (PCR) em tempo-real.

Na Parte I da presente dissertação aborda a aplicabilidade terapêutica do tramadol assim como o estado de arte relativo à farmacocinética e farmacodinâmica do tramadol e M1. Procedeu-se também à revisão da literatura sobre as intoxicações por tramadol e métodos analíticos validados para a quantificação de tramadol e metabolitos em várias matrizes biológicas. Especial consideração é dada à importância da farmacogenómica na interpretação dos resultados toxicológicos forenses e clínicos. Na Parte II encontram-se definidos os objetivos gerais e específicos deste trabalho. Na Parte III o trabalho experimental é descrito de acordo com os objetivos propostos, assim como a validação do método desenvolvido, a sua aplicação em casos reais de sangue *post mortem* e análises de genotipagem e fenotipagem. Na Parte IV estão descritas todas as referências bibliográficas consultadas para a realização do presente trabalho.

O método de GC-IT/MS descrito neste estudo exibiu uma boa seletividade, baixo limite de detecção (LOD) e baixo limite de quantificação (LLOQ) (0.74 e 0.56 ng/mL para o tramadol e 2.24 e 1.70 ng/mL para o M1 respetivamente), numa matriz com importância relevante na análise toxicológica forense. As análises de regressão para ambos os analitos mostraram linearidade no intervalo 5-1000 ng/mL com coeficientes de determinação (r^2) que variaram de 0.9991 a 0.9999. Os coeficientes de variação (CV%) oscilaram entre 0,70 e 12,45%. Este método foi aplicado com sucesso para a quantificação do tramadol e M1 em amostras reais de sangue *post mortem* de cinco casos de suspeita de intoxicação fatal por tramadol.

Em relação aos estudos dos polimorfismos, quatro indivíduos foram classificados como metabolizadores extensos (EMs). No entanto, não foi possível estabelecer qualquer comparação entre os resultados dos polimorfismos e concentrações de tramadol e M1, devido ao número muito reduzido de amostras. Para além disso, as concentrações elevadas de tramadol não podem ser completamente explicadas apenas com base nos polimorfismos estudados. Além da farmacogenómica, a farmacocinética do fármaco pode ser afetada por muitos outros fatores, como por exemplo idade, doença, medicação concomitante, interações metabólicas e função renal ou hepática. Num dos cinco casos, não foi possível determinar o genótipo. Neste caso, é importante ter em conta a possibilidade de ser um sujeito metabolizador pobre (PM) para mutações que não são comuns em Caucasianos, que não puderam ser identificados com a análise realizada.

Em conclusão, foi desenvolvido e validado um método de GC-IT/MS para quantificar o tramadol e M1 em amostras de sangue *post mortem*. Além disso, quatro polimorfismos foram aplicados em amostras de sangue. Se outras amostras estivessem disponíveis, seria de esperar obter melhores correlações. No entanto, os métodos foram validados e estão prontos para ser utilizados rotineiramente em aplicações forenses.

Palavras-chave: Tramadol; O-desmetiltramadol; Cromatografia Gasosa; Espetrometria de Massa; Intoxicação; Polimorfismos; CYP2D6; MDR1; OPRM1; COMT.

ABREVIATION LIST

BSTFA, N,O-bis(trimethylsilyl) trifluoroacetamide;

CE-UV-LINF, capillary electrophoresis with ultraviolet laser-induced native fluorescence detection;

CNECV, Portuguese National Council of Ethics for Life Sciences;

CNS, central nervous system;

COMT, catechol-O-methyltransferase;

CV, coefficient of variation;

CYP, Cytochrome P450;

CYP2D6, Cytochrome P450 2D6;

GC-EI/MS, gas chromatography-electron impact mass spectrometry;

GC-IT/MS, gas chromatography-ion trap mass spectrometry;

GC-MS, gas chromatography-mass spectrometry;

GC-NPD, gas chromatography-nitrogen-phosphorus detector;

EMs, extensive metabolizers;

ESI, electrospray ionization;

$HClO_4$, perchloric acid;

HPLC, high performance liquid chromatography;

HPLC-DAD, high performance liquid chromatography-diode-array detector;

HPLC-FL, high performance liquid chromatography-fluorescence detection;

HPLC-UV, high performance liquid chromatography-ultraviolet detection;

IMs, intermediate metabolizers;

IS, internal standard;

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;

LLOQ, lower limit of quantification;

LPME, liquid-phase microextraction;

M1, O-desmethyltramadol;

M2, N-desmethyltramadol;

M3, N,N-didesmethyltramadol;

M4, N,N,O-tridesmethyltramadol;

M5, N,O-didesmethyltramadol;

M6, 4-hydroxy-ciclohexyltramadol;

M7, 4-hydroxy-ciclohexyl-N-desmethyl tramadol;

MDR1, multidrug resistance protein 1;

Met, methionine;

MS/MS, tandem mass spectrometry;

MSTFA, N-methyl-N-(trimethylsilyl) trifluoroacetamide;

m/z, mass-to-charge ratio;

NaOH, sodium hydroxide;

NS, not specified;

NSAIDs, non-steroidal anti-inflammatories;

OPRM1, μ-Opioid receptor gene;

PCR, polymerase chain reaction;

PMs, poor metabolizers;

SD, standard deviation;

SIM, selected ion monitoring;

SNPs, single nucleotide polymorphisms;

SPE, solid-phase extraction;

SPE-LC-MS/MS, solid-phase extraction liquid chromatography-tandem mass spectrometry;

SPME, solid-phase microextraction;

SSRIs, selective serotonin reuptake inhibitors;

TMCS, trimethylchlorosilane;

t_r, retention time;

UMs, ultrarapid metabolizers;

UPLC, ultra-performance liquid chromatography;

UPLC-MS/MS, ultra-performance liquid chromatography-tandem mass spectrometric;

Val, valine.

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PART I: General Introduction

1. History and use of tramadol

Tramadol is a synthetic opioid from the aminocyclohexanol group that was firstly synthesized in Germany by the pharmaceutical company Grünenthal GmbH, in 1962. It was introduced on the market in 1977 with the trade name Tramal® and thereafter, several pharmaceutical companies have introduced it with other trade names and different pharmaceutical forms were developed [1, 2].

Tramadol is marketed in the form of a racemic mixture of (+) and (-) two enantiomers [1, 3]. In Portugal, it is commercialized in the form of a salt - tramadol hydrochloride, as a single component or in combination with paracetamol [4]. Tramadol is a weak agonist on μ-opioid receptor and its analgesic effect is owed to the increase of the serotonergic transmission and noradrenaline reuptake inhibition. This is currently often used in clinical practice (hospital and ambulatory use) in acute and chronic pain treatment of moderate and in some cases, of severe intensity, due to its effectiveness and safety [1]. It is a viable therapeutic option and an alternative to other opioid analgesics since it has low potential for abuse, dependence, tolerance and low probability to cause adverse effects, including respiratory depression [5].

Tramadol has many pharmacological properties with therapeutic importance beyond their known analgesic effect. It has been shown to be useful in the treatment of several analgesic indications as postoperative, dental, abdominal, neuropathic, musculoskeletal, rheumatological, cardiac, renal colic, malignant and chronic pain (effectively similar to low doses of strong opioids), headache, fibromyalgia, premature ejaculation, anesthetics and pediatric applications. It has also been used in non-analgesic indications, such as psychiatry, nocturnal leg cramps and in postoperative shivering [6-14].

Tramadol has also an important role in patients where the administration of non-steroidal anti-inflammatories (NSAIDs) is contraindicated, where stronger opioids may be undesirable because of the potential for abuse or for patients who do not tolerate its adverse effects, which are generally more intense [7, 8]. However, there are still some concerns about the use of tramadol. The existing studies on the safety of tramadol when used in long term (more than two years) are still limited and their efficacy is variable due to the genetic polymorphisms of its metabolism. Another concern is that in most patients with chronic pain, antidepressants are also administered which leads to an increasing risk of serotonin syndrome [7, 15].

The extended-release formulation of tramadol (tramadol ER), a long-acting analgesic, has demonstrated efficacy in patients with moderate to severe chronic pain caused by osteoarthritis and low back pain with an acceptable tolerability and safety, and has proved to have advantages in patients with need of analgesic treatment in a longer period of time [16].

2. Physical and chemical properties

Tramadol ((1RS,2RS)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)-cyclo-hexanol); molecular mass 263.37 g/mol) has two stereoisomers and four enantiomers [1] (Fig. 1).

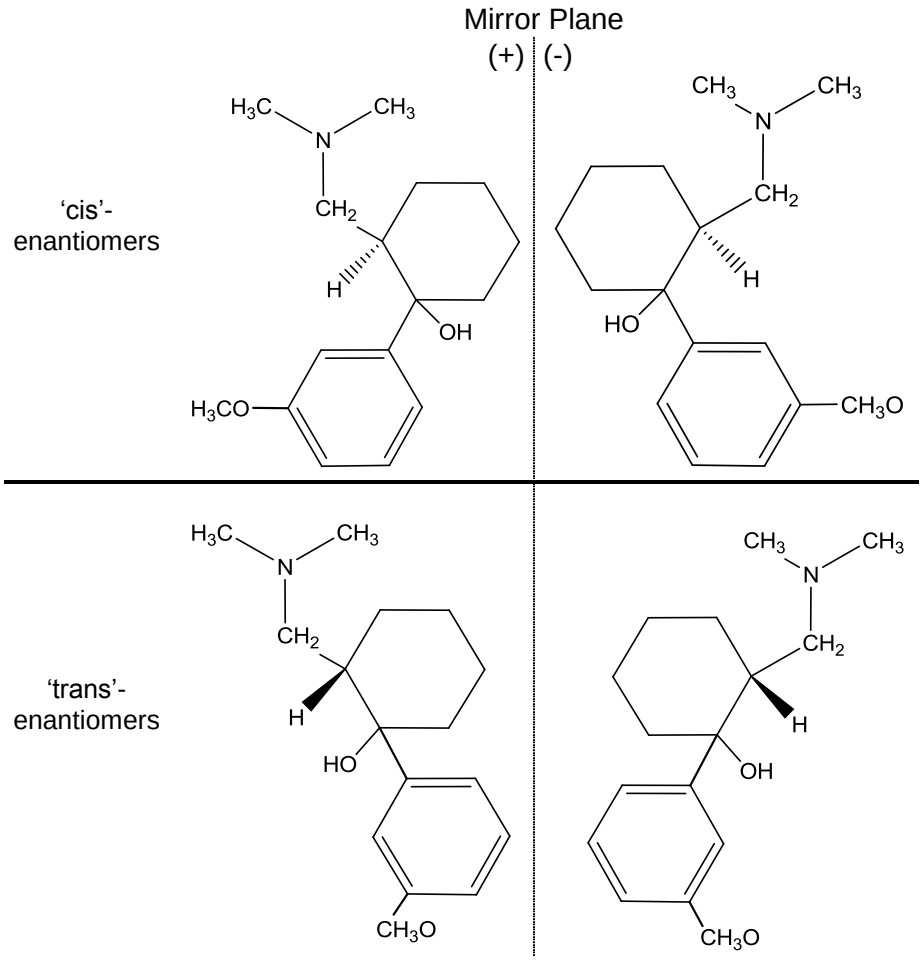
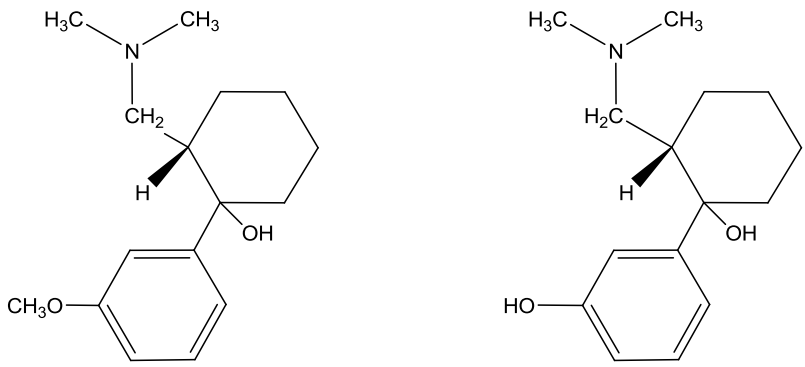


Figure 1. Enantiomers of tramadol.

In 1978, Frankus *et al*. developed a pharmacological study and observed that after dividing the compound 1-(m-Methoxyphenyl)-2-(dimethylaminomethyl)-cyclohexan-1-ol into cis- and trans-isomers, the resultant conformers were geometrically similar to morphine. However, the trans-isomer was more active than the cis-isomer as well as the (+/-) trans-isomer form was more active than (-/-) form [17, 18]. The enantiomers (+/-) and (-/-) are selective agonists of the μ-opioid receptors and act synergistically to improve the analgesia without increasing the adverse effects. The (+/-) enantiomer is about 4 times stronger than (-/-) in inhibiting serotonin uptake and (-/-) is approximately 10 times stronger than (+/-) in inhibiting noradrenaline uptake, thereby increasing the inhibitory effects in nociceptive transmission from the spinal medulla [19, 20].

Tramadol is mainly metabolized to O-desmethyltramadol (M1; (3-[2-(1-Amino-1-methylethyl)-1-hydroxycyclohexyl]phenol; molecular mass 249.34 g/mol)), a pharmacologically active metabolite, with an higher affinity for μ opioid receptors than the parent compound (Fig. 2) [21]. The (+/-) M1 enantiomer has 300-400 times greater affinity for these receptors than tramadol, whereas the (-/-) M1 mostly inhibits noradrenalin reuptake [22, 23].



Tramadol($C_{16}H_{25}NO_2$) **M1** ($C_{15}H_{23}NO_2$)

Figure 2. Chemical structures of tramadol and M1.

3. Pharmacokinetics of tramadol and O-desmethyltramadol

Tramadol can be administered by oral, intravenous, intramuscular or rectal, routes/vias, however in clinical practice, oral administration is the main route for tramadol [4].

Before excretion, tramadol is mainly metabolized by the cytochrome P450 2D6 (CYP2D6) isoenzyme in the liver, by the demethylation (mainly O- and N-demethylation) and by the conjugation reactions (mainly conjugation with glucuronic acid and sulfate). The primary metabolites O-desmethyltramadol (M1) and N-desmethyltramadol (M2) may be further metabolized to three additional secondary metabolites namely, N,N-didesmethyltramadol (M3), N,N,O-tridesmethyltramadol (M4) and N,O-didesmethyltramadol (M5). Only one of these metabolites, M1, is pharmacologically active [24, 25].

After oral administration, tramadol is rapidly absorbed and has a distribution volume of 3 L/Kg [26]. Approximately 90% of the drug is excreted in urine, 10% in feces, 20% are excreted as free and conjugated M1 and 17% as M2 [9, 27, 28]. The half-life is approximately 5-6 h for tramadol and approximately 8 h for M1, however, in patients with renal failure (creatinine clearance < 79 mL/min) there is a decreased excretion of these compounds compared to individuals with normal renal function (creatinine clearance > 100 mL/min). In patients with cirrhosis, there is a decrease in tramadol metabolism with a consequent increase of blood levels and double half-life time [1, 29-31].

4. Pharmacodynamics of tramadol and O-desmethyltramadol

4.1. Mechanism of action

Tramadol is a centrally acting opioid analgesic, which has a dual mechanism of action, as already mentioned: it is a weak agonist of μ-opioid receptors and inhibits serotonin and noradrenaline reuptake at the synapses of the spinal cord, acting on the pain transmission mechanism [27]. This drug has moderate affinity to μ-opioid receptor and a weaker affinity to δ and κ receptors [1]. Tramadol affinity to μ receptors is about 10 times weaker than codeine and 6,000 times weaker than morphine [32].

μ-opioid receptor is coupled to G-proteins, which are responsible for the transduction of response after activation of the receptors by opioids [33]. The activation of μ-opioid receptor in presynaptic membrane by tramadol or M1 activates the G-protein complex leading to an increase in membrane permeability to K^+ and a decrease of Ca^{2+} influx to the nerve endings occurring a decrease in the release of neurotransmitters related to the pain [34, 35]. The activation of μ-opioid receptors mediate supra spinal and spinal analgesia, sedation, respiratory depression, inhibition

of intestinal motility and the release of several neurotransmitters and hormones. However, tramadol is a weak agonist of these receptors, producing fewer adverse effects than those caused by other opioid analgesics (e.g. morphine and buprenorphine) [36-38]. The analgesic effect of tramadol is also dependent on the analgesic activity of M1, which is characterized by a higher affinity for the μ-opioid receptor [39]. The (+/-) M1 enantiomer has 300-400 times greater affinity for these receptors than tramadol, whereas the (-/-) M1 mostly inhibits noradrenalin reuptake [22, 23]. Other metabolites such as M2, M3 and M4 are pharmacologically inactive and do not have affinity for opioid receptors [40].

The opioid mechanisms account only for a part of tramadol analgesia. Inhibition of noradrenaline and serotonin reuptake also plays an important role and the consequences of reduced CYP2D6 activity for the clinical effects of tramadol are difficult to predict [32, 39].

4.2. Adverse effects

The most common adverse effects associated with tramadol are similar to those seen with other opioid receptor agonists (e.g. constipation, somnolence, pruritus, nausea, vomiting, dizziness, fatigue, sweating, dry mouth, drowsiness and orthostatic hypotension) [1, 41]. Besides these effects, it can also lead to hypertension, anaphylaxis, hallucinations and confusion [36, 42]. Rare adverse effects like hypoglycemia, hepatic failure, refractory shock and asystole have also been described in literature. Although tramadol has a low potential for respiratory depression (a common and potentially effect in most of the opioids), several fatal and non-fatal respiratory depression cases associated with tramadol administration are reported in literature [27, 43].

Concomitant administration of selective serotonin reuptake inhibitors (SSRIs) (e.g. venlafaxine, fluoxetine, sertraline, citalopram and paroxetine) with tramadol, can cause serotonin syndrome [44], which although not very often can lead to potentially fatal consequences. This syndrome is an iatrogenic disorder that results from excessive stimulation of serotonin receptors and can induce several changes and eventually coma and death (Table 1).

Table 1. Clinical features of serotonin syndrome [45-49].

Mental status	Autonomic hyperactivity	Neuromuscular disorders
Confusion	Tachycardia	Ataxia
Hypomania	Hyperthermia	Tremor
Hallucinations	Diaphoresis	Hyperreflexia
Agitation	___	Rigidity

Serotonin syndrome can also result from co-administration of tramadol with tricyclic antidepressants (e.g. amitriptyline) [1, 47]. However, at high concentrations of tramadol, this syndrome can occur only with tramadol administration itself [47, 50].

4.3. Dependency and addiction

Tramadol tendency to cause dependence and addiction is still controversial. Preston *et al*. (1991) [51] concluded that the development of dependence during tramadol treatment of up to 6 months is not significant, but the possibility of physical dependence during long-term treatment cannot be completely excluded. And thus, patients monitoring is required to prevent significant dependence [52].

While preclinical investigations suggest that abuse liability associated with tramadol use is low, there are increasing numbers of cases reported to the U.S. Food and Drug Administration of abuse, dependence and withdrawal associated with tramadol use [52]. Yates *et al*. (2001) [53] reported a clinical case of a 29 year-old female with no history of substance abuse that developed tramadol dependence after 3 years consuming up to 30 (50 mg) tablets of tramadol per day. In another study, Prakash *et al*. (2010) [54] described a case of a 37 year-old male with no history of substance abuse who developed tramadol dependence syndrome following a sprain injury in his leg. However, he started taking it on his own after any injury. On physical examination tremors, sweating, sleep disturbance, decreased psychomotor activity, euthymic mood with anxious affect and mild distractibility were observed without impairment of cognition. This demonstrates that tramadol has potential risk of abuse and physical dependence but it is lower than other opioids (e.g. morphine, hydrocodone) [51, 55].

Leo *et al*. (2000) [52] reported a case of a 46 year-old female with history of opioid (specifically pentazocine) and alcohol dependence with complaints on restlessness, diaphoresis, tremulousness and anxiety. The patient admitted to have a one year history of tramadol abuse, initially prescribed for analgesia, consuming up to 30 (50 mg) tablets of tramadol in divided doses daily. However, tramadol has been recommended as useful treatment option in patients undergoing opioid detoxification [56].

5. Tramadol intoxications cases

Although there is currently lack of information about acute tramadol intoxication, the number of cases reporting addiction, abuse or intentional overdose is increasing (Table 2). Fatal intoxications due to tramadol alone are not common. However, the administration of toxic doses of tramadol concomitantly with other central nervous system (CNS) depressants is one of the most common causes of severe or lethal acute intoxication.

Tramadol therapeutic blood concentration in adults range from: 0.01 to 0.25 mg/L. However, in toxic blood concentrations (0.8 mg/L), fatal complications can arise even in the absence of other drugs [57] .

Table 2. Review of tramadol intoxications.

Reference / number of cases studied	Main analytes	Samples	Peripheral blood concentration (mg/L)	Method
[27] / 2	Tramadol, M1 and M2	*Post mortem* blood and urine	**Case 1:** 7.7 (tramadol), 1.33 (M1) and 0.6 (M2); **Case 2:** 48.34 (tramadol), 2.43 (M1) and 10.09 (M2)	HPLC-FL
[36] / 1	Tramadol and M1	*Post mortem* blood, liver and kidney	5.2 (tramadol) and positive (M1)	HPLC-DAD: tramadol and M1 in blood; GC-MS: tramadol in liver and kidney
[37] / 1	Tramadol, M1 and M2	*Post mortem* peripheral and heart blood, urine, gastric content, liver, kidney and bile	9.6 mg/L (tramadol) and positive (M1 and M2)	HPLC-DAD: tramadol in all samples; GC-MS: tramadol, M1 and M2 in urine

[38] / 1	Tramadol	*Post mortem* blood	3.7	NS
[49] / 1	Tramadol	*Ante mortem* blood	9.5	GC-MS
[58] / 1	Tramadol	*Ante mortem* urine	Positive	GC-MS
[59] / 4	Tramadol	*Post mortem* blood	**Case 1:** 134; **Case 2:** 0.880; **Case 3:** 3.0; **Case 4:** 1.90	HPLC-DAD
[60] / 1	Tramadol, M1 and M2	*Post mortem* heart and peripheral blood, liver, urine, kidney, vitreous humor, lung, heart, brain, spleen, gastric content, bile and muscle	6.2 (tramadol), 0.68 (M1) and 0.20 (M2)	GC-MS
[42] / 1	Tramadol and M1	*Ante mortem* urine and blood	23,9 (tramadol) and positive (M1)	GC-MS: tramadol and M1 in urine; HPLC-DAD: tramadol in blood (quantification)
[50] / 1	Tramadol	*Ante mortem* serum	0.68	GC-MS
[61] / 12	Tramadol, M1 and M2	*Post mortem* heart and peripheral blood	0.03 to 22.59 (tramadol), 0.02 to 1.84 (M1) and 0.01 to 2.08 (M2)	GC-MS
[62] / 1	Tramadol	P*ost mortem* blood	13	GC-MS (quantification), HPLC-DAD (detection)
[63] / 1	Tramadol, M1 and M2	Peripheral blood, bile, liver and gastric contents	38.3 (tramadol), positive (M1 and M2)	GC-NPD (tramadol); GC-MS (M1 and M2);
[64] / 7	Tramadol	*Post mortem* heart and peripheral blood, urine, brain, liver and kidney	0.069 to 8.67	LC-MS/MS

GC-MS, gas chromatography-mass spectrometry; GC-NPD, gas chromatography-nitrogen-phosphorus detector; HPLC-DAD, high performance liquid chromatography-diode-array detector; HPLC-FL, high performance liquid chromatography-fluorescence detection; LC-MS/MS, high performance liquid chromatography- tandem mass spectrometric; M1, O-desmethyltramadol; M2, N-desmethyltramadol; NS, not specified.

The most common symptoms of acute tramadol intoxication are presented in Table 3 [7]. However, these symptoms vary according to the dosage, therapeutic formulation, administration route and an addiction/substance abuse history.

Table 3. Symptoms reported after tramadol overdose, (n=87 patients, including 15 children) [7].

Symptoms of overdose	Patients (%)
Lethargy	30
Nausea	14
Tachycardia	13
Agitation	10
Dizziness	9
Seizures	8
Vomiting	6
Coma	5
Hypertension	5
Confusion	3
Respiratory depression	2
Ataxia	2
Diplopia	1
Dry mouth	1
Diaphoresis	1

5.1. Case reports: intoxication due to single tramadol administration

Backer *et al*. [27] quantified in *post mortem* blood and identified in urine tramadol, M1 and M2 in 2 cases of fatal intoxication due to single tramadol administration. The tramadol concentration found in case 2 is one of the highest ever described in literature (48.3 mg/L) where the therapeutic range was exceeded in 160-fold. The authors concluded that in both cases, the underlying pathophysiological mechanism may be assumed to be respiratory depression.

In another fatal intoxication case described by Musshoff *et al*. [37], tramadol was quantified in several *post mortem* samples. Tramadol peripheral blood concentration of 9.6 mg/L exceeded at least 30-times the normal therapeutic range. Highest concentrations were measured in bile and urine (46.1 and 46.0 mg/L respectively). The concentration of tramadol in liver and kidney, in relation to blood, failed to suggest a major sequestration of drug in either specimen, which is consistent to the reported volume of distribution of 3 L/Kg. The authors concluded that in both

cases, the underlying pathophysiological mechanism could be assumed to be respiratory depression.

Levine *et al*. [26] analyzed tramadol distribution in four *post mortem* cases but none of the deaths were attributed to tramadol intoxication. However, blood concentrations of tramadol ranged from 0.27 to 6.5 mg/L. Loughrey *et al*. [38] described a case of accidental overdose of tramadol leading to fatal acute hepatic failure. Toxicological analysis revealed a blood tramadol concentration of 3.7 mg/L. Although well above the therapeutic range, tramadol concentration was in much lower levels than previously reported with fatal ingestion. It is possible that in these previous cases, death occurred at an early stage due to CNS or respiratory depression before liver injury became apparent. Lusthof and Zweipfenning [62] presented a suicide case due to tramadol (blood concentration of 13 mg/L), where 7-aminoflunitrazepam was also detected but in a very low concentration that could not be directly responsible for the death.

Multiple organ failure syndrome due to tramadol intoxication is rare but still documented. Decker *et al*. [36] described a case of a fatal intoxication due to single tramadol administration at a blood concentration of 8 mg/L. The mechanism of death was multiple organ failure syndrome. In the autopsy, pulmonary edema, diffuse hemorrhagic mucosa of the gastrointestinal tract and a shock liver were observed. Wang *et al*. [49] also presented a case of multiple organ failure syndrome due to tramadol intoxication alone (blood concentration of 9.5 mg/L). There is no evidence that the lung is a target organ of tramadol, however symptoms such as adult respiratory distress syndrome were observed in this case. This fact indicates that there are still some unknown mechanisms about tramadol.

5.2. Case reports: intoxication due to tramadol and other drugs

The concomitant use of tramadol with CNS depressants, particularly antidepressants, benzodiazepines, barbiturates and/or alcohol, other opioids analgesics and abused drugs may potentiate the adverse effects of this drug in the CNS, in several ways [59-61, 63]. For instance, tramadol is metabolized through O-demethylation (catalysed by the enzyme CYP2D6) to its active metabolite (M1). Such as tramadol, many selective serotonin reuptake inhibitors (SSRIs) and tricyclic antidepressants are substrates for the CYP2D6. Consequently, competitive inhibitions of isozyme prevent it to bind completely to tramadol, reducing the metabolism of the drug. Thus, there is an increase in the concentration of tramadol that can lead to an

acute intoxication [23, 61, 65] and in some susceptible individuals, idiosyncratic induction of serotonin syndrome [47]. Although a number of factors can increase or decrease the risk of an adverse drug reaction (e.g., genetic predisposition, diet and disease), the presence of drug combinations was considered the foremost risk and these other factors were taken into consideration as contributive factors [65].

Ripple *et al*. [66] reported a tramadol lethal intoxication but discovered multiple drugs with serotonin-effects. Tramadol blood concentration was 0.70 mg/L but death could not be attributed only to tramadol. Deaths involving the use of certain serotonin active drugs (tramadol, venlafaxine, fluoxetine, sertraline, citalopram and paroxetine), occurred between 2002 and 2008 were reviewed by Pilgrim *et al.* [65], to assess the incidence of contraindicated or ill advised drug combinations. This is important because contraindicated or inappropriate drug combinations can lead to adverse drug reactions and subsequent fatal toxicity. An example is the co-administration of tramadol and fluoxetine, which is likely to cause potentially fatal serotonin toxicity. In this study, tramadol was the most common drug usually detected, alongside a serotonergic antidepressant.

Four cases of lethal intoxication for tramadol and other CNS depressant drugs (particularly benzodiazepines) have been described by Clarot *et al*. [59]. The results demonstrated that in three of these four cases, tramadol blood concentration was greater than the toxic concentration limits. In one case, the concentration found of 134 mg/L was considered to be 62-times the toxic level. In two of these four cases, it was found a toxic co-ingestion of bromazepam and in three cases an enzymatic inducer (meprobamate, phenobarbital) was found at infra-therapeutic or therapeutic concentration. Daubin *et al.* [42] also described a tramadol overdose case in combination with other CNS depressants (hydroxyzine, gabapentin and clonazepam), leading to refractory shock. This case demonstrated that tramadol overdose (23.9 mg/L) may cause refractory shock and asystole when taken in combination with CNS depressants.

Bynum *et al*. [60] described a case of a fatal multiple drug overdose involving tramadol and amitriptyline and evaluated the distribution of these drugs and metabolites (nortriptyline, M1 and M2) in various tissues and fluids. A heart to peripheral blood ratio of 5.0 was observed. This fact can be explained by the existent diffusion from the stomach and/or gastrointestinal tract. Moreover, authors could not rule out the possibility of *peri mortem* aspiration, which can contaminate the airways and induce redistribution into the heart blood via the pulmonary vessels. In some

articles it has been suggested that tramadol is not sequestered in liver tissues to a significant degree [26, 63]. The largest amount of drug was discovered in the lungs followed by the spleen and muscle with 87, 29 and 22 mg/Kg respectively. The authors also observed that M1 concentrations in all tissues samples were higher than those of M2.

In a collaborative study, the frequency of fatal intoxications in individuals with dependence/abuse history was assessed by Simonsen *et al*. [67] in the Nordic countries, in 2007. The authors compared the results with previous studies conducted in 1991, 1997 and 2002. Tramadol has caused several deaths among drug addicts in 2002 and 2007, with a notable increase in Finland and Sweden in the latest study (9 to 14 and 2 to 12 cases respectively). Multiple drug use was common in all countries.

Goeringer *et al*. [61] reported 12 cases of tramadol-related deaths and intoxications involving tramadol. Analysis of 12 blood samples revealed concentrations ranging from 0.03 to 22.59 mg/L for tramadol and 0.02 to 1.84 mg/L for M1. The highest tramadol blood concentration (22.59 mg/L) was found in combined drug-intoxication (propranolol, desipramine and trazodone). Michaud *et al*. [63] reported fatal overdose of tramadol and alprazolam. Blood concentrations of alprazolam and tramadol were 0.21 mg/L and 38.3 mg/L (exceeded at least 100-times the normal therapeutic range) respectively, in association with alcohol at the concentration of 1.29 g/L.

Tjäderborn *et al*. [68] examined fatal unintentional tramadol intoxications cases. A total of 17 cases of fatal unintentional tramadol intoxications were identified. Intoxication with multiple drugs (other pharmaceutical substances, illicit drugs or ethanol) was considered to be the cause of death in 10 cases. However, in 7 cases tramadol was the only substance present at toxic concentrations. A history of substance abuse was identified in 14 subjects. These results suggest that fatal intoxications with tramadol may occur unintentionally and that subjects with a substance abuse history may be at certain risk. The reasons of tramadol intake were difficult to determine. The available data in 8 cases suggested the following reasons for abuse: to induce psychotropic effects, reduce withdrawal symptoms, misuse due to insufficient analgesia and wrong prescribing.

5.3. Case reports: tramadol intoxication in children

Tramadol has become widely used in recent years for treatment of chronic and acute pain, not only in the elderly but also in pediatric patients [15, 58, 69]. However, unintentional intoxication with seizures (after the inadvertent administration of 4 mg/Kg of tramadol) and severe CNS depression has been reported in children less than 1 year-old [58, 70]. Unintentional tramadol intoxication of a 4 year-old girl was reported by Grosek *et al*. [58]. Despite the negative history, the suspicion of opioid intoxication (CNS and respiratory depression, respiratory acidosis, cyanosis and miosis) led them to administer an opioid antagonist, naloxone. Urine toxicology screening was not completed, because tramadol was not part of the standard opioid urine toxicology screening. However, a toxicological analysis with GC-MS confirmed the presence of tramadol.

As described in adults, serotonin syndrome can occur in children intoxicated with tramadol. Severe case of unintentional tramadol intoxication in an 8 month old girl was reported by Maréchal *et al*. [50]. Tramadol serum levels confirmed the intoxication (680 mg/L). Clinical signs (agitation, tachycardia, hyperthermia, hyperreflexia and hypertension) were indicative of serotonin syndrome.

6. Treatment of tramadol intoxication

Treatment of acute tramadol intoxication consists in the administration of the opioids antagonists (e.g. naloxone), anticonvulsant drugs, if seizures occur, and other supportive measures such as intubation and mechanical ventilation in respiratory depression, for maintaining vital functions [58]. Naloxone is greatly important in opioid CNS and respiratory depression reversion, in opioids suspected intolerance diagnosis and in overdose cases. This opioid antagonist has been used to induce reversion of symptoms in this type of intoxication [49, 58, 71, 72]. However, naloxone should be administered with precaution in patients subjected to high doses of opioids, particularly in children (may initiate seizures) [50] or in patients that have an opioid physical dependence [58]. In the latter case, rapid reversal of opioid effects by naloxone may trigger the acute withdrawal syndrome [4].

Several cases are described in the literature, showing the successful use of naloxone. In a case of unintentional tramadol intoxication of a 4 year-old girl, the suspicion of opioid intoxication led to naloxone administration [49]. Wang *et al*. [49]

presented a case of a 19 year-old patient who had multiply organ dysfunction syndrome due to oral tramadol alone. Initially, the individual had developed deep coma, acute respiratory distress syndrome, hepatic and renal dysfunction, and shock. With the application of supportive measures and naloxone administration his overall status gradually improved. In the Marquardt *et al*. [72] study, naloxone improved CNS depression in seven out of eight patients.

Leo *et al*. [52] present a case of a patient with longstanding tramadol dependence that was successful treated with methadone detoxification. The patient tolerated well, with significant reduction in subjective symptoms of opioid withdrawal.

7. The importance of genetic polymorphism in tramadol pharmacokinetics and pharmacodynamics

Pharmacogenomics provides the hereditary basis for inter-individual differences in drug efficacy, side effects and toxicity [5].

Cytochrome P450 (CYPs) enzymes play an important role in drug metabolism, being responsible for about 80% of all metabolism [73]. The genes that encode the CYPs are functionally polymorphic and responsible for the appearance of different phenotypes. The enzyme encoded by the CYP2D6 gene plays an important role in the metabolism of several drugs (approximately 25% of all drugs administered in clinical practice including tramadol) [74]. More than 80 distinct allelic variants for CYP2D6 are known, which leads to a greater phenotypic and genetic heterogeneity with in populations [75].

The genetic variants give rise to different phenotypes in the drug metabolizing enzyme CYP2D6: poor metabolizers (PMs), homozygous for non-functional alleles and poor metabolism, intermediate metabolizers (IMs), heterozygous for a functional allele and one non-functional, ultrarapid metabolizers (UMs), multiple functional copies of the gene and substantially accelerated metabolism, or more common in the population the extensive metabolizers (EMs), homozygous for functional alleles [9, 76]. Phenotype distributions in European Caucasians show a frequency of 7 - 10% for PMs, 10 - 15% for IMs, 70 - 80% for EMs, and 3 - 5% for UMs [77]. In comparison to Caucasians, the incidence of PMs is much lower in Asian and African populations. Caucasians have a significantly increased frequency of three defective genes: CYP2D6*4, CYP2D6*3 and CYP2D6*6 whereas the frequency of CYP2D6*5 defective alleles are similar to that of other ethnic groups, all contributing to the PMs phenotype [78]. In contrast, other

polymorphisms like CYP2D6*10 are specifically frequent in Asian subjects and the *45 and *46 alleles in subjects from Black African origin [5].

Genetic variation in drug metabolizing enzymes can lead to inter-individual differences in drugs response, and also to adverse side effects, including death [76, 79]. As the analgesic effect of tramadol is dependent on the CYP2D6 activity, in individuals with PMs, the analgesic effect is lower because the drug is partially metabolized to M1 [80]. Another risk group consists on individuals whose metabolism is substantially accelerated, e.g. due to more than two functional copies of the CYP2D6 gene. They require higher doses than usual to achieve therapeutic parent drug levels in blood [76]. The IMs phenotype may also be relevant to the clinical effects of CYP2D6 substrates although to a lesser extent when compared with PMs and UMs phenotypes [9].

Other possible polymorphisms influencing tramadol pharmacokinetics are present in multidrug resistance protein (MDR1), μ-opioid receptor gene (OPRM1) and catechol-O-methyltransferase (COMT) gene.

The MDR1 gene with 28 exon is located in chromosome 7q21.12, and the coding region accounts for less than 5% of the total. Over 50 single nucleotide polymorphisms (SNPs) that have been identified in the human MDR1 gene [81-83]. Among them, C3435T may play a role in inducing drug resistance by altering the expression level of the MDR1 gene [83]. MDR1 gene codifies to Pg-glycoprotein which represents an important efflux transporter involved in bioavailability and elimination of several other drugs in humans. Tramadol is a substrate of P-glycoprotein, and a polymorphism, as C3435T in the MDR1 gene, may affect the pharmacokinetics of tramadol [84].

The COMT is an enzyme that catabolizes catecholamines and thus influences the dopaminergic and adrenergic/noradrenergic neurotransmission [85]. The COMT gene has been identified as a potential determinant of pain sensitivity in humans [86]. There are different SNPs in the COMT gene which induce important functional alterations of the enzyme [87]. The most studied SNPs in the COMT gene is Val158Met, that occurs in codon 158, resulting in a valine to methionine transition (Val/Met). The Val allele is associated with higher enzymatic activity than the Met allele [87, 88]. COMT Val158Met polymorphism could be important for the understanding of why certain individuals are more prone to develop opioid-induced hyperalgesia and tolerance to the antinociceptive actions of opioids [89].

Genetic polymorphisms involved in the μ-opioid receptor have been associated with an altered pain threshold and susceptibility to opioid drugs. The most common SNP in the OPRM1 gene is the A118G (adenine (A) to guanine (G) substitution) polymorphism. Individuals with the GG or GA genotype have shown an elevated sensitivity to pain and a reduced analgesic response to opioids compared with individuals with the AA genotype [90-93].

Besides pharmacogenomics, the pharmacokinetics of the drug can be affected by many other factors namely, age, disease, comorbidities, concomitant medication, metabolic interactions and kidney or liver function [5, 76].

8. Analytical methods to quantify tramadol and O-desmethyltramadol

Analytical Chemistry plays an important role in *ante mortem* and *post mortem* forensic and clinical toxicology. Therefore, the identification and quantification of tramadol and its main active metabolite M1 is of major importance. Several analytical methods of tramadol and M1 quantification in various biological matrices have been described. A synopsis is given in Table 4.

Table 4. Review of the analytical methods for qualitative and quantitative analysis of tramadol and metabolites.

Analytes/reference	Sample	Sample preparation	Derivatization	LOD (mg/L), (ng/mg - hair)	LLOQ (mg/L), (ng/mg - hair)	Method
Tramadol [94]	Human plasma	Liquid-liquid extraction with ethyl acetate	NS	0.009	0.017	HPLC-UV, spectroscopy at 225 nm, LiChrospher 60 RP-select B column;
Tramadol [95]	Rabbit plasma	Liquid-liquid extraction with ethyl acetate	NS	0.25	0.4	HPLC-DAD, Phenomenex Bondclone RP-18 column
Tramadol, M1, M2, M5, M6 and M7 [43]	Urine of dogs	Liquid-liquid extraction with diisopropyl ether / dichloromethane (1:1)	NS	**HPLC-FL:** 0.005 for tramadol, M1, M2 and M5; **LC-MS/MS:** NS	**HPLC-FL:** 0.01 for tramadol, M1, M2 and M5; **LC-MS/MS:** NS	HPLC-FL (tramadol, M1, M2 and M5): λ_{ex} 275 nm / λ_{em} 300nm; LC–MS/MS (M6 and M7), Phenomenex Luna C_{18} ODS2 column
Tramadol [96]	Human plasma	Liquid-liquid extraction with ethyl acetate / hexane (1:4)	NS	0.01	0.01	HPLC-UV, spectroscopy at 218 nm, LiChrosorb RP-18 column
Tramadol [97]	Hair	Solid-phase extraction using mixed-mode cation exchange columns	NS	0.5	NS	GC-MS
Tramadol, M1, M2 and M5 [98]	Rat perfused liver	NS	NS	NS	0.002 for all metabolites	HPLC-FL, Chromolith™ Performance RP-18 column

Tramadol [37]	Urine, heart and peripheral blood, liver, bile, kidney, gastric content	**GC-MS:** liquid-liquid extraction for urine with dichloromethane-isopropanol-ethyl acetate mixture (1:1:3; v/v); **HPLC-DAD:** liquid-liquid extraction for all tissues with n-butyl chloride	**GC-MS:** derivatization with acetic anhydride-pyridine mixture (1:1, v/v); **HPLC-DAD:** NS	NS	NS	GC-MS, HPLC-DAD, Lichrosorb RP-8 column
Tramadol, meperidine and oxycodone [99]	Human oral fluid	Solid-phase mixed mode extraction columns	BSTFA + 1% TMCS for tramadol	NS	0.01	GC-MS
Tramadol [100]	Human plasma	Reversed phase C_{18} solid-phase extraction columns	NS	0.001	0.002	GC-MS
Tramadol and M1 [101]	Human plasma	Reversed phase C_{18} solid-phase extraction columns	NS	NS	0.05	HPLC-UV, spectroscopy at 218 nm; C_{18} column
Tramadol and M1 [102]	Human plasma and urine	Liquid-liquid extraction with ethyl acetate	NS	NS	NS	HPLC-FL: λ_{ex} 216 nm / λ_{em} 308 nm, Zorbax RP-select B column
Tramadol [103]	Human plasma	Headspace solid-phase microextraction	NS	0.0002	NS	GC-MS
Tramadol and M1 [104]	Rat plasma	Liquid-liquid extraction with ethyl acetate-n-hexane (40:60, v/v)	NS	0.004 for tramadol and 0.003 for M1	0.015 for tramadol and 0.010 for M1	HPLC with electrochemical detection, Asahipack ODP-50 column
Tramadol, M1, buprenorphine, fentanyl, norbuprenorphine, norfentanyl, pethidine, piritramide and tilidine [105]	Urine and whole blood	Solid-phase extraction using mixed-mode cation exchange columns	NS	NS	**Urine:** 0.000025 for tramadol and M1; **Blood:** 0.00005 for tramadol and M1	UPLC-MS/MS, Acquity C_{18} column
Tramadol [106]	Human plasma	Liquid-liquid extraction with n-hexane	NS	0.005	0.005	GC-MS

Tramadol and M1 [107]	Human plasma	Liquid-liquid extraction with tert-butyl-methylether	NS	NS	0.05	HPLC-FL: λ_{ex} 280 nm / λ_{em} 310 nm, Spherisorb CN 5µ
Tramadol, M1 and M2 [26]	Heart and peripheral blood, urine, bile, liver and kidney	Liquid-liquid extraction with n-butyl chloride and methylene chloride	NS	0.05	0.10	GC-MS
Tramadol [108]	Human plasma	Liquid-liquid extraction with n-hexane	BSTFA + 1% TMCS	0.01	0.04	GC-MS
Tramadol and M1 [109]	Goat plasma	Liquid-liquid extraction with tert-butylmethyl ether	NS	NS	0.025 for tramadol and 0.01 for M1	HPLC-UV, 220nm, RP-18 column
Tramadol [110]	Human saliva	Liquid-liquid extraction with hexane-ethyl acetate (4:1)	NS	0.1	0.25	HPLC-DAD, Zorbax SB-C_{18} column
Tramadol and M1 [111]	Human plasma	The sample preparation consisted in plasma protein precipitation from 0.2 mL plasma using 0.2 mL solution of perchloric acid 7%	NS	NS	0.0021 for tramadol and 0.0022 for M1	LC-MS/MS, Zorbax SB-C_{18} column
Tramadol and M1 [112]	Human plasma	Analytes were extracted from 200 µL aliquots of human plasma via protein precipitation using acetonitrile	NS	NS	0.001 for tramadol and 0.0005 for M1	LC-MS/MS, Aquasil C_{18} column
11 opioids including tramadol [113]	Human plasma	Automated off-line solid-phase extraction system, with C_{18} columns	NS	0.0008	0.003	LC-MS/MS, Phenomenex C_{12} MAX-RP column
Tramadol and M1[114]	Human plasma	Liquid-liquid extraction with ethyl acetate	NS	NS	0.0041 for tramadol and 0.0032 for M1	HPLC-FL: $\lambda_{ex}/\lambda_{em}$ (200/300 nm, 200/295 nm, 212/305 nm), RP-18 column

Tramadol, M1, M2 and M5 [25]	Human plasma, saliva and urine	Liquid-liquid extraction with ethyl acetate	NS	NS	0.0025	HPLC-FL: λ_{ex} 200 nm / λ_{em} 301 nm, Chromolith^{TM} Performance RP-18 column
Tramadol [115]	Human urine and plasma	Three-phase hollow fiber liquid-phase microextraction	NS	0.00008	NS	GC-MS
Tramadol, morphine, 6-acetylmorphine, codeine and 6-acetylcodeine [116]	Hair	Solid-phase mixed mode extraction columns	MSTFA + 5% TMCS	40 for tramadol	0.05	GC-MS
Tramadol, M1, M2, amitriptyline and nortriptyline [60]	Heart and peripheral blood, liver, urine, kidney, vitreous humor, lung, heart, brain, spleen, gastric content, bile and muscle	Liquid-liquid extraction with n-butyl chloride / ethyl ether mixture (75:25)	NS	NS	0.25	GC-MS
Tramadol, M1 and M2 [61]	Heart and peripheral blood	Liquid-liquid extraction with n-butyl chloride;	NS	0.01	0.02	GC-MS
Tramadol, M1 and M2 [76]	*Post mortem* peripheral blood	Liquid-liquid extraction with dichloromethane isopropyl alcohol (M1 and M2) and butyl acetate (tramadol)	NS	NS	0.1 for tramadol, 0.01 for M1 and M2	GC-MS (tramadol); LC-MS/MS (M1 and M2), C_{18} column
Tramadol, metoprolol and midazolam [64]	*Post mortem* heart and peripheral blood, urine, brain, liver and kidney	Liquid-liquid extraction with dichloromethane	NS	NS	0.0078	LC-MS/MS, Synergy 4µ Polar-RP 80A column

Tramadol [59]	*Post mortem* blood	Liquid-liquid extraction with dichloromethane hexane ethylacetate (5:4:1, v/v/v)	NS	0.025	NS	HPLC-DAD, C_{18} BDS column
Tramadol, M1 and M2 [117]	Human plasma	Liquid-liquid extraction with ethyl acetate	NS	NS	0.0025 for tramadol, 0.00125 for M1 and 0.005 for M2	HPLC-FL: λ_{ex} 200 nm / λ_{em} 301 nm, Chromolith™ Performance RP-18 column
Tramadol [118]	Human urine	NS	NS	0.02	0.05	CE-UV-LINF, λ_{em} 257 nm, fused-silica capillaries of 75 cm
49 licit and illicit drugs including tramadol [119]	Oral fluid	Solid-phase extraction using bond elut certify® LRC cartridges	**GC-MS:** Ethyl acetate / pentafluoropropionic anhydride (3:2 v/v); **LC-MS/MS:** NS	0.0015	0.0049	GC-MS (tramadol); LC-MS/MS, Luna C_{18} column
Tramadol [120]	Human plasma	Liquid-liquid extraction with tert-butylmethyl ether	NS	NS	0.017	HPLC-FL: λ_{ex} 202 nm / λ_{em} 296 and 314 nm, C_{18} column; HPLC-UV/VIS spectroscopy at 275 nm, C_{18} column
Tramadol and acetaminophen [121]	Human plasma	Liquid-liquid extraction with ethyl acetate	NS	NS	0.005	LC-MS, Hanbon LiChrospher CN column;
Tramadol and M1 [122]	Human plasma	Liquid-liquid extraction with tert-butyl methyl ether	NS	0.0003 for tramadol and 0.0004 for M1	0.001 for tramadol and M1	HPLC-FL: λ_{ex} 202 nm / λ_{em} 296 and 314 nm, Kromasil 100 C_{18} column
Tramadol, M1, metoprolol and α-hydroxymetoprolol [123]	Human plasma and urine	Liquid-liquid extraction with ethyl ethanoate	NS	NS	0.0125 for tramadol and 0.005 for M1	HPLC-FL: λ_{ex} 216 nm / λ_{em} 312 nm, Zorbax RP-select B column

12 opioid agonists including tramadol [124]	Serum, blood, urine, cerebrospinal fluid, vitreous humor and bile	Reversed phase C_{18} solid-phase extraction columns	NS	0.0001 for tramadol	NS	LC-MS, Superspher RP 18 columns
Tramadol and M1 [125]	Human plasma	Liquid-liquid extraction with diethyl ether dichloromethane-butanol (5:3:2, v/v/v)	NS	0.001 for tramadol and 0.0005 for M1	0.003 for tramadol and 0.0015 for M1	HPLC-FL: λ_{ex} 275 nm / λ_{em} 300 nm, Hypersil C_{18} column
24 drugs including tramadol [126]	Hair	Headspace-solid phase microextraction	NS	0.1	0.4	GC-MS

BSTFA, N,O-bis(trimethylsilyl) trifluoroacetamide; CE-UV-LINF, capillary electrophoresis-ultraviolet laser-induced native fluorescence detection; GC-MS, gas chromatography-mass spectrometry; GC-NPD, gas chromatography-nitrogen-phosphorus detector; HPLC-DAD, high performance liquid chromatography-diode-array detector; HPLC-FL, high performance liquid chromatography-fluorescence detection; HPLC-UV, high performance liquid chromatography-ultraviolet detection; LC-MS, liquid chromatography-mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometric; LLOQ, lower limit of quantification; LOD, limit of detection; M1, O-desmethyltramadol; M2, N-desmethyltramadol; M5, N,O-didesmethyltramadol; M6, 4-hydroxy-ciclohexyl tramadol; M7, 4-hydroxy-ciclohexyl-N-desmethyl tramadol; MSTFA, N-methyl-N-(trimethylsilyl) trifluoroacetamide; NS, not specified; TMCS, trimethylchlorosilane; UPLC-MS/MS, ultra-performance liquid chromatography-tandem mass spectrometric.

Usually, an initial sample preparation step is essential for pre-concentration, isolation and extraction of tramadol and M1 in biological samples prior to its subsequent chromatographic analysis [115]. Liquid-liquid extraction (LLE) and solid-phase extraction (SPE) has been widely used as sample preparation step to extract tramadol and M1 from these samples. In the past few years special attention has been given to the sample preparation techniques which do not require the use of large volumes of organic solvents, have high sensitivity and speed, such as SPE. Another advantage of SPE is its capacity to automation, which allows a reduction in the sample preparation time and increase the accuracy of the method. However, despite its effectiveness, the costs are high and is sometimes incompatible with unstable samples [127]. Bjørk *et al*. [128] developed a method of automated solid-phase extraction liquid chromatography-tandem mass spectrometry (SPE-LC-MS/MS) for the analysis of 19 drugs and metabolites including tramadol and M1.

The most recent and promising techniques in the extraction of these compounds from biological matrices are the solid-phase microextraction (SPME) and liquid-phase microextraction (LPME). SPME is a simple, rapid, with high sensitivity and less spending of solvent extraction technique that has proved to be a powerful alternative to conventional methods. However, SPME fibers are fragile and relatively expensive and tend to degrade with repeated use. A simple, rapid and sensitive method for determination of tramadol in plasma samples was developed by Sha *et al*. [103] using SPME and gas GC-MS with limit of detection (LOD) of 0.0002 mg/L. Sporkert and Pragst [126] also developed a method of SPME for simultaneous extraction of 24 drugs including tramadol in hair samples and analysis by GC-MS with LOD of 0.1 ng/mg for tramadol. LPME is an emerging technique based on the use of small amounts of organic solvents to extract analytes from aqueous matrices [103, 115, 126]. Ghambarian *et al*. [115] developed a method of LPME for extraction of tramadol from plasma and urine samples and analysis by GC-MS, with good linearity and high sensitivity (LOD of 0.00008 mg/L).

Several authors have reported the analysis of tramadol and its active metabolite M1 simultaneously by high performance liquid chromatography (HPLC) with ultraviolet [101, 109], electrochemical [104], fluorescence [25, 43, 98, 102, 107, 114, 122, 123, 125, 129] and mass spectrometry detection [26, 60, 61, 76, 105, 111, 112, 128]. However, there are several disadvantages using HPLC-UV for determining tramadol and M1 concentrations in biological samples. Tramadol contains a weak absorbing chromophore in its molecule [95, 130], which makes determination of low tramadol

concentrations problematic [94], though there is a benzene ring present in tramadol and its metabolite molecules [114]. Furthermore, biological samples containing tramadol need to be extracted using multi-step pH-dependent procedures prior to HPLC. For the moment, only two types of detectors have reached low quantification levels: fluorescence and mass spectrometry, not only due to the detectors performances and chromatographic columns used but also to the optimized parameters of the methods involving the extraction processes [114]. Tandem mass spectrometry (MS/MS) has been used to enhance the sensitivity at minor concentrations.

The GC-MS and liquid chromatography-tandem mass spectrometric (LC-MS/MS) have also shown high sensitivity, specificity and efficiency. Although most of the procedures used for the determination of tramadol and M1 are based on GC-MS, the preparation of the sample is still time consuming and complex when compared to LC-MS/MS, because of the need for derivatization of these polar compounds. The derivatization of the compounds allows an increase in its volatility, improving the thermal stability and consequently the detectability of the derivative [131]. The most employed derivatizing agent for compounds analyzes is *N*,*O*-bis(trimethylsilyl) trifluoroacetamide + 1% trimethylchlorosilane (BSTFA + 1% TMCS). Tramadol and M1 have been analyzed by GC-MS in several biological samples, including blood [60, 61, 76], urine, liver, bile, kidney [26, 60], gastric content, vitreous humor, lung, heart, brain, spleen, and muscle [60].

Advanced and sophisticated methods have been recently described such as ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Verplaetse and Tytgat [105] presented a highly sensitive UPLC-MS/MS method for the analysis of various drugs and their metabolites (including tramadol and M1) in urine and whole blood. The alkaline wash step in the SPE protocol, the high pH of the mobile phase, the use of electrospray ionization (ESI) and the use of a column with small particle size (1.7 µm) were found to be crucial parameters warranting the high sensitivity of the method. However, despite ultra-performance liquid chromatography (UPLC) presented high specificity and selectivity and short runtimes with a higher resolution chromatographic separation, is a very expensive chromatographic technique, compared with LC-MS/MS and GC-MS [105].

Recently the capillary electrophoresis with ultraviolet laser-induced native fluorescence detection (CE-UV-LINF) emerged as a powerful analytical technique. CE enables rapid separations with high separation efficiency and compatibility with small

sample volumes. LINF can result in extremely low limits of detection in CE. The main problem in CE is the small inner diameter of the capillary which causes a low sensitivity with instruments equipped with a UV detector [132]. Soetebeer *et al*. [118] described a sensitive and highly selective CE-UV-LINF method for the direct determination of tramadol in urine samples without extraction or pre-concentration with LOD 0.02 mg/L.

PART II: General and specific objectives of the thesis

Objectives of the thesis

The general objective of this work was to validate an analytical method for qualitative and quantitative analysis of tramadol and M1 by GC-MS and to study the CYP2D6, MDR1, OPRM1 and COMT gene polymorphisms by real-time polymerase chain reaction (PCR) in five *post mortem* blood samples from cases of suspected tramadol fatal intoxications.

The specific objectives if this thesis were:

- To study different conditions of GC-MS method in order to obtain the best peak resolution and separation of the compounds;
- To study different conditions of sample preparation for GC-MS analysis;
- To optimize the extraction procedure;
- To study the sensitivity, selectivity, limit of detection (LOD), lower limit of quantification (LLOQ), accuracy and precision of the developed method;
- The application of the GC-MS method to real *post mortem* blood samples;
- The same *post mortem* blood samples were then genotyped for the presence of CYP2D6*4 allele (T1236T>C) SNP. MDR1 (C3435T), OPRM1 (A118G) and COMT (Val158Met) SNPs were also analyzed by real-time PCR.
- A final objective was to give a first insight (due to the limited number of samples) for the comparison of the genotyping results with concentrations of tramadol and M1 found in the samples. Further studies (with more samples) will be necessary to assess any correlation between variables.

PART III: Experimental Part

Chapter I

Materials and methods

1. Material and methods

1.1. Ethics statement

All research was approved by the Portuguese National Council of Ethics for Life Sciences (CNECV). According to the current Portuguese law for medico-legal autopsies and following the ethical principles of Declaration of Helsinki, no informed written or oral consent of the victim family is required for scientific research in routinely collected samples. Therefore the use of these samples beyond establishing the cause of death is foreseen by the law.

1.2. Reagents and standards

The analytical standard O-desmethyltramadol hydrochloride (3-[2-(1-Amino-1-methylethyl)-1-hydroxycyclohexyl]phenol hydrochloride); molecular mass 285.809 g/moL), were a generous gift from Grünenthal (Amadora, Portugal). Tramadol hydrochloride ((±)-*cis*-2-(Dimethylaminomethyl)-1-(3methoxyphenyl)cyclohexanol hydrochloride; molecular mass 299.84 g/moL) and phenacetin (*N*-(4-Ethoxyphenyl) acetamide); molecular mass 179.216 g/moL, internal standard, IS) were obtained from Sigma-Aldrich (St Louis, MO, USA) and LGC Standards respectively.

Methanol and N,O-bis(trimethylsilyl)trifluoroacetamide/trimethylchlorosilane (BSTFA+1%TMCS) were purchased from Sigma-Aldrich (St Louis, MO, USA), sodium hydroxide (NaOH) and perchloric acid ($HClO_4$) from Panreac (Barcelona, Spain). Ethanol and 2-propanol were acquired from Merck (Darmstadt, Germany). Nitrogen (99.99% purity) and helium (99.99%) were obtained from Gasin (Portugal).

Bond Elut®C_{18} cartridges (100 mg, 1 mL) were purchased from Varian (Sint-Katelijne-Waver, Belgium). FavorPrep™ genomic DNA mini kit (blood/cultured cell) was obtained from Favorgen® Biotech Corp. (Taiwan, China). CYP2D6 (T1236T>C, rs1128503), MDR1 (C3435T, rs1045642), OPRM1 (A118G, rs1799971) and COMT (Val158Met, rs4680) SNPs assays were obtained from Applied Biosystems™.

All the reagents used were of analytical grade or from the highest available grade.

1.3. Biological specimens

Post mortem blood samples (1 mL) of cases of suspected tramadol fatal intoxications were obtained from the North Branch of the Portuguese National Institute of Legal Medicine and Forensic Sciences, I.P.. The samples were collected according to the guidelines normally followed at this institution and were stored at -80 ºC prior to analysis [133].

1.4. Preparation of stock and working standard solutions

Stock solutions of tramadol and M1 were prepared in methanol at the concentration of 1 mg/mL. Tramadol and M1 concentrations of working standard solutions for the calibration curve were prepared at different concentrations by diluting of the stock solutions in methanol (5, 10, 50, 100, 500 and 1000 ng/mL). A working solution of the IS at 20 µg/mL was also prepared in methanol. All working solutions were prepared fresh daily. All stock solutions were stored at -80 ºC prior use.

1.5. Sample preparation for gas-chromatography mass spectrometry analysis

Blood samples were pre-treated by sonication for 20 minutes at room temperature. Then, 500 µL of $HClO_4$ (7%) were added, vortexed for 1 minute and centrifuged at 13000 rpm, 20 minutes at 4 ºC.

1.5.1. Tramadol and M1 extraction from biological sample

Different methods of LLE and SPE were tested to determine the optimal conditions of extraction but here it is only described the method that resulted in high recoveries.

SPE was performed using C_{18} cartridges connected to a vacuum manifold and conditioned twice with 1 mL of methanol, followed by thrice 1 mL of 0.001 M NaOH. The supernatant was then passed through the cartridges. The cartridges were washed with 1 mL of 0.001 M NaOH. After drying under full vacuum for 1 minute, the analytes were eluted with 2 mL of 2-propanol. The eluate was evaporated to dryness at 50 ºC under a gentle stream of nitrogen (Fig. 3).

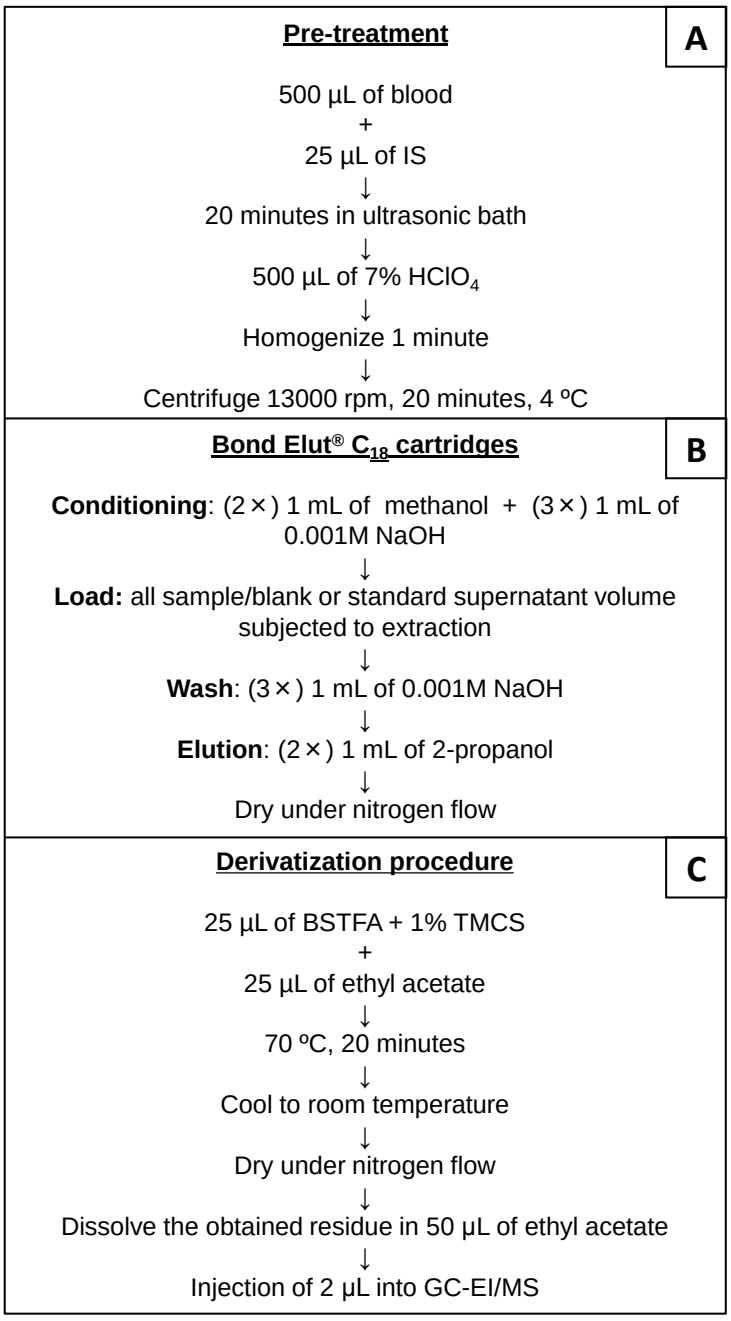


Figure 3. Sample preparation procedure. (A) Sample pre-treatment. (B) Extraction/purification with solid-phase extraction (SPE). (C) Derivatization procedure. BSTFA, N,O-bis(trimethylsilyl) trifluoroacetamide**;** GC-EI/MS, gas chromatography**-**electron impact/mass spectrometry**;** $HClO_4$, perchloric acid; IS, internal standard; NaOH, sodium hydroxide; TMCS, trimethylchlorosilane.

1.5.2. Derivatization procedure

In this study, tramadol and M1 were derivatized by silylation, reacting with BSTFA and TMCS. BSTFA is the silylation reagent that reacts with tramadol and M1

and replaces active hydrogens with a $–Si(CH_3)_3$ (trimethylsilyl) group (Fig. 4 and 5). TMCS increases the reactivity of BSTFA.

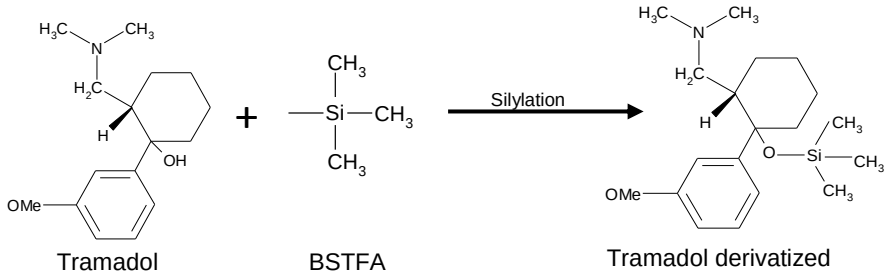


Figure 4. Derivatization reaction of tramadol.

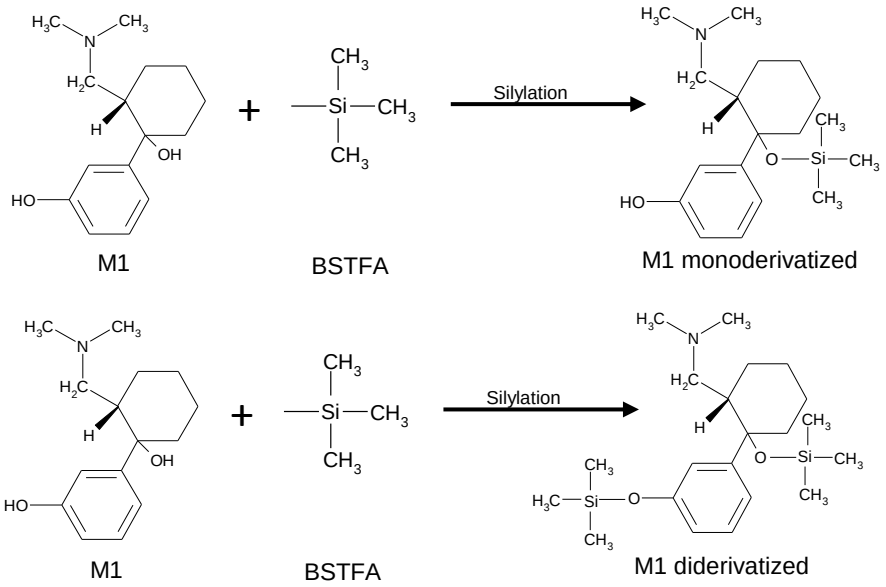


Figure 5. Derivatization reaction of M1.

Tramadol and M1 derivatization was performed with the addition of 25 µL of BSTFA + 1% TMCS and 25 µL ethyl acetate to the evaporated solution, mixed and heated for 20 minutes at 70 ºC. After cooling to room temperature, the samples were evaporated to dryness under nitrogen flow, reconstituted with 25 µL of ethyl acetate and 2 µL of each derivatized sample were injected into the GC-MS system.

1.6. Gas chromatography-mass spectrometry conditions

Quantitative GC-MS analysis were performed on a Varian CP-3800 gas chromatographer (USA) equipped with an ion-trap Varian GC-MS Saturn 2200 mass detector. Chromatographic separation was achieved using a capillary column Agilent®VF-5ms (30 m × 0.25 mm i.d. × 0.25 µm) and a high-purity helium C-60 carrier gas. An initial temperature of 90 ºC was maintained for 2 min, increased to 300 ºC at 20 ºC/min, and held for 3 min, giving a total run time of 16 min approximately. The flow of the carrier gas was maintained at 1.0 mL/min. The injector port was set at 280 ºC. Analyses were performed in fullscan in splitless injection mode.

The obtained full scan chromatogram was reprocessed using the following selected qualifier ions and retention times for each analyte, presented in Table 5. The underlined ions were used for quantification.

Table 5. Detection parameters of tramadol, M1 and IS by GC-MS.

Analytes	Retention time (t_r) (minutes)	Fragments (*m/z*)
Tramadol	Derivatized: 10.375	Derivatized: <u>58</u>; 336
	Without derivatization: 10.392	Without derivatization: <u>58</u>; 264
M1	Diderivatized: 10.665	Diderivatized: <u>58</u>; 394
	Monoderivatized: 10.801	Monoderivatized: <u>58</u>; 322
IS	8.900	109; 137; 179

The integration of the chromatographic peaks for quantitative analysis was performed by monitoring the fullscan chromatogram with specific selected ion monitoring (SIM) mode, allowing a more precise peak integration [133].

1.7. Method validation

A full method validation should be performed for any analytical method whether new or based upon literature [134]. Analytical method validation is a mandatory step to evaluate the ability of developed methods to provide accurate results for their routine application. The validation of the method was performed accordingly European Medicines Agency (EMA) [135] and other authors [136, 137]. EMA guidelines relies on the Food and Drug Administration guidance [138] which is now almost generally

accepted by the biopharmaceutical industries as the gold standard method validation approach [139].

The LOD, LLOQ, precision, accuracy, recovery and linearity of the method were determined. In order to obtain these validation data, calibration curves were prepared by spiking blank whole blood with appropriate volumes of tramadol and M1 standard solutions.

1.5.3. Selectivity

Six blank samples with no analytes or IS added were extracted by SPE as described previously and analyzed by GC-MS to detect possible chromatographic interferences with tramadol and M1. Chromatographic selectivity was evaluated by the presence or absence of co-eluting peaks at the retention times of the analytes. Three independent experiments were performed.

1.5.4. Linearity

The method linearity was determined by evaluation of the regression curve (ratio of analyte peak area and IS peak area *versus* analyte concentration) and expressed by the determination coefficient (r^2) using spiked samples. Three independent calibration curves (y = mx + b) were obtained using six different concentrations of tramadol and M1 (5, 10, 50, 100, 500 and 1000 ng/mL) and mean slopes were obtained for calculating the concentration of real samples (unknown concentrations). These concentrations were prepared daily as mentioned before.

1.5.5. Limit of detection and lower limit of quantification

In our study, LOD and LLOQ were obtained based on the standard deviation of the response and the slope of the calibration curve. The LOD is expressed accordingly to equation 1.1 and LLOQ accordingly to equation 1.2., in which σ is the standard deviation of the response and *S* is the slope of the calibration curve.

(1.1) $$LOD = \frac{3.3\sigma}{S}$$

(1.2) $LLOQ = \frac{10\sigma}{S}$

1.5.6. Precision and accuracy

The precision of the analytical method describes the closeness of repeated individual measures of analyte. Precision is expressed as the coefficient of variation (%CV). Intraday precision data was quantified by analyzing the areas of three replicates of three concentrations (low, 10; medium, 100; and high, 1000 ng/mL) and calculating the %CV. The areas of the same three concentrations, injected on three consecutive days, were used to calculate the interday repeatability (%CV). A %CV value of ≤15% for interday and intraday analysis was considered satisfactory. The accuracy of an analytical method describes the closeness of the determined value obtained by the method to the true concentration of the analytes (expressed as a percentage). The accuracy of the method was evaluated by spiking blank matrix with three different tramadol and M1 concentrations (low, 10; medium, 100; and high, 1000 ng/mL) and through the calculation of the percentage deviation between the calculated value and the nominal value [accuracy (%) = (experimental concentration/theoretical concentration) × 100]. A deviation percentage of ≤15% was considered satisfactory.

1.5.7. Recovery

The recovery was evaluated by analyzing two sample groups of the same concentrations (10, 100 and 1000 ng/mL) in triplicate, but differently processed. In the first group, tramadol, M1 and IS were analyzed following the extraction procedure mentioned above. In the second group, tramadol, M1 and IS were added to the 2-propanol before drying. The recovery was evaluated by the comparison of the mean response of the two groups. The response of the unextracted group represents 100% recovery. A deviation percentage of ≤20% was considered satisfactory.

1.5.8. Proof of applicability

Post mortem blood samples from five cases of suspected tramadol fatal intoxications were analyzed in this study to validate the method on real samples. Blood samples were obtained from the North Branch of the Portuguese National Institute of Legal Medicine I.P.. The samples were stored and processed as described above.

1.8. Genetic polymorphism

Genomic DNA was isolated and purified using FavorPrep™ genomic DNA mini kit (blood/cultured cell), according to the manufacturer's protocol (Fig. 6).

Isolation and purification of genomic DNA

Sample Preparation:
200 µL blood + 30 µL Proteinase K
+
Mix and incubate
↓
Cell Lysis:
200 µL FABG Buffer
+
Mix and incubate
↓
Binding:
200 µL ethanol
+
Transfer the sample mixture to FABG Column
+
Centrifuge
↓
Washing:
400 µL W1 Buffer
+
Centrifuge
+
600 µL Wash Buffer
+
Centrifuge
↓
Elution:
100 µL Elution Buffer
+
Centrifuge

Figure 6. DNA extraction procedure for real-time PCR.

All genotypes were determined by direct allelic discrimination using direct primers and probes in the ABI Prism Real Time PCR System 7300 and Taqman™ Allelic Discrimination. Genotyping of CYP2D6 (rs1128503) [76, 107], MDR1 (rs1045642) [84, 140], OPRM1 (rs1799971) [90] and COMT (rs4680) [87, 89] gene, was performed as previously described.

For rs1799971 probe sequences were as follows for VIC/FAM GGTCAACTTGT CCCACTTAGATGGC[A/G]ACCTGTCCGACCCATGCGGTCCGAA. For rs4680, probe

sequences were as follows for VIC/FAM CCAGCGGATGGTGGATTTCGCTG G[A/G]TGAAGGACAAGGTGTGCATGCCTGA. For rs1128503 probe sequences were as follows for VIC/FAM GCCCACTCTGCACCTTCAGGTTCAG[A/G]CCCTTCAAGATC TACCAGGACGAGT. For rs1045642, probe sequences were as follows for VIC/FAM TGTTGGCCTCCTTTGCTGCCCTCAC[A/G]ATCTCTTCCTGTGACACCACCCGGC.

Allelic discrimination PCR reactions were carried out in 6 µL volumes using 2.5 µL of TaqMan® Universal PCR Master Mix (2×), 0.125 µL of 40× assay mix, 2.375 µL of sterile H_2O and 1 µL of genomic DNA. Amplification of DNA was carried out on an ABI 7300 using the following conditions: 95 ºC for 10 min, followed by 45 cycles of 95 ºC for 15s and 60 ºC for 1 min. Data capture and analysis was performed through the ABI 7300 real-time PCR System (Applied Biosystems) with allele-specific probes for the common and variant SNPs (FAMand VIC, respectively) by the Sequence Detection Systems software (Applied Biosystems version 1.2.3).

Chapter II

Results and discussion

1. Results and discussion

1.1. Sample preparation for gas-chromatography mass spectrometry analysis

1.1.1. Pre-treatment of samples and extraction

Blood is the matrix of choice in toxicological analysis for the detection and quantification of xenobiotics, since there is a good correlation between blood concentration and the toxic effect [133]. However, it is a complex biological matrix, with the presence of many interferents, in particular, proteins, hormones, blood cells and possible clots.

The separation of the analytes of interest from the matrix and elimination of possible interferences is crucial for subsequent chromatographic analysis. Nevertheless, few methods have focused on the inherent problems associated to *post mortem* specimens, particularly blood which is often haemolysed and degraded frequently causing column blockage and reducing extraction efficiency and recovery [141]. To solve this problem, before the SPE procedure, we pre-treated blood samples by sonication for 20 minutes at room temperature.

Different intervals of time were tested (15 and 30 minutes), but 20 minutes proved to be the appropriate time, facilitating the passage of the sample along the cartridge during the extraction process. In the next step, 500 µL $HClO_4$ (7%) was added, to achieve protein precipitation. Also 5% and 10% $HClO_4$ were tested as well as common solvents such as methanol and acetonitrile. However, 7% $HClO_4$ proved to lead to a more efficient purification.

In SPE, the best results were obtained with a modified procedure, published by Merslavič and Zupančič-Kralj [100]. Comparatively, LLE methods were not so efficient and the loss of analytes was more evident. The chosen extraction method for this study proved to be simple and rapid in the preparation of samples prior to analysis by GC-MS. Suitable extracts were obtained for chromatographic analysis with the use of smaller volumes of organic solvents compared with LLE and with the efficient removal of interferents of the biological matrix. The extraction efficiency was considerable, given the low volume of sample used (500 µL) and also presented a good recovery of the analytes of interest.

1.1.2. Derivatization

Tramadol and M1 are polar organic compounds with low volatility, which limits its analysis by GC. The derivatization of the analytes allowed an increase in its volatility, improving the thermal stability and consequently the detectability of the derivative [131]. Tramadol and M1 quantification by GC-MS was not possible due to the presence of one and two hydroxyl groups, respectively in its chemical structure, leading to need of implementation of derivatization step in sample preparation procedure.

1.2. Method validation

1.1.3. Detection by gas-chromatography mass spectrometry

Preliminary tests were performed to determine the best conditions of chromatographic separation and detection in order to obtain the best peak resolution and separation of tramadol and M1.

In the chromatograms it is possible to identify two peaks for both tramadol and M1.

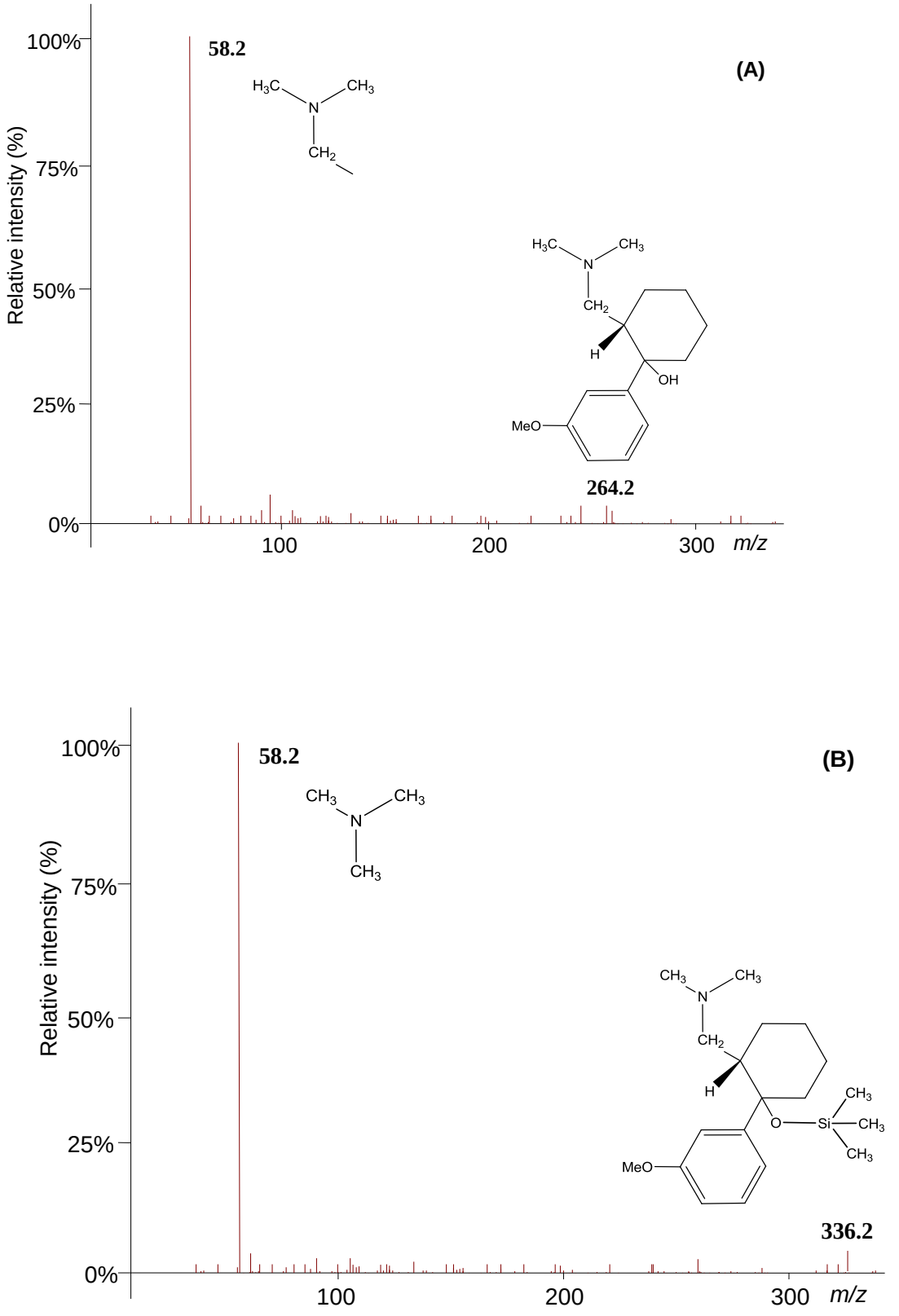


Figure 7. Reconstructed mass spectrum of tramadol. (A) First peak, tramadol without derivatization. (B) Second peak, tramadol derivatized.

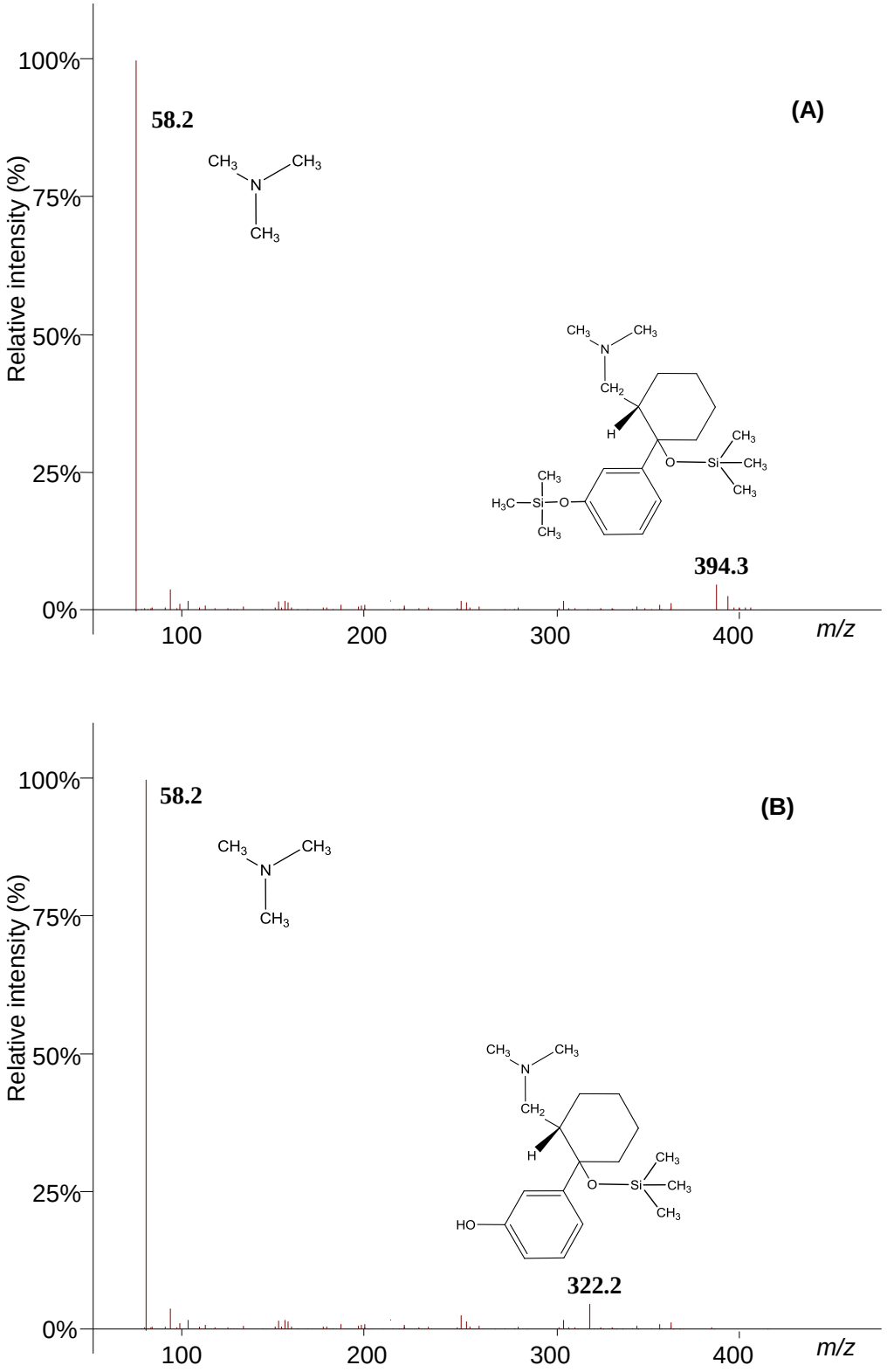


Figure 8. Reconstructed mass spectrum of M1. (A) First peak, M1 diderivatized. (B) Second peak, M1 monoderivatized.

Based on mass spectrum of each peak, for tramadol the first peak represents tramadol derivatized and the second peak tramadol without derivatization (Fig. 7). In the case of M1, the first peak represents M1 diderivatized and second peak M1 monoderivatized (Fig. 8).

For tramadol and M1 three ions were used. The most abundant ion (*m/z* 58 for both analytes) was used for quantification, and the other ions were used for the proper identification of each analytes, corresponding to the confirmation ions: *m/z* 264 and 336 for tramadol and *m/z* 322 and 394 for M1.

Analyzing the mass spectra (Fig. 7 and 8), it was possible to verify that there is an abundant ion at *m/z* 58 in both peaks of tramadol and M1. The mass spectra of tramadol (first peak) revealed low abundance product ion at *m/z* 264 and 336, representing the protonated and the derivatized molecule of tramadol, respectively. The second peak of tramadol has a fragment with *m/z* 58 and *m/z* 264 with low abundance. Regarding the mass spectra of M1, the *m/z* 58 was also found in high abundance in the two peaks. On the other hand, first peak revealed low abundance product ion at *m/z* 394, representing the protonated diderivatized molecule of M1. The mass spectra of second peak showed lower abundance at *m/z* 322, representing the protonated monoderivatized molecule of M1.

1.1.4. Selectivity

The GC-MS chromatograms of spiked samples were compared with the chromatograms obtained with a blank blood sample. No interference peaks were detected in the retention times of tramadol and M1, or in the IS and selected ions (Fig. 9).

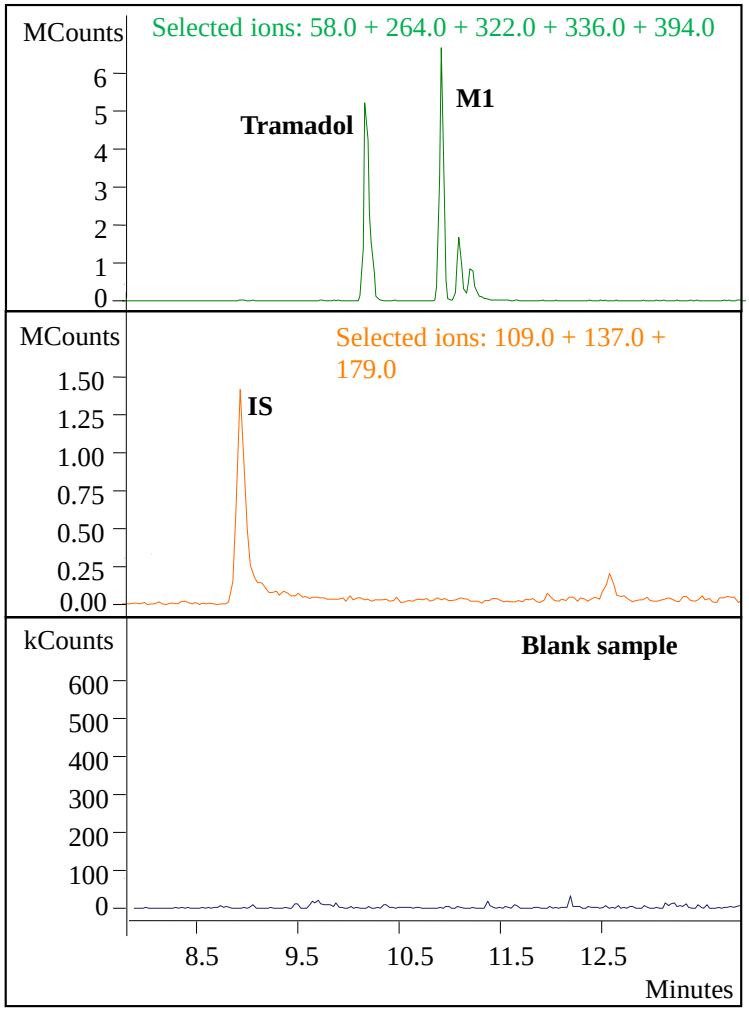


Figure 9. Reconstructed GC-MS (SIM mode) chromatogram of a blank and spiked blood sample (1000 ng/mL) of tramadol (*m/z* 58+336+264), M1 (*m/z* 58+394+322) and internal standard (IS; *m/z* 109+137+179).

1.1.5. Linearity

In the present work, the linearity of tramadol and M1 was evaluated in triplicate in the range of 5 ng/mL to 1000 ng/mL and all samples were analyzed according to the procedure previously described (Chapter I). The three independent calibration curves were obtained with six concentrations (5, 10, 50, 100, 500 and 1000 ng/mL). The weighted least squares regression equations and coefficients of correlation were calculated from these curves. The GC-MS chromatogram peak area ratios of tramadol/IS and M1/IS were determined to establish calibration equations (Fig. 10).

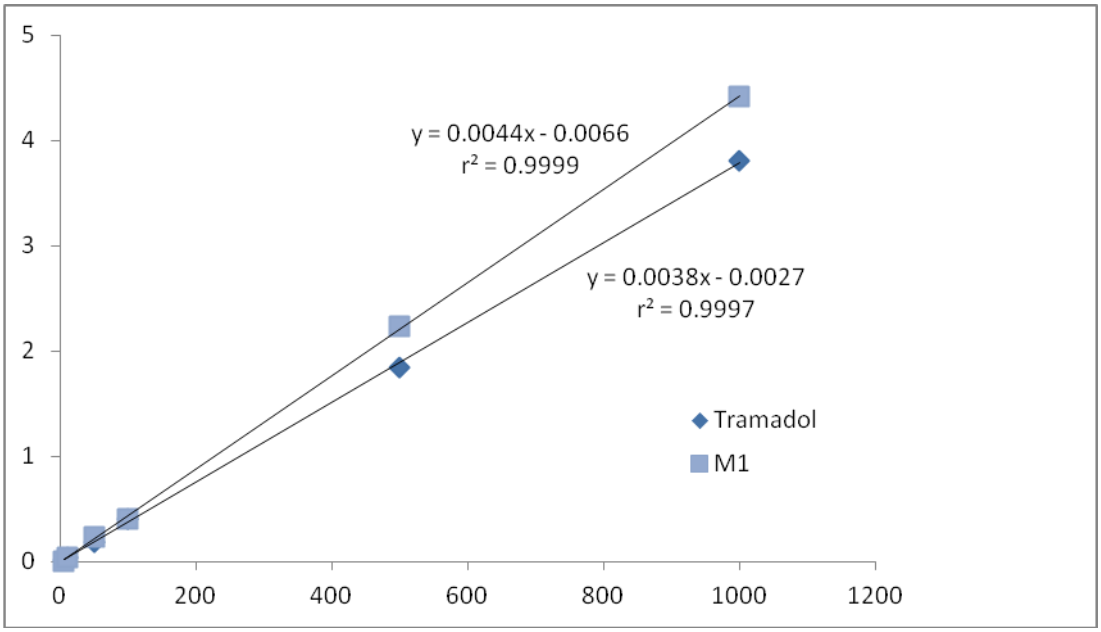


Figure 10. Calibration curves of tramadol and M1. Plotted peak areas of the analytes/IS peak areas *versus* concentrations (5, 10, 50, 100, 500 and 1000 ng/mL).

The method was linear at the concentration range established, with determination coefficients (r^2) greater than 0.99 for the calibration curves of tramadol and M1 (Table 6).

Table 6. Blood linear regression analysis of tramadol and M1 standard solutions (5-1000 ng/mL) performed on three different days.

Xenobiotic	n = 3	Y = mx + b	Concentration range (ng/mL)	r^2	LOD (ng/mL)	LLOQ (ng/mL)
Tramadol	Day 1	y = 0.0035x + 0.0188	5 - 1000	0.9993	0.74	2.24
	Day 2	y = 0.0038x - 0.0027		0.9997		
	Day 3	y = 0.0038x - 0.0055		0.9994		
M1	Day 1	y = 0.0045x - 0.0065	5 -1000	0.9998	0.56	1.70
	Day 2	y = 0.0045x - 0.0169		0.9991		
	Day 3	y = 0.0044x - 0.0066		0.9999		

LOD, limit of detection; LLOQ, lower limit of quantification.

1.1.6. Limit of detection and lower limit of quantification

The LOD and LLOQ (0.74 and 0.56 ng/mL for tramadol and 2.24 and 1.70 ng/mL for M1 respectively) (Table 6) suggest a good capacity of this method for the quantification of both compounds, even in low concentration.

1.1.7. Precision and accuracy

As the %CV values calculated for intra and inter-day precision studies of tramadol and M1 did not exceed 15%, the developed method was considered precise for both analytes. Accuracies in the range 85.79 - 110.24% for tramadol and 85.67 - 106.19% for M1 were determined (Table 7), which are within the proposed acceptance limits for this parameter (100 ± 15%) [135].

Table 7. Precision, accuracy and recovery (%) for tramadol and M1.

Xenobiotic	Concentration (ng/mL)	Intra-day precision (%, n = 3)	Intra-day precision (%, n = 3)	Accuracy (%, n = 3)	Recovery (%)
Tramadol	10	4.1	12.1	103.9	109.8
	100	3.3	4.9	106.4	
	1000	4.4	5.0	100.0	
M1	10	7.5	8.6	85.0	107.8
	100	3.2	4.6	94.3	
	1000	6.7	7.6	94.7	

1.1.8. Recovery

At three different concentrations of tramadol and M1 (10, 100, 1000 ng/mL), the results obtained indicated an efficient clean-up procedure, with extraction recoveries of 109.8 and 107.8% for tramadol and M1, respectively, which are within the proposed acceptance limits for this parameter (100 ± 20%) (Table 7) [135].

1.3. Genetic polymorphism

Genetic factors play, in general, a dominant role over other factors in the metabolism of individual drugs [76]. In the present study, five *post mortem* blood samples from cases of suspected tramadol fatal intoxications were analyzed for COMT,

MDR1, CYP2D6 and OPRM1 SNPs. Genotyping of these SNPs allows the identification of UMs and PMs individuals, which is important to better understand the efficacy, side effects and toxicity of this drug and thus adjust therapy in these subjects depending on their genotype. For most drugs, the effect and treatment outcomes are determined by the interplay of multiple genes [140]. Table 8 shows the results of the four SNPs for all five samples:

Table 8. The genotypes identified by real-time PCR for polymorphisms in study.

nº	COMT	MDR1	CYP2D6	OPRM1
		Genotype		
1	Val/Met	TT	CC	GA
2	Val/Val	CT	CC	AA
3	Val/Val	CT	CC	AA
4	Val/Val	TT	Indeterminated	AA
5	Val/Met	CT	CC	GA

A, adenine; C, cytosine; COMT, catechol-O-methyltransferase; CYP2D6, cytochrome P450 2D6; G, guanine; MDR1, multidrug resistance protein 1; Met, methionine; OPRM1, μ-opioid receptor gene; T, thymine; Val, valine.

The enzyme encoded by the CYP2D6 gene plays an important role in the metabolism of tramadol [74]. More than 80 distinct allelic variants for CYP2D6 are known, which leads to a wide spectrum of metabolic capacity and phenotype diversity within populations [9, 76, 81]. Subjects who did not possess any of the common inactivating alleles or gene duplications are considered to be homozygous EMs, carriers of one variant allele are considered to be IMs, and carriers of two variant alleles are classified as PMs [142]. The allelic frequencies of the *3, *4, *5 and *6 alleles in Caucasians, account for the majority of the CYP2D6 PMs [78]. The duplication or multiduplication of the CYPD6 gene (mostly CYP2D6*1 and CYP2D6*2 alleles in Caucasians) is associated with an ultrarapid metabolism of some compounds including tramadol [9].

In this study, all subjects were genotyped for the presence of CYP2D6*4 allele. In Caucasians, CYP2D6*4 allele occurs due to a mutation resulting in a defective splice and a premature stop codon [143]. In the molecular analysis of this allele, it was verified that four subjects are homozygous (CC). All these subjects with at least one

functional allele were categorized as EMs. For one sample, it was not possible to determine the genotype.

The studied COMT SNP (Val158Met) occurs in codon 158, resulting in a Val to Met transition. The Val/Val genotype gives rise to an effective enzyme, whereas the Met/Met genotype produces a defective enzyme, with lower enzymatic activity [87, 88]. The analysis of the SNP demonstrated that two subjects have intermediate enzyme activity with heterozygous (Val/Met) genotype and three subjects exhibit high enzyme activity with wild-type homozygous (Val/Val) genotype. So, in subjects with less activity COMT enzyme, it is possible that these have more pain due to decreased endogenous opioid, comparatively with the subjects who exhibit higher enzyme activity [144]. This SNP may then explain part of the inter-individual difference in the adaptation and response to pain and may be involved in this opioid dosing requirements and side effects [144, 145].

A well-known mechanism responsible for drug resistance is over-expression of ABC-transporter genes such as MDR1. Over 50 SNPs have been identified in the human MDR1 gene. Among them, C3435T SNP on exon 26 is a silent mutation and may play a role in inducing drug resistance by altering the expression level of the MDR1 gene [81-83]. Tramadol is a substrate of P-glycoprotein, an important efflux transporter involved in bioavailability and elimination of several other drugs in humans. A polymorphism, as C3435T in the MDR1 gene, may affect the pharmacokinetics of tramadol [84]. In this study, three subjects are heterozygous (CT) and two homozygous (TT) for polymorphisms of MDR1 C3435T. Alteration in function of the P-glycoprotein transporter could alter the relative amounts of tramadol in the CNS, and thereby influencing the prevalence and severity of tramadol related central side effects. Studies have shown that the T allele is linked to weaker expression/activity of P-glycoprotein, and consequently less drug resistance [140].

The μ-opioid receptor is the primary binding site for tramadol. The most studied SNP in OPRM1 (A118G), results in an amino acid exchange from asparagine to aspartate at position 40 [90, 92]. In this study, three subjects have AA and two GA genotypes for this SNP. In general, individuals with the GG or GA genotype show an elevated sensitivity to pain and a reduced analgesic response to opioids compared with individuals with the AA genotype [90, 91, 93].

In Chapter III the studied polymorphisms results are discussed jointly with M1 and tramadol concentrations determined in these cases.

Chapter III

Application of the developed GC-MS method to real *post mortem* blood samples

1. Proof of applicability

This method was applied for toxicological analysis of real *post mortem* blood samples of five suspected cases of tramadol fatal intoxications. Two peripheral blood samples, two heart blood samples and one blood with no indication of the sampling were obtained from the North Branch of the Portuguese National Institute of Legal Medicine and Forensic Sciences I.P..

For proper forensic interpretation it is necessary to take into account not only the therapeutic, toxic and fatal range but the clinical history, circumstances of death, data obtained during the autopsy and other relevant aspects [105, 146]. The blood samples collection site is another important aspect. Peripheral blood is the preferential sample, as this collection site allows a reduction of the impact of *post mortem* redistribution/diffusion in quantitative interpretation of results [147].

The concentrations were calculated by using the linear regression obtained from the validated method. All samples analyzed were positive for tramadol and metabolite M1 (Table 9).

Table 9. Concentration of tramadol and M1 in real *post mortem* blood samples.

The blood samples collection site	nº	Concentrations (ng/mL)	
		Tramadol	**M1**
Peripheral blood	1	2389.8	148.21
Peripheral blood	2	533.52	180.17
Blood with no indication of the sampling	3	490.55	44.39
Heart Blood	4	3995.9	461.29
Heart Blood	5	427.03	86.17

Analysis of blood samples revealed concentrations ranging from 427.03 to 3995.9 ng/mL for tramadol and 44.39 to 461.29 ng/mL for M1. The tramadol therapeutic blood concentration in adults range from: 100 - 250 ng/mL [57]. Reconstructed chromatogram of real blood peripheral sample (subject number 3) is shown in Fig. 11. However, as previously mentioned, for the correct interpretation of the results, other aspects should be taking into account beyond the concentrations of the compounds, but this information was not available.

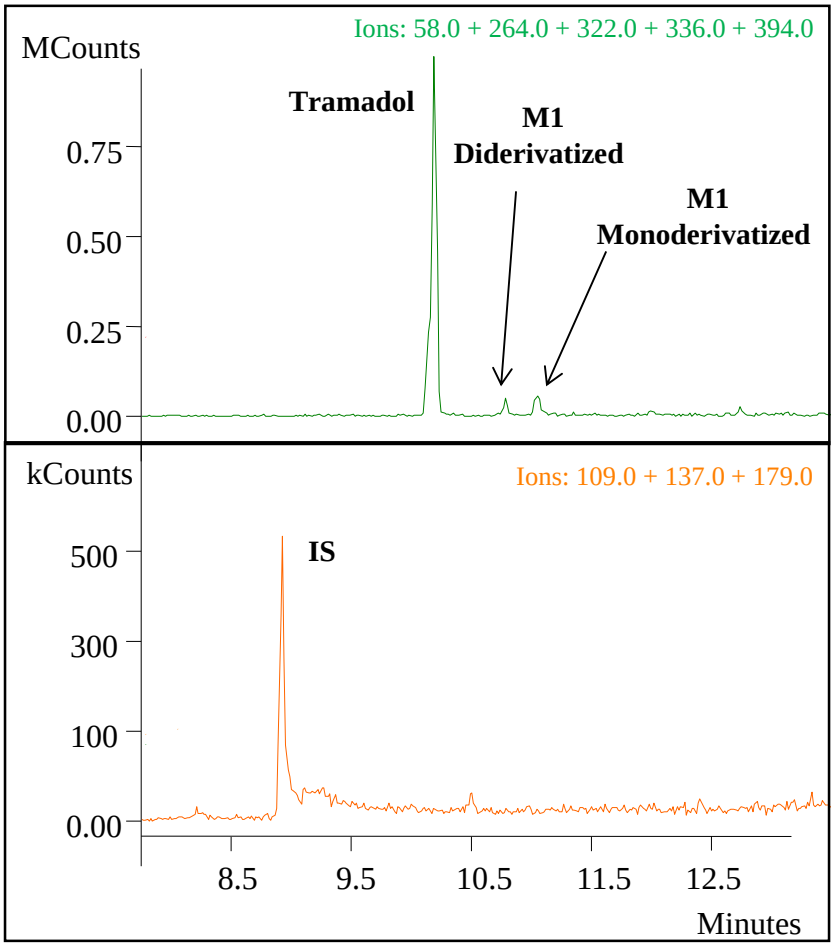


Figure 11. Reconstructed GC-MS (SIM mode) chromatogram of a real blood sample positive for tramadol and M1 (subject number 3). It is represented the ion chromatogram of tramadol (*m/z* 58+336+264), M1 (*m/z* 58+394+322) and IS (*m/z* 109+137+179).

What is important to mention, is the relevance of considering M1 quantification when interpreting tramadol concentrations. In addition, M1 contributes to toxicity due to its analgesic activity, which is twice that of the parent compound. The higher μ-opioid receptor affinity also contributes to toxicity through depression of the CNS [61]. Moreover, comparing the concentrations of the parent drug and metabolite it is possible to obtain information on whether the drug was chronically or acutely ingested. High concentrations of tramadol are indicative of acute intoxication because, before excretion. Tramadol is mainly metabolized by the cytochrome P450 isoenzyme CYP2D6 in its main pharmacologically active metabolite M1.

In the cases studied, four subjects with at least one functional allele were categorized as EMs. Nevertheless it was not possible to establish any comparison between polymorphic results and concentrations of tramadol and M1, due to the much reduced number of samples. In addition, high tramadol concentrations could not be completely explained only based on polymorphisms studied. Besides

pharmacogenomics, the pharmacokinetics of the drug can be affected by many other factors namely, age, disease, comorbidities, concomitant medication, metabolic interactions and kidney or liver function [5, 76], but this information was not accessible. One of the five cases was not possible to determine the genotype. In this case, it is important to consider the possibility of being a PM subject for rare mutations, which could not be identified with the assay used (e.g. *11, *12, *13, *14, *15, *16). These mutations are extremely uncommon in Caucasians, with an allele frequency of less than 0.001% [75].

In case 1, Val/Met genotype for COMT shows intermediate enzyme activity and GA genotype for OPRM1 exhibits an elevated sensitivity to pain and a reduced analgesic response to opioids. Moreover, as it is homozygous TT for the polymorphism of MDR1, the expression/activity of P-glycoprotein is lower and consequently less drug resistance. In this case, the tramadol and M1 were quantified in peripheral blood concentrations of 2398.8 and 148.21 ng/mL, respectively. The results of COMT and OPRM1 genotypes can explain a lower analgesia and by this may have contributed to higher doses ingested, despite being EM. However, as it is homozygous TT, it should be expected less resistance to treatment. Further studies and more cases are necessary to access the individual contributive effect of each polymorphism.

Cases 2 and 3 have similar concentrations of tramadol (533.52 ng/mL for case 2 and 490.55 ng/mL for case 3) and lower concentrations of M1 (180.17 ng/mL for case 2 and 44.39 ng/mL for case 3). Both are EMs and have AA genotypes that show a reduced sensitivity to pain, and consequently greater analgesia. However, both have CT genotypes which demonstrate greater expression/activity of P-glycoprotein and consequently greater drug resistance. Similarly to the explanations given for case 1, further studies and more cases are necessary to access the individual contributive effect of each polymorphism. However, the case 3 exhibit high enzyme activity with wild-type homozygous Val/Val genotype, comparatively with the case 2 which is Val/Met. In these cases, the dose is also unknown and the pharmacogenomics and pharmacokinetics of the drug also may have been affected by various factors such as renal and/or liver insufficiency.

The case 4 shows the highest concentration of tramadol and M1, 3995.9 and 461.29 ng/mL respectively, but this may be explained by the fact that they have been quantified in heart blood and not in peripheral blood, as previously reported. In this case, it was not possible to determine the CYP2D6 genotype, as previously reported.

The same happens for the case 5 wherein the concentration of tramadol and M1 were quantified in heart blood, but at concentrations less than those of the case 4, 427.03 and 86.17 ng/mL, respectively. In this case, the subject has CT genotype for MDR1, Val/Met genotype for COMT and GA genotype for OPRM1. These genotype results can explain a lower analgesia, the elevated sensitivity to pain and a reduced analgesic response to opioids greater and consequently greater expression/activity of P-glycoprotein drug resistance. This may have contributed to the subject to take a higher dose. However, such as in the other cases described here, other factors may affect the pharmacogenomics and pharmacokinetics of the drug, but this information was not available.

Chapter IV

Conclusions and future perspectives

1. Conclusions and future perspectives

I. A sensitive, reproducible, precise, accurate and inexpensive GC-MS method was developed and validated to quantify tramadol and M1 in *post mortem* blood samples;

II. Few studies are published exploring the quantification of tramadol and M1 in of *post mortem* blood samples and very few have evaluated the importance of the metabolite M1 fatal cases of tramadol intoxication;

III. The proposed GC-MS method was successfully applied in the quantification of these analytes in real *post mortem* blood samples and shown to be appropriate for routine analysis. Blood samples represents the most used matrix with relevant importance in toxicological *post mortem* analysis;

IV. The GC-MS method described in this study exhibited a good selectivity, lower LOD and LLOQ (0.74 and 0.56 ng/mL for tramadol and 2.24 and 1.70 ng/mL for M1 respectively). In addition, the regression analysis for both analytes showed linearity in the range 5-1000 ng/mL with r^2 ranging from 0.9991 to 0.9999. The %CV oscillated between 0.70 and 12.45%.

V. Although the sample preparation is more time-consuming, due to the derivatization step, it was possible to obtain a simple and efficient method using only 500 µL of sample;

VI. The same blood specimens were addressed to study CYP2D6, MDR1, OPRM1 and COMT SNPs by PCR;

VII. Regarding polymorphism studies, four subjects with at least one functional allele were categorized as EMs. Nevertheless it was not possible to establish any comparison between polymorphic results and concentrations of tramadol and M1, due to the much reduced number of samples;

VIII. Moreover, high tramadol concentrations could not be completely explained only based on polymorphisms studied. Besides pharmacogenomics, the pharmacokinetics of the drug can be affected by many other factors namely,

age, disease, concomitant medication, metabolic interactions and kidney or liver function;

IX. One of the five cases was not possible to determine the genotype. In this case, it is important to consider the possibility of being a PM subject for rare mutations that are extremely uncommon in Caucasians, which could not be identified with the assay used;

X. The genotyping analysis are relevant in forensic field in order to better understand the causes that contributed to the tramadol intoxication and how genetic factors may contribute;

XI. Future projects will be needed to better understand the role of genetic factors in acute tramadol intoxications, since this have a high impact (e.g. how quickly the xenobiotic it is cleared from the blood and metabolized in the liver);

XII. As it was not possible to establish any comparison between polymorphic results and concentrations of tramadol and M1, be interesting to analyze the CYP2D6, MDR1, OPRM1 and COMT polymorphisms using an higher number of blood *post mortem* samples;

XIII. Moreover, other factors may also affect the pharmacogenomics, the pharmacokinetics of the drug and it will be interesting correlate them with concentrations of tramadol and M1.

PART IV: References

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