Postmortem Redistribution of Tramadol and O-Desmethyltramadol

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Tramadol is a widely used analgesic opioid for moderate-to-severe pain due to its efficacy and safety. Although tramadol induces less adverse effects compared with other opioids, an increased number of documented cases of dependence, abuse, intentional overdose or intoxication have been described. In fatal intoxication, the interpretation of the probable cause of death often relies on the measurement of the tramadol concentration in blood. However, postmortem redistribution (PMR) may affect the results and therefore bias the autopsy report. In the present study, the postmortem cardiac and femoral blood samples from 15 cases of fatal tramadol intoxication were obtained to assess the PMR of tramadol and its main active metabolite, O-desmethyltramadol (M1). Toxicological analysis was performed by the gas chromatography–electron impact-mass spectrometry (GC–EI-MS) method, previously developed and validated for the quantification of both analytes. The cardiac-to-femoral blood ratios of 1.40 and 1.28 were obtained for tramadol and M1, respectively. Results were compared with those in the literature and it was possible to conclude that femoral blood should be considered for quantitative interpretations in fatal cases of tramadol intoxication.

Introduction

Tramadol is a widely used therapeutic alternative to other opioid analgesics since it has low potential for abuse, dependence and tolerance, and low probability to cause adverse effects, including respiratory depression (1). However, the number of cases reporting dependence, abuse, intentional overdose or intoxication by tramadol is increasing. Fatal intoxications due to tramadol alone also exist, but are not common (2–6). 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 (7–15). Tramadol is a centrally acting opioid analgesic, which has a dual mechanism of action: it is a partial agonist of μ-opioid receptors and inhibits serotonin and noradrenaline reuptake at the synapses of the spinal cord, acting on the pain transmission mechanism (3). After oral administration, tramadol is rapidly and almost completely absorbed (16). Plasma protein binding is ~20% and is rapidly distributed in the body with distribution volume (Vd) of 3 L/kg (7, 8, 10, 16). Using a rodent model, Bamigbade and Langford (17) demonstrated that tramadol was particularly distributed into the lungs, spleen, liver, kidneys and brain. Tramadol is mainly metabolized in the liver by O- and N-demethylation, catalyzed by the cytochrome P450 (mainly isoenzyme CYP2D6), and followed by conjugation with glucuronic acid and sulfate. The primary metabolites O-desmethyltramadol (M1) and N-desethyltramadol (M2) may be further metabolized to three additional secondary metabolites namely, N, N-didesmethyl tramadol (M3), N, O-tridesmethytramadol (M4) and N, O-didesmethytramadol (M5), but several other metabolites have also been described (16, 18–21). The therapeutic efficacy and toxicity of tramadol is dependent on the analgesic activity of its main pharmacologically active metabolite M1, which is characterized by a higher affinity for the μ-opioid receptor than the parent compound. M5 is also an active metabolite, but its affinity for the opioid receptor is ~30-fold lower than that of M1 (16, 22, 23). Approximately 90% of the drug is excreted in urine, 10% in feces, 20% as free and conjugated M1 and 17% as M2 (3, 24, 25). The elimination half-lives of tramadol and M1 are 6.3 and 7.4 h, respectively (9).

After fatal intoxications, drug concentrations may vary according to the sampling site and the interval between death and postmortem specimen collection (26, 27). These site- and time-dependent variations are designated as postmortem redistribution (PMR) (27). Different mechanisms underlying PMR of drugs can occur: diffusion through blood vessels and transsplanchnic diffusion toward the surrounding organs (such as the gastrointestinal tract, liver, lungs and myocardium), cell lyses and putrefaction (27). These mechanisms may be affected by the drug’s lipophilicity, volume of distribution and pH (28). Drugs with high lipid solubility and a high Vd (>4 L/kg) are more susceptible to PMR (12, 27, 29, 30). The release of basic drugs from solid tissues into the blood compartment within some hours after death can occur due to a decrease in blood pH, which may be responsible for a return of the basic molecules into this compartment, due to an increased concentration gradient in nonionized forms (27). The putrefactive process may also contribute to PMR since, after death, metabolism of xenobiotics by bacteria is possible (27, 31, 32). Nevertheless, the PMR of a drug cannot be predicted based only on these factors. Other factors such as the absorption route, pKa value, postmortem interval between death and autopsy, dose or particular affinity of the drug for some tissues and the possibility of a residual metabolic activity in the first hours after death also must be envisaged (27, 33).
Femoral blood appears to be the specimen of choice for post-mortem toxicological analysis since it is the least subject to PMR (27, 34, 35). Drug concentrations in cardiac blood are generally higher than in femoral blood (35). The position and manipulation of the corpse and regurgitation of drugs from the stomach into the airways or thoracic trauma may explain differences in post-mortem blood concentrations from different anatomic places (27, 36). For example, the contamination of airways can induce the redistribution of drugs into the cardiac blood via the pulmonary vessels (36, 37). Drugs that are also more concentrated in the liver, lungs or myocardium redistribute more quickly into cardiac blood, causing an increase of their concentrations (28). However, in a few cases, femoral blood concentrations were found to be higher than cardiac blood concentrations. This can occur in cases where resuscitation was attempted, presumably causing a shift of cardiac blood into the femoral vessels (27, 38, 39). The difference between the cardiac and femoral vessels is known as the cardiac-to-femoral blood ratio (26). Drugs with higher ratios have a great potential for redistribution (40).

This work aimed at the optimization and validation of an analytical method for the quantification of tramadol and M1 by gas chromatography–electron impact-mass spectrometry (GC–EI-MS) to evaluate the PMR of these analytes in postmortem cardiac and femoral blood samples from 15 cases of suspected fatal tramadol intoxication. It is expected that the obtained results will help to define the best conditions of blood collection for the measurement of tramadol and M1 in fatal cases resulting from tramadol intoxications.

Materials and methods

Ethics statement

All research was approved by the Portuguese National Council of Ethics for Life Sciences. 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's 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.

Reagents and standards

Tramadol hydrochloride ([(+)-cis-2-(dimethylaminomethyl)-1-(3-methoxyphenyl) cyclohexanol hydrochloride; molecular mass 299.84 g/mol], methanol and bis(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane (BSTFA + 1% TMCS) were acquired from Sigma-Aldrich (St Louis, MO, USA). Phenacetin [N-(4-ethoxyphenyl) acetamide; molecular mass 179.216 g/mol, internal standard, IS] was obtained from LG Standards. O-Desmethytramadol hydrochloride [M1, 3-(2-[(1-amino-1-methyl)1-4-hydroxycyclohexyl)phenolhydrochloride]; molecular mass 285.809 g/mol] was a generous gift from Grünenthal (Amadora, Portugal). Sodium hydroxide (NaOH) and perchloric acid (HClO₄) were obtained from Panreac (Barcelona, Spain) and ethanol and 2-propanol from Merck (Darmstadt, Germany). Nitrogen (99.99% purity) and helium (99.99%) were obtained from Gasin (Portugal). Bond Elut C₁₈ cartridges (100 mg, 1 mL) were purchased from Varian (Sint-Katelijne-Waver, Belgium). All the reagents used were of analytical grade or from the highest available grade.

Collection of blood samples

Postmortem cardiac and femoral blood samples (1.5 mL each) of 15 cases of suspected fatal tramadol intoxication were obtained from the Centre Branch of the Portuguese National Institute of Legal Medicine and Forensic Sciences, I. P. Samples were collected according to the Institutional rules and previously proposed guidelines (34).

Preparation of stock and working standard solutions

Stock solutions of the tramadol, M1 and IS were prepared in methanol at the concentration of 1 mg/mL. Working standard solutions of tramadol and M1 for the calibration curve were prepared at different concentrations by diluting stock solutions in methanol (0.005, 0.01, 0.05, 0.1, 0.5 and 1 μg/mL). A working solution of the IS at 20 μg/mL was also prepared in methanol. Working solutions were prepared fresh daily and stock solutions were stored at −80°C prior to use. Dilutions of blood samples (tramadol and M1 concentrations >1 μg/mL) with distilled water were performed to ensure quantification within the limits of the calibration curve.

Extraction step

Tramadol and M1 extraction from postmortem blood samples were performed according to Merslavic and Zupancic-Kralj (41) with modifications (Figure 1A and B). Solid-phase extraction (SPE) was performed using solid-phase C₁₈ cartridges connected to a vacuum manifold and conditioned twice with 1 mL of methanol, followed by thrice with 1 mL of 0.001 M NaOH. The supernatant was then passed through the cartridges. The cartridges were washed twice with 1 mL of 0.001 M NaOH. After drying under full vacuum for 1 min, the analytes were eluted with 2 mL of 2-propanol. The eluate was evaporated to dryness at 50°C under a gentle stream of nitrogen.

Derivatization procedure

Tramadol and M1 are polar organic compounds with low volatility, which limits their analysis by gas chromatography. To overcome these limitations, a derivatization step was introduced in the sample preparation procedure. In our study, tramadol and M1 derivatization was performed according to Pinho et al. (42). In this process, 25 μL of BSTFA + 1% TMCS and 25 μL of ethyl acetate were added to dry residue, mixed and heated for 20 min at 70°C (Figure 1C). After cooling to room temperature, the samples were evaporated to dryness under nitrogen flow and reconstituted with 50 μL of ethyl acetate, and 2 μL of each derivatized sample was injected into the GC–EI-MS system.

Gas chromatography–mass spectrometry conditions

Quantitative GC–EI-MS analyses were performed on a Varian CP-3800 gas chromatograph equipped with an ion-trap VARIAN Saturn 4000 mass detector. Chromatographic separation was achieved using a capillary column (30 m × 0.25 mm × 0.25 μm film thickness) from Varian® VF-5 ms and a high purity helium C-60 as carrier gas. An initial temperature of 90°C was kept for 1 min, increased to 300°C at 20°C/min and held for 3 min giving a total run time of 12 min. The flow of the carrier gas was maintained...
at 1.0 mL/min. The injector port was set at 280°C. Analyses were performed in a FullScan mode with splitless injection.

Method validation
The validation of the method was performed accordingly to European Medicines Agency (43) and other authors (42, 44, 45). The guidelines of the European Medicines Agency have been harmonized with the guidance by the Food and Drug Administration (46), which is now generally accepted by the biopharmaceutical industries as the gold standard method of validation (47). The limit of detection (LOD), lower limit of quantification (LLOQ), intra- and inter-assay precision, accuracy, recovery and linearity of the method were determined. To obtain these validation data, calibration curves were prepared by spiking blank blood with appropriate volumes of tramadol and M1 standard solutions.

Results and discussion
Preparation of blood samples
Blood is the matrix of choice in toxicological analysis for the quantification of xenobiotics, since there is a good correlation between blood concentration and the toxic effect, and is a useful specimen to establish acute ingestion (34). 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 interferents is crucial for subsequent chromatographic analysis. Nevertheless, few methods have focused on the inherent problems associated with postmortem specimens, particularly blood, which is frequently hemolyzed and degraded, causing column blockage and reducing extraction efficiency and recovery (48). In our study, to solve this problem, the postmortem blood samples (500 µL of blood and 25 µL of IS) were pretreated by sonication for 20 min at room temperature. Then, 500 µL of HClO₄ (7%) was added to achieve protein precipitation, vortexed for 1 min and centrifuged at 13,000 rpm for 20 min at 4°C (Figure 1A). The obtained supernatant was subsequently submitted to the SPE procedure (Figure 1B).

Method validation
The proposed GC–EI-MS method proved to be selective as there were no interferences of endogenous compounds with the same retention time of tramadol, M1 and IS. The obtained carry-over results were <20% for the LLOQ and 5% for the IS, which are within the proposed acceptance limits for this parameter (43). The regression analysis for both analytes was found to be linear in the range of 0.005–1 µg/mL, with correlation coefficients of 0.9997 and 0.9999 for tramadol and M1, respectively. The LOD and the LLOQ were 0.00074 and 0.00056 µg/mL, and 0.0022 and 0.0017 µg/mL for tramadol and M1, respectively. The comparison of the obtained results with values of LOD and LLOQ documented in the literature (7–10, 36, 49) suggests a good capacity for quantification of both analytes, even at low concentrations. The method provided an accurate and simple assay with adequate precision for the quantification of tramadol and M1 with coefficients of variation of 1.15–12.45%. The recovery was also evaluated and the results obtained indicated an efficient clean-up procedure, with extraction recovery rates of 109.8 and 107.8% for tramadol and M1, respectively.

Gas chromatographic and electron impact-mass spectrometry analysis
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. The study of chromatographic conditions revealed the presence of two peaks for each analyte. Based on the fragmentation pattern of each peak produced by electron impact-mass spectrometry, for tramadol, the first peak corresponds to tramadol derivatized and the second peak for tramadol nonderivatized, with retention times of 10.57 and 10.39 min, respectively. In
the M1 case, the first and second peaks correspond to M1 di-derivatized (10.66 min) in both OH and mono-derivatized in only one OH group (10.80 min), respectively. The hydrogen atom of the OH group of tramadol and M1 was replaced for a Si(CH₃)₃ (trimethylsilyl) group. The integration of the chromatographic peaks for quantitative analysis was performed by reconstructing the full-scan chromatogram with the selective m/z ions, allowing more precise peak integration (34). The IS chromatogram exhibited only one peak (phenacetin nonderivatized), with a retention time of 8.90 min. Quantitative analysis was performed taking into account some target or qualifier ions: m/z of 58, 264 and 336 for tramadol, 58, 322 and 394 for M1 as well as 109, 137 and 179 for IS.

**Postmortem redistribution**

The PMR of tramadol and M1 determinations were evaluated in postmortem cardiac and femoral blood samples from 15 cases of suspected fatal tramadol intoxication. The concentrations were calculated by using the linear regression obtained from the validated method GC–EI-MS. The analysis of femoral blood samples revealed concentrations ranging from 0.26 to 2.60 µg/mL for tramadol and 0.06 to 0.38 µg/mL for M1 (Table I). Cardiac blood samples concentrations ranged from 0.45 to 4.01 µg/mL for tramadol and 0.09 to 0.72 µg/mL for M1. According to the literature, therapeutic blood concentrations of tramadol in adults range from 0.01 to 0.25 µg/mL (50). Toxic concentrations of tramadol have been reported to be 0.80 µg/mL. Until now, therapeutic and toxic concentrations for the M1 metabolite have not been described. However, it is very important to consider M1 metabolite quantification when interpreting tramadol concentrations since it has greater affinity (300–400 times higher for (+) M1 enantiomers) for the μ-opioid receptor and therefore more potential for central nervous system depression (51). Moreover, comparing the concentrations of the parent drug and its metabolite (M1), it is possible to obtain information on whether the drug was chronically or acutely ingested (9). High concentrations of tramadol and low metabolite concentrations are indicative of acute intoxication because, before excretion, tramadol is mainly metabolized by the cytochrome P450 isoenzyme CYP2D6 in M1 (9, 52). In this study, tramadol concentrations were higher than those of the M1 metabolite, as previously described in other studies (3, 13, 36, 52, 53).

Depending on the sampling site and the postmortem interval, differences in postmortem concentrations of tramadol have been described (Table II) (4, 8, 10–12, 36). Nevertheless, tramadol has low-to-moderate PMR and this is relatively uniform in all organs and blood with the exception of urine (5, 8, 10, 13, 15). In urine, the concentration of tramadol in the liver and kidneys, compared with that in blood, does not suggest differences in postmortem concentrations of the drug (4, 5, 8). In cases of tramadol intoxication, postmortem arterial blood concentrations are often higher than in venous blood concentrations (4, 8, 10, 36).
In the brain, the tramadol concentration is similar to that in femoral blood, with little PMR (10, 36). However, the lipophilicity facilitates its distribution and penetration into the central nervous system (10), and therefore, the M1 metabolite concentration is lower than the parent compound to the same extent as tramadol (16, 22). PMR can, thus, complicate the interpretation of analytical data in forensic toxicology and make difficult the determination of the cause of death (27, 36).

The differences observed in this study between femoral and cardiac blood concentrations are partly due to the physicochemical properties of tramadol and M1. Both analytes are organic bases with similar molecular structures and pKₐ values (9.13 and 9.12 for tramadol and M1, respectively) (54). Nevertheless, tramadol has a higher lipophilicity coefficient (logDₖ₋ₒ 1.13) than M1 (logDₖ₋ₒ 0.4) (54). Perhaps due to this fact, higher tramadol concentrations were found in cardiac than in the femoral blood, and the M1 concentrations were identical in cardiac and femoral blood, which shows that tramadol has a greater PMR. However, the cardiac-to-femoral blood ratios for tramadol and M1 are 1.40 and 1.28, respectively, and this does not suggest a significant PMR for both analytes. These results are consistent with those obtained in other published studies for tramadol and M1. Levine et al. (8) analyzed the PMR of tramadol in four postmortem cases and obtained a ratio of 0.8 (between cardiac and femoral blood). Musshoff and Madea (4) obtained a ratio of 1.4 for tramadol (also between cardiac and femoral blood). Bynum et al. (36) described a fatal multiple drug overdose case involving tramadol and amitriptyline and evaluated the PMR of these drugs and metabolites (nortriptyline, M1 and M2) in various tissues and fluids. The cardiac-to-femoral blood ratios of 4.9 for tramadol and 1.3 for M1 were observed. The high cardiac blood concentration and, therefore, the high cardiac-to-femoral blood ratios were explained by the drugs diffusion from the stomach and/or gastrointestinal tract. Moreover, authors did not exclude the possibility of perimortem aspiration, which can contaminate the airways and induce redistribution into the cardiac blood via the pulmonary vessels. Moore et al. (55) evaluated the PMR of tramadol and M1 and M2 metabolites in an overdose fatality. In our work, by analyzing 15 cases, it was possible to better define the PMR ratio between tramadol and its main metabolite M1 for femoral and cardiac blood. Even in the presence of a reduced PMR, our findings show that femoral blood should be considered for quantitative analysis in order to reduce the influence of PMR and therefore errors in the interpretation of toxicological results.

Conclusions

The developed and validated GC–EI-MS method that was applied for toxicological analysis of postmortem cardiac and femoral blood samples of suspected cases of tramadol fatal intoxications proved to be accurate and precise. This method is also selective and sensitive, and requires a small sample volume (500 μL).

Higher tramadol concentrations were found in cardiac than in femoral blood, and the M1 concentrations were identical in cardiac and femoral blood, which shows a greater PMR for tramadol. This could be expected since tramadol has a lower polarity (higher lipophilicity) than the M1 metabolite. The cardiac-to-femoral blood ratios of tramadol and M1 were 1.40 and 1.28, respectively, and therefore femoral blood should be considered for quantitative analysis in forensic toxicology.

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