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Postmortem analysis of famprofazone and its metabolites, methamphetamine and amphetamine, in porcine bone marrow

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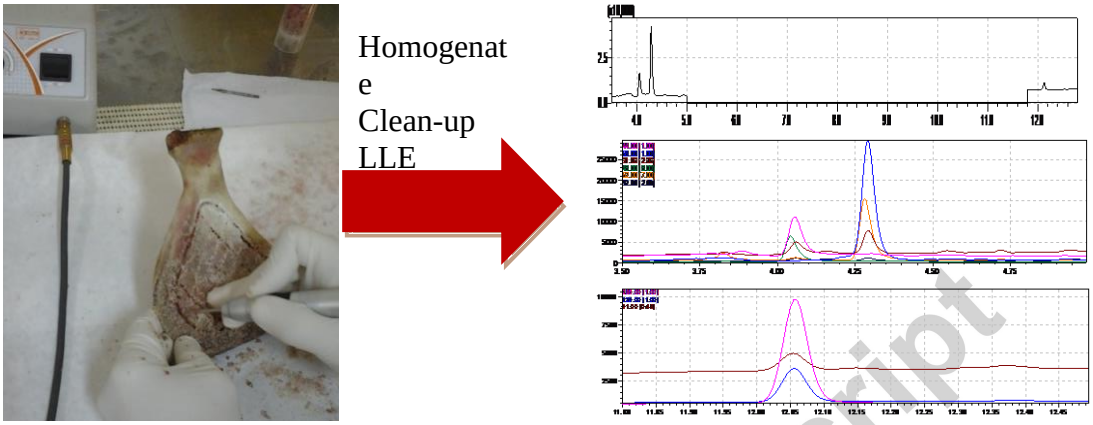
ABSTRACT

Forensic toxicologists typically work with body fluids, such as blood and urine, or visceral tissues. The analysis of alternative samples, such as bone marrow, can be requested when the commonly used samples are unavailable due to an extended time lapse between the time of death and collection of the material to be analysed. In this study, a method for the analysis of the lipophilic drug famprofazone (FA) and its metabolites, methamphetamine (MA) and amphetamine (AM), in bone marrow was developed, validated and applied to bone marrow from pigs given controlled doses of famprofazone. This method involves enzymatic bone-cleaning, fragmentation of the bones with the assistance of a micro electric motor, optimization of clean-up and LLE (liquid/liquid extraction) conditions and determination by GC/MS. After evaluation through statistical tests, such as Shapiro Wilk for normality and Cochran for homoscedasticity, a linear model was applied in the range of 100 (LOQ) – 2000 ng g^{-1}. Inter-day precision and bias was always < 4.6 %. In real sample analysis, bone marrow FA and MA concentrations ranged from 105 to

211 and from 102 to 148 ng g^{-1}, respectively; AM was not detected. The obtained results are useful for application in forensic toxicological protocols (human autopsy cases) and as a starting point for the development of further analytical tools.

GRAPHICAL ABSTRACT



Keywords:
Bone Marrow; Forensic Science; Toxicology; Drugs of Abuse; Gas Chromatography-Mass Spectrometry; Amphetamines

1. Introduction

In forensic sciences, bone marrow has been described as an alternative matrix in postmortem toxicological analyses because it is a well-preserved medium that is protected by the bone and a good repository of xenobiotics due to its large vascularization and high lipid content [1]. However, forensic toxicological analyses must consider that xenobiotics can undergo such processes as postmortem redistribution, biotransformation, chemical degradation, evaporation and neo-formation [2]. Despite these difficulties, bone marrow is a highly useful matrix in forensic autopsy, as it is often difficult to collect adequate blood specimens [3]. In a middle-aged adult, red marrow consists of 40% to 60% lipids, 30% to 40%water and 10% to 20% proteins [1].

This high lipidic matrix favours partitioning of lipophilic substances from blood into bone marrow [4].

Due to their structural and functional similarity to humans, pigs have been used as a model in biomedical research for evaluation of chronic and acute exposure to xenobiotics. Selegiline and metabolites have been investigated in porcine plasma [5], amitriptyline and citalopram and their respective metabolites have been detected in porcine bone and bone marrow [6], and cannabinoids have been examined in porcine serum [7]. Another study using bone and bone marrow samples from pigs evaluated amitriptyline, diazepam and pentobarbital [8].

Famprofazone($C_{24}H_{31}N_3O$), is a non-steroidal anti-inflammatory, analgesic and antipyretic drug. Famprofazone was selected as a model compound in this study because of its metabolic profile (a precursor of amphetamines) and its chemical features (a lipophilic drug) [9,10]. Although famprofazone has been analysed in biological fluids [11,12], there are no studies on famprofazone in bone marrow.

Different analytical methods have been proposed to determine xenobiotics in bone marrow. Sample pretreatment includes maceration in solvent [13,14] and dissolution in alkaline [4] or in acidic conditions [15,16]. Prior to chromatographic determination, preparation protocols often include an evaporation step. However, it has been reported that volatile compounds, such as amphetamines, may volatilize during the evaporation process. To prevent the loss of the amphetamines, a derivatization step may be used before evaporation [16]. To circumvent this problem, a method to analyse amphetamines in whole blood and urine without evaporation or a derivatization step was recently described [17].

The aim of this study was to present and validate a fast, simple liquid-liquid extraction (LLE)/GC/MS method for identification and quantification of famprofazone and its metabolites, methamphetamine and amphetamine, in porcine bone marrow after controlled oral administration of famprofazone. Detection and quantification of the analytes in authentic samples indicates that the method could be a useful tool in forensic science applications, especially for basic lipophilic drugs.

2. Experimental

2.1. Reagents and standards

Famprofazone was purchased from Sigma-Aldrich (Saint Louis, MO, USA), amphetamine, methamphetamine, amphetamine-D5 and methamphetamine-D5 (deuterated substances were used as internal standards, IS) were purchased from Cerilliant (Round Rock, TX, USA) and stored at -14 °C. Solvents (GC grade) used for analysis, methanol, n-hexane and methyl tert-butyl ether (MTBE), ethyl acetate, diethyl ether and 1-chlorobutane, were purchased from Tedia (Fairfield, OH, USA). The following reagents (analytical grade) were purchased from their respective manufacturers: sodium hydroxide (NaOH) and 37% hydrochloric acid (HCl) from Vetec (Duque de Caxias, RJ, Brazil) and tris-(hydroxymethyl)-aminomethane (TRIS buffer) from Isofar (Duque de Caxias, RJ, Brazil). Food-grade Alcalase® (2.4 L) purchased from Novozymes (Araucária, PR, Brazil). The standard stock solutions (SS, 1 mg mL^{-1}): amphetamine, methamphetamine, amphetamine-D5 and metamphetamine-D5 were purchased ready to use; famprofazone was prepared by weighing 10 mg (analytical balance, AUY-220 Uniblock, 0.0001 g precision, Shimadzu Corporation, Kioto, Japan), quantitatively transferring it to 10 mL volumetric flasks, and making up the volume with methanol. For each analyte, a working standard solution (WS) was prepared by diluting the corresponding SS solution with methanol to a final concentration of 100 µg mL^{-1}. MIX solutions (MS) containing the analytes were prepared in three levels - MS1 (25 µg mL^{-1}), MS2 (10 µg mL^{-1}) or MS3 (1 µg mL^{-1}) - and a MIX IS solution was prepared at 5 µg mL^{-1}, using methanol as the solvent. All solutions were transferred to amber glass flasks and stored at -14 °C.

2.2. Samples

The experiment was performed according to standard procedures in pig farming (Animal Production Department of UFRRJ). This project was approved

by the Ethics Research Committee of Universidade Federal Rural do Rio de Janeiro (COMEP/ UFRRJ), in accordance with the opinion N° 066/2010.

2.2.1. Blank samples

Drug-free bone marrow (from scapulae, ribs and vertebrae of 20 different animals) were obtained from a slaughterhouse of Universidade Federal Rural do Rio de Janeiro (UFRRJ), where healthy pigs were slaughtered for consumption.

2.2.2. Real samples

The animals (*Sus scrofa domesticus*) were housed and 100 mg (pig 602) or 200 mg (pig 721) of famprofazone was orally administered jointly with feed for a period of five days. Pigs were euthanized by cutting of the jugular vein within 2 hours of drug exposure. Bones (scapula, rib and vertebrae) were collected from animals, carefully identified and stored in a freezer at -30 °C prior to analysis.

2.3. Sample preparation

2.3.1. Enzymatic bone cleaning and fragmentation of bone pieces

Enzymatic bone cleaning was performed according to a previous work [16]. Briefly, a solution of 1 mol L^{-1} TRIS buffer was prepared by dissolving 121.14 g of TRIS buffer in 1 L of deionized water, and the pH 8.5 was adjusted with a 1 mol L^{-1} HCl solution. Next, 1 mL of Alcalase® enzyme solution was added. Bone specimens (scapula, rib and vertebrae) were immersed in the TRIS buffer solution containing the enzyme in a plastic heat-resistant container and warmed at 60 °C in a water bath for 6 hours (SL-150, Solab, Piracicaba, SP, Brazil). After the reaction time, the pieces were washed with distilled water and dried at room temperature.

Fragmentation was performed using an electric micro-motor Beltec LB 100 coupled to a straight nose hand piece and slow speed tungsten carbide burs ball shape (Araraquara, SP, Brazil). Bone marrow was collected by

scraping the bone with a stainless-steel spatula (Laborglass, São Paulo, SP, Brazil).

2.3.2. Obtaining bone marrow homogenate

Bone marrow homogenization was performed in acidic medium [16] per the following method: 50 µL of 1 ng μL^{-1} methamphetamine-D5 and amphetamine-D5 (internal standards) and 500µL of 3 mol L^{-1} hydrochloric acid were added to a test tube containing 100 mg of the bone marrow. After each addition step, the test tube was gently stirred (Vortex Lab Dancer, IKA, Wilmington, NC, USA). The test tube was incubated in a water bath (NT 245, Nova Técnica, Piracicaba, SP, Brazil) at 55 °C for 2 hours to obtain a bone marrow homogenate.

2.3.3. Optimization of the clean-up procedure

Bone marrow samples (n=6) were fortified at three analyte concentration levels (150, 600 e 1200 ng g^{-1}) by the addition of the MIX solutions prior to obtaining the homogenate.

Fortified bone marrow homogenate (n=6) was combined with 300µL of either n-hexane or diethyl ether. The mixture was stirred for 3 minutes on a vortex and later centrifuged (Centrifuge Mini Spin, Eppendorf, Hamburg, Germany) at 12000 rpm for 5 minutes. The organic layer was discarded, and extraction was performed as in 2.3.4 using ethyl ether as the extraction solvent in these tests. GC/MS analysis was performed according to 2.4. Student's t-test was performed to compare the performance of the solvents.

2.3.4. Optimization of the Liquid-liquid extraction (LLE)

This evaluation was initially performed comparing diethyl ether (EE) to MTBE. Bone marrow was fortified at three analyte concentration levels (150, 600 e 1200 ng g^{-1}), using six replicates per level. After homogenization and clean-up with n-hexane, 190 µL of 10mol L^{-1} NaOH was added, and the test tube was stirred for 10 seconds. Next, 150µL of the extraction solvent was added, and the test tube was vortexed for 3 minutes. After centrifugation at 12000 rpm for 5minutes, the upper layer of the extract was transferred directly

to a suitable container (2 mL vial with a 200 μL insert), and 1 μL was injected into the GC-MS system (GCMS-QP2010 Ultra system, Shimadzu Corporation, Kioto, Japan). In this first comparison step, Student's t-test was performed to evaluate a better performance.

A second experiment compared MTBE to 1-chlorobutane (1-CB) and ethyl acetate (EA) performances. The results were compared by one-factor ANOVA and Tukey test.

2.4. Gas chromatography-mass spectrometry (GC-MS)

The analyses were performed using a Shimadzu GCMS-QP2010 Ultra system, equipped with a RTX-5 MS column (30 m, 0.25 mm i.d., 0.25 μm film thickness) purchased from Restek Corporation (Bellefonte, PA, USA). The GC temperature programme was 60 (hold for 1 min) to 200 °C at 40 °C min^{-1}, increased to 250 °C by 25°C min^{-1} and to 300 °C by 15 °C min^{-1} and held at 300 °C for 6 min, resulting in a total run time of 15.83 minutes. Ultrapure Helium (purity grade 5.0, purchase form White Martins, Rio de Janeiro, RJ, Brazil) was used as the carrier gas at a constant linear velocity of 37.2 cm sec^{-1} and a total flow of 14.4 mL min^{-1}. Injection was at 260 °C in splitless mode (1 min). The interface temperature was set to 300 °C. The mass spectrometer was operated in electron ionization mode at 70 eV, the MS ion source was 230 °C, and the data were acquired in selected-ion monitoring (SIM) mode. The internal calibration of the detector was performed using a PFTBA solution. The characteristic ions selected were *m/z* 58 and 91for methamphetamine; *m/z* 44 and 91 for amphetamine; *m/z* 286, 229 and 91 for famprofazone; for the internal standards, *m/z* 48 and 92 (amphetamine-D5); and *m/z* 62 and 92 (methamphetamine-D5). The underlined ions presented the highest intensity and were used for quantification.

2.5. Validation procedure

Method validation was performed in accordance with the guidelines of Scientific Working Group for Forensic Toxicology (SWGTOX) published for

quantitative analysis [18]. Method validation parameters included selectivity, linearity, precision (within- and between-run), limit of detection (LOD), limit of quantification (LOQ), accuracy, carryover and stability.

The bone marrow samples used in the validation experiments were prepared according to the following protocol: 100mg of blank bone marrow was placed in a test tube and spiked with different volumes of the MIX solution for final concentrations of 100 to 2000 ng g^{-1} famprofazone, amphetamine and methamphetamine followed by vortex homogenization. The concentration of the internal standard in these samples was 500 ng g^{-1}. For LOD and LOQ, two additional concentrations were prepared at 25 and 50 ng g^{-1}.

2.5.1. Selectivity

The presence of interfering peaks in the elution region of the analytes and of the internal standard was evaluated by analysing twenty blank bone marrow samples. Blank matrices were also fortified with both analytes at 250 ng g^{-1} and with the internal standard at 500 ng g^{-1} and were analysed.

2.5.2. Linearity

Blank bone marrow was spiked at seven different concentration levels with five replicates for each level (100, 250, 500, 1000, 1250, 1500 and 2000 ng g^{-1}). Five curves were prepared on different days. All replicates were extracted and analysed as described previously. Linear regression analysis was performed on the peak area ratios of the analyte to the internal standard versus the analyte concentrations. The limits of acceptability for the linearity were as follows: a normal distribution of residues, data homoscedasticity, the coefficient of determination (r^2) should equal or exceed 0.990, and the deviation of the calculated from the theoretical values should not exceed 20%.

2.5.3. Precision

Precision, expressed as the relative standard deviation (% RSD), was studied at three concentration levels, 300 ng g^{-1} (low), 900 ng g^{-1} (medium) and 1800 ng g^{-1} (high), for the target analytes. The maximum acceptable RSD was 20% at each level. The levels were evaluated using four replicates over five

different runs. Within-run precision was evaluated for each level of the five runs. Between-run precision was assessed for each level over the five runs. Both within-run and between-run precisions were calculated using the one-way ANOVA approach, with the run number as the grouping variable, to verify if experiments performed on different days were statistically equivalent.

2.5.4. Accuracy

Accuracy was assessed using four replicates of fortified bone marrow at the same concentration levels used to evaluate precision (see item 2.4.3) over five different runs. Accuracy was measured as the relative percentage deviation (% error) from the nominal concentration at each concentration level. The maximum acceptable accuracy was ± 20% at each level.

2.5.5. Limit of detection (LOD)

The LOD was estimated using four different blank bone marrows fortified at 25, 50 and 100 ng $g^{-1}$of target analytes. The analyses were performed in duplicate for each level over four runs. LOD was defined as the lowest concentration that produces an identifiable peak with a signal-to-noise ratio greater than or equal to three times the background signal from the blank matrix (S/N > 3).

2.5.6. Limit of quantification (LOQ)

The LOQ was determined as the lowest concentration of the analytes that could be quantified with a %RSD ≤ 20%, an accuracy (%error) of 20% of the spiked value and a signal-to-noise ratio > 10 for all of the diagnostic ions. Blank bone marrow samples (triplicate) were fortified at three different concentrations (50, 100 and 150 ng g^{-1}) over four runs. The ion of greater intensity for each analyte was used for quantification.

2.5.7. Carryover

Carryover was evaluated by injecting a blank bone marrow immediately after a high concentration fortified sample (3000 ng g^{-1}) to verify if the injection

of positive samples would interfere with subsequent analyses. This procedure was realized using triplicates of fortified samples and blank matrices.

2.5.8. Stability

Stability tests were carried out by injecting replicate samples (n = 4) at low (300 ng g^{-1}) and high (1800 ng g^{-1}) concentration levels over a period of 48 hours. The samples were prepared in four sets. A set was analysed to establish time zero responses, and the remaining sets were analysed as described below.

2.5.8.1. Stability – Freeze/Thaw

Stability of the spiked samples was determined after three freeze and thaw cycles. The remaining three sets were frozen at -20 °C for 24 hours. This step was followed by an unassisted thaw at room temperature, and the first set of samples was analysed. The other samples were refrozen for 12 to 24 hours under the same conditions, and the freezer/thaw cycle and analysis were repeated two more times. The analysis results were compared with time zero and the analytes were considered stable according the method's acceptable accuracy (± 20%).

2.5.8.2. Stability – Processed sample

Spiked samples were prepared and aliquoted in accordance with the previous description. Stability at room temperature (autosampler stability) and after freezing were evaluated. In the first group of sets, the samples were analysed at different time intervals (12, 24 and 36 hours). The second group of sets were frozen, and each set was analysed after the corresponding time interval (12, 24 or 36 hours). The results are expressed as a relative percentage deviation (%error) and values smaller than 20% were considered acceptable.

2.6. Application to authentic samples

The validated method was applied to real samples obtained as described in section 2.2.2, and prepared/analysed in accordance with that specified in sections 2.3 and 2.4. Each sample was analysed in duplicate.

3. Results and discussion

Because of its metabolic conversion to methamphetamine and amphetamine, famprofazone can result in a positive drug-test [9]. Famprofazone's lipophilic characteristics (logarithmic octanol-water partition-coefficient, XLogP3 AA = 5) [19] should confer good affinity for the bone marrow. Certain lipophilic compounds have been analysed in the bone marrow, such as benzodiazepines [8,15], antidepressants [6,8] and opioids [4,14], but no studies involving famprofazone detection in this matrix were found. Previous studies of amphetamines in bone marrow and bone have been reported: one was a controlled study with rabbits involving the intravenous administration of methamphetamine [20], and the other was a methamphetamine abuser homicide case [21].

Considering the large heterogeneity of bone marrow, new studies about the detection of substances in this matrix are relevant. In this work, a new method of bone marrow preparation was proposed. A full validation of the method and the determination of famprofazone and its metabolite methamphetamine in this matrix were performed.

3.1. Choice of samples and sample preparation

The literature reports that different bones have different concentrations of stored drugs [6]. The scapula (shoulder blade), vertebra and rib meet this study's criteria and were chosen due to their high blood supply [6].

3.1.1. Bone cleaning and fragmentation

Connective and soft tissues were completely removed, and bones were fully cleaned according to a previous study [16]. Enzymatic bone cleaning was a simple step and was performed in a shorter time compared to techniques that

involve the use of water maceration or chemical products [22,23]. Normally, bones are fragmented by crushing and/or pulverization in grinder [8], which can be labourious. In this work, an excellent cutting performance and ease of effective decontamination were obtained, by using an electric micro-motor in association with a stainless-steel spatula for the fragmentation of bones and the removal of the bone marrow.

3.1.2. Bone marrow homogenization and clean-up

Based on an earlier study [16], homogenization was performed in an acidic medium (3 $molL^{-1}$ HCl). In this instance, the quantity of homogenate to be processed was reduced. Amphetamines are basic drugs; at acidic pH, they remained in their ionized forms, are soluble in the aqueous phase and thus have lower affinity for the lipophilic solvent used subsequently for the clean-up step.

Clean-up is an important pretreatment step. Interfering substances (i.e., lipids) can affect the chromatographic baseline, hindering detection of the analytes. Diethyl ether and n-Hexane are recommended in the literature for lipid extraction [24]. The areas of the chromatographic peaks obtained after clean-up with one of these solvents (2.3.3) were compared (Table 1).

Application of Student's t-test for six replicates (critical t =2.228; α=0.05) resulted in t values that were higher than the critical value for all concentration levels of amphetamine and for two levels of methamphetamine (600 and 1200 ng g^{-1}) and famprofazone (150 and 1200 ng g^{-1}), showing that the mean values were different. Therefore, hexane was chosen as clean-up solvent.

3.1.3. Choice of extraction solvent

This evaluation was performed in two steps. Initially, diethyl ether, used in a previous study [16], was compared with MTBE. The mean peak areas obtained according to 2.3.4 (Table 2) were compared by application of Student's t-test for six replicates(critical t =2.228;α=0.05). The t values obtained for the three analytes at all concentration levels (t between 3.29 and 20.02)

were higher than critical t, except for famprofazone at 600 ng g^{-1} (t=0.74), showing that the mean values were different, resulting in the choice of MTBE.

Next, MTBE was compared to 1-chlorobutane (1-CB) and ethyl acetate (EA) (Table 3). Guo and collaborators [17] tested 1-CB and EA for extraction of amphetamine and methamphetamine from the blood and observed a better performance of 1-CB. The results in Table 3 indicate a higher efficiency for MTBE. One-factor ANOVA confirmed that the mean peak areas were significantly different among solvents (F between 84 and 2414 >> critical F=3.098, α=0,05) and Tukey's test ($p<0.05$) proved that the three solvents were different from each other at all concentration levels. MTBE was chosen as the extraction solvent.

3.2. Determination of famprofazone and its metabolites in bone marrow

Drug concentrations found in the bone marrow are low because this matrix is not as extensively vascularized as organs (liver and kidneys), and the drug administration and/or metabolism of each substance is different. To detect famprofazone and its metabolites in bone marrow, the pH was adjusted to 12, changing these analytes to their non-ionized form, enabling liquid-liquid extraction with MTBE. Based on a study developed by Guo et al. [17], the amphetamines were analysed without evaporation or derivatization. Fig. 1 shows the chromatograms obtained after the fortification of bone marrow with amphetamine, methamphetamine and famprofazone at final concentrations of 250ng g^{-1} and the subsequent analysis of the matrix by the following experimental procedures: generation of bone marrow homogenate (section 2.3.2), clean-up (section 2.3.3) and liquid-liquid extraction (section 2.3.4).

3.3. Validation parameters

The levels used for validation were based on previous studies [25,26].

3.3.1. Selectivity

Blank bone marrow samples evaluated for endogenous interference (n=20) did not reveal the presence of any interfering compounds co-eluting with the analytes (Figs. 1 and 2). The clean-up step performed before liquid-liquid extraction improved the signal/noise ratio (low background), increasing the sensitivity. In addition, no interference was observed for blank bone marrow either with added internal standard at 500 ng g^{-1} (Fig. 2 A, B and C) or spiked with 250 ng g^{-1} of each analyte (Fig. 1 A, B and C).

3.3.2. Linearity

Linearity is the ability of a method to provide results that are directly proportional to the concentration of the analytes of interest within a working range. Shapiro-Wilk test showed that the data were normal for all analytes with p-values (AM= 0,3953; MA= 0,2144; FP= 0,1423) greater than 0.05 and W values (AM= 0,9682; MA= 0,9591; FP= 0,9532) higher than $W\alpha$ (0.05; 35) = 0.934. For all analytes, the Cochran test presented C values (AM= 0,3915; MA= 0,2896; FP= 0,3526) lower than the critical C value = 0.4310, which confirmed the data homoscedasticity. Grubbs test was performed for all samples, and no aberrant values were detected in the data sets; calculated G values were lower (maximum value calculated was 1,6880) than the Grubbs' critical value (α = 0.05) = 1.71.

The method was found to be linear from 100 to 2000 ng g^{-1} for the analytes. Previous studies in bone marrow presented mean levels on the order of ng g^{-1} for benzodiazepines [8, 26], antidepressants [13,26], benzoilecgonine and opioids [13]. The coefficients of determination (r^2) were > 0.990 for all calibration curves (Table 3). The lower end of the working range was limited by the value of the LOQ, which was 100 ng g^{-1} for all analytes (LOD = 50 ng g^{-1}). This result is comparable to the LOQ obtained by Nakao et al. [27] using SPE-LC-MS/MS for detection of methamphetamine and amphetamine in bone and bone marrow (50 ng g^{-1}), and lower than the one obtained by Santos et al. [16] using LLE, derivatization and GC-MS for detection of fenproporex and amphetamine in bone marrow (5 ng mg^{-1}). The present method has the advantage of being executed in a shorter time than the studies mentioned above.

3.3.3. Precision

Both the between-run and within-run precision (%RSD) were < 5% at all concentration levels for famprofazone and its metabolites (Table 4). These results are < 20%, which are in accordance with the SWGTOX guidelines [18] and are satisfactory due to the complexity of the matrix and are comparable to the precision obtained in other studies using bone marrow for forensic purposes [26,28].

3.3.4. Accuracy

The accuracy assays showed an average error below 5% at all levels (Table 3) in accordance with the SWGTOX guidelines [18], which recommend that the accuracy not exceed 20% at each level.

3.3.5. Carryover

Carryover tests report the contamination of a sample containing analytes of interest to a subsequently analysed sample. In this study, the test was performed with blank bone marrow injected immediately after a sample fortified at 3000 ng g^{-1}. Even after the injection of all spiked samples, the subsequent analysis of the blank bone marrow showed no carryover under the conditions tested.

3.3.6. Stability

Unexpected events can occur during instrumental analyses that prevent samples which have undergone routine preparation from being immediately analysed. Analytes and samples should be stable during the whole analytical procedure [18]. Thus, it is important to evaluate how much time a processed sample can be maintained while promoting reliable analyte detection, identification, or quantitation before it undergoes unacceptable changes. Stability experiments were performed in terms of the area ratio between the analytes and the internal standard. Considering 20% to be the maximum acceptable error [18], the processed samples were stable for at least 36 hours at room temperature and when frozen, with %error values smaller than 20%.

After three freeze/thaw cycles, all of the samples remained stable with %error values smaller than 15%. (Table 5).

3.4. Application to authentic samples

The LLE-GC-MS method was applied to the quantitative analysis of bone marrow samples collected from pigs, which received either 100 mg (pig 602) or 200 mg (pig 721) of famprofazone for five days. Famprofazone was detected in different bone marrow samples (scapula, vertebra and rib) and in variable concentrations (Table 6). These results are in accordance with a previous study, which reports that different bones have different concentrations of stored drugs [6]. On the other hand, methamphetamine was only quantified in samples collected from pig 721. Amphetamine was not quantified in any of the analysed authentic samples, which can be explained by the metabolic profile of famprofazone, where a low percentage of amphetamine (below 4%) was reported [10,29]. Methamphetamine presents higher lipophilicity than amphetamine, which may also explain its higher bone marrow levels. Compounds with lipophilic properties were reported to accumulate in bone marrow [30]. Chromatograms of an authentic sample are presented in Fig. 3.

4. Conclusions

An analytical method for the simultaneous quantification of famprofazone, methamphetamine and amphetamine in bone marrow is presented. Fragmentation of the bone with an electric micro-motor was fast and efficient, allowing the withdrawal of bone marrow without losses or contamination. Hexane exhibited a better performance as a clean-up solvent for the homogenate obtained by acidic digestion. Extraction with MTBE was more efficient than with diethyl ether, 1-chlorobutane or ethyl acetate. GC-MS was performed without the need for derivatization of the analytes. The method was fully validated and enabled the extraction, identification and quantification of famprofazone and its metabolites, methamphetamine and amphetamine, in bone marrow. Samples from pigs treated with famprofazone for five days were

analysed, and it was possible to detect and quantify famprofazone and one metabolite, methamphetamine.

This method represents a useful and reliable tool for application in the forensic analysis of human tissues when conventional matrices are not available and an alternative matrix is required.

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Table 1: Comparison of clean up solvents. Mean values of the analytes chromatographic areas after the cleaning step. Bone marrow samples with addition of analytes at three concentration levels (150, 600 and 1200 ng g^{-1}); six replicates per level. Analyses performed by GC-MS.

Analyte	**Theoretical concentration (ng g^{-1})**	**Clean up solvent**	
		HX	EE
		Mean analyte area ± SD	
AM	150	11606 ± 573	9455 ± 976
	600	38351 ± 829	34487 ± 2309
	1200	67801 ± 874	62458 ± 1961
MA	150	28403 ± 1269	26569 ± 2400
	600	165909 ± 9056	143111 ± 3414
	1200	282689 ± 8945	191291 ± 10668
FP	150	3651 ± 97	3346 ± 22
	600	11603 ± 1025	11351 ± 791
	1200	22218 ± 1124	18944 ± 1039

HX: n-hexane; EE: diethyl ether; AM: amphetamine; MA: methamphetamine; FP: famprofazone; SD: standard deviation.

Table 2: Comparison of extraction solvents. Mean values of the analytes chromatographic areas after the extraction step. Bone marrow samples with addition of analytes at three concentration levels (150, 600 and 1200 ng g^{-1}); six replicates per level. Analyses performed by GC-MS.

Analyte	Theoretical concentration (ng g^{-1})	Extraction solvent	
		MTBE	EE
		Mean analyte area ± SD	
AM	150	11914 ± 736	10035 ± 468
	600	38239 ± 1124	33335 ± 2014
	1200	68492 ± 772	63836 ± 1935
MA	150	29288 ± 1014	23889 ± 1122
	600	159791 ± 1466	140100 ± 1639
	1200	286024 ± 6893	227634 ± 13713
FP	150	3891 ± 215	3363 ± 123
	600	11313 ± 901	10989 ± 368
	1200	22075 ± 1132	20117 ± 699

MTBE: methyl tert-butyl ether; EE: diethyl ether; AM: amphetamine; MA: methamphetamine; FP: famprofazone; SD: standard deviation.

Table 3: Comparison of extraction solvents. Mean values of the analytes chromatographic areas after the extraction step. Bone marrow samples with addition of analytes at three concentration levels (150, 600 and 1200 ng g^{-1}); six replicates per level. Analyses performed by GC-MS.

Analyte	Theoretical concentration (ng g^{-1})	Extraction solvent		
		MTBE	1-CB	EA
		Mean analyte area ± SD		
AM	150	11121 ± 576	9709 ± 232	2912 ± 398
	600	37750 ± 921	11234 ± 858	5337 ± 191
	1200	67979 ± 971	44100 ± 2030	8358 ± 212
MA	150	29627 ± 814	21220 ± 3870	9515 ± 233
	600	159410 ± 1872	67343 ± 5605	13268 ± 157
	1200	235577 ± 2417	188509 ± 27098	21115 ± 627
FP	150	3648 ± 75	2206 ± 27	1052 ± 151
	600	11432 ± 717	5426 ± 286	2259 ± 167
	1200	21831 ± 1577	8719 ±397	3454 ± 195

MTBE: methyl tert-butyl ether; 1-CB:1-chlorobutane; EA: ethyl acetate; AM: amphetamine; MA: methamphetamine; FP: famprofazone; SD: standard deviation.

Table 4: Summary of validation results: calibration curves, accuracy, recovery and precision at low, medium and high levels.

	Analyte		
	Famprofazone	**Amphetamine**	**Methamphetamine**
Straight-line equation	y= 0.009x + 0.462	0.029x + 1.408	y= 0.024x - 0,511
r^2	0.996	0.998	0.998
LOD (ng g^{-1})	50	50	50
LOQ (ng g^{-1})	100	100	100
Low level (300 ng g^{-1})			
Accuracy (% error)	-1.8	2.0	-4.3
Within-run precision (% RSD)	3.5	4.6	3.9
Between-run precision (% RSD)	2.3	3.7	3.4
Medium level (900 ng g^{-1})			
Accuracy (% error)	2.0	-1.3	1.6
Within-run precision (% RSD)	3.1	2.5	3.0
Between-run precision (% RSD)	2.6	1.8	2.0
High level (1800 ng g^{-1})			
Accuracy (% error)	1.2	-2.3	1.7
Within-run precision (% RSD)	2.4	3.3	2.5
Between-run precision (% RSD)	1.3	2.5	1.8

r^2: Coefficient of determination; RSD: Relative Standard Deviation

Table 5: Accuracy data for stability tests under different conditions in low and high concentration levels (n =4). CV (%) are shown in brackets.

	Analyte		
	Famprofazone	**Amphetamine**	**Methamphetamine**
LOW LEVEL (300 ng g^{-1})			
freeze/thaw accuracy			
Time zero(% error)	-0.1 [0.8]	-2.7 [3.1]	-14.9 [4.5]
24 hours (% error)	3.7 [3.7]	6.3 [6.6]	3.7 [5.7]
36 hours (% error)	-0.9 [6.2]	0.2 [8.5]	-2.0 [2.5]
48 hours (% error)	-0.7 [8.8]	9.0 [5.2]	-2.5 [2.6]

room temperature accuracy			
Time zero(% error)	0.9 [2.6]	-2.9 [3.2]	-9.2 [3.0]
12 hours (% error)	-1.1 [1.9]	0.8 [3.8]	-7.7 [7.6]
24 hours (% error)	1.0 [1.5]	-2.1 [7.2]	-4.5 [3.2]
36 hours (% error)	-10.2 [3.6]	-18.0 [6.3]	-15.5 [4.1]
freezer (-30 ºC) accuracy			
Time zero(% error)	1.2 [2.3]	4.3 [4.9]	-6.4 [0.9]
12 hours (% error)	0.4 [2.3]	-5.9 [8.6]	-5.3 [6.8]
24 hours (% error)	0.2 [1.7]	-2.9 [5.4]	-8.5 [1.3]
36 hours (% error)	-0.5 [4.6]	0.6 [1.9]	-12.3 [9.4]
HIGH LEVEL (1800 ng g^{-1})			
freeze/thaw accuracy			
Time zero(% error)	0.5 [1.8]	4.1 [3.0]	-4.3 [3.9]
24 hours (% error)	0.1 [3.0]	0.9 [5.9]	-2.2 [2.5]
36 hours (% error)	-0.6 [2.2]	1.2 [4.4]	-2.3 [1.5]
48 hours (% error)	-1.1 [5.9]	1,0 [2.8]	-3.0 [5.9]
room temperature accuracy			
Time zero(% error)	-3.5 [4.0]	-0.5 [6,4]	-7.8 [2.5]
12 hours (% error)	0.3 [4.1]	4.0 [4.4]	0.2 [2.5]
24 hours (% error)	8.8 [3.7]	4.1 [5.9]	2.5 [1.9]
36 hours (% error)	-10.4 [1.7]	-10.5 [1.2]	-10.3 [1.5]
freezer (-30 ºC) accuracy			
Time zero(% error)	0.4 [0.4]	4.1 [3.0]	-2.1 [1.1]
12 hours (% error)	0.5 [2.2]	6.2 [4.3]	-0.2 [3.4]
24 hours (% error)	0.7 [1.6]	1.8 [7.7]	-0.4 [3.2]
36 hours (% error)	-0.4 [2.4]	-1.9 [6.7]	8.2 [4.4]

Table 6: Analysis of authentic bone marrow samples collected from pigs treated with famprofazone.

	Analyte (ng g^{-1})		
Sample	**Famprofazone**	**Amphetamine**	**Methamphetamine**
Pig 602			
Rib	152	nd	<LOQ
Scapula	105	nd	<LOQ
Vertebra	103	nd	<LOQ
Pig 721			
Rib	232	<LOQ	267

Scapula	134	<LOQ	192
Vertebra	139	<LOQ	174

nd: not detected; <LOQ: below the limit of quantification; Pig 602 was treated daily with 100 mg de famprofazone; Pig 721 was treated daily with 200 mg de famprofazone.

Figure Captions

Fig. 1: Evaluation of spiked bone marrow sample: A- TIC of bone marrow spiked with famprofazone, methamphetamine and amphetamine at 250 ng g^{-1} and with added IS at 500 ng g^{-1}; B- SIM for amphetamine (AM; *m/z* 44, 91; retention time 4.046 minutes), methamphetamine (MA; *m/z* 58, 91; retention time 4.285 minutes) and internal standards – amphetamine-D5 (AM-D5; *m/z* 48, 92; retention time 4.033 minutes), methamphetamine-D5 (MA-D5; *m/z* 62, 92; retention time 4.274 minutes); C- SIM for famprofazone (FP; *m/z* 286, 229, 91; retention time 12.133 minutes).

Fig. 2: Chromatograms for evaluation of bone marrow samples: A- TIC of blank bone marrow spiked with added IS at 500 ng g^{-1}; B- SIM chromatogram of amphetamine-D5 (*m/z* 48, 92; retention time 4.033 minutes), methamphetamine-D5 (*m/z* 62, 92; retention time 4.274 minutes); C- SIM chromatogram of detection window of famprofazone. None analyte was detected at the corresponding retention time.

Fig. 3: Evaluation of authentic rib bone marrow from pigs treated with famprofazone: A- Total ion chromatogram; B- amphetamine (*m/z* 44 and 91; retention time: 4.046 min) and methamphetamine (m/z 58 and 91; RT: 4.285 min); C- famprofazone (*m/z* 286, 229 and 91; retention time: 12.133 min). IS: amphetamine-D5 (*m/z* 48 and 92; RT: 4.033 min) and methamphetamine-D5 (*m/z* 62 and 92; retention time: 4.274 min).

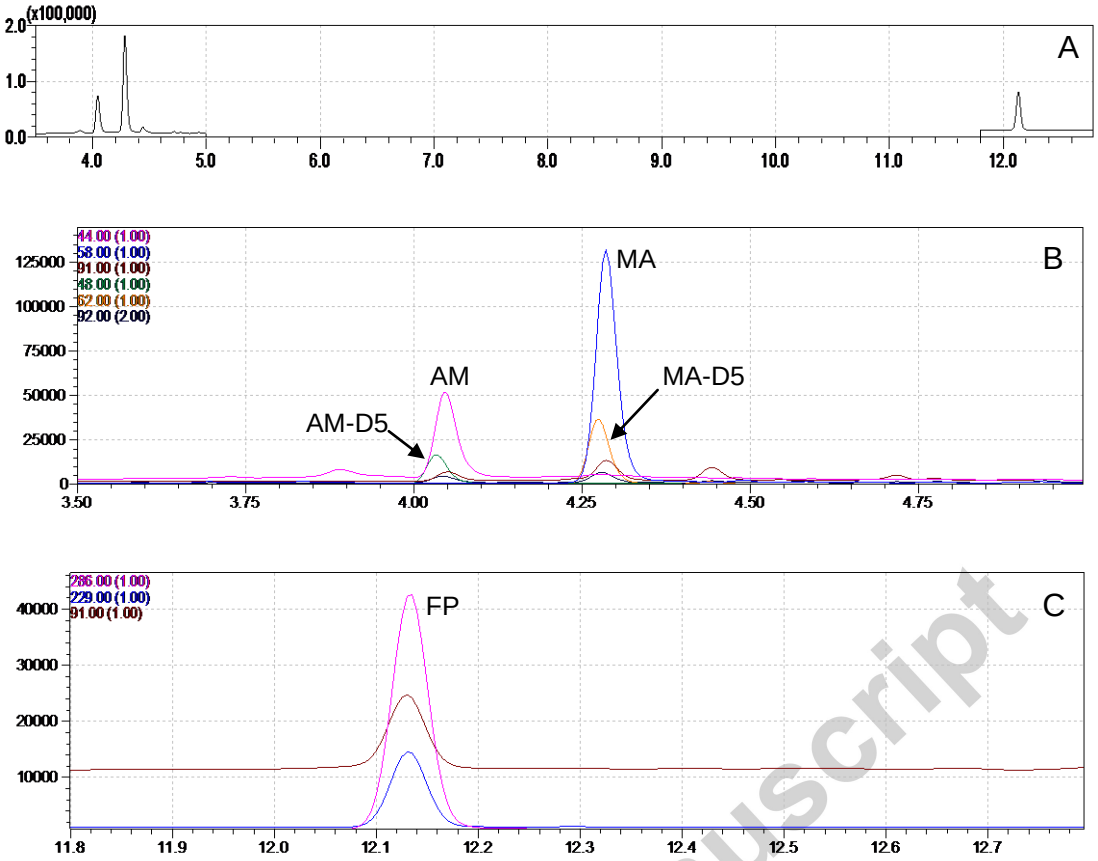
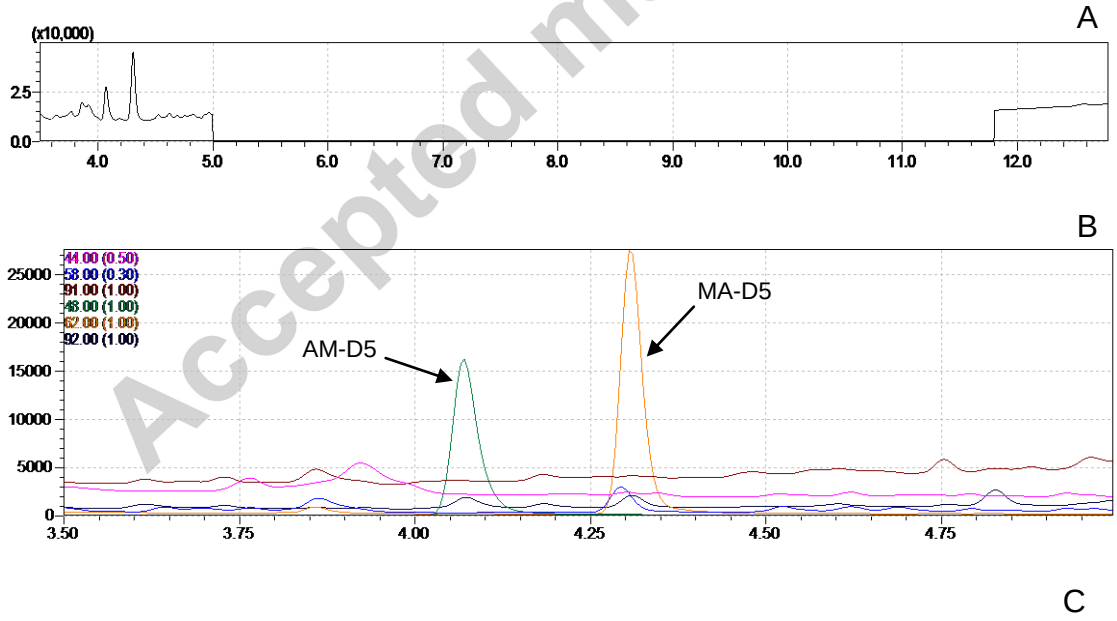


Figure 1



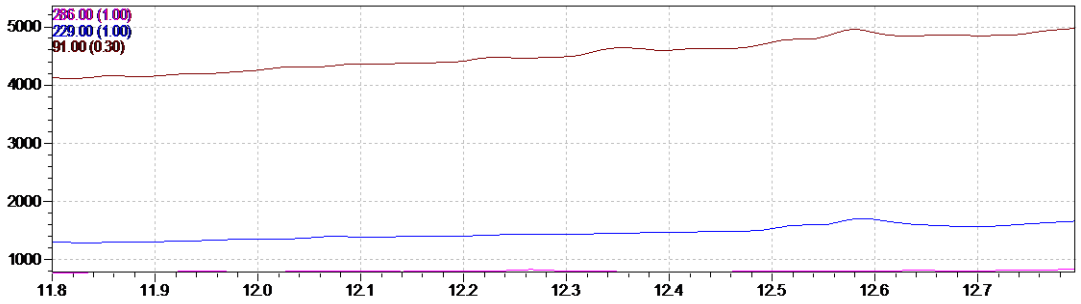


Figure 2

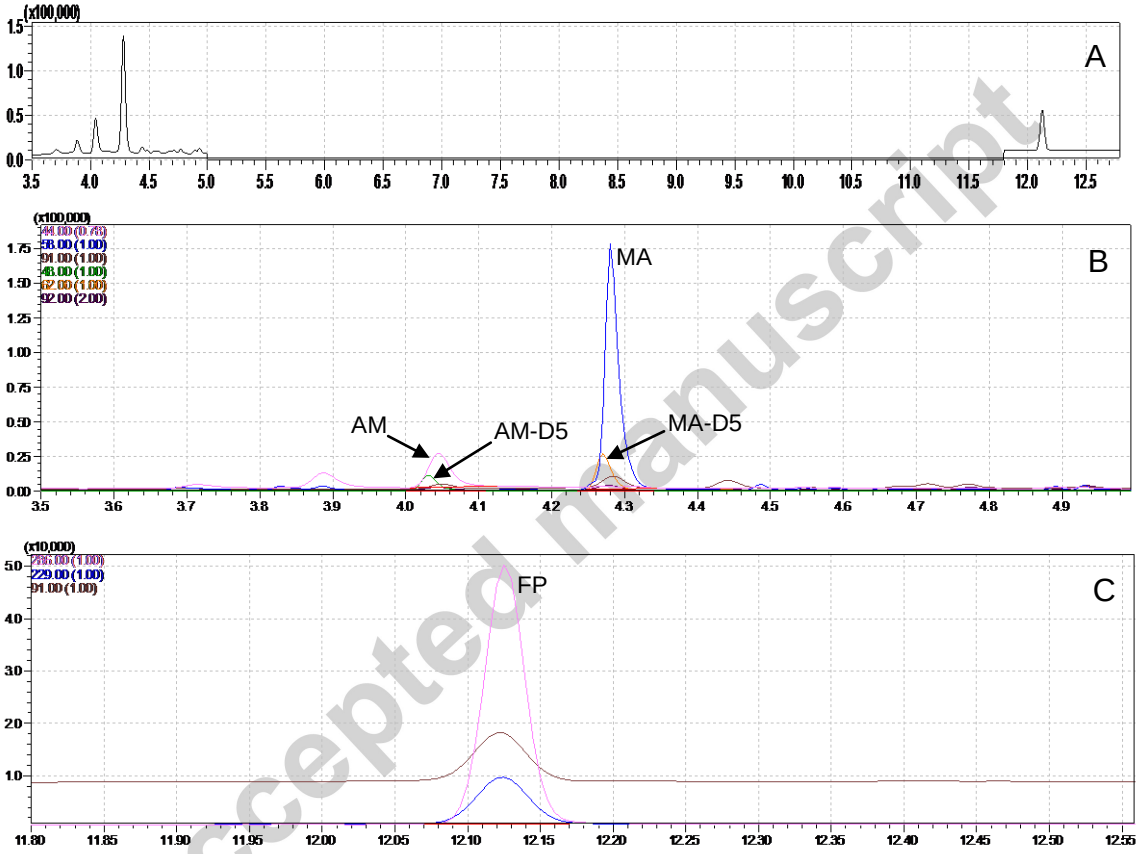


Figure 3

HIGHLIGHTS:

- A new method to analyse amphetamines in bone marrow is presented.
- The pretreatment showed be a very efficient method for analyzing amphetamines.
- Amphetamines were analysed by GC-MS without the evaporation or derivatization steps.
- The analytical method presented good selectivity, linearity, accuracy and precision.
- Processed samples remained stable at room temperature and refrigerated.