Technical Note

Solid-Phase Extraction and Gas Chromatographic–Mass Spectrometric Determination of the Veterinary Drug Xylazine in Human Blood

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Abstract

This paper presents a method for the determination of xylazine in whole blood using solid-phase extraction and gas chromatography–mass spectrometry. This technique required only 0.5 mL of sample, and protriptyline was used as internal standard (IS). Limits of detection and quantitation (LOQ) were 2 and 10 ng/mL, respectively. The method was found to be linear between the LOQ and 3.50 µg/mL, with correlation coefficients higher than 0.9922. Precision (intra- and interday) and accuracy were in conformity with the criteria normally accepted in bioanalytical method validation. The analyte was stable in the matrix for at least 18 h at room temperature and for at least three freeze/thaw cycles. Mean recovery, calculated at three concentration levels, was 87%. To the best of our knowledge, this is the first time that solid-phase extraction is used as sample preparation technique for the determination of this compound in biological media. Because of its simplicity and speed when compared to other extraction techniques, the herein described method can be successfully applied in the diagnosis of intoxications by xylazine.

Introduction

Xylazine is an alpha-2 adrenergic agonist developed in the 1960s (1). It is widely used in veterinary practice as a sedative, analgesic, muscle relaxant, or general anesthetic for large animals, such as deer, ruminants, and horses (2–4). Its use in humans as sedative-hypnotic/analgesic/anesthetic drug was also investigated, but rejected because of its frequent association with severe hypotension (5).

This compound is chemically and pharmacologically similar to clonidine, an antihypertensive agent (6), and acts by stimulation of alpha-2 receptors in the central and peripheral nervous system (4). Consequently, inhibition effects on the brain stem vasomotor center occur, which results in bradycardia and transient hypertension, followed by sustained hypotension (6). Xylazine has also peripheral autonomic effects, reducing the centrally mediated sympathetic tone by interneural blockade of norepinephrine release, resulting in bradycardia, hypotension, and reduced cardiac output (7). More recently, it has also been found that this compound has affinity for cholinergic, serotonergic, dopaminergic, alpha-1 adrenergic, H_2-histaminergic, and possibly opiate receptors (5,8).

Xylazine can be administered intravenously, intramuscularly, subcutaneously (in cats), or orally, often in combination with other anesthetic agents (e.g., barbiturates, chloral hydrate, halothane, and ketamine) (4,9). It distributes rapidly after intravenous administration, primarily to the central nervous system and kidneys (10), being metabolized in the liver and up to 70% of the dose is excreted in the urine in dogs (8,9).

Various antagonists have been used for the treatment of intoxication. Tolazoline may be administered in unresponsive bradycardia and hypotension, but has been associated with hypertension, arrhythmias, and tachycardia (8). Yohimbine, another alpha-2 antagonist, has been shown to antagonize the sedative effects of xylazine in humans (7,11). Atropine has been used and may reverse bradycardia and hypotension in humans (10), and naloxone was administered without effect (12).

Several methods for the detection of xylazine in plasma, urine, and other biological fluids using liquid–liquid extraction (5,8,13,14) have been published. Recently, a method using protein precipitation for the determination of this compound in serum of different animal species was described (15). However, there are no published data on the use of solid-phase extraction (SPE) to determine xylazine in biological samples.

This paper describes a new analytical procedure using SPE and gas chromatography–mass spectrometry (GC–MS) for the determination of xylazine in human whole blood, utilizing

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only 0.5 mL of sample. This reduced sampling volume can be very useful when several exams are needed and the amount of sample available is small. An anecdotal case (a unique reported intoxication with this compound in Portugal) is presented to illustrate the method's applicability, and the obtained results are discussed in the light of previous publications on the matter.

Case History

A 44-year-old male was admitted to the hospital after an accidental subcutaneous self-injection of a 20 mg/mL solution of xylazine, a drug commonly used in veterinary practice as a sedative and anesthetic. He had diabetes mellitus and had been medicated subcutaneously. The man, a veterinarian helper, was chasing an ox, the loaded gun fired accidentally, and the anesthetic dart hit him in his right foot. When he arrived at the hospital, he was sleepy and complained about pain in his foot. Physical examination revealed an arterial pressure of 167/56 mm Hg, and a heart rate of 80 bpm, with rhythmic cardiac auscultation. Respiratory rate was 22 breaths/min, and pulmonary auscultation was normal.

No other abnormal features were observed at examination. The blood values and arterial blood gases were in the normal range. Other laboratory findings, including complete blood count and electrolyte panel, were normal except for hyperglycemia. Chest X-ray was normal. The electrocardiogram showed sinus bradycardia at 60 bpm. He received IV fluids, atropine (SOS), enoxiparin, flucloxacilin, and insulin. After 30 h under hospital observation, the patient was clinically asymptomatic with normal blood pressure and heart rate and was discharged without other treatment.

A blood sample was sent to the Laboratory of Forensic Toxicology (Delegation of Coimbra, National Institute of Legal Medicine, Portugal) and stored light-protected at –20°C until analysis.

Materials and Methods

Chemicals, reagents, and material

Analytical standards, xylazine and protriptyline hydrochloride (internal standard, IS), were purchased from Sigma Aldrich Quimica S.A (Sintra, Portugal). Methanol (HPLC grade) was supplied by Merck (Darmstadt, Germany). Water was purified by a Milli-Q system obtained from Millipore (Molsheim, France). Oasis® HLB (3 mL, 60 mg) extraction cartridges were obtained from Waters (Milford, MA), and a Vac-Elut SPS 24 system coupled to a vacuum pump (AKNF) were purchased form Varian (Harbor City, CA).

Instrumentation

Analysis was performed on a Hewlett-Packard (Waldbronn, Germany) 6890 GC equipped with a mass-selective detector (MSD). The capillary column was from J&W Scientific (Folsom, CA) and packed with 5% phenylmethylsiloxane (12 m * 0.25-mm i.d., 0.25-µm film thickness). The oven temperature began at 120°C for 1 min, increased by 20°C/min to 270°C, holding for 7 min. Total analysis time was 14.5 min. The temperatures of the injection port and detector were 200 and 280°C, respectively. The split injection (1:5) mode was used, and helium at a flow rate of 1 mL/min was used as the carrier gas. The MS was operated with a filament current of 300 µA and an electron energy of 70 eV in the electron ionization (EI) mode.

Quantitation was carried out in the selected ion monitoring (SIM) mode, and ions were monitored at *m/z* 205, 220, and 177 for xylazine and at *m/z* 191, 70, and 165 for protriptyline (quantitation ions are underlined).

The retention times were 5.91 and 7.23 min for xylazine and the IS, respectively, obtaining a good separation of both compounds.

Extraction procedure

A 25-µL aliquot of the IS solution (10 µg/mL) was added to 500 µL of sample. The sample was diluted with 2 mL of deionized water, vortex mixed, and centrifuged at 3000 rpm for 5 min. The supernatant was added to the extraction cartridges, which had been previously conditioned with 2 mL of methanol and 2 mL of deionized water. The interferences were washed with 2 mL of a 5% methanolic solution in deionized water, and the columns were dried under full vacuum for 15 min.

The analytes were eluted with 2 mL of methanol, and the extract was subsequently evaporated to dryness at 40°C under a gentle N_2 stream and reconstituted with 50 µL of methanol. The extracts were transferred to autosampler vials for analysis, and a 1-µL aliquot was injected into the system.

Results and Discussion

The method's selectivity was checked by analyzing 10 blank whole blood samples of different origin. The obtained chro-

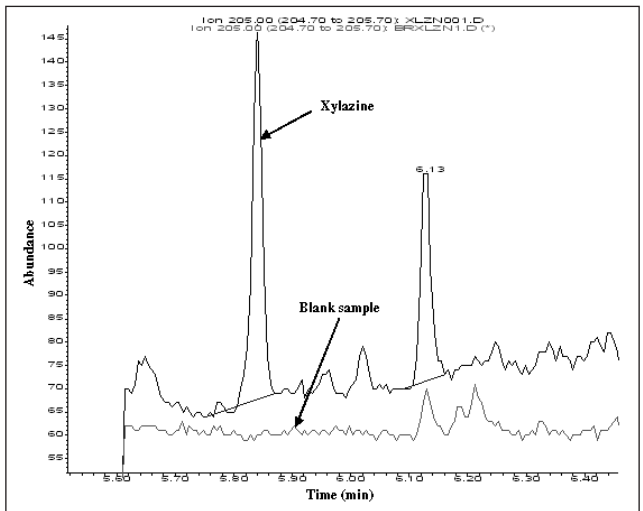

Figure 1. Merged fragmentograms (ion 205) of a blood sample spiked with xylazine at 0.01 µg/mL and a blank sample.

matograms were compared with those obtained by analysis of spiked samples. No interferences were observed at the retention times and selected ions for xylazine and IS (Figure 1).

Calibration curves were established using spiked blood samples prepared and analyzed using the described procedure between 0.01 and 3.50 µg/mL (12 calibrators evenly distributed), in two linear ranges. The r^2 values were higher than 0.9922. The calibrator's accuracy was within ± 15% of the nominal concentration [± 20% at the lower limit of quantitation (LOQ)] for all concentration levels and was considered acceptable according to the FDA's and ICH's guidelines for bioanalytical method validation (16,17). Calibration data are shown in Table I. The limit of detection (LOD) was defined as the lowest tested xylazine concentration yielding a signal-to-noise ratio higher than 3 and was 2 ng/mL. The LOQ was defined as the lowest xylazine concentration that could be measured with adequate precision [coefficient of variation (CV) of 20 % or less] and accuracy (measured concentration within ± 20% of the true value), and was found to be 10 ng/mL.

Intraday precision and accuracy were evaluated by same-day analysis of six replicates of blank blood samples spiked with xylazine at the LOQ, 0.025, 0.25, and 2.50 µg/mL. The obtained CVs were less than 9.22%, with an accuracy of 14.39% or better. Likewise, interday precision and accuracy were determined at the same concentration levels, with six replicates, over a period of 10 days. The CVs were no greater than 15% (20% at LOQ), with an accuracy of 1.50% or better. Precision and accuracy data are summarized in Table II. For recovery studies, six replicates of three intermediate concentrations (0.025, 0.25, and 2.50 µg/mL), in which the internal standard was only added after the extraction procedure, were analyzed by the described method. The obtained peak area ratios were compared with those obtained by spiking blank extracts with the same amounts of both compounds (100% recovery). The method's mean recovery was 87.2% (Table II).

Both short-term temperature stability and freeze and thaw stability of xylazine were evaluated accordingly to the criteria of FDA (16).

Short-term stability was evaluated at 0.01 and 2.50 µg/mL (n = 3). Blood samples were spiked and left at room temperature for 18 h. These samples were analyzed and the obtained results were compared to those obtained from samples prepared and analyzed on the same day; the CVs were inferior to 13.49% with an accuracy of 8.71% or better.

Freeze and thaw stability was also evaluated in triplicate at the same concentration levels. Blood samples were spiked with xylazine, and these aliquots were stored at –20°C for 24 h, after which they were thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 12 h under the same conditions. This freeze/thaw cycle was repeated twice more, and the samples were analysed after the third cycle. These samples were compared to samples prepared and analyzed in the same day, and the analytes were stable for at least three freeze/thaw cycles (the measured concentrations did not deviate more than 3.97% from the nominal concentration, with a precision of less than 5.85%).

The described method was applied to the described case, and the concentration of xylazine was found to be 1.50 µg/mL. The time between intake and blood collection is not known, and a urine sample was not provided, so one cannot conclude about the distribution of the drug in the victim's body fluids. According to published information (2), the doses of xylazine known to be toxic in humans vary from 40 to 2400 mg; however, in this case, it was not possible to know the accidentally self-administered dos because the medical report from the hospital did not include it.

The observed symptoms in our case are consistent to other published symptoms (7) and are in accordance with the pharmacological properties of the compound. The obtained xylazine concentration is higher than that reported by Stillwell (14) in a case of an impaired driver and that reported by Poklis (13) in a fatal situation. The disposition of xylazine in humans

Table I. Linearity Data

Calibration Range (µg/mL)	Linearity (n = 6)		
	Slope*	Intercept*	R^2 values*
0.010–0.25	1.8909 ± 0.2089	0.0037 ± 0.0008	0.9997 ± 0.0035
0.25–3.50	1.8228 ± 0.1050	0.0679 ± 0.0111	0.9922 ± 0.0020

* Mean values and standard deviations.

Table II. Precision and Accuracy for Xylazine in Blood

Concentration (µg/mL)	Recovery* (%)	Concentration (µg/mL)	Intraday Precision (n = 6)			Interday Precision (n = 6)		
			Concentration mean (µg/mL)	CV† (%)	Bias‡ (%)	Concentration mean (µg/mL)	CV† (%)	Bias‡ (%)
0.025	83.8 ± 9.14	0.010	0.011	7.77	14.39	0.010	19.78	–1.50
		0.025	0.027	7.08	7.31	0.026	14.95	2.19
0.25	83.7 ± 3.43	0.25	0.23	9.22	–7.57	0.25	10.29	–0.48
2.50	94.0 ± 5.44	2.50	2.46	3.66	–1.65	2.51	12.03	0.50

* Mean values and standard deviations.
† CV, coefficient of variation.
‡ Bias = [(measured concentration – nominal concentration)/nominal concentration] * 100.

is not reported, but it is likely that it undergoes extensive biotransformation (18). Concerning postmortem distribution of the compound, there are several tissue distribution reports (Table III).

To our knowledge, this is the first reported xylazine intoxication in Portugal. The typical chromatogram of the presented case is shown in Figure 2.

Human intoxications with this compound are a rare event. Nevertheless, this substance has gained popularity as drug of abuse, concomitantly with other veterinary anesthetics (19–21).

The first case of xylazine toxicity in a human was reported by Carruthers et al. in 1979 (22). The patient was a 34-year-old male who had been self-medicating for insomnia with intramuscular injection of about 1 g of xylazine. Several reports on the intoxication with this compound have been published ever since, which are summarized in Table III.

Concerning the extraction technique, the use of SPE for the determination of xylazine is not described in the literature. SPE is in general simpler than a multi-step liquid–liquid extraction (8,13,14), and provides cleaner extracts, which allows for the determination of low amounts of the analyte. Indeed, we present lower limits than those published elsewhere (8,13), despite using a smaller sample volume. On the other hand, SPE is easier to automate, providing greater sample throughput.

Conclusions

SPE coupled to GC–MS proved to be analytically suitable for the determination of xylazine in whole blood samples. It offers

Table III. Literature Review of Xylazine Intoxications

Reference	Age/Gender*	Usage†	Route‡	Dose (mg)	Toxicological Results/Analytical Technique	Observations
Carruthers et al., 1979 (22)	34 M	S	I.M.	1000	–	Non-fatal
Gallanosa et al., 1981 (10)	20 F	S	O	400	plasma: negative, urine: positive; GC	Non-fatal
Lewis et al., 1983 (23)	39 F	A	I.M.	–	serum (0.03 µg/mL), urine (1.7 µg/mL)	Fatal
Poklis et al., 1985 (13)	36 M	S	I.V.	–	blood (0.2 µg/mL), urine (7.0 µg/mL), brain (0.05 mg/kg), kidney (0.6 mg/kg), liver (0.9 mg/kg), lung (1.1 mg/kg), adipose (0.05 mg/kg); GC–NPD	Fatal
	29 F	–	I.M.	40 (0.73 mg/kg)	–	Non-fatal
Spoerke et al., 1986 (12)	29 F	–	I.V.	–	–	Non-fatal
	37 F	S	I.M.	2400 (22 mg/kg)	–	Non-fatal
Samanta et al., 1990 (24)	19 M	S	S.C.	200	–	Non fatal
Briellman et al., 1994 (25)	59 F	S	–	–	blood (16 µg/mL), urine (30 µg/mL), stomach (large amounts); GC–NPD	Fatal
Mittleman et al., 1998 (2)	23 F	H	–	–	liver (42 mg/kg); brain (19 mg/kg); kidney (28 mg/kg) GC–MS	Fatal
	33 M	H	–	–	liver (0.26 mg/kg), brain (0.16 mg/kg), kidney (0.15 mg/kg); GC–MS	Fatal
Capraro et al., 2001 (26)	16 M	–	I	–	blood (0.54 µg/mL); GC	Non-fatal
Moore et al., 2003 (5)	42 M	S	–	–	heart blood (2.3 µg/mL), peripheral blood (2.9 µg/mL), bile (6.3 µg/mL), urine (0.01 µg/mL), liver (6.1 mg/kg); kidney (7.8 mg/kg); GC–NPD	Fatal
Stillwell, 2003 (14)	23 M	I	I.M.	450	blood (0.57 µg/mL) GC–MS	Non-fatal
Elejalde et al., 2003 (19)	18 M	I	I	–	–	Non-fatal
Hoffman et al., 2004 (8)	27 M	S	I.M.	1500 (13 mg/kg)	serum (4.6 µg/mL); urine (194 µg/mL); gastric contents (446 µg/mL); HPLC–UV	Non-fatal
Arican et al., 2004 (20)	36 M	I	–	1500	–	Fatal

* Abbreviations: M, male and F, female.
† Abbreviations: A, accidental intoxication; S, suicide attempt; H, homicide; and I, intentional.
‡ Abbreviations: I.M., intramuscular; S.C., subcutaneous; I.V., intravenous; O, oral; and I, inhalation.

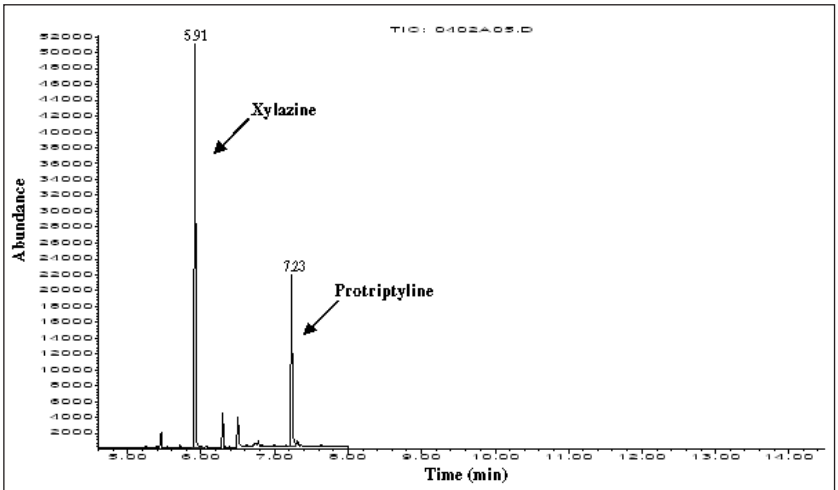

Figure 2. SIM chromatogram of the presented case.

time reduction and small sample volumes, simplicity and sensitivity for the quantitative determination of low concentrations of xylazine.

This validated methodology is appropriate for application in clinical and forensic toxicology laboratories for the determination of this compound in situations where it is involved.

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