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Measurement of ketamine and xylazine in rat brain by liquid-liquid extraction and gas chromatography-mass spectrometry

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(“How To” article)

Abstract

Introduction: In human and veterinary medicine, the injectable drugs ketamine and xylazine are mainly used in combination to induce, and then maintain general anaesthesia; they also provide pain and stress relief. Some side-effects have been reported on the auditory brainstem response, a method is therefore required to determine their concentrations in the brain.

Methods: This paper presents a method to determine nanogramme quantities of ketamine and xylazine in rat brain using liquid-liquid extraction and gas chromatography-mass spectrometry in selective ion monitoring mode. The technique requires only 0.5 g of sample, and uses xylazine d6 as an internal standard.

Results: The method was linear between 0.86 and 34.4 µg/g of brain. Limits of quantification were 378 and 87 ng (approximately 0.76 and 0.17 µg/g of brain) for ketamine and xylazine, respectively. The reliability of the method in terms of accuracy, within-day and between-day precision was also demonstrated. For xylazine, bias and intra-day precision were good (< 3.0%), as was between-day precision (< 10.5%); the equivalent values for ketamine were 7%, 11.1% and 20.9%, respectively. Stability of the analytes in the matrix at -80 °C was assessed over five months; both compounds were found to be stable for at least 1 month, even at very low concentrations. The procedure was successfully applied to determine (for the first time) the *in vivo* brain levels of both drugs in animals following systemic administration.

Discussion: The procedure will be useful in future studies of the side-effects of these drugs, and their interactions with other compounds.

Keywords: brain, ketamine, xylazine, GC/MS

Introduction

Ketamine (KET) is a cyclohexamine derivative with N-methyl-D-aspartate receptor antagonistic properties. Xylazine (XYL) is a thiazine derivative, which is an $\alpha 2$ adrenergic agonist. Both substances are injectable drugs used mainly in combination to induce and then maintain general anaesthesia in humans and animals. These drugs can also be used for sedation and analgesia (Stokes, et al., 2009).

KET and XYL are mainly metabolized by liver cytochrome P450 enzymes and their metabolic by-products are excreted in urine. Both drugs are rapidly absorbed and distributed to the central nervous system; in young rats, they have a reported half-life of approximately 1.3 hours (Veilleux-Lemieux, et al., 2013).

A mixture of KET/XYL can adversely modify the latency of the auditory brainstem response wave (Lima, et al., 2012). Recently, we demonstrated that toluene - an organic solvent widely used in industry - could counterbalance the inhibitory effects of anaesthesia on the amplitude of the middle-ear reflex in a dose-dependent manner (Campo, et al., 2013). These findings allowed us to demonstrate a competitive phenomenon when this solvent and the two anaesthetic agents are applied together. Ongoing research is investigating the origin of this competition: is it receptor specific or due to modifications of the fluidity of the phospholipid membrane?

To answer this question, it could be relevant to determine the relative proportions of solvent and anaesthetics in the brain. To our knowledge, the only methods describing the determination of these anaesthetic agents focused on blood or plasma concentrations of each drug separately, or, rarely, together (Barroso, et al., 2007; Li, et al., 2012; Lian, et al., 2012; Miksa, et al., 2005; Niedorf, et al., 2003; Rogstad & Yndestad, 1981; Veilleux-Lemieux, et al., 2012). Thus, no assay to determine cerebral KET and XYL concentrations has been reported in the literature.

The purpose of the present study, performed using Brown-Norway rats, was to develop and validate a simple method to measure the cerebral concentrations of KET and XYL after intraperitoneal administration of a mixture of the two drugs. The method is principally based on liquid-liquid extraction of brain homogenates followed by gas chromatography – mass spectrometry (GC/MS) analysis.

Experimental

Reagents

Xylazine hydrochloride ($\geq 99\%$) (XYL), xylazine-d6 (VETRANAL™, analytical standard) (XYLd6 = IS, internal standard), phosphate-buffered saline solution (pH 7.4. at 25 °C) (PBS), sodium hydroxide solution (1.0 M) (NaOH) and trichloroacetic acid solution (6.1 N) were all purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Chloroform (ACS. ISO. Reag. PhEur. $\geq 99.8\%$) was obtained from Merck-Millipore (Fontenay sous Bois, France). Ultrapure water (Type I) was produced by a Milli-Q water purification system (Merck-Millipore). Ketamine hydrochloride solution (1 g/L) (KET) used during the experiments was a pharmaceutical formulation (Clorketam 1000) acquired from Vetoquinol (Magny-Vernois, Lure, France).

Preparation of stocks, standard solutions, and Quality Control Samples

Stock solutions of KET.HCl (500 mg/L), XYL.HCl (500 mg/L) and XYLd6 (50 mg/L) were prepared in ultrapure water and stored at 4 °C. A 500 mg/L XYL.HCl (or KET.HCl) solution correspond approximately to a free base concentration of 430 mg/L. Standard solutions (free base) at 172, 86, 43, 21.5 and 4.3 mg/L for KET and XYL were prepared from these stock solutions by serial dilution with ultrapure water. Fresh standard solutions were prepared each week and stored at 4 °C prior to use.

To test recovery yield, standard mix solutions in chloroform at 86, 43, 21.5, 10.75 and 2.15 mg/L for KET and XYL and 25 mg/L for XYLd6 were prepared by serial dilution from neutralized KET (215 mg/L), XYL (215 mg/L) and XYLd6 (125 mg/L) stock solutions in chloroform. These solutions were stable for up to 1 month at 4 °C.

Samples for the calibration curves and quality controls (QCs) were prepared by adding 100 μ L of each standard to 500 μ L blank brain homogenate, yielding calibration spike standard quantities of 17.2, 8.6, 4.3, 2.15 and 0.43 μ g. These quantities corresponded to concentrations of 34.4, 17.2, 8.6, 4.3 and 0.86 μ g/g of brain. The lowest (0.86 μ g/g), medium (8.6 μ g/g) and highest (34.4 μ g/g) concentrations were also used for QC experiments. Samples of spiked brain homogenate (standards and QCs) were extracted as part of each analytical batch along with the samples of unknown concentration.

Animal experiment and sample collection

5 Brown-Norway rats (males weighing 270 ± 10 g) were used for the validation study. Animal investigations were performed in a French Ministry of Agriculture-accredited animal facility (Accreditation N° C 54-547-10) in line with the ethical regulations related to animal experimentation (European Directive 2010/63/EU). The present experiment series was approved by the local ethics committee.

Before exposure, 12-week-old Brown-Norway rats purchased from Janvier Labs (St Berthevin, France) were acclimatized to the experimental laboratory for 4 weeks with a 12:12 h night/day cycle. Temperature was maintained at 22 ± 2 °C with a relative humidity of $55 \pm 10\%$. Animals had free access to food and water.

Deep anaesthesia was induced with a single intraperitoneal injection of a mixture of ketamine and xylazine (45/5 mg/kg). Anaesthesia was subsequently maintained with a continuous intraperitoneal injection of a diluted mixture of ketamine and xylazine (33/3.7 mg/kg) for around 2 hours. At the end of the experiment, all rat brains were removed, quartered and rapidly frozen in nitrogen before storage at -80 °C.

Sample preparation

Each frozen brain quarter (~ 0.5 g) was placed in a 2-mL lysing matrix tube containing 1.4 mm ceramic spheres (FastPrep lysing matrix D from MP Biomedicals) with 500 µL of PBS solution (pH 7.4) and 100 µL IS (50 mg/L in water). The sample was homogenized in the FastPrep instrument for 30 seconds at a speed setting of 6.0. Proteins were precipitated by adding 10 µL of trichloroacetic acid (20%), the solution was vigorously mixed before centrifuging at 8000 g at 4 °C for 10 min. The supernatant was transferred to 2-mL Eppendorf tubes. After neutralising the samples by adding 40 µL of NaOH (1 M), they were extracted with 3 × 1 mL chloroform. For each extraction, the mixture was agitated for 5 min before centrifuging at 1000 g for 1 min, 975 µL of the lower organic layer was then removed with a Hamilton syringe. The three organic layers were combined and evaporated to dryness under nitrogen using a dry block heater set to 60 °C. The residue was reconstituted in 200 µL of chloroform in preparation for GC/MS analysis.

GCMS conditions

The GC analyses were developed and validated using a Shimadzu QP2010 Ultra GCMS equipped with a split/splitless injector and an AOC20i autosampler. GCMS Solution software (version 4.11 SU1) was used for data acquisition and to control the GC system. Samples were separated on a SLB-IL61 capillary column (30 m × 0.25 mm, film thickness 0.20 mm) (Supelco, France) with helium as the carrier gas at a constant linear velocity of 45 cm/s. The sample (1 µL) was injected in split mode with a split ratio of 1/30. The oven temperature was held at 250 °C for 8 min, then increased to 280 °C at a rate of 10 °C/min and maintained at 280 °C for 3 min (total time, 14 min). In these conditions, retention times for KET, XYL and XYLd6 were 3.83, 5.70 and 5.65 min, respectively. The temperatures for the injection port, transfer line and ion source were set to 250 °C, 280 °C and 200 °C, respectively.

The MS was operated by electron ionization (70 eV) in selected ion monitoring (SIM) mode. The following ions were chosen for SIM analyses (ions used for quantification are underlined): KT: 209. 180; XYL: 220. 205; XYL-d6: 226. 208.

Method validation

The analytical procedure was fully validated over a 5-day period, including linearity check, determination of limits of detection (LOD) and quantification (LOQ), estimation of recovery, accuracy and precision tests. The selectivity of the method and the stability of analytes over time in a fortified matrix were also checked.

Calibration samples were prepared in blank brain homogenate in four replicates, as described above. All samples were analyzed in duplicate. Calibration curves were established by linear least squares regression, using Statgraphics Centurion XVI software (version 16.2.04 – 32 bits) (Sigma Plus, Levallois-Perret, France), by plotting relative response (analyte/IS, in area) as a function of spiked analyte concentration (in μg). Assay linearity was tested by analysis of variance, and the significance of the intercept and slope were determined by t test. The validity of the linear calibration curves (lack-of-fit test) was tested using the Fisher–Snedecor F test ($p > 0.05$).

LODs and LOQs were calculated based on the standard deviation (σ_b) of the response and the slope (a) of the calibration curves reduced to the three lowest levels. LODs and LOQs were defined as $3.3 \sigma_b/a \times q_{IS}$ and $10 \sigma_b/a \times q_{IS}$, respectively, where q_{IS} is the amount of IS (in μg) initially added to the sample.

Bias (accuracy) and precision studies were performed concurrently using four separate QCs per concentration at low, medium and high concentrations over five different runs. Within-run and between-run precision was assessed using a one-way ANOVA test using the varied factor

(run number) as the grouping variable. ANOVA was calculated using Statgraphics Centurion XVI software. Relative standard deviation (%RSD) was used as an indicator of precision.

Matrix effects were assessed by comparing pure standard solution with standard extracted from blank brain homogenates. Recoveries were calculated at the three different QC levels by calculating the ratio between the peak areas for drugs measured after the complete procedure and the corresponding standards in chloroform.

The stability of KET and XYL in samples at $-80\text{ }^{\circ}\text{C}$ was investigated at three time points (at 1, 2 and 5 months) at low ($4.3\text{ }\mu\text{g/g}$ of brain) and high ($34.4\text{ }\mu\text{g/g}$ of brain) concentrations ($n = 3$ per condition).

Results and discussion

Method validation

Selectivity of the method was assessed by comparing the chromatograms acquired for the mixtures of standard solutions in chloroform and for samples extracted from blank brain samples with those from spiked brain homogenates and real samples (Fig. 2). Under the conditions indicated, no interfering peak derived from endogenous matrix components, or impurities in the solvents used was detected which would hamper detection of the KET, XYL and IS.

Calibration parameters, LODs and LOQs are summarized in Table 1. The method appeared to be linear for both anaesthetics over the full range of concentrations investigated (from 0.86 to 34.4 $\mu\text{g/g}$ of brain). In addition to coefficients of correlation (R^2) indicating excellent data fitting (≥ 0.995), p values for the lack-of-fit tests (> 0.05) demonstrated that the linear model adequately fit the data at a statistical significance of 95 %. These linear regressions were used to determine the LOD and LOQ for each analyte. For KET and XYL, respectively, LODs of 126 and 29 ng were found, along with LOQs of 378 and 87 ng (corresponding approximately to 0.76 and 0.17 $\mu\text{g/g}$ of brain). The LOQs for our method are higher than those obtained in blood with methods to simultaneously determine XYL and KET (between 5 – 100 and 5 – 10 ng/mL for XYL and KET, respectively) (Li, et al., 2012; Miksa, et al., 2005; Niedorf, et al., 2003; Veilleux-Lemieux, et al., 2012). This lower sensitivity may be due to low extraction efficiencies in brain. Indeed, in our case recoveries never exceeded 35%, with a mean recovery of around 27% (Table 2). Alternative extraction solvents, such as dichloromethane, diethylether, n-hexane, methyl ter-butyl ether have been successfully used for KET and/or XYL extraction from blood (Li, et al., 2012; Niedorf, et al., 2003; Rogstad & Yndestad, 1981). With our brain samples, these solvents did not significantly improve total recovery

over that achieved with chloroform (data not shown). Nevertheless, the procedure in the absence of brain gave satisfactory recoveries equal to $63.0 \pm 3.8\%$, $66.4 \pm 4.4\%$ and 66.4 ± 8.0 for XYL, XYLd6 and KET, respectively (knowing that the only drying-down step already reduced the yields to $94 \pm 10\%$, $91 \pm 10\%$, $78 \pm 9\%$ for XYL, XYL-d6 and KET). Perhaps improvements could be made to the protocol but it has not been investigated so far since the performance of the method was sufficient for our needs. Indeed, accuracy and precision tests gave very good results for XYL, with bias and intra-day precision below 3.0%, and inter-day precision below 10.5%. For KET, bias never exceeded 7% (at the lowest spiking level), and intra-day precision was good, with RSD values between 7.5 and 11.1. Inter-day precision was acceptable with an RSD < 20.9% at the lowest concentration (which was close to the LOQ determined).

The stability of the analytes in brain homogenates was studied at $-80\text{ }^{\circ}\text{C}$ over 5 months (Table 3). No loss of analyte was observed for the samples spiked with $34.4\text{ }\mu\text{g/g}$ of brain over the whole 5 months. With the samples spiked with $4.3\text{ }\mu\text{g/g}$ of brain, XYL was stable for at least 2 months, while losses were observed in the 2nd month for KET. As a consequence, for low-concentration samples, it is advisable to perform analyses within 1 month.

Application to real samples

The method was successfully used to determine brain concentrations of KET and XYL in five brains collected during the study from animals to whom the mixture of drugs had been administered intraperitoneally (Table 4). Mean KET and XYL concentrations were 19.6 and $7.3\text{ }\mu\text{g/g}$ of brain, respectively; the higher values noted for rats 4 and 5 were probably due to additional intra-muscular injections required during the experiments.

Conclusion

This is the first study describing simultaneous determination of the cerebral concentrations of ketamine and xylazine using GC/MS. The sample preparation procedure is simple, requiring no more than 0.5 g of brain. The method showed good specificity, accuracy and precision and an adequate limit of quantification for drug monitoring after systemic administration. This method will be useful in our studies to determine how solvents interact with anaesthetics in the brain.

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Declaration of interest

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Table 1. Linear calibration curve parameters, LOD and LOQ

Compound	Intercept ($\pm$ SE)	Slope ($\pm$ SE)	R ²	Lack-of-fit test (P value)	LOD (ng)	LOQ (ng)	LOQ (μ g/g of brain)
KET	-0.0019 ($\pm$ 0.0061)	0.1602 ($\pm$ 0.0030)	0.9950	0.5283	126	378	0.76
XYL	-0.0047 ($\pm$ 0.0107)	1.2171 ($\pm$ 0.0056)	0.9994	0.7752	29	87	0.17

SE: standard error

Table 2. Recovery, accuracy (bias), within-day and between-day precision for quality control samples

Compound	Nominal amount spiked (μg)	Recovery* (%)	Bias (n = 4) (%)	Within-day precision (n = 4) (% RSD)	Between-day precision (n = 5) (% RSD)
KET	0.43	23.9 $\pm$ 8.6	-7.0	11.1	20.9
	4.3	27.7 $\pm$ 7.0	-0.3	9.2	19.1
	17.2	31.8 $\pm$ 4.1	0.1	7.5	16.1
XYL	0.43	23.3 $\pm$ 4.5	2.9	3.0	10.5
	4.3	28.0 $\pm$ 4.8	0.2	2.5	8.8
	17.2	26.1 $\pm$ 3.4	-0.2	2.5	7.6
IS	5.0	26.6 $\pm$ 4.9	n.d.	13.7*	23.2*

* calculated from the peak area; n.d. = not determined

Table 3. Stability of KET and XYL over time in brain homogenates stored at - 80 °C (n = 3)

Compound	Spiking concentration ($\mu\text{g/g}$ brain)	% after 1 month	% after 2 months	% after 5 months
KET	4.3	103.2 ± 4.9	83.6 ± 6.8	82.7 ± 9.2
	34.4	111.6 ± 1.3	104.8 ± 6.7	100.0 ± 0.1
XYL	4.3	106.3 ± 1.0	99.0 ± 0.1	79.7 ± 16.7
	34.4	104.9 ± 0.2	106.4 ± 2.9	99.9 ± 0.1

Table 4. KET and XYL concentrations in the brains of rats after intraperitoneal administration (n = 5)

Rat number	[KET]	[XYL]
	($\mu\text{g/g brain} \pm \text{SD}$)	($\mu\text{g/g brain} \pm \text{SD}$)
1	15.75 ± 0.40	7.94 ± 0.10
2	15.51 ± 0.64	6.20 ± 0.32
3	13.30 ± 0.31	5.37 ± 0.21
4	26.62 ± 2.11	9.34 ± 0.14
5	26.78 ± 1.84	7.74 ± 0.23
Mean	19.60 ± 6.26	7.31 ± 1.47

Figure 1. Chemical structure of ketamine, xylazine and xylazine d6 (IS)

Figure 2. Typical individual mass ion chromatograms obtained with an extracted blank brain sample (a), with a spiked homogenate brain sample (KET and XYL at 34.4 $\mu\text{g/g}$ of brain; IS at 5 $\mu\text{g/g}$ of brain) and with a real sample (c).