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How to

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

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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.

1. Introduction

Ketamine (KET) is a cyclohexamine derivative with N-methyl-D-aspartate receptor antagonistic properties. Xylazine (XYL) is a thiazine derivative, which is an α2 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 h (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.

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2. Experimental

2.1. Reagents

Xylazine hydrochloride (≥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. ≥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-Vermois, Lure, France).

2.2. 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) (Fig. 1) 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 μL of each standard to 500 μL blank brain homogenate, yielding calibration spike standard quantities of 17.2, 8.6, 4.3, 2.15 and 0.43 μg. These quantities corresponded to concentrations of 34.4, 17.2, 8.6, 4.3 and 0.86 μg/g of brain. The lowest (0.86 μg/g), medium (8.6 μg/g) and highest (34.4 μg/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.

2.3. 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 ± 10%. Animals had free access to food and water.

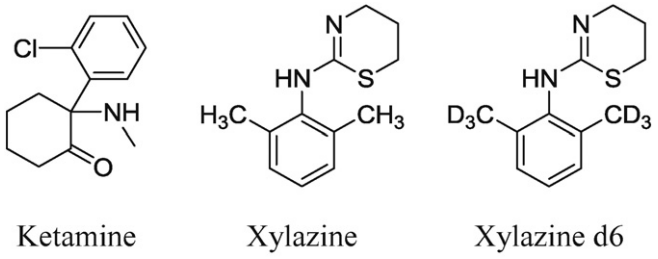

Fig. 1. Chemical structure of ketamine, xylazine and xylazine d6 (IS).

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 intra-peritoneal injection of a diluted mixture of ketamine and xylazine (33/3.7 mg/kg) for around 2 h. At the end of the experiment, all rat brains were removed, quartered and rapidly frozen in nitrogen before storage at −80 °C.

2.4. 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 s 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 neutralizing 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.

2.5. GC/MS conditions

The GC analyses were developed and validated using Shimadzu QP2010 Ultra GC/MS equipped with a split/splitless injector and an AOC20i autosampler. GC/MS 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.

2.6. 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 analysed 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 $3.3\ \sigma_b/a \times q_{IS}$