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J Pharmacol Toxicol 41 (1999) 91–95

Journal of Pharmacological and Toxicological Methods

Gas chromatography-mass spectrometry assay for determination of ketamine in brain

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Received January 7, 1999; revised and accepted May 7, 1999

Abstract

A sensitive and precise gas chromatography–mass spectrometry method with selected ion monitoring has been developed for determination of ketamine in the brain using chlorpheniramine as an internal standard. The assay is based on the acid extraction of brain homogenate with hexane and ethyl ether with subsequent alkaline ethyl ether extraction. The analytical procedure has a coefficient of variation of 3.0–5.3% and from 3.8 to 6.1% for extraction from water or spiked brain samples, respectively. The lowest detectable level of ketamine was 1 ng in any brain region. This level of detection was used to measure the ketamine concentrations in cerebellum, brain stem, midbrain, hypothalamus, and cortex of C57B1/6 mice at awakening following intraperitoneal injection of a hypnotic dose. The ketamine concentrations in mouse brain were in the range from 41.6 to 48.6 ng/mg of tissue.

Keywords: Ketamine assay; Pharmacokinetic/pharmacodynamic; Gas chromatography/mass spectrometry; Mouse brain assay

1. Introduction

Ketamine (2-chlorphenyl)-2-(methylamino-cyclohexanone) is a commonly used short-acting anesthetic agent. The underlying mechanisms of ketamine-induced anesthesia are not completely understood as is true for many other general anesthetics. A major area of investigation in the mechanism of anesthetic action involves defining multiple molecular targets associated with these drugs (Franks & Lieb, 1994). One approach for identifying molecular pathways directly relevant to anesthetic action is to identify and isolate genes that can modulate anesthetic sensitivity. On the experimental level this approach may be realized through measurement of the duration of loss of righting reflex (LORR) and parallel measurement of the brain concentration of anesthetic drug at the time of regaining righting response in various inbred mouse strains. Demonstrating interstrain differences in LORR requires a concomitant knowledge of brain levels of the anesthetic agent at regaining of LORR which will help differentiate pharmacokinetic differences from pharmacodynamic differences. Thus it is well known that the long-sleep and short-sleep mice are differentially sensitive to various CNS depressants and exhibit different LORRs and brain levels at awakening to a number of agents (McClearn & Kakihana, 1981; DeFiebre et al., 1992; Marley et al., 1986; Markel et al., 1996). Obviously, we are interested in pharmacodynamic differences in our genetic models in order to isolate genes that mediate anesthetic action at the level of the CNS. Accurate estimates of such variable parameters require a sensitive and specific analytical assay. There are several analytical methods for determination of ketamine in brain, plasma, or urine, but these methods use a toxic solvent (Cohen et al., 1973), lack specificity (Stiller et al., 1982; Geisslinger et al., 1991) and are less sensitive (Chang & Glazko, 1972; Kochhar, 1977), than a method necessary for our purposes. Recently, the highly sensitive gas chromatography–mass spectrometry method for determination of ketamine in plasma has been developed (Feng et al., 1995). This method uses the direct extraction of ketamine from plasma that usually is not appropriate for brain samples and electron impact ionization mode in recording the mass spectrum which is not routinely used in the most scientific laboratories. We have now developed a sensitive and specific gas chromatography–mass spectrometry method with selected-ion monitoring for identification and quantitative measurement of ketamine in experimental samples of mouse brain.

2. Materials and Methods

2.1. Materials

HPLC grade n-hexane, methanol, acetonitrile, and analytical grade anhydrous ethyl ether were purchased from Fisher (Fair Lawn, NJ, USA). The 30% ammonium hydrox-

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1056-8719/99/$ – see front matter
PII: S1056-8719(99)00023-4

ide solution (Baker "analyzed" reagent) was purchased from J.T. Baker (Phillipburg, NJ, USA). Trichloroacetic acid (A.C.S. reagent) was from Aldrich (Milwaukee, WI, USA). The ketamine-HCl and S(+)-dexchlorpheniramine maleate used as an internal standard came from Research Biochemical Inc. (Wayland, MA, USA). High-performance liquid chromatography (HPLC) grade water was used to prepare all solutions.

2.1.1. Standard solutions

The stock solutions of ketamine (2 mg /mL) and internal standard (2 mg /mL) were prepared by solubilizing the appropriate amounts of ketamine and dexchlorpheniramine in methanol.

2.1.2. Gas chromatography–mass spectrometry

Hewlett Packard Model 5890 gas chromatograph was fitted with J&W Scientific (Folsom, CA, USA.) 30 cm × 0.25 mm interior diameter, silica capillary column (DB-1, 0.25 μm film thickness) directly coupled to the mass selective detector HP-5970 (Hewlett Packard Co., Avondale, PA, USA.). Data handling was performed by VECTOR /ONE Workstation (Teknivent Corp., St. Louis, MO, USA) and HP LaserJet printer. Ultrapure helium was used as carrier gas. The operating conditions were as follows: purge flow rate was 40 mL/min; injector temperature was 270°C; detector temperature was 310 °C. Column temperature was increased from 100 ° to 220 °C at 15 °C/min, then to 300 °C at 25°C/min and maintained 1 min. Electron ionization (EI) mode was used in recording the mass spectrum: detection ions of m/z 180 and 203 were selected for quantitation; for estimation of spectrum of the ionic fragments the masses were acquired—50–300; electron multiplier: 2200 v. Total run time was 14 min.

2.2. Animal experiments

Adult male C57B1/6 mice weighing 20–22 g were used. The animals were housed with free access to food and water in a temperature-controlled room with a temperature of 22°–24°C under a constant 12:12 h light–dark cycle (lights on 7:00). All behavioral experiments were carried out between 10:00 and 17:00.

Mice were injected intraperitoneally with 150 mg /kg of ketamine ("Ketalar", Parke-Davis & Co., Chicago, IL.). The testing procedure consisted of placing the mice on their backs in a V-shaped trough. Sleep time was recorded as the time period between loss and regaining of the righting response. The animals were considered to have regained the righting response after they righted themselves three times in 30 s. At the moment of regaining the righting response, mice were decapitated, brains were quickly removed and brain regions (cortex, midbrain, hypothalamus, cerebellum, and brain stem) were dissected. The brain regions were frozen on dry-ice and stored at −80°C until assayed. The tissue samples were weighted and homogenized in water using an ultrasonic cell disruptor (40% continuous power for 20 sec; Model 200, Branson). For cortex, the 10% (w/v) homogenate was prepared. The 5% (w /v) homogenate was prepared from midbrain, cerebellum, and brain stem. The 0.1 mL of homogenate was prepared for extraction. The hypothalamus was homogenized completely in 1 mL of water. The vial for homogenization was rinsed with an additional 1 mL of water, and both volumes were combined and used for extraction.

2.3. Extraction procedure

Next, 2 mL of the aqueous phase were added to 10-mL glass tubes with 100-μL internal standard (2 μg) and mixed by vortexing with 1 mL of 10% Trichloroacetic acid (TCA). This mixture was centrifuged at 3000 rpm for 10 min at 5°C (JPR, Beckman, USA). The pellets were washed with 3 mL 3.3% TCA and centrifuged again. Both acid supernatants were combined, transferred to another clean 25-mL glass tube and vortexed for 1 min with 5 mL of *n*-hexane. After centrifugation at 1500 rpm for 10 min, the organic layer was removed by aspiration and 5 mL of ethyl ether were added to acid water phase. After shaking and centrifugation, the organic layer was removed by aspiration. Then, 0.6 mL of 30% ammonium hydroxide were added to the approximately 6 mL of acid water phase. After shaking, 5 mL of the ethyl ether were added, and the mixture was vortexed for 1 min and centrifuged for 10 min at 1500 rpm. The organic supernatant was transferred to clean glass tubes. To the aqueous layer, 5 mL ethyl ether was added and the extraction step was repeated. The combined ether supernatants were evaporated to dryness, and the residue was redissolved in 50 μL of acetonitrile. One μl of extract was injected into the gas chromatography—mass spectrometry system. Ketamine concentrations in the unknown brain samples were determined from peak–area ratio of each sample by references to the calibration curve.

2.4. Calculations

Calibration curves were constructed by plotting the peak-area ratios (ketamine/internal standard) as a function of the ketamine concentration. These data were then fitted to equation for linear regression with Inplot software package. The ketamine concentrations of unknown samples were calculated using the results of the regression analyses.

3. Results

On the preliminary step the absolute recovery for several organic solvents and their mixture has been studied. Direct extraction with methylene chloride, ethyl ether, or ethylacetate from basic solutions gave almost 100% of recovery of ketamine but led quickly to peak broadening of the internal standard as result of coextraction of contaminants. After extraction with methylene chloride:hexane (3:1) and chloroform: hexane (3:1) the 35–40% of added ketamine was lost. In comparison with these variants the proposed multistep ether procedure

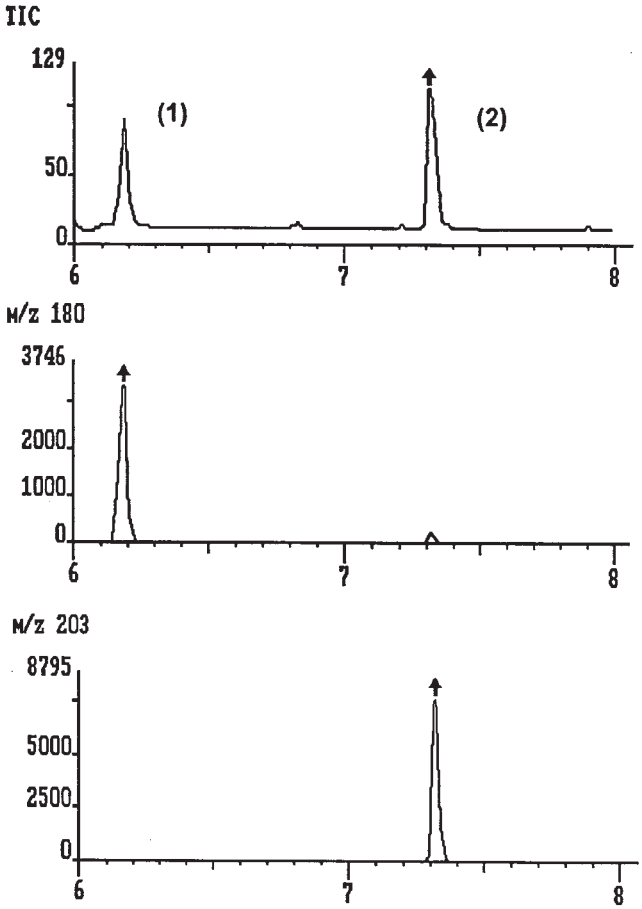

Fig. 1. Total ion current and selected ion monitoring chromatograms of a spiked brain sample. The data represent the results of extraction of ketamine and chlorpheniramine which have been added to the brain homogenate. Upper panel: Total ionic current (TIC). Middle panel: Selected ionic monitoring of fragment 180 m /z. Lower panel: Selected ionic monitoring of fragment 203 m /z. Peaks: 1 = ketamine (20 ng /μL); 2 = chlorpheniramine (40 ng/μL). Retention times are given in minutes.

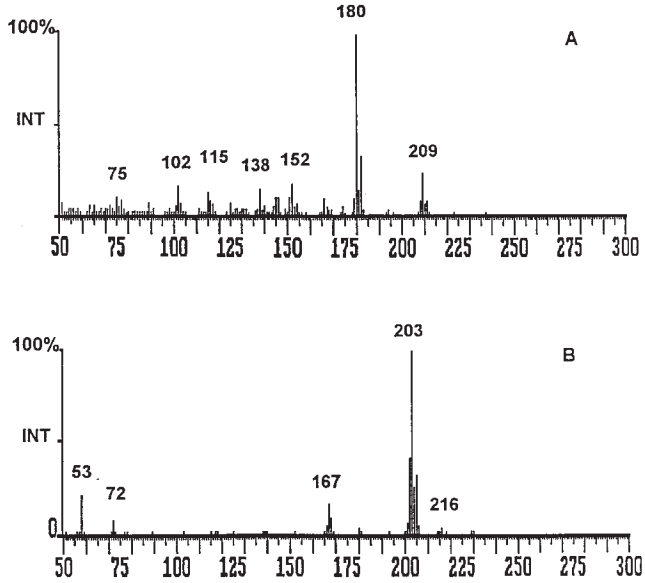

Fig. 2. Mass spectrum of ketamine and chlorpheniramine from a spiked brain sample. The data represent the results of extraction of ketamine and chlorpheniramine which have been added to the brain homogenate. A: Ketamine. B: Chlorpheniramine.

produced no visible residue after complete evaporation, gave very consistent extraction results, and resulted in high-percent recoveries of ketamine and internal standard.

Under chromatographic conditions described above, ketamine and internal standard were well separated with retention time 6.19 and 7.32 min, respectively (Fig. 1). There were no visible interfering peaks in the chromatograms of extracts from brain homogenate.

Under EI mode, no molecular ions of ketamine (m/z 238) and internal standard (m /z 275) were found (Fig. 2). The characteristic of EI mass fragmentation pattern was observed with major abundance corresponding to ionic fragment of ketamine at m/z 180 and ionic fragment of internal standard at m/z 203. Both these major ionic fragments were used for selective monitoring from 6 to 8 min. Chlorpheniramine gives a small fragment with mass 180 m /z that is seen as a small peak (Fig. 1) but this peak has a different retention time in the chromatogram and does not contribute to the quantification of ketamine.

Trichloroacetic acid was used for complete precipitation of proteins during the first phase of extraction of the brain samples. Theoretically, some percent of ketamine or internal standard could be lost due to protein binding. To check this possibility, we compared two different calibration curves. One of them was constructed after extraction from water samples with different concentrations of ketamine (10, 50, 100, 200, 500, 1000, 1500, and 2000 ng in extracted solution). The equation of the curve and its correlation coefficient were $y = 0.517\times - 0.004$ ($r = 0.9987$, $n = 8$, $p < 0.0001$). Another one was constructed after extraction of brain homogenate samples spiked with the same concentrations of ketamine. The equation of this curve and its correlation coefficient were $y = 0.533\times - 0.004$ ($r = 0.9982$, $n =$

Table 1
Day-to-day accuracy and precision for the determination of ketamine in spiked brain samples in comparison with extraction from water (Mean ± SEM)

Added amount of ketamine (ng/extraction)	Extraction from water (ng)	CV (%)	Extraction with brain samples (ng)	CV (%)
10	7.6 ± 0.4 $n = 5$	5.3	8.2 ± 0.5 $n = 4$	6.1
100	98.3 ± 4.9 $n = 5$	5.0	91.4 ± 4.8 $n = 6$	5.3
500	522.8 ± 23.0 $n = 6$	4.4	541.0 ± 24.4 $n = 6$	4.5
1000	1055.8 ± 31.2 $n = 12$	3.0	1021.4 ± 39.3 $n = 11$	3.8

n, number of experimental points; CV, coefficient of variation.

Table 2
The comparison of ketamine and internal standard recovery under extraction from water and spiked brain samples (Mean ± SEM)

Types of extraction	Without extraction (control)	Extraction of ketamine and internal standard (% of control)	Extraction of ketamine (% of control)	Extraction of internal standard (% of control)
From water	100 ± 3.87 $n = 5$	87.5 ± 1.52 $n = 5$	88.7 ± 2.91 $n = 5$	98.0 ± 4.70 $n = 5$
Spiked brain samples	100 ± 3.87 $n = 5$	83.4 ± 4.85 $n = 5$	84.2 ± 1.75 $n = 5$	97.8 ± 1.26 $n = 5$

In every experiment 1 μg of ketamine has been used. In the first column (without extraction), the 100 μL 10% ammonium hydroxide was added and after evaporating to dryness the residual was reconstituted in 50 μL of acetonitrile. The average value of ketamine was accepted for 100%.
n, number of experiments.

8, $p < 0.0001$). The slopes of both these equations were not significantly different.

Day-to-day accuracy and precision of the method were assessed by using different amount of brain samples spiked with four different concentrations of ketamine, the extraction of which was carried out on different days. The results are shown in Table 1. The precision was very good with the coefficients of variation between 3.0 and 5.3% for extraction from water and between 3.8 and 6.1% for extraction of spiked brain samples. The recovery of ketamine was about 88% under extraction from water and 84% under extraction with brain homogenate (Table 2).

This method has been applied in determination of ketamine concentrations at the time of regaining of righting response in different brain regions of C57B1 /6 inbred mice after i.p. injection of ketamine at dose of 150 mg /kg (Table 3). The concentrations of ketamine (ng per mg of tissue) were very close in different brain regions with values between 41.5 (midbrain) and 52.4 (hypothalamus). The average concentration of ketamine after awakening was 46.6 ng/mg of tissue and total amount of ketamine represented 0.54% ± 0.03% of injected dose. The individual correlation between concentrations of ketamine in different brian regions was very high with correlation coefficients between 0.913 and 0.958 (data not shown). Latent time for LORR was 2.99 ± 0.27 min, and the sleep time was 29.69 ± 1.62 min.

4. Discussion

The fragmentation pattern of ketamine completely coincides with results published earlier (Kochhar, 1977; Feng et al., 1995) with the major ionic fragment at m /z = 180. Chlorpheniramine has been used already as the internal standard (Feng et al., 1995), but unfortunately its fragmentation pattern has not been presented. Under our conditions the mass ion of chlorpheniramine of the maximum abundance represents the loss of a side $CH_2CH_2N(CH_3)_2$ group at m/z = 203. Taking into account the EI mode, which was used for fragmentation in our study, the absence of molecular ions for both compounds of interest is not surprising.

The minimum tested amount of ketamine was 10 ng. It means that after reconstitution in 50 μL of acetonitrile and injection of 1 μL into gas chromatograph we could detect 200 pg of ketamine. This is the quantification limit of our procedure. Theoretically, we can inject the 200 pg of ketamine in 2 μL of solution and reconstitute the residual after evaporation to dryness in as small an amount as 10 μL. This method allows recovery of about 1 ng of ketamine in any brain region. Another advantage of the assay is the use of relatively nontoxic solvents in comparison with benzene which was used in previous studies (Chang & Glazko, 1972).

The recovery of ketamine was similar after extraction from water and with brain homogenate. Both extraction methods lose about 15% of ketamine but no internal standard. It means that the loss of ketamine is not the result of protein binding and precipitation in the first step of the procedure. The most likely explanation of this loss is due to the acid form of ketamine dissolving in *n*-hexane or ethyl ether.

The average concentration of ketamine in whole brain of C57B1/6 mice was 46.6 ng /mg of tissue. This level is very close to ketamine concentration which has been obtained for Sprague-Dawley (Cohen et al., 1973) and Wistar (Liv-

Table 3
The level of ketamine in different brain regions of C567Bl/6 mice (Mean ± SEM)

Brain regions (ng/mg tissue)					Whole brain (ng/mg tissue)
Cerebellum	Brain stem	Midbrain	Hypothalamus	Cortex	
43.63 ± 4.09	48.57 ± 4.22	41.55 ± 2.36	46.58 ± 2.67	48.19 ± 2.71	46.58 ± 2.67

The means are the average from nine aminals.

ingston & Waterman, 1978) rats, but about 10–20 times higher, than was shown for HAS /LAS rats with differential sensitivity to ethanol (Liu & Dietrich, 1998). Large differences in brain ketamine level may be explained by interstrain differences in accumulation and /or metabolism of ketamine or problems with the experimental procedure for determination of ketamine concentration in brain. If this variability is explained by interstrain differences in sensitivity to ketamine, it is very likely that the analytical method for its determination in brain should cover the wide range of possible concentrations. The high individual correlation between concentrations of ketamine in different brain regions, which has been demonstrated in our study, marks the accuracy, consistency, and precision of proposed assay.

This work supported by a grant from National Institute of General Medical Sciences (GM55635).

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