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GAS CHROMATOGRAPHIC ANALYSIS OF KETAMINE AND NORKETAMINE IN PLASMA AND URINE: NITROGEN-SENSITIVE DETECTION*

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SUMMARY

A sensitive gas chromatographic method for quantitative analysis of ketamine and norketamine in human and animal biological fluids is described. The nitrogen-sensitive detection procedure used is more stable than electron-capture detection and reduced analysis time. The method used bromo-ketamine as an internal standard for quantitation and is linear from 10—25,000 ng/ml. No interferences were shown with drugs commonly associated with cardiac surgery with cardiopulmonary by-pass. This assay is sensitive, specific, using either native or derivatized drugs and can be used for routine analysis of ketamine and norketamine in plasma or urine.

INTRODUCTION

Ketamine (K), [2-(*o*-chlorophenyl)-2-(methylamino)] cyclohexanone, is used for induction and maintenance of anesthesia and analgesia [1, 2]. It is extensively metabolized in man and animals [1—4]. The present paper describes a sensitive and reliable gas chromatographic (GC) method for the quantitative assay of K and its major metabolite norketamine (NK) in plasma. Our single-dose pharmacokinetic studies of K require the measurement of

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drug levels in the range of concentration (10—25,000 ng/ml) used in patients and animals. The method is linear over this range. Of the metabolites, we analyzed only NK, because it is the only one with proven significant pharmacologic activity [5—7]. 5,6-Dehydronorketamine was identified by GC—mass spectrometry (MS) [3, 4, 8--10] as a metabolite. This structure was questioned [11] and later evidence indicated that it was an artifact due to non-enzymatic dehydration of one or both isomers of the metabolite 5-hydroxynorketamine [12].

There are several assays for K in biological fluids [3, 4, 8—10, 12—19], some of which also measure NK and other metabolites [3, 4, 8—12]. But, it has been shown that in the routine analysis of K in biological fluids by a number of methods endogenous biological substances have often interfered [9, 10]. One group deemed it necessary to resort to GC—MS techniques which they compared to GC with electron-capture detection procedures [9, 10].

Our method utilizes nitrogen—phosphorus selective detection and has resulted in greater specificity, rapidity and linearity over the concentration range usually encountered in plasma; we optimized reaction conditions and used more suitable column materials than those employed previously [8]. The method was also adapted for the assay of K and NK excreted in urine; the extraneous biological material that is extracted with the organic solvent was largely eliminated by purification of the extract by thin-layer chromatography (TLC) prior to GC analysis.

EXPERIMENTAL

Standards and reagents

K, NK and bromo-ketamine, the internal standard (IS) (Fig. 1) were obtained as hydrochlorides, as a gift from Drs. A.J. Glazko and T. Chang (Parke-Davis, Ann Arbor, MI, U.S.A.). All solvents used were either glass-distilled or HPLC grade; glassware was treated prior to each use with 10% dimethyldichlorosilane (Aldrich, Milwaukee, WI, U.S.A.) in toluene by soaking

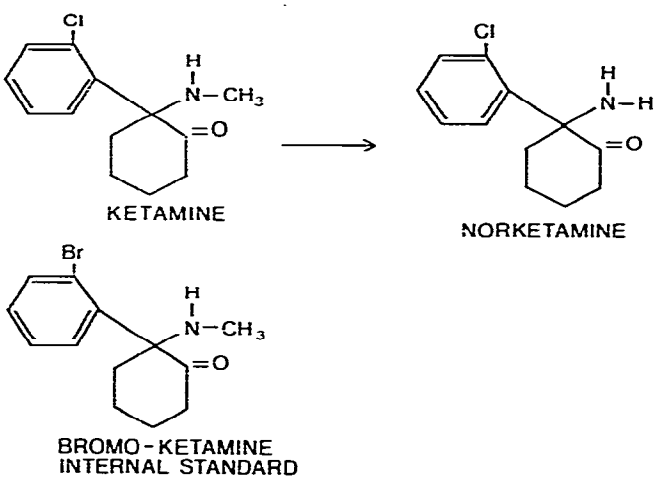

Fig. 1. Structures of ketamine, norketamine and the internal standard, bromo-ketamine.

overnight. After decanting, excess silane was removed with methanol and the tubes were dried at 110°C. Dimethylaminopyridine (DMAP) and heptafluorobutyric anhydride (HFBA) were purchased from Aldrich. Prior to first use and every six months DMAP was recrystallized with ethyl acetate and stored dry at 5°C. If DMAP becomes exposed to the atmosphere, it will decompose and cause interfering GC peaks. Solutions of DMAP and standards of K and analogue compounds were prepared monthly and stored at −20°C. To determine optimal conditions for the use of DMAP, standards of K, NK and IS were heated at different temperatures (45°C, 60°C and 80°C) and times (15–180 min) with HFBA to form the monoacyl derivatives. Identical experiments with pyridine [8] were carried out concurrently. Blood was collected in the presence of heparin in glass tubes or special Vacutainers (Becton-Dickinson, Rutherford, NJ, U.S.A.), fitted with stoppers without tris-2-butoxyethyl phosphate, TBEP; containing powdered Na_2EDTA, Lot OM 617. Plasma was obtained from blood by centrifugation at 500 *g* and was transferred into polyethylene capped tubes (Elkay Products, Shrewsbury, MA, U.S.A.) with a silanized borosilicate Pasteur pipet. Plasma and urine were stored at −70°C. Derivatized samples can be stored desiccated or in toluene at −20°C for at least six months.

Instruments

A 3711 Varian gas chromatograph equipped with a thermionic detector which is selective for nitrogen compounds was utilized. The parameters were: electrometer sensitivity, 10^{-12} A/mV; bead, 450; bias voltage, −4. Gas flow-rates were hydrogen, 4.6; air, 180; and carrier (nitrogen), 30 ml/min. Temperatures (Ultrabond 20M Carbowax column) were: injector 240°C, detector 250°C, and the column temperature was 180°C. The column temperature

TABLE I

RELATIVE RETENTION TIMES AND OPERATING TEMPERATURES FOR KETAMINE AND NORKETAMINE ANALYSIS AND THEIR HFBA DERIVATIVES

Column			Deriv-ative	Relative retention***		Retention (sec)	Correlation coefficient §
Type*	Temperature** (°C)			Ketamine	Norketamine	IS	(*r*)
	A	B					
Ultrabond	180	225	no	0.71	0.82	175	0.9956
	180	225	yes	0.75	0.60	278	0.9965
SE-30	155	275	yes	0.76	0.68	382	0.9992
OV-17	170	275	yes	0.68	0.36	585	0.9998

*Ultrabond 20M Carbowax, 100—120 mesh (Ultra, Mount Hope, RI, U.S.A.); SE-30, 2%, 800—100 mesh Chromosorb W HP; OV-17 2%, 100—120 mesh, Chromosorb W HP (Applied Science, State College, PA, U.S.A.). The bonded Carbowax had narrower peak widths, better symmetry and resolution than other stationary phases tested.

**A = Operating temperature; B = conditioning temperature.

***Relative to bromo-ketamine = 1.0.

§ Correlation coefficient of fit $y = mx + b$ for K (50—3000 ng/ml).

was maintained isothermally at 180°C for 8 min and then programmed to 225°C at 15°C/min and remained at the final temperature for 3 min. Data reduction was accomplished with a Spectra-Physics (Santa Clara, CA, U.S.A.) 4100 integrating microcomputer.

The dimensions of all columns were 1.8 m × 6.3 mm O.D., 2 mm I.D. The packings are given in Table I. In earlier experiments we also used 2% OV-17 and SE-30. Oxygen was excluded from the column to prevent deterioration. MS data were recorded on a Finnigan 3200 gas chromatograph—mass spectrometer in the chemical ionization mode. The column was 3% OV-17. The helium carrier gas flow-rate was 20 ml/min; column temperature, 180°C; electron energy, 70 eV; glass jet separator temperature, 210°C; reactant gas was methane.

Procedures

Blood (1—6 ml) was obtained from adults, children, monkeys and dogs given K. Blank specimens of blood and urine were obtained prior to the administration of K for control purposes. Analysis was carried out with plasma. The range of doses was 2—15 mg/kg. Plasma, 0.2—1.4 ml, was placed into a conical 13-ml ground glass centrifuge tube (Kontes, Vineland, NJ, U.S.A.). The appropriate amount of IS, in water (1 μg/ml), was added. The normal range of IS additions was 250—2000 ng per sample, added with a polypropylene Finn micro pipet. The IS usually was within 1/10 to 10 times the amount of the K and NK. Water was added to a total volume of 2 ml. The pH of the aqueous phase was brought to 9—10 with about 0.1 ml 0.1 *N* sodium hydroxide and 3 ml of *n*-heptane were added. In low level analysis, below 50 ng per sample, pH adjustments were made after the addition of heptane to prevent adsorption of the free bases. The mixture was shaken mechanically, 60 inversions per min for 15 min, and centrifuged at 500 *g* for 20 min at 5°C. The organic phase was transferred by a silanized pipet to a 5-ml centrifuge tube. The solvent was evaporated to almost dryness in vacuo with centrifugation (200-H Savant Speed-Vac, Hicksville, NY, U.S.A.). To ensure quantitative recoveries, two more serial extractions and evaporations were made. To the pooled material, 0.2 ml toluene was added in rinse fashion to concentrate the sample at the bottom of the tube and the mixture was evaporated to dryness in vacuo.

Derivative formation

The residue was dissolved in 1 ml of toluene and 0.1 ml of 0.1 *M* DMAP in toluene was added [20]. Then 20 μl of HFBA were introduced and the mixture was heated at 80°C for 2 h. Excess reagent and undesirable products were removed by two washes of 2 ml 5% ammonium hydroxide, followed by two washes of 2 ml of 1 *N* hydrochloric acid. Separation of phases after each wash was achieved by centrifugation at 500 *g* for 10 min. To the remaining organic phase, 1 ml of toluene was added in rinse fashion and the mixture was mixed and evaporated almost to dryness (Speed-Vac). The residue was dissolved in 10—50 μl toluene. It was then mixed by Vortex and 0.5—1.0 μl was injected into the gas chromatograph column.

For urine, the extract was purified by TLC before GC (Table II). Water,

TABLE II

THIN-LAYER CHROMATOGRAPHIC PARAMETERS

System A was used for purification of urine specimens prior to GC analysis; the appropriate zone was scraped and extracted twice with 2 ml warm methanol. The silica gel was separated by centrifugation; the pooled extracts were evaporated in vacuo. A separate side strip on the same plate was spotted with K and detected by exposure of the strip to iodine vapors in order to verify the migration of the drug. Radiochemical purification of [^{3}H]K was accomplished using System B (repeated three times), eluted as above, and evaporated to dryness with nitrogen. The residue was dissolved in 0.001 *M* hydrochloric acid—ethanolic solution. Purity was determined by all three Systems B, C and A.

System	Solvent	R_F value K + NK	TLC type*
A	Ethanol—methanol—water (6 : 6 : 1)	0.70	Silica gel 60
B	Ethanol—methanol—water (4 : 4 : 1)	0.81	Silica gel G
C	1,2-Dichloroethanol—ethanol (1 : 1)	0.77	Silica gel G

*Silica gel G was obtained from Supelco (Bellefonte, PA, U.S.A.) and silica gel 60 from E. Merck (Darmstadt, G.F.R.).

plasma or urine blanks from the experimental subjects and from drug-free volunteers were analyzed with each set of biological samples. Combined derivatized standards in toluene (at several concentrations; 50—2000 ng) were injected into the gas chromatograph at the beginning and end of runs. This was to check retention time and detector sensitivity. Recoveries were also analyzed with each set of samples. To verify the recovery of K and NK from biological fluids, blanks were spiked with aqueous standards of K, NK, IS and [^{3}H]K. These were randomly distributed among the unknown samples.

Synthesis of [^{3}H] ketamine·HCl

[^{3}H]K·HCl was synthesized by a modification of a published procedure [21]. K·HCl (50 mg) was labelled by the Wilzbach procedure (New England Nuclear, Boston, MA, U.S.A.). We carried out the purification as described [21], except that the exchange with 0.1 *N* sodium hydroxide at room temperature was carried out twice; others used heating with aqueous triethylamine [12]. Purification was carried out using TLC (Table II). The K carrier was added to the [^{3}H]K·HCl and it was recrystallized twice from ethanol. Purity was established by radio-TLC using three TLC systems (Table II). Radiochemical purity was greater than 99.3%. This was further confirmed by isotope dilution and high-performance liquid chromatography.

RESULTS AND DISCUSSION

Over 1000 plasma samples, mostly human and canine, were analyzed at least in duplicate by the described assay. The range of concentration was from 10—20,000 ng/ml. The calibration curves for K and NK extracted from plasma and urine were linear over the range studied (Table III). Reproducibility for

determination of K in plasma is given in Table IV. Details of the retention times and conditions are given in Table I; the coefficient of variation averaged 6% (Table IV).

TABLE III

CALIBRATION CURVES FOR K AND NK EXTRACTED FROM PLASMA AND URINE

Compound	IS (ng)	Range (ng/ml)	Regression equation $y=mx+b$*		r**	n
			m	b		
K	500	80—11,000	1.19	−0.037	0.9956	37
NK	500	10—700	0.761	−0.030	0.9965	41

*y = peak area ratio (K/IS and NK/IS); x = concentration ratio (K/IS and NK/IS).
**r = Correlation coefficient.

TABLE IV

REPRODUCIBILITY FOR DETERMINATION OF K IN PLASMA

Recovery over concentration range studied was 88 ± 3% (mean ± S.D., n = 8); precision was 4.0%

Added (ng/ml)	Found ± S.E.M.* (ng/ml)	S.E.M. (%)
50	54 ± 4	7.4
100	106 ± 7	6.5
150	153 ± 13	8.5
300	298 ± 6	2.0
450	423 ± 27	6.2
750	742 ± 38	5.3
1500	1530 ± 70	4.6
3000	3046 ± 126	4.1
		Average = 5.9

*S.E.M. = Standard error of the mean (n = 3).

Optimum conditions for derivatization were achieved with 40—60 min heating at 80°C; further heating for 68—180 min produced no significant change. Better yields and less interference with nitrogen detection and less deteriorization of the column were obtained with DMAP than with pyridine as the catalyst. Once derivatized, samples were stable for six months when kept dry and cold (−20°C). This is the first time DMAP was used as a catalyst for the formation of HFBA derivatives of K type compounds.

Recovery experiments using 100 ng of [^{3}H]K added to blank specimens of plasma and urine averaged 88%. This recovery is in agreement with the partition coefficient (K_p) > 100 for K and NK between aqueous buffer at pH 9 and heptane. With some plasmas containing high lipid concentrations and less than 150 ng/ml of K the recovery was not as complete; therefore, two extractions were needed. A number of drugs commonly used in a clinical anesthesia

were found not to interfere in the assays: morphine, diazepam, pancuronium and scopolamine. Quinidine partially co-chromatographed, but was minimally extracted. Propranolol and DMAP can interfere, if washes are omitted from either the derivatized or underivatized procedures. Blank equivalent for plasma and urine were less than 10 ng/ml. Specificity of the method was demonstrated by MS; it was shown that the individual peaks eluted with the retention times of K, NK, IS and their derivatives were due only to these individual compounds. MS data in the chemical ionization mode were the same as those published previously [4, 9, 10, 12]. The present method is a significant modification of a GC procedure with electron-capture detection [8]. By utilization of nitrogen-selective detection, it has been possible to achieve better linearity along with greater stability of the detector.

Two variants of the method have been used. For concentrations above 100 ng/ml the Ultrabond 20M column was suitable for underivatized samples. Below that level, the derivatization procedure should be used in order to increase the sensitivity and reduce low level noise commonly observed with

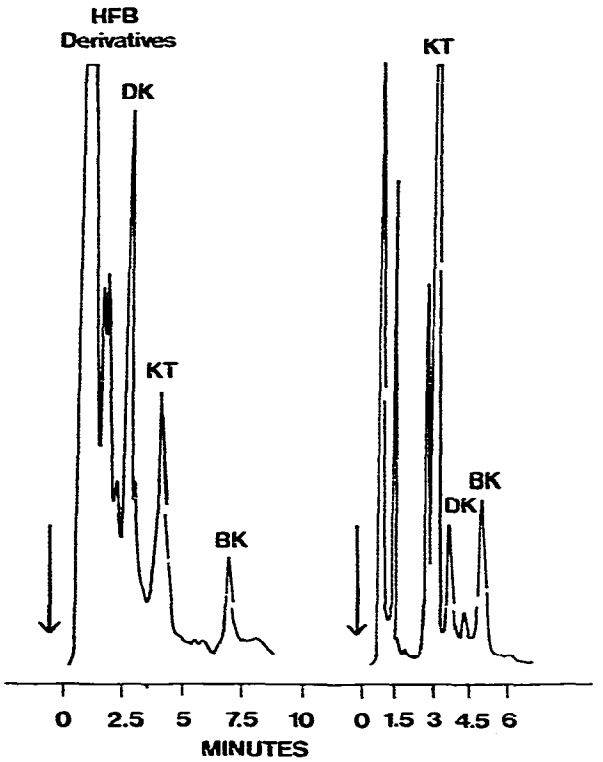

Fig. 2. (Left): gas chromatogram of an extract of plasma obtained from a dog (No. M63) given 10 mg/kg K (140 mg intravenously) not undergoing cardiac surgery with cardiopulmonary by-pass. (Right): gas chromatogram of an extract of plasma obtained from a patient (No. 6) given 2 mg/kg K (185.4 mg intravenously) and undergoing cardiac surgery with cardiopulmonary by-pass. Peaks: (left) DK = norketamine, 1025 ng/ml; BK = bromo-ketamine, 500 ng/ml; KT = ketamine, 1500 ng/ml; sample derivatized with HFBA. (right) DK = 185 ng/ml; KT = 1780 ng/ml; sample underivatized. Column: 20M Carbowax Ultrabond. Time in minutes.

biological samples. The derivatized samples are stable for at least six months.

While OV-17 and SE-30 columns provide separations of derivatized samples, the Ultrabond 20M column has several advantages. It gives still better resolution, results in symmetrical narrower peak widths, is more durable, and can be used without derivatization.

Attention was paid to the collection of blood samples. We had found that K is 20—45% bound to human plasma: 25% to 4% human albumin [22] and 28% to 0.1% alpha-1 acid glycoprotein. Also, TBEP is a constituent of stoppers of certain Vacutainer tubes and displaces K from the glycoprotein (unpublished results). The displaced K will penetrate red blood cells, thereby decreasing plasma levels, a falsely low plasma concentration is often obtained in the presence of TBEP [23—26].

Our studies of pharmacokinetics and distribution of K in man and animals employing the above-mentioned method have been described elsewhere [22, 27—29]. Typical data are given in Figs. 2, 3 and 4. The plasma concentration vs. time relationships in our patients were comparable to those found by other investigators [2, 3, 16]. The results in the dog were similar to those obtained with higher doses by Kaka and Hayton [30]. The method of analysis has also been applied by us to the study of the pharmacokinetics of K in infants and children (unpublished results). In these studies as little as 0.2 ml plasma (in some cases single specimens) were analyzed because of ethical limitations;

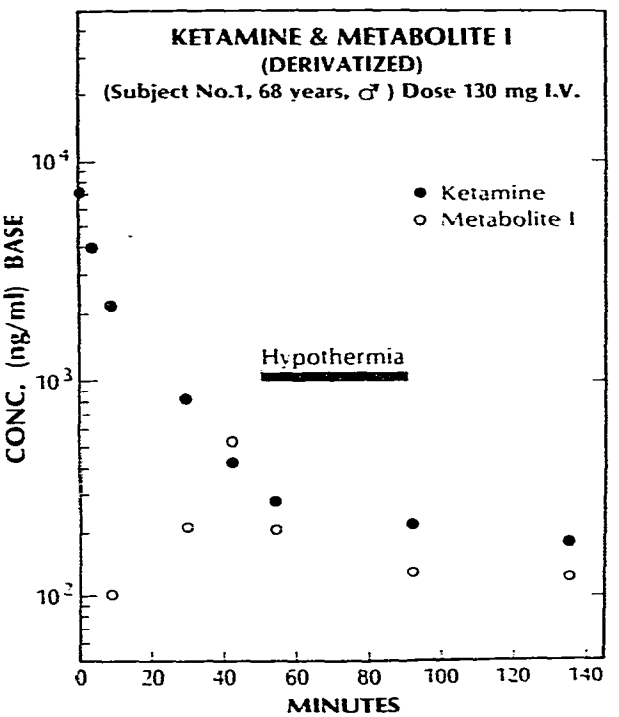

Fig. 3. Plasma concentrations of ketamine and norketamine (Metabolite I) in a patient over 2 h given 2 mg/kg (130 mg intravenously) ketamine, undergoing cardiac surgery with cardiopulmonary by-pass with hypothermia 55—90 min.

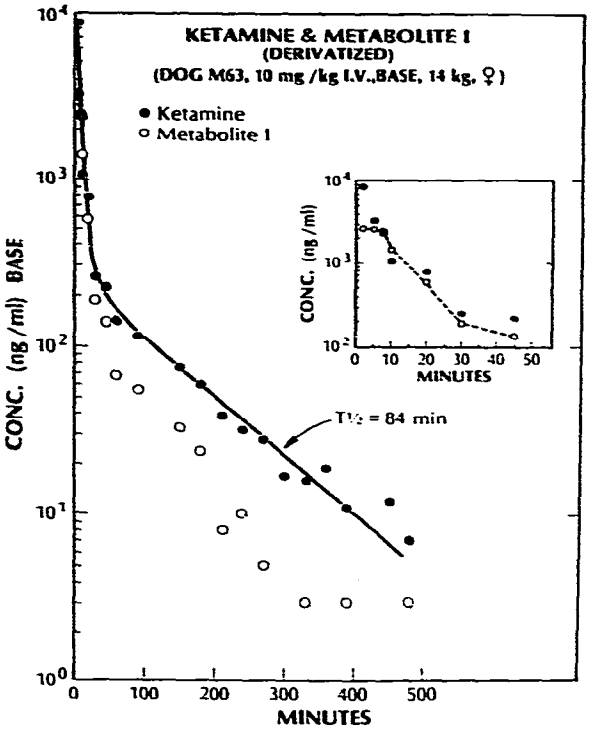

Fig. 4. Plasma concentrations of ketamine and norketamine (Metabolite I) in a dog over 6 h. Insert is for plasma levels for the first 45 min after the intravenous injection of K, 10 mg/kg samples were derivatized. Dose = 10 mg/kg intravenously of ketamine. Analyzed after derivatization. For abbreviations see Fig. 2.

the volume of blood withdrawn for analysis was less than 8% of the patients' estimated blood volume.

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