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Stereoselective high-performance liquid chromatographic determination of the enantiomers of ketamine and norketamine in plasma[a,b]

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ABSTRACT

An enantioselective high-performance liquid chromatographic assay for the quantitation of the enantiomers of ketamine and its major metabolite norketamine in human plasma is described (assay I). The procedure involved extraction of the compounds from alkalized plasma into cyclohexane. Stereoselective separation was achieved with a prepacked α_1-acid glycoprotein column without any derivatization procedure. A second assay using a conventional reversed-phase column to determine total (racemic) ketamine and norketamine is also described. Because of interfering plasma peaks (assay II) the cyclohexane solution was reextracted into 1 *M* hydrochloric acid. The detection wavelength was 215 nm for all substances. The limit of quantification of the method was *ca.* 40 ng/ml in plasma. The assays were sensitive and reproducible. The method was demonstrated to be sensitive for stereoselective pharmacokinetic studies of ketamine after clinical doses.

INTRODUCTION

In recent years, ketamine (KET) [*R,S*-2-(*o*-chlorophenyl)-2-(methylamino)cyclohexanone] has acquired a unique place as a "dissociative anaesthetic" in its racemic form [1,2]. Although KET (racemate) has a variety of desirable proper-

[a] Some of these results were reported at the *5th Annual Meeting of American Association of Pharmaceutical Scientists, Nov. 4–8, 1990, Las Vegas, NV, USA*, and were published as an abstract [*Pharm. Res.*, 7 (1990) 244].

[b] Dedicated to Professor Dr. Dr. E. Mutschler on the occasion of his 60th birthday.

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ties as an anaesthetic, it has also been associated with cardiovascular stimulant properties, sensory and perceptual illusions, and vivid dreams following anaesthesia ("emergence reaction") [3]. Recent findings have shown that there are quantitative and qualitative differences between the KET enantiomers in their effects on the central nervous system [4]. The *S*-enantiomer was found to show more marked anaesthetic and analgesic effects [4,5]. Emergence reactions, however, occurred more frequently after administration of *R*-KET or racemic KET [4].

The metabolism of KET has been well documented from both *in vitro* and *in vivo* experiments [6–12]. The lack of a stereoselective assay, however, means that correlations between pharmacodynamic and pharmacokinetic data are not conclusive. Several analytical methods to determine KET and its main metabolites norketamine (NORKET) [*R,S*-2-(*o*-chlorophenyl)-2-aminocyclohexanone] and dehydronorketamine [*R,S*-2-(*o*-chlorophenyl)-2-amino-5-cyclohexene-1-one] have already been described. KET concentrations in biological fluids have been determined by gas chromatography (GC) [7,13–16], thin-layer chromatography [14], colorimetry [17], high-performance liquid chromatography (HPLC) [18] and gas chromatography–mass spectrometry (GC–MS) [7,16,19,20]. With the exception of the GC and GC–MS methods, the assays lack either sensitivity or accuracy [7]. Although KET and its major metabolite are optically active compounds, to our knowledge no stereoselective method has been published so far. One reason for this may be that the enantiomers of KET react at different rates with the chiral reagents (N-trifluoroacetyl-S-prolyl chloride and S,S-N-trifluoroacetylproline anhydride) used for diastereomeric derivatization [21]. These problems, however, can be overcome by the use of a chiral HPLC column, which requires no derivatization procedure.

This paper describes a method to determine stereoselectively the enantiomers of KET and NORKET in human plasma, employing a commercially available α_1-acid glycoprotein (AGP) HPLC column [22] (assay I). Neither the separation nor the detection of the enantiomers of KET and NORKET requires any derivatization procedure. In order to compare the sum of the concentrations of the two enantiomers (assay I) and the amount of total (racemic) KET and NORKET, we developed a conventional reversed-phase HPLC procedure to quantify total KET and total NORKET (assay II).

EXPERIMENTAL

Chemicals

The KET and NORKET enantiomers as the pure compounds (as HCl salts), as well as the intravenous (i.v.) formulations containing racemic KET (Ketanest) or S-KET were gifts from Parke Davis (Freiburg, Germany). The composition of the i.v. solution of S-KET was identical with that of the commercially available product (Ketanest), except for the concentration. Racemic dehydronorketamine

(enantiomers not available), racemic nortilidine [ethyl DL-*trans*-2-(methylamino)-1-phenyl-3-cyclohexene-1-carboxylate, as HCl salt], used as the internal standard (I.S.) in assay II, as well as racemic bisnortilidine [ethyl DL-*trans*-2-amino-1-phenyl-3-cyclohexene-1-carboxylate, as HCl salt] were also supplied by Parke Davis. One enantiomer of bisnortilidine proved to be suitable as the I.S. in assay I. As no pure enantiomers of bisnortilidine were available, we separated a small amount of enantiomers via HPLC using the AGP column under the same chromatographic conditions as for KET described in assay I.

All other chemicals and organic solvents were of HPLC or reagent grade. The mobile phase was freshly prepared, filtered (0.45-μm filter), and degassed under vacuum prior to use.

The stock standard solutions of KET and NORKET enantiomers (HCl salts) were prepared by dissolving an appropriate amount of the compounds in distilled water. Working standards were prepared in drug-free plasma from the stock standard to yield concentrations of 50-5000 ng/ml of plasma and were kept at 5°C in darkness.

Stereoselective determination of ketamine and norketamine enantiomers in human plasma (assay I)

The HPLC system consisted of a Model SP 8810 pump (Spectra Physics, Darmstadt, Germany), a Model SP 100 UV monitor with a 10-mm analytical cell (Spectra Physics) fitted with a Model 231 diluter–autosampler (Gilson/Abimed, Langenfeld, Germany) and a D-2500 Chromato Integrator (Merck-Hitachi, Darmstadt, Germany). Stereoselective separation was achieved with a prepacked AGP column (100 mm × 4.0 mm I.D., Grom, Herrenberg, Germany). Owing to the temperature dependence of the chiral separation [23], a column thermostat (Chemdata, Sinsheim, Germany) set up at 25°C was utilized. The mobile phase was 2-propanol–0.02 *M* phosphate buffer, pH 7 (2.5:97.5, v/v). The usual flow-rate was 0.5 ml/min. The detection wavelength was 215 nm for all substances. The system was used in an air-conditioned room (20°C).

Determination of ketamine and norketamine in human plasma using a reversed-phase HPLC column (assay II)

The basic HPLC equipment used in assay II was similar in all respects to that employed in assay I. Chromatograms were recorded on a CR 3A Shimadzu integrator (Egling, Germany). Separation was achieved with a prepacked column (100 mm × 4.0 mm I.D., Nucleosil 3-μm RP 8; Macherey and Nagel, Düren, Germany). The eluent was acetonitrile–0.03 *M* phosphate buffer, pH 7.5 (30:70, v/v), adjusted to pH 7.0 with concentrated phosphoric acid. The flow-rate of the mobile phase was 1.3 ml/min. The detection wavelength was 215 nm. The system was used in an air-conditioned room (20°C).

Analytical procedure

Assay I. For the determination of the enantiomers of KET and NORKET, 1.00 ml of plasma (standard, quality control or a sample from a dosed patient) and 100 μl of the prepared solution (in mobile phase) of the I.S. were transferred to a conical glass tube and alkalized with 0.10 ml of 3 *M* NaOH. It was then extracted into 5.75 ml of ice-cooled cyclohexane by agitation for 15 min at 4°C. In order to ensure quantitative recovery, a second extraction with 3.00 ml of ice-cooled cyclohexane was performed. The combined organic layers (8.00 ml) were evaporated to dryness under a gentle stream of dry nitrogen. The residue was redissolved in 500 μl of cyclohexane, and the solvent was evaporated with a gentle stream of dry nitrogen so that the compounds were concentrated at the tip of the glass tube. The dry residue was redissolved in mobile phase (200 μl for concentrations less than 500 ng/ml, 500 μl for concentrations between 500 and 1000 ng/ml and 1000 μl for concentrations greater than 1000 ng/ml. The mixture was mixed for 30 s, allowed to stand for 2 min, then mixed again for 30 s. The sample was injected onto the HPLC column (100 μl for concentrations less than 500 ng/ml, 50 μl for concentrations greater than 500 ng/ml). Standard curves were prepared by injecting plasma extracts spiked with various amounts of the *R*- and *S*-enantiomers of KET and NORKET, respectively, simulating the concentrations following i.v. administration of racemic KET or *S*-KET. For the quantification of unknown plasma samples, peak-area ratios for KET and NORKET relative to the I.S. were used.

Assay II. The analytical procedure for assay II was similar to that used in assay I. Nortilidine was used as the I.S. After concentration of the compounds at the tip of the glass tube (with 500 μl of cyclohexane), the cyclohexane solution was reextracted into 2.20 ml of 1 *M* HCl on an automatic shaker. The cyclohexane solution was discarded, and 2.00 ml of the HCl solution were evaporated to dryness on a Speed Vac evaporation system (without heating) (Univapo 150 H, Speed Vac concentrator, Savant Instruments, Vaterstetten, Germany). The dry residue was redissolved in mobile phase as described above.

Precision of the assay

Four samples each of five plasma standards in the range 50–5000 ng/ml (KET, added as HCl salt; NORKET, 50–500 ng/ml, added as HCl salt) were analysed on three successive days with the same column. Peak-area ratios were plotted *versus* concentrations of the standards, and concentrations were back-calculated. The intra-day and inter-day variabilities were determined. The quantification limit definition adopted was that of the lowest concentration that could be determined during the inter-assay validation with either precision or accuracy of less than or equal to 15%.

Recovery values

Recovery values were evaluated by comparing extracted spiked plasma sam-

ples in the range 50–5000 ng/ml plasma (KET) or 50–500 ng/ml plasma (NOR-KET) with unextracted standard solutions.

Application

The utility of the method was demonstrated after i.v. administration of 2 mg/kg body weight of racemic KET (Ketanest) or 1 mg/kg body weight of *S*-KET to patients who were scheduled for minor surgery. After premedication with midazolam (Dormicum) the KET i.v. solution was administered into the left cubital vein over a period of 30 s. Blood was taken from an indwelling catheter of the right cubital vein. Then, 10 and 30 min after KET administration, anaesthesia was maintained with nitrous oxide and enflurane, respectively. Blood samples were collected prior to and up to 12 h after KET administration. The plasma was frozen immediately and stored at −30°C until analysis.

RESULTS

Assay I

Typical chromatograms of a blank human plasma sample and of real plasma samples obtained from patients after i.v. administration of 2 mg/kg body weight of racemic KET and 1 mg/kg body weight of S-KET are depicted in Fig. 1. A chromatogram of a standard solution containing the racemic compounds of KET, NORKET and dehydronorketamine, respectively, is also given in Fig. 1. One of the enantiomers of bisnortilidine (used as the I.S.) and one enantiomer of dehydronorketamine were eluted at approximately the same time. Nevertheless, this enantiomer of bisnortilidine proved to be suitable as the I.S. because in our real plasma samples, the concentrations of dehydronorketamine were below the detection limit. The capacity ratios of the I.S., *S*-NORKET, *S*-KET, *R*-KET and *R*-NORKET were *ca.* 4.6, 5.8, 7.6, 8.8 and 10.3, respectively. The separation factors for the chiral resolution of the enantiomers of ketamine and norketamine exceeded 1.15 and 1.75, respectively. The limit of quantification was *ca.* 40 ng/ml of plasma for all four substances. No reliance was placed on the detection limit (signal-to-noise ratio 3:1), which was *ca.* 20 ng/ml of plasma. The recovery values of the enantiomers of KET and NORKET are listed in Table I. The peak-area ratios of the compounds were linearly related ($r > 0.998$) to the amount of KET and NORKET enantiomers added (as HCl salts) to blank human plasma in the ranges 50–5000 and 50–500 ng/ml, respectively. The inter-day and intra-day precisions in plasma over three days are also summarized in Table I. In one case (*S*-KET, 43.3 ng/ml) we could not obtain an inter-assay precision of less than 15%.

Assay II

Dehydronorketamine, NORKET, KET and nortilidine (I.S.) were eluted after 3.3, 4.2, 7.1 and 10.2 min, respectively (Fig. 2). This assay also exhibited good

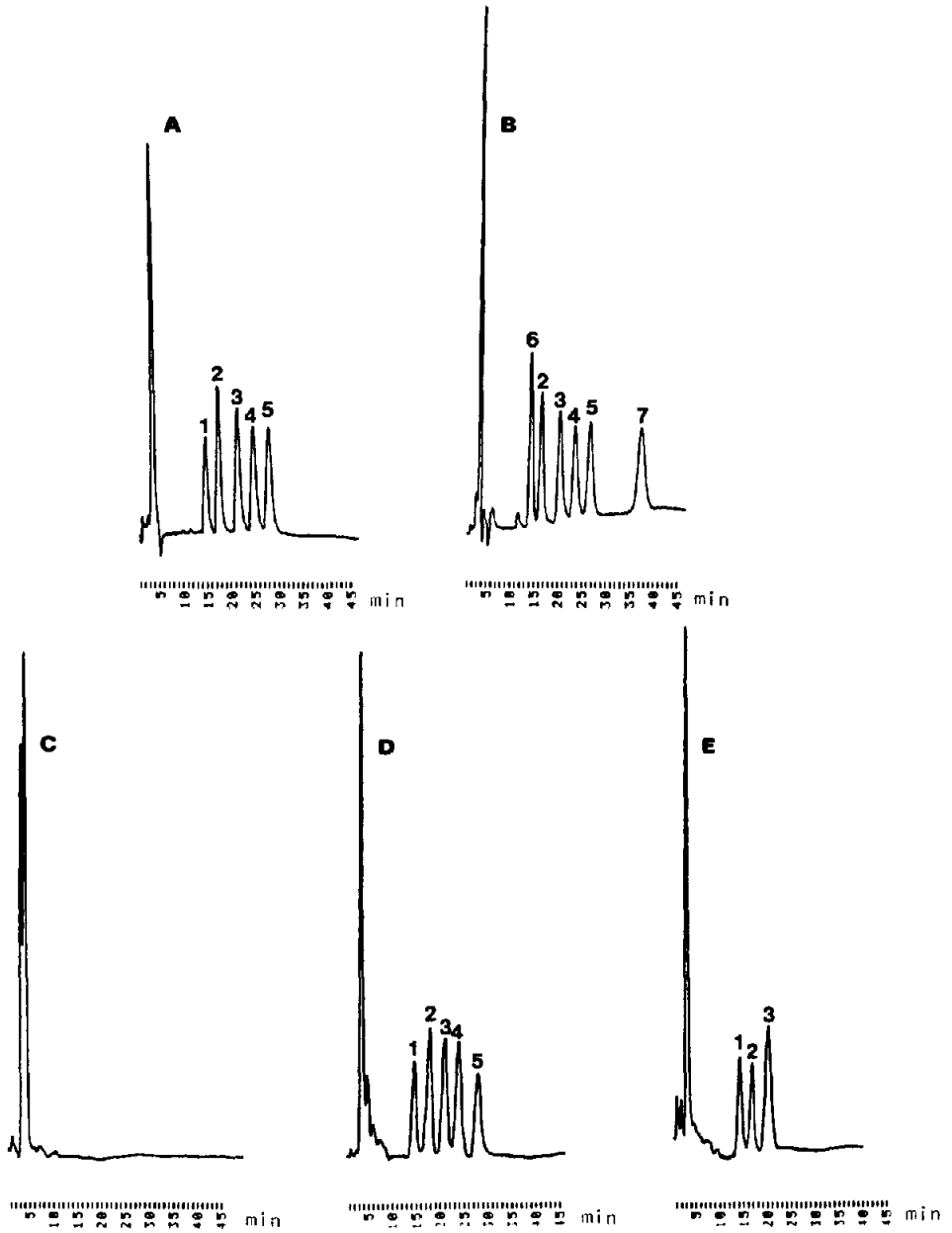

Fig. 1. Typical chromatograms of the enantiomers of ketamine, norketamine and dehydronorketamine (assay I). (A) Standard solution containing racemic KET, racemic NORKET and I.S.; (B) standard solution containing racemic KET, racemic NORKET and racemic dehydronorketamine; (C) blank human plasma; (D) human plasma obtained after i.v. administration of 2 mg/kg body weight of racemic KET (*S*-NORKET, 160 ng/ml; *S*-KET, 212 ng/ml; *R*-KET, 225 ng/ml; *R*-NORKET, 167 ng/ml); (E) human plasma obtained after i.v. administration of 1 mg/kg body weight of *S*-KET (*S*-NORKET, 94 ng/ml; *S*-KET, 220 ng/ml). Peaks: 1 = I.S.; 2 = *S*-NORKET; 3 = *S*-KET; 4 = *R*-KET; 5 = *R*-NORKET; 6 and 7 = enantiomers of dehydronorketamine. UV detection (215 nm, 0.002 a.u.f.s.).

TABLE I

ANALYTICAL RECOVERY AND INTER- AND INTRA-DAY PRECISION OF KETAMINE AND NORKETAMINE ENANTIOMERS OVER THREE DAYS (ASSAY I)

Concentration added (calculated as base) (ng/ml)	Recovery (%) (n = 4)	Concentration found (mean ± S.D.) (ng/ml)			
		Day 1 (n = 4)	Day 2 (n = 4)	Day 3 (n = 4)	Mean
S-Ketamine					
43.3	90.0 ± 9.8	48.9 ± 3.3	35.1 ± 0.9	48.4 ± 5.7	44.1 ± 7.8
86.7	95.7 ± 3.7	86.2 ± 4.8	77.5 ± 4.0	97.6 ± 7.9	87.1 ± 10.1
433.4	96.0 ± 5.7	401.0 ± 20.0	412.3 ± 12.6	440.9 ± 7.9	418.0 ± 20.6
866.8	90.3 ± 8.5	900.9 ± 9.3	871.3 ± 41.8	846.6 ± 12.5	872.9 ± 27.2
4334	92.2 ± 10.5	4324.0 ± 82.2	4322.0 ± 103.1	4331.0 ± 13.0	4325.7 ± 4.7
R-Ketamine					
43.3	97.5 ± 10.8	50.6 ± 6.5	38.9 ± 8.5	50.1 ± 3.7	46.5 ± 6.6
86.7	101.3 ± 8.4	89.6 ± 3.2	89.0 ± 2.2	94.4 ± 2.3	91.0 ± 3.0
433.4	99.8 ± 7.7	389.8 ± 29.7	443.9 ± 22.1	457.0 ± 2.3	430.2 ± 35.6
866.8	92.6 ± 9.8	885.0 ± 26.1	902.8 ± 38.7	820.0 ± 2.4	869.3 ± 43.6
4334	93.7 ± 11.1	4323.0 ± 133.4	4334.0 ± 108.4	4342.0 ± 98.4	4333.0 ± 9.5
S-Norketamine					
43	95.0 ± 7.5	48.8 ± 3.1	44.4 ± 0.6	40.5 ± 5.2	44.6 ± 4.2
85.9	96.0 ± 6.3	79.4 ± 0.8	84.8 ± 5.4	82.0 ± 6.6	82.1 ± 2.7
429.6	89.3 ± 5.7	430.0 ± 14.6	429.8 ± 1.7	428.5 ± 38.1	429.4 ± 0.8
R-Norketamine					
43	98.9 ± 11.2	45.4 ± 2.0	43.9 ± 5.2	41.6 ± 4.3	43.6 ± 1.9
85.9	101.4 ± 4.6	82.6 ± 5.6	78.7 ± 2.5	82.4 ± 5.6	81.2 ± 2.2
429.6	95.7 ± 8.4	429.9 ± 12.5	428.4 ± 3.0	428.4 ± 14.1	428.9 ± 0.9

TABLE II

ANALYTICAL RECOVERY AND INTER- AND INTRA-DAY PRECISION OF KETAMINE AND NORKETAMINE OVER THREE DAYS (ASSAY II)

Concentration added (calculated as base) (ng/ml)	Recovery (%) (n = 4)	Concentration found (mean ± S.D.) (ng/ml)			
		Day 1 (n = 4)	Day 2 (n = 4)	Day 3 (n = 4)	Mean
Ketamine					
43.3	103.9 ± 17.8	53.6 ± 9.3	39.2 ± 3.1	45.1 ± 3.3	46.0 ± 7.2
86.7	96.6 ± 5.7	92.7 ± 6.2	89.2 ± 6.9	80.9 ± 4.1	87.6 ± 6.1
433.4	100.8 ± 6.1	445.6 ± 10.3	416.7 ± 12.4	400.6 ± 11.0	421.0 ± 22.8
866.8	94.6 ± 9.6	877.3 ± 11.1	855.8 ± 9.2	872.4 ± 15.3	868.5 ± 11.3
4334	93.0 ± 3.5	4313.7 + 121.0	4389.0 ± 165.2	4402.0 ± 152.2	4368.2 ± 47.7
Norketamine					
43	74.8 ± 17.4	54.2 ± 4.0	44.0 ± 3.4	54.0 ± 6.6	50.7 ± 5.8
85.9	79.9 ± 9.6	85.2 ± 2.4	81.6 ± 6.0	89.9 ± 5.5	85.6 ± 4.2
429.6	94.0 ± 6.2	415.2 ± 20.0	422.9 ± 7.9	401.8 ± 13.1	413.3 ± 10.7
859.2	90.6 ± 3.6	849.2 ± 80.2	881.4 ± 36.4	865.4 ± 100.1	865.3 + 16.1

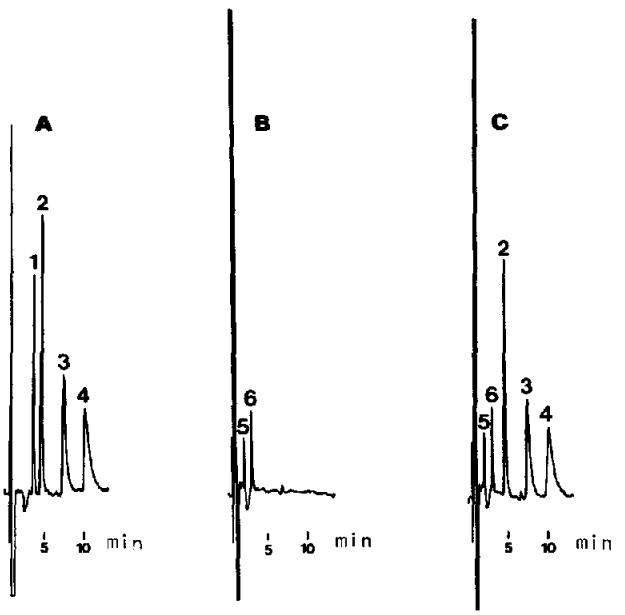

Fig. 2. Typical chromatograms of total KET, NORKET and dehydronorketamine (assay II). (A) Standard solution containing KET, NORKET and dehydronorketamine; (B) blank human plasma; (C) human plasma spiked with KET and NORKET (250 ng/ml). Peaks: 1 = dehydronorketamine; 2 = NORKET; 3 = KET; 4 = I.S.; 5 and 6 = unknown peaks. UV detection (215 nm, 0.02 a.u.f.s.).

accuracy and reproducibility. The accuracy for NORKET at the concentration of 43 ng/ml (118%) was beyond the limit that we defined prior to validation. Values for the recovery and the precision of the assay are listed in Table II. Standard curves were linear over the investigated concentration range. For KET and its main metabolite NORKET the coefficients of correlation exceeded 0.990.

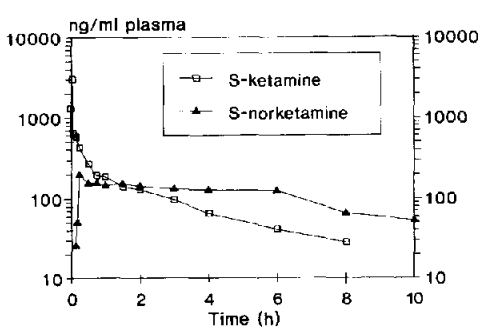

Fig. 3. Stereoselective plasma concentration–time profiles of KET and NORKET following i.v. administration of 1 mg/kg body weight of *S*-KET to a patient. No *R*-KET or *R*-NORKET could be detected.

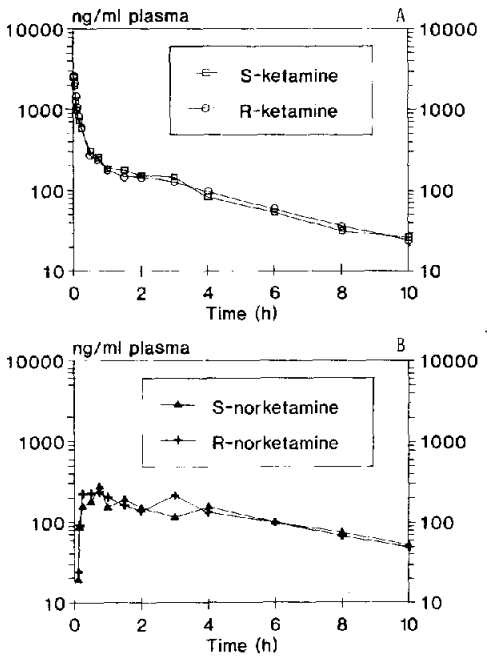

Fig. 4. Stereoselective plasma concentration time profiles of KET (A) and NORKET (B) following i.v. administration of 2 mg/kg body weight of racemic KET to a patient.

Application

Characteristic plasma concentration *versus* time profiles of KET and NORKET enantiomers following a single i.v. administration of racemic (2 mg/kg body weight) and *S*-KET (1 mg/kg body weight) to patients who were scheduled for minor surgery are shown in Figs. 3 and 4. The stereoselective pharmacokinetic behaviour will be described elsewhere.

DISCUSSION

A large number of investigators have measured plasma levels of KET and its major metabolites, employing several non-stereoselective methods [7,13–20]. GC and/or GC–MS appear to be the most practicable methods with respect to accuracy and sensitivity. As a chiral compound, however, KET should be determined stereoselectively [24]. For the separation of chiral compounds three methods are currently used: (i) the formation of diastereomeric derivatives [25–27]; (ii) the use of a chiral mobile phase [28,29]; and (iii) the use of a chemically bonded chiral stationary phase [28]. The enantiomers of KET react at different rates with chiral reagents [21]. An alternative approach to the direct resolution of KET enantiomers is the use of an AGP HPLC column [22]. Since KET is a poor absorber, Needham and Kochhar [18] developed a non-stereoselective HPLC

method based on the formation of the *p*-nitrobenzamide derivatives in order to detect low concentrations of KET and its metabolites. Recently, however, UV detection systems have become more and more sensitive. In our assays KET could be reliably detected down to *ca.* 40 ng/ml of plasma without attaching a chromophore; in our opinion, this sensitivity is sufficient for pharmacokinetic investigations.

The chiral separation using the AGP column depends to a high degree on the chromatographic conditions (pH, ionic strength, column temperature) [29]. Consequently, the range of optimum conditions to avoid interfering peaks from plasma was rather narrow, and depended on the plasma to be analysed. The optimum stability of the column was observed using constant chromatographic conditions. It could be shown, however, that the separation power differs from one AGP column to another, requiring methodological changes (mobile phase composition range, 1.3–2.8% 2-propanol; ionic strength range, 0.01–0.03 *M*; column temperature range, 18–25°C). Nevertheless, with a few columns (three out of nine), we could not achieve a good resolution between KET enantiomers and NORKET enantiomers, and especially between the I.S. and *S*-NORKET. In such cases, separation of KET and NORKET enantiomers was good enough to determine the ratio of *R* and *S*-enantiomers, but the correlation coefficients of the standard curves were only *ca.* 0.970. We, therefore, developed a reversed-phase assay to quantify total KET and total NORKET. Because of interfering plasma peaks we were forced to reextract the combined cyclohexane solutions into 1 *M* HCl.

Another difficult task was to find a suitable I.S. for assay I. The use of one enantiomer of bisnortilidine represents a practicable compromise: as mentioned above, the resolution between the I.S. and *S*-NORKET differed from column to column, but with most columns (six out of nine) we obtained good results (Fig. 1). The I.S. and one enantiomer of dehydronorketamine (metabolite II) were eluted at the same retention time (Fig. 1). Although earlier literature listed dehydronorketamine as an important breakdown product of KET in human plasma [6,14,18,19], our assay I did not reveal significant amounts of dehydronorketamine in plasma (without I.S.), and nor did our assay II. This finding is in line with the results of other investigators, suggesting that this substance is more likely an artifact of the GC analysis than an important metabolite *in vivo* [2,7,12].

In conclusion, the stereoselective HPLC assay described here provides a first approach for the stereoselective determination of KET and NORKET in human plasma after clinical doses for investigative purposes. Assay I proved to be sufficiently precise and sensitive, as indicated by a comparison of the sum of the concentrations of the two enantiomers and the amount of racemic KET or NORKET determined by assay II.

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NOTE ADDED IN PROOF

Since submission of this paper we could detect dehydronorketamine in plasma of some patients (concentration less than 50 ng/ml of plasma).

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