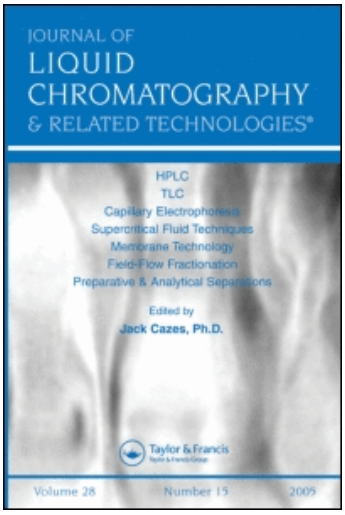


Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Determination of S-(-)-Cathinone and Its Main Metabolite R,S-(-)-Norephedrine In Human Plasma By High-Performance Liquid Chromatography and Photodiode Array Detection

R. Brenneisen[a]; K. Mathys[a]; S. Geisshüsler[a]; H. U. Fisch[b]; U. Koelbing[b]; P. Kalix[c]
[a] Institute of Pharmacy University of Berne, Berne, Switzerland [b] Department of Psychiatry and Social Medicine, University of Berne, Berne, Switzerland [c] Department of Pharmacology Centre of Medicine, University of Geneva CH-1211, Geneva, Switzerland

To cite this Article Brenneisen, R. , Mathys, K. , Geisshüsler, S. , Fisch, H. U. , Koelbing, U. and Kalix, P.(1991) 'Determination of S-(-)-Cathinone and Its Main Metabolite R,S-(-)-Norephedrine In Human Plasma By High-Performance Liquid Chromatography and Photodiode Array Detection', Journal of Liquid Chromatography & Related Technologies, 14: 2, 271 — 286
To link to this Article: DOI: 10.1080/01483919108049614
URL: http://dx.doi.org/10.1080/01483919108049614

JOURNAL OF LIQUID CHROMATOGRAPHY, 14(2), 271-286 (1991)

DETERMINATION OF S-(-)-CATHINONE AND ITS MAIN METABOLITE R,S-(-)-NOREPHEDRINE IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND PHOTODIODE ARRAY DETECTION

R. BRENNEISEN[1], K. MATHYS[1], S. GEISSHÜSLER[1], H. U. FISCH[2], U. KOELBING[2], AND P. KALIX[3]

[1]*Institute of Pharmacy*
University of Berne
CH-3012 Berne, Switzerland
[2]*Department of Psychiatry and Social Medicine*
University of Berne
CH-3010 Berne, Switzerland
[3]*Department of Pharmacology*
Centre of Medicine
University of Geneva
CH-1211 Geneva, Switzerland

ABSTRACT

A HPLC procedure is described that uses photodiode array detection (DAD) for the determination of *S*-(-)-cathinone and of its main metabolite *R,S*-(-)-norephedrine in human plasma. After addition of (±)-amphetamine as internal standard, extraction and clean-up are done on a cyano-bonded solid-phase column with water and methanol-phosphate buffer as mobile phases. HPLC analysis of plasma extracts is performed on a 3-μm ODS column with acetoni-

trile-water-phosphoric acid-hexylamine as mobile phase and detection at 192 nm. Peak identity and homogeneity are ascertained by on-line scanning of UV spectra from 190 to 300 nm. For the the differentiation of enantiomers as their diastereomers GC/MS is used. There is a linear relationship between the concentration and peak area ratio vs. internal standard ($r \geq 0.997$). The detection limit for cathinone and norephedrine is 25 and 12.5 ng/ml, respectively. The variation coefficient for inter-day precision ranges between 6.1 and 14.3%.

INTRODUCTION

Khat, a drug that consists of the fresh leaves of the evergreen shrub *Catha edulis* Forsk., is very popular in East Africa and the Arabian peninsula, and it is chewed habitually by many people. At present, khat begins to appear in certain developed countries, e.g. the United States, Great Britain, Italy and Switzerland. Phytochemical studies (1-5) and animal experiments (6,7) have shown that the phenylalkylamine *S*-(-)-cathinone (CA) is the main psychoactive alkaloid of khat, and that this substance has an amphetamine-like stimulating effect on the central nervous system. *R,S*-(-)-norephedrine (NE) and its diastereomer *S,S*-(+)-norpseudoephedrine (NPE) are always present in dried khat formed by an enzymatic reduction of CA and resulting in loss of psychotropic activity (4). Studies with humans have shown that NE is the main urinary metabolite of CA (8). Further experiments to characterize the pharmacodynamic and pharmacokinetic properties of CA in humans have recently been concluded (9).

The efficiency of HPLC-DAD in the field of analytical toxicology (10,11), forensic chemistry and phytochemistry of psychotropic drugs (12,13) as well as quality control of pharmaceutical preparations (14) has been demonstrated previously. It was the aim of this work to develop an improved high-performance liquid chromatography method with photodiode array detection (HPLC-DAD) that can be used for the pharmacokinetic profiling of CA and CA metabolite(s) in human plasma.

MATERIALS AND METHODS

Apparatus

Plasma analysis was performed on a Hewlett-Packard HPLC system (Waldbronn, F.R.G.) consisted of a 1090M liquid chromatograph, a 1090L autosampler, a 1040M photodiode array detector (DAD), a 79994A chemstation (software version 1.05), a 7470A x/y plotter and a 2225A thinkjet printer.

For the determination of enantiomers a Hewlett-Packard GC/MS system was used consisting of a 5990 gas chromatograph, a 5970 mass selective detector (MSD), a chemstation (Pascal Rev. 3.1), a 2225A thinkjet printer and a 7470A x/y plotter.

Chromatographic conditions

For the acquisition of the plasma profiles a 150 x 4.6 mm i.d. column directly coupled to a 20 x 4 mm i.d. pre-column and packed with Spherisorb 3 μm ODS-1 (Stagroma, Wallisellen, Switzerland) was used. The mobile phase was composed of 5% (v/v) acetonitrile and 95% water, containing 8.5 g/l o-phosphoric acid 85% and 140 μl/l hexylamine. The flow rate was 1 ml/min. The mobile phase was filtered under vacuum with a 0.45-μm nylon membrane filter (RC 55, Schleicher & Schuell) and degassed by sonication before and during use with a constant flow of helium. Methanol was used for column washing. All measurements were carried out at room temperature.

The separation of the enantiomers as their diastereomers was done on a J & W DB-5 bonded phase capillary column (J & W Scientific, Inc., Rancho Cordova, CA), 20 m x 0.18 mm i.d. and 0.40 μm coating that was inserted

directly into the ion source. The injector and transfer line temperature was 275 and 280°C, respectively. The oven temperature was programmed from 220 (3 min) to 280°C (7 min) at a rate of 10°C/min. The scan range was m/z 33-250, the scan rate was set at 1.97 scan/sec. Helium was used as the carrier gas at a flow of 0.7 ml/min (velocity of 49 cm/sec). The injection was done manually through a silanized split liner (split ratio 1:15) packed with 3% OV-1 on 80/100 mesh Supelcoport (Supelco SA, Gland, Switzerland) held in place with silanized glass wool.

Chemicals and Reagents

Optically pure (>98%) hydrochlorides of *S*-(-)-cathinone (CA) and *R*-(+)-cathinone were kindly donated by Dr. J.P. Wolf, Institute of Organic Chemistry, University of Berne. *R,S*-(-)-norephedrine (NE) and *S,R*-(+)-norephedrine hydrochloride were provided by Fluka Chemical Corp. (Buchs, Switzerland). *S,S*-(+)-norpseudoephedrine hydrochloride (NPE) and (±)-amphetamine sulphate (IS) were obtained from Siegfried & Co. (Zofingen, Switzerland), *R,R*-(-)-norpseudoephedrine hydrochloride and *S*-(-)-N-trifluoroacetyl prolyl chloride (TPC; 0.1 M in dichloromethane) from Sigma-Aldrich Chemical Comp. (St. Louis, MO, U.S.A.). All other chemicals and reagents were of HPLC or analytical grade and purchased from Merck and Fluka.

Solid-phase Extraction of Plasma Samples

Extraction and clean-up of human plasma samples (real, spiked, blank) were carried out using an Adsorbex SPU-Sample Preparation Unit (Merck,

Darmstadt, F.R.G.). Frozen plasma (stored at -20°C) was warmed up to room temperature in a ultrasonic bath. A 2-ml aliquot was transferred into a 2.5 ml vial and 100 ng/ml internal standard solution (IS; 10 μg/ml (±)-amphetamine sulphate in water) added. After vortexing for 1 min, the sample was applied on a Baker-10 SPE Cyano (CN) 3-ml column (P.H. Stehelin Cie AG, Basel, Switzerland) and the vial washed with 1 ml of water. The sorbent was preconditioned using 2 x 3 ml of methanol followed by 2 x 3 ml of water, not allowing to dry out at the end of the conditioning step. Plasma interferences were removed by washing the cartridge with 2 x 3 ml of water followed by centrifugation (4000 rpm/5 min). 2 x 500 μl eluent composed of 50% methanol and 50% phosphate buffer pH 3 (1.466 g sodium dihydrogenphosphate dihydrate and 0.197 g o-phosphoric acid 85% in 100 ml of water) was allowed to percolate through the column first without vacuum, then after about 15 min with slowly aspirating under vacuum. The combined eluates were concentrated to about 100 μl under a stream of nitrogen, filtered if necessary through a tip of a Pasteur pipette filled with cotton wool, and 10-μl aliquots were used for duplicate HPLC analysis. When frozen and stored at -20°C plasma samples and extracts are stable for at least 3 months.

Derivatization of Plasma Extracts and Standards for the GC/MS Determination of Enantiomers as Diastereomers

The remaining plasma extracts of each profile (about 500 μl) were combined, evaporated to dryness under a stream of nitrogen, redissolved in 100 μl methanol and filtered if necessary. The methanolic standard solutions of CA, NE and NPE enantiomers were prepared to give final concentrations of about 15

ng/μl. The on-column derivatization was made by filling 4 μl of each solution, 0.5 μl air and 1 μl TPC in a 10-μl syringe and rapidly injecting into the GC/MS.

Quantitation

Quantitation of plasma samples was done by the internal standard method, measuring the peak areas of CA, NE and IS at 192 nm. The calibration graphs (linear regression analysis) were obtained by analyzing five times pooled blank plasma spiked with 25, 50 and 100 ng/ml CA and NE and 100 ng/ml IS (aqueous solution, calculated as base). The extraction was done as described above.

Precision

The overall inter-day precision was determined by analyzing five replicates of three blank plasma samples spiked with 25, 50 and 100 ng/ml CA and NE and 100 ng/ml IS, and repeating the analysis on five different days during a two week-period. The extraction was done as described above.

Recovery Study

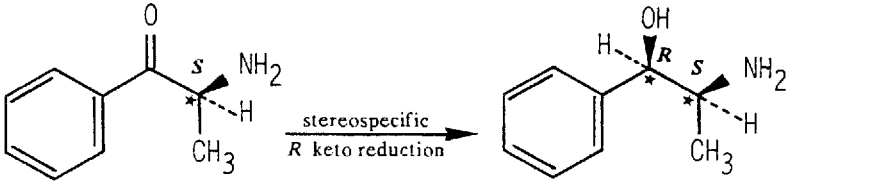
Three pooled blank plasma samples were spiked with 25, 50 and 100 ng/ml CA and NE and analyzed three times using the procedure described above except that after solid-phase extraction the eluate was concentrated to a

definite volume. The efficiency of extraction was determined by comparing the peak areas of CA and NE with those of similar aqueous standard solutions of CA and NE.

RESULTS AND DISCUSSION

CA as a ketoamine base is extremely unstable (4). Racemization and oxidative dimerization may occur during the concentration procedure in particular when CA is present as free base in basic solvents. It was therefore important to avoid any basic conditions during the *extraction procedure*. The standardized clean-up of low-volume plasma samples containing CA and its main metabolite NE (Fig. 1) can be done efficiently and rapidly by using short solid-phase extraction columns. As demonstrated in Figure 3 for the chromatogram of a pooled blank plasma extract, most of the potentially interfering endogenous matrix can be eliminated. The *recovery* for CA and NE for example at the 100 ng/ml level was determined as being 92.4 % ± 3.7% (4.0% CV, n = 6) and 88.2% ± 1.1% (1.2% CV). All data from the recovery study are summarized in Table 1.

The *chromatographic system* used was originally developed for the urine screening of cocaine and cocaine metabolites (11) as well as for the quality control of methadone preparations by HPLC-DAD (14). The efficiency of the 3-µm spherical reversed phase material is necessary for achieving symmetrical peak-shape and base-line separation of basic diastereomers like NE and NPE in complex matrices (Fig. 2, 4 and 5). Another advantage is the low UV cut-off of the mobile phase, which allows to detect at $\lambda < 200$ nm and thus to obtain the highest sensitivity for trace analysis of compounds with low ε-values at 254 nm (e.g. NE). It must also be noted that the selectivity of the chromatographic



S(-)-Cathinone (CA) R,S(-)-Norephedrine (NE)

FIGURE 1. Biotransformation of CA.

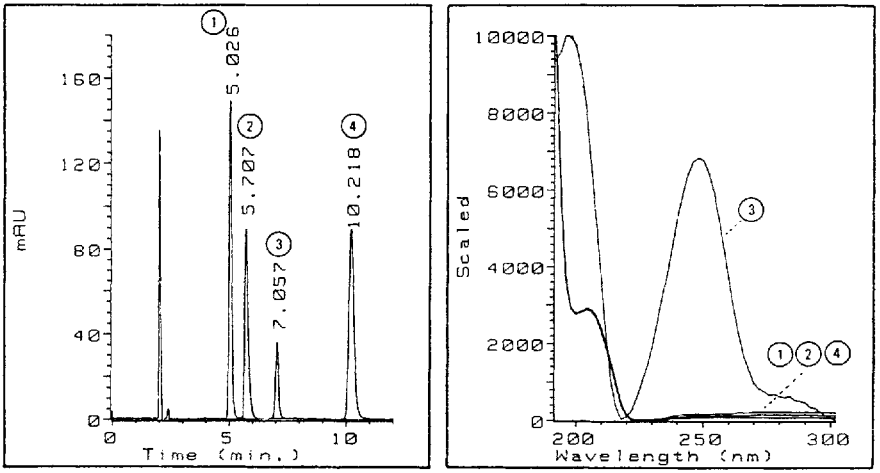


FIGURE 2. Chromatogram recorded at 192 nm and on-line photodiode array UV spectra of NE (1), NPE (2), CA (3) and IS (4). Chromatographic conditions as described under Materials and Methods.

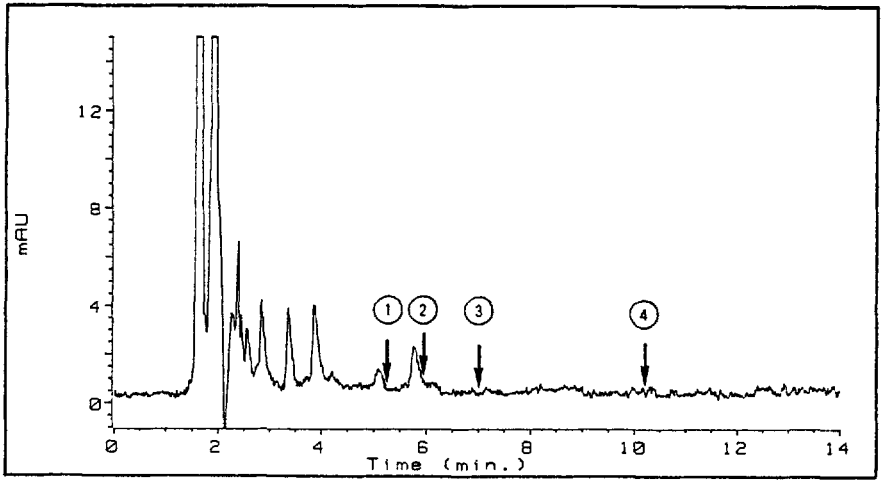


FIGURE 3. Chromatogram of pooled blank plasma. Arrows indicate the peak position of NE, NPE, CA and IS.

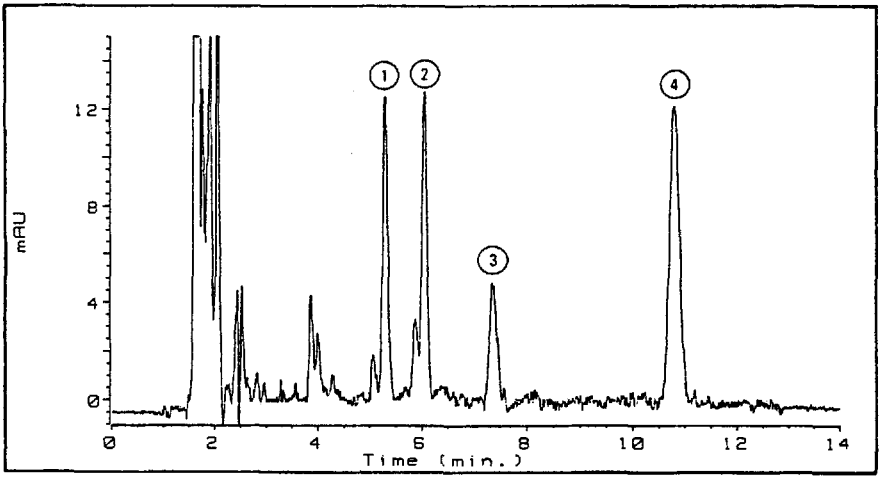


FIGURE 4. Chromatogram of pooled blank plasma spiked with 100 ng/ml NE, NPE, CA and IS. For peaks see Fig. 2.

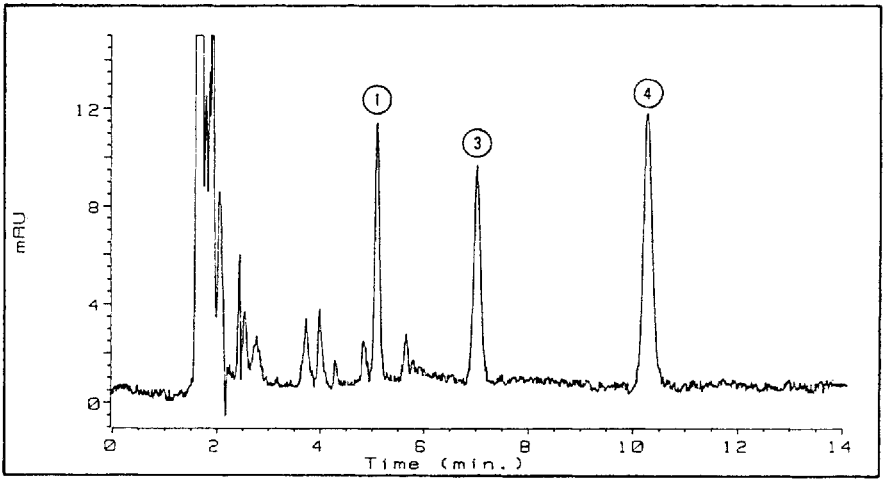


FIGURE 5. Chromatogram of a human plasma sample obtained 45 min after oral administration of 0.5 mg/kg CA.

system can be strongly affected by small changes in the ratio of the acetonitrile-water mixture as well as in the concentration of hexylamine. Hexylamine itself serves as a modifier and masking agent for residual silanol groups (15,16).

Peak *detection and identification* was done using a computer-aided high-speed spectrophotometer (photodiode array detector, DAD). Figure 2 shows the on-line UV spectra and the chromatogram of a standard mixture containing NE, NPE, CA and IS. The UV spectra of NE, NPE and IS (amphetamine) are characterized by a maximum at 205 nm. It was therefore possible to increase the sensitivity of the HPLC-DAD system for the detection of NE and NPE at 192 nm (log ε_{192}: 10.5) by a factor of about 200 as compared for example with the one at 257 nm (log ε_{257}: 5.2). This had been the detection wavelength used in an earlier study for monitoring the psychotropic phenylalkylamines in khat samples (5). The UV spectrum of CA shows maxima at 196 (log ε_{196}: 9.7) and 247 nm (log ε_{247}: 9.5). The high UV absorbance at 247 nm is due to the benzoyl

TABLE 1

Recovery of CA and NE in plasma

Spiked plasma		Amount determined ng/ml (mean; n = 6)	Recovery (%)	± SD	CV%
25 ng/ml	CA	16.8	67.0	6.6	9.8
	NE	20.0	80.0	2.5	3.1
50 ng/ml	CA	36.4	72.8	6.9	9.5
	NE	41.6	83.2	2.5	3.0
100 ng/ml	CA	92.4	92.4	3.7	4.0
	NE	88.2	88.2	1.1	1.2

TABLE 2

Inter-day precision of CA and NE in plasma

Spiked plasma (+ 100 ng/ml IS)		Concentration determined ng/ml (mean; n = 4)	± SD	CV%
25 ng/ml	CA	25.0	3.6	14.3
	NE	25.0	3.2	12.9
50 ng/ml	CA	49.4	3.6	7.3
	NE	47.3	4.3	9.1
100 ng/ml	CA	99.6	6.1	6.1
	NE	101.6	6.1	6.1

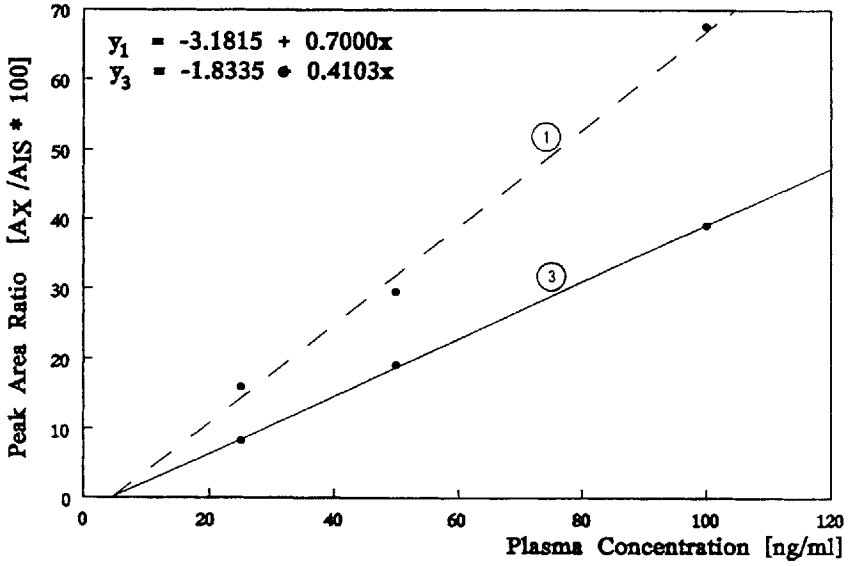


FIGURE 6. Calibration curve of CA (3) and NE (1) in plasma.

moiety of CA. An indication for peak homogeneity was given by the peak purity check (part of workstation software) which demonstrated an up-slope, apex and down-slope peak spectra match of > 990 for NE, CA and IS. The *detection limit* of NE at 192 nm and a signal-to-noise ratio of 6:1 was 2.5 ng (corresponding to 12.5 ng NE per ml plasma), whereas 5 ng (25 ng/ml plasma) was the minimal detectable amount of CA. The *linearity* was checked in the range of 25-100 ng/ml CA and NE (Fig. 6). A linear relationship was found between the peak-area ratio of CA and NE vs. IS and the concentration of CA and NE. The correlation coefficient r of CA and NE was 0.9998 and 0.9970, respectively. A satisfactory inter-day *precision* of the method for CA and NE quantitation was found at the low (25 ng/ml), medium (50 ng/ml) and high concentration level (100 ng/ml); the variation data are summarized in Table 2.

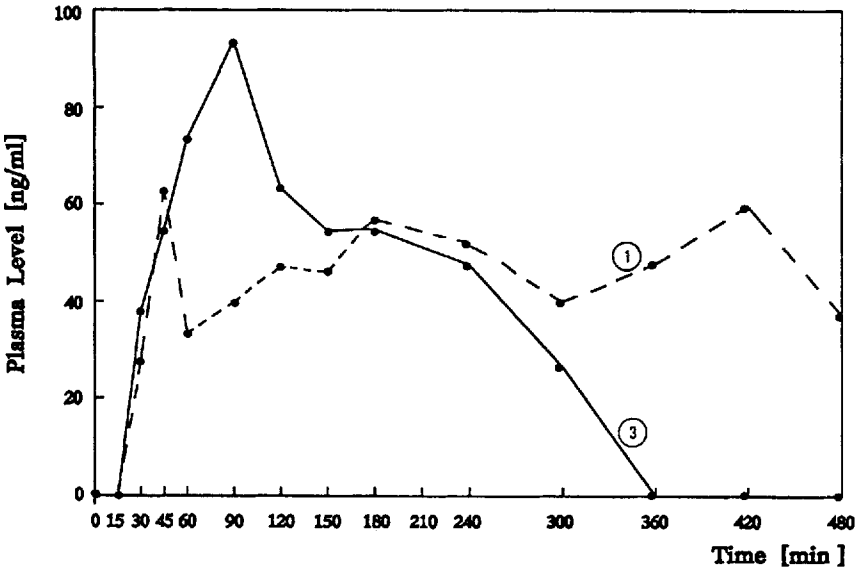


FIGURE 7. Plasma level of CA (3) and NE (1) vs. time following a single oral dose of 0.5 mg/kg CA in a healthy volunteer.

Figure 7 shows a typical *plasma profile* of CA and NE observed after oral administration of 0.5 mg/kg CA to a healthy male volunteer. CA is rapidly absorbed and is detectable in the plasma for about 300 min. Already 30 min after the administration of CA, its main metabolite NE appears in the plasma and fluctuates then between 30 and 70 ng/ml during the remainder of the test period. Design and results of the clinical study with CA in humans will be published elsewhere (9).

A direct chromatographic *determination of the enantiomers* of CA and NE in plasma samples by chiral HPLC could not be achieved. The system tested was a 100 x 4.0 mm chiral α_1-acid glycoprotein column (Chiral-AGP; Stagroma, Wallisellen, Switzerland) with 0.01M phosphate buffer pH 7-7.1 as mobile phase. The column did separate CA enantiomers in standard solutions, but was

found very sensitive to matrix interferences and overloading and thus inadequate for the analysis of plasma samples. Even by changing the mobile phase the enantiomers of NE and NPE could not be resolved. Instead, the determination of CA and NE isomers was performed by GC/MS after on-column derivatization with chiral TPC to the corresponding diastereomers. By comparing with pure standards it could be shown that CA in plasma has *S* configuration (CA-TPC: m/z 237, 194, 166, 105; t_R 7.10 min) and that the main metabolite of CA is the *R,S* configurated (-)-norephedrine (NE-TPC: m/z 238, 237, 194, 166, 139; t_R 7.14 min). The TPC derivatives of *R*-(+)-cathinone (t_R 6.62 min), *S,R*-(+)-norephedrine (t_R 6.87 min), *S,S*-(+)-norpseudoephedrine (t_R 7.21 min) and *R,R*-(-)-norpseudoephedrine (t_R 6.96 min) were not found in the plasma samples. This confirms our earlier postulate (8) that CA is mainly metabolized by a stereospecific *R* keto reduction to the *R,S* configurated (-)-aminoalcohol (Fig. 1). The absence of *R*-(+)-cathinone and the corresponding metabolite *R,R*-(-)-norpseudoephedrine shows that during absorption and distribution in the human body, CA is not racemized, or at least not to a detectable degree.

ACKNOWLEDGEMENTS

We thank Daniel Bourquin and Irene Schmid for technical assistance and Dr. J.P. Wolf for providing the cathinone isomers. Part of this work was supported by the Professor E. Steinegger Foundation for Medicinal Plant Research and the Foundation for Narcotics Research of the Swiss Federal Office of Public Health.

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