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Determination of (*S*)-(−)-cathinone and its metabolites (*R,S*)-(−)-norephedrine and (*R,R*)-(−)-norpseudoephedrine in urine by high-performance liquid chromatography with photodiode-array detection

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ABSTRACT

A high-performance liquid chromatographic (HPLC) procedure with photodiode-array detection (DAD) is described for the determination of (*S*)-(−)-cathinone (*S*-CA) and its metabolites (*R,S*)-(−)-norephedrine (*R*-NE) and (*R,R*)-(−)-norpseudoephedrine (*R*-NPE) in urine. Extraction and clean-up of 1-ml urine samples were performed on a cyano-bonded solid-phase column using (±)-amphetamine as internal standard. The concentrated extracts were separated on a 3-μm ODS-1 column with acetonitrile–water–phosphoric acid–hexylamine as the mobile phase. Peak detection was done at 192 nm. The detection limits for *S*-CA and *R*-NE/*R*-NPE in urine were 50 and 25 ng/ml, respectively. The differentiation of the enantiomers of cathinone and norephedrine was achieved by derivatization with (*S*)-(−)-1-phenylethyl isocyanate to the corresponding diastereomers followed by HPLC–DAD on a 5-μm normal-phase column. The *R* and *S* enantiomers of norpseudoephedrine were determined by gas chromatography–mass spectrometry after on-column derivatization with (*S*)-(−)-N-trifluoroacetylprolyl chloride. Following a single oral dose of 0.5 mg/kg of *S*-CA, the concentrations found in urine ranged from 0.2 to 3.8 μg/ml of *S*-CA, from 7.2 to 46.0 μg/ml of *R*-NE and from 0.5 to 2.5 μg/ml of *R*-NPE.

INTRODUCTION

Khat, the leaves or short tops of the evergreen shrub *Catha edulis* Forsk., is very popular in East Africa and the Arabian peninsula, where it is habitually chewed for its stimulating effects. As only fresh leaves are active, the habit of chewing khat has been confined to the areas where the plant grows. Mainly owing to the possibility of air transportation, fresh khat has recently been introduced in certain developed countries, *e.g.*, USA, UK, Italy and Switzerland.

Phytochemical studies [1–4] and animal experiments [5,6] have shown that the phenylalkylamine (*S*)-(−)-cathinone (see Fig. 7: *S*-CA) is the main psychoactive alkaloid of khat. Since 1985, *S*-CA has been scheduled as an internationally controlled substance. An experiment to study the effects and pharmacokinetic characteristics of *S*-CA in humans has recently been completed [7]. Therefore, a method for the pharmacokinetic profiling of *S*-CA and its main metabolite (*R,S*)-(−)-norephedrine (*R*-NE) in human plasma has been developed [8]. An earlier study on the metabolism of *S*-CA using 24-h urine samples showed *R*-NE and its corresponding diastereomer (*R,R*)-(−)-norpseudoephedrine (*R*-NPE) in a ratio of 8:1 to be the main metabolites of *S*-CA [9]. In that study it was not clear whether the occurrence of *R*-NPE was due to the use of optically impure *S*-CA or the partial racemization of *S*-CA to (*R*)-(+)-cathinone (*R*-CA) during absorption and partition.

The purpose of this work was to develop a method for the rapid and sensitive clean-up of small urine samples and an improved high-performance liquid chromatographic (HPLC) method with pho-

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todiode-array detection (DAD) for the pharmacokinetic profiling of *S*-CA and its metabolites in human urine.

EXPERIMENTAL

Instrumentation

Urine analyses were performed on a Hewlett-Packard (Waldbronn, Germany) HPLC system consisting of a Model 1090M liquid chromatograph, a Model 1090L autosampler, a Model 1040M photodiode-array detector, a Model 79994A Chemstation (software version 1.05), a Model 7470A *x–y* plotter and a Model 2225A Thinkjet printer.

For the determination of the *S* and *R* enantiomers of cathinone and norephedrine the same system was used, whereas the determination of the enantiomers of norpseudoephedrine was performed on a Hewlett-Packard gas chromatographic–mass spectrometric (GC–MS) system consisting of a Model 5990 gas chromatograph, a Model 5970 mass-selective detector, a Chemstation (Pascal Rev. 3.1), a Model 2225A Thinkjet printer and a Model 7470A *x–y* plotter.

Chromatographic conditions

For the analysis of urine samples a 150 mm × 4.6 mm I.D. column directly coupled to a 20 mm × 4 mm I.D. precolumn and packed with Spherisorb 3-μm ODS-1 (Stagroma, Wallisellen, Switzerland) was used. The mobile phase was acetonitrile–water (8.5:91.5, v/v) containing 8.5 g/l of orthophosphoric acid (85%) and 200 μl/l of 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 & Schüll) and degassed by sonication before use and with a constant flow of helium during use. Methanol was used for column washing. All measurements were carried out at room temperature. Peak detection was done at 192 nm, and peak identity and homogeneity were ascertained by on-line scanning of UV spectra from 190 to 300 nm.

The separation of the *S* and *R* enantiomers of cathinone and norephedrine as their diastereomers was done on a 250 mm × 4.6 mm I.D. column packed with LiChrosorb Si 60, 5 μm (Merck, Basel, Switzerland). The mobile phase was isooctane–isopropanol–acetic acid (>99.5%) (90:9:1, v/v/v). The flow-rate was 1 ml/min. Peak detection of the enantiomers after derivatization was done simultaneously at 254 and 236 nm.

The separation of the enantiomers of norephedrine and norpseudoephedrine was done after on column derivatization on a J & W DB-5 bonded-phase capillary column (J & W Scientific, Rancho Cordova, CA, USA), 20 m × 0.18 mm I.D. with a 0.40-μm coating, that was inserted directly into the ion source. The injector and transfer line temperatures were 275 and 280°C, respectively. The oven temperature was programmed from 220°C (held for 3 min) at 10°C/min to 280°C (held for 7 min). The scan range was *m/z* 33–250, and the scan rate was set at 1.97 scans/s. Helium was used as the carrier gas at a flow-rate of 0.7 ml/min (velocity 49 cm/s). Injection was done manually through a silanized split liner (split ratio 1:15) packed with 3% OV-1 on 80–100-mesh Supelcoport (Supelco, Gland, Switzerland) held in place with silanized glass-wool.

Chemicals and reagents

Optically pure (>98%) hydrochlorides of (*S*)-(−)-cathinone (*S*-CA) and (*R*)-(+)-cathinone (*R*-CA) were kindly donated by Dr. J. P. Wolf (Institute of Organic Chemistry, University of Berne). (*R,S*)-(−)-Norephedrine hydrochloride (*R*-NE), (*S,R*)-(+)-norephedrine (*S*-NE) and (*S*)-(−)-1-phenylethyl isocyanate (PEIC, >98%) were provided by Fluka (Buchs, Switzerland). (*S,S*)-(+)-Norpseudoephedrine hydrochloride (*S*-NPE) and (±)-amphetamine sulphate were obtained from Siegfried (Zofingen, Switzerland) and (*R,R*)-(−)-norpseudoephedrine hydrochloride (*R*-NPE) and (*S*)-(−)-N-trifluoroacetylprolyl chloride (TPC; 0.1 *M* in dichloromethane) from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals and reagents were of HPLC or analytical-reagent grade from Merck and Fluka.

Solid-phase extraction of urine samples

Extraction and clean-up of urine samples were carried out using an Adsorbex SPU sample preparation unit (Merck, Darmstadt, Germany). Frozen urine (stored at −20°C) was warmed to room temperature in an ultrasonic bath. After centrifugation (2000 *g*, for 5 min) a 1-ml aliquot was transferred into a 2.5-ml vial and 2 μg/ml of internal standard

solution [I.S.; 200 μg/ml (±)-amphetamine sulphate in water] were added. After vortex mixing for 1 min the sample was applied to a Baker-10 SPE cyano (CN) 3-ml column (Stehelin, Basel, Switzerland) and the vial washed with 1 ml of water. The sorbent was preconditioned using 2 × 3 ml of methanol followed by 2 × 3 ml of water, and was not allowed to dry out at the end of the conditioning step. Urine interferences were removed by washing the cartridge with 3 × 3 ml of water followed by drying the column for about 1 min under vacuum. For the elution 2 × 500 μl of phosphate buffer (pH 3) [1.466 g of sodium dihydrogenphosphate dihydrate and 0.197 g of orthophosphoric acid (85%) in 100 ml of water] and 2 × 500 μl of eluent consisting of methanol–phosphate buffer (pH 3) (50:50, v/v) were used. The eluents were allowed to percolate through the column first without vacuum and for complete elution with slow aspiration under vacuum. The combined eluates were concentrated to about 100 μl under a stream of nitrogen (which took about 1 h, but parallel working was possible), filtered if necessary through the tip of a Pasteur pipette filled with cotton-wool, and 10-μl aliquots were used for duplicate HPLC analyses. When frozen and stored at −20°C, urine samples and extracts were stable for at least 3 months.

Derivatization of urine extracts and standards for the determination of enantiomers as diastereomers

The urine extracts from one volunteer (about 300 μl) were combined, evaporated to dryness and the residue dissolved in 150 μl of tetrahydrofuran. After filtration through the tip of a Pasteur pipette filled with cotton-wool, 10 μl of PEIC and 5 μl of triethylamine were added. Immediately after vortex mixing the sample in an ultrasonic bath for 5 min, 10-μl aliquots were used for HPLC analysis. The PEIC derivatives were stable for only about 1 h. The minimum amounts of isomers of cathinone and norephedrine/norpseudoephedrine for the derivatization to take place were 10 and 100 μg, respectively.

For the determination of the *R* and *S* enantiomers of norpseudoephedrine, the extract of one urine sample was evaporated to dryness and the residue dissolved in 50 μl of methanol. Methanolic standard solutions of *R*-NE, *S*-NE, *R*-NPE and *S*-NPE were prepared to give a final concentration of about 20 ng/μl. On-column derivatization was effected by taking up 4 μl of each solution, 0.5 μl of air and 1 μl of TPC in a 10-μl syringe and rapidly injecting the mixture into the GC–MS system.

Quantitation

Urine samples were analyzed by the internal standard method, measuring the peak areas of *S*-CA, *R*-NE, *R*-NPE and the I.S. at 192 nm. Calibration graphs (linear regression analysis) were obtained by analyzing four times pooled blank urine spiked with 0.25, 0.75, 2.00, 6.00, 10.00 and 15.00 μg/ml of *S*-CA, *R*-NE and *R*-NPE and 2.00 μg/ml of the I.S. (aqueous solution, calculated as base). The extractions were done as described above.

Precision

The inter-day precision was determined by analyzing three replicates of three blank urine samples spiked with 0.75, 2.00 and 10.00 μg/ml of *S*-CA, *R*-NE and *R*-NPE and 2.00 μg/ml of the I.S. Duplicate analyses were repeated on three different days during a 2-week period. The extractions were performed as described above.

Recovery

Six pooled blank urine samples were spiked with 0.25, 0.75, 2.00, 6.00, 10.00 and 15.00 μg/ml of *S*-CA, *R*-NE and *R*-NPE and analyzed using the procedure described above. After the solid-phase extraction the eluates were concentrated to a definite volume. The efficiency of extraction was determined by comparing the peak areas of *S*-CA, *R*-NE and *R*-NPE with those of similar aqueous standard solutions.

RESULTS AND DISCUSSION

The sample clean-up of low urine volumes containing *S*-CA and its main metabolites *R*-NE and *R*-NPE can be done rapidly and effectively by the use of short cyano-bonded solid-phase extraction columns. The standardized extraction procedure avoids any basic conditions which could cause racemization or oxidative dimerization of the unstable ketoamine *S*-CA [3]. As demonstrated with the chromatogram of a pooled blank urine extract (Fig. 1), most of the interfering endogenous matrix can be eliminated. The recovery of *S*-CA and its metabolites at the 6 μg/ml level was determined to be 87.4

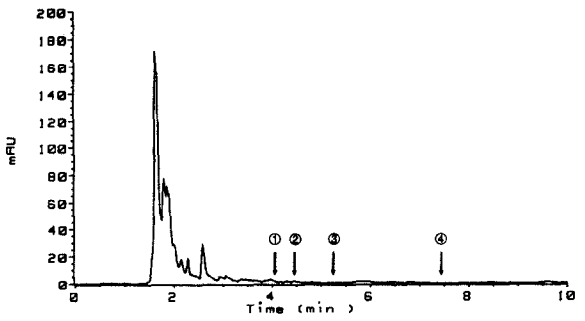

Fig. 1. Chromatogram of an extract of pooled blank urine recorded at 192 nm. Arrows indicate the peak position of *R*-NE (1), *R*-NPE (2), *S*-CA (3) and the I.S. (4). For chromatographic conditions, see Experimental.

± 2.0% (relative standard deviation, R.S.D. = 2.3%, n = 6) for *S*-CA, 90.2 ± 1.5% (R.S.D. = 1.7%) for *R*-NE and 97.9 ± 3.3% (R.S.D. = 3.4%) for *R*-NPE. All data from the recovery study are summarized in Table I. The recovery of amphetamine (I.S.) at the 2 μg/ml level was 99.2 ± 2.4% (R.S.D. = 2.4%, n = 6). Therefore, the method described could also be used for the determination of amphetamine and its derivatives in human urine.

The chromatographic system used was originally developed for screening urine for cocaine and its metabolites [10]. Hexylamine serves as a modifier and masking agent for residual silanol groups [11]. By making small changes in the concentration of hexylamine and in the ratio of the acetonitrile–water mixture, the potentially interfering matrix, which was not eliminated by the sample clean-up, could be separated chromatographically from the compounds of interest (Figs. 2 and 3).

For peak detection DAD was used. Peak homogeneity was ascertained by a peak purity check (part of the workstation software, up-slope, apex and down-slope peak spectra match). Owing to the very low UV cut-off of the mobile phase, it was possible to use 192 nm as the detection wavelength and to obtain a very high sensitivity. Fig. 4 shows the on-line UV spectra and the chromatogram of a standard mixture containing *S*-CA, *R*-NE, *R*-NPE and the I.S. At 192 nm and a signal-to-noise ratio of 5 the detection limit for *S*-CA was 5 ng (corresponding to 50 ng/ml), whereas 2.5 ng (25 ng/ml) was the minimum detectable amount of *R*-NE and *R*-NPE ($\log \varepsilon_{192}$ = 10.5).

Human urine samples were collected from six

TABLE I

RECOVERY OF *S*-CA, *R*-NE AND *R*-NPE FROM HUMAN URINE

Spiked urine (μg/ml)	Compound	Mean amount determined (μg/ml) (n = 6)	Recovery ± S.D. (%)	R.S.D. (%)
0.25	*S*-CA	0.20	78.7 ± 3.5	4.0
	R-NE	0.22	89.5 ± 4.9	5.5
	R-NPE	0.25	100.2 ± 7.9	7.9
0.75	*S*-CA	0.54	72.5 ± 2.0	2.8
	R-NE	0.62	82.5 ± 0.6	0.7
	R-NPE	0.70	93.6 ± 1.5	1.6
2.00	*S*-CA	1.45	72.3 ± 4.4	6.1
	R-NE	1.70	85.1 ± 1.6	1.9
	R-NPE	1.96	97.9 ± 2.2	2.3
6.00	*S*-CA	5.24	87.4 ± 2.0	2.3
	R-NE	5.41	90.2 ± 1.5	1.7
	R-NPE	5.87	97.9 ± 3.3	3.4
10.00	*S*-CA	9.49	94.9 ± 5.8	6.1
	R-NE	8.83	88.3 ± 3.5	4.0
	R-NPE	9.80	98.0 ± 10.8	11.0
15.00	*S*-CA	13.94	93.0 ± 5.0	5.4
	R-NE	12.40	82.6 ± 3.5	4.2
	R-NPE	14.73	98.2 ± 5.2	5.3

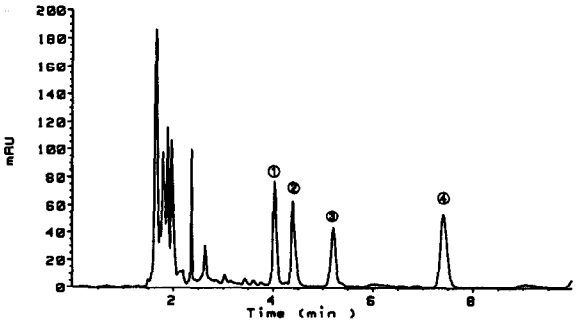

Fig. 2. Chromatogram of an extract of pooled blank urine spiked with 2 μg/ml each of *R*-NE (1), *R*-NPE (2), *S*-CA (3) and the I.S. (4).

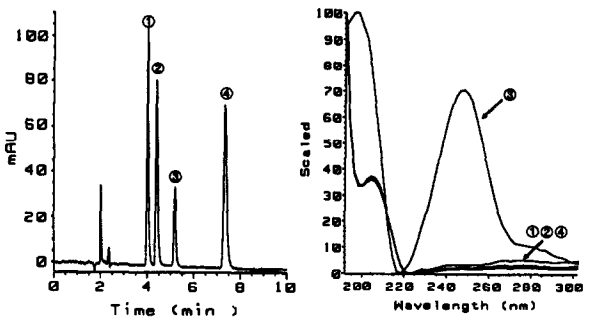

Fig. 4. Chromatogram and on-line DAD UV spectra of *R*-NE, *R*-NPE, *S*-CA and the I.S. Peaks as in Fig. 2.

male volunteers 2, 4, 6 and 8 h after the oral administration of 0.5 mg/kg of *S*-CA. Before admission to the experiment the subjects underwent medical and psychiatric examination and had agreed of refrain from any psychotropic drugs and medication for the 3 weeks preceding the experiment. The urine levels ranged from 0.2 to 3.8 μg/ml of *S*-CA, from 7.2 to 46.0 μg/ml of *R*-NE and from 0.5 to 2.5 μg/ml of *R*-NPE. Urine samples containing >20μg/ml of *R*-NE were reanalyzed after dilution with water (1:1) before the extraction. The *R*-NE/*R*-NPE ratio ranged from about 10 to 23. The excretion patterns are summarized in Table II.

The linearity between the peak-area ratios of *S*-CA, *R*-NE and *R*-NPE *vs.* the I.S. and the urinary concentrations of *S*-CA, *R*-NE and *R*-NPE was checked in the range 0.25–15.00 μg/ml. The correlation coefficient (*r*) for *S*-CA, *R*-NE and *R*-NPE was 0.9982, 0.9996 and 0.9994, respectively (Fig. 5). The

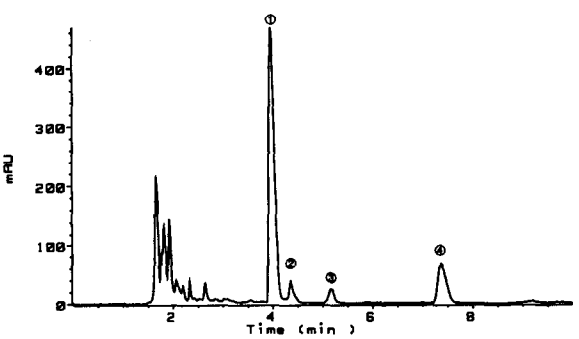

Fig. 3. Chromatogram of an extract of a human urine sample obtained 4 h after oral administration of 0.5 mg/kg of *S*-CA. Peaks as in Fig. 2.

TABLE II

EXCRETION OF *S*-CA AND ITS METABOLITES *R*-NE AND *R*-NPE IN HUMAN URINE AFTER ORAL ADMINISTRATION OF 0.5 mg/kg OF *S*-CA

Subject	Time after administration (h)	Compounds excreted in urine (μg/ml)			*R*-NE/*R*-NPE ratio
		S-CA[a]	*R*-NE	*R*-NPE	
1	2	n.d.	9.09	0.50	18.1
	4	1.79	40.68	2.34	17.1
	6	n.d.	16.44	1.03	16.0
	8	n.d.	14.53	1.14	12.8
2	2	0.20	17.08	1.05	16.3
	4	1.72	26.84	1.76	15.2
	6	0.78	18.80	1.54	12.2
	8	0.70	14.29	1.36	10.5
3	2	1.73	46.04	2.49	18.5
	4	3.81	30.48	2.01	15.2
	6	1.06	15.11	1.24	12.1
	8	0.16	12.59	1.16	10.9
4	2	0.27	28.04	1.22	23.0
	4	0.90	37.31	2.02	18.5
	6	0.70	32.48	1.92	16.9
	8	n.d.	27.40	2.40	11.4
5	2	0.24	8.26	0.54	15.4
	4	0.30	10.16	0.77	13.3
	6	0.31	7.67	0.64	12.1
	8	0.30	11.71	1.05	11.2
6	2	2.92	16.62	1.22	13.6
	4	1.99	7.20	0.64	11.2
	6	0.28	19.64	1.55	12.7
	8	n.d.	15.48	1.54	10.1

[a] n.d. = Not detectable (below detection limit).

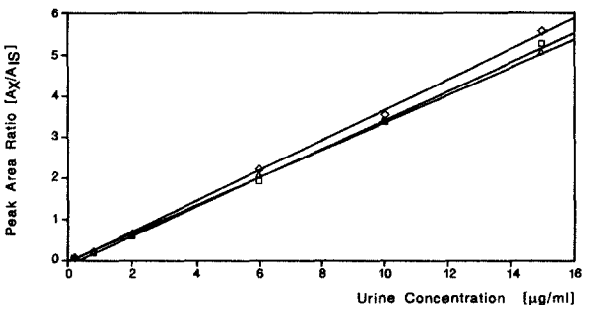

Fig. 5. Calibration graphs for (□) *R*-NE, (△) *R*-NPE and (◇) *S*-CA extracted from urine.

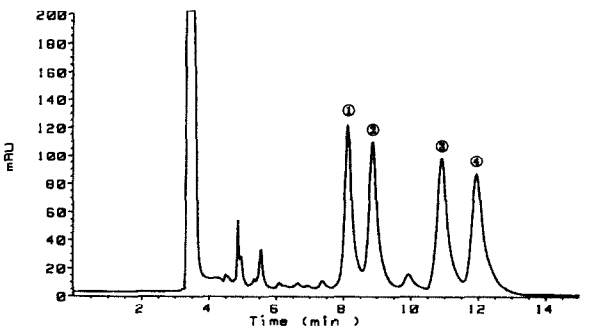

Fig. 6. Chromatogram of the PEIC derivatives of *S*-CA (1), *R*-CA (2), *R*-NE (3) and *S*-NE (4). For chromatographic conditions, see Experimental.

inter-day precision measured at low (0.75 µg/ml), medium (2.00 µg/ml) and high (15.00 µg/ml) concentration levels is summarized in Table III.

The determination of the enantiomers in urine by derivatization with PEIC was only practicable for cathinone and norephedrine and not for the very low concentrated norpseudoephedrine, as at least 100 µg of the amino alcohols was necessary for the reaction to take place. By comparing the results of the derivatization with the chromatogram for a standard solution containing derivatized *S*-CA, *R*-CA, *R*-NE and *S*-NE (Fig. 6), the enantiomers in urine samples were found to be *S*-CA and *R*-NE. The determination of the *R*- and *S*-NPE enantiomers was performed by GC–MS after on-column derivatization with chiral TPC to the corresponding diastereomers. To ensure that there was no interference with the simultaneously derivatized *R*-NE, the results were compared with those for a derivatized standard solution containing *R*-NE (*R*-NE–TPC: *m/z* 238, 237, 194, 166, 139; t_R = 7.14 min), *S*-NE (*S*-NE–TPC: t_R = 6.87 min), *R*-NPE (*R*-NPE–TPC: *m/z* 238, 237, 194, 166, 139; t_R = 6.96 min) and *S*-NPE (*S*-NPE–TPC: t_R = 7.21 min). The enantiomers found in the urine samples were *R*-NPE and *R*-NE. Therefore, it could be confirmed that, as shown in Fig. 7, orally administered *S*-CA is metabolized by a stereospecific 1*R* keto reduction to the corresponding amino alcohols [9].

As in this study the *S*-CA administered was optically pure (>98%) the simultaneous occurrence of

TABLE III

INTER-DAY PRECISION OF THE ASSAY FOR *S*-CA, *R*-NE AND *R*-NPE EXTRACTED FROM HUMAN URINE

Spiked urine (µg/ml)	Compound	Mean concentration determined ± S.D. (µg/ml) (n=6)	R.S.D. (%)
0.75	*S*-CA	0.77 ± 0.02	2.98
	R-NE	0.76 ± 0.04	4.63
	R-NPE	0.77 ± 0.02	2.87
2.00	*S*-CA	1.99 ± 0.06	2.97
	R-NE	1.92 ± 0.14	7.25
	R-NPE	1.97 ± 0.03	1.68
10.00	*S*-CA	10.20 ± 0.13	1.28
	R-NE	9.73 ± 0.18	1.82
	R-NPE	9.69 ± 0.13	1.31

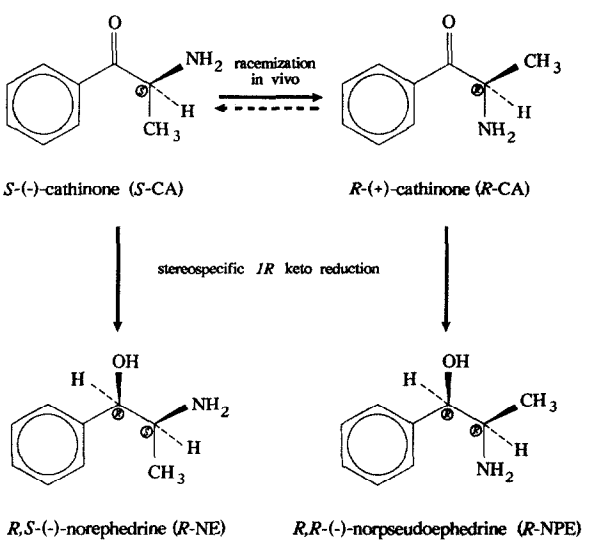

Fig. 7. Biotransformation of *S*-CA.

the corresponding diastereomeric amino alcohol *R*-NPE is probably the result of partial racemization of *S*-CA to *R*-CA during absorption and partition. The significant decrease in the *R*-NE/*R*-NPE ratio (see Table II) after oral administration also confirms this hypothesis. As only a small part (< 10%) of the administered *S*-CA is racemized, the presence of *R*-CA in urine samples could not be proved. Compared with the concentration of *R*-NE, the concentration of *R*-NPE found in human urine is very low (< 10%). This could explain why *R*-NPE (detection limit 12.5 ng/ml) could not be found in human plasma samples, where the maximum concentration of *R*-NE after oral administration was about 100 ng/ml [8].

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