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Enantioselective quantitation of the ecstasy compound (*R*)- and (*S*)-*N*-ethyl-3,4-methylenedioxyamphetamine and its major metabolites in human plasma and urine

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Abstract

An enantioselective HPLC method has been developed and validated for the stereospecific analysis of *N*-ethyl-3,4-methylenedioxyamphetamine (MDE) and its major metabolites *N*-ethyl-4-hydroxy-3-methoxyamphetamine (HME) and 3,4-methylenedioxyamphetamine (MDA). These compounds have been analyzed both from human plasma and urine after administration of 70 mg pure MDE-hydrochloride enantiomers to four subjects. The samples were prepared by hydrolysis of the *o*-glucuronate and sulfate conjugates using β-glucuronidase/arylsulfatase and solid-phase extraction with a cation-exchange phase. A chiral stationary protein phase (chiral-CBH) was used for the stereoselective determination of MDE, HME and MDA in a single HPLC run using sodium dihydrogenphosphate, ethylendiaminetetraacetic acid disodium salt and isopropanol as the mobile phase (pH 6.44) and fluorimetric detection (λ_{ex} 286 nm, λ_{em} 322 nm). Moreover, a suitable internal standard (*N*-ethyl-3,4-methylenedioxybenzylamine) was synthesized and qualified for quantitation purposes. The method showed high recovery rates (>95%) and limits of quantitation for MDE and MDA of 5 ng/ml and for HME of 10 ng/ml. The RSDs for all working ranges of MDE, MDA and HME in plasma and urine, respectively, were less than 1.5%. After validation of the analytical methods in plasma and urine samples pharmacokinetic parameters were calculated. The plasma concentrations of (*R*)-MDE exceeded those of the *S*-enantiomer (ratio *R*:*S* of the area under the curve, 3.1) and the plasma half time of (*R*)-MDE was longer than that of (*S*)-MDE (7.9 vs. 4.0 h). In contrast, the stereochemical disposition of the MDE metabolites HME and MDA was reversed. Concentrations of the (*S*)-metabolites in plasma of volunteers were much higher than those of the (*R*)-enantiomers.

Keywords: Enantiomer separation; Ecstasy; *N*-Ethyl-3,4-methylenedioxyamphetamine

1. Introduction

Ecstasy compounds such as *N*-methyl-3,4-methylenedioxyamphetamine (MDMA, street name ''Ecstasy'', ''Adam''), *N*-ethyl-3,4-methylenedioxyam-

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phetamine (MDE, ''Eve'') and 3,4-methylenedioxyamphetamine (MDA) are the most commonly abused illicit recreational drugs in the western world and their use has increased among young people since the late 1980s [1]. The relevant subjective effects that are described by users are different from classic hallucinogens (e.g., lysergic acid diethylamide, LSD) and stimulants (e.g., amphetamine) and consist of alteration in affect, perception, cognitive style (e.g., ''oceanic boundlessness''), and peaceful experiences of emotional closeness [2]. Controlled double blind clinical trials with healthy volunteers confirmed these observations [1,3–6]. Although abusers commonly believe in the innocuousness of these drugs, acute somatic adverse effects (e.g., hyperthermia, cardiovascular complications, renal and hepatic failure) occasionally even leading to death [7–12] as well as unwanted neuropsychiatric reactions [11,12] are increasingly reported. Pharmacologically these drugs affect both the catecholaminergic and the serotonergic system primarily through indirect monoaminergic mechanisms [13].

Ecstasy compounds act as entactogens [14] and have been used in psychiatric research in human experiments. MDE possesses a lower neurotoxicity than MDMA [15], hence MDE was used as ecstasy-analogue in several human studies [16,17]. We have already clarified the metabolic pathway of MDE in humans by means of gas chromatography–mass spectrometry (GC–MS) (Fig. 1) [16]. The biotransformation of MDE elucidates two routes of metabolism. On the one side there is degradation of the side chain by N-dealkylation to MDA and on the other side the methylenedioxy group is cleaved. This yields first the dihydroxymetabolite, which is then methylated in the 3 position of the aromatic ring

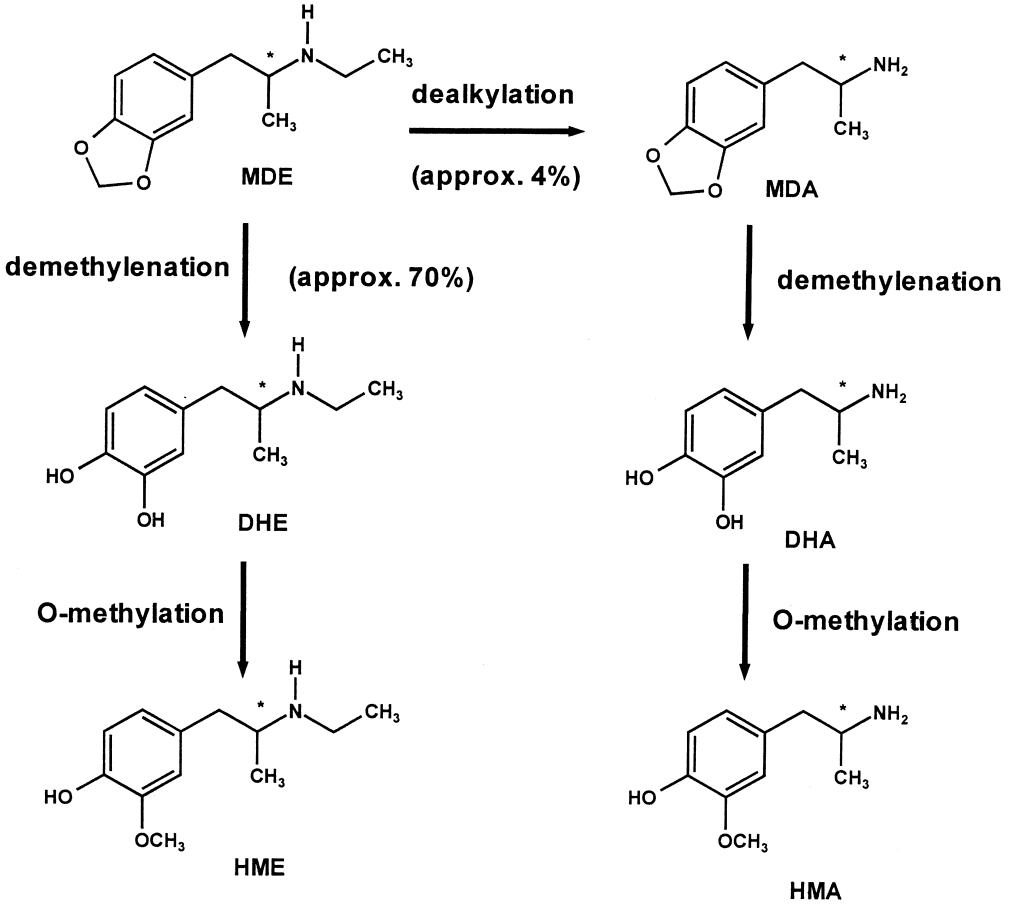

Fig. 1. Partial scheme of MDE metabolism [16].

yielding HME. Also, we have identified the human cytochromes P450 involved in the oxidative metabolism of ecstasy-related designer drugs. CYP2D6 is the most important enzyme for demethylenenation [18], but also CYP1A2 and CYP3A4+b5 are contributing to the metabolism. Dealkylation is mainly catalyzed by CYP1A2 and CYP2B6 [19]. Furthermore, we found a stereoselective metabolism after administration of racemic MDE and its metabolites in plasma. Significant differences were found in plasma concentrations: (*R*)-MDE was predominant over (*S*)-MDE, whereas the (*S*)-configured metabolites HME and MDA were showing higher plasma levels than (*R*)-HME and (*R*)-MDA [20]. This is in agreement with the results found by Fallon et al. [21] and Moore et al. [22] after administration of racemic MDMA: (*S*)-MDMA has a reduced area under the curve (AUC) and shorter half-life than (*R*)-MDMA [21,22]. In the study of Brunnenberg [20], plasma collection time was too short for the calculation of pharmacokinetic parameters of (*R*)- and (*S*)-MDE; Fallon et al. [21] did not determine the major hydroxy-methoxy metabolite HMMA. Enantioselective determination of MDMA in human fluids was also carried out by De Boer et al. [23], Lanz et al. [24] and Tagliaro et al. [25]. Stereospecific analysis of methylendioxyamphetamines in rodents is described by Fizgerald et al. [26], Cho et al. [27] and Hiramatsu and Cho [28]. The enantioselective cognitive and brain activation effects of (*R*)- and (*S*)-MDE in humans, is reported by Spitzer et al. [29].

To our knowledge, the pharmacokinetic properties after administration of the pure enantiomers of MDE in humans have not yet been reported in literature. In the present study we have developed a stereospecific high-performance liquid chromatography (HPLC) method for determination of MDE, HME and MDA in plasma and urine following oral administration of (*R*)- and (*S*)-MDE-HCl, respectively. In this paper, we report the enantioselective disposition of MDE and its major metabolites in humans.

2. Experimental

2.1. Materials

Acetonitrile and methanol of analytical grade were purchased from Rathburn (Zinsser Analytic, Frankfurt, Germany). Sodium acetate and sodium dihydrogenphosphate of analytical grade were obtained from Fluka (Deisenhofen, Germany). Ethylendiaminetetraacetic acid (EDTA) disodium salt was obtained from Fluka (Buchs, Switzerland). Orthophosphoric acid 85% of analytical-reagent grade, acetic acid 100% of analytical-reagent grade, isopropanol and β-glucuronidase (30 U/ml)/arylsulfatase (60 U/ml) were supplied by Merck (Darmstadt, Germany) and polyethylene glycol (PEG) 6000 in PhEur quality by Merck–Schuchardt (Hohenbrunn, Germany). Water was deionised and twice distilled. Human plasma for

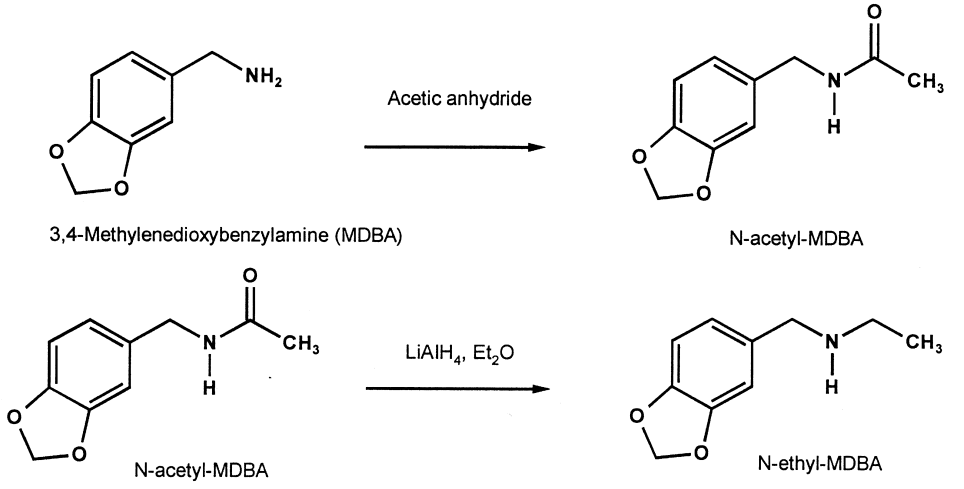

Fig. 2. Scheme of the synthesis of *N*-ethyl-3,4-methylenedioxybenzylamine (NEMDBA as internal standard, cf. Section 2.3).

reference was obtained from a blood bank (University Clinic, Tuebingen, Germany).

For purification of the enzyme solution, PD 10 columns (Pharmacia, Freiburg, Germany) were used. Solid-phase extraction was carried out with CBA columns (ICT, Bad Homburg, Germany).

The pure enantiomers of MDE, HME and MDA were synthesized in our laboratory as previously described [30].

2.2. Equipment

Plasma and urine drug and metabolite concentrations were determined by HPLC, using a Merck–Hitachi HPLC system comprising a gradient pump L 6200 A, an LaChrom autosampler L 7200, an LaChrom fluorescence detector L 7480 and an interface D-7000.

The MS analyses were carried out on a Finnigan TSQ70 ES-MS (ionization voltage 70 eV; source temperature 200 °C).

^{1}H nuclear magnetic resonance (NMR) spectra were obtained with a Bruker AC 250 spectrometer; ^{13}C NMR was carried out on a Bruker AC 62.5 spectrometer.

Infrared spectra were obtained with a Bruker Fourier transform (FT) IR IFS 48 equipment. X-Ray analysis was performed using a Enraf-Nonius CAD4 diffractometer with Cu Kα radiation. Elemental composition was analysed with Vario EL equipment.

2.3. Synthesis of the internal standard (*N*-ethyl-3,4-methylenedioxybenzylamine; NEMDBA; Fig. 2)

A 5-g amount of 3,4-methylenedioxybenzylamine (Fluka, Deisenhofen, Germany) was dissolved in 50 ml toluene. Then 75 ml anhydrous acetic acid was added and the mixture was stirred for 12 h. The solvent was then removed under vacuum. The residue was recrystallised from isopropanol. The formed crystals of *N*-acetyl-3,4-methylenedioxybenzylamine (N-AcMDBA) were removed by filtration [yield 4.212 g (66.1%); m.p. 85 °C; Anal. ($C_{10}H_{11}NO_3$) C, H, N].

A solution of 3 g N-AcMDBA in 100 ml anhydrous diethyl ether was prepared (Merck). This solution was added slowly to a well-stirred suspension of 1.47 g lithium aluminium hydride (Merck) in 100 ml anhydrous diethyl ether. This mixture was then brought up to a reflux and maintained there for 20 h, cooled by an external ice bath, and the excess hydride was destroyed by the cautious addition of 100 ml water. Finally, 50 ml 3 *M* NaOH was added to bring the pH above pH 9. The ether phase was separated and dried by the addition of $MgSO_4$. The drying reagent was removed by filtration, and the clear filtrate was removed under vacuum. The residue was then dissolved in analytical-reagent grade acetone (Merck) and neutralized with analytical-reagent grade hydrochloric acid (Merck). The formed crystals of *N*-ethyl-3,4-methylenedioxybenzylamine hydrochloride (NEMDBA-HCl) were removed by filtration and recrystallised from isopropanol [yield 2.48 g (74.2%); m.p. 183 °C, Anal. ($C_{10}H_{13}NO_2$-HCl)].

2.4. Sample preparation

2.4.1. Analysis of plasma samples

For the hydrolysis of the HME *o*-glucuronyl and sulfate conjugates, a 1000-μl aliquot of plasma was mixed with 875 μl 0.1 *M* sodium acetate buffer and with 125 μl of purified enzyme solution (cf. urine preparation). Then, the preparation was incubated at 37 °C for 24 h.

Plasma proteins were then precipitated by adding 1 ml of an aqueous solution of PEG 6000 (20%) under cooling in an ice bath for 5 min. The internal standard NEMBDA was diluted in this PEG solution; each plasma sample then contained 200 ng/ml NEMDBA in the resulting sample solution. After centrifugation at 2875 *g* for 5 min at 20 °C, the supernatants were subjected to solid-phase extraction on cation-exchange columns (Isolute CBA-columns 200 mg; ICT). After conditioning the columns with 1 ml acetonitrile, 1 ml 0.1 *M* hydrochloric acid and 3 ml 0.1 *M* sodium acetate buffer (pH 6.5) the samples were applied. The columns were then rinsed with 2 ml water and 2 ml water–methanol (75:25, v/v) and subsequently, the amphetamine derivatives were eluted from the columns with 2 ml acetonitrile–0.5 *M* HCl (9:1, v/v). The samples were then evaporated to dryness under nitrogen at 45 °C and taken up in 250 μl bidistilled water. Thus, plasma samples were concentrated by a factor of 4. A 20-μl

volume of this solution was used for injection into the HPLC system.

2.4.2. Analysis of urine samples

A 54-μl volume of the internal standard solution *N*-ethyl-3,4-methylenedioxybenzylamine-hydrochloride (NEMDBA; 200 ng/ml as free base in the resulting sample solution) was added to each 200 μl of the calibrator and volunteer urine sample.

For the hydrolysis of the conjugates in urine the β-glucuronidase/arylsulfatase solution was purified according the manufacturer's instructions by means of size-exclusion chromatography in 0.1 *M* sodium acetate buffer (pH 5.2; PD-10 columns, Pharmacia). The activity of the purified enzyme solution was 6 U/ml for the β-glucuronidase and 12 U/ml for the arylsulfatase. A 200-μl aliquot of urine was dissolved in 1550 μl 0,1 *M* sodium-acetate buffer and treated with 250 μl purified enzyme solution. After addition of 54 μl internal standard solution, the preparation was incubated at 37 °C for 24 h.

Then the samples were subjected to solid-phase extraction on cation-exchange columns (Isolute CBA-columns 500 mg; ICT). After conditioning the columns with 5 ml acetonitrile, 2 ml 0.1 *M* hydrochloric acid and 6 ml 0.1 *M* sodium acetate buffer (pH 6.5), the samples were applied. The columns were then rinsed with 4 ml water and 2 ml water–methanol (75:25, v/v) and subsequently, the amphetamine derivatives were eluted from the columns with 2 ml acetonitrile–0.5 *M* HCl (9:1, v/v). The samples were then evaporated to dryness under nitrogen at 45 °C and taken up in 5 ml bidestilled water. Thus, urine samples were diluted by a factor of 25. A 20-μl volume of this solution was used for injection into the HPLC system.

2.4.3. Calibration samples

Plasma calibrators (1000 μl) containing racemic MDE, MDA and HME were prepared to contain single enantiomer MDE concentrations of 5, 21, 37, 52, 68, 84, 100, 217, 334, 451, 568, 685 and 800 ng/ml, MDA single enantiomer concentrations of 5, 21, 37, 52, 68, 84 and 100 ng/ml, and HME single enantiomer concentrations of 10, 25, 40, 55, 70, 85, 100, 417, 734, 1051, 1368, 1685 and 2000 ng/ml.

Urinary calibrators (200 μl) containing racemic MDE, MDA and HME were prepared to contain the following single enantiomer concentrations: MDE: 5, 21, 37, 52, 68, 84, 100, 217, 334, 451, 568, 685 and 800 ng/ml; MDA: 5, 38, 70, 103, 135, 168 and 200 ng/ml; HME: 10, 25, 40, 55, 70, 85, 100, 417, 734, 1051, 1368, 1685 and 2000 ng/ml.

A solution of NEMDBA-HCl (concentration: 50 ng NEMDBA/ml in PEG-6000 for plasma and 18.52 μg NEMDBA/ml in water for urine) was used as the internal standard.

2.5. Volunteer study protocol

Four healthy, non-drug-using male Caucasian physicians with a mean (range) age of 36.8 (27–50) years (see Table 1 for demographic data) participated in a randomised, double-blind, cross-over pilot-study to investigate pharmacokinetics of (*R*)- and (*S*)-MDE and their metabolites as well as pharmacodynamic parameters (e.g., neuropsychological effects). The ethics committee of the Landesaerztekammer Baden-Wuerttemberg approved the study protocol and all volunteers gave written informed consent. Each volunteer received 70 mg of (*R*)- and (*S*)-MDE-HCl (equivalent of 60 mg of MDE base), respectively, in capsule form with 200 ml of water 30 min after a standardised breakfast.

Blood samples (8 ml) were collected from a cannulated forearm vein immediately before and at 10, 20, 30, 60, 90, 120, 180, 240, 360, 480, 600, 720, 840, 1440, 1560, 1800 and 2040 min after drug administration. The samples were centrifuged in EDTA-tubes at 2875 *g* for 10 min and plasma was stored deep-frozen at −20 °C until required for analysis.

Urine samples were collected in six fractions (12 h) over a period of 72 h. Aliquots of 100 ml were rapidly frozen and stored at −20 °C until analysis.

Table 1
Volunteer demographics

Number	Age (years)	Height (cm)	Body mass (kg)	Dose of MDE base (mg/kg)
1 (H.L.)	50	187	96	0.63
2 (M.T.)	42	170	81	0.74
3 (M.K.)	27	174	77	0.77
4 (S.F.)	28	185	80	0.75

2.6. Chromatography

A Chiral CBH column (Cellobiohydrolase) 150×4 mm, 5 μm in combination with a Chiral CBH guard column 10×3 mm, 5 μm (ChromTech, Haengersten, Sweden) was employed. Peaks were manually integrated, using Merck–Hitachi LaChrom D-7000 HPLC system manager software. The column temperature of 17.5 °C was kept by using a Techlab column oven.

Urine and plasma samples were both analyzed by means of the same HPLC method. The mobile phase consisted of 20 m*M* sodium dihydrogenphosphate, 50 m*M* EDTA disodium salt and isopropanol (7%, v/v). The pH was 6.44 and the flow-rate 0.7 ml/min. The natural fluorescence of the substances (MDE, HME, MDA and NMDBA) was used for fluorimetric detection (excitation wavelength 286 nm; emission wavelength 322 nm). All substances were identified and determined with qualified reference standards [30].

2.7. Method validation

Calibration curves were constructed by calculating peak areas of the analytes, which were adjusted by the internal standard. The analytical procedure was validated according to the ICH guidelines [31]. The evaluation of the statistical data was performed according Ebel [32] and Funk et al. [33]. Linear regression was used for the fitting of the calibration curves. Seven equidistant calibration points were measured after sample pre-treatment from spiked urine and plasma solutions, respectively. The recovery was calculated over the whole working range by determining 10 spiked urine and plasma samples, respectively, for the high working range as well as for the low working range. The limit of quantitation (LOQ) was determined by the signal-to-noise ratio, which was calculated according to the European Pharmacopoeia. The substances were added to the blank matrices and a signal-to-noise ratio of the chromatographic signals of 15 was established as the lowest point of the calibration graph. The working ranges of (*R*)- and (*S*)-MDE and HME, respectively, had to be divided, since homogeneity of variance was not obtained for the full working range. Linearity was verified with the Mandel-test and correlation coefficient. Ruggedness was controlled by screening parameters like retention time and resolution of a standard solution containing racemic MDE, HME, MDA and NEMDBA. Precision was evaluated by analyzing spiked samples for the high working range as well as for the low working range. For within-day precision, three replicates were analyzed on the same day; for total precision, seven replicates were analyzed on separate days. Accuracy (bias) was calculated with the same samples. The parameters necessary for statistical validation of the quantification are given in Tables 2 and 3.

The peaks in the chromatogram were identified by retention time and fluorescence spectra. Pure enantiomeric reference substances were determined by X-ray diffraction, NMR, MS, IR and elemental composition analysis. Purity was assured by HPLC. (*R*)- and (*S*)-MDE-HCl had a purity of 99.9% and met the requirements of the ICH guideline ''Impurities in New Drugs'' [34]. Enantiomeric purity of (*R*)-MDE and (*S*)-MDE-HCl was >99.5% at each case. Both substances were anhydrous.

2.8. Data analysis

Pharmacokinetic data were calculated according to the standard non-compartmental model with TopFit 2.0 program [35]. The following pharmacokinetic parameters were determined from plasma concentration–time data and urine concentration data for (*R*)- and (*S*)-MDE and their metabolites HME and MDA.

Peak plasma concentration (C_{max}; ng/ml) and time to reach peak plasma concentration (t_{max}; h) was obtained from the visual inspection of the plasma concentration–time curves. The MDE enantiomer elimination half-lives ($t_{1/2}$; h) was calculated according to $\ln 2/\lambda_z$. Areas under the plasma concentration curve (AUC_{0-24}; ng h/ml) were determined using the trapezoidal rule up to 24 h post drug administration. Additionally, the AUCs were extrapolated to infinity ($AUC_{0-\infty}$, ng h/ml) by adding the last concentration measured divided by the elimination rate constant. Apparent oral clearance of both MDE enantiomers was derived from the equation CL_0 = dose of enantiomer/AUC (CL; ml/min) and apparent volume of distribution (*V*; L) was calculated by dividing the CL by the slope of the last decay curve.

Table 2
Statistical parameters of validation from urine

	(*R*)-MDE	(*R*)-MDE	(*S*)-MDE	(*S*)-MDE	(*R*)-HME	(*R*)-HME	(*S*)-HME	(*S*)-HME	(*R*)-MDA	(*S*)-MDA
Working ranges (ng/ml)	5–100	100–800	5–100	100–800	10–100	100–2000	10–100	100–2000	5–200	5–200
Calibration standard points	7	7	7	7	7	7	7	7	7	7
Linearity, coefficient of correlation	0.9999	0.9998	0.9999	0.9997	0.9998	0.9993	0.9998	0.9998	0.9999	0.9999
Theoretical limit of quantitation (ng/ml)	4.36	42.31	3.62	52.99	4.72	77.06	4.70	97.38	4.94	4.75
Limit of quantitation (ng/ml) ($S/N>10{:}1$)	5	5	5	5	10	10	10	10	5	5
Recovery (%) (whole working range)	95.37	95.60	96.91	96.83	95.36	95.34	94.43	95.04	93.07	92.57
RSD (%)	0.923	1.001	0.764	1.258	0.929	0.813	0.923	1.029	0.539	0.559
Homogeneity of variance ($P=99.9\%$, performed)	7.94	8.81	8.45	7.77	8.62	8.21	7.35	8.10	8.15	8.07
Imprecision[a] high working range ($n=10$) (%)	0.60	0.16	0.61	0.17	1.29	0.16	1.16	0.16	0.36	0.39
Imprecision low working range ($n=10$) (%)	4.57	0.52	4.44	0.49	4.64	1.08	4.35	1.07	4.87	5.34
Inaccuracy[b] high working range ($n=10$) (%)	3.53	0.99	4.03	1.37	3.41	0.50	3.33	0.59	8.59	6.92
Inaccuracy low working range ($n=10$) (%)	7.40	5.12	9.40	4.17	5.70	6.13	7.20	8.03	9.80	9.80

[a] Imprecision of, e.g., 0.60% means precision of 99.4%.
[b] Inaccuracy of, e.g., 7.40% means accuracy of 92.6%.

Table 3
Statistical parameters of validation from plasma

	(*R*)-MDE	(*R*)-MDE	(*S*)-MDE	(*S*)-MDE	(*R*)-HME	(*R*)-HME	(*S*)-HME	(*S*)-HME	(*R*)-MDA	(*S*)-MDA
Working ranges (ng/ml)	5–100	100–800	5–100	100–800	10–100	100–2000	10–100	100–2000	5–100	5–100
Calibration standard points	7	7	7	7	7	7	7	7	7	7
Linearity, coefficient of correlation	0.9999	0.9997	0.9999	0.9997	0.9996	0.9999	0.9996	0.9998	0.9999	0.9999
Theoretical limit of quantitation (ng/ml)	4.57	60.06	4.73	62.45	4.68	88.34	4.99	97.32	3.61	2.50
Limit of quantitation (ng/ml) ($2S/N>10{:}1$)	5	5	5	5	10	10	10	10	5	5
Recovery (%) (whole working range)	96.54	95.74	96.07	96.51	93.66	94.06	94.86	92.86	95.46	98.40
RSD (%)	0.967	1.429	1.002	1.487	0.932	1.869	1.029	1.791	0.401	0.276
Homogeneity of variance ($P=99.9\%$, performed)	8.79	6.25	9.39	6.26	6.58	5.42	6.15	5.90	5.91	6.70
Imprecision high working range ($n=10$) (%)	0.62	0.19	0.59	0.18	1.06	0.12	1.03	0.13	0.60	0.78
Imprecision low working range ($n=10$) (%)	4.50	0.62	4.07	0.59	4.29	1.06	4.18	1.03	4.67	5.89
Inaccuracy high working range ($n=10$) (%)	6.70	7.18	5.71	0.48	1.81	0.51	2.83	0.65	9.39	7.13
Inaccuracy low working range ($n=10$) (%)	4.40	8.10	6.80	7.18	6.20	8.27	9.00	9.67	7.00	8.60

The cumulative amount (A_e; mg) represents the % of dose of urinary excreted enantiomers of MDE and their metabolites HME and MDA, respectively ($A_{e\ 0-24}$ and $A_{e\ 0-\infty}$; mg). The renal clearance of both enantiomers was calculated as A_e/AUC (CL_{ren}; ml/min) and metabolic clearances of (*R*)- and (*S*)-MDE to their metabolites (*R*)- and (*S*)-HME, and (*R*)- and (*S*)-MDA, respectively, were calculated as $A_{e\ 0-24}$ metabolite/AUC_{0-24} MDE ($CL_{MDE\rightarrow metabolite}$; ml/min).

3. Results and discussion

3.1. Analytical procedure

Plasma and urine samples were prepared according to Brunnenberg et al. [20]. In contrast to Brunnenberg et al., NEMDBA was established as internal standard and the chromatographic separation of all substances was carried out with one HPLC method on a chiral CBH stationary phase. This chiral protein phase contains covalently bonded cellobiohydrolase as a chiral selector. The change of pH, flow-rate, composition of mobile phase and column temperature led to acceptable resolution of MDE, HME, MDA and NEMBDA, whereas Brunnenberg's method was only suitable for the determination of HME. HPLC isocratic conditions resulted with overall runtimes of only 22 min. The pH control of the puffer is recommended (pH 6.4±0.02) and has a large effect on retention time and resolution. Moreover, column temperature has an effect on retention time too. The retention times for (*R*)-MDE, (*S*)-MDE, (*R*)-HME, (*S*)-HME, (*R*)-MDA, (*S*)-MDA and NEMDBA were 5.70, 6.49, 7.43, 8.07, 9.02, 11.95, and 16.50 min, respectively (Fig. 3). The method required only 1000 μl human plasma and 200 μl human urine for accurate determination due to an LOQ which is lower than in the previously described capillary electrophoresis (CE) [24,25] and HPLC methods [20,40]. The method described in this paper is the first method suitable for enantioselective determination HME together with MDE and MDA [36,37]. A further advantage of this method is that it does not require a derivatization step in contrast to the majority of methods previously described. Ring-substituted amphetamines exhibit a good native fluorescene, in particular, those which were the subject of the present study showed an excitation maximum of 286 nm and an emission maximum of 322 nm. The use of fluorescence detection provided greater selectivity compared with conventional ultraviolet detection and less interferences with endogen-

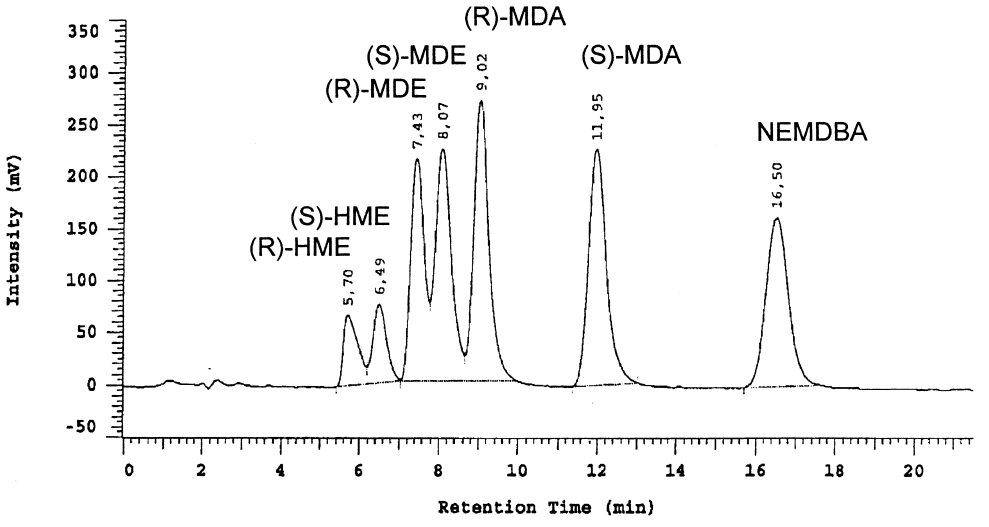

Fig. 3. HPLC–FD chromatogram of MDE, its major metabolites and the internal standard NEMDBA (aqueous solution). Concentrations of (*R*,*S*)-HME, (*R*,*S*)-MDE, and (*R*,*S*)-MDA: 300 ng/ml; NEMDBA: 200 ng/ml; CBH column 150×4.0 mm, 5 μm; mobile phase: 20 m*M* NaH_2PO_4–buffer (pH 6.44), and flow-rate 0.7 ml/min. Fluorescence detection: λ_{ex} 286 nm, λ_{em} 322 nm.

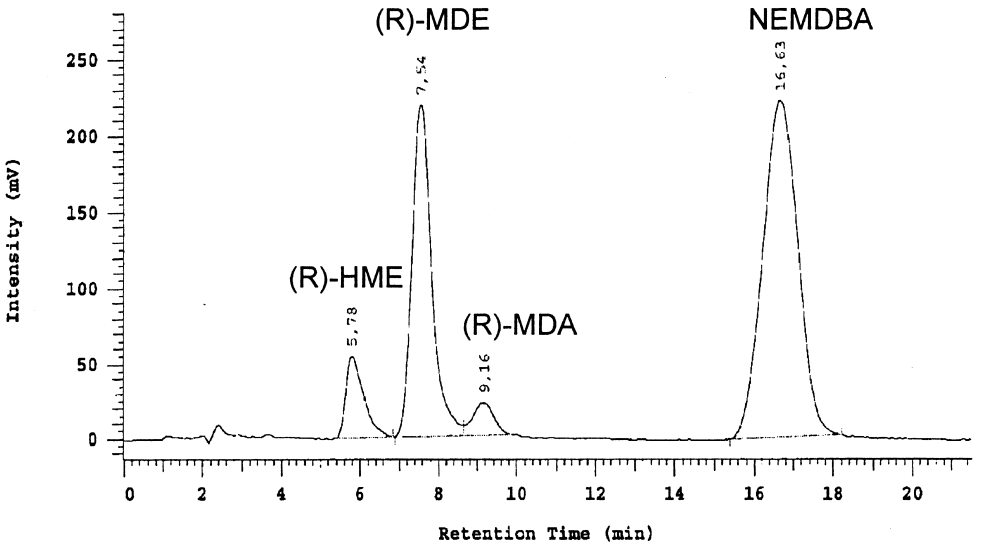

Fig. 4. HPLC–FD chromatogram of an authentic urine sample after (*R*)-MDE administration. Analytical conditions: cf. Fig. 3. Calculated concentrations of (*R*)-HME: 152 ng/ml, (*R*)-MDE: 110 ng/ml, (*R*)-MDA: 32 ng/ml.

ous blood or urine components [21,23,38,39]. Hence, a simple solid-phase extraction was sufficient to obtain chromatograms free from interferences of endogenous compounds in plasma as well as in urine (Figs. 4 and 5). Over the studied concentration ranges for all six analytes in urine and plasma, calibration curves over the studied concentration ranges from all six analytes in urine and plasma showed correlation coefficients ≥0.999 (Table 2).The extraction recoveries of the analytes were not less than 92% for all compounds in both urine and plasma. These results proved better than in methods

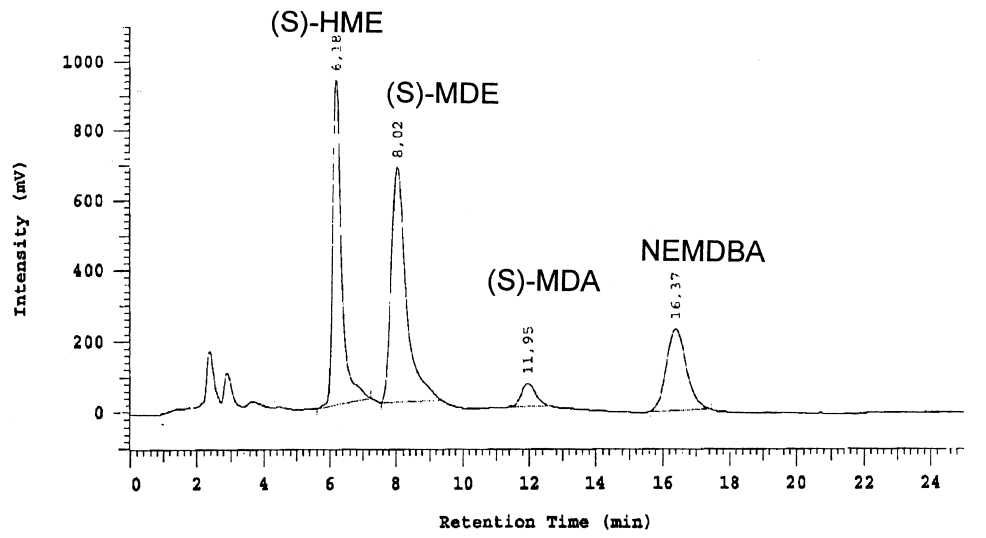

Fig. 5. HPLC–FD chromatogram of an authentic plasma sample after (*S*)-MDE administration. Analytical conditions: cf. Fig. 3. Calculated concentrations of (*S*)-HME: 1656 ng/ml, (*S*)-MDE: 368 ng/ml, (*S*)-MDA: 60 ng/ml.

previously described [21]. NEMDBA, which was used as internal standard, showed recoveries of 96% both from plasma and urine.

3.2. Identity and purity of the internal standard

A structurally related analog of MDE, MDA and HME was preferred as the internal standard, but none of the known MDA-analogs—such as MBDB or N-alkyl-derivatives—was suitable as an internal standard for the chromatographic method; thus, NEMDBA was synthesized in the laboratory and used as the internal standard. Identity of NEMDBA was confirmed on the basis of ^{1}H-NMR, ^{13}C-NMR, FT-IR, and ESI-MS. The following results were obtained:

^{1}H-NMR (250 MHz, $DMSO_{d6}$, δ) of NEMDBA-hydrochloride: 1.21 (t, 3H, J=7.25 Hz, $-CH_2-C\underline{H}_3$), 2.86 (q, J=7.20 Hz, $-C\underline{H}_2-CH_3$), 4.00 (s, 2 H, $-CH_2-$), 6.04 (s, $(-O-C\underline{H}_2-O-)$, 6.70 (dd, ^{3}J= 7.93 Hz, ^{4}J=1.62 Hz, 6-H_{arom}), 6.73 (d, ^{3}J=6.35 Hz, 5-H_{arom}), 6.87 (d, ^{4}J=1.56 Hz, 2-H_{arom}), 9.29 (s, $N\underline{H}_2^+$).

^{13}C-NMR (62.5 MHz, D_2O, δ) of NEMDBA-hydrochloride: 13.1 and 44.8 ($^{+}N-C_2H_5$), 53.0 ($^{+}N-CH_2-$), 104.2 ($-O-CH_2-O-$), 111.5, 112,5, 126.7, 127.1, 150.3, 150.7 (six aromatic C).

ESI-MS (70 eV) of NEMDBA-hydrochloride: m/z=178 (15.8%, $M^{+\cdot}$), 149 (3%, M–CO), 135 (100%, $M-C_3H_8N$), 105 (5.3%, $M-C_4H_{10}N$), 77 (14.3%, $^{+}C_6H_5$), 58 (9.0%, $^{+}C_3H_8N$), 44 (7.5%, $^{+}C_2H_6N$).

FT-IR (KBr-pellet of NEMDBA-hydrochloride): wave number (cm^{-1}): 2973–2717 ν ($^{+}N\underline{H}_2-$, $C\underline{H}$), 2796 ν $(-O-C\underline{H}_2-O-)$, 2436 Comb. ν ($^{+}NH_2-$), 1587, 1502 and 1487 ν (aromatic C=C), 1443 δ_{as} ($-CH_3$) and δ_{sy} ($-CH_2-$), 1253 ν (–O–C–O–), 1048 ν ($-H_2C-O-CH_2-$), 930 ν (C–O–C), 810 δ_{oop} (=C–H).

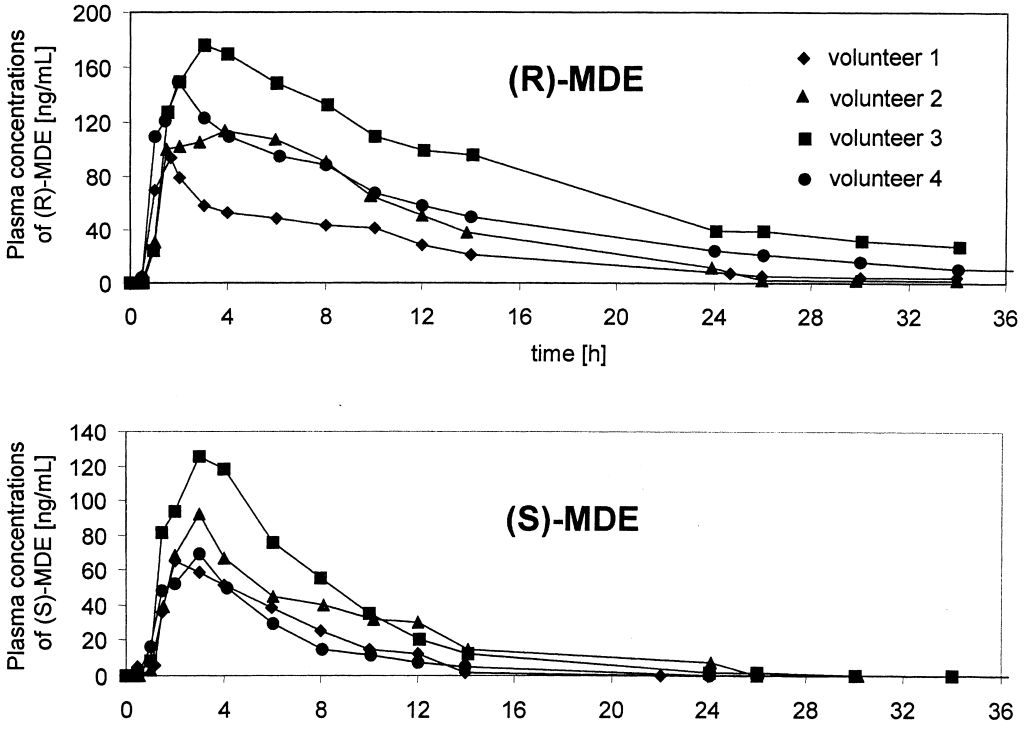

Fig. 6. Plasma concentration curves of (*R*)- and (*S*)-MDE, respectively.

Purity of NEMDBA-HCl was determined by reversed-phase HPLC [LiChroCart 125-4 LiChrospher RP-8 (5 μm); mobile phase: 20 m*M* KH_2PO_4(aqueous solution)–acetonitrile (90:10), pH 3.0, flow-rate 1.3 ml/min, detection: UV detection at 200 nm and diode array detection (DAD)] and found to be 99%. Moreover, fluorescence spectra of NEMDBA were taken to confirm that the compound had the same emission and excitation wavelengths as MDE, MDA and HME. On the basis of the above results, the identity of NEMDBA was verified and a purity of 99% was assigned. NEMDBA showed recoveries similar to the other compounds from the investigated biological fluids and was used throughout the analytical procedure to correct the peak areas of MDE, MDA and HME.

3.3. Plasma concentration curves, urine recovery and pharmacokinetic parameters

Figs. 6–8 show the single-dose plasma concentrations of (*R*)- and (*S*)-MDE and their metabolites HME and MDA, respectively, over a time period of 36 h for four volunteers. The mean C_{max} for (*R*)-MDE was 134 ng/ml (range: 94–176 ng/ml), whereas C_{max} of (*S*)-MDE was 88 ng/ml (range: 65–125 ng/ml). Plasma concentrations of (*R*)- and (*S*)-MDE displayed linear pharmacokinetic behaviour with a mean elimination half-life time for (*R*)-MDE of 7.9 h and 4.0 h for (*S*)-MDE. Differences between (*R*)- and (*S*)-MDE were also found in AUC, CL_T and CL to metabolite. Complete pharmacokinetic data of all patients are given in Tables 3

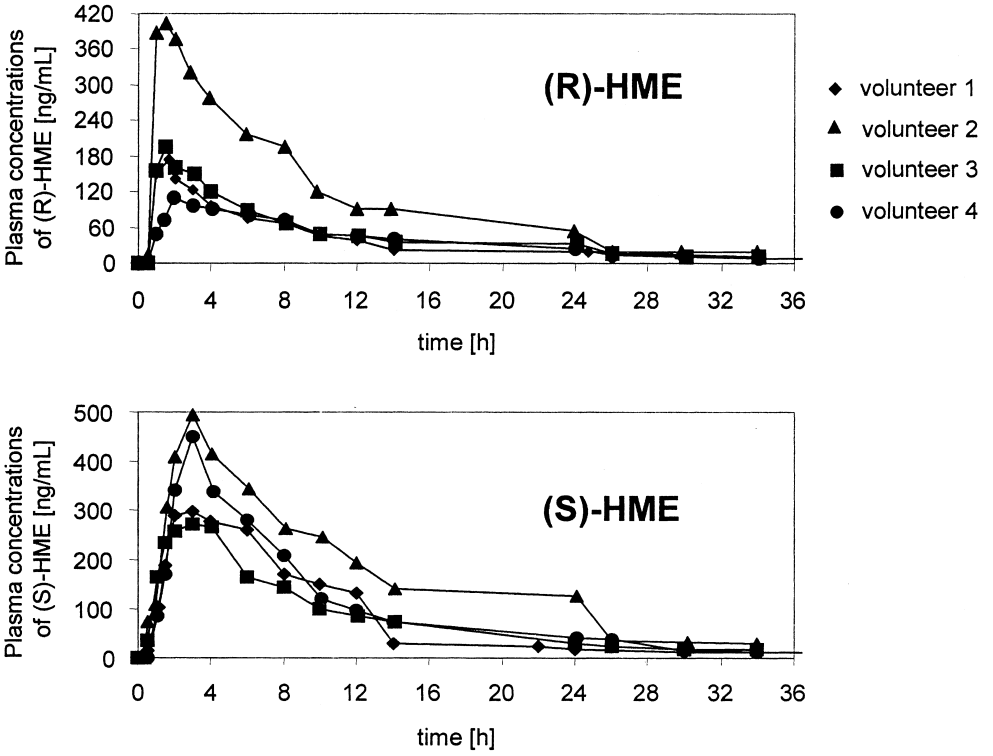

Fig. 7. Plasma concentration curves of (*R*)- and (*S*)-HME, respectively.

Table 4
Pharmacokinetic parameters of the individual enantiomers of MDE after administration of 60 mg (*R*)-MDE and (*S*)-MDE base, respectively

	C_{max} (ng/ml)		t_{max} (h)		$t_{1/2}$ (h)		$AUC_{0-\infty}$ (ng h/ml)		CL_T (ml/min)		CL_R[a] (ml/min)		V_D (l)		CL_{MDE}[b] (ml/min)		$CL^{b}_{MDE\rightarrow HME}$		$CL^{b}_{MDE\rightarrow MDA}$	
	(*R*)-MDE	(*S*)-MDE	(*R*)-MDE	(*S*)-MDE	(*R*)-MDE	(*S*)-MDE	(*R*)-MDE	(*S*)-MDE	(*R*)-MDE	(*S*)-MDE	(*R*)-MDE	(*S*)-MDE	(*R*)-MDE	(*S*)-MDE	(*R*)-MDE	(*S*)-MDE	(*R*)-MDE	(*S*)-MDE	(*R*)-MDE	(*S*)-MDE
1 (H.L.)	94	65	1.7	2.0	6.1	3.7	831	404	1200	2480	449	309	799	631	448	309	387	1960	37.2	36.5
2 (M.T.)	114	92	4.0	3.0	5.6	6.4	1383	743	723	1350	114	41	743	349	114	41.0	323	830	14.6	32.8
3 (M.K.)	176	125	3.1	3.0	10.8	3.0	3094	883	323	1130	121	253	291	303	121	253	93.5	535	10.3	29.6
4 (S.F.)	150	69	2.0	3.0	9.1	3.0	2063	373	485	2680	171	212	693	384	171	212	133	1472	33.4	124
Mean	134	88	2.7	2.8	7.9	4.0	1843	601	683	1910	214	208	632	417	214	204	234	1199	23.9	55.7

[a] $CL_{R\ 0-\infty} = A_{e\ 0-\infty}/AUC_{0-\infty}$.
[b] CL to metabolite = $CL_{MDE} \cdot A_{e\ 0-\infty}$ metabolite/60 mg MDE.

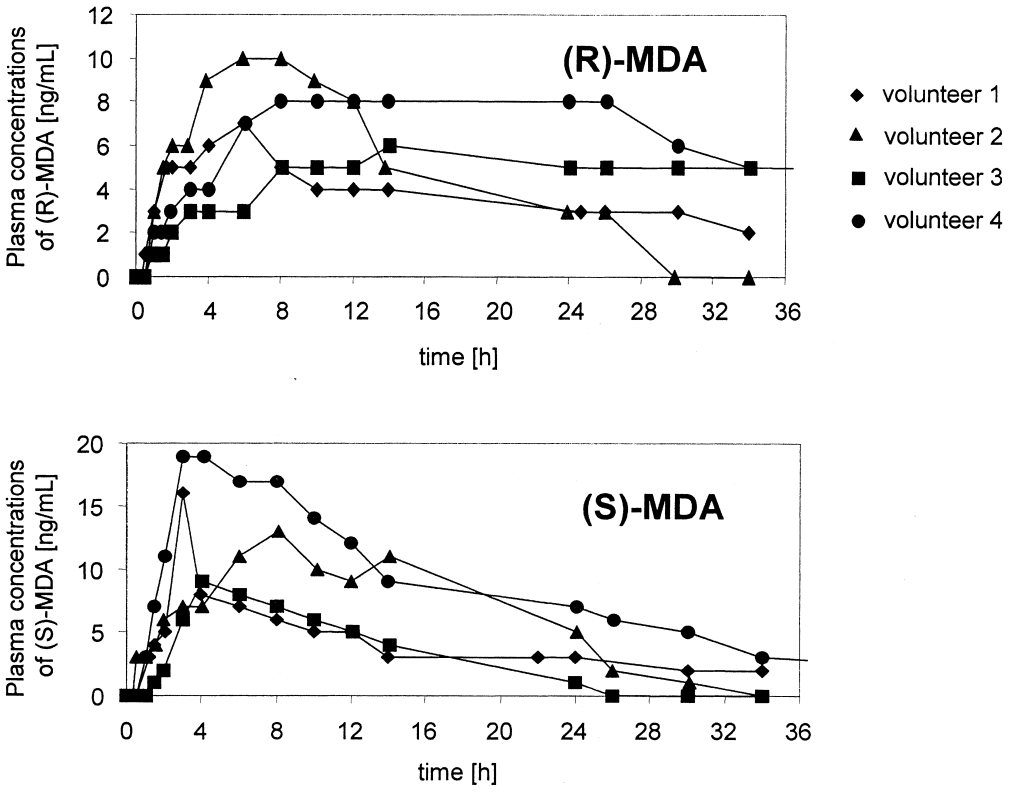

Fig. 8. Plasma concentration curves of (*R*)- and (*S*)-MDA, respectively.

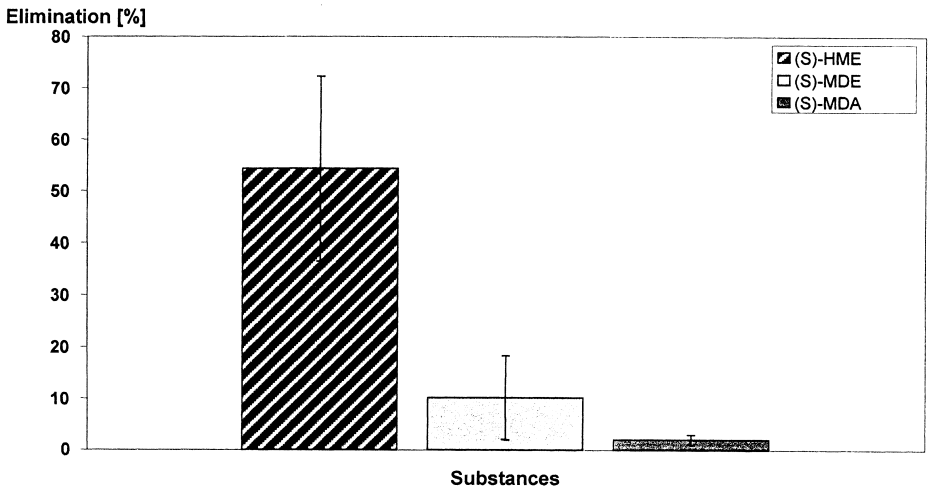

Fig. 9. Recovered material in urine after administration of (*S*)-MDE-HCl.

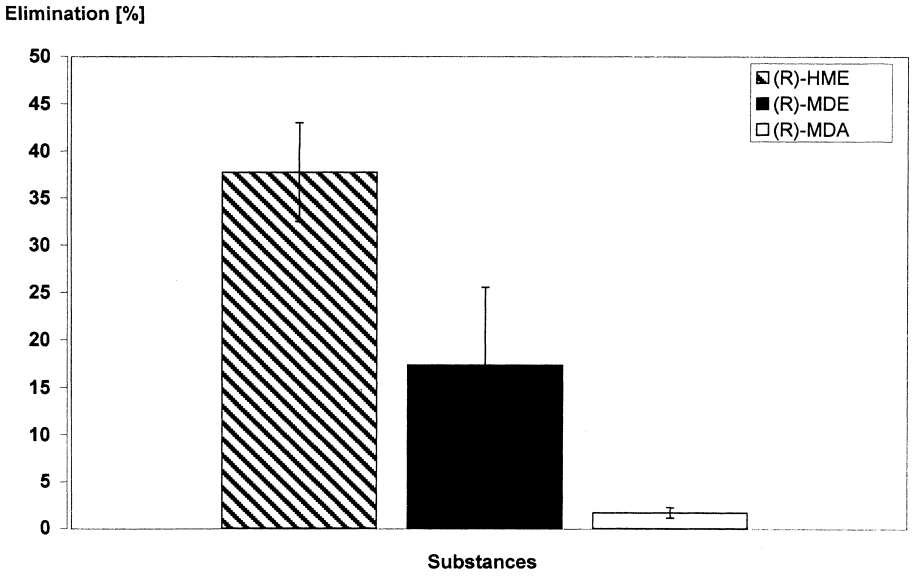

Fig. 10. Recovered material in urine after administration of (*R*)-MDE-HCl.

and 4. In general, (*S*)-MDE shows lower plasma concentrations and shorter plasma half-life than (*R*)-MDE. The C_{max} of (*R*)-configured metabolites are higher than (*S*)-HME and (*S*)-MDA, respectively. Moreover the AUC of (*S*)-HME is significantly higher than the AUC of (*R*)-HME, whereas the differences between the AUC of (*S*)- and (*R*)-MDA are less pronounced. The differences between MDA enantiomers are significant in C_{max} and t_{max}. Thus, (*S*)-MDA is produced faster than (*R*)-MDA, but over a period of 34 h the amounts of both substances determined in plasma are more or less comparable.

The data obtained by Brunnenberg et al. [20], Fallon et al. [21] and Moore et al. [22] after administration of racemic MDMA and MDE, respectively, indicated that the stereoselective disposition of the Ecstasy compounds in humans is comparable to the results of our study, even though we administered pure MDE enantiomers.

Analysis of urine samples showed that recovery of (*R*)-MDE is higher (27.7±8.2%) than that of (*S*)-MDE (10.2±8.1%). Again the amount and recovery of metabolites of (*S*)-MDE is higher than those of (*R*)-MDE: 54.3% (±17.9%) (*S*)-HME) vs. 37.7% (±5.3%) (*R*)-HME and 2.0% (±0.9%) (*S*)-MDA vs. 3.3% (±0.6%) (*R*)-MDA (Figs. 9 and 10). Fallon et al. [21] found similar results for MDMA and MDA urine recoveries, but could not determine HMMA (which is the major metabolite of MDMA) whereas Brunnenberg et al.'s plasma collection time was much too short for complete calculation of pharmacokinetic parameters [20]. Despite that, there is no difference between the results obtained after administration of racemic or pure enantiomeric MDMA and MDE, respectively.

Even though a statistical evaluation of our study was not possible because of the limited number of volunteers, the results shown in this paper show trends consistent with the results obtained from other investigations [20,21].

4. Nomenclature

A_e	Cumulative amount
AUC	Area under the curve
CBA	Carboxypropyl
CBH	Cellobiohydrolase
CL	Clearance
CL_0	Dose of enantiomer/AUC
CL_T	Total clearance
C_{max}	Maximum plasma concentration
CYP1A2	CytochromeP450 1A2
CYP2B6	CytochromeP450 2B6

CYP2D6	CytochromeP450 2D6
CYP3A4	CytochromeP450 3A4
DHA	3,4-Dihydroxyamphetamine
DHE	*N*-Ethyl-3,4-dihydroxyamphetamine
DMSO	Dimethylsulfoxide
EDTA	Ethylendiaminetetraacetic acid
ESI	Electrospray ionization
FD	Fluorescence detection
FT-IR	Fourier transformation infrared spectroscopy
HMA	4-Hydroxy-3-methoxyamphetamine
HMMA	4-Hydroxy-3-methoxymethamphetamine
HME	*N*-Ethyl-4-hydroxy-3-methoxyamphetamine
HPLC	High-performance liquid chromatography
KH_2PO_4	Potassium dihydrogenphosphate
ICH	International Conference on Harmonization
LOQ	Limit of quantitation
MBDB	[*N*-Methyl-1-(1,3-benzodioxol-5-yl)-2-butanamine]
MDA	3,4-Methylendioxyamphetamine
MDE	Eve, *N*-Ethyl-3,4-methylendioxyamphetamine
MDMA	Adam, *N*-Methyl-3,4-methylendioxyamphetamine
NEMDBA	*N*-Ethyl-3,4-methylendioxybenzylamine
NMR	Nuclear magnetic resonance spectroscopy
PEG	Polyethylene glycol
$t_{1/2}$	Elimination half-life time
t_{max}	Time to reach maximum plasma concentration
UV–DAD	Ultraviolet diode array detection

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