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# Stereospecific analysis of ecstasy-like *N*-ethyl-3,4-methylenedioxyamphetamine and its metabolites in humans

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**Abstract**

A chiral HPLC method has been developed for the ecstasy analogue (*R*,*S*)-*N*-ethyl-3,4-methylenedioxyamphetamine (MDE) and its metabolites *o*-glucuronyl-(*R*,*S*)-*N*-ethyl-4-hydroxy-3-methoxyamphetamine (HME) and (*R*,*S*)-3,4-methylenedioxyamphetamine (MDA) in human plasma. The chiral discrimination of the compounds was carried out with an enantioselective HPLC method using β-cyclodextrin in the mobile phase for MDE and MDA and a chiral protein phase (chiral-CBH) for HME. MDE and MDA were detected fluorimetrically at 322 nm, while the major metabolite HME was selectively determined by electrochemical detection at +600 mV. After hydrolysis of the conjugates using β-glucuronidase/arylsulfatase and solid-phase extraction with a cation-exchange phase for sample preparation high recovery rates of more than 95% were yielded. The limit of quantitation for the enantiomers of MDE and its metabolites in plasma were between 1.2 (MDA) and 16 ng/ml (HME) and the relative method standard deviations ($V_{x_0}$, Table 1) were less than 3%. The methods described have been used successfully in the enantioselective quantitation of the compounds in plasma samples obtained from six healthy volunteers in a clinical study after oral administration of 140 mg racemic MDE hydrochloride. Significant differences were found in the plasma concentrations of the examined stereoisomers. Whereas the *R*-enantiomer of the parent substance, MDE, was predominant in the plasma samples investigated, higher plasma concentrations of the *S*-enantiomers of the metabolites MDA and HME were measured. 

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

## 1. Introduction

The most frequently consumed psychoactive addictive substances are chemically derived from (*R*,*S*)-3,4-methylenedioxyamphetamine. These include (*R*,*S*)-3,4-methylenedioxyamphetamine (MDA), *N*-methyl-(*R*,*S*)-3,4-methylenedioxyamphetamine (MDMA) and *N*-ethyl-(*R*,*S*)-3,4-methylenedioxyamphetamine (MDE), which are also referred to as designer drugs [1]. As MDE seems to be less neurotoxic than MDMA (ecstasy) [2,3], it was used in several human studies [4,5]. In one of these studies, we have clarified the metabolic pattern of MDE in humans using gas chromatography–mass spectrometry (GC–MS) (Fig. 1) [4]. The preferred pathway of MDE metabolism is the oxidative degradation of the methylenedioxy group leading to the 3,4-dihydoxymetabolite followed by the methylation in position 3 of the aromatic ring yielding *N*-ethyl-4-

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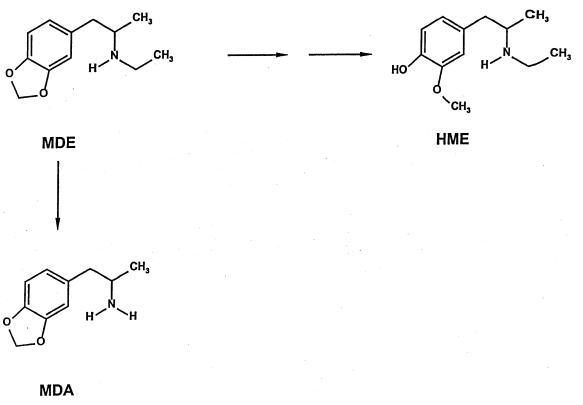


Fig. 1. Partial scheme of MDE metabolism [4].

hydroxy-3-methoxyamphetamine. The second pathway leads via *N*-desalkylation to the metabolite 3,4-methylenedioxyamphetamine.

Furthermore, plasma concentrations of racemic MDE and its metabolites MDA and, following hydrolysis of glucuronide and sulfate conjugates, HME have been determined previously by ourselves [5]. The enantioselectivity of metabolism of methylenedioxyamphetamines has been described in other human studies [6–9] and in some in vivo studies with rodents [10,11]. However, in these studies, the authors examined only the parent substance MDE and the minor metabolite MDA.

Therefore this paper describes two rapid, reliable and sensitive enantioselective high-performance liquid chromatography (HPLC) methods for the quantification of the stereoisomers of MDE, its major metabolite HME, and MDA in human plasma. Plasma concentration curves have been determined using plasma samples obtained from six healthy volunteers after oral administration of 140 mg MDE·HCl per person and pharmacokinetic parameters have been investigated.

## 2. Experimental

### 2.1. Materials

Racemic HME, MDA, MDMA, and MDE were synthesised according to Ref. [12]. *S*-MDA, *S*-MDE and *S*-HMA were synthesised according to Ref. [13]. Acetonitrile and methanol were purchased from Rathburn (Zinsser Analytic, Frankfurt, Germany). Potassium dihydrogenphosphate and sodium acetate of analytical grade were obtained from Fluka (Deisenhofen, Germany). Orthophosphoric acid 85% analytical-reagent grade, acetic acid 100% analytical-reagent grade, β-cyclodextrin, isopropanol and β-glucuronidase (30 U/ml)/arylsulfatase (60 U/ml) were supplied by Merck (Darmstadt, Germany) and poly(ethylene glycol) (PEG) 6000 in DAB quality by Merck–Schuchardt (Hohenbrunn, Germany). Water was deionized and distilled twice. Human plasma blanks were obtained from a blood bank (University Hospital, Tübingen, Germany). The real plasma samples were provided by a clinical study (Department of Psychiatry and Psychotherapy, University of Freiburg, Freiburg, Germany). Plasma samples were kept at −20°C until analysis.

For purification of the enzyme solution and for solid-phase extraction (SPE), PD 10 columns (Pharmacia, Freiburg, Germany) and CBA columns (ICT, Frankfurt, Germany) were used, respectively.

HPLC was carried out on a ChiraDex 250×4 mm I.D. and a LiChroCart Superspher 60 RP-select B column, 5 μm, 250 mm×4 mm I.D. using a guard column LiChrospher 60 RP-select B, 5 μm, 4×4 mm I.D. (Merck). In the second case the mobile phase consisted of 0.01 *M* β-cyclodextrin in 40 mmol potassium dihydrogenphosphate buffer, pH 3–acetonitrile (92:8, v/v). In addition, a Chiral CBH column 150×4 mm using a Chiral CBH guard column 10×3 mm (ChromTech, Hängersten, Sweden) was employed.

### 2.2. Equipment

The following HPLC systems were used for investigation of the samples:

A Merck–Hitachi (Merck) HPLC system comprising a gradient pump L 6200 A, an LaChrom autosampler L 7200, an LaChrom fluorescence detector L 7480 and a Chromointegrator D 2500.

A Hewlett-Packard (HP, Waldbronn, Germany) HPLC system containing the following components: an isocratic pump HP 1050, an autosampler HP, a programmable electrochemical detector HP 1049 and an integrator HP 3396 Series II.

### 2.3. Enzyme purification

The enzyme solution used, β-glucuronidase (30 U/ml)/arylsulfatase (60 U/ml) was purified according to the manufacturer's instructions by means of size-exclusion chromatography (SEC) in 0.1 *M* sodium acetate buffer (pH 5.2). The activity of the purified enzyme solution was 6 U/ml for the glucuronidase and 12 U/ml for the arylsulfatase.

### 2.4. Sample preparation

The frozen plasma samples were thawed in a waterbath at 30°C. Afterwards, a 1-ml aliquot was taken up in an Eppendorf pipette. This sample was treated with 875 μl of a 0.1 *M* sodium acetate buffer (pH 5.2) and 125 μl of the purified enzyme solution and incubated at 37°C for 24 h. The plasma proteins were precipitated by the addition of 1.0 ml of a solution of PEG 6000–water (2:8) and cooling the mixture on ice (10 min). MDMA (*N*-methyl-3,4-methylenedioxyamphetamine) (220 ng/ml) as internal standard for the fluorimetric determination of MDE and MDA was resolved in the precipitating reagent. The quantitation of HME by electrochemical detection was accomplished without an internal standard. Finally the sample was centrifuged for 5 min (2865 *g* at 20°C) and the supernatant was subjected to SPE on cation-exchange columns (CBA).

### 2.5. Solid-phase extraction

Isolute CBA columns (ICT) with 200 mg sorbent were used for the extraction of amphetamine derivatives from the plasma samples.

After conditioning and equilibration of the cation-exchange columns, the samples were applied with 2.0 ml acetonitrile, 1.0 ml of 0.1 *M* HCl, and 3.0 ml of a 0.1 N sodium acetate buffer, pH 6.5. In addition, the residue from the protein precipitation was washed twice with 200 μl of a 0.1 N sodium acetate buffer, pH 6.5 and the washings were also applied to the extraction column. Interfering substances were eluted in two washing steps with 2.0 ml water and 2.0 ml methanol–water (25:75); the amphetamines were eluted with 2.0 ml acetonitrile–0.5 *M* HCl (80:20). The samples were then concentrated to dryness at 45°C in a stream of nitrogen and resolved in 250 μl bidistilled water.

### 2.6. Chromatographic conditions for the determination of the enantiomers

#### 2.6.1. β-Cyclodextrin phases

The enantioseparation of MDE and MDA were also investigated on a ChiraDex 250×4 mm column at a column temperature of 20°C. In this chromatographic system β-cyclodextrins were covalently bonded on a RP phase. The mobile phase used was 40 mmol $KH_2PO_4$ buffer, pH 5.5–acetonitrile (91:9). At a flow-rate of 0.8 ml/min, the analysis time was 25 min. The volume of sample injected was 20 μl.

#### 2.6.2. Dynamic β-cyclodextrin phases

The enantiomeric separation of MDE and MDA was carried out on a Superspher 60 RP select B 240×4 mm column at a column temperature of 20°C. The enantioselectivity of the system was achieved by using a mobile phase of 0.01 *M* β-cyclodextrin dissolved in 40 mmol $KH_2PO_4$ buffer, pH 3–acetonitrile (92:8). At a flow-rate of 0.5 ml/min, the analysis time was 33 min. The volume of sample injected was 20 μl.

#### 2.6.3. Chiral CBH phase

HME was separated into its enantiomers on a chiral CBH 150×4 mm HPLC column at a column temperature of 20°C. The mobile phase consisted of 10 mmol $KH_2PO_4$ buffer pH 7.5, 50 μmol Na·EDTA–propan-2-ol (95:5). The flow-rate was 0.8 ml/min and the analysis time 15 min. The volume of the sample injected was 5 μl.

### 2.7. Detection and identification

When fluorescence detection was employed, the excitation wavelength for MDE and MDA was 286 nm and for HME 278 nm. The emission wavelength used for the detection of all three compounds was 320 nm. Nevertheless, HME was detected by electrochemical detection. The measurements were carried out at a cell potential of 650 mV. The retention times and the UV and fluorescence spectra of the compounds available were used for identification.

### 2.8. Quantitation

The analytical procedure was validated according to the ICH guidelines [14]. The evaluation of the statistical data were in accordance with Ebel [15] and Funk et al. [16]. Equidistant calibration points for all stereoisomers were measured first in aqueous solution and subsequently after sample pretreatment from spiked plasma solutions. Hence the recovery was calculated over the whole working range. In addition, 10 spiked plasma concentrations for the high working range as well as for the low working range were determined. The homogeneity of variance, the precision and the accuracy were calculated from the data obtained. In contrast to fluorimetric detection it was not possible to find an adequate internal standard for electrochemical detection. For this reason, a stock solution of HME was injected after each sample injection. The parameters necessary for statistical validation of the procedure are given in Table 1.

### 2.9. Study design and sampling

In the described clinical study, oral doses of 140 mg MDE·HCl per person were administered to six healthy volunteers (self-experimentation by physicians). The study was placebo controlled and double blind with randomised allocation and was approved by the Ethical Committee of the Department of Medicine in Freiburg, Germany. In order to study the enantioselective pharmacokinetics, blood samples were taken 0 to 240 min after administration. The blood samples were centrifuged in heparinized tubes for 10 min at 2875 *g* and 20°C. The supernatant plasma was stored frozen at −20°C.

## 3. Results and discussion

### 3.1. Analytical procedure

The preparation of the plasma samples described in this paper is the same as that described in Ref. [5]. The plasma extracts were separated with two different enantioselective HPLC methods. The methylenedioxyamphetamines MDE, MDA, and the internal standard MDMA were separated by a HPLC method described by Cabrera and Schwinn [17] after adjusting the method to separate the amino compounds. Hence it is necessary to use a special RP phase (LiChrospher 60 RP-select B), because no tailing of the amino compounds was observed. The chiral discrimination of the optically active compounds is obtained by the addition of β-cyclodextrin to the mobile phase. The separation of the methylenedioxyamphetamine enantiomer pairs was in the order of the homologues MDA, MDMA and MDE (Fig. 2). Another possibility for enantioseparation of the methylenedioxyamphetamines is the use of immobilised cyclodextrins as a stationary phase

Table 1
Comparison of statistical parameters[a]

| | *R*-HME | *S*-HME | *R*-MDA | *S*-MDA | *R*-MDE | *S*-MDE |
|---|---|---|---|---|---|---|
| $W$ | 332.2–16.1 | 332.2–16.1 | 20.3–1.2 | 20.3–1.2 | 175.9–10.1 | 175.9–10.1 |
| $N$ | 8 | 8 | 7 | 7 | 7 | 7 |
| $a_0$ | 23383.45 | 25603.76 | 100951.74 | 90624.97 | 88743.29 | 87182.76 |
| $a_1$ | 4354.59 | 33465.63 | −6638.45 | 1806.75 | −56444.31 | −28209.73 |
| $s_y$ | 137535.7 | 135472.7 | 6351.7 | 8779.2 | 76903.6 | 53146.7 |
| $s_{x_0}$ | 4.73 | 4.62 | 0.063 | 0.097 | 0.866 | 0.609 |
| $V_{x_0}$ | 2.14 | 2.41 | 0.58 | 0.90 | 0.93 | 0.66 |
| $R$ | 95.50 | 96.00 | 94.39 | 94.35 | 94.77 | 95.04 |
| $s_R$ | 3.0002 | 2.9918 | 2.8831 | 2.0349 | 2.1849 | 2.0211 |
| $V_R$ | 3.14 | 3.11 | 3.05 | 2.16 | 2.31 | 2.13 |
| $H_V$ | 7.51 | 7.20 | 6.93 | 6.37 | 10.27 | 9.10 |
| $P$ | 99 | 99 | 99 | 99 | 99 | 99 |

[a] $y=a_0-x+a_1$; $W$, working range (ng/ml); $N$, number of calibration standard points; $s_y$, residual standard deviation; $s_{x_0}$, method standard deviation; $V_{x_0}$, relative method standard deviation (%), $R$, recovery (whole working range) (%); $s_R$, standard deviation of recovery; $s_R^2$, variance of recovery; $V_R$, relative standard deviation of recovery (%); $H_V$, homogeneity of variance; $P$, power.

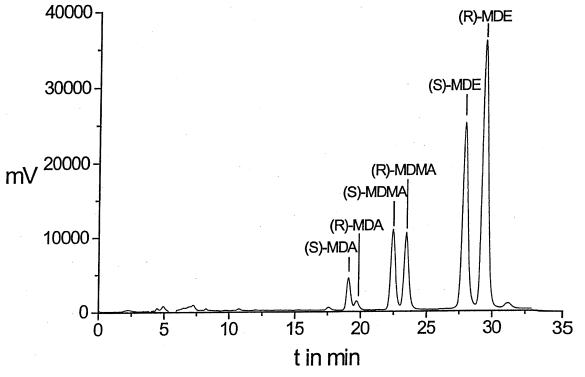


Fig. 2. HPLC chromatogram of an authentic sample (volunteer 5) via fluorimetric detection and β-cyclodextrin in the mobile phase.

(ChiraDex 250×4 mm, Merck). In comparison with the non covalently bonded cyclodextrins, an inversion of the order of enantiomers and a lower chromatographic resolution were observed (Fig. 3). Due to the lower chromatographic resolution it was not possible to use covalently bonded β-cyclodextrins for this separation problem, since the resolution between the enantiomer pairs MDA and MDMA (internal standard) was insufficient.

In addition, it was not possible to separate the stereoisomers of HME using β-cyclodextrin, since the spatial structure of the 4-hydroxy-3-methoxy analyte prevents interaction between HME and β-cyclodextrin. According to our investigation of 4-hydroxy-3-methoxy-ethylamphetamine and the related compounds 3-hydroxy-4-methoxy- and 3,4-dimethoxyethylamphetamine it seems that the methoxy group in position 3 of the phenyl substituent hinders the formation of a stable inclusion complex. The 3-hydroxy-4-methoxy compound is separated into its stereoisomers, the 3,4-dimethoxy compound acts like 4-hydroxy-3-methoxy-ethylamphetamine. For this reason, another stationary phase was selected. On this chiral protein phase, containing covalently bonded cellobiohydrolase as a chiral selector (Chiral CBH), the enantioselective separation of HME is possible (Fig. 4).

The plasma extracts separated by HPLC have been detected fluorimetrically or electrochemically. Methylenedioxyamphetamines were detected fluorimetrically at an excitation wavelength of 286 nm and an emission wavelength of 322 nm. HME could be detected in the same way, but the resolution of the used protein phase is not sufficient for the separation of HME and the methylenedioxyamphetamine MDE. For this reason, electrochemical detection for HME was selected. The major metabolite of MDE is oxidised by a potential of 650 mV at pH 6.1 of the mobile phase. Because the oxidation potential of phenolic compounds is dependent on the pH value, an increase of pH value produced a decrease in the oxidation potential. Under these conditions, the potential is too low for the oxidation of the methylenedioxy compounds, hence HME was selectively detected.

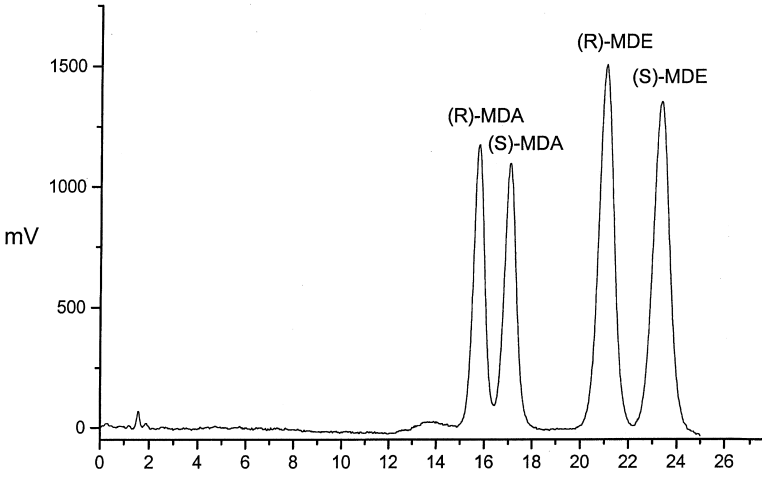


Fig. 3. HPLC chromatogram of racemic MDE and MDA with covalently bonded β-cyclodextrin (Chiradex 250×4 mm).

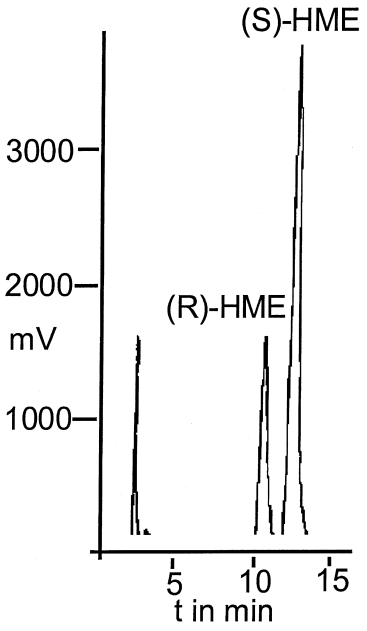


Fig. 4. HPLC chromatogram of an authentic sample (volunteer 5) via electrochemical detection.

Both described detection methods were, respectively, suitable to quantitate MDE, MDA and HME in plasma. The quantitation limit for MDE and MDA is down to less than 5 ng/ml by fluorescence detection and for HME less than 15 ng/ml by electrochemical detection. Reasons for the higher quantitation limit by electrochemical detection were, on the one hand, the absence of an internal standard and, on the other hand, the lower stability of the electrochemically detected signal. As consequence, a lower regression value of the calibration function and a wider confidential value were obtained. Nevertheless the validation of both methods yielded reliable and precise results (cf. Table 1). Another important advantage of the described methods is the possibility to analyse the compounds without derivatization. The direct determination reduces the time of analysis and removes a possible source of error.

### *3.2. Plasma concentration curves and pharmacokinetic parameters*

The validated analytical method was used to measure the concentrations of the stereoisomers of MDE and its metabolites HME and MDA in plasma samples from the six healthy subjects of the clinical study. The data obtained were used to construct plasma concentration curves for the stereoisomers of the individual substances (see Figs. 5–10). MDE and HME were already detectable in plasma samples within 0 to 20 min after ingestion, whereas formation of MDA was shown to occur not before 20 min after intake of MDE. It was still possible to determine

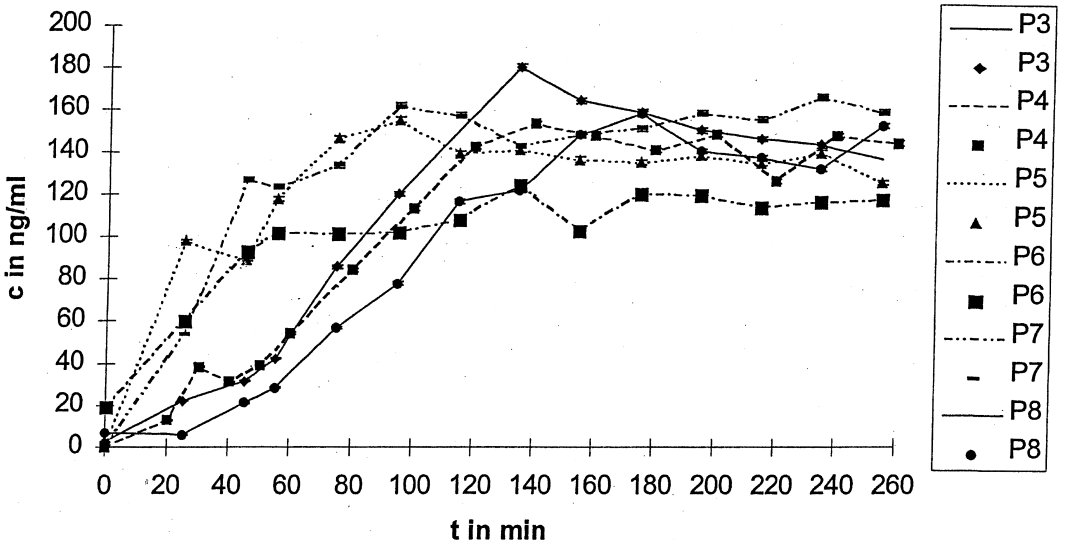


Fig. 5. Plasma concentration–time curves of *R*-MDE (volunteers 3–8).

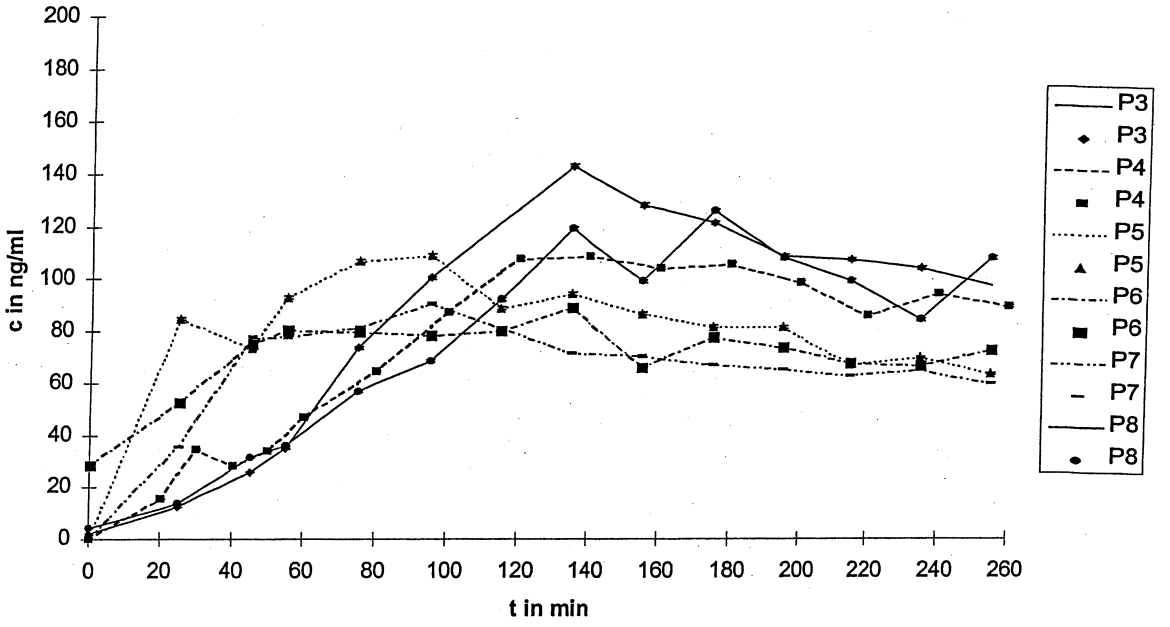


Fig. 6. Plasma concentration–time curves of *S*-MDE (volunteers 3–8).

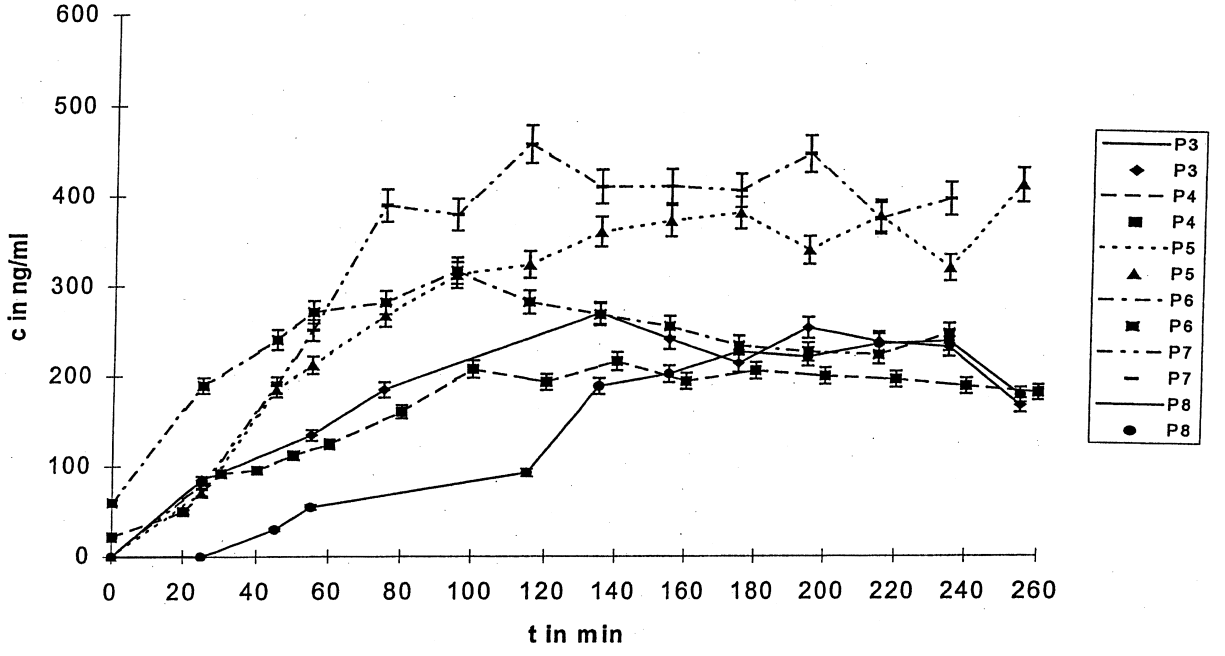


Fig. 7. Plasma concentration–time curves of *S*-HME (volunteers 3–8).

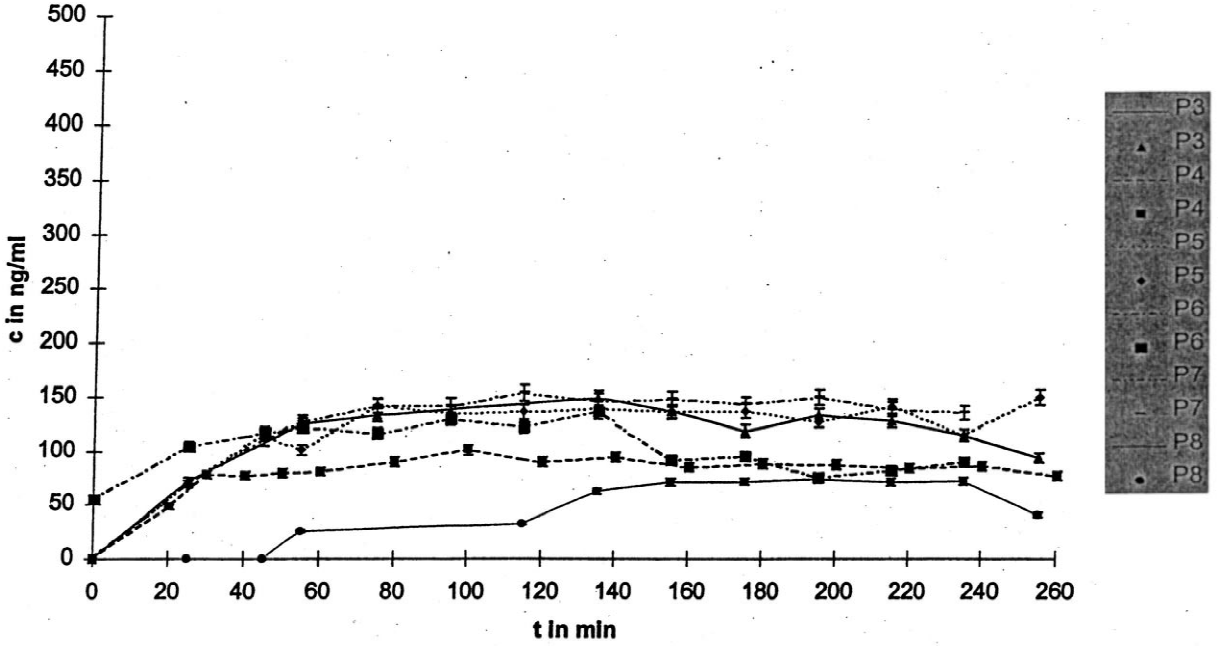


Fig. 8. Plasma concentration–time curves of *R*-HME (volunteers 3–8).

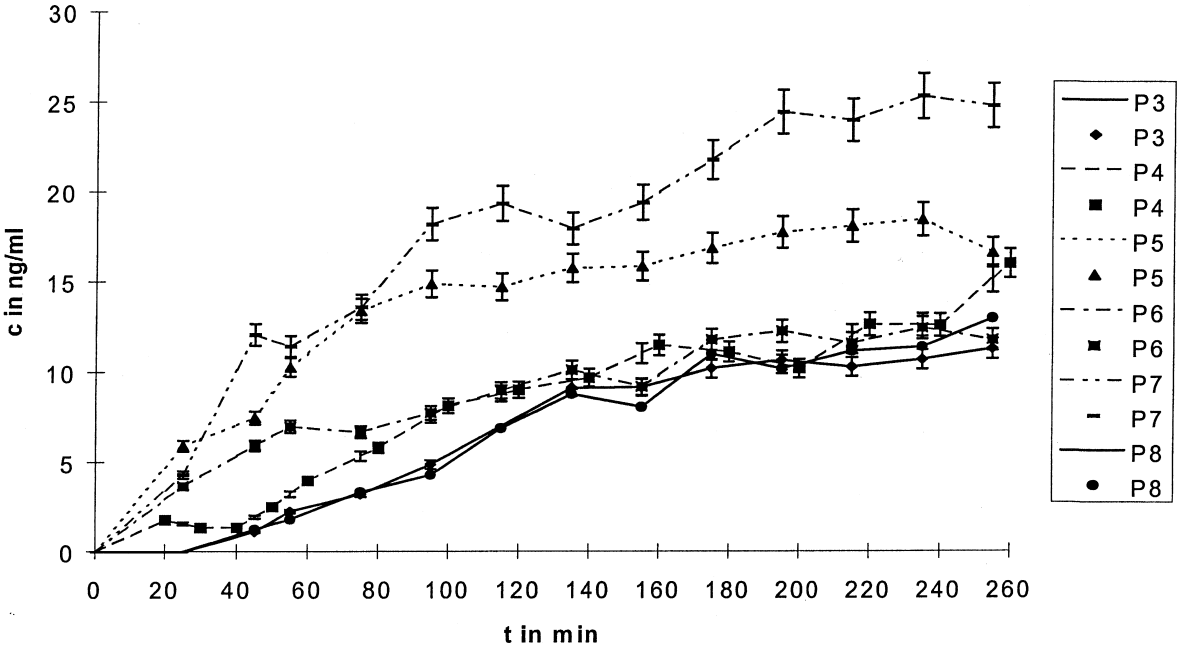


Fig. 9. Plasma concentration–time curves of *S*-MDA (volunteers 3–8).

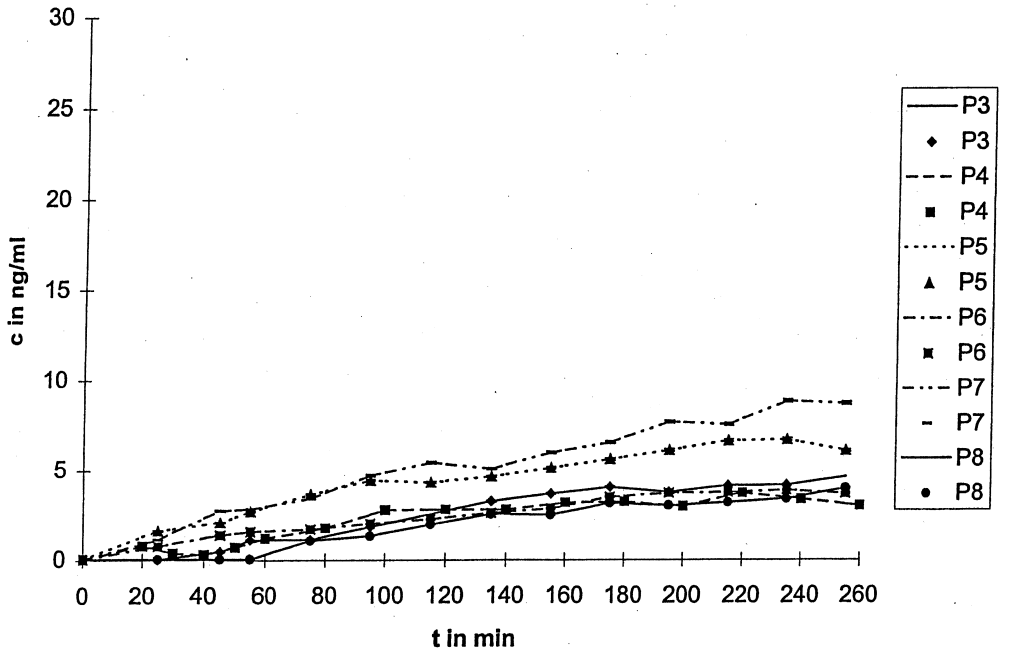


Fig. 10. Plasma concentration–time curves of *R*-MDA (volunteers 3–8).

MDE, HME and MDA in the blood samples of all the subjects at the end of the sampling period. The determination of the elimination phase was not possible, because the sampling period in the study was too short. For this reason, $C_{max}$ and $t_{max}$ were picked off the plasma concentration curves of the stereoisomers were investigated between 0 and 260 min. In all subjects, investigated $C_{max}$ values (Figs. 5 and 6) of *S*-MDE were lower than those of *R*-MDE. The maximum plasma concentrations ($C_{max}$) for *R*-MDE were between 110 ng/ml and 180 ng/ml after 80 to 120 min (Fig. 5). However, maximum plasma concentrations of *S*-MDE were found only between 90 ng/ml and 140 ng/ml (Fig. 6). The relation between the plasma concentrations of *S*-MDE and the time elapsed after ingestion of racemic MDE was similar of that for *R*-MDE. In contrast, significantly higher plasma concentrations were measured for the *S*-enantiomer of HME, the major metabolite of MDE. The highest plasma concentration of *S*-HME measured was 450 ng/ml (Fig. 8). Conversely, only low values of *R*-HME were found in the plasma samples. The highest observed plasma concentration of the *R*-stereoisomer was about 160 ng/ml (Fig. 7).

The ratio of the two enantiomers of the second metabolite MDA is similar to that found for HME (Figs. 9 and 10). The highest plasma concentration of *S*-MDA measured was about 25 ng/ml, and of *R*-MDA it was about 9 ng/ml.

### *3.3. Comparison of the pharmacokinetic results with other studies*

Pharmacokinetics of methylenedioxyamphetamines in men has been carried out by Lanz et al. [6], de Boer et al. [7], Fallon et al. [8] and Tagliaro et al. [9]. Lanz et al. determined the enantiomeric ratio of Ecstasy (MDMA) in urine samples of two male volunteers, further de Boer et al. and Tagliaro et al. elucidated the metabolites of MDMA in urine and hair samples. Only one of these publications [8] investigated the metabolism and disposition of racemic Ecstasy in plasma samples of men. The data obtained indicated that the stereoselective disposition of Ecstasy in humans is comparable to the results of our study. The enantiomeric ratio of the methylenedioxyamphetamine MDMA showed a surplus of *R*-enantiomer and its metabolite MDA a surplus of the *S*-enantiomer [8]. Different results were shown by the comparison of human and non-human phar-

macokinetics. The investigation of the metabolism of Ecstasy in rats [16] showed a similar enantioselectivity of the metabolic enzymes like in men. In contrast, the enantioselectivity of the metabolic enzymes in mice is inverse [18]. Inconsistent results were also shown by the in vitro investigation of human and rat liver microsomes [19]. On the one hand Tucker et al. [19] investigated a higher turnover of the *R*-enantiomer in rat liver microsomes on the other hand isolated human liver microsomes showed no surplus of the enantiomeric degradation of the used methylenedioxyamphetamine. In this case, data from non-human or in vitro experiments are not suitable to predict the enantioselectivity of the metabolism of methylenedioxyamphetamines in humans.

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