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Quantitation of *N*-ethyl-3,4-methylenedioxyamphetamine and its major metabolites in human plasma by high-performance liquid chromatography and fluorescence detection

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Abstract

A HPLC method has been developed for the analogue of Ecstasy MDE and its major metabolites *N*-ethyl-4-hydroxy-3-methoxyamphetamine (HME) and 3,4-methylenedioxyamphetamine (MDA) in human plasma. In the course of our investigations we found that the methylenedioxyamphetamines and HME exhibit fluorescence at 322 nm. Therefore the detection could be carried out with a fluorescence (FL) detector. Solid-phase extraction was used for sample preparation and yielded high recovery rates greater than 95%. The limit of quantitation for MDE and its metabolites in the extracts was between 1.5 and 8.9 ng/ml and the method standard deviations were less than 5%. This sensitive, rapid and reliable analytical method has been used successfully in the quantitation of the substances in plasma samples obtained from 14 volunteers in two clinical studies after p.o. administration of 100 to 140 mg MDE*HCl. The maximum plasma concentrations were 235–465 ng/ml (MDE), 67–673 ng/ml (HME) and 7–33 ng/ml (MDA), respectively. Pharmacokinetic parameters have been investigated using the plasma concentration curves. © 1998 Elsevier Science B.V. All rights reserved.

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

1. Introduction

N-Ethyl-3,4-methylenedioxyamphetamine (MDE), a designer drug of the ecstasy type [1], acts as an entactogen [2,3] and has been increasing in importance in recent years as an illegally consumed addictive substance. In addition, it is used in basic psychiatric research in human experiments, since MDE possesses a lower neurotoxicity than *N*-methyl-3,4-methylenedioxyamphetamine (MDMA, ecstasy) [4,5]. There is, therefore, a forensic and clinical analytical requirement for the quantitation of MDE and its metabolites in body fluids. Using human urine samples we have clarified the biotransformation with gas chromatography–mass spectrometry (GC–MS), high-performance liquid chromatography–UV detection (HPLC–UV) and high-performance thin-layer chromatography (HPTLC)–UV [6–8]. In this way it is possible to elucidate two routes of metabolism. In the one, there is degradation of the side chain by *N*-desalkylation to 3,4-methylenedioxyamphetamine (MDA) and, in the other, the

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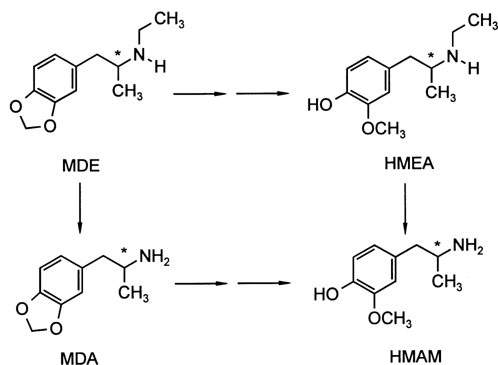

Fig. 1. Major metabolites of MDE (cf. Ref. [6]). The chiral C is marked with an asterisk.

methylenedioxy group is cleaved, yielding first the dihydroxymetabolite followed by methylation in the 3 position of the aromatic to yield *N*-ethyl-4-hydroxy-3-methoxyamphetamine (HME) (Fig. 1).

In the literature the quantitation of MDE in human plasma has been limited to two individual values obtained during road traffic checks, whereby the metabolites were not taken into account [9]. The procedure used was GC–MS, which necessitated derivatization of the samples.

Therefore this paper describes a rapid, reliable and sensitive HLPC–fluorescence detection (FL) method for the quantitation of MDE and its major metabolites HME and MDA in human plasma. Plasma concentration curves have been calculated using plasma samples obtained from 14 healthy volunteers after p.o. administration of 100 to 140 mg MDE-HCl per person and pharmacokinetic parameters have been investigated.

2. Experimental

2.1. Materials

MDE, HME, MDA and MDMA were synthesized according to Ref. [10]. Potassium dihydrogenphosphate and sodium acetate in analytical grade were obtained from Fluka (Buchs, Switzerland). Acetonitrile and methanol were purchased from Rathburn (Zinsser Analytic, Frankfurt, Germany), orthophosphoric acid 85% p.a., hydrochloric acid 1-molar p.a. and β-glucuronidase (30 U/ml)/arylsulphatase (60 U/ml) were supplied by Merck (Darmstadt, Germany) and polyethylene glycol (PEG) 6000 in DAB quality by Merck–Schuchardt (Hohenbrunn, Germany).

Water was deionized and twice distilled. Human plasma for reference was obtained from a blood bank (University Clinic, Tübingen, Germany). The real plasma samples were provided by two clinical studies (Departments of Psychiatry and Psychotherapy, University of Freiburg, Germany and Technical University of Aachen, Germany). Plasma was kept below −20°C until analysis. Under the described storage conditions the samples remained stable for the investigated period of time.

For purifying the enzyme solution PD10 columns (Pharmacia, Freiburg, Germany) and for solid-phase extraction (SPE) CBA columns (200 or 500 mg) were used (ICT, Frankfurt, Germany). HPLC was carried out on a LiChroCart Superspher 60 RP-select B column, 5 μm, 250×4 mm I.D. using a guard column LiChrospher 60 RP-select B, 5 μm, 4×4 mm I.D.

2.2. Equipment

The HPLC station comprised a gradient pumping system L 6200 A, a LaChrom autosampler L 7200, a LaChrom fluorescence detector L 7480 and a chromointegrator D2500 all from Merck (Darmstadt, Germany).

2.3. Sample preparation

For the hydrolysis of the conjugates in plasma the β-glucuronidase/arylsulphatase solution was purified according to the manufacturer's instructions by means of SEC (size-exclusion chromatography) in 0.1 *M* sodium acetate buffer (pH 5.2). The activity of the purified enzyme solution was 6 U/ml for the β-glucuronidase and 12 U/ml for the arylsulphatase. A 1-ml aliquot of plasma was treated with 125 μl purified enzyme and 875 μl 0.1 *M* sodium acetate buffer (pH 5.2) and then incubated at 37°C for 24 h. Protein was precipitated by adding 1 ml of an aqueous solution of PEG 6000 (20%) under cooling in an ice bath for 5 min. Two hundred and twenty ng/ml of the internal standard MDMA were solved in the PEG solution. Then the samples were cen-

trifuged at 2875 g/5 min/20°C. The supernatant was subjected to SPE on cation-exchange columns (CBA). After conditioning with 2 ml acetonitrile, 1 ml 0.1 M HCl and 3 ml 0.1 M sodium acetate buffer (pH 6.5) the sample was applied. Then followed rinsing with 2 ml water and 2 ml water–methanol (75:25, v/v). The elution, that followed, was carried out with 2 ml acetonitrile–water–1 M HCl (8:1:1, v/v/v). The samples were evaporated to dryness under nitrogen at 45°C and taken up in 250 μl water. Ten μl of the extract was used for injection.

2.4. HPLC conditions and detection

A LiChroCart Superspher 60 RP-select B column, 5 μm, 250×4 mm was used for separation. The mobile phase consisted of 20 mmol potassium dihydrogenphosphate buffer, pH 3.0–acetonitrile (85:15, v/v). The flow-rate was 700 μl/min. Detection was carried out with a fluorescence detector. The extinction wavelength was set at 278 nm for HME and 286 nm for MDA, MDMA and MDE and the emission wavelength was 322 nm for all substances.

2.5. Validation

The analytical procedure was validated according to the ICH guidelines [11,12]. Eight equidistant calibration points were measured first in aqueous solution and second after sample pretreatment from spiked plasma solutions. Hence the recovery was calculated over the whole working range. In addition ten spiked plasma concentrations of the high working range as well as of the low working range were determined. The homogeneity of variance, the precision and the accuracy were calculated from the data obtained. The parameters of validation are presented in Tables 1 and 2.

Table 2
Parameters of calibration

	a_0	a_1	s_y	s_{x0}	V_{x0}
HME	−1696	17 015	70 456	4.14	4.14
MDA	17 528	36 284	7724	0.31	1.47
MDE	47 433	34 770	61 223	1.76	0.79

$y=a_0+a_1x$.
s_y (residual standard deviation); s_{x0} (method standard deviation); V_{x0} (relative method standard deviation).

2.6. Study design and sampling

In the first clinical study oral doses of 140 mg MDE-HCl per person were administered to six healthy volunteers (self-experimentation by physicians). Some volunteers with low body mass experienced serious psychological effects. Therefore in the second clinical study with eight volunteers (physicians) the individual dose of MDE*HCl was adjusted to 2 mg per kg body mass, but maximum 140 mg per person. Both studies were placebo-controlled and double blind with randomized alloca-

Table 1
Statistical parameters

	HME	MDA	MDE
Working range (ng/ml)	32–680	2–40	20–480
Calibration standard points	8	8	8
Linearity, coefficient of correlation	0.9997	0.9998	0.9999
Limit of quantitation (ng/ml)	5.9	1.5	8.9
Recovery (%) (whole working range)	95.3	95.4	95.7
C.V. (%)	2.9	2.7	0.9
Homogeneity of variance	7.01	4.86	1.15
	(P=99.9%, performed)	(P=99%, performed)	(P=95%, performed)
Precision high working range (n=10) (%)	0.9	0.8	0.3
Precision low working range (n=10) (%)	6.0	5.7	4.2
Accuracy high working range (n=10) (%)	5.2	3.0	3.6
Accuracy low working range (n=10) (%)	15.5	4.5	8.8

tion. These studies have been approved by the Ethical Committees of the departments of medicine in Freiburg and Aachen, Germany. In order to study the pharmacokinetics blood samples were taken after administration (first study: 0 to 240 min; second study: −15 to 360 min, three volunteers 420 min, one volunteer 24 h). The blood samples were centrifuged in heparinized tubes at 2875 *g*/10 min/20°C. Then the supernatant plasma was stored deep frozen at −20°C.

3. Results and discussion

3.1. Analytical procedure

The testing of some plasma samples without enzymatic hydrolysis yielded very low concentrations of HME, the main metabolite in urine. Since HME in urine is primarily present in conjugated form [7], the plasma samples were subjected to enzymatic hydrolysis with β-glucuronidase/arylsulphatase. The comparison of both plasma concentrations of HME yielded a part of 80% of conjugated HME.

Since amphetamine derivatives can be expected to have a pK_a of between 9.5 and 10.5 [13], the weak cation exchangers, CBA columns, were used for the following SPE. These columns with a pK_a of 6.5 guarantee quantitative retention of amphetamines on the CBA sorbent at pH 6–7. The SPE yielded very high recovery rates of more than 95% here and it was very selective, since weaker bases with pK_a<9 were not retained by the ion-exchange column.

The plasma extracts separated by HPLC could then be detected fluorimetrically (Fig. 2). During development of the method it was established that both methylenedioxyamphetamines and HME possess analytically exploitable fluorescence (Fig. 3). The absorption maximum of the excitation wavelength for the FL detection of HME was 278 nm, thus, somewhat lower than that of the methylenedioxy compounds with 286 nm. The measured emission maximum was 322 nm for all compounds. This wavelength is presumably not dependent on the methylenedioxy ring system but is purely a function of the position and number of oxygen substituents on the aromatic. It was possible to quantitate MDE and its metabolites in plasma very sensitively down to less than 10 ng/ml by FL detection. On account of the higher determination

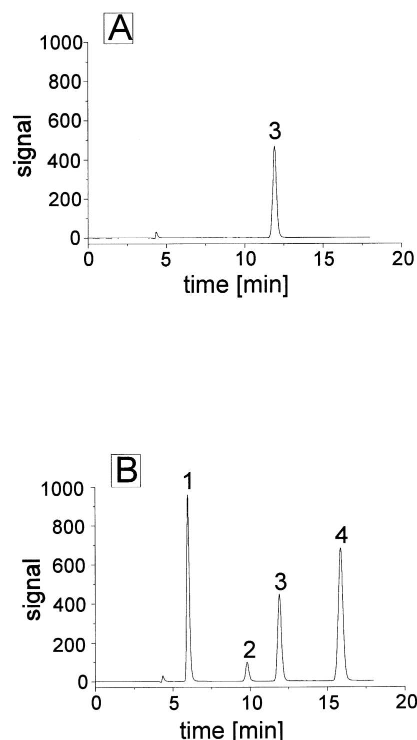

Fig. 2. HPLC chromatograms obtained from real plasma samples of volunteer 1 after 0 min (A) and 120 min (B) after p.o. administration of 140 mg MDE-HCl. (A) MDMA (**3**, t_R=11.8 min, internal standard). (B) HME (**1**, t_R=6.0 min), MDA (**2**, t_R=9.8 min), MDMA (**3**, t_R=11.9 min), MDE (**4**, t_R=15.9 min).

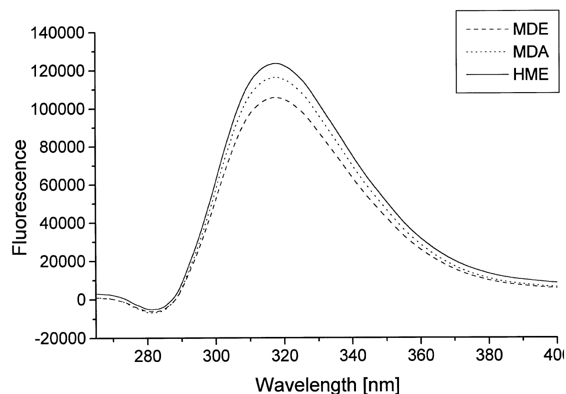

Fig. 3. Fluoresence spectra of MDE, HME, MDA in mobile phase.

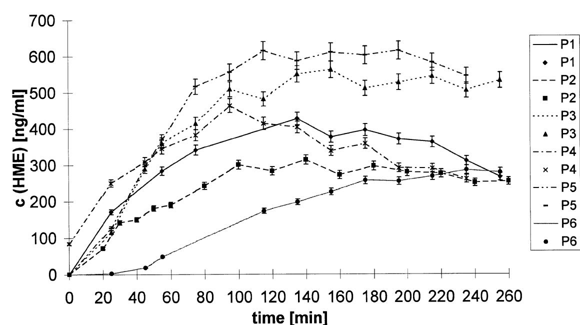

Fig. 4. Plasma concentration curves of HME (ng/ml); first study $n=6$, error bars=inter-individual S.D.

limits UV detection was not possible. This HPLC method has the advantage over a possible GC–MS measurement in that it is not necessary to derivatize the substances to be measured. Direct determination both reduces the analysis time and removes a possible source of error. The method validation yielded reliable and precise results (cf., Tables 1 and 2).

3.2. Plasma concentration curves and pharmacokinetic parameters

The validated analysis method was used to measure the concentrations of MDE and its metabolites HME and MDA in the plasma samples from the 14 subjects of two clinical studies. The data obtained were used to construct plasma concentration curves for the individual substances (see Figs. 4–9). The times from which it was possible to quantitate the substances in the plasma were between 15 and 50 min for MDE and HME and 30 and 140 min for MDA. It was still possible to determine MDE and MDA in the blood of all subjects at the end of the sampling period. Only in the case of two subjects was it no longer possible to detect HME after 360 min. In the case of one subject an additional sample was taken after 24 h. It contained 54 ng/ml MDE

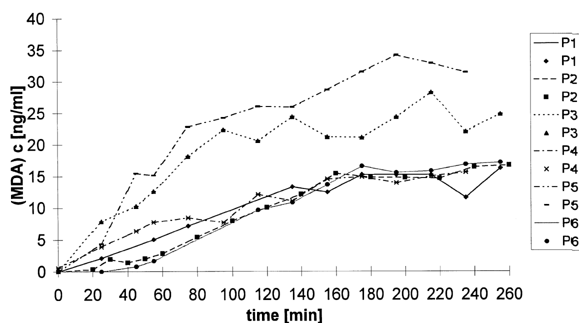

Fig. 6. Plasma concentration curves of MDA (ng/ml); first study $n=6$, error bars=inter-individual S.D.

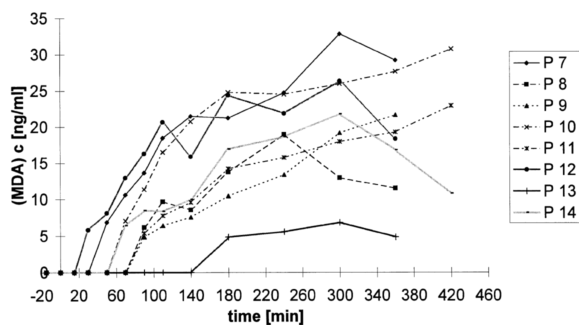

Fig. 7. Plasma concentration curves of MDA (ng/ml); second study $n=8$, error bars=inter-individual S.D.

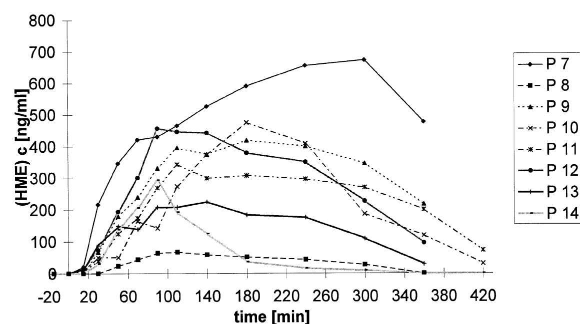

Fig. 5. Plasma concentration curves of HME (ng/ml); second study $n=8$, error bars=inter-individual S.D.

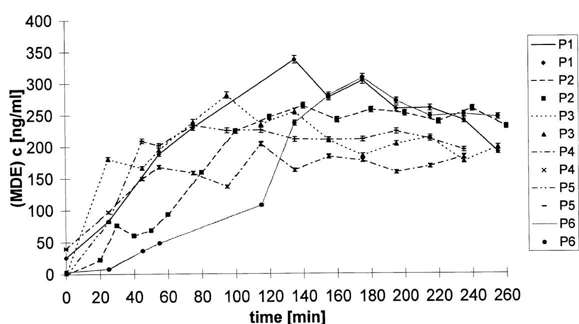

Fig. 8. Plasma concentration curves of MDE (ng/ml); first study $n=6$, error bars=inter-individual S.D.

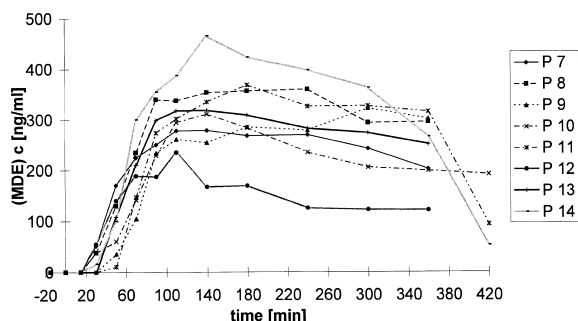

Fig. 9. Plasma concentration curves of MDE (ng/ml); second study n=8, error bars=inter-individual S.D.

and 16 ng/ml MDA. The phamacokinetic parameters c_{max} and t_{max} for MDE and its metabolites were determined by direct inspection of the plasma concentration curves obtained (Table 3). The AUC (area under the curve) was calculated by the trapezoidal rule until 4 h (first study) an 6 h (second study). The inter-individual variations are least for MDE with ca. 20% for the AUC and c_{max}, while these kinetic parameters are subject to appreciably more variation in the case of the metabolites. The highest concentration of HME measured was 673 ng/ml, the lowest c_{max} value was 67 ng/ml in plasma. The subject with the low HME concentration, thus, had a rate of metabolism that was lower by a factor of ten. In the case of the homologous MDMA the corresponding metabolic step by the enzyme cytochrome P-450 2D6 (CYP2D6) catalyses conversion to the dihydroxymetabolite in human liver microsomes [14], which is then methylated to the hydroxymethoxy metabolite. It is to be assumed that this reaction occurs in an analogous manner from MDE to HME. CYP2D6 is subject to great genetic variability [15]. It could be that the subject with the low plasma HME concentration is a member of the group of ‘‘poor metabolizers’’, who do not possess the enzyme CYP2D6 and who make up almost 10% of the population.

4. Conclusions

MDE and its metabolites HME and MDA, which are representatives of the two major routes of degradation, can be very sensitively detected in human plasma by means of HPLC–FL. The HPLC–FL method has the advantage over a GC determination in that the intrinsic fluorescence of the substances to be detected makes derivatization unnecessary. The result of the method validation shows that this method yields accurate analysis results rapidly, simply and reliably. It can, therefore, be employed for the forensic and clinical analysis of the methylenedioxyamphetamines (MDE, MDMA, MDA) and their metabolites. Here it is to be expected that the maximum plasma concentrations can be determined two to three h after administration and still be determinable in the plasma after 24 h.

Acknowledgements

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Table 3
Pharmacokinetic parameters

	First study (n=6)			Second study (n=8)		
	HME	MDA	MDE	HME	MDA	MDE
c_{max} (ng/ml)	285–615	15–32	203–333	67–673	7–33	235–465
$\bar{c}_{max}$ (ng/ml)	436	21	260	389	23	332
R.S.D. (c_{max}) (%)	31.2	34.8	17.4	49.4	35.2	20.6
t_{max} (ng/ml)	95–235	200–215	95–175	90–300	240–420	110–300
$\bar{t}_{max}$ (ng/ml)	166	203	129	159	330	174
R.S.D. (t_{max}) (%)	29.4	3.0	21.4	43.0	19.4	37.0
AUC (ng/ml h)	665–1976	38–95	657–875	218–3028	19–120	832–1935

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