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High-performance thin-layer chromatographic determination of N-ethyl-3,4-methylenedioxyamphetamine and its major metabolites in urine and comparison with high-performance liquid chromatography

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Abstract

The consumption of N-ethyl-3,4-methylenedioxyamphetamine (MDE, 1), an analogue of ecstasy, can be detected by direct in situ HPTLC–FTIR measurement of the main metabolite N-ethyl-4-hydroxy-3-methoxyamphetamine (HME, 2). HME (2) can, like the other important metabolite 3,4-methylenedioxyamphetamine (MDA, 3) and unchanged MDE (1), be determined quantitatively in urine by HPTLC–UV after two-step automatic development. The results have been compared with those obtained using an HPLC method. The differences were not generally significant. Small deviations were attributable to the different sample preparation methods necessary. The working range for the HPTLC method was between 0.1 and 8.2 μg/ml and for the HPLC method between 0.2 and 60.0 μg/ml. The method standard deviations were 2.66–4.91% (HPTLC) and 0.48–3.67% (HPLC).

Keywords: N-Ethyl-3,4-methylenedioxyamphetamine; N-Ethyl-4-hydroxy-3-methoxyamphetamine; 3,4-Methylenedioxyamphetamine

1. Introduction

N-Ethyl-3,4-methylenedioxyamphetamine (MDE, 1), which is a designer drug of the ecstasy type [1], is metabolized in the body via two major pathways [2]. The first and predominant pathway leads via ring degradation by O-dealkylation to the corresponding 3,4-dihydroxy metabolites, which are subsequently methylated at the hydroxyl group in position 3 of the aromatic ring. The second pathway leads via side chain degradation by N-dealkylation to the corresponding primary amines. Oxidative N-deamination forms the substituted phenylacetones, which are degraded to the corresponding benzoic acids and conjugated with glycine to substituted hippuric acids. The hydroxy metabolites are partly excreted in a conjugated form.

HME (2) (N-ethyl-4-hydroxy-3-methoxyamphetamine), which is the major metabolite quantitatively, and MDA (3) (3,4-methylenedioxyamphetamine) may be regarded as markers for these two pathways (Fig. 1). In a previous paper we described the identification of MDE (1) and its metabolites in urine by GC–MS and FPIA and the point of time until which they can be detected [3]. The purpose of this investigation was to identify HME (2) together with unchanged MDE (1) in urine samples, by thin-layer chromatography using direct

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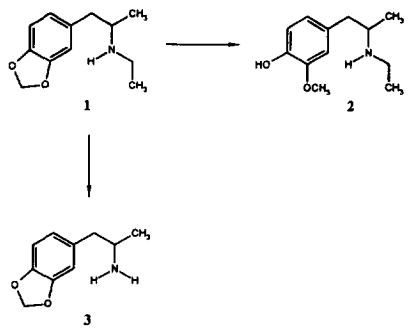

Fig. 1. Chemical structures of MDE and two of its metabolites.

FTIR coupling [4] and to quantitate them together with MDA (3) by densitometry. The results for the urine investigation of subjects, who had taken 140 mg MDE (1)-HCl during a study [5], were to be compared with those obtained by HPLC analysis.

2. Experimental

2.1. Materials

MDE (1), MDA (3), HME (2) and MDMA (N-methyl-3,4-methylenedioxyamphetamine) were synthesized according tothe procedure described in Ref. [6]. Solvents and mobile phases were of p.A. quality and were supplied, together with the β-glucuronidase (30 U/ml)/arylsulphatase (60 U/ml) solution (EC 3.2.1.31 and EC 3.1.6.1) and the Extrelut 3-columns for liquid–liquid extraction, by Merck (Darmstadt, Germany). Cation-exchange columns, Anlytichem Bond Elut PRS 3CC 500 mg (ICT, Frankfurt, Germany) were used for solid-phase extraction. A PD10 column (Pharmacia, Freiburg, Germany) was used for purifying the enzyme solution. The fluorescamine was obtained from Sigma (Deisenhofen, Germany).

The thin-layer chromatography was carried out on HPTLC silica gel 60 F_{254} plates with a layer thickness of 200 μm obtained from Merck. The HPLC was carried out on an LiChrospher 60 RP-select B-column, 5 μm, 125×4 mm (Merck).

2.2. Equipment

Sample application was carried out using a Linomat IV and sample development with an AMD apparatus, both from Camag (Muttenz, Switzerland). The HPTLC–FTIR spectra were recorded using an IFS 48 FTIR spectrometer in combinations with the external DRIFT (diffuse reflectance infrared Fourier transform) unit with a narrow-band nitrogen-cooled MCT detector, a globar as radiation source and GC-IR software (OPUS) (Bruker, Karlsruhe, Germany). The HPTLC–UV and fluorimetric measurements were made with a CD 60 densitometer (Desaga, Heidelberg, Germany). The HPLC station comprised a gradient pump L-6200 A, a programmable UV–Vis detector L 4250 and a Chromo-Integrator D-2500 (Merck).

2.3. Planar chromatography

2.3.1. Sample preparation

Sample preparation for the determination of HME (2) (with enzymatic hydrolysis) and of MDE (1) and MDA (3) (both without enzymatic hydrolysis) was carried out separately. The enzyme solution was prepurified 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 (β-glucuronidase) and 12 U/ml (arylsulphatase). A 0.5-ml aliquot of urine (or a smaller amount of urine made up to 0.5 ml with blank urine) was treated with 450 μl purified enzyme solution [for the determination of HME (2)] or 450 μl 0.1 *M* sodium acetate buffer (pH 5.2) [for the determination of MDE (1) and MDA (3)] and with 40 μl of the aqueous solution of internal standard (2 μg MDMA) and then incubated at 37°C for 24 h. The volume was then made up to 3 ml with borate buffer (pH 11), yielding a pH of 10. The liquid–liquid extraction was carried out as recommended by the manufacturer, by eluting twice with 6-ml portions of a mixture of diethyl ether–*n*-hexane (9:1). The solvent was evaporated off in a stream of nitrogen at 50°C and the residues were taken up in 50 μl methanol and the whole volume was sprayed onto the HPTLC plate in the form of a 3 mm-broad band. A total of 14 sample solutions and 7 standard solutions were applied to each plate.

2.3.2. Chromatographic conditions

HPTLC silica gel 60 F_{254} plates 20×10 cm with a layer thickness of 200 μm were employed. The

development was carried out in two steps each of 15 min with toluene–acetone–ethanol–ammonia 25% (45:45:7:3) followed by acetone–methanol (50:50) in the AMD apparatus. The total time spent in the AMD apparatus was 60 min, the total volume of mobile phase was 110 ml.

2.3.3. Detection and identification

Detection was carried out at a wavelength of 283 nm. The identification of MDE (1) and HME (2) was carried out by recording the HPTLC–FTIR spectra at the peak maxima of the chromatograms [7] and comparing the spectra obtained with the contents of an HPTLC–FTIR spectrum library. Our HPTLC silica gel library, containing 350 substances of forensic and pharmaceutical interest, was searched with Bruker 'Search' software.

2.3.4. Quantitative determination

The quantitation was carried out in UV or fluorescence mode. The internal standard (MDMA) and MDE (1) were measured at $\lambda = 283$ nm, HME (2) at 278 nm and MDA (3) by fluorescence after post-chromatographic derivatization with fluorescamine dipping reagent after excitation at 365 nm using a 450 nm cut-off filter. Derivatization reaction was a three-step process. First the plate was dipped in solution of 20 mg fluorescamine in 100 ml acetone, afterwards in a mixture of 10 ml triethylamine and 90 ml dichloromethane and finally in a mixture of 10 ml paraffin and 50 ml *n*-hexane [8]. The calibration standards were all subjected to the complete sample work-up procedure and applied to each plate.

2.4. Column liquid chromatography

2.4.1. Sample preparation

Aliquots (0.5 ml) of urine were incubated with 0.5 ml 0.1 *M* sodium acetate buffer (pH 5), the solution of the internal standard (7.8 μg MDMA) and 45 μl of the β-glucuronidase/arylsulphatase solution at 37°C for 24 h and then subjected to solid-phase extraction on cation exchange columns. After conditioning with 1 part by volume (pbv) methanol and 1 pbv sodium acetate buffer the sample was applied. This was followed rinsing with 1 pbv sodium acetate buffer, removal by suction for 5 min at maximum vacuum, then rinsing with 2 ml *n*-hexane followed by drying again. The elution, that followed, was carried out with four 500-μl portions of a methanol–water–ammonia 25% (45:45:10) mixture. The samples were evaporated to dryness in nitrogen at 50°C and taken up in 200 μl mobile phase.

2.4.2. Chromatographic conditions and detection

A LiChrospher 60 RP-select B-column, 5 μm, 125×4 mm was employed. The mobile phase was a mixture of 92 pbv KH_2PO_4 buffer pH 3 (2.7 g/l) and 8 pbv acetonitrile with a flow-rate of 1.25 ml/min. The analysis time was 16.5 min with a 13.5 min rinse with a mixture of 70 pbv of the above KH_2PO_4 buffer and 30 pbv acetonitrile between each HPLC run. Detection was carried out at 278 nm using a UV detector.

3. Results and discussion

Solid-phase extraction in aqueous medium was found to be unsuitable for sample preparation for thin-layer chromatographic quantification because interfering substances were co-extracted. These denatured when the residue was taken up in methanol and prevented quantitative sample application. Liquid–liquid extraction with a purely organic elutant proved more suitable. The enzyme solution for the hydrolysis of conjugates of HME (2) had to be prepurified before use for thin-layer chromatography, since the HPTLC–UV chromatogram of the pure enzyme extract was found to have three peaks, whose positions interfered with the determinations of MDE (1) and its metabolites. Purification was carried out by size exclusion chromatography on a Sephadex G25 filled column. In spite of this when using HPTLC, each sample was determined with and without enzymatic hydrolysis (Fig. 2), in order to exclude the possibility of interference by other hydroxy metabolites in the quantification of MDA (3) and MDE (1); in contrast HPLC analysis of all three substances was possible with the one sample (Fig. 3). Interferences by endogenous compounds were not observed as shown by extraction of blank urines under the same conditions (Fig. 2D and Fig. 3B). The identification of MDE (1) and HME (2) was carried out by direct in situ HPTLC–FTIR measurement followed by comparison of the spectra with the corresponding spectra in the HPTLC–FTIR library [4]. The HPTLC–FTIR spectra (Fig. 4)

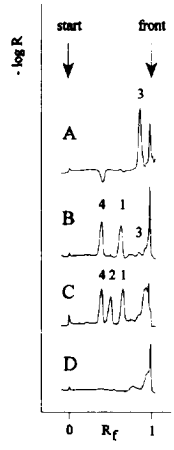

Fig. 2. HPTLC chromatograms obtained from urine containing MDMA (4), HME (2), MDE (1) and MDA (3). (A) After derivatization with fluorescamine (λ_{ex}=365 nm and λ_{fl}>450 nm); (B) detection with λ=283 nm; (C) after enzymatic cleavage, detection with λ=278 nm; (D) HPTLC chromatogram obtained from blank urine (λ=283 nm).

possess the advantage over HPTLC–UV spectra in that they are richer in bands and therefore considerably more suitable for the identification of substances [4]. Thus, the HPTLC–FTIR spectrum of HME (2) is characterized by the following bands: The bands at 2982 and 2946 cm^{-1} correspond to the

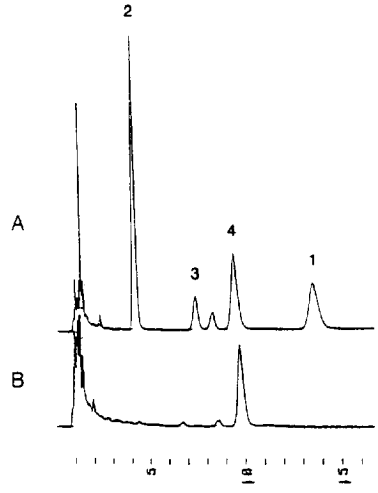

Fig. 3. (A) HPLC chromatogram obtained from urine after enzymatic cleavage containing HME (2), 4.06 min, MDA (3), 7.36 min, MDMA (4), 9.4 min and MDE (1), 13.52, (biomolecule 8.26 min). (B) HPLC chromatogram of blank urine spiked with MDMA (4), internal standard.

aliphatic CH-stretching vibrations, furthermore the CH-stretching vibration of the methoxy group is recognizable in the form of the band at 2848 cm^{-1}. The bands at 1610 and 1519 cm^{-1} are C=C stretching vibrations of the aromatic group, while the band at 1468 cm^{-1} represents the CH-bending vibration of the methoxy group. The CH-stretching vibration of the methylenedioxy group at 2782 cm^{-1} is particularly characteristic in the HPTLC–FTIR spectrum of MDE (1). It is possible, in this manner, to distinguish methylenedioxyamphetamines from each other and from their metabolites. Thus, it is possible to make identifications of quantities <1.3 μg/ml (<500 ng absolute). These quantities lie below the working range for MDE (1) and HME (2). The recognition of MDA (3) is more difficult because the working range for this compound is lower. However, the presence of MDA (3) is not characteristic for the consumption of MDE (1), since the presence of MDA (3) is also to be expected after the consumption of MDMA and of MDA (3) itself [9]. In order to be able to quantify at the low concentration range present MDA (3) was converted to a fluorescent product before determination by thin-layer chromatography.

It is necessary to dilute highly concentrated samples for quantitation by HPTLC in order to avoid reaching the saturation limits. In contrast, all HPLC determinations could be carried out using a 0.5-ml sample of urine. Here it was found that one working range sufficed for HPTLC, while two or three working ranges were necessary to guarantee variance homogeneity in the case of the HPLC method (Table 1). The working ranges lay between 0.1 and 8.2 μg/ml (HPTLC) and 0.2 and 60.0 μg/ml (HPLC). Then, in the case of HPTLC, chromatograms were evaluated and the calibration was carried out using the peak height which gave more reproducible results than the peak areas since the definition of the peak limits can lead to uncertainties. The linearity test according to Mandel [10] yielded a better fit with a second degree calibration function for all three substances. The HPLC calibration was carried out using the peak areas, when all three substances yielded linear calibration equations (Table 2).

Comparison of the method standard deviations for the two methods of determination by means of the *F*-test (P=99%) did not reveal any significant differ-

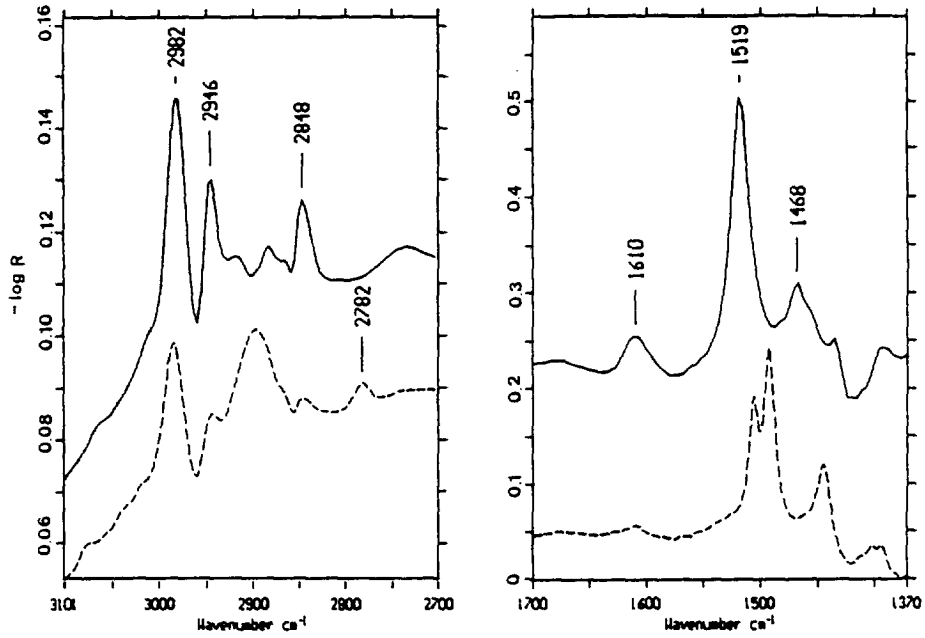

Fig. 4. HPTLC–FTIR spectra of MDE (- -) and HME (—).

ences, apart from one exception. Only in the case of the highest working range for HME (2) with the HPLC method it was found that the standard deviation for this method was significantly higher so that the HPTLC method yielded more precise results. The individual analysis results obtained by each method were compared for significant difference by a *t*-test of means (P=99%) (Table 3) after checking variance homogeneity. This test revealed no significant differences for the urine samples tested for MDE (1). In the case of MDA (3) the samples of subject 4 could not be distinguished, three urine samples of subject 8 were different. In the quantitation of HME (2) three and two urine samples of subjects 4 and 8 respectively were found to differ. This was attributed to the different sample preparation (solid-phase extraction for HPLC). Using HPLC, particularly for HME (2), the variations in the recovery rate were dependent on the amount and, hence, could not be compensated by the common internal standard (Table 4).

Table 1
Different working ranges

HPTLC	Working range (μg/ml)	HPLC	Working range 1 (μg/ml)	Working range 2 (μg/ml)	Working range 3 (μg/ml)
MDE	1.4–8.0	MDE	0.4–3.6	3.6–18.0	18.0–36.0
MDA	0.1–1.5	MDA	0.2–6.0	6.0–18.0	–
HME	1.3–8.2	HME	1.0–6.0	6.0–30.0	30.0–60.0

Table 2
Parameters of calibration

	a_0	a_1	a_2	s_{x0}	V_{x0}	N
HPTLC						
MDE	0.1638	0.4398	−0.0421	0.0625	2.66	7
HME	0.1142	0.3645	−0.0278	0.0634	2.66	7
MDA	−0.0051	5.0099	−2.3349	0.0188	4.91	7
HPLC						
MDE 1	−0.009	0.1254	0	0.037	3.67	5
MDE 2	−0.001	0.1194	0	0.1621	2.97	5
MDE 3	−0.003	0.1178	0	0.0657	0.48	6
HME 1	0.0174	0.1099	0	0.0377	2.14	6
HME 2	0.0445	0.109	0	0.2121	2.34	6
HME 3	0.078	0.1084	0	0.667	2.94	6
MDA 1	0.0023	0.1405	0	0.0235	1.56	6
MDA 2	−0.022	0.1458	0	0.054	1.21	6

$y=a_0+a_1x+a_2x^2$.
s_{x0} (standard deviation).
V_{x0} (relative standard deviation)$=(s_{x0}\times 100)/\bar{x}$.

Table 3
Analytical results of samples (μg/ml)

Sample	Volunteer 4				Sample	Volunteer 8			
	HPTLC		HPLC			HPTLC		HPLC	
	Result	CI	Result	CI		Result	CI	Result	CI
MDE									
4/2	5.41	0.45	5.03	1.25	8/2	2.71	0.29	2.46	0.26
4/3	5.94	0.50	5.68	1.22	8/3	9.24	0.79	9.2	1.14
4/4	7.29	0.74	7.54	1.17	8/4	18.72	1.60	18.88	0.44
4/5	12.08	1.02	12.48	1.14	8/5	21.6	1.81	22.74	0.41
4/6	1.80	0.28	1.8	0.26	8/6	13.98	1.31	15.14	1.19
4/7	2.07	0.28	2.17	0.26	8/7	11.02	0.92	10.81	1.13
HME									
4/2	10.96	0.88	11.8	1.55	8/2	2.29	0.31	2.21	0.24
4/3*	17.54	1.55	21.42	1.50	8/3	11.16	0.89	12.25	1.54
4/4	5.50	0.44	6.12	1.71	8/4*	19.05	1.63	23.11	1.52
4/5	5.91	0.65	6.83	1.68	8/5*	16.95	1.52	18.44	1.48
4/6*	1.19	0.34	1.66	0.19	8/6	11.33	0.90	11.36	1.56
4/7*	2.94	0.32	3.3	1.23	8/7	6.94	0.69	7.54	1.66
MDA									
4/2	0.15	0.09	0.19	0.16	8/2	0.10	0.09	0.10	0.16
4/3	0.29	0.09	0.32	0.16	8/3	0.66	0.18	0.58	0.16
4/4	0.59	0.11	0.59	0.16	8/4*	1.76	0.38	1.46	0.15
4/5	1.98	0.28	2.05	0.14	8/5	2.62	0.44	2.63	0.14
4/6	0.34	0.09	0.34	0.16	8/6*	1.86	0.27	2.13	0.14
4/7	0.57	0.10	0.6	0.16	8/7*	1.72	0.26	2.09	0.14

CI=confidence interval; *=significant differences between samples.

A comparison of the time and solvent requirement did not reveal any appreciable advantage for either method in spite of the doubling in the number of analysis samples for HPTLC. Thus, more organic solvent was required for sample preparation for HPTLC determination (12 ml per analysis sample) than for the solid-phase extraction used for HPLC (5 ml), but this relationship was reversed in the chromatography itself. Here the mobile phase consumption in HPTLC was almost 8 ml per analysis sample (110:14=7.9 ml) which was much lower than that required for liquid chromatographic analysis (almost 40 ml). This is valid especially if, like in the described study, many samples have to be analysed at the same time.

Table 4
Recoveries obtained from lowest and highest values of working ranges ($n=10$)

	MDE	HME	MDA	MDMA
HPTLC				
x (%)	81	79	86	79
VK (%)	8.9	7.5	13.1	4.2
HPLC				
x (%)	81	97	81	81
VK (%)	6.9	15.9	9.4	2.0

The time required for both methods is about the same in spite of the number of analysis samples being doubled for HPTLC. During HPTLC it is necessary to evaporate large quantities of solvent over a period of 1 h. The evaporation of aqueous samples for HPLC takes several h. Chromatography by HPTLC requires a time of ca. 4.5 min per sample while HPLC, including column rinsing, requires 30 min per analysis sample. The initial labour-intensive sample application for HPTLC is drastically reduced by use of a sample-applying apparatus. In the case of HPTLC account has also to be taken of the time required for detection and derivatization.

4. Conclusions

Thin-layer chromatography makes it possible to apply several nondestructive detection methods consecutively. The combination of the two coupling methods HPTLC–FTIR and HPTLC–UV makes rapid identification and quantitation of substances possible. In the investigation of the urine samples positive HPTLC–FTIR identification of MDE (1) and HME (2) makes it possible to prove the taking of MDE (1). The HPTLC–UV coupling was found to be as suitable a method as HPLC for the quantitation of the designer drug MDE (1) and its metabolites in urine. On account of the necessity for different sample preparation methods the amount of eluant required for HPTLC was greater than that required for HPLC, so that this, taken with the lower quantity of mobile phase required for thin-layer chromatography, means that the solvent consumption is about the same for both methods. This does not take conditioning and calibration during HPLC into account.

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