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Toxicological detection of the designer drug 3,4-methylenedioxyethylamphetamine (MDE, "Eve") and its metabolites in urine by gas chromatography–mass spectrometry and fluorescence polarization immunoassay[1]

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Abstract

Studies are presented on the toxicological detection of the designer drug methylenedioxyethylamphetamine [MDE, *rac*-N-ethyl-(3,4-methylenedioxyphenyl)-propane-2-amine] in urine after a single oral dose of 140 mg of MDE by GC–MS and fluorescence polarization immunoassay (FPIA). After acid hydrolysis, extraction and acetylation MDE and its metabolites could be detected by mass chromatography with the selected ions *m/z* 72, 86, 114, 150, 162 and 164, followed by identification of the peaks underlying full mass spectra by computer library search. The following metabolites could be detected: unchanged MDE and 3,4-dihydroxyethylamphetamine (DHE) for 33–62 h, 3,4-methylenedioxyamphetamine (MDA) for 32–36 h and 4-hydroxy-3-methoxyethylamphetamine (HME) for 7–8 days. 3,4-Dihydroxyamphetamine (DHA), 4-hydroxy-3-methoxyamphetamine (HMA), piperonyl acetone, 3,4-dihydroxyphenyl acetone and 4-hydroxy-3-methoxyphenyl acetone could only be detected in trace amounts within the first few hours. The Abbott TD_x FPIA assay amphetamine/metamphetamine II gave positive results in urine for 33–62 h. Therefore, positive immunoassay results could be confirmed by the GC–MS procedure which also allowed the differentiation of MDE and its homologues 3,4-methylenedioxymethamphetamine (MDMA) and MDA as well as other amphetamine derivatives interfering with the TD_x assay. Furthermore, this GC–MS procedure allowed the simultaneous detection of most of the toxicologically relevant drugs.

Keywords: Mass spectrometry; Fluorescence polarization immunoassay; 3,4-Methylenedioxyethylamphetamine

1. Introduction

Methylenedioxyethylamphetamine [*rac*-N-ethyl-(3,4 - methylenedioxyphenyl) - propane - 2 - amine; MDE; street name "Eve"] first described in 1980 [2] is a designer drug with psychedelic properties. MDE and its homologues 3,4-methylenedioxyamphetamine (MDA, "Love Pills") and 3,4-methylenedioxymetamphetamine (MDMA, "Adam", "Ecstasy") are able to enhance understanding, communicativeness and empathy while hallucinogenic effects are rare [3–5]. Nichols described these sub-

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[1]Dedicated to Professor Dr. Karl Pfleger, Homburg/Saar, on the occasion of his 70th birthday. Some of these results were reported at the EUROTOX '91, Annual Meeting of the European Society of Toxicology, Maastricht, September 1–4, 1991 [1].

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stances as entactogens, a new drug class different from hallucinogenic phenylethylamines and phenylpropanamines [6]. The use of these drugs was discussed for improving psychiatric explorations [7]. However, these substances have well documented neurodegenerative effects on the central serotonergic system, whereby the neurotoxic potency of MDE seems to be lower than that of MDA and MDMA [8–15]. The number of reports about abuse and intoxications with methylenedioxyamphetamine derivatives is increasing [16–22]. Within the scope of drug testing in clinical and forensic toxicology, procedures for screening and confirmation of MDE in biosamples are needed. The use of radio immunoassay [23], enzyme immunoassays [24,25] or fluorescence polarization immunoassays (FPIA) [24,26] for screening of methylenedioxyamphetamine derivatives was studied. The cross-reactivity values were published. More specific procedures are necessary not only for confirmation of the immunoassay results but also for differentiation of the amphetamines since the immunoassays also interfere with non-scheduled drugs like e.g. selegiline [27]. high-performance liquid chromatographic procedures have been published [28–30] but their specificity is not sufficient. In the following, studies are presented on the toxicological detection of MDE in urine by GC–MS and FPIA. The duration of detectability of MDE and its metabolites by GC–MS and FPIA was determined. This is necessary for the toxicological interpretation of the analytical result.

2. Experimental

2.1. Chemicals and reagents

All chemicals used were obtained from E. Merck (Darmstadt, Germany) and were of analytical grade. MDE and its metabolites were synthesized by Ensslin et al. as described in ref. [31].

2.2. Urine samples

Human urine was taken from healthy volunteers (psychiatrists or psychologists) who participated in a psychiatric study at the Department of Psychiatry, University of Freiburg (Germany) [3–5]. A single oral dose of 140 mg of *racemic* MDE was administered to nine volunteers. Urine samples were collected for 32 h. Three volunteers collected their urine samples for 10 days. All samples were stored at −20°C before analysis. Blank urine samples were collected before drug administration to control whether the samples were free of interfering compounds.

2.3. Sample preparation from urine

A 10-ml volume of urine was refluxed with 3 ml of 30% hydrochloric acid for 15 min. Following hydrolysis, the sample was basified with 4 ml of 10 mol/l aqueous sodium hydroxide and the resulting solution was mixed with 10 ml of 30% aqueous ammonium sulphate to obtain a pH between 8 and 9. This solution was extracted with 10 ml of a dichloromethane–isopropanol–ethyl acetate mixture (1:1:3 by volume). After phase separation by centrifugation (5 min, 3000 rpm), the organic layer was evaporated to dryness. The residue was derivatized by acetylation with 100 μl of acetic acid anhydride–pyridine (3:2 by volume) for 30 min at 60°C. After evaporation of the derivatization mixture, the residue was dissolved in 100 μl of methanol and 0.5–2 μl were injected onto the gas chromatographic system.

2.4. Gas chromatography–mass spectrometry

MDE and its metabolites were separated and identified in the acetylated urine extracts using a Hewlett-Packard (HP, Waldbronn, Germany) Series 5890 gas chromatograph combined with a HP MSD Series 5970 mass spectrometer and a HP MS ChemStation (DOS series) with HP G1034C software. The GC conditions were as follows: splitless injection mode; column, HP capillary (12 m×0.2 mm I.D.), cross-linked methylsilicone, 330 nm film thickness; injection port temperature, 270°C; carrier gas, helium; flow-rate 1 ml/min; column temperature, programmed from 100–310°C, 30°C/min, initial time 3 min, final time 8 min. The MS conditions were as follows: full scan mode; ionization energy, 70 eV; ion source temperature, 220°C; capillary direct interface heated at 260°C. For detection of MDE and its metabolites mass chromatography with the selected ions m/z 72, 86, 114, 150, 162 and 164

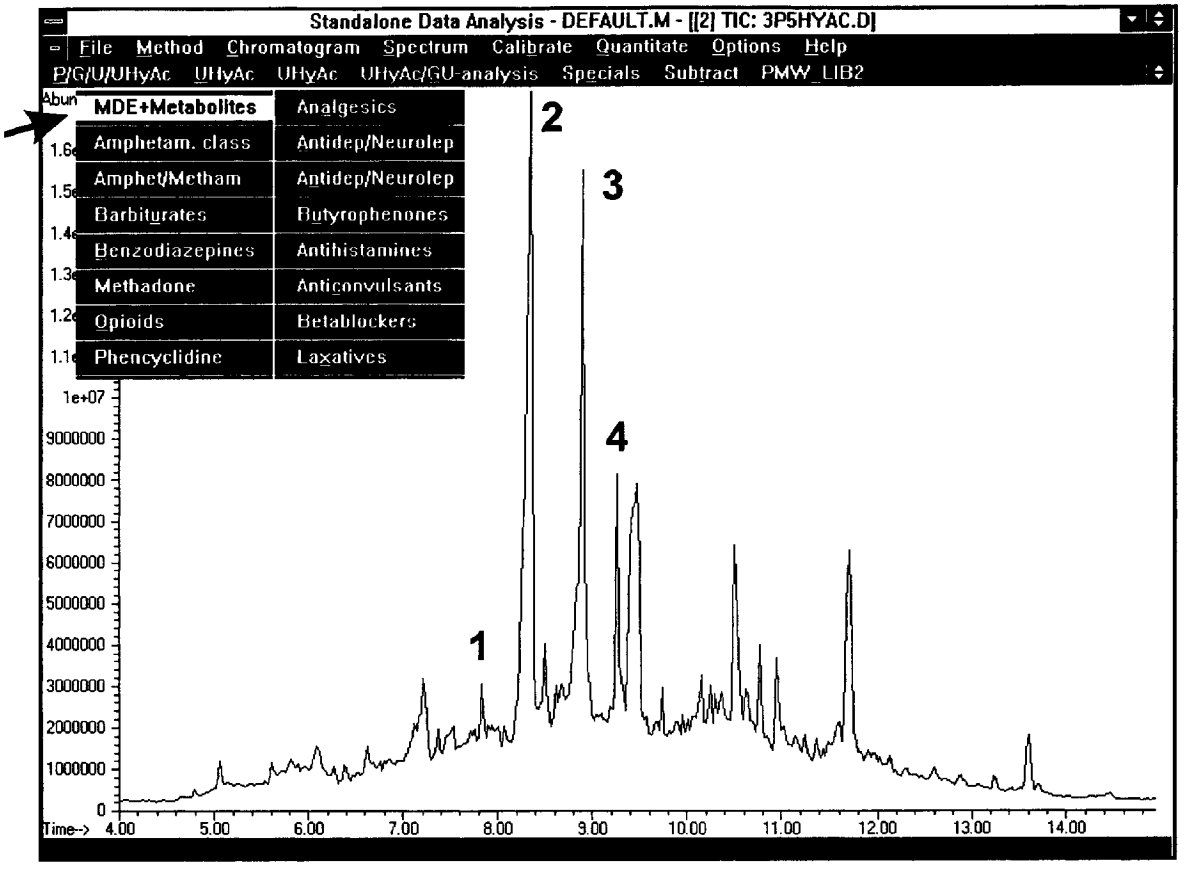

Fig. 1. Total ion chromatogram of an acetylated extract of a hydrolyzed urine sample collected 18 h after ingestion of 140 mg of MDE. Clicking the marked pull down menu for MDE and its metabolites allowed the generation of the mass chromatograms shown in Fig. 2. The peak numbers correspond to those of the peaks in Fig. 2.

was used. Generation of the mass chromatograms could be started by clicking the corresponding pull down menu which executes the user defined macros (the macros can be obtained from the authors). The identity of the peaks in the mass chromatograms was confirmed by computer library search of the underlying mass spectra [32].

2.5. Fluorescence polarization immunoassays (FPIA)

Urine samples from the volunteers were used for immunological determination. The TD_x system of Abbott (Irving, TX, USA) with the amphetamine/methamphetamine II assay (AM/MA II) was applied. The cut-off value and the detection limit recommended by the manufacturers were 300 and 100 ng/ml respectively. Blank urine samples were spiked with synthesized standards in concentrations of 100 and 1000 ng/ml for determination of the cross-reactivity of the assay with the MDE metabolites DHE, HME, 3,4-dihydroxyamphetamine (DHA) and 4-hydroxy-3-methoxyamphetamine (HMA).

3. Results and discussion

3.1. Sample preparation

For sample preparation, a procedure was used that has proved to be successful for several classes of drugs [36]. Since the hydroxy metabolites of MDE

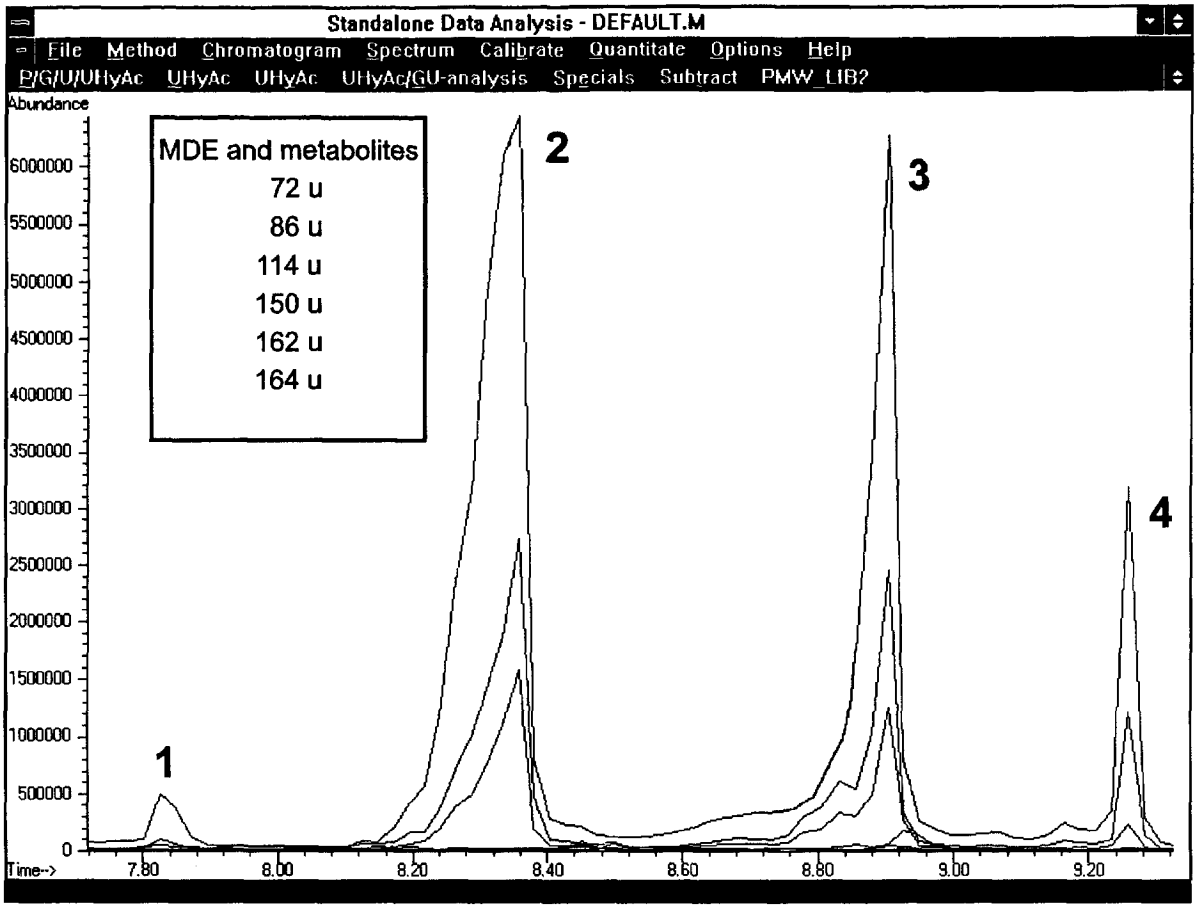

Fig. 2. Typical mass chromatograms with the ions *m/z* 72, 86, 114, 150, 162 and 164 indicating the presence of acetylated MDA (1), MDE (2), HME (3) and DHE (4). The merged mass chromatograms can be differentiated by their colours on a colour screen.

are excreted as conjugates and 4-hydroxy-3-methoxyethylamphetamine (HME) is the main metabolite [31], rapid acid hydrolysis was performed before isolation and derivatization in order to extend the duration of detectability. Derivatization was essential for sensitive detection of the polar MDE and its metabolites. Acetylation has been approved for the identification of numerous drugs and their metabolites as described in review articles [36,37]. It leads to stable derivatives with good gas chromatographic properties. The acetylation mixture can be evaporated before analysis so that the resolution power of capillary columns does not decrease in contrast to other derivatization reagents. The molecular mass does not increase very much, in contrast to silylation or perfluoroacylation, so that compounds with relatively high molecular mass and several derivatizable groups can be measured with low-priced mass selective detectors with a mass range only up to 650 u. The analytical recovery of MDE and its main metabolites ranged between 65–85%.

3.2. Detection by gas chromatography–mass spectrometry

The full mass spectra recorded during temperature-programmed gas chromatography were evaluated using mass chromatography. Generation of the mass chromatograms could be started by clicking the corresponding pull down menu (Fig. 1) which executes the user defined macros. The selected ions *m/z* 72, 86, 114, 150, 162 and 164 were used for

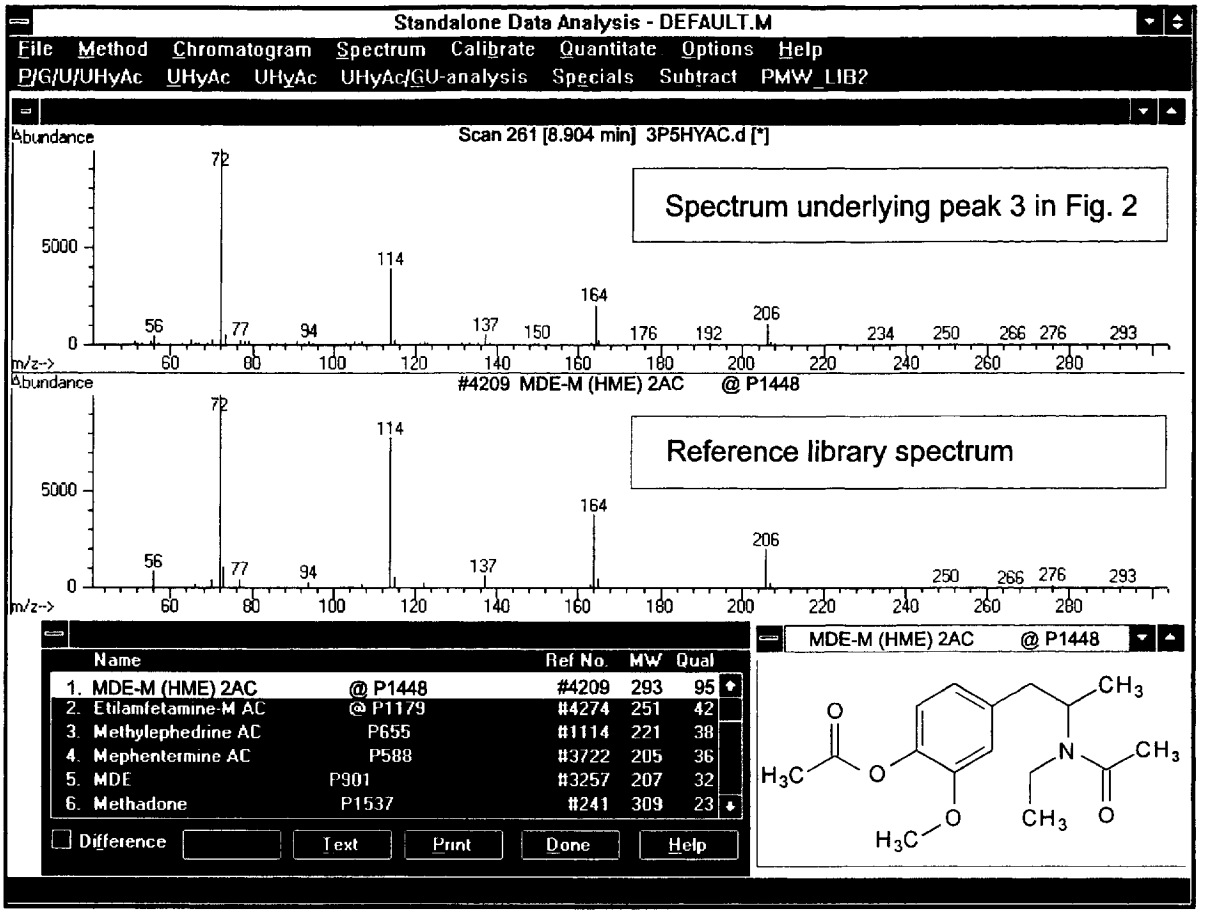

Fig. 3. Mass spectrum underlying peak 3 in Fig. 2, the reference spectrum, the structure and the hit list found by computer library search.

indication of the presence of acetylated N-desethyl MDE (MDA) (86, 162; peaks 1 in Fig. 2), acetylated MDE (72, 114, 162; peaks 2 in Fig. 2), acetylated 4-hydroxy-3-methoxyethylamphetamine (HME) (72, 114, 164; peaks 3 in Fig. 2) and acetylated 3,4-dihydroxyethylamphetamine (DHE) (72, 114, 150; peaks 4 in Fig. 2) in the urine samples. As shown in Fig. 3, the identity of peaks in the mass chromatograms was confirmed by computerized comparison of the underlying mass spectrum with those of standards [32]. The reference spectra, the gas chromatographic retention indices (RI) and the structures of acetylated MDE and its main metabolites are shown in Fig. 4. The numbers of the spectra correspond to those of the peaks in Fig. 2. In our experience, retention indices provide preliminary indications and may be useful to gas chromatographers without a GC–MS facility. The retention indices were recorded during the GC–MS procedure (Section 2.4) and calculated in correlation with the Kovats' indices [33] of the components of a standard solution of typical drugs which is measured daily for testing the GC–MS performance [34,35]. The reproducibility of retention indices measured on capillary columns was better using a mixture of drugs than that of the homologous hydrocarbons recommended by Kovats [35]. The spectra and the retention indices of the less abundant metabolites only described in Section 3.4 are presented elsewhere [32,38]. These minor metabolites are not essential for the screening procedure. The structures of all the MDE metabolites were confirmed by chemical synthesis [31]. As given

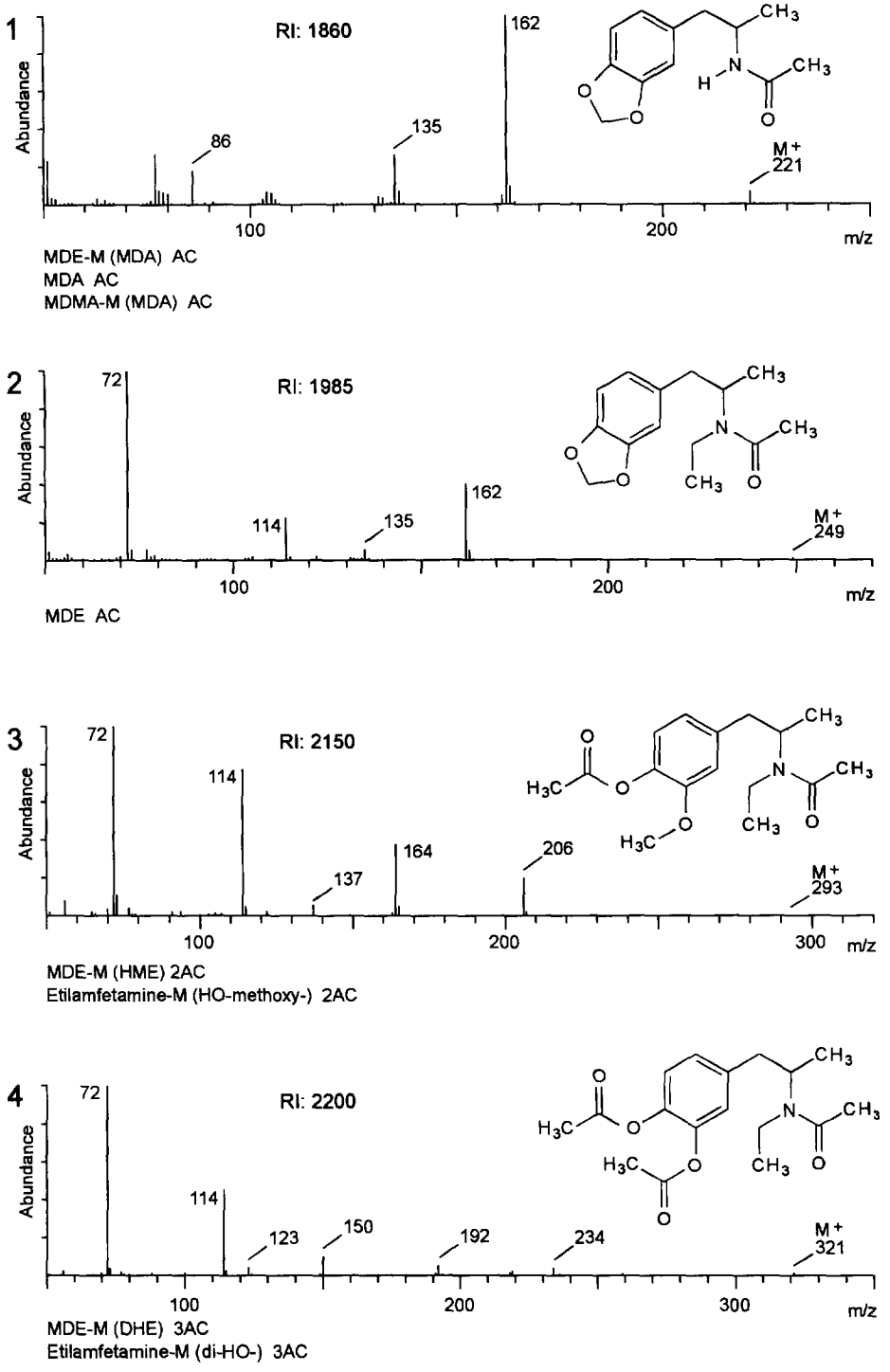

Fig. 4. Mass spectra, gas chromatographic retention indices (RI) and structures of MDE and its main metabolites after acetylation for precise identification. The numbers of the spectra correspond to those of the peaks in Figs. 1 and 2.

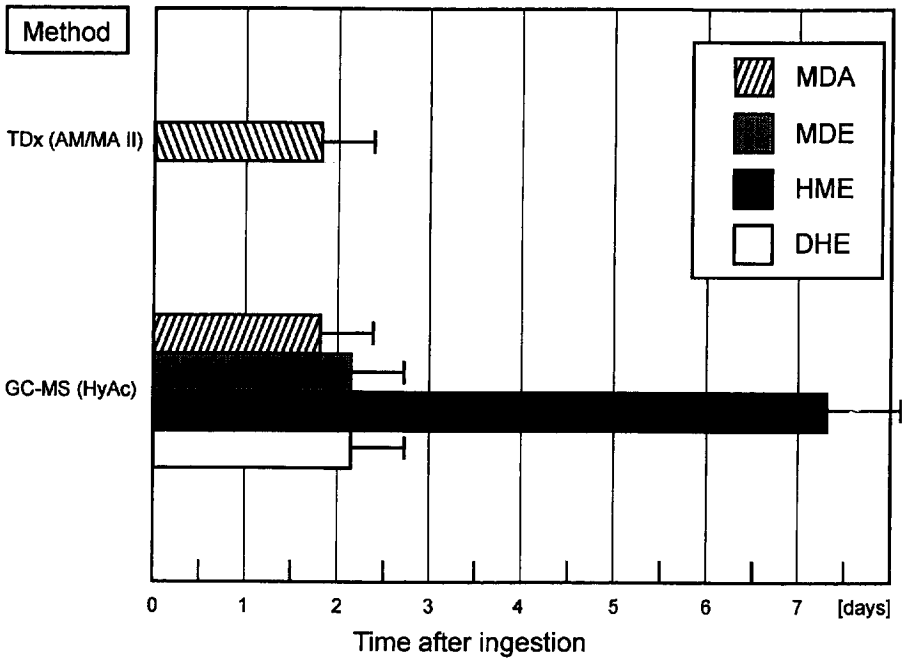

Fig. 5. Duration of detectability of MDE and its main metabolites MDA, HME and DHE (days after ingestion) by the TD_x assay AM/MA II and GC–MS in urine samples after ingestion of 140 mg of MDE ($n=3$).

in the legend to the spectra, the designer drug MDA is a common metabolite of MDE and of MDMA. HME and DHE are common metabolites of ethylamphetamine. However, the detection of the parent drugs and/or further unique metabolites allowed the differentiation of the drugs. Because of the mass spectral identification, interferences by biomolecules or further drugs could be excluded. The mass spectra of all the endogenous biomolecules detectable after the described procedure are included in our library and handbook [32,38]. The limit of detection of acetylated MDE was 5 ng/ml and of the acetylated main metabolite HME 10 ng/ml of urine.

3.3. Detection by immunoassays

The cross-reactivity values of the MDE metabolites DHE, HME, 3,4-dihydroxyamphetamine (DHA) and 4-hydroxy-3-methoxyamphetamine (HMA) with the AM/MA II assay ranged between 0.03–0.4% at 1000 ng/ml. Urine samples with 100 ng/ml of MDE metabolites gave no response. Since MDE and its nor metabolite MDA showed cross-reactivity values of 30–66% and 136–170% respectively with the AM/MA II assay [26], this assay gave positive results in urine after ingestion of MDE (cf. Section 3.4). The TD_x values measured during our excretion study ranged between 300–40 000 ng/ml.

3.4. Duration of detectability of MDE in urine by GC–MS and FPIA

The duration of detectability of MDE in urine by GC–MS and FPIA is shown in Fig. 5. After a single oral dose of 140 mg of MDE the AM/MA II assay gave positive results in urine for 33–62 h taking into consideration the cut-off value recommended by the manufacturer (300 ng/ml). The following metabolites could be detected in urine by the described GC–MS procedure during the given time: unchanged MDE and DHE for 33–62 h, MDA for 32–36 h and HME for 7–8 days. DHA, HMA, piperonyl acetone, 3,4-dihydroxyphenyl acetone and 4-hydroxy-3-methoxyphenyl acetone could only be detected in trace amounts during the first few hours. All the immunological results could be confirmed by GC–

MS detection of MDE, MDA, HME and DHE (Fig. 3). The variation of the time of excretion of MDE may be caused by interindividual differences e.g. relative body mass and/or renal function.

MDE is converted to MDA only in minor amounts. Therefore, the intake of MDE can be established by GC–MS detection of MDE and/or its metabolite HME. If the concentration of MDA is much higher than that of MDE, an additional intake of MDA must be assumed. Since HME can also be formed from ethylamphetamine a differentiation between MDE or ethylamphetamine is impossible in the late phase of excretion. Finally, the presented procedure also allowed the differentiation of MDE and other immunologically interfering amphetamine derivatives [27,39].

4. Conclusions

The GC–MS procedure described here allows the precise and sensitive detection of MDE and its metabolites in urine after therapeutic doses of MDE. The MDE homologues MDA, MDMA, BDB, MBDB and other amphetamine derivatives as well as most of the toxicologically relevant drugs [36] could also be detected and differentiated within the same procedure by clicking the corresponding pull down menu (e.g. "amphetamine class") followed by library search of the peaks underlying spectra. The TD_x immunoassay for amphetamines showed positive results in urine after ingestion of the MDE and its homologues. All the positive results could be confirmed by the described GC–MS procedure.

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References

[1] H.H. Maurer, H. Ensslin and K.-A. Kovar, Abstract Book to EUROTOX '91, Annual Meeting of the European Society of Toxicology, Maastricht, September 1991, p. 286.
[2] U. Braun, A.T. Shulgin and G. Braun, J. Pharm. Sci., 69 (1980) 192.
[3] E. Gouzoulis, A. Steiger, H.K. Ensslin, K.-A. Kovar and L. Hermle, Biol. Psych., 32 (1992) 1108.
[4] E. Gouzoulis, U.v. Bardeleben, A. Rupp, K.-A. Kovar and L. Hermle, Neuropsychopharmacology, 8 (1993) 187.
[5] L. Hermle, M. Spitzer, D. Borchardt, K.-A. Kovar and E. Gouzoulis, Neuropsychopharmacology, 8 (1993) 171.
[6] D.E. Nichols, J. Psychoactive Drugs, 18 (1986) 305.
[7] R.G. Greer and R. Tolbert, in S.J. Peroutka (Editor), Ecstasy: the Clinical, Pharmacological and Neurotoxicological Effects of the Drug MDMA, Kluwer Academic Publishers, Dordrecht, 1990, p. 21.
[8] D.M. Stone, M. Johnson, G.R. Hanson and J.W. Gibb, Eur. J. Pharmacol., 134 (1987) 245.
[9] D.L. Commins, G. Vosmer, R.M. Virus, W.L. Woolverton, C.R. Schuster and L.S. Seiden, J. Pharmacol. Exp. Ther., 241 (1987) 338.
[10] T.R. Insel, G. Battaglia, J.N. Johannessen, S. Marra and E.B. De Souza, J. Pharmacol. Exp. Ther., 249 (1989) 713.
[11] M. Johnson, I. Elayan, G.R. Hanson, R.L. Foltz, J.W. Gibb and H.K. Lim, J. Pharmacol. Exp. Ther., 261 (1992) 447.
[12] G.A. Ricaurte and U.D. McCann, Ann. N.Y. Acad. Sci., 648 (1992) 371.
[13] J. Brodkin, A. Malyala and J.F. Nash, Pharmacol. Biochem. Behav., 45 (1993) 647.
[14] S.F. Ali, G.D. Newport, A.C. Scallet, Z. Binienda, S.A. Ferguson, J.R. Bailey, M.G. Paule and W. Slikker Jr., Neurotoxicol. Teratol., 15 (1993) 91.
[15] M. Johnson, G.R. Hanson and J.W. Gibb, Biochem. Pharmacol., 38 (1989) 4333.
[16] C. Singarajah and N.G. Lavies, Anaesthesia, 47 (1992) 686.
[17] P. McGuire and T. Fahy, J. Psychiatry, 160 (1992) 276.
[18] J.A. Henry, K.J. Jeffreys and S. Dawling, Lancet, 340 (1992) 384.
[19] G.P. Dowling, E.T. McDonough III and R.O. Bost, JAMA, 257 (1987) 1615.
[20] E. Gouzoulis, D. Borchardt and L. Hermle, Arch. Gen. Psychiatry, 50 (1993) 75.
[21] M.T. Cregg and J.A. Tracey, Ir. Med. J., 86 (1993) 118.
[22] S. Manchada and M.J. Connolly, Postgrad. Med. J., 69 (1993) 874.
[23] J.T. Cody, J. Anal. Toxicol., 14 (1990) 321.
[24] G.W. Kunsman, J.E. Manno, K.R. Cockerham and B.R. Manno, J. Anal. Toxicol., 14 (1990) 149.
[25] A. Poklis, R.L. Fitzgerald, K.V. Hall and J.J. Saady, Forensic Sci. Int., 59 (1993) 63.
[26] J.T. Cody and R. Schwarzhoff, J. Anal. Toxicol., 17 (1993) 26.
[27] H.H. Maurer and T. Kraemer, Arch. Toxicol., 66 (1992) 675.
[28] L. Tedeschi, G. Frison, F. Castagna, R. Giorgetti and S.D. Ferrara, Int. J. Legal Med., 1058 (1993) 265.
[29] H.J. Helmlin and R. Brenneisen, J. Chromatogr., 593 (1992) 87.
[30] R.E. Michel, A.B. Rege and W.J. George, J. Neurosci. Methods, 50 (1993) 61.
[31] H.K. Ensslin, H.H. Maurer, E. Gouzoulis, L. Hermle and K.-A. Kovar, Drug Metab. Dispos., 24 (1996), in press.

[32] K. Pfleger, H.H. Maurer and A. Weber, Mass Spectral Library of Drugs, Poisons, Pesticides, Pollutants and their Metabolites, Hewlett Packard, Palo Alto, CA, 2nd rev., 1993.
[33] E. Kovats, Helv. Chim. Acta, 41 (1958) 1915.
[34] Deutsche Forschungsgemeinschaft, Senatskommission für Klinisch-toxikologische Analytik, J. Clin. Chem. Clin. Biochem., 20 (1982) 699.
[35] R.A. De Zeeuw, J.P. Franke, H.H. Maurer and K. Pfleger, Gas Chromatographic Retention Indices of Toxicologically Relevant Substances and their Metabolites (Report of the DFG commission for clinical toxicological analysis, special issue of the TIAFT bulletin), VCH publishers, Weinheim, New York, Basle, 3rd ed., 1992.
[36] H.H. Maurer, J. Chromatogr., 580 (1992) 3.
[37] G.B. Baker, R.T. Coutts and A. Holt, J. Pharmacol. Toxicol. Meth., 31 (1994) 141.
[38] K. Pfleger, H.H. Maurer and A. Weber, Mass Spectral and GC Data of Drugs, Poisons, Pesticides, Pollutants and their Metabolites, VCH publishers, Weinheim, New York, Basle, 2nd ed., 1992.
[39] H.H. Maurer, T. Kraemer, O. Ledvinka, C.J. Schmitt and A.A. Weber, J. Chromatogr. B, in press.