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Studies on the metabolism and toxicological detection of the amphetamine-like anorectic mefenorex in human urine by gas chromatography–mass spectrometry and fluorescence polarization immunoassay[1]

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Abstract

Studies on the metabolism and on the toxicological analysis of mefenorex [*R*,*S*-N-(3-chloropropyl)-α-methylphenethylamine, MF] using gas chromatography–mass spectrometry (GC–MS) and fluorescence polarization immunoassay (FPIA) are described. The metabolites were identified in urine samples of volunteers by GC–MS. Besides MF, thirteen metabolites including amphetamine (AM) could be identified and three partially overlapping metabolic pathways could be postulated. For GC–MS detection, the systematic toxicological analysis procedure including acid hydrolysis, extraction at pH 8–9 and acetylation was suitable (detection limits 50 ng/ml for MF and 100 ng/ml for AM). Excretion studies showed, that only AM but neither MF nor its specific metabolites were detectable between 32 and 68 h after ingestion of 80 mg of MF. Therefore, misinterpretation can occur. The Abbott TDx FPIA amphetamine/methamphetamine II gave positive results up to 68 h. All the positive immunoassay results could be confirmed by the described GC–MS procedure. © 1997 Elsevier Science B.V.

Keywords: Mefenorex; Anorectics; Amphetamine

1. Introduction

Mefenorex [*R*,*S*-N-(3-chloropropyl)-α-methylphenethylamine, Rondimen®, Pondinil®; MF] is a widely used anorectic. As for many other amphetamine like anorectics, central stimulant or habit forming properties are controversially discussed. It has been postulated that the presence of the chloropropyl side chain would prevent MF from N-dealkylation and the aromatic hydroxylation is the predominant metabolic pathway [2,3]. However, other authors reported metabolic N-dealkylation of MF to amphetamine (AM) [4–7]. AM has been found in urine or hair samples after ingestion of MF in some analytical studies on anorectics by immunoassay, high-performance liquid chromatography (HPLC), gas chromatography (GC) or GC–mass spectrometry (MS) [7–9]. Since we found MF and AM in urine of drug abusers, we have reinvestigated the metabolism of MF in humans using GC–MS in

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[1] Dedicated to Professor Dr. Ernst Mutschler, Frankfurt/Main, on the occasion of his 65th birthday. Part of these results was reported at the GTFCh Symposium, Mosbach, Germany, April 18–19, 1997.

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the electron impact (EI) and chemical ionization (CI) mode. Furthermore, we have studied the toxicological detection of MF and its metabolites in human urine by fluorescence polarization immunoassays (FPIA) and our systematic toxicological analysis (STA) procedure [10] by GC–MS in the EI mode. The aim of our studies was to investigate which metabolites are detectable in urine and for how long, and whether the intake of MF can be differentiated at any time from an intake of AM by detection of MF specific (non-N-dealkylated) metabolites.

2. Experimental

2.1. Chemicals and reagents

All chemicals used were obtained from E. Merck (Darmstadt, Germany) and were of analytical grade. Mefenorex was a kind gift of Asta Medica (Frankfurt/M, Germany). Rondimen® tablets (each containing 40 mg of MF) were used for human studies. Amphetamine was purchased from E. Merck.

2.2. Urine samples

After informing them according to the declaration of Helsinki and obtaining written consent, three healthy volunteers received a single oral dose of 80 mg of MF. Urine samples were collected every 4 h for 6 days. All samples were directly analyzed and the remainder were stored at −20°C before further analysis. Blank urine samples were collected before drug administration to check whether the samples were free of interfering compounds.

2.3. Sample preparation for metabolism studies

A 5 ml portion of urine was adjusted to pH 5.2 with acetic acid and incubated at 38°C for 12 h with 100 μl of a mixture of β-glucuronidase and arylsulfatase (100 000 Fishman units per ml), then adjusted to pH 8–9. In order to obtain the same conditions as after acid hydrolysis (cf. Section 2.4) the sample was mixed with a mixture of 1.5 ml of 37% hydrochloric acid, 2.5 ml of 2.3 mol/l aqueous ammonium sulphate and 2 ml of a 10 mol/l aqueous sodium hydroxide solution. The sample was cooled on ice and extracted with 5 ml of a dichloromethane–isopropanol–ethyl acetate mixture (1:1:3, v/v/v). After phase separation by centrifugation, the organic layer was transferred and evaporated to dryness, and the residue was acetylated with 50 μl of an acetic anhydride–pyridine mixture (3:2, v/v) for 30 min at 60°C. After evaporation, the residue was dissolved in 50 μl of methanol and 0.2 μl of this solution was injected into the gas chromatograph. The same procedure except for enzymatic hydrolysis was used to study whether metabolites of MF are excreted unconjugated.

2.4. Sample preparation for toxicological analysis

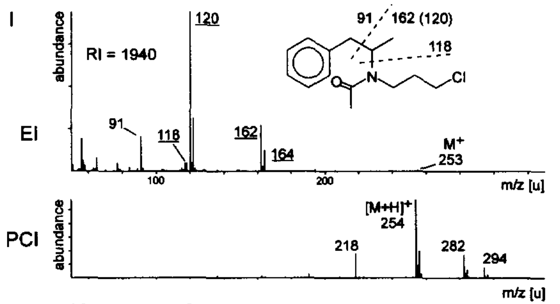
A 5 ml portion of urine was refluxed with 1.5 ml of 37% hydrochloric acid for 15 min. Following hydrolysis, the sample was basified with 2 ml of 10 mol/l aqueous sodium hydroxide and the resulting solution was mixed with 2.5 ml of 2.3 mol/l aqueous ammonium sulphate to obtain a pH between 8 and 9. This solution was extracted with 5 ml of a dichloromethane–isopropanol–ethyl acetate mixture (1:1:3, v/v/v). After phase separation by centrifugation, the organic layer was transferred and carefully evaporated to dryness. The residue was derivatized by acetylation with 50 μl of an acetic anhydride–pyridine mixture (3:2, v/v) for 30 min at 60°C. After evaporation of the derivatization mixture, the residue was dissolved in 50 μl of methanol and 0.2 μl were injected into the gas chromatograph.

2.5. Gas chromatography–mass spectrometry

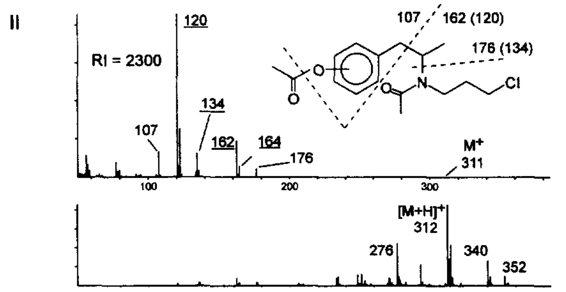
MF and its metabolites were separated and identified in acetylated urine extracts using a Hewlett-Packard (HP, Waldbronn, Germany) 5890 Series II gas chromatograph combined with an HP 5989B MS Engine mass spectrometer and an HP MS ChemStation (DOS series) with HP G1034C software. The GC conditions were 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, 280°C, carrier gas, helium; flow-rate 1 ml/min; column temperature, programmed from 100–310°C at 30°C/min, initial time 3 min, final time 8 min. The MS conditions were as follows: full scan mode; EI ionization mode:

ionization energy, 70 eV; CI using methane, positive mode (PCI): ionization energy, 230 eV; ion source temperature, 220°C; capillary direct interface heated at 260°C.

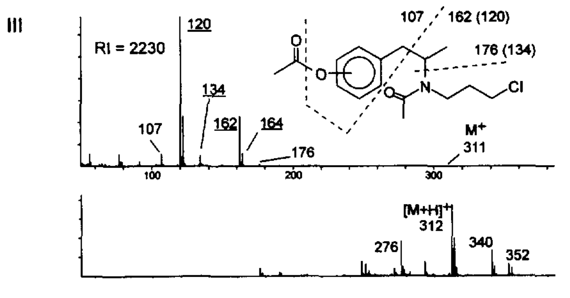
For toxicological detection of MF and its metabolites, mass spectrometry with the selected ions *m/z* 86, 118, 120, 134, 144, 162, 164 and 186 was used. Generation of the mass chromatograms could be started by clicking the corresponding pull down menu which executes the user defined macros [10] (the macros can be obtained from the authors: e-mail: pthmau@med-rz.uni-sb.de). The identity of the peaks in the mass chromatograms was confirmed by computerized comparison [11] of the peaks underlying mass spectra with reference spectra (Fig. 1) recorded during this study.



Mefenorex AC

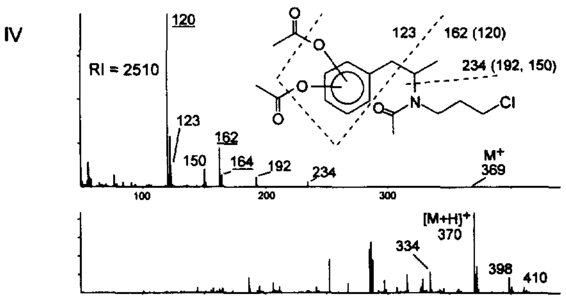


Mefenorex-M (HO-) isomer-1 2AC

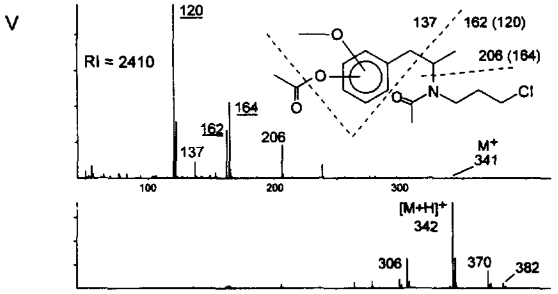


Mefenorex-M (HO-) isomer-2 2AC

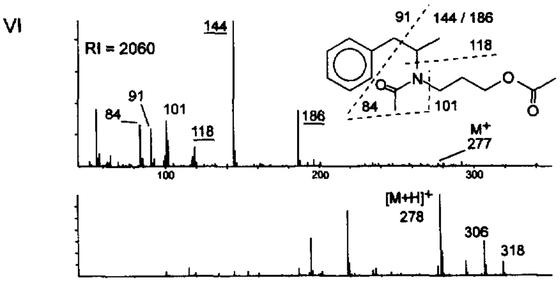
Fig. 1. Mass spectra (EI and PCI), predominant fragmentation patterns, structures and gas chromatographic retention indices (RIs) of MF and its metabolites after acetylation for precise identification. The numbers of the spectra correspond to those in Fig. 2. Ions selected for the toxicological detection are underlined. (The axes are only labelled for I).



Mefenorex-M (di-HO-) 3AC



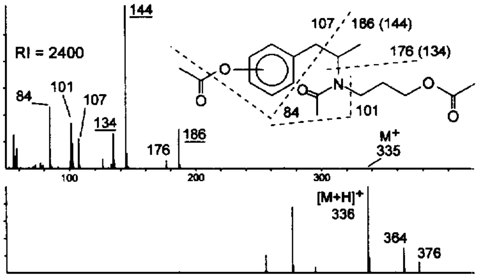
Mefenorex-M (HO-methoxy-) 2AC



Mefenorex-M (dechloro-HO-) 2AC

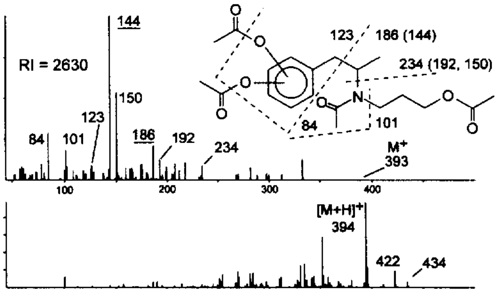
Fig. 1. (*continued*)

VII



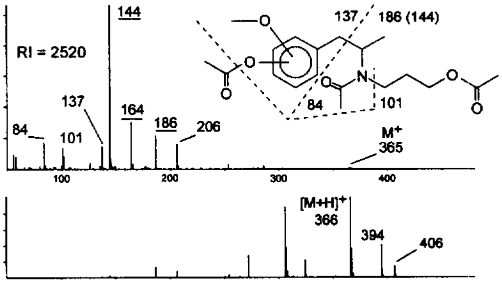
Mefenorex-M (dechloro-di-HO-) 3AC

VIII



Mefenorex-M (dechloro-tri-HO) 4AC

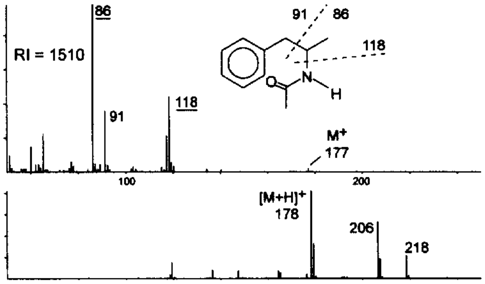
IX



Mefenorex-M (dechloro-di-HO-methoxy-) 3AC

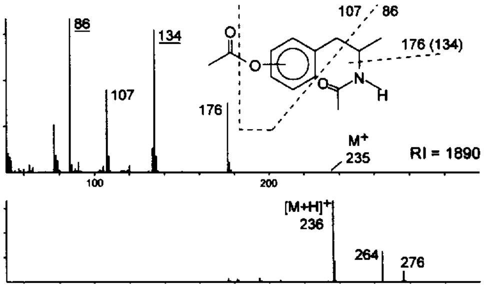
Fig. 1. (*continued*)

X



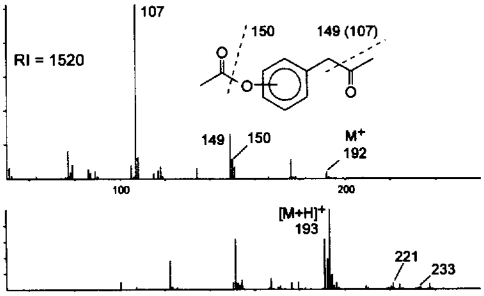
Mefenorex-M (N-dealkyl-) AC (Amphetamine AC)

XI



Mefenorex-M (N-dealkyl-HO-) isomer-1 2AC
Amphetamine-M (HO-) 2AC

XII



Mefenorex-M (deamino-oxo-HO-) AC

Fig. 1. (*continued*)

2.6. Fluorescence polarization immunoassays (FPIAs)

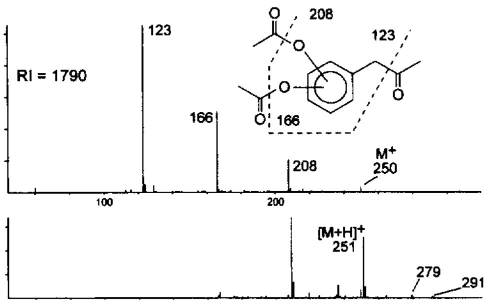
Native urine samples from the volunteers were used for immunological determination. The TDx system of Abbott (Irving, TX, USA) with the amphetamine/methamphetamine II assay (AM/MA II) was applied. The cut-off value and the detection limit recommended by the manufacturers were as follows: 300 and 100 ng/ml, respectively. To determine the cross reactivities of MF with this assay, blank urine samples were spiked with MF in concentrations of 100–1 000 000 ng/ml.

3. Results and discussion

3.1. Sample preparation

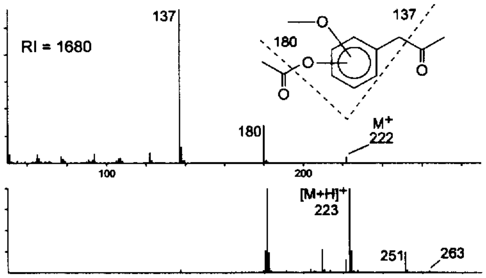
Cleavage of conjugates by enzymatic or acid hydrolysis was necessary before extraction since the expected hydroxy metabolites of MF were excreted

XIII



Mefenorex-M (deamino-oxo-di-HO-) 2AC

XIV



Mefenorex-M (deamino-oxo-HO-methoxy-) AC

Fig. 1. (*continued*)

mostly as conjugates, while for studies on the metabolism, gentle enzymatic hydrolysis was preferred. For studies on the toxicological detection rapid acid hydrolysis was performed. The (metabolic) introduction of an aromatic hydroxy group into a phenethylamine derivative increases the acidity of the compound and thereby changes the extractive properties of it. The resulting phenolbases were best extracted at pH 8–9. Use of a more alkaline pH for extraction of AM derivatives leads to the loss of such hydroxy metabolites which are often the main metabolites [12–15]. In our case, one isomer of hydroxy-MF was excreted for a much longer time (see Section 3.5) than the parent compound, thus resulting in a markedly increased time in which differentiation of the intake of MF from the intake of AM was possible. The high volatility of the amphetamines made very careful sample preparation indispensable for reproducible results. Derivatization of the extracts was indispensable for sensitive detection. The analytical recovery of MF and its metabolite AM determined after enzymatic hydrolysis at concentration levels of 500 and 1000 ng/ml, respectively was 97±12% for MF and 70±12% for AM. The analytical recovery of MF and its metabolite AM determined after acid hydrolysis at a concentration level of 500 and 1000 ng/ml, respectively was 77±7% for MF and 60±9% for AM. It has to be noted that the recoveries were determined under routine laboratory conditions. Higher recoveries and better precision for AM are possible using special precautions like ice cooling of the extracts as described elsewhere [16].

3.2. *Identification of metabolites*

The urinary metabolites of MF were identified by EI and PCI-MS after enzymatic hydrolysis, extraction, acetylation and GC separation. The EI mass spectra of the postulated metabolites were interpreted in correlation to that of the parent compound according to the rules described by McLafferty and Turecek [17]. In addition, the PCI mass spectra were used to ensure the identity of the metabolites, since they gave strong molecular peaks (M+H) with adduct ions typical for PCI using methane (M^+ plus 29 and 41 u, corresponding to C_2H_5 and C_3H_5) and in case of intact N-alkyl side chain a peak M^+ minus 35 corresponding to the loss the chlorine atom. The EI and PCI mass spectra, the structures and the predominant EI fragmentation patterns of MF and its metabolites after acetylation are shown in Fig. 1 [the gas chromatographic retention indices (RIs) were additionally given for GC detection as described below]. Besides MF (I), the following thirteen metabolites could be identified: two isomers of hydroxy-MF (II, III), dihydroxy-MF (IV), hydroxy-methoxy-MF (V), dechloro-hydroxy-MF (VI), one isomer of dechloro-dihydroxy-MF (VII), dechloro-trihydroxy-MF (VIII), dechloro-dihydroxy-methoxy-MF (IX), N-dealkyl-MF (amphetamine; X), one isomer of N-dealkyl-hydroxy-MF (hydroxy-amphetamine; XI), one isomer of deamino-oxo-hydroxy-MF (hydroxy-phenylacetone; XII), deamino-oxo-dihydroxy-MF (dihydroxy-phenylacetone; XIII) and deamino-oxo-hydroxy-methoxy-MF (hydroxy-methoxy-phenylacetone; XIV). Fig. 2 shows three partially overlapping metabolic pathways postulated by the aid of the identified metabolites: alteration of the phenyl ring by mono- and di-aromatic hydroxy-

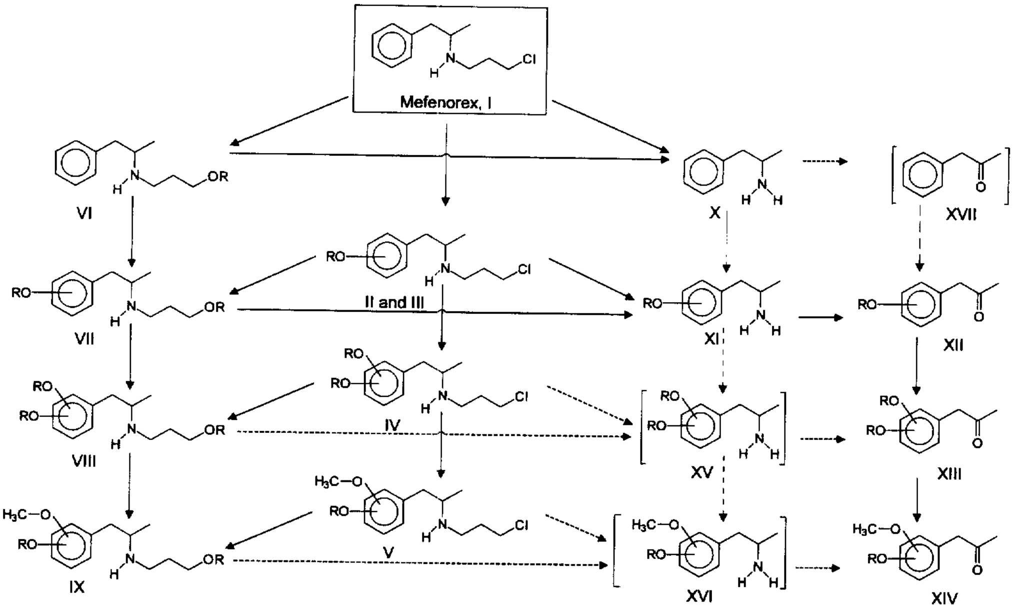


Fig. 2. Proposed scheme for the metabolism of MF in humans. The metabolites II–V, VII–IX and XI–XIV were also present as glucuronic and/or sulfuric acid conjugates in urine, indicated by the substituent R. The metabolites in brackets could not be found in this study.

lation followed by methylation of one of the hydroxy groups; side chain degradation by N-dealkylation to AM and hydroxy-AM with further degradation via oxidative N-deamination to deamino-oxo derivatives, and oxidative dechlorination of the chloropropyl side chain to the hydroxypropyl side chain. The metabolites XV, XVI and XVII could not be found in our urine samples, but they can be postulated as intermediate metabolites. They could possibly be detected in case of overdose. Norephedrine as a β-hydroxylation product of AM could not be detected. These studies showed that MF is indeed dealkylated to AM and that the chloropropyl substituent does not prevent MF from N-dealkylation. In this study the dechloro-hydroxy metabolites of MF are described for the first time. To check whether they could be generated during sample preparation, urine samples spiked with MF were treated in the same manner as the authentic samples. In fact, very small amounts (<0.1% peak area of the parent compound) of dechloro-hydroxy-MF could be found. However, this cannot explain the large amounts of dechloro-hydroxy metabolites relative to the corresponding parent compounds found in the urine of volunteers. For one volunteer the dechloro-dihydroxy metabolite of MF was even that specific metabolite which could be detected for the longest time. In addition, dechlorination of a chloroalkyl side chain has clearly been reported as a metabolic reaction for other substances like clomethiazole [18].

Rendic et al. [7] did find hydroxy-MF, hydroxymethoxy-MF, AM and hydroxy-AM as metabolites of MF, but they could not find the dihydroxy metabolites. In our experience this seemed due to the fact, that they did not directly analyze the urine samples. During our studies, the same urine samples were analysed twice: directly after the sampling and a second time after a one day storage in the deep freezer at −20°C. The dihydroxy metabolites were only detectable in the directly tested samples. This would be possibly due to high reactivity of the catechol (*o*-dihydroxybenzene) structure. One cycle of freezing and thawing was enough to destroy these metabolites.

3.3. Detection by GC–MS within the STA

The full mass spectra recorded during temperature-programmed GC were evaluated using mass spectrometry. Generation of the mass chromatograms could be started by clicking the corresponding pull down menu which executes the user defined macros. Fig. 3 shows the reconstructed mass chromatograms indicating the presence of MF and its metabolites in an acetylated extract of a urine sample of a volunteer taken 8 h after ingestion of 80 mg of MF. The selected ions *m/z* 120 ($C_5H_{11}NCl$) and 162 ($C_7H_{13}NOCl$) were used for indication of the presence of MF and its metabolites with unchanged side chain, the ion *m/z* 86 (C_4H_8NO) for the N-dealkylated metabolites (amphetamines) and the ions *m/z* 144 ($C_7H_{14}NO_2$) and 186 ($C_9H_{16}NO_3$) for the dechloro-hydroxy metabolites. In addition, ion *m/z* 118 (C_9H_{10}) was selected for indication of unsubstituted phenyl derivatives, ion *m/z* 134 ($C_9H_{10}O$) for hydroxy phenyl derivatives and ion *m/z* 164 ($C_{10}H_{12}O_2$) for hydroxy methoxy phenyl derivatives. The latter ion also indicates the compounds with unchanged side chain, because ion *m/z* 164 also represents the chlorine isotop peak of ion *m/z* 162. For each compound at least two fragment ions were selected so that at least two peaks must appear in the mass chromatogram at the same time. Screening for the deamino-oxo metabolites was not useful, since they were excreted only in minor amounts and since they are no typical metabolites of MF.

As shown in Fig. 4, the identity of peaks in the mass chromatograms was confirmed by computerized comparison of the underlying mass spectrum with reference spectra (Fig. 1) recorded during this study. In our experience, the gas chromatographic retention indices (RIs) provide preliminary indications and may be useful to gas chromatographers without a GC–MS facility. Therefore, they are also given in Fig. 1. The RIs were recorded during the GC–MS procedure (Section 2.5) and calculated in correlation with the Kovats' indices [19] of the components of a standard solution of typical drugs which is measured daily for testing the GC–MS performance [20,21]. The reproducibility of retention indices measured on capillary columns was better using a mixture of drugs than that of the homologous

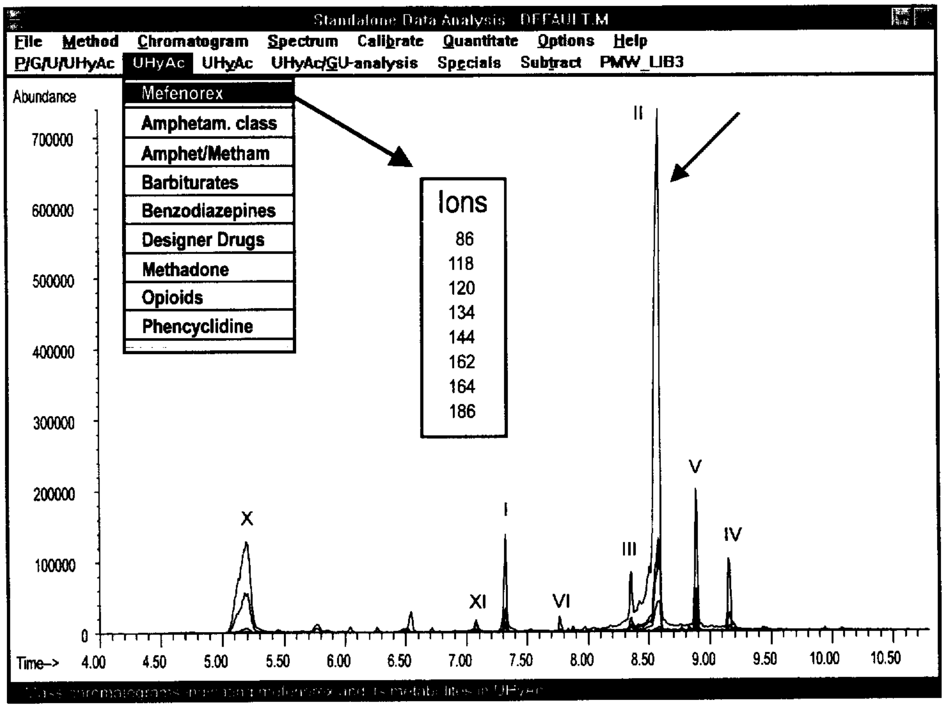
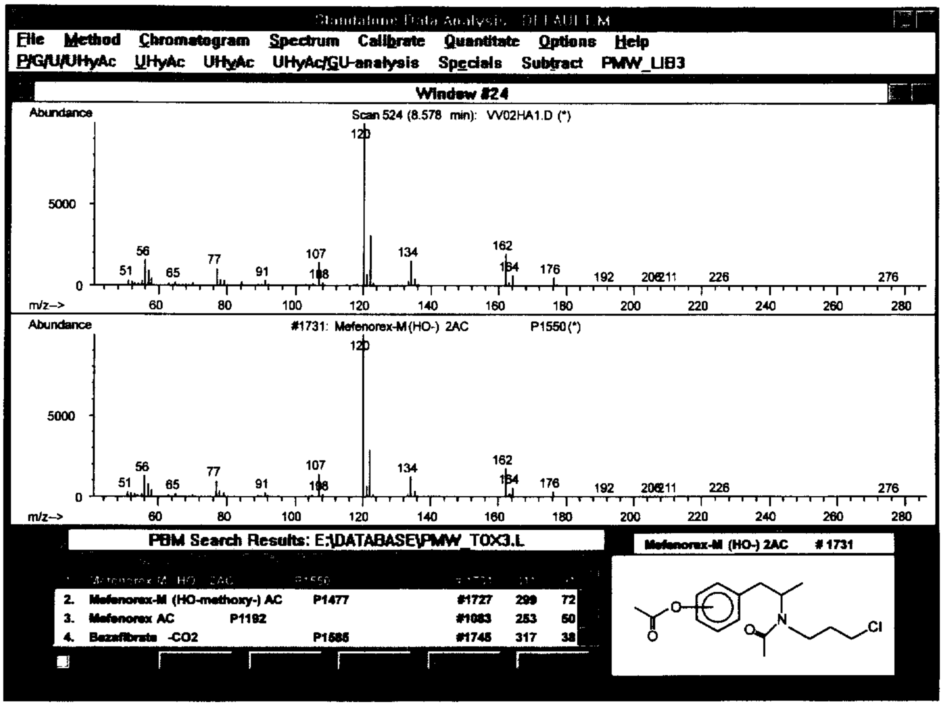


Fig. 3. Typical mass chromatograms with the ions *m/z* 86, 118, 120, 134, 144, 162, 164 and 186. They indicate the presence of MF and its metabolites in an acetylated extract of a urine sample taken 8 h after ingestion of 80 mg of MF. The merged chromatograms can be differentiated by their colors on a color screen.



1. Mefenorex-M (HO-) 2AC P1550 #1731
2. Mefenorex-M (HO-methoxy-) AC P1477 #1727 299 72
3. Mefenorex AC P1192 #1083 253 60
4. Bezafibrate -CO2 P1585 #1745 317 38

Fig. 4. Mass spectrum underlying the marked peak in Fig. 3, the reference spectrum, the structure and the hit list found by computer library search.

hydrocarbons recommended by Kovats [19]. Because of the mass spectral identification, interferences by biomolecules or further drugs could be excluded. It should be noted that AM and its metabolites can also be metabolically formed from its derivatives like amphetaminil, ethylamphetamine, fenethylline, methamphetamine [8,11,22], clobenzorex [15], fenproporex [1,23] or selegiline [24]. However, in urine samples, in which AM is not the only excreted metabolite, the taken drug can be differentiated by detection of unique metabolites. The GC and MS data of these are included in our handbook and library [11,22] together with the data of all the endogenous biomolecules detectable after the described procedure. The limit of detection of MF in urine was 50 ng/ml and that of the metabolite AM 100 ng/ml (*S*/*N* 3) under routine MS conditions.

3.4. Detection by FPIA

The cross-reactivity values of MF with the AM/MA II assay ranged between 0.1–0.4%. These data are in accordance with that of De-la-Torre et al. [8]. The TDx values measured during our excretion study ranged between 100 and 10 000 ng/ml. Since MF shows only minor cross reactivity with the AM/MA II assay, its dealkylated metabolites should be responsible for the positive results. As described in Section 3.5, AM is the metabolite which was excreted for the longest time. Therefore, in a late phase of excretion the TDx results corresponded to the presence of AM.

3.5. Duration of detectability of MF and its metabolites in urine by GC–MS and FPIA

The duration of detectability of MF and its metabolites in urine by GC–MS and FPIA is shown in Fig. 5. After a single oral dose of 80 mg of MF the AM/MA II assay gave positive results in urine up to 68 h taking into consideration the cut-off value recommended by the manufacturer. Using GC–MS, MF could be detected only for 16 to 20 h and its

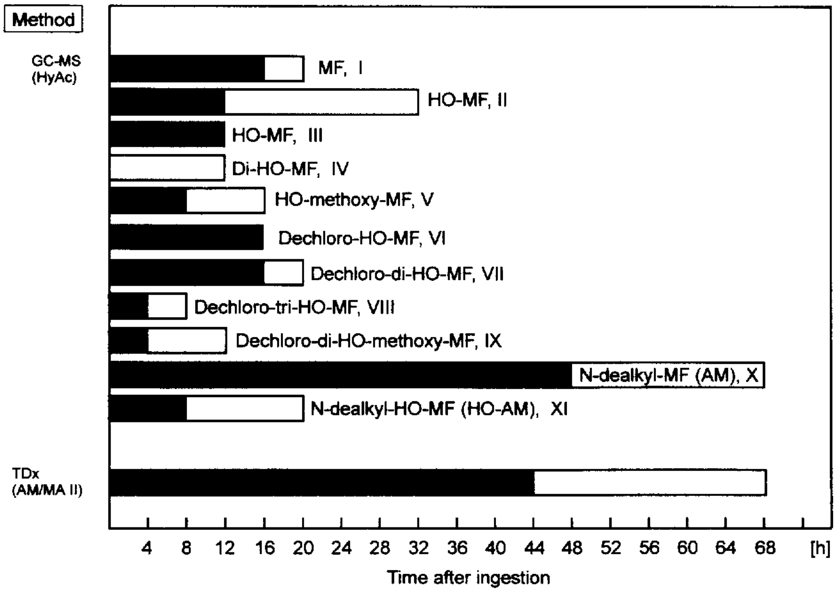


Fig. 5. Duration of detectability of MF and its main metabolites by GC–MS and by the TDx assay AM/MA II in urine samples after ingestion of 80 mg of MF ($n=3$). The black beams indicate the shortest and the white ones the longest time found for the three volunteers.

specific (non-dealkylated) metabolites for about 32 h. The metabolite AM could be detected for up to 68 h. All the immunoassay results can be confirmed by the described GC–MS procedure. The variation of the time of excretion of MF and its metabolites may be caused by inter-individual differences e.g., relative body mass and/or renal function. Since only AM can be detected from 32 h to 68 h after ingestion, a therapeutic intake of MF cannot be differentiated from an AM abuse during this period. Therefore, misinterpretation of positive immunoassay and even GC–MS results possibly happened. Studies on further substances causing similar problems are in progress and will be published elsewhere.

4. Conclusions

Our reinvestigation on the metabolism of MF confirmed the results published earlier [4–7] that MF is indeed metabolized to AM and, besides the known hydroxy metabolites, to dechloro-hydroxy metabolites.

The GC–MS procedure described here allowed the identification of the urinary metabolites of MF and precise and sensitive detection of MF and/or its metabolites in urine up to 68 h after ingestion of a double therapeutic dose. Other AM derivatives [1,15] as well as most of the toxicologically relevant drugs like barbiturates, benzodiazepines, opioids, analgesics, antidepressants, neuroleptics, antiparkinsonians, anticonvulsants, antihistamines, β-blockers, antiarrhythmics and laxatives could also be detected and differentiated by the same procedure by clicking the corresponding pull down menu (e.g., "amphetamine class") executing user defined macros followed by library search of the spectra underlying the peaks [10,25].

The AM/MA II assay showed positive results in urine up to 68 h after ingestion of MF. All the positive results could be confirmed by the described GC–MS procedure.

Misinterpretation of positive immunoassay and even GC–MS results possibly happen, since the parent compound MF or its specific metabolites became undetectable in a late phase of excretion. Since only AM is detectable, differentiation from intake of illicit AM is not possible in this late phase of excretion.

Acknowledgments

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