Studies on the metabolism and toxicological detection of the amphetamine like anorectic mefenorex in human urine by GC-MS and fluorescence polarization immunoassay (FPIA)

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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 GC-MS and fluorescence polarization immunoassay (FPIA) are described. The metabolites were identified in urine samples of volunteers by GC-MS. Besides *MF*, 13 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 (non-N-dealkylated) metabolites were detectable between 32-68 h after ingestion of 80 mg of *MF*. Therefore, misinterpretation concerning the origin of AM can occur. The Abbott TDx FPIA amphetamine/methamphetamine II gave positive results up to 68h. All the positive immunoassay results could be confirmed by the described GC-MS procedure.

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 controversely discussed. It was postulated that the presence of the chloropropyl side chain prevents *MF* from N-dealkylation and that the aromatic hydroxylation was the predominant metabolic pathway [1,2]. However, other authors reported metabolic N-dealkylation of *MF* to amphetamine (*AM*) [3-6]. *AM* was found in urine or hair samples after ingestion of *MF* in some analytical studies on anorectics by immunoassay, HPLC, GC or GC-MS [6-8]. Since we have found *MF* and *AM* for several times in urine of drug abusers, we have reinvestigated the

anorectics by immunoassay, HPLC, GC or GC-MS [6-8]. Since we have found *MF* and *AM* for several times in urine of drug abusers, we have reinvestigated the metabolism of *MF* in humans using GC-MS in the electron impact (EI) and chemical ionization (CI) mode. Furthermore, we have studied the toxicological detection of *MF* and its metabolites in human urine within our systematic toxicological analysis (STA) procedure by GC-MS in the EI mode and by fluorescence polarization immunoassays (FPIA). The aim of our studies was to investigate which metabolites are how long detectable in urine 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.

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 four hours for 6 days. All samples were directly analyzed and then 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 glucuronidase and arylsulfatase, then adjusted to pH 8-9. In order to get equal conditions as after acid hydrolysis (cf. 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-ethylacetate mixture (1:1:3; v/v/v). After phase separation by centrifugation, the organic layer was transfered 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 were injected into the gas chromatograph. The same procedure with the exception of 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 transfered and 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 50µl 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-1 capillary (12 m x 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°/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 chromatography 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 [9] (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 [10] of the peaks underlying mass spectra with reference spectra recorded during this study.

2.6 Fluorescence polarization immunoassays

Native urine samples from the volunteers were used for immunological determination. The TDx system of Abbott (Irving, TX) 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 as conjugates. For studies on the metabolism, gentle enzymatic hydrolysis was prefered. For studies on the toxicological detection rapid acid hydrolysis was performed. The samples were extracted at pH 8-9, to catch the hydroxy metabolites too. The analytical recovery of *MF* and its metabolite *AM* determined after enzymatic hydrolysis at concentration levels of 500 and 1,000 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 1,000 ng/ml respectively was 77±7 % for *MF* and 60±9 % for *AM*.

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 [11]. Unfortunately, the EI spectra gave no distinct molecular peaks. Therefore, the PCI mass spectra were additionally used to ensure the identity of the metabolites, since they gave strong molecular peaks (M+H) with adduct ions typical for PCI using methane. Besides *MF*, the following 13 metabolites could be identified: two isomers of hydroxy-*MF*, dihydroxy-*MF*, hydroxy-methoxy-*MF*, dechloro-hydroxy-*MF*, one isomer of dechloro-dihydroxy-*MF*, dechloro-tri-hydroxy-*MF*, dechloro-dihydroxy-methoxy-*MF*, N-dealkyl-*MF* (amphetamine), one isomer of N-dealkyl-hydroxy-*MF* (hydroxy-amphetamine), one isomer of deamino-oxo-hydroxy-*MF* (hydroxy-phenylacetone), deamino-oxo-dihydroxy-*MF* (dihydroxy-phenylacetone) and deamino-oxo-hydroxy-methoxy-*MF* (hydroxy-methoxy-phenylacetone). The EI and PCI mass spectra, the structures and the predominant EI fragmentation patterns of *MF* and its main metabolites hydroxy-*MF (isomer-1)* and *AM* after acetylation are shown in Fig. 1.

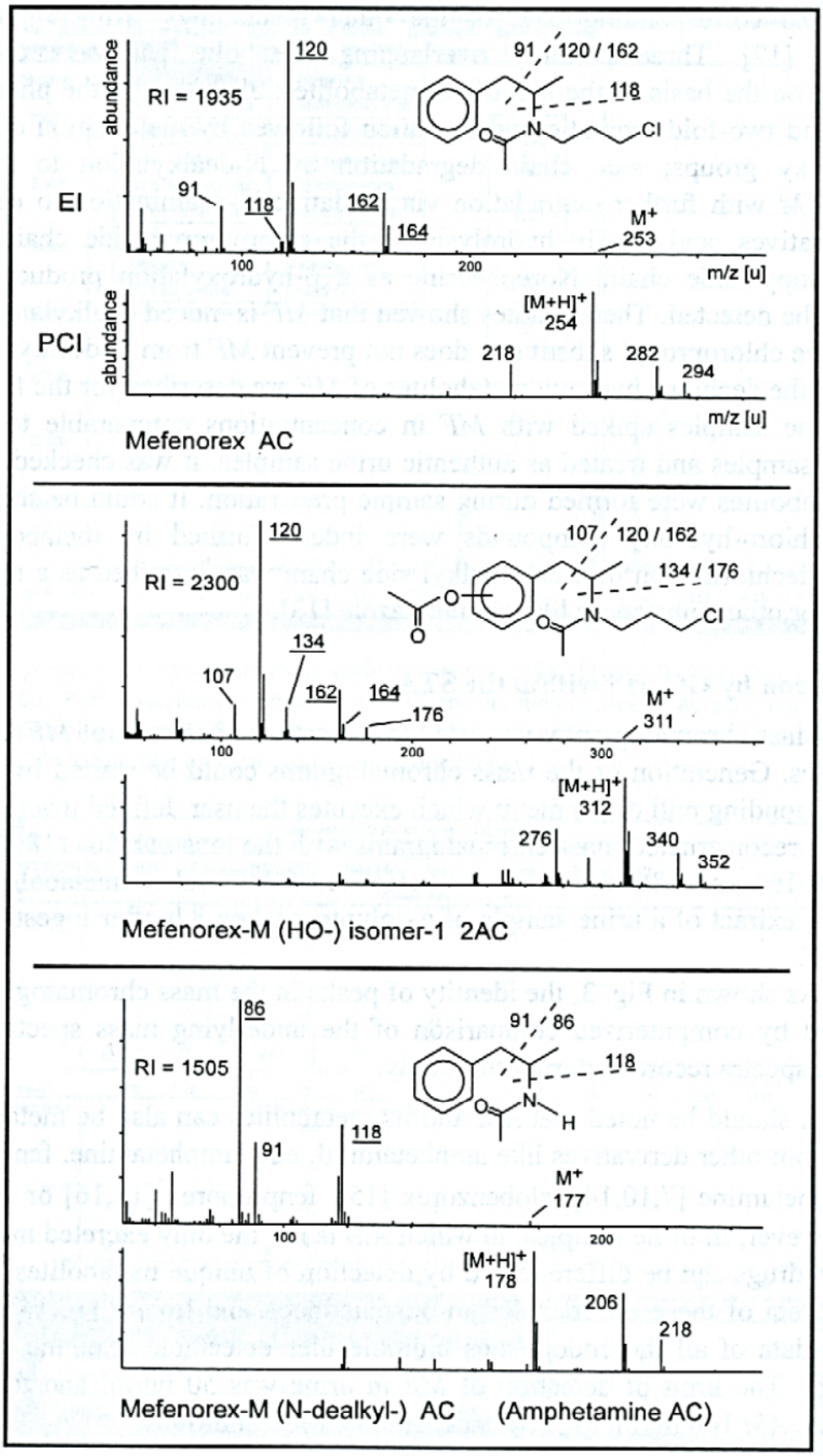

Fig. 1: Mass spectra (EI and PCI), predominant fragmentation patterns, structures and gas chromatographic retention indices (RI) of MF and its main metabolites hydroxy-MF and AM after acetylation.

The corresponding data of the other metabolites will be published elsewhere [12]. Three partially overlapping metabolic pathways could be postulated on the basis of the identified metabolites: alteration of the phenyl ring by one- and two-fold aromatic hydroxylation 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 finally hydrolysis of the chloropropyl side chain to the hydroxypropyl side chain. 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. Using urine samples spiked with *MF* in concentrations comparable to that of authentic samples and treated as authentic urine samples, it was checked whether these metabolites were formed during sample preparation. It could be shown that these dechloro-hydroxy compounds were indeed formed by metabolism. In addition, dechlorination of a chloroalkyl side chain was described as a metabolic reaction for other substances like clomethiazole [13].

3.3 Detection by GC-MS within the STA

Mass chromatography was used to indicate the presence of *MF* and/or its metabolites. Generation of the mass chromatograms could be started by clicking the corresponding pull down menu which executes the user defined macros. Fig. 2 shows the reconstructed mass chromatograms with the ions *m/z* 86, 118,120, 134, 144, 162, 164 and 186 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*.

As shown in Fig. 3, the identity of peaks in the mass chromatograms was confirmed by computerized comparison of the underlying mass spectrum with reference spectra recorded during this study.

It should be noted that *AM* and its metabolites can also be metabolically formed from other derivatives like amphetaminil, ethylamphetamine, fenethylline, methamphetamine [7,10,14], clobenzorex [15], fenproporex [12,16] or selegiline [17]. However, in urine samples, in which *AM* is not the only excreted metabolite, the taken drugs can be differentiated by detection of unique metabolites. The GC and MS data of these are included in our handbook and library [10,14] 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.

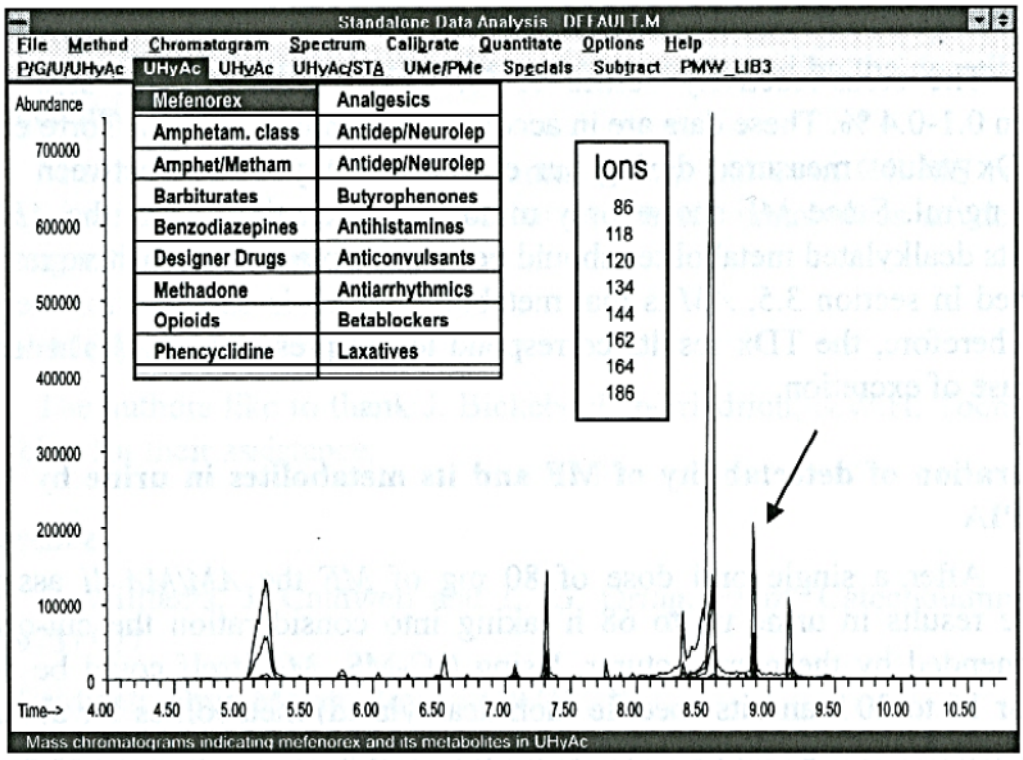

Fig. 2.: Typical mass chromatograms with the ions m/z 86, 118, 120, 134, 144, 162, 164, and 186. They indicate the presence of MF and its metabolites in an acetylated extract of a urine sample taken 8 h after ingestion of 80 mg of MF. The merged chromatograms can be differentiated by their colors on a color screen.

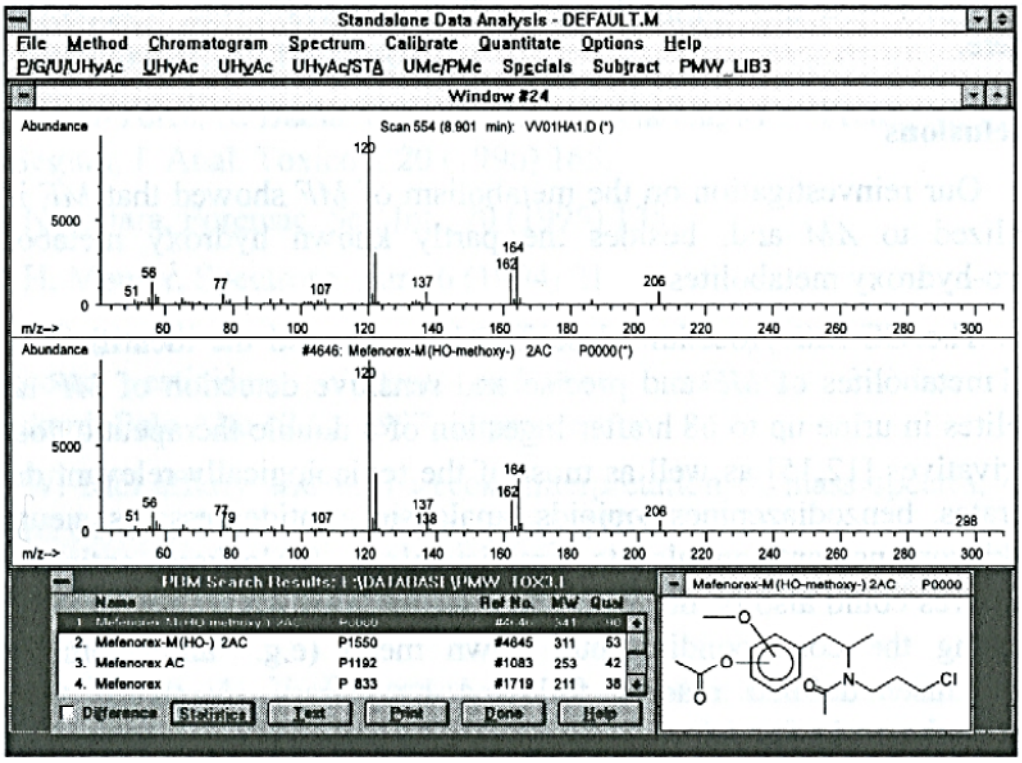

Fig. 3: Mass spectrum underlying the marked peak in Fig. 2, the reference spectrum, the structure, and the hit list found by computer library search.

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. [7]. 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 that metabolite which is excreted for the longest time. Therefore, the TDx results correspond to the presence of *AM*, at least in a late phase of excretion.

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

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* itself could be detected only for 16 to 20 h and its specific (non-dealkylated) metabolites for about 32 h. The metabolite *AM* could be detected for up to 68 h. All the immunoassay results could be confirmed by the described GC-MS procedure. Since only *AM* could be detected from 32 h to 68 h after ingestion, a therapeutic intake of *MF* could not be differentiated from an *AM* abuse during this period. Therefore, misinterpretation of positive immunoassay and even GC-MS results is possible. Studies on further substances causing similiar problems are in progress and will be published elsewhere.

4. Conclusions

Our reinvestigation on the metabolism of *MF* showed that *MF* is indeed metabolized to *AM* and, besides the partly 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 [12,15] 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 within 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 [10,17].

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 is possible, since the parent compound *MF* or its specific metabolites are detectable not as long as the metabolite *AM*.

5. Acknowledgment

The authors like to thank J. Bickeboeller-Friedrich, S.W.H. Toennes and A.A. Weber for their assistance.

6. References

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