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Studies on the metabolism and toxicological detection of the designer drug 4-methylthioamphetamine (4-MTA) in human urine using gas chromatography–mass spectrometry

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Dedicated to Dr. James Bäumler, Basel, at the occasion of his 80th birthday.

Abstract

4-Methylthioamphetamine (4-MTA) is a scheduled designer drug that has appeared on the illicit drug market and led to several non-fatal or even fatal poisonings. Only few data are available on its metabolism. The first aim of this study was to identify the 4-MTA metabolites in human urine and then to study whether the authors' STA procedure is suitable for screening for and identification of 4-MTA and/or its metabolites in urine. After enzymatic cleavage of conjugates, solid-phase extraction (SPE) and acetylation the following metabolites could be identified by full-scan gas chromatography–mass spectrometry (GC–MS): deamino-oxo 4-MTA, deamino-hydroxy 4-MTA, ring hydroxy and β-hydroxy 4-MTA. 4-MTA sulfoxide could be identified as possible artifact. In urine samples after enzymatic hydrolysis, acidic extraction, and methylation, 4-methylthiobenzoic acid could be identified. The authors' systematical toxicological analysis (STA) procedure using full-scan GC–MS after acid hydrolysis, liquid–liquid extraction (LLE) and acetylation allowed detection of 4-MTA as target analyte plus all the above-mentioned metabolites with the exception of 4-methylthiobenzoic acid. The extraction efficiency of 4-MTA was approximately 70% and the limit of detection (LOD) was 30 ng/ml (*S*/*N* 3).

Keywords: 4-Methylthioamphetamine; 4-MTA; Designer drug; Metabolism; GC–MS

1. Introduction

The amphetamine derivative 4-methylthioamphetamine (4-MTA) is a designer drug, which has appeared on the illicit drug market across Europe, especially in the dance scene [1,2]. There it has been distributed among drug abusers as tablets called 'ecstasy' or 'flatliner' and led to several non-fatal or even fatal poisonings [2–5]. Meanwhile, it has been scheduled in the lists of controlled substances of several countries. 4-MTA has been reported to be a non-neurotoxic serotonin releasing and reuptake inhibitory agent with monoamine oxidase-A inhibitory properties [6–9]. Its mode of action is similar to that of other designer drugs such as dimethoxymethylamphetamine (DOM) and of methylenedioxymethamphetamine (MDMA), but with a slower onset of the effects [9,10]. Only few data are available on the metabolism of 4-MTA [11]. Carmo et al. [12] identified a ring and a β-hydroxy metabolite and 4-(methylthio)benzoic acid in mouse urine after application of 4-MTA. In another study using hepatocytes of different animal species and humans, they could identify two side-chain hydroxy metabolites, 4-(methylthio)benzoic acid, and a sulfoxide [13]. The in-vivo metabolism of 4-MTA in humans has not been studied so far, although it is important for toxicological risk assessment based on extrapolation of animal to human data. It is also

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important for developing screening procedures for toxicological detection, especially in urine, because the excretion pattern allows selection of the most suitable target analyte. In reports on poisoning cases, gas chromatography–mass spectrometry (GC–MS) [2], liquid chromatography–tandem mass spectrometry (LC–MS–MS) [4], and liquid chromatography diode-array detection [3,14] were used for detection and/or determination of 4-MTA in various body samples. In addition, simultaneous determination of amphetamine derivatives including 4-MTA was described in human urine using HPLC-UV analysis [15] and in plasma using GC–MS [16]. Screening for the presence of 4-MTA in urine by commercial immunoassays and confirmation by GC–MS were described [17]. In the authors' laboratory, screening is performed using a so-called systematic toxicological analysis (STA) procedure based on full-scan electron ionization (EI) GC–MS that allows simultaneous screening for and identifying of various drug classes [18] in urine including classical and newer designer drugs [19–27].

The first aim of the present study was to identify the 4-MTA metabolites in human urine using GC–MS in the EI and positive-ion chemical ionization (PICI) mode. The second aim was to study whether the authors' STA procedure is suitable for screening for and identification of 4-MTA and/or its metabolites in human urine.

2. Experimental

2.1. Chemicals and reagents

4-MTA hydrochloride used for the extraction efficiency and limit of detection (LOD) studies was provided by the Bayerisches Landeskriminalamt (Munich, Germany) for research purposes. 4-(Methylthio)benzoic acid and vanillic acid were obtained from Fluka (Taufkirchen, Germany). Diazomethane was synthesized according to the procedure of McKay et al. [28]. All other chemicals and biochemicals used were obtained from E. Merck, Darmstadt (Germany) and were of analytical grade.

2.2. Urine samples

The investigations were performed using five authentic human urine samples that were taken about 8–15 h after reported ingestion of one to five pills of 4-MTA and submitted to the authors' laboratories for toxicological analysis. Blank urine samples were obtained from healthy young volunteers.

2.3. Sample preparation for metabolism studies

A 1-ml portion of urine was adjusted to pH 5.2 with acetic acid (1 M) and incubated at 50 °C for 1.5 h with 100 μl of a mixture (100,000 Fishman units per ml) of glucuronidase (EC no. 3.2.1.31) and arylsulfatase (EC no. 3.1.6.1). The sample was then diluted with 2 ml of water and loaded on a solid-phase extraction (SPE) cartridge (Isolute Confirm HCX, 130 mg, 3 ml), previously conditioned with 1 ml of methanol and 1 ml of water. After passage of the sample, the cartridge was washed with 1 ml of water and 1 ml of 0.01 M hydrochloric acid. The retained non-basic compounds were first eluted into a 1.5-ml reaction vial with 1 ml of methanol and gently evaporated under a stream of nitrogen at 56 °C. The basic compounds were eluted into the same vial in a second step with 1 ml of a freshly prepared mixture of methanol–aqueous ammonia (98:2, v/v). After evaporation to dryness under a stream of nitrogen at 56 °C, the sample was derivatized with 100 μl of an acetic anhydride–pyridine mixture (3:2, v/v) for 5 min under microwave irradiation at about 440 W. After evaporation under a stream of nitrogen at 56 °C, the residue was dissolved in 50 μl of methanol and 2 μl of this solution were injected into the GC–MS.

Another 1-ml portion of urine was adjusted to pH 5.2 with acetic acid (1 M) and incubated at 50 °C for 1.5 h with 100 μl of a mixture (100,000 Fishman units per ml) of glucuronidase (EC no. 3.2.1.31) and arylsulfatase (EC no. 3.1.6.1). The sample was then worked-up by liquid–liquid extraction (LLE) with 5 ml of dichloromethane–isopropanol–ethyl acetate (1:1:3, v/v/v). After phase separation by centrifugation, the organic layer was transferred into a glass flask and evaporated under a stream of nitrogen at 56 °C to dryness. The residue was dissolved in 50 μl of methanol and derivatized with 100 μl of a solution of diazomethane in diethyl ether. The reaction vial was sealed and left at room temperature for 30 min. Thereafter, the mixture was gently evaporated to dryness under a stream of nitrogen at 56 °C and dissolved in 50 μl of methanol and 2 μl were injected into the GC–MS.

2.4. Sample preparation for toxicological analysis

The urine samples (5 ml) were divided into two aliquots. One aliquot was refluxed with 1 ml of 37% hydrochloric acid for 15 min. Following hydrolysis, the sample was mixed with 2 ml of 2.3 M aqueous ammonium sulfate and 1.5 ml of 10 M aqueous sodium hydroxide to obtain a pH value of 8–9. Before extraction, the other aliquot of native urine was added. This mixture 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 into a glass flask and evaporated under reduced pressure at 70 °C to dryness. The residue was derivatized by acetylation with 100 μl of an acetic anhydride–pyridine mixture (3:2; v/v) for 5 min under microwave irradiation at about 440 W. After evaporation of the derivatization mixture under reduced pressure at 70 °C, the residue was dissolved in 100 μl of methanol and 2 μl were injected into the GC–MS system.

2.5. Gas chromatography–mass spectrometry

4-MTA and its metabolites were separated and identified in derivatized urine extracts using a Hewlett-Packard (Agi-

lent, 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 × 0.2 mm I.D.), cross linked methyl silicone, 330 nm film thickness; injection port temperature, 280 °C; carrier gas, helium; flow-rate 1 ml/min; column temperature, programmed from 100 to 310 °C at 30°/min, initial time 3 min, final time 8 min. The MS conditions were as follows: full scan mode, *m/z* 50–550 u; EI mode: ionization energy, 70 eV; PICI using methane: ionization energy, 230 eV; ion source temperature, 220 °C; capillary direct interface heated at 260 °C.

For toxicological detection of acetylated 4-MTA and its metabolites, mass chromatography with the selected ions *m/z* 164, 86, 137, 122, and 223 was used. The identity of the peaks in the mass chromatograms was confirmed by computerized comparison of the mass spectra underlying the peaks (after background subtraction) with reference spectra recorded during this study.

3. Results and discussion

3.1. Identification of metabolites

Cleavage of conjugates was necessary before extraction and GC–MS analysis of the suspected metabolites in order not to overlook conjugated metabolites. For studies on the metabolism, gentle enzymatic hydrolysis was preferred. 4-MTA and its metabolites were isolated from urine by SPE that led to cleaner extracts than the LLE procedure used for similar metabolism studies in the authors' laboratory in the past [22–27]. The metabolite 4-methylthiobenzoic acid was not extractable by this SPE, but by LLE at acidic pH followed by methylation. Derivatization of the extracts was indispensable for sensitive detection. The urinary metabolites of 4-MTA were separated by GC and identified by full-scan EI and PICI MS. The postulated structures of the metabolites were deduced from the fragments detected in the EI mode, which were interpreted in correlation to those of the parent compound according to the rules described by, e.g. McLafferty and Turecek [29] and Smith and Busch [30]. EI and PICI mass spectra, the gas chromatographic retention indices (RIs), structures and predominant fragmentation patterns of 4-MTA and its proposed metabolites are shown in Fig. 1. In order to verify the molecular mass of the postulated metabolites, PICI mass spectra were recorded, because they usually contain abundant peaks of the protonated molecule $[M + H]^+$ with the adduct ions typical for PICI using methane as reagent gas $[M + C_2H_5]^+$ and $[M + C_3H_5]^+$. However, the compounds containing an *O*-acetyl substituent (mass spectra nos. 3–5 in Fig. 1) did not show the protonated molecule $[M + H]^+$.

Besides 4-MTA (mass spectra no. 1 in Fig. 1), the following metabolites could be identified after SPE and acetylation: deamino-oxo 4-MTA (1-[4-(methylthio)-phenyl]propan-2-one; mass spectra no. 2 in Fig. 1), deamino-hydroxy 4-MTA (1-[4-(methylthio)phenyl]propan-2-ol; mass spectra no. 3 in Fig. 1), ring hydroxy 4-MTA ((2-aminopropyl)-(methylthio)phenol; mass spectra no. 4 in Fig. 1), and β-hydroxy 4-MTA (2-amino-1-[4-(methylthio)phenyl]-propan-1-ol; mass spectra no. 5 in Fig. 1). In addition, 4-MTA sulfoxide (mass spectra no. 6 in Fig. 1) could be identified as possible metabolite. However, this compound could also be detected in blank urine samples spiked with 4-MTA at a concentration of 1000 ng/ml, so that it can (also) be formed artificially during workup and/or analysis. The exact position of the hydroxy group in the phenyl ring (mass spectra no. 4 in Fig. 1) could not be determined by means of GC–MS. The two isomers of the β-hydroxy metabolite that have been detected by Carmo et al. [13] in hepatocytes, could not be detected in human urine, maybe due to their low urine concentrations.

In the urine samples after acidic extraction and methylation, 4-methylthiobenzoic acid (mass spectra no. 7 in Fig. 1), that was described as the main metabolite in hepatocyte incubations [13], could be identified. However, in some of the blank urine samples worked-up in the same way, a peak appeared with an underlying mass spectrum (mass spectra no. 8 in Fig. 1) that looked very similar to that of 4-methylthiobenzoic acid. The authors' mass spectral library [31] found as best hit methylated vanillic acid, a degradation product of several fruit or vegetable ingredients. As the sulfur isotope clusters of 4-methylthiobenzoic acid was not predominant enough in the EI spectrum for differentiation, both compounds were spiked alone or in combination into blank urine at concentrations of 1000 ng/ml, each, that was worked-up as described here for 4-methylthiobenzoic acid. Fortunately, the retention indices of both compounds differed markedly, which allowed differentiation of both.

Based on the identified metabolites of 4-MTA, the following metabolic pathway could be postulated (Fig. 2): oxidative deamination to the corresponding oxo metabolite (no. 2) followed by reduction to the corresponding alcohol (no. 3) or degradation of the side chain to 4-methylthiobenzoic acid (no. 7), ring hydroxylation to a phenolic structure (no. 4) and β–hydroxylation of the side chain to 4-methylthionorephedrine (no. 5). As the peak of the deamino-hydroxy metabolite was more abundant in urine after hydrolysis with glucuronidase and arylsulfatase, it can be concluded that it is partly excreted as glucuronide and/or sulfate. The pathways of 4-MTA are similar to those of structurally related 4-methoxyamphetamine (PMA) [32]. Three metabolites (nos. 4, 5 and 7) had already been identified in mouse urine [12] and one (no. 7) in incubations with hepatocytes from different species including humans [13]. The metabolic patterns were similar in all analyzed urine samples.

3.2. Detection by GC–MS within the STA

For cleavage of conjugates, rapid acid hydrolysis was performed that has proved to be very efficient and fast for

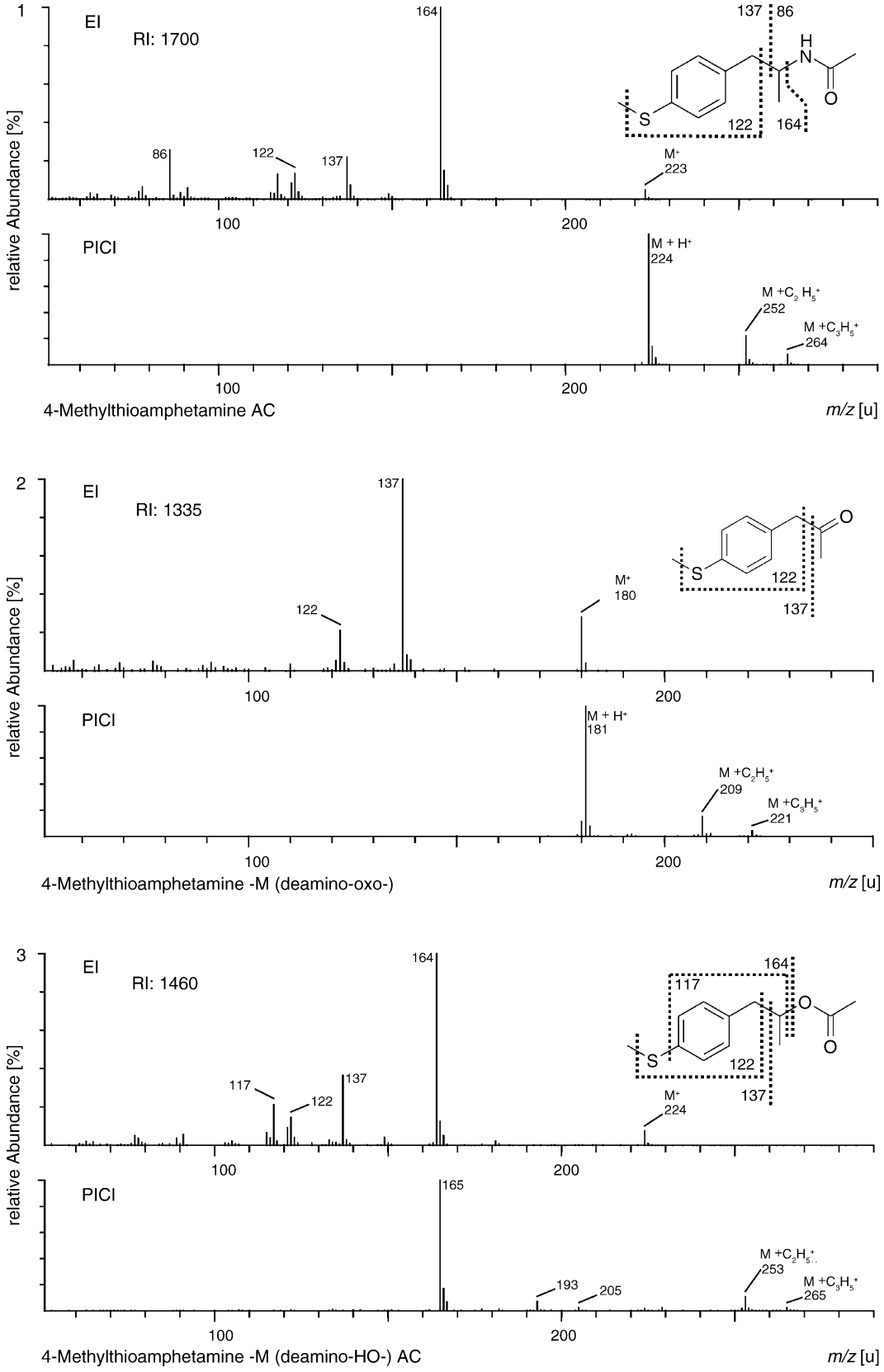
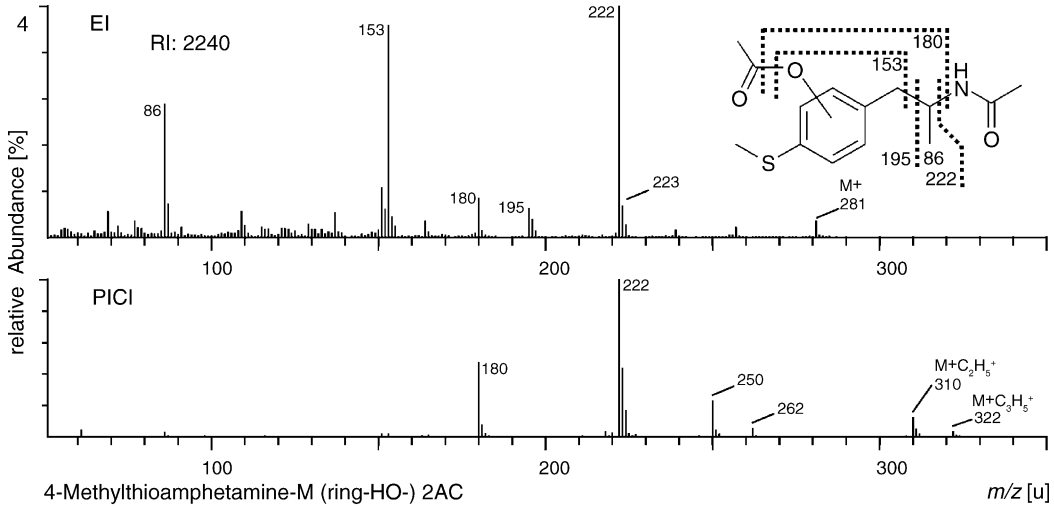
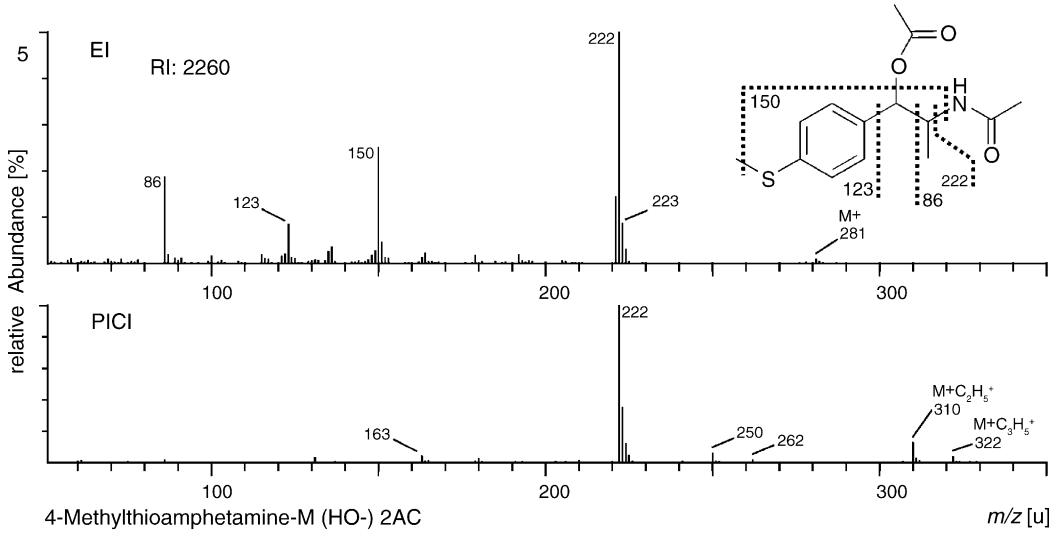


Fig. 1. EI and PICI mass spectra, the gas chromatographic retention indices (RI), structures and predominant fragmentation patterns of 4-MTA, its proposed metabolites and vanillic acid after acetylation or methylation.





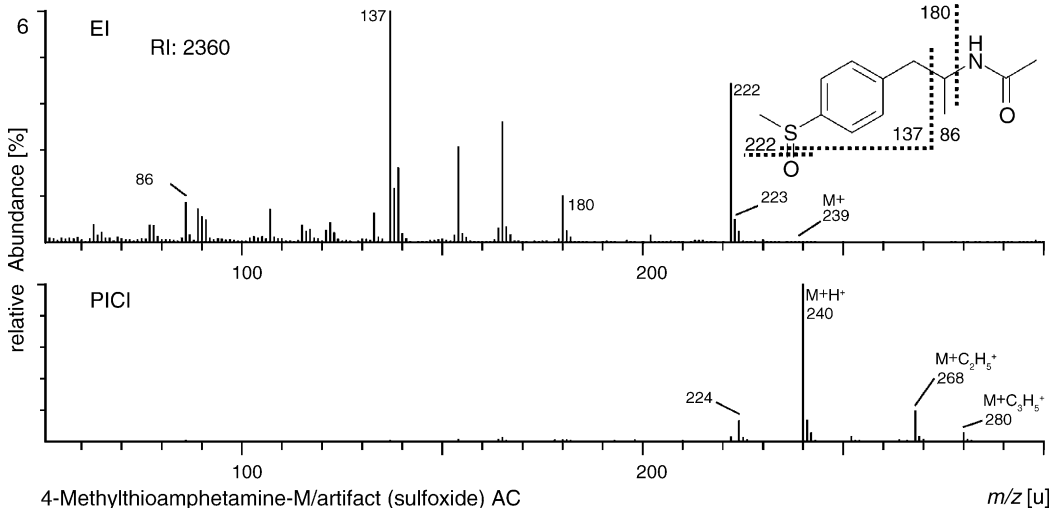
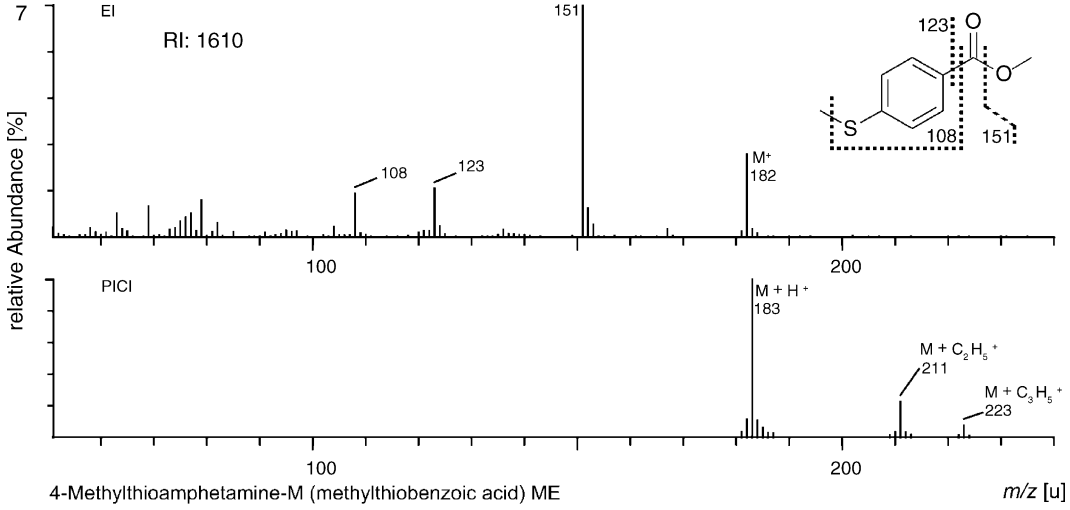


Fig. 1. (*Continued*)



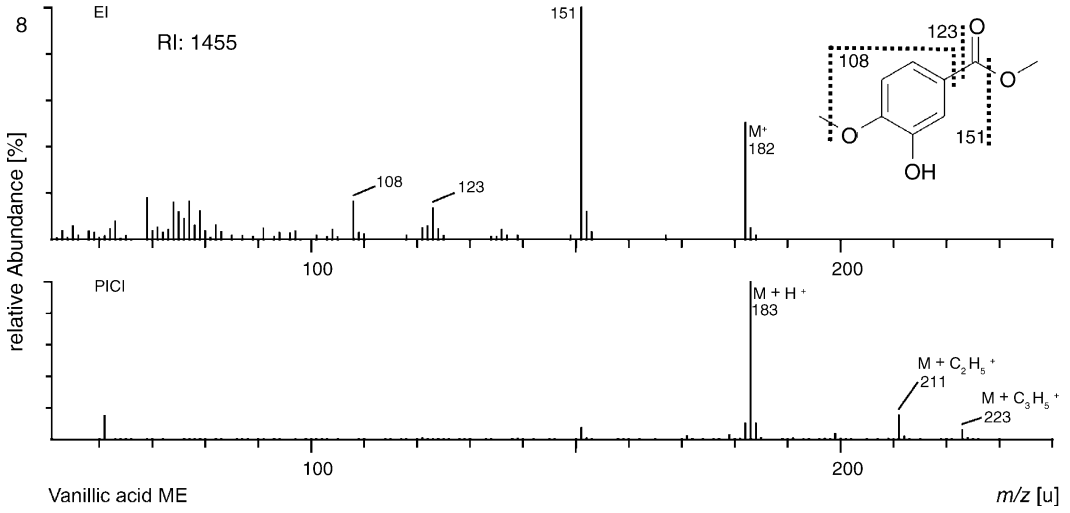


Fig. 1. (*Continued*).

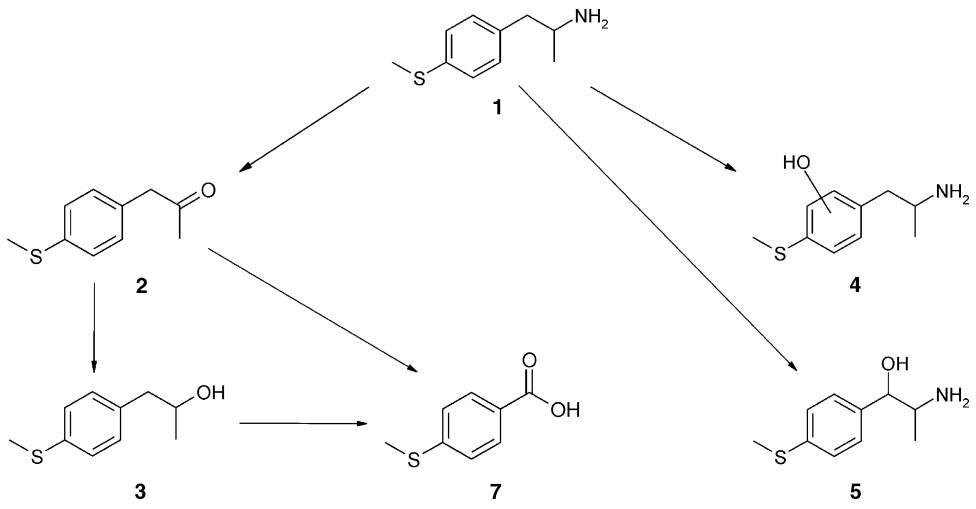


Fig. 2. Proposed scheme for the phase I metabolism of 4-MTA in humans. The numbering of the compounds corresponds to that of the mass spectra in Fig. 1.

this purpose [22–27]. However, one part of unhydrolyzed urine was added before extraction, because some analytes are altered or destroyed during hydrolysis [33]. 4-MTA and all its metabolites detected within the metabolism studies after enzymatic hydrolysis, SPE and acetylation could also be detected within the STA (mass spectra nos. 1–6 in Fig. 1). Only 4-methylthiobenzoic acid was not detected due to the basic extraction pH and the lack of methylation. As could be seen in all authentic cases, the target analyte for the STA is acetylated 4-MTA itself (mass spectrum no. 1 in Fig. 1). At a concentration of 1000 ng/ml, the extraction efficiency of 4-MTA was 69 ± 1.9% (mean ± S.D., $n = 5$). The LOD of acetylated 4-MTA was 30 ng/ml (S/N 3). Unfortunately, the excretion rates of the metabolites could not be determined due to the lack of reference substances. However, assuming similar chromatographic properties of the derivatives of 4-MTA and its metabolites, their peak areas allow assessing that the amount of the metabolites, detected in urine by STA, were about 10% of that of the parent drug. Nevertheless, detection of the metabolites might allow proofing an intake of 4-MTA and to exclude a manipulation of a urine sample by adding 4-MTA, e.g. to plead for diminished responsibility in case of crime after drug abuse.

The gas chromatographic RIs given in Fig. 1 provide preliminary indications, allow distinguishing between, e.g. 4-methylthiobenzoic acid and vanillic acid and may be useful for gas chromatographers without an MS facility. They were recorded during the GC–MS procedure and calculated in correlation with the Kovats' indices [34] of the components of a standard solution of typical drugs which is measured daily for testing the GC–MS performance [35,36]. The reproducibility of RIs measured on capillary columns was better using a mixture of drugs than that of the homologous hydrocarbons recommended by Kovats.

Interferences by biomolecules or further drugs indicated in the reconstructed mass chromatograms could be excluded, because these compounds have different gas chromatographic and/or mass spectral properties. The corresponding RIs and reference mass spectra are included in the used reference library [31].

For illustration of the method, Fig. 3 shows reconstructed mass chromatograms with the ions m/z 164, 86, 137, 122, and 223 indicating the presence of 4-MTA and its metabolites and artifact in an acetylated extract of an authentic urine sample of a patient after reported ingestion of two to four pills (the peak numbers correspond to those of the underlying mass spectra in Fig. 1). Ion m/z 164 was used for monitoring the presence of the 4-methylthiophenylisopropyl moiety, m/z 86 for the intact ethyl mono N-acetyl part, m/z 137 for the unchanged 4-methylthiophenylmethyl moiety, m/z 122 for the 4-thiophenylmethyl moiety, and m/z 223 for the molecular ion of the parent compound and the ^{13}C isotope mass of the hydroxy metabolites and the sulfoxide. The latter can be found in the STA as artifact of 4-MTA. The identity of the peak in the mass chromatogram was confirmed by computerized comparison of the underlying mass spectrum with reference spectra recorded during this study [37]. Fig. 4 shows the mass spectrum underlying the marked peak

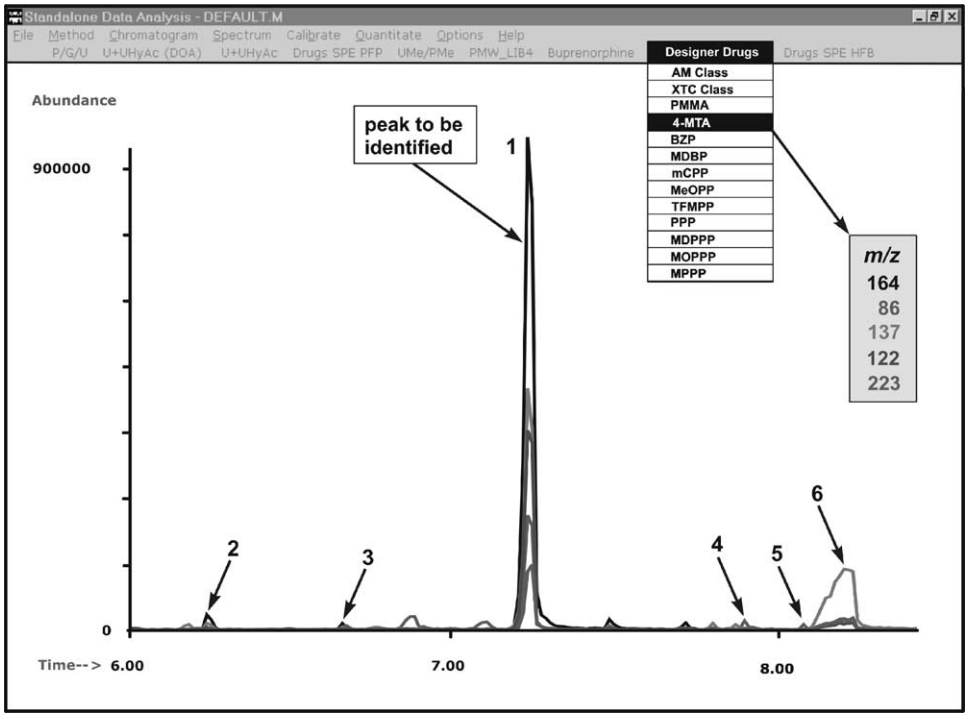


Fig. 3. Typical mass chromatograms with the ions m/z 164, 86, 137, 122, and 223. They indicate the presence of 4-MTA and its metabolites and artifact in an acetylated extract of an authentic urine sample of a patient after reported ingestion of 2–4 pills of 4-MTA. The merged ion chromatograms can be differentiated by their colors on a color screen.

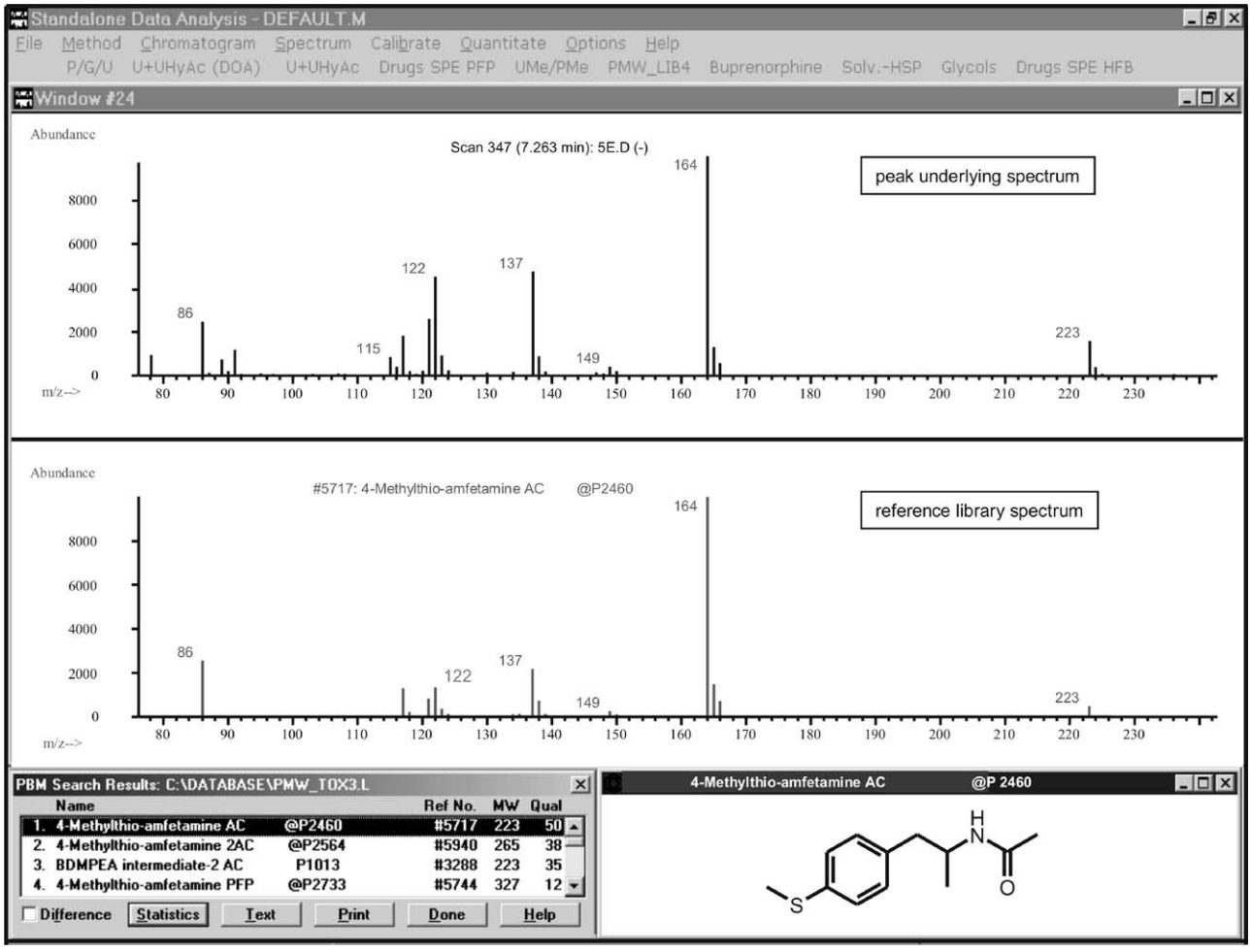


Fig. 4. Mass spectrum underlying the marked peak in Fig. 3 the reference spectrum, the structure, and the hit list found by computer library search.

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

4. Conclusions

The presented studies revealed that the designer drug 4-MTA was poorly metabolized by humans. Screening must, therefore, be focused on the parent compound. However, detection of metabolites may allow to exclude a manipulation of the urine sample by adding 4-MTA, e.g. to plead for diminished responsibility in case of crime after drug abuse. The described screening procedure was suitable for detection of 4-MTA and/or its metabolites in human urine in clinical or forensic cases.

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