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Studies on the metabolism and toxicological detection of the new designer drug 4′-methyl-α-pyrrolidinobutyrophenone (MPBP) in rat urine using gas chromatography–mass spectrometry

Frank T. Peters [a,*], Markus R. Meyer [a], Giselher Fritschi [b], Hans H. Maurer [a]

[a] *Department of Experimental and Clinical Toxicology, Institute of Experimental and Clinical Pharmacology and Toxicology, University of Saarland, D-66421 Homburg (Saar), Germany*
[b] *Hessisches Landeskriminalamt, D-65187 Wiesbaden, Germany*

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Abstract

The aim of the presented study was to identify the metabolites of the new designer drug 4′-methyl-α-pyrrolidinobutyrophenone (MPBP) in rat urine using GC–MS techniques. After enzymatic hydrolysis, extraction and various derivatizations, seven metabolites of MPBP could be identified suggesting the following metabolic steps: oxidation of the 4′-methyl group to the corresponding alcohol and further oxidation to the respective carboxy compound, hydroxylation of the pyrrolidine ring followed by dehydrogenation to the corresponding lactam or reduction of the keto group to the 1-dihydro compound. A previously published GC–MS-based screening procedure for pyrrolidinophenones involving enzymatic hydrolysis and mixed-mode solid-phase extraction of urine samples allowed detection of MPBP metabolites. Assuming similar metabolism and dosages in humans, an intake of MPBP should be detectable via its metabolites in urine.

Keywords: MPBP; 4′-Methyl-α-pyrrolidinobutyrophenone; Designer drug; Metabolism; Detection; GC–MS

1. Introduction

1-(4-Methylphenyl)-2-pyrrolidin-1-ylbutan-1-one (4′-methyl-α-pyrrolidinobutyrophenone, MPBP) is a new designer drug of the pyrrolidinophenone type. After α-pyrrolidinopropiophenone (PPP) [1,2], 4′-methyl-α-pyrrolidinopropiophenone (MPPP) [1–3], 4′-methyl-α-pyrrolidinohexanophenone (MPHP) [4], 4′-methoxy-α-pyrrolidinopropiophenone (MOPPP) [5], 3′,4′-methylenedioxy-α-pyrrolidinopropiophenone (MDPPP) [1,6], it was the latest of this new class of designer drugs to enter the illicit drug market in Germany. Their chemical structures are given in Fig. 1. MPBP was seized as powder by the German police. It is assumed to be taken orally as the other pyrrolidinophenones, which are distributed among drug abusers as tablets, capsules, or powders [1]. Statements on the frequency of their occurrence cannot be made, because they cannot be detected with usual routine analysis procedures [2–6] and might, therefore, have been overlooked. Nevertheless, PPP (1998), MPPP (1999), and MDPPP (1997) have been scheduled in the German Controlled Substances Act and possession is strictly prohibited.

So far, little information about the dosage as well as the pharmacological and toxicological effects of the pyrrolidinophenones is available. However, they may be expected to be very similar to those of pyrovalerone (4′-methyl-α-pyrrolidinovalerophenone) due to their close structural relation to this drug as shown in Fig. 1. Pyrovalerone is a psychostimulant which acts by releasing dopamine and norepinephrine from the respective nerve terminals [7,8]. It was first pharmacologically characterized in animal experiments by Stille et al. [9] in the early 1960s. These authors reported comparable central stimulatory effects but less influence

* Corresponding author. Tel.: +49 6841 16 26430; fax: +49 6841 16 26051.
E-mail address: frank.peters@uniklinikum-saarland.de (F.T. Peters).

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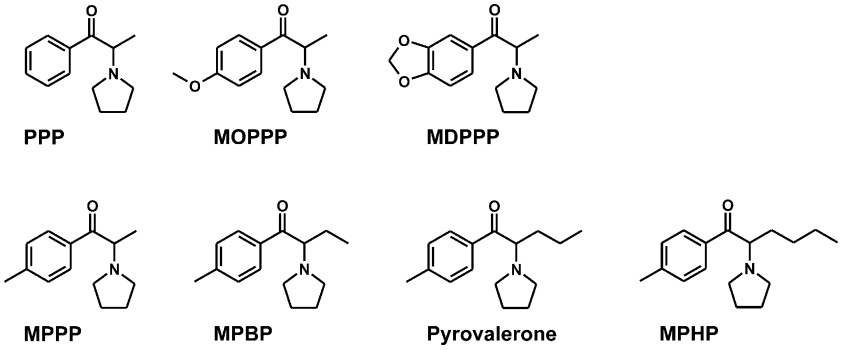


Fig. 1. Chemical structures of pyrrolidinophenone-type designer drugs and of the psychostimulant pyrovalerone.

on locomotor activity and autonomous function compared to amphetamine. The LD_{50} of pyrovalerone in mice was reported to be 350 mg/kg bodyweight (BW) per os. Comparable psychostimulatory effects [10,11] and smaller effects on motor function [11] of pyrovalerone in comparison to amphetamine were also found in controlled studies with humans. Pyrovalerone had been studied as a therapeutic drug [12–14], but was withdrawn from the market and scheduled as a controlled substance after reports of its intravenous abuse by polytoxicomaniacs [15]. Beside this, the chemical structure of pyrrolidinophenones is closely related to α-aminopropiophenone anorectics like amfepramone, drugs of abuse like cathinone/methcathinone and antidepressants like bupropion and might therefore evoke similar effects [16–19]. Clearly, such pharmacological profiles are in line with the abuse of pyrrolidinophenones as stimulant designer drugs.

While the metabolism and toxicological detection of PPP, MPPP, MPHP, MOPPP, MDPPP, and pyrovalerone have been described [2–6,20–22], MPBP has never been studied in this respect. However, the knowledge about metabolic steps is a prerequisite for toxicological risk assessment and for developing screening procedures for toxicological detection, as in both cases the metabolites may play a major role. Furthermore, determination or screening procedures for MPBP are necessary for confirmation of the diagnosis of an intoxication or drug abuse.

Therefore, the aim of the presented study was firstly to identify the MPBP metabolites in rat urine using GC–MS in the electron impact (EI) and positive-ion chemical ionization (PICI) mode and secondly to use this knowledge for incorporation of MPBP into the authors' EI GC–MS based toxicological screening procedure for the other pyrrolidinophenone-type designer drugs in urine [2,4–6].

2. Experimental

2.1. Chemicals and reagents

MPBP·HNO_3 from a drug seizure was provided from Hessisches Landeskriminalamt, Wiesbaden (Germany) for research purposes. The purity and identity had been proven by mass spectrometry, infrared and ^{1}H NMR spectroscopy. Isolute Confirm HCX cartridges (130 mg, 3 ml) were obtained from Separtis (Grenzach-Wyhlen, Germany). *N*-Methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) was obtained from Fluka (Steinheim, Germany). Diazomethane was synthesized in the authors' laboratory according to the procedure of McKay et al. [23]. All other chemicals and biochemicals used were obtained from Merck, Darmstadt (Germany) and were of analytical grade.

2.2. Urine samples

The investigations were performed using male rats (Wistar, Ch. River, Sulzfleck, Germany) for toxicological diagnostic reasons according to the corresponding German law. They were administered a single 20 mg/kg body mass dose (for metabolism studies) or a 1 mg/kg body mass dose (for development of the screening procedure) of MPBP-HNO_3 in an aqueous suspension by gastric intubation. Urine was collected separately from the faeces over a 24 h period. All samples were directly analyzed and then stored at −20 °C until 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 0.5 ml portion of urine was adjusted to pH 5.2 with acetic acid (1 M, approximately 10–50 μl) and incubated at 56 °C for 1.5 h with 50 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) from *Helix pomatia* L. (Roman Snail). The urine sample was then diluted with 2.5 ml of water and loaded on a Confirm HCX cartridge, 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 (fraction 1), whereas the basic compounds were eluted in a second step into a different vial with 1 ml of a freshly prepared mixture of methanol/aqueous ammonia (98:2, v/v; fraction 2). The eluates were gently evaporated to dryness under a stream of nitrogen at 56 °C and derivatized by methylation, acetylation, combined methyla-

tion/acetylation or trimethylsilylation according to published procedures [24]. Briefly, methylation was performed after reconstitution in 50 μl of methanol with 50 μl of a solution of diazomethane in diethyl ether. The reaction vials were sealed and left at room temperature for 30 min. Thereafter, the mixture was once again gently evaporated to dryness under a stream of nitrogen and redissolved in 100 μl of methanol. Acetylation was conducted 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, the residue was dissolved in 100 μl of methanol. A 3 μl aliquot each was injected into the GC–MS system. In case of combined methylation/acetylation, extracts were first methylated and subsequently acetylated. For trimethylsilylation, 50 μl of MSTFA were added to the extract previously reconstituted in 50 μl of ethyl acetate and derivatization was carried out for 5 min under microwave irradiation at 440 W. A 3 μl aliquot was injected into the GC–MS with an alcohol- and water-free syringe. The same experiments were repeated without the use of enzymatic hydrolysis to study which metabolites of MPBP were excreted as glucuronides/sulfates.

2.4. Sample preparation for toxicological analysis

The extraction procedure was the same as described under 2.3, but only the second eluate (fraction 2) was used for further workup. It was gently evaporated to dryness under a stream of nitrogen at 56 °C and then reconstituted in 50 μl of ethyl acetate. After addition of 50 μl of MSTFA the reconstituted extract was trimethylsilylated for 5 min under microwave irradiation at 440 W. A 2 μl aliquot of this mixture was injected into the GC–MS with an alcohol- and water-free syringe.

2.5. Gas chromatography–mass spectrometry

The MPBP metabolites were separated and identified in derivatized urine extracts using a Hewlett Packard (Agilent, 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 methylsilicone, 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 ionization mode: ionization energy, 70 eV; chemical ionization using methane, positive mode (PICI): ionization energy, 230 eV; ion source temperature, 220 °C; capillary direct interface heated at 260 °C.

For toxicological detection of MPBP and its trimethylsilylated metabolites, mass chromatography with the selected ions *m/z* 112, 126, 178 and 318 was used. Generation of the mass chromatograms could be started by clicking the corresponding pull down menu which executes the user defined macros [25,26]. The identity of the peaks in the mass chromatograms was confirmed by computerized comparison [27] of the mass spectra underlying the peaks (after background subtraction) with reference spectra recorded during this study (Fig. 2 and ref. [27]).

3. Results and discussion

3.1. Sample preparation

Cleavage of conjugates was necessary before extraction and GC–MS analysis of the suspected metabolites in order not to overlook conjugated metabolites. Gentle enzymatic hydrolysis was used because the analytes were destroyed during acid hydrolysis. The described cleavage procedure at elevated temperature (56 °C) for a short period of time (1.5 h) had been successfully used in routine screening procedures for other pyrrolidinophenones [2,4–6]. In the present study, this short procedure was also used for metabolism studies, since it had proven to yield similar results (i.e. same compounds detected at similar abundances) as the authors' standard conditions for metabolism studies (37 °C for 12 h).

The use of common liquid–liquid extraction under alkaline or acidic conditions followed by acetylation or methylation [24,26,28–30], respectively, was not appropriate, because the majority of the metabolites showed amphoteric properties. In addition, volatility of the free bases and the instability of the analytes under alkaline and high temperature conditions had caused difficulties with structurally related compounds [31,32]. In contrast, mixed-mode SPE had been successfully applied for extraction of amphoteric metabolites of other pyrrolidinophenones [2–6], and also proved to be applicable for extraction of MPBP and its metabolites.

Derivatization was needed to improve the GC properties of these relatively polar metabolites thus increasing the sensitivity of their detection. Methylation with diazomethane is well known to be versatile for derivatization of metabolites with phenolic hydroxy or carboxy groups. In contrast, aliphatic hydroxy groups remain unaffected thus allowing their distinction from phenolic hydroxyl groups. Moreover, the resulting derivatives often show favorable fragmentation properties in the EI mode. They yield easily interpretable fragment ions, which is advantageous for the elucidation of metabolite structures. In the present study, methylation was performed, because MPBP was expected to be metabolized to the respective 4′-carboxy compound as it had been described for other 4′-methyl-pyrrolidinophenones [2–4,22,33]. Acetylation has also proved useful in metabolism studies [28–30,34]. It can be employed for derivatization of primary and secondary amino groups as well as alcoholic and/or phenolic hydroxy groups. Generally, the fragmentation patterns of the resulting derivatives in the EI mode are also easy to interpret. In the present study, acetylation was used to allow

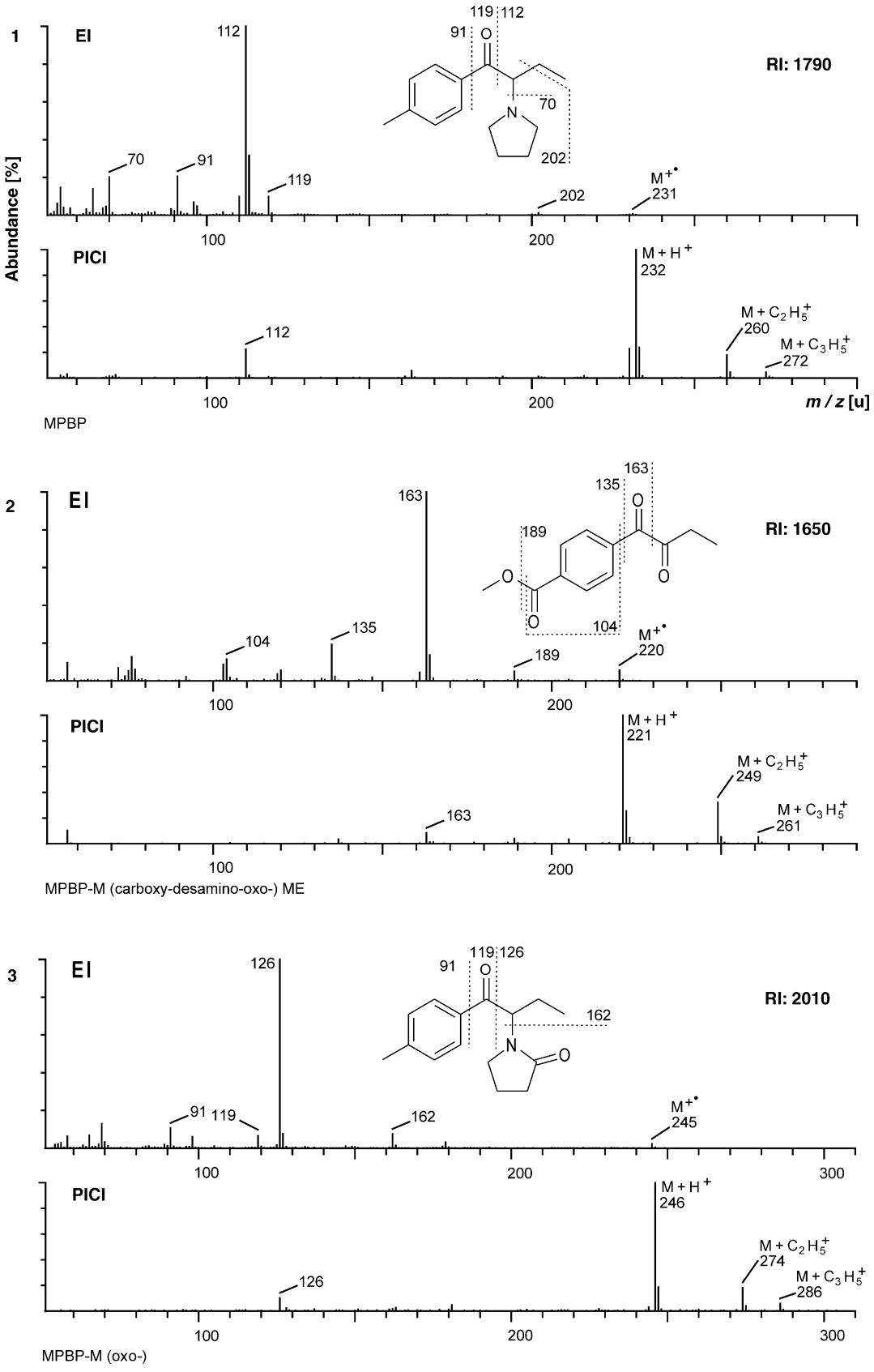
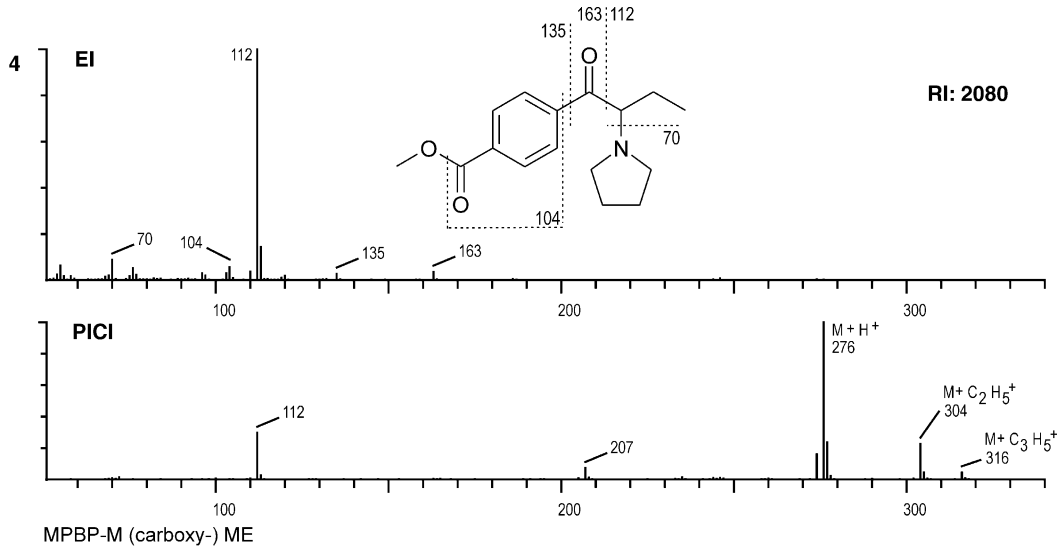
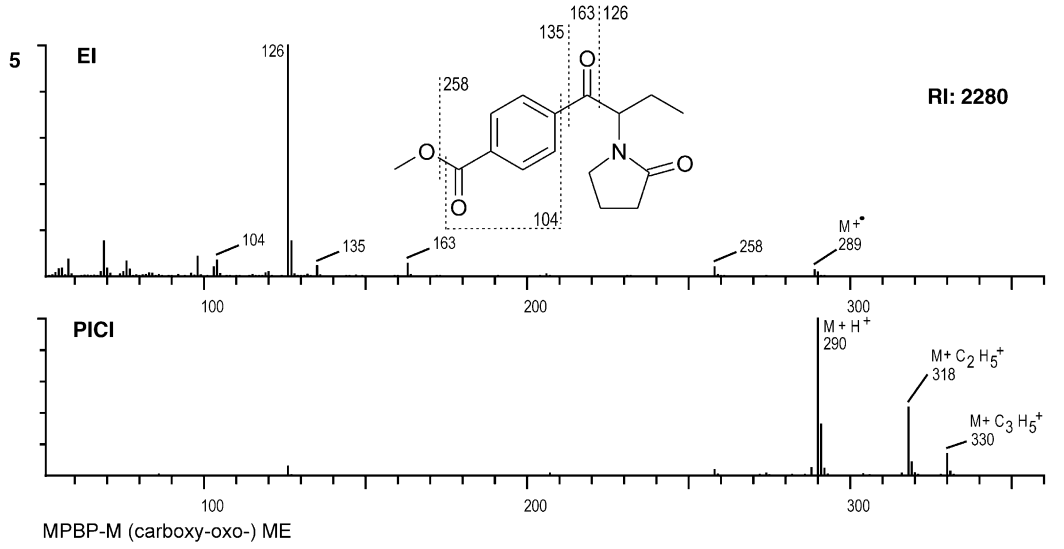
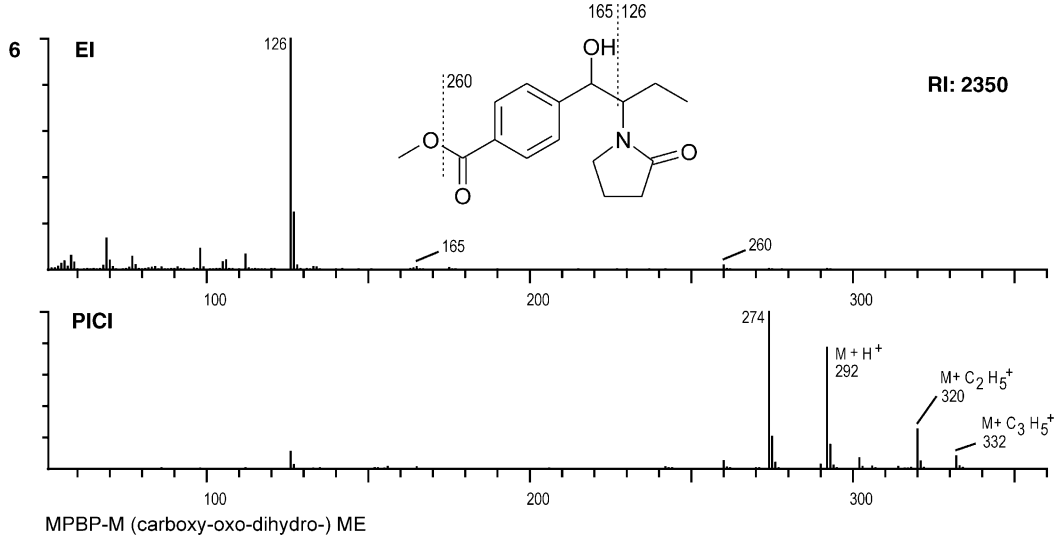


Fig. 2. EI and PICI mass spectra, gas chromatographic retention indices (RI), structures and predominant fragmentation patterns of MPBP and its metabolites after methylation or trimethylsilylation. The axes are only labeled for 1.







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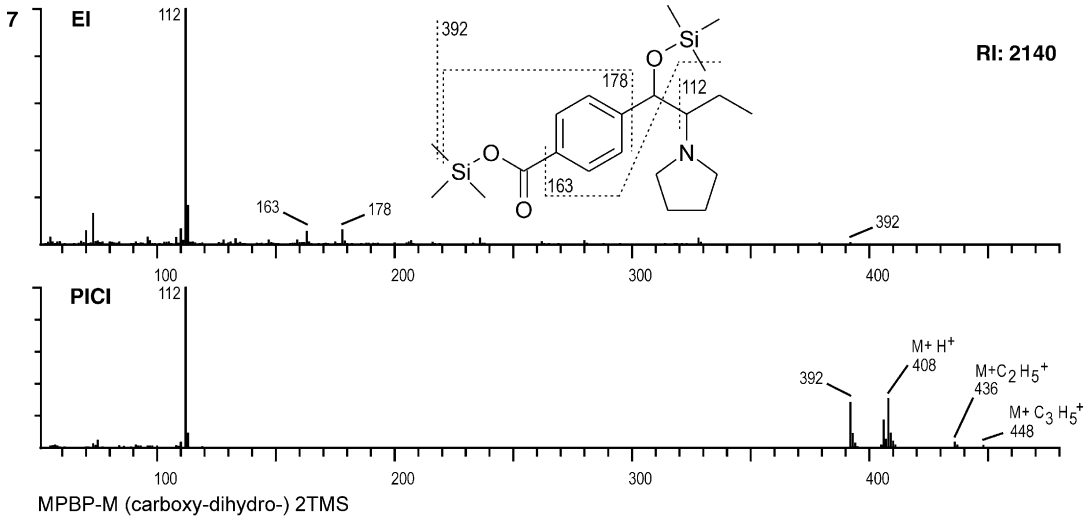

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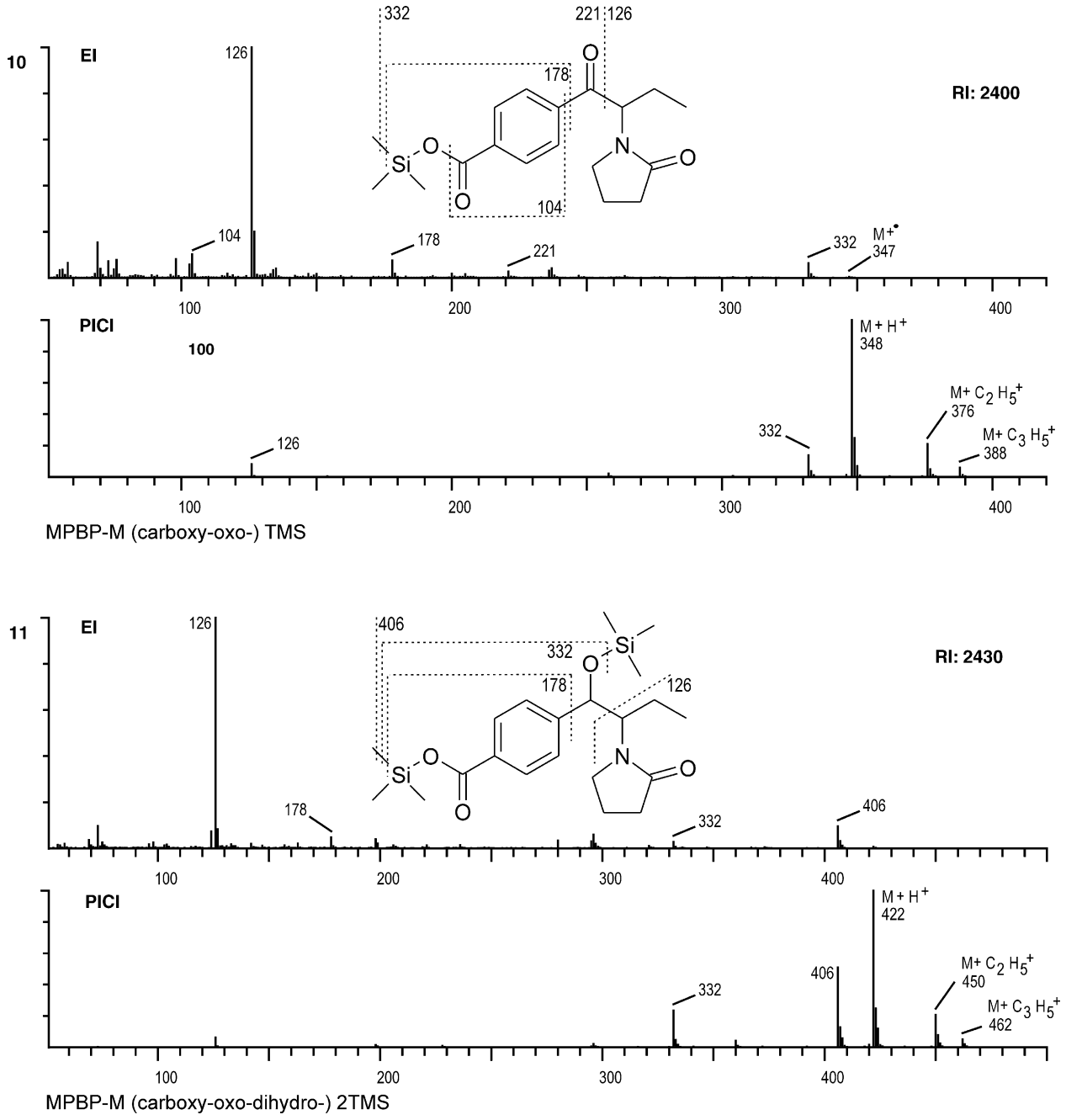

Fig. 2. (*Continued*).

detection of possible primary or secondary amine metabolites resulting from degradation of the pyrrolidine ring as it has been described for PPP [2]. Finally, trimethylsilylation was performed in order to record the mass spectra of the trimethylsilyl derivatives of the MPBP metabolites needed to incorporate MPBP into the authors' screening procedure for pyrrolidinophenones [2,4–6]. Trimethylsilylation, which is a common derivatization procedure in many routine methods, is safer and easier to handle than methylation with diazomethane and the reagent is commercially available. Even though the resulting mass spectra are generally less useful for elucidation of metabolite structures, the trimethylsilylated extracts were checked for the presence of additional metabolites.

3.2. Identification of metabolites

The urinary metabolites of MPBP were identified by full-scan EI and PICI MS after GC separation. 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 [35] and Smith and Busch [36]. In order to verify the molecular

mass of the postulated metabolites, PICI mass spectra were recorded, because they contain strong molecular peaks ($M + H^+$), in contrast to the EI spectra. In addition, adduct ions ($M + C_2H_5^+$, $M + C_3H_5^+$) are produced which are typical for PICI using methane as reagent gas.

EI and PICI mass spectra, the gas chromatographic retention indices (RI), structures and predominant fragmentation patterns of MPBP (mass spectra no. 1) as well as of its methylated and trimethylsilylated metabolites are shown in Fig. 2. From these mass spectra, the following metabolites (nos. in Fig. 2) could be deduced: 4′-hydroxymethyl-PBP (no. 8), 2″-oxo-MPBP (no. 3), 4′-carboxy-PBP (nos. 4 and 9), 4′-carboxy-2″-oxo-PBP (nos. 5 and 10), 2-oxo-4′-carboxy-BP (no. 2), 1-dihydro-4′-carboxy-PBP (no. 7), 1-dihydro-4′-carboxy-2″-oxo-PBP (nos. 6 and 11). Acetylation did not lead to detection of further metabolites, so degradation of the pyrrolidino-moiety of MPBP to secondary or primary amine structures obviously did not occur. With an estimated share of 40% of the excreted MPBP metabolites, 4′-carboxy-PBP was the metabolite in greatest abundance.

With the exception of 2″-oxo-MPBP, which carries no derivatizable moiety, one would have expected detection of all metabolites in the methylated(/acetylated) and in the trimethylsilylated extracts. However, 4′-hydroxymethyl-PBP and 1-dihydro-4′-carboxy-PBP were only detected in the trimethylsilylated extracts. This was most likely attributable to insufficient sensitivity after methylation and combined methylation/acetylation, because these two were only minor metabolites. In contrast, 2″-oxo-4′-carboxy-BP was only detected in methylated(/acetylated) extracts, which may indicate degradation of this compound during the trimethylsilylation procedure. The parent compound MPBP was not detected in any of the prepared samples, although the limit of detection (*S*/*N* 3) was 100 ng/ml. In addition, the extraction efficiency for MPBP was 77 ± 12% ($n = 5$) measured at 1000 ng/ml.

Concerning the structures of the metabolites, it must be mentioned that the exact position of the oxo group in the pyrrolidino-oxo metabolites of MPBP could not be deduced from the fragmentation patterns. However, the 2″-position seems to be the most likely as lactam formation is common in the metabolism of pyrrolidino compounds such as prolintane and nicotine [37]. Based on the same assumption, the corresponding lactam metabolites had also been postulated for other pyrrolidinophenones [2–6,22]. Only one peak was detected for the diastereomeric dihydro metabolites. On the one hand, this might be explained by enantioselective formation of only one diastereomer. On the other hand, more than one diastereomers might have been formed which were, however, not separated under the applied chromatographic conditions.

Based on the identified metabolites of MPBP, the following partly overlapping metabolic pathways could be postulated (Fig. 3): hydroxylation of the 4′-methyl group to the corresponding alcohol (no. 8) followed by oxidation to the corresponding carboxylic acid (no. 4/9); hydroxylation of the 2″-position of the pyrrolidine ring followed by dehydrogenation to the corresponding lactams (nos. 3, 5/10, 6/11); reduction of the keto group of 4′-carboxy metabolites to the corresponding secondary alcohols (nos. 7, 6/11); and oxidative deamination of 4′-carboxy-PBP to the corresponding 2-oxo compound (no. 2). As the peaks of the metabolites 2, 8 and 6/11 were more abundant after glucuronidase and sulfatase hydrolysis, it can be concluded that they are partly excreted as glucuronides and/or sulfates.

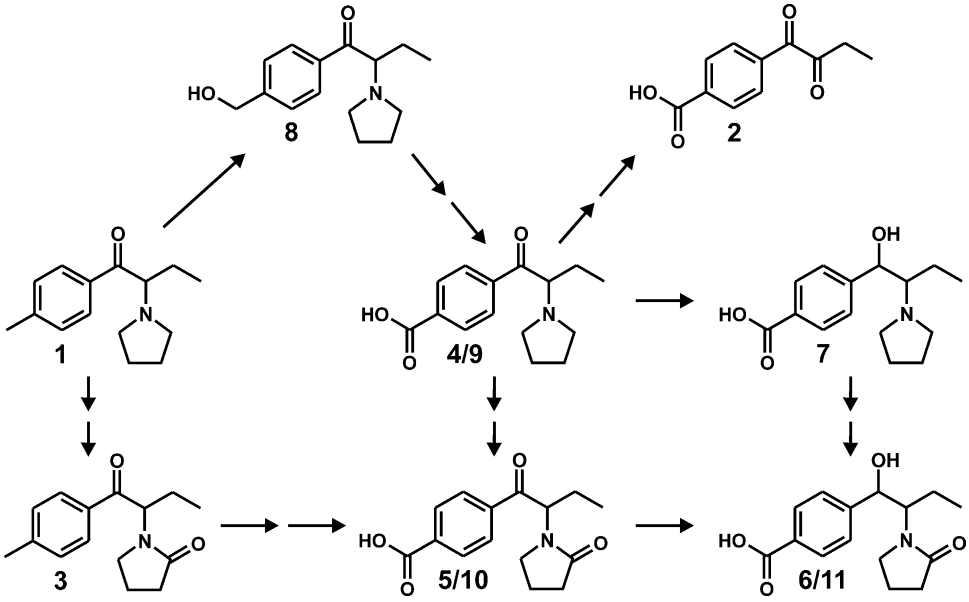

Fig. 3. Proposed scheme for the metabolism of MPBP in rats. The numbering of the compounds corresponds to that of the mass spectra of the corresponding derivative in Fig. 2.

3.3. Toxicological detection by GC–MS

MPBP metabolites were separated by GC and identified by full-scan EI MS after fast enzymatic hydrolysis, SPE and trimethylsilylation. Only fraction 2, where – among others – the MPBP main metabolite 4′-carboxy-PBP was eluted, was needed for the toxicological detection. Mass chromatography with the following ions was used to detect the presence of MPBP and/or its metabolites: *m*/*z* 112, 126, 178, 318. The selected ion *m*/*z* 112 was used for monitoring the presence of compounds with unchanged pyrrolidine ring (mass spectra nos. 7–9 in Fig. 2), *m*/*z* 126 for compounds with oxidized pyrrolidine ring (mass spectrum nos. 3, 10, and 11 in Fig. 2) and *m*/*z* 178, 318 for trimethylsilylated carboxy-metabolites (mass spectra nos. 7, 9–11 in Fig. 2).

Fig. 4 shows reconstructed mass chromatograms indicating the presence of MPBP metabolites in a trimethylsilylated extract of rat urine after administration of 1 mg/kg body mass of MPBP·HNO_3. For MPBP's structural homologue pyrovalerone, single doses of 20–75 mg of pyrovalerone were used in studies on pharmacological and therapeutic effects in humans [10,12,13,38], while intravenous drug abusers reportedly injected single doses of 60–140 mg of pyrovalerone [15]. Assuming a similar dosage for MPBP the above-mentioned 1 mg/kg body mass dose of MPBP should, therefore, approximately correspond to a dose ingested by abusers. This assumption is further supported by the fact that seized tablets of the related designer drug PPP contained approximately 40 mg.

The identity of the peak in the mass chromatograms was confirmed by computerized comparison of the underlying mass spectrum with reference spectra recorded during this study [27]. Fig. 5 illustrates the mass spectrum underlying the marked peak in Fig. 4, reference spectrum (no. 9 in Fig. 2), structure, and the hit list found by computer library search. The gas chromatographic RIs provide preliminary indications, allow distinguishing between positional isomers and/or diastereomers and may be useful to gas chromatographers without an MS facility. Therefore, RIs are also provided in Fig. 2. They were recorded during the GC–MS procedure (Section 2.5) and calculated in correlation with the Kovats' indices [39] of the components of a standard solution of typical drugs which is measured daily for testing the GC–MS performance [40,41]. The reproducibility of RIs measured on capillary columns was better using a mixture of drugs than that of the homologous hydrocarbons recommended by Kovats.

Unfortunately, no authentic human urine samples after intake of MPBP were available. However, based on the findings of Michaelis et al. [33], that the corresponding metabolite of pyrovalerone was the main metabolite of this drug in humans, one would expect 4′-carboxy-PBP to be an important metabolite of MPBP also in humans. Thus, the previously published screening procedure for

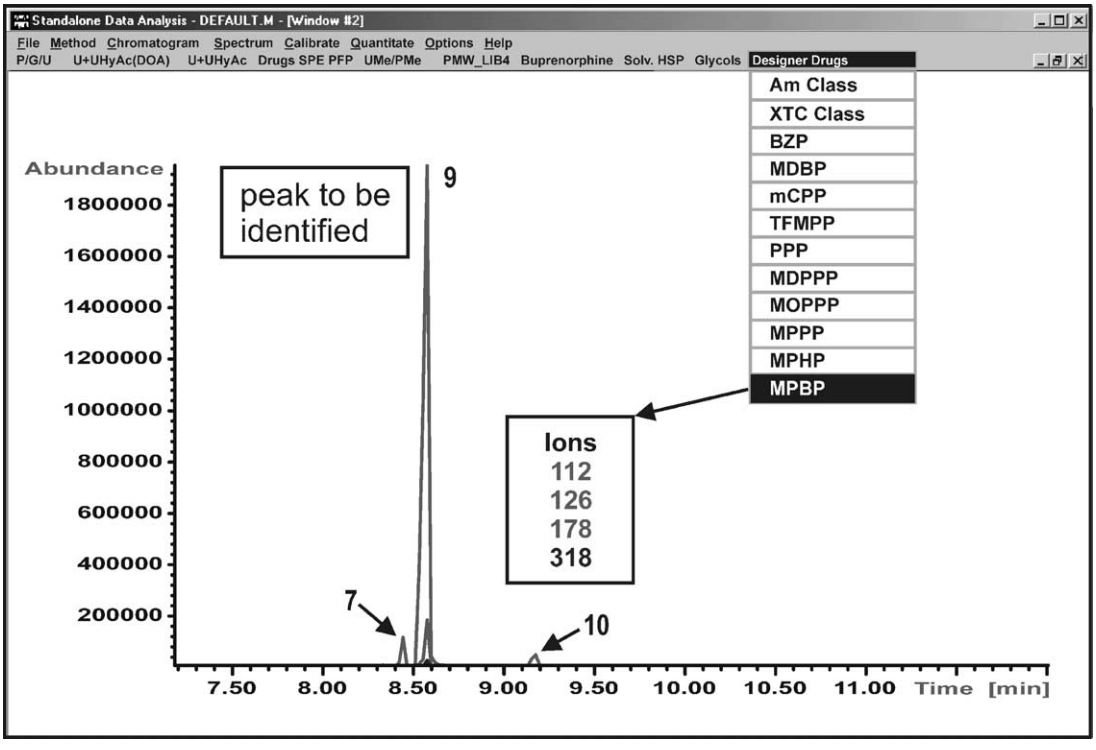

Fig. 4. Typical mass chromatograms with the ions *m*/*z* 112, 126, 178 and 318. They indicate the presence of MPBP metabolites in a trimethylsilylated extract of a rat urine sample collected over 24 h after ingestion of 1 mg/kg body mass of MPBP·HNO_3. The numbering of the peaks corresponds to that of the mass spectra of the corresponding derivative in Fig. 2. The merged chromatograms can be differentiated by their colors on a color screen.

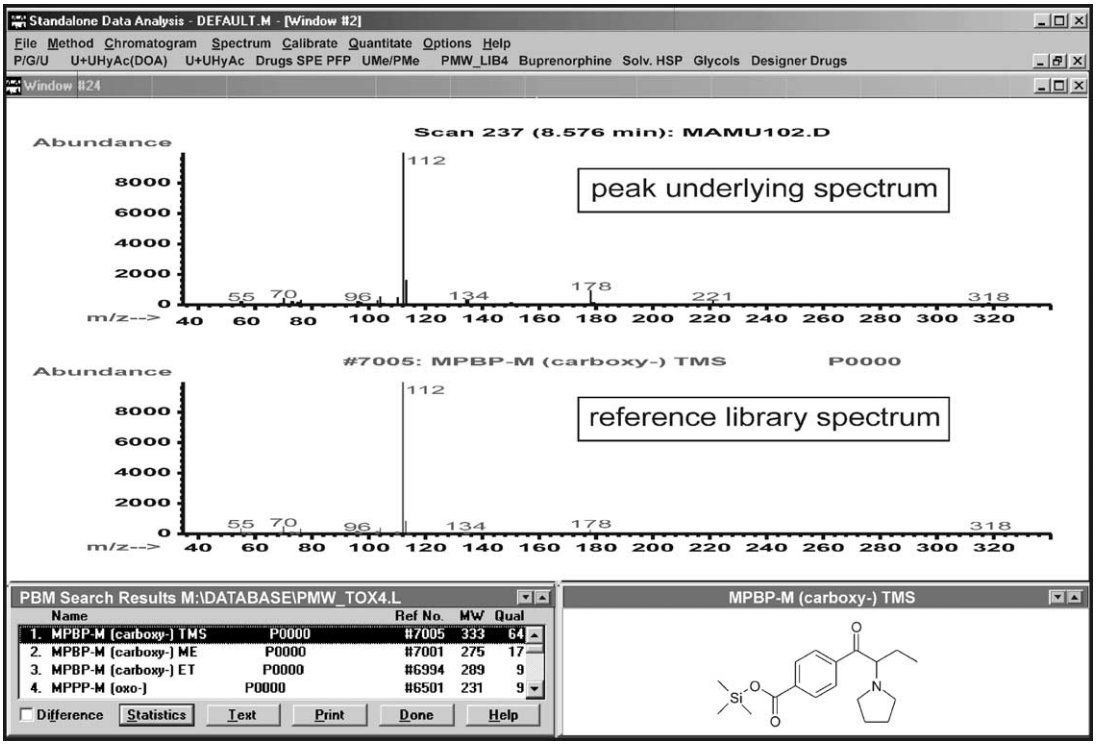

Fig. 5. Mass spectrum underlying the marked peak in Fig. 4 the reference spectrum, the structure, and the hit list found by computer library search.

pyrrolidinophenones [2,4–6] should also be applicable for detecting an ingestion of MPBP.

4. Conclusions

The presented studies revealed that the new designer drug MPBP was extensively metabolized via four pathways by the rat. Screening must, therefore, be focused on metabolites. The described screening procedure should be suitable for detection of MPBP and/or its metabolites in human urine in clinical or forensic cases.

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