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Metabolism of the new designer drug α-pyrrolidinopropiophenone (PPP) and the toxicological detection of PPP and 4′-methyl-α-pyrrolidinopropiophenone (MPPP) studied in rat urine using gas chromatography-mass spectrometry

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Abstract

R,S-α-pyrrolidinopropiophenone (PPP) is a new designer drug with assumed amphetamine-like effects which has appeared on the illicit drug market. The aim of this study was to identify the PPP metabolites using solid-phase extraction, ethylation or acetylation as well as to develop a toxicological detection procedure in urine using solid-phase extraction, trimethylsilylation and gas chromatography–mass spectrometry (GC–MS). Analysis of urine samples of rats treated with PPP revealed that PPP was extensively metabolized by hydroxylation of the pyrrolidine ring with subsequent dehydrogenation to the corresponding lactam, hydroxylation of the aromatic ring in position 4′ or double dealkylation of the pyrrolidine ring to the corresponding primary amine (cathinone) partly followed by reduction of the keto group to the corresponding secondary alcohol (norephedrines). As cathinone and the norephedrine diastereomers are also formed after intake of other drugs of abuse or medicaments, special attention must be paid to the detection of the unequivocal metabolite 2″-oxo-PPP as an unambiguous proof for the intake of PPP. The hydroxy groups were found to be partly conjugated. Based on these data, PPP could be detected in urine via its metabolites by full-scan GC–MS using mass chromatography for screening and library search for identification by comparison of the spectra with reference spectra. The same toxicological detection procedure can be applied to other designer drugs of the pyrrolidinophenone type, like MOPPP, MDPPP, MPHP, and MPPP. The detection of the latter will also be presented here.

Keywords: α-Pyrrolidinopropiophenone; 4′-Methyl-α-pyrrolidinopropiophenone

1. Introduction

α-Pyrrolidinophenone derivatives like *R,S*-α-pyrrolidinopropiophenone (PPP), *R,S*-4′-methyl-α-pyrrolidinopropiophenone (MPPP), *R,S*-4′-methyl-α-pyrrolidinohexanophenone (MPHP), *R,S*-4′-methoxy-α-pyrrol-

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idinopropiophenone (MOPPP) and *RS*-3′,4′-methylenedioxy-α-pyrrolidinopropiophenone (MDPPP) are new designer drugs which have appeared on the illicit drug market [1–7] and are available illicitly in tablet form. As the pyrrolidinophenones cannot be detected with usual routine analysis procedures, statements on the frequency of their occurrence cannot be made. Meanwhile, most of these substances including PPP are scheduled in the German Controlled Substances Act and possession is strictly prohibited. So far, little information about its pharmacological and toxicological properties is available. The chemical structure of PPP is closely related to anorectics like amfepramone and metamfepramone, drugs of abuse like cathinone and methcathinone, or antidepressants like bupropion. Having dopaminergic and noradrenergic properties, all these substances show amphetamine-like effects [8–11]. Therefore, it can be assumed that PPP shows similar effects, particularly since it is misused as a substitute for amphetamines. The metabolism of PPP has not yet been studied. 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. So far, determination of or screening procedures for PPP and/or its metabolites have not been published, although such procedures are necessary for confirmation of the diagnosis of an intoxication or drug abuse.

The aim of the presented study was firstly to identify the PPP metabolites in rat urine using gas chromatography–mass spectrometry (GC–MS) in the electron impact (EI) and positive-ion chemical ionization (PICI) mode and secondly to develop a toxicological screening procedure based on the identified metabolites using EI GC–MS. Metabolism and toxicological screening procedures after solid phase extraction, trimethylsilylation and full-scan EI GC–MS have already been described for MOPPP [6], MDPPP [7] and MPHP [5]. MPPP metabolism has also been described in a previous publication, but the proof of its intake was built upon ethylation as derivatization of the formed main metabolite, 4′-carboxy-PPP [4]. If sensitive detection of the main metabolites of PPP and MPPP could also be achieved after trimethylsilylation, a comprehensive screening for all pyrrolidinophenone-type designer drugs would be possible with a single toxicological detection procedure.

2. Experimental

2.1. Chemicals and reagents

All chemicals used were obtained from E. Merck, Darmstadt (Germany) and were of analytical grade. *R,S*-PPP-HCl and *R,S*-MPPP-HCl were provided from Hessisches Landeskriminalamt, Wiesbaden (Germany) for research purposes before they became controlled substances.

2.2. Urine samples

The investigations were performed using male rats (Wistar, Ch. River, Sulzfleck, Germany) which were administered a single 50 mg/kg body mass dose (for metabolism studies) or a 1 mg/kg body mass dose (for development of the screening procedure) of PPP 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. A urine sample from previous experiments of male rats, which were administered a 1 mg/kg body mass dose of MPPP, was thawed from −20 °C. 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 of PPP

A 0.5 ml portion of urine was adjusted to pH 5.2 with acetic acid (1 M) and incubated at 37 °C for 12 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). The urine sample was then diluted with 2.5 ml of water and loaded on an Isolute Confirm HCX cartridge (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 (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 ethylation or acetylation according to published procedures ([4] or [12]). Briefly, ethylation was performed after reconstitution in 50 μl of methanol, 50 μl of a solution of diazoethane in diethyl ether, synthesized according to the procedure of McKay et al. [13], the reaction vials were sealed and left at room temperature for 8 h. 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 [12,14,15]. After evaporation, the residue was dissolved in 100 μl of methanol. A 3 μl aliquot each was injected into the GC–MS. The same procedure was repeated without the use of enzymatic hydrolysis to study which metabolites of PPP are excreted as glucuronides and/or sulfates.

2.4. Sample preparation for toxicological analysis of PPP and MPPP

A 0.5 ml portion of the respective urine sample (PPP or MPPP) was adjusted to pH 5.2 with acetic acid (1 M) and incubated at 56 °C for 1 h with 50 μl of a mixture of glucuronidase and arylsulfatase (same as used in 2.3). This sample was diluted with 2.5 ml of water and loaded on an Isolute Confirm HCX cartridge (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, 1 ml of 0.01 M hydrochloric acid and 1 ml of methanol. The retained compounds were then eluted into a 1.5 ml reaction vial with 1 ml of a freshly prepared mixture of methanol/aqueous ammonia (98:2 (v/v)). The eluate was gently evaporated to dryness under a stream of nitrogen at 56 °C and then reconstituted in 50 μl of ethyl acetate and trimethylsilylated after addition of 50 μl of *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) 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 PPP and MPPP 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 100–310 °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; 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 PPP and its trimethylsilylated metabolites, mass chromatography with the selected ions *m/z* 98, 105, 112 and 193 was used. For toxicological detection of MPPP and its trimethylsilylated metabolites, mass chromatography with the selected ions *m/z* 98, 112, 119 and 221 was used. Generation of the mass chromatograms could be started by clicking the corresponding pull down menu which executes the user defined macros [16]. The identity of the peaks in the mass chromatograms was confirmed by computerized comparison [17] of the mass spectra underlying the peaks (after background subtraction) with reference spectra (Fig. 1, mass spectra nos. 1, 2, 6 and 7 for PPP; mass spectra nos. 8–12 for MPPP) recorded during this study.

3. Results and discussion

3.1. Sample preparation

Cleavage of conjugates by gentle enzymatic hydrolysis was necessary before extraction and GC–MS analysis of the suspected metabolites in order not to overlook conjugated metabolites. As usual for routine screening procedures, incubation was performed at a higher temperature and for a shorter time in contrast to the metabolism study, in which an almost complete cleavage can be achieved and temperature stress can

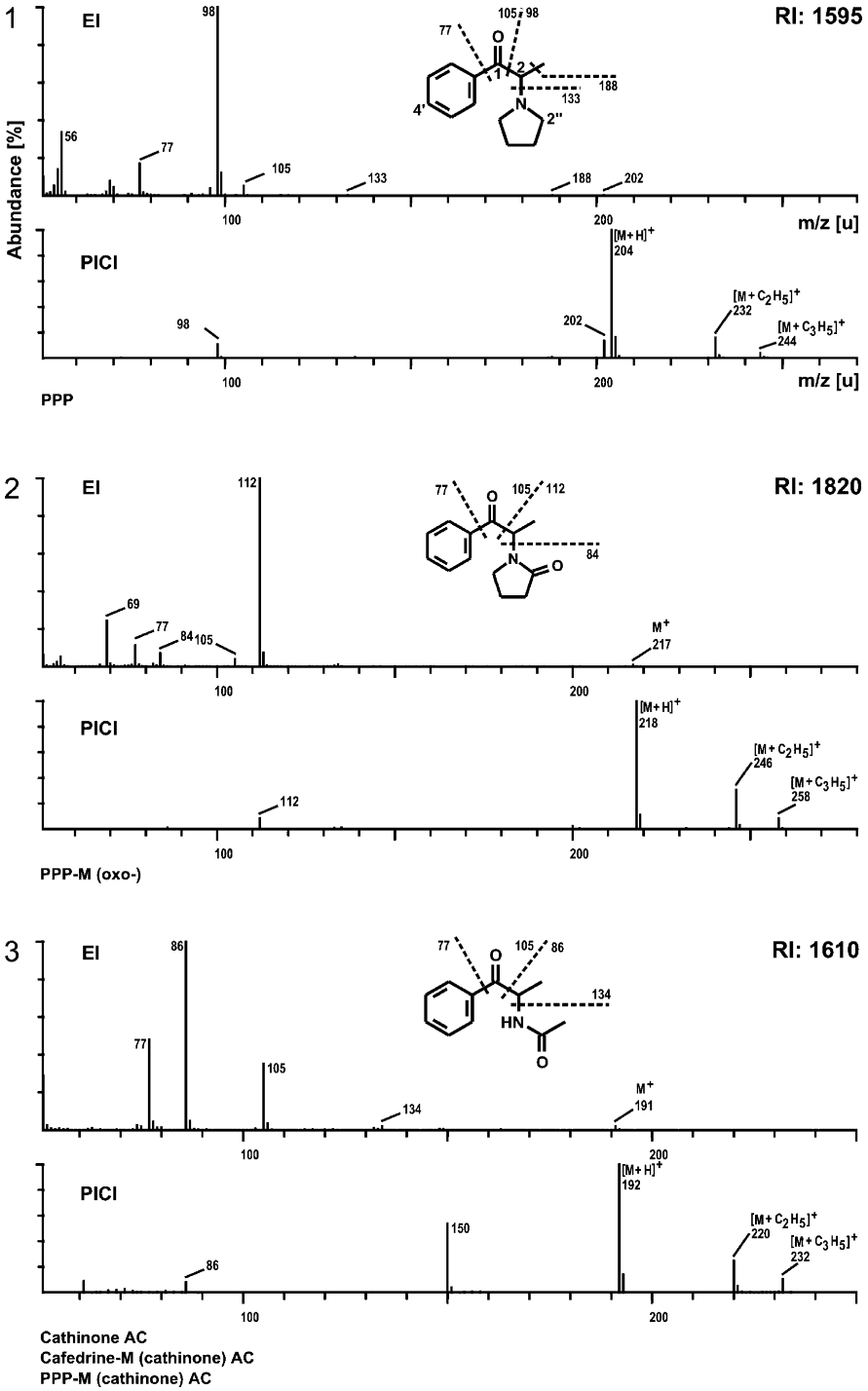
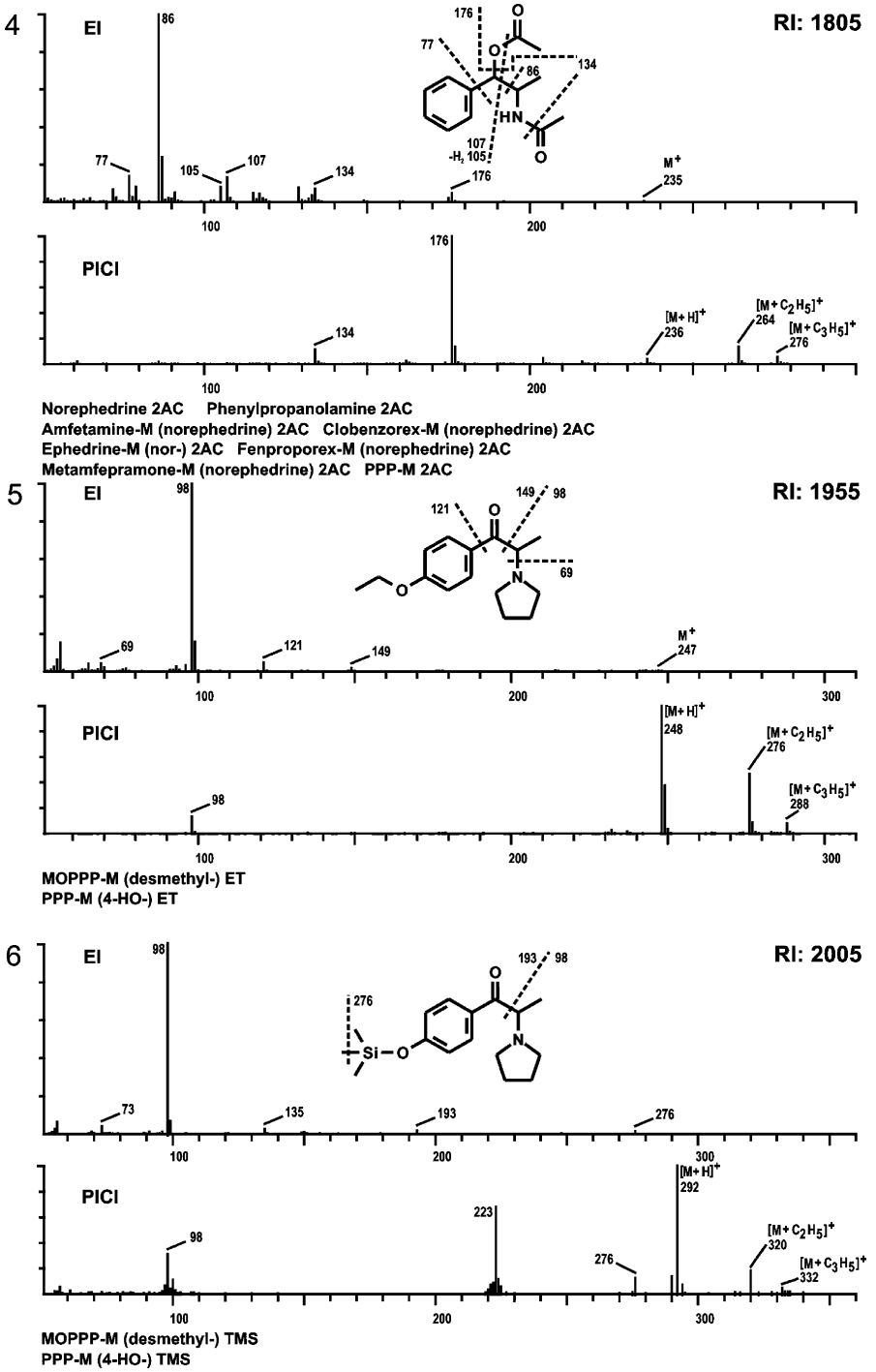


Fig. 1. EI and PICI mass spectra, the gas chromatographic retention indices (RI), structures and predominant fragmentation patterns of PPP and its metabolites after ethylation, acetylation or trimethylsilylation. The axes are only labeled for 1.



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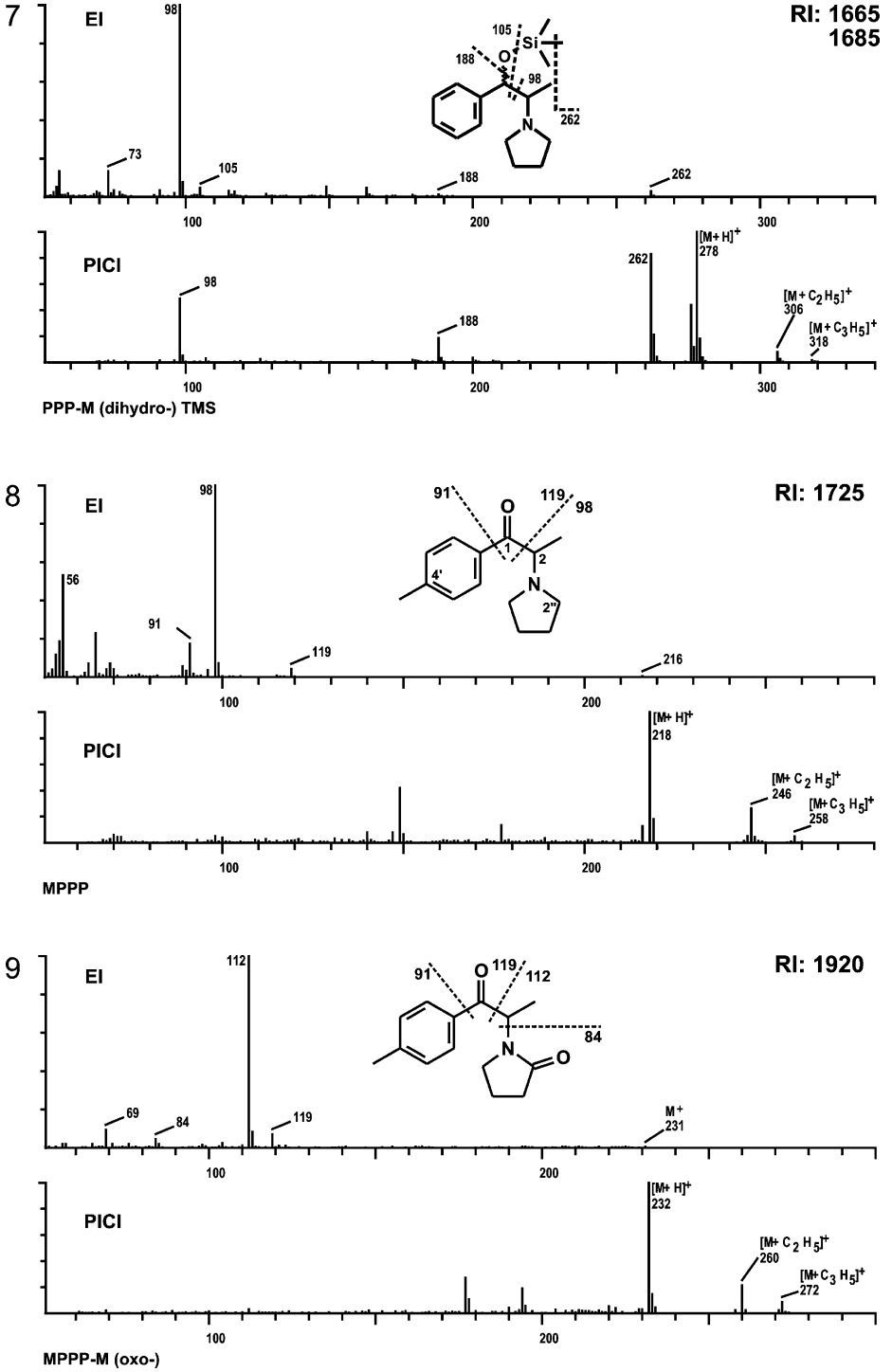

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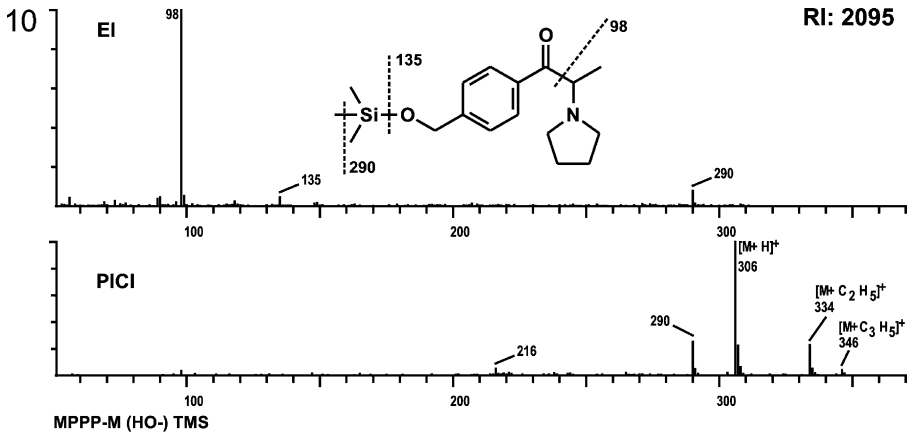

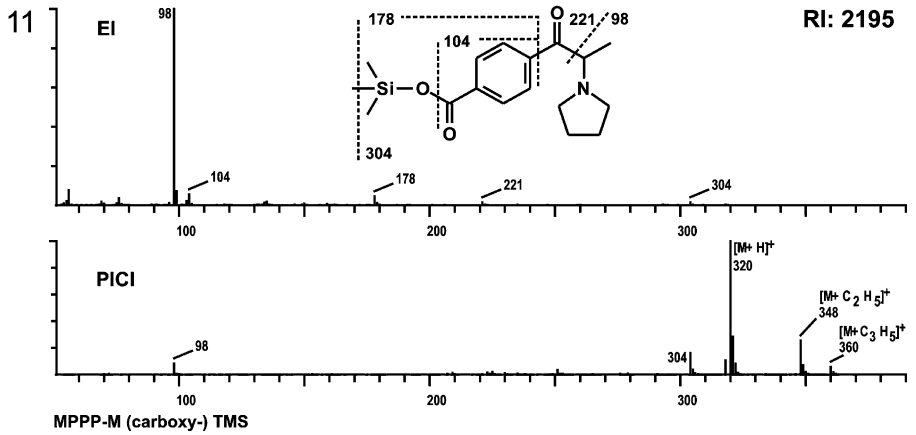

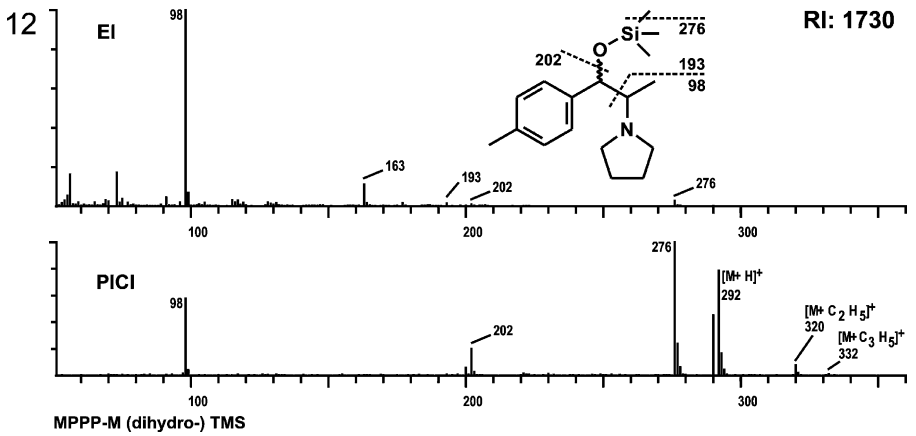

Fig. 1. (*Continued*).

be avoided. The use of common liquid–liquid extraction under alkaline conditions followed by acetylation [12,18–21] was not appropriate, because some of the metabolites showed zwitterionic properties. Even PPP and MPPP themselves were only poorly extractable under the liquid–liquid extraction procedure applied (pH 8–9, ethyl acetate/isopropanol/dichloromethane (3:1:1 (v/v/v)). In addition, volatility of the free bases and the instability of the analytes under alkaline and high temperature conditions caused difficulties [22,23]. In contrast, solid-phase extraction (SPE) demonstrated acceptable results due to mixed-mode SPE's ability to extract zwitterionic compounds [5].

Derivatization was needed for sensitive detection of metabolites after administration of lower drug doses. For metabolism studies, acetylation was preferred for derivatization of metabolites with primary and secondary amino groups as well as of alcoholic and/or phenolic hydroxy groups [18,24,25]. Ethylation was preferred for derivatization of metabolites with phenolic hydroxy and/or vinylogous carboxy groups [5]. In addition, ethylation by diazoethane has the further advantage that phenolic hydroxy groups can be derivatized without affecting alcoholic groups, thereby allowing a distinction between both types of hydroxy groups. Moreover, ethylation is favored over diazomethane methylation, allowing a distinction between metabolic methylation and derivatization. These procedures for sample preparation, extraction and derivatization have already proven to be appropriate for metabolism studies of other pyrrolidinophenone-type designer drugs [4–7].

However, for the toxicological detection procedure for PPP or MPPP, common trimethylsilylation was preferred. In routine work, trimethylsilylation is safer and easier to handle and the reagent is commercially available. All expected target analytes in urine after intake of PPP or MPPP showed good GC properties after trimethylsilylation.

3.2. Identification of metabolites of PPP

The urinary metabolites of PPP 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 [26] or Smith and Busch [27]. 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 are produced 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 PPP and its acetylated, ethylated or trimethylsilylated metabolites are shown in Fig. 1. Hydroxylation of PPP in position 4′ leads to the formation of a vinylogous carboxylic acid. Consequently, this metabolite could not be acetylated. Reduction of the keto group introduces a second chiral center into the molecule and diastereomers are created (mass spectra nos. 4 and 7). The two diastereomeric compounds with mass spectrum no. 7 could be separated under the applied GC conditions Only one mass spectrum of these diasteriomeric compounds is depicted in Fig. 1. Their spectra are very similar, so that one can be used for identification of both peaks considering the given two different RIs. The actual configurations of the compounds behind the two peaks, however, were not determined. The two diastereomeric compounds with mass spectrum no. 4 were not separated under the applied GC conditions, but their mass spectra should also look alike.

The following metabolites of PPP (mass spectrum no. 1 in Fig. 1) could be identified after high dose application: 2″-oxo-PPP (mass spectrum no. 2), cathinone (mass spectrum no. 3), nor-(pseudo-)ephedrine (mass spectrum no. 4), and 4′-hydroxy-PPP (mass spectrum no. 5). The 2″-oxo-PPP was the metabolite in greatest abundance. It was present in both fractions 1 and 2. All other metabolites were exclusively detected in fraction 2. 4′-Hydroxy-PPP accounted for about 10% of the metabolites formed.

Many studies by the authors have demonstrated a high degree of qualitative correspondence of rat and human metabolism [18,19,24,25,28–30]. However, substances like methcathinone [22], amfepramone [31] or metamfepramone [32] which are structurally related to PPP, were shown to be additionally excreted as dihydro metabolites (diasteromers) in humans to a considerable extent. Therefore, the data of dihydro-PPP (synthesized from PPP by sodium borohydride reduction according to [33]) were included in Fig. 1 (mass spectrum no. 7). The GC and MS data

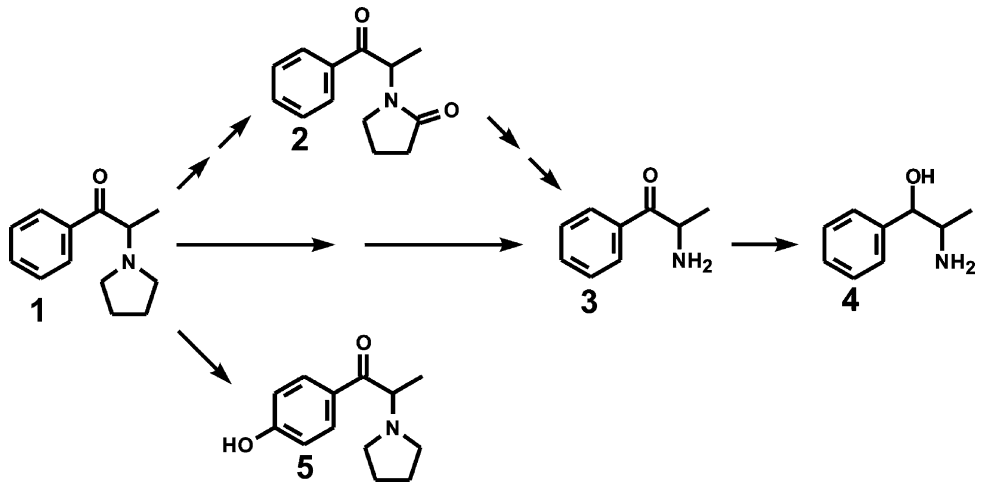

Fig. 2. Proposed scheme for the metabolism of PPP in rats. The numbering of the compounds corresponds to that of the mass spectra of the corresponding derivative in Fig. 1.

of those compounds and derivatives, which are not shown in Fig. 1, will be included in the forthcoming update of the authors' handbook and library [17,34].

The mass spectrum no. 2 did not identify the position of the carbonyl group in the pyrrolidine ring. However, as other compounds carrying a pyrrolidine ring are also excreted as their lactam metabolite (e.g. MPPP or nicotine), we postulate the same metabolic pathway for PPP. The mass spectrum no. 5 did not identify the position of the hydroxy group. However, the main metabolite of MOPPP, 4′-hydroxy-PPP [6], is eluted after the same time as this hydroxylated PPP. Ethylated 2′- or 3′-hydrdoxy-PPP would probably yield almost the same mass spectrum as ethylated 4′-hydroxy-PPP. Yet it is likely, that this compound would have a different RI. A second metabolite was not detected, so that 4′-hydroxy-PPP was the only positional isomer to be identified. However, NMR data are required for an unambiguous statement on the position of the hydroxy group. The limit of detection (S/N, 3) for the parent compound PPP was 100 ng/ml and the extraction efficiency was 90 ± 6% (mean ± S.D., $n = 5$) measured at 1000 ng/ml.

Based on the identified metabolites of PPP, the following metabolic pathways could be postulated (Fig. 2): hydroxylation of the 2″-position of the pyrrolidine ring followed by dehydrogenation to the corresponding lactam (no. 2), double dealkylation of the pyrrolidine ring to cathinone (no. 3) with partial subsequent reduction of the keto group to the corresponding nor-(pseudo-)ephedrine (no. 4) or 4′-hydroxylation of the phenyl ring (no. 5). In contrast to the metabolism of MPPP [4], MOPPP [6] and MDPPP [7], oxidative desamination to the corresponding 2-oxo metabolites did not result in excretion of detectable amounts of metabolite. Furthermore, hydroxylation of the side chain did not seem to take place, which was the case in MPHP metabolism [5]. The pathway(s) for the formation of cathinone from PPP could not be elucidated. Two possibilities are conceivable: firstly, hydroxylation in position 2″, ring opening, hydroxylation in position 5″, dealkylation. Secondly, lactam formation as described, hydrolysis of the lactam, hydroxylation in position 5″, dealkylation (cf. arrows in Fig. 2). As the peaks of the metabolites 4 and 5 were more abundant after enzymatic hydrolysis, it can be concluded that they are partly excreted as glucuronides and/or sulfates.

3.3. Toxicological detection of PPP and MPPP by GC–MS

PPP, MPPP and/or their 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 PPP main metabolite 2″-oxo-PPP and the MPPP main metabolite, 4′-carboxy-PPP, were eluted, was

needed for the toxicological detection. Although 2″-oxo-PPP cannot be derivatized, the urine extract was trimethylsilylated, so that a uniform detection procedure for all pyrrolidinophenones could be maintained. Derivatization did not decrease the sensitivity, which was found out by comparison of trimethylsilylated with underivatized urine extract GC–MS runs. Also for reasons of standard nature, the toxicological detection procedure for MPPP was changed from ethylation, as published earlier [4], to trimethylsilylation. Storage of the MPPP urine sample for 18 months at −20 °C did not seem to cause stability problems, as the GC–MS run recorded 18 months ago and the one recorded from the same urine sample after fresh working up according to the original procedure did not look different. Mass chromatography with the following ions was used to detect the presence of PPP and/or its metabolites: *m/z* 98, 105, 112 and 193. Mass chromatography with the following ions was used to detect the presence of MPPP and/or its metabolites: *m/z* 98, 112, 119 and 221.

For the detection of PPP and/or its metabolites, the selected ion *m/z* 98 was used for monitoring the presence of compounds with unchanged pyrrolidine ring (mass spectra nos. 1, 6 and 7 in Fig. 1), *m/z* 105 for compounds with unchanged or keto-reduced phenone group (mass spectra nos. 1, 2 and 7 in Fig. 1), *m/z* 112 for compounds with oxidized pyrrolidine ring (mass spectrum no. 2 in Fig. 1) and *m/z* 193 for trimethylsilylated hydroxy-metabolites (mass spectrum no. 6 in Fig. 1).

For the detection of MPPP and/or its metabolites, the selected ion *m/z* 98 was used for monitoring the presence of compounds with unchanged pyrrolidine

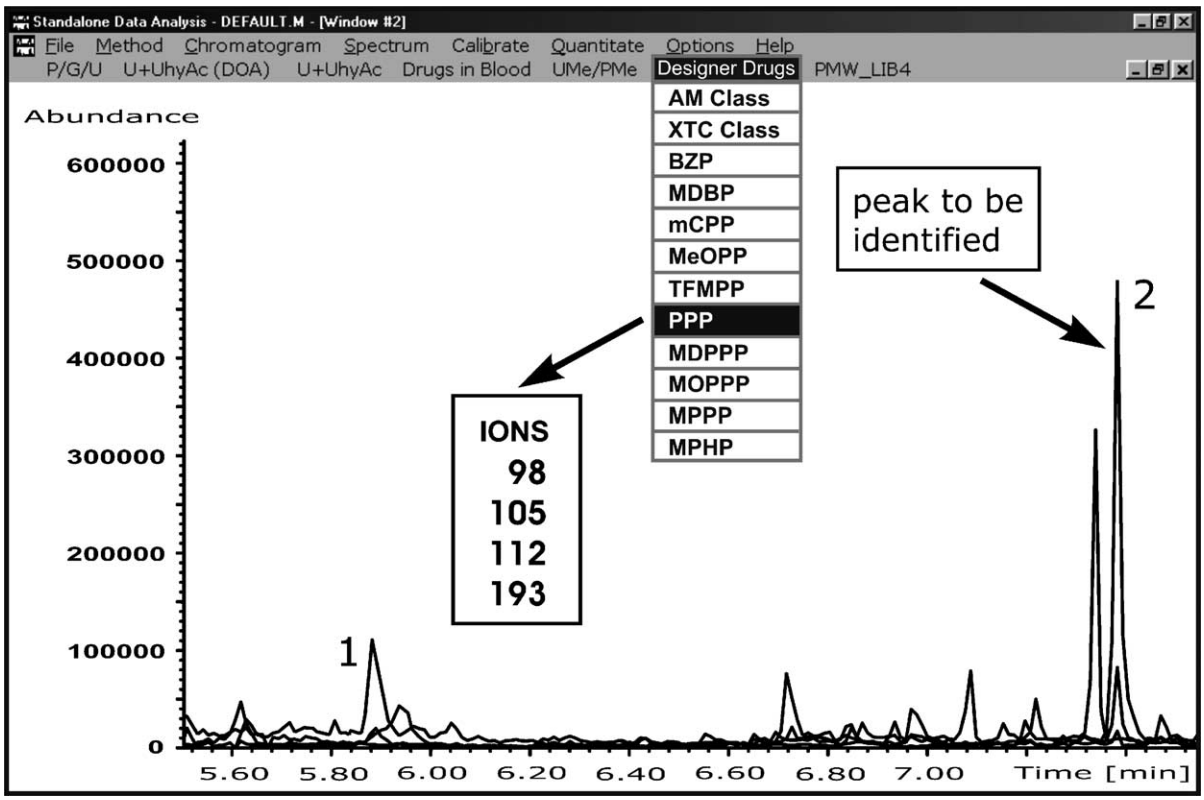

Fig. 3. Typical mass chromatograms with the ions *m/z* 98, 105, 112 and 193. They indicate the presence of PPP and its metabolites in a trimethylsilylated extract of a rat urine sample collected over 24 h after ingestion of 1 mg/kg body mass of PPP. The numbering of the peaks corresponds to that of the mass spectra of the corresponding derivative in Fig. 1. The merged chromatograms can be differentiated by their colors on a color screen.

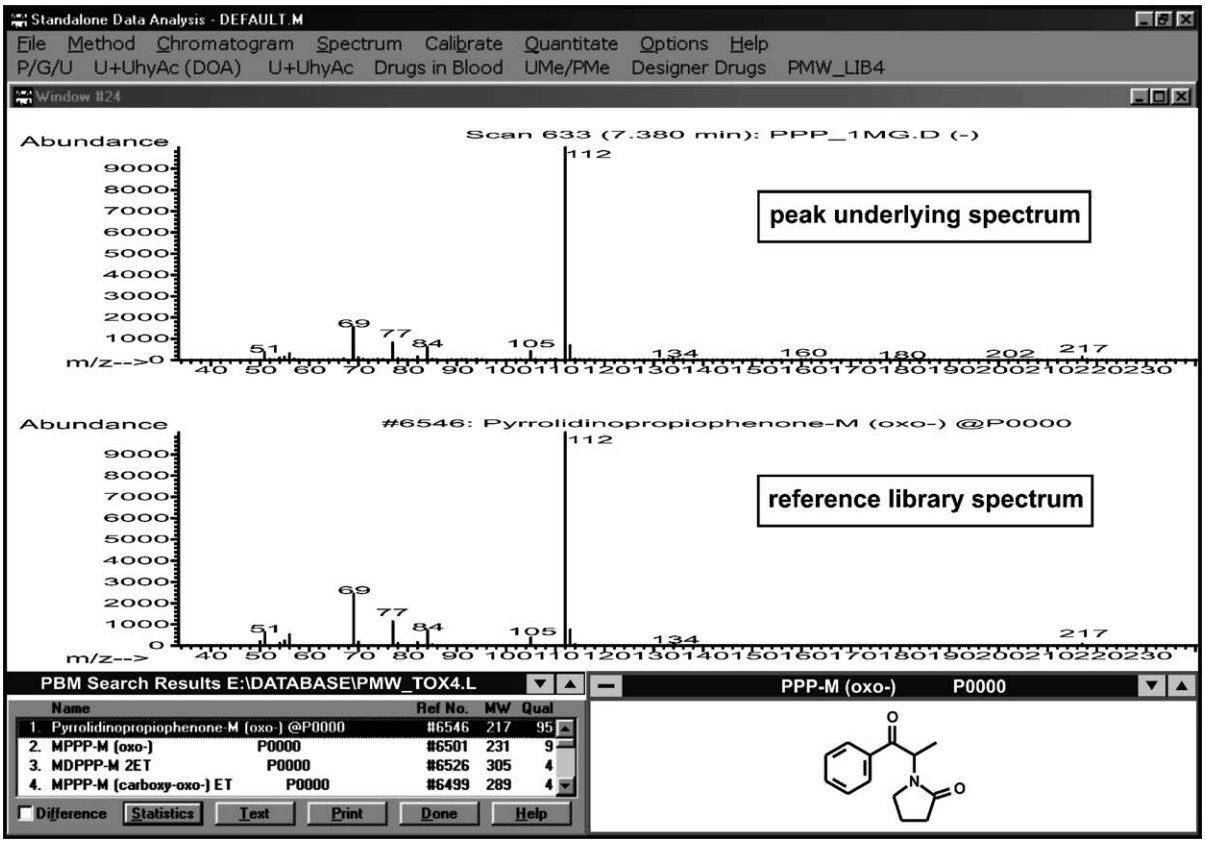

Fig. 4. Mass spectrum underlying the marked peak in Fig. 3, the reference spectrum, the structure, and the hit list found by computer library search.

ring (mass spectra nos. 8 and 10–12 in Fig. 1), *m/z* 112 for compounds with oxidized pyrrolidine ring (mass spectrum no. 9 in Fig. 1), *m/z* 119 for compounds with unchanged or keto-reduced 4′-methyl-phenone group (mass spectra nos. 8, 9 and 12 in Fig. 1) and *m/z* 221 for the trimethylsilylated carboxy-metabolite (mass spectrum no. 11 in Fig. 1).

Fig. 3 shows reconstructed mass chromatograms indicating the presence of PPP and its metabolites in a trimethylsilylated extract of rat urine after administration of 1 mg/kg body mass of PPP. This dose was chosen as it should approximately correspond to a dose ingested by abusers. Seized tablets of PPP always have been shown to contain approximately 40 mg. In addition, the chosen dose is in the same range as that of the structurally related medicament amfepramone. 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 [17]. Fig. 4 illustrates the mass spectrum underlying the marked peak in Fig. 3, reference spectrum (no. 2 in Fig. 1), structure, and the drug list found by computer library search.

Fig. 5 shows reconstructed mass chromatograms indicating the presence of MPPP metabolites in a trimethylsilylated extract of rat urine after administration of 1 mg/kg body mass of MPPP. Again, this dose was chosen as it should approximately correspond to a dose ingested by abusers. Fig. 6 illustrates the mass spectrum underlying the marked peak in Fig. 5, reference spectrum (no. 11 in Fig. 1), structure, and the drug list found by computer library search.

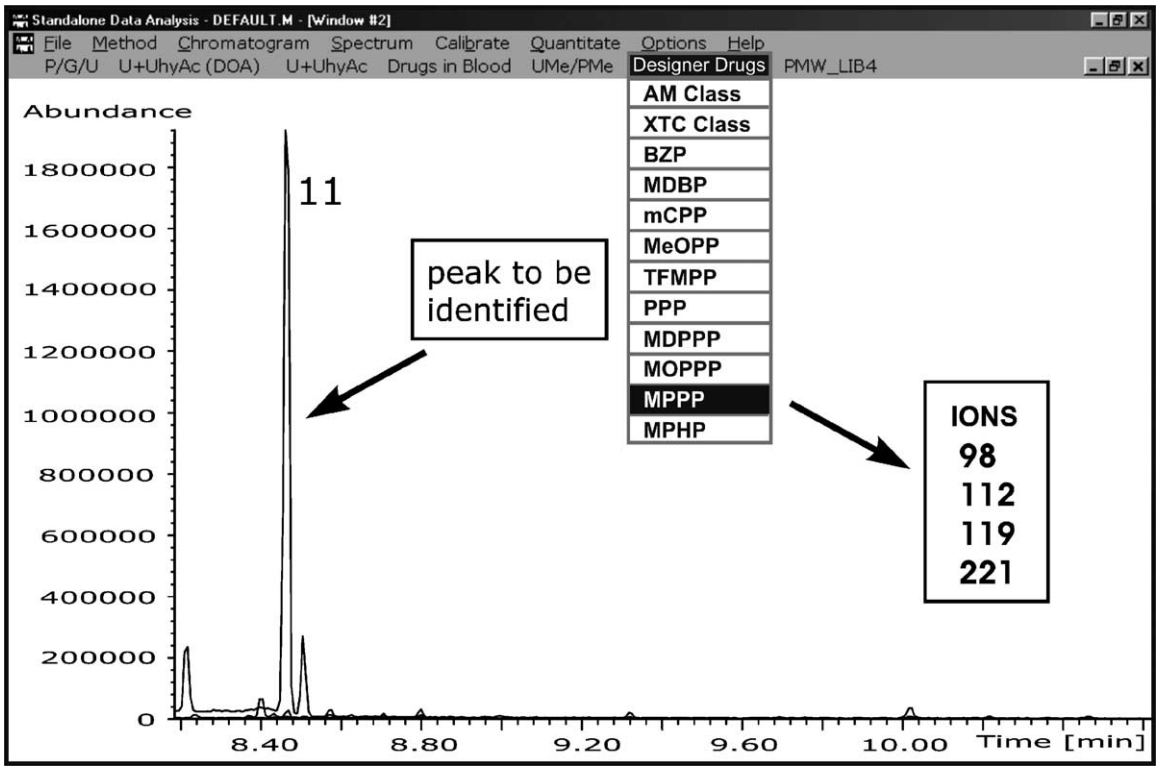

Fig. 5. Typical mass chromatograms with the ions *m/z* 98, 112, 119 and 221. They indicate the presence of MPPP and its metabolites in a trimethylsilylated extract of a rat urine sample collected over 24 h after ingestion of 1 mg/kg body mass of MPPP. The numbering of the peak corresponds to that of the corresponding mass spectrum in Fig. 1.

The gas chromatographic RIs provide preliminary indications, allow to distinguish between the above mentioned positional isomers and/or diastereomers and may be useful to gas chromatographers without an MS facility. Therefore, RIs are also provided in Fig. 1. They were recorded during the GC–MS procedure (Section 2.5) and calculated in correlation with the Kovats' indices [35] of the components of a standard solution of typical drugs which is measured daily for testing the GC–MS performance [36,37]. 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 PPP or MPPP were available. However, the same metabolites found in rat urine are also likely to be in human urine samples (cf., e.g. [18,19,25]). As already discussed above, the dihydro metabolite of PPP (mass spectrum no. 7) as well as of MPPP (also synthesized as mentioned above, mass spectrum no. 12) might additionally be detectable in human urine. However, as mass *m/z* 98 would also indicate the presence of dihydro-PPP and dihydro-MPPP, their mass spectra were included in Fig. 1. These metabolites are also likely to be detectable in human urine. The extraction efficiencies for trimethylsilylated dihydro-PPP were 80 ± 8% and 67 ± 6% for trimethylsilylated dihydro-MPPP ($n = 4$), respectively, measured at 1000 ng/ml. The limits of detection were 50 ng/ml (S/N, 3) for both. Extrapolating the rat data, the described screening procedures should be sensitive enough to detect an intake of an illicit dose of PPP or MPPP.

It should be mentioned, that 4′-hydroxy-PPP is a common metabolite of PPP and MOPPP [6]. Yet, differentiation of the intake of PPP in combination with

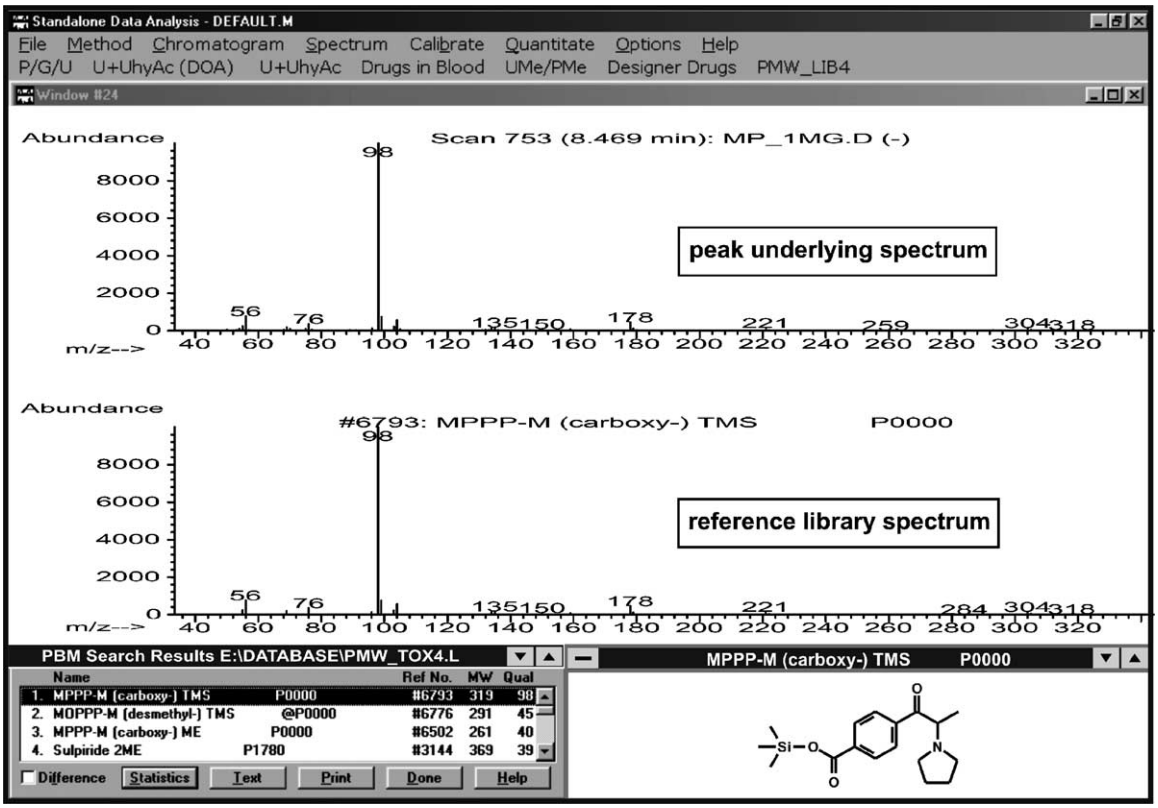

Fig. 6. Mass spectrum underlying the marked peak in Fig. 5, the reference spectrum, the structure, and the hit list found by computer library search.

MOPPP should be possible via the detection of the unique PPP main metabolite 2″-oxo-PPP or PPP itself. Differentiation of the intake of small doses of MOPPP in combination with PPP might become difficult, if 4′-hydroxy-PPP as the MOPPP main metabolite was present, as 4′-hydroxy-PPP is also detectable after the intake of PPP alone. Special attention must be paid to the detection of unique MOPPP metabolites in this case. If all members of the pyrrolidinophenone designer drug class were excreted as their respective dihydro metabolites to a considerable extent in humans as discussed above, incontestable statements on the ingested parent compound(s) can be made. As this study shows, the detection of cathinone, norpseudoephedrine and/or norephedrine (after different derivatization procedures) does not necessarily prove the intake of kath leaves and/or cathinone/nor-(pseudo-)ephedrine precursor drugs such as amfepramone, metamfepramone, methcathinone or (pseudo-)ephedrine. The abuse of PPP must also be considered. The presented screening procedure has already been proven to be suitable for other designer drugs of the α-pyrrolidinophenone type [5–7].

4. Conclusions

The presented studies revealed that the new designer drug PPP was extensively metabolized by the rat. Screening must, therefore, be focussed on metabolites. The described screening procedure should be suitable for detection of PPP, MPPP and/or their metabolites in human urine in clinical or forensic cases.

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