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Studies on the metabolism and the toxicological analysis of the nootropic drug fipexide in rat urine using gas chromatography–mass spectrometry

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Dedicated to Dr. Karel Macek, Prague, Czech Republic, at the occasion of his 75th birthday

Abstract

Qualitative studies are described on the metabolism and the toxicological analysis of the nootropic fipexide (FIP) in rat urine using gas chromatography–mass spectrometry (GC–MS). FIP was extensively metabolized to 1-(3,4-methylenedioxybenzyl)piperazine (MDBP), 4-chlorophenoxyacetic acid, 1-[2-(4-chlorophenoxy)acetyl]piperazine, *N*-(4-hydroxy-3-methoxy-benzyl)piperazine, piperazine, *N*-(3,4-methylenedioxybenzyl)ethylenediamine, and *N*-[2-(4-chlorophenoxy)acetyl]ethylenediamine. The authors' systematic toxicological analysis (STA) procedure using full-scan GC–MS after acid hydrolysis of one urine aliquot, liquid-liquid extraction and acetylation allowed the detection of FIP via its metabolites in rat urine after administration of a common FIP dose. Therefore, this qualitative procedure should also be suitable for detection of a FIP intake in human urine. Differentiation of an intake of FIP from that of other drugs which form common metabolites is discussed.

Keywords: Metabolism; Fipexide

1. Introduction

Fipexide (FIP, 1-[2-(4-chlorophenoxy)acetyl]-4-(3,4-methylenedioxybenzyl)piperazine) is a so-called cognitive enhancer, which was proposed for the treatment of asthenia and memory disorders in aging. Its pharmacological effects were discussed to be, at least partially, mediated by modulating dopaminergic transmission [1–7]. FIP has been associated with several severe side effects including hepatic failure with emergency liver transplantation and drug fever [8–10].

Although it is not marketed as a medicament any more, it is still mentioned on a great number of internet homepages specialized on so-called "smart drugs" (http://www.erowid.org; http://www.smartbasic.com) and is easily available from chemical supply companies. For this reason, it is likely that FIP is still consumed, and clinical and forensic toxicologists should be aware of the hepatotoxic effects of FIP, as they are often consulted for differential diagnosis in case of liver failure.

In clinical and forensic toxicology, comprehensive screening procedures in urine are necessary, because the taken drugs or toxicants can be detected for several hours or even days after ingestion, in contrast to blood analysis which often covers only a few hours [11,12]. Such urinalysis of FIP has not yet been published. A prerequisite for developing toxicological screening procedures in urine, is the knowledge of the metabolism, especially if the compounds are excreted in urine primarily or even exclusively as their metabolites [13,14]. Although FIP has been therapeutically used for several years, only very little data on its pharmakokinetic can be found in literature [3]. However, data on the metabolism are needed for studying the mechanisms of (hepato)toxic effects, as these might be caused by toxic metabolites. Furthermore, 1-(3,4-methylenedioxybenzyl)piperazine (MDBP, 1-piperonylpiperazine), which is abused as a designer drug [15,16], is a structural part of FIP.

Therefore, one aim of the study presented here was to identify the FIP metabolites in rat urine using GC–MS in the electron ionization (EI) mode in order to postulate metabolic

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pathways of FIP and to find out whether MDBP is a metabolite of FIP. Another aim was to study whether FIP is covered by the authors' routinely used qualitative systematic toxicological analysis (STA) procedure in urine by full-scan GC–MS and to find out whether an intake of FIP can unequivocally be differentiated from an intake of the designer drug MDBP [13,17–20].

2. Experimental

2.1. Chemicals and reagents

FIP-HCl was obtained from Sigma, Taufkirchen (Germany), MDBP, piperazine and 4-chlorophenoxyacetic acid were obtained from Lancaster Synthesis, Frankfurt (Germany). All other chemicals and biochemicals were obtained from E. Merck, Darmstadt (Germany) and were of analytical grade.

2.2. Urine samples

The investigations were performed using urine of male rats (Wistar, Ch. River, Sulzfleck, Germany) which were administered a single 50 mg/kg body mass (BM) for metabolism studies or a 2.8 mg/kg BM dose for STA of FIP in aqueous solution by gastric intubation ($n = 4$, two for each dose). The low dose corresponds to the content of FIP in tablets formerly marketed. Urine was collected separately from the feces over a 24 h period. All samples were directly worked up, derivatized, and analyzed by GC–MS as described below. Blank rat urine samples were collected before drug administration to check whether the samples were free of interfering compounds.

2.3. Sample preparation for identification of metabolites by GC–MS

A 5 ml portion of urine was adjusted to pH 5.2 with acetic acid (1 mol/l) and incubated at 37 °C for 12 h with 100 μl of a mixture (100,000 Fishman units/ml) of glucuronidase (EC no. 3.2.1.31) and arylsulfatase (EC no. 3.1.6.1) from *Helix pomatia*, then adjusted to pH 8–9 and extracted with 5 ml of a dichloromethane–isopropanol–ethyl acetate mixture (1:1:3, v/v/v). After phase separation by centrifugation, the organic layer was transferred into pear-shaped flasks and carefully evaporated to dryness at 56 °C and the residue was derivatized by acetylation [21]. 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 [18]. After careful evaporation, the residue was dissolved in 100 μl of methanol and 2 μl of this solution were injected into the GC–MS. The same procedure with the exception of enzymatic hydrolysis was used to study whether metabolites of FIP were excreted as conjugates.

Another urine sample was worked up as described above, but pH was adjusted to 4–5. The corresponding extract was analyzed after methylation and subsequent acetylation [21]. After reconstitution of the extraction residue in 50 μl of methanol, methylation was conducted with 50 μl of a solution of diazomethane in diethyl ether, synthesized according to the procedure of McKay et al. [22]. The reaction vials were sealed and left at room temperature for 15 min. Thereafter, the mixture was once again carefully evaporated to dryness under a stream of nitrogen, acetylated as described above and finally redissolved in 50 μl of methanol and 3 μl of this sample was injected into the GC–MS.

2.4. Sample preparation for toxicological analysis

The urine samples (5 ml) were divided into two equal 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 mol/l aqueous ammonium sulfate and 1.5 ml of 10 mol/l aqueous sodium hydroxide to obtain a pH value of 8–9. Before extraction, the aliquot of unhydrolyzed urine was added and this solution was extracted with 5 ml of a dichloromethane–isopropanol–ethyl acetate mixture (1:1:3, v/v/v). After phase separation by centrifugation, the organic layer was transferred and carefully evaporated to dryness. The residue was derivatized by acetylation with 100 μl of an acetic anhydride–pyridine mixture (3:2; v/v) for 5 min under microwave irradiation at about 440 W [17]. After evaporation of the derivatization mixture, the residue was dissolved in 100 μl of methanol and 2 μl of this sample were injected into the GC–MS.

2.5. Gas chromatography–mass spectrometry

FIP and its 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 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; EI mode: ionization energy, 70 eV; ion source temperature, 220 °C; capillary direct interface heated at 260 °C.

For toxicological analysis of FIP and its metabolites, mass chromatography with the selected ions *m/z* 135, 137, 141, 170, 262, and 306 was used. These ions were selected from the corresponding mass spectra (Fig. 1). Generation of the mass chromatograms could be started by clicking the corresponding pull down menu which executed the user defined macros [23]. 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 [24].

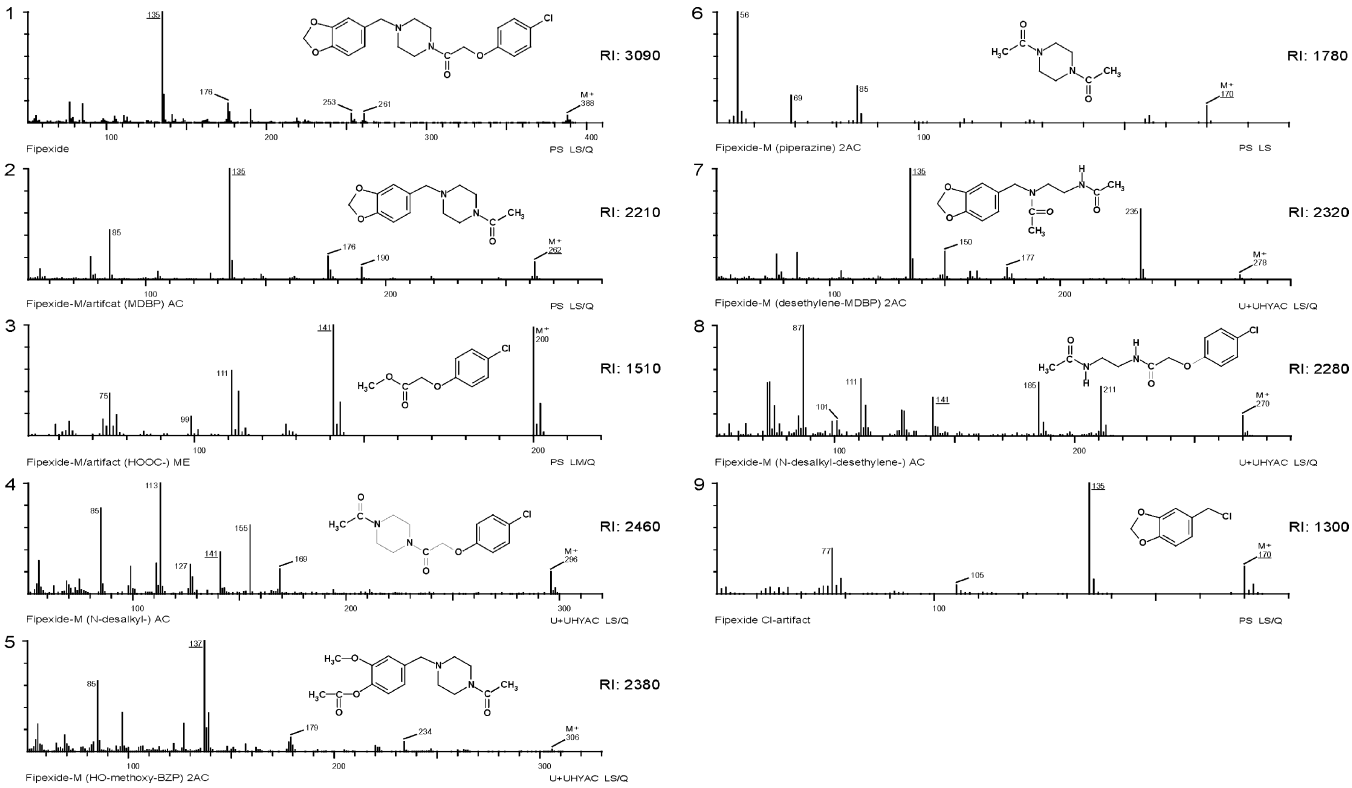

Fig. 1. EI mass spectra, the gas chromatographic retention indices (RI), and structures of FIP, its metabolites and its acid hydrolysis artifact, after acetylation or methylation. Axes only labeled for spectrum no. 1.

3. Results and discussion

3.1. Sample preparation

Cleavage of conjugates was necessary before extraction in order not to miss conjugated metabolites. For studies on the metabolism, gentle enzymatic hydrolysis was preferred, as this procedure did not lead to the formation of artifacts. For the study on the detectability of FIP within the authors' routinely used STA procedure, rapid acid hydrolysis was performed. This is important in emergency toxicology. However, this procedure led not only to the cleavage of conjugates but also to the cleavage of the acid amide bond of FIP to MDBP and 4-chlorophenoxyacetic acid. Another alteration of FIP and/or its metabolites during acid hydrolysis, was the formation of 3,4-methylenedioxy benzylchloride, a chloro artifact, which is also a known artifact of MDBP [25]. Alterations of compounds during acid hydrolysis have already been described for other compounds [18]. Therefore, the urine was divided into two equal aliquots. One of the two aliquots was left unhydrolyzed and was added to the hydrolyzed aliquot after pH adjustment before extraction, in order to avoid artifact formation during acid hydrolysis. This modified sample preparation was a compromise between the necessity of a quick cleavage of conjugates and the detectability of compounds destroyed during acid hydrolysis. Although the modification of the STA procedure led to lower extract concentrations of compounds excreted in conjugated form, this modified procedure was sufficient, because of the high sensitivity of modern GC–MS apparatus [17,18].

The analytes were isolated using a very universal liquid-liquid extraction at pH 8–9, because metabolic formation of aromatic hydroxy groups may lead to phenolbases which are best extracted at this pH. Using a more alkaline pH for extraction leads to decreased extraction efficacies of such hydroxy metabolites which are often excreted for a longer period of time than the parent compounds [26–32]. Derivatization of the extracts was indispensable for sensitive detection of the metabolites.

The extraction efficacy determined for FIP after STA working-up was $39 \pm 6\%$ ($n = 5$) at 1000 ng/ml. The rather low extraction efficacy might be explained by the above described alteration during sample preparation. However, as FIP is almost completely metabolized the determination of its extraction efficiency is of little use. Therefore, the extraction efficiency of its main metabolite MDBP was determined to be $39 \pm 15\%$ ($n = 5$ each). Again, the formation of artifacts during acid hydrolysis might be an explanation [25].

In order to check for acidic metabolites, the urine samples were extracted after cleavage of conjugates at acidic pH (4–5) and the corresponding extracts had been analyzed after methylation followed by acetylation [21].

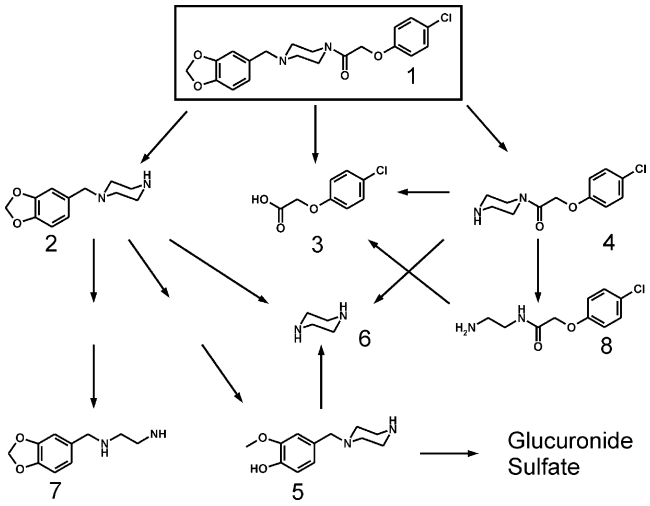

Fig. 2. Proposed scheme for the metabolism of FIP in rats. The metabolite 5 was also excreted as glucuronic and/or sulfuric acid conjugates in urine.

3.2. Identification of metabolites

The urinary metabolites of FIP were separated by GC and identified by EI MS after enzymatic hydrolysis, extraction at acidic and basic pH and derivatization. The basic extracts were acetylated, the acidic extracts were methylated followed by acetylation. Extractions at different pH values was conducted in order to find both, basic and acidic metabolites. The EI mass spectra, the gas chromatographic retention indices (RI) and the structures of FIP and its derivatized metabolites are shown in Fig. 1. The spectra are arranged according to the numbers given in Fig. 2. FIP (mass spectra no. 1 in Fig. 1) was extensively metabolized and could only be detected in very small amounts after the administration of the high dose. The following metabolites could be identified in rat urine: MDBP (mass spectra no. 2), 4-chlorophenoxyacetic acid (mass spectra no. 3), 1-[2-(4-chlorophenoxy)acetyl]piperazine (mass spectra no. 4), *N*-(4-hydroxy-3-methoxy-benzyl)piperazine (mass spectra no. 5), piperazine (mass spectra no. 6), *N*-(3,4-methylenedioxybenzyl)ethylenediamine (mass spectra no. 7), *N*-[2-(4-chlorophenoxy)acetyl]ethylenediamine (mass spectra no. 8).

The identity of MDBP, piperazine and 4-chlorophenoxyacetic acid could be confirmed by comparing their mass spectra and their gas chromatographic retention times with those of the reference substances. The structure of these metabolites could further be confirmed by spiking urine of FIP treated rats with either MDBP or piperazine before basic extraction and acetylation or with 4-chlorophenoxyacetic acid before acidic extraction and methylation. The corresponding FIP metabolites and the reference substances resulted in one single peak each. This fact together with the identical mass spectra is a confirmation of the structure.

The structures of the further metabolites were deduced from the fragments, which were interpreted in correlation to those of the reference substances of FIP, MDBP and 4-chlorophenoxyacetic acid according to the rules described by e.g. McLafferty and Turecek [33] and Smith and Busch [34]. In a study on the metabolism of MDBP, *N*-(4-hydroxy-3-methoxy-benzyl)piperazine, piperazine and *N*-(3,4-methylenedioxybenzyl)ethylenediamine were already identified as MDBP metabolites [25].

Based on these identified metabolites, the following metabolic pathways, shown in Fig. 2, could be postulated: alteration of FIP either by cleavage of the acid amide leading to MDBP (compound no. 2 in Fig. 2) and 4-chlorophenoxyacetic acid (no. 3) or by *N*-dealkylation at the benzyl carbon leading to 1-[2-(4-chlorophenoxy)acetyl] piperazine (no. 4). Likewise, *N*-dealkylation of MDBP or *N*-(4-hydroxy-3-methoxy-benzyl)piperazine (no. 5) led to piperazine (no. 6). Furthermore, the piperazine moiety of MDBP and 1-[2-(4-chlorophenoxy)acetyl]piperazine was degraded by double *N*-dealkylation to N-(3,4-methylenedioxybenzyl)ethylenediamine (no. 7) and to 1-[2-(4-chlorophenoxy)acetyl]ethylenediamine (no. 8), respectively. Cleavage of the acid amide bond in 1-[2-(4-chlorophenoxy)acetyl]piperazine or 1-[2-(4-chlorophenoxy)acetyl] ethylenediamine led also to 4-chlorophenoxyacetic acid. Comparison of urine samples with and without enzymatic conjugate cleavage showed that the phenolic metabolite 4-hydroxy-3-methoxy-*N*-benzylpiperazine was partially excreted as conjugates, because the peak areas were greater after enzymatic hydrolysis.

The metabolic steps of FIP are in accordance with those of other structurally related compounds. Although cleavage of the acid amide bond is a common metabolic reaction [35], it can not be excluded that partial acid hydrolysis already occurs in the stomach. Metabolic degradation of the piperazine heterocycle has been described for structurally related piperazine derivatives [19,20,36,37], and *N*-dealkylation at the benzyl carbon leading to the piperazine moiety is also a metabolic reaction of *N*-benzylpiperazine and MDBP [19,25].

3.3. Detection of FIP and its metabolites in urine by GC–MS within the STA

FIP and its metabolites were separated by GC and identified by full-scan MS after acid hydrolysis of one aliquot of the sample, extraction and acetylation within the authors' standard STA. In Fig. 1, the RIs are given besides the EI spectra, because they provide preliminary indications and may be useful to gas chromatographers without a GC–MS facility. The RIs were recorded during the GC–MS procedure and calculated in correlation with the Kovats' indices [38] of the components of a standard solution of typical drugs which is measured daily for testing the GC–MS performance [39,40]. The reproducibility of retention indices measured on capillary columns was better using a mixture of drugs than that of the homologous hydrocarbons recommended by Kovats.

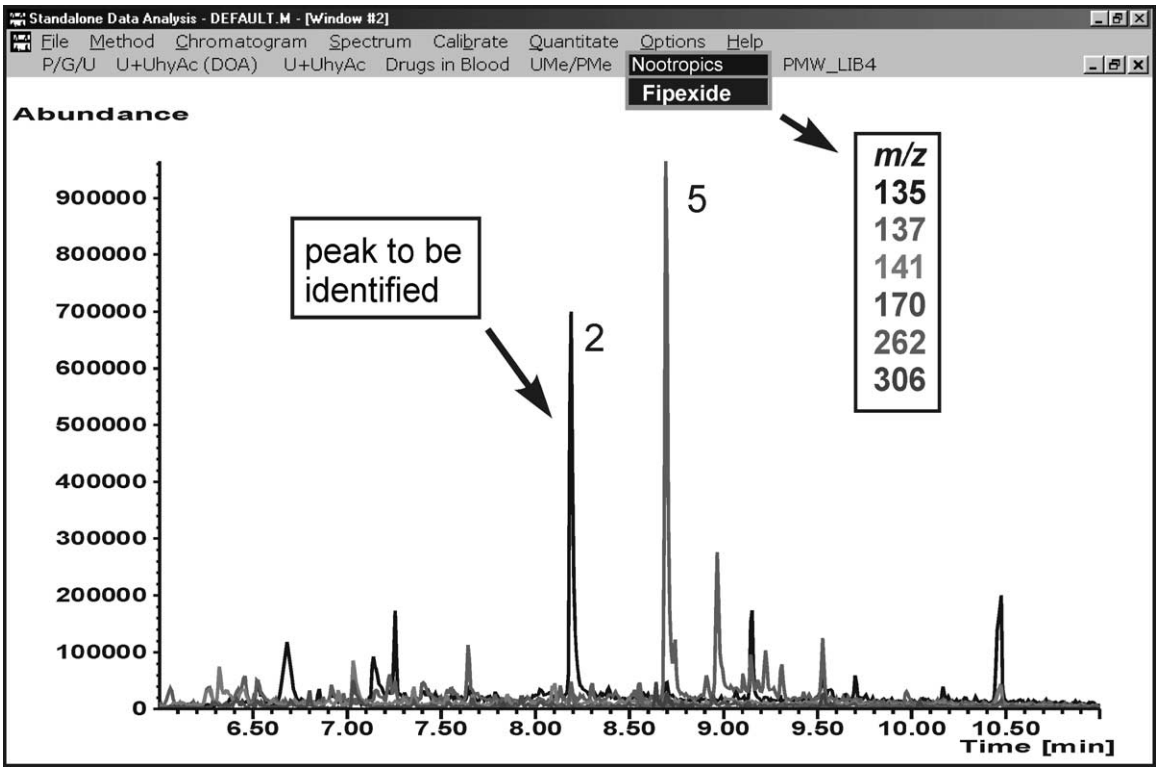

Fig. 3. Typical reconstructed mass chromatograms with the given ions of an acetylated extract of a rat urine sample taken 24 h after intake of 2.8 mg/kg BM of FIP. They indicate the presence of FIP metabolites. The peak numbers correspond to those used in Figs. 1 and 2. The merged chromatograms can be differentiated by their colors on a color screen.

Mass chromatography with the selected ions *m/z* 135, 137, 141, 170, 262, and 306 was used to indicate the presence of FIP and/or its metabolites. Generation of the mass chromatograms could be started by clicking the corresponding pull down menu which executed the user defined macros. Fig. 3 shows typical reconstructed mass chromatograms of the above mentioned ions of an acetylated extract of a rat urine sample taken after application of a dose of 2.8 mg/kg BM of FIP which corresponds to the content of FIP in tablets formerly marketed. The peak numbers correspond to the numbering in Figs. 1 and 2. The identity of peaks in the mass chromatograms was confirmed by computerized comparison of the underlying mass spectrum with reference spectra [24]. The ions *m/z* 135 and 262 were used for indication of the presence of the metabolite MDBP. Furthermore, the ion *m/z* 135 indicates the presence of all other metabolites with unchanged methylenedioxybenzyl moiety, such as the parent compound FIP, *N*-(3,4-methylenedioxybenzyl)ethylenediamine and the chloro artifact 3,4-methylenedioxy benzylchloride (mass spectrum no. 9 in Fig. 1). The ions *m/z* 137 and 306 were used for identification of *N*-(4-hydroxy-3-methoxybenzyl) piperazine. The ion *m/z* 141 was used for indication of 4-chlorophenoxyacetic acid and its derivatives 1-[2-(4-chlorophenoxy)acetyl]piperazine and 1-[2-(4-chlorophenoxy) acetyl]ethylenediamine. The ion *m/z* 170 was used for indication of piperazine. Together with the ion *m/z* 135, it indicates also the presence of the chloro artifact. After administration of a common dose of FIP, only MDBP and *N*-(4-hydroxy-3-methoxy-benzyl)piperazine could be found. Consequently, a differentiation between an intake of FIP and an intake of the designer drug MDBP was not possible. However, if higher doses of FIP had been administered, the other mentioned metabolites were also detectable in the STA urine extract. Although the limit of detection of FIP in urine was as low as 100 ng/ml (signal-to-noise $S/N > 3$) under routine conditions, the parent compound could not be detected, which means that FIP is excreted almost completely metabolized and/or altered during acid hydrolysis in the hydrolyzed aliquot. According to [25], the limit of detection of the metabolite and artifact MDBP was also 100 ng/ml ($S/N > 3$).

As illustrated in Fig. 4, the identity of the marked peak in the mass chromatograms was confirmed by computerized comparison of the underlying mass spectrum with reference spectra recorded during this study. The found reference spectrum is labeled as MDBP. In order to avoid misinterpretation of the analytical result of compounds which may be formed from different drugs, proper use of the Pfleger/Maurer/Weber library [24] is indispensable. The "@" sign indicates that the compound can also be found after intake of additional compounds (e.g. FIP) given in the corresponding handbooks [41,42]. Differentiation of an intake of FIP from an intake of other compounds is described in Section 3.4.

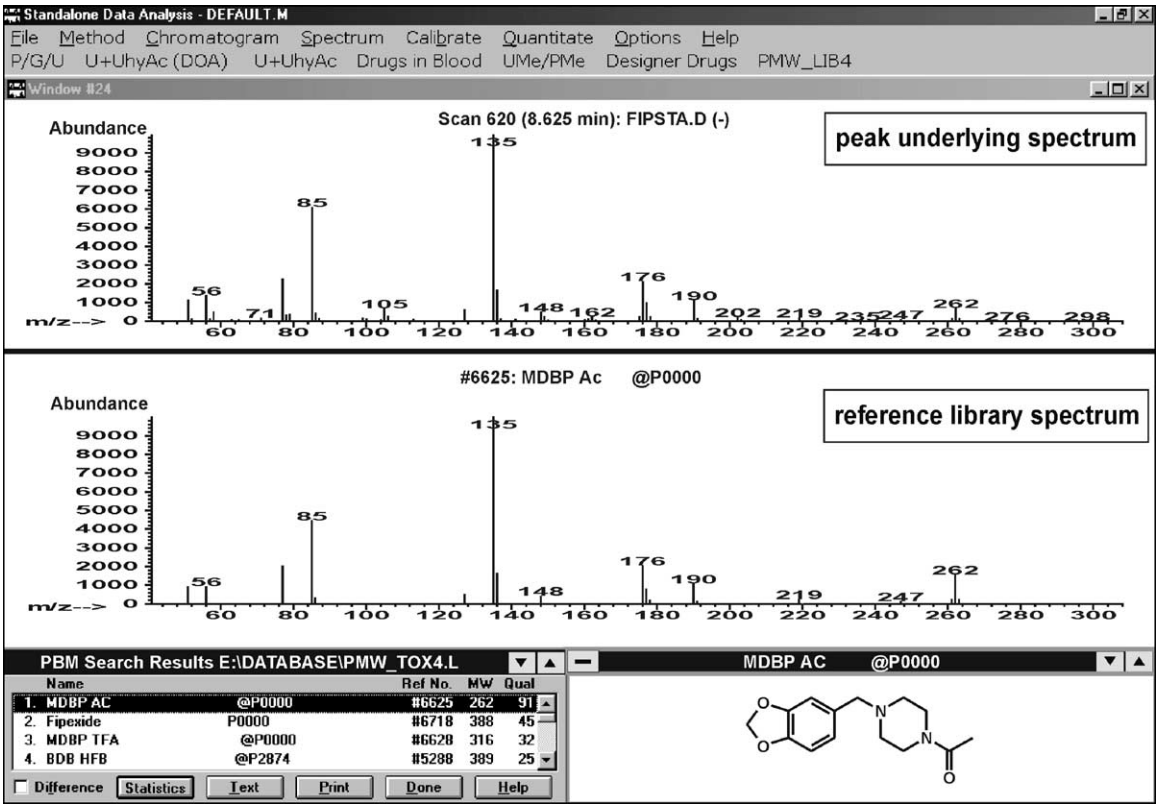

Fig. 4. Mass spectrum underlying peak 2 in Fig. 3, the reference spectrum, the structure, and the hit list found by computer library search.

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.

The authors' STA procedure allowed the detection of an intake of a common dose of FIP via its metabolites in rat urine. It can be concluded that it should also be detectable in human urine in a clinical or forensic case. According to the authors' experience in metabolism and analytical studies on rats and humans, it should be possible to detect the metabolites found in rat urine also in human urine samples [18,19,21,43].

3.4. Differentiation of an FIP intake from an intake of structurally related compounds

After intake of a common dose of FIP, only MDBP and *N*-(4-hydroxy-3-methoxy-benzyl)piperazine were detectable by the STA in urine. MDBP is a common designer drug and therefore, differentiation is necessary. Unequivocally, differentiation would be possible via the detection of FIP itself or via its unique metabolites 1-[2-(4-chlorophenoxy)acetyl]piperazine (compound no. 4) and 1-[2-(4-chlorophenoxy)acetyl]ethylenediamine (compound no. 8), but they could only be detected after administration of higher doses. The FIP metabolite 4-chlorophenoxyacetic acid which cannot be formed by MDBP would be detectable in an acid extract after methylation. However, its detection neither allows differentiation, because it is used as an herbicide and also found in urine after intake of the nootropic meclofenoxate [41] and probably after intake of further 4-chlorophenoxyacetic acid derivatives such as adafenoxate or iproclozide [44]. The FIP metabolites *N*-(4-hydroxy-3-methoxy-benzyl)piperazine and piperazine are also metabolites of the designer drug BZP [19]. As BZP is excreted mainly unmetabolized, a differentiation is possible via the parent compound BZP itself. Finally, it should be mentioned that the FIP metabolite piperazine itself is in use as an anthelmintic and can also be found in urine after intake of zopiclone, cinnarizine and cetirizine [41].

4. Conclusions

These studies showed that the nootropic FIP is a precursor drug of the designer drug MDBP. It was extensively metabolized mainly by cleavage of the acid amide bond to MDBP and 4-chlorophenoxyacetic acid. The authors' qualitative STA procedure allowed the detection of FIP via its metabolites in rat urine after administration of a common dose of FIP. Therefore, this procedure should also be suitable for detection of a FIP intake in human urine. However, differentiation of an intake of FIP from that of the designer drug MDBP was only possible after higher dosages, but

not after low therapeutic dosage. Further studies will show, whether plasma analysis will allow such differentiation.

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