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Phenethylamine-derived designer drug diphenyl-2-pyrrolidinemethanol (D2PM): Investigations of its biotransformation in rats

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M. R. Meyer, **S. Schmitt**, and H. H. Maurer:
Studies on the metabolism and detectability of the emerging drug of abuse diphenyl-2-pyrrolidinemethanol (D2PM) in rat urine using GC-MS and LC-HR-MS/MS

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1 ZUSAMMENFASSUNG

Diphenyl-2-pyrrolidinemethanol (D2PM), ein sogenanntes ‘legal high’, wurde zum ersten Mal 2007 in Großbritannien identifiziert. D2PM wird vor allem über Online-Shops vertrieben und als Stimulans missbraucht. Das Ziel der vorliegenden Arbeit war es, die Metaboliten des D2PM in Rattenurin mittels Gaschromatographie-Massenspektrometrie (GC-MS) oder Flüssigchromatographie-Hochauflösender-Tandem-Massenspektrometrie (LC-HR-MS/MS)-Techniken zu identifizieren. Nach entsprechender Probenvorbereitung konnten mittels GC-MS insgesamt fünf Phase-I-Metabolit identifiziert werden. Daraus konnten folgende Schritte im Abbau des D2PM postuliert werden: Hydroxylierung am Pyrrolidinring und Oxidation zum Keton, Einführung einer phenolischen Hydroxygruppe - auch in Kombination mit der Hydroxylierung am Pyrrolidinring. Neben den genannten Metaboliten konnte auch die unveränderte Muttersubstanz im Urin nachgewiesen werden. Alle postulierten Phase-I-Schritte konnten unter Verwendung von LC- HR-MS/MS bestätigt werden. Des Weiteren konnten mittels LC-HR-MS/MS zwei konjugierte Phase-II-Metabolite, Phenylhydroxy-oxo D2PM Glucuronid und Phenylhydroxy D2PM Glucuronid, detektiert werden. Der Nachweis eines missbräuchlichen Konsums sollte mittels GC-MS und LC-MS^n möglich sein.

2 SUMMARY

Diphenyl-2-pyrrolidinemethanol (D2PM) is a so-called ‘legal high’ and appeared for the first time in the United Kingdom in 2007. It can be ordered from online shops and is used as a recreational drug. The aim of this work was to identify its metabolites in rat urine using GC-MS and LC-high resolution-MS/MS (LC-HR-MS/MS).

After analysis of the urine samples, five phase I metabolites could be identified by GC-MS. The following metabolic steps could be postulated: hydroxylation at the pyrrolidine ring followed by oxidation to the ketone, introduction of a phenolic hydroxy group also in combination with hydroxylation at the pyrrolidine ring, and finally combination of hydroxylation at the phenyl ring and oxidation of the pyrrolidine ring. Besides these metabolites, the unchanged parent compound could be detected.

All postulated phase I metabolites could be confirmed using LC- HR-MS/MS. Additionally, the formation of two phase II metabolites, phenylhydroxy-oxo D2PM glucuronide and phenylhydroxy D2PM glucuronide, could be shown.

3 INTRODUCTION

More and more new psychoactive substances are sold via the internet as so-called "legal highs".[1,2] They are widely consumed although the content of the preparations is often unknown or not correctly declared making the health risks assessment difficult. Such drugs are detected frequently in clinical and forensic samples. Diphenyl-2-pyrrolidinemethanol (D2PM) is one of these substances that appeared for the first time in the United Kingdom in 2007.[3]

D2PM is a chiral structure analogue of phenethylamine (Fig. 1), a biogenic amine which can naturally be found in cocoa and bitter almond oil. Enantioseperation of (R-) and (S-)-D2PM has been described.[4] The desoxygenated form of D2PM, 2-(diphenylmethyl)pyrrolidine (DPMP, desoxypipradol) has also been found to be sold as drug of abuse. [5,6]

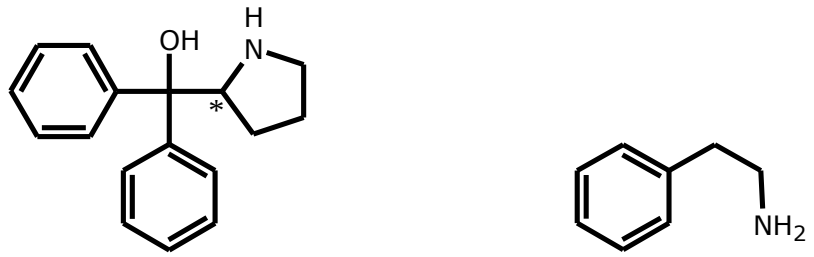


Figure 1 . *Chemical structure of D2PM and phenethylamine.*

3.1 Pharmacology and Toxicology of D2PM

The symptoms after ingestion of D2PM were of neuropsychiatric character and resulted in anxiety, paranoia, and prolonged insomnia. Furthermore, sympathomimetic effects could be observed, such as pupil dilation, tachycardia and hypertension.[7-9]

Ischemic-sounding chest pain as a consequence of ingestion of 'Head Candy', a D2PM containing preparation, has also been reported. Besides D2PM, Glaucine was detected in this preparation but was not held responsible for the cardiac symptoms.[7] According to other case reports, D2PM was detected in urine after consumption of diverse drugs without the awareness of its contents, namely 'Benzofury', 'NRG-3' (in combination with MDMA), 'Nytol', and further unknown substances.[8]

After ingestion of DPMP, consumers were reported to have shown symptoms such as tachycardia, tachypnea, elevated creatinine kinase (CK) concentration, leukocytosis and acute dystonia.[5,6]

The pharmacological properties of D2PM were recently reviewed by Wood and Dargan.[9] It was described to bind at the cocaine binding site of the dopamine transporter. The group of Wood also published case series, showing the neuropsychiatric effects after recreational use of D2PM. Lidder suggested cardiovascular toxicity in association with the intake of D2PM.[10]

3.2 Aim of the present work

The aim of this work was the elucidation of the biotransformation of D2PM in the rat using GC-MS and LC-high resolution-MS/MS (LC-HR-MS/MS) as basis for thorough screening procedures feasible in clinical and forensic toxicology.

4 EXPERIMENTAL PROCEDURES / MATERIAL AND METHODS

4.1 Chemicals and reagents

D2PM was obtained from Sigma-Aldrich (Steinheim, Germany), Isolute HCX cartridges (130 mg, 3 ml) from Biotage (Uppsala, Sweden), and all other chemicals and reagents from E. Merck, Darmstadt (Germany) and they were of analytical grade.

4.2 Urine samples

The investigations were performed using urine of male Wistar rats (Charles River, Sulzfleck, Germany) for toxicological diagnostic reasons according to the corresponding German law. They were administered a single 20 mg/kg body mass dose of D2PM for identification of the metabolites. Urine was collected separately from the feces over a 24 h period. All samples were directly analyzed and then stored at -20°C. Blank urine samples were collected before drug administration to check whether the samples were free of interfering compounds.

4.3 Sample preparation

4.3.1 Sample preparation for phase I metabolism studies

A 2.0 ml portion of urine was adjusted to pH 5.2 with acetic acid (1 M) and incubated at 56°C for 1.5 h with 50 µl of a mixture (100 000 Fishman units/ml) of glucuronidase (EC No. 3.2.1.31, Merck) and arylsulfatase (EC No. 3.1.6.1), from Helix Pomatia L. The urine sample was then diluted with 2.5 ml of water and loaded on a HCX solid phase extraction (SPE) 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, 1 ml of 0.01 M hydrochloric acid, and again with 1.0 ml of water. The retained non-basic compounds were first eluted into a 1.5 ml reaction vial with 1 ml of methanol (fraction 1). 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 32% (98:2 v/v, fraction 2).The elutes were gently evaporated to dryness under a stream of nitrogen at 70°C and derivatized by acetylation according to published procedures.[11] Briefly, acetylation was conducted with 50 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 50 µl of methanol and injected into the GC-MS system.

The same experiments were repeated without use of enzymatic hydrolysis to study which metabolites of D2PM were excreted as glucuronides / sulfates.

4.3.2 Sample preparation for phase II metabolism studies

For elucidating the formation of glucuronides and sulfates, 200 µl of urine were mixed with 200 µl of acetonitrile for protein precipitation, centrifuged at 14.000*g* for 5 min and the supernatant was transferred into an autosampler vial. A 10-µl aliquot of this solution was injected into the LC system.

4.4 GC-MS / LC-HRMS settings

4.4.1 GC-MS apparatus for metabolism studies

The extracts were analyzed using a Hewlett Packard (HP, Agilent, Waldbronn, Germany) 5890 Series II gas chromatograph combined with an HP 5972A MSD mass spectrometer and an HP MS ChemStation (DOS series) with HP G1034C software version C03.00. The GC conditions were as follows: splitless injection mode; column, HP-1 capillary (12 m x 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 100-310°C at 15°/min, initial time 3 min, final time 8 min. The MS conditions were as follows: full-scan mode, *m/z* 50-550 u; electron impact (EI) mode, ionization energy, 70 eV; ion source temperature, 220°C; capillary direct interface, heated at 260°C.

4.4.2 LC-HR-MS/MS apparatus for identification of phase I and II metabolites

D2PM and its metabolites were analyzed using a ThermoFisher Scientific (TF, Dreieich, Germany) Accela LC system consisting of a degasser, a high pressure quaternary pump and a HTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland), coupled to a TF Q Exactive system equipped with heated electrospray ionization II (HESI-II) source. The LC conditions were the same as described previously.[12] The MS settings were as follows. The mass spectrometer was operated in the positive ionization mode. The ionization voltage, capillary temperature, vaporizer temperature, sheath gas, and auxiliary gas were set to 3.0 kV, 380 °C, 350 °C, 60 and 20 au, respectively.

Nitrogen was used for spray stabilization, for collision-induced dissociation experiments in the higher energy collision dissociation (HCD) cell, and as the damping gas in the curved linear ion trap (C-trap). The mass spectrometer acquired full-scan data (*m/z* 50 – 750) at a resolution of 70,000 (full width at half maximum, FWHM at *m/z* 200) and a data-dependent MS/MS scan at a resolution of 35,000 (FWHM at *m/z* 200). The parent ion was selected in the quadrupole (isolation window 1.5 u) and subsequently fragmented in the HCD, operating at a collision energy set to 35 eV (*m/z* 400). A full scan (*m/z* 50-750) of all fragmented ions originating from the parent ion was performed.

5 RESULTS AND DISCUSSION

5.1 Identification of D2PM metabolites

After the experimental procedure of SPE and acetylation by full-scan EI after GC separation,[11] the phase I metabolites of D2PM could be identified in rat urine. To distinguish between metabolic acetylation and formation of the acetyl derivatives by derivatization, one non- derivatized extract was also analyzed.

In accordance to the fragmentation rules described by e.g. McLafferty and Turecek and Smith and Busch, the formation of fragments in the parent compound was interpreted and compared with the fragments detected in the EI mode of the urinary extracts.[13,14]
From this formation of fragments the structure of the postulated metabolites could be deduced.
Fig. 2 depicts the EI mass spectra of the metabolites and the corresponding gas chromatographic retention indices (RI). The assumed structures and the prevailing fragmentation pattern can be seen in Fig. 3.
HR-MS/MS was used to record ESI spectra with the intention to approve the postulated metabolites and the fragmentation pattern. Also phase II metabolites could be identified by HR-MS/MS. Table 1 shows the metabolites in their characteristic ionized fragments after HR-MS/MS.

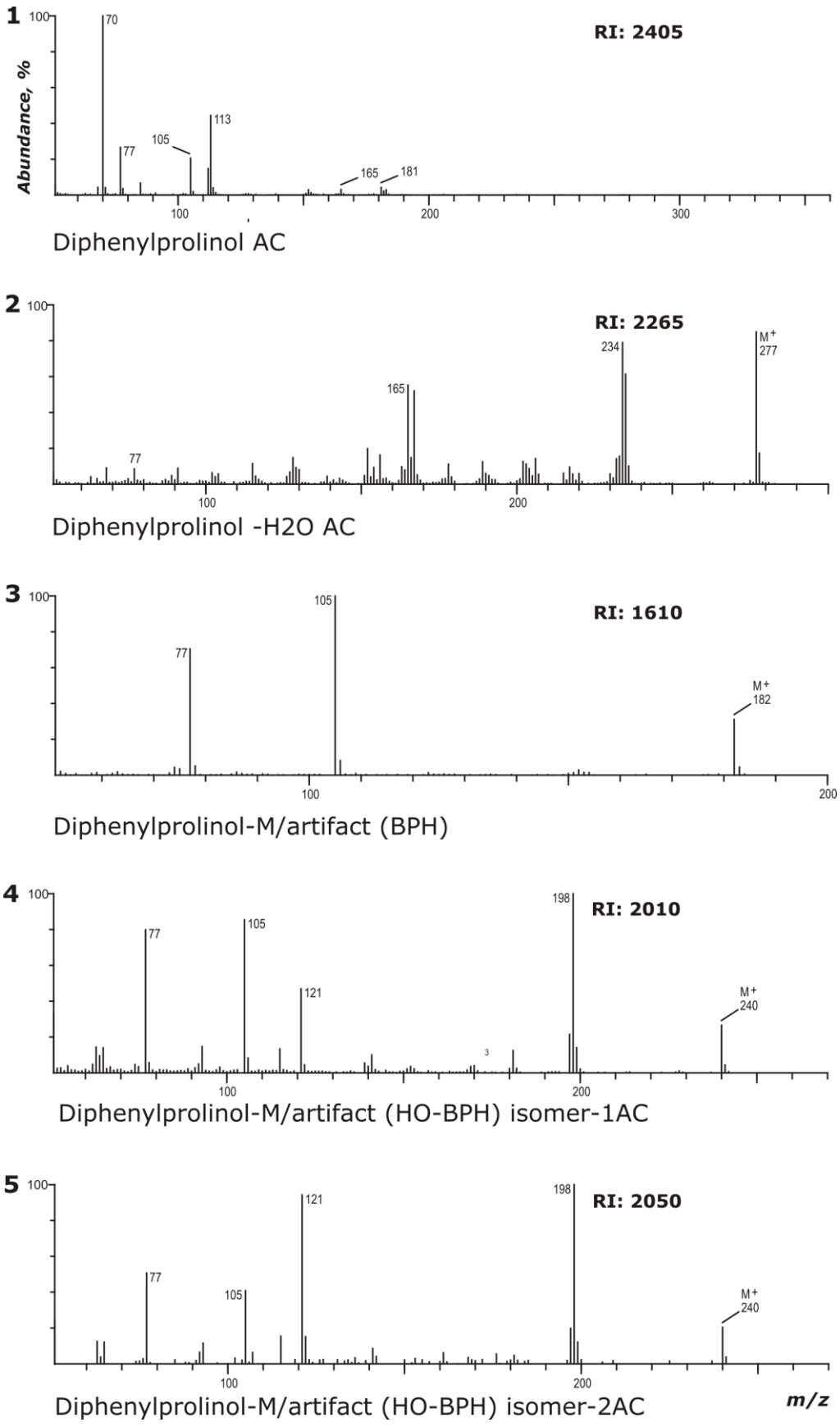


Figure 2. *EI mass spectra and gas chromatographic retention indices (RI) of D2PM and its metabolites arranged according to their RI.*

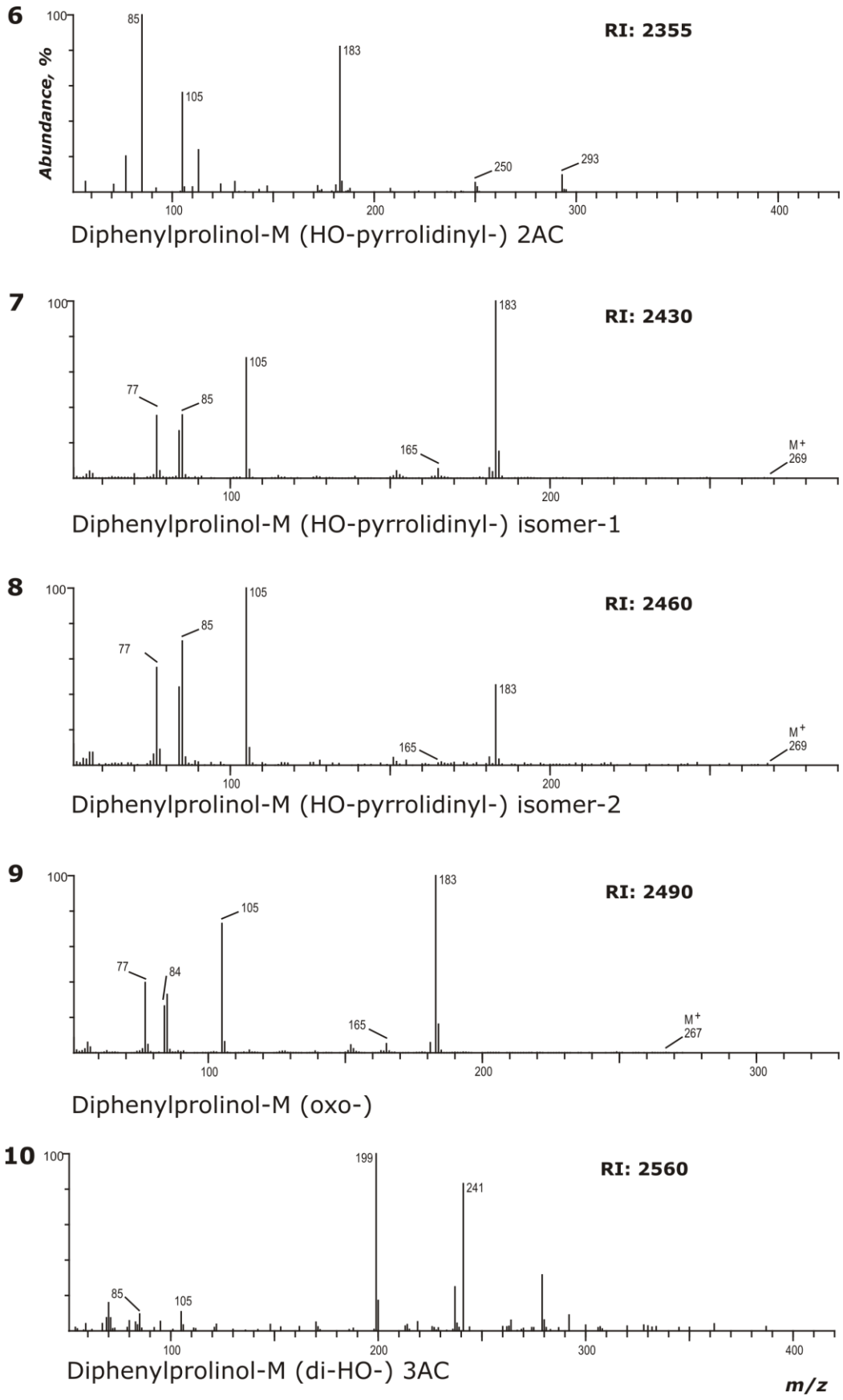
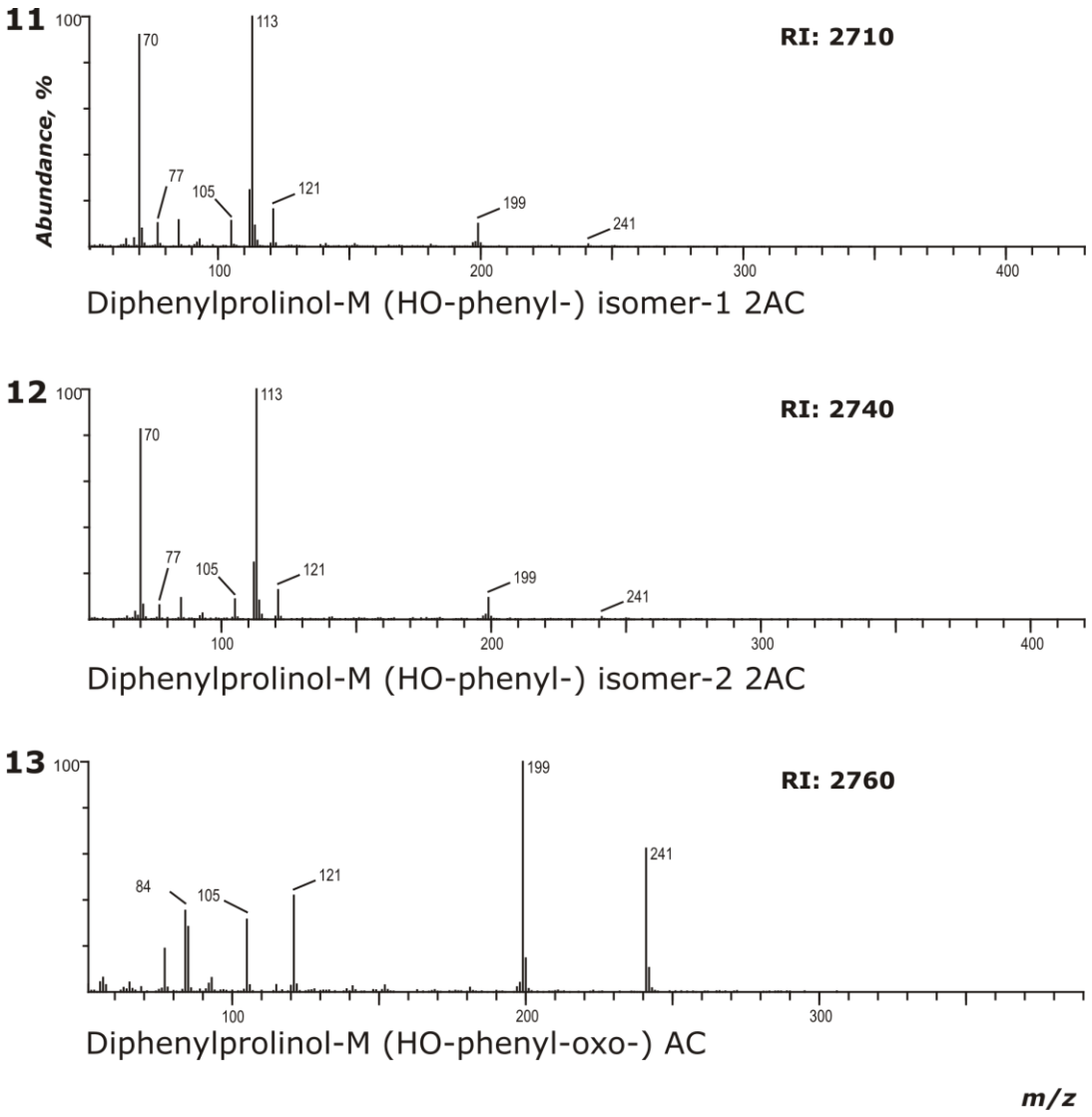


Figure 2 continued



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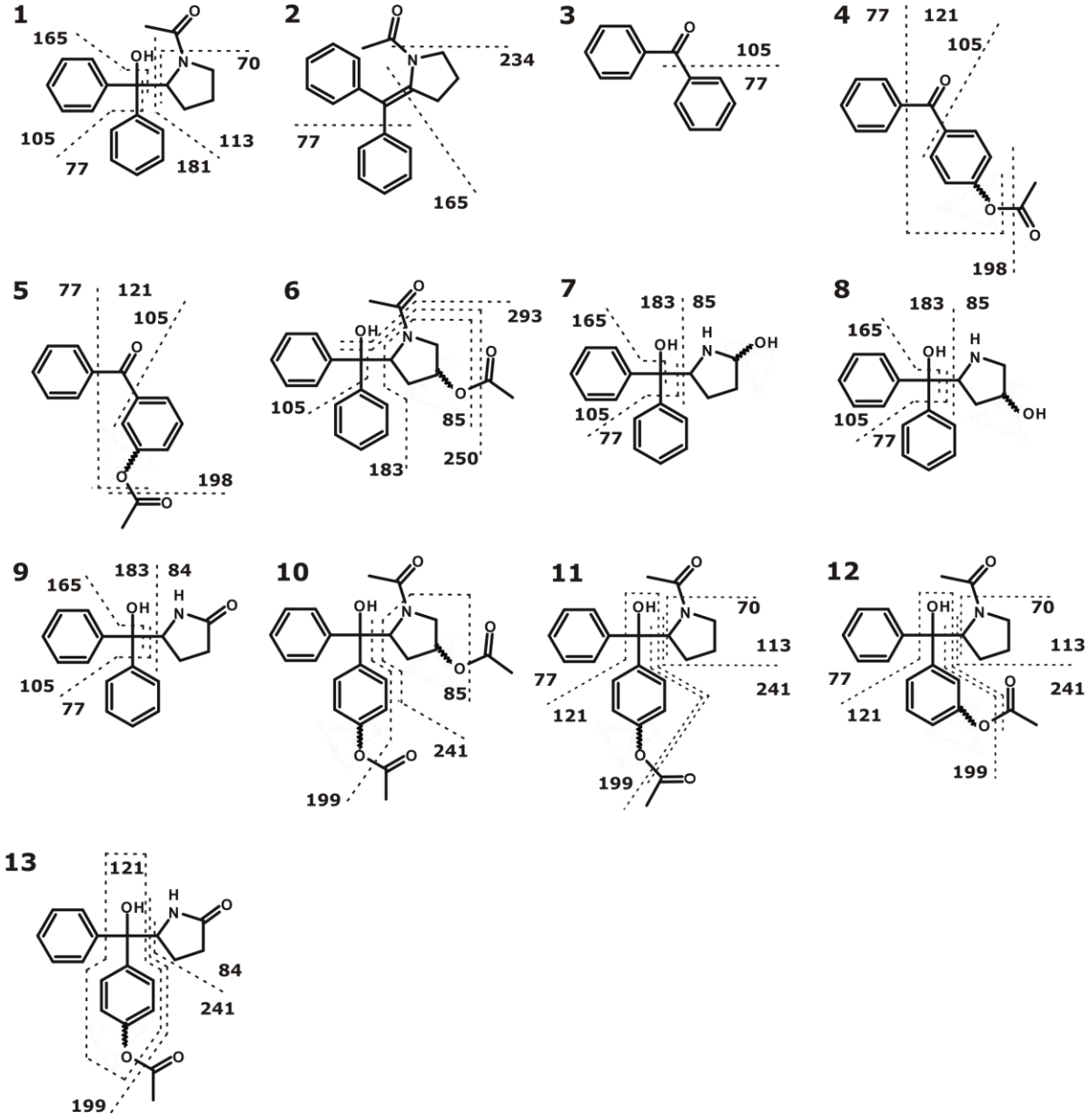

Figure 3. *Proposed structures and predominant fragmentation patterns of D2PM and its metabolites. The numbers correspond to them in Figure 2. The wavy bonds symbolize that a definite assignment to a particular isomer was not possible.*

5.2 Proposed fragmentation patterns for identification of phase I metabolites by GC-MS

The importance for the metabolic processes in relation to the characteristic EI-MS fragments is discussed here. Figs. 2 and 3 show the EI spectra of the metabolites and their proposed structure with the suggested fragmentation pattern, respectively. Suggesting the formation of intramolecular hydrogen bonds between the amine at the pyrrolidine ring and the aliphatic alcohol group, acetylation of the alcohol moiety was not observable in any of the recorded spectra. The same assumption applies for the acetyl group and the aliphatic alcohol group in the derivatized molecules.

Furthermore, the D2PM-HO-pyrrolidinyl-metabolites depicted in spectra and structure nos. 7 and 8 could also not be detected as acetyl derivatives. It can be supposed that the metabolic hydroxylation at the pyrrolidine ring takes place in position 2 of the pyrrolidine ring causing the formation of hydrogen bonds between the amine and the hydroxyl group. Therefore acetylation at the hydroxyl group or the amine might not be possible.

The spectrum no. 1 shows the acetylated D2PM. The most prominent signals are at *m/z* 70, also representing the base peak, 77, 105,113, 165, and 181. α-Cleavage leads to the formation of the two prominent ions at *m/z* 70 and *m/z* 181, representing the pyrrolidine ring and the diphenylmethanol part. The ion at *m/z* 77 stands for the phenyl rings. The diphenyl part of the molecule is represented by the ion at *m/z* 165 (no.1). The ion at *m/z* 105 represents the phenylmethanol part (no.1).

In spectra nos. 1, 11, and 12 prominent signals at *m/z* 70 and 113 could be detected. As the ion at *m/z* 70 represents the pyrrolidine ring, it can be suggested that the ion at *m/z* 113 represents the acetylated pyrrolidine ring before the loss of the acetyl group (43 u).

Besides the detection of the metabolites a formation of artifacts by the loss of water during the GC could be observed. Spectrum no. 2 shows the parent compound after dehydration during GC. The diphenyl part and the phenyl rings are again represented by the ions at *m/z* 77 and 165. The loss of the acetyl group is indicated by the ion at *m/z* 234. Furthermore, benzophenone artifacts (nos. 3-5) could be detected as it has been described before by Wood et al.[8]

The metabolites represented by spectra nos. 6-8 show single metabolic hydroxylation at the pyrrolidine ring resulting in the detection of the ion at *m/z* 85 instead of *m/z* 70. As the mass spectra no. 7 and 8 appear to be nearly identical the detection of isomers can be deduced. The exact position of the hydroxyl group cannot be determined.

Spectrum nos. 9 and 13 indicate the following oxidation of the pyrrolidinyl-hydroxyl group to the corresponding ketone. The pyrrolidine ring with the oxo group is represented by the ion *m/z* 84. As the metabolite no. 13 could not be acetylated at the nitrogen it can be suspected that the oxo group is located in position 2. Intramolecular hydrogen bonds might be the reason for the inability of derivatization. Besides that, the location of ketones in the pyrrolidine ring at position 2 has been described before. [15]

Hydroxylation at the phenyl ring could be observed in the metabolites represented by spectra nos. 10-13. The ion at *m/z* 241 represents the diphenyl-methanol part after acetylation at the metabolically hydroxylated phenyl ring. The ion at *m/z* 199 shows the corresponding part of the molecule after loss of the acetyl group. Spectra nos. 11 and 12 show two detected isomers with metabolic hydroxylation of the phenyl ring in different positions. Again, the exact position of the hydroxyl group cannot be deduced.

5.3 Identification of phase I and II metabolites by LC-HR-MS/MS

All the phase I metabolites that were identified by GC-MS could be detected and confirmed using LC-HR-MS/MS procedures. Furthermore, two phase II metabolites could be identified, namely phenylhydroxy-oxo-D2PM glucuronide and phenylhydroxy-D2PM glucuronide. Here the glucuronides were detected with a much lower signal abundance than the phase I metabolites. In Table 1, all the detected metabolites including the glucuronides are listed with the measured accurate masses of the protonated molecule and also the characteristic fragment ions. The corresponding calculated exact masses and the proposed elemental composition are also depicted.

Table 1. *List of D2PM and its phase I and II metabolites, the measured accurate masses of their protonated molecule and characteristic fragment ions, the corresponding calculated exact masses, and the proposed elemental*

Metabolites and characteristic ions [*m/z*]	Calculated exact masses [u]	Elemental compositions
D2PM, 254.1537	**254.1539 $[M+H]^+$**	C17H20NO
236.1431	236.1434	C17H18N
208.1119	208.1121	C15H14N
158.0963	158.0964	C11H12N
130.0651	130.0651	C9H8N
D2PM-M (oxo-), 268.1329	**268.1332 $[M+H]^+$**	C17H18NO2
250.1224	250.1226	C17H16NO
222.0912	222.0913	C15H12NO
196.1120	196.1121	C14H14N
179.0854	179.0855	C14H11
D2PM-M (HO-pyrrolidinyl-), 270.1485	**270.1489 $[M+H]^+$**	C17H20NO2
252.1379	252.1383	C17H18NO
210.1275	210.1277	C15H16N
168.0806	168.0808	C12H10N
100.0760	100.0757	C5H10NO
D2PM-M (HO-phenyl-), 270.1487	**270.1489 $[M+H]^+$**	C17H20NO2
252.1379	252.1383	C17H18NO
174.0910	174.0913	C11H12NO
158.0962	158.0964	C11H12N
130.0650	130.0651	C9H8N
D2PM-M (HO-phenyl-oxo-), 284.1278	**284.1281 $[M+H]^+$**	C17H18NO3
266.1171	266.1176	C17H16NO2
238.0860	238.0863	C15H12NO2
212.1068	212.1070	C14H14NO
195.0804	195.0804	C14H11O
D2PM-M (di-HO-), 286.1424	**286.1438 $[M+H]^+$**	C17H20NO3
268.1329	268.1332	C17H18NO2
250.1224	250.1226	C17H16NO
222.0911	222.0913	C15H12NO
196.1119	196.1121	C14H14N
D2PM-M (HO-phenyl-)glucuronide, 446.1810	**446.1810 $[M+H]^+$**	C23H28NO8
252.1381	252.1383	C17H18NO
274.0908	174.0913	C11H12NO
158.0963	158.0964	C11H12N
130.0650	130.0651	C9H8N
D2PM-M (HO-phenyl-oxo-)glucuronide, 460.1600	**460.1602 $[M+H]^+$**	C23H26NO9
266.1172	266.1176	C17H16NO2
238.0860	238.0863	C15H12NO2
212.1067	212.1070	C14H14NO
195.0804	195.0804	C14H11O

5.4 Proposed metabolic pathway

Fig. 4 shows all the metabolic steps that have been explained above. As initial metabolic steps the hydroxylation at the pyrrolidine ring and the phenyl ring could be deduced. The hydroxylation at the pyrrolidine ring was followed by the oxidation to the corresponding ketone. Combination of the introduction of a phenolic hydroxy group and the hydroxylation at the pyrrolidine ring as well as the oxidation to the corresponding ketone could be detected. Additionally the parent compound could be detected unchanged.

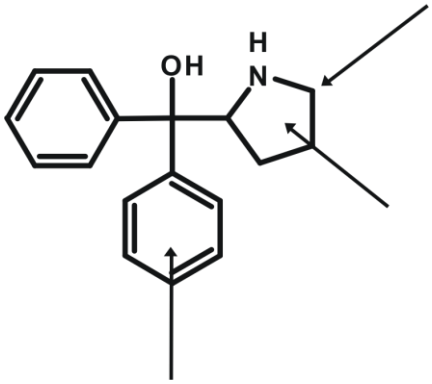

Figure 4. *Structure of D2PM with arrows indicating the areas of the described metabolic reactions.*

6 CONCLUSIONS

This study could reveal the extensive metabolism of the synthetic drug D2PM in the rat. Several phase I and II metabolites could be identified using GC-MS and LC-HR/MS techniques. Due to the huge increase of recreational drug abuse and the resulting health risks for the consumers, the importance of appropriate screening procedures has grown. Therefore and supposing that the kinetic processes in rats and humans are similar, this study could serve as a basis for developing suitable screening strategies for detection of a D2PM intake. This will be investigated in a further study.

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8 ABBREVIATIONS

D2PM	diphenyl-2-pyrrolidinemethanol
GC-MS	gas chromatography- mass spectrometry
LC-MS	liquid chromatography- mass spectrometry
LC-HRMS	liquid chromatography- high resolution- mass spectrometry
MS/MS	tandem mass spectrometry
IUPAC	International Union of Pure and Applied Chemistry

9 DANKSAGUNG

Diese Dissertation entstand unter Anleitung von Herrn Prof. Dr. Dr. h.c. Hans H. Maurer in der Abteilung für Experimentelle und Klinische Toxikologie der Medizinischen Fakultät der Universität des Saarlandes.

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