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Toxicokinetics of Emerging Drugs of Abuse: In vivo and in vitro studies on the metabolic fate of the cathinone-derived designer drug ß-naphyrone

Dissertation zur Erlangung des Grades eines Doktors der Medizin an der Medizinischen Fakultät der Universität des Saarlandes

2013

Von

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Geboren am 30.05.1987 in Stuttgart Bad Cannstatt

Teile dieser Dissertation sind Gegenstand folgender Publikation:

M. R. Meyer, **D. Prosser**, and Hans H. Maurer:

Studies on the Metabolism and Detectability of the Designer Drug β-Naphyrone in Rat Urine using GC-MS and LC-HR-MS/MS

Drug Test Anal. 2013 Jan 10. doi: 10.1002/dta.1443

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1. ZUSAMMENFASSUNG

Kurz nach Unterstellung des Mephedron, eine Cathinon-verwandte Designer-Droge, unter das Betäubungsmittelgesetz, wurde Naphyron (1-Naphthalen-2-yl-2-pyrrolidin-1-yl-pentan-1-on; Naphthylpyrovaleron, β-Naphyron) als Ersatz gehandelt. Inzwischen ist auch Naphyron dem Betäubungsmittelgesetz unterstellt. Daher war das Ziel dieser Dissertation die Identifizierung der Metaboliten von Naphyron in Rattenurin mittels Gaschromatographie-Massenspektrometrie (GC-MS) oder Flüssigchromatographie-Hochauflösender-Tandem-Massenspektrometrie (LC-HR-MS/MS)-Techniken. Die Hauptschritte im Metabolismus von Naphyron waren wie folgt: Oxidation des Pyrrolidinrings zum entsprechenden Lactam, Hydroxylierung der Propylseitenkette und des Naphthylrings, Abbau des Pyrrolidinrings zum primären Amin oder Kombinationen dieser Schritte. Die mittels GC-MS identifizierten Phase I Metaboliten konnten unter Verwendung der LC-HR-MS/MS bestätigt werden. Zusätzlich konnten drei Phase II-Metaboliten (Glucuronide) identifiziert werden. Ein weiterer Teil dieser Arbeit bestand in der Identifizierung der humanen Cytochrom P450 (CYP) Isoenzyme der Leber, die den initialen Schritt im Metabolismus von Naphyron katalysieren. Unter den zehn wichtigsten humanen CYP-Enzymen waren nur die Isoenzyme CYP2C19 und CYP2C9 in der Lage die Hydroxylierung am Naphthylring in deutlichem Ausmaß zu katalysieren.

2. SUMMARY

Rapidly after scheduling mephedrone, which was one the first cathinone-derived designer drugs to be brought on the drugs of abuse market, naphyrone (1-naphthalen-2-yl-2-pyrrolidin-1-yl-pentan-1-one; naphthylpyrovalerone, β-naphyrone) appeared as replacement. Currently naphyrone itself was scheduled. Therefore, the aim of this study was to study its in vivo and in vitro metabolic fate. By means of gas chromatography-mass spectrometry (GC-MS) and liquid chromatographic-high-resolution tandem-mass spectrometric (LC-HR-MS/MS) techniques, the following metabolic steps could be observed in vivo: degradation to the primary amines after opening of the pyrrolidine ring, oxidation of the pyrrolidine ring to the corresponding lactam, hydroxylation of the propyl side chain and the naphthyl ring, and combinations of these steps. In vitro, human cytochrome P450 (CYP) isozymes CYP2C19 and CYP2C9 were identified to catalyze the hydroxylation of the naphthyl ring.

3. INTRODUCTION

In recent years, a wide range of new cathinone designer drugs appeared on the drug of abuse market. They became popular as so called “legal highs”. The probably most important representative is mephedrone, which was scheduled in April 2010.[1-3] Since then, a dramatic increase of legal alternatives distributed online and sold as “bath salts” or “plant food” and labeled not for “human consumption” to evade drug legislation was observed.[2,4,5] These substances are consumed especially amongst young adults, homosexual men, and club people.[6-8]

However, there is only little information available about their pharmacological and toxicological risks and harmful long-term effects [2,4,5] because such drugs are marketed without any safety testing. It seems that the increased recreational use of legal highs is due to the widespread availability on the Internet and the advantages of being cheap and steadily available. As earlier studies have shown, this implicates the consumers that these new substitutes were pure, safe, and carrying a low risk for health.[1] However, recent research articles have shown that most of the products contain a mixture of different cathinones.[1] Consequently, the consumers often do not know exactly what kind of compound they are taking and how to dose it.

3.1 Chemical structure of naphyrone and its similarity to mephedrone

Naphyrone (1-naphthalen-2-yl-2-pyrrolidin-1-yl-pentan-1-one; naphthylpyrovalerone, β-naphyrone) is a cathinone-derived designer drug and was marketed as replacement for the synthetic cathinone derivative mephedrone. Naphyrone is sold in preparations such as NRG-1, Energy-1, or O-2482 and available online as so-called bath salt or plant food.[1,5] Its chemical structure in comparison to mephedrone is depicted in Figure 1.

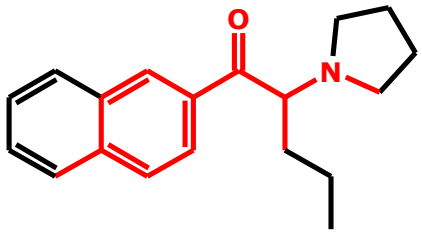

Figure 1. *Chemical structure of naphyrone. The structural part common with mephedrone is highlighted in red.*

3.2 Effects of naphyrone

Naphyrone inhibits the re-uptake of monoamines by inhibiting the serotonin, dopamine, and norepinephrine transporters with an approximately 10-fold higher potency than cocaine.[5] Entactogenic and sympathomimetic effects, such as feelings of empathy, increased energy, openness, and talkativeness are desired.[2,2,4,4,5,5] However, there is also a wide range of adverse effects such as cardiovascular risk, anxiety, hallucinations, insomnia, and nervousness.[2,4,5] According to reports found in online forums, the feeling of euphoria and high mood is supposed to be less after taking naphyrone in comparison to mephedrone and the rate of unwanted side effects is supposed to be higher.

Naphyrone showed a much higher potency with increased risk of overdose when used in amounts similar to mephedrone.[5] In general, the long-term effects of pyrrolidinophenones were not clearly defined. But especially naphyrone seemed to be even more harmful than its related derivatives, not least because of its naphthalene ring and the associated possible risk of carcinogenicity.[5]

3.3 Former publications on naphyrone

One publication is available presenting data on the metabolism of naphyrone by human liver microsomes. As with this approach only cytochrome P450 (CYP)-catalyzed metabolites could be formed, important reactions such as reactions catalyzed by alcohol or aldehyde dehydrogenase (ADH/ALDH) and the phase II reactions were missing.[9] A case report has been published about a patient suffering from acute sympathomimetic toxicity after ingestion of naphyrone.[5]

3.4 Aim of the presented work

The aim of the present study was the identification of naphyrone and its metabolites in rat urine using GC-MS and LC-HR-MS/MS. Additionally, the CYPs responsible for the initial metabolic step were identified.

4. EXPERIMENTAL PROCEDURES / MATERIAL AND METHODS

4.1 Chemicals and reagents

Naphyrone hydrochloride was obtained before scheduling from LGC (Luckenwalde, Germany), Isolute HCX cartridges (130 mg, 3 ml) from Biotage (Uppsala, Sweden) and all other chemicals and reagents from VWR, 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 naphyrone for identification of the metabolites. Urine was collected separately from the feces over a 24 h period.

4.3 Sample preparation

4.3.1 Sample preparation for phase I metabolism studies

The sample preparation was the same as described previously.[10] Briefly, a 2.0 ml portion of urine was adjusted to pH 5.2 and incubated at 56°C for 1.5 h with glucuronidase (EC No. 3.2.1.31, Merck) and arylsulfatase (EC No. 3.1.6.1). Afterwards, the urine was solid phase extracted (SPE) using HCX columns, derivatized by acetylation [11] and dissolved in 50 µl of methanol prior to injection into the GC-MS system.

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 urine dilution and desalinization, centrifuged at14.000*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 Enzymatic Studies

4.4.1 Microsomal Incubations

The microsomal incubations were the same as described previously.[10] Briefly, incubations were performed with HLM, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, or CYP3A5 at a substrate concentration of 25 µM for 30 min at 37°C.

4.5 GC-MS and LC-HR-MS/MS settings

4.5.1 GC-MS apparatus for metabolism studies

The extracts were analyzed using the same GC-MS setup as described previously.[10] Briefly, 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. a HP-1 capillary (12 m x 0.2 mm I.D.) and an injection port temperature of 280°C with a column temperature, programmed from 100-310°C at 15°/min, initial time 3 min, final time 8 min were used. The MS was operated in full-scan mode (*m/z* 50-550).

4.5.2 LC-HR-MS/MS apparatus for identification of phase I and II metabolites and for analysis of microsomal incubations

Naphyrone and its phase I and II 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.[10] 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 psi and 20 au, respectively. 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 higher energy collision dissociation (HCD) cell using

normalized collision energy of 35 eV. 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 naphyrone metabolites

The identification of the metabolites of naphyrone in rat urine resulted from full-scan EI after SPE, acetylation and GC separation. Analyzing one extract without acetylation allowed discriminating between *N*-acetyl derivatives formed by metabolism and *N*-acetyl derivatives formed by derivatization.

In accordance to common fragmentation rules and in correlation to the representative fragmentation of the unchanged naphyrone molecule, the postulated structures of the naphyrone metabolites could be detected in the EI mode.[12,13] The EI mass spectra, the main fragmentation patterns and corresponding structures of naphyrone and its acetylated metabolites are depicted in Figure 2 and 3, ranged after the ascending gas chromatographic retention indices (RI).

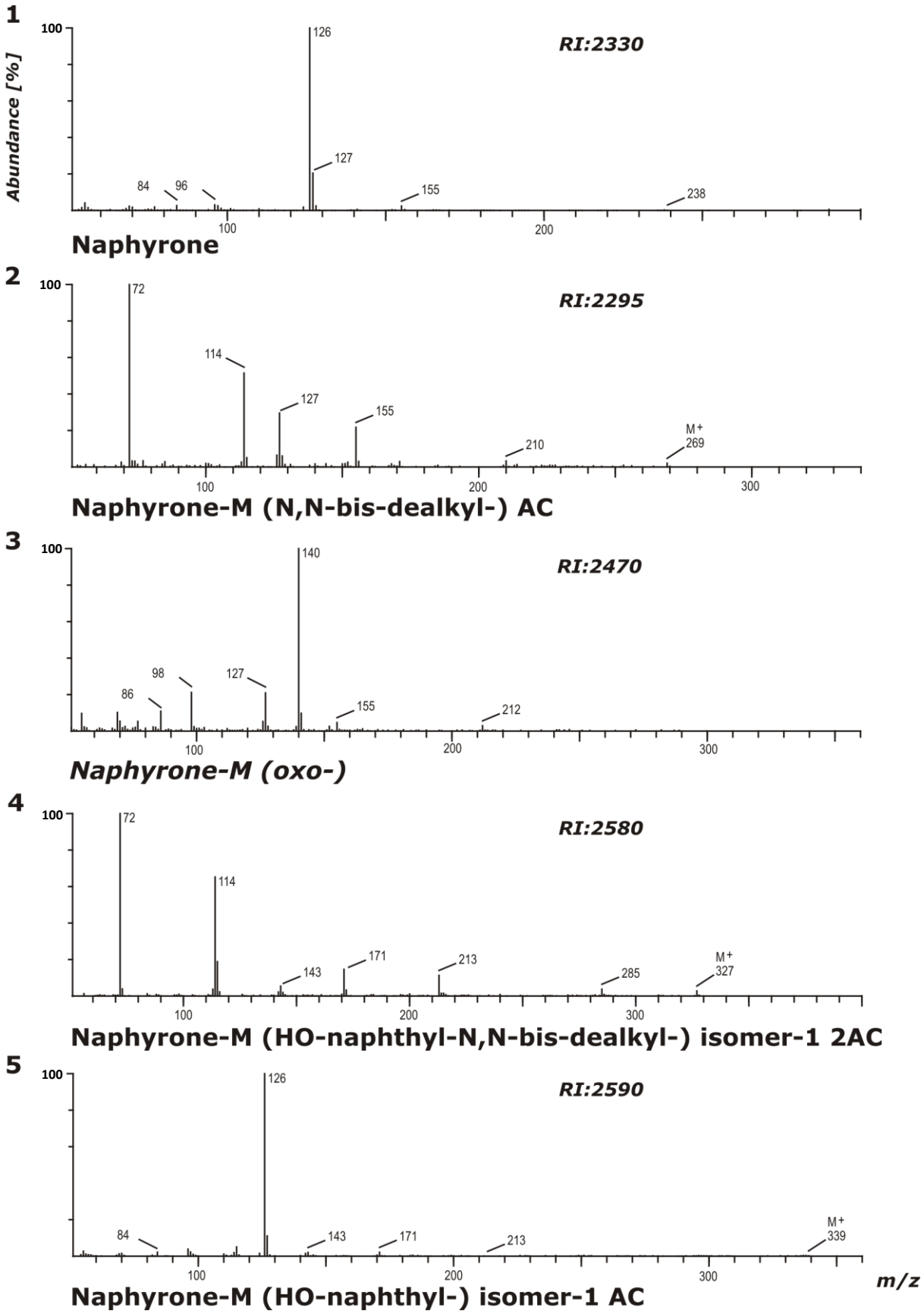

Figure 2. *EI mass spectra, gas chromatographic retention indices (RI) of naphyrone and its metabolites arranged according to their RI.*

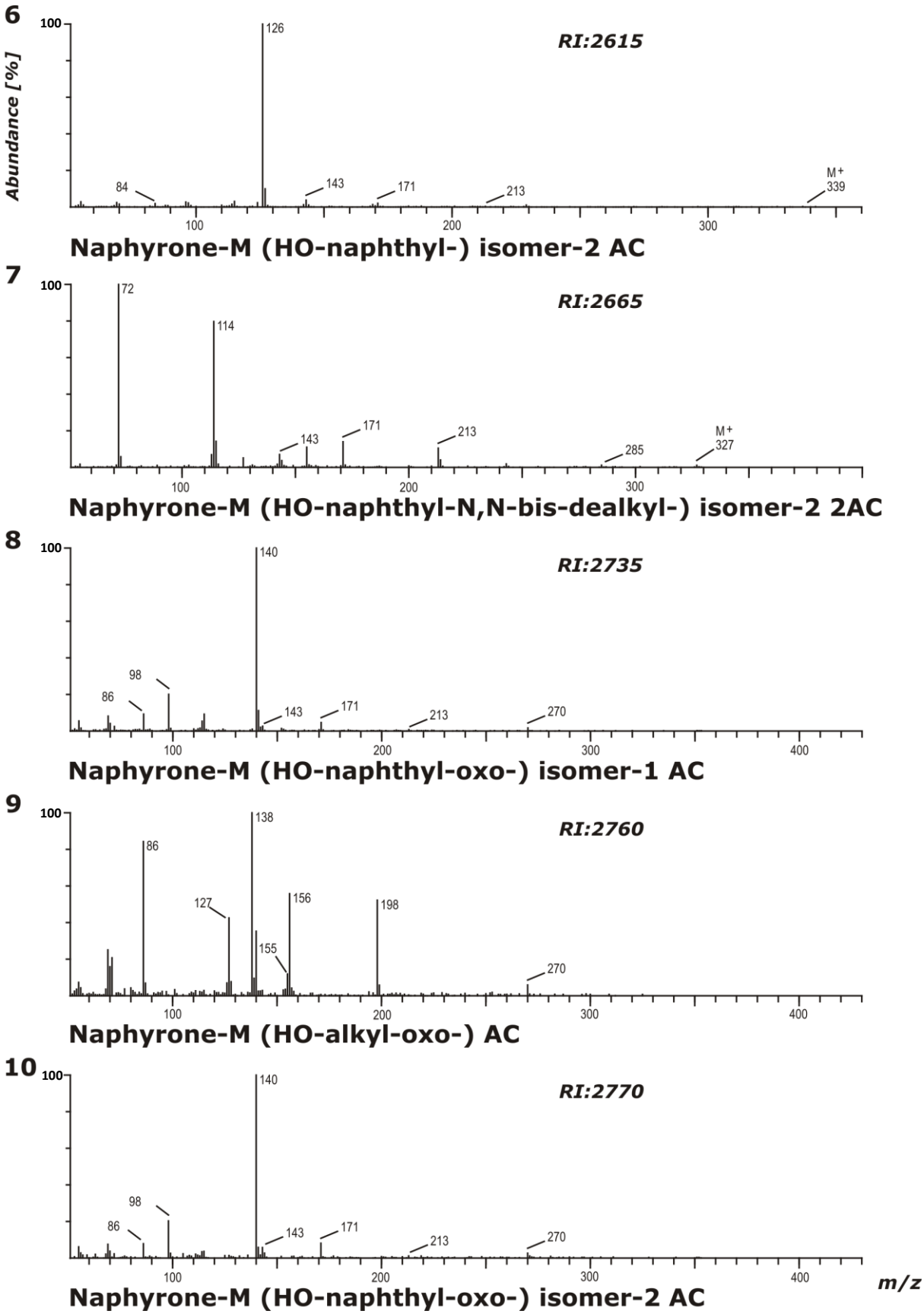

Figure 2 continued

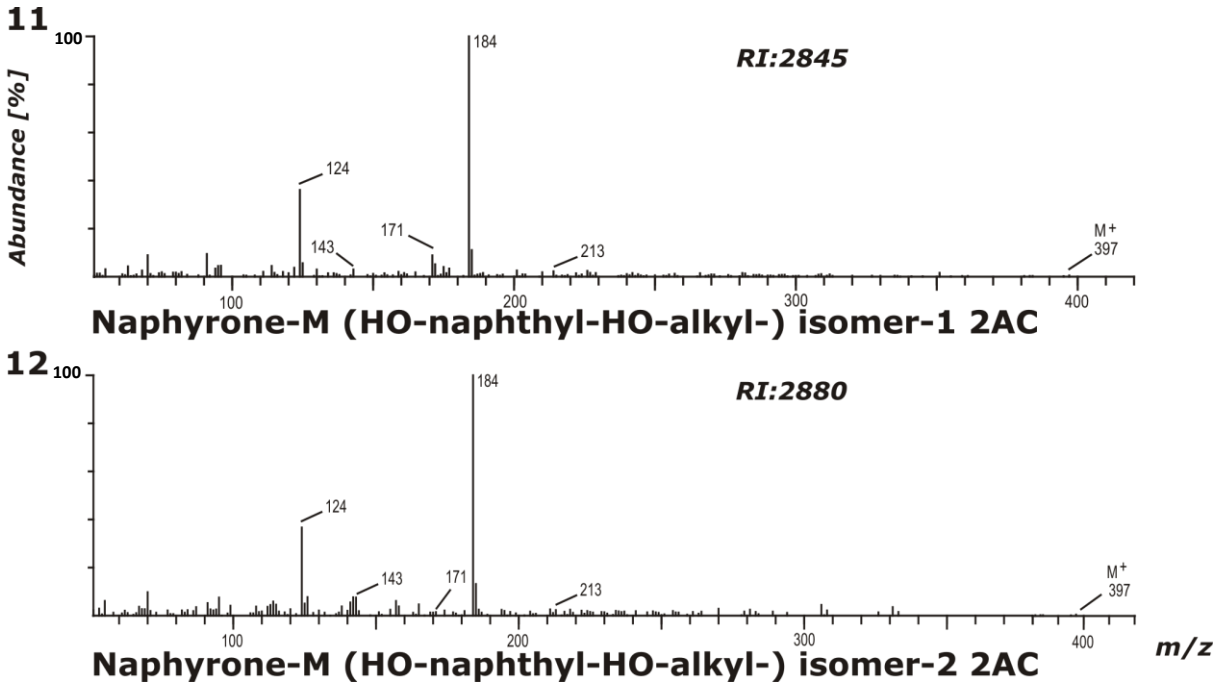

Figure 2 continued

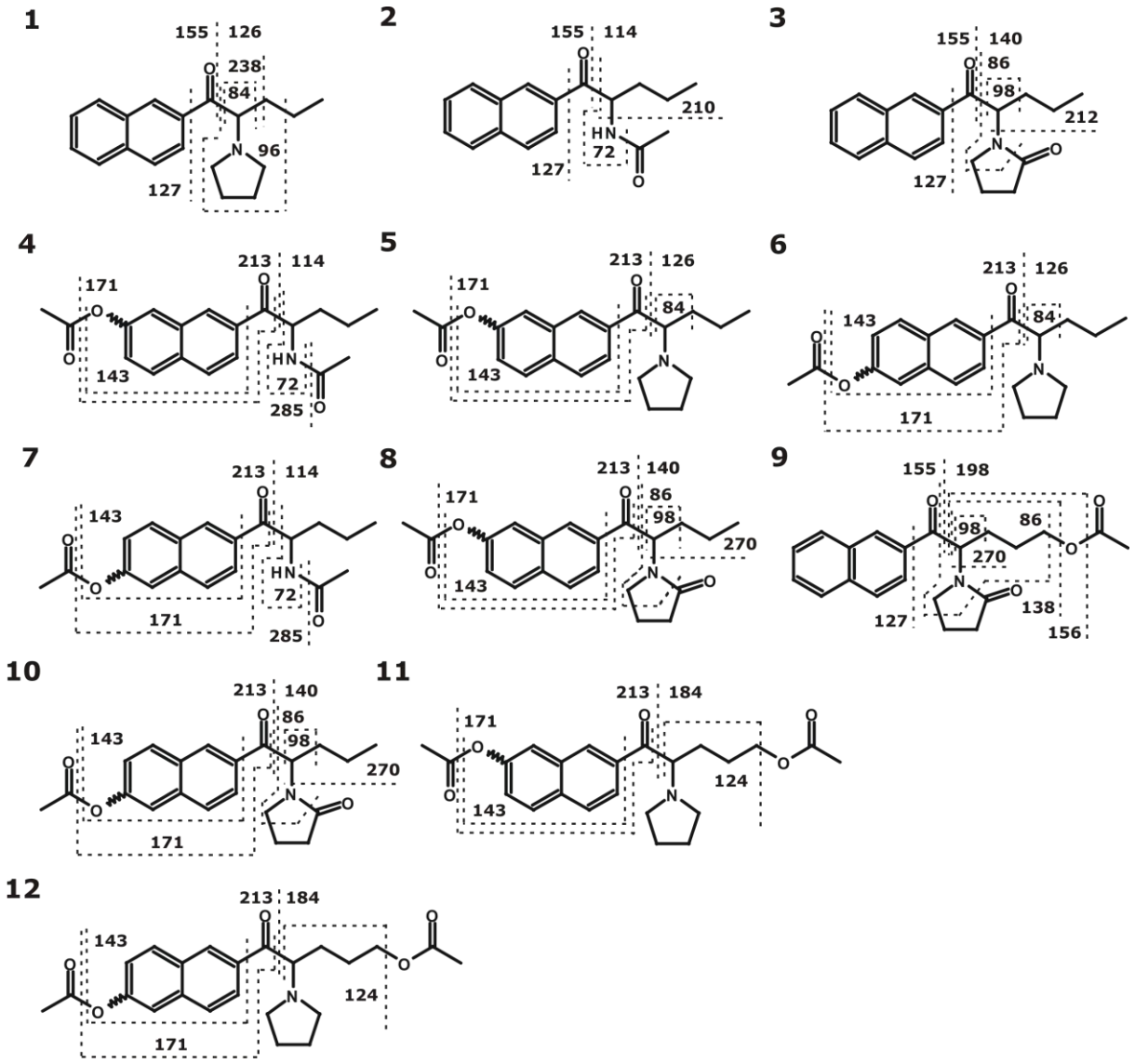

Figure 3. *Proposed structures and predominant fragmentation patterns of naphyrone and its metabolites. The numbers correspond to them in Figure 2. The wavy bonds symbolize that a definite assignment to a particular isomer was not possible.*

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

In consensus with previously published data on the fragmentation of the underivatized parent compound the expected fragmentation patterns of naphyrone metabolites could be discussed as follows.[3,5]

In the EI mass spectrum no. 1 in Figure 2 the fragmentation patterns of the unchanged parent compound are shown. A typical fragmentation step between the keto moiety and the tertiary amine is the detected α-cleavage. The base peak of this spectrum is represented by an

immonium ion at *m/z* 126. Optional α-cleavage at this position (between position 1 and 2) provides a naphthoyl ion at *m/z* 155, which is both stabilized by the mesomerism of the naphthyl part and of the carbonyl moiety. A naphthyl ion at *m/z* 127 indicates a subsequent CO loss. Another possibility for α-cleavage shows the ion at *m/z* 238, which is the result of the cleavage between the carbonyl group and the aliphatic side chain (between position 2 and 3).

Further, a combination of α-cleavage between position 1 and 2, followed by cleavage in the aliphatic side chain between position 3 and 4 yielded an ion at *m/z* 96. The ion at *m/z* 84 is the result of the loss of a methyl group from the fragment ion at *m/z 9*6. Via α-cleavage between position 1 and 2 corresponding to the parent compound in all acetylated metabolites the immonium ions at *m/z* 114 (nos. 2, 4, 7), 140 (nos. 3, 8, 10), 126 (nos. 5, 6), 198 (no. 9), or 184 (nos. 11, 12) were formed. In all of the following discussed spectra these ions always provide the base peak of the spectrum. The only exception can be seen in spectra nos. 2, 4, 7. In these spectra the ion at *m/z* 72 represents the base peak, which is the result of α-cleavage between position 1 and 2 leading to the ion at *m/z* 114 (in analogy to the formation of the ions representing the base peak) followed by a neutral loss of an acetyl part performing the base peak ion at m/z 72.

In accordance to the underivatized parent compound the naphthoyl ion at *m/z* 155 (nos. 2, 3, 9) indicates the unchanged naphthyl part. The appearance of the ions at *m/z* 213, 171, and 143 indicate the presence of the acetylated hydroxyl groups in the naphthyl part. A neutral loss of the acetyl part (distinctive shift of 42 units) leads to the ion at *m/z* 171, followed by the subsequent loss of the CO part yielding an ion at *m/z* 143 (nos. 4-8 and 10-12).

These metabolites carrying acetylated hydroxyl groups in the naphthyl part implicate the existence of at least two positional isomers. However, it must be mentioned that the exact position of the hydroxyl group in the naphthyl part cannot be deduced from the fragmentation patterns.

According to the distinctive 14 u shift (*m/z* 126 to 140) observed for the metabolites depicted in spectra nos. 3, 8, and 10, an ion at *m/z* 140 can be detected but not an additional neutral loss of an acetyl group or acetic acid. Consequently, theses metabolites implicate the existence of an oxo group in the propyl-pyrrolidine moiety. The fragment ion at *m/z* 140 leads after further cleavage (between position 2 and 3) to the fragment ion at *m/z* 98 representing the lactam part. However, it must me mentioned that the exact position of the oxo moiety in the pyrrolidino-oxo metabolites cannot be deduced from the presented fragmentation patterns.[14]

In spectrum no. 9 an absence of the distinctive 14 u shift (*m/z* 126 to 140) could be observed, though the ion at *m/z* 98 representing the lactam structure could be detected. However, based on the non-metabolic acetylation of the hydroxyl group in the propyl side chain the presence of *m/z* 198, 156, and 138 provides the recommended structure. In addition, the characteristic shift of 60 units from *m/z* 198 to 138 distinctively a neutral loss of acetic acid could be observed in spectrum no. 9, further in spectra nos. 11 and 12 (*m/z* 184 to 124). Therefore, these discussed fragmentation patterns of spectra nos. 9, 11, and 12 suppose the metabolic hydroxylation of the propyl side chain. However, it must be mentioned again that it is not possible to deduce the exact position of the hydroxyl group in the propyl side chain from the fragmentation patterns.

The fragmentation patterns of spectra nos. 2, 4, and 7 implicate the existence of metabolites containing a primary amine. Due to these non-metabolically acetylated metabolites a shift of 42 u distinctively the neutral loss of an acetyl part from the fragment ion at *m/z* 114 to 72 could be observed, after cleavage of the pyrrolidine part. The postulated metabolite structures concerning the propyl-pyrrolidine part were in accordance to previously published metabolism studies of the pyrrolidinophenone-type designer drugs containing the same structural part, such as MDPV and PVP.[[14,15]]

Due to these mass spectra, the following phase I reactions could be observed: *N*,*N*-bis-dealkylation (metabolite no.2 in Figure 3), oxidation (3), hydroxylation and *N*,*N*-bis-dealkylation (4, 7), naphthyl hydroxylation (5, 6), naphthyl hydroxylation and oxidation (8, 10), alkyl hydroxylation and oxidation (9) and naphthyl/alkyl bis-hydroxylation (11, 12).

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

The GC-MS-identified phase I metabolites of naphyrone could be verified using the LC-HR-MS/MS procedure. Furthermore, glucuronides of some phase I metabolites (nos. 5, 6, 8, and 10-12) were additionally identified. The calculated exact masses of the protonated and underivatized naphyrone and its corresponding phase I and II metabolites are shown in Table 1.

Table 1. *List of naphyrone and its phase I and II metabolites, the measured exact masses of their protonated molecule and representative fragment ions, the corresponding calculated exact masses, and the suggested elemental compositions.*

Metabolites and characteristic ions [*m/z*]	Calculated exact masses [u]	Elemental compositions
naphyrone, 282.1851	**282.1858 [M+H]$^+$**	C19H24NO
211.1116	211.1117	C15H15O
141.0967	141.0699	C11H9
126.1278	126.1283	C8H16N
***N,N*-bis-dealkyl naphyrone, 228.1376**	**228.1388 [M+H]$^+$**	C15H18NO
210.1276	210.1283	C15H16N
168.0806	168.0813	C12H10N
141.0698	141.0704	C11H9
oxo naphyrone, 296.1638	**296.1650 [M+H]$^+$**	C19H22NO2
141.0696	141.0699	C11H9
140.1069	140.1070	C8H14NO
98.0603	98.0600	C5H8NO
hydroxy-naphthyl-*N,N*-bis-dealkyl naphyrone, 244.1332	**244.1337 [M+H]$^+$**	C15H18NO2
226.1225	226.1232	C15H16NO
183.0679	183.0684	C12H9NO
157.0647	157.0653	C11H9O
hydroxy-naphthyl naphyrone, 298.1797	**298.1807 [M+H]$^+$**	C19H24NO2
227.1065	227.1072	C15H15O2
157.0647	157.0648	C11H9O
126.1277	126.1283	C8H16N
hydroxy-naphthyl-oxo naphyrone, 312.1591	**312.1600 [M+H]$^+$**	C19H22NO3
227.1065	227.1072	C15H15O2
157.0647	157.0648	C11H9O
140.1069	140.1070	C8H14NO
hydroxy-alkyl-oxo naphyrone, 312.1589	**312.1600 [M+H]$^+$**	C19H22NO3
270.1122	270.1130	C16H16NO3
252.1017	252.1024	C16H14NO2
224.1068	224.1075	C15H14NO
hydroxy-naphthyl-hydroxy-alkyl naphyrone, 314.1745	**314.1756 [M+H]$^+$**	C19H24NO3
227.1065	227.1072	C15H15O2
157.0647	157.0648	C11H9O
142.1226	142.1226	C8H16NO
hydroxy-naphthyl naphyrone glucuronide, 474.2121	**474.2128 [M+H]+**	C25H32NO8
298.1798	298.1807	C19H24NO2
227.1064	227.1072	C15H15O
157.0647	157.0648	C11H9O2
hydroxy-naphthyl-oxo naphyrone glucuronide, 488.1916	**488.1920 [M+H]+**	C25H30NO9
312.1592	312.1600	C15H15O2
157.0647	157.0648	C11H9O
140.1065	140.1070	C8H14NO
hydroxy-naphthyl-hydroxy-alkyl naphyrone glucuronide, 490.2069	**490.2077 [M+H]+**	C25H32NO9
314.1747	314.1756	C19H24NO3
227.1064	227.1072	C15H15O2
142.1225	142.1226	C8H16NO

5.4 Proposed metabolic pathways

From the above-described metabolites, the following partly overlapping metabolic pathways for naphyrone in rats could be postulated: hydroxylation of the pyrrolidine ring followed by dehydrogenation to analogous lactams (nos. 3 and 8-10), hydroxylation of the aliphatic side chain (9) or the naphthyl ring system (nos. 4-8, and 10), or both combined (nos. 11 and 12), and last-mentioned, degradation of the pyrrolidine ring to the corresponding primary amines (nos. 2, 4, and 7). In Figure 4, arrows are indicating areas of described metabolic reactions.

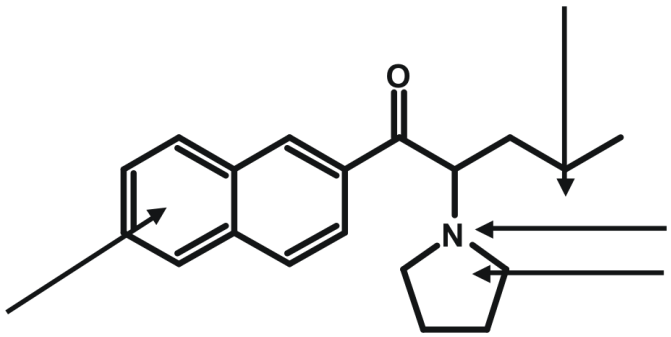

Figure 4. *Structures of naphyrone with arrows indicating the areas of the described metabolic reactions.*

5.5 Initial CYP screening

In order to identify the CYP enzymes, which were able to catalyze the hydroxylation at the naphthyl part of the molecule, an initial screening with the ten most abundant human hepatic CYPs was performed. Exclusively CYP2C19 and CYP2C9 were capable of catalyzing the hydroxylation of the naphthyl part.

6. CONCLUSIONS

The presented study revealed that naphyrone is extensively metabolized in rats and that the initial human metabolic reaction should be the hydroxylation of the naphthyl part of the molecule. This reaction could be shown to be catalyzed by CYP2C19 and CYP2C9, which may lead to severe interactions after co-ingestion of respective CYP inhibitors or in the case of a slow metabolizing phenotype. Due to the enormous increase of new Designer Drugs and the corresponding health risks, it is an important issue to identify and to study new emerged substances. In summary, this study could contribute to identification and detection of the recreational drug naphyrone by elucidating its metabolic pathways in both species. Supposing similar kinetic processes in rats and humans, this study could serve as a basis for developing suitable screening strategies for detection of a naphyrone intake. This will be investigated in a further study.

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8. ABBREVIATIONS

EI	Electron Ionization
FWHM	full width at half maximum
GC-MS	gas chromatography- mass spectrometry
HCD	Higher-energy C-trap dissociation
LC-HR-MS	liquid chromatography- high resolution- mass spectrometry
LC-MS	liquid chromatography- mass spectrometry
$LC\text{-}MS^n$	liquid chromatography- ion trap mass spectrometry
m/z	mass-to-charge ratio
RI	Gas Chromatographic Retention Index

9. DANKSAGUNG

Die vorliegende Dissertation entstand in der Abteilung für Experimentelle und Klinische Toxikologie an der Medizinischen Fakultät der Universität des Saarlandes in Homburg/Saar.

Herrn Prof. Dr. Dr. h.c. Hans H. Maurer danke ich für die Überlassung dieses interessanten Themas, die gute Aufnahme in seinen Arbeitskreis, und seine vielfältige Unterstützung.
Ebenso möchte ich mich für die von ihm bereitgestellten Materialien und Geräte bedanken.

Ganz besonders danke ich Dr. Markus R. Meyer für die hervorragende und kompetente Betreuung, seine stete Diskussionsbereitschaft und seinen allgegenwärtigen Optimismus bezüglich dieser Arbeit.

Dank gebührt auch allen wissenschaftlichen und technischen Mitarbeitern und Mitarbeiterinnen des Institutes für Ihre Hilfsbereitschaft und das angenehme Arbeitsklima. Besonders hervorzuheben sind Herr Armin Weber, der mir bei allen technischen Belangen eine große Hilfe war und Frau Gabriele Ulrich, die bei labortechnischen Fragen immer einen passenden Rat hatte.

Nicht zu Letzt danke ich all denen, die mich während meines Studiums stets unterstützten und an mich geglaubt haben. Sowohl meine Familie, insbesondere mein Opa, als auch meine Freunde haben alle auf ihre Weise maßgeblich zum Gelingen dieser Arbeit beigetragen.

Ein großes Dankeschön gilt außerdem Sara Schmitt für ihre Freundschaft und vielseitige Unterstützung während des gesamten Studiums. Sie hatte großen Anteil an der Fertigstellung dieser Arbeit.