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Studies on the metabolism and detectability of the designer drug β-naphyrone in rat urine using GC-MS and LC-HR-MS/MS

Markus R. Meyer,* Denise Prosser and Hans H. Maurer

Naphyrone (1-naphthalen-2-yl-2-pyrrolidin-1-yl-pentan-1-one; naphthylpyrovalerone, β-naphyrone) is a cathinone designer drug and was marketed as replacement for the synthetic cathinone derivative mephedrone. Meanwhile, naphyrone is also classified as a controlled drug in several countries. Therefore, the aim of this study was to identify the metabolites of naphyrone in rat urine using gas chromatography-mass spectrometry techniques and to show its detectability in urine samples. The following metabolic steps could be detected in rat urine: oxidation of the pyrrolidine ring to the corresponding lactam, hydroxylation of the propyl side chain and the naphthyl ring, degradation to the primary amines after opening of the pyrrolidine ring, and combinations of these steps. Assuming similar kinetics, an intake of naphyrone should be detectable in human urine mainly via its metabolites.

Keywords: designer drug; naphyrone; GC-MS; metabolism

Introduction

Naphyrone (1-naphthalen-2-yl-2-pyrrolidin-1-yl-pentan-1-one; naphthylpyrovalerone, β-naphyrone) is a novel pyrrolidinophenone-type drug of abuse and also belongs to the class of cathinone drugs. It is distributed in preparations such as NRG-1, Energy-1, or O-2482 and procurable online as so-called bath salt or plant food.[1] As it shows pharmacological similarity to mephedrone, it appeared on the drug of abuse market in the UK at the time when mephedrone was scheduled in April 2010.[1–3] In the meantime, naphyrone was also classified as a controlled drug in for example, UK and Germany.[4] Besides the mentioned β-isomer, the corresponding α-isomer is known as abused drug.[5]

After consumption of naphyrone, entactogenic and sympathomimetic effects were described as well as a wide range of adverse effects such as cardiovascular risk.[2,4,6] Naphyrone showed a high risk of overdose when used in amounts similar to mephedrone. Furthermore, one case about a patient suffering from acute sympathomimetic toxicity after ingestion of naphyrone has been published.[4] In general, the long-term effects of pyrrolidinophenones were not clearly defined. Naphyrone was recently characterized as a non-selective monoamine uptake inhibitor, similar to cocaine as previously shown also for other pyrrolidinophenones.[7,8] Concerning the metabolism of naphyrone, only one publication is available presenting data after its incubation with human liver microsomes. Unfortunately, this approach was only able to produce cytochrome P450 (CYP)-catalyzed metabolites lacking of important reactions such as reactions catalyzed by alcohol or aldehyde dehydrogenase (ADH/ALDH) and the phase II reactions.[9]

Therefore, the aim of the presented work was to identify the metabolites of naphyrone in rat urine using gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-high resolution-tandem mass spectrometry (LC-HR-MS/MS) and to show its detectability by the general GC-MS[10–12] and LC-MS^n[13,14] screening approaches described by the authors.

Experimental

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.

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 and a single of 1 mg/kg body mass dose for toxicological analysis. Urine was collected separately from the faeces over a 24-h period.

Sample preparation for phase I metabolism studies

The sample preparation was the same as described previously.[12] 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.

* *Correspondence to: Markus R. Meyer, Department of Experimental and Clinical Toxicology, Saarland University, D-66421 Homburg (Saar), Germany. E-mail: markus.meyer@uks.eu*

Department of Experimental and Clinical Toxicology, Institute of Experimental and Clinical, Pharmacology and Toxicology, Saarland University, D-66421 Homburg, Saar, Germany

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 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.

Sample preparation for the standard screening approaches

For GC-MS, urine was worked-up (hydrolysis, extraction, and microwave-assisted acetylation) according to published procedures.[11,15] For LC-MS^n, urine was worked-up (dilution and desalinization) according to previously published procedures.[14]

Microsomal incubations

The microsomal incubations were the same as described previously.[12] 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.

GC-MS apparatus for metabolism studies and standard screening approach

The extracts were analyzed using the same GC-MS setup as described previously.[12] Briefly, 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). For toxicological detection of the acetylated drug and its most abundant metabolites, mass chromatography was used with the selected ions *m/z* 126, 138, 140, 184, representing spectra Nos. 1, 3, 5, 6, and 8 – 12 in Figure 1.[10,11] Additionally, the data files acquired by the GC-MS system were evaluated by AMDIS (http://chemdata.nist.gov/mass-spc/amdis/) as described.[16]

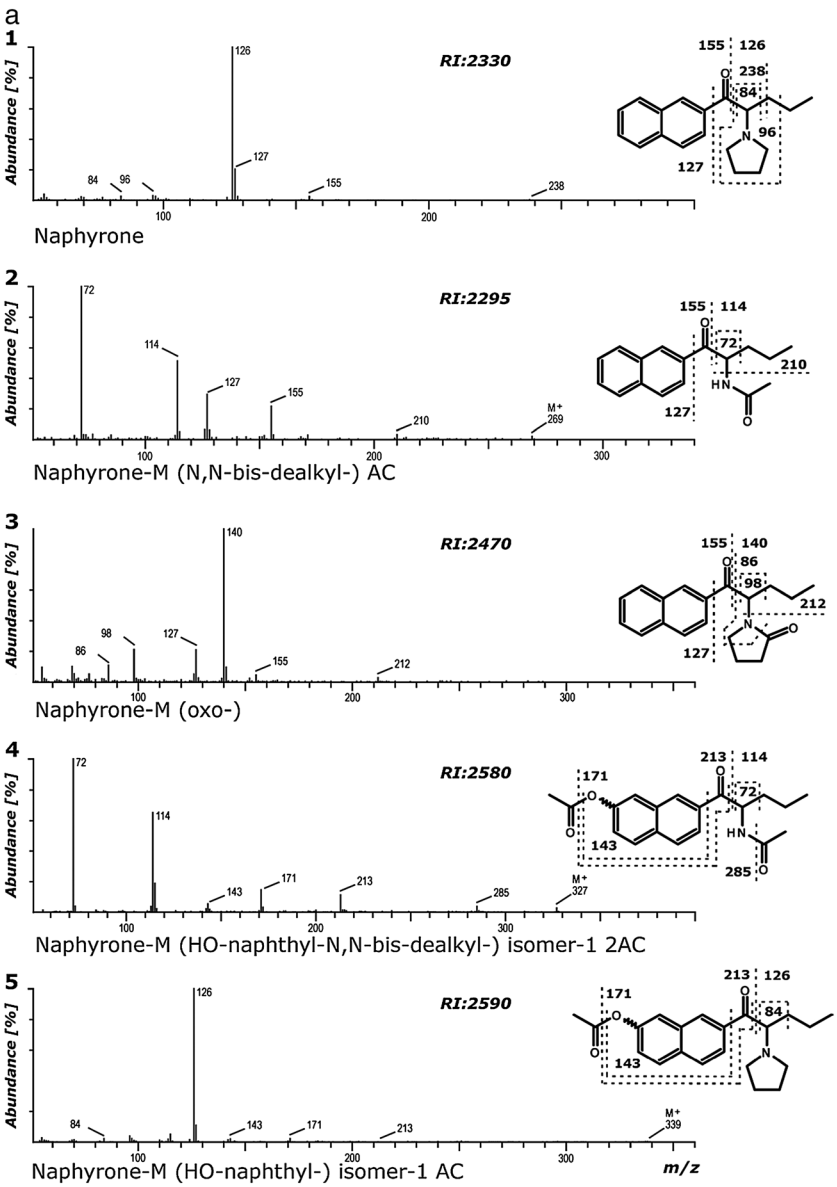

Figure 1. EI mass spectra, gas chromatographic retention indices (RI), proposed structures, and predominant fragmentation patterns of naphyrone and its metabolites arranged according to their RI. The wavy bonds symbolize that a definite assignment to a particular isomer was not possible.

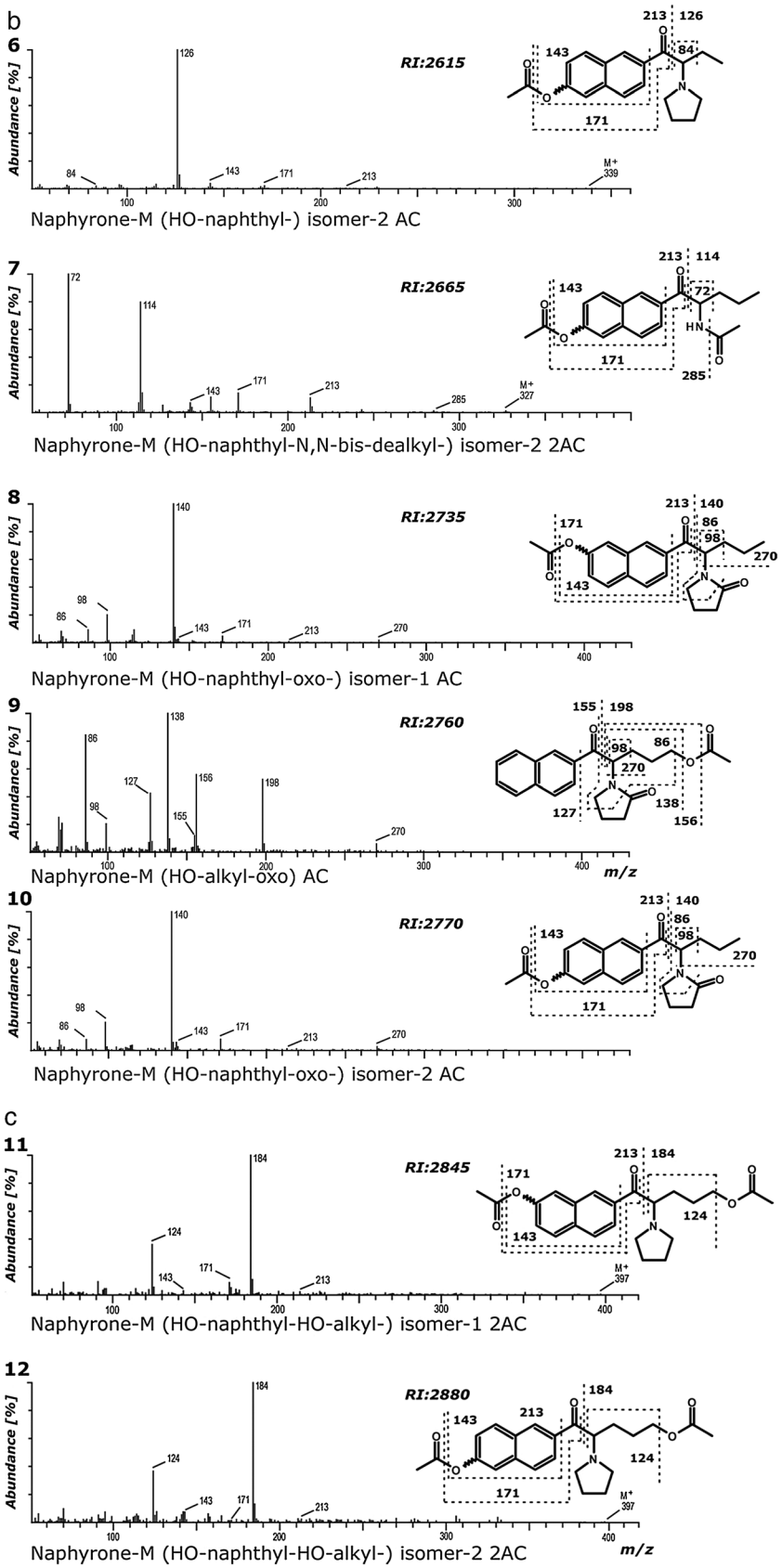

Figure 1. (Continued)

Table 1. List of naphyrone 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 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

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.[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 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.

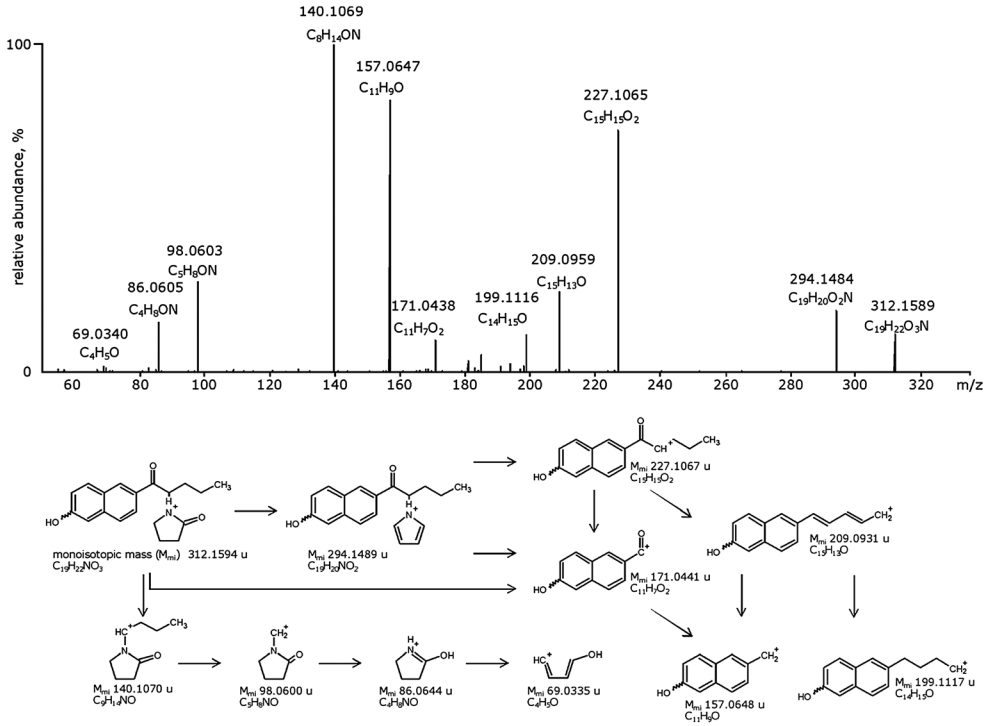

Figure 2. HCD mass spectrum and corresponding fragmentation patterns of one hydroxy-naphthyl-oxo naphyrone isomer.

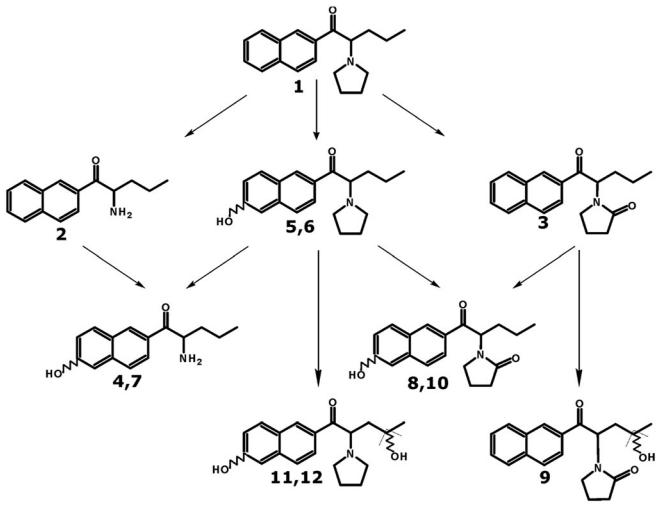

Figure 3. Proposed pathway for the phase I metabolism of naphyrone in rats.

LC-MS^n apparatus for standard screening approach

A TF linear ion trap LXQ coupled to an TF Accela LC was applied under conditions described previously.[14] Briefly, samples were worked-up by urine dilution using ACN. Data-dependent acquisition (DDA) on precursor ions selected from MS^1 was performed in the full scan mode. MS^2 and MS^3 were performed in the DDA mode: four DDA MS^2 scan filters were chosen to provide MS^2 on the four most intense signals from MS^1 and additionally, eight MS^3 scan filters were chosen to record MS^3 on the most and second most intense signals from the MS^2. MS^2 spectra were collected with a higher priority than MS^3 spectra.

Results and discussion

Identification of naphyrone metabolites

The urinary metabolites of naphyrone were identified after SPE and acetylation by full-scan EI after GC separation. Extracts were also analyzed without acetylation to differentiate whether *N*-acetyl derivatives were formed by metabolism or derivatization.

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 common fragmentation rules.[17,18] EI mass spectra, the gas chromatographic

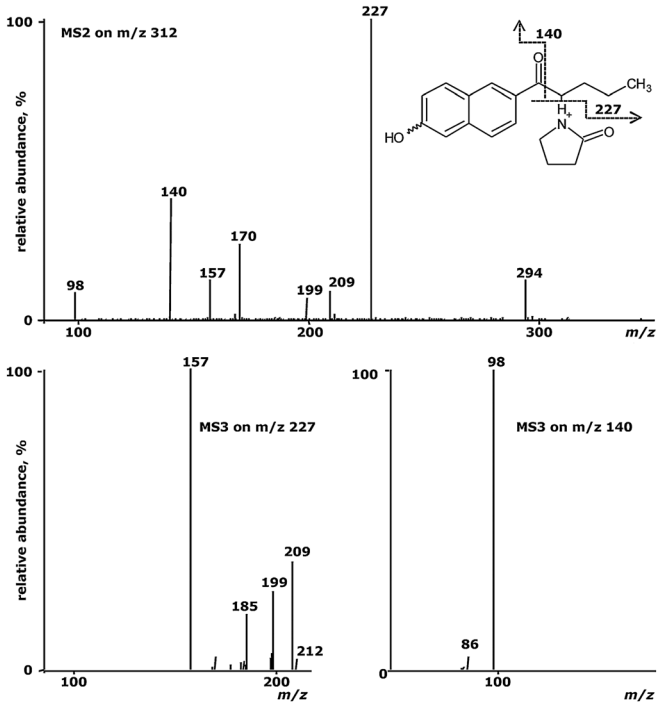

Figure 4. MS^2 and MS^3 linear ion trap mass spectra of one hydroxy-naphthyl-oxo naphyrone isomer detected using the LC-MS^n procedure.

retention indices (RI), structures and predominant fragmentation patterns of naphyrone as well as of its acetylated metabolites are shown in Figure 1.

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

The general fragmentation patterns of naphyrone metabolites were in accordance with previously published data on the unchanged parent compound.[3,5]

The EI mass spectrum of naphyrone (spectrum no. 1 in Figure 1) shows an α-cleavage between position 1 and 2 (between keto moiety and tertiary amine). This results in an immonium ion at *m/z* 126 representing the base peak of the spectrum. This α-cleavage also results in a naphthoyl ion at *m/z* 155, which is stabilized both by the mesomerism of the naphthyl ring and of the carbonyl part. As consequence of a subsequent CO loss, a naphthyl ion at *m/z* 127 is formed and represents the naphthyl part. Furthermore, α-cleavage between position 2 and 3 (between tertiary carbon and aliphatic side chain) leads to ion at *m/z* 238 and α-cleavage between position 3 and 4 (in the aliphatic side chain) followed by a cleavage between position 1 and 2 leads to ion at *m/z* 96. A further loss of a methyl part from the fragment ion at *m/z* 96 leads to ion at *m/z* 84.

In analogy to the parent compound, for all acetylated metabolites an α-cleavage between position 1 and 2 could be observed leading to 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), which are still representing the respective base peaks of the spectrum, with exception of *m/z* 114. All metabolites with an unchanged naphthyl part are represented by the naphthoyl ion at *m/z* 155 (Nos. 2, 3, 9) according to the parent compound. For acetylated hydroxyl groups in the naphthyl part, the ions at *m/z* 213, 171, and 143 (loss of the acetyl part and CO part, respectively) are characteristically formed after α-cleavage between position 1 and 2 (Nos. 4–8 and 10–12). These spectra also imply the existence of at least two positional isomers concerning this hydroxylation. However, the exact position of the hydroxyl group in the naphthyl ring system cannot be deduced from the fragmentation patterns.

In spectra 3, 8, and 10, an ion at *m/z* 140 can be observed but not a neutral loss of an acetyl group or acetic acid, implicating the metabolic introduction of an oxo-group into the molecule. After further cleavage between position 2 and 3, the fragment ion at *m/z* 140 leads to the fragment ion at *m/z* 98, which represents the lactam part. However, the exact position of the oxo group in the pyrrolidino-oxo metabolites cannot be deduced from the fragmentation patterns.[19]

Due to the non-metabolic acetylation of the hydroxy group in the propyl side chain besides the lactam structure, the distinctive 14 u shift (*m/z* 126 to 140) could not be observed in spectrum no 9. However, the presence of *m/z* 198, 156, and 138 supports the suggested structure. Furthermore, in spectrum no. 9, the neutral loss of acetic acid (60 u) is represented by a shift from *m/z* 198 to 138. This shift can also be observed in spectra Nos. 11 and 12 (*m/z* 184 to 124). Consequently, spectra Nos. 9, 11, and 12 indicate the metabolic introduction of a hydroxyl group in the propyl side chain. Again it is not possible to deduce the exact position of the hydroxyl group in the propyl side chain from the fragmentation patterns.

The non-metabolically acetylated metabolites represented by spectra 2, 4, and 7 are supposed to contain a primary amine. This is due to the neutral loss of an acetyl part corresponding to the loss of 42 u from the fragment ion at *m/z* 114 to 72 after cleavage of the pyrrolidine ring.

The metabolism of the above mentioned propyl-pyrrolidine part is comparable to the already described metabolism of other pyrrolidinophenone-type designer drugs containing the same structural part, such as MDPV and PVP.[19,20]

From these mass spectra, the following phase I metabolites could be deduced: *N,N*-bis-dealkyl naphyrone (spectrum No. 2 in Figure 1),

oxo naphyrone (3), hydroxy-naphthyl-*N,N*-bis-dealkyl naphyrone (4, 7), hydroxy-naphthyl naphyrone (5, 6), hydroxy-naphthyl-oxo naphyrone (8, 10), hydroxy-alkyl-oxo naphyrone (9) and hydroxy-naphthyl-hydroxy-alkyl naphyrone (11, 12).

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

Using the LC-HR-MS/MS procedure, the GC-MS-identified phase I metabolites could be confirmed. The metabolites Nos. 5, 6, 8, and 10–12 were additionally excreted as phase II glucuronides. In Table 1, all detected metabolites are listed together with the measured accurate masses of their protonated molecule and characteristic fragment ions, the corresponding calculated exact masses and their proposed elemental compositions. As example for the HCD fragmentation of the metabolites, the mass spectrum and corresponding fragmentation pattern of one hydroxy-naphthyl-oxo naphyrone isomer is depicted in Figure 2.

Proposed metabolic pathways

The following partly overlapping metabolic pathways in rat could be deduced (Figure 3): degradation of the pyrrolidine ring to corresponding lactams (Nos. 3 and 8–10), hydroxylation of either the propyl side chain (9) or the naphthyl ring system (Nos. 4–8, and 10), or combination of both (Nos. 11 and 12), and finally, degradation of the pyrrolidine ring to primary amines (Nos. 2, 4, and 7).

Toxicological detection by GC-MS or LC-MS^n

Naphyrone and its metabolites were separated and identified by GC full-scan EI MS after fast acidic hydrolysis, liquid-liquid extraction, and acetylation of the low-dose rat urine.[11,15] The main target of naphyrone identified in this rat urine sample was oxo naphyrone. Additionally, the full-scan data files acquired by the GC-MS system were evaluated by AMDIS allowing its detection using the previously described procedure.[16] Also using the general LC-MS^n screening procedure, the parent compound and its metabolites were detectable.[14] The MS^n mass spectra of one hydroxy-naphthyl-oxo naphyrone isomer, one of the most abundant metabolites detected using the LC-MS^n procedure, are shown in Figure 4.

Initial CYP screening

The initial screening studies were adequate to make a statement on the general involvement of a particular CYP enzyme in the initial metabolic step of naphyrone, which was observed to be the hydroxylation at the naphthyl part of the molecule. Among the tested CYPs, only CYP2C19 and CYP2C9 were markedly capable to catalyze this reaction.

Conclusion

The presented study showed that the new designer drug naphyrone was extensively metabolized. The GC-MS and LC-MS^n standard screening approaches should be suitable for detection of naphyrone and/or its metabolites in human urine after intake of a recreational user's dose assuming similar kinetics.

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