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ORIGINAL PAPER

Beta-keto amphetamines: studies on the metabolism of the designer drug mephedrone and toxicological detection of mephedrone, butylone, and methylone in urine using gas chromatography–mass spectrometry

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Abstract In recent years, a new class of designer drugs has appeared on the drugs of abuse market in many countries, namely, the so-called beta-keto (bk) designer drugs such as mephedrone (bk-4-methylmethamphetamine), butylone (bk-MBDB), and methylone (bk-MDMA). The aim of the present study was to identify the metabolites of mephedrone in rat and human urine using GC-MS techniques and to include mephedrone, butylone, and methylone within the authors' systematic toxicological analysis (STA) procedure. Six phase I metabolites of mephedrone were detected in rat urine and seven in human urine suggesting the following metabolic steps: *N*-demethylation to the primary amine, reduction of the keto moiety to the respective alcohol, and oxidation of the tolyl moiety to the corresponding alcohols and carboxylic acid. The STA procedure allowed the detection of mephedrone, butylone, methylone, and their metabolites in urine of rats treated with doses corresponding to those reported for abuse of amphetamines. Besides macro-based data evaluation, an automated evaluation using the automated mass spectral deconvolution and identification system was performed. Mephedrone and butylone could be detected also in human urine samples submitted for drug testing. Assuming similar kinetics in humans, the described STA procedure should be suitable for proof of an intake of the bk-designer drugs in human urine.

Keywords Mephedrone · Butylone · Methylone · Designer drug · Metabolism · GC-MS · AMDIS

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Introduction

In recent years, a new class of designer drugs has appeared on the drugs of abuse market in many countries, namely, the so-called beta-keto (bk) designer drugs such as butylone (2-methylamino-1-(3,4-methylenedioxyphenyl)butan-1-one, bk-MBDB), ethylone (2-ethylamino-1-(3,4-methylenedioxyphenyl)propan-1-one, bk-3,4-methylenedioxyethylamphetamine, bk-MDEA), methylone (2-methylamino-1-(3,4-methylenedioxyphenyl)propan-1-one, bk-3,4-methylenedioxymethamphetamine, bk-MDMA), and mephedrone (2-methylamino-1-*p*-tolylpropane-1-one, 4-methyl-methcathinone, bk-4-methylmethamphetamine) [1–3]. In Fig. 1, their chemical structures are depicted. Due to their chemical similarity to amphetamines or methcathinone and the use as alternatives for these drugs, a similar stimulant effect of the bk-designer drugs could be postulated. Additionally, methylone showed strong inhibitory effects on the re-uptake of dopamine, serotonin, and norepinephrine [4]. It increased

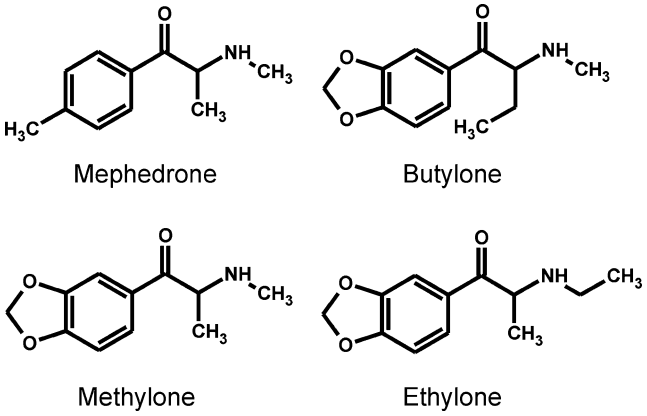


Fig. 1 Chemical structures of beta-keto-type designer drugs

serotonin and norepinephrine release, but had little effect on dopamine release using rat brain synaptosomes [4]. Mephedrone was recently scheduled in Germany because of its potential for addiction and the associated health risks. Therefore, an intake of such drugs must be monitored in clinical and forensic toxicology and doping control. As many psychotropic drugs are extensively metabolized, they can be detected particularly in urine only via their metabolites [5]. In contrast to mephedrone, the metabolism of butylone, ethylone, and methylone has already been described [2, 6].

Therefore, one aim of the present study was to identify the mephedrone metabolites in rat and human urine using GC-MS in the electron impact (EI) and positive-ion chemical ionization (PICI) mode. The second aim was to include mephedrone, butylone, and methylone into the authors' systematic toxicological analysis (STA) procedure for drugs in urine using full-scan EI GC-MS [5, 7].

Experimental

Chemicals and reagents

Mephedrone-HCl, butylone-HCl, and methylone-HCl were purchased from www.EU-Legals.com (Austria) before it was scheduled. Isolute Confirm HCX cartridges (130 mg, 3 ml) were obtained from Separtis (Grenzach-Wyhlen, Germany). Diazomethane was synthesized in the authors' laboratory according to the procedure of McKay et al. [8]. All other chemicals and biochemicals used were obtained from Merck, Darmstadt (Germany) and were of analytical grade.

Urine samples

The investigations were performed using male rats (Wistar, Ch. River, Sulzfleck, Germany) for toxicological diagnostic reasons according to the corresponding German law. They were administered a single 20 mg/kg body mass dose (for metabolism studies) or a 1 mg/kg body mass dose (for development of the screening procedure) of drugs in an aqueous suspension by gastric intubation. Urine was collected separately from the feces over a 24-h period. All samples were directly analyzed and then stored at −20 °C until further analysis. Blank urine samples were collected before drug administration to check whether the samples were free of interfering compounds.

Human urine samples were submitted for clinical toxicological analysis to the authors' laboratory. The patient claimed to have orally taken mephedrone and butylone powder.

Sample preparation for metabolism studies

A 0.5-ml portion of urine was adjusted to pH 5.2 with acetic acid (1 *M*, approximately 10–50 µl) and incubated at 56 °C for 1.5 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) from *Helix pomatia* L. (Roman snail). For solid-phase extraction, the urine sample was then diluted with 2.5 ml of water and loaded on a Confirm HCX 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 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 methylation, acetylation, or combined methylation and acetylation [5, 7]. After evaporation of the derivatization mixture, the residue was dissolved in 100 µl of methanol, and 2 µl was injected into the GC-MS system.

Methylation was performed after reconstitution in 50 µl of methanol with 50 µl of a solution of diazomethane in diethyl ether. The reaction vials were sealed and left at room temperature for 30 min. 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. After evaporation, the residue was dissolved in 100 µl of methanol. A 3-µl aliquot each was injected into the GC-MS system. In case of combined methylation/acetylation, extracts were first methylated and subsequently acetylated. The same experiments were repeated without the use of enzymatic hydrolysis to study which metabolites of mephedrone were excreted as glucuronides/sulfates.

Sample preparation for STA

A 5-ml portion of urine was worked up as previously described [5, 9]. The urine samples were divided into two 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 M aqueous ammonium sulfate and 1.5 ml of 10 M aqueous sodium hydroxide to obtain a pH value of 8–9. Before extraction, the other aliquot of native urine was added. This mixture was

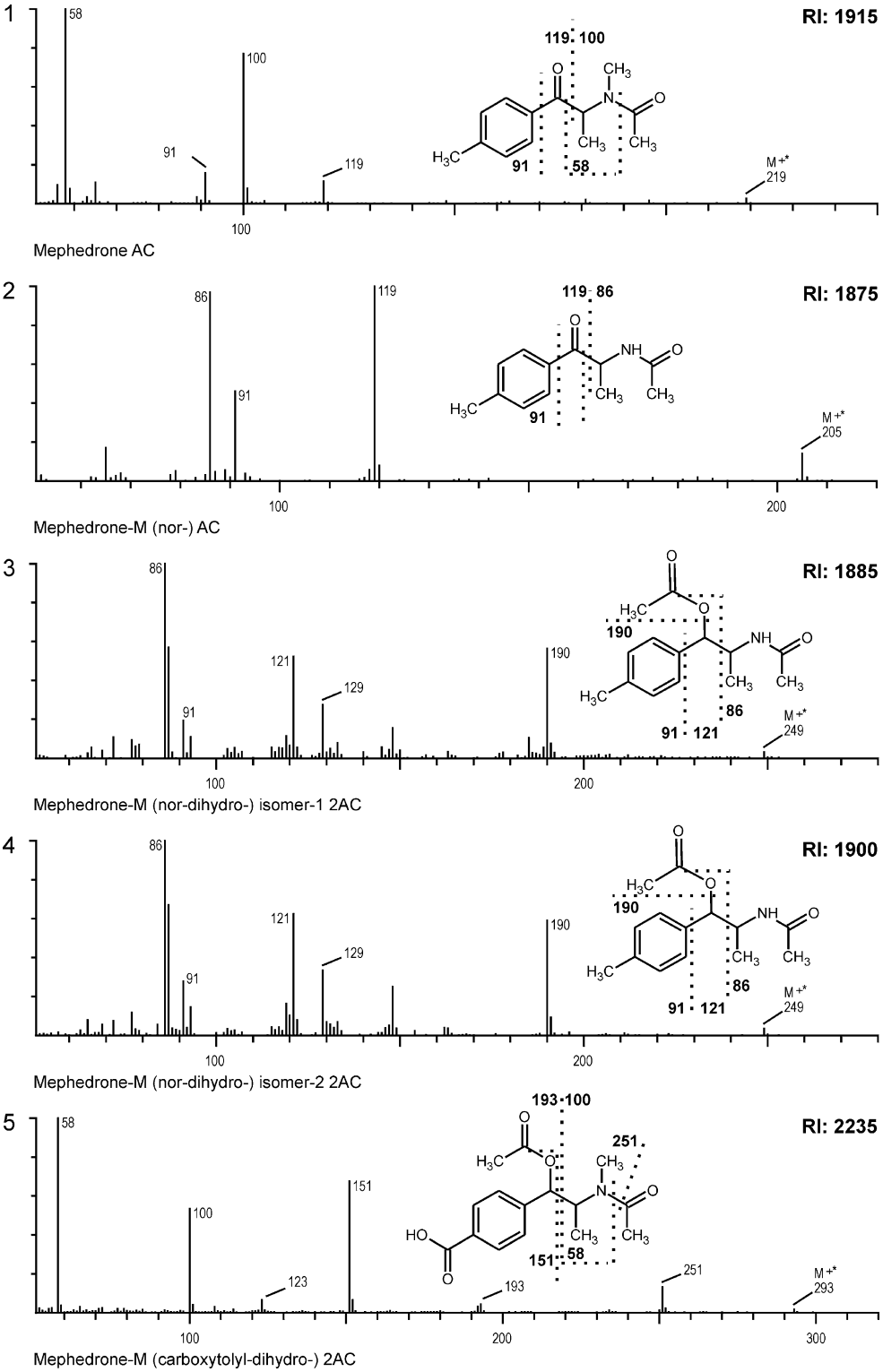


Fig. 2 EI mass spectra, gas chromatographic retention indices (*RI*), structures, and predominant fragmentation patterns of acetylated mephedrone and its main rat and human metabolites (nos. 1–7), butylone and its main human metabolites (nos. 8–11), and methylone and its main human metabolites (nos. 12–14)

Fig. 2 (continued)

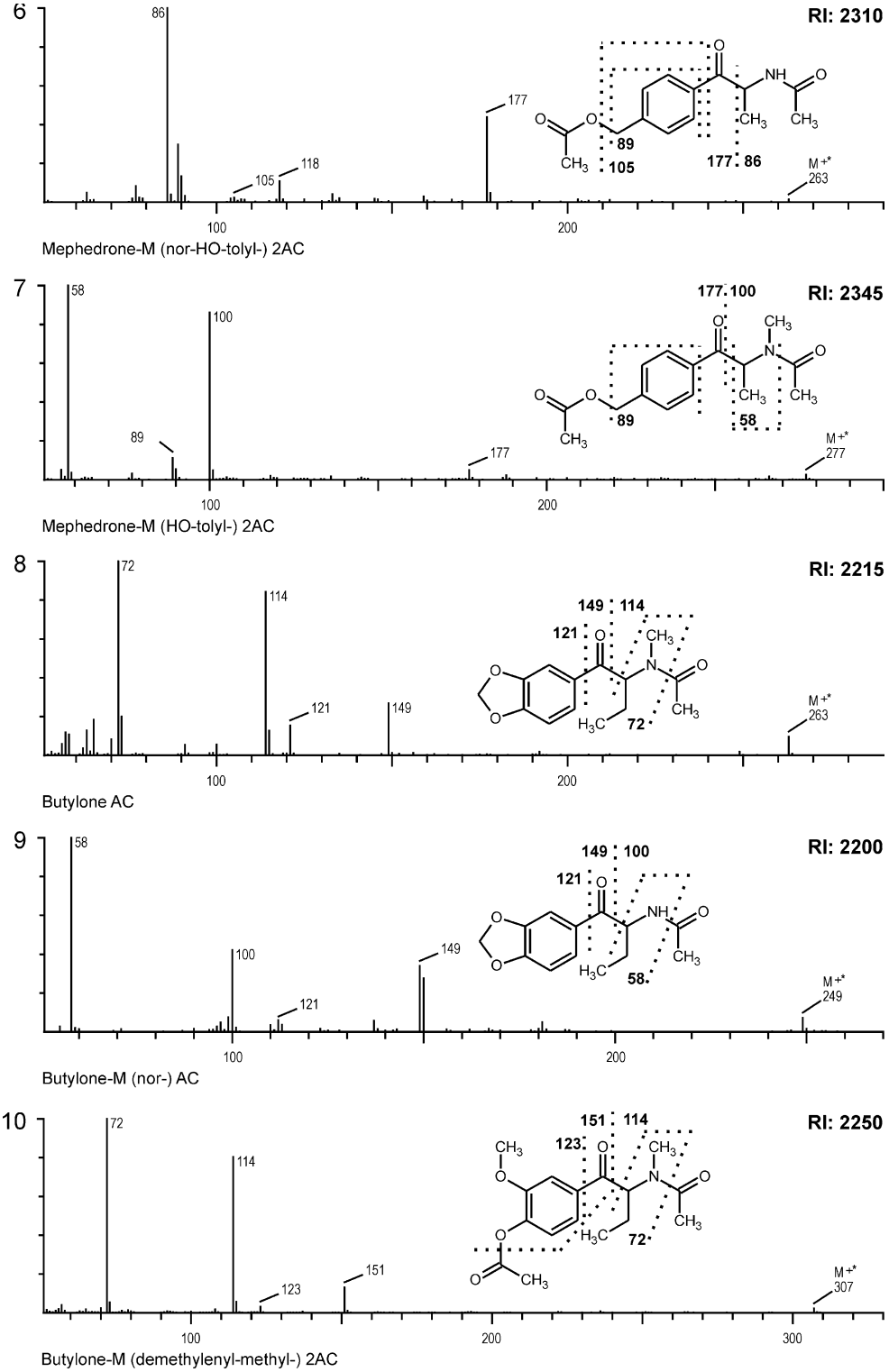
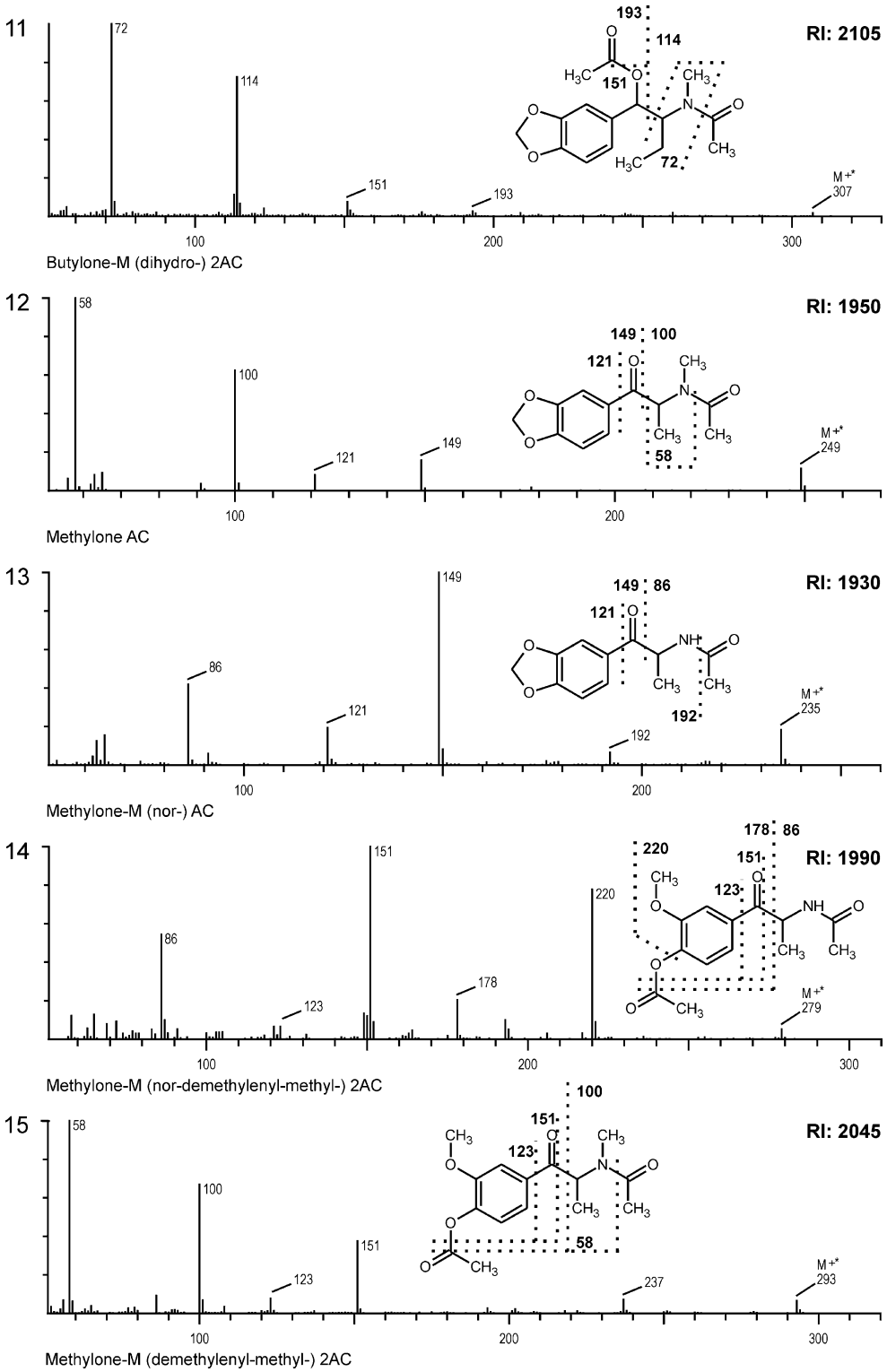


Fig. 2 (continued)



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 a glass flask and 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. After evaporation of the derivatization mixture, the residue was dissolved in 100 µl of methanol, and 2 µl was injected into the GC-MS system.

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 a HP 5989B MS Engine mass spectrometer and a 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×0.2 mm ID), 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 30°/min, initial time 3 min, final time 8 min. The MS conditions were as follows: full-scan mode, *m/z* 50–800 u; EI mode, ionization energy, 70 eV; PICI mode using methane, ionization energy, 230 eV; ion source temperature, 220 °C; capillary direct interface, heated at 260 °C.

GC-MS apparatus and procedure for STA

An HP 5890 Series II gas chromatograph combined with a HP 5972A MSD mass spectrometer was used. The GC conditions were the same as for the metabolism studies. The MS conditions were as follows: full-scan mode, *m/z* 50–550 u; EI mode, ionization energy, 70 eV; ion source temperature, 220 °C; capillary direct interface, heated at 280 °C.

For toxicological detection of acetylated drugs and their metabolites, mass chromatography was used with the selected ions *m/z* 58, 86, 100, and 119 (mephedrone), *m/z* 149, 114, 72, and 58 (butylone), or *m/z* 149, 100, 86, and 58 (methylone). Generation of the mass chromatograms could be started by clicking the corresponding pull down menu, which executes the user defined macros [5]. 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 recorded during this study [10].

Additionally, the full-scan data files acquired by the GC-MS system were evaluated by automated mass spectral deconvolution and identification system (AMDIS) (http://chemdata.nist.gov/mass-spc/amdis/) in simple mode. The used target library was a modified version of the Maurer/Pfleger/Weber MPW_2007 library [11], from which all mass spectra of silylated and perfluoroacylated compounds had been eliminated and the spectra of (acetylated) mephedrone, and its metabolites were added using the "build one library" option contained in the AMDIS main program. The used deconvolution parameter settings were as follows: width 32, adjacent peak subtraction two, resolution high, sensitivity very high, and shape requirements low. The minimum match factor was set to 50 [12].

Results and discussion

Identification of mephedrone metabolites

The urinary metabolites of mephedrone were identified by full-scan EI after GC separation after solid-phase extraction, acetylation, and/or methylation. Methylation was performed, because mephedrone was expected to be metabolized to the respective 4′-carboxy compound as it had been described for other compounds with a tolyl moiety [13–16]. Acetylation was used as standard derivatization reagent [5] providing good GC properties and spectra, which are easily interpreted. For differentiation, whether the *N*-acetyl derivatives are formed by derivatization or metabolism, one extract each was analyzed without 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 the rules described by, e.g., McLafferty and Turecek [17] and Smith and Busch [18]. 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 ($M+C_2H_5^+$, $M+C_3H_5^+$) are produced typical for PICI using methane as reagent gas.

EI mass spectra, the gas chromatographic retention indices (RI), structures, and predominant fragmentation

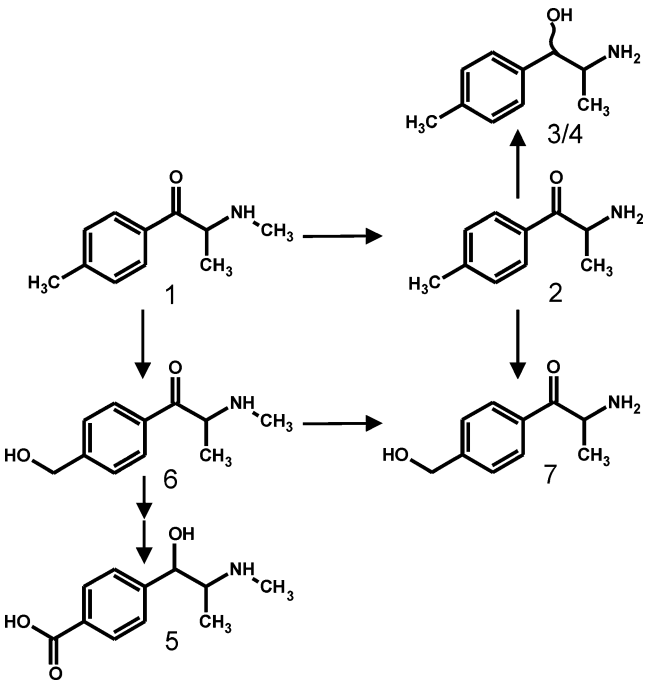


Fig. 3 Proposed scheme for the phase I metabolism of mephedrone in rats and humans. Metabolite no. 5 could only be detected in human urine samples

patterns of acetylated mephedrone (mass spectra no. 1) as well as of its acetylated metabolites are shown in Fig. 2. From these mass spectra, the following metabolites could be deduced: nor mephedrone (no. 2 in Fig. 2), nor-dihydro mephedrone (nos. 3 and 4), hydroxytolyl mephedrone (no. 7), and nor-hydroxytolyl mephedrone (no. 6). Besides these metabolites, the parent drug mephedrone could also be detected.

Based on the identified metabolites of mephedrone, the following partly overlapping metabolic pathways could be postulated (Fig. 3): *N*-demethylation to the primary amine (nos. 2–4 and 6), reduction of the keto moiety to the respective alcohol (nos. 3, 4, and 5), and oxidation of the tolyl moiety to the corresponding alcohols (nos. 6 and 7). As the peaks of the metabolites 6 and 7 were more abundant after glucuronidase and sulfatase hydrolysis, it could be concluded that they were partly excreted as glucuronides and/or sulfates.

Studying the human urine samples, a further metabolite, namely, 4-carboxy-dihydro mephedrone (no. 5 in Fig. 2), could be identified. Concerning the formation of the dihydro metabolites of mephedrone, it is obvious that only the primary amines are reduced to both diastereomic alcohols. In the case of the dihydro metabolites of the secondary amines, only one diastereomer was detectable. This might be due to the fact that the *N*-methyl group is sterically hindering the enzymatic reaction to one of the isomers.

Extraction efficiencies of 65±9%, 79±15%, and 72±10% were determined for mephedrone, butylone, and methylone (n=5 at 100 ng/ml, each), and the limit of detection for all drugs was 1 ng/ml with a signal to noise ratio of 3.

Toxicological detection of mephedrone, butylone, and methylone intake by GC-MS

Mephedrone and its metabolites were separated by GC and identified by full-scan EI MS after fast acidic hydrolysis,

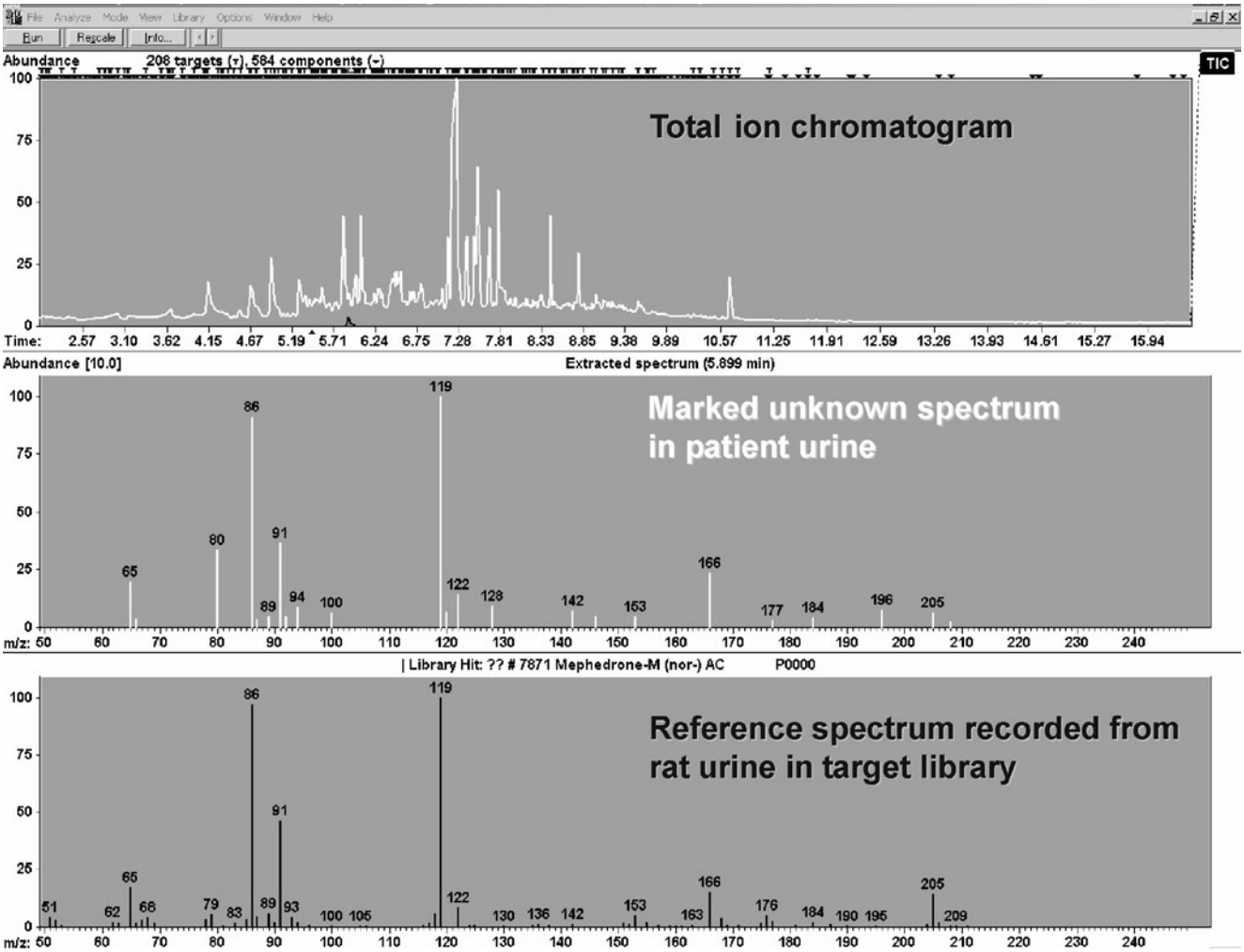


Fig. 4 Identification of nor mephedrone in human urine as an example for the power of AMDIS-based data evaluation (settings: width 32, adjacent peak subtraction two, resolution high, sensitivity very high, and shape requirements low, minimum match factor set to 50)

liquid–liquid extraction, and acetylation (authors' STA) of rat and human urine. Mass chromatography with the following ions was used to detect the presence of mephedrone and/or its metabolites: *m/z* 119, 100, 86, and 58. The selected ion *m/z* 119 was used for monitoring the presence of compounds with unchanged 4-methylphenone ring (mass spectra nos. 1 and 2 in Fig. 2), *m/z* 100, 58 for compounds with the unchanged acetylated amine side chain (mass spectrum nos. 1, 5, and 7 in Fig. 2), and *m/z* 86 for acetylated nor metabolites (mass spectra nos. 2, 3, 4, and 6 in Fig. 2).

Intake of butylone could also be monitored rat and human urine using the authors' STA procedure using mass chromatography with the following ions: *m/z* 149, 114, 72, and 58. Ion *m/z* 149 was used for monitoring the presence of compounds with unchanged 3,4-methylendioxyphenone ring (mass spectra nos. 8 and 9 in Fig. 2), *m/z* 114 and 72 for compounds with the unchanged acetylated amine side chain (mass spectrum nos. 8, 10, and 11), and *m/z* 58 for acetylated nor metabolites (mass spectra nos. 9). These findings are in line with the previously published results by Zaitsu et al. [6].

Intake of methylone could also be monitored in rat urine using mass chromatography with the following ions: *m/z* 149, 100, 86, and 58. Ion *m/z* 149 was also used for monitoring the presence of compounds with unchanged 3,4-methylendioxyphenone ring (mass spectra nos. 12 and 13), *m/z* 100, 58 for compounds with the unchanged acetylated amine side chain (mass spectrum nos. 12 and 15), and *m/z* 86 for acetylated nor metabolites (mass spectra nos. 13 and 14). These rat metabolites correspond to those described for humans [2].

The identity of the peaks indicated in the various mass chromatograms was confirmed by computerized comparison of the underlying full mass spectrum with reference spectra recorded during this study [10, 19]. The gas chromatographic RIs provided in Fig. 2 allowed differentiation of isomeric compounds. They were recorded during the GC-MS procedure and calculated in correlation with the Kovacs' indices [20]

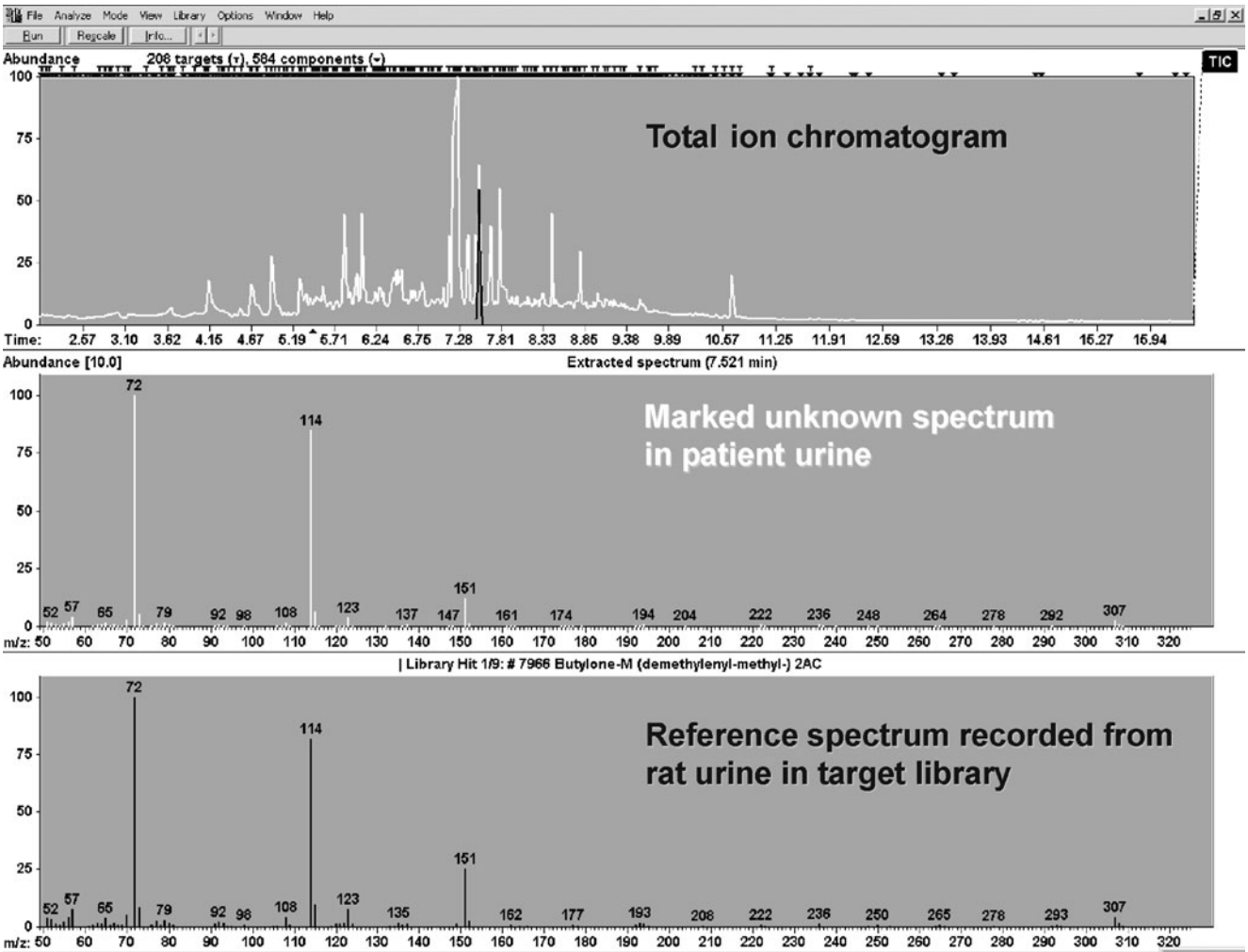


Fig. 5 Identification of demethylenyl-methyl butylone in human urine as an example for the power of AMDIS-based data evaluation (settings as given in Fig. 4)

of the components of a standard solution of typical drugs, which is measured daily for testing the GC-MS performance [21, 22]. The reproducibility of RIs measured on capillary columns was better using a mixture of drugs than that of the homologous hydrocarbons recommended by Kovacs.

Besides the above-mentioned macro-based data evaluation, an automated evaluation using AMDIS was performed. This software was able to detect and identify the metabolites in human urine, which were integrated into the target library after rat metabolism studies. Fig. 4 shows as an example identification of nor mephedrone and Fig. 5 of demethylenyl-methyl butylone in the same human urine. The deconvolution and identification settings of AMDIS used here have been found to be best for STA [12].

As the human urine samples used were single samples submitted for drug testing without information on dosage and time after ingestion, the question cannot be answered whether a common user's dose can be detected. Therefore, this was tested using urine samples of rats treated with doses corresponding to those reported for abuse of amphetamines [23]. Assuming similar toxicokinetics in humans, the described STA procedure should be suitable for proof of intake of the bk-designer drugs in human urine as shown for example for MDEA [24, 25].

Conclusions

The presented studies revealed that the new designer drug mephedrone was metabolized via three phase I pathways by rats and humans. The authors' STA procedure should be suitable to prove an intake of mephedrone, butylone, and methylone urine. These examples showed again that the used rat model was suitable to predict the qualitative metabolism and detectability of drugs in human urine

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