Research Article

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Studies on the metabolism of mitragynine, the main alkaloid of the herbal drug Kratom, in rat and human urine using liquid chromatography-linear ion trap mass spectrometry

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Mitragynine (MG) is an indole alkaloid of the Thai medicinal plant *Mitragyna speciosa* (Kratom in Thai) and reported to have opioid agonistic properties. Because of its stimulant and euphoric effects, Kratom is used as a herbal drug of abuse. The aim of the presented study is to identify the phase I and II metabolites of MG in rat and human urine after solid-phase extraction (SPE) using liquid chromatography-linear ion trap mass spectrometry providing detailed structure information in the MS^n mode particularly with high resolution. The seven identified phase I metabolites indicated that MG was metabolized by hydrolysis of the methylester in position 16, *O*-demethylation of the 9-methoxy group and of the 17-methoxy group, followed, via the intermediate aldehydes, by oxidation to carboxylic acids or reduction to alcohols and combinations of some steps. In rats, four metabolites were additionally conjugated to glucuronides and one to sulfate, but in humans, three metabolites to glucuronides and three to sulfates.

Keywords: mitragynine; Kratom; metabolism; LC-MS; urine; rat; human

Introduction

Mitragynine (MG) is an indole alkaloid of *Mitragyna speciosa* Korth. (Rubiaceae). This plant is native in Thailand and other southeast Asian countries and its Thai name is ''Kratom''.[1,2] The leaves of *M. speciosa* have been used in Thailand for its opium-like effect and its coca-like ability to combat fatigue of and enhance tolerance to hard workers under a scorching sun.[3] In addition, it has been used as a traditional medicine for common illnesses such as coughing, diarrhea, muscle pain, hypertension and to cure morphine addicts.[4,5]

MG is the most abundant of the more than 20 alkaloids of Kratom (66% of the crude base)[6] and is responsible for the substance's opioid effects.[1,7] Pharmacological *in vivo* and *in vitro* investigations revealed that MG shows anti-nociceptive effects on supraspinal opioid receptors, mainly of the μ- and δ-subtypes.[8–11] The μ-receptor mediates analgesia, euphoria, and respiratory depression, which accounts for the analgesia activity of MG, as well as its amelioration of opiate withdrawal symptoms.[12] MG has also been postulated to be involved in the activation of descending noradrenergic and serotonergic pathways in the spinal cord.[9] However, there are no reports of MG being screened for affinity at these specific receptors.[12] The clinical effects of Kratom are dose-dependent and consist of stimulant effects at lower doses and opiate effects at higher doses in humans.[3,4] These effects have also been witnessed in animal models.[13] However, there is little information about the MG toxicity and the dose required in humans to produce stimulation, analgesia, and toxicity.[3,4,12] In animal models, MG has been shown to cause less respiratory depression than other narcotics.[14] Nevertheless, users who combine Kratom with central nervous system depressants may experience respiratory depression.[15] In this case, the opioid antagonist naloxone should be considered in addition to supportive care.[12]

Use of Kratom is illegal in Thailand since 1946 and in Australia since 2005. It is currently not scheduled in the United States and Europe. The wide availability of Kratom via the internet reflects extensive demand for this product. For example, opiate addicts may attempt to mitigate opioid withdrawal symptoms.[12]

Janchawee *et al.*[16] described an high-performance liquid chromatographic (HPLC) method for determination of MG in rat serum for studying its pharmacokinetics. However, there is only little information about the metabolism of MG. In 1974, Zarembo

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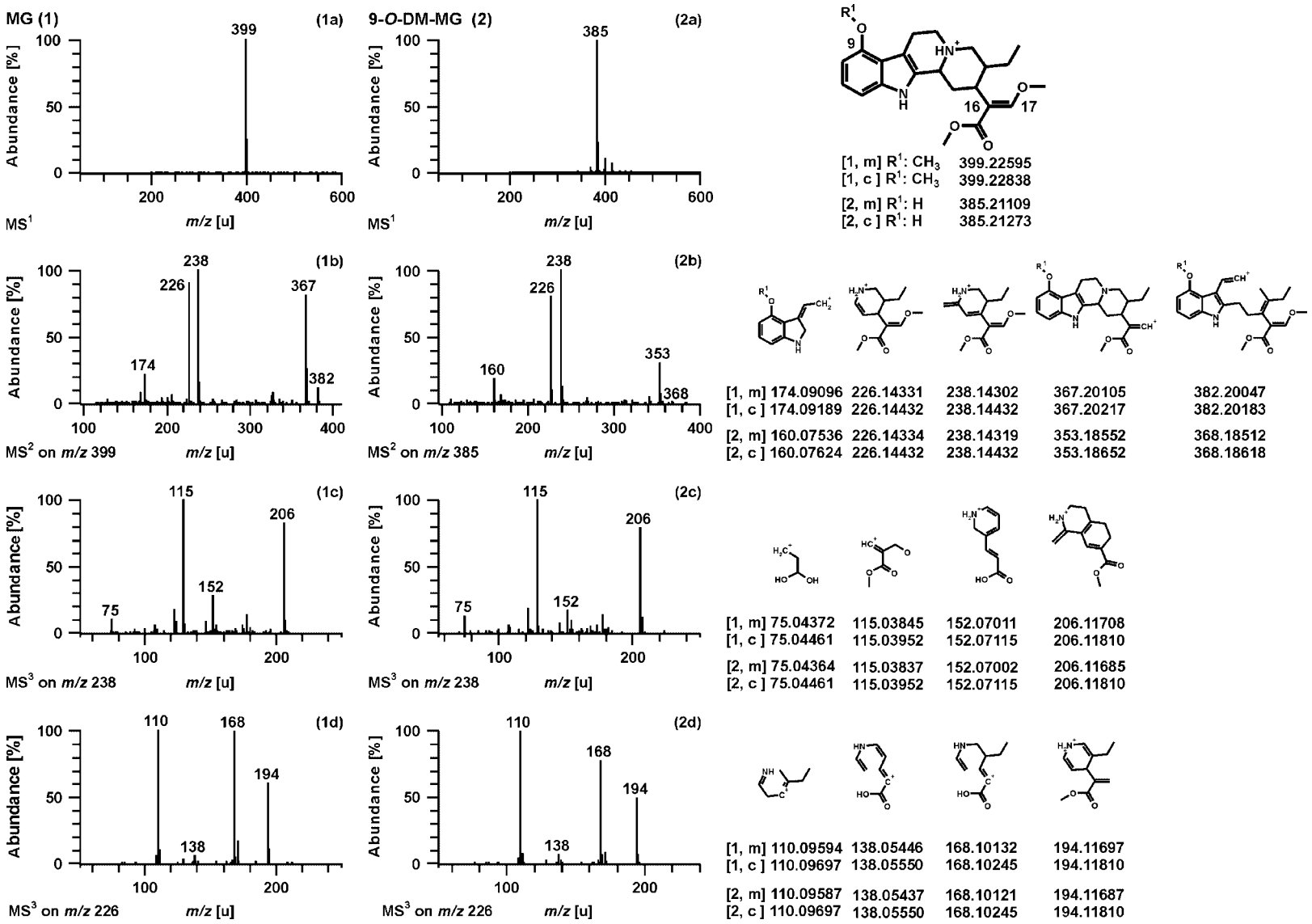

Figure 1a. MS^1 spectra of MG and phase I metabolites, MS^2 spectra of the protonated molecular ions in the MS^1 spectra, MS^3 spectra of the two most abundant fragments in the MS^2 spectra, structures of MG, its metabolites and fragments, and their accurate masses measured [m] and calculated [c].

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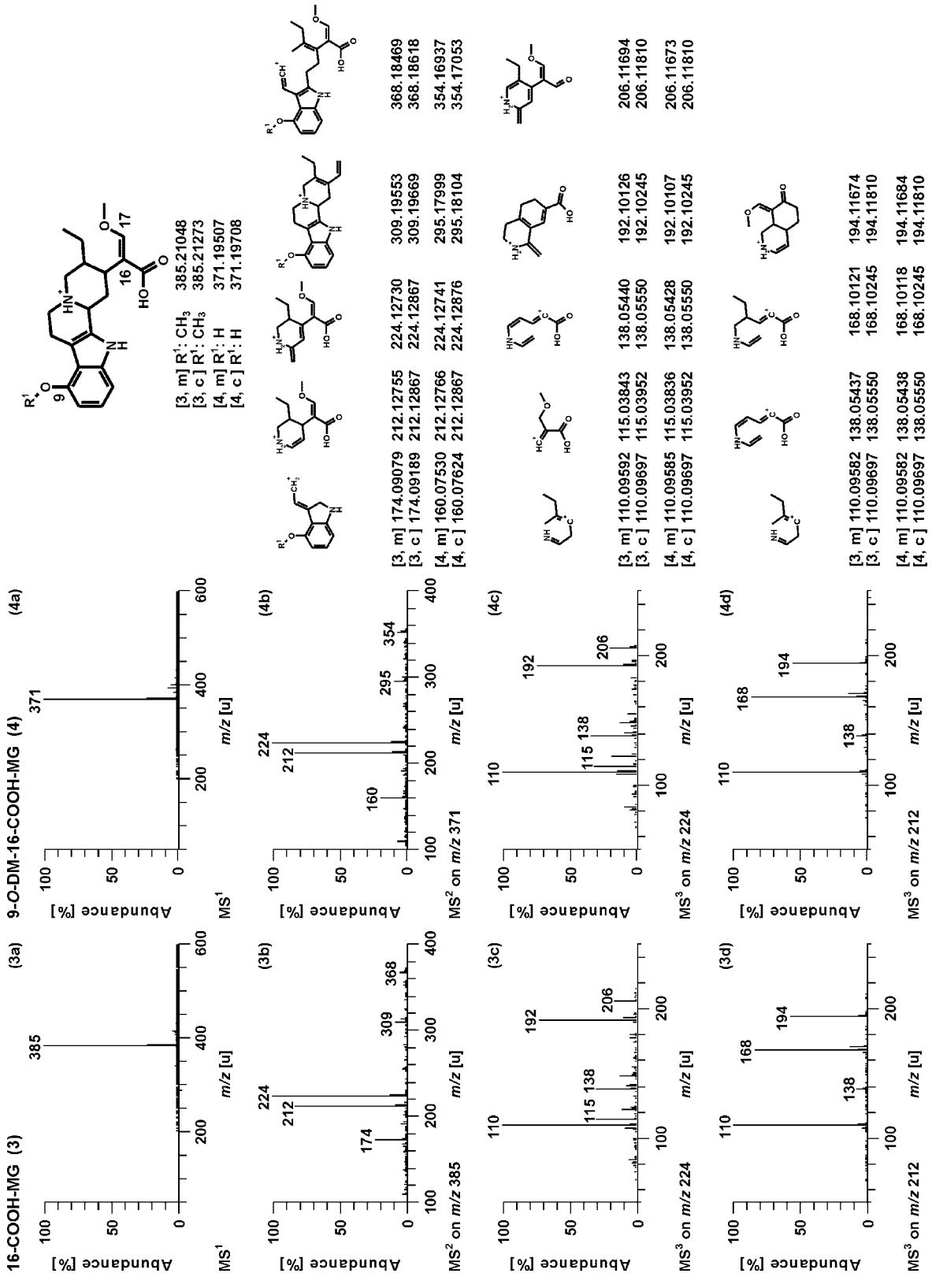

Figure 1b. *(Continued).*

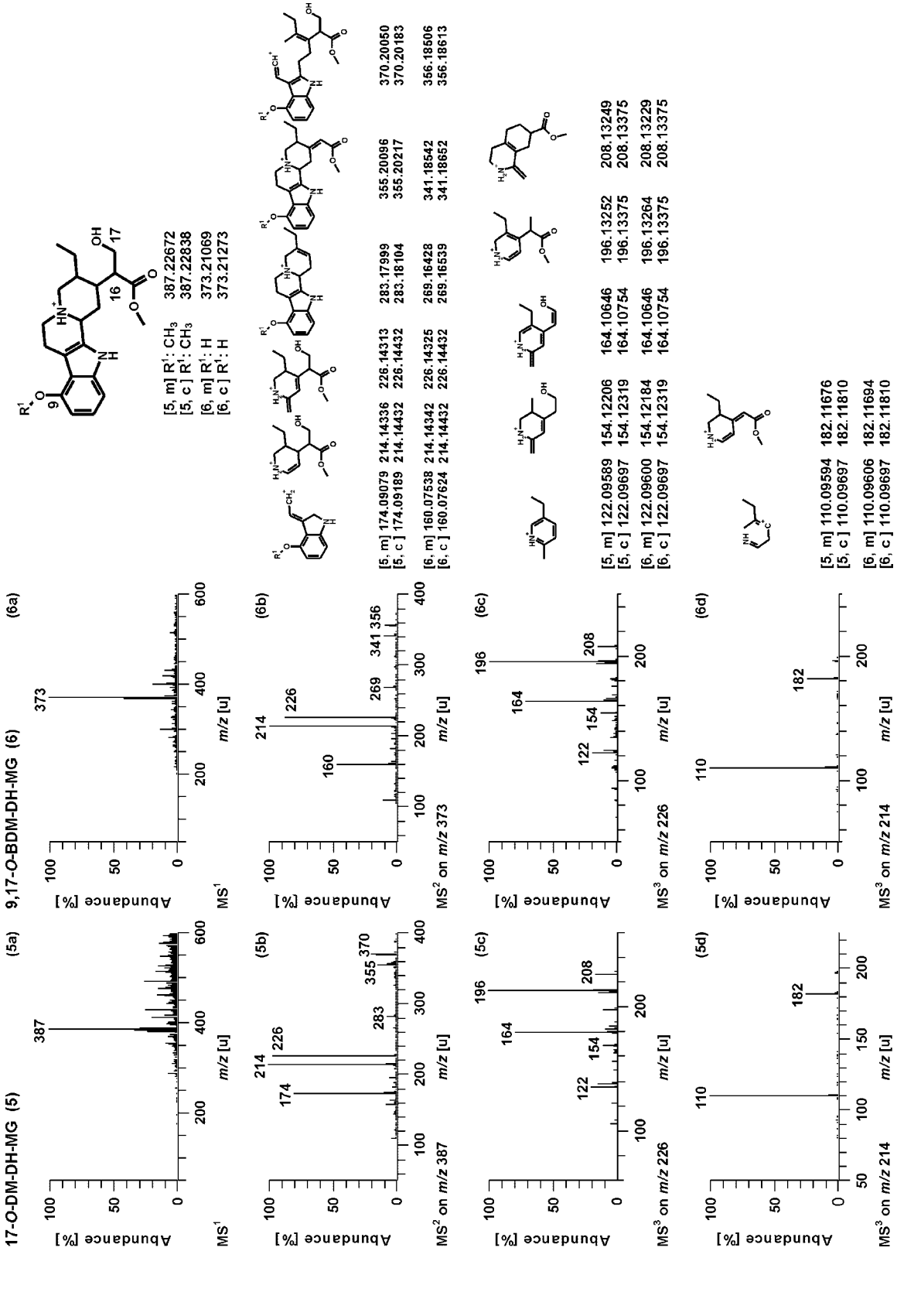

Figure 1c. (*Continued*).

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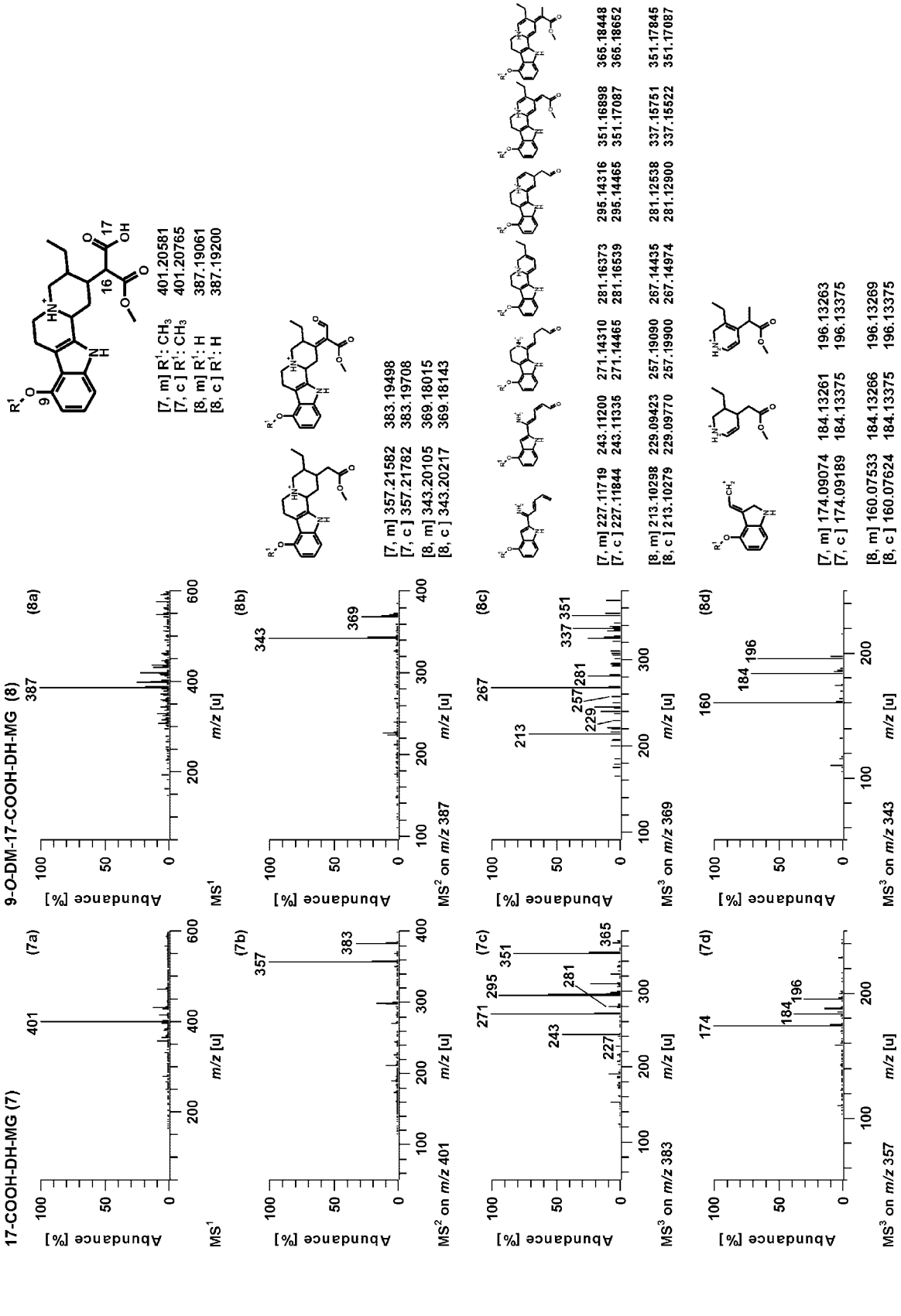

Figure 1d. *(Continued).*

et al.[17] studied the metabolism of MG by microbial transformation using *Helminthosporum* sp. However, the authors could not exclude that the two isolated metabolites were formed simply by air oxidation during the fermentation. Beckett *et al.*[18] investigated the metabolism of corynantheidine and 9-methoxycorynantheidine-type alkaloids using liver microsomes of rats, guinea pigs, and rabbits. MG, one of the tested corynantheidine alkaloids, was metabolized only in rabbit liver microsomes by *O*-demethylation of the methoxy group in position 17. Therefore, the aim of this study was to identify the phase I and II metabolites of MG in rat and human urine using liquid chromatography-mass spectrometry (LC-MS) with a linear ion trap (LIT) analyzer providing detailed structure information in the MS^n mode and to confirm these finding using high resolution mass spectrometry with an Orbitrap (OT) analyzer providing the empirical formula of the corresponding fragments.

Experimental

Chemicals and reagents

MG was delivered from the Department of Forensic Medicine, Johannes Gutenberg University, Mainz, Germany, where it was isolated from Kratom leaves obtained from head&nature (Regensburg, Germany). Purity and identity were checked by mass spectrometry, ^{1}H-NMR spectroscopy, and ^{13}C-NMR spectroscopy.[19] Isolute Confirm HCX cartridges (130 mg, 3 ml) and Isolute Confirm C18 cartridges (500 mg, 3 ml) were obtained from Biotage (Grenzach-Wyhlen, Germany). Ammonium formate (analytical grade) and formic acid (for mass spectrometry) were obtained from Fluka (Neu-Ulm, Germany). Acetonitrile and water (both LC-MS grade) were obtained from Fisher Scientific (Schwerte, Germany). All other chemicals and biochemicals used were obtained from Merck (Darmstadt, Germany) and were of analytical grade.

Urine samples

The investigations were performed using urine of male Wistar rats (Ch. River, Sulzfleck, Germany) for toxicological diagnostic reasons according to the corresponding German law. They were administered a single 40 mg/kg body mass (BM) dose in aqueous suspension by gastric intubation. Urine was collected separately from the faeces over a 24-h period stabilized with sodium fluoride. The samples were directly analyzed or stored at −20 °C until further analysis. Blank rat urine samples were collected before drug administration to check whether they were free of interfering compounds.

Several human urine samples of different patients were submitted to the authors' laboratory for toxicological analysis.

Sample preparation for identification of phase I metabolites

Urine (1 ml) was diluted with 2 ml of purified water, adjusted to pH 5.2 with acetic acid (1 mol/l), and incubated at 50 °C for 1.5 h with 100 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*. Then the urine sample was loaded on an Isolute 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, 1 ml of 0.01 M hydrochloric acid, and 1 ml of methanol. Elution was performed using 1 ml of a freshly prepared mixture of methanol/aqueous ammonia (98 : 2 v/v). The organic layer was carefully evaporated to dryness at 56 °C under a stream of nitrogen. The residue was dissolved in 50 µl of methanol and 5-µl aliquot of this solution was injected into the LC-MS system.

Sample preparation for identification of phase II metabolites

A 1-ml portion of urine was diluted with 2 ml of purified water. Then the sample was loaded on an Isolute Confirm C18 cartridge, previously conditioned with 1 ml of methanol and 1 ml of water. After passage of the sample, the cartridge was washed with 2 ml of water. Elution was performed using 1.5 ml of methanol. The organic layer was carefully evaporated to dryness at 56 °C under a stream of nitrogen. The residue was dissolved in 100 µl of a mixture of methanol and eluent A (ratio 1 : 1) and 5-µl aliquot of this solution was injected into the LC-MS system.

Another urine sample was worked up as described above. After reconstitution of the extraction residue in 50 µl of methanol, methylation was conducted with 100 µl of a solution of diazomethane in diethyl ether, synthesized according to the procedure of McKay *et al.*[20] The reaction vials were sealed and left at room temperature for 5 min or for 24 h. Thereafter, the mixture was again carefully evaporated to dryness under a stream of nitrogen and redissolved in 100 µl of a mixture of methanol and eluent A (ratio 1 : 1). Again, 5 µl was injected into the LC-MS system.

LC-MS apparatus

An Accela LC system (Thermo Fisher Scientific, TF, Dreieich, Germany) was used consisting of a degasser, a quaternary pump and an autosampler coupled to a TF LXQ LIT or to a TF LTQ Orbitrap XL MS system both equipped with a heated electrospray ionization (ESI) source.

The LC conditions were as follows: Agilent (Waldbronn, Germany) Zorbax strong bond (SB) C18 column (50 × 2.1 mm, 5 µm); column temperature, 35 °C; mobile phase A consisted of 10 mmol/l aqueous ammonium formate buffer containing 0.1% (v/v) formic acid (final pH 3), mobile phase B consisted of acetonitrile containing 0.1% (v/v) formic acid; flow rate, 0.5 ml/min, solvent gradient for phase I metabolism studies: 0.00–3.00 min: 98% mobile phase A, 3.01–20.00 min: continuous increase of mobile phase B to 100%. The total run time was 20 min. For phase II metabolism studies, the following gradient was used: 0.00–1.00 min: 98% mobile phase A, 1.01–25.00 min; continuous increase of mobile phase B to 100%; 25.01–35.00 min 100% mobile phase B, 35.01–45.00 min re-equilibration to start conditions. The total run time was 45 min.

The MS conditions for the LIT were as follows: ESI, positive mode; sheath gas, nitrogen at flow rate of 40 (arbitrary units (AU)); auxiliary gas, nitrogen at flow rate of 25 AU; vaporizer temperature, 300 °C; source voltage, 3.00 kV; ion transfer capillary temperature, 380 °C; capillary voltage, 4.7 V; tube lens voltage, 42 V. Collision-induced dissociation (CID)-MS/MS experiments were performed on precursor ions selected from MS^1 using information-dependent acquisition (IDA): MS^1 was performed in the full scan (FS) mode (*m/z* 100–800). MS^2, MS^3, and MS^4 were performed in the IDA mode: MS^2 on the most and second most intense signals from MS^1; MS^3 on the most and second most intense signals from MS^2; MS^4 on the most and second most intense signals from MS^3. Normalized collision energies were 35%, 40%, and 45% in MS^2, MS^3 and MS^4, respectively. Other settings were as follows: minimum signal threshold: MS^1: 500 counts, MS^2: 250 counts,

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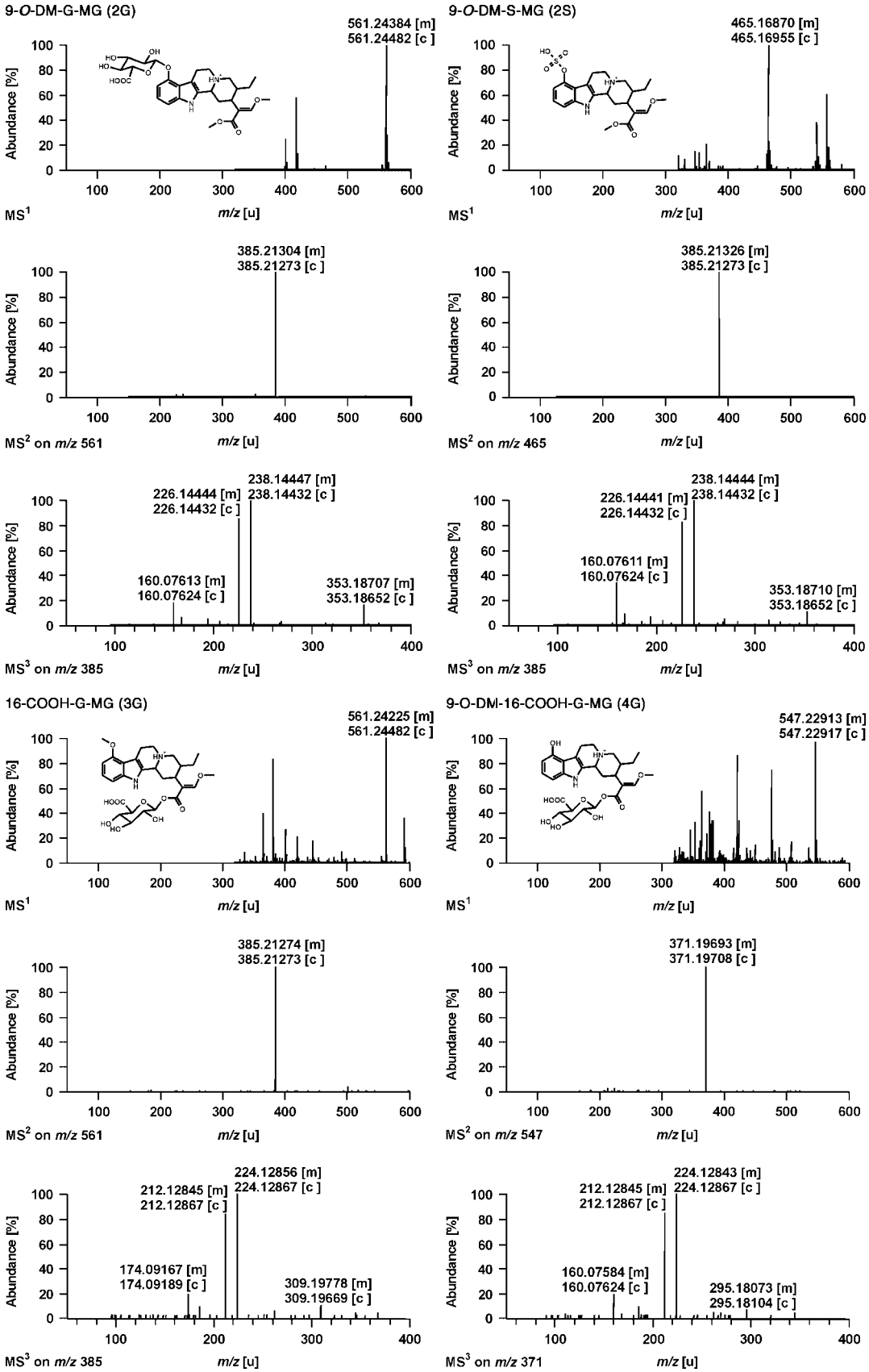

Figure 2a. MS^1 spectra with structures of the glucuronides (G) and sulfates (S) of MG metabolites, MS^2 and MS^3 spectra the most abundant ions in the corresponding MS^1 or MS^2 spectra, and the accurate masses of predominant ions measured [m] and calculated [c].

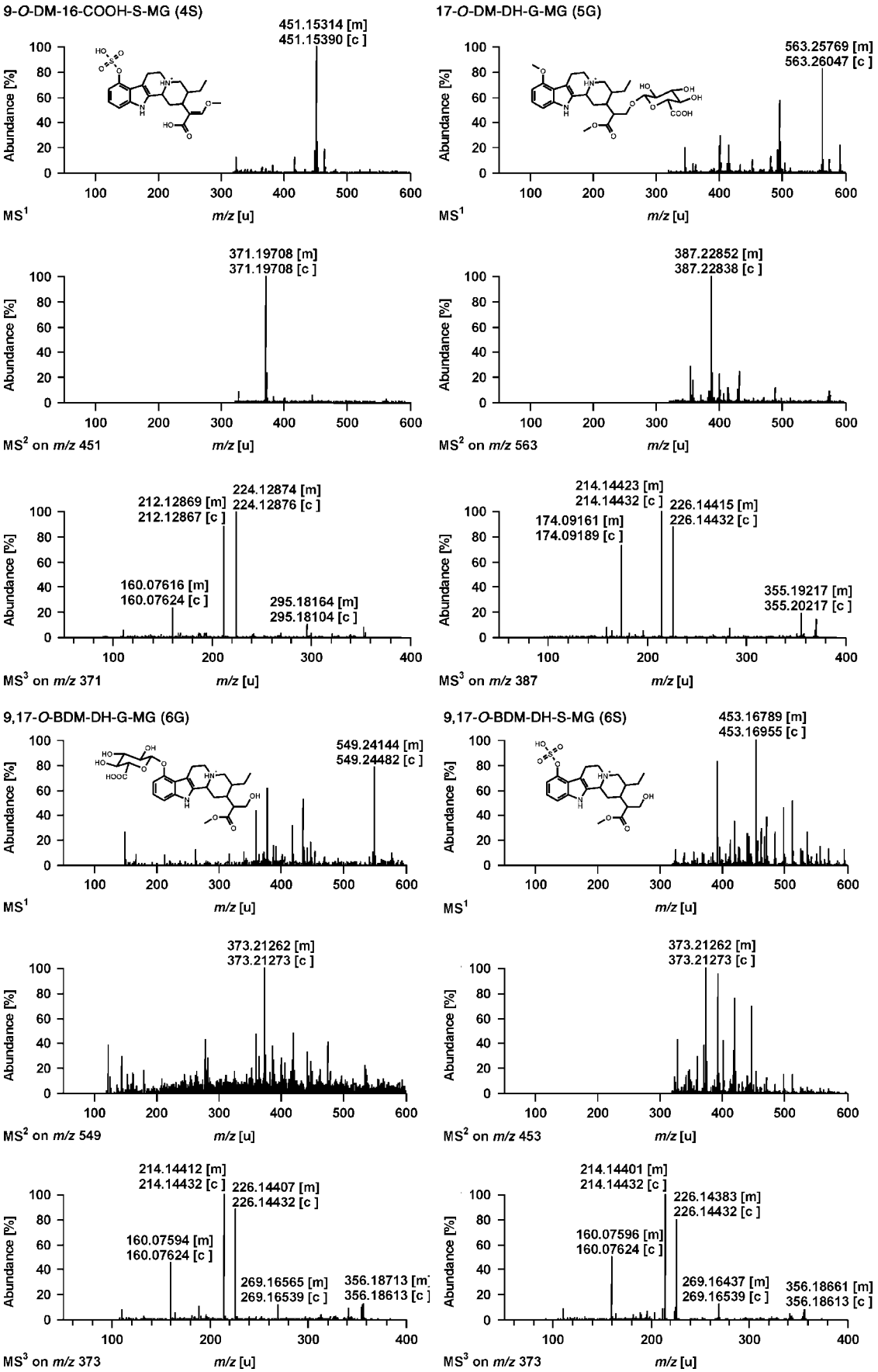

Figure 2b. (*Continued*).

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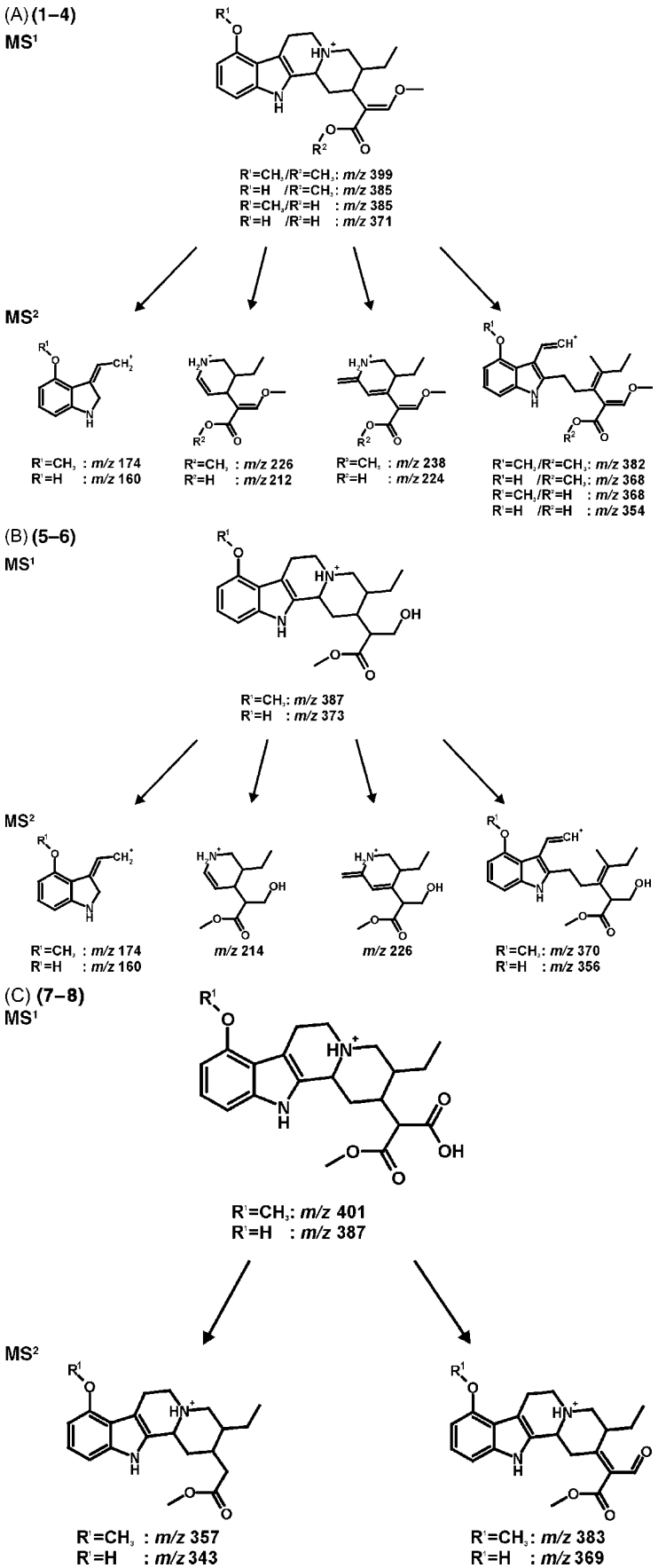

Figure 3. Proposed fragment ions in the MS^1 and MS^2 spectra characterizing metabolites 1–4 (A), 5–6 (B), and 7–8 (C).

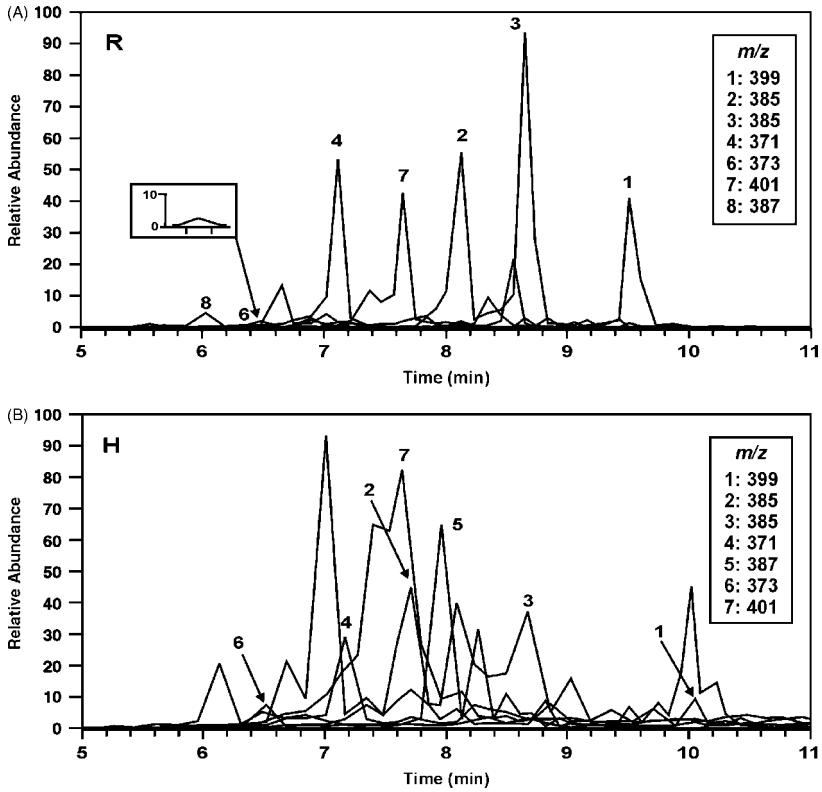

Figure 4. Reconstructed chromatograms of ions selected according to the protonated molecular ions of expected phase I metabolites in rat (R) urine (A) and human (H) urine (B).

MS^3: 125 counts, MS^4: 60 counts; isolation width, 1.5 u; activation Q, 0.25; activation time, 30 ms; dynamic exclusion mode, repeat counts 2, repeat duration 10 s, exclusion list 50, exclusion duration 10 s.

The MS conditions for the OT were as follows: ESI, positive mode; sheath nitrogen gas, flow rate of 30 AU; auxiliary gas, 25 AU; vaporizer temperature, 300 °C; source voltage, 3.5 kV; ion transfer capillary temperature, 300 °C; capillary voltage, 35 V; tube lens voltage, 100 V. CID-MS/MS experiments were performed on precursor ions selected from MS^1 using IDA: MS^1 was performed in the FS mode (*m/z* 320–800) with a resolution of 60.000. MS^2 and MS^3 were performed in the IDA mode: MS^2 on the most and second most intense signal from MS^1 and MS^3 on the most and second most intense signal from MS^2. Normalized collision energies were 35% in MS^2 and 40% in MS^3, respectively. Other settings were as follows: minimum signal threshold: MS^2: 250 counts with a resolution of 15.000, MS^3: 125 counts with a resolution of 15.000, isolation width, 1.5 u; activation Q, 0.25; activation time, 30 ms; dynamic exclusion mode, repeat counts 2, repeat duration 5 s, exclusion list 50, and exclusion duration 5 s.

Screening for and identification of metabolites

Two procedures were used for metabolite screening. In procedure I, FS was acquired in MS^1 for searching of metabolites in urine according to their expected molecular ions. The fragmentation patterns in the stage of MS^2 and MS^3 (in case of phase II metabolites additionally in the stage of MS^4) were compared to those of MG in order to elucidate the most probable metabolites structures. The interpretation of the fragments was confirmed using the high-resolution OT analyzer providing their elemental composition.

In procedure II, reconstructed ion chromatograms of typical fragments in MS^2 and/or MS^3 were generated in order to screen for unexpected metabolites that contain a particular partial structure of already identified metabolites. Their possible structures were elucidated as described above.

Results and Discussion

Identification of the metabolites

The MG metabolites were first identified in rat urine after a dose also used for rat pharmacokinetic studies.[16] This corresponds to a common user's dose. These identified metabolites were screened for in urine samples of potential MG users submitted for toxicological analysis, because urine samples of a controlled human study were not available.

The phase I metabolites were isolated after gentle enzymatic cleavage of conjugates using mixed-mode solid-phase extraction (SPE) and the phase II metabolites without cleavage using C18 SPE, as these have been used successfully in the authors' laboratory.[21–28] The HCX extraction being very suitable for basic compounds was not suitable for extraction of the conjugates, because the column was washed using HCl solution and the analytes were eluted from the cation exchanger using a basic solution. As the expected sulfates and acyl glucuronides are esters, their cleavage should have been avoided during the different pH steps. For the C18 extraction, no pH change was necessary, but it led to less cleaner extracts. However, this was the smaller problem than the risk of conjugate hydrolysis. As no metabolite standards were available, recovery of the metabolites could not be determined.

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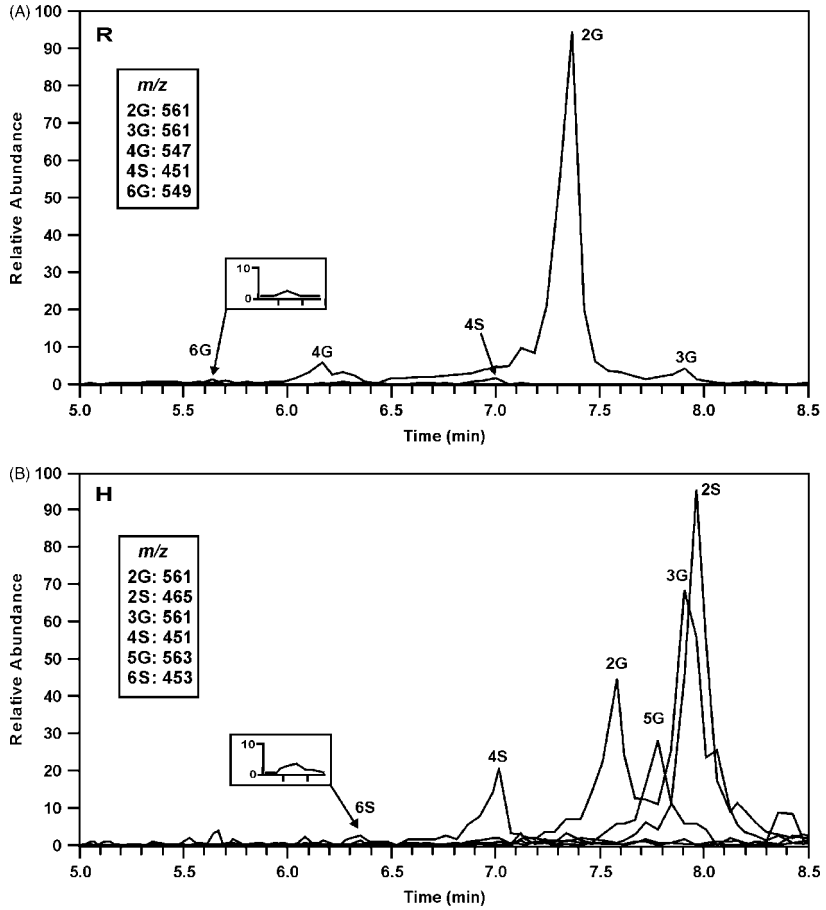

Figure 5. Reconstructed chromatograms of ions selected according to the protonated molecular ions of expected phase II metabolites in rat (R) urine (A) and human (H) urine (B).

The presence of phase I metabolites was screened by reconstructed ion chromatography according to procedures I and II. In Fig. 1, the underlying MS^1 spectra, the MS^2 spectra of the protonated molecular ions in the MS^1 spectra, and the MS^3 spectra of the two most abundant fragments in the MS^2 spectra are depicted. In addition, the structures of MG, its postulated metabolites and of the above-mentioned postulated fragments are shown. The accurate masses measured [m] by reanalysis of the samples using the OT analyzer allowing confirming the elemental composition of the fragments as well as the corresponding calculated theoretical accurate masses [c] of the depicted fragments are given. The mass errors ranged between −1.2 and −5.3 ppm. The given metabolite structures were deduced by comparing the fragmentation patterns in the stage of MS^2 and MS^3 of the metabolites with those of MG. The proposed fragmentation patterns are discussed below.

Phase II metabolites (glucuronides or sulfates) were searched for using procedure I detecting the calculated protonated molecular ions of conjugates of the identified phase I metabolites. Figure 2 shows the MS^1 spectra with structures of the glucuronides (G) and sulfates (S) of MG metabolites, MS^2 and MS^3 spectra, the most abundant ions in the corresponding MS^1 or MS^2 spectra, and the accurate masses of predominant ions measured [m] and calculated [c]. The structures could be confirmed by comparing the corresponding MS^2, MS^3 and MS^4 spectra with the MS^1, MS^2 and MS^3 spectra of the corresponding phase I metabolites (Fig. 1). However, the position of the glucuronic or sulfuric acid in metabolites 4 and 6 could not be determined. In case of metabolite 4G, short time methylation with diazomethane would lead only to methyl ester because of its low reactivity.[29] A reaction time of 24 h would allow distinguishing between phenolic and alcoholic hydroxy groups, because the latter cannot be methylated by diazomethane. In fact, the glucuronide 4G was not methylated during short-time methylation but during long-time methylation, confirming that the carboxylic acid and not the phenolic hydroxy group was conjugated. Metabolites 6G and 6S were not methylated during long-time methylation confirming that the phenolic hydroxy group was conjugated.

Proposed fragmentation patterns

In the following, possible fragmentation patterns of the MS^n spectra of MG and its metabolites will be discussed in relation to the postulated metabolite structures depicted in Fig. 1. The numbers of the corresponding mass spectra in Fig. 1 are given in brackets. MG (1) showed in the MS^1 spectrum the protonated molecular ion of *m/z* 399 (1a). Fragmentation of this ion in the stage of MS^2 lead to the following fragments (1b): Elimination of ammonia of the 2H-quinolizine ring lead to fragment *m/z* 382, elimination of methanol in position 17 lead to fragment *m/z* 367, cleavage of the 2H-quinolizine ring to ion *m/z* 238 and 226, and loss of the indole ring to ion *m/z* 174. Based on these fragmentation patterns, the MG metabolites 2–6 could be identified by comparing the different MS^n spectra considering the mass shifts according to

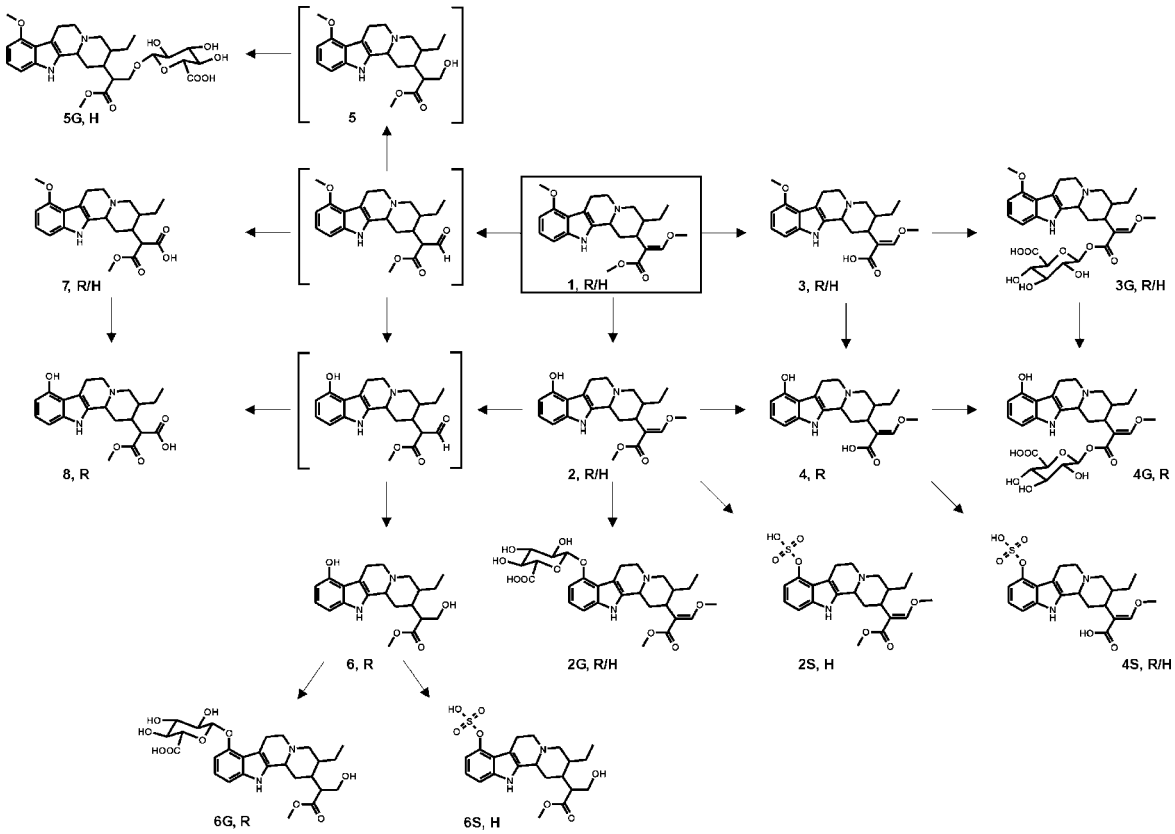

Figure 6. Postulated metabolic pathways of MG in rats (R) and humans (H). Assumed intermediate metabolites in square brackets.

the different elemental composition. This is exemplified for 9-*O*-demethyl MG (2). The protonated molecular ion of *m/z* 385 (2a) indicates demethylation by loss of 14 u. This mass shift is observed also in 2b for all fragments containing the corresponding partial structure (*m/z* 368, 353, and 160). The ions *m/z* 226 and 238 are unchanged (2b) and therefore, the corresponding MS^3 is identical (2c, 2d). The metabolites 7 and 8 showed different fragmentation patterns (Fig. 1, 7b–d, 8b–d) most probably because of the loss of water after cleavage of the carboxylic acid at position 17. The fragments without this molecule part (*m/z* 174 or 160) are the same as in MG (1b) and its *O*-demethyl metabolite (2b). The main fragmentation patterns of the MS^1 and MS^2 spectra characterizing metabolites 1–4 (A), 5–6 (B), and 7–8 (C) were summarized in Fig. 3 for better understanding.

Proposed metabolic pathways

As shown in Fig. 1 and indicated in the typical reconstructed MS^1 mass chromatograms, besides MG (1), the following phase I metabolites could be identified in rat urine (R, Fig. 4, upper part) or human urine (H, Fig. 4, lower part): 9-*O*-demethyl MG (9-*O*-DM-MG, 2); 16-carboxy MG (16-COOH-MG, 3); 9-*O*-demethyl-16-carboxy MG (9-*O*-DM-16-COOH-MG, 4); 17-*O*-demethyl-16,17-dihydro MG (17-*O*-DM-DH-MG, 5); 9,17-*O*-bisdemethyl-16,17-dihydro MG (9,17-*O*-BDM-DH-MG, 6); 17-carboxy-16,17-dihydro MG (17-COOH-DH-MG, 7); and 9-*O*-demethyl-17-carboxy-16,17-dihydro MG (9-*O*-DM-17-COOH-DH-MG, 8). The retention times in the runs of the rat or human urines were recorded at different times and therefore they varied. Nevertheless, the mass spectra allowed identifying the corresponding metabolites in the urine samples of both species.

As shown in Fig. 2 and indicated in the typical reconstructed MS^1 mass chromatograms, the following phase II metabolites could be identified in rat urine (R, Fig. 5, upper part) or human urine (H, Fig. 5, lower part): 9-*O*-demethyl MG glucuronide (9-*O*-DM-G-MG, 2G); 9-*O*-demethyl MG sulfate (9-*O*-DM-S-MG, 2S); 16-carboxy MG glucuronide (16-COOH-G-MG, 3G); 9-*O*-demethyl-16-carboxy MG glucuronide (9-*O*-DM-16-COOH-G-MG, 4G); 9-*O*-demethyl-16-carboxy MG sulfate (9-*O*-DM-16-COOH-S-MG, 4S); 17-*O*-demethyl-16,17-dihydro MG glucuronide (17-*O*-DM-DH-G-MG, 5G); 9,17-*O*-bisdemethyl-16,17-dihydro MG glucuronide (9,17-*O*-BDM-DH-G-MG, 6G); and 9,17-*O*-bisdemethyl-16,17-dihydro MG sulfate (9,17-*O*-BDM-DH-S-MG, 6S).

As can be seen in Figs 4 and 5, not all metabolites detected in rat urine could be detected in human urine and vice versa. Metabolites 4, 6, and 8 could be detected in unconjugated form only in rat urine. The major differences were observed in the phase II metabolism. Metabolites 4G and 6G could only be detected in rat urine and metabolites 2S, 5G (completely conjugated) and 6S only in human urine. These differences may be caused by species differences in metabolism and/or by the fact that the human urine samples may have been taken after different dosages and at different times after ingestion.

On the basis of the identified metabolites, the following metabolic pathways of MG, shown in Fig. 6, could be postulated for rats and humans: hydrolysis of the methylester in position 16 (3, 4), *O*-demethylation of the 9-methoxy group (2, 4, 6, 8) and of the 17-methoxy group (5–8), followed, via the corresponding aldehydes, by oxidation to carboxylic acids (7, 8) or reduction to the correspondent alcohols (5, 6), and combinations of some of these steps (4, 6, 8). Finally, in rats, four phase I metabolites were

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conjugated to glucuronides (2G, 3G, 4G, 6G) and one to sulfate (4S), but in humans, three metabolites to glucuronides (2G, 3G, 5G) and three to sulfates (2S, 4S, 6S).

Conclusions

The metabolism studies presented here showed that MG was extensively metabolized in rats and humans with some differences, particularly in phase II metabolism. The applied LIT technology was suitable for elucidation of the phase I and II metabolites, particularly in combination with the high-resolution OT technology.

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