Metabolism studies of the *Kratom* alkaloids mitraciliatine and isopaynantheine, diastereomers of the main alkaloids mitragynine and paynantheine, in rat and human urine using liquid chromatography–linear ion trap-mass spectrometry

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ABSTRACT

Mitragyna speciosa (*Kratom* in Thai), native in Southeast Asia, is increasingly misused as a herbal drug of abuse. During metabolism studies on the *Kratom* alkaloids mitragynine, its diastereomers speciogynine and speciociliatine as well as paynantheine in rats and humans, further isomeric compounds were detected in *Kratom* users' urine. The question arose whether these compounds were formed from the low abundant, isomeric alkaloids mitraciliatine (MC) and isopaynantheine (ISO-PAY). Therefore, the aim of the presented study was to identify using liquid chromatography–linear ion trap-mass spectrometry their phase I and II metabolites in rat urine after administration of pure MC or ISO-PAY, to confirm their formation in humans, and finally to confirm whether the above-mentioned isomeric compounds in human urine represent MC and ISO-PAY and/or their metabolites. The metabolic pathways of both alkaloids in rats were found to be comparable to those of their corresponding diastereomers. In the human urines tested, not all metabolites found in rats could be detected because of the much lower amounts of MC and ISO-PAY in *Kratom*. However, all the above-mentioned so far unknown isomeric compounds could be identified in the human urine samples as MC, ISO-PAY and/or their metabolites. The used LC separation was also suitable for the differentiation of all other *Kratom* alkaloids and their metabolites in human urine.

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1. Introduction

Mitragyna speciosa Korth. (Thai name *Kratom*; Rubiaceae) is a medical plant native to Thailand and other Southeast Asian countries [1,2]. Various alkaloids in varying concentrations could be identified in several plant species and/or plant parts [3–5]. For example, *M. speciosa* leaves from adult plants in Thailand have been reported to contain as main alkaloid approximately over 60% mitragynine (MG), those from Malaysia only over 10%. Paynantheine (PAY) and the MG diastereomer speciogynine (SG) were the second most abundant alkaloids and the MG diastereomer speciociliatine (SC) was the third abundant alkaloid in both plants

[5]. Shellard et al. [4] described the distribution and probable biogenetic route of *M. speciosa* in young and mature leaves from Thailand. The alkaloidal pattern is different in the young plants from that in the older trees. The leaves of young Thai plants contain in addition besides MG, PAY, SG, and SC, the MG diastereomer mitraciliatine (MC) and the PAY diastereomer isopaynantheine (ISO-PAY). The chemical structures and configurations at the chiral centers at position 3, 15, and 20 are given in Fig. 1. SG was found to be the main alkaloid in young plants. In fruits of Malaysian plants, MG, PAY, and all their diastereomers could be detected with SC being the main alkaloid [3]. The configuration of the different alkaloids is as follows: MG: 3S, 15S, 20S; SG: 3S, 15S, 20R; SC: 3R, 15S, 20S; MC: 3R, 15S, 20R; PAY: 3S, 15S, 20R; ISO-PAY: 3R, 15S, 20R.

Some alkaloids from *M. speciosa* have opiate- and cocaine-like effects, which is why *Kratom* has traditionally been used as a stimulant or as a substitute for opiate addicts [6,7]. Because of these effects, *Kratom* is misused as herbal drug of abuse, which is illegal in Thailand since 1946 and since recent years in Australia and Denmark. As is currently not scheduled in the United States and in most European countries, a broad range of *Kratom* products is available e.g. via the Internet.

For metabolism studies of SG [8] in humans, the liquid chromatographic (LC) separation used for the metabolism studies of
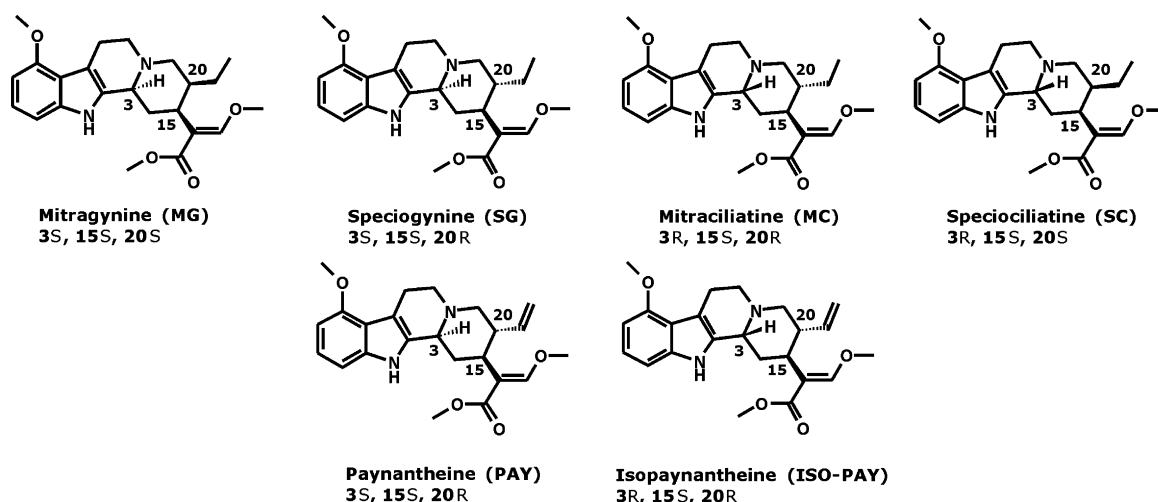
Fig. 1. Structures and configurations at the chiral centers at position 3, 15, and 20 of all mentioned *Kratom* alkaloids.

MG and PAY [9,10] had to be improved markedly allowing the separation of SG and its metabolites from its diastereomer MG and its metabolites. Using this modified LC separation, several isomeric compounds of MG and PAY and their metabolites were detected in *Kratom* users' urine, which were not found in rat urine after administration of each pure single alkaloid. In order to differentiate these isomers, the alkaloids MG, SG, SC, and PAY, most abundant in the *Kratom* preparations, were administered as pure single alkaloid to rats and their metabolites identified using low and high resolution LC–linear ion trap (LIT)-mass spectrometry (MS) [8–11]. So, most isomeric compounds detected in human urine could be identified by their mass spectra and retention times. The question arose whether the remaining unknown compounds were formed from the low abundant, isomeric alkaloids MC and ISO-PAY. This suspicion was strengthened by a recent case report [6], in which MG and its diastereomers SG, SC, and MC as well as PAY and an unknown isomer could be detected in a *Kratom* users' urine by LC–tandem–MS. This approach did not allow detecting any metabolites of the *Kratom* alkaloids. Another LC–MS/MS procedure also focused on the determination of only MG [12] in urine.

Therefore, the aim of the presented study was to isolate MC and ISO-PAY from *Kratom* leaves, to study using LC–LIT-MS with their phase I and II metabolites in rat urine after administration of pure single alkaloids, to confirm their formation in humans, and finally to confirm whether the above-mentioned isomeric compounds in human urine represent MC and ISO-PAY and/or their metabolites.

2. Experimental

2.1. Chemicals and reagents

MC and ISO-PAY were isolated as described below from shredded *Kratom* leaves (Thai plant) obtained from Azarius (Amsterdam, The Netherlands). Silica gel for column chromatography (particles of 35–70 μm and 50–200 μm) was obtained from Sigma–Aldrich (Steinheim, Germany). 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.

2.2. Isolation of MC and ISO-PAY from Kratom leaves

MC and ISO-PAY isolation and purification were performed according to Ponglux et al. [1] and Philipp et al. [8,10]. Briefly, the dried, shredded leaves (123 g) were extracted with 2.1 l of methanol (MeOH) using Soxhlet extraction. The residue was dissolved in 500 ml of 10% aqueous acetic acid solution. The aqueous layer was washed with 4× 200 ml n-hexane, separated and basified with 10 M NaOH (pH 8–9) at 0 °C and extracted with 4× 170 ml of chloroform (CHCl₃).

The organic layer was evaporated and purified by silicon dioxide (SiO₂) column chromatography (3 cm × 21 cm, particles of 50–200 μm) using the following mixtures: CHCl₃/ethylacetate (EtAc) (9:1; 500 ml; fraction A (A1–10)); CHCl₃/EtAc (4:1; 150 ml; A11–13), CHCl₃/EtAc (1:1; 150 ml; A14–16), EtAc (150 ml; A17–19), EtAc/MeOH (19:1; 150 ml; A20–22), EtAc/MeOH (4:1; 150 ml; A23–25), and dichloromethane (CH₂Cl₂) (250 ml; A26–30). The fraction (A) volume of the first SiO₂ column chromatography was 50 ml.

The fractions A16–26 of the first column chromatography were subjected again to SiO₂ column chromatography (3 cm × 24 cm, particles of 50–200 μm) using CHCl₃/EtAc (9:1; 500 ml; B1–10), CHCl₃/EtAc (4:1; 150 ml; B11–13), CHCl₃/EtAc (1:1; 150 ml; B14–16), EtAc (150 ml; B17–19), EtAc/MeOH (19:1; 150 ml; B20–22), EtAc/MeOH (4:1; 150 ml; B23–25), CH₂Cl₂ (250 ml; B26–30), MeOH (100 ml; B31–32), MeOH/Water (H₂O; 1:1; 100 ml; B33–34). The fraction (B) of the second SiO₂ column chromatography was 50 ml.

ISO-PAY: The fractions of B22 and B31 of the second column chromatography were subjected again to SiO₂ column chromatography (1 cm × 28 cm, particles of 35–70 μm) using CHCl₃/EtAc (4:1; 100 ml; C1–10), CHCl₃/EtAc (1:1; 100 ml; C11–20), EtAc (100 ml; C21–30), EtAc/MeOH (1:1; 100 ml; C31–40), MeOH (50 ml; C41–45), MeOH/H₂O (1:1; 50 ml; C46–50). The fraction volume (C) of the third SiO₂ column chromatography was 10 ml. The ISO-PAY (C27–29) containing fractions were evaporated to dryness.

MC: The fractions of B32 of the second column chromatography were subjected again to SiO₂ column chromatography (1 cm × 27 cm, particles of 35–70 μm) using CHCl₃/EtAc (4:1; 100 ml; D1–10), CHCl₃/EtAc (1:1; 100 ml; D11–20), EtAc (100 ml; D21–30), EtAc/MeOH (2:1; 60 ml; D31–36), EtAc/MeOH (1:1; 70 ml; D37–43), and MeOH/H₂O (1:1; 50 ml; D44–48). The fraction volume (D) of the fourth SiO₂ column chromatography was 10 ml. The MC (D29–30) containing fractions were evaporated to dryness.

As given below, gas chromatography–mass spectrometry (GC–MS) in the electron ionization (EI) mode was used for the detection of MC and ISO-PAY in the eluate fractions.

2.3. Identity confirmation of the isolated MC and ISO-PAY

As described below, MC and ISO-PAY were identified by GC–MS and LC–MS. For further structure confirmation by NMR spectroscopy, a solution of MC, ISO-PAY, MG, SC, or PAY (3 mg/0.7 ml each) were prepared in $CDCl_3$ and SG in acetone-d_6. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) together with 2D NMR-^{1}H-^{1}H correlated spectroscopy (COSY), and heteronuclear single quantum coherence (HSQC) were recorded on a Bruker DRX 500 (Bruker, Rheinstetten, Germany) at 300 K. The chemical shifts were given in δ values (ppm) relative to $CHCl_3$ at δ_H 7.24 or $CDCl_3$ at δ_C 77.00 and for acetone at δ_H 2.05 and acetone-d_6 at δ_C 29.80.

2.4. Urine samples

The investigations were performed using urine of three 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 of MC or ISO-PAY in aqueous suspension by gastric intubation. Urine was collected separately from the faeces over a 24 h period stabilized with 5 mg of potassium fluoride. The samples were directly analyzed or stored at $-20\,^\circ$C until further analysis. Blank rat urine samples were collected before drug administration to check whether they were free of interfering compounds.

In addition, human urine samples of various patients submitted to the authors' laboratory for toxicological analysis were used.

2.5. Sample preparation for the identification of phase I and II metabolites

The samples were worked up as described in Refs. [8–11]. Briefly, for the metabolism study, a 1-ml portion of urine was worked-up after enzymatic conjugate cleavage by solid-phase extraction (SPE, HCX) or directly by SPE (C18).

For elucidating the structure of the glucuronides, another urine sample was worked up directly by SPE (C18) and methylation was conducted with 100 μl of a solution of diazomethane for 5 min or for 24 h as described in Refs. [8–11].

2.6. GC–MS apparatus

The eluate fractions of the MC and ISO-PAY isolation were analyzed using a Thermo Fisher Scientific (TF, Dreieich, Germany) Trace GC Ultra gas chromatograph combined with a TF DSQ II MS (EI mode, TF TR-5MS capillary, 15 m × 0.25 mm I.D.) with TF XCalibur 1.4 software. For details see Ref. [8].

2.7. LC–MS apparatus

The samples were analyzed using a TF Accela LC system consisting of a degasser, a quaternary pump, and an autosampler coupled to a TF LXQ LIT equipped with a heated electrospray ionization (ESI) source. Collision induced dissociation (CID)-MS/MS experiments were performed on precursor ions selected from MS^1 using data-dependent acquisition (DDA): 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 DDA 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. For details see Refs. [8,11].

The LC was equipped with a TF Hypersil Gold C18 column (100 mm × 2.1 mm, 1.9 μm). Gradient elution using mobile phase A: 10 mmol/l aqueous ammonium formate buffer containing 0.1% (v/v) formic acid (final pH 3) and mobile phase B: acetonitrile containing 0.1% (v/v) formic acid. For details see Refs. [8,11].

2.8. Screening for and identification of metabolites

In procedure I, full scan was acquired in MS^1 for searching metabolites in urine according to their expected protonated 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 MC or ISO-PAY in order to elucidate the most probable metabolites structures. 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.

3. Results and discussion

3.1. Isolation and structure confirmation of MC and ISO-PAY

MC and ISO-PAY were commercially not available and had first to be isolated from Thai *Kratom* leaves according to Ponglux et al. [1] and Philipp et al. [8,10]. The toxic solvent $CHCl_3$ was unfortunately the only organic solvent that provided sufficient extraction and separation power for the alkaloids. All collected fractions of all four SiO_2 column chromatography systems were analyzed by GC–MS for in-process control. The identity and purity of the isolated alkaloids were proved by NMR, GC–MS, and LC–MS.

The ^{1}H NMR spectra of MC and ISO-PAY recorded in different solvents at ambient temperature showed the same extensive line broadening of the aliphatic resonances as described for dihydrocorynantheine [13], their 9-deoxy derivative. This is due to the fact, that at room temperature, several conformers were present in slow exchange on the NMR time scale. The ^{1}H NMR and ^{13}C NMR data ($CDCl_3$, Suppl. Tables 1 and 2) were close to those of MC and ISO-PAY from literature [3], but not all chemical shifts matched perfectly. For example, the NH singlet of both compounds appeared in the spectra around 1 ppm downfield compared to that of all the 9-methoxycorynantheidine-type alkaloids (MG, SG, SC, MC, ISO-PAY) published by Kitajima et al. [3]. This could be an effect of different pH values or concentrations of the applied solutions. Therefore, to find unambiguous evidence for the structures of our isolated products, the stereochemistry of C-3, C-15 and C-20 of both compounds was assigned directly from our NMR data.

By comparing the NMR literature data of 9-methoxycorynantheidine-type alkaloids [3,10], the chemical shift for C-3 was found to be an ideal marker for the stereochemistry at this position. In the 3S series (MG, SG, and PAY), the signal for C-3 appeared around δ 60, whereas for the 3R analogues (SC, MC and ISO-PAY) C-3 was found 5 ppm upfield around δ 55. δ 55.30 was measured for MC and δ 55.78 for ISO-PAY, respectively, clearly indicating that both belonged to the 3R series. The stereochemistry at C-15 and C-20 was deduced from the coupling values of the corresponding protons. H-15 in our MC (δ 2.32, *brdd*, *J* = 12, 12) as well as in our ISO-PAY (δ 2.49, *ddd*, *J* = 12, 12, 3) presented two diaxial couplings to H-14$_{ax}$ and H-20. Therefore, H-15 and H-20 were in axial positions for both compounds, indicating a 15S and a 20R configuration. Altogether both compounds showed the required 3S, 15S, 20R configuration. Their structures were therefore elucidated as MC and ISO-PAY.
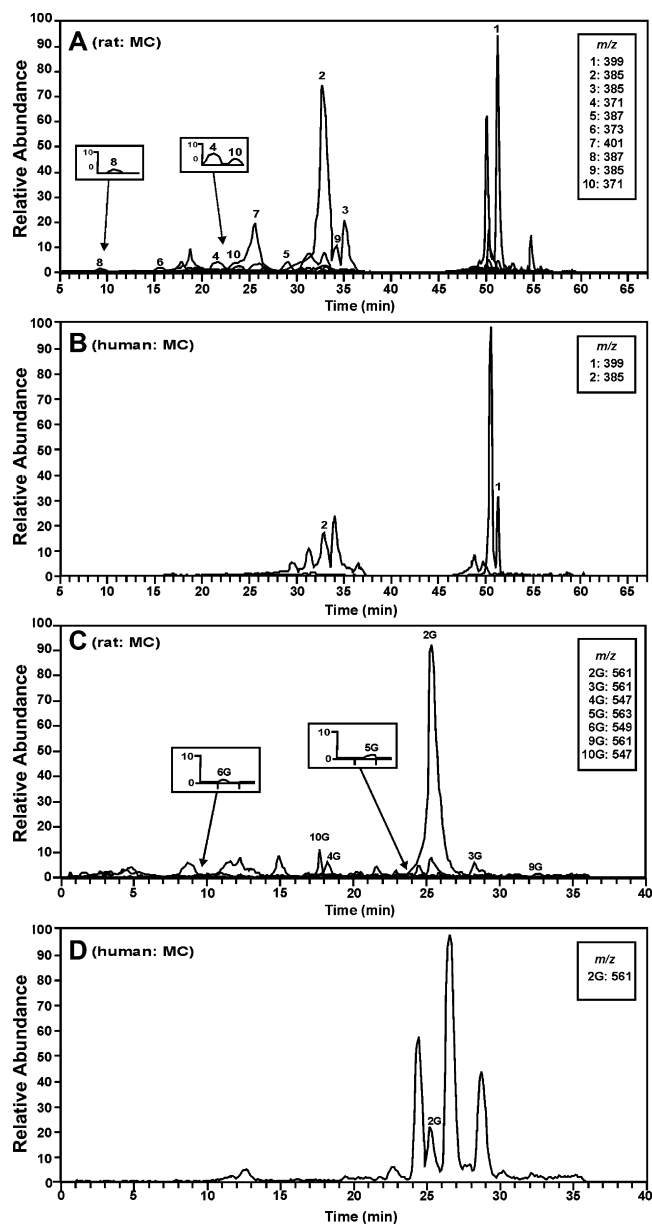
Fig. 2. Reconstructed chromatograms (excerpt) of the protonated molecular ions of expected phase I metabolites of MC in rat (A), and human (B) urine as well as phase II metabolites of MC in rat (C), and human (D) urine.

3.2. Identification of the metabolites

The MC and ISO-PAY metabolites were identified in rat urine after the same dose (40 mg/kg BM dose) as used for the MG, SG, SC and PAY studies [8–11]. The phase I metabolites were isolated after gentle enzymatic cleavage of conjugates using mixed-mode SPE and the phase II metabolites without cleavage of conjugate using C18 SPE [8–11]. The same LC separation as already mentioned for the metabolism studies for SG [8] and SC [11] was used, which allowed the separation of MC and ISO-PAY and its metabolites from other diastereomeric compounds in the human urine samples.

The presence of phase I metabolites was screened for by reconstructed ion chromatography according to procedure I and II. In Suppl. Tables 3 and 5, the retention times, empirical formulas, protonated molecular ions in the MS^1 spectra of MC or ISO-PAY and their phase I metabolites, their typical fragments and intensities in the MS^2 spectra of the protonated molecular ions in the MS^1 spectra, and their typical fragments and intensities in the MS^3 spec-
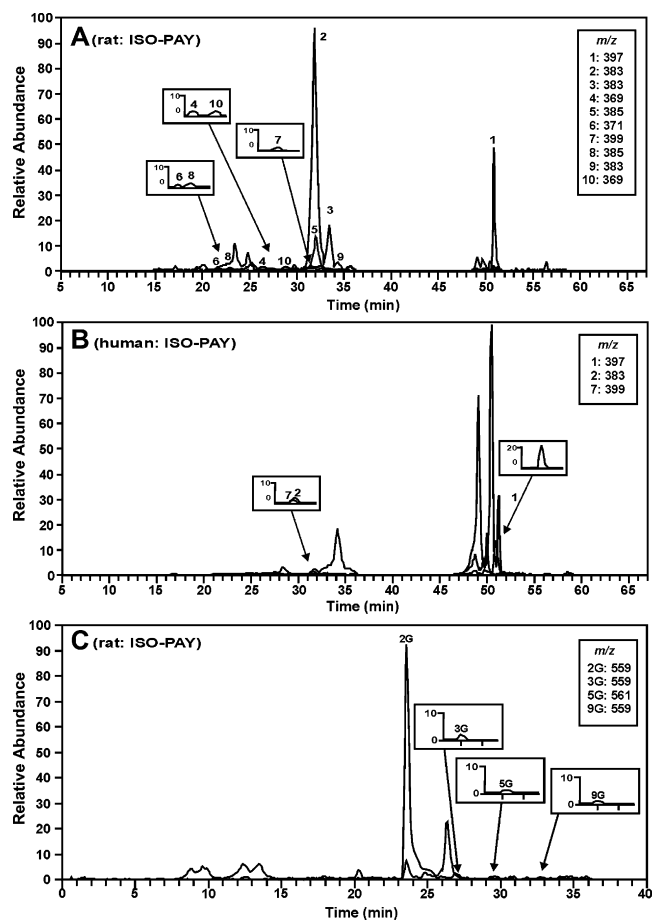

Fig. 3. Reconstructed chromatograms (excerpt) of the protonated molecular ions of expected phase I metabolites of ISO-PAY in rat (A), and human (B) urine as well as phase II metabolites of ISO-PAY in rat (C).

tra of the two most abundant fragments in the MS^2 spectra are depicted. 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 MC or ISO-PAY.

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. In Suppl. Tables 4 and 6, the retention times, empirical formulas, protonated molecular ions in the MS^1 spectra of MC or ISO-PAY phase II metabolites, their protonated molecular ions and intensities in the MS^2 spectra, their typical fragments and intensities in the MS^3 spectra of the protonated molecular ions in the MS^2 spectra, and the typical fragments and intensities in the MS^4 spectra of the two most abundant fragments in the MS^3 are given. The structures of the phase II metabolites (Suppl. Tables 4 and 6) 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 (Suppl. Tables 3 and 5). The position of the glucuronic acid in metabolites 4, 6 and 10 (only detected for MC) were determined using selective derivatization with diazomethane as described for MG, SG, SC, and PAY [8–11]. Only carboxy, but no hydroxy groups could be methylated within 5 min. Metabolite 4G was conjugated at the carboxylic acid and metabolites 6G and 10G at the phenolic hydroxy group.

The glucuronides of the metabolites 3 and 4 are acyl glucuronides, which are known to undergo intramolecular acyl migration [14]. As already discussed for speciogynine [8], the LC separation system allowed separating diastereomers of various
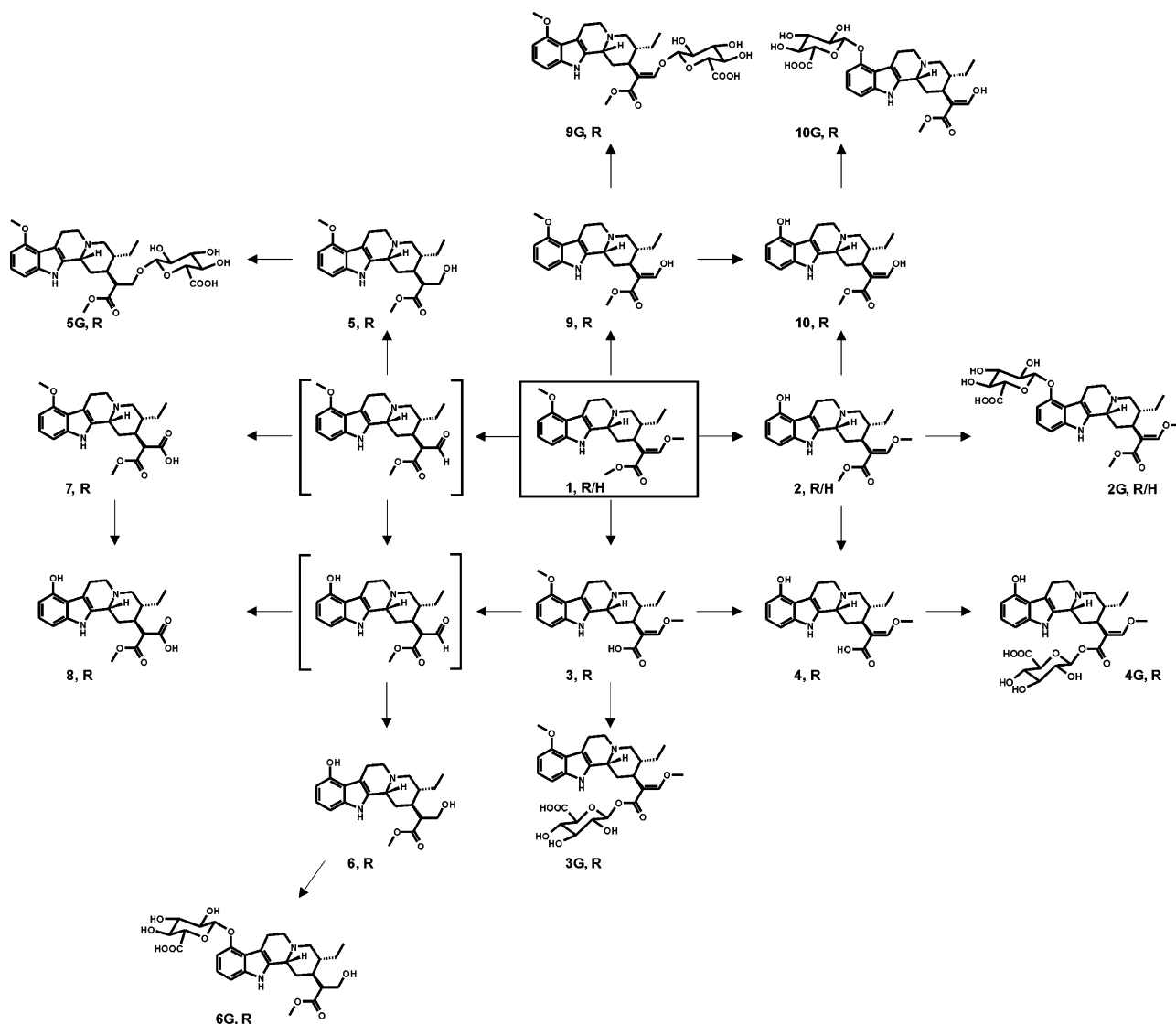
Fig. 4. Postulated metabolic pathways of MC in rats (R) and humans (H). Assumed intermediate metabolites in square brackets.

metabolites including glucuronides so that it could be assumed that such isomeric glucuronides should also have been separated. However, no additional peaks for such isomers were detected. If they were not separated, the problem could arise that they were not cleavable by the specific beta-glucuronidases. However, this was not relevant for this qualitative study for which the conjugates were only cleaved to facilitate the identification of the unconjugated metabolites.

3.3. Proposed fragmentation patterns

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 MC or ISO-PAY. MC and its metabolites showed comparable fragmentation patterns with the diastereomer SG and its metabolites [8], which might be explained by the same configuration in position 20 (both 20R). This was also the fact for ISO-PAY and PAY (both 20R) and their metabolites. Therefore, it seemed to be not necessary to confirm the elemental composition of the fragments by high resolution mass spectrometry as performed for MG [9], SG [8], and PAY [10].

3.4. Proposed metabolic pathways

As shown in Suppl. Table 3 and indicated in the typical reconstructed MS^1 mass chromatograms (Fig. 2A), besides MC (1), the following MC phase I metabolites could be identified in rat urine: 9-O-demethyl MC (2), 16-carboxy MC (3), 9-O-demethyl-16-carboxy MC (4), 17-O-demethyl-16,17-dihydro MC (5), 9,17-O-bisdemethyl-16,17-dihydro MC (6), 17-carboxy-16,17-dihydro MC (7), and 9-O-demethyl-17-carboxy-16,17-dihydro MC (8), 17-O-demethyl MC (9), 9,17-O-bisdemethyl MC (10).

As shown in Suppl. Table 4 and indicated in the typical reconstructed MS^1 mass chromatograms (Fig. 2C), the following phase II metabolites of MC could be identified in rat urine: glucuronides (G) of 9-O-demethyl MC (2G), 16-carboxy MC (3G), 9-O-demethyl-16-carboxy MC (4G), 17-O-demethyl-16,17-dihydro MC (5G), 9,17-O-bisdemethyl-16,17-dihydro MC (6G), 17-O-demethyl MC (9G), and 9,17-O-bisdemethyl MC (10G).

As shown in Suppl. Table 5 and indicated in the typical reconstructed MS^1 mass chromatograms (Fig. 3A), besides ISO-PAY (1), the following ISO-PAY phase I metabolites could be identified in rat urine: 9-O-demethyl ISO-PAY (2), 16-carboxy ISO-PAY (3), 9-O-demethyl-16-carboxy ISO-PAY (4), 17-O-demethyl-16,17-

Fig. 5. Postulated metabolic pathways of ISO-PAY in rats (R) and humans (H). Assumed intermediate metabolites in square brackets.

dihydro ISO-PAY (5), 9,17-O-bisdemethyl-16,17-dihydro ISO-PAY (6), 17-carboxy-16,17-dihydro ISO-PAY (7), and 9-O-demethyl-17-carboxy-16,17-dihydro ISO-PAY (8), 17-O-demethyl ISO-PAY (9), 9,17-O-bisdemethyl ISO-PAY (10).

As shown in Suppl. Table 6 and indicated in the typical reconstructed MS^1 mass chromatograms (Fig. 3C), the following phase II metabolites of ISO-PAY could be identified in rat urine: glucuronides (G) of 9-O-demethyl ISO-PAY (2G), 16-carboxy ISO-PAY (3G), 17-O-demethyl-16,17-dihydro ISO-PAY (5G), and 17-O-demethyl ISO-PAY (9G).

Considering the MS^2, MS^3, and/or MS^4 spectra (Suppl. Tables 3 and 4) and the retention times, in the urine samples of *Kratom* users, besides MC (1), only the following phase I and II metabolites could be detected (Fig. 2B and D): 9-O-demethyl MC (2) and the glucuronide of 9-O-demethyl MC (2G).

Considering the MS^2, MS^3, and/or MS^4 spectra (Suppl. Tables 5 and 6) and the retention times, in the urine samples of *Kratom* users, besides ISO-PAY (1), only the following phase I metabolites could be detected (Fig. 3B): 9-O-demethyl ISO-PAY (2) and 17-carboxy-16,17-dihydro ISO-PAY (7). For ISO-PAY no phase II metabolites could be observed in the urine samples of *Kratom* users. On the basis of the identified metabolites, the postulated metabolic pathways are depicted in Fig. 4 (MC) and Fig. 5 (ISO-PAY). They are comparable with those of the corresponding diastereomers MG, SG, and SC, or PAY [8–11].

At the end of these metabolism studies, the various peaks in the LC–MS chromatograms of extracts of *Kratom* users could finally be confirmed by addition of a suitable amount of the corresponding rat urine extract to the human urine extract. The peaks

Fig. 6. Reconstructed chromatograms of the protonated molecular ions of MG, SG, SC, MC, PAY, and ISO-PAY (upper part) and their main phase I and II metabolites in a human urine sample (zoomed excerpt, lower part).

of the corresponding diastereomeric alkaloid and its metabolites increased and could therefore, be identified. Isomeric metabolites such as 2G and 3G could be differentiated by different fragmentations as already discussed for MG [9]. In Fig. 6, reconstructed chromatograms are depicted of the protonated molecular ions of MG, SG, SC, MC, PAY, and ISO-PAY (upper part) and their main phase I and II metabolites in a human urine sample (zoomed excerpt, lower part). In our experience, the relation of the alkaloids and also of their metabolites can vary from case to case depending on the used *Kratom* preparation (tea, resin, extract, young/old plant, species etc.), dosage, administration route, individual differences in pharmacokinetics, and/or sampling time.

4. Conclusions

This metabolism study showed that the so far unknown isomeric compounds detected in *Kratom* users' urine samples could be identified as MC, ISO-PAY and/or their metabolites. The used LC separation was suitable for the differentiation also of all other *Kratom* alkaloids and their metabolites in human urine.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.03.005.

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