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***In Vitro and in Vivo* Study of the Gastrointestinal Absorption and Metabolisation of Hymenocardine, a Cyclopeptide Alkaloid**

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Abstract

Hymenocardine is a cyclopeptide alkaloid, present in the root bark of *Hymenocardia acida*. In traditional African medicine, the leaves and roots of this plant are used to treat malaria and moderate *in vitro* antiplasmodial activity has been reported for hymenocardine. However, in view of its peptide-like nature, potential metabolism after oral ingestion has to be taken into account when considering *in vivo* experiments. In this study the stability and small intestinal absorption of hymenocardine was assessed, using an *in vitro* gastrointestinal dialysis model (GIDM). In addition, potential liver metabolism was investigated *in vitro* by incubation with a human S9 fraction. Moreover, hymenocardine was administered to rats *per os*, and blood and urine samples were collected until 48 and 24 h after oral administration, respectively. All samples resulting from these three experiments were analyzed by LC-MS. Analysis of the dialysate and retentate, obtained from the GIDM, indicated that hymenocardine is absorbed unchanged from the gastrointestinal tract, at least in part. After S9 metabolism, several metabolites of hymenocardine could be identified, the major ones being formed by reduction, and/or the loss of an *N*-methyl group. The *in vivo* study confirmed that hymenocardine is absorbed from the gastrointestinal tract unchanged, since it could be identified in both rat plasma and urine, together with hymenocardinol, its reduction product.

Key words

Hymenocardia acida, Phyllanthaceae, hymenocardine, absorption, metabolism

Abbreviations

GIDM Gastrointestinal dialysis model

Introduction

Hymenocardia acida Tul. (Phyllanthaceae) is a shrub or small tree that is indigenous to the African Savannah. In traditional African medicine, its leaves and roots are used to treat malaria and moderate *in vitro* antiplasmodial activity has previously been reported for extracts of the leaves of *H. acida*, as well as for hymenocardine, a cyclopeptide alkaloid isolated from the root bark of *H. acida* [1,2]. Cyclopeptide alkaloids are polyamidic macrocyclic compounds, containing a 13-, 14- or 15-membered ring [3,4]. Apart from hymenocardine, *in vitro* antiplasmodial activity has been shown for several other cyclopeptide alkaloids, namely ziziphines-N and -Q, mauritine-M, hemsine-A, hymenocardinol, hymenocardine *N*-oxide, nummularines-H and -R, *O*-desmethylnummularine-R and oxyphylline-F [1,5-7]. However, no *in vivo* studies concerning the antiplasmodial activity of this type of compounds have been performed yet, and in view of their peptide-like nature, the question can be raised whether cyclopeptide alkaloids are stable in the gastrointestinal tract, and whether they will be absorbed after oral administration; these are essential prerequisites to exhibit *in vivo* antiplasmodial activity.

Up till now only a few reports exist, which describe the pharmacological (sedative and anxiolytic) effects of cyclopeptide alkaloids *in vivo*, after oral administration [8-10]. As for the absorption and metabolic stability of this class of compounds, Suh et al. reported that frangufoline is converted by enamide degradation, both *in vitro*, after incubation under acidic conditions, and *in vivo*, after IV injection to rats, leading to the formation of a substituted linear tripeptide [11,12]. No other cyclopeptide alkaloids were investigated for their stability and/or gastrointestinal absorption. Since hymenocardine does not contain an enamide bond, a similar conversion as observed for frangufoline is not possible. Therefore the metabolism and absorption of hymenocardine was investigated, *in vitro* as well as *in vivo* (for a general experimental overview, see Supporting information, Fig. S1).

In the first part of this study, hymenocardine was submitted to a gastrointestinal dialysis model (GIDM), in order to determine whether it can withstand the conditions of the digestive system and in order to assess possible absorption from the gastrointestinal tract. In addition, the liver metabolism of hymenocardine was investigated by incubation with a human S9 fraction, a liver homogenate containing both microsomes and cytosolic enzymes. In order to deduce which metabolites were formed, LC-HRMS profiles were recorded, and data were compared with a list of metabolites that was predicted *in silico*. The MS fragmentation pathway of hymenocardine was studied, which provided useful information for the interpretation of the fragment ions of the metabolites. Moreover, an *in vivo* study was performed, where hymenocardine was administered to rats *per os* and plasma and urine samples were analyzed for the presence of hymenocardine and its metabolites.

Results and Discussion

In the first part of our study, hymenocardine was submitted to an *in vitro* continuous flow GIDM. The compound was first incubated in a gastric phase, simulating the adult gastric environment, followed by a small intestinal phase. The continuous flow system that was applied in the small intestinal phase of the model, eliminates compounds diffusing through a semi-permeable membrane, thus simulating absorption by passive diffusion. This resulted in two fractions: a retentate (fraction remaining in the gastrointestinal tract) and a dialysate (fraction absorbed in the small intestinal phase). Analysis of hymenocardine itself, and the retentate and dialysate by UPLC-HRMS in positive ion mode, revealed the presence of hymenocardine in both fractions obtained with the dialysis model ($C_{37}H_{51}N_6O_6$, m/z 675.3992, $[M+H]^+$). This indicated that hymenocardine was at least in part absorbed from the small intestine. In addition, no signals due to metabolism products could be detected, neither in the dialysate, nor in the

retentate. This suggests that hymenocardine, in spite of its peptide-like character, is stable in gastric and small intestinal conditions and is not degraded.

In vitro liver metabolism of hymenocardine was investigated by incubation with human S9 fraction and analysis of the samples by UPLC-HRMS in both positive and negative mode. Moreover, hymenocardine was subjected to UPLC-HRMS fragmentation to determine the fragmentation pattern of this compound and to obtain insight in the expected fragmentation pattern of its metabolites. Two software programs for drug metabolism prediction, namely Meteor Nexus and Metaprint2D-React Web Server, were used to generate phase-I metabolites from hymenocardine *in silico*. The list of *in silico* metabolites was matched with the list of metabolites retrieved from the S9 metabolism experiment and this resulted in 14 possible metabolites. The spectral and chromatographic data of the metabolites are listed in Table S1, Supporting Information. For six of the metabolites, a tentative structure could be defined (Fig. 1).

In positive ion mode, hymenocardine (**1**) seems to be fragmented according to general peptide fragmentation rules, as described by Maleknia et al. [13] (Fig. 2). For example, the product ion at m/z 114, by far the most abundant product ion, corresponding to a most probable molecular formula $C_7H_{16}N$, originates from the breakage of the amide bond at the terminal isoleucine-like moiety, resulting in the formation of an a ion.

The product ion at m/z 241 ($C_{13}H_{25}O_2N_2$) results from a breakage of the second amide bond of the side chain of hymenocardine. The product ion at m/z 338 ($C_{18}H_{32}O_3N_3$) seems to arise from a breakage of the amide bond in the macrocyclic ring that is closest to the side chain, and an additional breakage at the ether bond. The product ions at m/z 185 ($C_{11}H_9ON_2$), m/z 170 ($C_{11}H_8ON$) and m/z 159 ($C_{10}H_{11}N_2$) originate from a combination of breakages of amide, C-CO, or C-NH bonds, resulting in the formation of product ions containing the indole moiety.

In negative ion mode, several intense product ions were formed: m/z 336 ($C_{19}H_{18}O_3N_3$), m/z 433 ($C_{24}H_{25}O_4N_4$), m/z 544 ($C_{28}H_{42}O_6N_5$), m/z 207 ($C_{10}H_{11}O_3N_2$), m/z 150 ($C_8H_8O_2N$) and m/z 93 (C_6H_5O). The tentative structures of these product ions are depicted in Fig. 3.

Taking into account the fragmentation pathways of hymenocardine and the structural differences between hymenocardine and its metabolites, depicted in Fig. 1, fragmentation of the metabolites should theoretically result in different, and therefore diagnostic product ions. Thus, the MS² spectra of the metabolites were investigated with the objective to confirm their tentative structures.

Metabolite **2** was identified as hymenocardinol ($C_{37}H_{52}O_6N_6$, m/z 677.40166, $[M+H]^+$), formed by a reduction of the keto group of the *p*-hydroxy- ω -amino acetophenone moiety of hymenocardine. During fragmentation of this metabolite in positive ion mode, various product ions were observed that were also detected during fragmentation of hymenocardine (m/z 338, m/z 185, m/z 159 and m/z 114, Fig. 2), indicating that the metabolic change did not occur at the side chain, the indole moiety, or the β -hydroxyvaline moiety.

Other product ions were detected at m/z 659 ($C_{37}H_{50}O_5N_6$) and m/z 437 ($C_{24}H_{29}O_4N_4$). These ions confirmed the metabolic change of the carbonyl group next to the phenyl group into a hydroxyl group. The former is formed by a loss of water, the latter by a breakage of the amide bond between the β -hydroxyvaline moiety and the valine moiety.

Fragmentation in negative ion mode also resulted in the occurrence of several diagnostic product ions, including the ions at m/z 191 ($C_{10}H_{11}O_2N_2$) and m/z 134 (C_8H_8ON). They correspond to product ions formed during fragmentation of hymenocardine in negative ion mode, but with a mass difference of approximately 16 Da (m/z 207 and m/z 150), owing to a loss of oxygen. This additional loss originates from the metabolic change of the carbonyl group into a hydroxyl group, which is easily lost during fragmentation. Several other product ions in Table S1 provided additional structural confirmation. Comparison by LC-MS of a standard

solution of hymenocardinol with compound **2** confirmed the identity of this metabolite. Hymenocardinol (**2**) is predicted as a possible metabolite of hymenocardine by the Meteor Nexus software; this conversion is catalyzed by the enzyme ADH (alcohol dehydrogenase). The metabolite tentatively identified as *N*-monomethyl hymenocardine ($C_{36}H_{48}O_6N_6$, m/z 661.37029, $[M+H]^+$) (**3**) results from a loss of one of the *N*-methyl groups in the isoleucine-like moiety in the side chain, thereby losing CH_2 . Fragmentation in positive ion mode rendered several diagnostic product ions. A very intense product ion was observed at m/z 100 ($C_6H_{14}N$), resulting from a fragmentation pathway similar to that of the product ion of hymenocardine at m/z 114, described before. The difference in mass between the product ions arises from the change of the tertiary amine group to a secondary amine during metabolism. Moreover, other product ions that were also diagnostic for the demethylation during metabolism were observed, namely an *a* ion at m/z 199 ($C_{11}H_{23}ON_2$) and a *b* ion at m/z 227 ($C_{12}H_{23}O_2N_2$). In addition to that, the presence of several other product ions that also occurred during fragmentation of hymenocardine (at m/z 159, m/z 170 and m/z 185) confirmed that the indole moiety in the 14-membered ring was not changed during metabolism. During negative ion fragmentation of the metabolite tentatively identified as *N*-monomethyl hymenocardine, also a large amount of structure revealing product ions were formed. Most of the product ions were also found in the MS^2 spectrum of hymenocardine (m/z 93, m/z 150, m/z 207, m/z 336 and m/z 433) (Fig. 3), thereby confirming that the metabolic change did not occur at the *para*-cyclophane part. Additionally, a product ion at m/z 530 ($C_{27}H_{40}O_6N_5$) was found, resulting from a fragmentation pathway similar to that of the product ion of hymenocardine at m/z 544. The mass difference between the product ion at m/z 544 and m/z 530 corresponds to the demethylation of the tertiary amino group in the side chain into a secondary amine, thereby confirming its structure depicted in Fig. 1.

The tentative structure of the metabolite corresponding to $C_{36}H_{50}O_6N_6$ (*N*-monomethyl hymenocardinol, compound **4**) is the result of a combination of both reduction of the *p*-hydroxy- ω -amino acetophenone moiety (as in compound **2**) and *N*-demethylation (as in compound **3**). Fragmentation of this metabolite in positive ion mode resulted in a multitude of diagnostic product ions (Fig. 4). For instance, the product ions at m/z 100, m/z 199 and m/z 227, which were encountered during fragmentation of the metabolite tentatively identified as *N*-monomethyl hymenocardine (**3**), were also detected as fragments of this metabolite. These product ions are in agreement with the structure obtained by demethylation of the tertiary amine group in the side chain, resulting in a secondary amine group. Product ions confirming the metabolic change of the carbonyl group next to the phenyl group into a hydroxyl group were detected at m/z 437 ($C_{24}H_{29}O_4N_4$), and m/z 419 ($C_{24}H_{27}O_3N_4$). The first one was also detected in the product ion spectrum of compound **2**, the second one is formed by an additional loss of water. Similar to the fragmentation of compound **2**, in negative ion mode product ions at m/z 191 ($C_{10}H_{11}O_2N_2$) and m/z 134 (C_8H_8ON) were detected, resulting from the loss of the hydroxyl group during fragmentation.

Due to the low abundance of the metabolite tentatively identified as *N*-demethyl hymenocardine ($C_{35}H_{46}O_6N_6$, 647.35469, $[M+H]^+$) (**5**) (Fig. 1), no product ions were detected in positive ion mode. Fragmentation in negative ion mode did, however, result in some product ions that were also detected during fragmentation of hymenocardine (m/z 336 and m/z 150, Fig. 3). These product ions originate from the indole moiety and the *p*-hydroxy- ω -amino acetophenone moiety, indicating that the metabolic change most probably occurred at the side chain of hymenocardine, which is in agreement with the proposed structure. Single and double *N*-demethylation were predicted by Meteor Nexus and Metaprint2D-React Web Server. According to the Meteor software, this type of metabolism is catalyzed by enzymes belonging to the CYP450 class.

Another metabolite that was predicted by both Meteor and Metaprint2D-React Web Server, and that could be identified in the metabolised hymenocardine samples, is the product of the hydrolysis of the amide bond linking the valine with the isoleucine-like moiety in the side chain. Fragmentation in positive ion mode of the metabolite tentatively labelled as ‘hymenocardine – terminal isoleucine-like moiety’ ($C_{29}H_{35}O_5N_5$, 534.27120, $[M+H]^+$) (**6**), resulted in several product ions that were also found during fragmentation of hymenocardine (Table S1). Others were different from hymenocardine fragmentation. These product ions confirmed that the substructure of hymenocardine containing the 14-membered ring and the indole moiety remained unmodified during metabolisation into compound **6**. The most intense product ion formed during hymenocardine fragmentation, m/z 114, originating from the breakage of the amide bond at the terminal isoleucine-like moiety (Fig. 2), is however absent during fragmentation of compound **6**. This confirms its tentative structure. Fragmentation in negative ion mode resulted mostly in the formation of product ions that were also observed during hymenocardine fragmentation, except for m/z 403 ($C_{20}H_{27}O_5N_4$), resulting from a fragmentation pathway similar to that of the product ion of hymenocardine at m/z 544. The mass difference between the fragment at m/z 544 and m/z 403 corresponds to the absence of the terminal isoleucine-like moiety in the metabolite $C_{29}H_{35}O_5N_5$, thereby confirming its structure depicted in Fig. 1.

Compound **7** was tentatively labelled as ‘hymenocardinol - terminal isoleucine-like moiety’ ($C_{29}H_{37}O_5N_5$, m/z 536.28640, $[M+H]^+$). Likewise to the fragmentation observed in positive ion mode for compound **6**, fragmentation of **7** resulted in the absence of a product ion at m/z 114. Furthermore, a diagnostic product ion was observed at m/z 447 corresponding to a fragment containing the three moieties taking part in the 14-membered ring, after the loss of the hydroxyl group ($C_{25}H_{27}O_4N_4$), thereby confirming the proposed structure in Fig. 1.

Large differences in intensities were observed between the detected metabolites **2-7** (Table S1). Notwithstanding the fact that responses of similar structures often differ in LC-MS analysis, in our experience the observed differences are too large to be caused only by the differences in ionization efficiency. It can therefore be stated that three major metabolites are formed during metabolism of hymenocardine (**1**) with human S9 fraction, namely those corresponding to $C_{37}H_{52}O_6N_6$ (**2**, hymenocardinol), $C_{36}H_{48}O_6N_6$ (**3**, *N*-monomethyl hymenocardine) and $C_{36}H_{50}O_6N_6$ (**4**, *N*-monomethyl hymenocardinol). Apart from compounds **2-7**, several other lower abundant metabolites were detected during UPLC-HRMS analysis, namely the metabolites corresponding to $C_{37}H_{48}O_6N_6$ (**8**), $C_{37}H_{52}O_8N_6$ (**9**), $C_{37}H_{52}O_7N_6$ (**10**), $C_{36}H_{50}O_7N_6$ (**11** and **12**), $C_{37}H_{50}O_7N_6$ (**13** and **14**), and $C_{36}H_{52}O_7N_6$ (**15**). Proton adducts of both $C_{36}H_{50}O_7N_6$ and $C_{37}H_{50}O_7N_6$ were detected at two different retention times, indicating that two structural isomers are formed, and thus, they correspond to two different metabolites.

A metabolite corresponding to the dehydrogenation of hymenocardine ($C_{37}H_{48}O_6N_6$, m/z 673.37002, $[M+H]^+$) (**8**) was detected (Table S1). Because of its relatively low intensity, only a small number of product ions were observed during fragmentation in positive ion mode. All product ions detected for this metabolite (m/z 241, m/z 159 and m/z 114) were also found during hymenocardine fragmentation (Fig. 2). As these product ions originate from the side chain and the indole moiety, it can be concluded that the dehydrogenation reaction must have occurred at either the β -hydroxy valine moiety or the *p*-hydroxy- ω -amino acetophenone moiety. Nonetheless, no other product ions were detected, neither during negative ion fragmentation, so no further fine-tuning of the location of the dehydrogenation reaction could be done based on LC-HRMS fragmentation. Moreover, this metabolite was not predicted by the *in silico* tools. Another low abundant metabolite corresponding to $C_{37}H_{52}O_8N_6$ (**9**) was detected both in positive (m/z 709.39124, $[M+H]^+$) and negative ion mode (m/z 707.37840, $[M-H]^-$) (Table S1). Targeted fragmentation resulted in several product ions in positive ion mode at m/z 114, m/z

241, and m/z 338: these product ions originate from the side chain. The metabolite with m/z 693.39640 (**10**, $C_{37}H_{52}O_7N_6$, $[M+H]^+$) showed several product ions, resulting from the side chain too (m/z 114, m/z 241, and m/z 338). These findings suggest that the metabolic changes most probably occurred at the *p*-cyclophane part.

The *in silico* tools Meteor Nexus and Metaprint2D-React Web Server predicted several possible metabolic conversions, which, when combined, can lead to the formation of **11-15** (Supporting Information, Fig. S2). These reactions include the reduction of the carbonyl group of the *p*-hydroxy- ω -amino acetophenone moiety (as reported for compound **2**), opening of the 14-membered ring by oxidative *N*-demethylation, hydroxylation, epoxidation, and loss of an *N*-methyl group. However, given the low abundance of compounds **11-15**, it was not possible to obtain product ion spectra of these metabolites and no tentative structures could be provided for metabolites **8-15**.

In the third part of this study, hymenocardine was orally administered to rats and blood and urine samples were collected up to 48 and 24 h after administration, respectively, in order to obtain information about the *in vivo* absorption, metabolism and excretion of hymenocardine. The plasma and urine samples were analyzed by UPLC-QqQMS (triple quadrupole MS); purified hymenocardine and hymenocardinol, isolated before [1], were used as analytical standards for quantification.

Hymenocardine was detected in the plasma from 1 to 12 h after administration, with the highest concentration, 1.43 ng/mL plasma, measured 1 h after oral administration (Fig. 5). Apart from hymenocardine, also hymenocardinol (**2**) could be detected from 1 to 24 h after administration of hymenocardine, and thus, it is present in plasma longer than hymenocardine itself. Based on the results of the non-spiked vs. the spiked samples, the average recovery of hymenocardine was found to be $106 \pm 8\%$ and $82 \pm 5\%$ for hymenocardinol.

The maximum concentration of hymenocardinol (0.52 ng/mL), was detected in plasma after 1 h, which is less than half of the maximum concentration of hymenocardine (1.43 ng/mL), measured at the same time point. The concentration of hymenocardine decreased more quickly in time, and could be measured up to after 12 h, while the concentration of hymenocardinol decreased more slowly and can still be measured after 24 h. This slower decrease of the hymenocardinol level, compared to the level of hymenocardine, can be explained by the fact that, although both compounds are excreted in urine, causing a decrease in the plasma levels of both compounds, the metabolism of hymenocardine into hymenocardinol will proceed as long as hymenocardine is present. This metabolism will have an additive effect on the elimination rate of hymenocardine, while at the same time the formation of more hymenocardinol will partly outweigh its urinary excretion. Therefore, hymenocardinol can persist in plasma for a longer period of time than hymenocardine.

Urine of the rats was collected between 2 and 24 h after administration of hymenocardine. Analysis of these samples also resulted in the identification of both hymenocardine and hymenocardinol, with a concentration of 11.8 ± 0.3 ng/mL and 16.0 ± 0.6 ng/mL, respectively. Based on the results of the non-spiked vs. the spiked samples, the average recovery was found to be $87.2 \pm 2.0\%$ for hymenocardine and $85.8 \pm 0.3\%$ for hymenocardinol.

These data suggest that hymenocardine is partly excreted unchanged, and at least in part as hymenocardinol. No additional metabolites were detected in plasma and urine, and also a specific search for the molecular ions corresponding to the metabolites identified in the S9 experiment and their glucuronide and sulphate adducts was negative. In the S9 experiment, *N*-monomethyl hymenocardine and *N*-monomethylhymenocardinol were identified as major metabolites, together with hymenocardinol. However, the former two were not detected in the plasma and urine samples. Perhaps the specific CYP450 enzyme, which catalyzes the oxidative

N-demethylation, is absent in rats, and thus, this reaction cannot take place. Other metabolites that were identified in the S9 experiment, were only considered as minor metabolites, and taking into account the fact that the levels of hymenocardine and hymenocardinol in plasma and urine are relatively close to the LOD, it seems likely that the analysis of the *in vivo* samples is not sufficiently sensitive to detect any minor metabolites.

The results indicate that, as observed in an *in vitro* gastrointestinal dialysis model, a metabolisation study with human microsomal S9 fraction, and an *in vivo* study in rats, reduction of hymenocardine to hymenocardinol is by far the most important metabolisation pathway. Interestingly, hymenocardinol showed a comparable IC₅₀ value against *Plasmodium falciparum* as hymenocardine [1], and the reduction of hymenocardine will not interfere with its potential *in vivo* antimalarial activity.

Materials and Methods

Chemicals and reagents

Acetonitrile and methanol (HPLC-quality) and ammonia (25%) were supplied by Fisher Chemical. Pepsin (P-7000, from porcine stomach mucosa, 800-2500 U/mg protein), bile salts (B-8631, porcine), pancreatin (76 190, from porcine pancreas, 149 USP U/mg amylase), sodium dihydrogen phosphate anhydrous (NaH₂PO₄), disodium phosphate dihydrate (Na₂HPO₄ • 2 H₂O) and tris(hydroxymethyl)aminomethane (TRIS) were purchased from Sigma-Aldrich. Hydrochloric acid (HCl, 32%), sodium bicarbonate (NaHCO₃) and sodium hydroxide (NaOH) were obtained from Merck. Formic acid was purchased from Acros Organics. Human liver S9 fraction and NADPH RS (Regenerating System) were purchased from XenoTech. All chemicals and reagents were of analytical grade and in all experiments deionized water (milliQ, Merck Millipore) was used. Hymenocardine (94%) and hymenocardinol (82%) were isolated from root bark of *Hymenocardia acida*. The purity of these two compounds was determined by HPLC analysis. The analysis was performed using a XBridge C₁₈ column (4.6 × 250 mm, 5µm,

Waters) and water + 0.1% formic acid (A) and acetonitrile (B) as mobile phase. The flow rate was 1.0 mL/min and the following gradient was applied: 0 to 5 min 80% A and 20% B, 40 to 45 min 100% B. UV detection was done at 201 nm.

***In vitro* gastrointestinal dialysis model (GIDM)**

Hymenocardine (25 mg) was submitted to a GIDM (stomach and small intestinal phase), as reported by Breynaert et al. [14], with modifications. A detailed description of the methodology is available as Supporting Information.

Incubation with liver S9 fraction

Hymenocardine was incubated with a liver S9 fraction, as reported by Van den Eede et al. [15], with modifications. A detailed description of the methodology is available as Supporting Information.

Animal study

Five male Wistar rats, 9 weeks old, weighing approximately 300 g to 325 g, were purchased from Janvier. The study was approved by the institutional ethical committee for animal experiments (ECD file number: 2016-034, date of approval: May 9th 2016) and throughout the whole experiment, the guidelines for the accommodation and care of animals used for experimental and other scientific purposes, as described by the European Commission, were followed. Rats were housed for 1.5 week in a climate and light controlled environment with free access to water and food in order to acclimatize before the start of the experiment. By this time, their weight had increased to 369 g on average. Four of the rats were given a dose of 20 mg/kg hymenocardine, administered by oral gavage in the form of a suspension (5 mg/mL in water), while one rat was treated with only vehiculum. About 500 µL of blood was taken from the tail vein at time-points 0 (before administration), 1, 6, 12, 24 and 48 hours after

administration. Between time-point 2 and 24 h, rats were kept in a metabolic cage and urine was collected. All blood samples were centrifuged at 2,000 xg for 10 min at 4 °C and plasma samples were collected. The four plasma samples of the rats which were administered hymenocardine and which were obtained at the same time point were pooled. Also, urine samples of the rats which were administered hymenocardine were pooled and both plasma and urine samples were stored at -80 °C until analysis.

Sample preparation of plasma: To 100 µL of plasma, 300 µL of methanol was added and samples were vortexed for 15 s and centrifuged at 13,680 xg for 10 min at 4 °C. The supernatant was dried under a flow of nitrogen gas and was redissolved in 100 µL of 80% water/20% methanol. Samples were vortexed shortly again and centrifuged at 18,625 xg for 10 min at 4 °C. The supernatant was diluted five times and was transferred to HPLC vials for UPLC-HRMS analysis. In order to estimate the recovery of the sample preparation and possible effects of matrix suppression during the MS analysis, also four samples (plasma samples taken at time points 0, 12, 24 and 48 hours, of the rats that were administered hymenocardine) were spiked at the start of the sample preparation procedure with hymenocardine (82.5 ng/mL plasma) and hymenocardinol (70.0 ng/mL plasma).

Analysis of plasma samples: The plasma samples were analyzed on a UPLC-QqQMS instrument (Acquity UPLC with XEVO TQ-S mass spectrometry system, Waters). A detailed description of the methodology is available as Supporting Information.

Sample preparation of urine: 500 µL of 4% formic acid was added to 500 µL urine.

Sample clean-up was performed in triplo with Oasis MCX 3cc (60 mg) extraction cartridges (Waters). The cartridges were conditioned with 10 mL methanol, 10 mL water and 10 mL water with 4% formic acid. Next, the samples were loaded and the cartridges were washed with 2 mL water and 5 mL methanol. Finally, elution was done with two times 1 mL of 60:40 acetonitrile: methanol + 5% NH₄OH. In order to estimate the recovery of the sample preparation and

possible effects of matrix suppression during the MS analysis, also spiked samples were prepared in triplicate at the start of the sample preparation procedure. Spiking was done with both hymenocardine (50.0 ng/mL urine) and hymenocardinol (57.6 ng/mL urine).

Analysis of urine samples: Urine samples were analyzed with an Acquity UPLC-DAD-QqQMS instrument. A detailed description of the methodology is available as Supporting Information.

Supporting Information

An experimental overview (Fig. **S1**), the possible metabolisation reactions in hymenocardine, as predicted by Meteor and Metaprint2D-React Web Server (Fig. **S2**), and an overview of the spectral and chromatographic data of hymenocardine and the metabolites formed during *in vitro* metabolisation with human S9 fraction (Table **S1**) are available as Supporting Information. Furthermore, detailed descriptions of the methodology followed in the gastrointestinal dialysis experiment, the S9 experiment and the analysis of plasma and urine samples are available as Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

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Figures

Fig. 1 Structures of six putative metabolites (2-7) of hymenocardine (1), detected with LC-HRMS, following S9 metabolism.

Fig. 2 Product ion spectrum and proposed fragmentation mechanism of hymenocardine (**1**) during HCD fragmentation in positive ion mode. The relative abundance of the fragment ion at m/z 114.13 is 100.

Fig. 3 Product ion spectrum of hymenocardine (**1**) in negative ion mode.

Fig. 4 Product ion spectrum and proposed fragmentation mechanism of the diagnostic product ions of the metabolite corresponding to $C_{36}H_{50}O_6N_6$ (**4**) during HCD fragmentation in positive ion mode. The relative abundance of the fragment ion at m/z 100.11 is 100.

Fig. 5 Concentration of hymenocardine and hymenocardinol in rat plasma, determined by LC-QqQMS, before (time point 0 h) and at different time points after oral administration of hymenocardine. Each bar represents a single measurement of the pooled plasma samples.

*Plasma concentration < LOQ (0.07 ng/mL for hymenocardine and 0.03 ng/mL for hymenocardinol).