Herbal Drugs of Abuse
Glaucium flavum and *Sceletium tortuosum:*
Metabolism and toxicological detectability of their alkaloids glaucine, mesembrine and mesembrenone studied in rat urine and human liver preparations using GC-MS, LC-MS, LC-HR-MS^n, and NMR

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Für die,
die es interessiert!

Phantasie ist wichtiger als Wissen,

denn Wissen ist begrenzt.

Albert Einstein

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1. General Part

1.1 Introduction

1.1.1 Herbal drugs of abuse as legal highs

Since the implementation of the opium law in the 1960s by the United Nations (UN), modern societies tried to control the illicit drug market for reasons of health and to protect the community from outgrowths like drug-related crime. The legislative process is superior organized by the UN, but every associated country has to execute its national depended narcotic acts by its own. In Germany and in many other countries (e.g. USA, Switzerland, France), the substances controlled by state were defined in form of a whitelist. Consequently, every substance or plant species named by this list is a "controlled substance". In the first four decades, only few substances were controlled and the illicit drug market was very stable. For example in the 1970s, consumption of LSD and THC was the new trend, in the 1980s, heroin became popular, and in the 1990s, MDMA and amphetamine. At the beginning of the 21st century, analogue structures were developed to circumvent the legislative process. For instance, different derivatives of synthetic cannabinoids (Spice), amphetamines, phenethylamines, and cathinones were put on the market [1-9]. These chemicals were sold via the internet as bath salts, plant fertilizers, or party pills. Being not (yet) scheduled, they were called "legal highs". These legal highs are single compounds or mixtures of different compounds with more or less strong psychoactive effects [7], but without declared ingredients. In addition, different old or new "herbal highs" from natural source, often plant species from other cultures, were available in the age of globalization. Especially due to the internet advertisement and the availability in the trade in goods all around the world, the number of new herbal highs increased in the last few years. Examples were magic mushrooms, different cactus species, Ayahuasca, baby woodrose (*Argyreia nervosa*) seeds, Kratom (*Mitragyna speciosa*), yellow hornpoppy (*Glaucium flavum),* and Kanna (*Sceletium tortuosum*) [3, 7, 10-14]. Different preparations such as herbs, extracts, tinctures, as well as fresh plant material were offered from single plant species or mixtures. Mostly, the identity of such preparations is not confirmed and their pharmacological and toxic effects as well as their pharmacokinetic behavior are unknown. Therefore, studies on the metabolism and detectability of the metabolites in body samples of such "herbal highs" play an important role in clinical and forensic toxicology.

1.1.2 Drug metabolism

Besides the endogenous metabolism, the metabolism of xenobiotics plays an important role in biochemistry and toxicology. The metabolizing enzymes are not selective and transform various endogenous and exogenous compounds. The resulting metabolites can be pharmacologically and toxicologically inactive (biodeactivation/detoxification) or active (bioactivation/toxification). Most of these reactions are oxidative reactions, which result in increasing hydrophilic properties, which facilitate the renal elimination of these compounds. Not all reactions increase hydrophilicity, for example, methylation catalyzed by the catechol-O-methyltransferase (COMT) leads to increased lipophilicity. The metabolic steps are divided in phase I reactions (functionalization) and phase II reactions (conjugation with e.g. glucuronic, sulfuric, or amino acids).

The characterization of the metabolic fate of xenobiotics is important in clinical and forensic toxicology for development of metabolite-based screening approaches [15-18] and for assessing the risk of pharmacokinetic interactions or pharmacogenomic variations in the effect. In contrast to medicaments, such studies are missing for drugs of abuse before consumption. Different in vitro assays and animal models can be used for elucidation of the metabolic fate. For example, rat species provide a metabolite pattern (qualitatively) similar to humans [2, 19, 20]. These data should be confirmed e.g. by in vitro assay with human recombinant enzymes, liver preparations, or hepatocytes. Recombinant enzymes allow studying the formation kinetics of single enzymes [21-24]. For determination of such enzyme kinetics, the formed metabolite or the degradation of the substrate must be quantified [25]. For quantification of the formed metabolites, reference standards are needed. In most cases, especially for new drugs of abuse, such reference standards are commercially not available. One opportunity is the chemical [25] or biochemical synthesis [26-28] of metabolites. If not possible and only one metabolite is formed by the particular enzyme, a substrate depletion approach can be used [25, 29-31]. However, arbitrary units (e.g. the ratio of the LC-MS peak area of the formed metabolite vs. that of an internal standard) can also be used for assessing K_m, but not for V_{max} values.

1.1.3 *Glaucium flavum* alkaloid glaucine

Glaucine ((*S*)-5,6,6a,7-tetrahydro-1,2,9,10-tetramethoxy-6-methyl-4H-dibenzo[de,g] quinoline, structure given in Fig. 1) is an isoquinoline alkaloid with aporphine structure and main ingredient of *Glaucium flavum* (yellow hornpoppy, Papaveraceae). Other

glaucine containing plant species are: *Glaucium oxylobum*, *Croton lechlen*, *Corydalis yanhusu*, *Nandina domestica [32-36]*. Yellow hornpoppy is native in Western Europe, North America, and Asia. The latex, typical for *Papaveraceae*, contains the main alkaloids in varying concentration (up to 3 %) depending on environmental factors, species variations and plant parts such as root, stem or leave [35]. Other alkaloids of yellow hornpoppy are protopine, chelerythrine, magnoflorine, and other minor ones [37].

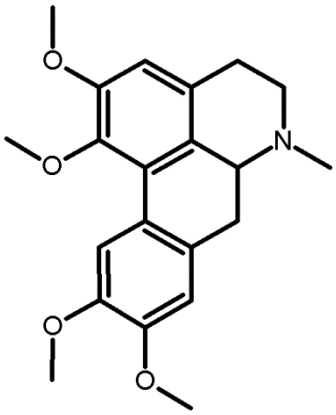

Fig. 1 Structure of glaucine

Glaucine has anti-inflammatory and antitussive properties, acts as a phosphodiesterase-4 (PDE4) inhibitor, bronchodilator, calcium channel blocker, and weak dopamine D_1 and D_2 receptor antagonist [38, 39]. The antitussive properties of glaucine in humans were investigated in three different clinical studies in comparison to codeine or dextromethorphan [40]. Glaucine showed a comparable decrement in cough besides less adverse effects and no signs of opiate withdrawal after longer intake [41]. On the other hand, case reports of recreational use [42, 43] or side effects of therapeutic use [10] are described and show the following symptoms: feeling of tiredness, hallucinations, vomiting, dizziness, and decreased blood pressure. Besides its use as antitussive e.g. in Bulgaria and Poland, glaucine is part of misused party pills or legal highs [10, 42].

1.1.4 *Sceletium tortuosum* alkaloids: mesembrine and mesembrenone

Sceletium tortuosum belongs to the plant family of *Aizoaceae,* which is mainly distributed in South Africa [44]. Besides the main alkaloids mesembrine ((3aR,7aR)-3a-(3,4-dimethoxyphenyl)-1-methyloctahydro-6H-indol-6-one) and mesembrenone ((3aR,7aS)-

3a-(3,4-dimethoxyphenyl)-1-methyl-1,2,3,3a,7,7a-hexahydro-6H-indol-6-one, structures given in Fig. 2), several structurally similar compounds were described in the literature, for example Δ7-mesembrenone, mesembranol, mesembrenol, tortuosamine, sceletium alkaloid A4 etc. [44-46]. These alkaloids were also identified in other *Mesembryanthemaceae* species [44, 45]. The total alkaloid content in *Mesembryanthemaceae* species is very low (1-1.5 %), but *Sceletium tortuosum* showed the highest levels [47]. Geographic and/or growing conditions, age of the plants, as well as the process of fermentation influence the alkaloid content and/or composition [48].

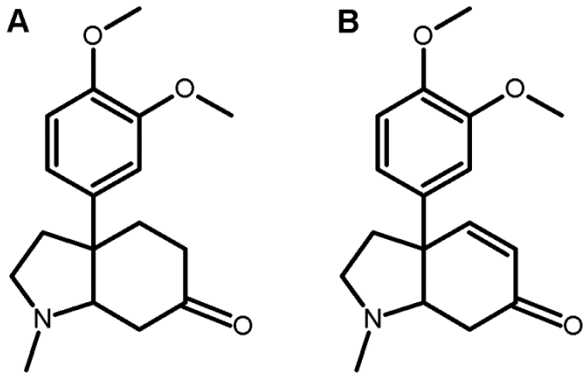

Fig. 2 Structures of mesembrine (A) and mesembrenone (B)

The KhoiSan, previously known as Hottentots, a tribe in South Africa, used the plant species after fermentation for their psychoactive effects [47, 48]. The alkaloids were tested by Harvey et al. in receptor and enzyme binding studies as well as in cell assays [49]. An ethanolic extract showed strong inhibition of 5-HT transporters (IC_{50}, 4.3 µg/mL) and of the PDE-4, (IC_{50}, 8.5 µg/mL). Further experiments concluded that mesembrine had the lowest K_i value (1.4 nM) for the 5-HT transporter, whereas mesembrenone showed the strongest inhibition effects against the PDE-4 (IC_{50}, <1 µM). Further clinical trials and experiments in humans confirmed these findings with antidepressive and anxiolytic effects of the extract [50, 51]. Khan et al. characterized the pharmacological activity of an extract of *Sceletium tortuosum* and mesembrine itself in Sprague-Dawley rats by conditioned place preference, hot plate, forced swim, elevated plus, and rotarod tests [52] for elucidating effects on nociception, depression, anxiety, ataxia, abuse liability. Differences between the single compound mesembrine and the administration of a plant extract containing several other substances were also tested. Mesembrine

caused analgesia, without abuse liabilities or ataxia, whereas the rats treated with the extract had higher tendency to fall from the rotating drum (ataxia), and showed higher activity swimming behavior (antidepressant activity) in the forced swim test. The authors interpreted these findings with the presence of further pharmacological active compounds in the extract, which were corresponding to the observed effects.

Besides the medical or traditional use, different Kanna or “Kougoed” preparations such as fermented and dried herbs, powders, capsules, extracts were administered by chewing, smoking, or sniffing. The preparations are available in so-called smart shops and are misused as legal highs [7].

1.1.5 Synthesis of phase I metabolites of glaucine

Due to the four methoxy and the *N*-methyl groups of glaucine, five isomeric demethyl metabolites could be expected as phase I metabolites. Reference standards of these metabolites were commercially not available and had to be synthesized. Full synthesis of such complex structures would have been too complicated, time consuming and expensive. Therefore, less complicated semi-synthesis by demethylation or methylation reactions of related structures was an alternative route of synthesis [53-56]. Non-selective reactions would yield in a complex mixture of isomers, which had to be isolated after synthesis. Hence, selective reactions could overcome these problems. In the literature, different selective *N*- or *O*-demethylation reactions were described for other substances [54-56]. An alternative route is the methylation of the phenolic compound boldine (2,9-dihydroxy-1,10-dimethoxyaporphine) [53], the main alkaloid of the boldo tree (*Peumus boldus*, *Monimiaceae*) [57], which was commercial available.

The corresponding syntheses were described in ref. [25] (see chapter 2.2)

1.1.6 Isolation of mesembrine and mesembrenone

In the literature, synthesis or isolation procedures for mesembrine and mesembrenone have been described [58-61]. The advantage of isolation from plant material was the possibility of yielding the main as well as the minor alkaloids in one step. In addition, the isolated compounds from plant material have the correct absolute configuration, which is not always achieved by chemical synthesis. So far, only few isolation procedures were described [60, 61]. Shikanga et al. described a method by using high-speed countercurrent chromatography (HSCCC) for the isolation of *Sceletium* alkaloids [61].

The all-liquid technique might avoid the reported irreversible absorption effects on packing material using column chromatography. In case of using acidic silica as stationary phase, these absorption effects led to strong tailing effects and therefore, to insufficient separation of the alkaloids [61]. Nevertheless, the authors separated the alkaloid extract after liquid-liquid extraction (LLE) by column chromatography (CC) with silica before separating the CC-fraction with HSCCC [61].

The adsorption effects on the silica packing material in CC might be avoided by using basic aluminum oxide as stationary phase. Hence, the more easily conducted CC might be effective enough to separate the alkaloids in acceptable yield, time and effort. To improve the overall yield of isolated alkaloids, the basic LLE were replaced using a Soxhlet apparatus, which allowed an exhaustive extraction.

The corresponding isolation was described in ref. [62] (see chapter 2.3)

1.1.7 Structure elucidation of the metabolites

For structure elucidation of unknown compounds, different analytical techniques can be used such as UV/VIS, IR, NMR, and MS [63]. Each technique has its advantages and disadvantages. The choice of the technique mainly depends on compound chemistry, the matrix, and the concentration therein. For identification of pure substances, all these techniques can be applied in parallel. In case of structure elucidations in drug metabolism studies, the application of different gas chromatographic (GC) or liquid chromatography (LC) techniques coupled to low or high resolution mass spectrometry (LR-MS or HR-MS) provide several advantages, such as high sensitivity and detailed structure information by the fragmentation patterns [64-68]. However, the exact position of a metabolically introduced or changed substituent cannot be elucidated. NMR would provide this information, but cannot be used for the low concentrated metabolites in biomatrix. Thus, only chemical synthesis of the proposed metabolites would help in this case.

The identification of the metabolites of the studied alkaloids was described in refs. [15, 62] (see chapters 2.1 and 2.3).

1.1.8 Toxicological detection

The detection of new drugs or legal highs in body samples of patients, victims, or suspects is a major challenge in clinical and forensic toxicology [5, 17, 20, 69, 70]. Urine

screening is the most comprehensive approach covering a broad range of (also low dosed) drugs for a longer time than blood, but mostly the metabolites are the analytical targets. Thus, the metabolism and the detectability of new drugs in urine have to be studied. After having elucidated the metabolite structures after high dose drug application and 24 h urine collection, their detectability in rat urine after a common user's dose using the standard urine screening approaches (SUSA) can be assessed [17, 20].

The detectability in urine of the studied alkaloids was described in refs. [15, 62] (see chapters 2.1 and 2.3).

1.2 Aims and scopes

So far, there are no studies available describing the metabolism of *Glaucium flavum* alkaloid glaucine or the *Sceletium tortuosum* alkaloids mesembrine and mesembrenone. Therefore, the aims of the presented study were:

- Identification of the phase I and II metabolites of glaucine in rat urine using GC-MS and LC-HR-MS^n
- Investigation of the detectability of glaucine by the standard urine screening approaches
- Confirmation of the phase I metabolites in human liver preparations and identification of the involved cytochrome P450 (CYP) isoenzymes
- Synthesis of the phase I metabolites of glaucine for structure confirmation and as reference standards for quantitative *in vitro* enzyme kinetics
- Determination of the CYP enzyme kinetics of glaucine in heterologically expressed single CYPs and in HLM and comparison of the results obtained by determination of the synthesized metabolites and using arbitrary units
- Isolation of mesembrine and mesembrenone from *Sceletium tortuosum* as reference standards for metabolism studies
- Identification of the phase I and II metabolites of mesembrine and mesembrenone in rat urine using GC-MS and LC-HR-MS^n
- Confirmation of the phase I metabolites of mesembrine and mesembrenone in human liver preparations and identification of the involved CYP isoenzymes
- Investigation of the detectability of mesembrine and mesembrenone by the standard urine screening approaches

2. Publication of the results

The results of the studies were published in the following papers:

2.1 Studies on the metabolism and toxicological detection of glaucine, an isoquinoline alkaloid from Glaucium flavum (Papaveraceae), in rat urine using GC-MS, LC-MS(n) and LC-high-resolution MS(n) [15] (DOI: 10.1002/jms.3112)

2.2 Studies on the in vivo contribution of human cytochrome P450s to the hepatic metabolism of glaucine, a new drug of abuse [25] (DOI: 10.1016/j.bcp.2013.08.025)

2.3 GC-MS, LC-MSn, LC-high resolution-MSn, and NMR studies on the metabolism and toxicological detection of mesembrine and mesembrenone, the main alkaloids of the legal high “Kanna” isolated from Sceletium tortuosum [62]

(DOI: 10.1007/s00216-014-8109-9)

3. Conclusion

The presented studies have shown that glaucine was extensively metabolized in rat by *O*- and *N*-demethylation, *N*-oxidation, hydroxylation, combinations of them as well as glucuronidation and/or sulfation of the phenolic metabolites [15]. In total, 26 phase I and 21 phase II metabolites could be identified. The synthesized demethylated metabolites, the structures of which were confirmed by NMR, allowed their identification in urine extracts by comparing their chromatographic retention times and mass spectra. The demethylated metabolites were also identified to be the main targets in both toxicological urine screening approaches [17, 20]. An intake of glaucine could be monitored in rat urine after administration of 2 mg/kg body mass (BM), corresponding to a 40 mg human single cough medication dose scaled by dose-by-factor approach according to ref. [71]. Using the GC-MS approach, the acetylated mono- and bis-demethylated metabolites could be detected whereas in the LC-MS^n screening approach the demethylated metabolites and their glucuronides were the analytical targets. The further in vitro studies with HLM confirmed the formation of the mono-demethylated metabolites in humans [25]. The involved CYP isoforms in the enzymatic demethylation of glaucine were as follows: CYP1A2, CYP2C19, CYP2D6, CYP3A4, and CYP3A5. All kinetic profiles could be modeled using the Michaelis-Menten equation. For all enzyme kinetics (CYPs, HLM), the metabolite formation approach using calibration with the corresponding synthesized reference standards was applied and compared to the results to those obtained by arbitrary units. The bias of the quantification approach for assessing the contribution of CYPs to hepatic clearance and therefore to the hepatic net clearance in vivo was negligible. In conclusion, the determination method had no substantial impact on the estimation of *in vivo* hepatic clearance of glaucine. Further inhibition experiments in HLM with specific and selective inhibitors confirmed the results of the contribution of the involved CYPs. Using the Michaelis-Menten equation and determined pharmacokinetic constants, concentration-dependent involvement of CYP isoforms was calculated for different glaucine concentrations (0.1-100 µM) according to Meyer et al. [72]. In a case report of recreational glaucine use [42], plasma levels of 0.7 mg/L (2 µM) were documented. Over the tested concentration range, only minor variations in the enzyme contributions were observed. The overall contribution of all CYP-catalyzed metabolic steps was as follow: CYP3A4 was about 79 %, of CYP1A2 18 %, of CYP2D6 2 %, and of CYP2C19 <1 % determined for a glaucine concentration of 5 µM. As glaucine was metabolized via three main steps and different CYP isoforms were

involved in the hepatic clearance of glaucine, a clinically relevant interaction with single inhibitors should not be expected. Over the last years, many studies have been published regarding the impact of interactions with drugs of abuse, therapeutically administered drugs, food ingredients, and/or tobacco smoke ingredients as well as of genetic variations [2, 73-75]. For assessment or prediction of all these variations, it is important to know whether an individual has a reduced or an increased turnover in these steps due to genetic variations or due to drug/food interactions by enzyme inhibition or induction. Such interactions are of general interest also in drug abuse because many drug users suffer from polytoxicomania with higher risk of drug interactions. Clinical or case studies are necessary for final assessment of the clinical relevance.

The developed isolation method for the Sceletium alkaloids allows simple and effective extraction and purification of both main alkaloids mesembrine and mesembrenone [62, 75]. The presented metabolism study has shown that mesembrine and mesembrenone were extensively metabolized by hydroxylation, dealkylation, dihydration, *N*-oxidation, and combinations of them. The main metabolites were confirmed in human liver preparations (HLM, HLC) and the following CYPs were determined: *O*-demethylation of mesembrine was catalyzed by CYP1A2, CYP2B6, CYP2C19, and CYP2D6 and the *N*-demethylation by CYP1A2, CYP2B6, CYP2C19, CYP2D6, CYP3A4, and CYP3A5. The *O*-demethylation of mesembrenone was catalyzed by CYP2C9, CYP2C19, and CYP2D6 and the *N*-demethylation by CYP2C19, CYP2D6, and CYP3A4 [62]. For the detectability studies, a 1 mg/kg BM dose were administered to rat and the urine was collected over a 24 h period. Using the GC-MS screening approach, the acetylated demethyl-dihydro and hydroxy metabolite of mesembrine, and the acetylated demethyl-dihydro mesembrenone metabolite could be detected. The main targets in the LC-MS^n screening approach for mesembrine were also the *O*- and *N*-demethyl-dihydro, and the *bis*-demethyl-dihydro metabolites, for mesembrenone only the *N*-demethyl- and the *N*-demethyl-dihydro metabolite and in addition their glucuronides.

4. Summary

In the presented studies, the metabolism and the toxicological detection of glaucine and the *Sceletium tortuosum* alkaloids mesembrine and mesembrenone were investigated. Glaucine was mainly *O*- and *N*-dealkylated in rats and further conjugated to glucuronides or sulfates. The phase I metabolites were synthesized and further in vitro experiments with HLM confirmed the formation of the metabolites in humans. The following CYP isoforms were mainly involved in these reactions: 1A2, 2C19, 2D6, 3A4, and 3A5. The kinetic profiles of all metabolite formations followed classic Michaelis-Menten behavior. The K_m values were between 25-140 µM and the V_{max} values between 0.10 - 1.92 pmol/min/pmol. Toxicological detection should be focused on the demethyl-dehydro metabolites and the corresponding glucuronides and/or sulfates.

Mesembrine and mesembrenone were also extensively metabolized in rat. The main metabolic pathway is a combination of demethylation and reduction of the keto function. The phenolic metabolites were partly excreted as glucuronides or sulfates. The phase I metabolites could also be found in human liver preparations. Demethylation of mesembrine was catalyzed by following CYPs: 1A2, 2B6, 2C19, 2D6, and 3A4 and the demethylation of mesembrenone by 2C9, 2C19, 2D6, and 3A4. The main analytical targets for the toxicological detection were the demethyl-dehydro metabolites and in case of LC-MS^n, the corresponding glucuronides and/or sulfates.

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6. Abbreviations

5-HT	serotonin
ADH	alcohol dehydrogenase
AU	arbitrary units
BM	body mass
CC	column chromatography
CNS	central nervous system
COMT	catechol-O-methyltransferase
CYP	cytochrome P450
DA	dopamine
DM	demethyl
EMEA	European Medicines Agency
FDA	US Food and Drug Administration
GC	gas chromatography
HLC	human liver cytosol
HLM	human liver microsomes
HR	high resolution
IR	infrared
IS	internal standard
LC	liquid chromatography
LLE	liquid-liquid extraction
MS	mass spectrometry
NAT	*N*-acetyltransferase
NMR	nuclear magnetic resonance spectroscopy
PDE	phosphodiesterase

SPE	solid-phase extraction
SULT	sulfotransferase
UGT	uridine diphosphate glucuronyltransferase
UN	United Nations

7. Zusammenfassung

Die vorliegende Dissertation umfasst Untersuchungen zum Metabolismus und Nachweisbarkeit der Alkaloide Glaucin, Mesembrin und Mesembrenon.

Glaucin wurde in der Ratte hauptsächlich zu vier Phase-I-Metaboliten *O*- und *N*-desalkyliert und anschließend zu Glucuronide oder Sulfaten konjugiert. Die Phase-I-Metaboliten wurden synthetisiert und deren Struktur mittels NMR-Techniken aufgeklärt. Weitere *in-vitro*-Experimente mit HLM bestätigten die Bildung der Metaboliten im Menschen. Folgende CYP-Isoformen waren an den Reaktionen beteiligt: 1A2, 2C19, 2D6, 3A4 und 3A5. Die resultierenden K_m Werte lagen zwischen 25 - 140 µM und die V_{max} Werte zwischen 0.1 - 1.9 pmol/min/pmol. Der analytische Fokus für die Nachweisbarkeit sollte auf den demethylierten Metaboliten und auf den zugehörigen Glucuroniden und/oder Sulfaten.

Mesembrin und Mesembrenon wurden von der Ratte stark metabolisiert. Beide Alkaloide werden hauptsächlich demethyliert und reduziert. Alle phenolischen Metaboliten werden teilweise als Glucuronide oder Sulfate ausgeschieden. Die Phase-I-Metaboliten konnten in *in-vitro*-Experimenten mit humanen Leberpräparationen belegt werden. Die Demethylierungen des Mesembrins wurden durch folgende CYP isoformen katalysiert 1A2, 2B6, 2C19, 2D6 und 3A4 und die des Mesembrenons durch 2C9, 2C19, 2D6 und 3A4. Der analytische Fokus für die Nachweisbarkeit sollte auf den demethylierten und reduzierten Metaboliten liegen und auf den zugehörigen Glucuroniden und/oder Sulfaten.