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Identification of benzo[*c*]phenanthridine metabolites in human hepatocytes by liquid chromatography with electrospray ion-trap and quadrupole time-of-flight mass spectrometry

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ABSTRACT

The metabolism of the benzo[*c*]phenanthridine alkaloids was studied using human hepatocytes which are an excellent model system for biotransformation studies. For analysis of the alkaloids and their metabolites, an electrospray quadrupole ion-trap mass spectrometry (ESI ion-trap MS) connected to a reversed phase chromatographic system based on cyanopropyl modified silica was used. The optimized experimental protocol allowed simultaneous analysis of the alkaloids and their metabolites and enabled study of their uptake into and interconversion in human hepatocytes. The results show that formation of the dihydro metabolite which may be followed by specific *O*-demethylenation/*O*-demethylation processes, is probably the main route of biotransformation (detoxification) of the benzo[*c*]phenanthridines in human hepatocytes. The structure of the main *O*-demethyl metabolite (2-methoxy-12-methyl-12,13-dihydro-[1,3]dioxolo[4′,5′:4,5]benzo[1,2-c]phenanthridin-1-ol; 336.1 *m*/*z*,) was proposed by the multi-stage MS and quadrupole time-of-flight MS methods using chemically synthesized standard.

1. Introduction

Sanguinarine (SG) and chelerythrine (CHE) are quaternary benzo[*c*]phenanthridine alkaloids (QBAs), a subgroup of isoquinoline alkaloids. QBAs have been isolated from plants of the Papaveraceae, Fumariaceae and Rutaceae families. Important QBA-rich sources include *Macleaya cordata*, *Sanguinaria canadensis*, *Dicranostigma lactucoides*, *Argemone mexicana* and *Eschscholzia californica*. For phytochemical characterization of *M. cordata* see Ref. [1]. SG and CHE have been extensively studied because of their anti-inflammatory, anti-microbial, anti-fungal and anti-parasitic activities. As a result, both are currently used in human and veterinary medicine as bioactive components of anti-microbial and anti-plaque preparations and in animal husbandary as components of feed additive Sangrovit. The widespread biological activities of the QBAs, their effects at a molecular level and the applications of QBAs in the pharmaceutical and biomedical fields, have been covered in recent reviews [2,3].

In aqueous solution at near physiological pH, SG and CHE can be present in pseudobase form (adduct of hydroxy group to the iminium bond) with tertiary nitrogen (Scheme 1A) and/or quaternary cationic form (Scheme 1B). Using spectral characterization, alkaloid pseudobases and quaternary cations have shown to be formed under alkaline and acidic conditions, respectively. Equilibrium of quaternary cations and corresponding pseudobase formation has been observed at pH 7.5–8.9 [4]. In the tissues and body fluids of living organisms, QBAs can undergo reduction leading to the formation of non-charged dihydro metabolites (dihydro derivatives)[i] [5] which are probably the main metabolites in mammals. The formation of the reduced metabolites, dihydrosanguinarine–DHSG and dihydrochelerythrine–DHCHE (Scheme 1C), has been found *in vitro* and also *in vivo* [5–8]. With the exception of the dihydro metabolites, detailed structural characterization of other SG and CHE

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[i] In the case of *in vitro* model experiments where SG and/or CHE are reduced chemically the general term 'dihydro derivative' for products of reduction is used. If reduction occurs in *in vitro* cell cultures or in *in vivo* systems, the term 'dihydro metabolite' for reduction products of the alkaloids is used throughout the text.

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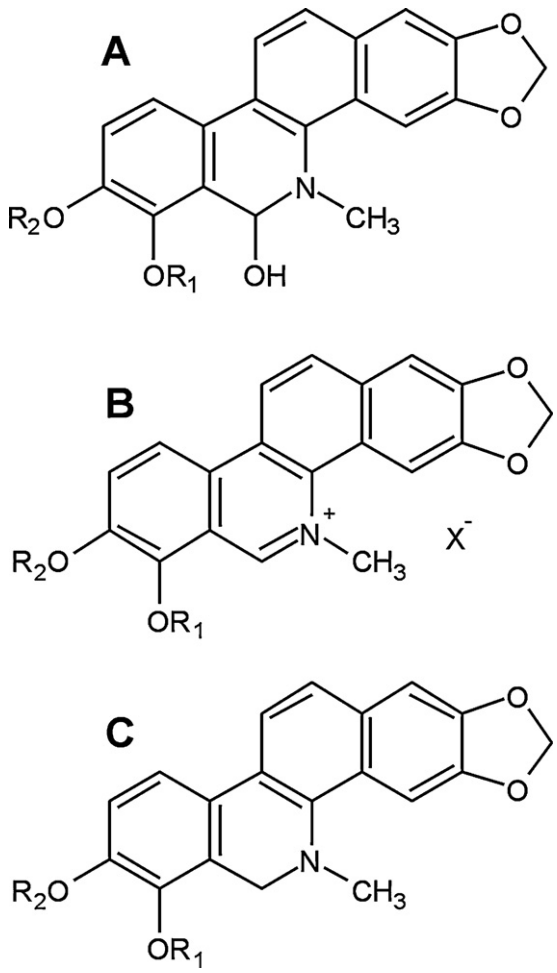


Scheme 1. Chemical structures of SG ($R_1 + R_2 = CH_2$) and CHE ($R_1, R_2 = CH_3$) as pseudobases (A), quaternary cations (B), and their dihydro derivatives (C). The quaternary structure of alkaloid NK-109 is presented in panel B (R_1=H, R_2=CH_3).

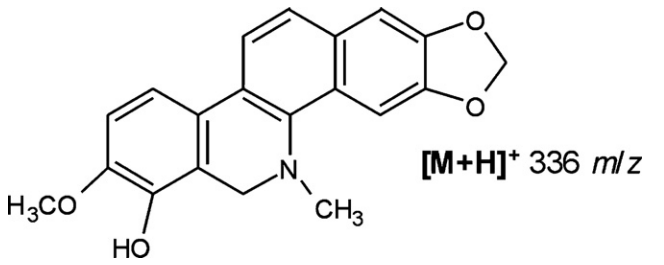
metabolites is still missing. This dearth of information formed the basis of our investigation, namely, the metabolism of the alkaloids in primary cultures and suspensions of human hepatocytes was studied by HPLC and mass spectrometry methods. Human hepatocytes which are considered a "gold standard" in the study of toxicity and the metabolism of xenobiotics [9–11] have not been used so far for study of the biotransformation of QBAs and their congeners.

The aims of this study were: (a) to apply HPLC with electrospray (ESI) ion-trap or quadrupole time-of-flight (QqTOF) mass spectrometry (MS) for the analysis of SG, CHE, and compounds with similar structures, (b) to study the interconversion between SG/CHE and their dihydro metabolites and, (c) to examine the uptake of the alkaloids by human hepatocytes and to identify their metabolites formed during the phase I of biotransformation.

2. Material and methods

2.1. Chemicals

SG and CHE were isolated from *M. cordata* (Willd.) R. Br. aerial parts. Dihydro derivatives of the alkaloids (DHSG and DHCHE) were prepared to 99% purity from SG and CHE by their reduction with $NaBH_4$ in methanol [12,13]. The *O*-demethylated metabolite of DHCHE (Scheme 2) was prepared from alkaloid NK-109 (Scheme 1B, R_1=H, R_2=CH_3) [14–16] which was reduced in the same way as the dihydro derivatives above [17]. Methanol was



Scheme 2. Chemical structure of the *O*-demethyl DHCHE (2-methoxy-12-methyl-12,13-dihydro-[1,3]dioxolo[4′,5′:4,5]benzo[1,2-c]phenanthridin-1-ol) found in human hepatocytes. The metabolite can be present as two possible isomers (hydroxy group at C7/C8). For other details see Section 3.4.

obtained from Merck (Darmstadt, Germany). Buffer components and other chemicals were purchased from Sigma–Aldrich, Inc. (St. Louis, MO, USA). All solutions were prepared using reverse-osmosis deionized water (Ultrapur, Watrex, Prague, Czech Republic). Nitrogen, argon and helium (99.999% for all) were obtained from Linde Gas (Prague, Czech Republic).

2.2. HPLC/ESI ion-trap MS

The HPLC chromatographic system used was Shimadzu (Shimadzu, Kyoto, Japan) equipped with a SCL-10Avp controller, a vacuum degasser, a binary pump (LC-10ADvp), an autoinjector (SIL-10ADvp), a column oven (CTO-10ACvp) and a UV-detector (SPD10Avp, at 280 nm). The system was coupled on-line to an ESI/ion-trap MS.

The chromatographic column (150 mm × 2.1 mm, 5 μm) Eclipse XDB-CN from Agilent (Santa Clara, USA) was used. The injection volume was 10 μL and the mobile phase consisted of methanol (solvent B)/1% acetic acid in a 10% methanol aqueous solution, linear gradient elution (%, *v*): 0–9 min (10–55% B), 9–12 min (55–60% B), 12–12.1 min (60–10% B), 12.1–16 min (10% B). The mobile phase flow rate was 0.4 mL/min, the temperature of the autosampler was 10 °C, and the column oven was set at 30 °C.

The quadrupole ion-trap MS instrument LCQ Fleet (Thermo Scientific, Waltham, MA, USA) operating in a positive ESI mode was used for the analysis. The ESI-MS parameters selected were: spray voltage (4.75 kV), capillary temperature (375 °C), and capillary voltage 30 V, if not stated otherwise. Nitrogen was used as sheath, auxiliary and sweep gas, and helium was used as the collision gas. The sheath, auxiliary and sweep gas flow rates were 50, 5, and 1 (as arbitrary units). The abundance of MS^2 fragment ions (SG 332.17 → 304.17, CHE 348.17 → 333.17, DHSG 334.17 → 319.17, and DHCHE 350.25 → 335.17 *m*/*z*) were monitored for the compound analysis in all cell samples.

2.3. HPLC/ESI-QqTOF MS

The second chromatographic system used was Waters Acquity UPLC (Waters, Milford, MA, USA) equipped with binary solvent manager, sample manager, column manager and PDA detector, which was coupled with a QqTOF mass spectrometer. The QqTOF Premier mass spectrometer (Waters, Milford, MA, USA) was used for confirmation of putative structures on the basis of the determination of elemental composition. For that purpose, MS and MS^2 experiments were performed. Optimized ESI-MS parameters for the mass spectrometer were as follows: capillary voltage 2.2 kV (positive mode), source temperature 120 °C, sampling cone 30 V, desolvation temperature 150 °C, cone gas flow rate, 38 L h^{-1} and a desolvation gas flow rate, 450 L h^{-1}. Nitrogen was used as a desolvation gas and argon as a collision gas. Other experimental conditions applied were the same as described in Section 2.2.

Table 1
HPLC ion-trap MS parameters for qualitative analysis of SG, CHE, and their dihydro derivatives.

	t_R (min)	MS → MS^2 → MS^3 → MS^4, m/z	MS^2 fragments, m/z (%)
SG	5.57	332.17 → 304.17 → 246.17 → 218.17	304.08, 274.12, 302.12 (100, 44.1,29.5)
CHE	6.68	348.17 → 333.17 → 330.17 → 302.08	333.07, 332.14, 304.14 (100, 80.8, 45.1)
DHSG	11.81	334.17 → 319.17 → 290.08 → 232.08	319.12, 304.14, 276.11 (100, 26.5, 8.2)
DHCHE	11.14	350.25 → 335.17 → 319.17 → 316.08	335.13, 319.17, 334.38 (100, 44.9, 12.1)

2.4. Human hepatocytes

2.4.1. Preparation of hepatocyte suspensions and incubation with the alkaloids

Liver samples ($n = 3$) were obtained from multi-organ donors. The tissue acquisition protocol conformed to the requirements of the Ethics Commission of the Faculty Hospital Olomouc. Three independent isolations of human hepatocytes (three multi organ donors; two men, 31 years and 61 years, one woman, 66 years) were carried out. Hepatocytes were isolated according to Pichard et al. [18]. The obtained cells were resuspended in serum-free medium, based on the mixture of Williams'E and Ham F12 (1:1) culture media with additives, fully defined in [18]. Human hepatocytes (4×10^6 cells mL^{-1}) were incubated with SG, CHE (5 μM) or DHSG, DHCHE (50 μM) on rotary equipment for 1 and 2 h at 37 °C. The cell viability at the end of the incubations was checked using the trypan blue test [19]. After incubation, the hepatocytes were centrifuged ($100 \times g$) for 3 min at room temperature, and cells and culture medium were separated.

2.4.2. Preparation of primary cultures and incubation with the alkaloids

Human hepatocytes ($n = 3$) isolated as described above (Section 2.4.1) were resuspended in culture medium (Williams'E and Ham F12, 1:1) with 5% bovine serum. Cells were seeded on collagen-coated six-well plates (area 9.4 cm^2) at a density of 1.5×10^5 cells cm^{-2}. The cell cultivation and treatment were carried out under sterile conditions, using a humidified incubator at 37 °C and an atmosphere containing 5% CO_2. After 12 h, the culture medium was changed for the serum free medium. After 24 h stabilization, the culture medium was replaced with fresh serum free medium and the alkaloids (5 μM) were applied on the cells. After 24 h incubation, the cells were scraped into the culture medium, centrifuged ($100 \times g$) for 3 min at room temperature, and the cells and culture medium were then separated.

2.5. Sample handling before HPLC/MS analysis

The hepatocytes were washed twice in 0.5 mL of phosphate buffered saline (PBS), resuspended in 0.5 mL of methanol (1% HCl, *v*) and sonicated (cycle 0.5, amplitude 50, 10-times) using the ultrasonic homogenizer UP 200S (Hielscher Ultrasonics GmbH, Teltow, Germany). The samples were then centrifuged ($14{,}000 \times g$) for 2 min at room temperature and supernatants (10 μL) were analyzed by HPLC/MS. The cultivation medium was diluted (1:1, v/v) in methanol (1% HCl, *v*), centrifuged ($14{,}000 \times g$) for 2 min at room temperature and supernatants (10 μL) were analyzed by HPLC/MS.

2.6. In vitro reduction of SG

SG (100 μM) was incubated with NADH, reduced glutathione (GSH) and/or L-ascorbic acid (AA) in a Britton–Robinson buffer (pH 7.4) under argon atmosphere at 37 °C for 20 min. The reducants were tested in a concentration range of 12.5–1000 μM. After incubation, the samples were diluted in methanol and analyzed by the HPLC/MS method (Section 2.2).

2.7. In vitro oxidation of DHSG

Incubation mixtures used to study the oxidation of DHSG with lactoperoxidase (LPO) contained in a final volume of 0.5 mL: 50 mM, sodium phosphate (pH 7.4); 100 μM, DHSG (5 μL of 10 mM DHSG ethanol stock solution was added to the incubation mixture); 1, 5 or 10 μg, LPO and 200 μM, hydrogen peroxide. The reaction was initiated by adding hydrogen peroxide and the reaction mixtures were incubated for 30 min at 37 °C. Control incubations were carried out (i) without LPO or (ii) without hydrogen peroxide. After the incubation, the mixtures were extracted twice with ethyl acetate (1 mL), and the extracts were evaporated under a nitrogen stream at 50 °C to dryness. Evaporates after dissolution in methanol (1% HCl, *v*) were analyzed by HPLC/MS as described in Section 2.2.

2.8. Method reproducibility

Intra- and inter-day HPLC/MS reproducibility was tested using standard solutions diluted in HPLC mobile phase. Series of analyses ($n = 6$) were performed for 6 consecutive working days to determine the inter-day reproducibility.

3. Results and discussion

3.1. ESI ion-trap MS and liquid chromatography of the alkaloids

Initially, we focused on use of the ESI ion-trap MS for the analysis of SG, CHE and their dihydro derivatives. The conditions for alkaloid ionization in positive ESI mode were optimized. ESI ionization is generally preferred due to the spontaneous ionization of alkaloids in acidic milieu resulting in formation of quaternary cations (see panel B in Scheme 1) [5,7]. The settings were carried out for ESI spray voltage (range 2.5–5.5 kV), capillary temperature (from 200 to 450 °C) and capillary voltage (range 0–100 V) (Fig. 1). The abundance of molecular ions M^+ of SG 332 m/z and CHE 348 m/z and pseudo-molecular ions $[M+H]^+$ of DHSG 334 m/z and DHCHE 350 m/z were monitored (for MS spectra, see Fig. 2). The best MS responses were observed for an ESI spray voltage of 4.75 kV, a capillary temperature of 375 °C and a capillary voltage of 20–30 V (Fig. 1). Ionization of dihydro metabolites was less effective than that of SG and CHE and this may have been caused by the greater solubility and ionic character of SG and CHE under the selected experimental conditions. The alkaloids were applied to the ESI using a flow-injection analysis (FIA) system. Standards were dissolved in methanol and then diluted in methanol/2% aqueous acetic acid (70/30%, *v*) and injected immediately into the ESI ion-trap MS. The solvents chosen were rich in methanol to avoid degradation of the compounds observable in aqueous solutions [20].

Typical m/z values of MS → MS^2 transitions and multi-stage MS (MS^n) fragmentation for individual compounds are presented in Table 1. To our knowledge, the MS^n fragmentations of the alkaloids examined in this study have not been unambiguously described before. MS → MS^2 transitions and specification of the three most abundant MS^2 fragments frequently used for better identification of the compounds were found (Table 1). The fragments corresponding to the following MS → MS^2 transitions were specified: SG 332.17 → 304.17, CHE 348.17 → 333.17, DHSG 334.17 → 319.17,

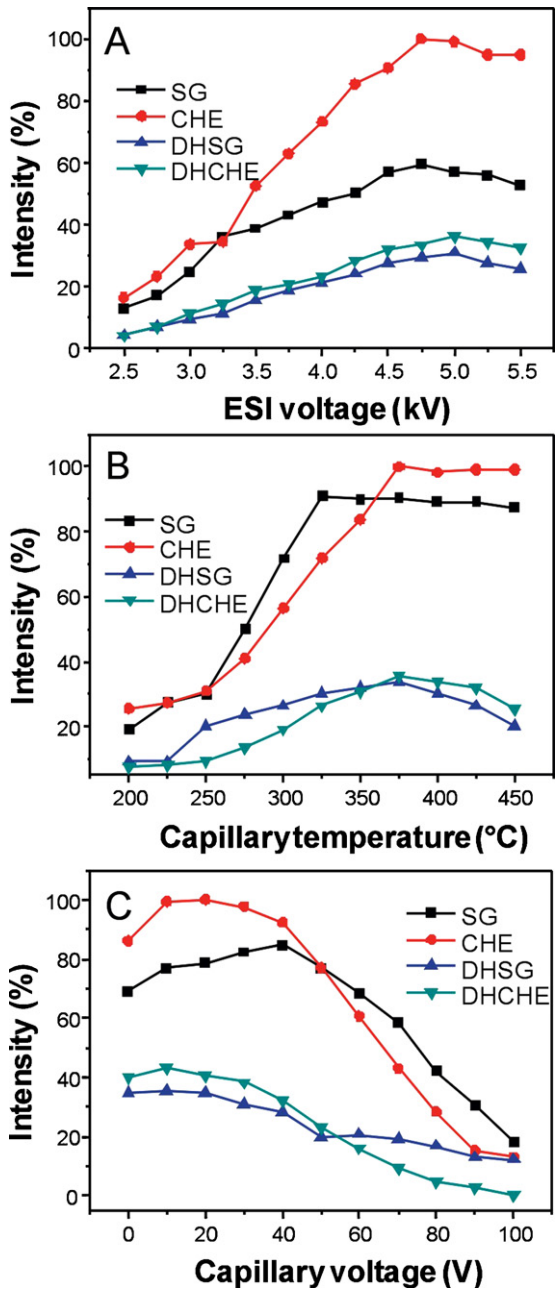


Fig. 1. Optimization of ESI parameters for MS analysis of SG, CHE, and their dihydro derivatives. Effects of ESI voltage (A), capillary temperature (B), and capillary voltage (C) on abundance of M^+ or $[M+H]^+$ of the alkaloids. The alkaloids were dissolved in methanol/2% acetic acid aqueous solution (70/30, %, *v*) and introduced into ESI ion-trap MS using flow injection system (flow rate 5 μL/min). For other details see Section 2.2.

and DHCHE 350.25 → 335.17 *m*/*z* (for MS^2 spectra see insets in Fig. 2). MS fragmentations of SG and CHE found in this study corresponded to those described by others [5–7]. Apart from QBA, MS studies have focused on other isoquinoline alkaloids, namely protopines [21,22] and protoberberines [23,24]. The $[M+H]^+$ of the alkaloids are generally subject to fragmentation associated with a mass loss of −16, −28 and −58 *m*/*z*, which corresponds to the production of $[M+H-CH_3-H]^+$, $[M+H-CO]^+$ and $[M+H-CH_2O-CO]^+$ fragments (Table 1).

In additional experiments, we used on-line connection of the ESI ion-trap MS with HPLC to separate the studied alkaloids and metabolites in biological samples. Using the reversed-phase chromatographic system based on cyanopropyl (CNP) modified silica, and linear gradient of the mobile phase which consisted of methanol/1% acetic acid in 10% methanol aqueous solution (see Section 2.2), the alkaloids were well separated. The CNP stationary phase (more polar than the frequently used C_{18}) in reversed-phase mode has already been successfully used in our laboratory for separation of several polar or less polar natural compounds using a methanol/water elution system [5,7,25–27]. For example, the CNP stationary phase was used to study plant phenolic secondary metabolites [25–27] and benzo[*c*]phenanthridine alkaloids and their dihydro metabolites [5,7]. Here, we used the CNP stationary phase for the complete separation of SG, CHE, and their dihydro derivatives in a single run up to 16 min. CHE (t_R 6.68 min) had stronger affinity for the surface of the stationary phase than did SG (t_R 5.57 min). As less polar derivatives of both alkaloids, DHCHE (t_R 11.14 min) and DHSG (t_R 11.81 min) were retained on the column longer than SG and CHE, it was necessary to use ~55% methanol in the mobile phase for their elution (Fig. 3). In addition to DHSG and DHCHE, some other less polar structural analogs of SG and CHE were also tested, i.e. 6-oxosanguinarine (t_R 10.44 min), 6-oxochelerythrine (t_R 10.19 min), norsanguinarine (t_R 12.58 min), and norchelerythrine (t_R 11.62 min) for separation by this system (data not shown).

After optimization of the HPLC separation and MS detection, a calibration procedure was carried out. The calibration curves (0.5 ng–10 μg mL^{-1} of SG, CHE and their dihydro derivatives) were linear with $R^2 > 0.996$. The limits of detection (LODs, 3S/N) for individual alkaloids were as follows: SG 1.2 ng mL^{-1}, CHE 0.83 ng mL^{-1}, DHSG 3.00 ng mL^{-1} and DHCHE 1.08 ng mL^{-1}. Reproducibility of HPLC/MS method was tested in the concentration range from 0.005 to 1 μg mL^{-1} of each alkaloid. Intra-day reproducibility of spiked SG/CHE/DHSG/DHCHE was: 97.4/97.4/97.8/97.7% for 0.005 μg mL^{-1}, 98.4/98.5/97.0/99.5% for 0.01 μg mL^{-1}, 99.3/100.1/97.1/100.1% for 0.1 μg mL^{-1}, and 100.2/102.4/101.0/96.9% for 1 μg mL^{-1}. Acceptable SG/CHE/DHSG/DHCHE reproducibility was also found in the case of inter-day measurements: 97.4/100.4/86.5/90.7% for 0.005 μg mL^{-1}, 108.6/108.7/93.2/97.0% for 0.01 μg mL^{-1}, 109.8/107.3/91.3/95.8% for 0.1 μg mL^{-1}, and 102.5/109.3/95.4/90.0% for 1 μg mL^{-1}.

3.2. Uptake of the alkaloids into human hepatocytes

Hepatocytes were incubated with 5 μM SG/CHE and/or 50 μM DHSG/DHCHE. Ten-fold higher concentrations of DHSG and DHCHE were used in the experiments due to their significantly lower toxicity than SG or CHE [28]. If human hepatocytes were incubated with the alkaloids for 1 and 2 h, cell viability did not drop below 90% of initial viability (data not shown). After incubation, cells were centrifuged, washed, and homogenized in methanol (1% HCl, *v*) and levels of SG, CHE and their dihydro metabolites in supernatants were identified and analyzed semiquantitatively using the HPLC/ESI ion-trap MS (for details, see Section 2.5). The rate of uptake of SG by hepatocytes was higher than that of CHE. The uptake rate for individual alkaloids (SG, CHE) as well, was generally similar to the uptake of DHSG and DHCHE. After 1 h incubation of human hepatocytes with 5 μM SG/CHE, and/or 50 μM DHSG/DHCHE, approx. 250 ng SG, 130 ng CHE, 3625 ng DHSG, and 2330 ng DHCHE per 4×10^6 cells (1 mL of cell suspension) were determined. HPLC/ESI ion-trap MS chromatograms of SG and DHSG in human hepatocytes are shown in Fig. 4. The amount of free alkaloids in human hepatocytes incubated for 2 h was lower than in hepatocytes incubated for 1 h and this could have been caused by their biotransformation to end-metabolic products.

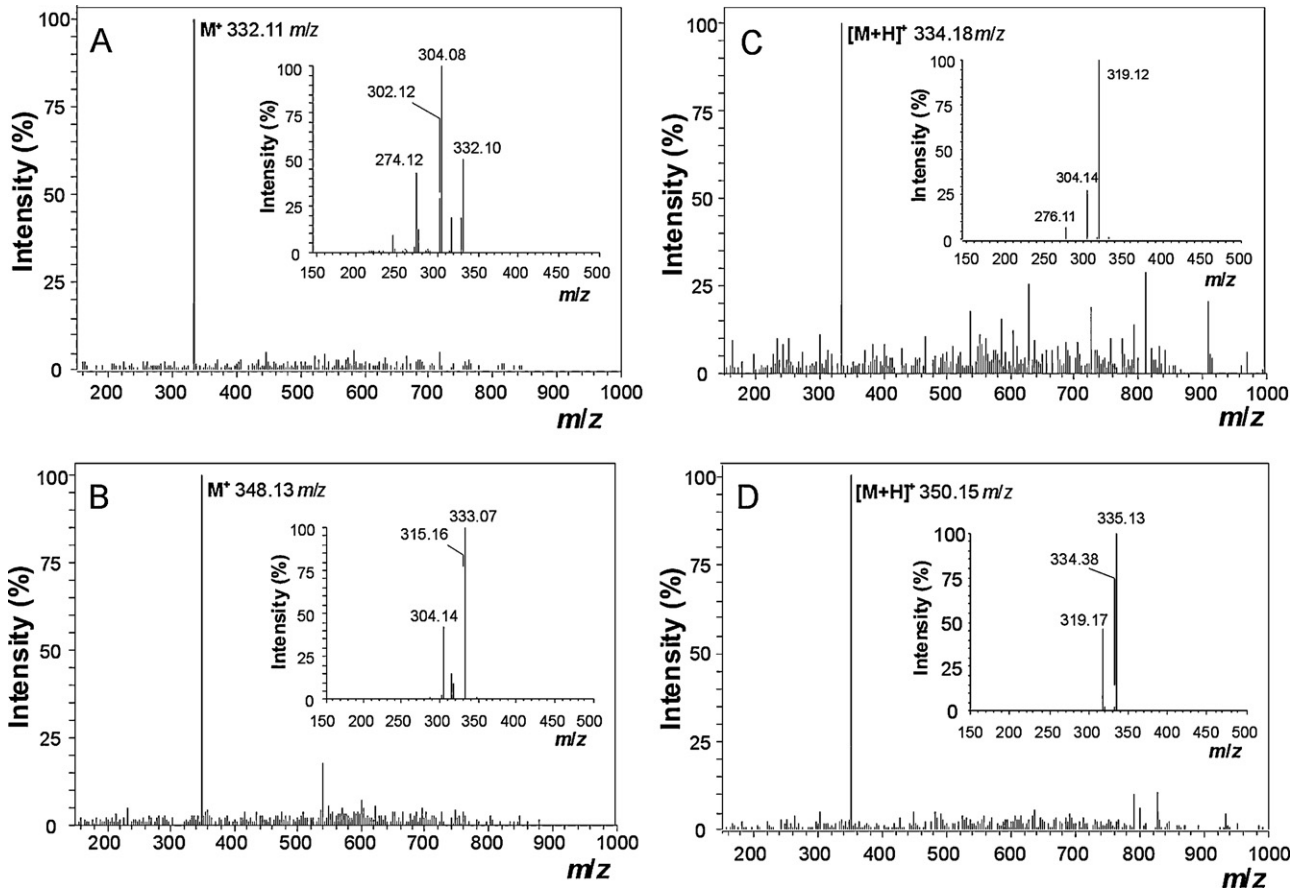


Fig. 2. MS spectra of SG (A), CHE (B), DHSG (C), and DHCHE (D). MS^2 spectra of the alkaloids are shown in insets. The MS spectra were subtracted from chromatographic peaks shown in Fig. 3. For other details, see Sections 3.1 and 2.2.

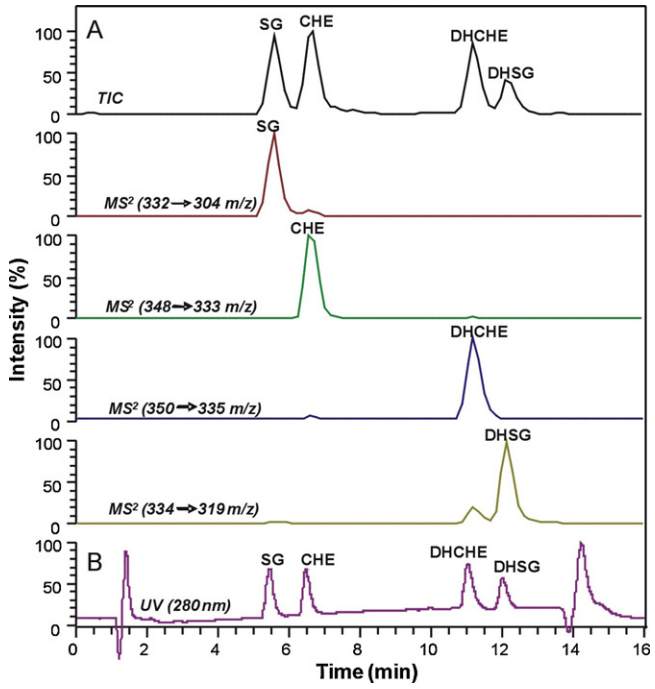


Fig. 3. HPLC chromatograms of SG, CHE and their dihydro derivatives model standard solutions prepared in methanol/2% aqueous acetic acid (70/30%, *v*) acquired using ESI ion-trap MS (A) and UV (B) detector. In addition to total-ion current (TIC) chromatogram, MS chromatographic records for individual alkaloids are shown in panel A. For other details see Sections 3.1 and 2.2.

3.3. Reductive and oxidative pathway in the metabolism of the alkaloids

The formation of dihydro derivatives of SG and CHE by ubiquitous reducing enzyme cofactors such as NADH and NADPH has already been demonstrated *in vitro* using UV–Vis spectrometry and fluorimetry for their detection [29]. The formation of dihydro derivatives was found especially under acidic and neutral pH, while their production under the alkaline conditions is limited [29]. The formation of dihydro metabolites has recently also been found in cell cultures and *in vivo* systems [3,5,7]. Using HPLC and ESI single quadrupole MS, DHSG formed from SG was found in blood serum and liver of rats after gastric application of 10 mg kg^{-1} body weight of SG [5].

We studied both formation of dihydro metabolites from SG or CHE in hepatocyte suspensions (Fig. 4A) and their reversible interconversion to SG and CHE in cells exposed to DHSG or DHCHE using the HPLC/ESI ion-trap MS method (Fig. 4B). As shown in Table 2, the reduction of CHE to dihydro metabolite in human hepatocytes is faster than that of SG. After 1 h incubations approx. 21% of SG and 37% of CHE were reduced to their dihydro metabolites. A similar trend was also found after 2 h incubation but the reduction was less marked for both alkaloids. In order to reduce the interindividual differences in the biotransformation pattern of hepatocytes based on variability in biotransformation enzymatic systems of individual donors, the experiments were carried out using three different samples of human hepatocytes, originating from three multi-organ donors (see Section 2.4.1). The total conversion of SG or CHE to their dihydro derivatives was achievable only under the optimal *in vitro* conditions. The dynamic interconversion between alkaloids and

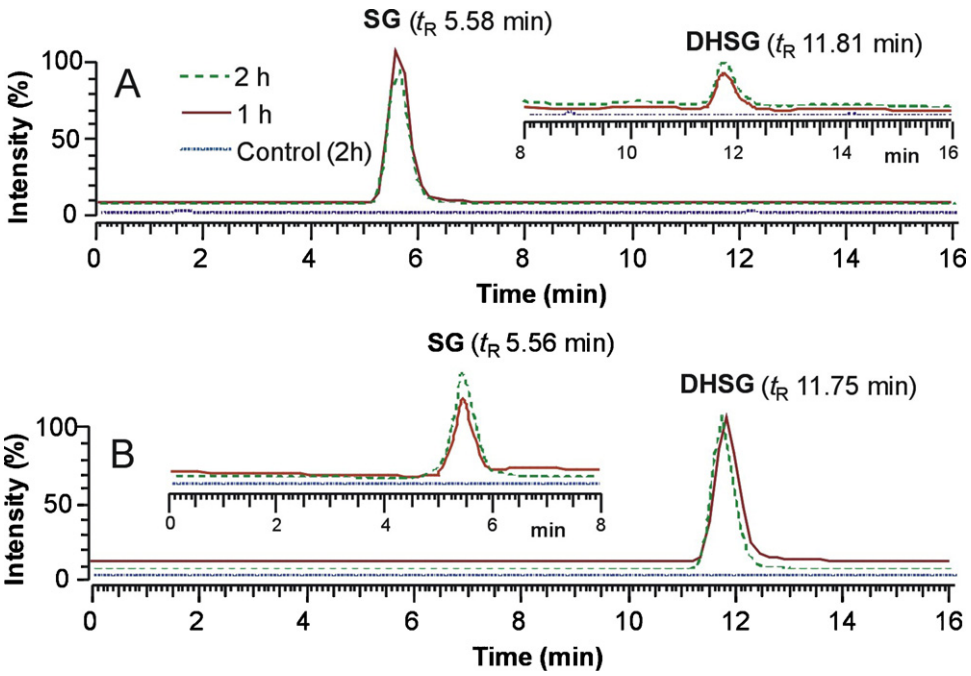


Fig. 4. HPLC/ESI ion-trap MS chromatograms of SG and DHSG in human hepatocytes after their incubation (full line: 1 h, dashed line: 2 h) with (A) 5 μM SG and (B) 50 μM DHSG. In addition, MS chromatographic records of DHSG (for A) and SG (for B) are shown in insets. Intensity scales for insets are 5-times lower than MS^2 chromatograms in panel A and B. For other details see Sections 3.2 and 2.2.

their dihydro metabolites probably occurs in cells and this leads to the establishment of the equilibrium state. The reduction may be due to low-molecular weight reducing agents and/or with non-specific reductases. As mentioned above, of non-enzyme systems, NAD(P)H is one of the main reducing powers in the reductive biotransformation of the alkaloids. Here, we show that glutathione (GSH) and L-ascorbic acid (AA) (additional physiologically important reducing agents) are unable to participate in the reduction of the alkaloids and this was confirmed using HPLC/ESI ion-trap MS in *in vitro* experiment with SG (Fig. 5A).

The reduction of the alkaloids to their dihydro metabolites in hepatocytes is not fully quantitative (Table 2). This finding can be explained by the reversible interconversion of dihydro metabolites to parent alkaloids (SG, CHE). We presuppose that oxidoreductases could be involved in this process. Here, we evaluated the potential of peroxidases to catalyze the oxidation of dihydro metabolites as peroxidases are known to catalyze one- and two-electron oxidations forming a wide range of products, including dehydrogenation products [30–32]. Lactoperoxidase (LPO) was used as a model to oxidize DHSG. As shown in Fig. 5B, LPO in the presence of hydrogen peroxide efficiently oxidizes DHSG, forming a parent compound, SG. Even though the mechanism of this reaction has not been studied in detail, we suggest that the reaction proceeds

Table 2
Procentual representation (±RSD) of the alkaloids and their dihydro metabolites (SG + DHSG or CHE + DHCHE = 100%) in human hepatocyte suspension after 1 and 2 h of incubation with 5 μM SG/CHE or 50 μM DHSG/DHCHE at 37 °C ($n = 3$).

Alkaloid (time of incubation)	SG	DHSG	CHE	DHCHE
SG (1 h)	78.6 ± 11.1	21.4 ± 11.1		
SG (2 h)	78.9 ± 15.1	21.1 ± 15.1		
DHSG (1 h)	3.0 ± 0.3	96.9 ± 0.3		
DHSG (2 h)	4.1 ± 0.3	95.9 ± 0.3		
CHE (1 h)			62.1 ± 9.3	37.8 ± 9.3
CHE (2 h)			72.6 ± 2.9	27.3 ± 2.9
DHCHE (1 h)			2.5 ± 0.1	97.5 ± 0.1
DHCHE (2 h)			4.6 ± 0.5	95.4 ± 0.5

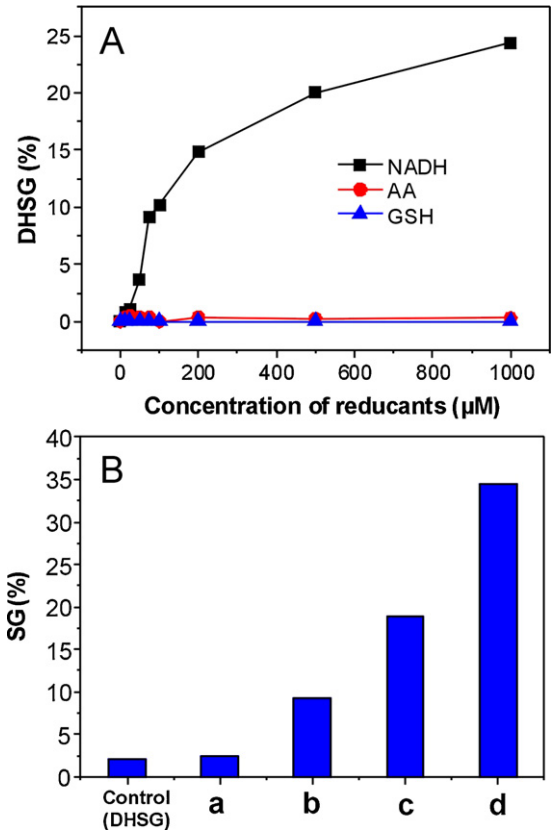


Fig. 5. (A) Reduction of SG by NADH, glutathione (GSH) and ascorbic acid (AA). (B) Oxidation of DHSG by lactoperoxidase (LPO). (a) DHSG + H_2O_2, (b) DHSG + H_2O_2 + 1 μg LPO, (c) DHSG + H_2O_2 + 5 μg LPO, (d) DHSG + H_2O_2 + 10 μg LPO. Experimental conditions are described in the Sections 2.6 and 2.7.

Table 3
ESI/QqTOF MS characterization of *O*-demethyl DHCHE.

Collision ramp energy	$[M+H]^+$ *m/z*	MS^2 fragments			Elemental composition	ppm
		m/z	Mass loss	%[a]		
10–45 eV	336.1231			60.3	$C_{20}H_{18}NO_4$	1.5
		321.1002	CH_3[b]	67.2	$C_{19}H_{15}NO_4$	0.3
		320.0926	CH_3[b], H[b]	100	$C_{19}H_{14}NO_4$	0.9
		305.0708	CH_3[b], CH_3[b], H[b]	49.7	$C_{18}H_{11}NO_4$	6.6
		304.0977	CH_3[b], OH[b]	28.2	$C_{19}H_{14}NO_3$	1.0
		277.0762	CH_3[b], H[b] CH_3[b], CO	13.5	$C_{17}H_{11}NO_3$	8.3
30–50 eV	336.1234			<3	$C_{20}H_{18}NO_4$	0.6
		320.0927	CH_3[b], H[b]	69.7	$C_{19}H_{14}NO_4$	1.2
		305.0692	CH_3[b], CH_3[b], H[b]	100	$C_{18}H_{11}NO_4$	1.3
		277.0749	CH_3[b], H[b] CH_3[b], CO	43.9	$C_{17}H_{11}NO_3$	3.6
		248.0728	CH_3[b], H[b], CH_3[b], CO, CHO[b]	12.5	$C_{16}H_{10}NO_2$	6.4

[a] 100% = predominant MS^2 fragment.
[b] Radical cleavage.

in two steps to form the SG. In the first step, DHSG is oxidized by one electron oxidation to a radical intermediate, followed by the next one electron oxidation, producing the DHSG dehydrogenation product, SG. However, this suggestion, as well as the peroxidative conversion of DHSG to SG by other enzymes such as cytochrome P450 2S1, known to oxidize several substrates in the presence of peroxides [33,34], awaits further investigation.

3.4. *O*-demethyl metabolites of the alkaloids formed in human hepatocytes

Apart from dihydro metabolites of SG and CHE, other metabolites which could be produced in phase I of biotransformation, were found in human hepatocytes. In addition, metabolism of DHSG and DHCHE in these cells was investigated here for the first time. In these experiments, suspensions of human hepato-

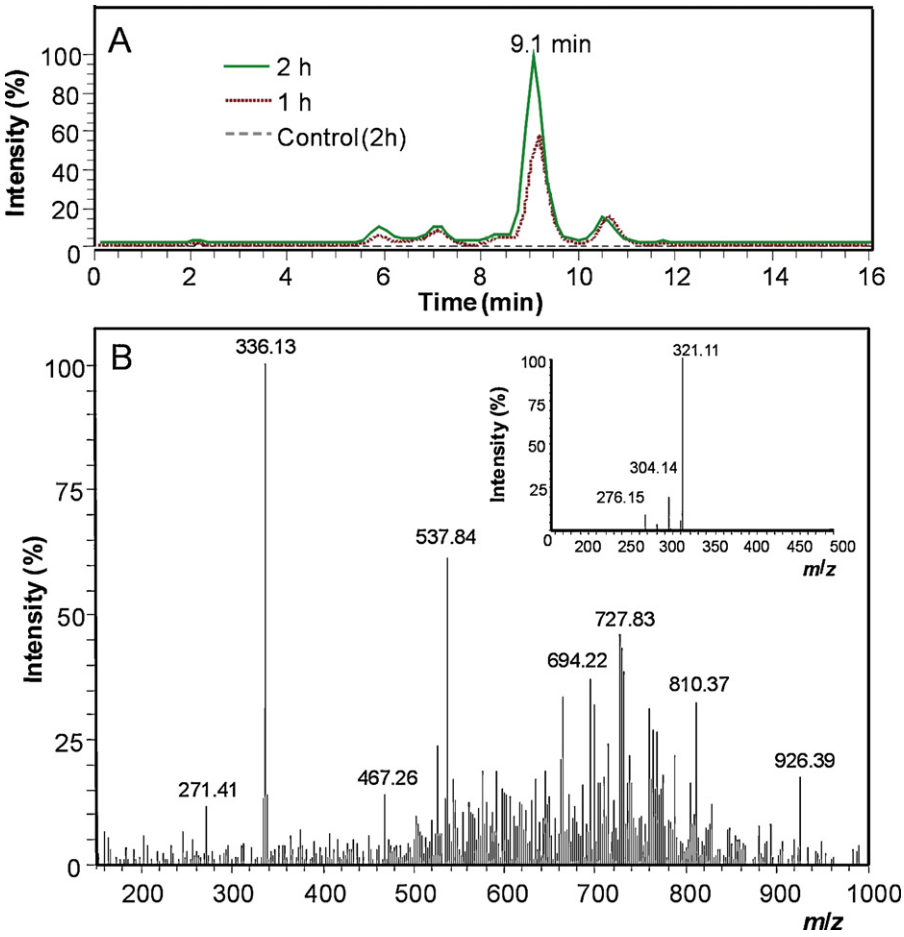
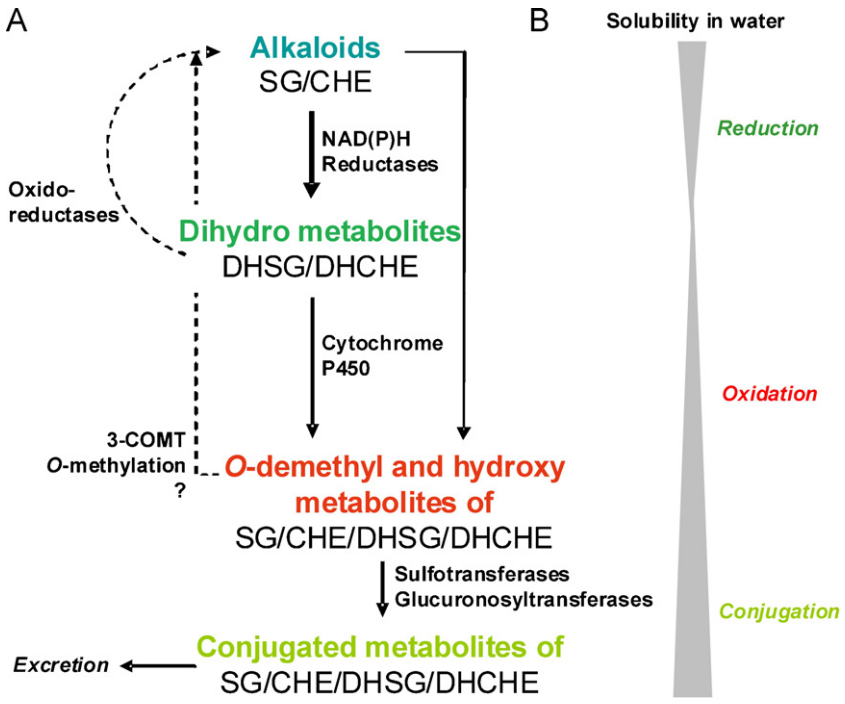


Fig. 6. HPLC/ESI ion-trap MS chromatograms of *O*-demethyl DHCHE (A) and its full MS spectrum (B) acquired in human hepatocytes after 1 and 2 h incubation with 50 μM DHCHE. MS^2 spectrum is shown in inset. Full MS spectrum (B) was subtracted from peak in panel A.



Scheme 3. (A) Proposed biotransformation pathways of SG and CHE. The alkaloids are reduced by low-molecular weight reducing agents and/or non-specific enzyme systems (reductases). In phase I of biotransformation, the dihydro metabolites or SG and CHE alone are subsequently oxidized by cytochrome P450 systems which lead to the formation of *O*-demethyl and hydroxy metabolites. In phase II of biotransformation, the metabolites with hydroxy group(s) are subjected to conjugation (e.g. glucuronidation) and water-soluble conjugates are excreted into the urine. The processes describe above are reversible via oxidoreductase enzyme systems and 3-COMT (3-catechol-*O*-methyltransferase), which can transform dihydro and *O*-demethyl metabolites back to the parental alkaloids or their previous metabolites (see dashed lines). Thus the equilibrium between the alkaloids and their metabolites can be observed up to complete elimination. (B) The water-solubility of the metabolites presented in panel A. The description of individual chemical processes in I and II phases of biotransformation is marked. The scheme was proposed on the basic of the results described in the present work and the results shown previously in the following Refs. [5,6,37].

cytes were exposed to the compounds studied for 1 and 2 h. The biotransformation of the alkaloids in primary cultures of human hepatocytes after 24 h-long incubation was also examined (see Section 2.4.2). Using the HPLC MS/MS, oxidative metabolites of SG and CHE with *m/z* 320, 348 and 366 were analyzed in rat liver microsomal fractions and Ad293 cell lines transfected with recombinant human cytochromes P450 (for putative structures of metabolites see Ref. [6]). The metabolites are probably produced by cleavage of methylenedioxy group(s) of the alkaloids and subsequent *O*-demethylation. The possible metabolites and conjugates (glucuronides) of the alkaloids have been found in the model cell systems and rats [6]. The oxidative metabolism (metabolic hydroxylations) of QBAs was described for plant tissue cultures in detail [35].

Here, we confirm that metabolites at *m/z* 320 and 336 are found in human hepatocytes incubated with SG for 1 and 2 h. As mentioned above, the metabolite at *m/z* 320 has been found [6], probably as SG *O*-demethylenated in position C-7,8. The metabolites (*m/z* 320 and 336) were found in hepatocytes in relatively low amounts, less than 5% of applied amounts of the compounds (data not shown).

DHSG was metabolized in human hepatocytes to the metabolites at *m/z* = 320, 336 and 306 which were found in lower amounts like SG. In the case of CHE but mainly of DHCHE, the production of phase I biotransformation metabolites was more extensive than in the case of SG and DHSG. This resulting from the absence of one methylenedioxy group in CHE unlike SG (Scheme 1), which can lead to the lower stability of CHE and its higher binding to biotransformation enzymes such as cytochromes P450. The metabolites at *m/z* 320, 336 and 366 were found after incubation of CHE/DHCHE with hepatocytes. The DHCHE metabolite at *m/z* 336.1 (t_R 9.1 min) was predominant and was produced as early as after 1 h incubation with human hepatocytes. After 2 h incubation, approx. 80% more of this metabolite was found than after 1 h incubation (Fig. 6A). This metabolite was identified as the *O*-demethylated derivative of DHCHE (*O*-demethyl DHCHE: 2-methoxy-12-methyl-12,13-dihydro-[1,3]dioxolo[4′,5′:4,5]benzo[1,2-c]phenanthridin-1-ol; see Scheme 2), which was confirmed on the basis of the MS fragmentation pattern (MS^n) and accurate mass measurement using ion-trap MS and QqTOF MS, respectively. The retention time (9.1 min) and MS data acquired in real samples was identical to qualitative parameters of *O*-demethyl DHCHE standard which was chemically synthesized for this purpose (Section 2.1). Namely, the following fragmentation pattern was found for *O*-demethyl DHCHE: $[M+H]^+$ 336.1 $m/z \rightarrow MS^2$ 321.1 $m/z \rightarrow MS^3$ 305.1 $m/z \rightarrow MS^4$ 277.1 *m/z*. For the $MS \rightarrow MS^2$ transition (Fig. 6B), the ratio of the three most abundant MS^2 fragments 321.1 *m/z*, 304.1 *m/z*, 276.1 *m/z* was determined (100%, 19%, 10%) including evaluation of their accurate *m/z* values. The accurate mass and elemental composition of the MS fragmentation products of *O*-demethyl DHCHE were measured at lower and/or higher collision energy (Table 3) using the previously optimized ESI-QqTOF MS method (Section 2.3).

O-demethyl DHCHE was identified using both MS methods in human hepatocyte suspensions and also in primary cultures of human hepatocytes incubated with 5 μM DHCHE or CHE for 24 h (data not shown). Our results suggest that the subject of biotransformation leading to the formation of *O*-demethyl metabolites may not only be the parent alkaloids (SG, CHE) but also their dihydro metabolites which were previously found as products of the first step in the biotransformation of SG and CHE. We assume that *O*-demethylenation and *O*-demethylation of DHSG and DHCHE allow them to participate in conjugation reactions and contribute to their

increased solubility in water, the key property for urinary excretion. The individual steps in the biotransformation of SG and CHE are shown in Scheme 3.

The biotransformation of SG and CHE and their structural analogs, berberine [36] and protopine type alkaloids [37–39] *in vitro* as well as *in vivo* has been studied. It has been shown that the oxidative pathway of biotransformation of these alkaloids is similar to the oxidative metabolism of SG and CHE, whereas the reductive pathway, leading to production of less polar dihydro metabolites, is typical only for SG and CHE (Scheme 3). During phase I biotransformation, generally, cleavage of methylenedioxy group(s) of the alkaloids and *O*-demethylations can be observed. The metabolites containing hydroxy group(s) formed in this process are probable subjects of conjugation. In this way, the polar metabolites of quaternary benzo[*c*]phenanthridine alkaloids in this study are subsequently excreted from the organism. This phenomenon has been well-documented for berberine and its metabolites which were identified in rat and human urine using HPLC, MS, and ^{1}H NMR [36]. The oxidative *O*-demethylenation of C-2,3 methylenedioxy group and *O*-demethylation of C-9,10 methoxy groups of berberine have also been found formed by microsomal fractions and S9 fractions of rat liver [40]. Similar metabolite formation to protoberberines was confirmed for protopine alkaloids using the GC/MS method. In these *in vivo* experiments, urinary metabolites were analyzed mainly in the form of glucuronides and sulfates [37,38,41]. The metabolites of protopine can be probably transformed up to tetrahydroprotoberberines whose production is connected with *N*-demethylation. However, we found no *N*-demethylated metabolites of SG and CHE generated in human hepatocytes.

4. Conclusion

Using the newly developed MS methods, isoquinoline alkaloids, SG, CHE, and their metabolites were analyzed in suspensions and/or primary cultures of human hepatocytes. This study is a continuation of our previous studies where we showed that the production of dihydro metabolites of SG and CHE was the first step in their metabolism *in vitro* and *in vivo* [5,7]. The metabolism of DHSG and DHCHE was studied for the first time. It was demonstrated that SG and CHE uptake or accumulation in hepatocytes is approximately the same as for significantly less polar DHSG and DHCHE. The biotransformation of the alkaloids to their metabolites was found to be higher for CHE than for SG. Equilibrium was found between alkaloids and their dihydro metabolites mediated by either low molecular weight reducing agents (e.g. enzyme cofactors NADH, NADPH) and/or oxidoreductases present in hepatocytes. SG, CHE and their dihydro metabolites could be subsequently subject to *O*-demethylenation or *O*-demethylation. For CHE and DHCHE, which were applied on human hepatocytes, the compound at *m*/*z* 336.1 was identified as the main *O*-demethyl metabolite. We think that our experimental protocols and optimized HPLC/MS method could be useful for further investigation of the metabolism of SG and CHE and/or other isoquinoline alkaloids. In addition, the methods proposed could be utilized for explanation of the reductive (dihydro metabolite formation) and oxidative (*O*-demethyl metabolite formation) biotransformation of the alkaloids in *in vitro* models of mammalian cells.

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