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Short communication

A liquid chromatographic–mass spectrometric evidence of dihydrosanguinarine as a first metabolite of sanguinarine transformation in rat

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Abstract

Adult rats were orally administered with a single dose of sanguinarine (10 mg SA per 1 kg body weight) in 1.0 ml water. In the plasma and the liver, dihydrosanguinarine (DHSA) was identified as a SA metabolite by high performance liquid chromatography–electrospray ionization mass spectrometry (HPLC/ESI-MS). Significantly higher levels of DHSA were found in both the plasma and the liver in comparison with those of SA. SA and DHSA were not detected in the urine. The formation of DHSA might be the first step of SA detoxification in the organism and its subsequent elimination in phase II reactions. Benz[c]acridine (BCA), in the literature cited SA metabolite, was found neither in urine nor in plasma and liver.

Keywords: Quaternary benzo[c]phenanthridine alkaloid; Sanguinarine; Rat; Metabolite; Liquid chromatography–mass spectrometry; Liver; Plasma; Dihydrosanguinarine; Benz[c]acridine

1. Introduction

Quaternary benzo[c]phenanthridine alkaloid (QBA) sanguinarine (SA) is found within the families Fumariaceae, Papaveraceae, Ranunculaceae and Rutaceae. SA originates from the aromatic amino acid tyrosine. An important intermediate of its biosynthesis is protopine. Dihydrosanguinarine (DHSA) is formed after hydroxylation of protopine in a reaction that depends on NADPH as a reduction cofactor, and molecular oxygen. This reaction is catalyzed by a microsomal cytochrome P450-linked monooxygenase. Then, DHSA is readily converted to SA by an oxidase.

The strong antimicrobial activity of SA indicates to its function as plant defense secondary metabolite or phytoalexine. If this alkaloid is administered to living organisms such as insects, fish and mammalian species, it can be absorbed, distributed, retained and/or metabolized either as a quaternary cation or a 6-hydroxydihydroderivative (pseudobase) [1]. In blood and organs, the equilibrium between these forms depends mainly upon the pH. The iminium bond in SA is susceptible to nucleophilic addition (of mainly SH-compounds) and is reduced by NADH/NADPH [2,3].

A benzo[c]acridine was described as the only metabolite of SA in in vivo experiments. The compound has been found in rabbit milk, rat, cat and monkey plasma after parentheral application of SA [4] and in rat after application by a probe [5]. Benz[c]acridine was extracted with organic phase after hydrolysis in 3 M HCl at 100 °C for 4 h and determined by paper electrophoresis and/or paper chromatography. Despite obvious absurdity of the suggested rearrangement of the benzo[c]phenanthridine skeleton into the benz[c]acridine structure (see Fig. 1 for chemical structures), this deduction has been quoted in the literature ever since [6]. This study deals with the transformation of orally administered SA in rats.

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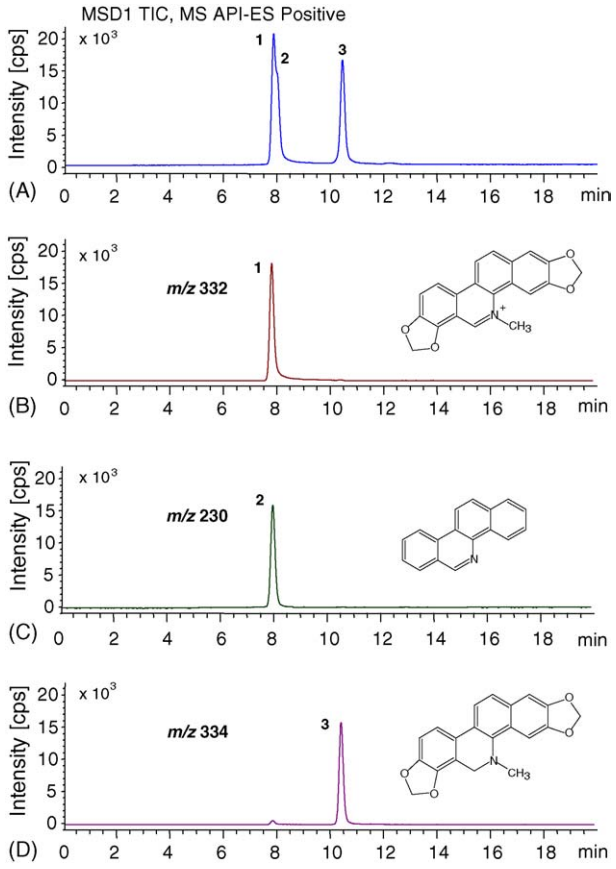

Fig. 1. HPLC/MS chromatograms of standards solution of sanguinarine, dihydrosanguinarine and benz[c]acridine; (A) total ion monitoring; (B) SIM mode for *m/z* 332 (sanguinarine); (C) SIM mode for *m/z* 230 (benz[c]acridine); and (D) SIM mode for *m/z* 334 (dihydrosanguinarine). Peak identification 1, sanguinarine (SA); 2, benz[c]acridine (BCA); 3, dihydrosanguinarine (DHSA). For chromatographic conditions, see Section 2.2.

2. Experimental

2.1. Chemicals

Sanguinarine was isolated from an extract of *Macleaya cordata* aerial part (provided by CAMAS Technologies Inc., Broomfield, USA) by column chromatography on alumina in 98.5% purity, MP 279–282 °C [7]; dihydrosanguinarine, 99% purity, MP 189–191 °C was prepared from SA by reaction with $NaBH_4$ in methanol [8]. Benzo[c]acridine was supplied by Fluka (Buchs, Switzerland). The stock standard solutions of SA, BCA and DHSA at 10 μg/ml were prepared in methanol and stored at 4 °C in dark. The working standard solutions were prepared daily by dilution of the stock solutions with methanol (1:10, v/v). All solutions were filtered through a 0.45 μm Teflon membrane filter (MetaChem, Torrance, USA) prior to HPLC/ESI-MS separations.

2.2. Animals

All procedures with animal were approved by the Ethics Committee of the Ministry of Education of the Czech Republic and were in accordance with the Czech Animal Protection Act No. 167/1993 Coll. and with the Good Laboratory Practice Regulations. Male Wistar rats (180 ± 10 g BW) were housed under standard laboratory conditions in a room maintained at 23 ± 2 °C with a common light/dark cycle. The rats ($n = 4$) were administered by gavage, after 10 h fasting with free access to water, a single oral dose of SA (10 mg/kg body weight) in 1.0 ml water. Urine was collected.

Animals were terminally narcotized i.m. anesthesia by fentanyl, medetomidin and diazepam (4, 20 and 500 μg/100 g BW, respectively) after 3 h. After opening the abdominal cavity, a macroscopic examination of the main organs, GI-tract, heart, kidney, liver, lungs, was performed. The blood samples were collected from the aortic bifurcation into a tube containing Na_2EDTA (1 mg/ml) and NaN_3 (0.1 mg/ml). The blood samples were centrifuged at 4 °C and 2500 × *g* for 10 min to obtain the plasma. The plasma aliquots and the phosphate buffer-washed liver were stored at −80 °C for the determination of the alkaloid content.

2.3. Sample preparation

Urine was diluted 1:1 with mobile phase, vortexed vigorously for 1 min, sonicated in an ultrasonic bath for 2 min, and centrifuged at 2500 × *g* for 1 min at room temperature. Supernatant (50 μl) was applied onto an HPLC column. For HPLC/ESI-MS analysis, standard stock solutions of SA, BCA and DHSA in methanol or plasma specimens (0.5 ml) were diluted by 1 ml of 0.01 M 1-heptanesulfonic acid (HEPES) in water, added to the conditioned (4 ml of methanol, 2 ml of water and 1 ml of 0.01 M HEPES in water) C_{18} SPE cartridges (Speed Octadecyl C18/18%, 200 mg/3 ml, Applied Separations, USA), washed by 2 ml of 0.01 M HEPES in water. QBA were eluted by 2 ml of 0.01 M HEPES in 95% methanol. After evaporation of the eluate and dissolution in the mobile phase, the samples were filtered through a 0.45 μm filter and applied (50 μl) onto an HPLC column. Liver (1 g) was homogenized with a mechanical homogenizer in 4 ml of a 0.01 M HEPES in 95% acetonitrile. The homogenate was vortexed vigorously for 1 min, sonicated in an ultrasonic bath for 2 min, and centrifuged at room temperature and 2500 × *g* for 1 min. The supernatant (3 ml) was evaporated under N_2 at 50 °C and dissolved in methanol.

2.4. HPLC/ESI-MS analysis and data evaluation

An HP 1100 chromatographic system (Hewlett-Packard, Waldbronn, Germany) was equipped with a vacuum degasser (G1322A), a binary pump (G1312A), an autosampler (G1313A), a column thermostat (G1316A) and a diode array detector (model G1315A). The system was coupled on-line to a quadruple mass-selective HP MSD detector (G1946A, Hewlett-Packard, Palo Alto, USA). The ChemStation software (Rev. A 07.01) controlled the whole system. The chromatographic column Zorbax SB-CN (7.5 mm × 4.6 mm, 3.5 μm, Agilent Technologies, USA) was used. A mobile phase consisted of 0.01 M (v/v) ammonium formate (A) and methanol (B). A linear gradient was applied as follows: from start to 1 min—60% B (v/v) to 60% B (v/v); from 1.0 to 7.0 min—60% B (v/v) to 71% B (v/v);

from 7.0 to 18.0 min—71% B (v/v) to 60% B (v/v). Flow rate was 0.7 ml min^{-1} and the temperature of the column oven was set at 15 °C. The signal was monitored with the diode array detector at 285 nm. Spectra were registered in the range of 190–400 nm (SBW 100 nm).

The column effluent was directly introduced into the single quadruple mass spectrometer operated in a positive ESI mode. The mass spectrometer was regularly calibrated according to the manufacturer recommendations. The nebulizer gas pressure was 50 psi, the drying gas was nitrogen at 12 l min^{-1}, the temperature was 350 °C and the capillary voltage was 1500 V. The fragmentor voltage was set to 100 eV (CID 200 V) and the gain was 1. The *m/z* spectra and data for the selected ion monitoring (SIM) mode were acquired at *m*/*z* for SA 332 → 274, *m*/*z* for BCA 230 → 200, *m*/*z* for DHSA 334 → 318 and for scan mode were 200–600 *m*/*z*.

2.5. Accuracy, precision and recovery

Accuracy, precision and recovery of the determination of the individual analytes (sanguinarine, dihydrosanguinarine and benz[c]acridine) were evaluated with the real samples spiked with 100 μl standards (sample concentrations varying from 10 to 100 ng ml^{-1}). Coefficients of variation (%C.V.) of the intra-day assay were determined in six homogenates. The inter-day precision was determined by analyses of six homogenates over a 5-day period. The homogenates were assayed blindly and analytes concentrations were calculated from the calibration curves. Accuracy was evaluated by comparing the estimated concentration with the known concentrations of the individual analytes.

2.6. Statistical analysis

A STATGRAPHICS® (Statistical Graphics Corp.®, USA) was used for statistical analyses. Results are expressed as the means ± S.D. unless stated otherwise. A value of $p < 0.05$ was considered significant.

3. Results and discussion

3.1. Procedure optimization

An HPLC/ESI-MS method has to be optimized with respect to the individual parameters of both steps—the separation of the HPLC part and the ionization conditions of the MS part. Type of the sorbent, flow rate of the mobile phase and its composition with respect to the ratio of eluent modifier (electrolyte, ammonium formate) and organic modifier (methanol) are the main parameters responsible for the chromatographic separation. The effectiveness of HPLC separation of the benzo[c]phenanthridine alkaloids was tested using the standard solutions of SA, BCA and DHSA. Several reverse phase chromatographic columns C_{18}, C_8 and CN were tested. The best results were obtained with CN column.

The gradient elution profile was optimized and the best resolution for all peaks was obtained using a linear gradient of the mobile phase containing methanol and 0.01 M ammonium formate (see Section 2.4). Under these chromatographic conditions, the retention times SA, BCA and DHSA were 8.10, 8.28 and 10.75 min, respectively (see Fig. 1 for a chromatogram from HPLC/ESI-MS analysis of SA, BCA and DHSA).

The operational parameters of MS detection were optimized for the positive ionization mode. These parameters and their operational range were: drying gas temperature (100–350 °C with 50 °C steps) and flow (3–13 l min^{-1} in 1 l min^{-1} steps), nebulizer pressure (20–60 psi in 5 psi steps), capillary voltage (1000–6000 V in 500 V steps) and fragmentor voltage (20–200 eV in 20 eV steps). To minimize fragmentation of analytes, gain 1 was kept constant throughout study. The optimization was carried out by flow injection analysis (FIA) of several solutions containing the individual compounds at a concentration of 1 ng μl^{-1} each (Figs. 2 and 3). The quadruple mass spectrometric detector was used with electrospray ionization (ESI-MS) in the positive ion selective mode (SIM: selected ion monitoring) in our experiments.

The mass spectrum of SA, DHSA and BCA shown Fig. 4 were scanned in the range 100–600 *m*/*z* at 1500 V capillary voltages and collision energy set at 100 and 200 eV, respectively. The molecular ion SA $[M + H^+]$ was measured at *m*/*z* 332 and specific products of fragmentation at *m*/*z* 317 and 274, the molecular ion DHSA $[M + H^+]$ was measured at *m*/*z* 334 and specific products of fragmentation at *m*/*z* 318, 260 and 232, and the molecular ion BCA $[M + H^+]$ was measured at *m*/*z* 230 and specific products of fragmentation at *m*/*z* 202. According to literature, the fragments were in good coincidence with so far published data [9–11].

The efficiency of ionization processes in HPLC/ESI-MS is seriously influenced by electrolyte composition and by fragmentor voltage. The effect of ammonium formate concentration (Fig. 3) on the ionization efficiency was tested. The highest intensity for SA and DHSA were recorded in 0.01 mmol l^{-1} ammonium formate. As for BCA, the most suitable conditions are 0.001 mmol l^{-1}, but the 0.01 mmol l^{-1} ammonium formate response is good enough for simultaneous determination of all three analytes.

Table 1
Quantification of SA and DHSA in plasma extracts with limits of detection, limits of quantitation, RSDs and calibration curves parameters

	Concentration	LOD		LOQ		R.S.D.	*r*	Calibration equation
	(ng/ml)	(fg)	(fmol)	(pg)	(fmol)	(%, $n = 8$)		
SA	9.54	598.08	1.77	17.9424	5.31	1.64	0.99987	$y = 497695.914x$
DHSA	14.29	858.28	2.57	25.7484	7.71	2.07	0.99986	$y = 333140.676x$
BCA	ND	518.03	2.25	15.5409	6.75	2.25	0.99995	$y = 1340833.660x$

ND: not detected.

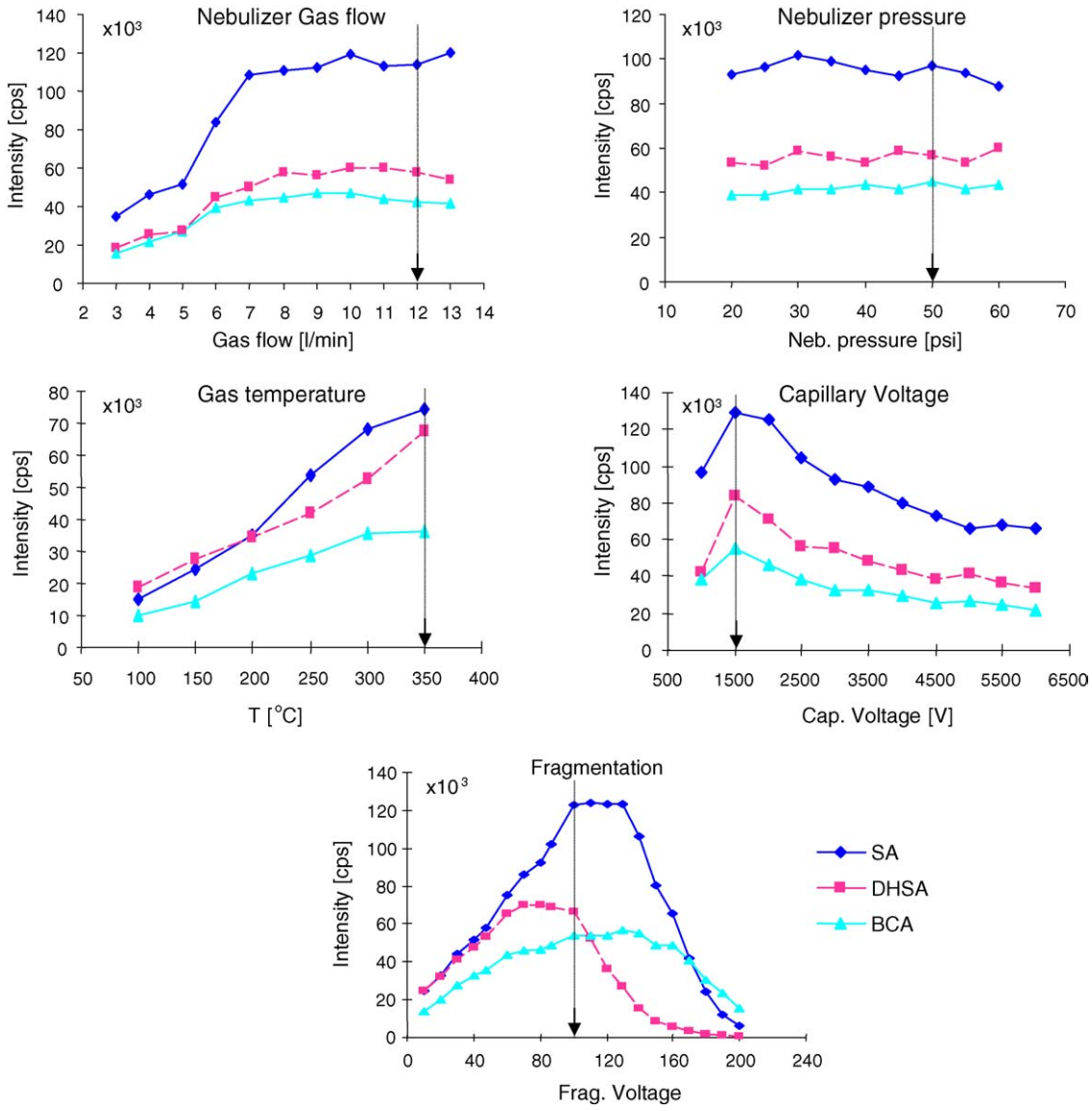

Fig. 2. FIA studies for optimization of the ESI-MS positive mode signal. Influence of nebulizer gas flow, nebulizer pressure, gas temperature, capillary voltage and fragmentation were examined.

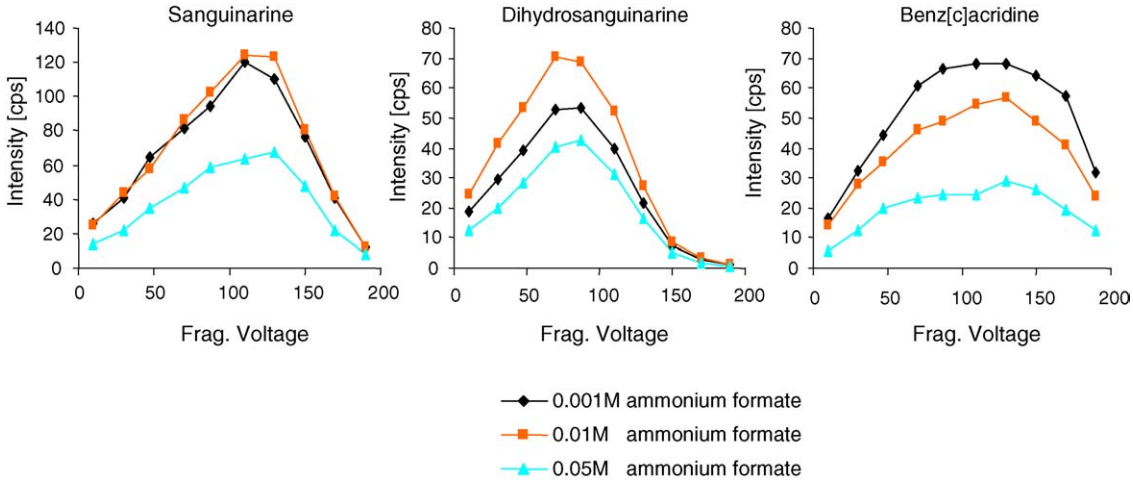

Fig. 3. Influence of both fragmentor voltage and ammonium formate concentration on the intensity of analytical ion current was examined.

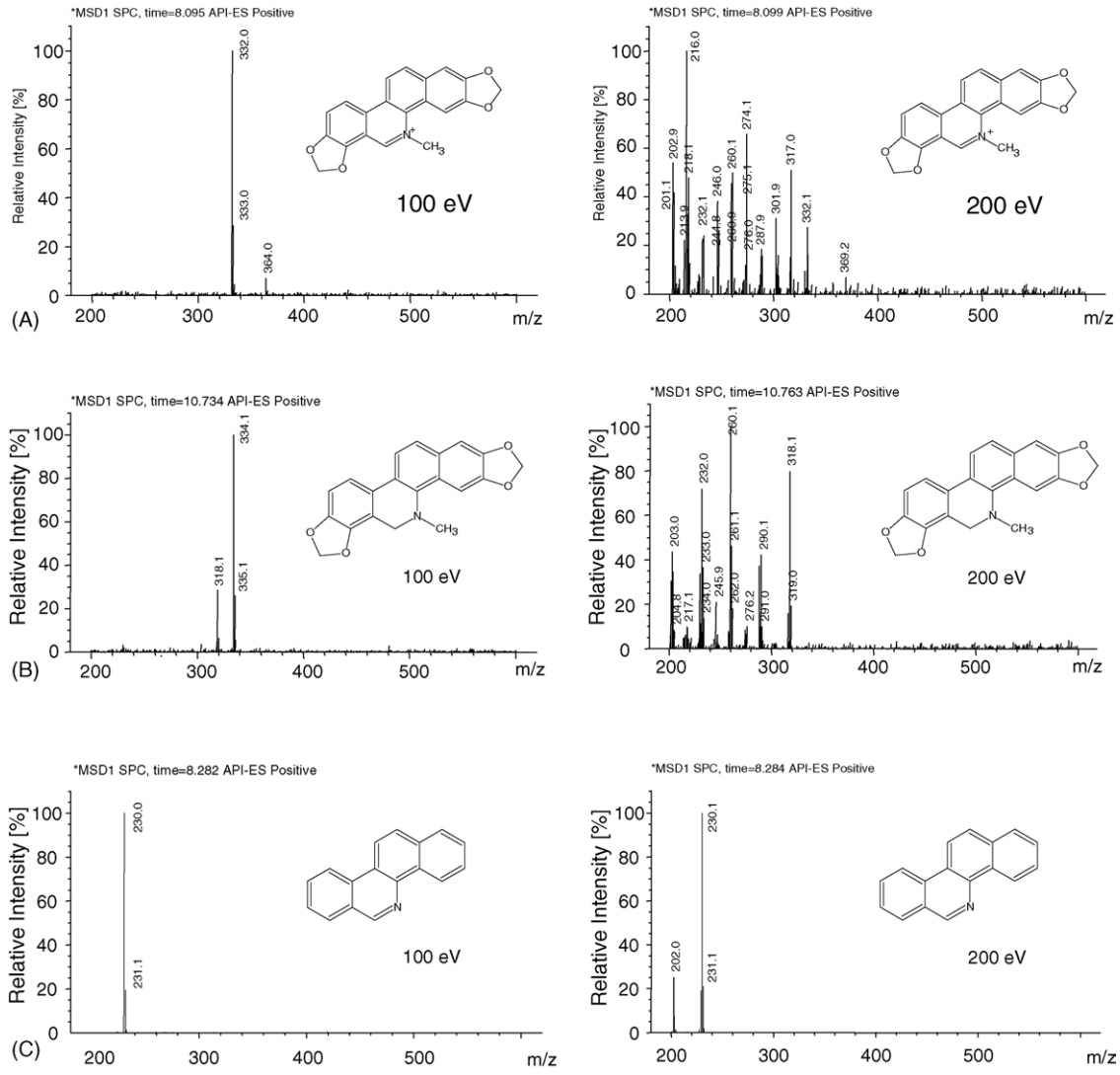

Fig. 4. ESI-MS spectral characteristics of (A) sanguinarine, (B) dihydrosanguinarine and (C) benz[c]acridine for the fragmentor voltage 100 and 200 eV. For chromatographic conditions, see Section 2.2.

Detection limits, parameters of calibration equations and correlation coefficients of calibration curves are given in Table 1. LODs in the range 598, 858 and 518 fg for SA, DHSA and BCA, respectively (i.e. 1.77, 2.57 and 2.25 fmol, respectively) enabled very sensitive determination. Also, calibration was very satisfactory, with correlation coefficients 0.99987, 0.99986 and 0.99995, respectively. Method was validated via spikes ($n = 6$) of three different concentrations of all investigated analytes into

Table 2
Method validation: recovery of SA and DHSA from plasma extracts in triplicate ($n = 6$)

	Plasma extract		Spiked analytes					
	Recovery (μg/ml)	R.S.D. (%)	Recovery (μg/ml)	R.S.D. (%)	Recovery (μg/ml)	R.S.D. (%)	Recovery (μg/ml)	R.S.D. (%)
SA	9.52	2.23	10.25	2.93	51.23	2.26	102.34	2.71
DHSA	14.34	2.47	10.30	1.67	50.71	1.88	98.67	2.47
BCA	Not detected	9.92	2.04	51.15	2.37	101.55	1.94	

	Plasma extract + spiked analytes						Recovery		
	Recovery (μg/ml)	R.S.D. (%)	Recovery (μg/ml)	R.S.D. (%)	Recovery (μg/ml)	R.S.D. (%)	Spike 10 (%)	Spike 50 (%)	Spike 100 (%)
SA	19.85	2.47	60.80	2.17	112.01	2.71	101.67	102.16	102.28
DHSA	24.33	2.04	65.32	2.48	113.17	1.65	99.97	101.52	98.98
BCA	10.09	2.09	49.92	2.39	99.30	1.58	100.95	99.84	99.29

Table 3
Precision and recovery of SA, DHSA and BCA analyses in plasma extracts ($n = 6$)

		Plasma extract	Spiked analytes	Total content	Recovery (%)
		Analyte content in μg/ml (R.S.D. in %)			
SA	Intra-day ($n = 6$)	9.53 ± 0.09 (1.00)	10.36 ± 0.21 (1.95)	19.29 ± 0.64 (3.29)	98.72
			99.64 ± 2.70 (2.73)	109.14 ± 1.28 (1.17)	99.63
	Inter-day ($n = 30$)	9.47 ± 0.20 (2.20)	10.11 ± 0.24 (2.39)	19.40 ± 0.57 (2.93)	99.28
			100.31 ± 2.94 (2.92)	109.95 ± 1.36 (1.24)	100.38
DHSA	Intra-day ($n = 6$)	14.36 ± 0.32 (2.21)	10.19 ± 0.17 (1.64)	24.44 ± 0.58 (2.37)	100.62
			98.97 ± 2.48 (2.51)	114.02 ± 2.53 (2.22)	99.76
	Inter-day ($n = 30$)	14.37 ± 0.31 (2.12)	9.91 ± 0.18 (1.78)	24.36 ± 0.82 (3.38)	100.27
			100.97 ± 1.83 (1.81)	115.05 ± 2.41 (2.10)	100.66
BCA	Intra-day ($n = 6$)	Not detected	9.81 ± 0.28 (2.96)	10.05 ± 0.12 (1.20)	100.45
			101.35 ± 2.81 (2.78)	100.94 ± 1.87 (1.86)	100.94
	Inter-day ($n = 30$)	Not detected	10.10 ± 0.23 (2.31)	10.13 ± 0.26 (2.52)	101.29
			100.55 ± 2.42 (2.40)	99.41 ± 1.76 (1.77)	99.41

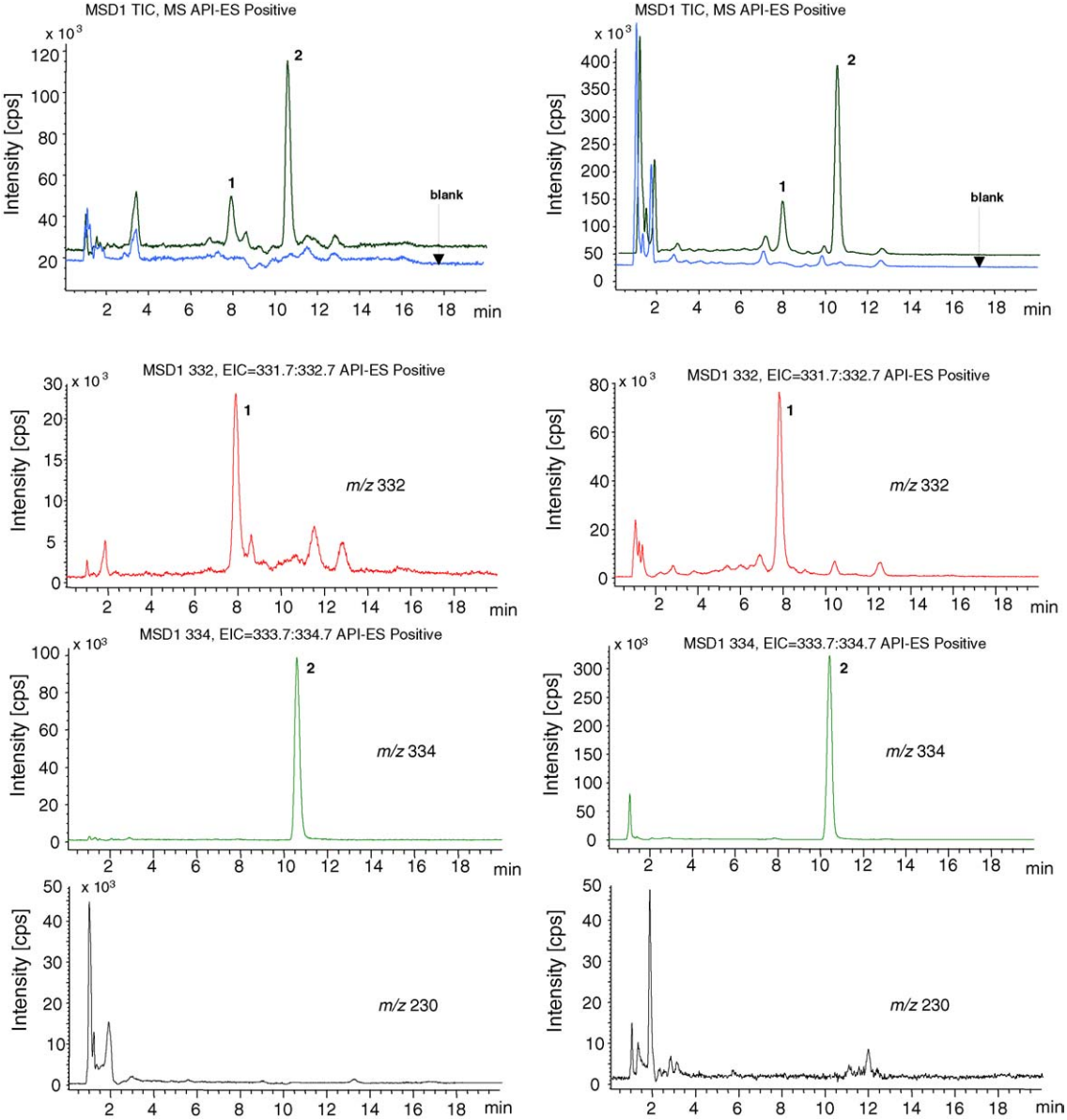

Fig. 5. HPLC/MS chromatograms (SIM mode for *m/z* 332 sanguinarine, for *m/z* 334 dihydrosanguinarine and for *m/z* 230 benz[c]acridine) of real samples. (I) rat plasma and (II) rat liver and their blanks. Peak identification 1, sanguinarine (SA); 2, dihydrosanguinarine (DHSA). For chromatographic conditions, see Section 2.2.

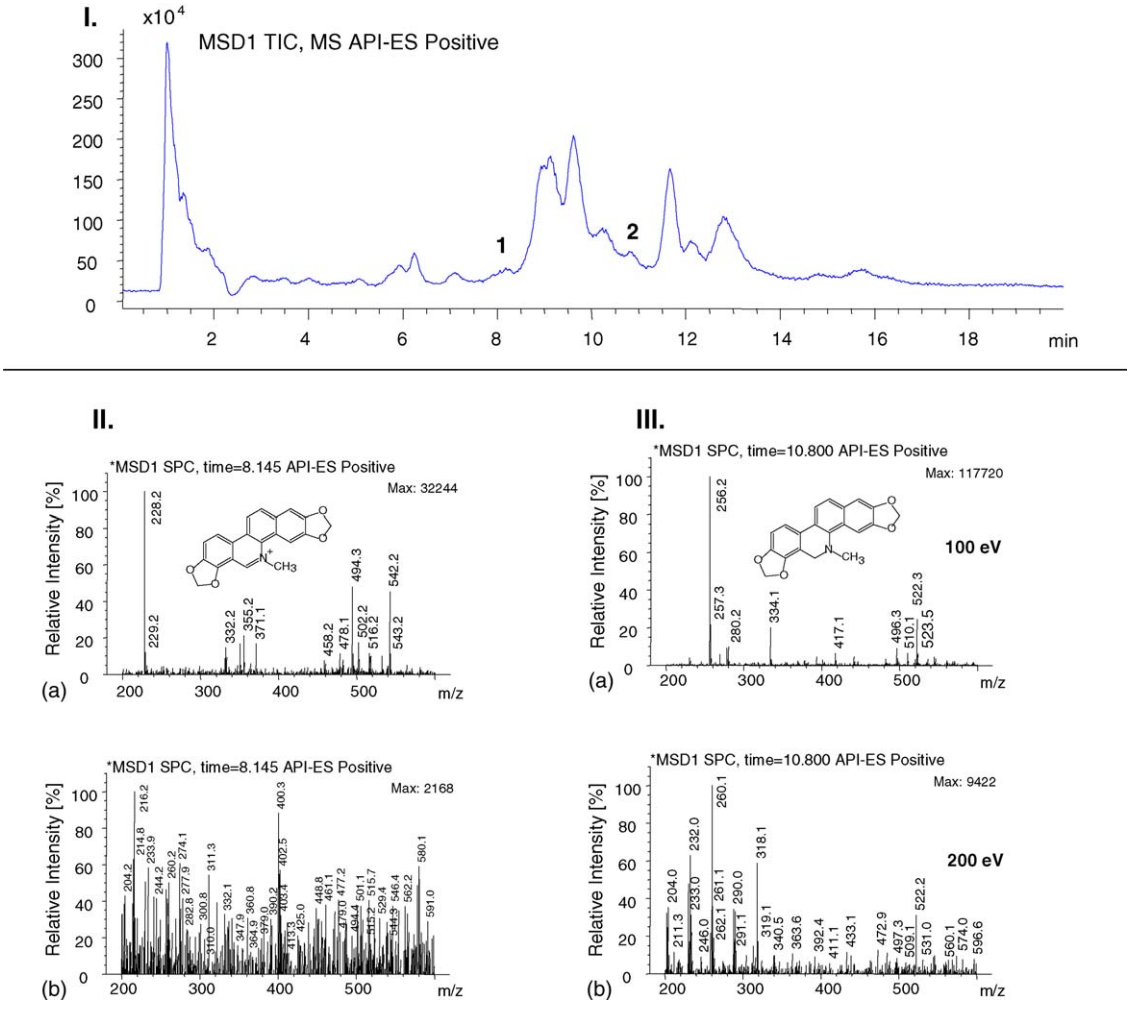

Fig. 6. HPLC/MS chromatograms (total ion current, full scan *m/z* from 200 to 600) of real samples (I) rat liver. MS spectral characteristics of (II) sanguinarine (fragmentor voltage (a) 100 eV and (b) 200 eV) and (III) dihydrosanguinarine (fragmentor voltage (a) 100 eV and (b) 200 eV). Peak identification 1, sanguinarine (SA); 2, dihydrosanguinarine (DHSA). For chromatographic conditions, see Section 2.2.

real samples of rat plasma (see Table 2). Inter-day and intra-day validation data are given in Table 3.

3.2. Analysis of real samples

Thanks to these exceptionally low detection limits, small changes of investigated analytes concentrations occurring in physiological processes can be determined. HPLC/ESI-MS analyses of the samples of rat liver and plasma extracts were performed in positive ion mode. Protonated molecular ions $[M + H]^+$ of SA and DHSA were registered in both the plasma and the liver, benz[c]acridine, in the literature cited SA metabolite, was found neither in urine nor in plasma and liver in detectable amounts (see Fig. 5).

Sensitive and selective detection was made possible by SIM of molecular and/or main fragment ions based on specific in-source collision-induced dissociation. SIM method, very precise and sensitive, was used as a main determination tool. For verification, also full scan mode was employed for all investigated samples (see Fig. 6). According to the mass spectra and the retention time agreement, the presence of quaternary benzo[c]phenanthridine alkaloids can be approved.

The biological activity of sanguinarine in vivo/in vitro is linked to two forms of the alkaloid: the cation form and the neutral 6-hydroxy-DHSA (pseudobase) form. A dynamic equilibrium between these two forms in the extra- and intracellular medium [12] is affected by instantaneous concentrations of H^+ ions and of interacting molecules (proteins, nucleic acids, polysaccharides, etc.).

The 6-hydroxy (pseudobase) form, which is more active than the cation form, is capable to penetrate the cell membrane. The reduction of the iminium bond in the cation form of SA is the detoxification reaction of an animal organism. DHSA formed this way can be further transformed into polar conjugates in the phase I and II of detoxication. Although the structures of the conjugates have not been fully elucidated, it has been evidenced that they are formed from their precursor dihydrosanguinarine the basic compound in the sanguinarine metabolism.

Despite the obvious absurdity of the suggested rearrangement of the benzo[c]phenanthridine skeleton into the benz[c]acridine

structure (see Fig. 1 for chemical structures), this deduction has been quoted in the literature ever since [6]. The presence of benz[c]acridine, in the literature cited SA metabolite [6], was indisputably rejected by our experiments.

Acknowledgement

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