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The toxicity and pharmacokinetics of dihydrosanguinarine in rat: A pilot study

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ABSTRACT

The quaternary benzo[c]phenanthridine alkaloid sanguinarine (SG) is the main component of Sangrovit®, a natural livestock feed additive. Dihydrosanguinarine (DHSG) has recently been identified as a SG metabolite in rat. The conversion of SG to DHSG is a likely elimination pathway of SG in mammals. This study was conducted to evaluate the toxicity of DHSG in male Wistar rats at concentrations of 100 and 500 ppm DHSG in feed for 90 days (average doses of 14 and 58 mg DHSG/kg body weight/day). No significant alterations in body or organ weights, macroscopic details of organs, histopathology of liver, ileum, kidneys, tongue, heart or gingiva, clinical chemistry or hematology markers in blood in the DHSG-treated animals were found compared to controls. No lymphocyte DNA damage by Comet assay, formation of DNA adducts in liver by ^{32}P-postlabeling, modulation of cytochrome P450 1A1/2 or changes in oxidative stress parameters were found. Thus, repeated dosing of DHSG for 90 days at up to 500 ppm in the diet (i.e. approximately 58 mg/kg/day) showed no evidence of toxicity in contrast to results published in the literature. In parallel, DHSG pharmacokinetics was studied in rat after oral doses 9.1 or 91 mg/kg body weight. The results showed that DHSG undergoes enterohepatic cycling with maximum concentration in plasma at the first or second hour following application. DHSG is cleared from the body relatively quickly (its plasma levels drop to zero after 12 or 18 h, respectively).

1. Introduction

The quaternary benzo[c]phenanthridine alkaloid (QBA) sanguinarine (SG) and its congener chelerythrine are elicitor-inducible secondary metabolites (phytoalexines) with antifungal and nematocidal activities in Caprifoliaceae, Fumariaceae, Meliaceae, Papaveraceae and Rutaceae plants (Wink, 1999). *In vitro* studies have shown that SG modulates a number of molecular targets in the metabolism of mammalian cells (Malikova et al., 2006a,b). SG is also used in toothpastes and mouthwashes for its antibacterial and anti-inflammatory activities and as an active component in the animal feed additive Sangrovit® used for pigs and chickens (Dvorak et al., 2006a). On the other hand, the toxicity of the oil of *Argemone mexicana* seeds has been attributed to SG and DHSG which putatively initiate oxidative stress (ROS formation leading to lipid peroxidation, depletion of GSH and decrease of total antioxidant capacity) and death of red cell via formation of methemoglobin (Das et al., 2005; Babu et al., 2006, 2007). Oxidative stress (production of oxygen and nitrogen reactive species) is closely connected with genotoxicity. In two previous studies we examined the safety of feeding pigs (Kosina et al., 2004) and rats (Psotova et al., 2006b) for 90 days with sanguiritrin, a mixture of SG and chelerythrine, extracted from *Macleaya cordata*. All animals remained in good health over the duration of both experiments and manifested no signs of the oxidative stress described for argemone oil intoxication. SG-derived DNA adducts and/or DNA damage in livers of pigs/rats exposed to sanguinarine were not detected. Recently we found that the first step in SG metabolism in rat is the reduction of the iminium bond leading to formation of DHSG (Vecera et al.,

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AOPP, advanced oxidation protein products; AST, aspartate aminotransferase; AUC, area under the plasma concentration–time curve; CYP, cytochrome P450; CHE, cholinesterase; DHSG, dihydrosanguinarine; GIT, gastrointestinal tract; GMT, gamma-glutamyl transpeptidase; GPX, glutathione peroxidase; GSH, glutathione; SG, sanguinarine; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

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2007). We also found that SG and DHSG were completely eliminated from plasma and liver after 24 h. We have never detected the formation of the genotoxic benz[*c*]acridine (Psotova et al., 2006a; Vecera et al., 2007), which had been suggested (Tandon et al., 1993) as a product of SG transformation.

In contrast to SG, practically no information about DHSG biological activity exists (Walterova et al., 1995). Only Vavreckova et al. (1994) reported that DHSG was nontoxic in rat hepatocyte primary cultures. The objective of this study was to assess the effects of 100 or 500 ppm DHSG in feed in a 90-day experiment in rats. The doses (100 ppm and 5 × 100 ppm) were selected in order to relate this study to that of Babu et al. 2006, who applied 1% of argemone oil (i.e. 53 mg of DHSG/SG mixture) in one kg of feed (toxicity reported) and to our previous experiments (Kosina et al., 2004; Psotova et al., 2006b), where we added 100 mg of benzo[*c*]phenanthridine alkaloids (SG/chelerythrine mixture) to 1 kg of feed (no toxicity found). The duration of the study was selected as 90 days, a standard time for subchronic toxicity. Moreover, farm animals consume feed with additives containing a mixture of QBA, mainly sanguinarine maximally for 3 months. Evaluated in this study were: health status, body weight, organ morphology, hematology, clinical chemistry parameters, oxidative stress markers in blood and liver. Modulation of liver microsomal cytochrome P450 and isoform 1A1/2 by DHSG and the effect of DHSG on hepatocyte and lymphocyte DNA and its distribution in organs were also investigated. To corroborate the findings, DHSG levels in plasma as well as the kinetics of DHSG elimination were determined in a pharmacokinetic study using oral doses of 9.1 or 91 mg/kg body weight.

2. Materials and methods

2.1. Test substance

Dihydrosanguinarine (DHSG), 99% purity, MP 189–191 °C was prepared from sanguinarine by reaction with $NaBH_4$ in methanol (Brossi and Borer, 1965). Benz[*c*]acridine was supplied by Fluka (Buchs, Switzerland) and sanguinarine from Sigma (USA).

2.2. Diets

Experimental diets: DHSG (100 mg or 500 mg) was blended with the powdered commercial diet (800 g), microcrystalline cellulose (190 g) and magnesium stearate (10 g) to prepare the feed pellets.

Control diet: The powdered commercial diet (800 g) was blended with microcrystalline cellulose (190 g) and magnesium stearate (10 g).

The diets were prepared monthly and analyzed by HPLC periodically to confirm concentration, homogeneity and stability of DHSG in the diet. The pellets were stored in paper bags and kept dry.

2.3. Animals

The study was approved by the Ethics Committee, Ministry of Education, Czech Republic and conducted in compliance with the Experimental Animals Protection Act No. 167/1993 L.C. Male Wistar rats (n = 18; 210 ± 10 g bw) were purchased from BioTest Ltd., Konarovice, Czech Republic. The rats were acclimatized 1 week before the experiment. They were kept in plastic cages containing dust-free sawdust, two animals per cage. On the day of treatment, the animals were 8 weeks old with a mean body weight ± SD of 240 ± 8 g (n = 18). They were randomized to three groups (6/group); control – Group 1 (241 ± 11 g bw); Group 2, 100 ppm DHSG (236 ± 7 g bw); Group 3, 500 ppm DHSG (243 ± 5 g bw). Group sizes of six were considered acceptable for this pilot investigation of toxicity.

During the acclimatization period and during testing, the conditions in the animal room were as follows: temperature (23 ± 2 °C; checked daily); relative humidity (30–70%); light/dark cycle 12 h/12 h. The animals had free access to their respective diets and water. The feed consumption was checked twice a week. The health of the animals was checked daily and body weights were monitored twice a week and prior to sacrifice.

Administration of DHSG: The animals consumed *ad libitum* either the standard diet or the diets containing 100 or 500 ppm DHSG, for 90 days.

Sample collection and preparation: The animals were deprived of food 12 h before terminal *i.m.* anesthesia by fentanyl (4 μg/100 g bw), medetomidin (20 μg/100 g bw) and diazepam (0.5 mg/100 g bw). After opening the abdominal cavity, macroscopic examination of the main organs (GIT, heart, kidneys, liver, lungs and other organs) was done. The urine was collected from the animal's bladder (to prevent urine components from oxidative processes and contamination by feces) and analyzed for DHSG content. The blood was collected from the aortic bifurcation into Na_2EDTA-tubes (Sarstedt, Germany) and Heparin-Lithium-tubes (Sarstedt, Germany). A part of the Na_2EDTA blood was used for lymphocyte isolation for genotoxicity assay and for blood count (0.5 ml). The rest of Na_2EDTA blood was centrifuged at 2500*g* for 10 min at 4 °C to obtain the plasma. The plasma aliquots were stored at −80 °C for determination of DHSG/SG content and parameters of antioxidant capacity. Heparin-lithium blood was centrifuged at 2500*g* for 10 min at 4 °C to obtain the plasma for the clinical chemistry. Na_2EDTA-erythrocytes were washed with phosphate-buffered saline and stored at −80 °C for determination of oxidative stress parameters. Liver, muscle, kidney, heart, ileum, tongue, thymus, and brain were removed, washed in cold phosphate-buffered saline and the selected organs were weighed and stored at −80 °C for determination of DHSG/SG content. Livers were utilized for DNA adduct formation analysis and parameters of oxidative stress and were frozen and stored at −80 °C prior to analyses. Histological examination was made of tongue, liver, ileum, kidney, gingiva and heart.

2.4. Determination of DHSG in diet, feces, urine, tissues and plasma

Diets and/or *feces* were extracted with acidified (1% HCl) methanol in a Soxhlet extractor for 12 h and the extracts after appropriate dilution by mobile phase were subjected to HPLC analysis.

Urine (0.4 ml) and/or *plasma* (0.4 ml) were mixed with methanol (0.6 ml or 0.8 ml, resp.), vortexed vigorously for 1 min and centrifuged at 12,000*g* for 15 min at 4 °C. The supernatant (30 μl) was applied to an HPLC column.

Tissues: Liver, kidney, muscle, ileum, tongue, brain and heart (1 g) were homogenized with a mechanical homogenizer in 4 ml of a 0.01 M 1-heptanesulfonic acid in 95% acetonitrile. The homogenate was vortexed vigorously for 1 min, and centrifuged at 12,000*g* for 15 min at 4 °C. After evaporation of supernatant (3 ml) under N_2 at 50 °C, it was dissolved in methanol (0.5 ml). The sample was then filtered through a 0.45 μm Teflon filter and applied (30 μl) to a HPLC column.

High performance liquid chromatography separation and mass spectrometry detection and identification of SG and DHSG were carried out as described elsewhere (Psotova et al., 2006a; Vecera et al., 2007).

2.5. Clinical chemistry

On day 90, following sacrifice, blood plasma analyses of sodium, potassium, chlorides, bilirubin, cholesterol, urea, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GMT), alkaline phosphatase (ALP), cholinesterase (CHE), total protein and prealbumin were analyzed on an Advia analyser 1650 (Bayer, USA).

2.6. Hematology

Hemoglobin, hematocrit, erythrocytes, mean erythrocyte volume, thrombocytes, leukocytes and differential leukocyte count were analyzed in Na_2EDTA blood.

2.7. Parameters of oxidative stress

Lipid peroxidation was assessed by measuring the presence of thiobarbituric acid reactive substances (TBARS) in the plasma, erythrocytes or liver homogenates (Buege and Aust, 1978). The level of glutathione (GSH) in erythrocytes and liver homogenate was determined according to Sedlak and Lindsay (1968) using Ellman's reagent. The plasma level of total SH-group was determined according to Hu (1994). An indirect spectrophotometric method used for assessment of superoxide dismutase (SOD) activity in erythrocytes and liver homogenate was based on the generation of $O_2^{\cdot-}$ by a mixture of nitro blue tetrazolium, NADH and phenazine methosulfate (Ewing and Janero, 1995). Glutathione peroxidase (GPX) activity in erythrocytes and liver homogenate was assayed by a modification of the method of Tappel (1978). Catalase activity in erythrocytes and liver homogenate was determined according to Beers and Sizer (1952) a method based on measuring the quantity of decomposed hydrogen peroxide in a unit of time. Advanced oxidation protein products (AOPP) in plasma were assessed spectrophotometrically at 340 nm. The plasma and liver homogenate total antioxidant capacity (TAC) was measured by cyclic voltammetry. The protein concentration was determined by the Bradford method.

2.8. Total and specific CYP1A1/2 content of cytochrome P450

Liver microsomes were isolated by ultracentrifugation and the cytochrome P450 content was measured according to Omura and Sato (1964). The microsomal protein concentration was measured according to Lowry. The levels of cytochromes P450 1A1/2 were assessed in pooled microsomal samples by immunoblotting after SDS–PAGE electrophoresis using a primary antibody against human CYP1A1 (CYP1A1 (G-18) goat polyclonal IgG; dilution 1/500) purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA) and a secondary antibody conjugated with horseradish peroxidase using chemiluminescence detection.

2.9. Histopathology

Liver, ileum, kidney, tongue, heart and gingiva specimens were fixed in Baker mixture, embedded in paraffin and 7 μm thick sections were cut on a rotary microtome. Sets of histological sections were stained with hematoxylin-eosin and PAS. The histological evaluation was performed on an Olympus BX 40 light microscope.

2.10. Genotoxicity

Detection of single-stranded DNA breaks: The DNA breaks were measured in peripheral lymphocytes using an alkaline version of Comet assay (Vodicka et al., 2001). Lymphocytes were isolated on Histopaque 1077 gradients. DNA damage was analyzed after staining by ethidium bromide on an Olympus IX 70 fluorescence microscope. One hundred cells per slide were analyzed and divided into four classes of DNA damage standard scale. Total DNA damage was calculated: (number of cells in class 1 × 1 + number of cells in class 2 × 2 + number of cells in class 3 × 3 + number of cells in class 4 × 4)/100. The maximal damage was given a value of 400.

Analysis of DNA adducts in liver: DNA was isolated from liver tissues of rats by the phenol/chloroform procedure and the ^{32}P-postlabeling assay was performed as described previously (Stiborova et al., 2002). The detection limit of relative adduct labeling was 0.1/10^8 nucleotides.

2.11. Pharmacokinetics of DHSG

The animals were starved for 10 h before gavage administration of DHSG. In total, 132 male Wistar rats (260 ± 10 g bw) were used in the pharmacokinetic study. If not stated, the conditions were the same as for the oral toxicity experiment. The gavage administration was used as an acceptable route in oral toxicity studies. The difference between the intragastric route (gavage) and the dietary route should not have been insignificant as most absorption takes place in the stomach and the GIT. Since there was no information on the pharmacokinetics of DHSG, we chose 36 h to increase the chances of detecting metabolites. The rats were given a single oral dose of DHSG suspension (9.1 or 91 mg/kg bw) in 1.0 ml water. After 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 16, 18, 20, 22, 24, 28, 32 and 36 h, three rats were terminally narcotized *i.m.* anesthesia by fentanyl (4 μg/100 g bw), medetomidin (20 μg/100 g bw) and diazepam (0.5 mg/100 g bw). Urine were collected at 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 16, 18, 20, 22, 24, 28, 32 and 36 h intervals. After opening the abdominal cavity, a macroscopic examination of the main organs, GIT, heart, kidney, liver and lungs was performed. The blood was collected from the aortic bifurcation into Na_2EDTA (1 mg/ml) and centrifuged at 10 °C and 2500*g* for 10 min to obtain plasma. The plasma aliquots and the liver samples washed with phosphate-buffered saline were stored at −80 °C for the determination of DHSG content. Experimental data were derived from three animals per time and dose.

Interruption of the enterohepatic cycle of DHSG: Immediately after oral application of DHSG (91 mg/kg bw) the rats were narcotized *i.m.* anesthesia by fentanyl (4 μg/100 g bw), medetomidin (20 μg/100 g bw) and diazepam (0.5 mg/100 g bw). The billiary duct was ligated and after 0.5, 1, 2, 3, 4, 5, 6, 7, 8 and 9 h the blood was collected from the aortic bifurcation into Na_2EDTA (1 mg/ml) and centrifuged at 10 °C and 2500*g* for 10 min to obtain plasma. At the end of the experiment, macroscopic examination was done of the main organs, GI-tract, heart, kidneys, liver and lungs. The plasma aliquots and phosphate-buffered saline washed liver were stored at −80 °C for the determination of DHSG content. Experimental data were derived from three animals.

2.12. Statistical analyses

All values were expressed as mean ± SD and ANOVA was used to analyze data ($p < 0.05$). A regression analysis (logarithmic regression model) was used to test the significance of differences in gain weight in the three groups of rats ($p < 0.05$).

3. Results

3.1. Diet analysis

The concentration, homogeneity and stability of DHSG in the prepared diets were periodically analyzed by HPLC. The DHSG concentration in diets was 97.5 ± 7.3 μg/g (Group 2) and 478.1 ± 20.6 μg/g (Group 3), respectively.

3.2. Feed consumption

The daily diet consumption was 40 g on average. Daily dose of DHSG for 100 ppm and 500 ppm (Groups 2 and 3), respectively, fell from 17 (Group 2) and 80.8 mg/kg/day (Group 3) at the beginning to 7.83 and 35.5 mg/kg/day at the end of the experiment, i.e. average doses over 90 days were 14 (Group 2) and 58 mg/kg/day (Group 3), respectively. No significant alteration in feed consumption or health status was found in any group.

3.3. Body and organ weight

The weight gain curves displayed very similar courses for Groups 1 and 2 (Fig. 1). There was a slightly greater weight increase in animals of Group 3 compared to Groups 1 or 2 and this difference was significant ($p < 0.05$), as evidenced by comparison of regression coefficients for the gain weight curves (Fig. 1).

There was no significant difference in weights of liver, right and left kidney, heart, brain and thymus for any of the groups of animals (Table 1).

3.4. Clinical chemistry parameters

The following parameters were investigated: sodium, potassium, chloride, bilirubin, urea, creatinine, cholesterol, ALT, AST, GMT, ALP, CHE, total protein and prealbumin (Table 2). Except for a significant decrease in ALT activity in Group 2, none of the tested parameters showed any significant difference between DHSG-treated and control animals.

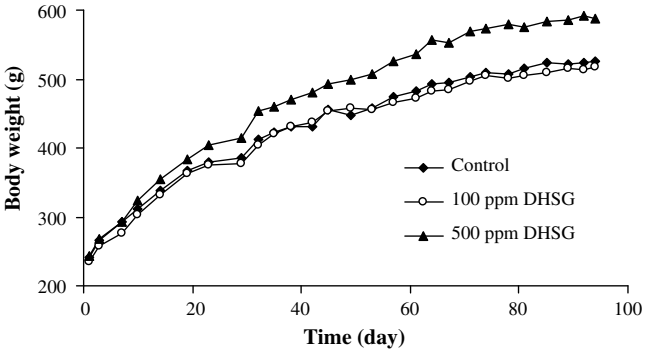

Fig. 1. Effect of dihydrosanguinarine on body weight of rats. The animals consumed *ad libitum* either standard diet (Group 1) or diets containing 100 ppm (Group 2) or 500 ppm (Group 3) DHSG, for 90 days as described in Section 2. Body weights were monitored twice a week and prior to sacrifice. *Body weight of animals in Group 3 was significantly different from that of Group 1 and 2 ($p < 0.05$).

Table 1
Rat body and organ weights on day 90

Weight (g)	Groups 1–3		
	DHSG dose level (mg/kg/day)		
	0	14	58
Body	526 ± 29	511 ± 23	553 ± 64
Liver	12.75 ± 1.58	12.17 ± 0.96	13.35 ± 1.75
Right kidney	1.63 ± 0.33	1.63 ± 0.21	1.69 ± 0.18
Left kidney	1.68 ± 0.32	1.61 ± 0.17	1.66 ± 0.16
Heart	1.38 ± 0.18	1.42 ± 0.08	1.42 ± 0.19
Thymus	0.41 ± 0.09	0.40 ± 0.16	0.44 ± 0.10
Brain	2.00 ± 0.13	1.95 ± 0.12	2.05 ± 0.10
Organ weight/body weight (%)			
Liver	2.42 ± 0.20	2.39 ± 0.26	2.41 ± 0.14
Right kidney	0.31 ± 0.06	0.32 ± 0.03	0.31 ± 0.03
Left kidney	0.32 ± 0.06	0.32 ± 0.02	0.30 ± 0.02
Heart	0.26 ± 0.02	0.28 ± 0.02	0.26 ± 0.04
Thymus	0.08 ± 0.02	0.08 ± 0.03	0.08 ± 0.01
Brain	0.38 ± 0.03	0.38 ± 0.02	0.38 ± 0.05

The animals were sacrificed on day 90 as described in Section 2. Body and organ weights were determined and are expressed as mean ± SD of six animals per group. An organ weights are normalized for body weight (lower part of Table).

Table 2
Clinical chemistry parameters in plasma

Parameter	Unit	Groups 1–3		
		DHSG dose level (mg/kg/day)		
		0	14	58
Sodium	mmol/l	146.0 ± 1.9	146.0 ± 2.6	145.2 ± 1.9
Potassium	mmol/l	3.85 ± 1.00	3.50 ± 0.10	3.97 ± 0.76
Chloride	mmol/l	95.7 ± 1.6	96.8 ± 1.0	95.2 ± 1.2
Bilirubin	μmol/l	1.57 ± 0.28	1.78 ± 0.40	1.42 ± 0.41
Cholesterol	μmol/l	1.43 ± 0.23	1.70 ± 0.41	1.38 ± 0.27
Urea	mmol/l	6.02 ± 1.66	6.60 ± 1.36	6.52 ± 1.35
Creatinine	μmol/l	54.5 ± 5.1	54.0 ± 7.3	58.5 ± 4.5
ALT	μkat/l	1.03 ± 0.11	0.76 ± 0.13*	0.90 ± 0.13
AST	μkat/l	1.50 ± 0.15	1.43 ± 0.29	1.64 ± 0.16
GMT	μkat/l	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
ALP	μkat/l	1.28 ± 0.29	1.13 ± 0.50	1.16 ± 0.15
CHE	μkat/l	5.7 ± 0.8	5.5 ± 1.9	5.5 ± 0.5
Total protein	g/l	73.0 ± 2.2	69.3 ± 5.0	69.5 ± 1.8
Prealbumin	g/l	0.062 ± 0.017	0.058 ± 0.010	0.070 ± 0.026

On day 90 the animals were sacrificed. The following parameters listed in the table were investigated.
* The value significantly different from control group ($p < 0.05$).

3.5. Hematological parameters

No effect of DHSG was found on monitored hematological parameters, i.e. hemoglobin, hematocrit, erythrocytes, mean erythrocyte volume, thrombocytes, leukocytes or differential leukocyte count (Table 3).

3.6. Oxidative stress parameters and content of cytochrome P450

We found no significant effect of DHSG on the selected parameters of oxidative stress in plasma, erythrocytes or liver. In addition, no alterations in level of total liver cytochrome P450 were found (Table 4). We also determined levels of CYP1A1/2 proteins in pooled samples of rat liver microsomes for each experimental group. We found no change in CYP1A1/2 protein levels in any of the three groups of animals, which is a positive finding from the point of view of chemically induced carcinogenesis (Fig. 2).

3.7. Genotoxicity

To examine the genotoxicity of DHSG, two different methods were used. First, the formation of single-stranded DNA breaks in peripheral lymphocytes was analyzed by Comet assay. In all three groups of animals, no significant differences were found (Table 5).

Next we examined the effect of DHSG on the formation of DNA adducts *in vivo* on samples of DNA isolated from rat livers, both those exposed to DHSG and the control group. The ^{32}P-postlabeling assays were carried out (Stiborova et al., 2002), no DNA adducts in the livers of all three animal groups were detected (data not shown).

Table 3
Hematology parameters

Parameter	Unit	Groups 1–3		
		DHSG dose level (mg/kg/day)		
		0	14	58
Hematocrit	l	0.45 ± 0.04	0.455 ± 0.02	0.458 ± 0.02
Hemoglobin	g/l	159.8 ± 10.2	159.2 ± 5.5	162.3 ± 7.6
Erythrocytes	tera/l	9.1 ± 0.87	8.9 ± 0.44	9.008 ± 0.3
Mean volume of erythrocytes	fl	49.7 ± 1.5	51.0 ± 2.2	51.0 ± 2.5
Leukocytes	giga/l	5.38 ± 1.23	5.02 ± 1.18	6.40 ± 1.63
Thrombocytes	giga/l	832.5 ± 114.1	881.3 ± 44.7	895.7 ± 103.0

On day 90 the animals were sacrificed. The parameters listed in the table were determined.

Table 4
Oxidative stress parameters in plasma, erythrocytes and liver

Parameter	Unit	Group 1–3		
		DHSG dose level (mg/kg/day)		
		0	14	58
TAC[a]	nA/g[d]	18.54 ± 3.69	19.61 ± 7.10	22.13 ± 3.8
AOPP[a]	μmol/l	35.85 ± 6.13	33.30 ± 3.44	40.37 ± 7.4
SH-groups[a]	mmol/g[d]	2.56 ± 0.46	2.74 ± 0.44	2.79 ± 0.5
TBARS[a]	nmol/g[d]	376.4 ± 33.1	397.9 ± 25.3	406.3 ± 37.4
GSH[b]	μmol/g[e]	10.06 ± 2.15	9.99 ± 1.18	10.58 ± 2.05
TBARS[b]	nmol/g[e]	379.2 ± 62.8	419.3 ± 59.7	417.0 ± 64.7
GPX[b]	μmol/min/g[e]	165.4 ± 29.9	148.1 ± 25.9	142.2 ± 29.9
Catalase[b]	μmol/min/g[e]	30.84 ± 6.96	28.64 ± 3.09	30.42 ± 4.0
SOD[b]	U/g[e]	1.17 ± 0.28	1.11 ± 0.17	0.92 ± 0.1
TAC[c]	μA/g[d]	0.135 ± 0.025	0.140 ± 0.024	0.150 ± 0.02
GSH[c]	μmol/g[d]	16.70 ± 1.50	16.50 ± 0.99	17.37 ± 2.3
TBARS[c]	nmol/g[d]	150.4 ± 10.8	158.0 ± 23.0	153.3 ± 15.8
GPX[c]	μmol/min/g[d]	263.4 ± 16.2	264.1 ± 34.6	220.8 ± 37.2
Catalase[c]	μmol/min/g[d]	29.80 ± 5.01	29.44 ± 3.9	26.79 ± 5.2
SOD[c]	U/g[d]	2.74 ± 0.57	2.54 ± 0.46	2.92 ± 0.8
Cytochrome P450[c]	μmol/g[d]	0.36 ± 0.05	0.33 ± 0.07	0.39 ± 0.09

On day 90 the animals were sacrificed. The parameters listed in the table were determined.
[a] Plasma.
[b] Erythrocytes.
[c] Liver.
[d] Values are expressed per 1 g of protein.
[e] Values are expressed per 1 g of hemoglobin.

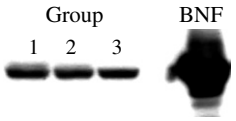

Fig. 2. Effect of dihydrosanguinarine on CYP1A1/2 protein levels. Hepatic microsomes were isolated after animal sacrifice as described in Section 2. Samples were pooled (i.e. six samples in each experimental group) and analyzed by immunoblotting. Western blot analysis of CYP1A1/2 proteins is shown.

Table 5
Effect of DHSG on DNA in peripheral lymphocytes by Comet assay

Parameter	Group 1–3		
	DHSG dose level (mg/kg/day)		
	0	14	58
Damaged lymphocytes	14.42 ± 16.30	17.58 ± 14.29	12.92 ± 5.88

At day 90 the animals were sacrificed, the peripheral lymphocytes were isolated and Comet assays were performed as described in Section 2.

3.8. Microstructure of tongue, heart, ileum, gingiva, kidney and liver

Tongue: Compared to the control, there were minor changes in Group 2, specifically a subtle increase in the number of lymphocytes in mucosal papillae and a slightly increased number of mitoses in sites without papillae. A similar effect of DHSG was observed on the tongue, i.e. a diminution of lymphocytes in the papillae and a slightly increased number of mitoses in sites without papillae in Group 3.

Heart: Heart was free of pathological findings in both DHSG-treated groups of animals. In Group 2 there was only vasodilatation of some coronary arteries. The heart muscle tissue was free of pathological findings.

Ileum: Compared to Group 1 (control), in Group 2 there were tiny foci of edema in the mucosal connective tissue closely under the epithelium in the proximal part of ileum villi. There was no evi-

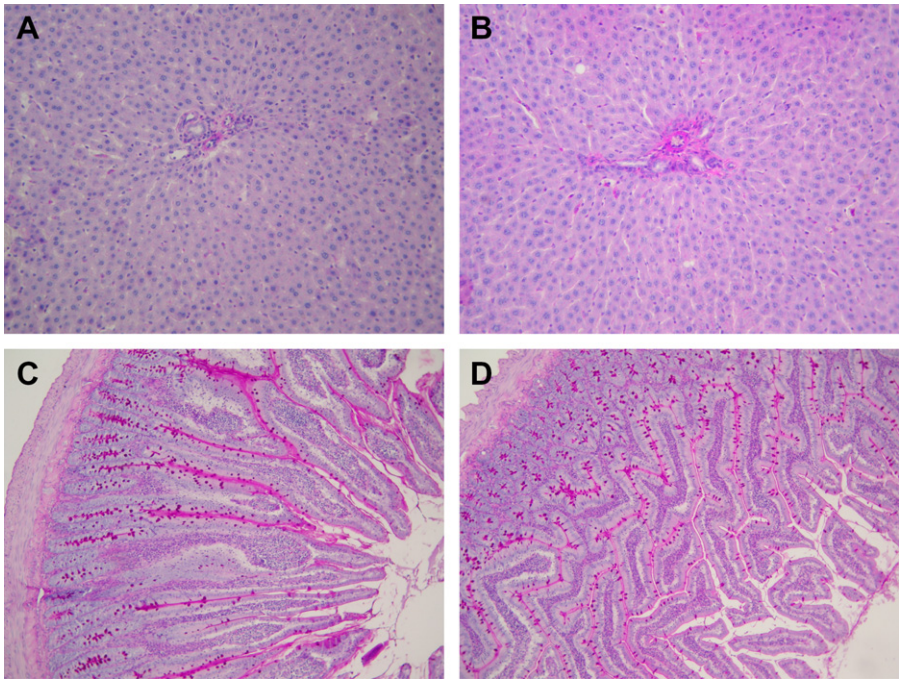

Fig. 3. Representative microphotographs of the liver and ileum of the rat from Group 1 and 3. Magnification 60×. Liver (A) and ileum (C) of the control rat, liver (B) and ileum (D) of the DHSG (500 ppm)-treated rat.

Table 6
Determination of DHSG/SG in plasma and organs

Sample	DHSG/SG (ng/g)[a]	
	Group 2	Group 3
Plasma[b]	nd/nd	0.50 ± 0.13[c]/nd
Liver	0.21 ± 0.22/0.19 ± 0.12	0.56 ± 0.33/1.34 ± 1.68
Kidney	0.32 ± 0.26/nd	1.77 ± 0.47/nd
Ileum	16.70 ± 4.80/nd	84.79 ± 36.11/nd
Muscle	0.02 ± 0.03/nd	0.08 ± 0.06/nd
Heart	0.05 ± 0.05/nd	0.31 ± 0.20/nd
Tongue	0.84 ± 0.56/nd	5.16 ± 2.46/nd
Brain	0.04 ± 0.02/nd	0.21 ± 0.08/nd

[a] Detection limit of DHSG in one gram of tissue was 178 fg, that of SG was 358 fg.
[b] ng/ml of plasma.
[c] A proportional decrease of the concentration with time of starvation during plasma collection was observed (0.71–0.38 ng/ml); nd – not determined.

dence of the infiltrate in any of the specimens. The ilea of animals in Group 3 displayed a picture similar to Group 2.

Gingiva: The gingiva of all animals in Groups 1–3 was free of pathological findings.

Kidney: In Groups 2 and 3 there were minor foci of hyperemia and capillary dilatation.

Liver: In the liver of animals of Group 3 there were foci of microvesicular steatosis in hepatocytes and very sporadic tiny foci of infiltrate formed by polymorphonuclears in the portobilliary spaces. In other specimens no pathological alterations were found.

Selected microphotographs of the liver and ileum of the rat of Groups 1 and 3 are shown in Fig. 3.

In all studied tissues (Group 2 and 3) morphological findings corresponded to those of control animals (Group 1).

3.9. Distribution of alkaloids

At the end of the 90-day experiment, in Group 2 and 3, feces, urine, plasma, liver, kidney, muscle, brain, tongue and heart were analyzed for the DHSG/SG content (Table 6). Both compounds were found in feces and liver of Groups 2 and 3. Quantities of DHSG/SG in feces were 109.0 ± 6.8/19.7 ± 5.3 μg/g (Group 2) and

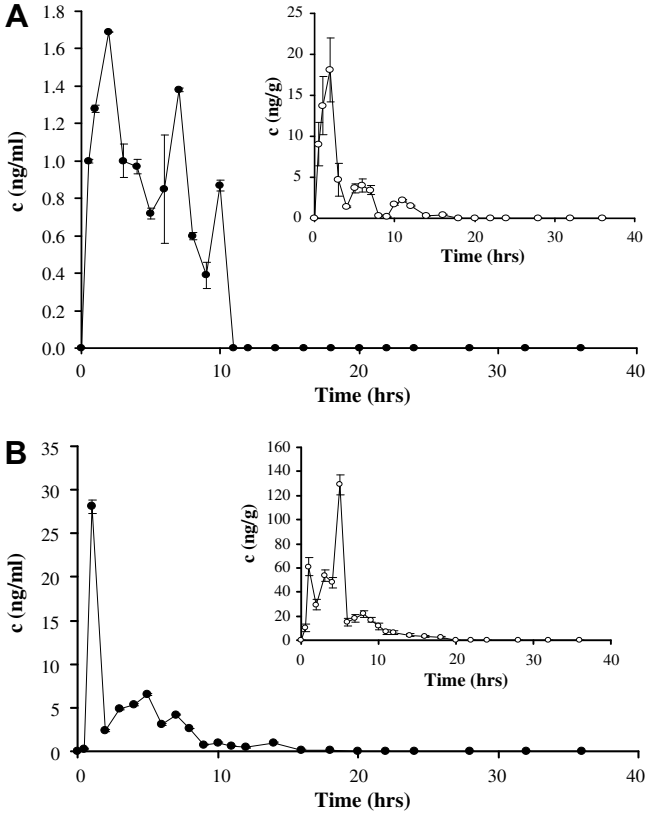

Fig. 4. Time course of dihydrosanguinarine in plasma and liver. Concentration profiles of DHSG (ng/ml) in rat blood plasma (•) and liver (○) after a single intragastric administration of DHSG (two doses, 9.1 mg/kg bw (A) or 91 mg/kg bw (B)). Experimental conditions are described in Section 2.

704.3 ± 22.5/127.4 ± 0.6 μg/g (Group 3). In all other body tissues of Groups 2 and 3 and the plasma of Group 3 DHSG only was determined. No detectable SG/DHSG quantities in urine were found, which confirmed the results of the pharmacokinetic experiment evidencing a rapid elimination of DHSG from the organism.

3.10. Determination of the pharmacokinetic parameters of dihydrosanguinarine (DHSG) in rat

In the final part of the experiment, we did a pharmacokinetic study. The time course for levels of DHSG (ng/ml) in plasma after a single intragastric administration dose of 9.1 or 91 mg/kg bw are shown in Fig. 4. DHSG was absorbed relatively rapidly. The plasma level peaked at the third sampling time (i.e. 2 h after administration for 9.1 mg/kg) and the second sampling time (i.e. 1 h after administration for 91 mg/kg). The time course for levels of DHSG in rat plasma showed for both doses multiple peaks suggesting the enterohepatic circulation of DHSG. For the lower dose, three peaks were observed at 2, 7 and 10 h after DHSG administration (Fig. 4A); with the higher dose, up to five peaks could be recognized in the corresponding curve at 1, 5, 7, 10 and 14 h (Fig. 4B). A plasma concentration–time course like this or similar is often indicative of an enterohepatic circulation showing multiple peaks and an extended half-life of the respective substance (Rang et al., 2001). In parallel, the liver DHSG content was also determined, and exhibited three to four maxima as well (see inserts in Fig. 4A and B).

A DHSG enterohepatic circulation was confirmed using rats with ligature of the biliary duct. The time course for levels of DHSG in plasma after a single intragastric administration (91 mg/kg) is shown in Fig. 5. DHSG was absorbed relatively slowly since the maximum plasma level was observed during the sixth sampling time (i.e. 5 h after administration). However, no multiple peaks were observed suggesting that ligation of the biliary duct had interrupted cycling.

The calculated values of c_{max}, t_{max}, and of the $AUC_{0\rightarrow\infty}$ for DHSG in plasma are given in Table 7. The data show that the $AUC_{0\rightarrow\infty}$ as well as the c_{max} values increase in rat plasma with the DHSG higher dose; comparison of data for lower and higher doses of DHSG indicate that the overall balance of administered substance was maintained.

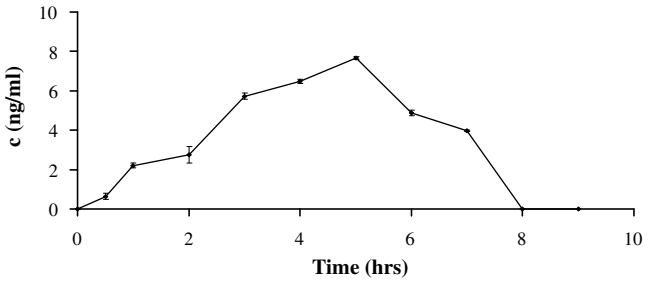

Fig. 5. Time course of dihydrosanguinarine in plasma with a ligature of the billiary duct. Concentration profile of DHSG (ng/ml) in rat blood plasma after a single intragastric administration of DHSG (91 mg/kg bw) using experimental animals with a ligature of the biliary duct. Experimental conditions are described in Section 2.

Table 7
Pharmacokinetic parameters of DHSG

Dose level (ng/kg bw)	9.1	91	91(ligature)
t_{max} (h)	2.0	1.0	5.0
c_{max} (ng/ml)	1.69	28.08	7.66
$AUC_{0\rightarrow\infty}$ (mg/ml h)	9.88 ± 0.36	51.86 ± 2.05	38.46 ± 0.48

4. Discussion

The higher dose of DHSG (58 mg/kg/day) resulted in a small (5% vs. control) increase in body weight gain after 3 months which reached statistical significance. Despite the small magnitude of this change and the small sample size ($n = 6$), the finding was considered as giving some support to the current use of QBA-containing additives as livestock feed stimulant.

DHSG in daily doses of 14 or 58 mg/kg over 3 months in rats had no significant effect on any of the parameters tested (Tables 1–5). However, long-term administration of 58 mg DHSG/kg/day (Group 3) caused a DHSG accumulation in various tissues (Table 6). Tissue DHSG concentration corresponded well with its intake from the feed (feed content 58 mg/kg/day, Group 3 vs. 14 mg/kg/day, Group 2). The tissues that contained the higher level of DHSG were those in contact with passage of the diet (tongue, ileum). A smaller quantity of SG together with DHSG was found in liver (Table 6). We currently have no plausible explanation for the SG presence in liver. SG/DHSG in ratio 1:5.7 were further found in feces. The presence of SG in feces may have been due to the metabolic activity of intestinal microflora. In contrast to feces, we found no detectable SG/DHSG quantities in urine (collected under experimental conditions used). No benz[*c*]acridine was found in any sample analyzed in this study, making questionable any explanation of DHSG toxicity (Das

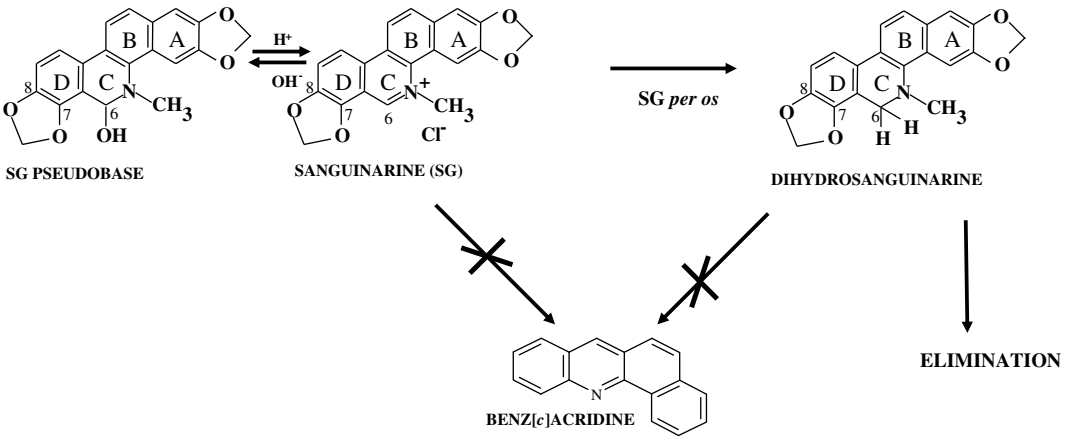

Fig. 6. *In vivo* transformations of sanguinarine.

et al., 2005) based on the assumption of benz[*c*]acridine formation. No histopathological changes in any of organs examined (Fig. 3) were observed.

In contrast to sanguinarine (SG, a precursor of DHSG, Fig. 6), the less polar dihydrosanguinarine undergoes enterohepatic cycling with maximum concentration in plasma in the first or second hour after application. Maximum level of DHSG (as well as SG) is reached by 2 h after administration and the compound is eliminated relatively quickly. This is in accord with Vecera et al. (2007) who found that DHSG plasma levels dropped to zero after 18 h, the more polar SG being cleared more quickly, i.e. in less than 10 h.

There are isolated reports in the literature that the biological activity of SG might be associated with the aryl hydrocarbon receptor (AhR) – cytochrome P450 CYP1A1/2 signalling pathway (Karp et al., 2005; Dvorak et al., 2005). Since AhR-CYP1A1/2 is involved in the process of chemically induced carcinogenesis, it is of major importance to determine whether SG/DHSG interferes with this pathway. We have reported in previous studies that SG does not activate the AhR-CYP1A1/2 pathway in human hepatoma cells HepG2 (Zdarilova et al., 2006) or rat hepatoma cells (Dvorak et al., 2006b). On the other hand, we have shown that activation of the AhR-CYP1A1/2 pathway by dioxin modulates SG toxicity in rat hepatocytes (Dvorak et al., 2006c; Dvorak and Simanek, 2007). Assuming the conversion of SG to DHSG in living systems (Vecera et al., 2007), it is unclear whether the biological effects attributed in the literature to SG are not actually results of a joint effect of SG and DHSG. In reality, there is no information about the effects of DHSG on the AhR-CYP1A1/2 signalling or *vice versa*. Hence an important finding of the present paper is the demonstration that DHSG had no effects on AhR-dependent CYP1A1/2 protein expression in rat liver after a 90-day use of DHSG (Fig. 2). In addition, DHSG displayed no genotoxicity in lymphocytes or liver of the rats receiving the higher dose of DHSG (Group 3) used in this 90-day study. This result is the same as for sanguiritrin (mixture of SG and chelerythrine) (Psotova et al., 2006b).

In a previous study *in vitro*, we found the formation of DNA adducts with SG in rat liver microsomes isolated from hepatocytes treated with AhR activators (Stiborova et al., 2002). Here we show, that DHSG does not produce genotoxic species in rat hepatocytes or lymphocytes.

In conclusion, dihydrosanguinarine is well tolerated in the rat, with no evidence for systemic toxicity at doses up to 58 mg/kg/day administrated orally for 3 months, its pharmacokinetics shows the relatively fast elimination of DHSG in the first 10 h most of the compound is eliminated and DHSG is not converted to SG and/or benz[*c*]acridine. Thus, the conversion of SG to DHSG recently described by Psotova et al. (2006a), represents the elimination pathway for SG in rat, as shown in the Fig. 6.

Conflict of interest statement

The authors declare that there are no conflict of interest.

Acknowledgements

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