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Short communication

HPLC quantification of seven quaternary benzo[*c*]phenanthridine alkaloids in six species of the family *Papaveraceae*

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Abstract

The content of the seven quaternary benzo[*c*]phenanthridine alkaloids (QBA) sanguinarine (SA), chelerythrine (CHE), chelirubine (CHR), chelilutine (CHL), sanguilutine (SL), sanguirubine (SR) and macarpine (MA) was determined in the underground part of six plant species of the family *Papaveraceae* (*Sanguinaria canadensis* L., *Dicranostigma lactucoides* HOOK.f.et THOMS, *Chelidonium majus* L., *Macleaya cordata* (Willd.), *Macleaya microcarpa* (Maxim) and *Stylophorum lasiocarpum* (Oliv.)). HPLC method with reversed phase column Synergi^{TM} Max-RP C-12 Phenomenex was used, mobile phase consisted of heptanesulfonic acid (0.01 mol/l) with triethanolamine (0.1 mol/l) in redistilled water, pH 2.5, acetonitrile gradient 25–60% during 25 min. Detection was performed at 280 nm. The highest content of SA and CHE was found in the roots of *D. lactucoides* (1.99%, resp. 3.43% of the dried roots). In rhizomes of *S. canadensis* was their content more then two times lower.

Keywords: Benzo[*c*]phenanthridine; Chelilutine; Chelirubine; Macarpine; Sanguirubine; Sanguilutine; Sanguinarine; Chelerythrine; RP-HPLC

1. Introduction

Quaternary benzo[*c*]phenanthridine alkaloids (QBA) occur mainly in the plants of families *Papaveraceae* and *Fumariaceae*. The main representatives are 2,3,7,8-tetrasubstituted alkaloids sanguinarine (SA) and chelerythrine (CHE). Their sources are especially species of the tribe *Chelidonieae*, e.g. *Sanguinaria canadensis* L., *Dicranostigma lactucoides* HOOK.f.et THOMS, *Chelidonium majus* L., *Macleaya cordata* (Willd.), *Macleaya microcarpa* (Maxim) and *Stylophorum lasiocarpum* (Oliv.) [1]. Biological activities of QBA have been summarized in many papers [2]. They display very wide antimicrobial, antifungal and antiinflammatory activity; they inhibit many enzymes, e.g. CHE is a known as a specific proteinkinase C inhibitor [3]. They also inhibit microtubule assembly and interact with DNA [4–7]. Many papers deal with cytotoxicity and induction of apoptosis by the both alkaloids [8–12]. SA and CHE are in plants very often accompanied by minor benzo[*c*]phenanthridine alkaloids chelirubine (CHR), chelilutine (CHL), sanguilutine (SL), sanguirubine (SR), and, in several plants, by macarpine (MA). The current knowledge on the distribution of these minor alkaloids in plants was closely summarized by Dostal and Slavik [1]. However, their quantity in plants has never been exactly determined, even though some plant extracts containing minor QBA are used commercially. The data on biological activity of minor QBA are rare however some remarkable effects have been described [12–14]. Therefore, an isolation of these alkaloids from plant sources based on the knowledge of their occurrence in plants might be in center of interest of some research groups in future. A number of studies have been published dealing with the determination of QBA using HPLC [15]. However, most of them have been focused solely on determination of SA and CHE. Until now, no literature has been reported on complete determination of minor QBA in the species used as sources of CHE and SA. The aim of this study was to introduce a method allowing to quantify SA and CHE together with the five minor QBA, to analyze the six plant species known by high content of SA and CHE and to select the best sources for isolation of the main and minor QBA. Structures of the alkaloids are given in Fig. 1.

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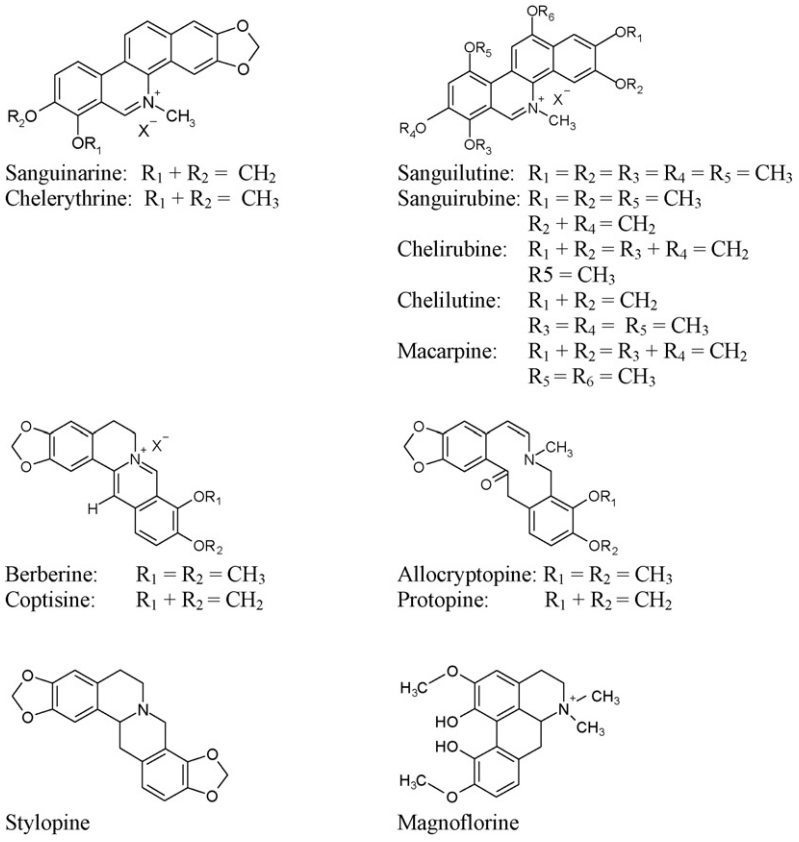

Fig. 1. Structures of alkaloids determined in extracts of six species.

2. Experimental

2.1. Plant material

The plants were cultivated in the Center of Medical Plants Faculty of Medicine, Masaryk University in Brno (Czech Republic) from the seeds obtained from various botanical gardens. Voucher specimens are deposited in the Department of Biochemistry Faculty of Medicine.

S. canadensis, *Ch. majus*, *M. cordata*, *M. microcarpa* and *Stylophorum lasiocarpum* (*Papaveraceae*) are perennial herbs. Their underground parts originated from the September 2004 harvest. *D. lactucoides* (*Papaveraceae*) is biennial plant. As in our preliminary experiments was estimated that the content of QBA is highest in roots harvested in spring of the second year of cultivation, the roots harvested in May 2005 were used.

2.2. Extract preparation

Five grams of the roots were macerated with 200 ml of methanol for 3–4 days. Subsequently methanol was poured off and new portion was added. This process was repeated for 4 weeks nine times. After the last extraction there was no residue after the evaporation of solvent and no peaks were detected by HPLC in the extract. Individual portions of the extracts were combined and the solvent was partially evaporated to the total volume 200 ml. Samples for HPLC analyses were taken from these modified extracts.

2.3. Instrumentation

The HPLC apparatus consisted of high pressure LCP 4100.2 pump with gradient programmer GP 3, LCD 2040 ultraviolet detector (ECOM, Czech Republic), Rheodyne 7125 syringe loading sample injector (Cotati, CA USA) and program for analysis Clarity^{TM} C 22 (Data Apex, Czech Republic). The identity of peaks was confirmed on HPLC system Shimadzu LC-10AVP with the SPD-M10AVP diode array detector. DAD detection was employed at the wavelength of 180–550 nm. Three types of column were tested: a Phenomenex C-12 column Synergi^{TM} Max-RP 80A (4 μ, 150 mm × 4.60 mm ID), a Phenomenex C-18 column Synergi^{TM} Fusion - RP 80A (4 μ, 150 mm × 4.60 mm ID) (Phenomenex, USA) and a Supelco C-18 column Supelcosil^{TM} LC-18 (5 μ, 150 mm × 3 mm ID) (Supelco, USA).

2.4. Reagents and chemicals

Phosphoric acid, heptanesulfonic acid were obtained from Sigma Chemical Co. (USA). Acetonitrile of HPLC grade and triethylamine were purchased from Merck (Germany). Deionized water was purified by Ultrapure water (Premier MFG Systems, Phoenix, AZ, USA). Standards of all alkaloids originated from isolations during the systematic studies of plants of families *Papaveraceae* and *Fumariaceae* in the laboratory of authors. Their identity was verified by EI-MS, ^{1}H NMR and ^{13}C NMR, their purity was not less 98% according to HPLC.

2.5. HPLC analysis

Standard solutions of alkaloids containing 10–50 mg/l of each alkaloid were prepared by dissolving of alkaloids in phosphate buffer (pH 2.5; 0.01 M). The solutions were filtered through 0.45 μm membrane filter (Millipore) and were stored at 4 °C in dark. Mobile phase was prepared from stock solution containing heptanesulfonic acid (0.01 M) and triethylamine (0.1 M) in redistilled water, pH 2.5 (H_3PO_4). A-solvent contained 25% acetonitrile and B-solvent 60% acetonitrile (v/v). Elution profile: 0–1 min 20% B in A; 1–10 min 50% B in A; 10–20 min 100% B in A; 20–25 min isocratically 100% B. Flow rate was 0.5 ml/min, the injection volume was 50 μl and detection was carried at λ = 280 nm. Extracts of roots were diluted 1:9 (v/v) by mobile phase before the injection. The peaks of alkaloids were identified on the base of co-chromatography with alkaloid standards and by comparison of their UV spectra measured by DAD with the reference values. The limit of detection was determined when the ratio of the testing peak signal-to-noise was greater than 3.

2.6. Validation parameters

Calibration curves based on six concentrations with a range 10–50 mg/l were obtained by plotting the peak area of the given alkaloid versus the concentration. Three determinations were carried out for each solution. The statistical parameters were calculated by regression analysis. Precision was evaluated by HPLC analysis with the standard solution five times in 1 day for intra-day variation and twice a day in three consecutive days for inter-day variation. The values were expressed as relative standard deviation (R.S.D. %) and were determined to be in range 0.27–2.18 and 0.37–2.40%, respectively.

3. Results and discussion

3.1. Optimization of separation

The method used in our previous work for separation of alkaloids in extracts of *Ch. majus* was used in the beginning [16]. As the peaks of some alkaloids remained unresolved (e.g. sanguirubine and chelerythrine) and the time of separation was longer than 30 min, the gradient of acetonitrile was modified as described in experimental part. Simultaneously, two reversed phase columns C-18 and one C-12 column were tested for separation. The optimum separation for the seven benzophenanthridine alkaloids was obtained with Synergi™ Max-RP 80A C-12 (4 μm) column. The sharp peaks, less tailing, the shortest retention times and the best resolution were obtained using this column. The complete separation was achieved during 25 min. It has been known that ionic interactions with free silanol groups on the silica surface can contribute to peak tailing during a separation on C-18 phase. This effect is particularly pronounced for basic compounds that can bind to un-protonated free silanol groups on the silica surface. Synergi™ Max-RP bonded phase provides better surface coverage reducing silanol activity and this is probably the main reason of improved separation of alkaloid mixture. The retention times of individual QBA obtained using the Synergi™ Max-RP column are given in Table 1.

Table 1
Retention times, linearity parameters and limits of detection of seven QBA and six other alkaloids

Alkaloid	t_R (min)	Calibration curve	r^2	LOD (ng ml^{-1})
CHE	17.23	$Y = 2085.22X$	0.9998	23.00
CHL	19.43	$Y = 2227.88X$	0.9999	10.00
CHR	16.94	$Y = 470.389X$	0.9995	64.00
MA	20.64	$Y = 776.92X$	0.9993	33.00
SL	18.88	$Y = 1854.82X$	0.9998	9.40
SA	14.82	$Y = 1901.09X$	0.9998	1.70
SR	18.04	$Y = 337.39X$	0.9993	68.00
ALL	13.98	$Y = 193.82X$	0.9998	20.20
BER	16.50	$Y = 1324.83X$	0.9999	17.00
COP	14.35	$Y = 1129.11X$	0.9997	34.00
MAG	8.39	$Y = 398.31X$	0.9995	9.00
PRO	13.59	$Y = 408.36X$	0.9995	6.00
STL	15.72	$Y = 341.44X$	0.9997	12.00

X, concentrations; Y, peak areas; t_R, retention time; r^2, correlation coefficient; LOD, limit of detection; CHE, chelerythrine; CHL, chelilutine; CHR, chelirubine; MA, macarpine; SL, sanguilutine; SA, sanguinarine; SR, sanguirubine; ALL, allocryptopine; BER, berberine; COP, coptisine; MAG, magnoflorine; PRO, protopine; STL, stylopine.

3.2. The validation data and quantification of QBA alkaloids in the plants extracts

Table 1 shows the equation of the regression line, correlation coefficient (r^2), retention time and limit of detection. All calibration curves exhibited good linear regression, limits of detection were in the range 1.70–34.00 ng.

The representative chromatograms of alkaloids in extracts from the roots of the six plants are shown in Fig. 2. The content of alkaloids in the individual extracts was calculated from the calibration curves and expressed as the mass percentage of the dry root. The main QBA alkaloids sanguinarine and chelerythrine were present in all plants analyzed (Table 2). The highest content of both the alkaloids (1.99% SA and 3.43% CHE) was found in the roots of *D. lactucoides*. This biennial herb is native to the subtropical region of the eastern Himalayas, but according to our long time experience can be cultivated also under climatic conditions of central Europe. The results are in accordance with those obtained on the base of isolation procedures [17]. The content of SA and CHE in rhizomes of *S. canadensis* was more then two times lower than in *D. lactucoides*. The literature data have mentioned 3–7% QBA in the dry rhizomes of this plant native in North America [1]. Possibly, the conditions of cultivation might cause the lower production of alkaloids in our case. The main QBA in roots of *D. lactucoides* was in accordance with the literature data CHE [17], while in rhizomes of *S. canadensis* was prevailing SA. Both alkaloids were the main alkaloids of these two plants at the same time.

The highest content of minor QBA (Table 2) was found in *S. canadensis* with exception of macarpine that was not detected

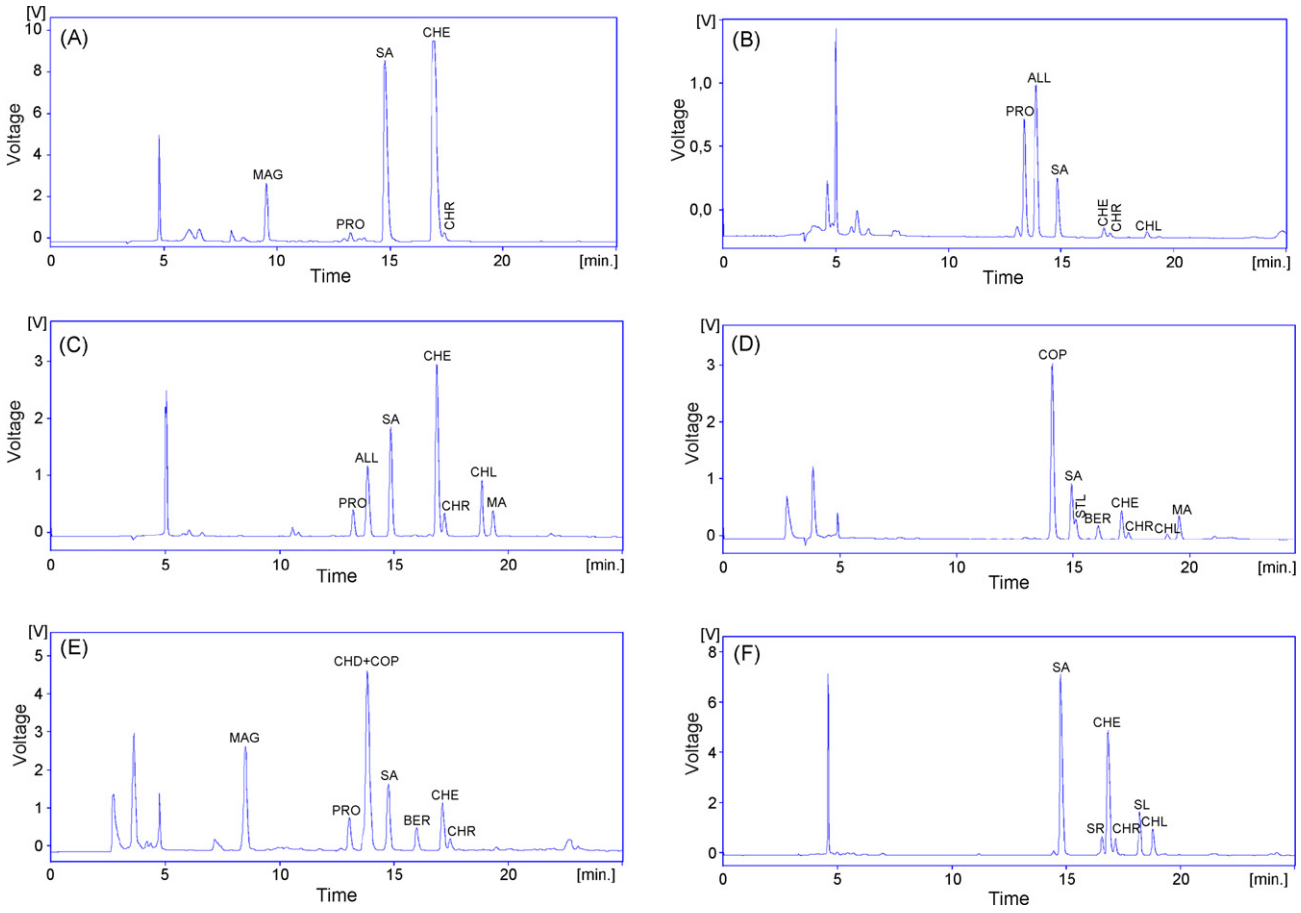

Fig. 2. Representative HPLC chromatograms of extracts from the roots of (A) *D. lactucoides*, (B) *M. cordata*, (C) *M. microcarpa*, (D) *Stylophorum lasiocarpum*, (E) *Ch. majus*, and (F) *S. canadensis*. SA, sanguinarine; CHE, chelerythrine; CHR, chelirubine; CHL, chelilutine; MA, macarpine; SR, sanguirubine; SL, sanguilutine; ALL, allocryptopine; BER, berberine; COP, coptisine; MAG, magnoflorine; PRO, protopine; STL, stylopine; CHD, chelidonine. Conditions: analytical column, Synergi^{TM} Max-RP 80A (4 μ, 150 mm × 4.60 mm ID); mobile phase, heptanesulfonic acid (0.01 M) and triethylamine (0.1 M) in redistilled water, pH 2.5 (H_3PO_4); the gradient was 25–60% acetonitrile during 25 min; flow rate, 0.5 ml/min and injection volume, 50 μl; concentration of samples, 0.5 g/200 ml.

in this plant. Sanguirubine and sanguilutine were detected only in this plant. Contrary, macarpine was found only in roots of *M. microcarpa* and *Stylophorum lasiocarpum* in relatively high yield about 0.2%. Even though our work was focused on the analysis of QBA in the selected plants, the other main alkaloids in the extracts were identified on the base of co-chromatography with the standards and comparison of their UV-spectra obtained by DAD. Their quantity was determined from calibration graphs.

Table 2
Content of alkaloids in extracts from roots of the six species expressed as the mass percentage of the dry root

Alkaloid	*M. cordata* (%)	*Stylophorum lasiocarpum* (%)	*M. microcarpa* (%)	*Ch. majus* (%)	*S. canadensis* (%)	*D. lactucoides* (%)
SA	0.134 ± 0.008	0.162 ± 0.005	0.462 ± 0.005	0.624 ± 0.052	1.329 ± 0.065	1.992 ± 0.048
CHE	0.031 ± 0.001	0.080 ± 0.003	0.734 ± 0.004	0.412 ± 0.002	0.804 ± 0.037	3.433 ± 0.035
CHR	0.019 ± 0.002	0.079 ± 0.013	0.228 ± 0.005	0.422 ± 0.035	0.350 ± 0.025	0.309 ± 0.006
CHL	0.008 ± 0.001	0.013 ± 0.001	0.137 ± 0.003	–	0.142 ± 0.010	–
MA	–	0.174 ± 0.007	0.198 ± 0.020	–	–	–
SR	–	–	–	–	0.594 ± 0.044	–
SL	–	–	–	–	0.300 ± 0.030	–
ALL	2.980 ± 0.248	–	2.555 ± 0.133	–	–	–
BER	–	0.060 ± 0.001	–	0.302 ± 0.020	–	–
COP	–	0.945 ± 0.039	–	–	–	–
MAG	–	–	–	5.237 ± 0.005	–	1.959 ± 0.007
PRO	0.807 ± 0.047	–	0.366 ± 0.013	1.421 ± 0.031	–	0.289 ± 0.013
STL	–	0.320 ± 0.005	–	–	–	–

CHE, chelerythrine; CHL, chelilutine; CHR, chelirubine; MA, macarpine; SL, sanguilutine; SA, sanguinarine; SR, sanguirubine; ALL, allocryptopine; BER, berberine; COP, coptisine; MAG, magnoflorine; PRO, protopine; STL, stylopine.

Magnoflorine (MAG), protopine (PRO), coptisine (COP), chelidonine (CHD) and berberine (BER) were detected in *Ch. majus*, (COP), (STL) and (BER) in *Stylophorum lasiocarpum*, (PRO) and allocryptopine (ALL) in *M. cordata* and *M. microcarpa*, (PRO), (ALL) and (MAG) in *D. lactucoides*. Only benzophenanthridine alkaloids were detected in *S. canadensis.* As *S. canadensis* and both species of the genus *Macleaya* are popular and traditional medicinal herbs, they are commonly used as sources SA and CHE. However, our result implicates that roots of the species *D. lactucoides* are at least equally effective source of the main QBA, namely of chelerythrine. On the other hand it is evident that the best source of four minor alkaloids with exception of macarpine remains *S. canadensis*. The only two species, *Stylophorum lasiocarpum* and *M. microcarpa*, are potential sources of macarpine. The presence of other (non benzophenanthridine alkaloids) in plants will also affect their utility to serve as a source for separation of any of these seven QBA. From this point of view *S. canadensis* is the best source with exception of macarpine. On the other hand, it should be strongly recalled, that commercial preparations based on the crude extracts contain not negligible amount of minor QBA in addition to SA and CHE and also other alkaloids, whose biological effects are not known in details.

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