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Solid-phase extraction and fluorimetric detection of benzophenanthridine alkaloids from *Sanguinaria canadensis* cell cultures

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ABSTRACT

A solid-phase extraction (SPE) method using Sep-Pak C_{18} reversed-phase cartridges was developed for the prepurification of benzophenanthridine alkaloids produced by *Sanguinaria canadensis* cell cultures. Efficient recoveries (>90%) of chelirubine, sanguinarine and chelerythrine were obtained from synthetic solutions using the SPE method. Up to 20 mg of alkaloids were extracted from spiked biomass and culture-medium samples on a 400-mg adsorbent cartridge. This extraction method was more reproducible and as selective as a conventional liquid–liquid extraction technique. Fluorimetric detection was used for the identification and quantification of benzophenanthridine alkaloids after high-performance liquid chromatographic separation. This detection method was compared to conventional UV absorption at 280 nm. The detection limits of both detectors were in the 100-pg range for chelirubine and sanguinarine and in the 1-ng range for chelerythrine. The main advantage of fluorimetry over UV spectrometry was its improved selectivity for the benzophenanthridine alkaloids.

INTRODUCTION

The *Sanguinaria canadensis* L. plant accumulates several benzophenanthridine alkaloids, mainly in the rhizome. The major alkaloids identified are sanguinarine, chelirubine, chelerythrine, sanguilutine, sanguirubine and chelilutine [1]. Sanguinarine is of particular interest as the active compound of some commercial toothpaste and oral rinse products exhibiting antiplaque properties [2,3].

The *S. canadensis* plant is one of the commercial sources of sanguinarine [3]. Plant cell cultures represent an alternative to agricultural supply of this product. Screening for productive *S. canadensis* cell lines required a rapid, simple, efficient and sensitive method for alkaloid quantification. Conventional procedures involve alkaloid extraction from culture samples into organic or aqueous acidic phases followed by several partitioning steps between different solvents to eliminate pigments, steroids, lipids and phenolic residues [4–6]. The disadvantages of these liquid–liquid extraction (LLE) techniques include time, limited reproducibility for small sample sizes and use of large volumes of organic solvents. Moreover, they involve steps such as evaporation and extreme pH conditions under which degradation and artefact formation may occur [7]. Some of these problems can be overcome by using

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solid-phase extraction (SPE) techniques [8,9]. The isolation of secondary metabolites using SPE methods has been reported for some plant products such as phenolics from *Indigofera heterantha* plant [10], phytoalexins from *Brassica* spp. plant [11] and indole alkaloids from *Catharanthus roseus* cells [6].

The SPE method developed for indole alkaloids [6] was adapted for the extraction of benzophenanthridine alkaloids obtained from *S. canadensis* cell cultures, evaluated on the basis of efficiency and selectivity and compared with a widely used LLE technique.

Routine high-performance liquid chromatographic (HPLC) analysis for the identification and quantification of plant secondary metabolites usually involves UV absorption [5,6,10–12]. Since fluoromicroscopy had already been used to identify some benzophenanthridine alkaloids in different cell cultures [13,14], the fluorescence property of these alkaloids was exploited for their detection after HPLC separation and compared to conventional UV absorption for selectivity and sensitivity.

EXPERIMENTAL

Chemicals

Chloride salts of chelirubine, sanguirubine, sanguilutine, chelerythrine and chelilutine were kindly provided by Vipont (Fort Collins, CO, U.S.A.). Sanguinarine chloride was purchased from Sigma (St. Louis, MO, U.S.A.). All solvents were purchased from Cadelon (Georgetown, Canada) and were HPLC grade except for diethyl ether which was analytical grade (BDH, Toronto, Canada). Other chemicals (*i.e.*, triethylamine, phosphoric acid and *n*-heptanesulfonic acid) were HPLC grade and were purchased from Fisher (Fair Lawn, NJ, U.S.A.). *S. canadensis* cells and culture media were collected from different suspension cultures and used as experimental alkaloid sources.

Sample preparation

Plant cells were separated from the culture medium by vacuum filtration using a qualitative filter paper (Whatman No. 1) and rinsed with water. Cells (*ca.* 5 g fresh weight) were disrupted at room temperature for 30 s into 20 ml acidic methanol [0.5% (v/v) HCl] using a Polytron homogenizer (Brinkmann, Westbury, NY, U.S.A.). Homogenates were centrifuged at 12 000 *g* for 15 min. Cell debris was re-extracted with 20 ml acidic methanol. The homogenates were centrifuged and the two methanolic fractions were combined. Samples (40 ml) of spent culture medium were centrifuged at 12 000 *g* for 15 min or filtered (0.45 μm) in order to remove colloidal matter which may hamper elution on the SPE cartridge.

Synthetic alkaloid samples (controls) were made of known quantities of chelerythrine, sanguinarine or chelirubine (chloride salts) dissolved in acidic methanol or in fresh sterile culture medium to simulate biomass crude extracts and culture-medium samples respectively.

Biomass crude extract and culture-medium samples to be extracted by SPE were diluted with *n*-heptanesulfonic acid aqueous solution (HS) by adding 30 ml HS (0.05 *M*) to 10 ml of biomass crude extract or by adding 10 ml methanol and 1.5 ml HS (1.0 *M*) to 30 ml culture medium. Controls were treated similarly.

Extraction methods

Solid-phase extraction. The SPE method takes advantage of the chemical structure of the benzophenanthridine alkaloids present in the plant (Fig. 1). These secondary metabolites have a quaternary ammonium function and are positively charged in acidic solutions. The SPE technique reported by Morris *et al.* [6] for indole alkaloids was adapted for benzophenanthridine alkaloids. A hydrophobic anion (*n*-heptanesulfonic acid) was used to form an ion-pair with the positive charge of the alkaloids so that they could be extracted on a reversed-phase C_{18} cartridge. The adsorbent was prepared by passing through the C_{18} cartridge (400 mg Sep-Pak cartridge, Millipore, Bedford, MA, U.S.A.) 5 ml of acetonitrile, 5 ml of 5% HS (0.05 *M*) in methanol and 5 ml of 100% HS (0.05 *M*). The adsorbent was not allowed to dry in between each solvent addition. The biomass crude extract or culture-medium solution, prepared as described above, was loaded onto the cartridge and allowed to flow through dropwise. The light-brown effluent was discarded. Subsequently, the cartridge was washed with 10 ml of a 25% HS (0.05 *M*) solution in methanol and was air dried. The alkaloids were recovered in a 2-ml fraction of 5% HS (0.05 *M*) in methanol.

Liquid liquid extraction. The LLE procedure used [5] was performed as follows. Samples (30 ml) of culture medium were adjusted to pH 9–10 with sodium hydroxide in order to allow pseudo-base formation of the quaternary ammonium alkaloid [15] which could then be extracted with ethyl acetate (3 × 30 ml). The organic extracts were combined and evaporated to dryness under nitrogen at 35°C (Reacti-therm, Pierce, Rockford, IL, U.S.A.). The residue was dissolved in 2 ml acidic methanol. Biomass crude extracts (10 ml) were evaporated to dryness. The residue was dissolved in 25 ml 1 *M* HCl and washed two times with 25 ml ethyl acetate followed by 25 ml diethyl ether. The aqueous phase was collected and extracted as for the culture medium.

Chromatographic systems

Alkaloid concentrations were measured using a HPLC system (Waters Assoc., Millford, MA, U.S.A.; Ultra Wisp automatic injector Model 715, pump Model 590) equipped with a UV-absorbance detector Model 481 set at 280 nm and a fluorimetric detector Model 420 with 338 nm excitation and a 425-nm long pass emission filters. Separation of alkaloids was performed using a guard column (15 mm × 3.2 mm I.D., 7 μm) and two Spheri-5 cyano columns (100 mm × 4.6 mm I.D., 5 μm) in series from Brownlee Labs. (Santa Clara, CA, U.S.A.). The mobile phase was a mixture (86:14, v/v) of methanol and of an aqueous solution containing triethylamine (5 m*M*) and

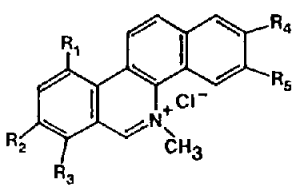

Fig. 1. Chemical structure of benzophenanthridine alkaloids. Chelirubine: $R_1 = OCH_3$; $R_2 + R_3$, $R_4 + R_5 = OCH_2O$. Sanguinarine: $R_1 = H$; $R_2 + R_3 = R_4 + R_5 = OCH_2O$. Sanguirubine: R_1, R_4, $R_5 = OCH_3$; $R_2 + R_3 = OCH_2O$. Chelilutine: R_1, R_2, $R_3 = OCH_3$; $R_4 + R_5 = OCH_2O$. Chelerythrine: $R_1 = H$; R_2, $R_3 = OCH_3$; $R_4 + R_5 = OCH_2O$. Sanguilutine: R_1, R_2, R_3, R_4, $R_5 = OCH_3$.

phosphoric acid (5 m*M*) adjusted to pH 5.6 with sodium hydroxide. The flow-rate and temperature were maintained at 1 ml min^{-1} and 40°C. All eluents and samples were filtered (0.45 μm) prior to injection.

The stability of the alkaloids in aqueous solution is improved under acidic conditions [16]. Prior to injection in the HPLC system, samples (10 μl) were acidified with 2 μl acidic methanol [33% (v/v) phosphoric acid in methanol]. The retention times of chelirubine, sanguinarine, sanguirubine, chelilutine, chelerythrine and sanguilutine standards were 12.0, 17.3, 17.8, 25.6, 31.0 and 31.5 min, respectively. Sanguinarine and sanguirubine as well as sanguilutine and chelerythrine were found to elute at similar retention times of *ca.* 17.5 min and *ca.* 31.2 min, respectively, when injected together. Their presence in the culture samples was confirmed by comparison with alkaloid standards using thin-layer chromatography (Whatman LK6DF silica plate) and a solvent system chloroform–ethyl acetate (70:30, v/v). All compounds were quantified by HPLC from their peak area using UV or fluorescence detection and a Spectra-Physics SP 4270 integrator.

RESULTS

Solid-phase extraction

The efficacy of the adsorption and desorption processes involved in the SPE method was verified using synthetic solutions of known alkaloid contents which simulated biomass crude extract and culture-medium samples. Results reported in Table I show that sanguinarine, chelerythrine and chelirubine (30–600 μg) were recovered from these synthetic solutions with a yield higher than 95%, 95% and 90%, respectively. The low quantity of chelirubine available prevented performance of a complete series of extractions. No difference in recovery was found between acidic methanol and culture-medium solutions, indicating that the adsorption and desorption of benzophenanthridine alkaloids onto the C_{18} cartridge were efficient even in the presence of fresh culture-medium components (salts, carbohydrates, hormones, vitamines).

Other extraction experiments were conducted on actual biomass crude extracts and culture-medium samples harvested at different culture times and spiked with

TABLE I

RECOVERY OF BENZOPHENANTHRIDINE ALKALOIDS FROM SYNTHETIC SOLUTIONS

Results are means ± standard deviation from two independent extractions.

	Recovery (%)						
	Acidic methanol loaded (μg)				Culture medium loaded (μg)		
	100	200	400	600	30	60	100
Sanguinarine	95 ± 5	100 ± 2	100 ± 2	99 ± 2	100 ± 2	101 ± 2	99 ± 3
Chelerythrine	100 ± 3	99 ± 2	100 ± 3	100 ± 2	101 ± 2	100 ± 3	95 ± 3
Chelirubine	90 ± 3	–	–		–	–	91 ± 3

TABLE II

RECOVERY OF SANGUINARINE ADDED TO CRUDE BIOMASS EXTRACTS PRIOR TO SPE

Results are means ± standard deviation from two independent extractions.

Initial (μg)	Added (μg)	Recovered (μg)	Recovery (%)
800	0	780 ± 30	98 ± 4
800	2400	3200 ± 100	101 ± 3
800	6200	6580 ± 70	94 ± 1
800	19 200	18 200 ± 900	91 ± 5

sanguinarine. Results presented in Table II show that the recoveries from spiked biomass extracts were higher than 91%. Therefore, it appears that the extraction of alkaloids on the reversed-phase cartridge was not affected by other extracted biomass components. This was observed even when the amount of sanguinarine extracted was as high as 20 mg. Similar results were obtained for SPE of spent culture-medium samples.

Comparison of SPE and LLE

The selectivity and efficiency of the SPE, LLE and crude extraction methods were compared on the basis of their capabilities to extract alkaloids from actual biomass and culture-medium samples. The selectivity was evaluated by comparing HPLC chromatograms obtained for samples treated according to these three methods. As shown in Fig. 2, unknown compounds from crude biomass extracts which coeluted with sanguinarine and chelerythrine were removed by SPE and LLE treatments of the samples. The compound(s) which coeluted with sanguinarine in crude extracts was (were) difficult to distinguish using UV absorption due to overlap but could be confirmed using fluorescence detection. For SPE- and LLE-treated samples, the same chromatographic patterns were observed for both biomass and culture-medium extracts. Among the alkaloids of interest, chelirubine, sanguinarine and chelerythrine were identified in these prepurified culture samples. Chelirubine, which was not detected in crude extracts, was detected in SPE- and LLE-treated samples because the extraction process allowed preconcentration of the alkaloids.

The efficiency of the SPE and LLE methods was evaluated by measuring the quantity of known alkaloids found in biomass and medium samples extracted by both methods. Quantities of chelirubine, sanguinarine and chelerythrine (70–170 μg) recovered from crude biomass extracts and culture-medium samples were in the same range using the two extraction techniques (Table III). The reproducibility of extraction was, however, significantly improved using SPE as indicated by their lower standard deviations (1–5% of the average quantities extracted and measured) compared to those of LLE (6–20%). The small volume treated (10 ml), the numerous steps and extreme pH involved in LLE of crude biomass samples may explain its poor reproducibility. For culture-medium samples, where the extraction procedure is simpler than for biomass extracts, the reproducibility of the LLE was improved (standard deviations of 2–11% of the average quantities extracted and measured) but was still worse than SPE (1–6%).

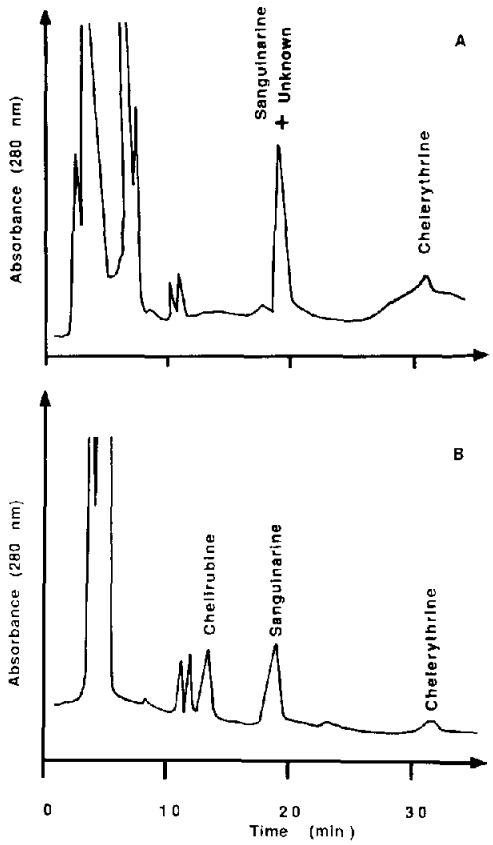

Fig. 2. Typical HPLC chromatograms of a biomass extract using UV detection. (A) Crude extract; (B) SPE extract (same pattern was obtained for LLE extract).

Detection methods

The alkaloids of interest were found to fluoresce at the fixed excitation (338 nm) and emission (425-nm long pass filter) wavelengths of the detector used. The sensitivity of this detector was compared to that of a UV detector set at 280 nm since all these compounds show maximum UV absorption near this wavelength [17]. The detection limits (lowest detectable quantity of product equal to twice the noise level) of the UV and fluorescence detectors were in the same range for either sanguinarine or chelirubine (0.8 and 0.4 ng, respectively). In the case of chelerythrine, the detection limit of the fluorescence detector (5 ng) was three times higher than for the UV detector (1.6 ng).

The selectivity of both detection methods was evaluated by comparing UV and fluorescence chromatograms of crude and SPE prepurified samples. In crude extracts, several unknown and interfering peaks observed using UV absorption were not detected by fluorescence (Fig. 3). For SPE-treated samples, both detection techniques gave similar peak patterns indicating that the extraction procedure removed most unknown compounds. The total alkaloid concentration, measured by fluorescence, of

TABLE III

COMPARISON OF TWO BENZOPHENANTHRIDINE ALKALOID EXTRACTION METHODS ON THREE CELL-CULTURE SAMPLES

Results are means ± standard deviation of three independent assays.

Sample No.	Alkaloid	Amount extracted (μg)			
		Cell extracts		Culture medium	
		SPE	LLE	SPE	LLE
1	Chelirubine	75 ± 2	80 ± 10	12.3 ± 0.6	12.0 ± 0.3
	Sanguinarine	79.3 ± 0.9	80 ± 10	13.5 ± 0.9	17 ± 4
	Chelerythrine	128 ± 4	120 ± 11	18.7 ± 0.7	21 ± 1
2	Chelirubine	91 ± 4	100 ± 20	6.9 ± 0.1	5.7 ± 0.6
	Sanguinarine	110 ± 5	120 ± 20	9.9 ± 0.2	8.3 ± 0.5
	Chelerythrine	157 ± 4	110 ± 20	9.6 ± 0.6	9 ± 1
3	Chelirubine	103 ± 5	110 ± 2	9.4 ± 0.4	10 ± 1
	Sanguinarine	101 ± 3	104 ± 8	7.4 ± 0.2	8.6 ± 0.5
	Chelerythrine	171 ± 5	160 ± 10	9.5 ± 0.5	11 ± 1

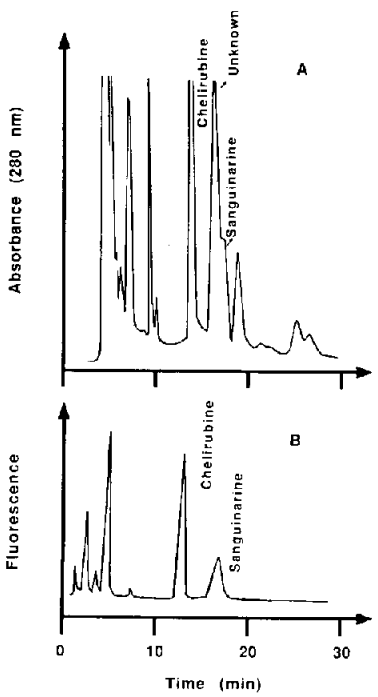

Fig. 3. Typical HPLC chromatograms of a crude cell extract. (A) UV detection; (B) fluorescence detection.

crude biomass extracts before and after SPE extraction are compared in Fig. 4. The results were linearly correlated (correlation coefficient of 0.997) by the following equation:

$$US = 1.12TS - 0.50 \quad (1)$$

where US is untreated sample alkaloid concentration (mg l^{-1}) and TS is treated sample alkaloid concentration (mg l^{-1}). The value of the slope of this line indicates that SPE treatment of samples resulted in 12% underestimation which can be accounted by experimental losses of products.

DISCUSSION

The SPE method presented in this study allowed rapid prepurification of sanguinarine, chelirubine and chelerythrine from *S. canadensis* cell-culture samples. This technique was found to be as selective and as efficient as conventional liquid–liquid extraction techniques. However, the SPE method offers several advantages over the latter technique. It involves mild extraction conditions. It is simple and allows good reproducibility (*ca.* 5% standard deviation over 3 analyses). This method is now routinely used in our laboratory for screening and production kinetics studies of different sanguinarine-producing cell systems. It was found to extract relatively high

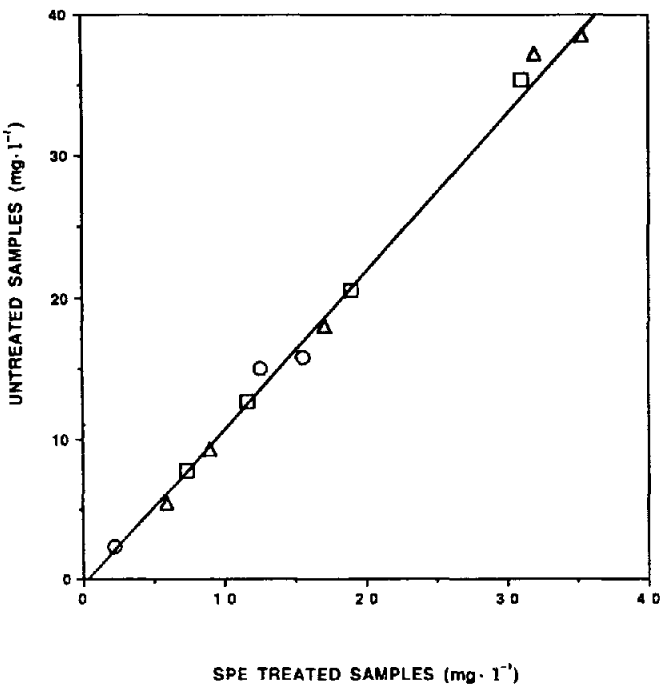

Fig. 4. Comparison of benzophenanthridine alkaloid concentration in crude extracts evaluated using fluorescence detection before and after SPE treatment. □ = Chelirubine; △ = sanguinarine; ○ = chelerythrine.

quantities of compound. In fact, up to 20 mg of sanguinarine could be efficiently extracted from spiked biomass (*ca.* 5 g fresh weight) and culture-medium samples (30 ml). This is more than 30 times the quantity of alkaloids found in actual culture samples. The application of SPE to the extraction of dihydro forms of benzophenanthridine alkaloids, which are often accumulated by plants [4,5], remains to be tested.

Fluorescence represents an interesting alternative to UV detection because of its improved selectivity without loss of sensitivity. The high selectivity of fluorescence for benzophenanthridine alkaloids allowed direct HPLC analysis of crude culture extracts which could not be achieved by UV absorption. However, it is recommended to treat crude samples by SPE or LLE for HPLC system cleanliness. In the case of dilute crude samples, extraction also allowed preconcentration of the alkaloids. The detection limits observed with the fixed-wavelengths fluorescence detector were similar to those found for UV detection for sanguinarine and chelirubine and were slightly higher for chelerythrine. The use of a programmable fluorescence detector might improve the sensitivity of fluorescence detection.

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