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Short Communication

Sanguinarine levels in biological samples by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic method is presented for the analysis of the benzophenanthridine alkaloid, sanguinarine, found in plant extracts. The method is demonstrated to be applicable to analyzing samples such as saliva and gingival crevicular fluid for sanguinarine following a simple acidified methanolic extraction step. The method utilizes an ethyl silane column with acidic and basic ion-pairing reagents in the mobile phase with a limit of detection of 3 ng of sanguinarine in a sample.

INTRODUCTION

Sanguinarine (CAS 557 807 304) exhibits several medically interesting properties such as antibacterial [1] and antiinflammatory qualities [2]. Consequently, it is being clinically investigated as an agent to treat periodontal disease. Clinical studies as well as *in vitro* research involving sanguinarine have required a very sensitive and highly specific method for the quantitation of the alkaloid in a variety of matrices. Several methods for the assay of sanguinarine have been noted and reported, including a high-performance liquid chromatographic (HPLC) method [3].

Sanguinarine is a major benzophenanthridine alkaloid found in an extract prepared from the leaves and stems of plants of *Macleaya microcarpa* and *cordata* as well as from the rhizome of the plant *Sanguinaria canadensis* L., commonly known as bloodroot. Sanguinarine comprises 40–45% (w/w) of the *Macleaya* spp. extracts. Chelerythrine, another benzophenanthridine alkaloid, is the greatest constituent at 50–55% (w/w). Structural details of these two benzophenanthridine alkaloids are found in Fig. 1.

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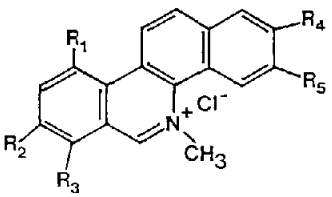

Fig. 1. Chemical structures of benzophenanthridine alkaloids.

Benzophenanthridine alkaloid	Structure
Sanguinarine	R_1 = H; R_2 + R_3, R_4 + R_5 = OCH_2O
Chelerythrine	R_1 = H; R_2, R_3 = OCH_3; R_4 + R_5 = OCH_2O

The HPLC method noted above employs a cyano type reversed-phase column (Waters Assoc., 5CN 10 μm Radial Pak column) with an acidified (pH 4.65) methanolic mobile phase which must be optimized to each column with triethylamine. An alternative HPLC method was developed due to the tedious nature of this method. An ion-pairing HPLC method proved to meet the demand for sensitivity, speed and resolution. The purpose of this paper is to present details of the new method and to demonstrate its application for the analysis of sanguinarine in the following matrices: (i) in extracts from biomass of the plant *Macleaya* spp.; (ii) in human saliva; (iii) in canine gingival crevicular fluid (GCF). The first is a source for sanguinarine; the last two are oral samples where sanguinarine can be found following the use of products containing the alkaloid.

EXPERIMENTAL

Reagents

Methanol, acetonitrile, phosphoric acid and concentrated hydrochloric acid (all ACS reagent grade from Fisher Scientific, Springfield, NJ, USA) were used throughout the tests. The ion-pairing reagents used were hexanesulfonic acid (HSA) and hexyltriethylammonium phosphate (HTAP), prepared as buffered solutions by Regis Chemical (Morton Grove, IL, USA).

Equipment

The HPLC system consisted of the following components: an LDC CM4000 pump, an LDC SM4000 UV detector (both from LDC Analytical, Riviera Beach, FL, USA); a Gilson 231/401 autosampler (Madison, WI, USA) and a Hewlett Packard 3396A computing integrator (Englewood, CO, USA). The column used was an Apex I ethyl column, 5 μm, 25 cm × 4.6 mm I.D. (Jones Chromatography, Lakewood, CO, USA). Purified water was prepared with a reverse-osmosis/deionization system (Polymetrics, San Francisco, CA, USA). To sonicate saliva samples a "Microultrasonic Cell Disrupter" (Kontes, Hayward, CA, USA) was

used. To transfer spiked serum samples a 1 μl Microcap pipette (Drummond, Broomall, PA, USA) was used. GCF in periodontal pockets was sampled with Periopaper gingival fluid collection strips (Interstate Drug Exchange, Amityville, NY, USA). GCF volume on Periopaper strips was electronically measured with a Periotron 6000 instrument (Interstate Drug Exchange). Octadecylsilane solid-phase extraction (SPE) columns (100 mg sorbent bed and stainless steel frits) were manufactured by Analytichem International (Harbor City, CA, USA). Syringe adapters as well as a manifold for multiple sample processing with SPE columns, a Vac-Elut, were manufactured by Analytichem International. Clean, non-sterile 10-ml syringes were obtained from Becton-Dickinson (Rutherford, NJ, USA). Test tubes, 150 mm × 13 mm, and the vortex mixer were made by Fisher Scientific. The shaker bath was from Precision Scientific Group (Chicago, IL, USA).

Chromatographic conditions

The alkaloids were monitored at 280 nm, or at 328 nm; both wavelengths are close to absorption maxima for sanguinarine (molar absorptivity coefficients equal to 32 300 and 27 700 mol^{-1} cm^{-1}, respectively, in acidified methanol).

The mobile phase was prepared with 2.75 mmol/l HSA and 2.25 mmol/l HTAP in purified water to which acetonitrile was added (60:40, v/v). Generally, the pH of the mobile phase was 2.7 after preparation. The ion-pairing reagents were purchased as 0.5 *M* solutions in a proprietary buffer which, after dilution, required no further pH adjustment. The flow-rate was typically 3 ml/min. The injection volume was 20 μl except when low-concentration samples were tested. In that case, 100 μl were injected. Fixed-volume loops were used in all applications. The system was operated at ambient conditions.

Standard reference material

Sanguinarine standard as the chloride salt was prepared by a contract laboratory from *Sanguinaria canadensis* L. (*Papaveraceae*). Identity was confirmed by infrared (IR) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy and elemental analysis, with reference to Krane *et al.* [4]. The purity was established by melting point (275–276°C), thin-layer chromatography (TLC), HPLC and fast atom bombardment mass spectrometry. The material assayed as 99.4% pure sanguinarine chloride.

Preparation of sanguinarine chloride standard solution

As sanguinarine is very hygroscopic, it was desiccated *in vacuo* at reduced pressure (16 kPa) at a temperature between 37 and 40°C over powdered phosphorous pentoxide for a minimum of 24 h prior to being used.

A stock solution of sanguinarine (160 μg/ml) was prepared in methanol with three drops of phosphoric acid added to ensure complete dissolution. Additional standards were made from this stock solution thereby avoiding errors associated with serial dilutions. Standard solutions for *Macleaya* spp. extract analyses were

prepared at 16, 40, 80 and 160 μg/ml. Standard solutions for analysis of sanguinarine from saliva were prepared at 2, 4, 8 and 10 μg/ml. Standard solutions for analysis of sanguinarine from GCF were prepared at 0.1, 0.2, 0.4 and 0.8 μg/ml. The standard solutions were found to be stable for at least a two-week period.

Determination of the chromatographic retention time for chelerythrine was made by using a preparation of chelerythrine chloride isolated from *Macleaya* spp. extract. Its identity was confirmed by reference to Krane *et al.* [4] with IR, NMR, UV–VIS, TLC and melting point. Analysis by HPLC showed the preparation to be at least 90% of the desired alkaloid.

Calculations

The concentration of sanguinarine in the samples was determined by comparing the peak area of sanguinarine in the sample chromatogram to a linear least-squares regression of standards. There were at least three standards in addition to a blank for every regression. The coefficient of correlation for the regression was at least 0.999.

Accuracy and reproducibility

Two analysts each accurately prepared a low-concentration solution (about 50 μg/ml) and a higher concentration solution (about 150 μg/ml) of sanguinarine in methanol. All samples were analyzed ten times every day for four days.

Sanguinarine in Macleaya spp. extract

A laboratory extract of sanguinarine and chelerythrine was produced from biomass of plants from *Macleaya* spp. A solution of this extract was prepared by dissolving 41.1 mg in methanol in a 100 ml volumetric flask with three drops of phosphoric acid.

Sanguinarine in saliva

The recovery of sanguinarine from saliva was determined by assaying saliva samples spiked at different levels. Unstimulated saliva was pooled from two healthy human volunteers. Aliquots of 1 ml were distributed into 22 test tubes. Seven aliquots were spiked with 30 μl, six were spiked with 40 μl and seven were spiked with 50 μl of a 173.7 μg/ml aqueous solution of sanguinarine. These yielded solutions of 5.21, 6.95 and 8.68 μg/ml sanguinarine, respectively. Seven tubes with 1.0 ml of purified water were spiked at the 6.95 μg/ml level. Two tubes with 1.0 ml of saliva were not spiked, these served as blanks. All tubes were covered, vortexed and incubated in a shaking water bath at 37°C for 1 h.

After incubation the samples were removed, and 10 ml of 0.5% HCl–purified water (v/v) were added to each tube. The contents were sonicated at full power for 2 min. Meanwhile, SPE columns were conditioned by passing 2 ml of methanol through each column followed by 2 ml of purified water. The barrel of a 10 ml syringe was affixed to each column (by means of the adapter) to serve as a reser-

voir. The diluted sample was transferred to the reservoir and drawn through the bed by vacuum over a 4 min period. The tube and reservoir were rinsed with two washes of 5 ml of purified water. Each SPE cartridge was eluted with 2 ml of 0.5% HCl–methanol (v/v) and transferred to an autosampler vial for analysis.

Sanguinarine in gingival crevicular fluid

GCF is a cellular exudate typically found in small amounts in the space or pocket between periodontally compromised gingival tissue (as well as healthy tissue) and the associated tooth. In appearance and composition it is similar to serum [5,6].

Volumes of GCF range from less than 1 to 3 μl. Due to the limited availability of GCF, a spike recovery of sanguinarine from blood serum was chosen to simulate GCF with sanguinarine present. Pooled serum (5 ml) was spiked with 0.5 ml of an aqueous 985 μg/ml sanguinarine solution. The sample was incubated for 1 h at 37°C. Recovery from the serum was examined by placing 1 μl of the spiked serum (with a Microcap pipette) into an autosampler vial with 400 μl of 0.05% HCl–methanol (v/v), capping it and allowing it to remain undisturbed at ambient temperature for a minimum of 3 h. Nine samples were treated in this fashion. Each vial was shaken and then analyzed. Recovery from a Periopaper strip was accomplished by using a Microcap pipette to spot 1 μl of the spiked serum onto a clean, dry Periopaper strip. The sample portion of the Periopaper strip was snipped off into an autosampler vial, excluding the handle part of the Periopaper strip. A 400 μl volume of the 0.5% HCl–methanol solution (v/v) was added, the vial capped and allowed to remain undisturbed at ambient temperature for a minimum of 3 h prior to analysis. Nine samples were treated in this fashion.

Suspensions of pharmaceutical grade sanguinarine (minimum purity of 97%) were prepared 10% (w/w) in a biocompatible, biodegradable carrier and transferred into a 1 ml syringe. The syringe was fitted with a 22 gauge cannula that was angled. A small dose of this suspension was administered subgingivally to two periodontal pockets in a canine laboratory subject to study *in vivo* release characteristics of SaCl. At periodic intervals samples of GCF were taken with Periopaper strips and the volume measured with a Periotron 6000. The Periopaper strips were submitted for HPLC analysis to determine the concentration of SaCl present in the GCF. Each Periopaper strip was extracted using 400 μl of the 0.5% HCl–methanol solution, following the same procedure as noted for the serum spike sample above.

RESULTS AND DISCUSSION

All samples and standards were prepared for analysis in an acidified matrix to put the sanguinarine in the iminium ion form needed for the ion-pairing chromatographic method. Apparently a mixed mode of retention of sanguinarine occurred when only the cation-pairing reagent (HSA) was used as significant tailing

TABLE I

INTRA-DAY REPRODUCIBILITY AND ACCURACY OF THE SANGUINARINE ANALYSIS

Analyst	Theoretical concentration (μg/ml)	Analyzed concentration (mean ± S.D., n = 10) (μg/ml)	R.S.D. (%)	Assay accuracy (%)
1	49.60	49.29 ± 0.34	0.7	99.4
2	50.00	49.46 ± 0.49	1.0	98.9
1	154.40	155.91 ± 1.56	1.0	101.0
2	150.00	150.85 ± 1.61	1.1	100.6

was observed in the chromatogram. When the anion-pairing reagent (HTAP) was used as a competing ion with the sanguinarine in the mobile phase, tailing was greatly reduced.

The accuracy and reproducibility of the method were determined by analyzing standard solutions of sanguinarine. The data for intra-day and inter-day analyses are presented in Tables I and II. The reproducibility as demonstrated by relative standard deviation (R.S.D.) ranged from 0.7 to 1.1% for intra-day analyses, and from 1.2 to 1.3% for inter-day analyses. The accuracy of the method varied from 98.9 to 101.0% for intra-day and from 99.1 to 101.3% for inter-day analysis. The limit of detection as used here describes the lowest level of analyte which can be reliably reported as being present in a sample where its signal is distinguishable from a blank signal, as described by Taylor [7]. It was determined to be equal to 3 ng of sanguinarine in a sample.

When the *Macleaya* spp. extract was analyzed by the HPLC system peaks corresponding to sanguinarine and chelerythrine retention times were observed in the chromatogram (see Fig. 2). The two peaks were clearly resolved. Twelve HPLC injections of this solution were made. The chromatogram peak corre-

TABLE II

INTER-DAY REPRODUCIBILITY AND ACCURACY OF THE SANGUINARINE ANALYSIS

Analyst	Theoretical concentration (μg/ml)	Analyzed concentration (mean ± S.D., n = 40) (μg/ml)	R.S.D. (%)	Assay accuracy (%)
1	49.60	49.33 ± 0.64	1.3	99.5
2	50.00	49.54 ± 0.61	1.2	99.1
1	154.40	156.36 ± 2.00	1.3	101.3
2	150.00	150.20 ± 1.84	1.2	100.1

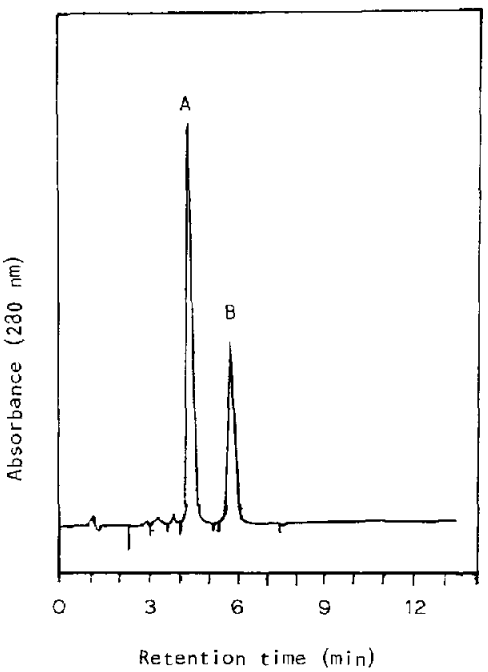

Fig. 2. *Macleaya* spp. extract. Peaks: A = sanguinarine (1.1 μg); B = chelerythrine. Injection volume, 20 μl; 0.1 a.u.f.s.

sponding to sanguinarine was found to be an average of 56.4 μg/ml with a relative standard deviation of 0.2%. This extract was 42.0% sanguinarine (w/w).

The recovery of sanguinarine from saliva was as follows: of seven aliquots spiked at 5.21 μg/ml, samples were assayed at 4.65 ± 0.17 μg/ml (mean ± S.D.), 3.7% R.S.D. with 89.3% recovery; of six aliquots spiked at 6.95 μg/ml, samples were assayed at 6.44 ± 0.23 μg/ml, 3.6% R.S.D. and 92.7% recovery; of seven aliquots spiked at 8.68 μg/ml, samples were assayed at 8.14 ± 0.20 μg/ml, 2.5% R.S.D. and 93.8% recovery. The blank saliva samples assayed at 0.0 μg/ml sanguinarine. The seven water spikes at 6.95 μg/ml assayed as 6.90 ± 0.1 μg/ml, 1.4% R.S.D. and 99.3% recovery. Without this SPE clean-up procedure, recovery of sanguinarine from saliva by extraction with acidified methanol was variable between 76 and 85%. Stainless steel frits were needed in the SPE columns as the standard polyethylene frits were found to retain sanguinarine.

Nine vials of serum spiked with sanguinarine were analyzed after preparation as described above. The calculated concentration from the spiked sample was 89.5 μg/ml sanguinarine. The analyzed value was 92.3 ± 8.1 μg/ml, 11.3% R.S.D. This value represents a 103% recovery of the sanguinarine. The nine Periopaper strips spotted with 1 μl of spiked serum were analyzed at 80.2 ± 1.4 μg/ml sanguinarine (1.7% R.S.D.). This represents a 90% recovery of the sangui-

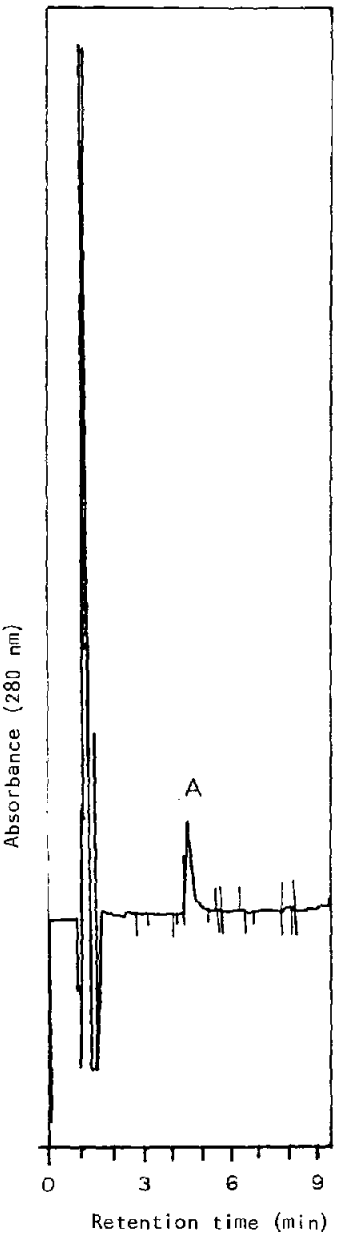

Fig. 3. Sanguinarine in GCF. Peak A = sanguinarine (0.005 μg). Injection volume, 100 μl; 0.001 a.u.f.s.

narine. It should be noted that the extraction of sanguinarine from serum and saliva required the use of a strong acid to ensure hydrolysis of the alkaloid from protein and other endogenous substances.

TABLE III

SANGUINARINE LEVELS IN GCF

Periodontal pocket No.	Time (h)	GCF volume (μl)	SaCl in GCF (μg/ml)
1	1	0.7	27
	2	0.9	126
	3	0.1	34
	4	0.4	1359
	5	0.8	329
2	1	0.5	194
	2	0.7	347
	3	0.6	535
	4	0.7	198
	5	1.0	149

Table III reports the data for the analyses of Periopaper strips from canine periodontal pockets. See Fig. 3 for a chromatogram of sanguinarine in GCF. The data demonstrate a burst in the release of sanguinarine within 4 h and then tapering off, which was the anticipated result. The low value of 34 μg/ml sanguinarine for pocket 1 at 3 h was apparently due to poor sampling, suggested by the low GCF value (0.1 μl).

CONCLUSION

This ion-pairing HPLC method is able to quantitatively separate the benzophenanthridine alkaloids sanguinarine and chelerythrine in extracts from *Macleaya* spp. A straightforward SPE procedure allows preparation of saliva samples for sanguinarine analysis. With a simple extraction procedure using acidified methanol sanguinarine is readily analyzed in a serum sample which is quite similar to GCF. This technique was used for canine GCF samples wherein release characteristics were examined. The data presented for sanguinarine standard preparations and samples of biological origin demonstrate that the method is accurate, reproducible and well suited for these applications.

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