

Research Article

Biosynthesis of Antitumoral and Bactericidal Sanguinarine

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A simple, rapid, and reliable TLC method for the separation and determination of sanguinarine has been established. This intensively studied biologically active alkaloid has a wide range of potentially useful medicinal properties, such as antimicrobial, antiinflammatory, and antitumoral activities. Sanguinarine has also been incorporated into expectorant mixtures and has a strong bactericidal effect upon gram-positive bacteria, particularly *Bacillus anthracis* and *staphylococci*. These medicinal properties are due to the interaction of sanguinarine with DNA. A fibre-optic-based fluorescence instrument for in situ scanning was used for quantitative measurements. The sanguinarine was determined over the range 5–40 ng and a detection limit of 1.60 ng. The method was applied to the quantification of sanguinarine in tissue culture extracts of *Chelidonium majus* L.

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INTRODUCTION

Continuing with our study of biologically active natural products [1–4], we developed a method for the biosynthesis, separation, and quantification of sanguinarine in tissue culture extracts.

Alkaloids represent one of the largest and most interesting groups of plant metabolites. One reason for the growing attention concerning these natural compounds is their pharmaceutical and therapeutical value. Sanguinarine is a quaternary benzo[c]phenanthridine alkaloid, widely distributed in the plants of the *Papaveraceae*, *Fumariaceae*, and *Rutaceae* families. These biologically active alkaloids have a wide range of potentially useful medicinal properties, such as antimicrobial [5, 6], antiinflammatory [7] and antitumoural activities [8]. Sanguinarine has a strong bactericidal effect on gram-positive bacteria, particularly on *Bacillus anthracis* and *staphylococci*. These medicinal properties are due to the interaction of sanguinarine with DNA [9, 10]. On the other hand, adverse effects (genotoxicity and hepatotoxicity) have been reported. The cytotoxicity of sanguinarine on hepatocytes is dose- and time-dependent [11]. Epidemic dropsy is a clinical state resulting from the use of edible oils adulterated with

Argemone mexicana oil which is characterized by oedema, cardiac insufficiency, renal failure, and glaucoma. Sanguinarine and dehydrosanguinarine are two major toxic alkaloids which cause widespread capillary dilatation, proliferation, and increased capillary permeability. Various epidemics have been reported in India, from Calcutta (1877) to Assam, Bihar, Orissa, Madhya Pradesh, Gujrat, and Delhi since then. Epidemics have also been reported from Rangoon, Fiji, Mauritius, South Africa, Madagascar, and Australia. The South African epidemic was unique, being associated with consumption of adulterated wheat flour. The organs chiefly affected in this disease are the eyes, heart, and the subcutaneous tissues [12].

Thin-layer chromatography (TLC) has been the most commonly used analytical technique for the separation of sanguinarine from plant extracts and pharmaceuticals. However, at present, high-performance liquid chromatography [13, 14] and capillary electrophoresis [15, 16] are the most widely applied methods to achieve facile separation and quantification. TLC offers significant advantages for the separation and identification of compounds of analytical interest [17] and has a great utility for applications in which many samples are analysed with minimal preparation [18, 19]. In

the quantification of analytes separated by TLC, densitometry has proved most useful [20]. Recently, as an alternative, the use of fibre optic sensors has been suggested since it allows the measurement of fluorescence emitted by fluorophors at some distance from the source of excitation and the detector [21–24]. The possibility of transporting light from one place to another, by means of optical fibres, facilitates taking readings of TLC plates and considerably reduces data acquisition times. Thus, it is possible to transmit useful spectral and chromatographic information for qualitative and quantitative analysis with minimal loss of precision and resolution.

In the present study, we describe a rapid, precise, and versatile TLC procedure for the separation and quantification of sanguinarine in tissue culture extracts using a commercially available fibre-optic-based instrument for the remote in situ scanning of sanguinarine. The method developed permits eliminating pretreatments of the samples.

MATERIALS AND METHODS

Reagents

Sanguinarine chloride was supplied by Sigma (St. Louis, Mo). Stock solutions of sanguinarine were prepared in ethanol (Merck, Darmstadt, Germany) and diluted as required. For chromatographic analysis, hexane (Merck), ethyl acetate (Merck), and ammonia (Merck) were used. Silica gel TLC aluminium sheets (Merck), without fluorescent indicator and with a layer thickness of 0.1 mm, were used as the stationary phase. All other used chemicals were of an analytical reagent grade and were used without further purification.

Apparatus

Fluorescence measurements and spectra were made with a Perkin-Elmer (Norwalk, Connecticut) LS-50 luminescence spectrometer equipped with a Perkin-Elmer fluorescence plate-reader accessory. A bifurcated fibre optic was used to transfer excitation and emission energy between the plate and the spectrometer. The spectrometer was connected via an RS232C interface to an Epson PCAX2e, containing fluorescence data manager software (FLDM) that controls the instrument.

Induction and establishment of the culture

The cultures were induced from parenchymatic cells from *Chelidonium majus* L in Murashige and Skoog basal culture medium, supplemented with 3% sucrose, naphthaleneacetic acid (NAA) (2 mg L^{-1}), and kinetin (K) (1 mg L^{-1}) and solidified with phytagel (2.5 g L^{-1}). The pH of the medium was adjusted to 5.8 before autoclaving at 120°C for 20 m. Cultures were grown in a culture room in darkness under a controlled temperature ($24 \pm 1^\circ\text{C}$). In this medium the cultures were replicated every 25 days. After that stage, the cultures were transferred to the Murashige and Skoog medium [25] or that of Gamborg et al [26], replacing the growth regulator NAA with 2,4-D and decreasing the concentration of K to 0.1 mg L^{-1} .

Preparation of samples for fluorescence microscopy

The selected culture fragments were submerged and disaggregated in an aqueous solution of sodium chloride 0.85% (w/v) and calcofluor white 0.01% (w/v). After 30 seconds squash preparations were mounted. Squash preparations were made of all the media utilized. The sanguinarine biosynthesis followed the same kinetics as the results obtained by fluorescence spectroscopy.

Quantification of sanguinarine in tissue culture extracts

From tissue cultures in solid medium obtained under Murashige and Skoog medium [25] and under that described by Gamborg et al [26], three samples were taken at random every ten days over a period of sixty days. The fresh weight and the dry weight of these samples were calculated, after maintaining them in a stove at 60°C until constant weight, usually 24 hours. The dry samples were homogenized in ethanol (Merck) with an Omni-Mixer Sorvall, at the rate of 1 mL of ethanol per 10 mg of dry weight. After a maceration of one week, the samples were centrifuged in a Sorvall RC-53 refrigerated superspeed centrifuge for 10 m at 1500 rev m^{-1} . The supernatant was poured into 10 mL vials and refrigerated at -20°C until their analysis. The samples were analysed as described above.

RESULTS AND DISCUSSION

In the studies reported below, the sanguinarine solutions and the samples of tissue culture extracts were prepared in ethanol. According to Dostál et al [27, 28], this alkaloid yields 6-ethoxy-5,6-dihydrosanguinarine in the presence of ethanol. We used the spectrofluorimetric characteristics of sanguinarine in ethanol and water solutions to check the formation of this compound, which was then confirmed by $^1\text{H-NMR}$ and MS. Sanguinarine presents a maximum excitation peak at a wavelength of 283 nm and a secondary peak at 326 nm in both solutions. In ethanol solutions, this alkaloid shows a maximum emission peak at 412 nm while, in water solutions, a maximum peak appears at 415 nm, with a secondary peak at 553 nm. Moreover, sanguinarine in ethanol solutions shows a higher fluorescence intensity than in aqueous solutions, due to the formation of its ethoxy derivative. These results, which are shown in Figure 1, are in agreement with those obtained by other authors [29–31]. The fluorescence emitted by a fluorophor largely depends on the nature of the medium in which it is found. In TLC the solid constituting the stationary phase is, at the same time, the medium in which solutes are retained after separation occurs. Consequently, selection of the stationary phase must be made bearing in mind its chromatographic properties and its possible influence on the spectrofluorimetric characteristics of the analyte.

Different solid surfaces and mobile phases were tested to obtain the greatest sensitivity for the spectrofluorimetric quantification and the best resolution for the separation of sanguinarine from other substances in tissue culture extracts.

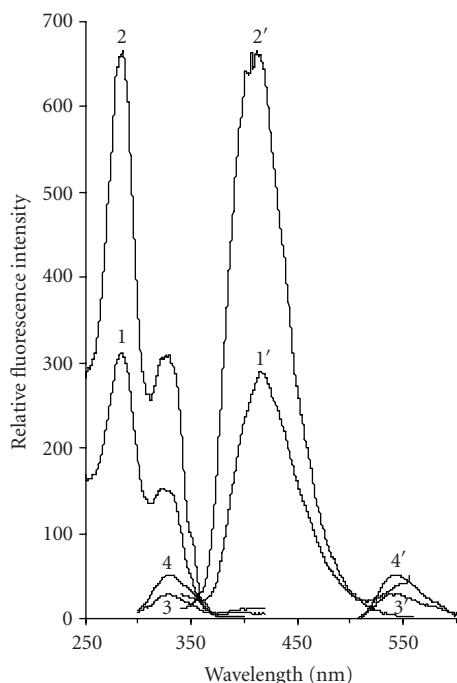


FIGURE 1: Excitation (1,2) and emission (1',2') spectra of sanguinarine. (1,1') 3 mg/L of sanguinarine in aqueous medium, slits 5 nm, $\lambda_{exc} = 283$ nm, and $\lambda_{em} = 415$ nm. (2,2') 1 mg/L of sanguinarine in ethanol medium, slits 2.5 nm, $\lambda_{exc} = 283$ nm, and $\lambda_{em} = 412$ nm. In situ excitation (3,4) and emission (3',4') spectra of sanguinarine, obtained with the fibre-optic sensor after elution on a silica gel plate. (3,3') 20 ng and (4,4') 100 ng of sanguinarine from ethanol and aqueous solutions, respectively. Slits 10 nm, $\lambda_{exc} = 326$ nm, and $\lambda_{em} = 545$ nm.

TABLE 1: Spectrofluorimetric and chromatographic characteristics of sanguinarine.

Medium	λ_{exc} (nm)	λ_{em} (nm)	E_s	S_s	S_{bk}	S/N
Water	283 (326)*	415 (553)	—	—	—	—
Ethanol	283 (326)	412	—	—	—	—
silica gel ^a	326	545	34.2	1.64	1.58	15.1

* Values of secondary peaks

^a 20.08 ng of sanguinarine.

From these studies, the best results were obtained when silica gel was selected as stationary phase and hexane : ethyl acetate (6 : 4) as mobile phase. To increase the fluorescence intensity of sanguinarine spotted on silica gel we studied the addition of ammonia to the mobile phase. The results show that the addition of 1% of ammonia to the mobile phase slightly increases the fluorescence intensity and improves the separation characteristics. Figure 1 shows the excitation and emission spectra of aqueous and ethanol solution of sanguinarine deposited on a silica gel plate and eluted with hexane : ethyl acetate : ammonia (25%) (6 : 4 : 0.1) (v : v : v). The main spectrofluorimetric and chromatographic characteristics of sanguinarine are summarized in Table 1. When

silica gel plates are developed with hexane : ethyl acetate : ammonia (25%) (6 : 4 : 0.1) (v : v : v) and protected from light and humidity, the fluorescence intensity emitted by sanguinarine remains stable for at least 2 hours. Analytical determinations on solid surfaces are strongly conditioned by the background signal and reproducibility of the measurements. Radiation reaching the detector consists of a background signal superimposed on fluorescence emitted by the components of the sample. Thus the ability of the system to detect and measure the sample emission is limited by the magnitude of the background signal and noise.

The use of a fibre optic to transport the radiation allows a certain amount of light from the surroundings to be added to the fluorescence emitted by the analyte, thus increasing the background signal. Table 1 gives the value obtained for the signal-to-noise ratio for sanguinarine, using the expression

$$S/N = E_s / (S_s^2 + S_{bk}^2)^{1/2}, \quad (1)$$

where E_s is the analytical signal obtained by subtracting the blank signal from the total and S_s and S_{bk} are the standard deviations of the analytical and blank signals, respectively. A minimum of nine determinations were performed for each parameter included in the above expression.

Quantitative determination of fluorescent compounds separated by TLC, measuring their radiation emitted when they are excited at appropriate wavelengths, is based on the measure of the peak size, area, or height. In our case, the results were similar in both cases, so we decided to base this study on the measure of peak height.

Figure 2 shows the three-dimensional chromatogram and its contour map for sanguinarine over the range 5–40 ng. As can be observed, the concentration profiles of spots exhibit a roughly Gaussian profile (cylinder character) [32]. The integral of the concentration density over the spot area was equal to the volume of the cylinder, which in turn is proportional to the peak concentration value. In this way we can substitute integration by measuring the maximum fluorescence intensity (or peak height) at the centre of the spot.

To establish the relationship that exists between the response of the detector and the concentration of analyte on the chromatographic plate, six samples, with different amounts of sanguinarine, were applied to the plate. The maximum fluorescence intensity for the analyte was read after the plates were eluted. To prove the robustness of the established methodology this relationship was also obtained in different days using two stock solutions deposited on different plates.

The calibration function was determined by second degree polynomial regression over the tested range of 5–40 ng of sanguinarine:

$$y = a + bx + cx^2. \quad (2)$$

The regression equations, which have low values of the fit standard error, are represented in Table 2 for one day and for different days. Considering the values of R^2 as a measure

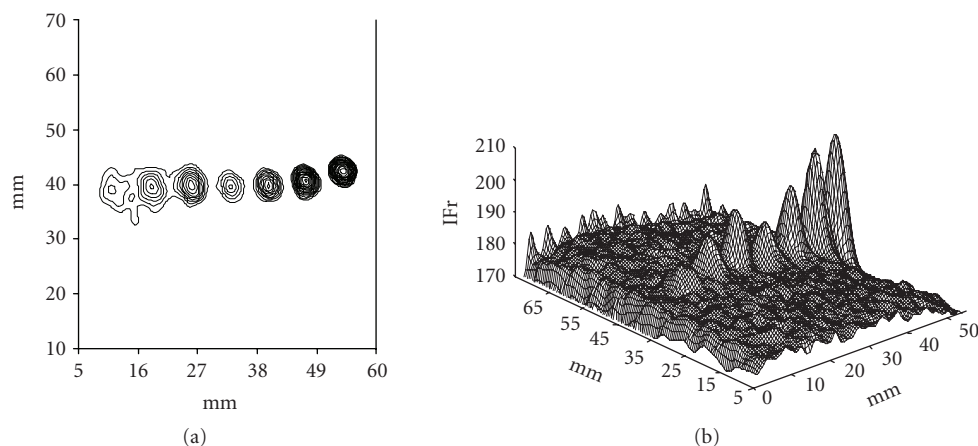


FIGURE 2: Contour map 2(a) and three-dimensional chromatogram 2(b) scanned at $\lambda_{\text{exc}} = 326 \text{ nm}$ and $\lambda_{\text{em}} = 545 \text{ nm}$ (slits 10 nm) for seven samples of sanguinarine ranged from 5 to 40 ng, after they were eluted with hexane : ethyl acetate : ammonia (25%) (6 : 4 : 0.1) (v : v : v) on a silica gel plate.

TABLE 2: Regression data and representative statistical parameters of the analytical method.

	One day	Different days
a	0.462 ± 0.546	0.664 ± 0.548
b	1.214 ± 0.075	1.262 ± 0.068
c	-0.003 ± 0.002	-0.004 ± 0.002
R^2	0.999	0.997
$S_{y/x}$	0.646	0.754
LOD (ng)	1.6	1.8
Added (ng)	20.08	19.96
(ng)	19.99	19.94
S (ng)	1.37	1.1
RSD (%)	6.86	5.54
E (%)	4.61	5.12
n	11	7
t_{tab}	2.23	2.45
t_{exp}	0.7	0.02
t'_{exp}	0.22	0.06

of the validity of the fit, it can be observed that the equations explain, in more than 99.7%, the relationship between the analytical signal and the amount of sanguinarine. In both cases, for 95% confidence level, there are no significant differences between the calibration functions and between the values obtained for the fit standard error. When the calibration functions were used for the quantification of series of samples containing 20.08 or 19.96 ng of sanguinarine, the relative errors were, for a 95% confidence level, 4.61% and 5.12%, respectively. Table 2 gives the most representative statistical parameters of the established analytical method and also the detection limit calculated through (3) (see [33]):

$$y = a + 3 \cdot S_{y/x}, \quad (3)$$

which is based on the recommendation of IUPAC using the calculated intercept as an estimation of the blank signal and $S_{y/x}$ instead of the standard deviation of the blank.

In accordance with the results shown, and taking a confidence level of 95%, it can be concluded that the method is correct and sufficiently accurate. Thus, the proposed method presents good repeatability, which permits high precision and robustness in the analysis. However, to ensure the accuracy, it is necessary to have strict external and internal quality controls. In the proposed method, the preparation of the stock and calibration solutions, the use of different plates, the mobile phase, and so forth are critical aspects. Many methods are currently used for quality control in analytical chemistry. In most of them control samples are analysed every day to recognize the presence of sources of variation that affect the measurements. In our case, this control was carried out by making use of a solution corresponding to a concentration of $5 \text{ ng } \mu\text{L}^{-1}$ of sanguinarine. This control solution was measured before each particular experiment, and a total of 32 control samples were introduced and controlled using a Shewhart chart [34, 35]. As usual in this kind of charts, two limits are established: an upper/lower warning limit (twice the SD of the values corresponding to the preliminary study) and an upper/lower action limit (three times that of the SD), which is never exceeded. Figure 3 shows the obtained chart. It can be observed that the control solution is always between the admissible limits, showing that the measurement process is clearly under statistic control. The chart shows different values for the same solution of sanguinarine, but it can be concluded that there is no systematic influence on the results since no clear trend appears in the measurements of the control solution plotted against time.

The method was applied to the quantification of sanguinarine (Figure 4) in tissue culture extracts. The cells in Murashige and Skoog basal culture medium [25], using as growth factors NAA (2 mg mL^{-1}) and K (1 mg mL^{-1}),

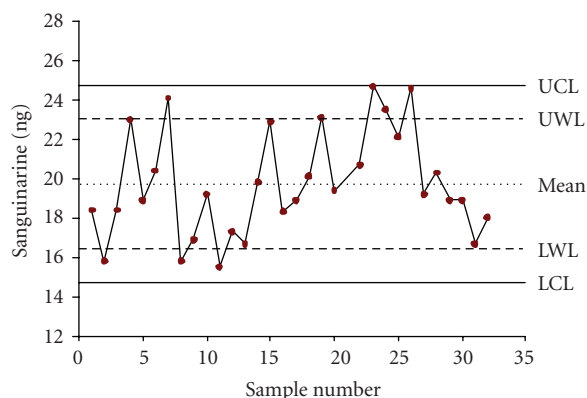


FIGURE 3: Shewhart chart for control of the chromatographic analysis of sanguinarine. UCL and LCL: upper and lower control limits. UWL and LWL: upper and lower warning limits. Mean = 19.72, $S = 1.66$.

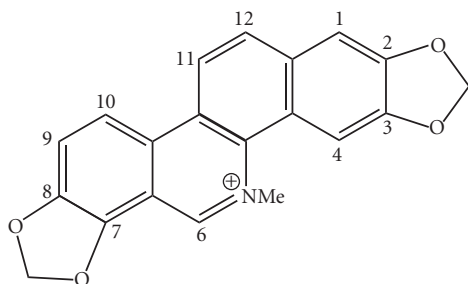


FIGURE 4: Molecular structure of sanguinarine. One of the best targets for testing interaction of a drug with its receptor is DNA. Antitumor antibiotics are known to act at the DNA level. Due to DNA's helical and relatively stable conformation in solution, DNA-drug complexes can conveniently be studied by high-resolution NMR-spectroscopy.

did not show alkaloid biosynthesis detectable by fluorescence microscopy. The formed root apices disappeared after the third replication. When the cultures were transferred to the Murashige and Skoog medium or that of Gamborg et al [26], substituting the growth regulator NAA with 2,4-dichlorophenoxyacetic acid (2,4-D) and decreasing the concentration of K, alkaloid biosynthesis was induced and later detected by fluorescence microscopy.

Table 3 summarizes the concentrations of sanguinarine found for the extracts obtained from the two different culture media used. Each value is the mean of extracts obtained from three samples which were taken at random every ten days during a period of sixty days. The differences obtained after 30 days of culture must be emphasized since the concentration of sanguinarine from Gamborg's medium was six times higher than that obtained from the Murashige-Skoog medium. The values obtained from the former medium were always higher but the observed tissue culture growth was lower.

TABLE 3: Sanguinarine levels (mg L^{-1}) in tissue culture extracts.

Days of culture	Medium ^a	
	Murashige and Skoog	Gamborg
10	1.04 ± 0.05	3.53 ± 0.17
20	2.86 ± 0.10	13.51 ± 0.67
30	5.95 ± 0.29	38.69 ± 1.93
40	10.23 ± 0.51	32.16 ± 1.60
50	11.48 ± 0.57	24.09 ± 1.20
60	11.03 ± 0.55	21.89 ± 1.09

^aMean values \pm standard deviation (three samples).

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