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Quantitative liquid chromatographic determination of sanguinarine in cell culture medium and in rat urine and plasma

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Abstract

Sanguinarine is a quaternary benzo[*c*]phenanthridine alkaloid, extracted from the argemone oil, which produced severe human intoxications. To investigate the sanguinarine biotransformation, we develop a simple extraction process and a high performance liquid chromatographic separation coupled to a sensitive fluorometric detection of sanguinarine in cell culture medium, as well as in rat urine and plasma. After extraction with an acidified organic solvent, sanguinarine elution is performed within 15 min on a Nucleosil C18 column with a gradient using 0.2% formic acid/water/acetonitrile as mobile phase. Extracted and standard sanguinarine are characterized by mass spectrometry. The extraction recovery of sanguinarine is about 80% in cell culture medium and in rat urine, but lower in plasma. This convenient high performance liquid chromatography (HPLC) method allows to quantify sanguinarine over concentrations ranged 10–2000 ng ml^{-1}. The limit of fluorometric detection is 0.5 ng. Under these conditions, the lower limit of quantification of sanguinarine is 50 ng ml^{-1} in cell culture medium and in rat urine and 100 ng ml^{-1} in rat plasma. This analytical HPLC method is specific, linear and reproducible in all media and is suitable for quantitative determination of sanguinarine in biological fluids.

Keyword: Sanguinarine

1. Introduction

In India, the consumption of edible oil, edulcorated by argemone oil, extracted from Argemone *mexicana Linn* (Papaveraceae), produces severe intoxication, referred to as epidemic dropsy [1]. A recent and large outbreak of epidemic dropsy appears in August 1998 in India involving 2900 hospitalized patients and more than 67 deaths reported [2–6]. Clinical manifestations in man are vomiting, diarrhea, nausea, swelling of limbs, erythema and cardiac arrest in extreme cases [1–4]. Toxic manifestations have been related to the presence of two alkaloids in argemone oil: sanguinarine and its dehydro-derivative. Sanguinarine is a benzo[*c*]phenanthridine alkaloid, also found in extracts prepared from *Sanguinaria canadensis Linn* or *Machaya mi-crocarpa* (Wild.) [7]. The properties of sanguinarine are the basis of its use in chemosurgery and skin cancer excision [8,9] and its antimicrobial activity explains its addition to toothpaste and oral rinse antiseptic solutions widely used in North America [10,11].

To prevent harmful toxicological consequences, a fast and sensitive monitoring is required to detect an excessive concentration of sanguinarine and its metabolites in biological fluids. Furthermore the development of a suitable method for the detection of sanguinarine metabolites will allow us to ascertain the metabolic pathway of sanguinarine which remains largely unknown.

Initially, quantitative methods were developed to determine sanguinarine concentration in various biological samples [12–14]. Later, high performance liquid chromatography (HPLC) methods used, respectively, methanol/water or methanol/acetonitrile/tetrahydrofuran/water mobile phase under isocratic conditions on a C-18 column [15,16]. Under these conditions, the separation of sanguinarine from

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medium components was not optimum and the collection of sanguinarine samples for mass spectrometry analysis was made difficult. Two other methods used mobile phases containing salts. The first used ion-pairing reagent and allowed the determination of benzophenanthridine in saliva and gingival crevicular fluid [17] and the second described by Ševčik et al. [18] used triphosphate buffer and ethanolamine in the mobile phase to determine the content of alkaloid in plant and hygiene products. All these HPLC conditions are incompatible with mass spectrometry analysis of sanguinarine samples after HPLC separation.

Herein, we report a sensitive and reproducible HPLC method to quantify sanguinarine isolated from rat urine and plasma and cell culture medium. The HPLC conditions are optimized to separate and detect sanguinarine and its metabolites for further in vivo and in vitro investigations. In addition, the simplicity of the extraction procedure and the accuracy, high sensibility and reproducibility of detection in comparison with previous determinations made this methodology a powerful tool for routine monitoring of sanguinarine in health products, such as toothpastes, ointments or food preparations.

2. Experimental

2.1. Chemicals and animals

Sanguinarine chloride (CAS 2447-54-3) was obtained from Sigma–Aldrich (Saint Quentin Fallavier, France). Formic acid and analytical grade HPLC solvents (acetonitrile, methanol) were provided by Carlo Erba (Val de Rueil, France). Water was purified and deionized on a Milli-Q system (Millipore, France).

The cell culture medium was Dulbecco's MEM supplemented with 4.5 g l^{-1} glucose (Invitrogen, Cergy-Pontoise, France).

Male Sprague–Dawley rats, weighing 150–175 g, were kept in metabolic cage. Rat urines were collected for 24 h. At the end of collection, plasma was obtained after intra-cardiac puncture from the same animals.

2.2. Standard solutions and calibration

A stock solution of sanguinarine was prepared in deionized water (acidified with a drop of formic acid to increase its solubility) at a concentration of 1 mg ml^{-1} (2.7×10^{-3} M) and stored in the dark at 4 °C. Working solutions were dilutions of the stock solution in water to the final concentration of 1 μg ml^{-1}. They were injected in triplicate between 5 and 500 ng and monitored by UV and fluorometric detection.

Six standard calibration solutions of sanguinarine were prepared to obtain final concentrations ranged from 10 to 2000 ng ml^{-1} ((0.27–54) $\times 10^{-7}$ M) by addition of working solutions to either water, rat urine or cell culture medium and from 40 to 1000 ng ml^{-1} ((1.08–27) $\times 10^{-7}$ M) to rat plasma. For rat urine and plasma, samples were incubated at 37 °C for 10 min before extraction.

For quality control, samples were freshly prepared in triplicate over three different days and analyzed twice to evaluate the linearity and the precision of the HPLC method to estimate intra-day and inter-day variations.

The calibration curves were obtained by plotting the peak area of sanguinarine against the known sanguinarine concentrations. The correlation coefficient was calculated using the linear regression with the weighting factor $1/x_2$ (Prism, Graphpad).

2.3. Extraction process

Ten microliters of formic acid was added to 1 ml of water, rat plasma or urine and cell culture medium containing sanguinarine to adjust the pH between 3 and 3.5. After vortexing, the sample was evaporated under nitrogen stream. Dry residues were dissolved in 250 μl of water and added to 5 ml of the mixture water/acetonitrile 50/50 (v/v) with 0.2% formic acid. The resulting mixture was shaken for 10 min and centrifuged 20 min at 6000 × *g* to discard denatured proteins. The supernatant was removed and evaporated to dryness under nitrogen. The residue was dissolved in 200 μl of solvent A (see below) and injected.

A blank sample without sanguinarine was prepared as above and analyzed to examine interference at the retention time of sanguinarine. The extraction recovery of sanguinarine in the different media was calculated as the ratio between the area of the UV or fluorescence signal of the extracted sample and the area of signal obtained from the same concentration of unextracted sanguinarine in water.

2.4. High performance liquid chromatography

The HPLC system included a Waters 600 MS controller, a 510 double pump fitted with a 712 WISP autosampler automatic injector. The detection was carried out at 327 nm by a 486 Waters UV detector and a 474 Waters fluorescence detector (excitation: 475 nm, emission: 577 nm). The HPLC system and detectors were controlled and results calculated by the Waters Millenium® 32 chromatography manager software.

The sample was injected on a Nucleosil C18 10 μm column (250 mm × 4.6 mm) (Hypersil, Saint Quentin en Yvelines, France) running at room temperature with a flow rate set at 1 ml min^{-1}. Eluent A consisted in water/acetonitrile 80/20 (v/v) with 0.2% formic acid and eluent B was water/acetonitrile 5/95 with 0.2% formic acid. A 15 min linear gradient started from 100% of eluent A to 100% of eluent B and then was followed by 100% eluent B for 5 min.

2.5. Mass spectrometry

Sanguinarine mass was measured by electrospray ionization/mass spectrometry (ESI/MS) in acetonitrile/water

(50/50) with 0.2% formic acid. Standard and extracted sanguinarine (5 ng ml^{-1}) were analyzed using a QUATTRO-LCZ mass spectrometer (Micromass, Manchester, UK). The MS spectrum was obtained by continuous scanning over the mass range (*m*/*z*: 200–380). The average positive mass spectra were processed with Masslynx NT software.

3. Results and discussion

3.1. Sanguinarine sample preparation

Several extraction methods of sanguinarine have been compared. The chloroform/acetic acid mixture was chosen by Tandon et al. [12] to extract sanguinarine and its metabolites from liver homogenate, lung, kidney, heart and spleen. Hakim et al. [13] used the mixture acetonitrile/methanol (50/50) with 1% formic acid and solid/liquid extraction on octadecyl columns to recover the parent compound and its metabolites from goat milk treated by sanguinarine. To obtain accurate and reproducible results and a yield higher than 90%, the salt form of sanguinarine must be preserved with a pH lower than 3 during HPLC analysis. Therefore, samples are dried and then diluted in the acetonitrile/water (50/50) mixture acidified with 0.2% of formic acid. In these conditions samples can be kept for 24 h at 4 °C without apparent degradation.

The presence of a quaternary nitrogen atom charged at physiological pH was responsible for the high binding capacity of sanguinarine to cellular proteins and DNA [19–21]. However, in biological fluids containing a large amount of proteins like in rat plasma, the binding to proteins severely reduced the yield of recovery and hampered the generalization of the method to plasma and probably to a majority of tissue homogenates.

Sanguinarine stability was not affected whatever the medium. The sanguinarine retention time in HPLC remained unchanged as well as its mass spectrum, strictly identical to those of authentic sanguinarine. The molecular ion $[M]^+$ 332 and the fragment ions generated in mass spectrometry were in good agreement with the quaternary structure of sanguinarine (Fig. 1).

3.2. Sanguinarine HPLC analysis and detection

Because of its charged quaternary structure, sanguinarine firmly stuck to the HPLC column and affected the performance of HPLC separation. We tested several reverse phase columns and only the Nucleosil C18 column allowed obtaining a reproducible retention time. However, in our conditions, it was requisite to inject a 200 μl volume of blank solvent, every five samples, to obtain a stable retention time and to preserve peak symmetry.

Sanguinarine can easily be detected by fluorescence after extraction from rat plasma and urine and culture medium. The fluorescence excitation and emission wavelengths of sanguinarine were optimized at 475 and 577 nm, respectively. The limit of fluorometric detection of sanguinarine was as low as 0.5 ng, in line with other methods using fluorometric detection [17,22].

Sanguinarine can also be monitored by UV detection at 327 nm in all media. Using the same conditions, the limit of detection was 5 ng in cell culture medium and rat urine. In rat plasma, the presence of endogenous components gave a high background level and therefore lowered the limit of detection to 10 ng. Nevertheless, this detection mode can be

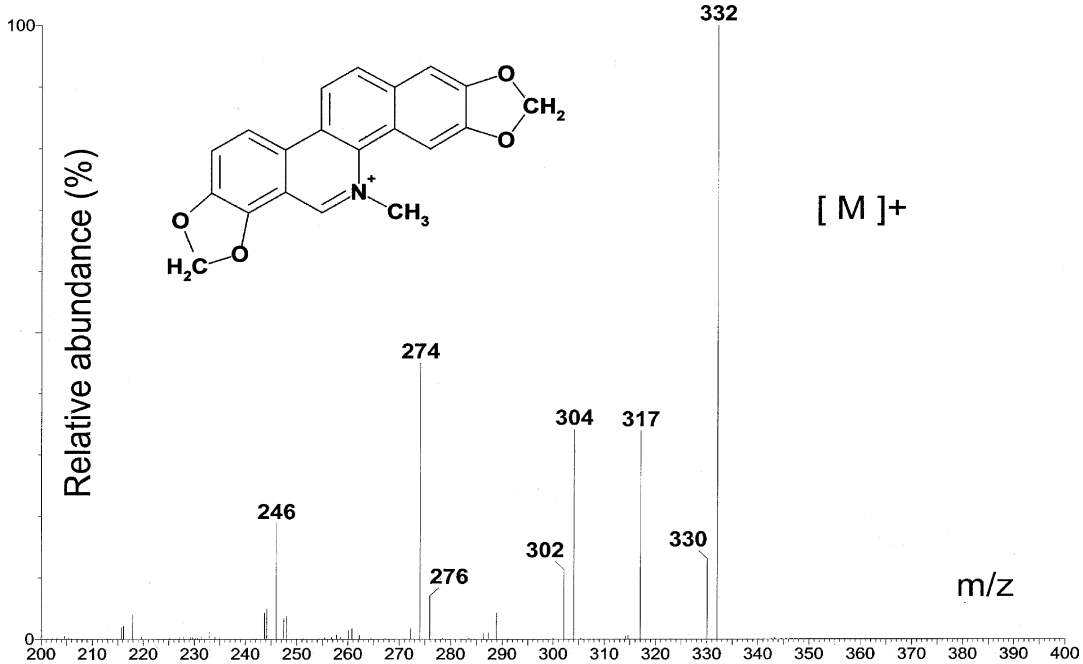

Fig. 1. Electrospray mass spectrum (ESI/MS) of sanguinarine standard.

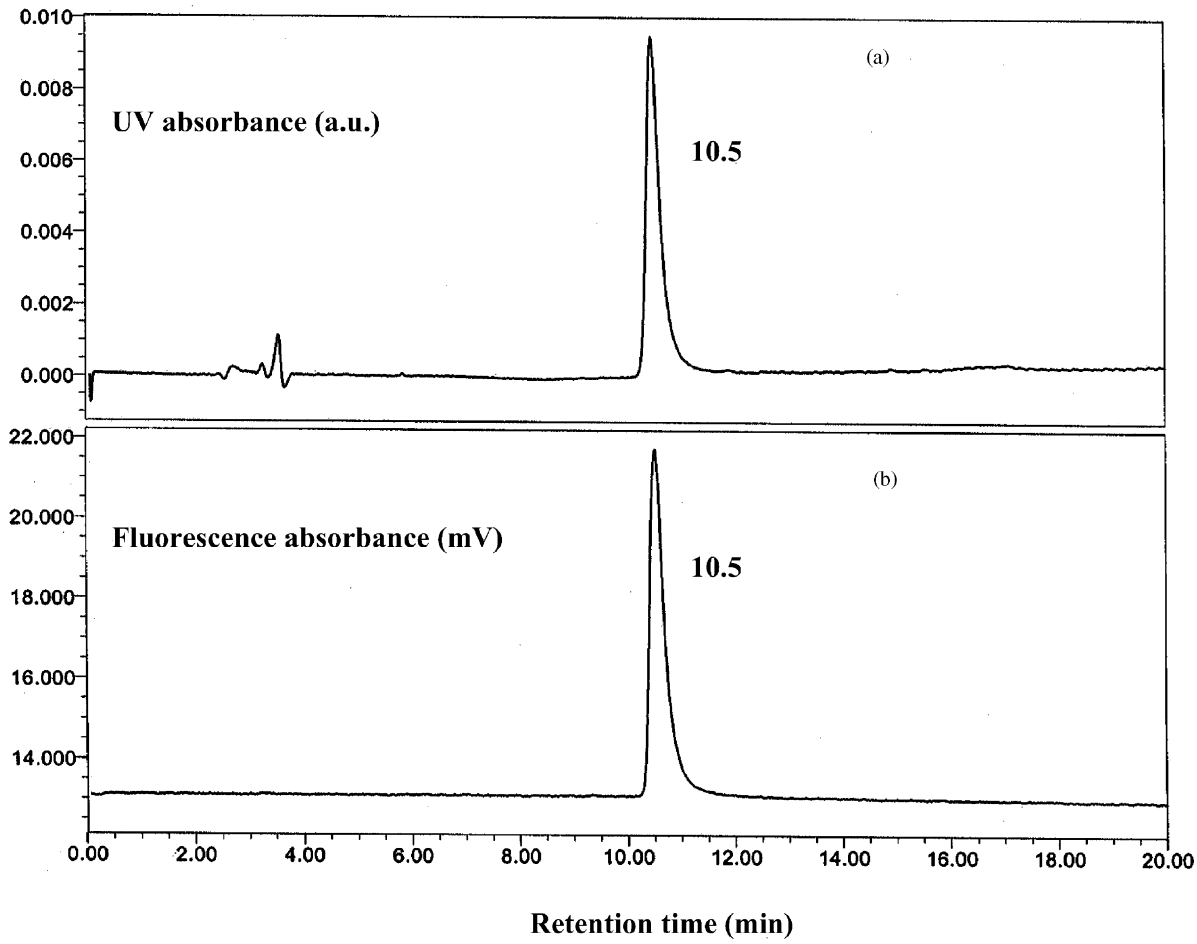

Fig. 2. UV (a) and fluorescence (b) HPLC chromatograms of sanguinarine. Chromatograms were monitored in line with UV ($\lambda = 327$ nm) and fluorescence ($\lambda_{ex} = 475$ nm, $\lambda_{emi} = 577$ nm) detections.

useful for sanguinarine derivatives devoid of fluorescence properties.

To be fitted with electrospray ionization/mass spectrometry, samples analyzed by HPLC must be salt free, excluding the use of salt buffer or ion pairing agents. Other criteria of importance were the high reproducibility of retention time, the stability of sanguinarine and the fast evaporation of eluted fractions. The mixture water/acetonitrile with 0.2% formic acid (pH = 2.5) was chosen on these criteria to keep stable the quaternary structure of sanguinarine during HPLC analysis.

With our HPLC conditions, the retention time of sanguinarine was 10.5 min (Fig. 2). The variation of the retention time did not exceed 5% from day-to-day and 3% from run-to-run within the same day whatever the sample medium extraction.

3.3. Cell culture medium

When sanguinarine was extracted from cell culture medium, a linear calibration curve was obtained with both fluorescence ($r^2 = 0.999$) (Fig. 3) and UV detection (Fig. 4). The recovery yielded from 70 to 86% when sanguinarine concentration in the cell culture medium varied from 50 to 2000 ng ml^{-1}. In that range, the mean accuracy varied from 99 to 111% (Table 1) with a precision >93%. Under these conditions, the limits of detection were 0.5 ng and 10 ng for fluorescence and UV detection, respectively, and the lower limits of quantitation were 3 ng and 30 ng ml^{-1} medium for fluorescence and UV detection, respectively.

3.4. Rat urine

In rat urine, the recovery of sanguinarine was 84% for concentrations of 50 and 100 ng ml^{-1} with fluorescence detection. The recovery varied from 80% for low and medium concentrations to 95% for high concentration with UV detection.

The correlation coefficients calculated from fluorescence (Fig. 3) or UV (Fig. 4) monitoring of calibration curves of sanguinarine ($r^2 = 0.985$ and 0.999, respectively) attested of the linearity of the detection. In the range 50–2000 ng ml^{-1} the mean accuracy yielded 99 to 110% with a precision >92% (Table 1).

The values obtained by UV detection were more scattered than those monitored by fluorescence. The important

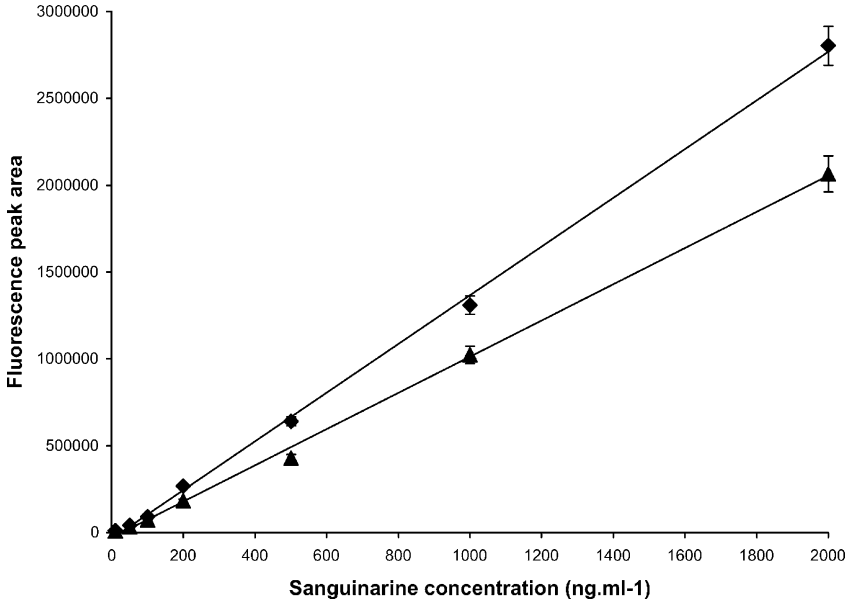

Fig. 3. Calibration curves of sanguinarine in cell culture medium (◆) and in rat urine (▲) with fluorescence detection.

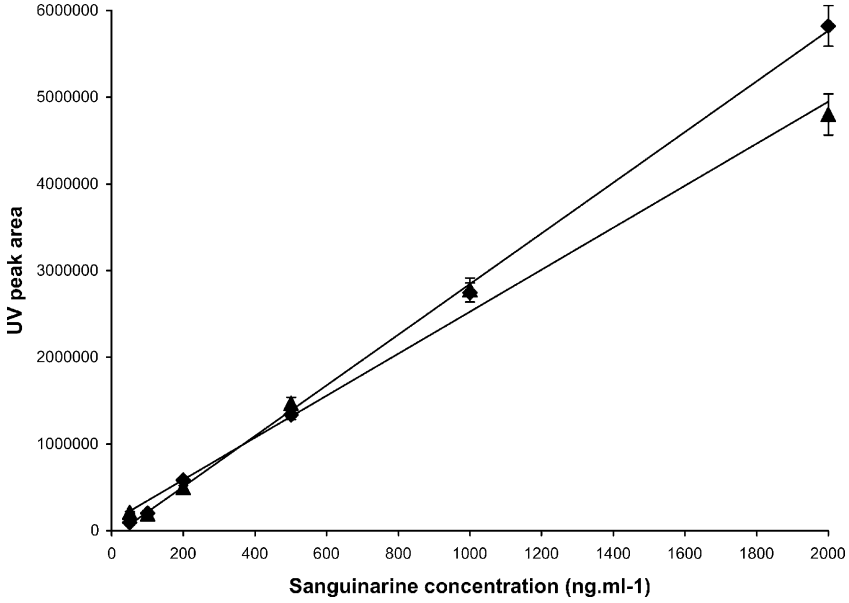

Fig. 4. Calibration curves of sanguinarine in cell culture medium (◆) and in rat urine (▲) with UV detection.

Table 1
Intra-day accuracy and precision of sanguinarine samples

Medium	Actual concentration ($ng\,ml^{-1}$)	Mean calculated concentration ($ng\,ml^{-1}$)	Extraction recovery (%)	CV (%)	Bias (%)	Accuracy (%)
Cell culture	50	55	70	2.4	10.4	111
	100	93	86	4	−7	93
	1000	987	85	5.8	5	99
Rat urine	50	57	83	7.5	14	114
	100	97	85	5.3	−3.3	97
	1000	980	85	6.1	2	98
Rat plasma	100	93	20	3.5	−7	93
	200	192	20	4.8	−4	96
	800	818	15	5.8	2.3	102

background in UV detection sample was due to the presence of endogenous compounds and was also observed in blank sample without sanguinarine. Different attempts to eliminate these endogenous compounds without loss of sanguinarine remained unsuccessful. These compounds did not interfere in fluorescence detection and thus the fluorescence detection appeared more sensible (detection limit: 10 ng) than UV detection (detection limit: 25 ng). In these conditions, the lower limit of quantification in rat urine was 50 ng ml^{-1} with fluorescence detection.

3.5. Rat plasma

The linearity of fluorescence and UV detection of sanguinarine is excellent ($r^2 = 0.999$) over the range tested (100–1000 ng ml^{-1}), the mean accuracy was estimated to 93–103% with a precision >93% (Table 1). However, the extraction recovery of sanguinarine from plasma was poor (20%) in relation with the presence of high protein content in samples, which bound sanguinarine in spite of the low pH and high polarity of the extraction solvent. The treatment of rat plasma with perchloric acid to separate sanguinarine from plasma proteins was unsuccessful. Under these conditions, the lower limit of quantification in rat plasma was 100 ng ml^{-1}, whatever the detection mode.

4. Conclusion

In the present paper, a reproducible extraction procedure and a sensitive HPLC separation coupled to fluorescence detection was described allowing quantifying sanguinarine in cell culture medium, as well as in rat urine and plasma. Alternatively, this HPLC separation coupled to mass spectrometry could be an appropriate tool for further identification of sanguinarine and its derivatives in in vivo and in vitro investigations presently in progress.

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