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Capillary electrophoretic studies of acid–base properties of sanguinarine and chelerythrine alkaloids

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Abstract

Capillary zone electrophoresis with UV detection was used for determination of dissociation constants of alkaloids sanguinarine and chelerythrine. Despite the limited solubility of the uncharged forms of the alkaloids resulting in insufficient analytical signal at higher pH the reliable dissociation constants were obtained when acidified samples containing low amount of the alkaloid were injected into the capillary. The precipitation of the alkaloid in the capillary induced by injecting sample of low pH into the background electrolyte of higher pH does not affect the mobility of the alkaloid if its concentration injected exceeds the solubility only to a small extent. Dissociation constants (pK_{R^+}) of sanguinarine and chelerythrine calculated to 8.3 ± 0.1 and 9.2 ± 0.1, respectively, are relevant to Good buffers of ionic strength of 30 mM.

Keywords: Dissociation constants; Sanguinarine; Chelerythrine; Alkaloids

1. Introduction

Sanguinarine (SA) and chelerythrine (CHE) are the most important members [1] of the family of quarternary benzo[c]phenanthridine alkaloids (Fig. 1) displaying a wide spectrum of biological activities [2,3]. The wide range of biological effects of SA and CHE is linked with their occurrence in two chemically different forms at the physiological pH 7.4 [4]. The pH-dependent (acid–base) equilibrium (Fig. 1) between the charged iminium Q^+ and the uncharged (pseudo-base) QOH forms of an alkaloid:

$$Q^+ + H_2O \leftrightarrow QOH + H^+ \quad (1)$$

may be characterized with an equilibrium constant K_{R+}

$$K_{R^+} = \frac{[H^+][QOH]}{[Q^+]} \quad (2)$$

in analogy to the acid–base dissociation constant K_a of Brønsted acids [5].

Reported pK_{R^+} values of SA and CHE in aqueous solutions range from 7.32 to 8.16 and from 7.53 to 9.02, respectively [6–9]. Spectrophotometry, fluorimetry or potentiometry was used for their determination [6–9]. The wide range of the values indicate a dependence on ionic strength of the liquid medium and, perhaps, on its composition. Unfortunately, the incomplete specification of the ionic strength of used buffers and of other experimental details in communications disables to judge the relevance of these indications.

The pK_{R^+} values of the alkaloids are necessary for the interpretation of any experiment with these alkaloids performed at physiological conditions. Evidently, the investigation of the interactions of these alkaloids with biological macromolecules, e.g. receptors, transport proteins, nucleic acids, etc., belongs to such experiments.

Capillary zone electrophoresis (CZE) proved to be highly effective in studies of some of these interactions [10,11]. Its experimental simplicity, speed, low consumption of investigated compounds and mild requirements on their purity are the main reasons. Moreover the cost of commercially available SA and CHE or their insufficient purity if prepared at laboratory conditions, their partial dissociation at physiological pH and their spectral properties made CZE superior to other techniques for the investigation of the interactions for biological purposes.

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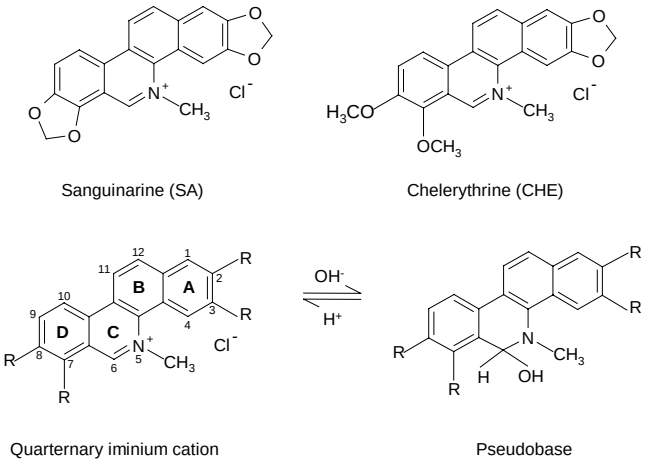

Fig. 1. The structural formulas of alkaloids sanguinarine and chelerythrine and the equilibrium between their charged and uncharged forms.

CZE was already successfully used for the determination of equilibrium constants of interactions of SA and CHE with compounds containing mercapto group in our recent electrophoretic study [12] although an unusual electrophoretic behavior of both alkaloids was observed in neutral solutions. A detailed study regarding the electrophoretic behavior of SA and CHE in almost neutral and weakly alkaline solutions [13] identified limited solubility of the alkaloids in these solutions as the primary reason for their anomalous electrophoretic behavior and describes conditions at which reliable electrophoretic measurements with these alkaloids might be performed.

The aim of this study is to extend the CZE method for the pK_{R+} determination of SA and CHE at conditions commonly used for electrophoretic interaction studies. Two different approaches are usually used to overcome difficulties of electrophoretic determination of dissociation constants of compounds with limited aqueous solubility: (i) increasing of detection sensitivity allowing working below the solubility limit of the respective compound [14]; and (ii) measurements with addition of an appropriate organic solvent to the background electrolyte in various concentrations and following extrapolation of the obtained constants to its zero concentration [15]. This work demonstrates that even if precipitation in limited extent occurs in the capillary reliable dissociation constants may be obtained by CZE. Due to the precipitation of the alkaloids in the capillary followed by slow dissolution of the precipitate, classical CZE method [16,17] was preferred to recently developed pressure-assisted CZE method [14,18,19].

2. Experimental

2.1. Chemicals

Sanguinarine and chelerythrine, isolated from the extract of *Macleya cordata* by preparative liquid chromatography [20], are the gift from the Institute of Medical Chemistry and Biochemistry, Medical Faculty, Palacký University Olomouc (Czech Republic). Their 2 mM stock solutions were prepared using freshly boiled distilled water acidified with hydrochloric acid to pH 5 and stored in the refrigerator.

Stock solutions of running buffers were prepared from 2-(*N*-morpholino)ethanesulfonic acid (MES), 3-(*N*-morpholino)propanesulfonic acid (MOPS), *N*-tris(hydroxymethyl) methyl-3-aminopropanesulfonic acid (TAPS) and 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CAPSO) (all from Sigma–Aldrich). Sodium hydroxide (Penta, Chrudim, Czech Republic) was used for the pH adjustment.

Mesityloxide (MO), 1-aminopyridinium iodide (API) (both Fluka, Buchs, Switzerland) and *N*-2,4-dinitrophenyl-DL-methionine (DNPM, Sigma) were used as the mobility standards. The concentrations of mobility standard stock solutions API, DNPM and MO were 5 mM, 1 mM and 1% (v/v), respectively. All used compounds were of analytical grade purity. Freshly boiled distilled water was used for the dissolving of all compounds used throughout the study.

The final pH of buffers (see Table 1 for the details on the composition) was controlled using a pH-meter model WTW pH 527 (Wissenschaftlich-Technische Werkstätten GmbH, Wilheim, Germany) using WTW SenTix 97T combined electrode.

2.2. Apparatus and conditions

A Beckman P/ACE System 5010 or an analogous Beckman P/ACE System 5510 (Beckman Instruments, Fullerton, CA, USA) equipped with filter UV detector was used for electrophoretic experiments. These two instruments differ in sensitivity being approximately 10 times better for Beckman P/ACE System 5510. The wavelength of 280 nm was selected for the detection as the best accessible alternative. The temperature of the cooling liquid was set at 25 °C. The constant power mode with the power input of 0.4 W was used for the measurements of the pK_{R+} constants in order to keep the temperature inside the capillary constant. In all measurements the analysis was co-electroosmotic, i.e. the cathode was placed at the detector.

Table 1
Used buffers

pH	Buffering acid[a]	C^b (mM)	pH	Buffering acid[a]	C^b (mM)
6	MES	68	8	TAPS	105
6.5	MES	42	8.5	TAPS	54
7	MOPS	78	9	TAPS	38
7.5	MOPS	45	9.5	CAPSO	68
			10	CAPSO	42

[a] pH adjusted by NaOH.
[b] Concentration of the buffering acid in final buffer corresponding to the ionic strength $I_S = 30$ mM. The ionic strength was calculated as $I_S = \sum_i c_i z_i^2$, where c_i and z_i are the concentration and the charge of ion i, respectively.

Fresh uncoated fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 50 cm separation length and 57 cm total length (75 μm i.d. × 375 μm o.d.) was used for all measurements. The new uncoated capillary was activated by successive flushing with 1 M nitric acid (30 min), with water (5 min) and with 1 M sodium hydroxide (30 min). The flushing pressure was 20 psi (ca. 138 kPa). Then, the capillary was flushed under reduced pressure 5 psi (ca. 34.5 kPa) with the background electrolyte (BGE) for 15 min and thereafter conditioned with the BGE until the stabilization of electroosmotic flow, which was measured by the injection of mesityloxide. The capillary was flushed with running buffer for 1 min between analyses and cleaned with 1 M hydrochloric acid and 1 M sodium hydroxide for 15 min before any change in the buffer composition. Stabilized capillary was stored overnight in running buffer. Distilled water was used for the longer storing period.

Sample solutions, injected for 5 s using the injection pressure 5 psi, were prepared just before the measurement from stock solutions. The aliquots of the alkaloid and of mobility standards were diluted with distilled water acidified with hydrochloric acid up to pH 2. The concentration of the alkaloid in the sample varied from 3 to 50 μM. The concentrations of mobility standards API and MO in the sample were 50 μM each.

The pK_{R^+} constants were calculated from the effective mobilities using the method of the Boltzman sigmoid [21] in the form:

$$u_{\text{eff}} = \frac{u - u_0}{1 + \exp(\text{pH} - pK_{R^+}/\text{dpH})} + u_0 \quad (3)$$

where u_{eff} is the effective mobility of the alkaloid and u, u_0 are the ionic mobilities of its charged and uncharged forms, respectively. Evidently, the mobility of the uncharged form is zero and the calculation provides the pK_{R^+} constant of the alkaloid as well as the mobility of its charged form as adjustable parameters giving the best fit.

The effective mobilities of the alkaloids were calculated from their migration times using the method of two mobility standards [22], which suppresses random changes in experimental conditions; the equation has the form:

$$u_x = u_A + (u_B - u_A)\frac{t_B}{t_B - t_A}\frac{t_A - t_x}{t_x} \quad (4)$$

where t_x is the migration time of the alkaloid, u_x is the alkaloid mobility, t_A, t_B are the migration times and u_A, u_B the mobilities of mobility standards A and B, respectively. 1-Aminopyridinium iodide and *N*-2,4-dinitrophenyl-DL-methionine were selected as the cationic and anionic mobility standards, respectively. Their mobilities are constant in the pH range 6–10. The mean mobilities of API and DNPM used in the calculation were 40.76 ± 0.09 and −18.76 ± 0.05, respectively. The migration times of both alkaloids were measured three times at each pH and mean effective mobilities from these measurements were used for the calculation of pK_{R^+}. Mobility data, corrected for electroosmosis, are expressed in $10^{-9}\,m^2\,V^{-1}\,s^{-1}$ units.

3. Results and discussion

Only the uncharged forms of alkaloids SA and CHE are less soluble in water [2] in contrast to their charged forms, which are well soluble. Therefore, the solubility (S) of these alkaloids is pH dependent and lowers with the increasing concentration of the uncharged form in the solution simultaneously with the increasing pH. At such a high pH where the well soluble charged form of the alkaloids disappears the solubility reaches its minimum and become independent of a further increase of pH. This minimum value is a constant giving the solubility of the uncharged form of the alkaloid (S_0). Knowledge of S_0 and pK_{R^+} values of the respective alkaloid allows then the calculation of S at any pH supposing side interactions with the solution constituents are absent.

Numerical data on solubility of SA and CHE are not available. However, first estimations of the solubility of the alkaloids given in the study on electrophoretic behavior of SA and CHE in neutral solutions [13] are in the order of tenth of micromoles in solutions of pH 7.4 being somewhat higher for CHE than for SA. With regard to the reported pK_{R^+} values of SA and CHE [6–9] the solubility of the uncharged form (S_0) is expected to be several micromoles or even lower.

The detection limit for SA and CHE was 50 μM in the instrument accessible for the initial measurements for pK_{R^+} determination. Such detection sensitivity is insufficient for the measurements with alkaloids concentrations below their solubility at higher pH. However, the precipitation of the alkaloids induced by injecting samples of pH 5 and lower into the sodium MOPS buffer of pH 7.4 does not influence the mobility of such a heterogeneous zone in concentration 50 μM and lower according to our previous experiments [13]. The decrease of the sample pH up to 2 made the alkaloid peak narrower and higher but the mobility of its zones did not change measurably in these experiments [13].

The series of biological (or so-called Good) buffers analogical to MOPS, in all cases adjusted to the final pH 6–10 by sodium hydroxide, was therefore chosen for the pK_{R^+} determination of SA and CHE (Table 1). Samples of pH 2 containing alkaloid in the concentration of 50 μM were injected into these buffers and their mobilities serving as a raw data for calculation of pK_{R^+} were recorded.

However, the precipitation in the capillary still could influence the mobilities of the alkaloids at pH > 7.4 where progressively higher concentrations of their less soluble forms are present. If this apprehension is true the pK_{R^+} constants of the alkaloids determined with the samples containing 50 μM of alkaloid will be lower than pK_{R^+} constants determined at identical conditions however with lower concentration of the alkaloid in the sample where the precipitation is less evident or absent. A CZE instrument with the detection limit for SA and CHE of 3 μM was used for the measurements with three different lower concentrations of the alkaloid in the sample 15, 5 and 3 μM, respectively (for comparison the pH dependencies of effective mobilities with 50 a 5 μM of

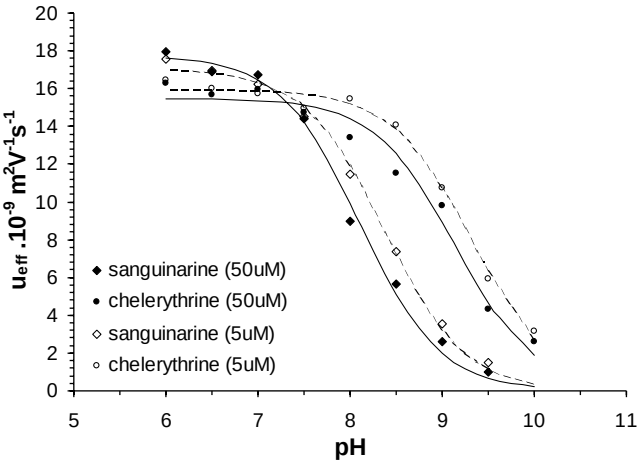

Fig. 2. Comparison of pH dependencies of effective mobilitites of sanguinarine and chelerythrine measured in zwitterionic Good buffers of ionic strength of 30 mM with samples containing alkaloid in the concentration of 50 and 5 μM. The concentrations of the alkaloids are given in the legend.

alkaloids see Fig. 2, the migration of CHE at various pH is shown in Fig. 3). The enormous broadening of MO peaks is probably connected to chemical interactions among the less soluble forms of analyte and some components of BGE could play an important role, too.

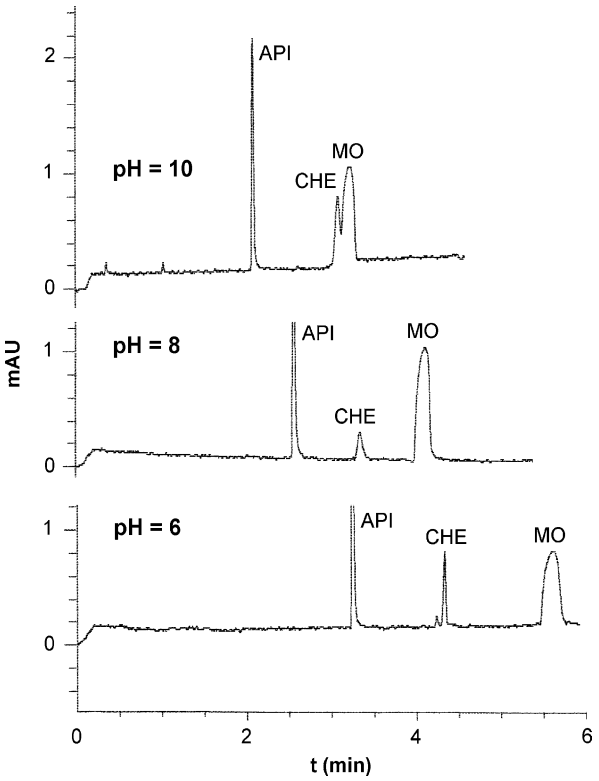

Fig. 3. Comparison of electropherograms of chelerythrine in MES buffer (pH 6), TAPS buffer (pH 8) and CAPSO buffer (pH 10). pH of the buffers is given as a parameter. The concentration of CHE in the sample is 5 μM, sample pH is 2. Mobility standards are 1-aminopyridinium iodide and mesityloxide (MO: as a marker of electroosmosis) at 50 μM each.

Table 2
Calculated pK_{R+} constants and ionic mobilities (u) of sanguinarine and chelerythrine (and their standard deviations) measured with the samples containing various concentration of the alkaloid

$C_{alkaloid}$ (μM)	Sanguinarine		Chelerythrine	
	u	pK_{R+}	u	pK_{R+}
3	17.19 ± 0.59	8.37 ± 0.08	16.06 ± 0.46	9.29 ± 0.08
5	17.06 ± 0.57	8.36 ± 0.08	15.96 ± 0.44	9.31 ± 0.08
15	17.09 ± 0.57	8.38 ± 0.08	15.84 ± 0.45	9.26 ± 0.08
50	17.42 ± 0.62	8.14 ± 0.08	15.30 ± 0.46	9.15 ± 0.09

Indeed, the pK_{R+} constant of SA determined with 50 μM of the alkaloid in the sample is somewhat lower than pK_{R+} constants from the measurements with 15, 5 and 3 μM, which are more or less comparable (Table 2). Therefore, the precipitation of SA in the capillary really affects the determination of its pK_{R+} constant when using samples containing 50 μM of the alkaloid. Only sufficiently low concentration of SA (15 μM and lower) in the sample yields "real" pK_{R+} constant valid in solutions of ionic strength of 30 mM. In the case of CHE all of its measured pK_{R+} constants are comparable (Table 2); thus, the measured pK_{R+} constants are the "real constants" for solutions of ionic strength of 30 mM. Mean pK_{R+} constants of SA and CHE calculated from the determinations not affected by limited solubility, 8.3 ± 0.1 and 9.2 ± 0.1, respectively, may be used as pK_{R+} constants valid in the Good buffers of ionic strength of 30 mM. The values are higher then those determined in static experiments by spectrophotometry and potentiometry that is in agreement with the data in literature [23]. However, general applicability of these pK_{R+} constants could not be recommended because our running experiments indicate their dependence on ionic strength, type of electrolyte and some other factors.

4. Conclusions

Electrophoretic mobilities and shapes of zones of sanguinarine and chelerythrine in nearly neutral and slightly alkaline aqueous media are affected by limited solubility of their uncharged forms and by pH-dependent chemical equilibrium between charged and uncharged forms [13]. The zones of the alkaloids in aqueous medium have the shape of tailed peak with concentration-independent mobility if the injected sanguinarine concentration is low ($\leq$50 μmol l^{-1}).

The presented experiments show that reliable dissociation constants of compounds with one of their coexisting forms less soluble in water may be obtained even if their precipitation in the capillary cannot be entirely eliminated. It is also true in the cases that injected concentration of this compound cannot be reduced due to the insufficient detection sensitivity of instrumentation and the concentration of the compound exceeds its solubility only to a small extent. This is the important finding mainly for the studies of interactions of biologically active substance that

cannot be conducted in the presence of organic solvents very commonly used to improve solubility of species.

The behavior of the alkaloids in aqueous solutions is more complex than may be expected from hitherto communications on their chemical properties since the investigated buffers interact with the alkaloids in very complex way. The complex chemical equilibriums have to be taken into account in studies of biological and/or biochemical activities of the species, especially when phosphate buffers are used [13,24].

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