Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 797 (2003) 357–366
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Electrophoretic investigation of interactions of sanguinarine and chelerythrine with molecules containing mercapto group

Radim Vespalec*, Petr Barták[1], Vilím Šimánek[2], Markéta Vlčková[3]

Institute of Analytical Chemistry, Academy of Sciences of Czech Republic, Veveří 97, CZ-611 42 Brno, Czech Republic

Abstract

Using mercaptoethanol and L-cysteine as representatives of mercapto compounds and capillary zone electrophoresis as experimental technique, it was evidenced that sanguinarine and chelerythrine do not react with the mercapto group of organic compounds at pH 7.4. Their interaction is fast and reversible complexation based on non-bonding intermolecular interaction in which enter uncharged forms of sanguinarine or chelerythrine. A negatively charged group, either bound to the mercapto ligand or supplied from solution, participates in the complexation. Simple 1:1 interaction scheme reported in literature holds therefore only for mercapto compounds bearing anionic group. Stoichiometric binding constants corrected for the abundance of the uncharged alkaloid form at pH 7.4 are of the order of magnitude of 10^4 l/mol and depend on both cations and anions of the background electrolyte. Interaction of sanguinarine and chelerythrine with human or bovine serum albumins does not qualitatively differ from their interaction with simple mercapto compounds. Stoichiometric binding constants for the binding of sanguinarine with human and bovine serum albumins in sodium phosphate buffer pH 7.4, corrected for the abundance of the interacting uncharged form, are $332\,000 \pm 38\,400$ and $141\,000 \pm 14\,400$ l/mol, respectively. The former agrees well with the value $K = 385\,000$ (or $\log K = 5.59$) from static photometric experiments. Constants for the complexation of uncharged chelerythrine with human and bovine serum albumins are $2\,970\,000 \pm 360\,000$ and $1\,380\,000 \pm 22\,600$ l/mol, respectively.
© 2003 Elsevier B.V. All rights reserved.

Keywords: Sanguinarine; Chelerythrine; Benzo[*c*]phenanthridine alkaloids; Albumins

1. Introduction

Benzo[*c*]phenanthridine alkaloids sanguinarine and chelerythrine (Fig. 1) exhibit variety of pronounced biologic effects in vertebrates' bodies [1–6]. Controversial reports on their hepatotoxicity [1,7] made the research of biological activity of these compounds still more attractive. It is clear that their transport to cells and to intracellular target molecules is affected by human serum albumin, disregarding the final effect of sanguinarine and chelerythrine in human body. The identification of the kind of sanguinarine and chelerythrine binding with albumin and its recognizing are therefore important.

The red and yellow colors of sanguinarine and chelerythrine, respectively, are ascribed to their quaternary cations [8,9] (Fig. 1). π-Electron densities on the heterocyclic nitrogen atom in position 5, and on the carbon atom in position 6 in sanguinarine, are 1.660 and 0.646, respectively, according to quantum chemistry calculation [10]. Thus, the double bond N(5)=C(6) of these quaternary cations is polar and sensitive to the attack by nucleophiles [10]. For hydroxide ion as nucleophile, the pH dependent equilibrium between the charged quaternary form and the uncharged, so-called pseudobase form (Fig. 1), is characteristic of both alkaloids. This equilibrium may be formulated as reversible complexation between heterocyclic cation, Q^+ and hydroxide ion:

$$Q^+ + OH^- \leftrightarrow QOH \quad (1)$$

or, more reasonably, as acidobasic equilibrium:

$$Q^+ + H_2O \leftrightarrow QOH + H^+ \quad (2)$$

* Corresponding author. Tel.: +420-53-229-0121; fax: +420-54-121-2113.
E-mail addresses: vespalec@iach.cz (R. Vespalec), bartak@prfnw.upol.cz (P. Barták), vilim@tunw.upol.cz (V. Šimánek).
[1] Present address: Department of Analytical Chemistry, Palacky University, 771 46 Olomouc, Czech Republic.
[2] Present address: Institute of Medical Chemistry and Biochemistry, 775 15 Olomouc, Czech Republic.
[3] Present address: Faculty of Agronomy, Mendel University of Agriculture and Forestry, Zemědělská 1, 616 00 Brno, Czech Republic.

1570-0232/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S1570-0232(03)00308-8

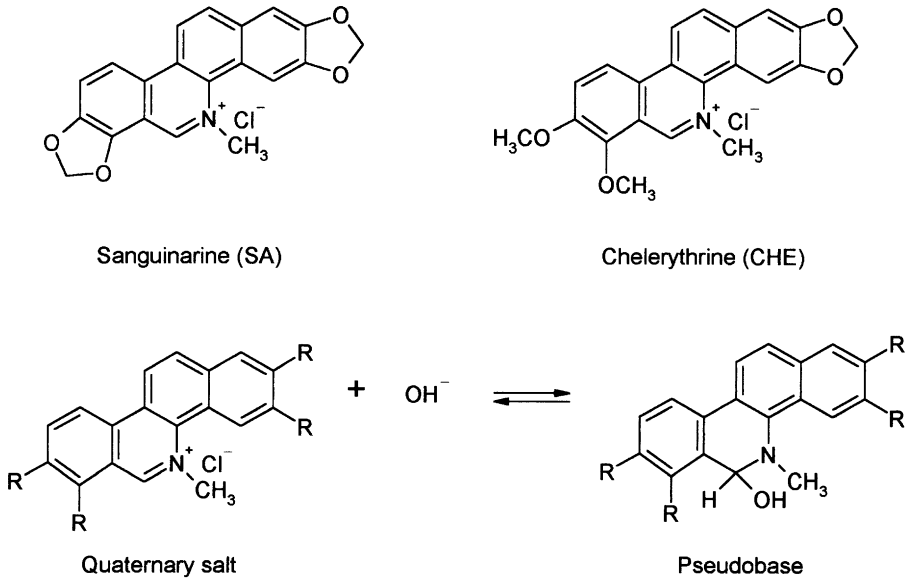

Fig. 1. Structural formulas of sanguinarine and chelerythrine chlorides and the scheme of interaction of their quaternary cations with hydroxyl.

with an equilibrium constant:

$$K_{R+} = [H^+][QOH]/[Q^+] \quad (3)$$

In analogy to Brønsted acids, the pK_{R+} denotes the pH at which the heterocyclic cation and the coupled uncharged pseudobase, QOH, are present in equal concentrations [10]. The alkanolamine structure of pseudobase (Fig. 1) has been generally adopted because quaternary hydroxides cannot exist [11]. The spontaneous formation of ether dimer between C(6) carbon atoms from two molecules of pseudobase has been reported for both sanguinarine and chelerythrine in weakly alkaline aqueous solutions [12]. pK_{R+} constants ranging between 7 and 9 [13–15] have been reported for sanguinarine and chelerythrine in aqueous solutions. Thus, both charged and uncharged forms of these alkaloids exist in more or less comparable concentrations in aqueous solutions and in blood at physiological pH 7.4.

Charged (iminium) form of sanguinarine and chelerythrine has been indicated as the form reacting with nucleophiles including mercapto nucleophile [16]; the latter was deduced from static photometric measurements. Based on this communication, iminium bond of these charged alkaloids is generally considered as the point of their chemical reaction with mercapto group [16–22]. This interpretation dominates in present biochemical research even if unequivocal evidence was not given for it till now to our knowledge, e.g. by the separation of some reaction product. Analogously, chemical reaction leading to a covalent product is considered between the iminium bond of charged sanguinarine or chelerythrine and simple organic reducing agents like cysteine, dithiothreitol or mercaptoethanol [21,22]. In contrast, the uncharged form of sanguinarine was indicated as the form, which interacts with albumin [23]. Thus, not only the identification of the kind of interaction of sanguinarine and chelerythrine with albumins but also the identification of the chemical form of these alkaloids, which enters in the investigated interaction, are important for biochemistry, medicine and related sciences.

We decided to utilize capillary electrophoresis for these purposes. Capillary electrophoresis can identify covalent product of chemical reaction by the separation of the product in discrete zone and differentiate it from a complex or from another interaction product [25]. Capillary electrophoresis is also proper for the investigation of complexation and another non-bonding interactions of dissolved species [25–29].

2. Experimental

2.1. Instrumentation

Beckman P/ACE System 5510 (Beckman Instruments, Fullerton, CA, USA) equipped with the Gold software and filter UV detector served for experiments described in Section 3. Detection wavelength 280 nm was used. Freshly activated uncoated thick-wall fused-silica capillary of 75 μm I.D. and 363 μm, O.D., (Supelco, Bellefonte, PA, USA) of 50 cm separation length and 57 cm total length, thermostated to 25 °C, was used for analyses of interaction and blank mixtures (see Section 3). Then, 100-μl aliquots were taken away and injected into separation capillary for 5 s by the injection pressure 5 p.s.i. 2-[*N*-morpholino]propanesulfonic (MOPS)-sodium buffer of pH 7.4 and $I = 30$ m*M* was background electrolyte. Acetone dissolved in the buffer was injected behind the zone of analyzed sample for the electroosmosis determination.

Electrophoretic set-up used in interaction experiments was based on a Spellman CZE 1000R high-voltage power supply (Plainview, NY, USA), and a Jasco 875 UV–Vis spectropho-

tometer (Tokyo, Japan) adapted for CZE experiments [30]. The adaptation allowed the regulation of temperature of the capillary with circulating liquid with precision better than ± 0.1 °C. An uncoated thin-wall fused-silica capillary (Capillary Columns, Bratislava, Slovak Republic) of 45 cm total length (35 cm separation distance), 75 μm I.D. and 150 μm O.D., was thermostated to 24.0 °C. Voltage applied on the capillary was adjusted with respect to the conductivity of the background electrolyte filling the capillary in order to keep constant the Joule heat input 0.2 W into the capillary. Detection wavelength 280 nm was selected. Hydrodynamic injection of samples by the difference of hydrostatic pressures between the capillary inlet and outlet was used. The length of the injected zone in the separation capillary, controlled by the injection time, was 2–5 mm.

2.2. Chemicals and procedures

Redistilled water was used for the preparation of stock buffers and other solutions. Stock buffers, stored at 4 °C, were prepared from calculated amounts of phosphoric, acetic and MOPS acids. Solid tris-(hydroxymethyl)aminomethane (Tris) or 1 *M* NaOH served for the adjustment of their pH. Concentration of stock buffers of ionic strength $I_s = 30$ m*M* depended on their chemical composition and pH (Table 1). Background electrolyte was prepared daily by dissolving cysteine (Cys), mercaptoethanol (MEt), and human serum albumin (HSA) or bovine serum albumin (BSA) in some stock buffer.

Sanguinarine (SA) and chelerythrine (CHE) were isolated from *Macleaya cordata* by one of the authors (VŠ) [31]. Alkaloids were dissolved in approximately 1 m*M* HCl. Their injected concentrations allowing reasonable detection at 280 nm did not exceed 5×10^{-5} *M*. Cysteine and mercaptoethanol were from Fluka (Buchs, Switzerland). HSA, Cohn fraction V, was from Exbio (Olomouc, Czech Republic), BSA, Cohn fraction V, was from Imuna (Šarišské Michalany, Slovak Republic). Mesityloxide and acetone, which served as the electroosmosis markers, and triphenyltetrazolium bromide, whose cation was the charged mobility standard, were from Sigma (St. Louis, MO, USA). Its diluted aqueous solution was stable up to 1 week. Commercially available chemicals except of HSA and BSA were of reagent grade purity.

Table 1
Background electrolytes of ionic strength $I = 30$ m*M* used in interaction experiments

pH	Buffering compound	*C* [m*M*]	Counterion
5.0	Acetic acid	47	$TrisH^+$
5.0	Acetic acid	47	Na^+
7.4	Phosphoric acid	13.5	$TrisH^+$
7.4	Phosphoric acid	13.5	Na^+
7.4	MOPS	49	$TrisH^+$
7.4	MOPS	49	Na^+
7.4	Tris	36	Acetate

C = concentration of the buffering compound.

The following flushing routine was used for the activation of fresh capillaries: water (60 min), HNO_3 1:1 (10 min), water (10 min), 1 *M* NaOH (60 min), water (10 min), 1 *M* HCl (60 min), water (10 min), 1 *M* NaOH (60 min) and 5 m*M* NaOH (60 min). Activated capillary was stored overnight in background electrolyte. Every morning before experiments and before the change in the buffer composition, the capillary was flushed with 1 *M* NaOH (10 min), water (3 min) and background electrolyte (10 min). First experiment was repeated till the relative standard deviation of three consecutive electroosmosis determinations decreased below 2%. Between analyses, the capillary was washed with background electrolyte for 1 min. After the change in the concentration of cysteine or mercaptoethanol in background electrolyte, the washing time was increased to 10 min. For washing procedure in experiments with albumins (see Section 4.3).

Sanguinarine and chelerythrine were injected as the complexation markers. Their mobilities as well as any other mobility data are given in 10^{-9} m^2/V/s units. The method of two mobility standards [32] with one uncharged mobility standard served for the conversion of their migration times to effective mobilities, μ_{eff}. Mobility of the triphenyltetrazolium cation determined from 16 measurements made within several days was $17.28 \pm 0.04\times10^{-9}$ m^2/V/s at 25 °C in 13.5 m*M* phosphate–Tris buffer of pH 7.40 and $I = 30$ m*M*. Stoichiometric stability constants, *K*, were calculated by a simple linearization procedure [33] based on the standard equation [28,29]:

$$\mu_{eff} = \frac{\mu_A + K\mu_{AX}c}{1 + Kc} \quad (4)$$

transformed for linearization in the form:

$$\mu_{eff} = \frac{1}{K}\left(\frac{\mu_A - \mu_{eff}}{c}\right) + \mu_{AX} \quad (5)$$

μ_A is the mobility of the complexation marker A in the absence of the interacting compound, X, *c* is the total concentration of X in background electrolyte, and μ_{AX} is the mobility of their complex.

3. Kind of interaction of sanguinarine and chelerythrine with mercapto compounds

Mercaptoethanol and cysteine that do not absorb UV light of 280 nm were chosen for the identification of the kind of interaction between simple, low-molecular-mass compounds and benzo[*c*]phenanthridine alkaloids sanguinarine and chelerythrine. The 1:1 interaction of an alkaloid and mercapto compound was considered in agreement with literature [10,16–23]. Sanguinarine or chelerythrine were mixed with mercaptoethanol or with cysteine in 1:2 molar ratio in MOPS-sodium buffer pH 7.4 and $I = 30$ m*M*. Concentration of alkaloids in these interaction mixtures was 0.02 m*M*. Blank mixtures containing either alkaloid or 0.04 m*M* mercapto compound in the buffer were prepared for comparison.

Interaction and blank mixtures, kept at the laboratory temperature, have been repeatedly analyzed within 28 h. Buffer used for preparation of interaction and blank mixtures served as background electrolyte. In another experiment, alkaloids and mercapto compounds were mixed in molar ratio 1:100, reaction mixtures were prepared using MOPS-sodium and phosphate sodium buffers pH 7.4 and $I = 30$ mM, and reaction time was extended to 48 h.

Neither meaningful change in the size of peaks of alkaloids in analyzed reaction mixtures nor additional peak that might be ascribed to some reaction product have been observed disregarding the dissolved alkaloid and mercapto compound added to it. This evidences that investigated alkaloids and mercapto compounds do not react at used experimental conditions.

In order to check the complexation of investigated alkaloids with mercaptoethanol or with cysteine, mercaptoethanol or cysteine was dissolved in two different millimolar concentrations in MOPS-sodium buffer and alkaloids were injected as complexation markers into this background electrolyte. Shift in migration times of alkaloids, dependent on the concentration of dissolved mercapto compound, was observed (Fig. 2).

These results evidence [24–29] that sanguinarine and chelerythrine do not react chemically with low-molecular-mass mercapto compounds at pH 7.4 on forming stable covalent compounds. Their interaction is based on non-bonding intermolecular interactions leading to kinetically labile complexes. Supposing the generally reported 1:1 interaction [10,16–23], the scheme:

$$A + L \leftrightarrow AL \quad (6)$$

where A is an alkaloid, L is a ligand and AL is their complex holds for this complexation. There is no information in literature that interactions of sanguinarine and chelerythrine with mercaptoethanol or with cysteine qualitatively differ from their interactions with another mercapto compounds.

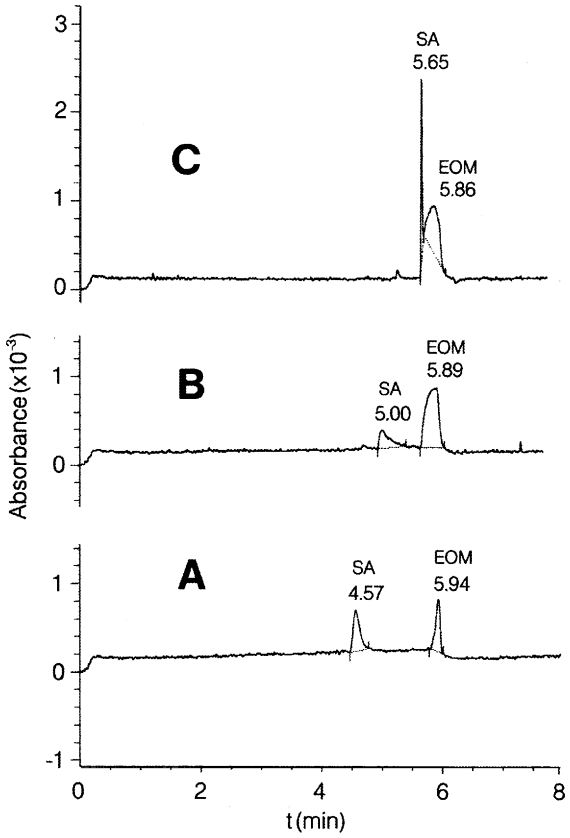

Fig. 2. Migration time of the sanguinarine peak read in its maximum at the absence of cysteine in 30 mM MOPS-sodium buffer pH 7.4 (A) and at the cysteine concentrations 0.5 mM (B) or 5 mM (C) in the buffer. SA = sanguinarine; EOM = electroosmosis marker. Instrument: Beckman P/ACE 5510 with uncoated fused-silica capillary 75 μM I.D. and 365 μM O.D, separation length 50 cm, total length 57 cm. Applied voltage 15 kV.

4. Results and discussion of interaction experiments

Data relating to so-called biological conditions, modeled by phosphate buffers of pH 7.4, are preferred in biochemistry. pI of human and bovine serum albumins vary around pH 5 depending on the ionic strength of liquid medium [34]. Mobility of isoelectric albumin is zero and its pronounced adsorption [35] on the capillary wall eliminates electroosmosis according to our experiments. Difficulties resulting from the simultaneous existence of the fractions of dissolved albumin, which is carried by electroosmosis, and adsorbed, immobile albumin are therefore absent at pH 5. Consequently, pH 7.4 and pH 5 were chosen for our experiments. Raw effective mobilities of alkaloids for the calculation of stability constants were measured at constant temperature and in liquid media of constant ionic strength [28,36]. The needs to keep sufficient buffering capacity of used buffers (Table 1) and to minimize their electric conductivity gave ionic strength $I_s = 30$ mM as reasonable compromise. Temperature difference between the capillary center and outer capillary wall was estimated to approximately 1 °C if the Joule heat input was 0.2 W [37,38]. As the outer capillary wall was thermostated to 24 °C, calculated constants are relevant to standard temperature 25 °C. Complex formation in the 1:1 ratio [10,16–23] was assumed.

4.1. Determination of interacting forms of alkaloids

The complexation of sanguinarine or chelerythrine with mercaptoethanol or cysteine was investigated photometricly [16]. Mercaptoethanol and cysteine were therefore chosen for our electrophoretic experiments. Reported pK_{R+} constants for equilibrium between the charged and uncharged forms of sanguinarine and chelerythrine in aqueous solutions range from 7.32 to 9.00 depending on the compound and on the experimental technique used [13–15] (Table 2). It is evident from published pK_{R+} constants that charged and uncharged forms of sanguinarine and chelerythrine coexist at pH 7.4 in more or less comparable concentrations.

Table 2
Reported pK_{R^+} constants for equilibria between charged and uncharged forms of sanguinarine and chelerythrine in aqueous solutions

Method	SA	CHE	Comment	Ref.
Spectrometry	8.05 ± 0.15	9.0 ± 0.2	Davies buffer; ionic strength not given	[13]
Fluorometry	7.95 ± 0.15	8.9 ± 0.2	Davies buffer; ionic strength not given	[13]
Fluorometry	7.92 ± 0.08	8.77 ± 0.07	$I = 100$ mM	[14]
Potentiometry	7.32 ± 0:07	7.53 ± 0.06	Without electrolytes	[15]
Capillary electrophoresis	8.10 ± 0.08	9.14 ± 0.08	Buffers $I = 30$ mM	[39]

At pH 5.0, the fractions of uncharged forms of these alkaloids are below 0.1%. The influence of the ionic strength of the used liquid medium and, perhaps, its composition may be the reasons for the spread of published values. Therefore, electrophoretically determined pK_{R^+} values 8.10 and 9.14 for sanguinarine and chelerythrine, respectively, found in buffers of $I_s = 30$ mM [39], have been used as the best values in our study.

Acetate buffers are reasonable alternative for phosphate buffers that lose buffering capacity at pH 5.0. However, acetate anion reportedly interacts as nucleophile with the charged form of investigated alkaloids [10]. In order to estimate influence of this interaction on the investigated complexation, conditional stoichiometric binding constants were measured in acetate–Tris buffer at pH 7.4, too (Table 3). No deceleration of sanguinarine and chelerythrine was measurable at pH 5.0 with concentrations of cysteine and mercaptoethanol that decelerate markedly both alkaloids at pH 7.4. Concentrations higher by two orders of magnitude were necessary to achieve measurable effect. Constants lower by two orders of magnitude are therefore obtained from Eq. (5) at pH 5 (Table 3). Decrease in calculated constants correlates with the decrease of concentrations of uncharged forms of these alkaloids if pH changes from 7.4 to 5.0. Conditional constant at pH 5 should be therefore ascribed to the residual fraction of uncharged sanguinarine or chelerythrine at pH 5. These results evidence that the uncharged form of these alkaloids is the single form that interacts with the mercapto group of simple organic compounds.

Mercaptoethanol is electrophoreticly uncharged in the pH range 5–7.4. Zero mobility of its complexes with the uncharged, pseudobase form of sanguinarine and chelerythrine must be therefore expected. Mobility of zones of sanguinarine and chelerythrine should approach zero with the increasing concentration of mercaptoethanol in background electrolyte. Surprisingly, the change of the cationic migration of these alkaloids to the anionic migration was observed in experiments with the highest concentrations of mercaptoethanol at pH 7.4 (Fig. 3). Such a change evidences that the creating complex is negative.

The zone of an alkaloid, which migrates at pH 7.4 in solution with mercaptoethanol, consists of three constituents. The uncharged form of an alkaloid, A, is bound by acidobasic equilibrium (2) with the charged form of the alkaloid, A^+. Simultaneously, the uncharged alkaloid is transformed to some complex with mercaptoethanol, supposedly to the complex AL by equilibrium (6). Consequently, these forms are not separable each from the other and effective mobility of their mixed zone, μ_{eff}, is [40,41]:

$$\mu_{eff} = \mu_{A^+}x_{A^+} + \mu_A x_A + \mu_{AL}x_{AL} \quad (7)$$

μ_{A^+}, μ_A, μ_{AL} and x_{A^+}, x_A, x_{AL} are mobilities and molar fractions of coexisting chemical forms of the compound A, A^+, A, AL, respectively. Mobility of uncharged A, μ_A, is zero. Experimentally found anionic migration of the mixed zone of compound A therefore requires negatively charged complex AL and the domination of its contribution over the contribution of the positively charged alkaloid form ($|\mu_{AL}|x_{AL} > \mu_{A^+}x_{A^+}$). Negatively charged complex can result from the complexation of uncharged alkaloid and mercaptoethanol only if an additional negative charge participates in its formation. It follows from coupled equilibria (2) and (6) that the complexation of A with the ligand L decreases x_{A^+}. The decrease of x_{A^+} and the increase of x_{AL} become more pronounced with the increasing concentration of mercaptoethanol (ligand L). Almost identical extrapolated mobilities of complexes of sanguinarine or

Table 3
Effects of pH and composition of background electrolyte on conditional binding constants for the complexation of sanguinarine or chelerythrine with cysteine or mercaptoethanol, K_c, and on mobilities of formed complexes, μ_{AX}, obtained from Eq. (5)

Ligand	Buffer	Sanguinarine		Chelerythrine	
		K_c	μ_{AX}	K_c	μ_{AX}
Cysteine	Acetate–Tris pH 5.0	8.9 ± 0.9	−0.02 ± 1.1	3.0 ± 1.2	1.5 ± 1.2
Cysteine	Acetate–Tris pH 7.4	1430 ± 160	0.72 ± 0.8	550 ± 90	1.6 ± 0.7
Cysteine	Phosphate–Tris pH 7.4	2970 ± 340	0.62 ± 0.9	385 ± 230	1.1 ± 1.1
Mercaptoethanol	Acetate–Tris pH 5.0	10.1 ± 0.8	−4.4 ± 1.2	2.1 ± 0.3	−5.0 ± 2.0
Mercaptoethanol	Acetate–Tris pH 7.4	3570 ± 170	−8.8 ± 0.4	370 ± 22	−8.1 ± 0.6
Mercaptoethanol	Phosphate–Tris pH 7.4	2080 ± 95	−7.6 ± 0.6	260 ± 24	−8.6 ± 1.1

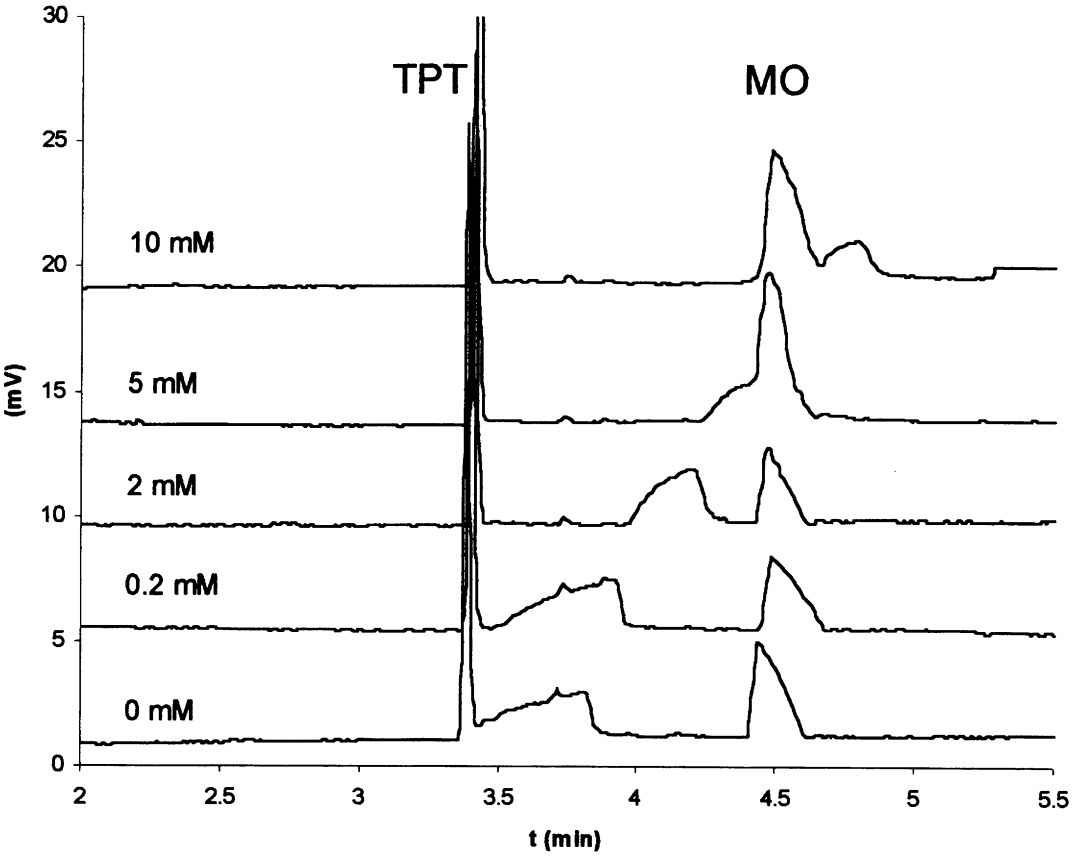

Fig. 3. Changes in the shape and migration time of the chelerythrine zone with the increasing concentration of the mercaptoethanol, given as parameter, in 13.5 m*M* phosphate–Tris buffer pH 7.4. TPT = triphenyltetrazolium cation; MO = mesityloxide. Instrument: laboratory set-up. For other details, see Section 2.

chelerythrine with mercaptoethanol in Table 4, close to −8, support the presented explanation.

With cysteine as the ligand, effective mobilities of both alkaloids approached zero with the increasing cysteine concentration. Different migration behavior of complexes of cysteine and mercaptoethanol may be explained by the supposition that the carboxylic group of the cysteine zwitterion supplies the necessary negative charge. In this case, the

Table 4
Parameters and constants characterizing the complexation of sanguinarine (a) or chelerythrine (b) with cysteine or mercaptoethanol in buffers of pH 7.4 and $I = 30$ m*M*

Ligand	Buffer	μ_{EOF}	μ_{eff}	K_c	K	μ_{AX}
(a) Sanguinarine						
Cysteine	Acetate–Tris	45.6 ± 0.2	13.6 ± 0.8	1430 ± 160	8600 ± 960	0.72 ± 0.8
Cysteine	Phosphate–Tris	52.2 ± 0.2	13.0 ± 0.3	2970 ± 320	17 900 ± 1930	0.62 ± 0.9
Cysteine	Phosphate–Na	65.3 ± 0.3	13.0 ± 0.4	1520 ± 150	9020 ± 890	0.18 ± 0.8
Cysteine	MOPS–Tris	43.7 ± 0.2	15.4 ± 0.4	3000 ± 350	18 000 ± 2100	1.5 ± 0.7
Cysteine	MOPS–Na	49.9 ± 0.2	15.3 ± 0.2	1400 ± 160	8420 ± 960	−1.6 ± 1.4
Mercaptoethanol	Acetate–Tris	45.6 ± 0.2	13.6 ± 0.8	3570 ± 170	21 500 ± 12 800	−8.8 ± 0.4
Mercaptoethanol	Phosphate–Tris	52.2 ± 0.2	13.0 ± 0.3	2080 ± 95	12 500 ± 570	−7.6 ± 0.6
(b) Chelerythrine						
Cysteine	Acetate–Tris	45.6 ± 0.2	12.0 ± 0.2	550 ± 90	30 800 ± 5040	1.6 ± 0.7
Cysteine	Phosphate–Tris	52.2 ± 0.2	11.0 ± 0.3	385 ± 230	21 500 ± 12 800	1.1 ± 1.1
Mercaptoethanol	Acetate–Tris	45.6 ± 0.2	12.0 ± 0.2	370 ± 22	20 700 ± 1230	−8.1 ± 0.6
Mercaptoethanol	Phosphate–Tris	52.2 ± 0.2	11.0 ± 0.3	260 ± 24	14 600 ± 1350	−8.6 ± 1.1

For details, see text.

Key of symbols: μ_{EOF} = electroosmotic flow; μ_{eff} = effective mobility in the used buffer at the absence of the interacting ligand; K_c = conditional binding constant obtained from effective mobilities measured at varying concentrations of the ligand using the linearization procedure [33] and Eq. (5); K = conditional binding constant, K_c = corrected for the abundance of the interacting pseudobase form using Eq. (8); μ_{AX} = mobility of the respective alkaloid–ligand complex obtained from Eq. (5) simultaneously with K_c.

resulting complex remains outwardly uncharged and effective mobility of the mixed alkaloid zone, μ_{eff}, approaches zero with increasing x_{AL}. If the negatively charged group is absent in the ligand, negative charge must be supplied from solution. In this case, the complex is negative.

Our electrophoretic experiments therefore evidence that the simple 1:1 interaction scheme, which is generally supposed in biochemistry [10,16–23], holds only for mercapto compounds bearing at least one anionic group. Albumins belong to such compounds.

4.2. Stability of complexes with mercaptoethanol and cysteine

Conditional binding constants measured in acetate–Tris and phosphate–Tris buffers of identical ionic strength (Table 3) proved the expected influence of the acetate anion on the stability of resulting complexes. The effect of the buffer composition was therefore investigated widely (Tables 3 and 4). Sodium was included as the usual alternative of Tris in buffers commonly used in biochemical studies. Pronounced influence of buffer cations on the complex stability was found in addition to the expected influence of buffer anions. This result and the magnitude of binding constants in Table 4 cast doubt on reported [10] nucleophilic interaction of the acetate ion with quaternary forms of sanguinarine and chelerythrine.

In order to avoid the distortion of conditional constants by the abundance of uncharged sanguinarine or chelerythrine, constants relevant to pH 7.4 have been corrected using the method of side-reaction coefficients [42,43] (Table 4). Side-reaction coefficient for alkaloid A, $\alpha_{A(H)}$, is:

$$\alpha_{A(H)} = 1 + \frac{[H^+]}{K_{R^+}} \quad (8)$$

because cysteine and mercaptoethanol do not interact with the charged form of both alkaloids as evidenced in Section 4.1. The correction coefficients, $\alpha_{A(H)}$, at pH 7.4 are $\alpha_{SA(H)} = 6.012$ and $\alpha_{CHE(H)} = 55.95$, for pK_{R^+} constants of sanguinarine and chelerythrine, 8.10 and 9.14, respectively.

Constants corrected in this way remain dependent on the composition of background electrolyte (Table 4). Side-interactions of the alkaloids with buffer constituents may be the reason as indicates the dependence of mobilities of alkaloids on the composition of background electrolyte (Table 4). It is worth of stressing that these processes take part in buffers whose constituents are commonly considered indifferent. The dependence of electroosmosis on the composition of buffers having identical ionic strength, included in Table 4, is another indication that indifferent buffers are scarce.

4.3. Complexation with albumins

Albumins adsorb on fused-silica at any pH relevant to electrophoretic experiments [35,44–46]. Adsorption increases mean albumin concentration in the capillary above the albumin concentration in background electrolyte, which enters the capillary [44–46], decreases the UV-transparency of the capillary and affects electroosmosis. We tried to determine the amount of HSA or BSA adsorbed on the inner wall of the fused-silica capillary from the delay of the albumin front to the front of mesityloxide dissolved in the albumin solution. No time difference was measurable at pH 7.4 with albumin concentrations that decelerated both alkaloids. However, the decrease of electroosmosis with the increasing concentration of albumins was easily measurable (Fig. 4). The decrease was almost constant for albumin concentrations exceeding 100 μ*M*. This implies that the increase of the adsorbed amount of albumin, or more precisely, the increase in the coverage of the inner capillary wall, with the increasing albumin concentration is negligible above 100 μ*M* albumin concentration at pH 7.4. Electroosmosis is not measurable in capillary coated with albumin at pH 5.0. This evidences that the layer of the electrophoretically uncharged albumin fully shields the surface charge of the capillary. The layer must be therefore dense and should consists at least of one monolayer of albumin macromolecules [35]. The albumin mobility was -18×10^{-9} $m^2/V/s$ at pH 7.4. Simultaneously, electroosmotic coefficient in the capillary coated with albumin was $+18\times10^{-9}$ $m^2/V/s$ at pH 7.4. Dissolved albumin does not therefore move with respect to the capillary wall at pH 7.4 equally with pH 5.

After flushing the 75 μm I.D. capillary with 100 μ*M* albumin pH 7.4, the UV-light absorption caused by adsorbed albumin equals that caused by approximately 0.5 μ*M* dissolved albumin. Correction for adsorbed albumin at any other concentration of dissolved albumin may be estimated from this limiting absorbance assuming that both the residual UV-light absorbance and the electroosmosis drop are directly proportional to the coverage of the capillary wall with albumin. Dashed line in Fig. 4 gives the limiting electroosmosis for such a correction. Correction determined in this way was unimportant starting from 15 μ*M* dissolved albumin. At pH 5.0, the concentration dependence of the electroosmosis drop was stronger (Fig. 4) and the UV-light absorption by adsorbed albumin corresponded to the light absorption of approximately 1.7 μm dissolved albumin. The limiting adsorbed layer was so stable at any used pH that at least 5–6 analyses, which included flushing with BGE for 1–2 min, were possible without measurable change in electroosmosis in difference to observations of Yang and Hage [44]. Pretreatment of capillary or properties of used albumins are possible reasons for this difference.

The formation of the adsorbed layer is fast. Coating times above 5 min usually affect neither electroosmosis nor the UV-light absorption disregarding the albumin concentration in solution. In order to safely standardize electroosmosis, the pre-coating of the capillary wall with 100 μ*M* albumin for 10 min was included in our experimental routine. Activated capillary was washed consecutively with 1 *M* NaOH for 10 min, with water for 3 min and with 100 μ*M* albumin

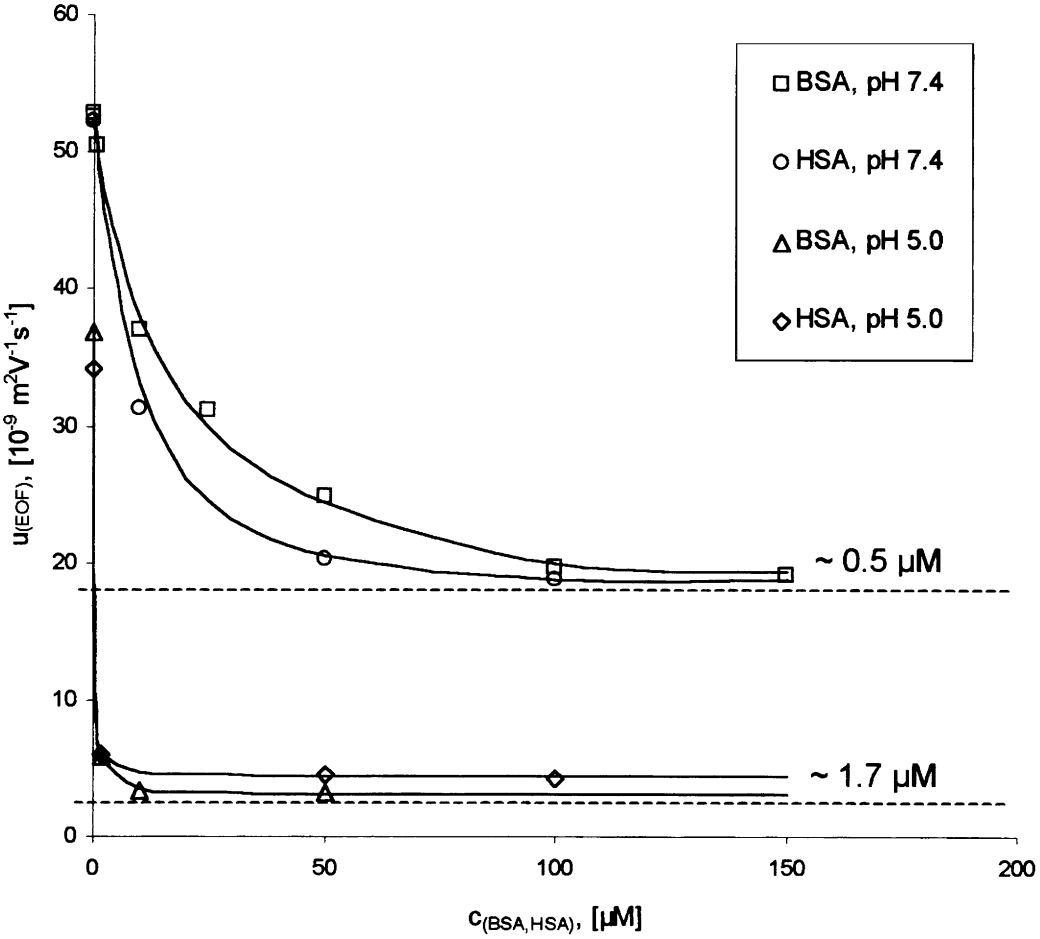

Fig. 4. The dependence of electroosmostic coefficient on the concentration of human or bovine serum albumins in background electrolytes of pH 7.4 and 5.0. Dashed lines are limiting electroosmostic coefficients estimated from experimental data. Concentration at these lines gives limiting increase in the mean concentration of dissolved albumin inside the separation capillary 75 μm I.D., which is caused by albumin adsorbed on the capillary wall. For details, see text.

dissolved in background electrolyte. If electroosmosis was unstable after this 10 min coating, the routine was repeated.

Mobility of alkaloids is identical in the uncoated capillary and in the albumin-coated capillary at pH 7.4. At pH 5.0, mobility of alkaloids is higher by 1.5 mobility unit in the albumin-coated capillary. This observation may be explained by identical coulombic interactions of the quaternary fraction of an alkaloid with unshielded negative charges of the capillary wall and with adsorbed albumin at pH 7.4. At pH 5≈pK_I, this retarding coulombic interaction is absent because the dense layer of uncharged albumin on the capillary wall shields the capillary charge completely. Mobility of sanguinarine and chelerythrine peaks in background electrolytes containing human and serum albumins depended on the concentration of albumins. It evidences that interaction of each of the alkaloids with HSA and BSA is reversible, rapid complexation. If the concentration of injected alkaloid was higher than approximately 0.02 m*M*, its peaks become narrower and more symmetrical with increasing concentrations of albumins like in experiments with cysteine and mercaptoethanol. This improvement of the peak shape may be explained by the complexation of the loosely soluble uncharged alkaloid form [12] with albumins.

In order to check the interaction of the charged form of sanguinarine and chelerythrine with albumins, their complexation was measured at pH 5. It was found that albumins interact much less with both alkaloids at pH 5 identically with cysteine and mercaptoethanol. For example, 100 μ*M* albumins decelerated the alkaloids by approximately 10% at pH 5.0. At pH 7.4, mobilities of the alkaloids dropped by 50% in 40 μ*M* albumins. This implies stability constants more than one order of magnitude lower at pH 5.0 than at pH 7.4. Therefore, at least millimolar concentrations of albumins have to be used at pH 5.0 in the measurement of raw effective mobilities of alkaloids for the stability constant calculation. So high concentrations of albumin cause pronounced experimental difficulties. Triphenyltetrazolium cation chosen as the charged mobility standard complexes with albumins. Stability constant of approximately 200 l/mol was estimated for this complexation. The hyperbolic decrease of effective mobilities of an interaction marker with the increasing concentration of the other complex constituent

Table 5
Binding constants for the interaction of uncharged pseudobase form of sanguinarine and chelerythrine with human and bovine serum albumins obtained from effective mobilities measured in 13.5 m*M* phosphate–Tris buffer, pH 7.4 ($I = 30$ m*M*)

Alkaloid	Ligand	K_c	K	log K_c	log K
Sanguinarine	BSA	23 500 ± 2400	141 000 ± 14 400	4.37	5.15
Sanguinarine	HSA	55 200 ± 6400	332 000 ± 38 400	4.74	5.52
Chelerythrine	BSA	27 400 ± 4500	1 380 000 ± 22 600	4.39	6.14
Chelerythrine	HSA	53 000 ± 7200	2 970 000 ± 360 000	4.72	6.47

Key of symbols: K_c = conditional binding constant obtained by the linearization procedure [33] and Eq. (5) from effective mobilities measured at the varying concentrations of the ligand; K = binding constant; K_c = corrected for the abundance of the interacting pseudobase form using Eq. (8). Logarithmic form of the constants are given only for their mean values for the sake of simplicity.

in BGE is standard if its deceleration is caused only by fast, reversible complexation. Approximately linear decrease of effective mobilities of sanguinarine and chelerythrine with increasing concentration of albumins at pH 5.0 indicates either a side effect or a contribution of another decelerating mechanism. This conclusion is supported by the impossibility to fit measured effective mobilities with Eq. (5) [33]. Therefore, the determination of binding constants was omitted at pH 5.0. Disregarding of this, only the pseudobase form of sanguinarine and chelerythrine should be identified as the form interacting with the mercapto group of albumins for reasons given in Section 4.2.

The effect of the buffer composition on conditional stability constants, observed with L-cysteine and mercaptoethanol, was also found with albumins. Constants obtained from linearized dependencies of effective mobilities of sanguinarine and chelerythrine on the HSA or BSA concentration in phosphate buffer pH 7.4 have been corrected for the abundance of their uncharged form (Table 5). Such a correction supplies conditional stability constants exceeding by two orders of magnitude constants for the interaction of uncharged alkaloids with simple mercapto compounds. Only the data relevant to 13.5 m*M* phosphate–Tris buffer ($I = 30$ m*M*) are in Table 5 owing to both the preference of phosphate buffers in biological assays and better electroosmosis stability in Tris buffers. Comparable data exist in the literature only for the statically investigated interaction of sanguinarine with human serum albumin [23]. Our conditional stability constant for interaction of sanguinarine with HSA, $K = 55\,200$ (or $\log K = 4.74$), corrected for the abundance of the interacting pseudobase forms by the method of side-reaction coefficients [42,43] gives $K = 331\,000$ (or $\log K = 5.52$). This value nicely agrees with the stability constant for the interaction of the pseudobase form of sanguinarine with human serum albumin, $K = 385\,000$ (or $\log K = 5.59$). No meaningful difference is reported in Ref. [23] in constants obtained with human serum albumin containing fatty acids and with albumin free of fatty acids.

5. Concluding remarks

Our results evidence that all investigated interactions must be classified as fast, reversible complexation disregarding the size and complexity of the mercapto compound. No chemical reaction between investigated constituents took place in aqueous solutions at room temperature and pH 7.4. The pH dependencies of electrophoretically determined conditional binding constants for the interaction of sanguinarine and chelerythrine with low-molecular-mass compounds bearing mercapto group, and with albumins evidence that only the uncharged form of the sanguinarine and chelerythrine interacts with mercapto group. These findings agree with the identification of the interacting form of sanguinarine in Ref. [23]. The simple, previously reported 1:1 complexation [10,16–23] is relevant to the ratio of an alkaloid and of a mercapto ligand with single mercapto group in the resulting complex. However, if the total complex composition is considered, the 1:1 ratio holds only for ligands with single mercapto group and with negatively charged group, which is located in such a way that it may participate in the complex formation. Calculated constants depend on both cations and anions present in the solution. Explanation of this dependence is not possible from our experiments.

Table 6
The comparison of conditional binding constants for the interaction of sanguinarine with human serum albumin from statistical measurement, $K_{c,exp}$, reported in Ref. [24], with constants $K_{c,calc}$, calculated by the method of side-reaction coefficients [41,42]

pH	$K_{c,exp}$	$K_{c,calc}$	log($K_{c,calc}$)	log($K_{c,exp}$)
5.4	1220	659	3.09	2.82
6.0	2190	2610	3.34	3.42
6.5	5200	8110	3.72	3.91
7.0	12 300	24 400	4.09	4.39
7.5	28 100	66 500	4.45	4.82
8.0	111 000	147 000	5.05	5.17
8.5	164 000	237 000	5.22	5.37
8.7	198 000	264 000	5.30	5.42
9.0	225 000	293 000	5.35	5.47
7.4	28 600 ± 500	55 000 ± 6400	4.46	4.74

For details, see text.
$K_{c,exp}$ values relate to Ref. [24]. Only binding constant at pH 7.4 and its error are given in Ref. [24]. Other $K_{c,exp}$ values in the table have been read from the graph published in Ref. [24] in which the error of experimental points is not given. $K_{c,calc}$ data relate to electrophoretic interaction measurements. In $K_{c,calc}$ data, only the value relevant to pH 7.4 was calculated using Eq. (5) from experimentally measured raw effective mobilities; other $K_{c,calc}$ values have been obtained from it by using Eq. (8). Error is therefore given only for binding constant relevant to pH 7.4.

If the interacting forms of the complex constituents are known, the method of side-reaction coefficients [41,42] allows the transformation of the conditional stability constant, measured at some pH, to that valid at any other pH (Table 6). The determination of the stability constant relevant to some particular form of an alkaloid is a . . . . . text.

Conditional binding constants obtained for the complexation of sanguinarine and chelerythrine with simple mercapto compounds at pH 5 reveal remarkable potential of capillary electrophoresis for the finding out and for the investigation of interactions of very low fractions of dissolved species. In our study, such constants have been obtained for the interacting fraction of the order of 10^{-5} if binding constant was 31 000 l/mol. It is reasonable to expect that complexation of still lower fractions of dissolved species may be identified and investigated for more stable complexes.

Acknowledgements

The research was supported by the Grant Agency of the Academy of Sciences of the Czech Republic, grant No. A-4031703, by the Grant Agency of the Czech Republic, grant No. 203/02/0023, by Ministry of Education, Youth and Sport, grant No. 151 100003, and by Internal Grant Agency of Ministry of Health of the Czech Republic, grant No. NL 5267-3/1999.

References

[1] C. Vavrečková, J. Ulrichová, Chem. Listy (in Czech) 88 (1994) 238.
[2] J. Ulrichová, J. Lenfeld, M. Cushman, P. Mohan, V. Šimánek, Acta Univ. Palacki Olomouc Fac. Med. (Olomouc) 113 (1986) 401.
[3] B.D. Krane, M.O. Fagbule, M. Shamma, B. Gözler, J. Nat. Prod. 47 (1984) 1.
[4] D. Walterová, J. Ulrichová, L. Válka, J. Vičar, C. Vavrečková, E. Táborská, R.J. Harkrader, D.L. Meyer, H. Černám, V. Šimánek, Acta Univ. Palacki Olomouc Fac. Med. (Olomouc) 139 (1995) 7.
[5] M. Wink, T. Schmeller, B. Latz-Bruning, J. Chem. Ecol. 24 (1998) 1881.
[6] J. Ulrichová, D. Walterová, C. Vavrečková, V. Kamarád, V. Šimánek, Phytother. Res. 10 (1996) 220.
[7] M. Stiborová, V. Šimánek, E. Frei, P. Hobza, J. Ulrichová, Chem.-Biol. Interact. 287 (2002) 231.
[8] E. Späth, F. Kuffner, Ber. Dtsch. Chem. Ges. 64 (1931) 370.
[9] E. Späth, F. Kuffner, Ber. Dtsch. Chem. Ges. 64 (1931) 1123.
[10] J. Dostál, M. Potáček, Collect. Czech. Chem. Commun. 55 (1990) 2840.
[11] V. Šimánek, Benzophenanthridine alkaloids, in: A. Brossi (Ed.), The Alkaloids, Vol. 26, Academic Press, Orlando, FL, 1985, p. 185.
[12] P. Sečkářová, R. Marek, J. Dostál, R. Dommisse, E.L. Esmans, Magn. Reson. Chem. 40 (2002) 147.
[13] J. Kovář, K. Šimek, E. Kožoušková, H. Klukanová, J. Slavík, Collect. Czech. Chem. Commun. 50 (1985) 1312.
[14] D. Walterová, V. Preininger, F. Grambal, V. Šimánek, F. Šantavý, Heterocycles 14 (1980) 597.
[15] M.E. Perelson, I.V. Persiyanova, T.S. Semenova, I.E. Konylova, Khim. Prirod. Soedin. (in Russian) (1984) 337.
[16] D. Walterová, J. Ulrichová, V. Preininger, V. Šimánek, J. Lenfeld, J. Lasovský, J. Med. Chem. 24 (1981) 1100.
[17] J. Wolff, L. Knipling, Biochemistry 32 (1993) 13334.
[18] L. Luhová, I. Frebort, J. Ulrichová, O. Adachi, V. Šimánek, P. Pec, J. Enzyme Inhib. 9 (1995) 295.
[19] M.D. Fadeeva, T.N. Beliaeva, Tsitologiya (in Russian) 30 (1988) 685.
[20] M.D. Fadeeva, T.N. Beliaeva, Tsitologiya (in Russian) 29 (1987) 576.
[21] L.B. Lombardini, C. Propps, Biochem. Pharmacol. 51 (1961) 151.
[22] M.M. Chaturvedi, A. Kumar, B.G. Darnay, G.B.N. Chiny, S. Agarwal, B.B. Agarwal, J. Biol. Chem. 272 (1997) 30129.
[23] H. Paulová, J. Slavík, Pharmazie 48 (1993) 555.
[24] B. Chankvetadze, Capillary Electrophoresis in Chiral Analysis, Wiley, Chichester, 1997.
[25] P. Camilleri (Ed.), Capillary Electrophoresis: Theory and Practice, 2nd ed, CRC Press, Boca Raton, FL, 1998.
[26] M.H.A. Busch, L.B. Carrels, H.F.M. Boelens, J.C. Kraak, H. Poppe, J. Chromatogr. A 777 (1997) 311.
[27] M.H.A. Busch, J.C. Kraak, H. Poppe, J. Chromatogr. A 777 (1997) 329.
[28] S.G. Pen, T. Bergström, I. Knights, G. Liu, A. Ruddick, D.M. Goodall, J. Phys. Chem. 99 (1995) 3875.
[29] R. Vespalec, P. Boček, J. Chromatogr. A 875 (2000) 431.
[30] P. Barták, P. Bednář, Z. Stránský, P. Boček, R. Vespalec, J. Chromatogr. A 878 (2000) 249.
[31] J. Dostál, E. Táborská, J. Slavík, Fitoterapia 63 (1992) 67.
[32] R. Vespalec, P. Gebauer, P. Boček, Electrophoresis 13 (1992) 677.
[33] P. Barták, P. Bednář, L. Kubáček, Z. Stránský, Anal. Chim. Acta 407 (2000) 327.
[34] T. Peters Jr., Serum albumin, in: F.W. Putnam (Ed.), The Plasma Proteins, 2nd ed, Academic Press, New York, 1975, p. 146.
[35] W. Norde, Proteins at solid surfaces, in: A. Baszkin, W. Norde (Eds.), Physical Chemistry of Biological Interfaces, Marcel Dekker, New York, 2000.
[36] F.J.C. Rossotti, H. Rossotti, Determination of Stability Constants and Other Equilibrium Constants in Solutions, McGraw-Hill, New York, 1961.
[37] J.H. Knox I.H, I.H. Grant, Chromatographia 24 (1987) 135.
[38] S.J. Gluck, K.P. Steele, M.H. Benkö, J. Chromatogr A 745 (1996) 117.
[39] R. Vespalec, M. Vlčková, P. Barták, in preparation.
[40] A. Tiselius, Nova Acta Reg. Soc. Sci. Uppsaliensis 7 (1930) 1.
[41] F. Foret, L. Křivánková, P. Boček, Capillary Zone Electrophoresis, VCH, Weinheim, 1993.
[42] L. Šůcha, S. Kotrlý, Solution Equilibria in Analytical Chemistry, Van Nostrand, London, 1972.
[43] J. Li, K.C. Waldron, Electrophoresis 20 (1999) 171.
[44] J. Yang, D.S. Hage, Anal. Chem. 66 (1994) 2719.
[45] J.K. Towns, F.E. Regnier, Anal. Chem. 63 (1991) 1126.
[46] J.S. Green, J.W. Jorgenson, J. Chromatogr. 478 (1989) 63.