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Interactions of sanguinarine and chelerythrine with molecules containing a mercapto group†

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ABSTRACT: Capillary zone electrophoresis was applied to the investigation of the interaction of sanguinarine and chelerythrine with mercapto compounds including albumins at pH 7.4. Mercaptoethanol and L-cysteine were chosen for the identification of the type of interaction and for the identification of the interacting chemical form of these alkaloids. It was evidenced that sanguinarine and chelerythrine do not react chemically with these mercapto compounds at pH 7.4 and that non-covalent products form in this interaction. Their interaction is a fast and reversible complexation and is based on non-bonding intermolecular interactions. Conditional binding constants measured at pH 7.4 and 5.0 indicate that only uncharged forms of sanguinarine and chelerythrine (pseudobases) participate in complexation. A negatively charged group, either bound to the mercapto ligand or supplied by the solution, enters in the complexation. The simple 1 : 1 interaction scheme holds, therefore, only for mercapto compounds bearing an anionic group. Constants corrected for the abundance of the uncharged alkaloid form are of the order of magnitude of $10^4\,l\,mol^{-1}$ and depend on the chemical composition of buffer. Interaction of sanguinarine and chelerythrine with human or bovine serum albumins is qualitatively identical with interaction of these alkaloids with simple mercapto compounds. Constants for the binding of uncharged form of sanguinarine with human and bovine serum albumins in sodium phosphate buffer at pH 7.4, corrected for abundance of the interacting uncharged form, are $332\,000 \pm 38\,400$ and $141\,000 \pm 14\,400\,l\,mol^{-1}$, respectively. The former agrees well with the value $K = 385\,000$ (or $\log K = 5.59$) reported from static experiments. For the uncharged form of chelerythrine, the constants are $2\,970\,000 \pm 360\,000$ and $1\,380\,000 \pm 22\,600\,l\,mol^{-1}$ for human and bovine serum albumins, respectively. Copyright © 2003 John Wiley & Sons, Ltd.

KEYWORDS: quaternary benzo[*c*]phenanthridines; sanguinarine; chelerythrine; mercapto compounds; albumin; capillary electrophoresis

INTRODUCTION

The quaternary benzo[*c*]phenanthridine alkaloids (QBA) sanguinarine and chelerythrine (Scheme 1) are usually isolated from Fumaraceae and Papaveraceae plants and belong to the elicitor-inducible secondary metabolites.[1] Both alkaloids display a plethora of species- and tissue-specific effects but the molecular basis of their pharmacological activities remains mysterious.[2] One of the prerequisites for their biological activity is the presence of an iminium bond C(6)=N$^+$(5). This bond is susceptible to nucleophilic attack. For hydroxide ion as a nucleophile, a pH-dependent equilibrium between the quaternary cation and the uncharged form, the so-called pseudobase (the 6-hydroxy-5,6-dihydro derivative, Scheme 1) is typical for sanguinarine and chelerythrine.[3] Regarding water protolysis the process may be formulated as a reversible acid–base equilibrium $Q^+ + H_2O \rightleftharpoons QOH + H^+$ with an equilibrium constant $K_{R^+} = [H^+][QOH]/[Q^+]$. In analogy to Brønsted acids, the pK_{R^+} value denotes the pH value at which the cation of alkaloid and pseudobase (the 6-hydroxy derivative) are present in equal concentrations.[4] The alkanolamine structure given in Scheme 1 has been almost universally adopted in aqueous alkaline medium because the quaternary hydroxide cannot exist.[5] The formation of a bimolecular aminoacetal structure from two molecules of pseudobase has been reported both for sanguinarine and chelerythrine only in less polar media (C_6H_6, $CHCl_3$). The data for the pK_{R^+} constants that characterize equilibria between charged and uncharged forms of sanguinarine and chelerythrine in water range between 7 and 9.[6–8] Thus, at physiological pH 7.4, both charged (cation) and uncharged (pseudobase) forms exist

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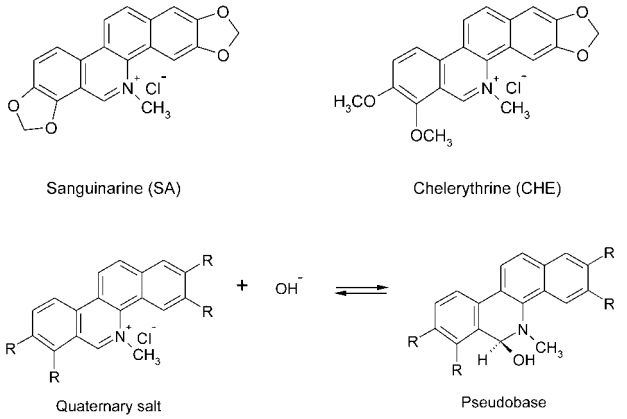


Scheme 1. Structural formulas of sanguinarine and chelerythrine chlorides and the reaction of the quaternary salt with hydroxide ion.

in aqueous solutions and in blood. Sanguinarine and chelerythrine form kinetically labile complexes with SH groups of simple organic compounds in a 1 : 1 ratio.[9] An analogous interaction was suggested with SH-enzymes. The iminium forms of sanguinarine or chelerythrine have been indicated as the form interacting with mercapto nucleophiles. The pseudobase of sanguinarine was denoted as the form interacting with human serum albumin in which a single free SH group is expected to be the interaction point.[10] However, the opinion that the interaction mechanism is a chemical reaction of the iminium bond of the quaternary cation of sanguinarine/chelerythrine with a mercapto group dominates in the recent literature.[11,12]

Disregarding their final biological effects, the transport of both alkaloids to target cells is effected by albumin, the main transport protein of blood. Thus, defining the nature of binding of sanguinarine and chelerythrine with albumin and the determination of the respective binding constants is of high importance. The aims of our study were (i) the unequivocal identification of the type of interaction between sanguinarine/chelerythrine and the mercapto group of various compounds including human and bovine albumins and (ii) the identification of the chemical form of these alkaloids which enters in this interaction. Capillary zone electrophoresis (CZE), which proved to be a powerful experimental technique for the analysis of complex mixtures and for the investigation of various interactions of solution constituents, was chosen as the experimental technique.[13–15]

RESULTS AND DISCUSSION

pH values of 7.4 and pH 5.0 were chosen for the experiments. Data relating to so-called physiological conditions, modeled by phosphate buffers of pH 7.4, are preferred in biochemistry. The p*I* values of human and bovine serum albumins vary around pH 5.0 depending on the ionic strength of liquid medium.[16] The mobility of albumin is zero at this pH value, and pronounced adsorption of isoelectric albumin on the capillary wall[17] eliminates electroosmosis according to our experiments. Raw experimental data for the calculation of stability constants were measured at constant temperature and constant ionic strength.[18] Interaction of alkaloids with mercapto compounds in an 1 : 1 ratio, was assumed.[9]

Type of interaction

Mercaptoethanol and cysteine were chosen for the determination of the type of interaction between a mercapto group and sanguinarine and chelerythrine. Sanguinarine/chelerythrine (0.02 mM) were mixed with mercaptoethanol or cysteine in an 1 : 2 molar ratio using 13.5 mM phosphate buffer and $I = 30$ mM adjusted to pH 7.4 with sodium hydroxide as the aqueous medium. Blank mixtures free of either alkaloid or mercapto compound were prepared for comparison. Interaction and blank mixtures kept at room temperature were repeatedly analyzed. The phosphate buffer that was used for the preparation of mixtures was the background electrolyte.

Electrophoresis separates stable solution constituents into discrete zones according to their mobility. If a reaction between the quaternary form of sanguinarine/chelerythrine and a mercapto compound takes place, at least the molecular weight of this interaction product must differ markedly from that of the alkaloid. Consequently, the mobility of this product must differ from that of the alkaloid present in the analyzed mixture. The alkaloid peak disappears and another peak, which belongs to the reaction product, is detected if the mercapto compound is in excess and if a fast reaction between an alkaloid and a mercapto compound is completed before first analysis. In this case, the product peak is of constant, time-independent height and area. The alkaloid peak decreases with time and the peak of the reaction product rises if the chemical reaction between the alkaloid and the mercapto compound is slower. Mercaptoethanol and cysteine do not absorb at 280 nm and are therefore not detectable.

No change in the size of alkaloid peaks in the analyzed reaction mixtures was observed within 24 h regardless of the dissolved alkaloid and mercapto compound added to it. Peaks of sanguinarine/chelerythrine in analyses of reaction mixtures and in blank mixtures were identical within the limits of the expected experimental error. No additional peak that might be ascribed to a product of a chemical reaction between some alkaloid and some mercapto compound added to it was found in the analyzed reaction mixtures within 24 h. This indicates that no reaction took place between these alkaloids and the added mercapto compounds, mercaptoethanol and cysteine, under these experimental conditions.

In another experiment, alkaloids and mercapto compounds were mixed in a molar ratio of 1 : 100; reaction

mixtures were prepared using 3-[*N*-morpholino]propanesulfonic acid (MOPS) and orthophosphoric acid whose pH was adjusted to 7.4 with sodium hydroxide; the ionic strength of the final buffers was again 30 mM. The reaction time was extended to 48 h. Qualitative results of analyses of both the reaction and blank mixtures remained identical in spite of the longer reaction time, drastically increased molar ratio of mercapto compounds to alkaloids and different compositions of the buffers used. Obviously, there is no chemical reaction.

In order to verify if the investigated alkaloids create kinetically labile complexes with mercaptoethanol or with cysteine, the following check was made. Mercaptoethanol or cysteine was dissolved at two different millimolar concentrations estimated by rule of thumb in MOPS buffer of pH 7.4 and alkaloids were injected as complexing markers into these background electrolytes. A shift in migration time of the injected alkaloid, which was dependent on the identity and concentration of the dissolved mercapto compound, was observed with both alkaloids (Fig. 1). Therefore, it was concluded that these experiments indicate that sanguinarine and chelerythrine do not react chemically at least with these low molecular weight mercapto compounds at pH 7.4 and room temperature. Their interactions are based on non-bonding intermolecular interactions, and kinetically labile complexes are products of these interactions.[13–15] Assuming the generally reported 1 : 1 interaction, the reaction scheme $A + X \rightleftharpoons AX$, holds for the complexation of an alkaloid A with a ligand X containing a mercapto group.[9] To our knowledge, there is no indication in the literature that interactions of mercaptoethanol and cysteine with sanguinarine or chelerythrine differ qualitatively from interactions of other investigated mercapto compounds.

Identification of the interacting form of alkaloids

The complexation of sanguinarine and chelerythrine with mercaptoethanol and cysteine has been investigated photometrically in static experiments.[9] These compounds were therefore used in electrophoretic studies to establish which chemical form of the alkaloids interacts with the mercapto group. Reported pK_{R^+} constants for the 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.[6–8] Therefore, electrophoretically determined pK_{R^+} values of 8.10 and 9.14 for sanguinarine and chelerythrine, respectively, valid for $I_s = 30$ mM, were used in our study.[19] It is evident from published pK_{R^+} constants[6–8] that conditional binding constants, K_c, are measured for complexes of both alkaloids at pH 7.4[15,18] and that the concentration of the uncharged forms of the alkaloids is below 0.1% at pH 5.0. The latter follows from pK_{R^+} values of 8.10 and 9.14 for sanguinarine and chelerythrine, respectively, which are relevant to the conditions of our experiments.[19]

Phosphate buffers lose their buffering capability at pH 5.0 and acetate buffers are their commonly used substitutes in electrophoresis. Nucleophilic attack of acetate ions upon sanguinarine/chelerythrine has been reported.[3] However, our previous experiments[19] evidenced that the influence of acetate ion on the stability constants is comparable to the influence of other common cationic or anionic buffer constituents.

Binding constants between sanguinarine/chelerythrine and mercaptoethanol/cysteine are lower by two orders of magnitude at pH 5 than at pH 7.4 (Table 1). This pH-related decrease of the constants correlates with the decrease in concentration of the pseudobase form of sanguinarine or chelerythrine, respectively, if the pH decreases from 7.4 to 5.0. Constants measured at pH 5 should, therefore, be ascribed to interactions of the resdual concentrations of the pseudobase of sanguinarine/chelerythrine with mercapto compounds dissolved in background electrolytes of pH 5.0.[19] Consequently, the pseudobase form of sanguinarine/chelerythrine is the form that interacts with the mercapto group of mercaptoethanol and cysteine.

Mercaptoethanol is electrophoretically uncharged in the pH range 5.0–7.4. Therefore, zero mobility of its complexes with the pseudobase of sanguinarine/chelerythrine has to be expected. Surprisingly, a change of cationic migration of an alkaloid zone to anionic migra-

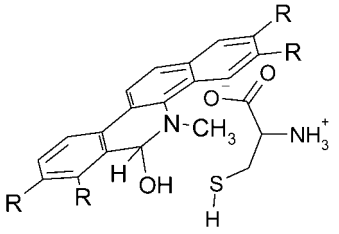
Table 1. Effects of buffer pH and composition on stoichiometric conditional binding constants for the complexation of sanguinarine and chelerythrine with cysteine and mercaptoethanol, K_c, obtained from Eqn (2)

Ligand	Buffer[a]	K_c	
		Sanguinarine	Chelerythrine
Cysteine	Acetate–Tris pH 5.0	8.9 ± 0.9	3.0 ± 1.2
Cysteine	Acetate–Tris pH 7.4	1430 ± 160	550 ± 90
Cysteine	Phosphate–Tris pH 7.4	2970 ± 340	385 ± 230
Mercaptoethanol	Acetate–Tris pH 5.0	10.1 ± 0.8	2.1 ± 0.3
Mercaptoethanol	Acetate–Tris pH 7.4	3570 ± 170	370 ± 22
Mercaptoethanol	Phosphate–Tris pH 7.4	2080 ± 95	260 ± 24

[a] Identified by its cationic and anionic constituents.

tion was observed in interaction experiments with mercaptoethanol at high mercaptoethanol concentrations. Such a change indicates the formation of a negatively charged complex in the investigated interaction. The existence of the complex becomes observable if the complex dominates in the migration of the alkaloid zone.[19] Migration of cysteine complexes remained cationic whatever the cysteine concentration in background electrolyte.

The structure of 1 : 1 complexes of cysteine and mercaptoethanol with uncharged monomeric forms of sanguinarine and chelerythrine was explored and modeled by using the software program HyperChem 2.0. The requirement of minimum total energy in aqueous solution was applied. Reasonable results were obtained for cysteine only. The twisted cysteine molecule, aligned by its carboxylic group to the vicinity of the C(6)—N(5) bond (Scheme 2), was the most probable result for each of the alkaloids. This result is in agreement with the knowledge from experiments with mercaptoethanol that participation of a negative charge is necessary for the complexation of uncharged sanguinarine or chelerythrine with a mercapto group. If cysteine is the ligand, its carboxylic group supplies the necessary negative charge (Scheme 2) and the resulting complex remains outwardly uncharged. If the anionic group is absent in the ligand, e.g. in mercaptoethanol, the negative charge may be supplied from solution only. In this case, the complex is negative. The simple 1 : 1 interaction of an alkaloid, A, with a ligand, X, therefore holds only for mercapto compounds bearing negatively charged groups. Albumins belong to such compounds.



Scheme 2. Schematic diagram of the 'intermolecular saturation' of the electron deficit on N(5) of sanguinarine or chelerythrine by the cysteine carboxylic group for the more probable conformer given by HyperChem 2.0.

In order to avoid the distortion of constants by the abundance of pseudobase of sanguinarine/chelerythrine, constants obtained from the fitting were corrected for charged fractions of the alkaloids at pH 7.4 (Table 2) using the method of side-reaction coefficients.[20,21] In our case, the coefficient $\alpha_{A(H)}$ is

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

because cysteine and mercaptoethanol do not interact with the charged form of sanguinarine/chelerythrine. Correction coefficients $\alpha_{A(H)}$ for sanguinarine and chelerythrine are $\alpha_{SA(H)} = 6.012$ and $\alpha_{CHE(H)} = 55.954$, respectively, at pH 7.4 for pK_{R^+} values of sanguinarine and chelerythrine of 8.10 and 9.14, respectively. However, constants corrected in this way remain conditional because of their dependence on the composition of background electrolyte.[18]

Complexation with albumins

Adsorption of albumins on the capillary wall has to be considered in electrophoretic interaction experiments.[17,22–24] Fortunately, electrophoretic movement of dissolved albumins with respect to adsorbed albumin need not be considered at pH 7.4 and 5.0.[19] Moreover, correction for albumin adsorbed in 75 μm i.d. fused-silica capillaries is unimportant for albumin concentrations of 15 μM and higher.[19] This markedly simplifies interaction experiments with human and bovine serum albumins and their evaluation.

Table 2. Stoichiometric constants characterizing the complexation of sanguinarine and chelerythrine with cysteine and mercaptoethanol in buffers of pH 7.4 and $I = 30$ mM

Alkaloid	Ligand	Buffer[a]	K_c[b]	K[c]
Sanguinarine	Cysteine	Acetate–Tris	1430 ± 160	8600 ± 960
		Phosphate–Tris	2970 ± 320	17 900 ± 1930
		Phosphate–Na	1520 ± 150	9020 ± 890
		MOPS–Tris	3000 ± 350	18 000 ± 2100
		MOPS–Na	1400 ± 160	8420 ± 960
	Mercaptoethanol	Acetate–Tris	3570 ± 170	21 500 ± 12 800
		Phosphate–Tris	2080 ± 95	12 500 ± 570
Chelerythrine	Cysteine	Acetate–Tris	550 ± 90	30 800 ± 5040
		Phosphate–Tris	385 ± 230	21 500 ± 12 800
	Mercaptoethanol	Acetate–Tris	370 ± 22	20 700 ± 1230
		Phosphate–Tris	260 ± 24	14 600 ± 1350

[a] Identified by its cationic and anionic constituents.
[b] K_c: constant obtained from effective mobilities measured at various concentrations of the ligand using the linearization procedure[29,30] based on Eqn (2).
[c] K = constant, K_c, corrected for the abundance of the interacting pseudobase form using Eqn (1).

Table 3. Binding constants for interaction of uncharged pseudobase form of sanguinarine or chelerythrine with human serum albumin and bovine serum albumin in 13.5 mM phosphate–Tris buffer, pH 7.4 ($I = 30$ mM)

Alkaloid	Ligand	K^a	Log $K_c^{b,c}$	Log K^c
Sanguinarine	BSA	141 000 ± 14 400	4.37	5.15
	HSA	332 000 ± 38 400	4.74	5.52
Chelerythrine	BSA	1 380 000 ± 22 600	4.39	6.14
	HSA	2 970 000 ± 360 000	4.72	6.47

[a] K = binding constant, K_c, corrected for the abundance of the interacting pseudobase form using Eqn (1).
[b] K_c = conditional binding constant obtained by the linearization procedure[29,30] based on Eqn (2) from effective mobilities measured at various concentrations of the ligand.
[c] Logarithmic form of the constant K given only for their mean values for the sake of simplicity.

In order to verify whether the pseudobase of sanguinarine and chelerythrine interacts with albumins, its complexation with albumins was tested at pH 5.0. Equally with cysteine and mercaptoethanol, albumins interact much less with both alkaloids at pH 5 than pH 7.4. For example, 100 μM albumins decelerate the alkaloids by ~10% at pH 5.0. At pH 7.4, the mobilities of the alkaloids drop by 50% in 40 μM albumins. This implies stability constants two orders of magnitude lower at pH 5.0 than at pH 7.4. Therefore, millimolar or even higher concentrations of albumins at pH 5.0 have to be used in the measurement of the raw effective mobility of the alkaloids that is necessary for the stability constant calculation. Such high concentrations of albumins cause pronounced experimental difficulties in interaction experiments and in their evaluation.[19] Therefore, the determination of stability constants for weak non-specific interactions of charged sanguinarine and chelerythrine with human and bovine serum albumins was omitted. This aside, only the pseudobase of sanguinarine and chelerythrine should be identified as the forms interacting with the mercapto group of albumins.

The effect of the buffer composition on the conditional stability constants observed with cysteine and mercaptoethanol was also found with albumins. However, only data relevant to 13.5 mM phosphate–Tris buffer ($I = 30$ mM) are presented in Table 3 owing to the preference of phosphate buffers in biological assays and better electroosmosis stability in Tris buffers. This stability improves the precision of the determined constants.

Linearized dependences of the effective mobilities of sanguinarine and chelerythrine on HAS/BSA concentrations in phosphate buffer of pH 7.4 (Fig. 2) were corrected for the abundance of their uncharged forms (Table 3). Constants obtained in this way exceed by approximately two orders of magnitude constants for interactions of uncharged alkaloids with simple mercapto compounds under identical experimental conditions. Comparable data exist only for the statically investigated interaction of sanguinarine with human serum albumin.[10] Our conditional constant for the interaction of sanguinarine with human serum albumin, corrected for the equilibrium concentration of the interacting pseudobase by the method of side-reaction coefficients,[20,21] is $K = 332\,000$ (or log $K = 5.52$). This value agrees surprisingly well

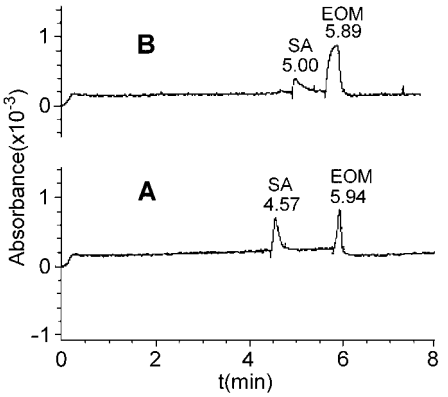


Figure 1. Migration times of sanguinarine in 13.5 mM phosphate buffer adjusted with Tris to pH 7.4 at 25 °C (A) and in the same buffer containing 0.5 mM mercaptoethanol (B). SA = sanguinarine; EOM = electroosmosis marker (mesityl oxide). Instrument: Beckman P/ACE 5510 with uncoated fused-silica capillary, 75 μm i.d. and 363 μm o.d., separation length 50 cm, total capillary length 57 cm; applied voltage, 15 kV.

with the reported stability constant for this interaction from static experiments, $K = 385\,000$ (or log $K = 5.59$).[10] No meaningful difference in constants obtained with human serum albumin containing fatty acids and after its defatting was reported in Ref. 10.

CONCLUSIONS

The difference between constants found for interactions of sanguinarine and chelerythrine with simple mercapto compounds and with albumins reveals that the protein milieu of albumins and effects linked with it are substantial for the strength of interactions of both alkaloids with albumins. The preferential binding of the uncharged pseudobase of these alkaloids to the mercapto group of albumins, and the higher stability of such complexes as compared with simple mercapto compounds, may be explained by the following hypothesis.

The absence of interaction of the quaternary ions of sanguinarine and chelerythrine with simple mercapto compounds indicates that this interaction cannot be expected with albumins. Positive charges inside the albumin globule, absence of water inside the globule and the strong

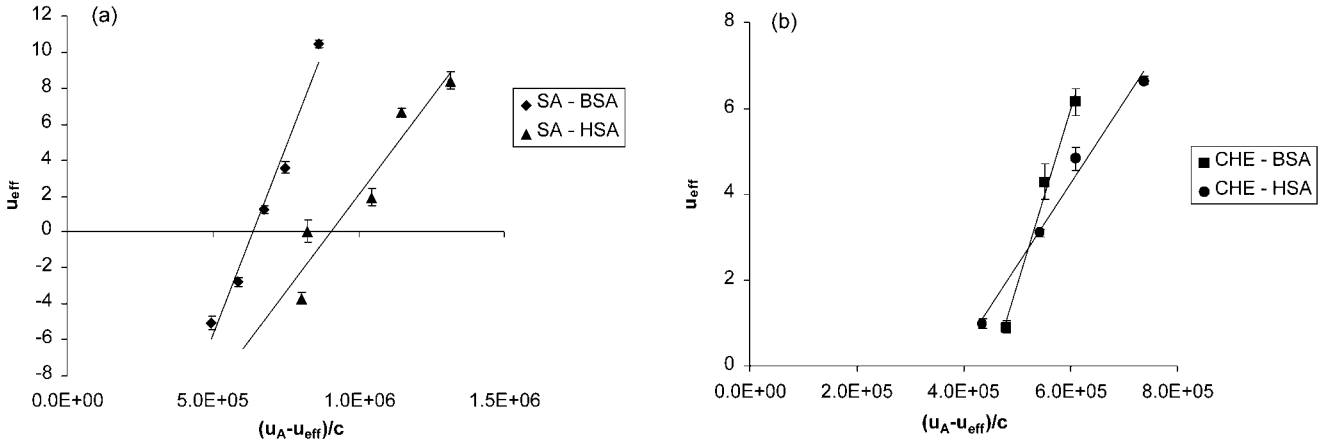


Figure 2. Linearized dependences of effective mobilities of sanguinarine (a) and chelerythrine (b) on the concentration of the human or bovine serum albumins. Raw data were measured in 13.5 mM phosphate–Tris buffer pH 7.4 ($I = 30$ mM) at 25 °C and at a Joule heat input of ~0.2 W in the electrophopretic capillary of 75 μm i.d. and 150 μm o.d., 50 cm separation length and 60.1 cm total length. For other detailes, including the description of the electrophoretic laboratory set-up used, see Experimental.

interaction of solvating water molecules with the positive charge act against the interaction of the charged alkaloid form with a mercapto group inside the albumin globule. The most probable mechanism of the measurable interaction of quaternary ions of the alkaloids with albumins is the coulombic attraction of these cations of sanguinarine/chelerythrine by the electrostatic field of negative domains on the surface of the albumin globule or close to it. The electrostatic field of domains of positive charges of albumin does not repulse the uncharged pseudobase alkaloid form. The pronounced hydrophobicity of pseudobase, which is manifested by its strong tendency to precipitate from aqueous solutions,[3,5] makes the elimination of the water molecules solvating the pseudobase form very easy and energetically almost undemanding. The uncharged and desolvated pseudobase easily penetrates the protein globule and inside it interacts with the mercapto group. The absence of water inside the albumin globule supports the stability of the complex formed. A mercapto group of a simple compound dissolved in water is hydrated to some extent because of the lone electron pairs of sulfur. The solvating water molecules have to be eliminated at least partially if the pseudobase form of an alkaloid complexes with the mercapto group. This water elimination consumes energy and, consequently, lowers the stability of the resulting complex in comparison with the analogical albumin complex.

The stability of complexes of the sanguinarine/chelerythrine pseudobase with mercaptoethanol or cysteine range from 8400 to 21 500 $l\,mol^{-1}$ (Table 2). Such stability is in the stability range of kinetically labile complexes, which create, e.g., in chiral separations with low molecular weight compounds chiral selectors such as cyclodextrins, macrocyclic antibiotics and oligopeptides.[22] Weak, unspecific interactions, which are determined by both the chemical composition and steric structure of both constituents of the complex, participate in its formation.[22] The same set of interactions has to be expected in mercaptoethanol and cysteine complexes. However, the identification of interactions that exist in a particular complex is impossible from electrophoretic experiments. Analogous discussion is possible for complexes with albumins whose stability is in the commonly reported range of 10^5–$10^6\,l\,mol^{-1}$ (Table 3).

The principal facts evidenced by our electrophoretic experiments is that the interaction of sanguinarine/chelerythrine with a mercapto group of a ligand is fast and reversible complexation of the alkaloid pseudobase with the mercapto group whatever the type of the ligand. Molecular weight and the complexity of the ligand affect only the stability of the complex formed. The complex is always in equilibrium with the dissolved and free (uncomplexed) fraction of the alkaloid.[18,19,22] This fraction contains both the pseudobase and the quaternary ion of the respective alkaloid owing to their fast and reversible acid–base equilibrium.[3] The abundance of these dissolved co-existing forms, which cannot be separated from each other under any conditions, is quantitatively given by the respective pK_{R^+} value of the alkaloid and by the solution pH. If the concentration of the dissolved pseudobase alkaloid exceeds somewhere, e.g., in a cell or at another target, its solubility limit[3,5] for any reason, the pseudobase form precipitates and, in this way, accumulates at this point. Quantitative data on the solubility of the pseudobase forms of sanguinarine and chelerythrine have not yet been reported.

It is reasonable to expect that the chain of mutually bound reversible equilibria of sanguinarine/chelerythrine plays important role in biological effects of sanguinarine and chelerythrine. Adequate recognition of each of these, including the determination of the respective equilibrium constant, is therefore important.

EXPERIMENTAL

A Beckman (USA) P/ACE System 5510 equipped with Beckman Gold software and a filter UV detector, which was operated at 280 nm, served for the identification of the type of interaction of sanguinarine and chelerythrine with mercaptoethanol and cysteine. An uncoated thick-walled fused-silica capillary of 75 μm i.d. and 363 μm o.d. (Supelco, USA) of 50 cm separation length and 57 cm total length, thermostated to 25 °C, was used for analyses of interaction and blank mixtures.

A laboratory set-up assembled with regard to special requirements of interaction measurements was preferred in another experiments. The instrument was based on a Spellman (USA) CZE 1000R high-voltage power supply and a JASCO (Japan) 875 UV–visible spectrophotometer adapted for CZE experiments. This set-up allowed temperature control of the capillary with circulating liquid with a precision better than ± 0.1 °C.[25] An uncoated thin-walled fused-silica capillary (Capillary Columns, Slovakia) of 45 cm total length (35 cm separation distance), 75 μm i.d. and 150 μm o.d., was kept at a constant temperature of 24.0 °C. The temperature difference between the capillary center and its outer wall was estimated to be ~1 °C for a Joule heat input of 0.2 W.[26,27] Constant temperature of the outer capillary wall (24.0 °C) therefore insured a constant temperature inside the capillary of ~25 °C at a Joule heat input of 0.2 W. The voltage applied on the capillary was controlled with respect to the conductivity of the background electrolyte filling of the capillary in order to keep this heat input constant. A detection wavelength of 280 nm was used. Hydrodynamic injection by the difference of hydrostatic pressure between the capillary inlet and outlet was used. Details of the activation of the capillary and the routine for the electroosmosis stabilization were described previously.[19]

Chemicals and procedures

Stock buffers of ionic strength $I = 30$ mM and pH 5 were prepared from 47 mM acetic acid; 30 mM acetic acid, 49 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) acid and 13.5 mM orthophosphoric acid were used for the preparation of buffers of pH 7.4. Acid was dissolved in ~900 ml of redistilled water and the buffer pH was adjusted with ~0.5 M NaOH or tris(hydroxymethyl) aminomethane (Tris) to the desired value. Then, the buffer volume was made up with water to 1000 ml. Stock buffers were stored at 4 °C. Background electrolyte was prepared daily by dissolving cysteine (Cys), mercaptoethanol (MEt), human serum albumin (HSA) or bovine serum albumin (BSA) in a stock buffer.

Sanguinarine (SA) and chelerythrine (CHE) were isolated from *Macleya cordata* by one of the authors (V.Š.). Sanguinarine of 98.1% purity, m.p. 279–282 °C, and chelerythrine of 95% purity, m.p. 200–204 °C, were obtained; their purity was determined by HPLC. The alkaloids were dissolved in ~1 mM HCl (stock solution). Injected concentrations of alkaloids allowing their reasonable detection at 280 nm were below 5×10^{-5} M. Cysteine and mercaptoethanol were obtained from Fluka (Buchs, Switzerland), HSA, Cohn fraction V, from EXBIO (Czech Republic) and BSA, Cohn fraction V, from IMUNA (Slovakia). Mesityl oxide used as the electroosmosis marker, and triphenyltetrazolium bromide, the cation of which served as the cationic mobility standard, were purchased from Sigma (USA). Commercially available chemicals apart from HSA and BSA were of reagent-grade purity.

Sanguinarine and chelerythrine were injected as the complexing markers. The method of two mobility standards[28] served for the conversion of their migration times to effective mobilities, u_{eff}, expressed in $10^{-9}\,\text{m}^2\,\text{V}^{-1}\,\text{s}^{-1}$ units. The mobility of the triphenyltetrazolium cation determined from 16 measurements was 17.28 ± 0.04 $10^{-9}\,\text{m}^2\,\text{V}^{-1}\,\text{s}^{-1}$ at 25 °C in 13.5 mM phosphate–Tris buffer, pH 7.40 and $I = 30$ mM. Mesityl oxide was the uncharged mobility standard.

The free, uncomplexed marker, A, cannot be separated from its complex, AX, which forms by the fast and reversible complexation of the marker A with the complex constituent, X, dissolved in background electrolyte. The free marker A and its complex AX migrate together in a common mixed zone. Its effective mobility, $u_{\text{eff,A}}$, depends on the mobility of the uncomplexed marker A, u_{A}, on the mobility of the complex, u_{AX}, on its stability given by the stoichiometric stability constant, K_{AX}, and on the concentreation of the other complex constituent X in the background electrolyte, c.[14,15] The mobilities of sanguinarine and chelerythrine at the absence of the investigated mercapto compound in the background electrolyte, u_{A}, and sets of data $u_{\text{eff,A}} = f(c_{\text{X}})$ have been measured for the determination of the conditional stoichiometric stability constants.[18] The constants, K_{AX}, were obtained by the linearization procedure from the equation[29,30]

$$u_{\text{eff,A}} = \frac{1}{K_{\text{AX}}}\left(\frac{u_{\text{A}} - u_{\text{eff,A}}}{c}\right) + u_{\text{AX}} \qquad (2)$$

assuming the fast and reversible 1 : 1 complexation of A with X. The mobility of the complex, u_{AX}, which cannot be measured experimentally,[14,15] was obtained simultaneously with K_{AX}.

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