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# Acid-base behaviour of sanguinarine and dihydrosanguinarine

**Research Article**

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**Abstract:** Acid-base and optical properties of sanguinarine and dihydrosanguinarine were studied in the presence of HCl, $HNO_3$, $H_2SO_4$, $H_3PO_4$, CAPSO and acetic acid (HAc) of different concentrations and their mixtures. The equilibrium constants $pK_{R+}$ of the transition reaction between an iminium cation $Q^+$ of sanguinarine and its uncharged QOH (pseudo-base, 6-hydroxy-dihydroderivative) form were calculated. A numerical interpretation of the *A-pH* curves by a SQUAD-G computer program was used. Remarkable shifts of formation parts of absorbance-pH (*A-pH*) curves to alkaline medium were observed. The shifts depend on the type and concentration of inert electrolyte (the most remarkable for $HNO_3$ and HCl ). The corresponding $pK_{R+}$ values ranged from 7.21 to 8.16 in the same manner ($\Delta pK_{R+}$ = 0.81 and 0.73 for $HNO_3$ and HCl, respectively). The priority effect of ionic species and ionic strength was confirmed in the presence of NaCl and KCl. The strength of interaction of SA with bioactive compounds (*i.e.* receptors, transport proteins, nucleic acids *etc.*) may be affected because of the observed influence of both cations and anions of the inert electrolytes.

**Keywords:** Sanguinarine • Dihydrosanguinarine • UV-VIS Spectrophotometry • Equilibrium constants

## 1. Introduction

Sanguinarine (SA) is one of the most important members [1] of the family of secondary plant metabolites, quaternary benzo[c]phenanthridine alkaloids (QBAs), displaying a wide spectrum of biological activities, *i.e.* antimicrobial and anti-inflammatory effects [2,3]. It is used as an antiplaque component [4], as remedies in myopathy [2] and as a constituent of veterinary preparations [5]. Dihydrosanguinarine (DHSA), its dihydro-derivative, has been recently identified as the first product in sanguinarine reductive metabolism [6] in rats.

The beneficial biological effects and/or adverse side effects are evidently connected with the occurrence of SA in the two structurally different forms at the physiological pH 7.4 [7]. The pH-dependent (acid-base) equilibrium (Fig. 1) between the charged iminium cation $Q^+$ and the uncharged QOH (pseudo-base, 6-hydroxy-dihydroderivative) forms of the alkaloid is totally reversible at very low concentrations. The reaction may be characterized by an equilibrium constant $K_{R+}$ = [H⁺][QOH]/[Q⁺] in analogy to the acid-base dissociation constant $K_a$ of Brønsted acids. The $pK_{R+}$ values range from 6.3 to 8.2 as determined by spectrophotometry, fluorimetry, potentiometry and capillary electrophoresis
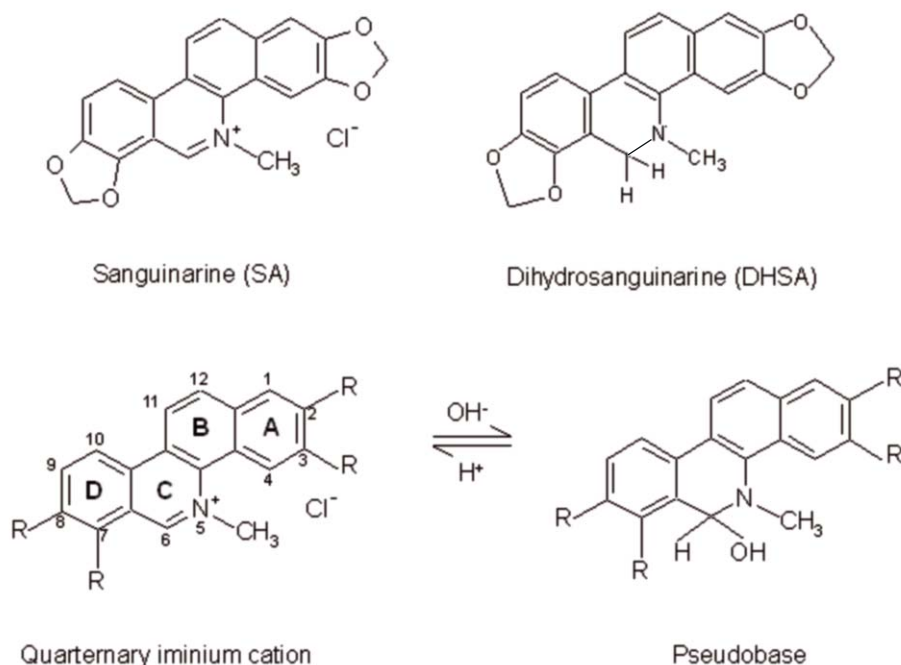
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**Figure 1.** The structural formulas of alkaloids sanguinarine (SA) and dihydrosanguinarine (DHSA) and the equilibrium between their charged and uncharged forms.

[7] indicates dependence on experimental conditions (ionic strength, liquid medium composition *etc.*).

The quaternary cationic form of SA is well soluble in water. Upon alkalization the intensive brown color of the solution disappears and a white opalescence and/or precipitate of a hydrophobic and sparingly soluble [7] uncharged pseudobase of SA appears at concentrations above 25 µmol L$^{-1}$. The limited solubility of the uncharged form of SA (not mentioned in most papers) influences the behaviour of the alkaloids in aqueous solution. The pseudobase easily dissolves in organic solvents of medium polarity [8,9]. Spontaneous dimerization of the uncharged form, which was found in polar organic solvents [8,9], fortunately, does not take place in aqueous solutions [10]. In addition, the pseudobase is photochemically oxidized on the C6 carbon atom to oxysanguinarine in strongly alkaline solutions [11].

These reactions may not only distort the determination of its p$K_{R+}$ values but also seriously influence interaction studies of SA with bio-macromolecules (proteins, peptides *etc.*). The exact knowledge of acid-base (protolytic) behaviour and the p$K_{R+}$ values of SA and DHSA are necessary for the interpretation of any investigation of the interactions of these alkaloids with biological macromolecules, *e.g.* receptors, transport proteins, nucleic acids, *etc.*

The main goals of our effort were (i) recognizing of the acid-base behaviour and time stability of SA and DHSA as the function of experimental conditions (pH, ionic strength, concentration of electrolyte and its composition, *etc.*), (ii) determination of true p$K_{R+}$ constants, (iii) identification of experimental conditions and requirements that qualify the possibility and correctness of interaction studies with these compounds in nearly neutral and weakly basic solutions.

## 2. Experimental

### 2.1. Chemicals

Stock solutions of sanguinarine chloride (SA, Sigma Aldrich) were prepared using freshly boiled distilled water acidified with hydrochloric acid to pH below 5. Working solutions ($c$ = 13 µmol L$^{-1}$) were prepared by dilution of the stock solution by freshly boiled distilled water. Dihydrosanguinarine (DHSA, 99% purity, MP 189-191°C) was prepared from SA by reduction with NaBH$_4$ in methanol [12]. Stock solutions of DHSA were prepared by dissolution of DHSA in ethanol or methanol. Working solutions ($c$ = 12 µmol L$^{-1}$) were prepared freshly by dilution of the stock solution by ethanol or methanol and/or freshly boiled distilled water. All the solutions were stored in the refrigerator and were protected from light.

Stock solutions of electrolytes were prepared from HCl, HNO$_3$, H$_2$SO$_4$, NaCl, KCl, (all Penta, Chrudim,

Czech Republic), $CH_3COOH$, $H_3PO_4$ (Lach-Ner, Neratovice, Czech Republic) all of p.a. purity and 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CAPSO, Sigma-Aldrich). Tris-(hydroxymethyl) aminomethane (TRIS – ultra pure grade, Amresco®, Solon, Ohio, USA), potassium or sodium hydroxide (both Penta, Chrudim, Czech Republic) solutions (1 – 0.001 mol L$^{-1}$) were used for the pH adjustment in a pH interval pH = 2 – 11 with steps $\Delta$pH = 0.3 – 0.5. All solutions were degassed with helium and kept under nitrogen.

## 2.2. Apparatus and conditions

All spectrophotometric measurements were performed using a UV/VIS Lambda 25 (Perkin Elmer, Shelton, USA) or Helios Beta UV-VIS spectrophotometers (Unicam, Cambridge, UK). The final pH of the solutions was controlled using a pH-meter model WTW pH 527 with a WTW SenTix 21 combined electrode. The electrode was regularly calibrated (several times per day, at least at the beginning and at the end of *A-pH* curve measurement) using a set of standard buffer solutions of pH = 4.01, 7.00, and 9.01 (all WTW GmbH, Wilheim, Germany).

The p$K_{R+}$ constants were calculated from the absorbance values at selected wavelengths between 270 and 350 nm using the SQUAD-G computer program [13]. The absorbance values of both alkaloids were measured three times at each pH and the mean values from these measurements were used for the calculation of p$K_{R+}$.

## 2.3. The SQUAD-G program

For a system comprising up to five interacting basic components, the SQUAD-G [13] program assembly makes it possible, based on a matrix analysis of absorbance data, to determine the number of absorbing species, the dissociation constants of the compound or the equilibrium constants, stability constants of complexes, the molar absorptivities of individual species and their standard deviations. The concentration proportions of the complexes present at a given pH, spectra of individual complexes or reagent species (even those that do not enable direct measurements), distribution diagrams of all species in the solution (with respect to the basic components of the system) and contributions of colored species to the total measured absorbance are also computed and printed out.

The input data include spectra in the form of an absorbance matrix for up to 170 solutions and 2 – 50 wavelengths, pH values for pH dependent reactions, total concentrations of components, composition of expected species and their constants estimates. The criteria used for adopting a model or for including the

species are: i) convergence of the calculation, ii) minimal value of the sum of squares of absorbance residuals $U = \Sigma_i (A_{exp,i} – A_{calc,i})^2$, where $i = 1-n$ is the total number of absorbance data for all solutions and wavelengths used, iii) minimal value of the average standard deviation of absorbance $s(A)$ over the whole data set, iv) for the standard deviation of calculated constant $k$ ($K_{R+}$, $\beta$), validity of the condition $s(\log k) < 0.1 \log k$.

# 3. Results and discussion

## 3.1. Time stability of SA and DHSA solutions

Time stability $A = f$(time) of the working solutions of DHSA was evaluated measuring absorption spectra (200-600 nm interval) in the aqueous solution with 4, 10, 30, 60, 100% (v/v) ethanol or methanol. The data were (in addition) collected at 274, 284, 322, 327 and 350 nm as the means of absorbance values (in relative %) for five replicates in 4% (v/v) ethanol at pH = 2 (0.01 mol L$^{-1}$ HCl) and pH = 7 (HCl + NaOH) and in the presence of formiate (pH = 2.8), acetate (pH = 4.2) and phosphate (pH = 6.7) buffers in addition.

The time stability of the DHSA working solutions was very short (a decrease to 93 and 85% of initial absorbance values in 30 min, respectively) in the less concentrated ethanol solutions (4 and 10%). The solutions were very stable at higher ethanol contents (≥ 30%) with a non-significant increase of absorbance values at 350 nm (up to 115% of the initial value) probably due to the increase of solubility in the mixed solvent. The highest stability of DHSA solution was observed in the strongly acidic medium (pH = 2, 10 mmol L$^{-1}$ HCl) while in all other cases (formiate, pH = 2.8, acetate, pH = 4.2, phosphate, pH = 6.7 and HCl/NaCl, pH 7.0) the stability was lower. A very similar behaviour was found for ethanol (≤ 30%) and methanol (≤60%).

On the contrary, working solutions of SA were stable over the whole pH intervals of 2 through 8. In the alkaline solutions (pH > 8) the solutions were less stable (*ca.* 10% decrease of the initial absorbance values in 60 min at pH 10.8) and a slow reduction of the intensity of the absorption bands was observed for at least two hours (a sharp isosbestic point (IP) at 346 nm indicates a reversible reaction). The instability of the DHSA solutions can be most probably explained by photochemical oxidation of DHSA similar to that described [14] for SA in a strongly alkaline medium. Absorption spectra of the final products of the photooxidation of SA and DHSA are very similar. The reactions take place in the pH regions in which the uncharged pseudobases prevail.
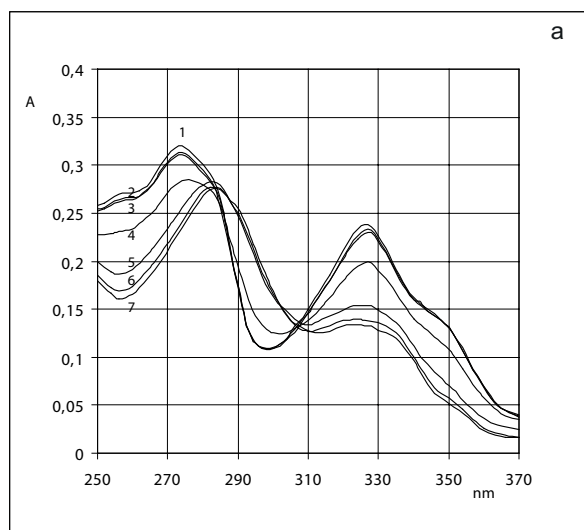
pH: 1 – 2.99; 2 – 3.97; 3 – 5.69; 4 – 7.46; 5 – 8.48; 6 – 8.96; 7 – 9.93
$c_{SA} = 1.3 \times 10^{-5}$ mol L$^{-1}$
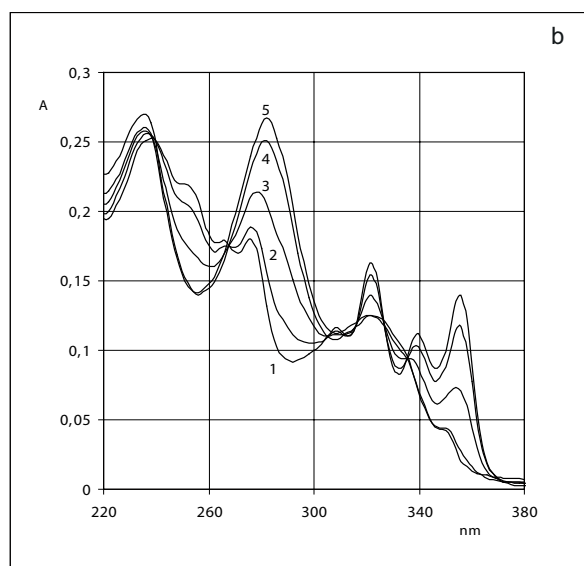


pH: 1 – 1,16; 2 – 2,16; 3 – 3,11; 4 – 4,12; 5 – 6,16 $c_{DHSA} = 6.10^{-6}$ mol L$^{-1}$

**Figure 2.** Absorption spectra of sanguinarine (a) in 100 mmol L$^{-1}$ HCl and dihydrosanguinarine (b) in 60% (v/v) methanol and 100 mmol L$^{-1}$ HCl

## 3.2. Absorption spectra

Marked precipitation of sanguinarine was observed when its more concentrated (≥50 µmol L$^{-1}$) stock solution was prepared by dissolution of sanguinarine chloride in neutral or alkaline solution or in sodium phosphate buffer of pH 7.4. The precipitate steadily dissolved at the lower concentrations (10 µmol L$^{-1}$ and lower) within several days while at the concentration 25 µmol L$^{-1}$ remained opalescent for several weeks. More concentrated solutions (≥50 µmol L$^{-1}$) did not change. This finding indicates that the total solubility of sanguinarine is below the 25 µmol L$^{-1}$. Thus 13 µmol L$^{-1}$ working solutions in water acidified to pHs of 1 through 5 were used as starting conditions in all experiments.

Absorption spectra of SA were registered in wavelength intervals from 250 to 600 nm at pH = 2.5 – 11.0 in the presence of HCl (see Fig. 2a), HNO$_3$, H$_2$SO$_4$, H$_3$PO$_4$, HAc and CAPSO (initial concentration c = 10 mmol L$^{-1}$). The spectra exhibited two distinct UV absorption maxima at 274 and 327 nm, three less promoted absorption maxima at 260, 398 and 470 nm and a broad shoulder at 350 nm in acidic media. The short wavelength maximum at 260 nm shifted to shorter wavelengths (≈ 245 nm) while the maximum at 274 nm shifted to longer wavelengths (284 nm, 288 nm for CAPSO) in alkaline medium of pH = 8 – 10. The absorption band with a maximum at 327 nm exhibited a broadening with a new maximum at 322 nm and with a broad shoulder in the 345 – 355 nm range. The intensity of other UV-VIS bands was reduced. The presence of isosbestic points at 286 and 307 nm confirmed a reversible equilibrium between cationic iminium Q$^+$ and the uncharged QOH forms in the pH interval of 5 through 10. The equilibrium is partly influenced by limited solubility of the uncharged QOH form. The molar absorptivities $\varepsilon_1$ a $\varepsilon_2$ of both forms of sanguinarine in HCl medium calculated by the SQUAD-G program are given in Table 1.

The absorption spectra of DHSA registered in the range 200 – 600 nm in the presence of 60% (Fig. 2b) and 4% (v/v) methanol or 4% (v/v) ethanol exhibited

**Table 1.** Molar absorptivities $\varepsilon_1$ and $\varepsilon_2$ (and their standard deviations) of cationic form ($\varepsilon_1$) and pseudobase ($\varepsilon_2$) of sanguinarine measured at different experimental conditions

| Conditions | $\varepsilon_1$ (274 nm) [L mol$^{-1}$ cm$^{-1}$] | $\varepsilon_2$ (274 nm) [L mol$^{-1}$ cm$^{-1}$] | $\varepsilon_1$ (327 nm) [L mol$^{-1}$ cm$^{-1}$] | $\varepsilon_2$ (327 nm) [L mol$^{-1}$ cm$^{-1}$] |
|---|---|---|---|---|
| 0.001 mol L$^{-1}$ HCl | 25 380 ± 290 | 18 300 ± 240 | 17 840 ± 230 | 10 520 ± 180 |
| 0.01 mol L$^{-1}$ HCl | 24 610 ± 80 | 18 460 ± 110 | 18 460 ± 70 | 10 150 ± 110 |
| 0.1 mol L$^{-1}$ HCl | 23 900 ± 50 | 19 540 ± 80 | 18 509 ± 60 | 10 810 ± 110 |

*30 700 L mol$^{-1}$ cm$^{-1}$ was given by [21] at 327 nm*

eight distinct absorption maxima at 238, 253, 268, 274, 308, 322, 338 and 355 nm and a broad shoulder at 214 nm with isosbestic points at 270, 303, 330 and 364 nm in the interval pH = 1 – 4. Due to the gradual destruction of the DHSA molecule the intensity of absorption bands continuously decreased and the isosbestic points disappeared at higher pH values. Absorption maxima at 237, 284, 327 nm, a less distinct maximum at 350 nm and a broad shoulder at 210 – 220 nm were present in the pH interval 4 – 10. With the increasing content of organic solvent (4, 10, 30, 60, 100% (v/v) ethanol or methanol) a distinct maximum at 284 nm and a less distinct maximum at 237 nm appear. The absorption maximum at 327 nm (1 – 10% (v/v) ethanol or methanol) was shifted to shorter wavelengths (322 nm) with increasing content of organic solvent (60 – 100% (v/v) ethanol or methanol).

## 3.3. Absorbance-pH curves

Influence of experimental conditions on acid-base behaviour of SA (type and concentration of anions of inorganic/organic acids) was studied by interpretation of absorbance-pH curves (*A-pH* curves) measured at a constant concentration of sanguinarine c = 13 µmol L$^{-1}$. The data were collected for HCl (see Fig. 3), HNO$_3$, H$_2$SO$_4$, H$_3$PO$_4$, acetic acid and CAPSO (not graphically presented) starting at the initial concentrations of acids 1, 10 and 100 mmol L$^{-1}$.
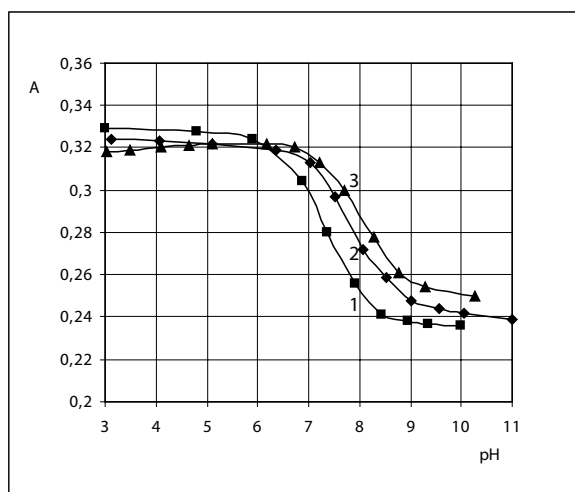
**Figure 3.** Absorbance-pH curves of sanguinarine at different HCl concentrations. Experimental conditions: 1 – 0.001 mol L$^{-1}$ HCl; 2 – 0.01 mol L$^{-1}$ HCl; 3 – 0.1 mol L$^{-1}$ HCl, $\lambda$ = 274 nm, c$_{SA}$ = 1.3.10$^{-5}$ mol L$^{-1}$

The formation parts of the *A-pH* curves (and of course the corresponding pK$_{R+}$ values) were shifted to the more alkaline medium with increasing concentration of acids and in dependence on type of anion. The corresponding pK$_{R+}$ values calculated using a numerical interpretation of the *A-pH* curves by the SQUAD-G program (see Table 2) changed from 7.21 to 8.16 in the same manner (in agreement with published data [15-22]). The most remarkable shift of pK$_{R+}$was observed in the presence of the strongest mineral acids HNO$_3$ ($\Delta$pK$_{R+}$ = 0.81) and HCl ($\Delta$pK$_{R+}$ = 0.73) and acetic acid ($\Delta$pK$_{R+}$ = 0.68) while a less remarkable one was recorded in the presence of CAPSO ($\Delta$pK$_{R+}$ = 0.34), H$_3$PO$_4$ ($\Delta$pK$_{R+}$ = 0.29) and H$_2$SO$_4$ ($\Delta$pK$_{R+}$= 0.23). Thus the pK$_{R+}$ values were influenced in increasing/decreasing order by the following anions: NO$_3^-$ ~ Cl$^-$ ~ Ac$^-$ > CAPSO ~ PO$_4^{3-}$ ~ SO$_4^{2-}$.

**Table 2.** pK$_{R+}$ constants of sanguinarine (and their standard deviations) measured at different experimental conditions

| Conditions | pK$_{R+}$ | s(A)[1] | U[2] |
|---|---|---|---|
| 0.001 mol L$^{-1}$ HCl | 7.33 ± 0.042 | 0.0063 | 0.0017 |
| 0.001 mol L$^{-1}$ HNO$_3$ | 7.21 ± 0.065 | 0.0102 | 0.0046 |
| 0.001 mol L$^{-1}$ H$_2$SO$_4$ | 7.52 ± 0.026 | 0.0039 | 0.0011 |
| 0.001 mol L$^{-1}$ H$_3$PO$_4$ | 7.50 ± 0.026 | 0.0041 | 0.0008 |
| 0.001 mol L$^{-1}$ CH$_3$COOH | 7.48 ± 0.037 | 0.0063 | 0.0001 |
| 0.001 mol L$^{-1}$ CAPSO | 7.25 ± 0.027 | 0.0030 | 0.0003 |
| 0.01 mol L$^{-1}$ HCl | 7.69 ± 0.032[3] | 0.0004 | 0.0013 |
| 0.01 mol L$^{-1}$ HNO$_3$ | 7.56 ± 0.065 | 0.0125 | 0.0069 |
| 0.01 mol L$^{-1}$ H$_2$SO$_4$ | 7.68 ± 0.039 | 0.0048 | 0.0015 |
| 0.01 mol L$^{-1}$ H$_3$PO$_4$ | 7.68 ± 0.031 | 0.0050 | 0.0014 |
| 0.01 mol L$^{-1}$ CH$_3$COOH | 7.88 ± 0.020 | 0.0032 | 0.0006 |
| 0.01 mol L$^{-1}$ CAPSO | 7.30 ± 0.032 | 0.0058 | 0.0011 |
| 0.1 mol L$^{-1}$ HCl | 8.06 ± 0.026 | 0.0023 | 0.0004 |
| 0.1 mol L$^{-1}$ HNO$_3$ | 8.02 ± 0.027 | 0.0030 | 0.0005 |
| 0.1 mol L$^{-1}$ H$_2$SO$_4$ | 7.75 ± 0.055 | 0.0088 | 0.0038 |
| 0.1 mol L$^{-1}$ H$_3$PO$_4$ | 7.79 ± 0.041 | 0.0035 | 0.0006 |
| 0.1 mol L$^{-1}$ CH$_3$COOH | 8.16 ± 0.051 | 0.0100 | 0.0028 |
| 0.1 mol L$^{-1}$ CAPSO | 7.59 ± 0.066 | 0.0084 | 0.0027 |

[1]*minimal value of the average standard deviation of absorbance s(A) over the whole data set;* [2] *the sum of squares of absorbance residuals U = $\Sigma_i$ (A$_{exp,i}$ – A$_{calc,i}$)$^2$;* [3] *7.65±0.04 is given for 0.01 mol L$^{-1}$ HCl,pK$_{R+}$ constants of SA 7.32–8.16 [8,9,11,15,16]*

Due to the low stability of solutions, the *A-pH* curves for DHSA were measured at a constant concentration of dihydrosanguinarine c = 12 µmol L$^{-1}$ in the presence of HCl in 60% (v/v) methanol and pK$_{R+}$ value of 2.32 was estimated in agreement with the value 2.3-2.6 acc. [21]).

## 3.4. Effect of electrolyte composition (M$^+$, X$^-$)

To confirm the influence of the type of anion, the *A-pH* curves were measured in the mixtures of acids at their constant total concentration 100 mmol L$^{-1}$. The pK$_{R+}$ values increased if the HCl, H$_2$SO$_4$ and H$_3$PO$_4$ (7.42±0.09, 7.79±0.09, 8.09±0.11) as the anions were

**Table 3.** $pK_{R+}$ values of sanguinarine in mixtures of inorganic acids (c = 0.1 mol $L^{-1}$, concentrations ratios 0.03 + 0.07 mol $L^{-1}$ and 0.07 + 0.03 mol $L^{-1}$, respectively))

| Conditions | $pK_{R+}$ | s(A)[1] | U[2] |
|---|---|---|---|
| 0.03 mol $L^{-1}$ HCl + 0.07 mol $L^{-1}$ $H_2SO_4$ | 7.96 ± 0.039 | 0.0046 | 0.0016 |
| 0.07 mol $L^{-1}$ HCl + 0.03 mol $L^{-1}$ $H_2SO_4$ | 8.17 ± 0.022 | 0.0028 | 0.0006 |
| 0.03 mol $L^{-1}$ HCl + 0.07 mol $L^{-1}$ $H_3PO_4$ | 8.06 ± 0.035 | 0.0049 | 0.0017 |
| 0.07 mol $L^{-1}$ HCl + 0.03 mol $L^{-1}$ $H_3PO_4$ | 8.11 ± 0.023 | 0.0027 | 0.0007 |
| 0.03 mol $L^{-1}$ $HNO_3$ + 0.07 mol $L^{-1}$ $H_2SO_4$ | 7.89 ± 0.074 | 0.0067 | 0.0031 |
| 0.07 mol $L^{-1}$ $HNO_3$ + 0.03 mol $L^{-1}$ $H_2SO_4$ | 8.12 ± 0.033 | 0.0043 | 0.0009 |
| 0.03 mol $L^{-1}$ $HNO_3$ + 0.07 mol $L^{-1}$ $H_3PO_4$ | 8.15 ± 0.030 | 0.0052 | 0.0016 |
| 0.07 mol $L^{-1}$ $HNO_3$ + 0.03 mol $L^{-1}$ $H_3PO_4$ | 8.30 ± 0.067 | 0.0056 | 0.0017 |
| 0.03 mol $L^{-1}$ HCl + 0.07 mol $L^{-1}$ $HNO_3$ | 8.11 ± 0.020 | 0.0021 | 0.0003 |
| 0.07 mol $L^{-1}$ HCl + 0.03 mol $L^{-1}$ $HNO_3$ | 8.05 ± 0.031 | 0.0034 | 0.0007 |
| 0.03 mol $L^{-1}$ $H_2SO_4$ + 0.07 mol $L^{-1}$ $H_3PO_4$ | 7.81 ± 0.048 | 0.0060 | 0.0027 |
| 0.07 mol $L^{-1}$ $H_2SO_4$ + 0.03 mol $L^{-1}$ $H_3PO_4$ | 7.89 ± 0.022 | 0.0029 | 0.0006 |

[1] minimal value of the average standard deviation of absorbance s(A) over the whole data set; [2] the sum of squares of absorbance residuals $U = \Sigma_i (A_{exp,i} - A_{calc,i})^2$

**Table 4.** $pK_{R+}$ values of sanguinarine in the presence of TRIS

| Conditions | $pK_{R+}$ | s(A)[1] | U[2] |
|---|---|---|---|
| 0.01 mol $L^{-1}$ HCl | 8.17 ± 0.037 | 0.0090 | 0.0051 |
| 0.10 mol $L^{-1}$ HCl | 8.24 ± 0.047 | 0.0059 | 0.0021 |
| 0.01 mol $L^{-1}$ $HNO_3$ | 8.27 ± 0.065 | 0.0098 | 0.0056 |
| 0.10 mol $L^{-1}$ $HNO_3$ | 8.66 ± 0.041 | 0.0095 | 0.0039 |
| 0.01 mol $L^{-1}$ $H_2SO_4$ | 8.05 ± 0.080 | 0.0090 | 0.0028 |
| 0.10 mol $L^{-1}$ $H_2SO_4$ | 8.44 ± 0.032 | 0.0055 | 0.0021 |
| 0.01 mol $L^{-1}$ $H_3PO_4$ | 7.96 ± 0.037 | 0.0055 | 0.0018 |
| 0.10 mol $L^{-1}$ $H_3PO_4$ | 8.26 ± 0.047 | 0.0083 | 0.0047 |

*[1] minimal value of the average standard deviation of absorbance s(A) over the whole data set; [2] the sum of squares of absorbance residuals $U = \Sigma_i (A_{exp,i} - A_{calc,i})^2$*

changed. The same trend was observed for 10 mmol $L^{-1}$ HCl with addition of 10 and 100 mmol $L^{-1}$ $H_2SO_4$ or $H_3PO_4$.

Due to the highest differences in $\Delta pK_{R+}$ values and in the most remarkable shifts of formation parts of *A-pH* curves, the mixtures of HCl + $H_2SO_4$, HCl + $H_3PO_4$, $HNO_3$ + $H_2SO_4$, $HNO_3$ + $H_3PO_4$, HCl + $HNO_3$ and $H_2SO_4$ + $H_3PO_4$ at the constant concentrations 0.1 mol $L^{-1}$ and at the concentration ratios 0.07 mol $L^{-1}$ + 0.03 mol $L^{-1}$ and *vice versa* were tested in further experiments. The increasing $pK_{R+}$ values and also their differences $\Delta pK_{R+}$ (see Table 3) documented the priority effect of anions of inorganic acids (HCl and $HNO_3$) in their mixtures with $H_2SO_4$ or $H_3PO_4$ with increasing concentration of HCl and $HNO_3$. The priority effects were less remarkable for the acids with very close $pK_{R+}$ values, *i.e.* HCl ($pK_{R+}$ = 8.06) and $HNO_3$ ($pK_{R+}$ = 8.02), or $H_2SO_4$ ($pK_{R+}$ = 7.75) and $H_3PO_4$ ($pK_{R+}$ = 7.79)

To verify the influence of cationic species, the pH of the sanguinarine solutions in HCl, $HNO_3$, $H_2SO_4$ and $H_3PO_4$ at the concentrations c = 10 mmol $L^{-1}$ and 100 mmol $L^{-1}$ was adjusted with TRIS and KOH. The corresponding

$pK_{R+}$ values (see Table 4) were again shifted to the more alkaline medium with increasing concentration of acids and they were significantly higher for TRIS compared to the values obtained for the *A-pH* curves with solutions neutralized with NaOH. The lowest differences $\Delta pK_{R+}$ were in the presence of HCl and, on the other hand, the differences were comparable for the other acids.

## 3.5. Influence of ionic strength

The changes in acid-base behaviour of sanguinarine in dependence on ionic strength were evaluated in the presence of NaCl and KCl at $I$ = 0.01, 0.10 and 1.0 and at the constant concentration of HCl, $HNO_3$, $H_2SO_4$, $H_3PO_4$ (c = 0.01 mol $L^{-1}$). The corresponding $pK_{R+}$ values are presented in Table 5 and in a graphical form in Figs. 4a and 4b. The formation parts of *A-pH* curves and also the corresponding $pK_{R+}$ values were shifted to the alkaline region with increasing ionic strength in the range $\Delta pK_{R+}$ = 0.35 – 0.78. The highest influence was observed in the presence of $HNO_3$ while the lowest in the presence of $H_3PO_4$. A slightly more remarkable effect was observed in the presence of KCl ($\Delta pK_{R+}$ = 0.38 – 0.89).
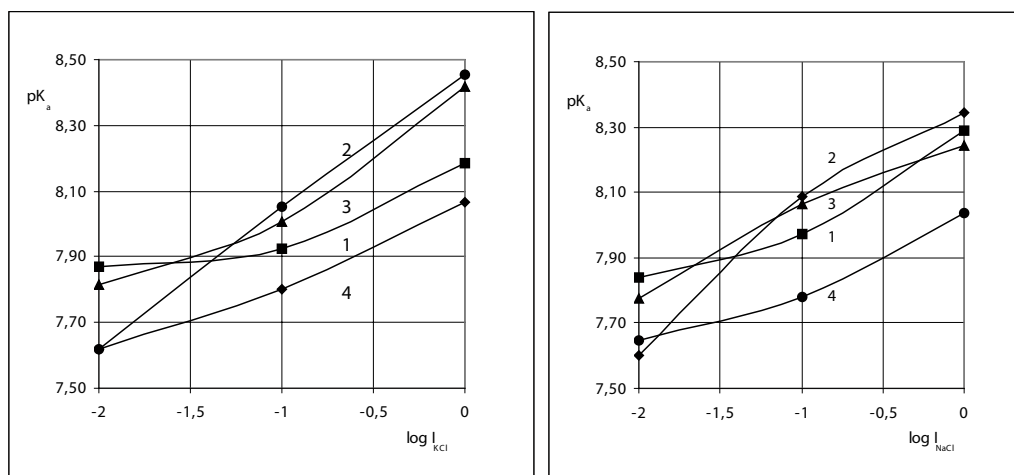
**Figure 4.** $pK_{R+}$ values of sanguinarine in dependence on a type and concentration of inert electrolyte (ionic strength) in the presence of NaCl (right) or KCl (left). Experimental conditions: 1 – 10 mmol L$^{-1}$ HCl, 2 – 10 mmol L$^{-1}$ HNO$_3$, 3 – 10 mmol L$^{-1}$ H$_2$SO$_4$, 4 – 10 mmol L$^{-1}$ H$_3$PO$_4$

**Table 5.** Influence of ionic strength (NaCl and KCl as inert electrolytes) on $pK_{R+}$ of sanguinarine

| Conditions | $pK_{R+}$ | | | |
|---|---|---|---|---|
| | 0.01 mol L$^{-1}$ HCl | 0.01 mol L$^{-1}$ HNO$_3$ | 0.01 mol L$^{-1}$ H$_2$SO$_4$ | 0.01 mol L$^{-1}$ H$_3$PO$_4$ |
| 0 | 7.69 ± 0.032 | 7.56 ± 0.065 | 7.68 ± 0.039 | 7.68 ± 0.031 |
| 0.01 mol L$^{-1}$ NaCl | 7.84 ± 0.014 | 7.60 ± 0.027 | 7.78 ± 0.018 | 7.65 ± 0.047 |
| 0.10 mol L$^{-1}$ NaCl | 7.97 ± 0.020 | 8.09 ± 0.023 | 8.07 ± 0.024 | 7.78 ± 0.049 |
| 1.00 mol L$^{-1}$ NaCl | 8.29 ± 0.024 | 8.34 ± 0.017 | 8.25 ± 0.026 | 8.04 ± 0.057 |
| 0.01 mol L$^{-1}$ KCl | 7.87 ± 0.056 | 7.62 ± 0.027 | 7.82 ± 0.020 | 7.62 ± 0.034 |
| 0.10 mol L$^{-1}$ KCl | 7.92 ± 0.065 | 8.05 ± 0.032 | 8.01 ± 0.030 | 7.80 ± 0.037 |
| 1.00 mol L$^{-1}$ KCl | 8.18 ± 0.019 | 8.46 ± 0.019 | 8.42 ± 0.030 | 8.07 ± 0.060 |

# 4. Conclusions

This study showed that interaction measurements are possible with SA in almost neutral and weakly alkaline solutions despite their limited solubility in such solutions. The necessary prerequisite for obtaining correct values is the use of the lowest possible concentrations of SA. The applied SA concentration should be below the solubility limit in the used buffer or, if this is impossible, close to it. Samples dissolved in water acidified with hydrochloric acid pHs of 1 through 5 are recommended to obtain further improvement in the studies.

The reported $pK_{R+}$ values of SA (7.32 – 8.16) [8,9,11,15-22] and our previous experience with electrophoretic behaviour of SA [20,23] imply that at least some of the published $pK_{R+}$ values are distorted by the abovementioned experimental conditions. Thus, in some cases an organic solvent was added to the solutions to improve the alkaloid solubility. Knowledge on the behaviour of QBAs in the solutions, particularly the ones containing an organic solvent, is therefore irrelevant to measurements performed in almost neutral and weakly basic solutions where the concentrations of uncharged forms of QBAs become comparable or prevail [24,25].

Different behaviour and solubility of SA in different media, which were adjusted to identical pH and ionic strength, must result from interactions of ionic species of acids and/or bases with investigated alkaloids. Recently we have found that MOPS and CAPSO anions form pseudo-micelles that can enhance dissolution of uncharged SA [23,26]. It has to be therefore expected that analogical interaction occurs between uncharged hydrophobic sanguinarine not only with complex molecular structures but similarly with the components of buffer solutions, electrolytes and non-electrolytes and other compounds present in solutions *etc*. Also adsorption/desorption processes on colloidal species, solid particles surfaces *etc*. can play an important role. The low solubility of the uncharged form of SA is the most probable reason for their extraordinary unfavourable behaviour we have observed in neutral and weakly alkaline region. To prevent precipitation of SA, the use of sufficiently sensitive techniques, *e.g.* fluorimetry, MS *etc*. (allowing application of the lowest concentration of SA) is recommended for the measurements for biological purposes.

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