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Determination of dissociation constants of labile drug compounds by capillary electrophoresis

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Abstract

The utility of capillary electrophoresis (CE) for determination of the negative logarithm of dissociation constants (pK_a) of labile compounds was investigated. In this study pyridinyl–methyl–sulfinyl–benzimidazoles (PMSB's), which have both an acidic and a basic pK_a, were selected as a first set of model drug compounds. This is a group of compounds that are known to degrade in aqueous solutions under neutral and acidic conditions which thus may impair their pK_a determination when using common batch techniques based on spectrophotometry or potentiometry. An additional set of model drug compounds, benzenesulfonic acid phenethyloxy–phenyl esters (BSAP's), which are labile at high pH, were also studied. It is demonstrated that pK_a values can be determined with high precision and accuracy by CE for both these sets of model compounds because decomposition products and impurities can be sufficiently separated from the main component. Based on the results in this study, a general strategy is proposed and discussed for determination of pK_a for labile compounds. Key steps comprise use of a stabilizing sample diluent, injection by electromigration, short analysis time, and characterization of the main component by UV–Vis spectra.

Keywords: Dissociation constant; Capillary electrophoresis; Pyridinyl–methyl–sulfinyl–benzimidazoles; Benzenesulfonic acid phenethyloxy–phenyl esters

1. Introduction

Knowledge of the physicochemical properties of a drug compound, e.g. its acid–base properties, are important in the optimization stage of a drug development project. Commonly dissociation constants (pK_a) of drug compounds are determined by techniques such as titration by potentiometry and UV–Vis spectrometry. Although highly useful, these techniques typically need sample amounts in the order of a few mg for analysis. Moreover, with these techniques there is no differentiation in analytical response between the analyte of interest and any analog impurity.

pK_a values can also be predicted by computational methods. These techniques have certain advantages, for example calculations can be per-

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formed on large virtual compound libraries. Still, erroneous data are often predicted for complex and flexible drug compounds containing several functional groups. Furthermore, these calculations are based on parameters in databases containing experimental data from the literature. Hence, sufficient data for new types of compounds, to give accurate predictions, may be missing.

In recent years capillary electrophoresis (CE) has been introduced as a technique for determination of dissociation constants [1–3]. Here, pK_a values can be calculated from electrophoretic mobility data after CE separation with a set of buffers having different pH. The CE technique offers advantages over traditional techniques because only a few ng or less of sample is used. Moreover, based on being an analytical separation technique, several substances can be measured simultaneously. The basis for determination of dissociation constants by CE is measurements of the migration time of the analyte in electrolytes with a well-defined pH. Because the mobility of an ionizable analyte is a direct function of the pH of the electrolyte, the pK_a value(s) of the analyte can be obtained by plotting analyte mobility versus buffer pH, analogous to a potentiometric titration curve, from which the pK_a can be determined. In practice, the mobility is calculated from the migration time for the analyte as well as for the electroosmotic flow. A number of studies have demonstrated the use of CE in applications to pK_a determinations for small drug compounds [3–27]. However, so far limited attention has been paid to the use of CE for determination of pK_a for labile compounds. Ishihama et al. have suggested the possibility of using CE for determination of compounds that are unstable in aqueous solutions, but have not demonstrated any experimental evidence [4]. To the best of our knowledge, there is so far only three reports on determination of pK_a of labile compounds by CE [10,26,28]. The action taken by Sheng et al. [10] was preparation of new sample solutions every 3 h. However, this introduce limitations for automated analyses. Takayanagi and Motomizu [26] determined acid dissociation constants of phenolphtalein and Örnskov and Folestad [28] determined dissociation constants of omeprazole analogues. In both studies advantage is taken of the CE separation to resolve the analyte of interest from related compounds such as degradation products. In spite of these reports, a general strategy for labile compounds is still missing.

The aim of this study was to extend the preliminary study by Örnskov and Folestad [28] to a wider range of drug compounds that are labile in aqueous solutions and also to outline a general methodology for determination of pK_a by CE. Both pyridinyl–methyl–sulfinyl–benzimidazoles (PMSB's) and benzenesulfonic acid phenethyloxy–phenyl esters (BSAP's), were used as model compounds. The PMSB is a group of drug compounds that has an acidic pK_a at 8–9 and a basic pK_a at 3–5 and that are known to degrade in aqueous solutions under neutral and acidic conditions [29]. As an example, the half-life of lansoprazole is 4 min in a methanol–phosphate (5:95, v/v) pH 3 buffer [30]. On the other hand, BSAP's represent compounds that are labile at the other end of the pH range, i.e. at high pH [31]. Various aspects on the developed methodology, with emphasis on injection and conditioning procedures as well as the effective length of the separation capillary length were investigated. Precision, accuracy and utility of the CE methodology for labile compounds are discussed.

2. Experimental

2.1. Apparatus

Separations were carried out on a model HP^{3D} CE instrument, (Agilent Technologies, Waldbronn, Germany) equipped with an UV absorption diode-array detector. The HP Chemstation CE software (G1601A, A.03.02) was used for instrument control, data acquisition and data analysis. Untreated fused-silica capillaries of 50 μm I.D., 375 μm O.D. from Polymicro Technologies (Phoenix, AZ, USA) were used for separations. The total capillary length was typically 35.0 cm, with a length to the detection window of 26.5 cm. Note that the CE separation was carried out in a reversed configuration. That is, injection was conducted from the detection end of the capillary.

This gives an effective length of the separation capillary of 8.5 cm. Buffer pH was measured at 22 ± 1 °C with a model PHM95 reference pH-meter (Radiometer, Copenhagen, Denmark).

2.2. Chemicals

All chemicals were of analytical grade if not otherwise stated. Acetonitrile (MeCN), methanol (MeOH) and Dimethyl sulfoxide (DMSO) of gradient grade for chromatography were obtained from MERCK LiChrosolv® (Darmstadt, Germany). Di-sodium hydrogen phosphate dihydrate (Na_2HPO_4), sodium dihydrogen phosphate monohydrate (NaH_2PO_4), tri-sodium phosphate dodecahydrate (Na_3PO_4), di-sodium tetraborate decahydrate ($Na_2B_4O_7$), sodium acetate-trihydrate (CH_3COONa), acetic acid (CH_3COOH) 100%, sodium hydroxide (NaOH) and hydrochloric acid (HCl) 37% were obtained from MERCK. Orthophosphoric acid (H_3PO_4) 85% was obtained from May & Baker LTD (Dagenham, England).

4-Hydroxybenzaldehyde was obtained from Fluka (Buchs, Switzerland). 4-Hydroxybenzoic acid, benzoic acid, nicotinic acid, pyridine and benzyl alcohol were all purchased from Merck. Salicylic acid was obtained from B.D.H (Poole, UK). Acetylsalicylic acid, alprenolol, atenolol, metoprolol, propranolol, H 168/66, lansoprazole, omeprazole, pantoprazole, and rabeprazole were obtained from AstraZeneca R&D (Mölndal, Sweden). The 11 BSAP's were synthesized in-house [31]. Purified water was obtained from a ELGA maxima ultra pure water system (Lane End, High Wycombe, Bucks, UK).

2.3. Procedures

New fused-silica capillaries were pretreated by flushing with 0.1 M NaOH (20 min), followed by rinsing with water (10 min). Prior to each run in an analytical separation sequence, the capillary was rinsed consecutively with 0.1 M NaOH (5 min), water (2 min), and background electrolyte (BGE) (3 min), respectively. Finally, the capillary was equilibrated by applying 300 V cm^{-1} for 30 s. New BGE vials were used for a new separation run. Before each analytical separation sequence, two pre-conditioning separations using the BGE with the highest pH in the specific series were performed to equilibrate the capillary and to allow the solutions on the autosampler tray to reach a constant temperature. Thermostatting of the capillary was achieved with regulated air (10 m s^{-1}) that passed through the capillary cartridge at 22.0 ± 0.1 °C, whereas thermostatting of the autosampler tray to 22–24 °C was carried out using an external water bath. For detection, the absorption was monitored at 191 and 303 nm. In addition, spectra (190–400 nm) were acquired for all eluting peaks and subsequently used for peak characterization. Sample solutions were injected with electromigration by applying 2 kV for 2 s, or hydrodynamically by applying 0.5 mbar for 5 s over the sample vial. This corresponds to an injection volume of approximately 0.1 nl. All separations were carried out using an applied voltage of 300 V cm^{-1}.

Table 1
Preparation scheme of buffers with the same ionic strength (I = 0.05) and of the desired pH

Buffer constituent	Stock solutions[a]	pH range
Phosphate	1.0 M NaH_2PO_4 and 1.0 M H_3PO_4	pH 2–4
Acetate	1.0 M CH_3COONa and 1.0 M CH_3COOH	pH 4–6
Phosphate	1.0 M NaH_2PO_4 and 0.5 M Na_2HPO_4	pH 6–8
Borate	0.1 M $Na_2B_4O_7$ and 0.1 M HCl	pH 8–9
Borate	0.1 M $Na_2B_4O_7$ and 0.1 M NaOH	pH 9–10
Phosphate	0.5 M Na_3PO_4 and 0.5 M Na_2HPO_4	pH 10–12

[a] Stock solutions were mixed and diluted to 100 ml with water and pH was measured at 22–23 °C.

Phosphate buffer solutions with different pH were prepared by mixing appropriate volumes of stock solutions (0.5 M Na_3PO_4 and 0.5 M Na_2HPO_4), or (0.5 M Na_2HPO_4 and 1.0 M NaH_2PO_4) or (1.0 M NaH_2PO_4 and 1.0 M H_3PO_4) and subsequently diluting with water to yield an ionic strength of I = 0.05. For example, a phosphate buffer of pH ~ 3.6 and ionic strength 0.05 was prepared by mixing 0.15 ml of the 1.0 M H_3PO_4 with 5.0 ml of 1 M NaH_2PO_4 and diluting to 100 ml with water. This gave a measured buffer

pH value of 3.63–3.64 at 22 °C. See Table 1 for details. Acetate buffer solutions with different pH were prepared by mixing appropriate volumes of stock solutions (1.0 M CH_3COONa and 1.0 M CH_3COOH) and subsequently diluting with water to yield an ionic strength of I = 0.05. Borate buffer solutions with different pH were prepared by mixing appropriate volumes of stock solutions (0.1 M $Na_2B_4O_7$ and 0.1 M NaOH or 0.1 M HCl) and subsequently diluting with water to yield an ionic strength of I = 0.05.

The phosphate buffer solution used for the dilution of PMSB's sample solutions was prepared by mixing 110 ml of 0.25 M Na_3PO_4 with 220 ml of 0.5 M Na_2HPO_4 and diluting to 1000 ml with water (pH 11, I = 0.5). Finally, the phosphate buffer solution was diluted ten times to yield an ionic strength of I = 0.05. Benzyl alcohol was used as marker of the electroosmotic flow. A stock solution of benzyl alcohol was prepared by adding 10 μl benzyl alcohol to 5 ml MeOH. Typically, samples were prepared by dissolving 0.2 mg of an analyte in 66 μl MeCN and thereafter the analyte stock solution was immediately diluted by 133 μl of the phosphate buffer solution (pH 11, I = 0.05). Finally, 4 μl of the benzyl alcohol solution was added to the sample solutions. The concentration of PMSB and BSAP analytes in the sample solution was approximately 1 mg/ml. Standard solutions of test compounds were used for precision and accuracy studies. Typically, stock solutions were prepared at a concentration of approximately 1–5 mg ml^{-1} by dissolving the test compounds in MeOH, ACN or DMSO and subsequently diluting with water. The standard solutions were prepared by mixing 40–300 μl of the respective stock solution of the test compounds with 40–50 μl of the benzyl alcohol solution and then diluting with water to yield a final concentration of 0.05–3 mg ml^{-1}.

2.4. Calculation of pK_a from CE data

The theory and procedures for determination of pK_a values using CE have been reported previously [1–3]. The effective electrophoretic mobilities of the analytes were calculated from the observed migration times of each analyte and migration times of the EOF marker (benzyl alcohol or DMSO) according to equation:

$$m_{eff} = m_{app} - m_{EOF} = \frac{L_{tot} \cdot L_{eff}}{V}\left(\frac{1}{t_{app}} - \frac{1}{t_{EOF}}\right) \quad (1)$$

where m_{eff} is the effective electrophoretic mobility ($cm^2\ V^{-1}\ s^{-1}$), m_{app} is the apparent electrophoretic mobility and m_{EOF} the mobility of the EOF. L_{tot} is the total length of the capillary and L_{eff} the length to the detection window. V is the applied voltage (V) and t_{app} is the observed migration time of each analyte and t_{EOF} the observed migration time of the EOF marker. The pK_a value and the effective electrophoretic mobility of a fully deprotonated acid (m_a) for a weak monoprotic acid can be described by:

$$m_{eff} = \frac{10^{(-pK_a + pH + A)} \cdot m_a}{1 + 10^{(-pK_a + pH + A)}} \quad (2)$$

where A is the activity correction term that can be calculated on basis of the Debye–Hückel theory as

$$A = \frac{0.5085 \cdot z^2 \cdot \sqrt{I}}{1 + 0.3281 \cdot a \cdot \sqrt{I}} \quad (3)$$

In Eq. (3), z is the charge of the ion, I the ionic strength of the BGE, and a is the hydrated analyte ion size parameter. The value of a was set to 5 Å according to Beckers et al. [1], i.e. the size of the analyte ion was assumed to be the same in all buffers and pH. Analogous to a weak monoprotic acid, the pK_a value and the effective electrophoretic mobility of a fully protonated base (m_b) for a weak monoprotic base can be calculated from

$$m_{eff} = \frac{10^{(pK_a - pH + A)} \cdot m_b}{1 + 10^{(pK_a - pH + A)}} \quad (4)$$

By fitting the respective non-linear Eq. (2), or Eq. (4), to data pairs of measured pH and m_{eff}, pK_a and m_a, or m_b, could be calculated. A statistical software package, SigmaPlot v.4.00, from Jandel Scientific Graphing Software (San Rafael, CA, USA), was used for the non-linear regression by means of the Marquardt–Levenberg algorithm.

Table 2
Comparison of dissociation constants determined by CE with reference data

Compound	pK_a CE[a]	pK_a Ref.
Acetylsalicylic acid	3.74 ± 0.1	3.5[b]
Alprenolol	9.38 ± 0.1	9.5[b]
Atenolol	9.42 ± 0.1	9.6[b]
Benzoic acid	4.17 ± 0.1	4.20[c]
4-Hydroxybenzaldehyde	7.58 ± 0.02	7.61[c]
4-Hydroxybenzoic acid	4.44/9.00	4.57[c]/9.46[c]
Metoprolol	9.44 ± 0.1	9.7[b]
Nicotinic acid	4.84 ± 0.1	4.8[b]
Propranolol	9.49 ± 0.2	9.5[b]
Pyridine	5.27 ± 0.03	5.23[c]
Salicylic acid	3.07 ± 0.4	3.0[b]

[a] Values calculated from experimental CE data, ±estimates of 95% CI for the estimates of the regression coefficients from non-linear fits to experimental data.
[b] Values from [44].
[c] Values from [42].

2.5. Calculation of theoretical pK_a

A software, ACD/Lab v.4.55, from Advanced Chemistry Development Inc. (Toronto, Ont., Canada), was used for the calculation of theoretical pK_a values [32].

3. Results and discussion

3.1. Test of CE methodology

Initial studies aimed at optimizing the CE methodology for determination of pK_a values and to establish figures of merit for stabile model compounds that later could be used as reference values in the study of labile compounds. Thus, a set of common model compounds was used to evaluate the accuracy and precision of the CE method. The results obtained here were compared with reference data and are compiled in Tables 2 and 3. A good agreement was observed between pK_a values determined by CE and literature reference data for all model compounds. Generally, lower pK_a values differed less than 0.1 pK_a units from literature data whereas the difference was less than 0.2 for higher pK_a values. Notably, most reference data were determined with different techniques in different media and often at a slight different temperature compared with the CE method. Therefore, reference data for the model compounds in the same media as in CE were not available. The CE-data were obtained at 22 °C and reference data mainly at 25 °C. A deviation in temperature is expected to affect pK_a of a basic compound more than for an acidic compound [33–35].

The precision of the CE method, expressed in terms of repeatability and reproducibility of the migration time, mobilities and pK_a, are shown in Table 3. The repeatability of effective electrophoretic mobilities and pK_a values were very good and slightly better than the corresponding reproducibility data, as expected. The precision in determination of pK_a is directly related to the precision in the measured effective electrophoretic mobilities, as is evident from Eqs. (2) and (4). Repeatability was studied using a capillary with an effective length of 41.4 cm (total length of 50.0 cm). Samples were injected by electromigration (1 kV for 2 s). The standard deviation of five repeated measurements of mobility for stabile

Table 3
Precision data for two model compounds

Model compounds	Effective mobility[a] (10^{-4} cm^2 V^{-1} s^{-1})		pK_a	
	Repeatability[b]	Reproducibility[c]	Repeatability[b]	Reproducibility[c]
4-Hydroxybenzaldehyde	2.64 (S.D. = 0.002)	2.66 (S.D. = 0.03)	7.62 (S.D. = 0.01)	7.57 (S.D. = 0.04)
Propranolol	1.78 (S.D. = 0.001)	1.78 (S.D. = 0.04)	9.52 (S.D. = 0.01)	9.53 (S.D. = 0.05)

[a] Effective electrophoretic mobility of fully ionized compounds.
[b] Five analysis sequences during 1 day.
[c] Five analysis sequences at different days during 9 months.

model compounds, pH range 4–9.5, were typically between 0.002 and 0.005 (10^{-4} cm^2 V^{-1} s^{-1}). This is in agreement with previous reports [1,36] that stated that measurements of effective mobility can be performed with very high precision. The precision of the overall methodology, determination of pK_a, showed that the reproducibility of the method between different days over a 9-month period of time was typically between 0.02 and 0.05 in terms of the standard deviation of pK_a.

The effective electrophoretic mobility and hence the experimentally determined pK_a and the actual analyte pK_a are influenced by any variation in BGE ionic strength. The robustness of the method was, therefore, tested by varying the ionic strength of the BGE's. Although a large deviation in ionic strength was deliberately introduced (I = 0.045, 0.050 and 0.055), the determined pK_a values varied only 0.02 pK_a-units or less which is at the same level as the S.D. of the mathematical non-linear regression procedure (data not shown). Also the number of BGE's sufficient for pK_a determinations were evaluated. That is, the effect of number of measured mobilities, using different electrolytes to span out the necessary pH range, above and below the pK_a value, was studied. The results showed that there were generally less than a ± 0.05 pH units difference between pK_a determined by using 14 instead of four BGE's, provided the BGE's were evenly distributed over the necessary pH range. This is in agreement with earlier reports by Smith et al. [37].

3.2. Strategy for determination of dissociation constants for labile compounds

Several aspects must be considered in the course to modify CE for determination of labile compounds. It is proposed that a general strategy should comprise the following three steps:

1. Compounds should be dissolved in a stabilizing medium to minimize analyte degradation in the sample container during the CE separation sequence.
2. Analysis times should be kept short to minimize analyte degradation in the capillary during the CE separation.

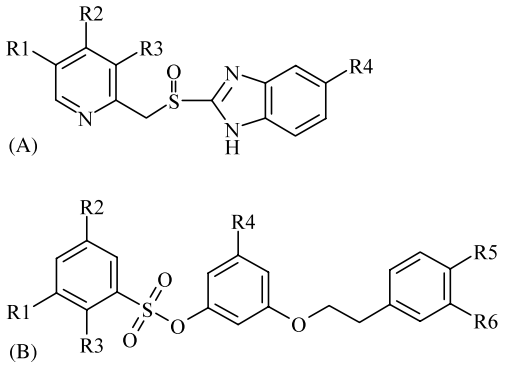

Fig. 1. (A) General structure of pyridinyl–methyl–sulfinyl–benzimidazoles (PMSB's). (B) General structure of benzenesulfonic acid phenethyloxy–phenyl esters (BSAP's). The substituents at the R5 position consist of a primary amine (–CH_2NH_2) or an amidine (–$CHNHNH_2$), the substituent at R6 consist of a hydroxyl-group or a hydrogen.

3. Any means that can enable peak characterization of the main component should be used, e.g. acquisition of UV–Vis absorption spectra or use of mass spectrometry detection. PMSB's were selected as a first set of model compounds in this study. PMSB is a group of drug compounds that are known to degrade in aqueous solutions under neutral and acidic conditions [38]. The general structure of PMSB's is shown in Fig. 1. Typical CE separations at different pH are shown in Fig. 2 for the rabeprazole compound. From these electropherograms it is obvious that degradation during CE separation increases at lower pH. In particular, at pH 3.5 degradation products interfere with the analyte peak of interest, Rabeprazole (R). Moreover, at this pH the electroosmotic flow is approximately four times lower at pH 10.6. Thus, the holding time is inevitably longer which enhance degradation of the labile compounds. Based on initial tests, lansoprazole and rabeprazole were selected for further investigations because they are the least stabile in the first set of model drug compounds studied here.

A number of key steps were evaluated in order to optimize the overall methodology. These experiments comprised sample matrix and injection, separation time and peak characterization. The

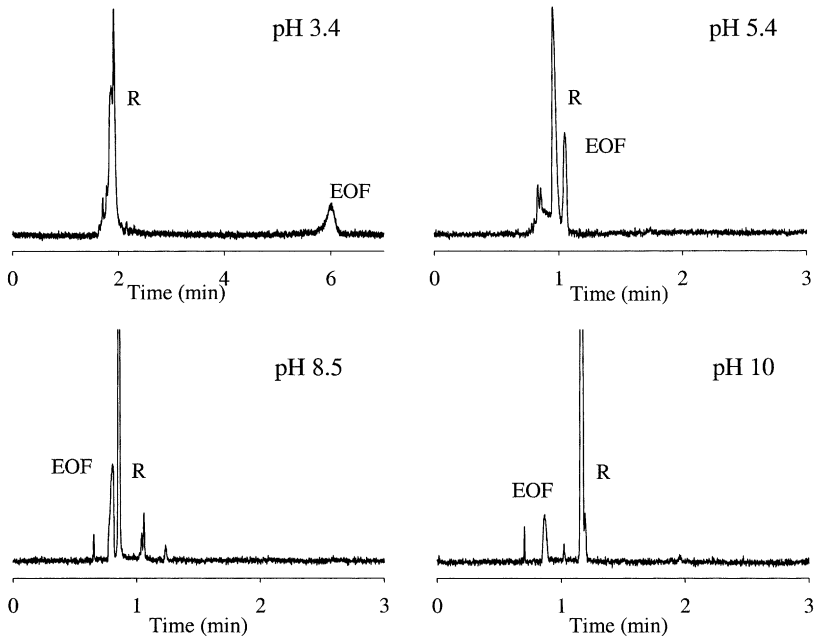

Fig. 2. CE separation of Rabeprazole that degrade at neutral and acidic pH. Electropherograms from CE separations in electrolytes of different pH. R, Rabeprazole; EOF, marker of electroosmotic flow (benzyl alcohol).

results from the tests of injection conditions on pK_a determination are shown in Fig. 3A–C. The effective electrophoretic mobility versus pH has been plotted for different sample diluents (A), injection volumes (B), and type of injection (C).

For the model compounds whose degradation accelerates at acidic conditions, an analyte can not be dissolved in the BGE, as is common in conventional CE, particularly not in separations using low pH electrolytes. The PMSB's are known

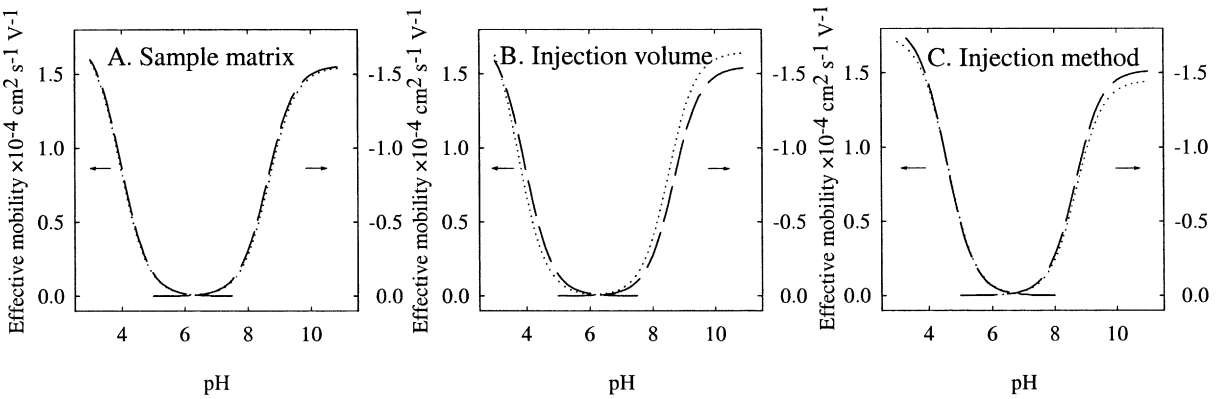

Fig. 3. Influence of injection conditions on determination of dissociation constants by capillary electrophoresis (CE). Effective electrophoretic mobility vs. electrolyte pH plotted as best fit of non-linear regression to experimental CE data. (A) Effect of sample matrix. Analyte stock solution diluted with water (— —) and pH 11 buffer (. . .); (B) Effect of injection volume 0.2 nl (— —) and 3.9 nl (. . .); (C) Effect of injection method, electrokinetic (— —) and hydrodynamic (. . .) when the same amount of analyte is injected. Lansoprazole and rabeprazole were used as model compounds in A, B and C, respectively. See Section 2 for further details.

to degrade rapidly in aqueous solutions under acidic conditions [38,39]. The half-life in methanol–phosphate (5:95, v/v) buffer solutions at pH 3 have been reported to be 9 min for pantoprazole and 4 min for lanzoprazole [30]. These compounds are stable in basic organic solvents such as *N*-methylformamide, dimethylformamide, dimethylacetamide and dimethylsulfoxide [40]. However, it is beyond the scope of this study to extend the methodology outside of aqueous electrolytes. The PMSB's were, therefore, dissolved in a high pH buffer (pH 11) to minimize any analyte degradation in the sample vial during the automated analysis sequence. Typically, a CE complete measurement sequence (conditioning, injections and analysis) could last 30–90 min while it was necessary to dissolve the PMSB's in a stabilizing diluent. Indeed, the PMSB's are stable in aqueous solutions at pH 11 since the half-life can be as long as 300 days [39]. Still, a potential risk at high pH is that a sample diluent may alter the separation conditions by introducing a too large zone of a different buffer into the separation electrolyte (inside of the capillary). In Fig. 3A are shown results for lansoprazole when comparing a sample matrix of water and a pH 11 buffer during hydrodynamic injection. No significant differences could be observed, provided that the injection volume is kept small. With larger injection volumes, peak broadening increases. In Fig. 3B is shown the influence on pK_a determination from an increase in injection volume (ca. a factor of 20) using hydrodynamic injection. A shift is observed towards lower pH in the mobility versus pH plot.

The next step was to investigate the effect of injection method on determination of pK_a. Here, electromigration was selected instead of hydrodynamic injection in order to minimize the volume of high pH buffer, used as sample diluent, to be introduced into the capillary (see Fig. 3C). This action improved the compatibility between the PMSB sample solution and the low pH electrolytes used for determination of the lower pyridinyl dissociation constants. The main advantage with electromigration is that a smaller volume of sample diluent is introduced into the capillary, in order not to alter the electrolyte conditions, i.e. the BGE pH. However, this effect may in practice be minimal because of the large difference between EOF mobility and analyte mobility once the high voltage is supplied. On the other hand, hydrodynamic injection is advantageous in that the sample is injected as a plug, which means that the analyte is maintained in a stabilizing sample matrix until the effective separation starts. Thus, the time spent in the capillary of the analyte in a non-optimal environment, here at low pH, is minimized, whereas with injection by electromigration, the sample will be introduced into the low pH electrolyte already during the injection step. Still, a potential advantage with electromigration over hydrodynamic injection is the built-in segregation between analyte and related substances. This may favor extraction of the analyte of interest over already existing degradation products in the sample, however, in this study no such advantage could be observed because the major degradation product had a similar mobility as the main drug compound.

The effect of injection method, electromigration or hydrodynamic injection, was further investigated during determination of the dissociation constants of lansoprazole, pantoprazole and rabeprazole (data not shown). The volume injected with hydrodynamic injection was adjusted to give the same analyte peak height as the analyte peak height obtained with electromigration injection. Generally, the lower pyridinyl dissociation constants were approximately 0.1 pK_a units higher when hydrodynamic injection was used. This implies that the pH 11 sample diluent increases the local pH in the capillary and, therefore, yields a slightly higher dissociation constant. The upper dissociation constant of the PMSB's were the same for both hydrodynamic and electromigration injection.

To minimize analyte degradation during CE separations, short analysis times were tested through use of a short separation capillary, effective length 8.5 cm compared with 26.5 cm. However, no significant effect regarding less degradation of PMSB's was observed. This emanates mainly from that the sample diluent was the most critical step in the analytical procedure for the PMSB's. In addition, the hold up time in the capillary of these model compounds was suffi-

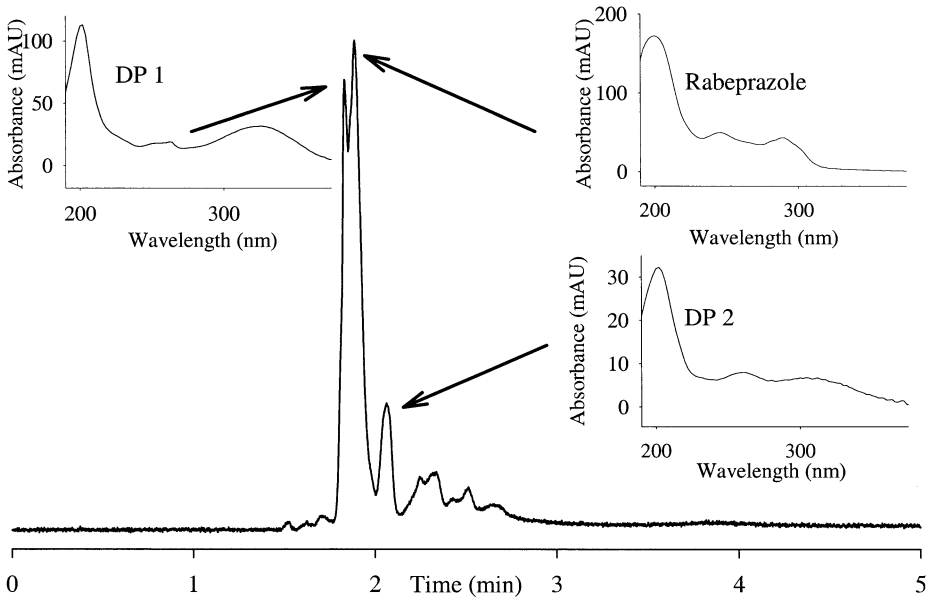

Fig. 4. Illustration of peak characterization using diode-array detection during determination of dissociation constant(s) by CE. DP 1 and DP 2 denotes the degradation products of Rabeprazole. See Section 2 for further details.

ciently short. Still, the shorter capillary significantly reduced the total analysis time.

A general problem with severe degradation of analytes during separation is the need for correct identification of peaks in the electropherogram. Typically, peaks for degradation products may overlap with the main analyte of interest, why then peak characterization is needed. In Fig. 4 is shown an illustration of the CE separation of Rabeprazole, where the main component is highly degraded. By means of UV–Vis spectra acquired online during separation, it was possible to distinguish between the main component (R) and two of its degradation products (DP1) and (DP2).

In addition to the PMSB's, a second set of model drug compounds, (BSAP's) benzenesulfonic acid–esters having a phenyl group substituted with primary amines, amidines and/or hydroxyl groups, was also investigated. The general structure of these BSAP's, is shown in Fig. 1. These compounds are ionizable and highly lipophilic and were found to be stable in pure DMSO and in mixtures of water and DMSO. However, in buffer at pH > 6 these compounds rapidly degrade as is shown in the electropherogram in Fig. 5. The main

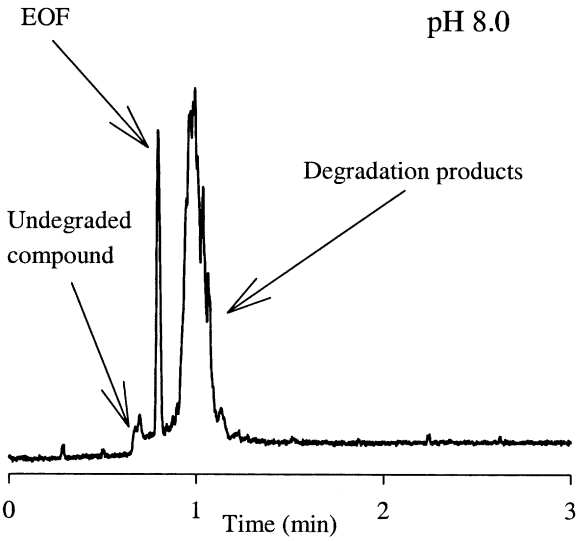

Fig. 5. Illustration of determination of pK_a values where the main analyte severely degrade during CE analysis in aqueous solutions at neutral to high pH. Electropherogram for a benzenesulfonic acid phenethyloxy–phenyl ester model compound from a CE separation in a BGE of pH 8. See Section 2 for further details.

Table 4
Dissociation constants of labile PMSB's determined by CE

Model compounds	pK_a CE[a]	pK_a Ref.
H 168/66	7.72±0.04/3.24±0.31	
Lansoprazole	8.73±0.12/3.82±0.08	8.78[b]/4.01[b]
Omeprazole	8.85±0.12/3.94±0.08	8.98[b]/4.13[b], 8.70[c]/3.98[c]
Pantoprazole	8.18±0.12/3.56±0.12	8.19[b]/3.92[b]
Rabeprazole	8.75±0.12/4.48±0.18	

[a] Values calculated from experimental CE data, ±estimates of 95% CI for the estimates of the regression coefficients from non-linear fits to experimental data.
[b] Values from [30], spectrophotometric 25 °C.
[c] Values from [41], potentiometric 37 °C.

Table 5
Dissociation constants of labile BSAP's determined by CE

Model compounds	pK_a CE[a]	pK_a calculated[b]
28	8.3±0.1	8.1±0.4
34	8.6±0.4	8.1±0.4
40	8.3±0.2	8.1±0.4
29	6.2±0.04	8.2±0.4
35	6.3±0.2	8.2±0.4
41	6.3±0.1	8.2±0.4
31	9.4±0.2	9.5±0.1
37	9.0±0.4	9.5±0.1
43	9.3±0.2	9.5±0.1
32	10.5±0.4	10.3±0.4
44	10.9±0.8	10.3±0.4

[a] Values calculated from experimental CE data, ±estimates of 95% CI for the estimates of the regression coefficients from the non-linear fit to experimental data.
[b] Values calculated using ACD/Lab [32], ±the 95% CI for the estimated pK_a values.

degradation products were found to be negatively charged at this pH because they migrated after the marker of the electroosmotic flow. The strategy employed here was to dilute the compounds dissolved in DMSO with water and use this solution as the stabilizing sample matrix during injection. In addition, a short analysis time was found to be crucial to minimize degradation during separation in buffers at pH > 6. The short analysis time was obtained by using an 8.5 cm effective capillary length. In this way the analyte yields a sufficient peak height to be distinguished from degradation products and the EOF marker. When using a longer effective length, 26.5 cm, the main analyte peak, could not be distinguished from other peaks (data not shown). Identification of the correct migration time through visual inspection of the electropherogram and the peak shape was important for the BSAP's, because of more severe degradation than with the PMSB's.

3.3. Dissociation constants of pyridinyl–methyl–sulfinyl–benzimidazoles and benzenesulfonic acid phenethyloxy–phenyl esters

Owing to the poor stability of PMSB's, a rapid potentiometric method has previously been developed for determination of their pK_a values [41]. With the optimized methodology described above, CE could be used to determine dissociation constants of five labile PMSB's (see Table 4). In Fig. 5 are shown plots of mobility versus BGE pH for 4 of the model compounds. Good agreement is shown between CE and potentiometric data. However, a small difference is indicated for the low pK_a between the CE and the reference data. For the low pyridinyl pK_a value the difference is still only 0.2 pK_a units. The difference in the pyridinyl pK_a value between CE and the reference data is not likely to be caused by a temperature effect. The temperature coefficient of pyridine is small while a decrease in temperature change from 25 to 20 °C would only be expected to increase the pK_a approximately 0.06 pK_a-units [33]. Thus, the small deviation between the CE and the reference data could emanate from several sources, e.g. different media used by the methods or interference from unpure substances or degradation products at low pH in the reference method.

For the second set of model drug compounds, BSAP's, CE enabled determination of pK_a values. Because only minute amount of sample was available in this study, and the BSAP's model compounds degrade at neutral to high pH, common techniques for pK_a determination could not be used [31]. This emphasizes the general utility of CE for determination of pK_a for labile compounds. Using the strategy described above CE enabled determination of pK_a values for the 11 model compounds although these partially de-

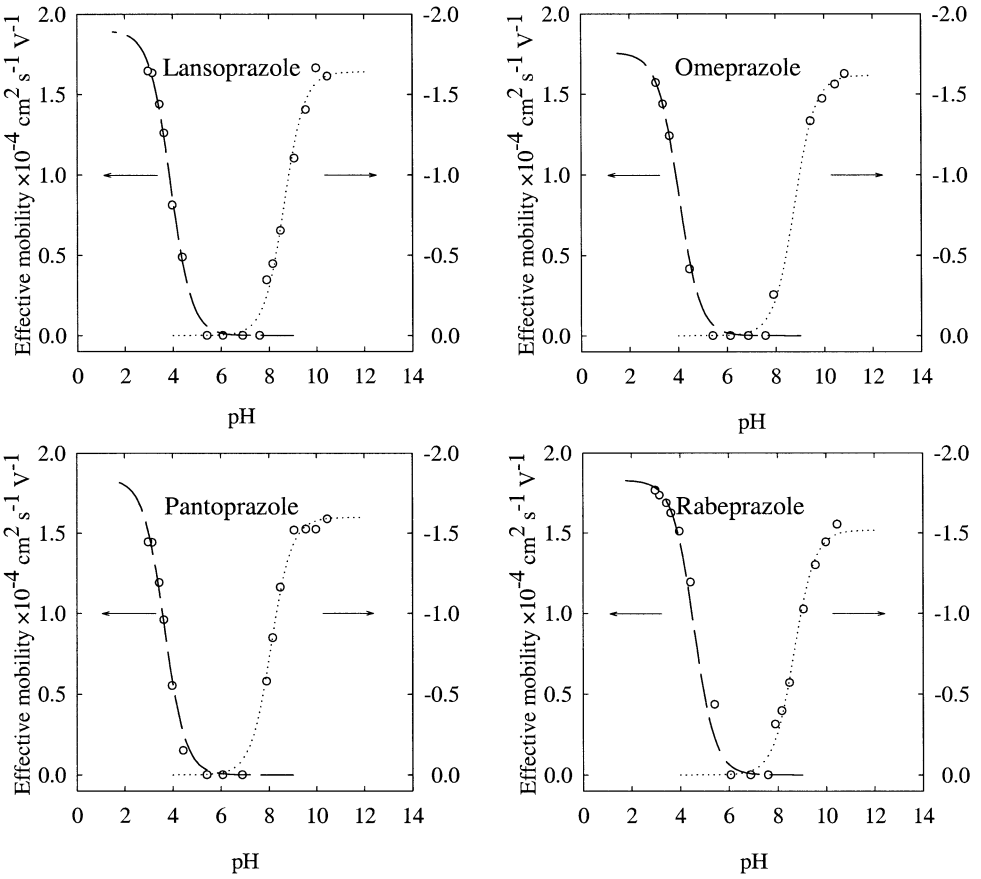

Fig. 6. Determination of dissociation constants for PMSB's by capillary electrophoresis (CE). Circles represent experimental CE data, and long dashed and dotted lines fitting expression 2 and 4, respectively, for modeling effective mobility as function of BGE pH.

graded during analyses (see Table 5). To our knowledge, no experimental pK_a values have been reported previously for this specific set of model compounds. However, the pK_a values determined by CE for the BSAP model compounds were found to be close to data reported for a simple benzamidine (p$K_a = 11.6$) and a simple benzylamine (p$K_a = 9.34$) [42]. Besides the hydroxyl-group, the benzamidine and the benzylamine are ionizable groups in these molecules. In addition, pK_a for amidine substituted thrombin inhibitors similar to these model compounds have been reported to be >12 [43]. Moreover, in order to get additional reference data, pK_a values were calculated using the ACD/Lab software (see Table 5). The CE determined pK_a values were in good agreement with the calculated pK_a values, except for the compounds containing a benzamidine substituted with a hydroxyl-group. This discrepancy between experimental pK_a data and calculated pK_a is here suggested to be due to the lack of comparable reference data for the calculation of theoretical pK_a values. However, it was expected that the hydroxy-group should lower the pK_a of the benzamidine group [31].

The estimated confidence intervals of the pK_a determined for the most labile model compounds, BSAP's, (see Table 5) was found to be within the same range as for the stable compounds (c.f. Table 3). This is only slightly larger than for the PMSB's (c.f. Table 4). Altogether, this demonstrates that pK_a can be determined even for labile compounds

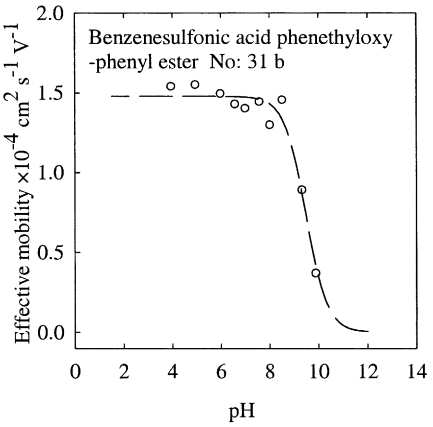

Fig. 7. Illustration of determination of pK_a values where the main analyte severely degrade during CE analysis in aqueous solutions of neutral to high pH. Determination of pK_a for the BSAP no 31b. Circles represents experimental CE data, and solid lines fitting of expression 4 (denoted by — —) for modeling effective mobility as function of BGE pH. See Section 2 for further details.

that degrade during the CE separation and, in addition, that a structured strategy is a means to obtain high precision and accuracy (Figs. 6 and 7).

4. Conclusions

The general methodology outlined in this study and the results from the application to the pyridinyl–methyl–sulfinyl–benzimidazole and benzenesulfonic acid phenethyloxy–phenyl ester model drug compounds demonstrate the power of CE as a general format for physicochemical characterization of drug compounds. In particular, the nanotechnological features of CE makes it interesting for use also in the early stage of drug discovery when compounds are often less pure and only available in minute amounts. As proposed here, by considering key steps in the CE procedure such as sample diluent, injection method and peak characterization, compounds that may degrade during separation in aqueous electrolytes may also be analyzed. Thus, pK_a can be determined with high precision and accuracy also for labile drug compounds.

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References

[1] J.L. Beckers, F.M. Everaerts, M.T. Ackermans, J. Chromatogr. 537 (1991) 407–428.
[2] J. Cai, J.T. Smith, Z.E. Rassi, J. High Resolut. Chromatogr. 15 (1992) 30–32.
[3] J.A. Cleveland, Jr, M.H. Benko, S.J. Gluck, Y.M. Walbroehl, J. Chromatogr. 652 (1993) 301–308.
[4] Y. Ishihama, Y. Oda, N. Asakawa, J. Pharm. Sci. 83 (1994) 1500–1507.
[5] J. Cao, R.F. Cross, J. Chromatogr. 695 (1995) 297–308.
[6] N. Chauret, D.K. Lloyd, D. Levorse, D.A. Nicoll-Griffith, Pharm. Sci. 1 (1995) 59–62.
[7] F. Lelievre, P. Gareil, J. Chromatogr. 735 (1996) 311–320.
[8] S. Bellini, M. Uhrova, Z. Deyl, J. Chromatogr. 772 (1997) 91–101.
[9] C.E. Lin, C.C. Chang, W.C. Lin, J. Chromatogr. 768 (1997) 105–112.
[10] C.L. Sheng, X. Zhou, S. Jin, Chromatographia 46 (1997) 555–559.
[11] P. Coufal, K. Stulik, H.A. Claessens, M.J. Hardy, M. Webb, J. Chromatogr. B. Biomed. Appl. 720 (1998) 197–204.
[12] J.P. Mercier, P. Morin, M. Dreux, A. Tambute, Chromatographia 48 (1998) 529–534.
[13] Y. Mrestani, R. Neubert, A. Munk, M. Wiese, J. Chromatogr. 803 (1998) 273–278.
[14] J. Barbosa, D. Barron, E. Jimenez-Lozano, J. Chromatogr. 839 (1999) 183–192.
[15] R. Jankowsky, M. Friebe, B. Noll, B. Johannsen, J. Chromatogr. 833 (1999) 83–96.
[16] D. Barron, E. Jimenez-Lozano, A. Irles, J. Barbosa, J. Chromatogr. A 871 (2000) 381–389.
[17] P. Bartak, D. Pechova, P. Tarkowski, P. Bednar, M. Kotoucek, Z. Stransky, R. Vespalec, Anal. Chim. Acta 421 (2000) 221–229.
[18] P. Bartak, P. Bednar, Z. Stransky, P. Bocek, R. Vespalec, J. Chromatogr. 878 (2000) 249–259.
[19] J. Barbosa, D. Barron, J. Cano, E. Jimenez-Lozano, V. Sanz-Nebot, I. Toro, J. Pharm. Biomed. Anal. 24 (2001) 1087–1098.
[20] J. Barbosa, D. Barron, E. Jimenez-Lozano, V. Sanz-Nebot, Anal. Chim. Acta 437 (2001) 309–321.
[21] G.A. Caliaro, C.A. Herbots, J. Pharm. Biomed. Anal. 26 (2001) 427–434.
[22] Z. Jia, T. Ramstad, M. Zhong, Electrophoresis 22 (2001) 1112–1118.
[23] W. Ketai, L. Huitao, C. Xingguo, Z. Yunkun, H. Zhide, Talanta 54 (2001) 753–761.

[24] C.E. Kibbey, S.K. Poole, B. Robinson, D. Jackson, D. Durham, J. Pharm. Sci. 90 (2001) 1164–1175.
[25] H.-X. Liu, G.-L. Yang, D.-X. Wang, S.-F. Sun, J.-J. Ma, Chin. J. Chem. 19 (2001) 675–680.
[26] T. Takayanagi, S. Motomizu, Chem. Lett. 1 (2001) 14–15.
[27] D.X. Wang, G.L. Yang, X.R. Song, Electrophoresis 22 (2001) 464–469.
[28] E. Örnskov, S. Folestad, Symposium on High Performance Liquid Phase Micro Separations Abstracts of Posters, Swedish Academy of Pharmaceutical Sciences, Lund, Sweden, 1998, p. 16.
[29] A. Brändstrom, P. Lindberg, N.A. Bergman, T. Alminger, K. Ankner, U. Junggren, B. Lamm, P. Nordberg, M. Erickson, et al., Acta Chem. Scand. 43 (1989) 536–548.
[30] B. Kohl, E. Sturm, J. Senn-Bilfinger, W.A. Simon, U. Kruger, H. Schaefer, G. Rainer, V. Figala, K. Klemm, J. Med. Chem. 35 (1992) 1049–1057.
[31] A. Linusson, J. Gottfries, T. Olsson, E. Örnskov, S. Folestad, B. Nordén, S. Wold, J. Med. Chem. 44 (2001) 3424–3439.
[32] A.C.D.I. ACD/Lab, in, 90 Adelaide Street Wet, Suite 702 Toronto, Ontario M5H 3V9, Canada.
[33] D.D. Perrin, Aust. J. Chem. 17 (1964) 489–490.
[34] A. Albert, E.P. Serjeant, The Determination of Ionization Constants, Chapman and Hall, New York, 1984, pp. 11–13.
[35] A. Albert, E.P. Serjeant, The Determination of Ionization Constants, Chapman and Hall, New York, 1984, pp. 199–202.
[36] S.J. Gluck, J.A. Cleveland, J. Chromatogr. 680 (1994) 49–56.
[37] S.C. Smith, M.G. Khaledi, Anal. Chem. 65 (1993) 193–198.
[38] M. Mathew, V.D. Gupta, R.E. Bailey, Drug Dev. Ind. Pharm. 21 (1995) 965–971.
[39] A. Pilbrant, C. Cederberg, Scand. J. Gastroenterol. Suppl. 108 (1985) 113–120.
[40] A. Tivesten, S. Folestad, V. Schönbacher, K. Svensson, Chromatographia 49 (1999) S7–S11.
[41] A. Brändström, N.-Å. Bergman, I. Grundevik, S. Johansson, L. Tekenbergs-Hjelte, K. Ohlson, Acta Chem. Scand. 43 (1989) 569–574.
[42] A. Albert, E.P. Serjeant, The Determination of Ionization Constants, Chapman and Hall, New York, 1984, pp. 137–166.
[43] C.T. Supuran, A. Scozzafava, F. Briganti, B.W. Clare, J. Med. Chem. 43 (2000) 1793–1806.
[44] A.C. Moffat, Clarke's Isolation and Identification of Drugs in Pharmaceuticals, Body fluids, and Post-Mortem Material, The Pharmaceutical Society of Great Britain, The Pharmaceutical Press, London, 1986.