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Rapid screening of pK_a values of pharmaceuticals by pressure-assisted capillary electrophoresis combined with short-end injection

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Abstract

A method applying pressure-assisted capillary electrophoresis combined with short-end injection has been developed for the rapid screening of the pK_a values of pharmaceuticals. The electrophoretic separation is performed on a short capillary length with short-end injection under an applied pressure, and the effective mobility is measured in a series of 10 different buffers with constant ionic strength ($I = 0.05$). The application of pressure not only reduces migration times, particularly in lower pH buffers, but also improves the repeatability of effective mobility measurements. The influence of pressure on the effective mobility was investigated at various pH values. It was observed for the first time that an increase in pressure resulted in a slight decrease in the effective mobility when the pH was above the pK_a for acidic analytes, whereas an increased effective mobility with increasing pressures was observed when the pH was below the pK_a. However, the observed effective mobility shift by the applied pressure did not significantly affect the determined pK_a values. The determined pK_a values were in good agreement with published data. Furthermore, a stacking condition was applied to increase the sensitivity, and a concentration down to 2 μM could readily be detected with UV detection using a 50 μm I.D. capillary. This technique is particularly suitable for measurement of pK_a values for compounds with poor aqueous solubility. The method also omits the commonly used preconditioning steps with sodium hydroxide and water. The exclusion of excessive preconditioning steps and the use of pressure reduces the total cycling analysis time, and makes it possible to determine the pK_a in less than 40 min per compound without loss of accuracy.
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1. Introduction

Recently, the rapid growth of combinatorial chemistry has generated a large number of compounds available for high-throughput screening as potential drug candidates. This has placed high demands on the development of rapid methods for characterization of the underlying physico-chemical properties, including parameters such as the dissociation constant (pK_a value). Traditional pK_a determination is primarily based on potentiometric titration and spectroscopic methods [1–5]. These methods require a relatively large quantity of sample and may give inaccurate results for compounds of low purity. In recent years, a new technique, capillary electrophoresis (CE), has been introduced for pK_a determination of diverse compounds [6–26]. This method, due to its distinct advantages in terms of minute sample consumption, not having to know the concentration of the solutes, ease of automation and,

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in particular, insensitivity to sample purity, has received increasing attention in the pharmaceutical area [22,24–26]. The fundamental principles of pK_a determination by CE rely upon measuring the ionic effective mobility of the solute as a function of pH. Consequently, the pK_a value is obtained by fitting an equilibrium equation to the effective mobility and pH data with a non-linear regression technique. The background of this technique and the equations that relate electrophoretic mobility to pK_a have been described in numerous publications [6–11]. More recently, a medium-throughput pK_a screening method based on pressure-assisted CE has been demonstrated by Jia et al. [22]. Their method is, in principle, similar to other CE methods with a normal inlet injection, but a pressure was applied during the electrophoretic separation to reduce migration times. The use of pressure-assisted CE for pK_a determination was reported earlier by Jankowsky et al. [18]. In addition, pressure-assisted approaches have been utilized in other separations, for instance CE–MS [27] and microemulsion electrokinetic chromatography (MEEKC) [28]. However, there have been only a few reports regarding pressure-assisted CE applications [18,22,25,27,28]. Also, the effect of pressure on the effective mobility has not been well investigated. In the present work, a method applying pressure-assisted capillary electrophoresis combined with short-end injection was explored for fast pK_a measurement. In this case, a short separation capillary length (8.5 cm from outlet to detection window) was employed and pressure was applied during the separation. The effect of pressure on the effective mobility was investigated. On-line stacking using pressure-assisted CE was demonstrated to be applicable for measurement of pK_a values at low concentrations. The developed method has been applied to pK_a determinations of compounds in the drug discovery phase.

2. Experimental

2.1. Apparatus

Capillary electrophoresis measurements were carried out with a HPCE^{3D} instrument (Agilent Technologies) equipped with a diode array detection (DAD) system. UV detection at 210 nm was used during the measurements. Untreated fused-silica capillaries (Skandinaviska GeneTec, Sweden) were used. The capillary was thermostated at 22 °C utilizing an external water bath connected to the sample tray. All pH values were measured with a pH meter (PHM240, pH Ion/meter, MeterLab, France) between 22 and 23 °C.

2.2. Procedures

2.2.1. Preparation of background electrolytes with the same ionic strength

A series of 10 different phosphate and acetate buffers were prepared using a laboratory developed computer program to calculate the appropriate electrolyte composition. The calculated and measured pH values are shown in Table 1. All buffers were filtered through 0.22 or 0.45 μm filters (Millex-GV, Millipore, Molsheim, France). The filtered buffers were tightly capped and kept at +4 °C until use. The three high-pH buffers (8.5, 10, 11) can only be used for 2 weeks due to dissolution of carbonate. The others can be used for more than 2 months.

2.2.2. Preparation of sample solutions

Each test compound was dissolved in water or acetonitrile–water (50:50) to give a stock concentration of ca. 10 m*M*. Injected samples were prepared by mixing 10 μL of the respective test compound (10 m*M*)+10 μL of 10% (v/v) dimethyl sulfoxide (DMSO) solution, then diluting to 1000 μL. The final concentrations of compounds and DMSO in the injected sample solution were 100 μ*M* and 0.1% (v/v), respectively. A mixture of three substances containing lidocaine (A), ibuprofen (B) and benzoic acid (C) was used to check the conditions.

2.2.3. Capillary pretreatment and electrophoretic conditions

New capillaries (42–50 cm×50 μm I.D.) were conditioned by flushing with 1.0 *M* NaOH for 30 min, then with purified water for 10 min. Between runs in the analytical sequence, the capillary was flushed only with running buffer for 1.5 min. Neither sodium hydroxide nor water was flushed between runs in this work. Reversed polarity of high voltage was applied. Samples were introduced by pressure

Table 1
BGE with the same ionic strength as determined by a computer program

Calculated pH	Ionic strength, I	Volume (mL)	Volume (mL)	Measured pH
		H_3PO_4 (0.5 M)	NaH_2PO_4 (1.0 M)	
2.50	0.05	4.408	4.630	2.50±0.03
3.00	0.05	1.393	4.882	3.00±0.04
		NaAc (1.0 M)	Hac (1.0 M)	
4.0	0.05	4.988	23.637	4.00±0.03
5.0	0.05	4.999	2.364	5.00±0.01
		Na_2HPO_4 (0.5 M)	NaH_2PO_4 (1.0 M)	
6.0	0.05	0.849	3.727	6.00±0.04
7.0	0.05	2.579	1.132	7.00±0.04
8.0	0.05	3.238	0.142	8.00±0.05
8.5	0.05	3.321	0.046	8.50±0.03
		Na_2HPO_4 (0.5 M)	Na_3PO_4 (0.1 M)	
10.0	0.05	3.246	0.214	10.00±0.06
11.0	0.05	2.605	1.718	11.00±0.02

Total volume 100 mL.
All pH values were measured at temperatures of 22–23 °C.

from the outlet. Sequence run orders were set from high pH to low pH to minimize the effect of carbonate. Other conditions are given in the figure legends.

2.2.4. Calculation of effective mobility

The effective mobility was calculated as:

$$m_{\text{eff}} = m_{\text{obs}} - m_{\text{eof}} = \frac{L_{\text{tot}} \cdot L_{\text{eff}}}{V} \cdot \left(\frac{1}{t_{\text{obs}}} - \frac{1}{t_{\text{eof}}} \right) \qquad (1)$$

where m_{eff} is the effective mobility of the analyte ($cm^2\ V^{-1}\ s^{-1}$), m_{obs} is the apparent or observed mobility of the analyte ($cm^2\ V^{-1}\ s^{-1}$), m_{eof} is the mobility of the electroosmotic flow (neutral marker) ($cm^2\ V^{-1}\ s^{-1}$), L_{tot} is the total length of the capillary (cm), L_{eff} is the effective separation length from injection to detector (cm), V is the applied high voltage (V), t_{obs} is the observed migration time of the analyte (s), and t_{eof} is the observed migration time of the neutral marker (DMSO) (s).

2.2.5. Calculation of pK_a by SigmaPlot and fit-pK_a

Equations relating pK_a and the effective electrophoretic mobility for a monoacid, monobase, monoacid/monobase, diacid and dibase are shown in Table 2. By fitting an equilibrium equation to measured m_{eff} and pH data with a non-linear regression by means of SigmaPlot (Jandel Scientific Graphing Software, San Rafael, CA, USA), the pK_a values and corresponding regression coefficients were obtained. In addition, a program, ''fit-pK_a'', written in our laboratory, was utilized for simultaneous calculations of pK_a values for a number of substances in a rapid manner.

3. Results and discussion

3.1. Choice of background electrolytes and ionic strengths

It is well recognized that an increase in ionic strength results in a decreased ionic mobility [11,29–31]. Theoretical pK_a model equations also reveal that the pK_a and ionic mobility of an analyte depend on the ionic strength of the background electrolyte. Therefore, it is important to keep the ionic strengths of the buffer series constant throughout the pK_a measurements. In the present work, a computer program was employed to calculate a series of 10 different pH buffers, which exhibited excellent agreement between calculated and measured pH values from pH 2.4 to 11 with constant ionic strength

Table 2
Model equations used for pK_a determination

Ionizable type	Model equation
Monoacid	$m_{eff} = \frac{m_a \cdot 10^{(-pK_a + pH + A)}}{1 + 10^{(-pK_a + pH + A)}}$
Monobase	$m_{eff} = \frac{m_b \cdot 10^{(pK_a - pH + A)}}{1 + 10^{(pK_a - pH + A)}}$
	$A = \frac{0.5085 \cdot z^2 \sqrt{I}}{1 + 0.3281 \cdot a \sqrt{I}}$
Monoacid/monobase	$m_{eff} = \frac{m_{a1} \cdot [10^{-pH}]^2 + m_{a2} \cdot 10^{-pK_{a1}} \cdot 10^{-pK_{a2}}}{[10^{-pH}]^2 + 10^{-pK_{a1}} \cdot 10^{-pH} + 10^{-pK_{a1}} \cdot 10^{-pK_{a2}}}$
Diacid	$m_{eff} = \frac{m_{a1} \cdot 10^{-pK_{a1}} \cdot 10^{-pH} + m_{a2} \cdot 10^{-pK_{a1}} \cdot 10^{-pK_{a2}}}{[10^{-pH}]^2 + 10^{-pK_{a1}} \cdot 10^{-pH} \cdot 10^{-pK_{a1}} \cdot 10^{-pK_{a2}}}$
Dibase	$m_{eff} = \frac{m_{a1} \cdot [10^{-pH}]^2 + m_{a2} \cdot 10^{-pK_{a1}} \cdot 10^{-pH}}{[10^{-pH}]^2 + 10^{-pK_{a1}} \cdot 10^{-pH} \cdot 10^{-pK_{a1}} \cdot 10^{-pK_{a2}}}$

A is the activity correction term, z is the charge of the ion, I is the ionic strength of the background electrolyte ($I = 0.5 \cdot \Sigma c_i \cdot z_i^2 = 0.05$), a is the hydrated analyte ion size parameter, set to 5 Å, and m_a and m_b is the maximum mobility (constants for a given ion).
For simplicity, the activity term A was not taken into account in some model equations.

(Table 1). As a consequence of the activity coefficients being taken into account, constant ionic strengths were obtained for all phosphate and acetate buffers used in the measurements. An ionic strength of 0.05 was chosen in this work because a higher buffer concentration can minimize negative effects from the dissolution of carbonate. Moreover, higher buffer concentrations favor electrophoretic stacking.

3.2. Effect of applied pressure on migration time and electrophoretic mobility

The effective mobility was measured on a short separation length (short-end injection) under an applied pressure. Therefore, it was interesting to see how the applied pressure influenced the effective mobility measurement at short migration times. As expected, an increase in pressure resulted in decreased migration times. This effect was particularly significant when acidic compounds migrated at lower pH. Fig. 1 shows that the migration times increased dramatically for benzoic acid and ibuprofen. Consequently, they did not elute before 40 min in the absence of pressure due to suppressed electroosmotic flow (EOF) at pH 3. It should be emphasized that our present method excluded the commonly used preconditioning step with sodium hydroxide and the separation capillary was only conditioned with running buffers between runs. Exclusion of the sodium hydroxide washing step reduces the preconditioning time and it is advantageous for direct coupling to mass spectrometry. Moreover, the sequence order was run from high pH to low pH buffers. These together might have resulted in the hysteresis of electroosmotic flow in the absence of pressure. As a result, much longer migration times for acidic compounds with low pH buffers was obtained compared with similar conditions with pressure (Fig. 1A). It was observed that the hysteresis of the electroosmotic flow occurred at low pH and this problem could be overcome by flushing the capillary with sodium hydroxide [8,24]. In our case, the hysteresis of the electroosmotic flow appeared to be negligible due to the applied pressure. The repeatability (RSD, $n = 8$) for rapid measurement of the effective mobility in a short time was under 2% in most circumstances. In general, the application of 25-mbar pressure on a capillary length from 42 to 50 cm resulted in an improved RSD for effective mobility measurement than that obtained without pressure (Table 3). A

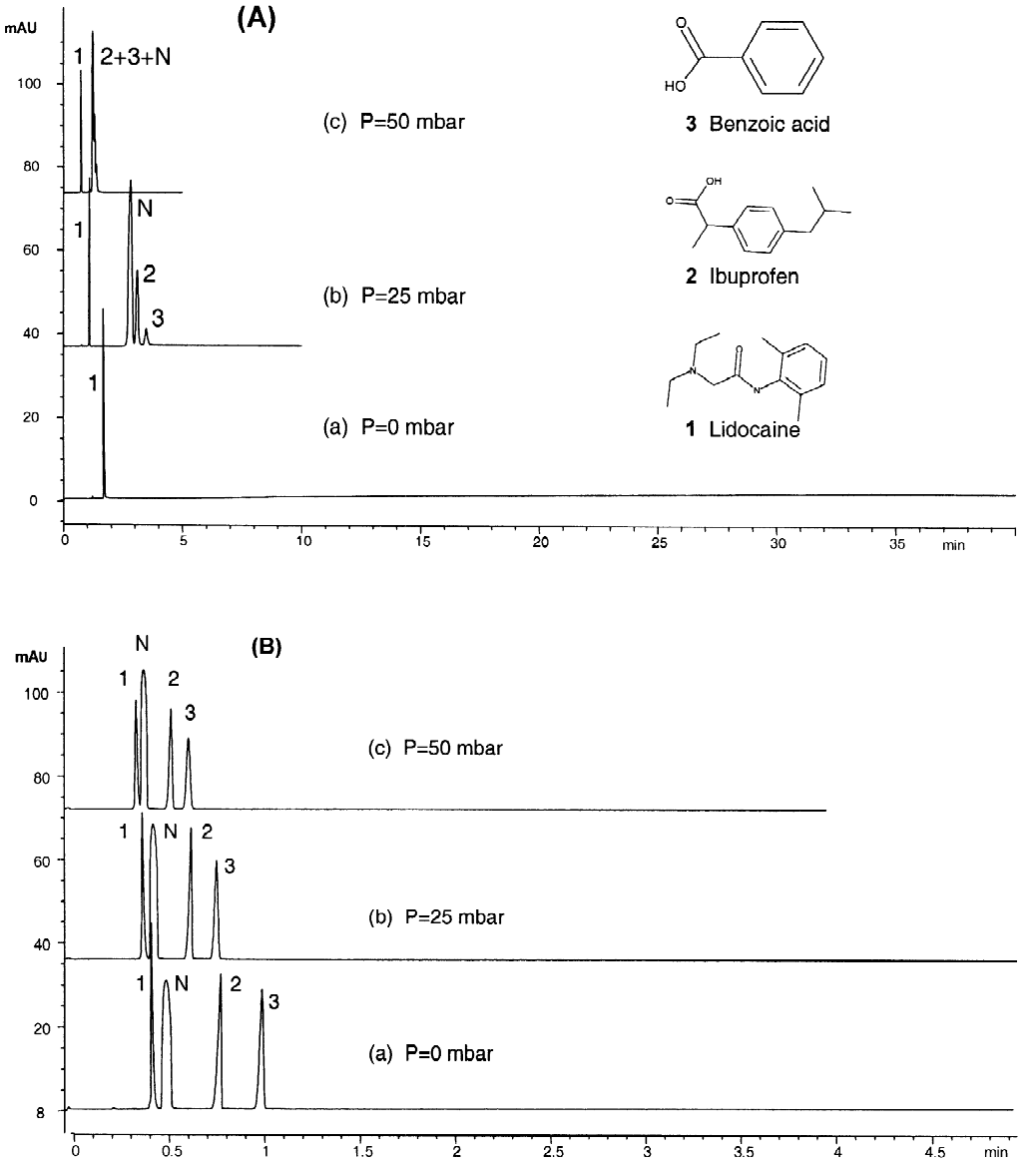

Fig. 1. Effect of pressure on the migration time and resolution at different pH. Conditions: capillary, 48.5 cm (effective separation length from outlet to detection window 8.5 cm)×50 μm I.D.; (A) NaH_2PO_4–H_3PO_4, pH 3.0, ionic strength, $I = 0.05$; (B) Na_2HPO_4–NaH_2PO_4, $I = 0.05$, pH 8.0; N, neutral marker DMSO; detection at 210 nm; temperature, 22 °C; high voltage, −20 kV; injection, 50 mbar, 4 s; sample concentration, ca. 100 μ*M*.

further increase in pressure led to a somewhat higher RSD and a deteriorated resolution (Fig. 1A). This can mainly be attributed to the decreased migration times. Clearly, the application of appropriate pressures not only reduced the electrophoretic migration times, particularly with lower pH buffers, but also improved the overall repeatability of the mobility measurements. It was also observed that an increase in pressure led to slightly decreased effective mobility values when the pH was above the pK_a for acidic analytes; the opposite trend was observed when the pH was below the pK_a. A typical example

Table 3
Effective mobility shifts from the applied pressure and the RSD ($n=8$)

Pressure (mbar)	Ibuprofen (pK_a 4.50)		Benzoic acid (pK_a 4.20)	
	Mobility	RSD (%)	Mobility	RSD (%)
Sodium phosphate (pH 8.0)				
0	2.14	0.64	3.02	0.54
10	2.13	0.59	3.01	0.44
25	2.12	0.43	2.98	0.32
40	2.08	0.47	2.94	0.33
50	2.08	0.79	2.94	0.63
Sodium acetate (pH 4.0)				
0	0.25	0.96	0.62	0.78
10	0.26	0.76	0.64	0.28
25	0.30	0.38	0.66	0.62
40	0.32	1.03	0.68	0.96
50	0.38	1.55	0.73	3.85

Short-end injection (effective separation length 8.5 cm); mobility units 10^{-4} cm^2 V^{-1} s^{-1}. Conditions: as in Fig. 3.

of effective mobility shifts as a function of the applied pressure is shown in Fig. 2. To the best of our knowledge, this interesting observation has not been reported before. The reason for this is most likely due to the mobility compromise between charged ions and the EOF ($1/t_{obs} - 1/t_{eof}$) rather than systematic measurement errors caused by pressure variations. As shown in Fig. 1, at a pH above the pK_a, an increase in pressure resulted in more decreased migration times for acidic solutes than for the EOF, thus giving rise to a decreased effective mobility (a decreased value of $1/t_{obs} - 1/t_{eof}$). In contrast, at a pH below the pK_a, an increase in pressure resulted in more decreased migration times for the EOF than for acidic solutes, thus giving rise to an increased effective mobility (an increased value of $1/t_{obs} - 1/t_{eof}$). The use of pressure introduces an additional hydrodynamic flow and generates a non-linear EOF, which shifts the effective mobility. Nevertheless, the observed effective mobility shifts had a negligible effect on the determined pK_a values.

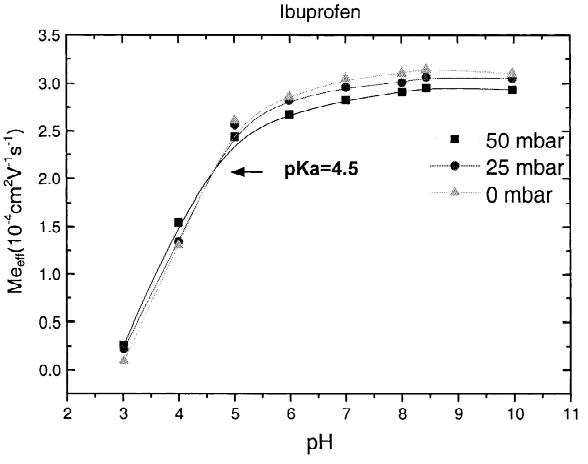

Fig. 2. Effective mobility shift due to the applied pressure for ibuprofen at different pH. Conditions: buffers, phosphate and acetate ($I = 0.05$, pH from 3 to 10, Table 1). Other conditions as in Fig. 1.

3.3. Determination of pK_a with pressure-assisted CE combined with short-end injection

A selection of 26 model compounds with pK_a values from 2.5 to 10.5 were determined by the currently reported method. The results are shown in Table 4. As can be seen from Table 4, the measured pK_a values coincide with reported literature data. In most cases, the differences between determined values and literature data are less than 0.2 units. Relatively greater discrepancies were observed for basic compounds with high pK_a values. This is probably due to the effect of carbonate, as we also observed that the dissolution of carbonate in high-pH buffers resulted in apparent pH shifts when freshly prepared buffers became aged. As a consequence, the determined pK_a values were sometimes higher than the literature data. This problem can be minimized by replacement with fresh buffers. Gluck and Cleveland [9] compared the CE method and the spectroscopic method for measuring the pK_a values of some basic compounds and found that the spectroscopic method also gave relatively higher pK_a values for basic compounds than those obtained by the CE method. They therefore questioned some historic literature values measured by other methods. Nevertheless, the currently reported CE method (mobility measured on a short separation capillary under an applied pressure) offers a reproducible and rapid approach for pK_a screening. The day-to-day reproducibility of pK_a measurements for three selected model compounds, lidocaine, ibuprofen and benzoic acid, is presented in Table 5. The total analysis time per compound for pK_a measurement is about 40 min.

Table 4
pK_a measured by pressure-assisted capillary electrophoresis with short-end injection

Compound	pK_{a1}/pK_{a2} (measured by CE–UV)	pK_{a1}/pK_{a2} (reference)[a]
Tryptophan	2.60/(8.97)	2.38/(9.39)
Nicotine	3.10/(8.02)	3.12/(8.02)
Quinidine	4.40/(8.52)	4.00/(8.54)
Quinine	4.14/(8.39)	4.11/(8.52)
Benzoic acid	4.22	4.20
4-Hydroxybenzoic acid	4.47/(9.03)	4.48/(9.32)
Aniline	4.54	4.60
Quinoline	4.88	4.80
Nicotinic acid	4.84	4.82
N-Methylaniline	4.79	4.85
Warfarin	5.06	5.03
Ibuprofen	4.51	4.55
Isoquinoline	5.38	5.40
Papaverine	6.38	6.39
Pilocarpine	6.88	6.85
4-Nitrophenol	6.93	7.15
4-Hydroxy-benzaldehyde	7.35	7.61
Lidocaine	7.92	7.90
Codeine	7.97	8.21
Benzylamine	9.71	9.59
Atenolol	9.61	9.58
Salicylamide	8.21	8.37
Phenylethylamine	10.26	10.3
3-Phenylpropylamine	10.85	10.36
4-Phenylbutylamine	10.95	10.59
Propranolol	9.49	9.50

Conditions: 25 mbar applied during electrophoretic separation. Other conditions as in Fig. 1.

[a] Data from Refs. [6,9,22]; other data from ACD database.

This is shorter than for the medium-throughput pK_a screening method reported by Jia et al., which required ca. 60 min for each compound for pK_a measurement [22].

3.4. Determination of pK_a for compounds with low aqueous solubility

Determination of the pK_a values for hydrophobic compounds is of particular interest since many drug candidates exhibit low aqueous solubility. Therefore, two different approaches, i.e. on-line stacking and dissolving the sample in an organic solvent, were investigated. First, on-line stacking conditions were applied in order to be able to detect lower concentrations with UV. Using this approach, the effective mobility was measured on a longer capillary length from the normal inlet injection to the detection window. The results were compared with those obtained by pressure-assisted CE combined with short-end injection. Fig. 3 shows an enhancement of UV sensitivity with an increased injection time or volume without apparent band broadening. Although the migration times decreased with increasing injection volume, the determined pK_a values are highly consistent with those obtained by pressure-assisted CE combined with short-end injection and also with literature data (Table 6). The detection limit ($S/N > 3$) was about 2 μM, indicating that the determination of pK_a values for compounds at concentrations down to 2 μM is feasible by pressure-assisted on-line stacking. This technique can be particularly useful for the determination of the pK_a of compounds with low aqueous solubility. Moreover, the solvent dissolving sample approach was attempted to directly measure the pK_a values for compounds of lower aqueous solubility using the same electrophoretic conditions. These relatively hydrophobic compounds were measured as other water-soluble compounds at normal concentrations (100 μM) and CE conditions without stacking by just dissolving them in acetonitrile–water (80:20). The measured pK_a values are comparable to literature data, indicating that water-insoluble compounds can also be measured using normal CE separation conditions by dissolving them in an appropriate organic solvent at a relatively high concentration. The solvent dissolving sample approach (acetonitrile or 2-propanol, 50–80%) tends to be a more rapid approach for the pK_a determination of hydrophobic compounds than on-line stacking. It can be concluded that low aqueous solubility, which often limits potentiometric titration and spectroscopic methods, is not a major problem in CE for pK_a determination.

4. Conclusions

This is the first reported study of the effective mobility shift which has been observed in the application of pressure-assisted CE for pK_a determi-

Table 5
Reproducibility of pK_a measurements by pressure-assisted capillary electrophoresis with short-end injection

	pK_a		
	Benzoic acid	Ibuprofen	Lidocaine
Literature data	4.20	4.5	7.90
14 September 2001	4.21	4.50	7.84
28 September 2001	4.20	4.52	7.92
15 October 2001	4.24	4.54	7.77
12 December 2001	4.25	4.55	8.02
13 December 2001	4.22[a]	4.48[a]	8.22[a]
9 January 2002	4.21	4.49	7.76
Mean values (95% confidence)	4.22±0.015	4.51±0.022	7.92±0.14
RSD (%) ($n=6$)	0.45	0.51	1.77

Conditions: capillary 42–50 cm (effective separation length from outlet to detection window 8.5 cm)×50 μm I.D.; buffers as in Table 1. Sample injection: 50 mbar, 10 s; sample concentration, ca. 100 μ*M*; other conditions as in Table 6.
[a] Data obtained after 24 injections using the same buffer; other data were obtained using freshly prepared buffers.

nation. The proposed pressure-assisted CE combined with short-end injection provides a reproducible approach for rapid screening of pK_a values ranging from 2.5 to 10.5. The use of appropriate pressures significantly reduced migration times, in particular with lower pH buffers. We found that the repeatability of effective mobility measurements was improved by the use of optimum pressures. At higher pressures, the RSD was increased. The suggested method excluded a preconditioning step with sodium hydroxide, which is advantageous for direct coupling to mass spectrometry for high-throughput screening of pK_a values of pooled samples. This study also demonstrated that the method is suitable for the determination of pK_a values of compounds with low aqueous solubility either by on-line stacking at lower sample concentrations or by dissolving the sample in a suitable solvent at a relatively high sample concentration. The method is expected to become a generic method for the coverage of widely diverse compounds and it is being applied to the screening of the pK_a values of selected compounds from drug discovery projects. In our opinion, pressure-assisted CE, by significantly reducing the analysis time and increasing the capacity of high-throughput physicochemical property screening, will play an important role in drug discovery.

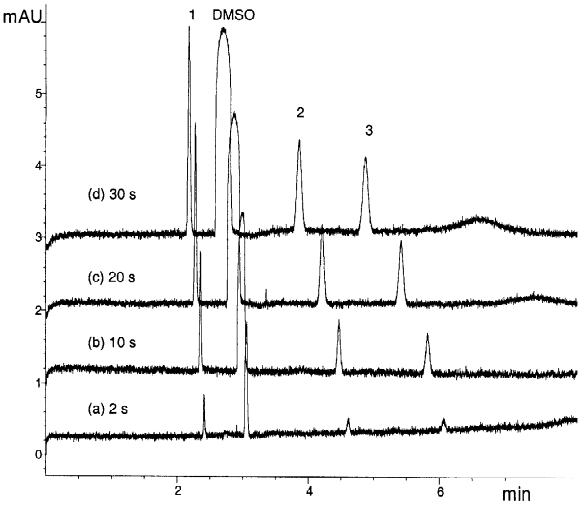

Fig. 3. Sensitivity enhancement by on-line stacking under applied pressure. Conditions: buffer, sodium phosphate (pH 7.0, $I = 0.05$). 1=Lidocaine, 2=ibuprofen, 3=benzoic acid. Capillary, 50 cm× 50 μm I.D. Normal concentration was performed by short-end injection (effective separation length from outlet to detection window 8.5 cm); −20 kV. Sample stacking was performed by a longer separation capillary (effective separation length from outlet to detection window 41.5 cm); 20 kV.

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Table 6
Comparison of pK_a values measured under normal conditions and with stacking at lower concentrations

Compound	pK_a (measured)	pK_a (reference)	Difference	Conditions
Ibuprofen	4.53	4.55	0.02	Normal concentration (100 μ*M*);
Benzoic acid	4.27	4.20	0.07	injection (50 mbar, 2 s);
Lidocaine	7.87	7.90	0.03	pressure 25 mbar
Ibuprofen	4.47	4.55	0.08	Sample stacking (10 μ*M*);
Benzoic acid	4.23	4.20	0.03	injection (50 mbar, 30 s);
Lidocaine	8.12	7.90	0.22	pressure 25 mbar
Ibuprofen	4.51	4.55	0.04	Sample stacking (10 μ*M*);
Benzoic acid	4.28	4.20	0.08	injection (50 mbar, 30 s);
Lidocaine	8.23	7.90	0.33	pressure 50 mbar

Conditions: capillary, 50 cm×50 μm I.D. Normal concentration was performed by short-end injection (effective separation length from outlet to detection window 8.5 cm); −20 kV. Sample stacking was performed by a longer separation capillary (effective separation length from outlet to detection window 41.5 cm); 20 kV.

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