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Journal of Chromatography A, 984 (2003) 283–290
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Short communication

Separation of negatively charged nonsteroidal anti-inflammatory drugs by reversed-phase capillary electrochromatography

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Received 12 July 2002; received in revised form 8 November 2002; accepted 8 November 2002

Abstract

Reversed-phase capillary electrochromatography in a 5-μm C_{18} fully packed capillary was employed to optimize the separation of negatively charged nonsteroidal anti-inflammatory drugs. The effect of the physico-chemical parameters and different analysis modes on the separation of 2-arylpropionic acids was studied and evaluated. The mobile phase composition, buffer type, concentration and pH differently influenced the peak efficiency and resolution, selectively modulating the analytes interaction with the stationary phase. The use of zwitterionic MES or acetate mobile phases strongly modulated the analytes migration order and peak efficiency. The optimum experimental conditions were found in MES buffer, pH 5.0, containing the 75% acetonitrile–methanol (1:1). All the analytes were baseline separated in a mixture in less than 13 min with peak efficiencies in the range of 78 500–84 200 *N*/m. Under these conditions the analytes were negatively charged and their effective electrophoretic mobilities played a role in the separation. The analysis of different pharmaceutical preparations containing anti-inflammatory drugs, e.g. drops and tablets, is also presented after a very simple sample pretreatment.

Keywords: Nonsteroidal anti-inflammatory drugs

1. Introduction

Capillary electrochromatography (CEC) was recently introduced as a capillary electrophoretic technique for the analysis of uncharged and/or hydrophobic compounds. In capillaries filled with stationary phase the separation occurs on the basis of the different partition of the analytes between the stationary and the mobile phase using the electroosmotic flow pump generated at the voltage application. However, under the suitable experimental conditions CEC can also provide the separation of charged compounds in the dissociated form. As recently outlined [1], the main target of CEC techniques is now the separation of charged compounds and the understanding of their retention mechanism. In fact, whereas other techniques provide satisfactory resolutions for uncharged and nonpolar analytes, CEC techniques can provide some unique selectivity

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0021-9673/02/$ – see front matter
PII: S0021-9673(02)01850-2

for the separation of charged analytes. In this case, together with hydrophobic interactions, the electrophoretic mobility of the analytes and several additional separation mechanisms, due to electrostatic and ionic exchange type interactions, can play an important role in the separation process [2–4].

Acidic compounds containing carboxylic groups were successfully analysed by reversed-phase CEC in the ion suppressed mode at acidic pH based on their different partitioning between the mobile and the stationary phases mainly due to hydrophobic interactions [5,6]. Very few papers have investigated the possibility of analysing negatively and positively charged compounds by reversed-phase CEC [3,7–12].

2-Arylpropionic acids (2-APAs) are a group of nonsteroidal anti-inflammatory drug compounds with different chemical structures. They are frequently used as an analyte test mixture for newly developed analytical methodologies. Recently they have been identified as potential environmental pollutants of the aquatic environment as a result of pharmaceutical industry manufacture, urine excretion after drug use, unused drugs disposal, etc. [13,14].

The development of fast and resolution powerful analytical methods is of primary importance for quality drug control in the pharmaceutical industry and for the identification and quantification of specific drug pollution rates in the environment.

The analytical separation of 2-APAs compounds by capillary electrophoresis was recently achieved by using different separation mechanisms, e.g. micellar electrokinetic capillary electrochromatography [15], capillary zone electrophoresis [16], capillary isotachophoresis [17], nonaqueous capillary electrophoresis [18] and capillary electrochromatography–mass spectrometry [6].

The aim of this paper was to investigate the possibility of using the high efficiency and selectivity power of CEC technique for the analysis of negatively charged 2-APA compounds in mixtures by studying the effect of several physico-chemical parameters on the separation. The analysis of different anti-inflammatory pharmaceutical preparations, namely tablets and drops, containing specific APA compounds, is also presented to show the possibility of CEC real applications.

2. Experimental

2.1. Chemicals

Formic acid (99%), glacial acetic acid and ammonia solution (30%) were purchased from Carlo Erba (Milan, Italy). Acetonitrile and methanol (both of HPLC grade were from J.T. Baker (Deventer, The Netherlands). 2-Morpholinethanesulfonic acid (MES) monohydrate was from Fluka (Buchs, Switzerland). Ibuprofen, indoprofen, ketoprofen, suprofen, fenoprofen and (+)-naproxen were purchased from Sigma (St. Louis, MO, USA). Concentrated analyte solutions (1 mg/ml) were prepared in methanol. Further dilutions were made with double distilled water (Menichelli, Rome, Italy).

2.2. Apparatus

A Hewlett-Packard HP^{3D} capillary electrophoresis automated apparatus (Waldbronn, Germany) equipped with diode array UV detection system and external nitrogen pressure (up to 12 bar) was used for CEC experiments using a 75 μm I.D., 375 μm O.D., fused-silica capillary (Composite Metal Services (Hallow, UK) totally packed with LiChrospher 100 RP_{18} (5 μm) (Merck, Darmstadt, Germany). The packed capillaries were prepared according to the procedure previously described [19]. The total capillary length was 31 cm with effective lengths of 23 or 8 cm depending if the longer or the shorter capillary end, respectively, was used for the separation. Detection was performed through-column at an output wavelength of 195 nm. During the run the capillary was pressurised at both ends applying 5 bar of the external pressure and air thermostated at 25 °C. The applied separation voltage was 30 kV or −30 kV depending if the normal of the reversed polarity mode was used to perform the separation in the longer or shorter capillary end, respectively. Analytes injection was made by the pressure method applying 12 bar for 0.5 min followed by mobile phase injection at 12 bar for 0.2 min to prevent sample loss at voltage application and to improve system precision. When the reversed polarity was used for short end injection, sample introduction was made applying −12 bar for 0.5 min followed by

−12 bar for 0.2 min mobile phase injection. Capillary zone electrophoretic (CZE) experiments were performed in 75 μm I.D., 375 μm O.D., fused-silica capillary (Composite Metal Services), 48.5 cm of total length (effective length, 40 cm) using the positive polarity mode, 25 kV of applied voltage and 25 °C of temperature. Samples were injected by pressure application of 2 bar for 0.01 min at the anodic end. Between runs the capillary was rinsed with water (0.5 min), 0.1 *M* sodium hydroxide solution (0.8 min), water (1 min) and filled for 1 min with the running buffer.

Concentrated (50 m*M*) MES or acetate buffers were prepared by weighing the appropriate amounts of MES or acetic acid compounds and titrating the aqueous solution to the desired pH with ammonium hydroxide before diluting to the final solution volume. These buffers were used for both the CZE experiments and for preparing the CEC mobile phase by mixing the appropriate buffer volume with water and organic solvent in order to obtain the final desired buffer concentration and organic solvent content. The buffer pH was not adjusted after the addition of the organic solvent.

2.3. Analysis of the pharmaceutical preparations

Different anti-inflammatory pharmaceutical tablet preparations, containing 200 mg of naproxen sodium salt or ibuprofen compounds, and drops containing 80 mg/ml of ketoprofen lysine salt, were purchased from the market and prepared as follows. Tablets were carefully grounded and weighed in order to prepare a 9 mg/ml concentrated APA solution by simply dissolving the tablet powder quantity in methanol. A 100-μl aliquot of anti-inflammatory drops was diluted in methanol to obtain 9 mg/ml ketoprofen lysine salt concentration. The methanolic solutions were further diluted with double distilled water to the final desired concentration before injection.

3. Results and discussion

Six different APAs, namely, fenoprofen, ibuprofen, indoprofen, ketoprofen, naproxen and suprofen, were employed as test compounds to study the effect of several physico-chemical parameters on the separation of negatively charged/chargeable compounds in reversed-phase CEC.

Based on our previous experience with CEC analysis of 4-hydroxybenzoic acid at pH 6.0 [19], the ammonium acetate and zwitterionic MES buffers, both at pH 6.0, were differently tested in mobile phases containing 80% of acetonitrile to investigate the effect of buffer type in CEC on APAs separation.

As already observed for 4-hydroxybenzoic acid [19], the use of the MES and the ammonium acetate buffers at pH 6.0, under the same experimental conditions, provided very different results in CEC on APAs separation. The ammonium acetate mobile phase produced poor resolution and very broad analytes peaks (Fig. 1a) whereas in MES buffer (Fig. 1b) very sharp peaks and shorter analysis times were obtained. In addition, in MES buffer a different elution order of the analytes and the co-elution of naproxen and ketoprofen were also observed. The acetonitrile, being used at the same concentration in the two mobile phases, was not considered to be an influencing parameter. In order to elucidate the different effects produced by the MES and acetate buffers on CEC APAs separation, CZE experiments in the same buffers, acetonitrile free, were separately performed. The CZE analysis confirmed that under both these experimental conditions all the studied compounds were negatively charged, according to their pK_a values [20] and migrated after the EOF. However, some important differences with the two buffers were observed also in this technique (see Table 1). In fact the APAs exhibited lower migration times in MES buffer than in ammonium acetate as a result of the combination of both the stronger EOF present and the analytes lower effective electrophoretic mobility (μ_{eff}). The μ_{eff} value was calculated using Eqs. (1) and (2):

$$\mu_{eff} = \mu_{app} - \mu_{eof} \quad (1)$$

$$\mu_{app} = \frac{Ll}{Vt_m} \quad (2)$$

where L and l are the total and the effective capillary lengths, respectively, V is the applied voltage and t_m is the analyte migration time. In CZE, with the

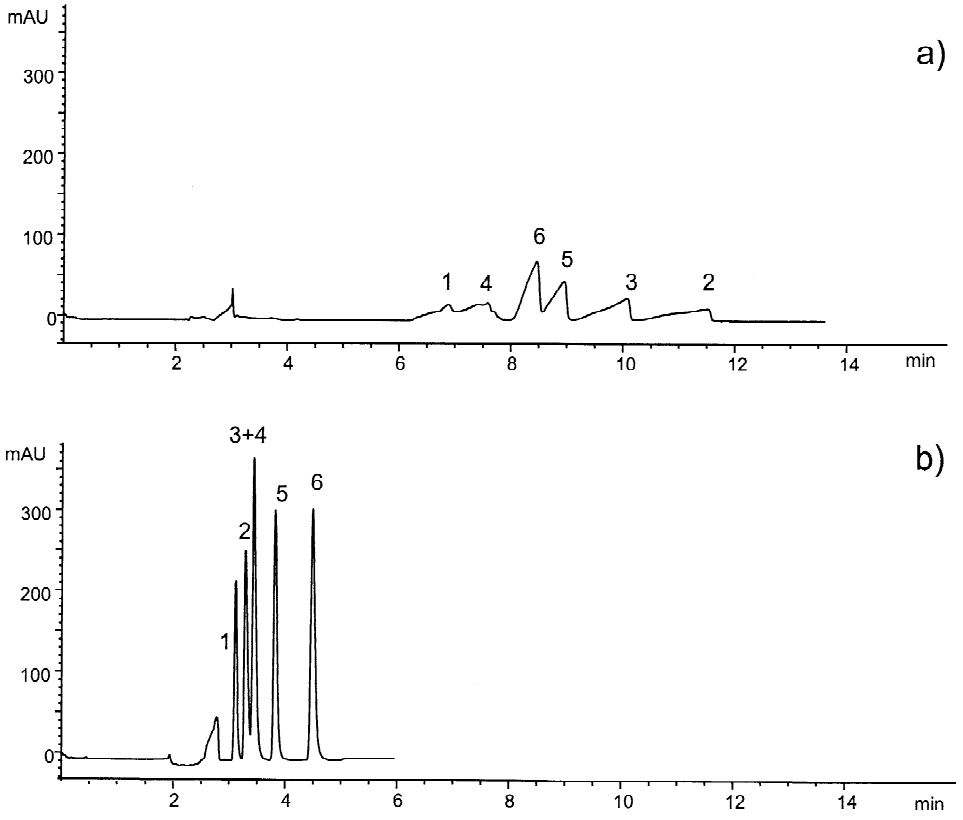

Fig. 1. CEC reversed-phase analysis of APAs compounds in different 5 m*M* (final concentration) ammonium buffers at pH 6.0 containing 80% of acetonitrile: (a) acetate buffer, (b) MES buffer. Capillary: 32 cm total length (23 cm effective length)×75 μm C_{18} μm fully packed; voltage, 30 kV; 12 bar for 0.5 min followed by mobile phase injection at 12 bar for 0.2 min. Analytes concentration: 0.1 mg/ml each. 1=Indoprofen; 2=suprofen; 3=ketoprofen; 4=naproxen; 5=fenoprofen; 6=ibuprofen.

exception of fenoprofen and ibuprofen, which were reversed, all the analysed compounds showed the same migration order in the two buffers. In CEC, a stronger difference in the analytes elution order was recognized being indoprofen>naproxen>ibuprofen>fenoprofen>ketoprofen>suprofen in

Table 1
Effect of different ammonium buffer types in CZE on APAs and EOF migration times (t_m) and effective electrophoretic mobility (μ_{eff})

Compound	t_m (min)		μ_{eff} or μ_{eof} ($\times 10^{-4}$ cm^2 V^{-1} s^{-1})	
	50 m*M* acetate	50 m*M* MES	50 m*M* acetate	50 m*M* MES
Fenoprofen	3.244	2.691	−1.158	−1.007
Ibuprofen	3.324	2.723	−1.152	−1.020
Indoprofen	3.060	2.571	−1.058	−0.944
Ketoprofen	3.321	2.664	−1.078	−0.989
Naproxen	3.260	2.699	−1.214	−1.036
Suprofen	3.298	2.681	−1.118	−1.001
EOF	1.834[a]	1.710[a]	2.592[a]	2.779[a]

[a] Average values of six runs.

ammonium acetate and indoprofen>suprofen> naproxen=ketoprofen>fenoprofen>ibuprofen in ammonium MES mobile phases. The type of buffer used in the mobile phase seemed therefore to strongly influence not only the analytes μ_{eff} but also their interaction with the stationary phase probably by promoting different types of interactions.

The 5 m*M* MES (final mobile phase buffer concentration) at pH 6.0 was therefore the buffer selected to evaluate the effect of mobile phase acetonitrile content in the range 60–80% (v/v).

The increase of acetonitrile mobile phase concentration produced higher peak efficiency (e.g. for fenoprofen compound from 65 600 to 82 900 number of theoretical plates per meter (N/m) at 60 and 80% acetonitrile, respectively) and shorter analysis time. At all the acetonitrile concentrations studied naproxen and ketoprofen were comigrating and, at 80% of organic solvent their coelution with suprofen also occurred. The almost linear dependence of log k' on acetonitrile mobile phase concentration (Fig. 2) could be ascribed to the prevalence of hydrophobic type interactions between the analytes and the stationary phase. However, the coexistance of additional interactions of electrostatic of ionic types, depending on the presence of ionizable moieties on the analytes molecule, could not be excluded.

An acetonitrile content of 65% provided the best compromise in terms of both analytes separation and total analysis time. In order to baseline resolve the naproxen and ketoprofen peaks, the effect of MES buffer mobile phase concentration in the range 1.25–30 m*M* was also studied.

The increase of MES buffer content produced several effects influencing both the analytes retention times, the peak efficiency and the analytes migration order. The APAs resolution improved at higher MES mobile phase concentrations but the too long migration times (6 and 18 min at 1.25 m*M* and 30 m*M* buffer, respectively) and poor peak shape impaired the separation (data not shown). The change of mobile phase buffer concentration modified the

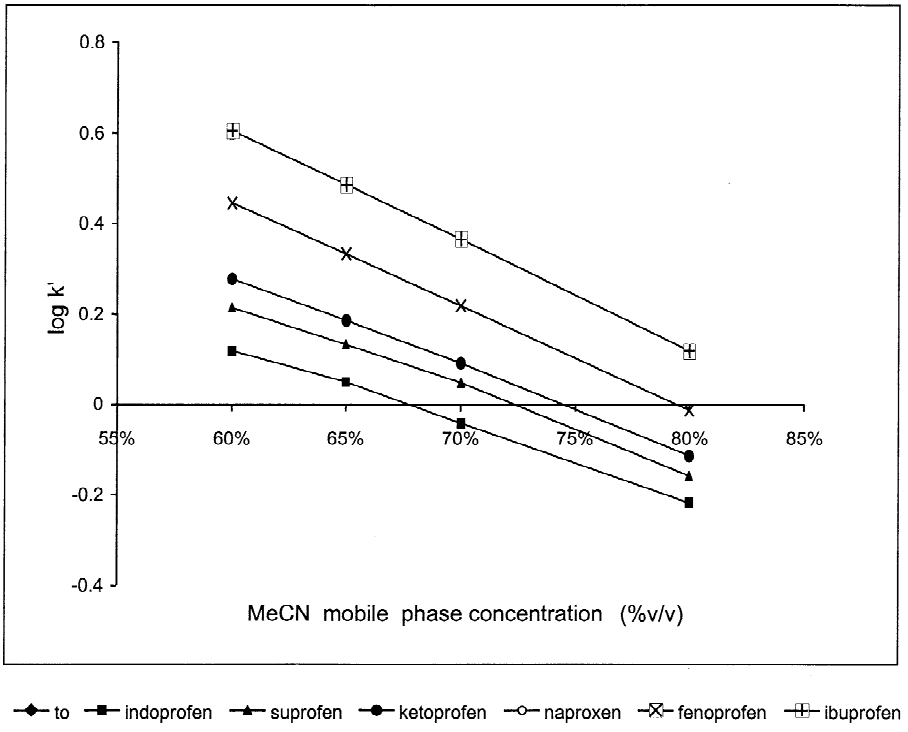

Fig. 2. Effect of acetonitrile mobile phase content on APAs logarithmic function of capacity factor (log k'). Mobile phase: 5 m*M* MES, pH 6.0, containing acetonitrile. Other conditions as in Fig. 1.

analytes' retention on the stationary phase—suprofen eluted before ketoprofen and naproxen at 5 m*M* MES, whereas at 30 m*M* it eluted after them.

Among the MES buffer concentrations studied the 5 m*M* provided good analytes resolution and fast analysis time (within 8.5 min) and was therefore chosen to study the effect of mobile phase buffer pH in the range 5–7 of MES buffering capacity.

The effect of mobile phase pH is particularly interesting in CEC when the separation of charged compounds has to be optimized. The buffer pH can influence several parameters, e.g. the analytes' dissociation degree and their effective electrophoretic mobilities, the dissociation of silanol groups on both the particles and the capillary wall and therefore the velocity of the electroosmotic flow.

Naproxen, ketoprofen and suprofen reversed their elution order from pH 5 to 7. According to the higher analytes μ_{eff} and therefore the stronger EOF opposite migration, at pH 7 longer retention times were observed and tailing or fronting peaks impaired the separation. At pH 5 and 6 the electrochromatogram profiles did not show strong differences, however the initial separation of ketoprofen and naproxen encouraged the use of the lower pH value (pH 5.0) for further optimize the baseline separation of all the compounds in mixture.

Maintaining 65% total organic solvent in the mobile phase the effect of different methanol content (10–30%) was studied in order to completely separate the naproxen and ketoprofen. The addition of methanol improved their resolution and the optimum separation was obtained at 30% methanol with 35% acetonitrile in 21 min analysis time. To speed the separation higher total organic solvent mobile phase contents (e.g. 70, 75 and 80%) containing acetonitrile–methanol 1:1 ratio were studied. A 5 m*M* final MES concentration containing 37.5% acetonitrile and 37.5% methanol (corresponding to 75% total organic solvent content) provided the fastest baseline separation of all the compounds in the mixture with peak efficiency values in the range 78 467–84 217 (N/m). Fig. 3 shows the electrochromatograms of the APAs mixture under optimum experimental conditions.

Finally, in order to elucidate the contribution rates of the electrophoretic migration and the sorption–partitioning interactions to the APAs stationary phase

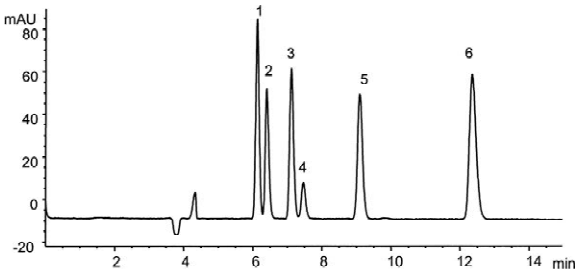

Fig. 3. Separation of an APAs mixture under the optimum experimental conditions. Mobile phase: 5 m*M* ammonium MES buffer, pH 5.0, containing 37.5% of acetonitrile and 37.5% of methanol. Other experimental conditions as in Fig. 1.

retention mechanism, the following different modes of separations were studied and compared using 5 m*M* buffer mobile phase, 37.5% acetonitrile, 37.5% methanol: (i) CEC APAs separation in ammonium MES buffer pH 5.0 (analytes in dissociated form); (ii) ammonium formate buffer at pH 2.5 (analytes in the uncharged form); (iii) nano-HPLC and CEC modes in MES buffer at pH 5.0 using the short end injection method to fast the HPLC separation.

At pH 2.5 (data not shown) the analytes were all in the uncharged form and the separation probably occurred mainly based on hydrophobic type interactions between APAs and the stationary phase. At pH 5 (Fig. 3) although a faster elution of analytes was observed, their separation improved. Probably the different buffer type or the possible dissociation of the analytes at this pH, especially of suprofen, the compound with the lower pK_a value [20], differently influenced their stationary phase retention providing a better analytes discrimination.

By comparing the electrochromatograms obtained in the nano-HPLC and CEC mode in MES buffer at pH 5.0, some interesting effects, especially on suprofen compound, were observed. In fact, whereas for most of the compounds the elution order was the same, for suprofen the elution order changed being the first eluting compound in HPLC mode and the second one after the indoprofen in CEC. These results supported the hypothesis of suprofen dissociation. The increased molecule polarity due to the presence of negative charge probably explained the lower analyte retention in HPLC and the slower

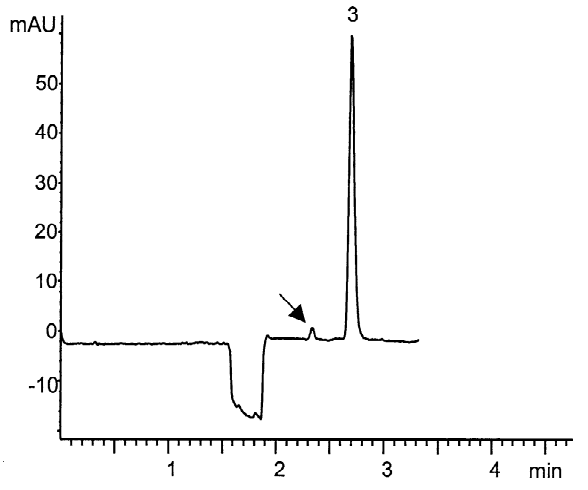

Fig. 4. CEC analysis of a pharmaceutical preparation of anti-inflammatory drops containing ketoprofen. Mobile phase: 5 m*M* MES buffer pH 5.0 containing 80% methanol. Other experimental conditions as in Fig. 1. 3 = Ketoprofen; the arrow indicates the methylparaben peak.

migration in CEC where a voltage is applied and EOF generated.

In order to demonstrate the real applicability of the optimized CEC method, the qualitative analyses of different pharmaceutical preparations of drops and tablets containing selected APAs were performed in 5 m*M* MES buffer, pH 6, containing 80% acetonitrile. The presence of single APA compound in the pharmaceutical formulations allowed the use of an acetonitrile mobile phase concentration higher than the optimum experimental conditions found to achieve a faster separation. As an example Fig. 4 shows the electrochromatogram of the analysis of pharmaceutical drops containing ketoprofen. In this sample the presence of the methylparaben preservative, as declared, was also evident. Analytes peaks were identified by spectra library comparison and the pure standard addition. Although the drug formulations only underwent, before CEC analysis, a very reduced sample pretreatment step, simply consisting of a sample dissolution and dilution, the relative electrochromatograms showed very clean profiles.

4. Conclusions

Reversed-phase CEC seemed to be a useful analytical tool for the separation of compounds with charged/chargeable moieties providing unique selectivity and separation mechanisms.

The optimization of CEC methods for the analysis of charged compounds particularly requires the careful investigation of different physico-chemical parameters as, e.g. the mobile phase composition, buffer type, pH and concentration. In fact the type and concentration of mobile phase buffer influenced the EOF, the analytes μ_{eff} values, the elution order and their partition separation mechanism. By using the zwitterionic MES buffer at pH 5.0 containing 75% (v/v) of acetonitrile–methanol (1:1), the optimized method provided the baseline separation of all the APAs compounds in the mixture in <13 min with high peak efficiency and was successfully applied to the real analysis of APAs in different kinds of pharmaceutical preparations.

The comparison of different CEC and nano-HPLC modes of separation for APAs compounds showed that, for compounds with ionizable groups, CEC in the reversed-phase mode can provide unique types of interactions different from the chromatographic partitioning of liquid chromatography.

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