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Research Article

CE-MS of antihistamines using nonvolatile phosphate buffer

Antihistamines were analyzed by CE-ESI-MS using phosphate buffer. The separation was performed in an acidic environment so that phosphate ions had a net velocity flowing toward the inlet reservoir instead of the ESI source. To further reduce the effect of ion suppression, the sodium ion in sodium phosphate was replaced with an ammonium ion. Furthermore, with the combination of reducing the concentration of acid added to the sheath liquid and the use of a low-flow interface, phosphoric acid could be added to the sheath liquid. Because of the use of the same counterion (phosphate ion) in running buffer and in sheath liquid, the separation integrity (resolution, elution order, and peak shape) was preserved. In addition, ion suppression was also greatly alleviated because a minimal amount of phosphate flowed into the ESI source.

Keywords:
CE-MS / Low-flow electrospray interface / Nonvolatile buffer / Phosphate buffer
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1 Introduction

Antihistamines are drugs used to treat the symptoms of allergies and allergic rhinitis by blocking the action of histamine, a chemical released by mast cells during an allergic response to an allergen [1]. CE separation using phosphate buffer and coupled with varies optical detector systems, such as UV [2, 3], diode array [4], and electrochemiluminescence [5] have been reported for the analysis of antihistamines.

The combination of CE with MS provides a powerful system for the analysis of complex mixtures of limited volume [6–9]. CE offers a number of practical advantages, including high separation efficiency (up to 10^7 theoretical plates), high speed, and small sample volume (a few nanoliters or less). MS is arguably the most powerful detection technique for CE, mainly because other common detection techniques (*e.g.* UV, visible, and fluorescence) provide limited information about an analyte's identity.

Both API [8–16] and MALDI/ionization [17–19] have been used for interfacing CE to MS. API allows a continuous delivery of liquid and is therefore directly compatible with separation methods, such as LC and CE. The coupling of CE to API-MS has the advantage of providing on-line mass analysis of the resolved components from complex mixtures. Among the API techniques, ESI is currently the method of choice for CE-MS because of its sensitivity, availability and ease of implementation. Its ability to generate gas phase ions from many kinds of analytes in solution makes it the most versatile ionization technique presently available.

The selection of running buffer is critical to the success of any CE separation [20]. In CE-UV separations, buffers containing sodium or potassium cations with phosphate or borate anions are common, and there are good reasons to use such buffers. Generally, these buffers are easy to prepare and often provide higher separation efficiency and better peak shape. In addition, phosphate/borate buffers are more UV-transparent, and thus baseline stability and detection limits are more favorable when employing UV detection [16, 21].

Phosphate buffer, a polybasic buffer, has more than one useful pK_a and thus can be used over a wide pH range. Although phosphate is a popular buffer for CE-UV applications, transferring methods developed for CE with UV detection to CE with ESI-MS detection is not an easy task, because it is known that phosphate buffer greatly suppresses ESI, produces several background cluster ions, and contaminates the ion optics of mass spectrometer [22]. The highly abundant background ions in the low-mass range have a severe negative impact on the ESI-MS sensitivity of low-mass analytes [23]. To alleviate these problems, volatile buffers such as ammonium acetate are often used to replace the nonvolatile phosphate buffers [24, 25]. Unfortunately, the separation efficiency is not always maintained when phosphate buffer is replaced with a volatile buffer such as ammonium acetate.

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Abbreviations: APCI, atmospheric pressure chemical ionization; **APPI,** atmospheric pressure photoionization

An alternative solution to the problem of nonvolatile buffer is the use of an ionization technique with less problem of ion suppression, such as atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI). Although ionization was achieved for some test compounds [13], the sensitivity of the CE-APCI-MS systems was in general poor. Further improvement was needed to make APCI a valuable technique for CE-MS applications. In comparison with APCI, APPI appeared to be a better ionization technique for alleviating the problem of ion suppression. APPI has been successfully applied both to CZE-MS [13, 16] and MEKC-MS [14]. However, considering the complementary roles of APPI and ESI as well as the much wider availability of ESI, it is still valuable to improve the performance of CE-ESI-MS when nonvolatile buffer is used in CE separation. Because of low sample dilution and higher salt tolerance due to the formation of smaller droplets with a higher surface to volume ratio, low flow interfaces have demonstrated its capability of coupling ESI with MEKC [26, 27] and CZE using nonvolatile running buffer [27, 28]. However, the performance of the low flow interfaces in CE-MS using nonvolatile buffer was not considered adequate. For example, in the analysis of gangliosides using borate buffer, the concentration used in CE-UV was too high to be directly applied to CE-MS. Even with the reduction of buffer concentration, the baseline was still rather noisy [27, 28]. A better approach is still needed for nonvolatile CE-ESI-MS applications.

In CE separation, the effective mobility of an ion is due to both its electrophoretic mobility and the electroosmotic mobility. For an anion such as a phosphate ion, the electrophoretic mobility and electroosmotic mobility are pointed in opposite directions. Therefore, it is possible that, under a condition of low electroosmotic mobility, the net velocity of the phosphate ion is directed towards the inlet reservoir rather than ESI source. Based on this strategy, an approach for coupling of CE with ESI-MS using phosphate buffer was proposed. The potential and limitations of this approach are described.

2 Materials and methods

2.1 Materials and preparation

All chemical standards, carbinoxamine (M_r 270), pheniramine (M_r 240), chlorpheniramine (M_r 274), brompheniramine (M_r 318), and doxylamine (M_r 290) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Hydrofluoric acid (48%) and ammonium hydroxide (28−30%) were also purchased from Sigma. Ammonium acetate, sodium dihydrogen phosphate, methanol (HPLC grade), sodium hydroxide, acetic acid (glacial), formic acid, and phosphoric acid (85%) were purchased from J. T. Baker (Phillipsburg, NJ, USA) and used without further purification. Deionized water (Milli-Q water system, Millipore, Bedford, MA, USA) was used in the preparation of the buffer solution and sheath liquid.

All chemical standards were dissolved in deionized water to a concentration of 1000 ppm as the stock solution. The stock solutions were diluted to the final concentration using separation buffer. For volatile buffers, a 0.9 M acetic acid solution was adjusted to pH 3.5 using ammonium hydroxide. For nonvolatile buffers, the running buffers contained 60 mM phosphoric acid, and the pH was adjusted to 3.5 using either sodium hydroxide or ammonium hydroxide. Two different sheath liquids were prepared, one was methanol-water-acetic acid (50:50:1 v/v/v), and the other was methanol-water-phosphoric acid (50:50:0.01 v/v/v)

2.2 Low-flow CE-ESI-MS interface

A low-flow sheath liquid sprayer was prepared based on a published procedure [26]. A 3 cm fused-silica capillary (700 μm id × 850 μm od, Polymicro Technologies, Phoenix, AZ) was tapered to a 25 μm orifice and was connected to a liquid reservoir (a microcentrifuge tube). In CE-MS operation, the liquid reservoir was filled with sheath liquid using a 500 μL syringe. The tapered CE capillary was then fully inserted into the electrospray tip. Electrical contact was achieved by inserting a platinum wire into the liquid reservoir.

2.3 CE and ESI-MS instruments

The CE instrument was configured in-house. Briefly, the setup consisted of a CZE1000R high-voltage power supply (Spellman, Plainview, NY, USA) connected to a platinum electrode in a vial containing CE running buffer and operated at constant-voltage mode. One end of the separation capillary was inserted into the buffer vial, and the other end was inserted into the low-flow CE-ESI-MS interface. All MS experiments were conducted on a classical LCQ ion trap mass spectrometer (Finnigan MAT, San Jose, CA, USA). A commercial *x-y-z* translation stage for LCQ API source (Protana Co., Odense, Denmark) was used for mounting the low-flow sheath liquid CE-ESI-MS interface. The position of the interface could be adjusted via the micrometer screws of the translation stage.

2.4 CE-UV analysis of antihistamines

In CE-UV analysis, the sample solution (200 ppm antihistamine mixture) was hydrodynamically injected by raising the sample reservoir 10 cm for 15 s. All separations were performed on a 100 cm × 50 μm id × 375 μm od fused-silica capillary with an effective separation length of 90 cm. The effective electrical field was 222 V/cm, and the wavelength of the UV detector (UV-C, Rainin, Emeryville, CA, USA) was set to 254 nm.

2.5 CE-MS analysis of antihistamines

In CE-MS analysis, the sample solution (20 ppm antihistamine mixture) was hydrodynamically injected by raising the sample reservoir 10 cm for 15 s. All separations were performed on a 90 cm × 50 μm id × 375 μm od fused-silica capillary, and the effective electrical field of separation was set to 222 V/cm. The liquid reservoir of the sprayer was filled with sheath liquid prior to CE-MS analysis. The ESI sprayer was positioned at a distance of ~2 mm from the orifice of the heated capillary. The CE-MS electropherograms were acquired in full scan mode (200–400 amu). The instrument was operated in positive ion mode with a spray needle voltage of 2.0 kV and a heated capillary temperature of 200°C.

3 Results and discussion

3.1 Separation of antihistamines by CE-UV

In CE applications, phosphate buffer is popular for CE-UV, whereas ammonium acetate buffer is preferred for CE-MS. To compare the separation efficiency, five antihistamines were analyzed by CE-UV using either phosphate buffer or acetate buffer. The results (Fig. 1) indicate that the five antihistamines can be successfully separated with either ammonium acetate or sodium phosphate. However, a comparison of the two electropherograms in Fig. 1 reveals that the peaks obtained using the acetate buffer were generally broader and had significant tailing compared to the peaks observed using phosphate buffer. Possible explanations for less peak tailing and broadening in the phosphate system include the effect of electromigration dispersion [29, 30] and the binding of phosphate ion to the silica surface of capillary wall thereby reducing the interaction between analytes and surface charge in the capillary wall [31]. For a reason not clear at this time, it was noticed that the acetate system provided better resolution for the first two peaks than the phosphate system.

3.2 Effect of EOF and buffer ion mobility

In CE, the running buffer is driven by EOF which is influenced by the pH of the buffer. At a basic condition of pH 9 and an electrical field of 222 V/cm, the EOF flow rate was measured to be ~170 nL/min (corresponding to a linear velocity of 8.7 cm/min). Theoretically, the velocity of the anions can be calculated by the product of electrical field strength and the effective electrophoretic mobility. The effective mobility is defined as the product of the ionic mobility and the degree of ionization [32]. Under an electrical field of 222 V/cm, the linear velocity of phosphate ions was calculated to be −7.7 cm/min at pH 9. Because the EOF is larger than the velocity of the phosphate ion, phosphate ions will have a good chance of flowing into the ESI ionization source and causing ion suppression. On the contrary, at an acidic condition such as

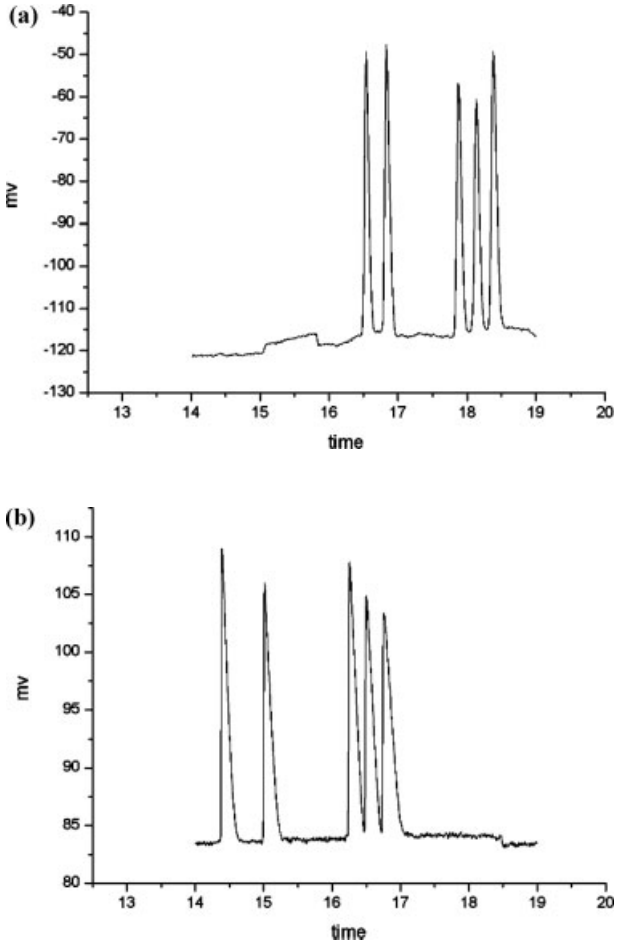

Figure 1. CE-UV analysis of a five antihistamines mixture using (a) 60 mM sodium phosphate, pH 3.5; (b) 0.9 M acetic acid adjusted to pH 3.5 using ammonium hydroxide.

pH 3.5, the EOF was measured to be about 20 nL/min (corresponding to a linear velocity of 1 cm/min). Under an electrical field of 222 V/cm, the linear velocity of phosphate ions was calculated to be −4.5 cm/min which is much larger than that of EOF. Hence, phosphate ions will flow towards the inlet reservoir instead of the ionization source. The above information suggests that phosphate buffer can be used in CE-MS without the problem of ion suppression if the CE separation is performed under acidic conditions.

3.3 Replacement of sodium ion with ammonium ion to CE separation and CE-MS analysis

Under neutral or acidic conditions, phosphate ions are not expected to flow into the ionization source avoiding the problem of ion suppression. However, sodium ions, the common counterion in phosphate buffer, are also known to cause ion suppression in ESI [23]. To alleviate the adverse effect of sodium ions, replacement by ammonium ions has been proposed [24]. Unlike sodium ions, ammonium ions

can be converted to ammonia and evaporated from the droplet. The effect of replacing sodium ion with ammonium ion was studied in a CE-MS experiment using pheniramine as the test compound. The antihistamine pheniramine (20 ppm) was injected hydrodynamically using either 60 mM sodium phosphate or 60 mM ammonium phosphate at pH 3.5 and the results are shown in Fig. 2. The signal obtained using ammonium phosphate appeared to be significantly better (S/N = 36) than the corresponding signal obtained using sodium phosphate (S/N = 3). The better S/N was due to an increase of analyte signal and a decrease in noise. Another issue that needs to be considered is the effect on separation. A CE-UV experiment involving analysis of the antihistamines mixture was performed using ammonium phosphate as the buffer. A very similar electropherogram (not shown) as that obtained using sodium phosphate (Fig. 1a) was obtained. These two experiments suggest that the use of ammonium phosphate instead of sodium phosphate can alleviate the problem of ion suppression caused by sodium ion while having little effect on the CE separation.

3.4 Analysis of antihistamines using ammonium phosphate as the running buffer and a volatile acid in the sheath liquid

CE-MS was performed using a low-flow sheath liquid interface. As mentioned earlier, a low-flow interface has the advantages of low sample dilution and higher tolerance to salts. In comparison with a flat tip low-flow interface, the low-flow interfaces with a beveled tip and a separated sheath liquid column [27, 28] have shown better performance in terms of alleviating the ion suppression effect. However, a flat tip [26] instead of a beveled tip interface was selected in this study mainly because the nonvolatile phosphate ion was not expected to flow into the ESI source. In addition, a flat tip interface is also easier to prepare.

In positive ion CE-MS, volatile organic acids, such as formic acid or acetate acid, are often added to the sheath liquid to enhance ionization efficiency. For the current application, a 20 ppm antihistamine mixture was hydrodynamically injected into the capillary using 60 mM ammonium phosphate as buffer and a sheath liquid containing 1% acetic acid in methanol/water (50:50 v/v). The results (Fig. 3) showed that the separation was degraded considerably in comparison with that from a CE/UV experiment (Fig. 1a). The degradation of separation is most likely due to the sheath liquid effect [32]. Sheath liquid effect has been studied by Karger and co-workers and is often observed in a system with low EOF particularly when the ions in sheath liquid and running buffer have different mobility. In the current situation, because the EOF is low and phosphate ions have a higher effective mobility (-34.1×10^{-9} m^2/Vs) than acetate ions (-3.1×10^{-9} m^2/Vs), the conductivity of the newly formed zone would be lower than that of the original one causing the formation of a sharp ionic boundary [33]. A similar phenomenon (data not shown) was observed when 0.1% formic acid instead of 1% acetic acid was added to the sheath liquid.

3.5 Analysis of antihistamines by CE-ESI-MS using ammonium phosphate as the buffer and 0.01% (1.5 mM) phosphoric acid in sheath liquid

In a CE-MS application, to avoid any complication (*e.g.* peak shape or elution order) to the separation, one should try to use the same solution as running buffer and sheath liquid. Sheath liquid effect due to sharp ion boundaries is the major problem one may encounter if the counterion in sheath liquid is different from the running buffer. Even under the

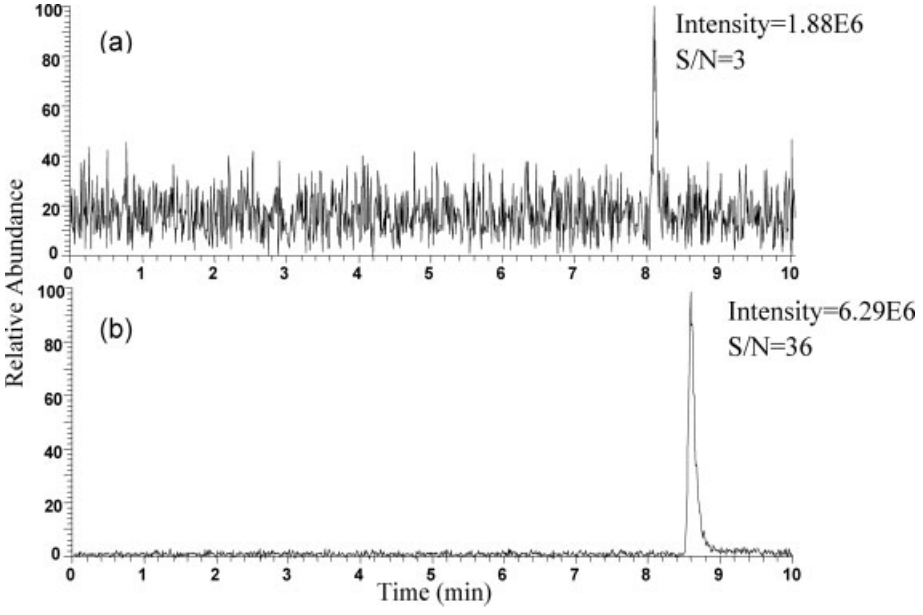

Figure 2. Mass electropherograms of pheniramine using (a) 60 mM sodium phosphate; (b) 60 mM ammonium phosphate as the running buffer.

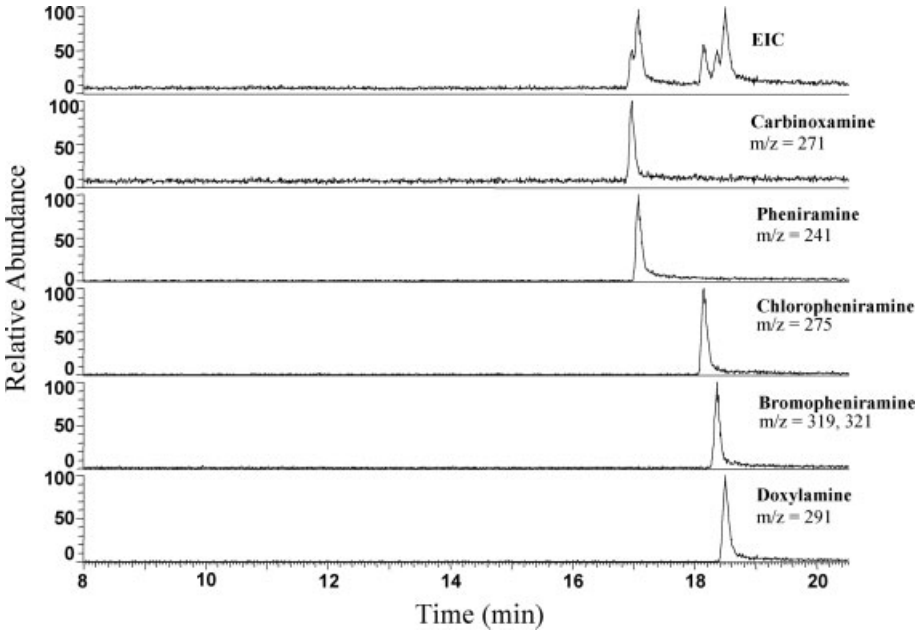

Figure 3. Mass electropherogram of a 20 ppm antihistamines mixture separation using ammonium phosphate as buffer and a sheath liquid containing 1% acetic acid in MeOH/H_2O (50:50 v/v).

conditions where no sharp ion boundaries are observed, the separation performance and/or migration order may still be affected because the phosphate ions in the column would be gradually replaced with the counterions (*e.g.* acetate ion) from the sheath liquid.

The use of a common ion (phosphate ion) in running buffer and sheath liquid was studied to alleviate the sheath liquid effect. However, phosphate added in the sheath liquid would certainly flow into the ionization source to cause ion suppression. To minimize ion suppression effect, the lowest permissible concentration of phosphoric acid should be used. In positive ESI, a major reason for adding volatile acid in the sheath liquid is to enhance ESI ionization efficiency. The use of 1% acetic acid or 0.1% formic acid is common in the preparation of sheath liquids. An examination of the pK_a values of acetic acid, formic acid, and phosphoric acid suggested that 1% acetic acid, 0.1% formic acid and 0.01% phosphoric acid (1.5 mM) would produce a solution with similar pH value. Therefore, 0.01% phosphoric acid in methanol/water (50:50 v/v) was selected as the sheath liquid.

Before the CE-MS experiment, the effect of using a much lower concentration of phosphate ion to CE separation was studied by a CE-UV experiment using 60 mM ammonium phosphate in anodic reservoir and 0.01% (1.5 mM) phosphoric acid in the cathodic reservoir. The result (Fig. 4) showed that although the concentration of phosphate ion in the sheath liquid was only 1/40 of that used in running buffer, it had little effect on the separation. During the CE-UV experiment, the CE current was found to be quite stable suggesting that the concentration of phosphate ions in the column was not changed to a significant level. One possible explanation for this phenomenon is that although the concentration of phosphate ions was low in the sheath liquid, the number of phosphate ions migrating into the column from the sheath liquid is similar to the number of phosphate ions entering the inlet reservoir.

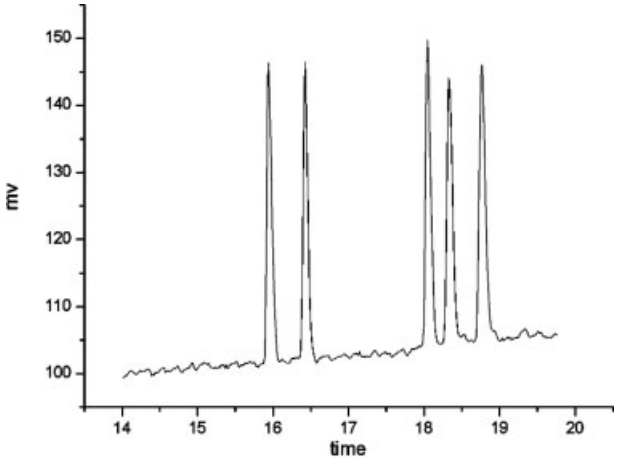

Figure 4. CE-UV analysis of antihistamines with 60 mM ammonium phosphate in the anodic reservoir and 0.01% (1.5 mM) phosphoric acid in the cathodic reservoir.

After the CE-UV experiment, the analysis was performed by CE-MS. The sum of extracted ion chromatograms of the five antihistamines using 0.01% (1.5 mM) phosphoric acid in sheath liquid and a low flow interface is shown in Fig. 5b. For a comparison, the five antihistamines were also analyzed using ammonium acetate as the buffer solution and 1% acetic acid in the sheath liquid (Fig. 5a). The performance characteristics of the two systems are listed in Table 1. As expected, the phosphate system on

Table 1. Performance characteristics of CE-MS in ammonium phosphate and in ammonium acetate buffer

Analyte	Ammonium phosphate buffer				Ammonium acetate buffer			
	Migration time (min)	S/N ratio[a)]	Plate number[b)]	Asymmetric factor[c)]	Migration time (min)	S/N[a)]	Plate number[b)]	Asymmetric factor[c)]
Carbinoxamine	15.14	64	350 000	1.7	16.09	65	180 000	4
Pheniramine	15.41	212	270 000	2	16.98	233	200 000	4.6
Chloropheniramine	16.86	108	320 000	2.3	18.74	270	300 000	6
Bromopheniramine	17.13	123	450 000	3.7	19.06	172	320 000	4.2
Doxylamine	17.46	152	350 000	3.7	19.38	325	330 000	4.5

a) Root-mean-square (rms).

b) $N = 5.55(t_m/w_{1/2})^2$

c) $A_s = B/A$, where A is the distance between the peak front and peak maximum, and B is the distance between the peak maximum and the peak end; both were measured at 10% of the peak height.

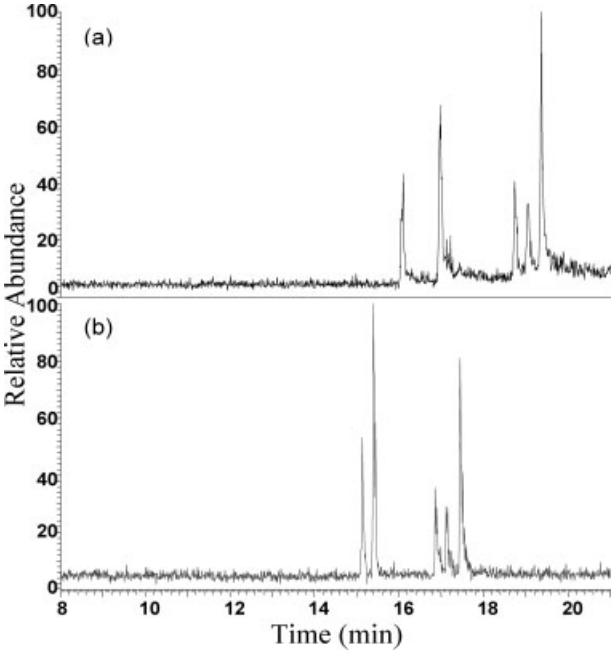

Figure 5. Sum of extracted ion chromatograms of a five antihistamines mixture using (a) 0.9 M acetic acid adjusted to pH 3.5 using ammonium hydroxide as the running buffer and methanol-water-acetic acid (50:50:1 v/v/v) as the sheath liquid; (b) 60 mM ammonium phosphate as the running buffer and methanol-water-phosphoric acid (50:50:0.01 v/v/v) as the sheath liquid. The sample was a 20 ppm mixture prepared in BGE. Separations were performed under an electrical field of 222 V/cm.

average provided better separation performance than the acetate system. Ion suppression was observed in the phosphate system. The S/N ratios were about one- to twofold poorer than the acetate system. However, it was noticed that the ion suppression in CE-MS was less severe in comparison with infusion experiment. In an infusion experiment (data not shown), the signals from a sheath liquid containing 0.01% phosphoric acid were about threefold poorer than that obtained using 1% acetic acid. One possible explanation for the less ion suppression observed in CE-MS is that the peaks in the acetate system had more tailing than the phosphate system and thus reduced the height of the peaks.

Although phosphoric acid was added to the sheath liquid, the amount of phosphate entering the ESI source was minimal because the concentration of phosphoric acid was low (1.5 mM) and the flow rate of the sheath liquid was only about 200 nL/min [26]. As a result, no salt was noticed on the sampling cone and the performance of the ESI source was not deteriorated after several days CE-MS operation.

4 Concluding remarks

Because of its better performance in separation, phosphate buffer was used in the analysis of antihistamines by CE-MS. In the coupling of CE with ESI-MS, several approaches were adopted to mitigate the associated ion suppression in ESI and at the same time to maintain the separation integrity. These approaches included the substitution of the sodium ion by ammonium ion, the use of an acidic separation condition, the use of a low-flow sheath liquid interface, and the use of a sheath liquid containing a low concentration of phosphoric acid. With these arrangements, the separation integrity was preserved and the ion suppression was significantly alleviated.

This work provides a solution for CE-ESI-MS analysis if phosphate buffer is used as the running buffer and the CE separation is performed at acidic conditions. Although APPI has shown its potential in CE-MS using nonvolatile buffer, considering the complementary roles of APPI and ESI as well as the much wider availability of ESI, this approach provides a valuable method for CE-MS applications. For CE separations performed at basic conditions, a CE-ESI-MS approach with minimum ion suppression effect should be highly valuable and remains to be developed.

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