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Ammonium hydrogencarbonate, an excellent buffer for the analysis of basic drugs by liquid chromatography–mass spectrometry at high pH

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Abstract

Ammonium hydrogencarbonate buffer has been found to be especially useful for high-pH HPLC analysis of samples from both combinatorial and medicinal chemistry sources. Satisfactory results were obtained by the standard diode array, evaporative light-scattering, and MS detection by using this buffer at a concentration of 10 m*M*. From a practical standpoint, ammonium hydrogencarbonate is an ideal buffer for chromatographers since it provides excellent chromatographic behaviour and reproducible separation. In addition to this, its volatility makes it an essential tool for rapid LC–MS product identification. Ammonium hydrogencarbonate was tested for a number of drug-like compounds analysed as mixtures, and data obtained were compared to those from the classical and MS-friendly buffers widely used by chromatographers: trifluoroacetic and formic acids. The results of this study revealed the suitability of this buffer for routine HPLC application in research laboratories.

Keywords: Buffer composition; pH effects; Ammonium hydrogencarbonate; Basic drugs

1. Introduction

Combinatorial chemistry has had a tremendous impact in pharmaceutical drug discovery research [1,2]. The major challenge of this approach is its application to the production of novel chemical entities as well as to lead optimisation [3,4]. This fact has led to the development of fast analytical methods for the analysis of large numbers of samples of wide structural diversity. Advances in the automation of liquid chromatography–diode array detection (HPLC–DAD) and liquid chromatography–mass spectrometry (HPLC–MS) methods have improved analytical productivity to monitor reaction progress, as well as the qualitative analysis and identification of synthesized compound [5,6]. In the same way, complementary detection methods such as evaporative light-scattering detection (ELSD) and chemiluminescent nitrogen detection (CLND) have recently emerged for high-throughput quantitation of combinatorial libraries [7–10].

It is well known that reaction products from combinatorial libraries contain mixtures of acidic, neutral and basic compounds (reaction by-products, excess reagents and contaminants). The analysis and purification of such samples by HPLC can be a critical task. In this regard, reversed-phase HPLC coupled to the above-mentioned detectors is the system of choice because of its availability for automation and flexibility with gradient generic methods [10,11]. Careful selection of both stationary and mobile phase parameters is essential in the

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development and optimization of analytical HPLC separation methods. Although silica-based C_{18} is the traditional and most commonly used stationary phase, sometimes it is not appropriate for this approach due to the narrow pH working range (pH 2–7) for most silica-based C_{18} phases, which reduces the ability to analyse basic compounds, the most abundant pharmaceutical substances [12]. It has been noted that the existence of residual silanols often interacts with these basic analytes producing poor peak shape and efficiency, unpredictable retention, and poor column-to-column reproducibility. Stationary phases suitable for analysis of basic substances are designed to cover a high-pH range (pH up to 12) and also to minimise the interaction between residual silanols responsible for poor peak shape, with the basic analytes. Alternative and robust HPLC columns based on highly pure silica, hybrid particle, bidentate phases, etc., have been introduced for rugged HPLC method development allowing accurate analysis and purification of basic compounds [13,14]. Prior to the existence of a new generation of HPLC columns, mobile phases and buffers suitable for analytical and preparative work remain constants. Thus, trifluoroacetic and formic acids are the traditional buffers for low pH work since their volatility makes them compatible with mass spectrometry detection and facilitates sample recovery after chromatographic separation. In the same way, acetate buffers are often used for intermediate pH values. However, the use of high-pH buffered eluents compatible with standard detectors appears to be a challenge for routine separation methods for chromatographers. Phosphate buffers have been successfully used at both intermediate- and high-pH ranges, but studies indicated that they should be avoided since they cause rapid column degradation [15]. Other organic (e.g. glycine, triethylamine, Tris) and borate buffers have been found useful for high-pH separations but all of them present several disadvantages for working in gradient conditions with MS detectors [16]. Therefore, chromatographers have to search continuously for alternative alkaline buffers.

In the present article we report our results with ammonium hydrogencarbonate as buffer of choice for alkaline (pH 8–10) work. In this study we have compared the behaviour of a mixture of ten drug-like compounds at low- and high-pH elution by using the traditional trifluoroacetic and formic acids buffers, and alkaline buffer. The compatibility of ammonium hydrogencarbonate with HPLC–DAD in combination with MS and ELSD is also described. The results of this study revealed the suitability of this buffer for routine HPLC application in research laboratories.

2. Experimental

2.1. Instrumentation

Chromatographic separation was carried out on an Agilent HP1100 liquid chromatography system equipped with a solvent degasser, quaternary pump, autosampler, column compartment and a diode array detector (Agilent Technologies, Waldbronn, Germany). The UV wavelength was set at 215 nm. Electrospray mass spectrometry measurements were performed on an MSD quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) interfaced to the HP1100 HPLC system. MS measurements were acquired simultaneously in both positive and negative ionisation modes. Data acquisition and integration for LC–UV and MS detection were achieved using HP Chemstation software (Agilent Technologies). The ELSD system was a PL-EMD from Polymer Labs. (Shropshire, UK). The drift tube temperatures and the nitrogen gas flow-rate were set at 45–65 °C and 1.7 l min^{-1}, respectively. The ELSD signal was collected through a 35900E interface (Agilent Technologies) and processed on the HP Chemstation.

2.2. Chemicals and reagents

HPLC-grade acetonitrile was obtained from Lab Scand (Dublin, Ireland), formic acid was from Merck (Darmstadt, Germany), trifluoroacetic acid and ammonium hydrogencarbonate were from Sigma–Aldrich (Steinheim, Germany) and water was purified in house with a Milli-Q plus system from Millipore (Bedford, MA, USA).

Table 1
Mobile phase gradient program of the chromatographic method used

Time (min)	% Solvent A (aqueous buffer)	% Solvent B (acetonitrile)
0	90	10
10	10	90
12	10	90
14	90	10

2.3. Test mixture

The drug-like compounds were obtained from Sigma–Aldrich, with the exception of oxprenolol which was purchased from ICN Biomedical (OH, USA). Each compound was dissolved in acetonitrile–water (50:50, v/v) to obtain stock solutions with a concentration of 0.1 mg ml^{-1}. A 100-μl sample of each one was used to prepare the standard solution to be analysed.

2.4. Analytical conditions

The chromatographic separation was performed on XTerra MS C_{18} columns 100×4.6 mm, 5 μm (Waters, Milford, MA, USA) unless otherwise stated. Polyether ether ketone (PEEK) tubing of 0.17-mm I.D. was used to connect the column to the pump as well as to the detectors. The tubing length was made as short as possible to minimize extra-column volume. The acidic mobile phases were water (solvent A) and acetonitrile (solvent B), both containing 0.1% formic acid (FA) or 0.05% trifluoroacetic acid (TFA). Meanwhile, the alkaline mobile phase was 10 m*M* ammonium hydrogencarbonate (NH_4HCO_3, solvent A) and acetonitrile (solvent B). Table 1 lists the mobile phase gradient program used in this study. The flow-rate prior to the mass spectrometer was 1 ml/min, which was split at a ratio of 3 to 1 in order to deliver 250 μl min^{-1} into the electrospray interface and 750 μl min^{-1} to the ELSD system.

3. Results and discussion

3.1. Standard mixture

Combinatorial and medicinal chemistry are continuously producing a great variety of compounds that have to be tested in several biological targets in the search for new chemical entities. The result of this is thousands of compounds designed to have maximum structural differences as well as molecular masses and polarities. In addition, the acidic or basic character of the compounds is also an essential parameter. All these features have been carefully considered in the selection of the ten components of the standard mixture. It mainly includes β-blockers and vasodilators of pharmaceutical relevance [17]. The molecular formulas, molecular masses, melting points and CAS numbers of these standards are given in Table 2. Their structures and pK_a values are shown in Fig. 1. A mixture containing almost equal amounts by weight of the standards was used to investigate the influence of the separation among trifluoroacetic acid, formic acid and ammonium hydrogencarbonate buffers on XTerra columns. In the same way, the performance of these buffers on mass spectrometry and ELSD was also evaluated.

Table 2
Standard compounds and their corresponding molecular formulas, molecular masses, melting points and CAS numbers

No.	Compound	Formula	M_r, Da	M.p. (°C)	CAS number
1	Diltiazem	$C_{22}H_{26}N_2O_4S$	414.98	207.5–212	33286-22-5
2	Dipyridamole	$C_{24}H_{40}N_8O_4$	504.63	165–166	58-32-2
3	Flunarizine	$C_{26}H_{26}N_2F_2$	405.42	251	30484-77-6
4	Lidoflazine	$C_{30}H_{35}N_3F_2O$	491.62	159–161	3416-26-0
5	Nifedipine	$C_{17}H_{18}N_2O_6$	346.33	172–174	21829-25-4
6	Oxprenolol	$C_{15}H_{23}NO_3$	265.81	78–80	6452-73-9
7	Pindolol	$C_{14}H_{20}N_2O_2$	248.32	167–171	13523-86-9
8	Procainamide	$C_{13}H_{21}N_3O$	235.79	165–169	614-39-1
9	Propranolol	$C_{16}H_{21}NO_2$	259.80	96	318-98-9
10	Verapamil	$C_{27}H_{38}N_2O_4$	455.06	142	152-11-4

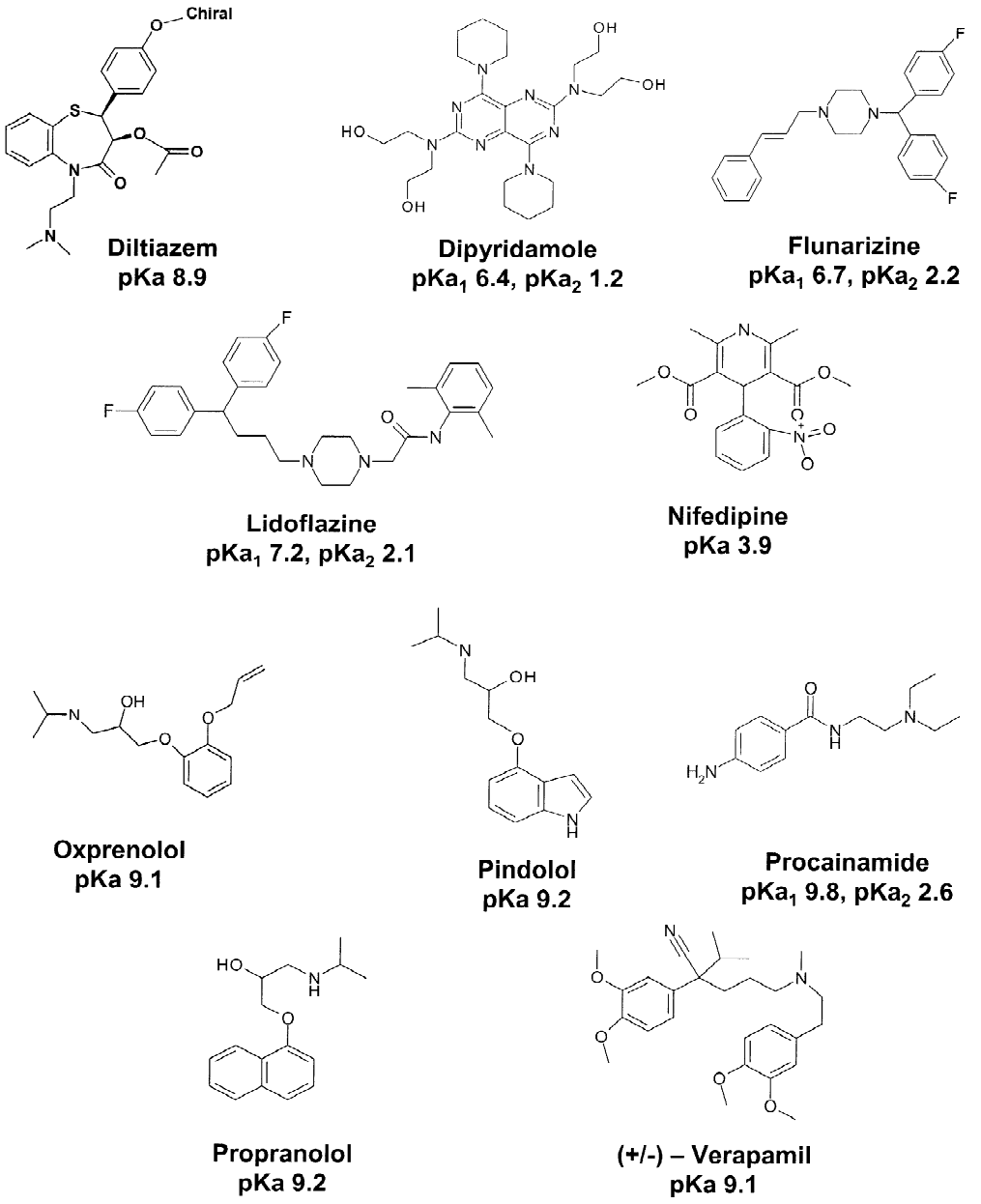

Fig. 1. Structures and pK_a values of the compounds used in this study.

3.2. Stationary phase

The HPLC analysis of these types of compounds obviously required control of the mobile phase pH by adding a buffer to obtain significant and reproducible retention. In order to achieve accurate results, it is recommended that retention is measured at least at low and high pH with organic or inorganic buffer [14]. Therefore, the selection of a suitable stationary phase is an essential parameter in the development and optimization of analytical HPLC separation methods. These methods need to be transferred to

preparative purification. Based on these issues, the XTerra column was selected for this study because of its acid- and base-resistant silica packing that allows separation in the pH range 1–12. It is also important to note the flexibility of XTerra packing materials in terms of availability for analytical, semipreparative and preparative HPLC work.

3.3. Influence of mobile phase buffer pH on compound separation

The retention of ionisable compounds is both mobile phase and buffer pH dependent [18,19]. Chromatograms illustrated in Fig. 2 highlight the retention of the ten standards in both low- and high-pH mobile phase buffers. Statistically, pharmaceutical compounds are mainly acidic or basic and normally there is no prior analytical information about the samples obtained from medicinal and combinatorial chemistry. It is reasonable to begin HPLC analysis with a gradient that use a low pH mobile phase in a first attempt. The benefits of these conditions are first to avoid analyte interactions with silanols on the stationary phase and second to protonate them in order to get positive ions. In our laboratories, trifluoroacetic ($pK_a < 1.0$) and formic (pK_a 3.71) acids are the preferred and standards buffers for routine HPLC analysis.

3.3.1. Trifluoroacetic acid and formic acid buffers

The chromatograms of the ten standards detected by UV are shown in Fig. 2. Fig. 2a corresponds to the mobile phase containing 0.05% TFA, while Fig. 2b depicts the mobile phase containing 0.1% formic acid. Almost all ten standards were separated and detected by UV at 215 nm under these low pH conditions. As expected, acceptable separation was observed between the two mobile phases for this set of compounds. However, the UV response exhibits some differences for some of the components. Thus compound 7 (pindolol) elutes clearly as a double tailing peak in the TFA chromatogram while in the

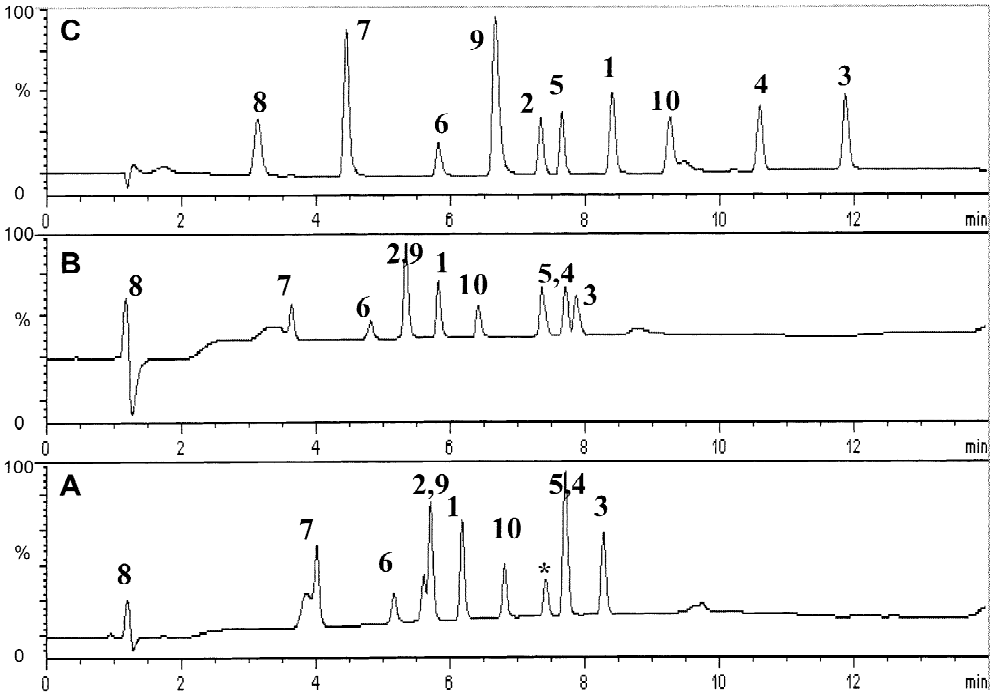

Fig. 2. DAD chromatograms of the standard mixture. Peaks: 1 = diltiazem; 2 = dipyridamole; 3 = flunarizine; 4 = lidoflazine; 5 = nifedipine; 6 = oxprenolol; 7 = pindolol; 8 = procainamide; 9 = propranolol, 10 = verapamil; * = nifedipine derivative. Buffers: A = 0.05% TFA; B = 0.1% formic acid; C = 10 m*M* NH_4HCO_3.

FA chromatogram it appears as a very broad tailing peak followed by a narrow peak. This effect is probably due to the influence of silanol activity of the packing at acidic pH eluent as has been discussed extensively in the literature [20–22]. An extra peak observed at a retention time of 7.5 min in the TFA chromatogram is attributed to a degradation of one of the components of the standard mixture. This extra peak as well as the anomalous behaviour of pindolol, were also observed when the standard mixture was analysed with acidic buffers in different stationary phases [23]. As expected under these low pH conditions, compound 8 (procainamide) was poorly retained, eluting with the solvent front in both chromatograms. A slight separation is observed between compounds 2 and 9 with TFA buffer while a single peak is attributed to these compounds in the FA buffer chromatogram. This was confirmed in the total ion current (TIC) chromatogram (Fig. 3). In contrast, peaks 4 and 5 are clearly resolved with FA buffer.

UV response varied greatly for the test compounds. No significant response variations were observed in the TIC chromatograms under the two mobile phases (Fig. 3). The relative intensities of individual peaks from UV and TIC chromatograms are in agreement, with the exception of compound 6 (oxprenolol), which shows the greatest response factor in the TIC chromatograms. The extra peak observed in the TFA chromatogram with a retention time of 7.5 min revealed an ion at m/z 329 $[M+H]^+$ attributed to a nifedipine derivative $[346+H\text{-}18]^+$ [24]. This compound was also detected in the TIC with FA between peaks 5 and 3. Chromatographers are reluctant to employ TFA ($pK_a<1$) buffer for LC–MS application because of its low sensitivity [25,26]. However, previous studies in our laboratories have revealed that 0.05% TFA buffer in generic HPLC methods allows high quality chromatography separation (purity assessment, method development, and UV guided HPLC preparative purification) as well as good mass spectra responses (flow injection and LC–MS analysis) as is evident in Fig. 3.

In terms of quantitation of small organic mole-

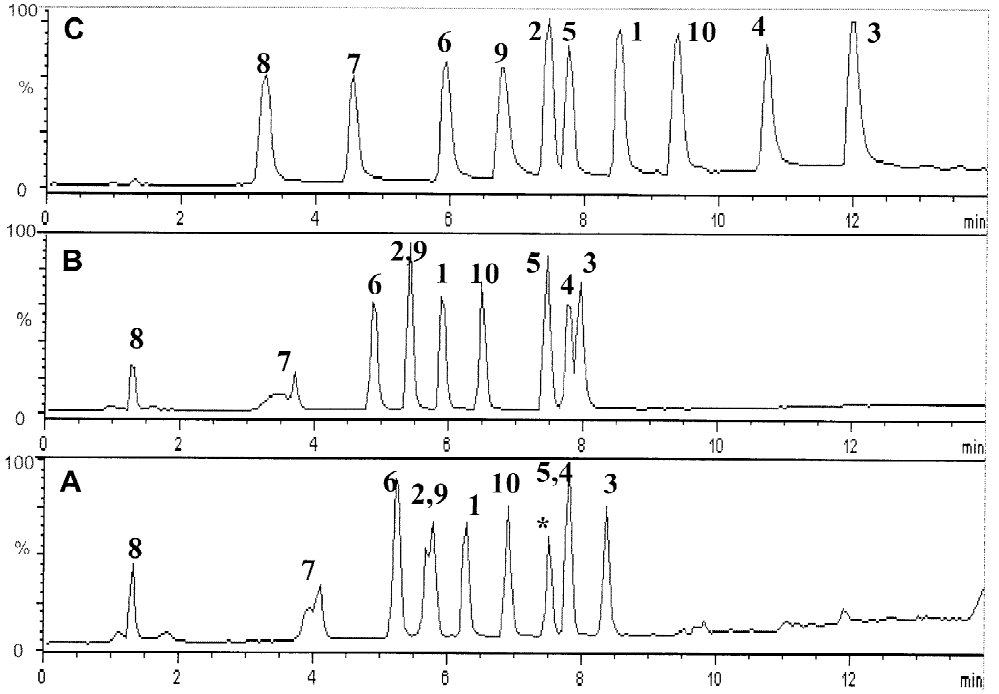

Fig. 3. TIC chromatograms of the standard mixture. Peaks: 1 = diltiazem; 2 = dipyridamole; 3 = flunarizine; 4 = lidoflazine; 5 = nifedipine; 6 = oxprenolol; 7 = pindolol; 8 = procainamide; 9 = propranolol, 10 = verapamil; * = nifedipine derivative. Buffers: A = 0.05% TFA; B = 0.1% formic acid; C = 10 m*M* NH_4HCO_3.

cules, ELSD has recently emerged as an attractive alternative detector since the detection response is mass dependent in contrast to absorptivity or ionisation efficiency characteristic of UV and MS detectors. ELSD coupled with both UV and MS detectors has become a versatile and precise tool for the characterization of combinatorial and medicinal chemistry samples. The advantages and disadvantages of this technique in the direct quantitation of small molecule libraries have been recently reported [7,10]. In this study we wanted to evaluate the response of this detector using acidic and basic buffers. The ELSD response of the standard mixture eluted with TFA and FA buffers is illustrated in Fig. 4a and b. Interestingly, an extra peak close to compound 8 (procainamide) was detected in both chromatograms. The retention time of this peak coincides with the negative peak observed in the UV chromatograms resulting from the injection volume. Apparently, the HPLC solvent front has no effect on the ELSD baseline since negligible mass is present there.

3.3.2. Ammonium hydrogencarbonate buffer

When low pH conditions give unpredictable retention and poor peak shape for the compounds of interest, it is advisable to perform HPLC analysis at intermediate or high pH (Fig. 2c). Since 75% of pharmaceutical compounds possess basic character, chromatographers have recently been forced to perform HPLC analysis using high-pH mobile phases. The most common buffers for high-pH HPLC analysis are listed in Table 3. Previously in these laboratories, several of these buffers were evaluated in order to find a suitable, volatile and high-pH mobile phase compatible with MS and ELSD and for both analytical and preparative HPLC purposes. Many factors where considered when developing high-pH mobile phase HPLC methods, including the influence of those buffers in drifting baselines with gradient elution at low wavelengths, buffer solubility, buffer natural pH, high buffering capacity, buffer sensitivity and compatibility with MS and ELSD. Based on these data, it was found that ammonium hydrogencarbonate (NH_4HCO_3) at a concentration of

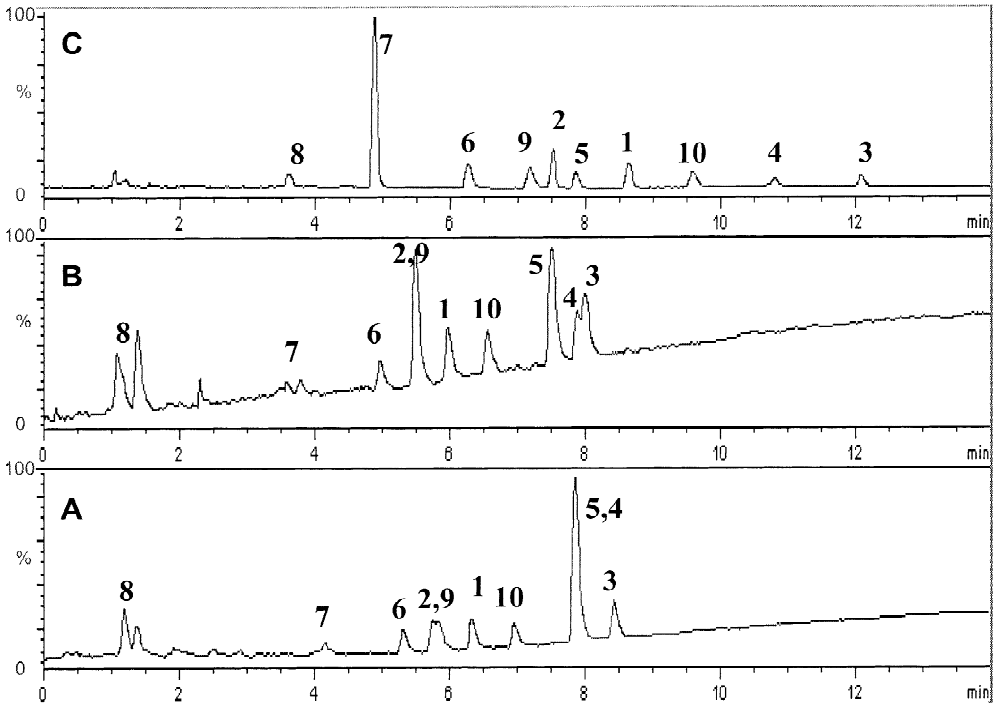

Fig. 4. ELSD chromatograms of the standard mixture. Peaks: 1 = diltiazem; 2 = dipyridamole; 3 = flunarizine; 4 = lidoflazine; 5 = nifedipine; 6 = oxprenolol; 7 = pindolol; 8 = procainamide; 9 = propranolol, 10 = verapamil. Buffers: A = 0.05% TFA; B = 0.1% formic acid; C = 10 m*M* NH_4HCO_3.

Table 3
Recommended buffers for high-pH HPLC analysis

Buffer	pK_a	Useful pK_a range	Volatile
Tris	8.1	7.1–9.1	
4-Methylmorpholine	8.4	7.4–9.4	Yes
Borate	9.2	8.2–10.2	No
Ammonia	9.2	8.2–10.2	Yes
Glycine	9.8	8.8–10.8	
1-Methylpiperidine	10.3	9.3–11.3	Yes
Diethylamine	10.5	9.5–11.5	Yes
Triethylamine	10.7	9.7–11.7	Yes
Pyrrolidine	11.3	10.3–12.3	Yes
Ammonium hydrogencarbonate		6.8–11.3	Yes
HCO_3^-	10.3		
NH_4^+	9.2		
$H_2CO_3^-$	7.8		

10 m*M* was the buffer of choice for high-pH work. Ammonium hydrogencarbonate has a natural pH of 8.4 and provides a broad alkaline pH range up to 10 (Table 3), and at concentration of 10 m*M* the pH ranges from 8 to 8.2. Earlier evaluation of this buffer carried out by Chandler et al. revealed the potential benefits of ammonium hydrogencarbonate on the semi-preparative HPLC purification of bis-anthracyclines. Column lifetime, sample recovery, column loading, as well as less product degradation were found to be the main advantages in comparison with other alkaline buffers [27].

Fig. 2c represents the UV chromatogram of the standard mixture obtained with 10 m*M* NH_4HCO_3. In terms of efficacy and resolution, the separation of the ten components has been achieved. In these alkaline conditions, with the exception of nifedipine, stronger retention for those compounds was achieved. Excellent peak shape is obtained for pindolol, oxprenolol and also for the most polar component of the mixture, procainamide. Although the retention times of peaks 4 and 3 are eluted at post gradient time (90% of acetonitrile), the separation of these compounds is greatly increased in comparison with the poor separation observed in the chromatograms with acidic buffers. In terms of HPLC purification, it is significant that a high concentration of acetonitrile in the collected fraction allows more rapid solvent removing by freeze-drying. Negligible baseline perturbation is observed in the gradient with NH_4HCO_3 as compared to gradient elution with TFA and FA buffer systems (Fig. 2a and b). These results confirm the benefits of this buffer concentration in the separation of drug-like compounds in gradient elution.

Several studies have demonstrated that mobile phase buffer volatility plays a critical role in standard LC–MS methods [28–30]. It is known that non-volatile buffers could block the MS source negatively affecting the signal detection and sensitivity of the system. Continuous contamination of the MS system requires regular cleaning of the source. Traditionally, chromatographers have used ammonium acetate and formate as the classic volatile buffers for MS detection [25,28,31–33]. However, these buffers present important drawbacks. At pH 7 (natural pH), they have no buffering capacity. Also, although these buffers are useful in MS detection, they offer low selectivity capabilities in chromatography separation. Finally, it should be considered that modifications might occur in pH and composition since such alkaline mobile phases absorb CO_2 from the atmosphere. In contrast, NH_4HCO_3 has a good buffering capacity and is stable at room temperature, decomposing at temperatures above 60 °C to form ammonia, carbon dioxide and water. These features together with the chemical properties previously mentioned, make NH_4HCO_3 compatible with MS spectrometers. Fig. 3c represents the TIC chromatogram of the standard mixture obtained with this alkaline mobile phase. The separation of the ten compounds with NH_4HCO_3 allows good correlation

between UV and TIC chromatograms as well as the MS characterisation for every compound. Using this weakly alkaline mobile phase, both acidic and basic compounds give good MS response. The greatest MS response factor was found to be for compound 3, which elutes at 90% of acetonitrile. In contrast, the more polar compound procainamide showed the lowest MS response. In terms of ionisation efficiency and chromatography, similar results were achieved when the standard mixture was analysed under more and less polar gradient conditions with the same mobile phase buffer contents. In summary, these results demonstrate the capabilities of NH_4HCO_3 in the MS ionisation process. It should be pointed out that NH_4HCO_3 buffer vaporises rapidly in the MS source. Therefore, no additional cleaning up of the MS source is required after several hours of pumping.

The ELSD response of the standard mixture used with NH_4HCO_3 mobile phase is illustrated in Fig. 4c. The chromatographic separation achieved with NH_4HCO_3 allowed identification of all the compounds. Surprisingly, pindolol (peak 7) afforded the highest response. This is interesting since the lowest response was observed for pindolol in chromatograms with acidic buffers (Fig. 4a and b). Excellent baseline signal-to-noise ratio was also obtained.

4. Conclusions

With the introduction of new stationary phases suitable for a wider pH range, numerous studies have been published. However, high-pH buffer applications with MS and ELSD are not routinely covered. The results from these studies provide information about the potential benefits of ammonium hydrogencarbonate buffer systems for routine HPLC analysis and preparative applications. The volatility and good buffering capacity of ammonium hydrogencarbonate as illustrated here indicate that it is a very useful buffer system in development of high-pH HPLC–DAD methods that may be directly transposable to HPLC–MS as well as to HPLC–ELSD.

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