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Optimization strategies for the analysis and purification of drug discovery compounds by reversed-phase high-performance liquid chromatography with high-pH mobile phases

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Abstract

Careful selection of both high-pH mobile phase as well as organic modifier, was performed in order to develop and optimize HPLC conditions for the separation of drug discovery compounds. High-pH mobile phases provide excellent chromatographic resolution and increased mass loading of basic compounds. The analytical methods so defined have been successfully transferred to preparative automated UV-directed purification, an important fact due to the increasing number of samples requiring purification. It should be noted that, the single prerequisite for this approach is an analytical LC–UV–MS run, therefore the system has the ability to collect only fractions likely to contain the target product. A cost-effective strategy for maximizing the purification of drug discovery compounds is proposed.

Keywords: Optimization; Mobile phase composition; Preparative chromatography; Pharmaceutical analysis; Drugs; Ammonium hydrogencarbonate

1. Introduction

The purity of compounds is a critical component in the whole process of drug discovery. In today's pharmaceutical organizations, close attention is being paid to the quality of compounds since more accurate characterization of compounds and more robust and reproducible biological data (minimize false positives as well as false negatives) are required [1]. The advent of high-throughput screening, automated parallel synthesis and combinatorial chemistry technologies have spawned a tremendous impact in analytical laboratories. Therefore, great effort in developing and integrating hyphenated analytical techniques is one of the major challenging objectives in modern drug discovery [2,3].

Due to recent instrumental developments in conjunction with significant reductions in the cost, HPLC–UV–MS have become the most powerful analytical tool for the detection, purity assessment and identification of pharmaceutical substances [4,5]. In an extensive controlled study, we have found that a combination of low- and high-pH, MS-compatible mobile phases, provide the best way to optimize analytical methods in drug discovery [6]. In this context, trifluoroacetic acid (TFA) (pH 2.5), formic acid (pH 3) and ammonium hydrogencarbonate (pH 8–10) are the preferred buffers in our laboratories because of their volatility and compatibility with most common detectors. Further on, excellent and reproducible results (e.g., peak shape, retention factor, MS ionization) are obtained with these buffers. The above-mentioned features have been carefully considered in the selection of stationary and mobile phases for the present study. This paper is firstly focused on the accurate high-pH HPLC–UV–MS analysis of samples from both medicinal and combinatorial chemistry laboratories. In addition, the influence of the organic modifiers (acetonitrile or methanol) as a powerful selectivity tool for reversed-phase separations is discussed. Next, we describe the development and optimization of automated preparative HPLC parameters for compound purification without the need of on-line MS detection. A cost-effective strategy for maximizing the purification of drug discovery compounds is proposed.

2. Experimental

2.1. Instrumentation

Analytical chromatographic separation was carried out on an Agilent HP1100 liquid chromatography system equipped

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with a solvent degasser, quaternary pump, auto sampler, 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 a MSD quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) interface to the HP1100 HPLC system. MS measurements were acquired simultaneously in both positive and negative ionization modes. Data acquisition and integration for LC–UV and MS detection were collected using a HP Chemstation software (Agilent Technologies).

Preparative HPLC separation was carried out on a Waters Delta Prep 4000 liquid chromatography system equipped with a fluid handling unit (pump heads), controller (for solvent gradient, flow rate, external events, and sparging process) and a 996 photodiode array detection (DAD) 996 system (Waters, Milford, MA, USA). The injector/fraction collector was a Gilson 215 liquid handler (Gilson, Middleton, WI, USA). The complete system was controlled by MassLynx software version 4.0 (Waters).

2.2. Chemicals and reagents

Acetonitrile and methanol (HPLC-grade) were obtained from Lab Scand (Dublin, Ireland), trifluoroacetic acid, ammonium hydroxide and ammonium hydrogencarbonate were from Sigma–Aldrich (Steinheim, Germany) and water was purified in the laboratory with a Milli-Q plus system from Millipore (Bedford, MA, USA).

2.3. Test mixture and Lilly proprietary compounds

The drug-like compounds employed in this study, has been described previously [6]. The names and structures of Lilly compounds used to illustrate chromatographic separations cannot be disclosed due to proprietary reasons. For analytical HPLC studies, Lilly samples were prepared as 1 mg/ml stock solution in CH_3OH and 5–10 μl of sample was injected into the HPLC columns. Samples for preparative HPLC were dissolved in a mixture of dimethyl sulfoxide (DMSO)–CH_3OH (1:1, v/v) at concentrations of 100–200 mg/ml. The standard sample injection was 250–500 μl.

2.4. Analytical conditions

The chromatographic separation was performed on XTerra MS C_{18} columns 100 mm × 4.6 mm, 5 μm (Waters) unless otherwise stated. The acidic mobile phase was water (solvent A) and acetonitrile (solvent B), both containing 0.05% TFA. Meanwhile, the alkaline mobile phase was 10 mM ammonium hydrogencarbonate (NH_4HCO_3, solvent A) at pH 8 and set to pH 9 and 10 using ammonium hydroxide and acetonitrile or methanol (solvent B). The gradient programs employed, are described in each chromatogram. The flow rate prior to the mass spectrometer was 1 ml/min, which was split at a ratio of 3:1 in order to deliver 250 μl/min into the electrospray interface and 750 μl/min to the waste reservoir.

2.5. Preparative conditions

The chromatographic separation was performed on XTerra MS C_{18} columns 100 mm × 19 mm, 5 μm (Waters) unless otherwise stated. Stainless steel tubing of 1.0 mm i.d. was used to connect the column to the pump as well as to the detectors. The mobile phases applied were the same as in the analytical conditions. Nevertheless, the refined gradient programs were slightly different. The collection parameters signal slope at the leading and tailing edge of the chromatographic peak as well as a minimum intensity threshold (dependent of the complexity of the mixture, peak spacing and purity requirements) were set up for each sample depending on the signal of the peak to be collected. The delay time used between the UV peak detection and the arrival of the compound at the collection valve was 17 and 34 s for 20 and 10 ml/min flow rate, respectively. In order to minimize the signal of the DMSO, the UV trigger collection was fixed at 230 nm. However, the on-line DAD/UV detector was monitoring between 210 and 400 nm with a scan speed of 1 s per spectrum.

3. Results and discussion

3.1. Chromatographic analysis at extended pH

The retention and separation of basic compounds is highly dependent of the mobile phase and proper buffer pH [7,8]. In a previous publication, we described the properties and benefits of low- and high-pH mobile phases on the analysis of ionizable compounds. It was found that ammonium hydrogencarbonate at a concentration of 10 mM at pH 8, influenced positively the peak shape and selectivity of basic analytes in comparison with the poor chromatography performance obtained with low-pH buffers. However, the most dramatic effect observed was the high retention times as well as the excellent ionization response from the MS detector [6]. It is well known that in order to improve retention of a basic analyte, it is advisable to adjust the pH of the mobile phase to at least two pH units above the pK_a of the target analyte since the analyte's basic ionizable functional groups are less ionized [9,10]. Chromatograms illustrated in Fig. 1 highlight the retention of the 10 standards at pH 8–10 with ammonium hydrogencarbonate. This mixture was eluted with a generic gradient from 10 to 90% of acetonitrile in 10 min. As it was expected, by extending the pH of the mobile phase the retention and selectivity of the most basic components: oxprenolol, pindolol, procainamide, propranolol and verapamil (peaks 6–10: pK_a range from 9.1 to 9.8) were dramatically modified. In contrast, this effect was not observed for compounds with lower pK_a values. For instance, the retention times for flunarizine and lidoflazine at

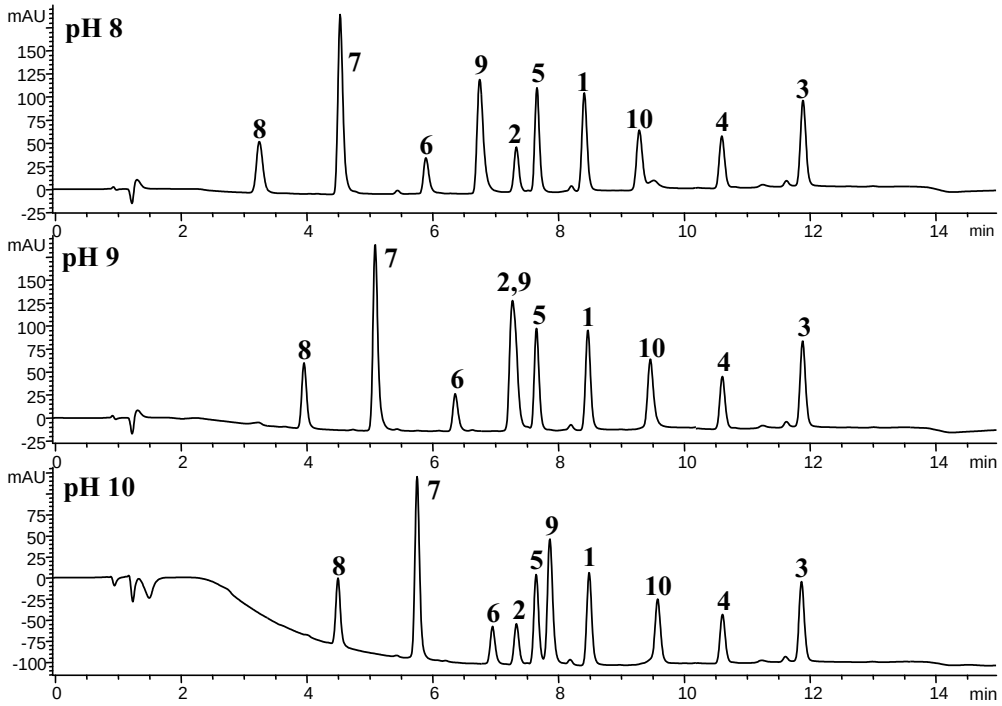

Fig. 1. UV chromatograms of the standard mixture. Peaks: (1) diltiazem; (2) dipyridamole; (3) flunarizine; (4) lidoflazine; (5) nifedipine; (6) oxprenolol; (7) pindolol; (8) procainamide; (9) propranolol; (10) verapamil. Gradient elution: mobile phase, 10 mM NH_4HCO_3 (A)–MeCN (B), from 10 to 90% B in 10 min, stay at 90% B for 2 min, and then 2 min to initial conditions.

higher pH (peaks 3 and 4: pK_a of 6.7/2.2 and 7.2/2.1, respectively) remained unchanged.

There is not doubt that the HPLC analysis and purification of drug discovery compounds is a challenging task since normally there is no prior analytical information about the samples obtained from medicinal and combinatorial chemistry. An example is shown in Fig. 2. Here, a crude sample containing a Lilly proprietary compound A (higher peak)

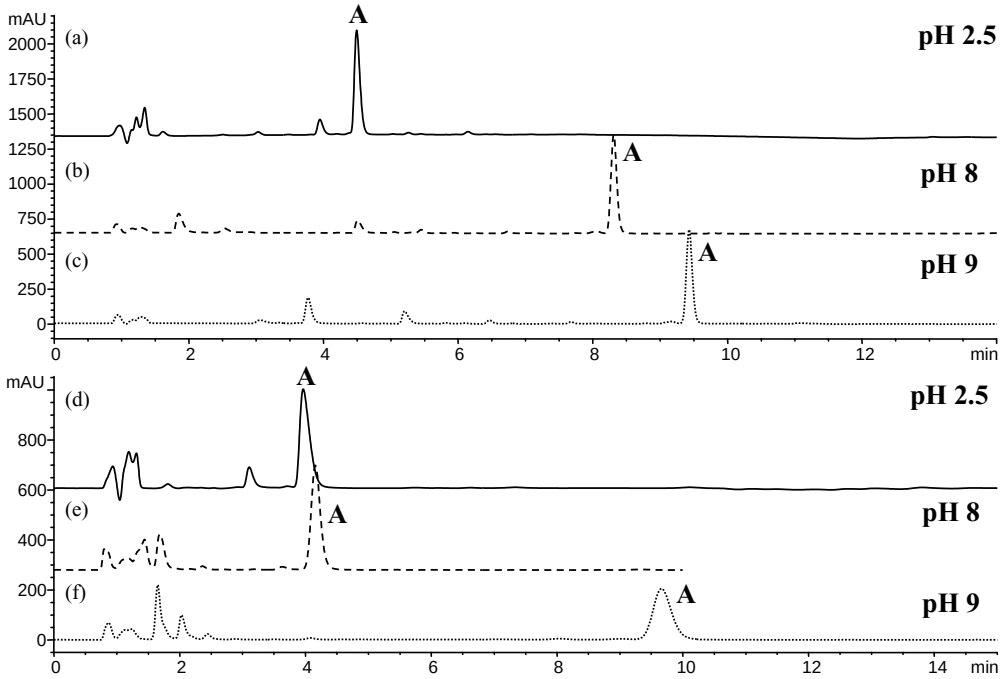

Fig. 2. UV Chromatograms of a crude mixture containing a Lilly proprietary compound A. Gradient elution (top chromatograms): mobile phase, aqueous buffer (A)–MeCN (B), from 30 to 99% B in 10 min, stay at 99% B for 2 min, and then 2 min to initial conditions. Isocratic elution (bottom chromatograms): mobile phase, aqueous buffer (A; 35%)–MeCN (B; 65%), Buffers: (a and d) 0.05% TFA, pH 2.5; (b and e) 10 mM NH_4HCO_3, pH 8; (c and f) 10 mM NH_4HCO_3, pH 9.

was analyzed with low- and high-pH mobile phases. In order to have an idea about the purity of the target compound and complexity of the mixture, a 10 min gradient from 30 to 99% of acetonitrile at pH 2.5 (TFA 0.05%) was employed. As it is shown in Fig. 2a, good separation of the target peak from the major and closer impurity was achieved. However, significant and striking changes in selectivity and retention times for all components were observed when the analysis was carried out at pH 8 (Fig. 2b) and 9 (Fig. 2c) with 10 mM of NH_4HCO_3. The retention time of the target peak was dramatically increased while a slight shift of the closer impurities was obtained. It is important to point out that this effect is consistent with the pK_a values found for the two basic functional groups of the compound of interest: 12.5 and 7.4. Further on, the highest retention time, 9.8 min at the end of the gradient, is observed at pH 9, where the compound elutes with the maximum concentration of the organic modifier. In terms of HPLC purification, it is significant since a high concentration of organic solvent in the collected fractions allows more rapid solvent removing by freeze-drying. In addition, and because of the wide peak spacing, mass loading and purification speed might be drastically improved. In fact, method development under isocratic conditions with the same mobile phases afforded similar results (see bottom chromatograms in Fig. 2). Depending of the purity requirements, any of the gradient/isocratic elution modes are suited for preparative HPLC purification. In order to reduce analysis/purification time, the isocratic conditions at pH 9 where compound A elutes as a single highly pure peak, is the method of choice (see Fig. 2f). Although in this chromatogram the compound looks as a broad peak, at this retention time the separation is optimal and a high increase in mass loading can be accomplished without risk of sacrificing chromatographic performance.

3.2. Mass loading at low and high pH

In order to maximize productivity and throughput preparative purifications, mass loading studies were carried out with both low-pH (TFA, pH 2.5) and high-pH (NH_4HCO_3, pH 10) mobile phases. A mixture of two standards propranolol (pK_a 9.5) and diltiazem (pK_a 8.9) were analyzed with tailored gradients from 30 to 60% (TFA) and from 40 to 65% (NH_4HCO_3) of solvent acetonitrile in 10 min (see Fig. 3). Because of its high efficiency and stability with acidic mobile phases, Kromasil C_{18} packing material was selected for this particular test [11]. In fact, the retention and separation of these basic compounds with TFA is quite acceptable although both compounds elute as band tailing peaks (Fig. 3a). Fig. 3c represents the preparative chromatogram under acidic conditions. Upon loading almost 3.0 mg, there is not good baseline resolution among these compounds because of their excessive peak broadening. This is in agreement with the analytical results, since compounds are in their ionized form and a strong interaction with silanols on the stationary phase affects their

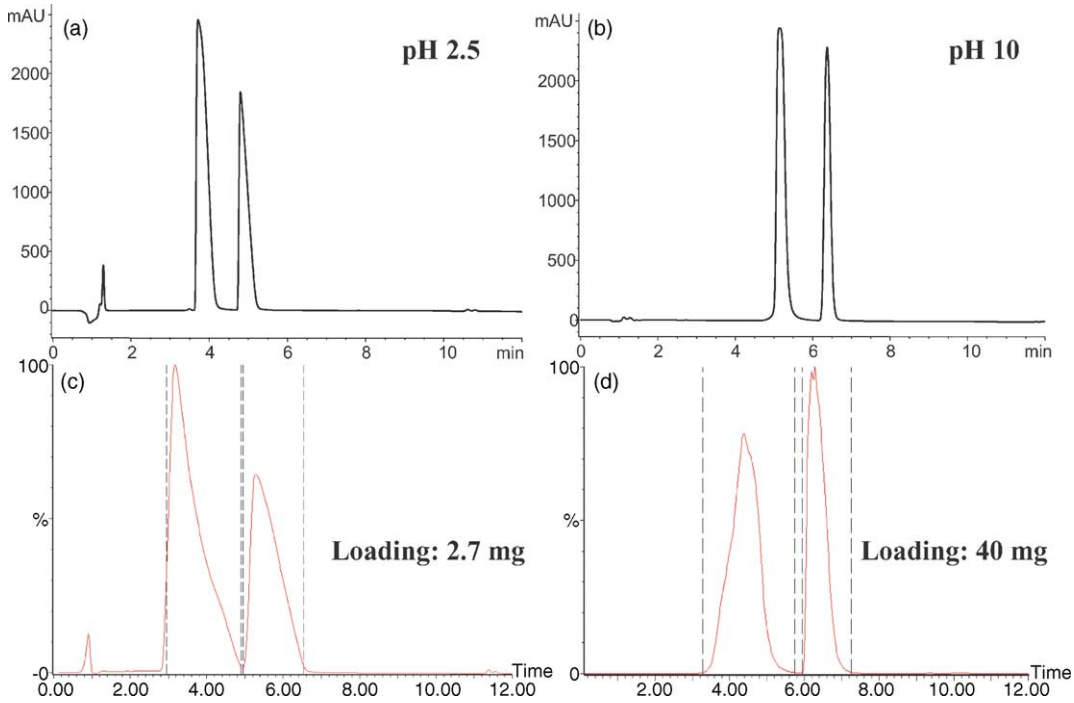

Fig. 3. Comparison of mass loading under acidic (a and c) and basic (b and d) conditions. Gradient elution (left chromatograms): mobile phase, aqueous buffer (A)–MeCN (B), from 30 to 60% B in 10 min, stay at 60% B for 2 min, and then 2 min to initial conditions. Gradient elution (right chromatograms): mobile phase, aqueous buffer (A)–MeCN (B), from 40 to 65% B in 10 min, stay at 65% B for 2 min, and then 2 min to initial conditions. Flow rate: (a and b) 1 ml/min; (c and d) 20 ml/min. Buffers: (a and c) 0.05% TFA, pH 2.5; (b and d) 10 mM NH_4HCO_3, pH 10. Time scales in min.

elution [9,12]. In contrast, much better retention and chromatographic resolution is obtained when the analysis was performed with NH_4HCO_3 at pH 10 on the hybrid packing material (Fig. 3b). As illustrated by Fig. 3d, under these alkaline conditions, a dramatic increase in mass loading in conjunction with smaller volume fraction were achieved in the preparative system. In fact, since compounds are in their non-ionized form, in a single injection, mass loading was enhanced 14 times without compromising chromatographic resolution. From these results, we can recognize the capabilities of NH_4HCO_3 as a buffer of choice to speed up and simplify purification of complex mixtures containing basic compounds [10].

3.3. Separation of basic isomers at low and high pH

Normal-phase liquid chromatography (NP-HPLC) provides good selectivity for the separation of samples containing positional isomers or stereoisomers [13,14]. However, due to the complexity of the isomer mixtures in addition to solubility issues, in some cases NP-HPLC cannot be applied. In terms of recovery and purity requirements, the analysis and purification of such kind of mixtures by RP-HPLC automated purification is a critical task. In this regards, high-pH mobile phase emerges as an alternative to the separation and purification of isomer mixtures possessing basic character. Fig. 4 depicts the UV chromatograms of a crude sample containing three positional isomers (Lilly proprietary compounds B1–B3, pK_a 9.5 and M_r 378). The initial HPLC analysis was carried out under acidic and alkaline conditions employing a tailored gradient from 25 to 40% of acetonitrile. The chromatogram in Fig. 4a corresponds to the mobile phase with TFA at pH 2.5 while chromatogram in Fig. 4b represents the mobile phase with NH_4HCO_3 at pH 8. The retention time and baseline resolution for these hydrophilic bases in acidic mobile phases was very poor as was confirmed by extracting the ion at 379 $[M + H]^+$ (see EIC chromatogram in Fig. 4a). Interestingly, part of the sample elutes also with the solvent front as a consequence of the mixed effect of the solvent strength and the grade of ionization of these compounds at low pH. It should be noted that some impurities are co-eluting with the target isomers. Conversely, the three isomers are well separated at pH 8 and the effect of the solvent strength is minimized due to lower ionization grade at this mobile phase pH as appear in Fig. 4b. Moreover, refined method by just increasing the pH of the mobile phase to 9, provided a great improvement on the peak residence time and baseline resolution among the target compounds, which is explained by the decrease in the grade of protonation as the mobile phase pH gets closer to the pK_a of the compounds. In the same way, accurate purity assessment is achieved since undesired peaks are not co-eluting with the target isomers (Fig. 4c). This fact supposes a big advantage for HPLC purification given that at pH 9, the mass loading can be increased by a factor of three in comparison to pH 8.

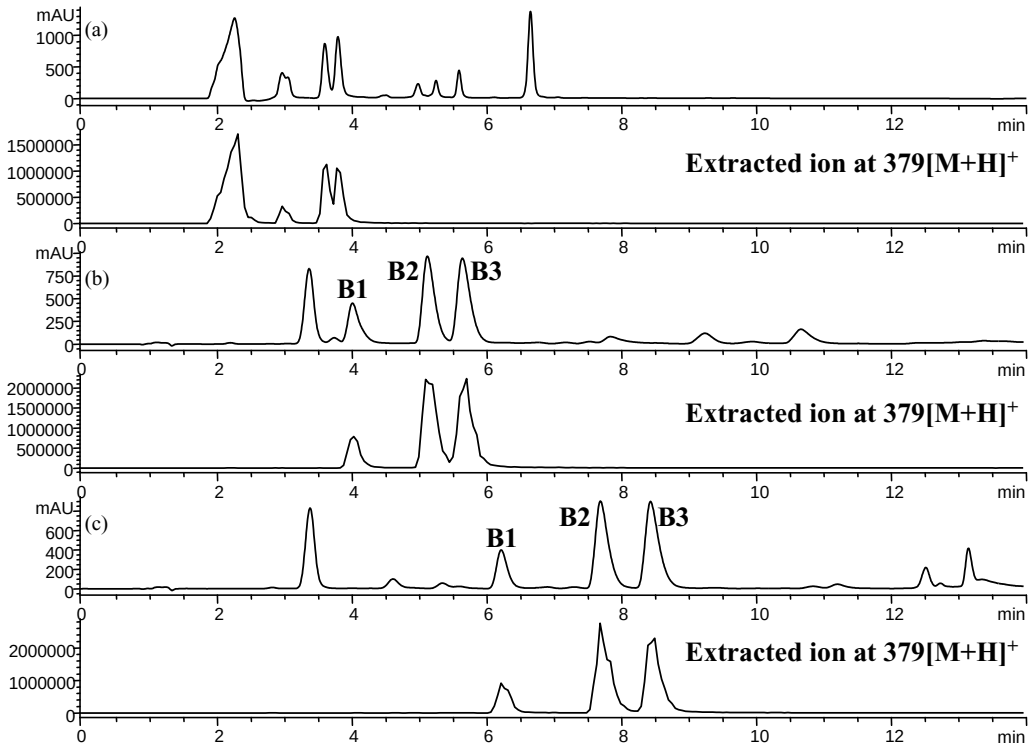

Fig. 4. UV and EIC chromatograms of a crude mixtures of isomers. Peaks B1–B3 are the target compounds. Gradient elution: mobile phase, aqueous buffer (A)–MeCN (B), from 25 to 40% B in 10 min, up to 70% B in 2 min, and then 2 min to initial conditions. Buffers: (a) 0.05% TFA, pH 2.5; (b) 10 mM NH_4HCO_3, pH 8; (c) 10 mM NH_4HCO_3, pH 9.

3.4. Effect of the organic modifier at high pH

We have discussed earlier the influence of mobile phase pH on the analysis and separation of drug discovery basic compounds. Besides, we have also evaluated the effect of the organic modifier as a powerful tool for selectivity of separations at high pH. Acetonitrile and methanol are the most common organic solvents used in combination with aqueous mobile phase in RP-LC. In this sense, their influence has been more clearly understood in recent years and more workers are turning to the use of pH measurements made directly in aqueous–organic solvents [15,16]. However, we have previously found that acetonitrile provides better chromatographic resolution than methanol when working at low pH. Despite this, because of its viscosity and hydrogen bonding ability with silanols, methanol emerges as an alternative to fine tune separation of difficult and unknown samples [17]. Fig. 5a depicts the DAD chromatograms of Lilly final product C with NH_4HCO_3 at pH 8 using acetonitrile (lower trace) and methanol (upper trace) as organic modifiers. In this case it is noted that acetonitrile gives higher efficiency than methanol since the peak width is slightly lower. However, the most important fact is the strong retention time obtained with methanol due to its mayor polarity in comparison to acetonitrile. Although, in the first general experiment carried out with methanol the first impurity co-elutes with the target compound (confirmed by LC–MS), striking selectivity changes were achieved by slightly refining the gradient. Thus, at a medium polar gradient from 30 to 70% of methanol with NH_4HCO_3 at pH 9 an excellent peak shape and peak spacing was obtained among the target peak and the impurities as it is shown in the chromatogram of Fig. 5b.

3.5. Preparative automated purification with high-pH mobile phases

It is well known that MS-directed collection is the most widely used purification approach in drug discovery laboratories [18–21]. However, some deficiencies and failures have arisen because of complex instrument configuration and buffer restrictions. Yet most of those strategies are time consuming as a consequence of limited mass loading, peak distortions or column breakthrough, among others [22]. Further on, time has to be invested in obtaining dry material and reconverting salts in free base forms. Our approach, based on the advantages of high-pH mobile phases, avoids those difficulties, simplifying preparative purification of drug discovery compounds. Once the target compound is identified by analytical HPLC–MS, and after optimizing UV collection parameters (signal slope, minimum intensity threshold, UV trigger, delay time, flow rate) all analytical methods are successfully transferred to preparative HPLC. In this context, highly pure samples (>95%) and recoveries (>90%) are obtained. Purification of samples B and C (Fig. 6a and b) show the efficiency of this approach. Because of the complexity of

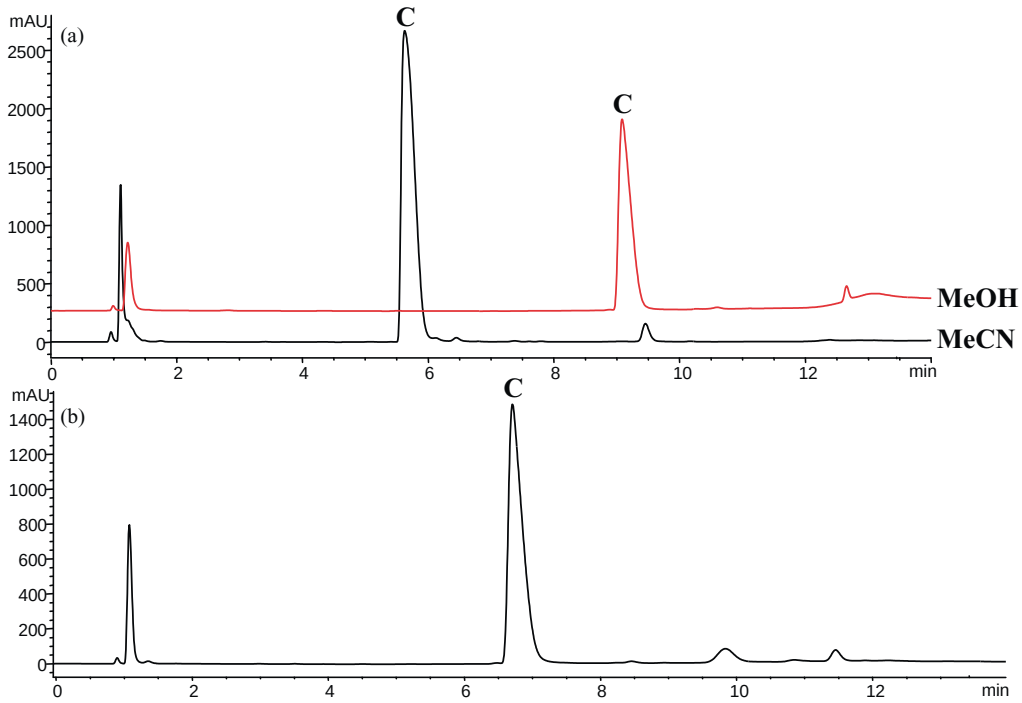

Fig. 5. UV Chromatograms of a crude mixture containing a Lilly proprietary compound C. Gradient elution (top chromatogram): mobile phase, aqueous buffer (A)–MeCN or MeOH (B), from 10 to 60% B in 10 min, up to 95% B in 2 min, and then 2 min to initial conditions. Gradient elution (bottom chromatogram): mobile phase, aqueous buffer (A)–MeOH (B), from 30 to 70% B in 10 min, up to 95% B in 2 min, and then 2 min to initial conditions. Buffers: (a) 10 mM NH_4HCO_3, pH 8; (b) 10 mM NH_4HCO_3, pH 9.

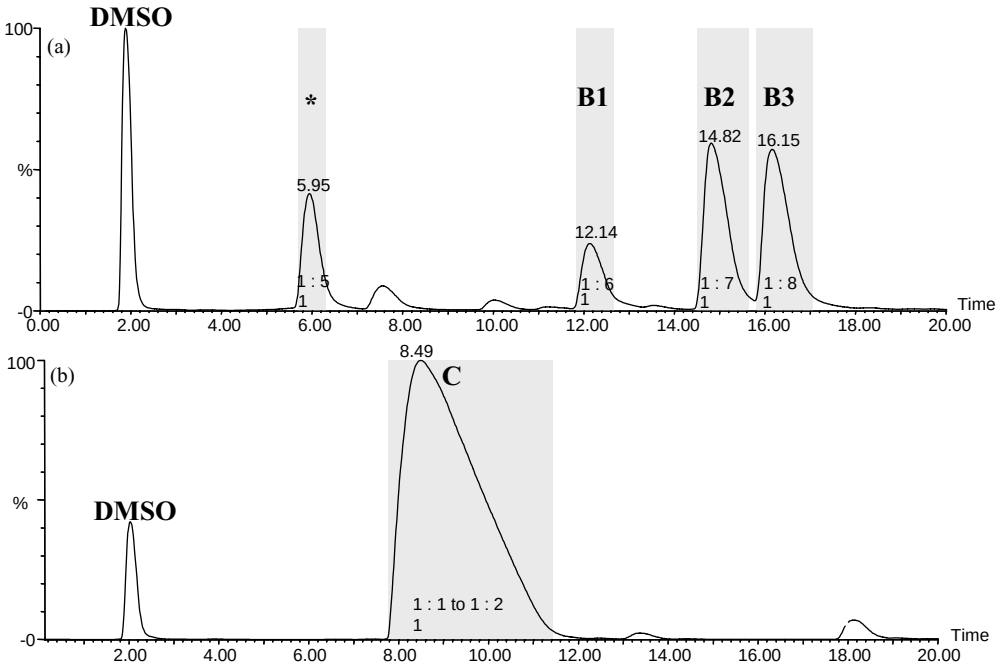

Fig. 6. UV Chromatograms of the automated-direct fraction collection of crude mixtures: (a) Lilly compounds B1–B3 and starting material denoted by an asterisk; (b) Lilly compound C. Gradient elution (top chromatogram): mobile phase, aqueous buffer (A)–MeCN (B), from 25 to 40% B in 15 min, up to 70% B in 2 min, and then 1 min to initial conditions. Gradient elution (bottom chromatogram): mobile phase, aqueous buffer (A)–MeOH (B), from 30 to 70% B in 15 min, up to 95% B in 2 min, and then 1 min to initial conditions. Flow rate: (a and b) 10 ml/min. Buffers: (a and b) 10 mM NH_4HCO_3, pH 9. Time scales in min.

sample B (isomers mixture) the gradient time and flow rate and were set up to 15 min and 10 ml/min (standard delay time for this flow rate was 34 s), respectively. Good reproducibility was obtained from analytical to preparative HPLC. In this regards, the UV collection was triggered to the isomers B1–B3 and to an extra peak corresponding to the starting material (marked with an asterisk). As it is indicated in the chromatogram in Fig. 6a, each peak was collected in a single tube. Similar results were achieved with sample C. Based on the better chromatographic resolution obtained with methanol the optimized analytical method was directly transferred to preparative HPLC (see chromatograms in Fig. 5a and b). It allowed us to maximize purification throughput and as consequence cost effective because of increasing mass loading, high organic solvent usage and faster solvent evaporation.

3.6. Optimized automated preparative purification strategy

Due to the increasing number of samples in higher quantities requiring purification, additional modifications and refinements to this preliminary automated preparative approach were carried out. To speed up flow rate, we carefully fixed the delay time to 17 s for a flow rate of 20 ml/min. After several tests, the recovery with a standard sample

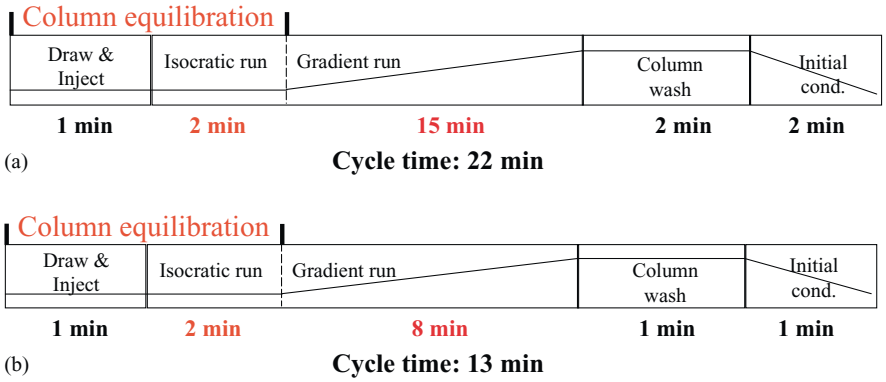

Fig. 7. Overall cycle time for preparative UV-directed fraction collection for a flow rate of: (a) 10 ml/min; (b) 20 ml/min.

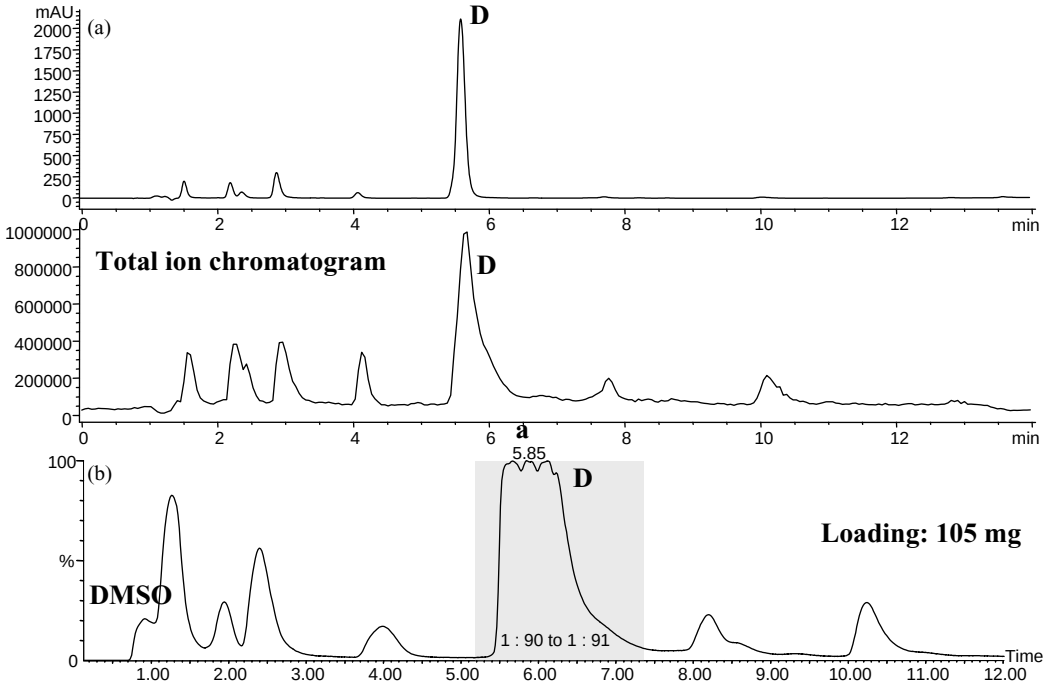

Fig. 8. Reproducibility from analytical (a) to preparative HPLC (b) of a crude mixture containing Lilly compound D. Gradient elution (top chromatogram): mobile phase, aqueous buffer (A)–MeCN (B), from 40 to 80% B in 10 min, up to 95% B in 2 min, and then 2 min to initial conditions. Gradient elution (bottom chromatogram): mobile phase, aqueous buffer (A)–MeCN (B), from 40 to 80% B in 8 min, up to 95% B in 1 min, and then 1 min to initial conditions. Flow rate: (a) 1 ml/min; (b) 20 ml/min. Buffer: 10 mM NH_4HCO_3, pH 8. Time scale in min.

(4-biphenylmethanol, CAS number 3597-91-9) was around 95%. This permits to combine two different flow rates (10 and 20 ml/min) with the same tubing length. On the bases to our experience, for a column size of 100 mm × 19 mm operating at a flow rate of 20 ml/min, 8 min gradient time is optimum. It is important to remark that, to minimize equilibration time, during the first 2 min of a run, the system is hold under initial conditions (isocratic). Thus, the elution

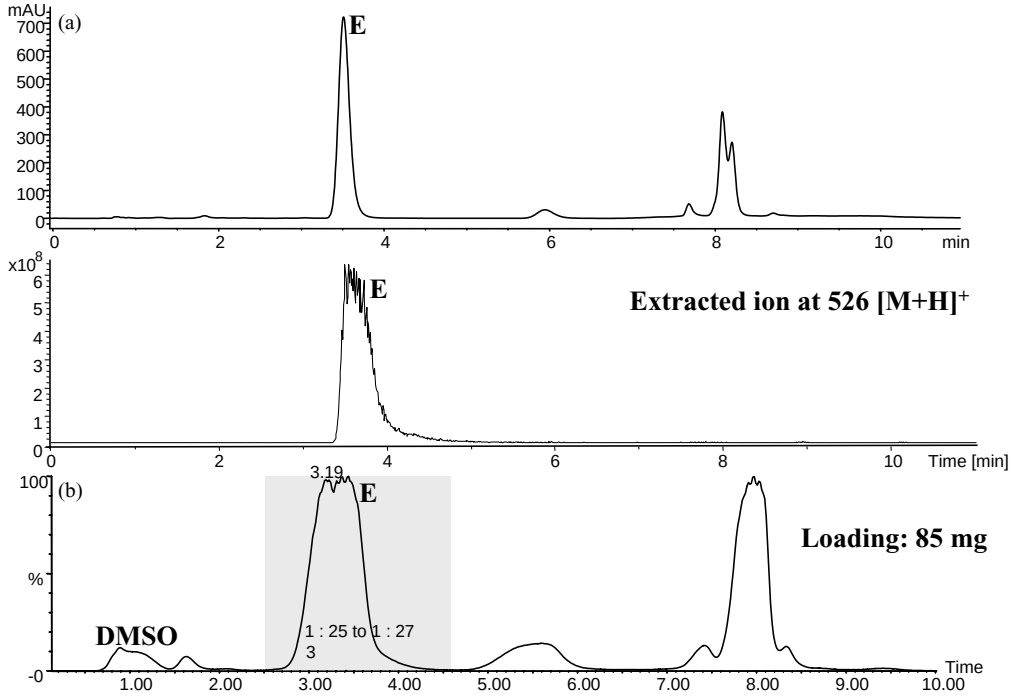

Fig. 9. Reproducibility from analytical (a) to preparative HPLC (b) of a crude mixture containing Lilly compound E. Isocratic elution: mobile phase, aqueous buffer (A; 40%)–MeCN (B; 60%) for 5 min, up to 90% B in 1 min, stay at 90% B for 2 min and then 1 min to initial conditions. Buffers: (a and b) 10 mM NH_4HCO_3, pH 9. Flow rate: (b) 20 ml/min. Time scale in min.

time is 10 min while the cycle time or injection-to-injection time is 13 min (see Fig. 7). This fully automated system enables processing 200–450 mg of crude material per hour. Fig. 8 shows an example of the fraction collection results for a crude sample containing a Lilly proprietary compound D. In terms of retention time and chromatographic performance, the reproducibility from analytical to preparative HPLC is excellent. An additional advantage of the system is its flexibility to operate under gradient/isocratic elution or combination of both without affecting the collection parameters and recoveries. An example is shown in Fig. 9. This particular sample was initially eluted under isocratic conditions (from 0 to 5 min). Once the target compound was eluted and collected, the concentration of the organic solvent was increased to 90% achieving a complete elution of undesired peaks in only 4 min. Thus, cross-contamination between sequential injections is avoided. The cycle time for this approach including column equilibration and injection time is 11 min.

4. Conclusions

These results reveal that mobile phases at extended pH used with the right combination of organic modifier are a powerful tool for the separation and purification of ionizable compounds. Ammonium hydrogencarbonate at concentrations of 10 mM (pH 8–10) reduces analysis time for wide polarity range of basic compounds. Further on, the selectivity changes produced by the organic modifier (acetonitrile or methanol) allows fining tune separations. The excellent chromatographic resolution, peak shape, retention factor as well as MS ionization obtained with these high-pH mobile phases, permits rapid identification of the desired peaks and direct transfer of the analytical methods to preparative HPLC. These HPLC variables combined with UV-directed fraction collection, allow us to optimize and maximize purification throughput because of significant improvements in mass loading, organic solvent usage and faster solvent evaporation. The automated purification strategy has been found to be efficient and robust and is now well established as separation and purification technique in our research laboratories. The results shown herein clearly support this assumption.

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