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Re-evaluation of anion-exchange HPLC for the analysis of acidic compounds

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Abstract

A number of weak and strong anion-exchange HPLC phases have been evaluated for the analysis of acidic drugs and related compounds. Using eluents with high organic content, reasonable chromatography could be obtained and widely differing compounds easily separated in isocratic mode. Retention was related to solute pK_a and concentration of ammonium acetate in the eluent. All the phases showed an unexplained loss in retention with repeated use, although with one material; nucleosil SAX, this was low (15%) and considered acceptable. This material was also shown to be relatively stable under typical bioanalytical conditions. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: High-performance liquid chromatography; Anion-exchange chromatography; SAX; Drugs; Acidic; Column stability

1. Introduction

Reversed-phase high-performance liquid chromatography (HPLC) continues to dominate the chromatography of drug compounds and other small molecules, despite the availability of other modes of HPLC such as ion-exchange or normal phase chromatography. Although, lacking the versatility of reversed-phase methods these other approaches can offer interesting selectivity differences, which could at the least, be used to complement the reversed-phase methods.

Anion-exchange HPLC has been used widely for the separation and analysis of biomolecules such as peptides and nucelotides. In contrast, however it has been very rarely used for the analysis of pharmaceuticals either as raw or formulated materials or in biological fluids [1–4]. Our own experience and success [5,6] as well as that of other workers [7] with strong cation-exchange HPLC, has lead us to carry out a re-evaluation of anion-exchange methods for analysis of acidic drugs and related compounds.

2. Experimental

2.1. Equipment

Chromatography was carried out using a Perkin Elmer LC 250 pump, a 135 diode array

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detector, and a ISS 200 autoinjector. Data was collected using a Hewlett Packard 3392A integrator or the Beckman Peak Pro system.

The HPLC columns were stainless steel (100 × 4.6 mm) packed with a range of materials: Spherisorb S5SAX (Phase Separations, Deeside, UK), Nucleosil SAX (Macherey-Nagel, GmbH, Durenrolsdorf, Germany), Hypersil SAX (Shandon Scientific, Runcorn, UK) Spherisorb APS2 (Phase Separations) and Hypersil APS2 (Shandon). The stationary phase in the three SAX columns were all bonded quaternary ammonium compounds, and in the APS columns, aminopropyl, all packings were 5 μm.

2.2. Materials

The following chemicals were used: ammonium acetate, Analar grade (BDH, Liverpool, UK); acetic acid and methanol both HPLC grade (Fisons, Loughborough, UK). The test compounds were obtained from a variety of commercial sources and were of the highest grade obtainable. These were dissolved in methanol at approximately 1 mg/ml and 5 μl injections were made.

2.3. Methods

The primary eluent consisted of methanol–water (900:100 v/v) containing ammonium acetate (15 mM) with an apparent pH of 7.35, unless stated otherwise. All experiments were carried out at room temperature, the flow rate was 1 ml/min with detection typically at 270 nm.

3. Results and discussion

3.1. Initial investigations

An eluent with a high organic content was selected since this had been shown in previous work using strong cation-exchange materials [6] to give improved efficiency over water rich eluents. This effect is probably attributable a reduction in viscosity [8].

A series of simple test compounds (weak acids, bases and neutrals) were first evaluated for there suitability as t_0 markers using the Spherisorb SAX column (Table 1). From this data caffeine (a very weak polar acid, pK_a 14) was selected since it gave lowest retention with good peak shape. Unlike some of the other compounds, e.g. benzamide and benzethonium chloride, the retention of caffeine was unaffected by small changes in the organic/aqueous ratio (data not shown).

Initial evaluation of a set of acidic drugs and simple aromatic compounds on the Spherisorb SAX column gave promising results in that a wide range of compounds could be eluted with good peak shapes and efficiencies using a single simple eluent (Fig. 1). The retention showed a reasonable correlation with pK_a with the most acidic compounds being the most highly retained (Fig. 2). The system also showed interesting selectivity. For example, the three isomeric methoxy benzoic acids were reasonably well separated with retention times of 10.0, 9.1 and 8.8 min for the 2-, 3-, and 4-isomers, respectively. The overlap in the physicochemical data for these compounds, log P, 1.59, 2.02, 2.02 [10] and pK_a, 4.0, 4.1 and 4.5 [9] for the 2-, 3-, and 4-isomers, respectively, may appear to preclude separation. However in combination, these resulted in good separation with the retention times being very well described as a function of these two properties

$$Rt = -0.75\,\mathrm{p}K_a - 1.92\log P + 16.05$$

$$R^2 = 1.000$$

Table 1
Compounds evaluated for their suitability as t_0 markers[a]

Compound	Retention time (min)	Peak shape
Caffeine	1.23	Good
Benzamide	1.25	Good
Benzethonium chloride	1.05	Poor
Biphenyl	1.25	Poor
Naphthalene	1.25	Good
Uracil	1.31	Good
Methanol	1.23	Good
Phenol	1.30	Good

[a] Column, spherisorb S5SAX, 100 × 4.6 mm, eluent, methanol–water (900:100 v/v) containing 10 mM ammonium acetate, flow rate 1 m/min.

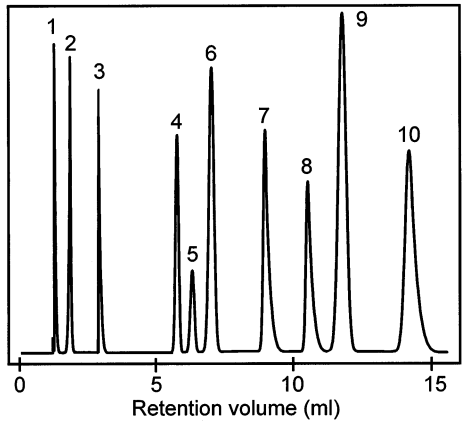

Fig. 1. A chromatogram showing the separation of a range of miscellaneous acids on a Spherisorb SAX column (100 × 4.6 mm). The eluent was of methanol–water (900:100 v/v) containing ammonium acetate (15 mM). Peaks 1, caffeine (t_0 marker); 2, phenolphthalein; 3, 4-nitrophenol; 4, ponalrestat; 5, ibuprofen; 6, indomethacin; 7, warfarin; 8, salicylic acid; 9, naphthalene-1-acetic acid; 10, 4-hydroxybenzoic acid.

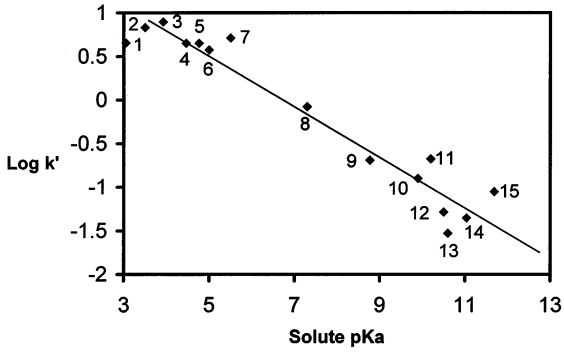

Fig. 2. Variation in retention with solute pK_a for a range of solutes chromatographed on a Spherisorb SAX column (100 × 4.6 mm) with an eluent of methanol–water (900:100 v/v) containing ammonium acetate (15 mM). 1, 2,5-dichlorobenzoic acid; 2, salicylic acid; 3, 4-aminobenzoic acid; 4, 4-chlorobenzoic acid; 5, 3,4,5-trimethoxybenzoic acid; 6, indomethacin; 7, warfarin; 8, 2-nitrophenol; 9, 3-nitrophenol; 10, chlorthalidone; 11, phenolphthalein; 12, phenol; 13, 3-cresol; 14, 4-methoxyphenol; 15, 4-aminophenol.

Similarly the isomeric 2, 4- and 3, 4-dimethoxy benzoic acids, with identical pK_a (4.36) [9]and similar log P values (1.58 and 1.46) [10]showed some separation with retention times of 8.8 and 8.4 min, respectively.

It was quickly observed however that the retentivity of the column fell off with continued use. The average drop in retention was around 30% although this was compound dependant with aminophenol showing a 11% decrease and 4-nitrophenol showing a 88% decrease over the same time period. Despite the relatively 'clean' nature of the samples that had been tested so far, it was assumed that the ion-exchange sites had become 'blocked' in some way. Attempts were then made to regenerate the column using ammonium acetate (1.0 M) or acetic acid (0.1 M). Neither of these approaches were successful, indicating that column deactivation was not the cause of the loss in retention.

3.2. Column comparisons and column stability

Four further ion-exchange columns were then also evaluated and compared with the Spherisorb SAX, both in terms of stability and general performance. Two of these were SAX and the other two were APS. The eluent was the same for all columns with the following two exceptions. The Hypersil SAX had lower retentivity and the eluent buffer concentration was reduced from 15 to 10 mM. The Nucleosil column was highly retentive and the buffer concentration was increased to 50 mM. Each column was evaluated with a set of 24 test compounds consisting mainly of simple aromatic acids and a number of drug compounds e.g. ibuprofen, warfarin, indomethacin. Flow was continued through each column, the performance of which were reassessed at regular intervals. The experiment was terminated when around 30–40 l of eluent had been passed through each column.

The results of this experiment are shown in Fig. 3. The loss in retention with each of the five columns is clearly seen, although with the Nucleosil column this only amounted to 15% over 37 l. Despite the loss in retentivity there was no significant change in column efficiency or peak asymmetry with any of the five columns.

The previously used Spherisorb SAX column was unpacked and the stationary phase returned to Phase Separations. Analysis showed the carbon content of the phase to be within the limits set at manufacture indicating that there was no loss of bonded phase. Given the negative results from the

regeneration experiment described above, this finding is somewhat difficult to understand since blocking of ion exchange sites or loss of bonded phase are the two obvious reasons for falling retention. At this time, the reason for loss in retentivity remains unresolved. One interesting fact emerged however in that the Nucleosil column which showed the lowest fall in retention was different to the other columns in respect of the bonded stationary phase. The four columns showing the greatest drop in retention all had the ion exchange group bonded to the silica via an alkyl chain. In contrast, the Nucleosil column incorporated a phenyl ring in the linker group. It is possible that this difference between the phases is responsible for the increased stability of the Nucelosil column.

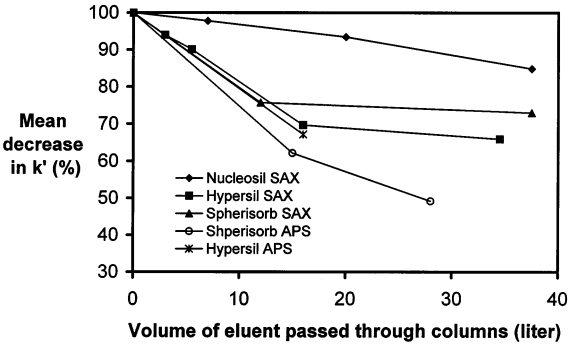

Fig. 3. The change in mean relative retention factor for a set of 24 test compounds with the volume of eluent passed through the columns. All columns were 100 × 4.6 mm and the eluents consisted of methanol–water (900:100 v/v) containing ammonium acetate (15 mM) except for the Hypersil SAX and the Nucleosil SAX where the ammonium acetate concentration was 10 and 50 mM, respectively.

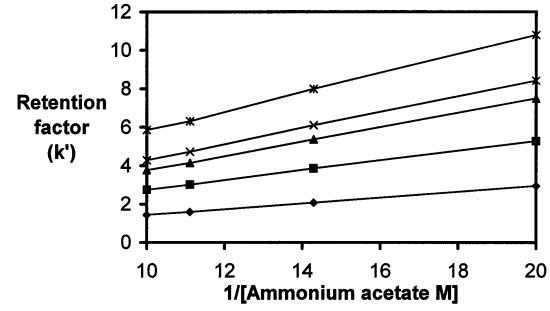

Fig. 4. The effect of mobile phase ammonium acetate concentration of the retention factor of five test compounds. The column was Nucleosil SAX (100 × 4.6 mm) and the eluents consisted of methanol–water (900:100 v/v) containing ammonium acetate (50–100 mM). Ibuprofen (◆), 3-naphthylpropanoic acid (■), 1-naphthoic acid (▲), 4-aminobenzoic acid (×) and warfarin (*).

3.3. Performance characteristics

Notwithstanding the problems with changing retention a full evaluation of the columns was carried out.

Fig. 4 shows the effect of the eluent ammonium acetate concentration on the retention of a selection of solutes using the Nucleosil SAX column. As expected with this ion-exchange system the retention is inversely proportional to the molarity of the buffer concentration. Doubling the buffer concentration from 50 to 100 mM resulted in a near halving of retention factor (k'). The fact that most of the compounds show a small positive intercept suggests that ion-exchange is not the only retention mechanism operating.

Changing the ammonium acetate concentration had no significant effect on efficiency or peak asymmetry. The absolute efficiency of all the columns was reasonably good, although the plate count was not as high as that seen with reversed-phase materials. Typical data for the Nucleosil SAX column are shown in Table 2.

A limited evaluation was carried out into the effect of the mobile phase solvents using the Nucleosil SAX column. In this part of the work, three additional mobile phase were investigated in addition to the standard mobile phase (A). These were all of reduced polarity and reduced viscosity and consisted of 100% methanol (B), methanol–acetonitrile–water (45:45:10, C) and methanol–acetonitrile–water (20:70:10, D). The four eluent were studied with a range of 34 acids, consisting mainly of simple aromatics and 11 drug compounds. Compared with the standard eluent (A), the two acetonitrile containing eluents gave a significant reduction in retention. To maintain a similar retention range as the other two eluents, the eluent buffer concentration was therefore reduced to 30 mM.

Compared with eluent A the three experimental eluents gave an increase in peak efficiency of

Table 2
Typical performance data for a Nucleosil SAX column (100 × 4.6 mm) using an eluent of methanol–water (900:100 v/v) containing ammonium acetate (50 mM) at a flow rate of 1 ml/min

Compound	Retention factor (k)	Efficiency plates/m	Peak asymmetry
Caffeine	0.0	29020	0.44
2-Nitrophenol	2.00	47800	1.28
Ibuprofen	2.74	40330	1.14
Naphthalene-1-acetic acid	8.32	41430	1.15
4-Aminobenzoic acid	9.16	40480	1.17
Warfarin	10.41	37640	1.23
3,5-Dinitrobenzoic acid	10.48	44570	1.20

around 7, 22 and 28% for eluents B, C and D, respectively. We attribute this change to a reduction in the eluent viscosity resulting in more rapid mass transfer. The selectivity with eluent B (100% methanol) was similar to that for A. The selectivity with the acetonitrile containing eluents (C and D) although similar to one another was very different to that with eluents A or B with significant changes in the order of elution.

3.4. Bioanalytical applications

To test the robustness of the system under typical bioanalytical conditions the following procedure was carried out. Plasma (1 ml) was acidified with hydrochloric acid (1 ml, 0.1 M) and extracted with the polar solvent ethyl acetate (5 ml) by tumble mixing for 20 min. Following centrifugation, an aliquot of the supernatant (4 ml) was removed, blown to dryness (N_2) and the residue dissolved in mobile phase (200 μl). An aliquot (100 μl) of the extract was then injected onto a Nucleosil SAX column which was protected with a guard column (30 × 2 mm) packed with Bond-elute SAX (60 μm) from Analytichem. The extracts were then chromatographed using an eluent of methanol–water (900:100 v/v) containing ammonium acetate (40 mM). At regular intervals the performance of the column was tested using the following protocol. At the beginning, end and five times during the evaluation period, injections (5 μl) were made of a test mixture containing the compounds ibuprofen, naphthalene-1-acetic acid, warfarin, ponalrestat and indomethacin.

In all 95 plasma extract injections were made, equivalent to a total plasma volume of 38 ml. Throughout the whole of the experiment, there was no significant change in retention time or retention factor. Although these results may appear to contradict those reported above, the eluent volumes used in this part of the work were relatively small. The average efficiencies for the test compounds were generally low, around 2500 plates, due to the effect of the guard column, which appeared to be non-optimal, efficiency and peak asymmetry also remained relatively constant.

4. Conclusions

In chromatographic terms, all the columns studied showed reasonably good efficiencies ($N \sim 40{,}000$ plates/m) and good symmetries (As $<$ 1.3). However, even with injections of pure standards, all columns showed a significant loss in retentivity ranging from approximately 10 to 50% with around 35 l of eluent. The reason for this remains unclear at this time, although it does not appear to be due deactivation of the ion-exchange site or loss of stationary phase. Of the five columns tested the Nucleosil SAX column was the most stable.

The Nucleosil column showed the expected linear relationship between retention factor and inverse of the eluent buffer concentration. Retention was also related to the pK_a of the analytes with the most acidic compounds being the most highly retained. Limited work indicated

significant changes in selectivity and an increase in efficiency when acetonitrile was added to the eluent.

Despite some loss in retentivity under prolonged usage, the Nucleosil SAX column appeared to be reasonably resistant to fouling by biological extracts.

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