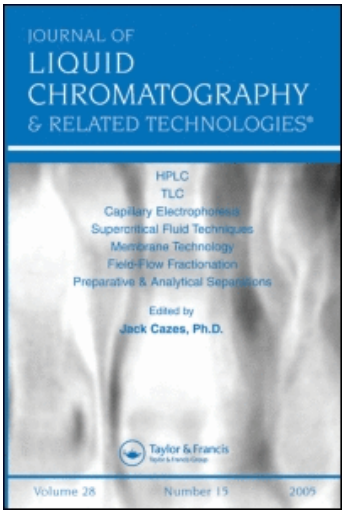

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THE EFFECT OF AMINE MODIFIERS ON THE CHROMATOGRAPHIC BEHAVIOR OF SALBUTAMOL ON REVERSED PHASE CHEMICALLY BONDED SILICA GEL

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ABSTRACT

The reversed phase chromatography of salbutamol in an aqueous phosphate buffer with quaternary and tertiary amine modifiers is described. The object was to control the retention and improve the asymmetry for use with electrochemical detection.

INTRODUCTION

Liquid chromatography with electrochemical detection is well suited to the separation and analysis of many bronchodilators in biological fluids. Electrochemical detection has the advantage of high sensitivity, a necessary requirement for this group of compounds. The mobile phase must be electrically conductive and an aqueous solvent without an organic modifier such as acetonitrile appears to provide improved sensitivity, lower background current and fewer problems.

However chromatographic peaks arising from compounds with a basic nitrogen are unsymmetrical and show extensive tails in reversed phase HPLC, particularly when the organic component of the solvent is present in low concentration (1).

Addition of organic amine compounds can improve the peak shape (1,2,3,4), and it may also be possible to make use of the concentration of the amine to adjust the retention of the compound of interest instead of adding an organic component such

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as methanol to the mobile phase. It is generally considered that tailing peaks arise from interactions between the solute and the adsorption sites on the silica matrix of the stationary phase. Apparently the organic amine preferentially occupies these active sites limiting the adsorption of solute. A wide range of amines have been used but this work examines the effects of tertiary and quaternary ammonium compounds on the reversed-phase chromatography of salbutamol using an aqueous mobile phase.

EXPERIMENTAL

Chemicals

Methanol was analytical grade (J. T. Baker, Phillipsburg, N.J. USA). Tetraethylammonium iodide (GPR, BDH), tetrapropylammonium iodide (Eastman Kodak), tetra-n-butylammonium iodide (GPR, BDH) and tetrapentylammonium iodide (Eastman Kodak) were converted into their hydroxides by precipitation of silver iodide with silver oxide and filtering. The iodide content was checked by measuring the absorbance at 254 nm. The quaternary ammonium hydroxide concentrations were confirmed by an acid-dye method (5). N,N-Dimethylhexylamine (DMHA), N,N-dimethyloctylamine (DMOA) and N,N-dimethyldecylamine (DMDA) were synthesized by a modified Eschwailer-Clarke reaction (6) and distilled. No impurities were found when examined by proton and carbon-13 NMR.

Salbutamol sulphate was of Pharmacopoeial grade and was supplied by Allen & Hanburys (Palmerston North, N.Z.).

All other substances were of analytical or reagent grade and used without purification.

Apparatus

A Waters Associates ALC/GPLC 244 liquid chromatograph with a Model 440 absorbance detector operating at 254 nm was used.

Column Packing Materials

The packing materials used were Zorbax CN (column I) and Zorbax ODS (columns II-V) (Du Pont Co. Wilmington, DE, USA), with mean particle diameters of 8 μm.

Column Tubing and Fittings

The columns consisted of 100mm x 4.6 mm i.d. or 150mm x 3.9mm i.d. (column V) stainless steel 316 tubing with a polished inner surface.

They were equipped with modified Swagelok compression fittings (#SS-400-6-1, Crawford Fitting Company, Cleveland, Ohio, USA). A 2µm removable frit (#716525, Alltech, Summer Hill, NSW, Australia) was placed at the outlet and an 8µm 316 stainless steel frit made from mesh (N. Greening Ltd, Warrington, U.K.) at the inlet. Teflon washers were inserted at each end.

Column Packing Technique

Columns were packed at 10,000 psi (except column I where the pressure was 2000 psi) using an upward slurry packing technique with the internal diameter of the reservoir being the same as the column. The slurry solvent was ethanol (95%)-n-propanol-toluene 1:1:1 (v/v) as described by Keller et al (7) for columns II-V and n-propanol for column I.

The quality of the columns was tested with anthracene and methanol-water (75/25) as eluent. The columns had a reduced plate height of less than 7.5 (8) and an asymmetry of less than 2.0 (except column I where the ASF was 4.0).

Asymmetry factors (ASF) were measured as follows: a perpendicular was drawn from the baseline to the vertex formed by the two peak tangent lines. A second line was drawn through the peak parallel to the baseline at 15% of the peak height. The ASF was calculated by dividing the length of the rear portion of this second line by the front part.

Chromatographic Conditions

Electrochemical detection of salbutamol tended to be more sensitive under acid conditions, however noisy traces attributed to the dissolution of iron (particularly from syringe needles) resulted. Thus ethylenediaminetetraacetic acid (10^{-4} M) was introduced to chelate any iron present and the optimum pH was

found to be 5.6. The solvent used in all experiments was sodium dihydrogen phosphate (0.1M) adjusted to pH 5.6 with sodium hydroxide (1M) plus 0.24 mmole of the appropriate amine (unless otherwise specified) per litre. The flow rate was 1.0 ml/min. Salbutamol (1mg/ml) dissolved in the mobile phase (less modifier) was injected in 10 ul amounts. Columns were taken as being equilibrated with the mobile phase when three replicate injections of salbutamol gave the same capacity factor (k'). The column void volume was estimated by an injection of sodium nitrate.

Procedures

a) Effect of tetra-n-butylammonium hydroxide (TBA) concentration on equilibration time and capacity factor.

The time taken for column (I) to equilibrate with the mobile phase was measured from the graph of the time of a salbutamol injection (the time was taken as zero when the mobile phase was switched from one without a modifier to one containing the appropriate concentration of TBA) versus the capacity factor of salbutamol for that injection. These plots showed a terminal straight line region (steady state conditions) and the time taken to reach this region was considered to be the equilibration time. The experiment was carried out with concentrations 0.03, 0.06, and 0.12 and 0.24 mM of TBA in the mobile phase and the equilibration time and capacity factor measured in each case.

b) Effect of amine modifiers on the k' & ASF of salbutamol.

Column V was equilibrated with mobile phase containing 0.12 mM of the appropriate amine and the capacity and asymmetry factors measured. The amines used were tetraethylammonium hydroxide (TEA), tetrapropylammonium hydroxide (TPrA), tetra-n-butyl ammonium hydroxide (TBA), tetrapentylammonium hydroxide (TPeA), dimethylhexylamine (DMHA), dimethyloctylamine (DMOA) and dimethyldecylamine (DMDA).

c) Adsorption of quaternary amine modifiers.

Three Zorbax ODS columns (II, III, V) were each equilibrated with a mobile phase containing the appropriate modifiers (TEA,

TPrA, TBA, TPeA). Methanol (100 ml) was then passed through (9) and collected. The methanol was evaporated and the residue dissolved in distilled water (10 ml). The concentration was measured by the method of Chatten and Okamura, using procedure c (5).

The concentration of amine modifier in the mobile phase used with column (V) was 0.12 mM. Columns (II) and (III) used a concentration of 0.24 mM.

Columns (III) and (V) had not been used with an amine modifier before whereas column (II) had been in use for two weeks with a quaternary ammonium salt.

After completion of these experiments, the equivalent amount of distilled water to two weeks use (6 litres) was passed through column (III). This column was then re-equilibrated with mobile phase containing 0.24 mM TBA and the amount of quaternary ammonium ion adsorbed was again estimated.

d) Adsorption of tertiary amine modifiers.

Column (V) was equilibrated with mobile phase containing 0.24mM of a tertiary amine modifier (DMHA, DMOA, DMDA), the modifier was eluted with methanol and an aqueous solution (200 ml) prepared as described for the quaternary amine modifiers.

The amount of amine absorbed in column (V) was measured by potentiometric titration which involved titrating a 10 ml sample with 1.0 mM hydrochloric acid and determining the end point with a pH meter (about 5.5).

The total amount of amine (amine + amine salt) was measured by gas chromatography using a 3 foot column of 10% carbowax 20M and 2% potassium hydroxide on Gas Chrom Q (Applied Science Laboratories, Inglewood, CA, USA), with the appropriate standards (ca 0.1 µg/ml).

e) Estimation of free silanol groups.

The method used is that described by Karch et al (11) and Tanaka et al (10). Column (IV) was new and had not been used with organic modifiers, column (III) was also new and had not been used with organic modifiers (except for procedure c), but

had the equivalent of two weeks use of distilled water passed through (6 litres). Column (II) had been well used with amine modifiers, as shown in table 3. in the extent to which the quaternary ammonium salts adsorbed.

RESULTS AND DISCUSSION

Although reversed phase coated silica is not quite so susceptible to attack by alkali as silica, quaternary ammonium compounds such as TBA can render a column useless within days (12). Hence it is desirable to maintain low TBA concentrations. However, at low solvent quaternary ammonium concentrations the time taken for the column to equilibrate with the solvent increases. This is shown in Table 1 along with the capacity factor for salbutamol after the column has equilibrated. The modifier TBA controls the retention and concentrations as low as 0.03 mM TBA are effective in improving the chromatography of salbutamol in aqueous solutions.

The effect of a series of quaternary ammonium compounds and tertiary amines on the asymmetry (ASF) and the capacity factors of salbutamol are shown in table 2.

Sokolowski and Wahlund (1) found that TBA and TPrA did not improve the ASF of a series of tricyclic antidepressants in 1:1 methanol, phosphate buffer pH 2-3.3 with 0.05M additive and Tilley-Melin et al (13) state that the adsorption sites for quaternary ammonium ions are not easily accessible to cations with bulky substituents such as TBA.

Our results (table 2) show that both TBA and TPrA do improve the ASF and there is an apparent trend towards the cations with bulky substituents showing the greatest improvement. An explanation for this apparently contradictory result is that the bulky quaternary ammonium ions favour the mobile phase when the methanol content is high and insufficient coverage of the active site occurs. In aqueous solvents hydrophobic bonding can be significant (14) and the greater the hydrophobicity of the quaternary ammonium ion, the higher the concentration in the

TABLE 1

The time taken for Column (I) to Equilibrate with the Mobile Phase containing Various Concentrations of TBA

TBA Concentration (mM)	Equilibration Time (min)	Capacity Factor
0	-	7.0
0.03	88	1.33
0.06	62	0.84
0.12	38	0.38
0.24	26	0.22

TABLE 2

The Effect of a Series of Amine Modifiers on the Retention and Asymmetry of Salbutamol

Modifier (0.12mM)	Capacity Factor	Asymmetry
None	86	7.6
TEA	47	3.2
TPrA	16	1.7
TBA	1.2	2.0
TPeA	1.0	2.0
DMHA	22	double peak
DMOA	2.6	2.2
DMDA	-0.6	1.7

stationary phase. The decreased retention is probably due to the stationary phase taking on cation exchange character.

Excessive tailing is caused by interaction with silanol groups on the stationary phase and it is likely that certain sites affect salbutamol more than others depending on their accessibility. Thus the modifier which has most effect would be the one which blocks those sites which affect salbutamol greatest. This would suggest that TPrA is effective at blocking those sites which cause tailing of salbutamol.

Peak tailing has been described by a two site theory (15) and in the case of reversed phase chromatography the second site is considered to be unprotected silanol groups (10). Under certain conditions the two sites can give rise to two peaks (16). DMHA is ineffective at covering sites active to salbutamol, presumably because either it has insufficient hydrophobic character and the concentration in the stationary phase is too low or it does not remain on the sites active to salbutamol for long enough.

Negative capacity factors as with DMDA have been explained as charge exclusion phenomena (17). When DMDA is adsorbed as the hydrogen phosphate the pores become electrically charged and tend to exclude similarly charged molecules making a portion of the solvent void volume inaccessible to salbutamol hydrogen phosphate.

The amounts of quaternary ammonium and tertiary amine adsorbed on reversed phase columns are shown in Table 3 and 4. As expected (18) the amount of quaternary ammonium ion adsorbed is greater with increasing hydrophobic chracter. However, if the concentration of quaternary ammonium ion in the mobile phase is halved the amount adsorbed does not change significantly (column (V) cf column (III)).

Iler (14), and Bijsterbosch et al (19) proposed that the mechanism of adsorption of the long chain aliphatic hydrocarbon amines to silica involves hydrophobic bonding. At low concentrations a single hydrophobic layer is adsorbed with the

TABLE 3

The Quantity of Quaternary Amine Modifier Adsorbed on Three Reversed Phase Columns

Quaternary Amine	Amount Adsorbed (mole x 10^{-5})		
	Column		
	III	V	II
TEA	0.03	0.17	0.51
TPrA	0.47	0.44	1.18
TBA	2.17	2.06	6.74
*TBA	3.89	-	-
TPeA	5.39	4.97	8.26

*This value was re-estimated after six litres of distilled water had been passed through Column (III).

nitrogen close to the silica surface and at higher concentrations a double layer forms. The second layer has the reverse orientation with the apolar chain towards the surface of the silica and hence the amine group has an associated counter ion.

The function of amine modifiers on reversed phase columns is to block sites that contribute to separation mechanisms other than liquid-liquid partition. Thus ideally a monolayer coverage with all sites occupied is required. If a bilayer is formed (with the amine group in the mobile phase), ion exchange as a separation mechanism would predominate and the separation characteristics of the column would change completely.

The adsorbed amine is eluted and measured by potentiometric titration. This gives an estimate of the proportion of monolayer formed (the amine contributing the double layer would be eluted as its phosphate salt and is not measured). Gas chromatography measures the total amine eluted and these results are shown in Table 4. The proportion of bilayer gives an estimate of the degree of ion-exchange character.

TABLE 4

The Extent and Type of Adsorption of Tertiary Amine Modifiers.

Tertiary Amine (0.24 mM)	Amount Absorbed		
	Total (mole x 10^{-5})	Monolayer (mole x 10^{-5})	Bilayer (percent of monolayer)
DMHA	3.49	3.58	0
DMOA	9.04	7.15	26
DMDA	24.9	17.2	45

Reversed phase packing materials are quite unstable towards quaternary ammonium compounds but less so towards the tertiary amines (1, 12). The amount of TBA adsorbed by the stationary phase was measured on a column previously unused (III) and a column used with a quaternary ammonium compounds in the mobile phase for two weeks (table 3, Column (II)). This column did not show a significant change in ASF or capacity factor for salbutamol when compared with the unused column. However, there is a considerable increase in the quantity of quaternary ammonium ions adsorbed for all the modifiers. Wehrli et al (12) have shown that the mechanism of breakdown of the column occurs by cleavage of the organic layer from the silicate particles and collapse of the silicate structure and thus presumably there would be more active sites for the quaternary ammonium ion. Water also hydrolyses silica and after the studies with the quaternary ammonium compounds, column (III) had six litres of water (the equivalent to two weeks use) passed through. The amount of TBA adsorbed increased but not to the same extent as column (II).

A method of estimating the polarity of a column, resulting from unshielded silanol groups is to measure the retention of small polar molecules in dry heptane (10,11). The more polar the column (i.e. the greater the number of available

TABLE 5

A Comparison of Three Columns for Free Silanol Groups

Column	State	Capacity Factor	
		Anisole	Methylbenzoate
IV	new	1.34	7.8
III	washed with water (61)	1.38	9.5
II	well used with amine modifiers	2.10	12.0

silanol groups) the more the solute is retarded. For a reversed phase column with no silanol groups the capacity factor should be one. The results for an unused column, a column which had used water as the mobile phase and a column which had used TBA, phosphate buffer as mobile phase are shown in table 5. The results confirm that less polar sites result from the use of an aqueous mobile phase than one containing TBA.

In summary the quaternary ammonium ions and tertiary amines studied are effective in controlling the ASF and retention of salbutamol. However the quaternary ammonium ions hydrolyse the silica stationary phase and are adsorbed on the resulting silanol groups. As the hydrophobicity of the tertiary amines increases a second layer is formed which can function in an ion exchange mode.

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