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MECHANISM OF ZWITTERION-PAIR CHROMATOGRAPHY

II. AMPICILLINE, LYSERGIC ACID, TRYPTOPHAN AND OTHER SOLUTES

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SUMMARY

The dependence upon pH of the column capacity ratio for the zwitterionic solutes ampicilline (zwitterionic from pH 2.5–7.3), lysergic acid (3.3–7.8) and L-tryptophan (2.3–9.3) has been determined on ODS Hypersil® using 1.25 m*M* acetate and 75 m*M* phosphate buffers with and without the addition of 1.25 m*M* 11-aminoundecanoic acid (C11AA) (4.4–10.0). Enhancement of retention is observed within the range of zwitterion overlap with maximum enhancement being observed at pH values corresponding to maximum zwitterion overlap. Outside the region of zwitterion overlap addition of C11AA either has no effect or causes a reduction in retention.

It is concluded that the enhanced retention within the region of zwitterion overlap arises from the formation of quadrupolar ion pairs, whereas the rejection outside this region arises from repulsive interactions of the like-charged forms of the solute and pairing species. The rejection mechanism is confirmed for simple amines and is exploited to provide a useful separation of cimetidine and metiamide. Addition of C11AA enhances the retention of some acids and provides a good separation of the urinary acids, vanilmandelic acid, 5-hydroxy-3-indoleacetic acid and homovanilic acid.

The results provide strong confirmatory evidence for the formation of quadrupolar ion pairs at an appropriate pH in zwitterion-pair chromatography

INTRODUCTION

In Part I of this study[1] the thermodynamic basis of zwitterion-pair chromatography was established with nucleotides as model solutes and with 11-aminoundecanoic acid (C11AA) and L-leucyl–L-leucyl–L-leucine (LLL) as ion-pairing agents. The study on the mechanism of this form of ion-pair chromatography followed our earlier report on the separation of nucleotides with C11AA as pairing agent[2]. The concept of zwitterion-pair chromatography had arisen previously in connection with the role of ethylenediaminetetraacetic acid (EDTA) in the separation of tetracyclines[3].

The major conclusions derived from this work were: (i) that k' increased with the adsorbed concentration of zwitterion-pairing agent; (ii) that as pH was changed

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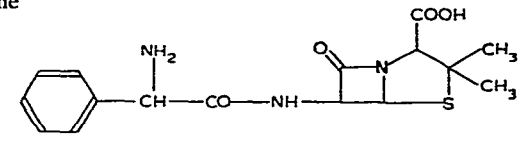
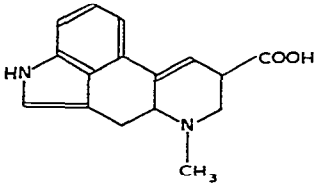
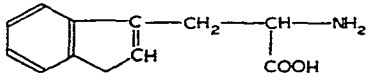
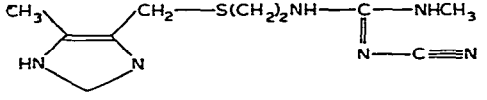
k' showed maximum value at pH of 4.0–4.2 with C11AA as pairing agent and 3.5 with LLL as pairing agent, these pH values being coincident with maximum overlap of zwitterionic forms; (iii) that as the ionic strength of the buffering salt mixture increased, the zwitterion-pairing effect of pairing agent was gradually suppressed.

While nucleotides are of great biochemical interest, they are not ideal test solutes for examining the thermodynamic basis of zwitterion-pair chromatography because of their complex ionisation patterns, and because of the small range of pH within which both the nucleotides and C11AA or LLL possess significant proportions of molecules in the zwitterionic form. In the present study with C11AA we have therefore sought solutes which will provide a greater range of zwitterion-pair overlap,

TABLE I

SOLUTES AND PAIRING AGENTS

Substance and formula	*pK_a*	*Charge**
Ampicilline 	+ 2.5 ± 7.3	−
Lysergic acid 	+ 3.3 ± 7.8	−
Tryptophan 	+ 2.3 ± 9.3	−
Cimetidine 	+ 2 + 6.8 N 12	−
Metiamide [as cimetidine]-NH-C-$NHCH_3$ (S)	As cimetidine	
11-Aminoundecanoic acid (C11AA) $NH_2(CH_2)_{10}COOH$	+ 4.4 ± 10.0	−

* The symbols +, ±, N and − refer to the charges on the molecules for the appropriate pH ranges: ± indicates a zwitterion, N indicates a neutral species with no charged centres.

and where the pH for maximal overlap would be significantly different from the value provided by the nucleotides. The substances examined comprise three amino acids, ampicilline, lysergic acid and tryptophan, and two bases, cimetidine and metiamide. Their pK_a values, formulae and ionic charges as a function of pH are listed in Table I. Some simple organic acids and bases have also been briefly examined.

The results again show that maximum enhancement of retention on the addition of the zwitterionic-pairing agent C11AA occurs at a pH corresponding to maximum overlap of the zwitterionic forms of the solute and pairing agent. They further support our contention that the enhancement of retention arising from addition of a zwitterionic-pairing agent is due to the formation of quadrupolar ion pairs in the stationary hydrocarbon phase.

EXPERIMENTAL

The chromatographic equipment and column packing procedure have been described previously[1]. ODS Hypersil (Shandon Southern Products, Runcorn, Great Britain) was used throughout as column packing material.

Methanol was HPLC grade solvent (Rathburn Chemicals, Walkerburn, Great Britain). C11AA was obtained from Aldrich (Gillingham, Great Britain); ampicilline was BP Pharmaceutical preparation; D-lysergic acid and L-tryptophan were obtained from Sigma (Poole, Great Britain); cimetidine, metiamide and cimetidine sulphoxide were obtained from Smith, Kline & French Labs. (Welwyn Garden City, Great Britain); 5-hydroxy-3-indoleacetic acid (5HIAA) was obtained from Koch-Light Labs. (Colnbrook, Great Britain); homovanillic acid (HVA) and vanilmandelic acid (VMA) were obtained from Hoffmann-La Roche (Basle, Switzerland).

RESULTS AND DISCUSSION

Zwitterionic solutes

The dependence of k' upon pH for the three solutes, ampicilline, lysergic acid and tryptophan is shown in Figs. 1 (ampicilline) and 2 (lysergic acid and tryptophan). A single concentration of C11AA, *viz.* 1.25 mM, and the same basic eluent, *viz.* water–methanol (88:12) has been used throughout. Two different buffers have been used to adjust pH, a weak acetate buffer of ionic strength 1.25 mM, and a stronger phosphate buffer of ionic strength 75 mM. In each figure the range of zwitterion overlap for the solute and pairing agent is shown either by an ionisation diagram or by a horizontal bracket; the ends of the bracket correspond to the pK_a values given in Table I; the central arrow within the bracket corresponds to the pH for maximum zwitterion overlap.

In Figs. 1 and 2 it is noted that with the weak buffer the k' values for ampicilline and lysergic acid in the presence of C11AA show maxima at or close to the position of maximum overlap. These maxima are not attributable to any similar change in the amount of C11AA adsorbed (C_{ads}) as is clearly seen by comparison of the curves for k' with those for C_{ads} taken from ref. 1.

Comparing k' values with and without added C11AA it is seen that within the main part of the region of zwitterion-overlap retention is enhanced by addition of C11AA, but outside this region retention is reduced. Thus C11AA, when not acting

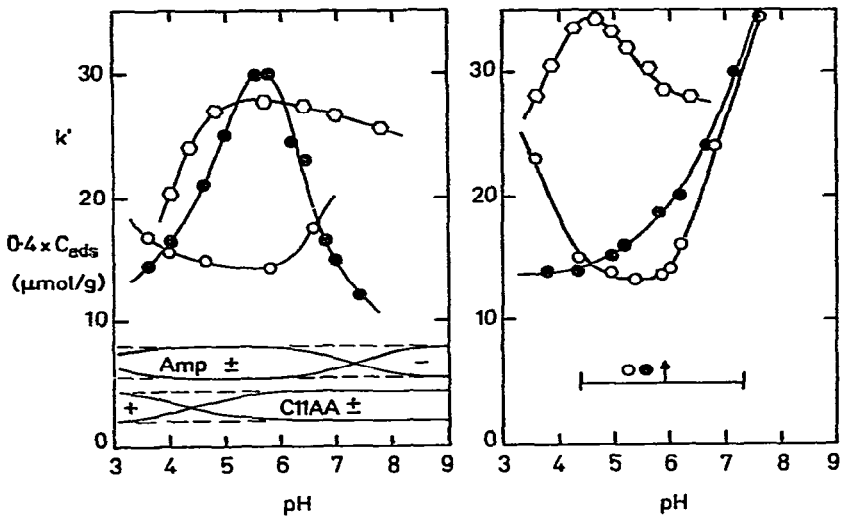


Fig. 1. Dependence of capacity ratio, k', of ampicilline and of concentration of adsorbed C11AA, C_{ads}, upon pH. Column, 100 × 5 mm I.D. Packing, 5 μm ODS Hypersil. Eluent, water–methanol (88:12) containing 1.25 mM acetate buffer (left hand side) or 75 mM phosphate buffer (right hand side). ●, k' for ampicilline with 1.25 mM C11AA added; ○, k' for ampicilline with C11AA absent; ○, C_{ads} for C11AA. The horizontal bar, indicates the range of zwitterion-pair overlap.

as a zwitterion-pair agent, has the effect of rejecting a charged or dipolar species.

When the stronger 75 mM phosphate buffer is used, retention of ampicilline and lysergic acid in the absence of C11AA is strongly dependent upon pH. Minimum retention occurs at a pH which corresponds closely to the isoelectric point of the solute (4.9 for ampicilline, 5.5 for lysergic acid). The strongly increased retention as the pH moves away from the isoelectric point presumably arises from ion pairing of

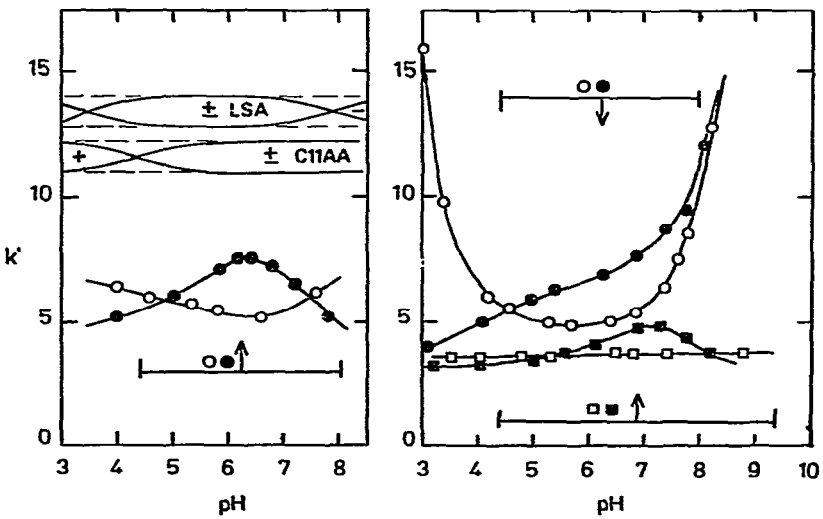


Fig. 2. As for Fig. 1 but for lysergic acid (●, ○) and L-tryptophan (75 mM buffer only) (■, □) as solutes. Filled symbols refer to eluent containing 1.25 mM C11AA; open symbols refer to eluent without C11AA.

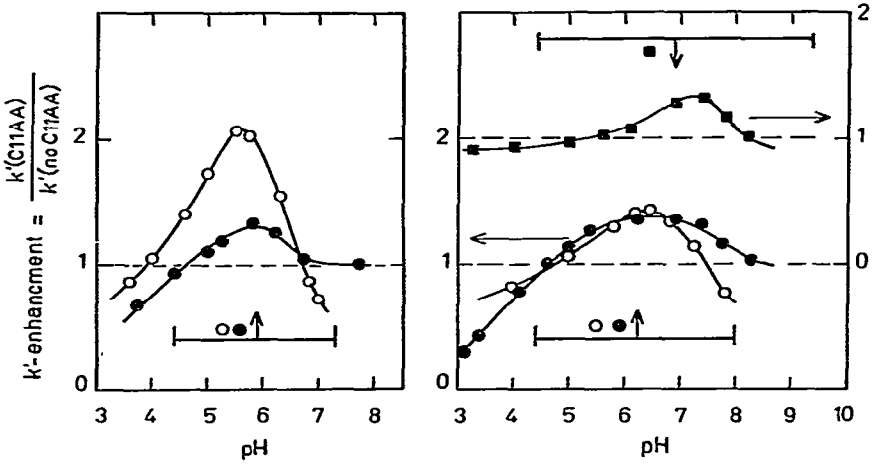


Fig. 3. Dependence of enhancement of retention upon pH when C11AA added to eluent: left, ampicilline; right, L-tryptophan (□) and lysergic acid (○): open symbols refer to 1.25 m*M* acetate buffer, filled symbols refer to 75 m*M* phosphate buffer.

the positively or negatively charged forms of the solutes with buffer ions. It should in passing be noted that at pH above 6 the peaks for ampicilline in the absence of C11AA became very wide; and ampicilline cannot therefore be effectively chromatographed with the buffer alone in this pH region.

On addition of C11AA retention is enhanced within the region of zwitterion overlap, but at lower pH (when C11AA is positively charged) rejection occurs. At higher pH (solutes negatively charged) the k' values tend toward those obtained when C11AA is absent.

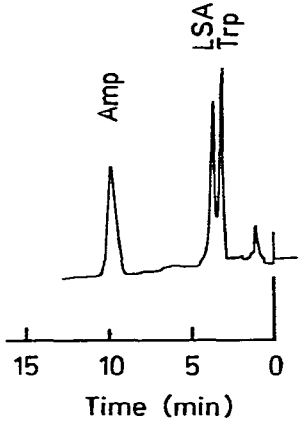
For tryptophan retention with 75 m*M* phosphate buffer in the absence of C11AA is by contrast independent of pH. On addition of C11AA, k' shows a maximum at pH 7.2, very close to the pH for maximum zwitterion overlap of 6.9.

In Fig. 3 the enhancement of retention, that is the ratio of k' in the presence of C11AA to k' without C11AA, is plotted against pH for the weak and strong buffers.

TABLE II

pH VALUES FOR MAXIMUM ENHANCEMENT OF RETENTION USING C11AA

Solute	*pH for maximum enhancement of retention*		*pH for maximum zwitterion overlap*
	1.25 mM phosphate	*75 mM phosphate*	
Adenosine monophosphate	4.0	4.4	4.1
Ampicilline	5.5	5.8	5.9
Lysergic acid	6.4	6.6	6.3
Tryptophan	—	7.2	6.9



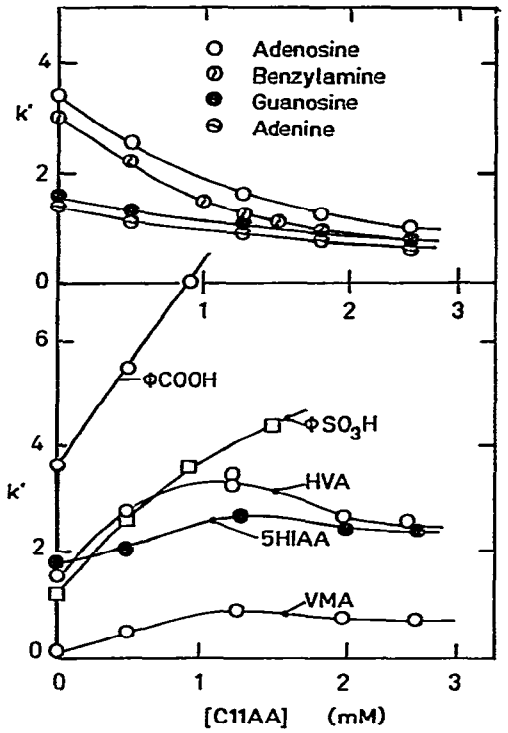


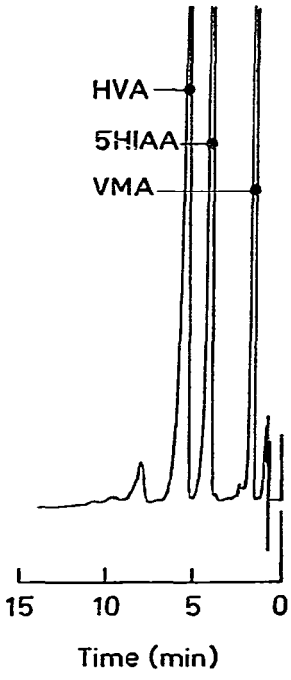
Fig. 4. Representative separation of L-tryptophan, lysergic acid and ampicilline. Conditions as for Fig. 1 with 75 m*M* phosphate buffer, pH 3. Detection, UV 254 nm.

Fig. 5. Dependence of k' for bases (upper) and acids (lower) upon concentration of C11AA in eluent. Conditions as Fig. 1 with 75 m*M* phosphate buffer, pH 5.8.

For ampicilline the enhancement is much greater with the weak buffer and rejection (giving an enhancement ratio below unity) occurs on both wings. On the other hand, with the strong buffer while a peak in k' still occurs, it is much lower and the maximum is relatively flat. Whereas rejection occurs at low pH, C11AA tends to have zero effect at high pH. These results are similar to those previously found with AMP[1]. That is the maxima in k' were much more pronounced with the weaker buffer solutions. For lysergic acid the maximum in the dependence of enhancement upon pH are comparable for the two buffers although the maximum is sharper for the weaker buffer. Again rejection is noted on both wings with the weaker buffer but only at low pH with the stronger buffer. With tryptophan the results with the strong buffer are similar to those with the other two zwitterionic solutes.

The pH for maximum enhancement of retention are compared in Table II with the pH for maximum zwitterion overlap. The table includes the data for adenosine monophosphate (AMP) from ref. 1. The pH for maximum enhancement of retention is about 0.2–0.4 units higher when using 75 m*M* buffers than when using weak buffers, but in all cases these pH values are very close to those for maximal zwitterion overlap. The values now cover approximately three pH units.

Fig. 4 shows a representative chromatogram of the three zwitterionic solutes



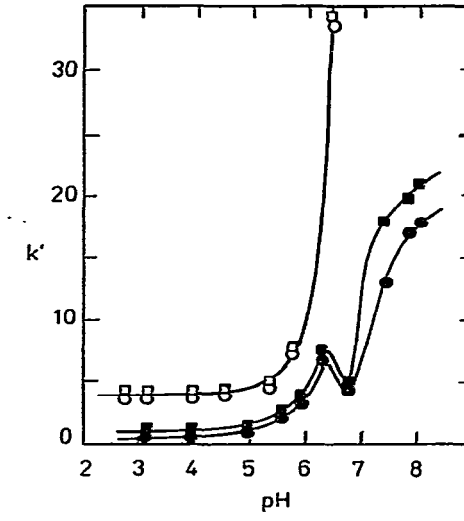


Fig. 6. Representative separation of urinary acids, VMA, 5HIAA and HVA. Conditions as Fig. 5 with 1.25 m*M* C11AA. Detection, UV 254 nm.

Fig. 7. Dependence of *k'* upon pH for metiamide (○) and cimetidine (□). Column and packing as for Fig. 1. Eluent: filled symbols, water–methanol (88:12) containing 75 m*M* phosphate buffer and 1.25 m*M* C11AA; open symbols, same but with no C11AA present.

and indicates the good plate efficiency which can be obtained. The new data obtained with ampicilline, lysergic acid and tryptophan provide further strong support for the view that a zwitterionic-pairing agent enhances the retention of zwitterionic solutes by the formation of quadrupolar ion pairs in the stationary phase.

Simple acids and bases

The phenomenon of rejection of ionized solutes by C11AA outside the range of zwitterion overlap appears to arise from like charge repulsion when both the pairing agent and the solute can exist with the same charge. We have examined this effect briefly with simple acids and bases at pH 5.8 using 75 m*M* phosphate buffer and the standard eluent of water–methanol (88:12). Fig. 5 shows that while benzylamine and some nucleic acid bases and nucleosides are rejected by addition of C11AA, retention of acids is either enhanced (benzoic and benzenesulphonic) or behaves indecisively (5HIAA, VMA and HVA). These results are broadly consistent with those found with the zwitterionic solutes in 75 m*M* buffer in that at low pH when the solutes and C11AA tended to be positively charged the solute was rejected while at high pH when the solutes and C11AA showed negative charge the effect of C11AA appeared to be minimal.

The marked difference between the behaviours of acids and bases in presence

of C11AA as pairing agent can only be understood as a result of higher polarity of NH_2^+ group as compared to COO^- group. The isoelectric point of C11AA lies around pH 7.4 and 7.6. Therefore C11AA can with respect to acidic compounds act as weak cationic pairing agent. This was proved, when k' *versus* pH dependence curve was studied for benzoic acid in eluents with dilute acetate buffer. The retention of benzoic acid was enhanced above pH 4.2 (the pK_a of benzoic acid) and reduced below pH 3.6.

Example of good separation of the key urinary acids: 5HIAA, HVA and VMA with C11AA is shown in Fig. 6.

Cimetidine and metiamide

The phenomenon of rejection of amines by addition of C11AA has proved useful in the liquid chromatography of the antihistamine drugs cimetidine and metiamide which cannot be separated using phosphate buffer alone. When 1.25 m*M* C11AA is added to the eluent the retention of the amines at all pH is much reduced as shown in Fig. 7. Without added C11AA retention remains unchanged between pH 3 and 5.5 then increases dramatically starting from pH 5.8 as the pH approaches the pK_a value of 6.8 corresponding to the formation of neutral form of the drug. The extent of the increase is suppressed in the presence of C11AA and a small maximum

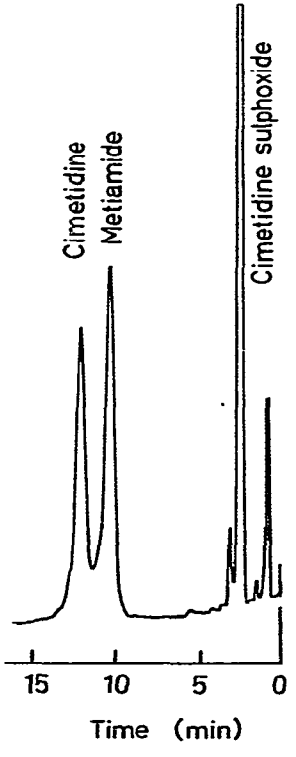


Fig. 8. Representative separation of cimetidine sulphoxide, metiamide and cimetidine. Eluent as for Fig. 7. pH, 7.4 containing 1.25 m*M* C11AA. Detection, UV 254 nm.

occurs at pH 6.2, which is not well understood. This enables chromatography to be carried out at a higher pH allowing separation of the two drugs to be achieved with reasonable k' values between pH 7.4 and 8. In clinical practice it is important to be able to assay both cimetidine and its main metabolite the sulphoxide. Fig. 8 shows that this separation, previously achieved by normal-phase chromatography[4,5], can readily be achieved using C11AA and 75 mM phosphate buffer.

CONCLUSIONS

The results obtained on the pH dependence of the retention of the zwitterionic solutes ampicilline, lysergic acid and tryptophan on a reversed-phase packing material in the presence of C11AA are described. Addition of millimolar concentration of C11AA cause a marked enhancement of retention in the pH range where both the solute and the pairing agent exist predominantly as zwitterions, and the pH for maximum enhancement of retention coincides within a fraction of a pH unit with the pH for maximum zwitterion overlap. The maxima are particularly sharp when weak buffers are used to adjust the pH. These results add further strong support to our contention made in previous papers[1,2] that enhanced retention caused by a zwitterionic-pairing agent arises from the formation of quadrupolar ion pairs.

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