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ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: THE USE OF LOW CONCENTRATIONS OF LONG-CHAIN ALKYLBENZYLDIMETHYLAMMONIUM CHLORIDES FOR RESOLVING ANIONIC SOLUTES

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SUMMARY

The qualitative and quantitative determination of large anionic solutes, including the drug sodium cromoglycate, by an ion-pair high-performance liquid chromatographic technique using alkylbenzyldimethylammonium chlorides as pairing ions is described. The method provides a sensitive and selective assay for the solutes examined in both simple aqueous solutions and in complex biological fluids, and requires no pre-extraction step. Retention mechanisms are discussed.

INTRODUCTION

Recently, the addition of long alkyl-chain surface active ions to reversed-phase high-performance liquid chromatography (HPLC) systems has received much attention[1–11] for resolving ionized solutes, as their use can be shown to lead to extremely efficient separating systems. Generally two models are proposed to explain the mechanism(s) of solute retention in such systems. First, that the stationary phase acts as a dynamically coated ion-exchanger due to an exchange of solute ions with the counter ions of adsorbed surfactant molecules, and second, that ion pairs are formed in the mobile phase and the formed "neutral" complex is then reversibly bound to the stationary phase. Experience of ion-pair formation between the cationic surface active agents, alkylbenzyldimethylammonium chlorides (ABDACs), and large anionic drug molecules[12,13], and a growing need for rapid, selective and sensitive assays for drugs of this type, have led us to develop an HPLC method which uses octadecyl reversed phases, ABDACs as pairing ions and aqueous methanol as the mobile phase. The purpose of this paper is to present that methodology and, by examining the retention behaviour of a series of acid red dyes and the dianionic drug sodium cromoglycate, to help elucidate further the retention mechanism(s). The

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applicability and efficiency of the method are demonstrated by the chromatography of sodium cromoglycate from both simple aqueous solutions and a complex biological fluid.

EXPERIMENTAL

Apparatus

The HPLC system was custom-built. Eluent was pumped from a 250-ml reservoir by a Jobling constant-flow-rate liquid delivery system (Model 709). All solute injections were made using a Specac injection valve (Type 30,001) with a 5-μl loop. The column end fitting was a modified 1/4 in./1/16 in. Swagelok reducing union drilled out so that the column end was as close as possible to the outlet tube. All column connections were made with stainless-steel (316) Swagelok fittings. The detector was a fixed-wavelength (254 nm) dual-beam spectrophotometer (Jobling, Model 1205J), fitted with 8-μl flow cells. The detector output was recorded on a potentiometric chart recorder.

Column materials and packing procedure

The stationary support was octadecylsilane bonded to silica (Spherisorb 55w ODS, Phase Separations, Queensferry, Great Britain). This was packed into a 50 × 5 mm I.D. stainless-steel column using a dilute slurry method[14] with *n*-hexane as solvent. After packing, a 5-mm depth of packed support material was removed and replaced with a steel mesh (5 mm diameter), which was pushed on to the top of the column. Glass beads (70–150 μm diameter) were then poured on to the mesh to a depth of 3 mm. A PTFE frit was placed above the beads and trimmed flush with the column end. This arrangement helped to effect an even flow of eluent.

Materials and samples

Alkylbenzyldimethylammonium chlorides were kindly supplied as the monohydrates by Dr. F. Nachod, Sterling-Winthrop, Rensselaer, N.Y., U.S.A., and their high purity was confirmed by mass spectral analysis, by the absence of minima in their surface tension *versus* concentration profiles and by the excellent linear relationship between the logarithms of their critical micellar concentrations and alkyl chain lengths[13].

Methanol and *n*-hexane (HPLC grade) were supplied by Rathburn Chemicals, Peebleshire, Great Britain. Water was doubly distilled from an all-glass still and further deionized before use. Chloroform was of analytical-reagent grade.

The acid red dyes used (I, II and III) have the structures shown in Fig. 1. Dye I (Acid Red 88) was lissamine Red J and dye II (Acid Red 18) was lissamine Scarlet 4R. Both were gifts from Imperial Chemical Industries (Macclesfield, Great Britain). Dye III (Acid Red 41) was Ponceau 6R and was a gift from Francolor, Munich, G.F.R.

Sodium cromoglycate (SCG), molecular weight 512.3, was donated by Fisons, Loughborough, Great Britain. Its structure is shown in Fig. 2.

Procedures

Alkylbenzyldimethylammonium chlorides of chain length C_8, C_{10}, C_{11}, C_{1}: and C_{13} were studied over the concentration range $1 \cdot 10^{-5}$–$6 \cdot 10^{-4}$ *M* using degassed

	R^1	R^2	R^3	R^4
Dye I	SO_3Na	H	H	H
Dye II	SO_3Na	SO_3Na	SO_3Na	H
Dye III	SO_3Na	SO_3Na	SO_3Na	SO_3Na

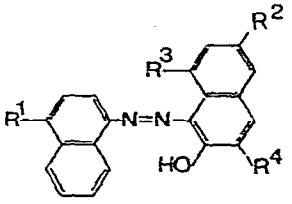


Fig. 1. Acid red dyes.

methanol–water (1:1) as the mobile phase. The concentration of ABDAC in the mobile phase was progressively increased with generally five or six concentration levels being examined for each ABDAC homologue studied. Each change in ABDAC mobile phase concentration resulted in an increase in recorder baseline after some period of time. When the rate of change of baseline stabilized at zero, the time taken was judged to be an indication of equilibrium conditions.

Knowledge of the ABDAC mobile phase composition, column flow characteristics and ABDAC column breakthrough times (*i.e.*, baseline equilibrium times) permitted the calculation of the amount of ABDAC retained within the column for each change in ABDAC mobile phase concentration. Fig. 3 is an idealized diagram illustrating the method used to calculate the amount of surfactant adsorbed, q, by the column at any particular mobile phase concentration C. For this example, at a concentration C_2 at equilibrium, as the ABDACs obey Beers law:

$$q = (C_2 - C_1)F_c(t_1 - t_0)\left(\frac{w_A + w_B}{w_A}\right) \quad (1)$$

where F_c is the flow-rate, t_0 is the retention time of a non-retarded solute and w_A and w_B refer to the weights of chart paper of areas A and B. After each homologue

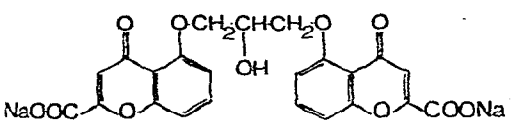


Fig. 2. Structure of sodium cromoglycate.

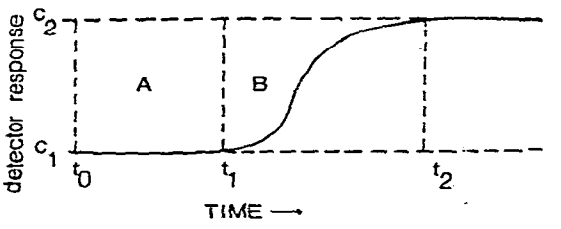


Fig. 3. Change in chart recorder baseline (solid line) with time for an alteration in ABDAC mobile phase concentration (c_1 to c_2). See text and eqn. 1 for detailed explanation and calculation of amounts of ABDAC uptake.

had been studied, the pairing ion was removed from the column by either passing through a $5 \cdot 10^{-4}$ *M* solution of sodium cromoglycate for 30 min followed by flushing with methanol–water (1:1) for 10 min, or by flushing through with pure methanol for an equivalent length of time. These procedures resulted in the SCG peaks being indistinguishable from the solvent front.

At each ABDAC concentration triplicate injections of dilute solutions of dye or SCG in methanol–water (1:1) were made. Column injection was generally $2.1 \cdot 10^{-4}$ *M* for SCG and *ca.* $1 \cdot 10^{-5}$ *M* for the dyes. Flow-rates were 1.0–1.2 ml·min^{-1}, achieved at a pressure of 50–100 bar. For the quantitative determination of the drug, the SCG concentration range was $3.9 \cdot 10^{-5}$–$2.0 \cdot 10^{-4}$ *M* and octylbenzyldimethylammonium chloride ($5.2 \cdot 10^{-4}$ *M*) was used as the pairing ion. For the assay of SCG in urine, drug was added to freshly collected urine, which was then injected directly on to the column. Water was used as the non-retarded solute.

The abilities of the acid red dyes and ABDACs to form ion pairs was assessed by examining for the bulk-phase extraction of the acid red dyes from water into chloroform in the absence and presence of ABDAC molecules. The procedure used was to shake 20 ml of chloroform-saturated water containing dye with 20 ml of water-saturated chloroform at 30° for 3 h. Experiments were performed in both the absence and the presence of various concentrations of ABDACs.

RESULTS AND DISCUSSION

The addition of long-chain alkylbenzyldimethylammonium chlorides to aqueous methanol–RP-18 systems results in the retention of all of the solutes examined. Fig. 4 shows how the addition of decylbenzyldimethylammonium chloride (up to

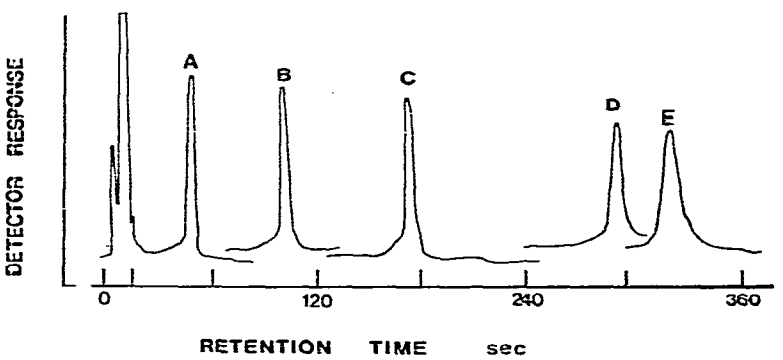
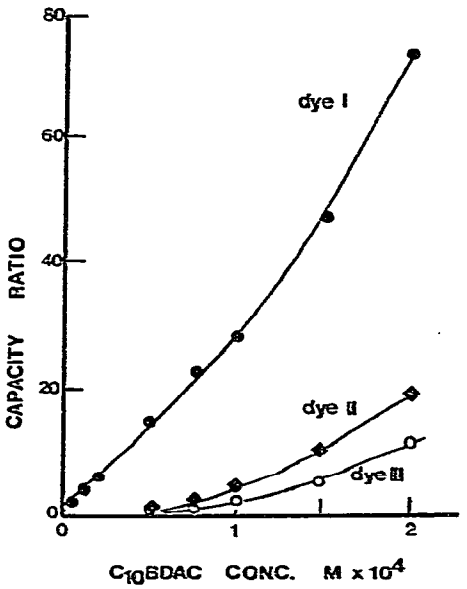


Fig. 4. Chromatograms showing the effect of different mobile phase concentrations of decylbenzyldimethylammonium chloride on sodium cromoglycate retention.

Peak	*C_{10}BDAC concentration (M)*	*SCG capacity ratio*
A	$0.9 \cdot 10^{-4}$	1.4
B	$1.8 \cdot 10^{-4}$	4.1
C	$3.0 \cdot 10^{-4}$	8.8
D	$4.5 \cdot 10^{-4}$	13.9
E	$5.0 \cdot 10^{-4}$	15.1

$3 \cdot 40^{-4}$ *M*) to a C_{18}/methanol–water (4:1) system causes the sodium cromoglycate peaks to be well removed from the solvent peak and leads to an increase in SCG capacity ratio from 0 to 15.1. Figs. 5–7 show how the capacity ratios (κ) alter with a change in both ABDAC concentration and alkyl chain length. The mean standard error for all κ values was $\pm$ 0.3.



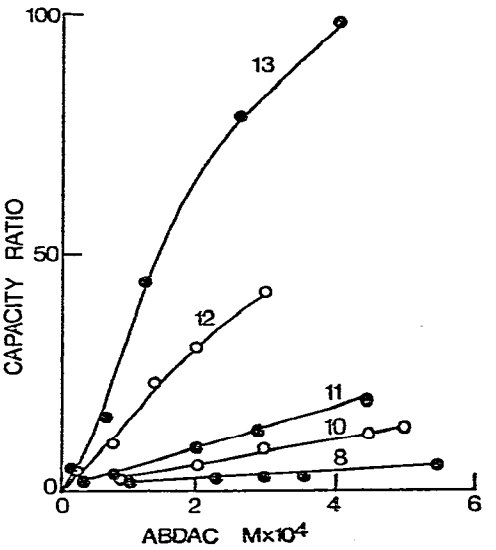


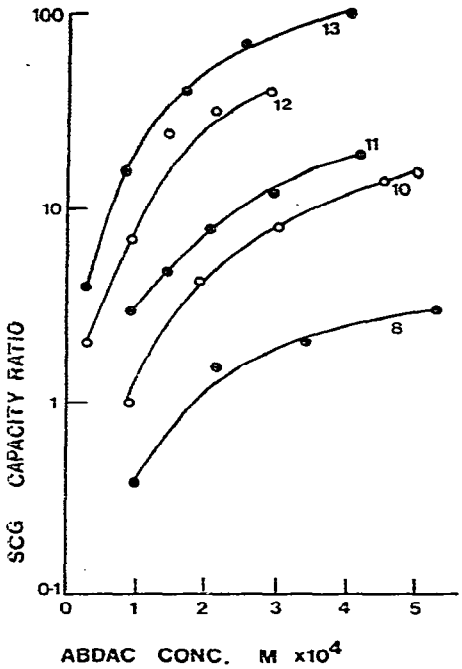
Fig. 5. Relationship between decylbenzyldimethylammonium chloride mobile phase concentration and acid red dye capacity ratios.

Fig. 6. Relationship between SCG capacity ratios and ABDAC mobile phase concentrations for a number of ABDAC homologues. The homologue number is given next to each data line.

A number of equilibria of various types would appear to be involved in the retention of ionizable solutes in ion-pair HPLC systems. Where the surface-active agent (A^+) concentration is changing a number of the more important physicochemical processes occurring may be shown by the following scheme, assuming that the solute molecules (B^-) do not adsorb on to the stationary support, *viz.*:

$$\begin{array}{ccccc} A_m^+ & + & B_m^- & \underset{K_{-2}}{\overset{K_2}{\rightleftharpoons}} & (A,B)_m \\ \Big\uparrow\Big\downarrow K_1 \quad K_{-1} & & & & K_{-4} \quad K_4\Big\uparrow\Big\downarrow \\ A_s^+ & + & B_m^- & \underset{K_{-3}}{\overset{K_3}{\rightleftharpoons}} & (A,B)_s \end{array}$$

where the subscripts m and s refer to the stationary and mobile phase, respectively. In this scheme ion-pair formation at the stationary support–mobile phase interface (K_3) assumes that the diffuse layer concentration of B^- around adsorbed A^+ molecules is the same as the bulk phase.



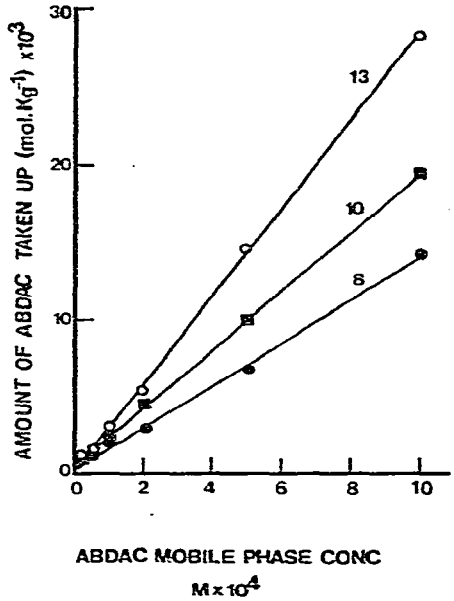


Fig. 7. Relationship between the logarithms of SCG capacity ratios and ABDAC mobile phase concentrations for a number of ABDAC homologues. The ABDAC homologue number is indicated next to each data line.

Fig. 8. Relationship between amount of ABDAC taken up by the stationary support *versus* ABDAC mobile phase concentration. The ABDAC homologue number is indicated next to each data line.

Uptake of pairing ion by the stationary support

The leveling-off effects shown in the SCG capacity ratio *versus* ABDAC concentration plots at high chain lengths (Figs. 6 and 7) are similar to those found with other surfactant ion-pair systems, and have been explained by Knox and co-workers[2,3] and others[8] as being due to a saturation of the stationary support–mobile phase interface. According to the above scheme this implies that the adsorption isotherm for ABDAC on to the stationary support should be curvilinear. Fig. 8 shows, however, that up to a mobile phase ABDAC concentration of $1.0 \cdot 10^{-3}$ *M* (which is in excess of the ABDAC concentrations used in Figs. 5–7) the amount of ABDAC retained per unit weight of stationary support material *in situ* is directly proportional to the ABDAC mobile phase concentration, with the longer chain length ABDAC being absorbed the greatest (suggesting that hydrophobic interactions are responsible for adsorption). The isotherms shown in Fig. 8 are dissimilar to those reported by Knox and Laird[2] for the uptake of cetrimide by SAS silica (Wolfson Unit reversed-phase material) as a function of cetrimide eluent concentration. In their study, using between 0 and 2% (w/v) of cetrimide, curvilinear adsorption isotherms could be described which obeyed a simple Freundlich expression. The isotherms in this study were obtained over low ABDAC concentration ranges, and their shape may be described as C1 (constant "partition") in the Giles *et al.* classification[15] of adsorption

behaviour. Assuming that the ABDAC molecule lies flat on the support surface, the extent of coverage of the support surface by the surfactant is between 1 and 4%, depending on the chain length.

Ion-pair partition

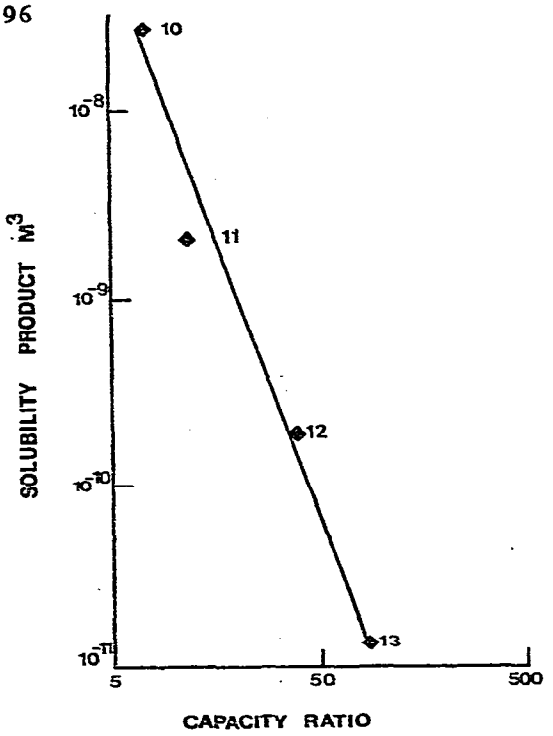
Using an automated conductimetric titration procedure, it has recently been shown that alkylbenzyldimethylammonium chlorides can form ion pairs with dianionic drug solutes in doubly distilled, deionized water[13]. The solute structural requirement for ion-pair formation in water is generally that the molecule should have some hydrophobic integrity, and this then permits hydrophobic interactions with the ABDAC molecule to overcome initial hydration shell repulsion[16].

Fig. 9 shows how the ability of SCG to complex with the ABDACs may be related to its chromatographic behaviour in ABDAC ion-pair HPLC systems, and is a plot of the logarithm of the SCG capacity ratios *versus* the logarithm of the 2:1 stoichiometric solubility constants[13] between SCG and the ABDAC homologues. Although this is a double logarithmic plot, a reasonable rectilinear relationship between the two is shown. Previously[17] it has been demonstrated that SCG–ABDAC ion pairs partition from water into chloroform. Evidence for ion pairing between the acid red dyes and the ABDAC molecules is presented in Fig. 10, where the transfer of dye II from water to chloroform is related to both the concentration and the alkyl chain length of the pairing ion. Apart from showing that the greater is the hydrophobicity of the formed dye–quaternary ammonium ion pair then the greater is the transfer to areas of lower dielectric constant, the bulk-phase experiments also show that the greater the dye charge number, the more difficult it is to transfer the formed ion pair into the chloroform phase at equivalent ABDAC concentrations (Fig. 11). This effect is similarly reflected by the relationship between capacity ratios for different dyes and ABDAC concentration (as shown in Fig. 5). The bulk-phase system is presented as a model for equilibria K_2 and K_4 (see the above scheme), with the presence of methanol in the aqueous phase increasing the tendency for equilibrium K_2 to occur.

Although ion pairs between the acid red dyes and ABDACs can form in water (and thus probably in the mobile phases used), ion pairs can also be formed between the ABDAC molecule adsorbed on to the stationary support phase and solute molecules in the pumped mobile phase. Some direct evidence we have for this is that removal of any particular ABDAC from the column may be effected by pumping through with acid red dye I in methanol–water (1:1) at a nominal concentration of $5 \cdot 10^{-4}$ *M* (see also Experimental). This causes complete removal of ABDAC from the column within a short period (*ca.* 20 min).

However, Fig. 12 shows how the capacity ratios for the monovalent dye I and the dianionic cromoglycate ion change with the carbon number of the pairing ion alkyl chain at an ABDAC concentration of $1.0 \cdot 10^{-4}$ *M*. The slope coefficients for these log-linear plots show an approximately doubling for the SCG slope compared with the dye slope.

This suggests that in the HPLC system there is a 2:1 stoichiometric interaction between the ABDACs and SCG, and a 1:1 interaction between the monovalent dye I and the quaternary ammonium ions. It is difficult to perceive that this could be occurring on the surface of the stationary support (*i.e.*, an *in situ* ion-exchange



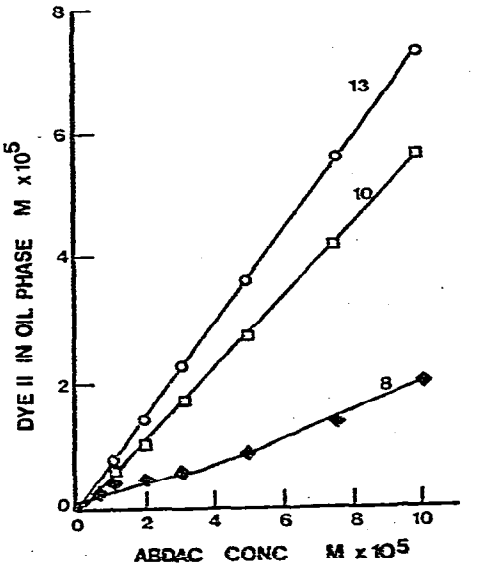
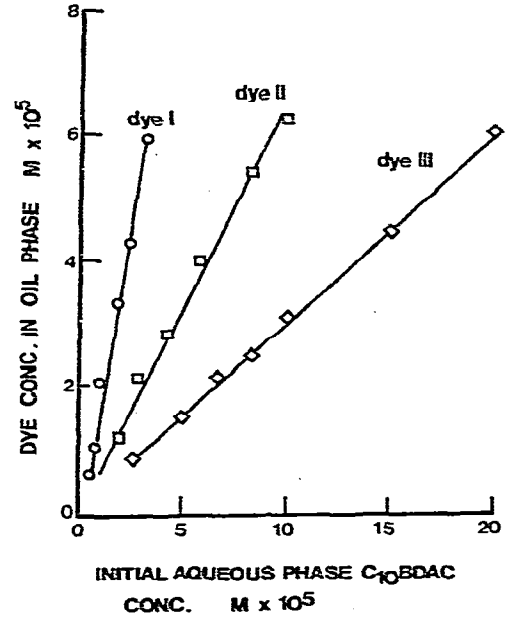


Fig. 9. Relationship between SCG capacity ratios at $3 \cdot 10^{-4}$ *M* ABDAC mobile phase concentrations and the 2:1 stoichiometric solubility products between SCG and ABDACs. The ABDAC homologue number is given next to each datum point.

Fig. 10. Extraction of acid red dye II from water into chloroform as ion pairs with different concentrations of ABDAC homologues. The ABDAC homologue number is indicated next to each data line. Initial dye concentration in the aqueous phase: $1 \cdot 10^{-4}$ *M*.



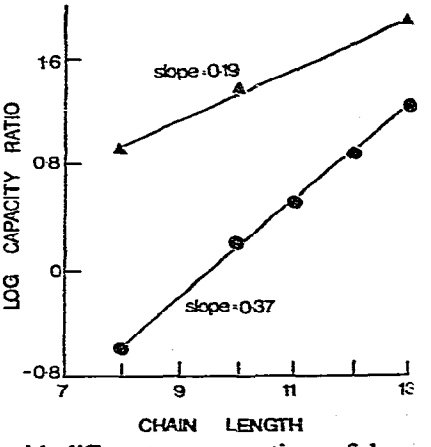


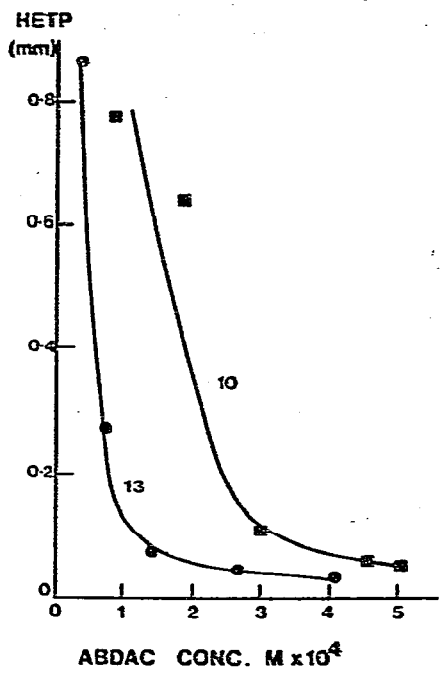
Fig. 11. Acid red dye extraction into chloroform as ion pairs with different concentrations of decyl-benzyldimethylammonium chloride. Initial dye concentration in the aqueous phase: $1 \cdot 10^{-4}$ *M*.

Fig. 12. Relationship between solute capacity ratio and ABDAC alkyl chain length (at an ABDAC concentration of $1 \cdot 10^{-4}$ *M*). ●, sodium cromoglycate; ▲, acid red dye I. Slope coefficients are given next to each data line.

mechanism), due to the stereochemistry needed for a 2:1 interaction and the total surface coverage by surfactant. By implication, the 2:1 (and 1:1) ion pairing take place in the mobile phase, followed by distribution to the stationary phase. The leveling-off in the capacity ratio *versus* ABDAC concentration profile seen for SCG is then explained by a limitation in the ability of the cromoglycate molecule to form ion pairs in water, which can be due to the size of solute sample (5 μl of $2.08 \cdot 10^{-4}$ *M*), in that for the higher ABDACs maximum ion-pair formation is reached at *ca.* $2 \cdot 10^{-4}$ *M*. The fact that the ABDAC and SCG concentrations are similar is not significant. These findings, which agree with those of Horvath *et al.*[9], make the suggestion[18] that in ion-pair HPLC the process of ion-pair "partition" to the stationary phase is insignificant somewhat hasty, especially when one considers the rather small amounts of ABDAC taken up by the stationary support and the residual ABDAC mobile phase concentrations.

Efficiency

Fig. 13 shows how the reduced plate heights for sodium cromoglycate change with both ABDAC mobile phase concentration and chain length. That is, an increase in ABDAC mobile phase concentration leads to a decrease in the value of the height equivalent to a theoretical plate (HETP). To take into account the changing capacity ratio the effective plate number (N_{eff}) has been plotted against ABDAC concentration (Fig. 14) and shows that over the concentration ranges studied there is an increase in N_{eff} with increase in surfactant concentration, with maximal N_{eff} values being achieved with the longer chain homologues.



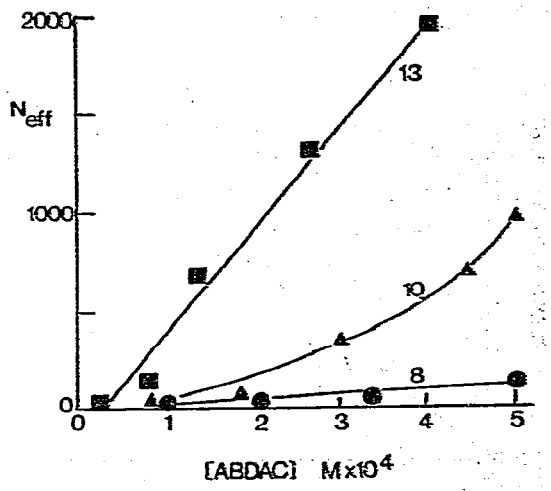


Fig. 13. Effect of ABDAC mobile phase concentration and chain length on HETP using sodium cromoglycate as sample. The ABDAC homologue number is given next to each data line.

Fig. 14. Effect of ABDAC mobile phase concentration and chain length on the effective plate number (N_{eff}) for SCG. The ABDAC homologue number is given next to each data line.

Quantitative determination

The sensitivity of our system for determining sodium cromoglycate at 254 nm ($\varepsilon = 16{,}800$) was determined using the C_{10} ABDAC homologue ($1.0 \cdot 10^{-4}$ *M*) by injecting progressively more dilute solutions of SCG (in 1:1 methanol–water) on to the column. A 5-μl injection of $2.2 \cdot 10^{-6}$ *M* solution of SCG was the minimum concentration detectable using our systems and is equivalent to a column loading of *ca.* $1.0 \cdot 10^{-11}$ mole.

For the determination of SCG, aniline hydrochloride was used as an internal standard, octylbenzyldimethylammonium chloride ($5.2 \cdot 10^{-4}$ *M*) as the counter ion and C_{18}/methanol–water (1:1) as the phase system. Aniline hydrochloride and sodium cromoglycate were well separated in this system, having capacity ratios of 1.2 and 7.9, respectively. The linearity of the detector response against SCG concentration was examined by constructing appropriate Beer's law calibration plots. A series of methanol–water (1:1) solutions containing identical amounts of aniline hydrochloride ($8.4 \cdot 10^{-4}$ *M*) and different amounts of SCG were injected into the chromatograph. Each injection was performed in duplicate. The ratios of peak heights of SCG to internal standard were then plotted against SCG concentration, as shown in Fig. 15. The average standard error for the mean peak height was $\pm$ 0.01. It is demonstrated that the ABDAC method leads to an excellent relationship between SCG concentration and peak-height ratio.

Determination in urine

The need for a sensitive and rapid assay for sodium cromoglycate in biological fluids and drug formulations has been apparent since the late 1960s. The usual methodology involves a time-consuming non-chromatographic ion-exchange resin procedure with a low sensitivity. To examine whether the ABDAC systems could be

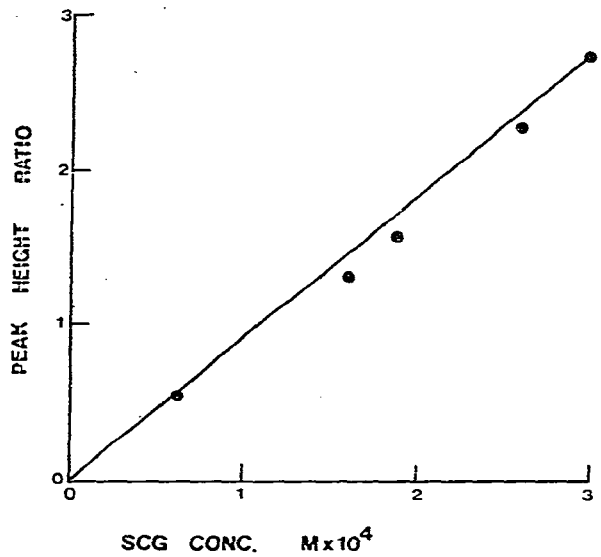


Fig. 15. Relationship between peak-height ratio of sodium cromoglycate to internal standard (aniline hydrochloride) and concentration of injected sodium cromoglycate solution.

used for the direct analysis of SCG in complex biological fluids, known amounts of SCG were added to freshly collected human urine, and the mixture was injected directly into the chromatograph. Fig. 16 shows three separate traces which combine to illustrate typical results. Trace A shows the chromatogram for the injection of neat urine (1 μl) in a C_{18}/methanol–water (1:1) phase system containing decylbenzyldimethylammonium chloride at a concentration of $3.06 \cdot 10^{-4}$ *M*. Trace B represents a similar injection but this time sodium cromoglycate ($1.0 \cdot 10^{-4}$ *M*) was added to the urine, and trace C shows the chromatogram after the injection of urine + sodium cromoglycate in a C_{18}/methanol–water (1:1) phase system (*i.e.*, in the absence of pairing ion). It can be seen from these traces that sodium cromoglycate can be well resolved in human urine without a prior extraction step.

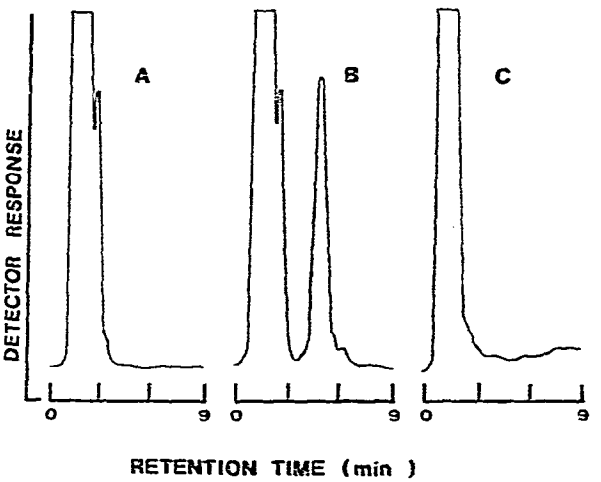


Fig. 16. Traces showing the effect of added ABDAC pairing ion on the chromotography of urine samples containing added amounts of sodium cromoglycate.

Column blockage due to injection of neat urine, as reported by Sharma *et al.*[19], did not occur with our systems even after repeated injections. Sharma *et al.* suggested that this blockage may be due to gelatinization of the urine; the pure methanol phases used in their study contrast with the aqueous methanol phases that we used and this could be the reason why our system did not exhibit such blockages.

The sensitivity of the method fell when using direct urine injections to a detection limit of $6.5 \cdot 10^{-10}$ moles column loading.

CONCLUSIONS

The addition of alkylbenzyldimethylammonium chlorides to reversed-phase HPLC systems can bring about the adequate resolution of anionic solutes. In this paper we have clearly demonstrated that both the qualitative and quantitative determination of sodium cromoglycate in both simple aqueous solutions and complex biological fluids (without prior extraction) is successfully handled by this methodology.

Using methanol–water as the mobile phase it has been found by altering either ABDAC concentration or chain length, or both, that extremely flexible phase systems can be produced. Although a surface coverage by ABDAC between 1 and 4% results when using the described conditions, we have argued that the mechanism of solute retention is primarily one of ion-pair formation in the mobile phase followed by distribution to the stationary phase.

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