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Studies on the retention behaviour of a group of organic anions of biochemical interest on quaternary bonded silica columns equilibrated with a functionally coherent series of counterions
Use of 2-(N-morpholino)ethanesulphonate as a counterion and N-tris(hydroxymethyl)methyl-3-aminopropanesulphonate as an eluent

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Abstract

The anions of a functionally coherent series of Good's buffers have been used to provide the counterions for quaternary bonded silica and the mechanisms of exchange with a test sample of organic anions of biochemical interest studied with the objective of finding strategies which might be suitable for the extraction of organic anions from biological fluids. A dual retention mechanism involving the electrical double layer and the pH related ionisation of the counterion is proposed. The MES [MES = 2-(N-morpholino)ethanesulphonic acid] anion is shown to be a useful counterion and the TAPS [TAPS = N-tris(hydroxymethyl)methyl-3-aminopropanesulphonic acid] anion a useful eluent in a series of compatible techniques which could be applicable in methods involving either solid-phase extraction or HPLC for separation purposes. An analytical protocol compatible with assay by capillary electrophoresis is described.

1. Introduction

The problems which may be encountered when attempting to apply capillary electrophoresis (CE) to the analysis of biological fluids have been summarised previously [1]. One of these problems is the high ionic strength of biological fluids relative to the ionic strengths of the buffers used in the electrophoresis. The high ionic strength of biological fluids is largely due to the presence of relatively large concentrations of strong electrolytes. When the ionic strength of the sample is higher than that of the running buffer peak spreading occurs because the electric field strength within the sample zone is less than in the running buffer and consequently electrophoretic velocities are less within the sample zone than in the running buffer. In these circumstances dilution of the sample or the use of a buffer with a higher ionic strength may be practical options but both are limited by other considerations. Dilution of the sample may compromise analytical sensitivity beyond a tolerable limit. Increasing the ionic strength of the running

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buffer will ultimately compromise the ability to maintain the capillary at a constant temperature. The ideal solution would be to isolate the analytes of interest in a solution of relatively low ionic strength thereby producing much greater flexibility in both the choice and strength of the buffer solutions available for the electrophoresis.

The technique of isolating analytes of interest from a biological fluid into a solid phase prior to subsequent chromatographic analysis was described in 1965 by Reid et al. [2,3] for the analysis of amino acids in human blood serum. A variety of commercially available kits and protocols now exist for the "solid-phase extraction" of analytes from fluids employing a range of bonded and unbonded chromatographic materials and some have found use in the extraction of analytes prior to analysis by CE or the related technique of micellar electrokinetic capillary chromatography [4–6]. Most workers have used octadecyl (C_{18})-bonded phases for the extraction of analytes which are essentially hydrophobic in character. As the majority of synthetic drugs which do not occur naturally are hydrophobic, extraction into a C_{18} phase has been particularly applicable to the analysis of drugs in biological fluids.

The principal objective of the present work was to establish techniques which might be generally applicable to the isolation of organic anions from the strong electrolyte environment in which they occur in biological fluids, to give extracts which were suitable for assay by CE. The techniques of ion-exchange chromatography appeared suitable for this purpose. The work presented here was aimed at achieving this solution for a test group of organic anions. The separation and assay of these organic anions by CE employing Good's buffers [7–9] has already been described [1]. Much of the data presented here would be equally relevant to the development of an HPLC system for the assay of some of these anions.

The solid adsorbent phase used consisted of macroporous silica particles covalently bonded to a strongly cationic quaternary amino nitrogen group giving a preparation which was a strong anion exchanger. Residual free silanol groups were largely eliminated by inert end-capping. This paper describes the results obtained when a functionally coherent series of the anions of a set of Good's buffers are used as counterions and exchange attempted with the anionic organic analytes. Good's buffers were chosen because their relatively low molar conductivities offered the hope of extracts with relatively low conductivities and because compatibility with existing methods of assay was assured [1]. The experimental observations are satisfactorily explained by an electrical double layer mechanism of the type proposed by Afrashtehfar and Cantwell [10] for styrene–divinylbenzene copolymer bonded to quaternary ammonium groups together with a counterion determined displacement mechanism. The proposed mechanism allows a number of experimentally verifiable predictions to be made which include a highly satisfactory method for the elution of retained analytes as an alternative to elution by acidification. Experimental conditions were found in which elution from the diffuse part of the electrical double layer can be distinguished from elution from the compact layer.

2. Experimental

2.1. Reagents and chemicals

Reference analyte solution

Reference preparations of creatinine, free base, anhydrous, crystalline; *trans*-urocanic acid [4-imidazoleacrylic acid; uric acid (2,6,8-trihydroxypurine)], monosodium salt; vanillylmandelic acid (VMA, *dl*-4-hydroxy-3-methoxymandelic acid); hippuric acid, (benzylaminoacetic acid), free acid; *trans*-cinnamic acid (3-phenylpropenoic acid); phenylacetic acid; orotic acid (6-carboxy-2,4-dihydroxypyrimidine), free acid, monohydrate; sodium benzoate; and sodium salicylate were obtained from Sigma, and used without further purification. Quantities of each of these analytes sufficient to give a 200 μM solution with respect to each analyte were transferred to a standard 500-ml volumetric flask

containing 200 ml water, 5 ml methanol, 2 ml 1 *M* sodium hydroxide solution and 1.0 ml 0.5 *M* disodium ethylenediaminetetraacetic acid and the mixture agitated until solution was complete. Water was added to the volumetric mark and the resulting reference solution divided into 20-ml aliquots in glass vials and stored at 4°C prior to use. Creatinine does not behave as a true analyte in the subsequent assay. It is included because it may occur in large quantities in urine and its behaviour needs to be followed. On assay by CE in the system employed here it migrates with the negative peak corresponding to the electroosmotic flow and accurate integration is impossible.

Solutions of Good's buffers

The free acid (zwitterionic) forms of 2-(N-morpholino)ethanesulphonic acid (MES), 3-(N-morpholino)-2- hydroxypropanesulphonic acid (MOPSO), 3-(N-morpholino)propanesulphonic acid (MOPS), N-(2-hydroxyethyl)piperazine-N″-(2-ethanesulphonic acid) (HEPES), N-tris(hydroxymethyl)methyl-3-aminopropanesulphonic acid (TAPS), 3-(cyclohexylamino)-2-hydroxypropanesulphonic acid (CAPSO) and the sodium salts of MES, MOPSO, MOPS, HEPES, TAPS and CAPSO were obtained from Sigma.

Buffers throughout this work were used at pH values at or close to their p*K* values with the exceptions of MES buffer at pH 7.30 and MOPS buffer at pH 6.07. These latter buffers, both at pH values approximately one unit from their respective p*K* values were made up as 50 m*M* solutions by dissolving the appropriate quantities of sodium salt and free acid in water. Stock solutions of other buffers were prepared by dissolving 50 mmol of the sodium salt and 50 mmol of the free acid in a small volume of water and making the solution volume up to 1 l. The working buffers of molarity 50 m*M* were prepared from the 100 m*M* stock solutions by the addition of an equal volume of water. The pH of a buffer was checked immediately before use at prevailing room temperature. Table 1 shows the essential parameters describing each of the buffers used in this study.

Table 1
Parameters describing the buffers used for establishing the counterion by equilibration with 1-g columns of quaternary bonded anion-exchange silica

50 m*M* Buffer	p*K*[a]	pH
MES	6.15	6.13
MOPSO	6.90	7.02
MOPS	7.15	7.23
HEPES	7.55	7.65
TAPS	8.55	8.65
CAPSO	9.60	10.1
MES[b]	6.15	7.30
MOPS[b]	7.15	6.07

[a] p*K* values are from Ref. [8].
[b] Buffers at pH values approximately one pH unit from their p*K* values used to provide data for the experiment illustrated in Fig. 4.

2.2. Adsorbent and preparation of chromatographic columns

The adsorbent used for these studies was quaternary amine bonded silica obtained from J.T. Baker UK, Hayes, UK (product No. 7043.00). The product is a strong anion exchanger and consists of irregular macroporous particles of average particle size 40 μm, average pore diameter 6 nm and with an exchange capacity of 0.7 mequiv./g and surface area of 500 m^2/g. As purchased the material is in the chloride state.

Aliquots (1 g) of the dry powdered adsorbent were poured into all glass columns (Bakerbond SPE glass columns, 8 ml capacity, 8 cm length, product No. 7328.06) containing approximately 5 ml chloroform. After removal of the residual chloroform from above the settled adsorbent, by suction from the bottom of the column, the column is washed with 2×5 ml chloroform followed by 2×5 ml methanol and finally 2×5 ml water or the appropriate buffer depending on whether the column is to be stored or used immediately. This washing protocol has been found to be essential for the successful subsequent analysis of eluates by capillary electrophoresis when monitoring in the UV region. It is of interest that the aqueous eluates which follow the chloroform and methanol washes of this

material have a pH of approximately 3! Columns prepared in this way have a void volume of 1 ml.

2.3. Equilibration with a new counterion

Columns were equilibrated and run by manual, stepwise application of 5-ml aliquots of eluent followed by manual removal of 5 ml eluate by suction from the bottom of the column at the approximate rate of 1 ml/min. Columns were equilibrated with counterion by repeated application of 5-ml aliquots of the appropriate buffer until the pH of two successive 5-ml aliquots of eluate was equal to the pH of the buffer. Before application of an analytical sample each column was washed once with 5 ml water.

When columns equilibrated with the MES^- anion came to be used frequently as analytical columns the equilibration process was greatly speeded up by employing MES buffers of strength 50 or 100 m*M* and with a pH approximately 1 unit above the p*K* of MES which is 6.15. Before application of sample the column was washed with 5 ml (5 void volumes) water.

2.4. Solid-phase extraction protocol

A common protocol was used for the chromatography of the test solution of organic anions irrespective of which buffer had provided the counterion for the quaternary bonded silica anion exchanger. The protocol was designed to be easily modified to provide a practical strategy for the solid-phase extraction of similar analytes from biological fluids. The reference analyte solution (5 ml) was applied to the top of the column and a corresponding 5 ml eluate removed from the bottom (E1). Two successive 5-ml aliquots of water were applied as eluents and the corresponding eluates collected (E2, E3). Elution was continued with four 5-ml aliquots 50 m*M* hydrochloric acid (E4–E7). Each of the successive eluent steps E1–E7 therefore comprised the equivalent of five void volumes giving a total eluent volume equivalent to 35 void volumes. In the later stages of this work 50 m*M* TAPS buffer, at a pH close to its p*K* of 8.55 replaced the hydrochloric acid as an eluent. Analysis of eluates by CE was carried out after transfer of each eluate step to a 5-ml glass sample phial and without any further treatment.

In the comparative studies in which different counterions were put in place by equilibration with the appropriate buffers hydrochloric acid was always employed in the elution stage as described above. In these latter studies each column was re-equilibrated with 50 m*M* MES buffer, pH 7.3 and re-run after loading the reference analyte solution in order to check that the ion-exchange properties of the bonded phase had not been degraded by either the equilibration or elution phases when using counterions other than MES^-.

2.5. Measurement of conductivities of buffers and eluates

These were done at a room temperature of 20°C using a Ciba-Corning portable M90 conductivity meter. All measurements were made in duplicate with frequent re-calibrations against a known commercial standard. The value for the cell constant was determined by measuring the conductance of a standard 100 m*M* solution of potassium chloride.

2.6. Analysis of eluates by CE

Eluates were analysed by CE in 50 m*M* MES buffer, pH 6.15 (or close to 6.15) at a temperature of 25°C with 25 kV applied across a capillary of 57 cm × 75 μm I.D. using Beckman P/ACE 2100 instrumentation as described previously [1].

3. Results and discussion

3.1. Quantitative and theoretical considerations

The buffering reaction for Good's buffers, using MES as an example is:

$$H^+ + MES^- = MES_{zwitterion}$$

and the pH in such a system is given by:

$$pH = pK + \log[MES^-/MES_{zwitterion}]$$

with the pK defined as the pH when the concentration of MES anion equals that of MES zwitterion.

The studies reported here employed 1-g columns of the quaternary bonded silica anion exchanger.These have an exchange capacity of 700 μmol/g for monovalent anions. The 50 mM buffers with pH values close to their pK values contain 125 μmol counter anion per 5-ml aliquot and therefore approximately six 5-ml aliquots of buffer contain sufficient anion to provide a complete counterion replacement on the 1-g columns. This is true in practice and after equilibration with six successive aliquots of buffer the eluate pH is equal to the pH of the eluent buffer.

The reference analyte solution is 200 μM with respect to each of ten analytes. Analytes are applied to the anion-exchange columns in a single 5-ml aliquot of the reference analyte solution which therefore contains 1 μmol each analyte per 5 ml or a total sample load 10 μmol anionic analyte (assuming monovalency which is approximately true). This is 1.43% of the total exchange capacity of 700 μmol and appears compatible with the trace conditions for both ion exchange and surface adsorption as defined by Afrashtehfar and Cantwell [10]. The anion-exchange activity of the quaternary bonded silica used in the present studies is assumed to occur at the surface of the bonded silica including the surface of the pore channels. There is no exchange within the material of the silica particles as occurs within the gel matrices of some ion-exchange resins.

3.2. The variation in the sorption and retention of analyte anions with the nature of the counterion at the bonded silica surface

The effect of the different counterions on the elution patterns of the reference analyte solution when the bonded silica columns are equilibrated, loaded and run as described in the experimental section is illustrated in Figs. 1 and 2. Recoveries refer to the summed recoveries of the analytes for each elution step (E1–E7). The data in Figs. 1 and 2 shows that as the pK of the buffer

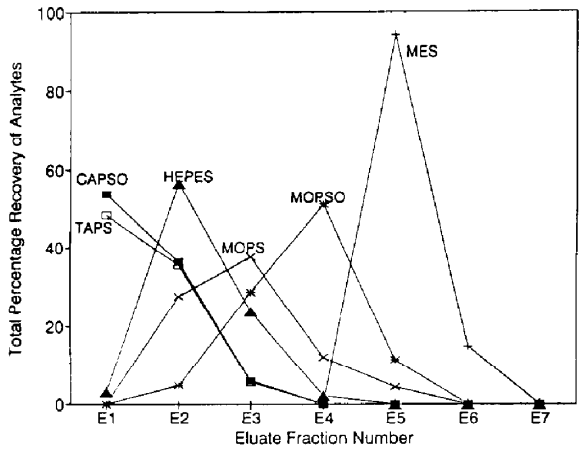

Fig. 1. Variation in the elution of the reference analytes from 1-g bonded silica anion exchange columns equilibrated with counterions from six different Good's buffers. The parameters describing the 50 mM buffers are given in Table I. Recoveries refer to the summed recoveries of analytes taken as a group in each eluate fraction. Seven 5-ml eluent steps E1–E7 were applied to each column giving seven 5-ml aliquots of eluent E1–E7. Key to eluents: E1 = 5 ml reference analyte solution (sample); E2, E3 = 5 ml water; E4–E7 = 5 ml 50 mM hydrochloric acid.

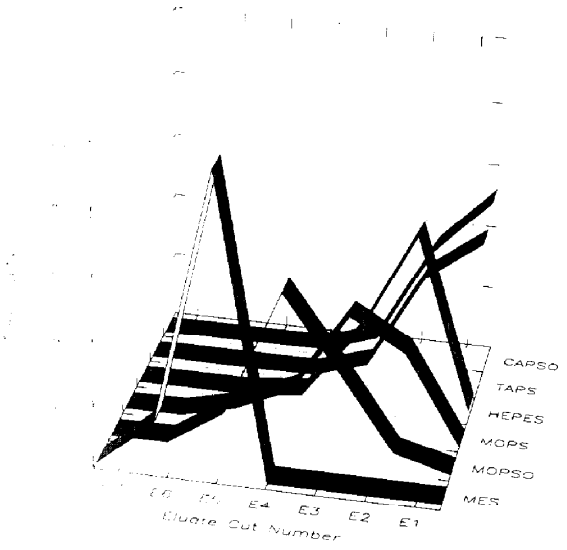

Fig. 2. The same data as in Fig. 1 presented as a pseudo-three-dimensional drawing emphasising the nature of the counterion as a determinant of the elution pattern. Note that to improve visibility E1–E7 are in reverse order. The counterions on the z-axis are arranged in order of increasing pK starting with MES. Elution details as in Fig. 1.

providing the counterion varies progressively from that of CAPSO at 9.6 to that of MES at 6.15 the retention of analytes by the bonded silica column increases. In all cases the total recovery of analytes by the elution procedure is approximately 100%. In the case of columns equilibrated with the MES counterion release of retained analytes does not occur until E5 after the column has been eluted with 50 m*M* hydrochloric acid at E4. By contrast, for columns equilibrated with the CAPSO counterion the elution of analytes is complete before the application of acid at E4. The counterions of TAPS, HEPES, MOPS and MOPSO, with progressively decreasing p*K* values, produce a continuum of elution patterns showing increasing retention of analytes between the limits exhibited by CAPSO and MES.

In Fig. 3 the pH profiles of eluate fractions E1–E7 corresponding to the six different counterions are shown. The eluate fractions E1–E3, corresponding to the loading and wash phases of the chromatographic protocol have pH values approximately equal to that of the buffer which provided the counterion while the post-acidification eluates E5–E7 have pH values of approximately 2. It is well known [11] that bonded silicas can behave unpredictably below an approximate pH of 3 when acidic degradation of the bonded phase occurs, and above an approximate pH of 8 when the alkaline dissolution of silica occurs. It was therefore important to be certain that the elution profiles illustrated in Figs. 1 and 2 were not, at least in part, pH-related artifacts. Each column was equilibrated with MES counterion after completion of its elution with 50 m*M* hydrochloric acid, re-loaded and re-run and the analyte elution pattern characteristic of the MES counterion obtained. This was considered to constitute adequate proof that under these experimental conditions the ion-exchange properties of the bonded phase remained essentially intact. This was a particularly relevant finding for the buffers TAPS and CAPSO with pH values over 8. No column in the above experiments was run more than twice as described, the second run always being to confirm the patency of the bonded phase. It appears that over periods of less than a few hours quaternary bonded silica can withstand exposure to solutions with pH values less than 3 or greater than 8 as determined by the exchange of trace amounts of analyte. It has been the subjective impression in the present studies that exposure to acidic conditions, for tens of hours, is more damaging to the bonded phase than similar exposure to relatively mild alkaline buffers.

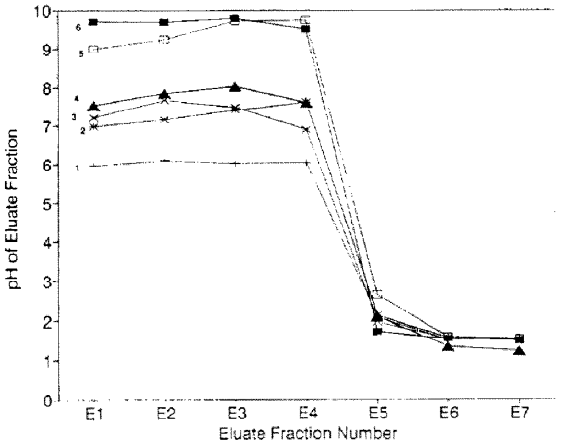

Fig. 3. The pH profiles of the eluate fractions from the columns whose analyte elution patterns are shown in Figs. 1 and 2. Key: 1 = 1 g quaternary bonded silica with MES counterion; 2 = MOPSO counterion; 3 = MOPS counterion; 4 = HEPES counterion; 5 = TAPS counterion; 6 = CAPSO counterion. Elution details as in Fig. 1.

It may appear obvious that at least for monovalent buffers (like Good's buffers) where there is only a single anionic species and a single p*K*, the elution pattern of exchanged analytes should be independent of the pH of the buffer which provides the counterion at the bonded silica surface, provided that the counterion can exist at that pH. The retention patterns of organic analytes illustrated in Figs. 1 and 2 show clearly that the retention pattern of analytes for the series of counterions used in this study appears to be determined by the pH (or p*K*) of the buffers which provide the counterion. Because of its relevance to subsequent mechanistic considerations it was important to determine the variation in retention pattern with pH when the counterion was fixed. This was done by the experiment illustrated in Fig. 4 in which the elution patterns

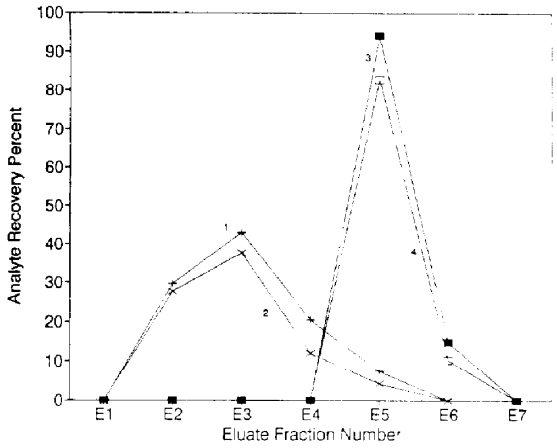

Fig. 4. Showing that when the counterion is fixed the elution pattern of the reference analytes is determined by the nature of the counterion and is, within limits, independent of the pH of the buffer used in placing the counterion. Curves: 1 = column equilibrated with 50 m*M* MOPS buffer, pH 6.07; 2 = column equilibrated with 50 m*M* MOPS buffer, pH 7.23; 3 = column equilibrated with 50 m*M* MES buffer, pH 7.30; 4 = column equilibrated with 50 m*M* MES buffer, pH 6.13. Eluents as in Fig. 1.

produced by equilibration of the quaternary bonded silica columns by MES and MOPS buffers, each at two different pH values, are compared. The data in Fig. 4 show that the elution pattern of the reference analytes is the same for MES anion irrespective of whether the counterion was achieved by equilibration with MES buffer at pH 6.13 (cf. a p*K* of 6.15) or at pH 7.30 (cf. a p*K* for MOPS of 7.15). Similarly, the elution pattern of the reference analytes was characteristic of the MOPS anion irrespective of whether the equilibrating buffer was at pH 6.07 (close to the p*K* of MES) or 7.23 (close to its own p*K*). These results suggest that for any given counterion the elution pattern of exchanged analytes is, at least over a limited pH range around the p*K*, independent of the pH of the buffer used to provide the counterion.

The fact that 50 m*M* MES buffer, pH 6.13 provides columns with elution patterns identical to 50 m*M* MES buffer, pH 7.30 has a useful analytical significance. The buffer with pH 7.30 has approximately ten times the anion concentration of the pH 6.13 buffer and will therefore bring a column to an equilibrated state much more rapidly than the buffer with the lower pH.

3.3. A hypothetical mechanism

The theoretical basis for the retention mechanism of organic anions on a low-capacity ion-exchange adsorbent was reported in 1982 by Afrashtehfar and Cantwell [10] using *p*-nitrobenzenesulphonate as a sample anion and a surface-quaternised macroporous styrene–divinylbenzene as the ion-exchange adsorbent ("QXAD"). These authors employed 1 m*M* aqueous ammonia containing concentrations of sodium chloride up to 1 *M* as mobile phases and studied the variation in the capacity factor for the anionic sample (k'_{s^-}) with the ionic strength of sodium chloride in the mobile phase. Afrashtehfar and Cantwell proposed a dual retention mechanism for the sorption of counterions by the exchange material in terms of the Stern–Gouy–Chapman model of the electrical double layer. The experimental results reported here are satisfactorily explained by this theory with minor modifications and in consequence the nomenclature used by these authors will be adopted. According to this theory sorption by the surface bonded anion-exchange material is determined by adsorption of counterion (including sample) by the charged surface and secondly by ion exchange of the sample ions for other counterions in the diffuse part of the electrical double layer. The compact region of the double layer according to these authors extends from the charged surface with its adsorbed counterions, up to but not including, the outer Helmholtz plane which is the start of the diffuse layer. Adsorption of counterion at the surface is dependent on the electrical potential at the surface while ion exchange between the bulk fluid and the diffuse layer is independent of the electrical potential at the surface. The authors emphasise that the structure of a sample molecule largely determines the relative extent of its partition between the compact and diffuse layers.

The results illustrated in Figs. 1 and 2 can be explained in terms of these concepts when the hydrogen ion concentrations of solutions of

Good's buffers in the region of their pK values are considered. All buffers for the experiment depicted in Figs. 1 and 2 were 25 mM with respect to the buffer anion and 25 mM with respect to the zwitterion (free acid) giving in each case a buffer with a pH close to or equal to the pK of the buffer. The hydrogen ion concentrations in the different buffer solutions varies widely however from 1000 nM at pH 6 (approximately that of MES) to 0.1 nM at pH 10 (approximately that of CAPSO). These pH values are only strictly applicable to the bulk buffer. All columns were equilibrated before use by exposure to approximately 30 column void volumes of each buffer until the eluate pH equalled the eluent pH. In the immediate vicinity of the adsorbed layer consisting of buffer anions, the adjacent diffuse layer will be enriched with buffer anions relative to the bulk buffer. There will also be a relative exclusion of hydrogen ions from this region (Donnan exclusion). These two effects would be expected to produce a pH in the region of the outer Helmholtz plane greater than that in the bulk buffer. It may be hypothesised that buffer anions are only displaced from the adsorbed layer after first accepting a proton thereby becoming zwitterions. Only then does a charged site become available for the adsorption of sample anions or indeed any anions. These arguments suggest that the rate at which charged sites become available for the re-adsorption of other anions is kinetically first order with respect to the hydrogen ion concentration. This means that an adsorption layer initially saturated with $CAPSO^-$ anions provides sites free for re-occupation by other anions at a rate approximately one thousandth of that for an adsorption layer initially saturated with MES^- anions. In both cases exchange occurs between analyte anions in the sample and buffer anions in the diffuse layer and in the case of MES equilibrated columns the analytes in the diffuse layer can enter and be retained by the adsorbed layer.

It seems that analytes retained in the adsorbed layer are not released by elution with 50 mM hydrochloric acid until the eluate is rendered acidic (at E5, cf. Figs. 1 and 2 with Fig. 3 for MES-equilibrated columns). There is no evidence for release by exchange with chloride ions in E4 when elution with hydrochloric acid is started. Acceptance of a proton may also be a pre-condition for the release of the reference organic anions from the adsorbed layer. It may be noted that all the analytes in the test solution have at least one pK value below the value of 6 and will be either ionised or strongly ionised under normal equilibrated column conditions.

The behaviour of the trace sample load of the reference analytes in bonded quaternised silica anion-exchange columns with the six counterions can now be explained. With CAPSO and TAPS equilibrated columns retention of analytes in the compact (adsorbed) layer is negligible but retention by ion exchange in the diffuse layer is evident (Fig. 1 and 2). At the lower limit of the pK range columns equilibrated with MES anion as the counterion exhibit strong retention of analytes predominantly in the adsorbed layer, from which they are released when the eluate pH approaches a value of 2. Columns equilibrated with the anions of HEPES, MOPS and MOPSO show progressively increasing retention of organic analytes with elution maxima at E2, E3 and E4 respectively. It seems reasonable to assume that the increased availability of hydrogen ions with decreasing pH in the vicinity of the compact layer is producing an increased proportion of sites in the adsorbed layer for occupancy by analyte ions.

The standard elution protocol in these studies employed water washes at E2 and E3. The purpose was twofold: firstly, to keep the chemistry simple and secondly to investigate the effect of washing on the retention of analytes prior to their deliberate elution at E4. The recoveries of analytes from the CAPSO and TAPS equilibrated columns are approximately 50, 35 and 5% in eluate cuts E1, E2 and E3, respectively, indicating weak retention in the diffuse layer in columns with void volumes of 1 ml. It is valid to ask what happens to the charged surfaces' requirement for counterion when some of that counterion is "leached" out by wash steps. The answer must be that more countercharge moves into the compact or adsorbed layer and more countercharge in the diffuse layer redistributes

closer to the compact layer. The diffuse layer becomes thinner according to the inverse square root of the ionic concentration in the bulk fluid as required by Debye–Huckel theory.

3.4. The use of TAPS buffer as an eluent

Inspection of the elution profiles in Figs. 1 and 2 and the matching eluate pH data in Fig. 3 show that columns with a MES counterion have qualities which are very suitable as the basis for a "solid-phase extraction" protocol for organic anions of the type present in the reference analyte solution. Columns equilibrated with the MES counterion can be rapidly prepared by elution with 50 or 100 m*M* buffer at a pH approximately 1 unit above the p*K* of 6.15. The organic analytes are quantitatively retained in the adsorbed layer, remain on the column after washing with 10 void volumes of water and can be rapidly recovered after acidification with 50 m*M* hydrochloric acid. This last step is unsatisfactory for several reasons. The acidic conditions limit the durability of the quaternary phase bonded to the silica surface. Secondly, if subsequent assay is to be by CE the presence of the chloride ion confers a relatively high conductivity on the sample. Thirdly, many biochemical substances might be labile in these acidic conditions.

The theoretical treatment outlined previously suggests that the anions of Good's buffers with a p*K* higher than that of MES should be successful as eluents for the removal of analytes. TAPS buffer (50 m*M*, pH 8.54) was tested and found to be much superior to acidification for this purpose. Apart from the avoidance of acidic eluates, elution with 50 m*M* TAPS at E4 results in the immediate elution of analytes at E4 with only a residual 10–20% eluting at E5. Table 2 shows in detail the recoveries of individual analytes at each of the steps in the elution procedure after analysis by CE. No data are available for creatinine which migrates with the electroosmotic flow. The negative solvent peak of the latter partly obscures the creatinine peak prohibiting its quantitation. The average recovery of the other analytes is approximately

Table 2
Recoveries of individual analytes from eluate fractions E1–E7 when sample is loaded onto 1-g quaternary bonded silica column equilibrated with MES counterion using 100 m*M* MES buffer, pH 7.2 followed by one 5-ml water wash and column run as described below

Analyte	Recovery (%)							
	E1	E2	E3	E4	E5	E6	E7	Total
Creatinine	–	–	–	–	–	–	–	–
Urocanic acid	0	0	0	73.7	15.9	0	0	89.6
Uric acid	0	0	0	89.1	8.4	0	0	97.5
VMA	0	0	0	75.9	28.3	0	0	104.2
Hippuric acid	0	0	0	87.5	15.5	0	0	103
Cinnamic acid	0	0	0	80.8	18.3	0	0	99.1
Phenylacetic acid	0	0	0	89.2	10.4	0	0	99.6
Orotic acid	0	0	0	93.8	7.1	0	0	100.9
Benzoic acid	0	0	0	82.7	15.3	0	0	98
Salicylic acid	0	0	0	79.2	15.0	0	0	94.2
Average total	0	0	0	83.5	14.9	0	0	98.4

Analytes loaded onto anion-exchange column in 5 ml reference solution containing 1 μmol of each analyte at E1. Elution continued with 2 × 5 ml aliquots water (E2, E3), 4 × 5 ml aliquots 50 m*M* TAPS buffer, pH 8.54 (E4–E7). Approximately 5 nl (5-s high-pressure injection) of each eluate analysed by CE at 25 kV in 50 m*M* MES buffer, pH 5.99 at 25°C with UV detection at 214 nm, capillary length 57 cm × 75 μm I.D. Conductivity and pH data for the eluates of the anion-exchange column and buffers involved in this experiment are given in Table 3.

98.5%. There are three recoveries, for VMA, hippuric and orotic acids which have values slightly over 100% and are easily accounted for by a computer integration strategy that was not entirely adequate.

3.5. Conductivity considerations in relation to analysis by CE

In 1991 Vinther and Soeberg [12] described a set of guidelines for determining the optimum relative conductivities of the sample and running buffer in CE. Their main recommendations were that the conductivity of the running buffer should be "low" to minimise Joule heating and that "moderate stacking" conditions should be employed in which the conductivity of the sample solution should be only slightly lower than that of the buffer solution. Good's buffers [7–9] were specifically designed to achieve buffering with the minimum of concurrent ionic strength and conductivity and were for that reason employed in both the present and previous studies [1] with the express intention of using CE as the final method of analysis. In the techniques described above analyte anions displace MES^- counterions on a quaternary bonded silica surface and both analyte anions and MES^- counterions are subsequently eluted by a TAPS buffer solution at a pH close to its pK of 8.55 in which half the TAPS molecules are anionic. It is to be expected that in the absence of a more complicated exchange mechanism $TAPS^-$ anions would displace MES^- anions on a one-for-one basis and in consequence the ionic strength of the eluates should not exceed that of the eluent TAPS buffer (the contribution from eluting analytes being minute in the present case). As the specific purpose of the techniques described in this work was to obtain conditions which closely resembled those suggested by Vinther and Soeberg [12] it was considered necessary to prove by appropriate conductivity measurements that this had in fact been achieved (prompted by referee).

Conductance (reciprocal resistance in units of μS) data together with other column and buffer parameters are summarised in Table 3 corresponding to the experiment described in Table 2 for 50 mM TAPS as the eluent buffer. The data in Table 3 show several interesting features. The first eluate (E1) has a conductance which obviously reflects the loss of cations from the 5-ml reference analyte solution with which the anion-exchange column was loaded. This analyte reference solution has a conductance of 645 μS. The next two eluates E2 and E3 have conductances that reflect the two 5-ml water washes which precede elution with TAPS buffer. The conductances and pH values of eluates E4–E7 show that

Table 3
Conductivity, pH and CE parameters for the eluates of the quaternary bonded silica anion-exchange column for which analyte recoveries were given in Table 2

Parameter	Eluate No.						
	E1	E2	E3	E4	E5	E6	E7
pH of eluate	6.23	5.95	5.62	7.78	8.49	8.54	8.53
Conductance (μS)	504	327	116	1036	1460	1479	1526
Recovery (%) of analyte after CE in 50 mM MES	0	0	0	83.5	14.9	0	0
Capillary current (μA) at 25 kV	34.0	34.0	34..0	34.2	34.2	34.2	34.2

Eluent was 50 mM TAPS, pH 8.54 from E4 to E7. Conductivities may be obtained in SI units (S m^{-1}) by multiplying conductances by the conductivity cell constant which is 83 in these studies. Conductance of 50 mM TAPS, pH 8.54 was 1553 μS. Conductance of the CE buffer, 50 mM MES, pH 5.99 was 1540 μS. Conductance of 100 mM MES buffer, pH 7.2, used for establishing the MES anion as the counteranion was 4510 μS.

the conductance and pH of the eluent TAPS buffer (1553 μS and pH 8.54, respectively) are not approached until the last eluate E7. The analytes are eluted sharply at E4–E5 when conductances have values relative to the CE buffer (50 m*M* MES, pH 5.99, conductance 1534 μS) which are as close to those required by the rules of Vinther and Soeberg as could reasonably be expected. The properties of MES buffer with respect to the CE of the analytes in this study has been fully described previously [1].

The data in Table 4 show the effect of changing the eluent buffer to 25 m*M* TAPS, pH 8.43. The conductance of the eluates is what might be predicted from the data for 50 m*M* TAPS and a lower molarity of MES buffer could be used for the CE which would be speeded up in consequence. There appears to be a delayed appearance of analytes in the eluates and this might not be considered a desirable feature.

It may be noted that the capillary currents during the CE of any of the eluates described in Tables 3 and 4 show little variation with conductance of the analysed eluate although these vary from 89 up to 1526 μS.

3.6. Experimental demonstration of retention in diffuse and compact layers

It is now generally accepted that when ion-exchange processes occur at surfaces as distinct from within microporous gel beds the mechanism of retention must be associated with the presence of the electrical double layer at the surface [10,11,13–15]. The theory of Afrashtehfar and Cantwell [10] requires two distinctly different retention modes when a sample anion is retained by a surface quaternised material: adsorption in a compact layer and ion exchange in a diffuse layer. The exact details of the behaviour of a sample molecule in this system depends critically on the nature of the sample and the nature of the electrolytes in the mobile phase [10]. The above authors did not demonstrate experimentally the separate elution of analyte or other ions from compact or diffuse layers, but showed that the theoretical total analyte capacity factor agreed with experiment and was equal to the sum of theoretically derived contributions from the compact and diffuse layers.

It was hypothesised that if retention of analyte or other counterion does occur by way of a dual mechanism, it should be possible to arrange conditions such that dual elution from the two retention sites could be demonstrated experimentally. The method chosen was to apply the sample analytes in a solution containing a relatively high concentration of other anions which would compete for available sites in the compact and diffuse layers. Columns equilibrated with the MES anion, in which the retention of analytes appears to be predominantly

Table 4
Analyte recovery, pH and conductivity data when the 1-g quaternary bonded silica anion-exchange column is eluted with 25 m*M* TAPS buffer, pH 8.43 at E4–E7 followed by analysis using CE

Parameter	Eluate No.						
	E1	E2	E3	E4	E5	E6	E7
pH of eluate	5.96	5.61	5.28	6.64	8.05	8.31	8.31
Conductance (μS)	406	198	89.2	579	807	886	882
Recovery (%) of analyte after CE in 50 m*M* MES	0	0	0	26.7	65.3	5.9	0
Capillary current (μA) at 25 kV	34.2	34.2	33.6	33.8	33.9	33.7	33.8

Column equilibrated, loaded and apart from the molarity and pH of the TAPS buffer, eluted and the eluates analysed as described in Tables 2 and 3. Conductivities may be obtained in SI units (S m^{-1}) by multiplying conductances by the conductivity cell constant which is 83 in these studies. Conductance of 25 m*M* TAPS, pH 8.43 was 851 μS.

by adsorption into the compact layer and in which competition with other ions might force retention of a proportion of sample anions in the diffuse layer, were chosen for this experiment. Fig. 5 shows the results of this experiment when the reference analytes are applied to the column in solution with 150 m*M* sodium chloride. The expected bimodal elution pattern corresponding to the bimodal retention mechanism is evident with analytes leaching out of the diffuse layer at E1–E3 with desorption from the compact layer in E4–E5 after elution with 50 m*M* TAPS is commenced at E4. Elution at E4 and onwards by 50 m*M* TAPS (pH 8.65) means that the sample analytes would be fully ionised throughout the entire elution protocol. Attempts to reduce the thickness of the double layer by washing the prepared column with 5 ml methanol [16] immediately prior to loading with sample and thereby varying the bimodal elution pattern gave an elution pattern not conclusively different from the untreated column (Fig. 5). It may be noted

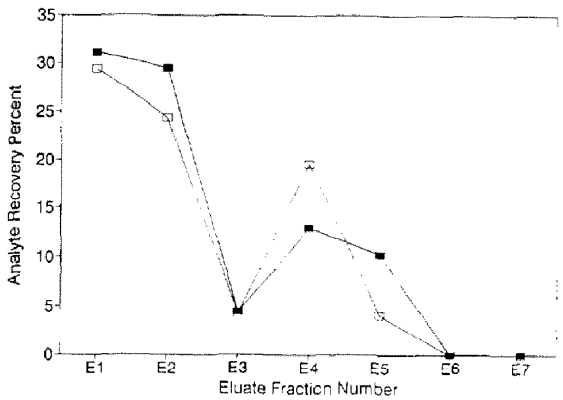

Fig. 5. Illustrating differential elution from the compact and diffuse regions of the electrical double layer when the reference analytes are applied to the 1-g bonded silica columns in solution with 150 m*M* sodium chloride. Columns equilibrated with MES counterion. Key: ■ = normal column preparation and elution procedure as described in text and in caption to Fig. 1 except that 50 m*M* TAPS, pH 8.6 was the eluent at E4 and onwards; □ = column washed once with 5 ml methanol before application of sample at E1 then eluted as described for Fig. 1 until E4 when elution continued with 50 m*M* TAPS, pH 8.6.

that in these experiments it required a 75-fold excess of chloride ions to partially displace the analyte anions from the adsorption layer. The organic analytes appear to have a considerable competitive advantage in occupying sites in the compact layer.

3.7. Analytical implications

The experimental protocol described above in which MES-equilibrated columns are loaded with sample, washed with water and eluted with 50 m*M* TAPS buffer provides extracts suitable for analysis by CE when tested against an artificial reference sample of organic anions. The transcript of the recorder tracing of the electropherogram obtained from the analysis by CE of the eluate E4 corresponding to the experiment described by Tables 2 and 3 for elution by 50 m*M* TAPS, pH 8.54 is illustrated in Fig. 6. The essentials of this protocol are summarised in Fig. 7 in diagrammatic form. In accordance with the mechanism described above the buffers HEPES, MOPS and MOPSO would be expected to be effective as eluents although with progressively decreasing efficiency when compared with TAPS. If direct assay by HPLC were intended the buffers with the lower p*K* values would appear to be good candidates as eluents. Future work will be aimed at adapting the protocol to the analysis of biological fluids such as urine and blood. There are many substances in both these fluids which are structurally similar to the reference analytes chosen for the present work and there appears to be no reason why these techniques should not be applicable. The present work deliberately employed worst-scenario large sample volumes of 5 ml and relatively small amounts of exchange material. These proportions could be varied significantly together with other parameters in a protocol suitable for fluids such as urine or blood in which the challenge from strong electrolytes is both considerable and variable.

Despite their many admirable properties Good's buffers are not often the choice of many

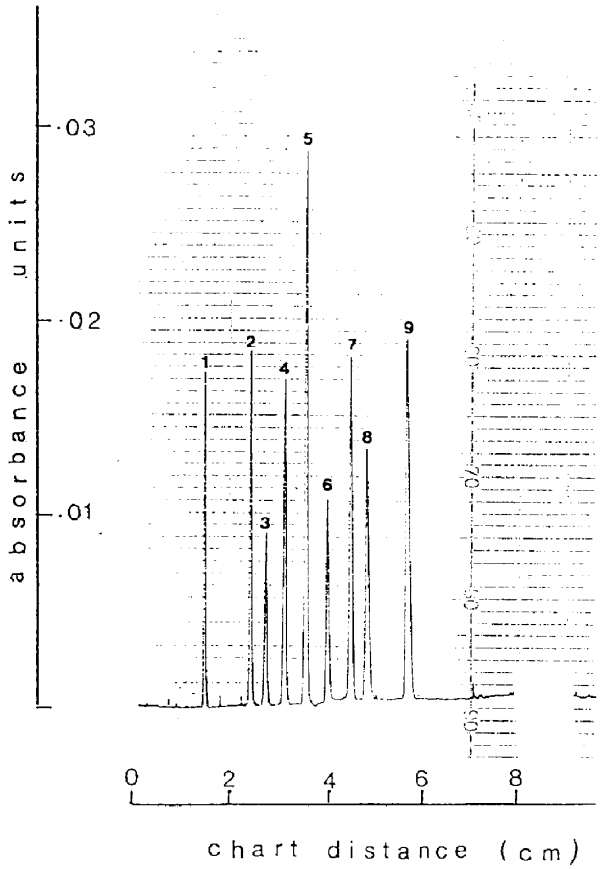

Fig. 6. The electropherogram obtained by the CE of eluate 4 (E4) of the experiment described in Tables 2 and 3 for elution of the anion exchange column with 50 m*M* TAPS buffer, pH 8.54. Peaks (retention times in minutes in brackets): 1 = urocanic acid (4.11); 2 = uric acid (4.55); 3 = VMA (4.72); 4 = hippuric acid (4.91); 5 = cinnamic acid (5.12); 6 = phenylacetic acid (5.35); 7 = orotic acid (5.59); 8 = benzoic acid (5.76); 9 = salicylic acid (6.17). Conditions for CE: capillary 57 cm × 75 μm, UV detection at 214 nm, high-pressure sampling for 5 s, running buffer 50 m*M* MES, pH 5.99, temperature 25°C, voltage 25 kV with 10-s ramp up time. Recorder chart speed 2 cm/min.

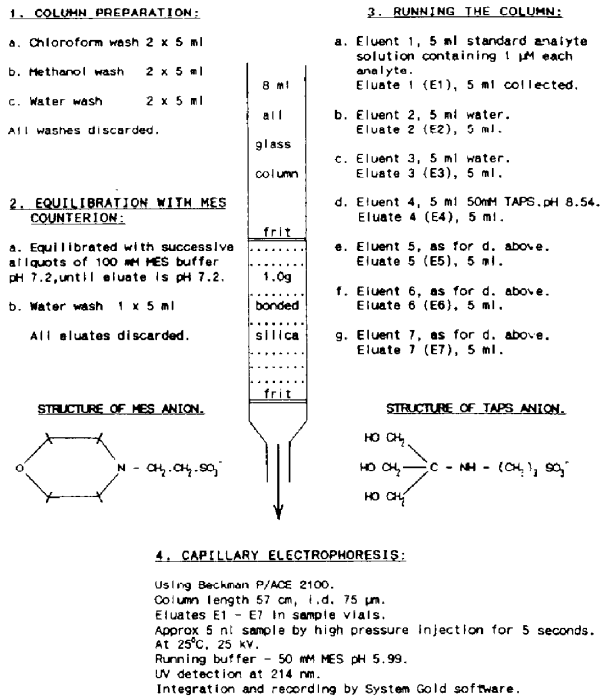

Fig. 7. Diagrammatic summary of the techniques described in the text when combined to give a method for the analysis of the organic anions present in a test solution at concentration 200 m*M*.

analysts for work in the pH range 6–10 and it is difficult to understand why this should be. In contrast a variety of other sulphonates such as hexyl-, heptyl- or octyl-sulphonate are widely used as anionic "pairing agents" in ion-pair chromatography [17]. Walton and Rocklin [18] in discussing eluents suitable for anion exchange, make no mention of the anions of Good's buffers as eluents.

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