Journal of Chromatography, 107 (1975) 25–33

CHROM. 8030

ION-EXCLUSION CHROMATOGRAPHY OF COMPOUNDS OF BIOLOGICAL INTEREST

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(First received August 12th, 1974; revised manuscript received October 18th, 1974)

SUMMARY

The principle of ion exclusion was examined as a method for the separation of small ionic compounds. The systems employed consisted of very porous column packings, substituted with fixed charges, which were eluted by buffer solutions of low ionic strength. DEAE-Sephadex A-50 was principally employed, and it was shown that there was a linear relationship between the net charge on a cation and its partition coefficient into the gel phase. A similar relationship existed in the chromatography of amino acids on various columns bearing fixed negative charges. It was concluded that this was an efficient form of chromatography, which gave results directly related to the ionic charge of the sample being examined. The charge characteristics of biologically active compounds could be determined by this method.

INTRODUCTION

The term "ion exclusion" has been used[1,2] to describe the separation of ionic from non-ionic solutes by passage over a column of ion-exchange resin. This was a class separation since resolution of the ionic components was not attempted. These were eluted from the column as a group, whereas the non-ionic solutes were eluted later and could be fractionated by virtue of their differential absorption by the resin. In these laboratories, interest in the phenomenon of ion exclusion arose from the observation that some anionic proteins were excluded from agarose gels[3] or porous glass particles[4] at low ionic strength due to repulsion by the negative charges on the solid phase. The elution position of a protein in these systems was governed by both ion exclusion and molecular size factors. However, if the factor of molecular size is eliminated by employing very porous, charged media for the chromatography of small ions of the same sign, then the variables controlling the elution should be purely ionic in nature. This should lead to a system of chromatography in which the results are readily interpretable in terms of the ionic properties of the solutes. Therefore the behaviour of small ionic drugs and natural metabolites on columns of the highly porous ion exchange materials Sephadex A-50 and C-50 has been examined. Other ion-exchange materials of a less porous synthetic resin type were also studied.

The method depends on the exclusion of ions from the pores of a matrix which bears fixed charges of the same sign, a principle well known for ion-exchange resins[5]. The greater the charge density on the ion, the less of the matrix is penetrated and hence the earlier the ion is eluted from the column. Low ionic strength of the eluting buffer is necessary, otherwise the repulsive effect of the fixed ions is partly shielded by the counter ions of the buffer. Results are presented which show that this mechanism can be realized in practice, leading to a useful method of separating ionic substances, and furthermore of gaining fundamental knowledge of the ionic properties of an unknown solute.

EXPERIMENTAL

Materials

The following compounds were synthesized at these laboratories: Toxogonin [bis(4-hydroxyiminomethylpyridinium-1-methyl) ether dichloride], TMB-4 [1,1'-trimethylene-bis(4-hydroxyiminomethylpyridinium bromide)] and the N-diethylmethyl analogue of edrophonium. Edrophonium (N-ethyldimethyl-3-hydroxyanilinium chloride), its N-triethyl analogue, paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride), and 2-PAM (2-hydroxyiminomethyl-1-methylpyridinium chloride) were obtained commercially and used without further purification. The 3- and 4-isomers of PAM were prepared by quaternization of the corresponding pyridine aldoximes by methyl iodide. Tetrodotoxin (TTX) was a product of Sankyo Co. Ltd. (Tokyo, Japan) and saxitoxin (STX) a gift from Dr. E. J. Schantz (Fort Detrick, Md., U.S.A.). The structures of some of the quaternary nitrogen drugs appear in an earlier publication[6].

Chromatographic columns and packings

Glass columns of 2.12 cm^2 cross-sectional area were used, packed to a height of approximately 22 cm. Weighed amounts of DEAE-Sephadex A-50 and CM-Sephadex C-50 (products of Pharmacia, Uppsala, Sweden) were allowed to swell for 18 h in the appropriate buffer solution, then packed into the columns under a pressure of 20–30 cm of water. The dry weight of material forming the gel of the packed bed was recorded. Controlled-pore glass particles, CPG-10 (from Corning Glass Works, New York, N.Y., U.S.A.), were degassed as described by the manufacturer and packed into the columns as above. The packed columns were eluted with the appropriate buffer until the effluent solution was of the same pH as the applied solution. The buffers employed were 5 m*M* Tris–HCl at pH 8.0 for cationic materials and 10 m*M* sodium tetraborate titrated to pH 9.0 with HCl for the anionic systems. Tris buffers of constant ionic strength but varied pH were prepared by the admixture of varied proportions of two solutions, one containing 5 m*M* Tris base with 5 m*M* NaCl, the second containing 5 m*M* Tris–HCl (pH between 3 and 4). The Amberlite ion-exchange resins and other materials with a high charge density on the matrix could not be brought to pH equilibrium using the dilute buffers described above. These materials were treated by successive suspension in changes of buffer of five times the operating strength until the supernatant pH had approached the required value. The resins were then washed in buffer of the working strength and packed into the columns. Other buffer systems are described at the appropriate point in the text.

Column operation

Chromatography was performed at 20° ± 1°, at a flow-rate of 32 ml/h. The sample volume was 0.5 ml and contained 1 μmole of each substance to be examined. Stock solutions in distilled water (1×10^{-2} *M*) were diluted to 0.5 ml with the buffer solution to be used on the column; not more than two substances were chromatographed at a time. Pyridinium and anilinium compounds and catecholamines were detected by passage of the effluent through a Gilson Medical Electronics UV-monitor fitted with a 280-nm filter. Other compounds were detected by analysis of 2.2-ml fractions collected in test tubes. Amino acids were assayed by the ninhydrin method[7], Blue Dextran and DNP-ethanolamine by measurement of extinction at 625 and 358 nm, respectively. Methanol in borate buffer was detected by the dichromate–sulphuric acid method described by Fischer[8]. The toxins STX and TTX were semi-quantitatively assayed by intraperitoneal injection of volumes between 0.2 and 1.0 ml into mice of approximately 20 g body weight. The smallest lethal volume of an extract was found and rated as one mouse unit.

The following symbols were used for the volumes used to characterize the elution position of a sample: V_0 = void volume of the column, or the elution volume of a totally excluded solute (see Results and discussion), V_t = total water space of column, *i.e.* elution volume of a neutral, low-molecular-weight sample, V_b = total volume of the column bed, and V_e = elution volume of the sample under examination. The distribution of the sample into the space within the gel pores was then calculated as $K_d = (V_e - V_0)/(V_t - V_0)$. In many cases the ratio V_e/V_b was used to compare the elution volumes of samples, with compensation for small changes in the column bed volume.

RESULTS AND DISCUSSION

General properties of the chromatographic systems

Applied samples were recovered from the chromatographic columns in good yield as moderately broad peaks. The recovery of amino acids from CM-Sephadex averaged 97%; this material was contained in a total of 11–14 ml, although over 50% was in less than 5 ml. A mean recovery of 80% was obtained for six samples of TTX chromatographed on DEAE-Sephadex. It was noticed that the trailing edge of the sample peak was generally sharper than the leading edge when the eluting buffer was Tris–HCl. The flow-rate of buffer through the column did not affect the elution position of samples when varied over a wide range of rates. The elution volume of 4-PAM varied from 30.7–30.8 ml when the flow-rate was changed through four values from 3.53–50.5 ml/h (1.66–23.8 ml/h/cm^2). These data also serve to show the excellent reproducibility of elution volumes for separate chromatographic runs.

Relationship between elution volume and net charge for chromatography on cationic dextran gels

Various cationic solutes of biochemical interest were chromatographed on DEAE-Sephadex A-50 columns, and the elution positions of the samples were compared with their ionic charges, either as directly determined or as reported in the literature, at the pH of 8.0 (Table I). Repacking of the column with fresh gel caused slight changes in the elution of a given sample, as seen for 2-PAM in Table I. The

TABLE I

RELATIONSHIP BETWEEN ELUTION POSITION AND NET POSITIVE CHARGE ON SAMPLES CHROMATOGRAPHED ON DEAE-SEPHADEX

Samples were chromatographed on DEAE-Sephadex A-50 at pH 8.0 as described under Experimental. Total column bed volume varied between 48.2 and 48.6 ml. After the first four results had been obtained, the column was repacked with fresh gel to obtain the subsequent results. Data on the ionization of adrenaline analogues were obtained from Barlow[9] on TTX and STX from refs. 10 and 11; the other substances were examined by spectrophotometry. Values of V_e/V_b are means of duplicate determinations.

Sample	V_e/V_b	*Net positive charge*
Paraquat	0.45	2.00
TMB-4	0.59	1.28
Toxogonin	0.69	0.98
2-PAM	0.795	0.49
2-PAM	0.76	0.49
4-PAM	0.65	0.74
3-PAM	0.555	0.92
TTX	0.595	0.86
STX	0.45	1.64
Edrophonium	0.76	0.56
N-Diethylmethyl analogue	0.73	0.575
N-Triethyl analogue	0.72	0.59
Noradrenaline	0.63	0.715
Adrenaline	0.605	0.72
Dopamine	0.57	0.77

results show that the elution position of the sample is closely related to its net charge. This is clearly seen for the positional isomers of PAM, which could quite readily be separated in this chromatographic system. Even where the differences in ionization are not marked, as for the catecholamine group, there are slight but consistent changes in elution. The column method was at least as sensitive as spectrophotometric titration in revealing differences in ionization between edrophonium and its analogues. The direction of change is as expected, that is an increase in positive charge results in earlier elution from the gel. If these results are plotted as V_e/V_b against net charge, there is no simple relationship between the points, beyond the general trend. This scatter may be due to changes in the column matrix (repacking with fresh gel could alter the fixed charge density) or differences in size or mobility of the various types of ions examined.

A second series of experiments was performed with 2-PAM, TMB-4 and paraquat, in which the pH of the eluting buffer was varied from 6.89–9.14 (ionic strength held constant) and resulting changes in the elution volumes of the samples were noted. Paraquat showed a slight increase in V_e/V_b (0.345–0.46) with increasing pH, although the ionization of the molecule could not change in this pH range. The effect was probably due to decreased dissociation of the diethylamino groups of the matrix at higher pH. The elution volume of paraquat at each pH value was taken to represent the effective void volume of the column (V_0), and the total bed volume was assumed to be equal to the total water space (V_t), since the solid phase was relatively small (about 1.5% w/v). These parameters enabled the distribution coefficients (K_d) of 2-PAM and TMB-4 at the various pH values to be calculated. When K_d was plotted

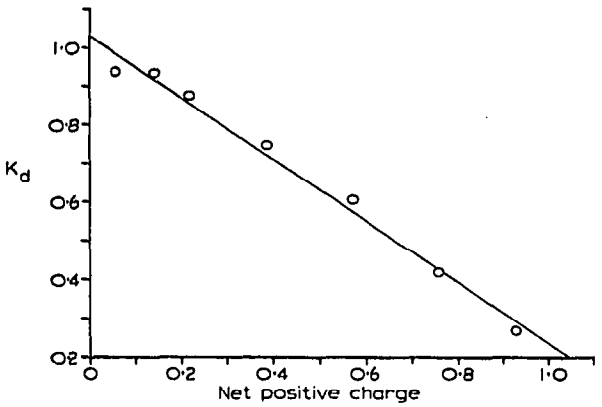

Fig. 1. The relationship between the distribution coefficient K_d and the net charge on 2-PAM when eluted from DEAE-Sephadex in Tris buffer at different pH values. The line was calculated to be the best fit by the method of least squares.

against net charge on the ions, good straight-line fits (by the least squares method) to the experimental points were obtained for both 2-PAM and TMB-4, as shown in Fig. 1 for the former. The slope of the line for 2-PAM was −0.799, the y intercept was 1.037 and the variance was 0.00125 ($n = 7$). For TMB-4 the corresponding figures were: slope −0.547, y intercept 0.913, variance 0.0005 ($n = 6$). The values for the y intercepts differ slightly from the theoretical value of 1.000 for neutral molecules, possibly due to errors in calculating net charges for which pK values become critically important. The slopes for the two compounds are different, showing that a factor other than net charge is involved.

A set of experiments at varied pH values was then performed in which the samples were paraquat, 2-PAM and the pufferfish toxin TTX. A series of values of K_d and net positive charge on the solutes could then be calculated as before, employing a pK_a value of 8.76 for TTX[10]. The results (Fig. 2) indicate that TTX is partitioned into the gel interior in a similar manner to 2-PAM. It is evident that the approximate

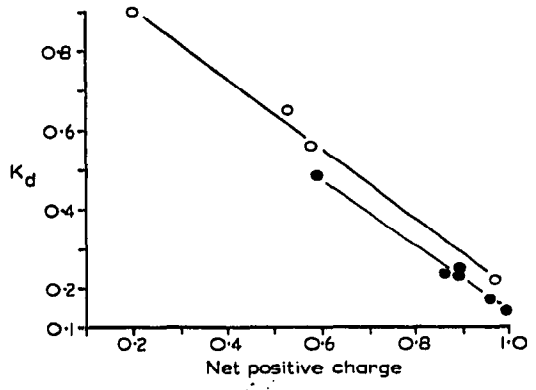

Fig. 2. A comparison between TTX (●) and 2-PAM (○) on elution from DEAE-Sephadex at different pH values. The pK of TTX (8.76) was obtained from the literature[10], that of 2-PAM (8.0) was found by spectrophotometry.

TABLE II

EFFECT OF IONIC STRENGTH ON THE ELUTION OF PYRIDINIUM COMPOUNDS FROM DEAE-SEPHADEX A-50

Buffer (pH = 8.0)	*I*	V_e/V_b			*Column packing (% w/v dry gel)*
		Paraquat	*TMB-4*	*2-PAM*	
5 m*M* Tris	0.0031	0.45	0.59	0.795	1.4
10 m*M* Tris	0.0062	0.43	0.59	0.79	1.7
25.8 m*M* Tris	0.0162	0.525	0.67	0.86	2.35
10 m*M* Tris, 10 m*M* NaCl	0.0162	0.56	0.69	0.87	2.1
10 m*M* Tris, 50 m*M* NaCl	0.0562	0.73	0.83	0.91	3.3
10 m*M* Tris, 150 m*M* NaCl	0.1562	0.83	0.90	0.94	4.4

p*K* of a group on an unknown toxin or other biologically active molecule could be estimated by plotting a graph of K_d *versus* pH. The resulting line would be in the form of a titration curve with an inflexion at the p*K* value. The main disadvantage would be that the method is tedious since the column has to be repacked when the pH of the buffer is changed. The results for the slope (—0.889) of the line and *y* intercept (1.089) for 2-PAM agree reasonably with those previously cited. Corresponding values for TTX were —0.846 and 0.982.

Effects of increasing ionic strength on chromatography in DEAE-Sephadex columns

The swelling of the Sephadex A-50 is markedly dependent on the ionic strength (*I*) of the medium, as demonstrated in Table II. For this reason, the column had to be repacked with fresh gel for each set of observations, making the examination of this effect tedious and the results subject to variation. However, it can be seen that the degree of exclusion of the three compounds tested decreased as the ionic strength was raised. These results, when plotted as V_e/V_b against log *I*, fitted straight lines reasonably well (except for the 5 m*M* Tris value). The lines when extrapolated to high *I* intersected the $V_e/V_b = 1.0$ line at $I = 0.35$ to 0.55, indicating that all exclusion effects would cease at this ionic strength. In practice, useful results would not be obtained above $I = 0.05$. The hyperbolic relationship found previously between *I* and elution volume for agarose gels[3] did not hold here, because the charge density of the gel varied consequent upon swelling changes in the present case. The manner in which the ionic strength was increased did not seem to affect the result, as can be seen for $I = 0.0162$, a value attained either by Tris alone or Tris and NaCl.

Comparison of the two grades of DEAE-Sephadex

A column of DEAE-Sephadex A-25 was prepared and several substances were chromatographed in 5 m*M* Tris on this material to compare the elution positions with those found from the A-50 grade. Paraquat, TMB-4 and 2-PAM were eluted at V_e/V_b ratios of 0.40, 0.50 and 0.61, respectively, as sharper peaks than observed when eluted from the A-50 gel. The diethylmethyl and triethyl analogues of edrophonium were eluted at 0.63 and 0.61, in the same order as from A-50 (Table I). The main difference between the gels was the charge density per unit volume of column. Thus for the more open-structured gel the charge density was approximately 5 mequiv./100 ml, whereas for the less highly swollen A-25 the figure was 50 mequiv./100 ml,

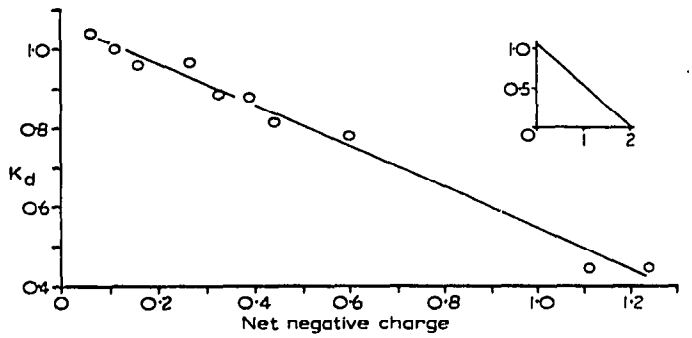

Fig. 3. The relationship between K_d and the net negative charge on a series of amino acids when eluted from a CM-Sephadex C-50 column at pH 9.0 in borate buffer. The small inset diagram shows the same graph with complete axes and the calculated line extended beyond the experimental points. The amino acids used were (in order from left to right): β-alanine, α-alanine, leucine, tryptophan, methionine, serine, threonine, asparagine, and aspartic and glutamic acids.

employing the manufacturer's figure of 3.5 ± 0.5 mequiv./g dry gel in each case. The higher charge density had two practical consequences, firstly that it was much more difficult to establish pH equilibrium when setting up the A-25 gel, and secondly from this gel the samples were eluted closer to one another as sharper peaks. In terms of separation efficiency, the two grades of gel seemed equivalent as far as this limited assessment could establish.

Anion-exclusion chromatography on dextran gels

Amino acids were chromatographed on the anionic gel CM-Sephadex C-50 at pH 9.0, and the elution volumes noted. K_d values were then calculated, using the elution position of Blue Dextran 2000 as a marker for V_0, and N-dinitrophenylethanolamine to indicate V_t. The graph of K_d *versus* net negative charge (calculated from data in literature[12]) for ten amino acids (Fig. 3) showed a straight-line relationship, with the slope equal to −0.524, a y intercept of 1.068, and a variance of 0.0005 (n = 10). The similarity to the results for the cation-exclusion system is evident. Steric considerations suggested that Blue Dextran was a true marker of V_0, and the elution volume of DNP-ethanolamine (44.5 ml for a bed volume of 45.5 ml) was consistent with the absence of interaction with the gel.

Anion-exclusion chromatography on controlled-pore glass particles

The same chromatographic system as was used for the CM-Sephadex work was then employed on columns packed with CPG-10 controlled-pore glass of pore diameter 8.9 nm. K_d values (calculated from Blue Dextran and DNP-ethanolamine volumes) for the ten amino acids were again plotted against net negative charge, and a very similar line to that for the CM-Sephadex experiment was obtained. The slope of the line was −0.612, the y intercept 1.122, and the variance was 0.0103 (n = 10). The increased scatter of the results compared with the previous experiment was due to the more unfavourable parameters of the system, as the solid phase occupied a much greater proportion of the column volume. A column of total bed volume 42.5 ml contained 9.3 ml of the solid phase, which might have allowed adsorption of

the amino acids in different degrees and thus caused variation in the results. The elution of the marker DNP-ethanolamine certainly was influenced by adsorption. Calculation of the internal pore volume of this column using the value given by the manufacturer (0.54 ml/g) resulted in a figure of 13.1 ml, whereas when measured using DNP-ethanolamine as a marker the figure was 17.65 ml. Use of methanol as a marker gave a value for the pore volume of 13.45 ml, showing that this material was not adsorbing to the glass. In the case of amino acids the elution position was probably determined both by ion-exclusion and by adsorption factors.

The column packing was then changed to a controlled-pore glass of wider pore diameter (43.7 nm). The solutes were eluted from this material as broad, distorted peaks, and no useful results could be obtained, as many amino acids were eluted together near the total bed volume of the column.

Exclusion chromatography employing other column packings

Anion-exclusion chromatography of glutamic acid and alanine on unsubstituted dextran gels (Sephadex G-10, G-25 and G-50) was tried using the techniques employed for the controlled-pore glass columns. No useful separation occurred, because of the very low charge density on the gels, and the tendency of the substances to adsorb on to the gel in the low ionic strength medium used. Similarly, an irregular relationship was found between K_d and the net charge of amino acids when chromatographed on Amberlite CG-50, a cross-linked polymethacrylic acid resin. No better results were obtained when 2-PAM and paraquat were chromatographed by cation-exclusion techniques on Amberlite IRA-400 or CG-4B resins. It was concluded that these resins were not suitable for ion-exclusion work as the more highly charged samples were totally excluded without resolution, whereas the samples bearing little charge were adsorbed to the solid phase.

CONCLUSIONS

It has been shown that the concept of ion exclusion can be extended to the fractionation of closely related small ions. The mechanism given in the introduction is sufficient to describe the effect qualitatively, but a quantitative analysis of the exclusion principle would require a study of the Donnan equilibrium between the inside and the outside of the gel particles.

The method can be used to separate biological compounds of low molecular weight on the basis of their ionic character. Examples of classes of compounds that could be separated are cholinergic and adrenergic drugs, amino acids, nucleotides, carboxylic acids and mixtures of oligomers of acidic or basic compounds. The separation of pyridinium oximes was good enough for practical use in preparative or analytical work. Determination of the charge characteristics of biologically active compounds of unknown structure, such as natural toxins, is possible by this method, and it is now being applied to the octopus toxin, maculotoxin, which is related to tetrodotoxin.

The main disadvantages of the method stem from the necessity of working at low ionic strength. Under these conditions adsorption of an ion on to a contaminating polyelectrolyte is possible, and also contaminants of opposite charge will adsorb to

the gel, masking its ionic groups. For these reasons the ion-exclusion chromatography should best be attempted after an initial clean-up of sample extracts.

The ideal gel for the column should have mechanical strength, a porous open structure and a moderate degree of ionic substitution —5 mequiv./100 ml of gel seems adequate. It is necessary to use as little solid phase as possible in the column to reduce adsorptive effects.

ACKNOWLEDGEMENTS

Dr. M. W. Jarvis and Mr. D. Amos synthesized some of the compounds used in this work. Mr. E. M. Smith is thanked for valuable technical assistance.

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