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CHROMATOGRAPHIC BEHAVIOUR OF AMINOPHOSPHONIC ACIDS ON LAYERS OF ANION AND CATION EXCHANGERS

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SUMMARY

The chromatographic behaviour of 16 aminophosphonic acids on anion and cation exchangers with polystyrene-, paraffin- and cellulose-based matrices has been investigated. The eluents used were buffer solutions, mineral and organic acid solutions and water–organic solvent mixtures. Interesting results were achieved with two successive developments in the same eluent. On polystyrene-based anion exchangers, an unusual pH gradient forms, which determines the chromatographic behaviour of the aminophosphonic acids at changing buffer concentrations.

INTRODUCTION

Ion-exchange chromatography has been widely employed for the isolation of aminophosphonic acids from biological systems[1–5] and to separate 2-aminoethylphosphonic acid (ciliatine) from phosphorylethanolamine[6,7]. With this technique, it has been possible to isolate 2-aminoethylphosphonic acid[1–3], its N-methyl, N,N-dimethyl and N,N,N-trimethyl derivatives[5], and 2-amino-3-phosphonopropionic acid (PAL)[4].

Corresponding work on the separation of natural and synthetic aminophosphonic acids, however, has not followed the wide application of ion exchangers to natural compounds. There appear to be only two papers concerning the use of cation exchangers on columns[8] and thin layers[9].

It was thought useful, therefore, to extend the investigation of the behaviour of aminophosphonic acids on thin layers by continuing our studies on cation exchangers and extending them to anion exchangers with polystyrene- and cellulose-based matrices.

EXPERIMENTAL

Dowex 50-X4 (H^+), AG 1-X4 in the acetate and propionate forms (AcO^-, PrO^-), Rexyn 102 (H^+) and alginic acid[10] thin layers (thickness 300 μm) were prepared by mixing 2 g of the exchanger with 6 g of microcrystalline cellulose in 40 ml of water. Thin layers of DEAE-cellulose (Cellex D) were prepared from 6 g of the exchanger

and 40 ml of water. A 0.2% solution of ninhydrin in ethanol, with 10% collidine for cation exchangers and 10% glacial acetic acid for anion exchangers, was used as developer. The usual loading of aminophosphonic acids was between 0.5 and 1 μg, with the exception of 2-aminoethylphosphonic acid (2 μg). The chromatographic measurements were carried out at 25° and the migration distance was 11 cm unless otherwise stated.

RESULTS AND DISCUSSION

Anion exchangers

In order to obtain a complete picture of the chromatographic behaviour of the aminophosphonic acids on anion exchangers, we employed both polystyrene (AG 1-X4) and cellulose (Cellex D) based anion exchangers.

Cellex D. On this exchanger, the behaviour of the aminophosphonic acids is not very interesting from an analytical standpoint, because with the eluents used (salt and buffer solutions) elongated spots are obtained and the differences in the R_F values are negligible for most compounds.

AG 1-X4. This exchanger was used in the acetate and propionate forms in order to increase its affinity towards the aminophosphonic acids, as these two organic anions are less retained by the exchanger than are inorganic anions with the same charge.

In Table I are reported the R_F values of the aminophosphonic acids on AG 1-

TABLE I

R_F VALUES OF AMINOPHOSPHONIC ACIDS AND OF THE CORRESPONDING AMINO ACIDS* ON AG 1-X4 (AcO^-) THIN LAYERS WHEN ELUTING WITH ACETATE BUFFER

Substance	*Acetate buffer concentration (M)*				
	0.01	*0.025*	*0.05*	*0.1*	*0.25*
1,4-Diaminophosphonic acid	0.96	0.96	0.96	0.96	0.96
1,3-Diaminopropylphosphonic acid	0.96	0.96	0.96	0.96	0.96
1,2-Diaminoethylphosphonic acid	0.96	0.96	0.96	0.96	0.96
3-Aminopropylphosphonic acid	0.86	0.93	0.93	0.96	0.96
2-Aminoethylphosphonic acid	0.61	0.76	0.81	0.86	0.93
1-Amino-2-(4-hydroxyphenyl)ethylphosphonic acid	0.12	0.23	0.33	0.46	0.53
1-Amino-2-phenylethylphosphonic acid	0.19	0.34	0.46	0.56	0.63
1-Aminomethylphosphonic acid	0.30	0.49	0.54	0.65	0.81
1-Aminoethylphosphonic acid	0.39	0.56	0.58	0.69	0.84
1-Aminopropylphosphonic acid	0.42	0.57	0.59	0.71	0.85
1-Aminobutylphosphonic acid	0.42	0.57	0.59	0.71	0.85
1-Aminopentylphosphonic acid	0.42	0.57	0.58	0.69	0.84
1-Amino-2-methylpropylphosphonic acid	0.43	0.59	0.60	0.74	0.87
1-Amino-1-methylethylphosphonic acid	0.44	0.60	0.61	0.74	0.87
2-Amino-4-phosphonobutyric acid	0.03	0.09	0.18	0.36	0.59
2-Amino-3-phosphonopropionic acid	0.00	0.04	0.09	0.25	0.52
Tyrosine	0.55	0.55	0.56	0.60	0.62
Phenylalanine	0.75	0.75	0.75	0.75	0.76
Glutamic acid	0.06	0.15	0.25	0.43	0.63
Aspartic acid	0.05	0.12	0.22	0.39	0.61

* The amino acids not reported migrate with the solvent front.

X4 (AcO^-) layers, eluting with acetate buffer solutions with concentrations between 0.01 and 0.25 *M*. The compounds with two amino groups run with the solvent front, independent of the buffer concentration in the eluent. Of the aminophosphonic acids with one amino group, those with an aromatic ring in the side-chain are retained more than those with an aliphatic side-chain. Of the latter compounds, the retention depends on the position of the amino group and the retention sequence is 1-amino > 2-amino > 3-amino. As expected, the two compounds with two acidic groups in the molecule show the greatest retention.

It should be noted that the influence of the number of carbon atoms in the aliphatic side-chain on the chromatographic behaviour of the 1-aminophosphonic acids is negligible because, with the exception of 1-aminomethylphosphonic acid, the acids have similar R_F values. Such behaviour is different from that observed for the same acids on cation exchangers on both columns[8] and thin layers[9] but is similar to that observed for the corresponding amino acids on polystyrene-based anion-exchange papers[11]. This behaviour is correlated with the greater influence of the ionic interactions compared with the non-ionic interactions between the side-chain of the compound and the matrix of the exchanger. The importance of the ion-exchange process was attributed by Knight[11] in the case of amino acids to the larger differences between the pK_2 values than those between the pK_1 values. This assumption was not supported by the experimental results, as the pK_2 values of glycine, valine and leucine differ less than the corresponding pK_1 values. In our opinion, the greater influence of the ion-exchange process is probably due to the decrease in the non-ionic interactions between the side-chain of the amino acids and the matrix of the exchanger as a result of the partial or total protonation of the amino group depending on the pH of the eluent. Owing to the close analogy between amino acids and aminophosphonic acids, we consider that the behaviour of the latter compounds may be explained on the basis of this same assumption. The similar behaviour observed on anion exchangers in the case of aromatic amino acids[12] supports such a hypothesis.

The behaviour of aminophosphonic acids on AG 1-X4 (AcO^-) may be used from an analytical standpoint. Of the separations that can be predicted on the basis of the R_F values, we effected the following: 2-aminoethylphosphonic, 3-aminopropylphosphonic and 1,2-diaminoethylphosphonic acids (0.01 *M* acetate buffer); 2-amino-2-(4-hydroxyphenyl)ethylphosphonic and 1-amino-2-phenylethylphosphonic acids (0.05 *M* acetate buffer); and 2-amino-4-phosphonobutyric and 2-amino-3-phosphonopropionic acids (0.1 *M* acetate buffer). From an analytical standpoint, the use of the exchanger in the propionate form is particularly interesting. On AG 1-X4 (PrO^-) the sequence of the affinities of the aminophosphonic acids is similar to that on AG 1-X4 (AcO^-) but, in some instances, separations not possible on AG 1-X4 (AcO^-) can be achieved. For instance, consider the separation of eight aminophosphonic acids reported in Fig. 1, obtained on AG 1-X4 (PrO^-) with two successive developments in 0.01 *M* propionate buffer. Such a separation can not be effected on AG 1-X4 (AcO^-) with 0.01 *M* acetate buffer as eluent because 2-amino-4-phosphonobutyric and 2-amino-3-phosphonopropionic acids can not be separated.

Comparison with amino acids. Table I reports the R_F values of the amino acids corresponding to the aminophosphonic acids, obtained under the same experimental conditions. The chromatographic behaviour of the aminophosphonic acids and the corresponding natural amino acids has been compared in order to establish whether

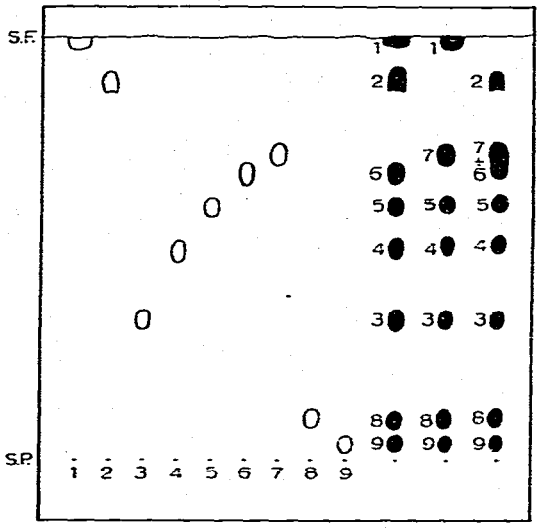

Fig. 1. Thin-layer chromatogram of some aminophosphonic acids on AG 1-X4 (PrO^-). Two successive developments in 0.01 *M* propionate buffer. Migration distance 14 cm. Spots: 1, 3-aminopropylphosphonic acid; 2, 2-aminoethylphosphonic acid; 3, 1-amino-2-(4-hydroxyphenyl)ethylphosphonic acid; 4, 1-amino-2-phenylethylphosphonic acid; 5, 1-aminomethylphosphonic acid; 6, 1-aminoethylphosphonic acid; 7, 1-amino-1-methylethylphosphonic acid; 8, 2-amino-4-phosphonobutyric acid; 9, 2-amino-3-phosphonopropionic acid. S.F. = Solvent front; S.P. = starting point.

or not on anion exchangers there was the same analogy as on cation exchangers[9].

It should be noted that most amino acids run with the solvent front, independent of the buffer concentration in the eluent; exceptions are the two compounds with an aromatic ring in the side-chain and the two dicarboxylic amino acids. Such behaviour, in accordance with the smaller possibilities of linking of the carboxylic group than of the phosphonic group, can be used in order to separate most aminophosphonic acids from the corresponding amino acids. Glutamic acid, however, cannot be separated from 2-amino-4-phosphonobutyric acid.

Tyrosine and phenylalanine behave differently from the corresponding aminophosphonic acids. The retention of these two amino acids is controlled by an adsorption process, as their R_F values do not change (or change very little) as the buffer concentration is increased. The behaviour of these two amino acids is similar also on AG 1-X4 (PrO^-) layers with propionate buffer as eluent, apart from different R_F values (0.83 for phenylalanine and between 0.59 and 0.63 for tyrosine).

Influence of the pH of the eluent. On elution with acetate and propionate solutions with concentrations between 0.01 and 0.25 *M*, the aminophosphonic acids mostly have lower R_F values than those observed on elution with the same concentrations of propionate and acetate buffers. The difference between the R_F values in the two cases decreases as the eluent concentration is increased and becomes negligible at concentrations above 0.1 *M*. It should be noted, however, that when the buffer is replaced with the salt solution, elongated spots are obtained, particularly with 1-aminophosphonic acids with an aliphatic side-chain. With 2-amino-3-phosphonopropionic and 2-amino-4-phosphonobutyric acids, the best results were achieved by eluting with

acetate solutions, as the resolution between the two compounds was improved and no elongated spots were observed.

As regards the formation of elongated spots when acetate or propionate solutions are used, this occurrence may be ascribed to a lower degree of protonation of the amino groups owing to the higher pH of the eluent.

Retention mechanism. In contrast to the extensive studies on cation exchangers[13–16], the retention mechanism on anion exchangers has been investigated very little and has given rise to inconsistent results. In particular, according to Ossicini[17], the relationship

$$R_M = -n \log a_{salt} + \text{constant} \qquad (1)$$

where n is the charge on the anion involved in the exchange process, does not give rise to reliable results with inorganic anions, while according to Cassol *et al.*[18], no disadvantages were found in the case of organometallic complexes. On applying eqn. 1 to the chromatographic data on the aminophosphonic acids when eluting with acetate buffer, the trends reported in Fig. 2 are obtained. No linear trend was found in the concentration range investigated and, in the case of curves (a) and (b), a curve with two steps was observed. As such a trend has not been noticed on cation exchangers, we tried to establish whether it could be attributed to a pH gradient along the layer. By carrying out pH measurements as described in a previous paper[19], we obtained the trends shown in Fig. 3, where the pH is plotted as a function of the distance from the origin. The pH value is never constant along the layer for any buffer concentration and, in every instance, a sharp increase in the pH value (about one pH unit) is observed at a distance from the origin between 6 and 8 cm, corresponding to R_F values between

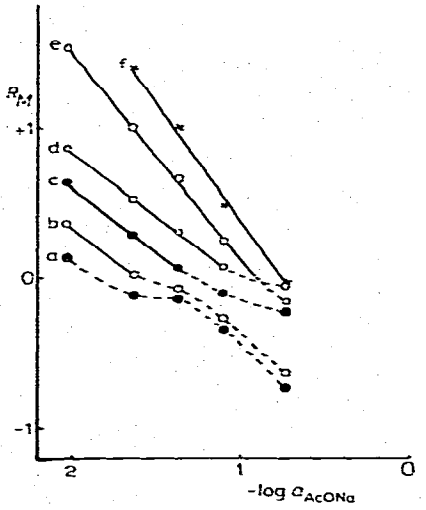

Fig. 2. R_M values *versus* $-\log a_{AcONa}$ on AG 1-X4 (AcO^-) thin layers. Curves: (a) 1-aminopentylphosphonic acid; (b) 1-aminomethylphosphonic acid; (c) 1-amino-2-phenylethylphosphonic acid; (d) 1-amino-2-(4-hydroxyphenyl)ethylphosphonic acid; (e) 2-amino-4-phosphonobutyric acid; (f) 2-amino-3-phosphonopropionic acid.

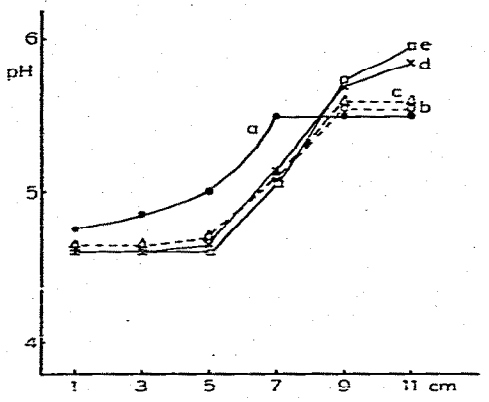

Fig. 3. pH values of AG 1-X4 (AcO^-) suspensions referred to the centre of the strips[19] (10 × 2 cm). Acetate buffer concentration: (a) 0.01 *M*; (b) 0.025 *M*; (c) 0.5 *M*; (d) 0.1 *M*; (e) 0.25 *M*.

0.50 and 0.66. The formation of the pH gradient may be attributed to the adsorption of hydrogen ions by the layer, whose adsorption capacity seems high as the pH is constant along the layer till $R_F = 0.50$ even if the buffer concentration in the eluent is increased from 0.025 to 0.25 *M*. The formation of the pH gradient and the overall pH increase observed at R_F values between 0.50 and 0.66 seems to cause the peculiar trend of the curves in Fig. 2. It may be noted, in fact, that the linear portion of these curves (continuous line) is limited only at R_F values between 0.0 and 0.5, that is, in the zone where the pH on the layer is equal to that of the eluent. At R_F values between 0.50 and 0.66, corresponding to the sharp increase in the pH on the layer, the curves deviate from linearity and tend to become parallel to the abscissa.

The chromatographic behaviour of the aminophosphonic acids in the R_F range 0.50–0.66 may be explained by assuming that the pH increase causes a decrease in the degree of protonation of the phosphonic group, which involves an increase in the affinity of the acid towards the exchanger: in fact, until $R_F = 0.50$ the prevailing species* is $A^{\mp}$, while at $R_F = 0.66$ the prevailing species is $A^{-\mp}$, which has a higher affinity towards the exchanger than $A^{\mp}$. Such species are the most probable if the pK values of the 1-aminophosphonic acids are assumed to be analogous to those known for 2-aminoethylphosphonic acid [pK_1($-PO_3H_2$) = 2.45 (ref. 20); pK_2($-PO_3H^-$) = 7.00 (ref. 20) or 6.4 (ref. 21); pK_3($-NH_3^+$) = 10.8 (ref. 20)].

On the basis of the above discussion, in order to calculate the charge on the anion involved in the exchange process, only the linear portion of the curves can be taken into account. The slope (0.85) for the aminophosphonic acids with an aromatic ring in the side-chain indicates that these acids behave in a similar manner to monovalent anions. With 2-amino-3-phosphonopropionic and 2-amino-4-phosphonobutyric acids, a slope between 1.35 and 1.56 is found, in accordance with the presence of two acidic groups in the molecule, one of which (the carboxylic group) may be deprotonated only partly. The slope for aspartic and glutamic acids is in fact 1.16 under the same experimental conditions.

$$^{*}\overbrace{A^{\mp}} = R{-}\underset{\displaystyle NH_3^+}{\underset{|}{CH}}{-}PO_3H^-;\quad A^{-\mp} = R{-}\underset{\displaystyle NH_3^-}{\underset{|}{CH}}{-}PO_3^{2-}.$$

On eluting with acetate solutions, the slope for 2-amino-3-phosphonopropionic and 2-amino-4-phosphonobutyric acids is between 1.7 and 1.9, which suggests that these compounds behave in a similar manner to divalent anions.

Cation exchangers

Dowex 50-X4 (H^+). In a previous paper[9] we employed Dowex 50-X4 (H^+) mixed with non-microcrystalline cellulose. As better results may be achieved with

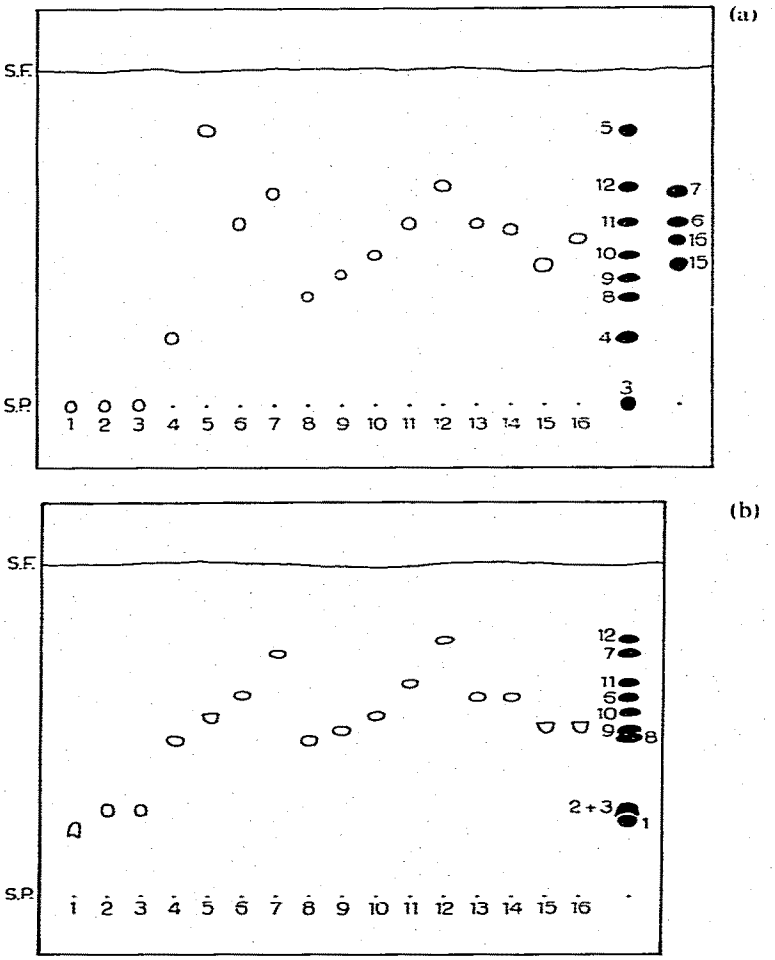

Fig. 4. Thin-layer chromatogram of aminophosphonic acids on (a) alginic acid and (b) microcrystalline cellulose. Two successive developments in water–isopropanol–*n*-butanol (1:1:1). Spots: 1, 1,4-diaminobutylphosphonic acid; 2, 1,3-diaminopropylphosphonic acid; 3, 1,2-diaminoethylphosphonic acid; 4, 3-aminopropylphosphonic acid; 5, 2-aminoethylphosphonic acid; 6, 1-amino-2-(4-hydroxyphenyl)-ethylphosphonic acid; 7, 1-amino-2-phenylethylphosphonic acid; 8, 1-aminomethylphosphonic acid; 9, 1-aminoethylphosphonic acid; 10, 1-aminopropylphosphonic acid; 11, 1-aminobutylphosphonic acid; 12, 1-aminopentylphosphonic acid; 13, 1-amino-2-methylpropylphosphonic acid; 14, 1-amino-1-methylethylphosphonic acid; 15, 2-amino-4-phosphonobutyric acid; 16, 2-amino-3-phosphonopropionic acid.

microcrystalline cellulose mixed with the different exchangers[12], we thought it would be useful to carry on a further investigation using Dowex 50-X4 (H^+) with microcrystalline cellulose. On elution with hydrochloric acid of concentrations between 0.1 and 1 *M*, the behaviour of the aminophosphonic acids was similar to that reported in the previous paper[9]. However, very compact spots were obtained, which are favourable from an analytical point of view. With the use of water–organic solvent mixtures as eluent, no satisfactory results were obtained. In fact, with two successive developments the aminophosphonic acids do not run to any great extent. A greater migration distance is obtained with water–ethanol (2:1) but the R_F values with this eluent are not greater than 0.25.

Rexyn 102 (H^+). On this paraffin-based exchanger, the sequence of the affinities of the aminophosphonic acids is similar to that on Dowex 50-X4 for most compounds. A different behaviour on the two exchangers is shown by 2-aminoethylphosphonic, 3-aminopropylphosphonic, 2-amino-3-phosphonopropionic and 2-amino-4-phosphonobutyric acids. On Rexyn 102 (H^+), in fact, on elution with both acetic acid solutions and water–ethanol mixtures, the first two compounds behave in a similar manner to the corresponding 1-aminophosphonic acids and the last two compounds run with the solvent front.

Alginic acid. This exchanger was used in a mixture with microcrystalline cellulose. On elution with acetic acid solutions there are no differences compared with the behaviour of the same compounds on alginic acid mixed with non-microcrystalline cellulose. The results obtained with water–organic solvent mixtures, which were not employed in the previous investigation, are very interesting. Fig. 4a shows the chromatogram of the 16 aminophosphonic acids obtained with two successive developments in water–isopropanol–*n*-butanol (1:1:1). The compactness of the spots is useful.

The separation of the five aminophosphonic acids with a linear side-chain is very interesting, as it cannot be achieved on either anion exchangers or cation exchangers with polystyrene- and paraffin-based matrices. By comparing such a chromatogram with that reported in Fig. 4b, obtained on microcrystalline cellulose under the same experimental conditions, the determining influence of alginic acid in the separation of these compounds can be seen. However, on microcrystalline cellulose, there are some advantages in the separation of the diaminophosphonic acids, which, on alginic acid, remain at the starting point.

On both layers 2-aminoethylphosphonic acid cannot be detected with ninhydrin reagent; its detection is possible by spraying with molybdate reagent and subsequently with a 1% tin(II) chloride solution in 2 *M* hydrochloric acid. The anomalous behaviour of 2-aminoethylphosphonic acid on layers of alginic acid should be noted. This compound, in fact, has a higher R_F value than those of 1-aminophosphonic acids and this behaviour cannot be explained on the basis of its acid–base characteristics.

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