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CHROMATOGRAPHIC AND ELECTROPHORETIC BEHAVIOR OF AMINOPHOSPHONIC ACIDS ON LAYERS OF CATION EXCHANGERS

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SUMMARY

The chromatographic and electrophoretic behavior of sixteen aminophosphonic acids was investigated on layers of weak and strong cation exchangers. The influence of the acidity of the eluent and of the form of the exchanger was also studied.

A comparison between the chromatographic characteristics of these aminophosphonic acids and the corresponding natural amino acids was made.

INTRODUCTION

Aminophosphonic acids have acquired importance in the past few years from a biological point of view[1,2]. They are not found in nature, with the exception of 1-aminoethylphosphonic acid[3]. In the pertinent literature we have not found any information about the chromatographic and electrophoretic behavior of the aminophosphonic acids. Furthermore, these compounds do not lend themselves to studies by adsorption or partition techniques due to their insolubility in the common organic solvents.

Ion-exchange chromatography seems particularly suitable for such compounds on account of their solubility in aqueous solvents and their acid–base characteristics. We have therefore deemed it useful, as a part of the research being carried on in our institute on the use of ion exchangers[4–7], to investigate the chromatographic and electrophoretic behavior of this class of compounds. The purposes of this study are: (a) to perfect methods for the separation of the aminophosphonic acids; and (b) to study their chemical properties by means of a comparison with the corresponding amino acids.

EXPERIMENTAL

Preparation of the layers

Layers having a thickness of 300 μ were used.

(a) Alginic acid. 6 g of the exchanger and 1.5 g of cellulose (No. 123 from Schleicher & Schüll) in 40 ml of water.

(b) Dowex 50 X4 (H^+ and Na^+ form). 4.5 g of 200–400 mesh resin and 4.5 g of cellulose in 40 ml of water.

(c) Dowex 50 X4 + CMCNa. 3 g of each exchanger in 40 ml of water.

(d) CMCNa (No. 132 from Schleicher & Schüll). 4.5 g in 50 ml of water.

Reagents and developers

Aqueous solutions (1%) of each aminophosphonic acid (Calbiochem) were prepared. These solutions were then diluted to obtain well-defined spots. The quantities of each compound (μg) are reported in Table I. A solution obtained by dissolving

TABLE I

R_F VALUES OF AMINOPHOSPHONIC ACIDS ON THIN LAYERS OF (1) ALGINIC ACID, (2) DOWEX 50 X4 (H^+), (3) DOWEX 50 X4 (Na^+), (4) CMCMa AND (5) DOWEX 50 X4 (Na^+) + CMCNa, WITH WATER AS ELUENT

Aminophosphonic acid	*1*	*2*	*3*	*4*	*5*	*Amount (μg)*
1,4-Diaminobutylphosphonic	0.03	0.00	0.00	0.37	0.06	1.5
1,3-Diaminopropylphosphonic	0.03	0.00	0.00	0.53	0.12	1.5
1,2-Diaminoethylphosphonic	0.03	0.00	0.00	0.80	0.25	2.0
3-Aminopropylphosphonic	0.49	0.03	0.34	0.96	0.93	2.0
2-Aminoethylphosphonic	0.68	0.12	0.65	0.96	0.94	2.0
1-Amino-2-(4-hydroxyphenyl)ethylphosphonic	0.74	0.19	0.40	0.96	0.94	1.5
1-Amino-2-phenylethylphosphonic	0.76	0.15	0.36	0.96	0.94	1.5
1-Aminomethylphosphonic	0.78	0.50	0.92	0.96	0.94	2.0
1-Aminoethylphosphonic	0.84	0.44	0.92	0.96	0.94	1.5
1-Aminopropylphosphonic	0.85	0.40	0.91	0.96	0.95	1.5
1-Aminobutylphosphonic	0.85	0.30	0.83	0.96	0.94	1.5
1-Aminopentylphosphonic	0.85	0.23	0.75	0.96	0.94	1.5
1-Amino-2-methylpropylphosphonic	0.85	0.32	0.92	0.96	0.94	1.5
1-Amino-1-methylethylphosphonic	0.85	0.32	0.91	0.96	0.94	1.5
2-Amino-4-phosphonbutyric	0.86	0.24	0.94	0.96	0.95	1.5
2-Amino-3-phosphonpropionic	0.95	0.62	0.96	0.96	0.96	1.5

0.1 g of ninhydrin in 50 ml of absolute alcohol, with addition of 10 ml of glacial acetic acid and 2 ml of collidine, was used as developer. After spraying, the plates were kept at 110° for 10 min. The only aminophosphonic acid to give a positive test with molybdate reagent was 2-aminoethylphosphonic acid.

Electrophoretic measurements

The electrophoretic measurements were made with a Camag apparatus for high potential electrophoresis, at a temperature of 18°. The electro-osmotic flow was measured with hydrogen peroxide.

RESULTS AND DISCUSSION

Effect of the exchanger

R_F values of sixteen aminophosphonic acids on layers of alginic acid, Dowex 50 X4 (H^+), Dowex 50 X4 (Na^+), CMCNa, and mixed layers of Dowex 50 X4 (Na^+) and CMCNa are reported in Table I.

On the alginic acid layers it can be observed that most aminophosphonic acids have an R_F value $\geqslant 0.68$. Only diaminophosphonic acids and 3-aminopropylphosphonic acid, although this latter to a lesser degree, are adsorbed to any extent on alginic acid. Their separation from all the others is therefore possible.

On Dowex 50 X4 (H^+) there is much greater retention than on alginic acid. It is interesting to note the unusual behavior of 1-amino-2-phenylethylphosphonic acid and 1-amino-2(4-hydroxyphenyl)ethylphosphonic acid, which are retained more than would be expected on the basis of their acid–base characteristics.

From an analytical point of view the separation of 2-amino-4-phosphonbutyric acid ($R_F = 0.24$) from 2-amino-3-phosphonpropionic acid ($R_F = 0.62$) is interesting; these two acids correspond to glutamic and aspartic acids in which a carboxylic group has been replaced by a phosphonic one. The behavior of the diaminophosphonic acids is identical on both the H^+ and Na^+ forms of Dowex 50 X4. All other acids are held back to a lesser degree, even though the two acids containing phenyl groups are retained in an anomalous way on the Na^+ form.

On mixed layers of Dowex 50 X4 and CMCNa all aminophosphonic acids, with the exception of the three diaminophosphonic acids, move with the solvent front. It is interesting to note that the mixture of the two exchangers is influenced by the characteristics of the weak exchanger, as shown by the R_F values of aminophosphonic acids on layers of CMCNa alone.

On this latter layer, as the chromatogram of Fig. 1 shows, an excellent separation can be effected between the three diaminophosphonic acids and between these and all the other acids. The separation is also made easier by the well-defined spots we can obtain on this layer[8].

Effect of the eluent

The data of Table I show that separations of aminophosphonic acids, with the exception of the three diaminophosphonic acids, are possible only on layers of Dowex 50 X4 in the acid form. For this reason the data in Table II, obtained by using HCl of different concentrations as eluent, refer to this exchanger. With respect to the values

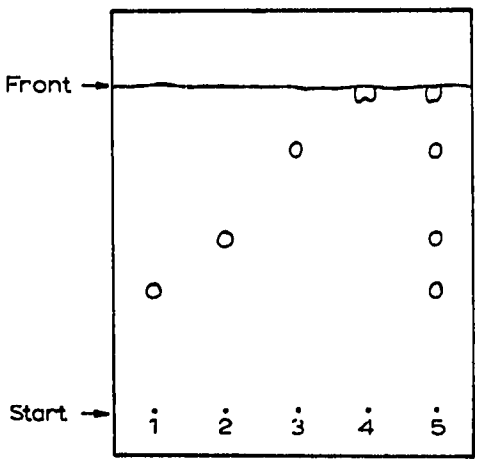


Fig. 1. Thin-layer chromatogram of aminophosphonic acids on CMCNa. 1 = 1,4-diaminobutylphosphonic; 2 = 1,3-diaminopropylphosphonic; 3 = 1,2-diaminoethylphosphonic; 4 = 3-aminopropylphosphonic; 5 = mixture.

TABLE II

R_F VALUES OF AMINOPHOSPHONIC ACIDS ON THIN LAYERS OF DOWEX 50 X4 (H^+)

Development distance, 14.5 cm. Eluents: (a) HCl, 0.1 *N*; (b) HCl, 0.25 *N*; (c) HCl, 0.5 *N*; (d) HCl, 1 *N*.

No.	*Aminophosphonic acid*	*a*	*b*	*c*	*d*
1	1,4-Diaminobutylphosphonic	0.01	0.05	0.11	0.37
2	1,3-Diaminopropylphosphonic	0.02	0.06	0.15	0.41
3	1,2-Diaminoethylphosphonic	0.03	0.09	0.19	0.44
4	3-Aminopropylphosphonic	0.15	0.26	0.45	0.64
5	2-Aminoethylphosphonic	0.25	0.37	0.50	0.71
6	1-Amino-2-(4-hydroxyphenyl)ethylphosphonic	0.21	0.22	0.26	0.38
7	1-Amino-2-phenylethylphosphonic	0.18	0.19	0.23	0.34
8	1-Aminomethylphosphonic	0.55	0.66	0.73	0.84
9	1-Aminoethylphosphonic	0.52	0.60	0.70	0.82
10	1-Aminopropylphosphonic	0.48	0.58	0.66	0.80
11	1-Aminobutylphosphonic	0.40	0.46	0.55	0.75
12	1-Aminopentylphosphonic	0.30	0.34	0.41	0.61
13	1-Amino-2-methylpropylphosphonic	0.44	0.50	0.55	0.76
14	1-Amino-1-methylethylphosphonic	0.44	0.50	0.55	0.76
15	2-Amino-4-phosphonbutyric	0.29	0.46	0.62	0.80
16	2-Amino-3-phosphonpropionic	0.48	0.59	0.69	0.86

obtained on eluting with water, we can observe a general increase in the R_F value, as was expected. Gradual resolution of the diaminophosphonic acids can be noted as the concentration of the acid in the eluent increases. It is interesting to draw attention to the behavior of the two aminophosphonic acids containing a phenyl group. These are not appreciably affected by a variation of the pH in the eluent, and this suggests that the mechanism determining their retention on the exchanger is not due solely to an ion-exchange process.

Correlation between R_F values and the characteristics of the aminophosphonic acids

The basicity of amino acids is intimately related to the position of the amino group in the molecule. It is therefore possible, without knowing the acid–base con-

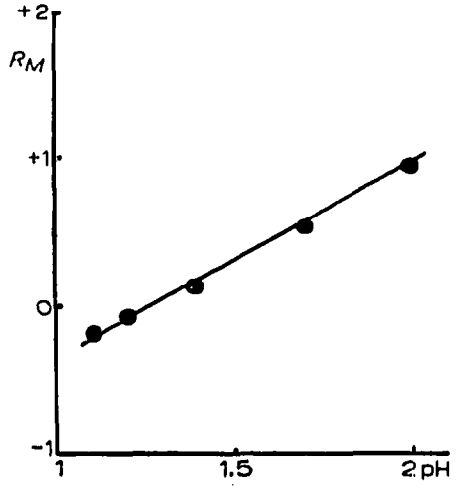


Fig. 2. R_M values *vs.* pH for diaminophosphonic acids on alginic acid thin layers. Eluents: HCl solutions.

stants, to forecast a basicity sequence for the various aminophosphonic acids on the basis of the position of the amino group with respect to the phosphonic group.

The sequence is: 1,4-diamino > 1,3-diamino > 1,2-diamino > 3-amino > 2-amino > 1-amino. R_F values on Dowex 50 X4 (H^+) are in agreement with this sequence, as the order of retention decreases starting with 1,4-diamino (see Table II), *i.e.* the R_F value increases with decreasing basicity. On the the other hand, on alginic acid the R_F values of the diaminophosphonic acids, though they are always lower than those of the monoamino acids, are equal to each other without depending on the acidity of the eluent (see Table I and Fig. 2).

On CMCNa, however, the differences in the acid–base characteristics of the diaminophosphonic acids are particularly evident, since they are distinctly separated on this layer (see Fig. 1).

With regard to the effect of the side chain, it is possible to observe with the increase in the number of carbon atoms a parallel increase in the retention capacity of the polystyrolic base exchangers.

On Dowex 50 X4 the R_F value sequence is: methyl > ethyl > propyl > butyl > pentyl.

Such a relationship is not respected with branched side chains, as the R_F values of 1-amino-2-methylpropylphosphonic acid and 1-amino-1-methylethylphosphonic acid show (see Table II). The sequence observed on Dowex is not respected on cellulose-type exchangers such as alginic acid.

Retention mechanism

Alginic acid. On this exchanger the relation[9]:

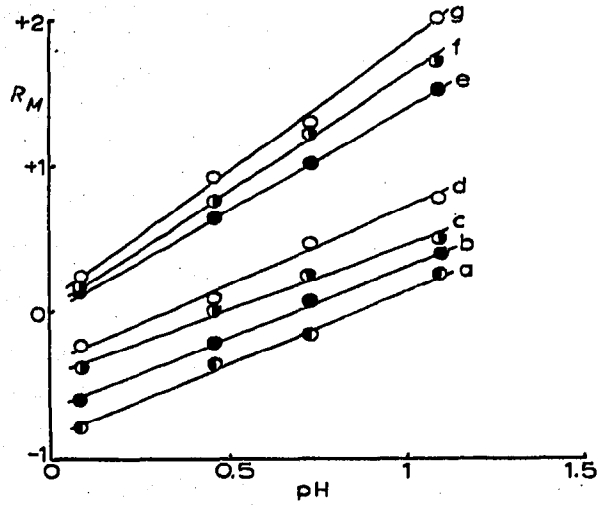
$$n\text{pH} = R_M + \text{constant} \qquad (1)$$

can be applied and the line in Fig. 2 is obtained, whose slope (1.32) indicates that these acids behave like divalent ions. The discrepancy between the theoretical and experimental values is probably due to the pH gradient along the layer[7]. We cannot affirm with certainty that the retention of the monoaminophosphonic acids is influenced only by the ion-exchange process, since eqn. 1 cannot be applied to them (due to their low retention with water), even if the agreement between the R_F values and the acid–base characteristics (3-amino > 2-amino > 1-amino) deems it probable.

Dowex 50 X4 (H^+). By applying eqn. 1 to chromatography on this exchanger it has been possible to show that the retention mechanism is not entirely an ion-exchange process. The progression of R_M/pH values for diaminophosphonic acids (curves e, f, g), 3-aminophosphonic acid (curve d), 2-amino-3-phosphonpropionic acid (curve a), and 2-amino-4-phosphonbutyric acid (curve b) are reported in Fig. 3. The slope for diaminophosphonic acids, falling between 1.4 and 1.7, indicates that on this exchanger too these amino acids behave like divalent ions. In the case of monoamino acids the slope, falling between 0.9 and 1.0, suggests that these compounds are present as monovalent ions.

As far as the other acids are concerned, it does not appear that the ion-exchange process is the predominant parameter in the retention mechanism, as shown by the curves reported in Fig. 4.

In particular, the greatest deviation from eqn. 1 is found for the two amino acids containing phenyl groups (curves f, g), and for acids having a linear side chain



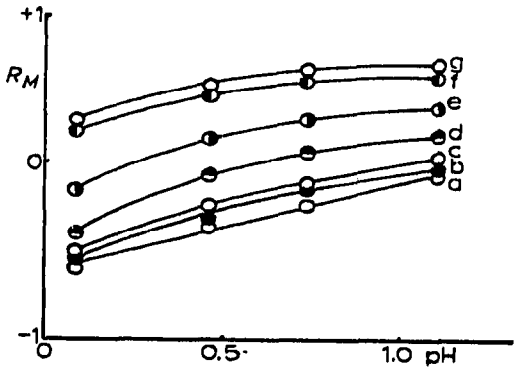


Fig. 3. R_M values *vs.* pH for some aminophosphonic acids on Dowex 50 X4 (H^+) thin layers. Eluents: HCl solutions. (a) 2-amino-3-phosphonpropionic; (b) 2-amino-4-phosphonbutyric; (c) 2-aminoethylphosphonic; (d) 3-aminopropylphosphonic; (e) 1,2-diaminoethylphosphonic; (f) 1,3-diaminopropylphosphonic; (g) 1,4-diaminobutylphosphonic.

Fig. 4. R_M values *vs.* pH for some aminophosphonic acids on Dowex 50 X4 (H^+) thin layers. Eluents: HCl solutions. (a) 1-aminomethylphosphonic; (b) 1-aminoethylphosphonic; (c) 1-aminopropylphosphonic; (d) 1-aminobutylphosphonic; (e) 1-aminopentylphosphonic; (f) 1-amino-2-(4-hydroxyphenyl)ethylphosphonic; (g) 1-amino-2-phenylethylphosphonic.

with five carbon atoms (curve d). As the length of the side chain decreases, the R_M/pH relation approaches its theoretical course. Nevertheless, the slope of the line (0.60) is distinctly less than its theoretical value of 1 (see curve a of Fig. 4) also in the case of 1-aminomethylphosphonic acid, which has only one carbon atom in the side chain. The other two acids having a branched side chain show the same curvilinear trend. Similar behavior was found by KNIGHT[10] in the separation of amino acids on ion-exchange papers consisting of polystyrolic matrices.

Comparison with amino acids

The use of aminophosphonic acids concerns, as far as can be discerned from the scarce data in the literature, their utilization in association with or as substitute for corresponding natural amino acids, and for this reason a comparison of the chromatographic behavior of these two classes of compounds was deemed useful.

The spots of natural amino acids (black) and the corresponding spots of aminophosphonic acids on layers of Dowex 50 X4 (H^+), eluted with 0.5 *M* HCl, are reported in Fig. 5. The distinct separation between the respective components of the two classes of substances appears evident from the chromatogram, as the aminophosphonic acids are retained less than the corresponding natural amino acids. This is in agreement with the more marked acid characteristics which the compound assumes when the carboxylic group is replaced by the stronger phosphonic group. Such a replacement does not however cause any variation in those chromatographic characteristics that depend upon the side chain of the molecule. In fact, in both classes of compounds, the sequence of R_F values for the five amino acids having a linear side chain containing from one to five carbon atoms follows an inverse order to the number of carbon atoms in the chain.

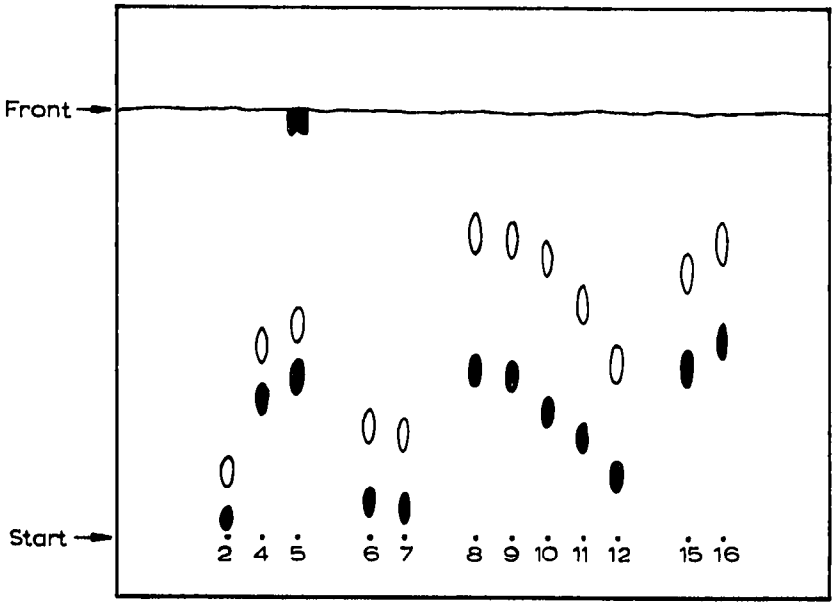


Fig. 5. Chromatographic behavior of aminophosphonic acids and the corresponding amino acids (black spots) on Dowex 50 X4 (H^+) thin layers. Eluent: 0.5 *N* HCl. The numbers refer to the aminophosphonic acids as reported in Table II. The spots relative to No. 5 refer, respectively, to β-alanine, 2-aminoethylphosphonic acid and taurine.

Moreover, considering the separation of β-alanine (R_F = 0.37) from 2-aminoethylphosphonic acid (R_F = 0.50) and from taurine (R_F = 0.96), we have a clear demonstration of the possibilities of ion-exchange chromatography where advantage is taken of the variations in the acid–base characteristics of the compounds due to the presence of a different acid group in the molecule.

TABLE III

MIGRATION DISTANCE (mm) OF AMINOPHOSPHONIC ACIDS ON THIN LAYERS OF ALGINIC ACID (AA) AND SODIUMCARBOXYMETHYLCELLULOSE (CMCNa)

Aminophosphonic acid	*AA*[a]	*CMCNa*[b]
1,4-Diaminobutylphosphonic	34	30
1,3-Diaminopropylphosphonic	34	25
1,2-Diaminoethylphosphonic	33	17
3-Aminopropylphosphonic	53	13
2-Aminoethylphosphonic	50	12
1-Amino-2-(4-hydroxyphenyl)ethylphosphonic	43	4
1-Amino-2-phenylethylphosphonic	43	2
1-Aminomethylphosphonic	43	−1
1-Aminoethylphosphonic	43	1
1-Aminopropylphosphonic	44	7
1-Aminobutylphosphonic	45	8
1-Aminopentylphosphonic	47	8
1-Amino-2-methylpropylphosphonic	46	9
1-Amino-1-methylethylphosphonic	46	8
2-Amino-4-phosphonbutyric	18	−16
2-Amino-3-phosphonpropionic	4	−35
H_2O_2	43	12

[a] Electric potential: 1100 V; time: 90 min; electrolyte: 1 *M* CH_3COOH.
[b] Electric potential: 600 V; time: 60 min; electrolyte: 0.1 *M* acetic buffer.

Electrophoresis

Migration distances of aminophosphonic acids on layers of alginic acid and CMCNa, using 1 *M* CH_3COOH and 0.1 *M* acetic buffer, respectively, as electrolytes, are reported in Table III. On both exchangers (and in particular on alginic acid) most aminophosphonic acids move only to a small extent in the applied electric field, taking into account the electro-osmotic flow (measured with H_2O_2). The behavior of 2-amino-4-phosphonbutyric acid and 2-amino-3-phosphonpropionic acid is an exception to this, as both show a distinctly anionic behavior and can be separated from all the others as well as from each other. The difference in the electrophoretic behavior of these two compounds increases as we use CMCNa instead of alginic acid as exchanger. On this latter exchanger most aminophosphonic acids show a predominately anionic character, with the exception of the most basic amino acids, such as the diamino acids, 3-aminopropylphosphonic acid, and 2-aminoethylphosphonic acid. This is also due to the higher pH of the electrolyte.

The fact that most aminophosphonic acids do not migrate, or at least migrate very little, under the influence of an electric field at pH = 4.85 indicates that, at such a pH, the amino acids, with the exception of the diamino acids, 2-amino-4-phosphonbutyric acid, and 2-amino-3-phosphonpropionic acid, are predominantly in the anionic form.

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