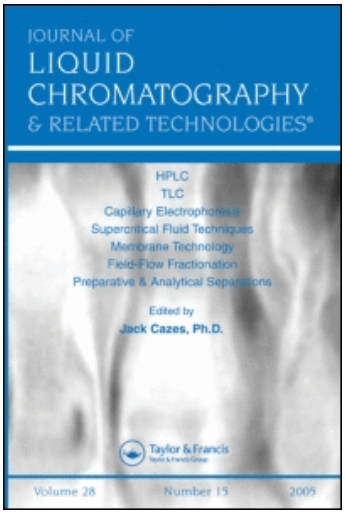

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SEPARATION OF ORGANOPHOSPHONATES BY ION CHROMATOGRAPHY WITH INDIRECT PHOTOMETRIC DETECTION

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ABSTRACT

An ion chromatographic method which utilizes lithium trimesate to separate three diphosphonate drugs and two process related organophosphonates is described. The lack of a chromophore on the five species and the high absorptivity of the trimesate anion at 254 nm allows for facile detection using indirect photometry. The effects of mobile phase concentration on the capacity factor of the species were investigated and a logarithmic relationship was established which was found to be dependent on the charge of the analyte anion and the mobile phase anion.

INTRODUCTION

A number of diphosphonates have found applications in the pharmaceutical industry for treatment of abnormal calcium metabolism such as osteoporosis,

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ectopic calcification, and various bone diseases. Diphosphonates are known to bind strongly to calcium hydroxyapatite, the major constituent of the mammalian skeleton, through chelation of calcium. This chelation inhibits bone resorption, as found with osteoporosis or various bone diseases (1), or at higher levels inhibits crystal growth through the prevention of further addition of calcium, orthophosphate, or hydroxide into the crystal lattice, leading to calcification in joints, arteries and various organs (2,3). Endogenous inorganic pyrophosphate performs such a role in mammals but tends to be hydrolysed quickly in the body (4).

Etidronate, pamidronate, and clodronate are some of the diphosphonates which are currently marketed. Their development was based on the observation that diphosphonates are more effective due to their increased stability towards hydrolysis in the body as compared to endogenous pyrophosphate (1-5). This extra stability is due to the presence of P-C-P bonds in these drugs as opposed to P-O-P bonds found in endogenous pyrophosphate. Alendronate, an amino diphosphonate (Fig. 1), is a new member of the class of diphosphonates which is specifically targeted for inhibition of bone resorption.

Routine chromatographic analysis of diphosphonates is complicated due to their high ionic character and general lack of a strong UV chromophore. Current methods of analysis include derivatization prior to chromatographic separation (6-9), post column reaction (10-13), ion conductivity detection (14), flame photometric detection (15), or inductively coupled plasma detection (16).

Previous chromatographic analysis for alendronate (9) consisted of pre-column derivatization of the amine group with 9-fluorenylmethyl chloroformate (FMOC-Cl) at pH 9 followed by elution from a polymeric phase (Hamilton PRP-1). FMOC-Cl reacts rapidly with primary and secondary amines to produce very stable derivatives. This derivatization is routinely used for reverse phase

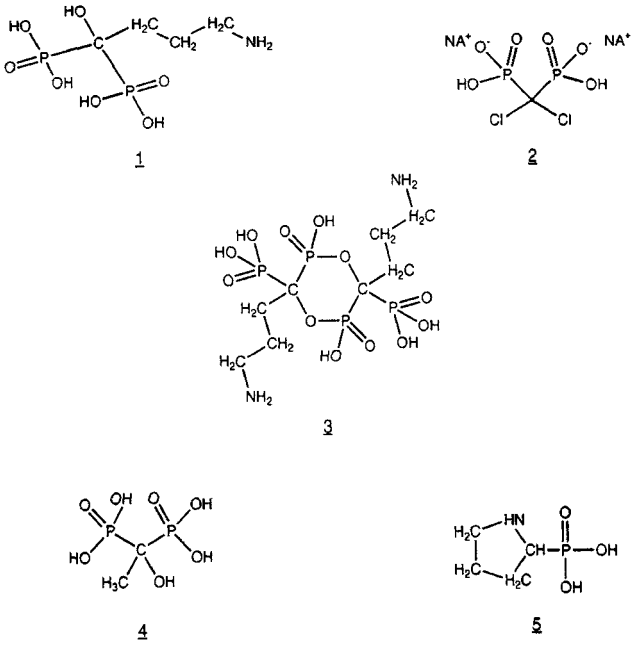

Fig. 1: Structures of the organophosphonates. 1 - Alendronate; 2 - Clodronate; 3 - Alendronate Dimer; 4 - Etidronate; 5 - 2-Phosphonopyrrolidine

chromatography of amino acids (17,18). The added FMOC group allows for better retention in the reverse phase mode and provides a strong chromophore suitable for UV or fluorescence detection.

There are two major disadvantages associated with the use of FMOC derivatization however. First considerable time and effort are devoted to sample preparation. Secondly, FMOC derivatization would be specific only for species which contain a primary or secondary amine group available for derivatization. The synthesis of alendronate (19) generates ionic by-products such as alendronate dimer, 2-phosphonopyrrolidine, chloride, phosphite, and methanesulfonate and could potentially generate de-aminated phosphonates as well. Some of these by-products would not be detected by the FMOC derivatization method.

In response to the above-mentioned disadvantages to the FMOC-HPLC method an ion chromatography method was developed. The use of ion chromatography with indirect photometric detection allows for separation and detection of diphosphonates with standard HPLC pumps and UV detectors and requires minimal sample preparation.

Indirect photometric detection (IPD) was introduced by Schill in 1981 (20) and has subsequently found wide application (21-29). It requires a mobile phase species which absorbs highly at a wavelength that the analyte does not. If the mobile phase species has equilibrated with the stationary phase, the detector senses a strong steady absorbance. When a non-absorbing analyte is injected onto an ion exchange stationary phase, it displaces mobile phase ions from charged sites on the stationary phase resulting in a localized zone of higher mobile phase ion concentration. This zone is manifested in the detector as a region of higher absorbance and produces a positive system peak. When the analyte ion is eventually eluted from the stationary phase, its transparency results in a localized region of lower absorbance and is manifested in the detector as a negative analyte peak.

Salts of carboxylic acids such as phthalic acid, citric acid and succinic acid tend to be good displacing anions for anion exchange chromatography and have been widely utilized. Salts of aromatic carboxylic acids such as phthalic acid, benzoic acid, trimesic acid, and p-hydroxybenzoic acid are suitable for ion chromatography with IPD because of their displacing strengths and their ability to strongly absorb light due to the presence of the aromatic ring. Lithium trimesate was chosen as the mobile phase for this particular separation.

An assay for etidronate using nitric acid as an additive in the mobile phase and IPD has been previously reported (30). The method does not, however, address the issue of impurities in the bulk drug. The method reported here, using

trimesate ion, can be used as an impurity profile as well as an assay. As a strong displacing agent, trimesate elutes both low-charged weakly retained analytes and high-charged strongly retained analytes without having to resort to a gradient method. Separation was achieved for three diphosphonate drugs (alendronate, etidronate, and clodronate) and two organophosphonates (2-phosphonopyrrolidine and alendronate dimer) which are by-products of the synthesis of alendronate (structures are given in Fig. 1). The analytes were detected by IPD. The retention characteristics of the organophosphonates were investigated as a function of eluent ion concentration, pH, and hydrophobicity. Results were found to closely match theoretical considerations.

EXPERIMENTAL

Chromatographic Equipment

The HPLC Equipment consisted of a Spectra Physics SP8800 ternary pump, a Spectra Physics SP8775 autosampler (Spectraphysics, Piscataway, New Jersey) and a Kratos Spectroflow 757 absorbance detector (ABI Analytical, Foster City, California) . The chromatograms were processed using PE Nelson Access Chrom version 1.7 software (PE Nelson, Cupertino, California). The column used was a Hamilton PRP-X100, 250 x 4.1 mm (Hamilton Co., Reno, Nevada) which consists of 10 μm spherical particles of poly(styrene-divinylbenzene) trimethylammonium base ion exchanger. This column is a low capacity (0.2 meq/g) ion exchanger.

Chromatographic Conditions

The mobile phase was trimesic acid (1,3,5-benzenetricarboxylic acid, Sigma, St Louis, Missouri) in water. Adjustments of pH were made with lithium

hydroxide (Baker analyzed, J.T. Baker, Philipsburg, NJ). Samples were prepared by dissolution in deionized water and were introduced into the chromatographic system through a 10 µL loop.

The column was equilibrated with the mobile phase until a steady baseline was obtained. The detector was zeroed at a higher wavelength than that used for detection such that the baseline absorbance at the detection wavelength is around 0.5 absorbance units. All chromatographs were performed at ambient conditions with a flow rate of 1.0 mL/min unless otherwise stated. Capacity factors were determined as defined by:

$$k' = (t_r - t_0)/t_0$$

where t_r is the retention time of the analyte peak and t_0 is the first perturbation in the baseline after injection of water.

pK_a Determinations

The pK_a's of alendronate dimer and 2-phosphonopyrrolidine were determined through potentiometric titrations using a Metrohm 665 dosimat and a 670 titroprocessor (Brinkmann, Westbury NY).

RESULTS AND DISCUSSION

System Optimization

The high ionic character of the diphosphonates render them incapable of being retained strongly on a reversed phase column without some form of derivatization. Adjustment of pH in order to protonate the phosphonate groups generally require pH conditions of less than 2 which is usually unacceptable for

these columns. They can be retained on silica based mixed mode columns but with very poor efficiency which is manifested in the form of very broad peaks. Polymer based anion-exchange columns provide mixed mode interactions without the tailing observed with silica based mixed mode columns. There are two types of sites available on polymer based anion exchange columns - hydrophobic and ion exchange sites. Separation is affected by ion exchange and by interaction of the organic analyte ion with the non-polar polymeric backbone provided the analyte ion possesses a hydrophobic moiety (31,32).

When using IPD, both the retention and the detection capabilities are dependent upon the nature of the mobile phase anion. Sensitivity is dependent upon the concentration of the highly absorbing mobile phase anion (28,33). The noise is directly proportional to the absorbance of the mobile phase anion and the absorbance increases with increasing mobile phase anion concentration. The signal is proportional to the concentration of the analyte anion and the difference in absorptivity between the analyte and the mobile phase anions. The signal to noise ratio is therefore inversely proportional to the concentration of the mobile phase anion. Specifically, sensitivity increases with decreasing concentration of the mobile phase anion. It is advantageous therefore to have a relatively strong displacing anion in the mobile phase so that one can obtain short elution times at lower concentrations while maximizing sensitivity for the analyte ions.

The optimal wavelength for detection is determined by two factors. First, it is best to choose a wavelength where the difference in the molar absorptivity of the mobile phase and the analyte anions is at its greatest (ideally at a maximum for the mobile phase anion and a minimum for the analyte anion). Secondly, the absorbance at this wavelength should not be too high as to saturate the detector.

Preliminary experiments using sulfobenzoic acid and p-hydroxybenzoic acid, which are strong displacing agents with strong chromophores, exhibited poor

selectivity for the separation of the organophosphonates. Trimesate ion, which is also a strong displacing agent with a strong chromophore, was chosen because it exhibited better selectivity.

The retention times of the five organophosphonates were monitored as a function of pH and trimesate concentration. The optimum mobile phase conditions were found to be 1 mM trimesic acid adjusted to pH 5.5 (Fig. 2). The samples were chromatographed under ambient conditions with a flow rate of 1 mL/min . There is minimal difference in the efficiency of the analyte peaks for flow rates varying from 0.25 to 1 mL/min. Detection was at 254 nm. Under these conditions, the method also resolves chloride, phosphite and methanesulfonate (byproducts from the synthesis of alendronate sodium) along with the five organophosphonates being studied (Fig. 3). The method also resolves unidentified thermal degradates from alendronate (Fig. 4).

Under the above conditions a linear detector response was observed from 0.1 mg/mL to 2 mg/mL of the five organophosphonates (correlation coefficients

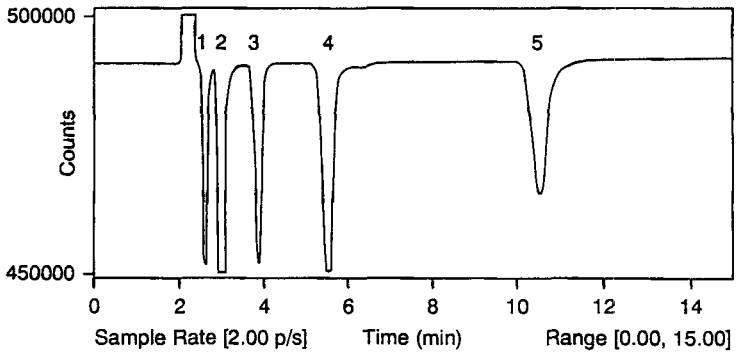

Fig. 2: Separation of the organophosphonates. Chromatographic conditions are 1 mM trimesic acid adjusted to pH 5.5 with detection at 254 nm. 1 - 2-phosphonopyrrolidine; 2 - alendronate; 3 - alendronate dimer; 4 - etidronate; 5 - clodronate.

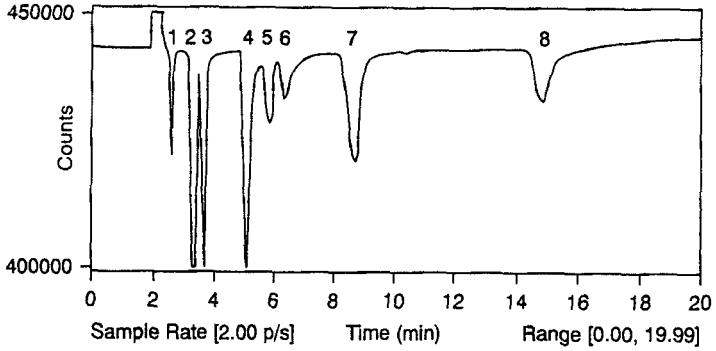

Fig. 3: Separation of the organophosphonates and process related components from the synthesis of alendronate. Chromatographic conditions are 1mM trimesic acid adjusted to pH 5.5 with detection at 254 nm. 1 - 2-phosphonopyrrolidine; 2 - alendronate; 3 - phosphite; 4 - chloride; 5 - methanesulfonate; 6 - alendronate dimer; 7 - etidronate; 8 - clodronate.

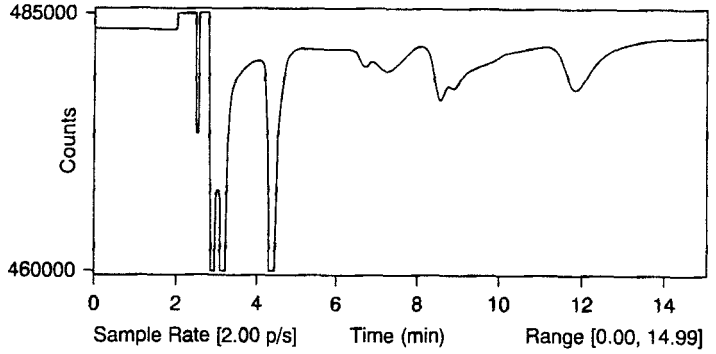

Fig. 4: Separation of alendronate from its thermal degradates. Chromatographic conditions are 1 mM trimesic acid adjusted to pH 5.5 with detection at 254 nm.

were all greater than 0.9997) and the limits of detection ranged from 50 ng for 2-phosphonopyrrolidine to 250 ng for alendronate dimer. The detection limits of the organophosphonates are an order of magnitude greater than that of species possessing small ionic masses such as Cl^- (20 ng) and this is a reflection of their larger ionic masses. If the analyte and displacing ion are of the same charge, each analyte ion replaces one ion of the mobile phase ion in the localized zone (in order to preserve electroneutrality) . Consequently, the response on a molar ionic basis should be the same for any analyte irrespective of any other properties of the analyte and the detection limit, on a mass basis, will be lower for analytes of small ionic mass.

Effect of Mobile Phase Anion Concentration on the Retention of the Analytes

The interaction between the mobile phase displacing anion and the analyte anion and their distribution between the stationary phase and the mobile phase can be described by:

$$yS_xM + xA \rightleftharpoons xS_yA + yM \quad \text{---------- K1} \quad \{1\}$$

where

y = charge on the analyte anion

x = charge on the mobile phase anion

M = mobile phase anion

S = stationary phase cation

A = analyte anion

Small and Miller (22) has previously derived the relation between the capacity factor and the mobile phase anion concentration at constant pH which is expressed as:

$$[S_yA]^x/[A]^x = K1[S_xM]^y/[M]^y \quad \{2\}.$$

Assuming that the stationary phase is saturated with respect to the mobile phase anion, its concentration can be assumed to be constant and:

$$[S_yA]/[A] = B/[M]^{y/x} \quad \{3\}$$

and

$$\log k' = -(y/x)\log[M] + \log C \quad \{4\}$$

where B and C are constants. Thus a logarithmic plot of capacity factor versus mobile phase ion concentration should give a straight line with a slope that is the ratio of the charges on the analyte and the mobile phase ion. This equation applies to a number of species eluted by various mono- and multivalent mobile phase ions (22,28,34).

The capacity factors of the five organophosphonates along with chloride ion were measured as a function of trimesate ion concentration at a constant pH of 5.0. At this pH the organophosphonates are each present as predominantly one solute species (Table 1). The logarithmic plot of capacity factor versus trimesate ion concentration proved to be linear for all six species (Fig. 5).

A slope of -0.5 was observed for chloride. Since chloride possesses a charge of negative one, the slope indicated (from Equation 4) that the displacing ion was the double charged trimesate species rather than the triple charged species at this pH. However, the pKa's for trimesic acid (Table 1) indicates that, at pH 5, the triple charged species should be the dominant trimesate species. One would thus anticipate that the triple charged species would be the displacing ion. The observed behavior can be explained by elaborating on Equation 4. Assuming the presence of a protonated species HM in the system, its interaction with the analyte can be expressed similarly to Equation 1:

$$yS_{x-1}HM + (x-1)A \rightleftharpoons (x-1)S_yA + yHM \quad \text{---------- K2} \quad \{5\}$$

TABLE 1

Acid Dissociation Constants

	pK1	pK2	pK3
Trimesic Acid (36)	3.10	3.90	4.70
Alendronate (37)	< 2	6.2	9.9
Alendronate Dimer	< 3	4.1	> 9
2-Phosphonopyrrolidine	< 3	5.6	
Etidronate (38)	< 2	2.5	6.89
Clodronate (38)	< 2	2.3	5.82

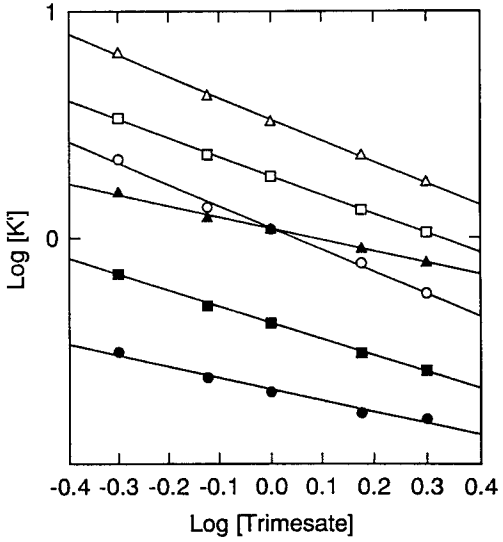

Fig. 5: Plot of log capacity factor vs log trimesate (mmolar) concentration at pH 5.0. Δ - clodronate; □ - etidronate; ○ - alendronate dimer; ▲ - chloride; ■ - alendronate; ● - 2-phosphonopyrrolidine.

In addition the interaction between the two anions, M and HM, in the mobile phase and in the stationary phase can be expressed as:

$$x\text{-}1S_xM + xH^+ + M \rightleftharpoons xS_{x\text{-}1}HM \text{ ---------- } K3 \quad \{6\}$$

and

$$H^+ + M \rightleftharpoons HM \text{ ---------- } K4 \quad \{7\}$$

Equation 7 indicates that at a fixed pH, the ratio of M to HM remains constant and is independent of total mobile phase anion (M_T). However such a linear relationship does not exist between S_xM and $S_{x\text{-}1}HM$. It is evident that even at constant pH, the relative amounts of S_xM and $S_{x\text{-}1}HM$ will vary in a non-linear fashion relative to the concentration of M_T.

Equations 1 and 5 can be combined such that:

$$K5 = K1 * K2 = ([S_yA]^{2x\text{-}1} [M]^y [HM]^y) / ([A]^{2x\text{-}1} [S_xM]^y [S_{x\text{-}1}HM]^y) \quad \{8\}$$

and

$$[S_yA]/[A] = (D [S_xM] [S_{x\text{-}1}HM]/[M] [HM])^{y/(2x\text{-}1)} \quad \{9\}$$

Where D is a constant. If the stationary phase is saturated with respect to S_xM only, then S_xHM and HM can be expressed in terms of S_xM and M by using Equation 3 and 4 which leads to:

$$k' \alpha ([S_xM]/[M])^{y/x} \quad \{10\}$$

Since the stationary phase is saturated with respect to S_xM, its concentration can be considered constant and thus:

$$\log k' = -y/x\log[M] + E \quad \{11\}.$$

Similarly, if the stationary phase is saturated with respect to S_xHM only, it can be shown that

$$k' \alpha ([S_{x-1}HM]/[HM])^{y/(x-1)} \quad \{12\}$$

and

$$\log k' = -y/(x-1)\log[HM] + F \quad \{13\}$$

where E and F are constants. If the stationary phase is saturated with respect to both S_xM and $S_{x-1}HM$, then:

$$\log k' = 2y/(2x-1)\log[M] + G \quad \{14\}$$

where G is a constant.

The slope observed for 2-phosphonopyrrolidine was -0.54 and for etidronate, clodronate and alendronate dimer -0.83, -0.93 and -0.95. Taking into account the pKa's of these species, the observed slopes are more in line with the theory that the doubly charged trimesate species is the species which is being exchanged with the analyte ion on the stationary phase. The only deviation was noted for alendronate which possessed a slope of -0.70 despite the fact that it would be predominantly singly charged at this pH.

Further evidence is provided by the data generated by Motimizu et al (28) using trimesate ion as the displacing ion. They observed that, using a IC-Anion PW column, slopes of -0.40, -0.39, and -0.73 were obtained for chloride, nitrate, and sulfate ion respectively. Similarly, using a IC Anion SW column, slopes of -0.46, -0.42, and -0.75 were obtained for chloride, nitrate, and sulfate ion respectively. These results are more compatible with the assumption that the

stationary phase is saturated with both negative two and three charged trimesate species than the assumption they made (saturation with respect to only a negative three charged trimesate ion). Saturation with respect to only the negative three charged trimesate ion would have given slopes of -0.33 for chloride and nitrate and a slope of -0.67 for sulfate. Saturation with respect to both the negative two and three charged trimesate would give slopes of -0.4 for chloride and nitrate and -0.8 for sulfate. No pH data was presented for their work, but their experimental section does indicate that they used just enough base to neutralize the trimesic acid which would place their pH range from 5 to 7. The authors also performed studies with many other carboxylate ion additives in the mobile phase and found similar results.

The dependence of the capacity factor on the mobile phase concentration was then investigated at a higher pH (6.0). The analytes used were chloride, alendronate dimer and etidronate as they would be present as predominantly one species at this pH. The slopes were found to be similar to those observed at pH 5.0 (Table 2).

Logarithmic plots of capacity factor versus displacing ion concentration can be applied to facilitate method development for the separation of ionic species. The retention time of the ionic species for which separation is desired can be monitored at two different concentrations and their retention times can be predicted from the line which joins the two points. The approximate region where separation is optimized (the concentration at which the species do not co-elute and are at maximal separation) can then be determined.

Effect of pH on the Retention of the Analytes

The dependence of the capacity factor of the organophosphonates on the pH of the mobile phase at constant mobile phase anion concentration was

TABLE 2

Slopes For log Trimesate Concentration vs log Capacity Factor

	pH 5.0	pH 6.0
Chloride	-0.50	-0.50
2-Phosphonopyrrolidine	-0.54	
Alendronate	-0.70	
Alendronate dimer	-0.95	-0.96
Etidronate	-0.83	-0.91
Clodronate	-0.93	

investigated. The capacity factor was monitored while varying the pH from 3.3 to 10 (Fig. 6). The capacity factor initially decreased with increasing pH indicating that deprotonation of the trimesic acid led to stronger displacing capability due to higher ionic strengths. A minima was observed at pH 4.5 after which the capacity factors again increased. This minima lends credence to the theory that the double charged species is the displacing agent since it occurs in the region where the double charged species is at its maximum concentration. The increase in the capacity factor can also be attributed to the deprotonation of the analyte ions which would cause them to become more tightly bound to the stationary phase. However such a correlation requires that the area where a minimum occurs should vary with the analyte and should also appear at a higher pH than 4.5. This factor thus appears to be minimal.

Effect of Organic Modifier on the Retention of the Analytes

Since the stationary phase is polymer, there are two possible types of interactions - ion exchange and adsorption. If adsorption plays a major role in the interaction with analyte ions, the addition of an organic modifier would affect the separation. Specifically the retention time of an analyte ion should decrease if

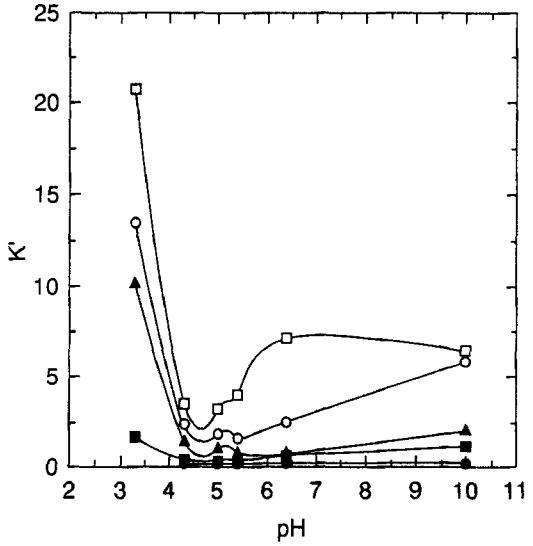

Fig. 6: Influence of pH on capacity factor. □ - clodronate; ○ - etidronate; ▲ - alendronate dimer; ■ - alendronate; ● - 2-phosphonopyrrolidine.

there was some type of adsorption interaction in effect. The retention time of an analyte ion could also increase if its retention is due primarily to ion exchange. As the mobile phase becomes more nonpolar the ion exchange sites would become more polar relative to the mobile phase increasing its attraction for species with little or no hydrophobic character and thus increasing their retention (35). It was indeed observed that chloride, which would have no hydrophobic interaction with the stationary phase, is increasingly retained with increasing percentage of acetonitrile in the mobile phase. The addition of 10% acetonitrile to the mobile phase decreased the retention of 2-phosphonopyrrolidine and alendronate and increased the retention of alendronate dimer and etidronate affecting a separation of the previously co-eluting alendronate and alendronate dimer (Figure 7). Increased amounts of acetonitrile up to 40% results in increased retention for 2-phosphonopyrrolidine, alendronate, alendronate dimer, and etidronate.

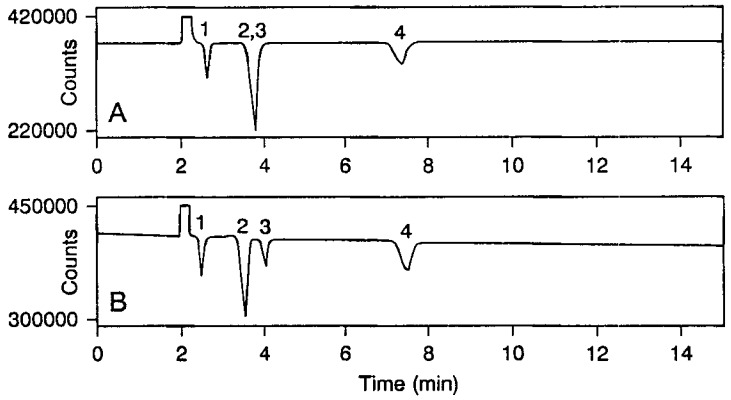

Fig. 7: Influence of organic modifier on retention of analytes. 1 mM trimesic acid adjusted to pH 6.5. 1 - 2-phosphonopyrrolidine; 2 - alendronate; 3 - alendronate dimer; 4 - etidronate. A: No organic modifier. B: Addition of 10% acetonitrile.

CONCLUSION

An ion chromatographic method has been developed for the separation of organophosphonates. This method utilizes trimesate ion as the displacing ion with indirect UV detection. It is suitable for the separation and detection of organic species which possess high ionic character and which do not possess an appreciable chromophore. It has also been demonstrated that a linear relationship exists between the log capacity factor of the analyte species and the log concentration of the displacing ion. This relationship is dependent on the charge on the analyte anion and the displacing anion and can be utilized to facilitate method development for the optimum separation of ionic species.

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