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High-performance liquid chromatographic method for simultaneous determination of clodronate and some clodronate esters

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ABSTRACT

A quaternary alkylmethylamine-bonded stationary phase has been used for the liquid chromatographic resolution of bisphosphonates. Clodronate and three of its esters were separated by this technique. Nitric acid (30 m*M*) was used as the mobile phase. The effect of the pH of the mobile phase on the retention, resolution, capacity factor and theoretical plates of the column was examined. Thorium–ethylenediaminetetraacetic acid–xylenol orange mixed ligand complex was used as a postcolumn reagent for bisphosphonates. Bisphosphonates react quantitatively with this complex in slightly acidic solutions, and a change in the absorbance of postcolumn reagent is used as a measure of the bisphosphonate concentration. The relative standard deviation (R.S.D.) values for samples in aqueous solutions were in the range 2.3–15.5% (area) and 1.7–5.9% (height). The detection limits for different compounds, C_{min}, varied from 0.3 to 1.4 mg/l (area) and from 0.3 to 0.5 mg/l (height). In urine samples the R.S.D. (%) varied from 3.1 to 18.9 (area) and from 1.1 to 6.3 (height). The linear dynamic range was from the detection limit up to 16 mg/l.

INTRODUCTION

Clodronate, disodium (dichloromethylene)bisphosphonate tetrahydrate (Na_2Cl_2MBP), and its esters belong to a group of geminal bisphosphonic acids of the type P-C-P [1–4]. Chemically bisphosphonates are related to pyrophosphates, and mimic their physiological behaviour. The most important feature of both classes of ligands is a very high affinity for calcium(II) in homogeneous solutions and at surfaces of minerals and bone. However, owing to the stability of bisphosphonates *in vivo*, many of these bisphosphonates are active as drugs in physiological conditions where inorganic pyrophosphates are rapidly enzymically hydrolysed by phosphatases [5]. Clodronate, together with conventional cancer therapy, has been used successfully for the treatment of hypercalcaemia related to osteolytic metastases and malignancies [6,7]. Clodronate has also yielded good results in the treatment of Paget's disease and primary hyperparathyroidism.

Several high-performance liquid chromatographic (HPLC) methods have been proposed for the determination of bisphosphonates, including reversed-phase ion-pair chromatography with refractive index or flame photometric detection [8,9], ion chromatography with postcolumn reaction and UV–visible detection [10], ion-exchange chromatography with refractive index or flame photomeric detection [11,12]; also bisphosphonates have been oxidized to orthophosphates and detected with a UV–visible detector as molybdenum blue [13], and postcolumn derivatization with fluorescence detection has also been used [14]. Some of these methods lack data for urine

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sample analysis [8,10,12,13]. Also data on the separation of different phosphonates is lacking [8,9]. The limitation of ion-exchange chromatography with flame photometric detection is the use of precipitation and the need to determine the precipitation recovery with the internal standard ^{14}C-labelled Cl_2MBP by scintillation counting [11]. Fluorescence detection [14] seems to be the best method of these, but because a fluorescence detector is not included in the conventional HPLC apparatus, this might limit the wider use of this method.

The aim of the present study was to develop a new, selective and sensitive HPLC method using conventional HPLC apparatus and postcolumn derivatization for the simultaneous determination of clodronate and its esters in aqueous and urine solutions. The esters are potential new pharmaceuticals, and no HPLC determination data are available. Recently, we reported an HPLC method for the determination of clodronate in aqueous solutions [15].

EXPERIMENTAL

Instrumentation

The chromatographic system consisted of a Spectra Physics (Santa Clara, CA, USA) SP 8700 solvent delivery system with a precision loop injector with a nominal volume of 100 μl (stainless steel), an HPLC AG7 (50 × 4 mm I.D., particle size 10 μm) anion-exchange guard column (Dionex, Sunnyvale, CA, USA), an HPIC AS7 (250 × 4 mm I.D., particle size 10 μm) anion-exchange column (Dionex), a postcolumn pump from Perkin Elmer (Norwalk, CT, USA) (Isocratic LC Pump 250), a postcolumn reactor unit, a Spectra Physics SP 8440 UV–visible detector, and a Hitachi (Tokyo, Japan) D-2000 Chromato-integrator. Ultrapure water was obtained from distilled water by a Water-I Model D 2200 (Barnstead, Division of Sybron, Boston, MA, USA). A Dionex DX-300 HPLC system was also used, consisting of a variable-wavelength detector (VDM-2) and an eluent degassing module (EDM-2), from Rheodyne (Cotati, CA, USA) an automatic sample injector (Model 9126-038) with a precision loop with a nominal volume of 100 μl (polyetheretherketone, PEEK), an HPIC AG7 (50 × 4 mm I.D., particle size 10 μm) anion-exchange guard column (Dionex), an HPIC AS7 (250 × 4 mm I.D., particle size 10 μm) anion-exchange column (Dionex), a postcolumn pump from Perkin Elmer (Isocratic LC Pump 250), a postcolumn reactor unit, and a Hitachi D-2000 Chromato-integrator.

Chemicals and reagents

Clodronate, disodium (dichloromethylene)bisphosphonate tetrahydrate (Na_2Cl_2MBP), was obtained from Leiras Oy (Turku, Finland). Esters of clodronate, monomethyl clodronate trisodium salt, monoethyl clodronate disodium salt, monoisopropyl clodronate trisodium salt, P,P-diisopropyl clodronate disodium salt, P,P′-diisopropyl clodronate disodium salt and triisopropyl clodronate monosodium salt, were also obtained from Leiras Oy. $Th(NO_3)_4 \cdot 4H_2O$ and ethylenediamine (EDA) were obtained from Fluka (Buchs, Switzerland). Ethylenediaminetetraacetic acid (EDTA) and xylenol orange (XO) were obtained from Merck (Darmstadt, Germany). Acids were obtained as analytical grade from Merck. Only ultrapure water (distilled and deionized) was used.

Procedures

The HPLC column was conditioned by washing it with water and then with at least 200 ml of degassed mobile phase.

The following reagent solutions were prepared:

(1) Thorium solution (4.0 m*M*): 1.104 g of $Th(NO_3)_4 \cdot 4H_2O$ was dissolved in 20 ml of 2.0 *M* nitric acid and diluted to 500 ml with distilled water. Further dilution was made to produce a 0.2 m*M* solution.

(2) EDTA solution (0.4 m*M*): 0.074 g of EDTA was dissolved in 500 ml of water.

(3) EDA buffer solution (0.6 *M*): 10.1 ml of EDA was added to ice-cold water, and 15 ml of concentrated hydrochloric acid were added slowly with stirring. The temperature was kept below 20°C. The pH was adjusted to 7.3 with dilute hydrochloric acid, and the solution was diluted to 250 ml with water.

(4) XO indicator solution (0.05%): 50 mg of xylenol orange were dissolved in 100 ml of water.

(5) Thorium postcolumn reagent: 4.5 ml of 0.2 m*M* Th solution was taken, and 100 ml of 0.4 m*M* EDTA solution were added and mixed. Then 60 ml of 0.6 *M* EDA buffer solution and 1.9 ml of XO solution were added, and the solution was shaken vigorously. The pH was adjusted with nitric acid solution to the desired value. The solution was diluted up to 500 ml with water. The postcolumn reagent solution was allowed to stand for at least 1 h before use, in order to ensure the complete formation of the Th–EDTA–XO complex.

(6) Mobile phase: 30 m*M* nitric acid solution was used as the eluent, and the pH was adjusted with dilute NaOH.

Mobile phase solutions were degassed before use by passing helium through them at lowered pressure for 15–20 min. The pH of the mixtures of mobile phase and postcolumn solutions was measured potentiometrically, and was adjusted to 6.0–6.3 by changing the pH of the PCR solution.

Standard clodronate solutions were prepared by dissolving a known amount of clodronate in water. Standard ester solutions were prepared by dissolving a known amount of ester in water.

Urine samples were prepared by adding a known volume of standard clodronate and/or ester solution to known volume of urine. Urine samples were injected into the column without any pretreatment or dilution.

The postcolumn reactor unit consisted of a T-piece and a PEEK tube (1.2 m × 0.25 mm I.D.) to ensure adequate reaction time before reaching the detector for the reaction between the eluent (analytes) and Th–EDTA–XO to take place.

The flow-rates of both the mobile phase and the postcolumn reagent were 1.0 ml/min. Absorbances were measured at 550 nm.

RESULTS AND DISCUSSION

This method for the separation and determination of bisphosphonate includes the liquid chromatographic separation of clodronate and some of its esters on an anion-exchange column with a mobile phase of nitric acid, followed by UV detection using a postcolumn technique.

In ion-exchange chromatography, the elution of the analytes depends on the relative affinities of these analytes for the ionogenic groups on the resin, as well as on the ionic concentrations in the mobile phase. The mobile phase was optimized by changing the pH of the eluent and keeping constant the nitrate concentration and the flow-rate of the mobile phase and postcolumn reagent (Fig. 1). The nitrate concentration in the mobile phase was optimized with clodronate [15]. A decrease of the nitrate concentration in the mobile phase increased significantly the retention time of clodronate, and the peak was broad and poorly shaped.

The effect of the pH on the sensitivity of detection was also tested. If the pH of the mixture of postcolumn reagent and mobile phase was below 6.0, the sensitivity of detection was drastically decreased or the reaction did not happen. The signal-to-noise ratio also decreased. At pH >6.3, the linearity of detection was less than at $6.0 <$ pH < 6.3. The sensitivity seemed to be poorer, perhaps because XO has a pK_a value of 6.4, and a part of free XO is red–violet coloured even at pH 6.3. The pK_a value of 6.4 also means that the free indicator (XO) is yellow (below 6.4) and the metal complexes are red–violet [16].

Because the pH of the mixed solution (mobile phase and postcolumn reagent) must be in the pH range 6.0–6.3, the pH of the postcolumn reagent was changed simultaneously with the pH of the mobile phase, even though the postcolumn reagent solution was buffered with EDA, to produce the desired pH value for the mixed solution. The change in the pH of the eluent effected the concentration of different acidic species of the studied compounds [17,18]. The retention time of a compound on an anion-exchange column is dependent on which acidic form it takes [15].

The detection technique is modified from the indirect spectrophotometric method proposed by Pribil and Vesely [19]. The method is based on the ligand-exchange reaction of bisphosphonate with the Th–EDTA–XO complex. The equilibria taking place are assumed to be [20]:

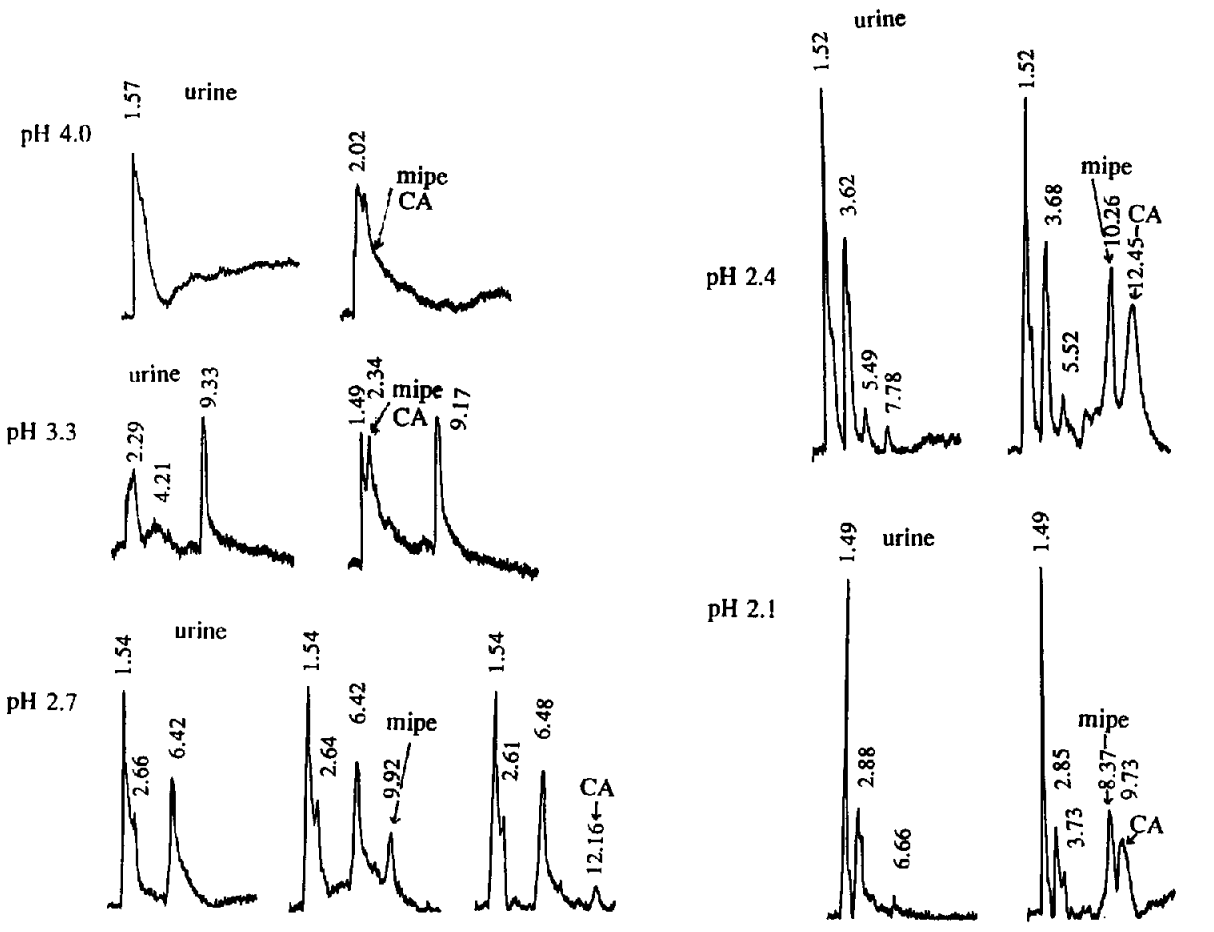

Fig. 1. Effect of the pH of the mobile phase on the separation and resolution of clodronate (CA) and its monoisopropyl ester (mipe) in urine. Chromatograms of urine and urine + compounds.

$$\mathrm{Th} + \mathrm{EDTA} \xrightarrow[\text{pH 2.5–3.5}]{} \mathrm{Th\text{–}EDTA} \xrightarrow[\text{pH 6.0–6.3}]{\text{XO}} \underset{\text{(red–violet)}}{\mathrm{Th\text{–}EDTA\text{–}XO}}$$

$$2\ \mathrm{Th\text{–}EDTA\text{–}XO} + \mathrm{Na_2bisphosphonate} \longrightarrow \underset{\text{(yellow)}}{\mathrm{Th_2(EDTA)_2(bisphosphonate)}} + 2\ \mathrm{XO}$$

The postcolumn reagent, Th–EDTA–XO, is mixed with the eluent and a steady baseline due the background absorption is provided by the Th–EDTA–XO complex at the observation wavelength. When an analyte, such as clodronate, elutes from the column, it reacts with Th–EDTA–XO to form a new mixed-ligand complex, and the absorbance is observed to decrease in the analyte zone. Therefore, negative absorbance peaks at the measured wavelength are obtained for the analytes that can react with the Th–EDTA–XO complex. The response factors vary for each analyte. The best signal-to-noise ratio cannot be observed at the λ_{max} (580 nm) of the postcolumn reagent. The best-shaped chromatograms and the best signal-to-noise ratio were obtained at 540 and 550 nm, and 550 nm was selected as the observation wavelength.

This method can be used to separate and determine clodronate and one of its three esters simultaneously in both aqueous and urine solutions. The three esters (monoisopropyl ester, monomethyl ester and monoethyl ester) cannot be separated from each other with this method because of their structural and chemical similarities. Symmetrical di- and unsymmetrical di- and triisopropyl esters of clodronate showed no response on the chromatogram. This is due to their weaker capability to undergo ligand-exchange reaction with the thorium postcolumn reagent.

In aqueous solution, each ester and clodronate can be determinated individually when the pH of the mobile phase is between 1.8 and 4.0. Better precision was obtained in both aqueous solution and urine, when peak-height measurements (R.S.D. 1.1–6.3%) were used instead of peak-area measurements (R.S.D. 2.3–18.9%). Also, the linearity of the calibration graph was better when the peak height was used. This is clearly seen in the corresponding R.S.D. values (Table I). The within-day reproducibility is good, but each time the mobile phase or the postcolumn reagent is renewed or changed, recalibration must be performed because the method is sensitive to changes in the concentration of the mobile phase and the Th–EDTA–XO complex in the postcolumn reagent. Daily calibration is needed for good between-day reproducibility. The detection limit for different compounds, C_{min}, varied from 0.3 to 1.4 mg/l (area) and from 0.3 to 0.5 mg/l (height). C_{min} was calculated from the equation [21] $C_{min} = [C_X \times 2s(X)]/\bar{x}$, where C_X is the concentration of the compound used in measurement, $s(X)$ is the standard deviation of the peak area or height, and $\bar{x}$ is the average peak area or height of four measurements.

The amount of clodronate found in the urine of patients usually varies between 2 and 100 mg/l [22]. The studied esters have not yet been used in clinical treatment, but their amounts are likely to

TABLE I

DETERMINATION OF CLODRONATE AND ESTERS

Compound	Amount (mg/l)	R.S.D. (%)		C_{min} (mg/l)	
		Area	Height	Area	Height
Aqueous solutions					
Clodronate	19.2	6.9	3.0		
	12	5.3	1.7	1.26	0.40
Monomethyl ester	18	7.2	2.2		
	12	5.9	3.4		
	6	11.6	2.2	1.39	0.27
Monoethyl ester	12	9.2	2.5		
	6	2.3	3.3	0.28	0.40
Monoisopropyl ester	12	4.6	3.5		
	8	7.6	4.0		
	4	15.5	5.9	1.24	0.47
Urine solutions					
Clodronate	12.8	6.1	6.3	1.56	1.61
Monomethyl ester	12[a]	3.1	2.8		
	9.6	9.7	2.7		
	6[a]	7.0	1.4		
	4[a]	18.9	1.1	1.51	0.10
Monoethyl ester	9.6	4.4	3.6	0.84	0.69
Monoisopropyl ester	9.6	11.3	2.0	2.16	0.38

[a] Measured with Dionex DX-300 HPLC system.

TABLE II

RETENTION PARAMETERS AND RESOLUTION BETWEEN CLODRONATE AND ESTERS

Compound	Aqueous solutions		Urine solutions	
	pH	R_s[a]	R_s[a]	k'(urine)[b]
Monoisopropyl ester	4.0	1.55	–[c]	–[c]
	3.3	1.09	–[c]	–[c]
	2.7	1.09	–[d]	5.66
	2.4	1.00	1.07	5.89
	2.1	0.88	0.93	4.85
	1.8	0.69	–[e]	3.89
Monomethyl ester	4.0	2.06	–[c]	–[c]
	3.3	1.38	–[c]	–[c]
	2.7	1.77	–[d]	5.50
	2.4	1.09	1.09	5.74
	2.1	1.01	0.54	4.80
	1.8	0.62	–[e]	4.08
Monoethyl ester	4.0	1.98	–[c]	–[c]
	3.3	1.29	–[c]	–[c]
	2.7	1.33	–[d]	5.45
	2.4	1.23	1.02	5.72
	2.1	1.12	0.88	4.65
	1.8	0.81	–[e]	3.90
Clodronate	4.0			–[c]
	3.3			–[c]
	2.7			7.16
	2.4			7.36
	2.1			5.82
	1.8			5.18

[a] Resolution between clodronate and ester.

[b] Capacity factor $k' = (t_r - t_0)/t_0$, where t_0 = 1.49 min. Flow-rate, 1 ml/min for both mobile phase and postcolumn reagent.

[c] Complexation in urine hinders separation.

[d] Partial complexation in urine.

[e] Ester not separated sufficiently from urine matrix.

be close to that of clodronate. Several different urine samples spiked with clodronate and/or esters were examined. R.S.D. values (three or four determinations) for eight samples were determined (Table I). Two samples spiked with monomethyl ester were determined against the calibration graph of urine samples spiked with monomethyl ester. Sample I (real value 6.80 mg/l) was found to contain 6.83 mg/l monomethyl ester (three determinations, R.S.D. 1.2%) and sample II (9.78 mg/l) was found to contain 10.40 mg/l monomethyl ester (one determination). The calibration graph was linear over the whole range from 16 mg/l down to the detection limits. The correlation coefficient, r, was 0.99986, and the detection limit, C_{min}, was 1.51 mg/l (area) and 0.10 mg/l (height). The linear dynamic range covers well the minimum concentration (2 mg/l) to be determined in practical urine samples. More concentrated samples (> 16 mg/l) can be diluted. The method seems to be sensitive enough for the analysis of urine.

Table II indicates that clodronate and its monoisopropyl ester can be determined simultaneously in aqueous solutions in the pH range 4.0–2.4 for the mobile phase, with the resolution greater than 1.0 (Fig. 2). Resolutions greater than 1.0 are usually sufficient for accurate quantification [23]. Monomethyl ester and clodronate can be determined simultaneously at 4.0 > pH > 2.1. Monoethyl ester and clodronate can be determined at the same pH range, 4.0–2.1.

Fig. 1 indicates that, when the pH of the mobile phase is 4.0 or 3.3, clodronate and its esters are likely to react with some substances in urine because they elute faster than in aqueous solutions. They seemed to elute with substances that cause main peaks in chromatogram. At pH 2.7, the peaks of clodronate and its esters were separated from urine substances, but their responses were weaker than expected. Clodronate and its esters seem partly to form compounds with some urine substances. At pH 2.7 clodronate and its monoisopropyl ester were injected individually because the clodronate peak was not clearly separated when they were injected simultaneously. The nature of the interaction of clodronate and its monoisopropyl ester at pH 2.7 or higher was not investigated. At pH 2.4 and 2.1, clodronate and the ester were separated from the urine matrix, and did not show any reactions with the urine components that might interfere with the determination.

In urine samples, clodronate and its esters can be separated and determined simultaneously at pH 2.4, with resolutions varying from 1.02 to 1.09. At pH 2.1, the resolution between clodro-

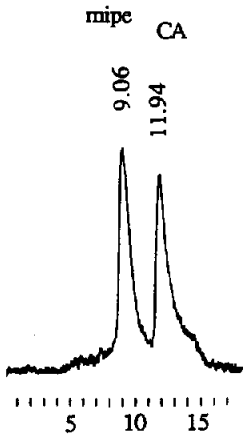

Fig. 2. Separation of the monoisopropyl ester of clodronate (mipe, 9.6 mg/l) and clodronate (CA, 12 mg/l). Mobile phase, 30 m*M* nitric acid, pH adjusted with sodium hydroxide. Flow-rate, 1 ml/min for both mobile phase and postcolumn reagent.

nate and its esters were not sufficient for simultaneous determination. The esters were not separated sufficiently from the urine matrix at pH 1.8.

The number of theoretical plates, N, was calculated, based on the length of the column (0.25 m). The deviation in N between determinations at the same pH was always below 18%. Fig. 3 shows that variation of the pH of the mobile phase only slightly affects N, and the effect is not linear.

Fig. 4 shows that capacity factors, k', for clodronate and its esters remain nearly constant in pH range 2.4–4.0. The variations in k' in aqueous solutions shown in Fig. 4 correlate well with the resolution values shown in Table II between clodronate and esters in this pH range. The three esters studied have quite similar values of k', which explains the poor (at best) separation between them. A decrease in the pH of the mobile phase from 2.4 to 1.8 causes a marked decrease in the capacity factors for all compounds. Also, the difference in k' for clodronate and it esters decreases significantly. This correlates with the decrease in the resolution values indicated in Table II in the same pH range. The decrease in k' values at this pH range is probably due to the acid–base equilibria of the compounds, *i.e.* a change from one acidic form to other occurs [17,18], which affects the ability of the anion-exchange column to retain the analyte.

CONCLUSION

The analytical method described is sensitive enough and selective for the simultaneous determination of clodronate and one of its esters in aqueous solutions and urine. This is sufficient for the method to be useful in clinical analysis, because patients are treated with clodronate and a maximum of only one ester at the same time [22].

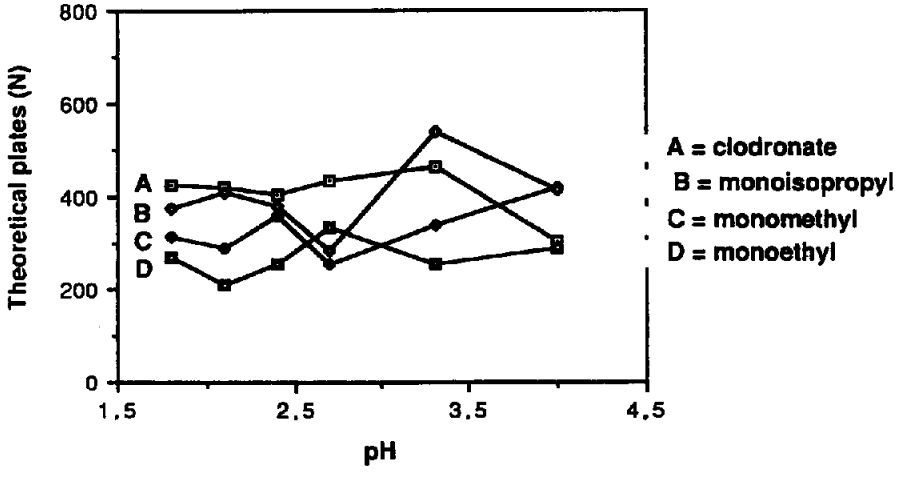

Fig. 3. Effect of the pH of the mobile phase on the number of theoretical plates (N). Peak efficiency in theoretical plates, calculated with the equation $N = 41.7(t_r/W_{0.1})^2 / [(B/A) + 1.25]$, where $W_{0.1}$ is the width at 10% of the height and B/A is the asymmetry factor [24]. Calculated to the length of the column (0.25 m).

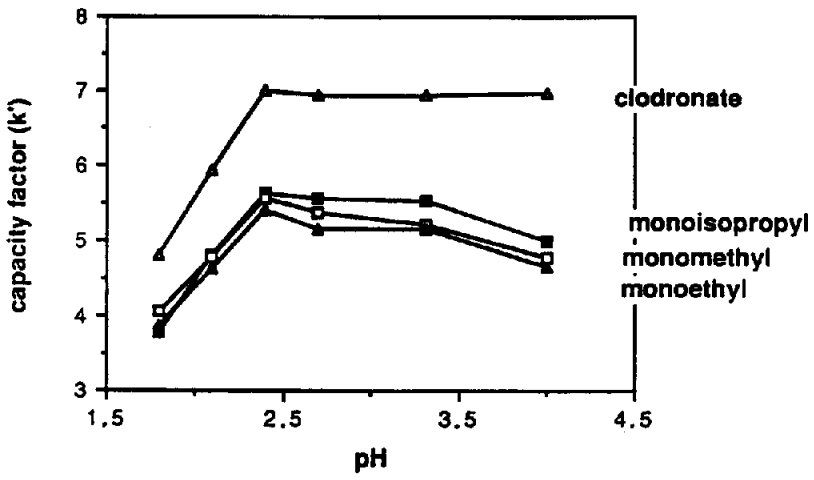

Fig. 4. Effect of the pH of the mobile phase on the capacity factor (k') in aqueous solution. Capacity factor $k' = (t_r - t_0)/t_0$, where t_0 = 1.49 min. Flow-rate, 1 ml/min for both mobile phase and postcolumn reagent.

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