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Determination of impurities in clodronic acid by anion-exchange chromatography

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Abstract

A validated ion chromatographic method for the determination of anionic impurities in clodronic acid or disodium clodronate is described. Separations are performed by using an anion-exchange column (IonPac AS5) and a sodium hydroxide gradient. Impurities are detected by suppressed conductivity without the need for derivatisation. The most important variable affecting the separation was shown to be the column temperature.

Keywords: Pharmaceutical analysis; Clodronic acid; Bisphosphonates; Inorganic anions

1. Introduction

Clodronate (Fig. 1) (disodium clodronic acid or disodium dichloromethylene bisphosphonic acid) is one of the most investigated entities in the bisphosphonate class of drugs. The drug is registered for use in the effective management of hypercalcaemia and bone pain associated with skeletal metastases in patients with multiple myeloma or carcinoma of the breast and has been clinically used for over ten years [1–5]. Clodronate is also an attractive candidate for Paget's disease [6] and osteoporosis [7].

Clodronic acid (V) has also found use as a starting material for novel phosphonate analogues of adenosine 5′-triphosphate, which have potential as antithrombotic agents [8,9].

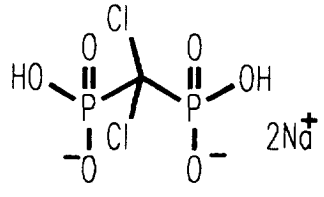

Fig. 1. Clodronate.

Clodronic acid is synthesised in a two-step reaction from tetraisopropylmethylenebisphosphonate (I) using sodium hypochlorite, followed by de-esterification with refluxing hydrochloric acid (Fig. 2) [10]. For the synthesis of nucleotide analogues, clodronic acid is subsequently neutralised with one equivalent of tributylamine to form the monotributylammonium salt.

Potential impurities arising from the synthesis of clodronic acid are chloride, the partial esters of (II), methylenebisphosphonic acid (VII) and monochloromethylenebisphosphonic acid (VI). Under basic conditions, degradation products may include phosphate and carbonylbisphosphonate [11].

Several chromatographic approaches have been proposed in the literature for the determination of clodronate, including gas chromatography–mass spectrometry (GC–MS) after extraction and derivatisation from urine [12], anion-exchange chromatography with flame photometric phosphorus-selective detection [13], anion-exchange chromatography with post-column derivatisation [14–16] and anion-exchange chromatography with indirect detection [17]. All of these procedures apart from that de-

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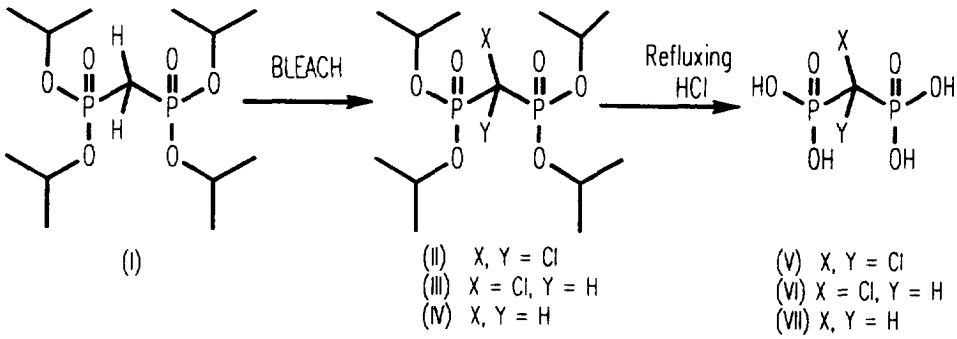

Fig. 2. Synthesis of clodronic acid.

scribed in Ref. [17] involve either post-column reactions where specialised equipment is necessary or pre-column derivatisation that requires time-consuming sample preparation.

Several chromatographic methods for other bisphosphonates also exist in the literature but, as with clodronate, most rely on either post-column reactions or pre-column derivatisation. Only one report in the literature dealt with direct detection; it described an assay of alendronate sodium (monosodium trihydrate salt of 4-amino-hydroxybutane-1,1-bisphosphonic acid) using anion-exchange chromatography with conductivity detection. As the procedures described were assays, little information was available on quantifying potential impurities.

As regulatory authorities typically require quantification and identification of drug impurities down to a level of 0.05 and 0.1% (w/w), respectively, before a drug product can be approved [18], the aim of this study was to develop a sensitive and selective high-performance liquid chromatography (HPLC) method using commercially available apparatus that could be used for either clodronate or for the quality control of monotributylammonium clodronate, which is used for nucleotide synthesis.

In order to develop an impurity method, it is necessary to ensure complete separation of the analyte from all likely process impurities, degradation products and possible anionic impurities. Whilst clodronic acid and its analogues, monochloromethylenebisphosphonic (VI) and methylenebisphosphonic (VII) acids are tetraprotic acids, other probable impurities may have charges varying from −3 (e.g. phosphate, triprotic ester of II) to −1 (e.g. chloride, nitrate, monoprotic ester of II). At the low pH values that have been used for assays [12–17], VI and VII are predominantly in the monovalent (−1) form, as are most of the other impurities: using a single isocratic mobile phase at low pH to separate such a complex mixture may prove difficult.

A pH gradient may prove more useful. An eluent of increasing acidity will first elute monoprotic acids, then, as their overall charges fall and they lose their affinity with the column, the bisphosphonates. However, the major obstacle to any approach is the lack of a useful UV chromophore in the molecules. Direct conductivity detection is not feasible, due to the high conductance of the acid eluents. Indirect detection, using nitric acid as the eluent, may be practical [17], however, an increasing concentration of nitric acid will cause an upward drift in the baseline, making integration difficult.

An alternative approach for clodronate that has not been reported in the literature is to use sodium hydroxide as the eluent. This has the advantage that under alkaline conditions all analytes will be fully ionised, thus maximising interaction with the anion-exchange column. Additionally, as sodium hydroxide is compatible with suppressed conductivity detection, direct detection becomes feasible.

This paper reports the development, validation and application of an anion-exchange method using sodium hydroxide eluent for the direct determination of impurities in clodronic acid. Typical validation procedures were carried out including specificity, injection precision, linearity and recovery. The performances of five different columns used in this work are also discussed.

Table 1
Structural and physical properties of evaluated columns

Column	IonPac AS4	IonPac AS5	IonPac AS5A-5μ	IonPac AS11	OmniPac PAX100
Particle Diameter (μm)	15	15	5	13	8.5
Substrate cross-linking (%)	2	2	2	55	55
Latex diameter (nm)	75	120	60	85	60
Latex cross-linking (%)	3.5	1	4	6	4
Capacity (per column) (μequiv.)	20	20	35	45	40
Functional group	Alkyl quaternary ammonium	Alkanol quaternary ammonium	Alkanol quaternary ammonium	Alkanol quaternary ammonium	Alkanol quaternary ammonium
Substrate resin	Ethylvinylbenzene cross-linked with 2% divinylbenzene	Ethylvinylbenzene cross-linked with 2% divinylbenzene	Ethylvinylbenzene cross-linked with 2% divinylbenzene	Ethylvinylbenzene cross-linked with 55% divinylbenzene	Ethylvinylbenzene cross-linked with 55% divinylbenzene
Column size (mm)	250×5	250×4	150×4	250×4	250×4
Hydrophobicity	Medium	Low	Low	Very low	Low

2. Experimental

2.1. Apparatus

All experiments were carried out with a DX 500 ion chromatographic system (Dionex, Sunnyvale, CA, USA), consisting of a quaternary gradient pump (GP40) [polyether ether ketone (PEEK) construction], a chromatography module (LC20), an electrochemical detector (ED40) with a thermostated DS3 cell (used in the conductivity mode) and an autosampler (AS3500) with a thermostated column compartment. A GecK-o-cil column cooler (C.I.L., Sainte-Foy-la-Grande, France) was used for low-temperature work. Eluents were kept under helium and were degassed by using a built-in vacuum solvent degassing device.

Separations were performed on the following anion-exchange columns: An Ionpac AS4A with an AG4A guard column, an Ionpac AS5 with an AG5 guard column, an Ionpac AS5A-5μ with an AG5A-5μ guard column, an Ionpac AS11 with an AG11 guard column and an OmniPac PAX100 with guard column (Table 1) (Dionex). Conductivity detection was carried out using an Anion Self Regenerating Suppressor (ASRS-1), either in the recycle mode or external mode. An anion trap column (ATC-1) and a transition metal ion-trap column (MFC-1) (Dionex) were placed in between the pump and the injector.

2.2. Chemicals and reagents

The ultra pure water (18 MΩ/cm resistivity at 25°C) used for the preparation of the eluents, samples and standards was obtained from a Milli-Q Plus water purification system (Millipore, Bedford, MA, USA). Sodium hydroxide 46–48% (w/v) solution, sodium chloride, potassium nitrate, sodium sulphate and potassium dihydrogenphosphate (all analytical-grade reagents) were purchased from Fisher Scientific (Loughborough, UK). HPLC-grade acetonitrile was purchased from Romil (Waterbeach, UK) or Fisher Scientific. Methylenebisphosphonic acid was purchased from Lancaster Synthesis (Lancaster, UK). A mixture of partial esters of tetraisopropyl dichloromethylenebisphosphonate and clodronate containing monochloromethylenebisphosphonic acid [10] were manufactured at Astra Charnwood (Loughborough, UK). The computer optimisation software, HIPAC-G, was purchased from Phase Separations (Deeside, UK).

2.3. Preparation of a standard solution

For quantification of impurities, a standard solution was prepared containing 5 μg/ml of sodium chloride, potassium nitrate, sodium sulphate, potassium dihydrogenorthophosphate and monotributylammonium clodronic acid.

2.4. Base-degraded mono tributylammonium salt of clodronic acid

The monotributylammonium salt of clodronic acid at 1.5 mg/ml in 100 m*M* sodium hydroxide was refluxed for 1 h. This produced the degradation product, carbonylbisphosphonate.

2.5. System suitability solution

A system suitability sample containing all known possible impurities was prepared. The solution contained monotributylammonium clodronate (830 μg/ml) containing 2.7% by area (VI), mixed partial esters (167 μg/ml), base-degraded monotributylammonium clodronate (167 μg/ml), methylenebisphosphonic acid (6 μg/ml), sodium chloride (5 μg/ml), potassium nitrate (5 μg/ml), sodium sulphate (5 μg/ml) and potassium dihydrogenorthophosphate (5 μg/ml).

2.6. Sample solution preparation

Clodronate i.v. solution (Bonefos, 60 mg/ml; Boehringer Ingelheim, Bracknell, UK) or monotributylammonium clodronate were diluted with water to yield a concentration equivalent to 850 μg/ml clodronic acid. The solutions were transferred to HPLC vials for analysis.

2.7. Recovery and sample determination

The system was equilibrated overnight and sample solutions were injected, bracketed by standard solutions. Expected impurities such as chloride were calibrated using external standards whilst unknowns

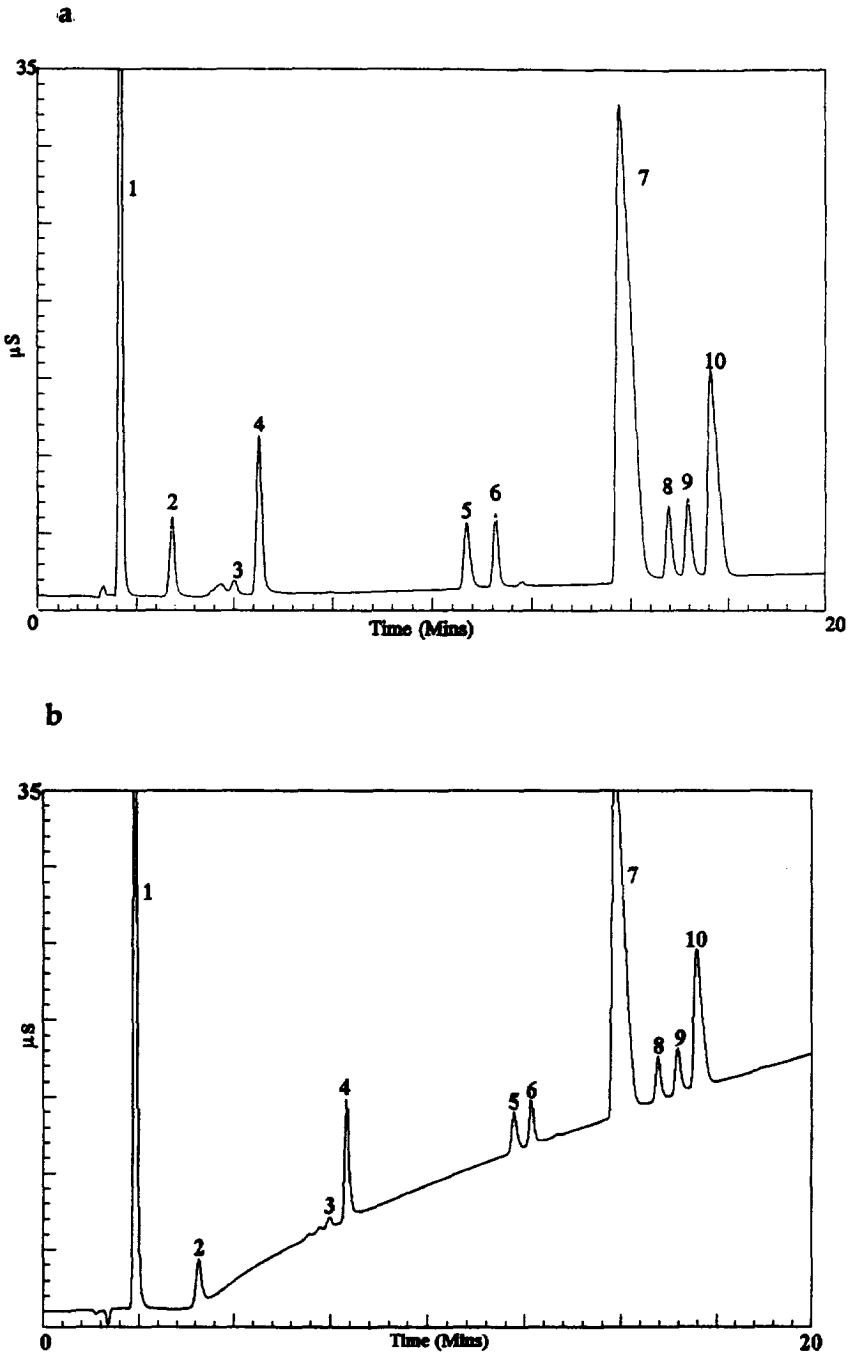

Fig. 3. System suitability on IonPac AS5. Eluent, 20 to 100 m*M* sodium hydroxide over 20 min; flow-rate, 1.0 ml/min; column temperature, 45°C; injection volume, 20 µl; detection, suppressed conductivity in recycle mode. Solutes: (1) Chloride, (2) nitrate, (3) diprotic acid of (II), (4) sulphate, (5) phosphate, (6) triprotic acid of (II), (7) clodronic acid (V), (8) monochloromethylenebisphosphonic acid, (9) methylenebisphosphonic acid and (10) carbonylbisphosphonic acid. (a) 0% (v/v) CH_3CN, (b) 10% (v/v) CH_3CN.

and the bisphosphonates VI and VII were quantified against clodronate.

3. Results and discussion

3.1. Choice of anion-exchange column

As adequate separation of the bisphosphonates may depend not only upon charge but on hydrophobic interaction with the column matrix, five anion-exchange columns of varying hydrophobicity were evaluated, i.e., IonPac AS4, AS5 and AS5A-5μ and solvent-compatible IonPac AS11 and OmniPac PAX 100. Column specifications from the manufacturer are shown in Table 1. All columns when used with aqueous sodium hydroxide used suppressed conductivity with a self-regenerating anion suppressor in the recycle mode, except where organic modifiers were used in the eluents, in which case, the external water mode was used.

Each column was used to chromatograph the system suitability, sample and standard solutions.

A sodium hydroxide gradient, when used with the IonPac AS5 column, gave adequate selectivity for the common inorganic anions and potential impurities. Fig. 3a shows a chromatogram of the system suitability mixture.

Other columns proved unsatisfactory, surprisingly, the AS5A-5μ column (5 μm particle size) gave significantly poorer resolution than the AS5 column (15 μm particle size). This may be due to differences in the diameter and latex cross-linking of the packing used in the two columns. The AS4 column, being the most hydrophobic, strongly retained clodronate and could only be eluted at a very high sodium hydroxide concentration, with the result that the less charged impurities eluted in the solvent front.

Fig. 4a was generated using an IonPac AS11 column, this produced the best chromatography for all impurities except for those of critical interest, the bisphosphonates IV and VII, which co-eluted. Similar chromatography was achieved when 10% acetonitrile was added as an organic modifier to the mobile phase, though, in this case, anions undergoing adsorption to the phase in addition to the anion-exchange process (nitrate and bisphosphonates) were less retained in the presence of the organic modifier (Fig. 4b).

The OmniPac PAX-100 gave the best resolution of the bisphosphonates (Fig. 5a–d), when acetonitrile was used as the organic modifier, and offered subtle changes in selectivity as the acetonitrile content was changed. Unfortunately, the background conductivity was higher using acetonitrile than would be found in a completely aqueous system and increased over time, suggesting that ionic contaminants were present in the commercially available HPLC-grade ace-

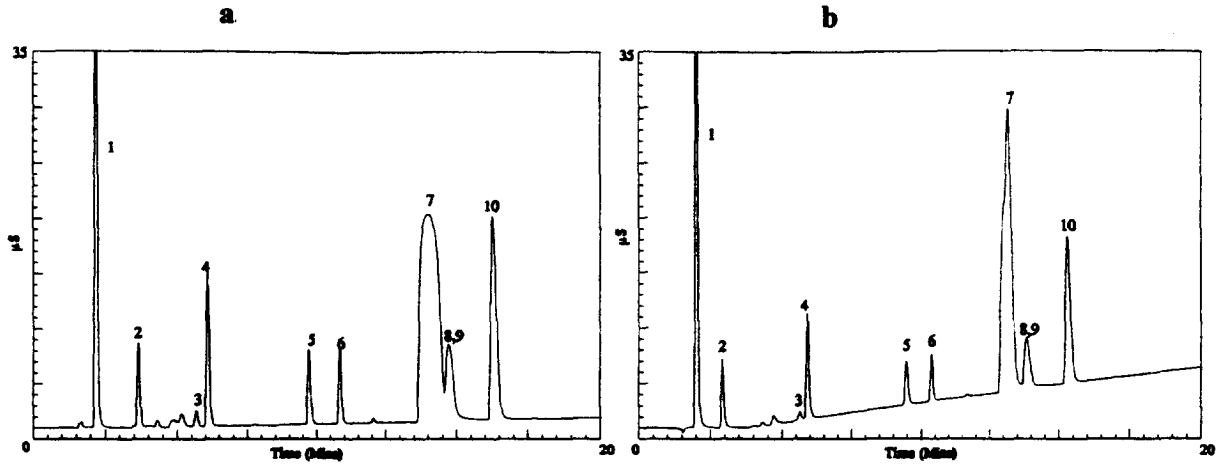

Fig. 4. Influence of organic solvents on the system suitability on the IonPac AS11 column. Eluent 20 to 100 m*M* sodium hydroxide over 20 min; flow rate, 1.0 ml/min; column temperature, 45°C; injection volume, 20 μl; detection, suppressed conductivity in recycle mode. Solutes are as in Fig. 3a. (a) 0% (v/v) CH_3CN, (b) 10% (v/v) CH_3CN.

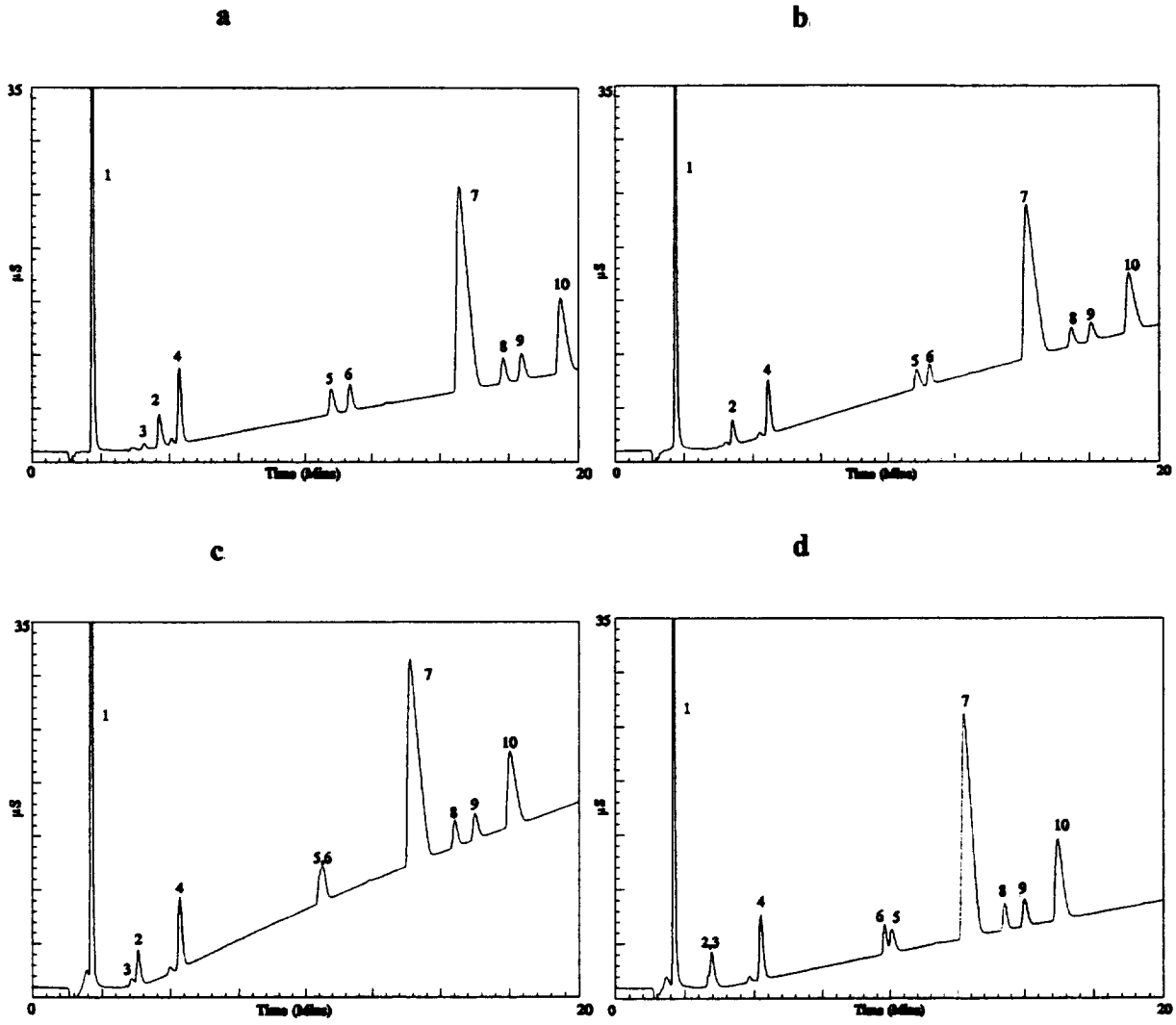

Fig. 5. Effect of organic solvents of the system suitability solution on the OmniPac PAX100 column. Eluent, 30 to 100 m*M* sodium hydroxide over 20 min; flow-rate, 1.0 ml/min; column temperature, 45°C; injection volume, 20 μl; detection, suppressed conductivity in external water mode. The solutes are as in Fig. 3a. (a) 5% (v/v) CH_3CN, (b) 10% (v/v) CH_3CN, (c) 15% (v/v) CH_3CN, (d) 20% (v/v) CH_3CN.

tonitrile. A change of supplier did not solve the problem. Furthermore, the anion self-regenerating suppressors used in external water mode proved to be less reliable in suppressing a sodium hydroxide gradient containing acetonitrile, which may be due to contaminants in the acetonitrile contaminating the suppressor.

Although not recommended by the column manufacturer, the effect of 10% (v/v) acetonitrile was also investigated on the IonPac AS5 column. Resolution of the bisphosphonates was slightly improved, but at the expense of high background conductivity (Fig. 3b).

As methodology capable of unattended operation was required, the IonPac AS5 column was chosen. This used aqueous eluent without organic modifiers and the self-regenerating anion suppressor in the more convenient auto-suppression mode.

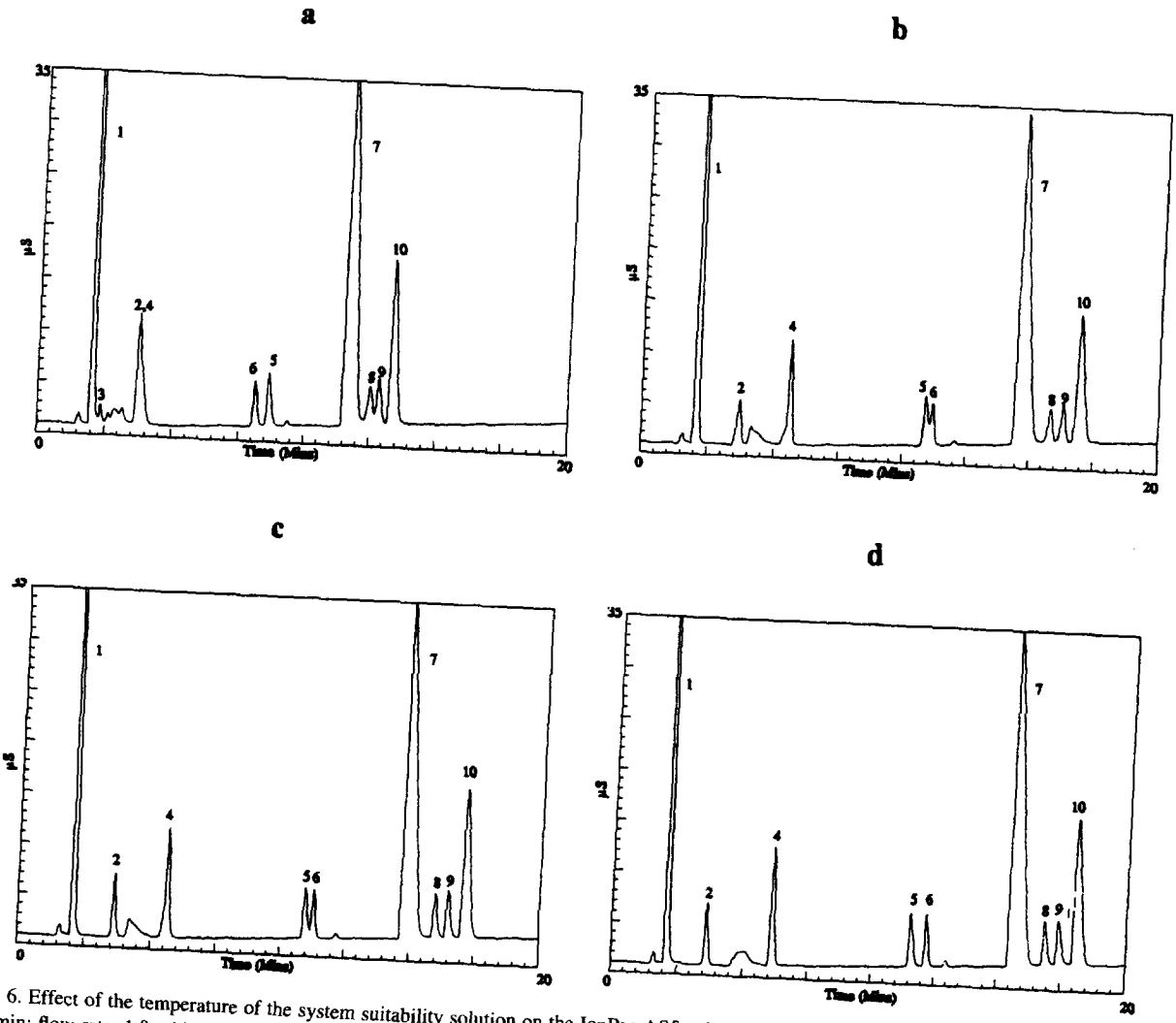

Fig. 6. Effect of the temperature of the system suitability solution on the IonPac AS5 column. Eluent, 20 to 100 m*M* sodium hydroxide over 20 min; flow-rate, 1.0 ml/min; column temperature, 45°C; injection volume, 20 μl; detection, suppressed conductivity in recycle mode. The solutes are as in Fig. 3a. (a) 4°C, (b) 25°C, (c) 35°C, (d) 45°C.

Table 2
Linearity of analytes using an IonPac AS5 column

Anion	Concentration range of anion (μg/ml)	Number of determinations	Coefficient of correlation
Chloride	24–0.12	10	0.9999
Nitrate	37.5–0.19	10	0.9999
Sulphate	51–0.25	10	0.9998
Phosphate	12.9–0.26	10	0.9994
Clodronate	1312–0.28	19	0.9999
	11.42–0.28	8	0.9982
Methylenebisphosphonate	8.0–0.49	9	0.9975

Table 3
Recovery of analytes using an IonPac AS5 Column

Anion	% (w/w) of nominal clodronate anion concentration at 850 μg/ml	Recovery (%)	No. of determinations
Chloride	0.848	100.7	3
	0.565	100.2	6
	0.283	101.5	6
	0.141	100.0	3
Nitrate	1.291	106.0	3
	0.861	104.6	6
	0.430	101.1	6
	0.215	98.4	3
Sulphate	1.794	107.6	3
	1.196	105.7	6
	0.598	102.5	6
	0.299	99.6	3
Phosphate	0.442	99.4	3
	0.295	96.7	6
	0.147	93.3	6
	0.074	94.9	3
Methylenebisphosphonate	0.283	85.9	3
	0.189	78.9	6
	0.095	70.0	6
	0.047	64.3	3

3.2. Effect of varying the gradient

The effect of varying the sodium hydroxide gradient was investigated with the IonPac AS11, AS11 and AS5A-5μ columns. Two gradients were run per column at 35°C, one using the same conditions as described in Fig. 3a and the other using exactly the same conditions but with the gradient extended to 60 min. Increasing the run time had no dramatic effects on resolution, but did decrease the detectability. Computer optimisation using HIPAC-G suggested that little improvement could be made by adjusting the gradient.

3.3. Effect of column temperature

The effect of column temperature on column selectivity was investigated with the IonPac AS11, AS5A-5μ and AS5 columns over a temperature range of 4 to 45°C. Whilst no significant improvements could be seen with the two former columns, the effect was remarkable on the IonPac AS5

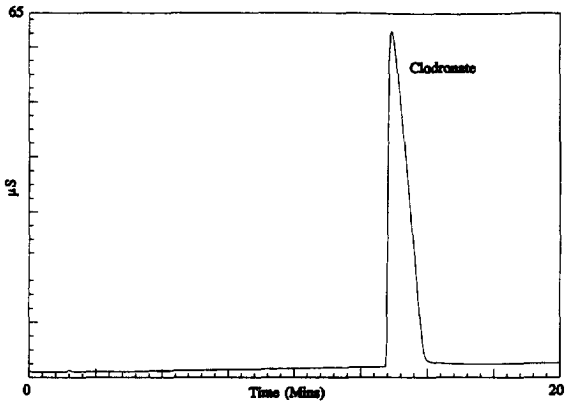

Fig. 7. Chromatogram of Bonefos. Eluent, 20 to 100 m*M* sodium hydroxide over 20 min; flow-rate, 1.0 ml/min; column temperature, 45°C; injection volume, 20 μl; detection, suppressed conductivity in recycle mode.

column. Fig. 6a–d shows the resulting chromatograms. With increasing temperature, polyvalent anions showed an increase in retention time, probably due to increasing ionisation of the analytes. Additionally, the positions of phosphate and triprotic (II) acid inverted with increasing temperature, showing no resolution near ambient temperature. The optimum temperature of 45°C was selected for reliable operation, as a cooled column required additional equipment.

3.4. The use of a transition metal ion-trap column

After prolonged use, it was noted that the chromatography of the bisphosphonates deteriorated, giving rise to poor peak shapes and splitting. This was believed to be due to transition metal ion contamination and was prevented by the introduction of a transition metal ion-trap column. Under severe cases of contamination, it was necessary to wash both the trap column and the analytical column with 100 m*M* hydrochloric acid and 10 m*M* disodium EDTA.

3.5. Validation

Linearity data for the IonPac AS5 column for the anions of interest are shown in Table 2. Significant deviations from linearity were only seen for clodronate and methylenebisphosphonate at low levels, which may be partly due to the slightly broader peaks expected for late-eluting compounds and an increase in the remnant sodium ion concentration, i.e., by the conversion efficiency of the suppressor. It has previously been suggested that the remnant ion not only affects the background conductivity but also seriously depresses the peak height of samples of very low concentration [19].

Recovery studies were performed by spiking a 1.5-mg/ml solution of monotributylammonium clodronate with stock mixtures of sodium chloride, potassium nitrate, sodium sulphate, potassium dihydrogenorthophosphate and methylenebisphosphonic acid (Table 3). The results were satisfactory for all of the common anions, however, methylenebisphosphonic acid showed the greatest deviation, which was probably due to poor linearity at this low concentration and the fact that methylenebisphosphonate may have a slightly different conductimetric response from clodronate. Ideally, if methylenebisphosphonate was found to be a common impurity, it could be included in the external standard. One other possibility that has not yet been investigated is the adsorption to glass of low concentrations of methylenebisphosphonate via chelation to metal ions on the glass surface.

The recovery of phosphate, at low levels, similarly showed some deviation from the ideal, which may be due to the difficulty in integration of a small tailing peak on a rising baseline.

3.6. Disodium clodronate

A sample of Bonefos was chromatographed (Fig. 7) and impurities of chloride and sulphate (both <0.05%, w/w) were detected, but neither VI or VII were detected.

4. Conclusions

A novel impurity screen using anion-exchange chromatography and suppressed conductivity has been developed and validated for monotributylammonium clodronate, which has been shown to be applicable to clodronate.

This work has shown the importance of selecting an anion-exchange column of the correct polarity. Two columns, the IonPac AS5 and the OmniPac PAX100, were found to give the required specificity, but as the OmniPac column could only be used with organic modifiers (which appeared to contain anionic impurities that prevented satisfactory chromatography), the AS5 column was selected. Effective optimisation of this column was found to be related to the temperature, which had more of an effect than altering the gradient.

References

[1] E. Jatunen, R. Lahtinen, M. Laakso, Leuk. Lymphoma 19 (1995) 207.
[2] I. Elomaa, C. Blomqvist, Acta Oncol. 34 (1995) 629.
[3] N. O'Rourke, E. McCloskey, F. Houghton, H. Huss, J.A. Kanis, J. Clin. Oncol. 13 (1995) 929.
[4] A.H. Paterson, T.J. Powles, J.A. Kanis, E. McCloskey, J. Hanson, S. Ashley, J. Clin. Oncol. 11 (1993) 59.

[5] R. Lahtinen, M. Laakso, I. Palva, P. Virkkunen, I. Elomaa, Lancet 340 (1992) 1049.
[6] S. Khan, E.V. McCloskey, K.S. Eyres, E.D. Fern, J.A. Kanis, Semin. Arthritis Rheum. 23 (1994) 271.
[7] J.A. Kanis, E.V. McCloskey, P. Sirtori, D. Fern, K. Eyres, J. Aaron, M.N. Beneton, Osteoporosis Int. 3 (1993) S23.
[8] R.G. Humphries, W. Tomlinson, J.A. Clegg, A.H. Ingall, N.D. Kindon, P. Leff, Drug Dev. Res. 31 (1994) 279.
[9] P. Leff, R.G. Humphries, B.E. Crack, C.E. Pollard, K. McKechnie, Drug Dev. Res. 31 (1994) 290.
[10] J.D. Curry, J.B. Prentice, O.T. Quimby, D.A. Nicholson, J.B. Roy, J. Organomet. Chem. 13 (1968) 199.
[11] J.D. Curry, J.B. Prentice, O.T. Quimby, D.A. Nicholson, J. Org. Chem. 32 (1967) 4111.
[12] S. Auriola, R. Kostiainen, M. Ylinen, J. Mökkönen, P. Ylitalo, J. Pharm. Biomed. Anal. 7 (1989) 1623.
[13] T.L. Chester, E.C. Lewis, J.J. Benedict, R.J. Sunberg, W.C. Tettenhorst, J. Chromatogr. 225 (1981) 17.
[14] V. Virtanen, L.H.J. Lajunen, J. Chromatogr. 617 (1993) 291.
[15] V. Virtanen, L.H.J. Lajunen, Talanta 40 (1993) 661.
[16] J.P. Kosonen, J. Pharm. Biomed. Anal. 10 (1992) 881.
[17] E.W. Tsai, S.D. Chamberlin, R.J. Forsyth, C. Bell, D.P. Ip, M.A. Brooks, J. Pharm. Biomed. Anal. 12 (1994) 983.
[18] Impurities in New Drug Products, The Third International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceticals for Human Use, Yokohama, 1995.
[19] Z.W. Tian, R.Z. Hu, H.S. Lin, W.L. Hu, J. Chromatogr. 439 (1988) 151.