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Determination of alendronate sodium by ion chromatography with refractive index detection

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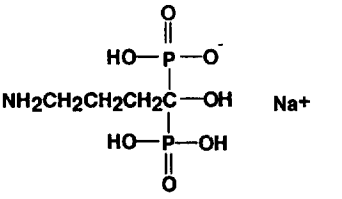
Abstract

Refractive index (RI) detection is applied to the ion chromatographic (IC) determination of alendronate sodium, an important drug for the treatment of bone diseases, using a Waters IC-Pak HR anion-exchange column with dilute nitric acid (6 m*M*) as the mobile phase. The method is validated and demonstrated to be accurate, precise, specific and robust. It is a good, alternative method for routine assay vs. the IC–indirect UV method. The detection limit ($4 \cdot 10^{-4}$ mg/ml) is comparable to or better than that of the IC–indirect UV detection ($1 \cdot 10^{-3}$ mg/ml). The mechanism of the IC separation was explored by the application of the Regnier retention model and van 't Hoff analysis.

1. Introduction

Alendronate sodium (**1**, see Fig. 1), an important aminobisphosphonate compound, was developed for the treatment of diseases of abnormal bone turnover, such as metastatic bone disease, hypercalcemia of malignancy, Paget's disease and osteoporosis [1,2].

The analysis of **1** in dosage formulations is challenging because it lacks a chromophore for UV or fluorescence detection. Three methods for the analysis of **1** in dosage formulations have been developed and validated in our laboratory [3–5]. The first method for introducing a chromophore into this molecule involves pre-column derivatization with 9-fluorenylmethyl chloroformate [3], which requires extensive sample preparations. The second method of ion chroma-



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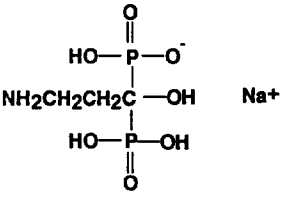

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Fig. 1. Structures of alendronate sodium (**1**) and its amino-hydroxyl-propyl analog (**2**).

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tography (IC) with conductivity detection [4] requires the use of a conductivity detector which is not routinely used in pharmaceutical analytical laboratories. The third method of IC with indirect UV detection [5] is simple and suitable for routine assay. Many other analytical methods for the analysis of **1** by pre-column or post-column derivatization and using special detectors have been described [3–5].

Since an alternative method is often needed in pharmaceutical development, e.g. in stability studies and in the validation of the first method, we have developed and validated another simple method for the determination of **1**. The method differs from IC–indirect UV chiefly in the mode of detection employed, namely, it employs a refractive index detector (RI). Haddad and Heckenberg [6] demonstrated that RI and indirect UV detection, when applied to IC, gave very comparable detection limits for many non-UV absorbing inorganic and organic anions and cations. However, no application of RI detection to the study of bisphosphonate drugs has been described in the literature. Here, we report such application to the study of alendronate sodium and demonstrate that IC–RI presents a good, alternative method for routine assay. We demonstrate that the detection limit for alendronate by IC–RI ($4 \cdot 10^{-4}$ mg/ml) is comparable to or even better than that by IC–indirect UV ($1 \cdot 10^{-3}$ mg/ml [5].

Although IC has been extensively used in the separation of alendronate sodium, its mechanistic aspect has not been fully explored so far. Recently, Thompson et al. [7] studied the relationship between the mobile phase concentration and the capacity factor (k') of several bisphosphonate species including alendronate sodium. They showed that a logarithmic plot of k' versus mobile phase concentration gave a straight line with a slope that is explained to be the ratio of the charges on the eluite and the eluent ion. However, they did not explain the deviation that the plot possessed a slope of -0.70 for the alendronate mono-anion (charge -1). We have also studied this relationship, using alendronate sodium as an example, by the retention model developed by Kopaciewicz et al. [8]. Although this model was originally developed for IC of proteins, we think that it may also be applicable to IC of other species such as alendronate sodium. In our opinion, it provides a better understanding of the alendronate retention on ion-exchange surfaces. Furthermore, we have applied van 't Hoff analysis to examine the temperature dependence of retention of alendronate, which showed nonlinear van 't Hoff plots in the temperature range 25–80°C.

2. Experimental

2.1. Chemicals and reagents

Alendronate sodium [**1**, MK-0217, 4-amino-1-hydroxybutane-1,1-bisphosphonic acid monosodium (alendronate sodium) trihydrate salt, $C_4H_{12}NO_7P_2Na \cdot 3H_2O$, M_r 325.1] and its amino-hydroxyl-propyl analog (**2**, Fig. 1) of pharmaceutical grade were manufactured by Merck Research Laboratories (Rahway, NJ, USA). Nitric acid (OPTIMA grade), citric acid, hydrochloric acid (reagent grade), methanol, and acetonitrile (HPLC grade) were purchased from Fisher (Pittsburgh, PA, USA). All solvents and reagents were used as received without further purification. Deionized water with at least 18 MOhm cm purified by a Milli-Q system was used for mobile phase, sample and standard preparations.

2.2. Equipment and assay conditions

Method development and validation were performed on a Hewlett-Packard (Avondale, PA, USA) 1090 HPLC equipped with a Perkin-Elmer (Norwalk, CT, USA) LC-30 RI detector. The anion-exchange column was a Waters (Milford, MA, USA) IC-Pak HR (6 μm particle size, 75×4.6 mm I.D.) with column temperature from 25°C to 80°C and dilute nitric acid (1.5–6 mM) as the mobile phase. The flow-rate was 0.5 ml/min. The injection volume was 50 μl. The packing material of the column is a spherical, highly cross-linked polymethacrylate resin with a quaternary ammonium functional group, with

the following characteristics: volume, 1.25 ml; capacity, 30 ± 3 μeq./ml (<100 ppm ions); plates per column, >2500; temperature range, 10–50°C (10–80°C if temperature is increased slowly); and back pressure limit, 2000 p.s.i. at flow-rate of 1 ml [9]. The HPLC column was equilibrated with the mobile phase by injecting the standard solution until reproducible retention times and peak areas were observed.

2.3. Standard and sample preparations

The standard solution was prepared by dissolving 52.7 mg of reference standard into 100 ml water to yield the assay concentration of 0.4 mg/ml (anhydrous free acid equivalent). Tablet (40 mg potency) samples of alendronate sodium were dispersed in 100 ml water by stirring for 30 min to yield a sample solution of 0.4 mg/ml. A portion of the resulting solution was filtered through a Millipore 0.22-μm filter unit and transferred to an HPLC vial for analysis. Capacity factors (k') were determined by $k' = (t_r - t_0)/t_0$, where t_r is the retention time of alendronate and t_0 is the retention time of an unretained substance (e.g. sodium cation).

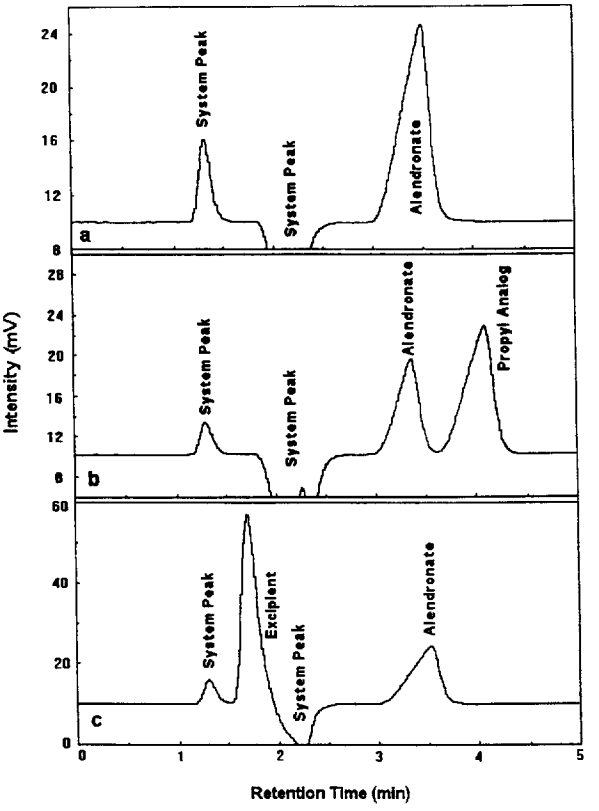

Fig. 2. Chromatograms obtained using the 6.0 m*M* mobile phase, including (a) chromatogram of a standard of alendronate sodium (**1**), (b) chromatogram of a standard spiked with the amino-hydroxyl-propyl analog (**2**), and (c) chromatogram of a tablet sample.

3. Results and discussion

3.1. Development of the IC–RI method

Fig. 2a shows a chromatogram of a standard solution of alendronate sodium obtained under the conditions used (see Experimental) with a mobile phase concentration of 6.0 m*M* nitric acid. Alendronate was well separated from sodium cation and eluted at retention times of ca. 3.5 min. In Fig. 2a, two system peaks were observed, which were also observed in solvent blanks and placebos under the same mobile phase concentration. Sodium cation co-eluted with the first system peak at about 1.3 min, which was not retained on the column and was independent of the mobile phase concentrations. This was confirmed by studying a sodium chloride solution. The second system peak may have resulted from an eluent-deficient zone formed by the sample injection and may depend on the pH of the injected sample [10].

Fig. 2b shows the separation of alendronate from its amino-hydroxyl-propyl analog (**2**) using the 6.0 m*M* mobile phase. Fig. 2c shows a chromatogram of tablet (40 mg potency) samples, which demonstrates the separation of alendronate and excipients.

The RI detector, Perkin Elmer LC-30, is a reflection-type refractometer, which monitors the difference in refractive index between the mobile phase (reference) and the column eluent (mobile phase plus analyte). It is different from indirect UV, which monitors the difference in UV absorption with the pre-requisite that the mobile phase possesses a strong UV absorption.

The IC–RI method was optimized by considering the following parameters:

pH of the mobile phase

The pK_a values of the parent alendronic acid, obtained by potentiometric titration in water with 0.1 *M* sodium hydroxide, are $pK_{a_1} < 2$, $pK_{a_2} < 2$, $pK_{a_3} = 6.3$, $pK_{a_4} = 9.9$ and $pK_{a_5} = 10.2$ [11]. Thus, alendronic acid can possess different ionization states, depending on the pH of the mobile phase. Below pH 2, it is a amine cation (+1). Between ca. pH 2 and 6, the amine group is ionized and positively charged while phosphonate groups have two negative charges. It forms a zwitterionic mono-anion (−1). Since the alendronate mono-anion (−1) dominates between ca. pH 2 and 6, this pH range was preferred. Above pH 6.3, the amine group is positively charged and phosphonate groups have three negative charges, and it forms a di-anion (−2). The di-anion would bound to the stationary phase more tightly, and was thus not preferred. Our experiments showed that the di-anion did not elute from the column even after one hour.

Different acids used in the mobile phase

Three mono-acids, citric acid, HCl and HNO_3, were studied and compared as the eluent in the mobile phase. At 6 m*M* concentration, the measured pHs of these three mobile phases were 2.7, 2.3, and 2.3, respectively, in which the zwitterionic mono-anion dominated. The pH difference of these mobile phases was slight, and had little effect on the ionization states. Particularly, the HCl and the HNO_3 mobile phases have the same pH 2.3. However, from peak-area measurement it was observed that the response factors of alendronate were different, and increased in the order of the mobile phase anion: $NO_3^- > Cl^- >$ citrate. Thus, nitric acid was chosen in the method.

Concentration of nitric acid

After nitric acid was chosen as the eluent, the effect of the concentration of nitric acid on the retention of alendronate was carefully investigated. Increasing the nitric acid concentration from 1.5 to 6.0 m*M* (pH from 2.9 to 2.3) resulted in a shorter retention of alendronate, and a more symmetrical and sharper peak shape of alendronate. Thus, 6 m*M* nitric acid is preferred in the method. However, quantitation of alendronate was satisfactory over the whole range from 1.5 to 6.0 m*M* (see Validation). Decreasing the concentration below 1.5 m*M* resulted in a worse peak shape and a much longer retention time, while increasing the concentration above 6 m*M* did not have a significant effect on the retention.

Organic modifier

Addition of 10% methanol to the 6.0 m*M* mobile phase resulted in a decrease of the alendronate peak height (64%) and area (72%), and a slight increase in the retention time (0.15 min, ca. 4%). Addition of 10% acetonitrile to the mobile phase resulted in large decrease in the alendronate peak height (38%) and area (44%), and a slight increase in retention time (0.14 min, ca. 4%). Thus, addition of organic solvents did not improve the separation nor the sensitivity.

Column temperature

Temperature effect was studied from 25°C to 80°C for the 1.5 and 6.0 m*M* mobile phases. When the 6.0 m*M* mobile phase was used, increasing temperature resulted in increase of the retention time of alendronate going from 25°C to 45°C, and then in a slight decrease of the retention time going from 45°C to 80°C. For the 1.5 m*M* mobile phase increasing temperature resulted in a slight increase of the retention time from 25°C to 70°C and then in a slight decrease of the retention time from 70°C to 80°C. These results were used for the temperature dependence of retention time by the van 't Hoff analysis (see below). However, both the peak height and the peak area of alendronate slightly decreased as the temperature increased in the two mobile phases. For example, the peak height at 45°C was 91.8% of that at 25°C using the 1.5 m*M* mobile phase, and 95.5% of that at 25°C using the 6.0 m*M* mobile phase, respectively. Thus, a temperature of 25°C was used in the method.

3.2. Retention mechanism of alendronate

The anion-exchange retention mechanism of alendronate is explored by studying the relationship between k' of alendronate and the nitric acid concentration using the retention model developed by Kopaciewicz et al. [8], and by studying the relationship between k' of alendronate and temperature using the van 't Hoff analysis.

The relationship between k' of alendronate and the nitric acid concentration

Kopaciewicz et al. [8] investigated charge–charge and other interactions between eluite and the surfaces of ion-exchange packing materials on the basis of a non-mechanistic model and obtained the following equation:

$$\log k' = 2Z \log (1/[\mathrm{M}])) + \log K_Z$$

where the parameter Z measures the number of charges interacting between the surface of the ion exchanger and the eluite ion (e.g., alendronate), and [M] is the concentration of the eluent (e.g., nitric acid), the anion of which is used as a displacing agent, and $\log K_Z$ is a constant. The equation is in excellent agreement with the experimental observations. Plotting of $\log k'$ against $\log 1/[\mathrm{M}]$, by the use of the experimental data listed in Table 1, gave a straight line with the correlation coefficient $r^2 = 0.999$. The linear relationship is described by:

$$\log k' = 1.35 \log [1/\mathrm{M}] + 1.28$$

From the slope of 1.35, a Z value (Z = slope/2) of 0.68 for the anion exchange of alendronate was obtained. This is about the same as the absolute value of (−0.70) reported by Thompson et al. [7], which is explained to be the ratio of the charges on the eluite and the eluent ion. According to the Regnier model [8], Z represents the charge–charge interaction in the ion-exchange process. The Z value of 0.68 was deviated from "net charge". This could be explained by the charge asymmetry of alendronate, the counter ion effect and solvation of the ions, which caused only a fraction of its surface to interact with the stationary phase in the ion-exchange process, as in the case of other zwitterionic molecules like proteins [8].

van 't Hoff analysis

Further information about the retention mechanism is obtained by examining the temperature dependence of retention by the van 't Hoff analysis:

$$\ln k' = (-\Delta H/RT) + (\Delta S/R) + \ln \Phi$$

where ΔH and ΔS represent the enthalpy and entropy of the anion-exchange process; R and T are the gas constant and absolute temperature, respectively; and Φ is the volume phase ratio (stationary volume/mobile volume).

The van 't Hoff analysis has been widely used in reversed-phase liquid chromatography (RPLC) for probing the thermodynamics of the partitioning process and investigating possible phase transition of the stationary phase [12]. When the retention mechanism is invariant throughout the temperature range and the enthalpy is constant, a plot of $\ln k'$ versus $1/T$ yields a straight line with the slope equal to $(-\Delta H/R)$, and the intercept equal to $(\Delta S/R + \ln \Phi)$. However, nonlinear van 't Hoff plots have sometimes been observed in RPLC, which is an indication of a change in the retention mechanism [12]. There have not been many studies of van 't Hoff analysis in anion-exchange chromatography of organic ions. Recently, Lee and Hoffman have reported the temperature effect on the retention of phenyl-substituted carboxylate anions and phenyl-substituted alcohols [13]. They obtained nonlinear van 't Hoff plots for

Table 1
k' of alendronate as a function of mobile phase (nitric acid) concentration (M)

k'	10.76	7.57	5.60	4.36	3.47	3.00	2.50	2.14	1.90	1.67
M (mM)	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0

carboxyl anion, which did not have simple shape. This was attributed to the difference in the effect of temperature on the energetics of transfer from the mobile phase to the stationary phase for the eluite and eluent ions [13].

In the present study, van 't Hoff analysis was performed from 25°C to 80°C using both the 1.5 and 6.0 m*M* mobile-phase concentrations. The obtained plots are shown in Fig. 3a (6.0 m*M*) and 3b (1.5 m*M*).

When using the 6.0 m*M* mobile phase, *k'* increased from 25°C to 45°C, and then decreased from 45°C to 80°C. When using the 1.5 m*M* mobile phase, *k'* increased from 25°C up to ca. 70°C and then decreased from 70°C to 80°C. Both plots are nonlinear. Both curves possess simple shapes with maxima. The maximum of the 6.0 m*M* plot (Fig. 3a) was at ca. 45°C, which was lower than that of the 1.5 m*M* plot (at ca. 70°C, Fig. 3b). These observations are different from the report by Lee and Hoffman [13], but are parallel to the observations of nonlinear plots in RPLC [12]. The temperature corresponding to the maxima is referred to as T_H in RPLC, the temperature at which ΔH is zero. The T_H is usually dependent on the mobile-phase compositions.

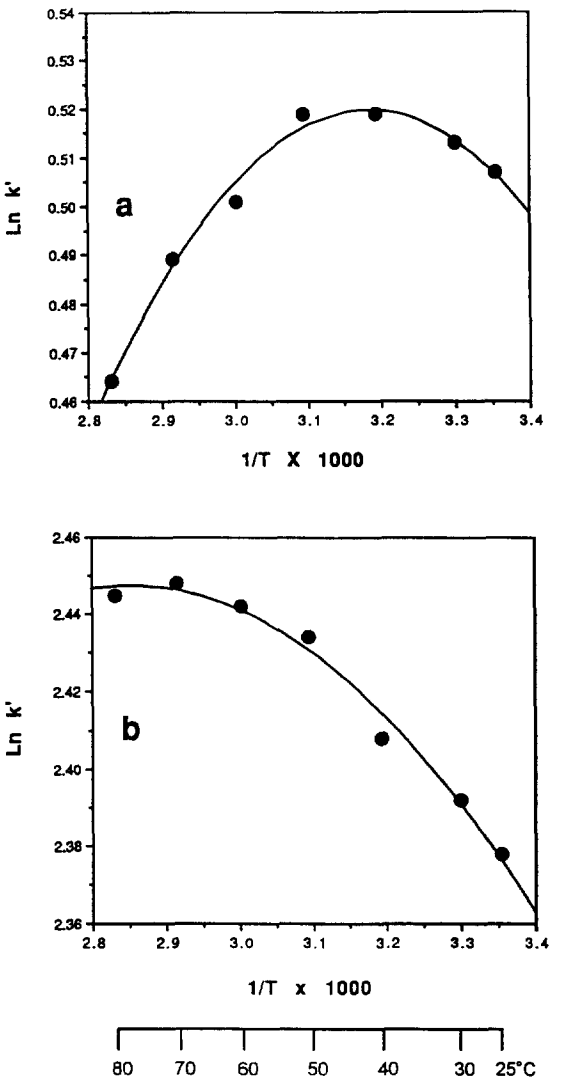

Fig. 3. Plot of ln *k'* vs. $1/T$ using (a) the 6.0 m*M* mobile phase and (b) the 1.5 m*M* mobile phase.

We may use T_H to designate the maxima in Fig. 3a and b. As can be seen, the van 'tHoff plots have negative slopes below the temperature of T_H ($\Delta H > 0$) and positive slopes above T_H ($\Delta H < 0$), indicating that the driving force of the anion exchange changed below and above T_H. This is probably attributed to the enhanced electrostatic interaction at higher temperature. The anion-exchange process is generally controlled by electrostatic interactions. In the most simplistic forms, the attractive force (F) between two oppositely charged species can be represented by Coulomb's law:

$$F = Q_1 Q_2 / 4\pi\varepsilon_0 \varepsilon r^2$$

where Q_1 and Q_2 are point charges of opposite sign, r is their separation distance, ε_0 is the permittivity of a vacuum, and ε is the relative permittivity or dielectric constant of the medium. Water may represent the dilute mobile phase as the medium approximately. As the temperature increases from 25°C to 80°C, the dielectric constant of water decreases [14], and the attractive force (absolute value) increases, which may cause the change of the driving force from positive enthalphy (entropy-driven) to negative enthalphy considering that ε_0 is different for the eluent and eluite anions. However, the exact dielectric constant of water may be dependent on the nitric acid concentration, and thus T_H is also dependent on the nitric acid concentration. More

detailed studies are needed to better understand the observations.

3.3. Validation of the IC–RI method

The IC–RI method was validated according to the USP guidelines [15] by use of the 1.5 and 6.0 m*M* mobile phases which represent the entire concentration range. The method was demonstrated to be accurate, precise, specific and robust.

Specificity

The method specificity was demonstrated by the separation of alendronate from its impurities, analogs and/or degradates formed only at very high temperatures, which can be achieved by easily controlling the retention of alendronate in the concentration range from 1.5 to 6.0 m*M*. As an example, Fig. 2b shows the baseline separation of alendronate from its amino-hydroxylpropyl analog (**2**, relative retention time, RRT, of 1.3, solid spiked into the sample) using the 6.0 m*M* mobile phase. The separation of these two species was satisfactory over the whole range from 1.5 to 6.0 m*M*.

The method was successfully applied to tablet (40 mg potency) samples. Except for the alendronate and the system peaks, an additional strong peak, eluting immediately after the first system peak and before the negative system peak, was observed as shown in Fig. 2c. This peak is due to the excipients of the tablet. Since alendronate always eluted after the negative system peak, alendronate was well separated from the excipients using all mobile phases.

Precision

The injection precision was determined by making 10 replicate injections of the standard solutions. R.S.D.s of 0.3% (1.5 m*M*) and of 0.2% (6.0 m*M*) were obtained. Method precision for the determination of alendronate was demonstrated by the analysis of 10 replicate tablet samples. The results gave 40.4 mg/tab (101.0%) with an R.S.D. of 1.9% using the 1.5 m*M* mobile phase and 39.6 mg/tab (99%) with an R.S.D. of 1.2% using the 6.0 m*M* mobile phase.

Linearity

The detector responses were found to be linear. The correlation coefficients, $r^2 = 1.000$ and $r^2 = 1.000$, were obtained in the range of 50–150% of the method concentration (0.4 mg/ml) for the 1.5 and 6.0 m*M* mobile phases, respectively.

Accuracy

The accuracy of the method was demonstrated by the recovery experiments, which were performed by spiking aliquots of a stock solution of the drug into a placebo tablet in duplicate followed by an appropriate dilution to yield amounts of drug equivalent to 50%, 75%, 100% and 125% of the potencies of formulations. The results are acceptable (average: 100.7% and R.S.D.: 0.46%) using the 1.5 m*M* mobile phase. Since the sample preparation was the same, the accuracy study was not repeated for other mobile phase concentrations.

Limit of detection

Under the method conditions, the limit of detection (LOD) was determined to be $4 \cdot 10^{-4}$ mg/ml (0.1% standard) at a signal-to-noise ratio of 4, using the 6.0 m*M* mobile phase, which was transferred to an absolute amount of ca. 20 ng. The detection limit was higher when the nitric acid concentration in the mobile phase was decreased because of broadening of the alendronate peak. The LOD is comparable to or better than that of the IC–indirect UV method [$1 \cdot 10^{-3}$ mg/ml (0.25% standard)] [5].

Robustness

The method has been validated over a wide range of mobile phase concentrations (1.5–6.0 m*M*) as demonstrated above. As also demonstrated, the slight variation of the pH in this range does not influence the ionic status of alendronate and its quantitation. The method was tested by different chemists using different HPLC instruments and columns on different days, and similar results in terms of separation,

precision, linearity, accuracy, and limit of detection were obtained. All these demonstrate the robustness of the method.

4. Conclusions

A simple IC–RI method using a Waters IC-Pak HR column has been developed and validated for the determination of non-UV absorbing alendronate sodium in dosage formulations. The method has a detection limit comparable to or better than that of IC–indirect UV. The IC separation mechanism has been studied by the application of the Regnier retention model and van 'tHoff analysis. It is thought that IC–RI can be applied to the determination of other bisphosphonate drugs.

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References

[1] G. Heynen, P. Delwaide, O.L.M. Bijvoet and P. Franchimont, Eur. J. Clin. Invest., 11 (1982) 29–35.
[2] H.P. Sleeboom, O.L.M. Bijvoet, A.T. van Oosterom, J.H. Gleed and J.L.H. O'Riordan, Lancet, 2 (1983) 239–243.
[3] J.D. DeMarco, S.E. Biffar, D.G. Reed and M.A. Brooks, J. Pharm. Biomed. Anal., 12 (1989) 1719.
[4] E.W. Tsai, D.P. Ip and M.A. Brooks, J. Chromatogr., 596 (1992) 217–224.
[5] E.W. Tsai, D.P. Ip and M.A. Brooks, J. Pharm. Biomed. Anal., 11 (1993) 513–516.
[6] P.R. Haddad and A.L. Heckenberg, J. Chromatogr., 252 (1982) 177–184.
[7] R. Thompson, N. Grinberg, H. Perpall, G. Bicker and P. Tway, J. Liq. Chromatogr., 17 (1994) 2511–2531.
[8] W. Kopaciewicz, M.A. Rounds, J. Fausnaugh and F.E. Regnier, J. Chromatogr., 266 (1983) 3–21.
[9] Communication with Technical Support of Waters.
[10] Y. Michigami and Y. Yamamoto, J. Chromatogr., 623 (1992) 148–152.
[11] Merck Research Lab, unpublished data.
[12] J.G. Dorsey and W.T. Cooper, Anal. Chem., 66 (1994) 857A–866A.
[13] H.K. Lee and N.E. Hoffman, J. Chromatogr. Sci., 32 (1994) 97–101.
[14] CRC Handbook of Chemistry and Physics, The Chemical Rubber Co., 75 ed., 1994.
[15] The United States Pharmacopiea, XXIII, 1982–1983 (1995).