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Ion-exchange liquid chromatographic analysis of bisphosphonates by on-line post-column photochemical reaction and spectrophotometric detection

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Abstract

A simple ion-exchange high-performance liquid chromatographic method was developed and employed for the analysis of bisphosphonate compounds in dosage formulations using on-line post-column photochemical reactions. The method used molybdate as the post-column reagent to react with the photolyzed bisphosphonate to form phosphomolybdate for enhanced spectrophotometric detection. A bisphosphonate compound, 2-thioethane-1,1-bisphophonic acid, was selected to evaluate the separation using both isocratic and gradient elution methods, along with the effects of other experimental parameters including mobile phase composition, flow-rate and post-column reagent concentration. The gradient elution method showed improved resolution and detection sensitivity compared to the isocratic elution method. The optimized gradient method was simple, reproducible, and specific to bisphosphonate compounds. It was successfully employed for the stability study of the bisphosphonate compound in pharmaceutical dosage formulations.

Keywords: Gradient elution; Bisphosphonates; Organophosphorous compounds

1. Introduction

Bisphosphonate compounds have been actively explored as drug candidates for the treatment of a variety of bone diseases [1,2]. Since many of these compounds do not contain strong chromophores, chromatographic analyses of the compounds are problematic. Different analytical methods have been developed for the determination of bisphosphonate-containing compounds in support of drug discovery and development. The high-performance liquid chromatographic (HPLC) measurement of bisphosphonate compounds without strong UV chromophores has been accomplished by indirect UV or indirect fluorescence detection by addition of a UV-absorbing reagent or a fluorescent additive in the mobile phase [3–5]. Direct detection techniques using UV, fluorescence, or electrochemical detectors have also been developed by introducing a chromophore, a fluorophore, or a electrochemical active group into these molecules through pre- and post-column derivatization [6–9]. A method based on gas chromatography with mass spectrometry detection was developed through sample derivatization [10]. In addition, other direct detection methods without derivatization have been reported utilizing ion-exchange chromatography with on-line flame photometric detection [11], conductivity detection [12], electrochemical detection [13], and mass spectrometry [14]. Several capillary electrophoresis-based methods are also described in the literature with the use of mass spectrometry [15], low-UV-wavelength detection [16], and UV detection

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by on-line formation of a chromophoric complex [17].

Here, we present an alternative method based on ion-exchange chromatography with UV–Vis detection using on-line post-column photochemical derivatization for routine determination of bisphosphonates in dosage formulations. Bisphosphonates can be photolyzed under UV irradiation to orthophosphate ions [18–20], these orthophosphate ions can then react with molybdate to form phosphomolybdate [7,21], the phosphomolybdate can further be reduced to mixed-valence phosphomolybdenum blue complex under UV irradiation [22], which can be detected at 750 nm. In our method, the bisphosphonate compounds are first separated by ion-exchange chromatography, then UV photolyzed post-column to orthophosphate ions and organic products in a photochemical reactor. The orthophosphate ions react with post-column reagent sodium molybdate to form phosphomolybdate which is further reduced to blue phosphomolybdenum complex and detected by a photodiode array detector at 750 nm. Compared to the previously reported post-column derivatization methods, this method is much simpler and easier to implement, involving a simple one-step post-column reaction procedure and minimal equipment requirement. As an attractive alternative to other methods for routine pharmaceutical analysis of bisphosphonates, this on-line post-column HPLC method is selective, reproducible and universal for bisphosphonate compounds. It was successfully employed for the degradation studies of bisphosphonate compounds in dosage formulations.

2. Experimental

2.1. Materials

The bisphosphonate compound, 2-thioethane-1,1-bisphosphonic acid (shown in Fig. 1), was synthesized by Procter & Gamble Pharmaceuticals (Mason, OH, USA). Nitric acid and sodium molybdate dihydrate were obtained from Aldrich (Milwaukee, WI, USA). Deionized water purified with a Milli-Q Ultra-Pure Water System (Millipore, Bedford, MA, USA) was utilized for all sample preparations.

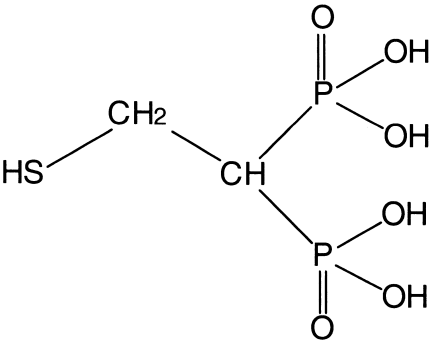

Fig. 1. Molecular structure of compound I.

2.2. Preparation of reagents, standards and samples

The post-column reagent was prepared by dissolving appropriate amounts of sodium molybdate dihydrate in Milli-Q water to yield a series of working solutions with concentrations ranging from 1 to 20 m*M*. The stock solution of the bisphosphonate compound was made by dissolving the compound in a blank dosage formulation to yield a 1.0 mg/ml solution. The standards were obtained by serial dilutions from the stock solution with the blank formulation to produce eight standard solutions in the range of 0.5 to 500 μg/ml. The degradation sample was prepared by dissolving an appropriate amount of the compound in the blank formulation to give a 1.0 mg/ml solution. Aliquots of the sample were incubated at 50°C and retrieved at various times for the forced degradation study. Each sample was diluted fourfold prior to chromatographic analysis and analyzed in triplicate. The calibration curve was generated by analyzing the standard samples immediately after they were made. Quality control (QC) samples were prepared by spiking the blank dosage formulation with the compound to yield three concentrations at 5, 50 and 300 μg/ml.

2.3. Equipment and chromatographic conditions

The HPLC system consisted of a Waters (Milford, MA, USA) 600S controller, a 616 pump, an in-line degasser, a 717 plus thermostatic autosampler, and a 996 photodiode array detector. The system was controlled by the Waters Millennium data system. A

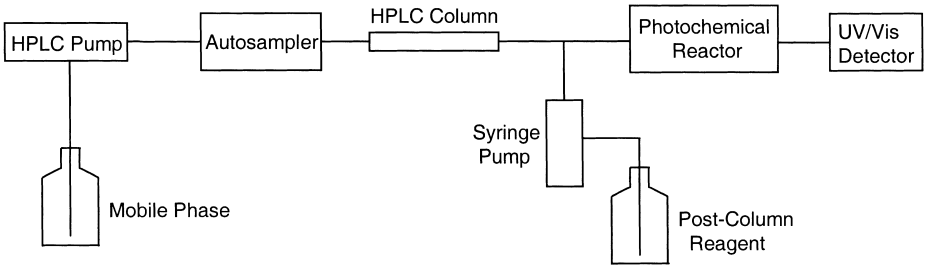

Fig. 2. Schematic diagram of the HPLC system with an on-line post-column photochemical reactor.

Waters anion-exchange column (IC-Pak Anion HR, 75×4.6 mm I.D., 6 μm) was utilized. The mobile phase systems were composed of different nitric acid concentrations ranging from 5 to 50 m*M* for isocratic elution and 5–400 m*M* for gradient elution. The mobile phase flow varied from 0.8 to 1.2 ml/min with an injection volume of 10–50 μl.

A schematic diagram of the on-line post-column photochemical reaction HPLC system is shown in Fig. 2. The post-column reagent was delivered at the flow-rate of 0.8–1.5 ml/min using an ISCO continuous flow syringe pump system (Lincoln, NE, USA), consisting of a series D pump controller and two Model 500D syringe pumps, and mixed with post-column effluent through a polyethylether ketone (PEEK) Y mixing tee (Upchurch Scientific, Oak Harbor, WA, USA). The mixed effluent was then passed through a knitted reactor coil that was made of PTFE tubing (10 m×0.50 mm I.D.) and placed inside a photochemical reactor (Aura Industries, Staten Island, NY, USA) containing a 254-nm UV lamp (8 W). Inside the reactor coil, the bisphosphonate compound in the post-column effluent reacted with the post-column reagent by continuous photolytic derivatization under the UV light. The final derivatized product, phosphomolybdate, was monitored and detected by photodiode array detection (DAD) at 750 nm.

3. Results and discussion

3.1. Optimization of separation conditions

The initial separation method was developed using isocratic elution. The effects of post-column reagent concentration on the separation were first evaluated. A series of isocratic separations of compound I at 100 μg/ml were performed using 10 m*M* nitric acid as the mobile phase while molybdate concentrations were varied in the range of 5–20 m*M*. The results indicated that the intensity of the analyte peak increased with increasing the molybdate concentration from 5 to 10 m*M*. However, no noticeable increase in intensity was observed when the concentration of nitric acid was further increased to higher than 10 m*M*. Therefore, 10 m*M* was selected as the optimal post-column reagent concentration for subsequent evaluations. The effects of the mobile phase strength on separation and sensitivity were assessed next. A series of nitric acid concentrations ranging from 5 to 50 m*M* were used as the isocratic mobile phase systems while the concentration of the post-column reagent, sodium molybdate, was kept constant at 10 m*M*. Fig. 3 shows the representative chromatograms of compound I obtained under the aforementioned conditions. As can be seen from Fig. 3, the analyte peak at 12.9 min was rather broad when 5 m*M* nitric acid was used. The analyte peak was sharpened and the retention time shortened as the nitric acid concentration was increased. The results revealed that the higher the nitric acid concentration, the better the detection sensitivity. Fig. 3 also indicated that the analyte peak height reached the maximum at 20 m*M* nitric acid concentration, but the peak was too close to the solvent front. Coelution of the analyte peak with the solvent front was observed when the nitric acid concentration was further increased to 50 m*M*.

To optimize both separation and detection sensitivity, gradient elution was employed using five different mobile phase systems in the following nitric

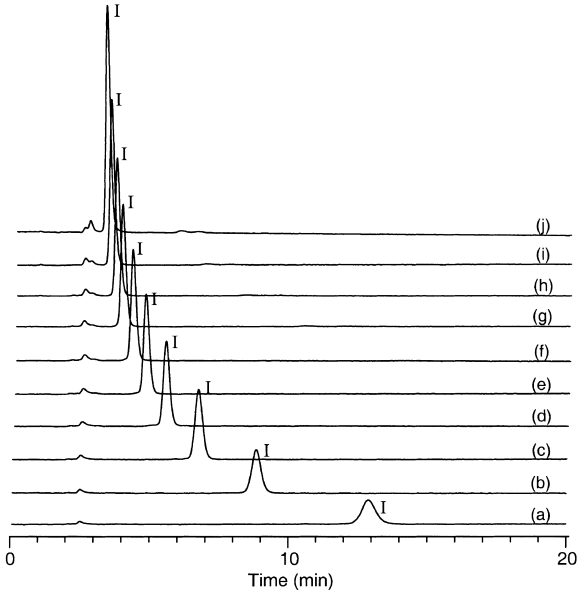

Fig. 3. Representative chromatograms of compound I (100 μg/ml) by isocratic elution at a constant nitric acid concentration of 5.0 m*M* (a), 8.0 m*M* (b), 9.5 m*M* (c), 11.0 m*M* (d), 12.5 m*M* (e), 14.0 m*M* (f), 15.5 m*M* (g), 17.0 m*M* (h), 18.5 m*M* (i), and 20.0 m*M* (j).

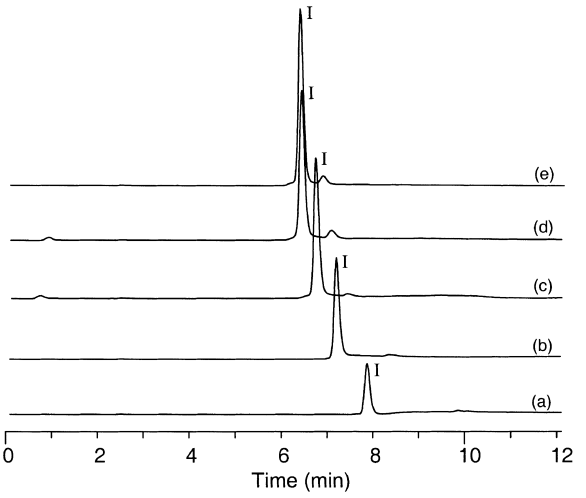

Fig. 4. Representative chromatograms of compound I (100 μg/ml) by gradient elution in the nitric concentration range of 5–20 m*M* (a), 5–100 m*M* (b), 5–200 m*M* (c), 5–300 m*M* (d), and 5–400 m*M* (e).

acid concentration ranges: 5–20 m*M*, 5–100 m*M*, 5–200 m*M*, 5–300 m*M* and 5–400 m*M*. As shown in Fig. 4, the results from linear gradient separations using these five different concentration ranges demonstrated that much better separations, in terms of optimal retention time, resolution and peak shape, were achieved in all cases compared to the isocratic elution. A small impurity peak eluted after the analyte was also found well separated from the main analyte peak as shown in Fig. 4. Additionally, the detection sensitivity was significantly improved using gradient elution as opposed to the isocratic elution. Because the reaction of molybdate with phosphate ions needs to be catalyzed under acidic conditions, the increased response in detection could be attributed to the faster and more complete reaction due to the increased nitric acid concentration under the gradient elution conditions evaluated. In the same time, the effects of the mobile phase and post-column reagent flow-rates on the separation and detection response were also investigated. The mobile phase flow was varied in the range of 0.8–1.2 ml/min while the reagent flow changed from 0.8 to 1.5 ml/min. The optimal separation and detection sensitivity were observed at the flow-rate ratio (the post-column reagent to the mobile phase) of 1.2 with the mobile phase flow at 1.0 ml/min. All chromatograms in Fig. 4 were obtained under the optimal flow-rate conditions. As can be seen from Fig. 4, little change in separation and detection sensitivity was observed beyond the nitric acid concentration range of 5–200 m*M* (Fig. 4c). Therefore, the mobile phase system consisting of 5–200 m*M* nitric acid and the mobile phase flow-rate of 1.0 ml/min, along with the post-column reagent concentration of 10 m*M* and the flow-rate of 1.2 ml/min, were chosen as the optimal gradient separation conditions for further method validation and application to degradation studies. Fig. 5 shows the representative chromatograms with and without post-column UV irradiation, illustrating the effect of UV light on the spectrophotometric detection.

3.2. Precision and accuracy

As shown in Table 1, the method precision and accuracy were investigated at three concentration levels: 5, 50 and 300 μg/ml in three assays on three

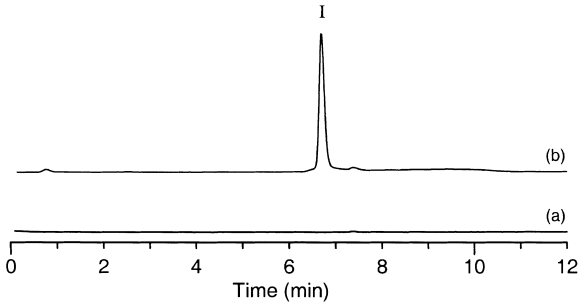

Fig. 5. Chromatograms of compound I (100 μg/ml) by gradient elution in the nitric concentration range of 5–200 m*M* without (a) and with (b) UV irradiation.

different days. The precision expressed as the relative standard deviation (RSD) based on six repetitive injections was less than 4.5% (intra-assay) and 5.2% (inter-assay) at all three levels. The accuracy was found to be in the range of 96.1–104.3% for all three drug concentrations.

3.3. Selectivity, linearity of calibration, and limit of detection

Fig. 6 shows the representative chromatograms of the degradation samples of the compound at 250 μg/ml in a dosage formulation. As can be seen from Figs. 4 and 6, the impurity or degradation product is well separated from the parent compound. No matrix interference was observed from either the blank dosing solution or degradation samples. The calibration curve was linear from 0.5 to 500 μg/ml with a correlation coefficient greater than 0.999. The detection limit, at a signal-to-noise ratio of 3, was about 0.15 μg/ml for the compound in the dosage formulation studied. The limit of quantitation is about 0.5 μg/ml, indicating that the method should be able to quantitatively measure impurities or degradation products above this level.

Table 1
Precision and accuracy data for the determination of compound I ($n=6$)

Concentration (μg/ml)	Precision (RSD, %)		Inter-assay accuracy (%)
	Intra-assay	Inter-assay	
5	4.5	3.8	96.1
50	2.7	5.2	98.5
300	2.1	4.1	104.3

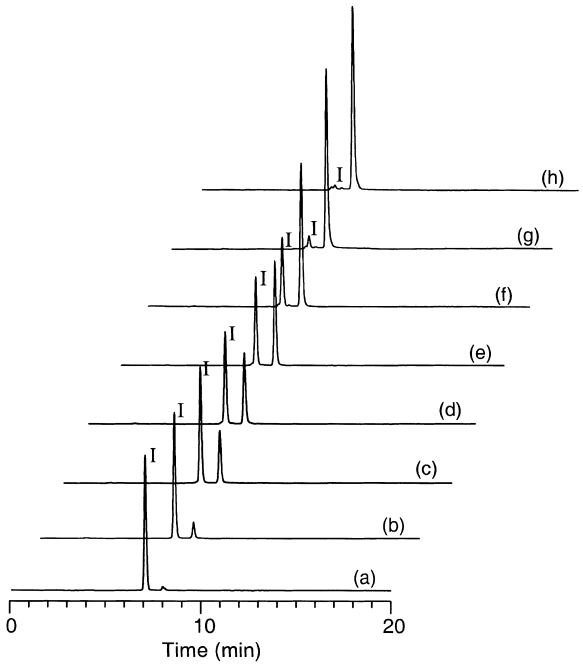

Fig. 6. Representative chromatograms of compound I (250 μg/ml) in the dosage formulation by gradient elution (5–200 m*M* nitric acid, 10 m*M* molybdate) at various incubation times: (a) 0 h; (b) 1 day; (c) 2 days; (d) 4 days; (e) 7 days; (f) 10 days, (g) 14 days; and (h) 21 days.

3.4. Degradation study

The forced degradation study of compound I in the dosage formulation was carried out by incubating the dosing solutions at 50°C. The samples were retrieved at various times and their concentrations were determined using the optimized gradient elution method.

Fig. 6 shows a series of representative chromatograms of compound I in the dosage formulation, representing a time–course study of the degradation samples. Clearly, the compound was degraded over time to one major degradation product, which was eluted just about 1 min after the analyte under the chromatographic conditions employed. The height and area of the analyte peak decreased while those of the degradant peak increased accordingly. The degra-

dation of the compound at 50°C was found to follow first-order reaction kinetics with a half-life of 6.5 days.

Although this method was developed and optimized based on compound I, it was quite general and successfully employed for the determination of many other bisphosphonate compounds having distinctively different structures, since all these bisphosphonates were photolyzed to a common product, orthophosphate, which reacted with molybdate and further photolytically generated a common final product, phosphomolybdenum blue, for spectrophotometric detection.

4. Conclusion

A simple on-line post-column ion-exchange HPLC method was developed and utilized for the routine determination of bisphosphonate compounds in dosage formulations. Both isocratic and gradient separations were evaluated, along with the effects of other experimental parameters such as mobile phase composition, flow-rate, and post-column reagent concentration. The optimal separation of the bisphosphonate compound and its impurity and degradation product was achieved using gradient elution with 5–200 m*M* nitric acid as the mobile phase system and 10 m*M* post-column molybdate reagent. Under these conditions, the calibration curve is linear in the range of 0.5–500 μg/ml with a detection limit of 0.15 μg/ml. This method is simple, reproducible, easy to implement, and specific to bisphosphonate compounds. It was successfully employed for the determination of other bisphosphonate compounds in dosage formulations.

References

[1] H. Fleisch, in: W.A. Peck (Ed.), Bone and Mineral Research, Annual 1, Excerpta Medica, Amsterdam, 1983, p. 319.
[2] H. Fleisch, in: Bisphosphonates in Bone Disease From the Laboratory to the Patient, Parthenon, New York, 1995, p. 67.
[3] R. Thompson, N. Grinberg, H. Perpall, G. Bicker, P. Tway, J. Liq. Chromatogr. 17 (1994) 2511.
[4] E.W. Tsai, S.D. Chamberlin, R.J. Forsyth, C. Bell, D.P. Ip, M.A. Brooks, J. Pharm. Biomed. Anal. 12 (1994) 983.
[5] M.J. Lovdahl, D.J. Pietrzyk, J. Chromatogr. A 850 (1999) 143.
[6] R.W. Sparidans, J. den Hartigh, P. Vermeij, J. Pharm. Biomed. Anal. 13 (1995) 1545.
[7] P.T. Daley-Yates, L.A. Gifford, C.R. Hoggarth, J. Chromatogr. 490 (1989) 329.
[8] L.E. King, R. Vieth, J. Chromatogr. B 678 (1996) 325.
[9] W.F. Kline, B.K. Matuszewski, J. Chromatogr. 583 (1992) 183.
[10] S. Auriola, R. Kostiainen, M. Ylinen, J. Monkkonen, P. Ylitalo, J. Pharm. Biomed. Anal. 7 (1989) 1623.
[11] T.L. Chester, E.C. Lewis, J.J. Benedict, R.J. Sunberg, W.C. Tettenhorst, J. Chromatogr. 225 (1981) 17.
[12] E.W. Tsai, D.P. Ip, M.A. Brooks, J. Chromatogr. 596 (1992) 217.
[13] T. Usui, T. Watanabe, S. Higuchi, J. Chromatogr. 584 (1992) 213.
[14] X.Z. Qin, E.W. Tsai, T. Sakuma, D.P. Ip, J. Chromatogr. A 686 (1994) 205.
[15] K. Huikko, R. Kostiainen, J. Chromatogr. A 872 (2000) 289.
[16] S.X. Peng, R. Takigiku, D.E. Burton, L.L. Powell, J. Chromatogr. B 709 (1998) 157.
[17] E.W. Tsai, M.M. Singh, H.H. Liu, D.P. Ip, M.A. Brooks, J. Chromatogr. 626 (1992) 245.
[18] R.A. Libby, Inorg. Chem. 10 (1971) 386.
[19] S. Bisaz, R. Felix, H. Fleisch, Clin. Chim. Acta 65 (1975) 299.
[20] S.R. Priebe, J.A. Howell, Anal. Lett. 16 (1983) 1219.
[21] F.A. Cotton, G. Wilkinson, in: Advanced Inorganic Chemistry, 5th ed., Wiley, New York, 1988, p. 815.
[22] I. Koshiishi, T. Imanari, J. Chromatogr. 358 (1986) 195.