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Determination of a new bisphosphonate, YM175, in plasma, urine and bone by high-performance liquid chromatography with electrochemical detection

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ABSTRACT

A high-performance liquid chromatographic method for the determination of disodium dihydrogen(cycloheptylamino)methylenebisphosphonate monohydrate (YM175) in plasma, urine and bone is described. Plasma obtained in high-dose animal studies is pretreated by Method A, a simple method using 1 ml of plasma, which is based on deproteinization of plasma followed by coprecipitation of the drug with calcium phosphate and removal of excess calcium ions by AG 50W-X8 resin. Plasma obtained in lower-dose clinical studies is treated by Method B, a more sensitive method using 10 ml of plasma, which is based on solid-phase extraction using a Sep-Pak C_{18} cartridge coupled with Method A. Urine and bone are treated similarly to Method B. The chromatographic system consists of a mobile phase at pH 11, an alkali-stable column and an electrochemical detector operating in the oxidation mode. The determination limit is 5 ng/ml for Method A and 0.5 ng/ml for Method B in plasma, 1 ng/ml in urine, and 25 ng/g in bone.

INTRODUCTION

Disodium dihydrogen(cycloheptylamino)-methylenebisphosphonate monohydrate (YM175, I) (Fig. 1), is a new bisphosphonate, which strongly inhibits bone resorption in animal models both *in vitro* and *in vivo* [1]. It is expected to be clinically useful in the treatment of hypercalcemia and osteoporosis.

Several methods for the determination of bisphosphonates in biological fluids have been reported. EHDP (ethane-1-hydroxy-1,1-diphosphonate) has been assayed by photolysis of the P–C–P bonds with UV light followed by spectrophotometric determination of the inorganic phosphate released [2]. Clodronate [(dichloromethylene)bisphosphonate] has been quantified by anion-exchange chromatography with a flame photometric detection to measure phosphorus emission [3], or by capillary gas chromatography–negative chemical ionization mass spectrometry (GC–NCI-MS) following trimethylsilyl derivatization of the phosphono group [4]. Pamidronate (1-hydroxy-3-aminopropylidene-1,1-bisphosphonate, APD) has been determined by anion-exchange chromatography coupled with a

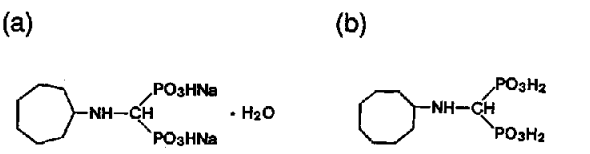

Fig. 1. Structures of I (YM175) (a) and the internal standard (b).

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two-step postcolumn reaction with ammonium persulphate followed by molybdenum ascorbate to generate the phosphomolybdate chromophore [5]. These methods are based on detection or derivatization of phosphono groups, and therefore seem in principle applicable to other bisphosphonates, including I.

Determination of I in biological samples, however, requires high sensitivity, because this agent is more potent than many other bisphosphonates [1], implying that its concentrations in plasma and urine at effective doses are lower. Methods for EHDP and clodronate using flame photometric detection lack sufficient sensitivity for assay of I, while those for clodronate using GC–NCI-MS and pamidronate using postcolumn reaction are more sensitive but require expensive or complicated instruments, which are inconvenient for routine applications. As for other assay methods for bisphosphonates, pamidronate has also been analysed by reversed-phase high-performance liquid chromatography (HPLC) with fluorometric detection after precolumn derivatization of the primary amino group with fluorescamine [6]. SR-41319 [(4-chlorophenyl)thiomethylene bisphosphonic acid], which contains a UV-absorbing chromophore, has been determined by reversed-phase HPLC with UV detection [7]. Compound I, however, lacks suitable functional groups for UV detection and derivatization.

We have shown that I is oxidized by using an electrochemical detector at an alkaline pH. Based on this principle, sensitive assay methods for I in plasma, urine and bone by reversed-phase HPLC using electrochemical detection (ED) have been developed.

EXPERIMENTAL

Chemicals and reagents

YM175 (I) and the internal standard (I.S.), cyclooctylaminomethylenebisphosphonic acid (Fig. 1), were synthesized at our Central Research Laboratories (Tokyo, Japan). Calcium phosphate (tribasic) was purchased from Nacalai Tesque (Kyoto, Japan) and dissolved in 0.1 *M* HCl at a concentration of 5 mg/ml. Distilled water was used for all procedures. Calf bovine serum was purchased from Flow Labs. (Costa Mesa, CA, USA) and membrane filters were from Nihon Millipore (Yonezawa, Japan). Cation-exchange resin, AG 50W-X8 (200–400 mesh, H^+ form) was obtained from Bio-Rad Labs. (Richmond, CA, USA) and converted into the K^+ form as follows. Three volumes of 1 *M* KOH were added to the resin. The mixture was stirred for 30 s, then the supernatant was decanted. This procedure was repeated twice. The resin was then washed five times with three volumes of water and stored in a dark bottle at 4°C. The resin was used after washing two or three times with three volumes of water in each analysis.

Standard solution

Stock solutions of I and the I.S. were prepared at a concentration of 1 mg/ml in water and stored at 4°C. These solutions were stable for at least 3 years after preparation. Standard solutions of I were prepared by diluting the stock solution with water to suitable concentrations. The I.S. solution was also prepared at 20 μg/ml in water.

Chromatographic conditions

The HPLC system consisted of a 655A-11 pump coupled with an L-5000 LC controller (Hitachi, Tokyo, Japan), a WISP712 autosampler (Waters Assoc., Milford, MA, USA), a Coulochem 5100A electrochemical detector (ESA, Bedford, MA, USA) with a Model 5020 guard cell and a 5010 analytical cell, and a C-R4AX integrator (Shimadzu, Kyoto, Japan). A PLRP-S column (5 μm, 100 Å, 15 cm × 4.6 mm I.D.; Polymer Labs., Shropshire, UK), which is packed with polystyrene divinylbenzene-based materials and applicable in the pH range 1–13, was used for separation. The guard cell, which reduces impurities in the mobile phase, was placed between the pump and autosampler and used at a potential of +0.60 V. The analytical cell consisted of two serial electrodes, detectors 1 and 2 (D1 and D2), together with associated reference and counter electrodes. D1, which removes easily oxidized interferences in the column eluate, was operated at a potential of +0.40 V.

D2, which quantifies the analytes, was operated at +0.55 V. The signal from D2 was monitored by the integrator.

The mobile phase was acetonitrile–50 m*M* Na_2HPO_4 buffer containing 1 m*M* tetrabutylammonium phosphate, adjusted to pH 11 with 2 *M* NaOH (7:93, v/v). Prior to mixing with acetonitrile, the buffer solution was filtered through a 0.22-μm membrane filter (type GS). The mobile phase flow-rate was 0.8 ml/min. The column temperature was maintained at 50°C.

Sample preparation

Plasma: Method A. To 1 ml of plasma in a 10-ml polystyrene tube, 50 μl of I.S. solution were added. The sample was deproteinized by adding 4 ml of 10% (w/v) trichloroacetic acid, and centrifuged at 2000 *g* for 10 min. The supernatant was transferred to a 15-ml polypropylene tube, then spiked with 0.25 ml of calcium phosphate and 0.4 ml of 10 *M* NaOH. The tube was vortex-mixed for 30 s and centrifuged at 2000 *g* for 10 min to precipitate the analytes with calcium salts. The supernatant was discarded. The precipitate was suspended in 4 ml of water, centrifuged at 2000 *g* for 10 min, and then resuspended in 4 ml of 50% (v/v) acetonitrile. After centrifugation at 2000 *g* for 5 min, the precipitate was dissolved in 0.2 ml of 0.2 *M* orthophosphoric acid. To remove excess calcium ions in the sample, 0.1 ml of AG 50W-X8 resin was added. After vortex-mixing and centrifuging, the supernatant was filtered through a 0.45-μm membrane filter (type HV) and alkalinized with 30 μl of 2 *M* NaOH. An 80-μl aliquot of the sample was injected into the HPLC column.

Plasma: Method B. To 10 ml of plasma in a 50-ml polypropylene tube, 50 μl of I.S. solution, 10 ml of water, 0.5 ml of calcium phosphate and 0.2 ml of 10 *M* NaOH were added. The mixture was vortex-mixed for 30 s and centrifuged at 2000 *g* for 10 min. The supernatant was discarded. The precipitate was suspended in 10 ml of 0.01 *M* NaOH and centrifuged at 2000 *g* for 10 min. Calcium salts in the precipitate were then dissolved in 4 ml of 0.1 *M* HCl. After centrifugation at 2000 *g* for 5 min, the supernatant was applied to a Sep-Pak C_{18} cartridge (Waters Assoc.), which was activated with 5 ml of acetonitrile and water. After application, the cartridge was washed with 10 ml of water. The analytes were eluted with 6.5 ml of 0.01 *M* NaOH. The first 0.5-ml fraction was discarded and the next 6 ml were collected into a 15-ml polypropylene tube. This fraction was treated in the same way as the supernatant obtained after deproteinization in Method A, except that 0.05 ml of 10 *M* NaOH was added.

Urine. To 10 ml of urine in a 50-ml polypropylene tube, 50 μl of I.S. solution, 0.1 ml of 2.5 *M* calcium chloride and 0.2 ml of 10 *M* NaOH were added. The mixture was vortex-mixed and centrifuged, and the precipitate suspended in NaOH and centrifuged in the same manner as plasma in Method B. The precipitate was then dissolved in 5 ml of 0.1 *M* HCl and applied to a Sep-Pak C_{18} cartridge. Treatment after application was identical with that in Method B, except that the cartridge was washed with 10 ml of water, 5 ml of 50% acetonitrile, then 5 ml of water.

Bone. Bone (250–500 mg) was digested in 4.7 volumes of conc. HCl for *ca.* 2 h at 50°C in a 10-ml glass tube with a cap. Compound I was stable under this treatment. Next, 4.3 volumes of 10 *M* NaOH were added to the digest to form a transparent liquid phase and a small amount of floating substances. To a 15-ml polypropylene tube, 2 ml of the liquid phase, corresponding to 200 mg of bone, were transferred and spiked with 50 μl of I.S. solution and 6 ml of water. The mixture was filtered through a 0.45-μm membrane filter (type HV) and then applied to a Sep-Pak C_{18} cartridge. Treatment after application was identical with that in Method B.

Preparation of calibration curves

A 50-μl portion of each standard solution of I was added to 1 ml of plasma and 10 ml of plasma and urine. These spiked samples were processed as described above. Bone was spiked with 0.2 volume of a standard solution of I and processed as described above, except that conc. HCl and 10 *M* NaOH were added at 4.6 and 4.2 volumes, respectively. Calibration curves were constructed by plotting the peak-height ratios of I to the I.S. *versus* concentrations of I.

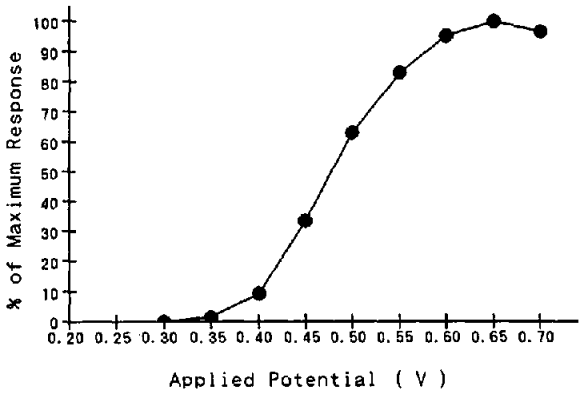

Fig. 2. Voltammogram of I expressed as a percentage of the maximum response with an electrochemical detector operating in the oxidation mode.

RESULTS

Selection of cell potentials

Fig. 2 shows the voltammogram of I using an electrochemical detector when the cell potential of D1 was set at 0 V and that of D2 was varied stepwise. Oxidation of I occurred from a potential of +0.35 V and reached a maximum at +0.65 V. The voltammogram of the I.S. showed the same profile as that of I, and the variation in peak-height ratio of I to the I.S. was within 5% when the potential of D1 was set at +0.40 V and that of D2 varied from +0.45 to +0.65 V. In this method, the potentials of D1 and D2 were set at +0.40 and +0.55 V, respectively, because a nar-

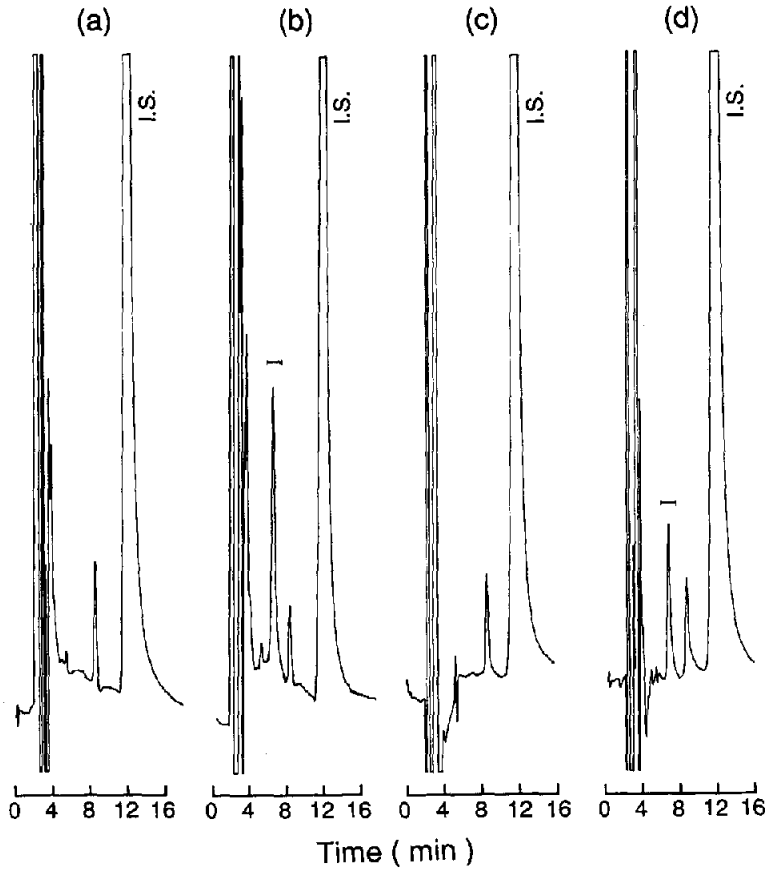

Fig. 3. Typical chromatograms of plasma samples treated by Method A [(a) and (b)] and Method B [(c) and (d)]: (a) control dog plasma containing 1 μg of I.S.; (b) dog plasma obtained 15 min after oral administration of 3 mg/kg I (109.4 ng/ml); (c) control human plasma containing 1 μg of I.S.; (d) human plasma obtained after intravenous infusion of 0.1 mg of I,at a constant rate for 2 h (3.56 ng/ml).

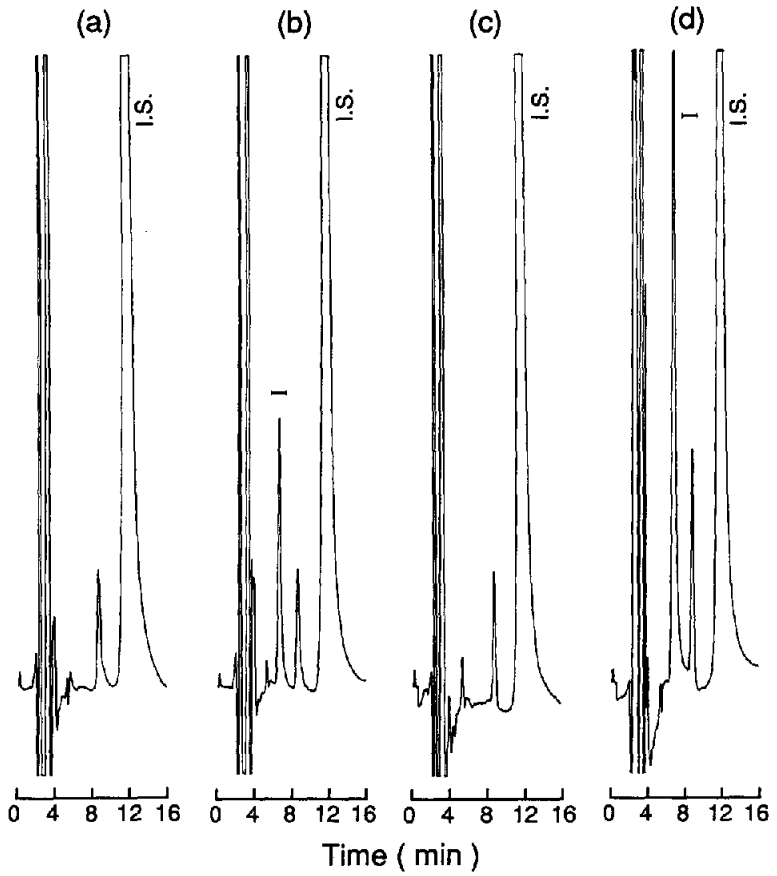

Fig. 4. Typical chromatograms of urine and bone samples: (a) control human urine containing 1 μg of I.S.; (b) human urine obtained from 12 to 24 h after intravenous infusion of 0.1 mg of I at a constant rate for 2 h (8.32 ng/ml); (c) control dog bone containing 1 μg of I.S.; (d) dog bone obtained 24 h after intravenous administration of 0.3 mg/kg I (1.16 μg/g).

row potential range between these electrodes made it possible to minimize the oxidation of interferences eluted from the column.

HPLC profiles

Typical chromatograms of plasma, urine and bone samples are shown in Figs. 3 and 4. Compound I and the I.S. were eluted at 6.7 and 11.8 min, respectively. The chromatograms obtained from control samples showed no interference peaks at the retention times of I and the I.S. (data not shown). The peak observed at 8.5 min may be a needle artifact peak with autosampler, because it was seen even after the injection of distilled water alone.

Calibration curves and limit of determination

Calibration curves were linear ($r > 0.999$) over the ranges 5–500 ng/ml (typical equation: $y = 0.0019x - 0.0016$) for Method A and 0.5–100 ng/ml ($y = 0.019x - 0.0056$) for Method B in plasma, 1–500 ng/ml ($y = 0.018x - 0.0088$) in urine, and 25–10 000 ng/g ($y = 0.00038x + 0.0046$) in bone. The limits of determination were 5 ng/ml for Method A and 0.5 ng/ml for Method B in plasma, 1 ng/ml in urine, and 25 ng/g in bone.

Recovery after sample preparation

The total recovery (mean ± S.D. of triplicate samples) of I following sample preparation was

TABLE I

ACCURACY AND PRECISION FOR THE DETERMINATION OF I IN HUMAN PLASMA, HUMAN URINE, AND RAT BONE

Prepared concentration (ng/ml or ng/g)	Concentration found (mean ± S.D. n = 4) (ng/ml or ng/g)	Coefficient of variation (%)	Percentage difference[a]
Plasma (Method A)			
5	5.3 ± 0.3	5.7	+ 6.0
10	10.1 ± 3.0	3.0	+ 1.0
50	49.9 ± 1.1	2.2	− 0.2
500	478.1 ± 8.4	1.7	− 4.4
Plasma (Method B)[b]			
0.5	0.47 ± 0.05	10.6	− 6.0
1	0.95 ± 0.04	4.2	− 5.0
5	4.94 ± 0.24	4.9	− 1.2
50	50.16 ± 1.20	2.4	+ 0.3
Plasma (Method B)[c]			
0.5	0.49 ± 0.05	10.2	− 2.0
1	0.98 ± 0.04	4.1	− 2.0
5	4.96 ± 0.24	4.8	− 0.8
50	50.92 ± 1.23	2.4	+ 1.8
Urine			
1	0.90 ± 0.03	3.3	−10.0
5	4.90 ± 0.14	2.9	− 2.0
50	48.91 ± 4.30	8.8	− 2.2
Bone			
25	27 ± 2	7.4	+ 8.0
50	47 ± 3	6.4	− 6.0
500	472 ± 20	4.2	− 5.6
5000	4875 ± 213	4.4	− 2.5

[a] Percentage difference (%) = [(mean observed concentration − prepared concentration) /prepared concentration] × 100.
[b] Determined by a calibration curve using control human plasma.
[c] Determined by a calibration curve using calf bovine serum.

56 ± 6% for Method A and 43 ± 4% for Method B in plasma, 56 ± 2% in urine, and 56 ± 4% in bone.

Accuracy and precision

As shown in Table I, the overall mean precision in Method A, as defined by the coefficient of variation (C.V.), ranged from 1.7% to 5.7% for human plasma. The analytical accuracy, expressed as a percentage difference of the mean observed value from the prepared concentration (Δ), varied from −4.4% to 6.0%. In Method B, calibration curves using control human plasma and calf bovine serum were simultaneously prepared to determine the concentration of I added to human plasma. Nearly identical values were thereby obtained using either calibration curve, and C.V. and Δ values ranged from 2.4% to 10.6% and from −6.0% to 1.8%, respectively. This result indicates that calf bovine serum, which is readily available and inexpensive, can be used as a subsitute for control human plasma in the preparation of the calibration curve. C.V. and Δ values in the assay of human urine ranged

from 3.3% to 8.8% and from −2.0% to −10.0%, and those of rat bone from 4.2% to 7.4% and from −6.0% to 8.0%, respectively.

Application to animal and human studies

Fig. 5 shows plasma concentrations of I determined by Method A after oral administration at 10 mg/kg to rats and by Method B at 5 mg to healthy volunteers. Plasma concentrations in rats and humans reached maximum levels at 0.5 and 1.0 h, and could be detected up to 3 and 6 h after administration, respectively.

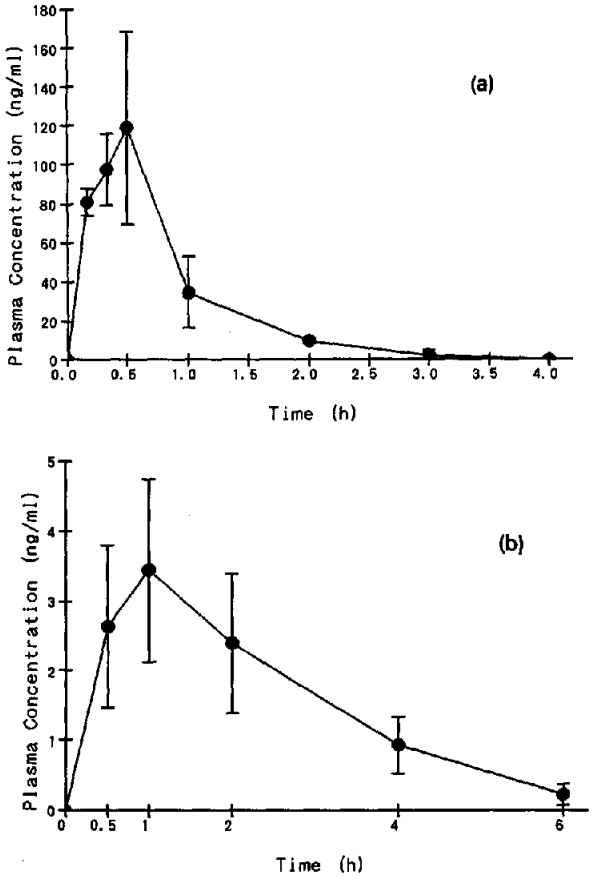

Fig. 5. Plasma concentrations of unchanged drug after oral administration of I at 10 mg/kg to rats, determined by Method A (a), and at 5 mg to healthy male volunteers, determined by Method B (b). Each point represents the mean ± S.E. of three rats (a) and six volunteers (b).

DISCUSSION

An HPLC method using a mobile phase at pH 11, an alkali-stable column and an electrochemical detector enabled the sensitive analysis of I in biological samples. Oxidation of I with the electrochemical detector was most reactive in the mobile phase at an alkaline pH, but was poor at pH 6.5 and even poorer at pH 4.0. Other bisphosphonates (EHDP, pamidronate, clodronate and SR-41319) were not oxidized under the same conditions. Cycloheptylamine was identified as an oxidation product of I (data not shown). In brief, when the peak fraction of I eluted from the detector was treated with fluorescamine, a fluorometric reagent for primary amines, the HPLC retention time of the derivative coincided with that of cycloheptylamine labeled with the reagent. This result indicates that I undergoes oxidative cleavage of the C–N bond in an electrochemical detector.

Sample preparation was based on coprecipitation of the drug with calcium phosphate, similar to the assay methods of other bisphosphonates [2,3,5–7]. Because calcium ions in the sample caused poor reproducibility, owing to the broadening of the peak on the chromatograms, we tried to remove them by adding EDTA solution. However, the peaks derived from the oxidation of EDTA and its impurities interfered with the analysis. A cation-exchange resin, AG 50W-X8, was therefore used instead of EDTA. The use of the commercially available H^+ form resulted in a low recovery of I, probably owing to adsorption of the secondary amino group of I on the resin. Therefore, we converted the H^+ form into K^+ in order to minimize ion-selectivity of the resin for amino groups. Recovery of I during the calcium-removal step using the K^+ form was 92%, higher than that using the H^+ form (77%).

Solid-phase extraction using a Sep-Pak C_{18} cartridge coupled with Method A enabled the extraction of I from a large volume (10 ml) of plasma and urine, and from bone samples containing a large excess of calcium. Compound I may be retained on this reversed-phase cartridge by a mechanism different from that of many other

compounds, namely via specific adsorption of bisphosphonic acid on materials in the cartridge, because I retained on the cartridge was eluted with NaOH solution but not with acetonitrile. Prior to solid-phase extraction, I in 10 ml of plasma was coprecipitated with calcium phosphate, whereas that in urine was precipitated by adding calcium chloride, because urine itself contained excess phosphate ions compared with calcium ions.

We established two methods for treating plasma samples. Method A uses 1 ml of plasma and is more simple, and is therefore useful for high-dose animal studies. Method B, being ten times more sensitive than Method A, is suitable for clinical studies at lower doses. Moreover, concentrations of bisphosphonates in bone, the target organ, have so far been determined by counting radioactivity following administration of labeled compounds [8–11]. The present method enables the determination of unchanged drug concentrations in bone after administration of unlabeled I, and is therefore useful for evaluating drug disposition in bone in long-term studies, such as toxicological or pharmacological studies.

REFERENCES

1 M. Kudo, T. Abe, K. Kawamuki, E. Yamaoka, Y. Isomura, M. Takeuchi and H. Kawashima, *J. Bone Mineral Res.*, 5, Suppl. 2 (1990) S166.
2 S. Bisaz, R. Felix and H. Fleisch, *Clin. Chim. Acta*, 65 (1975) 299.
3 T. L. Chester, E. C. Lewis, J. J. Benedict, R. J. Sunberg and W. C. Tettenhorst, *J. Chromatogr.*, 225 (1981) 17.
4 S. Auriola, R. Kostiainen, M. Ylinen, J. Mönkkönen and P. Ylitalo, *J. Pharm. Biomed. Anal.*, 7 (1989) 1623.
5 P. T. Daley-Yates, L. A. Gifford and C. R. Hoggarth, *J. Chromatogr.*, 490 (1989) 329.
6 G. Flesch and S. A. Hauffe, *J. Chromatogr.*, 489 (1989) 446.
7 J. P. Fels, J. Guyonnet, Y. Berger and W. Cautreels, *J. Chromatogr.*, 430 (1988) 73.
8 W. R. Michael, W. R. King and J. M. Wakim, *Toxicol. Appl. Pharmacol.*, 21 (1972) 503.
9 F. Wingen and D. Schmähl, *Arzneim.-Forsch.*, 37 (1987) 1037.
10 J. Mönkkönen and P. Ylitalo, *Eur. J. Drug Metab. Pharmacokin.*, 15 (1990) 239.
11 J. H. Lin, D. E. Duggan, I. W. Chen and R. L. Ellsworth, *Drug Metab. Dispos.*, 19 (1991) 926.