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Sensitive determination of a novel bisphosphonate, YM529, in plasma, urine and bone by high-performance liquid chromatography with fluorescence detection

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Abstract

A high-performance liquid chromatographic method for the sensitive determination of 1-hydroxy-2-(imidazo[1,2-a]pyridin-3-yl)ethane-1,1-bisphosphonic acid monohydrate (YM529) in plasma, urine and bone is described. Plasma obtained in high-dose animal studies is treated 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 dissolution of the precipitate in EDTA. Plasma obtained in low-dose clinical studies is treated by method B, a more sensitive method using 4 ml of plasma, which is based on direct precipitation of the drug prior to the deproteinization in method A. Urine and bone samples are prepared by solid-phase extraction using a Sep-Pak C_{18} cartridge coupled with method A. The drug is separated with a reversed-phase column using a mobile phase at pH 7, and detected with a fluorescence detector following postcolumn alkalization of the mobile phase to enhance fluorescence intensity. The limit of determination is 0.2 ng/ml for method A and 0.05 ng/ml for method B in plasma, 0.05 ng/ml in urine, and 5 ng/g in bone.

1. Introduction

1-Hydroxy-2-(imidazo[1,2-a]pyridin-3-yl)ethane-1,1-bisphosphonic acid monohydrate (YM529, I) (Fig. 1) is a newly synthesized bisphosphonate which is expected to prove clinically useful in the treatment of osteoporosis and hypercalcemia. In animal models, this agent shows a 100-fold greater potency against bone resorption than pamidronate, a second genera-

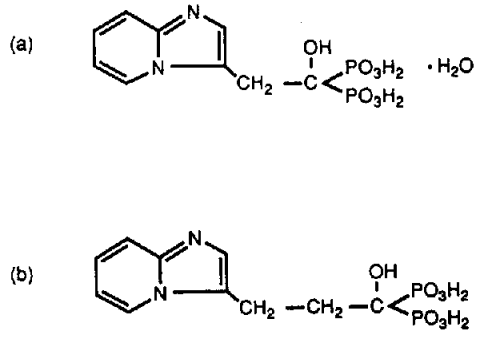

Fig. 1. Chemical structures of I (YM529) (a) and the internal standard (b).

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tion bisphosphonate [1], and is also 10 times more effective than disodium dihydrogen(cycloheptylamino)methylenebisphosphonate monohydrate (YM175, II), our previously developed bisphosphonate compound [1,2].

We previously reported an assay method for II by HPLC with electrochemical detection [3]. The limit of determination of II was 5 and 0.5 ng/ml in plasma, using samples of 1 and 10 ml, respectively, and 1 ng/ml in urine and 25 ng/g in bone. The determination of I in plasma and urine, however, was anticipated to require a 10-times higher sensitivity than that for II, because effective doses of I are as low as 1/10 of those of II. Although II lacks suitable functional groups for UV detection and derivatization, compound I possesses an imidazopyridin ring which emits intense fluorescence, especially at alkaline pH. We developed a sensitive assay method for I in plasma, urine and bone using reversed-phase HPLC with fluorescence detection.

2. Experimental

2.1. Chemicals and reagents

YM529 (I) and the internal standard (I.S.), 1-hydroxy- 2 -(imidazo[1,2-a]pyridin- 3 -yl)propane-1,1-bisphosphonic acid (Fig. 1), were synthesized at our Central Research Laboratories (Tokyo, Japan). Calcium phosphate (tribasic) was purchased from Nacalai Tesque (Kyoto, Japan) and used as a solution of 5 mg/ml in 0.1 *M* HCl. Distilled water was used for all procedures.

2.2. Standard solution

Compound I and the I.S. were each dissolved in a NaOH solution at two equivalents of these compounds, then diluted with water to a concentration of 1 mg/ml. These solutions were stored at 4°C for use as stock solutions. Standard solutions of I and the I.S. were prepared by diluting the stock solutions with water to suitable concentrations. Polypropylene tubes were used for the dilution because the studied compounds are readily adsorbed onto a glass surface in water at low concentrations.

2.3. 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), an RF-535 fluorescence detector (Shimadzu, Kyoto, Japan), a Develosil ODS-5 column (15 × 0.46 I.D. cm, Nomura Chemical, Tokyo, Japan), and a C-R4AX integrator (Shimadzu). In routine analysis, the column could used for 200–300 injections.

The mobile phase consisted of methanol – 10 m*M* sodium pyrophosphate containing 1 m*M* tetrabutylammonium phosphate, adjusted to pH 7.0 by orthophosphoric acid (5:95, v/v), and was delivered at a flow-rate of 1.0 ml/min. Column temperature was maintained at 40°C. The column eluate was mixed with 0.2 *M* NaOH using an LC-6A pump (Shimadzu) at a flow-rate of 0.1 ml/min, and then monitored fluorometrically at excitation and emission wavelengths of 281 and 391 nm, respectively.

2.4. Sample preparations

Plasma: method A

To 1 ml of plasma in a 10-ml polystyrene tube, 50 μl of 0.1 μg/ml I.S. solution was added. The sample was deproteinized by adding 4 ml of 6% (v/v) perchloric 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 mixture (pH > 12) 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. After centrifugation at 2000 *g* for 10 min, the precipitate was dissolved in 0.2 ml of 0.1 *M* ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) in 0.2 *M* NaOH (pH *ca.* 11). A 40-μl aliquot of the sample was injected onto the HPLC column. Compound I and the I.S. in the final solution

were stable for at least 72 h at room temperature.

Plasma: method B

To 4 ml of plasma in a 15-ml polypropylene tube, 50 μl of 0.2 μg/ml I.S. solution, 4 ml of water, 0.1 ml of calcium phosphate and 0.1 ml of 10 *M* NaOH were added. The mixture (pH > 12) was vortex-mixed for 30 s and centrifuged at 2000 *g* for 10 min. The supernatant was discarded. The precipitate was suspended in 1 ml of 0.1 *M* HCl, then deproteinized by adding 2 ml of 6% perchloric acid. After centrifugation at 2000 *g* for 10 min, the supernatant was treated in the same way as that obtained after deproteinization in method A, except that no calcium phosphate was added.

Urine

To 4 ml of urine in a 10-ml polystylene tube, 50 μl of 0.2 μg/ml I.S. solution, 0.1 ml of 2.5 *M* calcium chloride and 0.05 ml of 10 *M* NaOH were added. The mixture (pH > 12) was vortex-mixed for 30 s and centrifuged at 2000 *g* for 10 min. The supernatant was discarded, and the precipitate was dissolved in 3 ml of 0.1 *M* HCl and then applied to a Sep-Pak C_{18} cartridge, which was activated with 5 ml of acetonitrile and water. The cartridge was washed with 10 ml of water, 5 ml of 50% (v/v) acetonitrile, and 5 ml of water. The analytes were eluted with 6 ml of 0.2 *M* NaOH. The eluate was treated in the same way as the supernatant after deproteinization in method A, except that no 10 *M* NaOH was added.

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 capped glass tube. To the digest, 4.3 volumes of 10 *M* NaOH were added to form a transparent liquid phase. To a 15-ml polypropylene tube, 0.5 ml of the liquid phase (pH *ca.* 1), corresponding to 50 mg of bone, was transferred and spiked with 50 μl of 0.4 μg/ml I.S. solution and 3 ml of water, and then applied to a Sep-Pak C_{18} cartridge. The cartridge was washed with 10 ml of water and the analytes were eluted with 6 ml of 0.2 *M* NaOH. The eluate was spiked with 0.25 ml of calcium phosphate, vortex-mixed for 30 s, and centrifuged at 2000 *g* for 10 min. The precipitate obtained was dissolved in 4 ml of 0.05 *M* HCl and then spiked with 0.05 ml of 10 *M* NaOH. After mixing for 30 s and centrifugation at 2000 *g* for 10 min, the precipitate was dissolved in 0.1 *M* EDTA-2Na in 0.2 *M* NaOH, and 20 μl of the sample was injected onto the HPLC column.

2.5. Preparation of calibration curves

A 50-μl portion of each standard solution of I was added to 1 ml of plasma, 4 ml of plasma or urine, respectively. 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 4.6 and 4.2 volumes of conc. HCl and 10 *M* NaOH were added, respectively. Calibration curves were constructed by plotting the peak-height ratios of I to the I.S. *versus* the concentration of I.

3. Results

3.1. HPLC profiles

Typical chromatograms of plasma, urine and bone samples are shown in Figs. 2 and 3. Compound I and the I.S. were eluted at 6.2 and 9.2 min, respectively. The chromatograms obtained from control samples showed no interfering peaks at the retention times of I and the I.S. (data not shown).

3.2. Calibration curves and limit of determination

Calibration curves were linear ($r > 0.999$) over the ranges 0.2–100 ng/ml for method A and 0.05–10 ng/ml for method B in plasma, 0.05–10 ng/ml in urine, and 5–2000 ng/g in bone. The limits of determination were 0.2 ng/ml for method A and 0.05 ng/ml for method B in plasma, 0.05 ng/ml in urine, and 5 ng/g in bone.

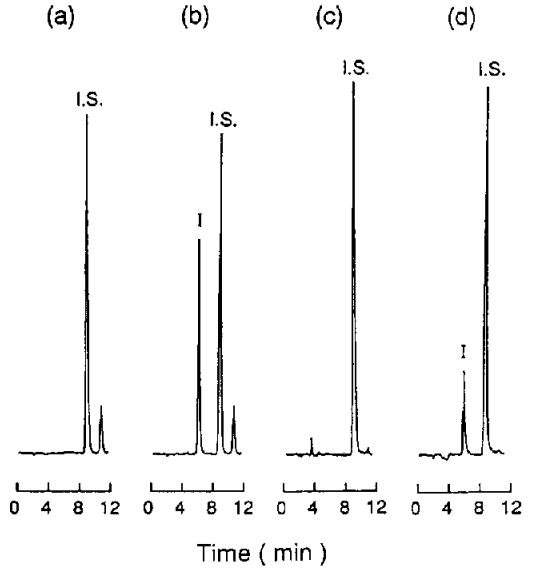

Fig. 2. Typical chromatograms of plasma samples containing I.S., treated by method A [(a) and (b)] and method B [(c) and (d)]: (a) control dog plasma; (b) dog plasma obtained 1 h after oral administration of 1 mg/kg of I (4.01 ng/ml); (c) control human plasma; (d) control human plasma spiked with 0.5 ng/ml of I.

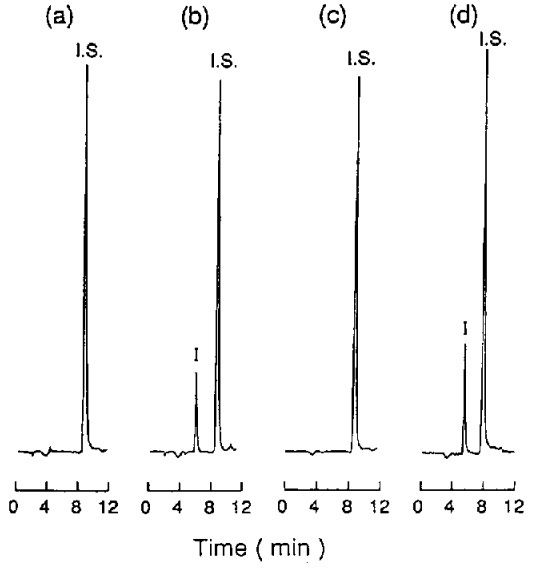

Fig. 3. Typical chromatograms of urine and bone samples containing I.S.: (a) control human urine; (b) control human urine spiked with 0.5 ng/ml of I; (c) control rat bone; (d) rat bone obtained 24 h after oral administration of 3 mg/kg of I (144 ng/g).

3.3. Recovery after sample preparation

Total recovery (mean ± S.D., $n = 3$) of YM529 following sample preparation was 90.1 ± 1.6% for method A and 45.6 ± 2.4% for method B in plasma at 5 ng/ml, 62.3 ± 3.5% in urine at 5 ng/ml, and 59.4 ± 0.8% in bone at 100 ng/g.

3.4. Intra-assay accuracy and precision

As shown in Table 1, overall mean precision in method A, as defined by the coefficient of variation (C.V.), ranged from 1.5% to 13.6% for human plasma. Analytical accuracy, expressed as a percent difference of the mean observed value from the prepared concentration (Δ), varied from 0% to +10.0%. In method B, C.V. and Δ values ranged from 3.9% to 10%, and from −2.0% to 2.0%, respectively.

C.V. and Δ values in assay of human urine ranged from 2.0% to 8.3%, and from −2.0% to 20%, and those for rat bone from 1.2% to 20%, and from −6.2% to 0%, respectively.

3.5. Application to the animal study

Fig. 4 shows plasma concentration of I determined by method A after oral administration at 0.3 mg/kg to dogs. Plasma concentration reached maximum levels at 2 h and could be detected up to 6 h after administration.

4. Discussion

Fluorescence of I in the alkaline mobile phase (pH *ca.* 11) was 4.5 times more intense than that in the mobile phase at pH 7. Although we tried reversed-phase HPLC using an alkaline mobile phase and several alkaline stable columns packed with polymer-based materials, sensitive analysis was not possible due to broadening of the peak. Therefore, compound I was separated using an octadecylsilica-based column with a neutral mobile phase, followed by mixing of the eluate with NaOH solution to enhance fluorescence intensity.

Sample preparation was based on coprecipita-

Table 1
Accuracy and precision for the determination of I in human plasma, human urine and rat bone

Sample	Prepared concentration (ng/ml or ng/g)	Concentration found (mean ± S.D., $n = 5$) (ng/ml or ng/g)	Coefficient of variation (%)	Percentage difference[a]
Plasma (method A)	0.2	0.22 ± 0.03	13.6	+10
	2	2.02 ± 0.03	1.5	+ 1.0
	20	20.0 ± 0.40	2.0	0
Plasma (method B)	0.05	0.050 ± 0.004	8.0	0
	0.5	0.51 ± 0.02	3.9	+ 2.0
	5	4.9 ± 0.5	10	− 2.0
Urine	0.05	0.060 ± 0.005	8.3	+20
	0.5	0.49 ± 0.02	4.1	− 2.0
	5	5.0 ± 0.1	2.0	0
Bone	5	5.0 ± 1.0	20.0	0
	50	46.9 ± 2.6	5.5	− 6.2
	500	495 ± 6	1.2	− 1.0

[a] Percentage difference = [(mean of concentration found − prepared concentration)/prepared concentration] × 100.

tion of the drug with calcium phosphate, in a way similar to the assay methods for other bisphosphonates [3–8]. This procedure involves a modification of the YM175 (II) assay [3], namely deproteinization of plasma, digestion of bone by HCl, and solid-phase extraction of urine and bone samples using a Sep-Pak C_{18} cartridge. Overall, the sample preparation in the assay for compound I is simpler than that for compound II, especially in the dissolution step of the final calcium precipitate. The precipitate obtained in the assay for I is merely dissolved in EDTA solution, while that for II is treated in 4 steps, *e.g.* dissolution of the precipitate in phosphoric acid, removal of excess calcium ions by a cation-exchange resin, filtration, and alkalization by NaOH.

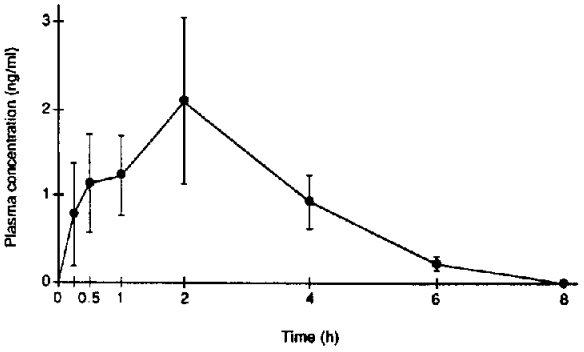

Fig. 4. Plasma concentration of unchanged drug after oral administration of I at 0.3 mg/kg to dogs, determined by method A. Each point represents the mean ± S.E. of four dogs.

We established two methods for the treatment of plasma samples, in the same manner as in the assay for II. Method A requires 1 ml of plasma, and is simpler and therefore useful for high-dose animal studies. Method B requires 4 ml of plasma, and is 4 times more sensitive than method A and therefore suitable for clinical studies at lower doses. Finally, the sensitivities in the assay of I are sufficiently higher than those for II, *i.e.* 25 times higher for method A and 10 times for method B in plasma, 20 times in urine, and 5 times in bone.

5. References

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