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Note

Determination of the bisphosphonate pamidronate disodium in urine by pre-column derivatization with fluorescamine, high-performance liquid chromatography and fluorescence detection

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Pamidronate disodium (Fig. 1), disodium 3-amino-1-hydroxypropylidenebisphosphonate pentahydrate (APD), is a bisphosphonate that inhibits bone resorption in vivo [1]. This compound is used in the treatment of bone diseases, such as Paget's disease [2] and hypercalcaemia of malignancy [3,4].

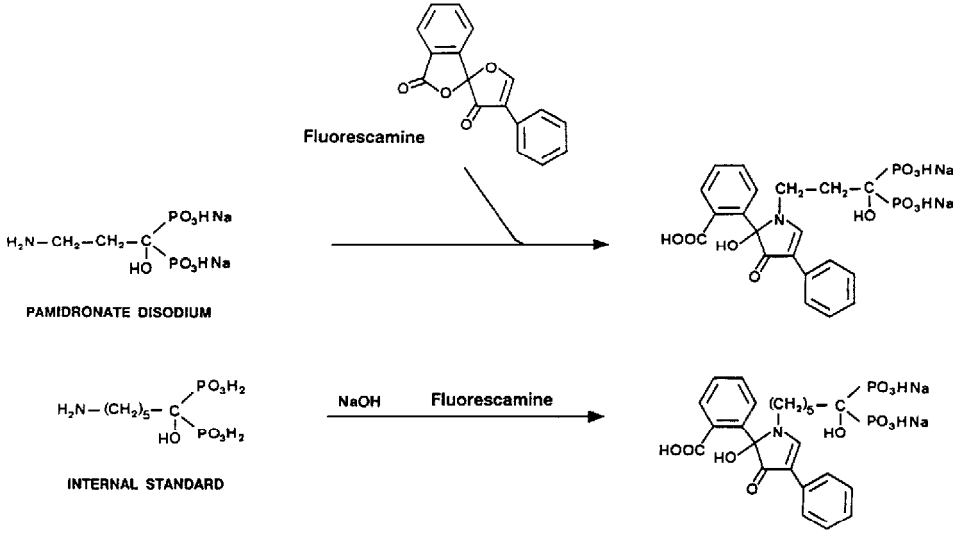

Fig. 1. Structures of pamidronate disodium and internal standard, and their probable reaction products with fluorescamine.

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A procedure for measuring another bisphosphonate, ethane-1-hydroxy-1,1-bisphosphonate (EHDP), in urine has been developed [5,6]. This procedure is based on a co-precipitation of the bisphosphonate with calcium phosphate, elimination of inorganic phosphate, decomposition of the P–C–P bond with UV light and spectrophotometric determination of the inorganic phosphate released. The limit of detection was 2.5 μmol/l in 5 ml of urine.

This paper describes a new technique for quantitation of APD in urine. The procedure is based on co-precipitation of pamidronate with calcium phosphate [7], derivatization with fluorescamine [8] and high-performance liquid chromatography (HPLC) on a Nucleosil C_{18} column with fluorescence detection. The method has been applied to the analysis of urine samples from dogs treated with a single dose of APD.

EXPERIMENTAL

Chemicals

All solvents and reagents were of analytical grade (Fluka, Buchs, Switzerland; Merck, Darmstadt, F.R.G.) and were used without further purification. Pamidronate disodium pentahydrate (CGP 23 339 A, $C_3H_9NO_7P_2Na_2 \cdot 5H_2O$, MW 369.11) and the internal standard (CGP 38 146, 6-amino-1-hydroxyhexylidene-bisphosphonate, $C_6H_{17}NO_7P_2$, MW 277.15) originated from CIBA-GEIGY (Basle, Switzerland).

Fluorescamine was obtained from Fluka and was dissolved in acetonitrile (1 mg/ml). Ethylenediaminetetraacetic acid disodium salt dihydrate (Na_2EDTA) was from Fluka. The pH of the 0.13 *M* Na_2EDTA solution was adjusted to 9 with a 6.25 *M* sodium hydroxide solution. These solutions were stored at 4 °C.

Calcium chloride (dihydrate), sodium dihydrogenphosphate (monohydrate), sodium hydroxide and hydrochloric acid were purchased from Merck.

Water was deionized and filtered through a 0.45-μm Millipore® filter before use.

Chromatographic conditions

A Hewlett-Packard binary HPLC system (Model 1090) equipped with an automatic sampling system and a fluorescence detector (Merck-Hitachi, F 1000) was used. The fluorescence detector was set at 395 nm for excitation and at 480 nm for emission. The peak heights were obtained through a Merck-Hitachi computing integrator (Model D-2000). The column (250 mm × 4 mm I.D.) was packed with Nucleosil C_{18} (10 μm particle size, Macherey-Nagel, Oensingen, Switzerland). Chromatography was performed at a temperature of 40 °C. The mobile phase contained an aqueous solution of 1 m*M* Na_2EDTA–methanol (97:3, v/v). The bisphosphonates have a strong affinity for metal ions, therefore Na_2EDTA, which is a cation-complexing agent, was used in the mobile phase. The pH of the Na_2EDTA solution was adjusted to 6.5 with a 1 *M* sodium hydroxide solution. The flow-rate of the mobile phase was 1 ml/min.

Preparation of standard solutions

Stock solutions of APD and internal standard were prepared by dissolving 4–8 mg of APD in 100 g of water and dissolving 4 mg of internal standard in 10 g of 0.1 *M* sodium hydroxide. Aliquots of these stock solutions were diluted with water to yield the appropriate concentrations. The solutions served to prepare spiked urine samples for calibration curves and validation analyses. Different amounts of internal standard were added to samples of low and high concentrations of APD.

Procedure

The co-precipitation procedure of pamidronate in urine was similar to that described by Bisaz et al. [5]. To 2.0 ml of human urine (in a 10-ml glass centrifuge tube), 0.1 ml of internal standard solution was added. The urine was adjusted to pH 3 by addition of concentrated hydrochloric acid to dissolve any calcium phosphate, which could absorb pamidronate. It was then filtered through a fluoromembrane (0.45 μm, Model Acro LC 13, Skan, Basle, Switzerland). The filtered samples were deproteinized with 1.5 *M* trichloroacetic acid (0.5 ml). The samples were centrifuged for 15 min at 1500 *g* at room temperature. After addition of 20 μl of a 2.5 *M* calcium chloride solution and 40 μl of a 0.5 *M* sodium dihydrogenphosphate solution to the protein-free samples, the pH was adjusted to 12.0 using first 6.25 *M* sodium hydroxide and finally 0.5 *M* sodium hydroxide. The precipitate was isolated by centrifugation for 15 min at 1500 *g* at room temperature, washed with 2 ml of deionized water and dissolved in 1 ml of 0.1 *M* hydrochloric acid. To remove impurities from the samples, a second precipitation was carried out using 0.5 *M* sodium hydroxide. The precipitate was separated by centrifugation for 15 min at 1500 *g* at room temperature, washed with deionized water and dissolved in 0.2 ml of a 0.13 *M* Na_2EDTA solution (pH 9). While the tube containing APD and the internal standard was shaken vigorously on a vortex-type mixer, 0.1 ml of fluorescamine dissolved in acetonitrile (1 mg/ml) was added. The mixture was extracted with 0.2 ml of dichloromethane and centrifuged for 3 min at 1000 *g* at room temperature. The aqueous phase was transferred to a micro injection vial which was sealed with a PTFE cap.

Calibration

To construct calibration curves, urine samples with known concentrations were prepared by adding APD to 2.0 g of drug-free human urine. After addition of the internal standard, the samples were processed as described above. A 10-μl volume of each sample was injected, and peak-height ratios of the compound to the internal standard were plotted against the APD concentrations. Calibration curves for APD were calculated by quadratic least-squares regression ($y=a+bx+cx^2$). By regression analysis the following terms for calibration curves in the range 0.5–21.9 μmol/l were obtained: $y=0.0516+0.1445x+0.001x^2$ ($Sb=0.0164$, $Sc=0.0008$), $r=0.9988$ (x denotes the independent variable, i.e. the concentrations of APD in urine in μmol/l; y denotes the dependent variable, i.e. the ratio of the peak-height values; S and r denote the estimated standard deviation and the coefficient of correlation).

RESULTS AND DISCUSSION

Derivatization

Fig. 1 shows the probable products of reaction of APD and internal standard with fluorescamine. These two compounds, on excitation at 395 nm, fluoresce at 480 nm.

Stability of fluorescence

Solutions of the fluorescamine derivatives of APD and internal standard were kept at room temperture. Aliquots of each solution were injected into the chromatograph over a period of 15 h. The peak-height ratios of APD to internal standard were constant for up to 15 h.

Selectivity

Urine samples containing no APD or internal standard were carried through the analytical procedure. Fig. 2 shows the chromatograms of drug-free human urine (A), drug-free dog urine (B) and urine from a dog receiving a single intravenous injection of 5 mg of APD in 0.15 *M* saline (C) carried out through the analytical procedure.

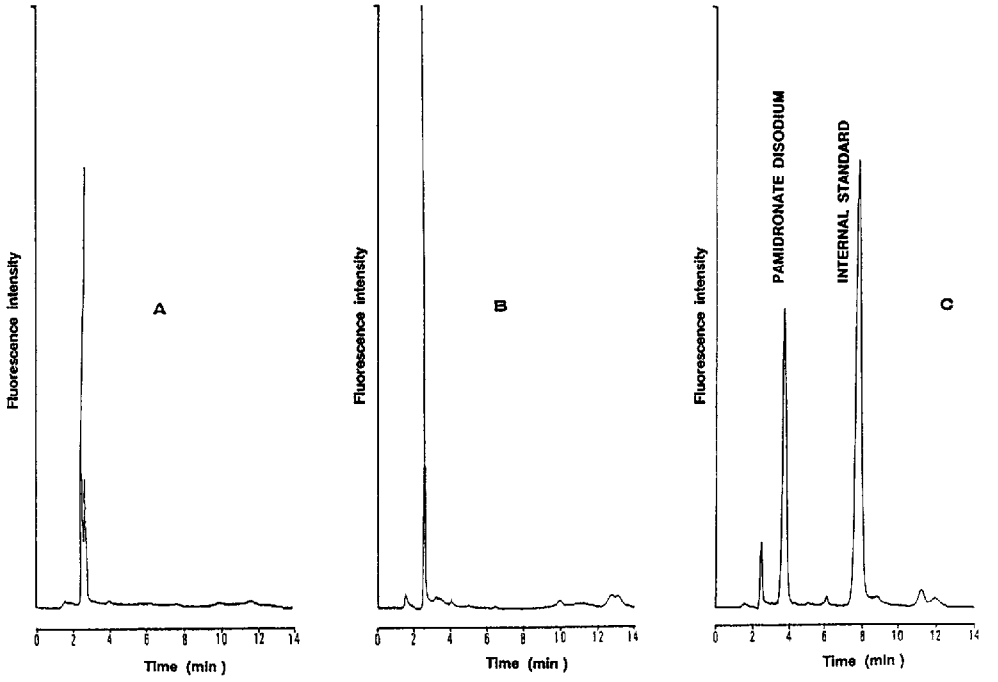

Fig. 2. Chromatograms of (A) drug-free human urine, (B) drug-free dog urine and (C) urine from a dog receiving a single intravenous injection of 5 mg of pamidronate disodium in 0.15 *M* saline.

TABLE I

RESULTS OF VALIDATION ANALYSES USING SPIKED URINE SAMPLES

Expected (μmol/l)	Found (mean $\pm$S.D., $n=3$) (μmol/l)	Inter-assay precision (C.V., %)	Deviation from theory (%)
0.33	0.28 $\pm$ 0.08	28.6	−15.2
0.55	0 47 $\pm$ 0.10	21.3	−14.6
1.00	0.98 $\pm$ 0.06	6.1	−2.0
2.85	2 83 $\pm$ 0.28	9.9	−0.7
4.05	4.08 $\pm$ 0.30	7.4	+0.7
5.66	5.51 $\pm$ 0.17	3.1	−2.6
7.57	7.70 $\pm$ 0.27	3.5	+1.7
8.03	8.20 $\pm$ 0.33	4.0	+2.1
9.69	10.11 $\pm$ 1.05	10.4	+4.3

Precision and accuracy

Validation samples of spiked human urine were analysed together with each series of analytical samples. Nine samples in the concentration range 0.33–9.69 μmol/l were analysed on three different days. The results of these analyses are summarized in Table I. The inter-assay coefficient of variation for the three repetitive determinations of one concentration ranged from 3.1 to 28.6%; the deviations of the mean found values from the given concentrations ranged from −15.2% to +4.3%.

Limit of quantitation and limit of detection

The limit of quantitation, defined as the lowest concentration that can be assayed with a relative standard deviation of $\pm$10%, is 1 μmol/l of urine, using a sample of 2 ml. The limit of detection was 50 nmol/l of urine for a signal-to-noise ratio of 3 : 1.

Application

APD was administered to two beagle dogs. First they received a single oral dose of 197 mg of pamidronate disodium pentahydrate as an enteric-coated tablet, corresponding to 150 mg of pamidronate disodium. Three weeks later one dog received a single intravenous injection of 5 mg of pamidronate disodium pentahydrate in 0.15 *M* saline. Urine was collected from each dog before and 0–4, 4–8, 8–24, 24–48 and 48–72 h after administration.

APD was determined in the urine samples by the method described, with one modification: dog urine was filtered through a paper filter (Schleicher and Schüll, Feldbach, Switzerland). The cumulative excretion of pamidronate as a percentage of the dose is shown in Fig. 3. In total, 61.2% of the dose was excreted in the 0–72 h urine of the dog receiving the intravenous dose. From the oral doses given as enteric-coated tablets, 0.4 and 0.5% of the dose were recovered in urine of the two dogs within 72 h. By reference to the renal elimination of pamidronate after the intravenous dose, 0.6 and 0.8% of the oral dose have been absorbed.

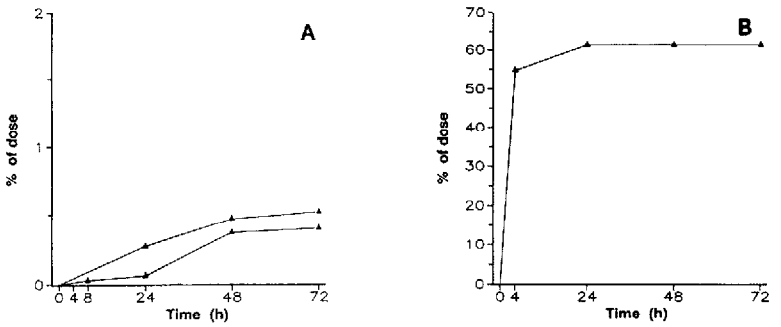

Fig. 3. Cumulative urinary excretion of pamidronate as a percentage of the dose. (A) Oral doses of 197 mg of APD pentahydrate, given as enteric-coated tablets to two dogs; (B) intravenous dose of 5 mg APD.

CONCLUSIONS

The analytical method described is sufficiently sensitive and selective for the determination of pamidronate in urine. The analysis has been used to measure urine levels after single oral or intravenous doses of pamidronate to dogs.

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