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Short Communication

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

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ABSTRACT

An improved method for the determination of 1-hydroxy-3-aminopropylidene-1,1-bisphosphonate (pamidronate) in human urine and plasma is described. The procedure is based on a co-precipitation of the bisphosphonates (pamidronate and 6-amino-1-hydroxypentilidene-bisphosphonate, used as internal standard) with calcium phosphate. After centrifugation the precipitate is redissolved in hydrochloric acid, followed by a second precipitation. Then the bisphosphonates are dissolved in ethylenediaminetetraacetic acid, derivatized with fluorescamine, and separated by high-performance liquid chromatography. Using fluorescence detection, the limit of quantitation for pamidronate was 0.8 μmol/l in plasma and 0.7 μmol/l in urine.

INTRODUCTION

Because pamidronate lacks functional groups that are readily detectable, it has to be derivatized before or after chromatography. Three high-performance liquid chromatographic (HPLC) methods are available for the determination of pamidronate disodium in biological fluids [1–3]. The first one to be published (developed for urine samples) was based on co-precipitation of pamidronate with calcium phosphate, derivatization with fluorescamine and HPLC on a Nucleosil C_{18} column with fluorescence detection [1]. In the second (developed for urine and plasma samples), pamidronate was isolated by precipitation as a calcium salt, chromatographed on an ion-exchange system and detected after reaction with ammonium persulphate and molybdenum ascorbate to yield the phosphomolybdate chromophore. Visible detection was performed at 820 nm [2]. In the most recently published method (developed for urine and plasma samples), pamidronate was derivatized using phenylisothiocyanate and analyzed by reversed-phase ion-pair chromatography. UV detection was performed at 240 nm [3].

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The method described here is based on the assay published earlier for the determination of pamidronate in urine samples [1], with essential improvements. The use of a new internal standard (I.S), structurally closely related to pamidronate, reduces the analysis time and makes for easier integration of the peak corresponding to the I.S. The derivatization step was also modified. A higher derivatization yield was obtained by increasing both the pH and the concentration of the fluorescamine solution, resulting in increased sensitivity and precision in both plasma and urine samples.

The method has been applied to the analysis of plasma and urine samples from a patient treated with a single intravenous infusion of pamidronate disodium at a constant infusion rate.

EXPERIMENTAL

Chemicals

All solvents and reagents were of analytical grade (Fluka, Buchs, Switzerland; Merck, Darmstadt, Germany) and 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 33 637, 6-amino-1-hydroxypentilidene-bisphosphonate, $C_5H_{15}NO_7P_2$, MW 263.12) originated from Ciba-Geigy (Basle, Switzerland). Fluorescamine was obtained from Fluka and was dissolved in acetonitrile (3 mg/ml). Ethylenediaminetetraacetic acid disodium salt dihydrate (Na_2EDTA) was also obtained from Fluka. The pH of the 0.13 *M* Na_2EDTA solution was adjusted to 10 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 (Darmstadt, Germany).

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

Chromatographic conditions

The chromatographic conditions were used as previously described [1]. The retention times of pamidronate and the derivative of the new I.S. under these conditions were 3.9 and 4.8 min, respectively.

Solutions

The solutions of pamidronate disodium and the I.S. were prepared by dissolving 4.0 mg of pamidronate disodium in 100 g of water, and dissolving 2.5 mg of the I.S. in 100 g of 0.1 *M* sodium hydroxide. Aliquots of these stock solutions served to prepare spiked plasma and urine samples for calibration curves and validation analyses.

Sample preparation

The co-precipitation procedure and the derivatization of the bisphosphonates and the I.S. in plasma and urine were similar to those described previously [1], with the following modifications: after the second precipitation, the precipitate was dissolved in 0.2 ml of a 0.13 *M* Na_2EDTA solution (pH 10), then the bisphosphonates were derivatized using 0.1 ml of fluorescamine dissolved in acetonitrile (3 mg/ml).

Calibration

Calibration curves in plasma and urine were prepared as follows: drug-free plasma samples (2.0 ml) were spiked with pamidronate disodium. After addition of the I.S., the samples were processed as described above. A 20-μl volume of each sample was injected. The peak area of pamidronate was divided by the peak area of the I.S. and plotted against the pamidronate concentrations. Calibration curves for pamidronate were calculated by quadratic least-squares regression ($y = a + bx + cx^2$).

In plasma, the calibration range was 0.64–8.26 μmol/l. The regression equa-

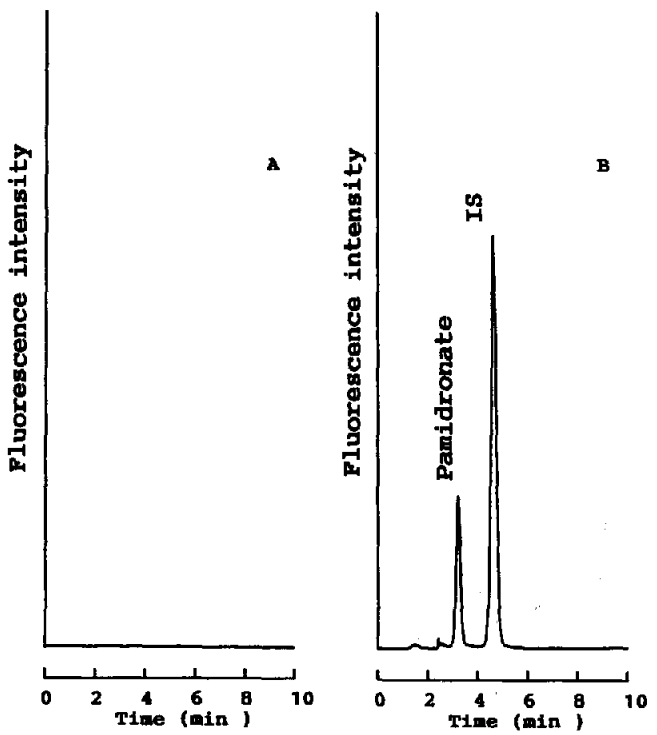

Fig. 1. Chromatograms obtained from (A) a drug-free plasma sample and (B) a patient's plasma sample after an infusion at constant infusion rate of 60 mg of pamidronate disodium in 1 h.

tion was: $y = 0.0086 + 0.1023x - 0.0007x^2$ ($S_b = 0.0020$, $S_c = 0.0001$), $r = 0.9999$ (x denotes the independent variable, *i.e.* the ratio of the peak area; S and r the estimated standard deviation and the coefficient of correlation).

In urine, the calibration range was 0.61–7.95 μM. The regression equation was: $y = 0.0072 + 0.1130x - 0.0007x^2$ ($S_b = 0.0023$, $S_c = 0.0001$), $r = 0.9999$.

RESULTS AND DISCUSSION

Derivatization

The two bisphosphonates produced after derivatization with fluorescamine a fluorophor with excitation and emission maxima at 395 nm and 480 nm, respectively.

Selectivity

The chromatograms of a drug-free plasma sample and a sample from a patient after a constant-rate infusion of 60 mg pamidronate disodium and processed as described above are shown in Fig. 1. No other plasma components were observed.

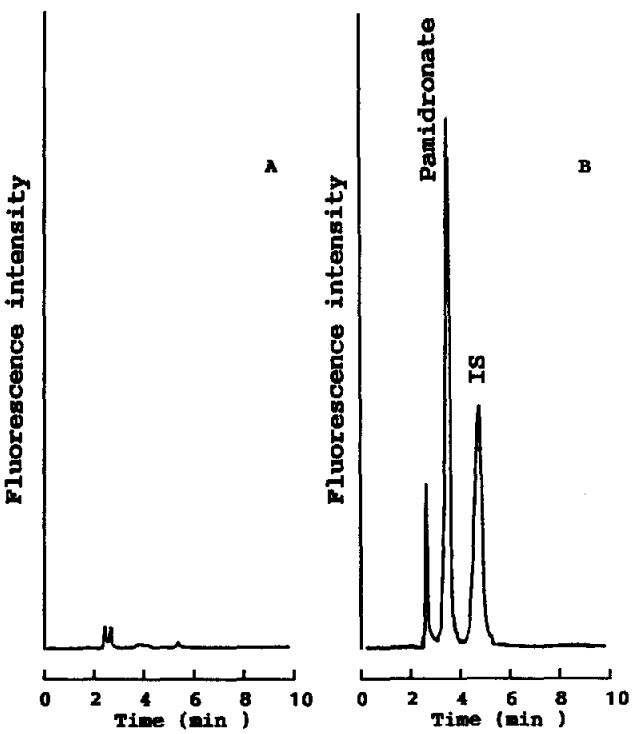

Fig. 2. Chromatograms obtained from (A) a drug-free urine sample and (B) a patient's urine sample after an infusion at constant infusion rate of 60 mg of pamidronate disodium in 1 h.

TABLE I

RESULTS OF VALIDATION ANALYSES IN PLASMA SAMPLES SPIKED WITH PAMIDRONATE DISODIUM

Added (μmol/l)	Found (mean ± S.D., n = 3) (μmol/l)	Inter-assay precision (C.V.,%)	Deviation from theory (%)
0.64	0.67 ± 0.03	4.5	+4.9
1.28	1.32 ± 0.05	3.9	+3.2
2.81	2.92 ± 0.08	2.7	+4.1
4.89	5.12 ± 0.20	3.9	+4.8
6.54	6.95 ± 0.38	5.5	+6.4
7.52	8.04 ± 0.63	7.9	+7.0
8.26	8.40 ± 0.92	11.0	+1.6

Fig. 2 shows chromatograms of a drug-free urine sample and a sample from a patient after a constant-rate infusion of 60 mg pamidronate disodium. There was no interference of urine components with the peaks of the bisphosphonate derivatives.

Accuracy and precision

Accuracy and precision were evaluated by analysing spiked plasma samples. Seven samples containing concentrations between 0.64 and 8.26 μmol/l pamidronate disodium were analysed on three different days. The inter-assay coefficient of variation (C.V.) ranged from 2.7 to 11.0%. The deviations of the mean found values from the given concentrations ranged from 1.6 to 7.0% (Table I).

TABLE II

RESULTS OF VALIDATION ANALYSES IN URINE SAMPLES SPIKED WITH PAMIDRONATE DISODIUM

Added (μmol/l)	Found (mean ± S.D., n = 3) (μmol/l)	Inter-assay precision (C.V., %)	Deviation from theory (%)
0.61	0.66 ± 0.04	5.9	+7.8
1.22	1.22 ± 0.03	2.6	−0.6
2.71	2.72 ± 0.14	5.0	+0.5
4.74	4.74 ± 0.22	4.7	+0.1
6.21	6.30 ± 0.27	4.2	+1.5
7.16	7.62 ± 0.31	4.1	+6.4
7.95	8.18 ± 0.90	11.0	+2.9

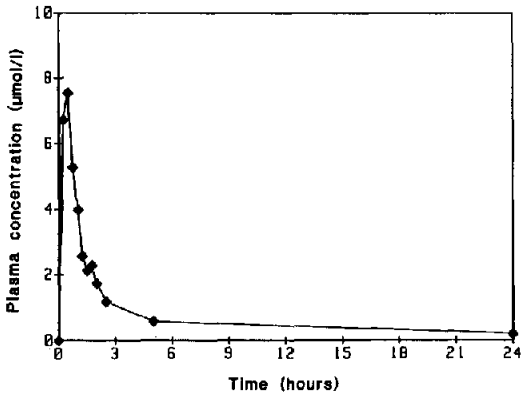

Fig. 3. Plasma concentration–time curve of pamidronate after a single intravenous infusion at constant infusion rate of 60 mg pamidronate disodium administered in 1 h to a patient with bone metastases.

Urine samples containing pamidronate disodium at seven different concentrations were analysed on three different days. The concentrations of pamidronate disodium ranged from 0.61 to 7.95 μmol/l. The inter-assay C.V. ranged from 2.6 to 11.0%. The deviations of the mean found values from the given concentrations ranged from −0.6 to +7.8%. The results are given in Table II.

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 ± 10%, is 0.8 μmol/l in plasma and 0.7 μmol/l in urine, using samples of 2 ml. The limit of detection was 10 nmol/l in both plasma and urine, at a signal-to-noise ratio of 3:1.

Application

Fig. 3 shows the plasma concentration–time curve of pamidronate in one patient with bone metastases after a single intravenous infusion of pamidronate disodium. This patient received 60 mg of pamidronate disodium in 1 h. Cumulative urinary excretion of pamidronate in the same patient after a single intravenous infusion of pamidronate disodium (60 mg in 1 h) was 55% of the dose.

CONCLUSION

The improved analytical method for pamidronate is sufficiently sensitive and selective for quantitative determination in human plasma, as well as in urine, after therapeutic doses.

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