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DETERMINATION OF (4-CHLOROPHENYL)THIOMETHYLENE BISPHOSPHONIC ACID, A NEW BISPHOSPHONATE, IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid and sensitive high-performance liquid chromatographic method for the assay of the bisphosphonate (4-chlorophenyl)thiomethylene bisphosphonic acid in plasma and urine is described. It requires selective precipitations and dissolutions of calcium salts prior to reversed-phase chromatography with UV detection. This method used semi-micro scale material and 200-μl biological aliquots. The limit for accurate quantification is 50 ng/ml. Data on reliability criteria and application to a pharmacokinetic study are presented.

INTRODUCTION

(4-Chlorophenyl)thiomethylene bisphosphonic acid (I) is a new bisphosphonate displaying antiosteolytic and antiarthritic activities in animals [1, 2], and is currently undergoing clinical trial in Paget's disease.

One of the new features of this drug is that it contains a functional group that can be readily detected with conventional liquid chromatographic detectors. This could be a decisive advantage, since the assay in biological media of bisphosphonates is still a technical challenge. Oral absorption of bisphosphonates has been reported to be very low and irregular [3], so researchers are attempting to adapt the drug dosage to an individual patient's need and to improve the bioavailability of the drug through new galenic formulations.

Few analytical methods have been described for the quantitative determination of bisphosphonates in biological fluids [3, 4], and none is sufficiently sensitive or quickly enough performed to allow the determination of bisphosphonates as needed in human therapy.

This paper reports the development and validation of a rapid and sensitive

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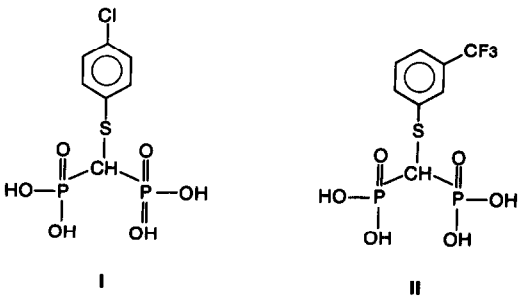

Fig. 1. Structures of (4-chlorophenyl)thiomethylene bisphosphonic acid (I) and (3-trifluoromethylphenyl)thiomethylene bisphosphonic acid (II).

high-performance liquid chromatographic (HPLC) assay with UV detection for unchanged I in plasma and urine.

EXPERIMENTAL

Chemicals and supplies

Compound I (SR 41319) and the internal standard (3-trifluoromethyl)-thiomethylene bisphosphonic acid (II) were supplied by Sanofi Recherche (Montpellier, France). ^{14}C-Labelled (4-chlorophenyl)thiomethylene bisphosphonic acid (960 MBq/mmol) was synthesized by Sanofi Labaz (Brussels, Belgium). All products were submitted to quality control. The chemical structures are shown in Fig. 1. Acetonitrile, HPLC grade, was purchased from Rathburn (Walkerburn, U.K.). All other chemicals were of analytical grade. Polypropylene micro test-tubes (2.2 ml capacity) from Eppendorf (Hamburg, F.R.G.) were used for preparation of biological samples.

Chromatographic equipment

The HPLC system consisted of a Model 5000 (Varian, Palo Alto, CA, U.S.A.) solvent-delivery pump, a Model 710 B WISP automatic injector (Waters Assoc., Milford, MA, U.S.A.) and a Model 160 UV detector (Beckman, Berkeley, CA, U.S.A.) equipped with a 280-nm wavelength filter. The output signal was fed to a Model SP 4270 integrator linked to a PC microcomputer, using Labnet-XT system (Spectra-Physics, Santa Clara, CA, U.S.A.) for recording and calculations.

Spectral comparisons were performed using a Waters 990 photodiode array detector.

The separation unit was a 150 mm × 4.1 mm I.D., 5 μm PRP-1 column (Hamilton, Reno, NV, U.S.A.). Chromatographic conditions are given in Fig. 2. Other equipment included a small centrifuge, Biofuge B (Heraeus-Christ, Osterode, F.R.G.), equipped with a drum rotor with a capacity of 80 micro-tubes.

Preparation of standards and quality control samples

Standards were prepared by dissolving compounds in deionised water. Two stock standard solutions were prepared each month, one containing 1 mg/ml I

and the other containing 1 mg/ml II. Working standard solutions at concentrations of 1–100 μg/ml were prepared weekly by appropriate dilution of the stock solutions.

Quality control samples were prepared by adding drug solution to biological control samples in order to obtain two levels of final concentration, namely 0.5–2 μg/ml in plasma and 1–2 μg/ml in urine. Enough of these samples were prepared to last throughout each study and were stored at −20°C pending analysis.

Procedure for separation of I from plasma or urine samples

To a 200-μl plasma or urine sample, 400 μl of water containing 2 μg of internal standard were added. A first precipitation of insoluble calcium salts was obtained after the addition of 100 μl of 1 *M* sodium hydroxide and 100 μl of 0.18 *M* calcium chloride. Before vortexing and centrifuging (at 11 600 *g*) to separate the precipitate, the medium was diluted with 1 ml of deionized water. Then the liquid phase was discarded and the solid residue was dissolved in 100 μl of 1 *M* hydrochloric acid. A second precipitation was obtained by adding 200 μl of 1 *M* sodium hydroxide (100 μl of 0.18 *M* calcium chloride were added only for plasma samples). Dilution, agitation and centrifugation were carried out under identical conditions, and the final residue was vortexed with 200 μl of mobile phase containing 0.1 *M* disodium ethylenediaminetetraacetate until complete dissolution. A 20–100 μl aliquot was injected into the HPLC column.

Quantification

The concentration of I in plasma and urine was determined from eight-point calibration curves of peak-area ratios (I/internal standard) versus the concentration of I added to control plasma and urine taken through the described procedures. A new calibration curve was made with each series, which included one complete biological control and four quality control samples (two levels in duplicate). If necessary, initial dilution of the biological samples was performed in order to obtain results within the calibration range.

RESULTS AND DISCUSSION

Method

Attempts to extract I from biological media with polar organic solvents containing counter ions led to a poor reproducibility with low recovery and chromatographic interferences. Taking advantage of the very low solubility of the calcium salt of I, a separation procedure was tried and finally the two-step precipitation of calcium salts in basic medium was shown to be a simple and reliable way to purify I. The recovery, measured by liquid scintillation counting prior to HPLC, was 74.4% ($n=7$, S.D.$=1.3$) in plasma and 93.0% ($n=7$, S.D.$=1.0$) in urine.

The ion-pair chromatographic system operated in reversed-phase mode proved more efficient than ion-exchange chromatography, especially when a strongly basic medium was used to increase the proportion of ionized bisphosphonate groups combined with the counter ion. A polystyrene–divinylbenzene resin, which is stable at this pH, was selected as stationary phase.

Calibration curves usually ranged from 0.05 to 2 μg/ml I. The equations of the curves were calculated by least-squares linear regression. Good linear relationships were obtained, with intercepts not significantly different from zero. Correlation coefficients for twelve standard curves averaged 0.999 ± 0.0001 (± S.D.).

The peaks representing I and the internal standard were symmetrical and well separated from the injection front (Fig. 2). Diode-array rapid-scan spectrophotometry was used to compare the UV spectra (230–310 nm) obtained at three points of the peak of I: at the apex, slope and valley. After baseline subtraction and normalization to eliminate concentration effects, superimposable spectra were obtained for the pure compound, for plasma from treated human subjects and for urine from mice treated at toxicological doses.

In addition, this procedure can be adapted to various biological samples such as soft tissues, cartilages and bones through a single initial acid digestion, although the data are not presented here.

The accuracy and precision of the method were estimated according to the following intra-day procedure: six series of spiked samples at seven concentration levels (0–2 μg/ml) were prepared from control plasma and urine. A linear regression using multiple dependent variables was then calculated and used as reference

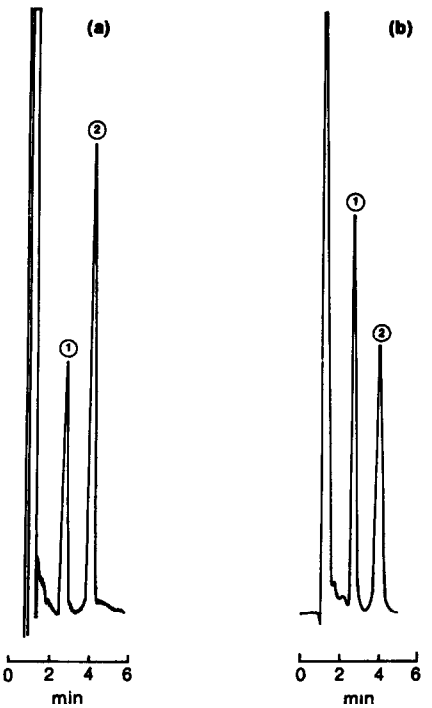

Fig. 2. (a) Typical chromatogram obtained of a plasma sample from a treated subject (1 μg/ml). (b) Typical chromatogram obtained of a urine sample from a treated subject (1.4 μg/ml). Column, Hamilton 5 μm PRP-1 (150 mm × 4.1 mm I.D.); column temperature, 22°C; mobile phase, 0.005 *M* tetrabutyl ammonium phosphate–0.05 *M* sodium hydrogenphosphate (pH 11.8)–acetonitrile (87:13). Flow-rate, 1 ml/min; injection volume, 20 μl; UV detection at 280 nm. Peaks: 1 = I; 2 = internal standard (II).

TABLE I

ACCURACY AND PRECISION FOR DETERMINATION OF I IN PLASMA (INTRA-DAY DATA)

Added amount (μg/ml)	Accuracy*	Precision**
0.020	−40.8	47.0
0.050	− 5.7	8.2
0.200	0.1	2.5
0.800	4.1	5.1
1.200	− 2.0	4.7
1.600	1.8	2.1
2.000	2.0	6.1

*Accuracy = 100 $(x-\bar{x}_p)/\bar{x}_p$, where x = added amount and $\bar{x}_p$ = mean measured plasma concentration.
**C.V.(%) = 100 S.D./$\bar{x}_p$.

TABLE II

ACCURACY AND PRECISION FOR DETERMINATION OF I IN URINE (INTRA-DAY DATA)

Added amount (mg/ml)	Accuracy*	Precision**
0.025	34.9	12.3
0.050	−4.0	8.9
0.250	9.6	6.9
0.500	9.9	1.5
1.000	0.5	2.2
1.500	−3.0	3.2
2.000	2.7	2.6

*Accuracy = 100 $(x-\bar{x}_u)/\bar{x}_u$, where x = added amount and $\bar{x}_u$ = mean measured urine concentration..
**C.V.(%) = 100 S.D./$\bar{x}_u$.

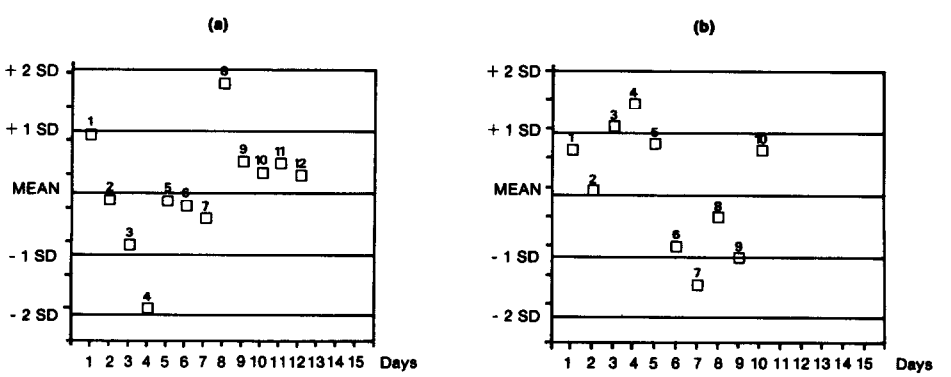

Fig. 3. Typical control charts. Each point is the mean of duplicate daily determination of I. (a) Plasma quality control samples (1 μg/ml); (b) urine quality control samples (2 μg/ml).

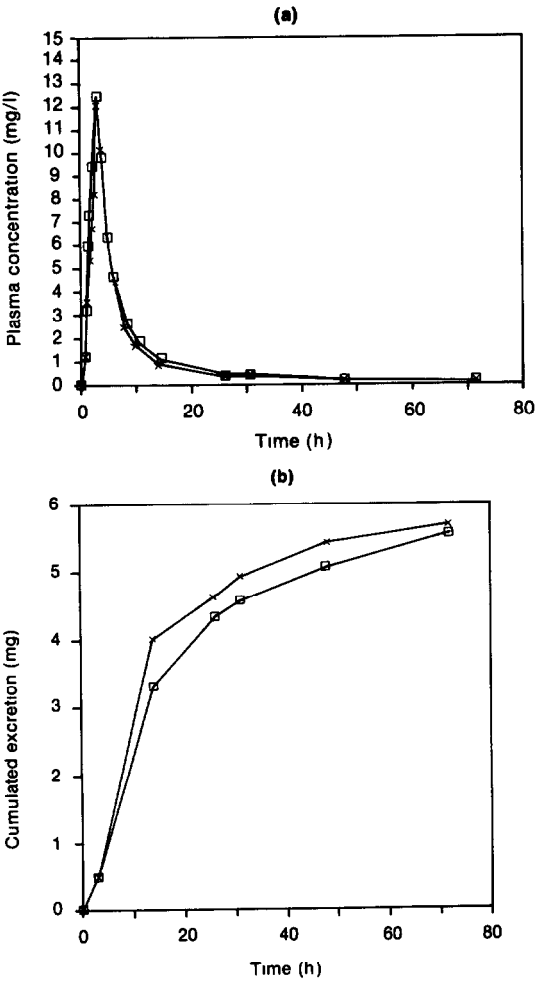

Fig.4. Data obtained from a baboon treated with 100 mg (1.85 MBq) of I. (a) Plasma concentration-time curves. Samples collected at the following times: 0.0, 0.5, 1.0, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 8.0, 10.0, 24.0, 32.0, 48.0 and 72.0 h after drug intake. (b) Cumulative urine excretion curves. Samples were collected at the following periods: 0-3 h, 3-14 h, 14-26 h, 26-31 h, 31-48 h, 48-72 h, 72-96 h. (□) Liquid scintillation counting determination (expressed as I equivalent); (×) HPLC determination (unchanged drug).

for quantification. The results obtained for plasma and urine are presented in Tables I and II. Based on these statistical data the lower limit for quantification was fixed at 0.050 μg/ml in plasma and urine. The inter-day variability was estimated using quality control samples introduced in each series. Control charting of these data allowed us to ensure that the measurement process had not shifted and had maintained an acceptable degree of variability [5] (Fig. 3). Routine analyses were performed for over a year, and control charts have shown the good reproducibility of the method over time.

Application to biological samples

The assay was applied to the determination of I in plasma and urine after administration to baboons. Animals received a single oral dose of 100 mg containing 1.85 MBq of ^{14}C-labelled drug. The plasma and urinary profiles are shown in Fig. 4. A good coincidence was observed between the determination of circulating total radioactivity measured by liquid scintillation counting and unchanged drug measured by the described HPLC procedure. This result is consistent with the non-metabolism of bisphosphonates [6, 7].

CONCLUSION

An HPLC assay has been developed for the analysis of I in plasma and urine. This method, which involves specific precipitations and dissolutions of calcium salts using semi-micro scale material, is optimized in terms of cost, rapidity, sensitivity and selectivity, and requires only 200 μl of plasma or urine. Our procedure has been shown to be a valuable tool for further elucidation of the human and animal pharmacokinetics of I.

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