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Journal of Chromatography B, 767 (2002) 111–116

JOURNAL OF CHROMATOGRAPHY B

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Determination of alendronate in human urine as 9-fluorenylmethyl derivative by high-performance liquid chromatography

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Received 20 July 2001; accepted 5 November 2001

Abstract

A high-performance liquid chromatographic method for the quantitation of alendronate as the 9-fluorenylmethyl derivative (FMOC) in human urine is presented. The sample preparation involved coprecipitation with calcium phosphate, separation on diethylamine (DEA) solid-phase extraction (SPE) cartridge and derivatization with 9-fluorenylmethyl chloroformate in citrate buffer pH 11.9. Liquid chromatography was performed on an octadecylsilica column (150×4.6 mm, 3 μm particles); a gradient method with starting mobile phase acetonitrile–methanol–citrate/pyrophosphate buffer (20:15:65 v/v) was employed. The total run time was 21 min. The fluorimetric detector was operated at the following wavelengths: 260 nm (excitation) and 310 nm (emission). Pamidronate was used as the internal standard. The limit of quantitation was 3.5 ng/ml using 5 ml of urine. Within-day and between-day precision expressed by relative standard deviation was less than 8% and inaccuracy did not exceed 9%. The assay was applied to the analysis of samples from a pharmacokinetic study.

Keywords: Derivatization, LC; Alendronate

1. Introduction

Alendronate (Fig. 1) is a bisphosphonate used for treatment of various diseases of bone metabolism. Bisphosphonates are incorporated to the bone matrix and act as its resorption inhibitor [1].

From the analytical point of view alendronate assay for pharmacokinetic studies with usual oral daily doses of 10 mg represents a difficult task due to the extremely low — less than 1% — bioavailability of the drug and resulting very low plasma levels.

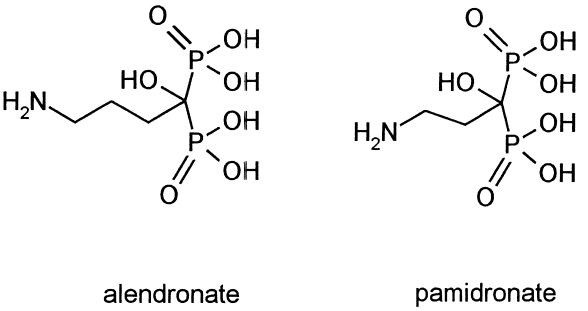

Fig. 1. Structure of alendronate and pamidronate (internal standard).

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1570-0232/02/$ – see front matter
PII: S0378-4347(01)00551-5

Moreover bisphosphonates are incorporated to the bone matrix and slowly released from it with resulting terminal half life greater than 10 years [2]. Thus evaluation of urinary excretion data seems to be a more promising approach for bioequivalence study than a standard procedure based on comparison of plasmatic concentrations [3].

The strategy of bisphosphonates determination in urine in so far published methods has similar features. Separation from the biological matrix is achieved by repeated coprecipitation with calcium phosphate under alkaline conditions and consequent calcium ions removal on various types of SPE column in all published procedures [4–13]. As bisphosphonates are with rare exceptions [4] compounds without chromophore, derivatization of the analyte is necessary. A precolumn derivatization of alendronate with 2,3-naphthalene dicarboxyladehyde [5,6], pamidronate with 1-naphtylisothiocyanate [7] or fluorescamine [8,9], olpadronate with 9-fluorenylmethylchloroformate [10] was described. Postcolumn derivatization techniques which detect fluorescent complexes with molybdenum [11] or ferrum (III) [12] were also used for bisphosphonates detection. Another possibility of detection is an electrochemical detector used either directly for bisphosphonate detection [13] or that of its derivative [6]. Various other methods of detection requiring expensive or complicated instruments were also used in combination with HPLC: negative ion electrospray ionization mass spectroscopy [14] or evaporative light-scattering detector [15].

The limit of quantitation (LOQ) of HPLC assays for alendronate ranges between 1 and 5 ng/ml and no internal standard is used [5,6]. The method is automated which is dictated by the demand of a precise control of the derivatization time and problems with limited sample stability. On the other hand it limits its usage to laboratories with more sophisticated equipment.

The aim of this study was to develop a robust HPLC method for alendronate determination in urine with LOQ at least 5 ng/ml to enable pharmacokinetic studies based on evaluation of urine excretion data. The variability caused by the sample preparation should be compensated for by a suitable internal standard and the derivatized samples should be stable in order to enable repeated injections.

2. Experimental

2.1. Chemicals

Alendronate sodium trihydrate was obtained from Léčiva, Czech Republic. Acetonitrile (for liquid chromatography) was Riedel de Haën (Seelze, Germany) product. Methanol (for liquid chromatography) and potassium dihydrogenphosphate (analytical grade) were manufactured by Merck (Darmstadt, Germany). The 9-fluorenylmethyl chloroformate (purris p.a.) and other chemicals (analytical grade) were Fluka products (Buchs, Switzerland). Pamidronate disodium (internal standard) was obtained in the local pharmacy in the form of injections (Aredia, Novartis Pharma AG Switzerland).

The DEA SPE cartridges were purchased from Varian (Bond Elut-DEA 100 mg/1 ml)

2.2. Apparatus

All HPLC instruments were obtained from Thermo Separation Products (Riviera Beach, FL, USA). The system consisted of a membrane degasser, pump ConstaMetric 4100, automatic sample injector AS 3000, fluorimetric detector FL2000 and datastation with PC1000 software, version 2.5. The separation was performed on a 150×4.6 mm I.D. column (Watrex, Prague, Czech Republic) filled with Nucleosil 100-3 C_{18} stationary phase, particle size 3 μm.

A simple gradient method was used: the starting mobile phase acetonitrile–methanol–buffer (25 m*M* citric acid and 25 m*M* sodium pyrophosphate without pH adjustment) mixture (20:15:65, v/v) was directly changed to acetonitrile–methanol–water mixture (13:57:30, v/v) at 7.5 min and hold at this composition for 9 min. Then the original mobile phase was again pumped to restore starting conditions. The total run time was 21 min. The flow-rate was 1 ml/min at 40°C. The excitation and emission wavelength was 260 and 310 nm, respectively and the time constant was set to 2 s.

2.3. Standards

Stock solutions of alendronate sodium trihydrate were made by dissolving approximately 15 mg in 25

ml of 0.2 *M* sodium citrate (conversion factor to the free base 0.76623). Separate solutions were prepared for the calibration standards and quality control samples. Further solutions were obtained by serial dilutions of stock solutions with citrate buffer. These solutions were added to drug-free urine in volumes not exceeding 1% of the urine volume.

The content of Aredia injection ampoule containing lyophilized mixture of 15 mg of pamidronate disodium and unspecified amount of manitol was weighted and conversion factor for pamidronate was calculated. An aqueous solution containing approximately 400 ng per 1 μl was prepared and 10 μl of this solution was added to 5 ml urine sample as the internal standard. The identical internal standard solution was used throughout the whole study.

All solutions were stored at 4°C and protected from light.

2.4. Preparation of the sample

The samples were stored in the freezer at −18°C and allowed to thaw at room temperature before processing. Ten μl of the internal standard solution (4000 ng of pamidronate disodium) were added to 5 ml of urine and the tube was briefly shaken. One hundred μl of 0.1 *M* KH_2PO_4 and the same amount of 0.1 *M* $CaCl_2$ was added and the sample was made alkaline with 200 μl of 1 *M* NaOH. The sample was centrifuged for 3 min at 2000 *g* and the supernatant was discarded. The precipitate was completely dissolved in 0.5 ml of 0.2 *M* acetic acid and 5 ml of water was added. Consequently the precipitation with sodium hydroxide was repeated twice. The resulting precipitate was finally dissolved in 1 ml of 0.2 *M* acetate buffer (pH 4.5) and diluted with 2 ml of water. The sample was then loaded on DEA SPE cartridge pre-washed with water. After washing the cartridge with 2× 0.5 ml water the drug was eluted with 1 ml of 0.2 *M* sodium citrate and an aliquot of the eluate was taken for the derivatization.

The derivatization procedure involved addition of 100 μl of 1 *M* sodium carbonate buffer (pH 11.9) to 270 μl of the sample and subsequent addition of 100 μl of FMOC solution (1 mg in 4 ml of acetonitrile). After 3 min 100 μl of 1 *M* citric acid was added to adjust pH and 50 μl of the sample was injected into the chromatographic system.

2.5. Calibration curves

The calibration curve was constructed in the range 3.5–300 ng/ml to encompass the expected concentrations in measured samples. The calibration curves were obtained by weighted linear regression (weighing factor $1/y^2$): the ratio of alendronate peak height to pamidronate peak height was plotted vs. the ratio of alendronate concentration to that of internal standard. The suitability of the calibration model was confirmed by back-calculating the concentrations of the calibration standards.

3. Results and discussion

3.1. Sample preparation

FMOC was chosen as the derivative reagent because of its superior properties compared with previously used ones. It reacts under mild conditions with amines and the resulting derivatives are stable for a very long period. The reaction mixture can be injected on standard silica based analytical column directly after simple pH adjustment and no preceding removal of unreacted reagent is required.

The yield of the derivatization reaction depends on pH, reagent concentration, reaction mixture composition and reaction time. It had to be optimised as only data on olpadronate FMOC derivative are available [10]. The presence of citrate ions in the reaction mixture was necessary probably due to prevention of alendronate adsorption looses. The amount of organic solvent in the derivatization mixture is also important due to occasionally occurring separation of phases and limited water solubility of FMOC derivatives. The derivative yield is strongly pH dependent, gradually increasing to pH 11.5 and rapidly falling down above pH 12. The derivatization is fast, prolonged reaction times had no effect on the reaction yield.

3.2. Chromatography

Alendronate adsorption is a severe problem as follows from published data. Various additives were used to avoid this phenomenon: EDTA, citrate buffer

or even bisphosphonate analog etidronate. We used combination of structurally similar pyrophosphate anion and chelating citrate ions in the mobile phase. Although most previous assays used polymeric reversed-phase columns and mobile phases with elevated pH values, conventional silica based reversed-phase C_{18} column produced symmetrical well resolved peaks with efficiency above 6000 theoretical plates. The gradient step was incorporated to speed up the elution of late derivatization by-products peaks resulting in the total run time 21 min.

The method selectivity was demonstrated on six blank urine samples obtained from healthy volunteers: the chromatograms were found to be free of interfering peaks. The typical chromatogram of blank plasma is shown in Fig. 2 and the chromatogram of a pooled urine sample (see Section 3.4 for details) is shown in Fig. 3. The concentration of alendronate was 59.10 ng/ml.

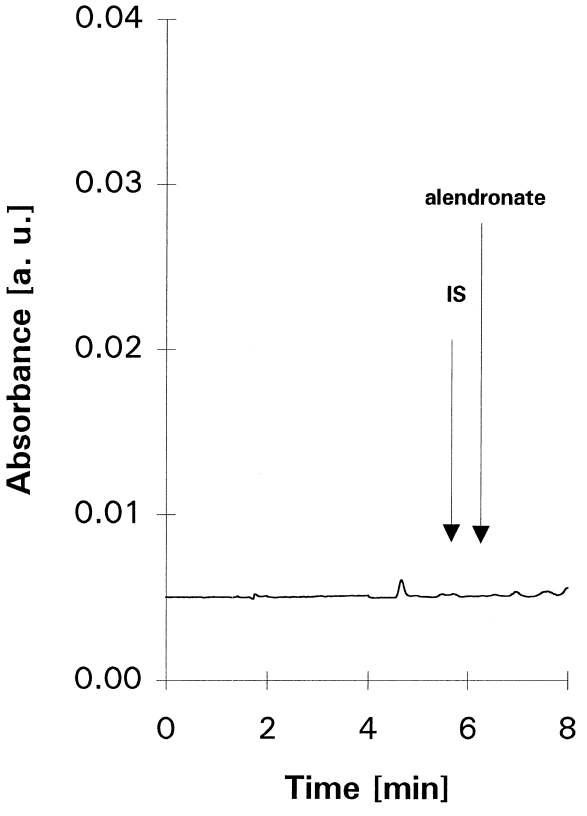

Fig. 2. Typical chromatogram of drug-free human urine.

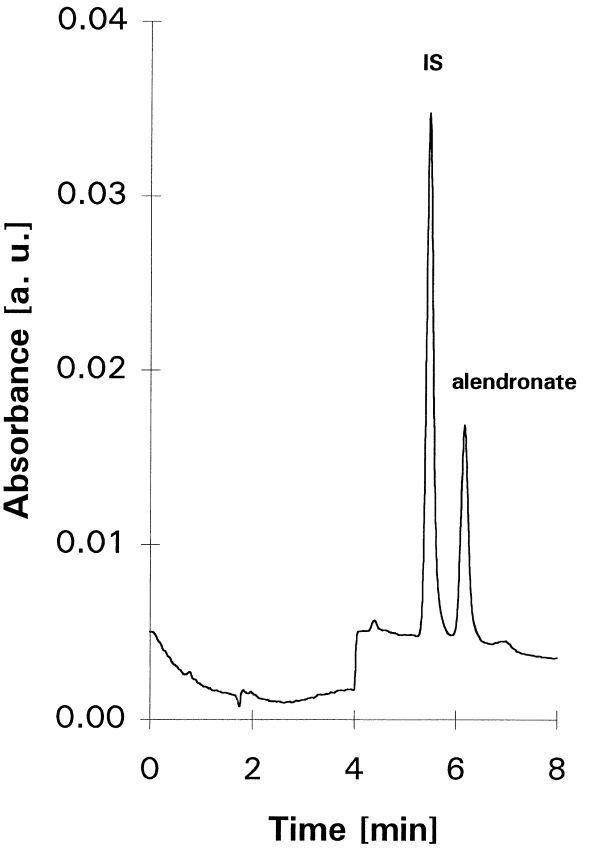

Fig. 3. Chromatogram of a pooled urine sample (collection period 72 h) from a volunteer after administration of 90 mg of alendronate during 3 days. The measured concentration of alendronate was 59.10 ng/ml.

3.3. Linearity and limit of quantitation

The calibration curves were linear in the studied range. The calibration curve equation is $y = bx + c$, where y represents the alendronate to pamidronate peak height ratio and x represents the ratio of alendronate concentration to that of internal standard. The mean equation (curve coefficients±standard deviation) of the calibration curve ($N=6$) obtained from 6 points was $y=5.08(\pm0.35)x+0.0005(\pm0.0017)$ (correlation coefficient $r=0.9995$).

The limit of quantitation was 3.513 ng/ml ($N=6$). This level was selected with respect to expected concentrations of the samples from the pharmacokinetic study. The precision, characterised by

Table 1
Intra-assay precision and accuracy

N	Concentration (ng/ml)		Bias (%)	RSD (%)
	Added	Measured		
6	6.826	7.212	5.4	7.3
6	32.85	32.55	−0.9	4.4
6	242.6	247.9	2.1	4.7

the relative standard deviation, was 9.2% and accuracy, defined as the deviation between the true and the measured value expressed in percents, was 8.2% at this concentration ($N=6$).

3.3.1. Intra-assay precision

Intra-assay precision of the method is illustrated in Table 1. It was estimated by assaying the quality control samples (low, medium and high concentration) six times in the same analytical run. The precision was better than 8% and the bias did not exceed 6% at all levels.

Table 2
Inter-day precision and accuracy

N	Concentration (ng/ml)		Bias (%)	RSD (%)
	Added	Measured		
6	6.826	7.389	8.2	6.9
6	32.85	33.00	0.5	2.7
6	242.6	252.3	4.0	7.2

3.3.2. Inter-assay precision and accuracy

Inter-assay precision and accuracy was evaluated by processing a set of calibration and quality control samples (3 levels analysed twice, results averaged for statistical evaluation) on six separate runs. The samples were prepared in advance and stored at −18°C. The respective data are given in Table 2. The precision was at most 8% and the inaccuracy was better than 9% at all levels.

3.3.3. Stability study

3.3.3.1. Freeze and thaw stability. Stock solutions of a low and high concentration sample were prepared. The solutions were stored at −18°C and subjected for 3 thaw and freeze cycles. During each cycle triplicate 5 ml aliquots were processed, analysed and the results averaged. The results are shown in Table 3. The concentrations found are well within the allowed limit ±15% of nominal concentration, indicating no significant substance loss during repeated thawing and freezing.

3.3.3.2. Processed sample stability. Two sets of samples with a low and a high concentration of

Table 3
Stability of the samples

Freeze and thaw stability

Sample		Cycle 1		Cycle 2		Cycle 3	
C (ng/ml)	N	Measured	Bias	Measured	Bias	Measured	Bias
14.83	3	14.04	−5.3%	15.56	4.9%	15.51	4.6%
242.6	3	258.2	6.4%	240.7	−0.8%	241.8	−0.3%

Processed sample stability

		N	Conc. found (ng/ml)	RSD	Difference
6.826	new	6	7.212	7.3%	
	3 days old	6	7.126	7.2%	−1.2%
242.6	new	6	247.9	4.7%	
	3 days old	6	222.1	4.7%	−10.4%

Long-term stability

		Bias
14.83	6	15.52	4.6%
242.6	6	254.2	4.8%

alendronate were analysed and left in the autosampler at ambient temperature. The samples were analysed using a freshly prepared calibration samples 3 days later. The results are presented in Table 3. The processed samples are stable at room temperature for 3 days.

3.3.3.3. Long term stability. Two sets of samples (low and high concentration of alendronate) were stored in the freezer at −18°C for 8 weeks. The samples were then analysed using freshly prepared calibration samples. The results are well within the acceptable ±15% limit of the nominal concentration (see Table 3). The samples are stable at −18°C for the studied period.

3.4. Application to biological samples

The proposed method was applied to the determination of alendronate in urine samples from a bioequivalence study. The volunteers ($N=30$) were administered 30 mg of alendronate daily as a single oral dose for three consecutive days. The urine collection started with the first drug administration and stopped 24 h following the last dose. Fig. 4 shows the total alendronate amount excreted during this period. The excreted amount corresponds with previously reported bioavailability below 1% of the oral dose [1].

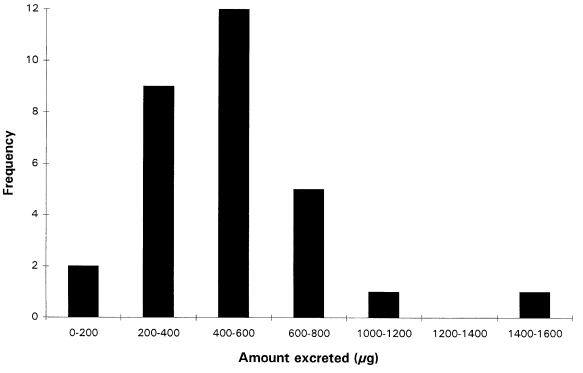

Fig. 4. Total amount of alendronate excreted in urine after administration 3×30 mg of alendronate in 3 days as an oral dose (30 healthy volunteers).

4. Conclusions

The validated method allows determination of urinary alendronate in the 3.5–300 ng/ml range. The analysis time is 21 min but this fact does not influence the total number of samples which can be analysed per day as the limiting step is rather the sample preparation than the HPLC analysis. The precision and accuracy of the method are well within the limits required for bioequivalence study methods. The limit of quantification 3.5 ng/ml permits the use of the method for pharmacokinetic studies based on urinary excretion data.

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