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Short communication

Application of a semi-automated 96-well format solid-phase extraction, column-switching, fluorescence detection protocol for the determination of alendronate in human urine samples obtained from a bioequivalence study

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Abstract

In the current study, a semi-automated, 96-well format, solid-phase extraction (SPE), analytical column-switching method for alendronate determination in human urine is developed, validated and applied to a bioequivalence study. The current protocol was a substantial improvement of an existing classical method. A robotic liquid handling system was employed to simplify and reduce the time of sample preparation procedure. Automated SPE was carried out using a 96-well cartridge plate and a vacuum control system. Urine samples were determined by applying a column-switching protocol with fluorescence detection. Analysis time, due to the column-switching procedure, was about half of the conventional LC approach (11.5 min instead of 21 min). The method application required the determination of alendronate in urine samples obtained from 96 healthy volunteers as part of a bioequivalence study of two 70 mg alendronate sodium tablets. All major pharmacokinetic parameters of the bioequivalence study were estimated and reported.

Keywords: Alendronate; Bioequivalence; Column-switching; 96-Well format; Urine

1. Introduction

Alendronate sodium is an important representative of the bisphosphonates used to treat metabolic disorders of calcium, such as osteoporosis, hypercalcemia and Paget's disease of bone [1,2]. Alendronate is selectively accumulated in the skeleton and its oral absorption is approximately 1% of the administered dose [3], since it is not lipid-soluble, its molecular weight is >150 and it is negatively charged. Therefore, extremely low plasma concentrations can be expected.

So far, several analytical methods have been reported for the determination of alendronate in formulations [4–8]. Regarding biological media, only one method has been reported for alendronate determination in plasma [9]. Bioavailability in urine is higher because the drug is not metabolized and it is renally excreted [10]. Urine samples, in all reported methods were prepared for analysis by co-precipitation of alendronate and pamidronate (internal standard, IS) along with calcium salts under alkaline conditions, followed by solid-phase extraction (SPE), so as for the calcium ions to be removed [11–16]. Another protocol reporting the determination of pamidronate with alendronate being the IS, following a similar pre-treatment procedure, has been published [17]. Detection of alendronate in urine samples has been mainly performed with fluorescence detector (FLD) after derivatization with 2,3-naphthalene dicarboxyaldehyde (NDA) [11,13], 9-fluorenylmethyl chloroformate (FMOC) [12,15], *o*-phthalaldehyde (OPA) [16] and fluorescamine [17] or with electrochemical detector [13]. All these methods included a complicated and labour intensive preparation procedure as a result of the sample clean up steps. Significantly long run times were also included, mainly because of the presence of derivatization by-products along with the derivatized analytes.

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In the present study, a substantial improvement of a classic protocol proposed by Ptacek et al. [12] is presented. Sample preparation time was reduced and the overall procedure was simplified by the utilization of robotic liquid handling system, Multiprobe II HT-EX. SPE, as well as the derivatization step were performed on 96-well format plates. As for chromatography, an analytical column-switching configuration was used, employing two valves (one 6-port and one 10-port switching valve) and two pumps, resulting in a total run time almost half of the conventional method (11.5 instead of 21 min).

The automation of liquid transfer as well as the use of the column-switching protocol, allowed the completion of a pharmacokinetic/bioequivalence study in a much shorter time. In fact, the current method was used to assess alendronate in human urine samples obtained from a bioequivalence study of two 70 mg alendronate sodium tablets administered to 100 healthy male and female volunteers.

2. Experimental

2.1. Chemicals and reagents

Alendronate sodium trihydrate was kindly donated by Rafarm (Athens, Greece) and pamidronate disodium used as the IS was purchased from Sigma–Aldrich (Athens, Greece). Acetonitrile, methanol and 9-fluorenylmethyl chloroformate (HPLC grade), potassium dihydrogen phosphate, calcium chloride, sodium hydroxide, glacial acetic acid, ammonium acetate, sodium pyrophosphate and sodium citrate monobasic anhydrous (analysis grade) were also purchased from Sigma–Aldrich. All aqueous solutions and buffers were prepared using de-ionized and doubly distilled water (Resistivity > 18 MΩ) from a Millipore Milli-Q Plus System (Malva, Athens, Greece).

2.2. Instrumentation

A Perkin-Elmer Multiprobe II HT-EX workstation (Perkin-Elmer, Downers Grove, IL, USA) was used for liquid transfer steps during sample preparation. Two hundred microliters and 1000 μL conductive disposable tip-boxes were purchased from E&K Scientific Products (Cambell, CA, USA). A SPE manifold, as well as a vacuum control system were obtained from Perkin-Elmer, while SPE DEA (diethylamine) Varian Versaplate cartridges (100 mg sorbent, 1 mL) were purchased from ALS (Athens, Greece). An Eppendorf 5810 R (Bacakos, Athens, Greece) centrifuge was also employed during sample preparation. Eppendorf 96-deepwell plates along with Eppendorf deepwell mats were purchased from Sigma–Aldrich. Polypropylene test tubes were obtained from Pnoi (Athens, Greece). The HPLC system included two Agilent 1100 series binary pumps, a degasser and a column oven/cooler (Hellamco, Athens, Greece). The CTC PAL autosampler (Hellamco) was equipped with two Varian valves: one 6-port and one 10-port. An Agilent 1100 series fluorescence detector (Hellamco) was coupled with the LC system and operated under Chemstation software (Version A.09.03). Finally, Nucleosil (C18) analytical columns were purchased from Tech-Line (Athens, Greece).

2.3. Chromatographic and fluorimetric conditions

A gradient method was developed, involving analytical column-switching and the following mobile phases for each binary pump: (i) eluting mobile phase (Mobile phase A): 20% ACN, 15% MeOH, 65% buffer (25 mM sodium pyrophosphate decahydrate and 25 mM citric acid) and (ii) washing mobile phase (Mobile phase B): 13% ACN, 57% MeOH, 30% water. Chromatography was performed on two Nucleosil (C18) analytical columns, S-3 μm, 100 Å (150 mm × 4.6 mm i.d.). The pressure of the system was about 169–175 bar. The temperature of the autosampler was maintained at 10 °C and the injection volume was 100 μL. Pamidronate and alendronate were eluted at about 6.8 and 7.6 min, respectively, with a total run time of 11.5 min. The fluorescence excitation and emission wavelengths were set at 260 and 310 nm, respectively (Fig. 2).

2.4. Column-switching protocol

The column-switching system (Fig. 1) consisted of two binary pumps (Pump 1 and Pump 2), an autosampler equipped with two valves, one 6-port injection valve and a 10-port switching valve, two analytical columns (COL 1 and COL 2) and the fluorescence detector. Two separate valve configurations were used according to the column-switching protocol (Configuration A and Configuration B).

- 0:00–7:10 min: 100 μL of the processed human urine were loaded on COL 1 with a flow rate of 1 mL/min, delivered from Pump 1, and the analytes were eluted for detection. During the same period, COL 2 received the flow from Pump 2 with a flow rate of 1.1 mL/min for column purification (Configuration A).
- 7:20–11:50 min: At 7:20 min, solvent composition and flow rate were changed to 100% Mobile phase B with a flow rate of 1.1 mL/min for Pump 1 and 100% Mobile phase A with a flow rate of 1 mL/min for Pump 2. During that period, derivatization procedure by-products were eluted from COL 1, while analytical column two was conditioned with elution mobile phase.
- At 11:50 min, the 10-port valve was switched so that the next sample was loaded on COL 2 by Pump 1, while at the same time purification mobile phase was delivered from Pump 2 onto COL 1 (Configuration B).

2.5. Standards preparation

Stock solutions of alendronate {100 μg/mL (SA_1)} and IS {100 μg/mL (IS_1)} were prepared by dissolving each of the accurately weighed reference compound in MeOH/water 90/10 (v/v). Working solutions of 50,000, 20,000, 10,000, 5000, 2000, 1000 and 500 ng/mL for alendronate were prepared by serial dilutions of SA_1 with sodium citrate 0.2 M. Four levels of QC working solutions, 40,000, 8000, 1500 and 500 ng/mL were also prepared. All working solutions were stored at 4 °C.

The calibration curve consisted of a blank sample, a zero sample and seven non-zero standards. Calibration standards, QC and MV samples were prepared in the same biological matrix

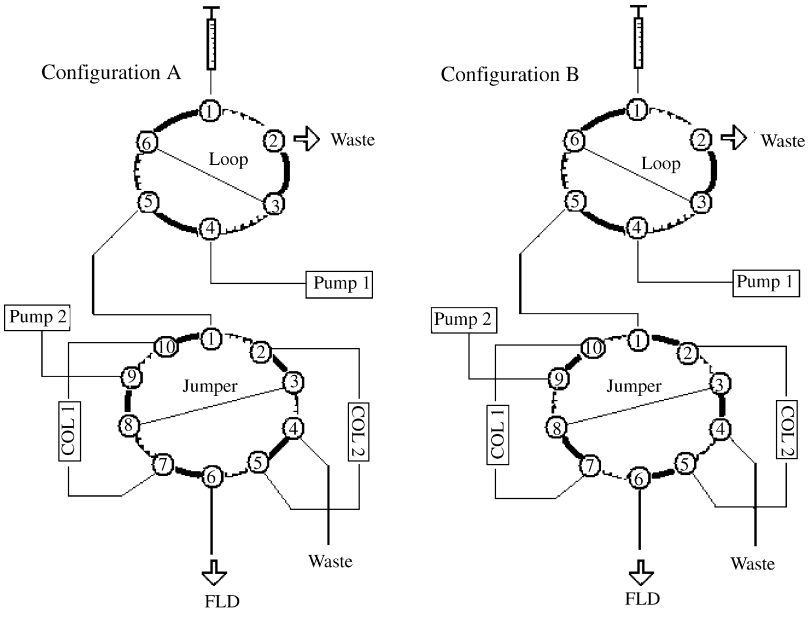

Fig. 1. Schematic representation of the dual column-switching system.

(human urine) as the samples to be analyzed. Working solutions were diluted 100 times, on a daily basis, with human urine obtaining final standard concentrations of 500, 200, 100, 50, 20, 10 and 5 ng/mL. Similarly, QC/MV samples concentrations were: MV_L (5 ng/mL), MV_1/QC_1 (15 ng/mL), MV_2/QC_2 (80 ng/mL) and MV_3/QC_3 (400 ng/mL).

2.6. Sample extraction and preparation

Urine samples were thawed at room temperature, vortex mixed, centrifuged at 2000 rpm for 5 min at approximately 4 °C and an aliquot of 5 mL from each sample was transferred inside the polypropylene test tubes. All tubes were placed into racks and all reagents to be added were placed inside reagent troughs on the deck of Multiprobe. The whole sample preparation procedure prior to SPE was similar with the one previously described [12], but much faster, due to the use of Multiprobe.

Automated SPE was performed according to the following protocol: the DEA 96-well format SPE tubes were conditioned twice by adding 500 μL of water. Samples were loaded on the SPE tubes according to the proforma sheet. Washing was performed twice with addition of 500 μL water and finally the loaded samples were eluted into a 96-deepwell plate by addition of 1000 μL sodium citrate 0.2 M.

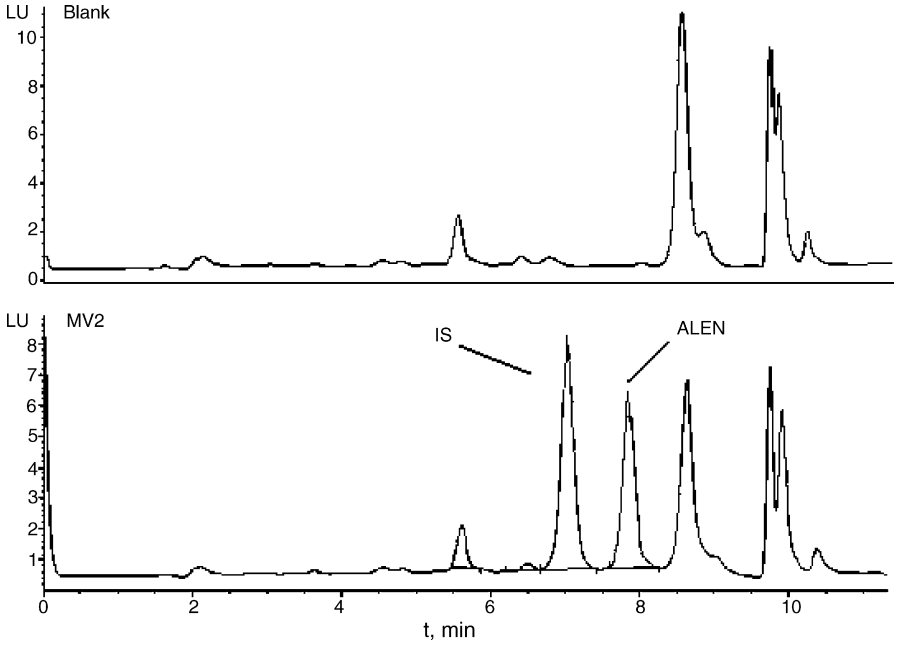

Fig. 2. Representative chromatograms obtained from a blank and a MV_2 sample.

Derivatization was performed by adding FMOC solution (2.5 mg in 10 mL ACN), prepared just before the derivatization procedure. Aliquots of 270 μL from each eluate were transferred to a new 96-deep well plate, followed by the addition of 100 μL sodium carbonate buffer 1 M (pH 11.9). Next, 100 μL of the freshly prepared FMOC solution were added and after 3 min 100 μL of citric acid 1 M were placed in each well. Then, the plate was covered with a 96-deepwell mat, vortex mixed and placed inside the autosampler for direct injection.

2.7. Bioequivalence protocol

A total number of 100 healthy male and female volunteers (96 plus 4 substitute volunteers) were enrolled in the bioequivalence study of alendronate sodium, comparing a test formulation (Tivarum/RAFARM Pharmaceutical Company) versus a reference formulation (FOSAMAX®/Merck Sharp & Dohme). It was conducted according to the approved protocol, the ethical principles that have origins in the Declaration of Helsinki and the Good Laboratory Practice (GLP) regulatory requirements. The study design was an open single-dose, two-treatment, two-period crossover with a washout period of 14 days between the two periods.

Alendronate sodium was administered as a single oral dose (70 mg/tab) under fasting conditions in accordance with the randomized schedule. Urine samples were collected and pooled at 1.5–1 h pre-dose and 0.25 (±3 min), 0.25–1, 1–2, 2–3, 3–4, 4–6, 6–8, 8–12, 12–24 and 24–36 h post-dose and stored at −20 °C pending for analysis. The following pharmacokinetic parameters were estimated: Ae_{0-36}, cumulative urinary excretion (amount recovered); R_{max}, the observed maximum urinary excretion rate; T_{max}, time of maximal urinary excretion. All calculations were performed using Pharsight WinNonLin 5.0.1 statistical software.

3. Results and discussion

The developed protocol was a modification and improvement of one of the most often cited methods regarding bisphosphonates analysis in human urine. One of the disadvantages of the specific protocol is the long sample preparation procedure with repeated clean-up steps. In the present study, a semi-automated approach was achieved by the utilization of the robotic liquid handling system Multiprobe II HT-EX. The latter performed all liquid transfer steps of sample preparation, simplifying thus the whole procedure and avoiding labor intensive and time consuming manual pipetting steps. The total time of sample preparation was reduced, as well as the possibilities of human error to occur. In addition, SPE was performed automatically onto Multiprobe deck, since a vacuum control system that switched on and off the vacuum pump was connected with the SPE manifold. SPE and derivatization procedure were performed in a 96-deepwell format, allowing thus the parallel sample preparation and greatly reducing the time of the procedure.

Another disadvantage of the conventional method, namely the long chromatographic run time (21 min), is a serious drawback especially when multi-sample analyses are the case. The column-switching configuration allowed the analysis of a plate containing 96 samples in 17 h time. The bioequivalence study (>2000 samples) was completed in a total time half of the required with the conventional method. Finally, even though column-switch configurations often cause carry over problems between consecutive samples, the current method presented no such interference.

3.1. Standard curve

Method validation was performed according to US Food and Drug Administration (FDA) bioanalytical method validaton guidance [18] by the preparation of five runs. Calibration curve consisted of seven non-zero standards ranging from 5 to 500 ng/mL. The regression coefficients (R-squared) for all five runs analyzed were greater than 0.996, average linear slope was 0.996 ($S_a = 0.00009$) and average intercept was −0.009 ($S_b = 0.020$).

3.2. Accuracy and precision

Accuracy and precision were assessed by analyzing six rows of four levels of MV samples in each one of the five runs. The percent accuracy was determined by calculating the deviations of the predicted concentrations from their nominal value. Results for both intra- and inter-run accuracy and precision are presented in Table 1. In all cases, the values were within the acceptable range.

3.3. Over-curve dilution

Because of extremely small urine excretion in some volunteer urine collections, samples with very high concentration values, exceeding calibration curve range, appeared after pre-study validation was completed. Therefore, an over-curve dilution pro-

Table 1
Intra- and inter-assay accuracy and precision results

MV sample	Intra-run accuracy[a] (%)	Inter-run accuracy[b] (%)	Intra-run precision[c] (%CV)	Inter-run precision[b] (%CV)
MV_L	109.8	109.7	9.3	7.3
MV_1	99.6	99.6	9.3	6.1
MV_2	96.8	96.8	8.8	4.3
MV_3	97.3	97.3	11	4.4

[a] $N = 6$, expressed as 100 × (mean calculated concentration)/(nominal concentration).
[b] Values obtained from all five runs ($N = 30$).
[c] $N = 6$.

Table 2
Pharmacokinetic parameters (mean values) of alendronate after oral administration of a single dose of a 70 mg tablet of each formulation in 96 volunteers

Parameter		Values
$Ae_{0–36}$ (ng)	Test	155950.9
	Reference	144482.3
R_{max} (ng/h)	Test	62988.9
	Reference	61821.6
T_{max} (h)	Test	1.03
	Reference	0.98

cedure had to be followed so as extrapolation of the calibration curve to be avoided. Spiked urine samples at four concentration levels were prepared (4000, 3000, 2000 and 1000 ng/mL) covering the full range of concentration values appearing among samples during the study. Curve dilution urine samples in triplicate, were extracted according to the procedure described earlier. Results were within the permitted range for both accuracy and precision (<15%).

3.4. Application to a bioequivalence study

Concentrations from urine samples of the 96 first subjects that completed the study were multiplied by the urine volume for its time interval to obtain the relevant excreted amount (ng). Data presented in Table 2 show that 90% geometric confidence intervals for $Ae_{0–36}$ and R_{max} were within the acceptance range (80–125%) set for bioequivalence studies.

Comparing the results obtained in this study with other bioequivalence studies of alendronate sodium (70 mg/tab), it is concluded that there are big deviations between males and females. For instance, there is an agreement, regarding pharmacokinetic parameters, with the study [15] that also includes both male and female volunteers, while when only male volunteers were used [19], $Ae_{0–36}$ and R_{max} values were much bigger. This conclusion is getting stronger when another bioequivalence study of alendronate [20] is considered. In this case, the study was also conducted with male and female volunteers and the results for $Ae_{0–36}$ and R_{max}, after oral administration of a 40 mg tablet, were in agreement with the current study, when taking into account the difference in dose.

4. Conclusions

A semi-automated 96-well SPE, LC/FLD method for the quantification of the bisphosphonate alendronate in human urine was presented. The current, modified and improved protocol had significant advantages over the conventional one due to the use of a liquid handling robotic workstation, which simplified sample preparation, along with an automated SPE procedure in a 96-well format. A column-switching configuration was also applied by the use of a system consisting of a 6-port and a 10-port switching valve as well as two LC pumps. All of the above resulted in a significantly shorter sample preparation and chromatographic run time, allowing the application of the method in a bioequivalence study.

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References

[1] G. Heynen, P. Delwaide, O.L.M. Bijvoet, P. Franchimont, Eur. J. Clin. Invest. 12 (1982) 29–35.
[2] H. Fleisch, Eur. Spine J. 12 (2003) S142–S146.
[3] A.G. Porras, S.D. Holland, B.J. Gertz, Clin. Pharmacokinet. 36 (1999) 315–328.
[4] E.W. Tsai, S.D. Chamberlain, R.J. Forsyth, C. Bell, D.P. Ip, M.A. Brooks, J. Pharm. Biomed. Anal. 12 (1994) 983–991.
[5] E.W. Tsai, D.P. Ip, M.A. Brooks, J. Chromatogr. 596 (1992) 217–224.
[6] J. De Marco, S.E. Biffar, D.G. Reed, M.A. Brooks, J. Pharm. Biomed. Anal. 7 (1989) 1719–1727.
[7] Y.-H.R. Han, X.-Z. Qin, J. Chromatogr. A 719 (1996) 345–352.
[8] X.-Z. Qin, E. Tsai, T. Sakuma, S.P. Ip, J. Chromatogr. A 686 (1994) 205–212.
[9] M.-H. Yun, K.-I. Kwon, J. Pharm. Biomed. Anal. 40 (2006) 168–172.
[10] J. Lin, Bone 18 (1996) 75–85.
[11] W.F. Kline, B.K. Matuszewski, W.F. Bayne, J. Chromatogr. 583 (1992) 183–193.
[12] P. Ptacek, J. Klima, J. Macek, J. Chromatogr. B 767 (2002) 111–116.
[13] W.F. Kline, B.K. Matuszewski, J. Chromatogr. 583 (1992) 183–193.
[14] P.T. Daley-Yates, L.A. Gifford, C.R. Hoggarth, J. Chromatogr. 490 (1989) 329–338.
[15] H.-S. Kang, S.-J. Hwang, J.-S. Park, C.-K. Kim, J. Liq. Chromatogr. R. T. 29 (2006) 1589–1600.
[16] S.K. Al Deeb, I.I. Hamdan, S.M. Al Najjar, Talanta 64 (2004) 695–702.
[17] J.A. Wong, K.W. Renton, J.F.S. Crocker, P.A. O' Regan, P.D. Acott, Biomed. Chromatogr. 18 (2004) 98–101.
[18] Guidance for Industry: Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Rockville, MD, 2001.
[19] E.J.A. Roldaň, O. Quattrocchi, D. Zanetti, E. Piccinni, J. Tessler, L.E. Caballero, A. Peřrez Lloret, Arzneim.-Forsch. 55 (2005) 93–101.
[20] A. Lainesse, Y. Ozalp, H. Wong, R.S. Alpan, Arzneim.-Forsch. 54 (2004) 569–572.