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Determination of disodium clodronate in human plasma and urine using gas-chromatography–nitrogen-phosphorous detections: validation and application in pharmacokinetic study

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Abstract

We present a specific method for the determination of disodium clodronate in human plasma and urine using a gas-chromatographic system with nitrogen phosphorus detector (NPD). The compound was extracted from plasma and urine samples by an anion-exchange resin and derivatizated with bistrimethylsilyltrifluoroacetamide (BSTFA). Sodium bromobisphosphonate was used as internal standard. The calibration curves were linear in both plasma and urine, with a regression coefficient $r > 0.9975$ in plasma and $r > 0.9977$ in urine.

The limit of quantitation was 0.3 μg/ml in plasma and 0.5 μg/ml in urine. The method was validated by intra-day assays at three concentration levels. During the study we carried out inter-day assays to confirm the feasibility of the method. The precision in plasma at 0.5, 15, and 45 μg/ml was 12.4, 0.2, and 6.5% ($n = 40$), respectively; in urine at 0.8, 8, and 40 μg/ml it was 8.6, 6.4, and 9.3% ($n = 40$), respectively.

The method was accurate and reproducible, and was successfully applied to determine the pharmacokinetic parameters of clodronate in healthy volunteers after intravenous infusion and intramuscular injection of 200 mg of the compound. The C_{max} after intravenous infusion and intramuscular injection was 16.1 and 12.8 μg/ml, respectively. $AUC_{0-48\,h}$ after infusion administration and intramuscular injection was 44.2 ± 18.0 and 47.5 ± 12.4 h μg/ml, respectively. The elimination half-life in both administrations was 6.31 ± 2.7 h.

Keywords: Validation; Pharmacokinetics; Disodium clodronate

1. Introduction

Bisphosphonates are compounds structurally related to the naturally occurring pyrophosphate which regulates mineralization of bone matrix. In bisphosphonates the P–O–P bond of pyrophosphate is replaced by a P–C–P bond that confers them resistance to enzymatic hydrolysis [1].

Clodronate (dichloromethylenebisphosphonate, Fig. 1A) belongs to the bisphosphonates and it has been widely investigated as an inhibitor of bone reabsorption as well as in calcium metabolic disorders [1]. In clinical it is used in the treatment of Paget's disease, malignant hypercalcemia, osteolytic metastasis [2] and in the treatment of osteoporosis [3] but due to its high water solubility and extensive ionization it crosses poorly the biological membranes [4]. For this reason clodronate is poorly absorbed from the gastrointestinal tract and its volume of distribution is rather small equating extracellular water volume [5].

Clodronate, like other bisphosphonates, is non-chromophoric and makes the use direct UV detection without pre- or post-column derivatization impossible. The pre- or post-column derivatization methods require extensive sample preparation or complicated and specialised equipment, even though in the literature detection based on derivatization of clodronate have been reported like an indirect UV detection which monitors the decrease in UV absorption [4].

For this reason there is a little information available on the pharmacokinetic of clodronate and several strategies have been followed, mainly based on the use of liquid chromatography, ion-exchange chromatography or the innovative chromatography–electrospray ionisation mass spectrometry [6] and gas-chromatography–mass spectrometry [7,8].

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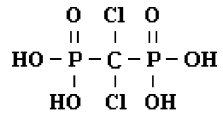
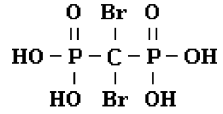


(A)



(B)

Fig. 1. (A) Chemical structure of dichloromethylene bisphosphonic acid Cl_2MBP. (B) Chemical structure of dibromomethylene bisphosphonic acid Br_2MBP (IS).

In this paper, we describe, validate and apply to pharmacokinetic study a selective and sensitive gas-chromatography (GC) method for extraction and quantitation of the clodronate in human serum and urine using nitrogen phosphorus detector (NPD). Moreover, the validation results including specificity, within- and between-day precision, limit of quantitation, accuracy, linearity and range, are reported.

2. Experimental

2.1. Chemicals and reagents

Sodium clodronate (dichloromethylene bisphosphonate sodium salt) was supplied by SPA-Societa' Prodotti Antibiotici (Milano, Italy), sodium bromobisphosphonate (Br_2MBP Fig. 1B) was generously provided by Professor F. Dosio (University of Torino, Italy). The reagent bistrimethylsilyltrifluoroacetamide (BSTFA) with 1% trimethylchlorosilane was obtained from Sigma (Milano, Italy), Dowex® 1 × 2 anion-exchange resin from Serva (Prodotti Gianni, Milano, Italy), acetonitrile from Carlo Erba (Torino, Italy) and chloridric acid from Merck SpA (Milano, Italy).

Stock solutions of sodium clodronate and sodium bromobisphosphonate (internal standard, IS) were prepared at concentration of 1 mg/ml in double-distilled water and stored at room temperature up to 3 months.

Standard solutions of clodronate were obtained from stock solutions by serial dilutions with water. All solutions were prepared in glass volumetric flasks. They were used to spike the plasma and urine samples prior to extraction; blank plasma supplied from AVIS (Torino, Italy) and urine samples from healthy volunteers were used to validate the method.

2.2. Sample extraction and derivatization

The preparation of the samples was performed according to the method described by Auriola et al. [9] with minor modifications. To 0.5 ml of plasma and 1 ml of urine samples 50 μg/ml of IS were added, then clodronate and IS were extracted from plasma and urine with 1 ml of Dowex 1 × 2 anion-exchange resin packed in an empty reservoir with a frit on the bottom (Alltech, Milano Italy).

The resin was washed with 2 ml of water, the sample was added and the resin was washed again with 10 ml of water and 10 ml 0.05 M of HCl. Finally, the bisphosphonates were eluted with 0.5 ml of 2 M HCl.

The eluted was transferred to the reaction vial and the solvent was evaporated to dryness overnight. 0.1 ml of acetonitrile and 0.1 ml of BSTFA were added into this vial, capped, shaken and heated at 80 °C for 2 h. The mixture was shaken vigorously by vortex for 5 min and 1 μl was injected into the GC. We verified the structure of derivatized sample components with mass spectra (MS) analysis.

2.3. Apparatus and chromatographic conditions

We performed a gas-chromatography analysis coupled with NPD detector (GC 6890 Agilent, Milano, Italy), employing a fused-silica capillary column (30 m × 0.32 mm diameter) coated with 0.1 μm film thickness of methyl silicone HP-5 (Hewlett-Packard, Milano, Italy).

The carrier gas was helium 2.0 ml/min, and the temperature program was as follows: initial temperature 100 °C, 10 °C/min to 200 °C (2 min).

The injector temperature was set to 250 °C mode splitless. NPD detector operating conditions were as following: bead temperature set at 330 °C, the Offset as 40 pA, H_2 flow 3 ml/min.

2.4. Assay validation

The calibration curves consisted of eight concentration points 0.3, 1, 3, 5, 10, 15, 30, 50 μg of clodronate per 1 ml of human plasma, and 0.5, 1, 3, 5, 10, 15, 30, 50 μg of clodronate per 1 ml of human urine.

These curves were prepared by adding 50 μg of IS and varying the concentrations of clodronate to human plasma and urine obtained from drug-free healthy volunteers.

The ratios of the peak-area of different concentrations of clodronate to that of IS were calculated and plotted by HP GC Chemstation software against the concentrations of clodronate.

2.4.1. Linearity

The linearity of the assay was performed with eight point calibration curve both in plasma and in urine. The slope and the intercept of the calibration graph were calculated through least squares by weighting linear regression (weighting factor $1/y^2$) of drug to IS peak-area ratio versus drug concentration. Experimental peak-area ratios were interpolated on the calibration curve and the concentrations back-calculated.

Intercept, slope and coefficient of correlation (r) were evaluated for five calibration curves of five independent sources of plasma and urine, and for each calibration curve performed daily. The linearity of the method between the

peak-area ratio and the concentration of clodronate was studied over the range 0.3–50 μg/ml in plasma and 0.5–50 μg/ml in urine. We checked if the slope of the linear calibration curve was statistically different from 0, if the intercept was not statistically different from 0 and if the regression coefficient was not statistically different from 1 [10].

2.4.2. Precision—accuracy

The intra-assay (intra-day) and inter-assay (inter-day) variability of the method were assessed analysing quality control (QC) samples. QC were prepared from a stock solution, that is separately prepared from that of the standards curve, at three different concentrations 0.5, 15, 45 μg/ml in plasma and 0.8, 8, 40 μg/ml in urine. We used these validation criteria for precision and accuracy to assess the suitability of the method.

The precision was evaluated by intra-day and inter-day percent relative standard deviation %(R.S.D.): within ±15% for all concentrations. The accuracy is a measure of the systematic error or bias and is defined as the agreement between the measured concentration and nominal value. Accuracy of the method is best reported as percentage bias which is calculated from the expression: % bias = [(measured value − true value)/true value] × 100, and should be within ±15% for all concentrations [11].

2.4.3. Limit of quantification (LOQ)

The LOQ of the method was defined as the lowest concentration of clodronate measured in six replicate with acceptable precision and accuracy (±20% R.S.D.).

2.4.4. Recovery

The recovery of clodronate was determined by comparison of peak-areas from plasma and urine samples spiked with known amounts of drug (5, 50 μg/ml) and IS (50 μg/ml), processed according to the described method versus non-extracted pure standards. Each concentration of plasma and urine samples was prepared in five replicates.

2.5. Pharmacokinetic study

We selected 20 healthy volunteers to enrol in the study. The Regional Ethics Committee approved the clinical protocol and all subjects gave written informed consent.

We conducted an open, balanced, randomized, two period cross-over pharmacokinetic study to assess the bioequivalence of 200 mg disodium clodronate (Difosfonal® 100 mg SPA Milano, Italy) after single intravenous infusion (i.v.) and intramuscular injection (i.m.). Blood samples were collected after i.v. infusion at 0, 15, 30 (end of infusion), 40, 50, 60, 75 and 90 min, 2, 2.5, 4.5, 8.5, 12.5, 24.5 and 48.5 h. Blood samples were collected after i.m. injection at 0, 5, 10, 20, 30, 45, 60 and 90 min, 2, 4, 8, 12, 24 and 48 h. The urine samples were collected after both administration at 0, 2, 4, 6, 8, 10, 12, 24 and 48 h. Blood samples were immediately centrifuged at 2500 × *g* for 15 min after collection, and plasma fraction were separated and stored in glass tubes at −28 °C until analysis. The fractions of urine samples were stored in polypropilene tubes at −28 °C until analysis.

3. Results

3.1. Linearity

The calibration curves were prepared over the concentration range of 0.3–50 μg/ml of clodronate in plasma and over the concentration range 0.5–50 μg/ml of clodronate in urine. The chromatographic peak-area ratios of clodronate/IS versus known concentrations of clodronate yielded linear relationship over the concentration range analyzed in plasma and urine samples. The mean regression parameters are given in Table 1. In both matrices, mean back-calculated concentrations are presented in Table 2. The linearity of this method was statistically confirmed. The residuals were normally distributed and centred around 0, $P > 0.05$ (Kolmogorov–Smirnov test). The slope of the linear calibration curves is statistically different from 0, $P < 0.05$ (*t*-test). The intercept is not statistically different from 0, $P > 0.05$ (*t*-test) and the regression coefficient is not statistically different from 1, $P < 0.05$ (*t*-test).

3.2. Precision and accuracy

Tables 3 and 4 show the intra-day and the inter-day precision and accuracy of the method, assessed by analysing quality control samples in plasma and urine.

Table 1
Regression data for the standard curve of clodronate in human plasma and urine

		r	Slope	Intercept
Plasma	Mean	0.9975	2.64	0.013
	R.S.D.	0.13	15.1	123
Urine	Mean	0.9977	2.16	0.007
	R.S.D.	0.13	11.5	114

Table 2
Reproducibility of the standard curve of clodronate in human plasma and urine

Spiking plasma concentration (μg/ml) ($n = 5$)	Back-calculated concentrations (mean ± R.S.D.)	Spiking urine concentration (μg/ml) ($n = 5$)	Back-calculated concentrations (mean ± R.S.D.)
0.3	0.3 ± 3.3	0.5	0.5 ± 8.0
1	1.1 ± 4.5	1	1.0 ± 11.0
3	3.1 ± 8.3	3	3.0 ± 9.3
5	5.1 ± 7.0	5	4.9 ± 5.5
10	10.2 ± 4.6	10	10.2 ± 5.1
15	14.6 ± 4.7	15	15.1 ± 5.2
30	29.7 ± 4.2	30	30.5 ± 4.1
50	50.2 ± 4.1	50	50.3 ± 2.5

Table 3
Intra-day precision and accuracy of the QC samples for clodronate

Intra-day reproducibility	Concentration calculated, mean (μg/ml)			Precision R.S.D.			Accuracy bias%		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Spiking plasma concentration (μg/ml) ($n = 5$)									
0.5	0.5	0.5	0.5	12.2	15.4	4.0	2.0	0.0	2.0
15.0	13.4	14.7	14.2	1.4	2.2	9.0	−10.5	−2.3	−5.4
45.0	44.9	42.1	46.1	1.9	1.2	14.9	−0.2	−6.5	2.4
Spiking urine concentration (μg/ml) ($n = 5$)									
0.8	0.9	0.9	0.9	2.3	8.2	5.6	13.8	8.8	1.3
8.0	8.6	8.3	7.8	11.1	0.5	6.7	7.3	3.6	−3.0
40.0	38.3	40.5	41.7	14.9	1.7	1.3	−4.4	1.3	4.4

3.3. Limit of quantification

LOQ was set at 0.3 μg/ml of clodronate in human plasma and 0.5 μg/ml in urine and at this level the R.S.D. was 13.0 and 13.4%, respectively.

3.4. Specificity

The specificity of the assay was established with six independent sources of plasma and urine and there were no chromatographic peaks interfering with that of clodronate or IS; as shown in Figs. 2A and 3A. Clodronate and IS were well resolved from each other, with retention times of 8.4 ± 0.03 and 9.7 ± 0.04 min ($n = 10$), Figs. 2 and 3. The additional peak at 8.6 min in chromatograms of spiked and unknown samples could be a byproduct of IS.

A representative chromatograms of volunteers after clodronate administration are shown Fig. 4A from plasma and in Fig. 4B from urine. Fig. 5A and B shows the MS of the trimethylsililated Cl_2MBP obtained by GC–MS (scan analyse). The MS by electron ionization in Fig. 5A shows molecular ion at *m/z* 532 for silylated Cl_2MBP and abundant (*M*-15)+ ions formed by loss of a methyl group from the trimethylsilyl group *m/z* 517. In Fig. 5B a chemical ionization spectrum of the silylated Cl_2MBP shows an abundant protonated molecular ion at *m/z* 533.

Table 4
Inter-day accuracy and precision of the QC samples for clodronate

Inter-day reproducibility	Concentration calculated, mean (μg/ml)	Precision R.S.D.	Accuracy bias%
Spiking plasma concentration (μg/ml) ($n = 40$)			
0.5	0.5	12.4	2.0
15.0	14.8	0.2	−1.1
45.0	43.4	6.5	−3.5
Spiking urine concentration (μg/ml) ($n = 40$)			
0.8	0.9	8.6	7.5
8.0	8.4	6.4	5.1
40.0	41.5	9.3	3.8

3.5. Stability

The stability of clodronate was evaluated by comparing fresh human blank plasma and urine fortified with 5 and 50 μg/ml of clodronate. The samples were stored at room temperature for 24 h, at 37 °C for 24 h, at −28 °C for 24 h, after two cycles of freezing-thawing at −28 and at −28 °C for 3 months. The calculations were made directly using the peak-areas ratio clodronate/IS. No degradations were observed (see Table 5).

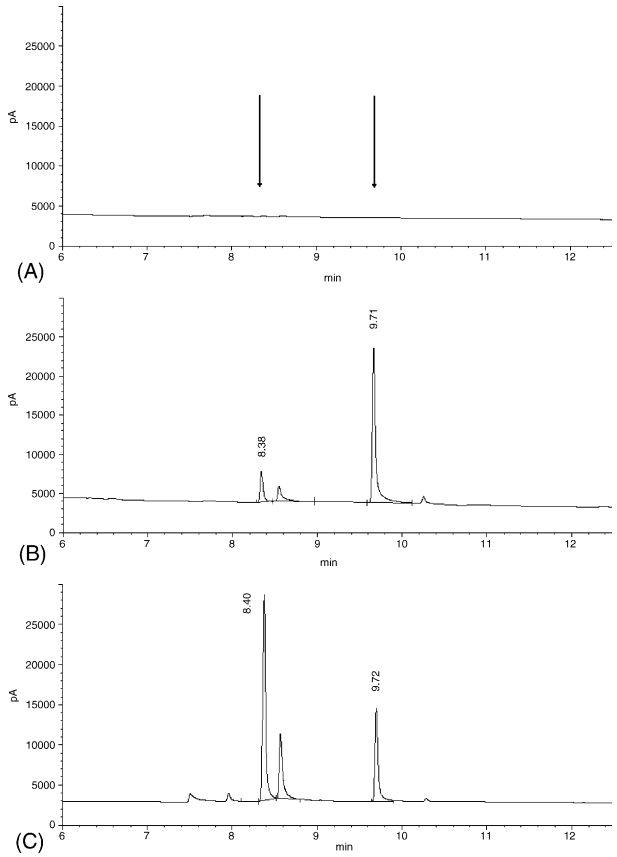


Fig. 2. (A) Plasma blank sample, (B) clodronate 5 μg/ml and (C) 50 μg/ml plasma spiked samples. Peak identification: clodronate (8.4 min), internal standard 50 μg/ml (9.7 min), byproduct (8.6 min).

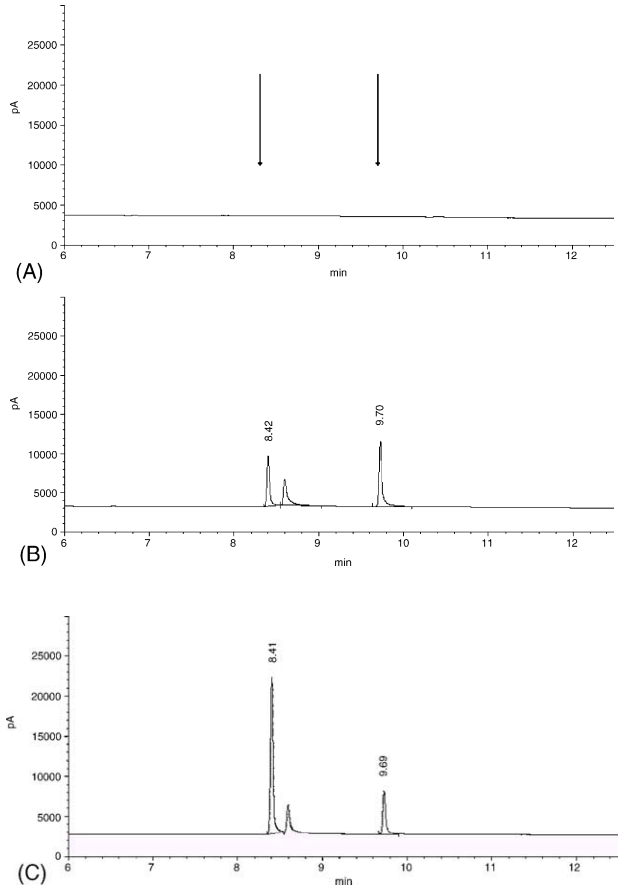


Fig. 3. (A) Urine blank sample, (B) clodronate 5 μg/ml and (C) 50 μg/ml urine spiked samples. Peak identification: clodronate (8.4 min), internal standard 50 μg/ml (9.7 min), byproduct (8.6 min).

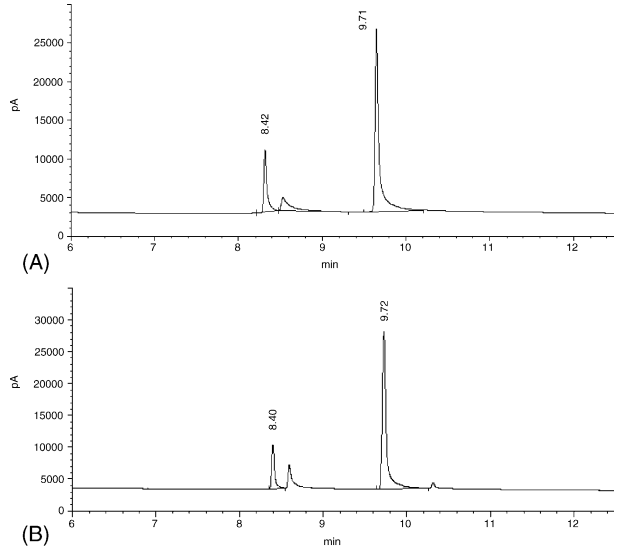


Fig. 4. (A) Plasma sample from volunteer treated with 200 mg disodium clodronate 4 h after i.v. administration. Peak identification: clodronate 11.4 μg/ml (8.4 min), internal standard 50 μg/ml (9.7 min). (B) Urine sample from volunteer treated with 200 mg disodium clodronate 2 h after i.v. administration. Peak identification: clodronate 6.9 μg/ml (8.4 min), internal standard 50 μg/ml (9.7 min).

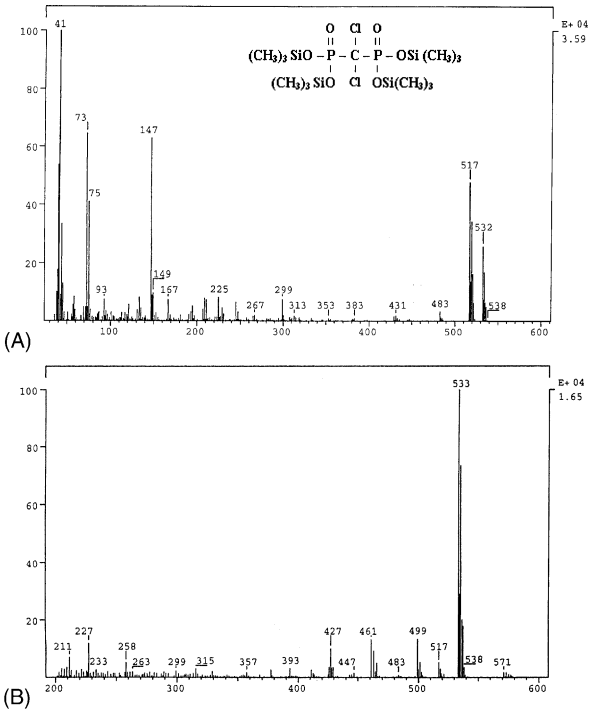


Fig. 5. Mass spectra of clodronate trimethylsilylated (Cl_2MBP). (A) Electron ionization mass spectrum. Molecular ion at *m*/*z* 532 for Cl_2MBP; the spectra show [*M*-15]+ ions formed by the loss of methyl group from the trimethylsilyl group *m*/*z* 517. (B) Chemical ionization spectrum of the Cl_2MBP shows the protonated molecular ion at *m*/*z* 533.

Table 5
Freezing and thawing stability of clodronate in human plasma and urine

	Mean area clodronate/IS plasma	Mean area clodronate/IS urine
Spiking concentration 5 μg/ml		
Fresh	0.28	0.30
24 h 21 °C	0.35	0.26
24 h 37 °C	0.34	0.25
1 freeze-thaw	0.32	0.24
2 freeze-thaw	0.31	0.27
3 months −28 °C	0.26	0.26
Mean ± S.D.	0.31 ± 0.04	0.26 ± 0.02
R.S.D. (%)	11.3	8.2
n	12	18
Spiking concentration 50 μg/ml		
Fresh	2.14	2.90
24 h 21 °C	1.82	3.26
24 h 37 °C	2.03	2.25
1 freeze-thaw	2.14	3.02
2 freeze-thaw	1.87	3.32
3 months −28 °C	1.80	3.16
Mean ± S.D.	1.98 ± 0.16	3.00 ± 0.39
R.S.D.	8.3	13.1
n	12	18

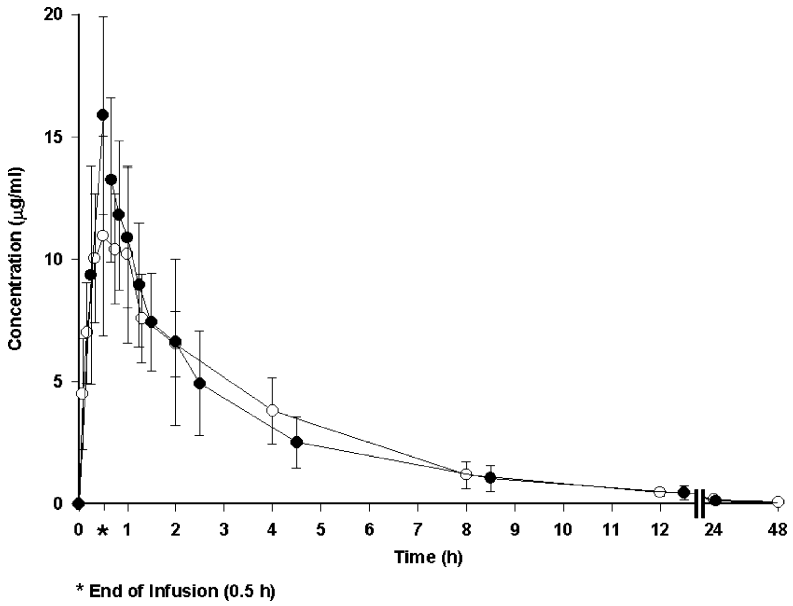


Fig. 6. Mean of S.D. of plasma concentrations after i.m. (○) and i.v. (●) administration of 200 mg disodium clodronate.

3.6. Recovery

The recoveries of 5 and 50 μg/ml of clodronate was prepared in five replicates. After extraction and derivatization procedures the recoveries were in plasma and in urine more than 50% for all concentrations.

3.7. Application to pharmacokinetic study

According to our validation parameters we applied the present method to determine the plasma and urine concentrations of clodronate during an open, balanced, randomized, two period cross-over pharmacokinetic study in twenty healthy volunteers to assess the bioequivalence of 200 mg of disodium clodronate (Difosfonal® 100 mg) after a single i.v. and i.m. administration. The limit of quantification of disodium clodronate in both matrices allowed plasma and urine concentrations to be followed up to 48 h after drug administration.

The pharmacokinetic parameters were calculated with the WinNonLin 3.0 Pro software (Pharsight Inc.).

The parameters obtained were as follows: the value of area under the plasma concentration–time curve from 0 to the last sampling time ($AUC_{0–48\,h}$) was 44.2 ± 18.0 h μg/ml after i.v. infusion and 47.5 ± 12.4 h μg/ml after i.m. injection. The i.v. maximum plasma concentration (C_{max}) was 16.1 μg/ml and the i.m. C_{max} was 12.8 μg/ml. The i.v. clearance was 4.99 ± 1.31 l/h and i.m. clearance was 4.47 ± 1.09 l/h.

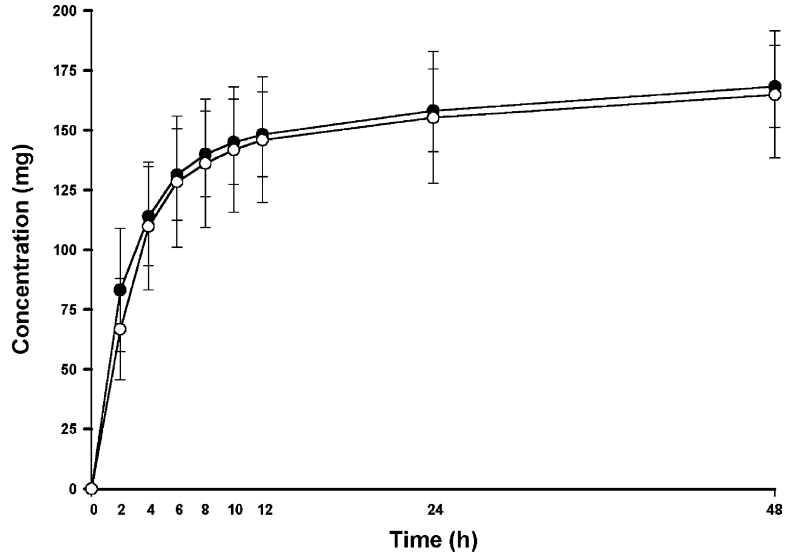


Fig. 7. Mean of S.D. of cumulative urinary excretion after i.m. (○) and i.v. (●) administration of 200 mg disodium clodronate.

The elimination half-life in both administrations was 6.31 ± 2.7 h.

The 48 h mean cumulative urinary excretion of clodronate was 84.1% after i.v. administration and 82.5% after i.m. injection.

Fig. 6 shows the mean and S.D. of plasma concentration–time curves following i.m. and i.v. administration of 200 mg of disodium clodronate.

Fig. 7 shows the mean and S.D. of cumulative urinary excretion curves following i.m. and i.v. administration of 200 mg of disodium clodronate.

4. Discussion

In recent years several studies on bisphosphonates have been published as these compounds have had new clinical applications [12–14]. This new interest comes from in vitro and in vivo studies, but there is little information available on the pharmacokinetic of disodium clodronate, this is mainly due to the chemical nature of bisphosphonates which causes several analytical difficulties.

The main approaches to develop a quantitative analytical method for bisphosphonates described in the literature are based on chromatography [1–7] and some of them by GC–MS [4,7].

Even though MS is considered to be the most promising technique in pharmacokinetics studies because of its sensitivity, specificity and versatility, the high cost and the feasibility of such instrument still do not allow the routine use and application of the methods based on this detector.

For these reasons we developed and validated a selective and sensitive gas-chromatography method for the extraction and quantification of clodronate in human plasma and urine using NPD detector, that was then used in a pharmacokinetic study.

The main step in the development of this analytical method was the extraction of the sample, not only from urine [9] but also from plasma because bisphosphonates are strongly polar and ionic, hindering, for example, a simple extraction from body fluids into an organic solvent. Therefore we used an anion-exchange resin packed with two washing steps to extract the compounds, the last one is critical for the goodness of the recovery.

Another important step was the derivatization of our sample because bisphosphonates are not volatile and the derivatizing agents for our GC analysis was the bistrimethylsilyltrifluoroacetamide, reacting with the phosphoric acid groups of clodronate [7,9].

With these improvements and changes compared to the other methods [5,7,9], the intra-day analyses carried out in the validation confirmed the reproducibility of the method and the obtained data satisfied the pre-defined acceptance criteria for accuracy and precision. The method was applied to the analysis of authentic plasma samples taken from volunteers in a pharmacokinetic study during which more than 800 samples were analyzed and a complete within-study assay performed. The linearity in urine cover the range 0.5–50 μg/ml but in three samples the concentrations was higher than the last point of our calibration curve and for this reason these samples were diluted. We found a good linearity over the entire range of calibration curves and the slope values remained quite similar to those obtained in linearity pre-study validation. This showed that the method was reproducible. The back-calculated concentrations of the calibration samples resulted, on average, within the acceptance criteria. No-runs were rejected, the results obtained in analysis of the quality control samples, injected daily with unknown samples, complied with the assumed acceptance criteria Table 4.

5. Conclusion

We have described an improved analytical method for the determination of clodronate in plasma and urine. We have also presented a validation procedure of the quality of the results. The method was demonstrated to be highly feasible and reproducible. The applicability of this method was evaluated in the analysis of unknown samples from volunteers in a pharmacokinetic study.

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