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An enzyme inhibition assay for the quantitative determination of the new bisphosphonate zoledronate in plasma

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Abstract

Zoledronate, 2-(imidazole-1-yl)-1-hydroxyethane-1,1-bisphosphonic acid is a new 3rd generation bisphosphonate. A specific enzyme inhibition assay was developed for the determination of zoledronate in plasma of animals and man. The multistep synthesis of cholesterol and some of its precursors (lanosterol, squalene) from ^{14}C-labeled mevalonic acid lactone is catalyzed by the S12 fraction of homogenized rat liver in the presence of ATP, NADH and Mg^{2+}. After hydrolysis of the reaction mixture with KOH, lipophilic reaction products were extracted with hexane and the overall yield determined by radiometry. Addition of zoledronate inhibited the formation of cholesterol and its precursors in a dose dependent manner. The described method is suitable to specifically and quantitatively measure concentrations of zoledronate down to 25 ng ml^{-1} in human and animal (dog, rat) plasma with acceptable reproducibility and accuracy. © 1997 Elsevier Science B.V.

Keywords: Enzyme inhibition assay; Bisphosphonates; Plasma

1. Introduction

Due to their inhibitory effect on bone resorption, bisphosphonates are used in the treatment of several bone disorders like Paget's disease, prevention of osteoporosis in postmenopausal women and the treatment of bone metastasis. Zoledronate, 2-(imidazole-1-yl)-1-hydroxyethane-1,1-bisphosphonic acid (Fig. 1) is a new bisphosphonate which is currently under development. The intended administration route is either intravenously or transdermally at very low doses (< 100 μg). The assessment of the pharmacokinetics in animals and in man thus requires a sensitive analytical method. Besides their action on bones some bisphosphonates also interfere with sterol biosynthesis. Pamidronate and aledronate have been found to be strong inhibitors of sterol biosynthesis and the compounds YM175, EB1053 and PHPBP very strongly inhibited squalene syn-

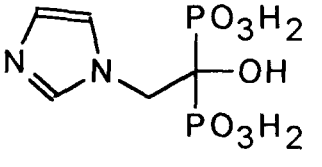

Fig. 1. Chemical structure of zoledronate.

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thase and cholesterol biosynthesis in a concentration dependent manner [1]. Therefore, zoledronate was likely to inhibit sterol biosynthesis, too. We report here on an assay which determines quantitatively zoledronate in animal and human plasma by the inhibition of sterol biosynthesis of S12 preparation of rat liver.

2. Experimental

2.1. Chemicals and reagents

Zoledronate was from Ciba-Geigy, relative molecular mass: 272.1. Mevalonic acid lactone, adensonine-5-triphosphate (ATP) and β-nicotinamide adenine dinucleotide phosphate (NADPH) were obtained from FLUKA, Buchs, Switzerland. ^{14}C-labeled mevalonic acid lactone (40–60 mCi nmol^{-1}) was obtained from New England Nuclear, Boston, Mass.(USA). Irgascint A 300 (Scintillation solution) was from Ciba-Geigy.

Assay buffer solution: 6.90 g $NaH_2PO_4 \cdot 2H_2O$, 2.033 g $MgCl_2 \cdot 2H_2O$ adjusted to pH 7.4. Mevalonic acid lactone solution: 50 μCi of ^{14}C-labeled mevalonic acid lactone (40–60 mCi nmol^{-1}) in 0.5 ml of ethanol were diluted with 16.1 mg of unlabeled mevalonic acid lactone and 2 ml of ethanol to obtain a specific activity of 0.4 mCi mmol^{-1} in 2.5 ml of ethanol. ATP-solution 88 mg ml^{-1}, to be prepared freshly just before use. NADPH-solution: 16.66 mg ml^{-1}, to be prepared freshly just before use. KOH-solution 15% (w/v) in ethanol. KCl/buffer: 0.1 M sodium phosphate buffer (pH 7.4) containing 0.15 M KCl (KCl/buffer solution may be stored for up to 4 weeks at 4°C.) S12 fraction: S12 fraction from rat liver homogenate was produced as follows: Freshly removed rat liver was immediately placed in a beaker containing KCl/buffer at 4°C. The tissue was then weighed and rinsed twice with cold KCl/buffer solution. The tissue was cut into fine strips and homogenized (at 0°C) using a 'Potter-Elvehjem' homogenizer with 4 strokes at 2000 rpm. The homogenate was centrifuged for 10 min at 12 000 × *g* at 4°C. The fat layer was aspirated, the supernatant, containing S12, transferred into a new tube and the pellet discarded. The protein content of this fraction was determined according to the method of Bradford [2]. Cytochrome *P*-450 content was measured photometrically [3] and in addition, the ethoxycoumarin-*O*-deethylase activity was determined [4]. The S12-fraction was then aliquoted into plastic ampoules and stored in liquid nitrogen.

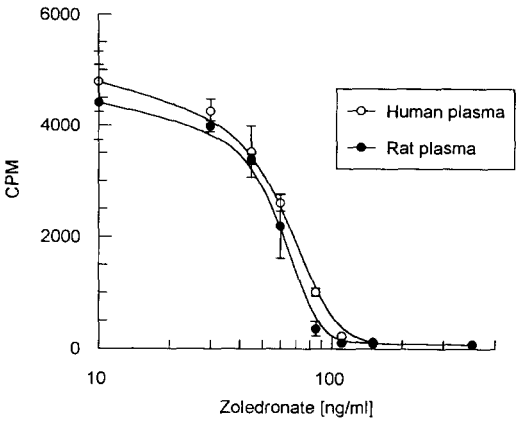

Fig. 2. Standard curves in pooled human and rat plasma. (CPM, counts per minute).

2.2. Assay procedure

Immediately before each assay an appropriate amount of assay buffer was flushed with O_2-gas for 15 min. To 1.2 ml of assay buffer were added: 0.1 ml of plasma sample (calibrator, control or unknown), 50 μl of ADP solution, 50 μl of NADPH solution and 60 μl of S12 homogenate in a glass tube (S12 aliquots were stored in liquid nitrogen and thawed immediately before use). Following this, 13 μl mevalonic acid lactone solution (0.38 μCi) were added and each tube was capped and incubated for 90 min at 37°C under gentle shaking. KOH solution (1 ml) was added followed by incubation at 75°C for 120 min. After cooling to room temperature the reaction solution was extracted with 5 ml of hexane by gentle shaking for 10 min. The aqueous fraction was discarded and the organic phase reduced to about 1.5 ml under a stream of nitrogen. The samples were dissolved in scintillation solution (16 ml) and counted (10 min) in the β-counter (TriCarb 2200

Table 1
Assay validation[a]

No.	Given (ng ml^{-1})	Mean found (ng ml^{-1})	±S.D.	%CV	Mean recovery
Human plasma					
8	25.0	25.4	6.4	25.12	101.72
8	25.0	31.6	6.2	19.47	126.35
4	40.0	43.0	8.7	20.23	107.5
14	40.0	43.0	4.2	9.77	107.5
8	40.0	41.0	4.4	10.73	102.5
8	50.0	46.8	6.3	13.43	93.58
8	50.0	53.3	3.9	7.25	106.65
8	75.0	70.7	5.7	8.12	94.22
8	75.0	71.5	2.5	3.97	95.17
4	80.0	82.6	4.1	4.96	103.3
15	80.0	83.0	6.8	8.19	103.8
8	80.0	85.4	5.7	6.67	106.8
3	80.0	77.1	1.7	2.50	96.4
3	90.0	88.4	4.4	5.02	98.2
3	100.0	100.9	5.8	5.79	100.9
3	120.0	119.1	1.2	1.01	19.3
Inter-assay precision				9.51 ± 6.82	
Intra-assay					102.7 ± 7.9
Dog plasma					
13	40.0	41.8	4.0	9.57	104.5
11	80.0	84.9	4.0	4.71	106.1

[a]Analysis of human and dog plasma spiked with zoledronate

from Canberra Packard). A calibration curve was fitted to the log-linear concentration-CPM plot using a spline function (software: RIASMAT by Canberra Packard). Each sample was assayed in triplicate.

3. Results and discussion

3.1. Assay principle

Mevalonic acid is an intermediate compound in the biosynthesis of steroid hormones. The multistep synthesis of cholesterol and some of its precursors (lanosterol, squalene) from ^{14}C-labeled mevalonic acid lactone was catalyzed by the S12 fraction of homogenized rat liver in the presence of ATP, NADH and Mg^{2+}. After hydrolysis of the reaction mixture with KOH, lipophilic reaction products were extracted with hexane. The total amount of radioactivity converted into lipophilic compounds was measured. Zoledronate inhibits this reaction in a dose dependent manner. Thus, an assay was set up by measuring the amount of radioactivity extracted into organic solvent as a function of added bisphosphonate. Typical inhibition curves in human and rat plasma are shown in Fig. 2. The range for zoledronate determination is narrow and plasma samples containing higher concentrations than 120 ng ml^{-1} have to be diluted carefully in order to obtain levels within the standard curve. The steepness of the curve also indicates that zoledronate inhibits steroid biosynthesis very efficiently.

3.2. Validation

Validation results for inter- and intra-assay variation in human and dog plasma and for the lower and upper quantitation limits of the calibration curve are shown in Table 1. The assay showed acceptable accuracy and precision in the range of 2.5–12 ng per sample (25–120 ng ml^{-1}), the lower limit of quantitation (LOQ) being 25 ng

Table 2
Intra-assay validation and lower limit of quantitation in human plasma

Sample no.	Given/Found (ng ml^{-1})		
	25	50	75
1	29.6	47.9	74.6
2	25.0	52.6	73.2
3	17.4	50.6	70.6
4	20.3	51.6	70.2
5	ns[a]	58.9	ns[a]
6	22.3	54.6	ns[a]
7	26.8	51.7	73.3
8	36.6	58.7	66.8
Mean	25.4	53.3	71.5
N	7	8	6
%CV	25.1	7.2	4.0
% recovery	102	107	95

[a]ns, no sample.

ml^{-1}. Intra-assay validation and lower limit of quantitation was performed between 25 and 75 ng ml^{-1} (Table 2). Parallel standard curves were obtained in human, dog and rat plasma. The assay is thus applicable for the determination of zoledronate in plasma samples of all three species. An increase of the proportion of plasma in the reaction mixture (> 100 μl) led to an inhibition of the enzyme reaction and was thus not suited for the enhancement of the sensitivity of the method. No other analytical method for the determination of zoledronate in biological fluids is currently available. A cross-check with an instrumental assay is therefore not possible yet.

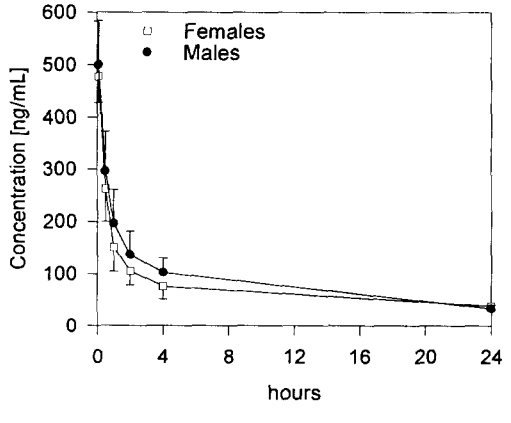

Fig. 3. Mean (± S.D.) plasma concentrations of zoledronate in dogs treated with a single intravenous dose of 0.1 mg kg^{-1} of zoledronate.

3.3. Analysis of plasma samples

Three male and three female beagle dogs were treated with single intravenous bolus doses of 0.1 mg kg^{-1} of zoledronate, a dose commonly used in toxicology studies in different species. The drug was administered as a suspension (10 ml kg^{-1}) composed as follows: 50 mg zoledronate, 395 mg mannitol, 10 mg trisodium citrate and sodium hydroxide for injection up to pH 6. Blood samples were collected before and 5 min, 0.5, 1, 2, 4, 24 h after dosing. The samples were centrifuged immediately and the plasma removed. Zoledronate was then determined as described above. The mean (± S.D.) plasma concentrations of the male and female dogs are shown in Fig. 3.

4. Conclusions

An enzyme inhibition assay for the direct determination of zoledronate in human, rat and dog plasma was developed. The lower and upper limits of the standard curve are 25 and 120 ng ml^{-1}, respectively. The sensitivity of this method is sufficient for the analysis of plasma samples from intravenous toxicity studies in various species but not for the utilization in dermal/human studies, where transdermal dosages of < 100 μg are foreseen.

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