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The determination of pamidronate in pharmaceutical preparations by ion-pair liquid chromatography after derivatization with phenylisothiocyanate.

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Abstract

An analytical method was developed for the determination of pamidronate [(3-amino-1-hydroxypropylidene)bisphosphonate] by ion-pair liquid chromatography. The analyte was derivatized with phenylisothiocyanate into an UV-absorbing derivative. The reaction product was cleaned-up by a double ion-pair extraction and treated with hydrogen peroxide prior to injection. Both, the detection limit and the lower limit of quantification of pamidronate in water were 0.1 μg ml^{-1} disodium pamidronate. The intra-day precision was 3% for a 5-μg ml^{-1} pamidronate standard solution and the inter-day precision 6% for a 3-μg ml^{-1} solution. The method was applied in the quality control of pamidronate injection concentrates and tablets. © 1997 Elsevier Science B.V.

Keywords: Bisphosphonates; Ion-pair liquid chromatography; Pamidronate; Phenylisothiocyanate; Derivatization

1. Introduction

In last three decades several bisphosphonates have been synthesized; these P-C-P structured compounds can interfere with the calcium metabolism because of their strong affinity to the hydroxyapatite bone matrix. They are specifically potent in inhibiting osteoclastic bone resorption [1]. Therefore, bisphosphonates are applied as drugs for indications like osteoporosis, Paget's disease and hypercalcaemia of malignancy. Pamidronate [(3-amino-1-hydroxypropylidene)bisphosphonate, APD)] is an important representative of the bisphosphonates. Intravenous and oral dosage forms of pamidronate are produced in the hospital pharmacy.

Analytical methods for the quality control of bisphosphonate preparations have been reported several times in the last decade. The first report by Ismail et al. [2] described a gas chromatographic (GC) method for etidronate [(1-hydroxyethylidene) bisphosphonate], the first bisphosphonate applied in clinical practice. After derivatization of etidronate with N,O-bis(trimethylsilyl)trifluoroacetamide, the derivative was chromatographed on a packed column. More recent methods are

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methods are based on liquid chromatographic and electrophoretic separation techniques. As most bisphosphonates in solution lack any specific detectable property; detection in high-performance liquid chromatography (HPLC) and electrophoretic separations in pharmaceutical analysis is often based on non-specific, not very sensitive, detection methods: refractive index [3,4], conductivity [5–7] and indirect [8] or low wavelength [7] UV-detection. ion-exchange chromatography (IEC), a rational choice for ionic compounds like bisphosphonates, is reported most often. Methods were developed for etidronate [6], pamidronate [3,5,9], alendronate [(4-amino-1-hydroxybutylidene)bisphosphonate] [4,8] and olpadronate [(3-dimethylamino-1-hydroxypropylidene)bisphosphonate] [9]. Electrophoretic methods were developed for alendronate based on capillary zone electrophoresis (CZE) [10] and for pamidronate based on capillary isotachophoresis [7]. The only known detection method specific for bisphosphonates is the in-line copper complexation with UV-detection of the complex in IEC [9] and CZE [10]. For reversed phase liquid chromatography (RPLC) a pre-column derivatization of the amino function can enhance the detectability of aminobisphosphonates. Such an application of RPLC for the pharmaceutical quality control of bisphosphonates was reported twice for alendronate and included a derivatization with *o*-phtalaldehyde and mercaptoethanol [11] or with (9-fluorenylmethyl)chloroformate [12].

A reversed phase (RP) mechanism will be preferred for analyses in more complex matrices because of a better selectivity. This approach has already been demonstrated for (amino-1-hydroxyalkylidene)bisphosphonates, also by a derivatization reaction of the amino group to obtain a sensitive detection, suited for bioanalysis based on RPLC. Fluorescamine [13,14] and 2,3-naphtalene dicarboxyaldehyde [15–17] have been employed as reagents in this type of assays to obtain a fluorescent bisphosphonate derivative.

An investigation into the derivatization of APD with phenylisothiocyanate (PITC) prior to analysis by HPLC was started to develop a method for RP separations as a possible sensitive and selective alternative for the IEC methods, developed previously in our laboratory [5,9]. PITC was chosen for its widespread use in the derivatization of amino acids and other primary amines prior to liquid chromatography [18].

2. Experimental

2.1. Chemicals

The disodium salt of pamidronate was obtained from Bufa B.V. (Uitgeest, The Netherlands), sodium etidronate originated from Nogepha (Alkmaar, The Netherlands) and neridronic acid [(6-amino-1-hydroxyhexylidene)bisphosphonic acid] was generously provided by Dr. C.W.G.M. Löwik (Department of Endocrinology, Leiden University Hospital, The Netherlands). Tetrabutylammonium-bromide (TBA-Br) and TBA-hydroxide (20% (w/w) in water) were of synthetic quality and were purchased from Merck-Schuchardt (Hohenbrunn, FRG). Acetonitrile (HPLC-grade) was supplied by Promochem (Wesel, FRG), PITC by Pierce (Rockford, Ill, USA) and triethylamine ($>99\%$ (w/w)) by Acros Chimica (Geel, Belgium). Hydrogen peroxide (30% (w/w)) of medical extra pure quality and all other chemicals (analytical grade) originated from Merck (Darmstadt, FRG). The pharmaceutical preparations of pamidronate and distilled water for HPLC were manufactured in the pharmacy of the Leiden University Hospital.

2.2. Equipment

Chromatographic analyses were performed on the following configuration: A Spectroflow 400 solvent delivery system (Applied Biosystems, Ramsey, NJ, USA) with a Marathon autoinjector with a built-in column thermostat (Spark Holland B.V., Emmen, The Netherlands), equipped with a 7010-80 Rheodyne injection valve (Rheodyne Inc., Cotati, CA, USA) and a 20-μl sample loop. The detector was a Spectroflow 773 variable wavelength detector (Kratos Analytical Instruments, Westwood, NJ, USA) and data were recorded on an IPC Dynasty HE 486DX personal computer

(IPC Corp. (PTE)LTD, Singapore), equipped with a Gynkosoft chromatographic data system (SOFTRON GmbH, Gräfelfing, FRG). Further, a Zymark TurboVap LV evaporator (Zymark Inc., Hopkinton, MA, USA), a Vortex-2Genie (Scientific Industries, Bohemia, NY, USA) and an Eppendorf 5416 centrifuge (Eppendorf GmbH, Hamburg, FRG) were used.

2.3. Chromatographic conditions

Flushed loop injections (20 μl) were made on a Microspher C_{18}-column (100 × 4.6 mm, average particle diameter = 3 μm, average pore diameter = 13 nm, Chrompack, Middelburg, The Netherlands) with a reversed phase (R2) guard column (10 × 2 mm, Chrompack). The column temperature was 30°C. The eluent comprised 77.5% (v/v) of a 30-mM phosphate buffer (pH 7.0), containing 5 mM TBA-hydroxide and 2 mM etidronate and 22.5% (v/v) acetonitrile. The eluent flow rate was 0.8 ml min^{-1} and the UV-detection wavelength was 240 nm.

2.4. Procedures

The procedure for aqueous reference samples started with 100 μl of a 0.5–4.5 or 5–45-μg ml^{-1} disodium pamidronate solution, made by appropriate dilution of a 0.5-mg ml^{-1} aqueous stock solution that was stored at 4–6°C. The pamidronate injection concentrate (3 mg ml^{-1}) was diluted a 100-fold before sampling 100 μl. Pamidronate 150-mg tablets were disintegrated in 100 ml water by ultrasonication during 15 min. After centrifugation and a 50-fold dilution 100 μl was sampled.

To the 100 μl reference or sample solution was added 10 μl neridronic acid as an internal standard (I.S.): 50 μg ml^{-1} for APD concentrations below 5 μg ml^{-1} and 300 μg ml^{-1} for higher APD concentrations. Next, 50 μl ethanol, 40 μl pyridine, 10 μl triethylamine and 2 μl PITC were added and the mixture was vortex-mixed a few seconds, yielding a clear solution. The tube was sealed and heated at 80°C during 5 min, followed by evaporation of the volatile compounds under nitrogen at the same temperature. The dry residue was reconstituted in 1 ml water; this solution was extracted twice by vortex-mixing for 30 s with 1 ml 2 g l^{-1} TBA-Br in chloroform; the organic solvent was discarded after 2 min centrifugation at 3.9×10^3 *g*. Next, 900 μl of the aqueous phase was heated for 5 min at 80°C in another sealed tube after adding 90 μl 0.06% (v/v) hydrogen peroxide. Finally, the sample was evaporated again at 80°C and redissolved in 100 μl mobile phase. Twenty μl of the sample was injected into the HPLC-system.

3. Results and discussion

For APD, a strongly ionic compound, water is actually the only applicable solvent. On the contrary, PITC dissolves in the other three solvents in the reaction mixture: ethanol, pyridine and triethylamine, but not at all in water. Therefore, the reaction mixture was a balanced compromise to dissolve both, APD and PITC. Besides for the dissolution of PITC, pyridine and triethylamine were also added to obtain the deprotonated amine function of APD, necessary for the derivatization reaction, at a pH > 12. Based on what is known from amino acid analysis [19], the reaction was expected to give the phenylthiocarbamyl derivative of APD (Fig. 1). The time and temperature dependence of the reaction is shown in Fig. 2. The APD conversion was determined directly by analysing the percentage of APD not derivatized by the IEC method of den Hartigh et al. [5]. To keep the method as fast as possible, heating during 5 min at 80°C was chosen as the final procedure.

After the initial experiments, the derivatization of pamidronate with PITC appeared not to result in one reaction product; in the chromatogram incidently two, hardly separated, peaks could be distinguished. Although the formation of a small amount of the phenylcarbamyl (PC) derivative together with the expected phenylthiocarbamyl (PTC) analogue had been reported earlier for amino acids [19], the formation of two products in comparable amounts in the PITC derivatization of pamidronate was not expected. However, assuming the formation of the PC derivative of

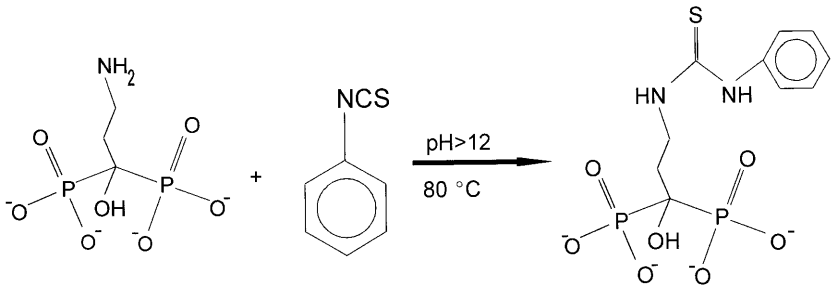

Fig. 1. Derivatization reaction of pamidronate with PITC into phenylthiocarbamyl-APD.

APD, it was decided to try to avoid the problem of the formation of two derivatives by performing an oxidative desulphuration of the PTC derivative into the PC derivative [20] with diluted hydrogen peroxide. Direct formation of NC-APD by derivatization with phenylisocyanate was not possible because of the reaction of this reagent with water. The approach with the treatment with hydrogen peroxide appeared to be succesfull. The result was a chromatogram with one sharp APD derivative peak. These observations supported the assumed formation of a PC derivative of APD. A disadvantage of the hydrogen peroxide treatment was the appearance of extra minor peaks in the chromatogram. The amount of hydrogen peroxide for the oxidation reaction was optimized experimentally: a too low amount could result in an incomplete conversion of PTC-APD, while a too high amount can cause further oxidation of the derivative. Although the optimum amount of hydrogen peroxide during the reaction was in between 0.002 and 0.004% for pamidronate, a little higher amount (0.0055%) was chosen to convert also the I.S. quantitatively into its PC derivative. Any instability of the derivatives under ambient conditions after the total pretreatment was not observed within 24 h. Further aspects of the reaction of different isothiocyates with different amino(bis)phosphonates will be reported seperately [20].

Initially, the liquid/liquid (L/L)-extraction of the reaction product after derivatization, cleaning up the aqueous sample solution, was performed with a chloroform/1-pentanol (v/v = 9/1) mixture. However, the emphatic presence in the chromatogram of disturbing peaks, due to byproducts of the derivatization, urged the development of an alternative cleaning-up method. After the unsuccessful investigation of several SPE sorbents, ion-pair L/L-extraction with a quaternary ammonium salt in chloroform was chosen. From different tetraalkylammonium salts tested, TBA-Br in chloroform provided the best results with respect to a clean blank chromatogram. The variation of the TBA-Br concentration between 1 and 10 g l^{-1} did not show any significant difference in extraction efficiencies. A sufficient long mixing time was essential during these extractions.

Liquid chromatographic (LC) analysis of derivatized APD necessitated the addition of an ion-pairing agent (IPA) to the eluent in order to obtain an acceptable retention behaviour; TBA-Br was selected. Different tetraalkylammonium IPAs influenced the retention behaviour of derivatized APD and I.S. strongly. The effect of several tetraalkylammonium IPAs in the LC analysis of several bisphosphonates, derivatized with different isothiocyanates, will be published elsewhere [20]. After optimization of the acetonitrile content of the eluent, both bisphosphonate derivatives were sufficiently separated from disturbing peaks. The acetonitrile percentage was especially critical for the separation of NC-APD from one specific unknown peak; this is shown in Fig. 3. In front of the APD derivative eluted a derivative of a contaminant of the I.S. (Fig. 4). The pH influence on retention was relatively small; lowering the pH from 7 to 4, decreased the retention time by a factor 2. The addition of etidronate severely improved the efficiency of the separation by sup-

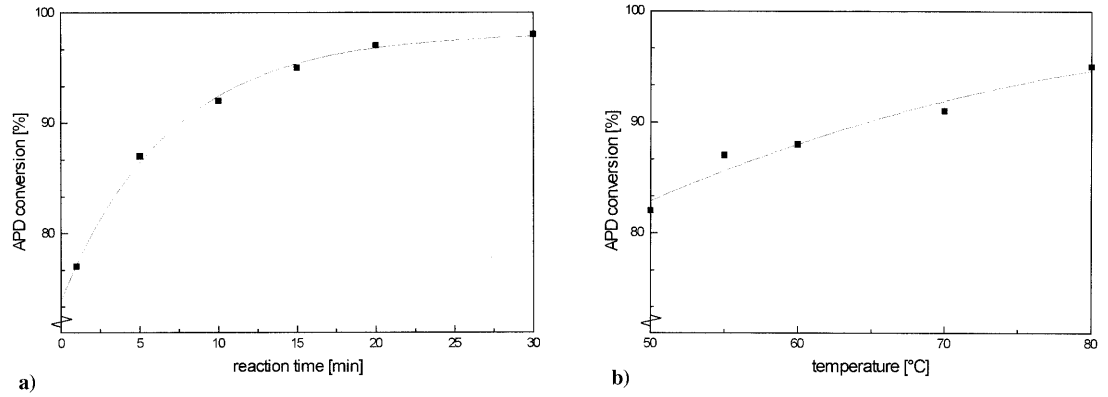

Fig. 2. APD conversion during a reaction with PITC. (A) as a function of time at 55°C, the points were fitted with the function $y = A + B[1 - \exp(-Cx)]$; (B) as a function of temperature during 5 min, the points were fitted with a second degree polynomial curve.

pressing adsorption effects of the analytes on the column. In an eluent without this adsorption suppressor, strongly tailing pamidronate peaks were observed.

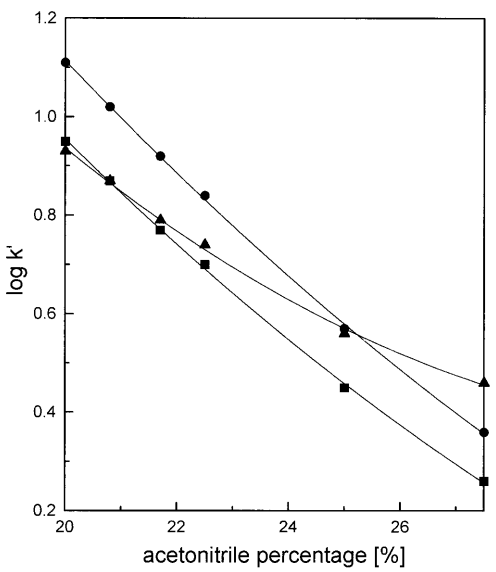

Fig. 3. Retention, expressed as the capacity factor (k'), as a function of the acetonitrile content of the eluent; the curves are obtained by second degree polynomial fitting. The retention time of an unretained compound (t_0) was estimated at the first disturbance of the baseline in the chromatogram. The phosphate concentration of the eluent buffer is 20 mM. (■) NC-APD; (●) NC-AHD; (▲) unknown.

The method was validated analysing series of six aqueous samples; examples of chromatograms are shown in Fig. 4. The intra- and inter-day precision and the accuracy are reported in Table 1. Table 1 shows, for example, a relatively small contribution of the chromatographic process to the total variance; the major part is probably due to the derivatization. Typical calibration lines in two different concentration ranges were:

$y = -0.0012\ (\pm 0.0165) + 0.1093\ (\pm 0.0054) \cdot x$
(range = 0–4.5 μg ml^{-1}, $n = 6$, $r^2 = 0.9987$),

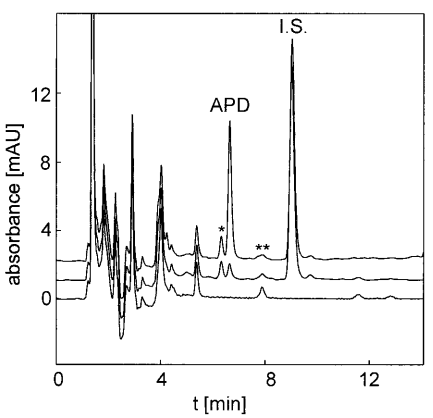

Fig. 4. Chromatograms of aqueous APD standard solutions, derivatized with PITC according to the standard procedure. The concentration of APD is respectively 0 (no I.S.), 0.5 and 4.5 μg ml^{-1}. * = Contaminant in I.S.; ** = unknown peak, corresponding to the one in Fig. 3.

Table 1
Precision and accuracy at different concentrations (c) of APD ($n = 6$)

c (APD) (μg/ml)	Chromatographic precision (multiple injection) R.S.D. (%)	Precision (repeatability) R.S.D. (%)
Intra-day data		
0		21
0.3		8
1.5	1.9	5
5	0.3	3
50	0.2	3
c (APD) (μg/ml)	Accuracy deviation (%)	Precision (reproducibility) R.S.D. (%)
Inter-day data with two-point calibration		
2.7	8	6
27	9	6

$y = -0.0041\ (\pm 0.0060) + 0.0266\ (\pm 0.0002) \cdot x$ (range = 0–45 μg ml^{-1}, $n = 6$, $r^2 = 0.9997$).

From the inter-day precision and the calibration line in the lower range, the lower limit of detection (LLD) and the lower limit of quantification (LLQ) were both estimated at 0.1 μg ml^{-1}. The LLD is the concentration level where the presence of APD is certain for more then 95%. At the LLQ, the relative standard deviation (R.S.D.) of the repeatability is not more than 20% of the concentration. Finally, the method was tested on two pharmaceutical preparations of pamidronate, using a calibration line in the higher concentration range (0–45 μg ml^{-1}). For the APD tablets a potency of 144.9 mg ($n = 10$; R.S.D. = 3.0) was found, resulting in a −3.4% deviation from the declared amount. The APD concentration in the injection concentrate was 3.00 and 3.01 mg ml^{-1} for two samples of one batch.

The presented method is, according to the validation results, sufficiently sensitive and selective for the analysis of pharmaceutical preparations of disodium pamidronate. Prior to the application of this method, one should take care of the preparation of the eluent; its composition can be very critical. With respect to sensitivity, this method is superior to the IEC methods reported previously [3–8]. Unfortunately, this method is rather laborious and can not meet the precision and accuracy of these IEC methods. Therefore, the new method will probably not displace other methods for routine applications. However, it can function as an alternative method in specific cases, for example in the validation of other pharmaceutical bisphosphonate assays and in case of interference by matrix components in a simple IEC method. The derivatization of bisphosphonates with isothiocyanates will be studied further for the development of other types of assays for aminobisphosphonates, for example bioanalytical, in the near future [20].

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