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Derivatization of pamidronate and other amino(bis)phosphonates with different isothiocyanates prior to ion-pair liquid chromatography

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Abstract

The derivatization of the amino group of pamidronate and other amino(bis)phosphonates with UV absorbing or fluorescent isothiocyanates (ITC) has been investigated. After derivatization at 80°C and pH>12 for 5–30 min and after liquid–liquid (LL) extraction, the reaction mixtures are analysed by ion-pair liquid chromatography with UV–Vis and/or fluorescence detection. The derivatization reaction results in the formation of both a thiocarbamyl and a carbamyl derivative of the respective amino(bis)phosphonates. Conversion of a thiocarbamyl-amino(bis)phosphonate into its carbamyl derivative can be achieved by oxidation using diluted hydrogen peroxide. The identity of the reaction products of pamidronate has been confirmed by fast atom bombardment mass spectrometry. A possible reaction mechanism for the oxidative desulphuration during derivatization, based on an autocatalytic effect, is postulated; supportive evidence is obtained by chromatographic analysis of different amino(bis)phosphonate derivatives after a reaction with phenylisothiocyanate. The most promising ITC reagent for bioanalysis of pamidronate is 1-naphthylisothiocyanate. © 1997 Elsevier Science B.V.

Keywords: Pamidronate; Amino(bis)phosphonates; Isothiocyanates

1. Introduction

Pamidronate disodium [(3-amino-1-hydroxypropylidene)bisphosphonate, APD] is an important representative of the bisphosphonates, a relatively new group of drugs, used in the treatment of Paget's disease, hypercalcaemia and osteoporosis [1,2]. Future clinical investigations, required to determine the long term efficacy and tolerance of APD, necessitate the availability of a reliable and sensitive bioanalytical method.

The acid form of the bisphosphonates is characterized by the $RC(PO_3H_2)_2R'$ structure. The polar ionic structure, as well as the absence of any fluorescent or UV–Vis absorbing chemical moiety in most of these molecules hamper the use of simple analytical methods. The first methods in bisphosphonate analysis, developed for etidronate [(1-hydroxyethylidene)bisphosphonate, EHDP] measurement in biological media, were based on either titration [3] or on detection of phosphate after decomposition of the bisphosphonate [4], respectively. Later, methods utilizing ion-exchange chromatography (IEC) with conductivity detection [5,6] or more specific detection methods [7–10] were applied for the analysis of bisphosphonates. All these methods are well

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suited for the determination of APD in, for example, bulk drugs and pharmaceutical preparations, however, they lack the necessary sensitivity and selectivity for the analysis of APD in biological matrices. More recently, assays based on GLC after silylation [11,12], RPLC with UV absorbance [13,14], fluorescence [15–19] or electrochemical [18–20] detection and capillary electrophoresis [21,22] were published. For APD and its analogue alendronate [(4-amino-1-hydroxybutylidene)bisphosphonate, 4-ABD] HPLC analysis, after derivatization of the amino group with a fluorescent reagent, seems to be the most promising approach regarding to detection limits. Fluorescamine was used as a derivatization reagent for APD [15,16]; *o*-phtalaldehyde (OPA) with mercaptoethanol [17] and 2,3-naphthalene dicarboxyaldehyde (NDA) [18,19] were used for alendronate.

At the same time, we started in our laboratory to investigate the potential use of phenylisothiocyanate (PITC) as a reagent to obtain a UV absorbing APD derivative suited for HPLC analysis [23]. This derivatization of APD, however, resulted in the formation of two reaction products in comparable amounts. For the PITC-derivatization of amino acids only some minor oxidative desulphuration of the phenylthiocarbamyl (PTC) derivative yielding its phenylcarbamyl (PC) analogue can occur [24]. Therefore, the relative amount of a second PITC derivative of APD was remarkable.

The first purpose of this investigation was to get more insight into the formation of carbamyl amino-(bis)phosphonates out of an amino(bis)phosphonate and a isothiocyanate reagent. For this purpose PITC-derivatizations of several (amino-1-hydroxyalkylidene)bisphosphonates (Table 1) and aminophosphonates were investigated using ion-pair LC. A hydrogen peroxide treatment was employed to convert thiocarbamyl-amino(bis)phosphonates, not desulphurated during derivatization, into their carbamyl analogues. Reaction products were characterised by ion-pair LC and fast atom bombardment mass spectrometry (FAB-MS).

Table 1
Applied (amino-1-hydroxyalkylidene)bisphosphonates, the basic structure (acid form) is $HOC(PO_3H_2)_2R$

R	Abbreviation	Non-proprietary name
$CH_2CH_2NH_2$	APD	Pamidronate
$CH_2CH(C_6H_5)NH_2$	APPD	
$CH_2CH_2CH_2NH_2$	4-ABD	Alendronate
$CH_2CH(NH_2)CH_3$	3-ABD	
$(CH_2)_5NH_2$	AHD	Neridronate
4-Aminophenyl	APMD	

Secondly, the chromatographic behaviour of the different derivatives after reaction with PITC, applying different ion-pairing agents, has been studied, as well as the usefulness of the fluorescent isothiocyanate (ITC) labelling reagents fluorescein 5-ITC (FITC), 1-naphthyl-ITC (NITC) and 4-dimethylamino-NITC (DNTC) for bisphosphonate analysis. The presented results are indispensable for further bioanalytical research of this type of bisphosphonate, following the approach of pre-column derivatization with fluorescent isothiocyanates.

2. Experimental

2.1. Chemicals

APD (disodium salt) and EHDP (monosodium salt) were obtained from Bufa (Uitgeest, The Netherlands). All other bisphosphonates (acid form) were generously provided by Dr. C.W.G.M. Löwik (Department of Endocrinology, Leiden University Hospital, The Netherlands). Aminomethylphosphonic acid (AMP) and 2-aminoethylphosphonic acid (AEP) were supplied by Sigma (St. Louis, MO, USA) and 4-aminophenylphosphonic acid (APP) by Aldrich (Milwaukee, WI, USA).

PITC was purchased from Pierce (Rockford, IL, USA), NITC and FITC from Sigma and DNTC from Janssen Chimica (Beerse, Belgium). The ion-pairing agent tetramethylammonium (TMA)-hydroxide (10% (w/w) in water) was obtained from Merck (Darmstadt, Germany), while tetraethylammonium (TEA)-hydroxide (20% (w/w) in water), tetrabutylammonium (TBA)-hydroxide (20% (w/w) in water) and TBA-bromide originated from Merck-Schuchardt (Hohenbrunn, Germany), tetrahexylammonium (THA)-hydrogen phosphate from Serva (Heidelberg, Germany) and tetraoctylammonium (TOA)-bromide from Sigma. Water was home-distilled, acetonitrile (HPLC-grade) was supplied by Promochem (Wesel, Germany), hydrogen peroxide (30% (w/w)) by den Hertogh (Woudenberg, The Netherlands) and tri-

ethylamine (>99% (w/w)) by Janssen Chimica (Geel, Belgium). All other chemicals were of analytical grade from Merck.

2.2. Equipment

Chromatographic analyses were performed on two configurations:

(A) A 2150 HPLC-pump, a 2152 gradient controller and a 11300 Ultrograd mixer driver (LKB, Bromma, Sweden), a KT-35 degasser (Shodex, Tokyo, Japan), a Rheodyne 7125 injection valve (Rheodyne Cotati, CA, USA), equipped with a 20-μl sample loop, a 1000S diode array detector (Applied Biosystems, Ramsey, NJ, USA) and a SP4270 integrator (Spectra Physics, San Jose, CA, USA).

(B) A Spectroflow 400 solvent delivery system (Applied Biosystems), a Marathon autoinjector with a built-in column thermostat (Spark Holland, Emmen, The Netherlands), equipped with a 7010-80 Rheodyne injection valve and a 20-μl sample loop, a Spectroflow 773 variable-wavelength detector (Kratos Analytical Instruments, Westwood, NJ, USA), a Schoeffel FS 970 L.C. fluorometer (Schoeffel Instrument, Westwood, NJ, USA), later replaced by a Jasco 821-FP spectrofluorometric detector (Jasco, Hachioji City, Japan) and a SP4270 integrator (Spectra Physics), later replaced by a IPC Dynasty HE 486DX personal computer (IPC, Singapore), equipped with a GYNKOSOFT chromatographic data system (Softron, Gräfelfing, Germany).

FAB-MS measurements were performed on a Finnigan MAT 900 with excitation by an ^{133}cesium source.

2.3. Chromatographic conditions

Flushed loop injections (20 μl) were made on a Chromspher C_{18} column (100×3.0 mm, d_p=5 μm, average pore diameter=13 nm) (Chrompack, Middelburg, The Netherlands). The column temperature was ambient using HPLC-configuration A and 30°C applying configuration B. The eluent (pH* 7–8) comprised a 20 m*M* phosphate buffer, containing 5 m*M* of a tetraalkylammonium ion-pairing agent and 0.5 m*M* EHDP as an adsorption suppressor and an appropriate amount of acetonitrile. The eluent flow was 0.4 ml/min. Typical acetonitrile percentages (v/v) combined with the different ion-pairing agents were 1% for TMA, 3% for TEA, 25% for TBA, 40% for THA and 60% for TOA. The detection wavelengths applied are listed in Table 2.

2.4. Sample preparation

Derivatization procedures were optimized for the reagents PITC, FITC and NITC using APD standard solutions in water. The sample handling was carried out in 10-ml conical glass tubes. The DNTC procedure was identical to the method followed for the NITC reagent.

The PITC procedure started with 100 μl 0.08–0.5 mg/ml of an amino(bis)phosphonate in water to which 50 μl ethanol, 40 μl pyridine, 10 μl triethylamine and 2 μl PITC were added; the mixture was vortex-mixed yielding a clear solution. The tube was then sealed and heated at 80°C for 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 with 2 ml 1-pentanol–chloroform (1:9, v/v), discarding the organic solvent.

The FITC procedure started with 100 μl 0.5 mg/ml APD in water to which 40 μl pyridine, 10 μl triethylamine and 50 μl 20 mg/ml FITC in acetone were added; the mixture was vortex-mixed giving a clear solution. The tube was then sealed and heated at 80°C for 30 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 with 2 ml 10 mg/ml TOA-bromide in chloroform, discarding the organic solvent.

The NITC (and DNTC) procedure started with 100 μl 0.5 mg/ml APD in water to which 15 μl

Table 2
Detection wavelengths, applied for different (thio)carbamyl labels

Reagent	UV–Vis	Fluorescence[a]	
λ	(nm)	λ_{ex} (nm)	λ_{em} (nm)
PITC	240		
NITC	285	285	390
DNTC	310	310	480
FITC	490	490	520

[a] Jasco spectrofluorometric detector.

triethylamine and 100 μl 20 mg/ml NITC (or DNTC) in pyridine were added; the mixture was vortex-mixed yielding a clear solution. The tube was then sealed and heated at 80°C for 15 min. This mixture was extracted twice with 1 ml 10 mg/ml TBA-bromide in chloroform, discarding the organic solvent.

The oxidative desulphuration of PTC-APD and naphthylthiocarbamyl-APD (NTC-APD) was performed by heating the final aqueous sample for 5 min at 80°C after adding 100 μl 0.02% (PTC-APD) or 10 μl 0.2% (NTC-APD) (v/v) hydrogen peroxide.

For the determination of detection limits of APD analysis after the different derivatizations, the APD standard was diluted until the detection limit was reached. For the fluorescent labels fluorescence detection was employed. To suppress irreversible adsorption of the analyte, 10 μl 1 mg/ml EHDP was added to the sample as an adsorption suppressor. In order to obtain a lower detection limit for APD with the PITC derivatization, this procedure was extended, after the LL-extraction, with evaporation of the sample at 80°C under a nitrogen stream and reconstitution in 100 μl water, prior to the HPLC analysis.

For the FAB-MS analyses a 100 mg/ml APD solution was subjected to the PITC-derivatization procedure and a 4 mg/ml APD solution to the NITC derivatization procedure, resulting in a 20 mg/ml and a 2 mg/ml APD-derivative solution, respectively, after derivatization and dilution. Both derivatizations were performed with and without a hydrogen peroxide treatment afterwards. From these four samples 1 μl was added to a drop of glycerol for the FAB-MS measurements in the negative ion mode.

3. Results and discussion

A substantial oxidative desulphuration during derivatization of the amino function in APD with PITC was already postulated previously [23]; for amino acids only a minor amount of a PC derivative could be formed [24]. Derivatization with an ITC of most other amino(bis)phosphonates investigated, resulted also in the formation of two reaction products with a comparable peak area in the UV chromatogram (Fig. 1, Table 3). These two peaks are expected to be the thiocarbamyl and carbamyl derivative. This is supported by the total conversion of the thiocarbamyl-derivative into its carbamyl analogue when treated with hydrogen peroxide (example shown in Fig. 1). Direct synthesis of a PC derivative by using an isocyanate is not possible, it would be quickly hydrolysed by the presence of water, necessary for the dissolution of a bisphosphonate, in the reaction mixture. To obtain complete certainty about the identity of the two products we performed FAB-MS analyses of the reaction products of APD with PITC and NITC with and without the hydrogen peroxide post-treatment. The mass spectra of the APD-NITC products are depicted in Fig. 2. As expected, the spectrum of the oxidized sample shows only the presence of one derivative: naphthylcarbamyl-APD (NC-APD) (m/z 403), while the spectrum of the non-oxidized sample also displays the other derivative: NTC-APD (m/z 419). The results for the APD-PITC products were similar (data not shown).

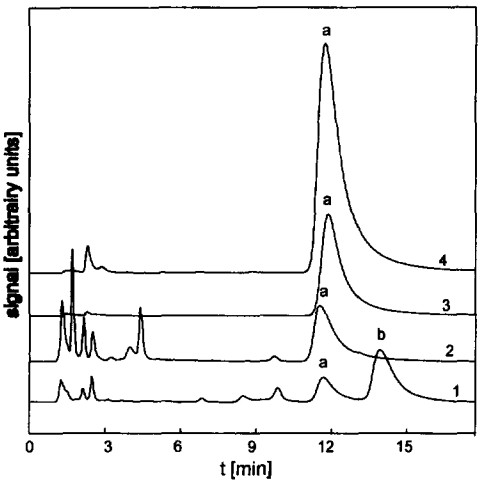

Fig. 1. Chromatograms of APD-NITC reaction products. HPLC-configuration B: eluent with TOA as the ion-pairing agent and 60% acetonitrile. UV signal of a sample (1) not treated, respectively (2) treated with hydrogen peroxide; fluorescence signal of a sample (3) not treated, respectively (4) treated with hydrogen peroxide. (a) naphthylcarbamyl-APD; (b) naphthylthiocarbamyl-APD.

In order to elucidate the formation of the unexpected large amounts of carbamyl-amino(bis)phosphonates we hypothesized an intramolecular catalytic effect, promoting the oxidative desulphuration, as demonstrated for APD and PITC in Fig. 3. The figure shows a nucleophilic attack on the sulphur

Table 3
Formation of a desulphurated derivative of the reaction between an amino(bis)phosphonate and PITC, relative to the PTC-derivative

Amino(bis)phosphonic acid	Position of NH_2 to the phosphorous atom	Ratio of the peak area of PC/PTC (λ=240 nm)
4-ABD	5	0.4
3-ABD	4	0.4
APD	4	0.5-0.7
AEP	3	0.5
AMP	2	0.19
APMD	2; *para*	0.07
APP	*para*	0.011

bonded carbon, resulting in a cyclic intermediate, and the reaction is followed by a nucleophilic reaction on the phosphorus atom involved. This reaction mechanism should lead to significant lower amounts of a carbamyl derivative if an amino(bis)-phosphonate is derivatized where the intramolecular interaction between the amino group and a phosphonate group is reduced or prohibited because of sterical reasons. For example, AMP would have to form a strained 5-ring intermediate, (4-aminophenyl-hydroxymethylene)bisphosphonate (APMD) a highly strained and unlikely cyclic structure and APP an impossible structure. Table 3 clearly shows a decreased ratio for the formation of the PC-derivatives of AMP, APMD and APP as we predicted. The degree of desulphuration of PTC-APP is comparable with that of some PTC-amino acids [24] for which the proposed intramolecular catalytic effect is also not possible.

The chromatography of the PITC derivatives of the (amino-1-hydroxyalkylidene)bisphosphonates was investigated using different eluent compositions. The selectivity of the chromatographic separation can be strongly influenced (Table 4), including inversion of elution orders by changing the strength

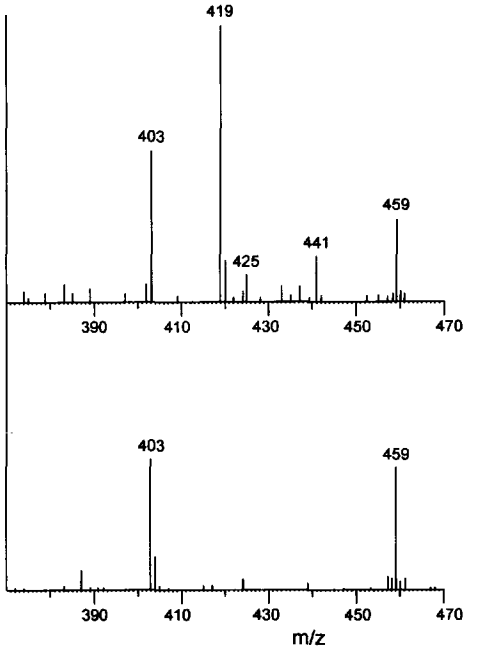

Fig. 2. FAB-MS spectra (negative mode) of APD-NITC reaction products (m/z=mass, relative to the charge of the ion). Upper spectrum: sample not treated with hydrogen peroxide; lower spectrum: sample treated with hydrogen peroxide. 403, NTC-APD; 419, NC-APD; 425, NTC-APD-Na; 441, NC-APD-Na; 459, glycerol adduct (n=5).

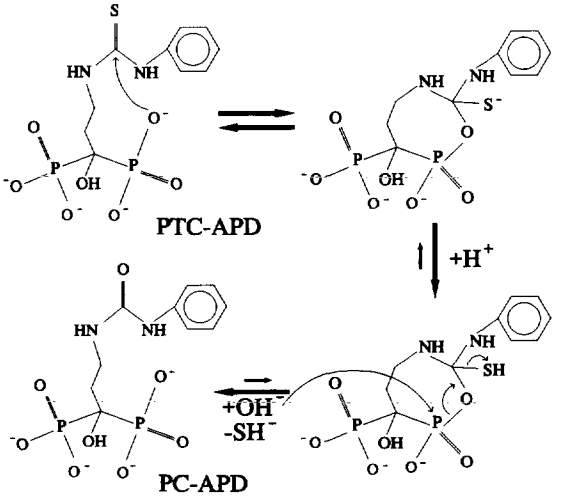

Fig. 3. Postulated reaction mechanism of an autocatalytic reaction for the oxidative desulphuration of PTC-APD into PC-APD.

Table 4
Influence of the ion-pairing agent on the relative retention of (amino-1-hydroxyalkylidene)bisphosphonate-PITC reaction products, expressed as the separation factor α to PTC-APD (t_0=1.3 min)

Components	TMA[a,c]	TEA[a]	TBA[b]	THA[b]	TOA[b]
PTC-APD	1	1	1	1	1
PC-APD	1.4	1.5	1	0.86	0.88
PTC-AHD		4.9	1.4	0.88	0.88
PC-AHD		5.0	1.4	0.84	0.79
PTC-APPD					2.0
PC-APPD					1.7
PTC-APMD		1.8			
PC-APMD		3.7			
PTC-3-ABD		1.6		1.07	1.07
PC-3-ABD		2.6		0.88	0.88
PTC-4-ABD		1.7		0.94	0.93
PC-4-ABD		2.2		0.80	0.82

[a] HPLC configuration A (ambient temperature).
[b] HPLC configuration B (30°C).
[c] Very unstable retention times.

of the ion-pairing agent. In order to keep acceptable retention times, the modifier content of the eluent was adjusted simultaneously. For example, PC-APD eluted after PTC-APD when TMA or TEA were applied as relatively weak ion-pairing agents but their elution order was reversed when the strong agents THA or TOA were applied. Results for the other derivatizing agents showed similar elution orders (data not shown).

UV–Vis chromatograms show comparable peak areas for corresponding carbamyl and thiocarbamyl derivatives when an amino(bis)phosphonate is derivatized with an ITC. The NTC- and 4-dimethylamino-NTC derivatives, however, show approximately fifty-fold higher fluorescence peak area compared to their, hardly detectable, NC and 4-dimethylamino-NC analogues; for both APD-NITC derivatives this is shown in Fig. 1. This large difference in fluorescence properties was not further investigated.

In order to select the most promising reagent for further development of a selective and sensitive method for the analysis of APD and other amino-(bis)phosphonates, the methods with different reagents were optimized and compared. The derivatizations (except for DNTC) were optimized for an 0.5 mg/ml APD standard solution with respect to the yield of the derivatization reaction and the efficiency and recovery of the clean-up by LL extraction. The reaction yield was measured by the determination of the underivatized APD with IEC [6]. With the reagent PITC, 93–94% of the APD standard was converted, with NITC 83–87%, with FITC about 70% and with DNTC about 60%. Derivatizing APD concentrations in the sub-μg/ml range leads to substantial losses of the analyte due to irreversible adsorption to the wall of the tubes, especially at an elevated temperature. Thus, 10 μl of 1 mg/ml EHDP as a not derivatizable, adsorption suppressor was added to the sample prior to derivatization at 80°C. Obviously, adsorption effects, comparable to those in the LC-column where EHDP is added to the eluent to avoid peak broadening by adsorption, are more pronounced at these low concentrations. The detection limit of APD in a 100-μl aqueous sample is 0.1 μg/ml using PITC [23], and 5 ng/ml using NITC. For FITC and DNTC, a detection limit of respectively about 1 ng/ml and 5 ng/ml may be expected, based on extrapolation of the results of derivatizations at higher APD concentrations. However, for these two derivatization reagents the extraction of by-products is less efficient compared to the NITC derivatization, resulting in a higher detection limit in practice. Based on these results, derivatization with NITC was chosen for further bioanalytical research with APD [25] as the reaction products can be very effectively cleaned up by ion-pair LL extraction and the NC derivative allows sensitive fluorescence detection in the HPLC system.

This approach has proven to be successful. For example, for a 2.5-ml urine sample, a detection limit of 1 ng/ml APD and an intra-assay precision of 5–7% was achieved. The bioanalytical procedure included calcium precipitation, solid-phase extraction (SPE) and evaporation prior to derivatization with NITC [25]. For serum samples a protein precipitation step was added to the procedure, as well as automated versions of the SPE, derivatization, LL extraction and hydrogen peroxide treatment [26].

Compared to other bioanalytical bisphosphonate assays based on derivatization of the amino function, the achievements of the new method are superior to the APD assay with fluorescamine [15,16] and comparable to the alendronate method with NDA [18,19]. The derivatization of alendronate with OPA

is not (yet) developed into a bioanalytical method [17].

4. Conclusions

During the ITC derivatization of several amino-(bis)phosphonates a significant part of the thiocarbamyl derivative can be oxidatively desulphurated into its carbamyl analogue, depending on the intramolecular interaction between the amino and a phosphonate group. An intramolecular catalytic reaction which involves a phosphonate group can explain the relative large amount of carbamyl-(bis)-phosphonate often formed during this derivatization. The carbamyl derivatives from a bisphosphonate after reaction with NITC or DNTC show superior fluorescence properties compared to the thiocarbamyl derivatives. The elution order of carbamyl and thiocarbamyl derivatives of (amino-1-hydroxy-alkylidene)bisphosphonates is strongly dependent on the strength of the specific tetraalkylammonium ion-pairing agent in the eluent. NITC is the ITC of first choice for derivatization of (amino-1-hydroxy-alkylidene)bisphosphonates and is also selected to be used in the development of bioanalytical methods for APD in both urine [25] and serum or plasma [26].

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