Journal of Chromatography, 583 (1992) 183–193
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6583

Improved determination of the bisphosphonate alendronate in human plasma and urine by automated precolumn derivatization and high-performance liquid chromatography with fluorescence and electrochemical detection

W. F. Kline and B. K. Matuszewski

Merck Research Laboratories, West Point, PA 19846 (USA)

(First received July 14th, 1992; revised manuscript received September 14th, 1992)

ABSTRACT

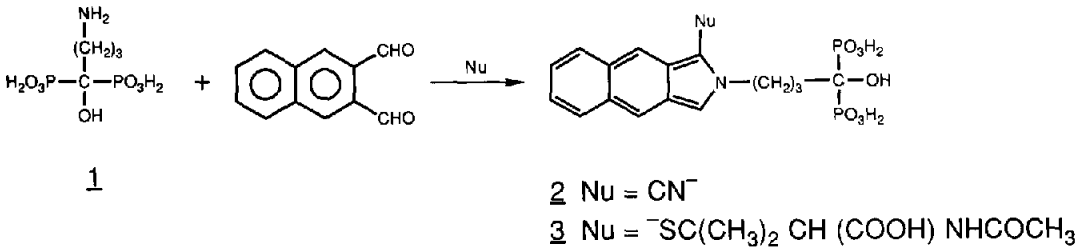
An improved method for the determination of 4-amino-1-hydroxybutane-1,1-bisphosphonic acid (alendronate) in human urine and an assay in human plasma are described. The methods are based on co-precipitation of the bisphosphonate with calcium phosphates, automated pre-column derivatization of the primary amino group of the bisphosphonic acid with 2,3-naphthalene dicarboxyaldehyde (NDA)–N-acetyl-D-penicillamine (NAP) or cyanide (CN^-) reagents, and high-performance liquid chromatography (HPLC) with electrochemical (ED) or fluorescence detection (FD). The feasibility of ED of the NDA–CN^- derivative of alendronate has been demonstrated, and a HPLC–ED assay in human urine has been validated in the concentration range 2.5–50.0 ng/ml. In order to eliminate the cyanide ion from the assay procedure, several other nucleophiles in the NDA derivatization reaction were evaluated. An NDA–NAP reagent was found to produce highly fluorescent derivatives of alendronate. The assay in urine based on NDA–NAP derivatization and HPLC–FD has been developed and fully validated in the concentration range 1–25 ng/ml. Based on the same NDA–NAP derivatization, an assay in human plasma with a limit of quantification of 5 ng/ml has also been developed. Both HPLC–FD assays were utilized to support various human pharmacokinetic studies with alendronate.

INTRODUCTION

An assay in urine for the determination of 4-amino-1-hydroxybutane-1,1-bisphosphonic acid (**1**, Fig. 1) has been previously developed in our laboratories [1]. The assay was based on pre-column derivatization of **1** with 2,3-naphthalene dicarboxyaldehyde (NDA), in the presence of cyanide ion (CN^-) as a nucleophile, to produce the fluorescent N-substituted cyanobenz[*f*]isoindole derivative **2**, which was determined by high-performance liquid chromatography (HPLC) with fluorescence detection (FD). The limit of quantitation (LOQ) in urine, defined as the lowest point on the standard curve for which the assay precision (coefficient of variation, C.V.) is lower than 10%, was 5 ng/ml when 5 ml of urine were utilized. Urine samples from a pilot bioavailability study, after dosing human subjects with 50 mg of **1**, were analyzed using this assay.

Although the LOQ of **1** using the method described in ref. 1 was far superior to all other procedures available for determination of structurally similar bisphosphonates [2–6], it became evident, after analysis of samples from the first clin-

Correspondence to: B. K. Matuszewski, Merck Research Laboratories, West Point, PA 19846, USA.



1

2 Nu = CN^-

3 Nu = $^-SC(CH_3)_2$ CH (COOH) $NHCOCH_3$

Fig. 1. Structures of **1** and derivatives **2** and **3**.

ical study, that the concentration of **1** in many subjects' urine samples after a 50-mg oral dose was below 5 ng/ml. Several pharmacokinetic studies with lower oral doses of **1** (20, 10 and 5 mg) were planned, and a more sensitive assay in urine was required. In addition, an assay for **1** in plasma was also highly desirable. Therefore, a series of studies were undertaken in order to: (1) improve at least five-fold the sensitivity of the assay of **1** in urine; (2) replace the CN^- ion, undesirable in the analytical laboratory, with a less toxic nucleophile in the NDA derivatization reaction; (3) develop an assay for **1** in plasma with an LOQ of 5 ng/ml.

In order to achieve these goals, several approaches including electrochemical detection (ED) rather than FD of the derivatives, utilization of a thiol instead of cyanide ion as a nucleophile in the derivatization reaction, and several modifications in the sample isolation procedure and in FD, were pursued and are described in this paper. Based on all of these studies the assay in urine with an LOQ of 1 ng/ml (using 5 ml of urine) and in plasma (LOQ = 5 ng/ml, using 1 ml of plasma) were developed. The 2,3-naphthalene dicarboxyaldehyde (NDA) derivatization reaction of **1** was shown to be effective with NAP as a nucleophile, and the CN^- ion has been successfully eliminated from the sample preparation procedure. The new methods, which were utilized for the analyses of more than 3000 urine and plasma samples from a variety of clinical studies, were based on NDA–NAP derivatization and HPLC–FD. These assays are almost two orders of magnitude more sensitive than the recently described method, based on pre-column derivatization with fluorescamine and HPLC–FD, for the structurally similar bisphosphonate, pamidronate [7].

In addition, our studies have demonstrated, that, similarly to other NDA–CN^--derivatized primary amino acids and amines which are ED-active [8–10], the benzo[*f*]isoindole derivatives of **1** are readily oxidized at favorable low potentials, making the HPLC–ED method a valid alternative for high sensitivity detection of **1**. Based on this approach an assay in urine, with an LOQ of 2.5 ng/ml, has been validated, and the details of this assay will also be presented.

EXPERIMENTAL

Materials

All solvents and reagents were of HPLC or analytical grade (Fisher, Fair Lawn, NJ, USA; Sigma, St. Louis, MO, USA). Alendronate monosodium trihydrate was obtained from Merck (Rahway, NJ, USA). The NDA was purchased from Molecular Probes (Eugene, OR, USA). N-Acetyl-L-cysteine (NAC), potassium thiocyanate and NAP originated from Fluka (Ronkonkoma, NY, USA). Water was deionized using a Milli-Q reagent water system (Millipore, Milford, MA, USA).

Instrumentation

A Varian HPLC system (Walnut Creek, CA, USA) interfaced to a Hewlett-Packard laboratory automation system (HP 3357 LAS, Palo Alto, CA, USA) was used as previously described [1]. As a fluorescence detector, a McPherson detector (Model FL-750B), equipped with xenon–mercu-

ry lamp, high-sensitivity attachment and autozero, was utilized (McPherson, Acton, MA, USA). As an electrochemical detector, the LC-17A ED flow cell with glassy carbon electrode, RE-4 Ag/AgCl reference electrode and LC-4B amperometric controller were employed (Bioanalytical Systems, West Lafayette, IN, USA).

The cyclic voltammograms were recorded with a Bioanalytical Systems Model CV-1B cyclic voltammograph, equipped with a Model VC-2 cell, a glassy carbon electrode and an Ag/AgCl electrode. Absorption and fluorescence spectra were taken using a diode-array spectrophotometer (HP 8452) and Perkin-Elmer Model 650-10S spectrofluorometer (Norwalk, CT, USA).

The polymeric reversed-phase analytical column and guard columns, as described previously [1], were used. For the clean-up of urine and plasma extracts, diethylamine (DEA) solid-phase extraction cartridges containing 200 mg instead of 500 mg of packing material (3 ml, Analytichem International, Harbor City, CA, USA), were utilized.

Chromatographic conditions

For the HPLC–ED assay of NDA–CN^--derivatized **1**, the same mobile phase as described previously was employed [1]. The eluent from the column was monitored by an electrochemical detector set at an oxidation potential of +0.65 V. This optimum potential was established by constructing a hydrodynamic voltammogram of **2** obtained by injecting the same amount of derivatized **1** at different potentials (0.4–0.8 V) of the electrochemical cell and recording the relative current generated.

The NDA–NAP derivatives of **1** were chromatographed using a mobile phase consisting initially of acetonitrile–0.025 *M* sodium citrate–0.025 *M* dihydrogenphosphate, adjusted to pH 6.3 with 85% phosphoric acid (15:85, v/v). The retention time for the derivative **3** (Fig. 1) was about 7 min. Late-eluting endogenous components from urine or plasma were removed by increasing the acetonitrile content to 32.5% in a gradient program from 10 to 15 min. The system was returned to the initial mobile phase conditions by 20 min, and after 10 min re-equilibration the next injection was made (total analysis time was 30 min).

The effluent from the column was monitored by a fluorescence detector with the excitation wavelength set at 436 nm. A cut-off filter with $\lambda > 440$ nm was placed in the emission path of the fluorescence detector. The mobile phase flow-rate was 1 ml/min.

The mobile phase components were measured separately, mixed and filtered through a 0.2-μm Nylon 66 filter (Rainin Instruments, Woburn, MA, USA).

Standard solutions

A stock standard solution of **1** (100 μg/ml) was prepared in water. This solution was further diluted to give a series of working standards with concentrations of 0.10, 0.25, 0.50, 1.0 and 2.5 μg/ml. Stock solutions were stored at −5°C and kept for a period of up to two weeks. A series of quality control (QC) samples in urine and plasma were prepared, by spiking 5-ml aliquots of urine and 1-ml aliquots of plasma with a separately prepared working stock standard of **1**. The QC samples were stored at −20°C until assayed.

*An HPLC–ED assay in urine using derivative **2** (method A)*

The sample preparation procedure was similar to the method described previously [1], with one important modification. The borate buffer used in the original assay was replaced with carbonate buffer (50 μl, 1.0 *M*, pH 10.7). When ED of **2** was attempted and borate buffer was used, in addition to noisy baseline and presence of some interfering peaks, a very wide solvent front peak extending to the elution area of the peak of interest was observed. Replacement of the borate buffer with carbonate buffer eliminated these interferences and improved the specificity of the ED assay.

*HPLC–FD assay using NDA–NAP derivative **3***

Urine (method B). Several major changes in the procedure [1] were made. The isolation of **1**

from urine (5 ml) including loading of the extract on the DEA SPE cartridge was unchanged except for the concentration of calcium chloride used for precipitation of **1** which was decreased from 2.50 to 1.25 *M*. After the initial isolation of the drug from urine, the characteristic pellet formed was dissolved in 100 μl of 1 *M* hydrochloric acid and precipitated again with 100 μl of 1 *M* sodium hydroxide. The isolated pellet containing **1** was reconstituted in 0.8 ml of 0.2 *M* acetic acid, 0.4 ml of 0.01 *M* EDTA, and 0.4 ml of 0.2 *M* sodium acetate. After addition of 3 ml of water the solution was applied to a DEA cartridge preconditioned with 3 ml of water and eluted with 1 ml of 0.2 *M* sodium citrate–0.2 *M* sodium phosphate (dibasic) (pH 8.5) (1:1, v/v). To the part of the sample eluent (250 μl), 50 μl of the 1 *M* carbonate buffer (pH 10.7) were added followed by the automatic addition of methanolic solutions of 10 μl NAP (1 mg/ml) and 10 μl NDA (1 mg/ml). A 50-μl aliquot of the mixture after derivatization was injected on the column.

Plasma (method C). Plasma (1 ml) was diluted with water (3 ml) and deproteinized with 10% trichloroacetic acid (1 ml). Direct precipitation of the calcium salt of **1** from plasma supernatant by the addition of 25 μl of 2.5 *M* calcium chloride and 0.5 ml of 1 *M* sodium hydroxide was ineffective. In order to induce co-precipitation of the calcium salt of **1**, 0.2 ml of 0.022 *M* sodium pyrophosphate in water was added to the plasma supernatant before the addition of calcium chloride and sodium hydroxide. Under these conditions the formation of a characteristic pellet, containing calcium pyrophosphate and calcium salt of **1**, was observed. Once the precipitation was achieved, the procedure in plasma was similar to the modified procedure in urine described above for NDA–NAP derivatives (method B). The protein precipitation with 10% trichloroacetic (1 ml) from plasma was done in four steps by the addition of 250 μl of the acid and vortexing after each addition. This stepwise protein precipitation contributed to the improvement in the recovery of **1**, probably by avoiding the entrapment of the drug in the precipitated protein.

Precision, accuracy, linearity, recovery and specificity

The precision of the method was determined by the replicate analyses ($n = 5$) of human urine or plasma containing **1** at all concentrations utilized for constructing calibration curves. A series of QC standards were prepared at the start of clinical studies to monitor the stability of **1** in plasma and urine stored at −20°C, and to assess assay performance and accuracy on the day-to-day basis. These QC samples were assayed daily with unknown samples using daily constructed standard lines. The accuracy of the assay was expressed by (mean observed concentration)/(expected concentration) × 100.

The linearity of each standard curve was confirmed by plotting the peak area of derivatized **1** *versus* drug concentration. Unknown sample concentrations were calculated from the equation $y = mx + b$, as determined by the weighted $(1/y)$ linear regression of the standard line.

The recovery was assessed by comparing the peak area of the derivatized **1** extracted from plasma and/or urine to that of derivatized standards injected directly. The recovery for the HPLC–ED assay utilizing derivative **2** was assessed by comparing the peak area of the synthesized **2** with the peak area of the derivative after extraction of the drug from urine and derivatization. In the latter case the recovery was >90%.

The assay specificity was assessed by analyzing pre-dose plasma and urine samples of various patients. No endogenous interference was encountered.

Attempted synthesis of derivative 3

The NDA–CN^- derivative **2** was previously synthesized and fully spectrally characterized [1]. Several attempts were made here to synthesize NDA–NAP derivative **3**, using a similar procedure as in the synthesis of **2**, and other primary amines derivatized with NDA [11]. The solvent composition of the reaction medium was modified to allow full solubility of NDA, NAP and **1** in the final reaction solution. The synthesis was performed first on a microgram scale and was

followed by a synthesis on a 50-mg scale.

In the initial attempt, 203 μg ($7.46 \cdot 10^{-4}$ mmol) of **1** in 2 ml of 0.02 *M* borate buffer (pH 10.1) were mixed with 73.8 μg of NDA ($4.0 \cdot 10^{-4}$ mmol) in 27 μl of methanol and 76.8 μg of NAP ($4.0 \cdot 10^{-4}$ mmol) in 14 μl of methanol. The progress of reaction was followed by monitoring the UV and fluorescence spectra of the reaction mixture. These spectra were compared with the spectra of **2** dissolved in the same solvent mixture except for the presence of **1**. A characteristic absorption band with maxima at 438 and 454 nm, and a fluorescence band with the emission maximum at 505 ± 5 nm (uncorrected) were observed, indicative of the formation of a characteristic benzo[*f*]isoindole chromophore of **3**. The solutions of the NDA–CN^- standard and of the NDA–NAP derivative with the same absorbance at λ_{exc} (400 nm) were prepared and the relative fluorescence intensities of the two solutions were measured [12]. From this comparison, an estimate of a relative fluorescence quantum efficiency ($\Phi_f^{rel} = 0.4$) of **3** *versus* **2** in the mixture directly after synthesis was made.

In an attempt to isolate **3** and confirm its structure by various forms of spectroscopy, a larger scale synthesis was attempted. To a stirred solution containing 54.4 mg (0.2 mmol) of the sodium salt of **1** in 4 ml of ethanol and 5 ml of water adjusted to pH 10 with 1 *M* sodium hydroxide, 38.2 mg (0.2 mmol) of NAP dissolved in 4 ml of the mixture of ethanol–water (1:1, v/v) was added. To this mixture a suspension of 36.8 mg (0.2 mmol) of NDA in 4 ml of ethanol–water (1:1, pH 9) were added dropwise. The progress of reaction was followed by UV and fluorescence spectroscopy. The formation of fluorescent, orange-greenish material in solution was immediately observed. The UV spectrum of the diluted mixture indicated the presence of a characteristic absorption band with maxima at 438 and 458 nm. Assuming the value of the molar absorption coefficient (ε) at 458 nm equal to $\sim 5000\ M^{-1}\ cm^{-1}$, a common value for the variety of NDA-derivatized primary amines [12], the initial yield of formation of **3** was estimated to be close to 100%. Both the intensity of fluorescence (λ_{max} = 505 nm) and absorption at 458 nm were decreasing with time after mixing, indicating poor stability of **3** in the reaction mixture. For example, a 20 and 72% decrease in the UV intensity at λ = 458 nm was observed after 20 min and overnight mixing of an aliquot of the reaction mixture kept at room temperature.

The mixture after synthesis was left overnight at −20°C, and 45 mg of practically non-fluorescent dark brown-greenish solid material were obtained. This non-fluorescent solid was not further analyzed. The fluorescent supernatant was split into two portions and extracted with ethyl acetate (15 ml) or a mixture of ethyl acetate–ethanol (8:1, v/v). Several attempts to isolate a fluorescent material from the organic or water layer extracts were unsuccessful, indicating the poor stability of **3** in solution.

RESULTS

HPLC–ED assay in urine based on NDA–CN^- derivative 2 (method A)

Cyclic voltammetry measurements indicated that the derivative **2** was ED-active with a half-wave potential of approximately +0.65 V (Fig. 2).

The electrochemical reaction was found to be

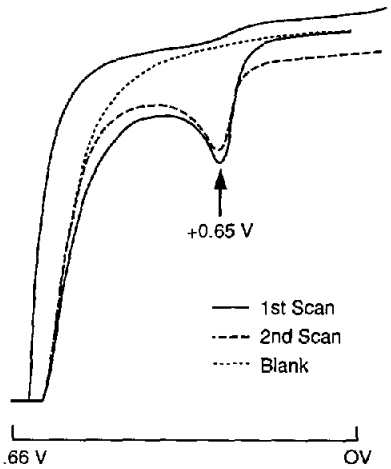


Fig. 2. Cyclic voltammogram of **2** in a 4:6 (v/v) mixture of methanol and 0.025 *M* sodium citrate–0.025 *M* dihydrogen phosphate (1:1, pH 8.5).

reversible, and the low oxidation potential of **2** was confirmed by measuring ED response at various ED potentials (hydrodynamic voltammogram) after injecting the same amount of **2** on column. The maximum response was observed at + 0.65 V and this potential of the ED cell was chosen for the assay.

The HPLC–ED assay was linear over the concentration range 2.5–50 ng/ml. The within-day precision was less than 9% for all concentrations within the calibration range (Table I). The accuracy of the assay was 97–104%, and a representative equation of the linear regression line was $y = 67786x - 30880$ (using peak area in arbitrary units for calculations) with a correlation coefficient of 0.9992. The specificity of the assay was confirmed by the analysis of urine blanks; impurities coeluting with **2** were not detected (Fig. 3).

HPLC–FD assay in urine and plasma based on NDA–NAP derivative 3 (methods B and C)

Following the procedure described in Experimental, the assay for **1** in urine (method B), with the improved LOQ of 1 ng/ml, has been developed and fully validated in the concentration range 1–25 ng/ml. The within-day and inter-day precision and accuracy data for this assay are presented in Table II. The representative equation of the standard line was $y = 186940x - 18810$ with a correlation coefficient of 0.9985.

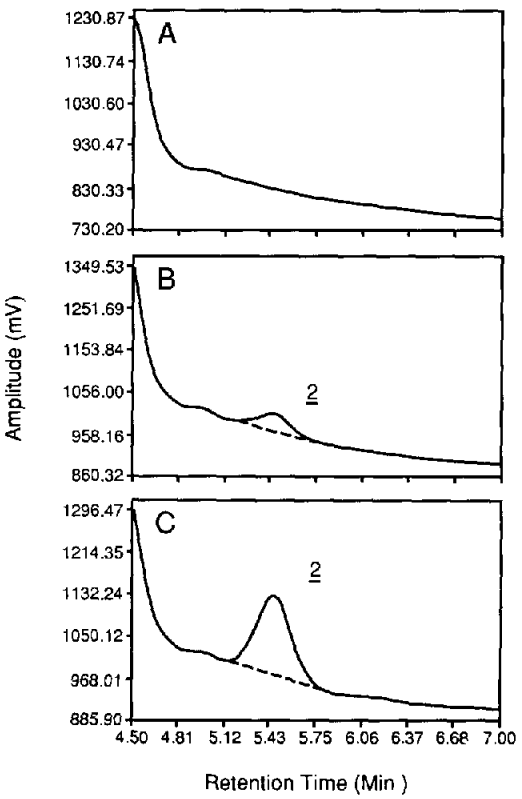


Fig. 3. Chromatograms of human urine spiked with **1**, derivatized to **2** with NDA–CN^-, and analyzed using HPLC–ED. (A) Blank control urine; (B) control urine spiked with 2.5 ng/ml; (C) urine spiked with 10 ng/ml. Only the region of elution of **2** (4.5–7.0 min) is shown in the chromatograms; total analysis time was 20 min.

TABLE I

INTRA-DAY VARIABILITY AND ACCURACY OF THE ASSAY OF **1** IN URINE USING DERIVATIVE **2** AND HPLC WITH ELECTROCHEMICAL DETECTION (METHOD A)

Concentration (ng/ml)	C.V. (n = 5) (%)	Accuracy[a] (%)
2.5	8.8	104
5.0	2.5	97
10.0	1.9	97
25.0	3.4	103
50.0	8.9	99

[a] Calculated as (mean observed concentration/nominal concentration) × 100.

The chromatograms of urine containing **1** are shown in Fig. 4. The limit of detection (LOD) of the method at a signal-to-noise ratio of 3:1 was 0.2 ng/ml.

Based on the formation of **3**, an assay for **1** in plasma (method C) has also been developed and fully validated in the concentration range 5–125 ng/ml. The typical equation for the standard line in plasma was $y = 8190x - 290$ with a correlation coefficient of 0.9986. The representative chromatograms in plasma and assay validation data are presented in Fig. 5 and Table III.

Analysis of samples from clinical studies

The HPLC–FD assay using derivative **3** was

TABLE II

INTRA- AND INTER-DAY PRECISION AND ACCURACY DATA FOR THE ANALYSIS OF 1 IN URINE AFTER DERIVATIZATION TO 3 (METHOD B)

Concentration (ng/ml)	Intra-day		Inter-day	
	C.V.[a] (%)	Accuracy[b] (%)	Assayed value[c] (ng/ml)	C.V.[c] (%)
1.0	3.7	94	–	–
2.5	5.5	100	2.4	5.7
5.0	8.3	109	–	–
10.0	5.3	100	–	–
20.0	–	–	20.2	6.9
25.0	3.4	99	–	–

[a] $n = 5$.

[b] Calculated as (mean observed concentration/nominal concentration) × 100.

[c] $n = 21$, over a period of eight weeks; quality controls were run daily and calculated from the daily standard curve.

used routinely for the analysis of urine and plasma samples from various human pharmacokinetic studies. The representative concentration data of **1** in urine for selected subjects participating in a food interaction study, are presented in Table IV.

The assay in plasma was used for determination of **1** in human subjects after intravenous dosing with ^{14}C-labelled **1**. The representative data are given in Table V.

DISCUSSION

The three major objectives of the present work were to improve sensitivity of determination of **1** in urine from 5 to 1 ng/ml, replacement of the cyanide ion in the derivatization reaction (Fig. 1) with an alternative and less toxic nucleophile, and development of a validated assay for **1** in

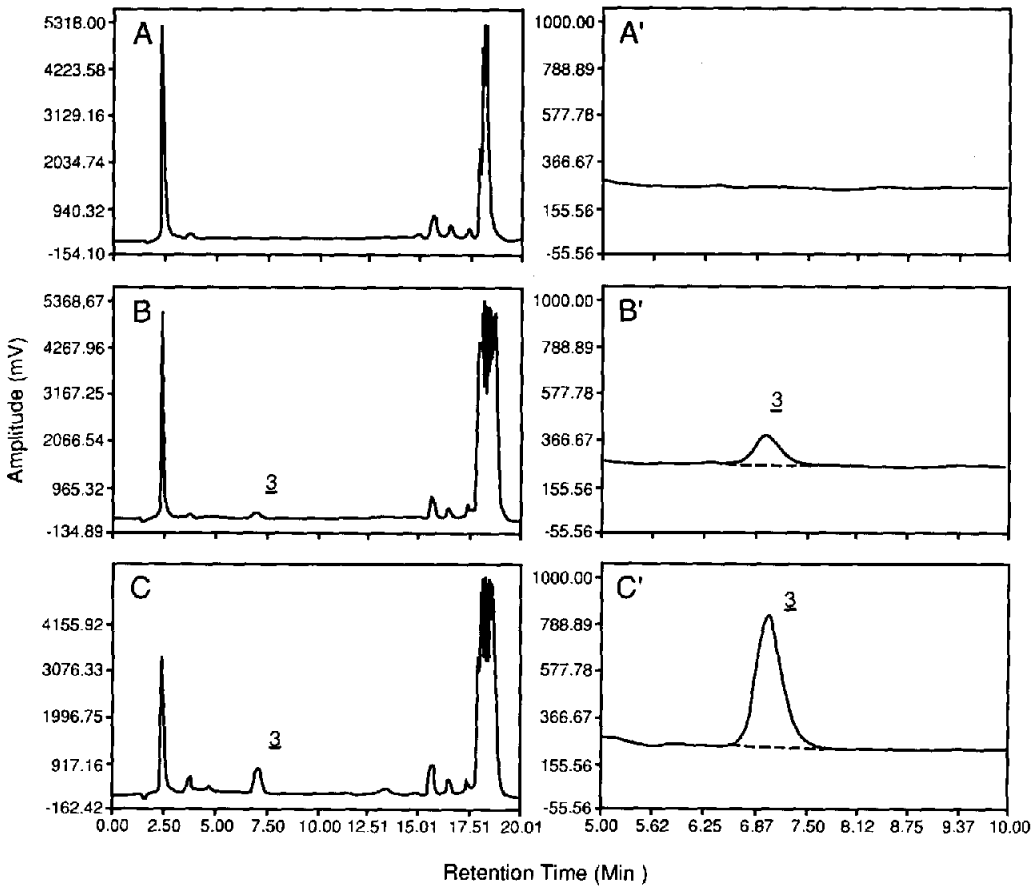


Fig. 4. Chromatograms of human urine spiked with **1**, derivatized to **3** with NDA–NAP, and analyzed using HPLC–FD. (A) Blank control urine; (B) control urine spiked with 2.5 ng/ml **1**; (C) urine sample of a subject (0–36 h collection) participating in a food interaction study after a 20-mg dose of **1** (food withheld for 2 h post-dose); the concentration of **1** in the sample analyzed was equivalent to 11.0 ng/ml. Chromatograms A', B' and C' show the elution region of **3**.

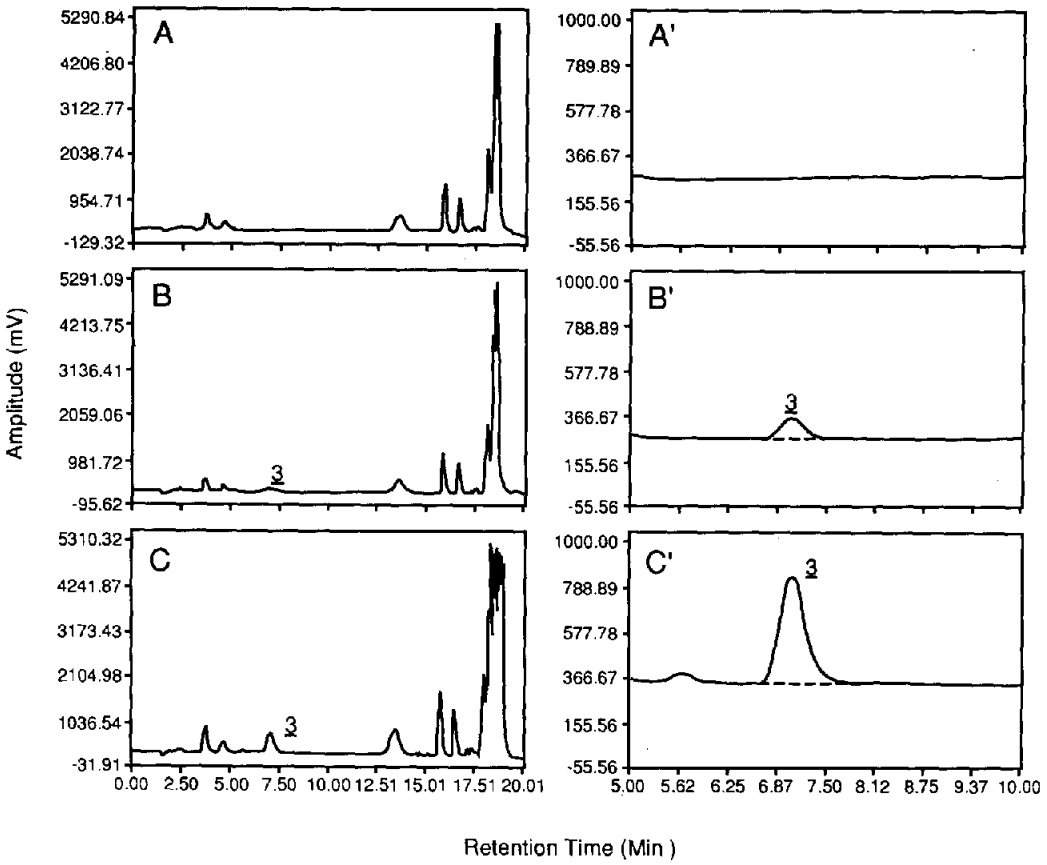


Fig. 5. Chromatograms of human plasma spiked with **1**, derivatized to **3** with NDA–NAP and analyzed using HPLC–FD. (A) Blank control plasma; (B) plasma spiked with 12.5 ng/ml **1**; (C) plasma sample of a human subject 1.5 h after the beginning of a 2-h infusion of 10 mg of **1**, the concentration of **1** in the analyzed sample was equivalent to 58.4 ng/ml. Chromatograms A′, B′ and C′ show the elution region of **3**.

TABLE III

INTRA- AND INTER DAY PRECISION AND ACCURACY DATA FOR THE ANALYSIS OF 1 IN HUMAN PLASMA AFTER DERIVATIZATION TO 3 (METHOD C)

Concentration (ng/ml)	Intra-day		Inter-day	
	C.V.[a] (%)	Accuracy[b] (%)	Assayed value[c] (ng/ml)	C.V.[c] (%)
5.0	4.2	94	–	–
12.5	4.3	102	–	–
25.0	7.1	102	23.1	12.6
50.0	3.5	105	–	–
100.0	–	–	102.7	4.5
125.0	3.0	98	–	–

[a] $n = 5$.

[b] Calculated as (mean observed concentration/nominal concentration) × 100.

[c] $n = 5$, over a period of seven days; quality control samples were run daily and calculated from daily standard curves.

TABLE IV

URINE CONCENTRATION OF ALENDRONATE AND TOTAL URINARY EXCRETION (0–36 h COLLECTIONS) MEASURED IN SUBJECTS AFTER A SINGLE ORAL DOSE OF 20 mg **1**

Food interaction study in postmenopausal healthy women, food withheld for 2 h after dosing, $n = 6$.

	Assayed concentration (ng/ml)	Amount excreted[a]	
		μg	% of dose
Mean	4.2	18.1	0.18
S.D.	1.8	4.6	0.04

[a]Amount excreted (μg) = [(ng/ml) × total urine volume]/1000.

TABLE V

REPRESENTATIVE CONCENTRATIONS OF 1 IN PLASMA FROM SUBJECTS RECEIVING 10 mg OF 1 AFTER 2-h INFUSION

Infusion at a constant rate; ^{14}C study in patients with breast cancer.

Time (h)	Concentration[a] (mean ± S.D.) (ng/ml)
2.00	309 ± 26
2.25	272 ± 41
2.50	203 ± 19
2.75	167 ± 21
3.00	127 ± 21
4.00	47 ± 21
8.00	12 ± 3
12.00	7 ± 1[b]

[a] $n = 4$.
[b] $n = 3$.

human plasma. The attainment of these goals required a series of studies which are summarized below.

Replacement of a cyanide ion with thiol in the derivatization reaction

It was demonstrated earlier that the NDA derivatization of compounds containing primary amino group leads to the formation of stable products when cyanide ion (CN^-), instead of 2-mercaptoethanol (2-ME) commonly utilized in the *o*-phthalaldehyde (OPA) derivatization reaction, was used as a nucleophile [11–13]. This finding led to the widespread use of the NDA–CN^- reagent for the derivatization of amines, amino acids and small peptides [8–10,14–16]. Based on this derivatization reaction, an assay for bisphosphonate **1** in urine was developed earlier in our laboratories [1]. However, in order to provide bioanalytical support for long-term clinical studies with **1**, it was desirable to eliminate the highly toxic cyanide ion from the assay procedure and replace it with an alternative and less toxic nucleophile, without compromising assay sensitivity.

Several nucleophiles, including isothiocyanate (NCS^-), NAC and NAP were screened against the CN^- in the derivatization reaction (Fig.1). Formation of fluorescent or ED-active product with **1** was not observed with NDA–NCS^-, whereas reaction with NDA–NAC was effective only at concentrations of **1** higher than 1 μg/ml. On the other hand, even at low nanogram concentrations of **1**, the highly fluorescent and ED-active derivative **3** was formed when **1** was reacted with NDA–NAP. This latter nucleophile (NAP) was chosen for further evaluation and assay development.

In the original mixture after derivatization, the stability of **3** was comparable or only slightly lower than for **2**, as indicated by the estimated relative fluorescence quantum efficiency (Φ_f) of **3** *versus* **2** ($\Phi_f = 0.4$) (see Experimental). This lower relative Φ_f could be attributed either to lower absolute Φ_f of **3** *versus* **2**, lower stability of **3** in the reaction medium within the time frame required for sample preparation and Φ_f measurements (~20 min), or a combination of both of these effects. However, since automated precolumn derivatization of **1** was performed in the final assay procedure and all samples were derivatized within the same, experimentally determined for maximum sensitivity, time period (1 min) before injection onto the HPLC system, the small differences in the stability between **3** and **2** were of no consequence to the overall assay sensitivity. The successful development of a highly sensitive assay (1–10 ng/ml) of a cyclic heptapeptide in human plasma, based on a similar NDA–NAP derivatization, has been recently reported by us in the literature [17].

On the other hand, the unsuccessful attempts at the isolation of **3** from the reaction mixture after synthesis on the miligram scale indicate that the long-term stability of **3** in solution was lower than for **2**, as observed for other thiols in the NDA or OPA amine derivatization reactions [13]. This lack of long-term stability of some NDA–thiol or OPA–thiol derivatives of primary amines should not be detrimental in their application for high sensitivity detection of the analytes, assuming an automated precolumn derivatization procedure is employed, as described in this and our previous papers [1,17].

Improvement in assay sensitivity

The five-fold improvement in the LOQ of **1** in urine (method B), utilizing the new (NAP) nucleophile in the derivatization reaction, was achieved through major modifications of the assay procedure described previously [1]. These changes included the replacement of a high-capacity 500-mg DEA SPE cartridge with 200-mg cartridge, allowing the decrease in the dilution of the sample before derivatization from 3 to 1 ml, replacement of a borate buffer (250 μl) with bicarbonate buffer (50 μl) in the final mixture before injection on column, and a change in the pH of the derivatization medium from 9.1 to 10.7, which was necessary to achieve the maximum conversion of **1** to **3**. Since the removal of calcium ions was necessary before derivatization and the DEA cartridge of lower capacity was utilized in the current procedure, the decrease in the amount of calcium ions present in the system from 50 μl of 2.5 *M* to 50 μl of 1.25 *M* calcium chloride was required and had no effect on the recovery of **1**. The replacement of the 1 *M* hydrochloric acid with 0.2 *M* acetic acid in the reconstitution solution was necessary for the full retention of **1** on the cartridge. Apparently, the high concentration of the chlorine ions from HCl, readily attaching to the DEA, prevented **1** to be fully retained on the cartridge. Several other changes including the modification of the mobile phase composition from methanol (40%) to acetonitrile (15%), adjustment of the pH of the mobile phase from 8.5 to 6.3, and gradient elution of late-eluting interference peaks, were all needed for achieving the required assay specificity and provided for uninterrupted multi-sample analyses of urine or plasma extracts. The retention times and resolution of **3** from plasma and urine impurities were very sensitive to the mobile phase pH, allowing the control of assay specificity at the expense of sensitivity. The peak area of **3** at pH 6.3 was only about 20% smaller than at pH 8.5, allowing quantification in the low ng/ml levels.

Since the pH of the mobile phase used in the assay was 6.3, several attempts were made to replace the polymeric analytical columns with more efficient silica-based C_{18}, CN, and C_8 columns. A very nice separation of **3** from interfering peaks was observed. However, on repeated injections, the performance of these columns quickly deteriorated, due to on-column injections of high volumes (50–100 μl) of highly basic solution (pH 10.7) directly after derivatization. The retention time of **3** was decreasing on the silica columns probably due to base hydrolysis and removal of aliphatic groups attached to the column silica material. Therefore, in order to preserve the day-to-day performance of the chromatographic system, the polymeric columns were utilized in the assay. On the other hand, the decrease in the volume of sample injected from 100 to 50 μl was required to preserve the adequate chromatographic peak shape of **3**. The latter compound exhibited front peak tailing when highly basic (pH 10.7) solution after derivatization was injected to the mobile phase of much lower pH (6.3). In addition, a McPherson fluorescence detector with xenon–mercury excitation lamp and high-sensitivity attachment was utilized, allowing high-intensity excitation in the region of maximum of absorption of **3** (438 nm), which overlapped with the emission of the mercury line (436 nm) from the source.

*Assay of **1** in plasma*

The crux of the assay in plasma (method C) was the addition of a phosphate salt (sodium pyrophosphate) to the mixture after protein precipitation, to induce the co-precipitation of calcium salts of **1** with other phosphates present in the system. In the presence of pyrophosphate salt, the formation of a characteristic pellet containing **1** was observed. The remaining steps in the assay were the same as in method B. The LOQ in plasma (5 ng/ml, Table III) was higher than in urine (1 ng/ml, Table II) since only 1 ml of plasma *versus* 5 ml of urine was processed. The sensitivity of the assay was sufficient to monitor the concentration of **1** in human plasma up to 12 h after intravenous dosing of human subjects with 10 mg of **1** (Table V). Based on the poor (< 1%) oral bioavailability of **1**, the assay sensitivity in plasma will probably be not sufficient to determine concentrations of alendronate in plasma after 10 mg oral administration of **1**.

Electrochemical detection of 2 and 3

Similar to other NDA-derivatized analogues [8–10], both derivatives **2** and **3** were found to be ED-active with a low half-wave oxidation potential of +0.6 to +0.7 V. This low half-wave potential makes these derivatives amenable to highly specific ED. The feasibility of development of an assay in urine using derivative **2** has been demonstrated (method A). The precision and accuracy of the method (Table I) were adequate in the concentration range 2.5–50.0 ng/ml, when the unmodified extraction methodology [1] was utilized. Since the long-term, day-to-day performance of the fluorescence detectors is usually more reliable than that of the electrochemical detectors, and comparable assay sensitivities were achieved using both detectors, in our long-term studies involving large number of clinical samples, a method based on FD was preferable. However, using a modified sample preparation procedure (method B) and ED of either **2** or **3**, the development of an assay in urine and plasma with similar LOQ as with FD seems to be feasible, but it was not explored here further.

CONCLUSION

A highly sensitive assay for the determination of **1** in human urine (1 ng/ml) and plasma (5 ng/ml) was developed. The cyanide ion in the NDA derivatization reaction was successfully replaced with a thiol nucleophile (NAP). The highly fluorescent derivative **3** was quantified by HPLC–FD. The assays were used routinely for the analyses of more than 3000 urine and plasma samples from various human pharmacokinetic studies with **1**.

Both derivatives **2** and **3** were found to be ED-active, and an assay in urine based on HPLC–ED of **2** has been also developed and fully validated (LOQ = 2.5 ng/ml).

REFERENCES

1 W.F. Kline, B.K. Matuszewski and W.F. Bayne, *J. Chromatogr.*, 534 (1990) 139.
2 S. Bisaz, R. Felix and H. Fleisch, *Clin. Chim. Acta*, 65 (1975) 299.
3 T. L. Chester, E. C. Lewis, J. J. Benedict, R. J. Sunberg and W. C. Tettenhorst, *J. Chromatogr.*, 225 (1981) 17.
4 G. Flesch and S. A. Hauffe, *J. Chromatogr.*, 489 (1989) 446.
5 P. T. Daley-Yates, L. A. Gillford and C. R. Hoggarth, *J. Chromatogr.*, 490 (1989) 329.
6 J. P. Fels, J. Guyonnet, Y. Berger and W. Cautreels, *J. Chromatogr.*, 430 (1988) 73.
7 G. Flesch, N. Tominaga and P. Degen, *J. Chromatogr.*, 568 (1991) 261.
8 M.D. Oates and J.W. Jorgenson, *Anal. Chem.*, 61 (1989) 432.
9 S.M. Lunte, T. Mohabbat, O.S. Wong and T. Kuwana, *Anal. Biochem.*, 178 (1989) 202.
10 M.D. Oates, B.D. Cooper and J.W. Jorgenson, *Anal. Chem.*, 62 (1990) 1573.
11 R.G. Carlson, K. Srinivasachar, R.S. Givens and B.K. Matuszewski, *J. Org. Chem.*, 51 (1986) 3978.
12 B.K. Matuszewski, R.S. Givens, K. Srinivasachar, R.G. Carlson and T. Higuchi, *Anal. Chem.*, 59 (1987) 1102
13 P. De Montigny, J. F. Stobaugh, R. S. Givens, R. G. Carlson K. Srinivasachar, L. A. Sternson and T. Higuchi, *Anal. Chem.*, 59 (1987) 1096.
14 M.C. Roach and M.D. Harmony, *Anal. Chem.*, 59 (1987) 411.
15 S.M. Lunte and O.S. Wong. *LC · GC Mag.*, 12 (1989) 743.
16 H. Koning, H. Wolf, K. Venema and J. Korf, *J. Chromatogr.*, 533 (1990) 171.
17 W.F. Kline, B.K. Matuszewski and J. Y.-K. Hsieh, *J. Chromatogr.*, 578 (1992) 31.