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ASSAY OF 1-HYDROXY-3-AMINOPROPYLIDENE-1,1-BISPHOSPHONATE AND RELATED BISPHOSPHONATES IN HUMAN URINE AND PLASMA BY HIGH-PERFORMANCE ION CHROMATOGRAPHY

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SUMMARY

A method is described for the analysis of 1-hydroxy-3-aminopropylidene-1,1-bisphosphonate and related bisphosphonates in human urine and plasma. Samples are spiked with 1-hydroxy-5-aminopentylidene-1,1-bisphosphonate as an internal standard and calcium chloride is added to precipitate the bisphosphonates. Following centrifugation the precipitate is redissolved in acetic acid, and the bisphosphonates are separated by high-performance ion chromatography on a Dionex AS7 column using nitric acid as mobile phase. The bisphosphonates are oxidised to orthophosphate using post-column addition of ammonium persulphate and this is followed by post-column reaction with molybdenum–ascorbate to yield the phosphomolybdate chromophore which is detected at 820 nm. A detection limit of 10 ng/ml is possible.

INTRODUCTION

Solutions to the problem of analysing polyphosphates and compounds with P–O–P and P–C–P bonds have been published by several groups [1–4]; however, these methods were not designed for the measurement of low concentrations in plasma and urine. Recently the interest in analysing these compounds in biological fluids has increased as a result of an expansion in their use as drugs particularly for Paget's disease and malignant hypercalcaemia [5,6].

To date most of the methods employed in the analysis of bisphosphonates have relied on the oxidative cleavage of the P–C bond and the colorimetric detection of the orthophosphate produced by conversion to the blue phos-

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phomolybdate complex. An exception to this is the use of ferric nitrate to form a complex directly with the phosphonate that absorbs in the ultraviolet spectrum; this method does not permit the determination of low concentrations of analyte due to the low extinction coefficient of the complex.

Hirai et al. [2] have described a complex method for the determination of phosphonate and phosphinates which employes sulphite as the oxidant for the cleavage of the P–O bonds. Sulphite is unsuitable for the oxidation of the less labile P–C bonds of the bisphosphonates; other workers [3] have used persulphate for this purpose. The use of persulphate prevents the method of Hirai et al. [2] from being adapted for the bisphosphonates since the heating of the sample simultaneously with persulphate and the molybdenum reagent leads to the oxidation of the molybdenum reagent. Waldhoff and Sladek [3] have described a method of analysis applicable to compounds such as 1-hydroxy-3-aminopropylidene-1,1-bisphosphonate (APD). They employed a three-stage post-column reaction which involved heating the sample in turn with persulphate, ascorbate and a molybdenum reagent. The ascorbate was added to reduce any unreacted persulphate and prevent oxidation of the molybdenum reagent. In our hands the molybdenum reagent employed by Waldhoff and Sladek [3] produced a less well defined end point than the reagent employed by Hirai et al. [2]. In addition the detection limit of 1 μg/ml claimed by Waldhoff and Sladek [3] was well above our expected requirements. This was in part due to the low-efficiency ion-exchange column chromatography they used. The method of Hirai et al. [2] employed a more efficient high-performance liquid chromatographic (HPLC) method; however, experience gained in the analysis of polyphosphates has demonstrated that metal ions leached from stainless-steel HPLC systems and at the concentrations present in some reagents can cause problems in the analysis of low concentrations of these compounds due to their ability to strongly chelate iron. More recently Vaeth et al. [4] have published a method for the measurement of polyphosphates and phosphonates in samples such as washing powders. This method employs molybdenum–vanadium reagent and ion chromatography.

This work was undertaken to develop a method which allows the analysis of sub μg/ml concentrations of APD and related bisphosphonates in urine and plasma. The analyte and the internal standard are precipitated together as their calcium salts. High-performance, metal-free, anion chromatography (HPIC) is used to separate the sample components. This is coupled with a simplified two-step post-column reaction which involves heating with persulphate to liberate orthophosphate followed by the simultaneous addition of ascorbic acid to neutralise excess persulphate and a molybdenum reagent to generate the phosphomolybdate complex.

We describe the application of the method to the measurement of APD in samples obtained from patients receiving the drug for Paget's disease.

EXPERIMENTAL

Chemicals and reagents

The following chemicals were obtained from BDH (Poole, U.K.): nitric acid (Aristar grade), ammonium molybdate tetrahydrate, sulphuric acid, hydrochloric acid, granular zinc, calcium chloride dihydrate, glacial acetic acid and disodium hydrogen orthophosphate anhydrous (Analar grade). Ammonium persulphate and ascorbic acid were obtained from Sigma (Poole, U.K.) The bisphosphonates were donated by Ciba-Geigy (Basle, Switzerland).

Doubly glass-distilled water was used to prepare all reagents. For the chromatography 1 m*M* nitric acid was used as eluent. Persulphate reagent comprised 5 m*M* ammonium persulphate for the on-line detection method and 50 m*M* ammonium persulphate for the manual method. Molybdenum–ascorbate reagent was a modification of the Mo^{5+}/Mo^{6+} reagent described by Hirai et al. [1] prepared as follows. A 35-g amount of ammonium molybdate tetrahydrate was dissolved in 400 ml of 6 *M* hydrochloric acid. Thereafter the solution was cooled in ice. Granular zinc (3 g) was added. Following complete dissolution of the zinc 200 ml of concentrated hydrochloric acid was slowly added followed by 400 ml of concentrated sulphuric acid yielding an emerald green solution. This solution was diluted 1:10 with 5 m*M* ascorbic acid prior to use. This reagent was stable for at least one week at room temperature.

Manual method for the measurement of bisphosphonates

For aqueous samples containing bisphosphonate and no orthophosphate the following method was used. To 0.1 ml of sample 0.1 ml of 50 m*M* ammonium persulphate was added and the sample heated for 5 min in a boiling water bath. A 1-ml volume of the molybdenum–ascorbate reagent, prepared as described above, was then added and the sample heated for a further 10 min in a boiling water bath. After cooling to 20°C the absorption at 820 nm was measured in a 1.5-ml plastic cuvette using an LKB ultraspec spectrophotometer.

Analytical apparatus and procedures

A diagram of the system is shown in Fig. 1. A Dionex 4000i inert HPIC pump was used to pump 1 m*M* nitric acid at 1 ml/min first through a Dionex MFC-1 column to remove trace metal ions from the eluent and then through a Dionex AG7 guard column and AS7 separator column. An inert Dionex valve was used to inject 50 μl of sample into the system; the sample did not pass through the MFC-1 column. Immediately post-column a PTFE "T" junction was used to introduce a stream of 5 m*M* ammonium persulphate pumped at 1.0 ml/min by a Dionex DQP-1 pump. After mixing with the persulphate the flow passed through a coil of PTFE tubing (5 m$\times$0.3 mm I.D.) immersed in silicon oil heated to 115°C in a Haake D8 thermostatically controlled oil bath. On emerging from the heating coil a further PTFE "T" junction was used to introduce

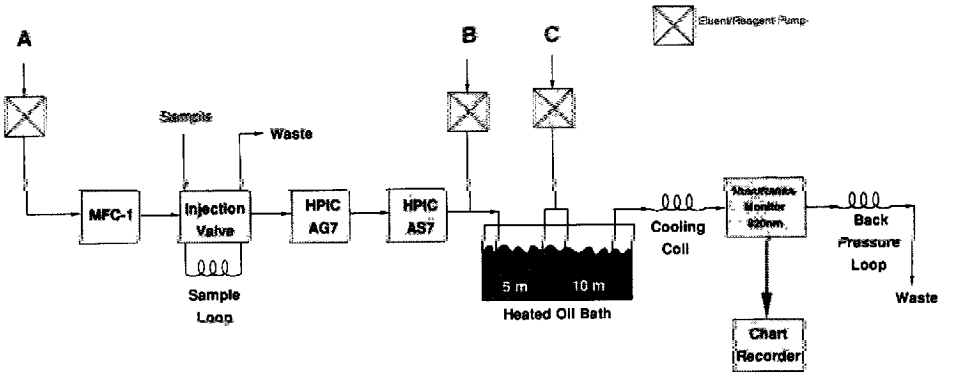


Fig. 1. Schematic diagram of the analytical apparatus. MFC-1, HPIC AG7 and HPIC AS7 are the Dionex metal-free, guard and separator columns, respectively. (A) 1 m*M* nitric acid; (B) 5 m*M* ammonium persulphate; (C) molybdenum–ascorbate reagent.

the molybdenum–ascorbate reagent at 1.0 ml/min via a Dionex DQP-1 pump. After mixing with the molybdenum–ascorbate reagent the flow passed through a coil of PTFE tubing (10 m × 0.3 mm I.D.) immersed in the silicon oil bath at 115 °C. A Spectraflow 757 spectrophotometer fitted with a PTFE flow cell was used to monitor the stream at 820 nm. The detector output was recorded on a flat bed chart recorder (Servoscribe RE 511) with a chart speed of 5 min/cm.

Sample treatment

1-Hydroxy-5-aminopentylidene-1,1-bisphosphonate (APDP) was used as an internal standard. To each 1.0 ml of urine 100 μl of a 50 μg/ml solution of APDP dissolved in 200 m*M* disodium hydrogenphosphate were added. After mixing 50 μl of 50 m*M* calcium chloride were added and following further mixing, the calcium salts were observed to precipitate. The sample was then centrifuged for 10 min at 2000 *g* and 20 °C. The supernatant was discarded and the precipitate redissolved in 1 ml of 100 m*M* acetic acid. Prior to injection into the HPIC system the sample was recentrifuged for 5 min at 2000 *g* and 20 °C to ensure the removal of any insoluble particulate material which may be present. Plasma samples were treated identically except that the concentration of internal standard was reduced by one tenth. Prior to injection into the chromatograph the urine samples were diluted 1:10 with distilled water. Urine and plasma samples were stored at −20 °C prior to analysis. Plasma was obtained from blood collected into lithium heparin tubes.

RESULTS AND DISCUSSION

Manual method

Initially the conditions for the formation of the molybdenum blue complex from orthophosphate were optimised. Using 0.1 ml of sample and 1 ml of mo-

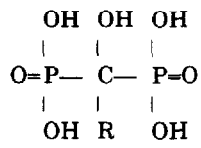
lybdenum–ascorbate reagent it was determined that heating for 5 min in a boiling water bath was necessary for the maximal development of the colour. The response was linear up to at least 500 μg/ml.

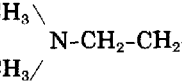
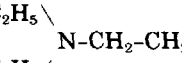
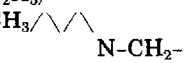
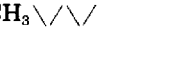
This method was modified to determine the optimum conditions for the analysis of bisphosphonates. Prior to reaction with the molybdenum–ascorbate reagent, 0.1 ml (250 μg/ml) of sample and 0.1 ml of 50 m*M* ammonium persulphate were heated for various times (0–60 min) in a boiling water bath. Ammonium persulphate oxidises the P–C bonds in the bisphosphonates quantitatively releasing two orthophosphate groups. A conversion of the bisphosphonates of 100% was achieved after heating for 5 min. Linear calibration plots for the analysis of bisphosphonates shown in Table I were obtained over the concentration range 0–500 μg/ml.

For subsequent assays a heating time of 5 min and a persulphate concentration of 50 m*M* were chosen followed by heating for 10 min with the molybdenum–ascorbate reagent.

TABLE I

STRUCTURE AND RETENTION TIMES OF SOME BISPHOSPHONATES AND ORTHOPHOSPHATE



R	Abbreviation	Retention time (min)
NH_2-CH_2-CH_2-	APD	8.5
NH_2-CH_2-CH_2-CH_2-CH_2-	APDP[a]	7.1
NH_2-CH_2-CH_2-CH_2-CH_2-CH_2-	AHDP	6.8
CH_3-	EHDP	10.0
	–	8.0
	–	8.7
	–	11.0
	–	50.0
–	PO_4^{3-}	12.5

[a]Internal standard.

Optimisation of ion chromatography and post-column reaction

Fig. 2 shows the separation of APD, APDP and orthophosphate using a Dionex AG7 guard column and a AS7 separator column. These columns are designed for the separation of polyvalent anions. An eluent of 1 m*M* nitric acid with a flow-rate of 1 ml/min was used. The low concentration of nitric acid was necessary to facilitate the separation of APD from the internal standard APDP. This separation is compromised by the use of samples of high ionic strength and high orthophosphate concentration such as untreated hypertonic urine samples. The presence of trace metals such as iron in the eluent leads very rapidly to loss of column performance. A badly contaminated column will retard bisphosphonates resulting in very severe tailing of the peaks. This is caused by binding to metals which have been retained by underivatised cationic sites on the anion-exchange resin. The performance can be recovered completely by washing the columns with 1 *M* nitric acid.

The post-column reaction was optimised initially for an aqueous solution containing orthophosphate (Na_2HPO_4, 5 μg/ml) and APD (5 μg/ml). Using an oil bath temperature of 140°C and heating coils of 10 and 20 m in length the total flow-rate was altered keeping the relative flow-rates of the persulphate and molybdenum–ascorbate the same. Low flow-rates resulted in a marked peak broadening and insufficient back-pressure to prevent boiling of the stream in the heating coil. A total flow-rate of 3 ml/min, comprising 1 ml/min of the eluent, 1 ml/min of the persulphate and 1 ml/min of the molybdenum–ascorbate reagent, produced the largest peak heights without excessive back-pressure. Next the temperature of the oil bath was reduced in 5°C steps: above 110°C the peak heights were unaffected, below 85°C no response was obtained. A temperature of 115°C was chosen for further analysis. Finally the length of the heating coils was reduced to 5 and 10 m, respectively. This re-

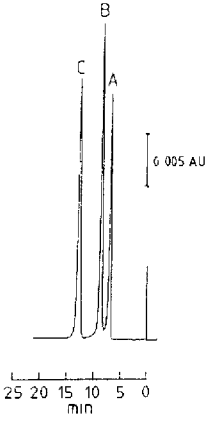


Fig. 2. Chromatogram of bisphosphonates and orthophosphate in water. Peaks: A = internal standard, APDP, 5 μg/ml; B = APD, 5 μg/ml; C = Na_2HPO_4, 5 μg/ml.

sulted in approximately 20% increase in peak height by reducing the band broadening. The bisphosphonates shown in Table I were also analysed by this method. Their retention times are shown in this table. For some of these compounds it may be necessary to alter the molarity of the eluent in order to further optimise the method.

Sample treatment

The chromatographic separation was found to be readily interfered with when samples of high ionic strength were assayed, the result being a dramatic reduction in peak height and very broad peaks for the bisphosphonates. One solution to this problem is sample dilution. For urine samples dilution of 1:50 was necessary. Two alternatives to this were investigated. Initially we employed Bond Elut SAX anion-exchange columns which bind APD, APDP and orthophosphate. However, the high concentration of orthophosphate and other anions in some urine samples result in poor recovery of the bisphosphonates due to the low capacity of the Bond Elut column. An additional problem with this method was the need to use 30 m*M* nitric acid to elute the bisphosphonates from the SAX column. Samples in 30 m*M* nitric acid required dilution 1:10 prior to chromatographic separation to prevent peak broadening.

The second method investigated, precipitation with calcium, proved very effective. Bisphosphonates have a high affinity for calcium ions and form insoluble calcium salts. The orthophosphate present in urine and plasma also precipitates on addition of calcium. This precipitation process was found to be more reproducible when both calcium chloride and disodium hydrogenphosphate were added to the sample, probably because the bisphosphonates coprecipitate with calcium phosphate. Initially EDTA was used to redissolve these calcium salts. However, EDTA at the concentrations required compromised the chromatography. Therefore, acetic acid was used to redissolve the calcium salts.

Linearity and reproducibility

All results are expressed as peak-height ratios of APD and the internal standard (APDP). The calibration plots are described by the following equations fitted by a least-squares method: for urine (range 0.3–20 μg/ml, internal standard 5 μg/ml) $y=0.227x-0.03$, $r=0.999$; for plasma (range 0.05–2.0 μg/ml, internal standard 0.5 μg/ml) $y=2.02x-0.03$, $r=0.995$. Table II shows the reproducibility of the method for various samples. The coefficient of variation for samples of high, medium and low concentration and for samples assayed on different days is also shown. The limit of quantitation of the assay using a 50-μl injection loop was approximately 10 ng/ml or 0.5 ng. This corresponds to a signal-to-noise ratio of 2.0.

TABLE II

REPRODUCIBILITY OF THE SAMPLE PRETREATMENT PROCEDURE AND HPIC ASSAY METHOD

Sample	*n*	APD found (mean ± S.D.) (μg/ml)	Coefficient of variation (%)
Replicate HPIC assays of a 10 μg/ml APD sample in water	7	10.1 ± 0.037	0.37
Replicate complete assays of a 10 μg/ml APD sample in water	7	10.3 ± 0.34	3.4
As above repeated on five consecutive days	5	10.5 ± 0.67	6.4
Replicate complete assays of a spiked urine sample containing 10 μg/ml APD	7	9.9 ± 0.32	3.3
Replicate complete assays of a patient urine sample	6	20.6 ± 0.68	3.3
Spiked urine sample, 15 μg/ml	6	15.65 ± 0.31	2.0
Spiked urine sample, 4.5 μg/ml	6	4.57 ± 0.13	2.8
Spiked urine sample, 0.45 μg/ml	6	0.42 ± 0.01	2.4

Patient samples

Local Ethical Committee approval was obtained prior to commencement of the study. Patients received 30–120 mg of APD (disodium salt, pentahydrate, Ciba-Geigy) dissolved in 250 ml of normal saline as an intravenous infusion over 2–8 h delivered via a constant-rate infusion pump. Patients were asked to empty their bladder prior to the start of the infusion. Fig. 3 shows a chromatogram of a blank urine sample (collected prior to administration of APD) analysed without addition of the internal standard and a urine sample from a patient collected 1 h post infusion. Fig. 4 shows a chromatogram of a blank plasma sample (collected immediately prior to drug administration) and a plasma sample collected 5 min post infusion of APD. Data from a patient showing typical plasma concentration–time and urine excretion rate–time profiles are shown in Fig. 5.

CONCLUSIONS

The method described is a selective and sensitive HPIC assay for bisphosphonates in biological fluid. The only identifiable endogenous substance that is detected by the method is orthophosphate. Currently the method has been

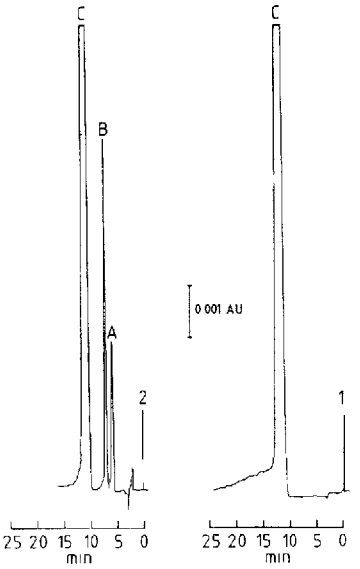


Fig. 3. Typical chromatograms of extracted urine samples. (1) Blank sample not containing internal standard; (2) urine sample collected from a patient containing 11.8 μg/ml APD (B), 5 μg/ml APDP (A) and PO_4^{3-} (C).

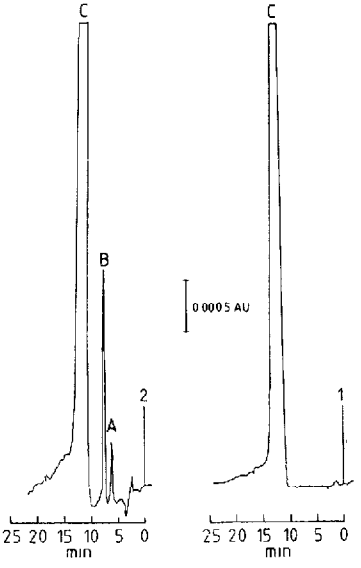


Fig. 4. Typical chromatogram of extracted plasma samples. (1) Blank sample not containing internal standard; (2) plasma sample collected from a patient containing 1.9 μg/ml APD (B), 0.5 μg/ml APDP (A) and PO_4^{3-} (C).

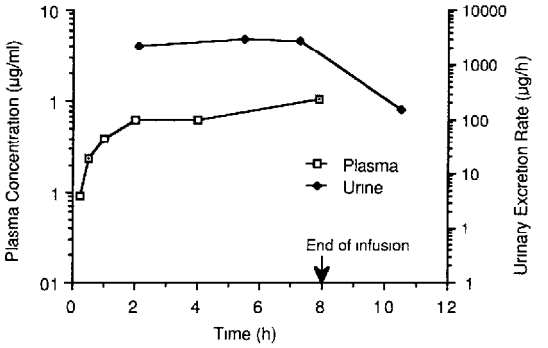


Fig 5. Plasma and urine excretion rate-time profiles for a patient receiving an 8-h infusion of APD.

applied to a study of the pharmacokinetics of APD in patients receiving the drug for Paget's disease. The manual method should be applicable to quality control assays of aqueous samples not containing orthophosphate.

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