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Determination of risedronate in human urine by column-switching ion-pair high-performance liquid chromatography with ultraviolet detection

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Abstract

An HPLC assay for the determination of risedronate in human urine was developed and validated. Risedronate and the internal standard were isolated from 5-ml urine samples in a two-part procedure. First, the analytes were precipitated from urine along with endogenous phosphates as calcium salts by the addition of $CaCl_2$ at alkaline pH. The precipitate was then dissolved in 0.05 *M* ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid and subjected to ion-pair solid-phase extraction using a Waters HLB cartridge (1 ml, 30 mg) with 1-octyltriethylammonium phosphate as the ion-pair reagent. Following extraction, the analytes were initially separated from the majority of co-extracted endogenous components on a Waters X-Terra RP18 (4.6×50 mm, 3.5 μm) column. The effluent from the X-Terra was ''heart-cut'' onto a Phenomenex Synergi Polar RP (4.6×150 mm, 4 μm) column for final separation. UV detection (λ=262 nm) was used to quantitate risedronate in the concentration range of 7.5–250 ng/ml. Mean recovery was 83.3% for risedronate and 86.5% for the internal standard. The intra-day precision of the assay, as assessed by replicate (n=5) standard curves, was better than 6% RSD for all points on the standard curve. Within-day accuracy for the standards ranged from 96.3 to 106.1% of nominal. Inter-day precision for quality controls assayed over a 3-week period was better than 5%, while inter-day accuracy was within 90% of nominal. The assay was employed to analyze samples collected during a clinical pharmacokinetics study.

Keywords: Risedronate

1. Introduction

Risedronate (1-hydroxy-2-(3-pyridinyl) ethylidine bisphosphonic acid monosodium salt) (Fig. 1) is a member of the bisphosphonate class of drugs. Bisphosphonates are potent inhibitors of osteoclast resorption and are widely used in the treatment of bone disorders such as osteoporosis and Paget's disease. A method to quantitate risedronate in urine was required to support human pharmacokinetics studies.

The development of assays for the quantitation of bisphosphonates in biological fluids presents a formidable challenge to the analyst. The difficulties associated with bioanalysis of bisphosphonates have been well documented [1]. Bisphosphonates are characterized by low bioavailability; typically 1% or less of the dose is absorbed. Hence, at therapeutic

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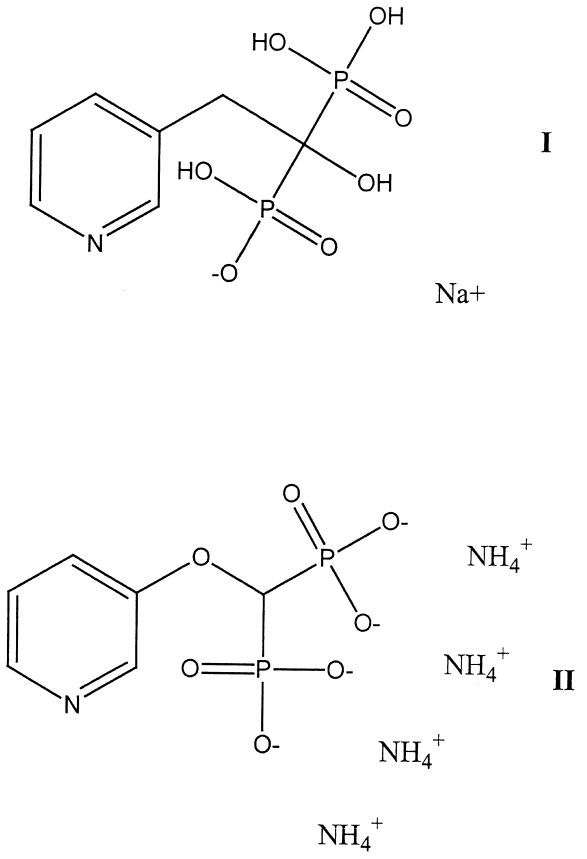

Fig. 1. Chemical structures of risedronate (**I**) and the internal standard (**II**).

doses, only low ng/ml levels are present in urine. Thus, assays with limits of quantitation below 10 ng/ml are required to support pharmacokinetic studies. In addition, these compounds are known to be extremely difficult to chromatograph. Bisphosphonates are strong chelators; they readily interact with metals in HPLC systems (e.g. in injection valves or HPLC columns), giving rise to poor peak shape and irreproducible chromatography. Furthermore, bisphosphonates are not typically amenable to analysis by HPLC with tandem mass spectrometric detection (HPLC–MS–MS). Experience has shown that bisphosphonates produce a distribution of multiply charged ions and are prone to adduct formation under electrospray ionization (ESI) conditions; these factors limit, in turn, assay sensitivity. The use of HPLC–MS–MS is further complicated by the fact that bisphosphonates are usually chromatographed under reversed-phase conditions with mobile phases containing ion-pair reagents. Most of the commonly used ion-pair reagents are nonvolatile and thus are incompatible with MS detection.

HPLC assays for bisphosphonates in biological fluids using detection based on the native UV absorbance [2], fluorescence [3], and electrochemical [4] properties of the analytes have been reported in the literature. Assays for bisphosphonates in biological matrices employing precolumn chemical derivatization [5,6], postcolumn phosphomolybdate complex formation [7], and indirect fluorescence detection schemes [8] have also been reported.

Risedronate has been determined in human urine using GC–MS following acylation and silylation to form a volatile derivative [9]. The limit of quantitation (LOQ) of this assay was reported to be 11 ng/ml. More recently, a highly sensitive assay (LOQ=0.15 ng/ml) for risedronate in human urine based on enzyme linked immunosorbent assay (ELISA) has been reported [10].

Although sensitive, the primary disadvantage of the ELISA approach is the complexity of method development. Initially, the analyte or a suitable analog must be linked to a protein in order to elicit an immune response in an animal. Antibodies must then be raised over a period of time ranging from weeks to months. These steps can be exceedingly complex and represent a significant investment of time and resources. As a rapid turnaround time was required for the present study, implementation of an ELISA assay was not feasible. In addition, derivatization of risedronate by acylation was explored and found not to be sufficiently reproducible for routine sample analysis.

To date, no HPLC-based assays for the determination of risedronate in biological fluids have appeared in the literature. In that risedronate lacks readily derivatizable functional groups and is not amenable to mass spectrometric detection, an HPLC–UV assay for the determination of risedronate in human urine based on the compound's native UV absorbance was developed and is described in this publication.

2. Experimental

2.1. Materials and reagents

Compounds **I** and **II** (Fig. 1) were obtained from the Chemical Data Department of Merck Research Laboratories (Rahway, NJ, USA) as the monosodium (**I**) and tetraammonium (**II**) salts. Compound **II** was used as an internal standard. Acetonitrile and methanol (Omni-Solv, HPLC grade) were purchased from EM Science. Etidronate (1-hydroxyethylidene-1,1-diphosphonic acid) was obtained from Strem Chemicals (Newburyport, MA, USA). Ion-pair reagent, 1-octyltriethylammonium phosphate (Q8), was obtained as a 0.5 *M* solution from Regis Technologies (Morton Grove, IL, USA). Ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) was obtained from Sigma–Aldrich (St. Louis, MO, USA). All other reagents were of ACS grade and were used as received. Drug-free control human urine was obtained in-house from volunteer donors. Deionized (18 mΩ/cm) water was generated in-house using a Millipore (Bedford, MA, USA) Milli-Q Plus system.

2.2. Instrumentation

The HPLC system consisted of a Varian ProStar 430 (Walnut Creek, CA, USA) or Agilent Technologies (Palo Alto, CA, USA) 1100 autosampler, Perkin-Elmer Series 200, or Agilent Technologies 1100 pumps, an Agilent Technologies 1100 diode array detection system or an Applied Biosystems (Foster City, CA, USA) 785A UV detector. A Valco (Houston, TX, USA) or Rheodyne (Rohnert Park, CA, USA) electrically actuated 10-port switching valve was employed.

2.3. Chromatographic conditions

The HPLC system is depicted schematically in Fig. 2. Column 1 (50×4.6 mm) contained Waters (Milford, MA, USA) X-Terra RP 18 (3.5 μm) packing. Mobile phase 1 consisted of 0.005 *M* Q8 ion pair reagent in 10 m*M* sodium phosphate, 1 m*M* etidronate–acetonitrile (92:8, v/v) pH_{app}=6.25, and was pumped at a flow-rate of 1.0 ml/min. Column 2 (4.6×150 mm) was packed with Phenomenex (Torrance, CA, USA) Synergi Polar RP (4 μm) material. Mobile phase 2 consisted of 0.005 *M* Q8 in 11 m*M* sodium phosphate, 1.1 m*M* etidronate–acetonitrile (87:13, v/v) pH_{app}=6.25, and was also pumped at a flow-rate of 1.0 ml/min The temperature of both columns was maintained at 30 °C. The injection volume was 100 μl and the detection wavelength was 262 nm.

The following valve switching program was used. Samples were injected onto column 1 while the switching valve was in position A. Prior to the elution of risedronate from column 1 (approximately 3.5 min after injection), the valve was switched to position B with the effluent from column 1 being directed into column 2. After the elution of the internal standard from column 1 (approximately 6.3 min after injection) the switching valve was returned to position A for the remainder of the run. Valve switching times were checked on a daily basis prior to analytical runs.

2.4. Preparation of standards

A 1 mg/ml stock solution of **I** was prepared by dissolving 10.8 mg of the monosodium salt of **I** in 10 ml deionized water. The solution was sonicated for several minutes to ensure complete dissolution of the solid. Fresh stock solutions were prepared monthly. A 100-μg/ml solution was prepared by diluting 2.5 ml of the stock solution to 25 ml with deionized water. Appropriate dilutions of the 100-μg/ml solution were performed to yield working standards at the following concentrations: 25, 15, 10, 5, 2.5, 1 and 0.75 μg/ml. All solutions were stored under ambient laboratory conditions.

Urine standards were prepared by spiking 50 μl of each working standard into 5 ml of human control urine. These standards were used to construct calibration curves for the quantitation of **I** in urine at concentrations ranging from 7.5 to 250 ng/ml. Samples found to contain **I** at concentrations above 250 ng/ml were diluted appropriately with control urine and reassayed. Dilution of the urine samples was found not to compromise assay accuracy.

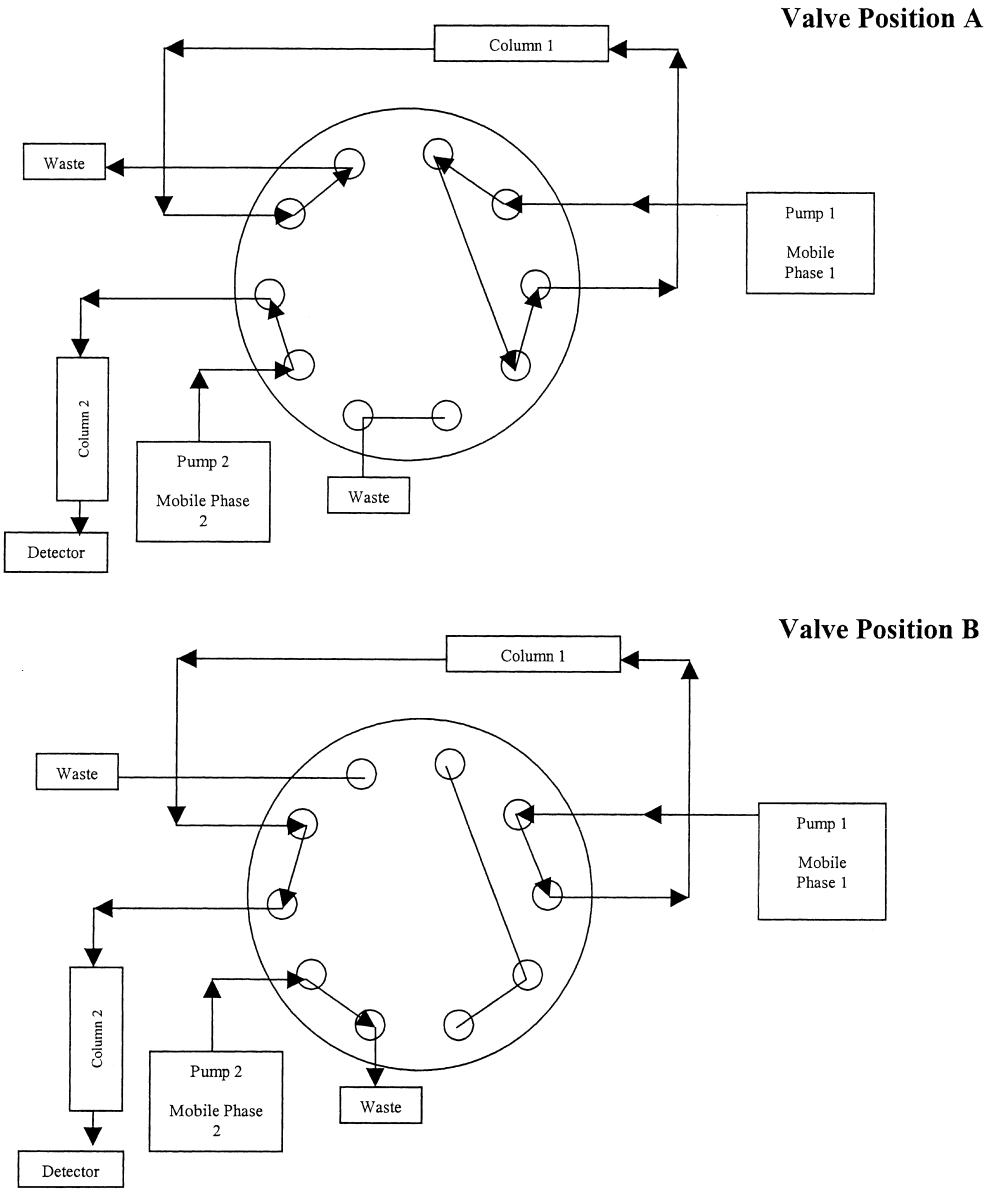

Fig. 2. Schematic diagram of the HPLC system. Arrows denote direction of the mobile phase flow.

2.5. Sample preparation procedure

2.5.1. Calcium phosphate co-precipitation

Urine samples were thawed and allowed to equilibrate to room temperature. After vortexing and centrifugation (2560 *g* for 5 min) of the sample tubes, 5-ml aliquots of urine were transferred into 16×125 mm disposable glass culture tubes. A 25-μl aliquot of internal standard (125 μg/ml **II** in water) was added to each tube. The initial step in the isolation of the analytes involved co-precipitation of the calcium salts of **I** and **II** with endogenous phosphates at alkaline pH. An adaptation of the method described by Bisaz et al. [11] was employed.

Briefly, a 50-μl aliquot of 1.25 *M* $CaCl_2$, followed by 65 μl of 30% (v/v) NaOH was added to induce the formation of a white precipitate. If, after thorough mixing of the sample with a vortex mixer, no precipitate was observed, additional 10-μl aliquots of 30% (v/v) NaOH were added until a visible precipitate was formed. Samples were then centrifuged for 10 min at 5020 *g*. The supernatant was aspirated to waste and the precipitate dissolved in 50 μl of 1 *M* HCl. Additional 25-μl aliquots of 1 *M* HCl were added, as necessary, to completely dissolve the solid. After dissolution of the precipitate, the sample was diluted with 5 ml deionized H_2O. A second precipitate was formed by the addition of 50 μl NaOH, followed by centrifugation and aspiration of the supernatant to waste. These steps were repeated to yield a third precipitate.

The precipitate pellet obtained after the third precipitation step was dissolved in 0.5 ml of 0.05 *M* EGTA. To ensure complete dissolution of the precipitate, the samples were placed in an sonicator for 5 min and thoroughly vortex mixed. After the addition of 4.5 ml deionized water to the sample tubes, a 100-μl aliquot of the IP reagent stock solution (0.5 *M* Q8) was added and the samples vortex mixed for 10 s.

2.5.2. Ion-pair solid phase extraction

Waters HLB extraction cartridges (30 mg, 1 ml) were conditioned with 2 ml MeOH followed by 1 ml deionized water. The cartridges were next conditioned with 1 ml 0.01 *M* Q8 reagent, which was drawn through the bed at a low flow-rate (approximately 0.3 –0.5 ml/min). Samples were then loaded onto the cartridges at the same flow-rate. After the loading step, the cartridges were washed with 1 ml deionized H_2O followed by 1 ml water–MeOH (95:5, v/v). Samples were eluted from the sorbent by drawing 1 ml MeOH though the cartridges by centrifugation (70 *g* for 5 min). The eluate was evaporated to dryness under a stream of N_2 in a TurboVap evaporator (Zymark, Hopkinton, MA, USA) with the temperature set to 50 °C. Samples were reconstituted in 0.5 ml 0.010 *M* sodium phosphate–0.001 *M* etidronate, pH 6.25, and transferred to polypropylene autosampler vials or a polypropylene 96-well plate for HPLC analysis.

2.6. Treatment of clinical samples

Previous experience with bisphosphonates in our laboratory has shown the need to acidify urine samples to avoid precipitation of the analyte during sample storage. Urine samples in this study were acidified at the clinical site immediately after collection by the addition of 25 μl of 6.0 *M* HCl per ml of urine collected. After acidification, the samples were stored at −20 °C. Urine QC samples were prepared in acidified urine and stored similarly.

3. Results and discussion

3.1. Assay selectivity

The selectivity of the assay for **I** was demonstrated by analyzing drug-free human control urine obtained from five different donors. No interferences were detected at the retention times of **I** or **II** in the five lots of control urine tested. Assay selectivity is illustrated in the representative chromatograms of a control urine double blank and a urine sample spiked with 10 ng/ml **I** and 625 ng/ml **II** (Fig. 3).

3.2. Sensitivity and linearity

The lower limit of quantitation (LLOQ) of the assay, defined as the lowest concentration on the standard curve that can be quantitated with precision not exceeding 15% RSD and accuracy within 15% of nominal, was 7.5 ng/ml.

Calibration curves were constructed by plotting the peak height ratio of **I** to internal standard versus nominal standard concentration. Weighted ($1/y$ where y = peak height ratio of **I** to **II**) linear least squares regression was employed. Linear calibration curves were obtained over the range of 7.5–250 ng/ml **I** in urine.

The use of weighted linear least squares regression minimized errors in the backcalculated standard concentrations. As shown in Table 1 for the analysis of replicate ($n=5$) standard curves, accuracy of the backcalculated standard concentrations ranged from 96.3 to 106.1% of nominal.

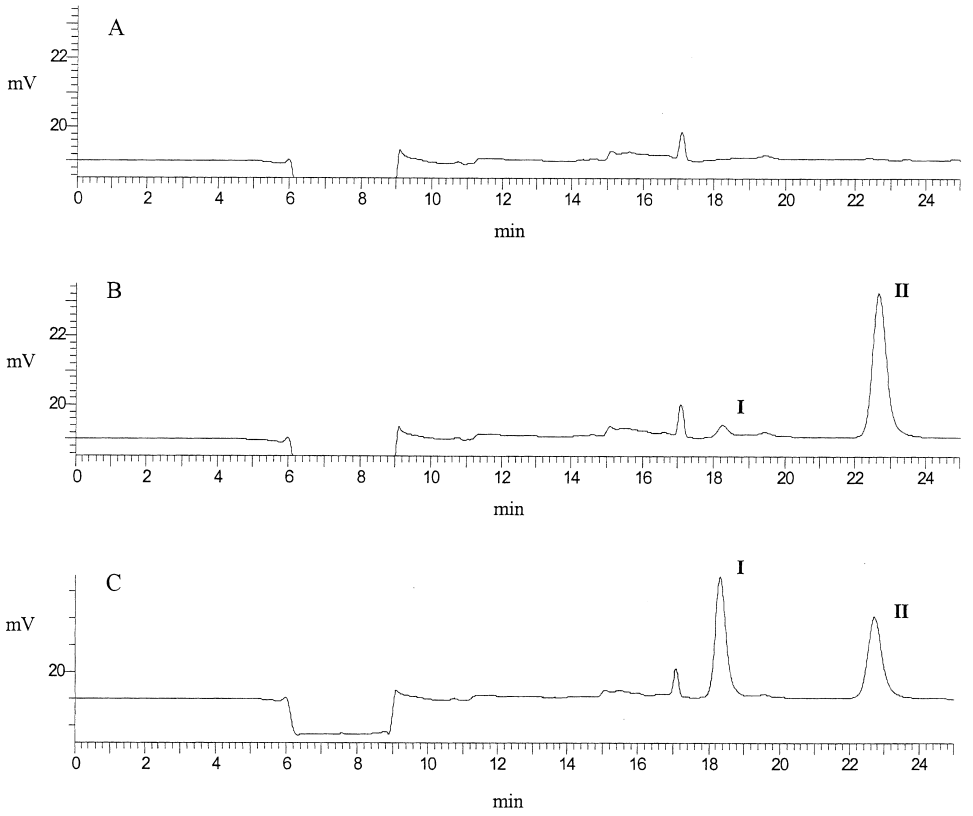

Fig. 3. Representative chromatograms from the assay of **I** in human urine. (A) Control urine double blank, (B) control urine spiked with 10 ng/ml **I** and 625 ng/ml **II**, and (C) 0–8 h postdose clinical sample. The assayed concentration of **I** in (C) was 191.3 ng/ml.

3.3. Assay accuracy and precision

An assessment of within-day assay accuracy and precision was conducted by preparing replicate sets ($n=5$) of standard curve samples in five different lots of human control urine obtained from five unique donors. Results of this analysis are illustrated in Table 1. The ruggedness of the assay and the absence of relative matrix effects on extraction and detection are further evidenced by the lack of variation of the slopes of the individual standard curves (Table 2).

Quality control (QC) samples were prepared at low (25 ng/ml), medium (75 ng/ml), and high (150

Table 1
Intra-day accuracy and precision of the assay

Nominal concentration (ng/ml)	Mean assayed concentration[a] (ng/ml)	Accuracy[b] (%)	RSD (%)
7.5	8.0	106.1	4.8
10	10.0	100.2	5.0
25	24.1	96.3	2.5
50	49.3	98.6	1.7
100	97.8	97.8	1.1
150	149.4	99.6	1.9
250	254.4	101.8	2.4

[a] $n=5$ in different lots of urine.
[b] Accuracy expressed as [(mean observed concentration)/(nominal concentration)]×100.

Table 2
Slopes of standard curves prepared in unique ($n=5$) lots of urine

Urine lot	Slope
A	0.00613
B	0.00611
C	0.00617
D	0.00626
E	0.00602
Mean	0.00614
RSD (%)	1.4

Table 3
Inter-day assay accuracy and precision as determined by the analysis of quality control samples

Analysis date	Assayed concentration (ng/ml)					
	25 ng/ml QC		75 ng/ml QC		150 ng/ml QC	
09 May	24.0	24.8	68.4	68.1	142.1	143.2
14 May	23.3	23.1	67.0	66.9	137.8	136.5
14 May	25.0	25.7	70.0	70.1	148.4	148.2
16 May	23.7	24.5	67.9	68.1	136.8	135.7
20 May	24.2	23.4	70.0	66.3	140.1	136.2
21 May	22.6	22.6	66.4	67.1	137.2	137.0
22 May	24.3	23.6	68.4	68.9	141.4	139.8
23 May	22.9	25.4	68.2	69.9	138.0	143.5
28 May	22.3	21.1	64.6	66.3	134.4	138.5
29 May	23.3	21.8	66.4	66.0	140.8	136.2
30 May	22.8	22.5	66.3	68.7	139.4	141.2
31 May	23.9	22.0	71.6	69.9	151.8	139.7
Mean	23.4		68.0		140.2	
Accuracy (%)	93.8		90.6		93.4	
RSD (%)	4.9		2.5		3.1	

ng/ml) concentrations and stored under the same conditions as the clinical samples for the purpose of evaluating sample stability and the inter-day accuracy and precision of the assay. Duplicate QC samples at each concentration were analyzed daily with each set of standard curve samples and clinical samples. The accuracy and precision data for QC samples analyzed over a 3-week period are provided in Table 3. The data demonstrate that **I** was stable in urine stored at −20 °C for at least 3 weeks.

3.4. Freeze–thaw stability

The stability of **I** in urine samples over multiple freeze–thaw cycles was investigated by subjecting QC samples to three freeze–thaw cycles, each cycle consisting of freezing at −20 °C for at least 24 h followed by a thaw and equilibration to room temperature (approximately 3 h). QC samples subjected to a single freeze–thaw cycle were used as controls. Results are summarized in Table 4.

3.5. Extraction recovery

The extraction recovery was determined by comparing the absolute peak heights of samples spiked with **I** and **II** prior to processing with that of drug-free control urine samples spiked postextraction with appropriate concentrations of **I** and **II**. The mean recoveries of **I** and **II** from urine were 83.3 and 86.5%, respectively.

3.6. Analysis of clinical samples

The assay was used to support a clinical study conducted in 20 volunteers. A chromatogram from a postdose clinical sample is shown in Fig. 3. Representative urinary excretion data from subjects receiv-

Table 4
Freeze–thaw stability of **I** in human urine

Nominal conc. (ng/ml)	Control assayed conc. mean ($n=5$) (ng/ml)	Three freeze–thaw cycles	
		Assayed conc. mean ($n=3$) (ng/ml)	Difference from control (%)
25	23.0 (2.5)	21.7 (0.7)	−5.6
75	69.1 (2.4)	67.7 (0.3)	−2.0
150	142.6 (1.6)	137.8 (1.1)	−3.4

Table 5
Representative urinary excretion data for **I** following a 30-mg single dose to human subjects

Subject	Mass excreted (μg)				
	Predose	0–8 h	8–12 h	12–24 h	24–36 h
A	ND	264.0	8.8	9.4	10.9
B	ND	223.2	20.5	18.0	4.7
C	ND	371.0	12.2	25.6	13.8

ing a single 30 mg oral dose of **I** is shown in Table 5.

3.7. HPLC detection conditions

In contrast to other bisphosphonates, risedronate cannot be readily derivatized and is neither natively fluorescent nor electroactive. In addition, because of its poor ionization properties, the compound is not suitable for sensitive tandem mass spectrometric detection. Hence, method development focused on the utilization of UV absorbance detection.

UV spectral analysis of risedronate showed an absorption band with a maximum at 262 nm and a molar absorption coefficient of approximately 3900 l mol^{-1} cm^{-1} at pH 7. Owing to the relatively weak chromophore of the compound, a tenfold preconcentration of urine samples was required to achieve the target LOQ. This large sample preconcentration factor in turn placed stringent demands on the selectivity of the extraction procedure.

3.8. Extraction procedure development

3.8.1. Calcium co-precipitation

Co-precipitation of bisphosphonates with the calcium salts of endogenous phosphates [10] is widely used as an initial step in the isolation of these compounds from urine. In the present work, it was found that the size of the precipitate pellet was critical in maximizing the recovery and selectivity of the sample isolation procedure. It was observed that samples having larger pellets exhibited low recovery. The inclusion of a third precipitation step resulted in a smaller pellet which improved selectivity as well as recovery. The improvement in recovery can be attributed to the effect of pellet size on the subsequent IP SPE step. Because sample mass and ionic strength are greater in samples having larger pellets, the capacity of the SPE sorbent under ion-pairing conditions is most likely exceeded when samples prepared from large pellets are applied to the columns, leading to breakthrough of analyte during the loading step.

Variations in the concentrations of endogenous phosphates in urine samples can arise from a number of factors (e.g. intersubject differences, time of day, extent of hydration, etc.). These variations directly impact the size of the precipitate pellet. It was found that obtaining uniform pellets in spite of these variations required very careful addition of NaOH and HCl during the precipitation procedure. For example, if a precipitate was not observed after the initial 50 μl addition of 1 *M* NaOH, then additional 25-μl aliquots were added until a visible precipitate was formed. Similarly, additional aliquots of 1 *M* HCl were added as necessary to completely dissolve the pellet.

Ethylenediamine tetraacetic acid (EDTA) and EGTA were investigated for pellet dissolution prior to SPE, with the latter providing fewer extraneous peaks in the chromatograms. The optimum concentration of EGTA was determined experimentally. Concentrations below 0.05 *M* were insufficient to consistently dissolve the precipitates while higher concentrations decreased recovery of the analytes during the SPE step.

3.8.2. Ion-pair solid phase extraction

Initial experiments were aimed at direct injection of the dissolved precipitate onto the HPLC system in a manner similar to that described by Fels et al. [2]. Although acceptable chromatography was obtained for **I** and **II** in neat solution, extremely poor peak shapes were obtained for the analytes in processed samples. In some instances, baseline disturbances in the extract chromatograms were so severe that the analyte peaks could not be discerned. Blank control

urine samples spiked with **I** and **II** postprecipitation (following dissolution in 0.05 *M* EGTA) exhibited the same degradation in peak shape.

SPE following co-precipitation was next investigated. A previously reported [6] anion-exchange SPE method developed in our laboratory for the isolation of alendronate, which employed a diethylamino sorbent, was evaluated for **I**. The high affinity of **I** for the DEA sorbent necessitated the use of a highly concentrated buffer for elution (0.2 *M* trisodium citrate, 0.2 *M* trisodium phosphate). However, neat solutions of **I** prepared in this elution buffer showed significant baseline disturbances in the chromatograms as well as poor peak shape for the analytes. Alternate counter ions were investigated for elution with none providing an acceptable combination of chromatography and recovery. The ion-exchange extraction procedure was further limited in that 1 ml of the citrate–phosphate buffer was required to elute the analytes from the 200 mg DEA cartridge. Evaporation of this aqueous buffer would be problematic, thereby limiting the extent to which samples could be concentrated.

It was hypothesized that the poor chromatography observed for the extracts was due the high ionic strength of the injected sample and, specifically, in the case of the direct injection of the dissolved precipitate pellet, the presence of residual Ca^{2+} ions disrupting the ion-pairing or ion-exchange retention mechanism.

In order to eliminate Ca^{2+} and to obtain extracts that were compatible with the ion-pair HPLC system, IP SPE was explored. IP SPE [12] is analogous to IP HPLC in that it employs reversed-phase sorbents conditioned with a solution containing an ion-pair reagent (an alkyl amine or acid). IP SPE was particularly advantageous in this case due to the ability to elute the analytes from the cartridge with methanol, which could readily be evaporated without contributing to the ionic strength of the final extract.

The same IP reagent used in the HPLC mobile phase, 1-octyltriethylammonium phosphate, was employed for IP SPE to ensure compatibility between the extracts and the mobile phase. The optimum concentration of IP reagent was determined experimentally. Concentrations ranging from 0.005 to 0.025 *M* were evaluated. Conditioning the Waters HLB cartridge with 1 ml of a 0.01 *M* solution of IP reagent was found to yield high recovery of **I** and **II** (overall recovery $>80\%$) reproducibly across multiple lots of control urine. Increasing the IP reagent concentration from 0.01 to 0.025 *M* provided no further increase in recovery or precision.

The analytes were eluted from the cartridges into glass test tubes with 0.5 ml of neat methanol. The elution solvent was drawn through the cartridges by centrifugation at 70 *g*. Samples were reconstituted in 0.01 *M* NaH_2PO_4, 0.001 *M* etidronate, pH 6.25. Reconstituting the samples in this buffer as opposed to mobile phase resulted in more consistent retention times on column 1.

Extracts prepared via the two part calcium co-precipitation–IP SPE procedure showed no degradation in peak shape for **I** and **II** when injected onto the IP HPLC system, eliminating a major obstacle to the development of the assay.

3.8.3. HPLC method development

Several different modes of chromatography were investigated during method development including ion-exchange, reversed-phase HPLC using columns designed to operate in 100% aqueous mobile phases, and ion-pair chromatography. Of these, ion-pair chromatography yielded superior results, particularly with respect to ruggedness and the ability to retain **I** with relatively high plate numbers and good peak symmetry. In addition, in ion-pair mode **I** and **II** were resolved, thereby enabling the use of **II** as an internal standard.

Results of initial IP HPLC experiments showed that acceptable chromatography could be obtained for **I** and **II** in urine extracts using a Waters X-Terra RP18 column. The addition of etidronate to the mobile phase at a concentration of 1 m*M* was found to significantly improve peak symmetry and chromatographic reproducibility. It is believed that the addition of a competing bisphosphonate to the mobile phase served to mask potential adsorption sites (e.g. metal surfaces) within the HPLC system. After numerous (i.e. >100) extracts prepared by the triple co-precipitation–IP SPE procedure were injected onto the system, no deterioration in peak shape for **I** or **II** was observed. Unfortunately, when multiple urine lots were analyzed during method validation, some of the lots were found to contain endogenous peaks that were not separated from **I** and

II. Efforts to separate these interfering peaks by adjusting mobile phase composition (i.e. organic composition, pH, and chain length of the ion-pair reagent) were unsuccessful.

In order improve selectivity, a number of other HPLC columns were screened. None of the columns tested yielded acceptable peak shapes for the analytes as well as adequate selectivity. During the course of the column screening experiments, however, it was found that **I** and **II** exhibited good peak shape and unusually strong retention on a Phenomenex Synergi Polar RP column. The stationary phase in this column is an ether-linked phenyl moiety with a hydrophilic endcapping group. The enhanced retention of **I** and **II** on this phase is potentially due to $\pi-\pi$ interactions between the aromatic ring on the analytes and the bonded phenyl ligand.

In light of the chromatographic behavior of **I** and **II** on Synergi Polar RP column, it was decided to evaluate the Synergi and X-Terra RP18 columns in tandem in column-switching mode. It was known from previous experiments that ion-pair HPLC system using the X-Terra RP18 was rugged with respect to retention time and analyte peak shape following the injection of several hundred urine extracts. For this reason, the X-Terra RP18 column was used as column 1 in the column-switching system, on which the analytes were separated from the majority of co-extracted endogenous components. Prior to the elution of **I** from the X-Terra column, a ''heart-cut'' was performed and the effluent directed into the Synergi Polar RP where final separation of the analytes occurred.

The enhanced retention of the Synergi Polar RP and the difference in organic composition between mobile phases 1 and 2 (8% versus 13%) enabled focusing of the analyte bands to occur during the heart cut, which served to increase not only separation, but detectability. In order to minimize baseline disturbances during the valve switching events, the concentrations of phosphate buffer and etidronate in the aqueous component of the mobile phase were adjusted such that the overall concentrations of each in the mobile phase were the same. The selectivities of the two HPLC columns were complimentary—extracts chromatographed on the system were free of interferences with the analytes.

Retention time drift was minimized by thermostating each column at 30 °C. Valve switch times were determined daily placing column 1 directly inline with the detector and injecting a neat solution containing **I** and **II**. No significant drift in retention times on column 1 during an analytical run, as evidenced by high errors in the assayed concentrations of QC samples at the end the runs, was observed.

4. Conclusion

The first HPLC–UV based assay for the determination of risedronate in human urine has been developed. The assay has been found to be accurate, precise, selective and suitable for the analysis of samples collected during human clinical studies.

The development of this method illustrates the challenges associated with the establishment of bioanalytical procedures for the determination of certain classes of analytes, for example, bisphosphonates, that are not amenable to mass spectrometric and other classical sensitive modes of detection (i.e. fluorescence or electrochemistry). Development of such non-MS-based assays requires considerable expertise and knowledge of the various modes of liquid chromatography as well as complex, multistep analyte extraction procedures. The value of these skills is often overlooked in the current era of HPLC–MS based assays; however, as the procedure described in this publication indicates, expertise in these areas is critical when assays for non-easily ionizable, ''difficult'', molecules are required to be developed.

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