UPLC-UV Method for Determination of Risedronate in Human Urine

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This study was designed to develop a sensitive and rapid method for the quantitation of risedronate in human urine using ultra-performance liquid chromatography with ultra-violet detector (UPLC-UV) and to compare bioavailability parameter of 5, 35 and 150 mg risedronate. The mobile phase consisted of sodium phosphate buffer, 1 mM etidronate–acetonitrile (95:5, v/v), pH 9.0, and was pumped at a flow rate of 0.3 mL/min. Detection of risedronate in human urine by the UPLC-UV was accurate and precise from 20 ng/mL to 5 μg/mL (a correlation coefficient of 0.99) with 97.16% in mean recovery. The intra-day accuracy was 89.17–110.43% with precision of 0.04–3.16% and the inter-day accuracy was 89.23–110.19% with precision of 1.63–9.72%. Aet (accumulated excretion amount) of risedronate in the urine after 5, 35 and 150 mg administration was 35.08, 246.67 and 1,413.85 μg within 36 h and Umax (maximal excretion rate) was 12.11, 77.7 and 374.24 μg/h, respectively. The assessed dose proportionality of Umax and Aet with three single doses of risedronate was found in an approximately linear manner. These results indicate that the developed simple, rapid and robust assay enables the complete processing of large samples for pharmacokinetic studies of risedronate in biological fluid.

Introduction

Bisphosphonates are a class of pharmacologically active chemical compounds that inhibit osteoclast action and the resorption of bone. Initially synthesized in the nineteenth century, these compounds were originally used as water softeners (1). From a clinical point of view, bisphosphonates are used for the treatment of osteoporosis, bone metastasis, Paget’s disease and other conditions that feature bone fragility. Among bisphosphonates, the most popular first-line drugs are alendronate and risedronate. In certain cases, intravenous pamidronate can be used as an alternative approach (2).

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

Risedronate (1-hydroxy-2-(3-pyridinyl) ethylidine bisphosphonic acid monosodium salt) is a nitrogen-containing bisphosphonate approved by the US Food and Drug Administration for the treatment and prevention of postmenopausal osteoporosis. From a chemical point of view, the pharmacological function of the active compound is determined by the P-C-P configuration, where two phosphate groups are covalently linked to a carbon atom as shown in Figure 1. The groups linked to the carbon atom of the P-C-P chain influence the pharmacokinetics and the mode of action/strength of the drugs. A method to quantitate risedronate in human sample was required to support human pharmacokinetics studies. One investigation developed a micellar HPLC method for the analysis of risedronate in raw material and a tablet formulation (9). However, owing to its high polarity and low oral bioavailability, low concentrations of risedronate are expected in human sample and, therefore, a sensitive assay is required to serve in pharmacokinetic studies (10).

Risedronate has been determined in human urine using GC–MS, following acylation and silylation to form a volatile derivative (11). The limit of quantitation (LOQ) of this assay was reported to be 11 ng/mL. More recently, a highly sensitive assay (LOQ: 50.15 ng/mL) for risedronate in human urine based on enzyme-linked immunosorbent assay (ELISA) has been reported (12). 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 ultra-performance liquid chromatography with ultra-violet detector (UPLC)-based assays for the determination of risedronate in biological fluids have appeared in the literature. Because risedronate lacks readily derivatizable functional groups and is not amenable to mass spectrometric detection, a UPLC-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 study.

Experimental

Chemicals and reagents

Risedronate was obtained from Yuhan Corporation (Seoul, Korea) as a form of monosodium. Ascorbic acid, ammonium acetate, sodium phosphate, potassium phosphate and acetic acid...
were purchased from Sigma (St Louis, MO, USA). Acetonitrile (HPLC grade) was purchased from Burdick & Jackson (Morristown, NJ, USA). Etidronate (1-hydroxyethylidene-1, 1-diphosphonic acid) was obtained from Waco (Tokyo, Japan). All other reagents were of ACS grade and were used as received. Deionized (18 mV/cm) water was generated in-house using a Millipore Milli-Q Plus system (Billerica, MA, USA). Bond Elute DEA Cartridge was obtained from Varian (Santa Clara, CA, USA).

For bioavailability study, we acquired drug-free human urine from five volunteer donors. The drug used in the bioavailability was Actonel® 5, 35 and 150 mg (Sanofi Aventis, Seoul, Korea).

**Instrumentation**
The HPLC system consisted of a Waters UPLC and TUV detector (Milford, MA, USA). Preparation instruments used were Centrifuge Union 32R (Seoul, Korea), Vortex mixer G560 (Bohemia, NY, USA), ULT Freezer 706 (Waltham, MA, USA), Cold Lab Chamber HB-603 CM (Seoul, Korea), Balance AB265-S (Greifensee, Switzerland), pH meter CG 843 (Göttingen, Germany) and SPE vacuum manifolds (St Louis, MO, USA).

**Chromatographic conditions**
The UPLC system contained Waters Acquity UPLC HSS T3 (1.8 μm, 100 mm × 2.1 mm² ID). The mobile phase consisted of sodium phosphate buffer, 1 mM etidronate–acetonitrile (95:5, v/v), pH 9.0, and was pumped at a flow rate of 0.3 mL/min. The temperature of the column was maintained at 30°C. The injection volume was 20 μL and the detection wavelength was 262 nm.

**Preparation of standards**
A 1 mg/mL stock solution of risedronate was prepared by dissolving 10.8 mg of the monosodium salt of risedronate in 10 mL of deionized water. The solution was sonicated for several minutes to ensure complete dissolution of the solid. Fresh stock solutions were prepared monthly. Appropriate dilutions of the 1000 μg/mL solution were performed to yield working standards at the following concentrations: 2, 5, 10, 20, 50, 100 and 500 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 risedronate in urine at concentrations ranging from 20 to 5000 ng/mL.

**Sample preparation procedure**
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 mm² disposable glass culture tubes.

The initial step in the isolation of the analytes involved co-precipitation of the calcium salts of risedronate with endogenous phosphates at alkaline pH. An adaptation of the method described by Bisaz et al. (13) was employed. Briefly, a 100 μL aliquot of 0.1 M CaCl₂, followed by 200 μL of 1 M NaOH and 0.1 M KH₂PO₄, was added to induce the formation of a white precipitate.

Samples were then centrifuged for 3 min at 3000 r.p.m. (3000 g). The supernatant was aspirated to waste and the precipitate dissolved in 0.5 mL of 0.2 M acetic acid. After dissolution of the precipitate, the sample was diluted with 5 mL of deionized H₂O. A second precipitate was formed by the addition of 200 μL of 1 M 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 1 mL aliquot of ascorbic acid (1 μg/mL in 0.2 M acetate). The ascorbic acid was used as internal standard (IS) because it was very stable and had proper retention time in this assay condition. To ensure complete dissolution of the precipitate, the samples were placed in a sonicator for 5 min and thoroughly vortex-mixed. After the addition of 2 mL of deionized water to the sample tubes, the sample vortex was mixed for 10 s.

Bond Elute DEA extraction cartridges (30 μg, 1 mL) were conditioned with 1 mL of deionized water. The cartridges were next conditioned with 3 mL of phosphate buffer, which was drawn through the bed at a low flow rate (~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 of deionized H₂O, followed by 1 mL of water–MeOH (95:5, v/v). Samples were eluted from the sorbent by drawing etidronate solution though the cartridges and transferred to 96-well plate for UPLC analysis.

**Bioavailability studies**
The bioavailability study was carried out in accordance with the International Conference on Harmonization Guideline for Good Clinical Practice and the ethical principles in the Declaration of Helsinki. The study protocol (permission number: 0010-025, expiration: 31 October 2012) was approved by the Ethics Committee of the Seoul Pharma Laboratories (Seoul, Korea).

One hundred and thirty healthy volunteers participated in the bioavailability studies and were divided randomly into three groups. The age range was 19–33 years and weight range was 46.6–86.1 kg in the three groups. The subjects were informed about possible risks and side effects of the drug and their written consent to participate was obtained.

The subjects were admitted to the hospital at 5 p.m. and provided with a standardized regular-calorie meal (920 ± 20 kcal: 65% carbohydrate, 20% protein and 15% fat). All participants fasted overnight for at least 10 h before drug dosing. The tablets of each dose (5, 35 and 150 mg) were administered at 8 a.m. next day. Food and beverage intake (other than water, which was allowed 2 h after study drug administration) was not...
permitted until 4 h after study drug administration. The subjects took a standardized lunch and dinner according to a regular time schedule. Urine from each volunteer was collected in plastic bottles just before drug administration and between 0 and 0.5, 0.5 and 1, 1 and 2, 2 and 3, 3 and 4, 4 and 6, 6 and 8, 8 and 12, 12 and 24 and 24 and 36 h. All urine samples were transferred to three labeled polyethylene tubes and frozen to at least −70°C until risedronate assay.

Throughout the study, the subjects were prohibited from consuming alcohol or beverages containing xanthine derivatives, engaging in intense physical activity and smoking. The subjects remained under continuous medical supervision at the study site to monitor for adverse events during the study. Physical examinations and vital signs were taken before and at the completion of study.

Results

Bioanalytical method validation

Bioanalytical method validation was carried out in accordance with international guideline for human use and industry (14, 15).

Specificity

Specificity was evaluated by analyzing matrix blanks from six unique lots of matrix. Each blank sample was tested for interference, and specificity was confirmed by the absence of any peak >20% of the mean peak response of the lower limit of quantification (LLOQ) in intra-batch run. No significant interference in the blank plasma traces was seen from endogenous substances in drug-free urine, and chromatographic separation has been exhibited in Figure 2. To evaluate analytes carry-over, blank

![Figure 2. Representative chromatogram of risedronate and IS in human urine.](image)

- (A) Chromatographic profile of a blank urine sample.
- (B) Chromatographic profile of a sample of urine spiked with IS (ascorbic acid).
- (C) Chromatographic profile of a sample of urine spiked with ascorbic acid (IS) and risedronate 20 ng/mL (LLOQ).
- (D) Chromatographic profile of a sample of urine spiked with ascorbic acid (IS) and risedronate 5000 ng/mL.
plasma extract was injected immediately following upper LOQ. Analytes were considered stable if blank plasma extract demonstrated a lack of significant carry-over in the chromatographic regions of interest for the analytes that had response of no more than 20 and 5% of the mean peak response of the LLOQ and IS, respectively.

Recovery
The relative recovery of the analytes and IS were determined by comparing the peak response for the extracted plasma samples at all QC levels (LLOQ, low, medium and high) with peak response of the samples extracted from water instead of human urine. The extraction recovery from human urine was found to be >97.2% and ~98.0% for IS, respectively, and the dependence on concentration is negligible. The recovery of the analytes and IS was high and it was consistent and reproducible. The results are presented in Tables I and II.

Sensitivity
Sensitivity is defined as the lowest concentration on the standard curve if the following conditions are met: the analyte response at the LLOQ should be at least 10 times the response compared with blank response (S/N ratio = 10:1, peak-to-peak) and identifiable, discrete and reproducible. The precision should not exceed ±20%, and the accuracy should be within ±20% from the theoretical value. The LLOQ in the present study was 20 ng/mL on the standard curve.

Table I
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<tr>
<th>Recovery of risedronate from human urine (extracted) and deionized water (unextracted) using an extracting solvent</th>
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Table II
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<th>Recovery of ascorbic acid (ISTD) from human urine (extracted) and deionized water (unextracted) using an extracting solvent</th>
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<td>Recovery</td>
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<td>Recovery (%)</td>
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Linearity
The study of linearity was estimated by the analysis of extracts obtained from aliquots of urine with IS (ascorbic acid) in the concentration range of 20–5000 ng/mL. A calibration curve consisted of a blank sample (matrix sample processed without IS), a zero sample (matrix sample processed with IS) and six samples covering the expected range, including LLOQ. The calculation was based on peak area ratios of the analyte to that of IS. The coefficient of correlation (r²) must be ≥0.99. When five such linearity curves were analyzed, the r² of all calibration curves is >0.99.

Accuracy and precision
Accuracy and precision were evaluated from five replicates of QC samples at four different concentrations, i.e., 20, 60, 2000 and 4000 ng/mL (LLOQ, low, medium and high). Intra-day accuracy and precision were determined by analysis of at least five replicates at each QC level within a day. Inter-day accuracy and precision were determined by analysis of at least five replicates at each QC level in a minimum of 5 days (this can include the intra-day). The intra-day accuracy was 89.2–110.4% with precision of 0.04–3.16% and the inter-day accuracy was 89.2–110.2% with precision of 1.63–9.72% for risedronate (Table III).

Bioavailability studies
The concentrations of risedronate in the urine (substituted by C) were determined by the proposed UPLC-UV method. The medicine discharge capacity of this segment (substituted by X) is defined as urine volumes multiplied by C. The average discharge rate of risedronate in the urine (substituted by ΔX/Δt) is defined as the difference of X divided by the interval of dwell times (substituted by Δt). The relation of the average excretion rate of risedronate in the urine (substituted by ΔX/Δt) is shown in Figure 3. The maximal excretion rate (Umax) was 12.11, 77.7 and 374.27 μg/h after 5, 35 and 150 mg administration, respectively. The accumulated excretion amount (Aet) of risedronate in the urine after 5, 35 and 150 mg administration was 35.08, 246.67 and 1.41385 μg within 36 h, and each profile is shown in Figure 4. The evaluated pharmacokinetic parameters are shown in Table IV.

The linearity curves of Umax and Aet were linear over the dose range (5–150 mg). A correlation coefficient of 0.997 and 0.999 was obtained and the linear regression equation was Y = 9.686X – 48.30 and Y = 2.52X – 4.921, where Y and X represent the relationship between Umax, Aet and doses (Figure 5).

Discussion
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. Bisphosphonates are characterized by low bioavailability; typically 1% of the dose is absorbed. Hence, at therapeutic doses, only low ng/mL levels are present in urine. As risedronate systemically disappears rapidly (0.5–2 h after oral administration) in blood, resulting in low plasma bioavailability (0.7%) in the fasted state, this study examined the urine concentrations of risedronate after oral administration. Detection of risedronate in human urine by the UPLC-UV was precise with a quantitation limit of 20 ng/mL.

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 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 non-volatile and thus are incompatible with MS detection.

In the present study, UPLC-UV-based assay for the determination of risedronate in human urine has been developed for the first time. 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 analytical extraction procedures.

This study reveals that risedronate is almost completely cleared from the body within 36 h even when the dose is increased up to 150 mg. The pharmacokinetic parameters such as $U_{\text{max}}$ and $A_{\text{et}}$ were obtained and the dose proportionality of three single doses (5, 35 and 150 mg) of risedronate was assessed. These results demonstrated that dose-dependent parameters ($U_{\text{max}}$ and $A_{\text{et}}$) increased in an approximately linear manner.

**Conclusion**

A highly sensitive, rapid and specific UPLC-UV method for the determination of risedronate in human urine has been

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**Table IV**

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<th>Dose (mg)</th>
<th>BA parameter</th>
<th>$U_{\text{max}}$ (µg/h)</th>
<th>$A_{\text{et}}$ (µg)</th>
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<tbody>
<tr>
<td>5</td>
<td>$A_{\text{et}}$ (µg)</td>
<td>35.08 (28.06)</td>
<td>12.11 (11.97)</td>
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<tr>
<td>35</td>
<td>$U_{\text{max}}$ (µg/h)</td>
<td>246.67 (149.43)</td>
<td>77.7 (50.96)</td>
</tr>
<tr>
<td>150</td>
<td>$A_{\text{et}}$ (µg)</td>
<td>1413.85 (744.06)</td>
<td>374.27 (305.71)</td>
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$r^2$ values for the linearity plots are given.

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**Figure 3.** Urinary excretion rate curve after administration of 5, 35 and 150 mg of risedronate to volunteers.

**Figure 4.** Cumulative urinary excretion profile after administration of 5, 35 and 150 mg of risedronate to volunteers.

**Figure 5.** Linearity equation of the maximal excretion rate ($U_{\text{max}}$) and the accumulated excretion amount ($A_{\text{et}}$).
developed and validated, with a lower quantitation limit of 20 ng/mL. Validation experiments have shown that the assay has good precision and accuracy over a wide concentration range (20–5000 ng/mL). In the evaluation of pharmacokinetic parameters in human urine, the assessed dose proportionality of \( U_{\text{max}} \) and \( A_{\text{et}} \) with three single doses of risedronate was found in an approximately linear manner. This UPLC-UV assay enables the complete processing of large sample for pharmacokinetic studies of risedronate in biological fluids.

**Funding**

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