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Development and validation of a sensitive solid-phase-extraction (SPE) method using high-performance liquid chromatography/tandem mass spectrometry (LC–MS/MS) for determination of risedronate concentrations in human plasma

Sussan Ghassabian[a,*], Linda A. Wright[a], Andrew D. deJager[b], Maree T. Smith[a]

[a] Centre for Integrated Preclinical Drug Development, The University of Queensland, Brisbane, QLD, Australia
[b] Drug of Abuse Laboratory, Clinical Chemistry, Mater Adults Hospital, South Brisbane, QLD, Australia

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ABSTRACT

Risedronate is a commonly prescribed bisphosphonate for the treatment of bone disorders. Due to its high polarity and low oral bioavailability, low concentrations of risedronate are expected in human plasma and therefore a sensitive assay is required to serve in pharmacokinetic studies. Here, we describe the development and validation of an LC–MS/MS assay for the measurement of risedronate concentrations in human plasma. Risedronate and the internal standard, risedronate-d4, were derivatized on an anion exchange solid-phase extraction cartridge. Trimethylsilyl-diazomethane which is a thermally stable and relatively non-toxic derivatization agent was used to methylate the risedronate phosphonic acid groups and decrease analyte polarity. Following extraction, the analytes were separated on a Phenomenex Gemini C18 column (150 mm × 2.0 mm, 5 μm), using a gradient of ammonium acetate 10 mM and acetonitrile with a flow rate of 300 μL/min. The assay calibration range was 0.2–25 ng/mL. The calibration curve of risedronate standards spiked in six individual plasma samples was linear (r^2 = 0.9998). Accuracy (percent deviation from nominal) and precision (percent coefficient of variation) at concentrations 0.5, 5 and 20 ng/mL, and at the lower limit of quantification (LLOQ) of 0.2 ng/mL were excellent at <6%. Mean recovery was 54% for risedronate and 51% for the internal standard. Risedronate was stable in human plasma samples for at least 5 h at room temperature, 101 days frozen at −80 °C, 72 h in an autosampler at 10 °C, and for three freeze/thaw cycles. The validated assay method successfully quantified the concentrations of risedronate in plasma samples from informed consenting healthy volunteers administered a single 35 mg risedronate tablet.

1. Introduction

Risedronate (RDR) is a member of the bisphosphonate class of drugs that are widely used for the treatment of bone disorders such as osteoporosis, bone metastasis, and Paget's disease [1]. Osteoporosis affects almost 200 million women worldwide and 75 million people in Europe, USA and Japan. During the year 2000, it was estimated that there were nine million new osteoporotic fractures [2]. Bisphosphonates have been prescribed by clinicians since the 1970s and they are first-line therapy for patients with osteoporosis [3]. Alendronate and RDR are the most popular nitrogen-containing bisphosphonates in this therapeutic class [4].

Bioanalysis of bisphosphonates in complex matrices such as human plasma is challenging due to their high polarity (Fig. 1) (structurally similar to endogenous phosphorylated compounds), metal chelating properties and low bioavailability [5]. Previously reported methods have generally utilized a combination of multiple extraction steps, derivatization to less polar or more volatile compounds, together with ion pair chromatography [6] and capillary electrophoresis [7] using HPLC [8], GC [9], LC–MS, or ELISA [9] to improve the efficacy of the assays [4]. LC–MS/MS is preferable for measuring bisphosphonate concentrations in plasma samples due to higher sensitivity and selectivity, as well as shorter analytical run times. An LLOQ of less than 1 ng/mL is required for the quantification of RDR in plasma and urine samples. Solid phase extraction (SPE) has been used for sample clean-up, ion-pairing and derivatization of various bisphosphonates of interest. Due to their high polarity, liquid–liquid extraction is unsuitable for extraction of bisphosphonates from plasma; however, it may have a role for removal of excess derivatization agents [4].

The concept of derivatization of bisphosphonates whilst retained on an SPE cartridge followed by elution and quantitation

* Corresponding author at: Centre for Integrated Preclinical Drug Development, The University of Queensland, Level 7, Block 6, Herston Campus, Brisbane, QLD 4029, Australia. Tel.: +61 07 3346 5194; fax: +61 07 3365 5444.
E-mail addresses: s.ghassabian@uq.edu.au, susan.ghassabian@gmail.com (S. Ghassabian).

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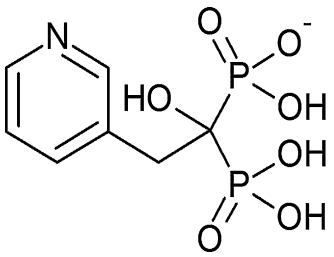

Fig. 1. Risedronate chemical structure.

of derivatized analytes using LC–MS/MS was first proposed by Zhu et al. [10]. Here, we report the development and validation of an improved LC–MS/MS method using SPE derivatization of RDR with a more efficient and less toxic derivatization agent, trimethylsilyldiazomethane (TMS-diazomethane). This method is both sensitive and robust for the quantification of RDR in human plasma samples.

2. Materials and methods

2.1. Chemicals

RDR sodium and RDR-d4 were purchased from Toronto Research Chemicals, (North York, Ontario, Canada). TMS-diazomethane and bovine serum albumin were bought from Sigma–Aldrich (Sydney, NSW, Australia). Sodium bicarbonate, sodium carbonate, ammonium acetate, formic acid were purchased from Lomb Scientific (Sydney, NSW, Australia). Methanol and acetonitrile (HPLC grade) were bought from Lab-Scan (Brisbane, QLD, Australia). Human blank plasma was purchased from BioCore Pty. Ltd. (Sydney, NSW, Australia). SPE cartridges (UCT, CHQAX1 100 mg/mL) were purchased from UCT Inc. (Bristol, PA, USA).

2.2. Methods

2.2.1. Standard solutions

RDR-d4 (RDR-d4) 25 ng/mL was prepared in 2% BSA solution, stored at 4 °C and protected from light. Stock standard solutions at 100, 10, 1 and 0.1 μg/mL and working standard solutions at 25, 20, 10, 5, 2, 1, 0.5 and 0.2 ng/mL were prepared in deionized water and drug free plasma, respectively. All these solution were stored in polypropylene tubes at −80 °C for six weeks. Quality control solutions (QCs) were prepared at concentrations of 0.5, 5 and 20 ng/mL in blank plasma and stored at −80 °C.

For system suitability assessment, two replicates of an RDR solution at 5 ng/mL in human plasma were extracted using the SPE method described in the next section. The extracts were combined to provide sufficient sample to allow six replicate of 10 μL injections.

2.2.2. Plasma sample extraction procedure

Following addition of 100 μL of internal standard solution (25 ng/mL) in BSA 2% and 500 μL of 10 mM sodium bicarbonate solution to 500 μL aliquots of plasma on ice, samples were vortexed briefly.

The UCT, CHQAX1 (quaternary amine/hydroxide, 100 mg/mL) cartridges were conditioned with methanol (1 mL, twice) and deionized water (1 mL) followed by sample (1.1 mL) loading. The cartridges were then washed successively with 1 mL aliquots of deionized water, 10 mM sodium carbonate and methanol. Next, 1 mL of freshly prepared derivatization reagent; TMS-diazomethane:methanol; (20:80) was added to each cartridge in a fume cupboard. The analytes and derivatization reagent were eluted from the cartridge under gravity over a 30 min period, followed by elution with methanol (1 mL). The collected methanol extracts were incubated for an additional 30 min period before evaporation to dryness. The dried residues were reconstituted in 100 μL aliquots of a mixture of acetonitrile and ammonium acetate 10 mM (20:80), followed by mixing for 2 min. The re-constituted samples were then transferred to a 96 well plate and sealed; 10 μL aliquots were injected.

2.2.3. LC conditions

The liquid chromatography system consisted of a Shimadzu LC-20AD pump, equipped with a Shimadzu DGU-20A3 degasser and a Shimadzu SIL-20AC autosampler. Analytical separation was achieved using a 150 mm × 2.0 mm (5 μm) Phenomenex Gemini C18 column and 4 mm × 2 mm guard column (Phenomenex, Sydney, NSW, Australia) placed in a Shimadzu CTO-20A column heater at 40 °C. The mobile phase comprised two phases, viz. A: 10% acetonitrile, 90% (v/v) 10 mM ammonium acetate and B: 10% ammonium acetate, 90% (v/v) acetonitrile, which was delivered using the following stepwise gradient elution program at a flow rate of 300 μL/min: A:B (v/v) 88:12 for 0.01–2.5 min; B: 100% for 2.55–3.90; and A:B (v/v) 88:12 for 3.90–6.5. To prevent the contamination of the detector, the flow was diverted to the waste after 5 min. The acquisition and processing of data were performed using Applied Biosystems/MDS Sciex Analyst™ software, version 1.4.2.

2.2.4. MS/MS conditions

Mass spectrometric analyses were performed using an Applied Biosystems/MDS Sciex API 4000 triple quadruple mass spectrometer equipped with an electrospray ionization source. The interface was operated in positive ionization mode. High purity nitrogen served both as collision-induced dissociation (CAD) gas and curtain gas (set at 6 and 30 psi, respectively). The nebulizer was supplied with ultra high purity nitrogen, regulated at 50 psi. The ion spray temperature was maintained at 600 °C, and the source voltage was optimized at 5000 V.

To tune the mass parameters for RDR and the internal standard, molecular ions were obtained by direct infusion, and the parameters were automatically acquired by the Analyst software version 1.4.2. Multiple reaction monitoring (MRM) in positive ionization mode was used to monitor transitions at m/z 340.19 → 214.20 and 344.25 → 218.20 for RDR and RDR-d4, respectively. The fragmentation pattern for derivatized RDR (m/z = 340) has been presented in Fig. 2.

The collision energy and collision cell exit potential (CXP) were optimized to generate the largest response for both analytes. Collision energy 25 V was found to be the best for both RDR and RDR-d4 ionization, and the collision cell exit potential (CXP) at 14 and 16 V for RDR and RDR-d4, respectively.

During last 1.5 min of the run (5–6.5 min), the curtain gas and capillary voltage was changed to 50 psi and 500 V, respectively, because no mobile phase flow was going to the detector.

2.2.5. Method validation

The method validation fulfilled the Food and Drug Administration Guidelines for Bioanalytical Methods (May 2001) and is detailed below. Full validation was performed for human plasma. Plasma was spiked with standard solutions to obtain 8 concentrations. The main QC concentrations were 0.5 (Low), 5 (Med) and 20 (High) ng/mL, and 0.2 ng/mL as LLOQ (lower limit of quantification).

2.2.5.1. Linearity. Linearity was assessed on six separate occasions using six different sources of plasma spiked by processing standard curves (0.2, 0.5, 1, 2, 5, 10, 20 and 25 ng/mL) for RDR over the concentration range 0.2–25 ng/mL. Each calibrator was also spiked with 100 μL IS containing 25 ng/mL RDR-d5. A linear least squares regression model was applied to all calibration curves. Daily

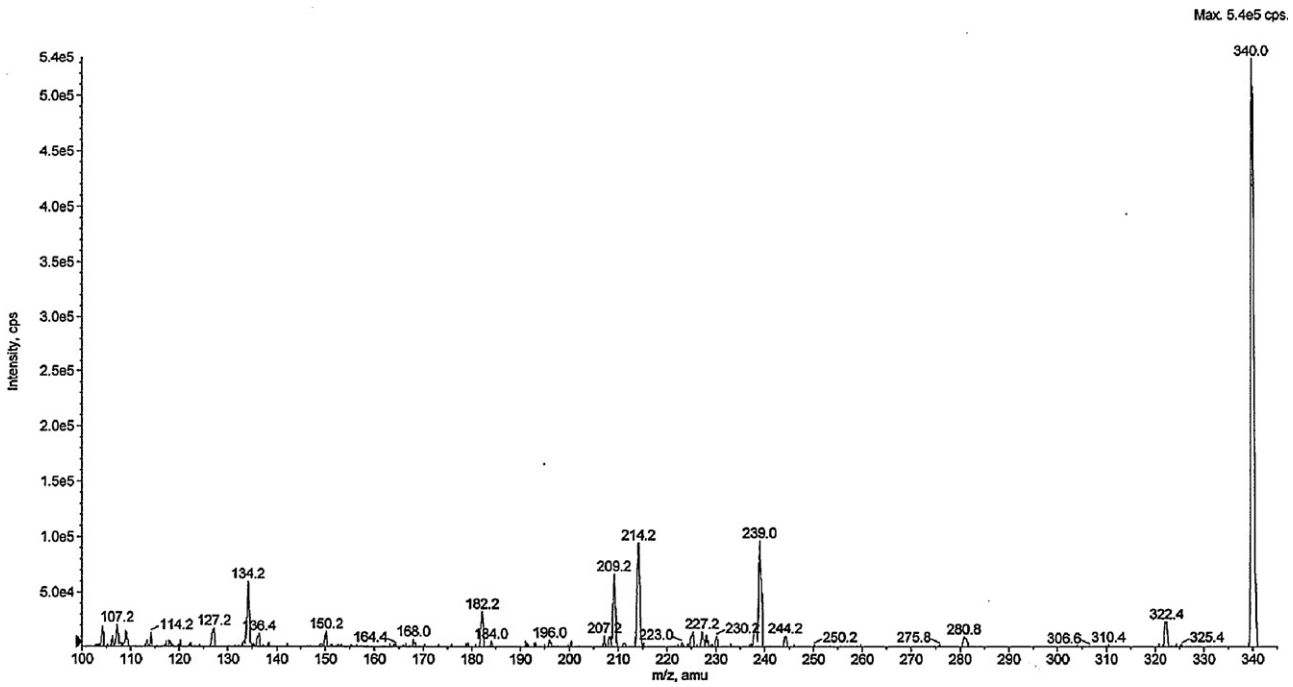

Fig. 2. Fragmentation pathways of derivatized risedronate.

calibration curves using the same concentrations were prepared for all following experiments.

2.2.5.2. Selectivity. Samples from different sources ($n = 6$), were extracted as described in Section 2.2.2 and checked for peaks that might interfere with the detection of the analytes or IS. All these plasma samples were collected in tubes containing lithium heparin as anti-coagulant.

2.2.5.3. Precision and accuracy. The intra-assay precision and accuracy were assessed using eight aliquots of each of the 0.5, 5 and 20 ng/mL QCs, and also at LLOQ (0.2 ng/mL) which were removed from frozen storage and assayed alongside a freshly prepared standard curve to include the possible influence of the daily variations of the calibration curve. Measured concentrations were inversely predicted from the standard curve obtained on that occasion. The inter-assay precision and accuracy were assessed on five separate occasions such that a duplicate of three QCs was assayed alongside a freshly prepared standard curve. The precision determined at each concentration level, as defined by the coefficient of variation (CV) should not exceed 15, except for LLOQ, where it should not exceed 20%. The deviation of the mean from the true value (accuracy) should also be within ±15% of the actual value at QCs, and ±20% at LLOQ.

2.2.5.4. Recovery and matrix effect. The extraction recovery of RDR from human plasma samples was assessed by comparison of the peak area ratio obtained for RDR/RDR-d4 ($n = 3$) for low, medium and high RDR concentrations (QCs). Recovery was quantified using the peak area ratio of the extracted QCs to the area ratio of the standards in aqueous solutions. The extent of recovery should be consistent, precise and reproducible.

To estimate the matrix effect, blank plasma samples from six different healthy volunteers were prepared in accordance with the assay method. The reconstituted extracts were spiked with RDR (at three QC concentrations) and RDR-d4 at the working concentration and injected onto the LC–MS/MS system. The precision of the peak area ratio (RDR/RDR-d4) across the six plasmas was determined.

2.2.5.5. Sensitivity. The LLOQ was assigned using the criterion that the analyte response at the LLOQ was 5 times the baseline noise and could be determined with a precision of ≤20% coefficient of variation (%CV) and an accuracy of 80–120% of the nominal concentration. Six different sources of plasma were spiked with several concentrations of RDR close to the LLOQ and were assayed alongside a freshly prepared standard curve. The concentration of RDR in each of these spiked samples was back-calculated from the standard curve. In addition, the accuracy and precision at LLOQ were estimated as explained in Section 2.2.5.3.

2.2.5.6. Dilution effect. The ability of the method to provide precise and accurate results after dilution of samples above the validated concentration range was tested. A QC at four times the highest standard concentration (100 ng/mL) was prepared. Eight replicates were diluted 1 in 10 with drug free blank plasma (to give a concentration within the standard curve range) and assayed alongside a freshly prepared standard curve. The concentrations were inversely predicted from the standard curve followed by application of the dilution factor. The dilution QC replicates must have a precision of ≤15% and an accuracy of ≤15% of nominal.

2.2.5.7. Ruggedness. The ruggedness of the method was assessed by an analyst not previously involved in the validation studies. Using the described assay procedure, the second analyst assayed a single standard curve in plasma and quality controls ($n = 2$) at each of the low, medium and high concentrations. These samples were processed using an analytical column of the same manufacturer and type, but different production batch number.

The results were assessed using our internally accepted criteria which is the co-efficient of determination must be greater than 98%, and four of the six QCs (at least one in each concentration) must assay to within 15% of the nominal concentration.

2.2.5.8. The impact of co-medications and haemolysis. The presence of common concomitant medications that may be co-administered in clinical trial samples with the potential to elute near the retention time of RDR or the internal standard, or manifest as interfering

Table 1
Linearity of the RDR standard spiked in six different plasma samples.

Calibrator concentration (nominal, ng/mL)	1	2	3	4	5	6	Mean	Precision (%CV)	Accuracy (%)
0.2	0.192	0.205	0.202	0.190	0.197	0.200	0.198	2.94	−1.17
0.5	0.509	0.490	0.498	0.523	0.507	0.533	0.510	3.10	+2.00
1	0.969	0.984	1.02	1.01	0.992	0.975	0.992	2.01	−0.83
2	2.12	2.02	1.95	2.02	2.00	1.92	2.01	3.45	+0.50
5	4.99	4.98	4.98	4.93	5.09	4.91	4.98	1.26	−0.40
10	10.0	10.1	10.0	9.88	9.87	10.1	9.99	1.01	−0.08
20	19.6	19.7	20.2	20.4	20.0	20.4	20.1	1.83	+0.50
25	25.3	25.2	24.9	24.7	25.0	24.6	25.0	0.93	−0.20
Slope	0.9983	1.0014	0.9990	0.9966	1.014	0.9997			
Intercept	−0.0014	−0.0051	0.0017	0.0091	−0.045	−0.0003			
r^2	0.9997	0.9999	0.9996	1.0000	0.9999	0.9996	Mean = 0.9998		

peaks were tested. Paracetamol and ibuprofen as the most likely medications were separately spiked (at clinically relevant concentrations; 50 μg/mL paracetamol and 10 μg/mL ibuprofen) in plasma samples that contained RDR (at LLOQ, medium and high concentration) and RDR-d4 (at the working concentration) alongside a freshly prepared standard curve. The impact of haemolysis on the RDR assay was also assessed. For RDR, the back calculated concentrations (as percent accuracy) of the samples spiked with paracetamol and ibuprofen were compared with the corresponding standards and it should be within ±10% of the nominal concentrations.

2.2.6. Stability tests

2.2.6.1. Room temperature stability. Triplicate samples of each of the low, medium and high QCs were prepared and allowed to stand at room temperature for 2 and 5 h prior to the assay. The concentrations of RDR for each time point in the experiment were compared to a triplicate set of 0 h control samples.

2.2.6.2. Freeze–thaw stability. Three cycles of freeze/thaw from −80 °C (for at least 12 h) to a mean (±SEM) temperature of 22 (±2) °C were applied to plasma samples that had been spiked with low, medium and high (QCs) concentrations ($n = 3$) of RDR and their concentrations were compared to freshly prepared samples in the same concentrations.

2.2.6.3. Frozen storage stability. The stability of samples of QCs (Low, Med and High) which were stored frozen at −80 °C was assessed at 13, 38 and 101 days with the concentrations of RDR inversely predicted from a freshly prepared standard curve on each occasion.

2.2.6.4. Stability of extracted samples and standard solutions. To assess the stability of extracted samples in a refrigerated autosampler (10 ± 2 °C) for up to 72 h, re-constituted extracts of low, medium and high QCs were pooled and divided into five replicate sets and analyzed at 0, 24, 48 and 72 h. The remaining set was placed in cold storage (4 ± 3 °C) and assayed after 193 h.

Stability of RDR and RDR-d4 in standard solutions was assessed by direct comparison of freshly prepared solutions with solutions that had been prepared 200 days earlier and stored refrigerated (4 ± 3 °C).

2.2.6.5. Stability during sample processing. In order to assess the stability of RDR in blood samples collected from healthy volunteers, samples drawn from two subjects at 1.25 and 6 h post-dose were treated in one of three different ways: (A) blood was centrifuged immediately post-collection, plasma separated without delay and frozen at −80 °C, (B) blood was centrifuged immediately post-collection and the separated plasma stored on ice for 2 h prior to freezing at −80 °C, and (C) blood was stored on ice for 2 h prior to centrifugation, with the separated plasma then immediately frozen at −80 °C.

In all stability experiments, analytes were assumed stable when the deviation of the mean concentrations of the test samples from the controls was within ±15%.

2.2.7. Application to biological samples

This bioanalytical method was used to measure RDR concentrations in plasma samples collected from informed consenting healthy subjects administered a single 35 mg tablet in a clinical study. The samples from patients were collected in tubes containing Li Heparin as the anticoagulant. The study was approved by the Local Medical Ethics Committee.

3. Results

3.1. Mass spectrometry and liquid chromatography

Using the method described herein, RDR and RDR-d4 were separated chromatographically with a 6.5 min overall run time. Retention times for RDR and RDR-d4 were 3.39–3.77 min and 3.35–3.72 min, respectively with the respective times being precise within-batches. Chromatograms of RDR and internal standard after extraction from a QC (5 ng/mL), plasma blank, at LLOQ, and extracted from a healthy volunteer plasma have been presented in Figs. 3–5. It was confirmed that drug-free plasma from six different sources was free from ions that could potentially interfere with the analytes of interest.

3.2. Assay performance

3.2.1. Linearity

For the calibration range, 0.2–25 ng/mL, the RDR assay was shown to be linear on six separate occasions using a linear model. The precision and accuracy of six replicates for each of the calibration curve concentrations (0.2, 0.5, 1, 2, 5, 10, 20 and 25 ng/mL) were excellent at <3.4%, and >98% of the nominal concentrations, respectively. The mean regression coefficient (r^2) for the six replicate standard curves was 0.9998 (Table 1). The data analyses show that the chosen regression model produced good accuracy over the measured concentration range on each of the inter-assay and intra-assay occasions for RDR.

3.2.2. Selectivity

The selectivity of this method in relation to endogenous compounds was established by confirming the absence of significant chromatographic peaks at the retention time of both RDR and RDR-d4 in six different individual human plasmas.

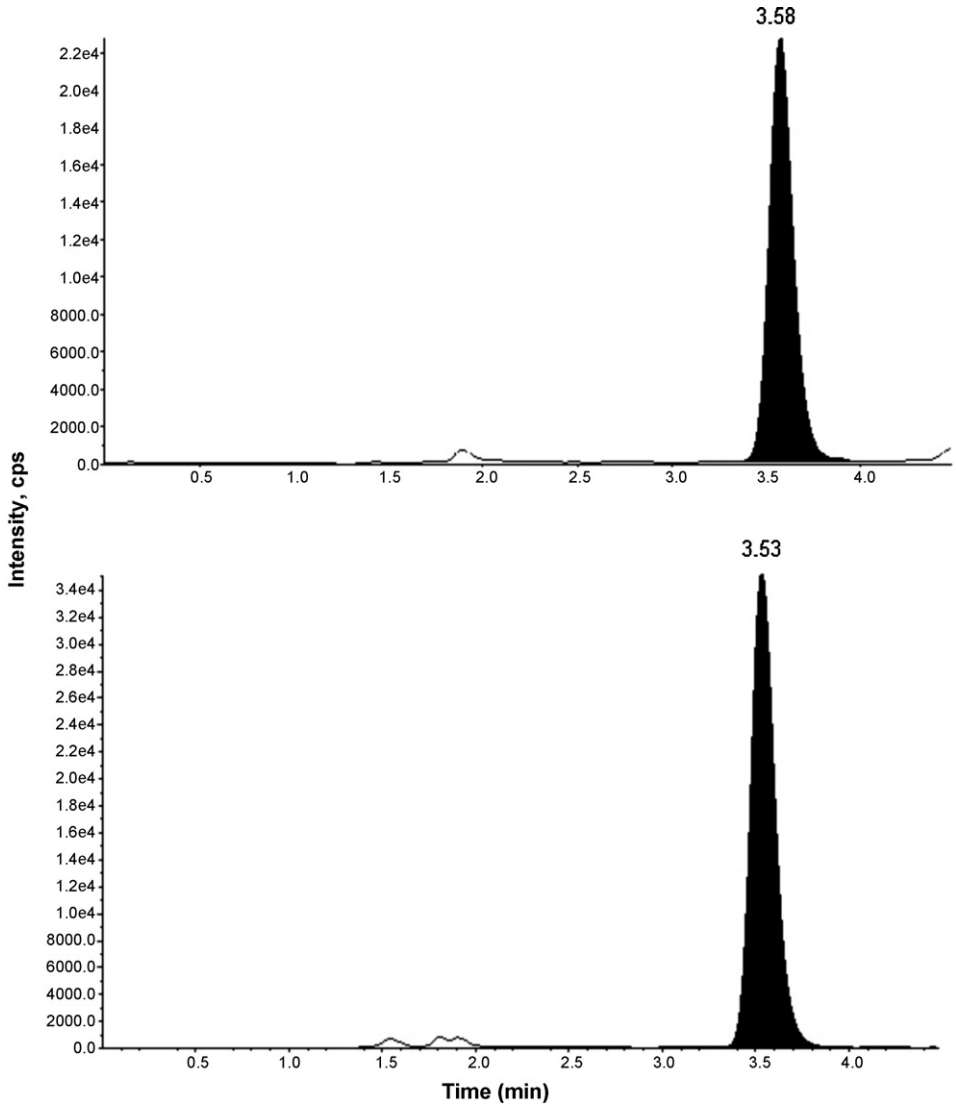

Fig. 3. Chromatogram of the medium QC sample (5 ng/mL) showing the RDR trace (upper) and the RDR-d4 trace (lower).

3.2.3. Precision and accuracy

For each of the low, medium and high QCs and also at LLOQ, intra-assay and inter-assay accuracy and precision are presented in Table 2. The precision and accuracy for intra-day and inter-day assays are smaller than 6% in Low, Med and High QCs with higher accuracy and less variability in higher concentrations.

3.2.4. Recovery and matrix effect

The mean recovery of RDR from human plasma was 54.5%, and the mean recovery of RDR-d4 at the working concentration in plasma was similar at 50.9% (Table 3).

The precision of the peak area ratios (RDR/RDR-d4) across the six different batches of human plasma were all within ±4.1%, thereby demonstrating that the method is not subject to significant matrix effects arising from different sources of plasma.

3.2.5. Sensitivity

For the six plasma samples spiked with various low concentrations of RDR, the LLOQ was confirmed at 0.2 ng/mL. The average of signal to noise ratio for the chromatogram of 0.2 ng/mL RDR spiked in six plasma samples was 5.2. The accuracy data at LLOQ lay within the acceptance interval of 20% of the nominal value and the precision value were smaller than 20% (Table 2).

Table 2
The Inter-day and Intra-day Accuracy and Precision of RDR quantification in human plasma samples.

RDR concentration (ng/mL)	Intra-day precision %CV	Intra-day accuracy[a] (ng/mL)	Inter-day precision %CV	Intra-day accuracy[a] (ng/mL)
0.2	2.9	11.2	4.85	−3.92
0.5	5.1	−3.1	3.1	−5.6
5	2.8	−0.5	5.7	−4.6
20	1.06	−2.5	5.1	−2.5

[a] % deviation from nominal.

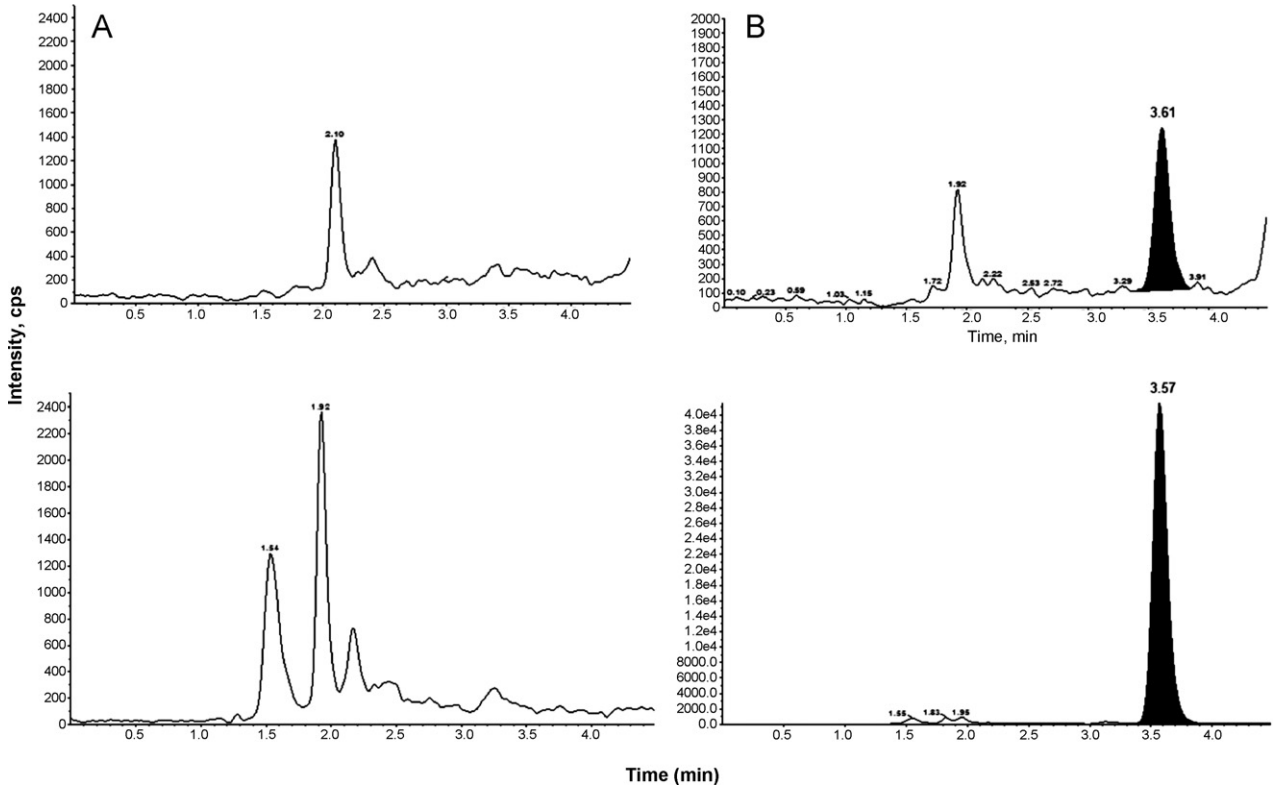

Fig. 4. Chromatogram of: (A) blank, and (B) LLOQ standard (0.2 ng/mL), showing the RDR trace (top) and the RDR-d4 trace (bottom).

3.2.6. Dilution effect

After a 10-fold dilution of a QC at a concentration four-fold higher than the highest standard concentration, mean accuracy and precision were satisfactory at 111% of the nominal concentrations and 0.75%, respectively.

3.2.7. Ruggedness

The standard curve regression co-efficient (r^2) for standard samples assayed by another analyst was >0.9976, and all standards were within ±91.5% of the nominal concentration. In all of the QCs, the deviation from nominal concentrations was within the ±15%. These findings together demonstrate that this method for the quantification of RDR in human plasma is suitably rugged.

3.2.8. The impact of co-medication and haemolysis

Paracetamol and ibuprofen at clinically relevant concentrations did not compromise the RDR assay with mean RDR concentrations within 6% of nominal (Table 4). Similarly, for haemolyzed plasma samples, mean RDR concentrations were within 8.0% of nominal (Table 4), further highlighting the robustness of this assay method.

Table 3
Recovery of RDR and RDR-d4 from human plasma samples.

RDR concentration (ng/mL)	Peak area ratio RDR/RDR-d4 (mean ± CV)	Peak area ratio RDR-d4/RDR (mean ± CV)
0.5	56.5 ± 1.1	
5	54.6 ± 10.4	50.9 ± 17.1
20	52.4 ± 8.8	

3.3. Stability tests

3.3.1. Room temperature stability

The mean concentration data (Table 5) for the 2 and 5 h samples at each of the low, medium and high concentrations showed less than 6% deviation from the concentrations of the control samples at time zero. Overall, the data shows that RDR is stable in plasma for periods of at least 5 h at ambient temperature.

3.3.2. Freeze and thaw stability

The plasma samples spiked with RDR in QC concentrations showed less than 15% deviation from the control samples in the first and third freeze–thaw cycles (Table 5), indicating good stability of the samples.

3.3.3. Frozen storage stability

The results for storage at −80 °C are shown in Table 5. The mean measured concentrations for all stored sample at −80 °C at each of the Low, Med and High concentrations were within ±9% of the mean concentrations of the freshly prepared samples. It can be concluded that the RDR is stable in plasma samples at −80 °C for at least 101 days.

3.3.4. Stability of extracted samples and standard solutions

Plasma sample extracts were stable in the autosampler (10 ± 2 °C) and when stored refrigerated (4 °C), for at least 72 and 193 h, respectively.

When stored refrigerated for 225 days, RDR and RDR-d4 stock solutions were within ±2.2 and ±6.9% of the respective concentrations for freshly prepared solutions demonstrating excellent solution stability of these analytes.

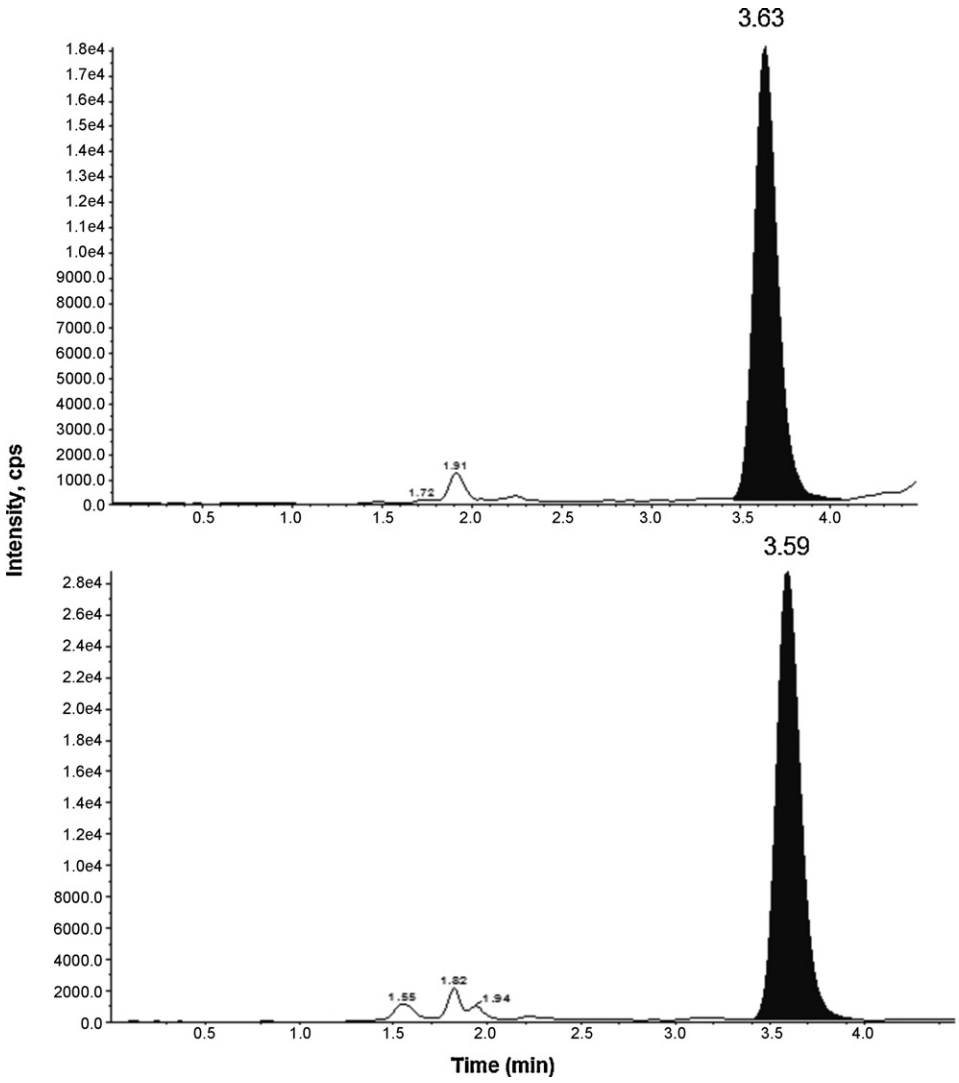

Fig. 5. Chromatogram of RDR in plasma sample collected from a healthy volunteer.

Table 4
Lack of effect of ibuprofen, paracetamol or red cell haemolysis on quantification of RDR in human plasma samples.

Nominal concentration ng/mL	Back-calculated concentrations (ng/mL) (% deviation from nominal)			
	Standard	+IBP[a]	+PCM[b]	Haemolysis
0.2	0.192 (−4.00)	0.201 (+0.50)	0.202 (+1.00)	0.184 (−8.00)
5.0	5.09 (+1.80)	4.71 (−5.80)	4.75 (−5.00)	5.08 (+1.60)
20	20.0 (0.0)	19.1 (−4.50)	159 (−3.00)	19.7 (−1.50)

[a] IBP: ibuprofen at 50 μg/mL.
[b] PCM: paracetamol at 10 μg/mL.

Table 5
Stability of low, medium, and high QCs at room temperature, −80 °C, and after freeze and thaw.

Nominal concentration (ng/mL)	Calculated concentration (ng/mL)				Calculated concentration (ng/mL)			Calculated concentration (ng/mL)		
	Stored at room temperature (h)				Stored at −80 °C (days)			Freeze/thaw cycles		
	0	2	5	0	13	38	101	0	1	3
0.5 (n = 3)	0.472	0.491	0.489	0.470	0.486	0.465	0.483	0.474	0.486	0.447
Precision (%CV)	±3.07	±3.87	±5.31	±2.25	±2.92	±8.64	±6.82	±2.25	±2.92	±8.84
% deviation from 0 h	–	+4.03	+3.60	–	+3.4	−1.13	+2.84	–	+3.40	−4.89
5 (n = 3)	5.11	5.12	4.99	4.59	4.97	4.74	4.66	4.59	4.97	4.82
Precision (%CV)	± 1.28	± 1.19	± 2.81	±0.98	±4.13	±1.48	±1.19	±0.98	±4.13	±5.46
% deviation from 0 h	–	+0.196	−2.35	–	+8.28	+3.42	+1.6	–	+8.28	+5.01
20 (n = 3)	21.3	21.1	20.2	18.5	21.2	19.0	18.8	18.5	21.2	19.90
Precision (%CV)	± 2.15	± 0.725	± 0.857	±1.43	±2.13	±3.43	±0.81	±1.43	±2.13	±3.20
% deviation from 0 h	–	−0.939	−5.16	–	+14.6	+2.52	+1.8	–	14.6	+7.57

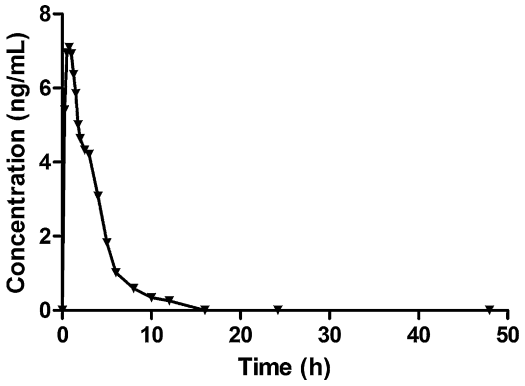

Fig. 6. Representative plasma concentration–time curve for one healthy volunteer administered a single oral tablet dose of RDR (35 mg).

3.3.5. Stability during sample processing

The results of this experiment showed that RDR is stable in plasma samples stored on ice for 2 h after separation from blood. Likewise, delays of up to 2 h before separation of plasma from collected blood samples stored on ice, did not significantly alter the measured RDR concentrations in human plasma samples.

3.4. Application

This validated LC–MS/MS bioanalytical method was successfully utilized for measurement of RDR concentrations in plasma samples. A representative plasma concentration versus time profile for one subject following oral administration of a single risedronate tablet (35 mg) is shown in Fig. 6.

4. Discussion

Measurement of low concentrations of bisphosphonates in biological matrices has been a challenge for analytical chemists. Various tedious, time-consuming, and not practical methods have been used by previous investigators including an enzyme-linked immunosorbent assay (ELISA) [11], ion-pair chromatography [5], co-precipitation of RDR with calcium in combination with a clean-up step such as protein precipitation [5], or ion-pair solid phase extraction [12]. Co-precipitation with calcium has also been combined with derivatization of ibandronate concentrations in human plasma and urine samples to be measured by GC–MS [9]. More recently, an LC–MS/MS method for the quantification of alendronate in human urine samples involving sequential derivatization of the amino and bisphosphonate groups followed by organic solvent extraction, enabled the tedious calcium precipitation steps of earlier methods to be avoided [13].

For risedronate and other bisphosphonates, methylation of phosphonic acid groups decreases polarity and its tendency to bind to metal ions, as well as improving chromatography and the ESI response in the MS [14]. On-cartridge derivatization of bisphosphonates proposed for measuring RDR in plasma and urine [10], greatly simplifies biological sample processing as it enables the multiple precipitation steps of earlier methods to be avoided whilst reducing matrix effects and enhancing the sensitivity of detection in the LC–MS/MS.

In present analytical methodology, the proposed approach by Zhu et al. [10] improved, validated and applied in a real clinical situation. An important step was using TMS-diazomethane instead of diazomethane as the derivitization agent. The advantages of TMS-diazomethane are that it is less toxic, thermally stable and non-explosive [15]. As phosphonic acid group methylation is more efficient, the reaction yield is potentially superior to that achieved with diazomethane [16]. Herein, we found that a 30 min on-cartridge exposure of derivatization reagent to RDR before complete elution using methanol, produced optimal methylation of the RDR phosphonic acid groups and that recovery of RDR from human plasma was >50%.

Our findings during method validation that RDR is stable in blood samples stored on ice for up to 2 h prior to centrifugation, and in plasma for up to 5 h at room temperature, highlight the practicality and applicability of our method with regard to clinical studies involving large cohorts of patients/volunteers. Importantly, we also found that commonly ingested analgesic agents such as ibuprofen and paracetamol did not interfere with the method which further enhances the utility of this method for measurement of RDR in plasma samples collected from patients taking this agent for the treatment of bone disorders.

5. Conclusions

Compared with previously published methods for the quantification of bisphosphonates in biological samples [4], the present SPE method using, on-cartridge derivatization with a relatively safe agent and LC–MS/MS, is simple, sensitive and robust. These features coupled with a short run time at 5-min, facilitates its application to high throughput measurement of RDR concentrations in human plasma samples collected from large cohorts of patients/volunteers in clinical studies. This derivatization approach can also be applied to the measurement of the concentrations of other bisphosphonates in biological matrices.

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