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Development and validation of ultra-high-performance liquid chromatography–mass spectrometry method for the determination of raloxifene and its phase II metabolites in plasma: Application to pharmacokinetic studies in rats

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Abstract

The aim of this study is to establish a reliable liquid chromatography–mass spectrometry method to simultaneously quantitate raloxifene, and its major metabolites, raloxifene-6-glucuronide, raloxifene-4'-glucuronide, and raloxifene-6-sulfate in rat plasma samples for pharmacokinetic studies. The separation of the analytes was achieved on a Waters BEH C₁₈ column. Water (0.1% formic acid) and acetonitrile were used as the mobile phases for elution. A one-step protein precipitation using a mixture solvent was applied for plasma sample preparation. The method was validated following the FDA guidance. The results showed that the linear range were 1.95–1000 nM for raloxifene-6-glucuronide, and raloxifene-4'-glucuronide, 0.195–100 nM for raloxifene-6-sulfate, and 0.195–200 nM for raloxifene, respectively. The lower limit of quantification was 1.95, 1.95, 0.195, and 0.195 nM for raloxifene-6-glucuronide, raloxifene-4'-glucuronide, raloxifene-6-sulfate, and raloxifene, respectively. Only 20 µl of plasma sample was required since the method is sensitive. The intra- and interday variance is <15% and the accuracy is within 85–115%. The variance of matrix effect and recovery were <15%. The method was successfully applied in a pharmacokinetic study in rats with oral administration of raloxifene.

Keywords

chromatography; mass spectrometry; metabolites; pharmacokinetic; raloxifene

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

ETHICAL APPROVAL

The study had been approved by the Institutional Animal Care and Use Committee at the University of Houston with approval number TR201800017.

1 | INTRODUCTION

Raloxifene (Ral, Figure 1A), a second-generation selective estrogen receptor modulator, is a prescription drug used by postmenopausal women to prevent and treat osteoporosis [1]. Ral increases the mechanical strength of the vertebral body without changes in bone density [2]. The drug suppresses osteoclast activity and bone remodeling in a manner similar to estrogen through high-affinity interactions with ER α 4 [3]. Clinical trials show that Ral decreases the incidence of breast cancer by 62~84% [4-7]. Studies also reveal that Ral can decrease plasma cholesterol levels. The major side effect associated with Ral is increment of risk for venous thromboembolism [8,9].

Ral is often used chronically for breast cancer prevention and treatment of osteoporosis for more than three years [1], drug exposure monitoring is necessary for safety issues.

The mechanism of efficacy and pharmacokinetic characteristics of Ral are still a hot topic as research papers are continuously published. It was reported that about 60% of the parent compound was absorbed in the gastrointestinal tract after oral administration, but only 2% reaches systemic circulation [10]. Further pharmaceutical studies revealed that low oral bioavailability is due to rapid metabolism as evidences have shown Ral is extensively metabolized by phase enzymes II (i.e. UDP-glucuronosyl transferases/UGTs, Sulfotransferases) into conjugates. The main metabolites are raloxifene-6-glucuronide (Ral-6-G), raloxifene-4'-glucuronide (Ral-4'-G), and raloxifene-6-sulfate (Ral-6-S) (Figure 1A) [11,12]. However, the disposition of Ral via the sulfonation pathway was not achieved enough studied [13]. Although the affinity of raloxifene's metabolites to estrogen receptors is only 1/20 of the parent drug [14], their concentrations are usually extremely high in different organs (including liver, lung, spleen, kidney, bone, and uterus) [15]. Whether Ral metabolites contribute to the efficacy is not well understood, but it is clear that metabolism plays an important role in lowering bioavailability. Therefore robust and sensitive methods not only for raloxifene but also for Ral metabolites quantification is required to study the pharmacological and pharmacokinetic characteristics of Ral [16].

There are a few studies reported quantification of Ral and some of its metabolite using LC-MS/MS methods. However, these methods either were not able to quantitate Ral-6-S or had poor assay recovery. For example, Trontelj et al. reported an LC-MS method to quantify Ral, Ral-6-G, and Ral-4'-G, but sulfate was not quantified. In addition, the extraction recovery of sample processing was only about 71% [17-20]. We report a sensitive, reliable ultra-high performance liquid chromatography (UHPLC)-MS/MS method that can simultaneously determine Ral and its three conjugates (Ral-6-G, Ral-4'-G, and Ral-6-S) in plasma sample with excellent extraction recovery.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Raloxifene hydrochloride, raloxifene-6-glucuronide, and raloxifene-4'-glucuronide were purchased from Toronto Research Chemicals (Toronto, Canada, all compounds purity 99%). Raloxifene-6-sulfate was synthesized in our laboratory using rat and mouse liver

S9 fraction, the purity of R-6-S was over 95% by ultra-performance liquid chromatography-UV. 3'-Phosphoadenosine-5'-phosphosulfate and sulfatase (without α -glucuronidase) were purchased from Sigma-Aldrich (St. Louis, MO). The MS grade water, methanol, and acetonitrile were obtained from EMD (Gibbstown, NJ, USA).

2.2 | Instruments and conditions

2.2.1 | Ultra-high performance liquid chromatography—The LC was performed with Exion LC analytical UHPLC systems (SCIEX company, CA, USA); BEH C₁₈ column (50 × 2.1 mm diameters, 1.7 μ m, waters, USA); The elution rate was 0.4 mL/min using a mobile phase containing 0.1% formic acid (mobile phase A, MPA) and 100% ACN (mobile phase B, MPB); the elution gradients were as follows: MPB, 0–2.0 min, 10–20% B, 2.0–3.0 min, 20–40% B, 3.0–3.5 min, 40–50% B, 3.5–4.0 min, 50–90 % B, 4–4.5 min, 90–90% B, 4.5–5.0 min, 90–10% B; column temperature, 40°C; injection volume, 5 μ L. Internal standard was formononetin.

2.2.2 | Mass spectrometry—The MS analysis was conducted on an AB SCIEX QTRAP 5500 System (Foster City, CA, USA) with a TurboIonSpray™ source. The biological samples were analyzed in Multiple Reaction Monitoring (MRM) negative mode. The ESI source was set as follows: ion spray voltage, 5.5 kV; ion source temperature, 450°C; nebulizer gas, nitrogen, 30 psi; turbo gas, nitrogen 25 psi; curtain gas, nitrogen 30 psi. Compound-dependent MRM parameters were followed as: Q1/Q3 transition masses were 648.1/472.1 for Ral-6-G, 648.2/472.4 for Ral-4'-G, 552.1/472.3 for Ral-6-S, 472.0/360.2 for Ral, and 267/252 for I.S; the declustering potential of Ral-6-S was –64 V, others were –100 V; the collision energy of Ral-6-G, Ral-4'-G, Ral-6-S, I.S and Ral were –48, –48, –40.6, –46, and –36 V, respectively. The collision cell exit potential of all compounds was –15 V.

2.3 | Biosynthesis of raloxifene-6-sulfate

2.3.1 | Preparation of mice and rat liver S9 fraction—Pooled liver S9 fraction was prepared from C57/BL6 mice (Male, 8 weeks, body weight 18–25 g, $n = 6$) or SD rat (Male, 8 weeks, body weight 180–250 g, $n = 6$) according to the published protocol and stored at minus 80°C freezer [21].

2.3.2 | Reaction—Biosynthesis of Ral-6-S was conducted according to the protocol used in our lab previously [22]. Briefly, 10 μ M Ral was typically incubated with PAPS and liver S9 fraction for 2 hours (final S9 concentration = 2 mg protein/mL) at 37°C. The reason we used the S9 fraction is that it contains the most sulfotransferases in comparison with the liver microsome.

2.3.3 | Raloxifene-6-sulfate purification—After incubation with the liver S9 enzyme, Ral-6-S was purified by liquid–liquid extraction. Due to the polarity difference between raloxifene and raloxifene sulfate, fourfold organic solvent dichloromethane was used to extract raloxifene from the reaction solvent twice, the two layers were separated using a separatory funnel. Then evaporate the water samples with nitrogen flow to afford Ral-6-S, which was dissolved in DMSO to prepare the stock solution.

2.3.4 | Structure determination—The biosynthesis was performed through sulfonation reaction with mice and rat liver S9 fraction following the published protocol [13]. After incubation with S9 fraction under sulfonation condition, an additional peak was afforded in the ultra-performance liquid chromatography with a diode array detector. Additional Structural confirmation was performed through UHPLC-MS/MS operating at both the positive and negative ion scan mode. We also analyzed the raloxifene mono-sulfates which were hydrolyzed to aglycone by sulfatase through UHPLC-MS/MS. Raloxifene mono-sulfates was incubated with 50 μ L sulfatase enzyme solution prepared in 1 M ammonium acetate buffer (pH=5) providing 10 units enzyme/ μ L at 37°C for 4 h. Hydrolysis was quenched by adding 100 μ L of methanol.

2.3.5 | Quantification of raloxifene-6-sulfate concentration—The concentration of Ral-6-S was determined using calibration curve of Ral, then divided by K (K is the conversion factor of extinction coefficients). The K value of the raloxifene sulfate metabolites was determined according to our previously published methods [23]. Briefly, after raloxifene sulfates hydrolysis by sulfatase, the decreasing peak area of sulfates divided by the increasing peak area of aglycone is equal to conversion factors K. Therefore, the concentrations of R-6-S equals the calculated concentrations of raloxifene divide by conversion factors K.

2.4 | Method validation

2.4.1 | Preparation of quality control and calibration curve samples—

Calibration curves and QC samples were prepared according to the procedure used previously [24]. Briefly, four stock solutions, each containing either 1 mM of Ral, 10 mM of Ral-6-G and Ral-4'-G, and 1 mM of Ral-6-S were prepared in DMSO: ethanol solvent (4:1, v:v). To prepare a working solution, the stock solutions were serially diluted in 50% methanol at concentrations of 4.88, 9.77, 19.50, 39.10, 78.10, 156.0, 313.00, 625.00, 1250.00, 2500.0, 5000.0, and 10 000.0 nM for R-6-G and R-4'-G; 0.488, 0.977, 1.95, 3.91, 7.81, 15.60, 31.30, 62.50, 125.00, 250.00, 500.0, and 1000.00 nM for R-6-S, and 0.977, 1.95, 3.91, 7.81, 15.60, 31.30, 62.50, 125.00, 250.00, 500.00, 1000.0, and 2000.0 nM for Ral, respectively. To prepare the calibration curve samples in plasma, the above working solutions (10 μ L) were spiked into 20 μ L blank rat plasma and were extracted with 300 μ L of methanol–acetonitrile (2:1, containing 50 nM formononetin as I.S.) by vortex-mixing for 1 min. Centrifuge the samples at $17\,000 \times g$ for 0.5 h at 4°C, then 80% supernatant was transferred to a new 1.5 mL tube and solvent was evaporated under N_2 flow. The residue was reconstituted in 100 μ L of 50% methanol and centrifuged at $17\,000 \times g$ for 0.5 h for LC-MS analysis.

Three quality control samples at high (500 nM for Ral-6-G and Ral-4'-G, 50 nM for Ral-6-S and 100 nM Ral), middle (62.5 nM for Ral-6-G and Ral-4'-G, 6.25 nM for Ral-6-S and 12.5 nM for Ral), and low concentrations (3.91 nM for Ral-6-G and Ral-4'-G, 0.39 nM for Ral-6-S and Ral) were prepared accordingly.

The calibration curve of raloxifene/its metabolites was carried out using Analyst 1.7.1 software supplied with the instrument (SCIEX, USA), weight $1/x^2$ was used to analyze the

slop. The LLOQ of the raloxifene and its conjugated considered at least signal-to-noise of 10:1 in plasma.

2.4.2 | Accuracy and precision—The quality control samples at different concentrations were on the same day to determine the intra-day precision and accuracy of the method. To evaluate inter-day precision and accuracy, the samples at different concentrations were injected into LC–MS on three different days.

2.4.3 | Recovery and matrix effect—The extraction recovery and matrix effect were determined using the QC samples at three different concentrations. Briefly, the peak area obtained from blank plasma spiked with the analytes was defined as A and that obtained from water spiked with the same amount of analytes was defined B. The peak area of blank plasma residue spikes with analytes was defined as C and that of water residue spiked with analytes was as D. The recoveries were calculated using B divided by A and matrix effect was calculated using C divided by D [23].

2.4.4 | Stability—The Short-term (room temperature for 24 h), long-term (–80°C for 15 days), and three freeze–thaw cycle (–80°C and 25°C) stabilities were determined using QC samples at low, middle, and high concentrations. Stability was expressed as the peak ratio of the mean peak area (triplicate samples) of an analyte at different time points to the mean peak area of the same analytes at time zero multiplied by 100.

2.5 | Pharmacokinetics study

2.5.1 | Animals—Female F344 Rats were obtained from Harlan Laboratory (Indianapolis, IN) and housed in the animal facility at the University of Houston within an environmentally controlled room (temperature, 23–27°C, humidity, 45–55%, and 12 h dark-light cycle) with free access of water.

2.5.2 | Experimental design—The animal protocols used in this study were approved by the IACUC at the University of Houston. Ral was given orally to rats at dose 10 mg/kg, the gavage volume was 1 mL. After the rats were anesthetized with isoflurane gas, blood samples (approximately 30–50 µL) were collected by snipping the tails at 0, 0.25, 0.5, 1, 2, 4, 6, and 24 h. Plasma and blood cells were separated by centrifugation (8000 rpm, 8 min). The plasma samples were kept at minus 80° C freezer.

2.5.3 | Plasma sample preparation—The 20 µL rat plasma was spiked with 300 µL of acetonitrile–methanol mixture (contained 50 nM formononetin (I.S.)). The plasma samples were prepared in the same procedure as the samples procedure described in Section 2.4.1. The supernatant (5 µL) was injected into LC–MS.

2.6 | Data analysis and statistical analysis

The rat pharmacokinetic data were analyzed by WinNon-lin 3.3 software (Pharsight, Mountain View, CA) with the non-compartmental model. In the statistical data analysis, data were presented as mean ± SD. All data analyses were conducted using Stata version

11.0 (StataCorp, USA), the paired Student's T-test was used to analyze the effect of different solvent on the extraction recovery, $P < 0.05$ was considered statistically significant.

3 | RESULTS AND DISCUSSION

3.1 | LC–MS chromatogram

The current method was reported as the first time to quantitate Ral-6-S, Ral, Ral-6-G, and Ral-4'-G simultaneously in biological samples. The developed method is specific, sensitive, and reliable and successfully applied to determine the plasma concentration of raloxifene and its conjugated in vivo. A typical MRM chromatogram of spiked raloxifene and its three metabolites in blank rat plasma sample were in Figure 1B. The retention time of Ral-6-G, Ral-4'-G, Ral-6-S, Ral, and formononetin (I.S.) were 2.88, 3.11, 3.27, 3.45, and 3.97 min, respectively.

3.2 | Identification of raloxifene-6-sulfate

The structure of Ral-6-S was identified using multiple approaches. When raloxifene was incubated with enzymes under sulfonation condition, an additional peak was afforded in LC–UV analysis (Figure 2). The additional peak can be hydrolyzed back to raloxifene by sulfatases (section 2.3.4), suggesting that this peak is a sulfate-conjugated peak. The UV spectra of this addition peak showed two peaks at 286 and 222.9 nm, which are similar with those of raloxifene (288.4 and 223.1 nm, Figure 2A), revealing that the backbone of this peak is as same as that of raloxifene. The LC-MS analysis showed that the pseudo molecular ion of this addition peak is m/z 552 $[\text{Ral}+80-\text{H}]^-$, MS/MS analysis showed a fragmental ion at m/z 472 $[\text{Ral}-\text{H}]^-$. In the positive mode, we also observed the $[\text{Ral}+80+\text{H}]^+$ at m/z 554.8 (Figure 2B, C). The neutral losses of 80 Da (SO_3) is indicative of sulfate metabolites. These findings demonstrated that this addition peak is a sulfate of raloxifene. It was reported that when raloxifene was incubated with rats S9 fraction, only Ral-6-S was afforded [13]. Therefore, the position is tentatively assigned at C-6 in the structure of raloxifene. When the reaction solution was incubated with sulfatase, a typical sulfate hydrolysis reaction, the additional peak was disappeared. The K value was 1.02 and the final concentration of Ral-6-S was 80 μM .

3.3 | Linearity and lower limit of the quantification

Ral, Ral-6-G, Ral-4'-G, and Ral-6-S standards extracted from rat plasma show a linear calibration curve over the dynamic range of 0.195–200 nM, 1.95–1000 nM, 1.95–1000 nM, and 0.195–100 nM with a correlation coefficient (r^2) of 0.9913 or better. The LLOQ was 0.195, 1.95, 1.95, and 0.195 nM for Ral, Ral-6-G, Ral-4'-G, and Ral-6-S, respectively. The LLOQ of raloxifene's glucuronides was similar to the published method [19]. The LLOQ of Ral-6-S in this method was 0.195 nM. The calibration equation were $Y = 0.000789X + 0.00014$ for Ral-6-G, $Y = 0.000978X - 0.000033$ for Ral-4'-G, $Y = 0.00146X + 0.000285$ for Ral-6-S and $Y = 0.00215X + 0.00271$ for Ral, respectively. The representative chromatograms of blank plasma and blank plasma spiked with the analytes at LLOQs are shown in Figure 3.

3.4 | Extraction recovery using different solvent

The extraction recovery was evaluated using methanol, acetonitrile, and methanol–acetonitrile. The result showed that a mixture of methanol and acetonitrile (2:1, v/v) enhanced the extraction recovery (Table 1). Methanol and acetonitrile are too common solvent used as protein precipitation solvents. Our results showed that the extraction recovery of raloxifene and its metabolites were different when these two solvents were tested. Briefly, when acetonitrile was used alone, only Ral-6-S can achieve the satisfying extraction recovery (i.e., >85%). The recovery for the other three analytes was less than the required value. However, when methanol was used alone, recovery for all the metabolites was acceptable. The maximal recovery for raloxifene was only 35%. When mixture solvent (methanol/acetonitrile, 2:1) was used, the recovery for all analytes were >85%, which is in the acceptable range. Therefore, we use a mixture of methanol and acetonitrile as the extraction solvent. For the reason why the recovery was different when different solvents were used, methanol is a polar-protic solvent, whereas acetonitrile is a polar-aprotic solvent. Therefore the protein precipitation solvent methanol is suitable for hydrophilic metabolites of Ral, acetonitrile is suitable for hydrophobic Ral. The lipophilicity value of Ral (LogP = 5.78) is higher than its metabolites (LogP of Ral-6-G, Ral-4'-G, and Ral-6-S are 3.9, 3.9, 3.6, respectively, which calculated by Marvin Sketch 6.0). Therefore, the mixture of methanol and acetonitrile is better.

3.5 | Precision, accuracy, and matrix effect

The results of precision, accuracy, and matrix effect are shown in Table 2. The intra-day, inter-day precision, and accuracy were in the acceptable range (85–115%), suggesting the method is accurate. The results also showed that the matrix effect of this method is in acceptable range.

3.6 | Stability

The results of stability studies were in Table 3. The shortterm (room temperature, 24 h), long-term freezer storage (−80°C, 15 days), and within three freeze-thaw cycles (−80 to 25°C) stability tests of raloxifene and its conjugated were determined using the QC samples. The results showed that all QC samples stability displayed between −12.5 and 14.2% variation. Taken together, the method validation meets the request for the analysis raloxifene and its conjugated in rat plasma. The validated LC-MS assay is sensitive, reproducible, and robust.

Our one-step protein precipitation with combined solvent is more simple and rapid than previously published assay which involved solid phase extraction procedure or Thermo Scientific™ SOLAμ™ SCX 96-well plate [17-20]. In addition, our method can simultaneously quantify both raloxifene glucuronide and sulfate in plasma. Previous method can either only quantify raloxifene and/or glucuronide, or not in plasma samples.

3.7 | pharmacokinetics study

We applied the validated LC–MS methods to a raloxifene pharmacokinetic study. The plasma concentration-time profiles of raloxifene and its three phase II metabolites after oral administration were shown in Figure 4. The PK parameters were calculated using

noncompartmental model (Table 4). The results showed that the C_{max} of Ral, Ral-6-G, Ral-4'-G, and Ral-6-S was 103.1 ± 66.7 , 1162 ± 625.3 , 59.4 ± 34.2 , and 10.6 ± 2.0 nM, respectively. The T_{1/2} for Ral, Ral-6-G, Ral-4'-G, and Ral-6-S was 12.9 ± 4.5 , 9.4 ± 6.2 , 6.3 ± 2.5 , and 20.2 ± 2.7 h, respectively. And the AUC of Ral-6-G was 18.4-fold higher than that of Ral-4'-G, 76-fold higher than that of Ral-6-S, 7.5-fold higher than Ral. Ral-6-G is the main metabolite in the rat; however, both Ral-6-G and Ral-4'-G are the main metabolites. The metabolite Ral-4'-G had the faster CL and V_z than other metabolites. In the PK study, the metabolite Ral-6-G was the major metabolite in rats, but the metabolite Ral-4'-G is the major in human. We also measure a few quantities of sulfate exposed in blood. In addition, the sulfonation is also the important excretion pathway of Ral and can be reabsorption into the blood after hydrolysis to aglycone by intestinal bacteria, therefore determine the disposition of raloxifene-sulfate in human is necessary.

4 | CONCLUSION

A highly reproducible, robust, and sensitive LC-MS method for quantification of raloxifene and its three phase II metabolites (R-6-G, R-4'-G, and R-6-S) was established and validated. We optimized the sample processing using protein precipitation with methanol and acetonitrile (2:1, v/v) as the solvent, which can significantly enhance the extraction recovery of the analytes. We determined the drug concentration in the plasma for at least 3 half-lives as required in rat p.o. administration of 10 mg/kg raloxifene, therefore the LLOQ of raloxifene and its three metabolites were well below the lowest concentrations. The method was successfully applied for rat pharmacokinetic study by using only 20 µL of rat plasma sample. Therefore, this method could be transferred and applied to raloxifene human clinical study, and simultaneously investigated the sulfonation and glucuronidation pathway of raloxifene.

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Article-Related Abbreviations:

I.S.	internal standard
LLOQ	lower limit of quantification
PK	pharmacokinetics
QC	quality control
Ral	raloxifene
Ral-4'-G	raloxifene-4'-glucuronide
Ral-6-G	raloxifene-6-glucuronide
Ral-6-S	raloxifene-6-sulfate

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FIGURE 1.

Chemical structures of raloxifene (Ral), raloxifene-6-glucuronide (Ral-6-G), raloxifene-4'-glucuronide (Ral-4'-G), and raloxifene-6-sulfate (Ral-6-S) (A), and the typical chromatograms of Ral(200 nM), Ral-6-G(1000 nM), Ral-4'-G(1000 nM), Ral-6-S(100 nM) and internal standard in LC-MS (B)

FIGURE 2.

The UV spectra and MS spectra of Ral-6-S. The UV profile of raloxifene (blue line) and Ral-6-S (red line) determined by a diode array detector from 200 to 400 nm (A). The MS spectra and fragmentation pathways of Ral-6-S in positive mode (B) and negative mode (C)

FIGURE 3.
Representative MRM chromatograms of (A) blank plasma spiked with the analytes at LLOQs and (B) blank plasma

FIGURE 4.

Time vs. blood concentrations of Ral (A) and Ral-4'-G (B), Ral-6-G (C), and Ral-6-S (D) in rats after oral administration raloxifene 10 mg/kg ($n = 4$)

TABLE 1
Extraction recovery for raloxifene and its three conjugates with different organic solvent ($n = 3$)

Analyte	QC concentrations (mM)	Extraction recovery, Average $\pm$ SD (%)		
		ACN	MeOH	MeOH: ACN (V:V = 2:1)
Ral-6-G	3.91	17.4 $\pm$ 6.9	93.9 $\pm$ 10.2	108.3 $\pm$ 7.5 [*]
	62.5	24.8 $\pm$ 6.9	103.3 $\pm$ 7.7	101.2 $\pm$ 10.9 [*]
	500	17.5 $\pm$ 0.2	113.1 $\pm$ 4.4	110.5 $\pm$ 10.7 [*]
Ral-4'-G	3.91	48.6 $\pm$ 7.7	116.4 $\pm$ 12.1	114.7 $\pm$ 2.6 [*]
	62.5	52.8 $\pm$ 14.8	107.4 $\pm$ 9.7	105.4 $\pm$ 11.3 [*]
	500	50.6 $\pm$ 5.2	123.6 $\pm$ 8.4	119.9 $\pm$ 7.6 [*]
Ral-6-S	0.39	103.3 $\pm$ 5.5	93.2 $\pm$ 8.7	93.6 $\pm$ 6.4
	6.25	93.3 $\pm$ 2.7	107.1 $\pm$ 11.0	107.8 $\pm$ 8.8
	50	95.1 $\pm$ 1.6	117.5 $\pm$ 4.1	112.4 $\pm$ 11.0
Ral	0.39	24.4 $\pm$ 7.3	34.9 $\pm$ 1.2	96.8 $\pm$ 7.6 ^{*#}
	12.5	26.8 $\pm$ 5.1	17.7 $\pm$ 0.5	103.5 $\pm$ 13.7 ^{*#}
	100	110.2 $\pm$ 0.3	20.2 $\pm$ 0.7	82.0 $\pm$ 10.4 ^{*#}

ACN, acetonitrile; MeOH, methanol;

^{*} means compared with ACN group;

[#] means compared with methanol group, $P < 0.05$.

TABLE 2

Inter-day and intra-day precision, accuracy, and matrix effect of raloxifene and its three conjugates in MRM mode for UHPLC-MS/MS analysis

Analyte	Concentration (nM)	Matrix effect Average $\pm$ SD (%)	Intra-day		Inter-day	
			Precision (RSD, %)	Accuracy	Precision (RSD, %)	Accuracy
Ral-6-G	3.91	120.0 $\pm$ 13.6	3.2	90.5	3.6	112.4
	62.5	100.2 $\pm$ 10.8	3.1	92.9	7.0	94.7
	500	97.4 $\pm$ 0.3	7.0	105	3.4	98.4
Ral-4'-G	3.91	118.6 $\pm$ 11.2	1.4	86.6	3.8	85.9
	62.5	96.8 $\pm$ 10.8	3.9	89.1	3.2	89.1
	500	104.6 $\pm$ 5.8	5.8	99.1	3.2	98.3
Ral-6-S	0.39	101.8 $\pm$ 11.9	2.1	82.9	4.5	87
	6.25	100.3 $\pm$ 1.4	2.3	90.9	6.7	90.6
	50	98.8 $\pm$ 3.7	1.3	96.4	1.4	93.6
Ral	0.39	101.0 $\pm$ 6.1	10.3	112.5	12.5	106.3
	12.5	98.4 $\pm$ 5.5	14.4	105.4	11.7	101.8
	100	107.6 $\pm$ 8.6	9.1	91.1	5.5	97.4

TABLE 3
Stability of raloxifene and its metabolites in rat plasma under different storage conditions ($n = 3$)

Analytes	Spiked concentration (nM)	4°C for 24 h			Three freeze-thaw cycles			Frozen for 15 days		
		Precision (RSD, %)	Accuracy (RE, %)		Precision (RSD, %)	Accuracy (RE, %)		Precision (RSD, %)	Accuracy (RE, %)	
Ral-6-G	3.91	8.9	13.7		13.7	12.9		8.9	13.2	
	62.5	3.6	14.2		3.8	11.9		3.1	13.1	
	500	4.2	12.1		5.3	13.3		1.5	10.3	
Ral-4'-G	3.91	11.7	8.8		13.9	11.1		11.7	7.9	
	62.5	1.0	12.0		12.9	11.3		1.0	8.1	
	500	9.4	11.5		10.5	15.8		9.4	7.5	
Ral-6-S	0.39	13.1	4.4		8.4	5.5		11.2	-5.6	
	6.25	8.7	-0.9		0.5	-12.5		8.5	-0.9	
	50	10.9	10.8		13.4	7.5		4.8	12.0	
Ral	0.39	15.1	3.6		14.2	13.9		14.1	6.4	
	12.5	5.7	14.0		11.9	-1.0		5.7	13.4	
	100	7.5	4.9		4.5	9.1		7.5	4.6	

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TABLE 4

Pharmacokinetic parameters of Ral and its metabolites after oral administration of raloxifene (10 mg/kg) to rat ($n = 4$)

Parameters	Ral-6-G	Ral-4'-G	Ral-6-S	Ral
T _{max} (h)	2.42 ± 3.13	3.7 ± 2.5	4.7 ± 1.2	2.67 ± 1.15
C _{max} (nmol/L)	1162 ± 625.3	59.4 ± 34.2	10.6 ± 2.0	103.1 ± 66.7
AUC ₀₋₁ (nmol h/L)	6654.2 ± 1276.9	361.5 ± 113.4	36.1 ± 13.2	891.6 ± 521.4
AUC _{0-∞} (nmol h/L)	7673.4 ± 943.5	453.8 ± 101.0	87.6 ± 18.4	1209.2 ± 527.4
MRT (h)	6.2 ± 1.2	6.3 ± 0.8	3.8 ± 0.6	8.4 ± 0.7
T _{1/2} (h)	9.4 ± 6.2	6.3 ± 2.5	20.2 ± 2.7	12.9 ± 4.5
CL (L/h/kg)	2.8 ± 0.34	49.8 ± 11.5	6.19 ± 0.11	20.5 ± 10.5
V _z (L/kg)	39.1 ± 29.3	476.6 ± 288.9	18.38 ± 27	422.9 ± 341.1