Journal of Bioanalysis & Biomedicine

Jadhav and Ramaa, J Bioanal Biomed 2012, 4:4
DOI: 10.4172/1948-593X.1000064

Research Article Open Access

Development and Validation of a UPLC-MS/MS Assay for Simultaneous Estimation of Raloxifene and its Metabolites in Human Plasma

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Abstract

An aim of the study is development and validation of a method for the simultaneous estimation of raloxifene (RAL) and its two active metabolites, raloxifene-4-glucuronide (R4G) and raloxifene-6-glucuronide (R6G) in human plasma samples using raloxifene-D4 as an Internal Standard. Sample preparation was performed by solid phase extraction (SPE) and was followed by separation of the analytes on a UPLC system with a linear gradient and a mobile phase consisting of acetonitrile and ammonium formate. Detection was achieved by tandem mass spectrometry operated in the electrospray positive ion mode. The method had a short sample preparation time, as well as a chromatographic run time of just 4.2 min, the shortest so far reported for RAL, R4G and R6G determination. It was validated and fulfilled all preset criteria for sensitivity, specificity, linearity, within inter- and intra-accuracy and precision, stability studies for all molecules. The method was linear in the concentration range of 0.040 to 1.5 ng/mL, 0.6 to 50.0 ng/mL and 0.6 to 50.0 ng/mL for RAL, R4G and R6G, respectively. The proposed method could be applied to the rapid and reliable simultaneous determination of RAL, R4G and R6G in a bioequivalence study.

Keywords: Raloxifene hydrochloride; Raloxifene glucuronides; UPLC-MS/MS; Plasma samples

Abbreviations: RAL: Raloxifene; R4G: Raloxifene-4'-Glucuronide; R6G: Raloxifene-6'-Glucuronide; IS: Internal Standard; UPLC-MS/MS: Ultra Performance Tandem Mass Spectrometry; AQ-LLOQ: Aqueous Lower Limit of Quantification; AQ-LQC: Aqueous Low Quality Control Sample; AQ-MQC: Aqueous Middle Quality Control Sample; AQ-LQC: Aqueous High Quality Control Sample.

Introduction

Raloxifene ([6-hydroxy-2-(4-hydroxyphenyl)-benzothiophen-3-yl]- [4-[2-(1-piperidyl) ethoxy] phenyl]-methadone) a non-steroidal selective estrogen receptor regulator, is currently applied to both the prevention and treatment of postmenopausal osteoporosis [1,2]. It acts as an estrogen agonist in bone and liver and in this way increases bone mineral density and decreases LDL-cholesterol [3]. Raloxifene is rapidly absorbed from the gastrointestinal tract and undergoes extensive first-pass glucuronidation, predominantly raloxifene-4'-glucuronide (R4G) and raloxifene-6'-glucuronide (R6G) [4-6]. Approximately 60% of an oral dose is absorbed; however, because of extensive presystemic glucuronide conjugation, absolute bioavailability is only 2%. Significant interpatient differences in bioavailability may result from alterations in the rate of glucuronide formation and enterohepatic recycling [7]. Various HPLC and LC-MS/MS methods, validated as effective and selective, have been for the detection of Raloxifene hydrochloride [8-10]. Trontelj et al. developed and validated raloxifene LC-MS/MS method along with its metabolites and the limit of quantification were 0.0880 to 60.0000 ng/ml, 0.2000 to 340.0000 ng/ml, and 1.6000 to 2720.0000 ng/ml for RAL, R4G and R6G, respectively with 16 min run time [11]. Therefore, the purpose of this study was to develop and validate a rapid and more sensitive UPLC-MS/MS method to quantify RAL, R4G and R6G in human plasma and apply it for the simultaneous determination of RAL, R4G and R6G in a bioequivalence study.

Materials and Methods

Chemicals and reagents

Raloxifene HCL (purity 99.64%), Raloxifene-4'-glucuronide (purity 97.50%), Raloxifene-6'-glucuronide (purity 97.30%), and Raloxifene-D_4 (purity 98.20%), were obtained from Varda Biotech (P) Ltd. (India). Acetonitrile (HPLC grade, J.T. Baker), methanol (HPLC grade, J.T. Baker), ammonium formate (GR, J.T. Baker) and formic acid (GR, Merck) were also purchased from Sigma. Purified water was for UPLC from Milli-Q system (Millipore, Germany).

Mass spectrometry

Mass spectrometry was performed using a Waters Quattro Premiere XE triple-quadrupole mass spectrometer (Micromass™ MS Technologies) equipped with an electrospray ionization (ESI) source. Multiple reaction-monitoring (MRM) modes were used for the determination of RAL, R4G and R6G, because of its high selectivity. The ion transitions monitored were Raloxifene *m/z*; 474.30 (precursor ion) and 112.06 (product ion), R4G *m/z;* 650.20 (precursor ion) and 474.40 (product ion), R6G *m/z;* 650.20 (precursor ion) and 474.40 (product ion) and Raloxifene-D_4 *m/z;* 478.40 (precursor ion) and 116.10 (product ion). Collision-induced dissociation (CID) was performed using 3.5×10^{-1} Pa argon. The cone potential 50, 45, 45 and 65V were the optimum values for RAL, R4G, R6G and RD4, respectively, in positive-ion mode. The capillary potential was 3.0 kV for all analytes and the entrance and exit energies of the collision cell were −1 and 1eV, respectively. Desolvation gas was used at flow rates of 1000 Lh^{-1}, respectively. The optimum source and desolvation temperatures were found to be 100 and 450°C, respectively. The dwell time for each transition was 200 ms and the interchannel delay was 20 ms. The system was controlled by Masslynx V 4.1 software (Waters, USA).

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Received July 29, 2012; **Accepted** August 20, 2012; **Published** August 23, 2012

Citation: Jadhav DH, Ramaa CS (2012) Development and Validation of a UPLC-MS/MS Assay for Simultaneous Estimation of Raloxifene and its Metabolites in Human Plasma. J Bioanal Biomed 4: 061-067. doi:10.4172/1948-593X.1000064

Liquid chromatography

Chromatographic separation was performed on a Waters Aquity UPLC system using an Aquity UPLC BEH® C18, 2.1×50 mm, 1.7 µm column with an installed guard column (Waters, USA) at 40°C. The injection volume was 8 µL. Mobile phase A was acetonitrile: methanol (50:50), and mobile phase B was ammonium formate solution (10 mM). The separation required gradient elution presented in Table 1. This gradient step greatly improved the reproducibility of MS detector response, as against 100% organic solvent.

Preparation of standard and quality-control solutions

Preparation of standard solutions: Weighed about 2.500 mg of each RAL, R4G, R6G and RD4 (internal standard) working standards. It was dissolved in methanol and made up the volume with the same, to produce a solution of 100 µg/mL of RAL, R4G, R6G and RD4. Corrected the above concentration of RAL, R4G, R6G and RD4 solution accounting for its potency, molecular weight and the actual amount weighed. Stored the stock solution in cooling cabinet set at -10°C and was used to prepare eight fresh working spiking solutions. Separately, from different weighing, primary stock solutions, a standard stock solution and working spiking solutions were prepared and used for quality control samples (QCs).

Preparation of aqueous calibration and quality control samples: Diluted the stock solution to suitable concentrations using a mixture of methanol: water, (75:25, v/v) to prepare aqueous calibration standards separately for RAL, R4G and R6G. The aqueous calibration standards were prepared in the range of 1.5684 ng/mL to 60.8896 ng/mL for RAL, 47.3616 ng/mL to 4025.7360 ng/mL for R4G and 47.9654 ng/mL to 4029.0934 ng/mL for R6G.

Prepared calibration standards consisting of Raloxifene, Raloxifene-4′-Glucuronide and Raloxifene-6′-Glucuronide at concentrations mentioned below:

Raloxifene Concentrations (ng/mL): 0.0392, 0.0923, 0.2306, 0.4613, 0.6919, 1.0379, 1.2685, 1.5222.

Raloxifene-4′-Glucuronide Concentrations (ng/mL): 0.5920, 1.1840, 2.9601, 5.9202, 17.7606, 29.6010, 40.2574, 50.3217

Raloxifene-6′-Glucuronide Concentrations (ng/mL): 0.5996, 1.1991, 2.9978, 5.9957, 17.9870, 29.9784, 40.7706, 50.3637.

The concentrations of aqueous quality control (QC) samples for RAL were 1.5812 ng/mL (AQ-LLOQ QC), 4.6507 ng/mL (AQ-LQC), 23.2536 ng/mL (AQ-MQC), and 53.4833 ng/mL (AQ-HQC). The concentrations of aqueous quality control (QC) samples for R4G were 47.4739 ng/mL (AQ-LLOQ QC), 123.4322 ng/mL (AQ-LQC), 1898.9568 ng/mL (AQ-MQC), and 3418.1222 ng/mL (AQ-HQC). The concentrations of aqueous quality control (QC) samples for R6G were 48.0854 ng/mL (AQ-LLOQ QC), 125.0221 ng/mL (AQ-LQC), 1923.4171 ng/mL (AQ-MQC), and 3462.1507 ng/mL (AQ-HQC). The aqueous quality control samples were stored in cooling cabinet set at -10°C. This concentration ratio of RAL: R4G:R6G was chosen so that that the spiked plasma samples would closely resemble the real plasma samples from subjects. The aqueous calibration and quality control sample solutions were kept in cooling cabinet set at -10°C and were used to prepare eight fresh working spiking solutions.

Preparation of calibration and quality control samples: Each of eight plasma calibration samples (CS) was prepared by spiking 950 µL of human plasma with 25 µL, 12.5 µL and 12.5 µL working spiking solutions of RAL, R4G and R6G, respectively. The concentrations of RAL, R4G and R6G ranged from 0.0392 to 1.5222 ng/mL, from 0.5920 to 50.3217 ng/mL and from 0.5996 to 50.3637 ng/mL, respectively. The quality control samples (Table 2) were prepared in the same way, and at four levels, LLOQ (LLOQ QC), low (LQC), medium (MQC), and high (HQC). The CS & QCs were aliquoted and stored in deep freezer below -70°C.

Sample preparation: Calibration standards, quality control samples and/or stability samples were removed from deep freezer and allowed them to thaw completely at room temperature. Pipetted 50 µL of 1000.0000 ng/mL RD4 solution as internal standard (I.S.) into pre-labeled polypropylene vials, except in vials labeled as blank samples wherein 50 µL of methanol: water (75:25, v/v) was added. 500 µL aliquot of plasma samples were added and 50 µL of 0.1% Formic Acid solution was added into these vials. The samples were subjected to a solid phase extraction (SPE) procedure using OASIS® HLB Cartridges (1 cc, 30 mg) on EZYPRESS™ 48 Positive Pressure Processor (Lombard, IL, USA). Before the samples were loaded, the SPE cartridges were sequentially conditioned with 1 mL of methanol and 1 mL of water. The Cartridges were washed sequentially with 1 mL water and 1 mL of 10% methanol in water, followed by drying with nitrogen gas for 2 min (25 psi). The elution was performed with 1mL of acetonitrile: methanol (50:50, v/v). The eluants were dried in a stream of nitrogen at 50°C in a Turbovap apparatus (Speedovap, India). The dried samples were reconstituted with 0.300 mL Acetonitrile: Water (10:90, v/v) and transferred to autosampler vials.

Method Validation Parameters and Procedures

Specificity, linearity and limits of detection and quantification

Specificity was determined by analyzing aqueous sample at middle (MQC) concentration. The presence or absence of any interfering peaks at the retention times of analytes or the internal standard was evaluated.

In order to assess the linearity of the detector response, a series of plasma calibration samples were prepared as described in previous section. On 3 days of validation, three standard calibration curves containing eight non-zero calibrators were prepared and analyzed by linear regression in the concentration range from 0.0392 to 1.5222 ng/mL, from 0.5920 to 50.3217 ng/mL and from 0.5996 to 50.3637 ng/mL,

Time (min)	Flow ml/min	%A	%B
0.00	0.300	10	90
1.00	0.300	30	70
3.80	0.300	90	10
4.20	0.300	10	90

Table 1: Gradient employed for successful separation of raloxifene metabolites.

	RAL (ng/mL)	R4G (ng/mL)	R6G (ng/mL)
LLOQ QC	0.0395	0.5934	0.6011
LQC	0.1163	1.5429	1.5628
MQC	0.5813	23.7370	24.0427
HQC	1.3371	42.7265	43.2769

Table 2: Concentrations of RAL, R4G and R6G in quality control samples (LLOQ QC, LQC, MQC, and HQC) achieved.

RAL, R4G and R6G, respectively. A correlation coefficient of more than 0.98 was set as acceptable, otherwise the calibration run would have been rejected. Back calculation of the concentration was made for each calibration sample. The detection limit was set as the dilution showing a signal-to-noise ratio of more than 10. The limit of quantification was not determined based on a signal-to-noise ratio (although this ratio exceeded 20), but was set as the lowest standard on the calibration curve that exhibits acceptable accuracy and precision (deviation from the nominal value of less than ± 20%) [12].

Accuracy and precision

Accuracy and precision were determined from the calibration curve and detector responses from six replicates of each quality control sample (LLOQ QC, LQC, MQC, and HQC) on each of the 3 days of validation. Within-day precision was calculated as the coefficient of variation of analysis of six replicate LLOQ QC, LQC, MQC, and HQC samples. For between-day precision, analyses of the same six replicate samples at LLOQ QC, LQC, MQC, and HQC concentration levels were performed on 3 different days. Accuracy was deemed acceptable when the calculated concentration was within ±15% of the nominal concentration, except at the limit of quantification where it should not deviate more than ±20%. Similarly, precision was acceptable when the coefficient of variation of replicates was smaller than ±15%, except at the LLOQ, where it should not exceed ±20% [12].

Recovery and matrix effects

The samples for recovery determination were prepared at three concentration levels in six replicates by spiking the analytes to blank plasma before extraction. The recovery reference samples were prepared by spiking the reconstitution solvent with the same amounts of working spiking solution as used for the plasma samples. Plasma samples were extracted with the described SPE method, while the recovery reference samples were left unextracted. Both types of samples were immediately analyzed by UPLC–MS/MS and the recovery was calculated as a ratio between the detector response of extracted plasma samples and the response of recovery reference samples.

In the development of every quantitative LC-ESI/MS method, the matrix effect should be thoroughly assessed [13,14]. Eight different blank plasma lots were used to evaluate whether different plasma matrices could suppress or enhance the signal of the internal standard or any of the analytes. For each of the LLOQ concentration levels, eight 500 µL aliquots of each plasma lot were extracted and then spiked with the analytes for subsequent analysis. The corresponding peak areas were compared to the responses of analytes spiked to the neat reconstitution solvent, at the same concentration level. The matrix effect was then calculated as a ratio of the former to the latter and multiplied by 100% [14].

Stability

The stability was evaluated by analyzing QC samples and comparing the concentrations found to the nominal values.

Stock solution stability

The stock solutions were aliquoted and kept at −10°C and five replicates were thawed on three different occasions, the last after 12 days, appropriately diluted with the reconstitution solvent and immediately analyzed by UPLC–MS/MS.

Short-term sample stability or bench-top stability

Similar to the long-term sample stability testing, six quality control samples at low and high concentration levels were analyzed after 6 h at room temperature (25 ± 3°C).

Autosampler stability

Six replicates of quality control samples at low and high concentration levels were extracted, dried, reconstituted and left in the autosampler for minimum 8 h at 5°C and then injected and quantified.

Freeze–thaw stability

Six replicates of quality control samples at low and high concentration level were stored at −80°C. Thawing was performed at room temperature, followed by freezing for 24 h. The samples were subjected to two more freeze–thaw cycles before being extracted and analyzed by UPLC–MS/MS.

Long term storage stability

Six replicates of quality control samples at low and high concentration level were stored at −80°C, thawed after 55 days, extracted and analyzed by UPLC–MS/MS.

Results

Performance of MS–MS

The electrospray interface was used to obtain good sensitivity, fragmentation, and linearity. We tested atmospheric-pressure ionization (APCI) and no obvious improvement was observed. The first step in developing the method of detection was to select the precursor ion to be fragmented. The analytes and the internal standard (IS) were monitored in positive-ion mode because of their high signal intensity. Because the $[M+H]^+$ ions of RAL (*m/z* 474.30), R4G (*m/z* 650.20), R6G (*m/z* 650.20), and RD4 (*m/z* 478.40) were the most intense they were chosen as the precursor ions for the determination. Capillary, cone, extractor, and RF potentials were optimized to obtain maximum signal intensity for these precursor ions. After optimization of these conditions the product-ion spectra of the analytes and the internal standard were obtained. The base peaks of the product ions were observed at *m/z* 112.06 for RAL, *m/z* 474.40 for both R4G and R6G in positive-ion mode. The chemical structures of analytes and the IS are presented in Figure 1. Collision energy was optimized in relation to the intensity of the selected product ions. The RAL, R4G, R6G and RD4 MS scan spectra are shown in Figure 2.

Performance of LC

Several analytical columns (Kromasil & Discovery C8, C18 embedded phase, Supelco; Xterra C18, Xbridge C18, and Aquity UPLC BEH C18, Waters) were tested to obtain the maximum response and good separation of RAL, R4G and R6G with reasonable analysis time. Symmetrical peak shapes of RAL, R4G, R6G and RD4 standards were not easily obtained on C8. The Aquity UPLC BEH® C18 (2.1×50 mm, 1.7 µm) was eventually selected for all assays because it furnished excellent peak shape and the best response for RAL, R4G, R6G and RD4 with acceptable analysis time. Selection of the mobile-phase components was also critical. We used 10 mM ammonium formate to adjust the ionic strength. Increasing the amount of buffer in the mobile phase enhanced analyte peak symmetry and resolution but simultaneously increased the retention times of RAL, R4G and R6G. Finally, a two-component linear gradient mobile phase containing acetonitrile: methanol and ammonium formate was used, with satisfactory results.

Method Validation

Specificity, selectivity, linearity, LOD and LLOQ

The chromatogram of aqueous samples from AQ MQC of analytes and working concentration of IS presented in (Figures 3a-3d), show good resolution. Specificity was confirmed by the absence of any peaks at the *m/z* and retention times of the analytes and IS in aqueous sample.

The limits of quantification achieved are shown in Table 1. The chromatogram of LLOQ plasma sample consists of RAL, R4G, R6G and RD4 presented in figure 4. The method showed good linearity over the entire concentration range, exhibiting a correlation coefficient (r^2) of 0.99 or higher (Table 3).

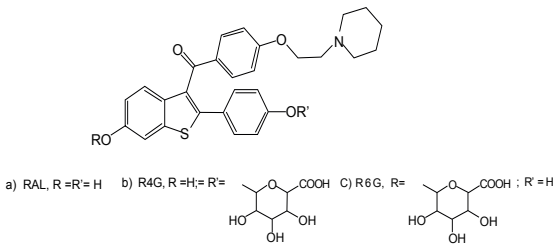

Figure 1: Structures of Raloxifene and its metabolites.

Accuracy and precision

The within-day and between-day precision and accuracy are within acceptable limits and are presented in Tables 4-6 for RAL, R4G and R6G, respectively.

Recovery and matrix effect

The recovery of RAL was somewhat lower: 72 and 67% at low and high levels, respectively. The recoveries of R4G and R6G at low and high levels ranged from 84 to 97% and the recoveries of RD4 was 76%.

The extraction efficiency of RAL and RD4 were lower than the R4G and R6G, hence they demonstrated comparatively lower recoveries. However similar and consistent recoveries were observed for RAL and RD4, because they are structurally similar. Due to precise, consistent and adequate recoveries of RAL and RD4, we accepted the method and performed the method validation.

The different plasma matrices did not have significant effects on the analyte signals. The matrix effect Analyte/ IS was found to be ranging from -0.69% to 8.55% for RAL, -22.20% to -16.86% for R4G and -22.75% to -16.01% for R4G. The matrix effect for IS/Analyte was found to be ranging from -8.20% to 1.22% for RAL, 19.97% to 28.53% for R4G and 19.13% to 29.39% for R6G. The figures relatively low,

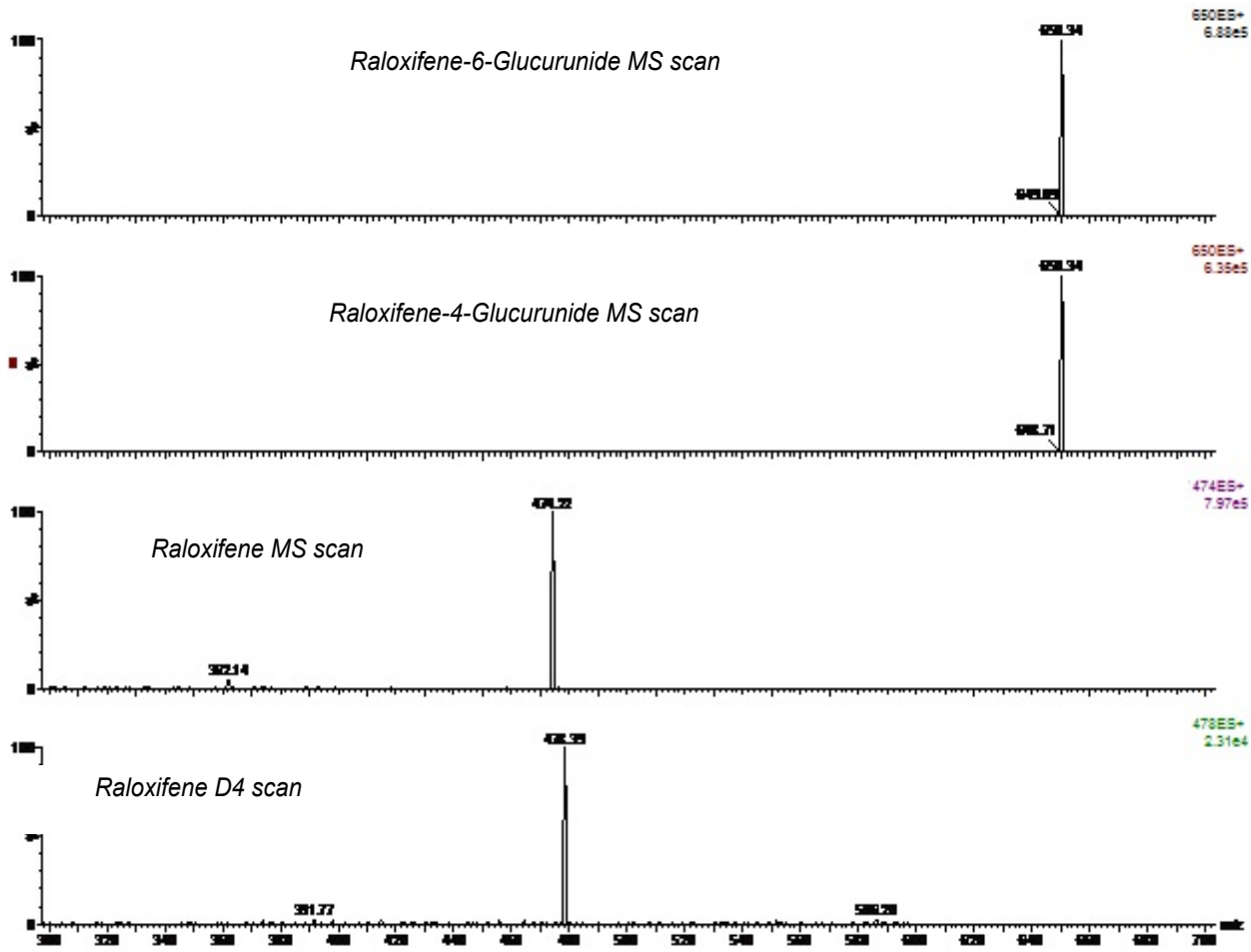

Figure 2: MS Scan spectra of RAL, R4G, R6G and internal standard (RD4).

showed that the matrix effect is low and reproducible, and would not interfere with the assay.

Calculate the matrix factor for analyte at each QC concentration and that of internal standard at working concentration.

Stability

The aqueous stock solutions were found to be stable for at least 12 days at −10°C. The changes in signal intensity after storage were less than 7% for RAL, R4G and R6G.

Autosampler stability (stability of RAL, R4G and R6G in the eluate) was estimated by analysis of QC samples (LQC and HQC level). Both sets of results differed by less than 15% from the nominal value; processed plasma samples of RAL, R4G, and R6G are considered stable, when retained in autosampler tray for at least 28 h.

The short-term stability (6 h) was considered acceptable, as the results differed by less than 15%. The freeze–thaw stability (3 cycles) was also acceptable, results differed by less than 15% from the nominal value.

Long term stability (stability of RAL, R4G and R6G in the plasma) was estimated by analysis of QC samples (LQC and HQC level). Both sets of results differed by less than 15% from the nominal value; plasma samples of RAL, R4G, and R6G are considered stable, when stored at −80°C for at least 55 days.

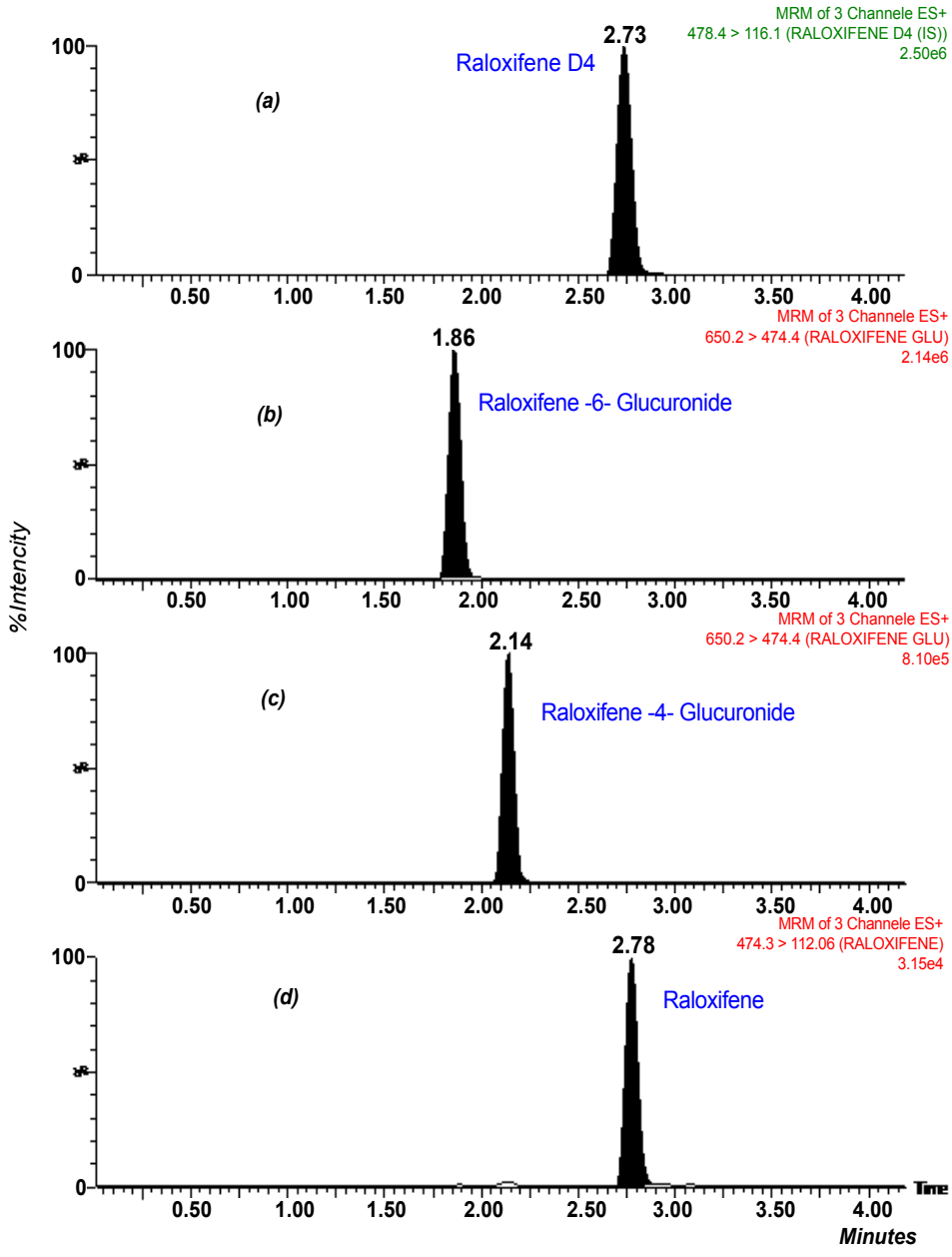

Figure 3: UPLC-MS/MS chromatograms of RAL (d), R4G(c), R6G (b) and Raloxifene D4 (a), when it was injected separately for specificity. No interference was observed from each other.

A summary of the stability tests for RAL, R4G and R6G is presented in Table 7.

Conclusion

A method has been developed and validated for simultaneous determination of raloxifene and its two metabolites, R4G and R4G. The LC-MS/MS method developed is sensitive, specific, accurate and precise. The limits of quantification achieved in plasma were 0.0392 ng/mL for RAL, 0.5920 ng/mL for R4G and 0.5996 ng/mL for R6G. The simple sample preparation procedure and short retention time with good separation between analytes. There were no interferences from

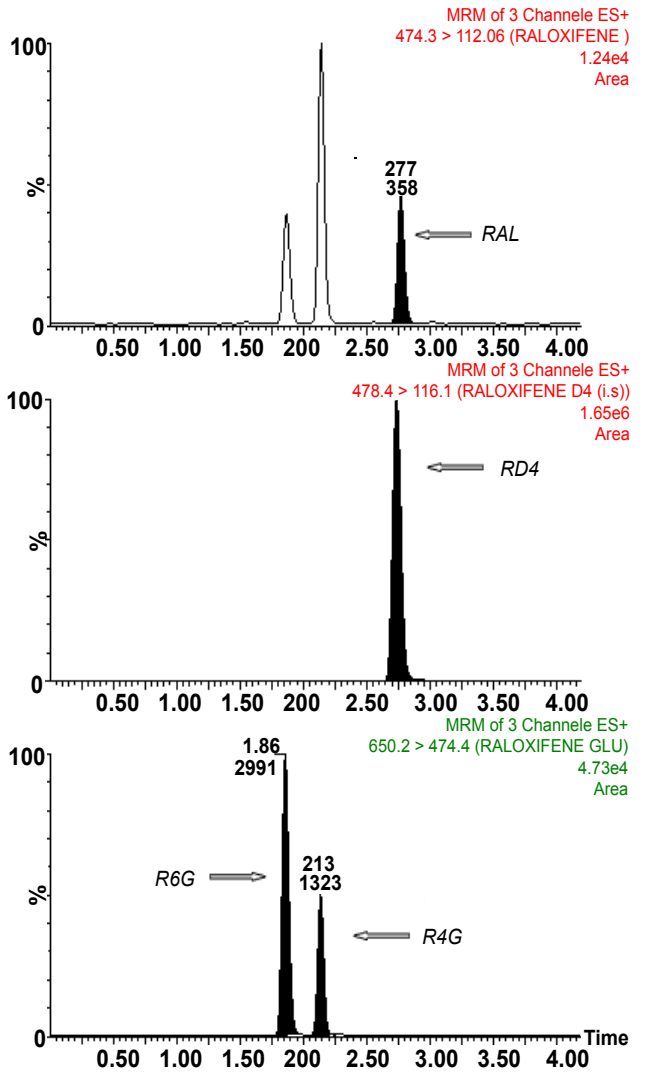

Figure 4: UPLC-MS/MS chromatogram of LLOQ plasma sample consists of RAL, R4G, R6G and RD4.

Analyte Name	Range(ng/mL)	Slope	Intercept	r	r^2
RAL	0.0392-1.5222	3051.59	47.327975	0.9976	0.9952
R4G	0.5920-50.3217	2535.34	757.37175	0.9993	0.9986
R6G	0.5996-50.3637	4172.04	172.3534	0.9989	0.9979

Table 3: Linear regression data for RAL, R4G and R6G.

	Sample	Nominal Concentration (ng/mL)	Mean Concentration Found (ng/mL)	Precision	Accuracy	n
Within-day	LLOQ QC	0.0395	0.0410	17.90	103.87	12
	LQC	0.1163	0.1043	9.79	89.70	12
	MQC	0.5813	0.5204	8.92	89.52	12
	HQC	1.3371	1.3073	12.73	97.77	12
Between Day	LLOQ QC	0.0395	0.0428	14.6	108.28	18
	LQC	0.1163	0.1106	9.78	95.06	18
	MQC	0.5813	0.5273	9.11	90.71	18
	HQC	1.3371	1.2199	7.20	91.23	18

Table 4: Within-day and between-day precision and accuracy for RAL.

	Sample	Nominal Concentration (ng/mL)	Mean Concentration Found (ng/mL)	Precision	Accuracy	n
Within-day	LLOQ QC	0.5934	0.6728	10.01	113.39	12
	LQC	1.5429	1.6328	7.29	105.83	12
	MQC	23.7370	24.5856	6.34	103.58	12
	HQC	42.7265	42.1166	6.10	98.57	12
Between Day	LLOQ QC	0.5934	0.6161	5.68	116.78	18
	LQC	1.5429	1.5284	5.33	95.72	18
	MQC	23.7370	23.2752	5.34	96.89	18
	HQC	42.7265	40.6725	3.50	94.8	18

Table 5: Within-day and between-day precision and accuracy for R4G.

	Sample	Nominal Concentration (ng/mL)	Mean Concentration Found (ng/mL)	Precision	Accuracy	n
Within-day	LLOQ QC	0.6011	0.6769	5.77	112.61	12
	LQC	1.5628	1.5443	4.18	98.81	12
	MQC	24.0427	25.2272	5.07	104.93	12
	HQC	43.2769	43.5019	5.19	100.52	12
Between Day	LLOQ QC	0.6011	0.6760	6.85	112.46	18
	LQC	1.5628	1.4922	4.11	95.48	18
	MQC	24.0427	24.1989	4.69	100.65	18
	HQC	43.2769	43.0417	2.76	99.46	18

Table 6: Within-day and between-day precision and accuracy for R6G.

	RAL	R4G	R6G
Short term [a]	104.37 to 111.13	88.39 to 92.36	110.83 to 113.09
Autosampler [b]	98.76 to 104.51	97.47 to 109.79	108.15 to 109.48
Freeze–thaw [c]	87.58 to 98.02	97.41 to 108.56	98.87 to 104.11
Dry Extract [d]	94.16 to 98.02	97.37 to 110.12	99.98 to 107.18
Long Term at -80°C [e]	92.86 to 93.09	100.98 to 106.64	99.45 to 104.40

The stability was evaluated in six replicates of low and high QC plasma samples.
[a] 6h at room temperature, n=6.
[b] 28 h at 5°C, n=6.
[c] Three freeze–thaw cycles, n=6.
[d] -10°C for 25 h, n=6.
[e] -80°C for 55 Days, n=6.

Table 7: Summary of stability tests [%] for RAL, R4G and R6G.

endogenous plasma components or from other sources, and no "cross-talk" effect was observed.

Moreover, the method described could easily be adapted to various other biological samples as it covers a high sensitivity, has a good precision, accuracy and high recovery. The bioanalytical method presented here will be useful in pharmacokinetic studies.

Acknowledgement

We are grateful to Bharati Vidyapeeth's College of pharmacy, India, for the excellent facilities and support provided during the project work, without which this work would not have been possible at academic level.

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