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SENSITIVE AND RAPID SIMULTANEOUS QUANTITATION OF RALOXIFENE AND ITS TWO MAJOR ACTIVE METABOLITES 4-GLUCURONIDE RALOXIFENE AND 6-GLUCURONIDE RALOXIFENE IN HEALTHY VOLUNTEERS USING A LIQUID CHROMATOGRAPHY COUPLED WITH TRIPLE QUADRUPLE MASS SPECTROMETER

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Keywords:

Interconversion, Raloxifene, Glucuronides, Mass spectrometer, Bioequivalence, Human plasma

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ABSTRACT: Simultaneous estimation of parent and its metabolite(s) in a single method poses a threat when one has to monitor one mass transition while running the other compound on liquid chromatography-mass spectrometer. This would again become critical once interference is observed at the retention time of compound of interest in-spite of its absence in the sample. In this paper, a selective and sensitive liquid chromatography-tandem mass spectrometry method for the simultaneous determination of Raloxifene and its two active metabolites, Raloxifene 4-Glucuronide and Raloxifene 6-Glucuronide in human plasma was developed and validated. The method involves a rapid solid-phase extraction from plasma, followed by reversed-phase chromatography with gradient flow condition and mass spectrometry detection. Validation parameters were selected ranging from 0.016 to 1.187 ng/mL for Raloxifene, 3.073 to 700.558 ng/mL Raloxifene 4-Glucuronide and 0.311 to 124.526 ng/ml for Raloxifene 6-Glucuronide. The mean recovery for Raloxifene, Raloxifene 4-Glucuronide, and Raloxifene 6-Glucuronide found to be 85.10, 86.30 & 87.20%, respectively. The peak concentration of Raloxifene, Raloxifene 4-Glucuronide, and Raloxifene 6-Glucuronide were 0.330 ng/mL, 153.047 ng/mL and 27.744 ng/mL respectively for the reference product, in fasted conditions.

INTRODUCTION: Raloxifene is a selective estrogen receptor modulator (SERM) having selective agonist or antagonist activities on tissues responsive to estrogen. In postmenopausal women with osteoporosis, Raloxifene reduces the incidence of vertebral fractures, preserves bone mass, and increases bone mineral density (BMD) [1-3].

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Raloxefine, [6-Hydroxy-2- (4-hydroxyphenyl) benzo [b]thien-3 -yl]-[4-[2- (1-piperidinyl) ethoxy] phenyl] methanone undergoes extensive first-pass metabolism by UGTs to the two active glucuronide conjugates: Raloxifene 4-Glucuronide and Raloxifene 6-Glucuronide which contribute to the therapeutic efficacy of Raloxifene.

Raloxifene belongs to a BCS and BDDCS class II compound that has high permeability and low solubility [4-5]. Metabolism of the drug is dominated by glucuronidation, which is responsible for 97 and 50% of the total intestinal and hepatic metabolism, respectively. Although Raloxifene 4-Glucuronide is the major metabolite, Raloxifene 6-Glucuronide is

also detected in human plasma. The formation of Raloxifene 4-Glucuronide has been shown to be catalyzed primarily by intestinal UGT1A8 and UGT1A10, whereas hepatic UGT1A1 preferentially forms Raloxifene 6-Glucuronide [6]. Although there are multiple active and inactive metabolites to Raloxifene however nonbinding Draft guidance published by USFDA mentions the need for assessment of Raloxifene along with its two active metabolites only for any bioequivalence study submission [7] hence in this paper only two metabolites were investigated. Very few reports are available on Raloxifene and its metabolite quantification. Earlier reports on HPLC [8-9] are mainly focused on the quantification of Raloxifene in solid oral dosage formulations, while studies based on LCMS methods only discussed the uses of β-Glucuronidase, the role of pH during processing and expected m/z of Raloxifene and its metabolites during MRM mode [10].

Jagadeesh *et al.*, have shown the structure elucidation of Raloxifene along with potential impurities using a mass spectrometer and other technique [11]. Likewise, Anil Kumar *et al.*, have worked on the estimation of Raloxifene (without metabolites) in human urine which is not very sensitive (LLOQ: 20 ng/ml) [12]. The preclinical study was performed for Raloxifene and its two active metabolites was also reported by Sun *et al.*, [13]. Surprisingly, only one report pertaining to a method quantifying Raloxifene in human plasma using LC-MS/MS with linearity 0.04-1.5 ng/ml has been developed and validated. However, this report did not envisage the interconversion aspect of drug and its metabolites and lacks in sensitivity to detect terminal concentration (high LLOQ) for Raloxifene and Raloxifene 6-Glucuronide compounds [14].

To the best of our knowledge, there are no bioanalytical methods published for simultaneous estimation of Raloxifene, Raloxifene 4-Glucuronide and Raloxifene 6-Glucuronide in a healthy population that could be employed for bioequivalence study of low dose (6omg) under fasting condition. In this work, we have tried to investigate the cause of interconversion between the parent compound and its active metabolites using two different analytical tools (UFLC-UV & UFLC-MS/MS). The extent of interconversion is well demanded by the regulatory agencies [15-16].

Upon confirmation of the existence of inter-conversion results, we have suggested a correction factor methodology to overcome it [17]. This developed method is highly sensitive (LLOQ: 0.016 ng/mL for Raloxifene) than any other published methods available in the literature. Further, the method's applicability has been assessed for the estimation of Raloxifene and its two active metabolites in human plasma in a pharmacokinetic study.

MATERIALS AND METHODS:

Chemicals and Reagents: Raloxifene, Raloxifene 4-Glucuronide, Raloxifene 6-Glucuronide, Raloxifene D4, Raloxifene 4-Glucuronide D4, and Raloxifene 6-Glucuronide D4 standards were procured from TLC (Canada). K2EDTA plasma was procured from Sai Labs Hyderabad. Acetonitrile (HPLC grade) and methanol were obtained from JT Baker, Germany. Milli-Q water (Millipore Co. MA, USA) purification system was used to obtain the purified water for the LC-MS & HPLC analysis.

Instrumentation: Chromatographic separation was performed at ambient temperature with a mobile phase consisting of acetonitrile: 0.1% formic acid under gradient flow condition **Table 1**. Before gradient programming, various isocratic methods were tested but none of them were able to provide a good peek resolution between Raloxifene and its two metabolites apart from endogenous plasma peaks. An ACQUITY UPLC BEH Shield RP18 1.7 µm × 2.1 × 100 mm column was used for chromatographic separation at a flow rate of 0.4 ml/min. The mobile phase was delivered by a UFLC pump and the sample injected by a UFLC auto-sampler (Shimadzu, Japan). Detection was performed by an API 5500 tandem Triple quadrupole mass spectrometer (AB Sciex, USA) fitted with electrospray ionization in positive ion mode. Oasis HLB 30 mg/1cc solid-phase extraction (SPE) cartridge for sample preparation was obtained from WATERS, USA). A 48 head positive pressure solid phase manifold from Orochem India was used for sample cleanup. API 5500 tandem Triple quadrupole mass spectrometer was operated at multiple reaction monitoring (MRM) mode, monitoring the transition of molecular ions m/z 474.169/112.000 (Raloxifene), 478.169 / 116.000 (Raloxifene D4), 650.158 /

474.100 (Raloxifene 4-Glucuronide), 654.202 / 478.100 (Raloxifene 4-Glucuronide D4), 650.167 / 474.100 (Raloxifene 6-Glucuronide), 654.208 / 478.200 100 (Raloxifene 6-Glucuronide D4).

TABLE 1: UFLC GRADIENT FLOW PROFILE

Time Program	Pump B conc.(Acetonitrile)	Pump A conc. (0.1% Formic acid)
0.01	20	80
1.00	25	75
3.50	35	65
5.00	40	60
6.00	20	80
7.00	20	80

Gradient programming used for the chromatographic separation and resolution between RAL and its two active metabolites

Electrospray ionization (ESI) was performed in the positive ion mode with source temperature set at 500°C, the ion-spray voltage at 5000, curtain gas at 35.00; CAD gas at 8; gas-1 flow at 50 l / min; gas-2 flow at 45 l/min. Optimization of the triple quadrupole settings of the instrument for detection of Raloxifene, Raloxifene 4-Glucuronide, Raloxifene 6-Glucuronide, and their respective internal standards were done by infusing a 10 ng/ml solution of each drug dissolved in methanol: water (50:50 v/v) solution at a constant flow rate of 5 µL/min.

Study Design: A study protocol was designed to conduct the bioequivalence study between the test formulation and reference formulation for 60 mg tablets. A total of fifteen healthy volunteers participated in the study in a fasting state. The protocol was approved by the Rational Independent Ethics Committee and Drug Controller General of India. A two way cross over study design was selected for the conduct of bioequivalence study. After overnight fasting of at least 10 h, a single dose of a drug product containing Raloxifene 60 mg was administered orally with at least 240 mL of drinking water. A pre-dose sample of 6 mL, plus 26 post-dose blood samples of 4 mL each was collected using K2EDTA vacutainer from each subject in each period. Samples was collected at pre-dose and post-dose at 0.167, 0.33, 0.50, 0.75, 1.00, 1.50, 2.00, 3.00, 4.00, 5.00, 6.00, 7.00, 8.00, 9.00, 10.00, 11.00, 12.00, 14.00, 16.00, 18.00, 24.00, 48.00, 72.00, 96.00, 120.00 and 144.00 hours in each period.

Preparation of Standards and Quality Control Samples: A stock solution of 1 mg / 10 mL was prepared by dissolving reference standard of Raloxifene, Raloxifene 4-Glucuronide, Raloxifene 6-Glucuronide, Raloxifene D4, Raloxifene 4-Glucuronide D4, Raloxifene 6-Glucuronide D4 in methanol. These stock solutions were further diluted by Methanol: Milli Q/HPLC grade water mixture (50:50) to a prepared working solution. Calibration curve standard (CC) and Quality control samples (QC) were prepared by spiking 1 % of aqueous dilutions for Raloxifene, 0.5% of aqueous dilutions for Raloxifene 4-Glucuronide and 0.5% of aqueous dilutions for Raloxifene 6-Glucuronide in human plasma. A total of 2% spiking was done for the preparation of CC & QC. The CC range between 0.016 to 1.187 ng/mL for Raloxifene 3.073 to 700.558 ng/mL Raloxifene 4-Glucuronide and 0.311 to 124.526 ng/ml for Raloxifene 6-Glucuronide were prepared. Similarly, QCs were prepared at four levels, lower limit of quantification (LLOQ), low-quality control (LQC), medium quality control (MQC), and high-quality control (HQC). QCs were prepared by spiking 2% of aqueous dilutions (1% Raloxifene & 0.5% each of Raloxifene 4-Glucuronide & Raloxifene 6-Glucuronide) in human plasma. QC samples between 0.016ng/mL (LLOQ QC), 0.043 ng/mL(LQC), 0.475 ng/mL (MQC), 0.950 ng/mL (HQC) for Raloxifene, 3.083 ng/mL (LLOQ QC), 8.446 ng/mL (LQC), 291.239 ng/mL (MQC), 582.478 ng/mL (HQC) for Raloxifene 4-Glucuronide & 0.313 ng/mL (LLOQ QC), 0.850 ng/mL (LQC), 51.486 ng/mL (MQC), 102.972 ng/mL (HQC) for Raloxifene 6-Glucuronide were prepared.

Extraction Procedure: Plasma samples (0.3ml) were pipetted into 5 ml ria vial followed by adding 50 µl of internal standard dilution mixture (approximately 10.000 ng/mL Raloxifene D4, 120.000 ng/mL Raloxifene 4-Glucuronide D4 & 15.000 ng/mL Raloxifene 6-Glucuronide D4). Samples were loaded in the SPE cartridges and applied the positive pressure to elute the plasma. Washed the cartridges with 1.0 ml of milli Q water. The sample was eluted by 0.5 mL methanol followed by 0.5 mL ammonia solution (1%). The eluted sample was further concentrated in a nitrogen evaporator till complete dryness (at 45 ºC temperature & 25 psi pressure). The dried samples were reconstituted in 0.3 mL volume with methanol: formic acid (1%) mixture (70:30 v/v).

Reconstituted samples were transferred into HPLC vial (0.5 mL). The vials were kept in the UFLC auto-sampler for injection (10 µL) for analysis.

Validation: Validation of the method was determined by analyzing quality control samples during three independent batches at eight different non zero concentrations of Raloxifene, Raloxifene 4-Glucuronide, Raloxifene 6-Glucuronide to determine the accuracy and precision of the method. Four concentration of Quality control samples (LLOQ, LQC, MQC and high-quality control HQC) were evaluated. A 1/ (concentration) 2 linear regression was used to build the calibration curves peak area ratios of the analyte / IS versus nominal drug concentrations.

RESULTS:

Chromatography, Retention Times and LLOQ: Chromatography plays a crucial role in any analysis on UFLC or HPLC. Parameters like resolution, retention time and symmetry are the major factors described in the pharmacopeia (USP, EP, BP, *etc.*) that must be looked into while monitoring any chromatogram. Resolution between the Raloxifene and its metabolites was greater than 2.0. Symmetry of all the peaks was close to 1.0. The LLOQ with acceptance precision (CV% < 20) and accuracy (bias<20%) was found to be 0.016 ng/mL for Raloxifene, 3.073 ng/mL Raloxifene 4-Glucuronide and 0.311 ng/ml for Raloxifene 6-Glucuronide respectively with a signal to noise ratio of more than 55. UFLC gradient programming was selected over an isocratic method to get well separation between all three analytes. The retention times for Raloxifene, Raloxifene 4-Glucuronide, Raloxifene 6-Glucuronide were 4.0, 3.3, 2.4 min respectively. The total chromatography run time of 7 min made it possible to analyze a large number of sample in a batch

Precision and Accuracy: The inter-day was determined over 5 days by analyzing 72 QC samples. The intra-day accuracy and precision of the assay was quantified by analyzing six spiked samples of Raloxifene, Raloxifene 4-Glucuronide, Raloxifene 6-Glucuronide at each QC.

The intraday accuracy and precision of the analytical method for Raloxifene, Raloxifene 4-Glucuronide, Raloxifene 6-Glucuronide is well within regulatory acceptance criteria **Table 2** and **3**.

TABLE 2: BACK CALCULATED CONCENTRATED OF RAL, RAL 4-G & RAL 6-G

	RAL										
	STD A	**STD B**	**STD C**	**STD D**	**STD E**	**STD F**	**STD G**	**STD H**	**Slope**	**Intercept**	**r2**
	0.016 ng/mL	**0.033 ng/mL**	**0.148 ng/mL**	**0.297 ng/mL**	**0.594 ng/mL**	**0.771 ng/mL**	**1.009 ng/mL**	**1.187 ng/mL**			
Mean	0.016	0.032	0.150	0.295	0.586	0.811	1.000	1.173	1.187225	-0.001484	0.9983
SD	0.000	0.001	0.003	0.005	0.033	0.018	0.018	0.052	0.020408	0.000734	0.0006
%CV	0.000	3.130	2.000	1.690	5.630	2.220	1.800	4.430	1.72	-49.46	0.06
%Bias	0.000	-3.030	1.350	-0.670	-1.350	5.190	-0.890	-1.580			
N	3	3	3	3	3	3	3	3	3	3	3

	RAL 4-G										
	STD A	**STD B**	**STD C**	**STD D**	**STD E**	**STD F**	**STD G**	**STD H**	**Slope**	**Intercept**	**r2**
	3.073 ng/mL	**6.146 ng/mL**	**87.799 ng/mL**	**175.598 ng/mL**	**351.196 ng/mL**	**453.156 ng/mL**	**595.474 ng/mL**	**700.558 ng/mL**			
Mean	3.037	6.281	88.878	181.909	341.552	465.585	577.990	680.202	0.000899	-0.000093	0.9982
SD	0.073	0.303	2.407	3.160	19.940	14.553	5.294	9.484	0.000047	0.000114	0.0011
%CV	2.400	4.820	2.710	1.740	5.840	3.130	0.910	1.390	5.23	-122.58	0.11
%Bias	-1.170	2.200	1.230	3.590	-2.750	2.740	-2.940	-2.910			
N	3	3	3	3	3	3	3	3	3	3	3

	RAL 6-G										
	STD A	**STD B**	**STD C**	**STD D**	**STD E**	**STD F**	**STD G**	**STD H**	**Slope**	**Intercept**	**r2**
	0.311 ng/mL	**6.22 ng/mL**	**15.547 ng/mL**	**31.094 ng/mL**	**62.187 ng/mL**	**80.973 ng/mL**	**105.847 ng/mL**	**124.526 ng/mL**			
Mean	0.309	0.629	15.855	32.672	60.181	80.476	103.352	122.864	0.059319	0.000227	0.9987
SD	0.006	0.026	0.063	0.867	2.178	1.145	1.462	2.525	0.001627	0.001298	0.001
%CV	1.940	4.130	0.400	2.650	3.620	1.420	1.410	2.060	2.74	571.81	0.10
%Bias	-0.640	1.130	1.980	5.070	-3.230	-0.610	-2.360	-1.330			
N	3	3	3	3	3	3	3	3	3	3	3

TABLE 3: INTRA-DAY ACCURACY OF THE METHOD FOR RAL, RAL 4-G AND RAL 6-G

	RAL							
	LLOQ 0.016 ng/mL		LQC 0.043 ng/mL		MQC 0.475 ng/mL		HQC 0.950 ng/mL	
	Cal. Conc.	%Bias	Cal. Conc.	%Bias	Cal. Conc.	%Bias	Cal. Conc.	%Bias
N:	18	18	18	18	18	18	18	18
Mean:	0.016	0.00	0.043	0	0.462	-2.74	0.927	2.42
SD (±):	0.001		0.002		0.010		0.026	
CV (%):	6.25		4.65		2.16		2.80	

	RAL 4-G							
	LLOQ 3.083 ng/mL		LQC 8.446 ng/mL		MQC 291.239 ng/mL		HQC 582.478 ng/mL	
	Cal. Conc.	%Bias	Cal. Conc.	%Bias	Cal. Conc.	%Bias	Cal. Conc.	%Bias
N:	18	18	18	18	18	18	18	18
Mean:	3.127	1.43	7.632	-9.64	271.974	-6.61	527.624	-9.42
SD (±):	0.173		0.288		9.134		17.649	
CV (%):	5.53		3.77		3.36		3.34	

	RAL 6-G							
	LLOQ 0.313 ng/mL		LQC 0.850 ng/mL		MQC 51.486 ng/mL		HQC 102.972 ng/mL	
	Cal. Conc.	%Bias	Cal. Conc.	%Bias	Cal. Conc.	%Bias	Cal. Conc.	%Bias
N:	18	18	18	18	18	18	18	18
Mean:	0.297	-5.11	0.829	-2.47	50.020	-2.85	94.592	-8.14
SD (±):	0.022		0.032		1.229		3.351	
CV (%):	7.41		3.86		2.46		3.54	

Note: Raloxifene (RAL), Raloxifene 4-Glucuronide (RAL 4-G), Raloxifene 6-Glucuronide (RAL 6-G)

Stability: The stability of the Raloxifene-family in human plasma under different temperatures and time duration was examined. Stored plasma aliquots were thawed and kept at room temperature for around 12 h to analyze benchtop stability of the samples. The freeze-thaw stability was assessed on the fifth cycle comparing with freshly prepared quality control samples & calibration curve standard. Stocks were stable in solution (methanol) at 2-8 °C for 15 days.

TABLE 4: STABILITIES FOR RAL, RAL 4-G AND RAL 6-G IN HUMAN PLASMA

	RAL		RAL 4-G		RAL 6-G	
			% Bias			
	LQC	HQC	LQC	HQC	LQC	HQC
Bench Top (12 h at 25 ºC),(n=6)	4.65	-0.53	-10.11	-8.43	4.01	-4.64
Freeze-Thaw (5-Cycle),(n=6)	4.68	-1.26	-9.24	-8.46	1.41	-5.61
Wet Extract (72 h at 2-8 ºC),(n=6)	4.62	1.47	-7.98	-4.56	2.24	-5.33
Auto-sampler (72 h at 5 ºC),(n=6)	4.67	-2.00	-8.42	-6.70	1.88	-4.60
Dry Extract (72 h at -65 ºC),(n=6)	6.98	-1.88	-8.44	-7.54	2.36	-5.61
Long Term (75 days at -65 ºC),(n=6)	7.02	2.18	-4.93	-2.45	4,23	-5.67
			% Recovery			
Mean Recovery (Drug: LQC,MQC & HQC)	85.10%,	86.30%	87.20%	Mean Recovery (Drug: LQC,MQC & HQC)	85.10%,	86.30%
Mean Recovery (IS)	83.40%	86.10%	88.90%	Mean Recovery (IS)	83.40%	86.10%
			% Change			
Stock Solution Stability (15 Days at 2-8 ºC)	2.40%	2.60%	4.12%	4.45%	3.15%	3.67%

Note: Raloxifene (RAL), Raloxifene 4-Glucuronide (RAL 4-G), Raloxifene 6-Glucuronide (RAL 6-G)

Long-term stability in the biological matrix kept at –65 °C was assessed over a period of 75 days at two different concentrations (LQC & HQC). RAL-family was tested for processed samples stability under three different conditions (auto-sampler stability at 5 °C; dry extract stability at –65 °C & wet extract stability at 2-8 °C) at two different concentrations levels (LQC & HQC). The stability of compounds was found to be within ±15% under the above-processed condition **Table 4**.

Method Application: This assay method was also employed to analyzed plasma samples containing Raloxifene and two metabolites from male volunteers after administrating a single dose of 60 mg tablet of Raloxifene under fasting condition. The C_{max} and AUC parameters have been presented are comparable to the test and reference formulation **Table 5**.

TABLE 5: PHARMACOKINETIC PARAMETERS FOR RAL, RAL 4-G AND RAL 6-G IN HUMAN PLASMA UNDER FASTING CONDITION

	RAL		RAL 4-G		RAL 6-G	
	Test	Reference	Test	Reference	Test	Reference
C_{max} (ng/mL)	0.332 ± 0.131	0.320 ± 0.142	205.836 ± 92.533	153.047 ± 74.794	33.066 ± 13.075	27.744 ± 10.156
AUC_{0-t}(ng*hr/mL)	10.303 ± 5.756	8.612 ± 4.595	2474.152 ± 1200.974	2193.124 ± 1293.383	658.453 ± 296.932	592.384 ± 302.781
N	15	15	15	15	15	15

Note: Raloxifene (RAL), Raloxifene 4-Glucuronide (RAL 4-G), Raloxifene 6-Glucuronide (RAL 6-G)

Incurred Sample Reanalysis: As per recent regulatory recommendations and various publications [18-20], random repeat study sample analysis is required to be performed as the incurred samples. A validated method, which is reproducible and shows satisfactory data of the long-term stability of the drug in the matrix, may not adequately indicate the stability and reproducibility of actual subject samples (incurred samples). Hence, the suitability of the Bioanalytical methods cannot solely rely on surrogate QC samples alone; demonstration and proper evaluation of incurred sample reproducibility and stability are essential to produce a reliable study data. In the bio-study, 10% of samples (from the close to T_{max} and elimination phase) were randomly selected for incurred samples reanalysis for all the compounds (Raloxifene and metabolites). Out of 10% samples, 98% of samples met (Data not shown) the acceptance criteria, which demonstrate the reproducibility of the method and stability of the compound in the plasma matrix.

Recovery: The complete recoveries were evaluated for Raloxifene, Raloxifene 4-Glucuronide, Raloxifene 6-Glucuronide, and their respective IS by comparing the peak area of the extract samples with the neat aqueous standard solutions peak area at three QC levels (LQC, MQC, HQC). The absolute recovery determination for all compounds was shown to be consistent and reproducible. Mean recovery ranged from 85.10%, 86.30% & 87.20 % respectively at the three QC levels for Raloxifene, Raloxifene 4-Glucuronide, Raloxifene 6-Glucuronide. Absolute mean analytical recoveries of internal standards were 83.40%, 86.10% and 88.90% for Raloxifene D4, Raloxifene 4-Glucuronide D4, Raloxifene 6-Glucuronide D4 respectively.

DISCUSSION: Raloxifene and its family belong to a very slightly soluble pharmacopeia category hence it was a challenge to overcome the remaining traces of these compounds in subsequent injection. At the beginning of the experiments, we were experiencing a lot of carryover at the retention time of Raloxifene. To rectify this, the carry-over effect, many groupings of solvents (Isopropyl alcohol, Acetonitrile, Methanol, DMSO, Water) were tested along with different cleaning methods. Even front end UPLC was also changed, but no improvement observed from the carryover problem. After investigating various trials, a method was finalized to nullify the carry-over effect where three rinsing modes were working in a sequential manner, starting from the acidic wash (0.5% formic acid), basic wash (0.5% ammonia solution) to methanol-water solution wash (70:30 v/v). The total rinsing time 4.5 min was running parallel to the analysis time. Notably, pH and temperature do contribute to conversion and back conversion between drug and metabolites; however, this phenomenon was not

noticed in Raloxifene and its metabolites [21]. For the simultaneous estimation of Raloxifene and its two metabolites, specificity and interconversion experiments were performed. Significant interconversion was observed from Raloxifene 4-Glucuronide to Raloxifene and Raloxifene 6-Glucuronide to Raloxifene **Fig. 1**. Considering working standards the culprit, fresh standards were procured from two more vendors and specificity, interconversion experiments were again performed but the results did not change much. In order to confirm whether the interference at the RT of Raloxifene was due to Interconversion or working standard impurity, the vendor (TRC-Canada) was asked to run the experiment with our developed method on LC-MS/MS. They performed the experiment on HPLC instead of LC-MS/MS by running an individual compound of Raloxifene and its two metabolites and sample mixture of all three compounds along with an appropriate diluent sample. They showed the experiment outcome from their lab & proved that these compounds are free from interference of each other. Hence, it was definite that Raloxifene and its metabolites working standard compounds were unadulterated however; the problem (of interconversion) was persisting.

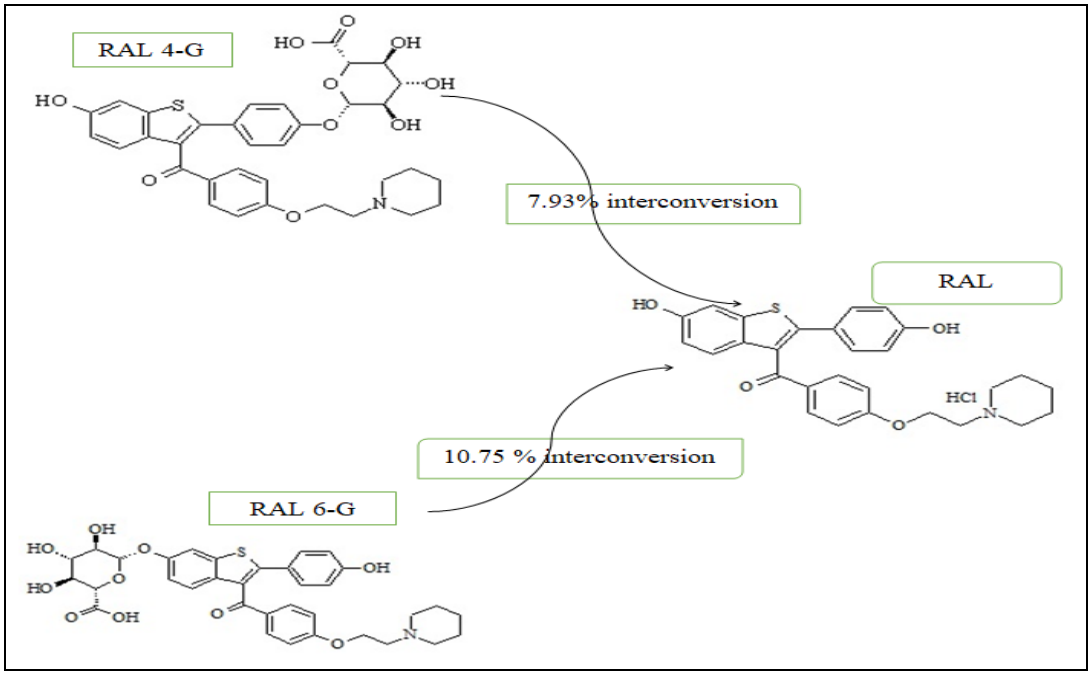

FIG. 1: INTERCONVERSION OF OBSERVED FROM TWO ACTIVE METABOLITES RAL 4-G & RAL 6-G TO RAL. Note: raloxifene (ral), raloxifene 4-glucuronide (ral 4-g), raloxifene 6-glucuronide (ral 6-g)

To eliminate any experimental error, three different application labs contacted for the specificity & interconversion experiments. They performed these crucial experiments, shared the results. These results were in agreement with our results. Instrument parameters like Source and compound parameters were changed to very mild conditions (source temperature kept at 0 ºC, collision energy & CAD gas were set to its minimum limit) to minimize the extent of interconversion results, but the outcome did not change. It was also tried to change the m/z fragments 474.169/112.000, 474.169 / 268.520 for Raloxifene; 650.158/ 474.100, 650.158 / 268.420, 650.158/112.200 for Raloxifene 4-Glucuronide & Raloxifene 6-Glucuronide that is known to alter the ionization pattern and might help in removing the interconversion impact but no improvement observed in the result of interconversion. It is seen during the MRM optimization that the m/z fragment of Raloxifene (474.169/112.000) was also noticeable in metabolites spectra. This pattern was perceptible to all the mass spectra obtained from three different vendors as well as in our laboratory. It could be allied with the circumstance that glucuronide tends to get fragment in a source of mass spectrometer even at mild or very low source and compound parameter conditions. Since the RT of Raloxifene 4-Glucuronide & Raloxifene 6-Glucuronide were much before the RT of Raloxifene, hence it was possible to identify the in-source mass fragmentation pattern of these

metabolites and its back conversion to Raloxifene. The extent of interconversion was studied by using one more analytical tool *i.e.* instead of connecting UFLC with MS (API 5500) detector; UV detector (at 286 nm) was connected. The chromophores of Raloxifene and its metabolites are very intense at wavelength 286 nm. Due to the low sensitivity of UV detector high-concentrated samples of Raloxifene and its metabolites were prepared and injected under the same chromatographic condition by keeping the only detector changed *i.e.* UV instead of MS detector. The results obtained on a UV detector (at wavelength 286 nm) were thrilling and astonishing. No specificity & interconversion problem was observed when the detector changed from MS to UV **Fig. 2**.

Our UFLC-UV results were matching with vendor (TRC Canada) experiment results. This signposted that all the interconversion was happening inside the MS source due to the labile nature of the Glucuronide bond to the Raloxifene hydroxyl group linked with glucose moiety.

Now it was visualized that interconversion was related to UFLC-MS and not with UFLC-UV. However, due to the high sensitivity (LLOQ: 0.016 ng/ml for Raloxifene) requirement of in the analytical method, UFLC-MS analytical technique was selected over UFLC-UV. A correction factor was consequent to overcome the effect of interconversion [17] **Table 6**.

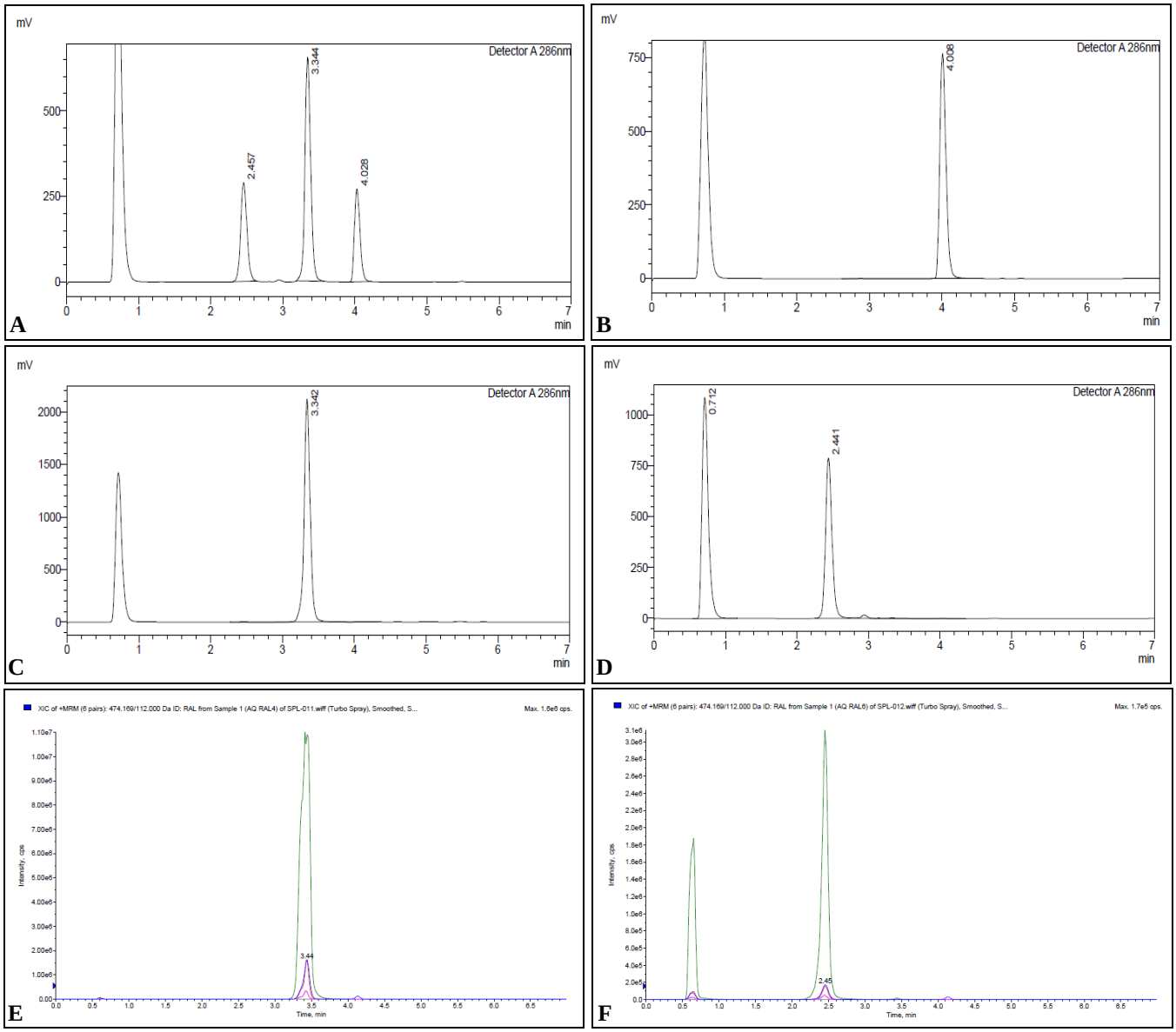

FIG. 2: INTERCONVERSION BETWEEN RAL, RAL 4-G & RAL 6-G BY UFLC-MS & UFLC-UV TECHNIQUES.
Note: raloxifene (ral), raloxifene 4-glucuronide (ral 4-g), raloxifene 6-glucuronide (ral 6-g)

Figure A: The UFLC-UV chromatograms of aqueous mixture of RAL & its two metabolites.

Figure B: The UFLC-UV chromatograms of aqueous sample of RAL alone. No interconversion observed from RAL to its metabolites (4-G and 6-G).

Figure C: The UFLC-UV chromatograms of aqueous sample of RAL 4-G alone. No Inter-conversion observed from RAL 4-G to RAL 6-G & RAL on UFLC-UV.

Figure D: The UFLC-UV chromatograms of aqueous sample of RAL 6-G alone. No Inter-conversion observed from RAL 6-G to RAL 4-G & RAL.

Figure E: The UFLC-MS/MS chromatograms of RAL 4-G alone. 7.93% interconversion observed from RAL 4-G to RAL.

Figure F: The UFLC-MS/MS chromatograms of aqueous mixture of RAL 6-G alone. 10.75% Inter-conversion observed from RAL 6-G to RAL.

TABLE 6: INTERCONVERSION OF RAL, RAL 4-G & RAL 6-G

	%	Total interconversion	Correction factor
Mean interconversion from RAL 4-G (LLOQ-3.073 ng/mL to ULOQ-700.558 ng/mL) to RAL	7.93	18.68	0.81
Mean interconversion from RAL 6-G (LLOQ-0.311 ng/mL to ULOQ-124.526 ng/mL) to RAL	10.75		
Mean interconversion from RAL (LLOQ-0.016 ng/mL to ULOQ-1.187 ng/mL) to RAL 4-G	0.00	0.02	1.00
Mean interconversion from RAL 6-G (LLOQ-0.311 ng/mL to ULOQ-124.526 ng/mL) to RAL 4-G	0.02		
Mean interconversion from RAL (LLOQ-0.016 ng/mL to ULOQ-1.187 ng/mL) to RAL 6-G	0.00	0.62	0.99
Mean interconversion from RAL 4-G (LLOQ-3.073 ng/mL to ULOQ-700.558 ng/mL) to RAL 6-G	0.62		

Interconversion observed from RAL to metabolites and vice-versa. The proposed correction factor suggested overcoming interconversion impact. Note: Raloxifene (RAL), Raloxifene 4-Glucuronide (RAL 4-G), Raloxifene 6-Glucuronide (RAL 6-G)

The problem was not yet over as during the conduct of various experiments, we observed an exclusive problem where parent compounds were contributing interference to their respective internal standard retention time (RT). Raloxifene was contributing to Raloxifene D4, Raloxifene 4-glucuronide was contributing to Raloxifene 4-glucuronide D4 and Raloxifene 6-glucuronide was contributing to Raloxifene 6-glucuronide D4. On judgmental review of various parameters, it was concluded that the compound isotopic mass pattern responsible for this interference. As per the mass calculator (available in Sciex Analyst software) the molecular mass of Raloxifene would be 473.2 however we could notice other isotopic masses 474.2, 475.2, 476.2, 477.2 and 478.2 that are less prominent but contribution from these less intense masses were causing the interference problem at the RT of Raloxifene D4. Likewise, the molecular mass of Raloxifene 4-glucuronide & Raloxifene 6-glucuronide would be 649.2 however the other less prominent isotopic masses 650.2, 651.2, 652.2, 653.2 and 654.2, were producing interference problem at the RT of Raloxifene 4-glucuronide D4 & Raloxifene 6-glucuronide D4 **Fig. 3** and **Table 7**.

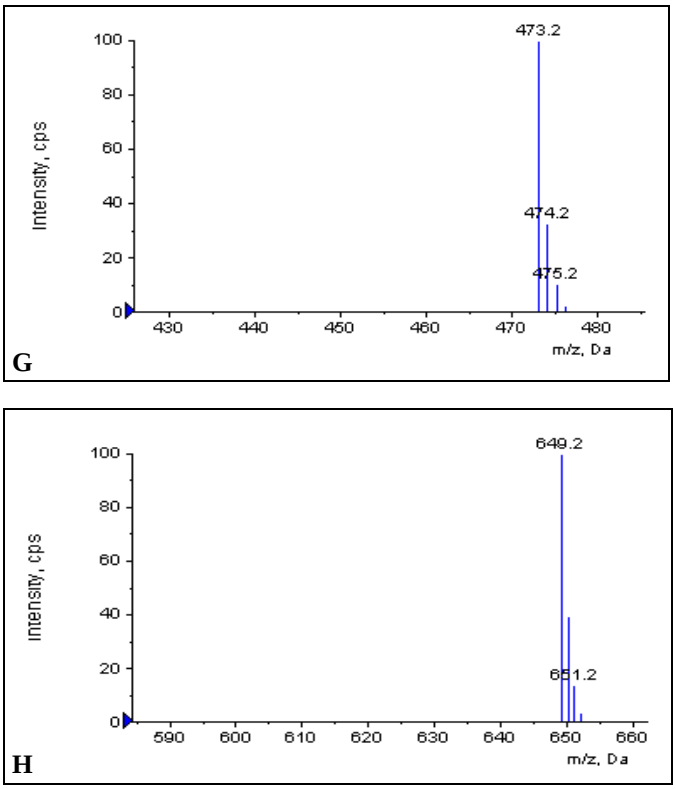

FIG. 3: ABSOLUTE MASS AND ITS ISOTOPIC ABUNDANCE OF RAL, RAL 4-G & RAL 6-G Note: raloxifene (ral), raloxifene 4-glucuronide (ral 4-g), raloxifene 6-glucuronide (ral 6-g)

TABLE 7: ISOTOPIC ABUNDANCE OF RAL, RAL 4-G AND RAL 6-G & INTERFERENCE TO STABLE LABELED IS

RAL & its isotopic abundance masses	RAL interference to stable labeled IS	RAL 4-G or RAL 6-G & their isotopic abundance masses	RAL interference to stable labeled IS
473.2 (100%)	NAP	649.2 (100%)	NAP
474.2 (32.7%)	RAL-D1	650.2 (39.7%)	RAL 4-G or RAL 4-G -D1
475.2 (10.4%)	RAL-D2	651.2 (14.1%)	RAL 4-G or RAL 4-G –D2
476.2 (2.2%)	RAL-D3	652.2 (3.4%)	RAL 4-G or RAL 4-G –D3
478.2 (0.3%)	RAL-D4	653.2 (0.6%)	RAL 4-G or RAL 4-G –D4
479.2 (0.0%)	RAL-D5	654.2 (0.1%)	RAL 4-G or RAL 4-G –D5
480.2 (0.0%)	RAL-D6	655.2 (0.0%)	RAL 4-G or RAL 4-G –D6

Absolute mass & its isotopic abundance obtained from Analyst software of AB Sciex instrument for RAL and its two active metabolites Note: Raloxifene (RAL), Raloxifene 4-Glucuronide (RAL 4-G), Raloxifene 6-Glucuronide (RAL 6-G)

Figure G: Absolute mass and its isotopic abundance of Raloxifene (RAL)

Figure H: Absolute mass and its isotopic abundance of Raloxifene 4-G or 6-G (RAL 4-G or RAL 6-G)

It is evident from the isotopic mass pattern of parent compounds, stable labeled IS (D4) would get interference from their parent compounds by 0.3% for Raloxifene and 0.6% for Raloxifene 4-glucuronide or Raloxifene 6-glucuronide. To deal with this problem two approaches were considered (a) either change the internal standard from D4 to higher stable labeled isotope & (b) or increase the internal standard concentration. Since no other stable labeled internal standard was available other than D4 to any vendor site, hence the second option was finalized for further work. The concentration of the internal standard was increased by a factor of 5. The subsequent IS areas were higher but consistent and had abolished the isotopic impact from parent compounds. The impact of increased internal standard concentration was evaluated in matrix effect & selectivity experiments. Matrix effect experiment was also performed through post column infusion technique [22] was an indicator to see whether interference of endogenous peaks was present at the RT of Raloxifene & metabolites or not. Due to the gradient programming, no interference seen at the RT of all analytes in the chromatograms obtained after the post column infusion experiment.

These experiment results were meeting the SOP criteria & regulatory (FDA & EMEA) acceptance criteria. There was no interference at the RT of Raloxifene-family and internal standard in the selectivity experiment. The post column infusion experiment did not show any suppression and enhancement at the RT of Raloxifene-family and IS. After incapacitating the above problems (Inter-conversion, Isotopic mass pattern), the method was hunted with a systematic tactic. Statistical parameters in three decimals for, mean, standard deviation, and two decimals for % CV, % recovery, % Change & % Bias were considered over the significant figure for reporting purpose.

To the best of our knowledge, there is no publication available for a bioanalytical method which has linearity ranged designed to cater to sensitive terminal sample concentration for Raloxifene and its two active metabolites.

CONCLUSION: With preparer planning and scientific rational,e it is always possible to sail through the various problems (*e.g.,* interconversion and isotopic impact) encountered during experimental work. This newly developed assay method was used in a pharmacokinetic study. The assay method allowed for much higher sample throughput due to short chromatographic time (7.0 min) and a simple sample preparation technique.

A single analytical column was used to chromate-graph about 5000 extracts and ion source did not require to be cleaned during the entire study. This method is an excellent analytical option for the rapid quantification of Raloxifene, Raloxifene 4-Glucuronide, and Raloxifene 6-Glucuronide simultaneously in human plasma.

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CONFLICTS OF INTEREST: This is to declare that there are no conflicts of interest in publishing this manuscript.

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