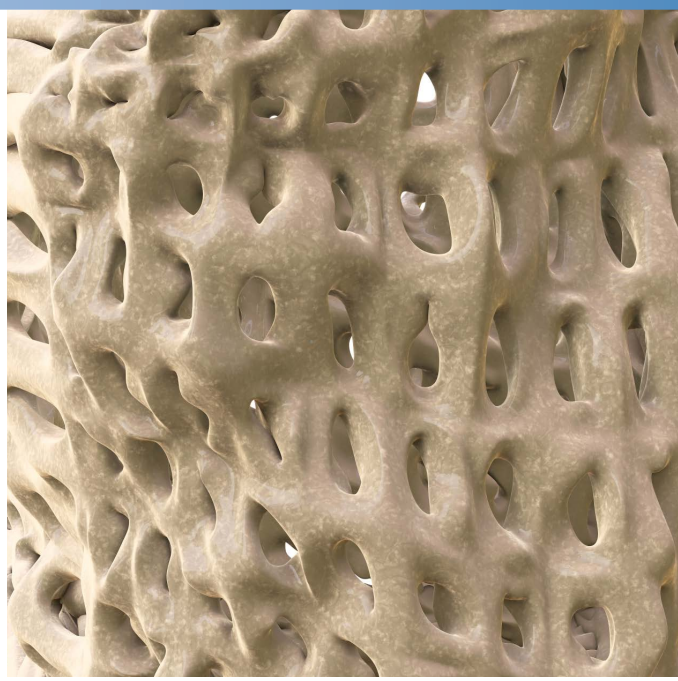
LC-MS/MS Method for the Determination of Raloxifene and its Glucuronide Metabolites from Human Plasma Using SPE Micro Elution

Krishna Rao Dara, Dr. Tushar N. Mehta, Centre of Excellence for Asia Pacific Laboratory
Thermo Fisher Scientific, Ahmedabad, India

Application Note 21002

Key Words
SPE, SOLAμ, Hypersil GOLD PFP, raloxifene, raloxifene glucuronide metabolites

Abstract
A simple, rapid, and sensitive method for the determination of raloxifene (RAL) and its two active metabolites, raloxifene-4-glucuronide (R4G) and raloxifene-6-glucuronide (R6G), in human plasma by liquid chromatography-tandem mass spectrometry using raloxifene-d4 as an internal standard was developed and evaluated. The drug and its metabolites were extracted from a plasma matrix using a Thermo Scientific™ SOLAμ™ SCX 96-well plate. The resultant extracts were separated on a Thermo Scientific™ Hypersil GOLD™ PFP column under reversed-phase, gradient conditions. Detection was performed on a triple quadrupole Thermo Scientific™ TSQ Vantage™ mass spectrometer using positive polarity, heated electrospray ionization (HESI) conditions operating in selected reaction monitoring (SRM) mode. The method was linear in the concentration range of 0.02 to 2 ng/mL, 3 to 300 ng/mL, and 0.6 to 60 ng/mL for RAL, R4G, and R6G, respectively, with excellent separation of two glucuronide metabolites.



Introduction

Raloxifene, 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 levels of 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, but because of extensive presystemic glucuronide conjugation, the absolute bioavailability is only 2%. Significant interpatient differences in bioavailability may result from alterations in the rate of glucuronide formation and enterohepatic recycling [7].

The purpose of this particular study is to demonstrate the effectiveness of combining SOLAμ as solid phase extraction and a Hypersil GOLD PFP HPLC column for the determination of raloxifene and its two metabolites in human plasma with tandem mass spectrometry detection. The structures of raloxifene and its two metabolites are shown in Figure 1.

SOLAμ plates provide reproducibility, robustness, and ease of use at low elution volumes by utilizing the revolutionary SOLA solid phase extraction (SPE) technology. This removes the need for frits, delivering a robust, reproducible format that ensures highly consistent results at low elution volumes.

SOLAμ plates deliver:

- Lower sample failures due to high reproducibility at low elution volumes
- Increased sensitivity due to lower elution volumes
- The ability to process samples which are limited in volume
- Improved stability of compounds susceptible to adsorption and solvation issues

Thermo SCIENTIFIC

Hypersil GOLD PFP (pentafluorophenyl) columns build on the performance of the Hypersil GOLD silica by providing excellent peak shapes while also offering alternative selectivity in reversed phase chromatography compared to alkyl chain phases.

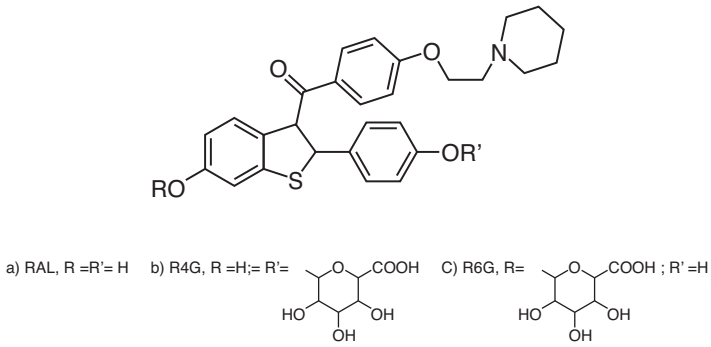


Figure 1: Structures of raloxifene (RAL), raloxifene-4-glucuronide (R4G) and raloxifene-6-glucuronide (R6G)

Experimental Details

Consumables	Part Number
Fisher Chemical™ Optima™ LC-MS grade acetonitrile	A955-4
Ultra pure water	
SOLAμ SCX 2 mg/1 mL 96-well plate	60209-002
Hypersil GOLD PFP 3 µm, 100 × 3 mm	25403-103030
Fisher Chemical Optima formic acid, 90%, LC-MS grade	A117-50
Raloxifene and its two metabolites, kindly supplied by a customer	
Thermo Scientific 96-well square well microplate	60180-P212
Thermo Scientific webseal mat	60180-M122

Sample Handling Equipment	Part Number
Thermo Scientific™ FinnPipette™ (100–1000 µL)	4642090
Thermo Scientific FinnPipette (20–200 µL)	4642080
Thermo Scientific FinnPipette (2–20 µL)	4642060
Thermo Scientific™ Finntip™ Flex™ 1000	94060720
Thermo Scientific Finntip Flex 200	94060320

Sample Pretreatment
A standard spiking stock solution of RAL, R4G, and R6G was prepared in methanol at a concentration of 0.1 mg/mL separately. An internal standard stock solution (d4-raloxifene) was prepared in methanol at a concentration of 0.1 mg/mL.
Blank human plasma (295 µL) was added to 300 µL of 2.0% formic acid. For standards and quality control (QC) samples, 6 µL of standard spiking solution and 20 µL of internal standard solution were added to 295 µL of human plasma. For blanks, 26 µL of water was added.

Extraction Procedure	
Condition:	200 µL methanol
Equilibrate:	200 µL water
Application:	Load pre-treated sample
Wash 1:	200 µL water with 2.0% formic acid
Wash 2:	200 µL methanol
Elution:	2 × 75 µL methanol with 5.0% ammonia
Dilution:	Add 50 µL of water with 6.0% formic acid to each sample

Separation Conditions	
Recommended instrumentation:	Thermo Scientific™ Dionex™ UltiMate™ 3000RS Rapid Separation System
Mobile phase A:	Water + 0.1% formic acid
Mobile phase B:	Acetonitrile + 0.1% formic acid
Mode:	Gradient (refer to Table 1)
Flow rate:	0.5 mL/min
Column temperature:	30 °C
Injection details:	10 µL

Time (min)	% B
0.0	20
6.0	80
6.2	20
7.5	20

Table 1: Mobile phase gradient

MS Conditions	
Instrumentation:	TSQ Vantage MS

The MS conditions and compound transition details are given in Tables 2 and 3.

Parameter	Setting
Ion Source Type	HESI-2
Polarity	Positive
Spray voltage (V)	4000
Vaporizer temperature (°C)	400
Sheath gas pressure (Arb)	45
Ion Sweep gas pressure (Arb)	0
Auxiliary gas pressure (Arb)	12
Capillary temperature (°C)	375
Declustering voltage (V)	0
Collision pressure (mTorr)	1.5
Scan width (*m/z*)	0.2
Scan time (s)	0.1
Q1 (FWHM)	1.2
Q3 (FWHM)	1.2

Table 2: TSQ Vantage MS conditions

Compound	RAL	R4G	R6G	d4-RAL (IS)
Parent (*m/z*)	474.2	650.2	650.2	478.2
Products (*m/z*)	112.1	112.0	112.0	116.1
Collision energy	28	40	40	28
S-lens	203	145	145	111

Table 3: Compound transition details

Data Processing	
Software:	Thermo Scientific™ LC QUAN™ version 2.6 quantitative software

Results

RAL, R4G, and R6G standards extracted from human plasma gave a linear calibration curve over the dynamic range of 0.02 to 2 ng/mL, 3 to 300 ng/mL, and 0.6 to 60 ng/mL with an r^2 coefficient of 0.995, 0.996, and 0.995, respectively (Figures 2, 3, and 4 and Tables 4, 5, and 6). The chromatography at the limit of quantitation (LOQ) is shown in Figure 5.

QC samples were analyzed in replicates of six (Tables 7, 8, and 9).

Overspikes (of RAL, R4G, and R6G) were analyzed and used to calculate recovery and matrix effects (Tables 10 and 11).

The Hypersil GOLD PFP column gave a good separation of RAL, R4G, and R6G.

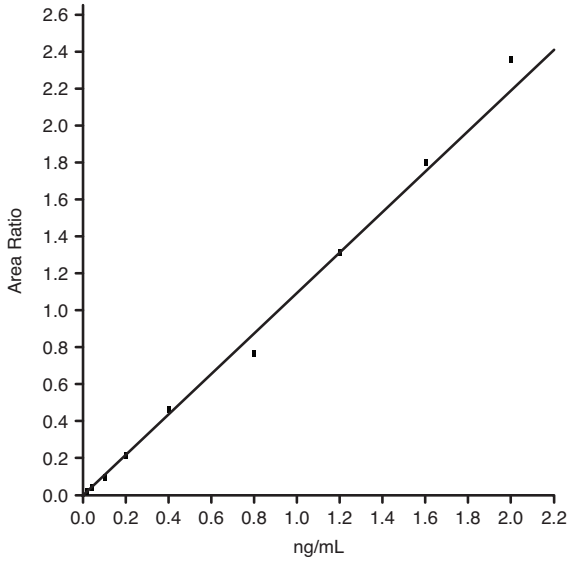


Figure 2: Raloxifene (RAL) linearity over the dynamic range 0.02–2 ng/mL

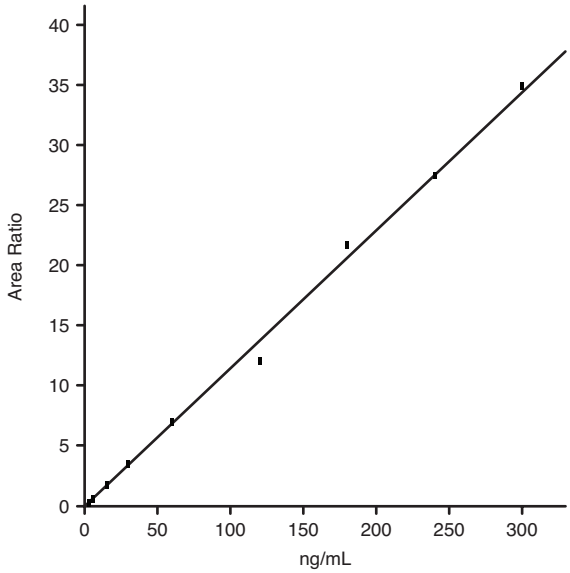


Figure 3: Raloxifene-4-glucuronide (R4G) linearity over the dynamic range 3–300 ng/mL

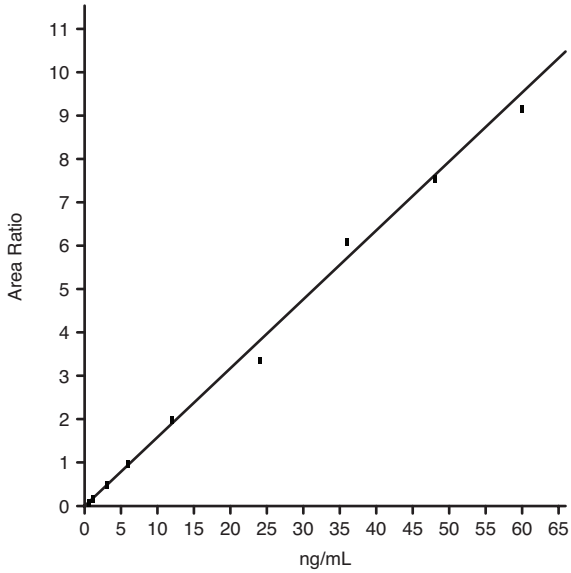


Figure 4: Raloxifene-6-glucuronide (R6G) linearity over the dynamic range 0.6–60 ng/mL

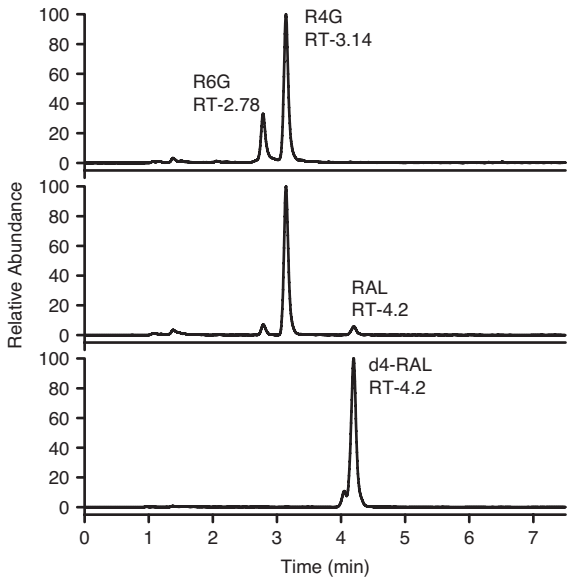


Figure 5: Representative SRM chromatograms of R4G and R6G (top) and RAL (middle), extracted from human plasma at the respective LLOQ levels along with d4-RAL (bottom) (ISTD)

Accuracy and Precision

Standard	Specified Concentration [RAL], ng/mL	Calculated Concentration [RAL], ng/mL	%Diff
S1	0.02	0.02	0.3
S2	0.04	0.04	2.0
S3	0.1	0.09	-6.8
S4	0.2	0.20	-0.6
S5	0.4	0.43	6.6
S6	0.8	0.70	-12.2
S7	1.2	1.20	0.2
S8	1.6	1.65	3.0
S9	2.0	2.15	7.6

Table 4: Accuracy data for extracted RAL standards over the linear range 0.02–2 ng/mL

Standard	Specified Concentration [R4G], ng/mL	Calculated Concentration [R4G], ng/mL	%Diff
S1	3	3.1	2.9
S2	6	5.5	-7.5
S3	15	15.4	2.7
S4	30	30.9	3.1
S5	60	61.7	2.8
S6	120	106	-11.5
S7	180	190	5.6
S8	240	240	0.1
S9	300	305	1.8

Table 5: Accuracy data for extracted R4G standards over the linear range 3–300 ng/mL

Standard	Specified Concentration [R6G], ng/mL	Calculated Concentration [R6G], ng/mL	%Diff
S1	0.6	0.6	1.3
S2	1.2	1.1	-5.8
S3	3.0	3.2	6.2
S4	6.0	6.2	3.5
S5	12	12.6	4.7
S6	24	21.2	-11.7
S7	36	38.4	6.6
S8	48	47.6	-0.9
S9	60	57.7	-3.9

Table 6: Accuracy data for extracted R6G standards over the linear range 0.6–60 ng/mL

Standard	Concentration [RAL], ng/mL	Number of Samples (N)	Peak Area (%RSD)	Peak Area Ratio (%RSD)
QC Low	0.06	6	10.2	6.2
QC Medium	0.7	6	9.6	9.9
QC High	1.4	6	4.1	4.6

Table 7: Average precision data for six replicate QCs for RAL

Standard	Concentration [R4G], ng/mL	Number of Samples (N)	Peak Area (%RSD)	Peak Area Ratio (%RSD)
QC Low	9	6	10.2	6.5
QC Medium	105	6	11.1	11.7
QC High	210	6	8.1	7.3

Table 8: Average precision data for six replicate QCs for R4G

Standard	Concentration [R6G], ng/mL	Number of Samples (N)	Peak Area (%RSD)	Peak Area Ratio (%RSD)
QC Low	1.8	6	10.2	6.4
QC Medium	21	6	10.2	4.6
QC High	42	6	8.0	6.4

Table 9: Average precision data for six replicate QCs for R6G

Recovery

Compound	% Recovery at QCL	% Recovery at QCM	% Recovery at QCH	Average % Recovery
RAL	106	113	116	112
R4G	55	61	53	56
R6G	58	66	56	60

Table 10: Recovery data for RAL, R4G, and R6G

Matrix Effects

Compound	% Signal Suppression (Matrix Effects) at QCL	% Signal Suppression (Matrix Effects) at QCM	% Signal Suppression (Matrix Effects) at QCH
RAL	5	1	5
R4G	-5	-8	15
R6G	-15	-13	10

Table 11: Matrix effects data for RAL, R4G, and R6G

Conclusion

- SOLAμ SPE plates and Hypersil GOLD PFP HPLC columns used with the TSQ Vantage mass spectrometer allow for simple and effective extraction, separation, and quantification of RAL, R4G, and R6G from human plasma.
- The method exhibited good linearity.
- Good accuracy and precision with and without IS correction were observed for RAL, R4G, and R6G at each QC level (Tables 7, 8, and 9). This highlights the benefit of the SOLAμ design in facilitating robust analytical workflows.

References

[1] Chmel, R.; Rob, L.; Strnad, P. What can we expect of raloxifene in the treatment of postmenopausal osteoporosis-views of a gynecologist. *Ceska Gynekol*. **2002**, *67*, 187-191.

[2] Siris, E.; Adachi, J.D.; Lu, Y.; Fuerst, T.; Crans, G.G. *et al*. Effects of raloxifene on fracture severity in postmenopausal women with osteoporosis: results from the MORE study. Multiple Outcomes of Raloxifene Evaluation. *Osteoporos Int*. **2002**, *13*, 907-913.

[3] Barrett-Connor, E.; Mosca, L.; Collins, P.; Geiger, M.J.; Grady, D. *et al*. Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. *N. Engl. J. Med*. **2006**, *355*, 125-137.

[4] Knadler, M.P.; Lantz, R.J.; Gillespie, T.A. The disposition and metabolism of 14C-labeled raloxifene in humans. *Pharm. Res*. **1995**, *12*, 372.

[5] Ni, L.; Allerheiligen, S.R.B.; Basson, R. Pharmacokinetics of raloxifene in men and postmenopausal woman volunteers. *Pharm. Res*. **1996**, *13*, S-430. (Abstract)

[6] Jeong, E.J.; Liu, Y.; Lin, H.; Hu, M. Species- and disposition model-dependent metabolism of raloxifene in gut and liver: role of UGT1A10. *Drug Metab. Dispos*. **2005**, *33*, 785-794.

[7] Evista (raloxifene hydrochloride) package insert (1998) Indianapolis. IN: Lilly.

[8] http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf

For research use only.

thermoscientific.com/chromatography

USA and Canada +1 800 332 3331
Australia 1300 735 292 (free call domestic)
China 800 810 5118 (free call domestic)
400 650 5118
France +33 (0)1 60 92 48 34
Germany +49 (0) 2423 9431 20 or 21
India +91 22 6742 9494
+91 27 1766 2352
Japan 0120 753 670 (free call domestic)
0120 753 671 fax
United Kingdom +44 (0) 1928 534 110
New Zealand 0800 933 966 (free call domestic)
Singapore +65 6289 1190
All Other Enquiries +44 (0) 1928 534 050

Technical Support
For advice and support, please visit our website:
www.thermoscientific.com/chromexpert

Thermo SCIENTIFIC
A Thermo Fisher Scientific Brand

AN21002-EN 0914S