Goal
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 HPLC 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 (pentfluorophenyl) 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

Hypersil GOLD PFP 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.

**Experimental details**

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

**Consumables**

<table>
<thead>
<tr>
<th>Cat. no.</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS-51101</td>
<td>Thermo Scientific™ Pierce™ LC/MS Grade Acetonitrile (ACN)</td>
</tr>
<tr>
<td>50131211</td>
<td>Thermo Scientific™ Barnstead™ GenPure™ water purification system</td>
</tr>
<tr>
<td>60209-002</td>
<td>SOLAµ SCX 96-well plate, 2 mg/1 mL</td>
</tr>
<tr>
<td>25403-103030</td>
<td>Hypersil GOLD PFP HPLC column, 3 µm, 100 x 3 mm</td>
</tr>
<tr>
<td>28905</td>
<td>Thermo Scientific™ Pierce™ Formic Acid, LC-MS Grade</td>
</tr>
<tr>
<td>-</td>
<td>Raloxifene and 2 metabolites</td>
</tr>
<tr>
<td>60180-P212</td>
<td>Thermo Scientific™ WebSeal 96-well non-coated plastic microplates</td>
</tr>
<tr>
<td>60180-M122</td>
<td>Thermo Scientific™ WebSeal Nonsterile Mat</td>
</tr>
</tbody>
</table>

**Sample handling equipment**

<table>
<thead>
<tr>
<th>Cat. no.</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4642090</td>
<td>Thermo Scientific FinnPipette™ F2 Variable Volume Single-Channel Pipette, 100 to 1000 µL</td>
</tr>
<tr>
<td>4642080</td>
<td>Thermo Scientific FinnPipette F2 Variable Volume Single-Channel Pipette, 20 to 200 µL</td>
</tr>
<tr>
<td>4642060</td>
<td>Thermo Scientific FinnPipette F2 Variable Volume Single-Channel Pipette, 2 to 20 µL</td>
</tr>
<tr>
<td>94060720</td>
<td>Thermo Scientific Finntip™ Flex™ Pipette Specific Tips, 1000 µL</td>
</tr>
<tr>
<td>94060320</td>
<td>Thermo Scientific Finntip Flex Pipette Specific Tips, 200 µL</td>
</tr>
</tbody>
</table>

**Sample pre-treatment**

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**

<table>
<thead>
<tr>
<th>Condition</th>
<th>200 µL methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equilbrate</td>
<td>200 µL water</td>
</tr>
<tr>
<td>Application</td>
<td>Load pre-treated sample</td>
</tr>
<tr>
<td>Wash 1</td>
<td>200 µL water with 2.0% formic acid</td>
</tr>
<tr>
<td>Wash 2</td>
<td>200 µL methanol</td>
</tr>
<tr>
<td>Elution</td>
<td>2 x 75 µL methanol with 5.0% ammonia</td>
</tr>
<tr>
<td>Dilution</td>
<td>Add 50 µL of water with 6.0% formic acid to each sample</td>
</tr>
</tbody>
</table>

**Separation conditions**

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

**Table 1: Mobile phase gradient**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>20</td>
</tr>
<tr>
<td>6.0</td>
<td>80</td>
</tr>
<tr>
<td>6.2</td>
<td>20</td>
</tr>
<tr>
<td>7.5</td>
<td>20</td>
</tr>
</tbody>
</table>

**MS conditions**

<table>
<thead>
<tr>
<th>Instrumentation</th>
<th>TSQ Vantage MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>The MS conditions and compound transition details are given in Tables 2 and 3.</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2: TSQ Vantage MS conditions**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ion source type</td>
<td>HESI-2</td>
</tr>
<tr>
<td>Polarity</td>
<td>Positive</td>
</tr>
<tr>
<td>Spray voltage (V)</td>
<td>4000</td>
</tr>
<tr>
<td>Vaporizer temperature (°C)</td>
<td>400</td>
</tr>
<tr>
<td>Sheath gas pressure (Arb)</td>
<td>45</td>
</tr>
<tr>
<td>Ion sweep gas pressure (Arb)</td>
<td>0</td>
</tr>
<tr>
<td>Auxiliary gas pressure (Arb)</td>
<td>12</td>
</tr>
<tr>
<td>Capillary temperature (°C)</td>
<td>375</td>
</tr>
<tr>
<td>Declustering voltage (V)</td>
<td>0</td>
</tr>
<tr>
<td>Collision pressure (mTorr)</td>
<td>1.5</td>
</tr>
<tr>
<td>Scan width (m/z)</td>
<td>0.2</td>
</tr>
<tr>
<td>Scan time (s)</td>
<td>0.1</td>
</tr>
<tr>
<td>Q1 (FWHM)</td>
<td>1.2</td>
</tr>
<tr>
<td>Q3 (FWHM)</td>
<td>1.2</td>
</tr>
</tbody>
</table>

**Table 3: Compound transition details**

<table>
<thead>
<tr>
<th>Compound</th>
<th>RAL</th>
<th>R4G</th>
<th>R6G</th>
<th>d4-RAL (IS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent (m/z)</td>
<td>474.2</td>
<td>650.2</td>
<td>650.2</td>
<td>478.2</td>
</tr>
<tr>
<td>Products (m/z)</td>
<td>112.1</td>
<td>112.0</td>
<td>112.0</td>
<td>112.1</td>
</tr>
<tr>
<td>Collision energy</td>
<td>28</td>
<td>40</td>
<td>40</td>
<td>28</td>
</tr>
<tr>
<td>S-lens</td>
<td>203</td>
<td>145</td>
<td>145</td>
<td>111</td>
</tr>
</tbody>
</table>

**Data processing**

| Software | Thermo Scientific™ LCQUAN™ quantitative software, version 2.6 |
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 R² 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.
### Accuracy and precision

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

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

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

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

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

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

<table>
<thead>
<tr>
<th>Standard</th>
<th>Concentration [RAL], ng/mL</th>
<th>Number of samples (N)</th>
<th>Peak area (%RSD)</th>
<th>Peak area ratio (%RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>QC Low</td>
<td>0.06</td>
<td>6</td>
<td>10.2</td>
<td>6.2</td>
</tr>
<tr>
<td>QC Medium</td>
<td>0.7</td>
<td>6</td>
<td>9.6</td>
<td>9.9</td>
</tr>
<tr>
<td>QC High</td>
<td>1.4</td>
<td>6</td>
<td>4.1</td>
<td>4.6</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Standard</th>
<th>Concentration [R4G], ng/mL</th>
<th>Number of samples (N)</th>
<th>Peak area (%RSD)</th>
<th>Peak area ratio (%RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>QC Low</td>
<td>9</td>
<td>6</td>
<td>10.2</td>
<td>6.5</td>
</tr>
<tr>
<td>QC Medium</td>
<td>105</td>
<td>6</td>
<td>11.1</td>
<td>11.7</td>
</tr>
<tr>
<td>QC High</td>
<td>210</td>
<td>6</td>
<td>8.1</td>
<td>7.3</td>
</tr>
</tbody>
</table>

Table 8: Average precision data for six replicate QCs for R4G
Table 9: Average precision data for six replicate QCs for R6G

<table>
<thead>
<tr>
<th>Standard</th>
<th>Concentration [R6G], ng/mL</th>
<th>Number of samples (N)</th>
<th>Peak area (%RSD)</th>
<th>Peak area ratio (%RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>QC Low</td>
<td>1.8</td>
<td>6</td>
<td>10.2</td>
<td>6.4</td>
</tr>
<tr>
<td>QC Medium</td>
<td>21</td>
<td>6</td>
<td>10.2</td>
<td>4.6</td>
</tr>
<tr>
<td>QC High</td>
<td>42</td>
<td>6</td>
<td>8.0</td>
<td>6.4</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Recovery at QCL</th>
<th>% Recovery at QCM</th>
<th>% Recovery at QCH</th>
<th>Average % recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAL</td>
<td>106</td>
<td>113</td>
<td>116</td>
<td>112</td>
</tr>
<tr>
<td>R4G</td>
<td>55</td>
<td>61</td>
<td>53</td>
<td>56</td>
</tr>
<tr>
<td>R6G</td>
<td>58</td>
<td>66</td>
<td>56</td>
<td>60</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Signal suppression (Matrix effects) at QCL</th>
<th>% Signal suppression (Matrix effects) at QCM</th>
<th>% Signal suppression (Matrix effects) at QCH</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAL</td>
<td>5</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>R4G</td>
<td>-5</td>
<td>-8</td>
<td>15</td>
</tr>
<tr>
<td>R6G</td>
<td>-15</td>
<td>-13</td>
<td>10</td>
</tr>
</tbody>
</table>

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 design the benefit of the SOLAµ plates in facilitating robust analytical workflows.

References