Quantification 25-Hydroxyvitamin D_2 and D_3 and 24R,25-dihydroxyvitamin D_3

25-hydroxyvitamin D_2 ($25(OH)D_2$), 25-hydroxyvitamin D_3 ($25(OH)D_3$), and 24R,25-dihydroxyvitamin D_3 ($24R,25(OH)_2D_3$) were measured by Ultra High Performance Liquid Chromatography (UHPLC) Tandem Mass Spectrometry. Briefly, standards were prepared by spiking known amounts of $25(OH)D_2$, $25(OH)D_3$, and $24R,25(OH)_2D_3$ (Sigma-Aldrich, St. Louis, MO) to pseudo plasma. Samples and standards (150ul for $25(OH)D_2$ and $25(OH)D_3$; 200ul for $24R,25(OH)_2D_3$) were spiked with 10 uL of a solution of internal standards (IS; hexa-deuterated $25(OH)D_2$ and $25(OH)D_3$; Medical Isotopes, Pelham, MA). $25(OH)D_3$ was used as IS for $24R,25(OH)_2D_3$. Proteins were precipitated with the addition of 1 sample volume of 0.2M $ZnSO_4$ and 2 sample volumes of methanol. Samples and standards were subsequently extracted with either 750ul of hexane (for $25(OH)D_2$ and $25(OH)D_3$) or 1000µl of 60% hexane/40% dichloromethane (for $24R,25(OH)_2D_3$). The organic phase was evaporated and reconstituted in 50µl of 70% methanol for $24R,25(OH)_2D_3$ and 100µl of 70% methanol for $25(OH)D_2$ and $25(OH)D_3$ analysis.

5µl of each sample was injected onto a 2.1 x 50 mm Zorbax Eclipse Plus C18 1.8um column (Agilent, Santa Clara, CA) run isocratically at 50°C for 4.5 minutes (78% 7.5mM ammonium formate in methanol; 0.3ml/min) followed by a 2 minute rinse (95% 7.5mM ammonium formate in methanol) and a 1.5 minute re-equilibration period (total run time: 8 minutes) using a 1290 UHPLC (Agilent, Santa Clara, CA). Samples were detected by positive electrospray ionization with a 6410 triple quadrupole mass spectrometer

(Agilent, Santa Clara, CA). The source temperature was set to 250°C, gas flow 10 l/min, nebulizer pressure 45 psi, and capillary set to 4000V. $25(OH)D_2$, $25(OH)D_3$, and $24R,25(OH)_2D_3$ and the IS were optimized with a fragmentor voltage of 96V. $25(OH)D_2$, $25(OH)D_3$, $24R,25(OH)_2D_3$, and IS were quantified using multiple reaction monitoring of the H+ ion with the transition 413.3 to 395.3 (collision energy = 2V), 401.3 to 383.4 (collision energy = 3V), 417.6 to 121.2 (collision energy = 13V) and 407.3 to 389.3 (collision energy = 2V), respectively. Quantification of analytes was calculated using the spiked pseudo plasma standard curves (response of analyte/response of IS).

The method is linear from 1.3ng/ml to 100 ng/ml, 0.7ng/ml to 100ng/ml and 0.5ng/ml to 100ng/ml, and inter-assay precision is 3.4%, 4.8% and <10% for $25(OH)D_2$, $25(OH)D_3$, and $24R,25(OH)_2D_3$, respectively.