

Lab Specimen Documentation

Quantification of 25-hydroxy Vitamin D2 [25(OH)D2], 25-hydroxy Vitamin D3 [25(OH)D3], and Epimeric Vitamin D3 [epi-25(OH)D3] in Human Serum or Plasma Using Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS).

Patient serum or plasma, calibrators, and controls (200 µL) are alkalized with 200 µL 1 N sodium hydroxide in a 96-deep well plate, covered with a silicone cover (MicroMat, Varian) vortexed for 15 s, and incubated at room temperature for 15 min. Internal standard [200 µL; 121.3 nmol/L (50 ng/mL) 25(OH)D2 and 25(OH)D3 in methanol] is added to each well and the plates are covered and vortexed for 15 sec. Samples are extracted with 1 mL n-heptane (5 min vortex, covered) and the plates are centrifuged at 1,100xg for 4 min at room temperature in a Beckman Allegra X-22 centrifuge equipped with a 96-well plate rotor. To seal the two plates together, a transfer gasket and another 96-deep well plate are fitted on top of the extraction plate. The sealed plates, held together by the gasket, are then placed in a dry ice-acetone bath for 50 min to freeze the lower aqueous layer. The entire organic layer is then transferred to the new plate by inverting and gently tapping the assembly on the benchtop. The extracts are dried under forced nitrogen at room temperature (Turbovap) and the residue is reconstituted in 200 µL 75% methanol in water.

Samples may also be extracted and pipetting performed using a liquid handling station. The process is similar to what is described above, but all of the liquids are handled with an automated pipetting device. In addition, after centrifuging the 96-well plate, the upper layer may be removed using the automated liquid handler rather than using the liquid transfer gasket.

A portion of the dissolved extracts (40 µL) is injected and developed using penta-fluorophenyl propyl chromatography (Restek PFPP, 100x3.2 cm 5 µm, 3.5 µm column with an integrated guard column) with isocratic mobile phase (2 mM ammonium acetate, 0.1% formic acid in 78.8% methanol in water, 0.4 mL/min) and analyzed using isotope dilution-tandem mass spectrometry (Quattro Micro with a 2795 HPLC, Waters). Concentrations of 25-hydroxy D2 and D3 are then calculated using a calibration curve made in 2% bovine serum albumin. Two blinded control samples will be interspersed in each batch.