LC-MS/MS Method for the Measurement of Free 25-OH Vitamin D_3

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The measurement of total 25-OH Vitamin D_3 is suboptimal with serum concentrations correlating poorly with PTH. For this reason we wished to explore the possibility of quantifying the free fraction employing ultrafiltration at 37°C and LC-MS/MS.

An AB SCIEX TRIPLE QUAD 6500 tandem mass spectrometer equipped with Atmospheric Pressure Chemical Ionization (APCI) source and Shimadzu HPLC system was employed to perform the analysis using isotope dilution with deuterium labeled internal standard 25-OH Vitamin D_3-d_6. 600 µL of 20 pg/mL internal standard in MeOH was added to the collection cup of a Sartorius VIVASPIN 2 HY ultrafiltration device (10,000 MW cut-off) in advance. 500 µL of human plasma/serum was pipetted to the VIVASPIN 2 ultrafiltration device for centrifugation at 2200 g and 37°C for about 8.5 minutes, when just 300 µL of sample was filtered through the ultrafiltration device. After centrifugation, ultrafiltrate and internal standard mixture was transferred directly to a glass sample vial and vortexed for 10 seconds. 300 µL aliquot was injected onto an Agilent Poroshell 120 SB-C8 column where both 25-OH Vitamin D_3 and internal standard undergo an on-line extraction, gradient chromatographic separation and elution. Quantitation by multiple reaction-monitoring (MRM) analysis was performed in the positive mode. The transitions selected were: mass-to-charge (m/z) 383.3 →229.2 for 25-OH Vitamin D_3 and 389.3 →211.2 for 25-OH Vitamin D_3-d_6. Nitrogen served as curtain and collision gas. The main working parameters of the mass spectrometer were: collision gas 7, curtain gas 35, ion source gas (GS1) 60, nebulizer current 3, probe temperature 350 °C, entrance potential 10 V, and dwell time 50 msec.

The between-day coefficients of variation (CVs) were below 10% for free 25-OH Vitamin D_3 at all concentration tested. Accuracy ranged between 90% and 110%. Good linearity was also obtained within the concentration range of 1-25 pg/mL for free 25-OH Vitamin D_3 ($r \geq 0.995$). The range of results from 34 healthy volunteers was 1.5 to 17.9 pg/mL. This cohort was supplemented with 8 patients with elevated parathyroid hormone (PTH). The free 25-OH Vitamin D_3 concentration correlates excellently with the concentration of PTH and poorly with

the total 25-OH Vitamin D_3 concentration. A poor correlation was observed between total 25-OH Vitamin D_3 and PTH.

We describe the first simple, accurate, and fast isotope dilution tandem mass spectrometry method for the measurement of free 25-OH Vitamin D_3 in human serum/plasma samples employing a high sensitivity tandem mass spectrometer. We can now evaluate the role of free 25-OH Vitamin D_3 in patients with bone and/or a variety of malignant diseases.