Metabolism and Pharmacokinetics of Vitamin D in Patients with Cystic Fibrosis

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**Supplemental Material and Methods**

**Chemicals and materials**

25(OH)D3-S, 25(OH)D3-G and *d6*-25(OH)D3 were purchased from Toronto Research Chemicals (North York, Ontario, Canada). (Diacetoxyiodo)benzene, 3'-phosphoadenosine-5'-phosphosulfate (PAPS), uridine 5′-diphosphoglucuronic acid trisodium salt (UDPGA), alamethacin, 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride (Tris HCl), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Corp. (St. Louis, MO). 4-(4'-dimethylaminophenyl)-1,2,4-triazolidine-3,5-dione (DAPTAD precursor) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX). HPLC grade acetonitrile, ethyl acetate, sodium acetate, ammonium hydroxide (28% w/w), methanol, methyl tert-butyl ether (MTBE), heptane, isopropanol, hexanes, formic acid, acetic acid, dichloromethane, and magnesium chloride were purchased from Fisher Scientific (Pittsburgh, PA). Vitamin D free serum (Seratrol™) was purchased from Golden West Biologicals, Inc. (Temecula, CA). Human liver microsomes and human liver cytosol were generated at the University of Washington.

Quantification of vitamin D metabolites

Vitamin D metabolites (i.e., 25(OH)D3, 1α,25(OH)2D3, 24,25(OH)2D3, 25(OH)D2, and 1α,25(OH)2D2) and circulating levels of fibroblast growth factor 23 (FGF), parathyroid hormone (PTH), albumin, and VDBP were measured in serum samples by liquid chromatography with tandem mass spectrometry (LC-MS/MS) by the Department of Laboratory Medicine at the University of Washington. *VDBP* haplotype was determined from DNA samples by the Department of Laboratory Medicine at the University of Washington.

A 4β,25(OH)2D3 calibration curve was prepared in BSA. After thawing, 500 µL of serum was aliquoted and an internal standard solution (20 µL of 60 pg/mL of *d6*-1α,25(OH)2D3) was added to each sample. Samples were vortexed, allowed to rest at room temperature for 25 minutes to allow for equilibration of the internal standard and centrifuged at 13,362 *g* for 10 min at 4°C following the addition of 1 mL of acetonitrile to precipitate soluble proteins. The supernatant was transferred to silanized glass tubes. After the addition of 1.5 mL of 0.1 M phosphate buffer (pH 10.5), analytes were extracted using two solid phase extraction (SPE) steps using Agilent (Santa Clara, CA) Bond Elut C18 OH SPE cartridges (500 mg, 3 mL) and Agilent Bond Elut SI cartridges (100 mg, 1 mL). Sample analytes were eluted with 5% isopropanol in MTBE (v/v) into silanized glass culture tubes and dried under N2 gas at 37°C. The residue was reconstituted in 50 µL of acetonitrile, followed by the addition of DAPTAD (50 µL). Samples were shaken at room temperature for 45 minutes before the reaction was quenched by drying under N2 gas at 37°C. Following reconstitution in 45 µL of methanol and 20 µL of water, the derivatized samples were centrifuged at 13,362 *g* for 5 min to remove insoluble particulates and excess DAPTAD. The supernatant was transferred to a 96-well plate for LC-MS/MS analysis.

Liquid chromatography-mass spectrometry (LC-MS/MS) analysis was performed on a Sciex QTRAP 6500 hybrid triple quadrupole/linear ion trap mass spectrometer (Framingham, MA) coupled with a Shimadzu liquid chromatography system (Kyoto, Japan). Chromatographic separation was achieved using an Ascentis Express RP-Amide column (2.1 mm x 150 mm x 2.7 µm) (Sigma-Aldrich Corp.), attached to a SecurityGuard™ ULTRA cartridge (2.1 mm x 2 µm) guard column (Phenomenex, Torrance, CA). The column temperature was maintained at 20°C with a mobile phase flowing at a rate of 0.275 mL/min. The autosampler was maintained at 4°C and the injection volume was 10 μL. A gradient was used to elute the analytes with a mobile phase consisting of 0.1% formic acid in water (A) and 100% methanol (B)The total run time was 26 minutes. Mass spectrometric conditions are summarized in Table S1. The electrospray ionization (ESI) source of the mass spectrometer was operated in the positive ion mode. Multiple reaction monitoring (MRM) was used for quantitation. The retention time, precursor and product ions, declustering potential, entrance potential, collision energy, and collision cell exit potential for each analyte are presented in Table S1.

Peak integration was performed using MultiQuant software (version 3.0.2) from AB Sciex. The peak height of 4β,25(OH)2D3 was normalized to*d6*‑1α,25(OH)2D3. A quadratic equation with 1/x2 weighting was used to estimate the relationship between the peak height ratio and concentration. The lower limit of detection (LLOD) was set at 5 times above background.

The conjugated metabolites, 25(OH)D3-S and 25(OH)D3-G, were quantified by the LC-MS/MS method described in Gao et al. (51). In brief, the calibration curve and QC samples were prepared in vitamin D-free serum. The internal standards, *d6*-25(OH)D3-S and *d6*-25(OH)D3-G, were generated by enzymatic incubations with *d6*-25(OH)D3. The *d6*-25(OH)D3-S internal standard was prepared by incubating 25 µM of *d6*-25(OH)D3, 0.1 M PAPS, and 4 mg/mL of pooled human liver cytosol in 50 mM Tris HCl buffer (pH: 7.5) (final volume 1 mL) at 37°C. The reaction was quenched after 3 to 5 hours with the addition of 1 mL of cold acetonitrile. The *d6*-25(OH)D3-G internal standard was prepared by incubating with 25 µM *d6*-25(OH)D3, 50 µg/mL alamethacin, 5 mM MgCl2, 5 mM UDPGA, and 1 mg/mL pooled human liver microsomes in 50 mM Tris HCl buffer (pH 7.5) (final volume 1 mL) at 37°C. The reaction was quenched after 3 to 5 hours with 1 mL of cold acetonitrile. Precipitated proteins were removed from the internal standard products by centrifuging the samples at 13,000 g for 10 min at 4°C. Following derivatization with DAPTAD as described below, the abundance of *d6*-25(OH)D3-S and *d6*-25(OH)D3-G in the internal standard solutions was estimated by comparing LC-MS/MS peak heights with undeuterated 25(OH)D3-S and 25(OH)D3-G standards.

Human serum (200 µL) was combined with approximately 4 pmol of *d6*-25(OH)D3-S and 1.6 pmol of *d6*-25(OH)D3-G. Proteins were precipitated with 1 mL of acetonitrile and centrifuged at 13,000 *g* for 10 min at 4°C. The supernatants were combined with 1 mL of 0.1 M sodium acetate (pH 3.2). The samples were subjected to SPE using Waters Oasis WAX (1 cc, 30 mg, 60 μm) anion exchange cartridges (Waters (Milford, MA). The analytes were eluted from the column with the addition of 28% w/w ammonium hydroxide in methanol (3:97 v/v). Samples were dried under N2 at 37°C and reconstituted in 50 µL of acetonitrile and derivatized with 50 µL of DAPTAD solution for 1 hour. Solutions were dried under N2 at 37°C, reconstituted in 100 μL of 30% acetonitrile in water and centrifuged at 20,000 *g* at 4°C for 5 min to remove excess DAPTAD.

Chromatographic separation was achieved using a Hypersil Gold (2.1 × 100 mm, 1.9 μm) column (Thermo Fisher Scientific, Waltham, MA) maintained at 45°C. LC-MS/MS analysis was performed on a Sciex QTRAP 6500 hybrid triple quadrupole/linear ion trap mass spectrometer with ESI operated in the positive mode coupled with a Shimadzu liquid chromatography system. The MRM transitions used for quantification, standard curve range, and the limits of quantification are presented in Table S2.

Peak integration was performed by MultiQuant software (version 3.0.2). Peak area ratios were determined by taking the peak areas of 25(OH)D3-S or 25(OH)D3-G and normalizing to the respective deuterated metabolite peak areas. A linear equation with 1/x2 weighting was fit to estimate the relationship between peak area ratios and concentrations of 25(OH)D3-S or 25(OH)D3-G.

**Table S1:** Retention time, precursor molecular ion/product ion for quantification and detection parameters for 4β,25(OH)2D3 and its internal standard

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Analyte | Retention Time  (min) | Precursor Ion | Product Ion | DPa  (V) | CEb  (V) | LLOQc  (pg/mL) | Standard Curve Range (pg/mL) |
| 4β,25(OH)2D3 | 15.5 | 635.2 | 357.1 | 146 | 33 | 1.6 | 2-800 |
| *d6-*1α,25(OH)2D3 | 15.6 | 641.2 | 357.1 | 146 | 33 | - | - |

a declustering potential

b collision energy

c lower limit of quantification

**Table S2:** Retention time, precursor molecular ion/product ion for quantification and detection parameters for 25-dihydroxyvitamin D3-sulfate, 25-dihydroxyvitamin D3-glucuronide, and their internal standards

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Analyte | Retention Time  (min) | Precursor Ion | Product Ion | DPa  (V) | CEb  (V) | LLOQc  (ng/mL) | Standard Curve Range (ng/mL) |
| 25(OH)D3-S | 10.78 | 699.5 | 323.0 | 100 | 45 | 0.5 | 2.4 – 96 |
| *d6-*25(OH)D3-S | 10.72 | 705.5 | 323.0 | 100 | 45 | - | - |
| 25(OH)D3-G | 9.59 | 795.5 | 341.1 | 100 | 42.5 | 0.2 | 0.3 – 11.5 |
| *d6-*25(OH)D3-G | 9.56 | 801.5 | 341.1 | 100 | 42.5 | - | - |

a declustering potential

b collision energy

c lower limit of quantification

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**Figure S1:** Partial metabolic schemes of A) vitamin D2 and B) vitamin D3