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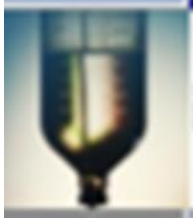
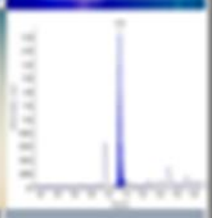




SecoSet Derivatization Kit For Vitamin D Analysis

Sample Preparation Manual for:

Dried Blood Spots

Dried Plasma Spots

Centrifuged Plasma

Novilytic LLC

1. Introduction

Vitamin D is actually a prohormone and the active hormone plays a role in chronic diseases such as osteoporosis, hypertension, autoimmune disorders and cancer. Contemporary research strategies often require quantitation of vitamin D and its metabolites at the nutritional and molecular level to facilitate broader understanding of various bio-regulatory functions unique to this hormone-like vitamin.

SecoSet by Novilytic is a derivatization kit intended for use to determine the concentration of 25-hydroxy-vitamin D2 and D3 in plasma or serum samples. Vitamin D and its metabolites are members of the class of 9, 10-secosteroids. The patented (US **9,068,995)** reagent in the SecoSet kit specifically targets secosteroids and forms a derivative that enhances signal intensity in tandem LC/MS. The derivatization reaction requires less than 2 minutes and no further sample preparation is required after the addition of reagents.

2. **Contents -** Derivatization Reagents and Optional Internal Standards

Warning: Wear disposable gloves while handling specimens or kit components and wash hands thoroughly afterwards. Follow the MSDS datasheet for safe treatment. Do not interchange reagent kit components from prior lots.

For Research Use Only. Not for use in diagnostic procedures.

1. **Derivatization Reagents include 2 vials of DR1, DR2, and DR3, respectively**. Store in the dark at -20˚C until use. Remove the vials from the freezer and allow to come to room temperature at least 15 min before use.
   - DR1; each containing initiator for chemical reaction in powder-dry form. Each vial to be reconstituted in 2.0 mL methanol.
   - DR2, each containing the SecoSet reagent in powder-dry form. Each vial to be reconstituted in 2.0 mL methanol.
   - DR3, each containing chemical to stop the reaction in powder-dry form.. Each vial to be reconstituted in 2.0 mL H_2O.

   Note: Each vial of reconstituted DR reagent is enough to run 40 solvent-extracted plasma/serum samples. The kit can derivatize up to 80 samples.

2. **Optional Internal Standards - Sold Separately**
   The Vitamin D2 and D3 isotopically labeled standards are sold separately.
   Vitamin D2 and D3 Internal Standard; store in the dark at below-20˚C.
   Single vial containing $^2H_6$-25-OH Vitamin D2 and $^2H_6$-25-OH Vitamin D3 in methanol (see the label of the vial for reference concentration (ng/mL) which may vary lot by lot). May be aliquoted for multiple use. Allow the vials to come to room temperature prior to any transfer operation.

   Caution: The concentrations of the Vitamin D2 and D3 will vary slightly with temperature. The recommended temperature range is 20 - 24°C. Internal standard may be aliquoted into separate amber glass containers for use with multiple separate runs. Vials must be re-sealed upon use.

Required Equipment or Reagent (Not provided)

1. 1.5 ml reaction tubes (Eppendorf)
2. Borosilicate glass tubes 13x100mm
3. Centrifuge
4. Various pipettes
5. Water bath (30°C) or evaporator
6. HPLC column (Suggested or compatible)
7. Vortex mixer
8. Solvent (Methanol, Water, and MTBE (methyl *tert*-butyl ether))

3. Sample Derivatization Procedures

3.1 Protocol for Plasma Prepared by Centrifugation

Note: Deviations from the method presented below may affect accuracy of results.

First Step: Liquid-liquid extraction

1. Transfer 100uL of plasma/serum sample or standard into an appropriate tube. **Note:** It is recommended to use borosilicate glass tubes 13 x 100mm or a 2 mL polypropylene tube
2. Add 50 µL Mixed Internal Standard to sample in tube.
3. Add 50 µL 1M potassium phosphate buffer, pH 7.4
4. Vortex vigorously, then allow to equilibrate in the dark at room temperature for 1-2 hours.
5. Add 1 mL methyl tertiary butyl ether **(**MTBE); vortex vigorously for 2 minutes.
6. Centrifuge (>2000Xg) for 10 minutes
7. Freeze at -80°C for >30 minutes.
8. Collect MTBE supernatant into a separate tube.
9. Evaporate under regulated nitrogen at 30°C until dry

Second Step: Derivatization

Note: The following steps are time-sensitive. Each step must be performed immediately upon completion of the previous step. The reagents must be fully dissolved before executing the remainder of this procedure. The dried tubes are sealed and may be stored at -20°C until derivatization.

1. Add 50 µL of DR1 solution (2 mg/mL in MeOH) to the dried residue. Vortex for 10 seconds.
2. Add 50 µL of DR2 solution (2 mg/mL in MeOH). Vortex for 60 seconds.
3. Add 50 µL of DR3 (8 mg/mL in H_2O) solution. Vortex for 10 seconds.
4. Centrifuge (> 10,000*X*g) as necessary to drop any precipitate.
5. Transfer supernatant to an appropriate autosampler vial for LC/MS/MS analysis.

Note: The derivatized samples may be analyzed immediately or stored at -20˚C.

3.2 Protocol for Dried Blood Spot Cards

1. Apply a single drop of blood onto the DBS card or pipette 10 µL of whole blood inside the circle
2. Dry the DBS card overnight in the dark
3. Excise or punch out the entire plot spot and place the cutout into a tube
4. Add 50 µL of research grade MeOH to precipitate protein
5. Add 4µL of internal standard containing $^{2}H_{6}$-25-OH Vitamin D2 and $^{2}H_{6}$-25-OH Vitamin D3
6. Add 50 µL of SecoSet reagent (DR1, Caution: concentration of DR1 for DBS card should be 4mg/mL because of step 4.), vortex for 10 seconds
7. Add 100 µL of Oxidizing reagent (DR2), vortex for 1 minute
8. Add 100 µL of Stopping reagent (DR3), vortex for 10 seconds
9. Centrifuge @10,000xg for 2 min
10. Transfer supernatant to autosampler vial and inject 15 µL into an autsampler vial.

3.3 Protocol for the Noviplex Plasma Card

1. Apply a single drop of blood onto the DBS card or pipette 35 µL of blood as directed
2. After 3 minutes remove the top layer with the blood cells and discard in a biohazard container
3. Dry the bottom layer with the plasma collection disc for 15 minutes
4. Transfer the plasma collection disc to a 2 mL polypropylene tube.
5. Add 50 µL of research grade MeOH to precipitate protein
6. Add 4µL of internal standard containing $^{2}H_{6}$-25-OH Vitamin D2 and $^{2}H_{6}$-25-OH Vitamin D3
7. Add 50 µL of SecoSet reagent (DR1, Caution: concentration of DR1 for plasma card should be 4mg/mL because of step 5.), vortex for 10 seconds
8. Add 100 µL of Oxidizing reagent (DR2), vortex for 1 minute
9. Add 100 µL of Stopping reagent (DR3), vortex for 10 seconds
10. Centrifuge @10,000xg for 2 min
11. Transfer supernatant to autosampler vial and inject 15 µL into an autsampler vial.

4. **Protocol for LC/MS Analysis**

Ensure the mass spectrometer is properly tuned or calibrated for both normal and deuterated forms of 25-hydroxy vitamin D2 and D3 for optimal sensitivity. Utilize the optimization technique recommended by the manufacturer of the mass spectrometer.

Vitamin D Species		MRM Transition	Collision Energy
			AB Sciex 4000
25 (OH)VD2	Native Species	617.4/149.1	85
d6-25 (OH)VD2	Deuterium Labeled	623.4/149.1	89
25 (OH)VD3	Native Species	605.4/149.1	93
d6-25(OH)VD3	Deuterium Labeled	611.4/149.1	85

LC Method

- Temperature at autosampler: 4˚C
- Temperature at column oven: 40˚C

Time(min)	% B
0	25
6	35
14	50
15	100
18	100
18.1	25
21	stop

- Inject 10 to 15 µL of sample
- Solvent A: 100% H_2O/0.05% formic acid
- Solvent B: 100% ACN/0.05% formic acid
- C18 column (150 x 2.1 mm, 2.7µm)HALO^{TM}; gradient from 25% water (0.05% formic acid) to 50% acetonitrile (0.05% formic acid); flow rate of 0.2 mL/min

LC Gradient Program

Tuning Parameters for a SCIEX API 4000 Triple Quad

- Curtain gas: 20
- Collision gas: high
- Ion spray voltage: 5000
- Temp: 450
- Ion source gas 1: 30
- Ion source gas 2: 50

Note: For each analyte, two peaks may be seen, choose either two peaks or the most abundant peak for the integration of the area after MRM (Please make sure to be consistent for both analyte and IS).

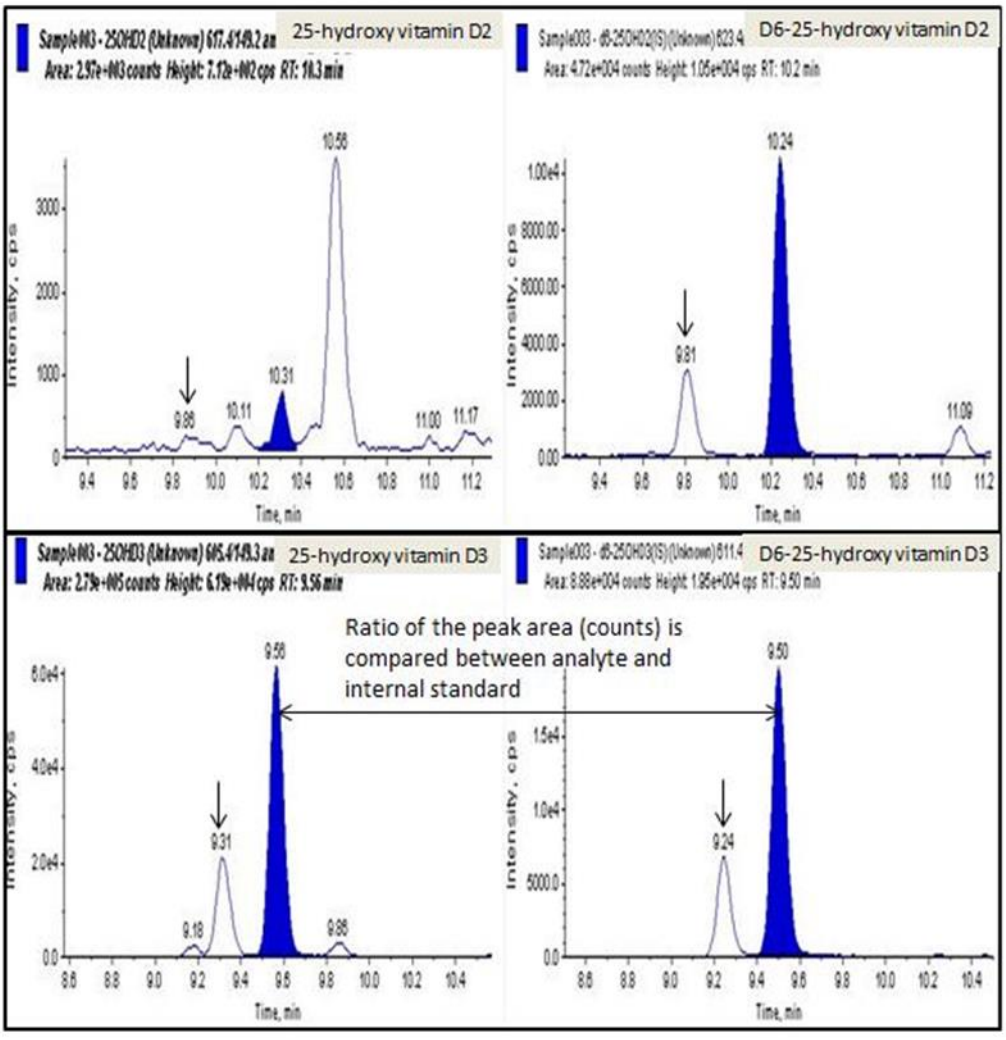
5. LC/MS Results

The derivatized vitamins elute at a concentration of between 35 and 50% acetonitrile. Retention time is depending on flow rate and column. Retention times may shift slightly

between runs due to normal operating variability. Generally, deuterated (heavy) samples are eluted a slightly earlier than non-deuterated (light), the difference of retention time is less than 0.1min in the system described above. It is highly recommended to zoom time axis within 5min to view peak at the expected retention time in the spectrum window.

Caution: Retention time may vary from those above due to variability between hardware, systems and condition. The peak intensity and background noise are very dependent on your system.

Extracted Ion Chromatograms of Native and Deuterated Vitamin D2 and D3



Example shows an extracted ion chromatogram using Multiple Reaction Monitoring of 25-hydroxy vitamin D2, and 25-hydroxy vitamin D3 in plasma, treated with D6-25- hydroxyl vitamin D2, and D6-25- hydroxyl vitamin D3 as an internal standard, Followed by liquid-liquid extraction and SecoSet tagging to enhance ionization. In this example, retention time has approximately 0.06min difference between light and heavy form of the vitamin. Two vitamin peaks may be observed in an equivalent LC system described here with a high resolution. The highlighted peak is more abundant peak of the vitamin and arrow indicates the second peak of the vitamin.

When analyzing 25(OH) VD2 in plasma samples, a few other peaks may be observed eluting at retention times close to (before and after) that of the vitamin's abundant peak, sometimes making it difficult to discriminate the abundant peak from the adjacent peaks. The abundant 25(OH)VD2 can be positively identified at system set-up by spiking a plasma sample with light tuning standard (LD2) prior to LC-MS/MS run and observing the retention time exhibiting a relative increase in peak area. It is recommended that only the abundant peak integrated for 25(OH)VD2. The user must also consider the potential for interference from other diene containing molecules that could be tagged during derivatization.

6. Calculation of Vitamin D Concentration

When replicates from a sample are measured, averaged formula I value is used for calculation below.

1. Formula I= (Peak Area of Analyte)/(Peak Area of Internal Standard)

2. Concentration of the internal standard (nM) in a LLE sample=(Given concentration (ng/ml)of the internal standard)/(Molecular weight of internal standard)

   Or
   Amount of the internal standard (pmol) in a DBS or plasma card=(Given concentration (ng/ml) of the internal standard * (Applied μL volume of internal standard))/(Molecular weight of internal standard)

   Note: The molecular weight of D6-25hydroxy vitamin D2 and D6-25hydroxy vitamin D3 is 623.4 and 611.4, respectively. The concentration of the internal standard can be found on the vial.

3. Concentration of the analyte (nM) in a LLE sample=[((Formula I)/R) * (Concentration of the internal standard (nM) in a LLE sample above)]
   R=Volume ratio of plasma/internal standard

   Or
   Amount of the analyte (pmol) in a DBS or plasma card = (Formula I) * (Amount of the internal standard (pmol) in a DBS or plasma card above)

4. Concentration of the analyte (ng/mL) in a LLE sample
   = (Concentration of the analyte (nM) * Molecular weight of the analyte)/1000

Or
Concentration of the analyte (ng/mL) in a DBS or plasma card = [(Amount of the analyte (pmol) in a DBS or plasma card above) * (Molecular weight of the analyte)]/(Volume of plasma collected (μL)**
**plasma volume may be varied by hematocrit, manufacturer, and lot.

Note: The molecular weight of 25hydroxy vitamin D2 and 25hydroxy vitamin D3 is 617.4 and 605.4, respectively.