Comparison of several approaches for vitamin D metabolite analysis

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Introduction

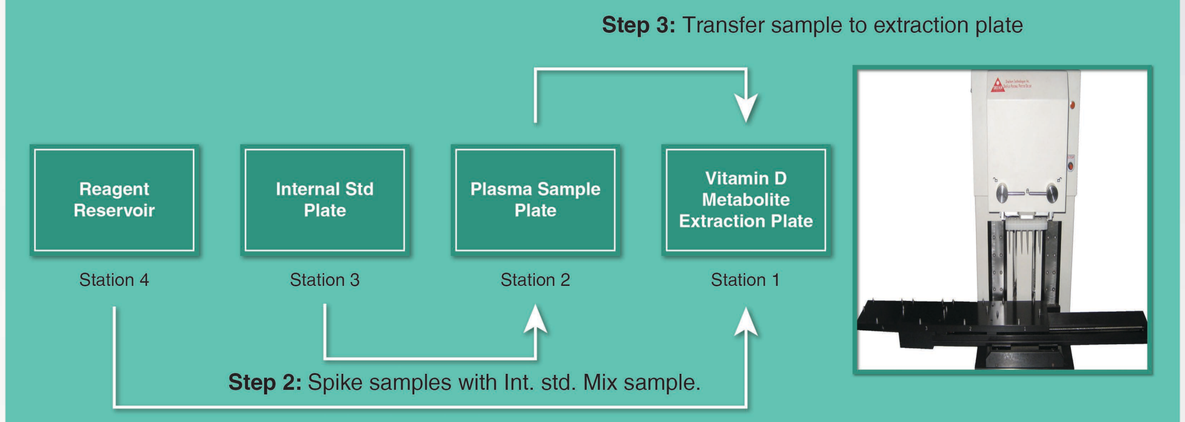
Vitamin D is a fat soluble vitamin responsible for regulating many physiological functions, among them, the absorption of calcium and phosphate. While the importance of Vitamin D has been recognized in maintaining the overall health and well being of individuals, increased clinical awareness of the prevalence of vitamin D deficiency and insufficiency has led to a need for large volume vitamin D metabolite assay.

Vitamin D and its metabolites circulate in blood bound to serum proteins. Thus to be able to analyze the metabolites, one will need to dissociate them from the serum proteins. LC-MS/MS has emerged as a reliable method of choice for vitamin D metabolite analysis due to its high specificity and high accuracy at low ng/mL level. However, LC-MS/MS is easily affected by phospholipids content in the serum samples.

We present two different approaches here for efficient vitamin D analysis:

1. Vitamin D metabolites extraction plate, It is simple and fast, while provides reasonable recoveries.
2. Sagacity HL SPE products, provides high recoveries and reproducibility of mono- and di- hydroxyl vitamin D.

Oroflex Robotic Workflow



Experiments

Instruments All automated extractions were carried out using Orochem's Oroflex Personal Pipettor robot. All LC-MS/MS method used a Shimadzu HPLC system coupled to an API 3000 mass spectrometer with a turbo spray ESI source operated in positive mode.

Materials Orochem's 96-well Vitamin D Metabolite Extraction plate, and Sagacity HL SPE plate were used for all extractions. The 25-hydroxyvitamin D2 and D3, 1,25-dihyroxyvitamin D3 standards were purchased from Sigma-aldrich. An EZYPRESS HT 96-well plate positive pressure manifold unit was used for conditioning, washing and processing the Vitamin D metabolite extraction plate and Sagacity HL SPE plate. Vitamin D and metabolites free human serum was purchased from Golden West Biologicals (Temecula, CA)

Procedures

Vitamin D metabolites extraction plate

1. Aliquot extraction reagent into each well
2. Add 0.1 mL of fortified serum sample
3. Wait about 1-2 minutes, then apply positive pressure to the plate such that fluid flow through the extraction plate drop by drop, total process is less than 10 minutes for 96-well plate
4. The vitamin D eluate is then evaporated and reconstituted with 0.1 mL of a solution of 80% methanol in water

Sagacity HL SPE plate

1. Condition the plate with 1 mL of acetonitrile followed by 1m of water.
2. Pretreat the fortified serum 0.1 mL with 50 µL of isopropyl alcohol first, then add 70 µL of water.
3. Load pretreated serum to the SPE plate, and wash with 1 mL of water followed by 1mL of 30% acetonitrile in water.
4. The metabolites are then eluted with 1 mL of acetonitrile. The vitamin D eluate was evaporated and reconstituted with 0.1 mL solution of 50% methanol in water.

LC-MS/MS analysis used Orochem's Reliasil C18 column, 3 µm, 50 x 4.6 mm with a 87% Methanol (0.1% Formic acid, 15 mM Ammonium Acetate) mobile phase.

Results

1. Extraction Reagent and Recovery of Vitamin D Metabolites

We evaluate variations of extraction reagents (methanol, acetonitrile, different ratio of methanol and acetonitrile, with or without formic acid) and different ratio of reagent to serum (3:1, 4:1). The results show that methanol/acetonitrile 1:1 and reagent to serum 4:1 ratio provides the best recovery and phospholipids removal rate.

2. Comparison of Vitamin D Metabolite Extraction Plate with Commercial Phospholipid Removal Plate

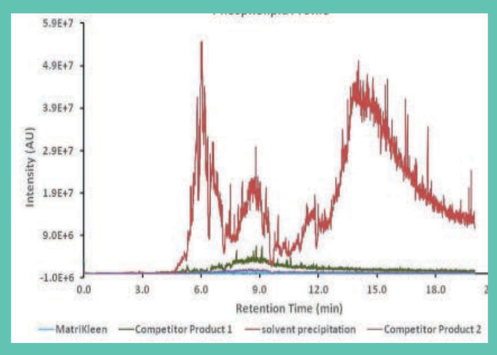
We evaluate three commercial phospholipid removal plates (B-D) with Vitamin D Metabolites Extraction Plate (A). For our plate, we use 4:1 reagent mixture (methanol/acetonitrile, 1:1) to serum ratio. We followed the instructions of each other commercial plates for its extraction.

Table 1 - Comparison of Vitamin D Recovery for Commercial Kits

Kit	1,25-dihydroxy vitamin D3 Recovery	25-hydroxy vitamin D2 Recovery
A	74.0%	73.0%
B	36.9%	14.0%
C	52.8%	40.5%
D	63.5%	67%

Compared to some commercial phospholipid depletion plate, vitamin D metabolites gave the best recoveries.

Figure 1 - Phospholipid profile of serum extracts.



3. Sagacity HL SPE Plate

When using Sagacity HL SPE product, recoveries for all three metabolites were all above 86%. We tested several different pretreatment method, found out 2:1 serum/IPA ratio could sufficiently release protein bounded vitamin D metabolites, while not enough precipitation to block the SPE. Additionally, certain volume of water needed to obtain 1,25 dihydroxy vitamin D on the Sagacity HL sorbent.

Conclusions

Two different types of vitamin D metabolite extraction methods have been established. Vitamin D extraction plate provides reasonable recovery while it is fast and simple. It is very easy to operate by clinical technicians. It is great for 25-hydroxy vitamin D test.

Sagacity HL SPE plate method is high recovery, and still high through put. It could be used for testing low concentration 1,25-dihydroxy vitamin metabolites.