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Rapid HPLC method and sample extraction procedures for measuring 25-hydroxyvitamin D3 concentrations in human breast milk

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Abstract

Background; Human breast milk is the milk produced by the breasts (or mammary glands) of a human female for her infant offspring. Milk is the primary source of nutrition for newborns before they are able to eat and digest other foods. Vitamin D describes a group of fat-soluble steroids. Vitamins D2 and D3 can be converted to the active steroid hormone in the human body. The active form of vitamin D is hydroxylated in two places. The most accurate measurement of vitamin D levels in the body is a blood test that detects the levels of circulating 25-hydroxylated vitamin D.

Aims of the study; the purpose of the present study was to develop a protocol for the extraction of cholecalciferol from human breast milk for analysis by HPLC using retinyl acetate as internal standards.

Methods: The HPLC proposed enables successful separation and quantitation of Vitamin D3 (cholecalciferol) and their respective internal standards (retinyl acetate) in less than 10 minutes; RP- C18 column (100 x 4.6 mm I.D.; particle size, 5 micron) at a flow-rate of 1 ml/min, the mobile phase was methanol. The eluate was monitored with a photodiode-array detector with wavelengths 265 nm.

Results: No interference was found from other fat soluble vitamins (vitamin A) that are commonly presents with vitamin D. Reproducibility studies carried out with pooled breast milk showed a within day and between day precision of the analysis did not exceed 2.6% and 4 % respectively for cholecalciferol. The detection limits were 2.8ng/ml, the linearity of the standard was excellent (r2 > 0.999), over the concentration range of 0-100ng/ml.

Conclusions: This method separates fat-soluble vitamins in human breast milk, including cholecalciferol using retinyl acetate as internal standards. HPLC method is a rapid determination and quantification of vitamins D in human breast milk, time-consuming steps and has been shown to be sensitive, and reliable.

Keywords: Breast milk, Cholecalciferol, HPLC, Retinyl acetate, Vitamin D.

1 Introduction

The most important physiological functions of vitamin D are the stimulation of the Intestinal resorption of Ca2+ and phosphate and their incorporation in the bon. While breastfeeding is the recommended method of infant feeding and provides infants with necessary nutrients and immune factors, most breastfed infants are able to synthesize additional vitamin D through routine sunlight exposure (1, 2).
A number of factors decrease the amount of vitamin D ,These factors include; Living at high latitudes (closer to the Polar Regions), particularly during winter months, Air quality conditions: high levels of air pollution, Weather conditions: dense cloud covering, the degree to which clothing covers the skin, Skin pigmentation: darker skin types(3,4,5).
D-vitamins or calciferols arise from provitamins by the UV radiation of sunlight, In the skin formed Vitamin D3 is bound to a vitamin D binding-protein in the plasma, transported in to the liver and hydroxylated in position 25 to form 25-OH vitamin D3. Another Hydroxylation is performed in the kidney to yield 1, 25 (OH) 2 vitamin D3 .It is proofed that 1, 25 (OH) 2 vitamin D3 is the metabolic Most active form of vitamin D. Nevertheless, more than 95% of 25-OH vitamin D is25-OH vitamin D3 (6, 7, 8).

Vitamin D levels should be monitored and contained within acceptable limits: 40-60 ng/mL or 100-150 nmol/L of 25 (OH) Vitamin D. (Conversion ratio: 1ng/mL equal to 2.5nmol/L)(9,10).
A lack of vitamin D leads in growing humans to rachitis, hypocalcaemia, and secondary hyper parathyroidism, in adults to osteomalasia.
HPLC-application for 25-OH vitamin D3 makes it possible to determine the vitamin in an easy, fast and precise method (11, 12).
Because there is a need for a fast reverse-phase high pressure liquid chromatography (HPLC) method than the methods currently available. We developed an HPLC method designed to be easy to use, sensitive, and rapid with simple sample preparation. Separation and quantification and compared several extraction methods for vitamin D3 in human breast milk.

Expected values

Normal ranges for 25-OH-Vitamin D_3

1 ng/ml = 2.5 nmol/l
1 nmol/l = 0.4 ng/ml

Information from ASBMR 2006

Deficiency (seriously deficient) < 12 ng/ml resp. < 30 nmol/l
Insufficiency (deficient) 12 - 30 ng/ml resp. 30 - 75 nmol/l
Sufficiency (adequately supplied) > 30 ng/ml resp. > 75 nmol/l
Society of Osteology SACHSEN E. V.
http://osteologie-sachsen.de/aktuelles_vitamin_d.html (12, 13, 14)

2 Subjects and methods

2.1 Milk sampling

Milk samples were collected from mothers'~5mL, Whole breast milk aliquots were prepared by gentle swirling of the sample using a vortex mixer before pipetting the whole milk into 2-mL aliquots. Milk aliquots were frozen and stored at −70°C
Long-term storage. Exposure of the samples to high temperature and bright light were avoided and the samples were analyzed as soon as possible.

2.2 Sample preparation

Breast milk vitamin D3 was measured in breast milk using HPLC. Samples were saponified in NAOH with gentle rotation for 2 min. Samples were extracted with hexane and dried under nitrogen. Once dried, the extracts were resuspended in methanol.

2.3 Chromatographic settings

Column dimension: 100 mm x 4.6 mm
Flow rate: 0.5 ml/min
UV-detection: 265 nm
Injection volume: 20 µl
Running time: 10 min
Temperature: 30 °C

3 Results

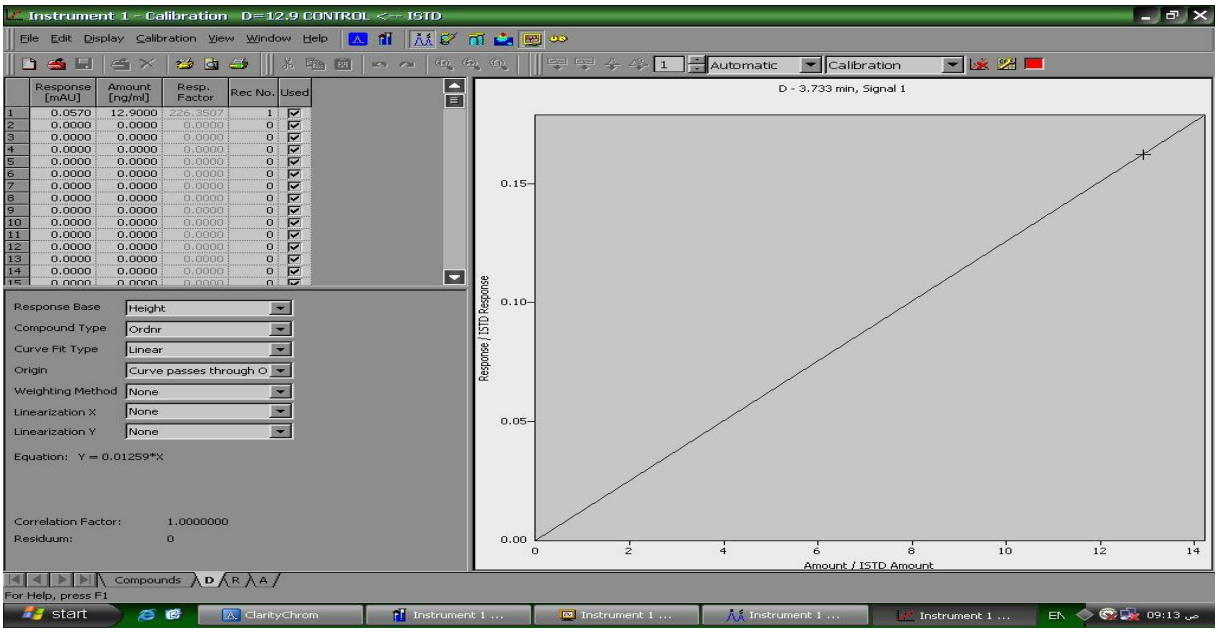

Fig. 1: Typical chromatograms of calibrators, Linearity fit, Concentration (0–100) ng/ml, r (0.999)

3.1 Typical chromatogram

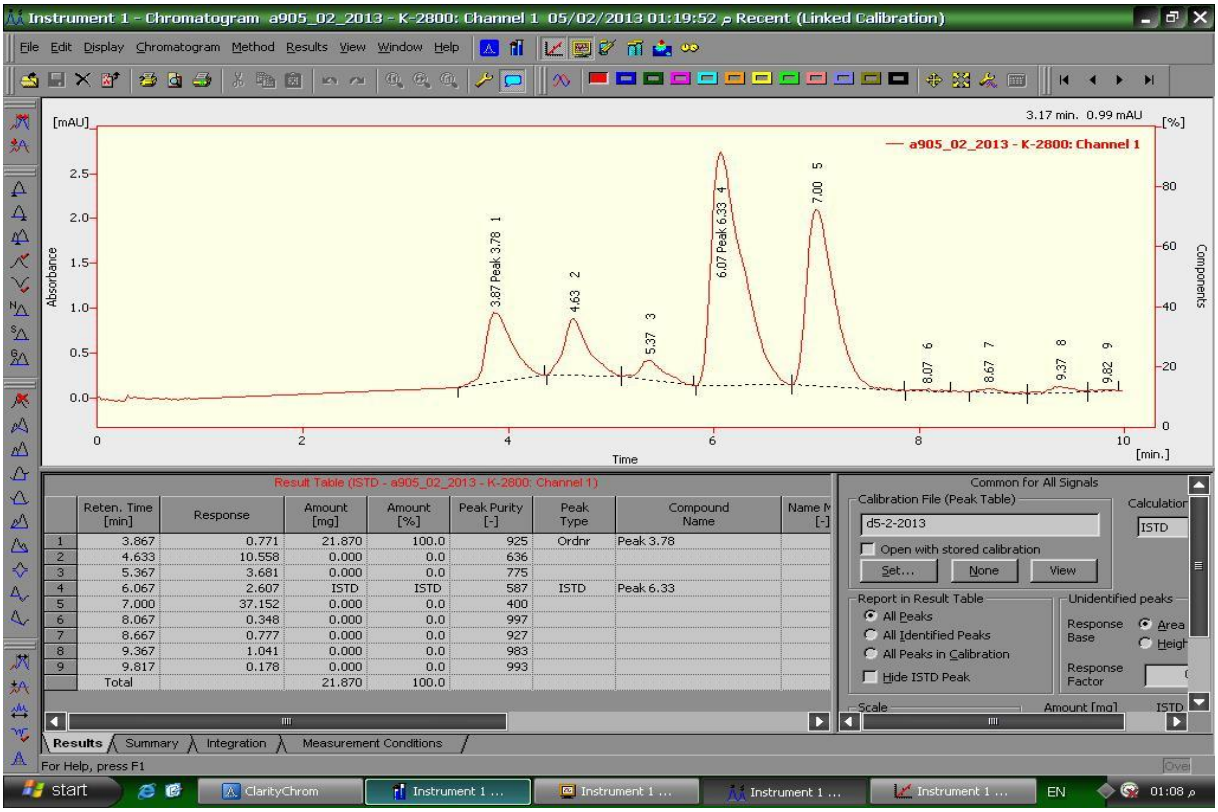

Fig. 2: Chromatogram of human breast milk analyzed by HPLC, breast milk 25(OH) D3 had a retention time of 3.7min minutes and 6.2min minutes for the internal standard

4 Method validation

4.1 Precision and reproducibility

Intra-Assay CV: 2.6 % (20.5 ng/ml) (n = 6)
Inter-Assay CV: 4.0 % (20.1 ng/ml) (n = 6)

4.2 Linearity

Calibration Curve was prepared by plotting vit.D3 concentrations (ng/ml) versus the high ratio of vit D3 to I.S (retinyl acetate). Each of these standard solutions was injected twice onto the HPLC-column and the peak height ratios were calculated. Linearity of the calibration curve was excellent (r2 > 0.999) for the concentration
Range 0 – 100 ng/ml.

4.3 Detection limit

2.8ng/ml

4.4 Specificity test

The possible interference with the retention times of the vitamins under investigation was evaluated for the vitamins most frequently present, using the same HPLC conditions. No interferences were observed as shown in figure 2.
In all experiments the mobile phase was used as the dissolving solvent,. Analyses were performed induplicate.

5 Discussion

There is a need for a fast reverse-phase high pressure liquid chromatography (HPLC) method than the methods currently available. Efficient extraction procedures for biological samples are valuable to save time, materials, and cost. We developed an HPLC method and compared several extraction methods for vitamin D3 in human breast milk. The method uses aknauer system equipped with c18 column (4.6 × 100 mm 5 μm). Mobile phase of methanol was run at 0.5mL min^{-1}, and run times were complete in approximately 10 min. This rapid method uses little solvent and provides excellent results in the analysis of these fat-soluble nutrients. But in previous study the method for determination of vitamin D3 in milk was established by high-performance liquid chromatography (HPLC) using reversed-phase and straight-phase columns(15).. 25.0 ml of a sample of milk was taken and the lipid was extracted with a solvent mixture of petroleum ether and ethyl ether (1:1). The extracted lipid was subjected to the first preparative HPLC using a Nucleosil C18 column with acetonitrile-methanol (1:1) as the mobile phase, and a fraction containing vitamin D3 was isolated. The fraction was subsequently subjected to the second analytical HPLC using SIL column (straight-phase type) with 0.4% isopropanol in n-hexane as the mobile phase. Vitamin D3 was assayed by estimating the peak height on the chromatogram.

6 Conclusion

We developed a rapid, simple, and very selective HPLC method for the determination of 25(OH) D3 in human breast milk. Using simple HPLC with diod-arry detection. This method provides excellent sensitivity, precision, and accuracy.

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