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Simultaneous determination of 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 in human serum by ultra performance liquid chromatography: An economical and validated method with bovine serum albumin

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ABSTRACT

A simple and economical method has been developed for simultaneous determination of human serum 25-hydroxyvitamin D_2 ($25OHD_2$) and 25-hydroxyvitamin D_3 ($25OHD_3$) using Ultra Performance Liquid Chromatography (UPLC). Non-human matrix of 4% BSA was used to construct the calibration curve and in quality control samples' preparation to avoid interference of the endogenous 25-hydroxyvitamin D (25OHD) present in the human serum. $25OHD_2$, $25OHD_3$ and dodecanophenone (internal standard, IS) were separated on a CORTECS solid-core particle column and monitored by photodiode array detector at wavelength of 265 nm within five min run time. The relationship between 25OHD concentration and peak area ratio (25OHD:IS) was linear over the range of 12.5 – 200 nM with mean correlation coefficients (r^2) > 0.998. The limit of detection (LOD) for $25OHD_2$ and $25OHD_3$ was 3.00 nM and 3.79 nM, while the lower limit of quantification (LLOQ) was 9.11 nM and 11.48 nM, respectively. High repeatability was obtained for both isomers with intra-day CV % < 5.6% and < 5.3% for inter-day assay. This method was further tested with a commercial lyophilized serum control with an accuracy of 92.87–108.31% and applied on 214 human serum samples. In summary, this validated method with BSA can be reliably applied for routine quantification of 25OHD in adults.

1. Introduction

Serum 25-hydroxyvitamin D (25OHD) is well accepted as a valid biomarker for Vitamin D status due to its long serum half-life, approximately 3 weeks [1]. It has been demonstrated that the circulating serum 25OHD is highly stable, hence its usefulness for long-term epidemiologic studies [2].

Various methods have been published describing Vitamin D determination using HPLC. It is a direct detection method for determining circulating 25OHD and capable to separate and quantitate circulating $25OHD_2$ and $25OHD_3$ individually [1, 2]. HPLC with ultraviolet detection has been regarded as the standard technique for quantifying Vitamin D [1, 3] and is very accurate if validated and performed by experienced personnel [4].

For the purpose of method validation, it is necessary to obtain 25OHD-free serum for the preparation of calibration curve and quality control samples, which is extremely difficult. Furthermore, it has been reported that 25OHD in serum samples stored at −20 °C are very stable with no decrease or loss of the analyte from 3 months [5] and up to 1 year [6]. Therefore, endogenous 25OHD in pool serum must be taken into account when assigning the final concentration to the calibrators [3]. Previously, the endogenous $25OHD_3$ levels were corrected by using the difference in peak area ratios between each consecutive concentration as the response (rather than peak area ratio) and plotted against the concentration [7]. Besides the pooled serum, human serum albumin has also been used for standard curve generation in the determination of serum 25OHD [8, 9]. Additionally, the use of bovine serum albumin (BSA) as calibrators has also been introduced [10–12] as an alternative approach for a 25OHD-free matrix. However, to date, none has reported the use of BSA as calibrators and quality control samples in a detailed method development and validation.

In this paper, we report a validated UPLC-UV-Visible method for the measurement and routine analysis of serum $25OHD_2$ and $25OHD_3$ using bovine serum albumin.

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2. Materials and methods

2.1. Chemicals

HPLC-grade solvents acetonitrile (ACN), *n*-hexane and methanol (MeOH) were obtained from Merck (LiChrosolv; Merck, Darmstadt, Germany). Bovine serum albumin (BSA), 25-hydroxyvitamin D_2, 25-hydroxyvitamin D_3 and Dodecanophenone (98%) were from Sigma (St. Louis, MO). Ultrapure water (18.2 MΩcm) was obtained from Sartorius arium® pro Ultrapure Water System (Sartorius AG, Germany).

2.2. Chromatographic condition

Chromatography was performed on Waters Acquity™ UPLC® (Waters Corp., Milford, MA) consisting of a binary pump, autosampler, column thermostat, and photodiode array detector. A CORTECS® UPLC® C18$^+$ column (2.1 × 100 mm, 1.6 μm particle size) and a CORTECS® UPLC® C18$^+$, VanGuard™ Pre-Column (2.1 × 5 mm, 1.6 μm particle size) operated at 35 °C were used for the separation. Empower 2 Software (Waters Corp., Milford, MA) was used for system control, data acquisition and processing. Chromatograms were recorded at 265 nm with a run time of five min.

2.3. Standard solutions and quality control samples

Stock standard solutions of 25OHD$_2$ (500 μM), 25OHD$_3$ (500 μM) and the internal standard (IS) dodecanophenone (1 mM) were each prepared in ACN. Both 25OHD stocks were stored at −20 °C and IS stock was stored at 4 °C. Working standard solutions of 10 μM 25OHD$_2$, 10 μM 25OHD$_3$ and 25 μM IS were prepared fresh daily in ACN. Six calibration standards were then prepared by spiking working standards of 25OHD$_2$ and 25OHD$_3$ into 4% BSA in distilled water to give final concentrations ranging from 12.5 nM to 200 nM.

Three levels of quality control (QC) samples were prepared from 4% BSA in distilled water; low (37.5 nM), medium (80 nM) and high (160 nM). The solutions were vortexed and aliquoted into 1.5 mL microcentrifuge tubes and stored at −20 °C until use.

2.4. Sample preparation

For sample preparation, 0.5 mL of each calibrator or QC sample was placed into its respective 13 × 125 mm borosilicate glass culture tube followed by 15 μL of working IS solution each. The tubes were capped with PTFE-lined caps (Pyrex®, Corning, NY) and vortexed for 5 s. Next, 0.5 mL MeOH was added for deproteinization, and the tubes were vortex-mixed for 10 s. The hydrophobic compound was extracted twice with 1 mL of *n*-hexane by vigorous mix for 10 s and the phases were separated by centrifugation at 3400 rpm for 5 min. The upper organic phase was carefully transferred and pooled into a 10 × 100 mm disposable glass test tube and dried under vacuum (CentriVap®, Labconco). The dry extract was then reconstituted with 100 μL ACN and vortex-mixed for 5 s. The samples were filtered through a 0.2 μm PTFE 4 mm syringe filter (Phenomenex, Torrance, CA) into a sample vial each and 10 μL was injected into the UPLC system for analysis. All procedures were performed under normal laboratory lighting condition.

2.5. Method validation

The method was validated according to the guidelines by International Council on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) [13] and FDA [14]. Quantification was performed by calculating the peak-area ratios of 25OHD$_3$ and 25OHD$_2$ respectively to dodecanophenone (IS).

2.5.1. System suitability

The system suitability of the method was assessed by the repeatability of peak area, retention time and theoretical plate number of five replicates of standard 25OHD and IS at concentration 150 nM. The % CV values were calculated for each case.

2.5.2. Selectivity

We performed the analysis on five replicates of extracted blank 4% BSA to assess the possible interference with 25OHD and IS. Peak purity was checked and confirmed on each obtained peak from the chromatograms to ensure no co-elution of other components within the retention time of interest.

2.5.3. Sensitivity

The limit of detection (LOD) and lower limit of quantification (LLOQ) of 25OHD$_3$ and 25OHD$_2$ in 4% BSA were determined from the calibration curve by calculating the equation of LOD = σ/S × 3.3 and LLOQ = σ/S × 10, where σ is the noise and S is the slope for the calibration curve as were described previously [10].

2.5.4. Linearity

Linearity was evaluated by analyzing a series of standard concentrations (calibration curves) generated over the range of 12.5–200 nM in 4% BSA. The peak area ratios were subjected to linear regression analysis and data are presented as the mean ± SD.

2.5.5. Recovery

Recovery analysis of the extraction method was performed at three 25OHD concentrations (Low, 37.5 nM; Medium, 80 nM; and High, 160 nM) in three replicates each. The percent recoveries of both 25OHD and ID were calculated and presented as mean recovery ± % CV.

2.5.6. Within-run and between-run precision and accuracy

Precision and accuracy were determined using three replicates of each calibrator in the standard curve and each of the three QC levels over three different days. Results were expressed as % CV of the measurements for precision and as % bias for accuracy. The acceptance range for precision was set at 15% or lower for the calibrators and QC samples whereas 20% or lower for LLOQ concentration level. The acceptance mean value for accuracy was set at < 15% bias of the nominal value and < 20% bias at the LLOQ level [14].

2.5.7. Stability

The stability of 25OHD in 4% BSA during analysis and usual storage condition was investigated from the following parameters: Stock solution stability, freeze-thaw cycle stability, long-term stability, pre-extraction stability at room temperature (RT), post-extraction stability at RT and post-extraction stability in autosampler. Each condition for stability testing was assessed from three replicates of low QC (37.5 nM) and high QC (160 nM) samples except for stock solution stability. All stability samples were tested against daily calibration curve, and the acceptable mean concentration range set for each level is 15% or lower than the nominal concentration. Data were expressed as percent recoveries ± % CV.

Stock solution stability of 25OHD$_3$, 25OHD$_2$ and IS were assessed by performing three replicates of each concentration in the calibration curve (12.5, 25, 50, 100, 150 and 200 nM) prepared in mobile phase. The back-calculated concentrations obtained from the curve were compared to the nominal values for Week 0, 1, 2, 3, 4 and 8.

For freeze-thaw cycle stability, the stability of 25OHD in 4% BSA was determined over three freeze and thaw cycles. Nine aliquots of each of the two QC samples were stored at −20 °C. After 24 h, all aliquots were left to thaw unassisted at room temperature. When completely thawed, three aliquots of each QC sample were analyzed. The remaining aliquots were returned to −20 °C and kept for 24 h. The same procedure was repeated twice with three aliquots being analyzed at each cycle.

Long term stability was assessed during storage at −20 °C for 1, 2,