HPLC method for measuring the circulating levels of 25-hydroxy vitamin D: Validation and comparison with ID LC/MS/MS and immunoassay

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Abstract

Twenty-five-hydroxy-vitamin D (25OHD) levels are accepted as an indicator for vitamin D status and their measurement is still a challenge. The aim of the present study was to develop a robust and easy to use HPLC-method for simultaneous analysis of 25OHD2, and 25OHD3, in human plasma and to assess its analytical performance. Protein precipitation followed by liquid-liquid extraction with n-hexane was used for separation from the matrix substances. A HPLC-system with UV detector (Thermo Scientific Spectra System, USA) was used. HPLC-UV analysis was performed on a Synergy hydro-RP column 4,6x250 mm, 4.0 µm particle size with mobile phase consisting of methanol/acetonitrile/water (70/25/5) under isocratic elution at a flow rate of 1.2 mL/min and UV-detection at 265 nm. Our HPLC-method was compared with a validated ID-LC-MS/MS method and with a commercial immunochemical method. Linear regression was used for data analysis. Linearity of the HPLC-assay was between 5-160 ng/ml for each analyte ($r^2=0.99$) with lower limit of detection 4 nmol/L, and limit of quantification 10 nmol/L. Intra-assay precision was determined by extracting and quantifying 30 replicates of 4 QC levels (RSD ≤ 9%). Inter-assay precision was determined over 21 consecutive days analyzing 30 replicates of all QC samples (RSD=10%). All results were within 14.2% deviation of the 25OHD LC-MS/MS method mean. Method comparison demonstrated good agreement between HPLC and LC-MS/MS ($r=0.85$) and disparity with the commercial immunoassay ($r=0.48$). This assay demonstrates excellent linearity, acceptable accuracy and precision, a good agreement with a validated ID-LC-MS/MS method. The simple sample preparation and ease of determination make it useful for the routine clinical laboratory.

Introduction

After discovering the vitamin D receptor protein in tissues not related to calcium homeostasis, numerous pleiotropic effects of vitamin D were established. Vitamin D deficiency is connected with various clinical disorders beside bone diseases [1,2]. As a consequence, the evaluation of vitamin D status is of great importance for making decision for clinical management of vitamin D deficiencies. It is widely accepted that the circulating form of vitamin D, 25-hydroxyvitamin D (25OHD), is the best indicator for assessment of individual’s vitamin D status [3,4]. In the last ten years there is an exponential increase in blood testing for 25OHD. A variety of methods, based on two main principles – indirect immunochemical and direct chromatographic are nowadays used for assessment of vitamin D status. The immunochemical methods are mostly used for routine testing due to their simplicity and expediency, while the chromatographic assays are more accurate and reliable [5]. The main limitation of immunochemical methods is the cross reactivity of the antibodies used in the assay. Another restriction is related to their inability to discriminate the D2 and D3 forms of the 25OHD metabolite [6]. The chromatographic methods are too sophisticated and require high-qualified laboratory staff. On the other hand they demonstrate excellent analytical performance and selectivity. Recently one of the chromatographic methods, the LC-MS/MS method, is proposed as a reference for measuring 25OHD in blood [7]. The aim of the present study is to develop a robust and easy to use in-house HPLC-method for simultaneous analysis of 25OHD2, and 25OHD3, in human serum and to assess its analytical performance.

Material and methods

Reagents and standards

The reagents and standards used were HPLC grade. Absolute ethanol, methanol, ultrapure water, hexane and acetonitrile were supplied by Sigma Aldrich (St. Louis, MO, USA). 1-alpha cholecalciferol, used as internal standard, cholecalciferol (vitamin D3) and ergocalciferol (vitamin D2) were supplied from Sigma Aldrich, USA. Stock standard solutions of vitamin D3 (2 mg/ml), vitamin D2 (2 mg/ml), and internal standard (2 mg/ml) were prepared in absolute ethanol and stored at $-20^\circ$C. From these individual standard solutions working standards were prepared by appropriate dilution with methanol.

Calibrators and controls

Serum calibrators and controls (bi-level I +II) for 25OHD2, and 25OHD3, were purchased from Chromsystems Instruments and Chemicals GmbH, Germany. The commercial lyophilized serum calibrators and controls (bi-level I +II) for 25OHD2, and 25OHD3, were purchased from Chromsystems Instruments and Chemicals GmbH, Germany. The commercial lyophilized serum

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controls and calibrators were reconstituted in water according to manufacturer's instruction and aliquoted. The aliquots were stored at −20°C.

**Blood samples**

For evaluation of analytical performance and for comparison studies 30 serum samples from the Clinics of Urology and 17 serum samples from the Pediatric ward at the University hospital were used. An informed consent was obtained by the participants. Vacuette with serum clot activator (Greiner Bio One International GmbH) were used for collection the blood samples. The blood was centrifuged at 3000 rpm for 10 min and the blood serum was immediately separated and stored at −20°C until analysis.

**Pre-analytical procedure**

In brief the sample preparation procedure includes: to 0.5 ml serum (calibrator, control) was added 1 ml absolute ethanol for protein precipitation. After mixing for 1 min the sample was centrifuged at 5500 rpm for 10 min. The clear supernatant was separated and extracted twice with 3 ml hexane. The combined extract was evaporated under gentle steam of nitrogen. The dry residue was dissolved in 0.2 ml methanol and subjected to HPLC analysis.

**HPLC analysis**

An HPLC system (Spectra System, Thermo Scientific, USA) equipped with UV detector (UV 2000) was used for chromatographic analysis. The separation of the analytes was performed on RP C18 analytical column Synergy hydro-RP5 4.6×250 mm, 4.0 µm (Phenomenex Inc., USA). The mobile phase consisted of 70% acetonitrile, 25% methanol and 5% water and used in isocratic elution mode with a flow rate of 1.2 ml/min. injections were done using a Rhodents type 7125 six-port valve equipped with 20 ul loop. The detection was carried out at 265 nm. Instrument control, data collection and quantitation were performed by the help of ChromQuest 4.2.34 software.

For method comparison studies an ID LC-MS/MS and immunocromiluminescent assay using the platform of DiaSorin Liaison were performed.

**ID LC-MS/MS**

Determination of 25OHD was performed by a validated LC-MS/MS method. Briefly, 100 µl of human plasma or serum were precipitated with 150 µl acetonitrile containing the internal standard, d3-25-hydroxyvitamin D3 (d325D3).Then 25OHD, 25OHD2, and d325D3 were extracted with 1.0 ml of hexane. Chromatographic separation was performed on a C18 analytical column with isocratic elution at a flow rate of 0.1 ml/min, utilizing a mobile phase consisting of 85% aqueous methanol/ammonium acetate/formic acid buffer. Positive-ion electrospray ionization at 25 KV and selected reaction monitoring were used to follow the predominant transitions: collision energy (CE) =10 at m/z 401→383 for 25OHD, CE=10 at m/z 413→395 for 25OHD2, and 404→386 for d325D3. Method was validated according to FDA guidance requirements. Calibration curves were constructed using 6 replicates for each concentration. The analytical recoveries varied form 57-64% for 25OHD3, 69-73% for 25OHD 2, 55-56% for d325D3; linearity range for both 25OHD, and 25OHD2, was 1.2-120.0 ng/ml, R2>0.99. Freeze-thaw stability was determined for three cycles each lasting 24 h, post-preparative stability was documented for 96 h at 10°C, short-term stability at ambient temperature was proven for 24 h in the dark and for 2 h at daylight; stock solution stability and long term stability in plasma – for 5 days at 4-8°C, and for 99 days at −20°C. With run time of less than 10 min, a throughput of over 100 samples per 24 h was achieved, with instrument running unattended overnight.

**DiaSorin Liaison automated system**

The levels of 25OHD in patients' samples were measured using commercial controls and calibrators according to the manufacturer's instructions [8].

**Results**

A typical chromatogram of a calibrator and patient sample illustrating the separation of 25OHD2, 25OHD3, and internal standard 1alpha-25OHD3, from the other serum components are given in Figures 1 and 2. The retention times were 9.14 min for 25OHD, 10.08 min for 25OHD2, and 16.03 min for the internal standard. The total analysis time was 22 min typical for conventional HPLC assay.

The analytical performance was evaluated by the precision data, limit of quantitation (LOQ), limit of detection (LOD), linearity and average recovery for both 25OHD1 and 25OHD2, using commercial control serum. The analytical accuracy was estimated for two target concentrations: 37 ng/ml and 51 ng/ml. The percent relative errors were 1.08% and 1.27%, respectively. The precision data were summarized in Table 1. The LOQ for both analytes was 10 ng/ml and the LOD=4 ng/ml. The assay was linear form 5-160 ng/ml for 25OHD, with the following regression equation y=0.04318X + 0.08295 (r²=0.9996). For 25OHD2, the line range was from 5-160 ng/ml and regression line equation was y=0.04464X + 0.1430 (r²=0.9935). In each case, x represents the concentration added to a serum sample and y the analytical result. The analytical recoveries based on standard addition to patient samples were calculated for the concentration range 10-100 ng/ml in three replicates for each concentration. The analytical recoveries varied form 82-110% for 25OHD, and form 87-101% for 25OHD2.

In order to ensure the analytical reliability of our method we participated in the Vitamin D External Quality Assessment Scheme (DEQAS). In the concentration range 19 ng/ml to 40 ng/ml our results were within average value ±1SD. The target value of the lowest tested concentration was 11.7 ng/ml, R2>0.99. Freeze-thaw stability was determined for three cycles each lasting 24 h, post-preparative stability was documented for 96 h at 10°C, short-term stability at ambient temperature was proven for 24 h in the dark and for 2 h at daylight; stock solution stability and long term stability in plasma – for 5 days at 4-8°C, and for 99 days at −20°C. With run time of less than 10 min, a throughput of over 100 samples per 24 h was achieved, with instrument running unattended overnight.

**Discussion**

In the last two decades high rates of vitamin D deficiency have been reported not only in adults but also in children worldwide. It's...
been estimated that more than half of all children and adults in the U. S. and Europe are at risk [9]. Therefore the estimation of vitamin D status attracted the attention of many researchers and clinicians. The best determinant for vitamin D status is accepted the circulating form of vitamin D, 25OHD [5]. Two forms of 25OHD can be detected in the circulation, 25OHD$_3$, mainly formed in the skin after sun exposure, and 25OHD$_2$ derived from the diet. Administration of vitamin D analogs and supplements for treatment of vitamin D deficiency requires a follow-up assessment of vitamin D status. Therefore a simple, precise and inter-laboratory consistent method for measuring the 25OHD levels is needed.

Being a lipophilic compound 25OHD is transported by the blood as a complex with vitamin D binding protein (DBP). Competitive binding to DBP derived from rat serum was the basis for the first immunoassay for measurement of 25OHD levels. Recently a numerous competitive immunochemical assays were introduced differing in binding agent and method of detection [10,11]. A common disadvantage of these methods is their limited selectivity towards 25OHD$_2$, 25OHD$_3$ and other polar vitamin D metabolites [12]. Nowadays the immunochemical assays are widely used in the routine laboratory practice for their convenience, speed, turnaround and cost [13].

Most of the disadvantages of immunochemical methods were overcome by the direct chromatographic methods. These assays quantitatively and selectively measure both 25OHD$_2$ and 25OHD$_3$ [6,14].

In the last few years the interest in testing 25OHD levels was exponentially increased in Bulgaria. For that reason several clinical laboratories introduced routine immunochemical assays. In Bulgaria a chromatographic method using liquid chromatography coupled to tandem mass spectrometry was developed for first time in 2012 at the National Reference Laboratory for Drug Monitoring. Since the LC-MS/MS method is quite sophisticated and requires skilled personnel and higher cost equipment, another more conventional chromatographic method was needed for reliable determination of vitamin D status. In our in-house method a simple sample preparation procedure based on protein precipitation followed by liquid-liquid extraction ensures effective release of 25OHD from the binding protein and obtaining a “visibly” clean extract. The smallest sample size for the method was

Figure 1. HPLC chromatogram of a commercial serum calibrator.

25OHD$_3$=37 ng/ml; 25OHD$_2$=28.2 ng/ml; ISTD=100 ng/ml
Peak 1–25OHD$_3$, retention time=9.14 min.; peak 2–25OHD$_2$, retention time=10.8 min.; ISTD–internal standard 1-alpha-OHD$_3$, retention time=16.03 min.
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Figure 2. HPLC chromatogram of a patient sample.
25OHD$_3$=14.30 ng/ml; ISTD=100 ng/ml
Peak 1–25OHD$_3$, retention time=9.10 min.; ISTD–internal standard 1-alpha-OHD$_3$, retention time=15.71 min.

Figure 3. Comparison of 25OHD$_3$ values obtained by the proposed HPLC-UV method and ID LC-MS/MS method.
n=17 patient samples, $r^2=0.48$, regression equation $y=0.82x + 7.00$

Figure 4. Comparison of 25OHD$_3$ values obtained by the proposed HPLC-UV method and DiaSorin Liaison 25OH vitamin D Total assay.
n=30 patient samples, $r^2=0.85$, regression equation $y=0.70x + 5.15$
0.5 mL, the LLQ was 10 ng/mL for both 25OHD$_3$ and 25OHD$_2$. The linearity range (5-160 ng/mL) covers the clinically significant interval for detection of vitamin D deficiency, insufficiency and sufficiency. The within-run precision varied between (12.7 ng/mL-100 ng/mL) RSD for 25OHD$_3$ and (11.4 ng/mL-100 ng/mL) RSD for 25OHD$_2$. The between-run precision was between (100 ng/mL-12.7 ng/mL) RSD for 25OHD$_3$ and (100 ng/mL-11.4 ng/mL) RSD for 25OHD$_2$. The analytical performance of our method in terms of LOD, LOQ, linearity, precision was in agreement with the performance of other published HPLC procedures [5].

Recently, the National Institute of Standards and Technology (NIST) developed an isotope-dilution (ID) method coupled with LC-MS/MS for the determination of serum 25OHD$_3$ and 25OHD$_2$--a candidate as reference method for 25OHD measurement. The use of stable-isotope-labeled internal standard identical to the analyte guarantees highest analytical accuracy of ID LC-MS/MS method and is a basis for the selection of this method as reference [5,15,16].

We have compared our in-house HPLC method with an ID LC-MS/MS method developed by the National Reference Laboratory for Drug monitoring in Bulgaria. The comparison study showed very close values ($r^2=0.85$) for 30 patients’ samples containing 25OHD in the range 7.48 ng/mL. A very good correlation between HPLC and ID LC-MS/MS assay ($r^2=0.99$) was reported also by others [16].

The immunochemical methods are the most routinely used for analysis of 25OHD in clinical laboratory practice due to their simplicity, speed and short turnaround time. The latest modified version of DiaSorin for testing 25OHD Liaison Total assay was accepted as one of the most reliable amongst immunochemical assays [16]. The comparison of results obtained by our HPLC method and DiaSorin Liaison Total assay showed poor correlation ($r^2=0.48$). The DiaSorin Liaison Total method overestimated total 25OHD concentrations by an average of 7%, having in mind that in our samples the 25OHD$_3$ was the only form in serum. This worse agreement may be due to cross-reactivity of the antibodies typical for immunoassays, to random errors related to antibody binding or matrix effects, whereas these limitations are not an issue in the direct chromatographic methods due to their high selectivity. Poor correlation between HPLC and Liaison assay ($r^2=0.735; n=203$) was reported also by others [17,18].

In summary our HPLC method enables reliable quantification of both 25OHD$_3$ and 25OHD$_2$. The relatively short analysis time make it useful for routine clinical laboratory practice. The HPLC procedures would be the preferred techniques for measuring these two metabolites due to its high selectivity and better analytical performance. The use of commercial serum calibrators and controls diminishes the variability of the results obtained with different chromatographic technics and allows better traceability.

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