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OPTIMISATION AND VALIDATION OF ULTRAHIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR QUANTIFICATION OF 25-HYDROXYVITAMIN D IN MATERNAL PLASMA

(Pengoptimuman dan Pengesahan Kaedah Kromatografi Cecair Berprestasi Ultra Tinggi untuk Kuantifikasi 25-hidroksivitamin dalam Plasma Ibu Mengandung)

Siew Siew Lee and Su Peng Loh*

Department of Nutrition,
Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

**Corresponding author: sploh@upm.edu.my*

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Abstract

This study aimed to optimise and validate a simple and efficient sample preparation and extraction method for the quantification of 25-hydroxyvitamin D (25OHD) in the maternal plasma sample. Different sample preparation methods and precipitation reagent-to sample ratio used by previous studies were compared. An ultrahigh performance liquid chromatography method was developed and validated for simultaneous quantification of $25OHD_2$ and $25OHD_3$. The chromatographic separation was achieved using the COSMOCORE 2.6Cholester Column and methanol and 0.1% formic acid (79:21, %v/v) as mobile phase at a flow rate of 0.3 mL/min and diode array detection at 264 nm. The results demonstrated that a recovery of approximately 100% could be achieved by extracting samples using 1 mL of hexane, vortex for 10s and a total number of three extraction steps. The precipitation reagent-to-sample ratio of 2.8 was optimum for the quantification of $25OHD_2$ and $25OHD_3$ in a pooled maternal sample. The value obtained for validation parameters meets the criteria of the Recommendations and Acceptance Criteria for Bioanalytical Method Validation by the Food and Drug Administration. The results showed that this method could be applied for routine quantification of 25OHD, particularly in the maternal plasma sample.

Keywords: 25-hydroxyvitamin D, maternal, ultrahigh performance liquid chromatography, extraction

Abstrak

Kajian ini bertujuan mengoptimumkan dan mengesahkan kaedah penyediaan dan pengekstrakan yang mudah dan cekap untuk pengkuantitian 25-hidroksivitamin D (25OHD) di dalam sampel plasma ibu mengandung. Kaedah kromatografi cecair berprestasi ultra tinggi telah dibangunkan dan disahkan untuk pengkuantitian $25OHD_2$ and $25OHD_3$ secara serentak. Pemisahan kromatografi telah dicapai melalui turus COSMOCORE 2.6Cholester dan metanol: 0.1% asid formik (79:21, %v/v) sebagai fasa bergerak pada kadar aliran 0.3 mL/min dan pengesahan susunan diod pada 264 nm. Keputusan kajian menunjukkan bahawa kaedah pengekstrakan terbaik yang memberi kadar pemulihan yang hampir dengan 100% adalah mengekstrak sampel dengan menggunakan 1mL heksana, vorteks selama 10 s dan sejumlah 3 kali bagi langkah pengekstrakan. Kadar reagen pemendakan kepada sampel sebanyak 2.8 adalah optimum bagi pengekstrakan $25OHD_2$ dan $25OHD_3$ dalam sampel plasma ibu mengandung. Nilai yang diperolehi untuk parameter validasi adalah memenuhi kriteria cadangan dan kriteria penerimaan untuk pengesahan kaedah bioanalitikal oleh

pentadbiran makanan dan ubat-ubatan (FDA). Hasil kajian menunjukkan bahawa kaedah yang diperolehi boleh diguna untuk rutin pengkuantitian 25OHD dalam sampel plasma, terutamanya sampel plasma dari ibu mengandung.

Kata kunci: 25-hidroksivitamin D, ibu mengandung, kromatografi cecair berprestasi ultra tinggi, pengekstrakan

Introduction

It is generally agreed that 25-hydroxyvitamin D (25OHD) is the biomarker that best reflects total vitamin D exposure-from food, supplement and skin synthesis [1, 2]. There are a variety of assays available for measuring serum or plasma 25OHD. These assays can be categorized into two method types: antibody-based methods (immunoassays) and liquid-chromatography (LC)-based methods. The strengths and weaknesses of each assay have been widely discussed in previous literature [3].

The complex nature of the biological matrix makes the development of assays for the quantification of blood 25OHD challenging. In the circulation, 25OHD is tightly bound to vitamin D binding protein (VDBP) [4]. Hence, the complete dissociation of 25OHD from VDBP in the blood sample before analysis is crucial to ensure accurate quantification of total 25OHD [5]. The complete dissociation of 25OHD is concerned in the blood sample from pregnant women as pregnancy is characterized by a two- to three-fold increase in VDBP [6-8]. Previous studies have reported the inaccuracy of measuring total 25OHD via automated immunoassay in populations with different levels of VDBP [9-11]. In automated immunoassays, releasing agents, which are proprietary, were used to release 25OHD from VDBP [3]. These releasing reagents may not be as good as the organic solvent in terms of their ability to dissociate 25OHD from VDBP. The incomplete dissociation of 25OHD from the VDBP could be a source of high variability between automated immunoassays and LC-based methods [5].

The complete dissociation of 25OHD from VDBP is also important for extraction-based assays such as LC-based methods. In previously published LC-based methods, different extraction methods have been used. The differences in terms of type and volume of solvent were used for protein precipitation and extraction as well as the duration of extractions. In an investigation of the consequences of differential extraction characteristics to the quantification of 25OHD in the serum sample, Lankes et al. found that the extracted concentration of 25OHD varied with the 70% methanol-to-sample ratio and the sample-to-hexane ratio used [12]. These could be a source of interlaboratory variability and bias of 25OHD results in LC-based methods. No previous study has investigated the consequences of differential extraction methods in the blood sample from pregnant women, which has high VDBP levels. Therefore, this study aimed to optimize a simple and efficient sample preparation and extraction method for the quantification of 25OHD in maternal plasma sample. Then, this study reports a validated ultrahigh performance liquid chromatography diode array (UHPLC-DAD) detection method for the routine measurement of plasma 25OHD using the optimised method.

Materials and Methods

Reagents and chemicals

25-hydroxyvitamin D_2, 25-hydroxyvitamin D_3, Dodecanophenone (98%) and phosphate-buffered saline (PBS) tablet were purchased from Sigma-Aldrich. Bovine serum albumin (BSA) was purchased from Nacalai Tesque (Kyoto, Japan). HPLC grade methanol (MeOH) was from JT Baker (Phillipsburg, NJ, USA). HPLC grade-water, ethanol and hexane were from Fisher Scientific Ltd. (Loughborough, UK).

Instrument

UHPLC analysis was conducted on the Agilent 1290 Infinity liquid chromatography system (Agilent Technologies, Wilmington, DE, USA), which comprised of a binary pump, autosampler, and column thermostat, coupled to a Diode Array Detector (DAD). A COSMOCORE 2.6Cholester Column (2.1 mm ID × 150 mm, 2.6 µm particle size) (Nacalai Tesque, Kyoto, Japan) was used. The column was protected by using a 2.1 mm UPLC C_{18} SecurityGuard ULTRA guard cartridge (Phenomenex, Torrance, CA, USA). The

injection volume was 20 µL, and the column temperature was maintained at 50 °C. ChemStation (Agilent Technologies, Wilmington, DE, USA) was used for system control, data acquisition, and processing.

Preparation of reagents, standards, calibration solution and quality control materials

Individual calibrator stock solution of $25OHD_3$ and $25OHD_2$ was prepared at 25 µmol/L in ethanol and stored in 2 mL glass vial at -20 °C. The concentrations of each calibrator stock solution were confirmed by measuring the absorbance (AU) of each stock solution at 264 nm (UV-1800 UV-VIS Spectrophotometer, Shimadzu, Kyoto, Japan).

From the calibrator stock solutions, a combined stock solution of $25OHD_2$ and $25OHD_3$ at 5,000 nmol/L was prepared. Then, six calibrators in the range from 12.5 to 200 nmol/L were prepared by spiking the combined stock solution into 4% BSA in PBS. Each of the calibrators was prepared freshly at the beginning of each run. Working internal standard (IS), dodecanophenone was prepared at 2,000 ng/mL in 80% of methanol, which was stored in 2 mL glass vial at -20 °C.

Optimisation of chromatographic conditions

The chromatographic conditions and mobile phase composition suggested by the manufacturer (Nacalai Tesque, Kyoto, Japan) were used: isocratic elution with methanol and 0.1% formic acid (80:20, v/v) as mobile phase, run at a flow rate of 0.4 mL/min and column temperature of 50 °C. The chromatographic conditions, which include flow rate, column temperature, and mobile phase composition, were subsequently optimised to give better resolution, peak shape and retention time for the plasma sample.

Extraction procedure

Five different sample extraction methods (methods A-E) modified from previous studies [13-15] were compared at 25OHD concentration at the medium concentration (80 nmol/L). The percent recoveries of both 25OHD were calculated and presented as mean recover ± standard deviation (SD) and percent of the coefficient of variation (CV).

Optimisation of the volume of precipitation reagent

The precipitation reagent contained methanol and 0.2M of $ZnSO_4$ (70:30, v/v). The different volume of precipitation reagents (700-2,100 µL) was tested using the pooled maternal sample collected for our cross-sectional study to determine vitamin D status among pregnant women. Maternal plasma was prepared by centrifuge maternal blood at 3,500 rpm and 4 °C for 15 minutes using Kubota 2810 Centrifuge (Tokyo, Japan). Plasma from 20 respondents was pooled. The pooled maternal sample was spiked with $25OHD_2$ standard because the pooled plasma had a very low concentration of $25OHD_2$.

Sample preparation

For sample extraction, 500 µL of plasma sample or calibrator was pipetted into a 13 × 100 mm glass tube. Fifty microliters of a working IS solution was added, and the tube was vortexed for 5 s. Next, 1,400 µL of precipitation reagent was added into each tube, and the tube was allowed vortex mixing. The mixture was extracted with 1 mL of hexane by vigorous mixing using a multi-tube vortexer for 10 sand then centrifuged at 3,000 rpm for 5 minutes. The extraction process was repeated for a total of 3 extraction steps. The hexane layer (supernatant) from each tube was transferred and pooled into another 13 × 100 mm glass tubes and dried under nitrogen gas flow. The dry extract was reconstituted with 120 µL of 79 % methanol and mixed for 10 s. The samples were transferred into a sample vial with a vial insert. Twenty microliters were injected into the UHPLC system for analysis.

Method validations

The guidelines by the US Department of Health Human Services Food and Drug Administration [16] and ICH Expert Working Group [17] were used for validating this method. Three replicates of blank (4% BSA) and blank spiked with 25OHD and IS were analyzed. Chromatograms were examined for possible chromatographic interference from 25OHD.

Linearity was evaluated by constructing a six-point (concentrations) calibration curve over the range of 12.5-200 nmol/L. The peak area ratios of 25OHD relative to IS were plotted against the corresponding

concentrations. The curve was not forced through zero. The correlation coefficient (r^2), y-intercept and slope of the calibration curve were reported.

The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the calibration curve according to the following formulas of Equations 1 and 2 [15], respectively.

$$LOD = 3.3 \times (SD/m) \quad (1)$$

$$LOQ = 10 \times (SD/m) \quad (2)$$

where SD corresponds to the standard deviation of response (y-value) and m corresponds to the slope of the calibration curve.

The recovery of the sample preparation method for $25OHD_2$ and $25OHD_3$ was evaluated at three concentrations (40, 80, and 160 nmol/L for low, medium and high concentrations, respectively). These samples were prepared by spiking an appropriate volume of standard solution into 4% BSA before sample extraction (pre-spiked sample) and after sample extraction (post-spiked sample). Three replicates at each concentration were assayed. The recovery was calculated by comparing the area of the pre-spiked sample to the area of the post-extracted spiked sample (Equation 3).

$$\% \text{ Recovery} = \frac{\text{Peak area of the pre-spiked sample}}{\text{Peak area of the post-spike sample}} \times 100\% \quad (3)$$

Intra-day precision was determined by extracting and analyzing three replicates of each calibrator in the calibration curve (12.5-200 nmol/L) one run. By contrast, the inter-day precision was determined by extracting and analyzing each calibrator (12.5-200 nmol/L) in triplicates in three different days. Results were expressed as CV and percent bias for intra-and inter-day precision and accuracy, respectively. The accuracy of the method was further evaluated using the Tri-level of serum control, purchased from the UTAK Laboratories, Inc. (Valencia, CA, USA). Each level was evaluated in five replicates. Our measured values were compared with the target values provided by the manufacturer and expressed in percent.

Two levels of serum control from UTAK, Vitamin D plus Low and Vitamin D Plus Level 2 were used to evaluate post-extraction stability. After extracting the control sample, the sample was subjected to injection (0 hour) into the system for analysis. After the first injection (0 hour), sample vials were stored at -20 °C. After 18 hours, sample vials were subjected to the second injection and stored again at -20 °C. The vials were subjected for the third injection after 120 hours. The stability was calculated by comparing the peak area ratio of the sample at 18 hours (or 120 hours) with the respective peak area ratio of the sample at 0 hour.

Results and Discussion

Optimisation of chromatographic conditions

Under the chromatographic conditions suggested by the manufacturer [isocratic elution with methanol and 0.1% formic acid (80:20, v/v) as mobile phase, run at a flow rate of 0.4 mL/min and column temperature of 50 °C], $25OHD_2$ and $25OHD_3$ were separated well from each other in standard dissolved in the mobile phase and BSA but not in the pooled maternal plasma sample. Then, different ratios of methanol and 0.1% formic acid, flow rate, column temperature, and gradient elution systems were considered.

It was found that as the ratios of water in the mobile phase increased (from 20% to 21-25%), the resolution for $25OHD_2$ and $25OHD_3$ was improved, but the analytes were eluted late. Thus, the peak appeared to be broad and showed reduced sensitivity because of the reduced peak height. Given that an increase in the ratio of water could compromise the peak shape, a ratio of methanol to 0.1% formic of 79:21 (%v/v) was chosen, which produced an acceptable separation time and resolution.

The effects of flow rate and column temperature were also investigated. Different mobile phase flow rates (0.1 to 0.4 mL/min) and column temperature (40-55 °C) showed no differences in the resolution of $25OHD_2$ and $25OHD_3$ visually. As the flow rate decreased, the retention times also increased, and the peak would be broad. However, as the flow rate increased, the column backpressure also increased. Thus, a flow rate of 0.3 mL/min and a column temperature of 50 °C were used,

which produced an acceptable retention time and column backpressure.

Hence, the optimum chromatographic condition for $25OHD_2$ and $25OHD_3$ in BSA and pooled maternal sample were mobile phase composition, methanol: water of 79:21 (% v:v); column temperature, 50 °C; and flow rate, 0.3 mL/min. Under this condition, the retention times for $25OHD_3$, $25OHD_2$, and IS were 8.4, 9.2, and 16.4 minutes. The total run time was 18 minutes (Figure 1).

Optimisation of the extraction procedure

Liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are two common methods used to extract 25OHD from plasma or serum. Previous studies have demonstrated that SPE was efficient in removing the interferences compounds [18-20]. However, the SPE column is expensive and required extensive optimisation. With an optimum downstream analysis, interferences compounds in the LLE sample are still can be resolute from the analyte of interest. Thus, LLE is still a preference and has been used in recent candidate reference measurement procedures (RMPs) [21, 22] and Centres for Disease Control and Prevention (CDC) refined method to access serum 25OHD.

In LLE, hexane, a non-polar solvent, is frequently used in published methods [5, 14, 15, 23, 24]. However, the volume of non-polar solvent, the total number of extraction steps and the time mixing (vortexing) varied from study to study. The volume of hexane used ranged from 1-5 mL [14, 15, 23, 24]. The total number of extraction steps is two to three times. The time of mixing ranged from 10 s to 3 minutes [13-15, 24]. Nevertheless, most studies mixed for 1 minute [13-15, 24]. These studies reported a satisfactory recovery for sample extraction, optimised an extraction method that provides good recovery, and used a minimum amount of solvent; thus, lesser time and cost spent are important.

In this study, we tested the four combinations of these extraction characteristics. The results revealed that the increase in the total number of extraction steps, from two to three times increased the recovery (Table 1). There was no significant difference in the recovery for increasing the volume of hexane used and time of mixing. The recovery of 25OHD was significantly lower when LLE was conducted twice (method A) compared to a total of three times (method B). This may indicate incomplete extraction of 25OHD from the plasma sample if the sample was extracted twice. The volume of hexane used and the time of vortex did not affect the recovery of 25OHD. The recovery of approximately 100% and satisfactory of % of CV (80-120%) can be achieved by using 1 mL of hexane, vortex for 10 s and a total number of three extraction steps. These extraction characteristics were subsequently applied to all optimisation and validation experiments.

Optimisation of the volume of precipitation reagent

In the previous published methods, precipitation reagent that was frequently used to dissociate and precipitate protein in plasma included ethanol [26, 27], acetonitrile [19, 28, 29], 70% methanol [13], methanol-2-propanol (80:20 v/v) [14, 24, 25] and zinc sulphate [30]. Before our experiment, we have tried a number of these precipitation reagents. We found that the use of methanol, ethanol or 70% methanol would make the reconstitute sample (sample before injecting into the system) very cloudy, which cannot be filtered by syringe filter nor precipitate by centrifugation. The use of acetonitrile would cause the “balling” of protein which would affect the recovery of 25OHD metabolites. We found that precipitation reagent contained methanol and 0.2M of ZnSO4 (70:30, v/v) was best for the sample (plasma).

In the absence of precipitation reagent, no $25OHD_3$ and $25OHD_2$ are detected and quantified (Table 2). This observation indicated the importance of precipitation reagent in releasing 25OHD from binding protein. Our results showed differential in precipitation reagent-to-sample-ratio affect the extracted $25OHD_2$ but not $25OHD_3$. As indicated in the values of 25OHD calculated based on the area under the peak, the use of 2,100 µL of PP reagent reduced the extracted $25OHD_2$. However, the increase in the volume of precipitation did not increase the extracted $25OHD_3$. Likewise, the differential in precipitation agent-to-sample-ratio also affects the quantification of the IS. This finding suggested the binding of IS (dodecanophenone) with

plasma protein. Overall, the results appeared that precipitation reagent-to-sample ratio of 2.8 is optimum for quantification of $25OHD_2$ and $25OHD_3$ based on peak area ratio. This finding is in agreement with the finding from Lankes et al. [12].

Method Validation

The chromatogram showed that there was no other interference at the retention time of $25OHD_2$, $25OHD_3$ and IS (Figure 2). BSA is suitable to be used as a matrix for calibrator.

The linearity of the method was evaluated in the concentration range from 12.5 to 200 nmol/L. The range was chosen based on the range of 25OHD in human plasma reported in previous studies. Table 3 shows the regression analysis of the 25OHD calibration curve. The calibration curves were linear over the concentration of 12.5-200 nmol/L with a correlation coefficient of 0.99 for both $25OHD_3$ and $25OHD_2$. The calculated LOQ and LOD for $25OHD_3$ were 11.25 nmol/L and 3.75 nmol/L, respectively. The LOQ and LOD for $25OHD_2$ were 10.25 and 3.25 nmol/L, respectively. The results were consistent with precision and accuracy results wherein the lowest concentration on the calibration curve of 12.5 nmol/L showed acceptable precision and accuracy limit, CV < 20% and accuracy ±20% of nominal concentration (Table 4).

The recovery of 25OHD was evaluated to determine the efficiency and reproducibility of the extraction method. Table 5 shows the recovery of 25OHD in a 4% BSA spiked sample. Satisfactory recovery was achieved with all mean recovery values were within ± 20% of 100%. The recovery was consistent and reproducible, which the % CV all recovery tests were <10%.

According to the FDA guidelines, acceptance criteria for precision and accuracy were CV ≤ 15%, bias = ± 15% of nominal concentrations. However, CV < 20% and bias = ± 20% for calibrator at LOQ concentration are acceptable. The Table 4 presents the inter- and intra-day precision and accuracy data. For $25OHD_3$, the intra-day CV ranged from 2.5% to 6.4% and the inter-day CV ranged from 1.8% to 10.5%. For $25OHD_2$, the intra-day CV ranged from 0.4% to 13.0%, and the inter-day CV ranged from 1.3% to 7.3%. The % of bias for both $25OHD_3$ and $25OHD_2$ was < 8% for all concentrations except for 12.5 nmol/L. The % of bias for both analytes was < 16%. The precision and accuracy of the method in this study were satisfactory. These results were consistent with the accuracy results obtained using commercial serum control (Table 6). The accuracy was approximately 91% to 99% for $25OHD_3$ and 82% to 87% for $25OHD_2$. These ranges were acceptable as they were within ± 20% of the target value provided by the manufacturer.

Table 7 shows the stability of extracted 25OHD after storing at −20°C for 18 and 120 hours. The stability ranged from 94.6% ± 1.2 % to 103.7% ± 9.4%, indicating that the extracted $25OHD_3$ and $25OHD_2$ were stable at least up to 120 hours when stored at -20 °C.

Overall, the optimised method showed satisfactory recovery, precision and accuracy. The method appeared to meet with the Recommendations and Acceptance Criteria for Bioanalytical Method Validation by the US Department of Health Human Services Food and Drug Administration [16]. The LOD and LOQ of our method are comparable with those of the published HPLC method [15, 24, 28] and with other automated immunoassays [5]. Although the LOD and LOQ of our method by our present method were higher compared with LC-MS/MS method [31, 32], this value still enable us to classify individuals with vitamin D deficiency defined as 25OHD < 25 nmol/L [1].

Strengths and limitations

Recently, a C3-epimer of $25OHD_3$ (3-epi-$25OHD_3$) has been highlighted to the confound quantification of total 25OHD [33-35]. In addition, 3-epi-$25OHD_3$ was found to contribute to approximately 6% in maternal [34, 36]. The biological significance of 3-epi-$25OHD_3$ is uncertain. The separate measurement of 3-epi-$25OHD_3$ is important to understand the biological role of the C3-epimer further. LC-MS/MS method with a specific column has been developed for separation of $25OHD_3$ from 3-epi-$25OHD_3$. However, we acknowledged our inability to separate and quantify C-3 epimers in our method as a limitation. However, this limitation does not defeat the validity of the assay.

Notably, LC-MS/MS may not be available and affordable to laboratories in developing countries. Moreover, LC-MS/MS requires highly trained operators to operate the instruments and significant instrument maintenance, which further increases the cost of the test [5]. A vitamin D assay capable of yielding accurate and reproducible results is sufficient for routine measurement of plasma 25OHD. The developed method uses low volumes of extraction solvent and time; therefore, it can be used to measure plasma 25OHD, particularly in laboratories that cannot afford to have an LC-MS/MS system. Many methods have been developed and validated previously. However, one critical aspect related to the extraction of 25OHD, particularly, in the maternal sample, which is high in VDBP was overlooked. The optimized method will be used as a reference in future studies. The effects of high VDBP will be further evaluated for an LC-based system that used MS as a detector.

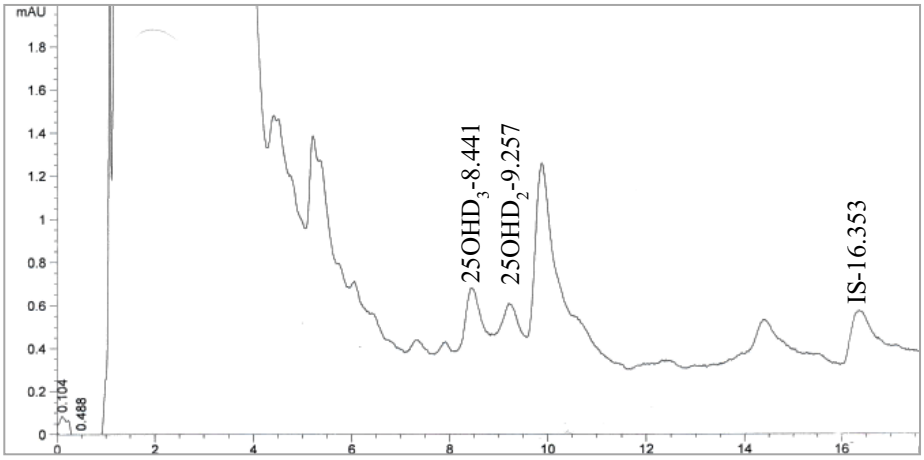

Figure 1. Representative UHPLC-chromatogram of pooled maternal sample spiked with $25OHD_2$

Table 1. Recovery of 25OHD by different extraction method

Method	Volume of Hexane (mL)	Time Vortex	Times of Extract	$25OHD_3$		$25OHD_2$	
				Recovery, %[a]	CV, %	Recovery, %[a]	CV, %
A	1	10 s	2	79.2 ± 8.6*	10.9	87.1 ± 7.8*	9.0
B	1	10s	3	99.2 ± 6.1†	6.1	100.6 ± 5.5*	5.5
C	1	3 min	3	89.2 ± 7.6*†	8.5	93.0 ± 6.6*	7.1
D	2	10s	3	100.6 ± 5.8†	5.8	99.2 ± 14.0*	14.1
E	2	3 min	3	93.6 ± 0.5†	0.53	94.4 ± 2.8*	3.0

[a] Mean followed by a different symbol within the same column indicate a significant difference (p <0.05)

Table 2. Differential in mean extracted 25OHD concentration (nmol/L) in the pooled maternal sample with different volume of precipitation reagents

Volume of Precipitation Reagent (µL)	$25OHD_3$ Concentration (nmol/L)		$25OHD_2$ Concentration (nmol/L)	
	Based on the Area[a]	Based on IS[a]	Based on the Area[a]	Based on IS[a]
0	0*	0*	0*	0*
700	31.0 ± 2.0†	45.6 ± 7.7†	20.9 ± 1.1†	36.4 ± 8.7†
1400	31.0 ± 1.0†	31.6 ± 1.9‡	20.8 ± 0.4†	23.2 ± 1.1†
2100	29.3 ± 0.6†	27.3 ± 1.5‡	17.7 ±0.6‡	17.6 ± 1.4‡

[a] Mean followed by a different symbol within the same column indicate a significant difference (p <0.05)

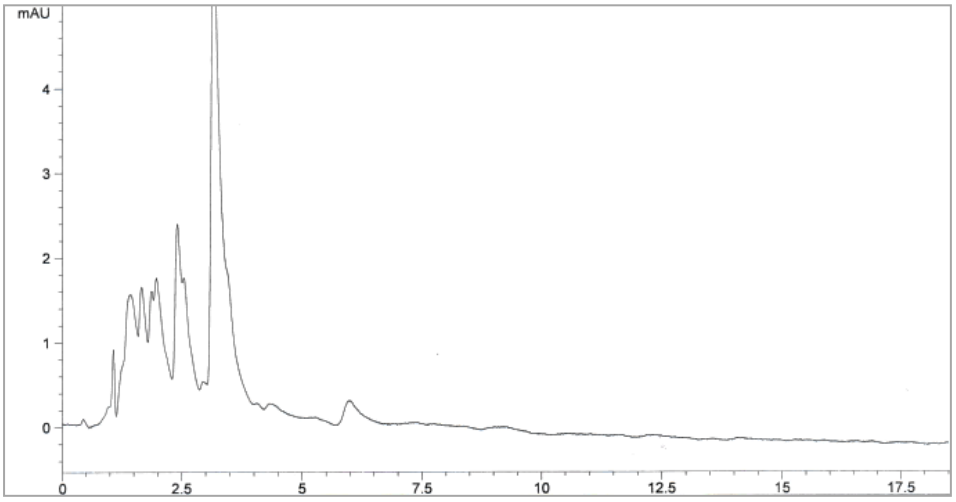

Figure 2. Representative UHPLC-chromatogram of a blank sample (4% BSA in PBS)

Table 3. Regression analysis of 25OHD calibration curve

Parameters	$25OHD_3$	$25OHD_2$
Number of concentrations per curve	6	6
Regression equation	y=0.015x + 0.027	y=0.017+ 0.053
Mean slope SD	0.015 ± 0.003	0.017 ± 0.002
Mean intercept SD	0.027 ± 0.007	0.0578 ± 0.007
Mean regression coefficient SD	0.991 ± 0.0012	0.993 ± 0.005
Limit of detection (LOD) (nmol/L)	3.75	3.25
Limit of quantification (LOQ) (nmol/L)	11.25	10.25

Table 4. Inter-intraday precision in standard

Nominal Concentrations (nmol/L)	25OHD$_3$				25OHD$_2$			
	Mean	SD	CV, %	Bias, %	Mean	SD	CV, %	Bias, %
Intra-day								
12.5	11.25	0.23	5.11	-9.94	13.05	0.68	13.0	4.45
25	25.10	0.64	6.35	0.40	26.93	0.57	5.28	7.7
50	46.35	0.85	4.56	-7.30	51.13	1.44	7.04	2.25
100	94.15	1.19	3.17	-5.86	93.0	0.28	0.74	-7.03
150	159.03	1.58	2.48	6.02	148.13	0.92	1.56	-1.26
200	195.95	2.07	2.64	-2.02	203.88	0.31	0.38	1.94
Inter-day								
12.5	14.40	0.61	10.54	15.26	13.63	0.35	6.5	8.96
25	25.50	0.18	1.74	1.97	26.4	0.77	7.27	5.63
50	48.35	1.10	5.68	-3.28	50.9	1.39	6.82	1.78
100	94.95	0.69	1.82	-5.06	97.23	1.47	3.79	-2.77
150	152.15	3.36	5.52	1.44	146.63	2.58	4.39	-2.24
200	200.43	1.85	2.31	0.22	203.15	1.05	1.29	1.57

Table 5. Recovery of 25OHD in 4% BSA spiked sample

Nominal Concentrations (nmol/L)	Mean Recovery, %	CV, %
25OHD$_3$		
40	85.8 ± 1.7	2.0
80	99.7 ± 5.2	5.2
160	89.1 ± 6.2	7.0
25OHD$_2$		
40	84.4 ± 2.1	2.5
80	100.2 ± 3.9	4.0
160	91.9 ± 5.9	6.4

Table 6. Validation of method with the commercial serum control at three concentration levels (n = 5)

	Target Value (nmol/L)	Measured Value (nmol/L)	Accuracy, %	CV, %
25OHD$_3$				
Level low	25	22.8 ± 1.5	90.9	5.5
Level L1	75	74.3 ± 4.0	99.0	5.2
Level L2	182.5	169.5± 4.5	92.9	2.5
25OHD$_2$				
Level low	25	21.8 ± 1.3	87.1	6.0
Level L1	75	64.0 ± 2.5	85.4	4.0
Level L2	182.5	149.5± 8.0	81.9	4.4

Table 7. Stability of 25OHD in commercial serum control

	18 hours		120 hours	
	Mean	SD	Mean	SD
25OHD$_3$				
Level low	100.4	0.7	103.5	3.2
Level L2	99.9	0.5	99.3	0.2
25OHD$_2$				
Level low	101.9	5.7	103.7	9.4
Level L2	97.8	1.5	94.6	1.2

Conclusion

In conclusion, it is important to optimise extraction and protein denaturation (or precipitation) procedure in the LC-based assay, particularly in the maternal sample, which is high in binding protein. Future studies on developing and applying methods for quantification of plasma sample, particularly maternal sample, should consider the type and volume of precipitation as well as the total number of extractions. We have developed a UHPLC-DAD method that was simple, rapid, and cost-effective. It provides good recovery and satisfactory reliability and accuracy, and is suitable to be applied to routine quantification of 25OHD, particularly in laboratories that cannot afford to have an LC-MS/MS system.

References

1. Institute of Medicine. (2011). Dietary Reference Intakes for Calcium and Vitamin D. The National Academies Press, Washington, DC: pp. 96.
2. Scientific Advisory Committee on Nutrition (2016). SACN vitamin D and Health report. https://www.gov.uk/government/publications/sacn-vitamin-d-and-health-report. [Access online 20 January 2019].
3. Wallace, A. M., Gibson, S., de la Hunty, A., Lamberg-Allardt, C. and Ashwell, M. (2010). Measurement of 25-hydroxyvitamin D in the clinical laboratory: Current procedures, performance characteristics and limitations. *Steroids*, 75(7): 477-488.

4. Bikle, D., Gee, E., Halloran, B., Kowalski, M. A., Ryzen, E. and Haddad, J. G. (1986). Assessment of the free fraction of 25-hydroxyvitamin D in serum and its regulation by albumin and the vitamin D-binding protein. *The Journal of Clinical Endocrinology & Metabolism*, 63(4): 954-959.
5. Le Goff, C., Cavalier, E., Souberbielle, J. C., Gonzalez-Antuna, A. and Delvin, E. (2015). Measurement of circulating 25-hydroxyvitamin D: A historical review. *Practical Laboratory Medicine*, 2: 1-14.
6. Bikle, D. D., Gee, E., Halloran, B. and Haddad, J. G. (1984). Free 1,25-dihydroxyvitamin D levels in serum from normal subjects, pregnant subjects, and subjects with liver disease. *The Journal of Clinical Investigation*, 74(6): 1966-1971.
7. van Hoof, H. J., de Sevaux, R. G. and van Baelen, H. (2001). Relationship between free and total 1,25-dihydroxyvitamin D in conditions of modified binding. *European Journal of Endocrinology*, 144(4): 391 – 396.
8. Jones, K. S., Assar, S., Prentice, A. and Schoenmakers, I. (2016). Vitamin D expenditure is not altered in pregnancy and lactation despite changes in vitamin D metabolite concentrations. *Scientific Reports*, 6, 26795.
9. Freeman, J., Wilson, K., Spears, R., Shalhoub, V. and Sibley, P. (2014). Influence of vitamin D binding protein on accuracy of 25-hydroxyvitamin D measurement using the ADVIA centaur Vitamin D total assay. *International Journal of Endocrinology*, 2014: 1-12.
10. Heijboer, A. C., Blankenstein, M. A., Kema, I. P. and Buijs, M. M. (2012). Accuracy of 6 routine 25-hydroxyvitamin D assays: Influence of Vitamin D binding protein concentration. *Clinical Chemistry*, 58(3): 543-548.
11. Cavalier, E., Wallace, A. M., Knox, S., Mistretta, V. I., Cormier, C. and Souberbielle, J. C. (2008). Serum vitamin D measurement may not reflect what you give to your patients. *Journal of Bone and Mineral Research*, 23(11): 1864-1865.
12. Lankes, U., Elder, P. A., Lewis, J. G. and George, P. (2015). Differential extraction of endogenous and exogenous 25-OH-vitamin D from serum makes the accurate quantification in liquid chromatography-tandem mass spectrometry assays challenging. *Annals of Clinical Biochemistry*, 52(Pt 1): 151-160.
13. Centers for Disease Control and Prevention (2010). Laboratory procedure manual; 25-hydroxyvitamin D3, 3-epi-25-hydroxyvitamin D3, 25-hydroxyvitamin D2. Access from https://wwwn.cdc.gov/Nchs/Data/Nhanes/2009-2010/LabMethods/VID_F_met_Vitamin_D.pdf. [Access 30 September 2018]
14. Turpeinen, U., Hohenthal, U. and Stenman, U. H. (2003). Determination of 25-hydroxyvitamin D in Serum by HPLC and Immunoassay. *Clinical chemistry*, 49(9): 1521 – 1524.
15. Chin, S. F., Osman, J. and Jamal, R. (2018). Simultaneous determination of 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3 in human serum by ultra-performance liquid chromatography: An economical and validated method with bovine serum albumin. *Clinica Chimica Acta*, 485: 60-66.
16. US Department of Health Human Services Food and Drug Administration, Center for Drug Evaluation and Research, Center for Veterinary Medicine. (2001). Guidance for Industry: Bioanalytical method validation (Revised May 2018).
17. International Conference on Harmonization (ICH) of Technical Requirement for the Registration of Pharmaceuticals for Human Use. ICH Harmonised Tripartite Guideline: Validation of analytical procedures: text and methodology Q2 (R1).
18. Kand'ar, R. and Zakova, P. (2009). Determination of 25-hydroxyvitamin D3 in human plasma using HPLC with UV detection based on SPE sample preparation. *Journal of Separation Science*, 32(17): 2953-2957.
19. Chen, H., McCoy, L. F., Schleicher, R. L. and Pfeiffer, C. M. (2008). Measurement of 25-hydroxyvitamin D3 (25OHD3) and 25-hydroxyvitamin D2 (25OHD2) in human serum using liquid chromatography-tandem mass spectrometry and its comparison to a radioimmunoassay method. *Clinica Chimica Acta*, 391(1-2): 6-12.

20. Abu el Maaty, M. A., Hanafi, R. S., Aboul-Enein, H. Y. and Gad, M. Z. (2015). Design-of-experiment Approach for HPLC Analysis of 25-hydroxyvitamin D: A comparative assay with ELISA. *Journal of Chromatographic Science*, 53(1): 66-72.
21. Mineva, E. M., Schleicher, R. L., Chaudhary-Webb, M., Maw, K. L., Botelho, J. C., Vesper, H. W. and Pfeiffer, C. M. (2015). A candidate reference measurement procedure for quantifying serum concentrations of 25-hydroxyvitamin D(3) and 25-hydroxyvitamin D(2) using isotope-dilution liquid chromatography-tandem mass spectrometry. *Analytical and Bioanalytical Chemistry*, 407(19): 5615- 5624.
22. Stepman, H. C., Vanderroost, A., Van Uytfanghe, K. and Thienpont, L. M. (2011). Candidate reference measurement procedures for serum 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 by using isotope-dilution liquid chromatography-tandem mass spectrometry. *Clinical Chemistry*, 57(3): 441-448.
23. Hymoller, L. and Jensen, S. K. (2011). Vitamin D analysis in plasma by high performance liquid chromatography (HPLC) with C(30) reversed phase column and UV detection-easy and acetonitrile-free. *Journal of Chromatography. A*, 1218(14): 1835-1841.
24. Nurmi, T., Tuomainen, T. P., Virtanen, J., Mursu, J. and Voutilainen, S. (2013). High-performance liquid chromatography and coulometric electrode array detector in serum 25-hydroxyvitamin D(3) and 25-hydroxyvitamin d(2) analyses. *Analytical Biochemistry*, 435(1): 1-9.
25. Maunsell, Z., Wright, D. J. and Rainbow, S. J. (2005). Routine isotope-dilution liquid chromatography-tandem mass spectrometry assay for simultaneous measurement of the 25-hydroxy metabolites of vitamins D2 and D3. *Clinical Chemistry*, 51(9): 1683-1690.
26. Franke, A. A., Morrison, C. M., Custer, L. J., Li, X. and Lai, J. F. (2013). Simultaneous analysis of circulating 25-hydroxy-vitamin D3, 25-hydroxy-vitamin D2, retinol, tocopherols, carotenoids, and oxidized and reduced coenzyme Q10 by high performance liquid chromatography with photo diode-array detection using C_{18} and C_{30} columns alone or in combination. *Journal of Chromatography. A*, 1301: 1-9.
27. Hrvolová, B., Martínez-Huélamo, M., Colmán-Martínez, M., Hurtado-Barroso, S., Lamuela-Raventós, R. M. and Kalina, J. (2016). Development of an advanced HPLC-MS/MS method for the determination of carotenoids and fat-soluble vitamins in human plasma. *International Journal of Molecular Sciences*, 17(10): 1719.
28. Lensmeyer, G. L., Wiebe, D. A., Binkley, N. and Drezner, M. K. (2006). HPLC method for 25-hydroxyvitamin D measurement: Comparison with contemporary assays. *Clinical Chemistry*, 52(6): 1120 – 1126.
29. Garg, U., Munar, A., Frazee, C. nd Scott, D. (2012). A simple, rapid atmospheric pressure chemical ionization liquid chromatography tandem mass spectrometry method for the determination of 25-hydroxyvitamin D2 and D3. *Journal of Clinical Laboratory Analysis*, 26(5): 349-357.
30. Bruce, S. J., Rochat, B., Beguin, A. Pesse, B., Guessous, I., Boulat, O. and Henry, H. (2013). Analysis and quantification of vitamin D metabolites in serum by ultra-performance liquid chromatography coupled to tandem mass spectrometry and high-resolution mass spectrometry-a method comparison and validation. *Rapid Communications in Mass Spectrometry*, 27(1): 200-206.
31. Eyles, D., Anderson, C., Ko, P. Jones, A., Thomas, A., Burne, T. and McGrath, J. (2009). A sensitive LC/MS/MS assay of 25OH vitamin D3 and 25OH vitamin D2 in dried blood spots. *Clinica Chimica Acta*, 403(1-2): 145-151.
32. Zhang, S. W., Jian, W., Sullivan, S. Sankaran, B., Edom, R. W., Weng, N. and Sharkey, D. (2014). Development and validation of an LC-MS/MS based method for quantification of 25 hydroxyvitamin D2 and 25 hydroxyvitamin D3 in human serum and plasma. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*, 961: 62-70.

33. Karras, S. N., Kotsa, K., Angeloudi, E., Zebekakis, P. and Naughton, D. P. (2017). The Road Not So Travelled: Should Measurement of Vitamin D Epimers During Pregnancy Affect Our Clinical Decisions? *Nutrients*, 9(2): 90.
34. Aghajafari, F., Field, C. J., Rabi, D., Kaplan, B. J., Maggiore, J. A., O'Bierne, M., Hanley, D. A., Eliasziw, M., Dewey, D., Ross, S. and Apron T. (2016). Plasma 3-epi-25-hydroxycholecalciferol can alter the assessment of vitamin d status using the current reference ranges for pregnant women and their newborns. *The Journal of Nutrition*, 146(1): 70-75.
35. Yazdanpanah, M., Bailey, D., Walsh, W., Wan, B. and Adeli, K. (2013). Analytical measurement of serum 25-OH-vitamin D(3), 25-OH-vitamin D(2) and their C3-epimers by LC-MS/MS in infant and pediatric specimens. *Clinical Biochemistry*, 46(13-14): 1264-1271.
36. Kiely, M., O'Donovan, S. M., Kenny, L. C., Hourihane, J. O., Irvine, A. D. and Murray, D. M. (2017). Vitamin D metabolite concentrations in umbilical cord blood serum and associations with clinical characteristics in a large prospective mother-infant cohort in Ireland. *Journal of Steroid Biochemistry and Molecular Biology*, 167: 162-168.