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Development and optimization of simplified LC–MS/MS quantification of 25-hydroxyvitamin D using protein precipitation combined with on-line solid phase extraction (SPE)☆

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ABSTRACT

25-Hydroxyvitamin D, the most useful marker of the vitamin D status of an individual, has seen an exponential growth of its routine measurement in recent years. Several methods are currently offered but the most specific is LC–MS/MS. However, the routine use of this technique in the clinical laboratory makes it essential to improve key steps of this method for high throughput delivery. Importantly, the preanalytical steps of this assay and the efficacy of the separation system need to be optimized prior to MS detection. In this report we replaced the standard and time consuming liquid–liquid extraction method of vitamin D metabolites with hexane (LLE) combined with centrifugation (LLE/centrifugation) by a simpler protein precipitation with extraction (PPE) in acetonitrile combined with a fast separation process using a 96-well plate filtration system (PPE/filtration). This rapid extraction was then followed by an on-line solid phase extraction (SPE) using a selective chromatographic separation. We also optimized the operational and consumable costs, by using an inexpensive guard column as a trapping column to significantly enhance the lifespan of the analytical column two to three times as compared to conventional chromatography. The LC–MS/MS technique permits the measurement of both 25-hydroxyvitamin D_2 (25-OH D_2) and the 25-hydroxyvitamin D_3 (25-OH D_3) metabolites in electrospray ionization (ESI) mode. The chromatographic system consisted of a 2.1 mm × 50 mm C18 3.5 μM column with a 2.1 mm × 20 mm C18 3.5 μM guard column connected with two 6 ports switching valves. Quantifications were done using the isotopic dilution technique with hexadeutered 25-OH D_3 and 25-OH D_2.The ion suppression problem with phospholipids was also evaluated and optimized to minimize this effect through the chromatography process and the on-line SPE trapping. Calibration curves were prepared by diluting a commercial high calibrator Chromsystems (München, Germany) with either pure triple stripped blank serum or diluted in 6% phosphate buffer saline at pH 7.2. Linearity was tested up to 160 nmol/L for 25-OH D_3 and 75 nmol/L for 25-OH D_2. Low limit of quantification (LLOQ) were established at 3 nmol/L for 25-OH D_2 and 4 nmol/L for 25-OH D_3. Inter-assay and intra-assay precision (CV%) was determined using 3 levels of commercial controls (Utak, CA, USA) for 25-OH D_2 and 25-OH D_3. Results obtained for intra-assay and inter-assay precision (CV%) were 1.1–3.4% and 5–8.9% respectively for the PPE/centrifugation technique and 2.0–3.1% and 4.6–6.6% for the PPE/filtration technique. Accuracy was estimated with the same commercial controls: % bias was −11.2 to 4.9% with PPE/centrifugation and −3.2 to 6.1% with PPE/filtration. 25-OH D_2 and 25-OH D_3 concentrations in human serum with LLE were compared to the new extraction methods using either PPE/centrifugation or PPE/filtration. Correlations comparing the two methods revealed a slope approximately 1.0 ± 0.3 with $R \geq 0.98$ with a bias < 1 nmol/L. In summary, the new LC–MS/MS method described in this report using an on-line SPE technique with a simple off-line pre-treatment is faster, cost-effective, more reliable and more robust than current and widely used LLE/centrifugation methods coupled with LC–MS/MS.

1. Introduction

During the past decade, requests for vitamin D measurement in the clinical laboratory have increased exponentially [1]. Vitamin D deficiency or insufficiency is a risk factor for metabolic bone diseases such as osteoporosis, osteomalacia and rickets [2].

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Vitamin D metabolism involves two major steps: conversion to 25-hydroxyvitamin D (25-OH D) in the liver and activation to 1α,25-dihydroxyvitamin D (1α,25-OH D) in the kidney [3]. 25-OH D is an index of the vitamin D status and the primary metabolite measured in clinical laboratories. 1α,25-OH D is less frequently requested in the context of specific metabolic bone disorders.

25-OH D exists in 2 forms: 25-hydroxyvitamin D_2 (25-OH D_2) and 25-hydroxyvitamin D_3 (25-OH D_3). Measurement of 25-OH D can be done by radioimmunoassay (RIA) [4,5], enzymoimmunoassay (EIA) [4], chemiluminescence immunoassay (CI) [4,5], high performance liquid chromatography (HPLC) [6–12] or LC–MS/MS [3,4,13]. Monitoring serum concentrations of 25-OH D by LC–MS/MS is now considered the gold standard because of its sensitivity and specificity [3,14,15]. It has also the distinct advantage to measure simultaneously 25-OH D_2 and 25-OH D_3.

A potential drawback, however, is the presence of the C3 epimers; 3-epi-25-OH D_3 and 25-OH D_2 which have been shown to elute within the 25-OH D regions by LCMS using standard columns [3,16–18]. Although its separation is possible by LCMS it will significantly prolong chromatographic time and therefore reduce the throughput of this method [16,18]. The clinical significance of these metabolites is presently unknown but it appears to be present at high levels in a significant number of neonates and therefore their measurement would be useful in this population as indicated by recent reports [14,15].

Currently off-line extraction methods of 25-OH D measurement in use include, protein precipitation with extraction (PPE) [19,20], liquid–liquid extraction (LLE) [16,20–24] and/or solid phase extraction (SPE) [25–32] applied to samples before injection in the column.

However, using a partial or complete on-line SPE for sample pre-treatment [33–37] makes LC–MS/MS more attractive, despite the need for additional hardware such as extra pumps and valves. The throughput depends on the hardware and the configuration of the chromatographic system with on-line pre-treatment. The on-line SPE technique could be either a strict solid phase extraction (SPE) [34–36], a two dimensional LC [33] or a the turbulent flow chromatography (TFC) techniques [37,38].

Although a labor intensive partial off-line pre-treatment method is adequate for a small daily workload higher volumes require alternate solutions for sample preparation such as an automated off-line pre-treatment or an on-line pre-treatment. With the number of vitamin D requests increasing dramatically in the clinical laboratory it becomes urgent to optimize technical resources for this type of analysis. In particular, optimization of on-line and off-line sample pre-treatment is needed to achieve cost-effectiveness.

We are reporting here a fast off-line sample pre-treatment method based on a combination of PPE and well-plate filtration technology combined to a simple direct on-line SPE pre-treatment technique. This method is not only faster for off-line sample preparation but also has the advantage of an on-line SPE pre-treatment allowing chromatographic separation within the same chromatographic turnaround time as those used by the LLE method. We also compared this new method to both the original LLE method and manual PPE method.

2. Material and methods

2.1. Chemicals and reagents

25-OH D_2 (26,26,26,27,27,27-d6) (IS1) and 25-OH D_3 (26,26,26,27,27,27-d6) (IS2) was obtained from Medicalisotopes (Pelham, NH, USA). USP grade ethyl alcohol was obtained from Les Alcools de Commerce (SAQ, Quebec, Canada). LC–MS grade water; acetonitrile and methyl alcohol were obtained from Fisher scientific. Ammonium acetate, formic acid, Chromasolv grade hexane and activated charcoal (untreated powder, 100–400 mesh) were obtained from Sigma–Aldrich. Human serum 25-OH D (25-OH D_2 and 25-OH D_3) calibrator cat. # 62028 was purchased from Chromsystems (München, Germany). Human serum Tri-Level Vitamin D Plus controls were purchased from Utak (CA, USA). Stripped human serum cat. # 1131-00 was purchased from Biocell (CA, USA). Sirocco 96-well plates were obtained from Waters (MA, USA).

2.2. Vitamin D stripped serum preparation

Biocell serum was stripped two more times using a method adapted by Carter [39]. A 45 mL aliquot of Biocell serum was mixed with 0.8 g of activated charcoal (untreated powder, 100–400 mesh) by agitation at room temperature overnight. The serum was then centrifuged at 3000 RPM for 10 min. Next the supernatant was filtered with a syringe connected to an "Acrodisc Syringe" Supor membrane 0.2 μM (Pall, NY, USA). The final triple stripped Biocell serum (pure serum) was tested with the LC–MS method currently in use to confirm that 25-OH D (25-OH D_2 and 25-OH D_3) was undetectable, i.e. less than 2.5 nmol/L.

2.3. Stock solutions, calibration standard and controls

An internal standard solution containing two standards (IS1 and IS2) was prepared at the concentration of 50 nmol/L in ethyl alcohol for the LLE method or acetonitrile for the PPE methods. The calibration curve was established using the Chromsystems calibrator diluted in triple stripped Biocell serum at 6 different concentrations above the zero calibrator (4–160 nmol/L (25-OH D_3) and 2–75 nmol/L (25-OH D_2)). The diluent tested was pure (100%) or diluted (6% in phosphate buffer saline at pH 7.2 (PBS)) triple stripped Biocell serum. Three levels of controls (Utak, CA, USA) were reconstituted as per company's recommendations.

2.4. Patients sample (serum)

All patient samples were from unused left over serum. Blood was collected and processed following our routine clinical laboratory procedure. The unused serum was aliquoted, anonymized and stored at −20 °C until assayed.

2.5. Sample preparation

2.5.1. LLE method

The LLE method was adapted from Thibeault et al. [40]. Briefly, 200 μL of sample, calibrator or control was transferred in a 1.5 mL plastic conical screw-cap tube (Sarstedt). After addition of 200 μL of IS1/IS2 in ethyl alcohol, the mixture was shaken for 5 min at 1500 RPM. Hexane (1000 μL) was added and the mixture shaken for 10 min at 2500 RPM. Following centrifugation (10 min, 10 000 RPM), the supernatant was transferred into a new plastic conical tube and solvent was then evaporated in a Turbovap LV (Caliperls), under nitrogen at room temperature. The crude residue was resuspended in 100 μL of a methyl alcohol: water (85:15) solution, mixed 3 min at 2500 RPM and sonicated 2 min for thorough reconstitution, then transferred into a glass insert for injection into the LC–MS. Chromatography was done using the chromatographic conditions defined for the LLE method.

2.5.2. PPE method with centrifugation (manual PPE method)

A 150 μL aliquot of IS1/IS2 solution in acetonitrile was transferred into a 1.5 mL plastic conical screw-cap tube (Sarstedt). After adding 100 μL of sample, calibrator or control, the mixture was shaken 10 min at 1500 RPM, then centrifuged (10 min at 10 000

Table 1
Quantification and qualification MRM precursor/product ion transitions selected for measurement of 25-OH D.

MRM type	Analyte	Q1 mass (Da)	Q3 mass (Da)	Δ mass (Da)	DP (V)	CE (V)	CXP (V)
Quantification	25-OH D_2	413.4	355.3	58.1	51	13	10
Quantification	25-OH D_3	401.4	365.3	36.1	45	17	10
Quantification	25-OH D_2 d6 (IS1)	419.4	355.2	64.2	46	15	10
Quantification	25-OH D_3 d6 (IS2)	407.5	371.3	36.2	45	17	10
Qualification	25-OH D_2	413.4	337.3	76.1	51	15	10
Qualification	25-OH D_3	401.4	257.3	144.1	45	21	14
Qualification	25-OH D_2 d6 (IS1)	419.4	337.2	82.2	46	15	8
Qualification	25-OH D_3 d6 (IS2)	407.5	263.3	144.2	45	21	6

RPM) prior to transfer into a glass insert for injection. Chromatography was done using the chromatographic conditions for the PPE methods.

2.5.3. PPE method with filtration on Sirocco 96-well plate (96-well plate PPE method)

Using the same procedure described for the PPE with centrifugation, samples, calibrators or controls were dispensed in each well of 96-well plate containing IS1/IS2 acetonitrile solution. The well plate, (Sirocco, Waters) was sealed with a vented cap mat and shaken for 10 min at 1500 RPM. It was subsequently placed on a vacuum manifold and filtered. The filtrate was collected into a well plate filled with glass inserts, which was then sealed with cap mat, shaken and loaded onto the autosampler for injection. Chromatography was done using the chromatographic conditions for the PPE methods.

2.6. Chromatography

2.6.1. LC–MS/MS configurations

A Shimadzu Prominence LC system (Mandel, Guelph, Ontario, Canada) was used for HPLC chromatography. The system includes an autosampler SIL-20AC, oven CTO-20AC with two 6/2 port switching valves FCV-12H and two pumps. Pump A (binary pump) consisted of combination of 2 isocratic pumps LC-10ADVP connected to a semi-micro mixer 100 μL and pump B (quaternary pump) was a LC-20AD (see Fig. 1).

For MS/MS analysis, an API 4000Q trap was used with a Tubololon-Spray interface (AB Sciex, Concord, Ontario, Canada). The instrument was operated on positive ion mode using ESI probe. The ion spray voltage was set at 5500 V, the source temperature to 425 °C, the curtain gas to 30 psi, the ion source gas # 1 at 70 psi and ion source gas # 2 at 80 psi. Table 1 summarizes the selected MRM transitions for quantification Analyst 1.5 software was used for the data treatment and operation of LC system.

2.6.2. Chromatographic conditions for LLE method

A Sunfire 2.1 mm × 10 mm 3.5 μm C18 guard column was connected to a 2.1 mm × 50 mm 3.5 μm C18 Sunfire column (Waters, MA, USA). Mobile phase A contained methanol:water (98:2) with 0.1% formic acid and 2 mM ammonium acetate. Mobile phase B contained water with 0.1% formic acid and 2 mM ammonium acetate. Chromatography was done with an injection of 15 μL at an isocratic flow 0.8 mL/min at 35 °C, using a mixed mobile phase A:B (85:15) with pump A. The column was washed with 100% mobile phase A between samples. Total chromatographic time was 5 min.

2.6.3. Chromatographic conditions for the PPE methods

A X-Terra 2.1 mm × 20 mm 5 μm C18 guard column and a column Sunfire 2.1 mm × 50 mm 3.5 μm C18 (Waters, MA, USA) were installed on the switching valves system as described in Fig. 1. The heating temperature and mobile phase A and B used were identical to that described with the chromatographic conditions for LLE method. The chromatographic process began by loading 80 μL of sample on the guard column which was equilibrated before with a mix of the two mobile phases with a ratio A:B (68:32) at a flow of 1.6 mL/min (pump A). At the same time the sample was cleaned on the guard column, the analytical column was equilibrated with a mix of the two mobile phases with a ratio A:B (85:15) at 0.8 mL/min (pump B). Between 2.0 and 2.5 min, the guard column was then connected to the column with the switching valves system to start the chromatography. While the chromatography continued (time 2.5–4.0 min), the guard column was washed with 100% of mobile phase A. Finally, the analytical column was disconnected from the MS detector at 4.2 min and washed between 4.0 and 5 min with 100% of mobile phase A, followed a re-equilibration with the starting mobile phase (Fig. 2).

2.7. Method validation

The MS method was validated by determining low limit of quantification (LLOQ), linearity, accuracy, precision, recovery, correlations and ion suppression. Linearity was estimated using a curve with 6 non-zero calibrators in duplicates and a best fit curve was established using linear or quadratic weighting $1/x$ mode, respecting a least squares linear regression with an $r > 0.98$. Accuracy was verified using the tri-level quality control obtained from Utak. LLOQ was determined using a very low level calibrator prepared in triple stripped Biocell and established using a minimal ratio signal/noise of 10/1 and/or a precision CV% maximal of 20% as recommended in best practices for bio-analytical methods [41]. Intra-assay precision was determined using duplicates (repeat extraction and analysis of the same samples) injected in the same chromatographic run. Inter-assay precision was determined by running duplicates every day over 2 weeks. Recovery assay for the standards when using the LLE and PPE approaches were not repeated since over 90% sample recovery had been previously reported [22,34]. Correlations were done using the same patient's samples. Methods comparisons were performed between LLE, manual PPE and PPE with filtration on the Sirocco 96-well plate. Best fit curves were determined for 25-OH D_2 and 25-OH D_3 using least squares linear regression. For all samples with results expected beyond the calibration range, samples were diluted with triple stripped Biocell (blank) before treatment. Ion suppression testing consisted of monitoring qualitatively control serums (Utak

Table 2
MRM precursor/product ion transitions selected for the phospholipids monitoring.

Phospholipids	Abbrev.	Q1 mass (Da)	Q3 mass (Da)
Phospholipids (all)	ALL_PC1	184	184
Phospholipids (all)	ALL_PC2	184.1	184.1
Lysophosphatidylcholine	LYSO1	496	184
Lysophosphatidylcholine	LYSO2	524	184
Phosphatidylethanolamine	PE	716.4	575.2
Sphingomyelin	SM	731.6	184.1
Phosphatidylcholine	PC1	704	184
Phosphatidylcholine	PC2	758	184
Phosphatidylcholine	PC3	806	184
Phosphatidylcholine	PC	758.6	184

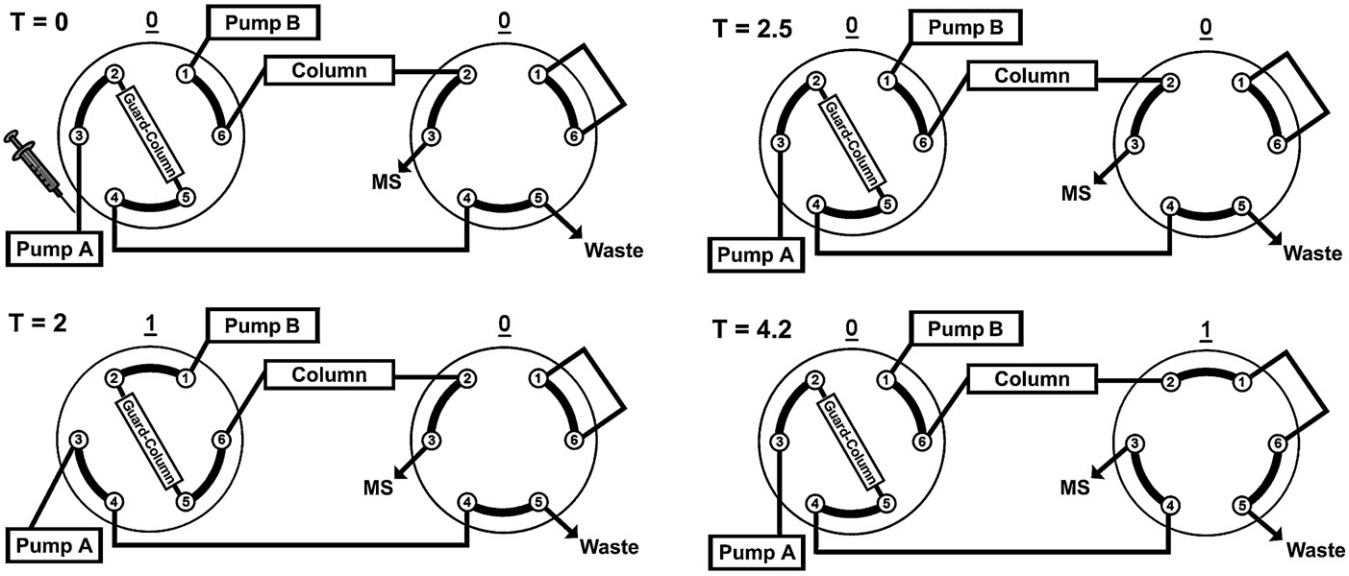

Fig. 1. Design of switching valves applied for the chromatography with the PPE methods. The first valve works for the trapping column (on-line pre-treatment) and the second one works like a diverting valve.

controls) for the different transitions (see Table 2) that represent the principal phospholipids families [42,43], i.e. lysophosphatidylcholine and phosphatidylcholine.

3. Results and discussion

3.1. Method development

The original method used in our laboratory involved a LLE technique with reconstitution of residual extract before injection onto a column. This technique was then replaced by a simplified off-line preparation combined with an on-line SPE pre-treatment, equivalent to a trapping column technique (chromatographic PPE method).

The considered off-line preparation consisted of a one step protein precipitation and extraction of the 25-OH D. The chosen sample pretreatment involved a manual protein precipitation using acetonitrile with a ratio of sample:acetonitrile of 1:1.5, described by Polson et al. [44], followed by a manual centrifugation (PPE method with centrifugation). However, the last step was advantageously replaced later by filtration on 96-wells plate (PPE with filtration on Sirocco 96-wells plate).

During the development of the on-line preparation step, we had to choose the best trapping technique between a small column, a SPE column or a guard column. A commercial SPE column was tested first for the trapping: Oasis HLB 2.1 mm × 20 mm (Waters, MA, USA). However, this approach did not meet our criteria. Despite the fact that this SPE column was specially designed for fast washing at a high flow with a low backpressure and could be reused for many injections, this approach did not provide a good separation of the 25-OH D_2 and 25-OH D_3 and the back-flushing necessary for this technique lead to contamination of the column and MS detector. Therefore, we opted for a longer guard column to avoid these disadvantages (Waters 5 μm X-Terra 2.1 mm × 20 mm C18) and minimize the operating costs.

3.2. Linearity and LLOQ

Calibration curves obtained for 25-OH D_2 and 25-OH D_3 gave correlation coefficients over 0.999. For 25-OH D_2, linear regression

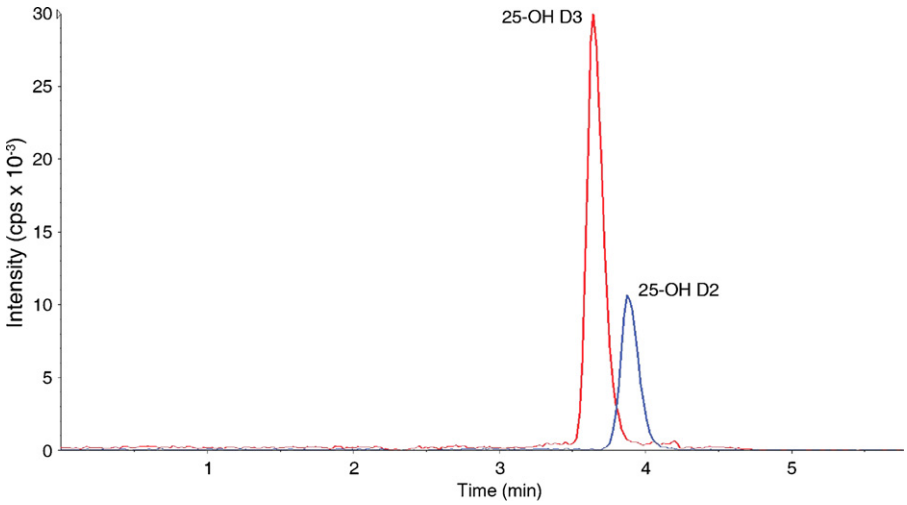

Fig. 2. Example of chromatogram with a standard of 25-OH D treated with the manual PPE method.

(no weighting) produced a slope of 0.0374 and intercept of 0.00209 (r = 0.9994). For 25-OH D_3, a simple linear regression model did not work. Consequently a quadratic regression ($1/x$ weighting) was applied. Saturation of the MS detector at high concentrations can explain the trend of 25-OH D_3 curve at a concentration of 160 nmol/L. The quadratic regression equation estimate ($1/x$ weighting) gave $y = -1.53 \times 10^{-5}X^2 + 0.0218X + 1.03 \times 10^{-8}$ (r = 0.9997).

The calibration curve prepared using the Chromsystems calibrator with 6% triple stripped Biocell serum, diluted in phosphate buffer saline at pH 7.2 (PBS), gave the same results for the controls and patients as 100% triple stripped Biocell did.

LLOQ was determined by running five repeats of a 80 μL injection of a very low calibrator with PPE ratio serum:acetonitrile of 1:1.5. The LLOQ for 25-OH D_2 was found to be 3 nmol/L with a signal to noise (S/N) ratio of 10/1, coefficient of variation (CV) < 10% and inaccuracy < 15%. Similarly, for 25-OH D_3 the calculated LLOQ was 4 nmol/L with a CV < 10% and inaccuracy < 15%.

3.3. Comparison of the methods

During the development of the on-line SPE method, the PPE method using centrifugation was compared with the original laboratory method (LLE). We used 116 serum samples from anonymized patients with concentrations covering a wide range, 25-OH D_3 (Table 3; assay # 1). We obtained a bias of less than 4% with an insignificant intercept (0.5) and an excellent correlation coefficient of 0.994. Less than 10% of the patient samples contained 25-OH D_2 and most of the results were very low. Nevertheless, we tested 22 patients and obtained less than a 3% bias with an intercept of 0.6 and a correlation coefficient of 0.992 (Table 3; assay # 2).

Thereafter, we compared the manual PPE method with the 96-well plate PPE method (Sirocco well plates) (Table 3; assays # 3 and 4). The % bias were less than 1% with an excellent correlation (R = 0.9995 for 25-OH D_3 and R = 0.998 for 25-OH D_2).

Replacement of the centrifugation of supernatant step with the Sirocco plates technique improved the sample preparation turnaround time, since the manual PPE method (centrifugation) requires that the supernatant be retransferred into another tube prior to injection. The final set of correlation's studies compared the manual PPE and PPE using filtration on well plates (Table 3; assays # 5 and 6). The regression equations for 25-OH D_3 and 25-OH D_2 were $y = 0.9974x + 0.2344$ (R = 0.997 for 67 patients) and $y = 1.0048x + 0.365$ (R = 0.998 for 48 patients), respectively.

3.4. Precision, accuracy, specificity and recovery

The intra-assay precision testing done with the PPE method without using Sirocco plate showed an excellent coefficient of variation (CV% < 4%) for 25-OH D_2 and 25-OH D_3 at various concentration levels (Table 4). For the inter-assay precision testing (injection of duplicates over 2 weeks), the CV% was estimated to be between 5 and 9% depending on the concentration level and the analyte. Finally, accuracy was evaluated using different levels of the Utak controls and the majority of results with the exception of level 1 control were within 6% of the assigned value (reported target recommended by the company). The level 1 for 25-OH D_3 had a bias of −11.2%.

The precision assays done with the Sirocco 96-well plate technique produced results similar to the centrifugation PPE method. The intra-assay precision had a CV% < 4% for 25-OH D_2 and 25-OH D_3 while the inter-assays precision testing (daily injection of duplicates over 4 weeks) the CV% was between 5 and 7% (Table 5). For accuracy, the estimated bias varied between −3.2 to 6.1%.

Maunsell et al. [22,45] reported the existence of two biologically relevant compounds with similar molecular mass as 25-OH D that are present in biological samples and that could potentially interfere with the assay. Therefore, we examined these two possible interfering compounds: 1α-hydroxyvitamin D_3 and 7α-hydroxy-cholestene-3-one. Our chromatographic procedure clearly separated these two compounds, and 25-OH D_3. The chromatographic peak of 7α-hydroxy-cholestene-3-one came off the column during the washing step while 1α-hydroxyvitamin D_3 never came off the column because the compound was retained in the trapping guard column and was eliminated through the washing procedure.

Finally, recovery of vitamin D metabolites with the trapping technique was evaluated qualitatively by the signal obtained using a sample injection volume ranging from 20 to 100 μL, taking into consideration the variability and complexity of the ion suppression produced by the matrix. It showed linearity up to approximately 80 μL, depending on the matrix effect intensity produced by the sample. The importance of the matrix effect is likely linked to the ion suppression effect produced by compounds such as phospholipids [46,47].

3.5. Selection of MRM transitions

MRM transitions (see Table 1) were selected considering the known problems with possible contaminants produced by a loss of 1 water molecule, i.e. m/z of 18 such as 413/395 (25-OH D_2) and 401/383 (25-OH D_3). We found that the Sirocco well plate produced a contaminant peak with transition 401/383 at the same chromatographic position as 25-OH D_3. Only transitions 401/365 for 25-OH D_3 and 407/372 for 25-OH D_3 d6 (IS2) (loss of 2 water molecules: 36 Da) can be retained for their quantification. Background was also lower with this transition.

Table 3
Method comparison results by linear regression.

Assays	Compared methods (x vs y)[a]	Analyte	Concentration range (nmol/L)	Slope	Intercept	n	r
1	LLE vs PPE with centrifugation	25-OH D_3	6.3–197	0.9657	0.4905	116	0.994
2	LLE vs PPE with centrifugation	25-OH D_2	4–106	0.9846	0.5685	22	0.992
3	LLE vs PPE (filtration on Sirocco 96-well plate)	25-OH D_3	6.3–137	0.9933	0.7134	65	0.9995
4	LLE vs PPE (filtration on Sirocco 96-well plate)	25-OH D_2	4.0–106	1.03	0.3984	17	0.998
5	PPE (centrifugation) vs PPE (filtration on Sirocco)	25-OH D_3	5.5–146	0.9974	0.2344	67	0.997
6	PPE (centrifugation) vs PPE (filtration on Sirocco)	25-OH D_2	4.1–106	1.0048	0.365	48	0.995

[a] See text.

Table 4
Accuracy and precision (intra-assay and inter-assays) obtained for the PPE method combined with centrifugation.

Analyte	Level	Accuracy[a] Expected values, nmol/L; (% bias)	Intra-assay[b] ($n=5$) Mean ± SD, nmol/L; (CV%)	Inter-assays (2 weeks)[c] Mean ± SD, nmol/L; (CV%)
25-OH D_2	L1	95.5 ± 15 (4.3%)	99.5 ± 1.8 (1.8%)	99.6 ± 5 (5%); $n=32$
	L2	235.6 ± 36 (−3.1%)	223.8 ± 6.3 (2.8%)	228.3 ± 18.7 (8.2%); $n=29$
	L3	32.9 ± 5 (4.9%)	32.5 ± 0.4 (1.1%)	34.5 ± 3.1 (8.9%); $n=30$
25-OH D_3	L1	68.9 ± 11 (−11.2%)	59.8 ± 2 (3.4%)	61.2 ± 3.9 (6.3%); $n=31$
	L2	162.5 ± 25 (−5.6%)	162.9 ± 4.7 (2.9%)	153.4 ± 8.5 (5.5%); $n=30$
	L3	27.7 ± 5 (−5.1%)	26.3 ± 0.4 (1.4%)	26.3 ± 2.3 (8.9%); $n=29$

[a] Accuracy was estimated using inter-assays results with recommended targets of Utak controls.
[b] Duplicates of the same sample treated before the injection in the same run.
[c] Injection of duplicate samples over 2 weeks.

Table 5
Accuracy and precision (intra-assay and inter-assays) obtained for the PPE method combined with filtration on Sirocco plate.

Analyte	Level	Accuracy[a] Expected values, nmol/L; (% bias)	Intra-assay[b] ($n=10$) Mean ± SD, nmol/L; (CV%)	Inter-assays (4 weeks)[c] Mean ± SD, nmol/L; (CV%)
25-OH D_2	L1	95.5 ± 15 (6.1%)	95.2 ± 3 (3.1%)	101.3 ± 5.9 (5.9%); $n=16$
	L2	235.6 ± 36 (2.5%)	215.5 ± 4.2 (2%)	241.4 ± 11.2 (4.6%); $n=17$
	L3	32.9 ± 5 (1.8%)	33.5 ± 1.1 (3.2%)	33.5 ± 1.8 (5.3%); $n=17$
25-OH D_3	L1	68.9 ± 11 (−3.2%)	62.3 ± 1.8 (3%)	66.7 ± 4 (5.9%); $n=16$
	L2	162.5 ± 25 (−2.6%)	158.2 ± 5 (3.2%)	158.2 ± 10.4 (6.6%); $n=17$
	L3	27.7 ± 5 (5.8%)	27 ± 0.7 (2.6%)	29.3 ± 1.6 (5.5%); $n=17$

[a] Accuracy was estimated using inter-assays results with recommended targets of Utak controls lot # 2896.
[b] Duplicates of the same sample treated before the injection in the same run.
[c] Injection of duplicate samples over 4 weeks.

We also observed the same potential isobaric contamination reported by Bunch et al. [37], with the transition 401/383 for two compounds with a different molecular mass: 25-OH D_2 d6 (IS1) and 25-OH D_3. However, this phenomenon did not affect our results because our current chromatographic method had enough resolution to separate and identify the contaminant peaks of 25-OH D_2 d6 (IS1) with 25-OH D_3.

3.6. Ions suppression and phospholipids

Phospholipids are known to produce a matrix effect, through ion suppression, i.e. they can decrease the signal intensity for measured analytes if the phospholipids migrate at same position as the analytes during the chromatography [42,46]. Using different MRM transitions that represent a large phospholipids family (see Table 2), we evaluated the impact of the switching valves technique on phospholipids peaks during the chromatography.

As represented in Fig. 3a, injection of a representative sample treated by the PPE method and simple chromatography (no switching valves, no special washing), show many distinct phospholipids' compounds. Using the general transitions 184/184 and 184.1/184.1 (ALL_PC1 and ALL_PC2) with different parameters, we observed that the maximum signal for phospholipids was approximately 1–3 min (position of LYSO1). LYSO2 was also present but an important group of others phospholipids were also detected after the chromatographic peaks of vitamin D: PE, SM, PC1, PC2, PC3 and PC. The use of on-line SPE technique coupled to the switching valves technique eliminated most phospholipids from passing through the column and reducing the between-injection column wash time (see Fig. 3b).

3.7. Robustness of the method

Our LC–MS method using on-line SPE technique was tested over a 12 months period. Simple protein precipitation with acetonitrile combined with filtration on a Sirocco well plate required only 2 h of technical work for the preparation of two 96-well plates with one calibration curve, controls and patient samples. Chromatography of a batch of two full 96-well plates took less than 24 h including analysis of the raw data with the Analyst software and send out of the results to the laboratory information system (LIS) by electronic transfer.

For the practical reasons described below, the ratio of serum:acetonitrile for PPE was modified from 1:1.5 to 1:2. 100 μL of serum could produce 200–250 μL supernatant. Considering the dead volume of injection and loss during protein precipitation, using a 1:2 ratio would result in enough supernatant volume to permit repeat injections despite a small loss of sensitivity.

The chromatographic system ran at a maximum of 3000–3500 psi with a flow of 0.8 mL/min at 35 °C, using a Waters guard column X-Terra 2.1 mm × 20 mm 5 μm C18 with an Sunfire column 2.1 mm × 50 mm 3.5 μm C18. The guard column did not get clogged if it was replaced every 800 injections. With this set up, the column could last for up to 6000–8000 injections with excellent peak shape and separation. According to the manufacturer's maintenance recommendations, the MS detector ESI source needs to be cleaned every week and preventive maintenance done every 6 months. With this protocol the signal intensity is stable for a workload of 600 samples per week.

3.8. Comparison of methods for throughput

Using the off-line sample preparation, the LLE method requires approximately 10 h of manual work to produce two 96-well plate (182 patients) with a calibration curve and controls (see Table 6) whereas our new method using PPE with centrifugation, permits the preparation of the same number of samples in 5 h. Replacing the

Table 6
Estimated working time used to measure 25-OH D on LC–MS/MS with two full 96-well plates (182 patients).

LC–MS technique	LLE (h)	PPE/centrifugation (h)	PPE/filtration (h)
Off-line pre-treatment	10	5	2
LC–MS analysis	17	17	17
Total	27	22	19

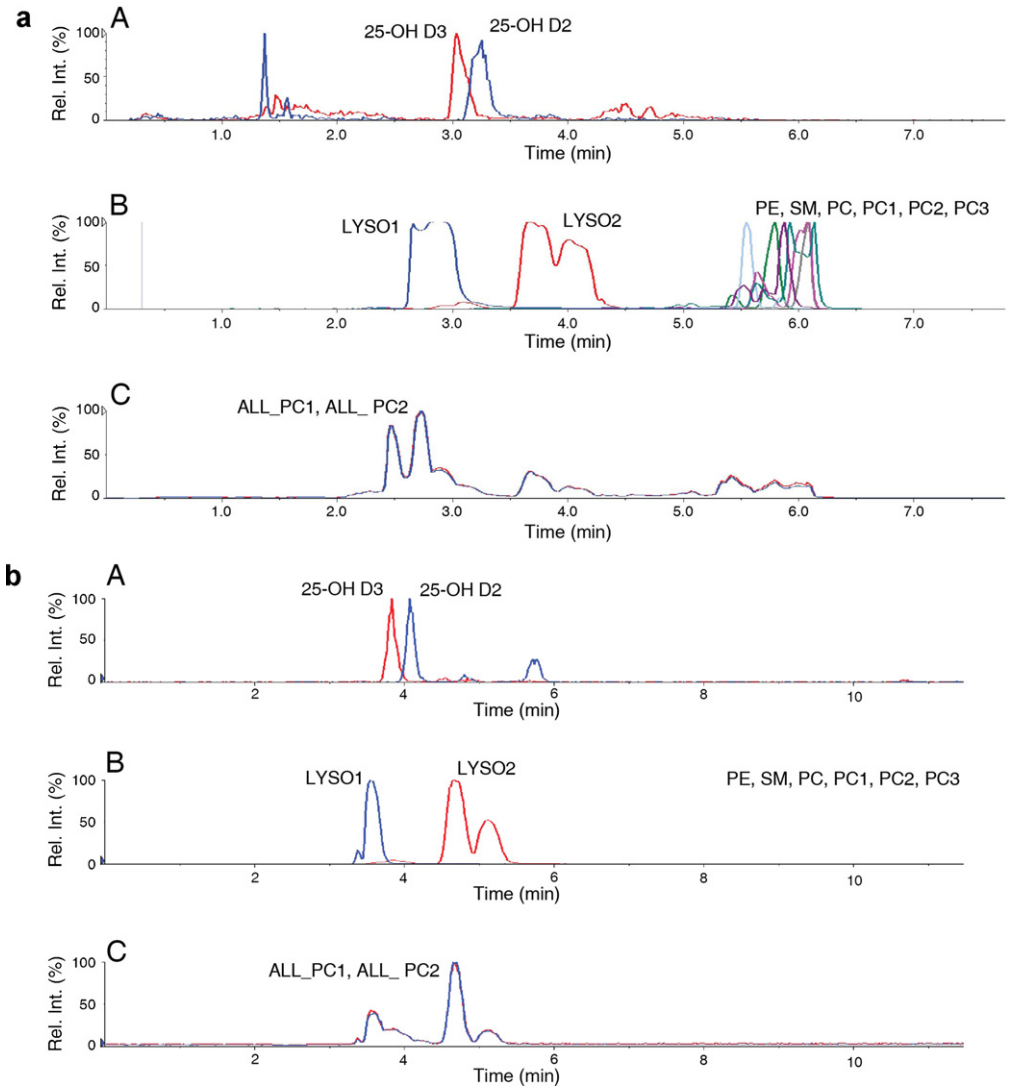

Fig. 3. Chromatograms of serum control with MRM transitions of 25-OH D and different phospholipids (see Tables 1 and 2 for description of MRM transitions). (a) and (b) represent respectively the chromatography of the same sample injected by the chromatographic PPE technique without use of the switching valves system (a) and the chromatographic PPE technique without the diverting valve (valve 2) (b). For this experiment, the chromatographic time was extended with a longer isocratic/washing time and the sample used in (a) was also concentrated following the LLE method to achieve the appropriate injection volume recommended for the column.

centrifugation step with filtration on a well plate further reduces the preparation time to 2 h.

The optimization process with the PPE method using filtration on 96-well plate led to a decrease in the turnaround time (TAT) necessary to produce patient's results by 30%, leading to an estimated throughput of 9.5 samples per hour compared to 6.7 samples per hour for the original LLE method. Despite a 30% decrease in TAT, the major limiting factor remained the off-line pre-treatment of samples since the chromatographic time remains similar for the LLE and PPE methods.

4. Conclusion

Switching from the LLE to the PPE method considerably reduced the technical time for sample preparation required to measure 25-OH D in human serum. Replacement of supernatant centrifugation by filtration using well plates eliminated manual transfer and saved additional time.

Use of the on-line SPE technique allowed faster sample pre-treatment, although the LLE technique was more efficient for removing phospholipids. However, the majority of phospholipids were removed before chromatography and detection using the on-line SPE technique, therefore preventing ions suppression and greatly reducing the contamination of the MS detector.

The use of a 2.1 mm × 20 mm C18 guard column rather than a short column with same configuration or a SPE column greatly decreased the operating costs. Furthermore, addition of a guard column as a trapping column extended the life of the chromatographic column by at least two to three times as compared to simple chromatography.

Use of a direct on-line SPE technique rather than a back-flushing technique, along with a switching valve system also allowed for optimal separation efficiency. 25-OH D_2 and 25-OH D_3 metabolites were well separated and possible problems of isobar contaminations were avoided.

Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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