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Short communication

A rubber transfer gasket to improve the throughput of liquid–liquid extraction in 96-well plates: Application to vitamin D testing

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ABSTRACT

Background: The unmitigated rise in demand for the assessment of vitamin D status has taxed the ability of clinical mass spectrometry laboratories to preserve turn-around times. We aimed to improve the throughput of liquid–liquid extraction of plasma/serum for the assay of 25-hydroxy vitamin D.
Methods: We designed and fabricated a flexible rubber gasket that seals two 96-well plates together to quantitatively transfer the contents of one plate to another. Using the transfer gasket and a dry-ice acetone bath to freeze the aqueous infranatant, we developed a novel liquid–liquid extraction workflow in a 96-well plate format. We applied the technology to the mass spectrometric quantification of 25-hydroxy vitamin D.
Results: Cross-contamination between wells was ≤0.13%. The interassay imprecision over 132 days of clinical implementation was less than 10%. The method compared favorably to a standard liquid–liquid extraction in glass tubes (Deming slope = 1.018, $S_{x|y}$ = 0.022). The accuracy of the assay was 102–105% as assessed with the recently released control materials from NIST.
Conclusions: The development of a plate-sealing gasket permits the liquid–liquid extraction of clinical specimens in a moderate-throughput workflow and the reliable assay of vitamin D status. In the future, the gasket may also prove useful in other sample preparation techniques for HPLC or mass spectrometry.

1. Introduction

There are many features of liquid chromatography–tandem mass spectrometry (LC–MS/MS) that make it an extremely attractive platform for the clinical analysis of small molecules in biological fluids. For example, the sensitive and specific multiplexed detection of analytes adds information over single-analyte assays, which can be clinically beneficial [1]. Sample preparation of serum and plasma for the mass spectrometric measurement of these small molecules can include protein precipitation or liquid–liquid extraction with organic solvent, solid phase extraction, and immunoaffinity purification. Liquid–liquid extraction has a long history of providing excellent separation of analytes from sample matrices. In some situations, it may be superior to solid phase approaches, although the throughput of the workflow is generally limited.

The assay of vitamin D status is particularly well suited to an LC–MS/MS platform and over the past two years clinical laboratories have seen a significantly increased demand for these assays [2]. Vitamin D, which comprises the biochemically and biologically distinct vitamins D_2 and D_3, is a prohormone formed via photolysis of sterols in vertebrates, plants, and fungi. It is converted to 25-hydroxy vitamin D in the liver, which is subsequently converted to 1α,25-dihydroxy vitamin D, or calcitriol, in the proximal tubules of the kidney and other tissues [3,4]. Calcitriol has long been recognized to play an important role in calcium metabolism. But more recent studies that suggest it may also have important roles in cancer [5–7], cardiovascular disease [8,9], and the regulation of inflammation [7,9,10] have helped drive the increased demand for clinical assessment of vitamin D status. The most widely accepted laboratory test to evaluate vitamin D status in patients is serum or plasma 25(OH)D. This is due to the long half-life of 25(OH)D in plasma, compared with calcitriol, the active metabolite (~21 d and 4–6 h, respectively) [3].

Medium-chain alkanes can be used to quantitatively extract vitamin D metabolites from foods, serum, and plasma [11–14]. We

Abbreviations: LC–MS/MS, liquid chromatography–tandem mass spectrometry; 25(OH)D, 25-hydroxy vitamin D; 1α,25(OH)2D, 1α,25-dihydroxy vitamin D or calcitriol; NIST, National Institute of Standards and Technology; $S_{x|y}$, standard deviation of Deming residuals.

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adapted previously described methodologies to include *n*-heptane as the solvent and a freezing step for the separation of organic and infranatant aqueous phases in glass tubes. For improved throughput without detriment to the limit of quantification, it became apparent that the extraction and freezing steps could be carried out in a 96-deep well plate, provided one could quantitatively transfer the organic layer from the extraction plate for evaporation and suspension of the residue. To accomplish the transfer, we designed and manufactured a rubber liquid-transfer gasket. Liquid handling equipment could also be used to transfer a fraction of the organic layer to a new plate. However, this would result in reduced analyte recovery and higher limits of detection due to incomplete recovery of the organic layer. We demonstrate the acceptable performance of the novel transfer gasket in the assay of 25(OH)D in human plasma/serum.

2. Methods and materials

2.1. Construction of 96-well plate liquid-transfer gasket

The process of constructing a gasket is shown schematically in Fig. 1. It started with pouring rubber (Dow Corning 3110 RTV Silicone Rubber with S TIN NW catalyst) into a Plexiglas mold and setting a 2 mL polypropylene 96-deep well collection plate (2 mL well volume, Greiner Bio-One) onto the surface of the rubber, which was allowed to cure for 24 h. The half-gasket was then removed from the plate, turned upside down, and a new liquid layer was poured onto the flat, hardened surface. Another 96-well plate was set down onto the surface of the liquid rubber and allowed to cure for 24 h. The gasket, now identical on the two faces, was removed from the mold and 96 holes were bored with a sharp borer (Fig. 1).

2.2. Sample preparation

Patient serum or plasma, calibrators, and controls (200 μL) were alkalinized with 200 μL 1 N sodium hydroxide in a 96-deep well plate, covered with a silicone cover (MicroMat, Varian) vortexed for 15 s, and incubated at room temperature for 15 min. Internal standard [200 μL; 121.3 nmol/L (50 ng/mL) 25(OH)D_2 and 25(OH)D_3 in methanol] was added to each well and the plates were covered and vortexed for 15 s. Samples were extracted with 1 mL *n*-heptane (5 min vortex, covered) and the plates were centrifuged at 1100 × *g* for 4 min at room temperature in a Beckman Allegra X-22 centrifuge equipped with a 96-well plate rotor. Next, a transfer gasket and another 96-deep well plate were fitted on top of the extraction plate. The sealed plates, held together by the gasket, were then placed in a dry-ice acetone bath for 50 min to freeze the lower aqueous layer. The entire organic layer was then transferred to the new plate by inverting and gently tapping the assembly on the benchtop. The extraction plate was removed and discarded. The transfer gasket could be washed and reused 20 times before the edges of the gasket that fit into the wells began to crack. The extracts were dried under forced nitrogen at room temperature (Turbovap) and the residue was reconstituted in 200 μL 75% methanol in water.

2.3. Liquid chromatography–tandem mass spectrometry

A portion of the dissolved extracts (40 μL) were injected and developed using standard reverse phase chromatography (XTerra C18, 2.1 × 50 mm, 3.5 μm column with XTerra MS C8, 2.1 × 10 mm, 3.5 μm guard column) with isocratic mobile phase (2 mM ammonium acetate, 0.1% formic acid in 95:5 methanol:water, 0.2 mL/min) and analyzed using isotope dilution-tandem mass spectrometry (Quattro Micro with a 2795 HPLC, Waters). The transitions analyzed were (analyte, precursor > product, cone voltage, collision energy): 25(OH) D_3-d_6 IS, 407.3 > 371.3, 35, 20; endogenous 25(OH) D_3, 401.3 > 365.3, 15, 12; 25(OH) D_2-d_6 IS, 419.3 > 355.3, 18, 12; endogenous 25(OH) D_2, 413.3 > 355.3, 15, 10. The dwell time for each analyte was 200 ms and the delay between transitions was 30 ms. The cycle time from injection to injection was 3 min.

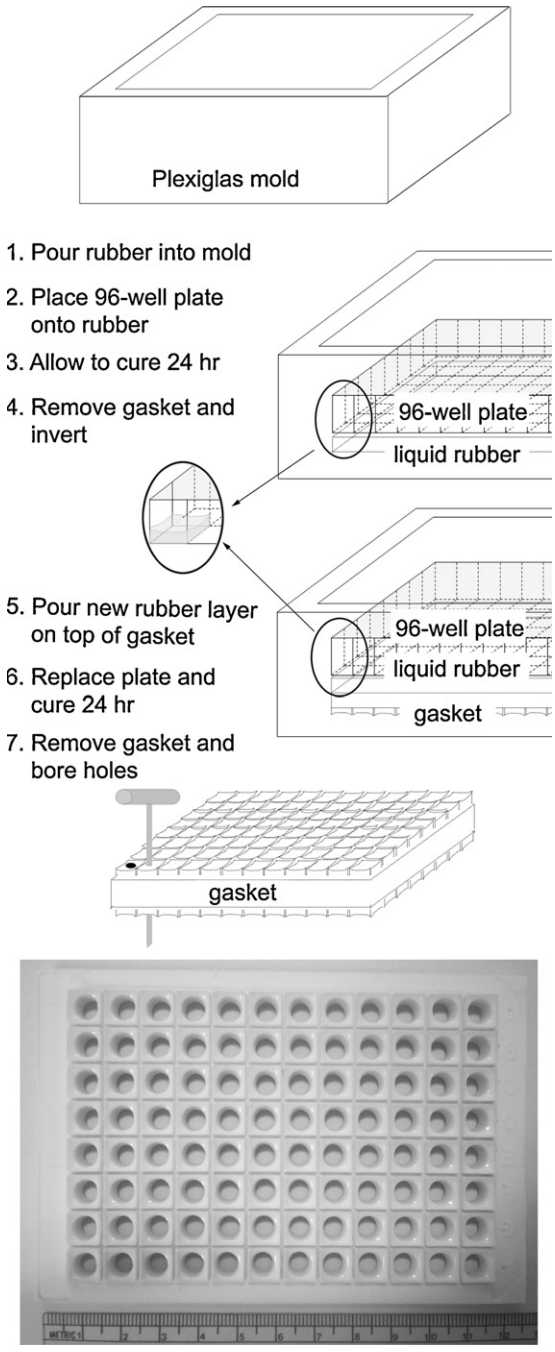

Fig. 1. Schematic presentation of the construction of a 96-well plate liquid-transfer gasket. A Plexiglas mold holds the liquid rubber in place as it is allowed to cure around the openings of a 96-well plate. The rubber rises into the plate openings by capillary action (inset). An example gasket is shown at the bottom of the schematic.

2.4. Method comparison

Comparison was made with an extraction procedure in borosilicate glass tubes as previously described [13] and modified to include the alkalinization, freezing steps, and internal standards described above. Deming regression was performed in the R statistical programming language [15] using the MethComp package.

Table 1
Operating characteristics of a moderate-throughput vitamin D assay using a 96-well plate-transfer gasket[a].

		25(OH)D_2		25(OH)D_3	
		Concentration	%CV	Concentration	%CV
Intraassay imprecision[b]		34.2 (14.1)	2.82%	23.0 (9.5)	2.89%
		86.3 (35.6)	3.35%	61.6 (25.4)	3.25%
Interassay imprecision[c]		37.6 (15.5)	6.18%	25.2 (10.4)	9.63%
		90.5 (37.3)	6.07%	68.6 (28.3)	8.40%
Limit of quantification[d]		0.24 (0.10)		0.78 (0.32)	
Cross-contamination[e]		0.03% (0–0.10%)		0.07% (0–0.13%)	
Recovery[f]		102.7% (94–115%)		98.2% (80–116%)	
		25(OH)D_2		25(OH)D_3	
		Observed[g]	Expected	Observed[g]	Expected
Accuracy	Level 1	<2.4 (<1.0)	<2.4 (<1.0)	62.8 (25.9)	59.6 (23.9)
	Level 2	4.1 (1.7)	4.1 (1.7)	32.7 (13.5)	30.8 (12.3)
	Level 3	64.3 (26.5)	64.1 (26.4)	47.8 (19.7)	46.2 (18.5)
	Level 4	6.8 (2.8)	5.8 (2.4)	202.5 (83.5)	82.3 (33.0)

[a] Concentrations are presented as nmol/L (ng/mL). Imprecision presented as the coefficient of variation (%CV).
[b] Results are calculated from 20 samples run on the same day in the same 96-well plate.
[c] Results are calculated from two samples, one each with high or low 25(OH)D_2 and 25(OH)D_3 for 132 consecutive days of running the assay.
[d] Limit of quantification is defined as the concentration at which the intraassay imprecision was 20% by interpolation/extrapolation from a dilution series. Clinically, samples are not reported below 2.4 nmol/L (1.0 ng/mL).
[e] Cross-contamination was determined by surrounding two samples containing high concentrations of 25(OH)D_2 and 25(OH)D_3 (727.6 nmol/L, 300 ng/mL each) with 10 wells containing 2% BSA in 0.9% saline. Average carryover is calculated from the 10 wells and the range is presented in parentheses.
[f] Ten grossly lipemic specimens were spiked with 24.3 nmol/L (10 ng/mL) 25(OH)D_2 and 25(OH)D_3. Total cholesterol ranged 3.60–6.68 mmol/L (139–258 mg/dL), triglycerides 3.8–8.1 mmol/L (335–716 mg/dL), and HDL cholesterol 0.62–1.06 mmol/L (24–41 mg/dL). There was no correlation between recovery of 25(OH)D_2 or 25(OH)D_3 with any lipid parameters except for an inverse correlation between recovery of 25(OH)D_2 and non-HDL cholesterol ($r^2 = 0.56$; $p = 0.013$). Average recovery is presented with the observed range (in nmol/L) in parentheses.
[g] Mean results from three independent analyses of each NIST control sample are presented.

Standard error of the estimate was calculated using 1000 boot-strap iterations. The variance ratio for the methods was set to 1.

3. Results and discussion

3.1. Assay characteristics

The operating characteristics of the 96-well plate liquid–liquid extraction–LC–MS/MS assay of 25(OH)D_2 and 25(OH)D_3 are presented in Table 1. Representative chromatograms are presented in Fig. 2A. The method had acceptable limits of quantification for both analytes, which scaled linearly with sample volumes down to 50 μL (data not shown). In routine clinical practice over three months, the interassay imprecision was <10% for both analytes at concentrations within and below the normal range. Although leakage of sample around the gasket with resulting inter-well contamination was an initial concern when developing the sealing gasket, the cross-contamination was acceptably low (≤0.13%; see footnote of Table 1 for method of determination). To test recovery, we used lipemic samples, which are most likely to interfere with extraction of lipophilic molecules and interfere with mass spectral analysis. Average recoveries were 102.7% for 25(OH)D_2 and 98.2%

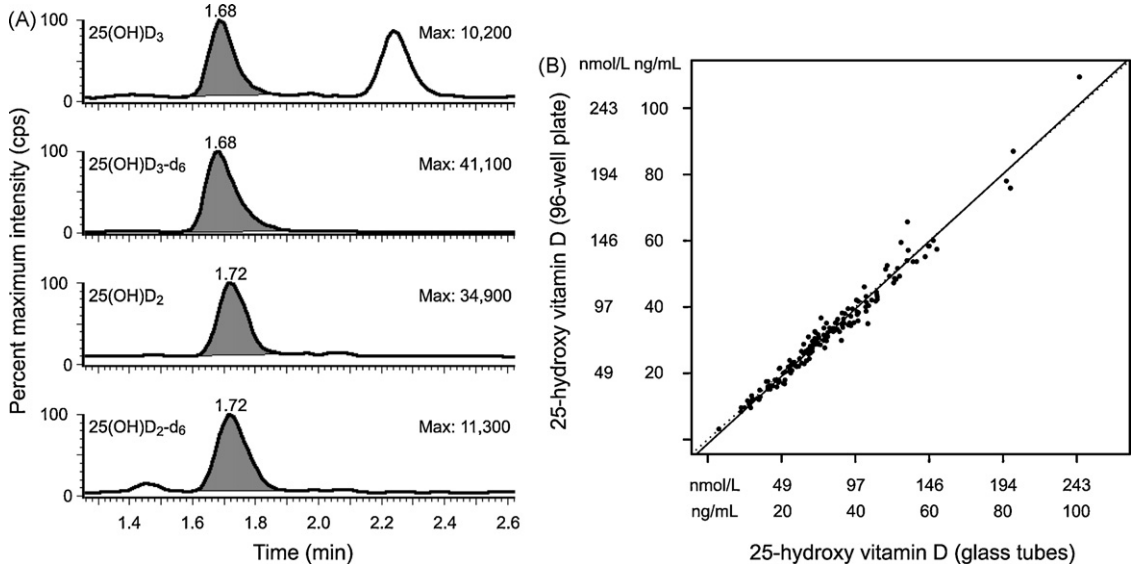

Fig. 2. Representative chromatograms and method comparison of the 96-well plate assay. (A) Representative chromatograms from a patient with 25.2 nmol/L 25(OH)D_3 (10.4 ng/mL) and 13.1 nmol/L 25(OH)D_2 (5.4 ng/mL) are presented for each analyte as percent maximal intensity. Retention times are labeled. (B) Over the course of four days, samples (total $N = 161$) were extracted using both a 96-well plate and a borosilicate glass tube workflow. Total vitamin D concentrations [25(OH)D_2 + 25(OH)D_3] are presented.

for 25(OH)D_3. We also compared the novel 96-well plate assay format with a traditional liquid–liquid extraction using *n*-heptane in glass tubes and found good agreement with a slope of the Deming regression of 1.018 and a standard error of the estimate ($S_{x|y}$) of 0.022 (Fig. 2B).

3.2. Comparison with NIST controls

To assess the accuracy of the method employing the 96-well gasket, we took advantage of control materials recently released by the National Institute of Standards and Technology (NIST) to help standardize assays across laboratories [16]. Four levels of controls were manufactured and contained different concentrations of 25(OH)D_2, 25(OH)D_3, and the C-3 epimer of 25(OH)D_3. It was important to have control materials that contained 25(OH)D_2 and the epimer of 25(OH)D_3 because these molecules have different biological and clinical properties. For example, the C-3 epimer of 25-hydroxy vitamin D_3 has reduced effects on calcium flux in cell culture experiments [17] and vitamin D_2 has a distinct therapeutic profile from vitamin D_3 [18]. The expected concentrations for 25(OH)D_2 and 25(OH)D_3 for each of the four levels of control material are listed in Table 1.

Measured concentrations of 25(OH)D_2 for Levels 2–4 were on average 104.8% of those expected. The amount of 25(OH)D_2 in Level 1 was below the clinically reportable range. There was a small contribution of the C-3 epimer to the total 25(OH)D_3 concentration in Levels 1–3 (~5.7% of total 25(OH)D_3), for which our assay detected 108.3% of the expected values (Table 1). If the expected concentration of 25(OH)D_3 was instead taken to be the sum of the native 25(OH)D_3 and its epimer, we measured on average 102.2% of the expected values for Levels 1–3 (data not shown). For control Level 4, into which a significant amount of epimer was spiked (94.2 nmol/L; 37.7 ng/mL), the accuracy of the assay improved from 253% to 118% if the epimer was included in the calculation (data not shown). Because LC–MS/MS methods do not typically resolve native 25(OH)D_3 and its epimer (Ref. [19] and unpublished observations), the presence of the epimer in normal adult serum (from which the control materials were derived) could, in part, explain the reported positive bias of LC–MS/MS methods when compared with traditional radioimmunoassays [20].

3.3. Conclusions

We designed, manufactured, and employed a novel rubber 96-well plate liquid transfer gasket to improve the throughput of liquid–liquid extraction of serum/plasma samples for the clinical measurement of 25(OH)D, the most important marker of vitamin D status. The assay had acceptable operating characteristics, which were comparable to or better than previously published LC–MS/MS methods [12,13,21], and was accurate when assessed with NIST reference materials [16]. There was no significant cross-contamination between wells.

The transfer gasket has potential applications in other liquid–liquid extractions that need to be adapted to moderate-throughput workflows. The type of inert rubber used resulted in a highly flexible mat providing an airtight seal between two 96-well plates. Using the Plexiglas mold, the gasket can be constructed to fit any commercially available plates. The ability to transfer supernatants from one plate to another without significant loss could be an important step in many future HPLC and LC–MS/MS sample preparation methodologies and represents an important alternative to liquid handling stations for moderate-throughput laboratories.

Conflict of interests

A.N. Hoofnagle has served as a consultant to Thermo-Fisher Scientific.

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