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Journal

Journal of Lipid Research, 58(4)

ISSN

0022-2275

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Publication Date

2017-04-01

DOI

10.1194/jlr.d073536

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A new sensitive LC/MS/MS analysis of vitamin D metabolites using a click derivatization reagent, 2-nitrosopyridine^S

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Abstract There is an increased demand for comprehensive analysis of vitamin D metabolites. This is a major challenge, especially for 1 α ,25-dihydroxyvitamin D [1 α ,25(OH)₂VitD], because it is biologically active at picomolar concentrations. 4-Phenyl-1,2,4-triazoline-3,5-dione (PTAD) was a revolutionary reagent in dramatically increasing sensitivity of all diene metabolites and allowing the routine analysis of the bioactive, but minor, vitamin D metabolites. A second generation of reagents used large fixed charge groups that increased sensitivity at the cost of a deterioration in chromatographic separation of the vitamin D derivatives. This precludes a survey of numerous vitamin D metabolites without redesigning the chromatographic system used. 2-Nitrosopyridine (PyrNO) demonstrates that one can improve ionization and gain higher sensitivity over PTAD. The resulting vitamin D derivatives facilitate high-resolution chromatographic separation of the major metabolites. Additionally, a liquid-liquid extraction followed by solid-phase extraction (LLE-SPE) was developed to selectively extract 1 α ,25(OH)₂VitD, while reducing 2- to 4-fold ion suppression compared with SPE alone. ^SLLE-SPE followed by PyrNO derivatization and LC/MS/MS analysis is a promising new method for quantifying vitamin D metabolites in a smaller sample volume (100 μ L of serum) than previously reported methods. The PyrNO derivatization method is based on the Diels-Alder reaction and thus is generally applicable to a variety diene analytes.—Wan, D., J. Yang, B. Barnych, S. H. Hwang, K. S. S. Lee, Y. Cui, J. Niu, M. A. Watsky, and B. D. Hammock. A new sensitive LC/MS/MS analysis of vitamin D metabolites using a click derivatization reagent, 2-nitrosopyridine. *J. Lipid Res.* 2017. 58: 798–808.

Supplementary key words 1,25-dihydroxyvitamin D • major vitamin D metabolites • matrix effect • smaller sample volume • quantification

This work was supported by the National Institutes of Health/National Eye Institute grant R01 EY021747 (to M.A.W.), National Institute of Environmental Health Sciences (NIEHS) grant R01 ES002710, NIEHS Superfund Research Program grant P42 ES004699 (to B.D.H.), the West Coast Metabolomics Center at UC Davis (National Institutes of Health/National Institute of Diabetes and Digestive and Kidney Diseases grant U24 DK097154), and National Institutes of Health/NIEHS grant 1K99ES024806-01 (to K.S.S.L.).

Manuscript received 22 November 2016 and in revised form 23 January 2017.

Published, *JLR Papers in Press*, February 1, 2017
DOI 10.1194/jlr.D073536

In 1943, vitamin D was recommended as an essential nutrient for the US public. People can acquire the precursors to the bioactive forms, vitamin D₃ and vitamin D₂, from diet, supplements, and sunlight. In the liver, both forms of vitamin D are rapidly hydroxylated to corresponding 25-hydroxyvitamin D [25(OH)VitD]. Being a predominant metabolite, circulating 25(OH)VitD serves as the preferred surrogate biomarker of vitamin D nutritional status (1, 2). Low levels of 25(OH)VitD indicate low vitamin D nutritional intake, which is related to many diseases, such as osteomalacia, multiple sclerosis, Parkinson's disease, asthma, and other health risks, including muscle weakness, diabetes, and cognitive decline or Alzheimer's disease (3–7). Consequently, analytical techniques for measurement of 25(OH)VitD have been developed (8–12). In the kidney, 25(OH)VitD is further hydroxylated to 1 α ,25-dihydroxyvitamin D [1 α ,25(OH)₂VitD]. 1 α ,25(OH)₂VitD is the major biologically active metabolite and plays an important role in decreasing the risk of many diseases, including common cancers, cardiovascular disease, autoimmune disease, and chronic kidney disease (13–17). Most recently, we have demonstrated that 1 α ,25(OH)₂VitD also appears to be important for the health of the eye (18). The influence that these biologically active forms of vitamin D have on these numerous diseases and associated organ systems

Abbreviations: 1 α ,25(OH)₂VitD, 1 α ,25-dihydroxyvitamin D; 25(OH)VitD, 25-hydroxyvitamin D; 1 α ,25(OH)₂VitD_{3-d6}; 26,26,26,27,27-hexadeuterium-1 α ,25(OH)₂VitD₃; 24R,25(OH)₂VitD₃; 24R,25-dihydroxyvitamin D₃; 25(OH)VitD_{3-d6}; 26,26,26,27,27-hexadeuterium-25(OH)VitD₃; ALTm, all laboratory trimmed mean; CUDA, 12-(3-cyclohexyl-ureido)-dodecanoic acid; DEQAS, Vitamin D External Quality Assessment Scheme; LLE-SPE, liquid-liquid extraction followed by solid-phase extraction; LLOQ, lower limit of quantification; MRM, multiple reaction monitoring; PTAD, 4-phenyl-1,2,4-triazole-3,5-dione; PyrNO, 2-nitrosopyridine; QC, quality control.

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^SThe online version of this article (available at <http://www.jlr.org>) contains a supplement.

has significantly increased scientific interest in their quantitative analysis (19–25).

Among the numerous analytical measurement techniques, Food and Drug Administration-approved DiaSorin RIA and Liaison assays (DiaSorin, Stillwater, MN) are the most widely used for quantifying 25(OH)VitD and other nonhydroxylated vitamin D metabolites, but do not include $1\alpha,25(\text{OH})_2\text{VitD}$. Furthermore, the accuracy and precision of these assays are unsatisfactory (26–28). Therefore, the National Health and Nutritional Examination Survey recommended liquid chromatography tandem mass spectrometry (LC/MS/MS) as the best method for quantifying vitamin D metabolites due to improved sensitivity, accuracy, and reproducibility (29). The 25(OH)VitD₃ is present at relatively high levels (50–125 nM) in human serum and thus can be accurately quantified by LC/MS/MS (30). Additionally, a 25(OH)VitD test is included in the Centers for Disease Control and Prevention Vitamin D Standardization-Certification Program, set up to assure accurate and reliable clinical measurements. By contrast, the $1\alpha,25(\text{OH})_2\text{VitD}_3$ is challenging to directly quantify using LC/MS/MS. One challenge associated with $1\alpha,25(\text{OH})_2\text{VitD}_3$ quantification is that its human serum concentration ranges from 35 to 150 pmol/l (14–60 pg/ml), approximately 1,000-fold lower than 25(OH)VitD₃ (31). This serum concentration is significantly lower than the general instrumental detection limit of conventional LC/MS/MS methods. Second, the poor ionization efficiency of $1\alpha,25(\text{OH})_2\text{VitD}_3$ further weakens the advantage of LC/MS/MS methods. Finally, less-than-optimal sample preparation techniques may cause high ion suppression and loss of the targeted analytes. To overcome these challenges, accurately measuring $1\alpha,25(\text{OH})_2\text{VitD}$ requires both a superior sample preparation method and a selective derivatization reagent to achieve good recovery and increased ionization efficiency, respectively. In previous studies, solid-phase extraction (SPE) sample preparation followed by 4-phenyl-1,2,4-triazole-3,5-dione (PTAD) derivatization proved to be a relatively more comprehensive, accurate, and sensitive method for quantitative analysis of major vitamin D metabolites (21, 22, 32). However, it is still difficult to quantify the low endogenous levels of $1\alpha,25(\text{OH})_2\text{VitD}$ in small sample volumes. In an attempt to further increase the method sensitivity, new effective dienophiles, 4-(4'-dimethylaminophenyl)-1,2,4-triazoline-3,5-dione; 4-[2-(3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxo-2-quinoxalanyl)ethyl]-3H-1,2,4-triazole-3,5(4H)-dione; and other “triazoline-diones,” have been developed (25, 33–37). Among them, the positively charged derivatization reagent, Amplifex-Diene and SecoSET, are reported to have a 10-fold ionization efficiency improvement over PTAD (25, 36, 38). Compared with PTAD, the SecoSET and Amplifex-Diene reagents are commonly used to detect 25(OH)VitD and $1\alpha,25(\text{OH})_2\text{VitD}$, respectively. However, they are more challenging for comprehensive profiling of vitamin D metabolites. It is acknowledged that the size and the fixed charge of derivatization reagents dramatically change the chromatography and can lead to poor separation of the derivatized vitamin D metabolites (25). In addition, some

vitamin D derivatives will share the same MS product ion (25, 38), which may cause a cross-interference for the poor separating analytes {for example, 24R,25-dihydroxyvitamin D₃ [$24\text{R},25(\text{OH})_2\text{VitD}_3$] and $1\alpha,25(\text{OH})_2\text{VitD}_3$ }. PTAD is therefore the common choice for comprehensive quantification of vitamin D metabolites. 2-Nitrosopyridine (PyrNO), a click or Diels-Alder derivatization reagent and an excellent dienophile, offers the enhanced sensitivity of the SecoSET and Amplifex reagents, while retaining the excellent chromatographic properties.

Besides the choice of derivatization reagent, the accurate detection of vitamin D metabolites may be improved by optimizing the sample extraction method to decrease the ion suppression. For example, using immunoextraction proved to be more superior than SPE for purifying $1\alpha,25(\text{OH})_2\text{VitD}$ from interfering compounds, where a lower limit of quantification (LLOQ) for $1\alpha,25(\text{OH})_2\text{VitD}$ (3 pg/ml) was dramatically improved after PTAD derivatization (23, 24). However, the immunoextraction method was developed only for $1\alpha,25(\text{OH})_2\text{VitD}$ and did not include other metabolites, 25(OH)VitD and $24\text{R},25(\text{OH})_2\text{VitD}$. These metabolites are also important to quantify, because the $24\text{R},25(\text{OH})_2\text{VitD}$ has proved to be a critical component for healing processes in tissues and bones, and knowing the levels of both 25(OH)VitD and $24\text{R},25(\text{OH})_2\text{VitD}$ is useful for epidemiological studies (35). In addition, immunoextraction required a relatively large sample volume (400–500 μl) compared with other methods. This suggests that a more sensitive and comprehensive method for quantification of major vitamin D metabolites will have value.

For this study, we developed a new method for quantification of vitamin D metabolites using liquid-liquid extraction followed by solid-phase extraction (LLE-SPE) and PyrNO as a derivatization reagent. The method combines both LLE and SPE to reduce the serum ion suppression of dihydroxyvitamin D. Organocnitroso compounds and their rich chemistry have been studied for more than 60 years, and broad applications have been found in organic synthesis, as derivatization reagents for drug discovery and other applications (39–42). To the best of our knowledge, their utility for improving ionization efficiency has not been explored. The PyrNO reagent contains a reactive dienophile and results in better multiple reaction monitoring (MRM) signal intensity than the previously reported PTAD. As a result, the combination of the LLE-SPE and derivatization procedure followed by LC/MS/MS analysis can accurately quantify the major vitamin D metabolites from a small sample volume (100 μl). For example, we quantify $1\alpha,25(\text{OH})_2\text{VitD}$; 24R, 25(OH)₂VitD; and 25(OH)VitD in 100 μl of serum.

MATERIALS AND METHODS

Chemicals and reagents

The $1\alpha,25(\text{OH})_2\text{VitD}_3$, $1\alpha,25(\text{OH})_2\text{VitD}_2$, 25(OH)VitD₂, and 25(OH)VitD₃ were purchased from Sigma-Aldrich (St. Louis, MO). The $24\text{R},25(\text{OH})_2\text{VitD}_3$ was from BIOMOL (Plymouth Meeting, PA). Deuterated surrogates of vitamin D metabolites,

$1\alpha,25(\text{OH})_2\text{VitD}_3\text{-}d_6$ and $1\alpha,25(\text{OH})_2\text{VitD}_2\text{-}d_6$, were from Medical Isotopes (Pelham, NH), and $25(\text{OH})\text{VitD}_3\text{-}d_6$ was from Synthetica (Oslo, Norway). Pooled human serum was prepared in our laboratory. The 12-(3-cyclohexyl-ureido)-dodecanoic acid (CUDA) was synthesized in our laboratory. Hexane (mixture of isomers), acetonitrile, methanol, glacial acetic acid, and potassium hydrogen phosphate (K_2HPO_4) were purchased from Fisher Scientific (Pittsburgh, PA). Deionized water (18.1 M Ω /cm) was prepared in-house and used for mobile phase preparation and SPE. Oasis HLB 1-ml (30 mg of sorbent) SPE cartridges were purchased from Waters Corp. (Milford, MA). PyrNO was synthesized in the laboratory (see supplemental data).

Stock and calibration solutions

Calibration solutions of $1\alpha,25(\text{OH})_2\text{VitD}_3$ and $1\alpha,25(\text{OH})_2\text{VitD}_2$ were prepared in 4% BSA in PBS buffer at levels of 0.01, 0.02, 0.05, 0.1, 0.5, 1, 5, 10, 20, and 50 nM. Similarly, calibration solutions of $24\text{R},25(\text{OH})_2\text{VitD}_3$, $25(\text{OH})\text{VitD}_3$, and $25(\text{OH})\text{VitD}_2$ were at the levels of 0.2, 0.4, 1, 2, 10, 20, 100, 200, 400, and 1,000 nM. The internal standard solution was prepared in methanol containing 20 nM $1\alpha,25(\text{OH})_2\text{VitD}_3\text{-}d_6$, 20 nM $1\alpha,25(\text{OH})_2\text{VitD}_2\text{-}d_6$, and 200 nM $25(\text{OH})\text{VitD}_3\text{-}d_6$. Analysis of $24\text{R},25(\text{OH})_2\text{VitD}_3$ and $25(\text{OH})\text{VitD}_2$ relied on $1\alpha,25(\text{OH})_2\text{VitD}_3\text{-}d_6$ and $25(\text{OH})\text{VitD}_3\text{-}d_6$ as internal standards for quantification, respectively.

Sample preparation

The stock standard solution (50 μL) or serum/plasma samples (100 μL) were spiked with an internal standard solution (10 μL), mixed, and incubated for 30 min. After that, two LLE and one SPE methods were used in vitamin D extraction. The first LLE method was involved in protein precipitation. The vitamin D metabolites were released from the binding protein with the addition of 600 μL methanol/acetonitrile (80/20), followed by mixing with a Vortex® and centrifugation at 20,000 g (Eppendorf centrifuge 5804R) for 10 min.

The supernatant from protein precipitation was transferred into 2-ml microcentrifuge tubes, followed by the addition of 600 μL of 0.4 M K_2HPO_4 . Then, 600 μL hexane was added for another LLE. The tubes were mixed for 5 min and centrifuged for 5 min at 20,000 g. As a result, all dihydroxy vitamin D metabolites [$1\alpha,25(\text{OH})_2\text{VitD}$ and $24\text{R},25(\text{OH})_2\text{VitD}_3$] remained in the aqueous phase. Therefore, the aqueous layer [$1\alpha,25(\text{OH})_2\text{VitD}$ and $24\text{R},25(\text{OH})_2\text{VitD}_3$] was further processed by SPE as described in supplemental data. The hydroxyvitamin D metabolites [$25(\text{OH})\text{VitD}$] were extracted to the hexane phase. Finally, the hexane layer [$25(\text{OH})\text{VitD}$] was transferred and evaporated on an RC10.22 vacuum concentrator (Jouan, Winchester, VA). The two dried samples, hydroxyvitamin D and dihydroxyvitamin D, were derivatized with PyrNO and analyzed separately.

Derivatization

PyrNO in methanol (2.5 mM, 40 μL) was added, and it redissolved the dried samples. Samples were subsequently transferred into an amber glass vial and were heated in the sand bath at 70°C for 1 h to complete derivatization. These samples were subsequently mixed with CUDA (10 μL , 200 nM), transferred to HPLC vials with 100 μL volume inserts, and stored at -20°C until analysis. Here, CUDA was added to account for instrument variability.

LC/MS/MS analysis

Separation of vitamin D metabolites was performed on an Agilent 1200 SL Liquid Chromatography series (Agilent Corp., Palo Alto, CA). The samples were kept in the autosampler at 4°C, and 10 μL samples were injected on the column. The Eclipse Plus C18 2.1 \times 150 mm 1.8 μm column (Agilent) was kept at 50°C. Mobile

phase A was water with 0.1% glacial acetic acid. Mobile phase B consisted of acetonitrile with 0.1% glacial acetic acid. Gradient elution was performed at a flow rate of 250 $\mu\text{L}/\text{min}$. Chromatography was optimized to separate all analytes from each other and the chief interferents in 13.0 min. The HPLC gradient is given in supplemental Table S1. The optimized instrument parameters are given in supplemental Table S2. The analytes were quantified by using a 4000 Q-Trap tandem mass spectrometer (Applied Biosystems Instrument Corp., Foster City, CA) equipped with an electrospray source (Turbo V), operating in a positive MRM mode. The LC/MS/MS conditions are described in Materials and Methods and supplemental Tables S1 and S2.

Derivatization and extraction method comparison

The improved ionization after PyrNO derivatization was evaluated by comparing MS signals between PyrNO- $1\alpha,25(\text{OH})_2\text{VitD}_3$ and PTAD- $1\alpha,25(\text{OH})_2\text{VitD}_3$ in the MRM mode. The sensitivity of the LLE-SPE method was also compared with that from SPE alone. Finally, the PyrNO method was compared with the PTAD method by using the same pooled serum samples.

Method validation: calibration and sensitivity

LLOQ in PBS (Table 1) was the minimum concentration giving a ratio of signal to noise (S/N) > 10 in 4% BSA in PBS buffer. To calculate the LLOQ in serum samples, known concentrations of five vitamin D metabolites were diluted with 4% BSA in PBS buffer and tested by using our analysis protocol.

Recovery

Analytical recovery was determined by spiking both 10 μL $1\alpha,25(\text{OH})_2\text{VitD}_3\text{-}d_6$ (20 nM) and 10 μL $25(\text{OH})\text{VitD}_3\text{-}d_6$ (200 nM) into 100 μL pooled human serum before each step in the extraction method, i.e., protein precipitation (precrash), LLE (preLLE), SPE (preSPE), vacuum dry (predry), and postvacuum dry (postdry). Recoveries were calculated by dividing the peak areas of precrash, preLLE, preSPE, and predry by the peak areas of postdry.

Accuracy and precision

Method accuracy and precision were established by analyzing quality control (QC) samples. The QC samples included spiked samples in BSA prepared in PBS buffer and fortified QC samples, prepared by spiking standard compounds in pooled human serum. For intraday accuracy, four extraction replicates of each sample were analyzed within 24 h to provide percent difference between the measured mean and the expected concentration. For interday accuracy, each sample was tested to show percent difference over 3 days. The coefficient of variation provided the precision.

Matrix effect

Three serum samples with deuterated internal standards spiked at three different concentration levels [$1\alpha,25(\text{OH})_2\text{VitD}_3\text{-}d_6/1\alpha,25(\text{OH})_2\text{VitD}_2\text{-}d_6/25(\text{OH})\text{VitD}_3\text{-}d_6$: set 1, 5/5/25 nM; set 2, 10/10/50 nM; and set 3, 20/20/100 nM] were prepared. The potential matrix effect present in human serum was calculated by dividing the response of IS spiked in methanol with the response of internal standards spiked in the pooled human serum (43). The absolute ion suppression caused by matrix was given by 100% minus the obtained value of matrix effect.

Verification of the developed method by Vitamin D External Quality Assessment Scheme samples

Twenty human serum samples (code nos. 456-465 and 346-355) from the Vitamin D External Quality Assessment Scheme (DEQAS)

QC program were selected to test the reliability of our method for analysis of 25(OH)VitD₃ and 1 α ,25(OH)₂VitD. All the samples were analyzed by the newly established method in quadruplicate. The known all laboratories trimmed mean (ALTM) values were compared with the resulting values obtained from our method.

RESULTS

Derivatization and LC/MS/MS analysis

VitD₂ was chosen as a representative conjugated diene to first test the click or Diels-Alder reaction with PyrNO. NMR, Fourier transform-infrared spectroscopy, and MS were used for product characterization and structure elucidation (see supplemental data). The reaction between PyrNO and VitD₂ is illustrated in Fig. 1. The reaction was then optimized for 1 α ,25(OH)₂VitD₃ because it was the analyte of the lowest endogenous abundance. Several rounds of optimization were conducted. The best reaction solvent was found to be methanol (supplemental Fig. S1A). This reaction was optimized by using a surrogate solution containing 2.0 nM 1 α ,25(OH)₂VitD₃ in methanol and by varying the following conditions: reaction temperature, reaction time, and PyrNO concentration (supplemental Fig. S1B–D). The best yield and consistency were obtained with 2.5 mM PyrNO at 70°C for 1 h. When 1 μ M vitamin D metabolites were derivatized by 2.5 mM PyrNO, there were no remnant vitamin D metabolites (limit of detection = 5 nM) that could be detected. The derivatization yield was therefore assumed to be near 100%.

The stability of the derivatized 1 α ,25(OH)₂VitD₃ was tested by storing it at -80°C , -20°C , 4°C , and room temperature (RT) over 1 week. Supplemental Fig. S2 shows that, in contrast to 15% loss at 4°C and 20% loss at RT after 1 week when exposed to ambient light, there was no significant analyte loss at -20°C and -80°C . These data on the short-time stability of PyrNO-1 α ,25(OH)₂VitD₃ under various conditions (supplemental Fig. S3) reveal that the main reason behind degradation regardless of temperature is the light exposure. This result is consistent with previous report on light sensitivity of 1 α ,25(OH)₂VitD₃ (22). Therefore, all derivatized samples were stored below -20°C and less than 1 week before analysis. Derivatization was carried out in the dark.

Figure 2A shows the major MS peak at m/z 525.4 and minor MS peak at m/z 547.6, corresponding to $[\text{M} + \text{H}]^+$ and $[\text{M} + \text{Na}]^+$ of PyrNO-1 α ,25(OH)₂VitD₃, respectively, in the direct-infusion full-scan mass spectrum. To avoid formation of sodiated ions, 0.1% glacial acetic acid was added in the HPLC mobile phase. The product ion scan of $[\text{M} + \text{H}]^+$ (m/z 525.4) (Fig. 2B) shows the major fragment ion at m/z 247.2 with the collision energy at 30 eV, derived from the cleavage of C6-C7 bond in the 1 α ,25(OH)₂VitD₃ skeleton. The same fragmentation pattern is also observed for the other vitamin D metabolite derivatives. As a result, the ion transitions in supplemental Table S3 were selected for the quantification of vitamin D metabolites as their PyrNO derivatives. Collision energy (CE), declustering potential (DP), and collision cell exit potential (CXP) were optimized by direct infusion using a syringe pump. Because the DP value is affected not only by the chemical properties, but by the solvent composition and HPLC flow speed, the DP value was further optimized under the optimized HPLC conditions. The optimized MS/MS ion transition, CE, DP, and CXP value are listed in supplemental Table S3.

Chromatography

In theory, a total of four isomers may be produced in the Diels-Alder reaction between PyrNO and VitD. The reaction could produce two regioisomers (A and B in Fig. 1), and each of the regioisomers is composed of two diastereoisomers (A1 and A2; B1 and B2). However, it has been suggested that the overall structure and the nature of the substituent on the unsymmetrical dienes and dienophiles affect the regioselectivity of the Diels-Alder reaction. Leach and Houk (44) recently published an excellent study on the mechanisms of the hetero-Diels-Alder reaction with nitroso species in which they drew generalizations on the regioselectivity in this reaction based on the literature data and quantum mechanical calculations. According to their results, the distal isomer (regioisomer A; Fig. 1) should be favored in the reaction between vitamin D and PyrNO. This suggested regioselectivity was further supported by the model reaction between vitamin D₂ and PyrNO. The nuclear Overhauser effect experiments on the isolated major product revealed that it is a single regioisomer comprising two diastereoisomers (A1 and A2) (see NMR data in

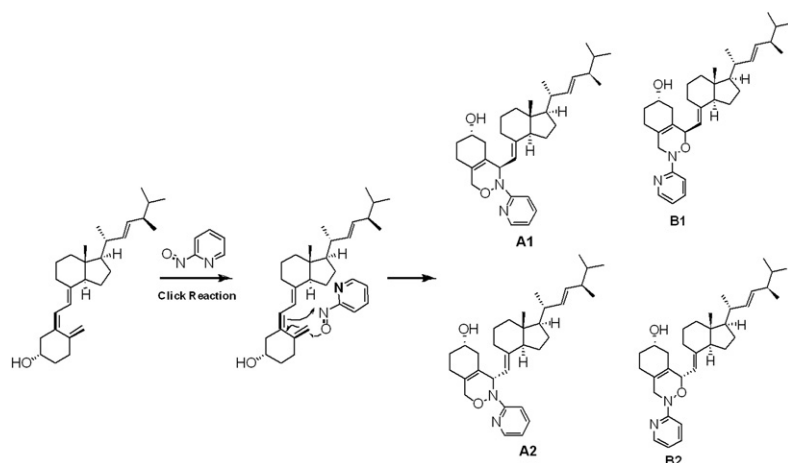


Fig. 1. Diels-Alder or click reaction between VitD₂ and PyrNO. Chemical reactions that are so highly favored that they proceed under a number of conditions are commonly referred to “click” reactions. Click reaction includes a class of biocompatible reactions intended primarily to join substrates of choice with specific biomolecules.

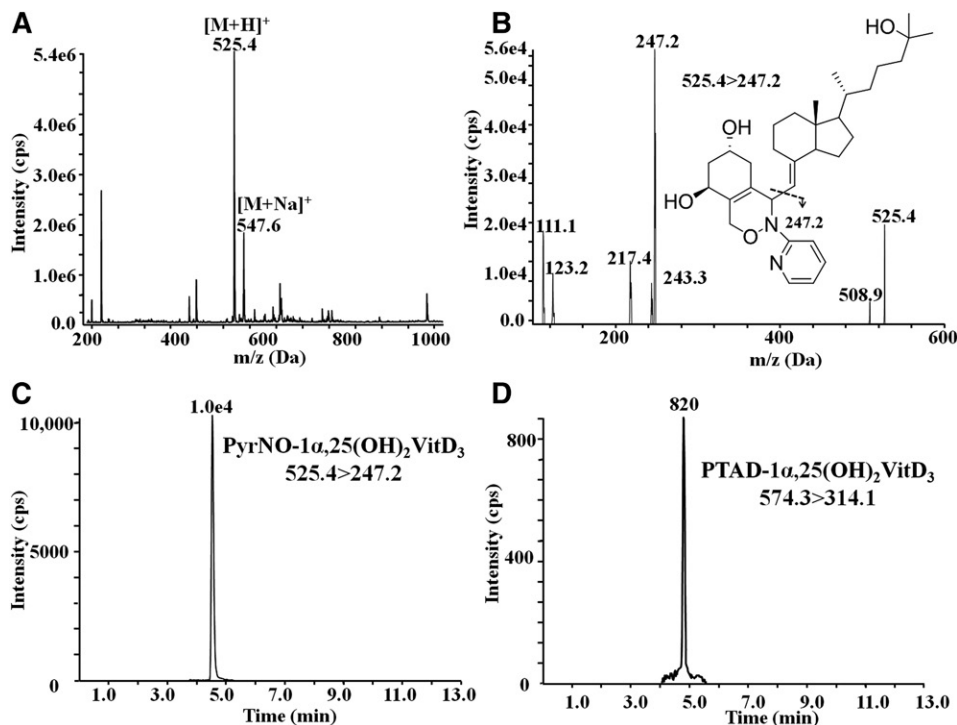


Fig. 2. A: Direct infusion full-scan mass spectrum of the PyrNO-1 α ,25(OH)₂VitD₃ product. B: Fragmentation of protonated PyrNO-1 α ,25(OH)₂VitD₃. C: Comparison of MRM intensity between PyrNO-1 α ,25(OH)₂VitD₃ and PTAD-1 α ,25(OH)₂VitD₃. The concentration of PTAD and PyrNO were 4.3 and 2.5 mM, respectively. The concentrations of 1 α ,25(OH)₂VitD₃ for PTAD and PyrNO derivatization are both 1.5 nmol/l.

the supplemental data). The ratio between the two diastereoisomers is approximately 1:4 (supplemental Fig. S4).

Column temperature, mobile phase composition, and HPLC gradient were optimized to separate all five targeted vitamin D metabolite derivatives, i.e., 25(OH)VitD₃, 25(OH)VitD₂, 1 α ,25(OH)₂VitD₃, 1 α ,25(OH)₂VitD₂, and 24R,25(OH)₂VitD₃. A representative chromatogram of the targeted vitamin D metabolites is illustrated in **Fig. 3**. The separation of the two diastereoisomers of each vitamin D metabolite depends on the mobile phase and analyte structure. As shown in Fig. 3, diastereoisomers of PyrNO-24R,25(OH)₂VitD₃, PyrNO-25(OH)VitD₃, and PyrNO-25(OH)VitD₂ can be separated as twin peaks under the optimized gradient condition (supplemental Table S1). In contrast, only one peak was detected for PyrNO-1 α ,25(OH)₂VitD₃ and PyrNO-1 α ,25(OH)₂VitD₂ with this gradient system, presented in Fig. 3. Although the separation of two diastereoisomers of PyrNO-1 α ,25(OH)₂VitD₃ can be achieved (supplemental Fig. S5), the separation does not provide any improvement on quantification analysis.

Advantage of PyrNO compared with PTAD and Amplifex-Diene

Because of the presence of a *cis*-conjugated diene group in vitamin D and its metabolites, a highly reactive dienophile can be used to enhance electrospray ionization. Traditionally, dienophiles like PTAD were commonly used, followed by detection using LC/MS/MS, leading to 100-fold increases in ion intensity over the underivatized analytes and allowing for a relatively accurate quantification of

1 α ,25(OH)₂VitD₃ (20, 31). In our previous research, PTAD derivatization after SPE had achieved a LLOQ of 60 pM (25 pg/ml) for the major metabolites using 0.5 ml of serum. However, the sensitivity of the method based on its reported LLOQs is sometimes not adequate to quantify low levels of 1 α ,25(OH)₂VitD accurately, particularly with the small sample volume.

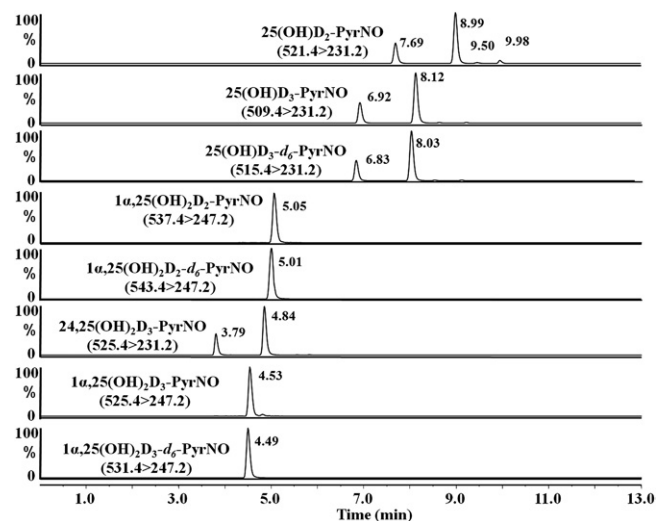


Fig. 3. Chromatography of PyrNO derivatives of major vitamin D metabolites and two deuterated surrogates of vitamin D metabolites. The separation condition is described in Materials and Methods. The HPLC gradient for separation of vitamin D metabolites is shown in supplemental Table S1.

We demonstrate here the use of PyrNO as a highly effective derivatization reagent for LC/MS/MS detection of Vitamin D metabolites. PyrNO is a click or Diels-Alder reaction reagent for derivatizing diene-containing compounds that, as shown here, provides a better ionization efficiency than PTAD. From the available proton affinities of functional groups of PTAD-VitD and PyrNO-VitD in supplemental Table S4, we deduce that the PyrNO-VitD complex is more likely than PTAD-VitD to capture a proton. Additionally, the available pKa value of the conjugated acids of functional groups in PyrNO-vitamin D and PTAD-VitD (supplemental Table S4) also indicate that the PyrNO-VitD complex has a higher proton affinity than PTAD-VitD. These data in Fig. 2 C, D show an approximately 5-fold increase in signal intensity for the PyrNO derivative as compared with the PTAD derivative, each analyzed under their own optimized experimental condition.

The reaction rate of PyrNO was evaluated by comparing PyrNO- $1\alpha,25(\text{OH})_2\text{VitD}_3$ and PTAD- $1\alpha,25(\text{OH})_2\text{VitD}_3$ in the MRM mode. Supplemental Fig. S6 shows that PTAD reacts more quickly than PyrNO at 22 and 50°C. Supplemental Fig. S1C shows that PyrNO reacts quickly at 70°C and the reaction is completed in an hour.

The Ampliflex-Diene reagent is reported to result in 10-fold ionization efficiency improvement over PTAD. However, the Ampliflex-Diene reagent results in a large change of the hydrophobicity of the vitamin D complex, and the chromatographic retention will be dominated by the contribution of the reagent. As is shown in supplemental Fig. S7A and Fig. 3, when similar chromatographic conditions for the Ampliflex-Diene and PyrNO derivatives are used, the permanently charged derivatives elute earlier than PyrNO derivatives. The smaller difference of polarity among the permanently charged derivatives requires more carefully optimization of separation. Even when chromatography is optimized for the more polar Ampliflex-Diene derivatives (supplemental Fig. S7B), it is still challenging to obtain baseline resolution of the early eluting peaks with the permanently charged reagent. Therefore, the separation of the Ampliflex-Diene-derivatized vitamin D metabolites will be more challenging, especially given that the Ampliflex-Diene derivatives of vitamin D will share the same MS product ion (25, 38), potentially causing a cross-interference, especially for $24\text{R},25(\text{OH})_2\text{VitD}_3$ and $1\alpha,25(\text{OH})_2\text{VitD}_3$ (supplemental Fig. S7B). This limitation does not detract from the excellent sensitivity of that for the advertised use of the Ampliflex-Diene reagent in the quantitative analysis of just $1\alpha,25(\text{OH})_2\text{VitD}$.

The data in supplemental Fig. S8 show that derivatization of vitamin D metabolites with PyrNO is water-tolerant. By comparison of PyrNO derivatization, the complete removal of water before Ampliflex-Diene derivatization was found to be critical to obtain a high level of reaction completeness (supplemental Fig. S9).

Newly developed extraction method to reduce the matrix effect (ion suppression)

Another approach to promote the accurate detection of $1\alpha,25(\text{OH})_2\text{VitD}$ is by choosing a highly effective sample

preparation method to decrease the ion suppression. Previous researchers found significant interfering compounds with retention time similar to $1\alpha,25(\text{OH})_2\text{VitD}_3$ (20–23). These interferences were regarded as a derivatization product from the matrix. In this regard, immunoaffinity extraction is among the best techniques for purifying $1\alpha,25(\text{OH})_2\text{VitD}$ from interfering compounds (23, 24). However, $25(\text{OH})\text{VitD}$ and $24\text{R},25(\text{OH})_2\text{VitD}$ will be lost during the sample preparation process. A growing number of publications illustrate that knowing the levels of both hydroxyvitamin D and dihydroxyvitamin D are important. Although SPE preparation is a more comprehensive extraction method that can extract all major vitamin D metabolites, and proved to cause lower ion suppression than LLE preparation, there are still interfering peaks with similar retention times to $1\alpha,25(\text{OH})_2\text{VitD}_3$, thereby impairing the sensitivity-improving effects of derivatization (20).

Here, we described a new sample preparation technique to extract all these major vitamin D metabolites, consisting of protein precipitation to release the vitamin D metabolites from serum protein and LLE-SPE to decrease the ion suppression. The extraction solution after protein precipitation was composed of methanol/acetonitrile/water (4:1:5.8). Hexane was the extraction solvent, and it was immiscible with methanol/acetonitrile/water. The combination of the hexane extraction and SPE will benefit in increasing the sensitivity by eliminating the most matrix effect. This partition takes advantage of the fact that hexane and methanol/acetonitrile are not miscible, and quite similar compounds can be partitioned between them by altering the amount of water in the methanol/acetonitrile layer.

During hexane extraction, hydroxy-vitamin D [$25(\text{OH})\text{VitD}_3$ and $25(\text{OH})\text{VitD}_2$] were extracted into the hexane layer, whereas dihydroxy-vitamin D [i.e., $1\alpha,25(\text{OH})_2\text{VitD}_3$, $1\alpha,25(\text{OH})_2\text{VitD}_2$ and $24\text{R},25(\text{OH})_2\text{VitD}_3$] remained in the methanol/acetonitrile/water layer after LLE. The hypophase was further processed by SPE before PyrNO derivatization. Fig. 4 shows the comparison of the samples processed by using SPE and LLE-SPE. The introduction of the LLE step in the sample preparation procedure proved to be beneficial for decreasing the ion suppression because the baseline noise resulting from LLE-SPE was significantly lower than that resulting from the SPE samples, at 200 and 600 counts, respectively. After the LLE-SPE procedure, matrix interferences associated with PyrNO- $1\alpha,25(\text{OH})_2\text{VitD}_3$ were reduced in both blank (Fig. 4C) and spiked (Fig. 4D) pooled human serum. After SPE alone, no signal for PyrNO- $1\alpha,25(\text{OH})_2\text{VitD}_3$ was observed until the spiked concentration reached 200 pM (Fig. 4A, B). Thus, it is evident that LLE-SPE is better at purifying $1\alpha,25(\text{OH})_2\text{VitD}_3$ than SPE alone, because it can reduce most of the interfering components, including $25(\text{OH})\text{VitD}$. This LLE-SPE purification procedure can also provide higher S/N values in the PTAD method by comparison with the normal SPE preparation procedure. As shown in supplemental Table S5, either PyrNO- or PTAD-derivatized dihydroxyvitamin D metabolites resulted in better sensitivities after LLE-SPE purification than after SPE alone. It is obvious that preparing

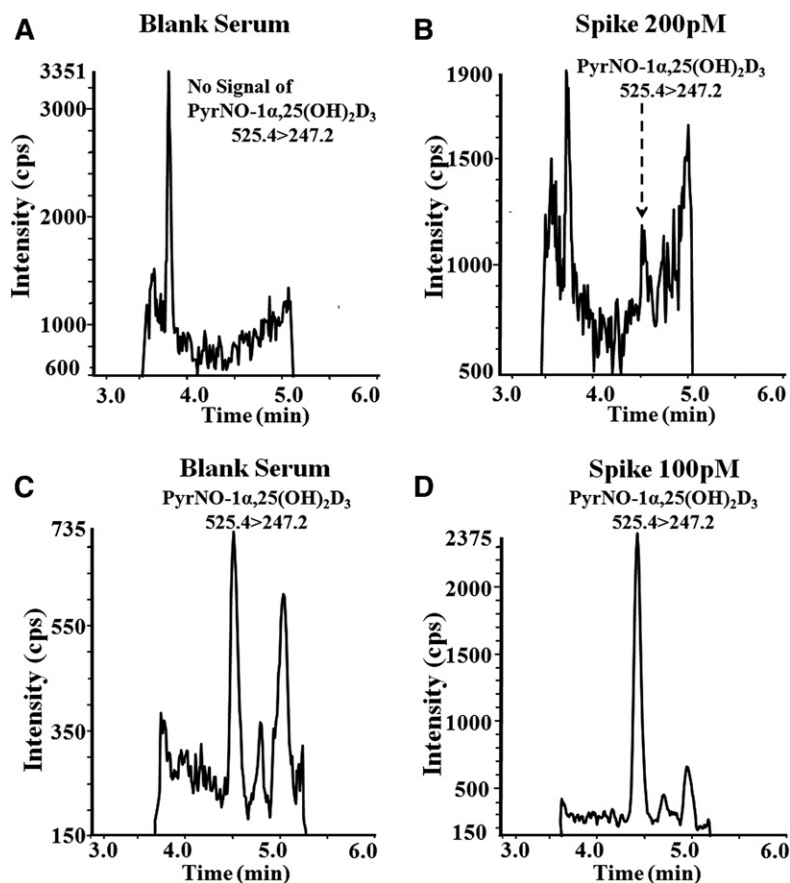


Fig. 4. Comparison of LLE-SPE and SPE methods of decreasing the ion suppression. Detection of PyrNO-1 α ,25(OH) $_2$ VitD $_3$ in 100 μ l of serum after LLE-SPE and SPE purification. A: SPE purification of pooled human serum. B: SPE purification of spiked human serum. The spiked concentration is 200 pM. C: LLE-SPE purification of pooled human serum. D: LLE-SPE purification of spiked human serum. The spiked concentration is 100 pM.

samples by using LLE-SPE followed by derivatization with PyrNO can provide the most sensitive approach to do quantitative analysis of all dihydroxyvitamin D metabolites. In the LLE step, hydroxyvitamin D was extracted into the hexane layer along with other interfering compounds that could compete with ionization of dihydroxyvitamin D, which will further help increase the ionization of dihydroxyvitamin D. The remaining dihydroxyvitamin D in the lower layer (methanol/acetonitrile/water layer) was further purified by using SPE. By a comparison of the absolute ion intensity of 1 α ,25(OH) $_2$ VitD $_3$ - d_6 , the LLE-SPE method provided a 2-4-fold increase in the detection sensitivity of dihydroxyvitamin D over the SPE alone. Finally, the LLOQ of the new assay is approximately 10-fold better than that of the PTAD assay (supplemental Table S5).

Linearity, limit of quantification, recovery, accuracy and precision, matrix effect

Method validation results are presented in **Table 1**. Excellent linearity ($R^2 > 0.99$) was obtained for the 10-point calibration curve over the 0.01–50 nM concentration range for both 1 α ,25(OH) $_2$ VitD $_2$ and 1 α ,25(OH) $_2$ VitD $_3$ and between 0.2 and 1,000 nM for 24R,25(OH) $_2$ VitD $_3$, 25(OH)VitD $_3$, and 25(OH)VitD $_2$.

In 100 μ l 4% BSA in PBS buffer, the LOQ of 1 α ,25(OH) $_2$ VitD $_3$, 1 α ,25(OH) $_2$ VitD $_2$, 24R,25(OH) $_2$ VitD $_3$, 25(OH)VitD $_3$, and 25(OH)VitD $_2$ are determined to be 5 pM (2 pg/ml), 5 pM (2 pg/ml), 20 pM (8 pg/ml), 10 pM (4 pg/ml), and 10 pM (4 pg/ml), respectively. Because of ion suppression in human blood samples, the LLOQ of

1 α ,25(OH) $_2$ VitD $_3$ and 1 α ,25(OH) $_2$ VitD $_2$ was validated as 25 pM (10 pg/ml), in 100 μ l human serum (supplemental Fig. S10). This LLOQ was obtained by diluting DEQAS samples with known concentrations of these analytes.

The recoveries of each of the extraction procedures of 1 α ,25(OH) $_2$ VitD $_3$ - d_6 and 25(OH)VitD $_3$ - d_6 are listed in supplemental Fig. S11A, B. The recoveries of preLLE and pre-SPE are similar, indicating no analyte loss during LLE. The analytical recovery (precrash) of 1 α ,25(OH) $_2$ VitD $_3$ - d_6 and 25(OH)VitD $_3$ - d_6 were both over 85%.

The intraassay and interassay accuracy of PBS buffer QC samples are greater than 80%, and the precision (relative SD) does not exceed 20%, as shown in **Table 2**. These data indicate that the developed method has a satisfactory reproducibility. The spiked pooled serum samples show good accuracy that ranges from 2.6% to 16.14% (**Table 3**). All relative SD values of the spiked pooled serum samples were below 16%.

A deuterated internal standard presents a similar matrix effect to the targeted analytes and is therefore usually used to normalize the ion suppression caused by matrix (43). Supplemental Table S6 shows that the average of serum matrix effects is more than 68%, depending on different analytes at different levels. The ion suppression of 1 α ,25(OH) $_2$ VitD $_3$ - d_6 , 1 α ,25(OH) $_2$ VitD $_2$ - d_6 , and 25(OH)VitD $_3$ - d_6 were tested to be between 14.3% and 25.6%, 29.3% and 31.4%, and 12.5% and 22.5%, respectively (supplemental Table S7). It is worth mentioning that deuterium-labeled compounds are commonly subject to chromatographic shifts relative to the targeted analytes,

TABLE 1. LLOQ and linear ranges of vitamin D metabolites

Vitamin D Metabolites	Limit of Quantification in BSA [pmol/l (pg/ml)]	LLOQ in human serum [pmol/l (pg/ml)]	Linear Range (nmol/l)
1 α ,25(OH) ₂ VitD ₃	5.0 (2.1)	25 (10)	0.01–50
1 α ,25(OH) ₂ VitD ₂	5.0 (2.1)	25 (10)	0.01–50
24R,25(OH) ₂ VitD ₃	20 (8.4)	25 (10)	0.2–1,000
25(OH)VitD ₃	10 (4)	100 (40)	0.2–1,000
25(OH)VitD ₂	10 (4)	100 (40)	0.2–1,000

known as the chromatographic isotope effect (45). In this paper, 1 α ,25(OH)₂VitD-d₆ and 25(OH)₂VitD₃-d₆ were shifted 0.04 and 0.09 min from 1 α ,25(OH)₂VitD and 25(OH)₂VitD₃, respectively. This may result in a small impact on the overall method accuracy.

Method comparisons

We validated our method using the reference samples from DEQAS, which was established to ensure the analytical reliability of 25(OH)VitD₃ and 1 α ,25(OH)₂VitD. The average mean values of DEQAS samples were calculated from different testing methods, including Cusabio ELISA (Cusabio, College Park, MD), DiaSorin RIA, DIASource RIA (DIASource, Louvain-la-Neuve, Belgium), IDS RIA (Immunodiagnostic Systems, Tyne and Wear, UK), IDS enzyme-linked immunosorbent assay (Immunodiagnostic Systems), IDS-Isys (Immunodiagnostic Systems), Immundiagnostik ELISA (Immundiagnostik, Bensheim, Germany), and LC/MS/MS. Twenty random DEQAS samples were analyzed by the developed method in quadruplicate. The new method was verified by using these samples to compare the concentration of vitamin D metabolites obtained by our method with the reported DEQAS data (Fig. 5). Figure 5A shows an excellent linear correlation ($R^2 = 0.936$) of our results with DEQAS data for 25(OH)VitD₃. The proportional and constant biases were 1.06 and 0.386, respectively. Figure 5B shows good correlation for 1 α ,25(OH)₂VitD ($R^2 = 0.893$). However, we obtained a proportional bias of 0.831 and constant bias of 16.4 for this analyte. This may simply reflect the methodological

differences, because the ALTM is derived predominantly from the immunoassay data. In general terms, the correlation analysis of 25(OH)VitD₃ and 1 α ,25(OH)₂VitD provide R^2 values of 0.936 and 0.893, respectively, which illustrated the good performance of the new method reported here.

DISCUSSION

The quantification of endogenous vitamin D metabolites is critical to further understanding their effects on human health and disease. Most techniques for the previous analysis focused on analysis of 25(OH)VitD, the major circulating form of vitamin D, as a surrogate for the bioactive metabolites. However, the major biologically active form of vitamin D is the 1 α ,25(OH)₂VitD metabolite. The accurate measurement by LC/MS/MS presents a great challenge due to the lack of good ionization and 1,000-fold lower circulating concentrations compared with 25(OH)VitD. Therefore, derivatization to increase the ionization efficiency has been used to increase the sensitivity of LC/MS/MS. PTAD-based methods have been commonly used, followed by detection using LC/MS/MS, leading to 100-fold increases in ion intensity over the underivatized analytes, and they allow for relatively accurate quantification of 1 α ,25(OH)₂VitD₃ (20, 31). However, the sensitivity of the method based on its reported LLOQs was sometimes not adequate to detect low levels of 1 α ,25(OH)₂VitD. Additionally, there was a strong interference signal around the

TABLE 2. Accuracy and precision of the PyrNO method for quantification of vitamin D metabolites in 4% BSA in PBS buffer

Vitamin D Metabolites	Spiked Concentration (nmol/l)	Detected Concentration [Mean (SD), nmol/l]	Intraday Accuracy (%)	Intraday Precision (%)	Interday Accuracy (%)	Interday Precision (%)
1 α ,25(OH) ₂ VitD ₃	0.01	0.010 (0.001)	3.75	11.9	4.75	14.9
	0.1	0.086 (0.004)	14.2	4.32	14.8	13.8
	1	0.863 (0.011)	13.7	1.28	14.9	2.65
	10	8.16 (0.103)	18.3	1.26	14.8	4.39
1 α ,25(OH) ₂ VitD ₂	0.01	0.009 (0.002)	8.12	18.7	11.1	17.0
	0.1	0.082 (0.002)	18.0	1.68	13.8	7.86
	1	0.897 (0.011)	10.3	1.20	7.85	3.67
	10	8.26 (0.119)	17.4	1.44	14.3	5.69
24R,25(OH) ₂ VitD ₃	0.4	0.330 (0.007)	17.6	2.00	4.25	17.4
	4	4.01 (0.152)	0.313	3.77	10.4	12.3
	40	37.1 (0.700)	7.19	1.89	0.281	8.65
	25(OH)VitD ₃	2	1.77 (0.190)	11.3	10.6	10.4
25(OH)VitD ₃	20	21.6 (0.637)	7.938	2.95	8.55	3.33
	200	195 (2.92)	2.50	1.50	1.92	1.77
	25(OH)VitD ₂	2	1.83 (0.153)	9.88	8.49	7.90
25(OH)VitD ₂	20	21.1 (0.606)	5.62	2.87	5.25	2.14
	200	199 (5.58)	0.750	2.81	0.081	3.39

PBS buffer was 100 μ l.

TABLE 3. Accuracy and precision of the PyrNO method for quantification of vitamin D metabolites spiked in pooled human serum

Vitamin D Metabolites	Spiked Concentration (nmol/l)	Detected Concentration [Mean (SD), nmol/l]	Intraday Accuracy (%)	Intraday Precision (%)	Interday Accuracy (%)	Interday Precision (%)
1 α ,25(OH) ₂ VitD ₃	0.1	0.061 ^a (\pm 16.6%)	3.32	8.44	6.40	14.7
	1	0.108 ^b (0.009)	8.71	4.80	10.0	5.51
1 α ,25(OH) ₂ VitD ₂	0.1	0.103 ^b (0.016)	14.0	15.3	10.3	11.9
	1	1.15 ^b (0.040)	12.7	3.48	6.19	8.88
24R,25(OH) ₂ VitD ₃	0.1	7.15 ^a (\pm 8.07%)				
	1	1.00 ^b (0.122)	7.26	12.1	14.7	5.22
25(OH)VitD ₃	10	10.5 ^b (0.708)	15.9	6.71	15.9	13.0
	4	28.3 ^a (\pm 5.56%)				
25(OH)VitD ₂	4	3.77 ^b (0.342)	5.83	9.08	8.54	1.70
	40	33.8 ^b (1.65)	15.5	4.88	14.2	1.51
25(OH)VitD ₂	4	8.62 ^a (\pm 17.9%)				
	40	3.71 ^b (0.191)	7.21	5.14	2.62	5.32
		33.5 ^b (1.06)	16.1	3.18	15.5	1.12

Pooled human serum was 100 μ l.

^aConcentration of targeted vitamin D metabolites in pooled human serum.

^bThe endogenous level of targeted vitamin D metabolites have been subtracted from the measured value.

1 α ,25(OH)₂VitD₃ derivative, reducing accuracy of the method. Larger sample sizes and more extensive cleanup could be helpful, but sample sizes are generally limited in epidemiological studies.

In this paper, we used LLE-SPE and PyrNO to reduce the ion suppression and enhance the ionization efficiency of dihydroxy vitamin D and related metabolites. The strategy has been optimized with emphasis on 1 α ,25(OH)₂VitD₃, the targeted analyte having the lowest endogenous abundance. The PyrNO derivatization markedly improved the ionization efficiency of the analytes. A new extraction method combining the LLE and SPE was developed to selectively extract hydroxyvitamin D and dihydroxyvitamin D to different fractions with high recoveries, while minimizing the ion suppression of dihydroxyvitamin D and achieving an ultrahigh sensitivity that enables a comprehensive quantification of all major vitamin D metabolites. Based on the combination of protein precipitation, LLE-SPE, and PyrNO derivatization, this highly sensitive method allows for the robust and comprehensive quantification of the major VitD metabolites by using lower volumes of serum/plasma (100 μ l).

For validation, the newly developed method was applied to test the DEQAS samples. Because of the increased sensitivity, both 1 α ,25(OH)₂VitD₃ and 25(OH)VitD₃ can be accurately quantified in 100- μ l serum. As shown in Fig. 5, this

method showed a good correlation with the ALTM on DEQAS samples.

Compared with previous methods, the method reported here offers several advantages. The comparisons among PyrNO, PTAD, SecoSET, and Amplifex-Diene derivatization reagents are shown in supplemental Table S8. The sensitivity after PyrNO derivatization improved 10-fold for quantification of 1 α ,25(OH)₂VitD compared with the PTAD derivatization. In this study, PyrNO shows even higher ion intensity of 1 α ,25(OH)₂VitD₃-d₆ than the corresponding permanently charged derivative (supplemental Fig. S12). Theoretically, the Amplifex-Diene derivatives can provide higher ionization efficiency than PyrNO derivatives. However, as advertised, all Amplifex-Diene derivatives of vitamin D metabolites are eluted out in a very narrow window (<1.0 min; supplemental Fig. S7B). Therefore, the permanent charge on the Amplifex-Diene derivatization reagent offers the advantage of high sensitivity and rapid analysis in separately detecting the 25(OH)VitD and 1 α ,25(OH)₂VitD₃. However, the PyrNO reagent offers advantages for more comprehensive profiling of all major vitamin D metabolites on a common C18 reversed column, including 1 α ,25(OH)₂VitD₃, 1 α ,25(OH)₂VitD₂, 24R,25(OH)₂VitD₃, 25(OH)VitD₃, and 25(OH)VitD₂. The immunoextraction method is reported in the literature to be the best extraction method for 1 α ,25(OH)₂VitD, but it

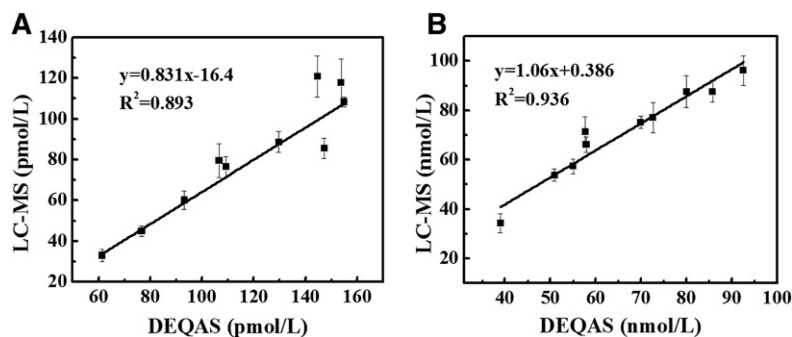


Fig. 5. A: Comparison of results obtained by the PyrNO method versus DEQAS mean value for analysis of 1 α ,25(OH)₂VitD. B: Comparison of the result obtained by our method versus DEQAS mean value for analysis of 25(OH)VitD₃. DEQAS was established to ensure the analytical reliability of 25(OH)VitD₃ and 1 α ,25(OH)₂VitD.

only targeted $1\alpha,25(\text{OH})_2\text{VitD}$ and excluded important vitamin D metabolites. Thus, the method is more comprehensive in that it can detect all the major vitamin D metabolites. It is worth mentioning that the sensitivity of the PyrNO method could be further enhanced by combing it with immunoextraction.

In summary, we developed a new method that allows comprehensive, sensitive, and simultaneous quantitative profiling of all the primary vitamin D metabolites. This method allows the routine measurement of primary vitamin D metabolites in 100- μl aliquots of serum/plasma, including $1\alpha,25(\text{OH})_2\text{VitD}$. The PyrNO method offers a comprehensive and highly sensitive quantification for vitamin D metabolites that may be generally applicable to various diene analytes. Currently, we are also screening other functionalized nitroso compounds to determine whether the ionization efficiency can be further improved.

We thank Amy Rand for detailed discussion and revision. We also thank Julia Jones for providing us with the DEQAS samples.

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