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Characterizing and harnessing antibody cross-reactivity for the immunoaffinity purification of analytes prior to multiplexed liquid chromatography-tandem mass spectrometry

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Abstract

Background—Immunoassays for 1α,25-dihydroxyvitamin D [1α,25$(OH)_2$D] lack specificity. We aimed to characterize the cross-reactivity of an anti-1α,25$(OH)_2$D antibody using purified vitamin D metabolites and to use these data to map the chemical features of 1α,25$(OH)_2$D that are important for antibody binding. Additionally, we hypothesized that when combined with isotope dilution-liquid chromatography-tandem mass spectrometry (LC-MS/MS), antibody cross-reactivity could be used to semi-selectively enrich for structurally similar metabolites of vitamin D in a multiplexed assay.

Methods—Sample preparation consisted of immunoaffinity enrichment with a solid-phase anti-1α,25$(OH)_2$D antibody and derivatization. Analytes were quantified using LC-MS/MS. Spike-recovery studies were performed using eleven vitamin D metabolites. A novel method for quantifying 25$(OH)D_2$, 25$(OH)D_3$, 24,25$(OH)_2D_3$, 1α,25$(OH)_2D_2$ and 1α,25$(OH)_2D_3$ simultaneously was developed and evaluated, which included deuterated internal standards for each analyte.

Results—The important chemical features of vitamin D metabolites for binding to the antibody were (1) native orientation of the hydroxyl group on carbon C3 in the A-ring, (2) the lack of substitution at carbon C4 in the A-ring, and (3) the overall polarity of the vitamin D metabolite. The new multiplexed method had lower limits of quantification (20% CV) of 0.2 ng/mL, 1.0 ng/mL, 0.06 ng/mL, 3.4 pg/mL and 2.8 pg/mL for 25$(OH)D_2$, 25$(OH)D_3$, 24,25$(OH)_2D_3$, 1α,25$(OH)_2D_2$ and 1α,25$(OH)_2D_3$, respectively. Method comparisons to three other LC-MS/MS methods were acceptable (r^2>0.9, intercept<lower limit of quantification, slope statistically indistinguishable from 1.0).

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Conclusions—LC-MS/MS can be used to characterize antibody cross-reactivity. We developed and evaluated a multiplexed assay for five vitamin D metabolites using immunoenrichment in a targeted metabolomic assay.

Keywords

Liquid chromatography-tandem mass spectrometry; immunoaffinity enrichment; multiplexed assay; specificity; hapten mapping; vitamin D metabolism

Vitamin D is present in blood in two chemically distinct forms, vitamin D_2 and vitamin D_3. While both are sterol prohormones, only vitamin D_3 is produced *in vivo*. Vitamin D_2 is instead obtained through diet and supplementation in the thermally stable form of ergocalciferol. Vitamin D_3 is synthesized in the skin by the photolysis of 7-dehydrocholesterol to an unstable previtamin D_3 which is then converted by isomerization to thermally stable cholecalciferol.(1) Vitamin D is then transported to the liver where it undergoes hydroxylation by CYP2R1 and other cytochrome P450 enzymes to 25-hydroxyvitamin D [25(OH)D],(2,3) the most abundant vitamin D metabolite in blood with a half-life of approximately 1 month.(4) As such, 25(OH)D is the most commonly measured metabolite of vitamin D to assess vitamin D stores.(1) The prohormone 25(OH)D undergoes further hydroxylation by CYP27B1 and CYP24A1 in the kidney into two different dihydroxyvitamin D metabolites, 1α,25$(OH)_2$D and 24,25$(OH)_2$D, respectively.(5–8) It is well-established that 1α,25$(OH)_2$D plays an important role in calcium metabolism. Studies on the function of 24,25$(OH)_2$D are more inconsistent, some suggesting that the metabolite is simply a catabolic breakdown product of 25(OH)D and others demonstrating a putative role in bone formation.(7) Other vitamin D metabolites have been described, including 25,26$(OH)_2$D, which may have a regulatory role in intestinal calcium transport,(9) a metabolite hydroxylated at carbon 4 by CYP3A4, which may explain certain drug-induced disorders of bone,(10) and an epimer at carbon C3 in the A-ring, whose concentration appears to be relatively constant throughout the human lifespan but has unclear biological function after 1α-hydroxylation.(11–13)

The concentrations of vitamin D metabolites in plasma span more than 3 orders of magnitude. For example, the concentration of the most active vitamin D metabolite, 1α,25$(OH)_2$D is 1,000-fold lower than the prohormone 25(OH)D. Previous experiments in our laboratory demonstrated significant cross-reactivity of an antibody used in a commercially available (IDS, Scottsdale, AZ) competitive RIA for 1α,25$(OH)_2$D,(14) which might raise concern regarding the utility of previous clinical and epidemiologic studies that have investigated 1α,25$(OH)_2$D as a biomarker in human disease. Using LC-MS/MS to resolve potential interfering analytes and directly detect the analytes of interest, we previously demonstrated that it is possible to greatly improve the specificity of the antibody-based assay.(14) Since the publication of that assay, we have confirmed the results of another study that demonstrated the utility of a different solid-phase antibody (ALPCO, Salem, NH) to immunopurify analyte prior to LC-MS/MS,(15) which does not need sample preparation prior to immunoaffinity enrichment (data not shown). The simpler workflow and preliminary data demonstrating saturation of the IDS solid-phase reagent with moderate concentrations of spiked 25(OH)D (data not shown) led us to transfer our clinical workflow to the new solid-phase reagent from ALPCO. The chromatographic gradient was similar to that described,(14) with the exception of the starting conditions being revised to 56% mobile phase A [Optima water (Fisher, Pittsburg, PA)/0.1% formic acid (VWR, Randor, PA)/ 0.5mM methylamine (Sigma-Aldrich, St. Louis, MO)], 44% mobile phase B [Acetonitrile (Fisher)/0.1% formic acid/0.5mM methylamine]. The chromatographic separation of multiple vitamin D analytes is shown in Figure 1.

To characterize the cross-reactivity of the solid-phase antibody, we determined the extraction efficiency (analytical recovery) of the antibody for different vitamin D metabolites. Each analyte (*see* Table 1 for a complete list of the metabolites and their structures and Supplemental Table 1 for the source of each analyte) was individually added to a vitamin D-depleted human serum matrix (MSG-4000, Golden West Biologicals, Temecula, CA), extracted using the immunoaffinity reagent from ALPCO, derivatized, and quantified using LC-MS/MS. The chromatographic peak areas obtained from the extraction were compared with the peak area observed after adding each analyte individually to the resulting extract of a non-spiked MSG-4000 sample (Table 1). There were three important features of the vitamin D metabolites that seemed to determine their affinity for the antibody. First, the extraction efficiency of $25(OH)D_2$ and $25(OH)D_3$ was significantly lower than the majority of the dihydroxyvitamin D metabolites, suggesting that overall polarity was important for binding. Second, vitamin D metabolites with an epimeric orientation of the C3 hydroxyl group of the A-ring had significantly reduced affinity for the antibody, suggesting that this was an important part of the molecule for specific binding. Third, $4\beta,25(OH)_2D_3$ had very little affinity, suggesting that this part of the A-ring was also important and further highlighting the importance of the A-ring and the region near the C3 carbon for antibody affinity. To further support these observations, we determined the apparent dissociation constant (K_d) in serum for several of the analytes using standard Scatchard analysis (K_d equals the negative slope of [bound]/[free] *vs.* [bound] analyte). The apparent K_d of $1\alpha,25(OH)_2D_3$ was 0.10 μM. $1\alpha,25(OH)_2D_2$ was approximately 4-fold lower (0.41 μM), which was similar to that observed for the dihydroxylated $24,25(OH)_2D_3$ metabolite (0.39 μM). The monohydroxylated $25(OH)D_3$ metabolite had a lower affinity (14 μM) and the affinity of the C3 epimer of $25(OH)D_3$ could not be determined exactly but was noted to be >140 μM. Taken together, these data suggest that specific A-ring substitutions and overall molecular polarity are important for hapten binding.

Our chemical characterization of the hapten complementarity of the antibody has two important implications. First, the C3-epimer of $25(OH)D_3$ is not well-recognized by the antibody. Because the epimer is not easily resolved from the native $25(OH)D_3$ in rapid chromatographic methods, the immunoextraction step could lead to shortened LC-MS/MS methods without interference from the epimer.(13) Similarly, $4\beta,25(OH)_2D_3$, which is present at similar concentrations to $1\alpha,25(OH)_2D_3$, is not well-recognized by the antibody. It is difficult to resolve these two analytes in short chromatographic methods (16,17) and as a result, methods to quantify $1\alpha,25(OH)_2D_3$ without immunoaffinity extraction need to be carefully evaluated for interference from $4\beta,25(OH)_2D_3$.(18)

Given the favorable affinities of many vitamin D metabolites, we decided to evaluate the possibility of using the immunoextraction of vitamin D metabolites as a step in a multiplexed assay of $25(OH)D_2$, $25(OH)D_3$, $1\alpha,25(OH)_2D_2$, $1\alpha,25(OH)_2D_3$, and $24,25(OH)_2D_3$. Such an assay could simultaneously evaluate vitamin D stores, production levels of active metabolite, and inactivation levels of metabolites. The multiplexed assay used 400 μL of calibrators, controls, or patient sample, 20 μL internal standard mixture in methanol [containing 500 ng/mL each of $25(OH)D_2$-d3, $25(OH)D_3$-d6, $24,25(OH)_2D_3$-d6 and 4 ng/mL each of $1\alpha,25(OH)_2D_3$-d6 and $1\alpha,25(OH)_2D_2$-d6], and 100 μL immunoaffinity beads (the commercial sources of the deuterated internal standards are listed in Supplemental Table 1). The plate was then covered and incubated for 2 h at 45°C while shaking at 800 rpm in a Thermomixer (Eppendorf, Hauppague, NY). After immuno-extraction, the beads were quantitatively transferred to a 2 mL filter plate (Strata Impact, Phenomenex, Torrance, CA) and the beads were washed ten times with 1 mL Optima grade water (Fisher, Pittsburg, PA). The analytes were eluted from the beads with 0.25 mL of acetonitrile into a 1 mL 96 deep-well collection plate (Waters, Milford, MA) and the eluate was evaporated in a Turbovap concentrator (Biotage, Charlotte, NC) at 30°C under nitrogen

(20 ft^3/hr). The residue was reconstituted in 50 μL of acetonitrile containing 0.7 mg/mL 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD, Sigma-Aldrich, St. Louis, MO). Following a 30-minute incubation at ambient temperature, the excess PTAD was quenched with 70 μL of water (Optima) and 40 μL was injected onto a Waters Acquity LC system coupled to a Waters Xevo TQ MS tandem mass spectrometer. Relevant mass spectrometer parameters are listed in Supplemental table 2. The experiments described spanned five lots of affinity beads.

The new multiplexed assay had lower limits of quantitation (LLOQ, 20% CV) of 1.0 ng/mL, 0.2 ng/mL, 0.06 ng/mL, 3.4 pg/mL and 2.8 pg/mL for 25(OH)D_3, 25(OH)D_2, 24,25$(OH)_2D_3$, 1α,25$(OH)_2D_3$, and 1α,25$(OH)_2D_2$, respectively. Total assay imprecision for each analyte is listed in Table 1 and was ≤17.1% for all analytes at low concentrations (at or below clinical decision points). We compared the new multiplexed method with liquid-liquid extraction methods optimized for 25(OH)D_2, 25(OH)D_3, and 24,25$(OH)_2D_3$, which have been previously described (19) or are described in Supplemental Material/ Supplemental Table 3, respectively. We also compared the new multiplexed method with the previously described immunoaffinity assay for 1α,25$(OH)_2D_2$ and 1α,25$(OH)_2D_3$ that uses IDS solid-phase antibody.(14) Descriptive data for the method comparisons are presented in Table 1 and the data are plotted in Supplemental Figures 2–4. The methods compared acceptably (defined as r^2>0.9, intercept<lower limit of quantification for each analyte, and slope statistically indistinguishable from 1.0). In terms of identifying low total 25(OH)D or low total 1α,25$(OH)_2$D concentrations in plasma (<20 ng/mL or <17 pg/mL, respectively), the new method reclassified 3.3% and 1.9% of people compared with the existing methods, respectively. We also compared the new method with a liquid-liquid extraction method for 1α,25$(OH)_2D_3$ that has been previously described.(17) The correlation was relatively poor between the two methods, which may be due to the improved specificity of the immunoaffinity purification approach (Supplemental Fig. 5). Ion suppression for the analytes ranged −1.2%—26.3%. We did not perform an extensive mapping experiment with the IDS solid-phase antibody, but the peak areas from a single patient sample were similar with both reagents (Supplemental Fig. 6). Although there is an increased cost of the new assay compared with standard liquid-liquid extraction, there are important advantages to the immunoaffinity approach: (1) interferences from 4β,25$(OH)_2D_3$ and epi-C3-25(OH)D_3 are eliminated, which can permit shorter chromatographic runs (16) and (2) the LLOQ for 1α,25$(OH)_2D_3$ is lower,(17) which permits the use of smaller volumes of plasma in the assay.

Competitive assays rely on antibody specificity or in the case of dihydroxyvitamin D analytes on extensive sample preparation prior to analysis (e.g. protein precipitation and chromatographic resolution of interfering substances).(8,20) The thermodynamics of the interference of related compounds with reagent antibodies are most often characterized by equilibrium inhibition studies. In this study we have used equilibrium binding studies in a human serum matrix to map the chemical features of the vitamin D hapten that most strongly dictate binding to the reagent antibody from a commercially available reagent antibody. This work complements competitive binding studies that have been performed in the past and the many epitope mapping experiments performed with overlapping synthetic peptides.

The obvious lack of specificity of reagent antibodies in small molecule immunoassays can be an advantage in multiplexed mass spectrometric assays, simplifying specimen preparation and greatly enriching for target analytes. It should be noted that the success of this multiplexed method relies on the large differences in concentration between each of the analytes being quantified. For example, if the plasma concentration of 25(OH)D_3 were similar to 1α,25$(OH)_2D_3$, we might not be able to quantify 25(OH)D_3 using this approach

due to the 140-fold lower affinity of the antibody for 25(OH)D_3 compared with 1α, 25$(OH)_2D_3$. In addition, the antibodies for the same analyte used in immunoaffinity enrichment steps prior to mass spectrometry could differ in significant ways between manufacturers and as a result, competition with similar analytes could differentially affect recovery of the analytes of interest. While this did not appear to be a significant problem between ALPCO and IDS, it could be important in other systems. Projecting forward from our findings in this study, it will be interesting to see if combining antibodies in a single assay will permit the quantification of different classes of analytes simultaneously.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

25(OH)D	25-hydroxyvitamin D
24,25$(OH)_2$D	24,25-dihydroxyvitamin D
1,25$(OH)_2$D	1,25-dihydroxyvitamin D

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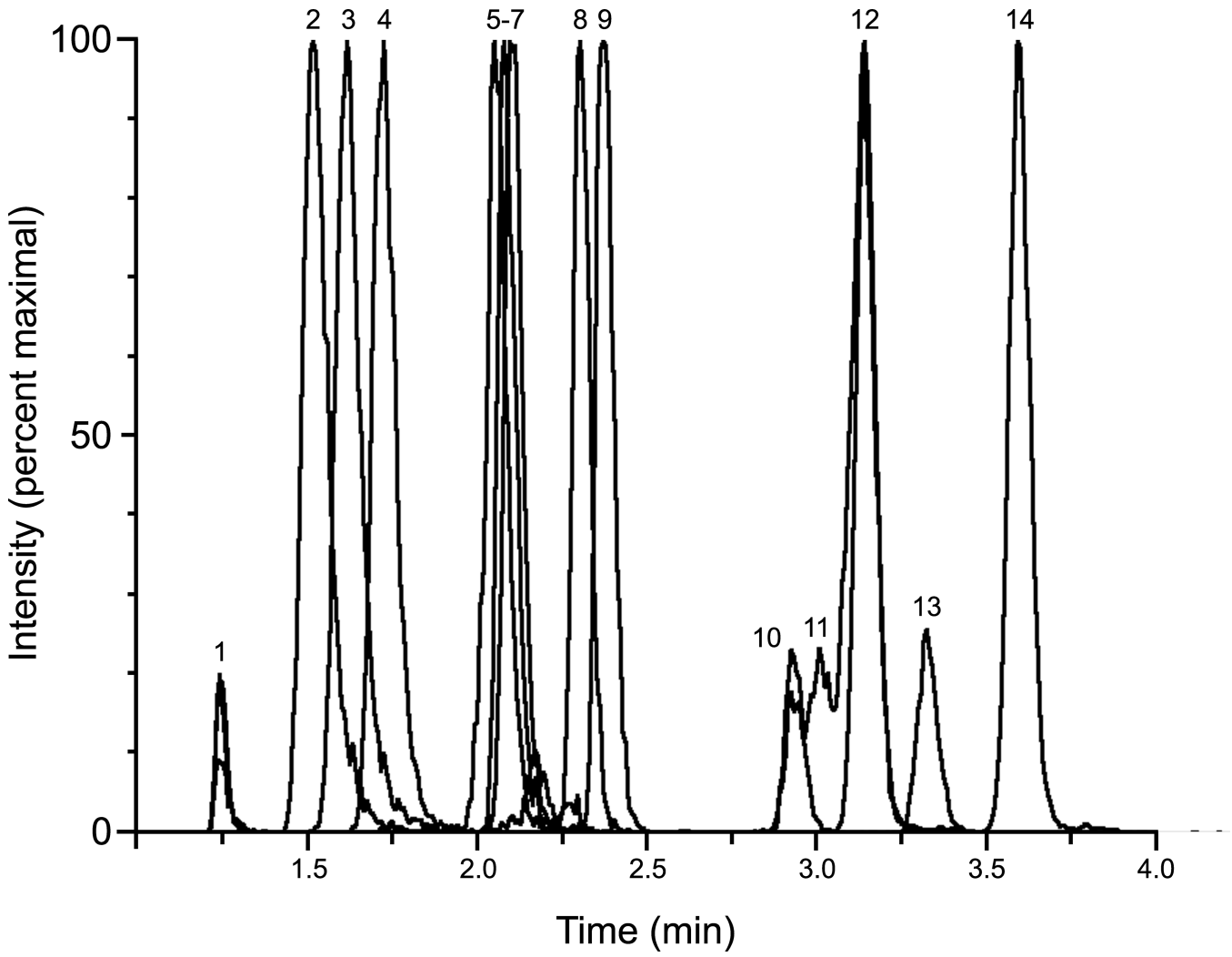
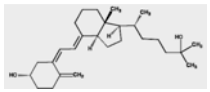
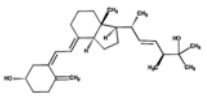
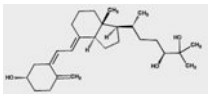
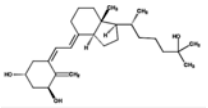
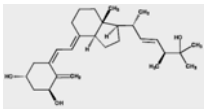
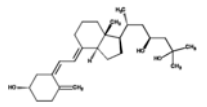
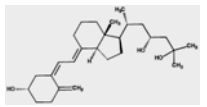


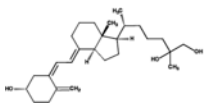
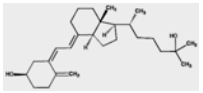
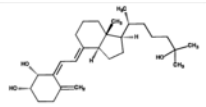
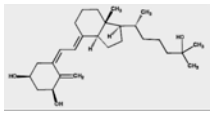
Figure 1. Composite chromatogram

Analytes were individually added to an immunoaffinity extract of stripped serum, derivatized, and chromatographically resolved as described for the multiplexed assay. The MRM transition for peaks 1, 2, 3, 4 and 5 is 623.5>298.2; peaks 6, 7, and 8 is 623.5 > 314.2; peak 9 is 635.5 > 314.2; peak 10, 11 and 12 is 607.6 > 298.2; peak 13 and 14 is 619.6 > 298.2. Peak 1: Minor 24,25$(OH)_2D_3$ PTAD isomer, peak 2: 23(S),25$(OH)_2D_3$, peak 3: 25,26$(OH)_2D_3$, peak 4: 24,25$(OH)_2D_3$, peak 5: 23(R),25$(OH)_2D_3$, peak 6: 4β,25$(OH)_2D_3$, peak 7: 1α25$(OH)_2D_3$, peak 8: 3-epi-1α,25$(OH)_2D_3$, peak 9: 1α,25$(OH)_2D_2$, peak 10: Minor 25$(OH)D_3$ PTAD isomer, peak 11: 3-epi-25$(OH)D_3$, peak 12: 25$(OH)D_3$, peak 13: Minor 25$(OH)D_2$ PTAD isomer, peak 14: 25$(OH)D_2$. A chromatogram of unsupplemented immunoaffinity extract of a stripped serum sample is shown in Supplemental Figure 1. The derivatization efficiency of 25$(OH)D_2$ and 25$(OH)D_3$ is 99.4% and 99.8%, respectively.

Table 1

Summary data of the new multiplexed vitamin D metabolite method.

Compound	% Recovery (SD)[d]	Structure	Regression[a]	r^2 [a]	Conc	Intra-assay[b] %CV	Total[b] %CV	LLOQ[c]
$25(OH)D_3$	43.3 (2.1)		Y=1.04x+0.08	0.955	12.3 ng/mL	3.0	3.7	1.0
$25(OH)D_2$	32.2 (3.3)		y=0.94x−1.00	0.981	10.6 ng/mL	4.7	10.2	0.2
$24,25(OH)_2D_3$	70.8 (9.8)		Y=0.96x−0.23	0.922	1.6 ng/mL	2.6	6.4	0.06
$1\alpha,25(OH)_2D_3$	79.4 (3.5)		y=0.96x−2.97	0.901	14.6 pg/mL	10.0	15.6	3.4
$1\alpha,25(OH)_2D_2$	78.2 (12.4)		y=0.89x−0.54	0.976	12.8 pg/mL	10.9	17.1	2.8
$23(S),25(OH)_2D_3$	64.0 (2.1)							
$23(R),25(OH)_2D_3$	67.0 (3.2)							

Compound	% Recovery (SD)[d]	Structure	Regression[a]	r² [a]	Conc	Intra-assay[b] %CV	Total[b] %CV	LLOQ[c]
25,26(OH)$_2$D$_3$	69.2 (4.0)							
3-epi-25(OH)D$_3$	3.2 (1.0)							
4β,25(OH)$_2$D$_3$	3.0 (0.01)							
3-epi-1α,25(OH)$_2$D$_3$	15.0 (0.4)							

[a] The equation of the Deming regression (y and x are the new and reference method, respectively) and Pearson correlation coefficient are presented.

[b] Intra-assay (N=10) and total-assay CV (sqrt[(intra-assay CV)^2 + (between-day CV)^2]) at the concentrations listed.

[c] Five replicates of linear dilutions were analyzed and the lowest dilution at which CV≤20% is listed. For 25(OH)D$_2$, 25(OH)D3, and 24,25(OH)$_2$D3 units are ng/mL. For 1α,25(OH)$_2$D$_2$ and 1α, 25(OH)$_2$D3 units are pg/mL.

[d] Analytical recovery was calculated as the analyte peak area when spiked before divided by the analyte peak area spiked after extraction.