VITAMIN D METABOLITE PROFILING USING
LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY (LC-MS/MS)

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Abstract

Liquid Chromatography tandem mass spectrometry (LC-MS/MS) has emerged as the latest technology to be used to assay the metabolites of vitamin D. The method uses molecular mass as a detection technique after straightforward extraction and chromatography steps. LC-MS/MS assay provides a level of accuracy and reproducibility not seen before with other methods and is beginning to rival antibody-based methods in terms of sensitivity and convenience. Methods for detection of underivatized and DMEQ-TAD derivatized vitamin D metabolites are evaluated. Sensitivity is improved by 10-100 fold with derivatization and allows for the simultaneous assay of multiple vitamin D metabolites, a process termed vitamin D metabolite profiling. Clinical and research applications of vitamin D metabolite profiling are discussed.

Keywords:

Metabolomics

25-OH-D₃

vitamin D assay

LC-MS/MS

DMEQ-TAD

History of Vitamin D Assays

The assay of vitamin D metabolites in clinical and animal serum samples is now almost 50 years old [1, 2]. It began with the application of radioactive vitamin D preparations, which though useful in identifying new metabolites, was limited by constraints posed by the use of radio-isotopes in humans and problems with endogenous body pools reducing the specific activity of administered radioactive vitamin D compounds [3,4,5]. By the mid-1970s, reliable clinical assays had been developed based upon extraction, with and without a step of chromatography and detection by a competitive-protein binding technique using DBP (Vitamin D binding protein) or VDR (vitamin D receptor) [1,2,6]. Such CPBA (competitive protein binding assay) methods are limited by the specificity of the protein involved. The late 1970s saw the introduction of high-pressure liquid chromatography(HPLC)-based methods that used high-performance small particle columns to separate vitamin D metabolites and then quantitation by a UV detector [7,8]. This approach takes advantage of the unique vitamin D chromophore derived from the cis-triene system ($\lambda_{\max}$ 265 nm; $\lambda_{\min}$ 228nm, ϵ 18,300) possessed by most vitamin D metabolites. Diode-array spectrophotometric detectors allowed for the optimization of the HPLC assays [9] but these approaches still lack the convenience of radioimmunoassays [10]. HPLC assays must be run sequentially through the same apparatus; require good resolution of vitamin D metabolites from UV interfering substances; and have sensitivity limitations in the low nanogram/mL range.

Application of LC-MS/MS to Vitamin D Assay

The emergence of liquid chromatography-tandem mass spectrometry (LC-MS/MS) is the latest step in the evolution of (HP)LC-based technologies and has been made possible by development of LC-MS interfaces to separate solvent from solutes and newer techniques to ionize lipid-soluble compounds, such as vitamin D metabolites [11]. The tandem MS portion of the LC-MS/MS provides the ultimate detector for the quantitation of vitamin D metabolites with

the minimum of interference. Based upon a combination of specificity, sensitivity and convenience, LC-MS/MS assays of vitamin D metabolites are now on the verge of superseding antibody-based techniques in both clinical and research fields. Whereas, antibody-based techniques measure one vitamin D metabolite at a time [10], the latest LC-MS/MS methods measure multiple metabolites simultaneously, in some situations negating the advantage of antibody-based methods to be automated for use on clinical platforms or plate readers. However, the main advantage of LC-MS/MS is its specificity for each individual vitamin D metabolite, a feature that provides its accuracy, quantitation being based upon comparison to a deuterated internal standard.

Advantages and disadvantages of LC-MS/MS in Serum 25-OH-D Analysis

It is becoming clear from several years of distributions by DEQAS (Vitamin D External Quality Assessment Scheme) that, in general, antibody-based 25-OH-D methods can be biased by as much as 10-15%, whereas the bias of LC-MS/MS methods for 25-OH-D₃ and 25-OH-D₂ assay is much lower, at around 5-7% [12]. For example, a recent January 2015 distribution of 5 DEQAS reference serum samples containing a range of 25-OH-D values (44.4 – 118.4 nmol/L) allowed for the comparison of results from 977 analysts from around the world with those from a NIST (LC-MS/MS) reference method (Table 1). The results show that 155 users of LC-MS/MS exhibited an average bias ranging from +0.3 to +2.7% for the 5 samples compared to 257 users of the most popular antibody-based method (Diasorin-Liaison) who showed an average bias ranging from -1.1% to -21.2%. The majority of the other antibody-based methods fared no better in this January 2015 distribution sometimes showing a bias > 10%. While LC-MS/MS clearly produces more accurate results than antibody-based approaches, it still has the disadvantages compared to antibody-based assays in that it requires costly instrumentation and involves sequential sample analysis.

Standard LC-MS/MS Approach to Serum 25-OH-D Analysis

The original approach to the LC-MS/MS of serum 25-OH-D, epitomized by the NIST reference method [13], involves the same three steps: extraction, chromatography and (mass spectrometry-based) detection employed in the earliest CPBA or HPLC assays. The LC-MS/MS steps usually involve reverse-phase chromatography using bonded phases and methanol-water gradient solvent systems, since these are more compatible with the mass spectrometry steps. The objective is to optimize instrument settings to maximize ionization of the 25-OH-D₃ and 25-OH-D₂. The molecular ions at m/z 401 and 413 respectively fragment in MS1 and then selected parent ions from the MS1 spectrum are collided with argon gas in the collision cell and daughter fragments separated in the MS2. The analyst selects parent and daughter ions for quantitation that maximize the signal and minimize potential interferences. In the case of the NIST reference method, the ions selected were m/z 401->383 for 25-OH-D₃ and m/z 413->395 for 25-OH-D₂, both of which represent simple dehydration products. But analysts are not confined to these ions and often select smaller size, more abundant fragments e.g. at m/z 383->257 and 395->269 from 25-OH-D₃ and 25-OH-D₂ respectively.

The development of a NIST reference LC-MS/MS method for 25-OH-D [13] paid particular attention to resolution of a minor, but significant impurity identified as 3-epi-25-OH-D₃, which is an isomer of 25-OH-D₃ with a 3 α - rather than a 3 β -hydroxyl. This 3-epi-25-OH-D₃ metabolite, of unknown origin and biological function, was first detected in early LC-MS/MS assays of neonatal serum samples by Singh et al [14]. While 3-epi-25-OH-D₃ is usually present in adult serum samples in a range of 4-6% of the total 25-OH-D [15,16], it can reach as high as 25-40% of the total 25-OH-D during the first 2 years of life. 25-OH-D₃ and 3-epi-25-OH-D₃ possess identical mass spectra so that they can only be distinguished by their retention time on the LC step. While the NIST reference method involves a 30 min runtime to provide baseline resolution of 25-OH-D₃ and 3-epi-25-OH-D₃, newer LC-MS/MS methods use columns that

resolve the two epimers within a 5 min run time [17]. 25-OH-D₂ has a similar retention time to 3-epi-25-OH-D₃ but can be resolved on the basis of its different mass spectral properties.

Use of Derivatization and Simultaneous Assay of Multiple Vitamin D Metabolites.

Although the sensitivity of underivatized 25-OH-D by LC-MS/MS is adequate for routine clinical analysis into the low ng/mL range, it is limiting for analysis of other less abundant vitamin D metabolites. Several groups have experimented with use of Cookson reagents, also known as dienophiles, which form adducts with all vitamin D compounds possessing the cis-triene system [17,18,19]. Several dienophiles, including PTAD and DMEQ-TAD, improve sensitivity by 10- to 100-fold by virtue of their adding ≈ 350 mass units to the vitamin D metabolite and moving it to a region of the mass spectrum where the background is lower. Because dienophiles react quantitatively with all vitamin D compounds in a sample, these reagents simultaneously tag all the clinically important vitamin D metabolites at the same time. One feature of TAD-tagging is that two adduct peaks are formed by dienophile attack from either plane of the cis-triene. Accordingly, if the resultant adduct peaks can be adequately resolved and are present in sufficient concentration for detection, the LC-MS/MS method can assay multiple metabolites simultaneously.

Recently, we published a method [17] for the simultaneous assay of several common vitamin D metabolites, including 25-OH-D₃, 25-OH-D₂, 3-epi-25-OH-D₃, 24,25-(OH)₂D₃, 24,25-(OH)₂D₂ and 25-OH-D₃-26,23-lactone, in a 100 microlitre sample after DMEQ-TAD derivatization. The published method actually makes three separate LC-MS/MS runs on the same lipid extract making it possible to use a single LC-MS run on serum volumes as low as 25 microlitres, when sample size is limiting. Using this method, we detect two DMEQ-TAD derivative peaks for each vitamin D metabolite and most metabolites require monitoring using a different daughter ion, a process known as multiple reaction monitoring (MRM). For monohydroxylated vitamin D₃ metabolites we use $m/z = 746 \rightarrow 468$; for dihydroxylated vitamin D₃

metabolites we use $m/z = 762 \rightarrow 468$; and for 25-OH-D₃-26,23-lactone we use $m/z = 774 \rightarrow 468$, all monitored simultaneously. The use of DMEQ-TAD allows for the detection of metabolites into the picogram/mL range.

Applications of Vitamin D Metabolite Profiling using LC-MS/MS

Vitamin D metabolite profiling using LC-MS/MS permits a rapid and convenient analysis of some of the rare vitamin D metabolites in clinical samples including 24,25-(OH)₂D₃, 24,25-(OH)₂D₂ and 25-OH-D₃-26,23-lactone. Although it was popular to measure such metabolites using laborious competitive protein binding assays in the early 1980s, the clinical value of assaying these metabolites has waned of late [20, 21]. However, the discovery of idiopathic infantile hypercalcemic (IIH) patients with mutations in CYP24A1 function [22] has caused a resurgence of interest in this topic. In IIH patients with CYP24A1 mutations, the serum level of 24,25-(OH)₂D₃ is <1.25 nmol/L and the ratio of 25-OH-D₃:24,25-(OH)₂D₃ is >80, whereas in normal individuals with serum 25-OH-D₃ levels in the 50-200 nmol/L range, serum 24,25-(OH)₂D₃ is between 5-20 nmol/L and the ratio of 25-OH-D₃:24,25-(OH)₂D₃ is between 5-25 [17].

Even vitamin D deficient patients with serum 25-OH-D₃ values <30 nmol/L rarely have 25-OH-D₃:24,25-(OH)₂D₃ ratios exceeding 50. Several research groups have noted that plots of serum 24,25-(OH)₂D₃ versus serum 25-OH-D₃ reveal a linear relationship where 24,25-(OH)₂D₃ levels are approximately 10% of 25-OH-D₃ levels and there is an intercept on the 25-OH-D₃-axis (x-axis) of about 20-25 nmol/L [17,23,19]. It has been proposed that the x-axis intercept is evidence of the true threshold to vitamin D deficiency and provides a more reliable inflection point than the conventional serum PTH v serum 25-OH-D plot [23]. Another condition in which serum 24,25-(OH)₂D₃ levels are compromised is chronic kidney disease, presumably due to loss the nephrons containing the CYP24A1 protein, although there is school of thought that elevated FGF23 levels due to phosphate retention should induce higher CYP24A1 enzyme activity since it leads to higher CYP24A1 mRNA expression [24]. It is our experience that

dialysis patients have elevated 25-OH-D₃:24,25-(OH)₂D₃ ratios due to lack of 24,25-(OH)₂D₃ production [25,26].

The other application of vitamin D metabolite profiling using LC-MS/MS has been in animal research especially in study of knockout mouse models. The utility of measuring multiple vitamin D metabolites simultaneously in small serum volumes from individual animals is attractive, especially if it enables sampling without sacrificing the animals and avoidance of pooling of samples. We have now applied our method for the common vitamin D metabolites to several mouse models including: CYP2R1-null, CYP27A1-null, CYP2R1/CYP27A1-double null, CYP27B1-null, CYP24A1-null and VDR-null [17,27-29]. We have been impressed by the amazingly small inter-animal reproducibility in any of these models, presumably reflecting the similarities of genetic background, gender, dietary intake and age of the animals. **Table 2** presents the similarity between individuals in two groups of mice representing CYP24A1-null mice and their CYP24A1 heterozygous littermates. The biological variability for most vitamin D metabolites averages <10%.

Another aspect brought out by LC-MS/MS-based animal studies is the species difference in vitamin D metabolism between various rodents and humans. *In vitro* research into polymorphisms and mutations of human and rodent CYP24A1 [29,30] has previously revealed that there are versions of CYP24A1 that primarily 23-hydroxylate 25-OH-D₃ and generate 25-OH-D₃-26,23-lactone (e.g. opossum and guinea pig); whereas there are other versions of CYP24A1 that primarily 24-hydroxylate 25-OH-D₃ and generate 24,25-(OH)₂D₃ and other side-chain truncated products (e.g. mouse and human) [30, 31]. These observations are also mirrored in the *in vivo* serum metabolic profiles from humans and rodents where the 25-OH-D₃-26,23-lactone is a trace metabolite in humans; is easily detectable in mice; and is the dominant product of 25-OH-D₃ in guinea pigs (**Figure 1**). The MRM m/z 762->468 for dihydroxyvitamin D₃ reveals two adduct peaks that co-migrate with both 23,25-(OH)₂D₃ and 24,25-(OH)₂D₃. In the case of all three species: human, mouse and guinea pig, these peaks are sensitive to sodium

m-periodate, an oxidizing agent that cleaves vitamin D compounds with vicinal hydroxyls [32], suggesting that these peaks are mainly derived from 24,25-(OH)₂D₃. While there is little doubt that animal studies have benefitted our understanding of vitamin D metabolism in all mammalian species, it is important to note the small differences between species that are highlighted by the LC-MS/MS-based vitamin D metabolite profiling.

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Figure Legend:

LC-MS/MS-based vitamin D metabolic profiles of serum extracts of representative normal human, mouse and guinea pig. Vitamin D metabolites are extracted, derivatized with DMEQ-TAD and then subjected to LC-MS/MS as described in [17]. Depicted are selected multiple reaction monitoring (MRMs) representing human (top traces), mouse (middle traces) and guinea pig (lower traces):

Left panels: the monohydroxyvitamin D₃ daughter fragments at m/z 746->468;

Middle panels: the dihydroxyvitamin D₃ daughter fragments at m/z 762->468; and

Lower panels: the 25-OH-D₃-26,23-lactone daughter fragment at m/z 762->468.

Note that each vitamin D metabolite generates two adduct peaks when derivatized with DMEQ-TAD. Retention times of metabolites, based upon authentic standards, and quantitation is assessed by use of deuterated internal standards. Concentrations of vitamin D metabolites:

Human: 25-OH-D₃=31.8 nmol/L; 24,25-(OH)₂D₃=1.5 nmol/L; 25-OH-D₃-26,23-lactone=0.3 nmol/L

Mouse: 25-OH-D₃= 10.8 nmol/L; 24,25-(OH)₂D₃=3.3 nmol/L; 25-OH-D₃-26,23-lactone=1.0 nmol/L

Guinea Pig: 25-OH-D₃=27.0 nmol/L; 24,25-(OH)₂D₃=4.5 nmol/L; 25-OH-D₃-26,23-lactone=11.3 nmol/L

Table 1: Average 25-OH-D values and % bias of different methods for serum samples # 466-470 circulated by DEQAS in January 2015 compared to a NIST Reference method.

n	NIST Ref. Method 4	DEQAS ALTM [†] All users 977	DEQAS LC-MS Users 155	DEQAS Liaison Users 257	DEQAS IDS-iSYS Users 114	DEQAS Roche Users 147
#466	64.5	66.2 (bias+2.7%)	65.9 (bias+2.2%)	63.8 (bias-1.1%)	70.9* (bias+9.9%)	61.5 (bias-4.7%)
#467	44.4	42.3 (bias-4.7%)	45.3 (bias+2.0%)	38.2* (bias-14.6%)	49.8* (bias+21.6%)	37.8* (bias-14.8%)
#468	68.3	64.2 (bias-6.0%)	69.1 (bias+1.2%)	53.8* (bias-21.3%)	72.4 (bias+6.0%)	70.5 (bias-1.0%)
#469	69.3	64.6 (bias-6.8%)	69.5 (bias+0.1%)	58.9* (bias-15.0%)	72.1 (bias+4.0%)	63.1 (bias-8.9%)
#470 [§]	118.4	128.4 (bias+8.4%)	121.6 (bias+2.7%)	115.6 (bias-2.4%)	134.7* (bias+13.8%)	145.5* (bias+22.9%)

All 25-OH-D values are in nmol/L

[†] ALTM = All Laboratory Trimmed Mean of all methods

* Bias is equal to or greater than $\pm 10\%$

[§] Sample #470 contained a high endogenous level of 24,25-(OH)₂D₃

Table 2: Vitamin D Metabolic Profiles of Individual homozygous or heterozygous CYP24A1-null mice

Genotype	Gender	25-OH-D ₃ nmol/L	24,25-(OH) ₂ D ₃ nmol/L	25-OH-D ₃ : 24,25-(OH) ₂ D ₃ Ratio	25-OH-D ₃ - 26-23-lactone nmol/L	3-epi-25-OH-D ₃ nmol/L
CYP24A1+/-	M	30.3	14.8	2.0	9.3	3.3
CYP24A1+/-	M	38.0	19.5	1.9	10.8	4.0
CYP24A1+/-	M	43.3	21.8	2.0	10.3	3.5
CYP24A1+/-	M	40.0	20.5	2.0	9.0	3.5
CYP24A1+/-	F	35.0	18.8	1.8	10.5	3.8
CYP24A1+/-	F	38.5	21.3	1.8	9.8	3.8
CYP24A1+/-	F	36.8	23.0	1.6	12.0	3.3
CYP24A1+/-	F	38.3	19.8	2.0	10.0	4.0
Average (n=8)		37.5	20.0	1.9	10.3	3.8
SD		3.5	2.3	0.1	1.0	0.3
CYP24A1-/-	M	251.0	2.8	90.4	0.3	7.5
CYP24A1-/-	M	259.5	2.5	102.0	0.3	7.5
CYP24A1-/-	M	247.8	3.3	78.4	0.3	8.8
CYP24A1-/-	F	260.0	3.5	75.7	0.3	7.8
CYP24A1-/-	F	277.3	3.3	87.1	0.3	7.8
CYP24A1-/-	F	289.8	3.3	86.5	0.3	9.5
CYP24A1-/-	F	211.0	3.0	73.4	0.3	6.3
Average (n=7)		256.5	3.0	84.8	0.3	7.8
SD		23.0	0.3	9.2	0.0	1.0

Adapted and expanded from data presented in reference 17.
Animals and serum courtesy of Rene St-Arnaud, McGill University.

Figure