INFANT FORMULA AND ADULT NUTRITIONALS

A Rapid Method for the Determination of Vitamin D_3 in Milk and Infant Formula by Liquid Chromatography/Tandem Mass Spectrometry

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A rapid method for the determination of vitamin D_3 applicable to milk and infant formula products is described. Samples are saponified at high temperature, and lipophilic components are extracted into isooctane in a single tube. Vitamin D_3 is derivatized with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) to form a Diels–Alder adduct, which is re-extracted into a small volume of acetonitrile and analyzed by UHPLC-MS/MS with quantification accomplished by an internal standard technique utilizing deuterium-labeled vitamin D_3. The analysis of vitamin D_3 as the PTAD adduct offers a significant increase in sensitivity and selectivity, allowing for rapid sample preparation and short chromatographic run times. The method was shown to be accurate, with spike recoveries of 94.7–104.7% and no statistical bias against both a certified reference material ($P = 0.37$, $\alpha = 0.05$) and a reference LC-UV analytical method ($P = 0.09$, $\alpha = 0.05$). Acceptable precision was confirmed with a repeatability RSD of 1.4–4.5% and corresponding HorRat values of 0.1–0.2. This high-throughput method is ideal for routine compliance testing, with more than 50 samples/day achievable by a single analyst.

The two main forms of vitamin D of significance to human health are vitamin D_2 (ergocalciferol), which is obtained solely from plant-based food, and vitamin D_3 (cholecalciferol), which is obtained from both animal-based food and exposure of 7-dehydrocholesterol to UV radiation in the skin. Both vitamins D_2 and D_3 are further metabolized to their respective 25-hydroxy vitamers in the liver, which are the dominant circulating forms in blood. The main biological function of vitamin D is calcium homeostasis, controlling the absorption, transport, and deposition of calcium and phosphorus as part of bone mineralization. Vitamin D is currently a high profile area of research because of the association of vitamin D deficiency with increased rates of cancer, diabetes, and cardiovascular disease (1, 2).

Milk and infant formulas are commonly fortified with vitamin D, generally as vitamin D_3, and are subject to strict regulatory control. For routine compliance analysis of these products in high-throughput product release laboratories, accurate and precise analytical methods need also to be reliable, robust, rapid, and cost-effective. Many current methods for the analysis of vitamin D in foods are based on HPLC-UV. These methods require protracted sample preparation procedures to extract vitamin D from the sample matrix, remove interferences, and concentrate the low levels of vitamin D prior to chromatographic analysis (3–11).

LC-MS methods for the determination of the vitamin D content in foods have proliferated recently due to the commercial availability of stable isotope-labeled standards and because of the increased selectivity compared with the HPLC-UV techniques traditionally deployed (12–19). Commonly, these LC-MS methods involve saponification, solvent extraction, and concentration to achieve sufficient sensitivity during MS analysis. Despite the inherent selectivity of MS/MS, isobaric interferences from components such as sterols can still cause problems during analysis because of their structural similarity to vitamin D (20). Although issues related to accurate quantification using LC-MS may be addressed through the use of stable isotope-labeled internal standards, deuterium labeling remote from the conjugated triene of vitamin D_3 is essential due to an isotope effect on the thermal equilibrium between vitamin D_3 and previtamin D_3 (21).

Derivatization of vitamin D with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) occurs via a Diels–Alder mechanism involving the 1,3-conjugated diene structure of the secosteroid and has been previously described for the analysis of vitamin D and its hydroxylated metabolites in biological tissues. Derivatization improves both MS sensitivity through increased ionization efficiency of the adduct compared to unmodified vitamin D, and MS selectivity through increased analyte mass, thereby avoiding isobaric matrix interferences and eliminating the need for extensive cleanup or concentration prior to analysis (22–25). The use and attributes of this derivatization strategy have recently been described for the analysis of vitamin D in infant formulas (26).

The method described includes modifications to the method reported by Abernethy (26) and incorporates saponification prior to solvent extraction, PTAD derivatization, and UHPLC-MS/MS analysis. The method has been subjected to single-laboratory validation and has been found to be suitable for the compliance analysis of a range of milk and infant formulas fortified with vitamin D_3.

Experimental

Apparatus

A Nexera X2 UHPLC system consisting of two LC30AD pumps and a SIL-30AC autosampler, CTO-20AC column oven,

Received August 31, 2014. Accepted by SG October 23, 2014.
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DOI: 10.5740/jaoacint.14-183

Table 1. Chromatographic gradient elution program with oven temperature 35°C

		Mobile phase composition	
Time, min	Flow rate, mL/min	A, %[a]	B, %[b]
0	0.6	25	75
3.3	0.6	0	100
3.7	1.0	0	100
4.8	1.0	0	100
4.9	0.6	25	75

[a] Mobile phase A = formic acid (0.1%, v/v).

[b] Mobile phase B = methanol.

CBM-20A control module, and DGU-20A5R degasser unit (Shimadzu, Kyoto, Japan) was used and incorporated a Kinetex 50 × 2.1 mm, 2.6 µm core-shell RP C_{18} column (Phenomenex, Torrance, CA). MS was performed using a 6500 QTrap triple quadrupole detector (ABSciex, Foster City, CA). Analyst software (ABSciex) version 1.6 was used for instrument control and data processing.

Reagents

Reagent grade formic acid (Thermo Scientific, Waltham, MA) and potassium hydroxide and pyrogallol (Merck, Darmstadt, Germany) were obtained. Vitamin D_3 (cholecalciferol) and PTAD were supplied by Sigma-Aldrich (St. Louis, MO). Stable isotope-labeled vitamin D_3, 26,26,26,27,27,27-d6 cholecalciferol (d6-vitamin D_3), was supplied by Chemaphor Services (Ottawa, Canada). LC grade ethanol, acetone, and isooctane and LC-MS grade methanol and acetonitrile were supplied by Merck. Water was purified to 18.2 MΩ resistivity using a Barnstead Genpure water system (Thermo Scientific).

Standards

A d6-vitamin D_3 stock standard (10 µg/mL) was prepared by dissolving 1 mg in 100 mL ethanol; aliquots (approximately 1.3 mL) of this solution were stored at –30°C. A d6-vitamin D_3 internal standard solution was prepared by diluting 1 mL stock standard in 10 mL acetonitrile. A vitamin D_3 stock standard (1 mg/mL) was made by dissolving 50 mg in 50 mL ethanol; this solution was stored at <–15°C. A vitamin D_3 purity standard was prepared fresh each run by diluting 1 mL vitamin D_3 stock standard to 100 mL with ethanol. The accurate concentration of this solution was determined by UV absorbance measurement ($E^{1\%}_{1\,cm}$: 485 dL/g.cm at 265 nm; 27). A vitamin D_3 working standard was prepared by diluting 5 mL vitamin D_3 purity standard in 50 mL acetonitrile. Calibration standards were made by pipetting 250 µL d6-vitamin D_3 internal standard and 10, 50, 250, 500, or 1250 µL vitamin D_3 working standard into separate 25 mL volumetric flasks and diluting to volume with acetonitrile.

Samples

Whole milk powder samples, a range of over 40 different vitamin D_3 fortified infant and follow-on formulas, and National Institute of Standards and Technology (NIST) 1849a (Gaithersburg, MD) certified reference material (CRM) were used in this study.

Sample Preparation

Approximately 2 g of powder was weighed accurately into a 60 mL boiling tube, dispersed in 10 mL ethanolic pyrogallol (1%, w/v) to which 500 µL internal standard solution and 2 mL potassium hydroxide solution (50% w/v) were added, and vortex-mixed thoroughly. For samples in which homogeneity may be an issue, a 10 g aliquot of an aqueous slurry (20 g powder dissolved in 80 g water) was diluted into 10 mL ethanolic pyrogallol (1%, w/v), and internal standard and potassium hydroxide solutions were added. Saponification was performed at 70°C for 1 h with vortex mixing every 15 min. After cooling to room temperature, 10 mL isooctane was added and the mixture was shaken on a horizontal shaker for 10 min. A 20 mL aliquot of water was added, and the tube was centrifuged at 250 × *g* for 15 min. A 5 mL aliquot of the upper layer was transferred to a 15 mL disposable centrifuge tube, 5 mL of water was added to wash the isooctane extract, and the tube was centrifuged at 2000 × *g* for 5 min. The entire lower aqueous layer was removed to waste with a Pasteur pipet prior to the addition of 75 µL PTAD solution with vortex mixing. Derivatization was complete after 5 min at room temperature, and a 1 mL aliquot acetonitrile was then added and vortex-mixed. A 0.5 mL aliquot of the lower acetonitrile layer was transferred to a 2 mL Eppendorf vial containing 167 µL of water. After vortex mixing, the extract was syringe-filtered into an LC vial ready for analysis.

LC-MS Analysis

The mobile phases were formic acid (0.1%, v/v) and methanol, with high-pressure binary gradients formed in a manner similar to that described previously (26) and presented in Table 1. A switching valve was timed to divert column eluate to the ion source between 1.5 and 3.7 min, with the flow otherwise directed to waste. Analyte detection was achieved by electrospray ionization (ESI) in positive mode and multiple reaction monitoring (MRM) under the MS conditions presented in Table 2. Infusion experiments confirmed that fragmentation of PTAD adducts of vitamin D_3 and d6-vitamin D_3 produce a common major fragment of *m/z* 298, as has been previously reported (22–26). Unambiguous identification of both vitamin D_3 and d6-vitamin D_3 PTAD adducts was ensured by selecting a specific elution time window for monitoring characteristic quantifier and qualifier ions, with performance criteria for the ion ratios set at ±25%.

Method Validation

Detector linearity was assessed by analysis of five vitamin D_3 standard solutions covering the expected working range. Linearity was evaluated by least-squares regression analysis of peak area ratio versus concentration ratio, with a minimum value of 0.997 for the correlation coefficient (r^2) considered to be acceptable. Each standard concentration was estimated against the line of best fit with plots of residuals assessed as a further test of linearity.

Table 2. Parameters for MS

Analyte	Precursor ion, *m/z*	Product ions, *m/z*	DP,[a] V	EP,[b] V	CE,[c] V	CXP,[d] V	Dwell time, ms
D_3[e]	560.2	298.0[f]	151	10	21	18	120
		279.8[g]			37		
d6-D_3[h]	566.2	298.0[f]			21		
		279.8[g]			37		

[a] DP = Declustering potential.

[b] EP = Entrance potential.

[c] CE = Collision energy.

[d] CXP = Collision cell exit potential.

[e] D_3 = Vitamin D_3-PTAD derivative.

[f] Quantifier ion.

[g] Qualifier ion.

[h] d6-D_3 = Hexadeuterated vitamin D_3-PTAD derivative.

Method accuracy was determined as recovery, bias against the CRM, and bias against an independent reference method. Thus, recovery was determined by spiking unfortified whole milk powder with vitamin D_3 at 50 and 150% of the vitamin D_3 concentration (10 µg/hg) found in typical infant formulas. Bias against a CRM was evaluated by replicate analyses (n = 9) of NIST 1849a, and bias against a reference method (9) was evaluated by replicate analyses (n = 40) of a wide range of infant formula, follow-on formula, and whole milk powders (28).

Precision was evaluated as repeatability of five replicate analyses of four different infant formulas (n = 20) and intermediate precision from duplicate analyses of an infant formula by a single analyst on 7 different days (n = 14), with precision values calculated as RSD.

The method limit (ML) is the smallest amount of an analyte that can be quantified with reliability and is calculated from the method detection limit (MDL) estimated from the precision of the complete method applied to a sample containing low levels of analyte (29). The MDL was therefore estimated by replicate analysis (n = 10) of a whole milk powder sample containing endogenous levels of vitamin D.

The robustness of the method was assessed by conducting a Plackett-Burman trial (30) in the manner described previously (31). The seven factors assessed were water bath temperature (75 and 65°C), saponification time (70 and 60 min), isooctane volume (11 and 9 mL), shaking time (12 and 8 min), isooctane volume transferred (5.5 and 4.5 mL), PTAD volume (80 and 70 µL), and derivatization reaction time (7 and 3 min).

Results and Discussion

Method Optimization

The detection and quantification by MS of the vitamin D_3-PTAD adduct, compared with underivatized vitamin D_3, offer a number of advantages: the adduct is more readily ionized under ESI source conditions and produces fewer collision-induced dissociation fragments leading to a significant increase in signal to noise; there are fewer isobaric matrix interferences such as plant sterols, which are excluded from detection since they lack the conjugated diene structure of vitamin D_3; and the adduct is stable at room temperature for at least a week prior to analysis (22–26).

The method described was based on the strategy of direct solvent extraction of lipid and PTAD derivatization, as reported recently (26). However, during initial implementation, it was found that acceptable precision was difficult to achieve for some products, and a statistically significant positive bias of approximately 10% was observed relative to the reference OMA **2002.05** LC-UV method (9). An additional problem was experienced with dry-blended products for which vitamin D_3 is incorporated in an encapsulated form, since direct lipid extraction may be insufficient to rupture the starch matrix and release vitamin D_3. A saponification protocol used previously (32) was therefore incorporated as part of the method, resulting in improved precision and an absence of bias relative to the reference LC-UV method. Further, a slurry sample procedure was implemented for dry-blended products in order to mitigate against the consequences to precision due to sample heterogeneity.

Optimization of the ion source parameters was performed by direct infusion of PTAD adducts of vitamin D_3 and d6-vitamin D_3 standards. The two most intense MRM transitions were selected for quantification and unambiguous identification of both the analyte and internal standard. The chromatography was modified from that of Abernethy (26), with 0.2% ammonium formate substituted by 0.1% formic acid for mobile phase A. Coelution of the target analyte and deuterated internal standard mitigates against the potential for variable sample matrix effects on the ionization efficiency of the vitamin D_3-PTAD adduct (Figure 1).

To minimize the potential for peak splitting, water was added to the acetonitrile extract to provide an injection solvent composition equivalent to initial eluent conditions. This step also resulted in precipitation of non-saponifiable sterols (derived from either milk fat or vegetable oils), which were removed by membrane filtration, thereby protecting the column and eliminating peak artifacts.

Pre-vitamin D_3 was not routinely quantitated since the pre-vitamin D_3-PTAD adduct, although chromatographically resolved, does not form a 298 *m/z* fragment during analysis (26). Further, although previous studies have reported the separation of the 6R and 6S vitamin D_3-PTAD adduct stereoisomers (23, 25),

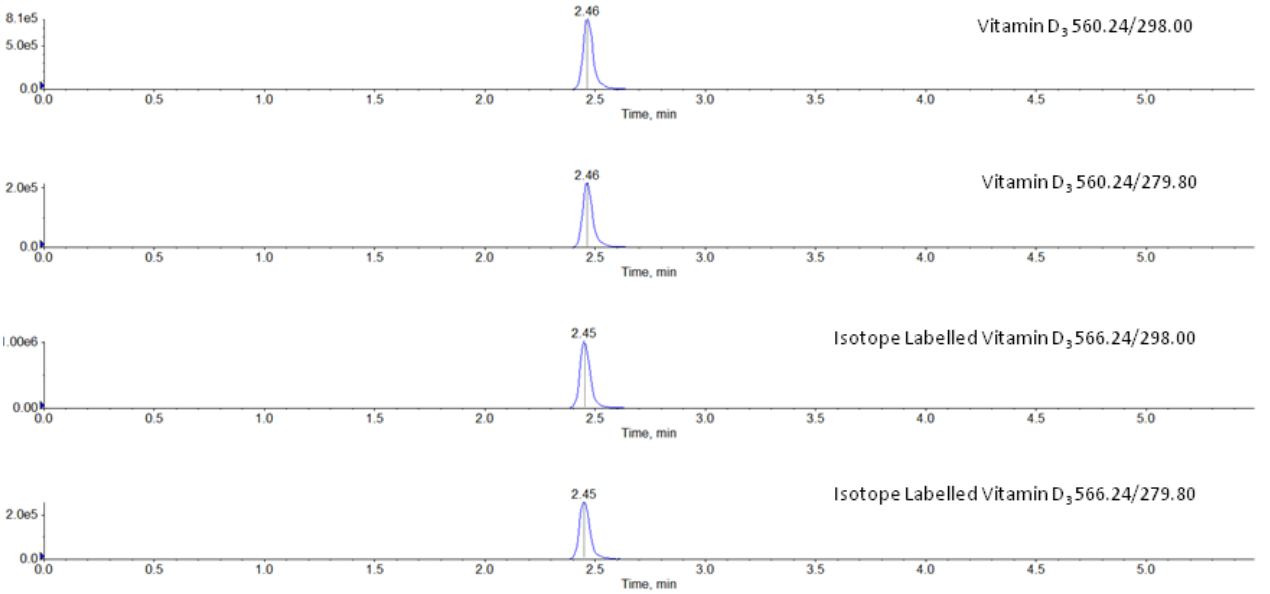

Figure 1. Chromatograms of quantifier and qualifier ions of PTAD-derivatized vitamin D_3 and PTAD-derivatized hexadeuterated vitamin D_3.

these coeluted under the conditions described in the current method. Furthermore, since the structures of vitamins D_3 and D_2 differ only in the alkyl side chain, the adaption of this method to vitamin D_2 should be readily achievable, provided that stable isotope-labeled vitamin D_2 is used as an internal standard.

The method described is capable of yielding a single result in approximately 4 h. Thus, in comparison with alternative LC-UV methods, the procedure is capable of significantly increasing sample throughput, with more than 50 samples/day completed by a single analyst.

Method Validation

Acceptable values for calibration linearity were obtained, with correlation coefficient $r^2 \geq 0.9990$, residuals showing no systematic pattern, and deviation from the line of best fit of ≤ 3.6%. Spike recovery values were 94.7–104.7% (mean = 99.3%, SD = 4.2%, $n = 4$). A P-value of 0.37 ($\alpha = 0.05$) was obtained for the CRM, indicating an absence of bias between measured results and the certified value. A P-value of 0.09 ($\alpha = 0.05$) demonstrated an absence of bias between results obtained by the described UHPLC-MS/MS method and the reference LC-UV method. A graphical representation of the correlation between these methods is illustrated in Figure 2. The values for the MDL and the ML were calculated as 0.04 and 0.14 µg/hg, respectively, suggesting that the described method is suitable for application to fortified infant formulas as these typically contain vitamin D_3 concentrations that are two orders of magnitude higher (approximately 10 µg/hg). Precision was evaluated as repeatability RSD (1.4–4.5%) and intermediate precision RSD (4.2%) yielding a HorRat of 0.1–0.2 that exceed the limits recommended in accepted guidelines (33). The method was found to be robust for the seven method performance parameters studied, with results normally distributed and variances conforming to that expected by chance (Figure 3). As with similar methods exploiting internal standard quantification, critical parameters include

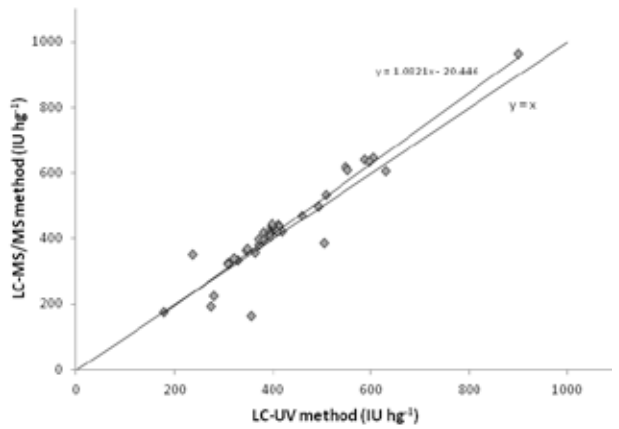

Figure 2. Comparison of vitamin D_3 in infant formula samples by LC-UV and LC-MS/MS.

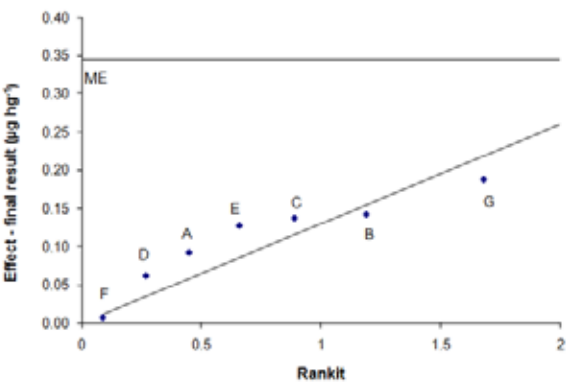

Figure 3. Half-normal plot of robustness results: A = water bath temperature; B = saponification time; C = isooctane volume; D = horizontal shaker time; E = isooctane transfer volume; F = PTAD volume; G = derivatization time; and ME = margin of error.

accurate measurement of sample weight and concentration and volume of the added internal standard.

Conclusions

A rapid method for the analysis of vitamin D_3 in milk and infant formulas is described. The analysis of a vitamin D_3-PTAD adduct by MS following derivatization offers significant advantages in sensitivity and selectivity over direct detection of vitamin D_3 by MS. The incorporation of a saponification procedure as part of this method gives improved precision and increases the method scope to a wider range of infant formula products. This method is intended for use in high-throughput laboratories as part of routine product compliance release during the manufacture of infant formulas and milk powders. The method was single-laboratory validated and found to be accurate, precise, and robust for the intended application of the analysis of milk and infant formula fortified with vitamin D_3.

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