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Quantification of vitamin D and 25-hydroxyvitamin D in soft tissues by liquid chromatography—tandem mass spectrometry

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Abstract

Inadequate data on tissue distribution of vitamin D and its metabolites remains a barrier to defining health outcomes of vitamin D intake and 25-hydroxyvitamin D (25(OH)D) status. The purpose of this study was to develop a method for the analysis of vitamin D₂ (ergocalciferol), vitamin D₃ (cholecalciferol), 25(OH)D₂, and 25(OH)D₃ in soft tissues, and determine distribution in select tissues from a dose-response study of vitamin D₂ and vitamin D₃ in rats. Liver, gastrocnemius muscle, and epididymal fat homogenates were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with electrospray ionization following liquid-liquid extraction, solid-phase extraction, and derivatization with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD). A dose-response was observed in most tissues for vitamin D and 25(OH)D from both vitamers. Vitamin D concentration was greater in epididymal fat than gastrocnemius muscle and liver, but 25(OH)D concentration was not significantly different between tissues. Soft tissues of rats fed crystalline vitamin D₃ had higher concentrations of total vitamin D than those of rats fed yeast-derived vitamin D₂, while total 25(OH)D concentrations were similar between vitamin D sources. This method is well suited to more complete studies of vitamin D bioavailability and metabolite tissue distribution.

Keywords

25-hydroxyvitamin D analysis; Vitamin D analysis; ergocalciferol; cholecalciferol; tissue distribution; LC-MS/MS

1. Introduction

Emerging evidence suggests that vitamin D may be involved in many non-skeletal health outcomes such as muscle and immune function, cardiovascular disease, type II diabetes, and cancer development in addition to its established role in the regulation of mineral balance and bone health [1]. A barrier to understanding the physiological role of vitamin D is the

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need to assess vitamin D status and vitamin D metabolite profiles beyond circulation in local tissues, where conversion to and action of bioactive metabolites can occur. Currently, the metabolism, storage, and functional roles of vitamin D are unclear due to lack of information on the tissue distribution of vitamin D and its metabolites.

Vitamin D₃ is derived from UV-induced cutaneous synthesis and animal-based dietary sources, while vitamin D₂ is derived from fungal and plant sources. Once in circulation, vitamin D is cleared by tissues and converted to 25-hydroxyvitamin D (25(OH)D) in the liver. The half-life of plasma 25(OH)D is 2–3 weeks, such that plasma 25(OH)D concentration reflects vitamin D status [2]. Evidence suggests that plasma 25(OH)D clears more rapidly from a single oral dose of vitamin D₂ than from vitamin D₃ [3,4], although bone outcomes [5] and plasma 25(OH)D concentration [6,7] with daily dosing of vitamin D₂ and vitamin D₃ have been similar. The active hormone form 1,25-dihydroxyvitamin D is produced in the kidney by 25-hydroxyvitamin D 1 α -hydroxylase (CYP27B1) under conditions of low serum calcium, and may also be produced in extrarenal tissues that express CYP27B1 to exert autocrine/paracrine action [8]. However, presence of 25(OH)D as a precursor for 1,25-dihydroxyvitamin D production in local tissues has not been adequately explored.

Many advances have been made towards the analysis of vitamin D metabolites from plasma/serum in recent years [9]. A national dialogue on the measurement of vitamin D status led by the NIH Office of Dietary Supplements has identified LC-MS/MS methodologies as the preferred approach [10] as immunoassays suffer from poor accuracy, poor repeatability, and interferences [11]. However, analysis of vitamin D and its metabolites from tissues by mass spectrometry remains a challenge due to poor ionization efficiency, matrix effects, and low concentrations relative to plasma. Diels-Alder derivatization with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) is a common approach to enhance sensitivity and reduce interferences [12,13]. Addition of methylamine to the mobile phase produces an $[M + CH_3NH_3]^+$ adduct with a greater response than the $[M + H - H_2O]^+$ ion achieved with ammonium mobile phase additives [14,15]. Solid-phase extraction is also commonly employed to reduce interferences from plasma [16,17], while stable isotope dilution allows for correction of matrix effects.

While advances in plasma/serum analysis have provided insight into vitamin D metabolism, tissue distribution of vitamin D metabolites has remained largely unexplored. Sensitive extraction and detection methods are required to measure these analytes in samples of limited size where vitamin D metabolite concentrations are low. Previous analyses of vitamin D and 25(OH)D have utilized large tissue samples [18,19] and preparative-HPLC [20], which limits application to routine analysis. While the vitamin D_{2/3} content of human adipose tissue has been examined by a more routine LC-MS/MS method [21,22], other tissues and metabolites of lower concentrations were not evaluated in those studies. The objectives of this study were to (1) develop methodologies for LC-MS/MS analysis of vitamin D_{2/3} and 25(OH)D_{2/3} in soft tissues, and (2) generate preliminary data on the distribution of these molecules in certain soft tissues. Quantification of vitamin D metabolites in soft tissues is critical towards understanding the metabolism of vitamin D and its subsequent physiological role.

2. Materials and Methods

2.1 Materials and Chemicals

Ergocalciferol (vitamin D₂), cholecalciferol (vitamin D₃), 25(OH)D₂, 25(OH)D₃, 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD), 40% w/w methylamine in water, dibasic potassium phosphate (K₂HPO₄), methyl *tert*-butyl ether (MTBE), acetonitrile (AcN), and

ethyl acetate (EtOAc) were purchased from Sigma-Aldrich (St. Louis, MO). Phosphate buffered saline (PBS) was purchased from Lonza (Walkersville, MD). Methanol (MeOH) was from JT Baker. Double distilled water (ddH₂O) was used for sample preparation and 18 M H₂O prepared with a MilliQ A10 system (Millipore, Bedford, MA) was used for LC-MS/MS. Isotopically labeled d6-vitamin D₃, d6-25OHD₂, and d6-25OHD₃ were from Medical Isotopes (Pelham, NH). Standard Reference Material (SRM) 1950 'Metabolites in Human Plasma' was obtained from the National Institute of Standards and Technology (NIST, Gaithersburg, MD).

2.1 Tissue Samples

Sprague-Dawley rat tissues were obtained from a previous vitamin D dose-response study by Hohman, et al. [5] that received approval from the Animal Care and Use Committee at Purdue University. Four-week old Sprague-Dawley rats were fed a vitamin D-deficient diet of 25 IU vitamin D₃ /kg diet (1 IU = 40 µg) for 7 weeks, then fed diets containing nominally 1000 or 25 IU or vitamin D/kg diet either from crystalline vitamin D₃ or vitamin D₂-rich yeast baked into bread for 8 weeks. After sacrifice, tissues were perfused with 180 to 240ml of saline pushed through the abdominal aorta, harvested, and stored at -80°C. Matched liver, epididymal fat, and gastrocnemius muscle tissues were utilized from n=4 animals in each group. Tissues were thawed, chopped finely, and then homogenized with a Branson 450 ultrasonicator in PBS to 10% w/w solutions for liver and epididymal fat or 5% solutions for gastrocnemius muscle, exact masses were recorded to determine mass fraction of tissue in the homogenate, and the homogenates were stored at -80°C until analysis. Plasma concentrations of 25(OH)D_{2/3} from the same n=4 rats per group were analyzed and reported previously [5].

2.2 Sample Preparation

Homogenates (400 µL), SRM 1950 plasma (100µL + 300 µL PBS), and method blank (400 µL PBS) were added to 2 mL eppendorf tubes, exact masses were recorded, samples were spiked with 10 µL of internal standard solution containing 100 ng/mL d6-vitamin D₃, 10 ng/mL d6-25-hydroxyvitamin D₂, and 10 ng/mL d6-25-hydroxyvitamin D₃ in AcN, vortex mixed, and equilibrated for 15 min. External calibration solutions of vitamin D₃, vitamin D₂, 25(OH)D₃, and 25(OH)D₂ from nominal ranges of 0.1–50 ng/mL were prepared, spiked with internal standard, and set aside. AcN (400 µL) was added before the mixture was vortex mixed for 5 min and centrifuged at 6,000×g for 1 min. MTBE (400 µL) was added, vortexed for 5 minutes, centrifuged, and the upper organic layer was collected. The extraction was repeated twice more with addition of 100 µL PBS, 400 µL AcN, and 400µL MTBE. The extract was dried under nitrogen, and then purified by solid phase extraction in a similar manner as Aronov et al. [17] and Ding et al. [15]. Oasis HLB cartridges (1 cc/30mg) were prepared with 1 mL each of EtOAc, MeOH, and ddH₂O. The samples were reconstituted with 700 µL MeOH, diluted with 300 µL 0.4 M K₂HPO₄, and then loaded on the cartridge at 1 ml/min. The cartridge was washed with 1 mL of each 30% and 70% aqueous MeOH, dried for 2 minutes under vacuum, and the tips were wiped dry. Analytes were eluted with 2 mL AcN then dried under nitrogen alongside external calibration standards for 1 hour at 35°C before derivatization. To derivitize the samples and external calibration standards, 100 µL of 2 mg/mL PTAD in AcN was added to the dried solutions and vortex mixed for 10 min, followed by an additional 100 µL, and vortex mixed for 10 minutes. The reaction was quenched by adding 20 µL ddH₂O and vortexing for 5 min. Samples were dried, reconstituted in 100 µL AcN, centrifuged for 5 min at 18,000×g, and transferred to HPLC vials.

2.3 LC-MS/MS

Separation was performed on an Agilent Rapid Res 1200 HPLC system using an Agilent Zorbax SB-C18 2.1×50mm 1.8µm column following a 2µm frit filter. The column compartment and autosampler were maintained at 25°C and 10°C, respectively. Mobile phase A was 18 M H₂O with 0.1% formic acid and 5mM methylamine, mobile phase B was AcN with 0.1% formic acid and 5 mM methylamine, and mobile phase C was EtOAc. Mobile phases A and B were prepared daily as degradation of methylamine resulted in a decrease of signal. Linear gradient elution was used at a constant flow rate of 0.5mL/min as follows: initial conditions 50% B; 0–2.5 min: gradient to 80% B; 2.5–6 min: gradient to 90% B; 6–7 min: gradient to 100%B; 7–9 min: hold at 100% B; 9–14 min: flush with 100%C; 14–18 min: hold at 100%B; 19–20 min: gradient to 50% B; 20–24 min. Eluent was transferred to the MS/MS from 2–9 min, and diverted to waste at all other times. The injection volume was 10 µL, and the needle was washed with 1:1 water:isopropanol for 10 seconds between runs to minimize carryover.

Analytes were quantified using tandem mass spectrometry (MS/MS) with an Agilent 6460 triple quadrupole with electrospray ionization (ESI) in positive ion mode. Selected Reaction Monitoring mass transitions are given in Table 1. All mass transitions used dwell time = 50 ms and fragmentor voltage = 100 V. Other parameters were as follows: electric multiplier voltage = 400 V, nitrogen gas flow rate = 9 L/min, nebulizer pressure = 40 psi, sheath gas temperature = 250°C, sheath gas flow rate = 7 L/min, capillary potential = 3000 V. Analyte responses were corrected for heavy standard recovery by Agilent MassHunter 1.4 software. Concentrations of analytes in solution were converted to tissue concentrations using mass fraction of tissue in the homogenate and mass of homogenate extracted.

2.4 Single Lab Method Validation

2.4.1 Accuracy—NIST SRM 1950 (metabolites in human plasma) was obtained for quality control since NIST 972 (vitamin D in human serum) was unavailable and pending re-issue. Accuracy was determined with n=3 repeated on 3 days by the following equation: (mean of measured value / NIST certified or reference value) × 100%.

2.4.1 Repeatability—Intraday repeatability was assessed by analyzing the same liver, epididymal fat, and gastrocnemius muscle homogenates n=5 times. Interday repeatability was determined across n=3 days with SRM 1950. Repeatability was determined as relative coefficient of variation (%CV): (standard deviation / mean) × 100%.

2.4.2 Recovery—Recovery was determined as (internal standard response from the sample / mean internal standard response from external calibration standards) × 100%, as external calibration curves were analyzed within each set. All samples and standards were analyzed by the ratio of the analyte to the corresponding isotopically labeled internal standard. Heavy isotopes d6–25(OH)D₃ and d6–25(OH)D₂ were internal standards for 25(OH)D₃ and 25(OH)D₂. d6-Vitamin D₃ served as the internal standard for both vitamin D₃ and vitamin D₂.

2.5 Tissue Pools

Tissue distribution was derived from a mass basis to a per tissue basis. Plasma pool size was estimated as 4.12 mL per 100 g body weight [23].

2.6 Data Analysis

Data are represented as mean ± standard deviation. ANOVA analysis was completed using SAS 9.3 software (Cary, NC) with a factorial model of dose, vitamin D source, and tissue

type. Pairwise comparisons were made with Tukey's test at significance level of $\alpha = 0.05$. Signal-to-noise ratio (S/N) was calculated by Agilent MassHunter software using the ASTM algorithm.

3. Results and Discussion

3.1 Chromatographic Separation

25(OH)D₃ (rt = 2.8 min) and 25(OH)D₂ (rt = 3.1 min) were resolved, and vitamin D₃ co-eluted with vitamin D₂ (rt = 6.7 min) but the vitamers were distinguishable by different mass transitions (see Figure 1). Even though vitamin D₃ and vitamin D₂ likely have very similar extraction efficiencies and co-eluted, use of d₆-vitamin D₃ as internal standard for both of these analytes may have contributed additional bias due to non-identical ionization. Flushing the column with EtOAc between each injection was found to minimize signal suppression after consecutive injections. Derivatization by PTAD formed 6R and 6S stereoisomers in a 4:1 ratio [14,15] that were resolved for each metabolite, and the minor 6S isomers were not used in quantification. C-3 epimers of metabolites were not confirmed to be separated, as their separation might have required two isocratic periods and increased chromatographic run time. Since 3-epi-25(OH)D₃ accounted for 0–20% of total 25(OH)D₃ in adult human serum [24], further investigation into the tissue distribution of C-3 epimers might be warranted.

3.2 Detection Limit

Limit of detection (LOD), defined as S/N = 3, was 0.1 ng/mL (0.26 nmol/L, or 2.6 fmol on column) for all analytes. The LOD after sample preparation was 0.1 ng/g tissue (0.26 picomol/g) when extracting 400mg of a 20% w/w tissue homogenate. Additional significant m/z transitions were not observed. Vitamin D and 25(OH)D were not detected in some tissues of animals fed a low dose of vitamin D (16 IU D₃ or 49 IU D₂/kg diet), but it is important to note that the 25(OH)D plasma levels of these animals were extremely low (<5 ng/g). Vitamin D and 25(OH)D were detectable in tissues of rats fed 1000 IU/kg diet, which corresponded to marginal 25(OH)D plasma levels [5]. Therefore, it is expected that this method will be suitable to assay vitamin D and 25(OH)D from tissues of free-living human donors with marginal to sufficient vitamin D status.

3.3 Linearity

Linearity for all analytes was confirmed from 0.1–1000 ng/mL, which covers well beyond the range of 25(OH)D in plasma. Routine standardization covered 0.1–50 ng/mL to focus on NIST SRM 1950 and tissue homogenates.

3.4 Method Validation

3.4.1 Accuracy—Due to the unavailability of NIST SRM 972 'Vitamin D in Human Serum' at the time of the study, accuracy was determined by comparison to NIST 1950 'Metabolites in Human Plasma' which has a certified value for 25(OH)D₃ and a reference concentration for 25(OH)D₂ with greater uncertainty. Observed values of NIST SRM 1950 were 19.0±1.5 ng/mL 25(OH)D₃ (78% of the certified value, –22% error) and 0.66±0.05 25(OH)D₂ (129% of the reference concentration, 29% error), as shown in Table 2. These errors are greater than recommended in the FDA's Guidance for Industry on Bioanalytical Method Validation [25]. Poor accuracy in comparison to the NIST certified value for 25(OH)D₃ was likely due to error in preparation of calibration solutions, and may not reflect the inherent accuracy of the method. Errors in calibration are often the source of disagreement between labs for vitamin D metabolite analysis, [11], and accuracy be improved with use of NIST SRM 2972 25-hydroxyvitamin D calibration solution. However,

this calibrant was not utilized in the present study because it does not include vitamin D_{2/3}, targeted analytes in this study. The observed value of 25(OH)D₂, though 22% from the reference concentration, was within large expanded uncertainty reported by NIST in this material. While use of in-house prepared quality control samples may yield greater accuracy, standard reference materials more fully portray inter-lab standardization [10]. Vitamin D₃ and vitamin D₂ were found in SRM 1950 at 3.7±0.8 and 0.4±0.4 ng/mL, respectively, but certified values for vitamin D₃ and vitamin D₂ are lacking for SRM 1950 and SRM 972.

3.4.2 Repeatability—Interday and intraday repeatability of vitamin D and 25(OH)D from plasma and soft tissues are shown in Table 3. Interday CV for SRM 1950 was 7.6–7.8% for 25(OH)D₂ and 25(OH)D₃, similar to that observed from other methods assaying 25(OH)D by PTAD derivatization (3.7–6.8% [15], 4–14% [26]). Intraday CV for 25(OH)D₂ and 25(OH)D₃ from SRM 1950 was 9.6–10%, comparable to previous assays (1.6–4.1% [15], 3–12% [26]) suggesting that the repeatability of the LC-MS method is reasonable both within and across days. Intraday CV was larger for tissue homogenates than for SRM 1950, 5–44%, suggesting that the poor precision relative to plasma was due to inhomogeneity within tissues and/or homogenate sampling. Vitamin D may be similar to vitamin A—another fat soluble vitamin—in that the distribution within a particular tissue is highly heterogeneous, as the CV for vitamin A was shown to be as large as 63% in human liver and 31% in rat liver [27]. Therefore, applications of this method using biopsy samples should proceed with caution. Increased matrix complexity for tissues in comparison to plasma may also explain, in part, the large observed repeatability from soft tissues. Regardless, repeatabilities such as 2–20% [28] and 5–22% [29] have been reported for LC-MS/MS analysis of steroids from soft tissues. Future improvements to this method may consider extracting greater sample size and additional enzymatic treatment of tissues before or after homogenization, though at a trade-off for additional cost, efficiency, and sample throughput.

3.4.3 Recovery—Recoveries of isotopically labeled internal standards are shown in Table 4. Recovery from method blanks averaged 96±19, 100±20, and 148±41 for d6-25(OH)D₃, and d6-25(OH)D₂, and d6-vitamin D₃, respectively. While the injector and column were flushed with EtOAc between each sample, variable recovery observed from method blanks may be due, in part, to carryover of interferences retained on the column causing intermittent ion suppression on subsequent injections. Recovery of d6-vitamin D from soft tissues was low (23–38%), but higher from SRM 1950 plasma (53±12%) and method blanks (148±41), suggesting poor recovery from tissues was due to matrix effects and not poor extraction recovery. Recovery of d6-25(OH)D_{3/2} from soft tissues ranged from 53–128%, which is comparable to observed recoveries of 58–85% for 25(OH)D from plasma [15] and 70–112% for steroids from soft tissues [28].

3.5 Tissue Distribution

Tissue concentrations of vitamin D₃, vitamin D₂, total vitamin D (vitamin D₃ + vitamin D₂), 25(OH)D₃, 25(OH)D₂, and total 25(OH)D are shown in Figure 2. Statistical analysis utilized an ANOVA factorial model of tissue type, dose, and vitamin D form, as well as pairwise-comparisons using Tukey's test. Total vitamin D accumulation was greater ($p<0.05$) in epididymal fat tissue (4.6–7.6 ng/g) than gastrocnemius muscle (not detected–2.0 ng/g) and liver tissue (0.1–3.9 ng/g) for high doses of both vitamin D₂ and D₃. Total vitamin D concentration was significantly greater ($p<0.05$) in rats fed 740 IU crystalline vitamin D₃ per kg diet than those fed 1064 IU vitamin D₂ per kg diet from enriched-yeast bread in epididymal fat and liver tissue. Most notably, vitamin D concentrations in epididymal fat were reflected by 25(OH)D concentrations in plasma when comparing across vitamin D sources of 1000 IU/kg diet: 7.6±0.3 ng/g vitamin D₃ vs 4.6±1.0 ng/g vitamin D₂ in

epididymal fat tissue, and 19 ± 3 ng/mL 25(OH)D₃ vs 11 ± 2 ng/mL 25(OH)D₂ in plasma. This is in great agreement with Heaney et al. [22], who reported the adipose concentration of vitamin D and plasma concentration of 25(OH)D was about 40% lower from vitamin D₂ than from vitamin D₃ in humans.

Conclusions on 25(OH)D tissue distribution are difficult to draw due to the limited concentrations observed relative to the sensitivity and repeatability of the assay. 25(OH)D concentrations of soft tissues ranged from 0.5–1.0 ng/g, over 10-fold lower than that of plasma. Pairwise comparisons between high dose vitamin D₃ vs high dose vitamin D₂ for total 25(OH)D concentration in epididymal fat, gastrocnemius muscle, and liver tissue were not significantly different, although the main effect of vitamin D source was significant overall in the model ($p=0.02$). A dose effect was significant ($p<0.05$) for total 25(OH)D concentrations in epididymal fat, gastrocnemius muscle, liver, and plasma. In contrast to vitamin D, 25(OH)D concentrations were not significantly different ($p>0.05$) between liver, gastrocnemius muscle, and epididymal fat tissues of rats fed ~1000 IU per kg diet, and the main effect of tissue was also not significant ($p=0.72$). In contrast, Jackobsen et al. [20] reported that 25(OH)D₃ concentrations were different across soft tissues of pigs fed 2000 IU vitamin D₃/day (adipose 1.9 ng/g, liver 4ng/g, muscle 1.1 ng/g). However, that study determined very similar levels of vitamin D₃ as the current study (adipose 7 ng/g, liver 2.7 ng/g, muscle 1 ng/g).

3.6 Tissue Pools

Tissue distribution of vitamin D and 25(OH)D derived on a per tissue basis are shown in Table 5. While extrapolating this data to entire tissue pools (i.e. all adipose tissue or muscle tissue) would be of ultimate interest, it cannot be assumed that the metabolite concentration is the same across different depots of the same tissue type. Plasma 25(OH)D seems to be the greatest pool of vitamin D metabolites, although some vitamin D seems to be deposited in epididymal fat (and liver for vitamin D₃) before being hydroxylated. Considering the 740 IU crystalline vitamin D₃/kg diet group, the liver appears to contain more vitamin D₃ than epididymal fat (48 ng vs 31 ng), although it is likely that additional adipose depots contribute to vitamin D storage. An unexpected observation is that muscle and liver did not retain as much vitamin D₂ as vitamin D₃, even after considering the reduced 25-hydroxyvitamin D status from vitamin D₂ in comparison to vitamin D₃. This raises the possibility that metabolism of vitamin D₂ may differ from that of vitamin D₃ in the liver.

4. Conclusions

The current study presents a method of vitamin D metabolite analysis from soft tissues that may be used to study the status, metabolism, and bioavailability of vitamin D. The advantage of this method is the simultaneous analysis of vitamin D in addition to 25(OH)D with a single sample preparation and chromatographic run. While most attention is given to circulating 25(OH)D levels, additional assessment of native vitamin D in soft tissues may better describe vitamin D status, and is necessary to fully assess vitamin D metabolism and bioavailability. The greatest limitation of this study is poor repeatability, which suggests that a greater number of replicates will be necessary to converge on the true mean. In application, an individual tissue would need to be analyzed multiple times to be compared to another. Further focus on the analysis of vitamin D from soft tissues may focus on tissue homogenization, which is not only a limiting step in sample throughput, but also could likely improve repeatability.

Despite these limitations, the current method shows promise in assessing vitamin D metabolism and bioavailability. Preliminary data from a dose-response study in rats contributes further evidence that adipose tissue accumulates vitamin D, and that circulating

25(OH)D is the primary vitamin D tissue pool. While vitamin D concentrations in soft tissues were greater from crystalline vitamin D₃ than similar doses of yeast-derived vitamin D₂, total 25(OH)D levels in soft tissues were similar from the two dietary sources. Further, these data suggest that adipose, muscle, and liver accumulate similar concentrations of 25(OH)D, which may serve as a temporary storage pool and/or substrate for production of the active hormone form 1,25-dihydrovitamin D. Further assessment of vitamin D and vitamin D metabolites in soft tissues will allow for more complete understanding of the biological actions of vitamin D intake and status.

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Abbreviations

PTAD	4-phenyl-1,2,4-triazoline-3,5-dione
PBS	phosphate buffered saline
IU	International Units

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Highlights

- Method developed for liver, muscle, adipose tissue, and plasma
- Dose response of tissue vitamin D reflected plasma 25(OH)D in most tissues
- Vitamin D₃ concentration was higher than D₂ in liver, muscle, and adipose
- Vitamin D concentrations were greater in adipose tissue than liver and muscle
- 25(OH)D was similar across soft tissues and an order of magnitude lower than plasma

External standard (1 ng/mL) **Extract of adipose tissue (1064 IU D2/kg diet)** **Figure 1.**

Chromatographic separation for each mass transition. Peak identification: a: 6S-PTAD-25(OH)D₃; b: 6R-PTAD-25(OH)D₃; c: 6S-PTAD-25(OH)D₂; d: 6R-PTAD-25(OH)D₂; e: 6S-PTAD-vitamin D₃; f: 6R-PTAD-vitamin D₃; g: 6S-PTAD-vitamin D₂; h: 6R-PTAD-vitamin D₂. Minor 6S-stereoisomers formed during derivatization were not used for quantitation

Figure 2.

Tissue Distribution of vitamin D and 25(OH)D in ng/g tissue from n=4 animals per group. Asterisks (*) represent significant pairwise comparisons ($p < 0.05$) between low and high dose groups of the same vitamin D source (dose-effect). Pound signs (#) represent significant pairwise comparisons ($p < 0.05$) between 740 IU vitamin D₃/kg diet group and 1064 IU vitamin D₂/kg diet group (source effect).

Table 1

MRM Transitions and Collision Energies

Analyte	[M-CH ₃ NH ₂] ⁺ m/z	[Fragment] ⁺ m/z	Collision Energy (V)
vitamin D ₃ -PTAD	591.4	298.1	15
d6-vitamin D ₃ -PTAD	597.4	298.1	15
vitamin D ₂ -PTAD	603.4	298.1	15
25(OH)D ₃ -PTAD	607.4	298.1	20
d6-25(OH)D ₃ -PTAD	613.4	298.1	20
25(OH)D ₂ -PTAD	619.4	298.1	20
d6-25(OH)D ₂ -PTAD	625.4	298.1	20

Table 2

Accuracy of NIST SRM 1950 Plasma.

Value	25(OH)D ₃	25(OH)D ₂	vitamin D ₃	vitamin D ₂
Observed (ng/g) (n=3×3)	19.0 ± 1.5	0.7 ± 0.1	3.7 ± 0.8	0.4 ± 0.4
NIST certified or reference value (ng/g)	24.3 ± 0.8	0.5 ± 0.2	-	-
Accuracy (% NIST value)	78%	129%	-	-

Table 3

Interday (n=3) and intraday (n=5) repeatability from select soft tissues and NIST SRM plasma.

Analyte	Liver	G. Muscle	E. Fat	NIST SRM 1950 Plasma
Intraday vitamin D ₃ (%CV) n=5	5.4	26.6	43.5	15.1
Intraday 25(OH)D ₃ (%CV) n=5	39.8	34.8	26.6	10.0
Interday vitamin D ₃ (%CV) n=3	-	-	-	21
Interday 25(OH)D ₃ (%CV) n=3	-	-	-	7.7

Table 4

Recovery of isotopically labeled internal standards.

Internal Standard	Liver n=8	G. Muscle n=8	E. Fat n=8	NIST SRM n=9	Method Blank n=4
d6-vitamin D ₃ (%)	38 ± 17	36 ± 11	23 ± 6	53 ± 12	148 ± 41
d6-25(OH)D ₃ (%)	103 ± 29	113 ± 31	128 ± 18	118 ± 5	96 ± 19
d6-25(OH)D ₂ (%)	58 ± 11	74 ± 21	126 ± 20	95 ± 1	100 ± 20

Table 5

Vitamin D and 25(OH)D on a per tissue basis from n=4 animals (ng/tissue)^{1,2}

Source			16 IU crystalline D ₃ /kg diet				740 IU crystalline D ₃ /kg diet			
Tissue	E. Fat	G. Muscle	Liver	Plasma	E. Fat	G. Muscle	Liver	Plasma	E. Fat	G. Muscle
Total Vitamin D (ng)	5.5 ± 3.0	1.8 ± 1.2	0.9 ± 1.1	na	30.6 ± 1.3	5.5 ± 3.6	48 ± 24	na		
Total 25(OH)D (ng)	0.2 ± 0.3	nd	nd	51 ± 41	2.0 ± 1.0	1.5 ± 0.6	5.9 ± 2.6	335 ± 51		

Source			49 IU yeast derived D ₂ /kg diet				1064 IU yeast derived D ₂ /kg diet			
Tissue	E. Fat	G. Muscle	Liver	Plasma	E. Fat	G. Muscle	Liver	Plasma	E. Fat	G. Muscle
Total Vitamin D (ng)	6.4 ± 7.4	1.1 ± 2.2	nd	na	18 ± 4	nd	2.7 ± 5.3	na		
Total 25(OH)D (ng)	0.1 ± 0.3	0.7 ± 1.4	nd	13 ± 2	4.6 ± 1.4	2.8 ± 1.1	11.7 ± 9.9	199 ± 37		

¹ na: not analyzed
² nd: not detected