Vitamin D in human serum and adipose tissue after supplementation.

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Supplemental Methods

Quantification of cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2) in serum

Serum samples were prepared in a 2 mL deep 96 well plate (Greiner Bio-One). A volume of 200 µL of serum, calibrators, or control materials was added to 200 µL of 1 M NaOH. Samples were covered with a PTFE/silicone cover (Agilent) and vortexed for 15 s and then incubated for 15 min at room temperature (RT). Each sample was then spiked with 200 µL of internal standard (5 ng/mL), covered and vortexed for 15 s. Cholecalciferol (D_3) and ergocalciferol (D_2) were extracted with either 1 mL 90:10 *n*-heptane:ethyl acetate (Pilot Study 1) or 50:50 *n*-heptane:methyl tert-butyl ether (MTBE) (Pilot Study 2). Samples were covered and vortexed on a multitube vortexer (VWR, speed 10) for 5 min and then centrifuged at 2000 rpm (493 *g*) for 4 min (Beckman-Coulter Allegra X-22). The plate was fitted with a liquid transfer gasket *(15)* and another 96-deep well plate, and the bottom aqueous layer was frozen by placing samples in a dry-ice acetone bath. After 50 min, the top organic layer was transferred to the new 96-well plate by inverting and tapping the plate. The extraction plate was removed and discarded. The organic layer was dried using nitrogen in a TurboVap (Caliper Life Sciences, plate temperature 35°C, gas flow 30) for 40 min. The residue was derivatized with 100 µL PTAD in acetonitrile (0.5 g/L). The 96-deep well plate was covered, vortexed for 15 s, and incubated at room temperature for 15 min. A volume of 100 µL LC/MS-grade water was then added to quench the PTAD.

Quantification of cholecalciferol and 25-hydroxycholecalciferol (25(OH)D_3) in adipose tissue

Approximately 10 mg of subcutaneous adipose tissue was weighed out and placed in a 2 mL deep 96-well plate (Greiner Bio-One). A volume of 200 µL of each of 0.9 g/dL saline and 1M NaOH was added to each well. The plate was covered and vortexed briefly at setting 7 on a multi-tube vortexer (VWR). The plate was incubated in a ThermoMixer at 95° C and 1050 rpm for 1 hour. After letting the plate cool at room temperature for 5 min, 200 µL of internal standard was added, and the plate was covered and vortexed briefly at setting 7. Analytes were extracted with 1 mL of 50:50 *n*-heptane:MTBE. The plate was vortexed at setting 10 for 5 min, then centrifuged at 2000 rpm for 4 min to separate the layers. The plate was fitted with a liquid transfer gasket *(15)* and another 96 deep-well plate, and the bottom aqueous layer was frozen by placing samples in a dry-ice acetone bath. After 1 hour, the organic layer was transferred to the new 96-well plate by inverting and tapping the plate. The organic layer was dried using nitrogen in a TurboVap (plate temperature 40°C, gas flow 25) for 30-45 min until completely dry. The residue was reconstituted in 250 µL of acetonitrile, and the plate was covered with a clean blue cap mat and vortexed at setting 7 for 1 min. Samples were transferred to a 1 mL 96-well plate (Waters) and evaporated using nitrogen in a Turbovap (plate temperature 40°C, gas flow 20). The residue was derivatized with 50 µL PTAD (0.5 g/L). After 15 min, the reaction was quenched with 70 µL LC/MS-grade water, and the plate was covered with a sealing tap and vortexed briefly at setting 5.

Chromatography and mass spectrometry

Vitamins D_2 and D_3 were quantified using isotope dilution/multiple reaction monitoring on a Waters Xevo mass spectrometer (transitions: D_3, 591.40>298.09, 298.10, and 298.11; D_2,

603.4>298.09, 298.10, and 298.11; D_3-d6, 597.40> 298.09, 298.10, and 298.11; D_2-d6, 609.4>298.09, 298.10, and 298.11). Quantification was performed using MassLynx 4.0 software (Waters) using integrated peak area ratios of D_2/D_2-d6 or D_3/D_3-d6.

Supplemental Tables

Supplemental Table 1. Monitoring multiple synonymous transitions improves precision. [1]

	Ergocalciferol (D_2) %CV	Cholecalciferol (D_3) %CV
1 transition [2]	13.70%	13.60%
3 transitions (sum) [3]	5.90%	9.50%

[1] To evaluate the improvement of multiple synonymous transitions, a sample near 110% of the LLMI was used to determine the analytical variability of the LC-MS/MS step of the assay (imprecision of 10 injections is shown). This experiment was performed with the 90:10 *n*-heptane:ethyl acetate method.

[2] A single transition (precursor-fragment pair) was monitored in the mass spectrometer. For each analyte, the fragment 298.10 *m/z* was monitored.

[3] For comparison with a single transition, three transitions were monitored in the mass spectrometer and summed for each of the analytes and their internal standards. For each analyte, the fragments monitored were 298.09, 298.10, and 298.11.

Supplemental Table 2. Mass spectrometer parameters.

Parameter	Setting
Capillary voltage	3.50 kV
Desolvation Temperature	500 °C
Desolvation Gas	1000 L/h
Cone Gas	30 L/h
Collision Gas Flow	0.15 mL/min

Supplemental Table 3a. Determination of the lower limit of the measuring interval (90:10 *n*-heptane:ethyl acetate). [1]

Concentration (ng/mL)	Concentration observed (ng/mL)	SD (ng/mL)	Bias [2] (ng/mL)	TE [3] (ng/mL)	TE [4] (%)	CV_{total} [5] (%)
Ergocalciferol (D_2)						
0.05	0.055	0.012	0.005	0.028	56.75	37.78
0.1	0.110	0.019	0.010	0.047	47.29	26.24
0.125	0.131	0.019	0.006	0.045	35.88	21.57
0.15	0.160	0.022	0.010	0.054	36.03	19.48
0.25	0.265	0.023	0.015	0.062	24.78	13.36
Cholecalciferol (D_3)						
0.05	0.055	0.012	0.005	0.029	58.92	42.96
0.1	0.115	0.017	0.015	0.049	48.54	26.46
0.125	0.133	0.020	0.008	0.048	38.66	23.07
0.15	0.171	0.033	0.021	0.086	57.24	26.41
0.25	0.278	0.022	0.028	0.073	29.03	13.07

[1] Fetal bovine serum was spiked with cholecalciferol and ergocalciferol to make 5 different samples. Each was analyzed 8 times on each of 5 days. The concentration of the spiking solution was verified using spectrophotometry (Beckman) at 265 nm with an extinction coefficient of 18,900 and 18,300 for ergocalciferol and cholecalciferol, respectively.

[2] Bias was calculated as the expected concentration minus the observed concentration.

[3] Total error (TE) was calculated as the mean bias plus 2SD.

[4] TE% was calculated as the (TE/expected concentration)*100

[5] CV_{total}% was calculated as [[(mean within-run CV)^2+(mean between-run CV)^2]1/2]*100

Supplemental Table 3b. Determination of the lower limit of the measuring interval (50:50 *n*-heptane:MTBE). [1]

Concentration (ng/mL)	Concentration observed (ng/mL)	SD (ng/mL)	Bias (ng/mL)	TE (ng/mL)	TE (%)	CV_{total} (%)
Ergocalciferol (D_2)						
0.05	0.065	0.020	0.015	0.055	110.38	72.15
0.1	0.114	0.018	0.014	0.050	49.69	30.25
0.125	0.144	0.024	0.019	0.068	54.37	26.46
0.15	0.187	0.023	0.037	0.083	55.55	19.44
0.25	0.315	0.033	0.065	0.131	52.57	15.23
Cholecalciferol (D_3)						
0.05	0.066	0.066	0.016	0.148	296.53	79.06
0.1	0.092	0.012	-0.008	0.033	32.55	18.74
0.125	0.118	0.029	-0.007	0.065	52.20	22.42
0.15	0.146	0.020	-0.004	0.045	29.96	21.62
0.25	0.259	0.017	0.009	0.043	17.04	29.62

[1] After we discovered that there were leaks under the plate mat in the 90:10 *n*-heptane:ethyl acetate assay, we revalidated the assay using 50:50 *n*-heptane:methyl tert-butyl ether (MTBE). This experiment was performed and the data analyzed as in Supplemental Table 3a.

Supplemental Table 4a. Assay imprecision (90:10 *n*-heptane:ethyl acetate). [1]

Sample	Mean (ng/mL)	Within-Run CV(%)	Between-Run CV(%)	CV_{total} (%)
Ergocalciferol (D_2)				
Level 1	0.13	14.0	14.6	20.2
Level 2	0.98	6.1	6.4	8.9
Level 3	45.25	2.3	2.4	3.3
Level 4	84.79	4.1	4.3	5.9
Cholecalciferol (D_3)				
Level 1	0.12	22.0	19.5	29.2
Level 2	0.99	6.8	9.2	11.4
Level 3	48.84	3.5	4.5	5.7
Level 4	88.81	3.5	4.4	5.6

[1] Samples were made using fetal bovine serum (FBS) or MSG4000 and spiked with unlabeled vitamin D_2 and D_3. Each sample was assayed five times each day for five days. Total CV (%) is [[(mean within-run CV)^2+(mean between-run CV)^2]1/2]*100.

Supplemental Table 4b. Assay imprecision (50:50 *n*-heptane:MTBE). [1]

Sample	Mean (ng/mL)	Within-Run CV(%)	Between-Run CV(%)	CV_{total} (%)
Ergocalciferol (D_2)				
Level 1	0.67	5.8	6.4	8.7
Level 2	62.58	3.2	4.2	5.3
Level 3	121.74	3.1	4.8	5.7
Cholecalciferol (D_3)				
Level 1	0.53	5.8	8.2	10.1
Level 2	49.37	3.9	6.0	7.2
Level 3	95.26	3.7	5.6	6.8

[1] After we discovered that there were leaks under the plate mat in the 90:10 *n*-heptane:ethyl acetate assay, we revalidated the assay using 50:50 *n*-heptane:methyl tert-butyl ether (MTBE). This experiment was performed and the data analyzed as in Supplemental Table 4a but with three samples instead of four.

Supplemental Table 5. Assessment of background noise. [1]

Sample tested	Ergocalciferol (D_2)		Cholecalciferol (D_3)	
	Peak area	*IS Area*	*Peak area*	*IS Area*
Fetal bovine serum [2] ***(calibrator background)***	3	18	20	14
Lowest calibrator	293	55407	270	44587
Background/Lowest calibrator [3]	1.02%	0.03%	7.28%	0.03%

1 To determine if there is non-specific chromatographic noise in the assay, the signal from a double blank (no spiked analyte or spiked internal standard added) was determined and compared with the signal from the lowest standard.

2 Fetal bovine serum was used as the background matrix for the lowest calibrators and was determined to have no cholecalciferol or ergocalciferol.

3 The peak area of the fetal bovine serum (noise) was divided by the peak area of the lowest calibrator to determine the potential contribution of background in the assay. Target background is < 20% of the lowest calibrator.

Supplemental Table 6. Bias due to common potential interferences. [1]

Interference	Concentration tested	Bias (%)			
		n-heptane: ethyl acetate		*n*-heptane: MTBE	
		D_2	D_3	D_2	D_3
Protein	12 g/dL	-2	-1	-22	-20
Triglyceride	1000 mg/dL	5	-4	9	8
Hemolysate	500 mg/dL	-3	-1	6	12
Unconj. Bili	20 mg/dL	-1	-3	6	4
Conj. Bili	20 mg/dL	7	-2	1	2
Creatinine [2]	13.42 mg/dL	-1	-1	-1	2

[1] The bias due to potential interferences (except creatinine) was assessed using patient serum spiked using ASSURANCE™ Interference Test Kit. The bias of each interference was assessed for vitamins D_2 and D_3 using the calculation (test-control)/control.

[2] Creatinine interference was assessed using a mixing study involving three patient samples with high creatinine concentrations and a patient pool low in creatinine. The high creatinine samples were low in vitamin D_2 and D_3 concentrations and the low creatinine serum pool had high vitamin D_2 and D_3 concentrations. The expected concentration of vitamin D_2 and D_3 were calculated and compared against the observed vitamin D_2 and D_3 concentrations. Bias was calculated using: (observed-expected)/expected. Creatinine interference was assessed over many concentrations; the highest tested was 13.42 mg/dL. Bias at all concentrations were averaged.

Supplemental Table 7. Evaluation of potential bias from isobaric interferences (lumisterol and tachysterol). [1]

		Recovery (%)	
		Ergocalciferol (D_2)	**Cholecalciferol (D_3)**
1 ng/mL spike	Lumisterol	94	97
	Tachysterol	93	100
30 ng/mL spike	Lumisterol	93	98
	Tachysterol	97	99

[1] Evaluation of possible isobaric interference by lumisterol$_2$ and lumisterol$_3$ (lumisterol) or tachysterol$_2$ and tachysterol$_3$ (tachysterol) were evaluated using two spike solutions at 20 and 600 ng/mL concentrations (Toronto Research Chemicals, each of the four potential interferences mixed at the same concentrations). Spike solutions were prepared with MSG4000 and 1 µg/mL stock solution of lumisterol and tachysterol, separately. A 10 µL volume of each spike solution was added to an aliquot of the 2nd highest concentration calibrator (28.8 ng/mL each of vitamins D_2 and D_3) pre-extraction. The final spike concentrations tested were 1 and 30 ng/mL for both lumisterol and tachysterol. Recovery was calculated by dividing the concentrations of vitamin D_2 and D_3 from the spiked sample by the concentrations of vitamin D_2 and D_3 from the calibrator without the spike.

Supplemental Table 8. Tube type and stability study for cholecalciferol (vitamin D_3). [1]

Tube Type	Bias (%)				
	Influence of tube type [2]	Room Temperature	2-8°C	Frozen (-20°C)	Frozen (-80°C)
No anticoagulant (red top)	Ref	3	0	-8	-10
Serum Separator Tube (gold top)	2	1	-2	-12	-11
EDTA (purple top)	-3	-1	-2	-12	-8
Lithium Heparin (green top)	-3	3	1	-7	-6

[1] Three normal adults were phlebotomized. Blood was collected in 4 different tubes. Aliquots were prepared and analyzed immediately. Remaining sample was stored for 48 hr at each of the conditions listed, then thawed, prepared, and analyzed. Bias was determined by calculating (condition-fresh)/fresh for each tube type. The concentrations of ergocalciferol were all below the LLMI of the assay.

[2] The influence of tube type was determined by using the concentration determined for the fresh red top tube as the reference (Ref).

Supplemental Table 9. Freeze-thaw stability. [1]

	Bias (%)			
	48 hr (-20°C)		72 hr (-20°C)	
	D_2	D_3	D_2	D_3
QC Low	-9%	2%	3%	-6%
QC High	-7%	-5%	10%	-11%

1 Low QC sample was made with fetal bovine serum (FBS) and high QC sample was made with MSG4000. Unlabeled vitamins D_2 and D_3 were spiked into each low and high serum pool. Samples were frozen at -20°C and thawed and analyzed in triplicate at 24 hr. Remaining unprepared samples were refrozen and thawed and analyzed in triplicate at 48 hr. This process was repeated again for analysis at 72 hr. The result at 24 hr was used to calculate bias (24 hr freeze-thaw validated in Supplemental Table 8).

Supplemental Table 10. Stability of prepared samples.

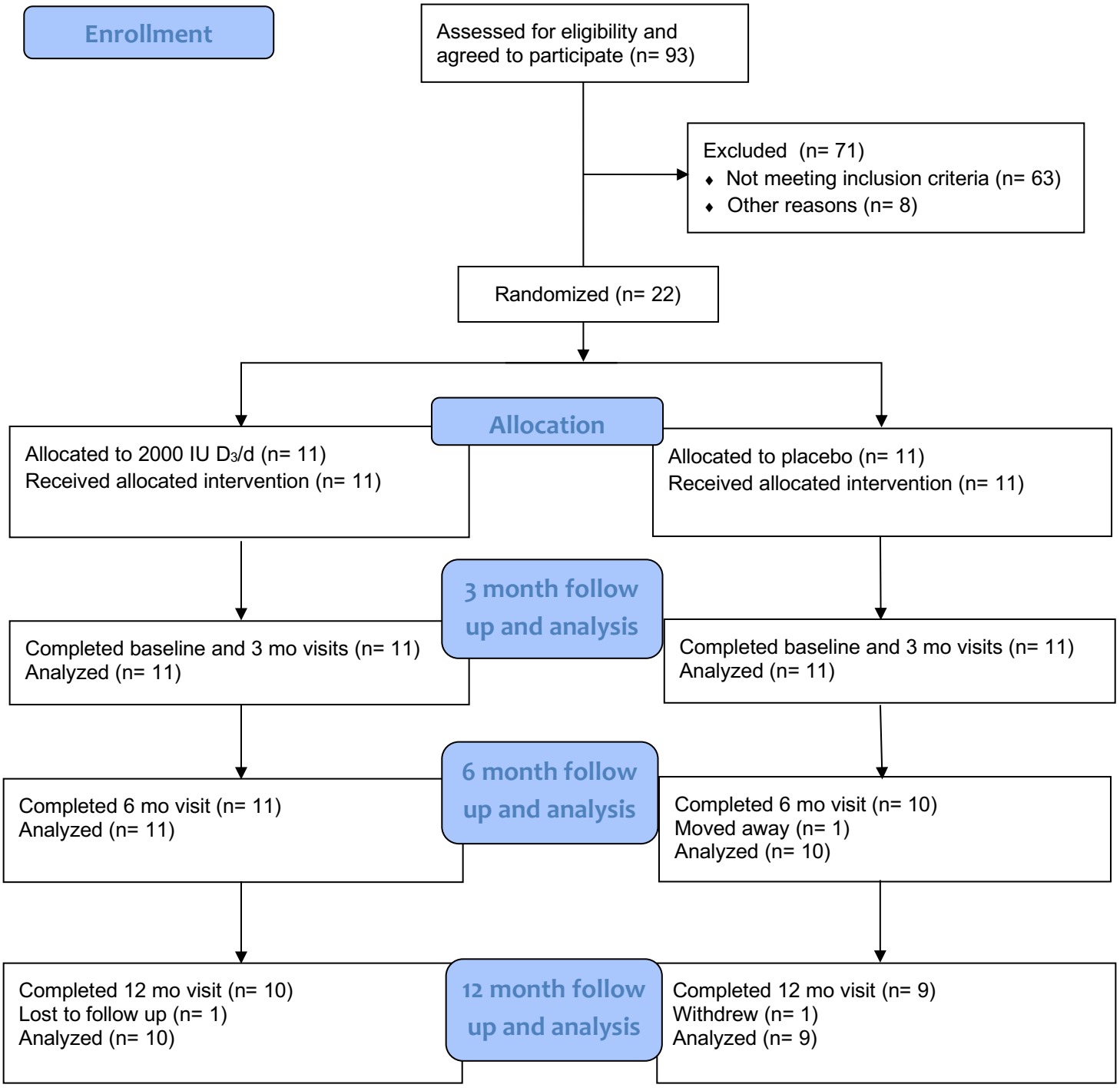
	Bias (%)	
	Ergocalciferol (D_2)	**Cholecalciferol (D_3)**
Autosampler, pierced [1]	13	10
Autosampler, unpierced [2]	-7	9
Freezer, dried, unpierced [3]	-11	5

[1] A set of 24 leftover clinical samples was prepared and analyzed on Day 1 and then left in the autosampler overnight after being sampled. They were reinjected on Day 2 and average bias across the 24 samples was calculated as $(Day2_{pierced} - Day1_{fresh})/Day1_{fresh}$.

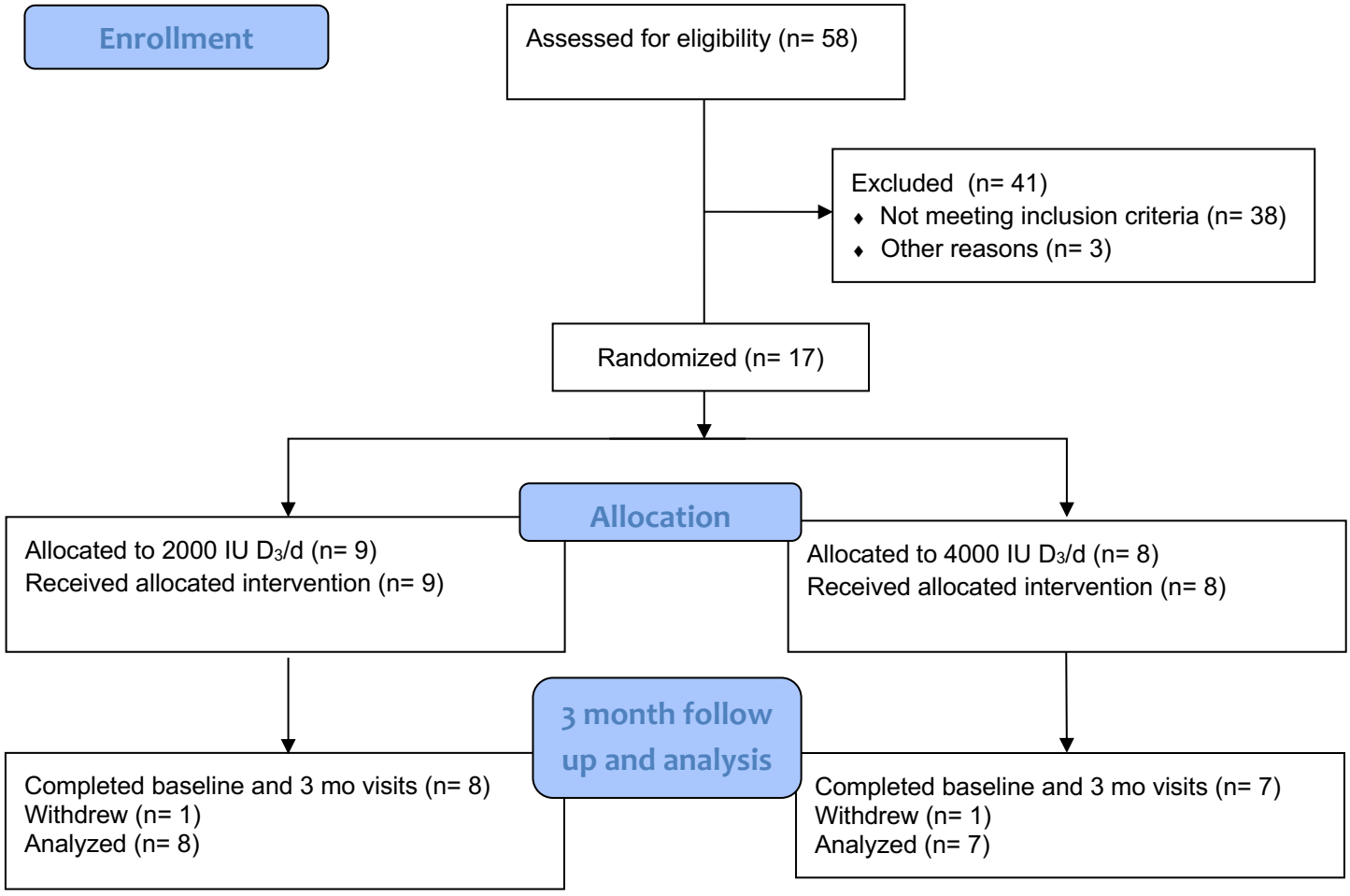
[2] The same set of 24 samples was prepared on Day 1 and stored in the autosampler overnight without being sampled. They were then injected on Day 2 and average bias was calculated as $(Day2_{unpierced} - Day1_{fresh})/Day1_{fresh}$.

[3] The same set of 24 samples was prepared on Day 1, but after evaporation and before derivatization, the plate was stored in the freezer overnight (-20°C). On Day 8, the plate was removed from the freezer and the evaporation step was repeated (to remove any condensation) and the samples were derivatized and injected. Average bias was calculated as $(Day8_{frozen} - Day1_{fresh})/Day1_{fresh}$.

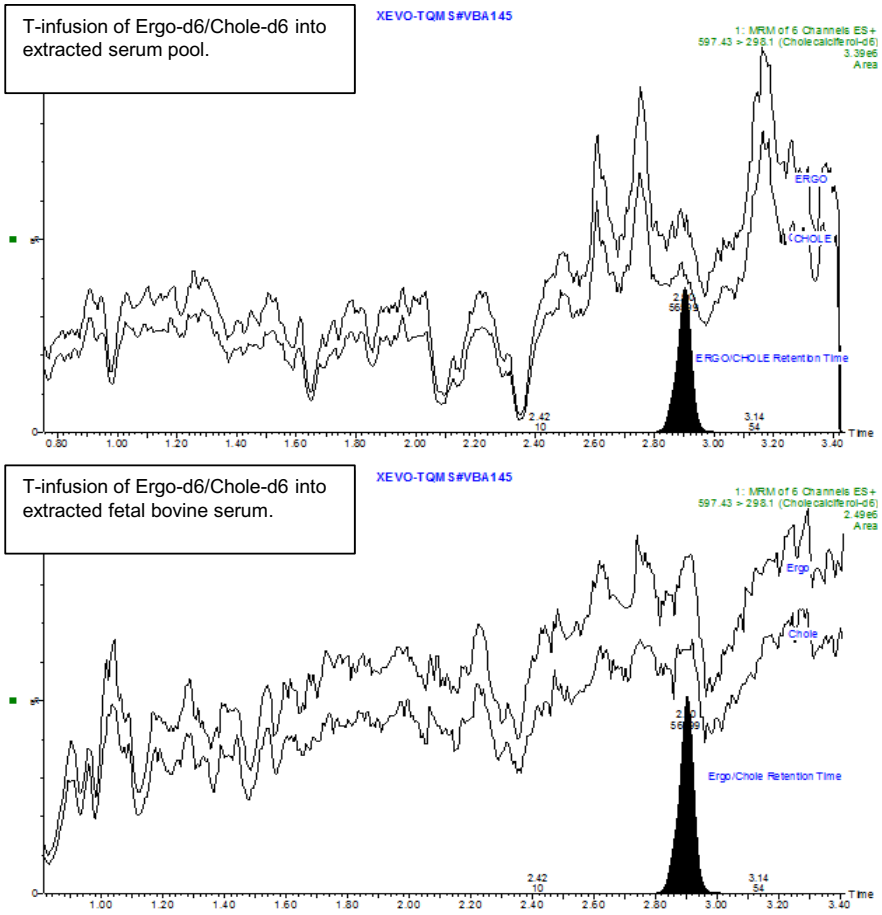
Supplemental Figures



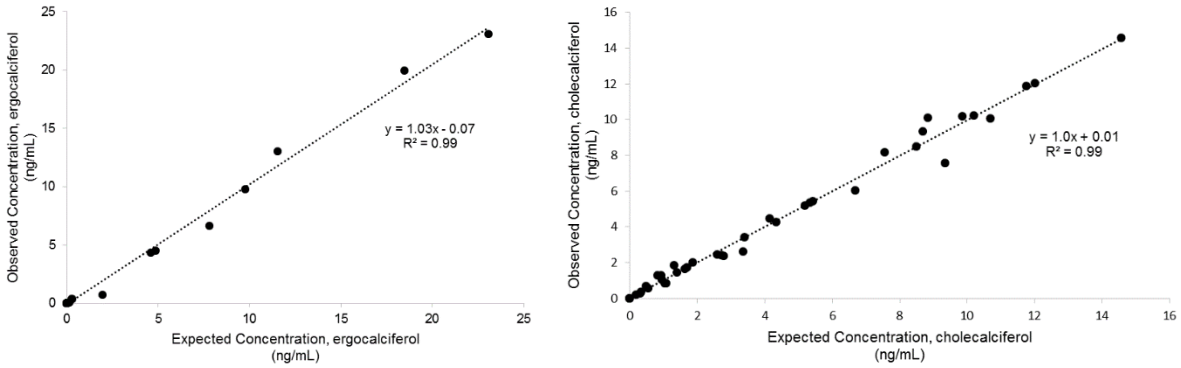
Supplemental Figure 1. Pilot study 1 participant flow diagram.



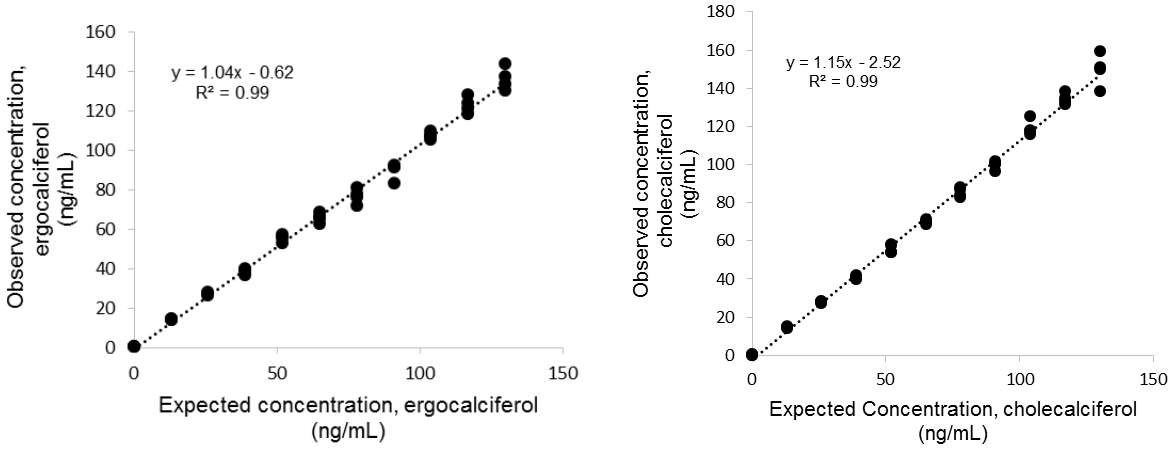
Supplemental Figure 2. Pilot study 2 participant flow diagram.



Supplemental Figure 3. T-infusion experiment. Derivatized deuterated analytes in acetonitrile were directly infused into the flow of the mobile phase post-column with a T-connector to establish a stable total ion count in the mass spectrometer. *(Top)* A pooled normal human serum sample or *(Bottom)* fetal bovine serum sample was extracted without internal standard and injected on the chromatographic column, which was developed normally. For reference, a representative chromatogram of unlabeled vitamin D_3/D_2 is overlaid in solid black.



Supplemental Figure 4. Parallelism study. Sixteen patient samples were mixed together in specific ratios. Eight samples were designated "A" and eight samples were designated "B" (*i.e.,* 1A, 1B, 2A, 2B, *etc.*), and the eight pairs of samples were mixed together to result in five different samples for each pair: A (100%), A:B (80%:20%), A:B (50%:50%), A:B (20%:80%), and B (100%). The observed concentration (y-axis) vs. expected concentration (x-axis) was plotted. A linear regression trendline was added. The mean recovery for *(Left)* ergocalciferol in the admixtures was 88% (N=24) and for *(Right)* cholecalciferol was 96% (N=24).



Supplemental Figure 5. Linearity. One sample was created using fetal bovine serum (0.07 ng/mL) and another using charcoal-stripped pooled human serum (130 ng/mL). These two samples were mixed to generate an 11-point dilutional series (sample concentration was equally spaced across the range). The results of linear regression are illustrated. In each case the intercept is not statistically different from zero.