Identifying Vitamin D2 and D3 and their 25-OH Metabolites and C3 Epimers in a Single LC-MS Run

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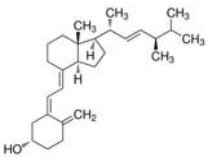
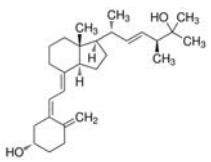
Introduction

The accuracy of current vitamin D measurements by immunoassays and LC-MS have been questioned due to overlapping LC peaks with identical m/z values for epimers. To solve this problem, we have developed a new HPLC method to achieve baseline separation of vitamin D_2/D_3 and their 25-OH metabolites and C3-epimers in one single run. A novel core-shell type reversed-phase HPLC column with cholesterol as the functional group (Cosmocore Cholester) was used in this study. The Cosmocore Cholester column has similar hydrophobicity to C_{18} columns, but has better steric selectivity. The baseline separation is so complete that it can be used for quantification by UV detector alone at low concentrations. Gradient conditions can be employed to further separate vitamin D_2/D_3 and their four metabolites/epimers, all in one LC-MS (or UV) run.

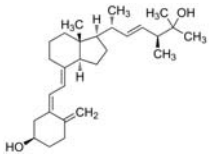
Vitamin D Structures



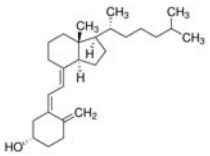
Vitamin D_2
MW 396.65



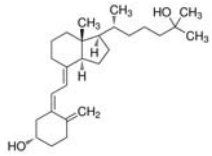
25-Hydroxyvitamin D_2
MW 412.65



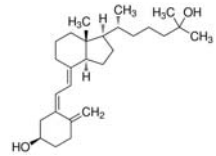
3-*epi*-25-Hydroxyvitamin D_2
MW 412.65



Vitamin D_3
MW 384.64

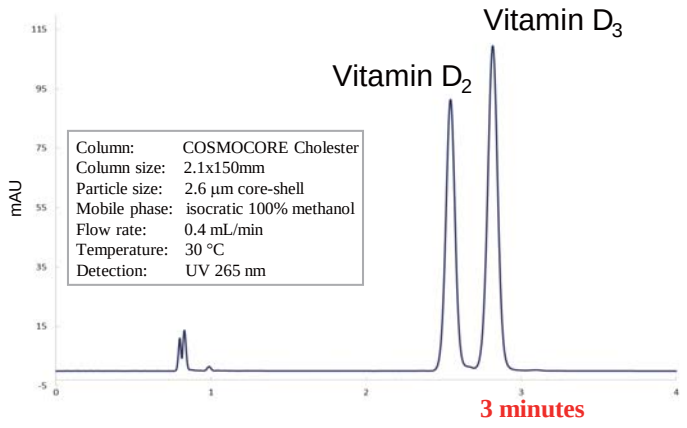


25-Hydroxyvitamin D_3
MW 400.64



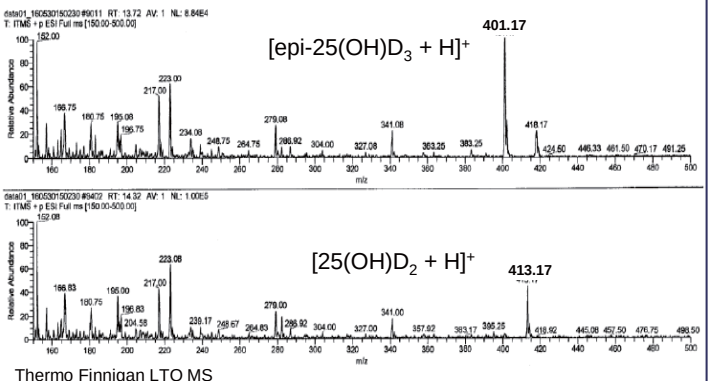
3-*epi*-25-Hydroxyvitamin D_3
MW 400.64

Vitamin D Separation



Column:	COSMOCORE Cholester
Column size:	2.1x150mm
Particle size:	2.6 µm core-shell
Mobile phase:	isocratic 100% methanol
Flow rate:	0.4 mL/min
Temperature:	30 °C
Detection:	UV 265 nm

Vitamin D Metabolite and Epimer LC-MS Data

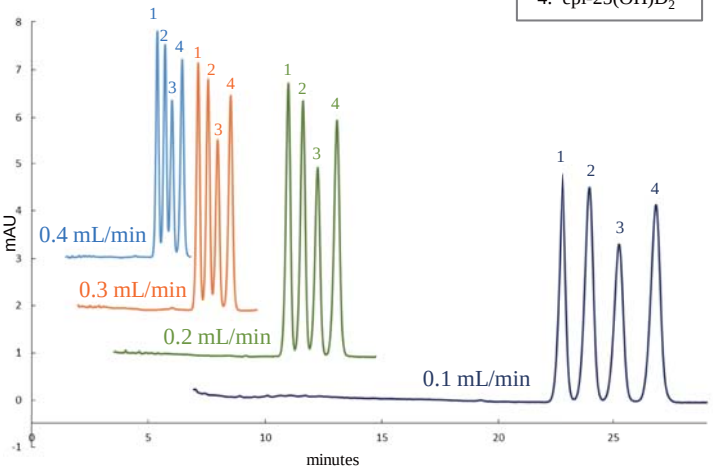


Thermo Finnigan LTQ MS
ESI positive mode, same HPLC condition as shown in UV
MS spectra obtain by BioPharmaDev, Corona, CA

Metabolite and Epimer Separation – Flow Rate Comparison

Column:	COSMOCORE Cholester
Column size:	2.1x150mm, 2.6 µm core-shell particles
Flow rate:	0.4 mL/min
Temperature:	50 °C
Detection:	UV 265 nm
Isocratic:	20A:80B A: 0.1% formic acid in H_2O B: methanol

Samples
1. 25(OH)D_3
2. epi-25(OH)D_3
3. 25(OH)D_2
4. epi-25(OH)D_2

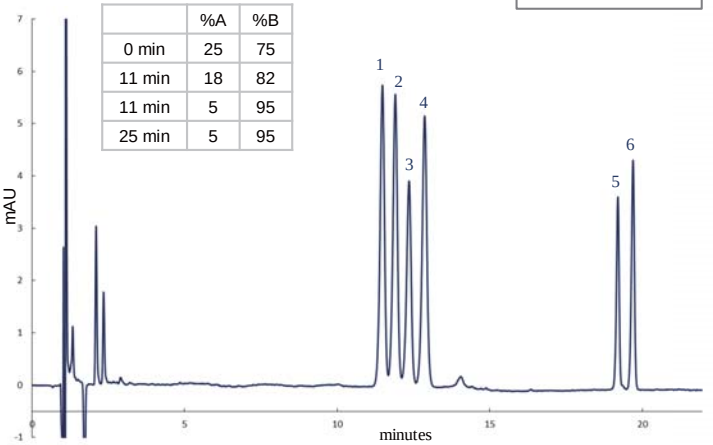


Vitamin D, Metabolite, and Epimer Separation

Column:	COSMOCORE Cholester
Column size:	2.1x150mm, 2.6 µm core-shell particles
Flow rate:	0.3 mL/min
Temperature:	50 °C
Detection:	UV 265 nm
Data:	Blank subtraction performed
Gradient:	A: 0.1% Formic acid in H_2O B: Methanol

	%A	%B
0 min	25	75
11 min	18	82
11 min	5	95
25 min	5	95

Samples
1. 25(OH)D_3
2. epi-25(OH)D_3
3. 25(OH)D_2
4. epi-25(OH)D_2
5. Vitamin D_2
6. Vitamin D_3



Conclusions

- Vitamin D_2 and D_3 isocratic separation under 3 minutes using 100% MeOH
- 25(OH) Vitamin D_2 and D_3 metabolites and C-3 epimers were baseline separated under isocratic conditions
- All six vitamin D and associated metabolites were separated in a single HPLC gradient run