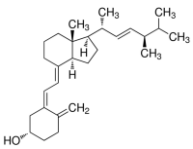
Separating Vitamin D_2 and D_3, their Metabolites and Epimers

Ken Tseng[1] (ken@nacalaiusa.com), Toshi Ono[1], Tsunehisa Hirose[2]
[1]Nacalai USA, Inc., San Diego, United States, [2]Nacalai Tesque, Inc., Kyoto, Japan

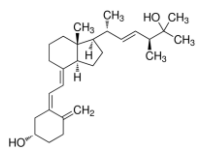
Introduction

The accuracy of current vitamin D measurements by immunoassays and LCMS have been questioned due to the overlapping LC peaks with identical m/z values epimers. To solve this problem, we have developed a new HPLC method to achieve baseline separation of vitamin D_2/D_3, 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) is used in this study. The Cosmocore Cholester column has similar hydrophobicity as regular C18 columns, so it is used in the same exact way. The baseline separation is so complete that it can be used for quantification by UV detector alone at low concentrations. Gradient condition can be employed to further separate vitamin D_2 and vitamin D_3 and their four metabolites/epimers, all in one LCMS (or UV) run.

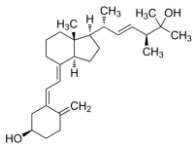
Vitamin D Structures



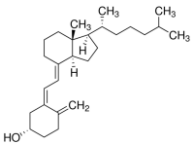
Vitamin D_2
MW 396.65



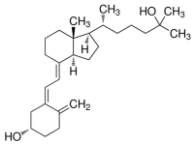
25-Hydroxyvitamin D_2
MW 412.65



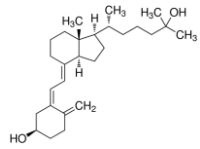
3-*epi*-25-Hydroxyvitamin D_2
MW 412.65



Vitamin D_3
MW 384.64

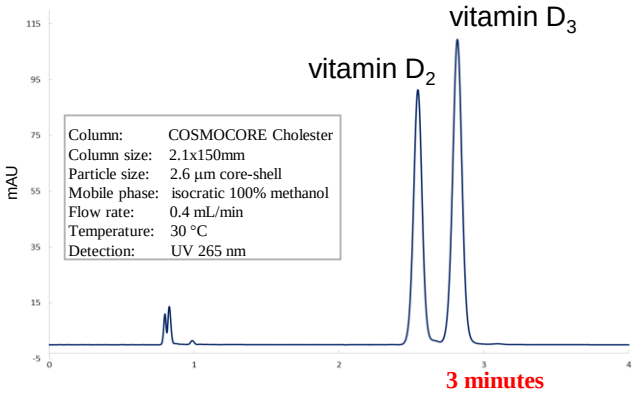


25-Hydroxyvitamin D_3
MW 400.64

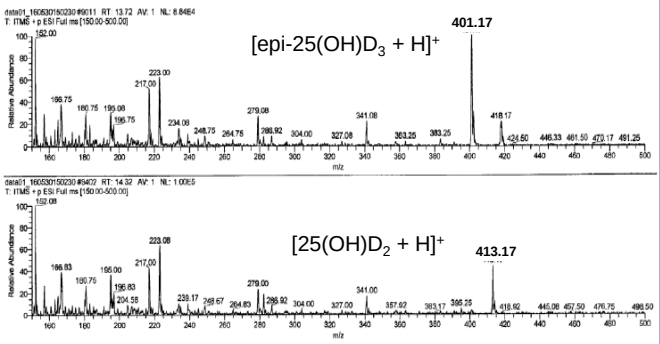


3-*epi*-25-Hydroxyvitamin D_3
MW 400.64

Vitamin D Separation



Vitamin D Metabolite and Epimer LCMS Data

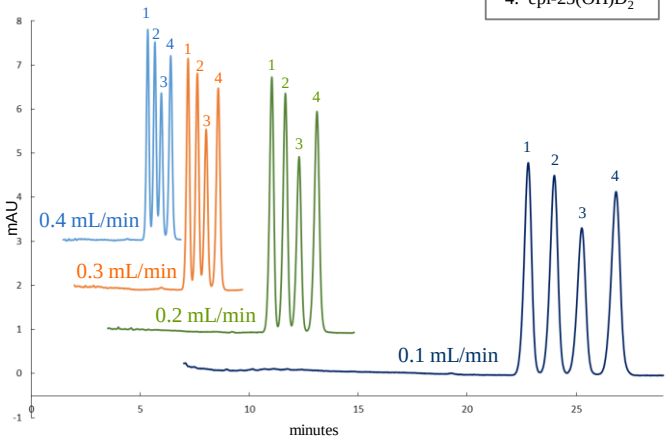


Thermo Finnigan LTQ MS
ESI positive mode, same HPLC condition as shown in UV
MS spectra obtain by BioPharmaDev, Corona, CA

Vitamin D 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

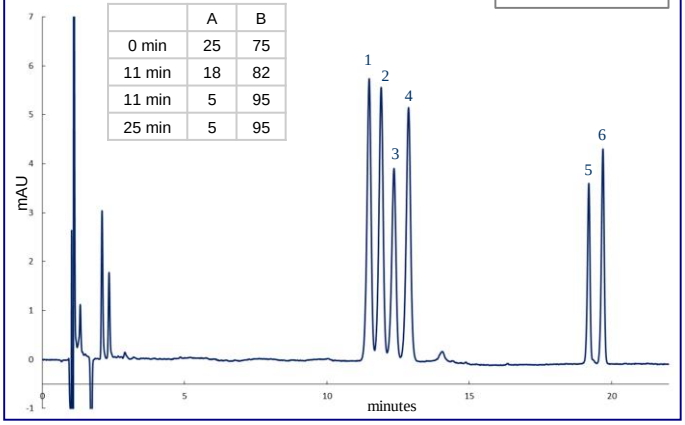


Vitamin D, Metabolite, and Epimer Separation

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



Conclusion

- 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 condition
- All six vitamin D and associated metabolites were separated in a single HPLC gradient run