# Determination of an Endogenous Biomarker - 4β-Hydroxycholesterol in $K_2$EDTA Human Plasma by LC-MS/MS

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## Novel Aspects

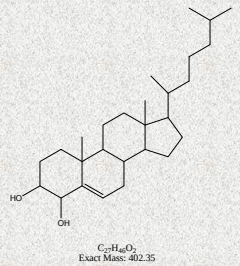
A sensitive LC-ESI-MS/MS method for determination of 4β-Hydroxycholesterol (4β-HC) as low as 5 ng/mL using picolinic acid as the derivatization reagent.

## Introduction

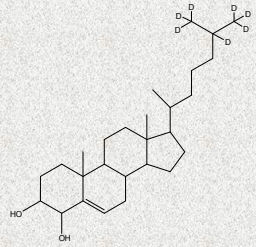
4β-HC has been proposed as a new endogenous biomarker for cytochrome P450 3A (CYP3A) activity with potential use in drug development. Therefore, a robust and reliable method for accurate determination of 4β-HC in human plasma is crucial. A successful method for accurate quantification of 4β-HC in $K_2$EDTA human plasma by LC-ESI/MS/MS was developed and validated in our lab using 4β-HC-$d_7$ as the internal standard (IS).

### Figure 1. Chemical Structures of 4β-HC and 4β-HC-$d_7$ (IS)

4β-Hydroxycholesterol




4β-Hydroxycholesterol-$d_7$ (IS)




## Methods

### Sample Preparation Procedure

4β-HC and IS in $K_2$EDTA human plasma (50 µL) were extracted with hexane after alkalizing with 1M potassium hydroxide (KOH) for 30 minutes at 37ºC. After completely drying of organic phase, 4β-HC and IS were derivatized with picolinic acid into picolinyl esters (30 min @ RT). The resulting sample was extracted again with hexane and 1000 µL of recon. solution was added after completely drying of organic phase. 5 µL was injected and analyzed using LC/MS/MS.

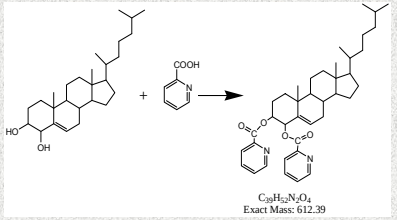
### Standard and QC sample Preparation

Working Standards and LLOQ samples were prepared in $H_2O$. Other QC samples were prepared in human plasma by spiking additional amount of 4β-HC after the baseline level was established in the pooled plasma.

### Interference test

4β-HC was completely separated from 8 commercially available isomers of 4β-HC and 4α-HC which was synthesized in our lab on Shimadzu UFLC system. There was no interference observed.

### Figure 2. Derivatization Reaction




### Chromatographic Conditions

Column: Thermo Hypersil Gold, 50 x 2.1 mm, 1.9 µm
Mobile Phase A: 0.1% Acetic Acid in $H_2O$
Mobile Phase B: 0.1% Acetic Acid in ACN
Flow Rate: 0.4 mL/min, gradient
Injection Volume: 5 µL
Column Temperature: 40°C
Run time: 12 min

### Mass Spectrometric Conditions

MS: Sciex API 4000 Triple Quadrupole

Interface: TurboIonSpray
Detection mode: Positive ion, MRM
MRM Transitions: 4β-HC m/z 613.5→490.4
4β-HC-$d_7$ (IS) m/z 620.5→497.4

## Results

### Linearity

- Validated conc. range: 5 - 500 ng/mL ($R^2 \geq 0.9929$).

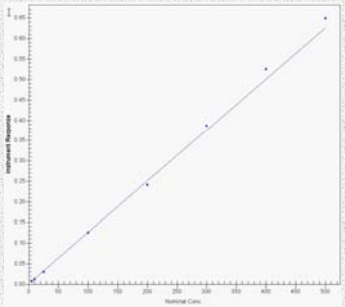
### Matrix Effects

- 1.06 ± 0.02 at 150 ng/mL (%CV 1.9%)

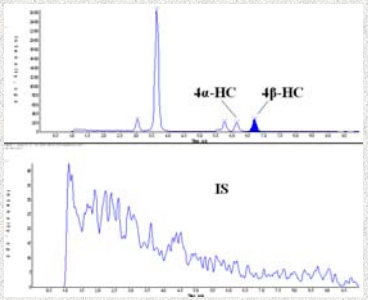
### Dilution Integrity

- 20-fold

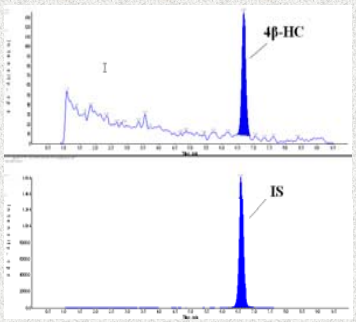
### Figure 3. Standard Curve of 4β-HC in water



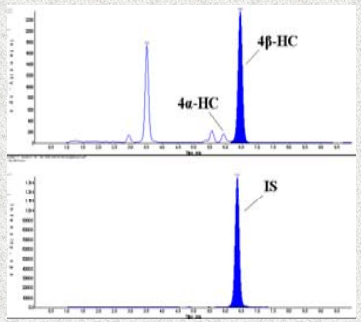
### Figure 4. Blank Plasma for 4β-HC




### Figure 5. Chromatogram of LLOQ (5 ng/mL)




### Figure 6. Typical Chromatogram of Mid-QC




### Table 1. Table 5. Back-Calculated Concentrations of 4β-HC Calibration Standards

| Run ID | 4β-Hydroxycholesterol Concentration, ng/mL | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 5 | 10 | 25 | 100 | 200 | 300 | 400 | 500 |
| Mean | 5.211 | 9.211 | 23.846 | 99.001 | 190.779 | 308.404 | 423.309 | 519.771 |
| SD | 0.103 | 0.441 | 0.731 | 1.469 | 6.949 | 9.600 | 12.638 | 9.632 |
| %CV | 2.0 | 4.8 | 3.1 | 1.5 | 3.6 | 3.1 | 3.0 | 1.9 |
| %Nominal | 104.2 | 92.1 | 95.4 | 99.0 | 95.4 | 102.8 | 105.8 | 104.0 |

### Table 2. Precision and Accuracy of QC Samples

| | Concentration (ng/mL) | 15 | 150 | 380 |
|---|---|---|---|---|
| Intrarun-1 | n | 6 | 6 | 6 |
| | Mean | 13.68 | 142.13 | 375.69 |
| | %CV | 3.4 | 3.5 | 3.0 |
| | %Nominal | 91.2 | 94.8 | 98.9 |
| Intrarun-2 | n | 6 | 6 | 6 |
| | Mean | 13.07 | 134.99 | 367.8 |
| | %CV | 8.1 | 1.8 | 2.0 |
| | %Nominal | 87.2 | 90.0 | 96.8 |
| Intrarun-3 | n | 6 | 6 | 6 |
| | Mean | 13.74 | 133.6 | 377.2 |
| | %CV | 5.7 | 5.4 | 4.4 |
| | %Nominal | 91.6 | 89.1 | 99.3 |
| Interrun | n | 18 | 18 | 18 |
| | Mean | 13.76 | 136.8 | 373.9 |
| | %CV | 6.2 | 4.1 | 3.0 |
| | %Nominal | 91.7 | 91.2 | 98.4 |

### Table 3. Stability Summary

| Stability Conditions | Minimum Stability |
|---|---|
| Processed Sample Stability | At least 74 Hours @RT |
| QC LT Stability | At least 145 days @ -20 °C |
| QC Bench-top Stability | At Least 16 hours @ RT |
| Refrigerator Stability in DBS | At Least 11 Days at 4 ºC |
| QC Freeze (-20 °C)/Thaw Stability | At least 3 cycles |
| Whole Blood Stability | At least 30 minutes @ RT<br>At least 120 minutes @ 0-4 ºC |
| Stock Solution in MeOH | At least 90 days @ -20 ºC<br>At least 16 hours @ RT |

## Conclusions

1. A highly sensitive, selective, and rugged LC-MS/MS method was developed and validated using picolinic acid as the derivatization reagent.
2. The derivatization conditions were optimized and reaction was completed with 30 min incubation at RT.
3. Water was used as the surrogate for human plasma to prepare working standards since 4β-HC is an endogenous compound. QCs were prepared in human plasma to mimic incurred samples.
4. 4β-HC was completely separated from 8 commercially available isomers of 4β-HC and 4α-HC which was synthesized in our lab.
5. Method validation data met the validation acceptance criteria defined in the method validation protocol.

## References

1. Honda A, et al. Highly sensitive analysis of sterol profiles in human serum by LC-ESI-MS/MS. *J Lipid Res. 2008 Sep;49(9):2063-73.*