Determination of an Endogenous Biomarker - 4β-Hydroxycholesterol in K_2EDTA Human Plasma by LC-MS/MS

FRONTAGE moving your compound forward

Weisheng Lin[1]; Wei Zhang[1]; Weimin Wang; Jing Ke[1]; Harry Zhao[1]; Zhongping (John) Lin[1]; Mike-Qingtao Huang[2]; Naidong Weng[2]

[1]Frontage Laboratories, Inc., Malvern, PA 19355; [2]Johnson & Johnson Pharmaceutical R & D, LLC, Raritan, NJ

Novel Aspects

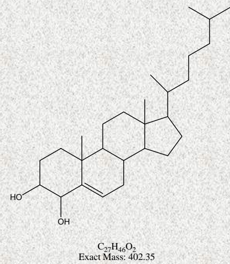
A sensitive LC-ESI-MS/MS method for determination of 4β-Hydroxycholesterol (4β-HC) as low as 5 ng/mL using picolinic acid as the derivatization reagent.

Introduction

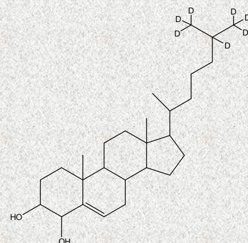
4β-HC has been proposed as a new endogenous biomarker for cytochrome P450 3A (CYP3A) activity with potential use in drug development. Therefore, a robust and reliable method for accurate determination of 4β-HC in human plasma is crucial. A successful method for accurate quantification of 4β-HC in K_2EDTA 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_2EDTA 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.

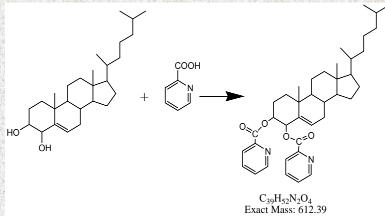
Standard and QC sample Preparation

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

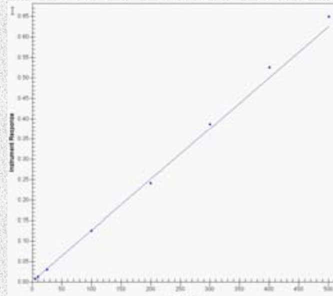


Figure 4. Blank Plasma for 4β-HC

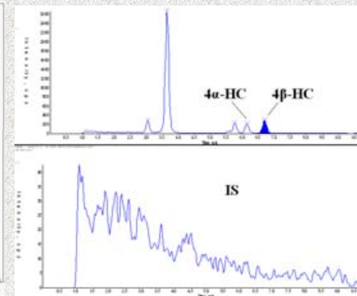


Figure 5. Chromatogram of LLOQ (5 ng/mL)

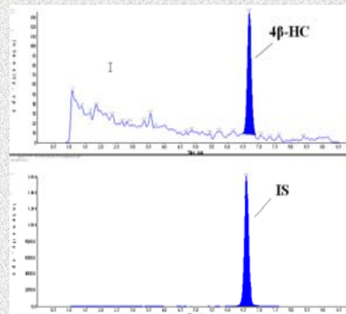


Figure 6. Typical Chromatogram of Mid-QC

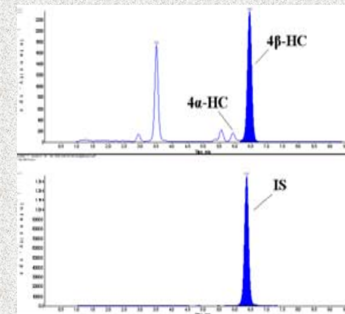


Table 1. Table 5. Back-Calculated Concentrations of 4β-HC Calibration Standards

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 At least 120 minutes @ 0-4 ºC
Stock Solution in MeOH	At least 90 days @ -20 ºC 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.*