A highly sensitive method for the quantification of key oxysterols in plasma using liquid chromatography with high resolution mass spectrometry

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Overview

Here we present a novel method for the analysis of 9 oxysterols in plasma using a liquid/liquid extraction procedure based upon Bligh/Dyer1 methodology and quantification using a Q ExactiveTM LC-HR-MS system, removing the need for any additional analyte derivatization,

Introduction

Oxysterols are oxygenated derivatives of cholesterol, which have been demonstrated to be present at increased levels in the plasma of patients with cardiovascular disease[2] and are considered as possible biomarkers for disease onset. High resolving power and mass accuracy afforded by mass spectrometers based on Orbitrap™ technology provides unique advantages for the screening and simultaneous quantification of oxysterols in complex biological matrices, which are normally present in trace amounts. However, the chromatographic separation remains crucial since many oxysterols are structural isomers. A sensitive method for the quantification of nine key oxysterols (Figure 1), using high resolution mass spectrometry is described.

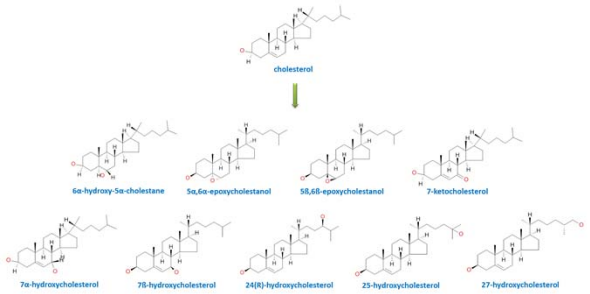

Figure 1. Molecular structures of cholesterol and the 9 target oxysterols

Materials and Methods

Sample Preparation

Liquid/liquid extraction according to an adapted procedure described by Bligh/Dyer[1] using dichloromethane and methanol was applied to 60 µl of mouse plasma spiked with labeled internal standards for each target compound. An alkaline hydrolysis with 10M potassium hydroxide solution was employed to cleave sterol-fatty acid conjugates. The organic phase was removed and evaporated and reconstituted with 400 µl of HPLC mobile phase.

LC-Conditions

LC System: Accela 1250™ (Thermo Scientific)
Injection volume: 5 µl
Solvent A: Methanol/acetonitrile/water (40:20:40, v/v/v), 0.1% formic acid
Solvent B: Methanol
Column: Kinetex™, Phenyl Hexyl, 150 mm x 2.10 mm, 2.6 µm (Phenomenex®)
Flow rate: 500 µl/min

Table 1. HPLC gradient

Time [min]	A (%)	B (%)
0	50	50
2	50	50
10	40	60
12	0	100
15	0	100
16	50	50
18	50	50

LC-HR-MS Conditions

MS Detection: Q Exactive™ (Thermo Fisher Scientific)
Ionization mode: APCI positive
Scanning mode: full scan
Scan range: 150 – 800 m/z
Resolution: 70.000 FWHM

Results

Data processing was performed using Xcalibur software (version 2.2; Thermo Fisher Scientific). Chromatograms were extracted with 5 ppm mass tolerance from the full scan data. Target analytes were confirmed using retention time and accurate mass and were quantified using peak area ratios of target and isotopically labeled oxysterols. For quantitative purposes ions with the highest intensity were chosen (Table 2).

Table 2. Retention time, theoretical mass and selected ions for oxysterols detection and quantification

Name	Formula	Rt [min]	*m/z*	$[M+H]^+$	$[M+H-H_2O]^+$	$[M+H-2H_2O]^+$
6α-hydroxy-5α-cholestane	$C_{27}H_{48}O_2$	6.64	404.3654	405.3727	387.3621	369.3516
7α-hydroxycholesterol	$C_{27}H_{46}O_2$	5.69	402.3498	403.3571	385.3465	367.3359
5α,6α-epoxycholestanol	$C_{27}H_{46}O_2$	8.47	402.3498	403.3571	385.3465	367.3359
7-ketocholesterol	$C_{27}H_{44}O_2$	6.78	400.3341	401.3414	383.3308	365.3203
7β-hydroxycholesterol	$C_{27}H_{46}O_2$	5.31	402.3498	403.3571	385.3465	367.3359
5β,6β-epoxycholestanol	$C_{27}H_{46}O_2$	7.91	402.3498	403.3571	385.3465	367.3359
24(R)-hydroxycholesterol	$C_{27}H_{46}O_2$	3.34	402.3498	403.3571	385.3465	367.3359
25-hydroxycholesterol	$C_{27}H_{46}O_2$	3.13	402.3498	403.3571	385.3465	367.3359
27-hydroxycholesterol	$C_{27}H_{46}O_2$	3.60	402.3498	403.3571	385.3465	367.3359

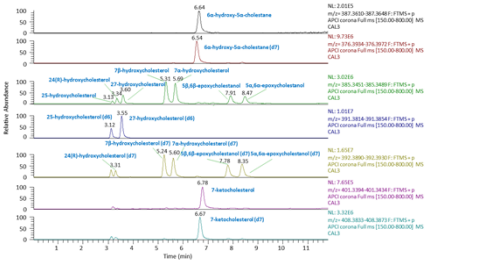

Figure 2. Chromatograms (mass extraction window: 5 ppm) for mouse plasma matrix spiked with 50 ng/ml of each standard oxysterol and 500 ng/ml of each corresponding deuterated internal standard

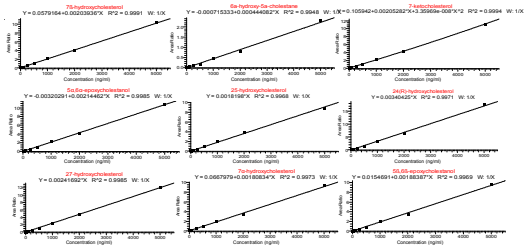

Figure 3. Calibration curves (9 levels from 5 ng/ml to 5000 ng/ml) in mouse plasma matrix

The method demonstrated analytical accuracy based on a standard addition recovery test within +/-10% and excellent linearity across the calibration range from 5 ng/ml to 5000 ng/ml (R^2>0.9948). Precision within 15% was achieved for the whole range of analytes. Based upon the calculation of the signal-to-noise ratio for each lowest calibration standard, LODs from 0.04 ng/ml to 1.76 ng/ml and LOQs from 0.12 ng/ml to 5.88 ng/ml were achieved.

Comparison between LC-MS/MS and LC-HR-MS approaches

The SRM/MRM mode using a triple quadrupole LC-MS/MS system is the conventional approach for quantitative analysis of oxysterols or similar molecules in body fluids. To increase sensitivity, derivatization of oxysterol species is routinely employed. For comparative purposes, plasma samples were also analyzed using a Triple Quad 5500™ (AB SCIEX) using the same chromatographic conditions as for the Q Exactive™.

Chromatograms obtained using the Q Exactive™ LC-HR-MS with accurate mass have shown baselines with low chemical background and improved signal-to-noise ratios, approximately 40 times higher compared to those achieved using traditional LC-MS/MS MRM approaches.

LC-MS/MS Conditions
Ionization mode: APCI positive
Scanning mode: MRM

LC-HR-MS Conditions
Ionization mode: APCI positive
Scanning mode: full scan
Scan range: 150 – 800 m/z
Resolution: 70.000 FWHM

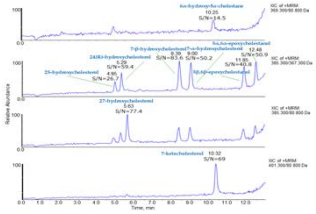

Figure 4. Extracted ion chromatograms (MRM acquisition mode) of the 9 oxysterols at a concentration of 20 ng/ml in mobile phase without matrix, acquired with a Triple Quad 5500 ™ (AB SCIEX)

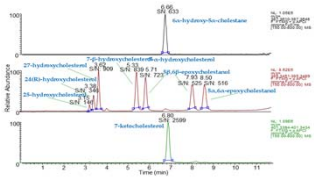

Figure 5. Extracted ion chromatograms (mass extraction window: 5 ppm) of the 9 oxysterols at a concentration of 20 ng/ml in mobile phase without matrix, acquired with a QExactive™ (Thermo Fisher Scientific)

Conclusion

An accurate and sensitive method for the simultaneous quantification of 9 oxysterols in plasma, using a Q Exactive™ LC-HR-MS System, has been developed.

- Compared to traditional LC-MS/MS approaches, this method demonstrated sufficient accuracy and sensitivity without any additional need for analyte derivatization.
- Decreased chemical background and increase of signal-to-noise ratios compared to LC-MS/MS MRM approaches were achieved.
- A wide dynamic range from 5 ng/ml to 5000 ng/ml could be achieved.
- The acquisition of full scan data maintained the opportunity for retrospective data evaluation to investigate related analyte species, which were not considered at the point of the analysis.

References

1) Bligh,E.G. and Dyer,W.J. A rapid method for total lipid extraction and purification. Can.J.Biochem.Physiol. 37:911-917 (1959).
2) Björkhem, I. Do oxysterols control cholesterol homeostasis? J. Clin. Invest. 110:725–730 (2002).

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