Sensitive quantification of key oxysterols in biological matrix using high resolution accurate mass spectrometry

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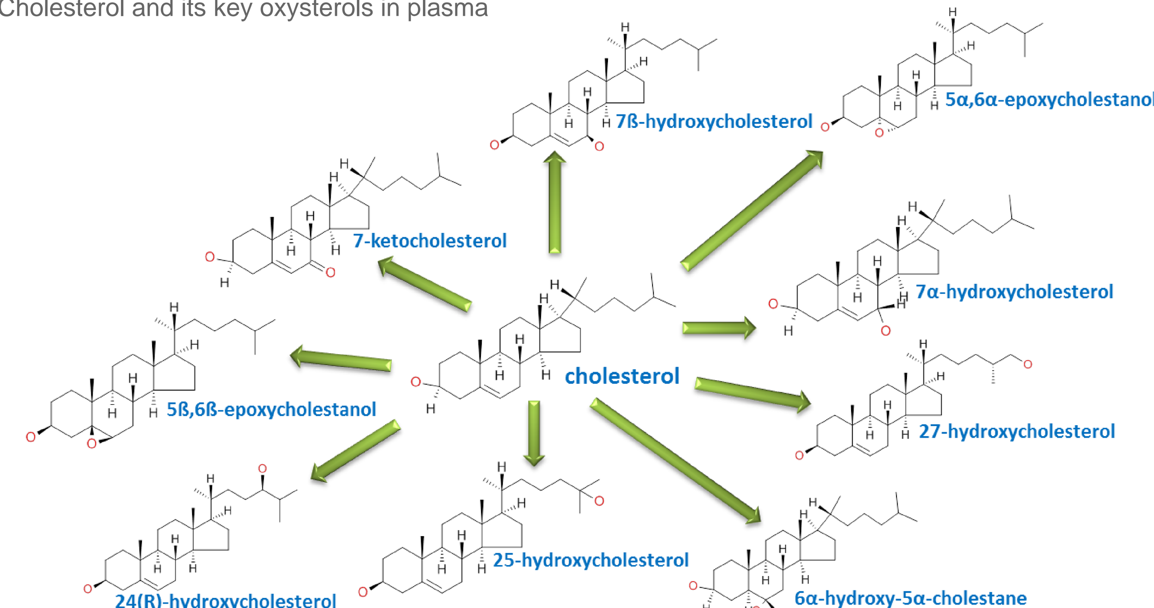
Overview

- This work presents a novel high sensitivity method for the quantification of 9 oxysterols in plasma samples using LC-HR-MS
  - No additional derivatization step is needed during sample preparation
  - Linearity over a wide dynamic range from 5ng/ml to 5000ng/ml could be achieved

Introduction

Oxysterols are oxygenated derivatives of cholesterol. Most of the known oxysterols are formed from cholesterol by enzymatic or free radical oxidation involving reactive oxygen and nitrogen species. Oxysterols play an important role in many biological processes, including cholesterol turnover, apoptosis, necrosis, inflammation, and immunosuppression. In addition, abnormal levels of certain oxysterols have been found in the blood of patients with cardiovascular disease, and therefore may be of use as an early indicator for disease onset. However, the levels of oxysterols present as oxygenated derivatives of cholesterol in blood and tissues are very low, hence determining them accurately requires advanced instrumentation and methods. A sensitive, robust and specific method for the quantification of key oxysterols using high resolution accurate mass spectrometry is described.

Figure 1. Cholesterol and its key oxysterols in plasma



Materials and Methods

LC-Conditions

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

Table 1. HPLC gradient

Time [min]	%B
0.0	50
2.0	50
10.0	60
12.0	100
15.0	100
16.0	50
18.0	50

LC-HR-MS Conditions

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

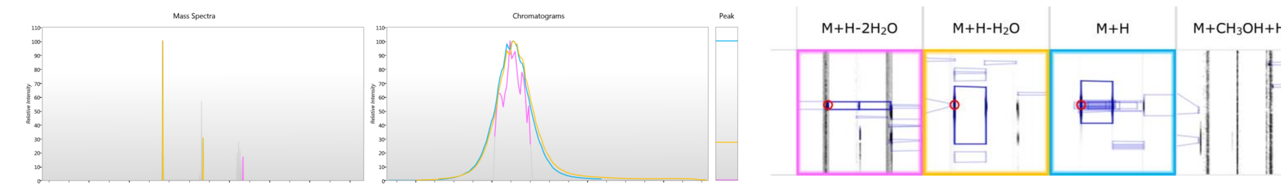
Sample Preparation

Liquid/liquid extraction (LLE) according to an adapted procedure described by Bligh/Dyer[1] was performed on mouse plasma samples spiked with labeled internal standards (one corresponding deuterated standard per compound) using dichloromethane and methanol. After protein precipitation the samples were treated with 10M potassium hydroxide to hydrolyze the oxysterol species. Samples were evaporated and reconstituted (400 µl Solvent A) without additional derivatization prior to analysis using LC-HR-MS. 9 calibration samples were prepared in mouse plasma using the same sample preparation protocol over the concentration range 5ng/ml to 5000ng/ml.

Results

A prescreening of the data set was performed using Progenesis CoMet™ software (Nonlinear Dynamics Ltd.). Most intense adduct ions were selected individually for each target compound.

Figure 2. Most intense adduct selection using Progenesis CoMet™ software

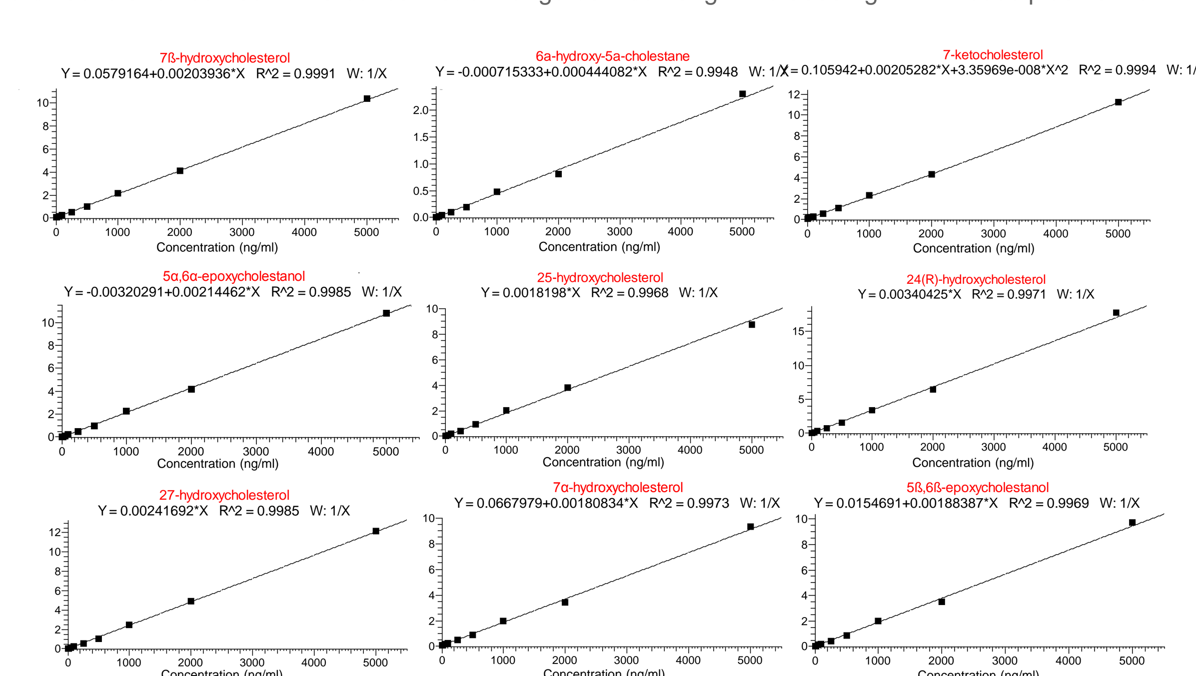


Data processing was performed using Xcalibur 2.2 (Thermo Scientific). Oxysterol target compounds were extracted from the full scan data using a mass window tolerance of 5 ppm. Target analytes were confirmed using retention time as well as a comparison of accurate mass and the proposed molecular formula. Absolute quantification for each oxysterol was performed using stable labeled internal standards for each specific target compound.

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

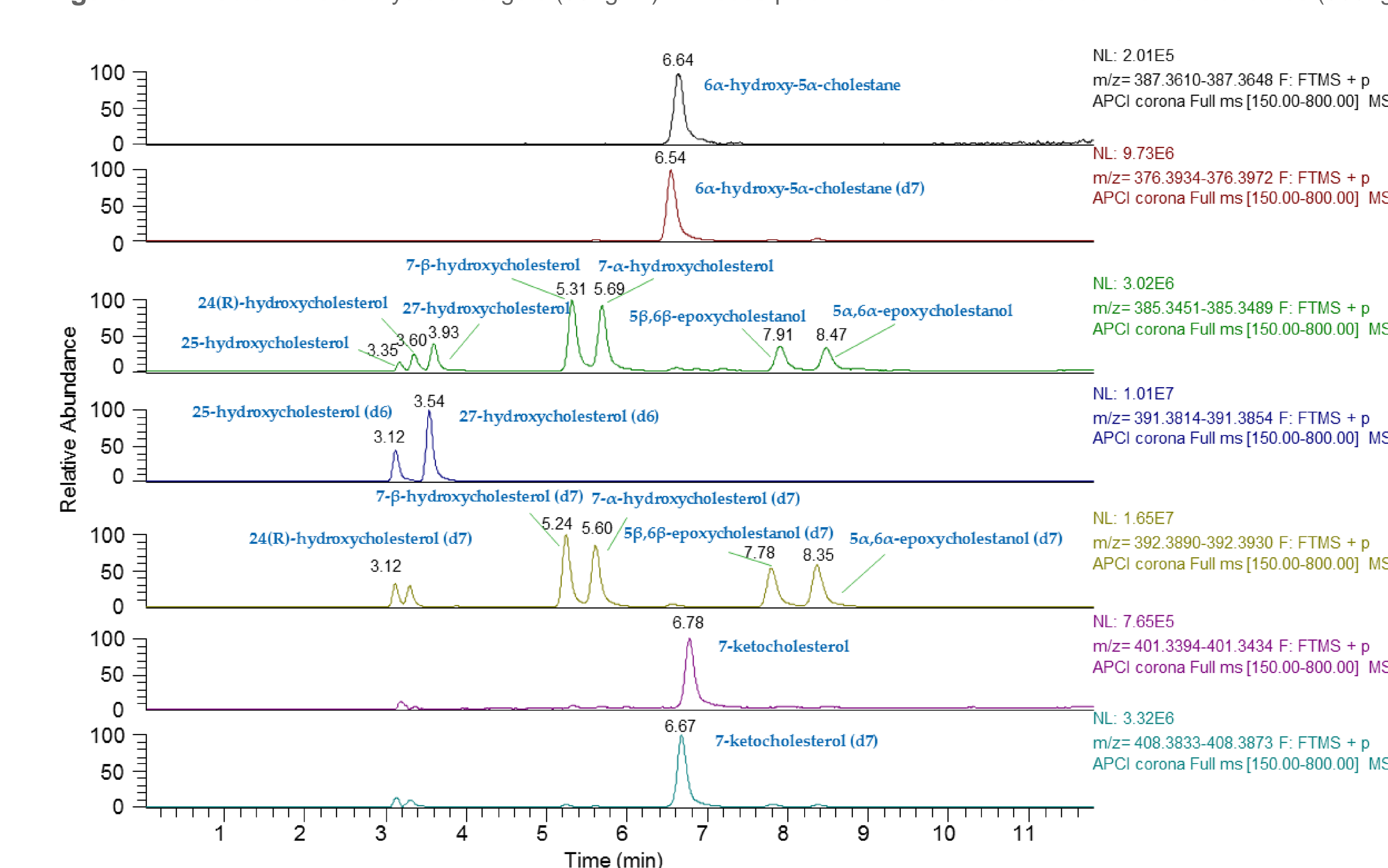
Name	Formula	Rt [min]	*m/z*	$[M+H]^+$	$[M+H-H_2O]^+$	$[M+H-2H_2O]^+$	$[M+H+CH3OH]^+$
6α-hydroxy-5α-cholestane	$C_{27}H_{48}O_2$	6.64	404.3654	405.3727	387.3621	369.3516	437.3989
7α-hydroxycholesterol	$C_{27}H_{46}O_2$	5.69	402.3498	403.3571	385.3465	367.3359	435.3833
5α,6α-epoxycholestanol	$C_{27}H_{46}O_2$	8.47	402.3498	403.3571	385.3465	367.3359	435.3833
7-ketocholesterol	$C_{27}H_{44}O_2$	6.78	400.3341	401.3414	383.3308	365.3203	433.3676
7β-hydroxycholesterol	$C_{27}H_{46}O_2$	5.31	402.3498	403.3571	385.3465	367.3359	435.3833
5β,6β-epoxycholestanol	$C_{27}H_{46}O_2$	7.91	402.3498	403.3571	385.3465	367.3359	435.3833
24(R)-hydroxycholesterol	$C_{27}H_{46}O_2$	3.34	402.3498	403.3571	385.3465	367.3359	435.3833
25-hydroxycholesterol	$C_{27}H_{46}O_2$	3.13	402.3498	403.3571	385.3465	367.3359	435.3833
27-hydroxycholesterol	$C_{27}H_{46}O_2$	3.60	402.3498	403.3571	385.3465	367.3359	435.3833

Figure 3. Calibration curves with a concentration range between 5ng/ml to 5000 ng/ml in mouse plasma matrix



The described analytical method demonstrated excellent accuracy in plasma samples based upon standard addition. The recovery values achieved in these tests were within ±10%. Linearity across the calibration range from 5ng/ml to 5000ng/ml was excellent, with R^2 values greater than 0.9948 for all analytes. Precision within 15% was achieved for the whole range of target analytes.

Figure 4. Extracted ions of oxysterol targets (50ng/ml) in mouse plasma matrix and deuterated internal standards (500ng/ml)



Based on the calculation of the signal-to-noise ratio for each lowest calibration standard, LODs between 0.04ng/ml and 1.76ng/ml and LOQs between 0.12ng/ml and 5.88ng/ml could be achieved. The signal-to noise ratios using accurate mass LC-HR-MS were approximately 40 times higher for all analytes, compared with traditional LC-MS/MS MRM approaches.

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.
  - The advantage of using accurate mass could be combined with absolute quantification.
  - Excellent chromatographic separation and mass spectrometric detection could be demonstrated, even for structural isomers.
  - Decrease of analytical background and increase in signal-to-noise compared with standard LC-MS/MS approaches could be achieved.
  - Wide dynamic range from 5ng/ml to 5000ng/ml could be achieved.
  - No further need for additional derivatization was required to obtain sufficient sensitivity for target analytes.
  - The acquisition of full scan data maintained the opportunity for retrospective data evaluation of related analyte species, which were not considered at the point of 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)*

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