Comparison of three platforms for absolute quantitation of oxysterols: LC-MS/MS, GC-MS/MS, GC-MS

Karolina M. Krasinska[a], Navdar Sever[b], Allis S. Chien[a]

[a]Vincent Coates Foundation Mass Spectrometry Laboratory; [b]Department of Developmental Biology; Stanford University, Stanford, CA

Overview

- This work compares the performance of three analytical platforms – LC-MS/MS, GC-MS/MS and GC-MS – for the absolute quantitation of oxysterols
- Each of the tested techniques, with its advantages and disadvantages, proves to be suitable for the quantitative analysis of oxysterols in biological matrix

Introduction

Historically, GC-MS has been the most common technique for analysis of steroids, but it is increasingly being replaced by LC-MS/MS. Now with GC-MS/MS emerging on the market, researchers have even more analytical choices with which to perform analysis of steroids. All these approaches come with inherent advantages and disadvantages, making selection of the most suitable method a complex matter (when not simply limited to instrument availability).

This study compares three different analytical platforms – single quad GC-MS, triple quad GC-MS/MS, and triple quad LC-MS/MS – for the quantitative analysis of sterols, cholesterol and its derivatives in particular. Criteria include sensitivity, selectivity, chromatographic resolution, speed of analysis and sample preparation; these are evaluated by obtaining standard calibration curves with isotope dilution in biological matrix.

Instrument platforms

LC-MS/MS

LC system: Thermo Fisher Scientific Accela 1250 LC
Injection volume: 5 µL
Solvent A: 50% MeOH, 10% ACN, 1% AcOH
Solvent B: MeOH
Column: Waters Acquity UPLC BEH C18,
1.7 µm, 2.1 x 100 mm
Flow rate: 350 µL/min
MS detection: Thermo Fisher Scientific
TSQ Vantage triple quadrupole MS
Ionization mode: APCI
Scanning mode: Selected Reaction Monitoring (SRM)
Analysis time: 22 min

Time [min]	%B
0	60
2	60
10	70
12	100
20.5	100
21	60
22	60

GC-MS/MS

GC System: Bruker 450-GC
Injection: 1µL, pulsed splitless
Inlet temperature: 250°C
Column: BR-5ms, 30 m x 0.25 mm ID, 0.25 µm film thickness
Column He flow: 1 mL/min
Oven temperature program:
180°C(1min) $\overset{20°C/min}{\rightarrow}$ 250°C $\overset{5°C/min}{\rightarrow}$ 300°C(8min) $\overset{5°C/min}{\rightarrow}$ 310°C(3min)
Transfer line temperature: 280°C
MS detection: Bruker Scion TQ triple quadrupole MS
Ionization mode: EI, 70eV, 250°C
Scanning mode: Selected Reaction Monitoring (SRM)
Analysis time: 27.50 min

GC-MS

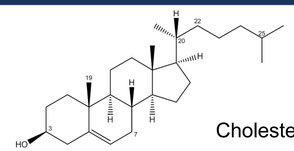
GC System: Agilent 7890A GC
Injection: 1µL, pulsed splitless
Inlet temperature: 250°C
Column: DB-5MS, 30 m x 0.25 mm ID, 0.25 µm film thickness
Column He flow: 1 mL/min
Oven temperature program: 120°C(1min) $\overset{80°C/min}{\rightarrow}$ 250°C(3.5min) $\overset{8°C/min}{\rightarrow}$ 300°C(6min) $\overset{20°C/min}{\rightarrow}$ 310°C(3min)
Transfer line temp: 280°C
MS detection: Agilent 5975C single quad MS
Ionization mode: EI, 70eV, 230°C
Scanning mode: Selected Ion Monitoring (SIM)
Analysis time: 22 min

Sample preparation

Extraction: Ten calibration samples were prepared in mouse plasma over a concentration range of 0.01 nM to 5000 nM. 200 µL of each was spiked with an internal standard solution (500 nM final concentration) and were extracted using the Bligh & Dyer protocol[1] ($CHCl_3$-MeOH liquid-liquid extraction). After drying, samples either were derivatized for GC analysis or reconstituted in 200uL solvent A and analyzed by LC-MS/MS.

Derivatization: For GC-MS and GC-MS/MS analysis, dried samples were silylated with 40 µL of MSTFA (N-Methyl-N-(trimethylsilyl)-trifluoroacetamide) for 60 min at 60°C. Samples were dried under N_2 and reconstituted in 200 uL hexane prior to analysis.

TABLE 1. Analytes. Cholesterol and 10 of its metabolites were evaluated. Two stable isotope-labeled compounds were used as internal standards (IS): 25-HCd6 and CHOLd7. On the LC-MS/MS and GC-MS/MS platforms, two SRM transitions per compound were selected whenever possible as quantifiers. One SIM ion was used for GC-MS analysis.



Cholesterol

Analyte	Abbreviation	MW	SRM LC-MS/MS	SRM GC-MS/MS	SIM GC-MS
Cholesterol	CHOL	386.6	369.2 > 147.06, 161.08	329.0 > 121.1, 109.0	329.3
Epicholesterol	EPI	386.6	369.2 > 147.06, 161.08	329.0 > 203.0, 121.0	329.3
Cholesteryl acetate	CHOL ACET	428.7	369.2 > 147.06, 161.08	-----	-----
7α-hydroxycholesterol	7α-HC	402.6	367.3 > 105.04, 91.04	456.0 > 233.0	456.4
19-hydroxycholesterol	19-HC	402.6	355.2 > 91.04, 147.07	353.2 > 145.1	353.3
20(S)-hydroxycholesterol	20(S)-HC	402.6	367.3 > 105.04, 91.04	201.3 > 117.0, 111.0	456.4
22(S)-hydroxycholesterol	22(S)-HC	402.6	367.3 > 105.04, 91.04	173.0 > 83.0, 73.0	173.1
22(R)-hydroxycholesterol	22(R)-HC	402.6	367.3 > 105.04, 91.04	173.0 > 83.0, 73.0	173.1
25-hydroxycholesterol	25-HC	402.6	367.3 > 105.04, 91.04	131.0 > 73.0	131.0
7-keto-25-hydroxycholesterol	7-KETO	416.6	383.3 > 81.04, 105.05	131.0 > 73.0	131.0
24(S)-25-epoxycholesterol	EPOXY	400.6	383.3 > 91.04, 105.05	382.2 > 362.0, 145.0	143.0
25-hydroxycholesterol IS	25-HC IS	408.6	373.4 > 105.1, 91.1	137.0 > 73.0	137.1
Cholesterol IS	CHOL IS	393.7	376.4 > 147.1, 105.1	336.4 > 121.1, 109.0	336.6

Results

TABLE 2. Calibration curve range and linearity. Solvent vs. biological matrix. Calibration samples were analyzed and observed to give linear responses from 2 nM to10 uM. LLOQs, defined as signal to noise (S/N) ratio of 10 to 1, were measured in both matrix free solvent and plasma (bold).

Analyte	Lower Limit of Quantitation (buffer/plasma) [fmol on column]			Calibration curve range/r^2 in plasma		
	LC-MS/MS	GC-MS/MS	GC-MS	LC-MS/MS	GC-MS/MS	GC-MS
CHOL*	10/**50**	25/**10**	50/**50**	0.998	0.998	0.992
EPI	50/**2500**	2.5/**10**	50/**50**	0.990	0.999	0.993
CHOL ACET**	1000/**Not measurable**	---	---	<0.8	Not measurable	Not measurable
7α-HC	100/**100**	0.1/**0.1****	5/**25**	0.996	0.998	0.990
19-HC	250/**200**	1/**1**	25/**50**	0.996	0.999	0.996
20(S)-HC	25/**100**	5/**200**	250/**250**	0.999	Poor signal - interfering peak	0.989
22(S)-HC	25/**100**	0.2/**1**	20/**50**	0.999	0.999	0.990
22(R)-HC	10/**50**	0.1/**0.5**	10/**25**	0.999	0.998	0.989
25-HC	10/**50**	0.05/**0.01**	50/**50**	0.999	0.996	0.988
7-KETO	25/**250**	0.1/**0.5**	1000/**1000*****	0.997	0.999	0.993
EPOXY	10/**25**	5/**10**	1000/**1000*****	0.999	0.999	0.994

* Stable isotope labeled cholesterol (CHOL IS) was used for determination of LLOQ in plasma due to the high endogenous levels of cholesterol in plasma used for this study
** Poor extraction yield – extraction method not suitable for this analyte
*** Poor GC signal
**** Endogenous level of analyte in plasma = LLOQ value estimated

FIGURE 3. Total Ion Chromatograms (TIC). LC-MS/MS (A), GC-MS/MS (B) and GC-MS (C) chromatograms show very good base peak separation of all tested analytes. All methods allow successful chromatographic resolution of epimers.

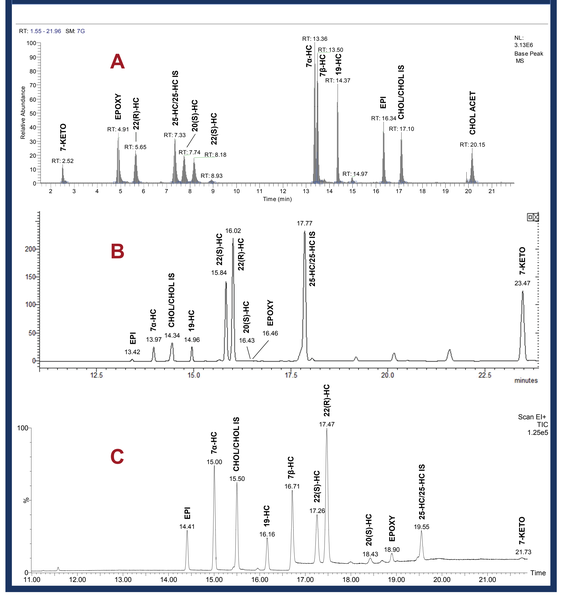


TABLE 3. Comparison of technical features. Sensitivity, stability and matrix interference are all highly analyte dependent. Sensitivity of the method varies depending on the matrix. While it is possible to shorten significantly the analysis time, chromatographic resolution will be compromised and potential interferences from endogenous isomers or other isobaric coeluters of the analytes should be considered.

Comparison criterion	LC-MS/MS	GC-MS/MS	GC-MS
Sensitivity - Solvent	Very good	Excellent	Excellent
Sensitivity - Matrix	Good	Very good	Moderate
Specificity	Excellent	Excellent	Moderate
Time – Sample preparation	Short	Long	Long
Time – Sample analysis	Comparable (long vs. typical LC-SRM)	Comparable	Comparable
Derivatization	Not necessary	Necessary, analyte dependent	Necessary, analyte dependent
Stability	> 7 days @10°C	< 3 days @ 10°C	< 3 days @ 10°C
Chromatographic resolution	Good to very good	Excellent	Excellent
Epimer separation	Moderate to good	Very good	Very good
Detection of sterol conjugates	Very good	Not possible	Not possible
Structural characterization	Limited	Possible	Possible
Matrix interference	Some	Minimal	Significant

Conclusions

- Each of the tested platforms proved to be suitable for absolute quantitation of numerous oxysterols – however, none of the methods worked equally well for all of the analytes as a whole.
- The choice of the most suitable technique should be made on a case by case basis, with the following primary considerations:
  - LC-MS/MS seems to be applicable to a wider range of analytes, but is not as sensitive as GC based techniques for most of the analytes.
  - GC-MS/MS provides the best overall sensitivity but requires more laborious sample preparation and has limited analyte stability, which makes it less suitable for analyzing large batches of samples.
  - GC-MS, even though highly sensitive, lacks the selectivity often necessary for analysis of biological samples and suffers from significant matrix interferences.
  - In the GC methods, EI provides a unique fragmentation profile of molecules allowing greater selection of quantifier ions.
  - All techniques achieve epimer separation, but have limited high throughput capabilities.
- All techniques - LC-MS/MS, GC-MS/MS and GC-MS - should be considered complementary rather than competing methods.

References

1) Bligh,E.G.; Dyer,W.J. *Can.J.Biochem.Physiol.* **1956**, 37:911-917
2) Krone,N.;Hughes,B.A.; et al. *J.Ster.Biochem.Mol.Biol.* **2010**, 121:496-504

Acknowledgements

Thanks to the Vincent and Stella Coates Foundation, to Pavel Aronov from SUMS for help with GC-MS setup as well as Helen (Qingyu) Sun, Ed George and Kefei Wang from Bruker Chemical and Applied Markets for help with GC-MS/MS analysis setup

This poster may be downloaded from the Stanford University Mass Spectrometry website at http://mass-spec.stanford.edu/Publications.html