Fast Profiling of 39 Bile Acids in Plasma, Urine and Feces, by Automated Extraction and LC-MS/MS.

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Complete solution for the analysis of 39 primary, secondary and conjugated Bile Acids, in plasma, urine and feces, with ready-to-use methods and automated sample extraction.

1. Introduction

Bile Acids (BA) are essential for cholesterol metabolism and play a key role in absorption of fats in the small intestine. Additionally, they serve as signaling molecules that regulate the metabolism. Their concentrations are used as markers of several hepatic diseases. Acute and chronic liver failure concern more than 12 million people worldwide, estimated by the WHO. Blood enzyme activity test and total BA assay are widely conducted as early examinations. However, these tests cannot provide a definitive interpretation and the monitoring of individual BA was recently proposed for injury differentiation. Therefore, a profiling method was needed to support the clinical research.

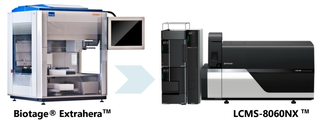
In this context, the aim of this work was to develop a LCMS quantitative method for individual BA in various biological matrices. Their wide structural diversity, associated with chemical similarities, leads to great challenges to separate and detect them. Also, large scale studies require an easy and robust sample preparation protocol, and a fast analytical workflow.

2. Methods

The final method is a quantitative analysis of 39 bile acids in biological samples: human plasma, human urine and mouse feces. *LC/MS/MS Method Package for Bile Acids version 2* was used (Shimadzu Corporation, Kyoto). It includes optimized conditions for LC-MS/MS, and an automated sample preparation protocol.

The sample preparation protocol (*Figure 1*) consists of an automated extraction using Biotage® Extrahera™. 96 biological samples are prepared simultaneously in a total of 45 min (so 0.5 min per sample). This is 4 times faster than manual extraction, which takes about 3h for the same number of samples. It has also the advantages of a higher reproducibility and a reduced cost.

Pretreated samples were analyzed under the conditions detailed in *Table 1*. System used was Nexera™ X2 UHPLC coupled to LCMS-8060NX™. Ready to use method files from the Method Package were used. Standards were 39 primary, secondary and conjugated BA, and 10 internal standards (Alsachim), covering all BA classes and selected to achieve high accuracy. Limited number of internal standards was used to reduce the cost of the analysis. MS acquisition time was 10 min. Particular attention was paid to maintain good resolution between isomers. Example Bile Acids isomers MRM chromatograms are shown in *Figure 2*.



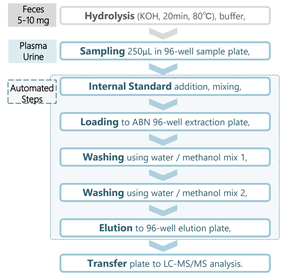


Figure 1. Pretreatment Protocol (Plasma, Urine, Feces).
Details of this protocol are provided in the Method Package.

Table 1. Analytical conditions.

UHPLC (Nexera ™ X2 system)	
Column	ACE Excel C18 Amide (2)
Mobile phase	Aqueous : Formic acid in Water Organic : Acetonitrile / Methanol
LC run time (MS time)	10.95 min (10 min)
Flow rate	0.65 mL/min
Column temperature	45 °C
MS TQ (LCMS-8060NX™)	
Ionization	Ion Focus ESI (Negative)
Mode	MRM
Nebulizing gas flow	3.0 L/min
Drying gas flow	5.0 L/min
Heating gas flow	15.0 L/min
DL temperature	250 °C
Heat Block temperature	500 °C
Interface temperature	400 °C

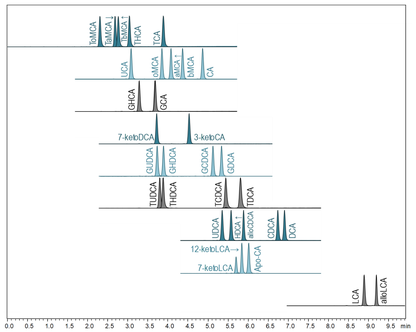


Figure 2. Bile Acids Isomers MRM Chromatograms.
Standard solutions at 10 ng/mL. Quantitative MRM transitions.

Calibration.

Regressions were built using 7 calibration points, in the range 0.5-50 ng/mL. Fully automated integration was performed using LabSolutions Insight software, with no manual re-integration. Example calibration data is presented in *Figure 3*. Accuracy was in the range 80-120% for all calibration points (data not shown).

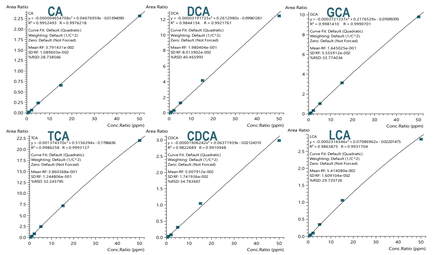


Figure 3. Example Calibration Curves.

4. Results

Evaluation of the method was conducted on plasma (human), urine (human) and feces (mouse). The samples were spiked at 10 ng/mL for plasma and urine, or 2.3 ng/mg for feces (in addition to endogenous concentrations). Unspiked samples were also analyzed (blank).

Method Precision.

Precision was confirmed using spiked plasma (retention time and area). Repeatability (intra-day) was evaluated by doing 5 extractions of the same plasma sample, over the same day. It was checked 4 times over 4 days for Intermediate precision (inter-day). Retention times repeatability was excellent (RSD<0.5%), also intermediate precision (RSD<0.3%). Area repeatability (RSD<12%) and intermediate precision (RSD<15%) were also very good. Results are detailed in *Table 2*.

Table 2. Repeatability and Intermediate Precision (Plasma).

	Intra-day RSD (n=5)				Inter-day RSD (n=4)		
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Min.	Max.	Av.
Ret. Time	0.06%	0.16%	0.13%	0.10%	0.03%	0.28%	0.09%
Peak Area	5.7%	5.6%	6.5%	5.8%	1.2%	14.8%	5.4%

Sample Quantification.

Bile acids of interest were quantified in plasma, urine and feces. Standard addition (of 10 ng/mL) was used as the reference method for quantification. Two additional methods were evaluated: (i) internal calibration (1-point calibration at 10 ng/mL in water, with ISTD ratio correction) and (ii) direct isotopic dilution (direct calculation by ISTD ratio). Example unspiked plasma chromatograms are presented in *Figure 4*.

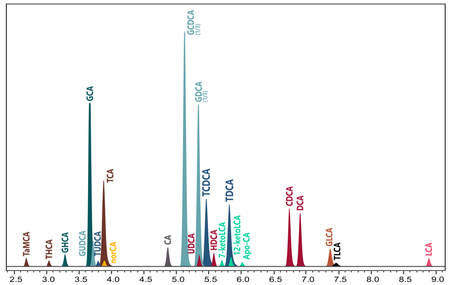


Figure 4. Example Chromatograms in Human Plasma.

Quantification by internal calibration.

Excellent performances were observed, for both unspiked and spiked samples, for all BA. Accuracies were within [80-120] % in feces, within [82-119] % in urine, and within [82-119] % in plasma. This quantification method proved its fitness for purpose to assay the selected BA. Calculated concentrations in all blank matrices and their accuracies are detailed in *Table 3*.

Table 3. Calculated Concentrations and Accuracies.
Standard addition was used for accuracy calculation.

Compound Abbreviation	Plasma Unspiked		Urine Unspiked		Feces Unspiked	
	Conc. (ng/mL)	Accuracy (%)	Conc. (ng/mL)	Accuracy (%)	Conc. (ng/mL)	Accuracy (%)
TaMCA	ND	N/A	ND	N/A	28.2	84%
TbMCA	6.6	84%	10.3	84%	35.2	80%
7,12-DiketoLCA	ND	N/A	ND	N/A	179.8	N/A*
DHCA	ND	N/A	ND	N/A	40.7	95%
THCA	4.6	89%	0.9	98%	7.3	88%
UCA	ND	N/A	2.1	101%	ND	N/A
GHCA	12.2	97%	ND	N/A	ND	N/A
GCA	181.8	N/A*	6.2	105%	0.8	99%
7-KetoDCA	1.0	104%	3.7	93%	60.8	81%
GUDCA	27.8	116%	0.9	101%	0.2	100%
TUDCA	2.6	106%	5.3	97%	12.2	100%
oMCA	ND	N/A	ND	N/A	160.3	N/A*
NorCA	2.7	99%	19.9	101%	0.3	98%
THDCA	ND	N/A	ND	N/A	1.4	101%
TCA	64.5	82%	4.6	87%	517.6	N/A*
GHDCA	ND	N/A	ND	N/A	ND	N/A
aMCA	ND	N/A	ND	N/A	17.5	83%
NorUDCA	ND	N/A	2.9	97%	ND	N/A
bMCA	ND	N/A	1.8	82%	149.4	N/A*
3-KetoCA	0.2	104%	0.6	105%	4.1	96%
CA	18.7	101%	7.5	110%	100.0	N/A*
GCDCA	673.5	N/A*	1.0	102%	ND	N/A
UDCA	13.4	104%	ND	N/A	6.1	100%
GDCA	470.2	N/A*	1.0	118%	0.4	116%
TCDCA	64.2	99%	0.3	115%	3.4	98%
HDCA	14.7	102%	ND	N/A	26.5	108%
7-KetoLCA	6.4	111%	ND	N/A	7.0	98%
TDCA	54.2	97%	4.2	100%	24.4	95%
12-KetoLCA	8.9	107%	ND	N/A	82.6	103%
AlloCDCA	ND	N/A	ND	N/A	ND	N/A
ApoCA	3.2	106%	ND	N/A	3.9	95%
CDCA	58.6	108%	ND	N/A	3.0	118%
DCA	57.6	116%	0.4	117%	67.3	120%
GLCA	17.9	119%	ND	N/A	ND	N/A
TLCA	3.0	114%	2.2	119%	1.3	120%
DHLCA	ND	N/A	ND	N/A	5.3	97%
LCA	10.0	116%	ND	N/A	6.1	104%
AlloLCA	ND	N/A	ND	N/A	ND	N/A

* Standard addition concentration is too low compared to endogenous value (1/10 or lower). Quantification by standard addition is not accurate. Accuracy calculation is not possible (no reference concentration).

Quantification by direct isotopic dilution.

Excellent performances were observed for both unspiked and spiked samples, for all Bile Acids. Accuracies were within the range [90-113] % in feces (Figure 10), within [84-118] % in urine (Figure 11), and within [81-120] % in plasma (Figure 12). This quantification method proved its fitness for purpose to assay the selected Bile Acids.

Both quantification approaches, internal calibration and direct isotopic dilution, have proved their fitness for purpose to assay the selected BA, with high performances. The correlation with standard addition is also very good for both methods (Figure 5).

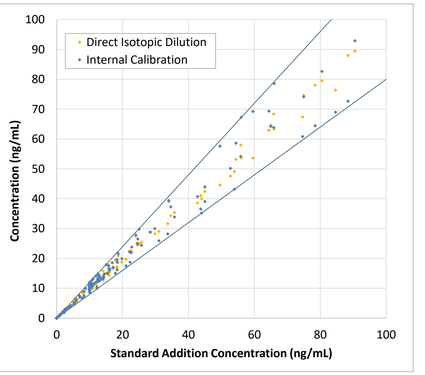


Figure 5. Correlation of Internal Calibration and Direct Isotopic Dilution with Standard Addition method.
Concentration range [0-100]ng/mL, for a clearer data visualization

5. Conclusion

This method proved its fitness for purpose to assay selected BA (primary, secondary and conjugates) in biological matrices of interest such as plasma, urine and feces. Extraction is automatized and can process 96 samples at once, and the MS acquisition is as fast as 10 min, including isomers resolution. Both the fast LC-MS/MS method and the automated sample extraction will enable robust routine analysis of Bile Acids with a high throughput and at a lower cost for the end user.

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