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Development and Validation of an LC-MS/MS Method for the Simultaneous Quantitation of Fifteen Bile Acids in Human Serum

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PURPOSE

- In the past few decades, bile acids have been well recognized as important biomarkers for various liver diseases.
- For diagnostic purposes, clinical laboratories perform enzymatic assays for estimation of the total bile acid levels, while individual bile acid levels are typically measured by methods based on chromatography combined with mass spectrometry.
- Efficacy biomarkers used during drug development require adaptation of concentration ranges suitable for the disease indication as well as comprehensive method validation according to regulatory guidelines.
- Herein, we report a sensitive and robust LC-MS/MS method for the quantitation of a previously established panel of fifteen bile acids in human serum: five major bile acids as well as their glyco- and tauro- conjugates.

METHODS

- Fifteen bile acids, including five major bile acids (cholic acid, deoxycholic acid, ursodeoxycholic acid, chenodeoxycholic acid and lithocholic acid), and their respective glyco- and tauro-conjugates were extracted by protein precipitation using methanol.
- Reversed-phase HPLC separation was achieved on a Waters Acquity, BEH C18 column (100 X 2.1 mm, 1.7 micron).
- Electrospray ionization in the positive ion mode and MS/MS monitoring was used for analysis of extracts.
- Because of the endogenous nature of these bile acids in human serum, surrogate matrix (PBS buffer) was used to prepare calibration standards.
- Individual stable-isotope labeled internal standards for each bile acid were utilized for quantitation.

RESULTS

- Due to the expected difference of concentration levels among bile acids, two assay ranges were applied (high-range (0.200-100.0 µmol/L) for 8 bile acids and low-range (0.0100-5.00 µmol/L) for the other 7 bile acids. High and low ranges differed by 20-fold. For each bile acid, linear regression with 1/x2 weighing was used.
- Good precision and accuracy was demonstrated. Inter run precision (CV%) were within 17.1% at LLOQ, and within 10.0% for all other QC levels, across all bile acids. Inter run accuracy (bias%) were between -6.0% to 8.0% at LLOQ level and between -8.3% to 4.0% for all other QC levels for all bile acids.
- Disproportionate QC sample testing was conducted to confirm the absence of interference between bile acids. High recovery for each bile acid was achieved.
- Matrix effects (both internal standard normalized and absolute) were carefully evaluated during method development and interferences reduced or eliminated. Automation was used to further improve the throughput of the assay.

Table a. Analyte Names and Assay Ranges

Bile Acid Name	Abbreviation	Assay Range (µmol/L)
Taurocholic Acid	TCA	0.200 – 100
Tauroursodeoxycholic acid	TUDCA	0.200 – 100
Taurochenodeoxycholic acid	TCDCA	0.200 – 100
Taurodeoxycholic acid	TDCA	0.0100 – 5.00
Taurolithocholic acid	TLCA	0.0100 – 5.00
Glycocholic acid	GCA	0.200 – 100
Glycoursodeoxycholic acid	GUDCA	0.200 – 100
Glycochenodeoxycholic acid	GCDCA	0.200 – 100
Glycodeoxycholic acid	GDCA	0.0100 – 5.00
Glycolithocholic acid	GLCA	0.0100 – 5.00
Cholic Acid	CA	0.0100 – 5.00
Ursodeoxycholic acid	UDCA	0.200 – 100
Chenodeoxycholic acid	CDCA	0.0100 – 5.00
Deoxycholic acid	DCA	0.0100 – 5.00
Lithocholic acid	LCA	0.0100 – 5.00

Figure. Representative Chromatograms of Bile Acid

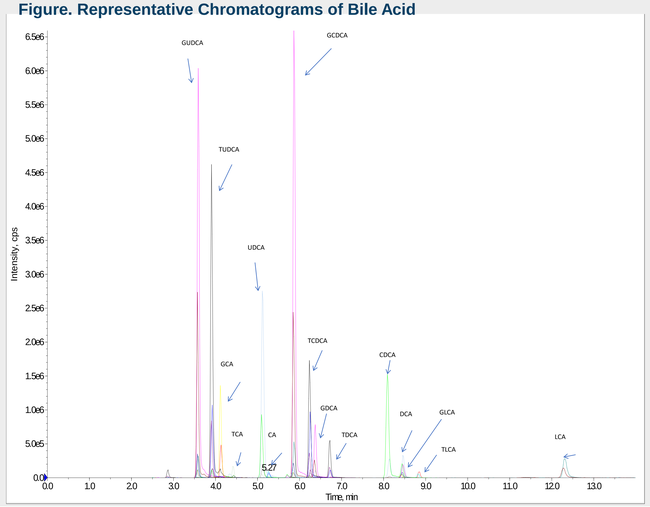

Table b. Intra-run and inter-run Accuracy and Precision Result

Analyte	Statistics	QC LLOQ	Intra-run QC Low	Intra-run QC Mid	Intra-run QC High
CDCA	Inter-run %CV	10.0	10.0	3.7	3.5
	Inter-run %Bias	8.0	0.0	-3.1	-5.0
CA	Inter-run %CV	9.2	6.2	5.2	4.6
	Inter-run %Bias	2.0	-3.3	-3.8	-3.1
DCA	Inter-run %CV	9.0	6.1	4.1	4.8
	Inter-run %Bias	-1.7	-1.3	-2.2	-4.2
GCDCA	Inter-run %CV	6.6	4.7	5.2	2.9
	Inter-run %Bias	-3.5	1.3	-2.4	-8.3
GCA	Inter-run %CV	4.8	6.1	4.2	4.1
	Inter-run %Bias	-1.5	-2.3	-1.0	-6.5
GDCA	Inter-run %CV	6.0	6.4	5.1	6.1
	Inter-run %Bias	2.0	-5.9	-4.4	-6.4
GLCA	Inter-run %CV	17.1	9.6	4.1	3.3
	Inter-run %Bias	-3.5	-5.7	-3.8	-6.3
GUDCA	Inter-run %CV	11.0	6.7	4.2	3.7
	Inter-run %Bias	-4.0	-4.3	-1.9	-6.8
LCA	Inter-run %CV	8.0	4.8	3.3	3.8
	Inter-run %Bias	1.0	-1.0	-1.0	-3.2
TCDCA	Inter-run %CV	7.8	6.6	3.6	6.2
	Inter-run %Bias	3.5	-3.7	-5.1	-4.9
TCA	Inter-run %CV	7.7	6.1	4.8	8.3
	Inter-run %Bias	0.0	-6.7	-4.5	-1.7
TDCA	Inter-run %CV	14.8	9.6	4.7	4.6
	Inter-run %Bias	-2.8	-4.7	-3.6	-6.0
TLCA	Inter-run %CV	10.0	5.7	4.6	3.2
	Inter-run %Bias	-3.2	-3.0	-5.8	-7.2
TUDCA	Inter-run %CV	14.6	7.1	5.1	5.9
	Inter-run %Bias	-6.0	-3.7	-3.9	-4.0
UDCA	Inter-run %CV	6.1	5.3	5.0	4.6
	Inter-run %Bias	1.0	1.8	4.0	-2.3

- To prepare each type of the disproportionate QC's, a stock solution containing only one bile acid with appropriate concentration (ex: 100 fold of its High QC counterpart) was spiked into the freshly prepared Low QC (in charcoal stripped human serum) in order to achieve the High QC concentration of the spiked analyte in human serum. The QC's were divided into two portions, one extracted upon preparation in triplicate, against a freshly prepared calibration standards; and the other portion was stored on the bench top at room temperature for a minimum of 24 hours and then extracted against a freshly prepared calibration curve. The first set of QC's obtained the T0 concentration and the second set of QC's obtained the T1 concentration of each type of bile acid in each type of disproportionate QC (see below).
- %Bias = (Mean Concentration at Tx - Mean Concentration at T0)/ Mean Concentration T0
- The results are within acceptance criteria that the mean of T1 concentration is within 100 ±20% of the nominal T0 Low QC concentration and the %CV is within 20%.

NOVEL ASPECT

Highlights of Frontage bile acid method comparing with published methods

- Method with highest number of bile acid analytes under regulated setting
- Higher overall recovery of bile acids (near 100%)
- High-throughput using automation and shorter injection time
- Two different assay ranges applied for the 15 bile acid (high range for 8 analytes and low range for the other 7 analytes). High and low range differ by 20-fold
- Low sample volume (25 µL of human serum) used for extraction, making the method feasible for pediatric study, if applicable

CONCLUSION(S)

- An LC-MS/MS method suitable for quantitation of serum bile acid biomarkers in clinical studies was developed and fully validated in accordance with health authority requirements for bioanalytical method validation.
- Disproportionate QC was conducted to verify the potential interference between analytes.
- Method is robust and high-throughtput and is suitable to support clinical studies.

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