Validation of a Bioanalytical Method for the Quantification of Serum Bile Acids by LC-MS/MS

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Abstract

Summary: A simple, fast, 1-D LC-MS/MS method has been developed to allow quantitative measurements of the most abundant species of Bile Acids in serum.

Introduction: Bile Acids are formed in the liver from cholesterol, stored and concentrated in the gallbladder, and excreted into the intestines in response to food intake. The liver synthesizes two primary bile acids, cholic acid and chenodeoxycholic acid from cholesterol. The primary bile acids are converted to the secondary bile acids, deoxycholic acid and lithocholic acid by intestinal bacteria. A fraction of chenodeoxycholic acid is also transformed into the tertiary bile acid, ursodeoxycholic acid (ursodeoxycholic Acid is also used as a therapy for intrahepatic cholestasis of pregnancy, ICOP). Elevated concentrations of bile acids often suggest impaired hepatic clearance due to liver disease. In this method, twelve of the most abundant bile acids (the unconjugated, glycine conjugates and taurine conjugates of ursodeoxycholic acid, cholic acid, chenodeoxycholic acid and deoxycholic acid) are independently quantified

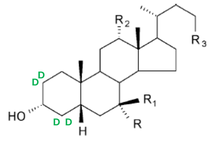
Methods : An analytical method was developed using a Thermo/Cohesive TX-4 HPLC system (Thermo-Fisher/Cohesive Technologies) with Agilent® 1200SL pumps (Agilent Technologies, Inc.) and an AB Sciex® 5000 (AB Sciex PTE. LTD.) triple quadrupole mass spectrometer. Independent calibration curves were prepared for all twelve metabolites (UDCA, CA, CDCA, DCA, GUDCA, GCA, GCDCA, GDCA, TUDCA, TCA, TCDCA, TDCA) in depleted serum (Golden West Biologicals). Sample preparation consisted of isotope dilution using a cocktail of eleven internal standards followed by protein precipitation. A Thermo Accucore® C18 analytical column (100 x 2.1mm, 2.7um, 100A) was used with an alkaline mobile phase and methanol gradient to achieve full baseline chromatographic separation of all bile acid isomers. Negative mode Electrospray Ionization (ESI) was used for detection in Multiple Reaction Monitoring (MRM) mode.

Validation Data: Analytical sensitivity was 0.1 umol/L per analyte. Inter-Assay precision ranged from 3.7 – 8.4%. Dynamic range was up to 10 umol/L for each analyte (200 umol/L with dilution). Correlation with enzyme cycling method (total bile acids only) was good (R2 = 0.91, slope 0.74x + 1.2 umol/L, n=25). Reference intervals were developed for each bile acid class as well as total bile acids. Proficiency materials from CAP (TBLA-01 through 03) were tested and found to be mostly unconjugated CDCA. Internal testing demonstrated the importance of fasting prior to specimen collection.

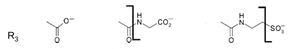
Clinical Significance: In cases of intrahepatic cholestasis of pregnancy treated with ursodiol (UDCA) measurement of bile acids by enzyme cycling methods cannot distinguish the endogenous production of bile acids from the therapeutic UDCA; therefore measuring total bile acids will not help judge the therapeutic efficacy. Fractionated Bile Acids testing can improve patient management and outcomes in several ways, illustrated in the following cases:
(1) A mother carrying twins at 29 weeks gestation developed ICOP and was treated with ursodiol. At 31 weeks signs of preeclampsia developed. Fractionated bile acids documented that although the total bile acids was elevated at 16 umol/L, 10 umol/L of the total was UDCA and conservative treatment allowed continuation of the pregnancy until 35 weeks at which time healthy 4.5 pound twins were delivered.
(2) At 33 weeks gestation ICOP in a 20 year old mother was treated with UDCA, resulting in resolution of symptoms (pruritus). At 36 weeks gestation an amniocentesis was performed and lungs were immature. At 36 weeks mild itching returned but a repeat amniocentesis was avoided when fractionation of bile acids demonstrated that an abnormally high total bile acids concentration was due solely to elevated UDCA. A normal delivery occurred at 37 weeks.

Analytes and Internal Standards

UDCA	Ursodeoxycholic Acid
CA	Cholic Acid
CDCA	Chenodeoxycholic Acid
DCA	Deoxycholic Acid
GUDCA	Glycoursodeoxycholic Acid
GCA	Glycocholic Acid
GCDCA	Glycochenodeoxycholic Acid
GDCA	Glycodeoxycholic Acid
TUDCA	Tauroursodeoxycholic Acid
TCA	Taurocholic Acid
TCDCA	Taurochenodeoxycholic Acid
TDCA	Taurodeoxycholic Acid

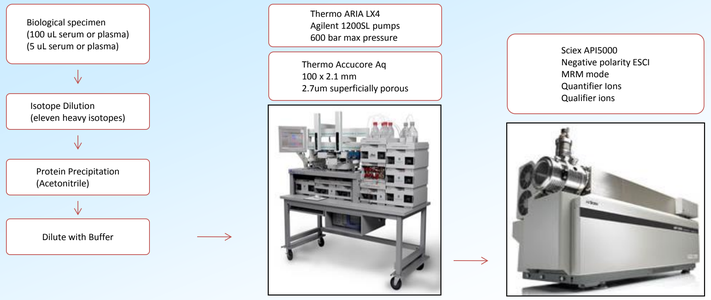


	R	R_1	R_2
Cholic acid (CA)	–OH	–H	–OH
Chenodeoxycholic acid (CDCA)	–OH	–H	–H
Deoxycholic acid (DCA)	–H	–H	–OH
Ursodeoxycholic acid (UDCA)	–H	–OH	–H



Internal standards were purchased from CDN (Unconjugated &Glycine-Conjugated) and Medical Isotopes (Taurine-Conjugated). A stable isotope of TDCA was not available at time of validation. All internal standards are isotopically labeled in the 2,2,4,4 positions on the A-ring (shown as "D" above).

Method Summary



Analytical Performance

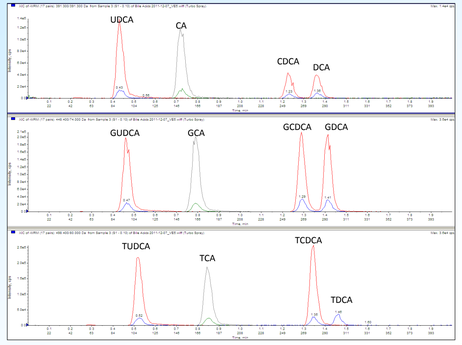
Chromatographic Resolution of Twelve Bile Acids

Calibrator mass chromatogram:

Analytes:
0.1 umol/L
(blue, green traces)

Internal Standards:
2.0 umol/L
(red, grey traces)

Note:
*Unconjugated Bile Acids do not produce suitable product ions in CID.
*Glycine-Conjugated Bile Acids produce the product ion 74 m/z in CID.
*Taurine-Conjugated Bile Acids produce the product ion 80 m/z in CID.



Reference Intervals

Fraction	Range	N
Ursodeoxycholic Acids	< 1.9 umol/L	44
Cholic Acids	< 2.2 umol/L	81
Chenodeoxycholic Acids	< 5.8 umol/L	100
Deoxycholic Acids	< 3.3 umol/L	96
Total Bile Acids	< 9.2 umol/L	105

To establish reference intervals, 130 specimens from routine wellness testing were analyzed (values <LLOQ and non-fasting specimens were not used in range calculations). The most abundant species in this population were the Chenodeoxycholic Acids and Deoxycholic Acids.

None of the specimens tested had the Cholic Acid fraction as the most abundant species of Bile Acids.

Routine patient specimens frequently have Cholic Acid fraction as the most abundant species.

Case Studies Showing Clinical Utility of Fractionation

Case Study #1 – No amniocentesis necessary

At 33 weeks gestation intrahepatic cholestasis of pregnancy in a 20 year old mother was treated with UDCA, resulting in resolution of symptoms (pruritus). At 36 weeks gestation an amniocentesis was performed and lungs were immature. At 36 weeks mild itching returned but a repeat amniocentesis was avoided when fractionation of bile acids demonstrated that an abnormally high total bile acids concentration was due solely to elevated UDCA. A normal delivery occurred at 37 weeks.

Case Study # 2 – Bringing multiples to term

A mother carrying twins at 29 weeks gestation developed intrahepatic cholestasis of pregnancy and was treated with ursodiol. At 31 weeks signs of preeclampsia developed. Fractionated bile acids documented that although the total bile acids was elevated at 16 umol/L, 10 umol/L of the total was UDCA and conservative treatment allowed continuation of the pregnancy until 35 weeks at which time healthy twins (4 pounds, 11 ounces and 4 pounds, 7 ounces) were delivered.

Proficiency (CAP April 2012 Challenge)

	UDCA	CA	CDCA	DCA	GUDCA	GCA	GCDCA	GDCA	TUDCA	TCA	TCDCA	TDCA	UDCA Total	CA Total	CDCA Total	DCA Total	Sum Total
TBLA-1	<0.1	0.2	**9.9**	0.4	0.2	0.2	1.1	0.5	<0.1	<0.1	0.2	0.1	0.2	0.3	**11.2**	1.1	**12.7**
TBLA-2	<0.1	0.2	**26.1**	0.8	0.1	0.2	1.2	0.5	<0.1	<0.1	0.1	<0.1	0.1	0.3	**27.4**	1.2	**29.1**
TBLA-3	<0.1	0.2	**81.2**	<0.1	0.2	0.2	1.1	0.5	<0.1	<0.1	0.2	<0.1	0.2	0.4	**82.4**	0.5	**83.4**

TLBA-01 Method Mean 10.8 uM (80% CDCA)
TLBA-02 Method Mean 19.5 uM (90% CDCA)
TLBA-03 Method Mean 49.4 uM (97% CDCA)

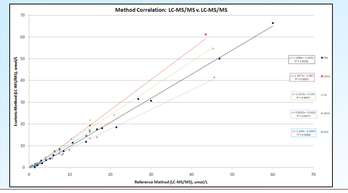
Analytical Performance

Precision (Inter-Assay, n=18, six replicates on three separate days), umol/L

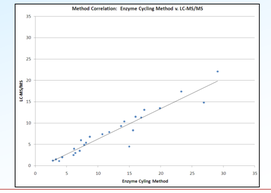
	Ursodeoxycholic Acids (UDCA + GUDCA + TUDCA)				Cholic Acids (CA + GCA + TCA)				Chenodeoxycholic Acids (CDCA + GCDCA + TCDCA)				Deoxycholic Acids (DCA + DCA + DCA)			
	QC1	QC2	QC3	QC4	QC1	QC2	QC3	QC4	QC1	QC2	QC3	QC4	QC1	QC2	QC3	QC4
Average	0.85	2.5	8.3	20.3	0.84	2.6	8.6	19.9	0.84	2.6	9.2	20.2	0.88	2.7	8.4	19.7
CV%	5.7	4.2	3.9	3.8	6.5	4.2	4.6	3.7	5.5	5.3	4.1	4.1	8.4	7.4	5.2	3.0

Method Comparisons

1. LC-MS/MS Correlation (Fractionated, n=15)
Total, R^2 = 0.9936, Y = 1.11 – 1.64 (Deming)
Individual fractions: R^2 >0.99 (All), Slopes ranged from 0.90 - 1.40 (Linear)



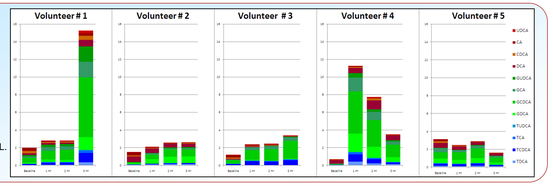
2. Trinity Biotechnology Enzyme Cycling Method (Total, n=25)
R^2 = 0.9086, Y = 0.74 – 1.2 (Deming)



The Importance of Fasting

Serum from five volunteers collected at hourly intervals after fasting, then consuming a breakfast consisting of fast food egg sandwich and hash browns along with microwave bacon. These specimens were analyzed in the fractionated Bile Acids method.

It was found that:
1. GCDCA and GDCA were the most abundant.
2. Total bile acids ranged from <2.6 to >15.3 umol/L.
3. Individual response was highly variable.



Summary

Results

1. A fast, simple 1-D LC-MS/MS method has been validated to individually quantify the most abundant twelve bile acids from serum.
2. Non-fasting specimens can be highly variable with regard to total and fractionated bile acid concentrations.
3. Correlation between enzyme cycling method (total bile acids) and LC-MS/MS method (fractionated bile acids) was acceptable.
4. Fractionation of bile acids is important for patients being treated with ursodeoxycholic acid for pruritis due to cholestasis of pregnancy.

Future Directions

1. Establish normal pregnancy ranges and characterize value of fractionated bile acids for diagnosis of intrahepatic cholestasis of pregnancy.
2. Evaluate opportunity for additional measurement of minor species such as lithocholic acids, sulfated or glycoside species of bile acids.

References and Acknowledgements

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