Exploring Different HPLC Column Chemistries for Optimal Separation of 13 Bile Acids by LC-MS/MS

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Introduction

The analysis of bile acids in human plasma is an important diagnostic tool for the detection of liver disease and can also be used as indicators of potentially harmful side effects of new drugs. There are two main types of bile acids based upon their functional groups: free (unconjugated) bile acids and conjugated bile acids, primarily glycine- or taurine-bound. Quantitation of bile acids in matrix can be very challenging due to several factors. These include structural similarities, varying polarity and stereochemistry, the presence of isomers, limited fragmentation of unconjugated bile acids in a mass spectrometer, high endogenous levels, and matrix effects caused by phospholipids or triglycerides.

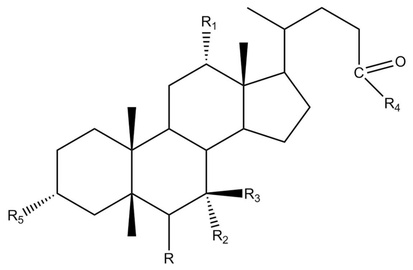
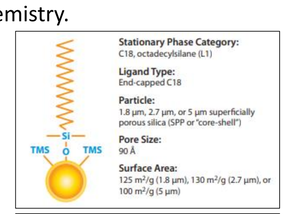
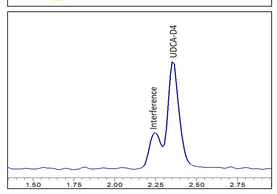


Figure 1: Base chemical structure of bile acids.

In this study, 13 bile acids were analyzed by LC-MS/MS using a Raptor C18 50 x 2.1 mm, 1.8 µm column. Through routine validation, a matrix interference was identified to be co-eluting with one of the bile acids standards and causing issues with quantitation. It was not possible to resolve this interference on the Raptor C18 column, requiring a new method be developed on an alternative column chemistry.





Column:	Raptor C18, 50 x 2.1 mm, 1.8 µm		
MPA:	5 mM ammonium acetate in H_2O		
MPB:	50:50 MeOH:ACN		
Column Temp:	60°C		
Inj. Volume:	3 µL		
Gradient:	Time (min)	Flow Rate (mL/min)	%B
	0.00	0.5	35
	2.00	0.5	40
	2.50	0.5	45
	3.50	0.5	50
	4.60	0.5	55
	5.70	0.5	80
	5.90	0.8	85
	6.50	0.8	85
	6.51	0.5	35
	8.50	0.5	35

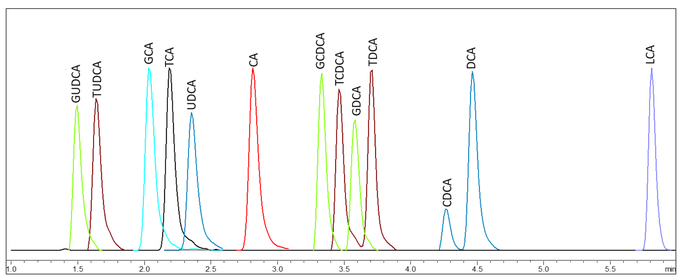
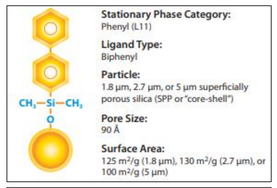


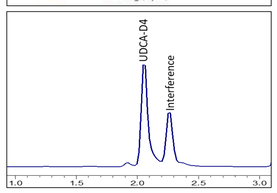
Figure 2: Bile acids analyzed on C18 column. All analytes, including isomers (GUDCA/GCDCA/GDCA, TUDCA/TCDCA/TDCA, UDCA/CDCA/DCA), are well resolved. A matrix interference with D4-UDCA is present.

Scouting Methods

Three alternative column chemistries were investigated for the method: Biphenyl, FluoroPhenyl, and ARC-18. These three stationary phases were selected as they offer alternative selectivity to the C18 phase. A scouting gradient was run to determine compound elution on each stationary phase. Methods were further optimized to determine which was best suited to meet analysis goals. The final method was required to resolve the matrix interference from D4-UDCA as well as adequately resolve the three pairs of isomers in the analyte list. The scouting gradient started at 10% B, ramped up to 100% B over 8.0 minutes, and then returned to starting conditions and held until 9.5 minutes for ample re-equilibration.

Results





Column:	Raptor Biphenyl, 100 x 2.1 mm, 2.7 µm		
MPA:	5 mM ammonium acetate in H_2O		
MPB:	50:50 MeOH:ACN		
Column Temp:	50°C		
Inj. Volume	3 µL		
Gradient:	Time (min)	Flow Rate (mL/min)	%B
	0.00	0.5	40
	7.00	0.5	70
	8.00	0.5	100
	8.50	0.5	100
	8.51	0.5	40
	9.50	0.5	40

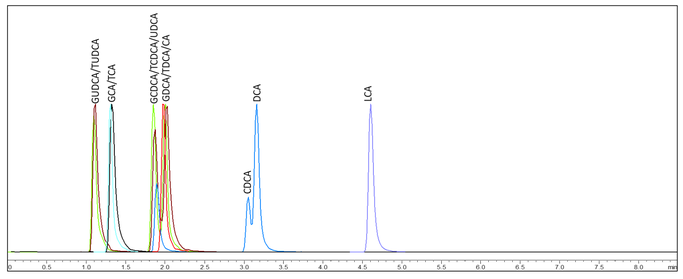
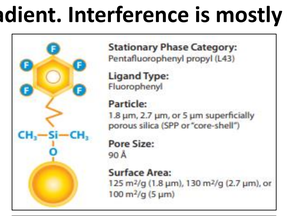
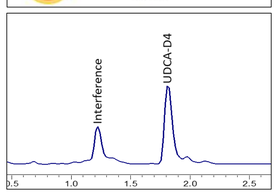


Figure 6: Bile acids analyzed on Biphenyl column. Some selectivity is observed for glycine/taurine isomers, but limited selectivity is observed for unconjugated isomers with CDCA/DCA unable to be fully resolved even with a very shallow gradient. Interference is mostly resolved from D4-UDCA.





Column:	Raptor FluoroPhenyl, 100 x 2.1 mm, 2.7 µm		
MPA:	5 mM ammonium acetate in H_2O		
MPB:	50:50 MeOH:ACN		
Column Temp:	50°C		
Inj. Volume:	3 µL		
Gradient:	Time (min)	Flow Rate (mL/min)	%B
	0.00	0.5	35
	7.00	0.5	60
	8.00	0.5	100
	8.50	0.5	100
	8.51	0.5	35
	9.50	0.5	35

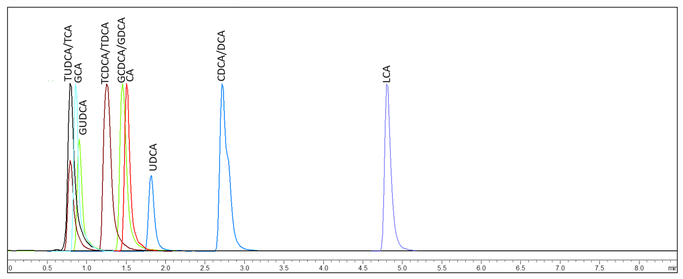
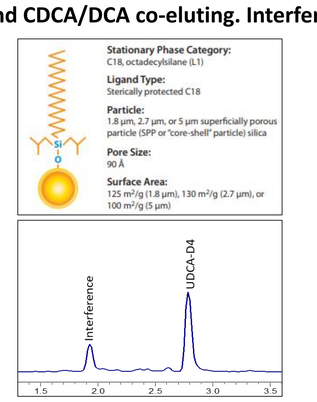


Figure 7: Bile acids analyzed on FluoroPhenyl column. No selectivity is shown for the glycine, taurine, or unconjugated isomers, with GCDCA/GDCA, TCDCA/TDCA, and CDCA/DCA co-eluting. Interference is resolved from D4-UDCA.



Column:	Raptor ARC-18, 100 x 2.1 mm, 2.7 µm		
MPA:	5 mM ammonium acetate in H_2O		
MPB:	50:50 MeOH:ACN		
Column Temp:	50°C		
Inj. Volume:	3 µL		
Gradient:	Time (min)	Flow Rate (mL/min)	%B
	0.00	0.5	40
	7.00	0.5	80
	8.00	0.5	100
	8.50	0.5	100
	8.51	0.5	40
	9.50	0.5	40

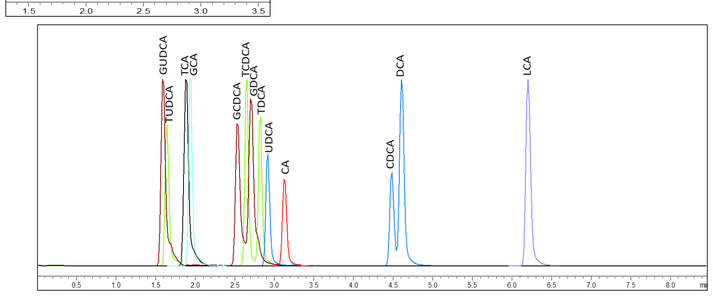
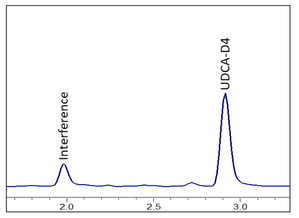


Figure 8: Bile acids analyzed on ARC-18 column. Selectivity is shown for all three isomer sets but further optimization is needed for full resolution. Interference is resolved from D4-UDCA.

Final Method



Column:	Raptor ARC-18, 100 x 2.1 mm, 2.7 µm		
MPA:	5 mM ammonium acetate in H_2O		
MPB:	50:50 MeOH:ACN		
Column Temp:	50°C		
Inj. Volume:	10 µL		
Gradient:	Time (min)	Flow Rate (mL/min)	%B
	0.00	0.5	40
	6.00	0.5	70
	7.00	0.5	80
	7.50	0.8	100
	8.10	0.8	100
	8.20	0.5	40
	9.50	0.5	40

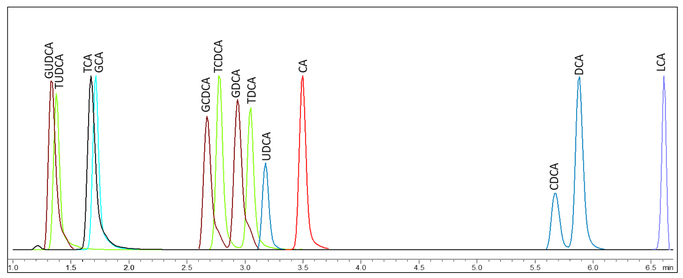


Figure 9: 13 bile acids further optimized on ARC-18 column. With a shallower gradient, analytes are well resolved. All three sets of isomers are resolved. The interference is well resolved from D4-UDCA, with a minute difference in retention time. The flow rate was increased from 0.5 mL/min to 0.8 mL/min from 7.50-8.10 min to flush phospholipids from the column and reduce matrix effects.

Discussion

Three columns were investigated in this study for the analysis of 13 bile acids. An alternative column chemistry was needed when a matrix interference could not be resolved from an analyte of interest on a C18 column.

C18 Stationary Phase

The original method used a C18 stationary phase, which showed excellent selectivity for all analytes. While this phase was able to affectively separate the analytes, a matrix interference was co-eluting with internal standard D4-UDCA. Even with a very shallow gradient, it was not possible to separate the interference from D4-UDCA.

Biphenyl Stationary Phase

The biphenyl phase column showed selectivity for the glycine and taurine isomers, but not for the unconjugated isomers. It was also unable to produce baseline resolution between the matrix interference and D4-UDCA. It is possible to increase the resolution of both the unconjugated isomers and the interference from D4-UDCA, but doing so would have resulted in an unreasonably long analytical run time.

FluoroPhenyl Stationary Phase

The FluoroPhenyl stationary phase was able to resolve the matrix interference from D4-UDCA but did not show selectivity for any of the isomer sets and resulted in analytes co-eluting.

ARC-18 Stationary Phase

The ARC-18 stationary phase showed selectivity for all three isomer sets and resolved the matrix interference from D4-UDCA. A method was further optimized on this column which allowed for analysis of all 13 bile acids in a total cycle time of 9.5 minutes. Because the Raptor C18 and Raptor ARC-18 are both C18 stationary phases, it may be expected that they would show similar selectivity for these analytes. The Raptor C18 is an end-capped C18 ligand whereas the Raptor ARC-18 is a sterically protected C18 ligand, which affects the retention and selectivity of the stationary phase. In this instance, the difference in selectivity allowed the Raptor ARC-18 column to resolve all the analytes as well as the matrix interference which was not possible on the Raptor C18 column.

Conclusions

While most method developments focus strongly on the analytes of interest, it is also necessary to give attention to potential matrix interferences. Matrix interferences can result in invalid quantitation if not properly resolved. Additionally, this study demonstrates the use of alternative column chemistries to resolve matrix interferences. While the Raptor C18 and Raptor ARC-18 are both C18 ligands, the difference in end-capping allowed increased selectivity to resolve the interference with the ARC-18 phase.

References

1. Li, D., & Carroll, F. *Rapid Analysis of 17 Bile Acids in Human Plasma by LC-MS/MS.* https://www.restek.com/articles/rapid-analysis-of-17-bile-acids-in-human-plasma-by-lc-msms. (accessed Jan 17, 2024)