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TITLE: **FAST LCMS SEPARATION OF BILE ACIDS AND THEIR CONJUGATES USING A HALO® C18 COLUMN**

MARKET SEGMENT: CLINICAL/TOXICOLOGY

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ABSTRACT

Bile acids break down fats, aid digestion, absorb important fat-soluble vitamins, and eliminate toxins; however, high levels of bile acids have been shown to cause serious medical conditions. High exposure to bile acids has been linked to gastrointestinal cancers, cholestasis and other liver diseases, particularly in people that consume a diet that is high in fats. LCMS has become one of the primary methods for bile acid analysis; however, the isobaric nature of bile acids is a challenge for identification due to multiple acids producing identical fragments. Therefore, peak resolution via the LC separation is critical for identification. Here we present the separation and identification of 13 bile acids using the HALO® C18 column. Multiple isobaric species were separated and identified, and linearity was well established.

INTRODUCTION

Bile acids, formed in the liver from cholesterol, are steroidal acids that facilitate the digestion and absorption of fats and fat-soluble molecules and vitamins in the small intestine, and are increasingly shown to have hormonal activity. Cholic acid (CA) and chenodeoxycholic acid (CDCA), are the primary bile acids in humans, and can be converted to secondary bile acids deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA) via bacteria found in the gut. The secondary bile acids are then reabsorbed and taken back to the liver, where they are conjugated with glycine or taurine.

The concentration of bile acids in patients has been used as a sensitive and specific indicator of liver disease, with elevated levels indicative of the inability of the liver to extract bile acids efficiently from the blood. The nominal level of bile acids in a non-pregnant adult, usually range from 0.3-4.8 µmol/L, with a concentration of 10-20 µmol/L considered high normal, and over 20 µmol/L considered high. In the case of pregnancy, intrahepatic cholestasis of pregnancy (ICP), is a result of increased levels of bile acids in the blood, and often requires induction of birth, as left untreated can lead to loss of child due to still birth.

Increases in bile acid levels in the blood has been linked to liver diseases such as viral hepatitis and cirrhosis. Additionally, cells in the gastrointestinal tract that have been in contact with high concentrations of bile acids are designated risk factors for gastrointestinal cancers.

In recent years, the metabolic effects of bile acids have been investigated, and four bile acids have been used in the treatment of metabolic diseases. Cholic acid is used in the treatment of bile acid synthesis disorders due to single enzyme defects, which results in the formation of abnormal bile acids. The diagnosis of a bile acid synthetic disorder is achieved using mass spectrometry to detect abnormal bile acids and intermediates associated with each genetic defect and by a lack of normal primary bile acids. The addition of cholic acid helps to decrease the formation of abnormal bile acids, and regulate the synthesis.

Chenodeoxycholic and ursodeoxycholic acid have been employed to dissolve gallstones, and obeticholic and ursodiol have been used for the treatment of biliary cirrhosis. Although incurable, biliary cirrhosis can be treated by these acids, which facilitate the movement of bile through the liver, which has shown to improve liver function and reduce liver scarring.

KEY WORDS:

Bile acids, clinical market, LC MS/MS, HALO® C18, isobaric species

EXPERIMENTAL

A Shimadzu 8040 triple quadrupole mass spectrometer was coupled to a Shimadzu Nexera X2 (Shimadzu Scientific Instruments, USA). Bile acid standards were provided by Millipore Sigma (Burlington, MA), and were prepared in SigMatrix serum at a concentration of 12.5µg/ml. Methanol (HPLC grade), water (HPLC grade) and ammonium formate were purchased from Millipore Sigma (Burlington, MA). A HALO 90 Å C18, 2.7 µm, 2.1 × 150 mm column (Advanced Materials Technology, Wilmington, DE) was used. The column flowrate was set at 0.4µL/min and thermostated at 40 °C.

Mobile phase A - 5 mM ammonium formate and 0.012% formic acid in water.

Mobile Phase B - 5 mM ammonium formate and 0.012% formic acid in methanol.

The gradient conditions are listed in Table 1

Table 1. Gradient

Time	*%B*
0	30
10	95
15	95
15.10	30
18	30
18	Stop

Table 2. MS Source Conditions

Mass Spectrometer	Shimadzu 8040
Ion mode:	Negative Electrospray
Heat Block Temperature	400 °C
Drying line	300 °C
Nebulizing Gas Flow:	3 L/min
Drying Gas Flow	18 L/min
Spray Voltage:	-4000 V
Q1/Q2 Resolution:	High

RESULTS

The analysis of bile acids by tandem mass spectrometry MS/MS is difficult based on a number of factors. As multiple bile acids have the same molecular weight and elemental composition; they cannot be identified by MS/MS alone and require chromatographic separation in both unconjugated and conjugated forms. Figure 1 demonstrated the separation of the 13 standards achieved by the HALO 90 Å C18, 2.7 µm, 2.1 × 150 mm column.

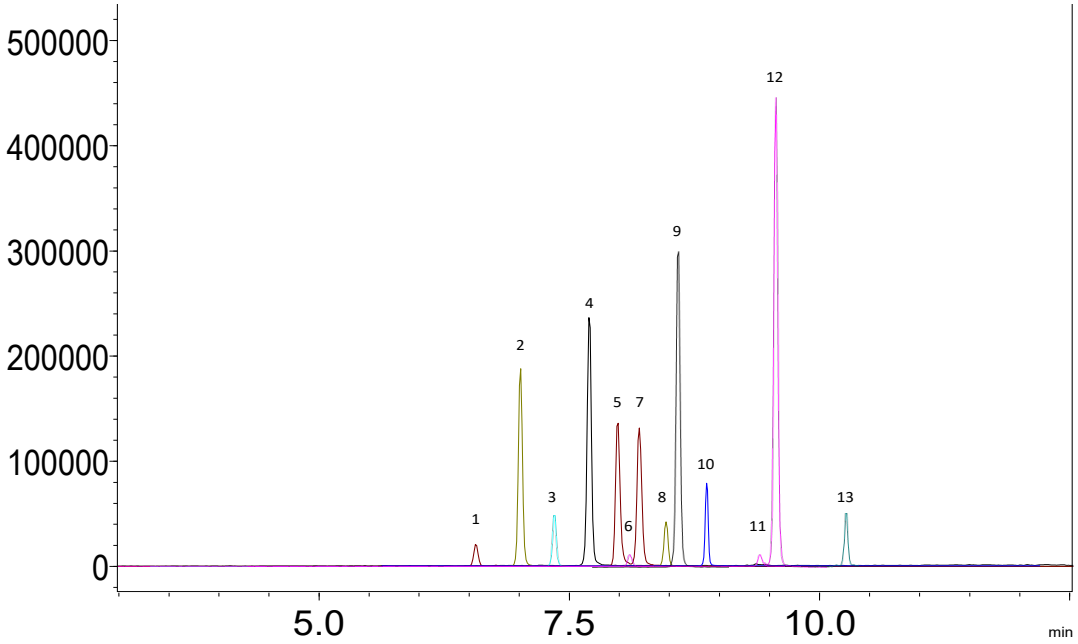

Figure 1. TIC of 13 bile acids showing full resolution of all isobaric compounds

The HALO® C18 separation of the 13 bile acid species (Figure 1.), included 3 groups of isobaric species. UDC, CDC, and DC (peaks 6, 11, & 12), GDC and GCDC (peaks 2 & 8), and TUDC, TCDC, and TDC (peaks 1, 5, & 7).
This is especially beneficial as DC and CDC are primary bile acids. In addition, fragments generated are not useful for identification purposes, as the same fragments are seen for all isobaric species. As DC and CDC are isomers the chromatographic separation is paramount for identification, and the resolution provided by the HALO® C18 allows for these acids to be identified based upon retention time.

Analyte	MH^-	Peak Number	Transition	CE	Concentration
Sodium-tauroursodeoxycholate (TUDC)	498.7	1	498.7>124.1	51	12.5µg/ml
Glycoursodeoxycholic acid (GDC)	448.2	2	448.2>74.1	34	12.5µg/ml
Taurocholic acid sodium salt hydrate (TCA)	514.3	3	514.3>80	35	12.5µg/ml
Glycocholic acid hydrate (GCA)	464.2	4	464.28>402.3	34	12.5µg/ml
Sodium taurochenodeoxycholate (TCDC)	498.7	5	498.7>124.1	52	12.5µg/ml
Ursodeoxycholic acid (UDC)	391.5	6	391.5>391.5	8	12.5µg/ml
Sodium-taurodeoxycholate hydrate (TDC)	498.2	7	498.7>124.1	52	12.5µg/ml
Sodium glycochenodeoxycholate (GCDC)	448.2	8	448.2>74.1	30	12.5µg/ml
Cholic acid (CA)	407.5	9	407.5>407.5	8	12.5µg/ml
Sodium-taurolithocholate (TLC)	482.2	10	482.2>124.1	50	12.5µg/ml
Chenodeoxycholic acid (CDC)	391.5	11	391.5>391.5	8	12.5µg/ml
Deoxycholic acid (DC)	391.5	12	391.5>391.5	8	12.5µg/ml
Lithocholic acid (LC)	375.5	13	375.5>375.5	8	12.5µg/ml

Table 3. Bile acids separated and corresponding peaks

LINEARITY

In order to test the validity of the method and column, the linearity was investigated to assess the range of concentrations for which the method can reliably function. A concentration of over 20 µmol per liter is considered high, so concentrations of the mixture were prepared (6.25 µg/ml, 12.5 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml) to cover from the normal-high range, up to the very high range. Measurements were done in triplicate and the intensities vs concentration was plotted. Figure 2 shows a plot of lithocholic acid as a representative plot of the entire set, with an R2 value 0.9987, demonstrating good linearity of the column. In addition, linearity of all the bile acids surveyed (Table 4) shows the linearity holds for all of them and demonstrates this column can be used for the analysis in clinically relevant concentrations (6.5 µg/ml, 15 µmol/L), which is designated the normal-high range and enabling quantitation at these levels. This trend held throughout the analysis of all 13 compounds in the mixture, showcasing the rugged reliable performance that is a hallmark of the HALO® brand.

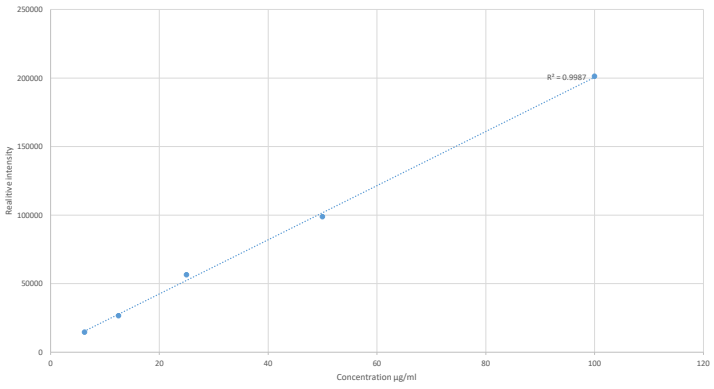

Figure 2. Linearity of Lithocholic Acid

Analyte	Peak number	R^2
Sodium-tauroursodeoxycholate (TUDC)	1	0.9998
Glycoursodeoxycholic acid (GDC)	2	0.9988
Taurocholic acid sodium salt hydrate (TCA)	3	0.9986
Glycocholic acid hydrate (GCA)	4	0.9978
Sodium-taurochenodeoxycholate (TCDC)	5	0.9982
Ursodeoxycholic acid (UDC)	6	0.9993
Sodium-taurodeoxycholate hydrate (TDC)	7	0.9971
Sodium glycochenodeoxycholate (GCDC)	8	0.9981
Cholic acid (CA)	9	0.9957
Sodium taurolithocholate (TLC)	10	0.9973
Chenodeoxycholic acid (CDC)	11	0.9955
Deoxycholic acid (DC)	12	0.9986
Lithocholic acid (LC)	13	0.9987

Table 4. for the HALO® C18

CONCLUSION

An LC MS/MS method was developed for the analysis of bile acids by the HALO® C18 column. The column demonstrated excellent performance in the separation of multiple isobaric compounds and rugged reliability with excellent linearity, enabling clinically relevant concentrations to be analyzed. The linearity shown allows identification and quantitation of the bile acids into the normal-high range, which would enable physicians to accurately determine bile acid levels, and help to diagnose liver disease. CD and CDC are isomers, and the two primary bile acids that are found in humans. The main limitation with identification by MSMS is associated to indistinguishable transitions, so the chromatographic separation is paramount for identification, and the performance of the column will dictate the success of the assay. As the data has shown, the resolution, precision and narrow peak widths provided by the HALO® C18 allows for these acids to be clearly separated and identified, and the linearity shows that these acids can be detected and quantitated at clinically relevant levels. The HALO® C18 is an ideal column for clinical applications as the Fused-Core® design, being less prone to clogging, lends itself to longer column lifetimes for difficult matrices, such as serums, providing excellent resolution and enabling multiple isobaric species to be detected and identified in clinically relevant ranges.

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