A Novel Separation for Ethyl Glucuronide and Ethyl Sulfate using a Multi-Mode Reversed-Phase Column

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Abstract:

Ethyl glucuronide and ethyl sulfate were separated using a multi-mode C_{18} column. The analytes were eluted of the column using an formic acid/ammonium formate/acetonitrile gradient mobile phase. The separation was primarily affected by the mobile phase buffer pH, salt concentration and organic modifier composition. Increasing the pH and the buffer concentration caused a decrease in retention time for both analytes. In addition, changing the composition of the organic modifier affected the retention of ethyl glucuronide and ethyl sulfate. Changing the initial concentration of the organic modifier gave a U-shaped retention for ethyl glucuronide. Greater retention of ethyl glucuronide and ethyl sulfate was achieved using this system compared to traditional the retention of the these analytes using reversed-phase methods.

Introduction:

Ethanol (alcohol) is one of the most commonly used drugs in the United States. When ethanol is used in conjunction with prescription narcotics, it can result in adverse affects to the person. Therefore, when testing pain management patients for prescription compliance, it is important to make sure they are not using alcohol in conjunction with their prescription. When ethanol is consumed 90% of the dose that is consumed is metabolized. The primary urine metabolites are ethyl glucuronide and ethyl sulfate. Traditional confirmation LC/MS methods use a C18 stationary phase along with an organic modifier and an aqueous buffer as the mobile phase. Under these conditions, both analytes elute off the column very early and can often give broad peaks. The resulting poor chromatography can lead to poor results for both analytes. Using this mixed mode C18 column we were able to obtain greater retention of both ethyl sulfate and ethyl glucuronide. The separation was affected by mobile phase pH , buffer concentration and organic modifier composition. Mobile phase pH and buffer concentration had the greatest effect on the retention of the analytes under reversed phase conditions. We now report on a chromatographic method for ethyl sulfate and ethyl glucuronide using these conditions. This method gave better retention over traditional reversed phase methods.

Materials and Equipment:

Reagents:

Ethyl sulfate, ethyl glucuronide, ethyl sulfate-D_5 and ethyl glucuronide-D_5 were purchased from Cerilliant Corp., Round Rock, TX. All solvents were purchased from VWR International, Visalia, CA. The Scherzo SM-C18 column 100 x 2.0mm 3µm was purchased from Imtakt USA, Portland, OR. Drug free human urine was purchased from Utak Labs, Valencia, CA, USA. Strata-X 33µm 30mg/3mL SPE cartridges were purchased from Phenomenex Corp., Torrance, CA.

Equipment:

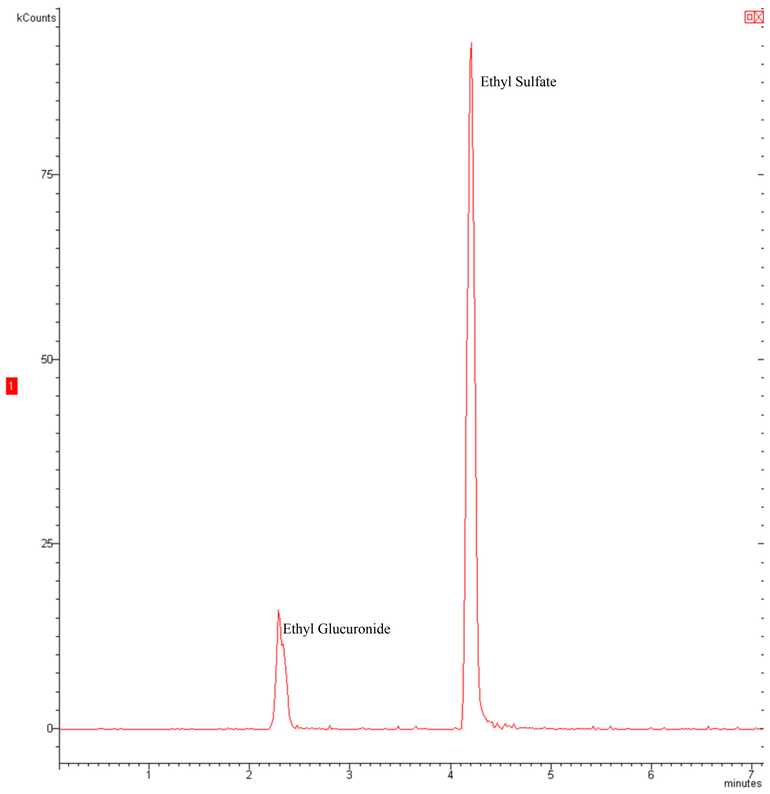
The samples were analyzed on a Varian 325 MS system equipped with Varian 212 pumps, Varian 460 autosampler and a ProStar 500 column switching valve. All the data was collected using the Varian workstation software v 6.9.3.

Experimental:

A stock solution (20 µg/mL) of ethyl sulfate and ethyl glucuronide were prepared in methanol.

Ethyl sulfate and ethyl glucuronide were spiked into human urine so that the final concentration was 100 ng/mL. Samples were removed from the sample matrix by solid phase extraction.

After the samples were dried, they were reconstituted in 5mM formic acid.



Chromatogram of ethyl glucuronide and ethyl sulfate separated on a Scherzo SM-C18 100 x 2.0mm column 3µm. The separation was obtained using a gradient mobile phase. Pump A) 5mM Formic acid Pump B) 50:50 Acetonitrile/5mM Ammonium formate pH 6.8. Gradient:, 0% B ramp to 100% B in 4 min hold 100% B for 1 min ramp to 0% B in 0.1 min hold for 2 min. The flow rate was 450 µL/min. The column temperature was 35° C. The compounds were detected using electrospray ionization in the negative mode.

Results

The SM-C18 column separates analytes using three mechanisms, reversed phase normal phase and ion exchange. The stationary phase contains both cation and anion exchange ligands in addition to C18 ligands. Ethyl sulfate and ethyl glucuronide could be separated by all three mechanisms. The reversed phase separation was affected by the mobile phase pH buffer concentration and organic modifier composition. Mobile phase pH provided the greatest change in selectivity. Table 1 shows the effect of pH on the retention of ethyl sulfate and ethyl glucuronide. As the mobile phase pH is increased the retention time of ethyl sulfate and ethyl glucuronide are decreased. Ethyl sulfate did not elute off the column when aqueous modifier was composed of only formic acid. Increasing the gradient to 100% acetonitrile did not elute ethyl sulfate off of the column.

Changing the buffer concentration also had an effect on the retention time of ethyl sulfate and ethyl glucuronide. Increasing the buffer concentration decreased the retention times for both analytes. The effect of buffer concentration on the retention time of ethyl sulfate and ethyl glucuronide is summarized in Table 2.

Table 1. Effect of Mobile Phase pH on Retention Time

pH	Ethyl Glucuronide Retention Time (min)	Ethyl Sulfate Retention Time (min)
2.5	2.5	-
4.2	1.7	2.9
5.0	1.3	2.0
6.8	0.8	0.8

Chromatography of ethyl sulfate and ethyl glucuronide at various pH values using the Scherzo SM-C18 column 100 x 2.0mm 3µm with Pump A) 5mM Ammonium formate, various pH's, pump B) Acetonitrile, Flow rate 450 µL/min column temp 35° C. Gradient conditions were 0% B to 60% B in 2 min hold for 60% B for 1min ramp to 0% B in 0.1min hold for 1 min The compounds were detected using ESI in the negative mode.

Table 2. Effect of Buffer Concentration on Retention Time

Buffer Conc. (mM)	Ethyl Glucuronide Retention Time (min)	Ethyl Sulfate Retention Time (min)
5	1.7	3.4
20	1.4	1.8
50	0.9	1.3
100	0.72	0.72

Chromatography of ethyl sulfate and ethyl glucuronide at various ammonium formate buffer concentrations using the Scherzo SM-C18 column 100 x 2.0mm 3µm with Pump A) Ammonium formate, pH6.8, pump B) Acetonitrile, Flow rate 450 µL/min column temp 35° C. Gradient conditions were 0% B to 60% B in 2 min hold for 60% B for 1min ramp to 0% B in 0.1min hold for 1 min The compounds were detected using ESI in the negative mode.

Optimal separations were obtained when using gradients that changed pH and the organic modifier over the course of the run. Figure 1 shows the separation of ethyl sulfate and ethyl glucuronide using a pH and an organic modifier gradient at the same time. Good peak shape and retention were obtained under these conditions. Increasing the initial concentration of acetonitrile had a different effect. At low initial concentrations of acetonitrile (≤ 50%), the separation mechanism was reversed phase. Under these conditions ethyl glucuronide eluted earlier than ethyl sulfate, and the retention times decreased when the initial concentration of acetonitrile was increased. When the initial acetonitrile concentration was increased to 80% or greater, the separation used a normal phase mechanism. The retention time of ethyl glucuronide increased. At initial concentrations of 90% acetonitrile the elution order was reversed. Under these condition ethyl glucuronide can be highly retained. However, the ethyl glucuronide peak was much broader than what was obtained using reversed phase conditions. This made quantitation of ethyl glucuronide more difficult. When the acetonitrile concentration was increased to above 95%, ethyl glucuronide did not elute off of the column as a measurable peak.

Conclusion:

The SM-C18 stationary phase gave good resolution of ethyl sulfate and ethyl glucuronide. Better retention was obtained using a pH/acetonitrile gradient. Starting at a low pH and increasing the pH over time allowed for better retention of both analytes. Changing the organic modifier also decreased the retention times. However, this worked best in conjunction with changing the pH. At very high initial acetonitrile concentrations ethyl glucuronide was retained longer than ethyl sulfate.

The disadvantage to using this stationary phase was the possibility of longer equilibration times. Since the separation involves pH the stationary phase can require slightly longer retention times than other columns. We found that we switching between pH values, buffer composition and concentration or separation mode. That the column needed approximately 20 minutes to equilibrate.