LC-MS/MS Analysis of Ethyl Glucuronide and Ethyl Sulfate in Urine: A Comparison of Sample Preparation Techniques.

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Abstract

Introduction: Ethyl glucuronide (EtG) and ethyl sulfate (EtS) are phase II metabolites of ethanol formed by conjugation of ethanol with glucuronic or sulfonic acid. These metabolites can be detected in urine for several days after last alcohol ingestion and are used as biomarkers of alcohol use in treatment or compliance programs. These compounds can be challenging analytically due to their small molecular weight, high water solubility and their propensity to form negatively charged ions under electrospray conditions.

Objective: Compare three different sample preparation techniques to determine if any provided the cleanest sample for LC-MS/MS analysis considering analyte recovery and minimal ion suppression.

Methods: We compared two solid-phase extraction (SPE) chemistries and a dilution protocol. The matrix was a pool of EtG/EtS-free urine collected from ten different donors. SPE #1 was a strong anion exchange sorbent (EVOLUTE AX, Biotage), SPE #2 was an amino-propyl sorbent (ISOLUTE NH2, Biotage). The dilution protocol consisted of raw urine diluted into purified water. The preparation protocols were optimized to provide equivalent amounts of analyte injected onto the LC-MS/MS system to ensure equivalent comparison. The LC-MS/MS system was an Agilent 1200 HPLC stack with a CTC-DLW autosampler coupled to an API4000 LC-MS/MS (Applied Biosystems). The HPLC column was a Phenomenex Synergi HydroRP (100Å, 2 x 100mm), The TurboSpray source was operated in the negative ion mode and all electrospray and CID parameters were optimized for MRM analysis of ethyl glucuronide, ethyl sulfate and their pentadeuterated analogs.

Results: All sample preparations allowed reproducible, quantitative analysis of EtG and EtS in human urine. Fortified calibration curves were linear from 100 – 10000 ng/mL. Ion suppression was evident in each preparation, but we controlled this with the use of deuterated internal standards for each analyte. A large endogenous peak eluted adjacent to EtS (*m/z* 125 → 80) with each preparation. The AX SPE preparation (reconstituted in mobile phase) was able to resolve this peak most efficiently. The NH2 SPE preparation (eluted with 10mM NH_4HCO_3) provided the cleanest chromatogram with the least amount of endogenous background. The straight dilution protocol provided the greatest amount of signal intensity compared to the SPE preparations.

Conclusions: No preparation was clearly superior to the other, but each offered distinct advantages. The dilution approach was by the far the fastest, but runs the risk of introducing unwanted matrix into the LC-MS/MS system. The AX SPE preparation offers the advantage of varying the reconstitution volume of the sample extract prior to analysis offering more control of the amount of material injected on the column. The NH2 SPE preparation provided a clean sample that did not require dry-down or reconstitution of the extract.

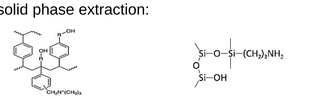
Keywords: Ethyl glucuronide, Ethyl sulfate, LC-MS/MS, Solid-phase extraction

Introduction

- Ethyl glucuronide and Ethyl sulfate are minor phase II metabolites of ethanol formed by enzymatic conjugation of ethanol with glucuronic or sulfonic acid that can be detected in urine for several days after last ingestion of alcohol.
- These compounds can be challenging analytically due to their small molecular weight, high water solubility and their propensity to form negatively charged ions under electrospray conditions.
- The objective of this study was to compare three different sample preparation techniques to determine if any provided a superior sample preparation for LC-MS/MS analysis considering analyte recovery and minimal ion suppression.

Experimental

- 10 patient samples were pooled to create the matrix used for comparisons.
- Ion suppression was evaluated by post column infusion.
- Biotage EVOLUTE AX50 (#613-0010) and ISOLUTE NH2 (#470-0010) were used for solid phase extraction:



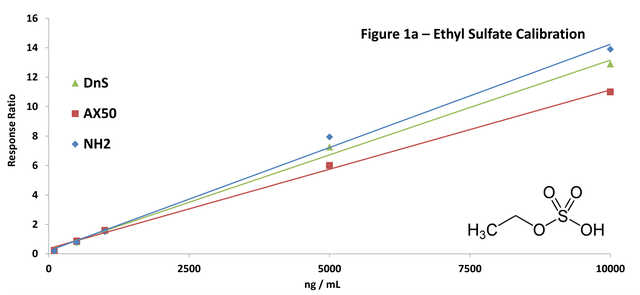
- Sample preparation details are summarized & compared:

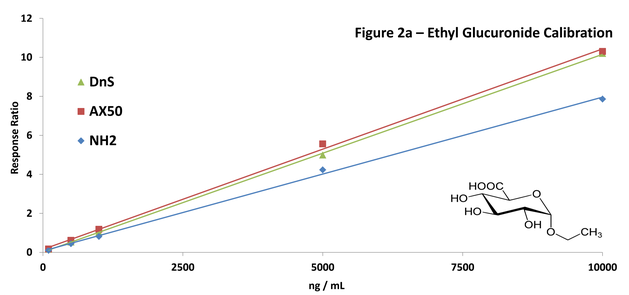
	Sample Prep		
	dilute and shoot	EVOLUTE AX50	ISOLUTE NH2
step			
sample pre-treatment	Urine + Water	Urine + ACN	Urine + 6N HCl + ACN
SPE prep	na	MeOH, Water, MeCN	MeOH, Water, 0.2% HOAc in ACN
SPE wash 1	na	ACN	Hexane
SPE wash 2	na	MeOH	na
SPE dry	na	na	10 minutes
SPE elute 1	na	2% HCl in ACN	10 mM NH_4HCO_3/HCOOH, pH 3
SPE elute 2	na	na	10 mM NH_4HCO_3/HCOOH, pH 3
dry/concentrate	na	~15 min	na
reconstitute	na	variable	na
inject	10	10	10

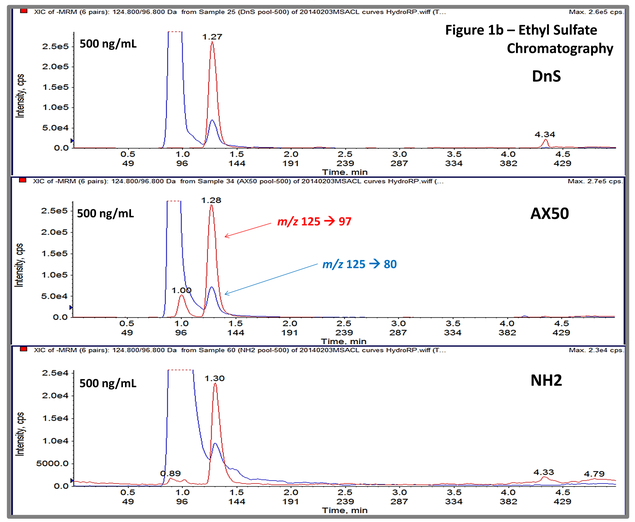
- Chromatography & Mass Spectrometry:
  - Agilent 1200 HPLC with CTC DLW autosampler
  - Phenomenex Synergi 2.5 HydroRP 100 Å, 2x100mm
  - Mobile phase A (0.1% HCOOH) & Mobile phase B (Acetonitrile), 0.3 mL/min in a nonlinear gradient at 30 °C
  - ABSciex API4000 triple quadrupole mass spectrometer Negative ion electrospray at 700 °C, -4500 V in MRM mode

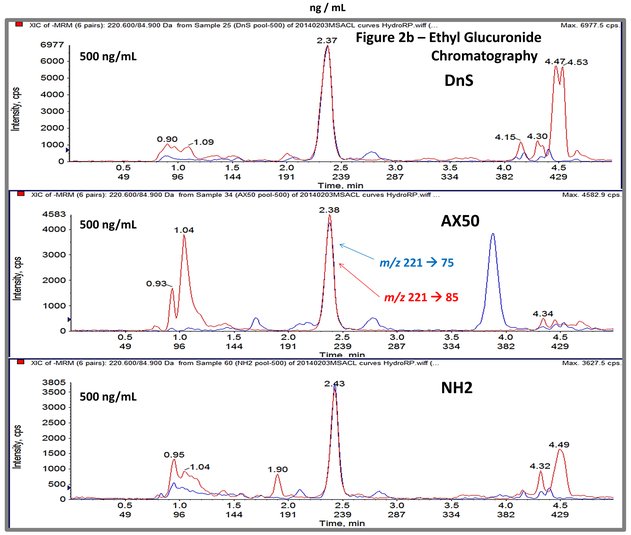
Part Number: P115

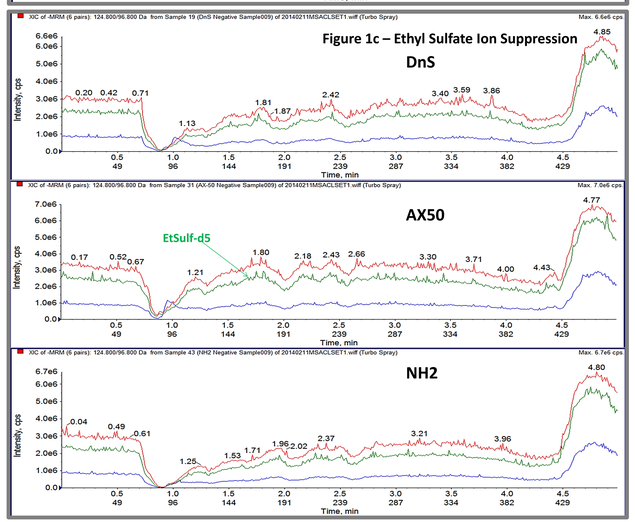
Results

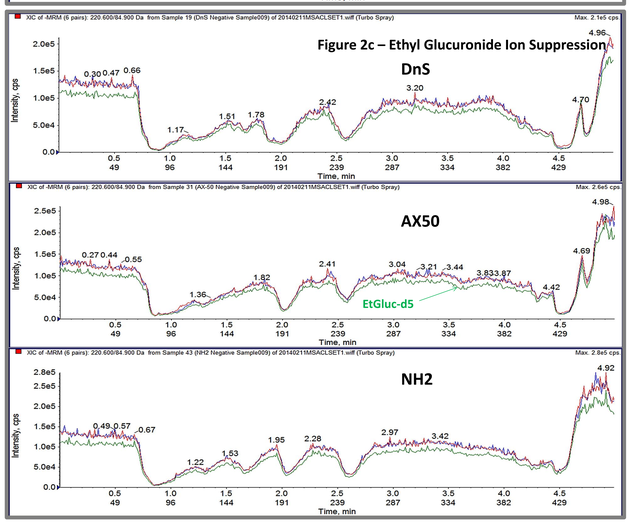












Discussion

- A pool of negative patient urine was used for all experiments to provide a realistic sample matrix.
- Figures 1a-c shows ethyl sulfate data for dilute-and-shoot (DnS), EVOLUTE AX50 (AX50) and ISOLUTE NH2 (NH2) sample preps.
  - Calibration curves show a linear response with r^2 values exceeding 0.99 for each preparation.
  - Chromatograms are generally clean with the exception of a large endogenous peak eluting prior to ethyl sulfate's qualifier ion (*m/z* 125→80). The intensity of this peak (and other contaminants) may be controlled by optimizing the elution volume (NH2) or the reconstitution volume (AX50) of the SPE preparations.
  - Reproducible ion ratios can be calculated although the NH2 prep may be challenged at the LOQ of 100 ng/mL with dirty samples under the conditions described here; this can be optimized as above.
  - Ion suppression is present here in all three preparations, but is reasonably controlled with internal standard and may be further controlled by optimizing elution volume (NH2) or the reconstitution volume (AX50).
- Figures 2a-c shows ethyl glucuronide data for dilute-and-shoot (DnS), EVOLUTE AX50 (AX50) and ISOLUTE NH2 (NH2) sample preps.
  - Calibration curves show a linear response with r^2 values exceeding 0.99 for each preparation.
  - In general, the NH2 prep seemed to give the cleanest chromatogram in terms of additional contaminant peaks (e.g. ~1 minute & ~ 4 minutes).
  - Ion suppression is present here in all three preparations, but is reasonably controlled with internal standard and may be further controlled by optimizing elution volume (NH2) or the reconstitution volume (AX50).

Conclusions

- No sample preparation was clearly superior, but each offer advantages.
- Dilution was fastest/simplest but runs the risk of adding unwanted contaminants to the analytical system.
- EVOLUTE AX SPE allows varying the final reconstitution volume thus controlling the amount of analyte injected into the system independent of injection volume.
- ISOLUTE NH2 SPE resulted in perhaps the cleanest chromatogram and did not require a dry-down step.
- Ion suppression was not eliminated under any sample prep conditions tested.
- Chromatographic separation of co-eluting matrix should be a priority in this analytical method.

References

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