Fast, Simple Method for the Analysis of Benzodiazepines in Meconium and an Inter-laboratory Method Comparison

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HIGHLIGHTS: High reproducibility

WAX-S XTR

ABSTRACT

A novel method for the quantitation of 10 commonly prescribed benzodiazepines and/or their metabolites in meconium was developed using enzymatic hydrolysis, Dispersive Pipette XTRaction (DPX) + SALLE, and LC-MS/MS analysis.

DPX + SALLE combines Dispersive Pipette XTRaction and SALLE (Salting-out Assisted Liquid-Liquid Extraction) for a novel cleanup mechanism. XTR tips contain Weak Anion Exchange (WAX) for cleanup and salt (S) necessary for SALLE. This methodology can remove matrix interferences in less than one minute. The method was evaluated for linearity, precision, extraction efficiency, and limits of detection and quantitation. To test the validity of our method, a blind study was done with a collaborative laboratory including 35 meconium patient samples tested for ten benzodiazepines and/or metabolites.

INTRODUCTION

Monitoring benzodiazepines in meconium is important for identifying potential health risks and treatment options for newborns. Meconium analysis is complex as a result of its heterogeneous composition. Meconium is a formulation of epithelial cells, mucus, lanugo, bile acids and salts, sugars, lipids, pancreatic and intestinal secretions, and more. When using liquid chromatography coupled with mass spectrometry, this difficult matrix can cause ion suppression or ion enhancement of the analytes of interest. Therefore, it is imperative to perform sample preparation to minimize these matrix effects. Reducing matrix effects with sample preparation involves extracting target analytes from the endogenous biological matrix, a mission that is often time consuming and labor intensive. Benzodiazepines are extensively metabolized, producing many glucuronide conjugates. Glucuronides often have poor LC-MS/MS sensitivity so enzymatic hydrolysis is employed to cleave the glucuronide moiety, leaving free parent compounds for improved detection.

Dispersive Pipette XTRaction incorporates a loosely contained sorbent in a pipette tip between a frit at the bottom and a barrier at the top of the tip. Aspirate and dispense steps mix sample solution and loose sorbent material. Dissolved salt (S) facilitates phase separation during extraction as shown in Figure 1.

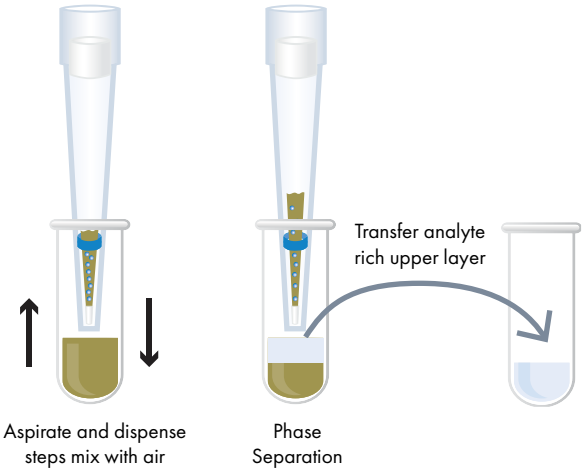

Figure 1. Cleanup process using WAX-S XTR tips.

MATERIALS AND METHODS

All drug standards were purchased from Cerilliant Corporation (Round Rock, TX, USA). WAX-S XTR tips were purchased from DPX Technologies, LLC (Columbia, SC). IMCSzyme™ was obtained from Integrated Micro-chromatography Systems, LLC (Columbia ,SC)

Analyses were performed using a Thermo TSQ Vantage triple quadrupole system with an Agilent 1100 HPLC equipped with an Agilent Poroshell EC-C18 column (3.0 x 50mm, 2.7 µm). Sample injections of 20 µL were made using a 6 port (0.25mm) Cheminert C2V injection valve incorporated on a dual rail GERSTEL MPS autosampler.

The mobile phase used 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The initial gradient was 70% A for 0.25 min, which ramped to 5% A at 2 min. The gradient remained at 5% A for 1 min, then back to 70% A for a total run time of 6.5 min. The eluent was diverted to waste during the intervals of 0-0.5 and 5-6.5 min after injection. The column flow rate was 0.4 mL/min. The electrospray voltage was 4000V, and the gas pressure was 60 psi.

Table 1. Sample Preparation

1	• Add 25 mg Meconium + 215 µL H_2O in vial • Vortex until homogenous
2	• Add 130 µL of mix: buffer, enzyme, internal standard and vortex • Hydrolyze for 1 hr at 55 °C
3	• Add 600 µL ACN, vortex and centrifuge • Transfer supernatant to clean vial
4	• Aspirate and dispense 2x with WAX-S XTR tips
5	• Transfer 500 µL of ACN upper layer • Solvent Evaporate • Reconstitute in 100 µL of 10% methanol
6	• Inject onto LC-MS/MS

RESULTS

The method (Shown in Table 1) was evaluated for percent recovery and percent ion suppression for each benzodiazepine. The percent recovery was calculated using three replicate extracted spiked samples and three replicate matrix matched (post-extraction spiked) samples. The percent ion suppression was calculated using the same three matrix matched samples compared to a neat sample (spiked solvent). All recoveries were greater than 50% and percent ion suppression did not exceed 45%.

A ten point calibration curve covering the range of 5 ng/g to 1000 ng/g with four replicates at each concentration was used to evaluate the linear regression for this method, data shown in Table 3. All compounds had correlation coefficients above 0.994 with slopes ranging from 0.9948 to 1.0414 and y-intercepts below the limit of quantitation. The limit of detection (LOD) was determined using the equation LOD=3.3*s/slope, where s is the standard deviation of the lowest non-zero calibrator. The limit of quantitation (LOQ) is simply three times the LOD. The LODs ranged from 0.5 to 2.1 ng/g and the LOQs ranged from 1.5 to 6.4 ng/g.

A precision study was performed over three days with three replicates of 100 ng/g and 1000 ng/g fortified samples each day. There was a total of nine samples at each concentration. The average with-in run precision, calculated in percent coefficient of variation, was calculated by the average of each day's concentration standard deviation, 10% or below for each benzodiazepine at each concentration. The average between-run precision was calculated by taking the standard deviation of the average concentration for each concentration on each day, which was 10.2% or below for each benzodiazepine at each concentration.

Table 2. Linear Regression Data and Limits of Detection and Quantitation.

Compound	R^2	Equation	LOD (ng/g)	LOQ (ng/g)
7-Aminoclonazepam	*0.9997*	*y = 1.0056x – 1.3848*	*1.5*	*4.4*
Midazolam	*0.9965*	*y = 1.0414x – 10.344*	*0.5*	*1.5*
α-hydroxyalprazolam	*0.9948*	*y = 1.0133x – 3.3349*	*1.8*	*5.3*
Alprazolam	*0.9953*	*y = 0.9971x + 0.7392*	*1.6*	*4.8*
Oxazepam	*0.9956*	*y = 1.0167x – 4.2001*	*1.2*	*3.7*
Nordiazepam	*0.9971*	*y = 0.9979x + 0.5054*	*1.9*	*5.8*
Lorazepam	*0.9979*	*y = 0.9948x + 1.2532*	*2.1*	*6.4*
Clonazepam	*0.9993*	*y = 1.0099x – 2.4967*	*1.4*	*4.2*
Temazepam	*0.9950*	*y = 1.0176x – 4.4042*	*0.6*	*1.8*
Diazepam	*0.9984*	*y = 1.0142x – 3.5546*	*0.7*	*2.1*

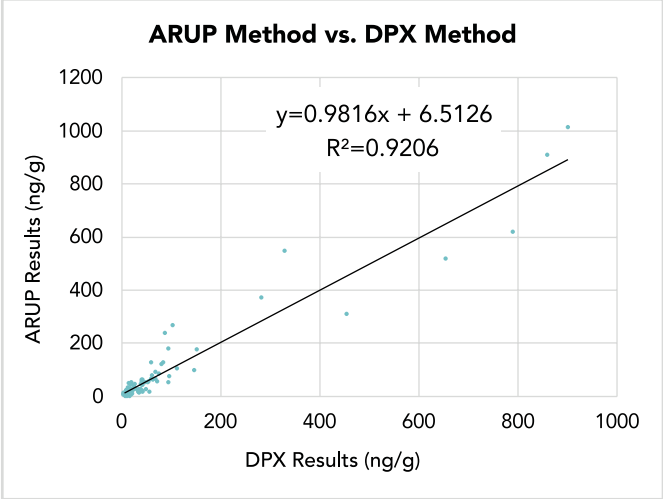

Figure 2. Correlation of positive patient sample results from the current method and the ARUP method.

Lastly, a blind study of 35 patient samples was done with a collaborating lab. We evaluated the patient samples in triplicate. The relative standard deviations ranged from 0.04% to 40% with an average of 8.0%. The correlation of this method's results with the corresponding lab's results was over 92%. (Shown in Figure 2) The success of this blind study signifies the validity of this quick and easy method compared to a more intricate, lengthy method.

CONCLUSIONS

The method established herein is characterized by simple vortexing for sample homogenation, fast and reliable IMCSzyme for hydrolysis in situ minimizing hydrolysis time to one hour, and WAX-S XTR tips for extraction that produces a small amount of clean, analyte rich acetonitrile to minimize sample preparation and solvent evaporation times. This method is quick yet effective. Correlation coefficients for each benzodiazepine were above 0.99. All precision values were below 15%. LODs and LOQs were below 5 and 10 ng/g, respectively.

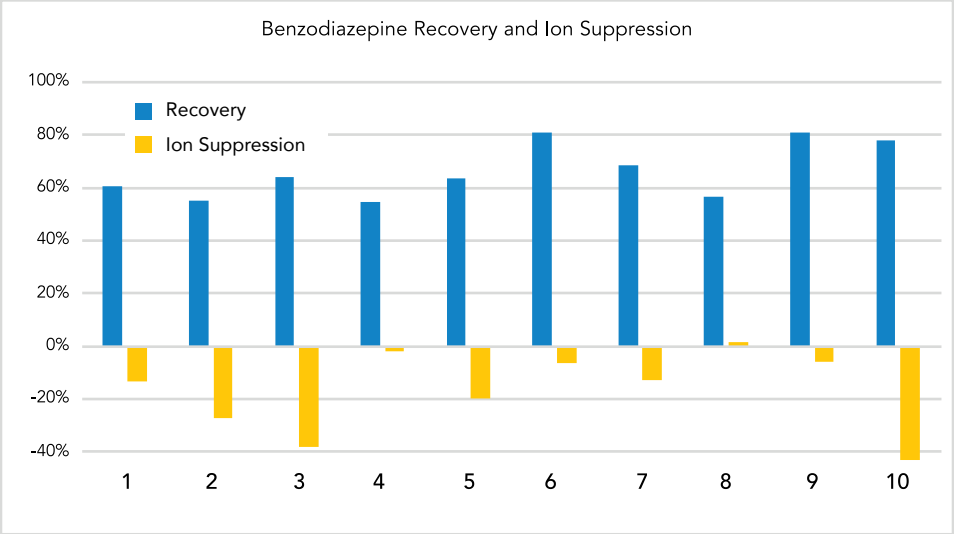

Figure 3. Percent recovery and percent ion suppression for each benzodiazepine: 1. Nordiazepam, 2. Diazepam, 3. 7-Aminoclonazepam, 4. Oxazepam, 5. Temazepam, 6. Alprazolam, 7. Clonazepam, 8. Lorazepam, 9. a-hydroxyalprazolam, 10. Midazolam

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Table 3. Average within-run precision, average between-run precision, and total precision for two concentrations expressed in percent coefficient variation (%CV).

	Avg. Within-Run Precision (%CV)		Avg. Between-Run Precision (%CV)	
Compound	**100 ng/g**	**1000 ng/g**	**100 ng/g**	**1000 ng/g**
7-Aminoclonazepam	*3.8*	*1.6*	*0.5*	*2.8*
Midazolam	*6.4*	*1.1*	*2.1*	*1.7*
α-hydroxyalprazolam	*10.0*	*8.1*	*7.4*	*0.9*
Alprazolam	*5.5*	*3.9*	*3.1*	*3.0*
Oxazepam	*3.0*	*3.0*	*2.8*	*3.0*
Nordiazepam	*5.9*	*4.7*	*8.7*	*3.9*
Lorazepam	*3.9*	*2.4*	*4.8*	*10.2*
Clonazepam	*1.8*	*2.2*	*1.6*	*1.9*
Temazepam	*4.9*	*2.3*	*2.3*	*3.7*
Diazepam	*3.8*	*3.1*	*2.4*	*1.7*