Extraction Techniques to Analyze Synthetic Benzodiazepines in Various Biological Matrices

Jillian Neifeld, Jeremy Smith, and Elena Gairloch
Biotage, 10430 Harris Oaks Blvd., Suite C, Charlotte North Carolina 28269, USA

Introduction

New, novel benzodiazepines have become some of the most abused unique psychoactive substances seen today. In the United States, these compounds are not approved for prescription use. Therefore, the demand for testing of these drugs has rapidly increased, both in the United States and worldwide. Urine and whole blood are common matrices of choice for forensic laboratories as they can be easy to collect, and they provide relevant information regarding recent or active use of illicit materials. Obtaining optimal analytical results from either matrix will require adequate sample preparation to remove interferences and isolate compounds of interest. Several options exist for effective preparation of whole blood and urine. Some may involve minimal effort, such as dual-mode extraction (DME+), while others may require more complex methodologies, such as solid phase extraction (SPE) with mixed-mode polymeric ion exchange sorbents. Each method of sample preparation will yield extracts of different levels of cleanliness. The results of different extraction procedures for both whole blood and urine fortified with 12 novel benzodiazepine compounds were collected via LC-MS/MS and compared to identify practical considerations for optimal workflows.

Methods

Reagents and Materials

Standards, Chemicals, Extraction Hardware

All standards were purchased from Cerilliant (Round Rock, TX). LC/MS grade water and methanol (MeOH) were purchased from Honeywell Chemicals (Chicago, IL). HPLC Plus grade ethyl acetate (EA) and *tert*-Butyl methyl ether (MTBE) was purchased from Sigma-Aldrich (St. Louis, MO). LC/MS Optima grade dichloromethane (DCM), 2-propanol (IPA), and formic acid (FA) were purchased from Fisher Scientific (Waltham, MA), as well as HPLC grade acetonitrile (ACN) and ammonium hydroxide (NH_4OH). A Raptor Biphenyl 2.7µm 50 x 3.0mm analytical column was provided by Restek (Bellefonte, PA). Drug-free human whole blood was provided by UTAK (Valencia, CA). Drug-free human urine was collected from a willing donor. EVOLUTE® EXPRESS CX (30 mg bed) extraction plate (601-0030-PX01), ISOLUTE® HYDRO DME+ (400 mg bed) extraction plate (970-0400-PZ01), ISOLUTE® SLE+ (400 µL) extraction plate (820-0400-P01), Biotage® PRESSURE+ 96 position positive pressure manifold (PPM-96), and Biotage® SPE Dry 96 (SD-9600-DHS-NA) were supplied by Biotage.

Sample Preparation

Whole Blood and Urine Sample Preparation

Each sample matrix was spiked at two known concentrations with all 12 target analytes resulting in stocks of 100 ng/mL and 10 ng/mL.

Compounds Included in the Panel

Clobazam, bromazepam, phenazepam, estazolam, clonazolam, prazepam, flubromazepam, etizolam, delorazepam, pyrazolam, diclazepam, nimetazepam

Sample Pretreatment

Each extraction protocol utilized a different pretreatment for both whole blood and urine workflows while maintaining a 1:1 dilution of raw sample. For whole blood and urine on ISOLUTE® SLE+, a 1% ammonium hydroxide buffer was used for pretreatment. The EVOLUTE® EXPRESS CX workflow utilized a 0.1% formic acid solution for pretreatment. It is reported that the novel benzodiazepine compounds do not undergo any significant conjugation during normal metabolism, so hydrolysis steps were not necessary for the urine samples. Samples were prepared in triplicate sets along with no matrix controls, extraction blanks, and unextracted standards for use in the calculation of analyte recoveries and matrix effects.

Extraction Procedures

Following pretreatment, extraction was performed. Data was obtained for the extraction of the urine samples with ISOLUTE® HYDRO DME+, ISOLUTE® SLE+, and EVOLUTE® EXPRESS CX, whereas whole blood extraction data was collected using ISOLUTE® SLE+, and ISOLUTE® HYDRO DME+ products. In all experiments, the initial urine or whole blood sample volume was 100 µL. For ISOLUTE® HYDRO DME+, samples were divided into sets. One set was pretreated with 10 µL of formic acid. No formic acid was added to the other set. The resulting sample was then added to the ISOLUTE® HYDRO DME+ plate, followed by 600 µL of ACN to each sample well. The contents of each well were mixed via aspiration and dispense 5 times before positive pressure was applied to push the samples through the ISOLUTE® HYDRO DME+ media. Urine and whole blood samples on ISOLUTE® SLE+ were first pretreated with 100 µL of 1% NH_4OH. Each pretreated sample was loaded onto the ISOLUTE® SLE+ plate with positive pressure and permitted to absorb for 5 minutes. After this waiting period, samples were eluted with 2 aliquots of 750 µL of one of four elution solvents: DCM, 95:5 DCM/IPA, MTBE, or EA. The more complex workflow on EVOLUTE® EXPRESS CX is detailed in table 1.

EVOLUTE® EXPRESS CX Urine Extraction				
Step	**Volume (µL)**	**Solvent**	**Time (min)**	**Pressure (psi)**
Condition	0	N/A		
Equilibrate	0	N/A		
Sample Load	200	Pretreated Sample	1-2	2-4
Wash #1	1000	H_2O	1-2	2-4
Wash #2	1000	0.1% FA	1-2	2-4
Wash #3	1000	MeOH 50:50 MeOH/H_2O	1-2	3-5
Plate Dry	N/A	N/A	1	20
Elute	2 x 750	DCM/IPA/NH_4OH EA/ACN/NH_4OH [78:20:2]	1-2	2-4

Table 1. *Biotage 96 Positive Pressure Processing Parameters for urine samples on EVOLUTE® EXPRESS CX plates. Elution was completed with 2 aliquots of 1 of 2 different complex mixtures.*

Dry Down and Sample Reconstitution: Eluates were collected into a collection plate. All samples were evaporated to dryness at 40°C with 20 L/min of nitrogen using a Biotage® SPE Dry. Extracts were then reconstituted with 50 µL of 50:50 mobile phase A/mobile phase B and analyzed via LC-MS/MS.

Chromatography Parameters

UPLC	Parameter
Column	Restek Raptor Biphenyl 2.7 µm, 50 x 3.0 mm
MPA	0.1% formic acid (aq)
MPB	0.1% formic acid in MeOH
Flow Rate	0.4 mL/min
Column Temp.	40°C
Sample Temp.	15°C
Injection Volume	2.5 µL

Table 2. *Shimadzu Nexera X2 SIL-30AC UPLC.*

A mobile phase gradient was used over a 9.25-minute data window to achieve chromatographic separation. Table 3 shows the mobile phase gradient that was used.

Time	% Mobile Phase B
0.01	5
0.5	10
5.25	70
7.50	95
7.70	95
7.75	5
9.25	STOP

Table 3. *Mobile phase gradient*

Mass Spectrometry Parameters

Instrument: A SCIEX 5500 triple quadrupole mass spectrometer with Turbo Ionspray® Ion interface (Redwood City, CA) was used. Source parameters were optimized and can be found in table 4. Acquisition was conducted by scheduled MRM (transition information not presented here but is available upon request). Data window for each sMRM was set at 60 seconds, with target scan time at 1.0 seconds.

Ionization Spray Voltage	+1500(V)	**CAD**	8
Source Temp	600 °C	**GS1**	50
Curtain	30	**GS2**	50

Table 4. *SCIEX 5500 Triple Quadrupole ESI (+) Turbo Ionspray® Source Parameters.*

Results

Extraction Recoveries

Recoveries varied greatly for some analytes, depending on the sample matrix, as well as the extraction technique and elution solvent applied. Figure 1 illustrates the recoveries of all 12 analytes from whole blood using each different applicable extraction technique.

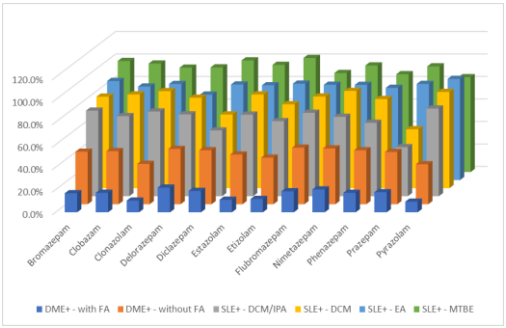

Figure 1. *Variations in recoveries for whole blood extraction of benzodiazepine compounds using different techniques.*

Similarly, the extraction of the benzodiazepine compounds from urine demonstrated variation in recovery with respect to the choice of technique and wash/elution solvent applied. The urine extraction results are found in figure 2. As can be seen, bromazepam and clobazam were almost completely removed from the EVOLUTE® EXPRESS CX sorbent when using a 100% methanol wash.

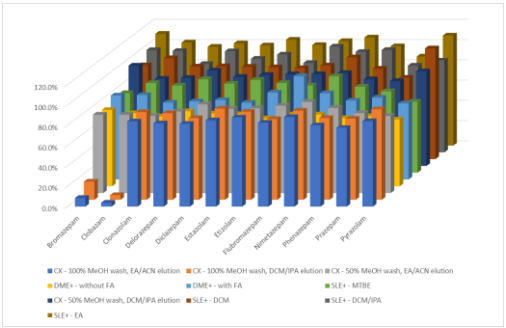

Figure 2. *Variations in recoveries for urine extraction of benzodiazepine compounds using different techniques.*

Extraction Matrix Effects

The measured matrix effects for each extraction and sample matrix did display notable variation. Figure 3 illustrates the matrix effects for each of the analytes in the whole blood extractions, while figure 4 contains the results of the urine protocols. Some of the compounds demonstrated either ion suppression or ion enhancement, specifically with the flow-through technique of ISOLUTE® HYDRO DME+. This indicates these extracts were simply not as clean as other approaches, such as the EVOLUTE® EXPRESS CX or ISOLUTE® SLE+ methods.

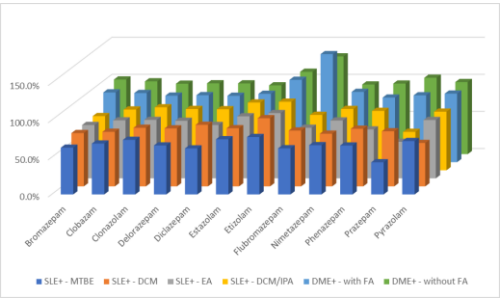

Figure 3. *The measured matrix effects for each whole blood extraction.*

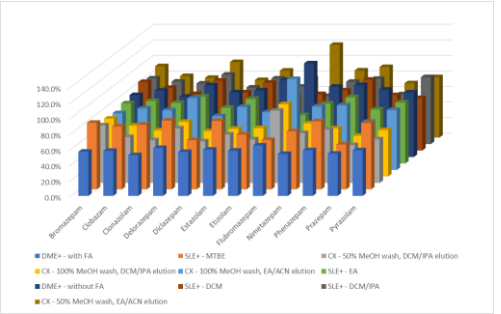

Figure 4. *The measured matrix effects for each urine extraction.*

Conclusions

- For the urine extractions, ISOLUTE® SLE+ is a fast and clean option for sample preparation that results in recoveries of at least 80% for all benzodiazepine compounds.
- The whole blood extractions produced qualitatively similar results. The ISOLUTE® HYDRO DME+ methods resulted in lower recoveries than the other techniques used. ISOLUTE® SLE+ resulted in higher recoveries with decreased matrix effects.
- The ISOLUTE® HYDRO DME+ extraction was the fastest, easiest, most inexpensive extraction method. However, it did result in lower recoveries than when using some other techniques. It also resulted in some ion enhancement for some compounds (nimetazepam in whole blood).
- Although each extraction method is suitable, the ISOLUTE® SLE+ sample preparation method with any of the four elution solvents tested provided the best recoveries of our target analytes with the least amount of matrix effects.

SOFT 2020
Part Number: P225