Drugs of Abuse with Multivariate Intermolecular Properties Analyzed by Polymeric Mixed-Mode Cation Exchange

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Introduction

With prescription abuse rising concomitantly with licit pain management, the need to expand a wider degree of drug monitoring within a single method has been increasingly sought after. With the incidence or prevalence of drug abuse typically restrained to various classes of opioids, benzodiazepines, cannabinoids, and amphetamines, the opportunity to isolate and identify analytes within these classes becomes effortless. This is in part due to the high degree of structural homology within each respective Drug of Abuse (DOA) class. Although the subtle dissimilar intermolecular traits can offer a remarkably different analgesic, anxiolytic or other off-label effects, their similarities often provide an opportunity for their isolation via pH modulation through common functional groups such as amines (opioids and stimulants) or imines (benzodiazepines).

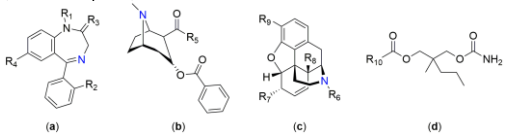

Figure 1. General scheme illustrating various generalized drug classes: benzodiazepines (a), stimulants (b), opioids (c), and carbamates (d). R-groups represent moieties that vary within each drug class

Herein, we demonstrate that a large urine panel, comprised of 43 DOA's, from multiple drug classes, can be simultaneously screened by mix-mode cation exchange despite their disparate intermolecular traits, by thoughtfully selecting appropriate organic wash and elution conditions that simultaneously enable sample isolation and detection along with minimizing sample matrix effects.

Experimental

Reagents & Materials

Standards: all standards were purchased from Cerilliant (Round Rock, TX). IMCSzyme β-glucuronidase and buffer were generously provided by IMCS. HPLC grade water, methanol (MeOH), and acetonitrile (ACN) were purchased from Sigma Aldrich (St. Louis, MO) in addition to reagent grade isopropyl alcohol (IPA), dichloromethane (DCM), phosphoric acid, formic acid and ammonium hydroxide (NH_4OH). Urine was supplied from healthy drug-free personnel. EVOLUTE® EXPRESS CX (30 mg bed) 96-well plates (601-0030-PX01), Biotage® Extrahera™ Sample prep station (414001), and Biotage® SPE Dry 96 (SD-9600-DHS-NA) were supplied by Biotage.

Sample Preparation

Standards and Enzyme Hydrolysis

All extracted samples were supplied from a 20 mL working stock of DOA free urine spiked with all analytes to yield a final concentration of 50 ng/mL. For each sample analyzed, 200 µL of spiked urine was loaded into a 96-position, 2 mL well plate with 200 µL of IMCS buffer along with 25 µL (1250 units, 50K Units/mL) of IMCSzyme β-glucuronidase. All samples were incubated for 30 minutes at 55°C and allowed to reach room temperature prior to pre-treatment

EVOLUTE® EXPRESS CX SPE Procedure

96-well Plates: All extractions were performed using 30 mg mixed-mode cation exchange EVOLUTE® EXPRESS CX 96-well plates (P/N: 601-0030-PX01).

Biotage® Extrahera™ Automated Sample Preparation Platform

The optimized extraction protocol was carried out using Extrahera, an automated sample preparation platform equipped with an 8 channel pipetting head and positive pressure processing functionality. The precise protocol is detailed below and in ***Table 1***.

Sample Pre-treatment: All samples were acidified with 200 µL of 4% phosphoric acid after reaching room temperature, post hydrolysis.

Table 1. *Extrahera Processing Parameters.*

Step	Volume (µL)	Solvent	Time (sec)	Pressure (Bar)
Sample Mix	600	N/A	N/A	N/A
Condition	500	MeOH	40	2
Equilibration	1000	4% H_3PO_4	40	2
Sample Load	600	Sample	50	0.8
Wash #1	1000	4% H_3PO_4	60	0.5
Wash #2	1000	0-100% MeOH	60	0.5
Plate Dry	N/A	N/A	60	5 bar at 600 mL/min
‡Elution #1	500	DCM/IPA/NH_4OH [78:20:2]	40	0.8
‡Elution #2	500	DCM/IPA/NH_4OH [78:20:2]	50	0.8
Plate Dry	N/A	N/A	45	5 bar at 600 mL/min

‡Elution: Samples were then eluted with two sequential 0.5 mL aliquots of DCM/IPA/NH_4OH [78:20:2] unless otherwise noted.
Dry Down and Sample Reconstitution: The elution solvent was evaporated under a stream of heated (40 °C) nitrogen at 80 L/min using a Biotage® SPE Dry 96. All extracts were subsequently reconstituted with 150 µL of 10% methanol and immediately analyzed via LC/MS-MS.

Chromatography Parameters

Table 2. *Shimadzu Nexera X2 UPHLC Parameters.*

Column	Phenomenex Kinetex Pheynl-Hexyl 2.6 µm, 50 x 4.6 mm
MPA	0.1% Formic Acid (aq)
MPB	0.1% Formic Acid in MeOH
Flow Rate	0.6 mL min^{-1}
Column Temp	40 °C
Sample Temp	15 °C
Inj Volume	2 µL

Mass Spectrometry Parameters

Instrument: SCIEX 5500 triple quadrupole Mass Spectrometer with Turbo Ionspray® Ion interface (Foster City, CA). Optimized source and sMRM parameters detailed in ***Tables 3 and 4,*** *respectively*. Retention window for sMRM set at 60 seconds with target scan time at 1 second.

Table 3. *SCIEX 5500 ESI (+) Turbo Ionspray® Source Parameters.*

Ionization Spray Voltage	+1500(V)	CAD	8 (V)
Source Temp	600 °C	GS1	50
Curtain	20 (V)	GS2	50

Table 4. *sMRM parameters for 4 of 43 DOA analytes (other transitions not shown).*

Analyte	RT	Q1	Q3	DP	CE	CXP
Pregabalin	2.29	160.2	142.2/55	20/30	15/35	10/10
Gabapentin	2.40	172.1	137.1/154.1	30/30	20/30	10/10
Meprobamate	4.12	219.2	158.2/97.1	100/50	10/20	14/10
Carisoprodol	4.71	261.2	97.2/176.2	30/30	20/10	10/12

Results

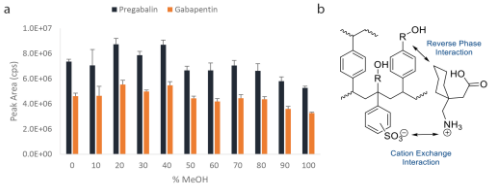

*Figure 2. (**a**) integrated peak area for 50 ng/mL extracted carisoprodol and meprobamate under methanol washes ranging from 0 to 100%. (**b**) EVOLUTE® EXPRESS CX sorbent's proposed non-covalent affinity with meprobamate. Error bars represent standard deviation (n = 4).*

Represented in **figure 1a** is the relationship between the percent methanol used for wash step #2 and each compounds relative peak area. Both demonstrate their resistance to the wash step at all intervals while maintaining excellent signal. Alternatively, **figure 2a** shows decrease in peak area response of carisoprodol and

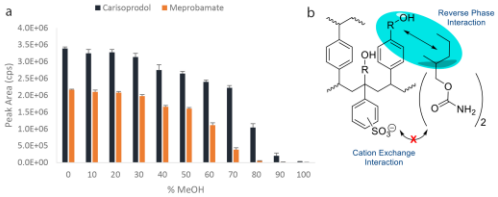

*Figure 1. (**a**) integrated peak area for 50 ng/mL extracted gabapentin and pregabalin under methanol washes ranging from 0 to 100%. (**b**) EVOLUTE® EXPRESS CX sorbent's proposed columbic complexation with pregabalin. Error bars represent standard deviation (n = 4).*

meprobamate as the ratio of organic to aqueous increases during the same wash step. These analytes lack Bronsted-Lowry moiety's or functional groups capable of pH manipulation, and thus their retention is based on the reverse phase or non-covalent mechanisms of the sorbent (**figure 2b**). **Figure 3a** demonstrates the relationship between urinary pigmentation upon increasing percentage of methanol in wash step #2. **Figures 4a-f** show the peak area of meprobamate decreases with increasing percentage of methanol in wash step 2. Moreover, both S/N and peak area substantially decrease as the percentage of methanol increases above 50% **(red dashed line in figure 4g)**.

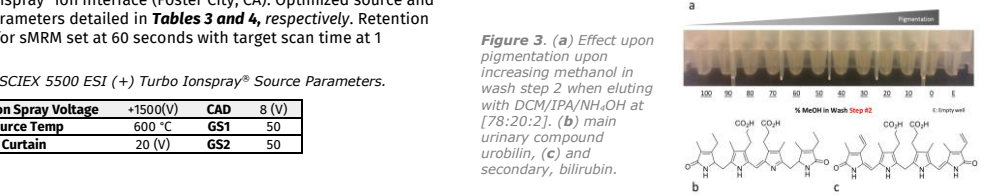

*Figure 3. (**a**) Effect upon pigmentation upon increasing methanol in wash step 2 when eluting with DCM/IPA/NH_4OH at [78:20:2]. (**b**) main urinary compound urobilin, (**c**) and secondary, bilirubin.*

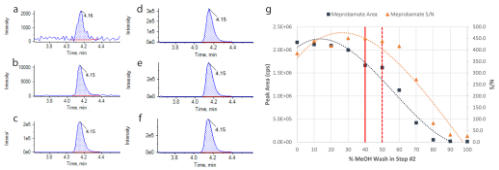

Figure 4. Integrated peak areas for the extraction of 50 ng/mL of meprobamate after (a) 100%, (b) 80%, (c) 60%, (d) 40%, (e) 20%, and (f) 0% methanol used in wash #2. All peaks collected using sMRM. (g) Integrated peak areas for methanol washes from 0 to 100%.

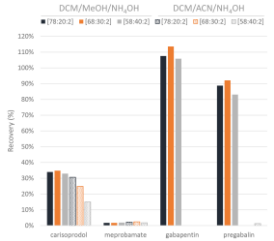

Figure 5. Effect upon the percent recovery eluting with solvents with low (DCM/IPA/NH_4OH) and high (MeOH/ACN/NH_4OH) dielectric capacity with increasing percentage of NH_4OH (n=3).

Substituting methanol and acetonitrile for DCM/IPA, the dielectric profile of the elution solvents closely matched that of the analytes engendering their release and recovery. Thus, the combination of columbic complexation and reverse-phase (or non-covalent) interactions governed the capture and release of these two analytes (**Figure 5**). Increasing the ratio of methanol in the elution volume from 0% to 20, 30, or 40% resulted in enhanced recovery of both gabapentin (> 100%) and pregabalin (> 85%), whereas the same increase with acetonitrile was deleterious (**figure 6**).

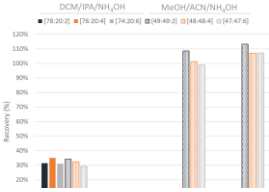

Figure 6. Effect upon the recovery eluting with MeOH or ACN with DCM and NH_4OH.

Conclusions

- EVOLUTE® EXPRESS CX isolated analytes with disparate non-covalent and columbic profiles among a 43 analyte panel.
- Carbamate functional group(s), acting as "ester-amide" hybrids are unable to participate in ion-exchange due to the resonance stabilization of the co-planar amide N-C=O atoms.
- Both gabapentin and pregabalin require at least a 20% polar-protic solvent for enhanced recovery and are insensitive to organic washes under a cation-exchange mechanism.

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