Current Methodologies for Drugs of Abuse Urine Testing

A White Paper from Biotage

Abstract

Analysis of drug panels in urine samples can be challenging, and the trend towards larger panels including multiple drug classes compounds the issues faced during method development.

This white paper examines a number of aspects of sample preparation, and their impact on the success of subsequent LC-MS/MS analysis of broad urine panels.

Section 1 examines the applicability of various sample preparation techniques: supported liquid extraction, reverse phase SPE and mixed-mode SPE, to the various classes of drugs extracted. In addition, hydrolysis approaches: enzyme type and protocol used (time, temperature), are compared.

Mixed-mode reverse phase/cation exchange SPE is widely used for extraction of basic drug classes from urine, but the inclusion of drugs and metabolites that exhibit 'non-typical' functionality within urine panels can be problematic. **Section 2** examines the impact of various parameters (interference wash strength, elution solvent composition) on analyte retention, elution and extract cleanliness with particular focus on zwitterionic (gabapentin, pregabalin) and non-ionic (carisoprodol, meprobamate) drugs.

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Section 1.

Practical Considerations for LC/MS Method Development of a Comprehensive Urine Pain Panel

By Stephanie J. Marin Ph.D., Dan Menasco Ph.D., Jillian Neifeld and Elena Gairloch.

Urine drug testing to support pain management is a mainstay of the clinical toxicology laboratory. Reduced reimbursement has continued to put increasing pressure on laboratories. Many toxicology labs are moving to larger drug panels to increase throughput and efficiency, while reducing turnaround time and cost. LC-MS methods with 50 or more drugs and metabolites are common. While "dilute and shoot" (D&S) methods are easy and affordable, they can result in shortened LC column lifetimes and increased MS instrumentation downtime. Matrix effects can also affect sensitivity and overall method performance.

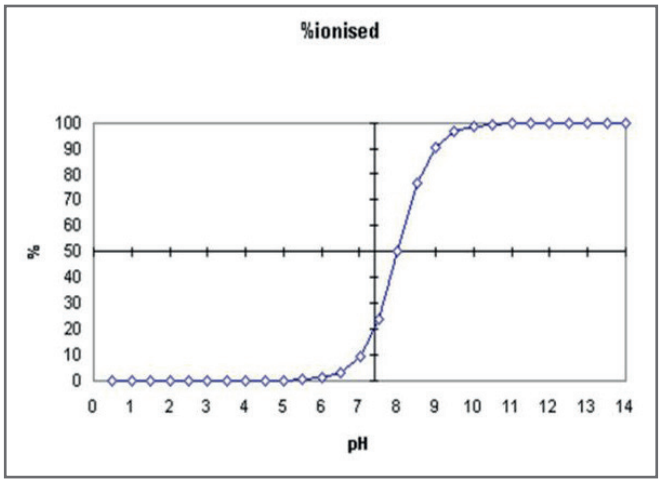
Clean-up of hydrolyzed urine specimens can reduce matrix effects, increase LC column lifetimes, and keep MS instrumentation cleaner. The result is an overall increase in efficiency and productivity because of reduced downtime. Samples can also be concentrated, resulting in improved sensitivity.

Here, we present sample preparation considerations for a panel of 56 drug analytes by three different sample preparation methods: supported liquid extraction (ISOLUTE® SLE+), polymeric reverse phase solid phase extraction SPE (EVOLUTE® EXPRESS ABN), and mixed-mode polymeric reverse phase strong cation exchange SPE (EVOLUTE® EXPRESS CX). Details of the sample preparation methods used are shown on page 5.

Part I.
Importance and Role of logP and pH

The selection of sample preparation products and protocols should be determined based on the chemical properties of the compounds of interest. These properties determine how the compounds are retained and eluted using different sample preparation methods. The octanol-water partition coefficient (logP) is a measure of the hydrophobicity of an analyte. It is an indication of an analyte's reverse phase retention behavior and its ability to partition into an organic solvent when using supported liquid extraction (SLE). The higher the logP, the more hydrophobic the compound. The acid dissociation constant (pK_a) is the pH where a compound is 50% ionized and 50% non-ionized. Figure 1 demonstrates behavior of an acidic and basic compound under different pH conditions. Pretreatment of specimens should be ±2 pH units away from the pK_a to make sure a compound is completely ionized or neutral, depending upon the mechanism of retention of the sample preparation product.

Acid Analyte Plot



Base Analyte Plot

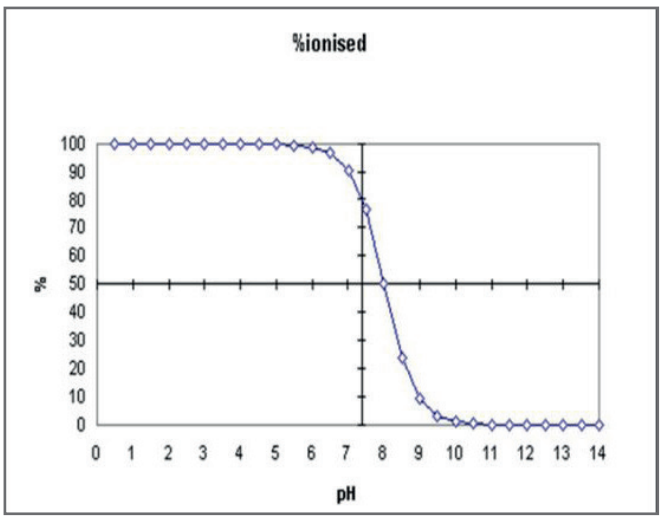


Figure 1. Ionization at different pH conditions for an acidic and basic analyte with a pK_a of 8.0.

Part II.

ISOLUTE SLE+ for Sample Preparation

Supported Liquid Extraction (SLE)

The mechanism for SLE is similar to liquid-liquid extraction, but instead of partitioning between an aqueous and organic phase in a tube or vial, aqueous samples are absorbed onto a refined diatomaceous earth based sorbent (see Figure 2). The aqueous sample is loaded onto the SLE column and dispersed as small droplets. A water-immiscible organic solvent, typically ethyl acetate (EtOAc), dichloromethane (DCM) or methyl tert-butyl ether (MTBE) is used to elute the analytes of interest. Compounds of interest partition into the elution solvent and are collected. The addition of a polar modifier such as 2-propanol (IPA) can aid in the elution of more hydrophilic compounds. Interfering or undesirable water-soluble compounds are retained on the SLE column, providing a clean extract. This extraction method works well for acidic, basic and neutral compounds, and is based on the logP of the compound.

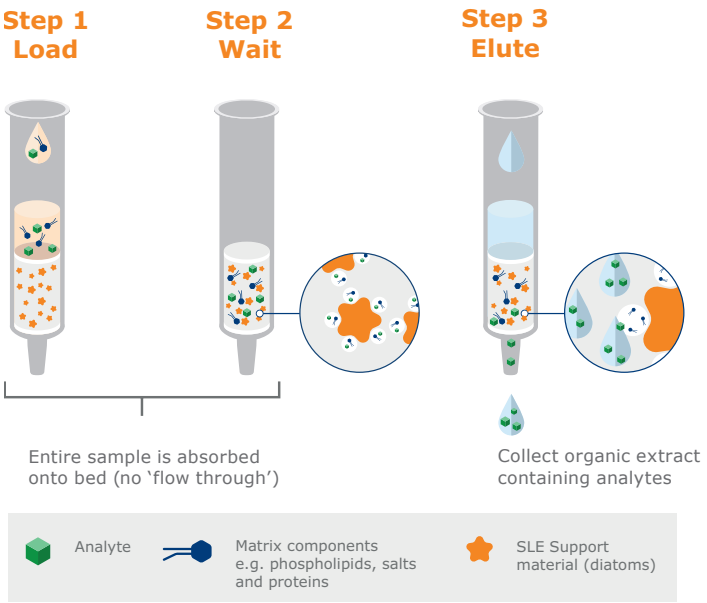


Figure 2. Mechanism for supported liquid extraction (SLE). Diagram illustrates individual well of 96-well plate.

Part III.

EVOLUTE EXPRESS for Sample Preparation

Solid Phase Extraction (SPE)

SPE methods depend upon retention of the analytes of interest, removal of interferences by washing with aqueous and/or organic solvents, followed by elution of the targeted drugs and metabolites (see Figure 3). Retention and elution are based on normal, reverse phase, or ion exchange mechanisms. EVOLUTE® EXPRESS ABN is a polymeric reverse phase SPE sorbent. EVOLUTE® EXPRESS CX is a mixed mode polymeric strong cation exchange product, which can exhibit both reverse phase and ion exchange retention behavior. Sample pretreatment and wash steps must be carefully controlled and understood. The retention mechanism of the compounds of interest must be known to ensure that analytes are not lost during the load step, washed away during interference wash steps, or retained on the column during elution, especially when compounds are retained and eluted by both mechanisms.

With a large panel of 50 or more drugs and metabolites, it will be very difficult to find conditions where all compounds are in the same ionization state. The method must be able to accommodate acidic, basic and neutral compounds in a single sample preparation method.

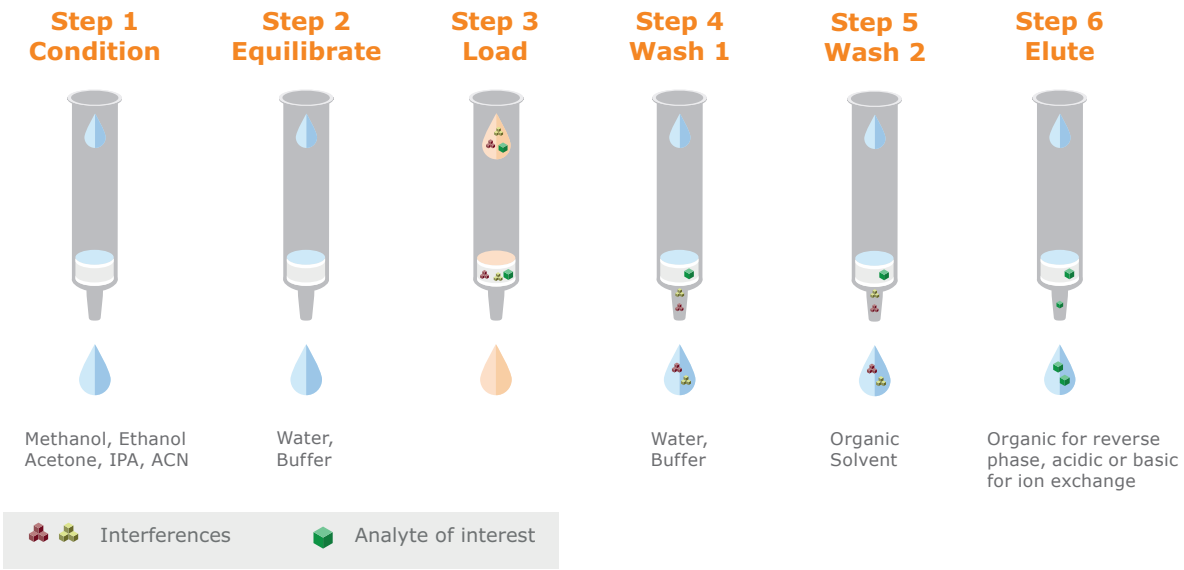


Figure 3. General SPE method procedure.

Part IV.

Properties of Analytes

The compounds evaluated for this section and their logP and pK_a values are listed in Table 1. The logP and pK_a were sourced from chemicalize.com or the Human Metabolome Database (1–2).

Table 1. 56 Drug analytes evaluated in urine drug panel.

Drug Class	Compound	Formula	LogP	pK_a
Anesthesia	Ketamine	C13H16ClNO	3.35	7.5
Anesthesia	Norketamine	C12H14ClNO	2.91	7.5
Anticonvulsant	Gabapentin (Neurontin)	C9H17NO2	-1.27	4.6, 9.9
Anticonvulsant	Pregabalin (Lyrica)	C8H17NO2	-1.35	4.8, 10.2
Barbiturate	Butalbital	C11H16N2O3	1.59	8.5
Barbiturate	Pentobarbital	C11H18N2O3	1.89	8.5
Barbiturate	Phenobarbital	C12H12N2O3	1.41	8.1
Barbiturate	Secobarbital	C12H18N2O3	2.03	8.5
Benzodiazepine	7-aminoclonazepam	C15H12ClN3O	0.49	3.0, 5.0
Benzodiazepine	Alpha-hydroxyalprazolam	C17H13ClN4O	1.53	5.0, 13.7
Benzodiazepine	Alprazolam (Xanax)	C17H13ClN4	3.02	1.4, 5.0
Benzodiazepine	Chlordiazepoxide (Librium)	C16H14ClN3O	3.05	6.5
Benzodiazepine	Clonazepam (Klonopin)	C15H10ClN3O3	3.15	1.9, 11.7
Benzodiazepine	Diazepam (Valium)	C16H13ClN2O	3.08	2.9
Benzodiazepine	Lorazepam	C15H10Cl2N202	3.53	10.6, 12.5
Benzodiazepine	Nordiazepam	C15H11ClN2O	3.21	2.9, 12.3
Benzodiazepine	Oxazepam	C15H11ClN2O2	2.92	10.6, 12.5
Benzodiazepine	Temazepam	C16H13ClN2O2	2.79	10.7
Cannabinoid	11-nor-9-carboxy-delta-9-THC	C21H28O4	5.14	4.2, 9.3
Carbamate hypnotic	Meprobamate	C9H18N2O4	0.93	>12.0
Carbamate muscle relaxant	Carisoprodol	C12H24N2O4	1.92	15.0
Cocaine	Benzoylecgonine	C16H19NO4	-0.59	3.2, 9.5
Cocaine	Cocaine	C17H21NO4	2.28	8.9
Hallucinogen	Phencyclidine (PCP)	C17H25N	4.49	10.6
Methadone	EDDP	C20H23N	4.63	9.6
Methadone	Methadone	C21H27NO	5.01	9.1
Non benzo hypnotic	Zolpidem (Ambien)	C19H21N3O	3.02	5.7
Non benzo hypnotic	Zolpidem-phenyl-4-carboxylic acid	C19H19N3O3	0.61	3.4, 5.7
Opioid	6-AM (heroin marker)	C19H21NO4	0.61	8.1, 9.7
Opioid	Buprenorphine (Suboxone, Butrans)	C29H41NO4	3.55	9.6
Opioid	Codeine	C18H21NO3	1.34	9.2
Opioid	Dihydrocodeine	C18H23NO3	1.55	9.3
Opioid	Fentanyl	C22H28N2O	3.82	8.8
Opioid	Hydrocodone (Vicodin)	C18H21NO3	1.96	8.6
Opioid	Hydromorphone (Dilaudid)	C17H19NO3	1.62	8.6, 10.1
Opioid	Meperidine	C15H21NO2	2.46	8.2
Opioid	Morphine	C17H19NO3	0.90	9.1, 10.3
Opioid	N-desmethyltapentadol	C13H21NO	2.31	10.6
Opioid	Norbuprenorphine	C25H35NO4	2.30	10.5
Opioid	Norfentanyl	C14H20N2O	1.42	10.0
Opioid	Norhydrocodone	C17H19NO3	1.58	10.0
Opioid	Normeperidine	C14H19NO2	2.07	9.3
Opioid	Noroxymorphone	C16H17NO4	0.12	9.4, 10.2
Opioid	Norpropoxyphene	C21H27NO2	4.52	10.7
Opioid	O-desmethyltramadol	C15H23NO2	1.72	9.0
Opioid	Oxycodone (Oxycontin, Percoset)	C18H21NO4	1.03	8.1
Opioid	Oxymorphone	C17H19NO4	0.78	8.2, 10.0
Opioid	Tapentadol (Nucynta)	C14H23NO	2.96	10.2
Opioid	Tramadol	C16H25NO2	2.46	9.3
Opioid agonist	Naloxone (Narcan)	C19H21NO4	1.62	7.8, 10.7
Sympathomimetic amine	Amphetamine (Adderall)	C9H13N	1.80	10.0
Sympathomimetic amine	MDMA (Ecstasy, Molly)	C11H15NO2	1.86	10.1
Sympathomimetic amine	Methamphetamine	C10H15N	2.24	10.2
Sympathomimetic amine	Ritalinic acid	C13H17NO2	-0.36	3.7, 10.1
Tricyclic antidepressant	Amitriptyline	C20H23N	4.81	9.8
Tricyclic antidepressant	Nortriptyline	C19H21N	4.43	10.5

Part V.

Experiments in Hydrolysis

Urine Hydrolysis Considerations

Most drugs are metabolized prior to excretion in the urine or feces. Many drugs and metabolites are conjugated as a glucuronide to increase water solubility and improve elimination from the body. Hydrolysis of urine specimens using a beta-glucuronidase enzyme to convert the metabolites to their "free" form for analysis increases sensitivity.

Three different sample preparation techniques for extraction of free drugs from hydrolyzed urine were investigated in this study. In addition, we evaluated three different beta-glucuronidase enzymes with four different glucuronide compounds over several incubation times and temperatures to determine optimal hydrolysis conditions for selected drug classes.

Sample Preparation Methods

200 µL of urine spiked at 50 ng/mL or 100 ng/mL with each drug was pretreated with 200 uL of 0.1M acetate buffer, pH 4.0 or IMCS buffer, depending on the enzyme used. 6,250 units/mL of beta-glucuronidase enzyme were added. Samples were hydrolyzed for 30 minutes at 55 °C. Next, 200 µL of 4% phosphoric acid (H_3PO_4) was added for EVOLUTE® EXPRESS CX methods and 200 µL of 0.1% NH_4OH was added for both ISOLUTE® SLE+ and EVOLUTE® EXPRESS ABN methods. The extraction methods used are detailed below. Samples were then loaded onto one of three different extraction columns (all 96-well plate format):

ISOLUTE® SLE+ 400 µL 96-well plate, (820-0400-P01)

EVOLUTE® EXPRESS ABN 30 mg plate (600-0030-PX01)

EVOLUTE® EXPRESS CX 30 mg plate (601-0030-PX01)

Hydrolysis Evaluation Procedure

Three beta-glucuronidase enzymes were evaluated: Red abalone (BG100, Kura Biotec, Los Angeles, CA), abalone (Campbell Scientific, Rockford, IL), and recombinant (IMCSzyme, Irmo, SC); to determine which provided the most complete hydrolysis of glucuronide metabolites without affecting the overall recovery of non-conjugated compounds. Four glucuronides were included in a urine glucuronide control to determine the extent of hydrolysis by each enzyme: morphine-3-beta-D-glucuronide, norbuprenorphine glucuronide, oxazepam glucuronide, and 11-nor-9-carboxy-THC glucuronide (THC-COOH) (Cerilliant, Round Rock, TX). The control was prepared so that the amount of non-conjugated drug would equal 100 ng/mL upon complete hydrolysis.

A spiked urine sample containing 56 non-conjugated drugs and metabolites at 100 ng/mL was also analyzed to calculate hydrolysis efficiency and compare differences in matrix effects among the 3 enzymes and different hydrolysis conditions. 200 µL of buffer (IMCS buffer for the IMCSzyme or 0.1M ammonium acetate buffer pH 4.0 for the Kura and Campbell enzymes) were added to 200 µL of sample. Next, enzyme at 6250 units/mL was added (25 µL of IMCSzyme or 13 µL of Kura or Campbell). The samples were incubated at either 55 °C or 65 °C for 30 or 60 minutes. The samples were extracted using EVOLUTE® EXPRESS CX method described to the right.

ISOLUTE® SLE+ Method

Step	Details
Load	Load hydrolyzed sample (400 µL) onto the ISOLUTE SLE+ column, and apply gentle pressure to initiate flow.
Wait	Allow to absorb for 5 minutes.
Elute	Elute with 90:10 (v/v) dichloromethane:2-propanol (DCM:IPA) (2 x 0.75 mL). Allow first aliquot of elution solvent to flow by gravity for 5 minutes, then apply gentle pressure. Repeat with second aliquot of elution solvent.
Post Elution	Dry under nitrogen (N_2) at 40 °C. Reconstitute in 90:10 (v/v) 0.1% formic acid (FA) in water/0.1% FA in methanol (MeOH).

EVOLUTE® EXPRESS ABN Method

Step	Details
Condition	Condition column with MeOH (1 mL).
Equilibrate	Equilibrate column with 0.1% NH_4OH (1 mL).
Load	Load hydrolyzed sample (400 µL) onto column.
Wash 1	Wash with 0.1% NH_4OH (1 mL).
Wash 2	Wash with 10% MeOH in water (1 mL).
Elute	Eluted with 90:10(v/v) DCM:IPA (2 x 0.75 mL).
Post Elution	Dry under nitrogen at 40 °C. Reconstitute in 90:10 (v/v) 0.1% FA in water/0.1% FA in MeOH.

EVOLUTE® EXPRESS CX Method

Step	Details
Condition	Condition column with MeOH (1 mL).
Equilibrate	Equilibrate column with 4% H_3PO_4 (1 mL).
Load	Load hydrolyzed sample (400 µL) onto column.
Wash 1	Wash with 4% H_3PO_4 (1 mL).
Wash 2	Wash with 50% MeOH in water (1 mL).
Elute	Eluted with either: a. 78:20:2 (v/v) DCM:IPA:NH_4OH (2 x 0.75 mL), or b. 78:20:2 (v/v) DCM:MeOH:NH_4OH (2 x 0.75 mL).
Post Elution	Dry under nitrogen at 40 °C. Reconstitute in 90:10 (v/v) 0.1% FA in water/0.1% FA in MeOH.

Results

Urine Hydrolysis Results

The results indicate that there was no one enzyme or incubation time/temperature that was optimal for all four glucuronides tested (see figure 4). The Campbell enzyme did not fully hydrolyze morphine under any conditions. Hydrolysis of THC-COOH was temperature and time dependent for all three enzymes (the degree of hydrolysis was higher at lower times and temperatures). Oxazepam was completely hydrolyzed under all conditions.

Recovery of most of the analytes in the 56 compound "free" control was consistent (within ±10%) among the three enzymes at the various times and temperatures. Carisoprodol, hydromorphone, and zolpidem-phenyl-4-COOH showed some variability among different enzymes and incubation parameters. Figure 5 shows the recoveries for these compounds for the three enzymes under all hydrolysis conditions.

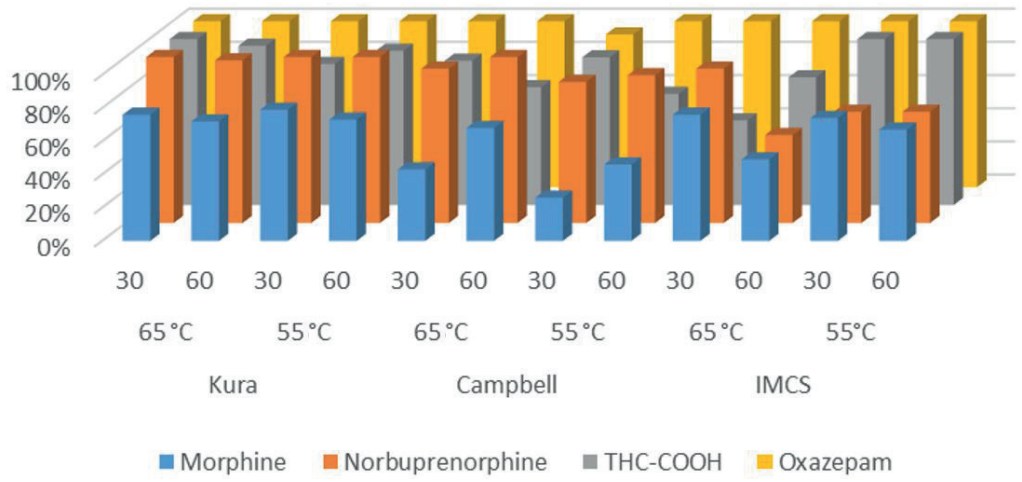


Figure 4. Percent hydrolysis (calculated as the ratio of glucuronide/free) for each of the compounds in the glucuronide control for all enzymes and hydrolysis conditions.

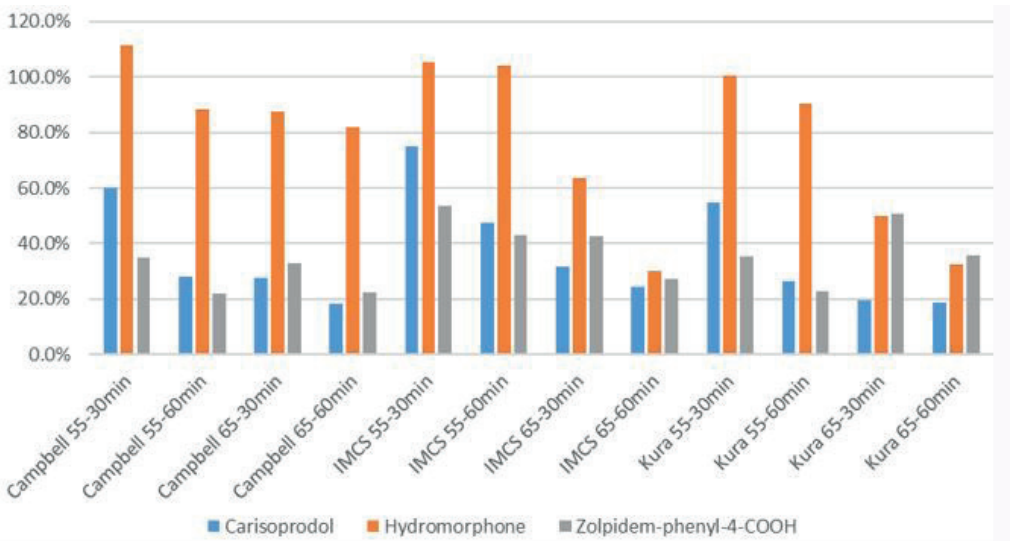


Figure 5. Recoveries of carisoprodol, hydromorphone and zolpidem-phenyl-4-COOH for all enzymes and hydrolysis conditions.

Based on these results, the Campbell enzyme provided adequate results for most of the glucuronide compounds but hydrolysis of morphine glucuronide was low. The Kura and IMCSzyme enzymes resulted in the most complete hydrolysis of all four glucuronides.The majority of compounds in the non-conjugated control yielded consistent recovery among all enzymes under all hydrolysis conditions.

Part VI.
Results By Drug Class

Opiates/Opioids I
Codeine, morphine, hydrocodone, hydromorphone, oxycodone, oxymorphone, 6-monoacetylmorphine (6-AM) and metabolites.

Greater than 90% recovery was achieved using EVOLUTE® EXPRESS CX with acid pretreatment, 50% MeOH wash, elution 78:20:2 (v/v) DCM:IPA:NH_4OH. ISOLUTE® SLE+ provided >90% recovery for all compounds except 85% recovery for (n-desmethyltapentadol) and 70% recovery for norhydrocodone and morphine using elution with 90:10 (v/v) DCM:IPA. EVOLUTE® EXPRESS ABN yielded 30-60% recovery for 6-AM, hydrocodone, norhydrocodone, codeine, dihydrocodeine, oxycodone and <10% recovery for morphine, hydromorphone, oxymorphone, using basic pretreatment, 10% MeOH wash, and eluting with 90:10 (v/v) DCM:IPA.

Opioids and Opioid Agonist
Methadone, buprenorphine, fentanyl, meperidine, tramadol, tapentadol, naloxone and metabolites

Better than 80% recovery of all compounds was observed using the described protocol with 78:20:2 (v/v) DCM:IPA:NH_4OH elution and EVOLUTE® EXPRESS CX. ISOLUTE® SLE+ produced >90% recovery for all compounds except for norfentanyl and n-desmethyltapentadol (80% recovery) with a 90:10 (v/v) DCM:IPA elution. EVOLUTE® EXPRESS ABN showed higher recovery for this group of compounds than the opiates/ opioids I group with >80% recovery for all compounds except for 50–60% recovery for norbuprenorphine, norfentanyl, tapentadol and n-desmethyltapentadol, and 25% recovery for naloxone. Basic pretreatment and 10% MeOH wash with an elution with 90:10 (v/v) DCM:IPA were used.

Benzodiazepines and Z-drugs
Alprazolam, chlordiazepoxide, clonazepam, diazepam, lorazepam, midazolam, zolpidem and metabolites

90% recovery or better was attained using EVOLUTE® EXPRESS CX and ISOLUTE® SLE+, using previously described protocols, except for recovery of 7-aminoclonazepam (80%) and the recovery of zolpidem-phenyl-4-COOH (80% when using CX and 50% when using SLE+). The elution solvent for EVOLUTE® EXPRESS CX was 78:20:2 (v/v) DCM:IPA:NH_4OH. EVOLUTE® EXPRESS ABN gave recoveries ›80% recovery for all compounds except for 7-aminoclonazepam, which had a recovery of 50%.

Barbiturates
Butalbital, pentobarbital, phenobarbital, secobarbital

EVOLUTE® EXPRESS CX produced poor recovery for all barbiturates under the conditions evaluated. These drugs are weakly acidic and would not be expected to perform well on a cation exchange SPE phase and acid pretreatment. Recovery of >90% was achieved using ISOLUTE® SLE+ and EVOLUTE® EXPRESS ABN.

Stimulants
Amphetamine, methamphetamine, ritalinic acid, MDMA, cocaine, benzoylecgonine (BZE)

EVOLUTE® EXPRESS CX had recoveries ›90% for all compounds except ritalinic acid which had 30% recovery using acid pretreatment, 50% MeOH wash and elution with 78:20:2 (v/v) DCM**:IPA**:NH_4OH. Recovery for ritalinic acid was increased to 80% by changing the elution solvent to 78:20:2 (v/v) DCM:**MeOH**:NH_4OH. These samples were not as clean as samples eluted with DCM:IPA:NH_4OH. ISOLUTE® SLE+ provided recoveries of >80% for methamphetamine, MDMA, cocaine and BZE, 50% recovery of amphetamine and <10% ritalinic acid. EVOLUTE® EXPRESS ABN had >80% recovery for cocaine and BZE, <30% recovery for amphetamine and methamphetamine, and 50% recovery of ritalinic acid. No other elution solvents or pretreatment were evaluated.

Illicit Drugs and Tricyclic Antidepressants (TCAs)
11-nor-9-carboxy-delta-9-tetrahydrocannabinol (9-carboxy-THC), phencyclidine (PCP), ketamine, norketamine, amitriptyline, nortriptyline

EVOLUTE® EXPRESS CX and ISOLUTE® SLE+ yielded recoveries of >90% for all analytes except 9-carboxy-THC (80%). EVOLUTE® EXPRESS ABN produced >80% recovery ketamine, norketamine, and PCP, 70% recovery for 9-carboxy-THC, and approximately 70% recovery for amitriptyline and nortriptyline.

Anticonvulsants, Carbamates
Pregabalin, gabapentin, meprobamate, carisoprodol

The recovery of these compounds is difficult in a large drug panel. Extensive work was conducted to find the best conditions for recovery of these analytes, and is the subject of section 2 of this white paper. Less than 20% recovery of pregabalin, gabapentin, carisoprodol and meprobamate was observed using EVOLUTE® EXPRESS CX with acid pretreatment, 50% MeOH wash and elution with 78:20:2 (v/v) DCM:IPA:NH_4OH. Lowering the organic wash to 30% MeOH improved recovery of meprobamate. Replacing IPA in the elution solvent with MeOH improved recovery to 80% for pregabalin, 100% for gabapentin and 30% for carisoprodol, but samples are not as clean as those eluted with IPA. ISOLUTE® SLE+ gave >90% recovery carisoprodol and meprobamate, but <20% recovery pregabalin and gabapentin. EVOLUTE® EXPRESS ABN provided >80% recovery of carisoprodol and meprobamate and <20% recovery of pregabalin and gabapentin.

Part VII.

General Recommendations

We have presented three approaches for extraction and clean-up of 56 drugs and metabolites. The choice of the final method depends on the drugs of interest and their properties.

ISOLUTE® SLE+ is recommended if opiates, opioids, benzodiazepines, stimulants (except ritalinic acid), PCP, barbiturates, 9-carboxy-THC, TCAs, meprobamate, carisoprodol, ketamine and norketamine are in the panel.

If the drugs and metabolites in the urine panel are mostly basic: opiates, opioids, benzodiazepines, PCP, stimulants (except ritalinic acid), TCAs, ketamine, norketamine, and 9-carboxy-THC; then the EVOLUTE® EXPRESS CX method with sample pretreatment using 4% H_3PO_4, a 50% MeOH wash, and elution with 78:20:2 (v/v) DCM:IPA:NH_4OH is recommended. If ritalinic acid, gabapentin and pregabalin are required, the same protocol, except elution with 78:20:2 (v/v) DCM:MeOH:NH_4OH should be used.

EVOLUTE® EXPRESS ABN works well for some opioid drugs and metabolites, most benzodiazepines, ketamine, norketamine, PCP, 9-carboxy-THC, amitriptyline, nortriptyline, carisoprodol, meprobamate, cocaine and BZE. The organic wash must be limited to 10% MeOH because of the reverse phase retention mechanism. Amphetamine, methamphetamine and ritalinic acid had lower recoveries (30–50%) but this could be adequate depending upon the sensitivity required.

Developing a single sample preparation method for a large panel of drugs and metabolites can be challenging. Finding a single sample preparation protocol for 50+ compounds requires knowledge of the hydrophobicity and acid-base properties of the drug analytes. Compromises in recovery and sample cleanliness are inevitable when multiple drug classes with vastly different properties are required in a single method. Methods should be optimized to provide sufficient recovery for required sensitivity and sample cleanliness. Several approaches should be investigated and evaluated to provide the most rugged, robust and sensitive method. Urine specimens should undergo enzymatic hydrolysis to maximize recovery of drug analytes that are conjugated prior to elimination in the urine. The enzyme and conditions for both hydrolysis of glucuronide metabolites and recovery of non-conjugated compounds should be selected based on the compounds of interest and the required limits of detection.

Part VIII.

References

1. Chemicalize.com, http://www.chemicalize.com, accessed August 1–10, 2016.
2. Human Metabolome Database, http://www.hmdb.ca/metabolites, accessed August 1–10, 2016.

Section 2.

Multivariate Intermolecular Properties Analyzed by Polymeric Mixed-Mode Cation Exchange SPE (focus on Pregabalin, Gabapentin, Carisoprodol and Meprobamate)

By Dan Menasco, Ph.D., Jillian Neifeld, Stephanie Marin, Ph.D., and Elena Gairloch.

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 confined to various classes of opioids, benzodiazepines, cannabinoids, and amphetamines, the opportunity to isolate and identify analytes within these classes becomes straightforward. This is in part due to the high degree of structural homology within each respective Drug of Abuse (DOA) class. Although subtle dissimilar intermolecular traits can offer remarkably different analgesic, anxiolytic or other off-label effects, their similarities often provide an opportunity for their isolation via pH adjustment through common functional groups such as amines (opioids and stimulants) or imines (benzodiazepines).

In this study, we investigate this approach for the anticonvulsants pregabalin and gabapentin, along with two carbamate drugs: carisoprodol and meprobamate, as these analytes are often problematic in large urine panels.

Solid Phase Extraction (SPE) functionalized with cation exchange provides clinicians with an opportunity to isolate compounds with imines or primary, secondary, and tertiary amines with robust analyses (Figure 1a–c). However, other DOA classes lack these functional groups and remain pH insensitive, e.g. carisoprodol and meprobamate (Figure 1–d). As a result, separate methods are necessary, which can increase turnaround time for clinicians and pain management facilities. Increasing the scope of diagnostic panels to include the array of both licit and illicit DOAs has become difficult as not all drug classes are capable of isolation and detection using the same workup method.

To work around this, alternative methods are sometimes used to directly analyze patient specimens with minimal sample clean up. While such procedures can be effective, they compromise sample cleanliness, with consequences on instrument downtime and data quality.

Part I.

Structures of Drug Classes

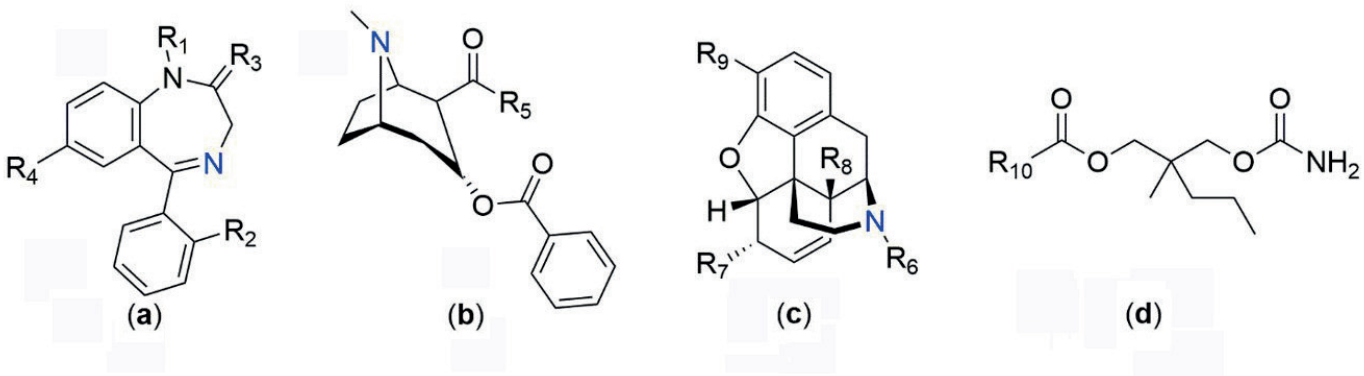


Figure 1. General scheme illustrating various generalized drug classes: benzodiazepines (a), stimulants (b), opioids (c), and carbamates (d). R-groups represent moieties that vary within each drug class.

Table 1. Drugs of abuse investigated in this study, with LC-MS/MS conditions used.

Compound ID	Quant (m/z)	Qual (m/z)	Retention Time (min)	DP (V)	EP (V)	CE (V)	CXP (V)
6-AM	328.1 > 165.2	211.2	2.68	30	10	60/30	10
7-aminoclonazepam	328.1 > 121.2	222.2	3.66	30	10	50/30	12
alpha-hydroxyalprazolam	325.1 > 297.0	216.1	5.03	30	10	40/60	12
Alprazolam	309.1 > 281.1	205.1	5.10	30	10	40/60	12
Amitriptyline	278.1 > 105.1	202.2	4.51	30	10	50/70	12
Amphetamine	136.1 > 119.0	91.0	2.42	30	10	20/20	12
Benzoylecgonine	290.2 >168.1	105.0	3.68	30	10	30/50	12
Buprenorphine	468.3 > 396.2	414.2	4.12	50/100	10	60/50	10
Carisoprodol	261.2 > 97.2	176.2	4.71	30	10	20/10	12
Codeine	300.1 >152.1	115.1	2.48	30	10	70/80	14
Methamphetamine	150.1 > 91.2	119.2	2.62	30	10	20/10	12
Phencyclidine (PCP)	244.3 > 91.0	159.3	4.00	30	10	60/20	12
Pregabalin	160.2 >142.2	55.0	2.29	20	10	15/35	10
Ritalinic Acid	220.1 > 84.1	56.1	3.30	30	10	50/60	12
EDDP	278.3 > 234.2	186.2	4.28	30	10	40/50	14
Fentanyl	337.2 > 105.1	188.1	4.06	50	10	50/40	10
Gabapentin	172.1 > 137.1	154.1	2.40	30	10	20/30	10
Hydrocodone	300.1 > 199.1	128.1	2.72	100	10	40/70	10
Hydromorphone	286.2 > 185.1	128.0	2.00	100/50	10	40/70	10
Ketamine	238.1 > 125.1	179.2	3.18	50	10	40/50	10
Lorazepam	321.0 > 275.1	229.1	4.93	50	10	50/40	14
MDMA	194.1 > 163.2	105.2	2.79	50	10	20/40	10/14
Meperidine	248.2 > 220.0	174.1	3.59	150	10	30/30	14
Meprobamate	219.2 > 158.2	97.1	4.12	100/50	10	10/20	14/10
Methadone	310.2 > 265.2	105.0	4.45	150/50	10	20/20	12
Temazepam	301.1 > 255.1	177.1	5.16	150/100	10	50/60	12
Tramadol	264.2 > 58.1	42.1	3.34	100	10	60/80	12/10
Zolpidem	308.1 > 235.1	236.2	3.82	100	10	50/40	10/12
Morphine	286.2 > 152.0	165.0	1.68	50/100	10	80/60	14/10
Naloxone	328.0 > 128.2	115.0	2.35	100	10	80/80	14
N-desmethyltapentadol	208.2 > 107.1	121.1	3.42	100	10	50/20	14/12
Norbuprenorphine	414.3 > 83.1	101.1	3.86	150	10	70/50	14/12
Nordiazepam	271.1 > 140.0	165.1	5.10	100	10	50/50	14/12
Norfentanyl	233.2 > 84.1	150.0	3.20	100	10	20/20	12
Norketamine	224.2 > 125.1	179.2	3.09	50/100	10	50/20	12
Normeperidine	234.2 > 160.1	188.1	3.60	100/50	10	20/20	10
Nortriptyline	264.2 > 91.1	117.1	4.46	50/100	10	60/20	14
Oxazepam	287.1 > 241.0	269.1	4.97	100	10	30/20	14
Oxycodone	316.2 > 241.0	256.0	2.62	150	10	50/30	14
Oxymorphone	302.1 > 227.0	198.1	1.82	100	10	50/60	14/12
Tapentadol	222.2 > 107.1	121.1	3.44	100	10	50/30	12
Zolpidem-phenyl-4-COOH	338.1 > 265.1	266.1	3.05	100	10	50/40	12
Clonazepam	316.1 >102.1	123.3	2.76	26	7	32/32	6
11-nor-9-carboxy-delta-9-THC	343.0 > 299.0	245.0	5.66	-125/-75	-10	-30/-40	-14

Part II.

EVOLUTE® EXPRESS CX Extraction Protocol Using the Biotage® Extrahera™ Sample Preparation Automation System

Here, we demonstrate that a large urine panel of 43 DOAs, from multiple drug classes, can be simultaneously extracted using mixed-mode cation exchange despite their disparate intermolecular traits. By carefully selecting the appropriate organic wash and elution conditions we simultaneously enable sample isolation and detection along with minimizing sample matrix effects.

Standards and Enzyme Hydrolysis

All extracted samples were supplied from a 20 mL working stock of 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 acidification with 200 µL of 4% phosphoric acid.

Biotage® Extrahera™ Extraction Parameters

Briefly, samples were loaded onto the Extrahera™ and extracted using a 30 mg EVOLUTE® EXPRESS CX 96-well plate. The sorbent was pre-treated with 0.5 and 1.0 mL of methanol and water, respectively, and 600 µL of spiked urine sample (prepared as described above) was loaded. The sorbent was washed twice: first with 4% phosphoric acid and second, with varying amounts of methanol ranging from 0 to 100% aqueous in 10% intervals. Samples were then eluted with two sequential 0.5 mL aliquots of DCM/IPA/NH_4OH (78:20:2, v/v) unless otherwise noted. 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 (aq) and immediately analyzed via LC/MS-MS.

Chromatography and Mass Spectrometry Parameters

A Sciex 5500 triple quadrupole mass spectrometer (Sciex, Foster City, CA.) equipped with a Turbo Ionspray® interface for mass analysis was used for direct injection/infusion and extracted urine analyses, respectively. Experimentally determined transitions were acquired under scheduled Multiple Reaction Monitoring (sMRM) mode and their corresponding optic voltages and gas metrics were collected under ESI positive and negative ionization conditions. Finalized chromatographic and mass spectrometric parameters were applied to all samples, which consisted of amphetamines (3), benzodiazepines (7), opioids (19), dissociative anaesthetics (3), carbamates (2), stimulants (2), TCAs (2), anticonvulsants (2), z-drugs (2), and one cannabinoid. LC-MS/MS conditions are shown in table 1.

Part III.

Mechanisms of Interaction for Gabapentin, Pregabalin, Carisoprodol and Meprobamate Using Mixed-Mode Strong Cation Exchange SPE

The anticonvulsants gabapentin and pregabalin are notoriously difficult to extract due to their zwitterionic nature, however, by adding a sufficient amount of phosphoric acid in the pre-treatment solution it was possible to breach the buffering capacity of the IMCS buffer and reach below the pK_a of gabapentin and pregabalin (4.6/9.9 and 4.8/10.2, respectively). This stabilized the positive charge on each compound's primary amine group while neutralizing their respective carboxylates, which led to a cation exchange interaction with the negatively charged sulfonic acid moiety on the backbone of the EVOLUTE® EXPRESS CX sorbent (Figure 2b). Represented in figure 2a is the relationship between the percent methanol used for wash step #2 in the SPE protocol and the relative peak area for each compound. Although gabapentin shows an approximately 20% lower signal compared to pregabalin, both demonstrate their resistance to the wash step at all intervals enabling the user to tailor their organic wash strength accordingly while maintaining excellent signal.

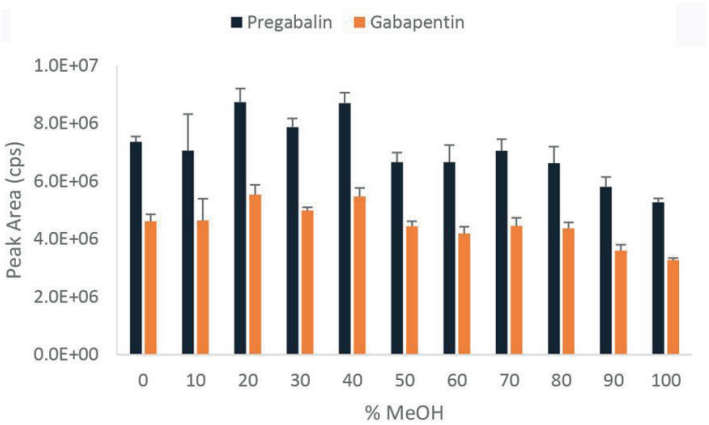


Figure 2a. Integrated peak area for 50 ng/mL extracted gabapentin and pregabalin under methanol washes ranging from 0 to 100%. Error bars represent standard deviation (n = 4).

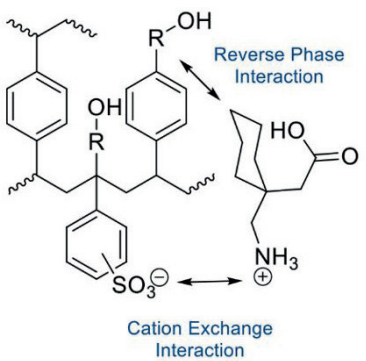


Figure 2b. EVOLUTE® EXPRESS CX sorbent's proposed columbic complexation with pregabalin.

Alternatively, analytes that either lack any Bronsted-Lowry moieties or functional groups capable of pH manipulation are incapable of ion-exchange, thus the main interaction between analyte and sorbent is left to any reverse phase mechanisms. For this panel both meprobamate and its pro-drug analog, carisoprodol, fall within this category where hydrophobic interaction is the primary means of capture on the EVOLUTE® EXPRESS CX sorbent.

As shown in figure 3a, both carisoprodol and meprobamate peak areas are inversely proportional to that of the concentration of methanol applied in wash step number 2. This is due to the disruption of the hydrophobic interaction between the reverse phase character of the EVOLUTE® EXPRESS sorbent and the methylene side chain of each analyte (Figure 3b). While both possess at least one carbamate functional group, his "ester-amide" hybrid does not behave as an acid or base within the recognized pH range of 1–14 (Figure 1d). Thus, like most amides, they are unable to participate in ion-exchange due to the resonance stabilization of the co-planar amide N-C=O atoms. Therefore, neither carbamate functional group directly contributes to the analyte's retention via ion-exchange.

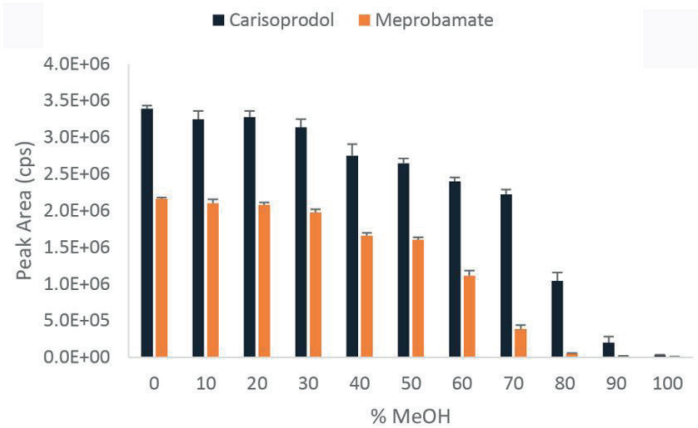


Figure 3a. Integrated peak area for 50 ng/mL extracted Carisoprodol and Meprobamate under methanol washes ranging from 0 to 100%. Error bars represent standard deviation (n = 4).

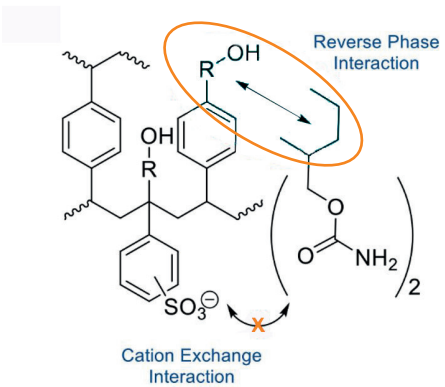


Figure 3b. EVOLUTE® EXPRESS CX sorbent's proposed reverse phase affinity with Meprobamate.

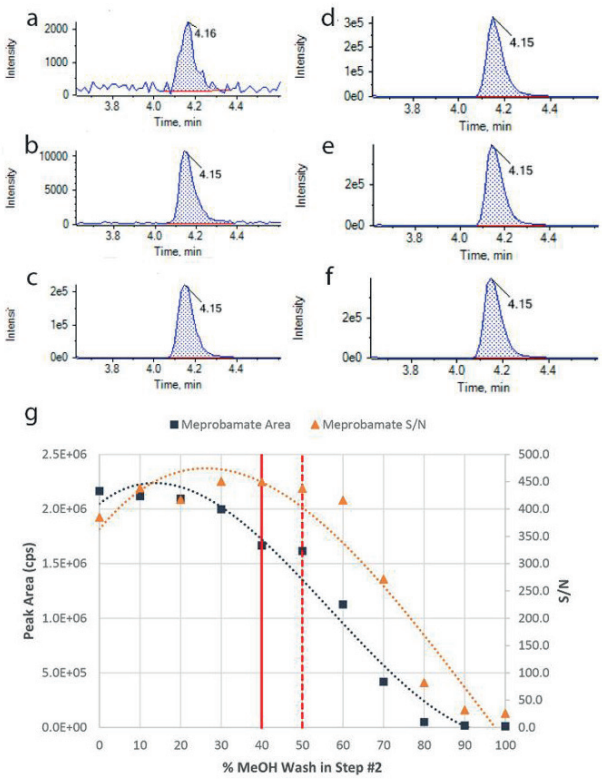


Figure 4. Integrated peak areas for the extraction of 50 ng/mL of Meprobamate after (a) 100%, (b) 80%, (c) 60%, (d) 40%, (e) 20%, and (f) 0% methanol used in wash #2. All peaks collected using sMRM. (g) integrated peak areas for methanol washes from 0 to 100% with each peaks' corresponding S/N using 30 mg EVOLUTE® EXPRESS CX sorbent.

This phenomenon is demonstrated in figures 4a-f, where the peak area of meprobamate decreases with increasing percentage of methanol in wash step 2. Moreover, both signal-to-noise (S/N) and peak area begin to decrease significantly as the percentage of methanol increases above 50% (red dashed line in figure 4g). As illustrated, the zone within the red lines represents the amount of methanol required to maintain maximum peak area and signal-to-noise for this compound (figure 4g). Moreover, a clean retention window and negligible matrix effects are maintained using a 50% methanol wash at 25, 50, and 100 ng/mL (figure 5).

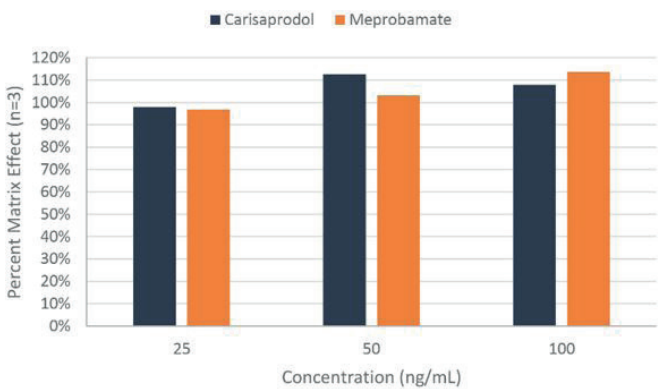


Figure 5. Matrix Effect for 50 ng/mL extracted carisoprodol and meprobamate in urine where > 100% constitutes enhancement and < 100% indicates suppression (n=3).

Nonetheless, even at high levels of methanol, meprobamate still maintains a reasonable signal, with a clean retention window (figure 4a–c). Like meprobamate, carisoprodol also yielded the same retention window for organic washes and maintains negligible matrix effects at 25, 50, and 100 ng/mL (figure 5, retention window not shown). Although hydrophobic retention seemingly restricts the protocol to a lower % organic wash, thus implying limited clean-up and a higher composition of pigmentation (Figure 6), it does not limit the sorbent's ability to successfully maintain analyte retention nor prevent an analyst from reaching the lower limits of quantitation for either carisoprodol or meprobamate.

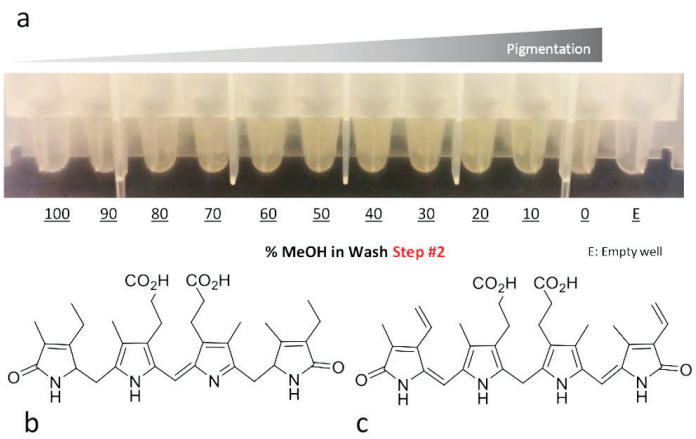


Figure 6. Effect upon pigmentation the percent methanol in wash step 2 upon elution with DCM/IPA/NH_4OH at [78:20:2]. (b) Structure of main urinary pigmentation, urobilin, responsible for the yellow color of urine. (c) Structure of secondary urinary pigment, bilirubin.

Additionally, retention mechanism plays a significant role in the subsequent recovery of analytes via the disruption of their non-covalent interactions with the sorbent. Specifically, both the disruption of cation exchange and reverse-phase interactions directly affects the solubility of each analyte, and therefore their release, recovery, and ultimately, their level of detection. For example, although the reported intensity for both gabapentin and pregabalin was substantial, their recovery was poor when using a 50% MeOH wash (figure 2a and 7, respectively) followed by elution using DCM/IPA/NH_4OH. In an effort to determine whether the mechanism controlling analyte elution was steered by ion-exchange, we examined the effect of varying the concentration of ammonia in the elution solvent on recovery of gabapentin and pregabalin (figure 7).

Interestingly, the effect of increasing ammonia, distributed as NH_4OH, had no impact upon the recovery of the analytes indicating that the relatively low recovery was not due to insufficient disruption of the electrostatic mechanism. Furthermore, analyzing post-sample load and both wash steps revealed only a small amount of gabapentin and pregabalin (1–20%, data not shown), suggesting the majority of the analytes were still bound to the sorbent by an alternate non-covalent mechanism. The reverse-phase character of the CX sorbent under increasing concentration of ammonia was evaluated by using elution solvents with high dielectric constants. By altering the intermolecular landscape between solvent, sorbent, and the analytes, both gabapentin and pregabalin were readily recovered at levels >98% (figure 7). Therefore, by substituting methanol and acetonitrile, in equal portions, for dichloromethane and isopropyl alcohol, the dielectric profile of the elution solvent (a proportional mixture of both polar-protic and aprotic solvents) closely matched that of the analytes, prompting their subsequent release and recovery. Thus, the combination of ion exchange and reverse-phase interactions governed the capture and release of these two analytes.

Carisoprodol and meprobamate remained insensitive to both solvent systems despite relying on the reverse-phase component of EVOLUTE® EXPRESS CX (figures 3a and 7).

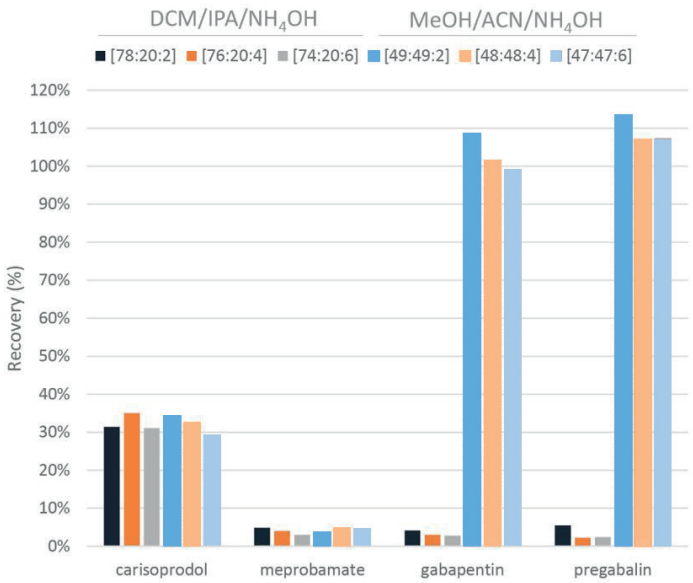


Figure 7. Effect upon the percent recovery eluting with solvents with low (DCM/IPA/NH_4OH) and high (MeOH/ACN/NH_4OH) dielectric capacity with increasing percentage of NH_4OH (n=3).

While both methanol and acetonitrile provided excellent recoveries for both antiepileptic compounds, as well as opioids and other analyte classes, the elution of urinary pigmentation (urobilin) was also shown to increase (data not shown). Previously, it was determined dichloromethane and isopropyl alcohol reduced the release of urobilin when combined with modest organic washes (figure 6); however, it also reduced analyte recoveries. In an effort to maintain high analyte recoveries and simultaneously suppress urobilin release, we evaluated the solubility of the same compounds by using dichloromethane in combination with various ratios of methanol (polar-protic) and acetonitrile (polar-aprotic) at 2% NH_4OH (figure 8).

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 did not show the same effect (figure 8).

This experiment demonstrated that these compounds specifically require a polar-protic solvent for enhanced recovery. Again, neither carisoprodol nor meprobamate showed any pronounced response in recovery. While both gabapentin and pregabalin showed remarkable recoveries when eluting with methanol, compared to acetonitrile or DCM/IPA, using dichloromethane allows for a balance in terms of sample recovery and maintaining low levels of urine pigmentation in the final extract.

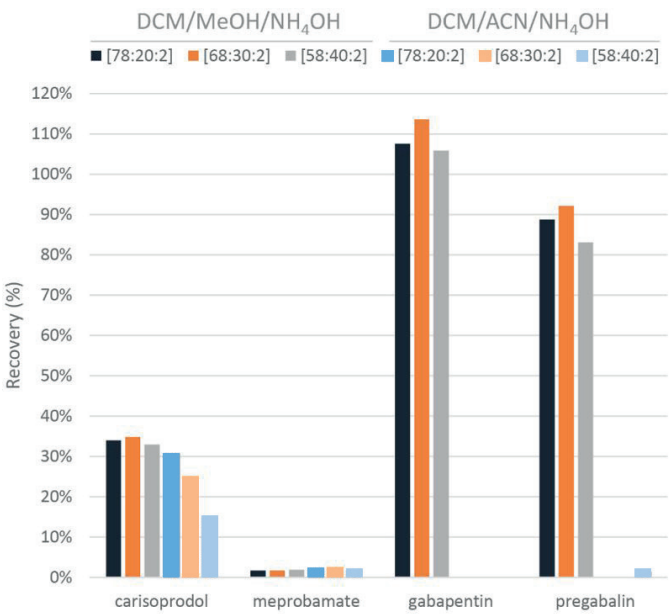


Figure 8. Effect upon the percent recovery eluting with DCM/MeOH/NH_4OH and MeOH/ACN/NH_4OH with increasing percentage of either polar-protic (MeOH) or polar-aprotic (ACN) solvents with high dielectric capacity.

Part IV. Summary of Optimum Retention and Elution Conditions

Most drugs of abuse classes can be extracted using the well understood mixed-mode reversed phase/cation exchange approach utilizing their basic amine or imine functional groups. However, special attention should be paid to both retention and elution protocols for those with non-typical molecular characteristics, for example:

- Gabapentin and pregabalin (zwitterionic, with both acidic and basic groups)
- Carisoprodol and meprobamate (no ionizable functional groups)

Retention Considerations

Gabapentin and pregabalin

Providing these analytes are loaded under low pH conditions, ensuring their basic group is ionized and their acidic group is neutralized, these analytes are retained by both cation exchange and reversed phase interactions. High concentrations of aqueous methanol in wash steps do not significantly reduce analyte recovery.

Carisoprodol and meprobamate

These analytes are retained through reversed phase interactions only, so wash solvents with higher % methanol can lead to reduced recovery. However, retention is sufficient that a clean retention window using moderate concentrations of methanol can be identified.

Elution Considerations

Gabapentin and pregabalin

Recovery of these analytes depends on disruption of the dual retention mechanisms, and choice of solvent in which these analytes are highly soluble. Analyte recovery is improved using an elution solvent consisting of MeOH/ACN/NH_4OH compared to the less polar combination of DCM/IPA/NH_4OH.

However, choice of elution solvent should also be made with consideration to the cleanliness of the final extract. Urinary pigments are co-eluted with polar elution solvent combinations, leading to yellowish discoloration in the final extract. This can be avoided through the use of a modified elution solvent consisting of DCM/MeOH/NH_4OH, without impacting analyte recovery.

In summary, maintaining a specific level of organic wash in addition to formulating the proper ratio of elution solvents will have a profound effect upon the recovery of your analytes and, ultimately, the longevity of your LC/MS system.

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