

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

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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.

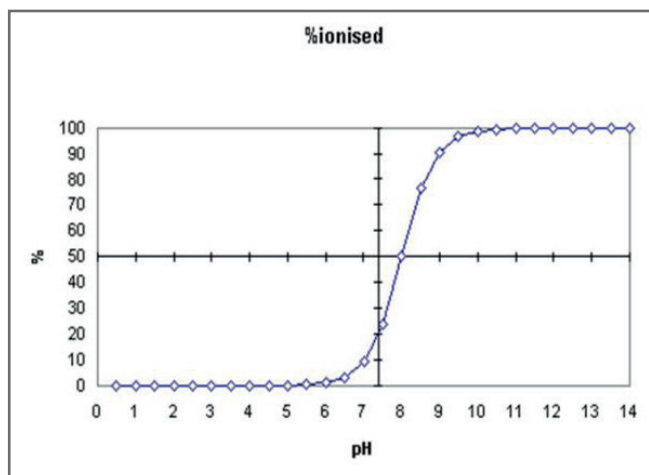
Here, we present sample preparation considerations for a panel of 56* drug analytes by three different sample preparation methods: supported liquid extraction (ISOLUTE® SLE+), polymeric reverse phase solid phase extraction SPE (EVOLUTE® EXPRESS ABN), and mixed-mode polymeric reverse phase strong cation exchange SPE (EVOLUTE® EXPRESS CX). Details of the sample preparation methods used are shown on page 6.

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.

*Since the original publication of this white paper, approximately 50 new drugs have been added to the drugs of abuse panel. See Addendum (page 18) for details).

Acid Analyte Plot



Base Analyte Plot

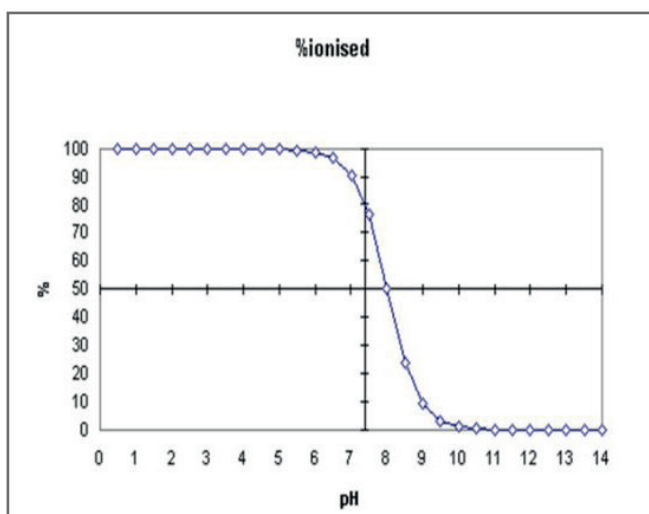


Figure 1. Ionization at different pH conditions for an acidic and basic analyte with a pK_a of 8.0.

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 ISOLUTE® 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 ISOLUTE 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.

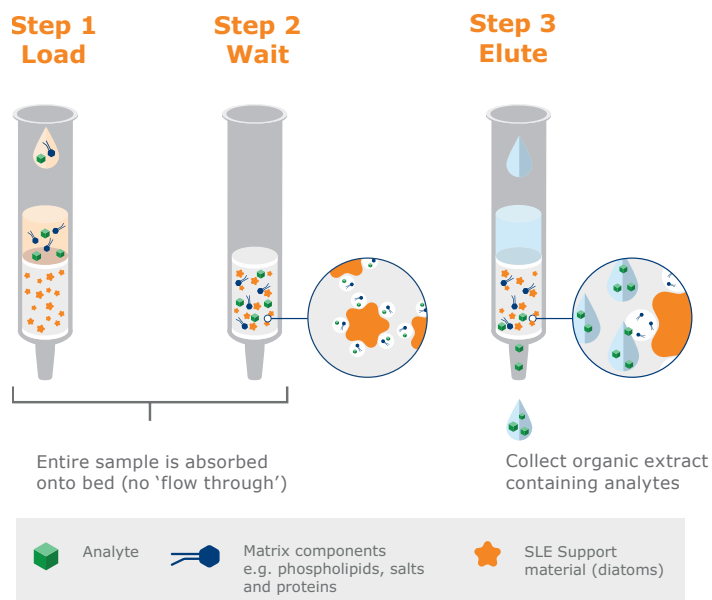


Figure 2. Mechanism for supported liquid extraction (SLE) using ISOLUTE® SLE+ products. Diagram illustrates a column or individual well of 96-well plate.

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.

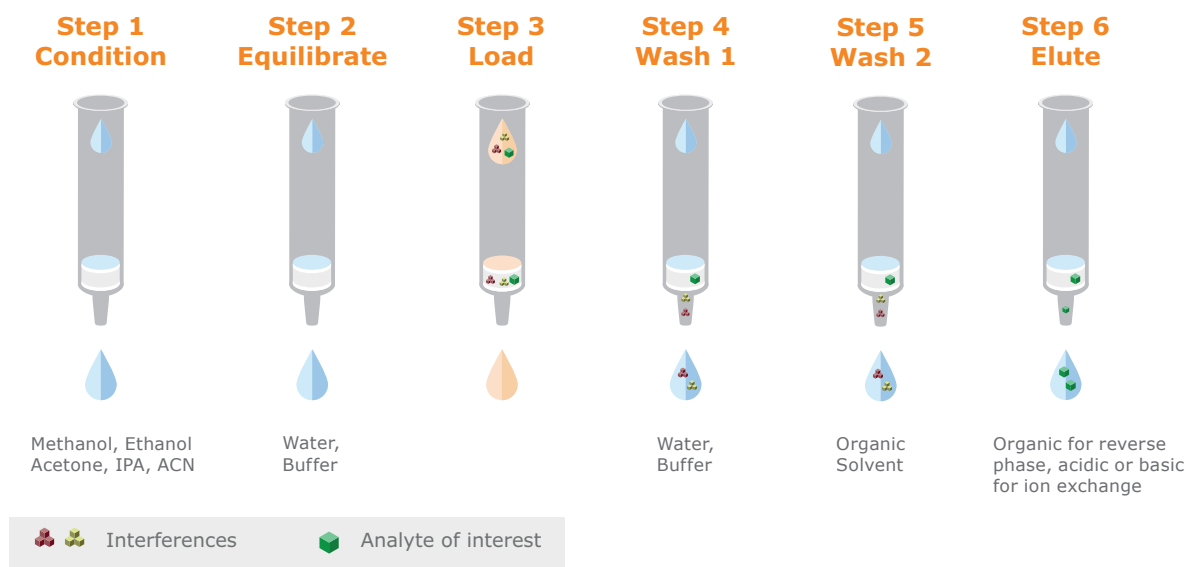


Figure 3. General SPE method procedure.

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	C15H10Cl2N2O2	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	Benzoyllecgonine	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 assay sensitivity.

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

Sample Preparation Methods

For EVOLUTE® EXPRESS CX methods, 100 µL of urine spiked at 100 ng/mL with each drug was combined with 100 µL of 0.1M acetate buffer, pH 4.0, 0.15M sodium phosphate buffer, pH 6.8, or 25 µL IMCS buffer, depending on the enzyme used. Enzyme was added. Various hydrolysis conditions were evaluated (see Hydrolysis Evaluation Procedure). Following hydrolysis, 100 µL of 4% phosphoric acid (H_3PO_4) was added to all samples before proceeding with the extraction.

For ISOLUTE® SLE+ and EVOLUTE EXPRESS ABN methods, 100 µL of urine spiked at 100 ng/mL with each drug was combined with 100 µL of 0.1M acetate buffer, pH 4.0, 0.15M sodium phosphate buffer, pH 6.8, or 25 µL IMCS buffer, depending on the enzyme used. Enzyme was added. Various hydrolysis conditions were evaluated (see Hydrolysis Evaluation Procedure). Following hydrolysis, 100 µL of 0.1% NH_4OH was added to all samples before proceeding with the extraction.

The extraction methods used are detailed on page 6. Samples were 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 Science, Rockford, IL), along with a recombinant enzyme (IMCSzyme, Irmo, SC). An additional recombinant enzyme was added for later studies (BGTurbo, Kura Biotec, Los Angeles, CA). The enzymes were evaluated 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). Four additional controls were added to later studies: codeine-3-beta-D-glucuronide, hydromorphone glucuronide, oxymorphone glucuronide, and lorazepam glucuronide, (Cerilliant, Round Rock, TX). The control was prepared so that the amount of non-conjugated drug would equal 100 ng/mL upon 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 or 4 enzymes and hydrolysis conditions. Sample volume for all enzymes was 100 µL. For the Campbell and BG100 enzymes, 100 µL of 0.1M ammonium acetate buffer, pH 4.0 was added. Next, 20 µL of enzyme was added. For the IMCSzyme, 25 µL of IMCS buffer and 55 µL of water was added to the sample. Next, 20 µL of enzyme was added. For the BGTurbo enzyme, 100 µL of 0.15M sodium phosphate buffer, pH 6.8 and 55 µL of water was added to each sample. Next, 30 µL of enzyme was added. For analysis of the Campbell, BG100 and IMCS enzymes, samples were incubated at either 55 °C or 65 °C for 30 or 60 minutes. All enzymes, including the BGTurbo enzyme, were also analyzed at room temperature for 30 minutes and at 55 °C for 10 minutes. The samples were extracted using EVOLUTE® EXPRESS CX method described on page 6.

ISOLUTE® SLE+ Method

Step	Details
Load	Load hydrolyzed, pre-treated sample onto the ISOLUTE SLE+ column, and apply gentle pressure to initiate flow.
Wait	Allow to absorb for 5 minutes.
Elute	Elute with 95:5 (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. Apply a 30 second, 20 psi push to ensure all elution solvent has flowed through.
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 (Optional)	Condition column with MeOH (1 mL).
Equilibrate (Optional)	Equilibrate column with 0.1% NH ₄ OH (1 mL).
Load	Load hydrolyzed, pre-treated sample onto column.
Wash 1	Wash with 0.1% NH ₄ OH (1 mL).
Wash 2	Wash with 10% MeOH in water (1 mL). Dry plate for 1–2 minutes at 20 psi.
Elute	Elute with 90:10(v/v) DCM:IPA (2 x 0.75 mL). Apply a 30 second, 20 psi push to ensure all elution solvent has flowed through.
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 (Optional)	Condition column with MeOH (1 mL).
Equilibrate (Optional)	Equilibrate column with 4% H ₃ PO ₄ (1 mL).
Load	Load hydrolyzed, pre-treated sample onto column.
Wash 1	Wash with 4% H ₃ PO ₄ (1 mL).
Wash 2	Wash with 50% MeOH in water (1 mL). Dry plate for 1–2 minutes at 20 psi.
Elute	Elute with either: a. 78:20:2 (v/v) DCM:IPA:NH ₄ OH (2 x 0.75 mL), or b. 78:20:2 (v/v) DCM:MeOH:NH ₄ OH (2 x 0.75 mL). Apply a 30 second, 20 psi push to ensure all elution solvent has flown through.
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

For the 65 °C hydrolysis, the results indicate that no one enzyme performed better for the four initial glucuronides tested. The 55 °C, 60 minute incubation did not show improved hydrolysis efficiency for most enzymes or glucuronides (see Figure 4). When adding in the BGTurbo enzyme for comparison, as well as additional glucuronide compounds, the room temperature incubation was not sufficient for hydrolysis, except in the case of lorazepam and oxazepam (see figure 5). The 55 °C, 10 minute incubation showed similar results, in that lorazepam and oxazepam were close to fully hydrolyzed while other glucuronides, particularly opiates, were not well hydrolyzed (see figure 6). When hydrolyzing at 55 °C for 30 minutes, close to complete hydrolysis was observed for most glucuronidated compounds (see figure 7). Therefore, this 55 °C, 30 minute incubation was used for all extraction techniques. Hydrolysis of THC-COOH was temperature and time dependent for the enzymes tested (the degree of hydrolysis was higher at lower times and temperatures).

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 8 shows the recoveries for these compounds for three enzymes (Campbell, BG100, and IMCSzyme) under all hydrolysis conditions.

Based on these results, the Campbell enzyme provided adequate hydrolysis efficiencies for most of the glucuronide compounds when a 30 min, heated incubation was used. The BG100, BGTurbo, and IMCS enzymes provided more complete hydrolysis efficiency of the opiate compounds. However, a heated incubation of at least 30 minutes is needed to achieve adequate hydrolysis.

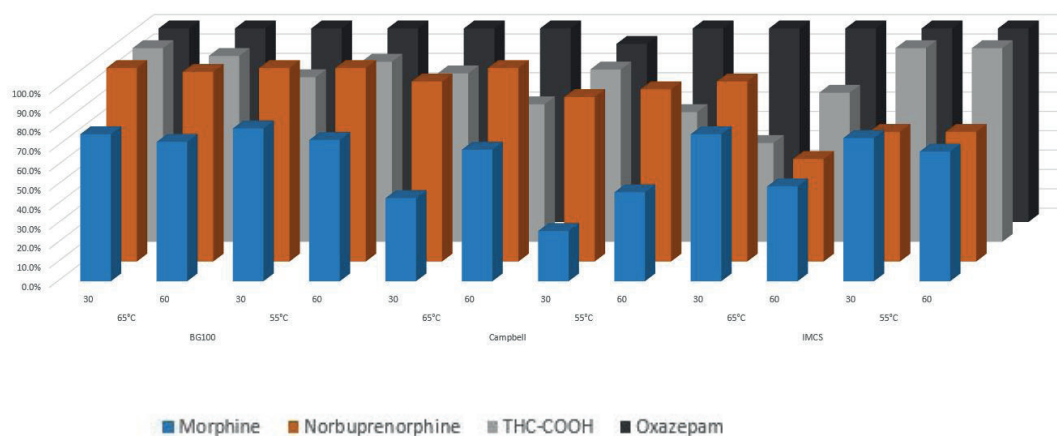


Figure 4. Percent hydrolysis (calculated as the ratio of glucuronide/free) for each of the compounds in the glucuronide control for three enzymes and hydrolysis conditions.

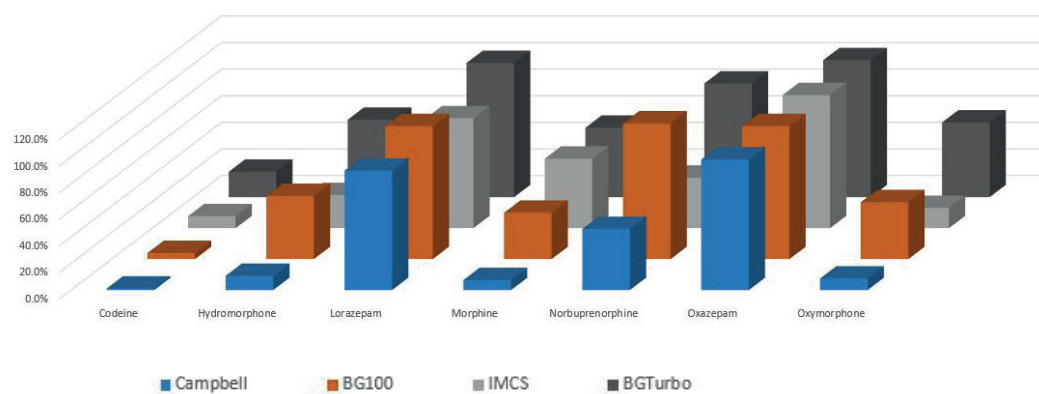


Figure 5. Percent Hydrolysis (calculated as the ratio of glucuronide/free) at room temperature for 30 minutes. (Figure includes data for additional glucuronide analytes and BGTurbo enzyme).

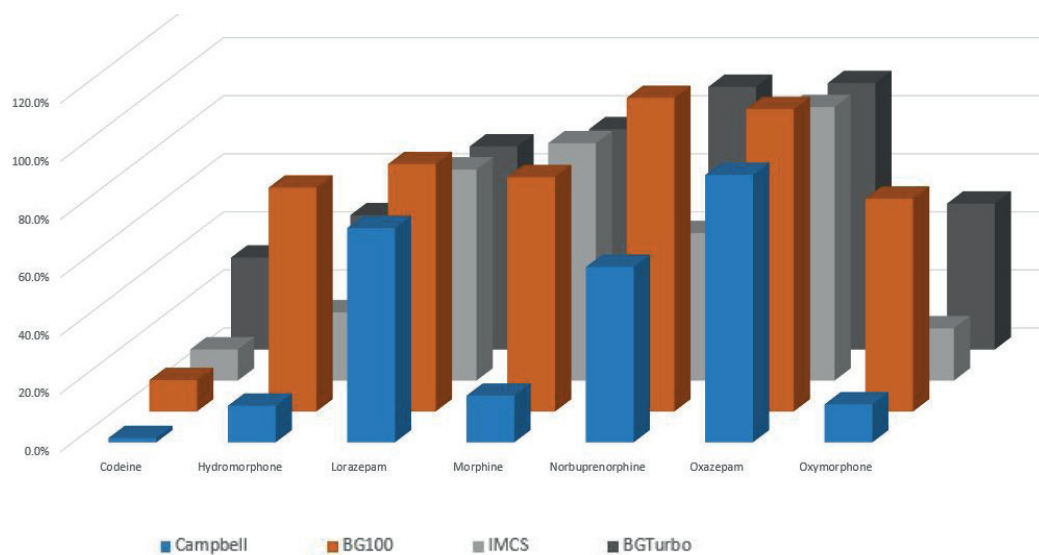


Figure 6. Percent Hydrolysis (calculated as the ratio of glucuronide/free) at 55 °C for 10 minutes. (Figure includes data for additional glucuronide analytes and BGTurbo enzyme).

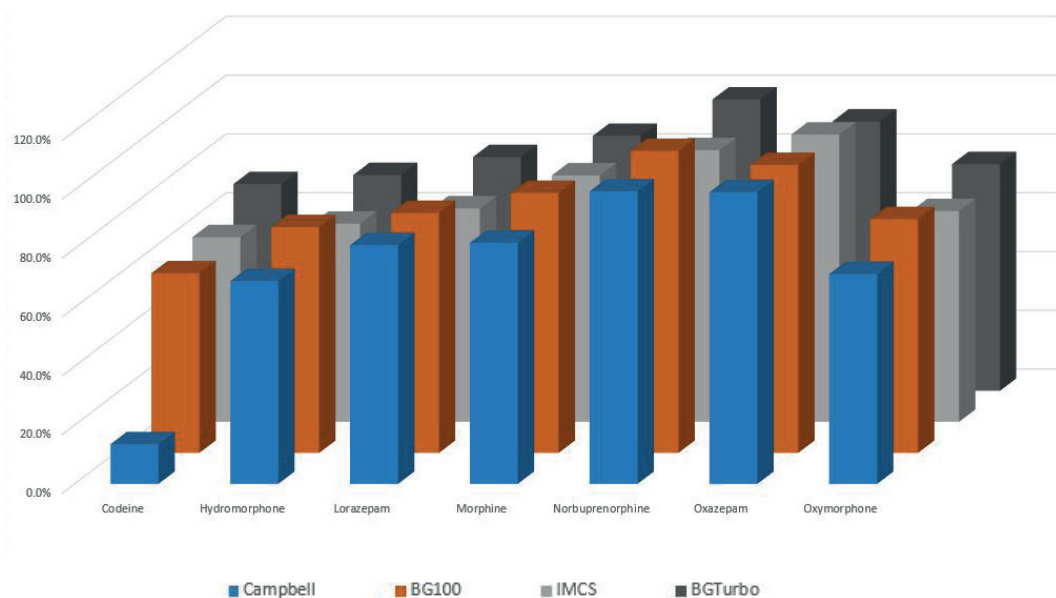


Figure 7. Percent Hydrolysis (calculated as the ratio of glucuronide/free) at 55 °C for 30 minutes. (Figure includes data for additional glucuronide analytes and BGTurbo enzyme).

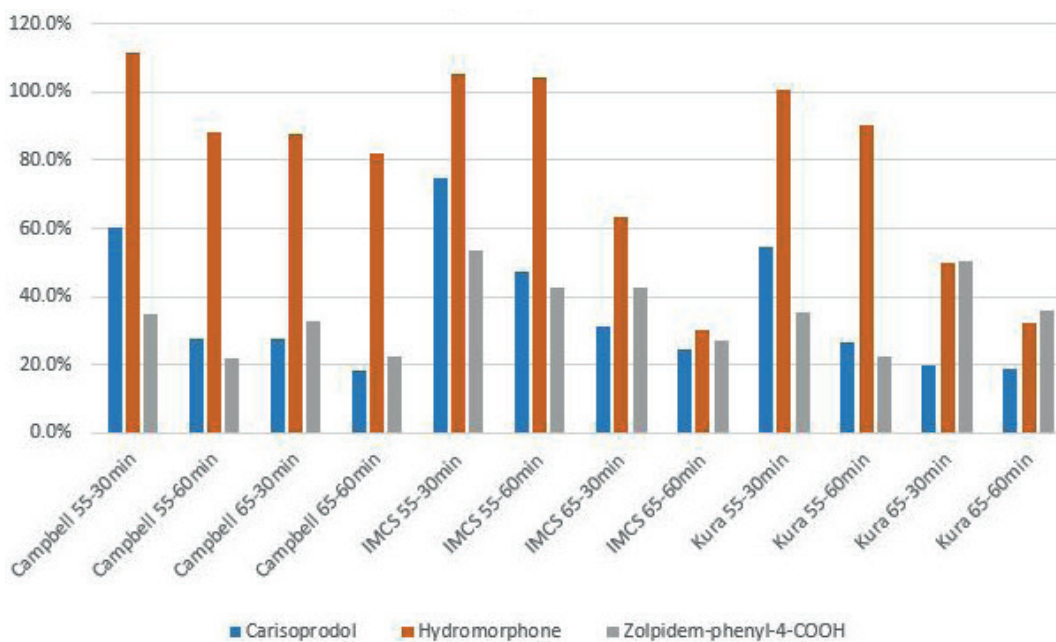


Figure 8. Recoveries of carisoprodol, hydromorphone and zolpidem-phenyl-4-COOH for three enzymes and all hydrolysis conditions.

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, and eluting with 78:20:2 (v/v) DCM:IPA:NH₄OH. ISOLUTE® SLE+ provided >90% recovery for all compounds except 85% recovery for n-desmethyltapentadol and 70% recovery for norhydrocodone and morphine using elution with 95:5 (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 Agonists

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:NH₄OH elution and EVOLUTE® EXPRESS CX. ISOLUTE® SLE+ produced >90% recovery for all compounds except for norfentanyl and n-desmethyltapentadol (80% recovery) with a 95:5 (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:NH₄OH. EVOLUTE® EXPRESS ABN gave recoveries of >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 with 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 of >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:NH₄OH. Recovery for ritalinic acid was increased to 80% by changing the elution solvent to 78:20:2 (v/v) DCM:MeOH:NH₄OH. These samples were not as clean as samples eluted with DCM:IPA:NH₄OH. 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 pretreatments 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 of 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:NH₄OH. 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 of carisoprodol and meprobamate, but <20% recovery of pregabalin and gabapentin. EVOLUTE® EXPRESS ABN provided >80% recovery of carisoprodol and meprobamate and <20% recovery of pregabalin and gabapentin.

Part VII.

Removal of Conditioning and Equilibrating for EVOLUTE® EXPRESS ABN and EVOLUTE® EXPRESS CX

ABN and CX are polymeric sorbents with hydrophilic (water wettable) characteristics. Because of this, the conditioning and equilibrating steps can be removed. This provides a reduction in both cost and volume of solvents used. Figure 9. shows recoveries for several compounds with and without conditioning and equilibrating for the IMCSzyme when

extracting using ABN. As can be seen, there is little difference between conditioning and no conditioning. The no conditioning extraction tends to have slightly higher recoveries than the conditioning extraction. Figure 10. shows recoveries for several compounds extracted using CX with and without conditioning and equilibrating. Similar to ABN, the recoveries are very similar between the two extractions.

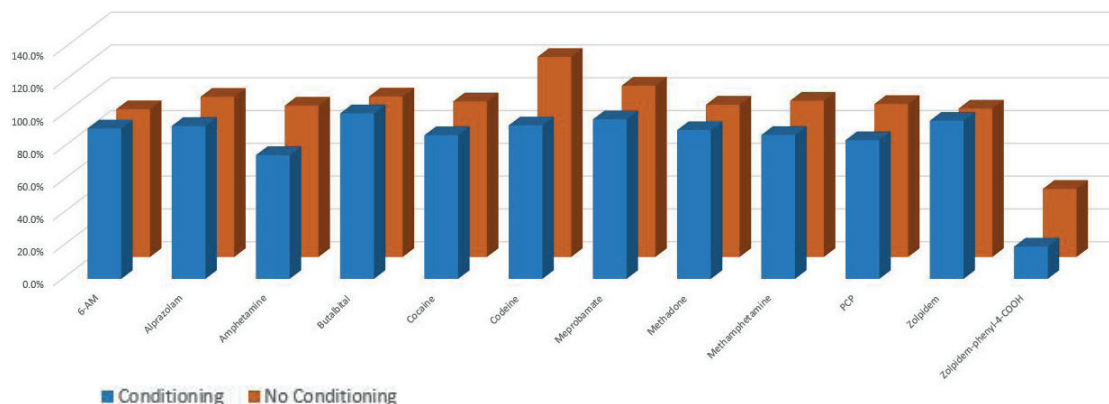


Figure 9. Selected analyte recoveries with and without conditioning and equilibrating steps using the IMCSzyme enzyme for hydrolysis, on EVOLUTE® EXPRESS ABN.

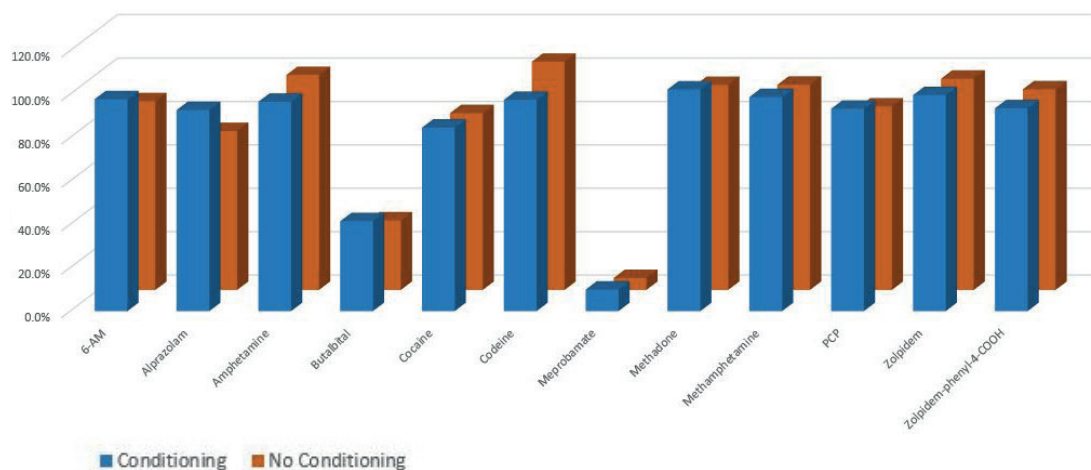


Figure 10. Selected analyte recoveries with and without conditioning and equilibrating steps using the IMCSzyme enzyme for hydrolysis, on EVOLUTE® EXPRESS CX.

Part VIII.

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% H₃PO₄, a 50% MeOH wash, and elution with 78:20:2 (v/v) DCM:IPA:NH₄OH is recommended. If ritalinic acid, gabapentin and pregabalin are required, the same protocol, except elution with 78:20:2 (v/v) DCM:MeOH:NH₄OH 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.

Lastly, the conditioning and equilibrating steps are not necessary to achieve maximum recoveries when extracting using EVOLUTE® EXPRESS ABN or EVOLUTE® EXPRESS CX. The elimination of those steps can save time and money.

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 IX.

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

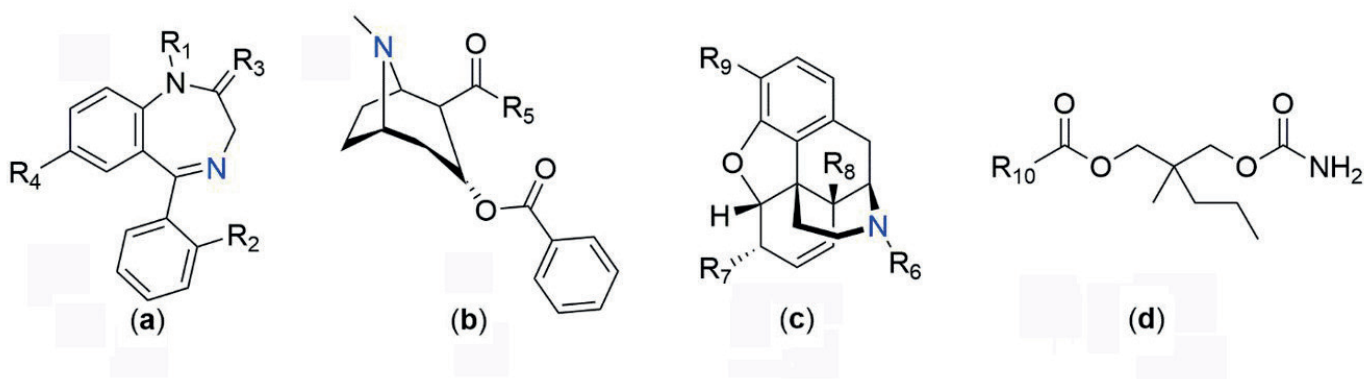


Figure 1. General scheme illustrating various generalized drug classes: benzodiazepines (a), stimulants (b), opioids (c), and carbamates (d). R-groups represent moieties that vary within each drug class.

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
Benzoyllecgonine	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 μ L of spiked urine was loaded into a 96-position, 2 mL well plate with 200 μ L of IMCS buffer along with 25 μ L (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 μ L of 4% phosphoric acid.

Biotage® Extrahera™ Extraction Parameters

Briefly, samples were loaded on 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 μ L 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 μ L 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 anesthetics (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.

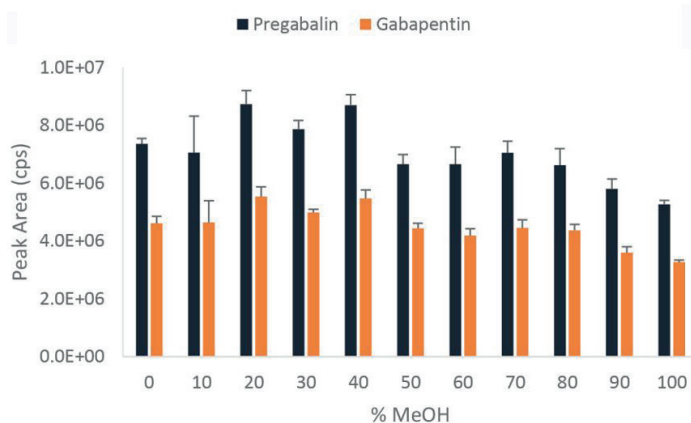


Figure 2a. Integrated peak area for 50 ng/mL extracted gabapentin and pregabalin under methanol washes ranging from 0 to 100%. Error bars represent standard deviation (n = 4).

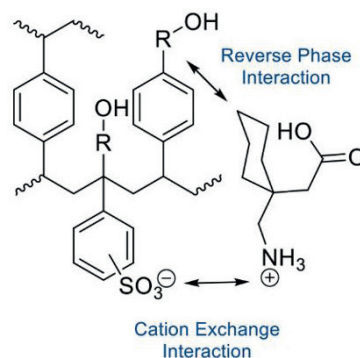


Figure 2b. EVOLUTE® EXPRESS CX sorbent's proposed columbic complexation with pregabalin.

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, this “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.

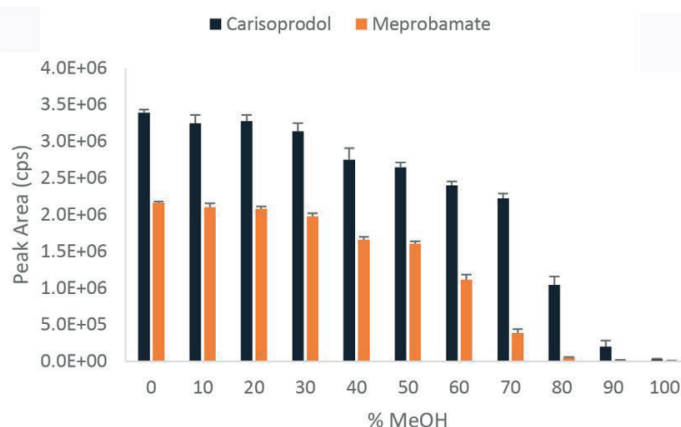


Figure 3a. Integrated peak area for 50 ng/mL extracted Carisoprodol and Meprobamate under methanol washes ranging from 0 to 100%. Error bars represent standard deviation (n = 4).

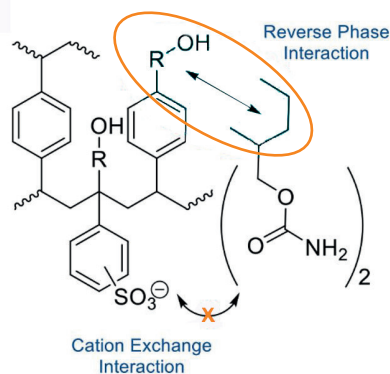


Figure 3b. EVOLUTE® EXPRESS CX sorbent’s proposed reverse phase affinity with Meprobamate.

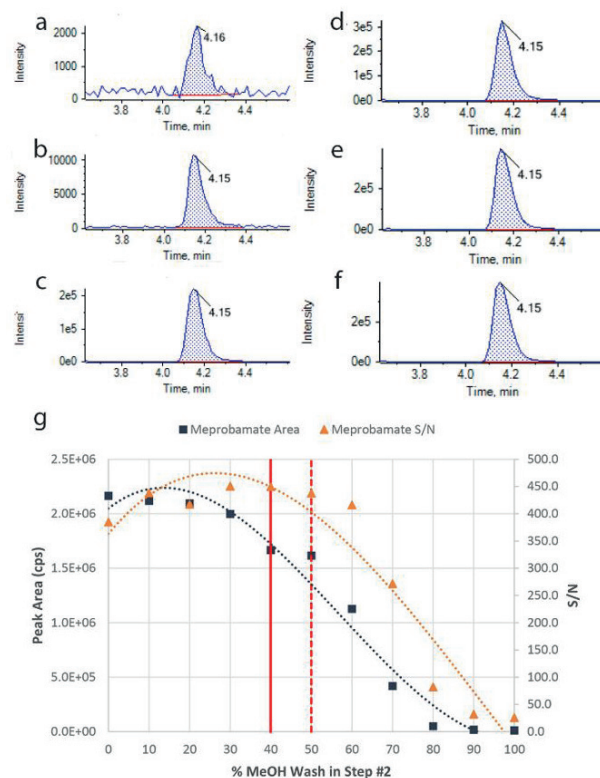


Figure 4. Integrated peak areas for the extraction of 50 ng/mL of Meprobamate after (a) 100%, (b) 80%, (c) 60%, (d) 40%, (e) 20%, and (f) 0% methanol used in wash #2. All peaks collected using SMRM. (g) integrated peak areas for methanol washes from 0 to 100% with each peak’s corresponding S/N using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

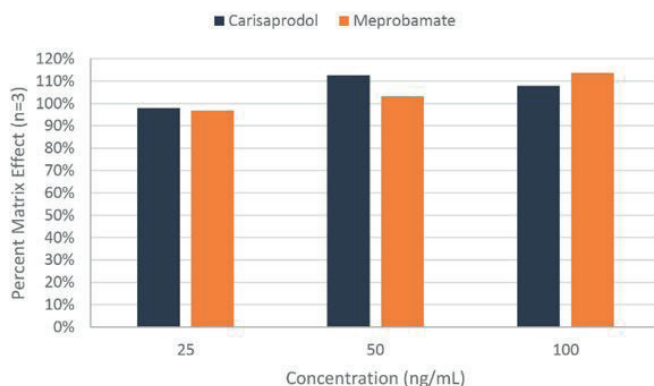


Figure 5. Matrix Effect for 50 ng/mL extracted carisoprodol and meprobamate in urine where > 100% constitutes enhancement and < 100% indicates suppression (n=3).

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.

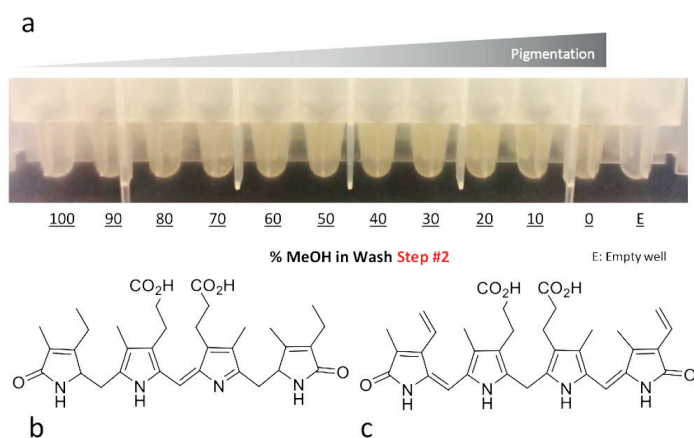


Figure 6. Effect upon pigmentation the percent methanol in wash step 2 upon elution with DCM/IPA/NH₄OH at [78:20:2]. (b) Structure of main urinary pigmentation, urobilin, responsible for the yellow color of urine. (c) Structure of secondary urinary pigment, bilirubin.

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/NH₄OH. 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 NH₄OH, 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).

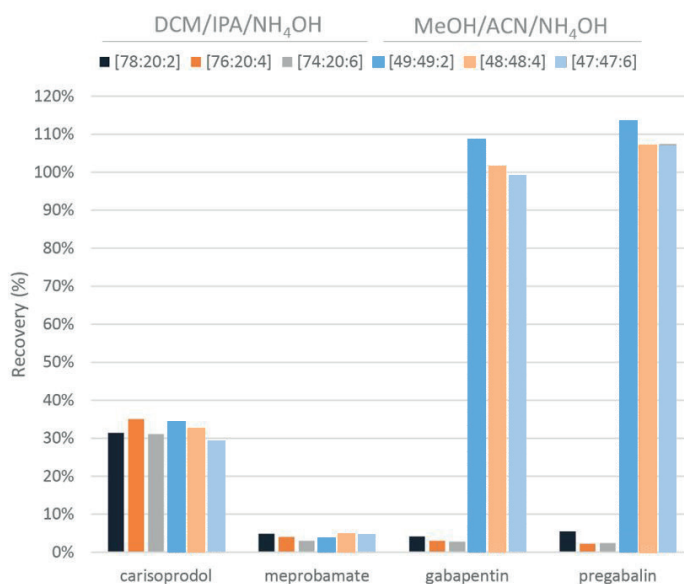


Figure 7. Effect upon the percent recovery eluting with solvents with low (DCM/IPA/NH₄OH) and high (MeOH/ACN/NH₄OH) dielectric capacity with increasing percentage of NH₄OH (n=3).

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.

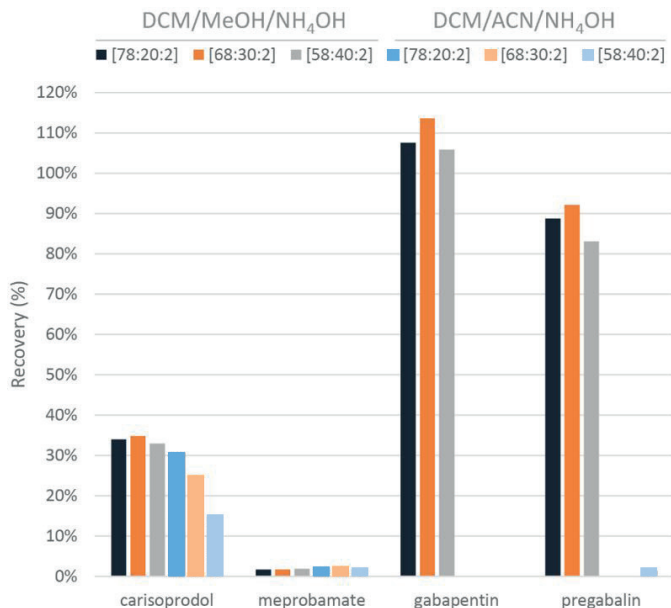


Figure 8. Effect upon the percent recovery eluting with DCM/MeOH/ NH_4OH and MeOH/ACN/ NH_4OH with increasing percentage of either polar-protic (MeOH) or polar-aprotic (ACN) solvents with high dielectric capacity.

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.

Addendum

Additional Analytes

Since the original publication of this white paper, approximately 50 compounds have been added to the drugs of abuse panel. These compounds are from all drug classes, including opioids, benzodiazepines, antipsychotics, and antidepressants. Table 1 shows the added compounds, their chemical formulas, LogPs, and pK_as.

Table 1. Details of additional analytes.

Drug Class	Compound	Formula	LogP	pK _a
Opioid	6-acetylcodeine	C20H23NO4	1.38	9.01, 11.72
Benzodiazepine	7-aminoflunitrazepam	C16H14FN3O	1.75	3.32
Antipsychotic	Aripiprazole	C23H27Cl2N3O2	4.9	7.46, 13.51
ADHD drug	Atomoxetine	C17H21NO	3.81	9.8
Antidepressant	Bupropion	C13H18ClNO	3.27	8.22, 18.29
Antidepressant	Buspirone	C21H31N5O2	1.78	7.62
Anticonvulsant	Carbamazepine	C15H12N2O	2.77	15.96, -3.8
Antihistamine	Chlorpheniramine	C16H19ClN2	3.58	9.47
Antipsychotic	Chlorpromazine	C17H19ClN2S	4.54	9.2
Antidepressant	Clomipramine	C19H23ClN2	4.88	9.2
ADHD drug	Clonidine	C9H9Cl2N3	2.49	8.16
Antipsychotic	Clozapine	C18H19ClN4	3.4	7.35, 15.9
Stimulant	Cocaethylene	C18H23NO4	2.64	8.77
Muscle relaxant	Cyclobenzaprine	C20H21N	4.61	9.76
Benzodiazepine	Desalkylflurazepam	C15H10N2OCIF	3.35	1.8, 12.29
Opioid	Dextromethorphan	C18H25NO	3.49	9.85
Antidepressant	Duloxetine	C18H19NOS	4.2	9.7
Opioid	EDDP	C20H25N	4.63	9.64
Alcohol	Ethyl Sulfate	C2H6O4S	-0.11	-2.1
Antidepressant	Fluoxetine	C17H18F3NO	4.17	9.8
Antipsychotic	Haloperidol	C21H23ClFNO2	3.66	8.05, 13.96
Antidepressant	Hydroxybupropion	C13H18ClNO2	2.22	7.65, 14.79
Benzodiazepine	Hydroxymidazolam	C18H13ClFN3O	2.48	4.99, 13.95
Benzodiazepine	Hydroxytriazolam	C17H12Cl2N4O	2.63	1.99, 10.97
Antidepressant	Imipramine	C19H24N2	4.28	9.2
Anticonvulsant	Lamotrigine	C9H7Cl2N5	1.93	5.87, 14.98
Antiepileptic	Levetiracetam	C8H14N2O2	-0.59	16.09, -1
Stimulant	Lidocaine	C14H22N2O	2.84	7.75, 13.78
Designer drug	mCPP	C10H13ClN2	2.15	8.87
Stimulant	MDA	C10H13NO2	1.43	10.01
Stimulant	MDEA	C12H17NO2	2.5	8.52
Opioid	Meperidine	C15H21NO2	2.46	8.16
Opioid	Methadone	C21H27NO	5.01	9.12, 18.78
Antidepressant	Methaqualone	C16H14N2O	3.17	-1.2
Designer drug	Methcathinone	C10H13NO	1.61	8.02, 18.52
Benzodiazepine	Midazolam	C18H13ClFN3	3.33	6.57
Antidepressant	Mirtazapine	C17H19N3	3.21	6.67
Antidepressant	n-desmethyldesmethylclomipramine	C18H21ClN2	4.5	10.02
Antidepressant	n-desmethyldesmethylmirtazapine	C16H17N3	2.82	8.75
Antipsychotic	Olanzapine	C17H20N4S	3.39	7.24, 14.17
Anticonvulsant	Oxcarbazepine	C15H12N2O2	1.82	12.92, -4.3
Antidepressant	Paroxetine	C19H20FN3O3	3.15	9.77
Antiepileptic	Phenytoin	C15H12N2O2	2.15	9.49, -9
Antipsychotic	Quetiapine	C21H25N3O2S	2.81	7.06, 15.12
Antipsychotic	Risperidone	C23H27FN4O2	2.63	8.76
Antidepressant	Sertraline	C17H17Cl2N	5.15	9.85
Antidepressant	Trazodone	C19H22ClN5O	3.13	7.09
Benzodiazepine	Triazolam	C17H12Cl2N4	2.89	4.32, 18.08
Antidepressant	Trimipramine	C20H26N2	4.76	9.42
Antidepressant	Venlafaxine	C17H27NO2	2.74	8.91, 14.42
Schizophrenia/Bipolar	Ziprasidone	C21H21ClN4OS	4.3	7.09, 13.18

Appendix

Biotage® Extrahera™ parameters for methods described in Section 1 Part V (Page 6).

ISOLUTE® SLE Method

ISOLUTE® 400 µL 96-well Plate

Part Number 820-0400-P01

Step	Solvent	Pressure	Time
Sample	100 µL 0.1% ammonium hydroxide (aq).		
Pre-treatment			
Load	Mix sample before loading.		
	Load 350 µL hydrolyzed, pre-treated sample.	5 bar	30 sec
	5 minute wait		
Elute	2 x 750 µL 95:5 (v/v) DCM/IPA.		
	Allow first aliquot to flow by gravity for 5 minutes.		
	Add second aliquot.		
	Allow to flow by gravity.		
	Plate dry.	5 bar	30 sec

EVOLUTE® EXPRESS CX Method

EVOLUTE® EXPRESS CX 30 mg 96-well Plate

Part Number 601-0030-PX01

Step	Solvent	Pressure	Time
Sample	100 µL 4% H ₃ PO ₄ .		
Pre-treatment			
Condition	1 mL MeOH.	2 bar	60 sec
Equilibrate	1 mL 4% H ₃ PO ₄ .	2 bar	60 sec
Load	Premix samples 3x Load 350 µL sample.	0.5 bar	60 sec
Wash 1	1 mL 4% H ₃ PO ₄ .	0.8 bar	60 sec
Wash 2	1 mL 50:50 (v/v) MeOH/H ₂ O.	0.8 bar	60 sec
	Plate dry	5 bar	60 sec
Elute	2 x 750 µL 78:20:2 (v/v) DCM/MeOH/NH ₄ OH.	0.5 bar	60 sec
	Plate dry.	5 bar	30 sec

EVOLUTE® EXPRESS ABN Method

EVOLUTE® EXPRESS ABN 30 mg 96-well Plate

Part Number 600-0030-PX01

Step	Solvent	Pressure	Time
Sample	100 µL 0.1% ammonium hydroxide (aq).		
Pre-treatment			
Condition	1 mL MeOH.	2 bar	60 sec
Equilibrate	1 mL 0.1% NH ₄ OH.	2 bar	60 sec
Load	Premix samples 3x Load 350 µL sample.	0.5 bar	60 sec
Wash 1	1 mL 90:10 (v/v) H ₂ O/MeOH.	0.8 bar	60 sec
Wash 2	1 mL 90:10 (v/v) H ₂ O/MeOH.	1.0 bar	60 sec
	Plate dry.	5 bar	60 sec
Elute	2 x 750 µL 90:10 (v/v) DCM/IPA.	0.5 bar	60 sec
	Plate dry.	5 bar	30 sec

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