Acidic, Basic, and Neutral Drug Screen of Hydrolyzed Urine Using Supported Liquid Extraction Prior to LC-MS/MS Analysis

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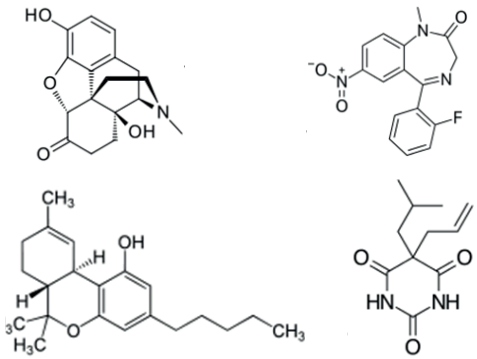

Figure 1. Structures of Oxymorphone, Flunitrazepam, Tetrahydrocannabinol and Butalbital

Introduction

This application note describes the extraction of acidic, basic, and neutral drugs from urine for screening purposes using ISOLUTE® SLE+ supported liquid extraction plates prior to LC-MS/MS analysis.

ISOLUTE SLE+ Supported Liquid Extraction products offer an efficient alternative to traditional liquid-liquid extraction (LLE) for bioanalytical sample preparation, providing high analyte recoveries, no emulsion formation, and significantly reduced sample preparation time.

In this application note, ISOLUTE SLE+ is used to quickly screen a panel of drugs from a small amount of urine (100 µL). The limit of detection was determined for each analyte and was found to range from 5 ng/mL to 1.25 ng/mL. The application note describes this quick robust screening assay for the extraction of 32 different drugs from a hydrolyzed urine matrix.

Analytes

Codeine, hydrocodone, oxycodone, norcodeine, oxymorphone, 6-acetyl codeine, alprazolam, clonazepam, diazepam, flunitrazepam, nitrazepam, oxazepam, temazepam, dextromethorphan, buprenorphine, norbuprenorphine, fentanyl, EDDP, benzoylecgonine, tetrahydrocannabinol, normeperidine, naltrexone, hydromorphone, propoxyphene, pentazocine, amphetamine, norfentanyl, MDEA, butalbital, pentobarbital, phenobarbital, secobarbital, 11-nor-9 –carboxy- Δ9-tetrahydrocannabinol, and d3-11-hydroxy-tetrahydrocannabinol.

Sample Preparation Procedure

Format:

ISOLUTE® SLE+ 400 µL Supported Liquid Extraction plate, part number 820-0400-P01

Sample Pre-treatment

To 100 µL of sample (calibrator, QC or patient sample), add 100 µL of 100 mM ammonium acetate, pH 5 (hydrolysis buffer). Then add a pre-determined amount of β-glucuronidase (enzyme) to solution to achieve a target concentration of 5000 U/mL. Incubate the sample at optimal temperature for enzymatic hydrolysis (follow vendor guidelines for optimal activity). Post-hydrolysis, add water (200 µL) to the hydrolyzed solution to pre-treat sample.

Sample Loading

Load up to 400 µL total volume of the pre-treated sample into each well and apply a pulse (3–5 seconds) of vacuum (using a vacuum manifold) or positive pressure (using a Biotage® PRESSURE+ 96 Positive Pressure Manifold).

Analyte Extraction

Elute analytes with two successive aliquots of dichloromethane: isopropanol (90:10 [v:v], 700 µL) per well. Elute solution slowly at a rate of 1 mL/min (~10-12 drops/min) under gravity or using minimal pressure/vacuum. Apply a pulse (5–10 seconds) of vacuum or positive pressure to pull through any remaining extraction solvent.

Post Extraction

Dry the extract in a stream of air or nitrogen using a Biotage® SPE Dry 96 (40 °C at 60 L/min) or TurboVap® 96 (40 °C at 1.0 bar/14.5 psi).

Reconstitution

Reconstitute the sample with of methanol:water with 0.1% formic acid (60:40 [v:v], 100 µL) in each well and let sample equilibrate for 15 minutes.

HPLC Conditions

Instrument
Agilent 1260 Liquid Handling System (Agilent, Santa Clara, CA.)

Column
Restek Raptor Biphenyl (50 mm x 2.1 mm, 3 µm)

Mobile Phase
A: Water with 0.1% formic acid

B: Methanol with 0.1% formic acid

Flow Rate
0.5 mL/min

Injection Volume
15 µL for positive analytes

20 µL for negative analytes

Column Temperature
50 °C

Table 1a. HPLC Gradient Conditions for Positive Analytes.

Step	Time (min)	Flow Rate (µL/min)	% A	% B
1	0.0	500	60	40
2	0.2	500	60	40
3	1.0	500	5	95
4	2.5	500	5	95
5	2.6	500	60	40
6	6	500	60	40

Table 1b. HPLC Gradient Conditions for Negative Analytes.

Step	Time (min)	Flow Rate (µL/min)	% A	% B
1	0.0	500	60	40
2	0.2	500	60	40
3	1.5	500	0	100
4	2.5	500	0	100
5	2.6	500	60	40
6	6	500	60	40

MS Conditions

A Sciex 4000 Q-Trap triple quadrupole mass spectrometer (Sciex, Foster City, CA) equipped with a Turbo Ionspray® interface was used for mass analysis. A dual injection was perfomed for each sample to analyze for both positive and negative analytes. Positive and negative ions were acquired in the multiple reaction monitoring (MRM) mode with the ion source temperature at 500 °C. The MRM transitions for all of the analytes of interest and their optimized collisional parameters in positive and negative mode are listed in **Table 2**.

Table 2. MS conditions and retention times for target analytes in positive and negative mode.

Analyte(s)	MRM Transition	Declustering Potential (DP)	Collision Energy (CE)	Retention Time (mins)
Codeine	300.2>215	40	25	1.11
Hydrocodone	300>199	40	25	1.37
Oxycodone	316>241	40	30	1.28
Norcodeine	286.1>225	40	25	0.94
Oxymorphone	302>227	40	30	0.59
6-acetyl codeine	342.4>215	40	30	2.99
Morphine	286.1>165	40	30	0.90
Alprazolam	308.8>280.5	40	30	3.96
Clonazepam	315.8>269.8	40	30	3.66
Diazepam	284.9>154	40	30	4.05
Flunitrazepam	313.9>267.9	40	30	3.87
Nitrazepam	282.1>180	40	30	3.67
Oxazepam	288>242	40	30	3.64
Temazepam	300.9>255	40	30	3.88
Dextromethorphan	272>215	40	30	3.32
Buprenorphine	468.2>396.2	55	55	3.23
Norbuprenorphine	414.1>83.1	55	55	3.09
Fentanyl	337>188	40	30	3.23
EDDP	278>234	40	30	3.39
benzoylecgonine	290>168	40	30	2.87
THC	315.2>193	40	28	4.17
Naltrexone	342>323.8	40	30	1.28
hydromorphone	286.2>185.1	40	30	0.58
Propoxyphene	340.3>266.3	40	30	3.29
Pentazocine	286.3>69.1	40	30	3.08
Amphetamine	136>91	40	30	1.88
Norfentanyl	233.1>84	40	35	1.02
MDEA	208.1>105.1	40	45	2.33
Butalbital	223.1>180.1	-50	-22	3.47
Pentobarbital	225.1>182	-50	-18	3.63
Phenobarbital	231.1>188	-50	-18	3.36
Secobarbital	237.1>193.9	-50	-23	3.72
THC-COOH	343.2>299	-50	-35	4.6
d_3-11-OH THC	332.2>314	-50	-30	4.42

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Results/Discussion

Chromatography

The target analytes were chromatographically separated via dual injections on a biphenyl column using the two linear gradients outlined in **Table 1a and 1b** for the positive and negative analytes, respectively. The extracted ion chromatograms (see **Figure 2a and 2b**) are shown for the positive and negative ions of interest. The observed retention times for all of the analytes are listed in **Table 2**.

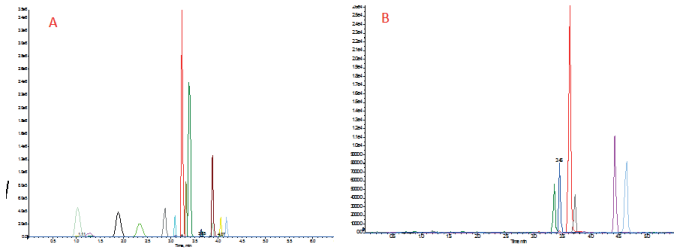

Figure 2. Extracted ion chromatograms for positive (A) and negative (B) target analytes.

Extraction Recoveries, Limit of Detection and Matrix Suppression

A 1000 ng/mL working stock solution of the target analytes was prepared in methanol from Cerilliant (Round Rock, TX) stock standards. The working stock solution was used to spike blank urine at three different concentration levels (25 ng/mL, 50 ng/mL and 100 ng/mL). The samples were allowed to equilibrate for a minimum of 30 minutes. The spiked urine samples were then extracted following the protocol outlined previously using the ISOLUTE® SLE+ Supported Liquid Extraction plate format. The observed recoveries at the three different levels are shown in **Figure 3**.

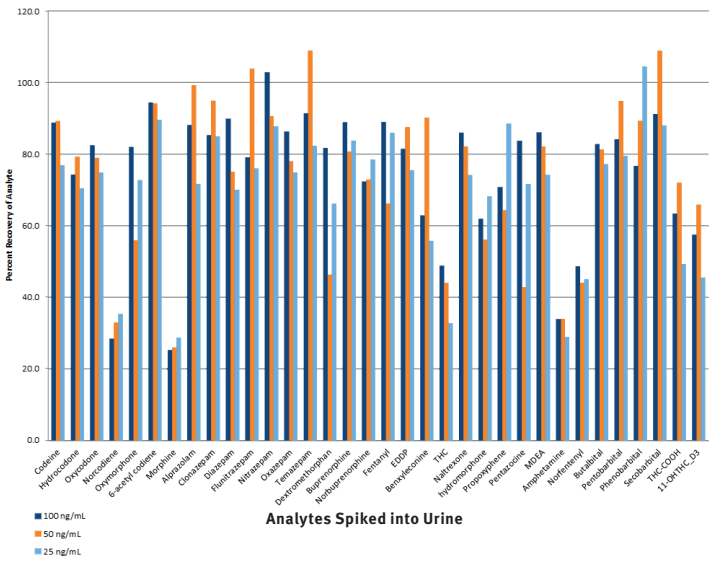

Figure 3. Plot of observed extraction recoveries for positive, negative and neutral analytes at 25, 50, and 100 ng/mL.

Most of the tested analytes were recovered at 70 percent or greater. The lower recovery of some analytes (<50%), was attributed to the use of water as a neutral pre-treatment solvent. The addition of water as a pre-treatment solution adjusts the pH of the sample to ranging from a pH 6.5 to 7. Typically, samples are pH adjusted to de-ionize acidic or basic functional groups present on the analytes. This is accomplished via the addition of either base or acid to the sample. It was found that raising the pH to optimize for the basic compounds negatively impacted the acidic compounds and vice versa. Hence, pre-treating the sample at a pH close to neutral allowed for the successful extraction and detection of all of the analytes of interest.

The limit of detection for each analyte was determined (LOD defined as observed signal to noise >3) and is shown in **Figure 4.** Despite lower recoveries for some of the analytes, detection limits for the panel is more than sufficient to employ an effective screening protocol using ISOLUTE SLE+.

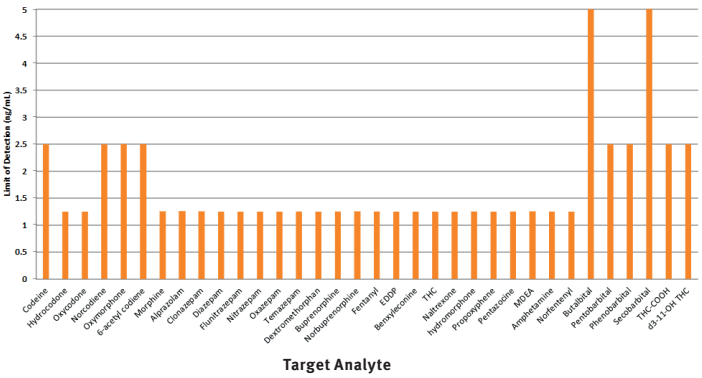

Figure 4. Plot of observed LODs for positive, neutral and negative analytes.

Matrix effects were also evaluated for each analyte using hydrolysed urine blanks extracted using the protocol described, and then fortified. The averaged response for three fortified blanks was compared to the averaged response of two neat aliquots of the elution solvent spiked with appropriate amount of working stock to determine either suppression or enhancement. The suppression and enhancement for each analyte is plotted in **Figure 5.**

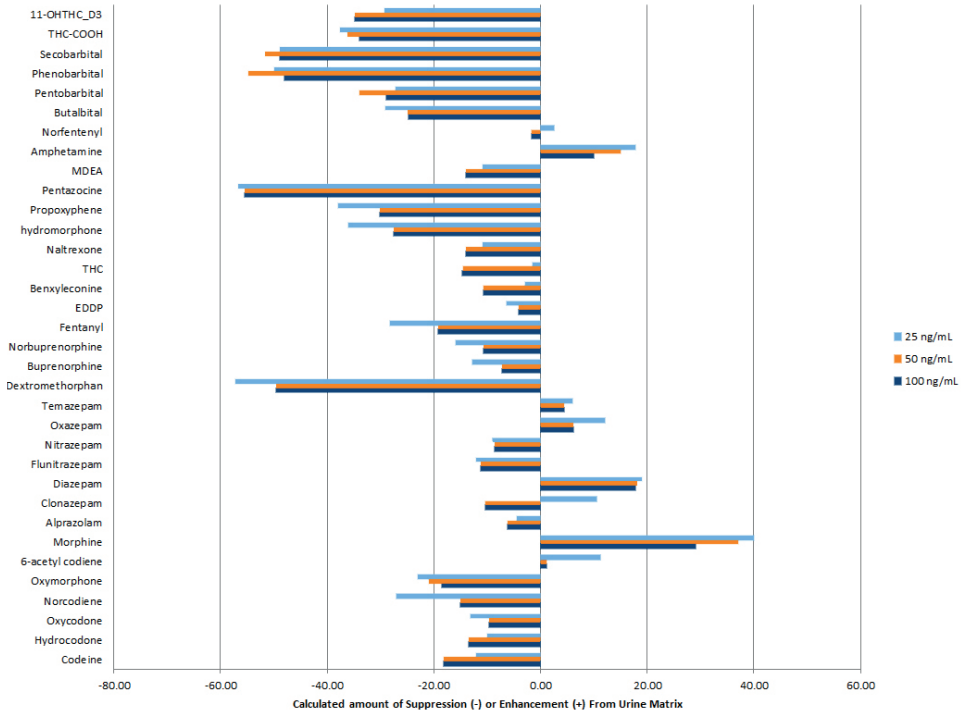

Figure 5. Plot of suppression/enhancement determinations for analytes at 25, 50, 100 ng/mL.

Suppression or enhancement for most analytes was observed between 20–25%, which is sufficient for a screening assay. For some analytes matrix effects were observed at >25%. The analytical response for the extracted analytes with higher matrix effects was sufficient to achieve adequate recoveries and LODs. This response, therefore, supports the use of ISOLUTE SLE+ for the screening of urine samples for various illicit and prescribed drugs.

Ordering Information

Part Number	Description	Quantity
820-0400-P01	ISOLUTE® SLE+ 400 Supported Liquid Extraction Plate	1
PPM-96	Biotage® PRESSURE+ 96 Positive Pressure Manifold	1
SD-9600-DHS-EU	Biotage® SPE Dry Sample Concentrator System 220/240 V	1
SD-9600-DHS-NA	Biotage® SPE Dry Sample Concentrator System 100/120 V	1
C103263	TurboVap®96, Evaporator 100/120V	1
C103264	TurboVap® 96, Evaporator 220/240V	1

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