Extraction of 22 Pain Management Drugs from Urine using ISOLUTE® SLE+ in 96-Fixed Well Plate Format Prior to LC-MS-MS

Introduction

This application note describes the extraction of 22 different drugs in urine which are typically screened for pain management panels using ISOLUTE SLE+ fixed well plates.

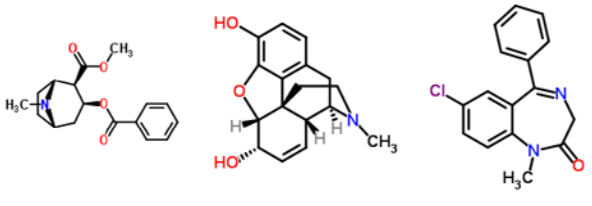

Figure 1. Structures of Cocaine, Morphine and Diazepam

The use of schedule I drugs for patient pain management therapy warrants constant monitoring of therapeutic levels in the patient. Screening patient urine samples for the free drugs is complicated by the metabolism process which converts the free drug to the β-glucuronide form. Patient urine samples can be enzymatically hydrolyzed and extracted to detect the drugs using Supported Liquid Extraction (ISOLUTE SLE+) which offers an efficient alternative to traditional liquid-liquid extraction (LLE) for bioanalytical sample preparation.

Analytes

Cocaine, Heroin , Morphine, Codeine, Oxymorphone, Hydromorphone, Oxycodone, Hydrocodone, Methadone, Clonazepam, Diazepam, Flunitrazepam, Oxazepam, Temazepam, Nitrazepam, Alprazolam, Methamphetamine, Fentanyl, Buprenorphine Meperidine, Naloxone, Naltrexone

ISOLUTE SLE+ Supported Liquid Extraction plates and columns 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.

ISOLUTE SLE+ Procedure

ISOLUTE® SLE+ Supported Liquid Extraction Plate (820-0400-P01)

Urine Hydrolysis: Add β-glucuronidase at a concentration of 5000 units/mL to urine and dilute sample with 100mM ammonium acetate (pH 5). Spike the matrix solution with internal standard. Incubate sample as per instructions with enzyme.

Sample pre-treatment: Mix 200 µL of hydrolyzed urine with 200 µL of 2% Ammonium Hydroxide (NH_4OH). Gently vortex solution.

Sample loading: Load pre-treated samples into fixed wells. Apply a short pulse of vacuum (VacMaster 96 Sample Processing Manifold **121-9600**) or positive pressure (PRESSURE+ 96 Positive Pressure Manifold **PPM-96**) to initiate flow and then allow sample to flow under gravity for 5 minutes.

Analyte elution: Apply 2 x 600µL of Ethyl Acetate to each well. Apply positive pressure or pull slight vacuum as needed during collection process at a flow rate of 1 mL per minute.

Post extraction: Evaporate sample to dryness (SPE Dry 96 –**SD-9600-DHS-NA**) and reconstitute in mobile phase (500 µL).

Additional Information: Dry down degradation of methamphetamine can be decreased by the addition of 50 uL of 50 mM HCl in methanol to elution collection wells prior to sample elution.

Sample Throughput: A full 96 well plate can be can be fully processed in 1 hour or less.

Application Note

HPLC Conditions

Instrument: Agilent 1200 Liquid Handling System (Agilent Technologies, Berkshire, UK)

Column: **Biotage Resolux 200 C_4**, 4.5µm analytical column (150 x 2.1 mm id) (Biotage, Uppsala, Sweden)

Mobile Phase: **Solvent A**: 0.1% Ammonium Hydroxide/ 0.01%Formic Acid in Water
Solvent B: 0.01% Formic Acid in Acetonitrile

Gradient:

Step	Time (min)	Flow Rate	%A	%B
1	0.0	500	80	20
2	0.50	500	80	20
3	4.0	500	0	100
4	6.0	500	0	100
5	7.0	500	80	20
6	10.0	500	80	20

Injection Volume: 5 µL

Temperature: 60 °C

Mass Spectrometry Conditions

Instrument: Applied Biosystems/MDS Sciex 4000 Q-Trap triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA.) equipped with a Turbo Ionspray® interface for mass analysis.

Ion Source Temperature: 400 °C.

Table 1. MRM Transitions for drugs in positive mode Atmospheric Pressure Ionization (API).

Scan Function	Analyte	MRM Transition	Declustering Potential (DP)	Collision Energy (CE)	Cell Exit Potential (CXP)
1	Cocaine	304>182	30	30	16
2	Heroin	370>165	30	30	16
3	Morphine	286>165	45	40	16
4	Codeine	300>199	30	40	16
5	Oxymorphone	302>227	30	30	16
6	Hydromorphone	286>185	30	35	16
7	Oxycodone	316>241	45	40	16
8	Hydrocodone	300>199	25	25	16
9	Methadone	310>265	30	30	16
10	Methamphetamine	150>91	30	40	16
11	Alprozolam	308.8>280.5	30	35	16
12	Clonazepam	315.8>269.8	30	30	16
13	Diazepam	284.9>154	30	30	16
14	Flunitrazepam	313.9>267.9	30	30	16

Mass Spectrometry Conditions

Scan Function	Analyte	MRM Transition	Declustering Potential (DP)	Collision Energy (CE)	Cell Exit Potential (CXP)
15	Oxazepam	288>242	30	40	16
16	Temazepam	300.9>255	30	30	16
17	Nitrazepam	282>180	40	40	16
18	Fentanyl	337>188	30	30	16
19	Buprenorphine	468>396	30	60	16
20	Meperidine	248>220	30	30	16
21	Naloxone	328>310	35	30	16
22	Naltrexone	342>323.8	30	40	16

Results

Blank urine was hydrolyzed with β-glucuronidase and spiked with a 22 drug mixture. The concentration of the stock solution was 100 ng/mL for all of the drugs except fentanyl which was at a concentration of 10 ng/mL. The final spike concentration was <5 ng/mL for all of the analytes except fentanyl which was spiked at concentrations <0.5 ng/mL. The average inter-run recoveries for the target analytes were above 80% with the overall intra-run RSDs <10%. The target analyte retention times as shown in **Table 2**.

Figure 2 shows a typical extracted ion chromatogram for the analysis of a 10 ng/mL mixed solution of the drug panel in urine and extracted using the 400 µl ISOLUTE SLE+ fixed well plate. **Figure 3** shows a plot of the average % recoveries for the drug panel spiked into human un-hydrolyzed urine at 5 ng/mL for all of the drugs except fentanyl which was spike into matrix at 0.5 ng/mL. **Figure 4** shows the average % recoveries for the target analytes spiked at a concentration of 2.5 ng/mL into hydrolyzed urine. Recoveries for methamphetamine showed loss due to dry down effects. This degradation effect was stabilized by the addition of 50 µL of 50 mM HCL into the bottom of the well prior to elution.

Table 2. Table of observed retention times for drug screen panel. Targets were separated using the Biotage Resolux C_4 HPLC column.

Analyte	RT (mins)	Analyte	RT (mins)
Cocaine	5.35	Clonazepam	4.57
Heroin	5.75	Diazepam	4.99
Morphine	3.27	Flunitrazepam	4.72
Codeine	4.92	Oxazepam	4.52
Oxymorphone	3.81	Temazepam	4.77
Hydromorphone	4.15	Nitrazepam	4.46
Oxycodone	3.81	Fentanyl	5.51
Hydrocodone	4.92	Buprenorphine	5.93
Methadone	9.14	Meperidine	5.59
Alprazolam	4.75	Naloxone	4.62
Methamphetamine	6.04	Naltrexone	4.86

Results

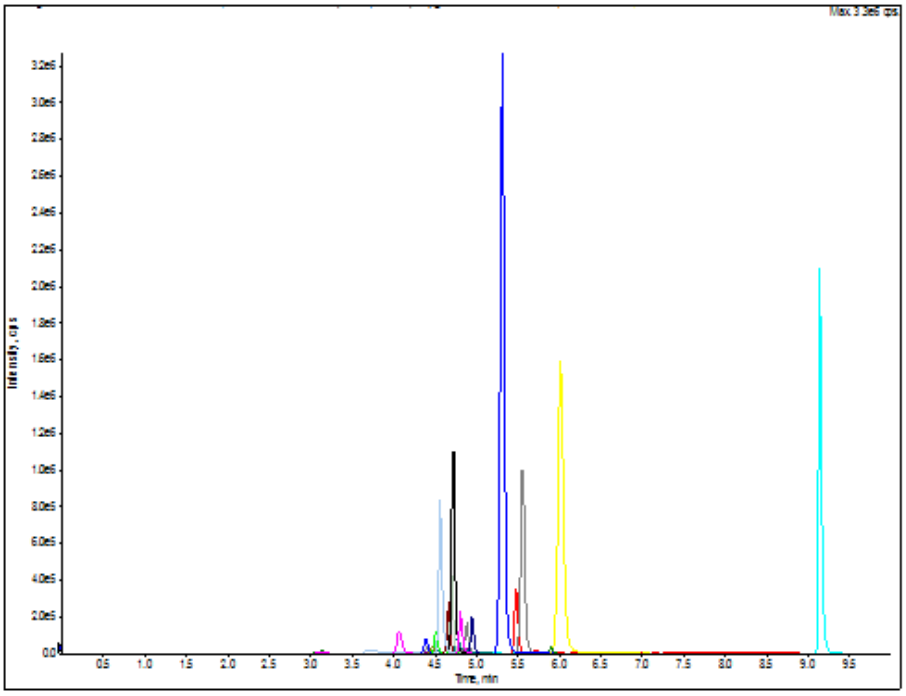

Figure 2. Typical extracted ion chromatogram for drug panel mixture spiked into urine at 10 ng/mL

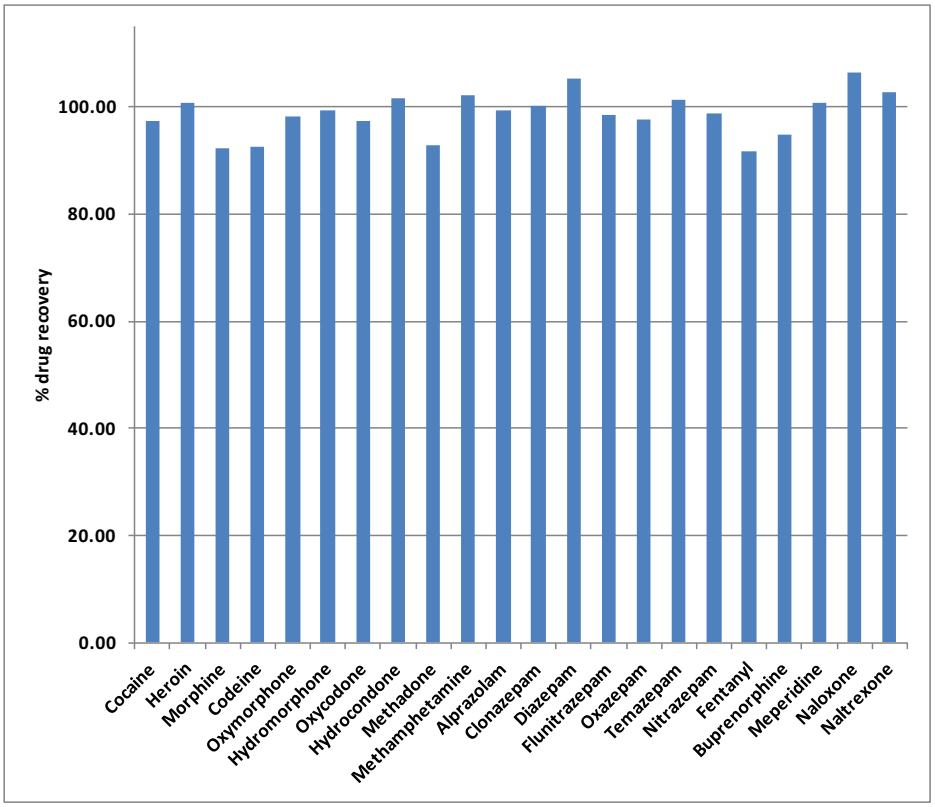

Figure 3. Plot of average % recoveries of drugs spiked into human urine and extracted on SLE+ 400 fixed well plates. All of the drug were spiked at a concentration of 5 ng/mL except fentanyl which was spiked at 0.5 ng/mL.

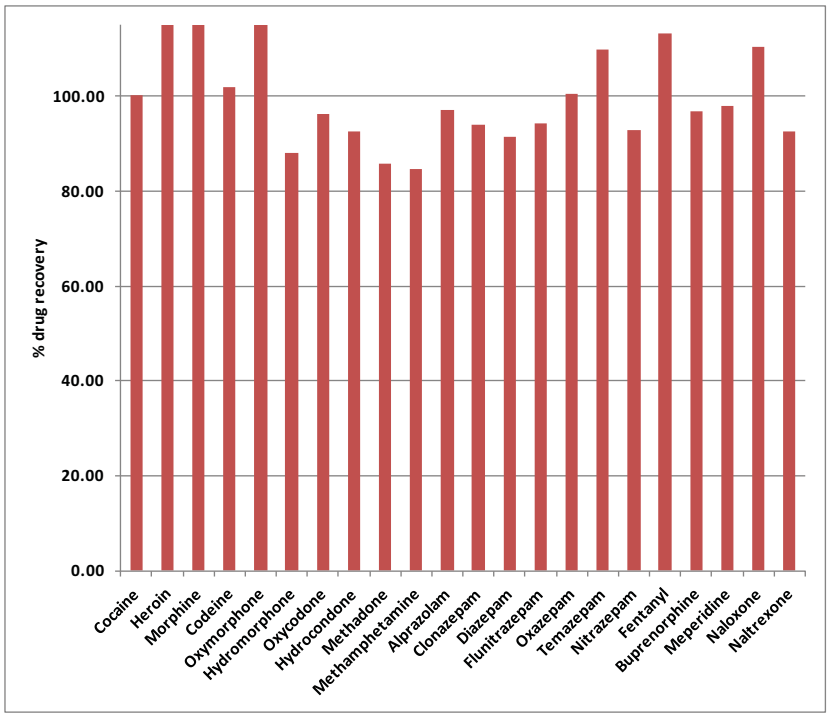

Figure 4. Plot of average % recoveries of drugs spiked into human urine hydrolyzed with β-glucoronidase and extracted on SLE+ 400 fixed well plates. All of the drug were spiked at a concentration of 2.5ng/mL except fentanyl which was spiked at 0.25 ng/mL.

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 96 Well	1
SD-9600-DHS-NA	SPE Dry 96 110 V, USA	1
R2-1521-2045	Resolux 200 HPLC Column C4	1

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