Extraction of a Drugs of Abuse Panel from Human Urine Using Biotage® Mikro ABN SPE Microelution Plates Prior to UPLC-MS/MS Analysis

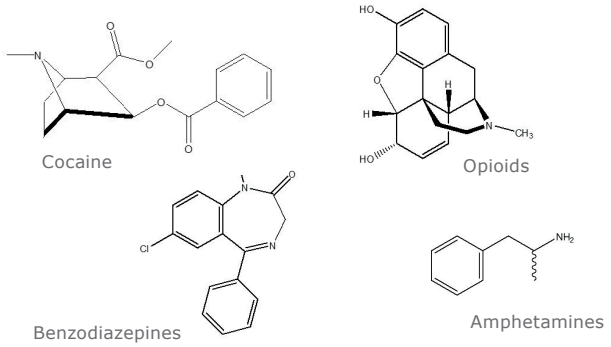

Figure 1. Example structures by class.

Introduction

This application note describes the extraction of 49 drugs of abuse from human urine using Biotage® Mikro ABN microelution plates, prior to LC/MS-MS analysis.

The simple sample preparation procedure delivers clean extracts and analyte recoveries mostly greater than 80% with RSDs lower than 10% for most analytes. Linearity of greater than 0.99 is achieved for all analytes from 1–1000 pg/mL.

Mikro plate extraction allows for very low elution volumes and enhanced workflow efficiency.

Analytes

Amphetamine, Methamphetamine, 3,4-Methylenedioxyamphetamine (MDA), 3,4-Methylenedioxymethamphetamine (MDMA), 3,4-Methylenedioxy-N-ethylamphetamine (MDEA), Hydromorphone, Morphine, Benzoylecgonine (BZE), Oxymorphone, Dihydrocodiene, Oxycodone, Mephedrone, Norfentanyl, 7-amino-flunitrazipam, 7-amino-clonazepam, Hydrocodone, Codeine, 6-Monoacetylmorphine (6-MAM), Cocaine, Norketamine, 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), Zaleplon, Zopiclone, Norbuprenorphine, Ketamine, Nitrazepam, Flunitrazepam, Clonazepam, α-OH-triazolam, Oxazepam, Estazolam, Temazepam, Zolpidem, Alprazolam, Methadone, Lorazepam, Bromazepam, α-OH-alprazolam, 2-OH-ethyl-flurazepam, Triazolam, Nordiazepam, Diazepam, Midazolam, Fentanyl, Flurazepam, Buprenorphine, Phencyclidine (PCP), Lysergic acid diethylamide (LSD).

Internal Standards

Amphetamine-D_5, Morphine-D_3, BZE-D_3, 6-MAM-D_3, Diazepam-D_5

Sample Preparation Procedure

Format

Biotage® Mikro ABN Plate, 2 mg, part number 600-0002-LVP

Sample Pre-Treatment

Spike urine (1 mL) with internal standard solution and allow to equilibrate for 1 hour. Dilute sample with 100 mM NH_4OAC pH 5 (950 µL) and add β-glucuronidase (50 µL). Incubate at 60 °C for 2 hours.

Internal standard solution consisted of a 10 pg/ µL methanolic solution. 100 µL of this was added to 1 mL of urine to give a 1 ng/mL spike concentration.

Condition (Optional)

Condition wells with methanol (100 µL)

Equilibration (Optional)

Equilibrate wells with 0.1% ammonium hydroxide (aq) (100 µL)

Sample Loading

Load 400 µL of the pre-treated urine sample

Wash 1

Elute interferences with 0.1% ammonium hydroxide (100 µL)

Wash 2

Elute interferences with H_2O:MeOH (90:10, v/v, 100 µL)

Elution

Elute analytes with DCM:MeOH (90:10, v/v, 30 µL)

Post Elution and Reconstitution

Dry the extract in a stream of air or nitrogen using a Biotage® SPE Dry at 40 °C, 20 to 40 L/min, or a TurboVap® at 40 °C, 1.5 L/min, for approximately 5 minutes.

Reconstitute evaporated samples with H_2O:MeOH (90/10, v/v) containing 0.1% formic acid (30 µL).

Vortex mix and cover plate with a sealing mat prior to injection.

Processing Conditions

Biotage® Mikro plates were processed using a Biotage® Pressure+ Positive Pressure Manifold.

Settings: Condition, equilibrate, load, wash and elute steps: 7–9 psi (fine control setting).

UHPLC Conditions

Instrument
Shimadzu Nexera UHPLC

Column
Restek Raptor™ Biphenyl 2.7 µm (100 x 2.1 mm)

Mobile Phase
A: 2 mM ammonium formate (aq) containing 0.1% formic acid

B: 2 mM ammonium formate (MeOH) containing 0.1% formic acid

Flow Rate
0.4 mL/min

Injection Volume
5 µL

Column Temperature
30 °C

Table 1. HPLC Gradient.

Time (min.)	%A	%B
0	80	20
2.00	80	20
7.50	40	60
11.25	40	60
12.75	0	100
13.50	0	100
13.51	80	20
15.00	80	20

MS Conditions

Instrument
Shimadzu 8060 Triple Quadrupole MS using ES interface

Nebulizing Gas Flow
3 L/min

Drying Gas Flow
3 L/min

Heating Gas Flow
17 L/min

Interface Temperature
400 °C

DL Temperature
250 °C

Heat Block Temperature
300 °C

CID Gas Flow
270 kPa

Table 2. MS conditions for target analytes in positive mode.

Analytes	MRM Transition	Collision Energy
Morphine-D_3	289.0 > 201.1 (289.0 > 152.1)	-26.0 -50.0
Morphine	286.0 > 152.1 (286.0 > 201.1)	-50.0 -25.0
Oxymorphone	302.00 > 227.1 (302.00 > 198.1)	-30.0 -45.0
Hydromorphone	286.0 > 185.0 (286.0 > 157.0)	-30.0 -40.0
Amphetamine-D_5	141.0 > 93.0 (141.0 > 124.15)	-15.0 -20.0
Amphetamine	136 > 91.05 (136 > 119.1)	-15.0 -14.0
Methamphetamine	150.0>90.95 (150>119.1)	-20.0 -14.0
MDA	180 > 105 (180 > 77)	-20.0 -40.0
Dihydrocodiene	302 > 119.05 (302 >171)	-35.0 -45.0
Codeine	300.0>215.1 (300.0>165)	-25.0 -40.0
6-MAM-D_3	331.0 > 165.1 (331.0 > 211.1)	-40.0 -25.0
6-MAM	328.0 > 165.1 (328.0 > 211.1)	-40.0 -25.0
MDMA	194.0 > 163.1 (194.0 > 105.0)	-15.0 -25.0
Oxycodone	316.2 > 241.2	-20.0
Mephedrone	178.00 > 145.05 (178.00 > 144.00)	-20.0 -30.0
Hydrocodone	300.0 > 199.05 (300.0 > 171.1)	-30.0 -40.0
MDEA	208 > 163.05 (208 > 105.05)	-15.0 -25.0
Nor-Ketamine	223.9 > 125 (223.9 > 179.05)	-20.0 -15.0
Nor-Fentanyl	233.0 > 84.05 (233.0 > 56.05	-20.0 -26.0
BZE-D_3	293.00 > 171.05 (293.00 > 77.00)	-20.0 -50.0
BZE	289.90 > 168.05 (289.90 > 105.00)	-20.0 -30.0
Ketamine	237.90 > 125.00 (237.90 > 207.05)	-30.0 -14.0
7-Aminoclonazepam	285.90 > 222.10 (285.90 > 121.10)	-25.0 -29.0
Cocaine	304.00 > 182.05 (304.00 > 82.05)	-20.0 -30.0
Zopiclone	388.90 > 245.05 (388.90 > 217.00)	-15.0 -35.0

Table 2. Continued.

Analytes	MRM Transition	Collision Energy
Norbuprenorphine	414.00 > 101.25 (414.00 > 187.20)	-39.0 -38.0
LSD	323.50 > 208.10 (323.50 > 223.25)	-29.0 -23.0
7-Aminoflunitrazepam	283.90 > 135.05 (283.90 > 227.05)	-30.0 -26.0
Zolpidem	308.00 > 235.10 (308.00 > 263.10)	-35.0 -25.0
Buprenorphine	468.10 > 396.25 (468.10 > 414.30)	-40.0 -35.0
Fentanyl	337.00 > 188.10 (337.00 > 105.00)	-20.0 -40.0
Flurazepam	388.00 > 315.00 (388.00 > 288.00)	-20.0 -26.0
PCP	244.00 > 91.05 (244.00 > 159.15)	-35.0 -14.0
Midazolam	325.90 > 249.10 (325.90 > 223.00)	-35.0 -40.0
Bromazepam	315.80 > 182.10 (315.80 > 209.10)	-31.0 -27.0
EDDP	278.00 > 234.00 (278.00 > 234.00)	-30.0 -45.0
Lorazepam	320.80 > 275.00 (320.80 > 229.05)	-22.0 -30.0
Oxazepam	320.80 > 229.05 (286.90 > 104.20)	-23.0 -35.0
Nitrazepam	286.90 > 104.20 (281.90 > 180.10)	-25.0 -35.0
Clonazepam	315.90 > 270.05 (315.90 > 214.05)	-25.0 -38.0
a-OH-Triazolam	358.90 > 331.10 (358.90 > 239.05)	-28.0 -44.0
2-OH-et-flurazepam	332.90 > 211.10 (332.90 > 109.00)	-37.0 -27.0
Methadrone	310.50 > 265.10	-16.0
a-OH-Alprazolam	324.90 > 216.10 (324.90 > 205.10)	-39.0 -46.0
Nordiazepam	270.90 > 140.05 (270.90 > 208.10)	-26.0 -28.0
Zaleplon	305.90 > 236.15 (305.90 > 264.20)	-28.0 -22.0
Flunitrazepam	313.90 > 268.10 (313.90 > 239.10)	-25.0 -35.0
Estazolam	294.90 > 267.05 (294.90 > 205.05)	-20.0 -40.0
Temazepam	300.90 > 255.05 (300.90 > 177.05)	-20.0 -39.0
Triazolam	342.90 > 308.10 (342.90 > 239.05)	-27.0 -41.0
Alprazolam	308.90 > 281.00 (308.90 > 205.05)	-25.0 -40.0
Diazepam-D_5	289.90 > 193.05 (289.90 > 154.00)	-32.0 -27.0
Diazepam	285.10 > 193.05 (285.10 > 154.00)	-32.0 -27.0

Biotage® PRESSURE+ 96 Positive Pressure Manifold.

Biotage®

Results

High (mostly › 80%) reproducible (RSD ‹ 10%) recoveries were achieved using the method described in this application note using the Biotage® Mikro plate format.

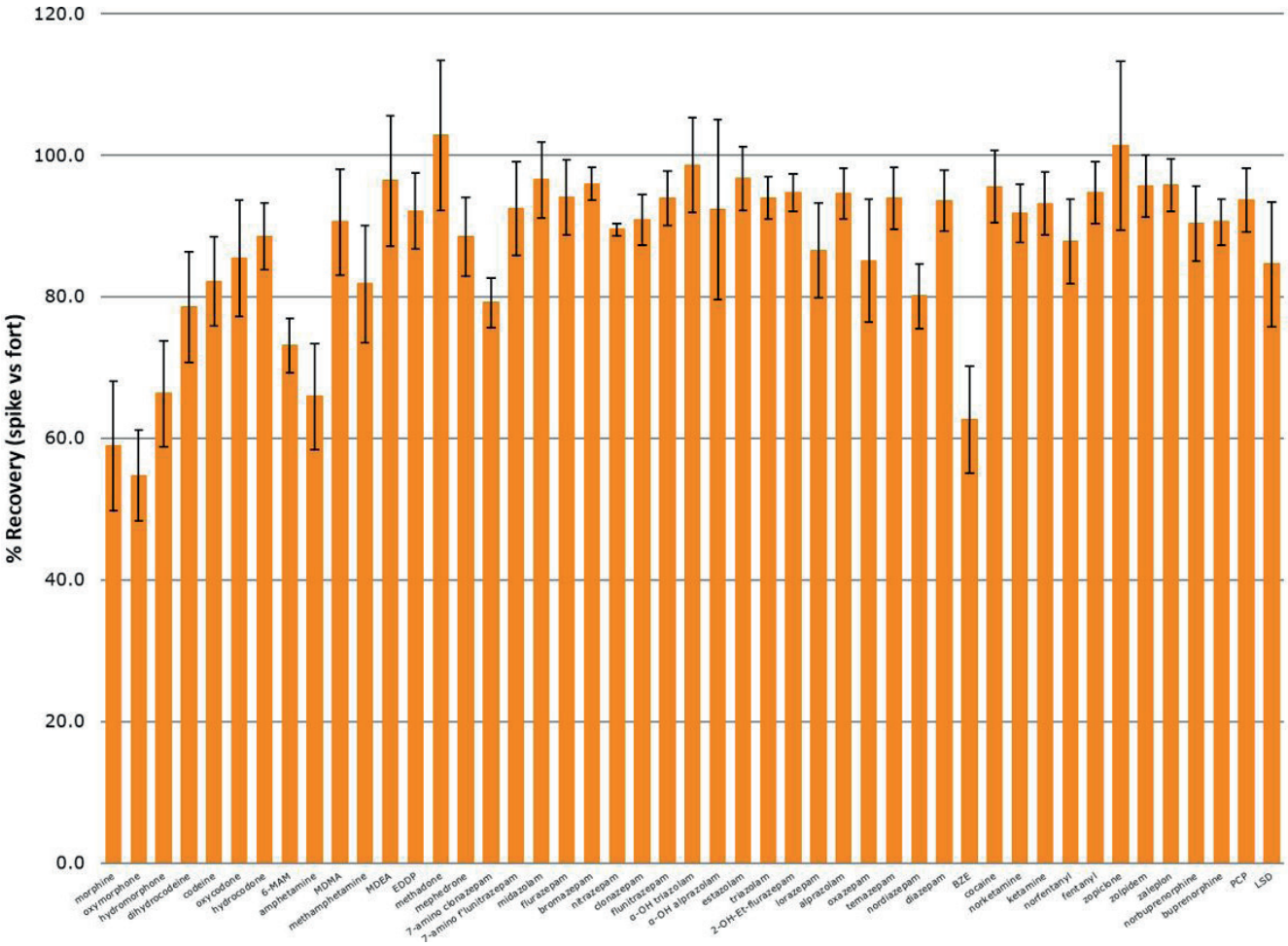

Figure 2. Shows analyte recoveries (1 ng/mL) using the optimized Mikro ABN protocol described in this application note. The time saving Load-Wash-Elute procedure gave similar analyte recoveries. See Discussion section for recovery data generated using the Load-Wash-Elute method.

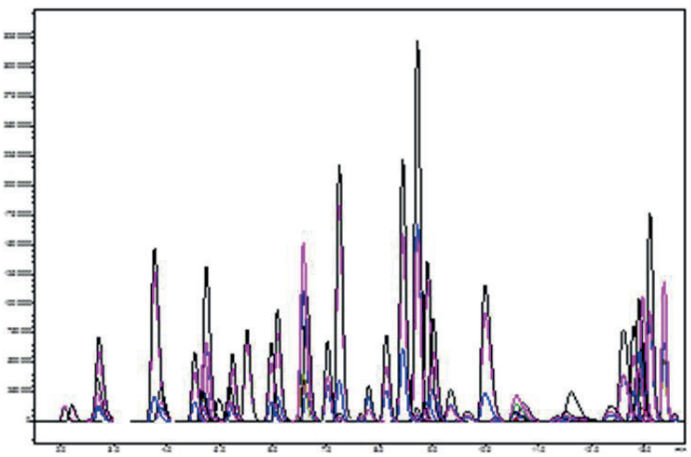

Figure 3. Representative chromatography for application analytes in urine spiked at 1 ng/mL.

Calibration curve performance was investigated from plasma spiked between 1–1000 pg/mL. Good linearity was observed for all analytes typically delivering r^2 values greater than 0.99. Table 3. details linearity performance and associated LOQ for each analyte.

Table 3. Analyte calibration curve r^2 and LOQ performance.

Analyte	r^2 200 µL Load	LLOQ (pg/mL) 200 µL Load
Morphine	0.9990	100
Oxymorphone	0.9991	25
Hydromorphone	0.9996	50
Amphetamine	0.9990	50
Methamphetamine	0.9995	50
Dihydrocodiene	0.9993	1
Codeine	0.9990	25
6-MAM	0.9993	50
MDMA	0.9993	5
Oxycodone	0.9996	25
Mephedrone	0.9993	50
Hydrocodone	0.9990	50
MDEA	0.9993	10
Nor-Ketamine	0.9993	10
Nor-Fentanyl	0.9992	5
BZE	0.9995	50
Ketamine	0.9995	5
7-Aminoclonazepam	0.9998	100
Cocaine	0.9996	25
Norbuprenorphine	0.9995	100
LSD	0.9995	25
7-Aminoflunitrazepam	0.9993	100
Zolpidem	0.9992	5
Buprenorphine	0.9991	10
Fentanyl	0.9990	50
Flurazepam	0.9990	1
PCP	0.9994	10
Midazolam	0.9997	50
Bromazepam	0.9995	100
EDDP	0.9990	1
Lorazepam	0.9996	< 250
Oxazepam	0.9990	250
Nitrazepam	0.9990	500
Clonazepam	0.9994	100
a-OH-Triazolam	0.9992	50
2-OH-et-flurazepam	0.9995	< 100
Methadrone	0.9990	250
a-OH-Alprazolam	0.9997	250
Nordiazepam	0.9990	50
Zaleplon	0.9991	5
Flunitrazepam	0.9991	50
Estazolam	0.9993	5
Temazepam	0.9995	< 250
Triazolam	0.9993	1
Alprazolam	0.9994	25
Diazepam	0.9996	5

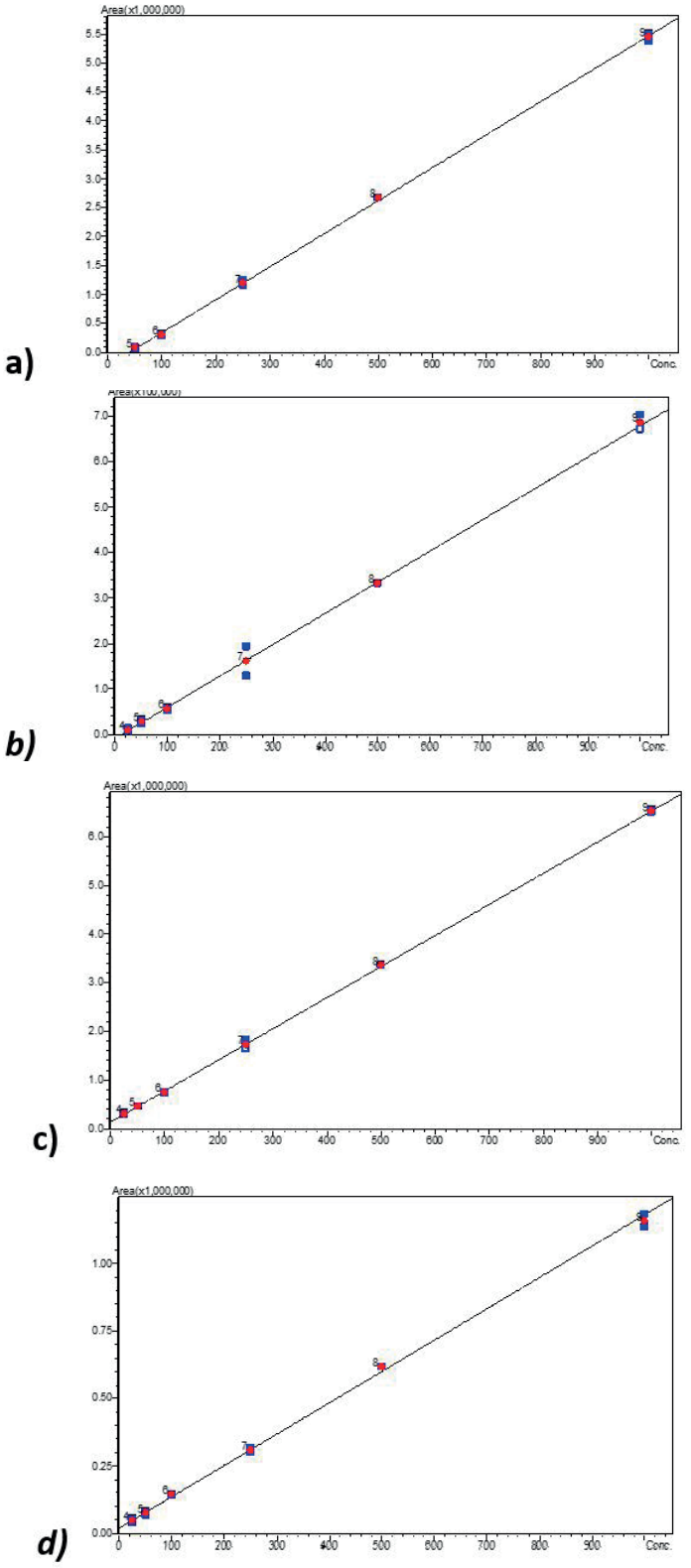

Figure 4. Calibration curves for Fentanyl (a), 6-MAM (b), Cocaine (c) and Methamphetamine (d) using the Biotage® Mikro plate to extract human urine.

Discussion and Conclusion

The Biotage® Mikro solid phase extraction microelution plate provided robust extraction of a large drugs of abuse panel from hydrolyzed urine samples. High, reproducible recoveries were achieved, with an overall processing time of approximately 22 minutes (including the evaporation step). An evaporation step was required in this application, as the elution solvent (DCM/MeOH) which gave the highest analyte recoveries was not compatible with the reversed phase analytical UPLC system. However, due to the low elution volume, evaporation time for 96 samples was ~ 5 minutes.

Compared to the equivalent procedure using a 10 mg EVOLUTE® EXPRESS ABN plate, a reduction in total processing time (~22 mins vs ~33 mins) was possible. In addition, higher, more reproducible recoveries for some analyte types (such as benzodiazepines) were achieved.

Comparison with EVOLUTE® Load-Wash-Elute Methodology

Elimination of the optional conditioning and equilibration steps in this method was investigated. A reduced overall extraction time (and solvent usage) was possible, and analyte recoveries were not adversely impacted (see figure 5).

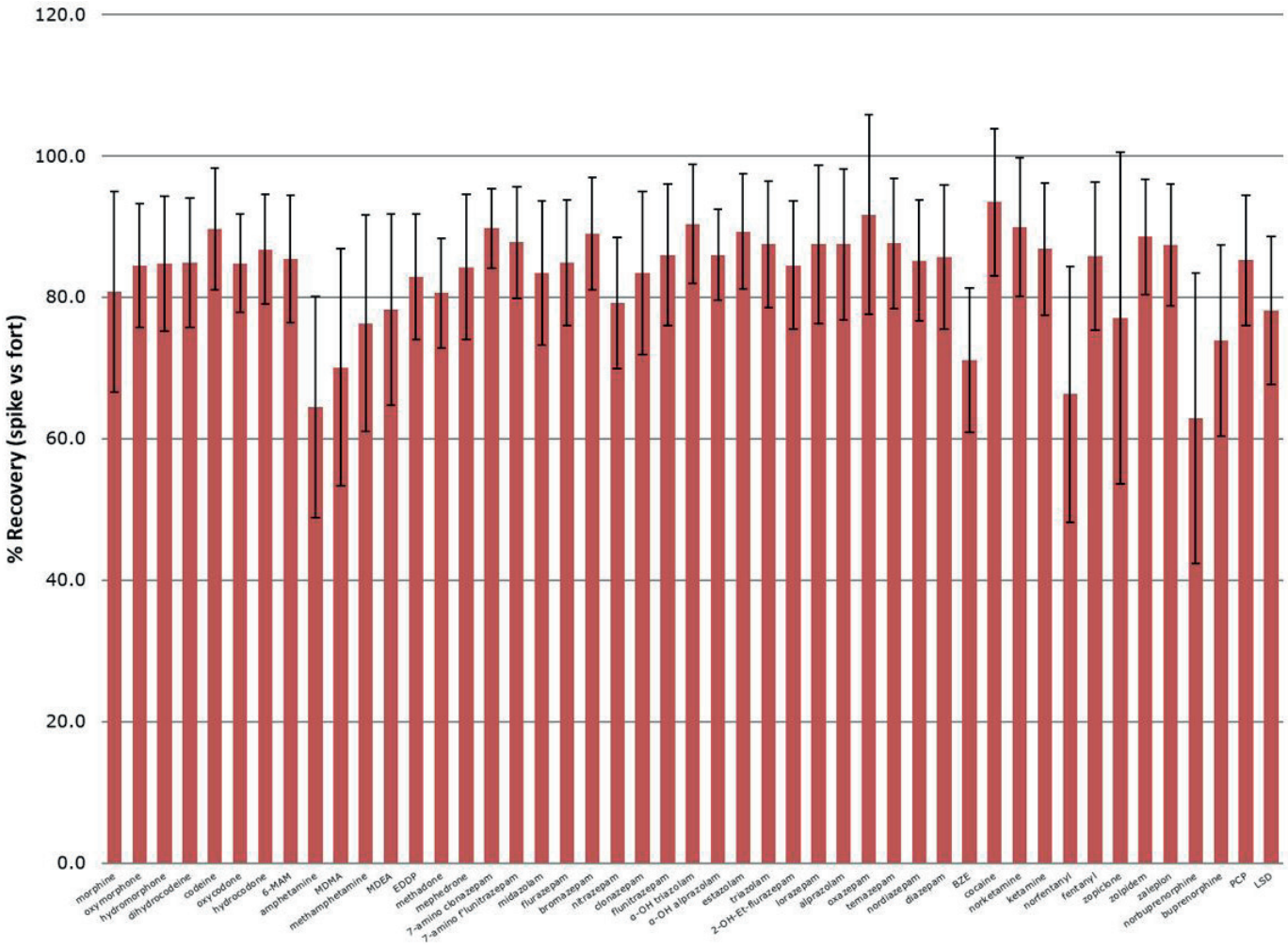

Figure 5. Analyte recoveries achieved using the Load-Wash-Elute methodology (where conditioning and equilibration steps are eliminated). This approach reduced the overall processing time by approximately 5 minutes (~17 mins for L-W-E method vs ~22 mins for full method).

Chemicals and Reagents

- Methanol (LC-MS grade), Ultra-Pure Methanol (Gradient MS), and dichloromethane (99.8%) were purchased from Honeywell Research Chemicals (Bucharest, Romania).
- All analyte standards, deuterated internal standards, ammonium acetate, ammonium formate, formic acid and ammonium hydroxide (27–30%) were purchased from Sigma- Aldrich Company Ltd. (Gillingham, UK).
- Water used was 18.2 MOhm-cm, drawn daily from a Direct-Q5 water purifier.
- Mobile phase A (2 mM ammonium formate (aq), 0.1% formic acid) was prepared by adding 0.126 mg of ammonium formate to 1 L purified water with 1 mL formic acid.
- Mobile phase B (2 mM ammonium formate (methanolic), 0.1% formic acid) was prepared by adding 0.126 mg of ammonium formate to 1 L ultra-pure MeOH with 1 mL formic acid.
- Internal standards (100 pg/µL) were prepared from a 10 ng/µL stock solution by adding 10 µL of each of to 950 µL of MeOH. 10 µL of this solution was then added to each calibration solution.
- Hydrolysis buffer solution (100 mM ammonium acetate) was made by adding 0.3854 mg of ammonuim acetate to 50 mL of water (18.2 MOhm-cm).
- Hydrolysis enzyme β-Glucuronidase from Helix pomatia, Type HP-2; ≥100,000 units/mL purchased from Sigma- Aldrich Company Ltd. (Gillingham, UK).
- Equilibration and wash 1 solvent (0.1% ammonium hydroxide) was made by adding 100 µL of ammonium hydroxide to 99.9 mL of water (18.2 MOhm-cm).
- Wash 2 solvent (H_2O:MeOH (90:10, v/v)) was made up by measuring out 90 mL of water (18.2 MOhm-cm) and 10 mL of methanol and adding both to a bottle.
- Elution solvent ((DCM:MeOH (90:10, v/v)) was made up by measuring out 90 mL of DCM (18.2 MOhm-cm) and 10 mL of methanol and adding both to a bottle.
- Reconstitution solvent was made by measuring out 90 mL of purified water (18.2 MOhm-cm) and 10 mL of MeOH and adding them to the same bottle with 100 µL formic acid.

Additional Information

- All data shown in this application note was generated using human urine donated by healthy human volunteers.

Ordering Information

Part Number	Description	Quantity
600-0002-LVP	Biotage® Mikro ABN Plate, 2 mg	1
PPM-96	Biotage® PRESSURE+ 96 Positive Pressure Manifold	1
SD-9600-DHS	Biotage® SPE Dry Sample Concentrator System	1
121-5202	Collection Plate, 1 mL Square	50
121-5204	Pierceable Sealing Mat	50

EUROPE
Main Office: +46 18 565900
Toll Free: +800 18 565710
Fax: +46 18 591922
Order Tel: +46 18 565710
Order Fax: +46 18 565705
order@biotage.com
Support Tel: +46 18 56 59 11
Support Fax: + 46 18 56 57 11
eu-1-pointsupport@biotage.com

NORTH & LATIN AMERICA
Main Office: +1 704 654 4900
Toll Free: +1 800 446 4752
Fax: +1 704 654 4917
Order Tel: +1 704 654 4900
Order Fax: +1 434 296 8217
ordermailbox@biotage.com
Support Tel: +1 800 446 4752
Outside US: +1 704 654 4900
us-1-pointsupport@biotage.com

JAPAN
Tel: +81 3 5627 3123
Fax: +81 3 5627 3121
jp_order@biotage.com
jp-1-pointsupport@biotage.com

CHINA
Tel: +86 21 68162810
Fax: +86 21 68162829
cn_order@biotage.com
cn-1-pointsupport@biotage.com

KOREA
Tel: + 82 31 706 8500
Fax:+ 82 31 706 8510
korea_info@biotage.com
kr-1-pointsupport@biotage.com

INDIA
Tel: +91 22 4005 3712
india@biotage.com

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