Simplified Sample Preparation for Drugs of Abuse Extraction from Urine Samples Prior to LC-MS/MS Analysis

Rhys Jones, Katie-Jo Teehan, Adam Senior, Alan Edgington, Lee Williams, Geoff Davies, Helen Lodder, Steve Jordan, Claire Desbrow and Paul Roberts.
Biotage GB Limited, Distribution Way, Dyffryn Business Park, Ystrad Mynach, Cardiff, CF82 7TS, UK

Introduction

Dilute and shoot (D/S) is the most common form of sample preparation for the high throughput analysis of drugs of abuse (DoA) in urine. Although superior in extract cleanliness, solid phase extraction is sometimes deemed too complex, expensive and time consuming especially when matrix hydrolysis is required. This poster aims to demonstrate a simplified sample preparation workflow utilizing on-plate urine hydrolysis, organic solvent precipitation followed by flow through filtration and matrix component scavenging. Although more complex and expensive than dilute and shoot approaches, the up-front cost is offset by superior extract cleanliness, robustness, LC column longevity ultimately leading to increased LC/MS lifespan between cleaning cycles.

Experimental

Reagents

Standards, ß-glucuronidase, ammonium acetate and formate and formic acid were purchased from Sigma-Aldrich Company ltd. (Gillingham, UK). LC/MS grade solvents were from Honeywell Research Chemicals (Bucharest, Romania). Water (18.2 MΩ.cm) was drawn fresh daily from a Direct-Q 5 water purifier (Merck Millipore, Watford, UK). Urine was donated by healthy human volunteers.

Sample Preparation

Blank human urine was spiked with a drugs of abuse standard and corresponding internal standards.

On-plate Urine Hydrolysis: 100-150 µL of urine was applied to the ISOLUTE® HYDRO DME+ (dual mode extraction) plate, mixed 1:1 with buffered enzyme mixture (95:5 100 mM ammonium acetate buffer at pH 5 and ß-glucuronidase) and incubated at 60 °C for 2 hours.

Post hydrolysis: samples were allowed to cooled, mixed with acetonitrile (ACN) and flowed through the plate using positive pressure at 5 psi for 2 minutes.

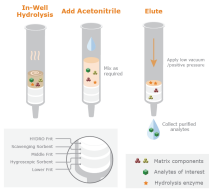

Figure 1. *Schematic of the ISOLUTE® HYDRO DME+ plate process.*

Post extraction: Extracts were either directly injected, diluted prior to injection or evaporated and reconstituted in mobile phase.

LC/MS Conditions

Instrument: Waters Acquity UPLC interfaced via electrospray ionization to a Quattro Premier XE triple quadrupole mass spectrometer (Waters Assoc., Manchester, UK). Positive ions were acquired in the multiple reaction monitoring (MRM) mode.
Desolvation Temp: 450 °C **Ion Source Temp:** 150 °C
LC gradient and MRM transitions: Details on Biotage.com

Creatinine & Urea Analysis

Column: Thermo Scientific BetaMax Acid 5 µm (100 x 2.1 mm) with C8 guard cartridge.
Mobile Phase: A: 10 mM Ammonium Acetate pH4 (aq)
Mobile Phase B: Acetonitrile (ACN).

DoA Analysis

Column: Restek Raptor™ Biphenyl 2.7 µm (100 x 2.1 mm) with EXP guard cartridge (Thames Restek UK Ltd., Saunderton, UK.)
Mobile Phase: A: 2 mM Ammonium Formate (aq) 0.1% formic acid
Mobile Phase B: 2 mM Ammonium Formate (MeOH), 0.1% formic acid

Results

Matrix Component Removal

Urinary drugs of abuse testing are dominated by dilute and shoot approaches due to time and cost. However, cost saving upfront can lead to more expensive downstream issues. Interfering matrix components: creatinine, urea, salts, pigment, and proteins for enzyme-hydrolyzed urine are simply diluted but not removed. Poor extract cleanliness will reduce expensive LC column lifetime and increase the requirement for LC/MS maintenance.

Pigment removal from hydrolyzed urine pre and post extraction using the dual mode extraction plate is demonstrated in ***Figure 2***.

Figure 2. *Hydrolyzed urine: pre-extraction (left); and post-extraction using ISOLUTE® HYDRO DME+ plate (right).*

Investigation of salt residue was performed by evaporation of urine extracts in culture tubes. ***Figure 3.*** illustrates the comparison of salt residue returned using D/S or dual mode extraction.

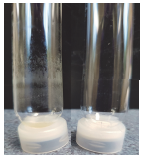

Figure 3. *Urinary salt residues: D&S evaporation (left) and ISOLUTE® HYDRO DME+ plate evaporation (right).*

Creatinine and urea found in significant concentrations in urine can exhibit suppression effects in LC/MS. To demonstrate the removal of these matrix components, MRM acquisition was performed with and without sample extraction. ***Figure 4.*** charts the extent of removal of these matrix components.

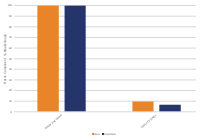

Figure 4. *Creatinine and Urea content chart for 1:9 dilute and shoot and ISOLUTE® HYDRO DME+ plate.*

Enzymatic hydrolysis using beta-glucuronidase adds considerable complexity to the sample. Investigation of protein content was performed using gel electrophoresis experiments. ***Figure 5.*** demonstrates protein content from urine during various stages of processing. Full protein removal was achieved that are otherwise not removed using dilute and shoot.

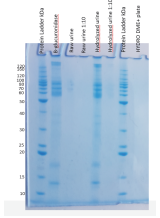

Figure 5. *Gel electrophoresis profile demonstrating protein content in various matrices and extracts.*

Analyte Recovery

Previous experiments indicated optimum urine:ACN ratio of 1:6 (data not shown). ***Figure 6.*** demonstrates maximum load volume investigation from 100-250 µL. Increasing load volumes subsequently increased recoveries. However, extract cleanliness can suffer. Optimum cleanliness was observed using 100-150 µL loads.

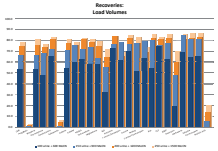

Figure 6. *Hydrolyzed urine recovery chart investigating load volumes.*

Subsequent work focused on increasing the recoveries of "problem analytes"; ritalinic acid, pregabalin and gabapentin. Acidification of the urine prior to extraction allowed increased recoveries for all compounds as demonstrated in ***Figure 7.***

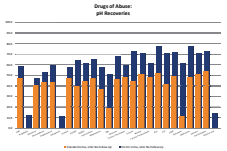

Figure 7. Hydrolyzed urine recovery chart investigating pH modified urine.

Further optimization involved the use of a subsequent solvent aliquot. ***Figure 8.*** demonstrates increased recoveries up to 30% when incorporating 100 µL of ACN as a second aliquot.

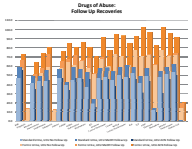

Figure 8. *Hydrolyzed urine recovery chart investigating additional solvent aliquot.*

Comparison of direct injection against standard processing involving evaporation and reconstitution of the extracts was demonstrated in ***Figure 9.*** Providing LOQs and chromatography allow direct injection, significant workflow advantages are attained.

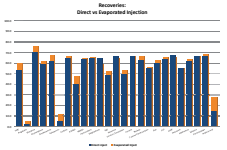

Figure 9. *Hydrolyzed urine recovery chart comparing direct injection.*

Calibration curves were constructed in blank human urine from 20-800 ng/mL. ***Figure 10.*** Demonstrates example calibration curves obtained using evaporated injections of extracted 100:600 urine:ACN while ***Table 1.*** summarizes the r2 and LOQs for ISOLUTE® HYDRO DME+ using 100:600 urine:ACN.

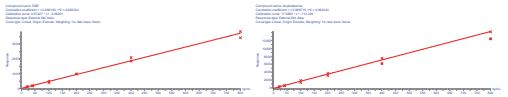

Figure 10*. demonstrates calibration curves for EME and amphetamine.*

Table 1*. Summary of method performance.*

Analyte	LLOQ ng/mL Direct	LLOQ ng/mL Evaporate	Analyte	LLOQ ng/mL Direct	LLOQ ng/mL Evaporate
EME	1.2	0.11	7-amino Clonazepam	0.81	0.24
Pregabalin	20.83	10.00	Cocaine	0.35	0.01
Morphine	12.5	0.36	Norbuprenorphine	2.78	0.24
Oxymorphone	12.5	0.36	7-amino Flunitrazepam	0.36	0.11
Amphetamine	0.8	0.05	Buprenorphine	10.00	1.85
Gabapentin	10.42	0.36	PCP	1.85	0.05
Codeine	2.78	0.24	EDDP	0.24	0.01
6-MAM	2.78	0.16	Oxazepam	6.1	4.17
MDMA	0.50	0.03	Methadone	0.36	0.01
Hydrocodone	1.22	0.07	Zaleplon	0.36	0.05
Mephedrone	31.25	4.17	Flunitrazepam	0.36	0.03
BZE	0.5	0.02	Ritalinic Acid	1.85	0.81
Ketamine	0.5	0.03			

Figure 9. provides rough assay cost breakdown comparing D/S, dual mode extraction and SPE. Improved sample preparation as expected increase upfront assay costs. However, instrument issues are not considered: For example, source cleaning, instrument downtime and impact on throughput needs; UPLC column replacement. These issues can offset sample preparation costs.

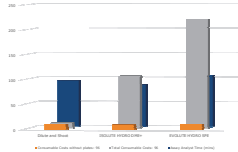

Figure 9. *Sample preparation costs.*

Conclusion

- This poster describes the use of a novel flow-through matrix scavenging plate for drugs of abuse extraction from urine.
- The removal of urinary matrix components and the associated benefits to quantitation are highlighted along with workflow advantages.

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