Automating Low-Volume 96-well SPE Assays for Forensic and Clinical Toxicology Prior to UHPLC-MS/MS Analysis

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Introduction

Early phase small animal drug trials have largely been the driver for miniaturization due to limited sample size. However, this trend is gaining popularity in forensic/clinical toxicology with respect to alleviating patient discomfort, particularly in paediatrics. This combination along with increased LC-MS/MS sensitivity has been invaluable in reigniting focus within the low volume sample preparation area. This poster will present an automated approach for low volume solid phase extraction (SPE) and its application within the clinical and forensic toxicology arenas.

Experimental

Reagents

Drug standards were purchased from LGC Standards (Teddington, UK). Steroids, catecholamine standards, ammonium fluoride, ammonium formate, ammonium acetate and formic acid were purchased from Sigma-Aldrich Company ltd. (Gillingham, UK). All solvents were HPLC grade from Rathburn Chemical Ltd (Scotland, UK) and Milli-Q (Merck Millipore, Germany) water used throughout. Urine samples were kindly donated by healthy human volunteers. Plasma was purchased from Welsh Blood Service (Pontyclun, UK). Serum was purchased from Golden West Biologicals, Inc. (Temecula CA).

Sample Preparation

Urine Drugs of Abuse:

Urine was spiked with standards/ISTDs. Hydrolysis was performed 1:1 with buffered enzyme 95/5 100 mM ammonium acetate pH 5 and 6-glucuronidase at 60 °C for 2 hrs.

Extraction: Biotage® Mikro CX Plate, 2 mg (601-0002-LVP)

Plasma catecholamines:

Plasma (100 µL) was spiked with standards/ISTDs and pre-treated 1:1 with 10 mM sodium citrate buffer at pH 7 (aq).

Extraction: Biotage® Mikro WCX Plate, 2 mg (602-0002-LVP)

Serum Steroid Hormones:

Serum (200 µL) was spiked with standards/ISTDs and pre-treated with 50 µL of MeOH and 150 µL 1% formic acid (aq).

Extraction: Biotage® Mikro ABN Plate, 2 mg (600-0002-LVP)

Table 1. Optimized Extraction Protocols.

Conditions	Steroids	DoA	Cats/Mets
Condition **100 µL**		MeOH	
Equilibrate **100 µL**	0.1% formic acid aq	4% phosphoric acid aq	10 mM ammonium acetate pH 6.0 aq
Load	**400 µL** Pre-treated serum	**400 µL** Pre-treated urine	**200 µL** Pre-treated plasma
Wash 1 **100 µL**	0.1% NH_4OH aq	4% phosphoric acid aq	10 mM ammonium acetate pH 6.0 aq
Wash 2 **100 µL**	0.1% NH_4OH 60:40 H_2O:MeOH	50:50 H_2O:MeOH	20/80 H_2O:MeOH
Wash 3 **100 µL**	/	/	DCM
Elution	**30 µL** MeOH or EtOAc	**30 µL** DCM:MeOH:NH_4OH 78:20:2	**100 µL** 0.1% formic acid 85:15 H_2O:IPA
*Recon	0.05% acetic acid 50:50 H_2O:MeOH	0.1% formic acid 90:10 H_2O:MeOH	0.1% formic acid 95/5 H_2O:MeOH

* Evaporation and reconstitution required for maximum sensitivity and/or when elution solvent not compatible with LC/MS mobile phase.

Biotage® Extrahera™ LV-200 Automated Sample Preparation Platform

Optimized extraction protocols were transferred to a dedicated low volume sample preparation platform, equipped with an 8 channel pipetting head capable of accepting 50, 200 or 1000 µL tips and positive pressure processing functionality. The Extrahera™ LV-200 platform is shown in ***Figure 1***.

Figure 1. *Biotage® Extrahera™ LV-200 automated sample preparation platform.*

LC/MS Conditions

Steroids & DoA

Instrument: Shimadzu Nexera X2 UHPLC and 8060 Triple Quad MS (Shimadzu Europa GmbH, Duisburg, Germany)

Steroid:

Column: ACE C18 1.7 µm (100 x 2.1 mm)

Mobile phase: A, 0.2 mM NH_4F (aq); B, MeOH **Flow rate:** 0.4 mL/min

Column temp: 40 °C **Injection volume:** 5 µL

DoA:

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

Mobile phase: 2 mM ammonium formate; 0.1% formic acid in (aq) and MeOH **Flow rate:** 0.4 mL/min

Column temp: 30 °C **Injection volume:** 5 µL

Catecholamines

Instrument: Shimadzu Nexera UHPLC (Shimadzu Europa GmbH, Duisburg, Germany) 5500 Triple Quad MS AB Sciex (Framingham, USA)

Column: ACE Excel C18-PFP 1.7 µm (100 x 3 mm)

Mobile phase: A, 2 mM ammonium formate 0.05% formic acid (aq); B, 0.5 mM NH_4F in MeOH **Flow rate:** 0.5 mL/min

Column temp: 30 °C **Injection volume:** 10 µL

For details on MS conditions and MRM transitions for each analyte panel visit Biotage.com.

Results

Previously developed SPE protocols for each analyte panel were converted from 10/30 mg 96-well plate to the corresponding chemistry using the Biotage® Mikro 2 mg (low volume plate) format. In all cases wash volumes were optimised to 100 µL.

Drugs of Abuse Analysis

Conversion of EVOLUTE® EXPRESS CX 10 mg to the 2 mg Mikro CX format using equivalent load volumes demonstrated subtly reduced analyte recoveries for most analytes (data not shown). Processing optimization was performed for manual positive pressure, PRESSURE+ 96 (6-8 psi), and the Extrahera™ LV-200 automated platform (1 bar). Excellent correlation in terms of analyte recoveries, matrix factors, and RSDs are demonstrated in ***Figures 2-4*** respectively.

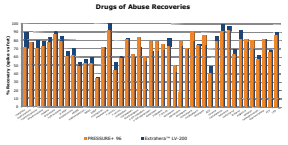


Figure 2. DoA *recovery profiles comparing manual vs automated processing using Mikro CX plates.*

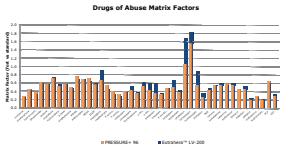


Figure 3. DoA *matrix factor profiles comparing manual vs automated processing using Mikro CX plates.*

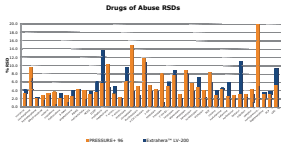


Figure 4. DoA *RSD profiles comparing manual vs automated processing using Mikro CX plates.*

The method was previously optimized to allow capture of amphoteric analytes such as gabapentin and pregabalin and other weakly basic analytes that do not retain using an ionic interaction. Organic wash solvents were therefore weaker than optimum and resulted in slightly lower matrix factors.

Catecholamine Analysis

Due to the polarity of catecholamines and low bed mass, processing pressures were reduced for maximum phase interaction (0.6 bar on the LV-200 system). Increased matrix loading volumes demonstrated breakthrough of NE so were limited to 200 µL (data not shown). ***Figures 5-7*** demonstrate analyte recoveries, matrix factors, and RSDs respectively.

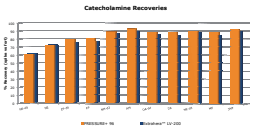


Figure 5. Catecholamine *recovery profiles comparing manual vs automated processing using Mikro WCX plates.*

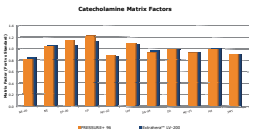


Figure 6. Catecholamine *matrix factor profiles comparing manual vs automated processing using Mikro WCX plates.*

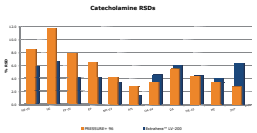
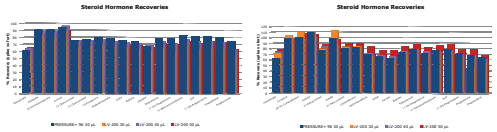


Figure 7. Catecholamine *RSD profiles comparing manual vs automated processing using Mikro WCX plates.*

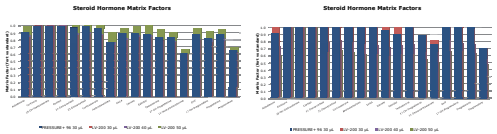
Optimized cleanliness required high % aq in the elution solvent which resulted in elevated elution volumes of 100 µL for this assay.

Steroid Hormone Analysis

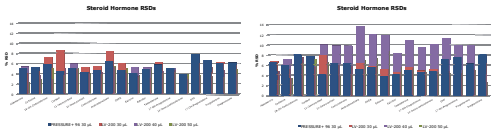
Subtly reduced recoveries were returned converting the 10 mg SPE methodology to the Mikro ABN format using equivalent sample loading. Two protocols for elution were evaluated; EtOAc is preferred when DHEAS is not in the target panel or MeOH when DHEAS is present. The latter affords an option of direct injection depending on sensitivity requirements. Respective recoveries, RSD, and matrix factor performance are demonstrated in ***Figures 8-13.***



Figures 8-9. Steroid *recovery profiles comparing manual vs automated processing using Mikro ABN plates: EtOAc LHS; MeOH RHS.*



Figures 10-11. Steroid *matrix factor profiles comparing manual vs automated processing using Mikro ABN plates: EtOAc LHS; MeOH RHS.*



Figures 12-13. Steroid *RSD profiles comparing manual vs automated processing using Mikro ABN plates: EtOAc LHS; MeOH RHS.*

Calibration curves constructed over the relevant ranges for each panel presented excellent linearity, coefficients of determination (r^2), and LOQ as demonstrated in ***Table 2.***

Table 2. Optimized Extraction Protocols.

Analyte Class	*r^2*	*Estimated LOQ*
Amphetamines & Ketamines	> 0.999	1-50 pg/mL
Benzodiazepines & Z Drugs	> 0.999	<5-500 pg/mL
Cocaines	> 0.999	5-25 pg/mL
Opioids	> 0.999	1-100 pg/mL
PCP & LSD	> 0.999	5-500 pg/mL
Steroid Hormones	> 0.999	5-500 pg/mL
Catecholamines	> 0.999	4-24 pg/mL
Metanephrines	> 0.999	< 6 pg/mL

Conclusion

- This poster demonstrates the combination of a dedicated low-volume automated sample preparation platform with miniaturized SPE formats.
- The Mikro SPE format allows sample extraction with elution volumes of 30 µL or below in a single aliquot.

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