“Prep-and-Shoot”: Complete automation of the hydrolysis and analysis of forensic urine samples by LC-MS/MS.

AB SCIEX QTRAP® 4500 LC/MS/MS System and Gerstel, Inc. MultiPurpose Sampler

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Overview

Here we describe, a completely automated, 96 well plate format “Prep-and-Shoot” workflow including enzymatic hydrolysis, dilution and injection. A GERSTEL MultiPurpose Sampler (MPS) coupled to an AB SCIEX QTRAP® 4500 LC/MS/MS system was used for a fast enzymatic hydrolysis process (15 minutes), dilution and injection of urine samples. The procedure was applied to the analysis of multiple drug classes (e.g., opiates, opioids, benzodiazepines, muscle relaxants, hallucinogens) in urine.

This automated workflow employed an ultra-pure β-Glucuronidase enzyme yielding hydrolysis efficiencies of glucuronide conjugates above 80% for the analytes tested. The methodology developed allowed the reproducible injection and analysis of over 960 samples on the same analytical column, with %RSDs ≤ 10%. Moreover, the combined automation of urine hydrolysis, injection and analysis allowed the system to process more than 200 samples in a day.

Introduction

The clearance of drugs, toxins, environmental contaminants and other waste products from the body often involves processing in the liver to form glucuronide conjugates which are more readily solubilized and excreted by the kidneys. Any studies monitoring the processing of these metabolites must either measure both free and conjugated forms of the analytes or the conjugates must be hydrolyzed to allow determination of total excreted analytes in the urine.

LC/MS/MS has been most commonly employed to quantify total analyte (such as drugs) present in urine samples due to the high sensitivity, selectivity, robustness, and low detection limits (e.g., 1 ng/mL) the technology provides. These assays typically involve forensic workflows that consist of lengthy sample handling steps such as hydrolysis, centrifugation, sample cleanup and concentration prior to analysis. Automating all of these steps would be beneficial for various reasons; better reproducibility, higher sample processing throughput, lower cost per samples and more efficient forensic results reporting.

Figure 1. GERSTEL MPS 2XL multi-purpose sampler configured with the incubator option and an AB SCIEX QTRAP® 4500 LC/MS/MS system for automated drug screenings.

Here we describe a completely automated “Prep-and-Shoot” workflow in a 96 well plate format for the forensic analysis of multiple drug classes (e.g., opiates, opioids, benzodiazepines, muscle relaxants, hallucinogens) in urine samples. A GERSTEL MPS autosampler coupled to an AB SCIEX QTRAP® 4500 LC/MS/MS system was used for a fast enzymatic hydrolysis process (15 minutes), dilution, and injection of urine samples. Over 40 drugs and their metabolites were monitored using the *Scheduled* MRM™ Pro algorithm programmed in the LC/MS/MS acquisition method. This technology combined with the automated hydrolysis and injection makes it possible to produce accurate and reproducible quantitation for multiple classes of analytes within a very short period of time. This automation strategy can also be adapted to other analyte-glucuronide analysis needs of low concentrations of the designer drugs.

Experimental

Materials

More than 45 neat reference standards (including 8 glucuronide conjugates) and a selected panel of deuterated analogues

solutions of different drug classes were purchased from Cerilliant (Round Rock,TX). Stock solutions were prepared by combining all the drugs with methanol, at appropriate concentrations to evaluate performance of the automated hydrolysis workflow. A detailed list of the drugs used for this study is available upon request.

β-Glucuronidase and hydrolysis buffer solutions were obtained from Integrated Micro-Chromatography Systems (Columbia, SC). Blank urine was obtained from a male volunteer and incurred samples with a known list of analytes present were donated by a local analytical toxicology laboratory. All other reagents and solvents used were reagent grade.

Instrumentation

The automated urine "Prep-and-Shoot" method was developed and tested using a GERSTEL MPS equipped with a well plate incubating station as shown in Figure 1. All analyses were performed using an Agilent 1260 HPLC coupled to an AB SCIEX QTRAP® 4500 LC/MS/MS System. Sample injections were made using a 6 port (0.25mm) Cheminert C2V injection valve outfitted with a 2 µL stainless steel sample loop.

Automated urine hydrolysis, dilution and injection

Prior to analysis, all urine samples were centrifuged to remove any particulates or insoluble proteins present in the samples. Three (3) empty, sealed well plates and the hydrolysis enzyme/buffer/internal standard mix were loaded onto the MPS in appropriate trays.

All steps of the automated "Prep-and-Shoot" workflow performed by the MPS are detailed in the flowchart shown in Figure 2.

LC/MS/MS Method Parameters

The analytical column used in this work was a Phenomenex Kinetex Biphenyl, (50 x 4.6 mm, 2.6 µm, 100 Å), plumbed to a Biphenyl Security Guard ULTRA Holder (4.6 mm ID).

Mobile Phase: A – 5 mM ammonium formate in H_2O (0.1% Formic Acid)
B – Methanol (0.1% Formic Acid)

LC Gradient:

Time (min)	Flow (mL/min)	% B
0.00	0.7	10
2.50	0.7	100
3.50	0.7	100
3.51	0.7	10
5.00	0.7	10

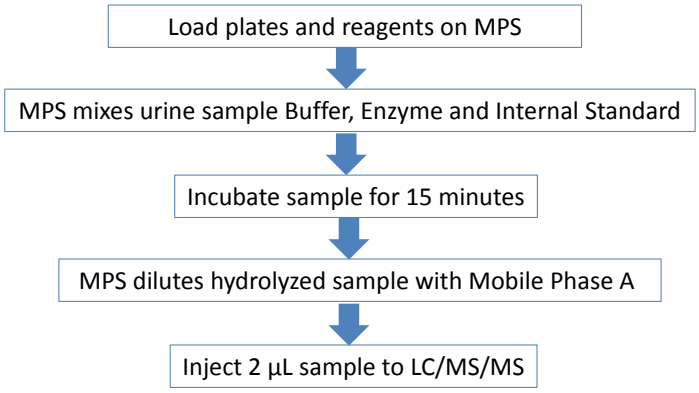

Figure 2. Automated "Prep-and-shoot" Urine Hydrolysis Workflow

Run time: 5.00
Injection volume: 2 µL (loop over-fill technique)
Column Temperature: 55 °C

Diverter Valve program:

Time (min)	Position
0.00	Waste
1	MS
3.5	Waste

Mass Spectrometer Parameters

Operation: ESI+ (THC-COOH was analyzed in ESI-)
Temperature: 650°C
Ion Source Gas 1: 60
Ion Source Gas 2: 50
IonSpray Voltage: 5500 V (-4000 V ESI-)
Curtain Gas: 30
CAD: Medium

The AB SCIEX QTRAP® 4500 LC/MS/MS System was operated with Turbo V™ source and Electrospray Ionization (ESI) probe. 105 MRM transitions were monitored in both positive and negative polarity. The *Scheduled* MRM™ Pro algorithm was used in combination with fast polarity switching using Analyst® 1.6.2 Software and MultiQuant™ 3.0 Software was used for quantitative data processing.

Results and Discussion

Automated enzymatic hydrolysis performance

The efficiency of the automated enzymatic hydrolysis was examined by spiking blank urine with 8 different glucuronide conjugates (Table 1).

Table 1. Glucuronide Conjugates tested with the automated "Prep-and-Shoot" workflow

Morphine-3-glucuronide
Oxymorphone-glucuronide
Codeine-6-glucuronide
Tapentadol-glucuronide
Buprenorphine-glucuronide
Oxazepam-glucuronide
Lorazepam-glucuronide
THC-COOH-glucuronide

The use of the β-Glucuronidase enzyme in combination with the optimized rapid buffering solution ensured completion of the hydrolysis procedure within 15 minutes. Representative MRM chromatograms of a spiked urine sample after automated hydrolysis is shown in Figure 3.

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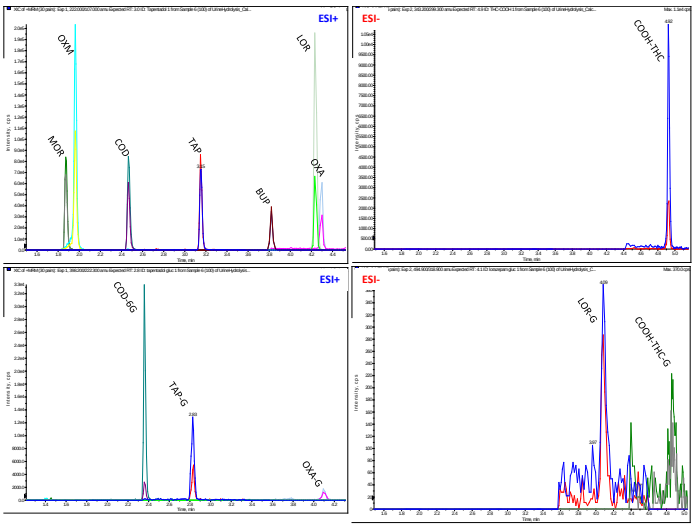

Figure 3. Overlay chromatograms of a spiked urine sample after automated hydrolysis. Top Chromatograms: MRM Transitions of deconjugated analyte forms. Bottom Chromatograms: MRM Transitions for glucuronide conjugates

Over 95% hydrolysis was achieved for most compounds with the exception of Codeine-6-Glucuronide (80.5% hydrolyzed) which required a longer incubation time for complete deconjugation. (Table 2)

Table 2. Hydrolysis efficiency of the glucuronide conjugated analytes tested

Analyte	% Hydrolysed
Morphine-3G	99.7%
Oxymorphone-G	99.8%
Codeine-6G	80.5%
Tapentadol-G	97.9%
Buprenorphine-G	99.6%
Oxazepam-G	95.8%
Lorazepam-G	99.3%
THC-COOH-G	94.4%

Analytical Column robustness

The robustness of the analytical column was tested by injecting approximately 960 hydrolyzed and diluted urine samples (10 x 96 well microtitre plates) and by measuring the column back pressure (psi) as a function of the number of injections made on the column (Figure 4). The Phenomenex Security guard column was replaced after the analysis of every 2 plates, resulting in an average backpressure of 1478.3 psi with a %RSD of 4.3% indicating no adverse pressure buildup due to fouling on the analytical column.

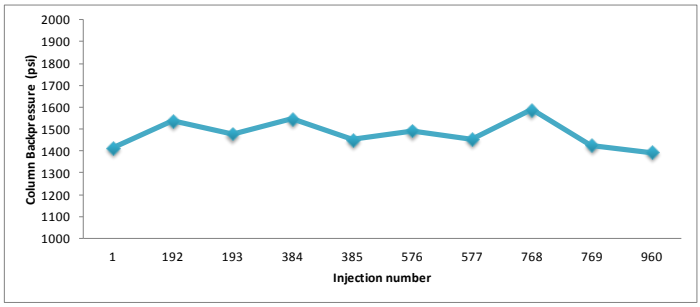

Figure 4. Analytical column backpressure plot after 960 injections.

Figure 5 shows the resulting MRM chromatograms of Sample 1 and Sample 960 injected using the same analytical column as well as the recorded backpressure logs from each sample's injection. No evidence of column performance deterioration is seen after 960 injections.

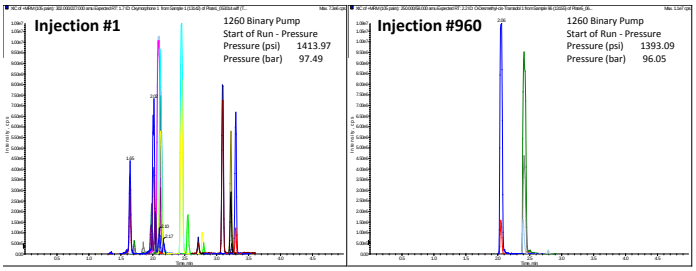

Figure 5. Chromatograms of hydrolyzed samples showing column backpressure logs

A qualitative assessment of the mass spectrometer inlet was performed before and after the robustness testing. Figure 6 shows the physical state of the mass spectrometer curtain plate after injection number 960. The simple source architecture and orthogonal spray design of the AB SCIEX Turbo V™ ion source provides outstanding robustness and sensitivity for complex biological matrices. The AB SCIEX Turbo V™ ion source, therefore, in combination with the ultra-clean hydrolysis enzyme, allows for this simple, high throughput "Prep-and-shoot" approach. The key reason for this is the strategic application of heated gas to the spray region to aid in the desolvation of analytes. By merging two orthogonal streams of hot gas in the ESI region, efficient de-solvation and hydrodynamic focusing of ions towards the orifice is achieved. Uniform temperature distribution and optimized curtain gas flow allow for this robustness and ruggedness.

Figure 7 shows a comparison of an incurred urine sample treated with three (3) different hydrolysis enzymes. The high purity of the β-Glucuronidase (95% enzyme in solution) allowed the sample to be diluted and directly injected, avoiding a final centrifugation step as commonly required with workflows that employ other enzymes from different sources (e.g., abalone, helix-pomatia).

The small injection volume and the use of the diverter valve were also key elements in maximizing the lifetime of the analytical column and keeping the mass spectrometer inlet clean. The robust, automated hydrolysis strategy enables high throughput for both the enzymatic hydrolysis of the urine samples, as well as sample analysis of the compounds tested.

Figure 6. 4500 QTRAP LC/MS/MS system's curtain plate after injection #960

Other compounds may require the addition of a sample cleanup or enrichment technique (e.g., dSPE, SPE, SLE) which can be combined with this method.

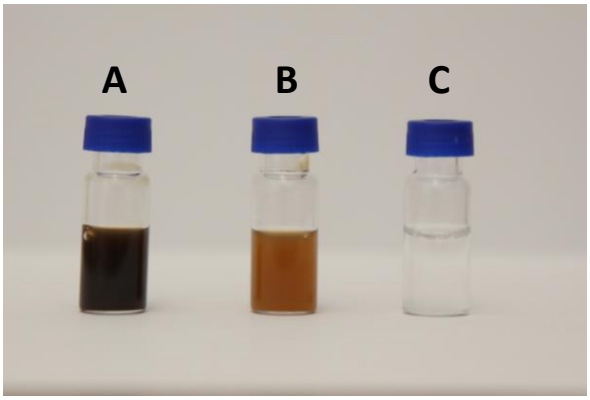

Figure 7. Commercially available enzyme solutions at 100 kU/mL A) Snail extract, B) Abalone extract, C) IMCSyme™

Incurred urine samples reproducibility testing

Ten (10) incurred urine samples with semi quantitative results were automatically hydrolyzed and injected into the LC/MS/MS system. Each sample was hydrolyzed, diluted and injected a total of 96 times. Figure 8 shows extracted ion chromatograms of samples ID=13188 and ID=13230. In each chromatogram the glucuronide conjugate and parent drug ions monitored were extracted to show the completion of the 15 minute enzymatic hydrolysis process.

Tables 3 and 4 list average concentrations obtained from urine samples (ID=13188 and ID=13230) after automated hydrolysis with %RSDs all less than 10.4%.

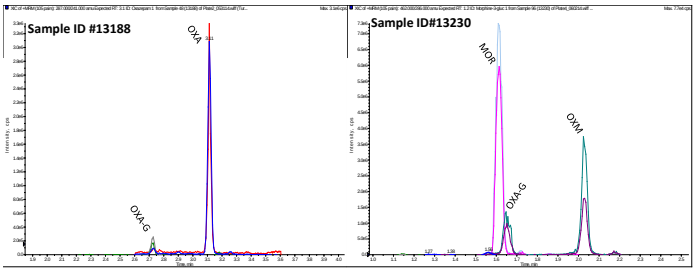

Figure 8. Chromatograms of incurred urine samples after automated hydrolysis

Table 3. Analyte concentrations obtained for Sample ID 13188 after automated hydrolysis

Analyte (Sample ID 13188)	Automated Hydrolysis Avg. Concentration (ng/mL) n==96	%RSD
Norfentanyl	857.9	*9.0*
Fentanyl	*412.6*	*6.8*
Oxazepam	*2444.1*	*8.0*
Temazepam	*1843.2*	*9.8*
Nordiazepam	*559.1*	*10.4*

Table 4. Analyte concentrations obtained for Sample ID 13230 after automated hydrolysis

Analyte (Sample ID 13230)	Automated Hydrolysis Avg. Concentration (ng/mL) n=96	%RSD
Morphine	39050.1	*6.3*
Oxymorphone	*7469.4*	*6.7*
Hydromorphone	*5316.5*	*5.6*
Oxycodone	*6603.0*	*7.1*

Workflow Throughput

The combined automation of urine hydrolysis, injection and analysis allowed the system to process more than 200 samples in a 24 hr span. A graphical representation of the automatically staggered “Prep-and-Shoot” workflow is displayed in Figure 9.

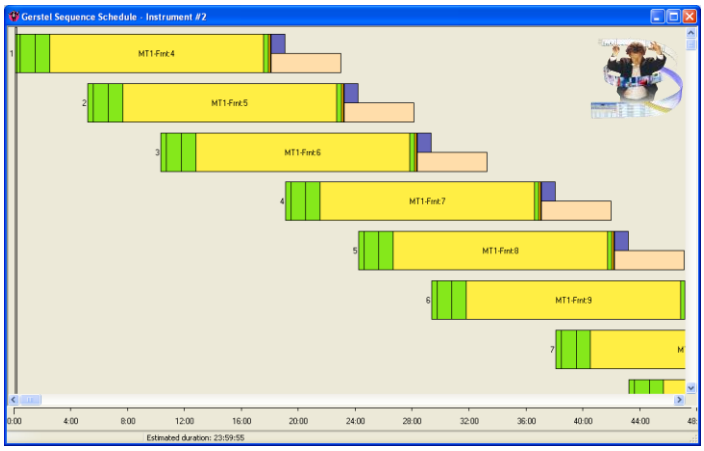

Figure 9. Graphical representations of the automated urine hydrolysis workflow using GERSTEL MAESTRO software.

Summary

As a result of this study, we were able to show:

- A fast and simple “Prep-and-Shoot” workflow was designed using the GERSTEL MPS robotic sampler coupled to an AB SCIEX QTRAP® 4500 LC/MS/MS System for the automated hydrolysis and forensic analysis of urine samples within a single run.
- The buffer, internal standard and β-Glucuronidase combination was optimized to perform the automated urine hydrolysis, yielding hydrolysis efficiencies above 80% with a 15 minute incubation time.
- The combination of LC-MS/MS analysis conditions, the ultra purity of the β-Glucuronidase and the design of the Turbo V™ ionization source, allowed the sample to be automatically diluted following hydrolysis and injected reproducibly (%RSDs ≤10%) over 960 urine samples without having to replace the analytical column.
- The combined automation of urine hydrolysis, injection and forensic analysis using the GERSTEL MPS allowed the system to process more than 200 samples in a 24 hour period.

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