Automation of Sample Clean-up for a Comprehensive Forensic Toxicology Hydrolyzed Urine Screening LC-MS/MS Method

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ABSTRACT

- The effectiveness of a novel beta glucuronidase enzyme in allowing a 30 minute urine hydrolysis in urine was investigated.
- The rapid hydrolysis procedure was combined with automated sample clean-up using Disposable Pipette Extraction (DPX) automated tips on a Tecan Freedom EVO® workstation to provide a high throughput urine drug screening method.
- The automated procedure has been used in a comprehensive LC-MS/MS screening and quantification workflow for the analysis of 157 drugs.
- The reproducibility of the automated procedure produced CVs less than 15% over the entire concentration range covered by the assay. The method displayed good linearity for all analytes, with R>0.99. Recoveries for over 60% of the analytes were greater than 80%.

INTRODUCTION

Lab automation around LC-MS/MS analysis is an increasingly popular concept in the modern day research laboratory. In the absence of complete method automation, results are susceptible to human error at many different stages, including sample preparation and data processing.

Benefits of Automation

- Eliminate human error, thus increasing reproducibility
- Eliminate subjectivity during data processing
- Has the potential to save a great deal of time.

The Tecan Freedom EVO® Liquid Handling Workstation

- Effectively used to alleviate the time consuming, error prone and labor intensive nature of manual sample pre-treatment by its ability to automate the entire process.

In this work the Tecan Freedom EVO® 150 liquid handling workstation was used with the Disposable Pipette Extraction (DPX) automated tips for rapid sample preparation of urine samples for comprehensive LC-MS/MS screening. DPX is a novel dispersive solid-phase extraction device that uses sorbent loosely contained in a pipette tip to efficiently mix with sample solutions and are amenable to automation.

Disposable Pipette Extraction (DPX) automated tips

- Rapid extractions and negligible solvent waste generation
- Direct extraction of hydrolyzed urine samples without the need for centrifugation which is costly to automate.

Automatic LC-MS/MS data acquisition, processing, and reporting were performed using the Cliquid® software, which enabled the direct import of batch lists from the Tecan Freedom EVO® Liquid Handling Workstation. Upon completion of data acquisition, the software automatically performed quantitation for the 157 analytes included in the method, and automatically generated and printed out reports.

MATERIALS AND METHODS

- 157 neat standard solutions of different drug classes and selected glucuronide standards were purchased from Cerilliant Corporation (Round Rock, Texas) to investigate the new β-Glucuronidase enzyme (codeine-6-β-D-glucuronide, oxymorphone-3-β-D-glucuronide, tapentadol-β-D-glucuronide, oxazepam glucuronide and morphine-3-β-D-glucuronide)
- A selected panel of deuterated analogues were purchased from Cerilliant Corporation, and used for quantification.
- IMCSzyme (β-Glucuronidase; *E. coli*, cat.#04-E1F-020, 54 KU/Ml) from IMCS was obtained from Hummingbird Research and Applications. Fresh urine was obtained from a male volunteer. All other reagents and solvents used were reagent grade.
- Urine was spiked at 0.5, 1, 5, 10, 50, 100, 500, 1000 and 2000 ng/mL

Combined 10 µL of the working internal standard solution, 20 µL of β-Glucuronidase, 20 µL of 0.2 M phosphate buffer; pH 6.8
↓
Vortex mixed for 30 seconds
↓
Added 100 µL urine, mixed and incubated at 55 ° C for 30 minutes
↓
Aliquots of hydrolyzed urine samples were added into 13 mm test tubes

} Hydrolysis

↓
150 µL of hydrolyzed sample was mixed with 510 µL acetonitrile
↓
DPX automated tip clean-up; Tecan EVO® Liquid Handling Workstation
↓
The final supernatant was further diluted 1:10 with water

} Automated sample clean-up

↓
15 µL injected on to the LC/MS/MS
↓
Cliquid® Software for automation of acquisition, data processing and reporting.

Figure 1. Tecan Freedom EVO® 150 Liquid Handling Workstation and AB SCIEX QTRAP® 4500 LC/MS/MS System with Eksigent ekspert™ ultraLC 100

HPLC Conditions:
An Eksigent ekspert™ ultraLC 100 was employed for LC separation using a Phenomenex Kinetex 2.6 µm C18, 100 Å, 50 x 3.0 mm column held at 40° C; 15 minute gradient of water and methanol with ammonium formate buffer; 0.4 mL/min flow rate; 15 µL injection volume.

MS/MS Conditions:
An AB SCIEX QTRAP® 4500 LC/MS/MS system with Turbo V™ source and Electrospray Ionization (ESI) probe was used for detection of the analytes, and the system was operated using *Scheduled* MRM™ algorithm.

RESULTS

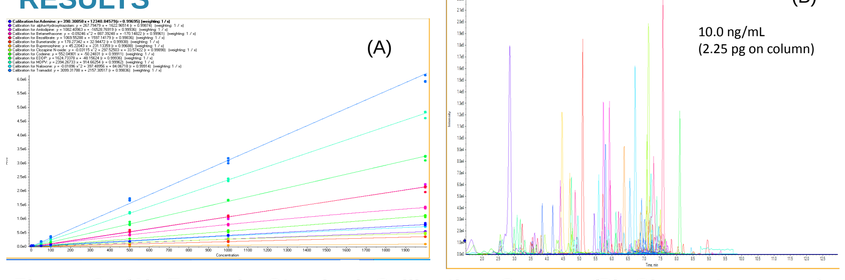

Figure 2. (A) Example Standard Calibration Curves. (B) Chromatogram of hydrolysed 10 ng/mL 157 drug spiked urine calibrator; Overlaid MRM ion traces for the quantifier MRM transitions

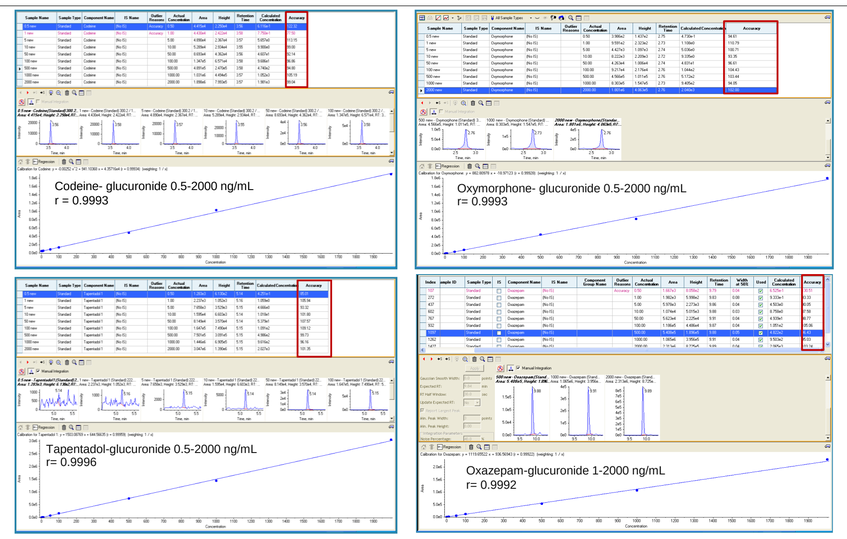

Figure 3. Calibration curves for select glucuronides in urine that had been hydrolyzed in 30 minutes using the IMCSzyme β-Glucuronidase; E. Coli

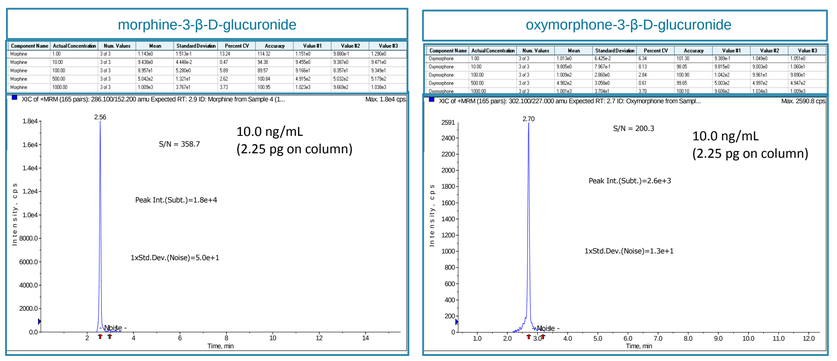

Figure 4. Selected Examples of Reproducibility (n=3) and Signal/Noise for glucuronidated drugs in urine undergone IMCSzyme β-Glucuronidase; E. Coli

Compound	Average recovery over 3 concentrations (%)
codeine-6-β-D-glucuronide	75
oxymorphone-3-β-D-glucuronide,	98
oxazepam glucuronide	88
morphine-3-β-D-glucuronide	98

Table 1. % Recovery of 30 minutes β-glucuronidase hydrolysis for selected glucuronides in urine; Processed 3 blanks; spiked at appropriate concentration to represent 10, 100 and 1000 ng/mL concentrations

CONCLUSIONS

- An LC-MS/MS method for the comprehensive screening of 157 drugs has been developed comprising a 30 minute β-glucuronidase enzyme hydrolysis.
- A Tecan Freedom EVO® 150 Liquid Handling Workstation was used to perform sample clean-up using DPX automated tips.
- Cliquid® Software allowed for the automatic LC-MS/MS data acquisition, processing and reporting of the results.
- The reproducibility of the automated procedure produced CVs less than 15% over the entire concentration range covered by the assay. The method displayed good linearity for all analytes, with R>0.99. Recoveries for over 60% of the analytes were greater than 80%.

TRADEMARKS/LICENSING