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"Prep-and-Shoot": The Completely Automated Workflow for the Hydrolysis and Analysis of Urine Samples by LC/MS/MS.

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Abstract

In this report, a completely automated, 96 well plate format "Prep-and-Shoot" workflow including enzymatic hydrolysis, dilution and injection is described. 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 workflows that consist of lengthy sample handling steps such as hydrolysis, centrifugation, sample cleanup and concentration prior to analysis. Automating all of these would be beneficial for various reasons: better reproducibility, higher sample processing throughput, lower cost per sample and more efficient results reporting.

This report describes a completely automated "Prep-and-Shoot" workflow in a 96 well plate format for the analysis of multiple drug classes (e.g., opiates, opioids, benzodiazepines, muscle relaxants, hallucinogens) in urine samples. 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. Over 40 drugs and their metabolites were monitored using the Scheduled MRM™ 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.

Experimental

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

Figure 1. GERSTEL MultiPurpose Sampler (MPS) configured with an AB SCIEX QTRAP® 4500 LC/MS/MS system.

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).

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.

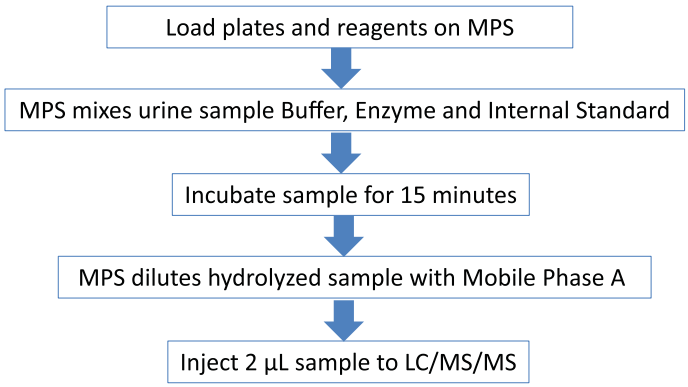

Figure 2. Automated "Prep-and-shoot" urine hydrolysis workflow.

LC/MS/MS Method Parameters

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

Run time: 5.00 min
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 in ESI-)
Temperature:	650°C
Ion Source Gas 1:	60 mL/min
Ion Source Gas 2:	50 mL/min
IonSpray Voltage:	5500 V (-4000 V ESI-)
Curtain Gas:	30 mL/min
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™ algorithm was used in combination with fast polarity switching using Analyst® 1.6.2 Software. 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). 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.

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

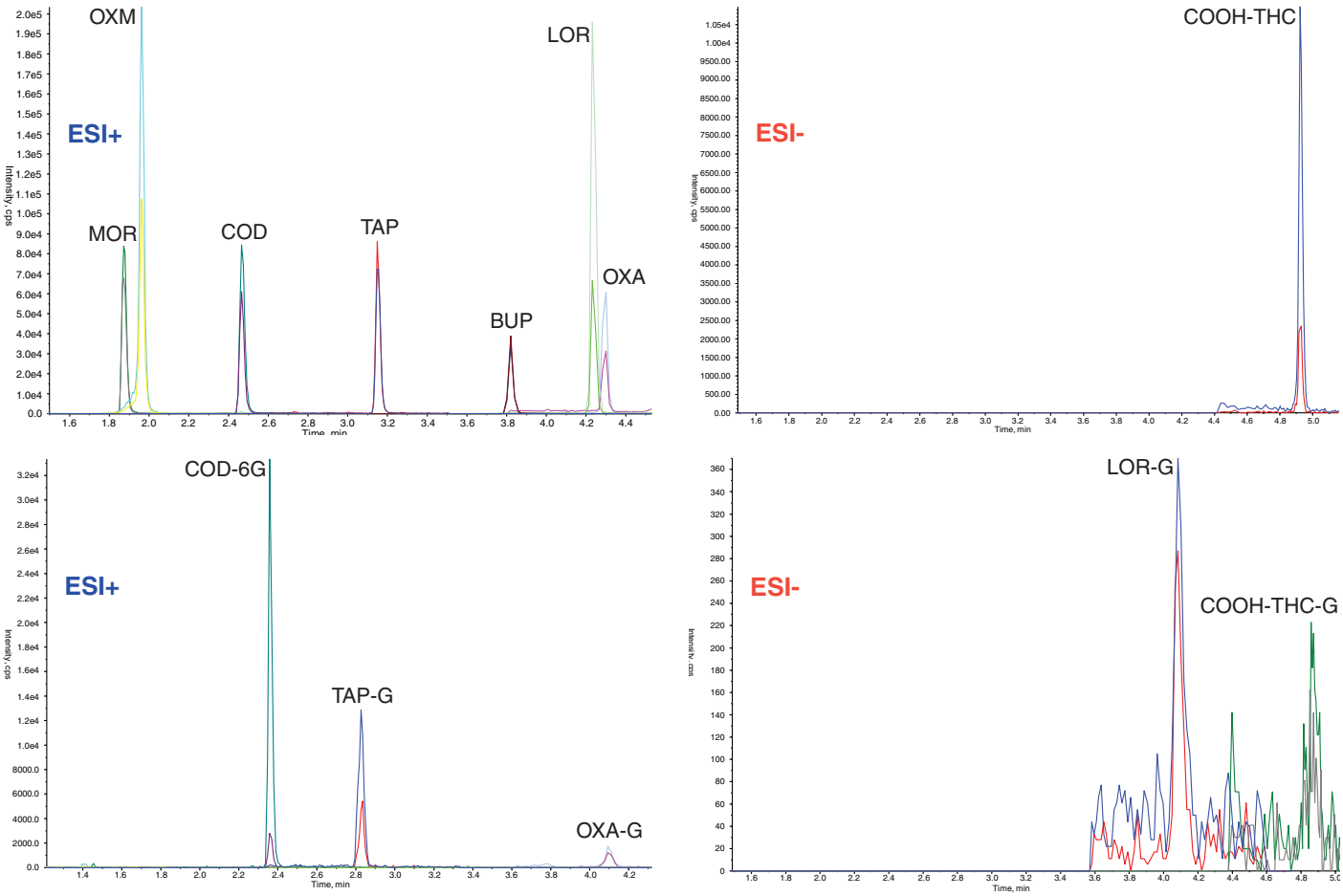

Figure 3. Overlay chromatograms of a spiked urine sample after automated hydrolysis. Top chromatograms: MRM transitions of deconjugated analyte forms. Bottom chromatograms: MRM transitions for glucuronide conjugates.

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	% Hydrolyzed
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.

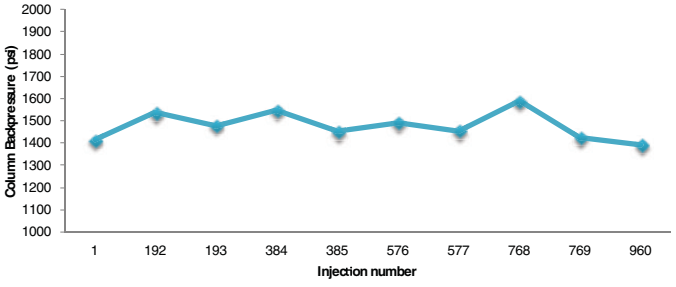

Figure 4. Analytical column backpressure plot after 960 injections.

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.

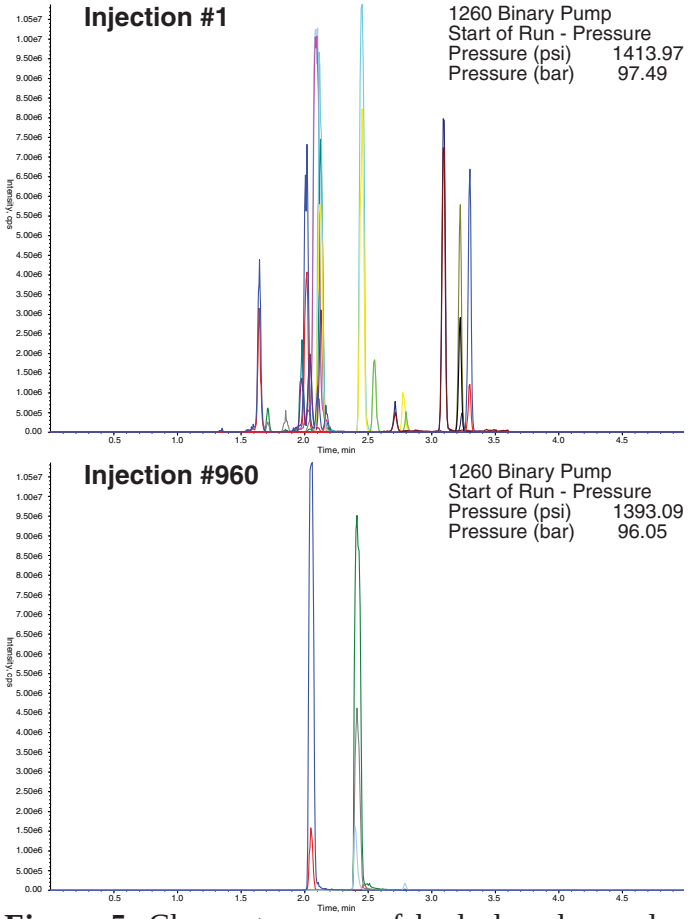

Figure 5. Chromatograms of hydrolyzed samples showing column backpressure logs.

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.

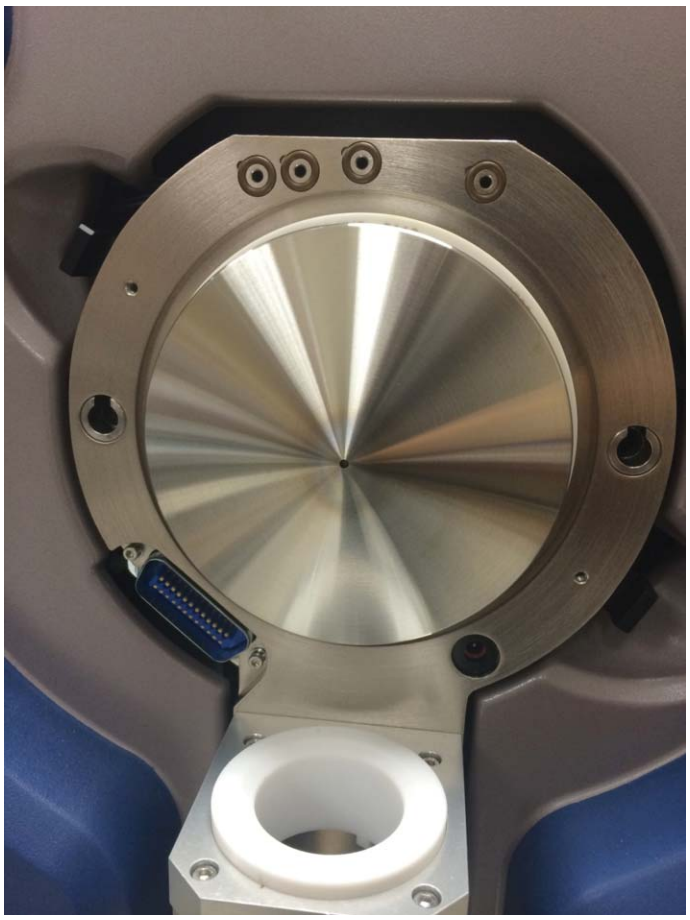

Figure 6. 4500 QTRAP® LC/MS/MS system's curtain plate after injection #960.

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) [1].

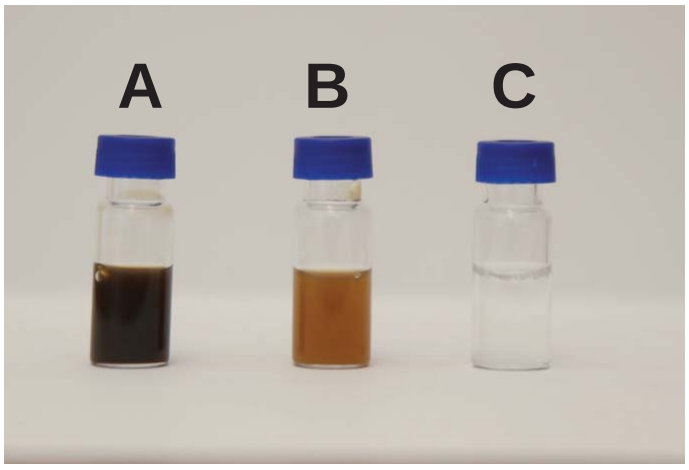

Figure 7. Urine Samples treated with different β-Glucuronidase enzymes. A) Helix-Pomatia, B) Abalone, C) Recombinant Protein .

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 shown enables high throughput for both the enzymatic hydrolysis of the urine samples, as well as sample analysis for the compounds tested. 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.

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.

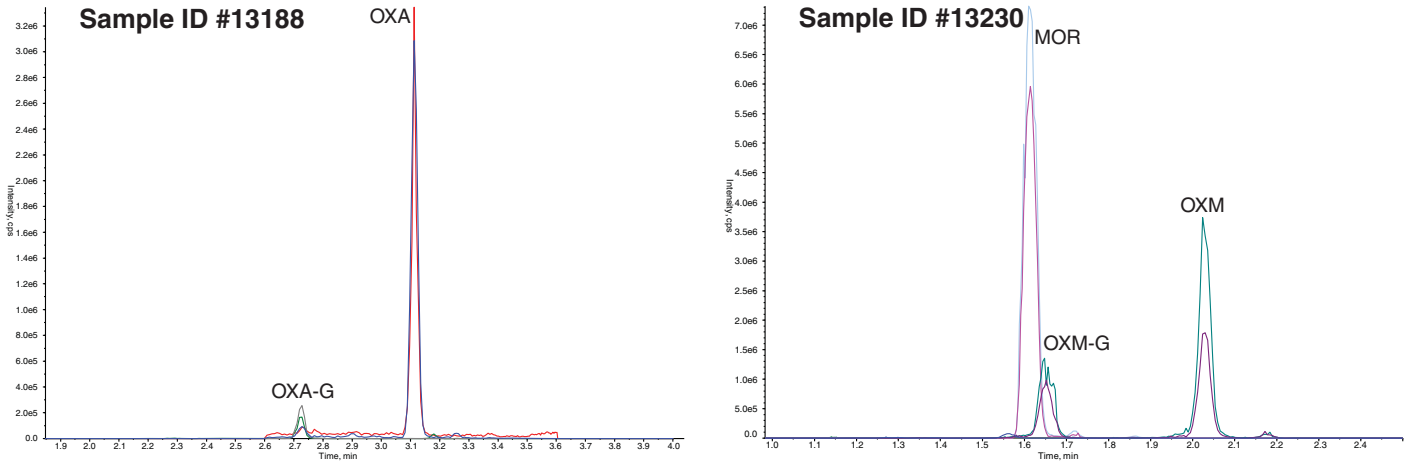

Figure 8. Chromatograms of incurred urine samples after automated hydrolysis.

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

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 h period. A graphical representation of the automatically staggered "Prep-and-Shoot" workflow is displayed in Figure 9.

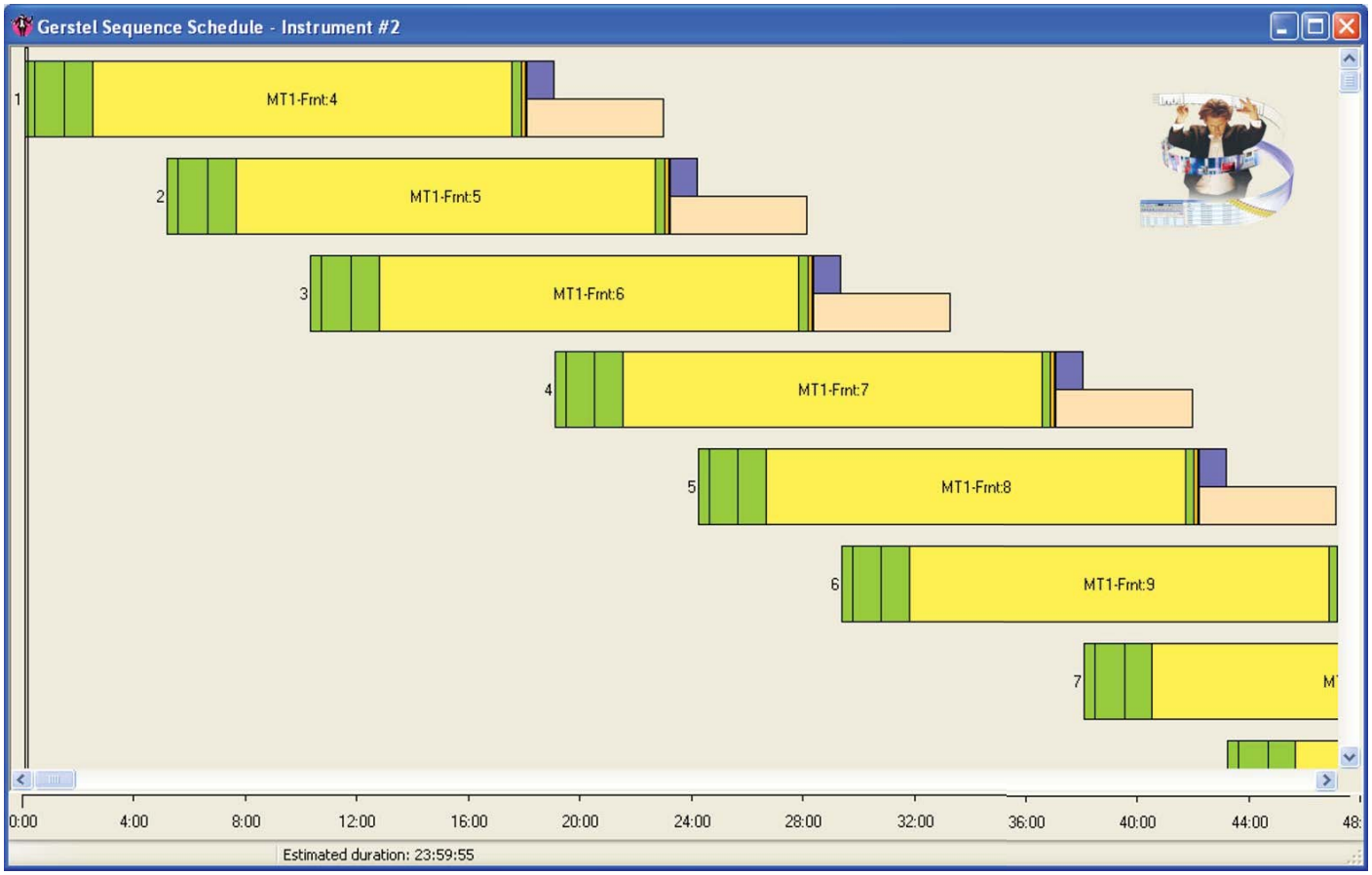

Figure 9. Graphical representation of the automated urine hydrolysis workflow in the GERSTEL MAESTRO software Scheduler.

Conclusions

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

- A fast and simple "Prep-and-Shoot" workflow was developed using the GERSTEL MPS autosampler and sample preparation robot coupled to an AB SCIEX QTRAP® 4500 LC/MS/MS System for the automated hydrolysis and analysis of urine samples within a single automated 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 high purity of the β-Glucuronidase and the design of the Turbo V™ ionization source, allowed over 960 urine samples to be automatically hydrolyzed, diluted injected, and analyzed without having to replace the analytical column while delivering reproducible results (% RSDs ≤10 %).
- The combined automation of urine hydrolysis, injection and analysis using the GERSTEL MPS allowed the system to process more than 200 samples in a 24 hour period.

References

[1] "Automated Hydrolysis, DPX Extraction and LC/MS/MS Analysis of Pain Management Drugs from Urine" GERSTEL AppNote AN-2014-01

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