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A High Throughput Automated Sample Preparation and Analysis Workflow for Comprehensive Toxicology Urine Screenings using LC/MS/MS

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Abstract
This application demonstrates the use of Disposable Pipette Extraction (DPX) for rapid, automated sample preparation of urine samples for comprehensive LC/MS/MS screening. The combination of automated sample cleanup and introduction with mass spectrometric detection using a *Scheduled* MRM™ (AB SCIEX) algorithm and fast MS/MS spectral acquisition allowed high confidence compound identification based on mass spectral library matching. The automated workflow enabled monitoring of large panels of analytes (100+ drugs); detecting and quantifying these compounds in a single run.

The new automated DPX-LC/MS/MS workflow provides rapid extractions, high recoveries, and minimized matrix interferences with complete automation capabilities towards high throughput chromatographic analysis.

Introduction

Due to the widespread use and abuse of drugs, comprehensive screening for the detection of pharmaceuticals and illicit drugs is an important part of toxicological analysis and it often requires a workflow that provides rapid, just-in-time sample preparation for high throughput analysis. As demand for monitoring of an ever increasing number of drugs continues to rise, so too does the need to detect and quantify these compounds in a single run.

Traditional solid phase extraction (SPE) techniques are regularly employed for sample preparation in drug testing and validation studies have demonstrated both high recoveries and a reduction of matrix effects when using these techniques. However these methodologies are often performed manually resulting in excessive sample processing times and high cost of analysis, directly affecting overall assay productivity.

Disposable Pipette Extraction (DPX) was developed as an alternative to traditional SPE, combining efficient and rapid extraction with significantly reduced solvent consumption. DPX is based on a dispersive solid-phase extraction device that uses sorbent loosely contained in a pipette tip where it is efficiently mixed with sample solution or extract. The main advantages of the DPX technology for toxicology monitoring are: Hydrolyzed urine samples can be extracted directly; extraction is rapid; only a small amount of solvent waste is generated; the extraction can be fully automated; and the eluate can be injected directly into the chromatography system making the approach ideal for high throughput comprehensive drug screening.

This report describes the rapid and automated cleanup of urine samples using DPX for high throughput LC-MS/MS screening, confirmation and quantification .The DPX extraction process has been shown to remove matrix interferences and efficiently clean up sample extracts yielding high recoveries for comprehensive screening of urine samples [1-3]. A GERSTEL MultiPurpose Sampler (MPS) equipped with DPX option coupled to an AB SCIEX QTRAP® 4500 LC/MS/MS System was used for the extraction of over 100 drugs and metabolites in a single urine specimen. The LC-MS/MS was operated in Multiple Reaction Mode (MRM) for detection. Dependent MS/MS spectra were acquired in the Enhanced Product Ion (EPI) mode after being triggered from a *Scheduled* MRM™ Information Dependent Acquisition (IDA) survey scan.

The speed of a *Scheduled* MRM™ algorithm allows for the best data quality covering the broadest range of drugs possible. To further increase confidence in analytical results QTRAP® technology is used to automatically acquire fast and sensitive MS/MS spectra in Enhanced Product Ion (EPI) mode and search them against mass spectral libraries for compound identification. The information of the complete molecular fingerprint saved into EPI spectra significantly reduces the risk of false positive and negative results [7-10]. This technology combined with the automated DPX sample clean-up make it possible to monitor large panels of analytes within a very short time with high confidence in identification. The method was successfully applied to quantify and confirm the identification of drug compounds in spiked urine samples with high confidence.

Experimental

Materials. 130 neat standard solutions of different drug classes were purchased from Cerilliant. An analyte stock solution containing all drugs at appropriate concentrations was prepared in methanol in order to evaluate the automated urine cleanup method for all compounds. A detailed list of the drugs used for this study is available upon request. A selected panel of deuterated analogues was purchased from Cerilliant and used for quantification. β-Glucuronidase, from abolone, (cat.#DR2100 1M Units) was purchased from Campbell Science. Fresh urine was obtained from a male volunteer. All other reagents and solvents used were reagent grade.

Instrumentation. All automated DPX PrepSequences and injections were performed using an MPS XL MultiPurpose Sampler in Dual Head configuration with the GERSTEL DPX Option as shown in Figure 1. All analyses were performed using an Agilent 1260 HPLC with a Phenomenex Kinetex column (C18, 3 x 50 mm, 2.6 µm, 100 Å), and an AB SCIEX QTRAP® 4500 LC/MS/MS System. Sample injections were made using a 6 port (0.25 mm) Cheminert C2V injection valve fitted with a 2 µL stainless steel sample loop.

Figure 1. GERSTEL MultiPurpose Sampler (MPS XL) in Dual Head configuration with DPX Option used for automated drug screening in combination with the LC/MS/MS system.

Sample pretreatment. Hydrolysis of urine consisted of combining 0.2 mL of urine, 40 µL of the working internal standard solution, 20 µL of β-Glucuronidase, 50 µL of 0.1 M acetate buffer; pH 4 and 230 µL sample diluent, vortex mixing for 30 seconds, and then incubating at 55°C for 2 hours. Aliquots of 260 µL of hydrolyzed urine samples were added into clean shell vials for automated cleanup and injection.

Figure 2 shows a graphical representation of the automated DPX-LC/MS/MS workflow. The automated DPX extraction was programmed using MAESTRO software coupled to AB SCIEX Analyst® 1.6 Software. The PrepAhead functionality in MAESTRO was enabled, allowing high throughput "just in time" sample preparation and analysis (Figure 3).

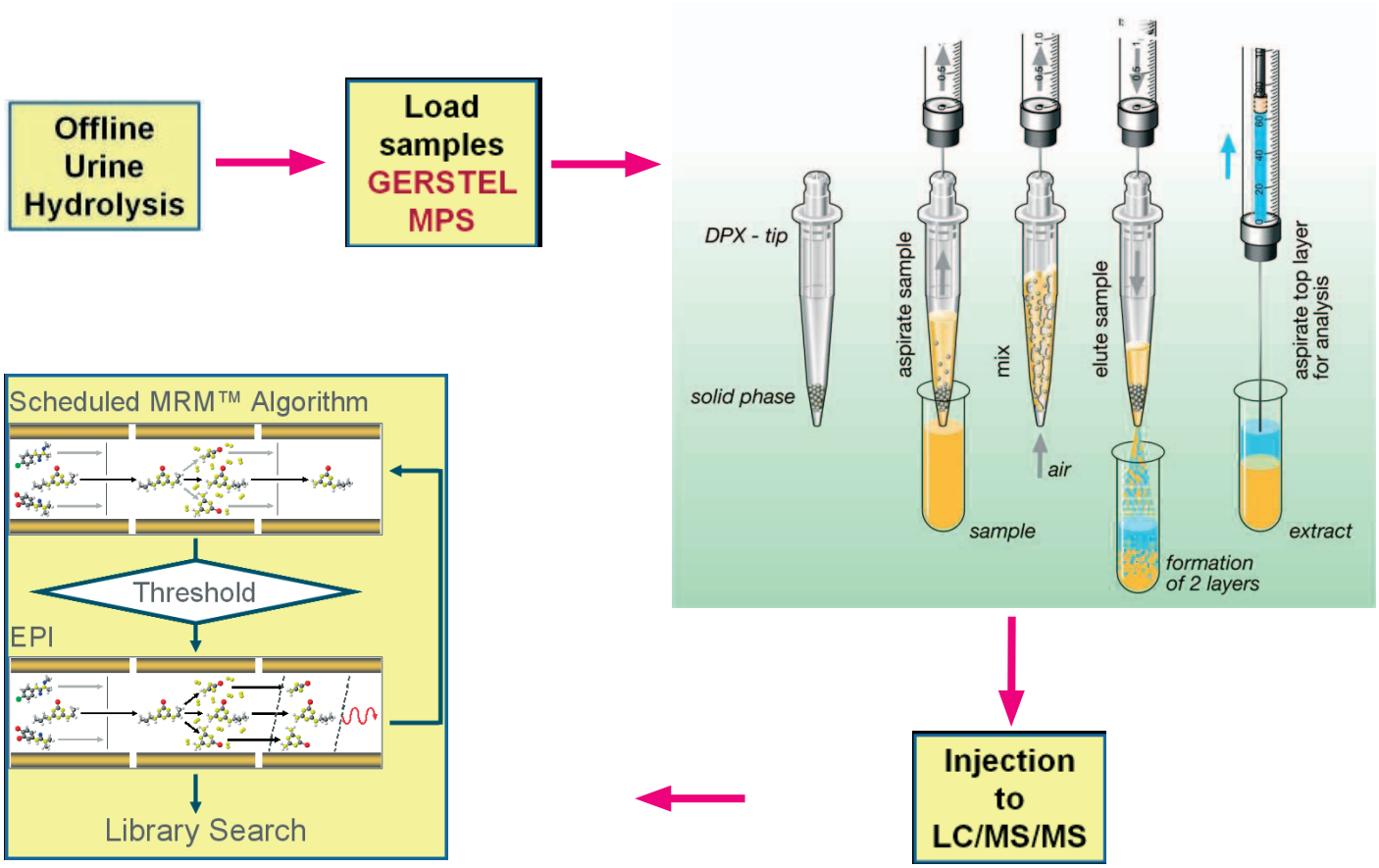

Figure 2. Graphical representation of the automated DPX-LC-MS/MS workflow.

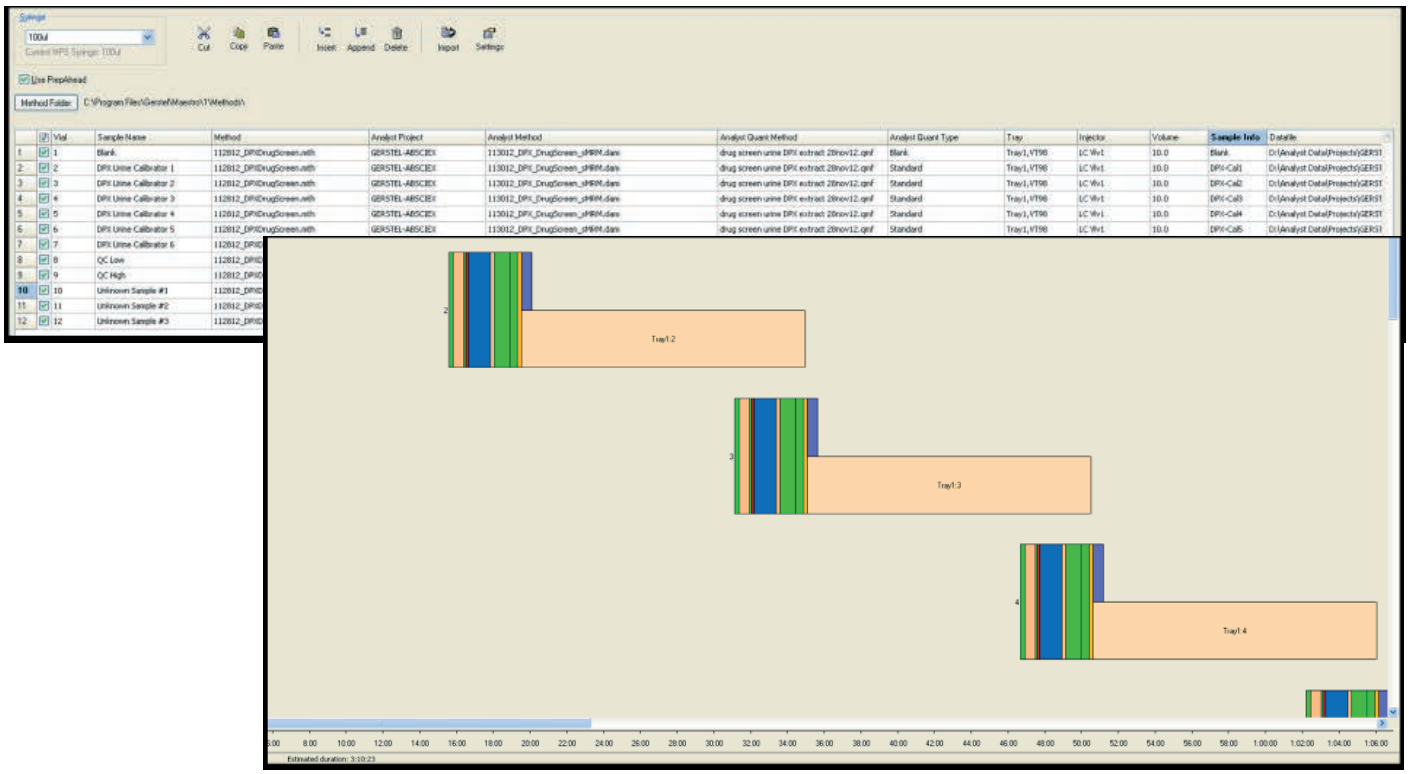

Figure 3. MAESTRO Sequence Scheduler with PrepAhead coupled to Analyst® Software MS acquisition software.

The automated DPX-LC-MS/MS urine cleanup and analysis method included the following parameters:

Automated DPX Prep Sequence:

1. Aspirate 750 µL of 100 % acetonitrile using the 2.5 mL DPX syringe.
2. Pick up a new DPX tip (DPX-RP-S) from the DPX tray.
3. Add 500 µL of 100 % acetonitrile through the DPX tip, into the urine sample located on the MPS sample tray.
4. Wait for 6 seconds to allow acetonitrile to completely wet the DPX sorbent.
5. Aspirate the sample and acetonitrile followed by 1400 µL of air into DPX tip.
6. After equilibrating for 5 seconds, dispense the contents of the DPX tip, as well as the remaining acetonitrile found within the DPX syringe, back into the original shell vial in the tray.
7. Dispose of the DPX tip at the PipWaste position.
8. Transfer 100 µL the upper organic layer located within the original shell vial, into a clean, empty, capped autosampler vial with septum.
9. Dilute the extract with 900 µL of water.
10. Inject 50 µL of the diluted extract into the HPLC injection valve.

Analysis conditions LC.

Pump:	gradient, flowrate = 0.4 mL/min	
Mobile Phase:	A - 10 mM ammonium formate in H_2O	
	B - Acetonitrile + methanol (1:1)	
Gradient:	Initial	2 % B
	1 min	2 % B
	10 min	100 % B
	13 min	100 % B
	13.1 min	2 % B
	15.5 min	2 % B

Injection volume:	2 µL (loop over-fill technique)
Column temperature:	40°C

Analysis conditions MS.

Operation:	positive mode
Temperature:	500°C
Ion Source Gas 1:	40
Ion Source Gas 2:	70
IonSpray Voltage:	4000 V
Curtain Gas:	30
CAD:	High

The AB SCIEX QTRAP® 4500 LC/MS/MS System was operated with AB SCIEX Turbo V™ source and Electrospray Ionization (ESI) probe. A total of 132 transitions in positive mode were monitored with an MRM pause time of 5 ms.The *Scheduled* MRM™ algorithm was used with an MRM detection window of 120 s and a target scan time of 1 s in Analyst®1.6 Software. For increased confidence in compound identification EPI spectra at a scan speed of 10000 Da/s were acquired using a dynamic fill time for optimal MS/MS quality.EPI spectra were generated using standardized Collision Energy (CE) of ±35 V with Collision Energy Spread (CES) of 15 V to ensure a characteristic MS/MS pattern independently of the compound's fragmentation efficiency. MS/MS spectra were searched against the AB SCIEX iMethod™ application AB SCIEX Forensic Library version 2.1.

Results and Discussion

DPX-LC-MS/MS with acquisition of MS/MS spectra for compound identification through mass spectral library searching. Figure 4 shows representative *Scheduled* MRM™chromatograms for over 100 different drugs and internal standards, from a hydrolyzed urine sample spiked sample at 100 ng/mL after the automated DPX cleanup procedure.

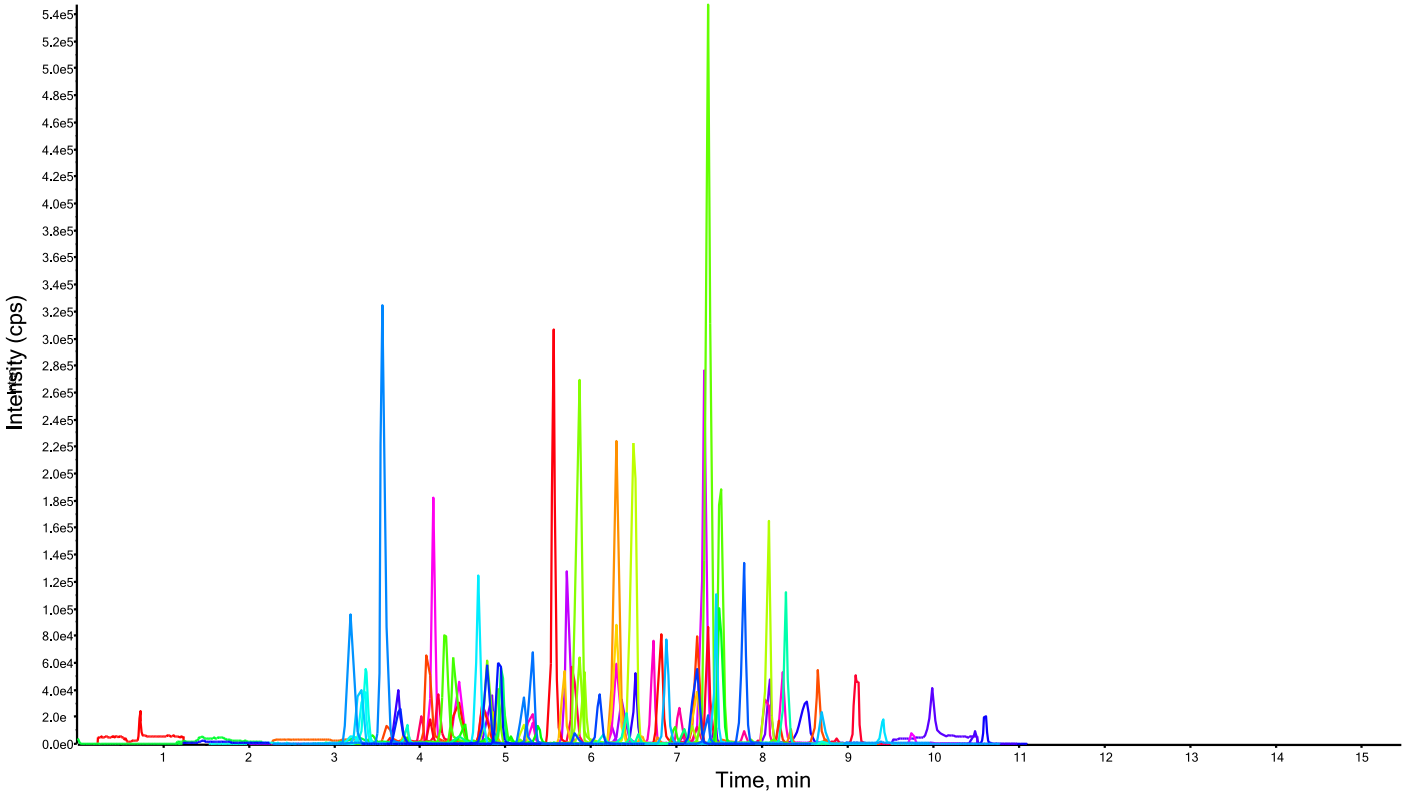

Figure 4. Overlay chromatograms for all *Scheduled* MRM™ transitions from a hydrolyzed urine sample at 100 ng/mL.

Despite the high selectivity that MRM provides, there is always a risk of false positive findings due to endogenous compounds that have the same mass. Typically a second MRM is monitored per analyte and the ratio of quantifier to qualifier transition is calculated for each unknown sample and compared to the MRM ratio of standards for identification. However, targeted compounds with low fragmentation efficiencies (i.e. Low Intensity Ions) have been reported to produce false positive results for compound identification [4-6].

For improved accuracy, compound identification was performed using full scan MS/MS experiments with automated library searching capabilities to compare spectra of unknown compounds with standard spectra. The dependent MS/MS spectra were acquired using the EPI mode of the QTRAP® system after being triggered from a *Scheduled* MRM™ IDA survey scan. The rapidly collected high quality MS/MS data were used in mass spectral library searching, using AB SCIEX LibraryView™ Software 1.0, to increase the confidence of detection. Extracted spectra and library search Purity Score values using an MS/MS library search algorithm are shown in Figures 5 and 6 for extracted urine samples with low analyte concentrations.

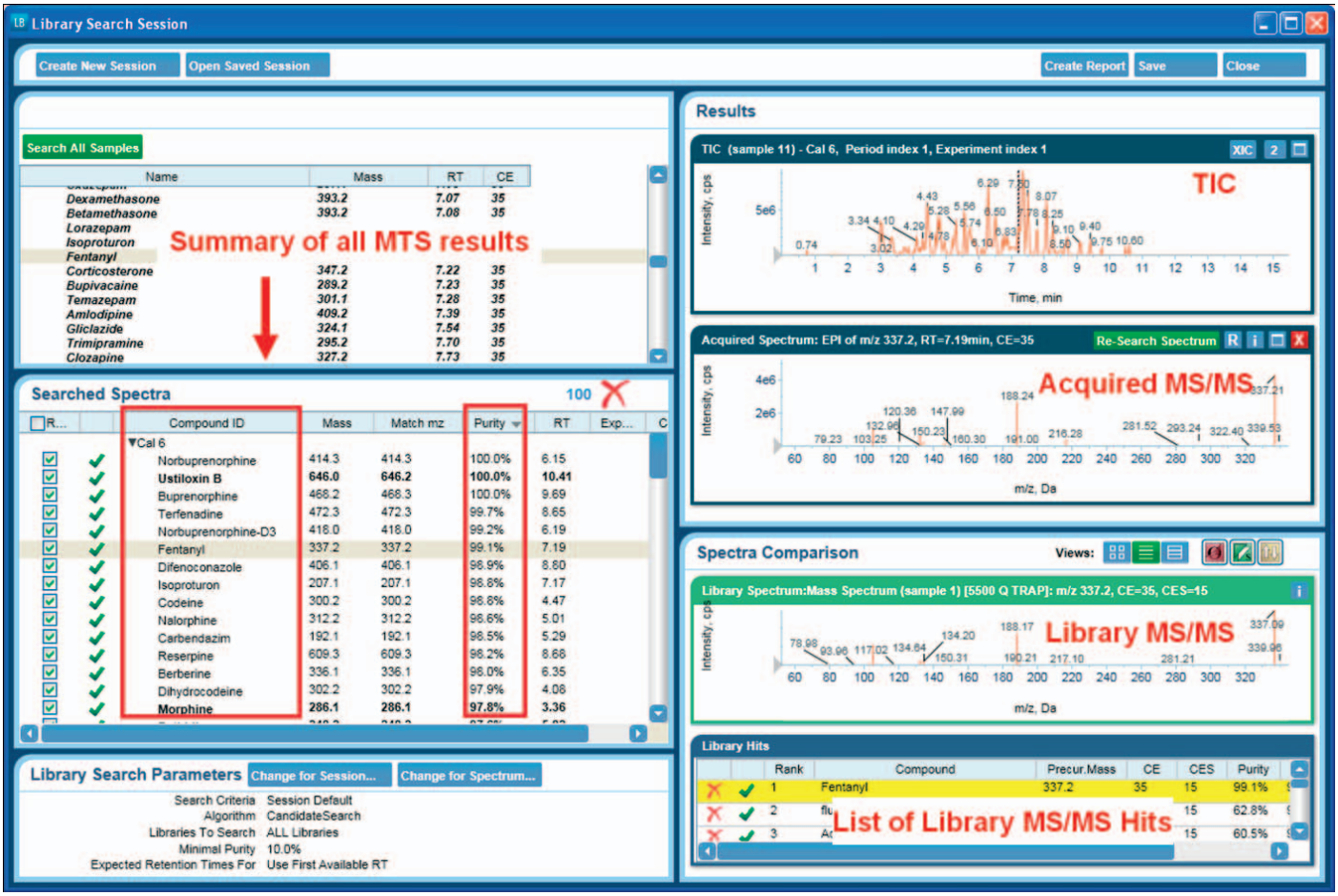

Figure 5. Extracted spectra and automated library search with Purity Score values for a low concentration urine sample.

Figure 6. Automated library confirmation for Salbutamol determined in a hydrolyzed urine sample after DPX cleanup.

Quantitative analysis was performed in the same run allowing both quantitative and qualitative data to be collected simultaneously. Figures 7 and 8 show example calibration curves (1 – 2000 ng/mL) created from the same run for two compounds identified from the library matching. Regression analysis for the analyzed samples within this method resulted in R^2 values of 0.99 or greater. Although extracted ion chromatogram peaks are maintained for accuracy and precision, % CVs averaged 15 % due to forced quantification below the compounds minimum reportable limits that have been reported in previous application notes [2,3].

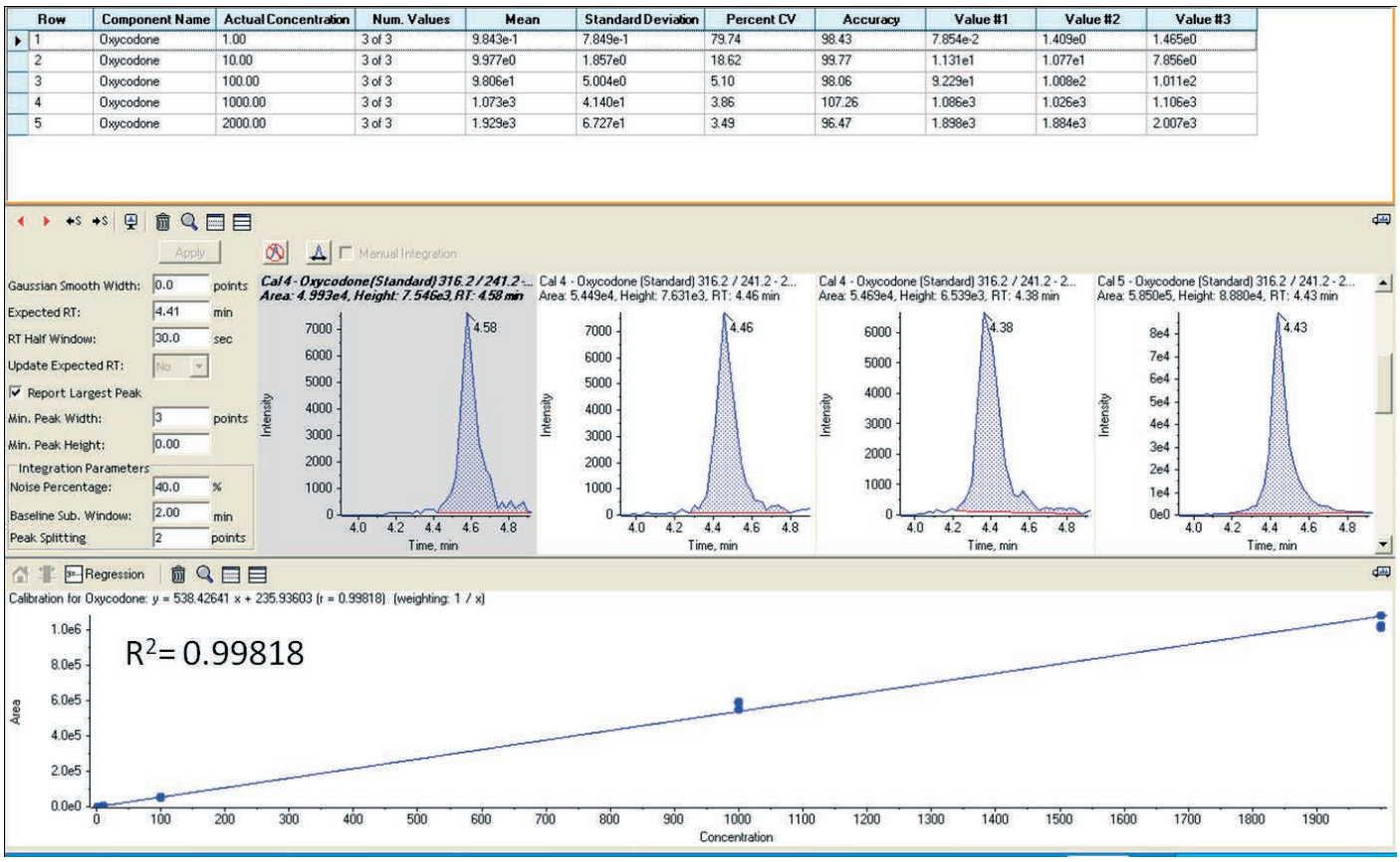

Figure 7. Example calibration curve for Oxycodone, generated from the same run in which qualitative information was obtained for confident identification through library searching. Linear range of 1-2000 ng/mL.

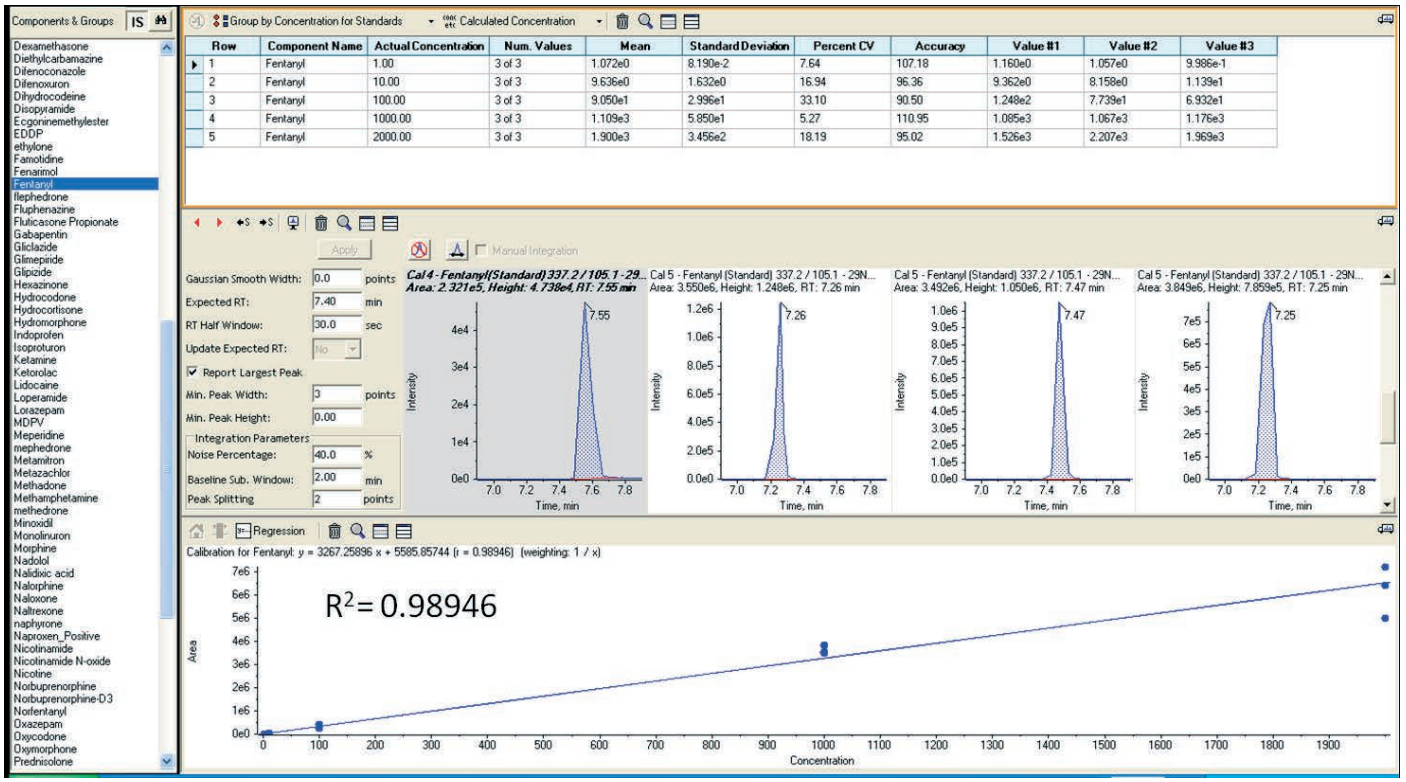

Figure 8. Example calibration curve for Fentanyl, generated from the same run in which qualitative information was obtained for confident identification through library searching. Linear range of 1-2000 ng/mL.

DPX-LC/MS/MS workflow throughput. With a single column configuration and automated DPX cleanup prep sequence, a 17.5 min/sample cycle time is achieved which allows the user to process over 82 samples per day. By integrating valve switching capabilities to bring a second conditioned column online while regenerating the first column, the system cycle time was reduced to approximately 15 min/sample, improving the throughput to over 95 samples per 24 hrs. This was achieved with "just in time" sample preparation using the MAESTRO PrepAhead function coupled to Analyst® Software.

If only analyte confirmation and quantification is desired, the DPX-LC-MS/MS method may be performed using a *Scheduled* MRM™ method with 3 MRM transitions (1 quantitative, 2 qualitative) and a shorter LC gradient (6.5 min). This allows a 7 min/sample cycle time, which subsequently allows the automated procedure for extraction and analysis to process over 200 samples per day.

Conclusions

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

- The DPX-LC-MS/MS workflow using the dual head GERSTEL MPS XL robotic sampler under MAESTRO PrepAhead control coupled to an AB SCIEX QTRAP® 4500 LC/MS/MS System enabled automated cleanup and injection of hydrolyzed urine samples for screening and confirmation of over 100 analytes in a single run.
- The QTRAP® allowed high accuracy compound identification by performing full scan MS/MS experiments using the Enhanced Product Ion mode after being triggered from a *Scheduled* MRM™ IDA survey scan with automated library searching capabilities to compare spectra of unknown compounds with standard spectra.
- Quantitative analysis was performed in the same run allowing both quantitative and qualitative data to be collected simultaneously. Linear calibration curves with R^2 values of 0.99 or greater were achieved for the samples analyzed.
- With a single column configuration and automated DPX cleanup prep sequence, a 17.5 min/sample cycle time is achieved, the addition of valve switching capabilities further increases the throughput to process over 95 samples/day using the automated DPX-LC-MS/MS drug screen method.

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