# Fast Screening Method for 86 Forensic Analytes in Human Urine

When a urine specimen is acquired for clinical analysis it is put through a presumptive, or screening, test prior to confirmation analysis. Traditionally enzyme linked immunosorbent assay (ELISA) has been used for screening to identify the presence of a compound class in a given sample. However, multiple ELISA kits are often required for a comprehensive panel increasing the cost of analysis. The difficulty of producing new antibodies for each assay also makes it a challenge for immunoassays to rapidly adapt to include new analytes.

While the ability of liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) to simultaneously analyze across multiple compound classes has made it the gold standard for confirmation analysis, it offers many benefits to screening as well. This screening analysis utilizes multiple reaction monitoring (MRM) scans as well as an Enhanced MS (EMS) scan.

For each MRM scan, a specified precursor ion is selected in Q1, which is fragmented to produce a unique product ion that is monitored in Q3; therefore, yielding sensitive and specific data for the analytes of interest. When the *Scheduled MRM*™ Algorithm is then used optimal cycle and dwell times can be established to produce high quality data in a short overall run time.

For this project, we have designed a rapid screening method of 86 compounds in human urine using a SCIEX QTRAP® system to collect both MRM and EMS data within a single injection. With it's simple to perform sample preparation and fast acquisition time (2.5 min), this method is optimal for forensic laboratory screening.

## Key Features of QTRAP Technology and Scheduled MRM™ Algorithm

- Rapid, robust approach allows direct detection of 86 forensic analytes in human urine with simple sample preparation and fast chromatography
- Targeted MRM workflow allows sensitive detection with high selectivity, reduced matrix effects and higher confidence in results
- Additional QTRAP® technology allows for enhanced scan functions that further improve confidence in results, enabling spectral identification and library searching.
- Scheduled MRM™ Algorithm enables the use of fast chromatography without loss of data integrity, allowing for faster throughput and turnaround times.

## Experimental Details

### *Compound list and spiking solutions*

Table 1 lists the 86 compounds plus 6 internal standards in the panel. Two spiking solutions in methanol were prepared: one for analytes (**SA**) and the other for internal standards (**SIS**).

### *Calibrator and Control preparation*

Blank human urine was spiked with solution **SA** to prepare calibrators. A single calibrator and three quality controls (QCs) were prepared. Actual concentrations varied for each compound; however, the calibrator was prepared at the cutoff (CO) and three QCs at 50% CO, 3xCO, and 10xCO.

### *Sample preparation*

- 100 µL urine sample was mixed with 25 µL IMCS RapidHydrolysis Buffer, 20 µL IMCSzyme and 10 µL **SIS**. Hydrolysis time was typically between 30 and 60 min at 55°C.
- After hydrolysis was complete, 0.8 mL of diluent was added to the mixture.
- The mixture was then centrifuged at 21,000 *g* for 15 min.
- The supernatant was transferred to a glass vial with insert for analysis by LC-MS/MS.

### *Liquid Chromatography*

Liquid Chromatography analysis was performed on a SCIEX HPLC system. Separation was achieved using a Phenomenex Luna C18 column. Mobile phase A (MPA) was ammonium formate in water. Mobile phase B (MPB) was formic acid in methanol. The LC flow rate was varied throughout the run from 0.2 mL/min to 1 mL/min to allow for necessary equilibration of the column in the 2.5 min LC run-time. The injection volume used was 5 µL.

## *Mass Spectrometry*

The system was run using ESI positive mode. Individual voltages were optimized for each individual component. Scheduled MRM Algorithm was optimized to ensure best data quality

Enhanced MS Scan, where used, was run over a 100-600Da window.

| Compounds | Compounds | Compounds | Internal Standards |
|---|---|---|---|
| *6-MAM* | *Gabapentin* | *Naltrexone* | *Amphetamine-d5* |
| *7-Aminoclonazepam* | *Hydrocodone* | *N-desmethyltapentadol* | *Fentanyl-d5* |
| *7-Hydroxymitragynine* | *Hydromorphone* | *Norbuprenorphine* | *JWH 018 4-OH pentyl-d5* |
| *Acetyl Fentanyl* | *Imipramine* | *Norcodeine* | *Methadone-d3* |
| *Alpha-Hydroxyalprazolam* | *JWH-018 4-OH pentyl* | *Nordiazepam* | *Methamphetamine-d5* |
| *Alpha-Hydroxymidazolam* | *JWH-018 pentanoic acid* | *Norfentanyl* | *Morphine-d6* |
| *Alpha-Hydroxytriazolam* | *JWH-019 6-OH hexyl* | *Norhydrocodone* | |
| *Alpha-PPP* | *JWH-073 3-OH butyl* | *Normeperidine* | |
| *Alpha-PVP* | *JWH-073 butanoic acid* | *Noroxycodone* | |
| *Alprazolam* | *JWH-081 5-OH pentyl* | *Norpropoxyphene* | |
| *AM-2201 4-OH pentyl* | *JWH-122 5-OH pentyl* | *Nortriptyline* | |
| *Amitriptyline* | *JWH-210 5-OH pentyl* | *O-Desmethyltramadol* | |
| *Amphetamine* | *JWH-250 4-OH pentyl* | *Oxazepam* | |
| *Benzoylecgonine* | *Lorazepam* | *Oxycodone* | |
| *Buphedrone* | *MDA* | *Oxymorphone* | |
| *Buprenorphine* | *MDEA* | *PCP* | |
| *Carisoprodol* | *MDMA* | *Pregabalin* | |
| *Clomipramine* | *MDPV* | *Propoxyphene* | |
| *Codeine* | *Meperidine* | *Protriptyline* | |
| *Cotinine* | *Mephedrone* | *RCS4-4-OH-pentyl* | |
| *Cyclobenzaprine* | *Meprobamate* | *Ritalinic Acid* | |
| *Desalkylflurazepam* | *Methadone* | *Sufentanil* | |
| *Desipramine* | *Methamphetamine* | *Tapentadol* | |
| *Desmethyldoxepin* | *Methedrone* | *Temazepam* | |
| *Dextromethorphan* | *Methylone* | *Tramadol* | |
| *Diazepam* | *Methylphenidate* | *Zolpidem* | |
| *Dihydrocodeine* | *Midazolam* | | |
| *Doxepin* | *Mitragynine* | | |
| *EDDP* | *Morphine* | | |
| *Fentanyl* | *Naloxone* | | |

**Table 1: List of compounds and internal standards analysed**

## Results and Discussion

### *Sample preparation*

IMCSzyme was selected as the hydrolysis enzyme for this study. Using this enzyme, the hydrolysis of glucuronide-conjugated analytes was completed in a shorter time frame compared to other traditional beta-glucuronidase enzymes. Furthermore, less interferences were observed when using IMCSzyme, which proved beneficial for LC column life and MS maintenance.

### *Calibrator preparation*

Human urine was established as the necessary matrix for the calibrator and all controls in this method. Due to the rapid nature of the gradient, interferences present in human urine more closely match that of unknown samples. The method was tested using both human urine and purchased synthetic urine; however, spiked samples in synthetic urine often fell outside of the expected range when compared against data points collected using human urine.

### *Scheduled MRM™ Data*

We designed the LC gradient to ensure that the retention times of the various compounds were evenly distributed throughout the gradient to reduce the MRM concurrency. This enabled maximum sampling of every LC peak, while maintaining optimal dwell times for each MRM transition. The re-optimized Scheduled MRM™ Algorithm ensured optimal data quality even during regions of the chromatogram when the MRM concurrency was very high. A minimum of 5 data points was achieved across every peak, providing the necessary peak definition for a screening method. A typical chromatogram is shown in Figure 1 below

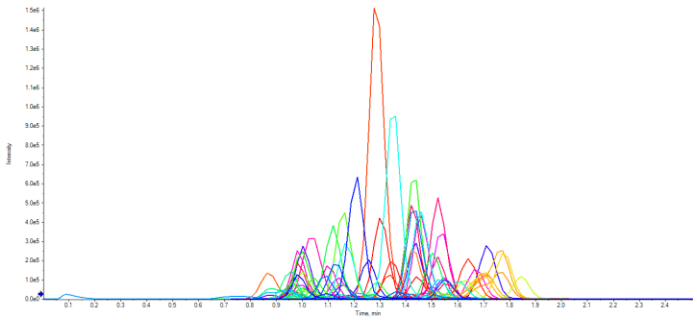

**Figure 1: Elution profile of all the 86 compounds in screening panel**

### *Enhanced MS Data*

The Enhanced MS (EMS) data was collected to assist in the identification of an unknown peak. Figure

Enhanced MS Scan, where used, was run over a 100-600Da window.

shows (A) total ion chromatogram (TIC) of EMS of the entire 2.5-min run, (B) 100-Da window extracted ion chromatogram (XIC) around 240 with EMS, and (C) the EMS mass spectrum at 1.15-min. In order to further identify the analyte of interested it would be necessary to run a secondary experiment in which an Enhanced Product Ion (EPI) spectrum is collected for comparison with a library.

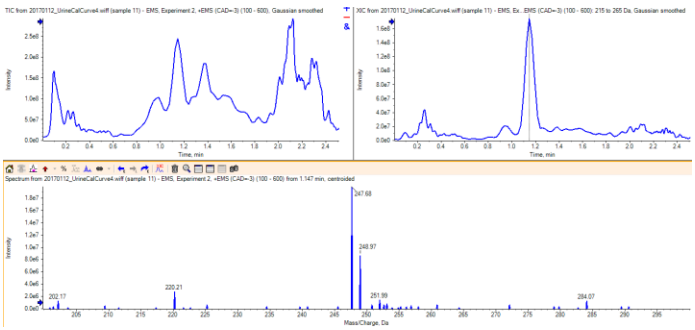

**Figure 2: Enhanced MS data. (Clockwise from top left) Total ion chromatogram (TIC) of EMS of the entire 2.5-min run, 100-Da window extracted ion chromatogram (XIC) around 240 Da with EMS, EMS mass spectrum at 1.15-min.**

### *Analytical sensitivity*

A single-point calibration was used for all 86 analytes, which yielded accurate results for low, medium, and high QCs. Figure 3, shows a typical calibration curves for alpha-PVP with excellent linearity fitted through zero. The XICs of the quality controls for alpha-PVP are shown over three replicate sets of injections (Figure 4).

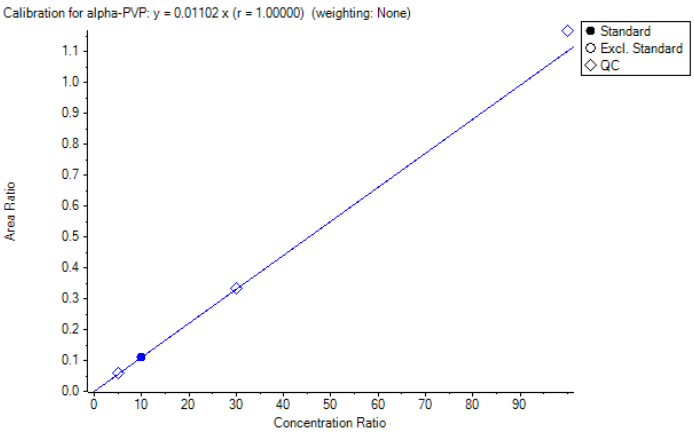


**Figure 3: Typical calibration curve of alpha-PVP plotted with quality controls.**

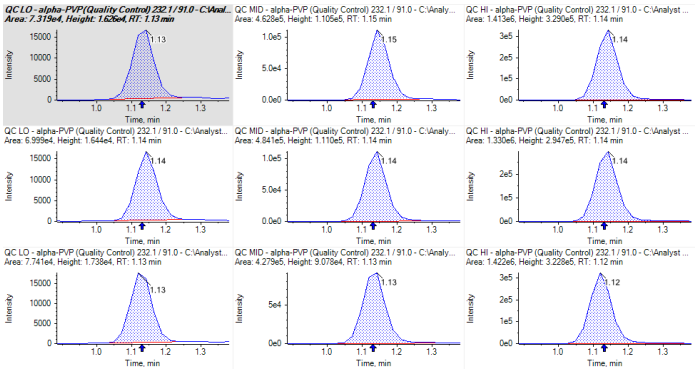

**Figure 4: Extracted ion chromatograms (XICs) of alpha-PVP (n=3, quality controls. (A) 5 ng/mL; (B) 30 ng/mL; (C) 100 ng/mL.**

## Conclusions

This method provides a fast screening technique for 86 forensic analytes making use of both the Scheduled MRM™ Algorithm and Enhanced MS scan in a single injection. The SCIEX QTRAP® technology produces high quality data for all analytes, while utilizing samples prepared by rapid enzyme hydrolysis and a simple dilute and shoot pretreatment.

## References

1. QTRAP® LC-MS/MS Technology: https://sciex.com/technology/qtrap-technology
2. Various spectral libraries available: https://sciex.com/products/spectral-library



**Headquarters**
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

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