Fast Screening Method for 86 Forensic Analytes in Human Urine using the QTRAP® 4500 LC-MS/MS System

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Overview

This technical note introduces the rapid screening analysis of an 86 compound panel. The analysis was accomplished in human urine using the ExionLC™ AC HPLC system and the QTRAP® 4500 LC-MS/MS system (Figure 1). The short LC runtime of 2.5 minutes and simple to perform sample preparation make this method appropriate for any high throughput forensic lab.

Introduction

When a urine specimen is acquired for forensic 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.

The EMS scan in the screening analysis makes use of the linear ion trap (LIT) function of the QTRAP® 4500 LC-MS/MS system; this is called EMS. During an EMS scan, Q1 and Q2 operate in RF-only mode transmitting only ions within a selected mass range. The LIT entraps the ions within the specified range, and subsequently scans them out (Figure 2). A major advantage of EMS scanning is that data can be collected at a fast scan rate (20,000 Da/s); despite the fast scan time data generated is of better mass spectral quality than a traditional triple quadrupole. This functionality is unique to the SCIEX QTRAP platform and allows for the collection of mass spectral data which can be further reviewed for potential analytes of interest that were not already collected by MRM.

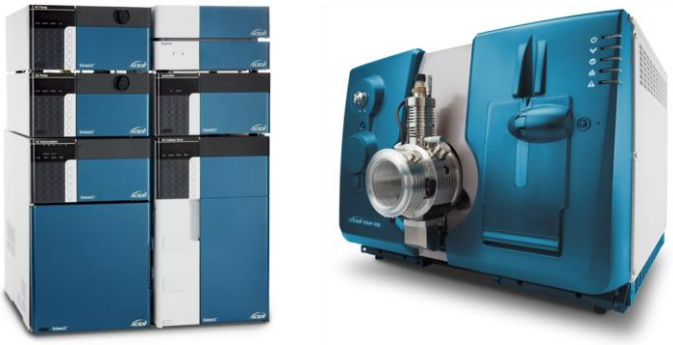

Figure 1: The SCIEX ExionLC™ AC HPLC system (left) and the SCIEX QTRAP® 4500 LC-MS/MS System (right).

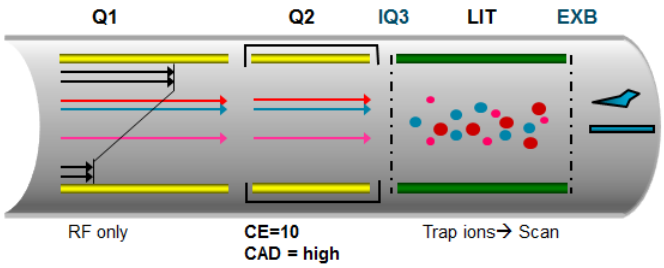

Figure 2: Depiction of how Enhanced MS (EMS) works on the QTRAP system.

For this project, we have designed a rapid screening method of 86 compounds in human urine using a QTRAP® 4500 LC-MS/MS 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.

Materials and Methods

Compound list and spiking solutions

Table 1 lists the 86 compounds plus 6 internal standards in the panel. All were procured from Cerilliant Corporation (Round Rock, TX). Two spiking solutions in methanol were prepared: one for analytes (**SA**) and the other for internal standards (**SIS**). Concentrations of all the analytes in the spiking solution **SA** are listed in Table 1.

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. For instance, fentanyl's only calibrator was 100 ng/mL and it's low, mid, and high QCs were 50, 300 and 1000 ng/mL.

Sample preparation

1. 100 µL urine sample was mixed with 25 µL IMCS Rapid Hydrolysis Buffer, 20 µL IMCSzyme and 10 µL **SIS**. Both IMCS Rapid Hydrolysis Buffer and IMCSzyme were acquired from IMCS (Columbia, SC). Hydrolysis time was typically between 30 and 60 min at 55°C.
2. After hydrolysis was complete, 0.8 mL of diluent was added to the mixture.
3. The mixture was then centrifuged at 21,000 *g* for 15 min.
4. The supernatant was transferred to a glass vial with insert for analysis by LC-MS/MS.

Liquid Chromatography

Liquid Chromatography analysis was performed on the SCIEX ExionLC™ AC HPLC system at 40°C. 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 uL.

As for the autosampler, the needle rinse solution was methanol: acetonitrile: isopropanol (1:1:3, v/v/v). The rinse sequence was as follows:

- Rinsing volume: 1 mL
- Rinsing speed: 35 µL/sec
- Sampling speed: 15 µL/sec
- Rinse dip time: 3 sec
- Rinse mode: before and after aspiration.

MS Source/Gas conditions

- Curtain gas (CUR): 30
- Collision gas (CAD): Medium
- IonSpray Voltage (IS): 2500 V
- Temperature (TEM): 550°C
- Ion Source Gas 1 (GS1): 60
- Ion Source Gas 2 (GS2): 50

MRM table and Scheduled MRM™ algorithm

The Declustering Potential (DP), Collision Energy (CE), and Collision Cell Exit Potential (CXP) voltages were optimized for each individual component, as described in Table 2 below.

- Target Scan Time = 1.0 sec
- MRM detection window = 30 sec

Enhanced MS scan

- Scan Rate = 20,000 Da/s
- Mass Range = 100 – 600 Da

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.

Table 1: List of analytes and internal standards, and their concentrations in spiking solution (for calibrator and control preparation)

Compounds	(ng/mL)	Compounds	(ng/mL)	Compounds	(ng/mL)	Compounds
6-MAM	*1000*	*Gabapentin*	*10000*	*Naltrexone*	*5000*	*Amphetamine-d5*
7-Aminoclonazepam	*5000*	*Hydrocodone*	*5000*	*N-desmethyltapentadol*	*5000*	*Fentanyl-d5*
7-Hydroxymitragynine	*1000*	*Hydromorphone*	*5000*	*Norbuprenorphine*	*2000*	*JWH 018 4-OH pentyl-d5*
Acetyl Fentanyl	*200*	*Imipramine*	*5000*	*Norcodeine*	*5000*	*Methadone-d3*
Alpha-Hydroxyalprazolam	*5000*	*JWH-018 4-OH pentyl*	*1000*	*Nordiazepam*	*5000*	*Methamphetamine-d5*
Alpha-Hydroxymidazolam	*5000*	*JWH-018 pentanoic acid*	*1000*	*Norfentanyl*	*200*	*Morphine-d6*
Alpha-Hydroxytriazolam	*5000*	*JWH-019 6-OH hexyl*	*1000*	*Norhydrocodone*	*5000*	
Alpha-PPP	*1000*	*JWH-073 3-OH butyl*	*1000*	*Normeperidine*	*5000*	
Alpha-PVP	*1000*	*JWH-073 butanoic acid*	*1000*	*Noroxycodone*	*5000*	
Alprazolam	*5000*	*JWH-081 5-OH pentyl*	*1000*	*Norpropoxyphene*	*10000*	
AM-2201 4-OH pentyl	*1000*	*JWH-122 5-OH pentyl*	*1000*	*Nortriptyline*	*5000*	
Amitriptyline	*5000*	*JWH-210 5-OH pentyl*	*1000*	*O-Desmethyltramadol*	*5000*	
Amphetamine	*10000*	*JWH-250 4-OH pentyl*	*1000*	*Oxazepam*	*5000*	
Benzoylecgonine	*5000*	*Lorazepam*	*5000*	*Oxycodone*	*5000*	
Buphedrone	*1000*	*MDA*	*10000*	*Oxymorphone*	*5000*	
Buprenorphine	*2000*	*MDEA*	*10000*	*PCP*	*2500*	
Carisoprodol	*10000*	*MDMA*	*10000*	*Pregabalin*	*10000*	
Clomipramine	*5000*	*MDPV*	*1000*	*Propoxyphene*	*10000*	
Codeine	*5000*	*Meperidine*	*5000*	*Protriptyline*	*5000*	
Cotinine	*5000*	*Mephedrone*	*1000*	*RCS4-4-OH-pentyl*	*1000*	
Cyclobenzaprine	*5000*	*Meprobamate*	*10000*	*Ritalinic Acid*	*5000*	
Desalkylflurazepam	*5000*	*Methadone*	*10000*	*Sufentanil*	*200*	
Desipramine	*5000*	*Methamphetamine*	*10000*	*Tapentadol*	*5000*	
Desmethyldoxepin	*5000*	*Methedrone*	*1000*	*Temazepam*	*5000*	
Dextromethorphan	*5000*	*Methylone*	*1000*	*Tramadol*	*5000*	
Diazepam	*5000*	*Methylphenidate*	*5000*	*Zolpidem*	*5000*	
Dihydrocodeine	*5000*	*Midazolam*	*5000*			
Doxepin	*5000*	*Mitragynine*	*1000*			
EDDP	*10000*	*Morphine*	*5000*			
Fentanyl	*200*	*Naloxone*	*5000*			

Table 2: MRM Table (part 1)

Analyte	Q1	Q3	RT (min)	DP	EP	CE	CXP
Amphetamine-d5 IS	*141.1*	*93*	*0.98*	*30*	*10*	*21*	*8*
Fentanyl-d5 IS	*342.3*	*105.1*	*1.25*	*90*	*10*	*50*	*8*
JWH 018 4-OH pentyl-d5 IS	*363.1*	*155.1*	*1.72*	*100*	*10*	*35*	*14*
Methadone-d3 IS	*313.2*	*105.1*	*1.42*	*60*	*10*	*35*	*14*
Methamphetamine-d5 IS	*155.2*	*92*	*0.99*	*45*	*10*	*25*	*6*
Morphine-d6 IS	*292.1*	*152*	*0.85*	*90*	*10*	*75*	*12*
6-MAM/ Naloxone	*328.1*	*152*	*0.98*	*76*	*10*	*89*	*10*
7-Aminoclonazepam	*286.1*	*121.1*	*1.16*	*80*	*10*	*37*	*13*
7-Hydroxymitragynine	*415.2*	*190.1*	*1.20*	*85*	*10*	*50*	*8*
Acetyl fentanyl	*323.1*	*188.1*	*1.17*	*90*	*10*	*31*	*10*
Alpha-Hydroxyalprazolam	*325.1*	*297.1*	*1.50*	*60*	*10*	*40*	*15*
Alpha-Hydroxymidazolam	*342.1*	*203.1*	*1.54*	*60*	*10*	*36*	*10*
Alpha-Hydroxytriazolam	*359.1*	*331.1*	*1.48*	*90*	*10*	*37*	*12*
alpha-PPP	*204.1*	*105*	*0.99*	*80*	*10*	*30*	*9*
alpha-PVP	*232.1*	*91*	*1.13*	*80*	*10*	*30*	*10*
Alprazolam	*309.1*	*281.1*	*1.54*	*80*	*10*	*35*	*10*
AM 2201 4-OH pentyl	*376.1*	*155.1*	*1.67*	*65*	*10*	*31*	*10*
Amitriptyline	*278.1*	*233.1*	*1.44*	*70*	*10*	*23*	*12*
Amphetamine	*136*	*119*	*0.98*	*40*	*10*	*12*	*10*
Benzoylecgonine	*290.1*	*168*	*1.11*	*80*	*10*	*37*	*12*
Buphedrone	*178.1*	*160*	*1.01*	*41*	*10*	*17*	*12*
Buprenorphine	*468.3*	*414.2*	*1.38*	*120*	*10*	*46*	*18*
Carisoprodol	*261.1*	*176*	*1.53*	*35*	*10*	*11*	*12*
Clomipramine	*315.1*	*86.1*	*1.51*	*26*	*10*	*23*	*12*
Codeine/Hydrocodone	*300.1*	*152*	*0.97*	*91*	*10*	*77*	*12*
Cotinine	*177*	*80*	*0.99*	*70*	*10*	*28*	*8*
Cyclobenzaprine	*276.1*	*215.1*	*1.41*	*80*	*10*	*58*	*12*
Desalkylflurazepam	*289.1*	*140*	*1.56*	*90*	*10*	*40*	*10*
Desipramine	*267.1*	*72*	*1.43*	*70*	*10*	*50*	*8*
Desmethyldoxepin	*266.1*	*107*	*1.34*	*70*	*10*	*29*	*8*
Dextromethorphan	*272.1*	*171.1*	*1.29*	*70*	*10*	*50*	*12*
Diazepam	*285.1*	*193*	*1.65*	*80*	*10*	*40*	*10*
Doxepin	*280.1*	*107*	*1.33*	*70*	*10*	*45*	*10*
EDDP	*278.1*	*234.1*	*1.28*	*80*	*10*	*50*	*12*
Fentanyl	*337.1*	*188.1*	*1.24*	*90*	*10*	*31*	*10*
Gabapentin	*172.1*	*137*	*0.85*	*50*	*10*	*20*	*10*

Table 2: MRM Table (part 2)

Analyte	Q1	Q3	RT (min)	DP	EP	CE	CXP
Imipramine	*281.1*	*86*	*1.42*	*80*	*10*	*25*	*6*
JWH 018 4-OH pentyl	*358.1*	*155.1*	*1.71*	*100*	*10*	*37*	*14*
JWH 018 Pentanoic acid	*372.1*	*127.1*	*1.77*	*90*	*10*	*71*	*10*
JWH 019 6-OH hexyl	*372.2*	*155.1*	*1.77*	*90*	*10*	*27*	*12*
JWH 073 3-OH butyl	*344.1*	*155.1*	*1.71*	*100*	*10*	*33*	*14*
JWH 073 Butanoic acid	*358.2*	*127.1*	*1.71*	*90*	*10*	*65*	*10*
JWH 081 5-OH-pentyl	*388.1*	*185.1*	*1.70*	*100*	*10*	*31*	*18*
JWH 122 5-OH pentyl	*372.1*	*169.1*	*1.79*	*90*	*10*	*32*	*14*
JWH 210 5-OH-pentyl	*386.1*	*183.1*	*1.86*	*100*	*10*	*45*	*18*
JWH 250-4-OH pentyl	*352.1*	*121.1*	*1.64*	*90*	*10*	*35*	*12*
Lorazepam	*321.1*	*275.1*	*1.53*	*80*	*10*	*40*	*10*
MDA	*180.1*	*133.1*	*1.00*	*40*	*10*	*25*	*10*
MDEA	*208.1*	*72*	*1.04*	*70*	*10*	*17*	*10*
MDMA	*194*	*51*	*1.01*	*36*	*10*	*81*	*10*
MDPV	*276.1*	*175.1*	*1.14*	*70*	*10*	*30*	*8*
Meperidine	*248.1*	*220.1*	*1.15*	*86*	*10*	*28*	*10*
Mephedrone	*178.1*	*145.1*	*1.04*	*46*	*10*	*26*	*6*
Meprobamate	*219*	*158*	*1.30*	*26*	*10*	*11*	*8*
Methadone	*310.1*	*265*	*1.42*	*80*	*10*	*25*	*18*
Methamphetamine	*150*	*119*	*0.99*	*60*	*10*	*15*	*8*
Methedrone	*194.1*	*161.1*	*1.00*	*50*	*10*	*26*	*6*
Methylone	*208.1*	*160.1*	*0.95*	*60*	*10*	*25*	*12*
Methylphenidate	*234.1*	*84.1*	*1.12*	*31*	*10*	*60*	*8*
Midazolam	*326.1*	*291.1*	*1.48*	*101*	*10*	*45*	*22*
Mitragynine	*399.2*	*174.1*	*1.26*	*71*	*10*	*59*	*12*
Morphine/ Hydromorphone/ Norcodeine/ Norhydrocodone	*286*	*152*	*0.87*	*90*	*10*	*75*	*12*
Naloxone	*328.1*	*310*	*0.95*	*50*	*10*	*27*	*20*
Naltrexone	*342.1*	*267.2*	*0.96*	*86*	*10*	*39*	*18*
N-desmethyl-Tapentadol	*208.1*	*107*	*1.15*	*120*	*10*	*30*	*10*
Norbuprenorphine	*414.3*	*83*	*1.23*	*141*	*10*	*70*	*12*
Nordiazepam	*271.1*	*140.1*	*1.61*	*71*	*10*	*37*	*10*
Norfentanyl	*233.1*	*84.1*	*1.08*	*50*	*10*	*23*	*8*
Normeperidine	*234.1*	*160*	*1.17*	*45*	*10*	*30*	*14*
Norpropoxyphene	*308.1*	*100*	*1.34*	*50*	*10*	*18*	*10*
Nortriptyline	*264.1*	*233.1*	*1.44*	*56*	*10*	*17*	*4*

Table 2: MRM Table (part 3)

Analyte	Q1	Q3	RT (min)	DP	EP	CE	CXP
O-Desmethyltramadol	*250.1*	*58.1*	*0.99*	*55*	*10*	*97*	*19*
Oxazepam	*287.1*	*241.1*	*1.54*	*76*	*10*	*31*	*18*
Oxycodone	*316.1*	*241.1*	*0.95*	*75*	*10*	*38*	*8*
Oxymorphone/ Dihydrocodeine/ Noroxycodone	*302*	*227.1*	*0.94*	*86*	*10*	*39*	*18*
PCP	*244.1*	*159.1*	*1.21*	*56*	*10*	*19*	*10*
Pregabalin	*160.1*	*55.1*	*0.70*	*58*	*10*	*31*	*8*
Propoxyphene	*340.1*	*266.1*	*1.39*	*46*	*10*	*20*	*10*
Protriptyline	*264.1*	*155*	*1.42*	*76*	*10*	*29*	*10*
RCS4-4-OH-pentyl	*338.2*	*135*		*90*	*10*	*40*	*8*
Ritalinic Acid	*220.1*	*84*	*1.10*	*50*	*10*	*60*	*8*
Sufentanil	*387.1*	*238*	*1.35*	*46*	*10*	*27*	*16*
Tapentadol	*222.1*	*107*	*1.13*	*100*	*10*	*40*	*8*
Temazepam	*301.1*	*255.1*	*1.56*	*70*	*10*	*50*	*8*
Tramadol	*264.1*	*58.1*	*1.09*	*66*	*10*	*103*	*8*
Zolpidem	*308.1*	*235.1*	*1.19*	*85*	*10*	*60*	*12*

LC performance

A Phenomenex Luna C18 column was used for LC separation. Since isobaric separation is not necesarry in screening tests, some analytes within the same class were reported together (Table 2). In Figure 3, the elution profile of all 86 compounds analyzed within the rapid 2.5 min gradient can be seen; the earliest eluting compound produced a peak at 0.7 min (pregabalin) and the latest at 1.86 min (JWH 210 5-OH-pentyl).

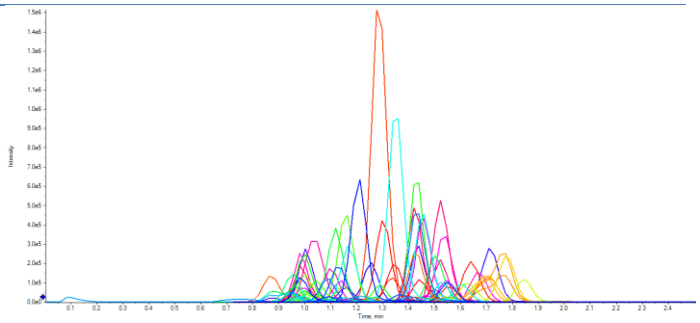

Figure 3: Elution profile of all the 86 compounds in screening panel.

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 in Analyst® 1.6.3 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.

Enhanced MS Data

The Enhanced MS (EMS) data was collected to assist in the identification of an unknown peak. Figure 4 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.

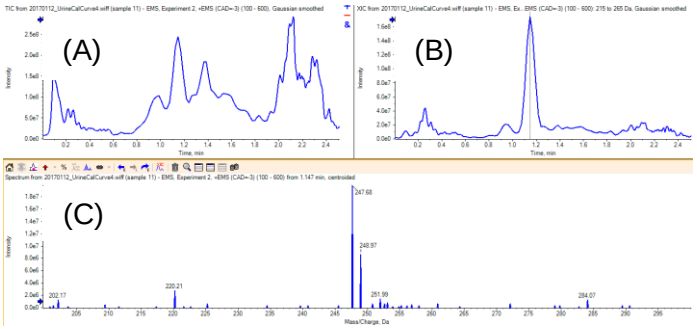

Figure 4: Enhanced MS data. (A) Total ion chromatogram (TIC) of EMS of the entire 2.5-min run. (B) 100-Da window extracted ion chromatogram (XIC) around 240 Da with EMS. (C) EMS mass spectrum at 1.15-min.

Analytical sensitivity

A single-point calibration was used for all 86 analytes, which yielded accurate results for low, medium, and high QCs. Figure 5, shows a typical calibration curves for alpha-PVP with excellent linearity fitted through zero. The XICs of the quality controls for alpha-PVP (232.1 → 91.0 m/z) are shown over three replicate sets of injections (Figure 6, row 1 to 3).

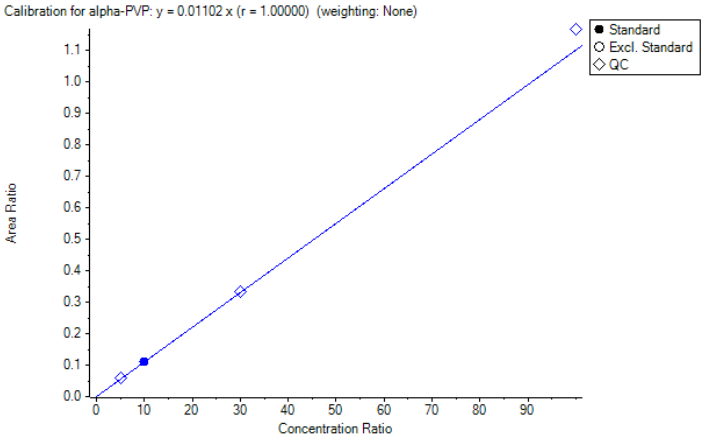

Figure 5: Typical calibration curve of alpha-PVP (232.1 → 91.0 m/z), plotted with quality controls.

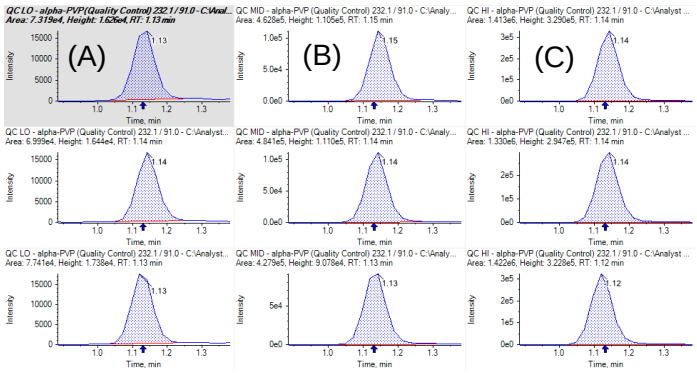

Figure 6: Extracted ion chromatograms (XICs) of alpha-PVP (n=3, 232.1 → 91.0 m/z) quality controls. (A) 5 ng/mL; (B) 30 ng/mL; (C) 100 ng/mL.

Conclusion

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 ExionLC™ AC HPLC system and the SCIEX QTRAP® 4500 pair to produce high quality data for all analytes, while utilizing samples prepared by rapid enzyme hydrolysis and a simple dilute and shoot pretreatment

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