

# 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.

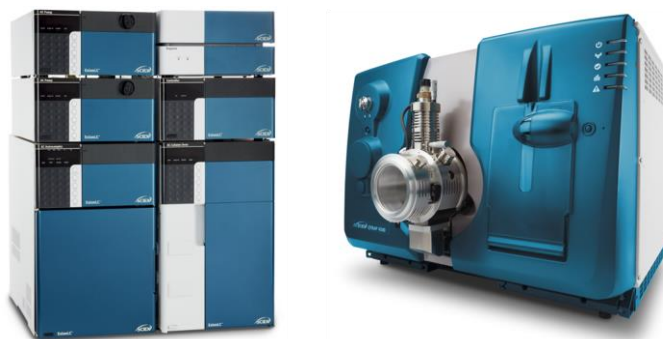


Figure 1: The SCIEX ExionLC™ AC HPLC system (left) and the SCIEX QTRAP® 4500 LC-MS/MS System (right).

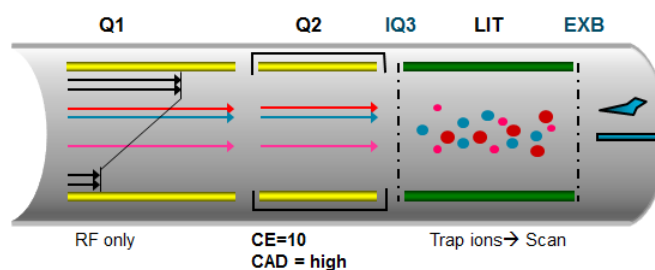


Figure 2: Depiction of how Enhanced MS (EMS) works on the QTRAP system.

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 its 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 its low, mid, and high QCs were 50, 300 and 1000 ng/mL.

### Sample preparation

1. 100  $\mu$ L urine sample was mixed with 25  $\mu$ L IMCS Rapid Hydrolysis Buffer, 20  $\mu$ L IMCSzyme and 10  $\mu$ L **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  $\mu$ L.

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  $\mu$ L/sec

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

**Table 2: MRM Table (part 2)**

Analyte	Q1	Q3	RT (min)	DP	EP	CE	CXP
<i>Imipramine</i>	281.1	86	1.42	80	10	25	6
<i>JWH 018 4-OH pentyl</i>	358.1	155.1	1.71	100	10	37	14
<i>JWH 018 Pentanoic acid</i>	372.1	127.1	1.77	90	10	71	10
<i>JWH 019 6-OH hexyl</i>	372.2	155.1	1.77	90	10	27	12
<i>JWH 073 3-OH butyl</i>	344.1	155.1	1.71	100	10	33	14
<i>JWH 073 Butanoic acid</i>	358.2	127.1	1.71	90	10	65	10
<i>JWH 081 5-OH-pentyl</i>	388.1	185.1	1.70	100	10	31	18
<i>JWH 122 5-OH pentyl</i>	372.1	169.1	1.79	90	10	32	14
<i>JWH 210 5-OH-pentyl</i>	386.1	183.1	1.86	100	10	45	18
<i>JWH 250-4-OH pentyl</i>	352.1	121.1	1.64	90	10	35	12
<i>Lorazepam</i>	321.1	275.1	1.53	80	10	40	10
<i>MDA</i>	180.1	133.1	1.00	40	10	25	10
<i>MDEA</i>	208.1	72	1.04	70	10	17	10
<i>MDMA</i>	194	51	1.01	36	10	81	10
<i>MDPV</i>	276.1	175.1	1.14	70	10	30	8
<i>Meperidine</i>	248.1	220.1	1.15	86	10	28	10
<i>Mephedrone</i>	178.1	145.1	1.04	46	10	26	6
<i>Meproamate</i>	219	158	1.30	26	10	11	8
<i>Methadone</i>	310.1	265	1.42	80	10	25	18
<i>Methamphetamine</i>	150	119	0.99	60	10	15	8
<i>Methedrone</i>	194.1	161.1	1.00	50	10	26	6
<i>Methylone</i>	208.1	160.1	0.95	60	10	25	12
<i>Methylphenidate</i>	234.1	84.1	1.12	31	10	60	8
<i>Midazolam</i>	326.1	291.1	1.48	101	10	45	22
<i>Mitragynine</i>	399.2	174.1	1.26	71	10	59	12
<i>Morphine/ Hydromorphone/ Norcodeine/ Norhydrocodone</i>	286	152	0.87	90	10	75	12
<i>Naloxone</i>	328.1	310	0.95	50	10	27	20
<i>Naltrexone</i>	342.1	267.2	0.96	86	10	39	18
<i>N-desmethyl-Tapentadol</i>	208.1	107	1.15	120	10	30	10
<i>Norbuprenorphine</i>	414.3	83	1.23	141	10	70	12
<i>Nordiazepam</i>	271.1	140.1	1.61	71	10	37	10
<i>Norfentanyl</i>	233.1	84.1	1.08	50	10	23	8
<i>Normeperidine</i>	234.1	160	1.17	45	10	30	14
<i>Norpropoxyphene</i>	308.1	100	1.34	50	10	18	10
<i>Nortriptyline</i>	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
<i>O-Desmethyltramadol</i>	250.1	58.1	0.99	55	10	97	19
<i>Oxazepam</i>	287.1	241.1	1.54	76	10	31	18
<i>Oxycodone</i>	316.1	241.1	0.95	75	10	38	8
<i>Oxymorphone/ Dihydrocodeine/ Noroxycodone</i>	302	227.1	0.94	86	10	39	18
<i>PCP</i>	244.1	159.1	1.21	56	10	19	10
<i>Pregabalin</i>	160.1	55.1	0.70	58	10	31	8
<i>Propoxyphene</i>	340.1	266.1	1.39	46	10	20	10
<i>Protriptyline</i>	264.1	155	1.42	76	10	29	10
<i>RCS4-4-OH-pentyl</i>	338.2	135		90	10	40	8
<i>Ritalinic Acid</i>	220.1	84	1.10	50	10	60	8
<i>Sufentanil</i>	387.1	238	1.35	46	10	27	16
<i>Tapentadol</i>	222.1	107	1.13	100	10	40	8
<i>Temazepam</i>	301.1	255.1	1.56	70	10	50	8
<i>Tramadol</i>	264.1	58.1	1.09	66	10	103	8
<i>Zolpidem</i>	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 necessary 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).

### 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.

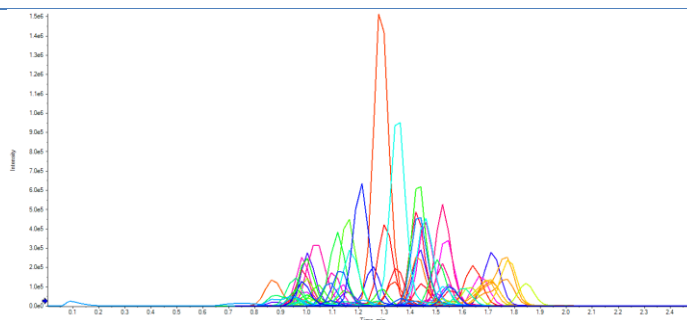


Figure 3: Elution profile of all the 86 compounds in screening panel.

### Enhanced MS Data

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

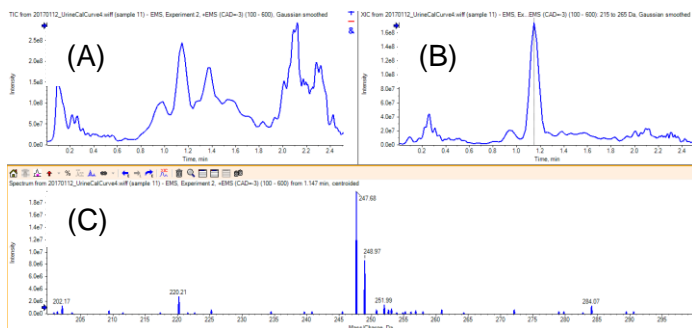


Figure 4: Enhanced MS data. (A) Total ion chromatogram (TIC) of EMS of the entire 2.5-min run. (B) 100-Da window extracted ion chromatogram (XIC) around 240 Da with EMS. (C) EMS mass spectrum at 1.15-min.

### 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).

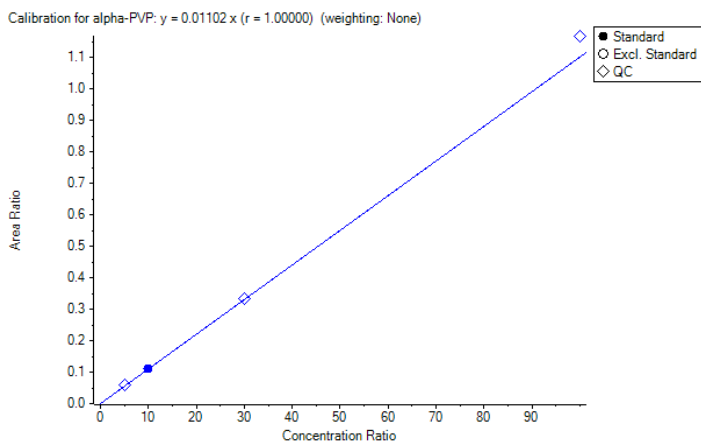


Figure 5: Typical calibration curve of alpha-PVP (232.1 → 91.0 m/z), plotted with quality controls.

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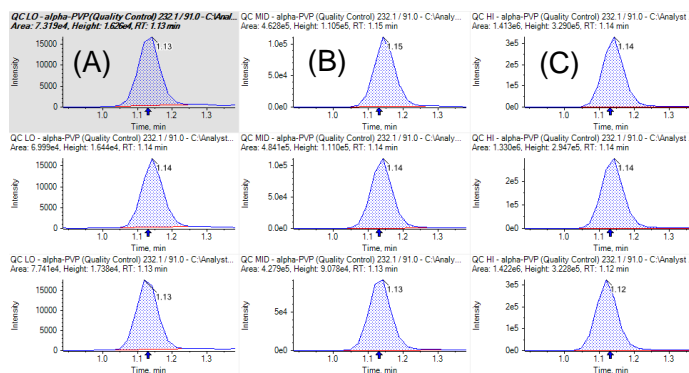


Figure 6: Extracted ion chromatograms (XICs) of alpha-PVP (n=3, 232.1 → 91.0 m/z) quality controls. (A) 5 ng/mL; (B) 30 ng/mL; (C) 100 ng/mL.

### 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 injection samples prepared by rapid enzyme hydrolysis and a simple dilute and shoot pretreatment.