Application Note 588

High-Throughput Quantitative LC-MS/MS Analysis of 6 Opiates and 14 Benzodiazepines in Urine

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Key Words
Opiates, benzodiazepines, Prelude SPLC

Goal
Develop a high-throughput, low solvent consumption, easy-to-run method for quantitative forensic analysis of six opiates and fourteen benzodiazepines in urine.

Introduction
Analyses of opiate and benzodiazepine panels are some of the highest-volume applications in forensic toxicology labs. In order to meet the need for high throughput, a fast, simple, and cost-effective method was developed, consisting of hydrolysis, simple urine dilution, separation by liquid chromatography (LC), and analysis by mass spectrometry (MS). The method incorporated the Thermo Scientific™ Prelude SPLC™ system (Figure 1), which features two independent channels of sample preparation and liquid chromatography (SPLC). With the Prelude SPLC system, LC methods can be executed in parallel with a different method on each channel (Figure 2) or the same method on both channels (Figure 3) and multiplexed into a mass spectrometer for serial detection. Serial MS detection of multiplexed methods improves mass spectrometer utilization time, increases throughput of forensic toxicology laboratories, and reduces analysis cost. The syringe pumps and high-pressure, low-volume gradient mixing used in Prelude SPLC system provide enhanced HPLC performance: improved peak shape and resolution as well as stable retention times.

Figure 1. Prelude SPLC system

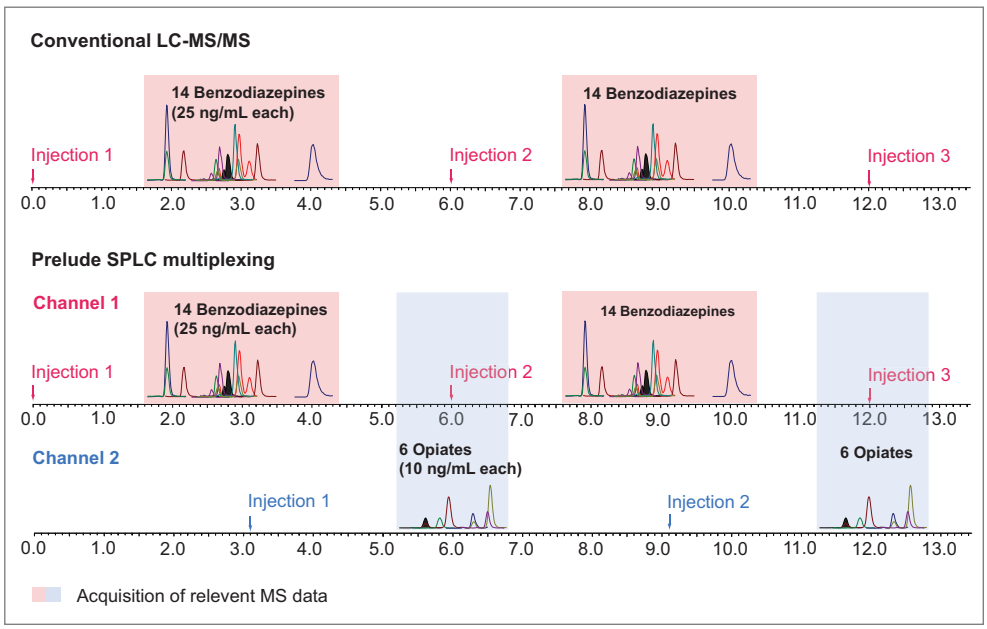

Figure 2. Parallel analysis of 6 opiates (10 ng/mL) and 14 benzodiazepines (25 ng/mL) in multiplexed mode

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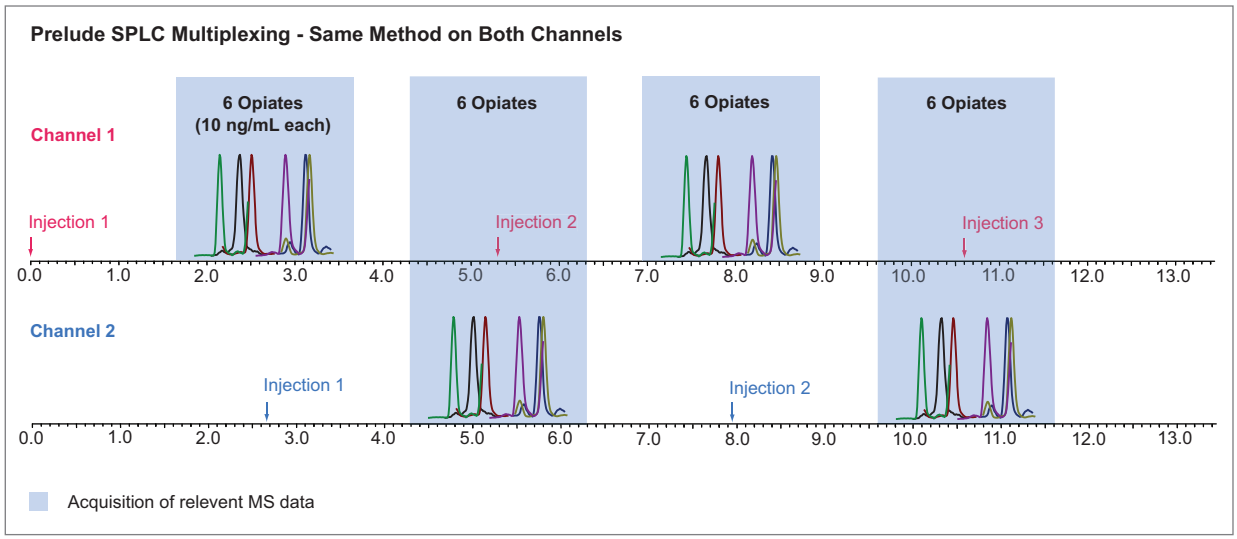

Figure 3. Analysis of six opiates (10 ng/mL) using both channels in multiplexed mode

Experimental

Sample Preparation

Tables 1 and 2 contain the lists of opiates and benzodiazepines analyzed. Sample preparation consisted of glucuronide hydrolysis followed by dilution. For each sample, a 200 µL aliquot of urine was spiked with 10 µL of internal standards solution and 100 µL of β-glucuronidaze enzyme in an ammonium acetate buffer (pH = 5.0). The samples were incubated at 60 °C for 2 hours. A 200 µL aliquot of methanol was added to each sample to stop enzymatic reaction. Samples were cooled, centrifuged, and diluted 20 times with deionized water. Then, 20 µL of sample was injected into the liquid chromatograph-mass spectrometer (LC-MS) system.

Table 1. SRM transitions for opiates method

Compound	Precursor Ion *m/z*	Quantifier Ion *m/z*	Qualifier Ion *m/z*
Morphine	286.1	152.1	185.1
Morphine-d_3	289.1	152.1	185.1
Oxymorphone	302.1	227.1	198.1
Oxymorphone-d_3	305.1	230.1	201.1
Hydromorphone	286.1	185.0	157.1
Hydromorphone-d_6	292.1	185.1	157.1
Codeine	300.2	152.1	165.1
Codeine-d_3	303.1	152.1	215.1
Oxycodone	316.2	241.1	256.1
Oxycodone-d_3	319.2	244.1	259.1
Hydrocodone	300.1	199.1	171.1
Hydrocodone-d_3	303.1	199.1	171.1

Table 2. SRM transitions for benzodiazepines method

Compound	Precursor Ion *m/z*	Quantifier Ion *m/z*	Qualifier Ion *m/z*
7-Aminoclonazepam	286.1	222.1	250.1
7-Aminoclonazepam-d_4	290.1	226.1	254.1
7-Aminonitrazepam	252.1	121.1	224.1
7-Aminoflunitrazepam	284.1	135.1	227.1
7-Aminoflunitrazepam-d_7	291.2	138.2	230.1
α-Hydroxytriazolam	359.0	331.0	239.0
Lorazepam	321.0	275.0	229.0
α-Hydroxyalprazolam	325.0	297.1	229.0
α-Hydroxyalprazolam-d_5	330.1	302.1	216.1
Oxazepam	287.1	241.1	221.1
Oxazepam-d_5	292.1	246.1	104.1
2-Hydroxyethylflurazepam	333.1	109.1	109.1
2-Hydroxyethylflurazepam-d_4	337.1	215.1	113.1
Desalkyflurazepam	289.0	140.0	226.1
Desalkyflurazepam-d_4	293.1	140.0	230.1
Temazepam	301.1	255.1	177.0
Temazepam-d_5	306.1	260.1	177.0
Nordiazepam	271.1	140.1	208.1
Nordiazepam-d_5	276.1	213.1	140.0
Alprazolam	309.1	281.1	205.1
Alprazolam-d_5	314.1	286.1	210.1
Diazepam	285.1	193.1	154.0
Diazepam-d_5	290.1	198.1	154.1
Midazolam	326.1	291.1	249.1
Midazolam-d_4	330.1	295.1	253.1

Liquid Chromatography

Chromatographic separations were performed with a Prelude SPLC system by direct injections onto Thermo Scientific™ Accucore™ PFP 50 x 2.1 mm, 2.6 µm analytical columns. The columns were maintained at room temperature. Mobile phases A and B consisted of 10 mM ammonium formate with 0.1% formic acid in water and methanol, respectively. Mobile phase usage was about 3.8 mL per sample. The total gradient run time was 5.3 min for opiates analysis (Figure 4) and 6 min for benzodiazepines analysis (Figure 5). The data acquisition windows were 2 min and 2.8 min for opiates and benzodiazepines, respectively.

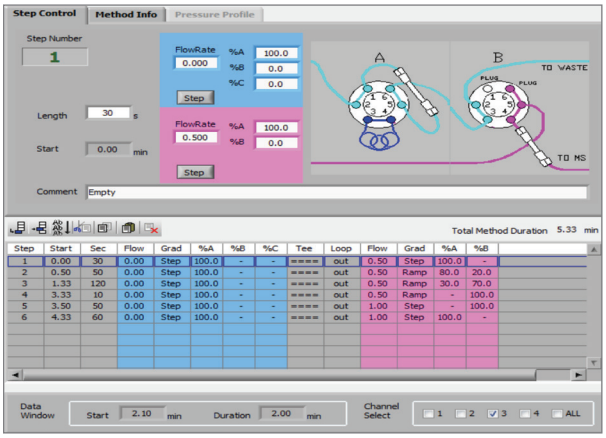

Figure 4. LC gradient for opiates analysis

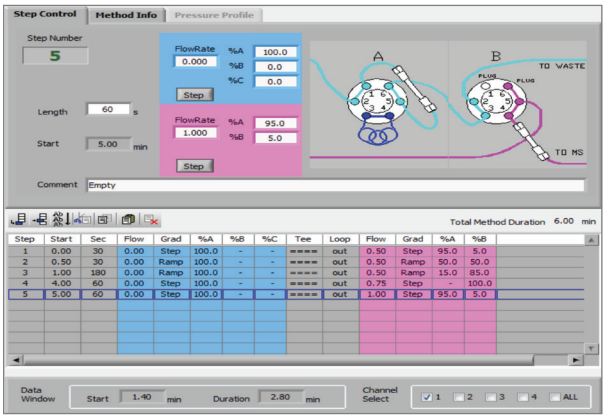

Figure 5. LC gradient for benzodiazepines analysis

Mass Spectrometry

MS analysis was carried out on a Thermo Scientific™ TSQ Quantum Ultra™ triple quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI-II) probe. The mass spectrometer was operated in selected-reaction monitoring (SRM) mode. Two SRM transitions were collected for each analyte and each internal standard (Tables 1 and 2) to calculate the ion ratio.

Validation

Standard curves were prepared by fortifying pooled blank human urine with analytes. Quality control (QC) samples were prepared in a similar manner at concentrations corresponding to the low (LQC), middle (MQC), and high (HQC) ranges of the calibration curve. Intra-run precisions were determined by processing six replicates of each QC level along with a calibration curve on three different days. Matrix effects were investigated by analyzing seven donated urine samples spiked at concentrations of 27.5 ng/mL for opiates and 50 ng/mL for benzodiazepines. The method performance was compared with method validated in a forensic toxicology lab by analyzing the same donor samples. Method validation experiments were run by executing opiates and benzodiazepines methods in parallel on two channels in multiplexed mode.

Results and Discussion

Opiates Analysis

The limits of quantitation were 10 ng/mL and calibration ranges were 10–6000 ng/mL for all opiates. Figure 6 shows representative calibration curves for selected opiates. Figure 7 shows representative chromatograms at 10 ng/mL for all opiates tested. Intra- and inter-assay quality control statistics shown in Table 3 demonstrate the method to be reproducible across the calibration range for the opiates. Limited matrix effects were seen, and those were largely mediated by deuterated internal standards (Table 4). The data collected with this method correlated well with data collected using an LC/MS method previously validated in a collaborating laboratory (Figure 8).

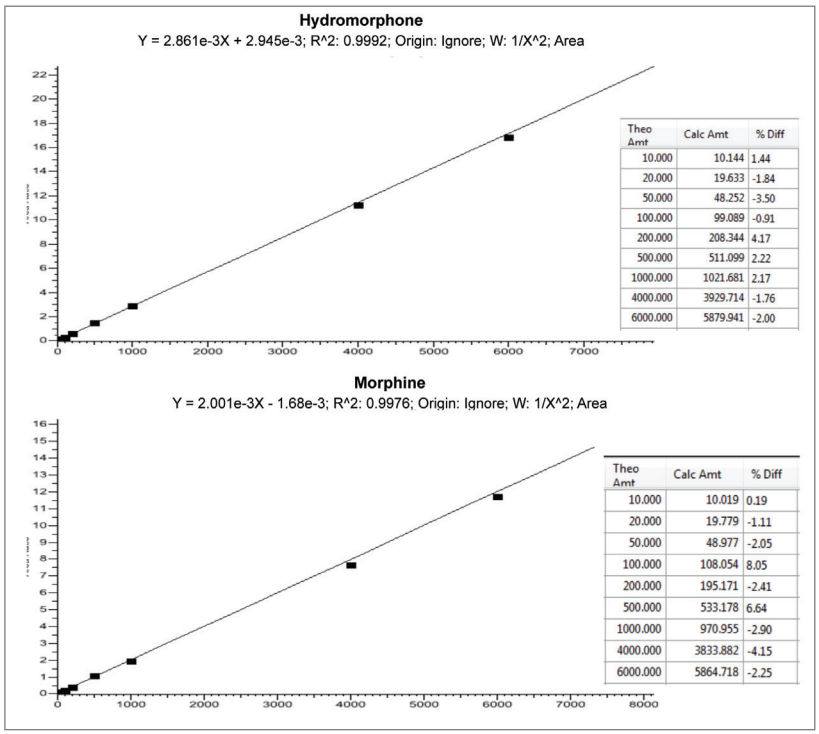

Figure 6. Calibration curves for selected opiates

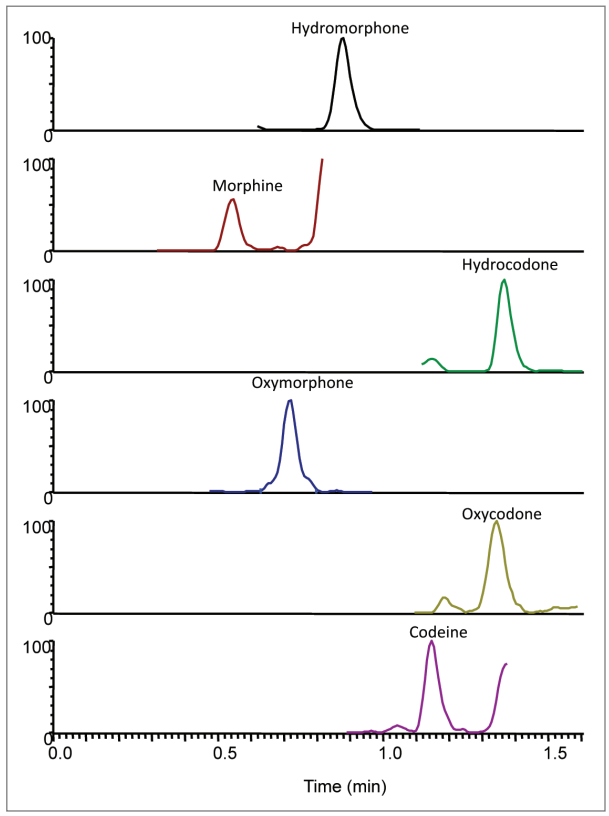

Figure 7. Chromatograms of the lowest opiates calibration standard (10 ng/mL)

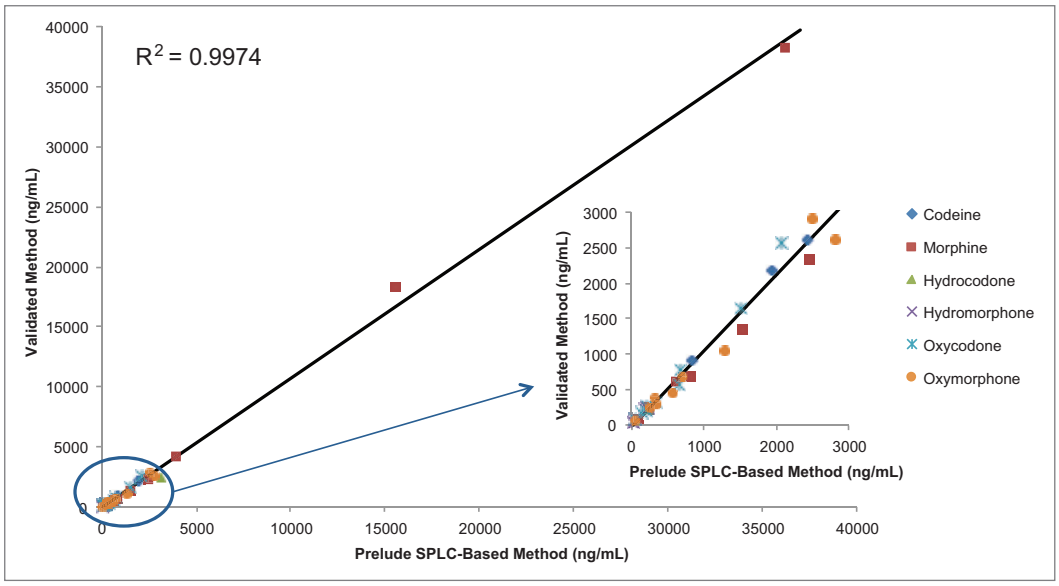

Figure 8. Data correlation between Prelude SPLC-based opiates method and a previously validated LC/MS method

Table 3. Intra- and inter-assay precision for opiates analyses

Compound	Precision % RSD					
	Intra-assay			Inter-assay		
	LQC	MQC	HQC	LQC	MQC	HQC
Morphine	<7.0	<2.8	<2.3	8.3	2.5	3.3
Hydromorphone	<3.7	<1.8	<1.7	4.7	2.2	2.7
Oxymorphone	<5.9	<4.8	<4.9	9.8	4.9	9.8
Codeine	<8.2	<11	<2.2	8.2	4.8	3.0
Hydrocodone	<4.7	<3.8	<2.8	4.7	3.9	4.2
Oxycodone	<7.4	<3.9	<2.8	7.1	3.8	3.6

Table 4. Results of matrix effect experiment showing percent recovery of opiates in spiked urine

Urine Lot#	% Recovery					
	Morphine	Hydromorphone	Oxymorphone	Codeine	Hydrocodone	Oxycodone
1	96.1	93.7	104	102	99.7	93.9
2	99.8	93.8	101	100	99.7	99.6
3	91.0	98.5	101	102	98.1	93.8
4	90.7	96.5	105	103	95.8	101
5	93.9	103	94.9	99.7	97.0	96.9
6	92.3	100	107	109	106	103
7	92.0	97.8	108	109	100	103

Benzodiazepines Analysis

The limits of quantitation were 25 ng/mL and calibration ranges were 25–2000 ng/mL for all benzodiazepines. Figure 9 shows representative calibration curves for selected benzodiazepines. Figure 10 shows representative chromatograms at 25 ng/mL for all benzodiazepines tested. Intra- and inter-assay quality control statistics shown in Table 5 demonstrate the method to be reproducible across the calibration range for these benzodiazepines. Use of deuterated internal standard eliminated the small matrix effects we experienced with the method (Table 6). The data collected with this method correlated well with data collected using an LC/MS method previously validated in a collaborating laboratory (Figure 11).

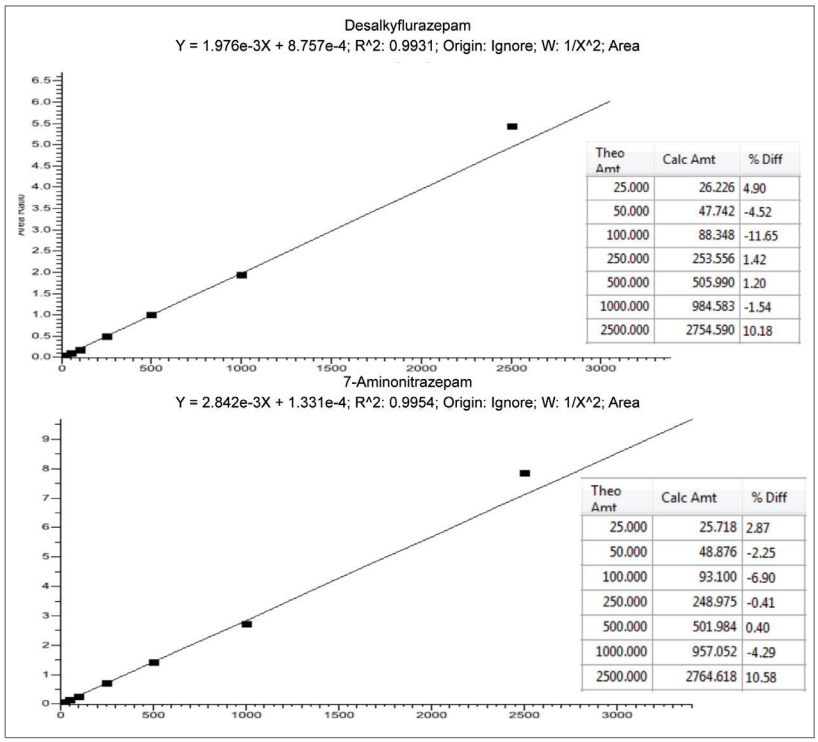

Figure 9. Calibration curves for selected benzodiazepines

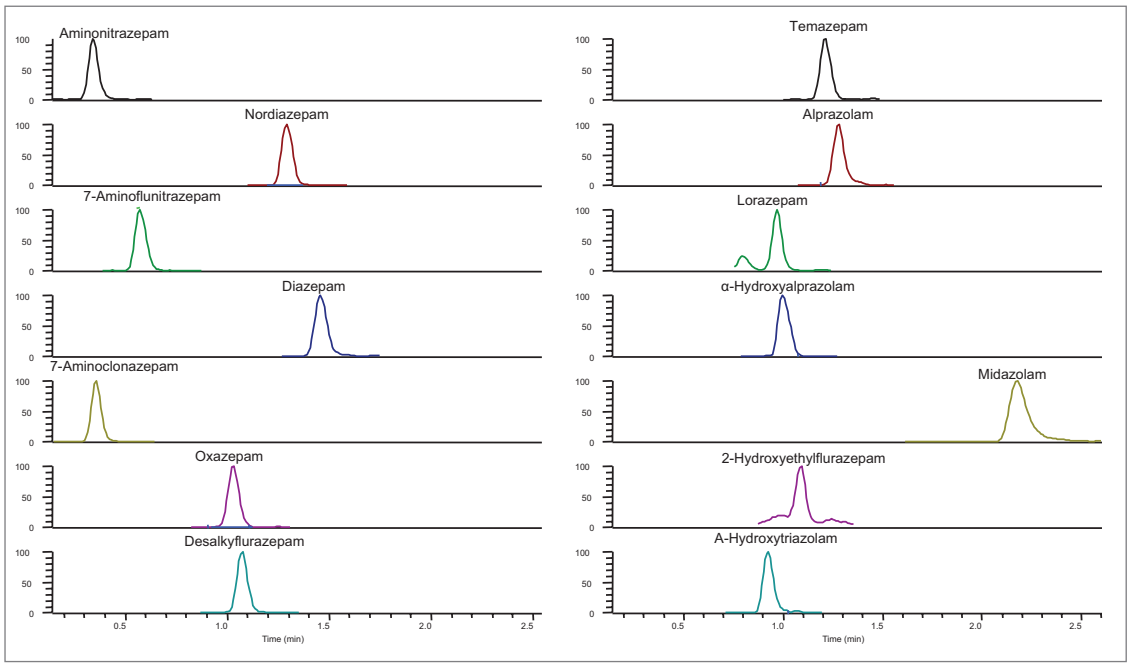

Figure 10. Chromatogram of the lowest benzodiazepines calibration standard (10 ng/mL)

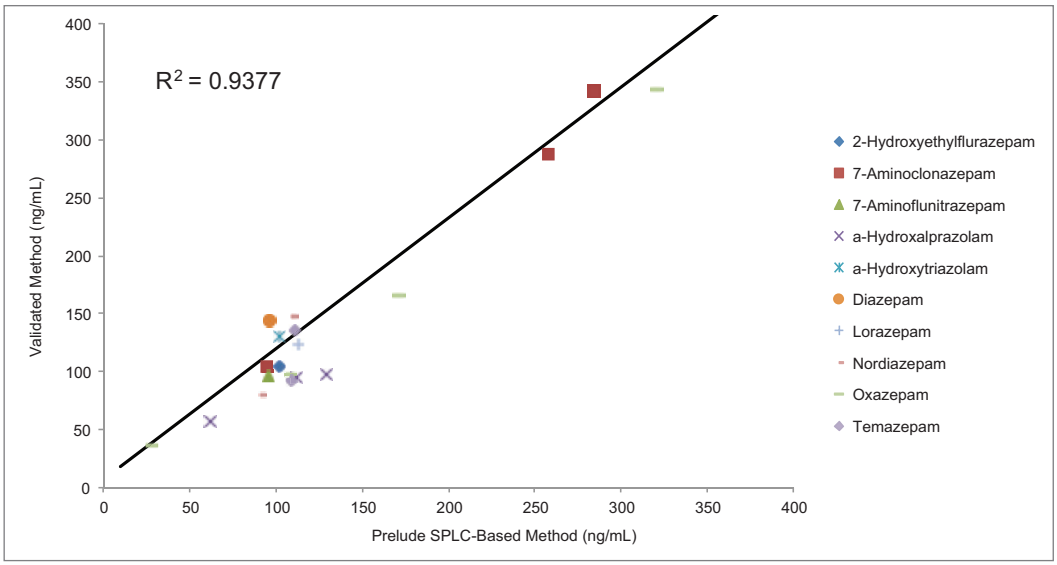

Figure 11. Data correlation between Prelude SPLC-based benzo-diazepines method and a previously validated LC/MS method

Table 5. Intra and inter-assay precision for benzodiazepines analyses

Compound	Precision % RSD			
	Intra-assay		Inter-assay	
	LQC	HQC	LQC	HQC
2-Hydroxyethylflurazepam	<10	<3.3	8.9	4.2
7-Aminoclonazepam	<2.5	<2.7	2.5	2.0
7-Aminoflunitrazepam	<3.6	<3.2	3.1	2.9
7-Aminonitrazepam	<2.7	<3.6	2.4	2.7
α-Hydroxyalprazolam	<5.8	<4.1	5.8	4.5
α-Hydroxytriazolam	<5.9	<4.1	7.2	3.8
Alprazolam	<5.2	<2.1	3.5	2.3
Desalkyflurazepam	<5.3	<5.9	3.6	2.3
Diazepam	<2.8	<3.0	3.1	2.2
Lorazepam	<5.3	<4.5	6.7	3.3
Midazolam	<1.2	<5.4	2.8	1.6
Nordiazepam	<4.0	<4.5	5.1	2.5
Oxazepam	<3.3	<3.2	3.3	3.9
Temezepam	<5.3	<3.1	4.3	3.6

Table 6. Results of matrix effect experiment showing percent recovery of benzodiazepines in spiked urine

Urine Lot#	% Recovery						
	2-Hydroxy-ethylflurazepam	7-Aminoclona-zepam	7-Aminoflunitra-zepam	7-Aminonitra-zepam	α-Hydroxy-alprazolam	α-Hydroxy-triazolam	Alprazolam
1	112	104	103	99.2	111	114	116
2	102	99.8	103	104	112	116	113
3	106	103	101	102	113	116	108
4	112	104	106	100	108	111	114
5	100	102	102	95.8	110	111	108
6	118	105	109	104	113	118	111
7	106	101	99.7	104	111	124	110
8	107	97.8	98.5	101	112	93.0	107

Urine Lot#	% Recovery						
	Desalkyflura-zepam	Diazepam	Lorazepam	Midazolam	Nordiazepam	Oxazepam	Temezepam
1	114	118	110	117	110	105	111
2	109	111	104	116	112	99.8	105
3	108	112	103	117	113	103	102
4	107	114	108	114	118	105	106
5	105	115	108	117	112	99.0	106
6	114	113	109	115	111	104	105
7	108	113	96.2	117	112	98.4	103
8	107	107	101	112	111	95.7	103

Conclusion

Using the Prelude SPLC system, high-throughput, cost-efficient solutions were developed for forensic analysis of opiates and benzodiazepines in urine. The methods met industry requirements for precision, accuracy, and robustness. Implementation of the method on a Prelude SPLC simplified the work flow and resulted in a 40–60% reduction of solvent usage due to the ability of the system to utilize high efficiency, small diameter columns. The mobile phase volumes in developed methods were approximately 3.8 mL per sample, which reduced cost of reagents and waste disposal. Multiplexing into a single mass spectrometer increased MS utilization and reduced overall system hardware costs relative to two independent LC-MS systems. The Prelude SPLC system makes multiplexing of two different methods, with or without on-line sample prep, possible and enabled a throughput of 480 samples in 24 hours. The implementation of methods was facilitated by the many ease-of-use features incorporated into the system.

Acknowledgement

Thanks to Kent Johnson, Director of Toxicology at Pacific Hospital of Long Beach, CA for providing donor urine samples and data collected with their validated LC-MS method.

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