Application Note 570

Quantitation of Six Opioids in Urine with Super-Dilution and Microflow LC-MS/MS

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Key Words

TSQ Vantage, Microflow, LC-MS/MS, Forensic Toxicology

Goal

To quantitate six opioids in urine with 500-fold urine dilution and microflow LC-MS/MS for forensic toxicology use, using the Thermo Scientific Dionex UltiMate 3000 RSLCnano LC system and the Thermo Scientific TSQ Vantage mass spectrometer.

Introduction

Morphine, codeine, hydromorphone, hydrocodone, oxymorphone and oxycodone are some of the most abused opioids in the United States. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been widely used for their quantitation in forensic toxicology. The analytical methods typically use normal LC flow rates (~0.5 mL/min) and sample preparation usually involves solid phase extraction (SPE) for sensitive detection. Microflow LC uses significantly lower flow rates (15 to 50 µL/min). With the same sample amount and identical LC peak width, the reduction in LC flow rate results in a much-improved detection limit for concentration-dependent detection techniques such as electrospray ionization (ESI) mass spectrometry. Because of this sensitivity increase, we can achieve a similar analytical performance for sensitive measurements of urine opioids for forensic toxicology purposes with a simple "dilute-and-shoot" approach.

Our goal was to use a super-dilution approach to improve the dilute-and-shoot detection of opioids in urine by minimizing matrix effects, and to compensate the sensitivity decrease from super-dilution by using microflow LC. We anticipated savings in solvent consumption and the cost of waste disposal, better environmental conservation, and improved longevity of the LC-MS/MS system.

Methods

Sample Preparation

Urine samples were spiked with internal standards (IS) and then mixed with β-glucuronidase and incubated at 60 °C for hydrolysis. Methanol was added to the mixture and the supernatant was diluted. The tested dilution factors were 100, 250 and 500. The mixture was centrifuged at 17,000 g for 5 minutes, and 20 µL of supernatant was injected for microflow LC-MS/MS analysis.

LC-MS/MS Conditions

LC-MS/MS analysis was performed on a TSQ Vantage™ triple stage quadrupole mass spectrometer coupled to an UltiMate™ 3000 RSLCnano LC system equipped with a microflow flow rate selector. The microflow LC plumbing was set up in "pre-concentration on a trapping column" mode (Figure 1). The temperature of the columns was maintained at 35 °C. The trapping column was a Thermo Scientific Hypersil GOLD PFP drop-in guard cartridge (10 × 1 mm, 5 µm particle size) in the guard holder, and the analytical column was a Hypersil GOLD™ PFP column (100 × 0.32 mm, 5 µm particle size). LC connections were made with Thermo Scientific Dionex nanoViper fingertight fittings. The LC gradients for sample loading and analytical elution are shown in Figure 2. The mass spectrometer was operated with a heated electrospray ionization (HESI-II) source in positive ionization mode. Data was acquired in selected-reaction monitoring (SRM) mode. Detailed source parameters and SRM settings are shown in Figure 3. For each analyte, two SRM transitions were monitored. One of them was used as the quantifier and the other as qualifier. The signal ratio between the qualifier and the quantifier was used to evaluate the validity of the results, and any ratio outside 20% (relative to the ratio) was considered an invalid data point.

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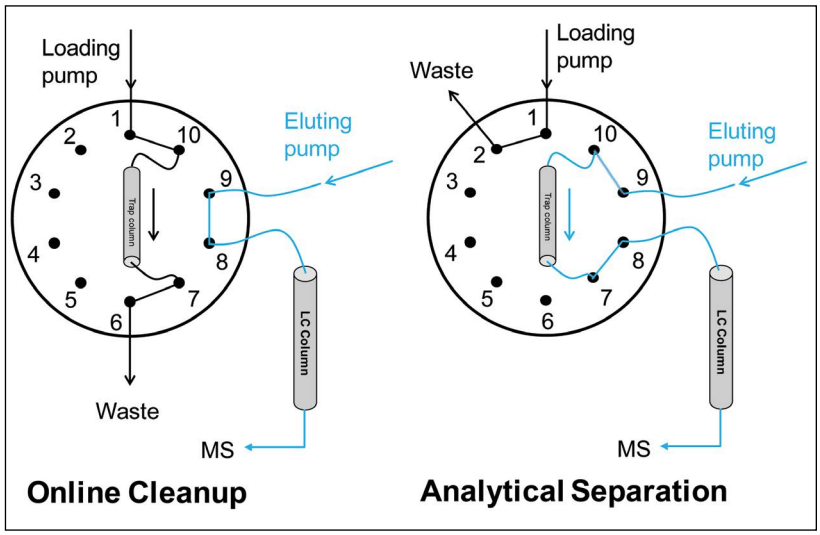

Figure 1. Microflow LC setup with pre-concentration trapping column

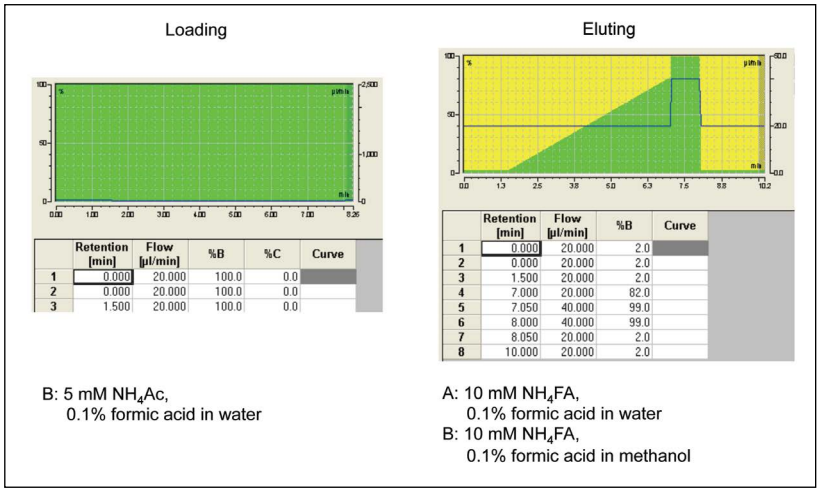

Figure 2. LC gradients of microflow LC with online clean-up

Polarity: positive
Spray Voltage (V): 4000
Vaporizer Temperature (°C): 150
Capillary Temperature (°C): 270
Sheath Gas (AU): 15
Aux Gas (AU): 2

Scan Mode: SRM
Scan Width (*m/z*): 0.02
Scan Time (Sec): 0.1
Q1 (FWHM, *m/z*): 0.7
Q3 (FWHM, *m/z*): 0.7
Collision Gas (Torr): 1.5

Analytes	Precursor (*m/z*)	Quantifier (*m/z*)	Qualifier (*m/z*)	Ion Ratio (%)	IR Window (%)
Morphine	286.1	152.2	201.1	90.0	18.0
Codeine	300.1	165.1	215.1	68.0	13.6
Hydromorphone	286.11	185.1	157.1	79.0	15.8
Hydrocodone	300.11	199.1	171.1	39.0	7.8
Oxymorphone	302.1	227	198.1	69.0	13.8
Oxycodone	316.1	298.1	256.1	22.5	4.5

Figure 3. MS source parameters and SRM transitions

Results and Discussion

Validation

The validation procedure includes tests for 1) recovery; 2) lower limit of quantitation (LLOQ), dynamic range, accuracy; 3) precision; and 4) carryover.

Recovery

First, we determined the optimal dilution factor for urine sample preparation. Twelve lots of blank human urine samples, six lots of donor urine samples, and two water samples were spiked with the IS, hydrolyzed, and diluted 100-, 250- and 500-fold with water. The SRM signals of the internal standards from the urine samples and the water samples were compared for absolute recovery. Table 1 shows the average recoveries (n=18) for the six opioids using different dilution factors. Clearly, the 500-fold dilution led to the highest recoveries for all six opioids.

We used the 500-fold dilution to determine the recoveries for unlabeled opioids spiked into 12 lots of blank urine samples. Two concentrations of opioids at 100 and 500 ng/mL were tested. The absolute recovery was determined by comparing the signals of unlabeled opioids in urine and water samples. The relative recovery was determined by comparing the analyte/IS ratio in urine and water samples. The recovery results are summarized in Table 2. There was minimum ion suppression for morphine, codeine, hydromorphone and hydrocodone. Although there was moderate ion suppression for oxymorphone and oxycodone even after 500-fold dilution, the relative recoveries against their IS were nearly 100% in both concentration levels after compensation from the IS.

Table 1. Dilution factor test results

Recovery (%, n=18)	500x	250x	100x
Morphine-d3	101.2	86.6	85.4
Codeine-d3	99.5	88.0	79.7
Hydromorphone-d6	85.9	73.1	63.7
Hydrocodone-d3	78.0	68.2	67.2
Oxymorphone-d3	59.9	45.1	43.2
Oxycodone-d3	68.2	52.3	42.3

Table 2. Summary of recoveries with 500-fold dilution

		100 ng/mL[a]		500 ng/mL[a]	
Analyte	Recovery (%)	Average (%, n=12[b])	Standard Deviation (%, n=12)	Average (%, n=12)	Standard Deviation (%, n=12)
Morphine	Absolute	76.4	6.8	78.6	5.4
	Relative	92.1	10.9	96.1	9.6
Codeine	Absolute	86.5	6.0	89.7	6.2
	Relative	88.7	10.6	95.6	8.2
Hydromorphone	Absolute	74.4	7.1	73.2	6.6
	Relative	92.8	8.1	89.9	7.0
Hydrocodone	Absolute	82.6	9.0	71.8	6.7
	Relative	101.9	17.1	83.6	13.4
Oxymorphone	Absolute	57.5	7.6	57.9	7.0
	Relative	103.7	17.8	103.0	15.1
Oxycodone	Absolute	63.4	9.9	68.7	8.1
	Relative	90.6	8.5	103.8	8.5

[a] Two levels of spiked opioids concentrations were tested.

[b] Twelve different individual urine lots were tested and compared to water samples (n=2).

Lower Limit of Quantitation (LLOQ), Dynamic Range, and Accuracy

Blank human urine samples were spiked with the six opioids and their IS. Concentrations of the opioids ranged from 20 to 5000 ng/mL. At each concentration level, three individually processed replicates were tested. The concentration of IS was 100 ng/mL for all samples. Linearity samples were analyzed in triplicate along with one set of calibrators, which were also prepared in blank human urine. The calibration curves for morphine and codeine (Figures 4 and 5) were constructed by plotting the analyte/IS peak area ratio vs. analyte concentration.

The linearity was determined to be 20 to 5000 pg/mL for all six opioids. The LLOQ for the six opioids were determined to be 20 ng/mL. At LLOQ, the accuracy (n=3) ranged from 99.2% to 115.5% for the six opioids and the precision (n=3) ranged from 3.9% to 8.8% (Table 3). Within the linear range, the accuracies (at higher than LLOQ levels) were within 11.2% for the six opioids (data not shown). Figures 4 and 5 show the calibration curves for morphine and codeine. Figure 6 shows the SRM chromatograms of the six opioids at their LLOQ in spiked human urine. The signal-to-noise ratios for all six opioids at their LLOQs were excellent.

Table 3. LLOQ, linear range and accuracy for the six opioids in urine

Analyte	LLOQ (ng/mL)	Linear range (ng/mL)	Accuracy at LLOQ (%, n=3)	Precision at LLOQ (%, n=3)
Morphine	20	20-5000	100.8	6.1
Codeine	20	20-5000	102.1	6.9
Hydromorphone	20	20-5000	115.5	8.8
Hydrocodone	20	20-5000	99.2	3.9
Oxymorphone	20	20-5000	102.3	6.2
Oxycodone	20	20-5000	107.4	4.4

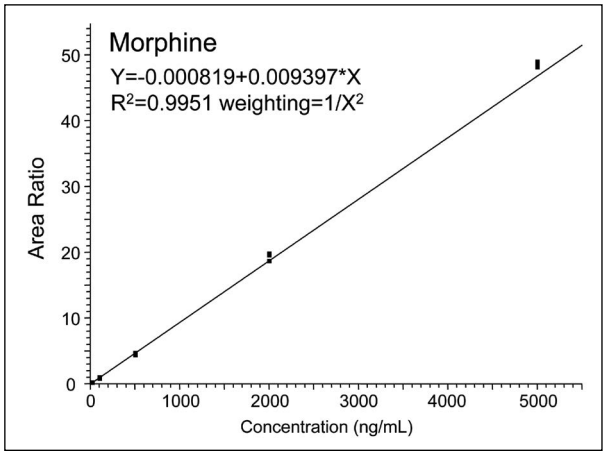

Figure 4. Calibration curve of morphine in human urine

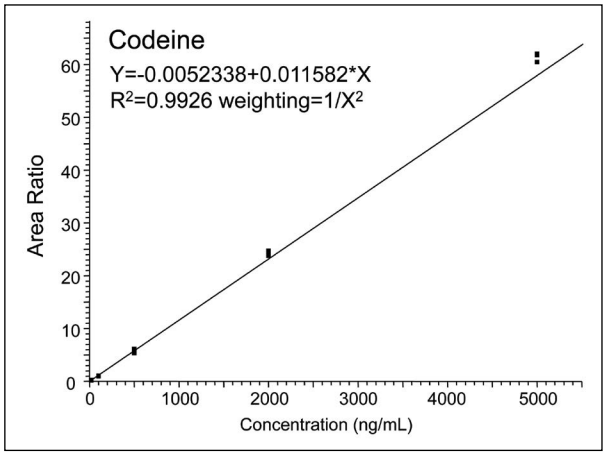

Figure 5. Calibration curve of codeine in human urine

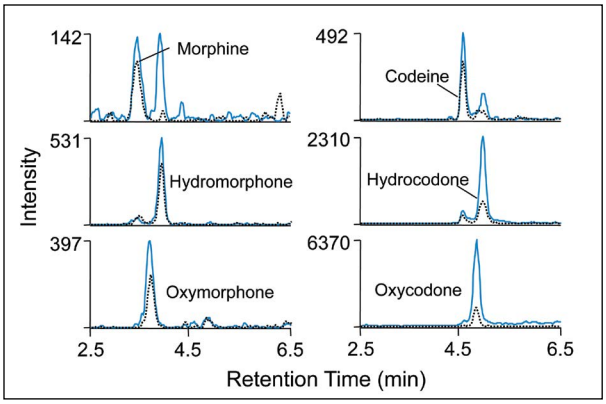

Figure 6. SRM chromatograms (quantifier: solid line; and qualifier: dotted line) of the six opioids at LLOQ in spiked human urine

Precision

Precision was assessed with spiked human urine at concentrations of 40 and 200 ng/mL. Inter- and intra-assay CV values at low and high quality-control concentrations varied between 5.0% and 12.9% (Table 4).

Table 4. Precision data

Precision (%)	Intra (n=5)	Inter (n=15)	Intra (n=5)	Inter (n=15)
Concentration (ng/mL)	40	40	200	200
Morphine	12.0	10.8	9.7	7.4
Codeine	6.8	6.4	9.3	8.0
Hydromorphone	7.0	7.7	5.9	5.0
Hydrocodone	8.3	8.2	12.9	10.0
Oxymorphone	14.1	11.4	7.9	6.4
Oxycodone	5.1	6.3	6.7	5.8

Carryover

No carryover was observed.

Solvent Usage

The method used only 5%–10% of the solvent amount used at a normal flow rate setting (0.5 mL/min). This dramatically lower solvent use will significantly lower both initial solvent cost and the cost of disposing of solvent waste.

Conclusion

We have used a novel approach for sensitive quantitation of six opioids in urine for forensic toxicology purposes. This approach used super-dilution to minimize frequently observed ion suppression in urine samples and used a microflow LC setup (Ultimate 3000 RSLCnano LC system and TSQ Vantage mass spectrometer) to compensate for sensitivity losses from super-dilution. This robust method was linear between 20 and 5000 ng/mL for the six opioids and highly accurate and precise. The method used only 5%–10% of the solvent amount used at a normal LC flow rates, significantly lowering both solvent purchase and waste disposal costs.

For forensic toxicology use only.

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