SAMHSA-Compliant LC/MS/MS Analysis of Opiates (Morphine and Codeine) in Urine with Agilent Bond Elut Plexa PCX and Agilent Poroshell 120

Application Note

Forensics & Drug Testing

Authors

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Abstract

New guidelines from the US Substance Abuse and Mental Health Services Administration (SAMHSA), effective October 2010, allowed LC/MS/MS methods to be used for confirmation of initial drug tests by government-certified workplace drug testing laboratories. LC/MS/MS methods are far less complicated than previously employed GC/MS methods because they do not require a derivatization step. We present a method for analysis of opiates that meets the most recent SAMHSA guidelines to demonstrate linearity, limit of detection (LOD), accuracy and precision, as well as measurement of matrix effects, extraction recovery and overall process efficiency. This is one of a suite of six simplified methods covering all classes of SAMHSA-regulated drugs and using premier Agilent products, including Agilent Bond Elut Plexa PCX mixed-mode polymeric SPE, Agilent Poroshell 120 EC-C18 2.7 µm superficially porous LC column, Agilent 1200 Infinity LC system and Agilent 6460 Triple Quadrupole LC/MS system with Agilent Jet Stream Technology (AJST) enhanced electrospray source.

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Introduction

Opiates (morphine and codeine) are natural alkaloids found in the resin of the opium poppy. Codeine is currently the most widely used opiate in the world. In addition to detection of morphine and codeine, guidelines from SAMHSA require the confirmation method to demonstrate the ability to distinguish these drugs from structurally related compounds, such as the semi-synthetic opioids: hydromorphone, oxymorphone, hydrocodone, oxycodone, and the codeine metabolite norcodeine. All of these drugs work by binding to opioid receptors in humans, and in addition to their prescribed use as analgesics, they are used recreationally and can cause opioid dependence; high dose of opiates/opioids can cause death by respiratory failure.

Both morphine and codeine are extensively metabolized in the body. Morphine is metabolized primarily into morphine-3-glucuronide and morphine-6-glucuronide. Codeine's major metabolites are morphine, codeine-6-glucuronide and norcodeine. Since both morphine and codeine are found in urine largely in the form of glucuronide conjugates, SAMHSA requires measurement of the total concentration of each compound. A full conversion of glucuronides back to parent species must be performed prior to analysis. The most reliable conversion method ensuring complete recovery of free opiates is acid hydrolysis. Frequently used enzymatic hydrolysis has been proved to provide incomplete recovery of parent compounds which could lead to false negative results (Wang *et al*., 2006)

The SAMHSA-established confirmation cutoff concentration for morphine and codeine is 2000 ng/mL. Since high concentrations of opiates (as well as amphetamines) can be expected in some urine samples, we chose to use a higher capacity 3-mm id Agilent Poroshell 120 column in preference to a 2-mm id column for all Agilent SAMHSA methods. With superficially porous 2.7-µm particles, Poroshell 120 provides similar efficiency to sub-2 µm UHPLC columns, but with about 40% less back pressure. It therefore allows users of even 400 bar LC systems to increase resolution and to shorten both analysis and re-equilibration times by applying a higher flow rate.

The extraction method described here provides reproducible high recoveries of morphine and codeine due to the unique properties of Agilent Bond Elut Plexa. Unlike other polymeric sorbents, Plexa possesses an amide-free hydroxylated particle surface that excludes protein binding. This results in minimized ion suppression and maximum sensitivity. Fast flow and reproducible performance are due to the narrow particle size distribution with no fines to cause blockages.

With a low sample injection volume of 2 µL and no sample preconcentration, the method demonstrates excellent signal-to-noise ratios for both morphine and codeine (>150:1 at 200 ng/mL, 10% of the SAMHSA confirmation cutoff) due to the enhanced sensitivity of the Agilent 6460 Triple Quadrupole LC/MS with the AJST electrospray source.

Previous methods from Agilent (Moorman and Hughes, 2010) utilized the Agilent 6410 Triple Quadrupole LC/MS system and other SPE/LC products and procedures.

Experimental

Analytes

Drug standards were purchased from Cerilliant Corporation as 1 mg/mL (morphine, codeine, hydromorphone, norcodeine, hydrocodone, oxycodone, oxymorphone and morphine-3-glucucronide) and 100 µg/mL (morphine-D_6, codeine-D_6) solution in methanol.

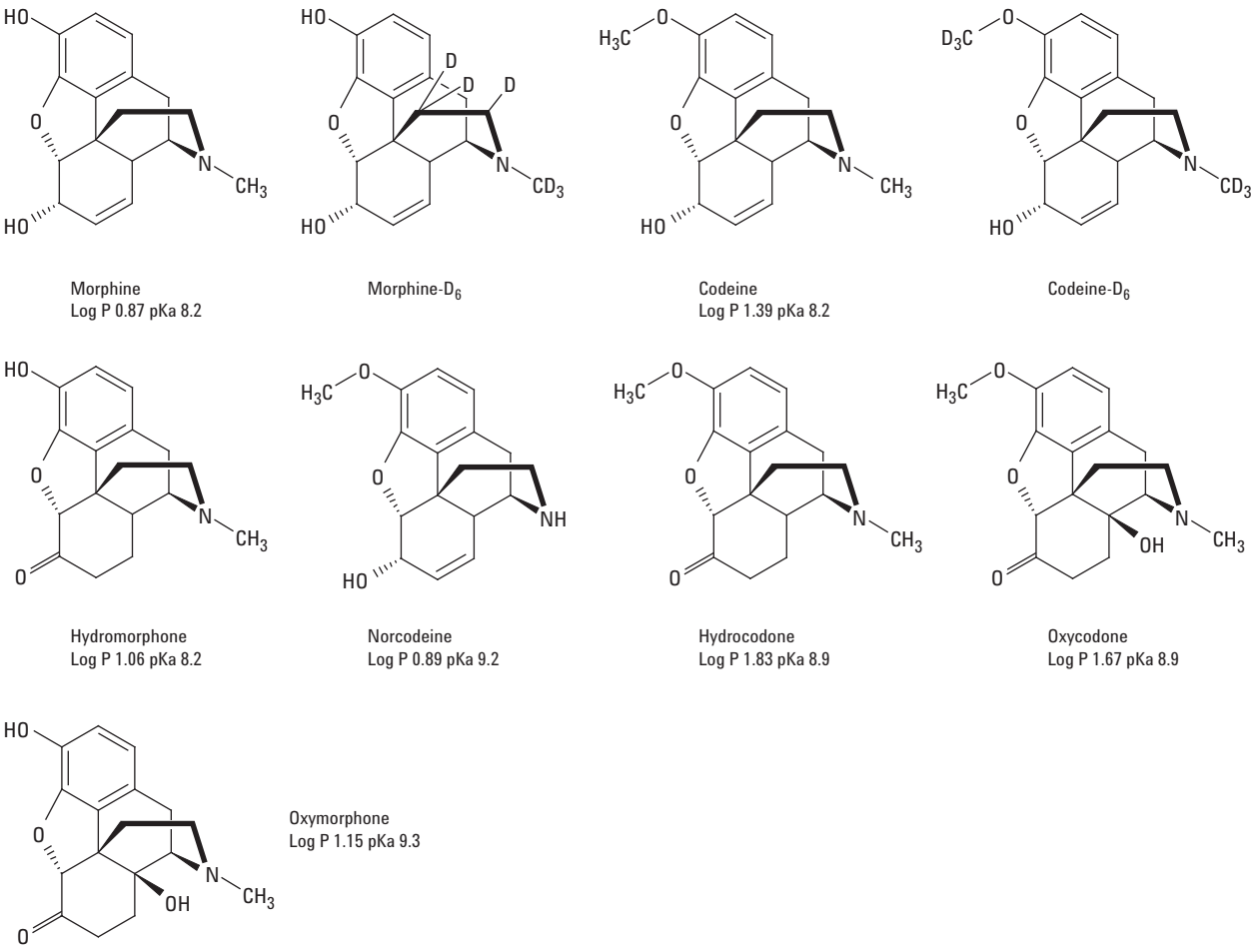

Figure 1. Opiate analytes and their structures.

Materials and instrumentation

SPE

- Agilent Bond Elut Plexa PCX cartridges 30 mg, 3 mL (p/n 12108303)
- Agilent vacuum manifold VacElut 20 (p/n 12234100)
- Agilent stopcock valves (p/n 12234520)
- Agilent 2-mL autosampler vials (p/n 5182-0716)
- Agilent screw caps for AS vials (p/n 5182-0717)

LC

- Agilent Poroshell 120 EC-C18 3 × 50 mm, 2.7 µm (p/n 699975-302)
- Agilent 1260 Infinity LC system (G1379B microdegasser, 1312B binary pump in low delay volume configuration, G1367E autosampler, G1330B thermostat)

MS

- Agilent 6460A Triple Quadrupole LC/MS system with AJST electrospray ionization source.

Sample preparation

Hydrolysis and sample pretreatment

1. Spike 0.5 mL of urine with ISTD at 1000 ng/mL; use of 12 × 75 mm glass tubes is recommended.
2. Add 125 µL conc. HCl.
3. Incubate in the heating block at 95 ±5 °C for 90 minutes.
4. Cool. Add 2 mL 0.1 M sodium acetate buffer (pH 4.5).
5. Neutralize with 250 µL 7 N KOH, vortex, test pH – it should be <6.
6. Centrifuge 20 minutes at 6000 rpm.

Extraction

1. Condition Bond Elut Plexa PCX column with 0.5 mL methanol – soak, then let drip.
2. Load sample/supernatants.
3. Wash 1: 1 mL 2% formic acid.
4. Wash 2: 1 mL of methanol.
5. Dry 5–10 minutes under vacuum (10–15 in Hg).
6. Elute with 2 mL methanol: ammonium hydroxide (100:20), freshly prepared. Let eluate drip into collection vials, then apply low vacuum (2–3 in Hg).
7. Evaporate to dryness at 40 °C.
8. Reconstitute in 0.5 mL initial mobile phase (5% methanol, 95% water, 0.1% formic acid).

LC/MS/MS

LC conditions

Mobile phase A	0.1% formic acid in water	
Mobile phase B	0.1% formic acid in methanol	
Flow rate	0.8 mL/min	
Gradient	Time (min)	% B
	0.0	5
	0.5	5
	1.5	25
	2.5	55
	2.6	90
	5.6	90
	5.7	5
Stop time	5.8 min	
Post time	2 min	
Max pump pressure	400 bar	
Injection volume	2 µL	
Injection with needle wash		
Needle wash	Flush port 75:25 methanol:water for 10 s	

Disable overlapped injection

No automatic delay volume reduction

MS conditions

ES Source Parameters

Ionization mode	positive
Capillary voltage	3000 V
Drying gas flow	10 L/min
Drying gas temperature	350 °C
Nebulizer gas	35 psi
Sheath gas flow	12 L/min
Sheath gas temperature	400 °C
Nozzle voltage	0 V

MS parameters

Scan type	Dynamic MRM
Pre-run script	SCP_MSDiverterValveToWaste() {MH_Acq_Scripts.exe}
Time segments	#1: 1.0 min - diverter valve to MS
Delta EMV (+)	0 V

Results and Discussion

At low pH morphine, codeine and their derivatives are protonated at the tertiary amine group and are strongly retained on Plexa PCX polymeric sorbent by a combination of hydrophobic retention and a strong cation exchange.

A 100% methanol wash eliminates most matrix interferences without loss of opiates from the SPE column. A strong base is added to the organic eluent in order to break ionic interaction between the analytes and the strong cation exchange sorbent. The opiates recovery is optimized with 20% NH_4OH added to methanol shortly before sample elution.

Agilent Poroshell 120 EC-C18 3 × 50 mm, 2.7 µm column provides excellent separation and peak shapes for opiates and potentially interfering compounds, with the analysis completed within 2.5 minutes (Figure 2). LC separation begins with a low fraction of organic solvent (5%) to allow salts and other polar components of urine to elute at the beginning of the sample run. Each sample run begins with diverting a first portion of flow (0 to 1 minutes) to waste to minimize source contamination. Data collection begins at 1.0 minutes, immediately after the diverter valve switch. A flow rate of 0.8 mL/min allows for short analysis and re-equilibration times.

The only partially unresolved pair in the chromatogram in Figure 2 is codeine and norcodeine (peaks 4 and 5), but since these compounds have different precursor ions and mass transitions, any possibility of interference of norcodeine signals with codeine quantitation is excluded.

In a separate experiment, Plexa PCX was tested for the possibility of norcodeine methylation and conversion to codeine. Test results were negative; no codeine was detected in negative urine samples that were spiked with norcodeine and then extracted using the method described here.

When testing for interferences, a dynamic MRM method using retention time and delta RT (time window) for a certain transition is recommended. However, when good separation from interferences is ensured, data collection for morphine and codeine and their ISTDs can be performed with normal MRM.

SAMHSA guidelines require the use of one quantifier and at least one qualifier ion for both target compound and ISTD. A 3rd transition for the target analyte is provided (Table 1) for additional confidence. Agilent MassHunter Quantitative software calculates qualifier ion ratios, automatically highlighting those out of acceptable range.

Table 1. MRM Transitions

Compound	Precursor	Product	Fragmentor	Collision energy
Codeine	300.2	215.1	130	23
Codeine	300.2	165.1	130	46
Codeine	300.2	153.1	130	50
Codeine-D_6	306.2	165.1	130	44
Codeine-D_6	306.2	218.1	130	23
Morphine	286.1	201.1	130	23
Morphine	286.1	181.1	130	40
Morphine	286.1	165.1	130	43
Morphine-D_6	292.1	181.1	130	40
Morphine-D_6	292.1	165.1	130	42
Morphine-3-glucuronide	462.2	286.1	162	45
Oxycodone	316.2	298.1	130	15
Oxymorphone	302.2	284.1	130	17
Hydrocodone	300.2	199.1	130	30
Norcodeine	286.1	225.1	130	20
Hydromorphone	286.1	185.1	130	28

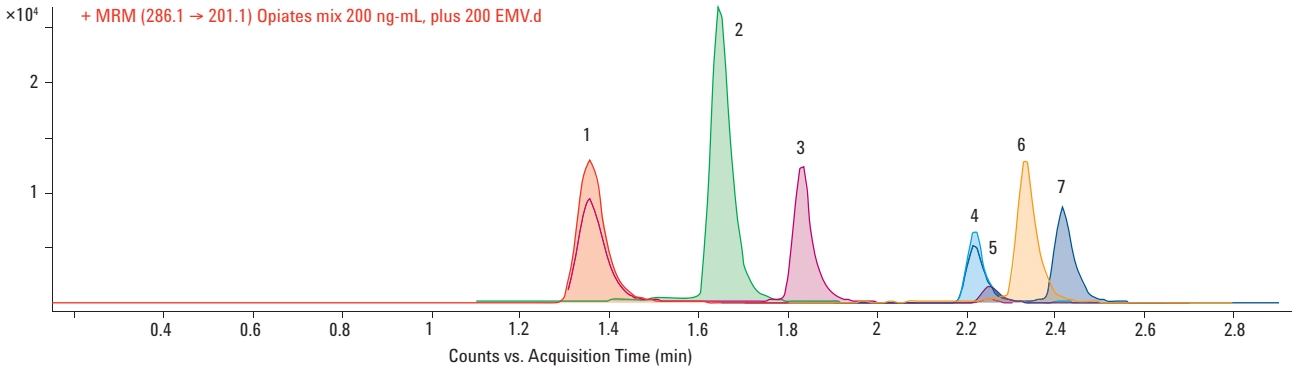

Figure 2. Separation of opiates and potential interferences on Agilent Poroshell 120 EC-C18 3 × 50 mm, 2.7 µm column - overlaid MRM extracted ion chromatograms. Concentration of each analyte is 200 ng/mL in urine. Peaks in order of their elution are:
1. morphine; 2. oxymorphone; 3. hydromorphone; 4. codeine; 5. norcodeine (small magenta peak); 6. oxycodone; 7. hydrocodone.

When processed according to the protocol, urine samples spiked with morphine-ß-3-glucuronide at 10,000 ng/mL showed 97 to 99.2% conversion to morphine. MS parameters for the detection of morphine-ß-3-glucuronide are included in Table 1 for analysts interested in testing the hydrolysis efficiency.

Signal-to-noise ratios exceeding 150:1 were obtained for quantifier peaks of morphine and codeine at 200 ng/mL (Figure 3, panel 1 and 2 from the top). This illustrates the state-of-the-art performance of the Agilent 6460 Triple Quadrupole LC/MS system, capable of reliably detecting opiates at a small fraction of the SAMHSA cutoff.

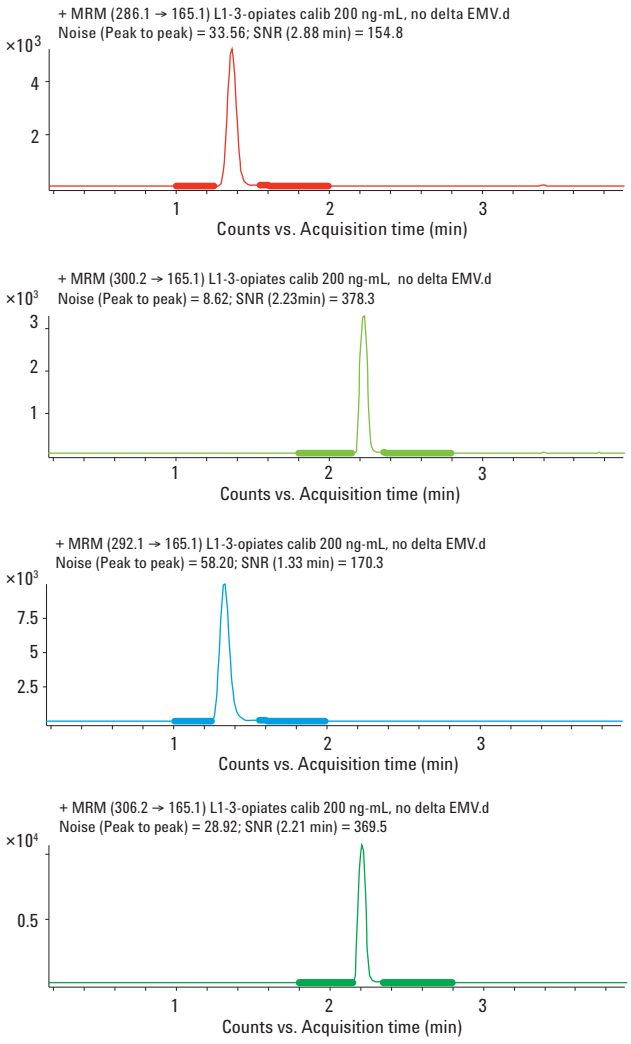

Figure 3. MRM extracted ion chromatograms for morphine and codeine quantifiers (200 ng/mL) and ISTD quantifiers (1000 ng/mL) in urine extract. Agilent Poroshell 120 EC-C18 3 × 50 mm, 2.7 µm column. Noise regions are shown in bold.

Figure 4 gives examples of calibration curves for extracted urine standards at 5 concentration levels. Calibration standards were prepared by spiking negative urine at 200, 1000, 2000, 10,000 and 20,000 ng/mL with morphine and codeine. Internal deuterated standard morphine-D_6 and codeine-D_6 were added at 1000 ng/mL. Excellent linear fit ($R^2 \geq 0.998$) to each of the curves demonstrates linearity of the method across a broad dynamic range of concentrations, as required by SAMHSA guidelines.

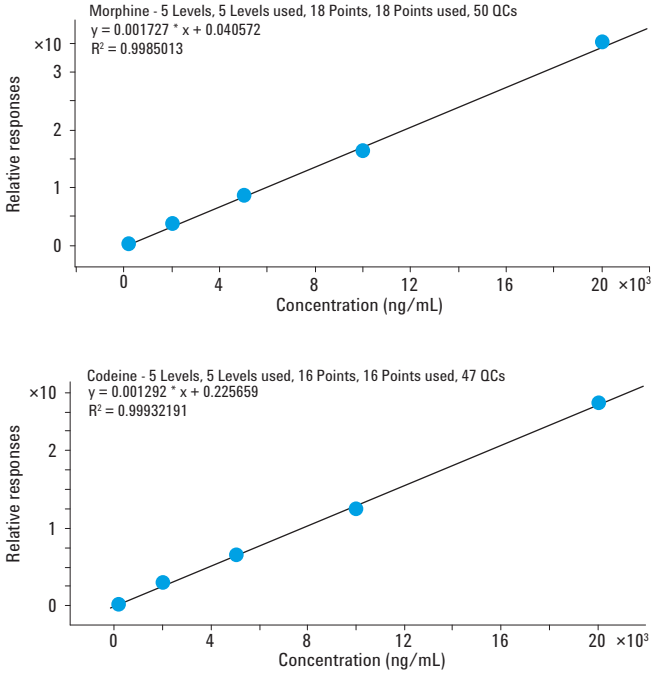

Figure 4. Examples calibration curves for morphine (upper panel) and codeine (lower panel) in urine extract. Concentration range 200 to 20 000 ng/mL. Linear fits, $R^2 \geq 0.998$.

Method evaluation

Method performance metrics in Table 2 were calculated according to the principles laid out in Matuszewski et al. (2003) and widely accepted as an industry standard approach for LC/MS/MS methods. Extraction procedure and LC/MS/MS measurement were performed for five replicates of negative urine spiked pre-extraction with morphine and codeine at the cutoff level, and five replicates of negative urine extract reconstituted in initial mobile phase and then fortified at 2000 ng/mL (spiked post-SPE). The third measurement was of initial mobile phase (the reconstitution solvent) fortified to correspond to the cutoff concentration of 2000 ng/mL in urine (spiked mobile phase).

Table 2. Method Evaluation of Opiates at the Cutoff Level, n = 5

Parameter	Morphine	Codeine
Process efficiency (%)	83	85
Extraction recovery (%)	85	86
Matrix effect (%)	98	99
Accuracy (%)	108	108
Precision (CV) (%)	0.6	0.7

Process efficiency (absolute recovery) is a ratio of a peak area of target analyte in urine sample spiked pre-SPE to its peak area in matrix-free spiked mobile phase. Extraction recovery is a ratio of a peak area of target analyte in urine extract spiked pre-SPE to its peak area in an extracted negative urine sample spiked post-SPE. Matrix effect is a ratio of a peak area of target analyte in urine spiked post-SPE to its peak area in spiked mobile phase. Accuracy is a ratio of a measured concentration calculated using the calibration curve to the expected concentration in a sample spiked with a known amount of target analyte. Precision or coefficient of variation (CV) is a measure of reproducibility and is calculated as a percent standard deviation over the mean of the five measurements

Table 2 shows high extraction recovery and process efficiency for morphine and codeine (around 85%). The high matrix effect value (98–99%) means only 1 to 2% signal reduction is due to ion suppression, thus confirming the exceptional cleanliness of Plexa-processed extracts. High accuracy (within 10% of the target) and excellent precision (CV<1%) are typical for the method.

Conclusions

The solid phase extraction procedure coupled with LC/MS/MS detection method described here is fully SAMHSA-compliant and provides highly reproducible results for workplace drug testing or other analytical environments with similar requirements for legally defensible data. The hardware setup is the same as in other 2011 SAMHSA methods from Agilent. These methods are intended for all users of Agilent 1100 and Agilent 1200 LC series since the back pressure in the LC system does not exceed 400 bar. Source parameters can be easily modified to use this method with other models of Agilent Triple Quadrupole LC/MS systems. Electronic copies of the LC/MS/MS acquisition and quantitation methods are available from Agilent Technologies.

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