

## Simultaneous Extraction and Quantitation of Morphine and Its Metabolites in Plasma Using Mixed-Mode SPE and UPLC-MS/MS

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*For forensic toxicology use only.*

## Abstract

In this application note we demonstrate, a UPLC-MS/MS method was developed to separate morphine and five of its metabolites on a 2.1 x 100 mm, 1.8  $\mu$ m ACQUITY UPLC HSS T3 Column in a single run using an ACQUITY UPLC System connected to a fast-scanning triple quadrupole MS detector (TQD).

## Introduction

Morphine is an effective pain-relieving drug that is primarily metabolized into morphine-3-glucuronide (M3G) and morphine- 6-glucuronide (M6G). The highly potent M6G may have adverse effects such as respiratory depression and renal failure if accumulated in the body. Other metabolites of morphine include morphine N-oxide, 6-acetylmorphine and 10-hydroxymorphine. As morphine abuse continues to affect modern society, an effective method must be established to analyze morphine and its metabolites in biological fluid samples.<sup>1</sup>

In this work, a UPLC-MS/MS method was developed to separate morphine and five of its metabolites on a 2.1 x 100 mm, 1.8  $\mu$ m ACQUITY UPLC HSS T3 Column in a single run using an ACQUITY UPLC System connected to a fast-scanning triplequadrupole MS detector (TQD). The method achieved adequate retention of these very polar compounds by reversed-phase (RP) chromatography in an 8-minute total run time. Mixed-mode solid-phase extraction (SPE) uses both reversedphase and ion-exchange mechanisms to separate analytes more selectively from matrix components. Therefore, sample preparation of porcine plasma was performed with the Oasis Mixed-mode Cation eXchange (MCX)  $\mu$ Elution Plate. The strong cation-exchange sorbent was chosen because morphine, its five metabolites and, their internal standards are basic compounds (pKa of morphine = 9.85). The structures of the six analytes are shown in Figure 1. The Oasis MCX SPE procedure requires a high-pH elution step that is not suitable for 6-acetylmorphine due to degradation. To avoid compound degradation prior to UPLC-MS/MS analysis, a neutralizing collection step was employed.<sup>2</sup> The SPE recovery and reproducibility of the method were determined, as well as the linearity and lower limit of quantitation (LLOQ) for each analyte.

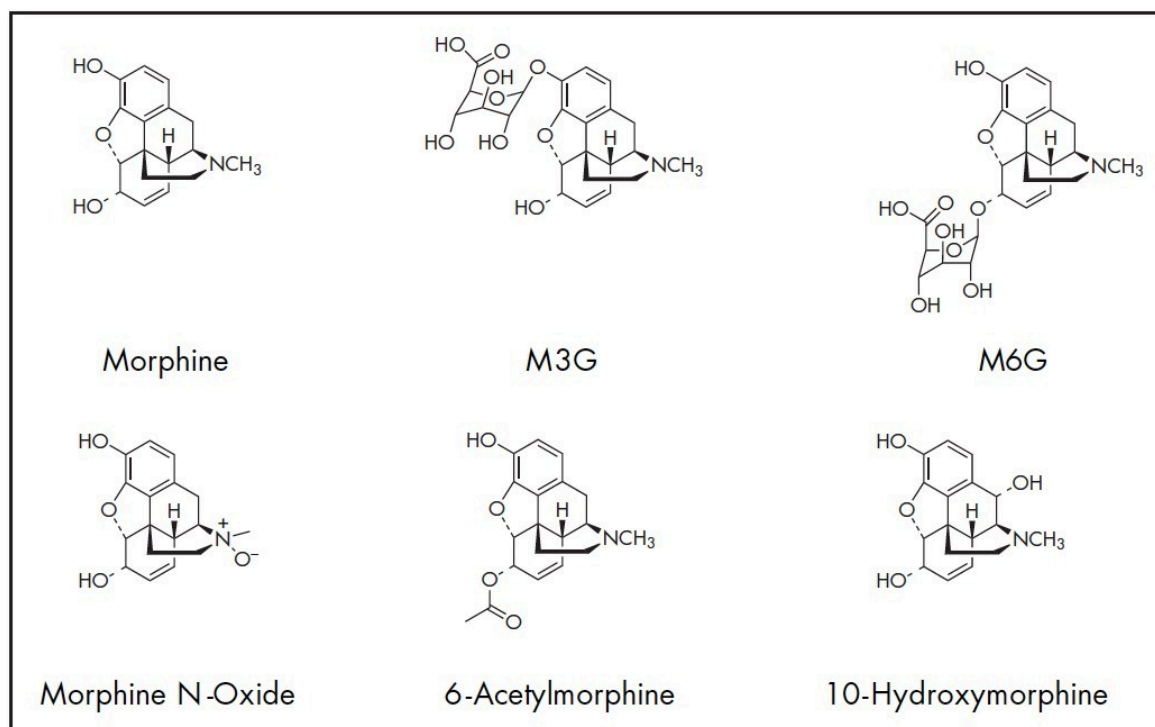


Figure 1. Structures of morphine and five metabolites.

## Experimental

### UPLC Conditions

System:	Waters ACQUITY UPLC System
Column:	ACQUITY UPLC HSS T3 Column 2.1 x 100 mm, 1.8 $\mu\text{m}$
Part Number:	186003539
Column Temp:	45 °C
Sample Temp:	4 °C
Flow Rate:	0.5 mL/min

Mobile Phase A:	0.1 % HCOOH in H <sub>2</sub> O
Mobile Phase B:	CH <sub>3</sub> OH
Injection Volume:	15 µL
Injection Mode:	Partial loop with needle overfill (PLNO; 20 µL loop size)
Weak Needle Wash:	95/5 (H <sub>2</sub> O/CH <sub>3</sub> OH)
Strong Needle Wash:	95/5 (CH <sub>3</sub> CN/H <sub>2</sub> O)

## Gradient:

Time(min)	Profile		Curve
	A (%)	B (%)	
Initial	98.0	2.0	-
3.0	40.0	60.0	6
3.5	2.0	98.0	6
5.0	2.0	98.0	6
5.1	98.0	2.0	6
8.0	98.0	2.0	6

## MS Conditions

MS System:	Waters ACQUITY TQD
Ionization Mode:	Electrospray Positive
Capillary Voltage:	1.0 kV
Cone Gas:	50 L/Hr
Desolvation Gas:	800 L/Hr
Collision Cell Pressure:	4.07e <sup>-3</sup> mbar
Desolvation Temp:	350 °C
Source Temp:	120 °C
Dwell Time:	10 ms
MRM Transitions:	See Table 1

Compound	Reaction (m/z)	Cone Voltage (V)	Collision Energy (ev)
M3G, M6G		30.0	35.0
M3G-D3, M6G-D3	462.0 > 285.9	45.0	36.0
6-Acetylmorphine	465.2 > 289.0	50.0	35.0
6-Acetylmorphine-D3	328.0 > 164.9	45.0	50.0
Morphine N-Oxide	331.1 > 165.0	50.0	30.0
10-Hydroxymorphine	302.0 > 161.9	50.0	25.0
Morphine	302.0 > 057.9	50.0	25.0
Morphine-D3	286.0 > 200.9	50.0	28.0
	289.1 > 201.1		

*Table 1. MRM transitions for 6 analytes and 4 deuterated internal standards.*

### Standard Solutions

All of the six standard and four internal standard (IS) solutions were received at a concentration of 100 µg/mL from the manufacturer. Working solutions were prepared by diluting and mixing each of the six standard solutions with 50:50 CH<sub>3</sub>OH:H<sub>2</sub>O (v:v) to give the appropriate concentrations for calibration. The four individual IS solutions were diluted with 50:50 CH<sub>3</sub>OH:H<sub>2</sub>O (v:v) to give an IS working solution of 1 µg/mL for each compound.

### Sample Preparation

Calibration curves for each of the six compounds in porcine plasma were generated with individual points at the following concentrations: 0.1, 0.25, 0.5, 1, 5, 10, 25, 50, 100, and 250 ng/mL. The internal standard working solution was spiked to a final concentration of 25 ng/mL each. Porcine plasma was spiked as follows for each concentration:

50 µL standard working solution and 50 µL internal standard working solution were added to 2 mL porcine plasma. The sample was then vortexed and diluted with 1.9 mL 1% H<sub>3</sub>PO<sub>4</sub> in water. These solutions are the

pretreated (diluted and acidified) plasma solutions which are ready for SPE. The plasma blank was prepared by adding 50  $\mu\text{L}$  of 50:50  $\text{CH}_3\text{OH}:\text{H}_2\text{O}$  (v:v) and 50  $\mu\text{L}$  internal standard working solution to 2 mL porcine plasma and 1.9 mL 4 %  $\text{H}_3\text{PO}_4$  in water.

## Extraction Procedure

Solid-phase extraction was performed with the Oasis MCX  $\mu\text{Elution}$  96-well Plate. Because acetylmorphine was found to be unstable at high pH, the eluates were collected into a collection plate containing 3 %  $\text{HCOOH}$  in  $\text{CH}_3\text{OH}$  in order to neutralize the basic elution solvent (final pH  $\sim 4.5$ ). These samples were then evaporated and reconstituted with water to ensure compatibility with the starting gradient conditions.

The detailed SPE procedure is described below:

Oasis MCX  $\mu\text{Elution}$  96-well plate (Part Number: [186001830BA <https://www.waters.com/nextgen/us/en/shop/sample-preparation--filtration/186001830ba-oasis-mcx-96-well--elution-plate-2-mg-sorbent-per-well-30--m-1-p.html>](https://www.waters.com/nextgen/us/en/shop/sample-preparation--filtration/186001830ba-oasis-mcx-96-well--elution-plate-2-mg-sorbent-per-well-30--m-1-p.html).)

1. Condition the wells with 200  $\mu\text{L}$   $\text{CH}_3\text{OH}$
2. Equilibrate with 200  $\mu\text{L}$  water
3. Load 500  $\mu\text{L}$  diluted plasma sample
4. Wash with 200  $\mu\text{L}$  2 %  $\text{HCOOH}$  in water
5. Wash with 2 x 100  $\mu\text{L}$   $\text{CH}_3\text{OH}$
6. Elute with 2 x 25  $\mu\text{L}$  5 %  $\text{NH}_4\text{OH}$  in 90:10  $\text{CH}_3\text{OH}:\text{H}_2\text{O}$  into a collection plate already containing 50  $\mu\text{L}$  3%  $\text{HCOOH}$  in  $\text{CH}_3\text{OH}$
7. Evaporate at room temperature until completely dry
8. Reconstitute with 50  $\mu\text{L}$  water

## SPE Recovery

Recovery for the analytes and internal standards was determined by comparing the MRM peak areas of pre-extracted spiked samples at 25 ng/mL to those of the post-extracted spiked samples at the same concentration. The pre-extracted samples were spiked with 50  $\mu\text{L}$  of the standard mixture at 1  $\mu\text{g}/\text{mL}$  each and extracted in the plate. Blank plasma samples were extracted in the plate and then spiked with 10  $\mu\text{L}$  of the standard mixture at

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625 ng/mL each. The pre-extracted and post-extracted spiked samples were evaporated to dryness and reconstituted with 50  $\mu$ L water. Extraction was performed on three different days (N = 4 each day).

## Calibration Curves

Calibration curves were generated using the Waters QuanLynx Application Manager for MassLynx MS Software by plotting the ratio of analyte to internal standard peak area for each concentration. The linear regression was constructed using a weighting of 1/x with the origin excluded. The percentage deviation for each calibration point was obtained from QuanLynx by comparing the concentration value calculated from the linear regression to the expected value.

## Results and Discussion

To investigate analyte stability, a standard mixture containing 20 ng/mL of each analyte was prepared in H<sub>2</sub>O, 5% NH<sub>4</sub>OH in H<sub>2</sub>O, 5% NH<sub>4</sub>OH:3% formic acid (1:1, v:v) and stored at 4 °C (set temperature of the autosampler) for 24 hours. Stability of the analytes was evaluated by comparing the area counts after 24 hours to the area counts obtained immediately after the solutions were prepared. In general, the compounds are stable in H<sub>2</sub>O. At high pH, however, 6-acetylmorphine degrades completely after 24 hrs, and area counts for morphine increase by 25% (Figure 2). It is expected that some of the 6-acetylmorphine hydrolyzes to morphine at high pH. All the six compounds were stable (<10 % change) in the neutralizing solution.

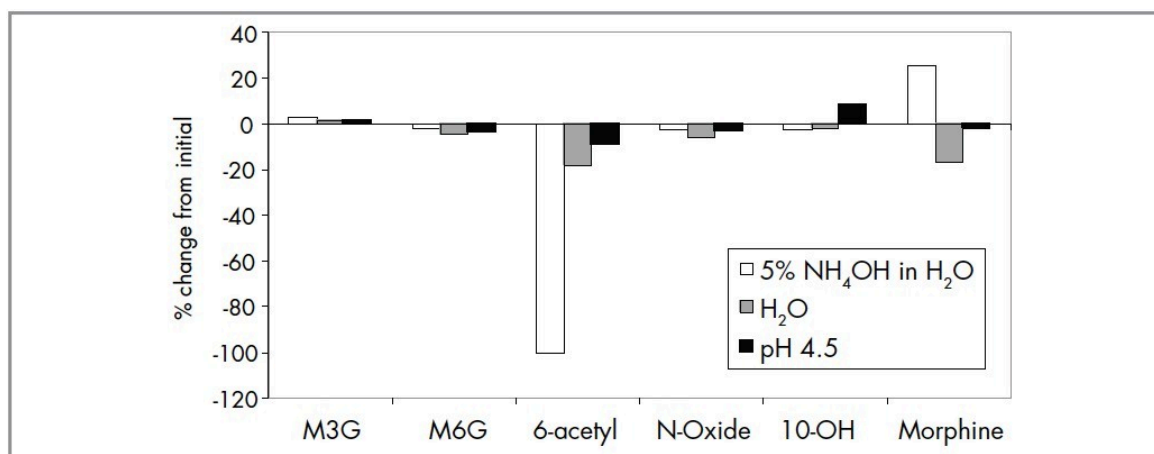


Figure 2. Twenty-four hour stability of morphine-related compounds incubated at 4 °C in various solutions (N = 3).

~~SPE recoveries for most of the compounds at 25 ng/mL were >90% except for the glucuronide metabolites, that~~



had slightly lower recovery (Table 2). Inter-day SPE reproducibility was better than 6%. A representative chromatogram of a pre-extracted spiked sample is shown in Figure 3.

Compound	Average SPE Recovery	Inter-Day % RSD
M3G	76.7	0.8
M3G-D3	74.7	5.4
M6G	87.3	5.8
M6G-D3	86.0	1.2
6-Acetylmorphine	117.7	0.5
6-Acetylmorphine-D3	118.7	2.6
Morphine-N-oxide	100.0	1.0
10-Hydroxymorphine	93.7	4.8
Morphine	119.7	2.1
Morphine-D3	119.7	1.3

Table 2. SPE recoveries and inter-day % RSD.

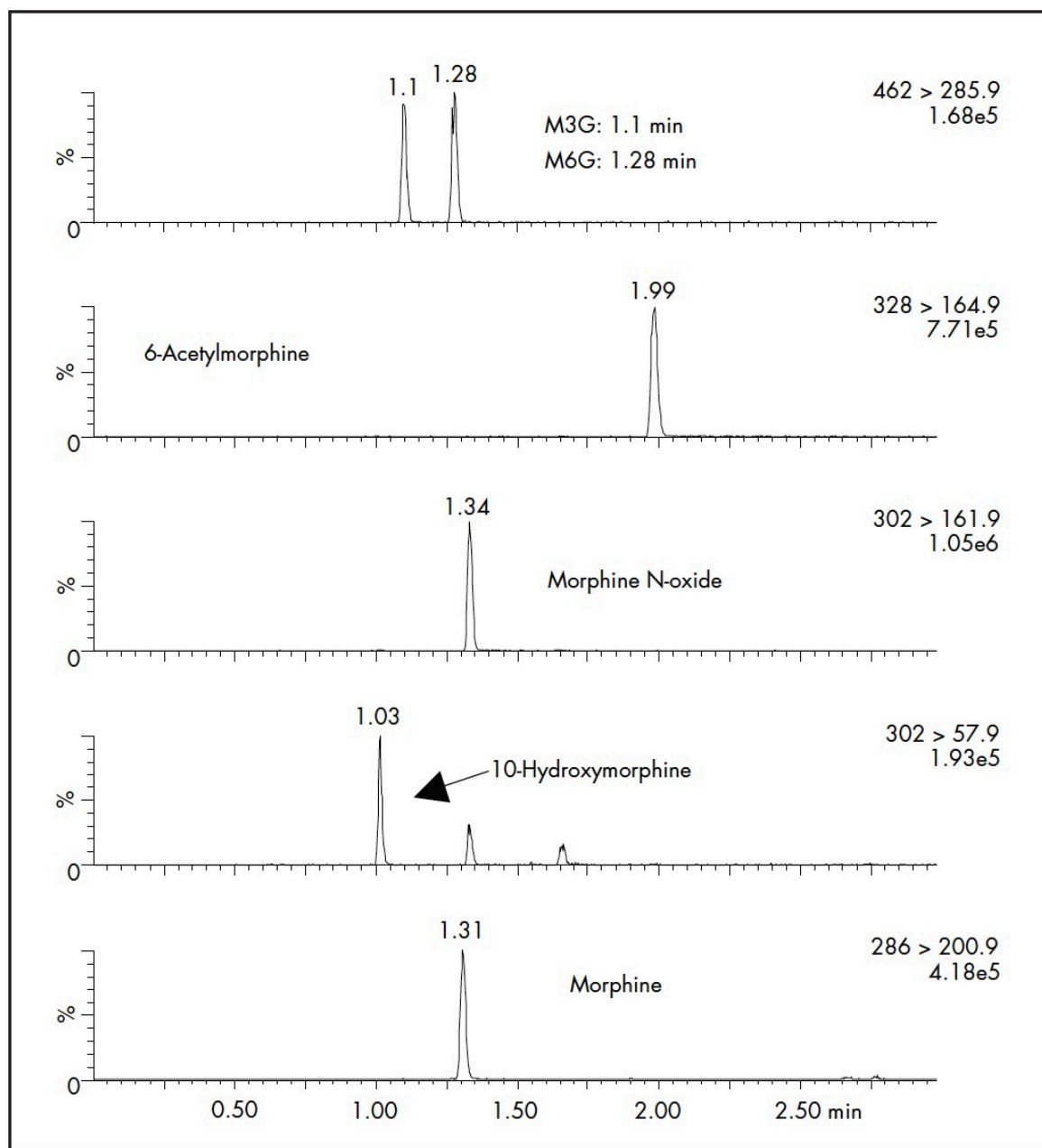


Figure 3. UPLC-MS/MS chromatogram of a pre-extracted spiked sample at 25 ng/mL of each analyte in porcine plasma. Retention times for each analyte are shown at each peak apex.

The results for linearity and LLOQ are shown in Table 3. All calibration curves had an  $R^2 \geq 0.998$ , and % deviation for each point was  $< \pm 15$  % of the expected values. The LLOQ values (5x level in blank 4) for all analytes were determined to be 0.1 or 0.25 ng/mL. The signal-to-noise (S/N) ratio of each analyte at the LLOQ was also determined. Two representative chromatograms showing the LLOQ for morphine and M6G are shown in Figure 4.

Analyte	Internal Standard	Range (ng/mL)	Linearity ( $R^2$ )	% Deviation Range	LLOQ (ng/mL)	S/N @ LLOQ
M3G	M3G-D3	0.1 to 250	0.999	-11.3 to 13.4	0.1	14
M6G	M6G-D3	0.25 to 250	0.998	-11.1 to 9.3	0.25	43
6-Acetylmorphine	6-Acetylmorphine-D3	0.1 to 250	1.000	-6.5 to 14.9	0.1	13
Morphine-N-Oxide	Morphine-D3	0.1 to 250	0.999	-12.6 to 7.0	0.1	14
10-Hydroxymorphine	Morphine-D3	0.1 to 100	0.998	-9.8 to 14.3	0.1	12
Morphine	Morphine-D3	0.1 to 250	1.000	-3.9 to 8.6	0.1	27

Table 3. Linearity, % deviation and LLOQ results for morphine and five metabolites.

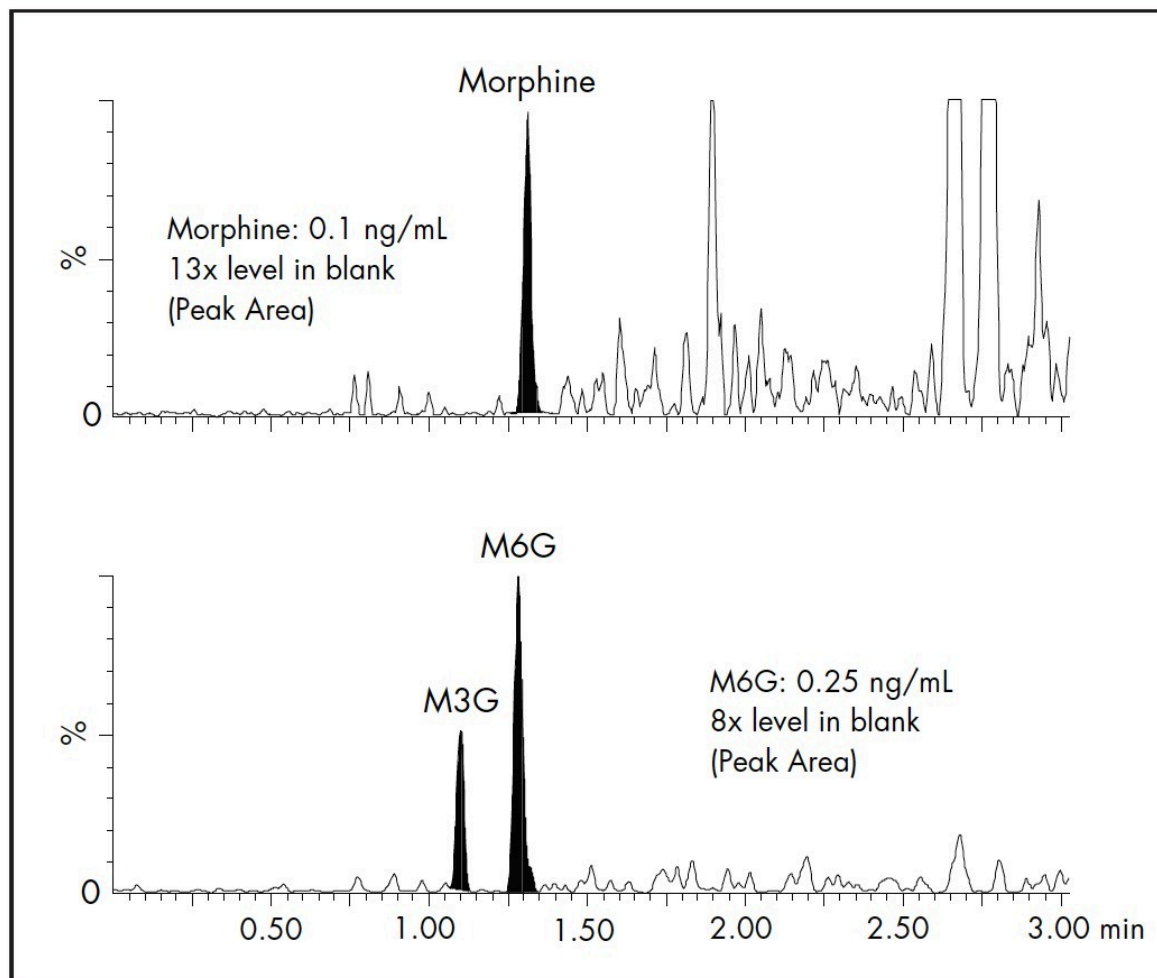


Figure 4. Representative XIC chromatograms at the LLOQ of morphine and M6G in porcine plasma.

## Conclusion

A method for the simultaneous extraction and quantitation of morphine and five of its metabolites in porcine plasma was developed. All six compounds were analyzed in a single UPLC-MS/MS run in 8 minutes. A

neutralizing collection step was used in the SPE protocol to prevent analyte degradation. The SPE procedure

using an Oasis MCX  $\mu$ Elution Plate was able to achieve consistent recoveries ranging from 77% to 120%, depending on the analyte. The method was linear over at least 3 orders of magnitude with  $R^2 \geq 0.998$ , and achieved LLOQ values in the range of 0.1 to 0.25 ng/mL. This method achieves the desired detection limits in less time than previously published methods, and addresses the issue of analyte instability for the selective extraction of polar compounds.

## References

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· [ACQUITY UPLC System <https://www.waters.com/514207>](https://www.waters.com/514207)

720002896, January 2009



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