# Analysis of Opiates in Oral Fluids Utilizing LC-MS/MS

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## Introduction

LC/MS/MS is a very selective and sensitive analytical technique for detection and quantification of analytes in complex matrices. As a result, the use of LC/MS/MS in forensic toxicological applications has been significantly increasing over the past decade. The use of oral fluid as an alternative biological matrix for drug testing has also increased. However, the sample volume is usually much smaller and drugs are present at lower concentrations in oral fluid when compared to urine or blood. The selectivity and sensitivity of LC-MS/MS make it ideally suited for analysis of oral fluid for the presence of opiates. A method for extraction, detection, and quantification of several opiates in oral fluid has been developed and discussed in this application note. The developed method demonstrates that the LLOQs are better than 0.5 ng/mL for all analytes, and the linear dynamic range is about three orders of magnitude. The ion ratios are reproducible throughout the entire dynamic range. LC-MS/MS has proven to be a powerful tool for oral fluid opiates sample confirmation and quantitation at low concentration levels.

## Experimental Method

### Standards

Analytical standards for morphine, oxymorphone, hydromorphone, 6-MAM, oxycodone, codeine,hydrocodone, and fentanyl, as well as deuterated internal standards, were all purchased from Cerilliant. Internal standards were d3 analogs, with the exception of 6-MAM and fentanyl, which used a d-6 and d-5 analog, respectively. Oxymorphone used hydromorphone-d3 as an internal standard and codeine-d3 is used as an internal standard for dihydrocodeine. Internal standards were added at a concentration of 50 ng/mL. All solvents were HPLC grade and obtained from Burdick and Jackson.

### Sample Preparation

Oral fluid (OF) was collected from donors known to be negative for opiates. Four hundred µL aliquots were spiked with standards and placed onto the collection pad of an OraSure Technologies Intercept® collection device. The collection pads were extracted using liquid-liquid extraction. A detailed description of the sample preparation procedure is illustrated in Figure 1.

**3200 QTRAP® LC/MS/MS system**

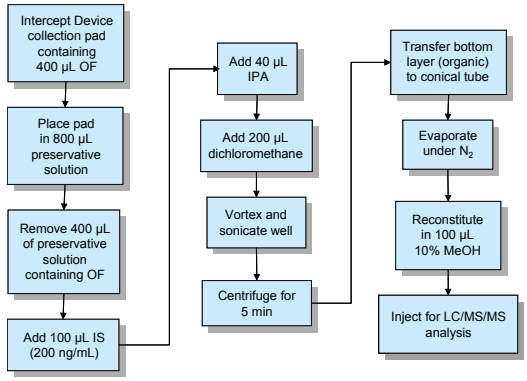


**Figure 1. Extraction Procedure for Oral Fluid Samples: Liquid-Liquid extraction was used for sample clean-up. The extraction procedure resulted in an approximately 33% concentration of the sample.**

### Instrumentation

The LCMS/MS system consisted of a Shimadzu Prominence LC stack interfaced to an AB SCIEX 3200 QTRAP® mass spectrometer. Electrospray positive ionization was used and the mass spectrometer was operated in multi-reaction monitoring mode (MRM). Two transitions per analyte and one transition per internal standard were monitored. Mobile phases and the LC gradient are shown in Table 1. Separation was achieved on a Restek 2.1 mm x 50 mm PFP Propyl column and 10 µL of sample was injected for analysis. Retention times and MRM transitions for all analytes and internal standards are listed in Table 2.

| Time (min) | %A | %B |
|---|---|---|
| 0.0 | 98 | 2 |
| 0.5 | 98 | 2 |
| 3.0 | 65 | 35 |
| 4.5 | 5 | 95 |
| 6.0 | 5 | 95 |
| 6.1 | 98 | 2 |
| 7.5 | 98 | 2 |

**Table 1. The LC Gradient used for Analysis: Mobile phases A and B are water and 90% ACN, respectively, with 0.1% formic acid and 2.5 mM ammonium acetate added to each. A flow rate of 0.500 mL/min is used.**

| Analyte | Q1 | Q3 (quant) | Q3 (qual) | RT (min) |
|---|---|---|---|---|
| Hydromorphone | 286 | 185 | 157 | 2.99 |
| Hydromorphone-d3 | 289 | 185 | N/A | 2.98 |
| Oxymorphone | 302 | 227 | 198 | 2.82 |
| Codeine | 300 | 152 | 115 | 3.34 |
| Codeine-d3 | 303 | 152 | N/A | 3.34 |
| Morphine | 286 | 152 | 128 | 2.65 |
| Morphine-d3 | 289 | 152 | N/A | 2.65 |
| Dihydrocodeine | 302 | 199 | 227 | 3.24 |
| 6-MAM | 328 | 165 | 211 | 3.69 |
| 6-MAM-d6 | 334 | 165 | N/A | 3.69 |
| Oxycodone | 316 | 241 | 256 | 3.66 |
| Oxycodone-d3 | 319 | 244 | N/A | 3.63 |
| Fentanyl | 337 | 188 | 105 | 5.64 |
| Fentanyl-d5 | 342 | 188 | N/A | 5.63 |
| Hydrocodone | 300 | 199 | 128 | 3.85 |
| Hydrocodone-d3 | 303 | 199 | N/A | 3.83 |

**Table 2. Analyte Table: List of analytes, their MRM transitions and retention times (RT). Two MRM transitions are monitored per analyte – a quantifier and qualifier ion. Only one MRM transition is monitored per internal standard. Hydromorphone-d3 is used as an IS for oxymorphone and codeine-d3 is used as an internal standard for dihydrocodeine.**

## Results

Representative chromatogram of a 5 ng/mL standard is shown in Figure 2. Figure 3 compares the extracted blank matrix spiked at 1 ng/mL to a 1 ng/mL solvent standard. There is no significant suppression except for fentanyl. The internal standard is used to correct for any analyte suppression and the required lower limits are achieved. The superior sensitivity of this method is illustrated by extracted MRM chromatograms presented in Figure 4. Signal-to-Noise (S/N) varies depending on the ionization and fragmentation efficiency of each analyte. Figure 4 shows the S/N for the quantifier ion for all analytes. The LLOQ for fentanyl is estimated to be 0.01 ng/mL and the LLOQ for all analytes is 0.5 ng/mL or better.

Figure 5 shows the precision, accuracy, and calibration curves for representative sample morphine and dihydrocodeine. All other analytes have similar linearity and statistics. Good linear dynamic range spanned from 0.1 ng/mL to 100 ng/mL for all analytes, except oxymorphone which has a lower limit of about 0.5 ng/mL.

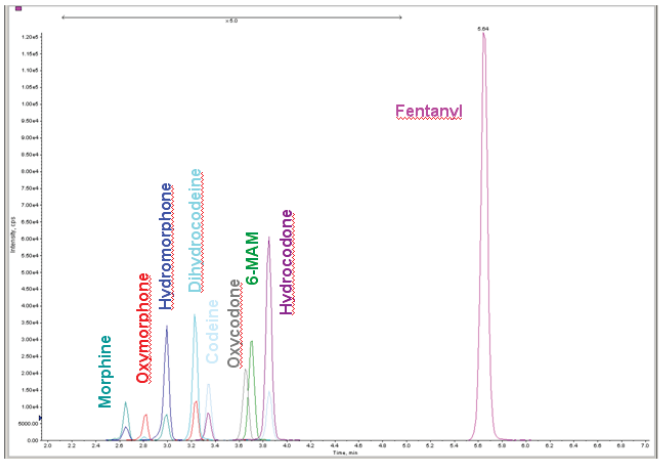


**Figure 2. Representative Chromatogram of a 5 ng/mL Standard: Note that the y-axis has been magnified 5x for all analytes except fentanyl. For quantitation, the run was divided into two periods. The period break is at 3.5 min between codeine and oxycodone.**

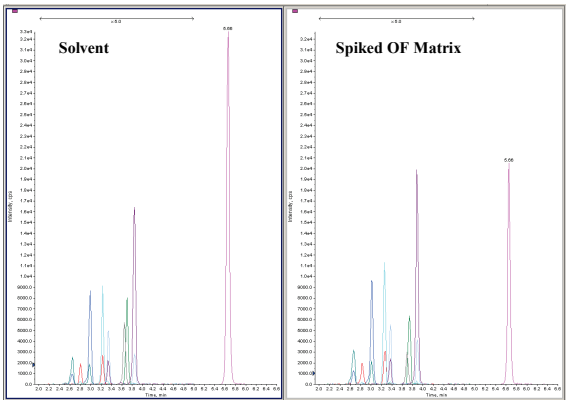


**Figure 3. Suppression Test: Extracted blank matrix spiked at 1 ng/mL and compared to a 1 ng/mL solvent standard.**

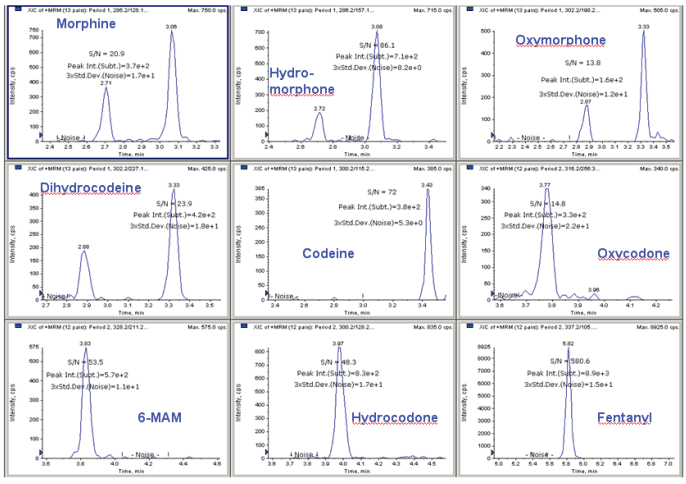


**Figure 4. Lower Limits: The S/N for the quantifier ion for all analytes is shown. Blank matrix was spiked at 0.5 ng/mL and 10 µL are injected. The LLOQ for all analytes was 0.5 ng/mL or better and the LLOQ for fentanyl is estimated to be 0.01 ng/mL.**

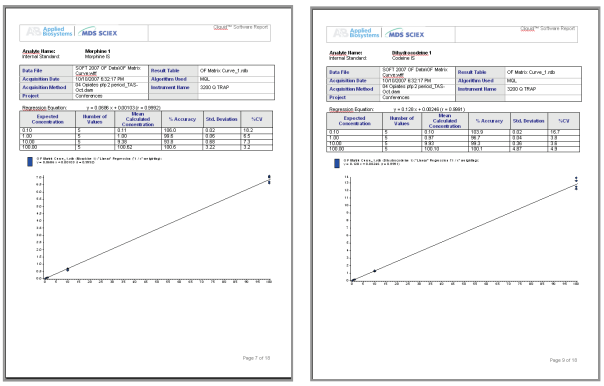

**Figure 5. Calibration Curves and Statistics: Report of precision, accuracy, and calibration curves for morphine and dihydrocodeine.**

Extraction efficiency is demonstrated by comparing the extracted analytes vs. analytes spiked into matrix after extraction (Figure 6). Extraction efficiency for most analytes is 50% or better, with the exception of dihydrocodeine and codeine. Table 3 lists the calculated extraction efficiencies. The required LLOQ takes into account the cutoff level, extraction efficiency, as well as the 1.3x concentration achieved from the sample preparation procedure. Table 3 shows the cutoff levels and required LLOQ values for the opiates, the result demonstrates that the observed LLOQs significantly exceed the required LLOQ.

The developed method allows reproducible quantitation even at low concentration levels. The ratios of the quantifier to qualifier ions are calculated for each analyte. The Coefficients of Variation (%CV) of the ion ratios are below 10% for most of the compounds and less than 20% for all compounds (Table 4).

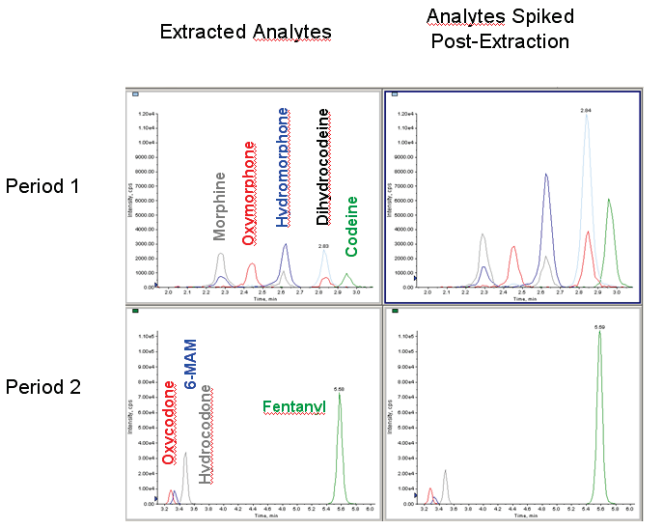


**Figure 6. Estimation of Extraction Efficiency A comparison of extracted analytes vs. analytes spiked into matrix after extraction.**

| Analyte | Cutoff Level | Extraction Efficiency (%) | Required LLOQ | Estimated LLOQ |
|---|---|---|---|---|
| Morphine | 10 | ~100 | 13 | 0.3 |
| Oxymorphone | 10 | 75 | 11 | 0.5 |
| Hydromorphone | 10 | 46 | 6 | 0.06 |
| Dihydrocodeine | 10 | 22 | 3 | 0.3 |
| Codeine | 10 | 20 | 3 | 0.07 |
| Oxycodone | 10 | 87 | 12 | 0.5 |
| 6-MAM | 1 | ~100 | 1 | 0.1 |
| Hydrocodone | 10 | ~100 | 13 | 0.1 |
| Fentanyl | <1 | 57 | 1 | <0.01 |

**Table 3. Required Cutoffs and LLOQs Cutoff levels and required LLOQs for the opiates. The estimated LLOQ is calculated from the data shown in Figure 4 and the LLOQ was defined as having a S/N≥10. The estimated LLOQ is based on a 10 µL injection.**

| Analyte | %CV | %CV* |
|---|---|---|
| Morphine | 14 | 7.3 |
| Oxymorphone | 7.0 | 4.8 |
| Hydromorphone | 9.0 | 7.2 |
| Dihydrocodeine | 19 | 14 |
| Codeine | 12 | 11 |
| Oxycodone | 7.0 | 5.1 |
| 6-MAM | 17 | 7.6 |
| Hydrocodone | 6.4 | 4.8 |
| Fentanyl | 2.1 | 1.6 |

**Table 4. Ion Ratios of the quantifier to qualifier ions were calculated for each analyte. Five replicate injections at four different concentrations (0.1, 1, 10, and 100 ng/mL) were measured. The %CV for the ratio is shown for all data (%CV).**

## Conclusions

An LC-MS/MS method for analysis of opiates in oral fluids was developed and greatly exceeded the limits required by the cutoff levels. Sensitivity of the method was better than 0.5 ng/mL for all analytes. Linearity for all analytes extended three orders of magnitude. The reproducibility of the ion ratios were proven to be well within 20% across the entire linear dynamic range.

Future work includes adding other analytes and improving the extraction efficiency for dihydrocodeine and codeine.

## References

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Publication number: 3570111-01

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