National Laboratory Certification Program
DRUG TESTING MATTERS

2019

Analysis of 6-Acetylmorphine in the Presence of Interfering Compounds

Introduction

This article provides information based on National Laboratory Certification Program (NLCP) Performance Testing (PT) errors in an effort to help laboratories understand how the PT errors occurred and how to prevent them. This article addresses five PT errors caused by the interference of opioids or their metabolites when analyzing 6-acetylmorphine (6-AM).

Before discussing each PT error case, a brief history of the analysis of 6-AM in federally regulated workplace drug testing is given, followed by a review of several potentially interfering compounds. Finally, an overview of a sodium bisulfite pre-treatment step that can be used to reduce interferences is described.

Brief History of 6-AM Analysis in Federally Regulated Workplace Drug Testing

The U.S. Department of Health and Human Services (HHS) originally allowed certified laboratories to test federal agency specimens for 6-AM only upon the request of a Medical Review Officer (MRO) to provide additional information for a specimen with a positive morphine result.[1] Effective December 1, 1998, HHS revised the Mandatory Guidelines[2] to require laboratories to test all morphine-positive specimens for 6-AM using a confirmatory test with a 10 ng/mL cutoff. Since October 1, 2010, the HHS Mandatory Guidelines[3,4] have required certified laboratories to conduct initial testing on all federal agency specimens for 6-AM, regardless of whether morphine is present. Laboratories must perform a 6-AM initial test and reflex specimens with positive results to 6-AM confirmatory testing, using a 10 ng/mL cutoff for both tests. For the confirmatory test, laboratories must establish a limit of detection (LOD)/limit of quantification (LOQ) that is *below* 40% of the cutoff (i.e., an LOQ *less than* 4 ng/mL).

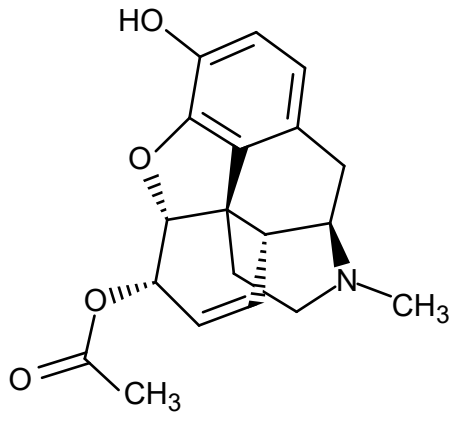
The NLCP Manual requires laboratories to perform interference studies for each confirmatory drug test with samples containing no target analyte and containing target analyte at 40% of the cutoff. The 2010 revisions to the NLCP Manual required 6-AM interference study samples containing free morphine, codeine, hydrocodone, hydromorphone, oxycodone, oxymorphone, and norcodeine at 5,000 ng/mL.[5] In 2017, HHS added hydrocodone, hydromorphone, oxycodone, and oxymorphone to the testing panel.[6] The 6-AM interference study requirements in the NLCP Manual were revised to specify that laboratories must demonstrate lack of interference from free morphine and codeine at 40,000 ng/mL; hydrocodone, hydromorphone, oxycodone, oxymorphone, norcodeine, and norhydrocodone at 5,000 ng/mL; and noroxycodone and noroxymorphone at 1,000 ng/mL.[7]

Interfering Compounds

In the late 1990s, several articles were written describing a method for reducing interference from hydrocodone, hydromorphone, oxycodone, and oxymorphone during analysis for codeine and morphine.[8-10] Hydrocodone, hydromorphone, oxycodone, and oxymorphone all contain a ketone (a carbon-oxygen double bond, with a carbon atom on both sides) functional group. The method employed the addition of hydroxylamine to convert the keto-opioids to oxime derivatives. This reduced the number of trimethylsilyl derivatives produced by preventing keto-enol tautomerization.

A recent NLCP PT sample has shown that some laboratories have difficulty with the analysis of low levels of 6-AM when other opioid metabolites are present. Many laboratories use methods for analysis of 6-AM that include the generation of a trimethylsilyl derivative using reagents such as *N,O*-bis-trimethylsilyl-trifluoroacetamide (BSTFA) and *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA). The PT errors may be due to the co-elution of trimethylsilyl derivatives of other opioids and their metabolites with 6-AM. This article focuses on the opioids and metabolites containing a ketone group (e.g., oxycodone, oxymorphone, hydrocodone, hydromorphone, norhydrocodone, noroxycodone, noroxymorphone) that may cause potential interference with 6-AM analysis.

Below are the structures of 6-AM and several opioids and metabolites that contain a ketone and may cause potential interference in analysis for 6-AM.



6-AM

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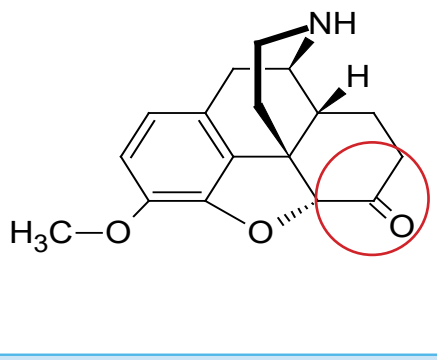
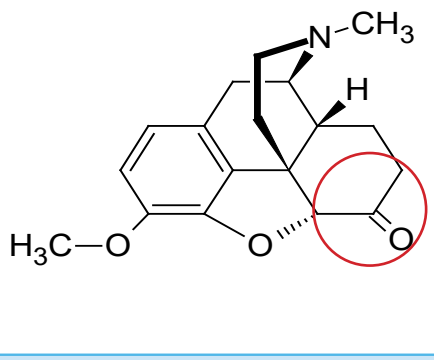
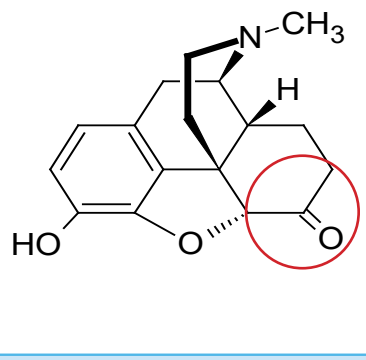
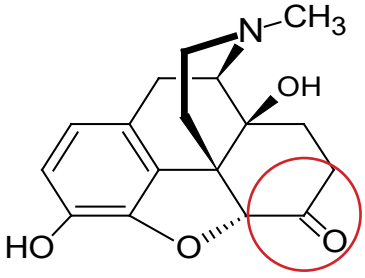
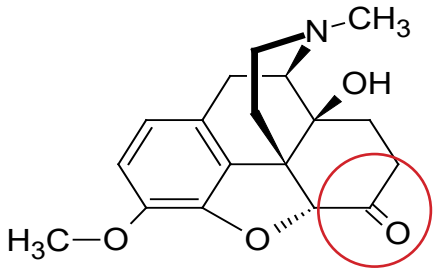
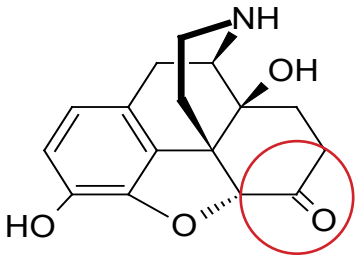
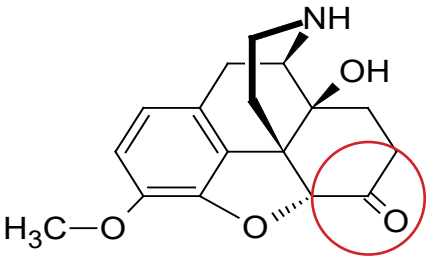
		
Norhydrocodone	**Hydrocodone**	**Hydromorphone**

	
Oxymorphone	**Oxycodone**

	
Noroxymorphone	**Noroxycodone**

PT Error Case Studies

In NLCP PT Occasion 129 (January 2019), five laboratories did not confirm 6-AM in a retest sample containing the analytes at the concentrations listed in Table 1.

Table 1. Analytes and concentrations present in the NLCP PT sample

Analyte	Group mean
6-AM	5.8 ng/mL
hydrocodone	54 ng/mL
hydromorphone	54 ng/mL
oxycodone	55 ng/mL
oxymorphone	53 ng/mL
norcodeine	Targeted at 5,000 ng/mL
norhydrocodone	Targeted at 5,000 ng/mL
noroxycodone	Targeted at 1,000 ng/mL
noroxymorphone	Targeted at 1,000 ng/mL

Each laboratory obtained acceptable results for all analytes in this sample except for 6-AM. Three of the five laboratories identified interference from norhydrocodone as the reason for their failure to confirm 6-AM.

Case # 1

This laboratory indicated that the 6-AM peak was present but did not meet acceptance criteria. The laboratory reported the PT sample as failed to reconfirm for 6-AM and invalid due to gas chromatography (GC)-mass spectrometry (MS) interference. The laboratory indicated that the interference studies for the GC-MS method showed interference when 5,000 ng/mL norhydrocodone was present.

The laboratory had previously begun validating a 6-AM liquid chromatography (LC)-tandem mass spectrometry (MS/MS) method to be used as an alternate method when interference was observed. The laboratory had submitted validation records for NLCP review as required but had chosen not to pursue approval at that time. When 2017 validation studies for the GC-MS assay revealed interference from 5,000 ng/mL norhydrocodone, the laboratory resumed validating the LC-MS/MS assay on a newer instrument.

During the PT remedial process, the laboratory was given permission to re-test the PT sample with the proposed LC-MS/MS method and obtained an acceptable result. The laboratory was reminded that it must continue to use its current 6-AM GC-MS method for regulated specimens until the NLCP has approved

the LC-MS/MS method and must continue to contact the MRO prior to reporting specimens as invalid due to interference. The laboratory submitted the completed validation records for the LC-MS/MS method for NLCP review and provided validation records for review by an on-site inspection team.

Case # 2

This laboratory observed chromatographic interference and transition ratios that were outside the acceptable range in the primary LC-MS/MS method. The laboratory reported this sample as failed to reconfirm for 6-AM. The sample was re-analyzed at a 1:2 dilution with similar results. The sample was then analyzed using the laboratory's alternate GC-MS method. The qualifier ion ratio from the GC-MS analysis did not meet acceptance criteria. The laboratory had previously identified interference with both the LC-MS/MS and GC-MS methods and revised the temperature program of the GC-MS method to resolve the interference. As part of the investigation into this PT error, the laboratory prepared and analyzed a sample designed to mimic the PT sample. The laboratory identified that the interference was caused by norhydrocodone.

It appeared that the ability to resolve the interference was dependent not only on the extended temperature program but also other system conditions, such as the GC column length and age. The laboratory performed further modifications to the temperature program and increased the length of the GC column from 15 to 30 meters. Upon re-analysis of the PT sample with the revised alternate GC-MS method, the laboratory obtained an acceptable 6-AM result.

Case # 3

This laboratory obtained qualifier ion ratios that did not meet acceptance criteria. One qualifier ion peak (i.e., 340) gave no response in the original data. The laboratory reported this sample as failed to reconfirm for 6-AM. The laboratory reprocessed the data to ensure that the 340 ion peak was integrated; however, the peak did not meet chromatographic acceptance criteria (i.e., resolution). The laboratory indicated that it had purchased the materials needed to validate a new alternate 6-AM method.

The laboratory submitted data from reanalysis of the PT sample with the new method; however, the 340 ion again failed to meet the program's requirement of at least 90% resolution. The laboratory was instructed to continue method revisions and was reminded to contact the MRO prior to reporting any donor specimens as invalid due to GC-MS interference. The laboratory prepared a sample designed to mimic the PT sample for use in further method revisions.

The laboratory changed the derivatizing reagent from BSTFA to MSTFA and also modified the temperature program and ions monitored for 6-AM. The laboratory re-analyzed the PT sample using the new method and obtained an acceptable result.

Case # 4

This laboratory analyzed the PT sample in three separate GC-MS batches for 6-AM. In the third batch, the laboratory analyzed one undiluted aliquot and one aliquot at a 1:2 dilution. In all four aliquots, the 340 qualifier ion ratio failed to meet acceptance criteria. The laboratory reported this sample as failed to reconfirm for 6-AM and invalid due to GC-MS interference.

In response to the PT error, the laboratory added a sodium bisulfite pretreatment step to their sample preparation process. They retested the PT sample with the revised procedure and obtained an acceptable result.

Case # 5

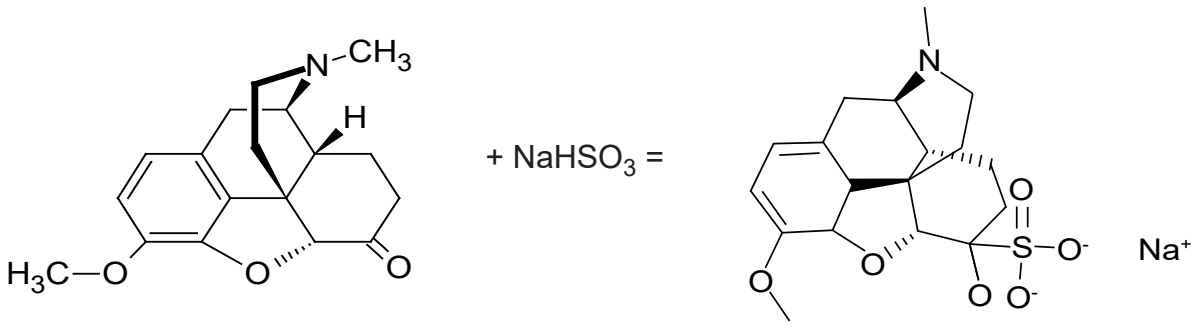
This laboratory also analyzed the PT sample for 6-AM in three separate GC-MS batches. Each batch was analyzed on two different instruments. The laboratory was unable to obtain acceptable resolution and peak shape. The laboratory reported this sample as failed to reconfirm for 6-AM. During the PT remedial process, the laboratory indicated that the sample should have also been reported as invalid due to GC-MS interference. The laboratory indicated that no interference was observed in the 2018 method validation; however, when the laboratory began the 2019 reverification during the same time period as the remedial process for the PT error, they observed interference from norhydrocodone.

The laboratory revised the temperature program for their GC-MS method to resolve the interference. The laboratory consumed approximately 32 mL of the PT sample during the testing process. To demonstrate that the laboratory's revised method could provide accurate analysis, the NLCP provided a new sample to the laboratory. The laboratory tested the new PT sample with the revised procedure and obtained an acceptable result. The laboratory also indicated that it is in the process of validating a new LC-MS/MS method to be used as an alternate method (once approved by the NLCP).

Possible Solution: Introduction of a Bisulfite Pretreatment Step

One possible solution to interference from other opioids during 6-AM analysis is the addition of a sodium bisulfite sample pretreatment step. When sodium bisulfite ($NaHSO_3$) is added to the sample, the bisulfite ion interacts with the carbonyl carbon through the nucleophilic addition of a bisulfite ion ($^-SO_3H$) to the carbonyl group to produce a water-soluble salt. Interferences are eliminated, or at least reduced, as the water-soluble bisulfite salts are removed during solid-phase extraction.

Below is the reaction that occurs during the bisulfite pretreatment step.



Hydrocodone + $NaHSO_3$ = Hydrocodone NaO_3S

In 2000, Singh et al. published a method for eliminating interferences to enable quantification of 6-AM within 20% of the target concentration with acceptable chromatography.[11] Five-milliliter aliquots of urine containing 4 ng/mL 6-AM and 10,000 ng/mL each of hydrocodone, hydromorphone, oxycodone, and oxymorphone were treated by the addition of 2 mL of a saturated solution of sodium bisulfite. The samples were allowed to incubate at room temperature for 15 minutes prior to solid-phase extraction and derivatization with BSTFA with 1% trimethylchlorosilane (TMCS). Analysis was performed by GC-MS.

As part of their corrective actions for the failure to confirm 6-AM error, two of the laboratories from the case studies described in this article investigated the use of this bisulfite pretreatment method. One laboratory implemented this step as a modification to their routine 6-AM assay, and the other laboratory added this step to their alternate method. The bisulfite pretreatment step appears to be a simple method for reducing interferences.

References

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