Performance Testing

Issues with Amphetamines Caused by Periodate Oxidation

2018

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 four PT errors caused by the production of amphetamine (AMP) from methamphetamine (MAMP) and methylenedioxyamphetamine (MDA) from methylenedioxymethamphetamine (MDMA). In each PT error, improper conditions during the periodate oxidation step prior to extraction led to the demethylation of MAMP and MDMA.

Before discussing each PT error case, a brief history of the introduction of periodate oxidation as a standard procedure for amphetamines extraction is given, followed by a description of the structural chemistry of several amphetamines and over-the-counter (OTC) sympathomimetic amines.

History

In the early years of the NLCP, the Department of Health and Human Services (HHS) sent Program Documents (PDs) to HHS-certified laboratories. A notice (PD002)[1] issued on October 12,1990 described a problem with gas chromatography-mass spectrometry (GC-MS) amphetamines confirmation using 4-carbethoxyhexafluorobutyryl (4-CB) derivative with specimens containing no AMP, a low MAMP concentration, and high concentrations of sympathomimetic amines (e.g., ephedrine, pseudoephedrine). The notice required a second confirmation test using a different derivative for all MAMP and other positive drugs derivatized with 4-CB. A follow-up notice on December 19, 1990 (PD003)[2] stated that several false positive MAMP results were reported by a certified laboratory using 4-CB derivatization. HHS rescinded the ban on reporting positive results using 4-CB derivatization and implemented the

requirement that to report a specimen as positive for MAMP, any specimen with a MAMP positive result (≥500 ng/mL) must also contain its metabolite AMP at ≥200 ng/mL. A third HHS notice on February 4, 1991 (PD004)[3] noted that certified laboratories using another derivatizing reagent—pentafluoropropionic acid anhydride (PFPA)—had also reported false positive MAMP results. Independent reference testing revealed these specimens had ephedrine and pseudoephedrine concentrations of 100,000 to 1,500,000 ng/mL. The notice reminded all HHS-certified laboratories of the need to ensure adequate validation of their assays, including interference studies evaluating analytical responses to large concentrations of compounds structurally similar to the compound of interest.

The scientific community immediately began to investigate and find solutions to prevent the generation of MAMP from sympathomimetic amines.

Thurman et al.[4] verified that unlike heptafluorobutyric anhydride (HFBA), which caused complete derivatization, 4-CB partially derivatized ephedrine and pseudoephedrine to ephedrine-4-CB and pseudoephedrine-4-CB, respectively. Thus, underivatized ephedrine and pseudoephedrine could be available for conversion to MAMP when the 4-CB derivatizing reagent is used. Their experiments using spiked samples containing ephedrine and pseudoephedrine generated MAMP and not AMP when 4-CB was used but not when HFBA was used. They believed that the ephedrine and pseudoephedrine 4-CB derivatives could be converted to the MAMP-4-CB derivative when the hydroxyl group was replaced by a hydrogen. They demonstrated, using a newly silanated injection port sleeve, the generation of MAMP in samples containing ephedrine or pseudoephedrine, with especially large yields in samples containing the latter.

Hornbeck et al.[5] reported that injector port temperatures of 300°C caused the thermal transformation of ephedrine and pseudoephedrine to MAMP with the 4-CB, HFBA, and N-trifluroacetyl-L-prolyl chloride (TFAP) derivatizing reagents. Using GC-MS, they identified pseudoephedrine and ephedrine at concentrations up to 1,116,000 ng/mL and 997,000 ng/mL, respectively, in donor urine specimens. MAMP was generated with each of the three derivatives. No MAMP was observed when the injection port temperature was lowered to 185°C.

ElSohly et al.6 provided an additional solution to the potential conversion of sympathomimetic amines to MAMP. They suggested that the confirmation procedure include an oxidation step prior to extraction by adding 1 mL of sodium periodate (0.35 M) and 1 mL of 40% phosphate buffer (pH 9) to 5 mL of urine with deuterated internal standard and using an oxidation time of 10 minutes at room temperature. The periodate treatment should oxidize ephedrine and pseudoephedrine and other related compounds to smaller fragments before derivatization. This oxidation step should remove the interfering peaks that affect MAMP and AMP when compound derivatization is not complete and prevent the generation of MAMP when high concentrations of sympathomimetic amines are present.

The Navy Drug Screening Laboratory[7] published a detailed review of the periodate oxidation procedure to include temperature, time, pH, and periodate concentration parameters to prevent a shortcoming in the procedure: the potential for demethylation of MAMP to AMP. Studies were performed using a spiked urine sample containing 2,000 ng/mL MAMP at a pH of 12.5 for the periodate oxidation, followed by extraction and derivatization with 4-CB. As shown in Table 1 (a modified table from the publication), when a urine sample was incubated with periodate (0.15 M) for 10 minutes at 50°C in a closed tube and analyzed in triplicate, AMP (average = 23.5 ng/mL) was formed, corresponding to approximately 1.2% of

MAMP (average = 1,983 ng/mL). Using the same conditions but increasing the incubation time from 10 to 60 minutes, the average percentage of AMP formed increased from 1.2% to 1.8% of MAMP. Using an incubation time of 60 minutes at a higher temperature (i.e., 80°C), the average percentage of AMP formed increased from 1.8% to 6% of MAMP. These experiments showed that the oxidation of MAMP to AMP occurs at a high basic pH (12.5) and that the amount of MAMP oxidized increased as the incubation time and temperature increased. Because the primary purpose of the periodate oxidation step was to remove the interfering OTC compounds and eliminate potential MAMP production, the laboratory conducted additional studies that showed complete oxidation of ephedrine, pseudoephedrine, and phenylpropanolamine at pH 5.2. Furthermore, no oxidation of MAMP to AMP occurred when the pH of the periodate oxidation step was below pH 9.1. The laboratory chose to buffer the 0.15 M sodium periodate and urine specimen solution to a pH of 6.2 with tubes capped and incubated for 15 minutes at 50°C to oxidize the interfering OTC sympathomimetic amines to break down products and prevent the oxidation of MAMP to AMP.

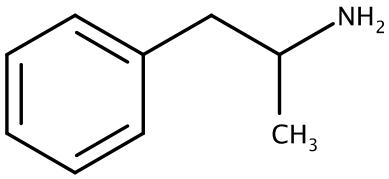
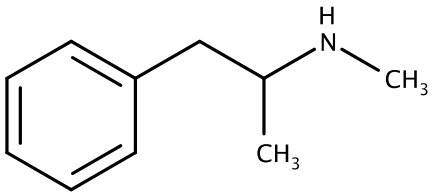
Table 1. Periodate Oxidation of MAMP (2,000 ng/mL) at pH 12.5 (Adapted from Reference #7)

Incubation		MAMP*	AMP*	AMP as % of MAMP*
Minutes	°C	(ng/mL)	(ng/mL)	
10	50	1,983	23.5	1.2
30	50	1,897	28.3	1.5
60	50	1,785	32.8	1.8
60	60	1,379	42.6	3.1
60	80	769	45.6	6.0

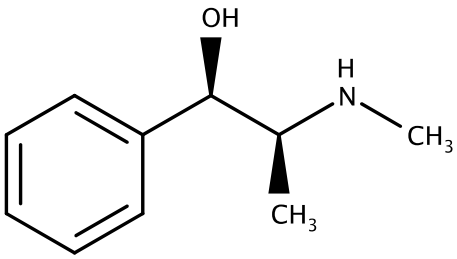
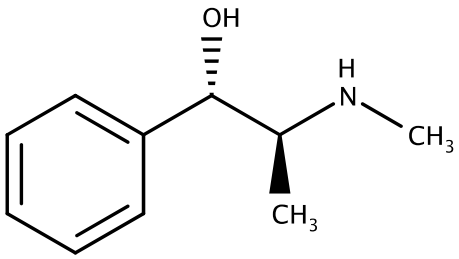
*Average of three samples

Structures

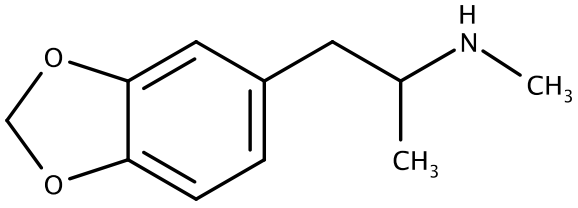
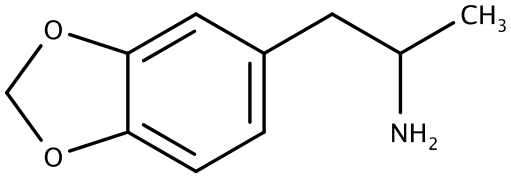
MAMP is metabolized to AMP by N-demethylation.

	
Amphetamine	**Methamphetamine**

Ephedrine and pseudoephedrine are optical isomers. They differ from MAMP by a hydroxyl group.

	
Ephedrine	**Pseudoephedrine**

MDMA and MDA are psychotropic derivatives of MAMP and AMP.

	
Methylenedioxymethamphetamine (MDMA)	**Methylenedioxyamphetamine (MDA)**

PT Case Studies Involving the Demethylation of MAMP

Case #1

This case is the first of two similar PT errors that occurred in the same laboratory a year apart with two different PT samples. Table 2 summarizes the analytical results of the certified laboratory compared to the laboratory group mean for the first PT error. The laboratory reported an AMP result (631 ng/mL) that was 70% higher than the group mean (371 ng/mL), a major quantitation error (>50% outside of the group mean). The reported MAMP result (5,183 ng/mL) was 4.7% higher than the group mean (4,949 ng/mL) and was acceptable (within 20% of the group mean). No other phenethylamines were included in the PT sample.

Table 2. Major Quantitative Error: AMP – Laboratory and Group Results

PT Sample #1	AMP (ng/mL)	MAMP (ng/mL)
Lab Results (ng/mL)	631 (70%↑)	5,183 (4.7%↑)
Group Mean (ng/mL)	371	4,949

The laboratory's confirmatory test method used a periodate oxidation, solid-phase extraction, and derivatization with N-methyl-bis-heptafluorobutyramide (MBHFBA). The laboratory's periodate oxidation step occurred at approximately pH 12. As presented above,[7] in vitro demethylation of MAMP to AMP has been demonstrated to proceed at pH >9.1.

As part of the laboratory's remedial investigation, they prepared and analyzed spiked samples, as shown in Table 3. A sample with only MAMP targeted at 8,000 ng/mL produced AMP (259 ng/mL) when the periodate solution was pH 12. When tested with no periodate, a sample spiked with AMP and MAMP to mimic the PT sample gave results (356 ng/mL AMP and 5,558 ng/mL MAMP) consistent with the group means (see Table 2). The laboratory lowered the pH of the periodate oxidation step to pH 6 and retested the original PT sample. The AMP result (431 ng/mL) was 16% higher than the group mean but within the acceptable range.

Table 3. Laboratory Investigation – Spiked and PT Sample Results

Lab Investigation Samples	AMP (ng/mL)	MAMP (ng/mL)
8,000 ng/mL MAMP + periodate @ pH 12	259	7,745
Lab PT Mimic Sample (no periodate)	356	5,558
NLCP PT Sample Retest + periodate @ pH 6	431 (16%↑)	4,464 (9.7%↓)

Case #2

A year later, a PT sample was designed for the certified laboratories with a similar target MAMP concentration but a lower target AMP concentration. Again, the same laboratory reported an AMP result (329 ng/mL) that was 114% higher than the group mean, a major quantitation error. The reported MAMP concentration was acceptable (Table 4).

Table 4. Major Quantitative Error: AMP – Laboratory and Group Results

PT Sample #2	AMP (ng/mL)	MAMP (ng/mL)
Lab Results (ng/mL)	329 (114%↑)	4,825 (7.6%↑)
Group Mean (ng/mL)	154	4,482

As described above, the Navy Drug Screening Laboratory[7] demonstrated that, when using 0.15 M sodium periodate, the amount of AMP formed because of MAMP oxidation increases as the incubation time and temperature increase. The laboratory with the AMP major quantitative error used a stronger periodate concentration—0.4 M—and incubation at 60°C for 30 minutes.

For its remedial investigation, the laboratory prepared a spiked sample targeted with AMP at 175 ng/mL and MAMP at 6,300 ng/mL and evaluated the effects of different periodate concentrations and incubation times at 60°C. As shown in Table 5, as the periodate concentration increased from 0.2 M to 0.4 M, the AMP concentration increased from 172 ng/mL to 305 ng/mL with a 10-minute incubation. In contrast, when an incubation time of 15 minutes was used, as the periodate concentration increased from 0.2 M to 0.4 M, the AMP concentration increased from 175 ng/mL to 668 ng/mL.

Table 5. Laboratory Investigation – AMP Concentrations of Spiked Samples (Target AMP = 175 ng/mL, MAMP = 6,300 ng/mL) with Different Periodate Concentrations and Incubation Times at 60°C

Incubation @ 60˚ C		AMP (ng/mL)
Periodate Concentration (M)	Minutes	
0.2	10	172
	15	175
0.3	10	247
	15	394
0.4	10	305
	15	668

As a corrective action to this second major quantitative error for AMP, the laboratory further refined their periodate oxidation by lowering the sodium periodate molarity from 0.4 M to 0.15 M and decreasing the incubation time from 30 minutes to 10 minutes at 60°C. When the original PT sample was retested with the new periodate oxidation parameters, the AMP result (167 ng/mL) was 8.4% greater than the group mean but within the acceptable range. To detect the formation of AMP by MAMP oxidation, the laboratory added a MAMP control of 5,000 ng/mL to all AMP/MAMP confirmation batches.

PT Case Studies Involving the Demethylation of MDMA

Case #3

As shown in Table 6, a laboratory reported an MDA result (554 ng/mL) that was 60% higher than the group mean (347 ng/mL), a major quantitative error. The MDMA result (4,434 ng/mL) differed by <2% from the group mean.

Table 6. Major Quantitative Error: MDA – Laboratory and Group Results

PT Sample #3	MDA (ng/mL)	MDMA (ng/mL)
Lab Results (ng/mL)	554 (60%↑)	4,434
Group Mean (ng/mL)	347	4,455

The laboratory's remedial investigation included a study of spiked samples containing MDMA and MDA, as shown in Table 7. Demethylation of MDMA to MDA occurred in all samples, and the percentages of MDA ranged from 1.3% to 2.8%. The extraction conditions included the addition of carbonate buffer at pH 10 prior to the oxidation step with 10% sodium periodate and incubation at room temperature for 40 minutes, followed by solid-phase extraction with HFBA derivatization.

Table 7. Laboratory Investigation – Spiked Sample Results with Periodate Oxidation at pH 10

MDMA (ng/mL)		MDA (ng/mL)		MDA as % of MDMA
Target	Result	Target	Result	
20,000	16,572	0	259	1.6
10,000	10,604	0	137	1.3
5,000	4,612	0	98	2.1
2,000	1,744	0	43	2.5
1,000	942	0	26	2.8
500	423	0	12	2.8

As part of the laboratory's investigation, they replaced the pH 10 carbonate buffer with a pH 6 phosphate buffer prior to periodate oxidation. As shown in Table 8, MDA generated from MDMA was present in one sample at a concentration that was 0.3% of the MDMA concentration. When the original PT sample was retested with the pH 6 phosphate buffer, the MDA result (354 ng/mL) was only 2% higher than the group mean. The laboratory revised and revalidated its amphetamines extraction procedure.

Table 8. Laboratory Investigation – Spiked Sample Results with Periodate Oxidation at pH 6

MDMA (ng/mL)		MDA (ng/mL)		MDA as % of MDMA
Target	Result	Target	Result	
20,000	17,809	0	0	0
10,000	8,305	0	22	0.3
5,000	4,612	0	0	0
2,000	1,894	0	0	0
1,000	1,078	0	0	0
500	572	0	0	0

Case #4

As shown in Table 9, a laboratory reported an MDA result (409 ng/mL) that was 68% higher than the group mean of 243 ng/mL, a major quantitative error. The MDMA result (4,978 ng/mL) differed by 5% from the group mean.

Table 9. Major Quantitative Error: MDA – Laboratory and Group Results

PT Sample #4	MDA (ng/mL)	MDMA (ng/mL)
Lab Results (ng/mL)	409 (68%↑)	4,978 (5%↑)
Group Mean (ng/mL)	243	4,744

The laboratory's remedial investigation included a study of spiked samples containing MDMA only, as shown in Table 10. Demethylation of MDMA to MDA occurred in all samples, and the average percentage of MDA was approximately 2.2%. The liquid-liquid extraction procedure used a 0.1 M bicarbonate buffer with the samples and 0.35 M periodate solution at pH >10. The incubation time was 20 minutes at room temperature, followed by extraction and derivatization using N-trifluoroacetylimidazole (TFAI).

Table 10. Laboratory Investigation – Spiked Sample Results with Periodate Oxidation at pH >10

MDMA (ng/mL)		MDA (ng/mL)		MDA as % of MDMA*
Target	Mean*	Target	Mean*	
20,000	21,873	0	542	2.5
15,000	17,137	0	364	2.1
12,500	14,623	0	313	2.1
10,000	11,873	0	251	2.1
5,000	6,038	0	130	2.2
2,500	2,974	0	68	2.3

*Average of three samples

The laboratory performed another study with a modified procedure: incubation of the sample in the periodate solution, then addition of 0.1 M phosphate buffer at pH 6, followed by solid-phase extraction and derivatization. The conversion of MDMA to MDA was reduced to <0.5% with all MDA values below the limit of detection (LOD)/limit of quantification (LOQ) of 50 ng/mL. Reanalysis of the PT sample with the new extraction procedure yielded an MDA result (235 ng/mL) that was 3% lower than the group mean. The laboratory completed the PT error remedial process and validated the revised AMP extraction procedure.

Summary

The periodate oxidation step has been used by HHS-certified laboratories for more than 20 years to remove interfering OTC drugs capable of generating MAMP in the amphetamines extraction. Periodate oxidation is also used during the extraction of the MDMA compounds that were added to the federal drug testing panel in 2010. Conditions for periodate oxidation must be such that the pH is < 9.1, the molarity of the periodate is not too strong, and the incubation times and temperatures are not excessive. Otherwise, demethylation of MAMP and MDMA may occur, as discussed in the four PT error cases. In 2010, the amphetamines cutoffs were lowered to 500 ng/mL for initial drug testing and 250 ng/mL for confirmatory drug testing. At this time, HHS revised the requirement for AMP to be present (at ≥100 ng/mL) for MAMP to be reported positive. Therefore, it is also important to prevent the generation of AMP from MAMP to avoid incorrectly meeting this positive reporting requirement. MDA production from MDMA may not be as consequential in regulated testing because MDMA can be reported positive with or without MDA. Nonetheless, the PT error cases show that proper conditions for periodate oxidation must be followed to prevent MDA production and, perhaps, a PT major quantitative error.

References

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