Forensic toxicologists consider detection of 6-acetylmorphine (6-AM) definitive evidence of heroin abuse. This study investigated the possibility that aspirin, when in solution with morphine, may acetylate morphine to produce acetylmorphine (AM). Morphine sulfate-extended release tablets (15 mg) and aspirin (325 mg) tablets were incubated in 50 mL postmortem gastric contents or deionized water at 37°C. One-milliliter aliquots were taken at timed intervals, extracted by solid-phase extraction, derivatized and analyzed by the gas chromatograph with a mass selective detector. Both 3- and 6-AM were detected in samples containing morphine and aspirin in combination; no heroin was detected. Production of AM was pH dependent with optimal formation at pH ≥ 4. In gastric contents, concentrations of 3-AM exceeded that of 6-AM by ~10-fold. Production of 3-AM in gastric contents was approximately twice as high as it was in water, while matrix did not appear to affect 6-AM production. Urine specimens (10,602) assayed at a pain management laboratory were extracted by solid-phase extraction, derivatized and analyzed by the GC/MS. Both 3- and 6-AM were detected in samples containing morphine and aspirin in combination; no heroin was detected. Unpublished urine data mentioned the possibility that aspirin, when incubated in blood (6), may be contaminated with 6-AM (5, 6), and finally, when aspirin is administered in conjunction with morphine, the aspirin may have the ability to acetylate the morphine to form 6-AM (6, 8).

Aspirin (acetylsalicylic acid, ASA) is prescribed in pure form or in combination with other drugs for its analgesic, antipyretic, anti-inflammatory and anticoagulant properties (9). Within minutes (half-life of aspirin is 12–20 min), the total dose of aspirin is metabolized by the liver and blood esterases to salicylic acid (salicylate). It is the salicylate, which accounts for most of the pharmacological effects of aspirin (9). Salicylic acid has a half-life of 3–20 h.

A Medical Review Officers (MRO) Alert published in October 2009 and a case study from the Maricopa County (AZ) Office of the Medical Examiner alluded to the possibility of in vitro synthesis of 6-AM by adding aspirin to a urine (8) or blood (6) sample containing morphine. Unpublished urine data mentioned in the MRO Alert determined that it was, in fact, possible to generate 6-AM in this manner. The data provided by the Maricopa County Medical Examiner suggested that synthesis of 6-AM did not occur via an acetylation reaction between morphine and aspirin when incubated in blood (6). It remained unclear whether this process can occur in vivo.

It is plausible that a person may be prescribed both aspirin and a product containing aspirin at the same time. Co-administration of ASA and morphine is likely to occur with elderly people who take morphine for pain management and use aspirin anticoagulant therapy to prevent heart attack and stroke. If in vivo formation of 6-AM from morphine and aspirin is possible, then the elderly population is an ideal group to investigate for non-heroin-derived 6-AM.

In 2011, the Cuyahoga County Medical Examiner’s Office (CCMEO) in Cleveland, Ohio, encountered an 85-year-old, white, female who tested positive for 6-AM. The decedent was under hospice care for failure to thrive and had a history of multiple strokes, syncope, hyperkalemia, osteoporosis, anemia and osteomyelitis. There was no history of illicit drug use, and the decedent was prescribed morphine sulfate elixir (Roxanol®). Whether she was taking aspirin was not recorded and salicylates were not detected in blood by colorimetry (limit of detection (LOD) = 40 µg/mL).
Tablets of extended release morphine sulfate (MS-ER), 15 mg (Watson\textsuperscript{8}, Mallinkrodt\textsuperscript{9}), were collected as evidence from deceased individuals. Aspirin (Bayer\textsuperscript{8}), 325 mg, was an OTC preparation obtained from a local grocery. The morphine sulfate powder (≥98% purity, TLC grade) used in this study was obtained from Sigma\textsuperscript{8} (St. Louis, MO, USA) and determined analytically (see below) to contain 0.02% 6-AM and 0.2% codeine as contaminants. 6-AM, 6-AM-D3 and 6-AM-D6 standards (1 mg/mL in acetonitrile) were obtained from Cerilliant\textsuperscript{8} (Austin, TX, USA). 3-AM (1 mg/mL in acetonitrile) was obtained from Lipomed\textsuperscript{8} (Cambridge, MA, USA). Organic solvents (high-performance liquid chromatography, HPLC, grade) were manufactured by Honeywell Burdick Jackson (Muskegon, MI, USA) and purchased from Fisher Scientific. All other chemicals were of reagent grade quality or better and obtained from various commercial sources.

**Materials and methods**

**Chemicals and reagents**

Tablets of extended release morphine sulfate (MS-ER), 15 mg (Watson\textsuperscript{8}, Mallinkrodt\textsuperscript{9}), were collected as evidence from deceased individuals. Aspirin (Bayer\textsuperscript{8}), 325 mg, was an OTC preparation obtained from a local grocery. The morphine sulfate powder (≥98% purity, TLC grade) used in this study was obtained from Sigma\textsuperscript{8} (St. Louis, MO, USA) and determined analytically (see below) to contain 0.02% 6-AM and 0.2% codeine as contaminants. 6-AM, 6-AM-D3 and 6-AM-D6 standards (1 mg/mL in acetonitrile) were obtained from Cerilliant\textsuperscript{8} (Austin, TX, USA). 3-AM (1 mg/mL in acetonitrile) was obtained from Lipomed\textsuperscript{8} (Cambridge, MA, USA). Organic solvents (high-performance liquid chromatography, HPLC, grade) were manufactured by Honeywell Burdick Jackson (Muskegon, MI, USA) and purchased from Fisher Scientific. All other chemicals were of reagent grade quality or better and obtained from various commercial sources.

**Matrix selection**

Gastric specimens used in this study were selected from recent medical examiner cases that had been autopsied and known to be free of opiates and salicylates. Four different gastric solutions were used in the preliminary experiments of this investigation. The final buffered pH study was performed on a pooled gastric solution made from eight separate postmortem gastric specimens. Ultrapure distilled water used in this study was prepared in-house.

**Incubations**

All incubations were carried out in the following manner: gastric samples (50 mL) were placed in a 37°C water bath and allowed to reach temperature before the addition of morphine sulfate (15 mg) and aspirin tablets (325 mg). The concentrations of morphine and aspirin in the incubation solutions were 1.05 and 36 mM, respectively. Negative control samples were evaluated using morphine sulfate with no aspirin added. A 5-min dissolution period with mixing was observed prior to removing 1 mL aliquots (Time 0) for extraction and analysis. Subsequent aliquots were removed at 10-min intervals from 10 to 60 min and then at 90 min, 120 min and 26 h. Incubation in the water bath was stopped after 120 min and the specimens were covered and allowed to sit at room temperature until 26 h when a final aliquot (1 mL) was removed for extraction as described below.

To determine whether the formation of AM was limited by the extended release formulation, the procedure was repeated using morphine sulfate powder in place of MS-ER tablets. In these experiments, duplicate analyses were compared and pH was adjusted to 5. Morphine sulfate powder results were normalized to account for the small amount of 6-AM contaminant (~0.02%) present in the powder.

Separate incubations were performed with sodium azide (NaN\textsubscript{3}, final concentration = 15 mM) to examine whether the AM formation was catalyzed by gastric or microbial enzymes. Additionally, because sodium fluoride and potassium oxalate are additives routinely used in forensic laboratories, incubations including these additives were also performed with final concentrations of 60 and 15 mM, respectively.

To determine the effect of pH on AM formation, incubations were performed on both gastric contents and water samples buffered with 2 M preparations of potassium iodide (KI/HCl), potassium hydrogen thalate/NaOH or potassium phosphate (K\textsubscript{2}HPO\textsubscript{4}/KH\textsubscript{2}PO\textsubscript{4}) buffers to control pH at 1, 2, 3, 4, 5, 6 and 7 (10). The pH of gastric contents and DI water samples (50 mL) were measured and/or adjusted using an Orion 370 pH meter (Thermo Electron Corporation). pH studies in gastric contents (pH 4–6) were performed in triplicate and 1 mL aliquots were removed for extraction at 0, 30, 60, 90, 120 min and 26 h.

**Extraction**

The AM analytes were extracted from 1 mL aliquots by solid-phase extraction using UCT Clean Screen\textsuperscript{10} ZSDAU020 extraction columns (State College, PA, USA) and a modified version of a previously published opiate drug extraction procedure (11). Aliquots (1 mL) to be extracted were treated first with a protein precipitation step [1 mL of acetonitrile was added and the sample was vortexed 7 s, allowed to stand for 5 min, and 3 mL of potassium phosphate buffer (pH 6)] and centrifuged for 5 min at 3,500 rpm. Supernatants were decanted onto previously conditioned columns (3 mL methanol, 2 mL DI water and 1.5 mL of 0.1 phosphate buffer, pH 6). Columns were rinsed in the following order: 2 mL DI water, 2 mL 100 mM acetic acid buffer (pH 4.5) and 2 mL methanol. After drying, opiate analytes were eluted with 5 mL of freshly prepared methylene chloride: isopropanol (80 : 20) v/v with 2% ammonium hydroxide elution solvent. One-milliliter samples of matrix-matched blank calibrators (6-AM: 4, 10, 20, 30 and 40 ng/mL; 3-AM: 10, 50, 200, 400 and 1,000 ng/mL, respectively) and a 20 ng/mL matrix-matched 6-AM control were also extracted. After dry down on a Biotage Turbo Vap\textsuperscript{8} LV evaporator, extracted samples were reconstituted with 50 μL acetonitrile and derivatized with 50 μL Selectra-Sil\textsuperscript{10} MSTFA (UCT) on a heating block (75°C) for 20 min.

**Instrumentation**

Analysis by the gas chromatograph with mass selective detector (GC–MSD) was performed in the selective ion monitoring (SIM) mode, on an Agilent Technologies (Wilmington, DE, USA) 7890A/5975C GC–MSD with an Agilent 7683B auto-injector. The GC–MSD was used for separation and identification of the 6- and 3-AMs, respectively. A Restek Rxi\textsuperscript{8} 5-ms capillary column (30 m x 0.25-mm I.D., 0.25-μm film thickness) was used with a helium gas flow rate of 1.276 mL/min.
Operating parameters were as follows: the injection port temperature was 239°C, the initial oven temperature was 150°C with an initial hold time of 2.00 min, a ramp rate of 50.0°C/min to 200°C for 1 min and then 10.0°C/min to 250°C for 10 min, resulting in a total run time of 19 min. The injector was operated in a splitless mode. The MSD was operated in the electron impact ionization mode, the interface temperature was 280°C and the quadrupole and ion source were set at 230 and 150°C, respectively. The sample injection volume was 1.5 μL and wash solvent was methanol, with a total of five pre- and five-post syringe washes between samples.

**Analysis**

These analytes were identified by their characteristic retention times and quantified from a calibration curve with target ion m/z 399 for 6-AM and 357 for 3-AM. Additionally, qualitative identification was made by ion ratio criteria with qualifying ions of 287 and 400 for 6-AM and 329 and 356 for 3-AM. When chromatography was poorly resolved for 6-AM using 399, 287 and 400 ions, alternate ions (340 and 204) were used to obtain satisfactory chromatographic resolution for quantification and qualification. In these circumstances, calibrators and controls were evaluated in the same fashion as unknown specimens. Retention times for analytes had a reference window of ±0.1 min. Alternating methanol and chloroform blanks were injected between samples to prevent carryover.

**Linearity/precision**

The limit of quantitation (LOQ) and the upper LOQ (ULOQ) for 6-AM were 4 and 160 ng/mL, respectively. The calibration curve was linear, with a coefficient of determination of \( R^2 = 0.996 \). The coefficient of variation (CV) for intra-run precision was 8.32%. The CV for inter-run precision was 10.77%. The LOQ for 3-AM was determined to be 10 ng/mL and the ULOQ was 1,200 ng/mL. The calibration curve was quadratic, with a coefficient of determination of \( R^2 = 1.000 \). No 3-AM control was available for analysis due to the inability to purchase 3-AM from a separate source.

**In vivo formation**

Cases from both the CCMEO (>6,000 postmortem cases) and Ethos Laboratory (10,602 antemortem urine samples) were reviewed to determine whether there were any incidences of unexplained 6-AM results. Three total cases were identified and were evaluated in more detail.

**Results and discussion**

**Initial in vitro formation of 6-AM**

Initial studies examined the formation of only 6-AM in gastric contents and DI water. 6-AM formation in gastric contents exceeded that in DI water during the 120-min incubation, and ultimately at 26 h. In negative control samples containing only morphine, no 6-AM was detected demonstrating that 6-AM had not come from an impurity in the morphine sulfate tablet, or as a result of the extraction process. Continuous formation of 6-AM in two separate postmortem gastric specimens (pH 4.74 and 5.27) was linear with respect to time with \( R^2 = 0.982 \) and 0.997, respectively. At 26 h, 124 and 121 ng/mL 6-AM had been formed in the gastric samples, respectively. Formation of 6-AM in DI water (pH 7) was not linear over time (\( R^2 = 0.630 \)) and occurred at a much lower concentration; at 26 h, only 27 ng/mL 6-AM had been synthesized in water. In the initial in vitro experiments, the pH of the samples was not controlled. The addition of aspirin to solutions caused a drop in pH. The water sample exhibited the most change in pH from 7 down to 2.88. The two gastric samples appeared to have a natural buffering ability and lowered to pH 3.92 and 3.86, respectively.

**Formation of 6-AM from morphine sulfate powder**

Morphine sulfate powder (15 mg) was substituted for the MS-ER tablet to determine whether the time release properties of the tablet had an effect on 6-AM formation. Duplicate analyses in postmortem gastric specimens were performed at pH 5. 6-AM formation was linear with respect to time from both the morphine sulfate powder (\( R^2 = 0.998 \)) and the MS-ER tablet (\( R^2 = 0.963 \)) during 120-min incubation. The concentration of 6-AM was significantly higher (paired t-test, \( P < 0.05 \)) from the morphine sulfate powder compared with that observed with the extended release tablets, indicating that the extended release properties of the MS-ER tablet did limit 6-AM formation. From these results, it was hypothesized that the formation of 6-AM was pH dependent and possibly catalyzed by gastric or microbial enzymes.

**Formation of 6-AM in the presence of inhibitors**

In two separate experiments, formation of 6-AM from aspirin and morphine in gastric contents was measured in the presence of enzymatic inhibitors NaN₃ and sodium fluoride. NaN₃ and sodium fluoride had no effect on 6-AM production compared with uninhibited controls. Throughout the initial 2-h incubation, the amount of 6-AM formed was equivalent in both the gastric solution containing the NaN₃ (\( R^2 = 0.979 \)) and the control (\( R^2 = 0.983 \)). After 26 h, the formation of 6-AM in the inhibited gastric contents (136 ng/mL) exceeded that of the non-inhibited gastric contents (100 ng/mL). Sodium fluoride/potassium oxalate (5 : 1) additives were also evaluated, because they are commonly used in forensic toxicology laboratories as antimicrobial/anticogulants to preserve the integrity of post-mortem specimens. Addition of sodium fluoride/potassium oxalate did not inhibit the formation of 6-AM (\( R^2 = 0.983 \)); at 26 h, 101 ng/mL 6-AM had been formed. Therefore, formation of 6-AM from aspirin and morphine does not appear to be an enzymatic reaction since addition of enzymatic inhibitors did not alter the formation of 6-AM.

**Detection of 3-acetylmorphine formation**

During SIM analysis for 6-AM (m/z 399), an additional peak was detected, which eluted immediately following 6-AM (Figure 1A). During SIM analysis for 6-AM (m/z 399), an additional peak was detected, which eluted immediately following 6-AM (Figure 1A). Detection of 3-acetylmorphine formation could not come from an impurity in the morphine sulfate tablet, or as a result of the formation of 6-AM from aspirin and morphine does not appear to be an enzymatic reaction since addition of enzymatic inhibitors did not alter the formation of 6-AM.
Effect of pH on formation of 3- and 6-AMs

The effect of pH on the production of 3- and 6-AMs was evaluated in buffered solutions of gastric contents and water. Gastric samples, pH range 4.17–6.85, were pooled with a final pH of 5.06. Aliquots (50 mL) of the pooled gastric contents were adjusted to pH values of 1–7 using appropriate buffers (10). When the addition of an aspirin tablet altered the adjusted pH, further titration with buffers was carried out to ensure the desired pH. Seven samples of DI water were prepared in the same fashion.

Formation of both 6- and 3-AM was pH dependent with production greatest at pH 4. Essentially, no 3- or 6-AM was formed in gastric contents or water at pH 1 and 2. At pH 3, and after 26 h (1,560 min), the formation of both 3- and 6-AM was detected. With incubation conditions the same, the formation of 6-AM in gastric contents and water was almost the same at 18 and 19 ng/mL, respectively, while that of 3-AM in gastric contents was 174 ng/mL and in water was 77 ng/mL. Production of 6-AM increased as the pH increased, for both gastric contents and water. Figure 2A–D shows the formation of 3- and 6-AM in gastric contents and water at pH 4, 5 and 6 (this pH range best represented the pH of post-mortem gastric contents used in this study). For 3-AM in gastric contents, the formation was greatest at pH 6 (1,022 ng/mL, at 26 h); formation of 6-AM in gastric contents was greatest at pH 7 (133 ng/mL, at 26 h). Formation of 6-AM in gastric contents paralleled its formation in water. After 26 h, the production of 3-AM in gastric contents was ~10-fold higher than that of 6-AM in gastric contents; 3-AM in water was ~5-fold higher than 6-AM in water. The factors causing the formation of 3-AM in gastric contents to be approximately twice what was detected in water are unknown. Formation of 6-AM in gastric contents was measured in triplicate, while measures of 3-AM in gastric contents and water and 6-AM in water were single determinations. Replicate analyses were prevented by a depleted supply of extended release morphine sulfate (15 mg).

Reaction mechanism

To determine whether AM was formed from a direct reaction with acetic acid, which had been hydrolyzed from aspirin, morphine sulfate-extended release tablets were incubated with acetic acid solution (0.036 M). Assuming complete hydrolysis, the concentration of acetic acid was calculated based on 325 mg
aspirin placed in 50 mL DI water. After 2 h of incubation at 37°C, no 3- or 6-AM was detected.

Formation of AM from aspirin and morphine is postulated to occur as diagramed in Figure 3, by the direct trans-esterification between the aspirin and morphine tablets. Figure 3A shows the products formed in the reaction between aspirin and morphine. Although both mono-acetylated products are formed in this transfer of the acetyl group from aspirin to the morphine molecule, as described above, the formation of 3-AM is preferential (12). Salicylic acid is the other product formed in this process. Figure 3B illustrates the chemical mechanism for the trans-esterification process using the major reaction product, the 3-AM. In this process, the phenolic hydroxyl group at the 3-position of morphine displaces the acetyl group from aspirin yielding the 3-AM and salicylic acid. The formation of 6-AM would occur in an analogous mechanistic process from the alcoholic 6-hydroxy group.

The formation of AM from aspirin and morphine follows the same pattern as the metabolism of heroin (diacetylmorphine), only in reverse (12). Hydrolysis of the 3-acetyl moiety is preferential to that of 6-AM, thus leaving the latter as the definitive heroin metabolite (13). Preferential hydrolysis at the 3-position most likely reflects the fact that esters of phenols (aromatic ring with the 3-position) are easier to hydrolyze than that of alcohols. In the formation of AM, preferential acetylation at the 3-position probably occurs first because of steric factors between morphine and aspirin.

In vivo formation
Approximately 6,000 CCMEO postmortem cases and 10,602 specimens from Ethos Laboratory (pain management laboratory) were investigated for the possibility of in vivo formation of 6-AM. There was only one unexplained 6-AM result at CCMEO, thus leaving the latter as the definitive heroin metabolite (13). Preferential hydrolysis at the 3-position most likely reflects the fact that esters of phenols (aromatic ring with the 3-position) are easier to hydrolyze than that of alcohols. In the formation of AM, preferential acetylation at the 3-position probably occurs first because of steric factors between morphine and aspirin.

The Ethos cases identifying only 6-AM (without the presence of morphine or codeine) may have contained conjugated...
morphine. Perhaps further hydrolysis of the specimens would produce the necessary morphine evidence to support heroin use (7). Other explanations may include administration of heroin too close to the time of urine collection (preventing systemic distribution), morphine concentrations below cutoff or polymorphism of heroin metabolism (5).

The human stomach seems like an anatomically reasonable location for in vivo formation of AMs. Normal pH of gastric contents during fasting is ~1.5–3.0 (14). In our study, postmortem gastric contents ranged in pH from 4.17 to 6.85. It is presumed that the presence of food stuffs caused the gastric contents to be more alkaline. This investigation demonstrated that a pH range of 4–7 was optimal for formation of both 3- and 6-AM.

Although AM may form in gastric contents when morphine and aspirin are co-administered, it is unlikely that detection of 6-AM will occur in blood analysis. Previous studies evaluating the pharmacokinetics of heroin demonstrated that, following administration of low oral doses of heroin, only measureable concentrations of morphine were detected; no 6-AM was present in serum after exposure (18–20).

It is expected that AM does not form in vivo to any forensically significant extent. In cases where there is concern of AM production, a careful case history and identification of 3-AM would be crucial in making that determination. If AM is formed in the stomach from co-administration of morphine and aspirin, it is likely that it is quickly hydrolyzed to morphine by enzymatic activity in the gastrointestinal tract (20).

Conclusions
In this work, it was demonstrated that in vitro production of 6-AM occurred when morphine and aspirin were incubated at 37°C in both postmortem gastric contents and DI water. Additionally, 3-AM was produced. Formation of both 3- and 6-AM was dependent on the pH. Under the conditions employed, 3-AM was the preferential product formed. The mechanism of production was found to be non-enzymatic and was expected to be the result of a direct trans-esterification between aspirin and morphine. 3-AM was the favorable product possibly due to steric factors. These findings are significant to toxicologists, because 6-AM detection is expected to be a result of heroin metabolism. No heroin was detected in any of the incubated samples in this study.

After examining over 16,000 ante- and postmortem specimens for evidence of AM production, it was determined that 6-AM does not form in vivo to any forensically significant extent. In cases where there is concern about AM production, a screen for opiates and salicylates should be performed and 3-AM should be monitored. When appropriate, stomach contents may be a suitable alternative sample for testing if case history indicates oral administration of both aspirin and morphine.

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