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Transformation of codeine and codeine-6-glucuronide to opioid analogues by urine adulteration with pyridinium chlorochromate: potential issue for urine drug testing

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RATIONALE: Pyridinium chlorochromate (PCC) is the active ingredient of 'Urine Luck', a commercially available *in vitro* adulterating agent used to conceal the presence of drugs in a urine specimen. The exposure of codeine and its major glucuronide metabolite codeine-6-glucuronide (C6G) to PCC was investigated to determine whether PCC is an effective masking agent for these opiate compounds.
METHODS: Following the addition of PCC to both spiked and authentic codeine and C6G-positive urine specimens, the samples were monitored using liquid chromatography/mass spectrometry (LC/MS). Stable reaction products were identified and characterized using high-resolution MS analysis and, where possible, nuclear magnetic resonance (NMR) analysis.
RESULTS: It was determined that PCC effectively oxidizes codeine and C6G, thus altering the original codeine-to-C6G ratio in the urine specimen. Four reaction products were identified for codeine: codeinone, 14-hydroxycodeinone, 6-*O*-methylcodeine and 8-hydroxy-7,8-dihydrocodeinone. Similarly, three reaction products were identified for C6G: codeinone, codeine and a lactone of C6G (tentative assignment).
CONCLUSIONS: Besides addressing the complications added to interpretation, more investigation is warranted to further determine their potential for use as markers for monitoring the presence of codeine and C6G in urine specimens adulterated with PCC.

The act of adulteration to mask the presence of drugs-of-abuse in urine is an issue that has been recognized by drug testing authorities for decades. Specifically, the use of *in vitro* adulterants is of great interest due to its oxidizing capabilities. Examples of commercial *in vitro* adulterants and their active ingredients include: 'Klear' (sodium nitrite), 'Whizzies' (potassium nitrite), 'Urine Luck' (pyridinium chlorochromate or PCC) and 'Stealth' (peroxidase/peroxide).[1–4] In studies conducted by Kuzhiumparambil and Fu,[5,6] it was shown that various oxidizing adulterants significantly altered the steroid profile that is naturally found in human male and female urine, potentially masking steroid abuse. Furthermore, Luong *et al.*[7,8] reported that the concentrations of 6-monoacetylmorphine (6-MAM), morphine and morphine-6-glucuronide (M6G) in urine specimens were significantly diminished upon nitrite adulteration. This was a result of the nitrite oxidant converting each of the opiates into their nitro analogues, forming 2-nitro-6-monoacetylmorphine (2-nitro-MAM), 2-nitromorphine and 2-nitromorphine-6-glucuronide (2-nitro-M6G), respectively. Interestingly, codeine was shown to be insignificantly affected by the nitrite adulteration process.

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Codeine is an alkaloid commonly derived from the opium poppy plant, *Papaver Somniferum*.[9,10] It possesses analgesic properties, and is often legally prescribed for the management of mild to moderately severe pain. Codeine is also available over the counter as an active ingredient in cough suppressant formulations due to its antitussive effects. It is also used for sedative purposes, as well as for the treatment of acute pulmonary edema and diahorrea.[11] Although codeine has many valuable therapeutic uses, it is also highly abused due to its psychotropic properties.[12] Drug testing programs routinely monitor codeine due to its high prevalence for abuse. In addition, the presence of codeine may also be linked to illicit heroin administration (codeine is present as an artifact of the manufacturing process).

In the human body, codeine is metabolized in the liver by P450 CYP2D6 enzymes to form morphine. The *N*-demethylation of codeine facilitated by CYP3A4 enzymes to produce norcodeine is also reported. However, conjugation of both the parent drug and the metabolites as the glucuronide remains a significant metabolic pathway. After an oral dose, 80–90% is excreted in urine as codeine or codeine-6-glucuronide (C6G); within this 80–90%, approximately 10% is codeine and 90% is C6G.[11–13]

The objective of this study was to determine the effect of PCC adulteration on codeine and C6G in urine. It has been reported that PCC contains the hexavalent chromium (Cr^{6+})

which significantly oxidizes 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol in urine.[3,4,14] On the contrary, it has also been suggested that the mechanism of interference by PCC appeared to be through the decrease of pH levels of the urine specimens, and not by chemically altering the target drug analyte.[15] Thus, the aims of our study were to expose codeine and C6G to PCC in urine and monitor the specimens over time. Liquid chromatography/mass spectrometry (LC/MS) allowed any stable reaction products that may be formed to be identified. Structural elucidation was also complemented by nuclear magnetic resonance (NMR) spectroscopy when possible.

EXPERIMENTAL

Drug standards and reagents

Codeine free base (1 mg/mL in methanol), codeine-6-β-D-glucuronide (1 mg/mL in acetonitrile/water 1:1), oxycodone hydrochloride monohydrate (1 mg/mL in methanol) and ethylmorphine (1 mg/mL in methanol) were sourced from Lipomed (Arlesheim, Switzerland). Codeine hydrogen phosphate solid was obtained from Macfarlan Smith Limited (Edinburgh, UK) and 6-*O*-methylcodeine solid was sourced from the National Measurement Institute (North Ryde, NSW, Australia).

PCC and oxalyl chloride were sourced from Sigma Aldrich (Castle Hill, NSW, Australia). Ammonium formate, acetic acid and deuterated chloroform ($CDCl_3$) were sourced from Sigma Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile was purchased from Honeywell (Muskegon, MI, USA). Sodium acetate was obtained from Merck (Darmstadt, Germany). Disodium hydrogen orthophosphate, sodium dihydrogen orthophosphate, sodium hydrogen carbonate and sodium carbonate were sourced from Ajax Chemicals (Sydney, NSW, Australia).

The carbonate/bicarbonate buffer (pH 9.5, 1.5 M) was prepared by dissolving 63.6 g sodium carbonate and 75.6 g sodium hydrogen carbonate in water, and made up to a 1 L volume.

Urine specimens

Urine from healthy individuals were collected using polypropylene urine specimen containers and pooled (n = 4) to create a representative blank urine matrix. Volunteers were selected randomly and had highly variable diets, both male and female, aged between 25–60 and from different ethnic backgrounds. The imposed condition was that they had not taken pain medication or eaten poppy seeds. The same combination of donors was not used for more than one experiment. Pooled urine was used on the day of voiding, and analyzed using the LC/MS methods developed in this study to ensure that it was negative for opiates prior to use.

Authentic urine specimens testing positive for codeine and C6G were supplied by the Drug Toxicology Unit, NSW Forensic and Analytical Science Service, after removal of sample identification. The specimens were stored in a freezer at –18°C before analysis.

Instrumentation

All samples were analyzed using a 1290 LC system coupled to a 6490 triple quadrupole (QQQ) mass spectrometer or a 6510 quadrupole time-of-flight (QTOF) mass spectrometer for high-resolution mass measurement. NMR data were recorded on a 500/54 premium shielded NMR spectrometer paired with a 7510-AS autosampler. These instruments were from Agilent Technologies (Forest Hill, VIC, Australia). The instrumental parameters for LC/MS and NMR are detailed below.

LC/MS

An electrospray ionization interface was utilized for LC/MS analysis. Analyte separation was achieved by injecting 1 µL of the sample onto an Agilent Zorbax Eclipse XDB-C18 column (2.1 mm × 50 mm × 1.8 µm) set at 40°C with a 0.25 mL/min flow rate. Mobile phase A consisted of ammonium formate (pH 6.3, 20 mM) and mobile phase B was 95% acetonitrile in water. Mobile phase A was diluted from a 2 M ammonium formate stock solution that was prepared by dissolving ammonium formate solid in water. Each analysis had a starting gradient of 2% B, which was increased to 5% at 6 min, 30% at 12 min, 70% at 17 min and 95% at 19 min; this gradient was held until 21 min was reached, and then decreased to 2% at 21.1 min. A 4 min post-run column equilibration at 2% B was the final step to ensure that the column was conditioned for the next analysis. Full scan MS (scan time = 500 ms) and product ion scan MS/MS analyses (scan time = 150 ms) were performed in positive ion mode. The protonated molecule of an analyte was used as the precursor ion for MS/MS experiments. For QQQ-MS, the default fragmentor voltage (380 V) was used, with a collision energy range of 25–45 eV. The sheath gas temperature and flow were 250°C and 11 L/min, respectively. For the QTOF-MS, targeted MS/MS was carried out (scan time = 200–500 ms) with the fragmentor and collision energy ranges falling within 150–250 V and 20–40 eV, respectively. Mass correction was carried out using *m/z* 121.0509 and *m/z* 922.0098 reference ions. The gas temperature and flow were maintained at 200°C and 14 L/min, respectively. The capillary and nozzle voltages were adjusted to 3000 V and 1500 V, respectively.

NMR

^{1}H-NMR (1024 scans; 1 s relaxation delay), ^{13}C-NMR (10 000 scans; 1 s relaxation delay), and ^{1}H-^{1}H COSY (8 scans per t1 increment, 512 t1 increments; 2 s relaxation delay) were performed.

Experimental procedures

Exposure of opiates to PCC

To monitor the effect of PCC on codeine and C6G in aqueous environments, a series of samples were prepared and monitored over time using LC/QQQ-MS. A 1 M PCC working solution was prepared by dissolving solid PCC in water. Another four PCC working solutions were then prepared (200, 20, 2 and 0.2 mM) by serial dilution. Codeine and C6G were spiked into water (at 10 000 ng/mL in the final 1 mL sample) and adulterated with each of the PCC working

Rapid Communications in Mass Spectrometry

solutions to give final oxidant concentrations of 100, 20, 2, 0.2 and 0.02 mM PCC in the samples (100 µL PCC working solution per 1 mL sample). For C6G, only final PCC concentrations of 100 mM and 20 mM were trialed. The reactions were allowed to proceed for 1 h at room temperature (22°C) prior to analysis, and then subsequently refrigerated at 4°C when not analyzed. The samples were monitored at 1 h, 1 day and 1 week, unless otherwise specified. A codeine standard (10 000 ng/mL), reagent controls (opiate negative), and a water blank were prepared and analyzed alongside the adulterated samples. The pH readings were measured for all samples (including the control samples) and recorded. A relatively high concentration of codeine and C6G was used in this study to facilitate easy monitoring of product formation.

In a parallel sample set, the codeine/C6G reactions with PCC were also replicated in blank urine. Samples were fortified with 100 mM and 20 mM PCC. The urine samples were centrifuged at 4500 *g* for 10 min and filtered through 0.22 µm hydrophilic syringe filter units (MicroAnalytix Pty Ltd, Taren Point, NSW, Australia) prior to analysis.

One authentic urine specimen positive for codeine and C6G was also adulterated with 100 mM and 20 mM PCC, and processed in the same manner as the other specimens. The samples were monitored at 1 h, 1 day and 5 days after adulteration.

Preparation of the reaction products

To produce enough material for structural elucidation of the reaction products present in the codeine and PCC reaction mixture by NMR, the reactant ratios detailed in the section above were proportionally scaled up in water to accommodate 5.62 mg codeine with 20 mM PCC. The reaction mixture was left for several weeks at room temperature and monitored by LC/QQQ-MS prior to extraction. Solid-phase extraction was performed to isolate the reaction products, which were adsorbed onto Clean Screen® CSDAU extraction columns (United Chemical Technologies, Bristol, PA, USA).[16] Anhydrous potassium carbonate was used to dry the combined final eluate fractions, before being dried down under a gentle stream of nitrogen at 30°C. The remaining residue was also placed in a vacuum desiccator overnight prior to reconstitution in 600 µL $CDCl_3$ and analysis by NMR spectroscopy.

In addition, a codeinone reference sample was synthesized via the Swern oxidation using a method adapted from Huang *et al*.[17] Codeine hydrogen phosphate (30 mg) was converted into the free base form by dissolving the solid in water, basifying the solution to pH 9.5 with carbonate/bicarbonate buffer, and extracting the aqueous fraction with dichloromethane (DCM). The organic solvent was evaporated under nitrogen and the codeine residue (19 mg) was re-dissolved in anhydrous DCM. Codeinone was then synthesized from the codeine base. The reaction was conducted under a nitrogen atmosphere at –78°C (dry ice/acetone cooling bath). A solution of oxalyl chloride (100 µL) in anhydrous DCM (580 µL) was added dropwise to a solution of dimethyl sulfoxide (170 µL) in anhydrous DCM (905 µL) in the reaction vessel over 20 min, with continual stirring. The reaction mixture was further stirred at –78°C for 1.5 h. This was followed by a dropwise addition of the codeine base solution over 15 min with continuous stirring extending over another 2 h. Finally, triethylamine (56 µL) and dry DCM (112 µL) were added and the reaction mixture stirred for another 10 min before being warmed up to room temperature. The sample was washed with six equivalent volumes of water. The organic fraction was isolated and dried using sodium sulfate. LC/MS analysis was carried out on this sample, in addition to the samples prepared above to further aid structural elucidation of the reaction products.

Immunoassay and GC/MS study

A batch of urine specimens consisting of two opiate negative blank samples and six opiate positive samples were adulterated with 100 mM and 20 mM PCC and left to react for 16 h (overnight) at 22°C. Each specimen was divided into two aliquots; the first aliquot was sent to the Drug Toxicology Unit for immunoassay screening and GC/MS confirmatory testing. The CEDIA® Opiate immunoassay (Microgenics Corp., Fremont, CA, USA) was performed on an Olympus AU 2700 analyzer (Olympus America Inc., Melville, NY, USA). The GC/MS confirmatory testing was performed using an in-house validated method that involved enzymatic hydrolysis by β-glucuronidase, extraction on Clean Screen® CSDAU columns, derivatization of the extract by bistrimethylsilyltrifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS), and MS analysis in selected ion monitoring (SIM) mode. Quantification was based on the use of internal standards codeine-d_6 and morphine-d_6. The second aliquot was concurrently analyzed on the LC/MS instrument using the conditions detailed above.

RESULTS AND DISCUSSION

Exposure of codeine and C6G to PCC

Codeine and C6G samples prepared in water and urine were spiked with PCC at various concentrations to mimic adulteration conditions. The oxidant working solution concentrations were chosen based on the PCC concentration found in the commercial product 'Urine Luck', which has been reported to contain 200 mM PCC.[18] In a real-life situation, the exact number of vials used is arbitrary and amount of urine voided is highly variable; therefore, a concentration range was trialed. During each LC/MS analysis conducted in the exposure studies, fresh codeine/C6G standards and reagent controls were successively analyzed with the adulterated specimens. This established that significant decreases in codeine and C6G peak area abundances were attributed to reaction progression, and not starting material degradation. The reagent controls ensured that any reagent peaks could be distinguished from peaks belonging to potential reaction products. Additionally, post-column infusion experiments did not indicate that PCC in the urine contributed any additional matrix effects in the analyte regions of interest (approximately 6–12 min) when compared to urine alone.

Reaction of codeine and PCC in water and in urine

The exposure of codeine ($[M+H]^+$ at *m/z* 300) to PCC in both water and urine resulted in the formation of multiple reaction products: one major product with $[M+H]^+$ at *m/z* 298

(product *m/z* 298), one minor product with $[M+H]^+$ at *m/z* 316 (product *m/z* 316) and another two minor products with $[M+H]^+$ at *m/z* 314 which is referred to as product *m/z* 314a and product *m/z* 314b in this study. It appeared that the same four reaction products were detected in both water and in urine under the LC/MS conditions employed. However, their relative abundances appeared to be affected by three factors: the oxidant concentration, the type of aqueous reaction medium and the time elapsed since the codeine samples were adulterated with PCC. It was found that all four reaction products were still detected in the urine specimens 1 week subsequent to PCC adulteration, demonstrating that they are stable enough to be detected in the time frame required for urinalysis. Furthermore, codeine is still detectable in the samples following PCC adulteration, albeit with a loss of abundance. This increases the difficulty in data interpretation of opiate test results. The observation of codeine and its analogues in the specimen may be an indication that the other reaction products may have originated from codeine modification.

A steady decrease in codeine concentration was observed in specimens fortified with PCC, with codeine detected at 9307 ng/mL (1 h), 4301 ng/mL (1 day) and 1330 ng/mL (1 week) in urine containing 20 mM PCC. This diminishment was more pronounced in urine containing 100 mM PCC, where the codeine concentration was detected at 7895 ng/mL (1 h) and 1985 ng/mL (1 day). At the end of the 1-week monitoring period, the codeine concentration was detected at less than 100 ng/mL.

Figures 1(a)–1(c) depict the TIC chromatograms of the codeine urine sample adulterated with 100 mM PCC over 1 week. Under the LC/MS conditions employed, the retention time (R_t) for codeine is 9.2 min, with major product ions at *m/z* 153, 165 and 181. Product *m/z* 314a (R_t = 10.3 min) has major product ions at *m/z* 239, 254 and 296, with product *m/z* 314b (R_t = 10.8 min) possessing major product ions at *m/z* 152, 165 and 181. Product *m/z* 316 (R_t = 9.5 min) and product *m/z* 298 (R_t = 11.3 min) have characteristic product ions at *m/z* 171, 185 and 199, and *m/z* 153, 165 and 181, respectively.

Table 1 is a summary of the analytes that were detected in water and in urine over 1 week. Upon comparison of the codeine specimens adulterated with 20 mM PCC, it appears

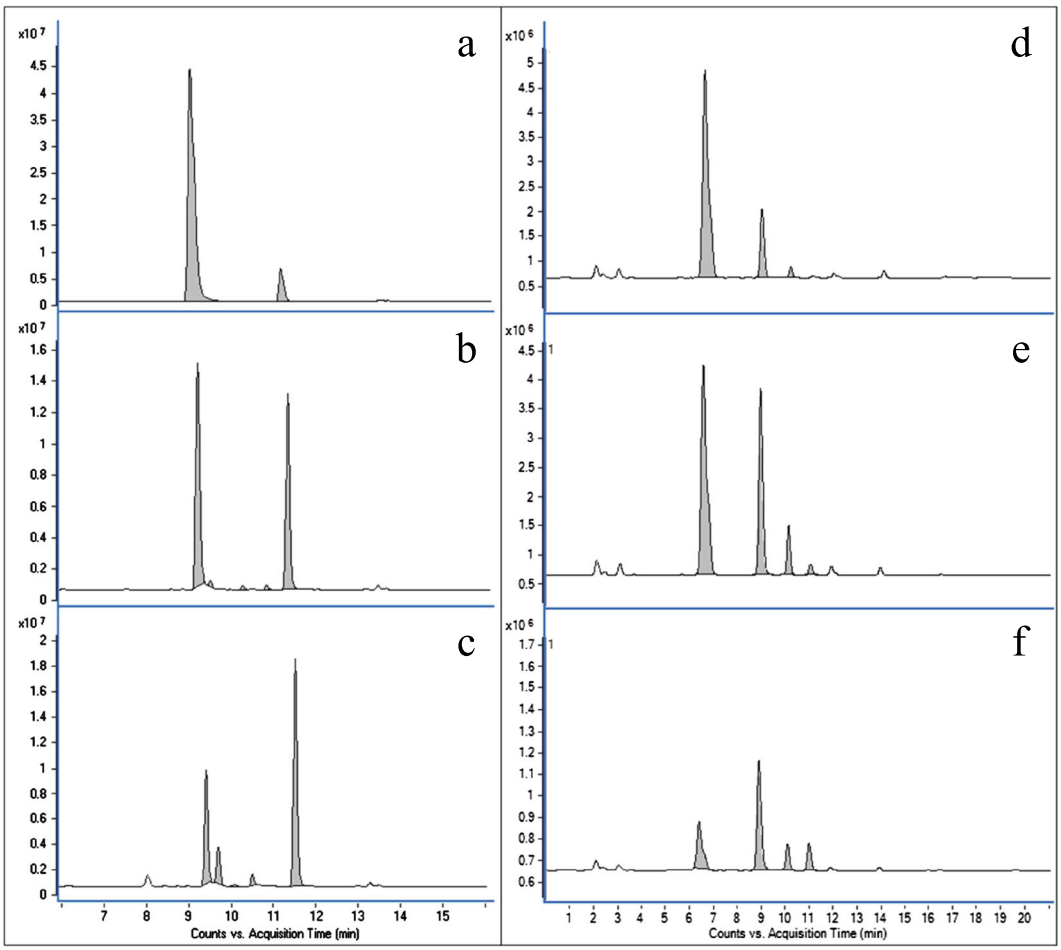

Figure 1. TIC chromatograms (product ion scan) of a codeine urine sample adulterated with 100 mM PCC (a) 1 h, (b) 1 day, and (c) 1 week after adulteration (the peak at 8.1 min was also present in the control samples and therefore determined not to be a reaction product); and TIC chromatograms of a C6G urine sample adulterated with 100 mM PCC (d) 1 h, (e) 1 day, and (f) 1 week after adulteration. Nb: R_t for codeine is 9.2 min; R_t for product *m/z* 316, product *m/z* 314a, product *m/z* 314b and product *m/z* 298 are 9.5 min, 10.3 min, 10.8 min and 11.3 min, respectively. R_t for C6G and the product *m/z* 416 are 6.5 min and 10.1 min, respectively.

Rapid Communications in Mass Spectrometry

Table 1. Relative peak areas of analytes (normalized to the most abundant analyte (100%)) detected in the codeine-adulterated samples after 1 h, 1 day and 1 week elapsed since commencement of the reaction using LC/MS (product ion scan) analysis

	20 mM PCC					100 mM PCC				
	Codeine	Product *m/z* 316	Product *m/z* 314a	Product *m/z* 314b	Product *m/z* 298	Codeine	Product *m/z* 316	Product *m/z* 314a	Product *m/z* 314b	Product *m/z* 298
Codeine in water										
1 hr	100.00	-	-	-	1.11	100.00	-	-	-	25.00
1 day	100.00	-	-	2.34	38.17	15.10	2.85	-	0.61	100.00
1 week	83.21	5.30	0.25	2.02	100.00	-	22.06	1.07	-	100.00
Codeine in urine										
1 hr	100.00	-	-	-	0.63	100.00	-	-	-	10.78
1 day	100.00	0.06	5.72	1.99	11.55	100.00	1.58	1.68	1.93	79.55
1 week	100.00	4.98	15.86	0.87	8.39	48.65	14.08	0.77	4.45	100.00

The collision energy used for codeine is 40 eV. Collision energies used for products *m/z* 316, 314a, 314b and 298 are 40 eV, 25 eV, 25 eV and 40 eV, respectively. Reactions were allowed to proceed for 1 h at room temperature (22°C) prior to analysis, and then subsequently refrigerated at 4°C when not analyzed.

that the urine matrix facilitates the reaction pathway for the formation of products *m/z* 316 and *m/z* 314a to a greater extent than water. This can be seen by the absence of both these products after 1 day of reaction, but present after 1 week, in water. In contrast, all four reaction products were detectable after 1 day and also 1 week in urine.

In terms of the effect of oxidant concentration on the formation of the major reaction product, the data suggested that higher PCC concentrations encouraged the formation of product *m/z* 298, relative to the lower concentrations trialed. This was evidenced by the relative peak areas of codeine to product *m/z* 298 at the same time period, when comparing the two different PCC concentrations (Table 1). This observation was further demonstrated with samples fortified with lower PCC concentrations (0.02–2 mM). In a simplified reaction system, it was expected that an increase in substrate (PCC) concentration would result in an increased rate of formation of product *m/z* 298.

Due to the numerous pathways of oxidation by PCC, it is quite difficult to determine the effect of the reaction matrix on the rates of the reactions, which is beyond the scope of this study. However, the findings of the exposure studies suggested that formation of product *m/z* 298 was more favored in water compared to urine. With adulteration using 20 mM PCC and sample monitoring after 1 day, abundances of codeine (7612 ng/mL and 4301 ng/mL in water and urine, respectively) were approximately three and nine times greater than product *m/z* 298. After 1 week, the abundance of product *m/z* 298 became 1.2 times greater than codeine itself (2044 ng/mL) in water. On the other hand, codeine (1330 ng/mL) remained the predominant analyte in urine after 1 week, approximately 12 times greater than product *m/z* 298 (Table 1). The same trend was observed with samples adulterated with 100 mM PCC. This suggested that the endogenous compounds in urine were competing against codeine for reaction with PCC.

Additional information can be derived from monitoring all four of the reaction products of interest in the codeine specimens adulterated with 100 mM PCC. In general, it was apparent that the PCC was exerting its oxidizing capabilities, with the depletion of the codeine starting material and formation of reaction products detected over time. The buffering capacity of the urine matrix appeared to stabilize the reaction products, which were all detected 1 day and 1 week after adulteration (Table 1).

A different scenario was observed in the corresponding specimen with water as the reaction matrix. After 1 day, codeine concentration was seen to have significantly decreased (less than 100 ng/mL), with product *m/z* 298 being the major analyte in the sample (approximately seven times greater than codeine, Table 1). After 1 week, codeine was found to be undetectable under the conditions of analysis. It may be worthwhile to mention that product *m/z* 314a was not detectable in this specimen after 1 day, but was detectable after 1 week; the contrary was observed for product *m/z* 314b. These findings support the idea that urine possesses a buffering role in the multiple oxidation pathways of codeine, and stabilizes the reaction products in the sample to some degree.

Since LC/MS monitoring showed that the same four reaction products were formed in both water and urine matrices, codeine-fortified water samples were adulterated with PCC at a lower concentration range (0.02–2 mM). In general, only two of the four reaction products (product *m/z* 314b and *m/z* 298) detected in the exposure studies were found to have formed; no additional reaction products were detected. The trend for their formation was consistent with the trend observed for the higher concentrations of PCC.

The pH measurements of both the water and urine samples (codeine and reagent control samples) spiked with PCC demonstrated the acidifying effect of the oxidant; the higher the PCC concentration, the lower the pH. The addition of 20–100 mM PCC to urine specimens with pH 6 resulted in a decrease to pH 4–5. With the corresponding water set, the pH dropped from 7 to 2 as expected, due to the absence of buffering capacity. It is possible that the oxidation process is facilitated by pH, and may account for the differences in the relative abundances of the reaction products observed in urine and water.

Reaction of C6G and PCC in water and in urine

Initially, the study involved the monitoring of reaction mixtures containing C6G (10 000 ng/mL) and PCC in water. The samples were monitored over a 1-week period. It was found that the exposure of C6G to both concentrations of PCC in this matrix resulted in the formation of three reaction products of interest; codeine, product *m/z* 298 (same as the reaction product yielded from the codeine reaction with PCC) and a new analyte with a protonated molecule of *m/z* 416 (product *m/z* 416, $R_t = 10.1$ min). Analysis of the parallel urine sample set revealed that these reaction products were also forming in urine (Figs. 1(d)–1(f)). Due to the detection of product *m/z* 298, the samples were also monitored for the minor reaction products (products *m/z* 314a, 314b and 316) which are yielded from the reaction between codeine and PCC. Surprisingly, these analytes were not formed upon reaction of C6G with PCC in both water and urine over this monitoring period.

Over the monitoring period, C6G concentration was found to decrease from 8468 ng/mL (1 h) to 5299 ng/mL (1 week) following 100 mM PCC exposure in urine. Exposure of C6G to 20 mM PCC in urine resulted in a less marked decrease in analyte abundance, with C6G concentration at 9063 ng/mL (1 day) and 8999 ng/mL (1 week). The trends observed for reaction progression with C6G and PCC were parallel to those that were observed for codeine and PCC. It was apparent that the reaction between C6G and PCC does not go to completion in urine. Although the abundance of C6G does show a decrease over time in both water and urine, it was detectable at all time points of analysis under the LC/MS conditions employed. On the other hand, the abundance of codeine was observed to increase within the first day after PCC adulteration in both aqueous environments. Therefore, it is quite possible that codeine is being formed via deconjugation of C6G by the PCC adulterant (relative peak area of free codeine in the C6G standard is <3%). Based on the relative abundances of C6G and codeine shown in Table 2, cleavage of the C6G glucuronide functional group appeared to be the favored route of reaction.

In general, an increase in PCC concentration and/or reaction time was also found to contribute to the formation of products *m/z* 416 and *m/z* 298. A comparison of the relative analyte abundances in the C6G urine specimens spiked with 20 mM PCC and 100 mM PCC showed that products *m/z* 416 and *m/z* 298 were present in the latter specimen only. Upon adulteration with 20 mM to 100 mM PCC, product *m/z* 416 was formed within the first hour after adulteration; product *m/z* 298 was detectable 1 day after adulteration. This was also observed with the corresponding water specimens, whereby product *m/z* 298 was detectable 1 week in the specimen adulterated with 20 mM PCC, compared to 1 hour in the specimen adulterated with 100 mM PCC. Codeine and products *m/z* 416 and *m/z* 298 appeared to be more stable in urine than in water.

PCC adulteration of an authentic specimen

Exposure of an authentic specimen positive for codeine (235 ng/mL) and C6G (4880 ng/mL) to both concentrations of PCC resulted in the formation of products *m/z* 298 and *m/z* 416 within 1 h. These reaction products were still found in the sample 1 day and 5 days subsequent to adulteration, in addition to codeine and C6G (Supplementary Fig. S1, Supporting Information). It was also noted that the addition of 20 mM to 100 mM PCC did not appear to significantly alter the pH of the sample (from pH 6 before adulteration to pH 4–5 after adulteration). Likewise, the pH of the authentic specimen adulterated with 20 mM PCC did not cause a substantial pH change in the sample (pH 5–6), and yielded formation of products *m/z* 298 and *m/z* 314b within 1 day. These reaction products could still be observed in the sample on the fifth day of monitoring, in addition to product *m/z* 314a, codeine and C6G. The overall results were consistent with those obtained during previous exposure studies with spiked samples. This study served as a proof of concept that addition of PCC to authentic codeine/C6G positive urine specimens does alter the codeine and C6G abundances, and also produces reaction products that may be used as markers for proving the act of adulteration with this oxidant.

Table 2. Relative peak areas of analytes (normalized to the most abundant analyte (100%)) detected in the C6G adulterated samples after 1 h, 1 day and 1 week elapsed since commencement of the reaction using LC/MS (product ion scan) analysis

	20 mM PCC				100 mM PCC			
	C6G	Codeine	Product *m/z* 416	Product *m/z* 298	C6G	Codeine	Product *m/z* 416	Product *m/z* 298
C6G in water								
1 hr	100.00	2.62	-	-	100.00	2.30	-	0.19
1 day	100.00	6.38	-	-	100.00	3.66	-	2.77
1 week	100.00	54.67	4.56	4.71	17.70	-	-	100.00
C6G in urine								
1 hr	100.00	3.46	-	-	100.00	20.88	3.23	-
1 day	100.00	5.43	-	-	100.00	61.85	13.83	3.22
1 week	100.00	6.08	-	-	60.17	100.00	20.41	23.45

The collision energies used for C6G and codeine are 45 eV and 40 eV, respectively. The collision energy used for products *m/z* 416 and 298 is 40 eV. Reactions were allowed to proceed for 1 h at room temperature (22°C) prior to analysis, and then subsequently refrigerated at 4°C when not analyzed.

Rapid Communications in Mass Spectrometry

Immunoassay and GC/MS study

Following the analysis of 24 urine specimens (Supplementary Table S1, Supporting Information), immunoassay screening indicated that PCC consistently decreased the response of the CEDIA® Opiate assay. Both PCC concentrations caused a reduction in response when compared to the original specimen without PCC fortification. A greater decline in response was caused by the presence of 100 mM PCC when compared to 20 mM PCC. This observation was also reflected in the immunoassay readings for the blank specimens. All patient samples positive for opiates remained positive following PCC adulteration (with 300 ng/mL cut-off concentration).

Morphine/codeine ratios are conventionally used to aid determination of heroin, morphine or codeine use of a test subject. However, such ratios are highly unreliable due to individual variations in metabolizing these drugs and are subject to debate.[19–24] In this study, GC/MS analysis showed that PCC adulteration caused the morphine/codeine ratios to change in a sporadic manner (Supplementary Table S2, Supporting Information). This was partly due to the significant loss of morphine-d_6 and codeine-d_6 internal standards observed in the presence of PCC (Fig. 2). LC/MS analysis of these specimens did indicate the presence of products *m/z* 298, 314a, 316 and 416, in addition to codeine and C6G.

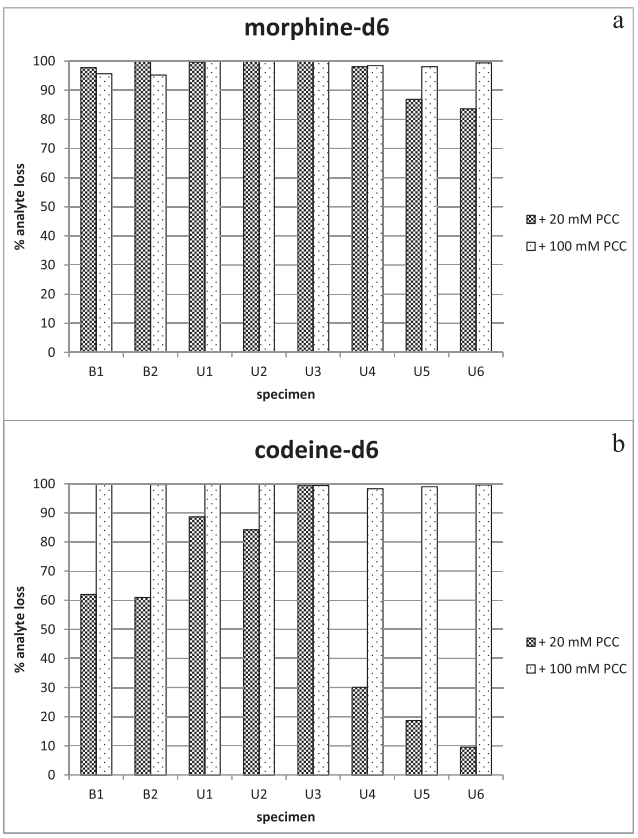

Figure 2. Plot of (a) morphine-d_6 loss and (b) codeine-d_6 loss in two urine blank specimens (B1, B2) and six opiate positive urines (U1 to U6) adulterated with PCC (analyte loss is expressed as a percentage relative to morphine-d_6 and codeine-d_6 abundance in the corresponding unadulterated specimen).

Structural elucidation of the reaction products

Structural elucidation of the reaction products of interest was based on high-resolution MS data obtained from LC/MS analysis. In addition, NMR spectroscopy was also utilized to aid the identification of the major reaction product (product *m/z* 298). Various mechanisms of reactions documented in literature were also consulted to lend support to the molecular structures proposed for the reaction products.

Characterization of product m/z 298

The most obvious difference observed between codeine and product *m/z* 298 was the loss of 2 Da in the latter product. One typical pathway for oxidation by PCC is through the conversion of primary and secondary alcohols into aldehydes and ketones, respectively.[25,26] Since the structure of codeine contains an –OH functional group at the C-6 position (Fig. 3(a)), it was hypothesized that product *m/z* 298 is codeinone (Fig. 3(b)), an α,β-unsaturated ketone derivative of codeine. This was unambiguously confirmed by ^{1}H-NMR and ^{13}C-NMR analyses. Direct injection of the NMR sample prepared as above into the LC/QQQ-MS system showed that although codeine and the other reaction products were present, product *m/z* 298 was the major analyte in the sample (Supplementary Fig. S2, Supporting Information). Therefore, chemical shift signals of significant intensity were generally attributed to product *m/z* 298.

The structural elucidation process for this compound was multifaceted. Firstly, it was expected that product *m/z* 298 shared the majority of its ^{1}H chemical shifts with the codeine starting material, except for the signals belonging to the H-5 to H-8 protons in product *m/z* 298. Comparison of the codeine ^{1}H chemical shifts to the chemical shifts belonging to product *m/z* 298 (Supplementary Table S3, Supporting Information) showed that this was the case. The signal attributed to the H-5 proton for product *m/z* 298 was found to have shifted slightly upfield (δ 4.65 ppm) compared to the codeine H-5 proton (δ 4.89 ppm). This could be explained by the diamagnetic shielding effect, due to the close proximity of the electron dense carbonyl bond (C=O) at the C-6 position found in the codeinone structure (Fig. 3(b)). Furthermore,

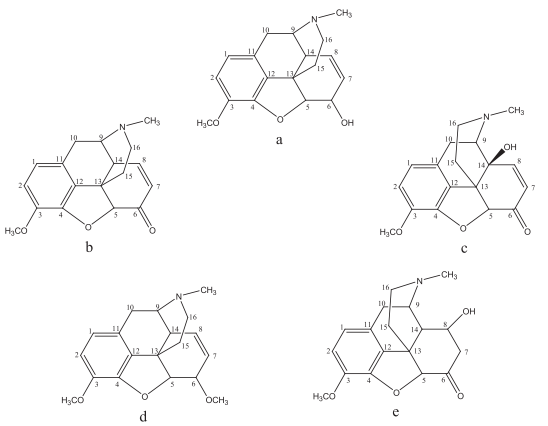

Figure 3. Molecular structures of (a) codeine, (b) codeinone, (c) 14-hydroxycodeinone, (d) 6-*O*-methylcodeine, and (e) 8-hydroxy-7,8-dihydrocodeinone.

the H-5 signal for product *m/z* 298 was a singlet, unlike the distinct doublet observed for the H-5 proton of codeine. This was consistent with a lack of a neighboring proton at the H-6 position in the codeinone molecule. On the other hand, the H-7 proton in product *m/z* 298 was observed to have exhibited a downfield shift (δ 6.09 ppm) when compared to the H-7 proton for codeine (δ 5.73 ppm). This deshielding effect was due to the anisotropy of the adjacent C=O group. Similarly, the signal belonging to the H-8 proton for the *m/z* 298 product was found to be further downfield (δ 6.65 ppm) than codeine (δ 5.30 ppm), and also coinciding with the aromatic region. This can be explained by the partial positive charge carried by the C-8 carbon in codeinone; the presence of a C=O group conjugated with a carbon-carbon (C=C) double bond, results in resonance within the structure (Supplementary Fig. S3, Supporting Information).[26] Therefore, a partial positive charge was carried by the C-8 carbon (also known as the β carbon), causing the H-8 proton to be deshielded. The ^{1}H chemical shifts correlated well with those described for codeinone in the literature (Supplementary Table S3, Supporting Information).

Complementary spectroscopic characterization with ^{13}C-NMR analyses also supported the identification of product *m/z* 298 as codeinone. The only differences when comparing the ^{13}C chemical shifts of codeine and product *m/z* 298 (Supplementary Table S4, Supporting Information) were the signals corresponding to the C-5 to C-8 carbons. This was expected for codeinone, especially the significant chemical shift of the C-6 carbon from δ 66.40 ppm to δ 194.51 ppm. This indicated the presence of a C=O group (aldehyde or ketone) within the structure of the compound. A comparison of the literature ^{13}C chemical shifts for codeinone and product *m/z* 298 showed that they were in good agreement.

MS/MS data was also obtained for codeine, product *m/z* 298 and the codeinone that was synthesized as a reference material for comparison (due to the incomplete oxidation of codeine to codeinone during this synthesis, there was not enough material for NMR analysis). In addition to the desired codeinone, a small amount of product *m/z* 314b was also present in the sample (data not shown). The distinct fragmentation patterns of codeine (Supplementary Fig. S4(a), Supporting Information) and product *m/z* 298 (Supplementary Fig. S4(b), Supporting Information) showed that they share common product ions at *m/z* 153, 165, 181 and 223, an expected observation since they share a common core structure. The identification of product *m/z* 298 as codeinone was strongly supported by comparison of Supplementary Figs. S4(b) and S4(c) (Supporting Information). Both fragmentation patterns appeared to be the same under the same analytical conditions employed, with common product ions at *m/z* 153, 165, 181, 183, 198, 211, 223 and 239, in addition to the precursor ion at *m/z* 298. It is worthwhile noting that a constitutional isomer of codeinone, known as neopinone (Supplementary Fig. S5, Supporting Information), was also considered as a possible structure for product *m/z* 298. In aqueous environments, codeinone exists in equilibrium with neopinone (3:1 ratio) in both acidic and alkali conditions (acid- and alkali-catalyzed isomerism).[27] Although their fragmentation patterns are very similar, they can be distinguished by the different intensity pattern. More importantly, it appears that the product ion at *m/z* 198 is present in the ESI-MS/MS spectrum of codeinone, but not neopinone.[28] Since this ion is quite prominent in the collision-induced dissociation (CID) spectrum of product *m/z* 298, the reaction product (at least the major isomer) was determined to be codeinone. Furthermore, the presence of the C=O and C=C conjugation found in codeinone, but not neopinone, was confirmed with NMR analyses.

Finally, the CID spectrum of the product obtained using HRMS showed major product ions at *m/z* 239.0694, 211.0756, 181.0648, 165.0695 and 153.0699. In addition, the proposed fragmentation pathways are exhibited in Supplementary Fig.S6 (Supporting Information). The $[M+H]^+$ ion at *m/z* 298.1423 was found to correlate the molecular formula for protonated codeinone ($C_{18}H_{20}NO_3$, –2.35 ppm mass accuracy error). Further, the MS peaks corresponding to the sodium and potassium adducts of codeinone were also found at *m/z* 320.1250 ($[M+Na]^+$, –2.19 ppm mass accuracy error) and *m/z* 336.0989 ($[M+K]^+$, –2.38 ppmmass accuracy error). Overall, the MS data correlated well with the literature,[28,29] with the mass accuracy determination within the dynamic range in line with the instruments' specifications.[30]

Characterization of product m/z 314a

HRMS identified product *m/z* 314a to be 14-hydroxycodeinone (Fig. 3(c)). The protonated molecule had a *m/z* 314.1380, with major product ions at *m/z* 296.1276, 281.1038, 264.1013, 254.1168 and 239.0942. It was determined that the mass of the protonated molecule corresponded to the molecular formula $C_{18}H_{20}NO_4$ (–3.82 ppm mass accuracy error). This indicated that there was an additional oxygen atom within the structure of product *m/z* 314a in comparison to codeinone. Such an observation could be explained by the substitution of an –OH group at the C-10 or C-14 carbons of codeinone, or the formation of an N-oxide of codeinone. It has been reported that the C-10 carbon is a viable site for reaction, with 10α-hydroxy analogues of codeine able to be recovered following chromium trioxide oxidation.[31] On the other hand, the production of 14-hydroxycodeinone from codeinone has also been documented, despite the relatively unreactive nature of the tertiary C-14 carbon found in morphine alkaloid structures. This hydroxylation reaction has been observed through the direct oxidation of codeinone to 14-hydroxycodeinone using various oxidizing agents,[17,32–34] as well as in biological systems, whereby codeine undergoes biotransformation by *Pseudomonas putida* M10 to produce 14-hydroxycodeinone.[35]

Product *m/z* 314a was identified to be 14-hydroxy-codeinone based on the major product ions and its intensities observed in the CID spectrum. The proposed fragmentation pathways of 14-hydroxycodeinone can be found in Supplementary Fig. S7 (Supporting Information), and is consistent with the product ions observed. Fragmentation of the protonated molecule at *m/z* 314.1380 resulted in the loss of water to yield the prominent ion at *m/z* 296.1276. The removal of a -CH_3 radical from this latter product ion produced the distinctive ion at *m/z* 281.1038. Following this, the observation of the *m/z* 264.1013 ion could be explained by a loss of CH_3OH or an –OH radical from *m/z* 296.1276 or *m/z* 281.1038, respectively. The

m/z 296.1276 ion could also be alternatively fragmented to yield a C_2H_2O neutral loss, resulting in the observation of the ion at *m/z* 254.1168. The appearance of the *m/z* 239.0942 ion could be explained by the removal of a -CH_3 radical from the *m/z* 254.1168 ion. Furthermore, the sodium and potassium adducts of 14-hydroxycodeinone could be observed at *m/z* 336.1220 (2.38 ppm mass accuracy error) and 352.0939 (–3.41 ppm mass accuracy error), respectively. Finally, ESI-MS/MS data for 14-hydroxycodeinone found in the literature[29] was consistent with the accurate mass data obtained in this study, further supporting the hydroxylation of codeinone at the C-14 site to produce product *m/z* 314a.

Characterization of product m/z 314b

Product *m/z* 314b had a $[M+H]^+$ ion at *m/z* 314.1751, corresponding well with $C_{19}H_{23}NO_3$ (0.10 ppm mass accuracy error). Compared to codeine, it appears that there is an addition of a –CH_2 group in the structure, an unexpected finding based on the oxidative mechanism of PCC. Nevertheless, two likely possibilities for product *m/z* 314b given the structure of the codeine starting material were 6-*O*-methylcodeine (Fig. 3(d)) and ethylmorphine (Supplementary Fig. S8, Supporting Information). LC/MS analysis of commercial standards identified product *m/z* 314b to be 6-*O*-methylcodeine, with both product

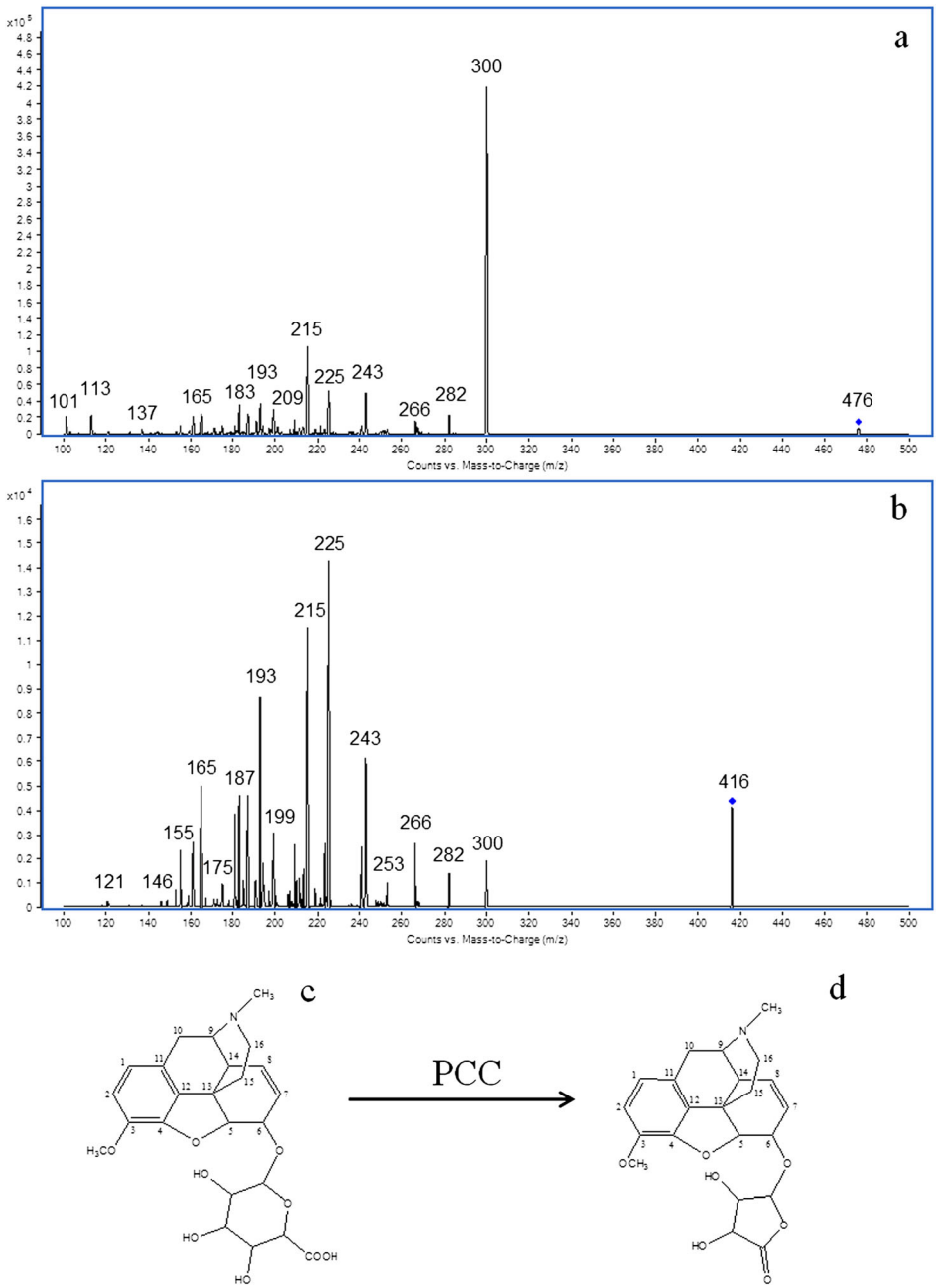

Figure 4. CID spectra of (a) C6G (CE = 45 eV) and (b) product *m/z* 416 (CE = 40 eV), with a reaction scheme depicting the proposed conversion of (c) C6G into (d) the tentative reaction product by PCC.

m/z 314b and the 6-*O*-methylcodeine standard sharing the same retention time and characteristic mass fragmentation pattern (Supplementary Fig. S9, Supporting Information). Further, the high-resolution MS/MS spectrum obtained for product *m/z* 314b showed that a protonated molecule at *m/z* 314.1751 was produced, followed by major product ions at *m/z* 280.0974, 266.1167, 252.1032, 239.0943, 225.0909, 210.0923, 193.0654, 181.0655, 165.0694 and 152.0624. The proposed fragmentation pathways (Supplementary Fig. S10, Supporting Information) are consistent with the product being 6-*O*-methylcodeine.

Characterization of product m/z 316

HRMS of product *m/z* 316 revealed a protonated molecule of *m/z* 316.1546. This was consistent with the molecular formula $C_{18}H_{22}NO_4$ (0.95 ppm mass accuracy error). Initially, the reaction product was hypothesized to be 14-hydroxy-7,8-dihydrocodeinone (commonly known as oxycodone). The preparation of oxycodone from codeine starting material has been achieved as an alternate process for the production of oxycodone from thebaine.[17,33,34] However, LC/MS/MS

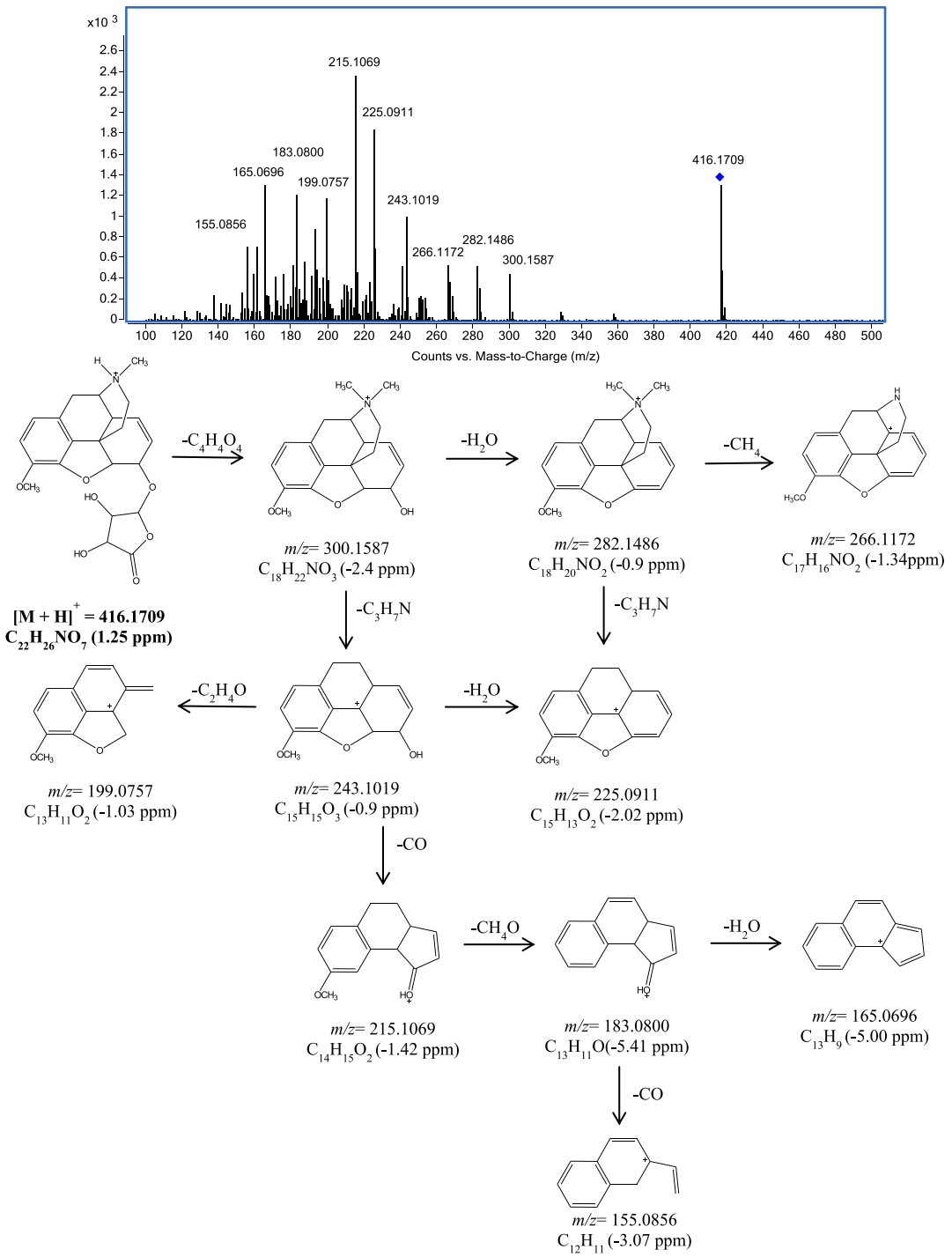

Figure 5. High-resolution CID spectrum (FE = 170 V, CE = 40 eV) and the proposed structures for the product ions for product *m/z* 416, tentatively identified as a C6G lactone derivative.

analysis of a commercial oxycodone standard under the same parameters employed for product *m/z* 316 indicated that they were not the same products (Supplementary Fig. S11, Supporting Information). Codeine-N-oxide and 14-hydroxycodeine were also considered as potential molecular structures; however, the fragmentation pathways proposed for these two compounds could not account for the observed product ions at *m/z* 270.1498 and 213.0542, respectively. Additionally, the CID spectrum for the reaction product did not correlate with those documented for 14-hydroxycodeine.[29] The first product ion at *m/z* 298.1445 indicated a neutral loss of water from the precursor ion, also observed for 14-hydroxycodeinone; this supported the hypothesis that the reaction product was hydroxylated. Thus, it is proposed that product *m/z* 316 is likely to be 8-hydroxy-7,8-dihydrocodeinone (Fig. 3(e)). The major product ions (*m/z* 213.0542, 199.0763, 185.0595 and 171.0815) observed in the CID spectrum appeared to correlate with those proposed for 8-hydroxy-7,8-dihydro-codeinone in the literature (Supplementary Fig. S12, Supporting Information[29]).

Tentative structure for product m/z 416

It was interesting to note that the MS fragmentation pattern of product *m/z* 416 bore resemblance to the fragmentation pattern belonging to C6G, with major product ions at *m/z* 300, 225 and 215 (Fig. 4). Comparison of the CID spectra obtained by QQQ-MS for the C6G starting material (Fig. 4(a)) and product *m/z* 416 (Fig. 4(b)) suggested that the latter structure had incurred a mass loss of 60 Da. Closer inspection of the fragmentation pattern belonging to product *m/z* 416 showed that it possessed the same prominent product ions as detected in the C6G fragmentation pattern, including *m/z* 300, 282, 266, 243, 225, 215, 209, 193, 183 and 165. Since the formation of *m/z* 300 (product ion) from *m/z* 476 (precursor ion) observed for C6G is due to the loss of the glucuronide entity from the structure, it appears that product *m/z* 416 shares the same common molecular skeleton as the starting material, with the PCC reaction occurring somewhere on the glucuronic acid moiety. Thus, it is hypothesized that the transformation of C6G (Fig. 4(c)) to the reaction product is carried out via ring cleavage at the C-O epoxy bond within the glucuronide group with a loss of $-C_2O_2H_2$, followed by ring closure. Additionally, an –OH group on the glucuronide is also oxidized to a carbonyl (C=O) group, with the position of the carbonyl group likely to be adjacent to the remaining oxygen (in line with lactone formation, see Fig. 4(d)). Further structural elucidation with HRMS analysis supported this hypothesis. The $[M+H]^+$ ion for product *m/z* 416 was measured to be *m/z* 416.1709, corresponding well with the formula of $C_{22}H_{26}NO_7$ (1.25 ppm mass accuracy error). The next major ion is found at *m/z* 300.1587, which corresponds with the mass of protonated codeine ($C_{18}H_{21}NO_3$, –2.41 ppm mass accuracy error). This corroborates with the suggestion that the site of oxidation is on the glucuronic acid moiety. Further proof that the codeine portion of the structure remains intact was obtained through examination of the remaining product ions; they are in agreement with the HRMS data published for codeine.[29] Figure 5 illustrates the MS/MS data and the proposed structures for the product ions for product *m/z* 416. Attempts at fragmenting the glucuronic acid entity to obtain further structural information proved difficult. However, as lactones are relatively stable, and based on the data obtained thus far, product *m/z* 416 is tentatively characterized as a C6G lactone derivative.

Proposed mechanism of action of PCC as a urine adulterant

Although pH was found to be slightly lowered by PCC, this study has shown that its main mechanism of action is through the oxidation of codeine and C6G in urine to various other analogues. Since these analogues exhibit different chromatographic and mass spectrometric behavior, they are not detected using LC/MS parameters employed for the parent compounds.

Upon exposure of codeine to PCC, the major route of oxidation results in the formation of codeinone. The proposed reaction mechanism is initiated by the co-ordination of chromium(VI) (existing as the chlorochromate ion) and the codeine –OH group (C-6 position), to form a chromium(VI) acid ester. Following intermolecular re-arrangement ultimately resulting in the removal of the codeine H-6 proton, codeinone is produced, with the reduction of chromium(VI) to chromium(IV) (Supplementary Fig. S13, Supporting Information, adapted from Bruckner[36]). For the formation of 14-hydroxycodeinone, it is viable to propose that it is produced by further oxidation of codeinone. This route (codeine → codeinone → 14-hydroxycodeinone) has been documented in the literature by Cr^{6+} and hydrogen peroxide, however the yield is not significant.[17,32,35] Finally, the Michael addition[37] is suspected as a possible pathway of reaction for the relatively minor production of 6-*O*-methylcodeine and 8-hydroxy-7,8-dihydrocodeinone.

CONCLUSIONS

The adulteration of codeine- and C6G-positive urine specimens with PCC results in the conversion of the parent drugs into various reaction products. Although codeine and C6G are still detectable in these samples, ambiguity may be introduced to the interpretation of the results; the presence of PCC decreases the concentrations of codeine and C6G, therefore altering the drug to metabolite ratios. Morphine/codeine ratios were also found to be affected by the presence of PCC. Consequently, the use of morphine/codeine ratios in result interpretation should be excised with care. It was also determined that although PCC did alter the urine specimen pH, its ability as an adulterant predominately lies with its oxidative capabilities. Furthermore, this study has shown that the presence of codeinone in a urine specimen may be due to adulteration with PCC and not as an impurity of hydrocodone synthesis from codeine. As these reaction products are stable for approximately 1 week after its formation, more investigation is warranted to further determine their potential for use as markers for monitoring the presence of codeine and C6G in urine specimens adulterated with PCC.

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