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Article

Fentanyl as a marker of illicit drug use in morphine-positive urine specimens from workplace drug testing

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Abstract

Total morphine is an important urinary marker of heroin use but can also be present from prescriptions or poppy seed ingestion. In specimens with morphine concentrations consistent with poppy seed ingestion (<4,000 ng/mL), 6-acetylmorphine has served as an important marker of illicit drug use. However, as illicit fentanyl has become increasingly prevalent as a contaminant in the drug supply, fentanyl might be an alternative marker of illicit opioid use instead of or in combination with 6-acetylmorphine. The aim of this study was to quantify opiates, 6-acetylmorphine, fentanyl and fentanyl analogs in 504 morphine-positive (immunoassay 2,000 ng/mL cutoff) urine specimens from workplace drug testing. Almost half (43%) of morphine-positive specimens had morphine concentrations below 4,000 ng/mL, illustrating the need for markers to differentiate illicit drug use. In these specimens, fentanyl (22% co-positivity) was more prevalent than 6-acetylmorphine (12%). Co-positivity of 6-acetylmorphine and semi-synthetic opioids increased with morphine concentration, while fentanyl prevalence did not. In 110 fentanyl-positive specimens, the median norfentanyl concentration (1,520 ng/mL) was 9.6× higher than the median fentanyl concentration (159 ng/mL), illustrating the possibility of using norfentanyl as a urinary marker of fentanyl use. The only fentanyl analog identified was para-fluorofentanyl ($n = 50$), with results from most specimens consistent with para-fluorofentanyl contamination in illicit fentanyl. The results confirm the use of fentanyl by employees subject to workplace drug testing and highlight the potential of fentanyl and/or norfentanyl as important markers of illicit drug use.

Introduction

In urine, morphine and morphine glucuronides are important markers of heroin use (1) and therefore an integral part of most urine drug testing programs. However, there are other reasons why morphine is detected in urine, including the ingestion of unwashed poppy seeds. Several studies on typical poppy seed ingestion report urinary morphine concentrations up to 860 ng/mL (2–5), but even higher concentrations have been reported after extreme ingestion of unwashed seeds (6, 7).

To distinguish between heroin use and poppy seed ingestion, there is a need for additional markers of intake. Heroin is rapidly metabolized to 6-acetylmorphine (1) and commonly included in drug testing panels.

In recent years, the heroin supply in the USA has changed, and heroin is frequently used in combination with illicit fentanyl. In 2018, fentanyl was present in 60% of heroin-involved overdose deaths (9,038/14,966) (8). Since then, fentanyl has become more prevalent and heroin less prevalent based on seized drug data from the National Forensic Laboratory Information System (NFLIS) (9). In 2022, fentanyl was detected 163,201 times compared to 41,227 detections of heroin.

Fentanyl is a highly potent opioid, mainly metabolized to norfentanyl, both of which can be detected in urine (1). A minor metabolite of fentanyl, 4-anilino-N-phenethylpiperidine (4-ANPP), also referred to as despropionyl fentanyl, is also a known precursor and byproduct of illicit fentanyl production. In two studies conducted by our group in 2017 and 2018, fentanyl was detected in 7 of 4,297 specimens (5/2,139 in 2017 and 2/2,158 in 2018), corresponding to a prevalence of 0.16% (0.04–0.29%, 95% confidence interval) (10), which would make it more prevalent than opiates (0.08% positivity in 2022) (11). The 2022 prevalence is likely higher, as both fentanyl drug seizures (95% increase) (9, 12) and fentanyl overdoses (138% increase, reported as T40.4) increased between 2018 and 2022 (13).

Another concern is the fentanyl analogs, which are a class of New Psychoactive Substances (NPS) designed to produce similar effects as fentanyl but with sufficiently different structure to avoid scheduling in different countries, and detection in drug testing. Recently, para-fluorofentanyl has been the most common fentanyl analog (9).

The purpose of this study was to measure the prevalence of fentanyl in urine specimens with a positive immunoassay test for opiates, and to determine if fentanyl identifies more

individuals who may be using opioids illicitly compared to 6-acetylmorphine.

Materials and methods

All specimens in this study were received by Clinical Reference Laboratory (Lenexa, KS) for workplace drug testing in the second half of 2022 or first quarter of 2023. Specimens were obtained from multiple states across the USA.

Aliquots of specimens with a positive immunoassay for opiates, which were to be discarded after initial test data review, were saved and deidentified. The laboratory used the DRI Opiate Assay (Thermo Scientific) calibrated to 2,000 ng/mL morphine. The assay has good cross-reactivity to codeine (210%) and 6-acetylmorphine (80%), and some cross-reactivity to hydrocodone (31%), hydromorphone (15%) and oxycodone (2%) (14).

All 504 included specimens were re-screened by immunoassay for opiates (due to deidentification) and opiates and semisynthetic opioids were quantified by LC–MS-MS (regardless of screening result). In addition, all specimens were screened by immunoassay for 6-acetylmorphine and fentanyl. Quantification 6-acetylmorphine by LC–MS-MS was only performed in specimens with positive immunoassay screen ($n = 106$). In specimens positive for fentanyl by immunoassay ($n = 113$), fentanyl and fentanyl analogs, including para- and ortho-fluorofentanyl, were quantified by LC–MS-MS. A flow-chart can be found in Supplemental Figure S1.

Immunoassay screening

All 504 included specimens were screened for 6-acetylmorphine by the CEDIA Heroin Metabolite (6-AM) Assay (Thermo Scientific) calibrated to 10 ng/mL 6-acetylmorphine, with no cross-reacting analytes reported (15).

In addition, all 504 specimens were screened by the ARK fentanyl II assay (ARK Diagnostics) calibrated to 1 ng/mL fentanyl. Cross-reactivity for norfentanyl was reported to be 7% and the cross-reactivity for evaluated fentanyl analogs ranged from <1% to 91% (16). Of note, cross-reactivity was reported for acryl fentanyl (77%), para-fluorobutyryl fentanyl (53%), para-fluorofentanyl (67%), para-fluoroisobutyryl fentanyl (53%) and furanyl fentanyl (67%). No cross-reactivity was reported for alfentanil (<0.001%).

LC–MS-MS analysis

Specimens with positive screening results were confirmed and quantified by four different LC–MS-MS methods, all using a Shimadzu Nexera Liquid chromatograph coupled to an AB Sciex 6500+ triple quadrupole mass spectrometer. Brief method descriptions are given below with more details provided in the Supplemental Materials. Analytes and limits of quantification (LOQs) are provided in Supplemental Table S-I.

Opiates and semisynthetic opioids

The method was used to quantify morphine and codeine with a 50 ng/mL cutoff, as well as hydrocodone, hydromorphone, oxycodone and oxymorphone with a 20 ng/mL cutoff. Urine samples (50 μL) were hydrolyzed using KuraBGTurbo β-glucuronidase (125 μL) and derivatized with methoxyamine to oxime derivates of the semisynthetic opioids (see Supplemental Figure S2). The samples were extracted using a methyl tert-butyl ether (MTBE) liquid–liquid extraction and reconstituted in 10 mM ammonium formate (750 μL). The analytes (2 μL) were separated on a Restek Raptor Biphenyl column (100 × 2.1 mm, 2.7 μm, 40°C) using a 5.5 min tertiary gradient with 10 mM ammonium acetate with 0.1% formic acid in water [A], 0.1% formic acid in water [B] and 0.1% formic acid in acetonitrile [C].

6-acetylmorphine

The method was used to quantify 6-acetylmorphine with a 2 ng/mL cutoff. Urine samples (50 μL) were diluted with 1 mL 10 mM ammonium acetate. The diluted sample (12 μL) was separated on a Waters Cortecs Shield RP18 (100 × 2.1 mm, 2.7 μm, 30°C) column using a 3.0 min gradient with 10 mM ammonium acetate with 0.1% formic acid in water [A] and methanol [B].

Fentanyl and fentanyl analogs

The method was used to quantify fentanyl, norfentanyl, 4-ANPP, acryl fentanyl, alfentanil, cyclopropyl fentanyl, para-fluorobutyryl fentanyl, para-fluoroisobutyryl fentanyl, furanyl fentanyl, methoxyacetyl fentanyl, 3-methylfentanyl and sufentanil using a 0.05 ng/mL cutoff. Urine samples (250 μL) were extracted with a basic MTBE liquid–liquid extraction. The organic phase was evaporated, and the sample reconstituted in 500 μL 0.1% formic acid with 10% methanol. The analytes (15 μL) were separated on a Waters Cortecs C18+ (100 × 2.1 mm, 2.7 μm, 40°C) column using a 6.5 min gradient with 0.1% formic acid in water [A] and 0.1% formic acid in acetonitrile [B].

Ortho- and para-fluorofentanyl

The method was used to quantify ortho- and para-fluorofentanyl using a 0.05 ng/mL cutoff. Urine samples (250 μL) were extracted with a basic MTBE liquid–liquid extraction. The organic phase was evaporated, and the sample reconstituted in 500 μL 0.1% formic acid with 10% methanol. The analytes (15 μL) were separated on a Waters Cortecs C18+ (100 × 2.1 mm, 2.7 μm, 40°C) column using a 6.5 min gradient with 0.1% formic acid in water [A] and methanol [B].

Results

In total, 504 specimens with a positive opiate immunoassay result were included in this study. Of those, 501 were positive for morphine. All three morphine-negative specimens were positive for 6-acetylmorphine (21–235 ng/mL), and one of them was also positive for oxycodone (1,907 ng/mL) and hydrocodone (489 ng/mL) but not oxymorphone and hydromorphone.

Observed concentrations

Concentrations for all analytes are shown in Table I, and the distribution of morphine concentrations is shown in Figure 1. Concentrations below 2,000 and 4,000 ng/mL morphine were observed in 135 (27%) and 214 (43%) of specimens, respectively. Morphine concentrations below the 2,000 ng/mL cutoff in the opiate immunoassay were expected as the immunoassay cross-reacts with morphine metabolites and other opiates.

Table I. Observed Drug Concentration in Opiate Screening Positive Specimens (ng/mL)

Concentrations (ng/mL) in specimens positive for opiates by immunoassay (cutoff 2,000 ng/mL, *n* = 504)							
Drug	***n*** **(%pos)**	**10%**	**25%**	**Median**	**75%**	**90%**	**Max**
Morphine	501 (99%)	995	1,870	5,500	18,600	55,100	381,000
Codeine	172 (34%)	76.5	153	441	1,830	4,240	29,700
6-Acetylmorphine	86 (17%)	11.3	19.0	61.1	363	1,080	4,540
Hydromorphone	302 (60%)	29.0	53.8	133	362	871	42,100
Fentanyl	110 (22%)	1.1	4.8	159	937	2,270	7,000
Norfentanyl	110 (22%)	4.7	32.9	1,520	4,410	14,900	34,600
4-ANPP	72 (14%)	1.4	10.1	40.7	75.2	290	1,630
Para-fluorofentanyl	50 (10%)	0.6	1.5	6.6	32.3	156	606
Specimens also positive for fentanyl or a related substance by LC–MS-MS (cutoff 0.05 ng/mL, *n* = 110)							
Normalized to number of specimens positive for fentanyl or a related substance (*n* = 110)							
Fentanyl	110 (100%)	1.1	4.8	159	937	2,270	7,000
Norfentanyl	110 (100%)	4.7	32.9	1,520	4,410	14,900	34,623
4-ANPP	72 (65%)	<1	<1	9.1	49.9	155	1,630
Para-fluorofentanyl	50 (45%)	<1	<1	<1	6.3	56.1	606

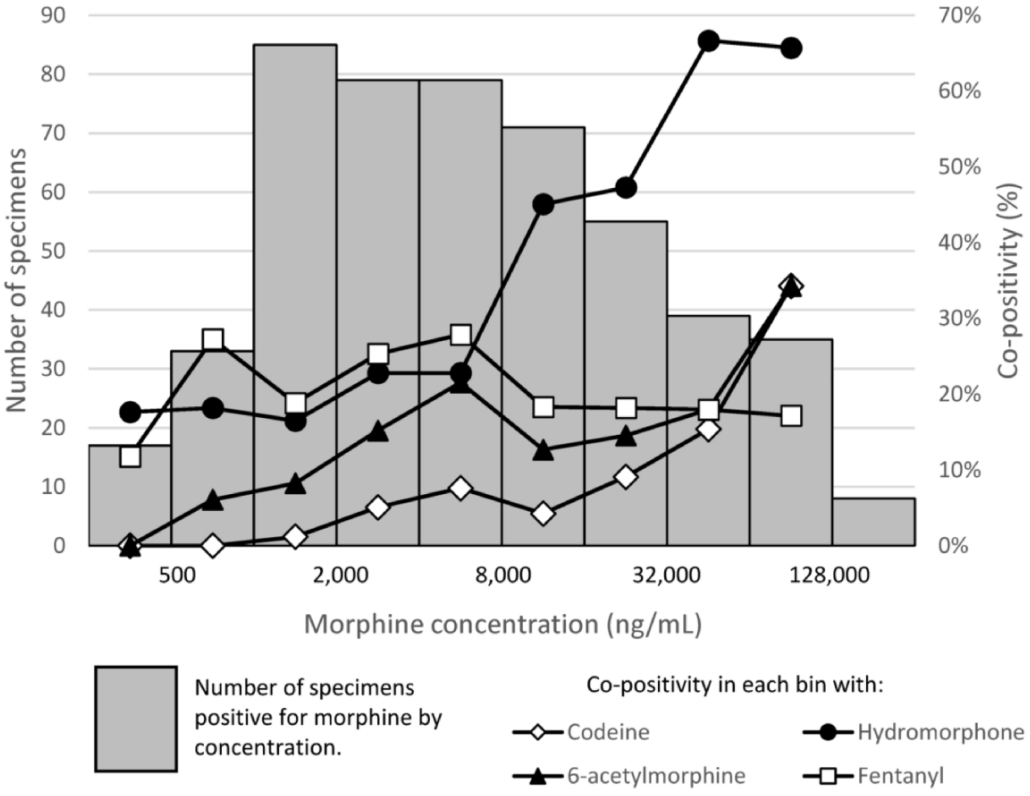

Figure 1. Distribution of morphine concentrations (grey bars) and co-positivity of 6-acetylmorphine (≥10 ng/mL, filled triangles), fentanyl (≥0.5 ng/mL, empty squares), hydromorphone (≥100 ng/mL, filled circles) and codeine (≥2,000 ng/mL, open diamonds). Increases in co-positivity with increasing morphine concentration were significant for 6-acetylmorphine (Chi-square, *P*-value 0.0046), hydromorphone (Chi-square, *P*-value <0.001) and codeine (Chi-square, *P*-value <0.001) but not fentanyl. Data points at the bin cutoffs counted towards higher bin. First bin includes specimens with concentrations above the method cutoff (300 ng/mL) but less than 500 ng/mL. Last bin contains all concentrations at or above 128,000 ng/mL (max 381,000 ng/mL). Co-positivity not reported in last bin due to low number of specimens.

Co-positivity of semisynthetic opioids, 6-acetylmorphine and fentanyl

Co-positivity of other drugs is shown in Figure 1 and Supplemental Table S-X as a function of morphine concentration. Co-positivity for fentanyl was 22% and not dependent on concentration. In contrast, co-positivity of 6-acetylmorphine increased by concentration from 0% in specimens with <500 ng/mL of morphine to 55% in specimens with ≥120,000 ng/mL of morphine. A similar positivity increase from 18% to 55% was also seen for the morphine metabolite (17) hydromorphone. In addition, 132 (26%) specimens were positive (≥100 ng/mL) for oxycodone (15%), oxymorphone (18%) and/or hydrocodone (17%). Of 86 specimens positive for 6-acetylmorphine (≥2 ng/mL), 57 (66%) were also positive for fentanyl and norfentanyl (≥0.5 ng/mL).

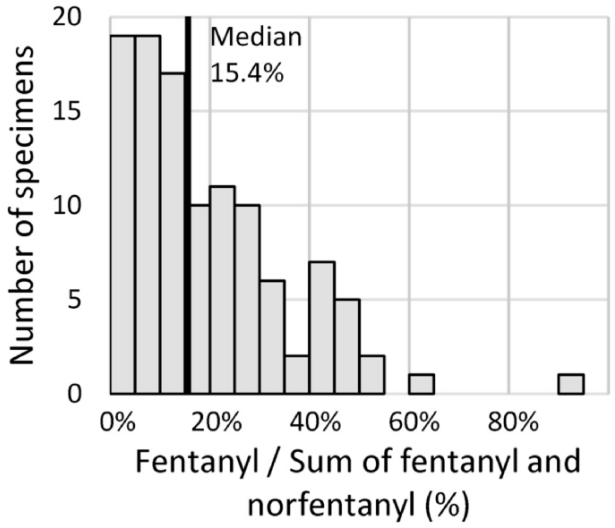

Figure 2. Proportion of fentanyl as the sum of fentanyl and norfentanyl. Data points at the bin cutoffs counted towards higher bin.

Fentanyl

Among 113 specimens with positive immunoassay results for fentanyl, 110 specimens with at least 1 ng/mL of fentanyl ($n = 102$) and/or norfentanyl ($n = 110$) were identified. Concentrations of fentanyl, norfentanyl, 4-ANPP and para-fluorofentanyl are shown in Table I. In most specimens, the norfentanyl concentration was higher than that of fentanyl. Fentanyl comprised 15.4% (median) of the sum of fentanyl and norfentanyl (Figure 2), and in only a single specimen did fentanyl make up more than two-thirds of the sum.

The median fentanyl concentration was 84 times higher in specimens also positive for 6-acetylmorphine (580 ng/mL, $n = 57$) compared to those negative for 6-acetylmorphine (7.0 ng/mL, $n = 53$). Similarly, the median norfentanyl concentration was 72 times higher in specimens also positive for the 6-acetylmorphine (2,900 ng/mL, $n = 57$ versus 40 ng/mL, $n = 53$). These differences were significant using a Student's t-test ($P < 0.05$). The distribution of concentrations shown in Figure 3 show that high fentanyl and norfentanyl concentrations were seen both with and without 6-acetylmorphine, while low fentanyl and norfentanyl concentrations were mainly observed without 6-acetylmorphine.

Para-fluorofentanyl and other fentanyl analogs

The only fentanyl analog observed was para-fluorofentanyl, which was observed in 50 specimens, all also positive for fentanyl. The median ratio of para-fluorofentanyl to fentanyl was 1.7% (Q1–Q3: 0.4–4.5%). In two specimens, the concentration of para-fluorofentanyl was higher than that of fentanyl.

Discussion

The study design, using deidentified urine specimens with a positive opiates initial test, allowed for an enriched data set where all specimens were positive for one or more drugs of interest. Some of the positive results, especially for morphine and the semisynthetic opioids, may represent legitimate prescriptions and not illegal use. Legitimate co-use of morphine and fentanyl by prescription cannot be ruled out either. The combination is used for operative analgesia (18) and for pain management, for example, combining a fentanyl patch with morphine for breakthrough pain (19).

Observed morphine concentrations

While morphine concentrations span a large concentration interval, most of the specimens quantitated in the lower end of the concentration range, with a median concentration of 5,500 ng/mL. The drop in prevalence observed below 1,000 ng/mL (Figure 1) is likely an effect of the specimens not having enough opiates to test positive using the opiate initial test (cutoff 2,000 ng/mL morphine).

Specimens positive for 6-acetylmorphine but negative for morphine have been reported previously so the identification of three such specimens in this study was not surprising (20). It appears that in some individuals, the conversion of 6-acetylmorphine is reduced, potentially through genetic polymorphism or inhibition of key enzymes. It has been reported that the conversion of 6-acetylmorphine can be catalyzed by human carboxy esterase 2 (hCE-2) (21), but it is possible that other enzymes are also involved. It is also possible that some specimens were collected quickly after heroin use without time for conversion to morphine in the body.

Co-positivity of semisynthetic opioids, 6-acetylmorphine and fentanyl

In morphine-positive specimens, the co-positivity of fentanyl (22%) was higher than that of 6-acetylmorphine (17%, P-value 0.037, Fisher's exact test), especially in specimens with <4,000 ng/mL morphine, where differentiation from poppy seed ingestion is most challenging (see Figure 1). In these specimens ($n = 214$), co-positivity of fentanyl and 6-acetylmorphine was 22% ($n = 47$) and 12% ($n = 25$), respectively (P-value 0.006, Fisher's exact test). Even though fentanyl and norfentanyl are not heroin metabolites, they were more sensitive in identifying illicit opioid use compared to 6-acetylmorphine. Testing for fentanyl would also allow detection of specimens positive for fentanyl without co-ingestion of opiates.

An increase in 6-acetylmorphine, hydromorphone and codeine co-positivity was observed with increasing morphine concentration, which is consistent with morphine being a metabolite of heroin and codeine, and hydromorphone being a metabolite of morphine (17). Street heroin often contains 6-acetylcodeine which is metabolized to codeine (22). More frequent heroin use will increase both morphine concentrations, and the likelihood of 6-acetylmorphine, codeine and hydromorphone being detected. Fentanyl co-positivity, on the other hand, showed no correlation to morphine concentrations, but remained high (22%) in the study specimens.

Fentanyl concentrations

As seen in Table I, there was a perfect correlation between specimens positive for fentanyl and norfentanyl. The lowest concentrations were 0.070 ng/mL fentanyl and 1.3 ng/mL norfentanyl, and Figure 2 similarly shows that norfentanyl concentrations in urine were five times higher (median) compared to fentanyl concentrations. Both these findings indicate that if norfentanyl is used as the urinary marker of fentanyl use, either with or instead of fentanyl, a similar sensitivity

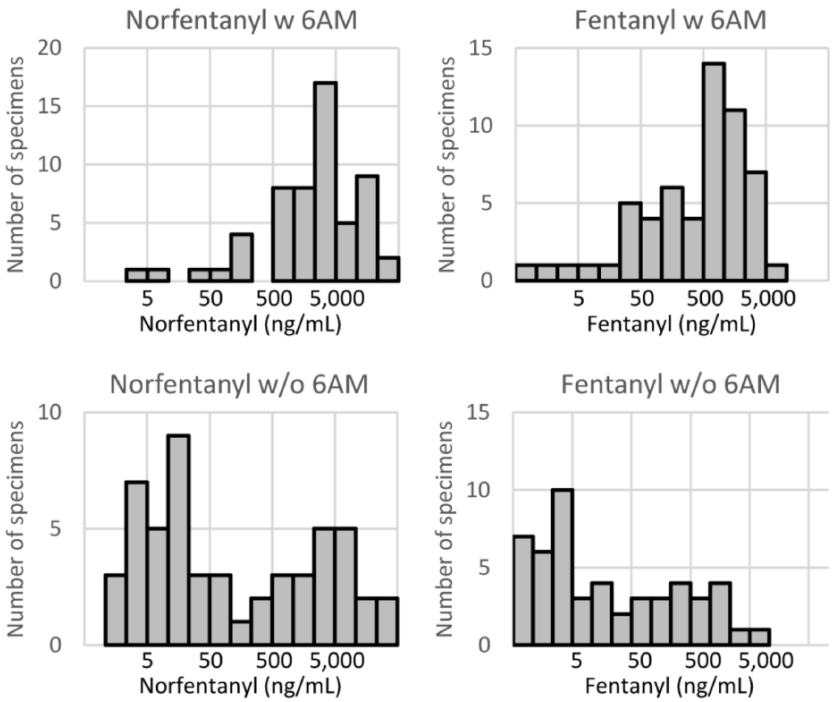

Figure 3. Concentration distribution of fentanyl and norfentanyl in specimens with (w) and without (w/o) 6-acetylmorphine (6AM). Data points at the bin cutoffs counted towards higher bin.

can be achieved using a higher cutoff compared to fentanyl. In turn, a higher cutoff might allow for more cost-efficient testing.

Higher fentanyl and norfentanyl concentrations were observed in specimens also positive for 6-acetylmorphine as seen in Figure 3. A possible explanation is that both high fentanyl/norfentanyl concentrations and the combined use of both heroin and fentanyl are associated with extensive illicit opioid use. However, such a hypothesis cannot be verified using deidentified data, as in the present study.

Para-Fluorofentanyl and other fentanyl analogs

The only fentanyl analog detected was para-fluorofentanyl, consistent with both reports from NFLIS (9) and the Center for Forensic Science Research & Education (CFSRE) (23). Para-fluorofentanyl is a drug of abuse in its own right, and higher ratios to fentanyl indicate likely use in combination with fentanyl in some cases. However, the low ratios observed in most specimens would instead indicate that para-fluorofentanyl is an adulterant or contaminant of illicit fentanyl. Finding fluorofentanyl as a minor component in fentanyl was also reported by the Drug Enforcement Administration (DEA) (24), and Centers for Disease Control and Prevention (CDC) reported that over 90% of overdose deaths involving para-fluorofentanyl also involved fentanyl (25).

Conclusions

Almost half (43%) of morphine-positive urine specimens had morphine concentrations below 4,000 ng/mL, illustrating the need for markers to differentiate illicit drug use from poppy seed ingestion. Fentanyl and/or norfentanyl appear to be more sensitive indicators of illicit opioid use compared to 6-acetylmorphine, especially in specimens with low morphine concentrations. For workplace drug testing, this increased ability to identify people who use illicit opioids would likely contribute to the deterrence effect of drug testing.

However, legitimate co-use of morphine and fentanyl by prescription cannot be ruled out by toxicology results alone and results should be evaluated in the light of prescription and medical history, clinical signs and symptoms of intoxication or illicit drug use, as well as other information available. Evaluations can be carried out by a medical professional such as a treating physician or a medical review officer.

Norfentanyl was detected in all fentanyl-positive specimens, typically at concentrations five times higher than fentanyl. This indicates that higher cutoffs could be used if norfentanyl was chosen as the primary marker of fentanyl ingestion.

The only identified fentanyl analog was para-fluorofentanyl. Even though the results from some specimens indicate the use of para-fluorofentanyl products, the low para-fluorofentanyl to fentanyl ratios observed in most specimens were consistent with para-fluorofentanyl being a contaminant in illicit fentanyl.

Supplementary data

Supplementary data is available at *Journal of Analytical Toxicology* online.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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