An Enzyme-Linked Immunosorbent Assay for Urinary Screening of Fentanyl Citrate Abuse*

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ABSTRACT

An enzyme-linked immunosorbent assay (ELISA) for quantitation of urinary fentanyl was evaluated as a screening tool for detecting abuse of this potent narcotic. The assay was found to have reproducible calibration curves from 0.5 to 10 ng/mL and a limit of detection of 0.5 ng/mL. Interference by proteins, glucose, or pH was negligible. The assay was specific for fentanyl with little cross-reactivity against despropionyl fentanyl and norfentanyl metabolites, other analgesics and common drugs of abuse. To evaluate its use in humans, urines were collected from 57 normal individuals, 48 patients seen in the Emergency Department, and 18 surgical patients receiving either low (50 μg) or moderate fentanyl dosage (200 and 250 μg) for routine anesthesia. In patients receiving 50 μg (a dose consistent with early abuse), urinary fentanyl was detectable for 3 to 10 h post administration. In patients receiving 200 or 250 μg (a dose more consistent with addiction), urinary fentanyl was detectable for longer time periods (>24 h). These results indicate that the ELISA is sensitive for the detection of recent fentanyl exposure under conditions likely to mimic those in abuse and addiction. The assay is simple to perform, reliable, and can be used to screen urine specimens prior to gas chromatography/mass spectrometry (GC/MS) confirmation.

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Introduction

Fentanyl citrate (Sublimaze) is a powerful synthetic narcotic (200 times morphine) routinely used operatively for the induction and maintenance of anesthesia and postoperatively for analgesia. Its rapid effect (1 to 2 min), short duration of action (0.5 to 1 h) and preservation of cardiovascular stability, make fentanyl the preferred anesthetic for surgical procedures (approximately 70 percent). Plasma concentration after therapeutic use ranges 1 to 3 ng/mL. The metabolism to norfentanyl and despropionyl fentanyl is rapid, with a $t_{1/2}$ of 3 to 4 h. Only 6 to 8 percent excreted as the parent compound. It is unfortunate that the pharmacologic properties which have made fentanyl the anesthetic of choice have also contributed to its popularity as a drug of abuse.

Current analytical methods for fentanyl include gas chromatography with flame ionization, electron capture, and nitrogen sensitive detection, and gas chromatography/mass spectrometry (GC/MS). These methods can be used for both parent and metabolites. They are, however, too costly and time consuming for use as screening tools. Traditional opiate immunoassays do not cross-react with phe nylpiperidine narcotics. Several fentanyl radioimmunoassays (RIAs) have been reported, but require radionuclide tracers (e.g., $^{125}$I and $^3$H) and in some, a preliminary extraction step. Radioimmunoassays are, however, very sensitive (0.1 ng/mL for the Coat-A-Count* assay). Enzyme-linked immunosorbent assays (ELISA) are a nonradioactive alternative, but there have been limited reports on the usefulness of ELISA in actual patient specimens. One group examined a prototype ELISA for fentanyl and its analogs in spiked human urine, but did not evaluate urine collected from patients. In another report, the use of fentanyl ELISA was evaluated in horses only.

A commercial fentanyl ELISA was evaluated by us as a means to provide more effective screening for diversion of this addictive drug. This assay was developed for veterinary use and was evaluated because there were no commercial nonisotopic immunoassays available for human urine approved by the Food and Drug Administration.

Materials and Methods

ANALYTICAL STUDIES

Preparation of fentanyl calibrators. A stock standard (100 μg/mL) of fentanyl† (Cat. No. F-3886), prepared by dissolving 5 mg of the crystalline citrate salt (FW = 528.6 g/mol) into 50 mL ultrapure water, was used for all subsequent dilutions. Calibrators for the fentanyl ELISA were prepared as intermediate standards, and stored at 4 to 6°C. On the day of the assay, the working calibrators were prepared by diluting the intermediates in human urine to a final concentrations of 0.5, 1, 5, and 10 ng/mL. Blank urine was obtained from apparently healthy individuals and commercially.§ The urine was tested by “dip-stick” urinalysis prior to use. Particulates were removed by centrifugation and the supernatant was used for subsequent analyses.

ELISA method. The fentanyl ELISA kit§ consisted of microtiter plates coated with sheep polyclonal fentanyl antibody and fentanyl-horseradish peroxidase conjugate. The sample volume was 20 μL.

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The assay has one 30-minute incubation step. The substrate was 3,3',5,5'-tetramethyl benzidine and hydrogen peroxide (H₂O₂, 0.1 mL/L). The absorbance of the product was monitored at 655 nm with a microtiter plate reader. Data were graphed using least squares linear regression and logistic analysis.

Precision and Sample Stability. Intra-assay precision was determined by repeated analysis (n = 12) of blank urines spiked with fentanyl to an approximate concentration of 1.0 and 5.0 ng/mL. Inter-assay precision was determined by analysis of these samples each of 11 days. These controls were stored at 4°C between runs. The inter-assay precision provided data on the short-term stability of fentanyl under refrigerated conditions. Long-term stability was assessed by analysis of 8 positive urine samples after 12 months of storage at 4°C.

Interferences. Interference was evaluated over the concentration range of the fentanyl calibrators (0.5, 1, 5 and 10 ng/mL) and was performed in human urine (matrix). Bovine serum albumin* (1 and 2 g/dL) and glucose* (500 and 1000 mg/dL) were added directly to urine. Intermediate standard fentanyl were added to achieve final concentrations of the calibrators. To evaluate the effect of pH, dilute NaOH or HCl was added to the calibrators to a range of pH 5 to 9.

Specificity. The manufacturer has shown that the cross-reactivity of this assay was 100 percent to fentanyl, p-fluorofentanyl, α-methylfentanyl, 3-methylfentanyl, and thienylfentanyl, 25 percent to carfentanil, 2.5 percent to sufentanil, 1.0 percent to lofentanil, 0.1 percent to alfentanil, and 0.01 percent to mazindol. The current authors did not attempt to verify these claims. Cross-reactivity of the fentanyl ELISA was evaluated against its two principal metabolites despropionyl fentanyl† (Cat. No. M-002) and norfentanyl‡ (Cat. No M-001) and other drugs including other narcotics (codeine, morphine, hydromorphone, hydrocodone, oxymorphone, and oxycodone), those with a piperidine ring (meperidine, normeperidine, and cocaine), and antihistamines (diphenhydramine, tripelennamine, and pheniramine). Drugs in the latter group were included because one experimental immunoassay for fentanyl metabolites showed significant cross-reactivity towards these amines. Stock standard solutions (1 mg/mL) of the fentanyl metabolites were prepared in methanol. For the metabolites, standards were diluted in the fentanyl calibrators (in urine matrix) to achieve final concentrations from 1-10,000 ng/mL. For the others, drugs were diluted in commercial drug free urine in concentrations of 1, 10, and 100 μg/L.

Clinical Studies

Limit of detection and validation of cutoff concentrations. The limit of detection (LOD) was determined by the mean + 3SD of negative urines. Blank urine was obtained from 57 apparently healthy laboratory volunteers who denied drug abuse. Urine from 48 patients seen in the Emergency Department with a suspicion of drug abuse was also assayed for fentanyl. In 38 of these samples, a positive result was obtained for one or more of the following: opiates, cocaine, benzodiazepines, barbiturates, or tetrahydrocannabinol. Because fentanyl is not readily available on the streets, it was presumed that fentanyl was absent in all of these specimens.

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Comparison to Gas Chromatography/Mass Spectrometry. Eight positive urine samples and 9 negative urines samples were screened by the ELISA technique, and confirmed results by GC/MS.§ (Medtox Laboratories, St. Paul, MN). The LOD and cutoff concentration for this assay is 0.5 ng/mL.

Fentanyl in patient urine. Urine specimens were collected through a Foley Catheter on 18 surgical patients who received fentanyl for anesthesia. In each case, the anesthetic consisted of a barbiturate for induction, with nitrous oxide and Isoflurane inhalation agents for maintenance of anesthesia. As a supplement, intravenous fentanyl citrate¶ (50 µg/mL) was administered as a bolus either a low dose, 50 µg (n = 7); or one of two moderate doses 200 µg (n = 5) and 250 µg (n = 6). Urine specimens were collected at various intervals after fentanyl administration as indicated in figure 5. Urines were stored at 4 to 6°C until analysis.

Results

Assay Calibration, Precision, and Sample Stability

The ELISA had a reportable range of 0.1 to 10 ng/mL (figure 1). The calibration curve was found to be highly reproducible (mean $r = 0.989$, range = 0.963–0.998, n = 10) and effectively spanned the range of fentanyl concentrations found in urine.¹⁴,¹⁵ The concentration corresponding to 50 percent binding occurred between 0.8 and 1.0 ng/mL. A four parameter logistic curve fit was also applied to these calibration curves and produced correlation coefficients of 1.000 for nine of the ten curves, further demonstrating the reproducibility of the calibrations (data not shown). Color development was rapid and linear over both short (2 to 3 min) or long time periods (15 to 30 min) after addition of substrate. For convenience, the absorbance at 15 min was chosen for routine analysis.

The intra- and inter-assay precision for the fentanyl assay are shown in table I. The data for intra-assay precision also demonstrated that fentanyl concentrations in urine were stable over the duration of the between-run trials. In addi-
TABLE I

<table>
<thead>
<tr>
<th>Intra-assay Precision</th>
<th>Inter-assay Precision</th>
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<tr>
<td>Control</td>
<td>Mean</td>
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<tr>
<td>I</td>
<td>0.97</td>
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<tr>
<td>II</td>
<td>5.14</td>
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The specificity of the ELISA for norfentanyl and despropionyl fentanyl is shown in figure 4. Approximately 200-fold more despropionyl fentanyl was required to provide a 50 percent level of competition (cross-reactivity 0.53 percent). The cross-reactivity of norfentanyl was <0.03 percent. No significant cross-reactivities were observed for any of the other drugs listed in the Materials and Methods section. In addition, studies conducted by the manufacturer have shown that this assay does not cross-react with phenylbutazone, furosemide, benzylecgonine, and cocaine.

Detection of Fentanyl in Urine

The specificity of the ELISA for fentanyl in urine of volunteers was 0.06, 0.12, and 0–0.453 ng/mL, respectively. Corresponding results of 48 urines from Emergency Department patients were 0.10, 0.09, 0–0.489 ng/mL, respectively. From this data, the limits of detection calculates to 0.42 and 0.37 ng/mL, respectively. Based on the LOD and the fentanyl concentration that resulted in 50 percent binding, the optimum cutoff concentration for this assay is 1.0 ng/mL. However, these results show that a low cutoff concentration of 0.5 ng/mL can be used to extend the length of time by which a urine sample remains positive after abuse. Although not observed in any of the negative urines collected in this study, use of a 0.5 ng/mL cutoff may produce some false positives results.
Results from the ELISA fentanyl assay were in agreement against GC/MS for the 17 samples sent to a reference laboratory (100 percent sensitivity and 100 percent specificity). GC/MS concentrations for the 8 positive fentanyl samples ranged from 1 to 20.8 ng/mL.

Serial urinary fentanyl concentrations from surgery patients are shown in figure 5. No attempt was made to correct for urine output or concentration in these random collections. As expected, the concentration of fentanyl was found to be highly variable in urines from both low (50 μg) (figure 5A) or moderate (200 and 250 μg) dosed patients (figures 5B and 5C). Peak urinary levels of fentanyl were achieved rapidly (0.5 to 2 h) in low dose patients and were somewhat delayed in patients receiving moderate dosage (0.5 to 4 h). Following peak levels, urinary fentanyl concentration decreased rapidly and approached the limit of sensitivity of the ELISA (within 3 to 10 h) in the low dose group (figure 5A). However, in patients administered a dose more consistent with addiction (200 and 250 μg), fentanyl was detectable for significantly longer time periods (6 to 24 h) and in one patient was detectable for approximately 35 h. The small number of patients studied (n = 18) and the wide variations in
urine output preclude rigorous statistical analysis. Nevertheless, the data demonstrate that the ELISA is valid over a time period long enough to detect fentanyl administration.

**Discussion**

Because of access, the abuse of natural and synthetic opiates by healthcare workers is a major problem. In a recent national survey of physicians, 39.9 percent and 7.5 percent responded that they had used minor (e.g., codeine and propoxyphene), and major opiates (e.g., meperidine and fentanyl) at least once. As reported in the 1990 NIDA survey, abuse of prescription drugs by doctors is 5 to 20 times higher than an age and sex-adjusted general population. Fentanyl abuse specifically has led to several deaths among residents, prompting the Association of Anesthesia Program Directors to produce a videotape warning residents of the dangers of this drug. Detecting abuse at early stages is important for rehabilitation. Physicians who turn up positive should be enrolled in drug treatment programs. An essential part of this program is regular testing of urine. However, the analysis of many prescription drugs is difficult because
Figure 5. Fentanyl excretion in surgical patients. Urines were collected from patients receiving an IV bolus of (A) 50 μg, (B) 200 μg, and (C) 250 μg of fentanyl citrate.
there are no FDA-approved assays for oxycodone, oxymorphone, hydrocodone, hydromorphone, meperidine, or fentanyl. Therefore, it was our attempt to use drug assays originally developed for testing of racehorses and greyhounds.

It was found that if urine is collected within 24 h, fentanyl ELISA has adequate sensitivity for detection of abuse and addition. Unfortunately, the assay had little cross-reactivity to fentanyl metabolites. Use of an antibody that cross-reacted with metabolites would have been more advantageous for screening, as norfentanyl remains in urine longer (48 to 96 h) than the parent drug. The lack of cross-reactivity to metabolites was unexpected as the sheep polyclonal fentanyl antibody used in this assay was not subjected to affinity purification prior to attachment to microtiter wells.* These findings are, however, similar to a previous report of fentanyl antibodies raised in rabbits.26

In our study, patients receiving low dosage (i.e., likely to resemble those at early stages of abuse) fentanyl was detectable for only 3 to 10 h in urine. These levels likely represent the low end of the excretion spectrum because substantially higher concentrations and wider ranges have been reported in urine collected from patients undergoing fentanyl anesthesia (87 to 485 ng/mL) and drug abusers in a methadone program (0.14 to 531 ng/mL).27 In patients receiving moderate doses (i.e., likely to resemble those in addiction), fentanyl was detectable for significantly longer periods of time (up to and greater than 24 h). These studies indicate that the timeframe for detecting recent exposure is appreciably longer (up to 10-fold) at higher doses, suggesting that terminal fentanyl elimination is dose-depen-


References


