

High-sensitivity radioimmunoassay screening method for fentanyl

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SUMMARY

A radioiodinated analog of fentanyl was synthesized for use with a commercially available radioimmunoassay for fentanyl. The sensitivity of the modified assay was at least 100 times greater than that of the original assay. Using this modified assay, concentrations of fentanyl as low as 1 pg/ml of fentanyl or fentanyl equivalents in equine urine were detected.

Doses of fentanyl 100 times smaller than the minimum dose for a pharmacologic effect were detectable and a pharmacologically effective dose of fentanyl was detectable for up to 96 hours or more. The high sensitivity of the assay indicated that large numbers of urine samples (ie, 10 to 20) probably could be pooled and screened simultaneously, which would result in an economical analysis for fentanyl in the urine of horses after a race. Sufentanil and its metabolites also were detectable, using this assay, but at only about 1% of the efficiency at which fentanyl was detectable.

Fentanyl (N-phenyl-N-[1-(2-phenylethyl)-4-piperidinyl]propanamide) is a synthetic opioid derivative of meperidine and is a potent narcotic analgesic, with about 80 to 150 times the potency of morphine.^{1,2} The narcotic actions are characterized by rapid onset and short duration of action. The pharmacologic actions are similar to those of morphine, and fentanyl is considered a pure opioid agonist.³

In man, the major pharmacologic action of fentanyl is analgesia, with euphoria and respiratory depression. High doses cause muscular rigidity.² In the horse, the most noticeable pharmacologic effect is a marked increase in locomotor activity.^{4,5} In Thoroughbred horses, IV injection of fentanyl citrate can increase locomotion 15-fold within a few minutes, and the effect can last up to 1 hour. These movements are well controlled and coordinated. Maximum locomotor activity develops at fentanyl plasma concentrations of about 50 ng/ml and the minimal plasma concentration associated with locomotion is about 5 ng/

ml. High doses of fentanyl (about 16 mg/horse) cause disorientation, incoordination, and collapse.^a

In the horse, the major urinary metabolites of fentanyl are fentanyl β -keto acid (N-[1-(2-phenethyl-4-piperidinyl)] malonanilic acid; 90%) and despropionyl fentanyl.^{6,a} During acid hydrolysis, which is used in most fentanyl detection techniques, the β -keto acid is converted to despropionyl fentanyl.⁷

Although fentanyl citrate has been used for anesthesia and sedation in man and animals, it also has been used in racehorses in attempts to improve their performance.

Control of the use of fentanyl in racehorses has been based on the use of a commercial radioimmunoassay^b (RIA) to detect this drug. Purposes of the present study were to evaluate the sensitivity of a modification of this RIA for fentanyl and to compare the sensitivity of the modified RIA with that of the original commercial RIA.

Materials and Methods

Fentanyl RIA standard curves were constructed, using RIA kits^b according to the manufacturer's instruction.

The [³H]fentanyl solution^b (50 μ l) was pipetted into 3-ml conical polypropylene tubes, then 50 μ l of 30% methanol/water were added to all tubes, except the fentanyl standard tubes. In duplicate, 50 μ l of the fentanyl standards,^b (serial dilutions of the stock fentanyl solution [40 ng/ml] in methanol/water [30:70]), were added to the standard tubes. The kit's assay buffer (300 mg of potassium phosphate monobasic, 3,120 mg of sodium phosphate dibasic dihydrate, 200 mg of sodium azide, and 2,000 mg of bovine serum albumin in 400 ml of distilled water; pH 7.5) was added to total count tubes (800 μ l), nonspecific-binding tubes (600 μ l), maximum-binding tubes (500 μ l), and the standard tubes (500 μ l). The lyophilized antiserum^b was dissolved in 10 ml of the assay buffer; 100 μ l of this solution was added to the maximum binding and standard tubes. The tubes were capped and mixed, using a rotating mixer, for 2 hours at room temperature. The kit's dextran-coated charcoal suspension^b (200 μ l) was added to all tubes, except the total count tubes. The tubes were rotated another 60 minutes at room temperature and centrifuged at 6,000 \times g for 8 minutes.

All of the supernatant solution of each tube was decanted into 20-ml glass scintillation vials, 10 ml of a scintillation cocktail,^c was added to each vial, and each vial was vortexed. Radioactivity in each vial was quantified, using a liquid scintillation counter^d (10-minute counts). Standard curves also were constructed, using [¹²⁵I]fentanyl instead of [³H]fentanyl.

Carboxyfentanyl-tyrosine methyl ester (TME) conjugate was prepared according to the procedure of Tai and Yuan⁸ for the

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^a Combie J. Studies on the locomotor responses and pharmacokinetics of fentanyl and other narcotic analgesics in the horse. MS Thesis, University of Kentucky, Lexington, 1979.

^b FEN-RIA-200, Janssen Life Sciences Products, Piscataway, NJ.

^c 3a70b, Research Products International, Mount Prospect, Ill.

^d LS 3801, Beckman Instruments Inc, Arlington Heights, Ill.

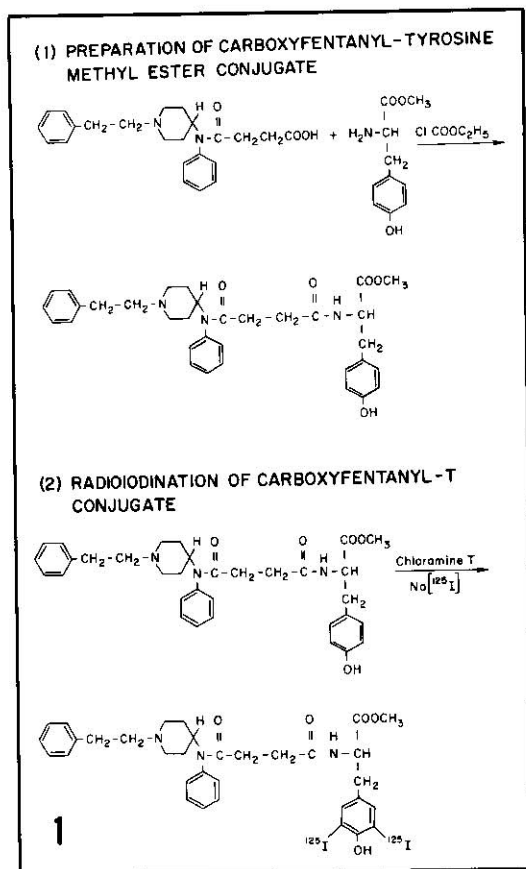


Fig 1—Synthesis of [125 I]fentanyl conjugate. The carboxyfentanyl-tyrosine methyl ester conjugate was prepared by reacting carboxyfentanyl with tyrosine methyl ester in the presence of ethyl chloroformate in dimethyl formamide and triethylamine.

conjugation of thromboxane B_2 to TME (Fig 1). Carboxyfentanyl (N-[1-(2-phenethyl-4-piperidinyl)]succinamic acid; 1 mg) was dissolved in 0.2 ml of dimethyl formamide at 0 C with 2 μ l of triethylamine. Ethyl chloroformate (0.5 μ l) in 0.1 ml of dimethyl formamide was added, and the reaction was allowed to proceed at 0 C for 15 minutes. Tyrosine methyl ester (1.3 mg) and triethylamine (0.6 μ l) in 0.2 ml of dimethyl formamide was added, and the reaction was allowed to continue for an additional 2 hours at room temperature with stirring. The reaction mixture was then evaporated with a stream of nitrogen. The product was purified, using thin-layer chromatography on a silica gel G plate with chloroform/methanol/acetic acid (85:15:1) as the developing solvent. The carboxyfentanyl-TME conjugate (R_f = 0.62) migrated ahead of carboxyfentanyl (R_f = 0.34) and TME (R_f = 0.0). The carboxyfentanyl-TME conjugate was scraped off the plate and extracted twice with 0.6 ml of ethanol. After removal of the gel (centrifugation), the conjugate was stored in

^c Biomol Research Laboratory, Philadelphia, Pa.

ethanol at -20 C. The yield was 80% with respect to carboxyfentanyl (Fig 1).

The conjugate (0.3 μ g) was dissolved in 50 μ l of 0.5M sodium phosphate buffer pH 7.5, and Na [125 I] (300 μ g) in 3 μ l of 0.001M sodium hydroxide was added to the conjugate solution. Iodination was initiated by the addition of 25 μ g of chloramine-T in 5 μ l of 0.05M sodium phosphate buffer, pH 7.5. After 30 s, the reaction was terminated by the addition of 250 μ g of sodium metabisulfate in 50 μ l of 0.05M sodium phosphate buffer, pH 7.5. The reaction mixture was extracted twice with 0.6 ml of ethyl acetate. The organic layer was evaporated to dryness with a stream of nitrogen. The residue was spotted on a silica gel G plate (2 \times 20 cm), with chloroform/methanol/acetic acid (85:15:1) as the developing solvent. The location of labeled conjugate was identified by use of autoradiography. Two labeled conjugates (presumably monoiodinated and diiodinated derivatives) were synthesized and were scraped off the gel. Labeled conjugates were extracted twice from the gel, using 0.6 ml of ethanol. After removal of gel by centrifugation (1,500 \times g), the labeled conjugates were stored in ethanol at -20 C. Only the monoiodinated conjugate (lower R_f) was used for RIA.

The [125 I]fentanyl RIA method was the same as the [3 H]fentanyl method, except for the following modifications. The [125 I]fentanyl stock solution (160,000 cpm/50 μ l) was diluted with assay buffer to about 10,000 cpm/50 μ l and added to each tube instead of the [3 H]fentanyl. The fentanyl standard solutions^b were used, but required further dilution. The antiserum^b was diluted 1:15 with assay buffer and 100 μ l of the diluted antiserum was used in the assay. The dextran-coated charcoal suspension used consisted of 0.25% dextran^f and 2.5% charcoal^g in assay buffer. When urine samples were assayed, 450 μ l of buffer was added to the tube, and 50 μ l of the urine sample was added to the tube. A standard curve for the assay also was generated, using 30% methanol/water serial dilutions of the stock sufentanil standard from a sufentanil RIA kit,^h using the [125 I]fentanyl RIA method.

Logit-log transformation (L) of the counts per minute values for each tube was performed, using the following equation:

$$L = \ln \left(\frac{B/B_0}{1 - B/B_0} \right)$$

where Ln = natural logarithm, B_0 = the maximum binding counts per minute minus the nonspecific binding counts per minute, and B = standard or sample counts per minute minus the nonspecific binding counts per minute. Standard curves were constructed by plotting the logarithmic fentanyl concentration of each standard vs the corresponding L values. For the urine samples, the fentanyl equivalent concentration for each sample was calculated from the standard curve for each run.

Urine samples from 6 horses given fentanyl citrateⁱ (200 μ g, 100 μ g, 20 μ g, 10 μ g, 2 μ g, or 1 μ g/horse) were analyzed for fentanyl equivalents, using the [125 I]fentanyl method. Urine

^f T-70, Sigma Chemical Company, St Louis, Mo.

^g Norit A Charcoal, Fisher Scientific, Fairlawn, NJ.

^h RIA-KIT for Sufentanil, Janssen Life Sciences Products, Piscataway, NJ.

ⁱ Sublimase, McNeil Pharmaceutical Co, Fort Washington, Pa.

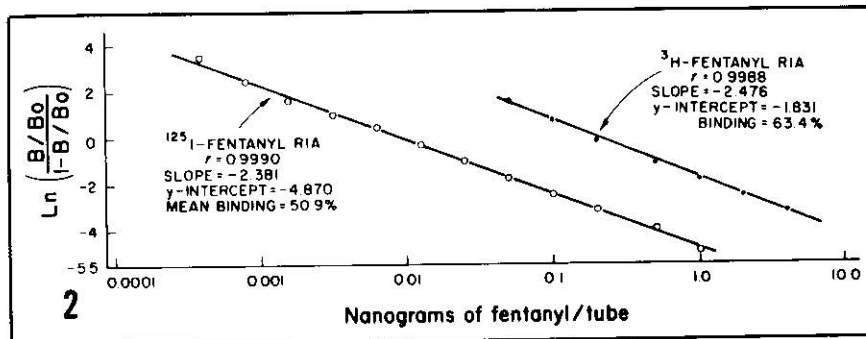


Fig 2—Standard curves for radioimmunoassay (RIA) with [3 H] and [125 I]fentanyl. Standard curves were constructed, using a fentanyl RIA kit, with [3 H]fentanyl (●) and with [125 I]fentanyl (○) as the labeled antigen. For the [125 I]fentanyl RIA curves, the antibody was diluted 1:15. The data were rearranged by logit-log transformation and the line of best fit was determined by linear regression. All data points are the means of 6 determinations.

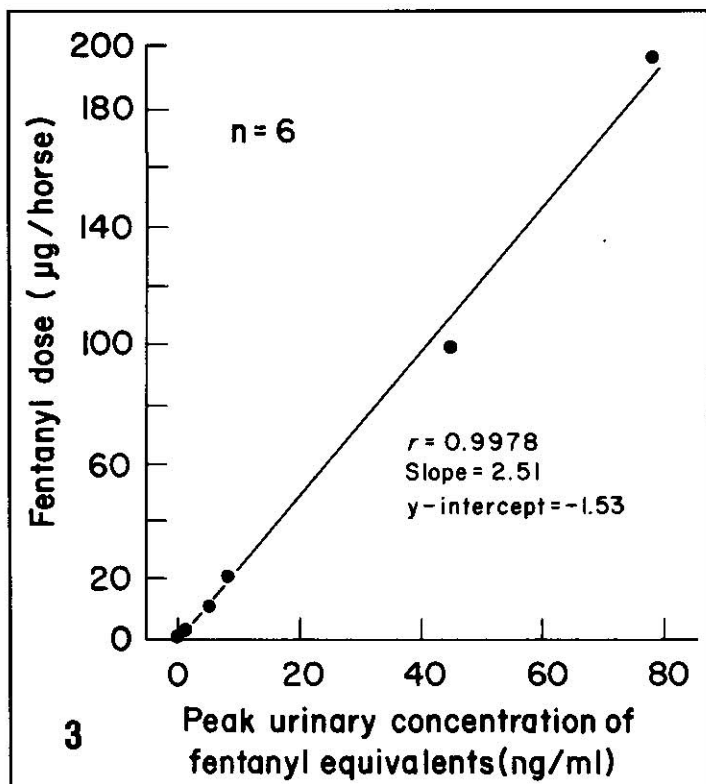


Fig 3—Correlation between fentanyl dose and peak urinary concentrations of fentanyl equivalents. Peak urinary concentrations of fentanyl equivalents were plotted against the dose of fentanyl administered iv to each horse. The least-squares regression fit for these data points yielded a correlation coefficient of 0.9978.

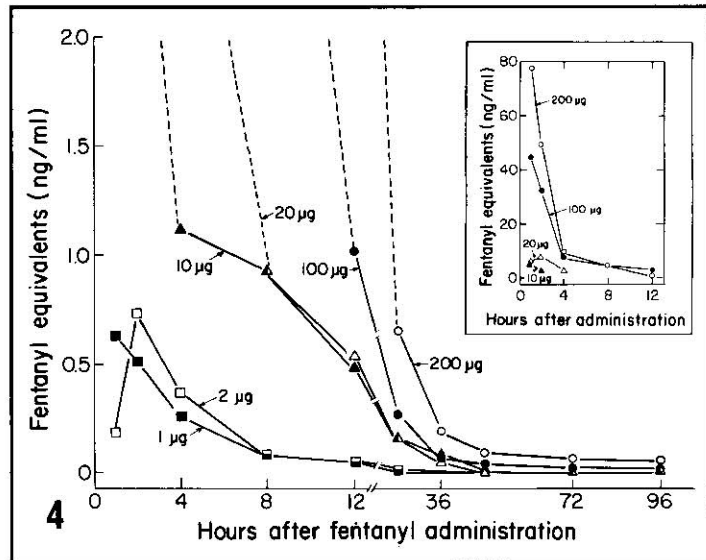


Fig 4—Fentanyl equivalent concentrations in 6 horses after iv administrations of fentanyl (200 µg/horse [○], 100 µg/horse [●], 20 µg/horse [△], 10 µg/horse [▲], 2 µg/horse [□], and 1 µg/horse [■]). Fentanyl equivalents were determined by [¹²⁵I]fentanyl radioimmunoassay. Each value represents individual values for each horse. Inset = data points from 0 to 80 ng/ml, whereas the main figure indicates only those data points < 2 ng/ml.

samples were obtained by bladder catheterization before fentanyl administration (treatment), 1 hour, 2 hours, 4 hours, 8 hours, and 12 hours after treatment and every 12 hours thereafter up to 96 hours after treatment. The urine samples were

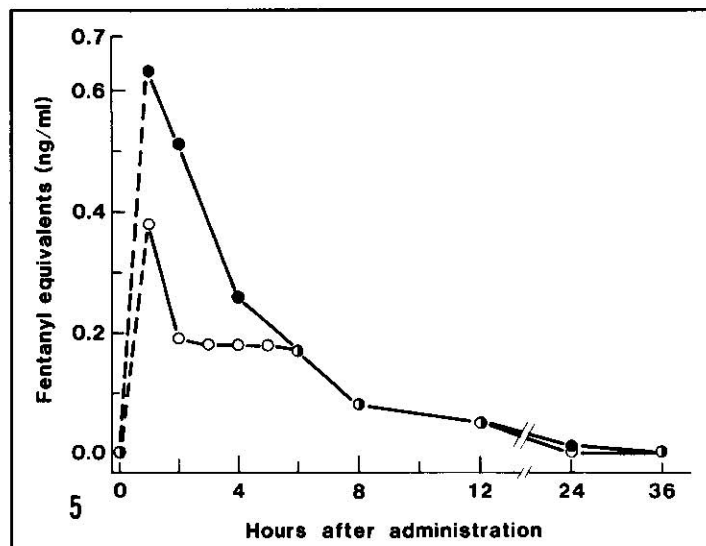


Fig 5—Urine concentrations of fentanyl in horses given fentanyl or sufentanil. Solid circles = urine concentrations of fentanyl after iv administration of 1 µg of fentanyl. Open circles = urine concentrations of fentanyl equivalents after iv administration of 40 µg of sufentanil.

analyzed directly, without extractions. When necessary, samples were diluted with assay buffer. One hour after treatment, all test samples and values were significantly different from control values ($P < 0.001$).

To further establish the validity and sensitivity of the assay, we plotted the peak concentration of fentanyl equivalents detected in each horse's urine against the dose of fentanyl administered. A highly significant correlation ($r = 0.998$) was found between the dose of fentanyl administered and the concentration of fentanyl equivalents detected in the corresponding urine, further supporting the efficacy and accuracy of the assay (Fig 3).

Urine samples from a horse given sufentanil also were analyzed for concentration of fentanyl equivalents, using the [¹²⁵I]fentanyl method. The horse was given sufentanil (40 µg, iv) as sufentanil citrate,¹ and urine samples were collected before sufentanil administration and 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 8 hours, 24 hours, and 36 hours after administration.

Results

Use of the radioiodinated analogue of fentanyl as the ligand in the commercially available RIA increased the sensitivity of the assay more than 100-fold. The useful region (80% to 20% inhibition) of the immunoassay, using the [³H]fentanyl as the radiolabeled ligand, was in the area of 0.2 ng/ml (Fig 2). However, when the radioiodinated analogue of fentanyl was used, the useful region of the immunoassay was about 0.002 ng/ml. This increased sensitivity allowed detection of small amounts (ie, 1 µg/horse up to 8 hours) of fentanyl administered to horses or, conversely, the detection of pharmacologically effective doses for long periods (ie, 200 µg/horse up to 96 hours).

After administration of 200 µg of fentanyl/horse, fentanyl equivalent concentrations of 80 ng/ml of horse urine were detected initially (Fig 4). These concentrations decreased rapidly to about 10 ng/ml in the first 4 hours after treatment. Thereafter, the fentanyl equivalent concentrations in urine decreased more slowly, and concentra-

¹ Sufenta Injection, Janssen Pharmaceutica Inc, Piscataway, NJ.

tions of about 50 pg/ml were detectable in the urine of the horses at 96 hours after treatment. After administration of 1 µg of fentanyl/horse, urinary concentrations of fentanyl (or its metabolites) were detectable in the urine for at least 24 hours, whereas fentanyl doses of 10 µg/horse were detectable for up to 48 hours.

More recently, a number of analogs of fentanyl have been synthesized, and some of these agents are in the final stages of clinical trials, or are about to be released for use in human medicine. One of these agents is sufentanil which is about ten times as potent as fentanyl.⁹ To test whether sufentanil or its metabolites in horse urine would yield a significant response in our fentanyl immunoassay, we tested the reactivity in urine from a horse dosed with 40 µg of sufentanil. Compared with the amount of fentanyl detectable in the urine of horses given the low dose (1 µg) of fentanyl, [¹²⁵I]fentanyl RIA enabled us to detect a small amount of reactivity in the urine of the horse given 40 µg of sufentanil (Fig 5). We also found about 1% of cross-reactivity of sufentanil with the [¹²⁵I]fentanyl RIA when substituted for fentanyl in the standard curve. Therefore, on the basis of these observations, sufentanil in racehorses may be detectable by use of the fentanyl antibody used in the present study.

Discussion

Fentanyl is a typical narcotic agonist in the horse, in that it produces locomotor activity and analgesia.¹⁰ These actions are potentially useful in horses with subclinical lameness and could serve to improperly improve the racing performance. For these reasons, the use of narcotic analgesics in racehorses has been forbidden for most of the twentieth century, and these regulations are enforced by chemical testing.¹¹

Chemical testing for fentanyl in urine samples of horses after racing is difficult because of the great potency of this drug.³ Fentanyl is about 100 times more potent than morphine or most of the other narcotic analgesics that are readily available. For this reason, the high-performance thin-layer chromatographic methods generally used to detect narcotic analgesics in horse urine are ineffective for detection of fentanyl. Therefore, detection of this drug in urine samples of horses after racing has been dependent on RIA screening.^{7,11}

A problem with the use of RIA as a routine screening test for fentanyl is that the test has to be run specifically for fentanyl. This is a cumbersome procedure, and unless the probability of fentanyl being in the sample is high, the cost of the procedure relative to the yield of positives is high. Therefore, use of RIA screening for fentanyl has tended to be restricted to circumstances where the probability of detecting fentanyl is high.

The RIA methodology used in the present study markedly increased the usefulness of the fentanyl RIA for routine screening, since it increased its sensitivity for fentanyl, while reducing its cost. Because of the increased sensitivity, concentrations of antibody used in the system could be reduced 15-fold,^{*} thereby extending the antibody. The

sensitivity of the assay was increased up to 100-fold. This increased sensitivity can be used to detect smaller concentrations of fentanyl in urine or, conversely, can allow pooling of urine samples, and the simultaneous screening of larger numbers of samples in horses after a race. Using this approach, a pool of urine samples (10 to 20) collected from horses during a day of racing could be screened for fentanyl, and, if necessary, the source of any detected fentanyl could be pinpointed by performing a second screening of each horse's urine.

Results of the present study indicate that the smallest dose of fentanyl that probably induces an effect on a racing horse probably is about 100 µg/horse, and that any clinical dose probably induces a pharmacologic effect within 4 hours after administration. Using the modified RIA, we were able to detect fentanyl in horses given 1 µg of fentanyl and could detect this dose for at least 24 hours after administration.

This large reserve of sensitivity indicates that the illicit use of fentanyl in individual horses could be detected in pooled urine samples obtained after racing. For example, about 0.25 ml of each sample from a day's urine samples obtained after racing could be pooled, and the pooled samples stored frozen until the end of the week. Then at this time, an aliquot of each day's pooled samples after racing would be screened for fentanyl. Because of the great sensitivity of the modified test, dosing with a pharmacologically significant amount of fentanyl would likely be detectable in the pooled samples by the modified RIA. In the event of a positive sample, each of the targeted day's individual samples can be screened individually and the source of the fentanyl readily identified. Using this methodology, 1 week's urine samples obtained after racing could be screened for fentanyl in 1 day.

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* Subsequent alterations have reduced these concentrations 60-fold.