Detection of Fentanyl and Fentanyl Derivatives Using Radioimmunoassay and Enzyme-Linked Immunosorbent Assay

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Abstract

We have developed and evaluated an ^{125}I radioimmunoassay (RIA), and a one step enzyme-linked immunosorbent assay (ELISA), for fentanyl as part of a panel of tests for narcotic analgesics in racehorses.

The ^{125}I-fentanyl RIA is highly sensitive, capable of detecting concentrations as low as 1 pg/ml of fentanyl equivalents in urine. This RIA will detect administration of doses that are 100 times less than that required to produce a measurable pharmacological effect. With this amount of sensitivity, 10 to 20 urine samples can be pooled and screened simultaneously. Pools flagged positive can be re-analyzed and the sample(s) containing fentanyl can be isolated.

The ELISA test detects fentanyl with an I-50 of about 100 pg/ml and readily detects the presence of fentanyl or its metabolites after administration of sub-therapeutic doses. Antibodies developed also cross-react with several analogs of fentanyl and likely detect these analogs in blood and urine shortly after their administration to horses. As such these immunoassays are capable of improving the quality and reducing the cost of pre-race and post-race testing for fentanyl and several fentanyl analogs in racehorses.

Introduction

Fentanyl (N-phenyl-N-[1-(2-phenylethyl)-4-piperidinyl]propanamide) is a synthetic opioid derivative of meperidine. As a powerful narcotic analgesic, the potency of fentanyl is estimated at 80 to 150 times that of morphine.[1,2] Narcotic actions of fentanyl 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.[2] In the horse, the most noticeable pharmacologic effect is a marked increase in locomotor activity.[4] Intravenous injection of fentanyl citrate can increase locomotion 15-fold within minutes, and the effect can last up to 1 hour.[5] Maximum locomotor activity develops at fentanyl plasma concentrations of about 50 ng/ml and minimal plasma concentrations associated with locomotion are about 5 ng/ml. Due to the analgesic and locomotor effects of fentanyl, this drug has been frequently reported as used illegally in racehorses.[6]

Recently, a number of licit and illicit derivatives of fentanyl have been synthesized, including the illicit 3-methyl and α-methylfentanyl analogs as well as the proprietary derivatives sufentanil and carfentanil. (Fig. 1) Some of these agents are in the final stages of clinical trials or have been licensed for use. One of these proprietary agents, sufentanil, is about ten times as potent as fentanyl.[7]

Most recently, control of the use of fentanyl in racehorses has been based on radioimmunoassay (RIA) screening.[8,9] The purpose of this study was to evaluate the sensitivity of an ^{125}I fentanyl RIA (^{125}I RIA) compared with the sensitivity of a commercially

available ^{3}H fentanyl RIA. Secondly, because RIA methodology is relatively slow, we have also developed and evaluated an alternate one step enzyme-linked immunoassay (ELISA) to detect fentanyl and its derivatives in blood and urine samples from racehorses.

Materials and Methods

Horses

Mature Thoroughbred and Standardbred mares (450-550 kg) were used throughout. Mares kept at pasture and allowed free access to food and water, then placed in standard box stalls (17 sq M) approximately 12 hours prior to dosing for acclimatization.

Post-race urine samples from racing horses were collected by the authorities in charge at the racecourses and delivered to the drug testing laboratory of the racing jurisdiction.

^{125}I Fentanyl RIA Protocol

Fentanyl RIA standard curves were constructed, using ^{3}H-Fentanyl RIA kit[a] components as described by Woods *et al.*[8] A carboxyfentanyltyrosine methylester (TME) conjugate was prepared according to the procedure of Tai and Yuan for the conjugation of thromboxane B_2 to TME.[10] Iodination of this carboxyfentanyl-TME conjugate was by the procedure described by Woods *et al.*[8] Standard curves also were constructed, using ^{125}I fentanyl instead of ^{3}H fentanyl.

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

RIA Dosing and Sampling

Urine samples from 6 mares given fentanyl citrate (200 µg, 100 µg, 20 µg, 10 µg, 2 µg, or 1 µg/horse) were analyzed for fentanyl equivalents, using the ^{125}I RIA method. Urine samples were obtained by bladder catheterization before fentanyl injection and 1, 2, 4, 8 and 12 hours after injection, and every 12 hours thereafter to 96 hours. The urine samples were analyzed directly without extraction. When necessary, samples were diluted with assay buffer.

Urine samples from a mare given sufentanil were analyzed for concentration of fentanyl equivalents, using the ^{125}I RIA method. The mare was given sufentanil (40 µg/horse, IV) as sufentanil citrate, and urine samples were collected before sufentanil administration and 1, 2, 3, 4, 5, 6, 8, 24, and 36 hours after administration.

One Step ELISA Test

The one step ELISA tests were performed as previously described.[11,12,13] Briefly, anti-fentanyl antibody was linked to flat bottom microtiter wells[c] as described by Voller.[12] Similarly, carboxyfentanyl was linked to horse radish peroxidase (HRP), as described by Wie and Hammock[14] to give rise to a covalently linked fentanyl-HRP complex. All reactions were run at room temperature (25°C).

The assay was started by adding 20 µl of the standard, test, or control samples to each well, along with 100 µl of the fentanyl-HRP solution. During this step, the presence of free drug or cross reacting metabolites competitively prevented the antibody from binding to the fentanyl-HRP conjugate. The degree of antibody-fentanyl binding was therefore inversely proportional to the amount of drug in the sample. After ten minutes of incubation the fluid was removed from the microtiter wells and the wells washed three times with buffer. Substrate solution[d] was then added to all wells, a 10 minute incubation period allowed, and absorbance values read at 650 nm in a microwell reader[e].

ELISA Dosing and Sampling

Authentic fentanyl, carfentanil, and sufentanil drug standards were obtained[f]. Fentanyl was injected (500 µg/horse) as fentanyl citrate. All administrations were by rapid IV injection into the jugular vein. Urine samples were collected by bladder catheterization, pre-

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Plasma samples were collected from the jugular vein into sterile evacuated tubes containing sodium oxalate and potassium fluoride.[8] Samples were collected pre-dose and at 5, 10, 20, 30, 45, 60 minutes and 2, 4, and 8 hours after dosing.

Equine plasma samples were treated with trichloroacetic acid (TCA) to denature and remove excess plasma proteins. In this step 300 µl of 4% TCA were added to 300 µl of plasma, vortexted, and allowed to react at room temperature for 20 minutes. The samples were then centrifuged at 8,800 x g for 10 minutes and the supernatant removed. To 50 µl of the supernatant 12 µl of 0.24 M sodium carbonate, pH 10, were added, the mixture votexed, and an aliquot taken for assay.

We evaluated the ability of the ELISA test described here to detect closely related congeners of fentanyl. Several polyclonal antibodies to carboxyfentanyl derivatives raised in rabbits were incubated in the presence of increasing concentrations of fentanyl, 3-methylfentanyl, α-methylfentanyl, carfentanil, sufentanil, and alfentanil.

We introduced both this fentanyl ELISA and ^{125}I RIA for fentanyl and derivatives carfentanil and sufentanil into routine testing as described previously.[9,12] The test was introduced in Oklahoma and adjacent Western states in response to reports that horsemen in these states were using sufentanil and that its use was going undetected.

Mass Spectral Confirmation of Sufentanil

Confirmation of the presence of sufentanil in urine samples was by gas chromatography/mass spectroscopy (GC/MS). To a 50 ml urine sample in a glass flask 20 ml of saturated sodium bicarbonate solution and 5 grams of moist nonionic resin[a] were added. The flask was mixed by shaking agitation for 20 min. The urine sample was aspirated off and the resin was washed with several portions of water. The resin was transferred to a plastic tub and was dried by sucking air through it using a vacuum pump. The sufentanil was eluted with 25 ml of 1% acetic acid in methanol; the eluent was concentrated to 5 ml under partial vacuum. To the concentrated eluent 6 ml of 3M hydrochloric acid (HCL) were added and the mixture was transferred to a glass tube for autoclaving at 120° C for 2 hrs. This procedure converted sufentanil to despropionyl-sufentanil. The solution was cooled and sufficient 50% sodium hydroxide (NaOH) was added to neutralize approximately 1/2 of the HCL. The pH was checked to assure that the sample was still strongly acidic.

The aqueous solution was washed with 20 ml of ethyl acetate and then with 20 ml dichloromethane (DCM)/isopropanol (10:1). The aqueous solution was made strongly basic (pH > 12) with 50% NaOH. The aqueous solution was extracted with 30 ml DCM. After centrifugation, the DCM phase was back-extracted with 4 ml of 1% sulfuric acid. The solution was made strongly basic with 50% NaOH was extracted twice with 15 ml portions of petroleum ether. The petroleum ether extracts were combined and evaporated to dryness in a water bath and the residue was dissolved in ethyl acetate for injection onto the GC/MS.

The GC/MS system employed consisted of a capillary gas chromatograph equipped with a mass spectroscopy detector and a data station.[i] GC/MS conditions were similar to those previously described.[13]

Results

RIA

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 [^{3}H]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). (Fig. 3)

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 concentrations of about 50 pg/ml were detectable in the urine of 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. (Fig. 3)

To evaluate whether sufentanil or its metabolites in horse urine would be detectable in our ^{125}I RIA, we tested the reactivity in urine from a mare dosed with 40 μg of sufentanil IV. Compared with the amount of fentanyl detectable in the urine of horses given 1 μg of fentanyl IV, the ^{125}I RIA enabled us to detect a small amount of reactivity in the urine of the mare given 40 μg of sufentanil (data not shown). We also found about 1% of cross-reactivity of sufentanil with the ^{125}I RIA when substituted for fentanyl in the standard curve (data not shown) On the basis of these observations, sufentanil in racehorses can be detected by the use of the fentanyl antibody used in the present study.

ELISA

In the absence of added fentanyl the one step ELISA reaction rapidly runs to completion, with an apparent absorbance value of about 1.6 being attained between 10 and 15 minutes after initiation. The addition of increasing concentrations of fentanyl inhibited the reaction, with virtually complete inhibition occurring after addition of 5 ng/ml of fentanyl. (Fig. 4) In our hands the optimal time to read the test is about 12 minutes after addition of substrate, when evident sensitivity is greatest. Under these conditions, apparent I-50 for fentanyl is about 100 pg/ml.

Since we wished to incorporate this test into a panel of pre-race tests, we examined the effect of the addition of equine plasma on this assay. Addition of untreated plasma to the ELISA reduced the test efficacy. (Fig. 5) However, treatment of the plasma with TCA substantially improved the quality of the assay, and its sensitivity was similar to that obtained in the absence of plasma. (Fig. 5)

Urine also acted to inhibit the ELISA reaction, but the effect was much less marked. The presence of horse urine slowed the reaction such that the maximum color was not obtained in control samples for at least 20 minutes. (Fig. 6) This effect is small however, impacting optimum reading time but not test efficacy.

The fentanyl ELISA test readily detected the presence of fentanyl in equine plasma. Three mares dosed with 500 μg fentanyl/horse I.V. (a subtherapeutic dose), resulted in inhibition of enzyme activity in our ELISA in plasma at 5 minutes after dosing and this inhibition remained substantial for ≤ 60 minutes. (Fig. 7) Based on the short duration of the pharmacological activity of fentanyl after IV administration, this short period of detection of parent drug in the plasma is consistent with the known pharmacokinetics of fentanyl.

Fentanyl was also readily detected in urine from these mares. In urine, virtually complete inhibition of the ELISA reaction was observed for the first 8 hours after dosing in all mares, and substantial inhibition was still observed at 24 hours after dosing. (Fig. 8) The data suggest that this test can readily detect the administration of sub-therapeutic doses of fentanyl from equine blood or urine samples.

Additionally, this ELISA based on polyclonal antibodies crossreacted with a number of fentanyl derivatives. (Fig. 9) The C-4 antibody exhibited somewhat greater sensitivity to the presence of fentanyl and its methylated derivatives, and also to carfentanil and sufentanil, than did the ELISA based on the T-3 antibody.

This ELISA also readily distinguished between urine samples from fentanyl dosed horses and control horses. Post-race urine samples from 47 horses racing in Kentucky yielded absorbance readings of about 1.8, while that from dosed horse urine yielded values in the order of 0.2. (Fig. 10) based on this large discrepancy between the control and test values, it is relatively easy to distinguish between urines from control and fentanyl treated horses.

Out of about 319 urine samples screened for fentanyl and its crossreacting derivatives by both ^{125}I RIA and ELISA methods over a two week period, 20 were flagged for fentanyl based on either ELISA or RIA data. When these flagged suspicious samples were submitted for GC/MS analysis, 10 were confirmed positive for the fentanyl derivative sufentanil. (Fig. 11)

Discussion

RIA

Fentanyl and its derivatives are typical narcotic agonists in the horse, in that they produce locomotor activity and analgesia. (Fig. 12) These actions are potentially useful in horses with subclinical lameness and could serve to improperly improve the racing performance.

Chemical testing for fentanyl in post-race urine samples is difficult because of the great potency of this drug. Fentanyl is about 100 times more potent than morphine or most of the other narcotic analgesics 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, recent methods for detection of fentanyl in post-race urine samples of horses after racing have been dependent on RIA screening.

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 with a high probability of detection.

The ^{125}I RIA method used here markedly increased the usefulness of RIA for routine screening. Results of a previous study[5] indicate that the smallest dose of fentanyl that induces an effect on a horse is ≥ 100 μg/horse, and that any clinical dose probably induces a pharmacological effect within 4 hours after administration. Using the modified ^{125}I RIA, we were able to detect fentanyl in horses given as little as 1 μg of fentanyl and could detect this dose for ≤ 24 hours after administration. Assay sensitivity is increased up to 100-fold relative to the ^{3}H RIA assay.

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 post-race samples. Using the pooling approach for example, about 0.25 ml of each sample from a day's post-race urine samples could be pooled, and pooled samples stored frozen until the end of the week. Then at that time, an aliquot of each day's collection of post-race samples could be screened for fentanyl. Because of the sensitivity of the ^{125}I RIA test, dosing with a pharmacologically significant amount of fentanyl would likely be detected in pooled samples. In the event of a positive sample, each of the targeted day's individual samples can be screened individually and the source of the positive readily identified. Thus, 1 week's urine samples obtained after racing could be screened for fentanyl in 1 day. (Table I)

ELISA

Effective routine screening for fentanyl would be more widely implemented if time and materials costs for the test were reduced. Based on these considerations there was a considerable incentive to develop this ELISA, as its availability enables the testing of post-race urines for fentanyl and cross-reacting derivatives rapidly and inexpensively.

This technique is even more important if pre-race testing is contemplated as time constraints in pre-race testing virtually eliminate the use of conventional RIA. In this situation one is limited to thin layer chromatography or to a rapid immunoassay technique. However, thin layer chromatography does not have the sensitivity necessary to effectively detect high potency narcotics such as fentanyl either post-race or pre-race. For this reason the only currently available technology with the requisite sensitivity for an effective pre-race testing system is an enzyme immunoassay based system.

The ELISA reported here readily detected fentanyl administration in blood and urine from mares given 500 μg fentanyl. Fentanyl was detected in equine blood within minutes after injection of 500 μg/horse and remained detectable for ≤ 1 hour. (Fig. 7) This short period for fentanyl detection in equine blood is consistent with fentanyl kinetics in horses and humans where its actions are terminated by redistribution. Similarly, the test detected the drug in urine for 24 hours, again consistent with our prior experience with fentanyl. (Fig. 8)

Introduction of this fentanyl ELISA into forensic screening was in response to requests from Industrial Testing Laboratories in Denver, Colorado. Reports of sufentanil abuse in Western racing jurisdictions had

been received and we chose a launch both this ELISA and ^{125}I RIA fentanyl-sufentanil-carfentanil test. Out of about 319 samples screened for fentanyl and cross-reacting fentanyl derivatives in a relatively short period, twenty were flagged for fentanyl or a fentanyl derivative by one or the other of these tests. All of these samples showed clearly on our ELISA test, as did urine samples from known horses injected with carfentanil, sufentanil, and fentanyl. (Table II) Our ELISA was therefore readily able to detect abuse of fentanyl under field conditions.

Of 20 samples "flagged" for fentanyl, ten were confirmed positive for sufentanil by MS analysis. (Fig. II) Of the remaining samples either an insufficient volume of urine was available for MS confirmation, or the time frame for reporting a positive had passed. These data indicate that the ELISA flagged fentanyl containing samples with a 50% or greater probability that the flagged sample could be confirmed as a true fentanyl/fentanyl derivative positive.

In summary, therefore, we have developed a one step ELISA test for fentanyl and its cross-reacting derivatives in horses. The test is extremely sensitive, and will detect fentanyl in the blood of horses after a 500 μg/horse I.V. dose. It readily detects fentanyl, carfentanil, sufentanil, and likely α-methylfentanyl and 3-methylfentanyl in the urine of horses dosed with clinically used doses of these drugs. When introduced into field testing this test flagged about twenty of 319 samples and about 50% of these samples were confirmed positive for sufentanil.

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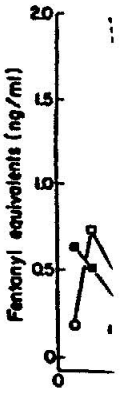

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Footnotes

[a] FEN-RIA 200, IRE Corp., Fleurs, Belgium
[b] Sufenta-RIA, Janssen Life Science Products, Piscataway, NJ
[c] Immulon Removeawells, Dynatech, Chantilly, VA
[d] TMB Microwell Peroxidase, Kirkegaard & Perry, Gaithersburg, MD
[e] International Diagnostic Systems Corp., St. Joseph, MI
[f] Janssen Life Science Products, Piscataway, NJ
[g] Vacutainer, Becton-Dickinson, Rutherford, NJ
[h] Amberlite XAD-2, Mallinckrodt, Paris, KY
[i] Hewlett-Packard #5890 GC, H-P #5970 MSD, Hewlett-Packard Data Station, Hewlett-Packard Instruments, Palo Alto, CA

Figure 1. *Representation of the chemical structures of fentanyl, carfentanil, sufentanil, 3-methylfentanyl and α-methylfentanyl.*

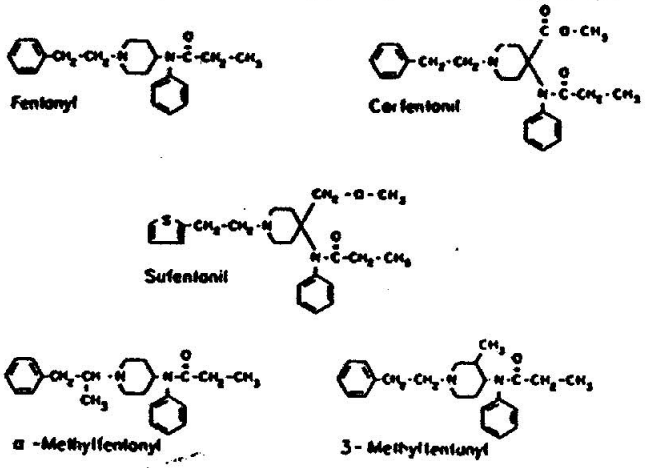

Figure 2. *Standard curves for ^{125}I and ^{3}H fentanyl RIAs.*

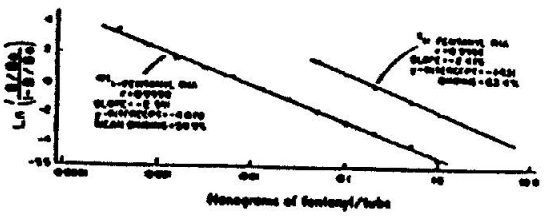

Figure 3. *Fentanyl equivalent concentrations in 6 horses after IV administration of fentanyl.*

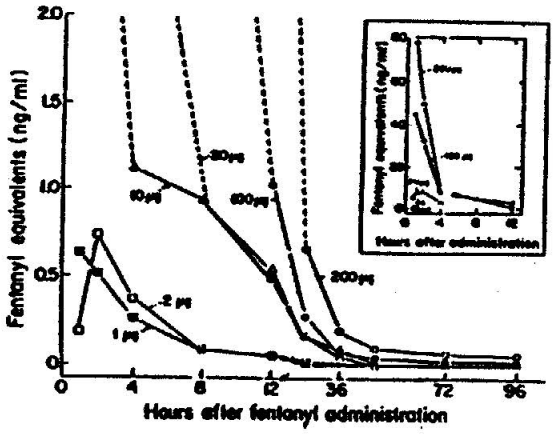

Figure 4. *Time course of the absorbance changes with increasing concentrations of fentanyl added to assay buffer.*

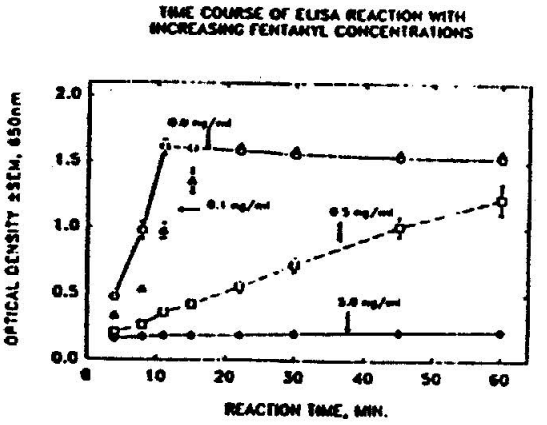

Figure 5. *Effect of plasma and TCA treated plasma on ELISA test for fentanyl.*

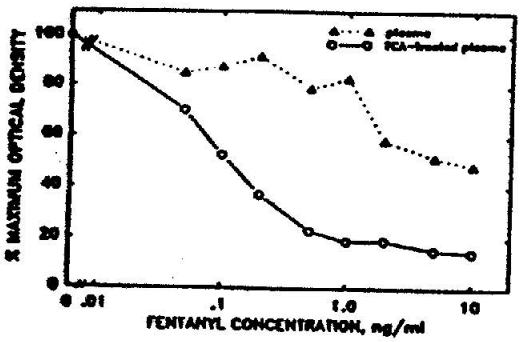

Figure 6. *Time course of ELISA reaction in the presence of added urine.*

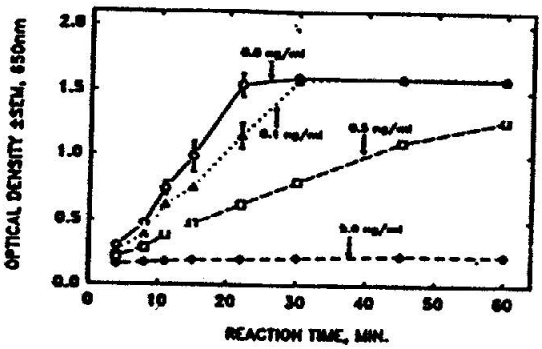

Figure 7. *Detection of fentanyl in equine plasma by ELISA.*

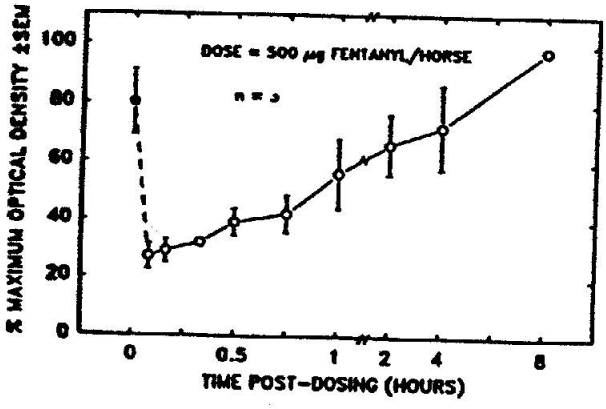

Figure 8. *Detection of fentanyl in equine urine by ELISA.*

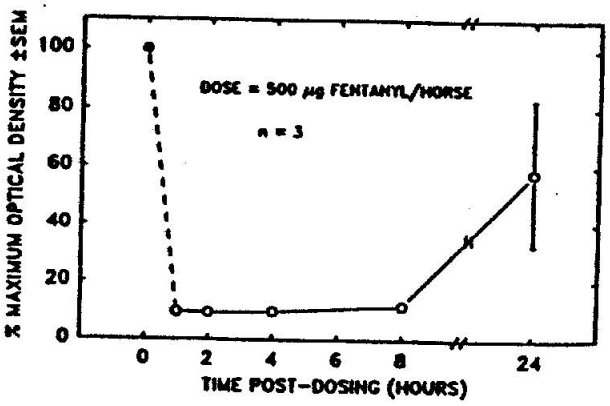

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Figure 9. *Cross-reactivity of T-3 and C-4 based ELISA tests.*

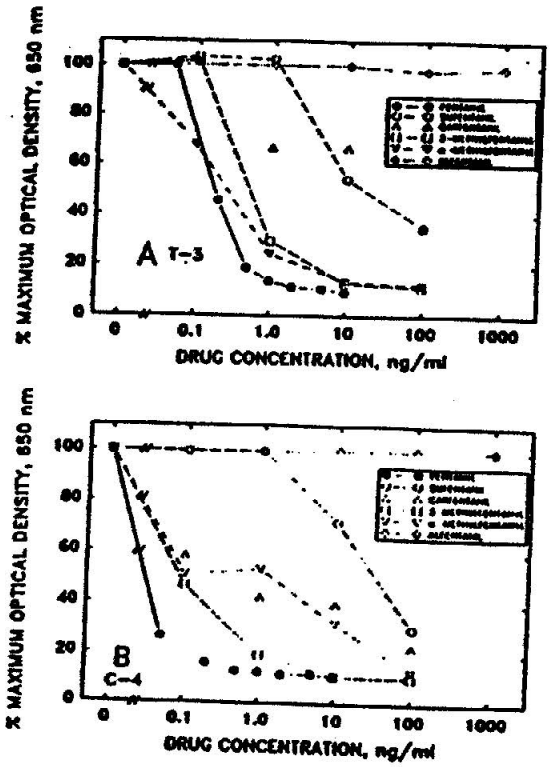

Figure 10. *Frequency distribution of fentanyl ELISA reactions of post-race track urine samples.*

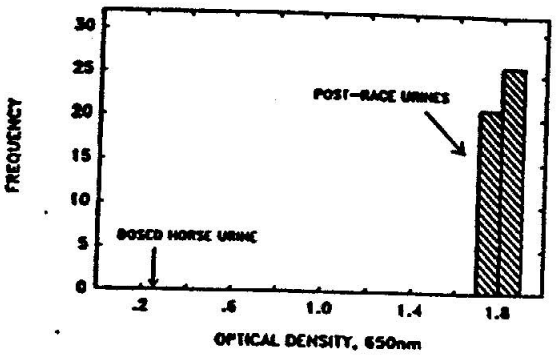

Figure 11. *Sufentanil mass spectral data.*

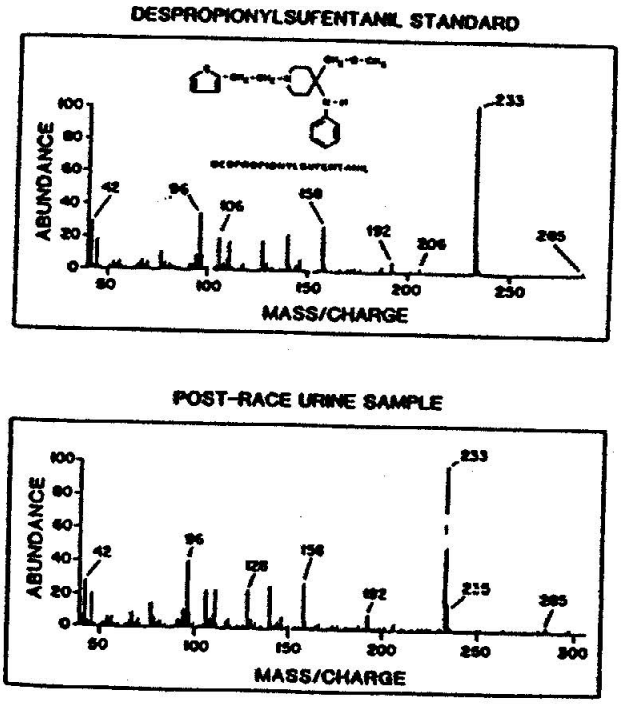

Figure 12. *Locomotor response to fentanyl and some key fentanyl derivatives after IV administration to horses.*

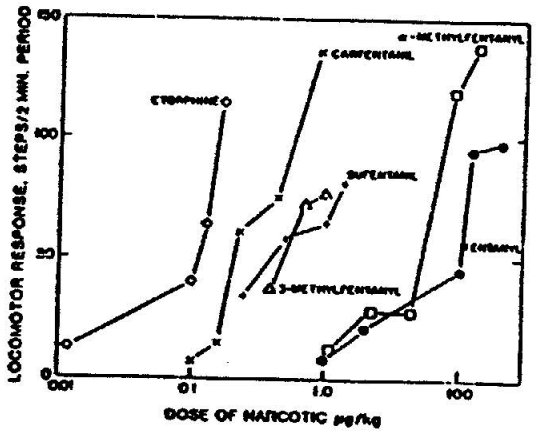

Table I. *Example of routine screening with ^{125}I fentanyl RIA in pooled blood urine samples.*

POOLED SAMPLES				INDIVIDUAL SAMPLES	
TRACK	DATE	#URINES	FENT. EQUIV. (PG/ML)	TRACK "C" 11/5/86	FENT. EQUIV. (PG/ML)
A	11/09/86	12	23.0	#1	<2.0
C	11/11/86	14	11.0	#2	<2.0
C	11/09/86	14	23.4	#3	<2.0
A	11/06/86	13	11.0	#4	1775.0
A	11/08/86	12	14.4	#5	25.4
A	11/05/86	11	13.6	#6	18.8
A	11/07/86	9	12.0	#7	<2.0
C	11/08/86	17	9.8	#8	6.6
C	11/07/86	13	16.8	#9	12.4
C	11/09/86	14	21.4	#10	<2.0
C	11/05/86	16	403.4	#11	13.0
				#12	<2.0
				#13	<2.0
				#14	7.4
				#15	3.8
				#16	6.4

Table II. *Absorbance values for control, drug treated, and flagged sufentanil positive urine samples.*

Sample #	Race Day	Absorbance	Status
1	---	.839	Quality assurance control
2	---	.272	Carfentanil, 125 μg, 1-2 hr urine
3	---	.182	Fentanyl, 100 μg, 0-1 hr urine
4	---	.300	Sufentanil, 100 μg, 1-2 hr urine
5	10-31-87	.345	Confirmed, GC-MS, Sufentanil
6	11-6-87	.240	Confirmed, GC-MS, Sufentanil
7	11-6-87	.345	Confirmed, GC-MS, Sufentanil
8	11-8-87	.335	Confirmed, GC-MS, Sufentanil
9	11-8-87	.485	Confirmed, GC-MS, Sufentanil
10	11-8-87	.269	Confirmed, GC-MS, Sufentanil
11	11-12-87	.339	Confirmed, GC-MS, Sufentanil
12	11-12-87	.467	Confirmed, GC-MS, Sufentanil
13	11-13-87	.302	Confirmed, GC-MS, Sufentanil
14	11-14-87	.416	Confirmed, GC-MS, Sufentanil

Urine samples from races run on the dates indicated in column 2 were screened for fentanyl by our ELISA test. The third column shows absorbance values obtained in control urine, dosed horse urines and track urine samples. Samples #5-14 confirmed positive for sufentanil.

Discuss

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TW That
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Discussion

S. KAMERLING Any evidence in the literature for cross-reactivity with endogenous opioid material to antibodies drawn against fentanyl or etorphine, for example?

TW That is something I do not know. We have not screened against endogenous compounds with these antibodies. That is something that could be investigated.

GERRY JOHNSTON I may have missed part of what you were saying; the positives you said that were confirmed by GC/MS were then actual racetrack samples?

TW Yes they were. That tabular data is hard to read in a presentation like this, but 300 samples run through on a field test basis. Twenty samples flagged by one or the other of the two methods. Ten samples confirmed out from ELISA flagging. That was the indication on that table. Those were actual post-race samples.