

IMMUNOASSAY DETECTION OF DRUGS IN RACING HORSES
IV. DETECTION OF FENTANYL AND ITS CONGENERS IN EQUINE BLOOD AND URINE
BY A ONE STEP ELISA ASSAY

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SUMMARY

We have developed and evaluated a one step enzyme-linked immunosorbent assay (ELISA) test for fentanyl as part of a panel of pre- and post-race tests for narcotic analgesics in racing horses. This ELISA test detects fentanyl with an I-50 of about 100 pg/ml. The test is economical in that it can be read with an inexpensive spectrophotometer, or even by eye. The test is rapid, and ten samples, a normal pre-race complement, can be analyzed in about twenty minutes. The test readily detects the presence of fentanyl or its metabolites in equine blood and urine from two and twenty-four hours respectively after administration of sub-therapeutic doses. The two antibodies evaluated also cross-react with the methylated analogs of fentanyl, sufentanil and carfentanil and the test detected these drugs shortly after their administration to horses. When introduced into routine screening, this test, in combination with another immunoassay test previously described, yielded 10 sufentanil positives. As such this test is capable of both improving the quality and reducing the cost of pre-race and post-race testing for fentanyl and a number of its congeners in racing horses.

INTRODUCTION

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 (Tobin *et al.*, 1979; Jaffe and Martin, 1985). Its 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 (Martin, 1984).

In man, the major pharmacologic action of fentanyl is analgesia, with euphoria and respiratory depression (Jaffe and Martin, 1985). In the horse, the most noticeable pharmacologic effect is a marked increase in locomotor activity (Combie et al., 1979). 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. Due to the analgesic and locomotor effects of fentanyl, this drug and its congeners, have been reported to be frequently used illegally in racing horses (Tobin, 1981).

Until recently, control of the use of fentanyl in racehorses has been based on the use of radioimmunoassays (RIA) to detect this drug (Weckman et al., 1987). This methodology, however, is relatively slow and time consuming. We have, therefore, been working on alternate enzyme immunoassay based strategies for the detection of this drug in racing horses. Recently, we have evaluated particle concentration fluorescence immunoassay (PCFIA) as a screening technique (McDonald et al., 1987), and in this communication we report on the development of a simple one step enzyme linked immunosorbent assay (ELISA) test for fentanyl in the blood and urine of racing horses.

MATERIALS AND METHODS

Horses

Mature Thoroughbred, half Thoroughbred and Standardbred horses (400-600 kg) were used throughout. The animals were kept at pasture and allowed free access to food and water. The horses were 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 individual racecourses after races and delivered to the drug testing laboratory of the racing jurisdiction.

Dosing and Sampling

Authentic fentanyl, carfentanil and sufentanil drug standards were obtained from Janssen Life Sciences (Piscataway, NJ). Fentanyl was injected as fentanyl citrate. All injections were by rapid IV injection into the jugular vein. All urine samples were collected by bladder catheterization, and were stored frozen until assayed.

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

One Step ELISA Test

The one step ELISA tests were performed as previously described (Voller *et al.*, 1976; Yang *et al.*, 1987; McDonald *et al.*, 1988). Briefly, anti-fentanyl antibody was linked to flat bottom Immulon Removawells® (Dynatech, Chantilly, VA) as described by Voller. Similarly, carboxyfentanyl was linked to horse radish peroxidase (HRP), as described by Wie *et al.* (1982) to give rise to a covalently linked fentanyl-HRP complex. All reactions were run at room temperature.

The assay was started by adding 20 μ l of the standard, test or control samples to each well, along with 100 μ l 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 HRP 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. TMB Microwell Peroxidase substrate solutions (Kirkegaard and Perry, Gaithersburg, MD) were then added to all wells and their absorbance read at 650 nm in an International Diagnostic Systems Corp. (St. Joseph, MI) microwell reader at 10 minutes after addition of substrate. Kits for this fentanyl test and for other tests reported in this series of papers are commercially available from International Diagnostic Systems Corp., St. Joseph, Michigan.

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 Amberlite XAD-2 nonionic resin (Mallinckrodt, Paris, KY) 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 despropionylsufentanil. 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 and 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.

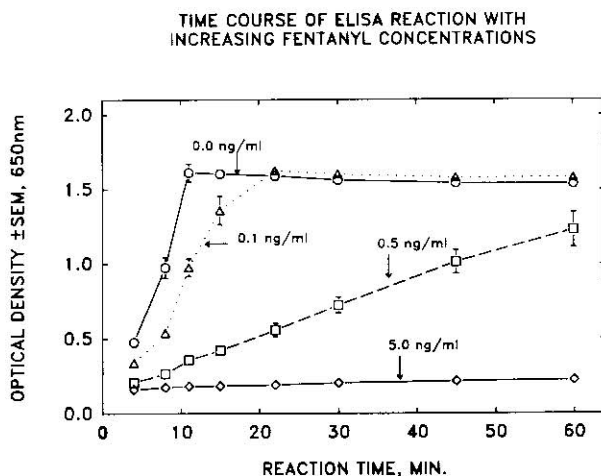
The GC/MS system employed consisted of a Hewlett-Packard Model #5890 capillary gas chromatograph equipped with a Hewlett-Packard Model #5970 mass spectroscopy detector (MSD) and a Hewlett-Packard data station (Hewlett-Packard Instruments, Palo Alto, CA). GC/MS conditions were similar to those previously described (McDonald, *et al.*, 1988).

RESULTS

The data of Fig. 1 show the time course and sensitivity to added fentanyl of our one step ELISA test. In the absence of added fentanyl the reaction rapidly runs to completion, with an apparent absorbance value of about 1.6 being attained between 10 and 15 minutes after starting the reaction. The addition of increasing concentrations of fentanyl acted to inhibit the reaction, with virtually complete inhibition of the reaction occurring after addition of 5 ng/ml of fentanyl.

Because of the progressive nature of the reaction, the time after addition of substrate at which the optical density is read is important. As shown in Fig. 2, reading the results before full color development in the blanks has occurred reduces the apparent sensitivity of the test. In our hands, therefore, the optimal time to read the test is about 12 minutes

Figure 1. Time course of absorbance change in the presence of increasing concentrations of fentanyl.



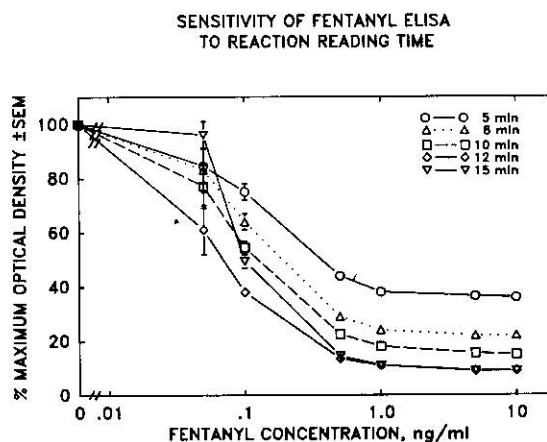
The symbols show the time course of the fentanyl ELISA reaction in the presence of the indicated concentrations of added fentanyl.

after the addition of substrate, when the apparent sensitivity of the test is greatest. Under these conditions the apparent I-50 for fentanyl in this test is about 100 pg/ml.

Because we wished to incorporate this test into a panel of pre-race tests, we tested the effect of the addition of equine plasma on this assay. As shown in Fig. 3 the addition of untreated plasma to the ELISA reduced the efficacy of the test. However, treatment of the plasma by the TCA method outlined in methods substantially improved the quality of the assay, and its sensitivity was similar to that obtained in the absence of plasma.

Urine also acted to inhibit the ELISA reaction, but the effect was much less marked. As shown in Fig. 1, in the absence of added urine, the color reaction had gone to completion by 10 minutes after starting the reaction.

Figure 2. Sensitivity of assay to reaction reading time.

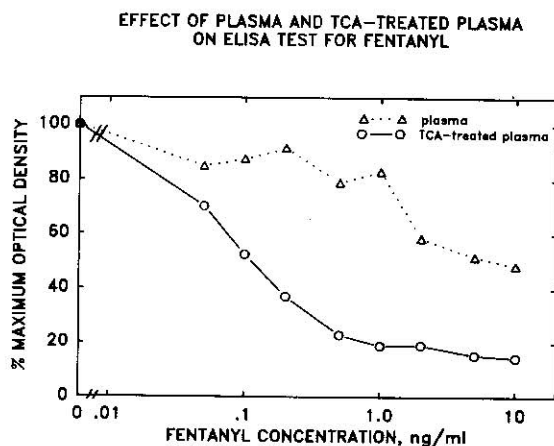


The symbols shows the family of fentanyl inhibition curves throughout the first fifteen minutes of the assay. Maximal sensitivity, with an I-50 of about 100 pg/ml for fentanyl, was found after 12 minutes incubation ($n = 2$).

The presence of horse urine, however, slowed the reaction such that the maximum color was not obtained in control samples for at least 20 minutes (Fig. 4). This effect is small, however, and does not interfere with the efficacy of the test.

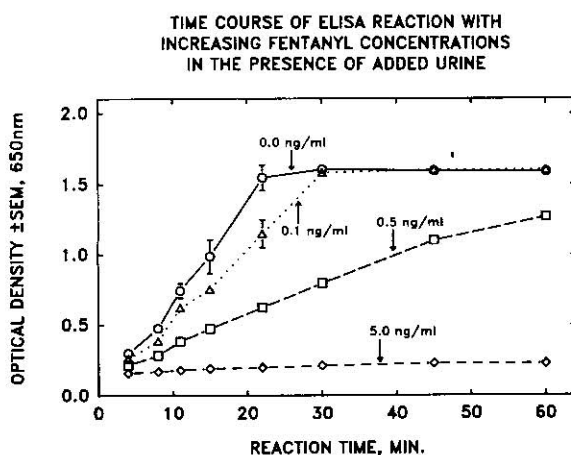
This ELISA test for fentanyl readily detected the presence of fentanyl in equine plasma and urine samples from dosed horses. When three horses were dosed with 500 μ g fentanyl/horse I.V., a sub-therapeutic dose, inhibitory activity in our ELISA test was observed in the plasma at five minutes after dosing and this inhibition remained substantial for at least sixty minutes (Fig. 5). Based on the short duration of the pharmacological

Figure 3. Effect of plasma and trichloroacetic acid treated plasma on ELISA test for fentanyl.



Fentanyl in the indicated concentrations was added to equine plasma and used directly in the ELISA assay or after treatment with trichloroacetic acid as outlined in methods. The open triangles (Δ - Δ) show inhibition of ELISA activity in the presence of plasma and the indicated concentrations of fentanyl, while the open circles (O-O) show inhibition after TCA treatment of the plasma.

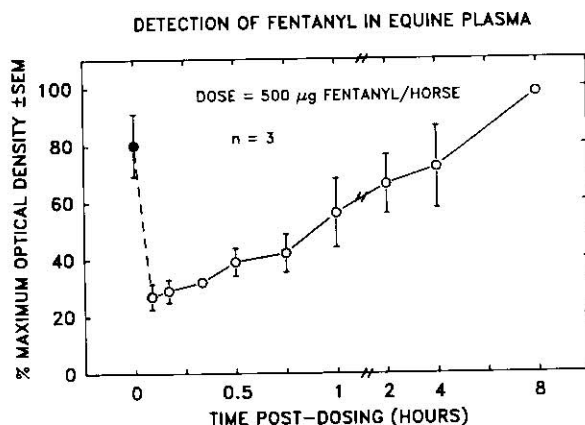
Figure 4. Time course of ELISA reaction in the presence of added urine.



The symbols show the time course of the ELISA reaction in the presence of the indicated concentrations of fentanyl, in the presence of 20 μ l of added horse urine ($n = 4$).

activity of fentanyl after IV administration of this drug this short period of detection of parent drug in the plasma is consistent with the pharmacokinetics of fentanyl in the horse.

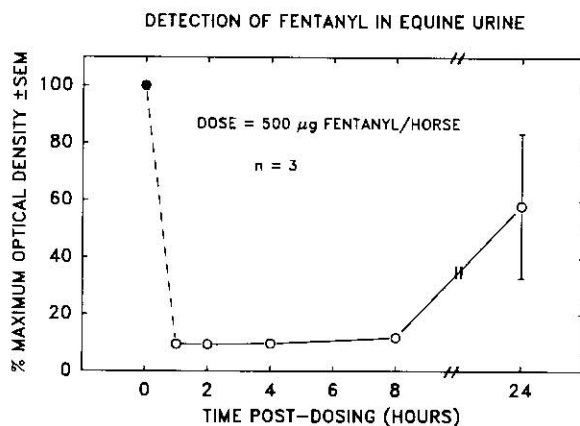
Figure 5. Detection of fentanyl in equine plasma by ELISA.



The figure shows ELISA activity in plasma from horses prior to (solid circle ●) and post dosing (open circles ○) with 500 μ g of fentanyl I.V. in three horses.

Fentanyl was also readily detected in urine from these horses. As shown in Fig. 6 virtually complete inhibition of the ELISA reaction was observed for the first 8 hours after dosing in all horses, and substantial inhibition was still observed at 24 hours after the last dose. The data suggest that this test should readily detect the administration of small doses of fentanyl to horses from either blood or urine samples.

Figure 6. Detection of fentanyl in equine urine by ELISA.

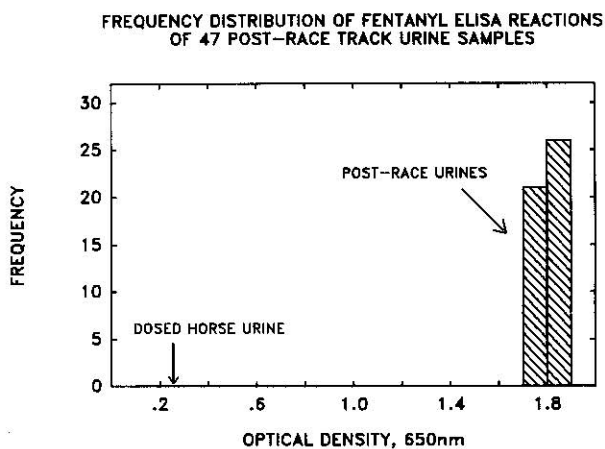


The figure shows ELISA activity in urine from horses prior to (solid circle ●) and post dosing (open circles ○ - ○) with 500 µg of fentanyl/horse I.V.

The data of Fig. 7 shows the ability of this test to distinguish between urine samples from fentanyl dosed horses and control horses. In this experiment, post race urine samples from 47 horses racing in Kentucky yielded absorbance readings of about 1.8, while that from dosed horse urines yielded values in the order of 0.2. 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.

We introduced this fentanyl ELISA into routine testing in combination with the ^{125}I RIA for carfentanil and sufentanil described previously (Weckman *et al.*, 1987; Yang *et al.* 1987). 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.

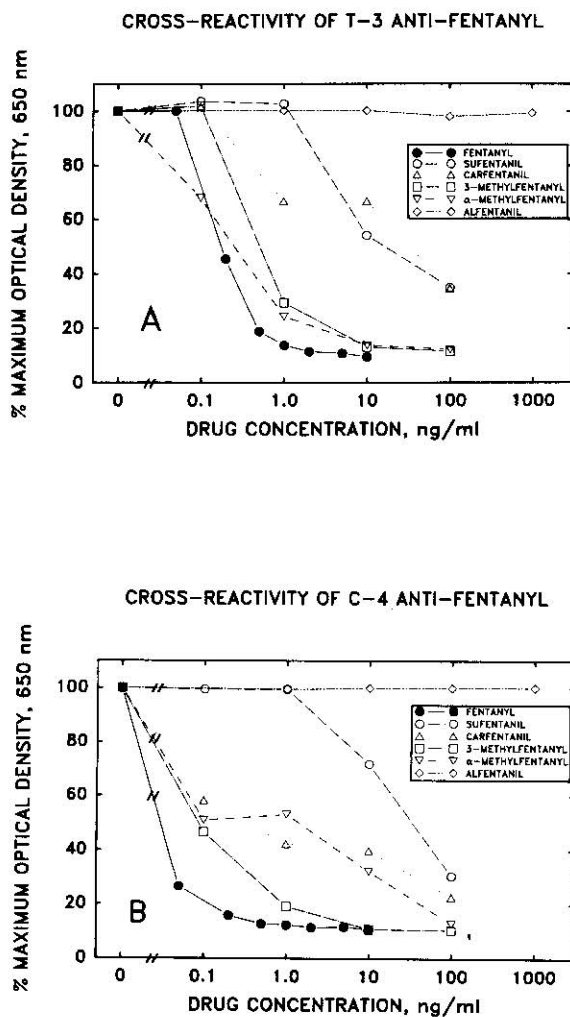
Figure 7. Frequency distribution of fentanyl ELISA reactions of post race track urine samples.



The hatched columns show a frequency distribution of 47 post race urine samples analyzed for fentanyl and read at 60 minutes. The arrow shows the optical density of a urine sample from a fentanyl dosed horse (500 μ g fentanyl/horse I.V.) sampling at two hours after dosing.

We also evaluated the ability of the ELISA test described in this paper to detect closely related congeners of fentanyl. As shown in Fig. 8, a test based on 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 test based on the T-3 antibody.

Figure 8. Cross reactivity of T-3 and C-4 based ELISA tests.



The symbols show the ability of the indicated congeners of fentanyl to inhibit ELISA reactions based on T-3 and C-4 antibodies to carboxyfentanyl. Optical densities were recorded after 20 min of color development.

Out of about 319 urine samples screened for sufentanil by both of these techniques over a two week period twenty were flagged for fentanyl based on either ELISA or RIA data. When these samples were submitted for Mass Spectral Analysis approximately ten of these samples confirmed positive for sufentanil, as shown in Table I.

TABLE I

Absorbance Values for Control, Drug Treated and Flagged Sufentanil Positives

<u>Sample #</u>	<u>Race Day</u>	<u>Absorbance</u>	<u>Status</u>
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 GG-MS, Sufentanil
6	11-6-87	.240	Confirmed GG-MS, Sufentanil
7	11-6-87	.345	Confirmed GG-MS, Sufentanil
8	11-8-87	.335	Confirmed GG-MS, Sufentanil
9	11-8-87	.485	Confirmed GG-MS, Sufentanil
10	11-8-87	.269	Confirmed GG-MS, Sufentanil
11	11-12-87	.339	Confirmed GG-MS, Sufentanil
12	11-12-87	.467	Confirmed GG-MS, Sufentanil
13	11-13-87	.302	Confirmed GG-MS, Sufentanil
14	11-14-87	.416	Confirmed GG-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.

DISCUSSION

Fentanyl is a typical narcotic agonist in the horse, in that it produces locomotor activity and analgesia. These actions are potentially useful in horses with subclinical lameness and could serve to improperly improve 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.

Chemical testing for fentanyl in urine samples of horses after racing is difficult because of the great potency of this drug. For example, fentanyl is about 100 times more potent than morphine. For this reason, the high-performance thin layer chromatographic methods generally used to detect narcotic analgesics in horse urine are unable to detect fentanyl. Detection of fentanyl in post race urine samples of horses has therefore been dependent on RIA screening.

The 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 an expensive, cumbersome and time consuming procedure, and unless the probability of a fentanyl being discovered is high, its cost relative to the yield of positives is high. The use of RIA screening for fentanyl has therefore tended to be restricted to circumstances where the probability of detecting fentanyl is high.

Screening for fentanyl would be much more cost competitive and effective if the time and expense of this test could be reduced. For example, an ELISA test such as is reported here can be completed in about 30 minutes and the cost of the reagents and instrumentation is minimal. Based on these considerations there was a considerable incentive to develop this ELISA test, as its availability enables us to test post-race urines for fentanyl and its congeners rapidly and inexpensively.

This technique is even more important if pre-race testing is the type of testing being utilized. The time constraints of 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 detect drugs 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. Pre-race testing technologies based on thin layer chromatography are ineffective with respect to medications as potent as morphine or fentanyl, and rapid and sensitive immunoassay tests are the only currently effective pre-race testing techniques.

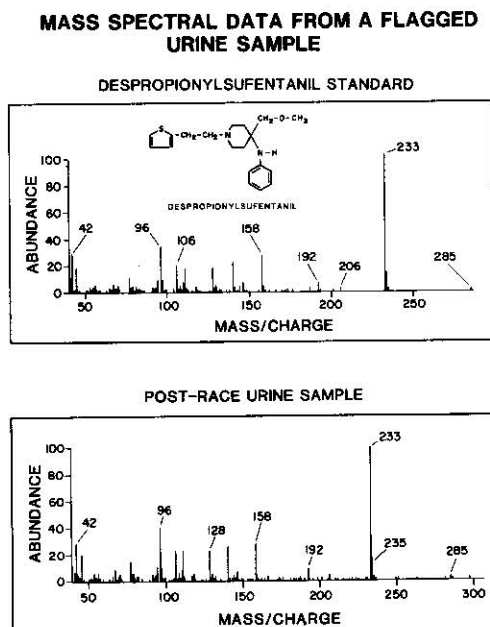
This fentanyl ELISA readily detected the administration of fentanyl in blood and urine from treated horses. As shown in Fig. 5 fentanyl was detected in equine blood within minutes after injection of this drug, and remained detectable for at least 1 hour. This short period for which fentanyl was detectable in equine blood is consistent with the pharmacokinetics of this drug in the horse and human 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 this drug.

We introduced this ELISA test for fentanyl into testing 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 to launch both this ELISA test and our previously reported ^{125}I RIA fentanyl-sufentanil-carfentanil test. Out of about 150 samples screened for sufentanil in a relatively short period, twenty were flagged for fentanyl or a fentanyl congener by one or other of these tests. As shown in Table I, all of these samples showed clearly on our ELISA test, as did urine samples from horses injected with carfentanil, sufentanil and fentanyl. Our ELISA test was therefore readily able to detect abuse of sufentanil under field conditions.

Of these twenty samples "flagged" for fentanyl, ten were confirmed positive for sufentanil by Mass Spectral Analysis. Of the remaining samples

either an insufficient volume of urine was available for Mass Spectral confirmation or the time frame for reporting a positive had passed. These data, therefore, show that the test flagged fentanyl containing samples with a 50% or greater probability that the flagged sample was a true fentanyl positive. Fig. 10 shows a typical Mass Spectral confirmation of one of these field positives developed as described in methods.

Figure 9. Sufentanil mass spectral data.



A urine sample flagged for sufentanil was subjected to acid hydrolysis and mass spectral analysis as described. The dispropionyl derivative of sufentanil was run as a standard to confirm the presence of sufentanil in the urine.

In summary, therefore, we have developed a one step ELISA test for fentanyl and its congeners in racing 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 -methyلفentanyl and 3-methyلفentanyl in the urine of horses dosed with clinically used doses of these drugs. When introduced into field testing this test flagged about twenty of 150 samples and about 50% of these samples were confirmed positive for sufentanil.

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