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IMMUNOASSAY DETECTION OF DRUGS IN RACING HORSES

II. DETECTION OF CARFENTANIL IN EQUINE URINE

BY RIA, PCFIA AND ELISA

J.-M. Yang, C.L. Tai, T.J. Weckman, H.-H. Tai, J.W. Blake,
and T. Tobin
The Maxwell H. Gluck Equine Research Center and the
Kentucky Equine Drug Research and Testing Programs
Department of Veterinary Science and School of Pharmacy
University of Kentucky
Lexington, Kentucky 40546-0076
and
John McDonald, Roy Gall, Paul Wiedenbach, Vivian D. Bass,
and B. DeLeon
Illinois Racing Board Laboratory
750 South State Street
EMCH Mendel Building
Elgin, Illinois 60120
and
Catherine Brockus, Diana Stobert, Siong Wie and Charles A. Prange
International Diagnostics Systems Corporation
2614 Niles Avenue
P.O. Box 799
St. Joseph, Michigan 49085

SUMMARY

Carfentanil is a high potency analog of fentanyl that acts as an analgesic and stimulant in racing horses. As the most potent of the fentanyl analogs its detection in racing horses is difficult, and no sensitive screening method for this drug has been reported.

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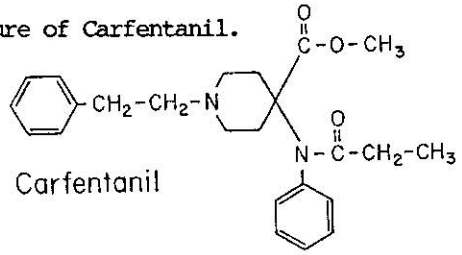
Correspondence should be addressed to Dr. Tobin

Because carfentanil cross-reacts well with a commercially available antibody to sufentanil, we tested the ability of an assay constructed from this anti-sufentanil antibody and an iodinated analog of fentanyl to detect carfentanil in horse urine. The system cross-reacted well with carfentanil and its analogs, with I-50's of about 450, 1000, and 4,000 pg/ml respectively for carfentanil, fentanyl and sufentanil. Using this methodology doses of carfentanil of as low as 25 µg/horse could be detected in horse urine for at least 4 hours after dosing. In addition a good correlation was obtained between the results from this test and that obtained by a Particle Concentration Fluorescence Immunoassay (PCFIA) previously reported and a one step ELISA method.

INTRODUCTION

Carfentanil (Fig 1) is an analog of fentanyl used for immobilization of large wild animals (DeVos, 1978; Haigh, et al., 1983; Jessup, et al., 1985) which has about ten times the potency of fentanyl in the horse (Weckman, et al., 1987b). As a μ agonist, it has the ability to produce both analgesic and locomotor responses in horses. While not readily available in North America, the possibility remains that this substance could be used to illicitly stimulate the performance of racing horses. There is, therefore, a need for a rapid and sensitive screening test for carfentanil in racing horses.

FIGURE 1. Structure of Carfentanil.



Carfentanil

A problem in developing a test for carfentanil is that it cross-reacts poorly with antibodies prepared to fentanyl. One approach to this problem has been to raise families of monoclonal antibodies to fentanyl and select out those that reacted with carfentanil. Taking this approach we developed an antibody that cross-reacted well with carfentanil, but its nonspecific reactivity was too high for it to be useful in routine post race testing (Weckman, et al., 1987b).

More recently we have investigated the use of our iodinated carboxyfentanyl analog (Weckman, et al., 1987b) and an antibody to sufentanil. As reported here, this combination allows detection of the presence of carfentanil and its analogs in urine samples from horses dosed with clinically effective levels of carfentanil. In addition, we compared the efficacy of this radioimmunoassay (RIA) method for carfentanil with a particle concentration fluoroimmunoassay (PCFIA) method previously reported (McDonald, et al., 1987) and also with a one step enzyme-linked immunosorbent-assay (ELISA) method under development by International Diagnostic Systems Corporation (St. Joseph, MI).

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.

Drugs

Carfentanil was obtained from a commercial supplier in an injectable solution (Wildnil®, 3 mg/ml,) and also as authentic carfentanil citrate standard from Wildlife Laboratories, Inc., Fort Collins, CO. Fentanyl and sufentanil standards were obtained from Janssen Life Sciences, Piscataway, NJ. All drug administrations were by rapid intravenous injection into the left jugular vein. Urine samples were collected by bladder catheterization, and were stored frozen until assayed. For PCFIA analysis all urine samples were filtered through Spin-X® microcentrifuge filters (Costar®, Cambridge, MA) prior to assay.

Radioimmunoassay Method

Concentrations of fentanyl, carfentanil and sufentanil were measured as carfentanil equivalents by a modified RIA employing ^{125}I-carboxyfentanyl-tyrosinemethylester conjugate as a labeled ligand and antibody from a commercial (Janssen) RIA kit (SUFEN-RIA-200)®.

^{125}I-carboxyfentanyl was prepared as previously described (Weckman, et al., 1987b). Carfentanil RIA standard curves were constructed using Janssen sufentanil antiserum, carfentanil standard and ^{125}I-carboxyfentanyl.

The standards, antiserum, and ^{125}I-carboxyfentanyl were diluted in RIA buffer (50 mM tris(hydroxymethyl)aminomethane, pH 7.5 containing 0.1% gelatin). Carfentanil standards were prepared from pure carfentanil citrate powder. The stock solution (1 mg carfentanil /ml) was further diluted to obtain standards of 40 to 4000 pg/ml. The Janssen lyophilized sufentanil antiserum was dissolved in 1 ml of

water and 0.7 µl was used for each assay tube. The RIA procedure was similar to that previously reported (Weckman, et al., 1987a). The incubation mixture contained 50 µl of carfentanil standard or sample, 100 µl of diluted antiserum, 100 µl of diluted ^{125}I-carboxy fentanyl (approximately 10,000 c.p.m.) and 150 µl of assay buffer. All samples were run in duplicate in 10 x 75 mm glass tubes. The tubes were allowed to incubate at room temperature for 90 minutes. At the end of incubation, 1 ml of water and 200 µl of gamma-globulin coated charcoal (1% bovine gamma-globulin, 3% charcoal in assay buffer) were added to the tube. The tubes were incubated for 5 minutes, then centrifuged for 5 minutes at 2000 x g at room temperature. The supernatants were pipetted into clean 10 x 75 mm glass tubes and counted on a gamma-counter (Beckman 5500 Gamma Counter, Beckman Instruments, Arlington Heights, IL). The data from the gamma counter was reduced on an IBM PC-XT (IBM Corp., Boca Raton, FL) using RIA-AID (Robert Maciel Assoc., Arlington, MA) software. The curve fitting was by four parameter logistic (Rodbard) statistics (Robdard and Lewald, 1970).

Non-Radiolabel Immunoassays

Our research groups are also developing rapid and sensitive non-radiolabel based immunoassays for drugs in horses. Among these assays are Pandex Laboratories (Mundelin, IL) based PCFIA and one step ELISA tests. Both of these tests use an antibody that is separate and distinct from the Janssen antibodies and are commercially available in International Diagnostic Systems Corporation kits.

Particle Concentration Fluoroimmunoassay

The basic functional unit in the Pandex® PCFIA is a 96 well plate with a filter base in each plate. To each well is added 20 µl of fentanyl-β-phycoerythrin (fentenyl-BPE) prepared as described by Wie and Hammock, 1982, 20 µl of anti-fentanyl antibody prepared by Tobin and co-workers, and 40 µl of blank, standard, or test sample. The system is allowed to equilibrate for about 10 minutes when a second antibody system is added. The second antibody consists of 20 µl of goat anti-rabbit antibody bound to latex beads. The system is allowed to react for another 10 minutes and then the fluid is drawn out of the system through the filter membrane. The reaction system is then washed with about 80 µl of phosphate buffer to resuspend the particles, and the system again drawn down with the vacuum. The filtration step has the effect of concentrating the latex beads 1000-fold, thereby increasing the sensitivity of the method. After the wash step, the fluorescence of the particles at 545 and 575 nm is measured. The mean response from control horse urines is usually about 25,000-30,000 arbitrary fluorescence units/well. In this test the presence of free drug or drug metabolites inhibits antibody-fentanyl-BPE binding, thereby reducing the amount of fluorescence observed in each inhibited well.

One Step ELISA Test

The one step ELISA test was performed as described by Voller, et al., 1976. Briefly, antifentanyl 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 and Hammock, 1982, to give rise

to a covalently linked fentanyl HRP complex. All reactions were run at room temperature.

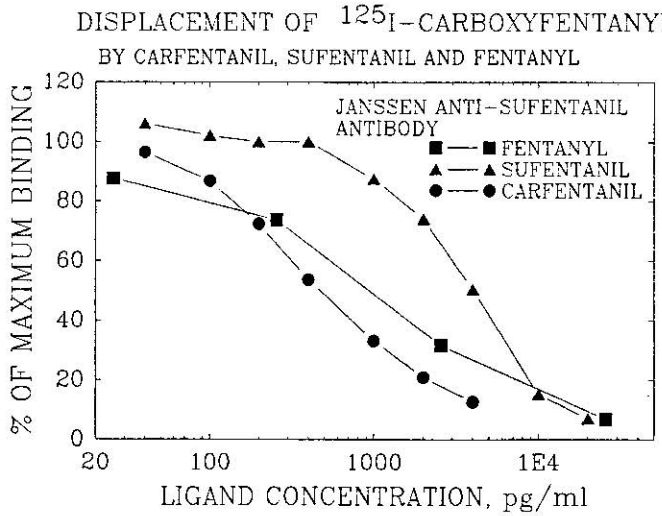
The assay was started by adding 20 μl of the standard, test or control samples to each well, along with 100 μl of the fentanyl-HRP solution. During this step, the presence of free drug or cross reacting metabolites competitively prevents the antibody from binding to the fentanyl-HRP conjugate. The degree of antibody-fentanyl HRP binding is 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. Tetramethylbenzamine substrate was then added to all wells and their absorbance read at 560 nm in a Dynatech microwell reader after 5 minutes.

RESULTS

Figure 2 shows the ability of carfentanil, fentanyl and sufentanil to displace ^{125}I carboxyfentanyl from the Janssen antibody to sufentanil. Carfentanil had the highest apparent affinity of any of the ligands tested, in that it displaced the iodinated fentanyl analog with an I-50 of about 450 pg/ml. Fentanyl was about 50% less effective than carfentanil in displacing the iodinated analog, with an apparent I-50 of about 1000 pg/ml. Sufentanil was substantially less potent than the others in that it required about 4000 pg/ml of sufentanil to displace our iodinated fentanyl analog from the system.

FIGURE 2. Displacement of ^{125}I fentanyl analog from sufentanil antibody by carfentanil, fentanyl and sufentanil.

The symbols show the displacement of ^{125}I-carboxyfentanyl from the Janssen anti-sufentanil antibody by carfentanil (●-●, I-50=450 pg/ml), fentanyl (■-■ I-50=1000 pg/ml), and sufentanil (▲-▲, I-50=4000 pg/ml).



These data suggest that it may be possible to detect the administration of carfentanil to horses by use of the sufentanil antibody and the iodinated analog of fentanyl. Figure 3 shows that the test will indeed detect administration of carfentanil to horses after administration of doses of between 25 and 100 µg /horse. In each case the background level in the urine of these horses was about 100 pg/ml of carfentanil equivalents. This level increased to between 400 and 550 pg/ml within the first two hours after administration of the drug, and then declined in a biphasic fashion. In at least one of the horses tested the level at 48 hours after testing was still significantly higher than in the control sample, suggesting the possibility of a relatively long clearance time for carfentanil in the horse.

Before one can estimate the probability that a sample contains carfentanil or any other fentanyl analog one needs to know the background levels of "carfentanil-fentanyl equivalents" likely to be found in post race horse urine samples. Figure 4 shows the background levels of carfentanil equivalents found in 51 post-race urine samples. The distribution is apparently exponential, and based on the highest-value observed any post race sample, readings above 200 pg/ml of carfentanil equivalents should be considered suspicious and further investigated.

FIGURE 3. Carfentanil equivalents in urine from horses treated with different doses of carfentanil.

The symbols show levels of carfentanil equivalents in urines of horses treated with 25 μg of carfentanil (●-●), 50 μg of carfentanil (▲-▲), and 100 μg of carfentanil (◆-◆).

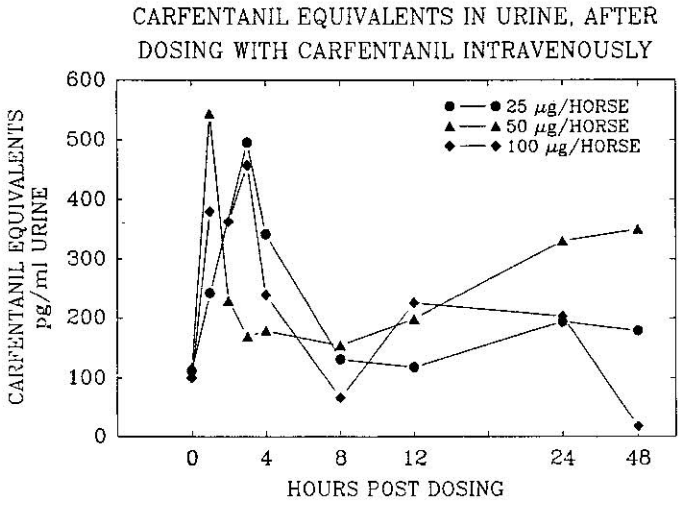
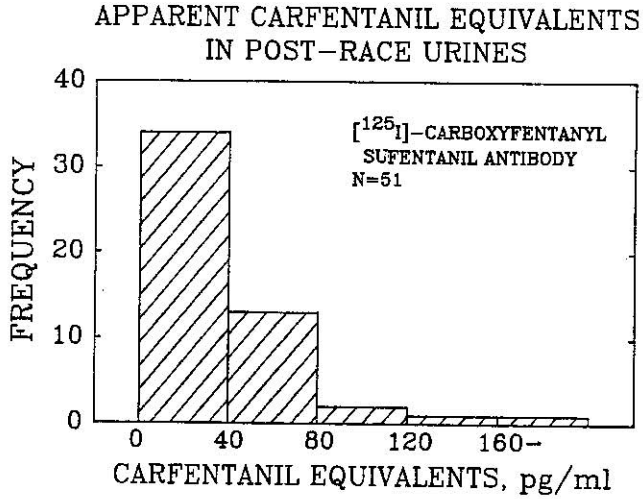


FIGURE 4. Apparent carfentanil equivalents in post race urines of horses.

Post race urines from 51 horses racing in Kentucky were analysed for levels of carfentanil equivalents and a frequency distribution developed.



In other work in progress our research groups are developing and evaluating the ability of other non-radiolabel immunoassays to detect the administration of fentanyl and its congeners to horses. Among these modes are PCFIA (McDonald, et al., 1987) and a one step ELISA to be described in detail in a forthcoming paper (Prange, et al., in draft). As shown in Table 1 we compared the ability of these tests to detect the administration of a series of fentanyls to horses under conditions approximating race track conditions. In addition, because of the possibility of interfering substances in track samples we included a number of post race samples in this system to evaluate the amount of horse to horse variability that we might expect to find in track samples. Table 1, therefore, presents the results of screening a series of fifty samples containing track samples and control and test samples from horses treated with different doses of fentanyl congeners.

TABLE I

COMPARISON OF ^{125}I FENTANYL RIA ASSAY, PCFIA ASSAY AND ONE STEP ELISA ASSAY ON AN UNKNOWN SERIES

		RADIOIMMUNOASSAY		PCFIA ASSAY			ONE-STEP ELISA ASSAY	
Sample Number	Treatment	Carfentanil Equivalents (pg/ml urine)	Status	Fluorescence	Inhibition (%)	Status	Absorbance	Status
1	--	<40	-	36,747	- 27	-	1.420	-
2	--	52	-	27,219	+ 6	-	.821	-
3	Carfent, 160 ug, 2-4 h	538	Pos	19,443	+ 33	Pos	.448	Pos
4	--	<40	-	52,698	- 83	-	1.194	-
5	Sufent, 100 ug, 0-1 h	3,960	Pos	22,344	+ 23	Pos	.309	Pos
6	Fent, 100 ug, 2-4 h	1,744	Pos	2,114	+ 93	Pos	.142	Pos
7	--	65	-	29,042	- 1	-	.548	-
8	--	<40	-	32,190	- 12	-	.588	-
9	--	140	-	27,549	+ 4	-	.526	-
10	--	44	-	27,472	+ 5	-	.458	-
11	--	46	-	23,619	- 17	-	.921	-
12	--	<40	-	28,338	+ 2	-	.559	-
13	--	<40	-	39,615	- 37	-	1.099	-
14	Carfent, 100 ug, 0-1 h	576	Pos	23,118	+ 20	Pos	.376	Pos
15	--	58	-	24,588	+ 15	-	.593	-
16	--	<40	-	55,401	- 92	-	1.634	-
17	--	<40	-	45,295	- 57	-	.982	-
18	--	<40	-	35,880	- 24	-	.877	-
19	Fent, 100 ug, 0-2 h	2,783	Pos	1,247	+ 96	Pos	.138	Pos
20	--	<40	-	13,677	+ 53	Pos*	.950	-
21	Carfent, 160 ug, 1-2 h	558	Pos	15,710	+ 46	Pos	.426	Pos
22	--	150	-	17,605	+ 39	Pos*	.494	-
23	--	<40	-	31,340	- 9	-	1.095	-
24	--	91	-	22,569	+ 22	Pos*	.513	-
25	--	<40	-	43,150	- 70	-	.908	-
26	--	110	-	20,774	+ 18	-	.449	Pos*
27	Carfent, 125 ug, 1-2 h	462	Pos	20,266	+ 20	Pos	.433	Pos
28	Sufent, 1000 ug, 0-1 h	>4,000	Pos	8,319	+ 28	Pos	.405	Pos
29	--	96	-	34,030	- 34	-	1.061	-
30	--	138	-	39,188	- 54	-	.630	-
31	--	115	-	41,805	- 65	-	.663	-
32	Carfent, 160 ug, 0-1 h	654	Pos	17,747	+ 30	Pos	.436	Pos

Sample Number	Treatment	RADIOIMMUNOASSAY Carfentanil Equivalents (pg/ml urine)	Status	PCFIA ASSAY Fluorescence	Inhibition (%)	Status	ONE-STEP ELISA ASSAY Absorbance	Status
33	Carfent, 125 ug, 0-1h	398	Pos	29,066	- 15	- ***	.562	- ***
34	Fent, 50 ug, 1-2 h	1,858	Pos	2,908	+ 89	Pos	.150	Pos
35	--	197	-	36,060	- 22	-	.550	-
36	--	104	-	42,683	- 68	-	.740	-
37	--	<40	-	48,460	- 91	-	.888	-
38	Carfent, 100 ug, 2-4 h	526	Pos	12,603	+ 50	Pos	.365	Pos
39	--	199	-	37,817	- 49	-	.450	Pos
40	--	166	-	16,326	+ 36	Pos	.498	-
41	--	93	-	34,239	- 35	-	.636	-
42	Carfent, 160 ug, 4-6 h	384	Pos	17,134	+ 32	Pos	.446	Pos
43	--	195	-	23,634	+ 7	-	.556	-
44	Carfent, 100 ug, 4-6 h	413	Pos	9,160	+ 64	Pos	.411	Pos
45	--	132	-	26,308	- 4	-	.538	-
46	--	130	-	49,629	- 96	-	.696	-
47	Carfent, 125 ug, 2-4 h	564	Pos	15,480	+ 39	Pos	.374	Pos
48	--	116	-	41,486	- 65	-	.533	-
49	--	193	-	33,180	- 31	-	.502	-
50	--	143	-	33,569	- 32	-	.547	-

A series of 50 unknown samples were provided by the Illinois Racing Board laboratory and shipped to Kentucky for analysis. The series consisted of samples from laboratory horses at Illinois dosed and sampled at the indicated times, control samples from these horses, and post race samples from horses racing in Illinois. Samples giving carfentanil values of above 200 pg/ml were designated positive in the RIA, samples showing greater than 20% inhibition were designated positive in the PCFIA and samples reading less than 0.450 were designated positive in the one-step ELISA test. The single asterick (*) refers to a false positive while the triple asterisk (***) refers to a false negative.

DISCUSSION

The RIA method reported here offers a useful quantitative method for detecting carfentanil administration to horses. The method utilizes the iodinated analog of fentanyl developed in our laboratory (Weckman, et al., 1987a), and an antibody to sufentanil raised by Janssen Life Sciences. When these two reagents were used to construct a RIA, good sensitivity to carfentanil was obtained, along with good detectability of both sufentanil and fentanyl (Weckman, et al., 1987b). The test also likely detects the methylfentanyl or designer analogs of fentanyl (Weckman, et al., 1987a). This listing includes the most readily available fentanyls, and thus members of this group of drugs most likely to be abused in horse racing.

Since carfentanil is the most potent of the fentanyl analogs it is the one most likely to be used in very small doses. Review of the dose-response data for carfentanil in the horse (Weckman, et al., 1987b) suggests that doses of less than 50 µg/horse are likely to be sub-therapeutic doses. Conversely, doses above this level are required for a pharmacological effect. However, as shown in Figure 3, doses of as little as 25 µg/horse are detectable in the urine of treated horses for up to eight hours after administration of carfentanil. These data suggest that this test should be readily able to detect the use of carfentanil in racing horses.

The utility of any test in a post race screening situation depends on the ability of the test to distinguish between background noise and a true carfentanil "positive". To enable this distinction to be made with confidence in the case of carfentanil we determined the background "noise" levels of "carfentanil-equivalents" in post race

urines from about 51 horses racing in Kentucky. As shown in Figure 4 the distribution of "carfentanil-equivalents" in post race urines follows an exponentially declining pattern, with very few samples yielding readings above 160 pg/ml of carfentanil equivalents. Based on this distribution of values we arbitrarily selected 200 pg/ml of carfentanil equivalents as the "cut-off" for a positive.

This arbitrary level for determination of a positive worked well in a series of blind tests. When a series of fifty unknown samples (Table I) prepared by one laboratory (Illinois Racing Board) was tested in our laboratory very good detactability of fentanyl administration was obtained. This experimental protocol included urines from horses dosed with sufentanil, carfentanil and fentanyl. All samples from horses dosed with fentanyl and its congeners were readily identified.

Review of the data of Table I shows that the fentanyl analogs other than carfentanil were easily and unambiguously detected in the RIA. In each case more than one thousand pg/ml of carfentanil equivalents were observed and the "positives" for these drugs were easily distinguished as samples yielding four figure readings. On the other hand, none of the carfentanil urines yielded four figure responses, although some were in the 500 pg/ml carfentanil equivalents range, and all were above 350 pg/ml. The RIA therefore, will readily detect carfentanil administration, although the level in a positive test may be only about twice that of the peak noise level to be expected. The test could therefore be substantially improved, presumably by the development of a specific anti-carfentanil antibody.

The PCFIA test data also detected all drug administrations, but in addition yielded some false positives. All the fentanyl administrations were readily detected, consistent with the fact that the antibody on which this assay is based was raised to fentanyl. Sufentanil was also readily detected in this test, although the percentages of inhibition observed were less than those seen after fentanyl administration. The inhibition of fluorescence seen after carfentanil administration were also smaller, but were still sufficient for screening purposes.

The responses of the PCFIA test to carfentanil were surprisingly strong. The dose of carfentanil was above 100 µg/horse and the time more than one hour post drug administration. The inhibition of fluorescence seen was generally greater than 35%, more than sufficient to draw attention to the sample. However, if the urine sample was taken at one hour or less after drug administration, then the test result was marginal or the administration was not detected (Sample 33). This is most likely because the concentration of carfentanil in the horse's bladder at the time of injection of the drug is zero, and this volume of drug free urine will dilute out the freshly formed post administration urine which urine contains carfentanil. It is clear, therefore that a horse dosed shortly before post with carfentanil and sampled within the first thirty minutes post-race would not be likely to produce a positive urine sample. On the other hand if the horse were held under pre-race testing conditions, where access to the horses is restricted for one hour or more pre-race, then the horse would be highly likely to produce a positive sample.

In the one-step ELISA assay the arbitrary cutoff for a positive was selected as the reading from a horse dosed with 100 µg of

carfentanil and sampled one hour after drug administration. This sample yielded an absorbance reading of 0.450 and any reading less than this was interpreted as a positive. Based on this cutoff, the only positive missed by the ELISA test was the one hour sample after 100 µg of carfentanil, which positive was also missed by the PCFIA. This, however, was the only false negative in the whole series of tests, and as pointed out earlier, would not occur in a situation where pre-race testing was in use.

In conclusion, all of the tests reported here easily detected fentanyl administration and the response to fentanyl in the RIA, PCFIA, and ELISA tests was such that the tests were virtually unambiguous. On the other hand, however, the ability of these tests to detect carfentanil and sufentanil was less sharply defined. This was because the inhibition values observed on the PCFIA and ELISA tests merged without a clear break into the low values for normal horses. Thus while these tests will readily detect fentanyl with a very high probability that each hit is a true hit, if the criterion for a positive is set high enough to allow low doses of sufentanil and carfentanil to be detected, a proportion of false positives are to be expected. As shown in the data of Table I, one false positive was seen in the ELISA test and three false positives in PCFIA test. However, this is not a problem that is inherent in this technology, but is primarily a problem with the reactivity of the antibody to fentanyl on which these tests are based. Incorporation of an antibody specific for carfentanil into these tests should allow very good detection and control of this agent in racing horses, with a minimum of interference from false positives.

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