A REVIEW OF THE PHARMACOLOGY AND DETECTION OF FENTANYL AND ITS CONGENERS IN THE HORSE

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Running Head: Fentanyl congeners in the horse

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SUMMARY

Fentanyl, a potent and short acting narcotic analgesic, has been used as an illegal medication in horse racing in North America since its introduction into human medicine. More recently carfentanil and sufentanil have been produced for the pharmaceutical market and the ∝-methylfentanyl and 3-methylfentanyl analogs of fentanyl have become available on the illicit market. Since these agents have the potential to be abused in horse racing we investigated their pharmacological actions and detection in horses. All appear to be typical μ-narcotic agonists, in that they stimulate locomotor activity at low doses. The locomotor responses of all the fentanyl congeners are qualitatively similar to that of fentanyl. Carfentanil is the most potent locomotor stimulant of any of the fentanyl congeners we examined. 3-Methylfentanyl and sufentanil are each about three times more potent than fentanyl, and ∝-methylfentanyl is approximately equivalent to fentanyl.

We studied the cross-reactivity of these fentanyl analogs with each of the fentanyl antibodies available to us. The methylated analogs of fentanyl cross-reacted well with a commercially available Janssen antibody. Cross-reactivety was also satisfactory to ∝-methylfentanyl when tested against two of five in house produced antibodies. None of these same five antibodies showed satisfactory cross-reactivity with 3-methylfentanyl. When the Janssen antibody was combined with an iodinated derivative of fentanyl developed in our laboratory, good detectability in terms of fentanyl equivalents was obtained. In blind tests this assay picked up fentanyl readily and sufentanil only at high doses. Carfentanil was not detected in the samples at the doses administered with the Janssen antibody.

We also determined the ability of this test to detect the use of fentanyl and its methylated analogs in pooled urine samples. The Janssen antibody-^{125}I fentanyl test allows ready detection of fentanyl and the methylated fentanyl analogs, even after pooling with up to 20 inactive samples. Rapid cost-effective testing of post race samples for these agents in post race urines is quite practical.

Pooling is potentially effective for detection of sufentanil and carfentanil but was not attempted as no satisfactory commercial antibody was available. A monoclonal fentanyl antibody was raised which exhibited good cross - reactivity to carfentanil. This antibody allows detection of carfentanil from dosed horses after administration of doses of 200 µg/horse.

INTRODUCTION

Fentanyl (N-phenyl-N-[1-(2-phenethyl)-4-piperidinyl]propanamide) is a synthetic opioid derivative of meperidine and a narcotic analgesic with about 80 to 150 times the potency of morphine.1,2 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 µ-opioid agonist.3

The pharmacological actions of the fentanyls in horses are different from their actions in man. In horses, fentanyls produce marked locomotor stimulation, along with their analgesic effects. The sum of these actions, in that they act to alleviate lameness and stimulate running, is quite likely to be useful in a racing horse. For these reasons, the fentanyls have been widely used in racing horses, despite the fact that their use is uniformly illegal.4

The development of radioimmunoassay (RIA) technology, which allows screening of large numbers of post race samples for fentanyl, has led to

control of the use of fentanyl in racing horses. More recently, however, other congeners of fentanyl have become available, and the ability of RIA to control use of these analogs is unclear.

Among the congeners that have become available is recent years are carfentanil, sufentanil and the so-called "designer" fentanyls. These "designer" agents include ∝-methylfentanyl [1-(1-methyl-2-phenethyl)-4-(N-propionylanilino) piperidine] and 3-methylfentanyl [3-methyl-1-(2-phenyethyl)-4-(N-propionylanilino)piperidine] which are produced illicitly. As fentanyl analogs, these agents have the potential to be used illegally in racing horses. However, no information is available as to the pharmacological actions of these agents in racing horses, nor to the ability of currently used systems to detect abuse of these agents.

We have obtained dose forms of carfentanil and sufentanil and also research amounts of two of these so-called "designer" fentanyls, ∝-methylfentanyl and 3-methylfentanyl (Fig 1). We have tested these agents in our behavioral model to determine their pharmacological potency in the horse and also their detectability in our immunoassay system for fentanyl. This report shows that most of these agents are readily detectable with existing or modified RIA test, suggesting that control of all of these fentanyl congeners in racing is readily obtainable.

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 the experimental session for acclimatization.

DRUGS

Carfentanil was obtained from a commercial supplier in an injectable solution (Wildnil®, 3 mg/ml, Wildlife Laboratories, Inc., Fort Collins, CO). Sufentanil was also obtained in an injectable form from a commercial source (Sufenta®, 50 ug/ml, Janssen Pharmaceutica Inc., Piscataway, NJ). 3-Methylfentanyl was dissolved in 10 ml of sterile physiological saline (pH 7.0) . ∝-Methylfentanyl was dissolved by adding the crystaline form to 10 ml of sterile physiological saline (pH 7.0) and heating to 58 °C with stirring. Drug administration was by rapid injection into the left jugular vein. These methylfentanyl analogs were generously supplied by Dr. R. L. Hawks of the National Institute of Drug Abuse, Rockville, MD.

LOCOMOTOR STUDIES

For this measurement horses were placed in box stalls which are enclosed on all sides. A window made of one-way mirrored glass located in each door permitted observers to record behavior without detection by the animal. Locomotor behavior was quantified by counting the number of footsteps taken per 2 min period. A foot step was scored each time the right foreleg was lifted off the ground and returned along with a positional change.5 Locomotor assays with carfentanil were based on doses of 0.08, 0.14, 0.20, 0.30 and 0.60 μg/kg. Sufentanil was administered at doses of 0.25, 0.50, 1.00, 1.33 μg/kg. Preliminary studies with 3-methylfentanyl indicated that intravenous doses at 4 μg/kg were in excess of tolerable limits. This dose produced severe excitement, tachycardia and tachypnea in one horse. This dose was antagonized with 8 mg naloxone i.m. (Narcan®, DuPont Pharmaceuticals Inc., Manati, Puerto Rico). Subsequent doses of 0.4, 0.7, and 1.0 μg/kg 3-methylfentanyl were well tolerated in all subjects. These subsequent three dose levels formed the basis of the 3-methylfentanyl

locomotor assay. ∝-Methylfentanyl was administered at doses of 1.0, 2.0, 4.0, 8.0, and 13.0 μg/kg and was well tolerated by all subjects.

Locomotor activity was quantified for 16 min prior to each injection to establish the pretreatment baseline. Footstep frequency was then recorded every 2 min for a minimum of 60 min post injection.

RADIOIMMUNOASSAY METHOD FOR METHYLFENTANYLS

∝-Methylfentanyl and 3-methylfentanyl urine concentrations were measured as fentanyl equivalents by a fentanyl RIA employing ^{125}I-carboxyfentanyl tyrosine methlyester conjugate as a labeled ligand and based on a modified commercial RIA kit (FEN-RIA-200, Janssen Life Science Products, Fleurus-Belgium).6 The ^{125}I-labeled fentanyl derivative was prepared similarly to a method previously described.6,7 Fentanyl RIA standard curves were constructed using Janssen fentanyl antiserum, Janssen fentanyl standard and ^{125}I-labeled fentanyl derivative. ^{125}I-labeled fentanyl derivative (approximately 10,000 cpm in 100μl assay buffer) was pipetted into 10X75 mm glass culture tubes. Assay buffer was 50mM tris(hydroxymethyl)aminomethane HCl, pH 7.5, containing 0.1% gelatin.

The Janssen stock fentanyl solution (40 ng/ml) was serially diluted with 30% methanol/water to obtain standards of from 0.5 to 64 pg/50 μl; 50 μl of these standards were added to the standard tubes. Urine (50 μl) was assayed without extraction. The Janssen lyophilized fentanyl antiserum was dissolved in 10 ml assay buffer and then diluted to give approximately 2.5 μl/tube (the dilution of antiserum was adjusted to give about 30% binding). The diluted antiserum (100 μl) was added to all but the total activity and non-specific binding tubes. The tubes were allowed to incubate at room temperature for 1 hr. After incubation 1 ml of water was added to each tube. Gamma-globulin coated charcoal (1% gamma-globulin, 3% charcoal in assay

buffer, 200µl) was pipetted into Luckham 12 mm plastic stoppers (Luckham LP3S, Luckham LTD., West Sussex, England, UK) . The Luckham caps were carefully placed on all but the total activity tubes. The tubes were inverted several times, allowed to stand 5 min, then centrifuged 5 min, 2000Xg 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 Inc., Arlington Heights, IL) with data transporter (Data Transporter DT064, Beckman Instruments, Inc., Arligton Heights, IL) or a liquid scintillation counter (Beckman LS 3801 Scintillation Counter, Beckman Instruments Inc.) using 10ml counting cocktail (3a70B cocktail, Research Products International, Mt. Prospect, IL).

The data from the gamma counter was reduced on an IBM PC-XT® (IBM Corp., Boca Raton, FL) using RIA-AID® software (RIA-AID, Robert Marciel Associates, Arlington, MA). The curve fitting was by four parameter logistic (Rodbard) statistics.8 The data from the scintillation counter was reduced by Data-Capture® software (Data-Capture, Beckman Instruments Inc., Lab Automation Operations, Irvine, CA) using Logit-log transformation as previously described.9

For the urine samples, the fentanyl equivalent level for each sample was calculated from the standard curve for each run.

DEVELOPMENT OF RADIOIMMUNOASSAY FOR CARFENTANIL

A specific carfentanil screening method was developed by producing a monoclonal fentanyl antibody with a high level of cross-reactivity to carfentanil and its metabolites. A carboxy-fentanyl-BSA conjugate was prepared and injected subcutaneously into a group of Balb-C mice. Each of five mice were immunized three times at monthly intervals and test bled 7

days after the last immunization. All mice had detectable antibody titers as judged by the binding assay with ^{125}I-fentanyl. Two mice were sacrificed 3 days after a final subcutaneous injection. Spleen cells from these mice were fused with the non-secretor murine myeloma line NS-1 using polyethyleneglycol (m. wt. 1540) (Koch-Light,Ltd., Haverhill, Suffolk, England) as fusogen. The hybridomas were screened for antibody producing ability by use of an antibody-isotope binding test.

Cross-reactivity studies with fentanyl, sufentanil, carfentanil and alfentanil were subsequently performed. Antibody producing hybridomas with desirable properties were selected and cloned twice by limiting dilution to insure a homogeneous antibody producing cell population. Desired monoclonal cell lines were expanded for antibody production. Of the 21 cell lines established, "AG9" produced the most cross-reactivity with carfentanil (24%). This antibody showed ability to detect a 200 µg/horse dose of carfentanil for about 8 hours post drug administration (FIG 2). High backgrounds proved to be the only constraint when using this cell line. The RIA method used with this antibody was identical to the one described above for the analysis of the methylfentanyls, the only modification being the substitution of the "AG9" antibody for the Janssen antiserum when called for in the assay scheme.

DOSING AND SAMPLING FOR PHARMACOKINETIC STUDIES

Urine samples from horses dosed with carfentanil and sufentanil were analyzed for fentanyl equivalents using the ^{125}I method as described above. One horse each received a dose of 0.08, 0.14, 0.20, 0.30, and 0.60 µg/kg carfentanil. One horse each received a dose of 0.02 µg/kg and 0.08 µg/kg of sufentanil for RIA testing. Samples were collected predose and at 1, 2, 4, 6, 8, 12, 24, 36 and 48 hours post dose. Urine samples from horses dosed

with 3-methylfentanyl and ∝-methylfentanyl were also analyzed for fentanyl equivalents using the ^{125}I method described above. Two horses each were dosed with 3-methylfentanyl at 0.4, 0.7, and 1.0 μg/kg. One horse received 3-methylfentanyl at 4.0 μg/kg. Two horses each were also dosed with ∝-methylfentanyl at 1.0, 2.0, 4.0, 8.0, and 13.0 μg/kg. Urine samples were taken pre-dose and at 1, 2, 4, 6, 24, 36 and 48 hrs and every 24 hrs thereafter up to 110 hrs. All the urine samples were collected by bladder catheterization and were analyzed directly without extractions. Dilution of samples, when necessary, was with each animal's respective control urine.

POOLED ASSAY METHOD

In the Kentucky Equine Drug Testing Program, samples from racing horses are received daily and about a 2 ml aliquot of each is pipetted off and stored frozen in an individually labeled tube. At the end of each week or upon accumulation of sufficient samples the racing samples are thawed and pooled. The pooling process involves pipetting 100 μl of each respective post-race sample into a common tube. This combined sample is a total of all horses racing at a particular track in a single day or a maximum of 10-20 individual samples. The assay of the pooled sample is then the same as the routine ^{125}I fentanyl RIA assay and 50 μl of the pooled sample is added when called for in the assay scheme.

RESULTS

The locomotor effects of carfentanil and sufentanil are shown in Fig 3 panels A and B respectively. The effects of 3-methylfentanyl and ∝-methylfentanyl on equine locomotor response are shown in Fig 4 panels A and B respectively. 3-Methylfentanyl produced a significant locomotor response, peaking at about 15 to 20 min after administration of the drug and then declining thereafter. The duration of the locomotor response to

3-methylfentanyl was somewhat longer than that to fentanyl in that substantial locomotor activity was still observed at 60 min. With fentanyl, the locomotor response is always back to baseline by 60 min post dosing, unless fentanyl was combined with another drug.4 Beyond this, the peak response observed, at about 75 steps/two minutes, was approximately the same as that observed with fentanyl.

The peak dose-response pattern to ∝-methylfentanyl was virtually indistinguishable from that to fentanyl (Fig 5). At doses of less than 1.0mg/454kg horse, little or no locomotor response was seen, when the dose was increased the locomotor response increased sharply, and in parallel with that of fentanyl. While it appears that the maximal locomotor response to ∝-methlyfentanyl is greater than that for fentanyl, the small number of horses used in these experiments suggests that this possibility should be further explored. The duration of the locomotor response to ∝-methylfentanyl was broadly similar to that of fentanyl, in that the bulk of the locomotor response was over within 40 minutes of drug administration (Fig 4B).

The response to carfentanil was significantly greater than that to either fentanyl or either of the methylfentanyls (Fig 5). Locomotor response approached that produced by etorphine4, peaking at about 10-12 min post dosing and producing about 135 steps/2 min period. The duration of stimulation produced at the high dose of carfentanil lasted about twice that of fentanyl or about 120 min (Fig 3A).

Sufentanil, in comparison, produced locomotor stimulation roughly equivalent to that seen with 3-methylfentanyl. The peak effect occurred within 10 min at about 100 steps/2 min. and the horses behavior returned to baseline activity by about 90 min post dose (Fig 3B).

Comparison of the dose response curves for the locomotor response to these four congeners of fentanyl showed that 3-methylfentanyl was between three and ten times more potent than fentanyl and α-methylfentanyl, which were about equipotent. Carfentanil has proven to be the most potent of any of the fentanyl congeners we have examined to date, being about 10-100 times more potent than fentanyl. Sufentanil is about 3-10 times more potent than fentanyl and in this locomotor assay about equipotent to 3-methylfentanyl.

Since these congeners of fentanyl produce a clearcut locomotor response in the horse, and likely also produce analgesia, screening tests for these agents in post-race urines are required. We therefore determined the cross-reactivity of these analogs of fentanyl with each of the antibodies available to us. As shown in Table 1, the Janssen antibody was the most effective of these antibodies, cross-reacting well with both α-methyl and 3-methylfentanyl analogs. Antibody made available to us by Dr. Larry Soma of the University of Pennsylvania and several antibodies raised in our laboratory to a carboxyfentanyl conjugate all reacted well with fentanyl but poorly with most other congeners of fentanyl. For this reason, the most satisfactory antibody for screening for fentanyl and its methylated analogs at this time appears to be the Janssen antibody.

Lack of a readily available commercial antibody to carfentanil prompted development of a useful screening method through the production of a monoclonal fentanyl antibody with good carfentanil cross-reactivity. Detection of fentanyl equivalents from horses dosed with carfentanil with 2 in-house generated antibodies and the Janssen commercially available fentanyl antibody is shown in Fig 2.

The effectiveness of a Janssen commercial antibody and an antibody produced in our laboratory ("AG9") in a blind screening test with blank and

dosed horse urines is shown in Table 2. While the Janssen antibody performed adequately in detecting a 100 μg dose of fentanyl for 6 hours and sufentanil doses of 50 μg and 1 mg for at least 6 hours it did not react with carfentanil. Only the "AG9" antibody was effective in revealing samples from horses dosed with carfentanil. Here doses of 125 μg carfentanil were detectable for up to four hours after a single i.v. administration.

Similarly, when a series of serum samples submitted from another lab in a blind sequence of blanks and dosed order were assayed, the Janssen antibody was again found to be effective in detecting the fentanyl dosed horses. Serums from horses given doses of fentanyl as low as 100 μg/horse could have fentanyl equivalents detected for at least 2 hours post injection. Detectability of fentanyl equivalents from horses dosed with 50 μg fentanyl was limited to the first 15 minutes post dose when assaying serum (Table 3). Background fentanyl equivalent values were high for all serum samples and a mean background value, derived from a set of blank serums was subtracted from all fentanyl equivalent evaluations. Carfentanil and sufentanil were, however, not detected in this fluid with the commercial Janssen fentanyl antibody. Recovery of fentanyl equivalents from serum after the 50 μg and 100 μg i.v. doses of fentanyl is displayed in Fig 6. Plotted as time vs log fentanyl equivalents the data from our RIA work yields a close to linear recovery relationship.

Use of the Janssen antibody, along with the iodinated analog of fentanyl developed in this laboratory, allowed very effective screening for both methyl analogs of fentanyl. At the threshold dose of α-methylfentanyl producing a pharmacological effect in these experiments (2.0 μg/kg) the level of fentanyl equivalents in urine was about 2 ng/ml at the peak urinary concentration, which occurred at about two hours after dosing. By five to

six hours post-dosing, when the pharmacological effect of the drug was likely to be minimal, there was still more than 1000 pg/ml of fentanyl equivalents in the sample (Fig 7). These levels are more than sufficient to allow easy detection of this analog in an undiluted sample for up to 48 hours after administration of the drug.

Similar results were obtained with 3-methylfentanyl. Administration of doses of this drug of 160 µg or more of 3-methylfentanyl/horse resulted in detection of fentanyl equivalents of this drug in urine for up to 24 hours (Fig 8). If pooled samples were used, the test would still detect administration of 3-methylfentanyl within 4 hours of post time at clinically effective doses.

The sensitivity of the fentanyl test suggests that it would be possible to detect residues of this drug and its methylated analogs in pooled samples. Urine samples from horses dosed with fentanyl, 3-methylfentanyl and/or α-methylfentanyl, and each containing approximately 2 ng/ml drug as fentanyl equivalents, were included in a series of track horse urine samples from the Kentucky Equine Drug Testing Laboratory and were pooled for routine fentanyl RIA screening. Table 4 shows an example of a routine screening assay with a fentanyl dosed horse urine sample included in one of the sample pools. The pool from track "C" of 11/5/86, which contained sixteen urine samples, had a fentanyl equivalent concentration of more than 400 pg/ml, well above the other pools. The data reduction software was set to flag "positive" any sample with a value greater than 50 pg/ml: any concentration less than this was considered background. Therefore, the track "C" 11/5/86 pool was flagged and the individual samples from this pool were assayed by RIA. As shown in Table 4 sample #4 was found to contain about 1800 pg/ml fentanyl equivalents. Any concentration value greater than 1280 pg/ml was

above the high standard in the assay and was therefore an extrapolation and a rough approximation of the concentration. From these data sample #4 from track "C" 11/5/86 was determined to actually be the fentanyl dosed horse urine.

Table 5 shows the results of a routine fentanyl screening when urines from a horse dosed with 3-methylfentanyl and one dosed with ∝-methylfentanyl were included in a pool of urines from racing horses. It can be seen that the pool from track "A" of 12/19/86 had a high concentration of fentanyl equivalents. This pool was flagged and the individual samples were tested. Samples #22 and #23 were found to contain high levels of fentanyl equivalents and were determined to be the dosed horse urine samples.

A summary of the detection of fentanyl and methylfentanyl in routine testing pooled urine samples is shown in Table 6. Pool sizes for these assays varied from 7 to 20 samples/pool. The pooled sample mean background fentanyl equivalents were from less than 2 pg/ml to about 15 pg/ml. The pooled samples flagged "positive" had fentanyl equivalents from 40 to 150 times the background levels. The individual sample mean background fentanyl equivalents were about the same as the mean background values for the pooled samples. The individual samples flagged "positive" had fentanyl equivalents levels from about 1.6 ng/ml to 2.5 ng/ml, in good agreement with the added fentanyl equivalent concentrations of 2 ng/ml.

DISCUSSION

Chemical testing for fentanyl and its congeners in the urine of horses is difficult because of the potency of these drugs. For example, fentanyl is about 100 times more potent than morphine and most of the other narcotic analgesics that are available. This means that the standard high performance thin layer chromatography (HPTLC) tests that are used in drug screening are of limited use for the control of high potency drugs such as fentanyl. To

further compound the problem, numerous analogs of fentanyl are available, both licit and illicit . For good control of these agents the only technology available today that offers good screening is RIA.

There are however, a number of problems with RIA as a screening mechanism in post race testing of horses. The first is that the tests tend to be limited to single drugs or closely related groups of drugs. The second is that compared with HPTLC, the technology tends to be expensive and labor intensive. While thin layer chromatography screens can detect a wide range of drugs, each RIA test can only detect a limited range of drugs, and is relatively expensive in terms of technician time and instrumentation. In addition, if reagents have to be purchased commercially, these costs can be significant. RIA therefore, as currently configured, is hampered by cost and technical limitations as a routine screening test.

One of the more useful RIA screening tests in equine drug testing has been the Janssen RIA for fentanyl. Use of this test, in conjunction with good mass spectrometry confirmation methods has allowed control of the use of fentanyl in racing horses. More recently, we have developed an iodinated derivative of fentanyl that has allowed us to improve the efficacy and sensitivity of this test, while at the same time reducing the cost to the user of this test substantially.

The increased sensitivity of the fentanyl test prompted us to investigate the ability of this test to detect other fentanyls in post race urines, and also to assess the ability of this test to detect these agents in pooled urine samples. It appeared likely that pooling and freezing of up to ten or more day's of urine samples, and then at some later date, testing this large number of pooled samples for fentanyl was possible. Pooling of samples in this way would allow a single day's work to screen perhaps a

weeks worth of samples, and greatly reduce the costs of testing while having little effect on its efficacy. To use a pooled test in this way one needs to know the concentrations of drug or drug metabolite likely to be found in post race urine after administration of doses likely to affect equine performance. We determined the doses of carfentanil, sufentanil and the methylated analogs of fentanyl likely to affect the performance of a horse. Fentanyl is available on the illicit market as at least two methylated analogs, ∝-methylfentanyl and 3-methylfentanyl. Carfentanil is available commercially as is sufentanil. The pharmacological potency and pharmacological actions of these agents were unknown in the horse.

When we tested these congeners, all agents produced significant locomotor responses, suggesting that they are typical μ-agonist narcotic analgesics in the horse. Of the four, carfentanil is the most potent, 3-methylfentanyl and sufentanil being about three and ten times more potent than fentanyl are about equivalent. ∝-Methylfentanyl appears to be very similar in both potency and duration of action to fentanyl, while 3-methylfentanyl appears to have a slightly longer duration of action than fentanyl. The strong locomotor response to all these agents, and the virtual certainty of a good analgesic response means that the abuse potential of carfentanil, sufentanil and the methylated analogs of fentanyl is likely to be similar to that of fentanyl.

To maximize the likelihood of detecting agents other than fentanyl in our screening test, we determined the cross-reactivity of all the fentanyl antibodies available to us with the different congeners of fentanyl. As shown in Table 1, the Janssen antibody was clearly superior in terms of cross-reactivity. It reacted well with ∝-methylfentanyl, and 3-methylfentanyl, and to a limited extent with carfentanil and sufentanil.

None of the other antibodies available to us showed this broad cross-reactivity, although some of the antibodies raised in our laboratory showed good cross-reactivity with α-methylfentanyl. The "AG9" antibody was satisfactory in detecting carfentanil.

Consistent with these results, both α-methylfentanyl and 3-methylfentanyl were readily detectable in horse urine using our modified fentanyl RIA. As shown in Fig 8, 3-methylfentanyl was readily detected in horse urine at one hour after dosing, and remained detectable (i.e. at more than 50 pg/ml fentanyl equivalents) for about 48 hours after the smallest dose used. These data suggest that 3-methylfentanyl would be readily detected by this modified fentanyl test in post race urine samples.

Broadly similar data were obtained with α-methylfentanyl which was also readily detectable in horse urine for at least 48 hours after dosing (Fig 7). These data therefore suggest that use of the modified fentanyl test should allow good detectability of these agents in post race urine after clinically effective doses of these agents.

The RIA methodology used in the present study markedly increases the usefulness of the fentanyl RIA for routine screening. It increases the sensitivity for the fentanyls, while decreasing the costs. Because of the increased sensitivity, concentrations of the antibody used in the system can be reduced 15-fold thereby extending the antibody. The sensitivity of the assay is increased up to 100-fold. This increased sensitivity can be used to detect smaller concentrations of fentanyls in individual urine samples or, conversely, can allow pooling of many urine samples, and the simultaneous screening of larger numbers of post-race samples. Using this approach, a pool of urine samples (10-20) collected from horses during a day of racing can be screened for fentanyls, and, if necessary, the source of

any detected fentanyls can be pinpointed by performing a second screening of each horse's urine from the flagged pool.

Results from a previous study indicate that the smallest dose of fentanyl likely to induce an effect on a racing horse is about 100 µg/horse, and that any clinical dose probably induces a pharmacological effect within 4 hours of administration.9 Using the modified RIA, we can detect fentanyl in horses given at 1 µg fentanyl/horse and could detect this dose for at least 24 hours after administration. Likewise, α-methylfentanyl can be detected for at least 48 hours after administration of a dose of 1 µg/kg using the modified RIA assay. 3-Methylfentanyl is also detectable for at least 48 hours after a dose of 0.4 µg/kg with the RIA assay described above. This large reserve of sensitivity and the good cross-reactivity of the available commercial antibody to the α-methyl and 3-methylfentanyl analogs of fentanyl indicates that the illicit use of these fentanyl analogs in racing horses is readily detectable in individual as well as pooled post race urine samples.

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TABLE 1.

Specificity of different fentanyl antibodies in detection of fentanyl and its congeners from equine urine samples.

Cross-reactivity
Value (pg) obtained at 50% max binding (% cross reactivity vs fentanyl)

Compound / Antibody	Fentanyl*	Sufentanil	Alfentanil	Carfentenil	3-methyl-fentanyl	-methyl-fentanyl
Janssen's	8.05	560 (1%)	>10,000 (<0.1%)	290.0 (3.7%)	80.0 (12.5%)	75.0 (14.3%)
Soma's	10.4	>10,000 (<0.1%)	>10,000 (<0.1%)	12,800 (<0.1%)	455.0 (1.7%)	296.0 (2.6%)
T2	8.0	>10,000 (<0.1%)	>10,000 (<0.1%)	>10,000 (<0.1%)	156.0 (6.3%)	660.0 (1.5%)
T3	8.7	>10,000 (<0.1%)	>10,000 (<0.1%)	>10,000 (<0.1%)	430.0 (2.3%)	66.0 (14.7%)
T4	9.35	6,000 (0.2%)	>10,000 (<0.1%)	5,800.0 (0.2%)	750.0 (1.2%)	180.0 (5%)
T6	10.9	>10,000 (<0.1%)	>10,000 (<0.1%)	1,450.0 (1%)	375.0 (3.7%)	87.0 (16%)
T7	11.9	>10,000 (<0.1%)	>10,000 (<0.1%)	850.0 (1.2%)	300 (3.3%)	100.0 (3.8%)

* average values of 2 assays.

The column under fentanyl shows the number of picograms of fentanyl required to inhibit the binding of the iodinated analog of fentanyl by 50%. The values in the other columns show the number of picograms of fentanyl analog added and in brackets the estimated percentage cross-reactivity of the antibody with the analog.

TABLE 2.

Comparative effectiveness of Janssen fentanyl antibody and a monoclonal fentanyl antibody (AG9) in detecting fentanyl, carfentanil or sufentanil from a series of blind samples submitted to us by the Illinois Racing Board. "Call" column shows our interpretation of RIA results. "Drug" column shows the actual dose administered as revealed by a key furnished post screening by the Illinois Racing Board.

Sample #	Call (TT)	Drug	Dose	Time	Fentanyl Equivalents pg/ml	
					Janssen Antibody	AG9 Antibody
1	-	Negative Control	--	--	< 2.0	318
2	+	Sufentanil	50 µg IV	1-2 hour	395	1132
3	-	Negative Control	--	--	22.4	372
4	+	Sufentanil	1 mg IV	2-4 hour	515	2328
5	+	Fentanil	100 µg IV	4-6 hour	4437.8	19038
6	+	Sufentanil	1 mg IV	1-2 hour	550.6	4132
7	-	Negative Control	--	--	64.2	384
8	-	Carfentanil	125 µg IV	4-6 hour	65.5	334
9	+	Fentanil	100 µg IV	2-4 hour	10355	26156
10	+	Sufentanil	50 µg IV	0-1 hour	425.4	1256
11	-	Carfentanil	125 µg IV	0-1 hour	25	400
12	-	Carfentanil	100 µg IV	1-2 hour	16	324
13	-	Negative Control	--	--	25.6	322
14	-	Negative Control	--	--	34.2	258
15	+	Sufentanil	1 mg IV	4-6 hour	507.2	1838
16	+	Carfentanil	125 µg IV	2-4 hour	56	656
17	+	Fentanil	100 µg IV	1-2 hour	10606	80930
18	-	Carfentanil	100 µg IV	2-4 hour	96.2	420
19	+	Sufentanil	50 µg IV	2-4 hour	300	702
20	-	Carfentanil	100 µg IV	0-1 hour	18.8	346
21	-	Carfentanil	100 µg/IV	4-6 hour	63.2	234
22	+	Carfentanil	125 µg IV	1-2 hour	18.2	522
23	+	Sufentanil	50 µg IV	4-6 hour	251.8	332
24	-	Negative Control	--	--	12	228
25	-	Negative Control	--	--	30.2	240
26	-	Negative Control	--	--	14.4	220
27	+	Fentanil	100 µg IV	0-1 hour	6401.4	41192
28	+	Sufentanil	1 mg IV	0-1 hour	634.6	2924

TABLE 3.

Table 3 shows the detection results when blind order dosed and blank horse serum samples were assayed against the Janssen fentanyl antibody for fentanyl equivalents. "Drug", "Dose", and "Time" columns detail the key revealed after RIA was conducted and raw fentanyl equivalents had been furnished to the Illinois Racing Board lab.

Sample #	Drug	Dose	Time	Fentanyl Equivalents pg/ml
1	Fentanyl	100 µg	30 min	213.9
2	Fentanyl	50 µg	2 hour	0.0
3	Negative Control			0.0
4	Negative Control			0.0
5	Negative Control			0.0
6	Fentanyl	50 µg	4 hour	0.0
7	Fentanyl	100 µg	2 hour	29.5
8	Fentanyl	50 µg	30 min	99.3
9	Negative Control			0.0
10	Negative Control			21.5
11	Negative Control			0.0
12	Fentanyl	50 µg	15 min	443.3
13	Negative Control			0.0
14	Negative Control			0.0
15	Fentanyl	100 µg	1 hour	108.9
16	Fentanyl	100 µg	6 hour	0.0
17	Fentanyl	50 µg	1 hour	43.1
18	Fentanyl	100 µg	15 min	370.1
19	Fentanyl	100 µg	4 hour	0.0

TABLE 4.

Example of a routine fentanyl RIA screening with an added fentanyl urine sample.

POOLED SAMPLES			FENT. EQUIV.	INDIVIDUAL SAMPLES TRACK "C"	FENT. EQUIV.
TRACK	DATE	#URINES	(pg/ml)	11/5/86	(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 5.

Example of a routine fentanyl RIA screening with added methylfentanyl urine samples.

POOLED SAMPLES			FENT. EQUIV.	INDIVIDUAL SAMPLES TRACK "A"	FENT. EQUIV.
TRACK	DATE	#URINES	(pg/ml)	12/19/86	(pg/ml)
T	01/01/87	17	3.0	#21	13.8
T	12/23/86	15	<2.0	#22	1597.6
A	12/17/86	12	3.0	#23	1604.6
T	12/20/86	20	6.8	#24	<2.0
A	12/18/86	10	4.2	#25	<2.0
T	12/21/86	18	6.4	#26	<2.0
T	12/27/86	17	<2.0	#27	<2.0
A	12/20/86	10	3.0	#28	<2.0
T	12/19/86	14	3.8	#29	36.6
T	12/18/86	15	6.4	#30	<2.0
T	12/30/86	14	<2.0		
T	12/26/86	13	2.6		
T	12/31/86	16	3.0		
T	12/28/86	17	<2.0		
T	12/29/86	17	8.2		
A	12/19/86	10	1874.0		

TABLE 6.

Summary of detection of fentanyl and analogs in routine testing pooled urine samples.

ADDED DRUG	POOL FENTANYL EQUIVALENTS (pg/ml) POOL POS.	$\bar{X}$ BKG	INDIVIDUAL FENTANYL EQUIVALENTS (pg/ml) INDIV. POS.	$\bar{X}$ BKG
Fentanyl	437.6	10.2	2258.2	11.1
Fentanyl	403.4	15.6	1775.0	7.2
Fentanyl	316.2	<2.0	2149.8	5.3
Fentanyl	257.8	12.0	2519.0	11.7
Methylfentanyls*	1874.0	3.9	1597.6 1604.6	7.8
∝-Methylfentanyl	521.8	12.0	2056.8	7.7
3-Methylfentanyl	311.2	12.0	1578.4	8.2

* Pool contained both a 3-methylfentanyl urine and an ∝-methylfentanyl urine.

Fig 1

Representation of chemical structures of fentanyl, carfentanil, sufentanil, ∝-methylfentanyl and 3-methylfentanyl.

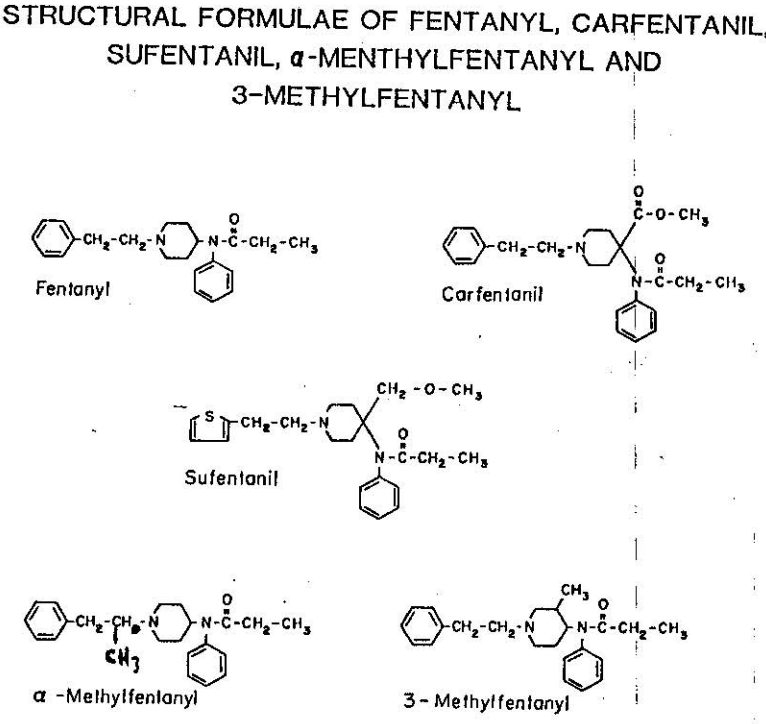

Fig 2

Figure 2 shows the effectiveness of a monoclonal fentanyl antibody (AG9) with good cross-reactivity to carfentanil when used to screen urine from a horse dosed with 200µg carfentanil i.v. Detection was still quite good at 4 hours post dose with about 900 pg/ml fentanyl equivalents being seen. Less effective was the P20 monoclonal antibody and the commercially available Janssen fentanyl antibody.

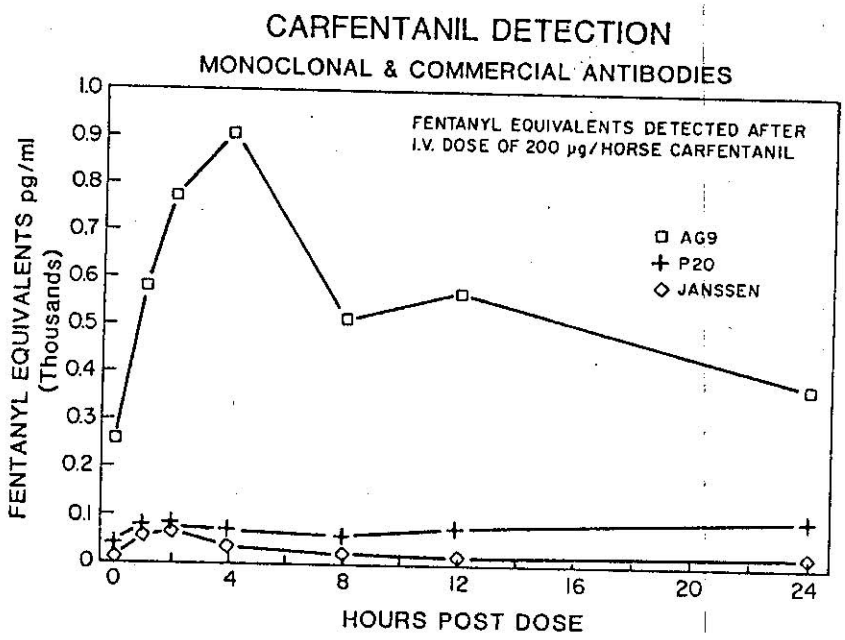

Fig 3

Figure 3 illustrates the locomotor effects of A) carfentanil and B) sufentanil in the horse after administration at the doses indicated. Each point on the carfentanil and sufentanil graphs represents the response of one horse.

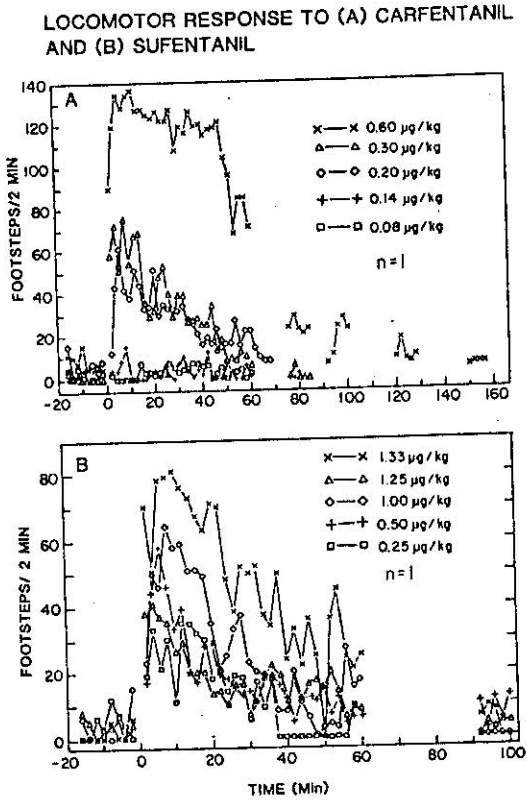

Fig 4

Figure 4A shows the spontaneous locomotor response to 3-methylfentanyl after administration to 2 horses at each indicated dose level.

Figure 4B shows the spontaneous locomotor response to α-methylfentanyl after administration to 2 horses at each of the indicated dose levels.

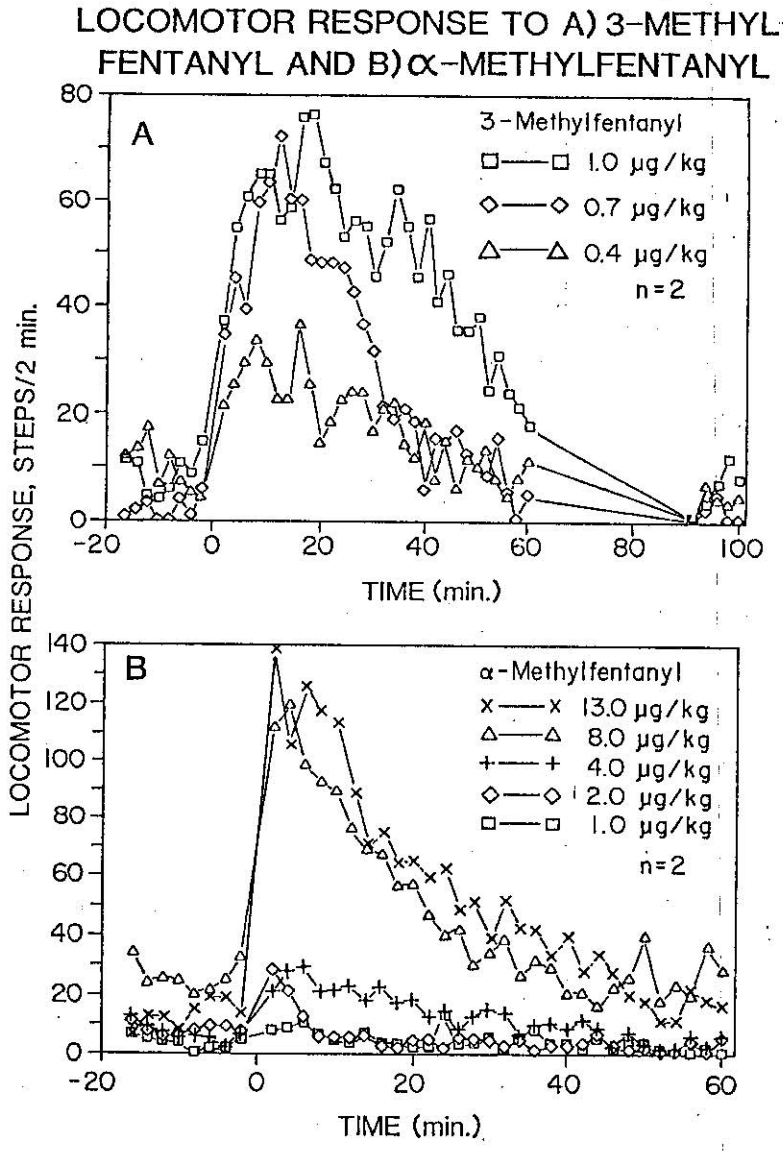

Fig 5

Figure 5 shows the relationship between the dose of fentanyl, etorphine or fentanyl congener and the peak locomotor response produced.

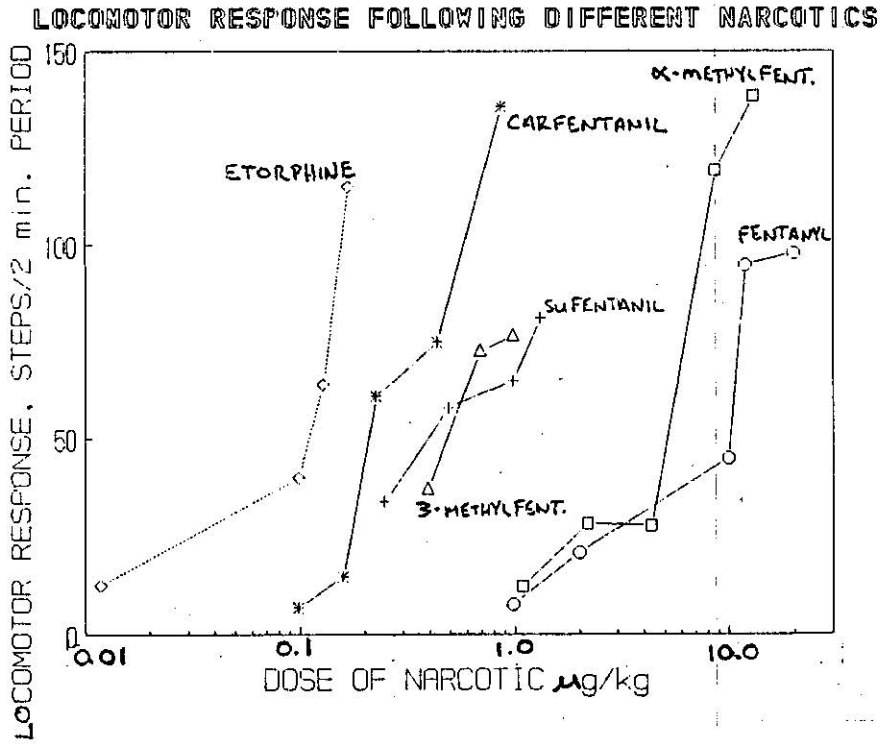

Fig 6

Figure 6 illustrates the pharmacokinetic profile of fentanyl equivalents recovered from horses dosed with 50 µg and 100 µg/horse using ^{125}I radioimmunoassay. Data points were plotted on a semi-logarithmic scale. The correlation coefficient (r) for the 100 µg and 50 µg dose was 0.9965 and 0.9385 respectively.

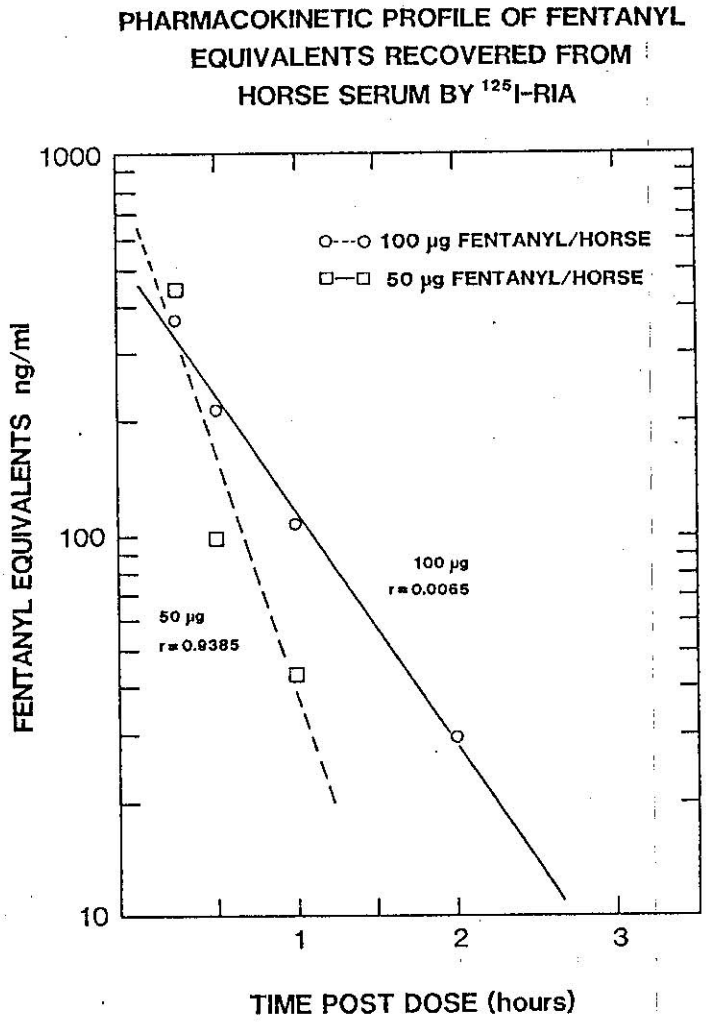

Fig 7

Figure 7 illustrates the urinary fentanyl equivalents after administration of ∝-methylfentanyl at the doses indicated. The inset shows the relationship between the dose of ∝-methylfentanyl and the peak measured fentanyl equivalents for each of the five doses administered.

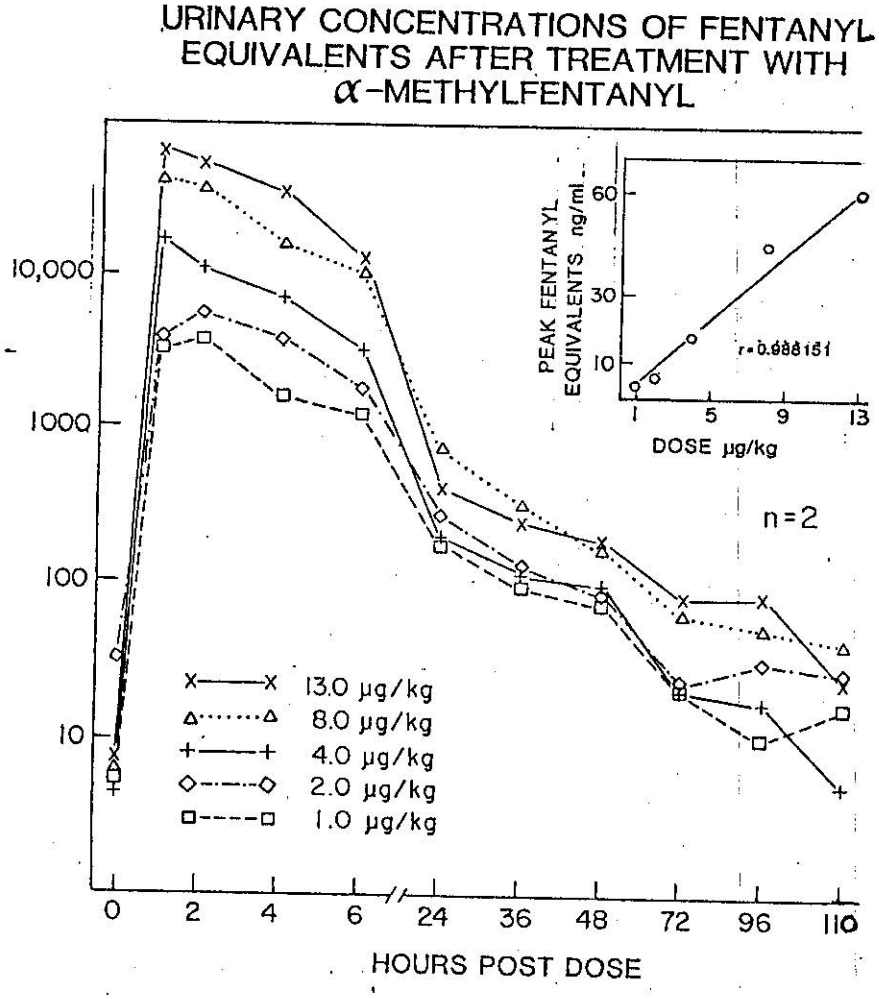

Fig 8

Figure 8 illustrates the urinary fentanyl equivalents after administration of 3-methylfentanyl at the doses indicated. The inset shows the relationship between the dose of 3-methylfentanyl and the peak measured fentanyl equivalents for each of the four doses.