Pharmacologic effects and detection methods of methylated analogs of fentanyl in horses

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SUMMARY

Pharmacologic effects of α-methylfentanyl and 3-methylfentanyl, analogs of fentanyl, were investigated in mares. The ability of an ^{125}I-labeled fentanyl radioimmunoassay (^{125}I-RIA) to detect these methylated fentanyl analogs in individual and pooled urine samples from horses was evaluated. Also, the ability of 7 fentanyl antibodies to react with fentanyl and fentanyl derivatives (sufentanil, alfentanil, and carfentanil) was investigated.

Mares were studied in a locomotor test to determine the amount of stimulation methylated fentanyl analogs might induce. Two mares each were given α-methylfentanyl at 1, 2, 4, 8, or 13 μg/kg of body weight, IV, or 3-methylfentanyl at 0.4, 0.7, or 1 μg/kg IV.

The cross-reactivity of sufentanil, alfentanil, carfentanil, α-methylfentanyl, and 3-methylfentanyl with 7 fentanyl antibodies was studied, using the ^{125}I-RIA. All fentanyl analogs, with the exception of alfentanil, cross-reacted well with a C1 antibody raised to fentanyl. Less satisfactory cross-reactivity was determined with 6 other antibodies raised to fentanyl derivatives. When the C1 antibody was combined with an iodinated analog to fentanyl, good detectability of α-methylfentanyl and 3-methylfentanyl, in terms of fentanyl equivalents, was obtained from urine samples of dosed mares.

The ability of the ^{125}I-RIA to detect methylated fentanyl analogs in forensic urine samples pooled in groups of up to 20 samples was evaluated. When these methylated analogs were administered to mares in doses that induced measurable locomotor stimulation, the analog's presence was readily detected in individual or pooled samples.

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

In horses, fentanyl induces marked locomotor stimulation, as well as analgesia.[4] Because fentanyl alleviates minor lameness and stimulates running, fentanyl has been used widely in racehorses, despite the fact its use is illegal.[5]

α-Methylfentanyl [1-(1-methyl-2-phenethyl)-4-(*N*-propionylanilino)piperidine] and 3-methylfentanyl [3-methyl-1-(2-phenethyl)-4-(*N*-propionylanilino)piperidine] analogs of fentanyl have become available. Both analogs appear to be typical morphine-like narcotic agonists; they stimulate locomotor activity at low doses and the locomotor response is qualitatively similar to the response induced by fentanyl.

The development of a radioimmunoassay (RIA), which allows routine screening of postrace samples for fentanyl, has led to control of the use of fentanyl in racehorses. However, the ability of ^{3}H-labeled fentanyl RIA (^{3}H-RIA) to detect methylated fentanyl analogs is unclear. Purposes of the study reported here were to determine pharmacologic potency of α-methylfentanyl and 3-methylfentanyl (Fig 1) in horses and to determine our ability to detect these drugs in an ^{125}I-labeled fentanyl RIA (^{125}I-RIA).

Materials and Methods

Mares—Eight nonpregnant, mature Thoroughbred or Standardbred mares (450 to 500 kg) were kept at pasture and were allowed free access to food and water. Mares were acclimated in standard box stalls (≈ 16 m^2) approximately 12 hours before testing sessions.

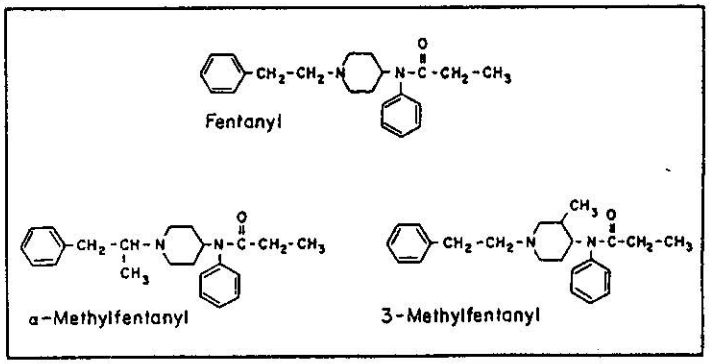

Fig 1—Chemical structures of fentanyl, α-methylfentanyl, and 3-methylfentanyl.

Received for publication Feb 25, 1987.

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Supported by a grant from the Kentucky Equine Drug Research Council and the Kentucky State Racing and Harness Racing Commissions.

Published as Kentucky Agricultural Experiment Station Article 87-4-37, with the approval of the Dean and Director, College of Agriculture and Kentucky Agricultural Experiment Station. Publication No. 133 from the Kentucky Equine Drug Testing and Research Programs, Department of Veterinary Science and the Graduate Center for Toxicology, University of Kentucky, Lexington.

The authors thank Dr. R. L. Hawks, National Institute of Drug Abuse, Rockville, Md, for methylfentanyl analogs, and Dr. Larry Soma, University of Pennsylvania, Kennett Square, for S1 fentanyl antibody.

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Drugs—3-Methylfentanyl and α-methylfentanyl were each dissolved in 10 ml of sterile isotonic saline solution (pH 7). Heat (30 C) and stirring were required to solvate α-methylfentanyl. All drug administrations were by rapid injection into the left jugular vein. Authentic drug standards[a] of fentanyl, sufentanil, alfentanil, and carfentanil were used in an in vitro antibody cross-reaction study.

Locomotor studies—Mares were placed in box stalls that were enclosed on all sides. A window of one-way mirrored glass in each door permitted observers to record each mare's behavior unseen. Locomotor behavior was quantified by counting the number of footsteps taken/2 min. A footstep was scored each time the right forelimb was lifted off the ground and was returned along with a positional change.[6]

A preliminary study with 3-methylfentanyl indicated 4 μg of 3-methylfentanyl/kg of body weight, IV, induced excitement, tachycardia, and tachypnea in mare 204. This dose was antagonized with 8 mg of naloxone,[b] IM. Locomotor data were not collected from this session.

Subsequent doses of 0.4 μg, 0.7 μg, or 1 μg of 3-methylfentanyl/kg, IV, were well tolerated in 6 mares. These doses formed the basis of the 3-methylfentanyl locomotor assays, with each dose of 3-methylfentanyl administered to 2 mares (Table 1). The preliminary session dose (4 μg of 3-methylfentanyl/kg) was administered to only mare 204.

α-Methylfentanyl was administered at doses of 1 μg, 2 μg, 4 μg, 8 μg, or 13 μg/kg, IV, to 2 mares/dose and was well tolerated by mares (Table 1). In 3-methylfentanyl and α-methylfentanyl dosings, a minimum of 2 weeks between treatments was allowed for any mare given > 1 treatment.

Locomotor activity was quantified for 16 minutes before each injection to establish a baseline. Footstep frequency was recorded every 2 minutes for 60 minutes after injection of α-methylfentanyl or 3-methylfentanyl. Prolonged stimulation after 3-methylfentanyl injection necessitated an additional 10 minutes of footstep counting at 90 to 100 minutes after dosing to ensure activity in treated mares had returned to baseline.

Radioimmunoassay—α-Methylfentanyl and 3-methylfentanyl urine concentrations were measured as fentanyl equivalents by a ^{125}I-RIA and were based on a modified ^{3}H-RIA[c] validated for use with equine urine samples.[7]

^{125}I-Labeled fentanyl derivative was prepared by a method similar to that described.[7,8] Fentanyl RIA standard curves were established, using fentanyl antiserum[c] and fentanyl standards[c] with ^{125}I-labeled fentanyl derivative. ^{125}I-Labeled fentanyl derivative (approx 10,000 counts/min) in 100 μl of assay buffer was pipetted into 10 × 75-mm glass culture tubes. Assay buffer was 50 m*M* tris (hydroxymethyl)-aminomethane HCl, pH 7.5, 0.1% gelatin.

The stock fentanyl solution (40 ng/ml) was serially diluted with 30% methanol in water to obtain 0.5 to 64 pg/50 μl standards. Of these standards, 50 μl was added to standard tubes. Urine samples (50 μl) were assayed without extraction. The lyophilized fentanyl antiserum was dissolved in 10 ml of assay buffer and was diluted to give approximately 2.5 μl/tube (amount of antiserum was adjusted to give 25 to 30% binding). Tubes were incubated at 25 C for 1 hour. After incubation, 1 ml of water was added to each tube. γ-Globulin-coated charcoal (1% γ-globulin, 3% charcoal in assay buffer, 200 μl) was pipetted into 12-mm plastic stoppers.[d] Caps were placed on all but total-activity tubes. Tubes were inverted several times and were allowed to stand 5 minutes and were centrifuged for 5 minutes (2,000 × g at 25 C). Supernatants were pipetted into clean 10- × 75-mm glass tubes and were counted on a gamma counter[e] with a data transporter[f] or a liquid scintillation counter,[g] using 10 ml of counting cocktail.[h]

Data from the gamma counter were reduced on a personal computer,[i] using RIA data-reduction software.[j] The curve fitting was by 4 parameter logistic statistics.[9] Data from the scintillation counter were reduced by data-reduction software,[k] using logit-log transformation as described.[10] For each urine sample, the fentanyl equivalent was calculated from the standard curve for each run.

Pharmacokinetic studies—Beginning at postdosing hour (PDH) 1, urine samples were collected from mare 204 dosed in the preliminary study and the 6 mares dosed with 3-methylfentanyl in the 3 other treatment regimens. For 6 mares dosed with α-methylfentanyl, using 5 treatment regimens, urine samples were also collected beginning at PDH 1. All urine samples were analyzed for fentanyl equivalents, using the ^{125}I-RIA. Urine samples were obtained by urinary bladder catheterization before dosing, and at PDH 1, 2, 4, 6, 24, 36, 48, 72, 96, and 120. Urine samples were analyzed directly without extractions. Dilution of samples, when necessary, was with each mare's urine collected before dosing.

Antibody evaluation—Seven antibodies (C1, S1, T2, T3, T4, T6, and T7) raised to fentanyl derivatives were evaluated in vitro for binding ability with fentanyl and fentanyl analogs (sufentanil, alfentanil, carfentanil, 3-methylfentanyl, and α-methylfentanyl). Antibody cross-reaction was evaluated by constructing standard curves with each of the 7 fentanyl antibodies and fentanyl or the 5 fentanyl analogs of interest added when called for in the assay scheme. The added fentanyl concentration or analog concentration required to induce 50% of maximum binding (I-50) for each antibody was thus deter-

[a] Janssen Pharmaceutica, Piscataway, NJ.
[b] Narcan (naloxone HCl), DuPont Pharmaceuticals Inc, Manati, Puerto Rico.
[c] FEN-RIA-200, Institut National Des Radioelements, Fleurus, Belgium.
[d] Luckham LP3S stoppers, Luckham Ltd, West Sussex, England, UK.
[e] Beckman 5500 gamma counter, Beckman Instruments Inc, Arlington Heights, Ill.
[f] Data transporter DT064, Beckman Instruments Inc, Arlington Heights, Ill.
[g] Beckman LS 3801 scintillation counter, Beckman Instruments Inc, Arlington Heights, Ill.
[h] Liquid scintillation cocktail 3a70B, Research Products International, Mount Prospect, Ill.
[i] IBM PC-XT, IBM Corp, Boca Raton, Fla.
[j] RIA-AID software, Robert Marciel Associates, Arlington, Mass.
[k] Data capture software, Beckman Instruments Inc, Lab Automation Operations, Irvine, Calif.

TABLE 1—Administration schedule for α-methylfentanyl and 3-methylfentanyl in eight mares

	Dose (μg/kg)								
	α-methylfentanyl					3-methylfentanyl			
Mare No.	1	2	4	8	13	0.4	0.7	1	4
203	X	...	X	X	X	...	...	...	...
204	...	X	...	...	X	...	X	...	X
205	...	...	X	...	...	...	...	X	...
208	...	...	...	X	...	X	...	...	...
209	...	...	...	...	...	X	...	...	...
210	X	...	...	...	...	...	...	...	...
217	...	...	...	...	...	...	X	...	...
226	...	X	...	...	...	...	...	X	...

X = Dose mare was given.

mined. Binding at I-50 was expressed as a percentage of binding, compared with the antibody's I-50 performance for binding with parent fentanyl (Table 2).

Pooled sample RIA—In the Kentucky Equine Drug Testing Program, samples from horses racing in Kentucky are received daily. A 2-ml aliquot of each sample is pipetted off and is stored frozen in an individually labeled tube. On accumulation of sufficient samples, or at the end of each week, samples are thawed and pooled. The pooling process involves pipetting 100 µl of each individual postrace sample into a common tube. This combined sample represents all horses racing at a particular track on a single day or a maximum of 10 to 20 individual samples. Assaying of the sample was then the same as the ^{125}I-RIA, and 50 µl of the pooled sample was used.

Urine samples from horses dosed with fentanyl,[1] 3-methylfentanyl, and/or α-methylfentanyl, containing approximately 2 ng of drug (as fentanyl equivalents), were included in a series of urine samples from the Kentucky Equine Drug Testing Laboratory and were pooled for ^{125}I-RIA fentanyl screening (Table 3). The dosed horse urine was added at random to sample pools to ensure blind screening.

Results

3-Methylfentanyl, as well as α-methylfentanyl, induced a dose-related increase from baseline values in locomotor response in all mares. For each of the 3 doses administered, 3-methylfentanyl induced a dose-related increase in locomotor activity, peaking within 20 minutes. Activity in mares given 0.4 or 0.7 µg of 3-methylfentanyl/kg returned to baseline values by postdosing minute (PDM) 60. In mares given 1 µg of 3-methylfentanyl/kg, baseline activity was not reestablished until PDM 90 to 100 (Fig 2).

At doses of ≤ 4 µg of α-methylfentanyl/kg, little or no locomotor response was seen; when the dose was increased to ≥ 4 µg/kg, the locomotor response increased sharply. With α-methylfentanyl, onset to peak effect developed within PDM 10. The largest dose administered, 13 µg of α-methylfentanyl/kg, induced a mean locomotor response of 138 steps/2 min at PDM 4 to 6. Activity in each mare given α-methylfentanyl returned to baseline by PDM 60.

Use of the C1 fentanyl antibody, along with our iodinated analog of fentanyl, allowed for effective screening for both methylated fentanyl analogs. At the threshold dose of α-methylfentanyl (4 µg/kg) inducing a detectable pharmacologic effect, the amount of urinary fentanyl equivalent was about 20 ng/ml at peak urinary concentration (PDH 2). By PDH 5 to 6, when the pharmacologic effect of the drug was likely to be minimal, ≥ 1,000 pg of fentanyl equivalents/ml was still in the sample (Fig 3). Similarly, administration of ≥ 0.4 µg of 3-methylfentanyl/kg resulted in detection of fentanyl equivalents in urine for up to 24 hours (Fig 4).

In in vitro antibody cross-reaction studies, 7 fentanyl antibodies at drug concentrations ≤ 12 pg had I-50 binding with parent fentanyl. For antibody C1, I-50 was detected at concentrations of 8.05 pg of added fentanyl. The C1 antibody also had cross-reaction with 3-methylfentanyl and α-methylfentanyl, resulting in 12.5% binding at 80 pg of added 3-methylfentanyl and 14.3% binding at 75 pg of added α-methylfentanyl. Antibody S1 reacted poorly with fentanyl derivatives sufentanil, alfentanil, and carfentanil; added drug concentrations ≥ 10,000 pg of each sufentanil, alfentanil, and carfentanil, resulted in ≤ 0.1% binding. However, S1 antibody did react with methylated derivatives of fentanyl at added drug concentrations of ≤ 455 pg. Antibodies T2 through T7 raised to a fentanyl derivative, also had negligible cross-reaction with sufentanil and alfentanil. The T2 antibody cross-reacted with 3-methylfentanyl at an added concentration of 156 pg, and T3 antibody cross-reacted with α-methylfentanyl at an added drug concentration of 66 pg (Table 2).

Routine ^{125}I-RIA screening with a urine sample from a fentanyl-dosed horse, which was added blind to a sample pool, allowed for detection of fentanyl equivalents (Table 3). A pool of 16 urine samples from track C of 11/5/86, contained fentanyl equivalent concentrations ≥ 400 pg/ml, well above the concentrations of the 10 other pools. Data reduction software was set to flag as positive any sample value ≥ 50 pg/ml; any concentration less than this was considered to be background. The track-C 11/5/86 pool was flagged and individual samples from this pool were isolated and reassayed by ^{125}I-RIA. Sample 4 of this 16-sample pool contained ≥ 1,775 pg of fentanyl equivalents/ml and was determined to be urine from a fentanyl-dosed horse.

Use of an ^{125}I-RIA in routine postrace screening for fentanyl also detected the presence of urine samples from mares dosed with 3-methylfentanyl or α-methylfentanyl when included in pooled urine samples (Table 4). Pool sizes for these assays varied from 7 to 20 samples/pool. Mean background fentanyl equivalents of pooled samples ranged from ≥ 2 to ≤ 16 pg/ml. Pooled samples considered positive had fentanyl equivalents ranging from 40 to 150 times these background values. Mean background fentanyl equivalents in individual samples considered positive were about the same as were those for pooled samples

[1] Fentanyl-dosed horse urine sample provided by Kentucky Equine Drug Testing Laboratory, Lexington, Ky.

TABLE 2—Specificity of fentanyl antibodies

Antibody	Fentanyl* (pg)	Cross-reactivity				
		Sufentanil	Alfentanil	Carfentanil	3-Methyl-fentanyl	α-Methyl-fentanyl
C1	8.05	560 (1)	>10,000 (<0.1)	290 (3.7)	80 (12.5)	75 (14.3)
S1	10.4	> 10,000 (<0.1)	> 10,000 (<0.1)	12,800 (<0.1)	455 (1.7)	296 (2.6)
T2	8.0	> 10,000 (<0.1)	> 10,000 (<0.1)	> 10,000 (<0.1)	156 (6.3)	660 (1.5)
T3	8.7	> 10,000 (<0.1)	> 10,000 (<0.1)	> 10,000 (<0.1)	430 (2.3)	66 (14.7)
T4	9.35	6,000 (0.2)	> 10,000 (<0.1)	5,800 (0.2)	750 (1.2)	180 (5)
T6	10.9	> 10,000 (<0.1)	> 10,000 (<0.1)	1,450 (1)	375 (3.7)	87 (16)
T7	11.9	> 10,000 (<0.1)	> 10,000 (<0.1)	850 (1.2)	300 (3.3)	100 (3.8)

* Fentanyl required to inhibit the binding of the iodinated analog of fentanyl by 50%. Mean values of 2 fentanyl assays.
Cross-reactivity data are exprssed as value obtained at 50% maximum binding (percentage of cross-reactivity vs fentanyl).

TABLE 3—Example of a routine ^{125}I-labeled fentanyl radioimmunoassay screening with an added fentanyl urine sample

Pooled Samples Track	Date (11/86)	No.	Fentanyl* (pg/ml)	Individual Track C 11/5/86	Fentanyl* (pg/ml)
A	9	12	23.0	1	< 2.0
C	11	14	11.0	2	< 2.0
C	11	14	23.4	3	< 2.0
A	6	13	11.0	4	1,775.0
A	8	12	14.4	5	25.4
A	5	11	13.6	6	18.8
A	7	9	12.0	7	< 2.0
C	8	17	9.8	8	6.6
C	7	13	16.8	9	12.4
C	9	14	21.4	10	< 2.0
C	5	16	403.4	11	13.0
...	...	...	...	12	< 2.0
...	...	...	...	13	< 2.0
...	...	...	...	14	7.4
...	...	...	...	15	3.8
...	...	...	...	16	6.4

* Equivalents.

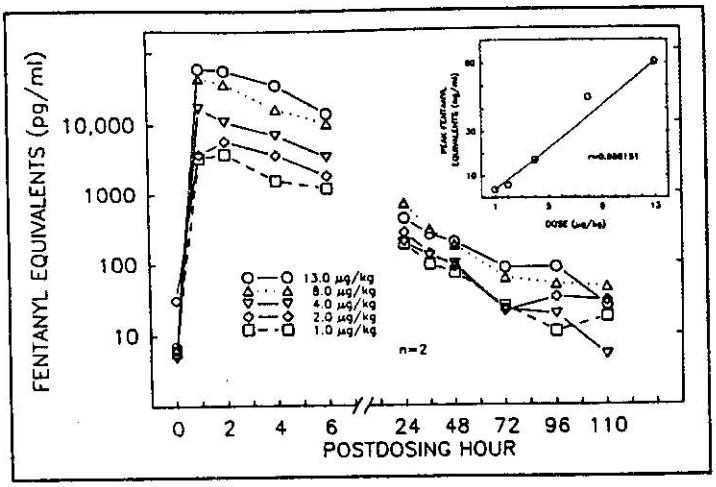

Fig 3—Urinary fentanyl equivalents after administration of α-methylfentanyl in 6 mares at doses indicated.
Inset—the relationship between dose of α-methylfentanyl and peak measured fentanyl equivalents for each of 5 doses.

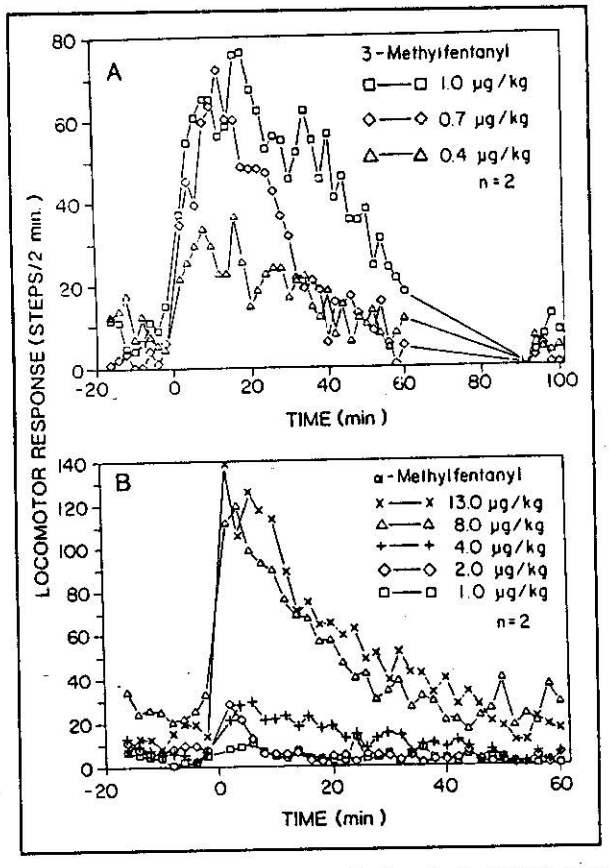

Fig 2—Spontaneous locomotor response to 3-methylfentanyl in 2 mares at each indicated dose (A). Spontaneous locomotor response to α-methylfentanyl in 2 mares at each dose (B). n = No. of mares

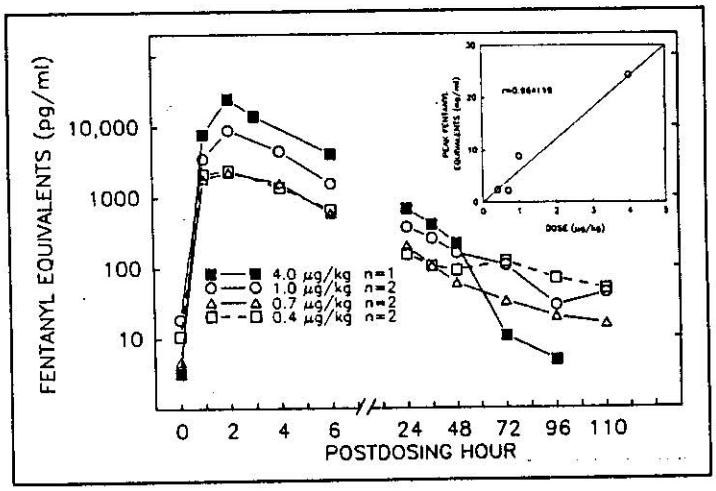

Fig 4—Urinary fentanyl equivalents after administration of 3-methylfentanyl in 6 mares at doses indicated.
Inset—the relationship between dose of 3-methylfentanyl and peak measured fentanyl equivalents for each of 4 doses.

TABLE 4—Detection of fentanyl and its analogs in routine testing of pooled urine samples

	Fentanyl equivalents			
	Pooled		Individual	
Added drug	Positive	X̄ Background	Positive	X̄ Background
Fentanyl	437.6	10.2	2,258.2	11.1
Fentanyl	403.4	15.6	1,775.0	7.2
Fentanyl	316.2	< 2.0	2,149.8	5.3
Fentanyl	257.8	12.0	2,519.0	11.7
Methylfentanyls*	1,874.0	3.9	1,597.6 1,604.6	7.8
α-Methylfentanyl	521.8	12.0	2,056.8	7.7
3-Methylfentanyl	311.2	12.0	1,578.4	8.2

* Pool contained a 3-methylfentanyl urine sample and an α-methylfentanyl urine sample.
Data are expressed as picograms per milliliter.

(range, 5.3 to 11.7 pg/ml). Individual samples considered positive had fentanyl equivalents 1.6 to 2.5 ng/ml, which agreed with the added drug concentrations of 2 ng/ml.

Discussion

Chemical testing for fentanyl and its analogs in equine urine samples is difficult because of the potency of these drugs and the resultant low concentrations of parent drug or metabolites excreted. Fentanyl is about 100 times more potent than are morphine and many other narcotic analgesics. The routine high-performance thin-layer chromatography tests used in drug screening are of limited use for the control of narcotics such as fentanyl. To further compound the problem, numerous analogs of fentanyl are available, licit, and illicit. For control of these

agents, the only currently available technique that offers effective screening is ^{3}H-RIA.

When we administered 3-methylfentanyl or α-methylfentanyl analogs of fentanyl to mares, both agents induced a locomotor response, indicating that they are typical morphine-like narcotic agonists in horses. 3-Methylfentanyl is the more potent, being 3 to 10 times more potent than is fentanyl (Fig 4). The duration of the locomotor response to 3-methylfentanyl also was somewhat longer than that to fentanyl; in mares given 3-methylfentanyl (1 μg/kg), locomotor activity was still detectable at PDM 60 (Fig 2). With fentanyl, the locomotor response always returns to base-line by PDM 60, unless fentanyl was combined with another drug.[11] Beyond this, the peak response observed with 3-methylfentanyl, ≥ 75 steps/2 min, was approximately the same as that observed with fentanyl.[4]

α-Methylfentanyl appears to be similar in potency and duration of action to fentanyl, and locomotor response to α-methylfentanyl was indistinguishable from that to fentanyl (Fig 5). The duration of the locomotor response to α-methylfentanyl was similar to that of fentanyl; most locomotor responses were over by PDM 40.

Because these analogs of fentanyl induce a locomotor response and likely analgesia in horses, postrace screening tests for the presence of these agents in urine samples are required. One of the more useful screening tests for fentanyl in equine drug testing has been the ^{3}H-RIA for fentanyl. Use of the ^{3}H-RIA, in conjunction with reliable mass spectrometry confirmation methods, has allowed the use of fentanyl in racehorses to be controlled. Development of the iodinated analog of fentanyl has allowed us to improve the efficacy and sensitivity of the basic ^{3}H-RIA, while reducing costs on a per-test basis.[10]

To determine the likelihood of detecting fentanyl analogs in our ^{125}I-RIA, we assessed the cross-reactivity of 7 fentanyl antibodies with analogs of fentanyl in vitro. The S1 antibody, as well as antibodies T2, T3, T4, T6, and T7 raised to a carboxyfentanyl conjugate, reacted well with fentanyl, but reacted poorly with other analogs of fentanyl (Table 2). The C1 antibody was superior in terms of cross-reactivity, and reacted well with α-methylfentanyl and 3-methylfentanyl, and to a limited extent, with carfentanil and sufentanil. None of the 6 other antibodies had this broad cross-reactivity, although antibodies T3 and T6 had good cross-reactivity with α-methylfentanyl.

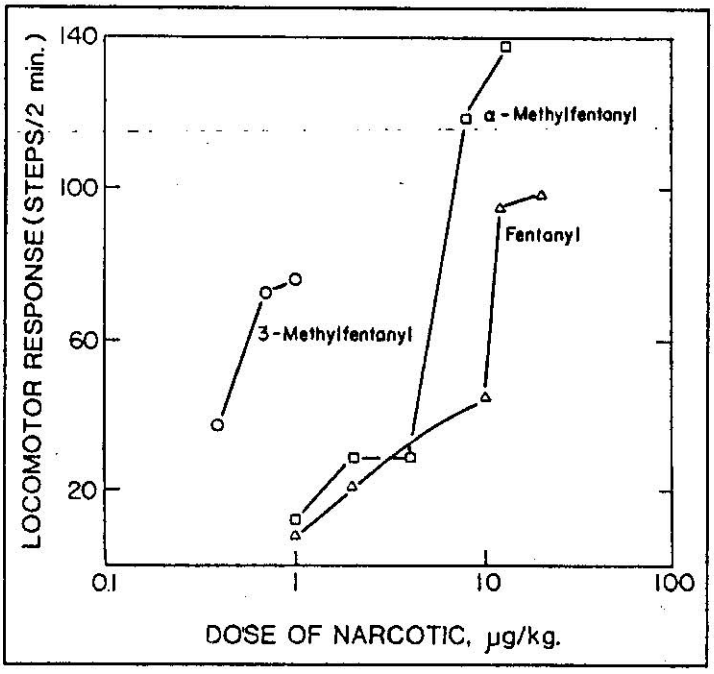

Fig 5—Relationship between the dose of fentanyl or its methylated analog and the induced locomotor response.

α-Methylfentanyl and 3-methylfentanyl were readily detected in urine samples from dosed mares, using C1 antibody in the ^{125}I-RIA. 3-Methylfentanyl was readily detected in urine samples from dosed mares at PDH 1, and remained detectable (ie, ≥ 50 pg of fentanyl equivalents/ml) at PDH 48, even after the smallest dose used (0.4 μg/kg; Fig 4). Seemingly, 3-methylfentanyl would be readily detected by this ^{125}I-RIA test in postrace urine samples.

Similar data were obtained with α-methylfentanyl, in that it also was detectable in urine samples from mares at ≥ PDH 48 after administration of 1 to 13 μg/kg, IV (Fig 3). These detection limits are more than sufficient to allow easy identification of α-methylfentanyl in an undiluted sample for ≤ PDH 48.

For the racing analyst, use of RIA in routine screening does pose some problems. As an antibody-based test, RIA is limited to single drugs or to closely related groups of drugs for which antibody has been developed. Compared with high-performance thin-layer chromatography, RIA instruments and reagents are expensive, and RIA is more technically demanding and time-consuming. High-performance thin-layer chromatography can detect a wide range of unrelated drugs simultaneously and at a relatively low cost per sample. For many racing jurisdictions, widespread implementation of RIA has been impeded by cost and technical considerations.

Development of increased sensitivity in the ^{125}I-RIA prompted us to investigate the ability of this test to detect fentanyl analogs in postrace urine samples, and also to assess the ability of the ^{125}I-RIA to detect these agents in pooled urine samples. Seemingly, urine samples could be pooled and frozen for ≥ 10 days and tested for fentanyl or its analogs at a later date. Pooling of samples allows a week's worth of samples to be screened in a single day, thereby greatly reducing testing costs while having little effect on efficacy.

Concentrations of drug or drug metabolite in urine samples after administration of performance-altering doses needs to be known. Doses of methylated analogs of fentanyl likely to affect the performance of a horse (Fig 2) and the excretion patterns of these methylated fentanyl analogs were determined (Figs 3 and 4). These findings allowed for establishment of required detectability limits necessary to make urine sample pooling a useful RIA screening alternative.

The smallest dose of fentanyl likely to induce an effect on a horse is about 0.2 μg/kg. Any clinical dose probably induces a pharmacologic effect within PDH 4.[4] Using the ^{125}I-RIA, we can detect fentanyl in horses given 0.002 μg of fentanyl/kg for at least 24 hours after administration.[10] α-Methylfentanyl can be detected for at least 48 hours after administration of 1 μg/kg, using the ^{125}I-labeled fentanyl RIA, and 3-methylfentanyl is also detectable for at least 48 hours after a dose of 0.4 μg/kg with the ^{125}I-labeled fentanyl RIA. This large reserve of sensitivity and

the good cross-reactivity of the C1 antibody to the α-methylfentanyl and 3-methylfentanyl analogs of fentanyl indicates that the illicit use of these fentanyl analogs in racehorses is readily detectable in individual, as well as pooled, postrace urine samples.

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