APPENDIX III

RADIOIMMUNOASSAY SCREENING FOR FENTANYL WITH AN IODINATED FENTANYL ANALOG

T Tobin, WE Woods, HH Tai,* C Tai, T Weckman, T Wood, and JW Blake

Department of Veterinary Science and School of Pharmacy*
University of Kentucky, Lexington, KY 40546-0076

ABSTRACT

A radio-iodinated analog of fentanyl was synthesized for use with a commercially available antibody for fentanyl. The sensitivity of the modified assay was at least 100 times greater than that of the original assay. Using this modified assay, concentrations of fentanyl as low as one picogram of fentanyl or fentanyl equivalents in equine urine were detected.

The sensitivity of this test for fentanyl is such that doses of fentanyl 100 times smaller than threshold dose for a pharmacological effect of this drug are readily detectable. Similarly, a pharmacologically effective dose of this drug can be detected for up to 96 hours or more. On the other hand, the high sensitivity of this test means that large numbers (10-20) urine samples can be pooled and screened simultaneously. This approach leads to substantial economies in the use of fentanyl RIA for analysis of post race urines. The test also detects sufentanil in post race urines, although at only about 1% of the efficiency with which it detects fentanyl.

Published as Kentucky Agricultural Experiment Station Article No. 85-4- with the approval of the Dean and Director, College of Agriculture and Kentucky Agriculture Experiment Station.

Publication #126 from the Kentucky Equine Drug Research and Testing Programs, Department of Veterinary Science and the Graduate Center for Toxicology, University of Kentucky.

Supported by a grant entitled "Immunoassay Tests for High Potency Narcotic Analgesics," from the Kentucky State Racing and Harness Racing Commissions and the Kentucky Equine Drug Research Council.

Correspondence should be addressed to Dr. Tobin.

INTRODUCTION

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

The major pharmacological action of fentanyl in man is analgesia, associated with euphoria and respiratory depression. High doses are known to cause muscular rigidity [2]. In the horse, the most noticeable pharmacological effect is a marked increase in locomotor activity [4,5]. In thoroughbred horses, IV injection of fentanyl citrate can increase spontaneous locomotion 15-fold within a few minutes, and the effect can last up to one hour. These movements are well controlled and coordinated. Maximum locomotor activity occurred at fentanyl plasma levels of about 50 ng/ml and the minimal plasma level associated with locomotion was about 5 ng/ml. High doses of fentanyl (about 16 mg/horse) cause disorientation, incoordination and collapse [6].

In the horse, the major urinary metabolites of fentanyl are fentanyl β-keto acid (90%) and despropionyl fentanyl [6,7]. During acid hydrolysis used in most detection techniques the β-keto acid is converted to despropionyl fentanyl [8].

Fentanyl citrate is used for intravenous anesthesia and sedation in human and veterinary surgery. As such, it is readily available on a prescription basis, and is widely used in this country. This ready availability has led to fentanyl being used in racing horses in attempts to improve their performance.

Control of the use of fentanyl in horses has been based on the use of a commercial radioimmunoassay (RIA) for this drug. In this report, we outline modifications to this RIA which increase the effectiveness of this test more than 100-fold. This enhancement in sensitivity of the commercial RIA makes very economical screening of post race samples for Sublimase® or fentanyl possible.

MATERIALS AND METHODS

Fentanyl RIA standard curves were constructed using kits (FEN-RIA-200®) purchased from Janssen Life Sciences Products (Piscataway, NJ) as described by the kit instruction manual.

The Janssen ^{3}H-fentanyl solution (50 µl) was pipetted into 3 ml conical polypropylene tubes, then 50 µl of 30% methanol/water was added to all except the fentanyl standard tubes. In duplicate, 50 µl of the fentanyl standards, 30% methanol/water serial dilutions of the Janssen stock fentanyl solution (40 ng/ml), were added to the standard tubes. Janssen kit assay buffer (300 mg potassium phosphate monobasic, 3120 mg sodium phosphate dibasic dihydride, 200 mg sodium azide, and 2000 mg bovine serum albumin in 400 ml distilled water, pH 7.5) was added to total count tubes (800 µl),

non-specific binding tubes (600 µl), maximum binding tubes (500 µl), and the standard tubes (500 µl). The Janssen lyophilized antiserum was dissolved in 10 ml of the assay buffer; 100 µl of this solution was added to the maximum binding and standard tubes. The tubes were capped and mixed on a rotating mixer for 2 hrs at room temperature. The Janssen kit dextran-coated charcoal suspension (200 µl) was added to all except the total count tubes. The tubes were rotated another 60 min at room temperature and centrifuged at 6000x G for 8 min.

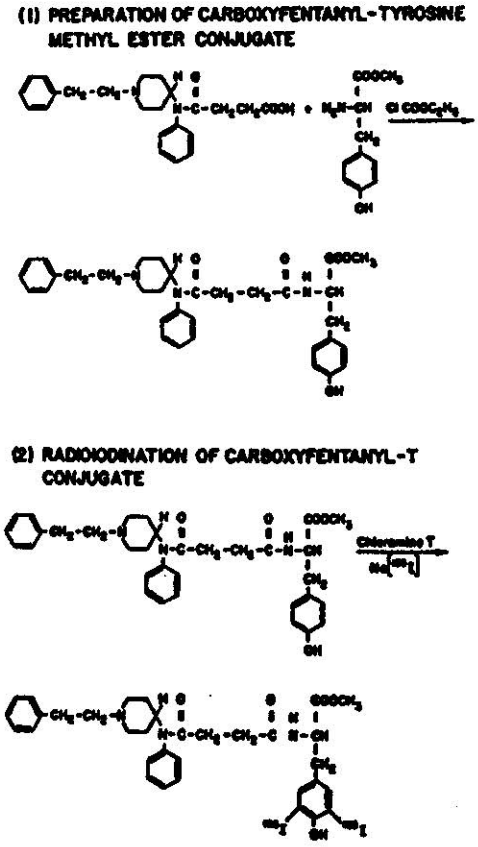
All the supernatant of each tube was decanted into 20 ml glass scintillation vials, and 10 ml of scintillation cocktail, 3a70b (Research Products International, Mount Prospect, IL) was added to each vial which was then vortexed. The samples were then counted 10 min on a Beckman (Arlington Heights, IL) LS 3801 liquid scintillation counter. Standard curves were also constructed using ^{125}I-fentanyl instead of ^{3}H-fentanyl.

Carboxyfentanyl-tyrosine methyl ester (TME) conjugate was prepared according to the procedure of Tai and Yuan for the conjugation of thromboxane B_2 to TME [9]. Carboxyfentanyl (1 mg) was dissolved in 0.2 ml of dimethyl formamide at 0° C with 2 µl of triethylamine. Ethyl chloroformate (0.5 µl) in 0.1 ml of dimethyl formamide was added, and the reaction was carried out at 0° C for 15 min. TME (1.3 mg) and triethylamine (0.6 µl) in 0.2 ml of dimethyl formamide was then added, and the reaction was continued for an additional two hours at room temperature with stirring. The reaction mixture was then blown dry with a stream of nitrogen. The product was purified by thin layer chromatography on a silica gel G plate using chloroform/methanol/acetic acid = 85:15:1 as the developing solvent. The carboxyfentanyl-TME conjugate (R_f 0.62 moved ahead of carboxyfentanyl (R_f = 0.34) and TME (R_f = 0.0). The carboxyfentanyl-TME conjugate was scraped off the plate and extracted twice with 0.6 ml of ethanol. After removal of gel by centrifugation, the conjugate was stored in ethanol at -20° C. The yield was 80% with respect to carboxyfentanyl (Fig. 1).

The conjugate (0.3 µg) was dissolved in 50 µl of 0.5 M sodium phosphate buffer pH 7.5. NA[^{125}I] (300 µg) in 3 µl of diluted sodium hydroxide was added to the conjugate solution. Iodination was initiated by the addition of 25 µg of chloramine T in 5 µl of 0.05 M sodium phosphate buffer, pH 7.5. After 30 seconds the reaction was terminated by the addition of 250 µg of sodium metabisulfate in 50 µl of 0.05 M sodium phosphate buffer, pH 7.5. The reaction mixture was extracted with 0.6 ml of ethyl acetate twice. The organic layer was evaporated to dryness with a stream of nitrogen. The residue was spotted on a silica gel G plate (2 x 20 cm) with chloroform/methanol/acetic acid (85:15:1) as the developing solvent. The location of labeled conjugate was identified by autoradiography. Two labeled conjugates (presumably mono- and diiodinated derivatives) were synthesized and were scraped off the gel respectively. The labeled conjugates were extracted from the gel with 0.6 ml of ethanol twice. After removal of gel by centrifugation, the labeled conjugates were stored in ethanol at -20° C. Usually monoiodinated conjugate (lower R_f) was used for radioimmunoassay (Fig. 1).

FIGURE 1. Synthesis of ^{125}I-fentanyl conjugate.

(1) Carboxyfentanyl-tyrosine methyl ester conjugate was prepared by reacting carboxyfentanyl with tyrosine methyl ester in the presence of ethyl chloroformate in dimethyl formamide and triethylamine. Purification was by thin layer chromatography. The yield was 80%. (2) The carboxyfentanyl-tyrosine methyl ester conjugate was radioiodinated by reaction with sodium iodide-^{125}I in the presence of chloramine T in 0.5M phosphate buffer pH 7.5. Purification was by thin layer chromatography, with location by autoradiography.



The ^{125}I-fentanyl RIA method was the same as the ^{3}H-fentanyl method except for the following modifications. The ^{125}I-fentanyl stock solution (~160,000 cpm/50 µl) was diluted with assay buffer to about 10,000 cpm/50 µl, which amount was added to each tube instead of the ^{3}H-fentanyl. The Janssen fentanyl standard solutions were used, but required much further dilution. The Janssen antiserum was diluted 1:15 with assay buffer; 100 µl of the diluted antiserum was used. The dextran-coated charcoal suspension used was 0.25% dextran T-70 (Sigma, St. Louis, MO) and 2.5% Norit A Charcoal (Fisher Scientific, Fairlawn, NJ) in assay buffer. When urine samples were assayed, the standard solution was omitted, only 450 µl buffer were added, and 50 µl of the urine sample was added. A standard curve was also generated using 30% methanol/water serial dilutions of the stock sufentanil standard from a Janssen Sufentanil RIA kit with the ^{125}I-fentanyl RIA method.

Calculation of the data was by Logit-Log transformation of the cpm values for each tube:

$$L=Ln\left(\frac{B/Bo}{1-B/Bo}\right)$$

where Bo = the maximum binding cpm minus the non-specific binding cpm, and B = standard or sample cpm minus the non-specific binding cpm. Standard curves were constructed by plotting the logarithmic fentanyl concentration of each standard versus the corresponding L values. For the urine samples, the fentanyl equivalent level for each sample was calculated from the standard curve for each run.

Urine samples from horses dosed with fentanyl citrate (Sublimase®, McNeil, Fort Washington, PA) were analyzed for fentanyl equivalents using the ^{125}I-fentanyl method. Six horses were dosed with fentanyl citrate at 200 µg, 100 µg, 20 µg, 10 µg, 2 µg and 1 µg/horse, respectively. Urine samples were taken by bladder catheterization pre-dose, and post-dose 1 hr, 2 hrs, 4 hrs, 8 hrs, 12 hrs and for every 12 hrs thereafter up to 96 hrs. The urine samples were analyzed directly without extractions. Dilution of samples, when necessary, was with assay buffer.

Urine samples from a horse dosed with sufentanil were also analyzed for concentration of fentanyl equivalents using the ^{125}I-fentanyl method. One horse was dosed with 40 µg IV of sufentanil as sufentanil citrate (Sufenta Injection,® Janssen, Piscataway, NJ) and urine samples were taken pre-dose and post-dose at 1 hr, 2 hrs, 3 hrs, 4 hrs, 5 hrs, 6 hrs, 8 hrs, 24 hrs and 36 hrs.

RESULTS

Use of the radio-iodinated analogue of fentanyl as the ligand in the commercially available RIA test increased the sensitivity of the assay more than 100-fold. As shown in Fig. 2, the useful region of the immunoassay using the tritiated fentanyl as the radiolabelled ligand was in the area of 0.2 ng/ml. However, when the radio-iodinated analogue of fentanyl was used the useful region of the immunoassay was about 0.002 ng/ml. This increased sensitivity allowed detection of very small amounts of fentanyl administered to horses, or conversely, the detection of pharmacologically effective doses for very long periods.

Fig. 3 shows the concentrations of fentanyl equivalents detected in equine urine after administration of different doses of fentanyl. At a dose of 200 µg/horse, concentrations of fentanyl equivalents in equine urine of 80 ng/ml were observed initially. These concentrations declined rapidly to about 10 ng/ml in the first 4 hrs after dosing. Thereafter, the concentrations of fentanyl equivalent in urine declined more slowly, and concentrations of about 50 picograms/ml were readily detectable in the urine of these horses at 96 hrs after dosing. Reduction in the dose of fentanyl to 1 µg/horse gave rise to urinary concentrations of fentanyl (or its metabolites) which were detectable in this horse's urine for at least 24 hrs, while doses of 10 µg/horse of fentanyl were detectable for up to 48 hrs.

FIGURE 2. Standard Curves with [^{3}H] and ^{125}I-Fentanyl.

Standard curves were constructed using the Janssen Fentanyl RIA Kit with ^{3}H-fentanyl (●) and with ^{125}I-fentanyl (O) as the labeled antigen. For the ^{125}I-fentanyl RIA curves, the Janssen antibody was diluted 1:15. The data was calculated by logit-log transformation and plotted by linear regression.

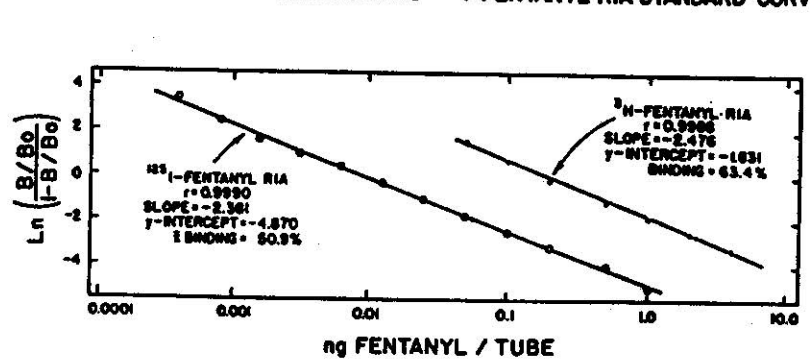
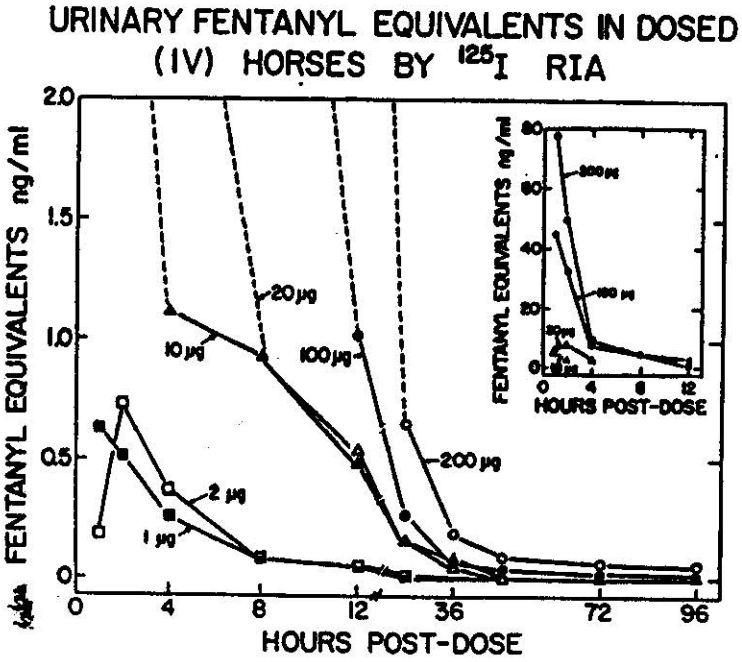


FIGURE 3. Apparent Urinary Fentanyl in Fentanyl Treated Horses.

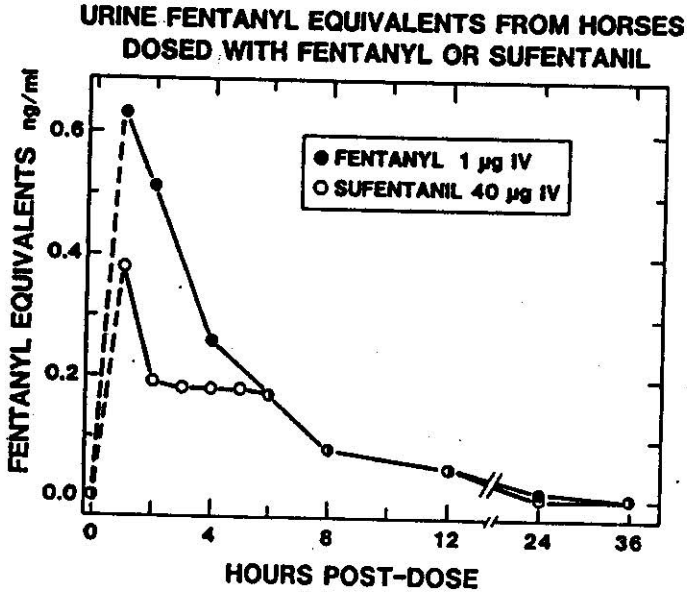
The symbols show the levels of fentanyl equivalents in horses after IV dosing with 200 µg/horse (O), 100 µg/horse (●), 20 µg/horse (Δ), 10 µg/horse (▲), 2 µg/horse (□), and 1 µg/horse (■). Fentanyl equivalents were determined by ^{125}I-fentanyl RIA. All values represent single experimental points.



More recently, a number of analogs of fentanyl have been synthesized, and some of these agents are in the final stages of clinical trials, or are about to be released for use in human medicine. One of these agents is sufentanyl which is about ten times as potent as fentanyl [10]. To test whether or not sufentanil or its metabolites in equine urine would cross-react in our fentanyl immunoassay, we tested the reactivity of urines from a horse dosed with a threshold dose (40 µg) of sufentanyl. As shown in Fig. 4, there was a small, though significant reactivity of urine from this horse with the ^{125}I-fentanyl RIA in comparison to the response of the urine from the low dose (1 µg) of fentanyl. We also found a small degree of cross-reactivity of sufentanil when substituted for fentanyl in our ^{125}I-fentanyl RIA standard curve. Based on these observations, therefore, it appears that use of sufentanyl in racing horses may be detected by use of the fentanyl antibody used in these experiments.

FIGURE 4. Apparent Urinary Fentanyl in Horses Dosed with Fentanyl or Sufentanyl.

The solid circles (●–●) show apparent urinary fentanyl in horse urine after 1 µg of fentanyl IV. The open circles (O–O) show apparent urinary fentanyl levels after 40 µg of sufentanil IV.



DISCUSSION

Fentanyl is a typical µ narcotic agonist in the horse in that it produces both locomotor activity and analgesia. These actions are potentially useful in horses with subclinical lameness and could serve to improve the racing performance of such horses. For these reasons the use of narcotic analgesics in racing horses has been forbidden for most of the twentieth century, and these regulations are enforced by chemical testing [11].

Chemical testing for fentanyl in post race urines is difficult due to the great potency of this drug [3]. Fentanyl is about 100 times more potent

than morphine or most of the other narcotic analgesics that are readily available. For this reason the High Performance Thin Layer Chromatographic (HPTLC) methods generally used to detect narcotic analgesics in horse urine are virtually ineffective for detection of fentanyl. Detection of this drug in post race urines has therefore been dependent on RIA screening [8,11].

A problem with the use of RIA as a routine screening test for fentanyl is that the test has to be run specifically for fentanyl. This is a cumbersome procedure, and unless the probability of fentanyl being present in the sample is high, the cost of the procedure relative to the yield of positives is relatively high. For this reason, the use of RIA screening for fentanyl has tended to be restricted to circumstances where the probability of detecting fentanyl is high. To our knowledge, the routine use of fentanyl RIA as a post race screening test is not common in North America.

The methodology reported in this paper markedly increases the utility of the fentanyl RIA for routine screening because it increases its scope while reducing its cost. In the first place, because of the increased sensitivity, it was possible to reduce the concentrations of antibody in the system by 15-fold. This has the effect of "extending" the antibody, which is relatively expensive when purchased commercially. Beyond this, however, the sensitivity of the test was increased up to 100-fold. This increased sensitivity can be used to detect smaller amounts of this drug in urine, or conversely, it can allow pooling of samples, and the simultaneous screening of larger numbers of samples post race. Using this approach, for example, it should be possible to pool a day's (10-20) samples, screen them for the presence of fentanyl, and then pinpoint the source of any fentanyl in a second screen, if this is necessary.

The rationale behind the pooling of samples depends on the sensitivity of the test. For example, to produce a measurable pharmacological response to fentanyl in the horse, it takes about 1000 µg/horse of fentanyl [4]. This figure is in good agreement with data obtained on humans, where the clinical dose to produce a pharmacological effect in a human is about 250 µg/human. Based on those figures, therefore, it appears that a dose of fentanyl of 100 µg/horse would be unlikely to have any significant pharmacological effect on a horse.

As well as being a very potent drug, the pharmacological actions of fentanyl are short lived. The effects of fentanyl on locomotor responses and pain perception, are over within one hr of administration of this drug, although the effects on respiratory rate and temperature appear to last longer [4]. It therefore appears likely that the effects of fentanyl on a racing horse that would be of significance for performance effects are like to be over within one hr of dosing, and certainly within 4 hrs of dosing.

Based on this information, it appears reasonable that the smallest dose of fentanyl likely to produce an effect on a racing horse would be about 100 µg/horse, and that any clinical dose is unlikely to produce a pharmacological effect by 4 hrs after dosing. There is, therefore, a very substantial reserve of sensitivity in this modified radioimmunoassay, since it can readily detect administration of doses of fentanyl of less than 1 µg/horse and can detect this dose for at least 24 hrs after administration.

This large reserve of sensitivity suggests that the illicit use of fentanyl in horses could be detected by pooled post race samples. For example, about 0.25 ml of each sample from a day's post race urines could be pooled, and the pooled samples stored frozen until the end of the week. Then at this time, an aliquot of each day's pooled post race samples would be screened for fentanyl. Because of the great sensitivity of the modified test, dosing with a pharmacologically significant amount of fentanyl would likely be detectable in the pooled samples by the modified RIA test. In the event of a positive, each of the targeted day's individual samples can be screened individually and the source of the fentanyl readily identified. Using this methodology, one week's post race urines could be screened for fentanyl in one day. The only requirement for this screening is that the original samples would have to be retained frozen for up to 7 days so that the presence of the drug could be confirmed in the sample. In this way, the costs of post race screening for fentanyl could be substantially reduced, and the scope of testing increased.

The great sensitivity of this assay also led us to determine whether this test would detect sufentanil in post race urines. As shown in Fig. 3 the reaction of antibody with sufentanil is sufficient to allow detection of administration of 40 µg/horse of sufentanil, although at only about 1/100 of the sensitivity with which it can detect fentanyl. Nevertheless the sensitivity of the test is such that it is likely capable of detecting sufentanil in clinically effective doses in post race urines.

REFERENCES

1. Tobin, Thomas; Combie, Joan; Miller, James; Crisman, Mark; Blake, Jerry. Irish Vet. J. 1979, 33, 169-176.

2. Jaffe, Jerome; Martin, William. IN Goodman, Louis; Gilman, Alfred (eds). "The Pharmacological Basis of Therapeutics", MacMillan: New York, 1975, pp 494-534.

3. Martin, William. Pharmacol. Rev. 1984, 35, 283-323.

4. Combie, Joan; Shults, Ted; Tobin, Thomas. IN Tobin, Thomas; Blake, Jerry; Woods, William (eds). "Proceedings of the Third International Symposium on Equine Medication Control", Lexington, KY, 1979; pp 311-317.

5. Tobin, Thomas. J. Eq. Med. Surg. 1978, 2, 397-399.

6. Combie, Joan. MS Thesis, University of Kentucky, Lexington, KY, 1979, pp 26-29.

7. Frinke, J; Henderson, G. Drug Metab. Disp. 1980, 8, 425-427.

8. Maylin, George. IN Tobin, Thomas; Blake, Jerry; Woods, William. "Proceedings of the Third International Symposium on Equine Medication Control", Lexington, KY, 1979; pp 281-287.

9. Tai, Hsin-Hsiung; Yuan, Barbara. Anal. Biochem. 1978, 87, 343-349.

10. Michiels, M; Hendriks, R; Heykants, J. J. Pharm. Pharmacol. 1983, 35, 86-93.

11. Tobin, Thomas. "Drugs and the Performance Horse", Thomas: Springfield, IL, 1981; pp 363-397.