FENTANIL GROUP ELISA FOR THE DETECTION OF REMIFENTANIL AND OTHER NARCOTIC ANALGESICS

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ABSTRACT

The Fentanil Group ELISA kit (Neogen Corporation, Kentucky, USA) is an effective screening device for detecting remifentanil and 4 other narcotics: sufentanil, norsufentanil, alfentanil and carfentanil, based upon standard curves and administration sample data. The sensitivity, or I_{50}, for remifentanil is: 1.1 ng/ml in buffer, 6.1 ng/ml in equine serum, 4.4 ng/ml in equine urine, 3.9 ng/ml in equine plasma and 1.8 ng/ml in canine urine. At a 1.0 mg iv injection, remifentanil was detectable for at least 8 h in post administration equine urine. Carfentanil, sufentanil and alfentanil were also detectable in post administration equine urine samples.

INTRODUCTION

Remifentanil (4-methoxycarbonyl-4-[(1-oxopropyl) phenylamino]-1-piperidinepropionic acid methyl ester) is part of the fentanyl narcotic family which includes sufentanil, alfentanil, carfentanil and fentanyl. These μ-opioid receptor agonists were first synthesised by Chesher (1990). They all have similar effects in man including analgesia, respiratory depression, nausea and vomiting (Bovill 1987). However, potency varies greatly within the group; for example fentanyl has 50–100 times the analgesic potency of morphine (Flannagan *et al.* 1996) and lofentanil and carfentanil are 20–30 times as potent as fentanyl (Clotz and Nahata 1991). In the horse, μ-opioid receptor agonists increase locomotor activity and stimulate movement while, at the same time, providing pain relief. The Association of Racing Commissioners International Uniform Classification Guidelines for foreign substances have classified these drugs as Class I agents (A.F. Lehner, unpublished data). Remifentanil, being the most recently synthesised, is the newest threat from this class of agents.

Current detection methods for remifentanil and the other opioids include the Fentanil Group ELISA kit, gas chromatography and mass spectrometry analysis (A.F. Lehner, unpublished data).

MATERIALS AND METHODS

Dosing and sample collection

Horse 733 was dosed with a 1.0 mg iv injection of remifentanil. Horse 873 was dosed with a 20 mg iv injection of alfentanil. Horse 697 was dosed with a 0.66 mg iv injection of sufentanil. Horse 552 was dosed with a 0.3 mg iv injection of carfentanil. Horse 695 was dosed with a 20 mg iv injection of fentanyl. Urine was collected at 0, 1, 2, 4, 6, 8 and 24 h post injection. All samples were frozen until the time of testing.

The background study used samples taken at the racecourse which had previously tested negative for a panel of drugs.

One step ELISA

The ELISA is a competitive assay in which the drug in the sample, if present, competes with the drug:enzyme conjugate for a limited number of previously bound antibody binding sites on a Costar (Costar Corporation, Massachusetts, USA) 96-well microplate. The first step is to place 20 μl of the sample or a standard into the wells. Next, 180 μl of the diluted drug:enzyme conjugate are added to each well and incubated for 45 min at room temperature. The solution in the wells is then discarded and the wells washed 3 times with a mild wash buffer and tapped dry. A 3,3',5,5'-tetramethylbenzidine (TMB) based substrate is added at 150 μl per well and incubated for 30 min at room temperature. The plate is read at 650 nm on a microplate reader (Bio-Tek Instruments, Inc., Vermont, USA). Colour development is inversely proportional to the amount of drug present in the sample. Therefore, the well is light blue to no colour in the presence of the drug and is blue in its

TABLE 1: Cross-reactivity in EIA buffer

Sufentanil	**270%**	**Remifentanil**	**76%**	**p-Fluorofentanyl**	**0.19%**
Norsufentanil	**119%**	**Fentanyl**	**2.0%**	**3-Methylfentanyl**	**0.08%**
Alfentanil	**100%**	**Thienylfentanyl**	**1.1%**	**Norfentanyl**	**0.04%**
Carfentanil	**88%**	**α-Methylfentanyl**	**0.77%**		
Acepromazine	<0.01%	Niacinamide	<0.01%	Prednisolone	<0.01%
Lidocaine	<0.01%	Dexamethasone	<0.01%	Furosemide	<0.01%
Acetaminophen	<0.01%	Nortriptyline	<0.01%	Primadone	<0.01%
Lofentanil	<0.01%	Dextromethorphan	<0.01%	Gemfibrozil	<0.01%
E-Amino-n-Caproic Acid	<0.01%	Orphenadrine	<0.01%	Procainamide	<0.01%
Mazindol	<0.01%	Dextromoramide	<0.01%	Gentisic Acid	<0.01%
Amitriptyline	<0.01%	Oxycodone	<0.01%	Procaine	<0.01%
Meperidine	<0.01%	Dezocine	<0.01%	Glipizide	<0.01%
Anileridine	<0.01%	Oxymorphone	<0.01%	Promazine	<0.01%
Metaproterenol	<0.01%	Diclofenac	<0.01%	Glutethimide	<0.01%
Ascorbic Acid (Vitamin C)	<0.01%	Oxyphenbutazone	<0.01%	Pyrantel	<0.01%
Methadone	<0.01%	Dihydrocodeine	<0.01%	Glycopyrrolate	<0.01%
Aspirin	<0.01%	Penicillin-G-Potassium	<0.01%	Pyrilamine	<0.01%
Methaqualone	<0.01%	Dimethyl Sulfoxide	<0.01%	Heparin	<0.01%
Benzoylecgonine	<0.01%	Penicillin-G-Procaine	<0.01%	Quinidine	<0.01%
Methocarbamol	<0.01%	Dipyrone	<0.01%	Hordenine	<0.01%
Buprenorphine	<0.01%	Pentazocine	<0.01%	Quinine	<0.01%
Methylene Blue	<0.01%	Doxepin	<0.01%	Hydrocodone	<0.01%
Butorphanol	<0.01%	Pentoxifylline	<0.01%	Salbutamol (Albuterol)	<0.01%
Methylphenidate	<0.01%	Erythromycin	<0.01%	Hydrocortisone	<0.01%
Chlordiazepoxide	<0.01%	Phenazocine	<0.01%	Salicylamide	<0.01%
6α-Methylprednisolone	<0.01%	Ethyl-p-amino-benzoate		Hydromorphone	<0.01%
Chlorpromazine	<0.01%	(Benzocaine)	<0.01%	Salicylic Acid	<0.01%
Morphine	<0.01%	Phencyclidine	<0.01%	Ibuprofen	<0.01%
Clenbuterol	<0.01%	Ethylmorphine	<0.01%	Theophylline	<0.01%
Nalbuphine	<0.01%	Phenothiazine	<0.01%	Imipramine	<0.01%
Codeine	<0.01%	Etorphine	<0.01%	Thiamine	<0.01%
Nalorphine	<0.01%	Phenylbutazone	<0.01%	Isoxsuprine	<0.01%
Cotinine	<0.01%	Fenoprofen	<0.01%	Trimipramine	<0.01%
Naproxen	<0.01%	Polyethylene Glycol	<0.01%	Levorphanol	<0.01%
Despropionylfentanyl	<0.01%	Flunixin	<0.01%		

absence (or in the presence of horseradish peroxide).

Results and Discussion

Matrix effects

Background studies were performed with the Fentanil Group ELISA to determine if any endogenous components contained in equine urine could interfere with the assay. Such interference would be capable of binding to the antibody, thus prohibiting a significant portion of the drug:enzyme conjugate from binding. This matrix-induced behaviour then gives a false appearance of drug in the sample. To eliminate the problem, a dilution of the sample with the buffer is performed where necessary. In this study, post race samples of equine urine, canine urine and equine plasma were examined. Equine urine was found to require a 1:1 dilution with enzyme immuno-assay (EIA) buffer. Of 40 equine samples diluted 1:1 the highest apparent alfentanil concentration was 0.52 ng/ml (52%B/B_0). Fifty canine urine and 40 equine plasma samples did not require any sample treatment. The highest apparent alfentanil concentrations were 0.55 ng/ml (56%B/B_0) and 0.22 ng/ml (71%B/B_0), respectively. Normal equine serum samples were not available for evaluation. The absorbance values for one sample of equine serum used for standard curve evaluation suggest that no sample treatment should be required when screening equine serum.

Sensitivity and cross-reactivity

The sensitivity of an ELISA is measured by its I_{50}, which is defined as the drug concentration that gives 50% less colour activity than the zero standard. Extensive screening was performed with the Fentanil Group ELISA kit to determine if any drugs showed the sensitivity required to be considered a cross-reactant to the kit. A variety of illegal and therapeutic drugs, fentanyl analogues, drug vehicles and potential masking agents were tested (Table 1). The percent cross-reactivity was calculated by dividing the I_{50} of the cross-reactant

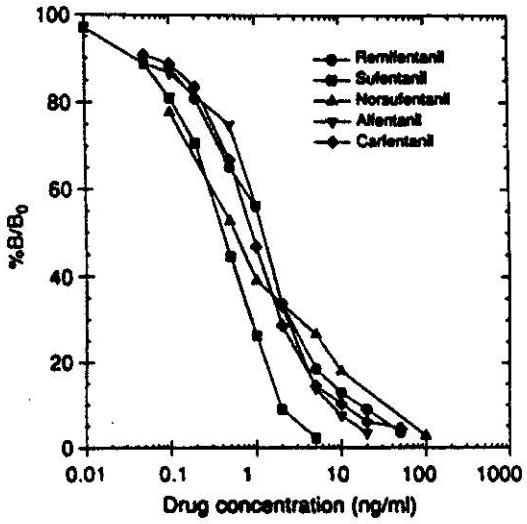

Fig 1: Standard curves in EIA buffer.

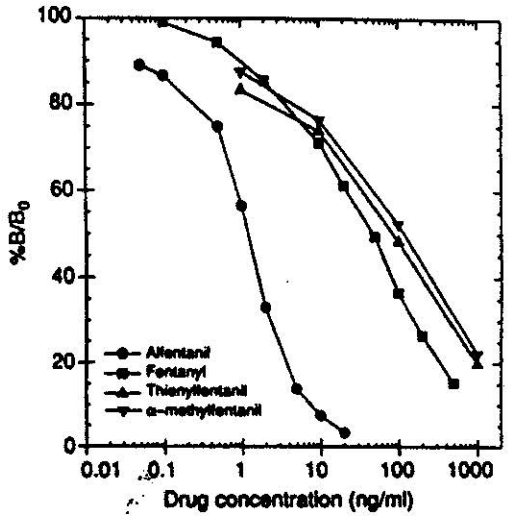

Fig 2: Standard curves in EIA buffer.

by the I_{50} of the parent compound and then multiplying by 100. Alfentanil was used as the parent compound. An alfentanil standard curve was performed each time a cross-reactant was assayed. Each I_{50} was calculated from a regression equation.

The Fentanil Group ELISA is extremely sensitive for remifentanil, sufentanil, norsufentanil, alfentanil, and carfentanil. Except for norsufentanil, standard curves were run in 5 media: EIA buffer, equine urine (diluted 1:1), canine urine, equine plasma and equine serum. A standard curve in EIA buffer was the only medium evaluated for norsufentanil because of lack of availability of this compound. The equine standard curves were made in neat equine urine and then diluted 1:1 with EIA buffer. Without changing the concentration during the I_{50} calculation, the shift in sensitivity from diluting the sample is illustrated (see Figs 1 and 3 for comparison of remifentanil standard curves). When screening samples are diluted 1:1, the actual concentration of drug in the sample will decrease due to dilution but the occurrence of false positives will also decrease by eliminating the matrix effects. Although diluting the samples will decrease possible drug concentration, this sample treatment will increase detection of the drug without obtaining false positives.

In EIA buffer, the I_{50} for remifentanil is 1.1 ng/ml, 0.3 ng/ml for sufentanil, 0.8 ng/ml for alfentanil and 0.9 ng/ml for carfentanil. In equine urine (diluted 1:1), the I_{50} for remifentanil is 4.4 ng/ml, 0.9 ng/ml for sufentanil, 2.5 ng/ml for alfentanil and 2.4 ng/ml for carfentanil. In canine urine, the I_{50} for remifentanil is 1.8 ng/ml, 0.3 ng/ml for sufentanil, 1.2 ng/ml for alfentanil and 0.7 ng/ml for carfentanil. In equine plasma, the I_{50} for remifentanil is 3.9 ng/ml, 1.1 ng/ml for sufentanil, 2.0 ng/ml for alfentanil and 5.9 ng/ml for carfentanil. In equine serum, the I_{50} for remifentanil is 6.1 ng/ml, 1.1 ng/ml for sufentanil, 2.3 ng/ml for alfentanil and 8.7 ng/ml for carfentanil. Figure 3 illustrates the differences in sensitivity with each medium using Remifentanil.

Other significant cross-reactants include fentanyl, norsufentanil, thienylfentanyl and α-methylfentanyl. Their I_{50}s in EIA buffer are 41 ng/ml, 0.7 ng/ml, 62 ng/ml and 92 ng/ml, respectively. Figures 1 and 2 illustrate the standard curve comparisons in EIA buffer.

Duration of detection

'Apparent' concentration of an administration sample refers to the result being assumed to be equivalent to the parent drug and its metabolites combined. All administrations were quantified against an alfentanil standard curve. Depending on the kit's sensitivity for the particular drug, the concentration would increase or decrease if the administration samples were compared against a standard curve for the same drug as the administration itself. Figure 4 illustrates each of the administration results as $\%B/B_0$ for easy comparison. The time point was considered detectable if less than 30% B/B_0 but this is not a recommended cut-off value. Assignment of cut-off values should be the responsibility of each drug-testing laboratory.

Pre-dose and post dose data from the iv injection of remifentanil indicated detectability of the drug for

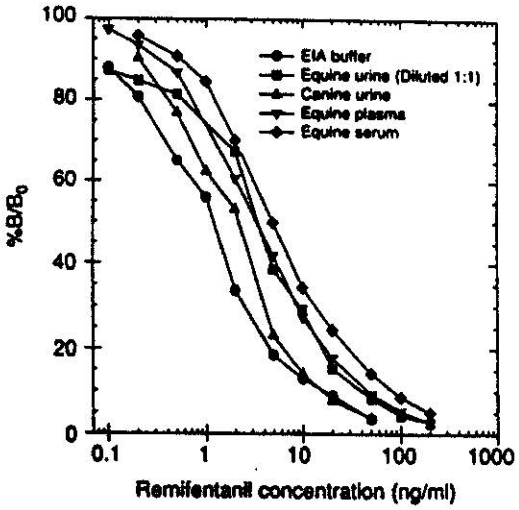

Fig 3: Remifentanil standard curves in 5 media.

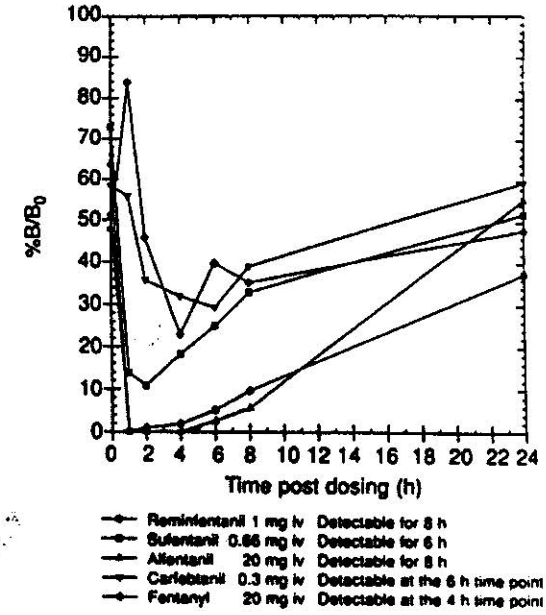

Fig 4: Duration of detection in equine urine.

at least 8 h in equine urine. The concentration vs dose curve peaked at 1 h with an apparent alfentanil concentration of 171 ng/ml, then decreased gradually to 14 ng/ml at 8 h. The urine was diluted up to 1:100 with EIA buffer to quantify the samples by bringing their absorbance values within range of the standard curve. The data were then back-calculated to the recommended sample treatment of 1:1. Alfentanil administrations were detectable for at least 8 h in equine urine. The peak level occurred at 2 h with an apparent alfentanil concentration of 419 ng/ml before decreasing gradually to 16 ng/ml at 8 h. The samples were also diluted up to 1:100 with EIA buffer and back-calculated to the 1:1 sample treatment. Sufentanil proved to be detectable for at least 6 h. Peak levels of sufentanil occurred at 2 h with an apparent alfentanil concentration of 5 ng/ml then decreased gradually to 2.9 ng/ml at 6 h. Sample treatment required up to a 1:10 dilution with EIA buffer to fit in the standard range. The data were then back-calculated to the recommended sample treatment of 1:1. Carfentanil was only detected at the 6 h time point having an apparent alfentanil concentration of 2.2 ng/ml. Fentanyl was detected at 4 h with an apparent alfentanil concentration of 3.1 ng/ml (Fig 4).

Because the fentanyl administrations were quantified against an alfentanil standard curve, fentanyl administrations gave a very low apparent alfentanil concentration. The fentanyl administrations would give an approximately 50 times greater concentration of apparent fentanyl if quantified against a fentanyl standard curve on the Fentanil Group ELISA kit.

Conclusion

Remifentanil is a new potential for abuse in the racing industry, having only been synthesised by GlaxoWellcome in 1996. The Fentanil Group ELISA kit provides an excellent screening tool for this drug. Although fentanyl was detected in the post administration samples, the kit cannot be recommended for routine screening due to its lack of sensitivity illustrated by the standard curve data. The Fentanil Group ELISA kit has been used successfully as a screening assay for sufentanil, alfentanil and carfentanil as demonstrated by the 5 media standard curves and duration of detection data for post administration samples. Further, this ELISA format provides the opportunity to screen for 5 narcotics simultaneously, creating cost- and time-efficient screening.

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