Utilization and comparison of immunochemical vs. classical techniques in the screening and confirmation of fentanyl in equine urine and plasma

by

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INTRODUCTION

Since the early 1900s, in organized pari-mutuel horse racing, some form of drug testing has been necessary to insure that the industry is operating "drug-free", and all horses have equal opportunity to win races and resultant purses. In the early part of this century, the temptation to "dope" a horse was extensive. In fact, the practice of doping was widespread due to the inadequate detection techniques available at the time. These inadequacies allowed some trainers to perform criminal acts (doping), while those trainers who did obey the rules were put at a severe disadvantage. In one of the earliest reported instances of doping around the turn of the century, horses were described as winning races with their eyes popping out of their heads, sweating profusely, and running as if possessed by the devil. One horse reportedly killed itself by running into a stone wall following a race. Testing of horses for drug presence by chemical analyses of bodily fluids was first introduced in Europe in 1910. In the first reported "positive", Bourba Rose was disqualified after winning France's golden cup in 1912 (Tobin, 1981).

As the years progressed, the ability of the chemist to analyze and detect various drugs and substances within the animal progressed to the point at which we are today. Using today's technology, chemists have the ability to detect an enormous number of drugs at extremely minute concentrations in biological fluids. Chemical analysis of biological samples, usually urine and plasma, is a three step process: extraction of exogenous agent from the biological sample; followed by screening of the sample for any suspected agents; and legal confirmation of the agent's identify within the sample.

Currently there are three commonly used screening procedures: 1) classical liquid-liquid extraction followed by thin-layer chromatography and visualization of the plate, 2) liquid-liquid extraction followed by high-performance liquid chromatography (HPLC) with ultraviolet absorption or gas chromatography with flame ionization or nitrogen selectivity, or 3) the newly developed immunoassay methodology such as radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and particle

concentration fluorescence immunoassay (PCFIA). Gas chromatography/mass spectrometry is the methodology commonly utilized to legally confirm analyte presence within a sample, regardless of the screening procedure used.

The purpose of the study was to determine the relative merits among several immunochemical techniques, and to compare the relative sensitivity of classical detection to immunoassay and instrumental methods in the screening and confirmation of the narcotic analgesic fentanyl in equine urine and plasma.

LITERATURE REVIEW

General Review of Fentanyl

Characteristics in man

Fentanyl, 1-(2-phenethyl)-4-N-(N-propionyl-anilino) piperidine, is an extremely potent synthetic narcotic analgesic with a very rapid onset of action. In man the major action is analgesia associated with euphoria and respiratory depression. Higher doses can produce muscle rigidity. Fentanyl is a 4-anilino-piperidine derivative of meperidine. First manufactured by Janssen in 1960, fentanyl was found to have pharmacological actions similar to morphine, and was approximately 400 times more potent as an opioid agonist in the tail-withdrawal reflex reaction of rats (Janssen et al., 1963). The citrate salt of fentanyl, fentanyl citrate, N-(1-phenethyl-4-piperidyl) propionanilide, commercially known as Sublimaze®, was also derived from meperidine and was first synthesized by Janssen in 1959 (Janssen et al., 1963; Janssen, 1964).

In man, fentanyl has been used since the early 1960s for the relief of pain and pre-operative anesthesia (Holderness et al., 1963; Dobkin and Su, 1966). Fentanyl has enjoyed widely accepted use due to a very fast onset and short duration of action, and a potency clinically estimated at approximately 150 times that of morphine (Janssen et al., 1963; Shephard, 1965; Finch and DeKornfeld, 1967; Grell et al., 1970; Hess et al., 1972; Martin, 1984; Jaffe and Martin, 1985). One of the major drawbacks of fentanyl is that high doses may cause severe respiratory depression either initially, or several hours after administration (Downes et al., 1967; Finch and DeKornfeld, 1967; Grell et al., 1970; Anderson et al., 1976; Becker et al., 1976; Harper et al., 1976; Adams and Pybus, 1978; McQuay et al., 1979; Stoeckel et al., 1979; Klausner et al., 1988). Fentanyl was implicated in the overdose deaths of several individuals in the United States who gave themselves very large intravenous injections, apparently dying from extreme respiratory depression (Garriott et al., 1984; Pare et al., 1987).

Characteristics in equine

As previously mentioned, in man the major action of fentanyl is analgesia, with euphoria and respiratory depression. When administered to the horse, however, a wide variety of effects occur including respiratory depression, analgesia, increased cardiac and respiratory rates, and most importantly stimulation of the central nervous system. Instead of sedating the horse, when administered at low levels fentanyl stimulated the horse to a brisk trot, while at the same time alleviating any lameness (Combie, 1979; Combie et al., 1979; Tobin et al., 1979a; Tobin et al., 1979b; Tobin, 1981; Kamerling et al., 1985).

The fact that fentanyl can stimulate the locomotor activity of the horse is of extreme importance to the pari-mutuel horse racing industry. Trainers may give their animal an injection of fentanyl hoping to gain an unfair advantage, leaving trainers who obey the rules at a disadvantage because they do not resort to criminal tactics.

Almost all racing jurisdictions in the United States have rules prohibiting the use of fentanyl in the racing equine. As previously mentioned, fentanyl is classified as a narcotic analgesic, and all narcotic analgesics stimulate the central nervous system of the equine. The classification of fentanyl, however, was not always as a stimulant within the horse. In 1977 the "California Rules of Racing" listed fentanyl as a depressant on its list of prohibited drugs. This classification dramatically changed in 1979, as Tobin demonstrated that a small dose of fentanyl stimulates the locomotor response in the horse upon administration (Tobin et al., 1979a; Tobin et al., 1979b).

Tobin et al. (1979b) developed a method to quantitate the presence of fentanyl in the horse. The method involved counting the number of times the animal lifted its left front foreleg every two minute period. It was reported that a dose of 2.0 μg/kg was being used in the racing industry to dope a horse. However, when this dosage was injected intravenously, no observable effects were noticed (Tobin et al., 1979b). A dose between 10.0 μg/kg and 20.0 μg/kg produced an effect that peaked at about 110 steps/2 minute period and then declined to control values at 60 minutes. When the dosage was increased to 40.0 μg/kg the horse became uncoordinated, staggered, and sometimes

fell within the first few minutes. The intensity of the trotting response was directly proportional to the level of fentanyl injected intravenously into the horse (Tobin et al., 1979b; Combie et al., 1979).

The maximum locomotor activity of fentanyl occurred at approximately five minutes following intravenous administration, corresponding to a concentration of 50 ng/ml in the blood stream (Combie, 1979; Combie et al., 1979; Tobin et al., 1979b; Kamerling et al., 1985). When administered intramuscular or subcutaneously, the response peaked between 20 and 30 minutes post-injection, was erratic, and much less intense than after an intravenous injection (Tobin et al., 1979b). This was particularly interesting, for intramuscular was the method of injection reported in the racing industry (Tobin, 1981).

Other symptoms reported to be produced upon fentanyl administration include tachycardia, decreased body temperature, and constriction of pupils (Kamerling et al., 1985). Fentanyl has also been shown to depress equine abdominal pain syndrome (colic), and has clinical application to alleviate colonic spasms (Roger et al., 1985). The narcotic antagonist naloxone, has been shown to counteract the effects of fentanyl in the equine. Pretreatment with naloxone prior to fentanyl injection will completely block the locomotive response (Combie et al., 1981).

Metabolism and elimination

Because this research involved the administration of fentanyl to horses, the detection of fentanyl metabolites was equally important to detection of the parent drug. Even when none of the original drug was present, metabolites indicated administration of the parent compound. Most racing jurisdictions judge metabolite presence as legal evidence that the parent compound was administered.

Because of fentanyl's high degree of lipophilicity, it requires biotransformation to more polar metabolites which are excreted more easily by the kidneys. The major metabolite discovered in the urine and plasma of the horse was N-[1-(2-phenethyl-4-piperidinyl)] malonanilic acid, called beta-keto acid, which is formed by oxidation of

the propionyl side chain of the parent fentanyl molecule (Maylin, 1979; Frincke and Henderson, 1980; Henderson et al., 1981). In addition to N-[1-(2-phenethyl-4-piperidinyl)] malonanilic acid, Maylin (1979) found approximately 10% of original administration excreted as despropionylfentanyl, 4-anilino-1-(2-phenylethyl)piperidine, a metabolite formed by amide hydrolysis of the parent fentanyl molecule (Figure 1).

It has been estimated that approximately 80-90% of the original dose is excreted in the equine urine as N-[1-(2-phenethyl-4-piperidinyl)] malonanilic acid within 24 hours, when a small dosage between 0.1 μg/kg to 0.5 μg/kg is administered. Larger doses (5.0 μg/kg) resulted in 50% of original dose being excreted in the urine as N-[1-(2-phenethyl-4-piperidinyl)] malonanilic acid within 24 hours post administration (Maylin, 1979; Frincke and Henderson, 1980; Henderson et al., 1981). The level of metabolites recovered seems to be dependent on the concentration of the original dosage administered.

The literature seems to indicate two major metabolic pathways within man and rodents for fentanyl. One pathway (Figure 2) is oxidative N-dealkyation of the parent molecule to norfentanyl, 4-N-(N-propionylanilino)piperidine (Van Wijngaarden and Soudijn, 1968; Van Rooy et al., 1981; Goromaru et al., 1982; Goromaru et al., 1984; Schneider and Brune, 1986). In addition to norfentanyl, other oxidative products of the propionyl or piperidine side chain have been identified as metabolites (Goromaru et al., 1982; Goromaru et al., 1984). The other pathway is the same as that found in the equine, amide hydrolysis forming despropionylfentanyl (Maruyama and Hosoya, 1969; Van Rooy et al., 1981; Schneider and Brune, 1986).

Pharmacokinetics

The locomotor effects on the equine can be reproduced in a very short period of time (approximately one hour), and there is one very good reason for this reproducibility. The action of fentanyl within vertebrates is ended by fentanyl being carried away from the brain in the plasma. Because an intravenous injection causes a

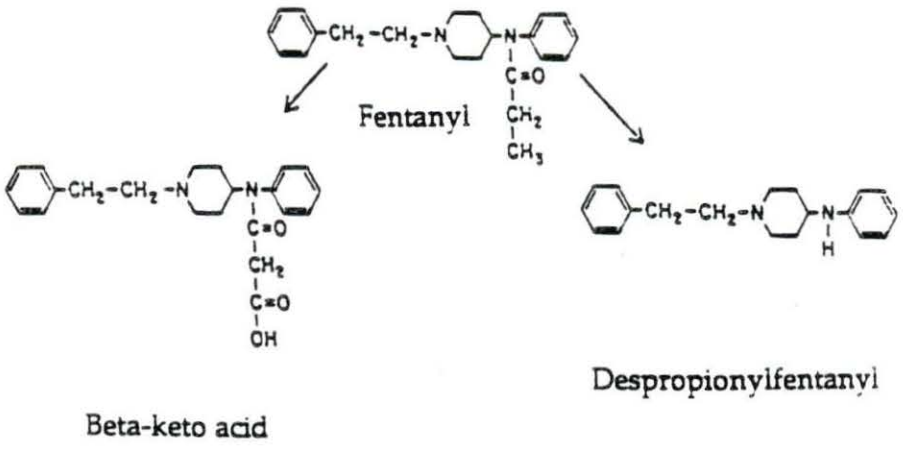


Figure 1. Metabolic pathway of fentanyl in the equine

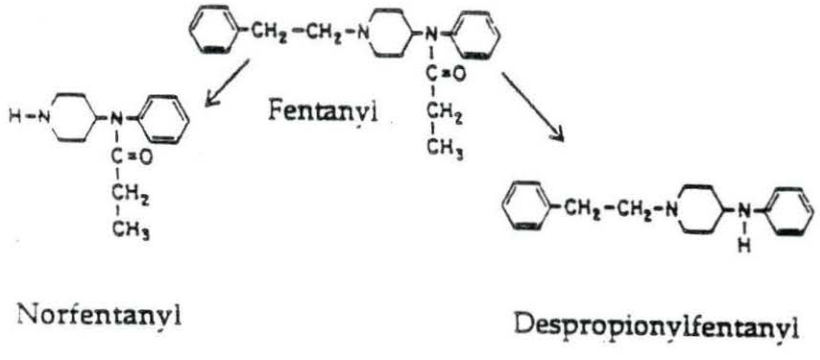


Figure 2. Oxidative and hydrolytic pathway of fentanyl

"bolus" of dose to be administered, and fentanyl is highly hydrophobic, high concentrations appear in tissues, organs, and the cerebrospinal fluid rather quickly, causing the rapid onset of action (Hess et al., 1971; Hess et al., 1972; Schleimer et al., 1976; Ainslie et al., 1979; Hug and Murphy, 1979; Combie et al., 1979; McClain and Hug, 1980). However, uptake rate of fentanyl by tissues and the blood brain barrier is regulated by the blood flow. The pharmacological action is stopped by the redistribution of fentanyl from the central nervous system (cerebrospinal fluid), rather than by metabolism (Tobin et al., 1979b; Combie et al., 1979). Thus, the increase in cardiovascular and locomotor parameters occur promptly, while the respiratory and analgesic responses occur somewhat later and are longer in duration (Kamerling et al., 1985).

Although much research has been done concerning the pharmacokinetics of fentanyl in man, very little information is available regarding the equine. Hess et al. (1972), utilizing human subjects found that 10 minutes following intravenous injection of fentanyl, approximately 98% of the original dose had already left the blood stream. The organs which attain the highest concentration of fentanyl 0.5 minutes following intravenous administration are the lung, heart, and kidneys. Five minutes after administration, the areas of largest concentration are the intestinal wall, skeletal muscle, and liver, followed by adipose tissue which occurs at approximately 30 minutes post administration (Hess et al., 1971). The concentration in gastric juice and stomach has also been determined, with approximately 3-4% of original dose being present in gastric juice, and 16% located in the stomach (Stoeckel et al., 1979). Fentanyl has been calculated as having a mean half-life of 20 minutes (Van Rooy et al., 1981).

Even after 96 hours following the administration of 200 µg/horse, urine concentrations in the picogram level of fentanyl equivalents could still be detected. At a low dose, such as 1 µg/horse, urinary concentrations of fentanyl equivalents up to 24 hours could still be detected with assurance, further indicating fentanyl's slow redistribution from tissues (Tobin et al., 1986; Weckman et al., 1988).

Fentanyl elimination kinetics from plasma has been proposed as a three compartment model (Henderson et al., 1975; Schleimer et al., 1976; Michiels et al., 1977; Combie et al., 1979; Hug and Murphy, 1981). In the equine, the 1/2-life of the initial phase is approximately 3 minutes, followed by a 1/2-life of 42 minutes for the second phase, and a 1/2-life of 180 minutes for the final phase (Combie et al., 1981). Hug and Murphy (1981) hypothesize that in a three compartment model, the central compartment is the blood rich tissues; such as brain, kidney, heart, and lung, and the two peripheral compartments are muscle and adipose tissue. Initial phase may be reuptake of fentanyl by blood rich tissues, followed by redistribution, and the last phase of excretion (Lin et al., 1981).

Analysis of Fentanyl

The analysis of biological fluids for the presence of fentanyl has been reported via a variety of methods ranging from the fairly basic, such as thin-layer chromatography, to the extremely sophisticated, such as gas chromatography/mass spectrometry. Forensic analyses, in general, can be catagorized as being either a screening technique or a confirmatory technique. Screening methods are fairly rapid, less sensitive, and contain a lower degree of accuracy and a corresponding higher degree of false positives. Confirmatory methods on the other hand are longer in duration, have a high degree of accuracy and sensitivity, and are less susceptible to false positive results.

Immunoassay techniques

Immunochemical techniques were developed to detect specific antigens of interest in biological samples through the interaction with antibodies. Specifically, animals are challenged with a particular drug-protein complex and the antibodies produced in response to this immunogen are collected and purified. These antibodies are then used in the particular assay to test for the presence of original compound. Antibodies with high specificity will produce accurate and sensitive immunochemical assays.

When utilizing immunoassay technology as a screening procedure, several advantages have been noted in comparison to the classical "wet" chemistry analyses. Extraction of the biological sample, both urine and plasma, is usually not necessary when employing immunoassay, unlike the classical methods which require an acid hydrolysis of urine followed by liquid-liquid extraction. Plasma samples require extraction only. Some antibodies used for immunoassay detection can detect drug metabolites with similar structures to the original parent molecule. However, these tests do not distinguish between parent and metabolite. Several different formats of immunochemical detection of fentanyl and related compounds have been developed.

Radioimmunoassay Radioimmunoassay was developed to determine fentanyl equivalents in plasma and urine through the interactions with antisera. Fentanyl equivalents are designated as the parent fentanyl molecule and its metabolites. The fentanyl equivalents within the sample compete with radioactive tracers for fentanyl binding sites on fentanyl antibodies. The radioactive tracers can be either ^{125}I, ^{3}H, or ^{14}C. The bound and free fentanyl are separated, and the degree of radioactivity in the bound or unbound portion is determined using a beta scintillation counter. When counting the radioactivity of the unbound portion, the quantity of fentanyl equivalents present in the original sample would be inversely related to the amount of radioactivity measured. A few drawbacks to performing radioimmunoassay include the prospect of laboratory personnel working with radioactive material, and proper disposal of radiotracer materials.

One of the earliest radioimmunoassays for fentanyl was developed by Henderson et al. (1975), who obtained fentanyl antibodies by immunizing rabbits with a carboxyfentanyl-bovine gamma globulin conjugate, and then precipatating out the antibodies for use in the assay. Early antibodies developed suffered from a rather poor level of sensitivity (approximately 10 ng total fentanyl), and limited cross-reactivity in identifying fentanyl metabolites and analogs. Since that time, however, various investigators succeeded in improving the sensitivity and cross-reactivity of fentanyl radioimmunoassays through various modifications. In 1977, Michiels et al. (1977)

increased the sensitivity of the assay to 60 pg/ml by using 4-oxo-4{phenyl-[1-(2 phenylethyl)-4-piperidinyl] amino} butanic acid (a fentanyl analog) conjugated to bovine gamma globulin as the immunogen.

One of the commercial assays currently available for the detection of fentanyl equivalents is produced by Janssen Life Sciences Products (Piscataway, N. J.). The assay has a detection limit of 0.2 ng/ml, and limited cross-reactivity with various fentanyl metabolites and analogs. The conjugate used to produce the assay antibodies was not listed in the accompanying product information.

In 1986, sensitivity of the fentanyl RIA was increased 100-fold down to 2 pg/ml by challenging with a similar immunogen to that utilized in the Janssen RIA (carboxyfentanyl-tyrosine methyl ester conjugate). More importantly, however, a radioiodinated (^{125}I), rather than a tritium labelled (^{3}H) analog was used in the competitive binding portion of the assay (Tobin et al., 1986; Woods et al., 1986; Weckman et al., 1988). The ^{125}I-fentanyl antibody has acceptable crossreactivity with sufentanil, 3-methylfentanyl, and alpha-methylfentanyl (Tobin et al., 1986; Weckman et al., 1988).

The order in which assay ingredients are added together can drastically affect the assay results. Schuettler and White (1984) compared results obtained when mixing antisera and sample followed by buffer and labelled antigen, with results when mixing sample and buffer followed by labelled antigen and antisera. Overestimations ranging from 29-76% were reporting employing the former addition sequence. The latter sequence allowed the labelled and unlabelled drug equal opportunity for binding sites on the antibody, thus producing more consistent results. Most radioimmunoassays recommend using this revised addition sequence.

Enzyme-linked immunosorbent assay One of the newer immunoassay techniques for fentanyl detection is called enzyme-linked immunosorbent assay (ELISA). It is superior to RIA in terms of expense, time, simplicity, instrumentation, and it does not require the handling of radioactive material. The test can usually be completed in one

hour and the cost for reagents and instrumentation is small compared to radioimmunoassay (Tobin and Blake, 1987).

Fentanyl equivalents compete with a drug-enzyme complex for high affinity antibodies that are bound to a microtitre well. The microtitre well is washed, substrate is added, and a color change is indicative of a negative response due to enzyme conversion of substrate over to colored product. No color change (known as "white-outs") denotes a sample as possibly positive for drug of interest, due to accumulation of non-labelled antigen on the antibodies and enzyme unavailable for substrate conversion (Tobin and Blake, 1987; Weckman et al., 1988).

The ELISA procedure is sensitive enough to detect minute quantities of fentanyl equivalents in plasma or urine shortly after administration. Weckman et al. (1988) showed that inhibition of the color change occurred at a concentration of 5.0 ng/ml. ELISA was shown to detect fentanyl metabolites and a fairly large number of fentanyl related compounds (Tobin and Blake, 1987; Weckman et al., 1988).

Various investigators have shown that fentanyl binds with a high degree of affinity to plasma proteins. A lipophilic base, such as fentanyl, commonly binds to albumin and lipoproteins (Bickel, 1975). Only the free, unbound drug can perfuse from the plasma, and it could lessen the amount of parent fentanyl available in the blood stream to perform its locomotor effects, and the possibility of protein binding could lessen the amount available for metabolism and ultimate elimination from the body. In addition, protein binding will lessen availability of free drug for antibody interactions, thus increasing the possibility of false negatives. To combat this problem, Weckman et al. (1988) added tricarboxylic acid to denature and remove the excess plasma proteins, and increase the sensitivity of the assay.

Particle concentration fluorescence immunoassay The newest addition to the immunoassay technology field is called particle concentration fluorescence immunoassay (PCFIA). Fentanyl equivalents compete with fentanyl-beta phycoerythrin conjugates for binding sites on fentanyl antibodies in a microtitre well. Following equilibration, a second antibody system is added, consisting of goat anti-

rabbit antibody bound to latex beads. After a second incubation step, the entire sample is vacuum filtered through a 0.2 micron membrane present in the bottom of the microtitre well, resulting in a concentration of the latex beads and any fluorescent phycoerythrin label. Following a wash step performed under vacuum, the degree of fluorescence of the particles are read at a particular wavelength, and the intensity of the fluorescence is inversely related to the amount of free drug in the sample (McDonald et al., 1987).

There is little information in the literature pertaining to fentanyl detection using particle concentration fluorescence immunoassay. McDonald et al. (1987) have estimated a threshold of sensitivity for the fentanyl PCFIA test in the order of 100 pg/ml. In addition, excellent cross reactivity was noted with various fentanyl analogs. Several problems have been noted with the PCFIA assay when utilized on horse urines. Blank horse urines contain some degree of endogenous fluorescent materials, enough to cause a 15% inhibition in the binding of the fentanyl antibodies (McDonald et al., 1987). This could possibly lead to several false positive results. Lastly, horse urines contain large quantities of precipitous and mucous material, requiring removal by filtration through a 0.22 micron membrane filter.

Classical chemistry methods

Fentanyl, as previously mentioned, is metabolized completely in the equine to two compounds at low doses, the beta-keto acid, and despropionylfentanyl. None of the parent compound is present in urine. Thus, when urine is utilized as the biological sample for the detection of drug presence, one of the two metabolites must be used for confirmation of fentanyl administration. Since the beta-keto acid accounts for approximately 90% of the original dosage, it would seem logical that this would be the compound in which to target. However, the beta-keto acid metabolite is very unstable, especially at high temperatures, and as such is rendered useless for underivatized gas chromatographic injections (Gallicano and Young, 1985). The other alternative is to use the despropionylfentanyl metabolite for ultimate confirmation. To overcome the

low level of despropionylfentanyl (10%) found in the urine, a "special" acid hydrolysis procedure is performed in which the urine is heated under pressure in the presence of an acid. This hydrolyzes the beta-keto acid and forms the despropionylfentanyl metabolite in large enough concentration for confirmation (McDonald and Ozog, 1978). The acid hydrolysis procedure is not performed on plasma samples because the parent fentanyl molecule can be ascertained in plasma due to the small degree of metabolism occurring within the blood stream.

Thin-layer chromatography Within the United States racing industry, the current method for initial screening of biological samples for the presence of exogenous constituents is liquid-liquid extraction followed by thin-layer chromatography (TLC). Following development of the chromatographic plate, the plate is visualized under short wave (254 nm) and long wave (366 nm) ultraviolet light for the presence of quenching and fluorescence, respectively. Lastly, the plate is subjected to a variety of chemical oversprays which highlight the presence of constituents within the original sample.

Thin layer chromatography is used to obtain qualitative information. It has been shown to be of poor sensitivity and is mainly used for initial forensic screening of biological samples. Maruyama and Hosoya (1969) report a detection limit of 2 ug for fentanyl by thin layer chromatography.

Utilizing thin-layer chromatography, the Rf values of fentanyl and its metabolites are predictable characteristics of these compounds. The Rf value is defined as the distance the drug of interest migrates up the chromatographic plate relative to the solvent front. The Rf value will vary depending on the mobile and stationary phase employed. The most common TLC developing solvent employed in forensic racing labs for fentanyl detection is ethyl acetate/methanol/acetic acid, 8:1:1 (Maylin, 1980). The Rf values of standards can then be used as references to isolate and detect fentanyl or despropionylfentanyl on the chromatographic plate. The chemical oversprays commonly utilized to detect the parent fentanyl molecule or its metabolite include

concentrated hydrochloric acid, followed by an iodoplatinate reagent. Fentanyl and despropionylfentanyl react and turn blue following the iodoplatinate reagent (Maylin, 1979).

High-performance liquid chromatography Two high performance liquid chromatographic (HPLC) methods for the separation and detection of fentanyl in biological samples have been reported: normal phase HPLC (Frincke and Henderson, 1980; Kumar et al., 1987), and reversed phase HPLC (Lurie et al., 1984a; Lurie et al., 1984b). The detection method most commonly used in conjuction with HPLC is ultraviolet spectrophotometric detection.

Standard fentanyl levels in the ppb range have been detected utilizing HPLC with the column effluent being monitored by a fixed wavelength ultraviolet detector at a wavelength of 195 nm (Kumar et al., 1987). In addition, Lurie et al. (1984a), have employed ultraviolet detection followed by absorbance ratioing of the chromatographic peaks to distinguish among twenty-six different fentanyl related compounds.

Gas liquid chromatography Gas liquid chromatography can be used for drug confirmation by utilizing various detection methods, such as flame ionization, nitrogen-phosphorus thermionic detection, or mass spectrometric detection. Of the three, gas chromatography/mass spectrometry is the method usually required as a minimum for legal confirmation of drug presence in urine or plasma by most pari-mutuel racing authorities.

Detection and confirmation of fentanyl and its metabolites have been reported by gas-liquid chromatography using packed glass columns (Gillespie et al., 1981; Lin et al., 1981; Phipps et al., 1983a; Van Rooy et al., 1981; Kowalski et al., 1987), normal capillary columns (Pare et al., 1987), and fused silica megabore columns (Kowalski et al., 1987). Kowalski et al. (1987) report that although packed columns gave adequate selectivity and sensitivity to below 1 ng/ml fentanyl, the sensitivity can only be maintained for approximately three weeks due to column degradation. On the other hand, fused silica megabore columns provide adequate sensitivity for over one year. In addition, it has

been noted that packed columns (OV-17) occasionally "bleed", causing problems with the chromatographic determination of fentanyl (Van Rooy et al., 1981).

The parent fentanyl molecule, as well as the despropionylfentanyl metabolite, can be detected by gas-liquid chromatography without derivatization. Several investigators, however, have employed derivatization to increase the sensitivity of their assay. Trimethylsilyl derivatives of fentanyl and its metabolites have been formed by reacting the sample residue with bistrimethylsilylacetamide (BSTFA) before injection into the gas chromatograph (Goromaru et al., 1982; Goromaru et al., 1984). Moore et al. (1986) were able to differentiate between fentanyl and twenty-five analogs and homologs upon derivatization with heptafluorobutyric anhydride in the presence of 4-(dimethylamino) pyridine. As previously mentioned, the beta keto-acid metabolite of fentanyl is thermally unstable, decarboxylating at temperatures greater than 150^{o}C. Gallacino and Young (1985) counteracted the thermal instability by preparing triflouroacetyl, trimethyl, and methyl derivatives of the compound. The derivatization prevented decarboxylation from occurring, resulting in useful chromatographic separation and detection.

Because fentanyl contains two tertiary nitrogen atoms it is possible to use a nitrogen-phosphorus thermionic detector for the detection of fentanyl. The nitrogen-phosphorus thermionic detector has been shown to be approximately 10-50 times more sensitive to molecules containing nitrogen atoms, and is considerably more useful in measuring low sample concentrations than a flame-ionization detector (Gillespie et al., 1981). The reported detection limit of fentanyl concentration employing a nitrogen-phosphorus detector ranged from 0.25 ng/ml (Woestenborghs et al., 1986) down to 0.02 ng/ml (Phipps et al., 1983a), and for a flame-ionization detector from 3.3 ng/ml (Van Rooy et al., 1981) down to .5 ng/ml (Phipps et al., 1983b). Various authors have utilized nitrogen-phosphorus and flame-ionization detectors in the analysis of fentanyl and its metabolites, employing various chromatographic parameters, columns, and obtaining various results (Tables 1 and 2).

Table 1. Operating conditions and detection limits of fentanyl concentrations measured by gas liquid chromatography using nitrogen-phosphorus or flame ionization detection.

ARTICLE	COLUMN SPECIFICATIONS	OVEN TEMP.	CARRIER GAS	DETECTION LIMIT	DETECTOR TYPE
Beaumier et al., 1979	10 m x 8 mm I.D.	260°C	NA[a]	NA	NPD
Gillespie et al., 1981	2 m x 2 mm I.D.	280°C	Helium	0.1 ng/ml	NPD
van Rooy et al., 1981	1.2 m x 2 mm I.D.	250°C	Helium	3.3 ng/ml	FID
Phipps et al., 1983a	3.05 m x 3.2 mm I. D.	290°C	Helium	0.02 ng/ml	NPD
Phipps et al., 1983b	6 ft. x 1/8 in. I.D.	290°C	Helium	1) 0.2 ng/ml 2) 0.5 ng/ml	NPD FID
Weldon et al., 1985	2 m x 2 mm I.D.	290°C	Helium	0.1 ng/ml	NPD
Woestenborghs et al., 1986	1 m x 2 mm I.D.	280°C	Nitrogen	0.25 ng/ml	NPD
	10 m x 0.32 m I.D.	235°C	Nitrogen	0.25 ng/ml	NPD
Kowalski et al., 1987	5 m, fused silica megabore	238°C	Nitrogen	0.1 ng/ml	NPD

[a]NA = Not available.

Table 2. Operating conditions and detection limits of fentanyl metabolite concentrations measured by gas liquid chromatography using nitrogen-phosporus or flame ionization detection

ARTICLE	COLUMN SPECIFICATIONS	OVEN TEMP.	CARRIER GAS	DETECTION LIMIT	DETECTOR TYPE	METABOLITE(S) IDENTIFIED
Gillespie et al., 1981	2 m x 2 mm I.D.	220°C	Helium	subnanogram	NPD	1) 4-N-anilinopiperidine 2) Norfentanyl 3) Despropionylfentanyl
van Rooy et al., 1981	1.2 m x 2 mm I.D.	230°C	Helium	1) .65 ng/ml 2) 5.4 ng/ml	FID	1) Norfentanyl 2) Despropionylfentanyl

Within most forensic racing labs, drug confirmation by gas chromatography/mass spectrometry is performed using positive ion collection with electron impact (EI) ionization. Fentanyl and despropionylfentanyl fragment to form characteristic mass spectra, and several ions are present in both fentanyl's and despropionylfentanyl's mass spectra. The three major ions formed by the fragmentation of fentanyl include m/z 245, which results from the loss of a benzyl radical, m/z 146, which has been identified as a N-phenethylpiperidine cation, and m/z 189, identified as a N-phenethylaziridium cation (Maruyama and Hosoya, 1969). The despropionylfentanyl compound undergoes fragmentation to three major ions as well. The m/z 280 (M^+) is a radical cation resulting from the loss of an unpaired electron from the parent despropionylfentanyl compound. The other two ions are those also found in the fragmentation pattern of the parent fentanyl molecule, m/z 146 and m/z 189 (Maruyama and Hosoya, 1969).

The lowest detection limit for gas chromatography/mass spectrometry has been reported as 0.2 ng/ml (Lin et al., 1981). Tobin et al. (1986) indicate that endogenous constituents present in equine urine may correspond to approximately 10 pg/ml fentanyl-like material and can reach as high as 50 pg/ml, thus it is necessary to take into consideration the possible presence of endogenous constituents when confirmation of fentanyl administration is attempted via mass spectrometry. Tables 3 and 4 list the detection limits and various other important chromatographic parameters employed with gas chromatography/mass spectrometry detection for fentanyl and its metabolites.

Comparative studies

Within the literature, various investigators have analyzed the detection capability of numerous procedures, on an individual basis, for fentanyl equivalents in biological samples. Two communications have compared gas liquid chromatography vs. radioimmunoassay. Utilizing sample spikes, Phipps et al. (1983a) chose gas liquid chromatography with nitrogen-phosphorus detection as the method of choice over

Table 3. Operating conditions and detection limits of fentanyl concentrations measured by gas liquid chromatography using mass spectrometric detection

ARTICLE	COLUMN SPECIFICATIONS	OVEN TEMP.	CARRIER GAS	DETECTION LIMIT
Lin et al., 1981	0.91 m x 2 mm I.D.	235°C	NA[a]	0.2 ng/ml
van Rooy et al., 1981	1.2 m x 2 mm I.D.	230°C	Helium	NA
Garriott et al., 1984	1.2 m x 2 mm I.D.	230-285°C	Helium	0.5 ng/ml
Goromaru et al., 1984	1 m x 3 mm I.D.	260°C	Helium	NA
Pare et al., 1987	50 m x 0.2 mm I.D.	150-270°C 20°/min	Helium	NA

[a]NA = Not available.

Table 4. Operating conditions and detection limits of fentanyl metabolite concentrations measured by gas liquid chromatography using mass spectrometric detection

ARTICLE	COLUMN SPECIFICATIONS	OVEN TEMP.	CARRIER GAS	DETECTION LIMIT	METABOLITE(S) IDENTIFIED
Maruyama & Hosoya, 1969	NA[a]	NA	NA	NA	Despropionylfentanyl
Maylin, 1979	3 ft. x 2 mm I.D.	220°C	NA	NA	Despropionylfentanyl
Frincke & Henderson, 1980	NA	NA	NA	NA	Beta-keto Acid
Henderson et al., 1981	NA	NA	NA	NA	Beta-keto Acid
van Rooy et al., 1981	10 m x 0.5 mm I.D.	210°C	Helium	NA	Despropionylfentanyl Norfentanyl
Goromaru et al., 1982	1 m x 3 mm I.D.	130-270°C	Helium	NA	Norfentanyl
Goromaru et al., 1984	1 m x 3 mm I.D.	190°C	Helium	NA	Norfentanyl

[a]NA = Not available.

radioimmunoassay, citing lower expense, and a greater application and ability to analyze a greater variety of compounds. Woestenborgs et al. (1986), using urine samples from an administered animal chose radioimmunoassay as their method of choice. Interference from packed columns (GC/NPD) overestimated the results in comparison to radioimmunoassay. These two communications indicate no difference in the ability to detect the presence of fentanyl equivalents in biological samples, but a preference is indicated for the detection of fentanyl or fentanyl equivalents between the two assay methods.

Few comparisons of immunoassay formats involving drugs of possible abuse have been reported. Hyde and Hill (1988) compared fluorescence polarization (FP), ELISA, and enzyme activity (EMIT) procedures for detection of opiates, barbiturates, amphetamines, benzodiazepines, and THC, and noted advantages and disadvantages of the methods. No comparison of classical to the new immunoassay methods including ELISA and PCFIA have been done to date.

Summary

Fentanyl is a very potent narcotic analgesic, used for clinical anesthesia in man. In the racing community, however, fentanyl is often used illicitly as a stimulant to the central nervous system of the equine, resulting in a significant increase in locomotor activity. This increase could possibly give an animal an advantage over non-drugged horses which it is competing against. Because of this, very sensitive and selective methods are necessary to ascertain the presence of fentanyl in order to control its criminal use in the racing industry.

With today's current technology, many drugs including fentanyl, are at best poorly or not detectable in urine and blood using thin layer chromatography. The lack of sensitivity, compounded by the initial minute dosage, make thin layer chromatography an undesirable method for detection of fentanyl within the equine. As Tobin (1988) stated ..."while a thin layer chromatographic method for fentanyl

exists, this method is marginally useful, and to this author's knowledge has never given rise to a positive call for fentanyl." While special extraction procedures for fentanyl do exist, they are cumbersome and time consuming and are therefore not practical as a daily laboratory routine.

Detection using immunoassay methodologies are sensitive, exhibit a great degree of cross-reactivity to analogs and metabolites, and are easily applied. Few studies have compared and elucidated the advantages and disadvantages of the classical methods relative to the newer immunoassay techniques. The object of this work was to compare characteristics of all methods for the detection of fentanyl in urine and plasma samples collected post the administration of the drug.

MATERIALS AND METHODS

Materials

Equine administration/collection materials and supplies

Fentanyl citrate was obtained from Janssen Pharmaceutica, Piscataway, NJ. Whirl-Pak bags for initial urine collection were purchased from Fisher Scientific, Fair Lawn, NJ. Graduated wide mouth specimen containers for final urine storage were obtained from ABCO, Milwaukee, WI. Heparin vacutainer blood collection tubes (16 X 125 mm) for initial blood collection were acquired from Bectin Dickinson, Rutherford, NJ. Falcon® 2057 polystyrene round bottomed tubes (17 X 100 mm) for final plasma storage were acquired from Bectin Dickinson, Lincoln Park, NJ.

Solvents, chemicals and reagents

All organic solvents used in the research were of reagent grade or better. All chemicals and reagents were acquired from Fisher Scientific, Fair Lawn, NJ. including: acetic acid, acetone, acetonitrile, dichloromethane, ethyl acetate, concentrated hydrochloric acid, hexane, methanol, sodium chloride, sodium hydroxide, petroleum ether, isopropyl alcohol.

Hexamethyldisilazine (HMDS) and pyridine for silanization of glassware were obtained from Pierce Chemical Company, Rockford, IL., and Aldrich Chemical Company, Inc., Milwaukee, WI., respectively.

All gases (nitrogen, air, helium, hydrogen, and high purity helium) for GLC and GC/MS were obtained from Air Products, Des Moines, IA.

General

Fentanyl citrate standard utilized for standard and spike preparations was obtained from Janssen Pharmaceutica, Piscataway, NJ. Screw top test tubes (16 x 125 mm) with PTFE lined caps used in the liquid-liquid extraction steps were purchased from Fisher

Scientific, Fair Lawn, NJ. Thin layer chromatographic separations were performed with Merck silica gel 60 plates with F_{254} fluorescence indicator purchased from Cobert Associates, St. Louis, MO.

Pipetman® precision microliter pipettes, utilized in all immunoassay procedures for liquid dispensation, were purchased from Rainin Instrument Company, Inc., Woburn, MA. A Branson 2200 Ultrasonicator, for sample sonication, was acquired from Branson Cleaning Equipment Co., Shelton, CT.

Radioimmunoassay materials and supplies

Two commercially available radioimmunoassay procedures were employed in the current research. Tritium labelled fentanyl RIA kits were donated by Janssen Pharmaceutica, Piscataway, NJ. Iodine-125 labelled fentanyl RIA kits were donated by Cambridge Medical Technology Corporation, Inc., Billerica, MA. Only the reagents supplied by the respective kits were used in the analyses.

Microcentrifuge tubes (1.5 ml) employed in the RIA analyses were acquired from USA/Scientific Plastics, Ocala, FL. Scintiverse® BD liquid scintillation cocktail was purchased from Fisher Scientific, Fair Lawn, NJ. Twenty milliliter disposable scintillation vials were purchased from Owens-Illinois, Toledo, OH. A Packard model 2425 Tri-Carb® liquid scintillation spectrometer system, Packard Instrument Company, Inc., Downers Grove, IL., was utilized in the detection of all RIA results.

ELISA materials and supplies

Two commercially available ELISA kits were employed in the present research. Two fentanyl assays were donated by the Tri-Tec Corporation, WestChester, PA. All other fentanyl ELISA materials were obtained from International Diagnostic Systems, Inc., St. Joseph, MI. Only the reagents supplied in the assay procedure were employed in the respective kits. Microtitre well strip holders, and a MR 600 Microplate® Reader for the reading of well optical density were obtained from Dynatech Laboratories, Inc., Chantilly, VA.

PCFIA materials and supplies

The only fentanyl PCFIA kits presently available to this study were obtained from International Diagnostic Systems, Inc., St. Joseph, MI. The ninety-six well vacuum filtration plates and the Fluorescence Concentration Analyzer (FCA) for data collection, were acquired from Baxter-Pandex, Mundelein, IL. Spin-X® centrifuge filter units for urine filtration prior to PCFIA analysis, were purchased from Costar®, Cambridge, MA.

High-performance liquid chromatography materials and supplies

HPLC was performed using a Hewlett-Packard (Waldbronn, W. Germany) model 1090 with diode array ultraviolet spectrophotometric detection. All data were stored and analyzed with a Hewlett-Packard series 300 chemstation data system. Samples were separated using a Zorbax® C-8 (4.6 mm x 25 cm) reverse phase column obtained from DuPont Company, Wilmington DE. Solvent A consisted of 0.1% H_3PO_4 (v/v), and 0.07% triethylamine (v/v). Solvent B consisted of 0.1% H_3PO_4 (v/v), 0.07% tetraethylamine (v/v), and 80% CH_3CN (v/v).

Gas chromatographic materials and supplies

A Hewlett Packard model 5890 gas chromatograph equipped with a nitrogen-phosphorus specific detector (NPD) interfaced to a computer data station was utilized in obtaining GC/NPD results. A Hewlett Packard model 5890 gas chromatograph coupled to a Hewlett Packard 5988 quadrapole mass spectrometer-data station equipped for electron ionization with positive ion collection was employed for mass spectral collection. All GC/NPD and GC/MS analysis was acquired using a DB-5 capillary column, 15 m X .25 mm fused silica with .25 phase loading, acquired from J & W Scientific, Rancho Cordova, CA.

Methods

Equine administration and sample collection

Two mares, designated #17 and #18, each weighing approximately 750 lbs (340.2 kg), were administered intravenously via the left jugular, 500 µg fentanyl base (as Sublimaze®). Thus, the dosage each animal received was approximately 1.47 µg/kg fentanyl base. Following fentanyl administration, urine and blood were collected at the times post administration indicated in Table 5. Urine was collected by bladder catheterization, and blood was collected by an intravenous catheter in the left jugular.

At each urine collection period, all urine present in the bladder was drawn out, transferred to Whirl-Pak bags, and then all urines for a particular collection time were pooled and mixed thoroughly. The urine was dispensed evenly in graduated specimen

Table 5. Total volume of urine and plasma collected following intravenous administration of 500 µg fentanyl

Time Post Dose	Urine		Plasma	
	#17	#18	#17	#18
0 Hour (Pre)	467	456	21	27
1 Minute	—	—	22	25
5 Minute	—	—	21	27
10 Minute	—	—	22	27
30 Minute	—	—	23	27
1 Hour	233	1156	21	24
2 Hour	380	72	23	24
4 Hour	288	625	—	—
8 Hour	1129	983	—	—
12 Hour	857	1083	—	—
24 Hour	80	433	—	—
48 Hour	697	961	—	—
72 Hour	746	514	—	—

containers, capped, labelled, and stored at -20°C. At each plasma collection period, 40 mls of blood was drawn through the catheter, placed in heparin vacutainer blood collection tubes, and immediately spun down at 10,000 rpm for 5 minutes. The plasma portion was then drawn off and all plasma for a particular collection time were pooled and mixed thoroughly. The plasma was then dispensed evenly in Falcon tubes, capped, labelled, and stored at -20°C.

General

Prior to use, all glassware was rinsed thoroughly with acetone to remove any interfering compounds. To prevent drug adsorption to glass, all conical vials utilized in HPLC, GLC and GC/MS analyses were initially silanized prior to use by heating at 69°C with a 5% HMDS in pyridine solution for approximately one hour. The vials were then thoroughly rinsed with acetone and used in the analyses.

RIA methods

When performing RIA analyses, the fentanyl standard accompanying the kit was used for all standard preparation. The exact procedure employed for both the RIA kits is presented in their entirety in the Appendix.

Although the Cambridge Medical Technology ^{125}I fentanyl RIA kit specifies to count the total activity of the bound portion using a gamma counter, due to a lack of access to a gamma counter total radioactivity presence in the unbound portion was determined by a beta counter and utilized in the assessment of data. The Janssen ^{3}H-fentanyl RIA kit specifies the counting of total tritium activity present in supernatant portion of dextran-charcoal suspension. All tritium activity was determined with a Packard liquid scintillation spectrometer system with a total counting time of 1 minute.

The mean net counts for each standard and sample were expressed as a percentage of the mean counts of the zero standard (B_0), with both corrected for the non-specific

response (NSB). The $\%B/B_0$ calculations were then utilized in all graphics and statistical analyses. The conversion was performed by the following equation (Eq. 1):

$$\text{Eq. 1} \quad \%B/B_0 = \frac{\text{standard or sample mean counts - NSB}}{\text{zero standard mean counts - NSB}} \times 100$$

The level of radioactivity in the supernatant was inversely related to the quantity of fentanyl equivalents present in the sample.

ELISA methods

No pretreatment after ultrasonication was necessary for plasma and urine sample ELISA analysis. Urine and plasma to be analyzed were added directly to microtitre wells. The complete ELISA procedures for both the Tri-Tec and the IDS fentanyl ELISA assays are listed in the Appendix.

Several differences were noted between the IDS and Tri-Tec methodologies. In the IDS ELISA, high-affinity fentanyl antibodies are bound to the bottom of the microtitre wells. In the Tri-Tec ELISA, parent fentanyl molecules are bound to the bottom of the wells. In addition, the Tri-Tec ELISA contains a stop solution allowing for the color reaction to be stopped at any time following substrate addition. The IDS ELISA does not contain a stop solution, and relies on readings being made at consistent times post substrate addition for accurate and reproducible results. Following substrate addition optical density of the microtitre well solution was read using a MR 600 Microplate® Reader at wavelengths 650 nm, and 490 nm for the IDS ELISA and Tri-Tec ELISA, respectively. The optical density of the solution was inversely related to the concentration of fentanyl equivalents present in the sample.

PCFIA methods

No pretreatment was performed on PCFIA plasma samples, however, urine samples were spin filtered at 10,000 rpm for 5 minutes using Spin-X™ filtration units. The filtration step removed precipitate and decreased viscosity of sample. The IDS PCFIA procedure utilized in the research is listed in the Appendix. All reagents used in the PCFIA analysis were supplied by International Diagnostic Systems, Inc. All standards were prepared in phosphate-buffered saline (from IDS) by dilution of fentanyl citrate stock solution at a concentration of 1 μg/μl fentanyl base in methanol.

After addition of all reagents in the PCFIA assay, any remaining empty wells in the ninety-six well assay plate were filled with distilled water. In the FCA machine, the entire plate was vacuum filtered at 20 mm Hg, the particles were concentrated at the bottom of the 0.2 micron membrane with a wash solution (from IDS), and the fluoresence of each well was read at an excitation/emission wavelength of 545/575 nm. The amount of fluorescence present was inversely related to the concentration of fentanyl equivalents in the sample.

Liquid-liquid extraction (administration urines)

For extraction of urines (both dosed and spikes) by classical liquid-liquid extraction, a revision of the acid hydrolysis extraction procedure described by McDonald and Ozog (1978) was used. Twenty-seven mls of urine for each collection time for both mare #17 and #18 were extracted when GLC and HPLC analyses were performed. Forty-five mls of urine were extracted by acid hydrolysis when thin-layer chromatographic analysis was conducted.

After thawing, samples were ultrasonicated for 5 minutes prior to analysis. To extract the fentanyl metabolites, 9 mls of urine were dispensed into each of 3 or 5 screw top test tubes depending on type of analysis as previously mentioned. The urines were acidified with concentrated HCl (saturated with NaCl) to approximately pH 2. Urines were extracted twice with 5 mls of dichloromethane:isopropyl alcohol (10:1). Extracts were combined and the dichloromethane:isopropyl alcohol phase evaporated to

dryness under nitrogen. To the dried residue 5 mls of 4 M HCl were added and heated at 15 psi (in a pressure cooker) for approximately 2 hours to allow for hydrolysis of beta-keto acid metabolite to despropionylfentanyl (Figure 1).

Following hydrolysis, the acid solution was cooled down and washed with two 5 ml portions of hexane (discard organic phase). Sample was alkalinized (pH 10) with 10 M NaOH and extracted twice with 5 mls dichloromethane. The combined extracts were evaporated to dryness under nitrogen. The residue was dissolved in 2 mls of 2 M HCl and washed twice with 5 mls of petroluem ether. Aqueous phase was made strongly alkaline with 10 M NaOH and extracted three times with 4 mls petroleum ether.

Following extraction, the petroleum ether portions were combined and evaporated to dryness under nitrogen. Two-hundred µls of petroleum ether were added, vortexed, and petroleum ether was transferred to a silanized conical vial where it was evaporated to dryness. Residue was dissolved in 20 µls of acetonitrile, an internal standard added (100 ng/µl meperidine), then capped and vortexed for GC/NPD and GC/MS analysis. Four µls of the same extract were injected for GC/NPD analysis. For HPLC analysis, 20 µls of acetonitrile was added to residue and four µls of the final extract were injected through the liquid chromatograph.

Liquid-liquid extraction (spiked urine)

As previously mentioned, no despropionylfentanyl standard was available at a known concentration. Despropionylfentanyl standard was diluted with methanol such that 1 µl injected through the GC/NPD produced a peak of equal height to a peak associated with a 1 µg injection of fentanyl standard at a concentration of 1 µg/µl. Thus, the despropionylfentanyl standard was at a concentration of approximately 1 µg/µl fentanyl, assuming similar ionization characteristics for the two homologs.

After thawing, 5 mls of blank urine was spiked at the following concentrations of despropionylfentanyl: 10 ppb, 50 ppb, 100 ppb, 250 ppb, 500 ppb, and 1 ppm. Acid hydrolysis of the urines was not performed as the despropionylfentanyl metabolite was the analyte spike, negating the usually necessary hydrolysis of fentanyl to

despropionylfentanyl (Figure 1). Extraction of the despropionylfentanyl metabolite spikes was performed using the same extraction procedure employed on the dosed urines following acid hydrolysis.

Liquid-liquid extraction (plasmas)

When extracting dosed plasmas for eventual analysis via gas-liquid chromatography and HPLC, two and four mls of plasma, respectively, at each collection time for both mares #17 and #18 were used. However, six mls of plasma were extracted when thin-layer chromatographic analysis was desired. Fentanyl citrate plasma spikes were prepared at the following concentrations of fentanyl base in 1 ml blank plasma: 1 ppb, 10 ppb, 25 ppb, 50 ppb, 100 ppb, 250 ppb, 500 ppb, 1 ppm, 2.5 ppm, and 5 ppm. The 1 ml spikes were then extracted by liquid-liquid extraction for ultimate gas-liquid chromatographic analysis.

After thawing, samples were ultrasonicated for 5 minutes prior to use. Fentanyl extraction was carried out in 16 X 125 mm screw top test tubes. One hundred microliters of 4 N NaOH was added for every ml of plasma extracted. Basic plasmas were extracted three times with hexane, and the extracts were combined and evaporated to dryness under nitrogen. Two-hundred µls of hexane were added, vortexed, and hexane was transferred to a silanized conical vial where it was evaporated to dryness. Residue was dissolved in 20 µls of acetonitrile with an internal standard (25 ng/µl meperidine), then capped and vortexed. Four µls of the final solution was utilized for GC/NPD and GC/MS analyses. For HPLC analysis, 20 µls of acetonitrile was added to residue and four µls of the final extract were injected through the liquid chromatograph.

Thin-layer chromatographic techniques

All TLC analysis was performed on Merck silica gel 60 TLC plates, and all extracts were developed on the plate for a distance of 5 centimeters from the origin with ethyl acetate:methanol:acetic acid (8:1:1). Following development, plates were visualized

under short wave and long wave ultraviolet light for quenching and fluorescence respectively. Lastly, the plates were sprayed with concentrated HCl, followed by an Iodoplatinate reagent (Appendix), then dipped in distilled water. Fentanyl and despropionylfentanyl reacted to give a dark purple band.

High-performance liquid chromatographic techniques and conditions

The ultraviolet range from 200-260 nm was monitored, with a linear solvent program over 20 minutes of 0% B/A to 100% B/A, then reversed back to initial conditions from 20-25 minutes. A solvent flow of 2 ml/min. was used and solvents were degased by a steady flow of helium before each analysis.

Gas-liquid chromatographic techniques and conditions

J&W DB-5 fused silica columns, 15 M X .25 mm with a .25 micron loading phase were used for all gas-liquid chromatographic analyses (both GC/NPD and GC/MS). All extracts were injected without derivatization and without thin-layer cleanup.

Meperidine was utilized as an internal marker to allow for easier identification of fentanyl and despropionylfentanyl by use of relative retention times. This allowed for identification of peaks of interest among many peaks present in resultant chromatograms. Meperidine was chosen primarily due to its similar structure to fentanyl.

GC/NPD analyses were performed with a Hewlett Packard 5890 GLC equipped with a nitrogen-phosphorus detector. The carrier gas and detector gas flows were set according to manufacturers specifications for maximum sensitivity.

Conditions for GC/NPD were:

Injector temp.	210°C
Oven temp. (programmed)	70(hold 1 min.)-300°C; 20°/min.
Detector temp.	220°C
Bead voltage	20 V
Carrier gas flow (helium)	30 ml/min.

Detector gas flows:	
Hydrogen	3.5 ml/min.
Air	100-120 ml/min.
Injection mode	Splitless

All GC/MS data were obtained with a Hewlett Packard 5890 GLC and a Hewlett Packard 5988 quadrapole MS in electron impact ionization (EI), with positive ion collection at 70 eV. Resolution was nominally 1 AMU with no more than 10% base overlap.

Conditions for mass spectrometer GLC were:

Injector temp.	260°C
Oven temp. (programmed)	70(hold 1 min.)-300°C; 20°/min.
Carrier gas	Helium
Carrier gas flow	1 ml/min.
Injection mode	Splitless

Conditions for mass spectrometer were:

Electron energy	70 eV
Source temp.	265°C
Ionizer vacuum	$< 10^{-6}$ torr

Dosed urines and plasmas were analyzed by GC/MS in full scan (40-550 AMU) as well as in single ion monitoring (SIMS) mode. Spiked urines and plasmas were analyzed by GC/MS using SIMS, with no full scan analyses as the most sensitive GC/MS technique was to be employed on the spiked samples. The ions targeted when employing SIMS analyses were m/e 146, m/e 189, and m/e 245 when analyzing plasma extracts. These three ions were used since these are the three major ions present in the mass spectra of fentanyl (parent). For urine extracts the ions monitored were m/e 146, m/e 189, and m/e 280, corresponding to the three major ions in the mass spectra of despropionylfentanyl.

RESULTS AND DISCUSSION

Immunoassay Techniques

Threshold values and sensitivity limits

Diagnostic immunoasssay tests involving the use of antibodies have characteristic saturation points, or threshold values. The threshold value is the concentration of antigen at which the antibodies become completely saturated, and have no additional antigen binding capacity. The sensitivity limit, or lowest analyte concentration which the assay can positively detect, is another immunoassay characteristic.

Resultant standard curves produced by the various immunoassays include three distinct regions: a region in which low sample concentrations are indistinguishable from a buffer blank response, a region in which the antibodies become completely saturated, and a region of the curve characterized as the useable portion. The useable portion is labelled as such because the concentration:response curve can furnish semi-quantitative information about the concentration of the analyte of interest.

Linear regression was performed on each region of the standard curves, producing three distinct lines within a standard curve for a particular assay. For this research, intersection of the linear regression line of the indistinguishable region with the linear regression line of the useable region was estimated as the sensitivity limit for the assay. Intersection of the linear regression line of the saturation region with the linear regression line of the useable region was estimated as the threshold value for the assay. The reproducibility of an assay is the deviation in the response of known standards on a daily, weekly, and monthly basis within assay lots and between assay lots. Two standard deviations (95% confidence level) of the percent buffer response, as indicated on the standard curves, were used to estimate the reproducibility of the respective assay on a daily/weekly basis.

Radioimmunoassay The Cambridge Medical RIA kit uses competitive binding between an ^{125}I-labelled antigen and non-labelled sample analyte for antibody sites.

The test was performed on two separate occasions using two individual kits, allowing for the construction of an overall standard curve for the assay (Figure 3). The sensitivity limit and threshold value of the radioimmunoassay kit was estimated as 5 pg and 350 pg absolute fentanyl concentration, respectively (Table 6). The fairly large, overlapping error bars (at the 95% confidence level) indicate results from different kits should not be anticipated, with a high degree of confidence, to remain constant. Intra-assay variability, however, or the variance among individual sample results on a daily performance was very minimal for the Cambridge Medical RIA.

The Janssen RIA kit uses a tritium(^{3}H)-labelled antigen in the competitive binding portion of the assay, and has been reported to be 100 times less sensitive than RIAs using an iodinated antigen (Tobin et al., 1986, Woods et al., 1986, Weckman et al., 1988). The Janssen assay was performed over four separate days, using a total of three separate assay kits. The sensitivity limit and threshold value estimated for the Janssen RIA were 20 pg and 5 ng absolute fentanyl concentration, respectively (Table 6). The error bars (at the 95% confidence level) were the smallest for any of the immunochemical techniques analyzed, implying the assay may be performed over several occasions with confidence the results will remain fairly constant over that period (Figure 4). In addition, the intra-assay variability was very small for the Janssen RIA.

Radioimmunoassays using iodinated labelled antigens reportedly have increased sensitivity in comparison to RIAs using tritium labelled antigens (Tobin et al., 1986; Woods et al., 1986; Weckman et al., 1988). The Cambridge ^{125}I assay showed an increase in sensitivity on the order of 4X when compared to the Janssen ^{3}H assay, although not the reported 100X. The Cambridge Medical ^{125}I assay had a lower threshold (onset to antibody saturation) in comparison to the Janssen kit, further illustrating the difference in antigen-antibody interaction among the RIA tests. Possible reasons for the differences indicated in the two tests include: antibodies produced in response to the immunogen utilized by Cambridge Medical may be more site-specific than the antisera utilized in the Janssen assay; iodination of the antigen for competitive binding may render the antigen molecule less accessible to antibody

Figure 3. Fentanyl standard curve (buffer) from Cambridge Medical ^{125}I-RIA performed on a total of 2 separate days with error bars indicating two standard deviations, n = 2 or 4 (zero corresponds to 0 pg concentration)

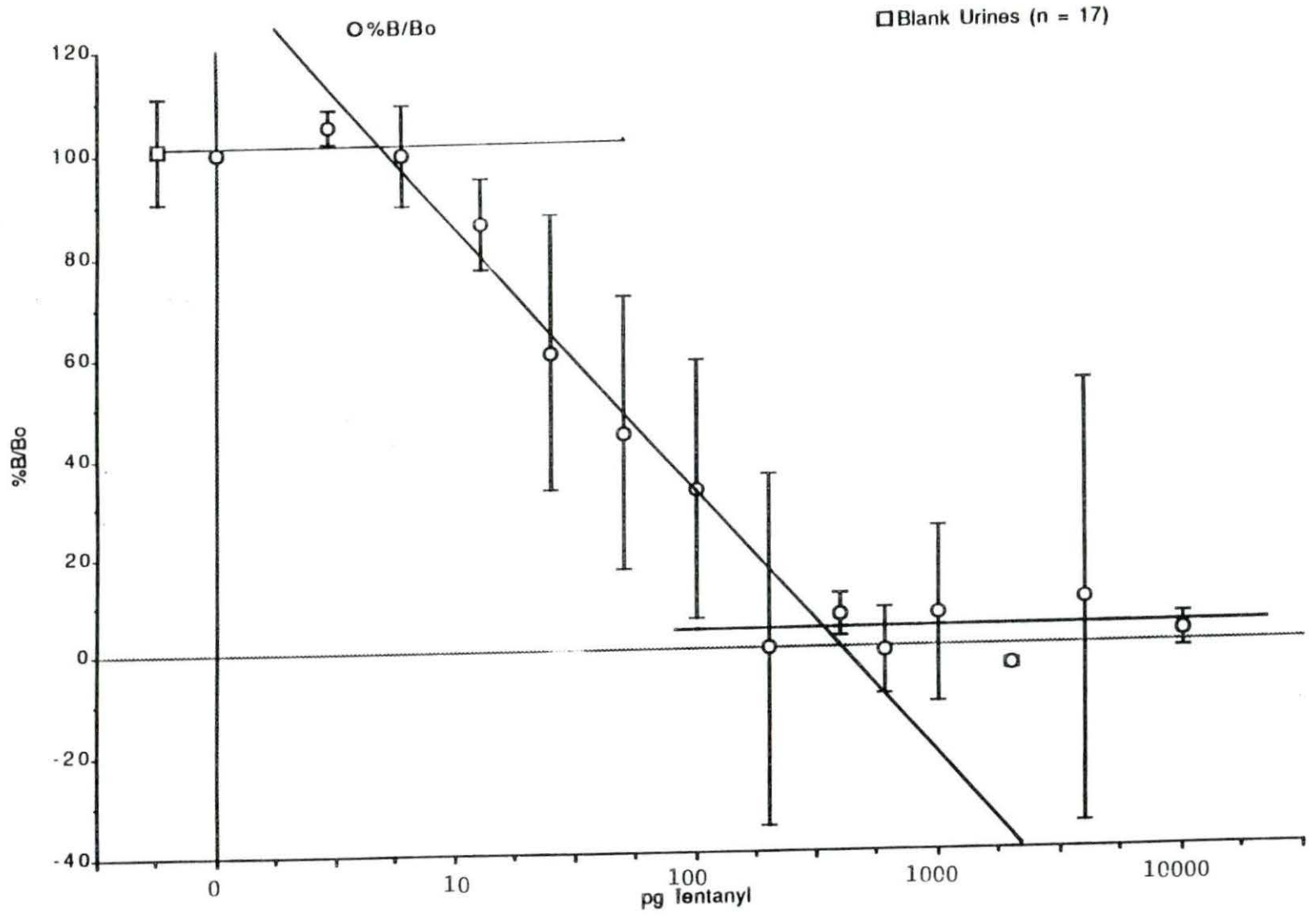
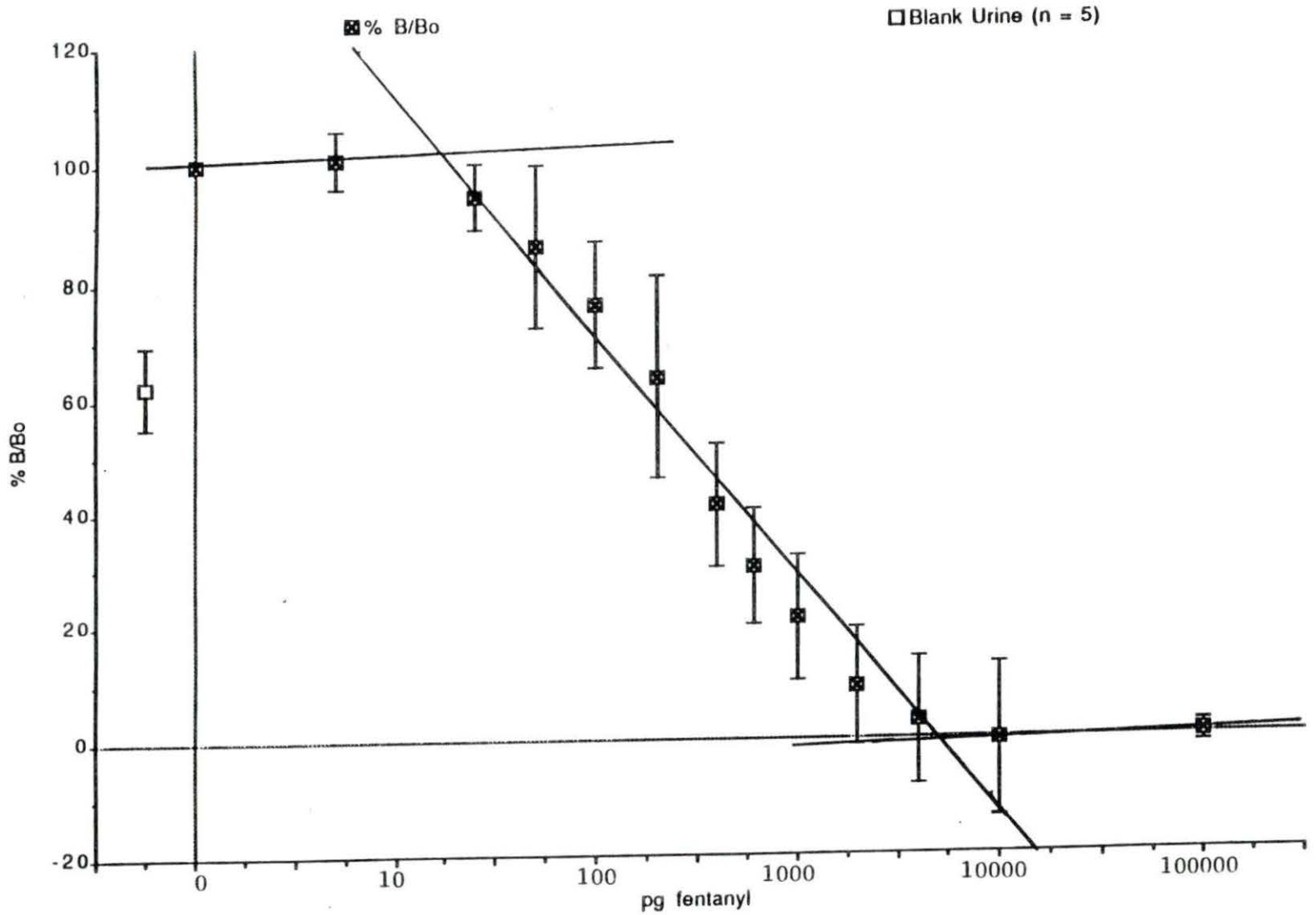

O%B/Bo
Blank Urines (n = 17)
120
100
80
60
40
20
0
-20
-40
%B/Bo
0
10
100
1000
10000
pg fentanyl

Figure 4. Fentanyl standard curve (buffer) from Janssen ^{3}H-RIA performed on a total of 4 separate days with error bars indicating two standard deviations, n = 6 or 10 (zero corresponds to 0 pg concentration)


% B/Bo
Blank Urine (n = 5)
120
100
80
60
40
20
0
-20
% B/Bo
0
10
100
1000
10000
100000
pg fentanyl

binding sites due to steric constrants caused by the larger iodine molecule, in turn allowing for more non-labelled antigen to bind to the antibody improving the overall sensitivity of the assay.

ELISA The Tri-Tec ELISA assay was performed on two separate occasions using two different assay kits. Thirty mls of substrate buffer per o-phenylenediamine (OPD) tablet and 60 mls substrate buffer per OPD tablet were used in the first and second assay performance, respectively. Increasing the volume of substrate buffer two-fold was indicated in the kit instructions to increase the sensitivity of the assay two-fold as well. Sensitivity limit and threshold value estimated for the initial assay execution was 100 pg and 750 pg total fentanyl well concentration, respectively (Table 6). A sensitivity limit of 20 pg total fentanyl was manifested for the second assay performance, revealing an increase in sensitivity with a two-fold increase in substrate buffer, as would be expected per the assay instructions. The intra-assay reproducibility of the Tri-Tec ELISA was excellent, indicated by the small error bars (95% confidence level) within the useable portion of the standard curves (Figures 5 and 6). The point at 1000 pg total fentanyl concentration in Figure 6, in relation to the other points in the useable region, contains large error bars and are noted but unexplained by the author.

The IDS ELISA procedure was analyzed on four separate instances, using three different assay lots. The sensitivity limit and threshold value estimated for the IDS ELISA were 1.5 pg and 100 pg absolute fentanyl well concentration, respectively (Table 6). The extremely large error bars (95% confidence level) indicate inter-assay results should not be anticipated to remain constant with a high degree of confidence, and the test should only be utilized for yes-no (positive/negative) determinations (Figure 7). Lack of a stop solution, for stopping substrate color reaction at a precise time post addition, may be a contributing factor to the large standard deviations shown. Substrate reacts in the well with antibody-antigen-enzyme complex until all substrate is converted to colored compound, unless the reaction is stopped by the addition of an acid solution. Thus, while reading the IDS ELISA microtitre strips, the substrate

Figure 5. Fentanyl standard curve (buffer) from Tri-Tec ELISA using 30 ml substrate buffer/OPD tablet with error bars indicating two standard deviations, n = 4 (zero corresponds to 0 pg concentration)

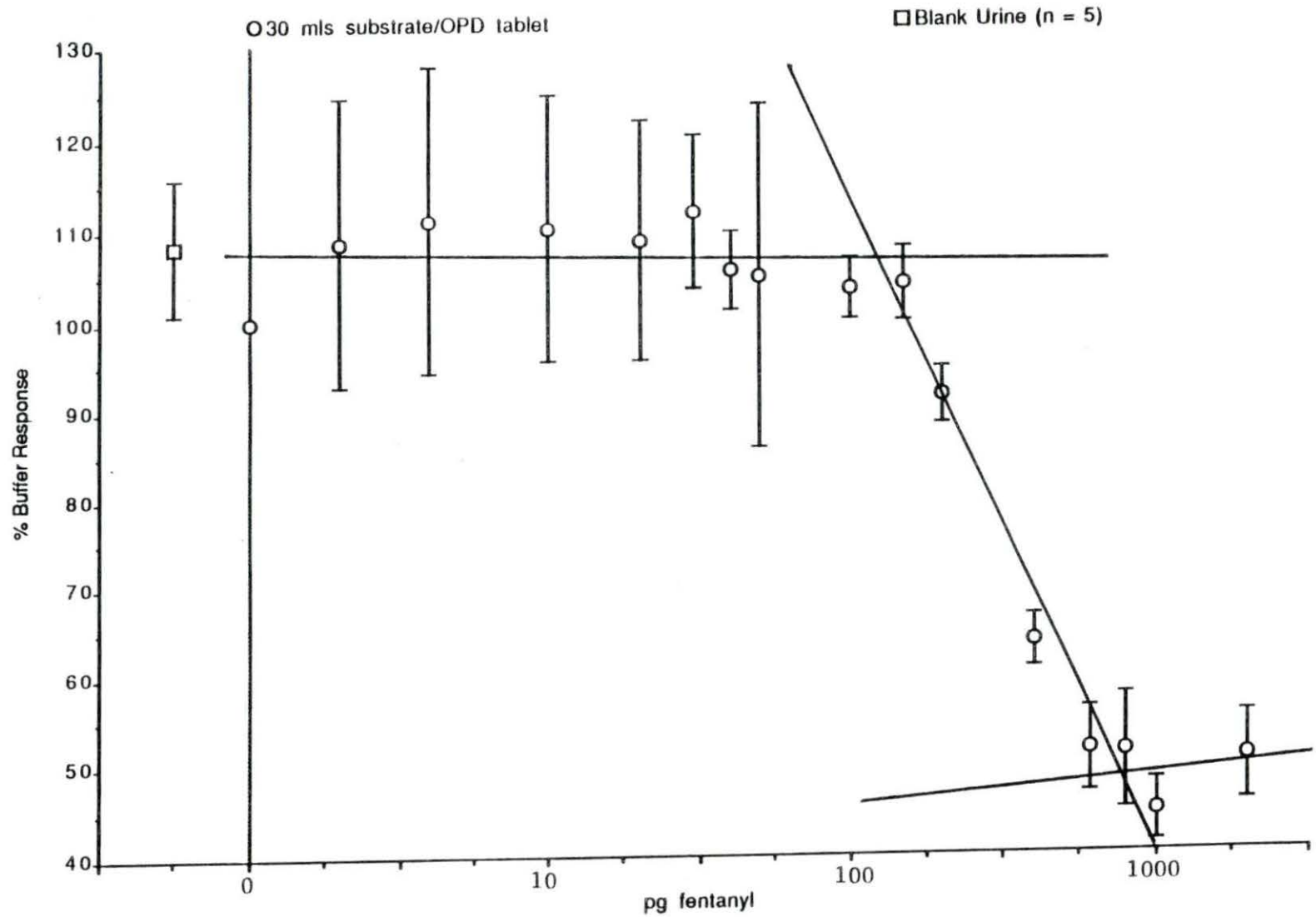

O 30 mls substrate/OPD tablet
□ Blank Urine (n = 5)
% Buffer Response
130
120
110
100
90
80
70
60
50
40
0
10
100
1000
pg fentanyl

Figure 6. Fentanyl standard curve (buffer) from Tri-Tec ELISA using 60 ml substrate buffer/OPD tablet with error bars indicating two standard deviations, n = 2 (zero corresponds to 0 pg concentration)

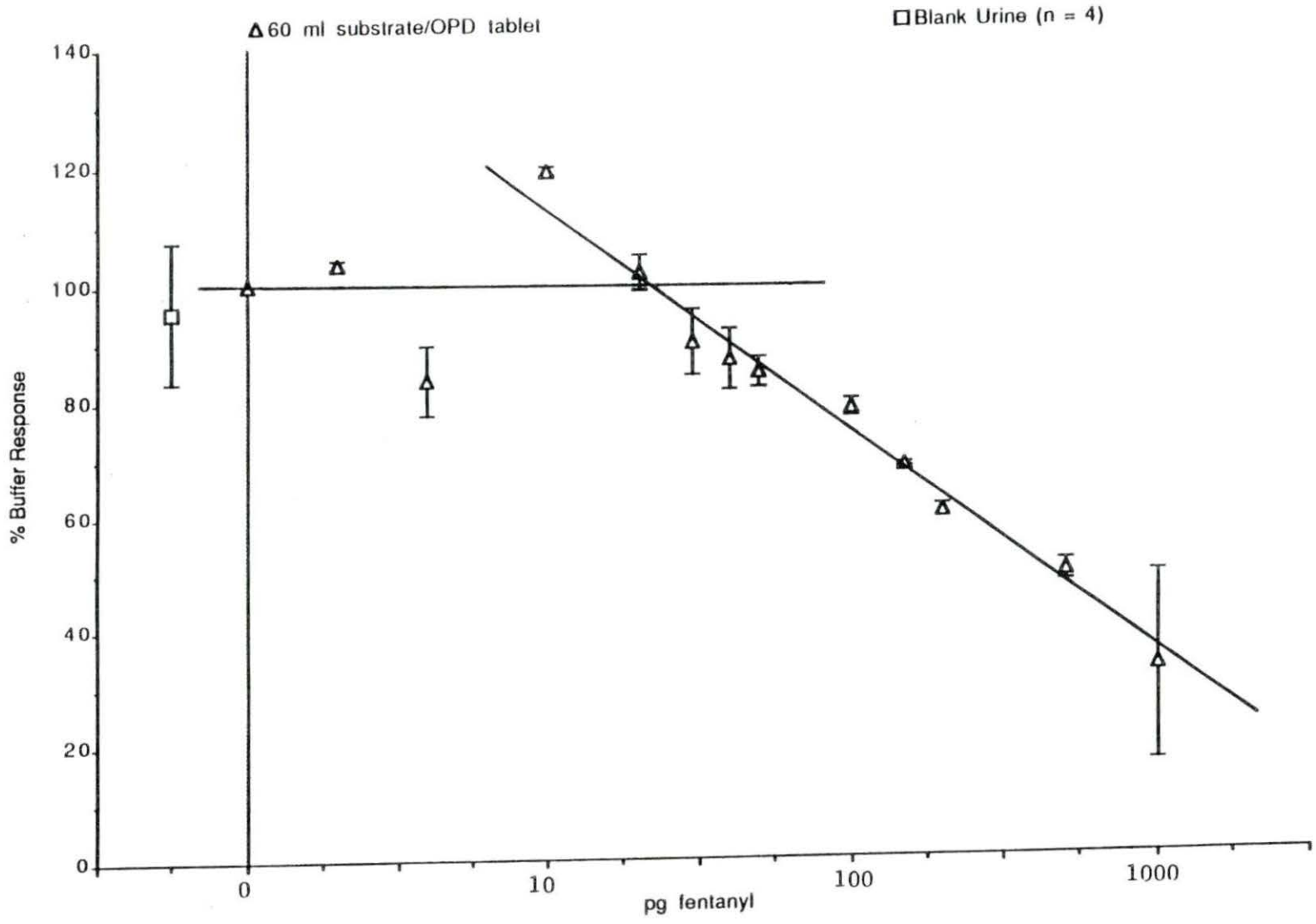

Δ60 ml substrate/OPD tablet
□Blank Urine (n = 4)
% Buffer Response
140
120
100
80
60
40
20
0
0
10
100
1000
pg fentanyl

Figure 7. Fentanyl standard curve (buffer) from IDS ELISA over a total of four separate days with error bars indicating two standard deviations, n = 4, 6, 7, 8, 9 or 13 (zero corresponds to 0 pg concentration)

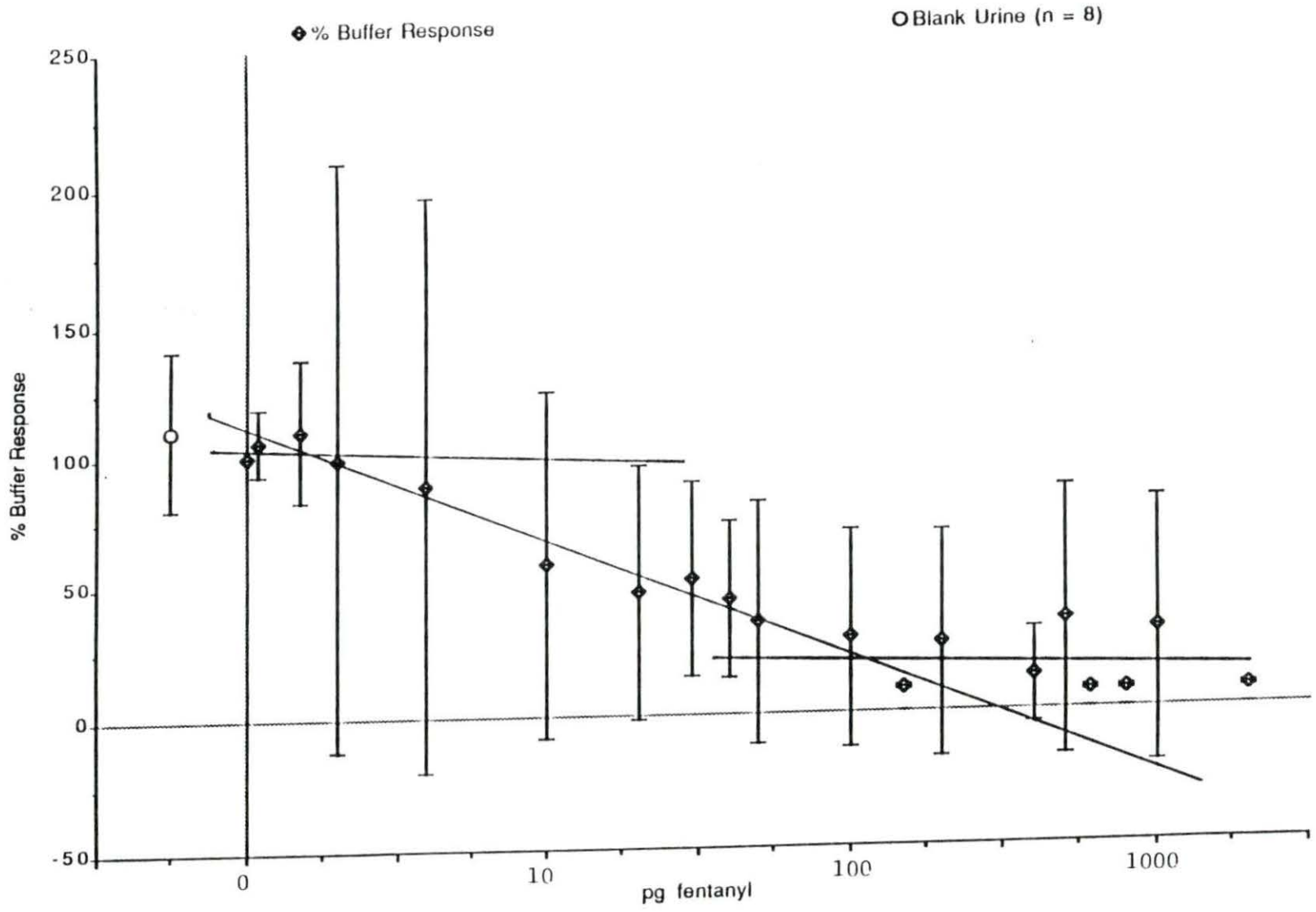

% Buffer Response
Blank Urine (n = 8)
250
200
150
100
50
0
-50
% Buffer Response
0
10
100
1000
pg fentanyl

Table 6. Estimated detection limits and threshold values for all immunoassays investigated; ^{3}H and ^{125}RIA, Tri-Tec and IDS ELISA, and IDS PCFIA (values reported as absolute fentanyl concentration, with numbers in parentheses corresponding to concentration of buffer standard)

Assay (mls substrate)	Detection Limit	Threshold Value
^{125}I RIA	5 pg (0.1 ppb)	350 pg (6 ppb)
^{3}H RIA	20 pg (0.4 ppb)	5 ng (100 ppb)
Tri-Tec ELISA (30 mls)	100 pg (2 ppb)	750 pg (15 ppb)
Tri-Tec ELISA (60 mls)	20 pg (0.3 ppb)	> 1000 pg (20 ppb)
IDS ELISA	1.5 pg (0.75 ppb)	100 pg (5 ppb)
IDS PCFIA	< 1 pg (0.05 ppb)	80 pg (4 ppb)

possibly was still reacting in the wells, leading to variation in the results. Intra-assay variability, however, was relatively minute for the IDS ELISA.

Due to competitive advantages (if the assay antibodies both had the same binding specificities) the Tri-Tec ELISA should have the greater degree of sensitivity. However, this was not the case when the standard curves for each assay were examined. Specifically, the Tri-Tec ELISA has well-bound fentanyl antigen in competition with sample analyte for HRP-labelled antibody binding sites. Thus, the sample analyte of interest and labelled antibody are mobile, and the antigen necessary for analyte competition is immobile. The mobile sample antigen has a greater probability of binding to the mobile labelled antibody than does the fixed antigen. In comparison, the IDS ELISA contains well-bound antibodies allowing for equal competition between labelled sample antigen and analyte of interest for binding sites.

PCFIA IDS PCFIA standard curves were generated on a total of five separate occasions, using two different assay kits. Sensitivity limit and threshold value of the assay were estimated as less than 1 pg and 80 pg absolute total fentanyl well concentration, respectively (Table 6). The IDS PCFIA assay had the lowest sensitivity limit and threshold value of all the immunochemical methodologies analyzed. The 1.5 pg absolute fentanyl corresponds to a concentration of 0.075 ng/ml which is

approximately 25X greater sensitivity than that reported by McDonald et al., (1987) who reported a sensitivity limit of 2 ng/ml for PCFIA. The error bars (95% confidence level) associated with the concentration:response curve reveal reproducibility is not as beneficial as the Janssen RIA, but superior to the IDS ELISA immunoassay (Figure 8). Similar to the other immunoassay methodologies, the intra-assay variation of the PCFIA results was exceptional, indicating results from a sample analyzed in duplicate, triplicate, etc, will remain fairly constant when analyzed by IDS PCFIA.

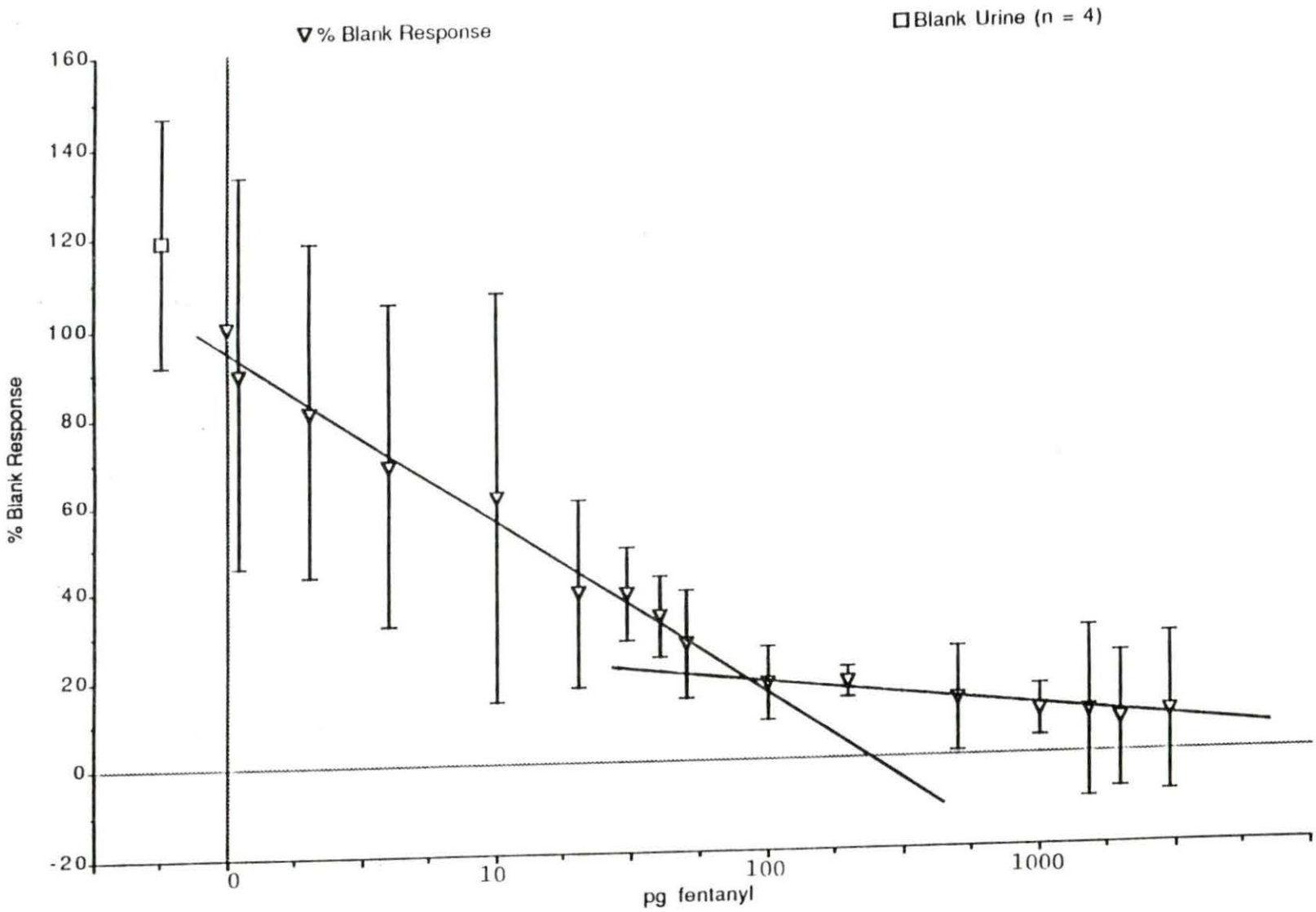
Non-specific response

It is imperative in any immunoassay technique to determine the non-specific response, that is the response of "clean" urine with the assay of interest. In an optimum situation, the antibodies present in the assay would register a "zero", or negative response with normal, non-medicated urines. However, antibodies are known to react to some degree with endogenous constituents present in equine urine, as well as plasma. This non-specific response may alter results, leading to inaccurate readings and possibly false positive responses.

Radioimmunoassay Blank urine response of the ^{125}I Cambridge Medical radioimmunoassay showed the assay antibodies do not react with endogenous constituents found in equine urine to a large degree (Figure 3). The average $\%B/B_0$ value manifested in the assay was approximately 101%, with the small error bars (95% confidence level) indicating the assay may be performed with a high degree of confidence of no false positive responses. In the ^{3}H RIA, results showed an average $\%B/B_0$ response for blank urine of approximately 62.4%, extrapolating out to a concentration of approximately 150 pg total fentanyl well concentration (Figure 4). Of all the immunoassays investigated, the ^{3}H Janssen RIA reacted to the greatest extent with "blank" urines, implying individuals must be aware of the possibility of false positive responses due to interaction of endogenous constituents with assay antibodies.

ELISA Both executions of the Tri-Tec ELISA indicated a "blank" response of little consequence to cause concern for false positive sample results. In the initial standard

Figure 8. Fentanyl standard curve (buffer) from IDS PCFIA over a total of five separate days with error bars indicating two standard deviations, n = 4, 6, 10 or 13 (zero corresponds to 0 pg concentration)


% Blank Response
Blank Urine (n = 4)
160
140
120
100
80
60
40
20
0
-20
% Blank Response
0
10
100
1000
pg fentanyl

curve (30 ml/OPD) the % buffer response was approximately 108.3%, and when the sensitivity of the assay was increased (60 ml/OPD) the response increased as well down to 95.4% buffer response (Figures 5 and 6). Response from "blank" urine within the IDS ELISA averaged approximately 110.8% of the buffer response (Figure 7). It would thus seem that endogenous components do not pose a significant threat to propagate false positive urine responses in the IDS or Tri-Tec ELISAs, although it is still possible.

PCFIA Endogenous compounds present in equine urine were not found to react with antibodies in the IDS PCFIA assay. The average % buffer response of blank urine was approximately 118.9% for the IDS PCFIA, and was the least reactive of all the immunoassays investigated with "blank" urines (Figure 8). The results infer the assay may be performed with a high degree of confidence no false positive sample responses will be propagated.

Consequence of urine and plasma on performance (sample spikes)

The analysis of spiked urines and plasmas to ascertain whether urine and plasma presence is detrimental to the assays ability to detect fentanyl and its equivalents was of interest in this study. The presence of urine/plasma may consequently react with the antibodies in such a way as to possibly amplify or attenuate the assay response, leading to erroneous results. It was also of interest to determine the percent recovery of spiked urines and plasmas in relation to standards prepared in buffer solution.

Cambridge medical RIA Analysis of spiked urines and plasmas was not performed on the ^{125}I-Cambridge Medical RIA due to limited kit availability.

Janssen RIA The percent recoveries of spiked urines for the Janssen RIA showed good recoveries at the lower concentrations, with the percent recovery decreasing as the concentration of the spike gradually increased (Table 7). Thus, at the higher concentrations, endogenous urine components possibly compete with the analyte for antibody binding sites, rendering the lower % recovery results observed. The plasma spikes on the other hand showed good recovery at all the concentrations analyzed, in contrast to the urine spikes (Table 8).

Tri-Tec ELISA Analysis of spiked urines and plasmas was not performed on the Tri-Tec ELISA due to limited kit availability.

IDS ELISA Results from the spiked urines indicates that the IDS ELISA had excellent recovery of fentanyl at all concentrations employed (Table 7). The plasmas, however, showed excellent recovery at the very low concentrations, but at the higher concentrations the recovery was much less, in the range of 20-40% recovery (Table 8). The relatively low recoveries revealed in the plasma spikes are of interest due to the fact that untreated plasmas are known to decrease the efficacy of the ELISA test. Pre-treatment of plasma with tricarboxylic acid has shown to substantially improve the quality of the assay (Weckman et al., 1988). Fentanyl is known to bind to a large extent to plasma proteins such as albumin and lipoproteins (Bickel, 1975), and the tricarboxylic acid hydrolyzes the fentanyl-protein bond leaving the fentanyl molecule available for antibody binding. It can only be assumed that upon addition to the "blank" plasmas, some of the fentanyl molecules bind to the plasma proteins rendering them invisible to the fentanyl antibodies. As no TCA pretreatment was performed on the plasma the overall recovery efficacy was poor, possibly leading to the low recovery values.

PCFIA Similar to the results seen in the IDS ELISA assay, the IDS PCFIA showed good to excellent percent recovery of non-treated urines (other than Co-star filtration) with all concentrations having greater than 70% recovery, except for one (Table 7). It has been noted that blank horse urines contain endogenous materials which may inhibit binding capability of the antibody by as much as 15%, as well as endogenous quenching materials, possibly leading to the high recoveries indicated (McDonald et al., 1987). Also similar, and possibly due to the same cause, the plasma spikes revealed good recovery at low concentrations, but at the higher concentrations the ability of the assay to recognize the fentanyl presence decreased significantly (Table 8). The poor response of the spiked plasmas may be due in part to the fact that blank urines and

Table 7. Percent fentanyl recovery ± SEM[a] from spiked urines employing Janseen RIA, IDS ELISA, and IDS PCFIA immunochemical techniques

Total Fentanyl	^{3}H RIA[b]	IDS ELISA[c]	IDS PCFIA[d]
1 pg	NDA[e]	110 ± 2.4	170 ± 51.9
1.5 pg	NDA	237 ± 58.9	NDA
2 pg	NDA	172 ± 24.2	166 ± 24.3
4 pg	NDA	149 ± 22.2	140 ± 7.5
5 pg	107 ± 0.2	NDA	NDA
10 pg	NDA	130 ± 19.9	113 ± 28.1
20 pg	NDA	101 ± 9.2	83.3 ± 13.1
25 pg	96.6 ± 0.8	NDA	NDA
30 pg	NDA	99.0 ± 11.3	86.5 ± 5.1
40 pg	NDA	69.4 ± 2.7	87.9 ± 5.7
50 pg	88.8 ± 1.0	75.5 ± 1.6	71.2 ± 10.6
.1 ng	82.9 ± 1.7	67.0 ± 1.8	48.6 ± 2.3
.2 ng	83.1 ± 0.0	79.5 ± 2.3	82.7 ± 5.8
.4 ng	62.7 ± 10.4	NDA	NDA
.5 ng	NDA	87.4 ± 1.9	72.8 ± 6.9
.6 ng	50.6 ± 0.2	NDA	NDA
1 ng	44.7 ± 4.7	NDA	70.2 ± 6.9
2 ng	37.3 ± 2.1	NDA	NDA
4 ng	38.2 ± 0.2	NDA	NDA

[a]SEM = Standard error of the mean.
[b]n = 2.
[c]n = 7.
[d]n = 5.
[e]NDA = No data available.

plasmas contain naturally fluorescent materials, decreasing the level of fentanyl equivalents indicated in the sample, leading to false negative results (McDonald et al., 1987).

Administration urine and plasma

The real test of an assay's usability is its capacity to detect the analyte of interest and its metabolites in urine or plasma from animals initially dosed with the drug. The longer an assay can initiate a positive response in terms of time post administration, the greater will be the usability and relevance to drug testing programs.

Table 8. Percent fentanyl recovery ± SEM[a] from spiked plasmas employing Janseen RIA, IDS ELISA, and IDS PCFIA immunochemical techniques

Total Fentanyl	^{3}H RIA[b]	IDS ELISA[c]	IDS PCFIA[d]
1 pg	NDA[e]	NDA	67.4 ± 0.35
1.5 pg	NDA	111 ± 3.9	NDA
2 pg	NDA	122 ± 3.1	70.0 ± 4.1
4 pg	NDA	96.8 ± 3.5	70.3 ± 1.1
5 pg	124 ± 0.7	NDA	NDA
10 pg	NDA	81.3 ± 9.4	69.5 ± 4.5
20 pg	NDA	57.3 ± 2.2	42.0 ± 0.67
25 pg	116 ± 0.0	NDA	NDA
30 pg	NDA	42.7 ± 3.8	NDA
40 pg	NDA	34.7 ± 2.2	36.6 ± 0.69
50 pg	107 ± 0.7	30.7 ± 1.5	37.3 ± 1.1
.1 ng	112 ± 1.7	21.7 ± 0.9	29.3 ± 2.0
.2 ng	121 ± 2.4	25.6 ± 1.8	36.8 ± 2.2
.4 ng	99.0 ± 3.7	NDA	NDA
.5 ng	NDA	31.2 ± 2.5	33.6 ± 2.0
.6 ng	90.5 ± 8.0	NDA	NDA
1 ng	130 ± 9.9	NDA	62.2 ± 6.0
2 ng	89.6 ± 4.1	NDA	NDA
4 ng	81.2 ± 4.8	NDA	NDA

[a]SEM = Standard error of the mean.
[b]n = 2.
[c]n = 7.
[d]n = 3.
[e]NDA = No data available.

All immunochemical assays utilized in the current research resulted in typical urine and plasma profiles. The fentanyl drug and equivalents were readily detected in the first few collection periods post administration, with the response gradually returning to the pre-administration values.

Radioimmunoassay Urine and plasma from administered horses were not analyzed by the Cambridge Medical ^{125}I RIA kit in the current research.

The response generated by "blank" non-medicated urines complete with error bars denoting two standard deviations (Figures 9 and 10), shows that the Janssen ^{3}H RIA

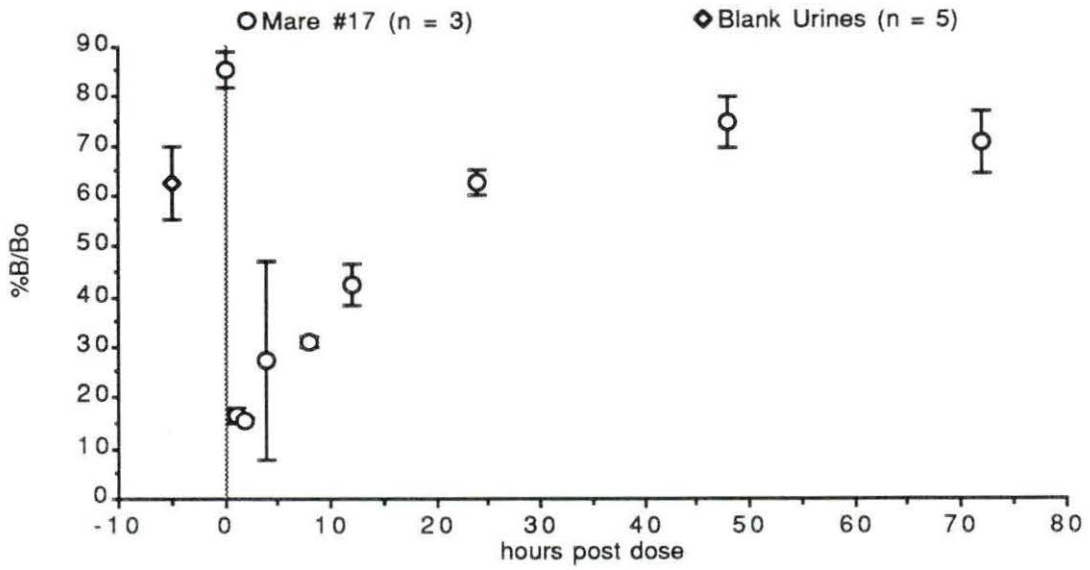


Figure 9. Urinary excretion profile produced by Janssen ^{3}H-RIA from mare #17 administered 500 µg fentanyl base with error bars indicating two standard deviations (blank response indicated in far left)

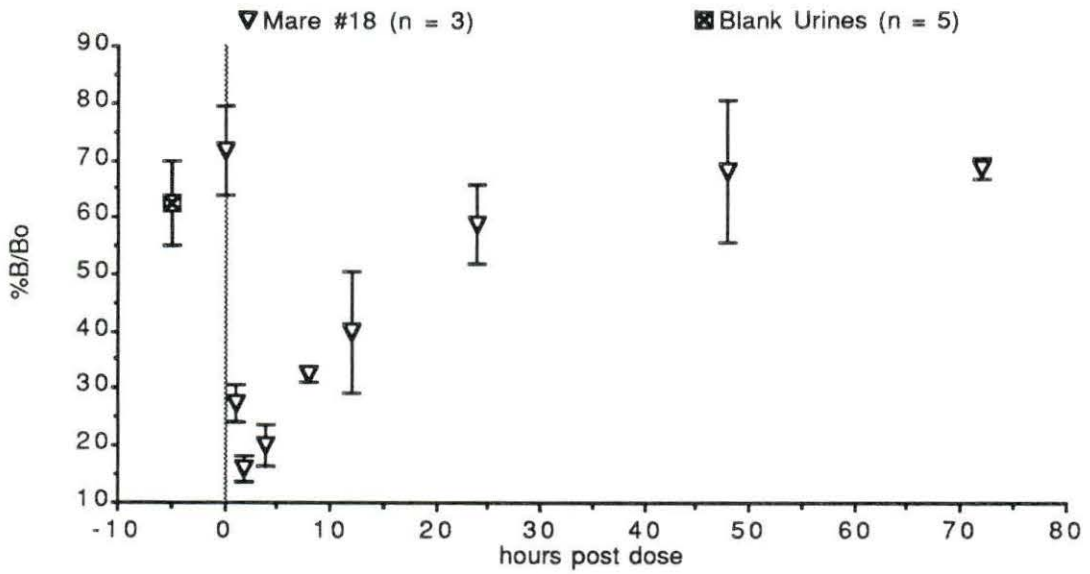


Figure 10. Urinary excretion profile produced by Janssen ^{3}H-RIA from mare #18 administered 500 µg fentanyl base with error bars indicating two standard deviations (blank response indicated in far left)

allowed for detection of fentanyl administration up to and including 12 hours post administration, at the 95% confidence level. Beyond 12 hours the error bars for both mares overlap with those from the "blank" urines, thus rendering the responses as indistinguishable from negative. The large error bars (95% confidence level) at the 1 hour collection period in mare #17 are noted, but unexplained by the author.

The response produced by "blank" non-medicated plasmas permitted the Janssen RIA kit to generate positive results up to and including 10 minutes post administration of fentanyl (Figures 11 and 12). The responses from the collection periods after 10 minutes contained over-lapping error bars at two sigma with the "blank" plasmas, in effect causing them to be labelled as indistinguishable from negative. The large error bars (95% confidence level) at the 30 minute collection time are noted, but unexplained by the author.

ELISA The Tri-Tec ELISA was performed on administration samples using the 60 mls/OPD format. The responses generated by the administered urines indicate that the Tri-Tec assay initiated positive results up to and including 12 hours post administration when compared to blank and pre-administration urines. However, beyond 12 hours post dose, overlapping error bars cause Mare #18 results to be designated as negative, similar to the Janssen ^{3}H RIA (Figures 13 and 14). Mare #17, however, showed a positive response at 24 hours, with the subsequent collection periods responding as indistinguishable from negative. The Tri-Tec assay produced positive responses for plasmas at the one and five minute post dose collection periods for both mares #17 and #18 (Figures 15 and 16). Subsequent collection times produced negative values for mare #18, while mare #17 generated a positive response at 10 minutes post dose, but beyond that overlapping error bars yielded negative results at the 95% confidence level.

The urinary excretion profile produced by the IDS ELISA show positive responses for all hours up to and including the 24 hour collection time (Figures 17 and 18). Past 24 hours, over-lapping error bars render the optical density values as negative to fentanyl presence. The responses generated by the equine plasmas show the IDS ELISA

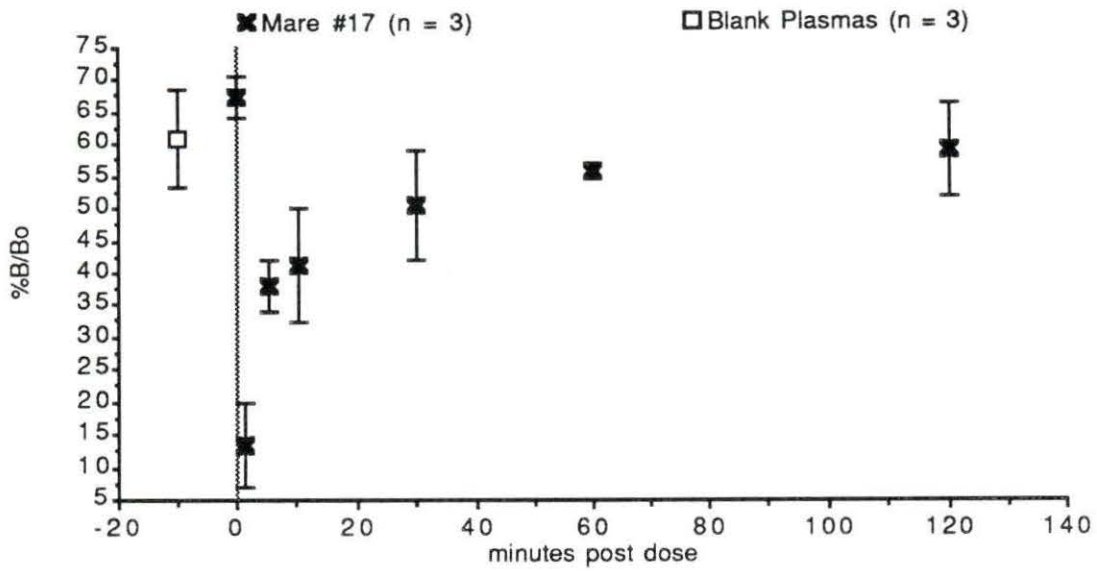


Figure 11. Plasma profile produced by Janssen ^{3}H-RIA from mare #17 administered 500 µg fentanyl base with error bars indicating two standard deviations (blank response indicated in far left)

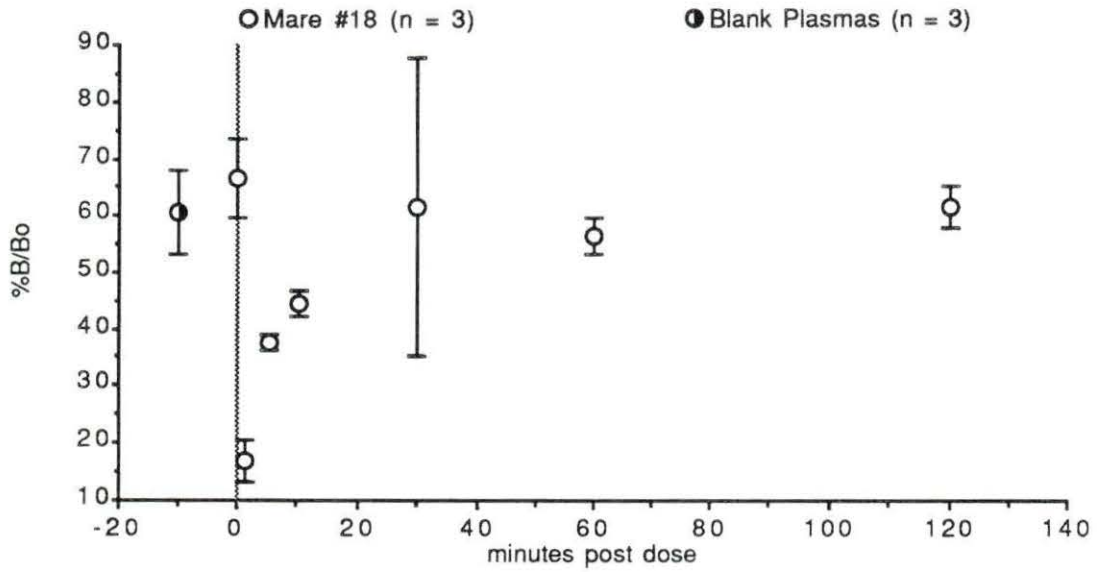


Figure 12. Plasma profile produced by Janssen ^{3}H-RIA from mare #18 administered 500 µg fentanyl base with error bars indicating two standard deviations (blank response indicated in far left)

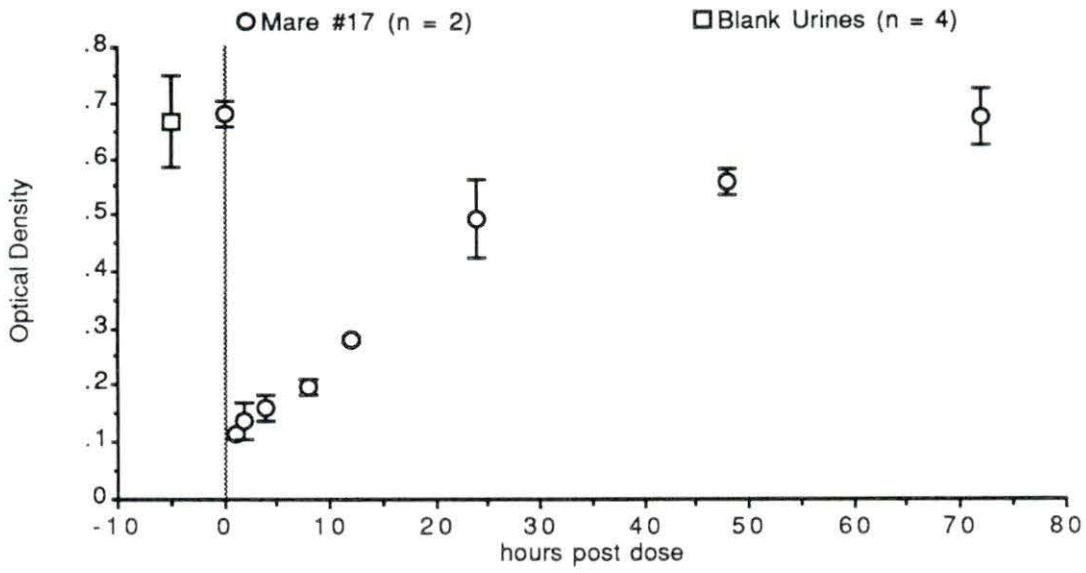


Figure 13. Urinary excretion profile produced by Tri-Tec ELISA from mare #17 administered 500 µg fentanyl base with error bars indicating two standard deviations

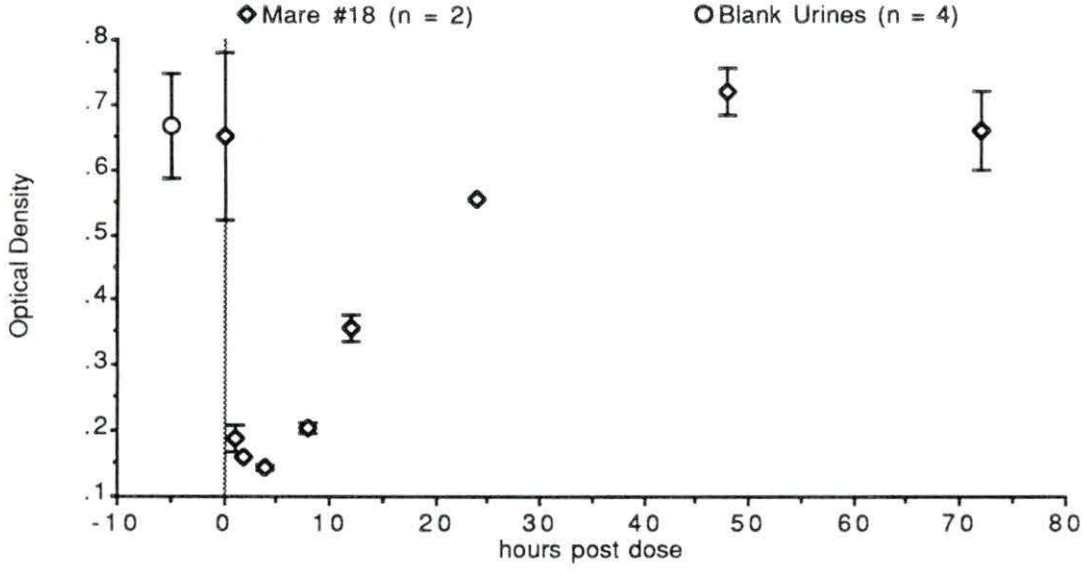


Figure 14. Urinary excretion profile produced by Tri-Tec ELISA from mare #18 administered 500 µg fentanyl base with error bars indicating two standard deviations

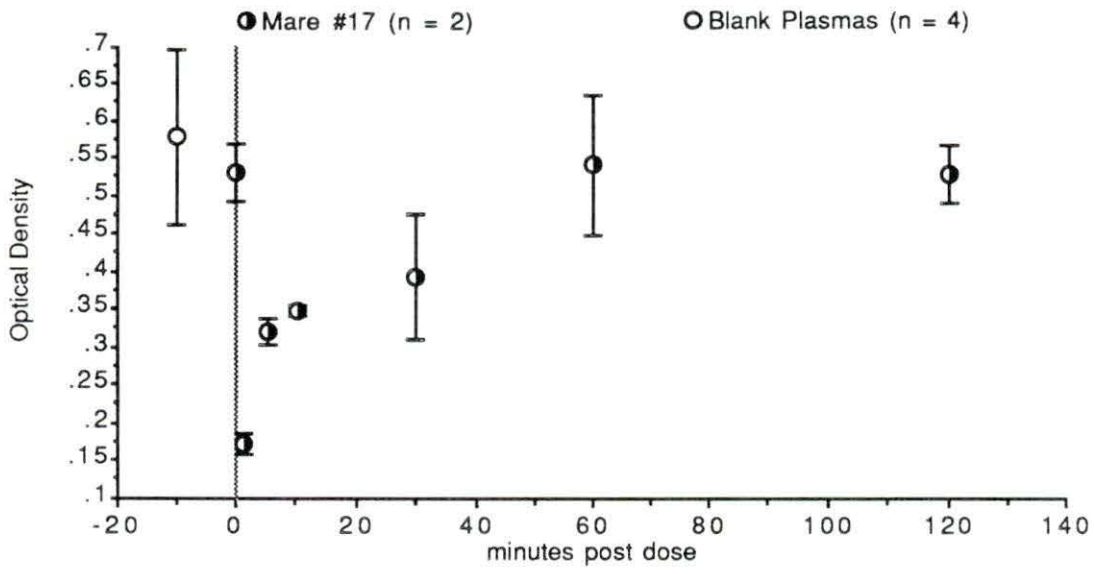


Figure 15. Plasma profile produced by Tri-Tec ELISA from mare #17 administered 500 µg fentanyl base with error bars indicating two standard deviations

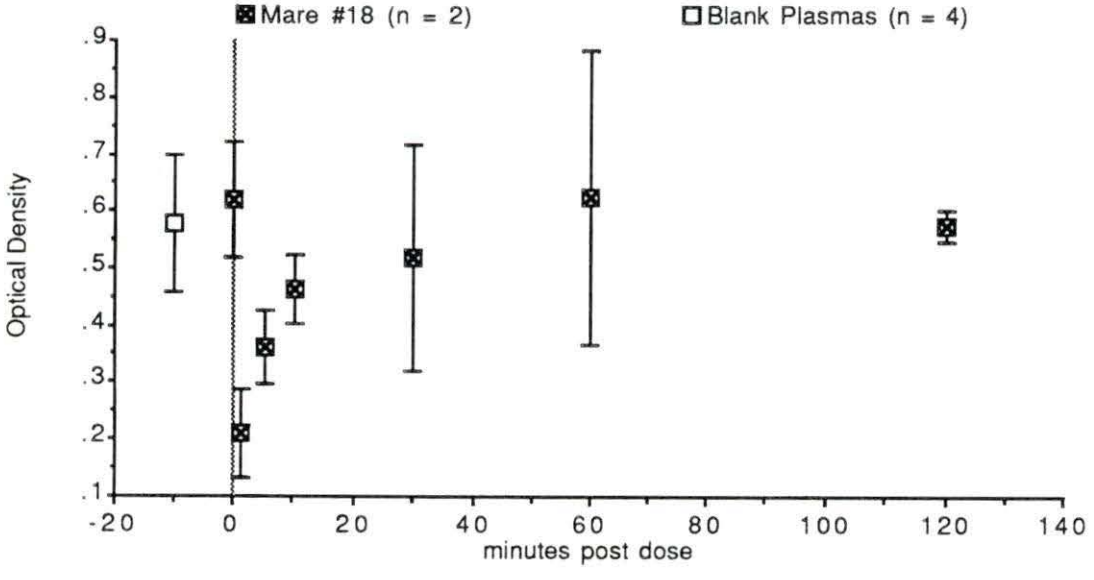


Figure 16. Plasma profile produced by Tri-Tec ELISA from mare #18 administered 500 µg fentanyl base with error bars indicating two standard deviations

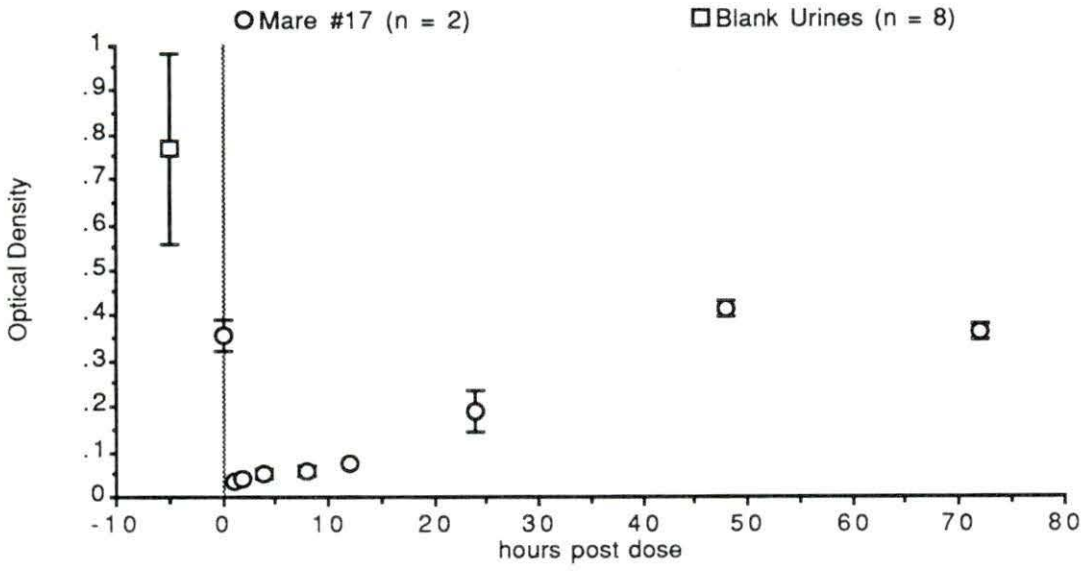


Figure 17. Urinary excretion profile produced by IDS ELISA from mare #17 administered 500 µg fentanyl base with error bars indicating two standard deviations (blank response indicated in far left)

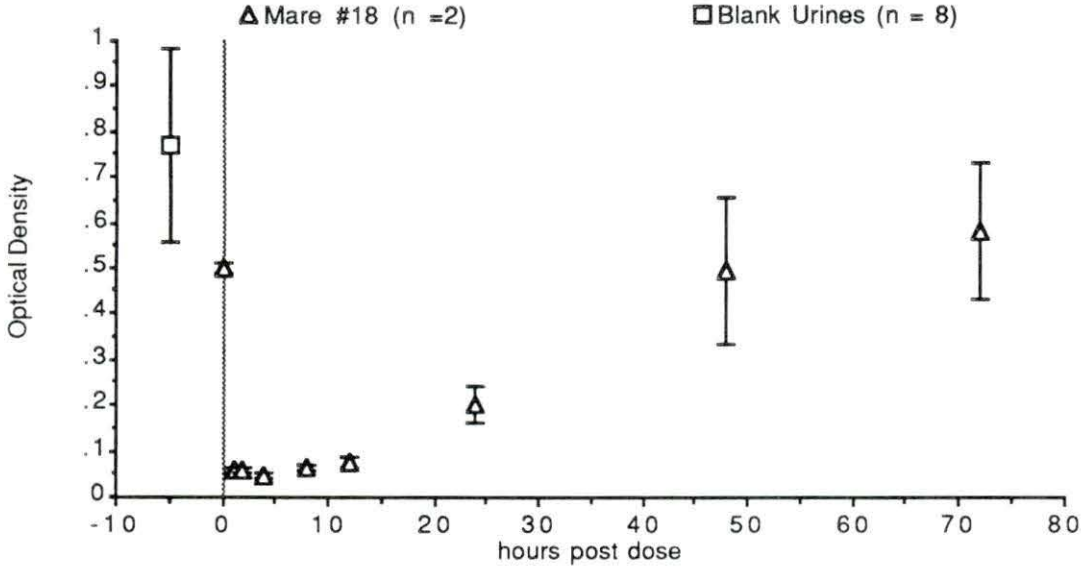


Figure 18. Urinary excretion profile produced by IDS ELISA from mare #18 administered 500 µg fentanyl base with error bars indicating two standard deviations (blank response indicated in far left)

indicating positive results for all collection times for mare #17, with mare #18 showing positive results up to and including the 1 hour collection period (Figures 19 and 20). The "blank" urine response only transformed the 2 hour response of mare #18 to a negative, due to over-lapping error bars. It is interesting to note the IDS ELISA results clearly showed positive responses (at the 95% confidence level) for the farthest time post administration among all immunoassay methodologies utilized.

PCFIA The administered urines produced positive responses up to and including the 24 hour post administration collection period (Figures 21 and 22). The responses for collection times beyond 24 hours contained overlapping error bars when compared to the pre-administration and "blank" urines. The large error bars (at the 95% confidence level) are noted, but unexplained by the author. No "blank" plasmas were analyzed by the IDS PCFIA assay, other than the plasma collected pre-drug administration. The dosed plasmas yielded positive responses at the one and five minute collection times for both mares #17 and #18 (Figures 23 and 24). Mare #17 showed a positive response at the 10 minute period, while mare #18 produced negative results from 10 minutes on upward. After 10 minutes, mare #17 generated responses deemed as negative as well. Table 9 summarizes all the immunochemical techniques and their individual ability to detect fentanyl presence in equine urine and plasma.

Time and ease of use

The rapidity and ease-of-use of an assay can greatly affect its usability in forensic testing laboratories. Assays allowing for minimal time and personnel while at the same time employing relatively simple instrumentation, can be extremely advantageous to the racing chemist. Of all the immunoassays analyzed, the IDS PCFIA was the most rapid in terms of time to completion, taking approximately 30 minutes from start to finish of the assay. In comparison, the IDS ELISA assay and Tri-Tec ELISA assay on average took approximately 90 minutes and 105 minutes, respectively to complete. The RIA techniques took greater than 5 hours and 6 hours on average

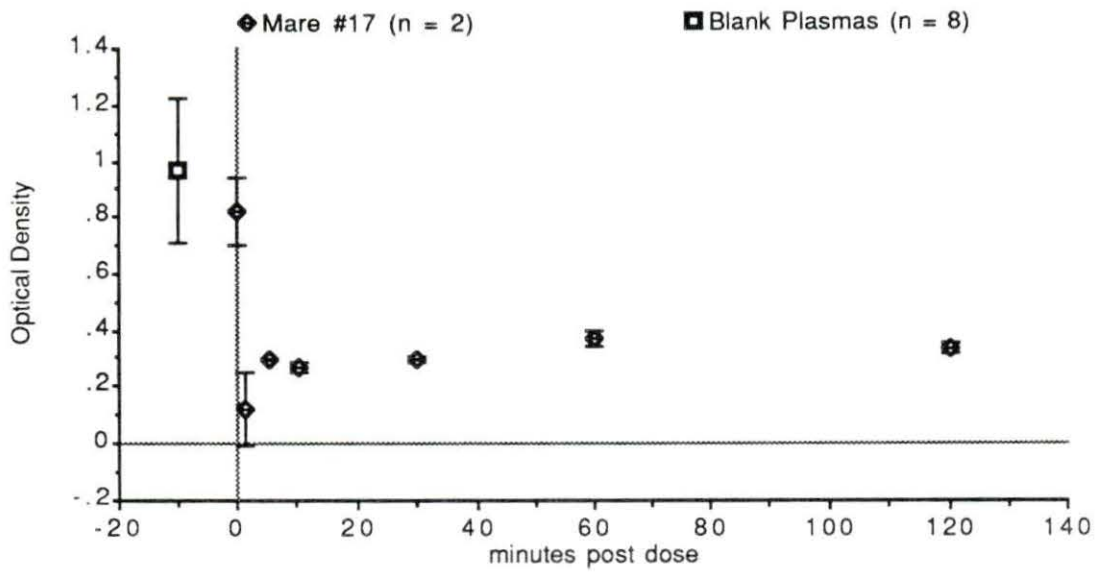


Figure 19. Plasma profile produced by IDS ELISA from mare #17 administered 500 μg fentanyl base with error bars indicating two standard deviations (blank response indicated in far left)

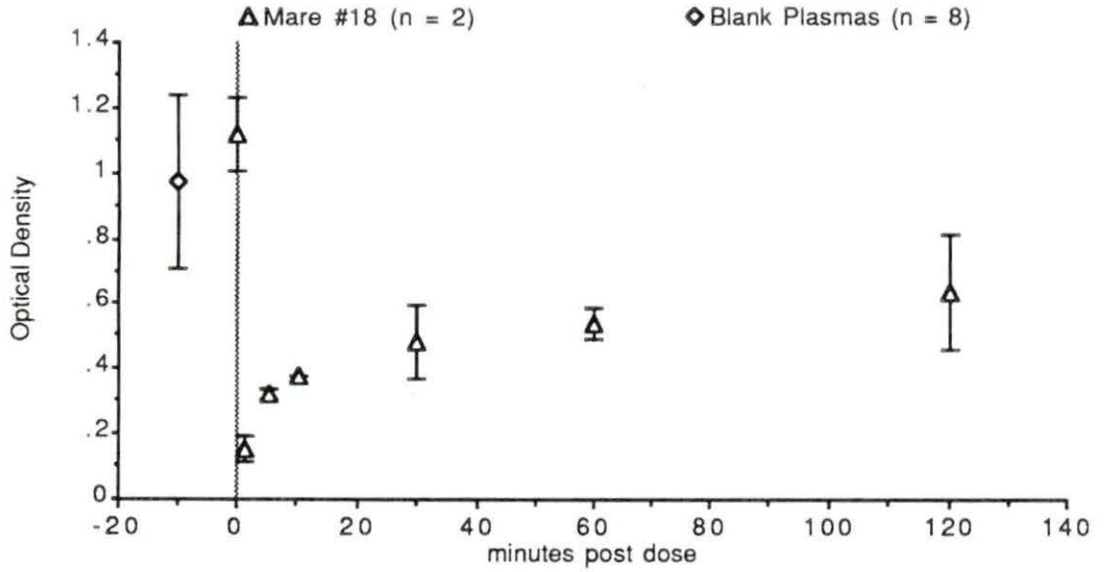


Figure 20. Plasma profile produced by IDS ELISA from mare #18 administered 500 μg fentanyl base with error bars indicating two standard deviations (blank response indicated in far left)

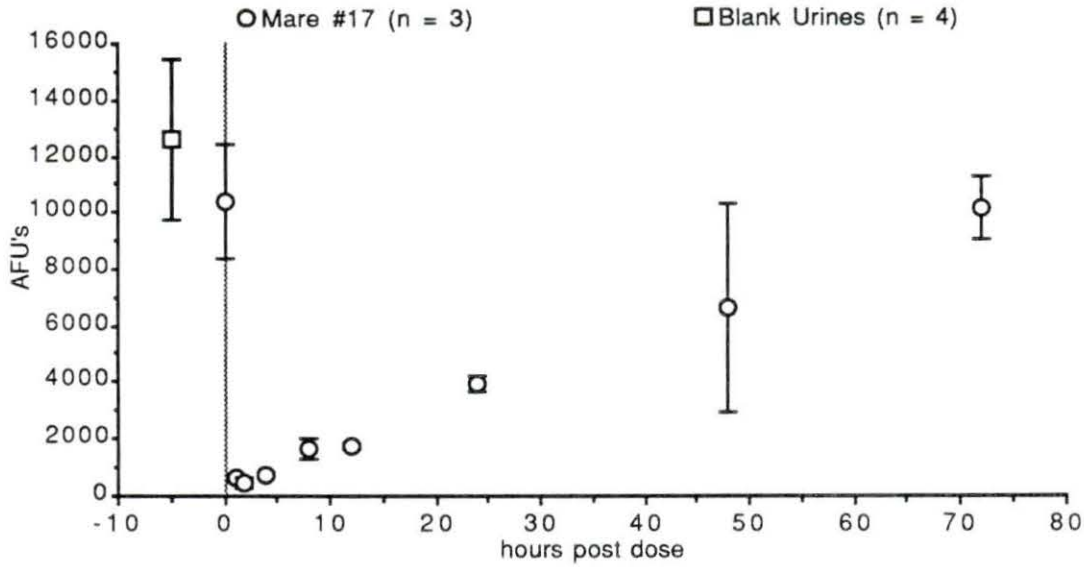


Figure 21. Urinary excretion profile produced by IDS PCFIA from mare #17 administered 500 µg fentanyl base with error bars indicating two standard deviations (blank response indicated in far left)

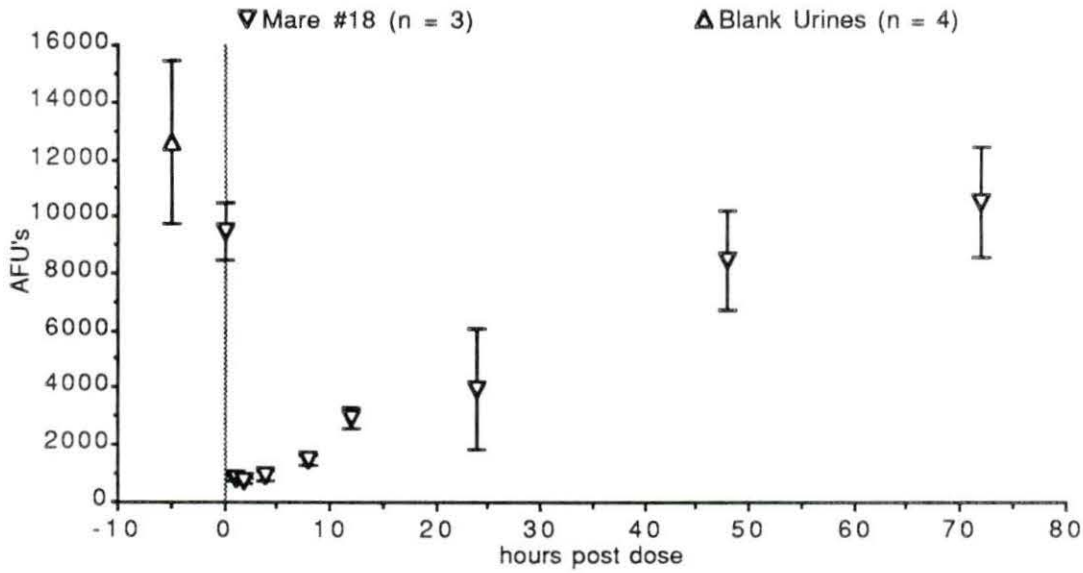


Figure 22. Urinary excretion profile produced by IDS PCFIA from mare #18 administered 500 µg fentanyl base with error bars indicating two standard deviations (blank response indicated in far left)

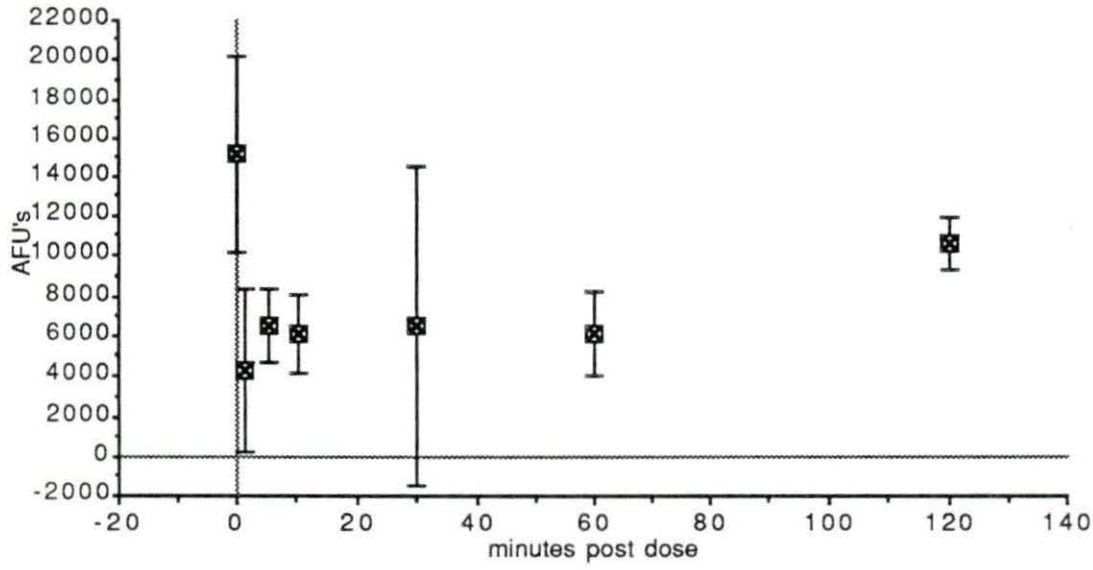


Figure 23. Plasma profile produced by IDS PCFIA from mare #17 administered 500 µg fentanyl base with error bars indicating two standard deviations, n = 3

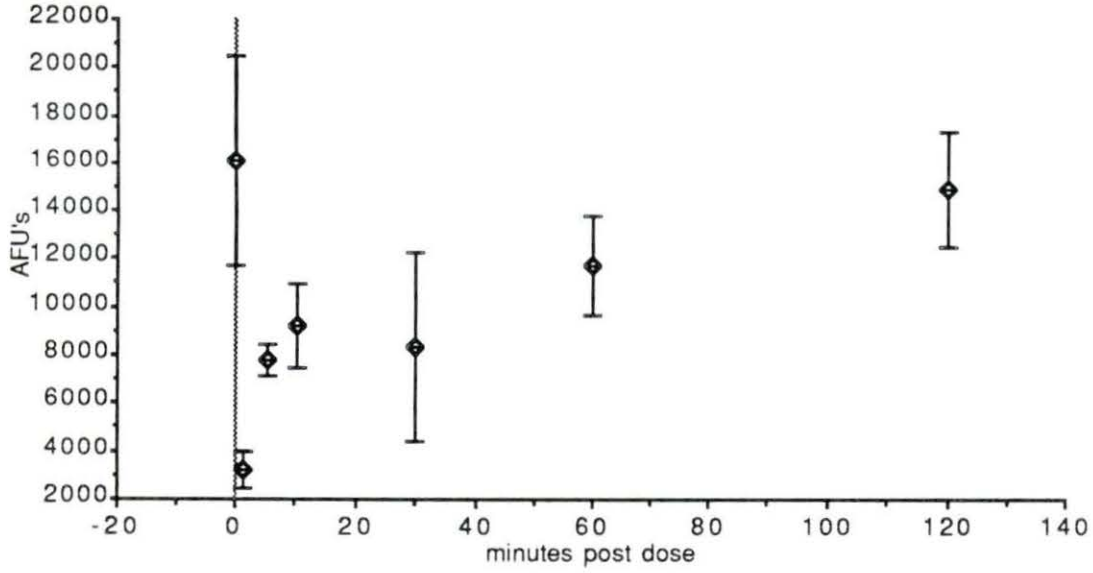


Figure 24. Plasma profile produced by IDS PCFIA from mare #18 administered 500 µg fentanyl base with error bars indicating two standard deviations, n = 3

respectively, for the ^{125}I-RIA and ^{3}H-RIA to generate results using liquid scintillation counting. Table 9 lists the respective assays and their average time to completion in the current research. The time necessary to complete an assay will vary according to the number of samples tested, and can be facilitated much more rapidly through the use of repeater pipettes for rapid reagent addition.

All the immunoassays were fairly easy to perform as they all were of a "recipe" format. The RIAs were the most difficult and cumbersome of the immunochemical assays to perform due to the fact that handling of radioisotopes was necessary for assay execution, requiring precautions to be employed because of the radioactive presence. Also, when comparing the RIAs individually, the Cambridge Medical assay was easier to perform than the Janssen assay as the directions included in the kit were much clearer, the reagents were easier to prepare, and was overall less time consuming. Due to a lack of radioactivity in the ELISA and PCFIA kits, these assays were able to be performed rapidly, with little difficulty in terms of assay execution.

In terms of instrumentation, the RIAs necessitated the use of a liquid scintillation counter, and although not difficult to operate it, does require some experience to generate accurate and reproducible results. An advantageous feature of ELISA methods is the ability to interpret results strictly "by eye", without the need of a reader

Table 9. Latest collection times post administration of fentanyl (500 µg) generating positive responses (2 S.D.) for both urine and plasma from mares #17 and #18, and total time to completion of the assay procedure for all immunochemical techniques analyzed

Assay	Urine	Plasma	Time to Completion
^{125}I-RIA	NDA[a]	NDA	> 5 Hours
^{3}H-RIA	12 Hour	10 Minute	> 6 Hours
Tri-Tec ELISA	12 Hour	5 Minute	1 Hour; 45 Minutes
IDS ELISA	24 Hour	1 Hour	1 Hour; 30 Minutes
IDS PCFIA	24 Hour	5 Minute	30 Minutes

[a]NDA = No data available.

to generate results. In this manner the slightest color indication in the well would constitute a negative response, with clear wells (white-outs) denoting possible positive responses. Although a reader is not necessary to perform the assay, it is highly recommended as unknown samples producing a slight color reaction would be interpreted as negative without a reader, but probably deemed suspicious when a reader is utilized. This type of instrument is easy to operate and can be programmed so that results can be automatically generated. The PCFIA utilizes an FCA machine, and although not extremely difficult to operate, does require some practice to become familiar with the various parameters available. This instrument is also programmed to automatically generate reports.

Classical Techniques

Within the forensic drug testing community, the accepted method of screening urine and plasma samples for illicit compounds is by thin-layer chromatographic separation, followed by visualization with various chemical oversprays. It has long been known that thin-layer chromatography (TLC), although the accepted method, is a limited methodology with unsatisfactory detection limits for a wide variety of compounds of interest to the racing chemist. High performance liquid chromatography (HPLC) with ultraviolet spectrophotometric detection, although superior to TLC techniques in the ability to ascertain drug presence, is generally utilized as a screening technique rather than a confirming one. Gas chromatography with nitrogen-phosphorus thermionic or flame ionization detection are used as screening techniques, and occasionally in a confirmatory capacity. Gas chromatography/mass spectrometry methods, however, are designated and utilized specifically as confirmatory analyses when samples have been deemed suspicious by screening procedures.

Limit of detection

By comparing the detection limits of the various methods, some generalities can be made regarding the usability of the respective chromatographic techniques. A despropionylfentanyl standard at a known concentration was unavailable for the determination of a detection limit by any of the chromatographic systems.

TLC For all thin-layer work, the TLC plates were developed for a distance of five centimeters with a solvent consisting of ethyl acetate:methanol:acetic acid (8:1:1). Following development the plates were sprayed with a chemical overspray sequence of concentrated HCl-iodoplatenate-water, and the spots of interest were then visible on the plate. Following plating and development of fentanyl and despropionylfentanyl standards on the TLC plate, quenching was observed under SWUV for the despropionylfentanyl compound, while the parent molecule indicated neither quenching nor fluorescence following UV visualization. The Rf values for fentanyl and despropionylfentanyl were 0.30 and 0.54 , respectively.

Concentrations of 20, 15, 10, 5, 2, 1, 0.75, 0.5, 0.25, and 0.1 µg fentanyl present on the TLC plate were developed, visualized under UV light, and then sprayed with the aforementioned spray sequence. All concentrations higher than, and including 1 µg, were plainly visible to the naked eye at the Rf value of interest. Below 0.75 µg fentanyl the ability to visualize the spot of interest with the naked eye was greatly impaired and extremely difficult. When taken into context that an actual TLC plate would contain a large number of samples, each with endogenous constituents becoming visible following spraying, the detection limit of fentanyl would have to be classified as approximately 1 µg on the TLC plate. This is consistent with the results of Maruyama and Hosoya (1969) who reported a detection limit of 2 µg for fentanyl by TLC.

HPLC The results seem to indicate the limit of detection for fentanyl by HPLC with ultraviolet spectrophotometric detection and the parameters listed in the Materials and Methods is equal to approximately 0.5 µg on column. Figure 25 shows a fentanyl standard curve by HPLC in terms of total quantity of fentanyl on column.

GC/NPD Gas chromatography with nitrogen-phosphorus thermionic detection had a detection limit for fentanyl equal to approximately 2.0 ng on column. However, the sensitivity may decrease when actual samples are injected, due to the possibility of endogenous interferences at the retention time of fentanyl in the chromatogram. Figure 26 shows a fentanyl standard curve by GC/NPD.

GC/MS Gas chromatography/mass spectrometry detection in full SCAN and SIMS mode had detection limits for fentanyl equal to approximately 10 ng and 2 ng on column, respectively. Figures 27 and 28 show fentanyl standard curves by GC/MS in full SCAN and SIMS mode, respectively.

Sample spikes

Extraction of despropionylfentanyl urine spikes and fentanyl plasma spikes was performed to determine if the extraction techniques employed were removing enough of the analyte of interest for adequate chromatographic analysis.

TLC No thin-layer analysis of urine or plasma spikes was performed in the current research.

HPLC No high-performance liquid chromatographic analysis of urine or plasma spikes was performed in the current research.

GC/NPD Five mls of blank equine urine was spiked with despropionylfentanyl and then extracted by the acid hydrolysis procedure indicated in the Materials and Methods without the hydrolysis step being performed under acidic conditions in the pressure cooker. Urines were spiked at the following concentrations: 10, 50, 100, 250, 500, and 1000 ppb despropionylfentanyl. Table 10 shows the percent recovery of the despropionylfentanyl spikes by GC/NPD. The results indicate that at all concentrations listed, recovery of despropionylfentanyl approached or exceeded 100% indicating adequate recovery of despropionylfentanyl from the equine urine.

One ml of blank equine plasma was spiked with fentanyl at 10, 50, 100, 250, 500, and 1000 ppb and then extracted by the base extraction procedure indicated in the Materials and Methods. Table 11 shows the percent recovery of the fentanyl plasma spikes by

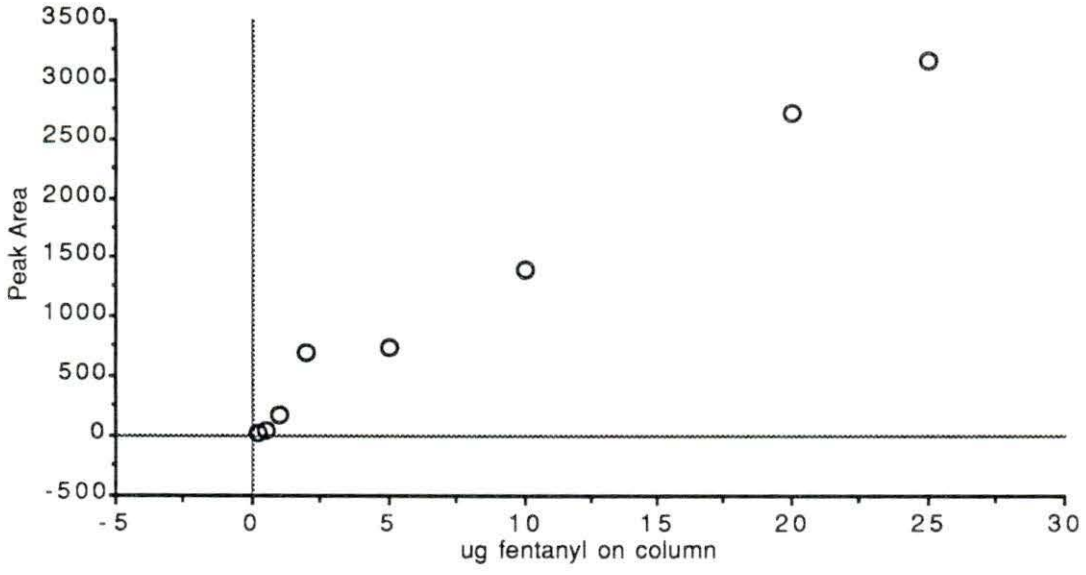


Figure 25. Fentanyl standard curve by high-performance liquid chromatography with ultraviolet spectrometric detection (200-260 nm), n = 1

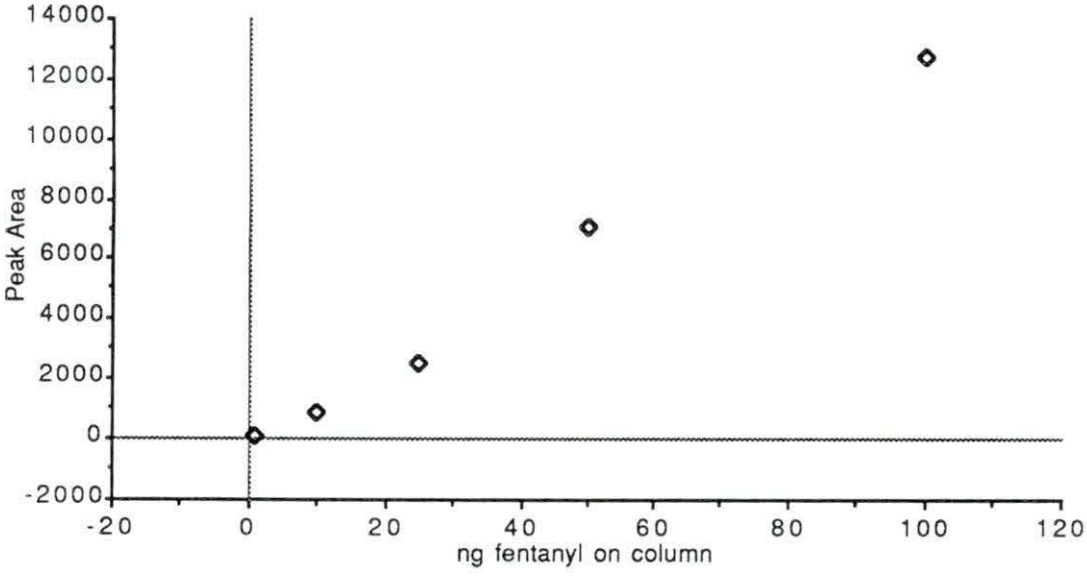


Figure 26. Fentanyl standard curve by gas chromatography using nitrogen-phosphorus thermionic detection, n = 1

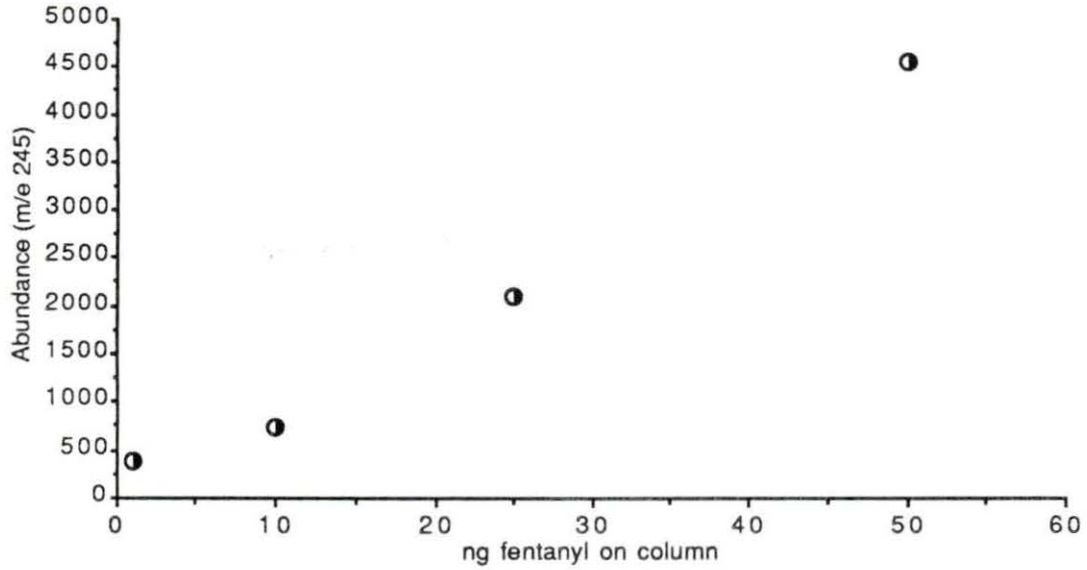


Figure 27. Fentanyl standard curve by gas chromatography with mass spectrometric detection in full scan mode, n = 1

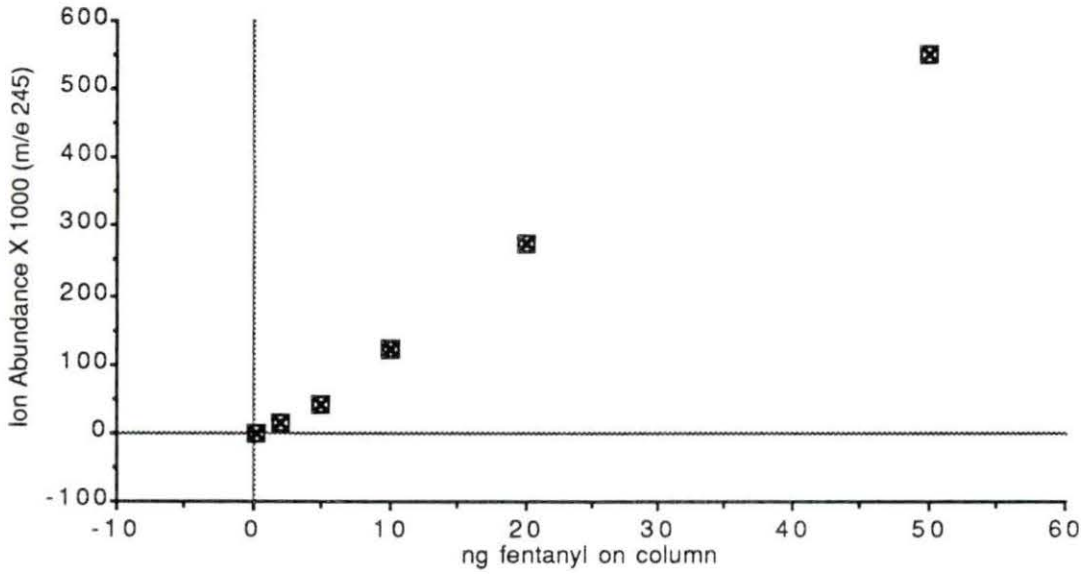


Figure 28. Fentanyl standard curve by gas chromatography with mass spectrometric detection in SIMS mode, n = 1

Table 10. Percent recovery of despropionylfentanyl urine spikes by GC/NPD (n = 1)

	10 ppb	50 ppb	0.1 ppm	0.25 ppm	0.5 ppm	1 ppm
Spike[a]	2.0	28.1	53.7	164.6	414.7	550.7
Standard[b]	3.5	16.7	137.9	167.9	440.9	552.6
% Recovery	56.9	168.3	256.7	98.0	94.1	99.7

[a]Sample spike (area X 100).
[b]Standard (area X 100).

Table 11. Percent recovery of fentanyl plasma spikes by GC/NPD (n = 1)

	10 ppb	50 ppb	0.1 ppm	0.25 ppm	0.5 ppm	1 ppm
Spike[a]	13.3	65.5	78.9	234.8	330.4	977.7
Standard[b]	15.5	72.8	93.4	205.1	390.2	989.7
% Recovery	85.6	90.0	84.5	114.5	84.7	98.8

[a]Sample spike (area X 100).
[b]Standard (area X 100).

GC/NPD following base extraction. The results indicate that at all concentrations listed, recovery of fentanyl approached or exceeded 100%, indicating adequate recovery of the parent fentanyl molecule from plasma using base extraction techniques.

GC/MS Within the forensic racing community, gas chromatography followed by mass spectrometric detection is the current accepted method of choice for legal identification and confirmation of drug presence within a particular sample of interest. For despropionylfentanyl urine spikes and fentanyl plasma spikes, abundance of ion m/e 146 was used to determine percent recovery by GC/MS in single ion monitoring mode (SIMS). Tables 12 and 13 indicate the percent recovery of the despropionylfentanyl urine and fentanyl plasma spikes by GC/MS in single ion monitoring mode following acid hydrolysis and base extraction, respectively. The results indicate that at all concentrations listed, recovery of fentanyl approached or

Table 12. Percent recovery of despropionylfentanyl urine spikes by GC/MS (n = 1)

	10 ppb	50 ppb	0.1 ppm	0.25 ppm	0.5 ppm	1 ppm
Spike[a]	71.4	586.9	2899.0	2842.1	5811.7	7237.6
Standard[b]	78.6	567.4	1309.2	2560.5	5298.7	6852.6
% Recovery	90.8	103.5	221.4	111.0	109.7	105.6

[a]Ion abundance (m/e 146) X 1000 of sample spike.
[b]Ion abundance (m/e 146) X 1000 of standard.

Table 13. Percent recovery of fentanyl plasma spikes by GC/MS (n = 1)

	10 ppb	50 ppb	0.1 ppm	0.25 ppm	0.5 ppm	1 ppm
Spike[a]	19.5	141.6	227.0	532.4	1164.9	2324.6
Standard[b]	16.0	153.8	170.2	582.8	1312.3	2377.8
% Recovery	121.9	92.0	133.4	91.3	88.8	97.8

[a]Ion abundance (m/e 146) X 1000 of sample spike.
[b]Ion abundance (m/e 146) X 1000 of standard.

exceeded 100%, indicating good recovery of despropionylfentanyl and parent fentanyl molecule from urine and plasma by the extraction techniques utilized, coinciding with the GC/NPD results.

Administration urines

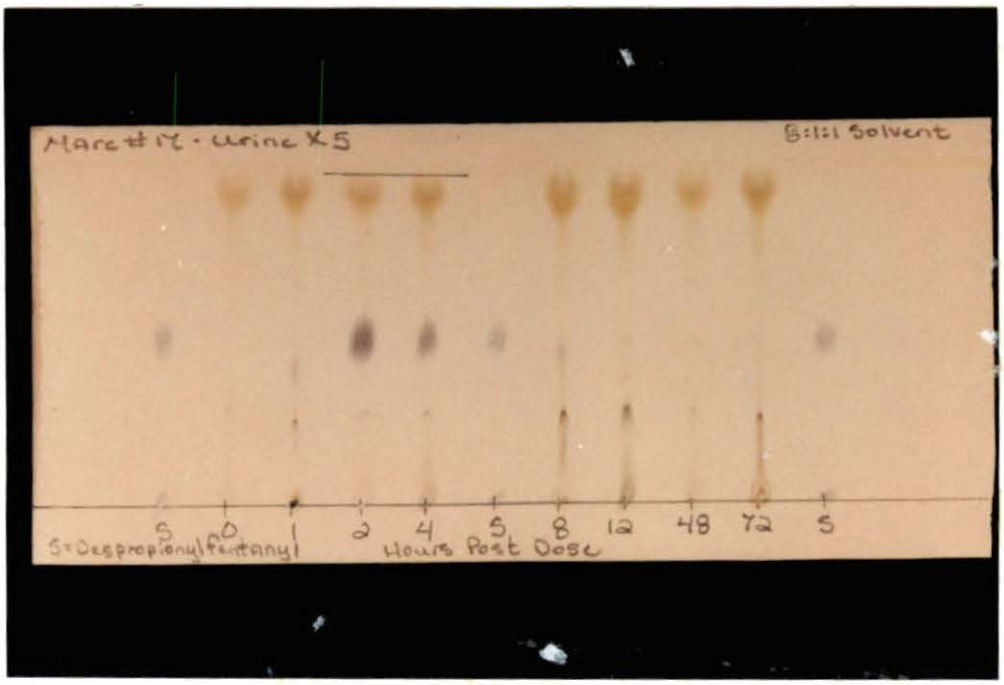
Thin-layer chromatography Analysis of administration urines from both mares #17 and #18, 45 mls of urine were acid hydrolyzed according to the revision of the McDonald and Ozog (1978) procedure previously described. The results indicate that at the administered dose (1.47 µg/kg), presence of the beta-keto acid hydrolysis product, despropionylfentanyl, can be detected according to the currently accepted thin-layer parameters up to approximately 8 hours post administration when 45 mls of urine are used in the analysis. From 1 hour to 8 hours post dose, visualization to the naked eye of original fentanyl administration (due to despropionylfentanyl presence) was possible in both animals. After 8 hours post dose, neither the presence of

despropionylfentanyl or parent fentanyl was indicated on the plate following TLC analysis (Pictures 1 and 2).

It must be kept in mind that the acid hydrolysis procedure and iodoplatenate spray sequence are unique techniques, designed specifically for the ascertation of fentanyl and fentanyl-like compounds in urine or plasma. Thus, these techniques are usually not part of the daily practices of forensic racing laboratories, and are only performed when samples enter the lab labelled as needing special attention for fentanyl-like compounds.

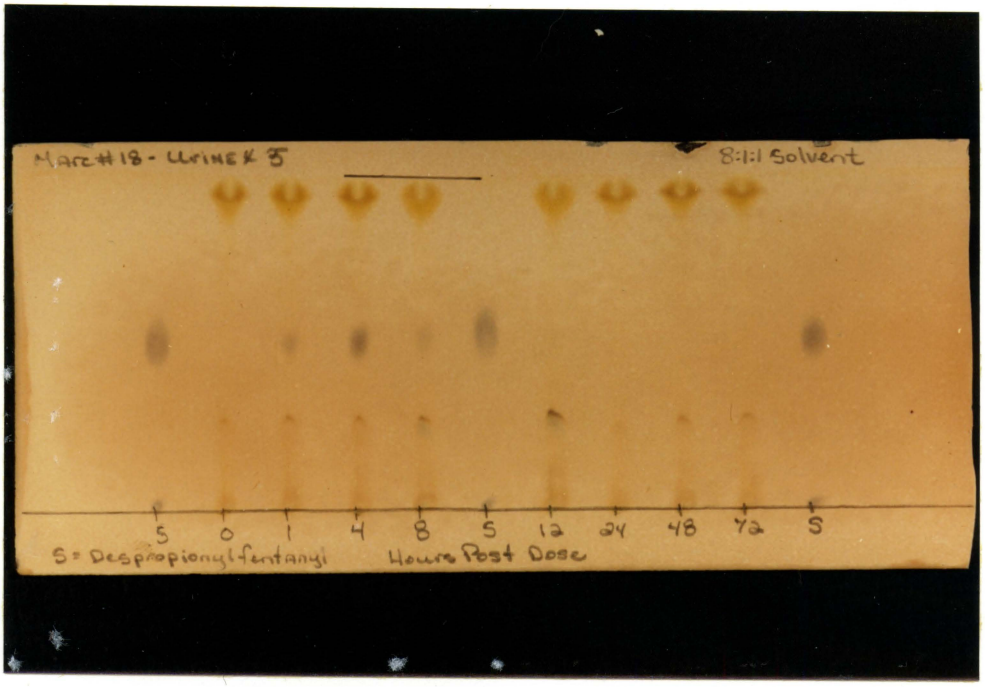
HPLC High performance liquid chromatographic analysis of the administration urines and plasmas was performed assuming no prior knowledge of previous fentanyl administration. Within the Racing Chemistry Laboratory at Iowa State University, all unknown samples requiring HPLC-UV diode array analysis are analyzed by monitoring the ultraviolet range of 200-260 nm, as most compounds will absorb ultraviolet light within that 60 nm range. Utilizing the procedure indicated in the Materials and Methods, the presence of despropionylfentanyl following hydrolysis of twenty-seven mls post administration urines (#17 and #18) was not indicated in any of the hours post dose. Thus, HPLC with ultraviolet spectrophotometric detection was not sufficiently sensitive to detect fentanyl administration (by despropionylfentanyl presence) when 500 µg fentanyl was the original administration dosage.

GC/NPD Acid hydrolysis of twenty-seven mls of urine was performed three times and the results were averaged for each hour post dose. When analyzing the total area under the despropionylfentanyl peak in the chromatogram following acid hydrolysis, mares #17 and #18 both seem to reach maximum excretion of fentanyl metabolites in the urine at 2 hours post administration (Figures 29 and 30). An extremely important factor as to whether fentanyl equivalents will be detected in urine would be the acid hydrolysis step, and complete hydrolysis of the beta-keto acid metabolite over to despropionylfentanyl at the appropriate psi is essential for the detection of fentanyl administration in urine. The farthest time post administration in which despropionylfentanyl was detected by GC/NPD was 24 hours post administration in



Spray sequence = concentrated HCl: iodoplatenate reagent: H_2O

Picture 1. Thin-layer chromatographic results from mare #17 following acid hydrolysis of forty-five mls urine



Spray sequence = concentrated HCl: iodoplatenate reagent: H_2O

Picture 2. Thin-layer chromatographic results from mare #18 following acid hydrolysis of forty-five mls urine

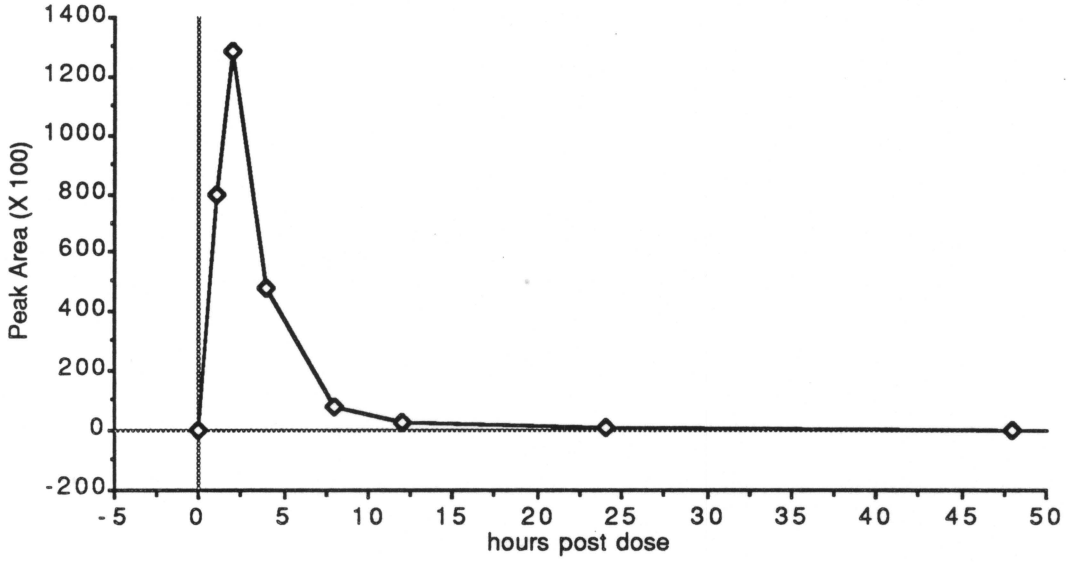


Figure 29. Urinary excretion profile of mare #17 by GC/NPD following acid hydrolysis, n = 3

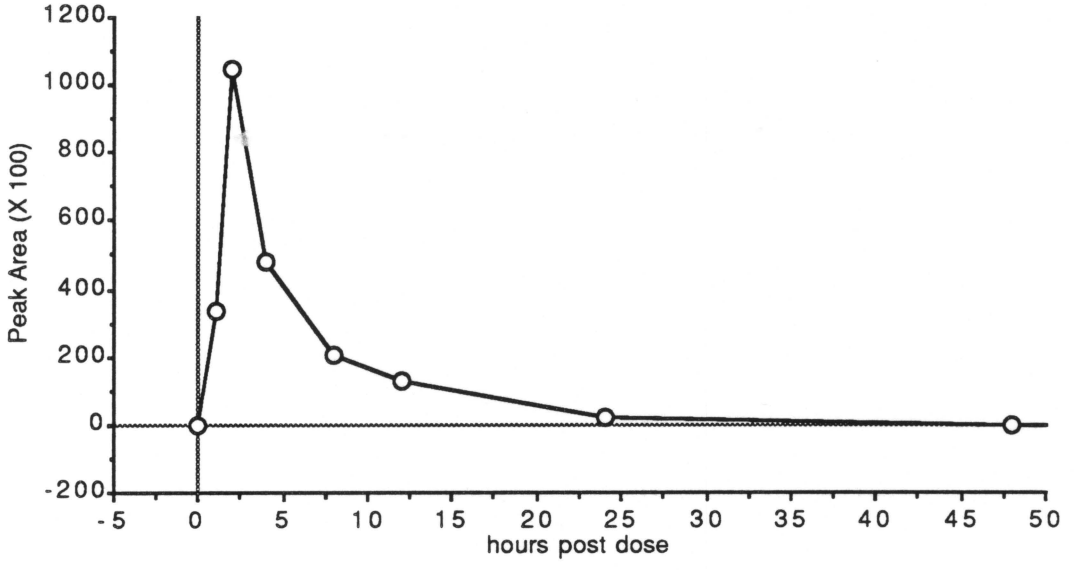


Figure 30. Urinary excretion profile of mare #18 by GC/NPD following acid hydrolysis, n = 3

both mares #17 and #18 (Table 14). Results from pre-administration urines indicate endogenous constituents elute from the column at approximately the same time as despropionylfentanyl and must be taken into account when analyzing GC/NPD chromatograms.

GC/MS When analyzing the total abundance of ions m/e 146, m/e 189, and m/e 280 in full SCAN and SIMS mode, mares #17 and #18 both reach maximum excretion of fentanyl metabolites in the urine at 2 hours post administration which is consistent with the GC/NPD results (Figures 31 through 34). Using full SCAN analysis, the farthest time post administration in which despropionylfentanyl was detectable by the presence of all three major ions (m/e 146, m/e 189, and m/e 280) was 12 hours post dose in both mares #17 and #18 (Table 15). However, using SIMS analysis, the farthest time post administration was increased to 24 hours in which the presence of the three major ions of despropionylfentanyl were present in the urine of both mares #17 and #18 (Table 16).

It must be kept in mind, however, that initial mass spectral analysis of suspicious samples is routinely performed in full SCAN mode. The SCAN run allows for all ions within the sample to be detected, and then when an exogenous drug is thought to be present SIMS analysis can be performed by selectively looking at specific ions within the chromatogram. For samples to be run initially in SIMS mode, an indication of drug presence must be known prior to analysis in order to select the ions of interest for mass spectral detection.

Administration plasmas

TLC For thin-layer analysis of the administration plasmas, 6 mls of plasma were extracted according to the base hydrolysis procedure previously described. As pictures 3 and 4 indicate, at the dose administered, no parent fentanyl was observed in the plasma at any of the collection times post dose following spraying. Two factors may have contributed to the results obtained: a) fentanyl is a very hydrophobic compound, and b) the initial dose (1.47 μg/kg) was small in relation to concentrations known to

Table 14. Average peak area (despropionylfentanyl) of urine from mares #17 and #18 by GC/NPD following acid hydrolysis (n = 3)

	Mare #17								
	0 Hr	1 Hr	2 Hr	4 Hr	8 Hr	12 Hr	24 Hr	48 Hr	72 Hr
Peak Area[a]	2.8	799.8	1290.2	478.0	83.6	26.7	9.8[b]	NDA[c]	NDA
% RSD[d]	86.8	25.1	29.7	41.6	53.9	45.7	1.4	N/A[e]	N/A
	Mare #18								
	0 Hr	1 Hr	2 Hr	4 Hr	8 Hr	12 Hr	24 Hr	48 Hr	72 Hr
Peak Area	NDA	338.9	1048.8	481.1	203.9	125.2	19.4	NDA	NDA
% RSD	N/A	90.3	104.1	115.4	118.7	106.2	70.6	N/A	N/A

[a]Peak area X 100.

[b]n = 2.

[c]No detectable amount.

[d]Relative standard deviation as % of the mean.

[e]Not available.

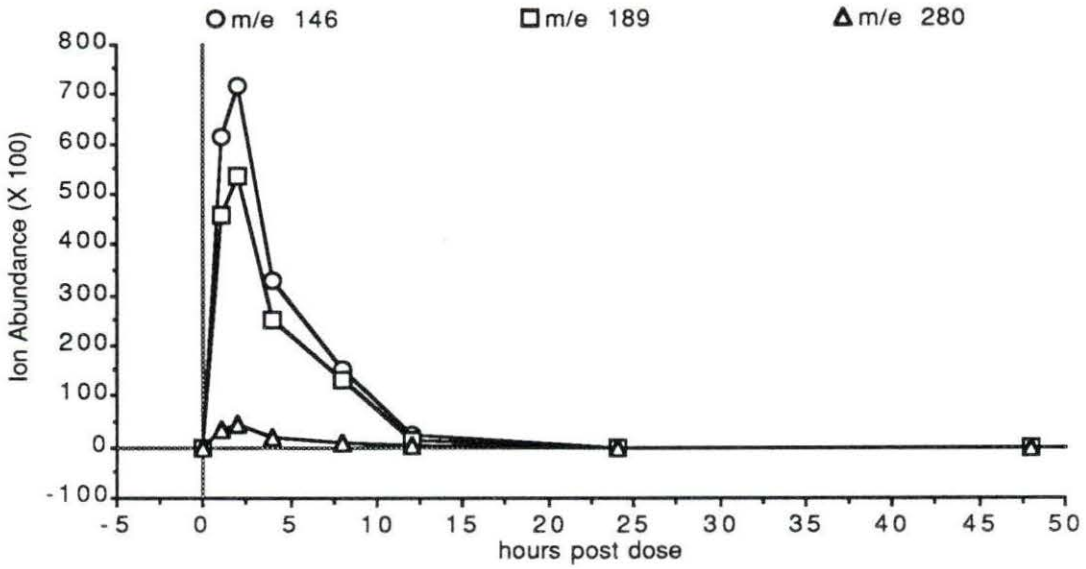


Figure 31. Mare #17 urinary excretion profiles following administration of 500 µg fentanyl i.v. by GC/MS in full SCAN mode, n = 3

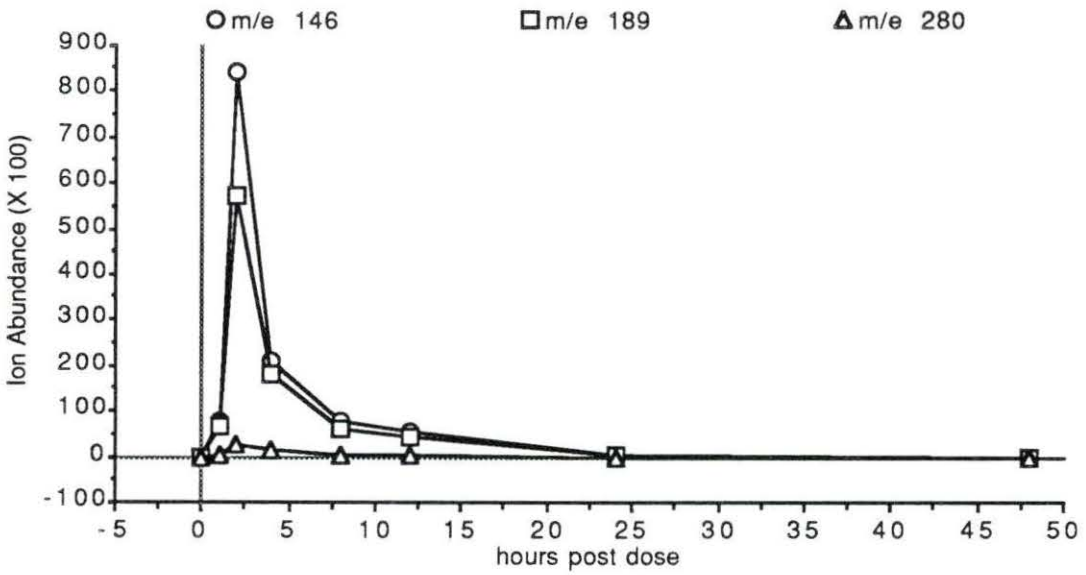


Figure 32. Mare #18 urinary excretion profiles following administration of 500 µg fentanyl i.v. by GC/MS in full SCAN mode, n = 3

Table 15. Average ion abundance (despropionylfentanyl) of urine from mares #17 and #18 by GC/MS in full SCAN mode following acid hydrolysis (n = 3)

Mare #17

					m/e 146				
	0 Hr	1 Hr	2 Hr	4 Hr	8 Hr	12 Hr	24 Hr	48 Hr	72 Hr
Ion Abundance[a]	NDA[b]	613.4	718.9	330.9	155.3	22.0	NDA	NDA	NDA
% RSD[c]	N/A[d]	73.2	23.3	58.6	81.9	100.4	N/A	N/A	N/A

					m/e 189				
	0 Hr	1 Hr	2 Hr	4 Hr	8 Hr	12 Hr	24 Hr	48 Hr	72 Hr
Ion Abundance	NDA	458.2	538.7	252.6	133.6	16.0	NDA	NDA	NDA
% RSD	N/A	12.0	28.1	56.5	82.2	106.9	N/A	N/A	N/A

					m/e 280				
	0 Hr	1 Hr	2 Hr	4 Hr	8 Hr	12 Hr	24 Hr	48 Hr	72 Hr
Ion Abundance	NDA	34.4	44.5	19.1	10.7	1.7	NDA	NDA	NDA
% RSD	N/A	15.7	30.8	64.9	94.4	173.1	N/A	N/A	N/A

Mare #18

					m/e 146				
	0 Hr	1 Hr	2 Hr	4 Hr	8 Hr	12 Hr	24 Hr	48 Hr	72 Hr
Ion Abundance	NDA	76.3	845.2[e]	210.8	76.7	55.6	4.7	NDA	NDA
% RSD	N/A	73.8	111.3	91.5	96.1	77.8	86.8	N/A	N/A

	m/e 189								
	0 Hr	1 Hr	2 Hr	4 Hr	8 Hr	12 Hr	24 Hr	48 Hr	72 Hr
Ion Abundance	NDA	65.4	570.5[e]	179.6	62.9	45.5	4.1	NDA	NDA
% RSD	N/A	69.9	109.1	100.8	97.2	78.9	87.2	N/A	N/A

	m/e 280								
	0 Hr	1 Hr	2 Hr	4 Hr	8 Hr	12 Hr	24 Hr	48 Hr	72 Hr
Ion Abundance	NDA	5.6	28.7[e]	16.7	4.9	4.8	NDA	NDA	NDA
% RSD	N/A	81.9	97.0	116.5	136.0	74.9	N/A	N/A	N/A

[a]Ion abundance X 100.

[b]No detectable amount.

[c]Relative standard deviation as % of the mean.

[d]Not available.

[e]n = 2.

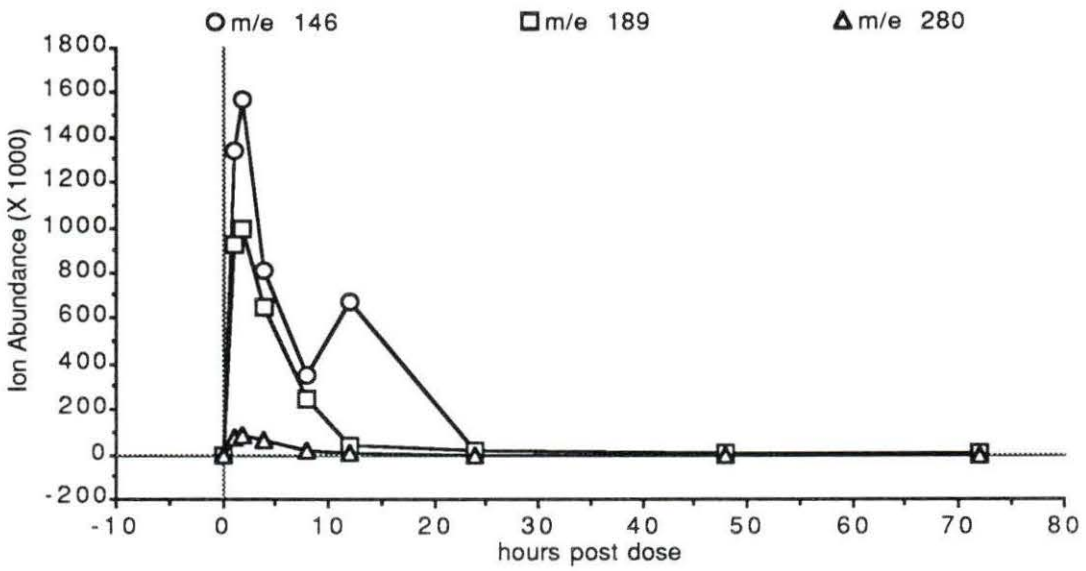


Figure 33. Mare #17 urinary excretion profiles following administration of 500 µg fentanyl i.v. by GC/MS in SIMS mode, n = 3

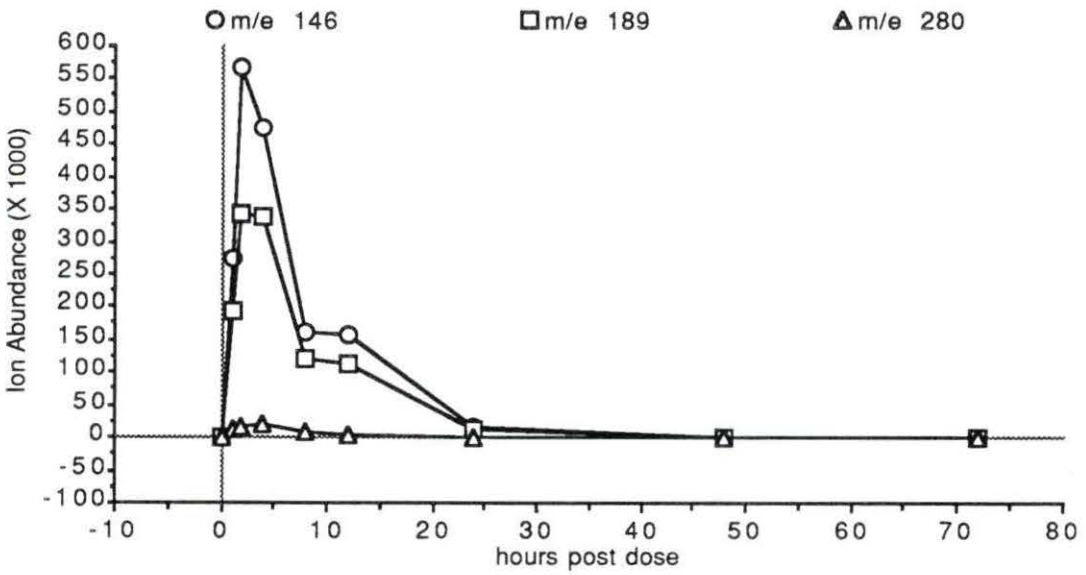


Figure 34. Mare #18 urinary excretion profiles following administration of 500 µg fentanyl i.v. by GC/MS in SIMS mode, n = 3

Table 16. Average ion abundance (despropionylfentanyl) of urine from mares #17 and #18 by GC/MS in single ion monitoring mode following acid hydrolysis (n = 3)

Mare #17

					m/e 146				
	0 Hr	1 Hr	2 Hr	4 Hr	8 Hr	12 Hr	24 Hr	48 Hr	72 Hr
Ion Abundance[a]	NDA[b]	1339.8	1571.6	809.5	354.3	67.4	23.0[c]	2.2	2.0
% RSD[d]	N/A[e]	31.9	19.4	68.6	82.1	82.7	N/A	65.4	95.1

					m/e 189				
	0 Hr	1 Hr	2 Hr	4 Hr	8 Hr	12 Hr	24 Hr	48 Hr	72 Hr
Ion Abundance	NDA	924.6	998.0	654.6	247.0	46.5	13.7[e]	1.6	1.2
% RSD	N/A	31.6	14.1	79.3	81.3	85.9	N/A	63.1	60.3

					m/e 280				
	0 Hr	1 Hr	2 Hr	4 Hr	8 Hr	12 Hr	24 Hr	48 Hr	72 Hr
Ion Abundance	NDA	80.6	82.9	64.4	22.0	3.7	NDA[e]	0.13	0.07
% RSD	N/A	41.9	25.4	87.4	83.3	90.9	N/A	172.5	172.0

Mare #18

					m/e 146				
	0 Hr	1 Hr	2 Hr	4 Hr	8 Hr	12 Hr	24 Hr	48 Hr	72 Hr
Ion Abundance	NDA	275.4	567.9[e]	473.5	161.0	156.4	15.6	1.2	0.6
% RSD	N/A	80.3	N/A	36.8	73.6	83.4	106.1	48.2	111.3

	m/e 189								
	0 Hr	1 Hr	2 Hr	4 Hr	8 Hr	12 Hr	24 Hr	48 Hr	72 Hr
Ion Abundance	NDA	194.7	344.4[e]	337.3	122.4	112.8	11.9	1.0	0.2
% RSD	N/A	83.2	N/A	32.2	78.2	82.6	103.8	47.8	173.3

	m/e 280								
	0 Hr	1 Hr	2 Hr	4 Hr	8 Hr	12 Hr	24 Hr	48 Hr	72 Hr
Ion Abundance	NDA	12.8	18.4[e]	20.5	8.6	6.6	0.7	NDA	NDA
% RSD	N/A	105.9	N/A	53.7	104.2	75.8	79.6	N/A	N/A

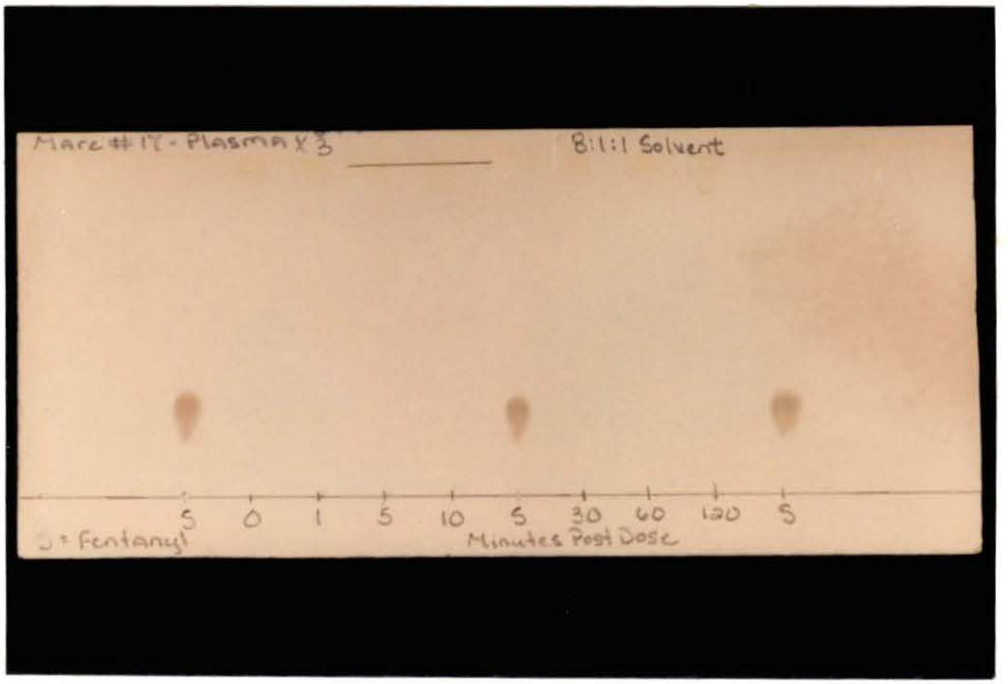
[a]Ion abundance X 1000.

[b]No detectable amount.

[c]n = 1.

[d]Relative standard deviation as % of the mean.

[e]Not available.



Spray sequence = concentrated HCl: iodoplatenate reagent: H_2O

Picture 3. Thin-layer chromatographic results from mare #17 following base extraction of six mls plasma

Table 16. Average ion abundance (despropionylfentanyl) of urine from mares #17 and #18 by GC/MS in single ion monitoring mode following acid hydrolysis (n = 3)

Mare #17

					m/e 146				
	0 Hr	1 Hr	2 Hr	4 Hr	8 Hr	12 Hr	24 Hr	48 Hr	72 Hr
Ion Abundance[a]	NDA[b]	1339.8	1571.6	809.5	354.3	67.4	23.0[c]	2.2	2.0
% RSD[d]	N/A[e]	31.9	19.4	68.6	82.1	82.7	N/A	65.4	95.1

					m/e 189				
	0 Hr	1 Hr	2 Hr	4 Hr	8 Hr	12 Hr	24 Hr	48 Hr	72 Hr
Ion Abundance	NDA	924.6	998.0	654.6	247.0	46.5	13.7[e]	1.6	1.2
% RSD	N/A	31.6	14.1	79.3	81.3	85.9	N/A	63.1	60.3

					m/e 280				
	0 Hr	1 Hr	2 Hr	4 Hr	8 Hr	12 Hr	24 Hr	48 Hr	72 Hr
Ion Abundance	NDA	80.6	82.9	64.4	22.0	3.7	NDA[e]	0.13	0.07
% RSD	N/A	41.9	25.4	87.4	83.3	90.9	N/A	172.5	172.0

Mare #18

					m/e 146				
	0 Hr	1 Hr	2 Hr	4 Hr	8 Hr	12 Hr	24 Hr	48 Hr	72 Hr
Ion Abundance	NDA	275.4	567.9[e]	473.5	161.0	156.4	15.6	1.2	0.6
% RSD	N/A	80.3	N/A	36.8	73.6	83.4	106.1	48.2	111.3

cause rapid and reliable pharmacological responses in the equine (Tobin et al., 1979b; Combie et al., 1979). Thus, the rather small quantity of fentanyl could have been absorbed into the tissues at such a rapid rate that after several minutes the quantity available in the blood stream was too minute for TLC analysis.

HPLC Presence of fentanyl following extraction of six mls post administration plasmas (#17 and #18) was not indicated in any of the collections post administration. Thus, HPLC with ultraviolet spectrophotometric detection at 200-260 nm was not sufficiently sensitive enough to detect fentanyl administration in plasma when 500 μg fentanyl was the original administration dosage.

GC/NPD Base extraction of two mls of plasma was performed three times and the results were averaged for each hour post dose. When analyzing the total area under the fentanyl peak in the chromatogram following injection, mares #17 and #18 both reach maximum fentanyl levels in the blood stream at 1 minute post administration (Figures 35 and 36). The presence of despropionylfentanyl was indicated in both mares up to and including 10 minutes post administration. Peak area decreased to near background values at 30 minutes post administration and remained at background in the subsequent collections. In addition, the GC/NPD results indicate the presence of endogenous constituents which elute at approximately the same retention time as fentanyl (Table 17).

GC/MS In agreement with the GC/NPD results, abundance of ions m/e 146, m/e 189, and m/e 245 in full SCAN and SIMS mode show mares #17 and #18 reach maximum plasma fentanyl concentration at 1 minute post administration (Figures 37 through 40). Using full SCAN analysis, the farthest time post administration in which fentanyl was detectable by the presence of all three major ions (m/e 146, m/e 189, and m/e 245) was 5 minutes post dose for both mares #17 and #18 (Table 18). Using SIMS analysis, the farthest time post administration was increased to 10 minutes for mare #17 and 30 minutes for mare #18 by the presence of all three major ions of fentanyl. (Table 19).

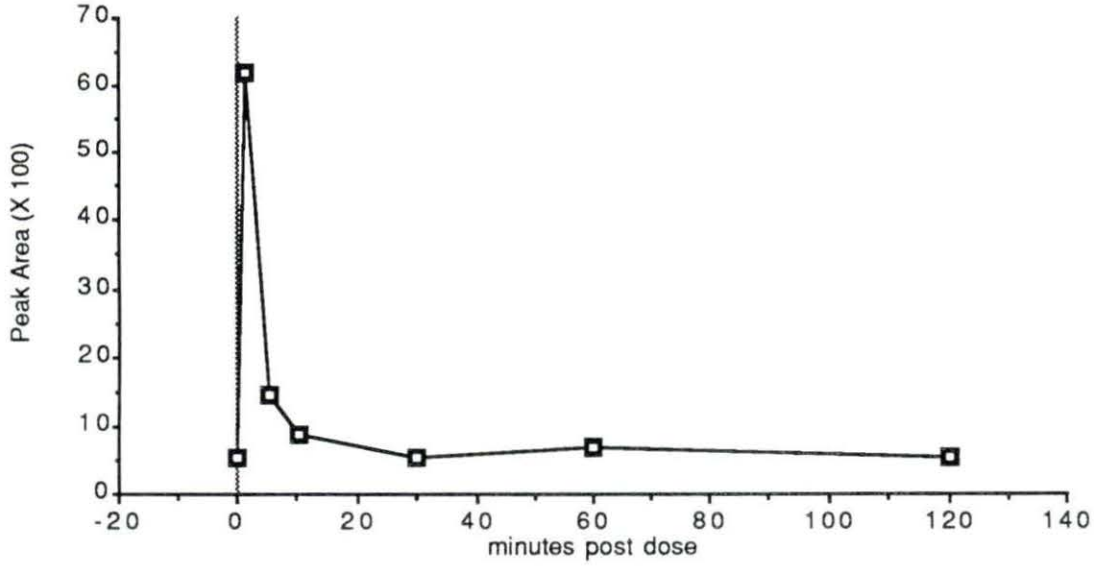


Figure 35. Plasma profile of mare #17 by GC/NPD following base extraction, n = 3

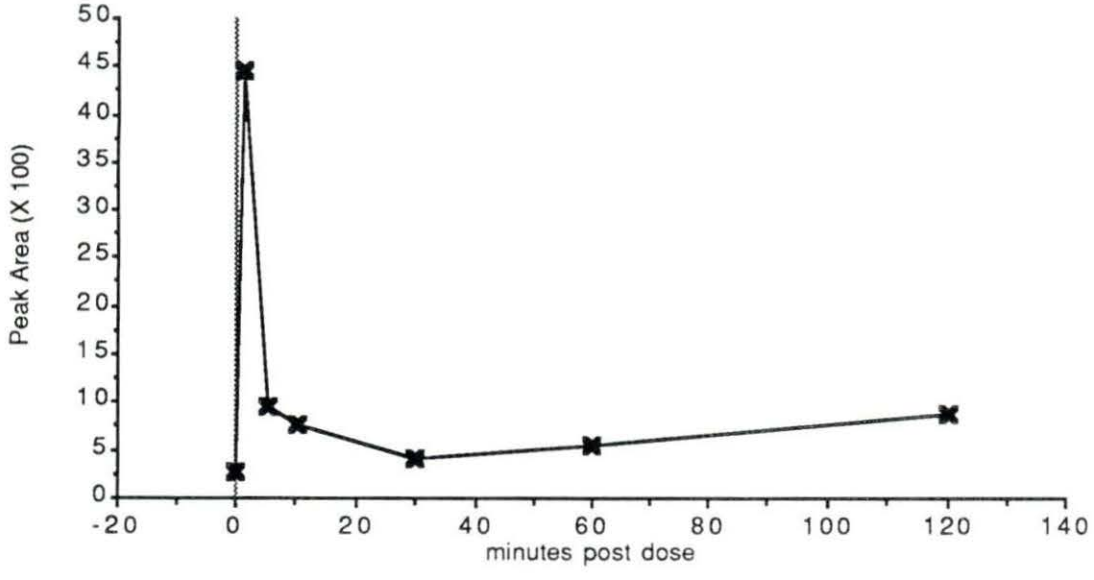


Figure 36. Plasma profile of mare #18 by GC/NPD following base extraction, n = 3

Table 17. Average peak area (fentanyl) of plasma from mares #17 and #18 by GC/NPD following base extraction (n = 3)

	Mare #17						
	0 Hr	1 Min	5 Min	10 Min	30 Min	1 Hr	2 Hr
Peak Area[a]	5.5	62.0	14.7	9.0	5.5	6.7	5.4
%RSD[b]	49.5	16.0	14.3	61.1	20.0	71.6	40.7
	Mare #18						
	0 Hr	1 Min	5 Min	10 Min	30 Min	1 Hr	2 Hr
Peak Area	2.8	44.5	9.5	7.8	4.1	5.3	8.7
%RSD	103.6	34.8	43.2	39.7	107.3	35.8	94.3

[a]Peak area X 100.

[b]Relative standard deviation as % of the mean.

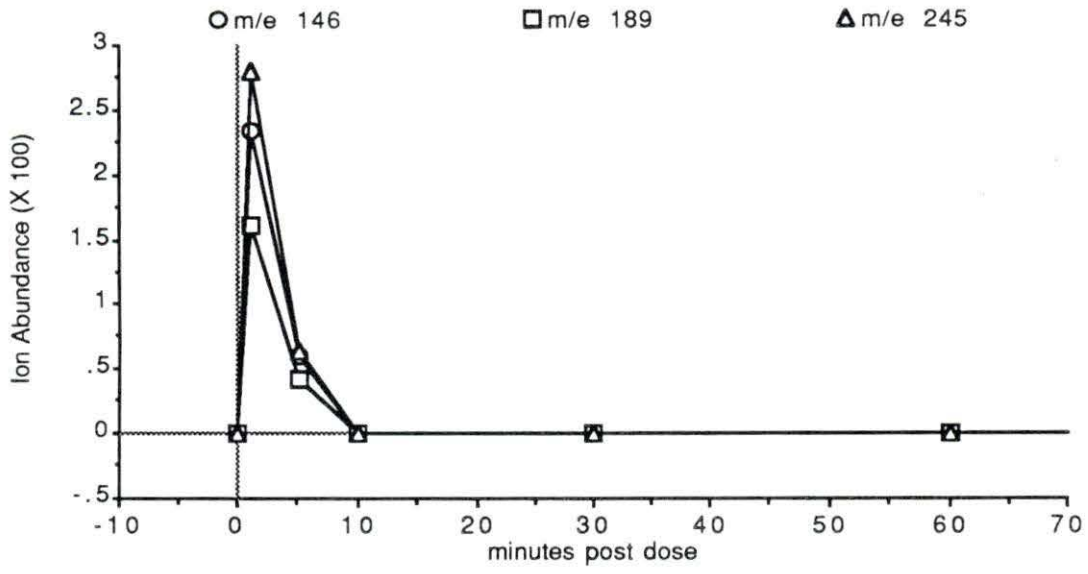


Figure 37. Mare #17 plasma profiles following administration of 500 µg fentanyl i.v. by GC/MS in full SCAN mode, n = 3

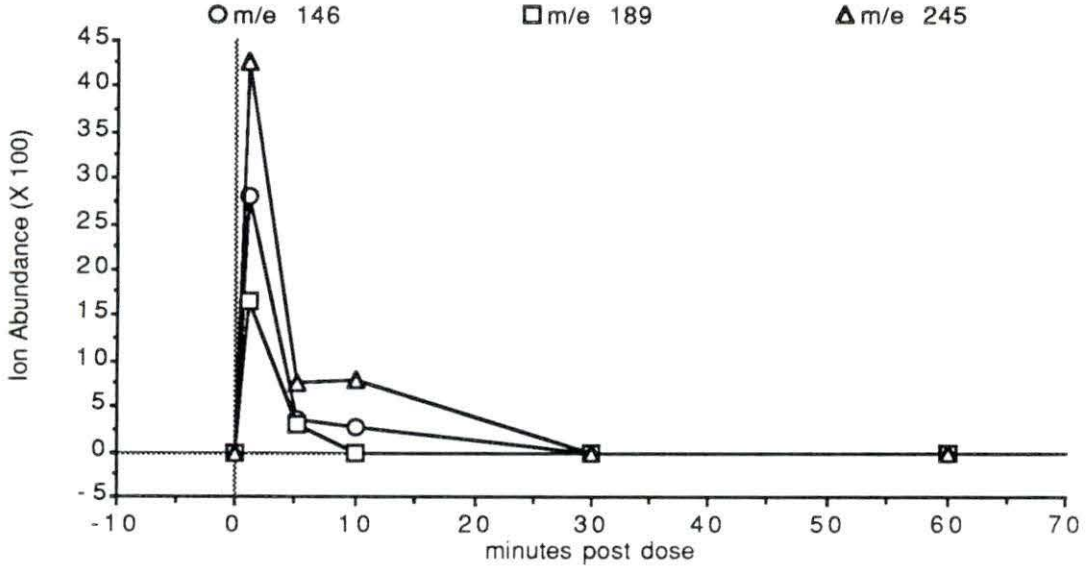


Figure 38. Mare #18 plasma profiles following administration of 500 µg fentanyl i.v. by GC/MS in full SCAN mode, n = 3

Table 18. Average ion abundance (fentanyl) of plasma from mares #17 and #18 by GC/MS in full SCAN mode following base extraction (n = 3)

Mare #17

				m/e 146			
	0 Hr	1 Min	5 Min	10 Min	30 Min	1 Hr	2 Hr
Ion Abundance[a]	NDA[b]	2.3	0.6	NDA	NDA	NDA	NDA
% RSD[c]	N/A[d]	14.6	93.3	N/A	N/A	N/A	N/A
				m/e 189			
	0 Hr	1 Min	5 Min	10 Min	30 Min	1 Hr	2 Hr
Ion Abundance	NDA	1.6	0.42	NDA	NDA	NDA	NDA
% RSD	N/A	9.9	85.7	N/A	N/A	N/A	N/A
				m/e 245			
	0 Hr	1 Min	5 Min	10 Min	30 Min	1 Hr	2 Hr
Ion Abundance	NDA	2.8	0.64	NDA	NDA	NDA	NDA
% RSD	N/A	22.9	92.7	N/A	N/A	N/A	N/A

Mare #18

				m/e 146			
	0 Hr	1 Min	5 Min	10 Min	30 Min	1 Hr	2 Hr
Ion Abundance	NDA	28.1	3.6	2.7	NDA	NDA	NDA
% RSD	N/A	54.6	173.0	170.7	N/A	N/A	N/A

				m/e 189			
	0 Hr	1 Min	5 Min	10 Min	30 Min	1 Hr	2 Hr
Ion Abundance	NDA	16.5	3.0	NDA	NDA	NDA	NDA
% RSD	N/A	44.8	173.2	N/A	N/A	N/A	N/A
				m/e 245			
	0 Hr	1 Min	5 Min	10 Min	30 Min	1 Hr	2 Hr
Ion Abundance	NDA	42.7	7.7	8.0	NDA	NDA	NDA
% RSD	N/A	78.7	102.6	44.3	N/A	N/A	N/A

[a]Ion abundance X 100.

[b]No detectable amount.

[c]Relative standard deviation as % of the mean.

[d]Not available.

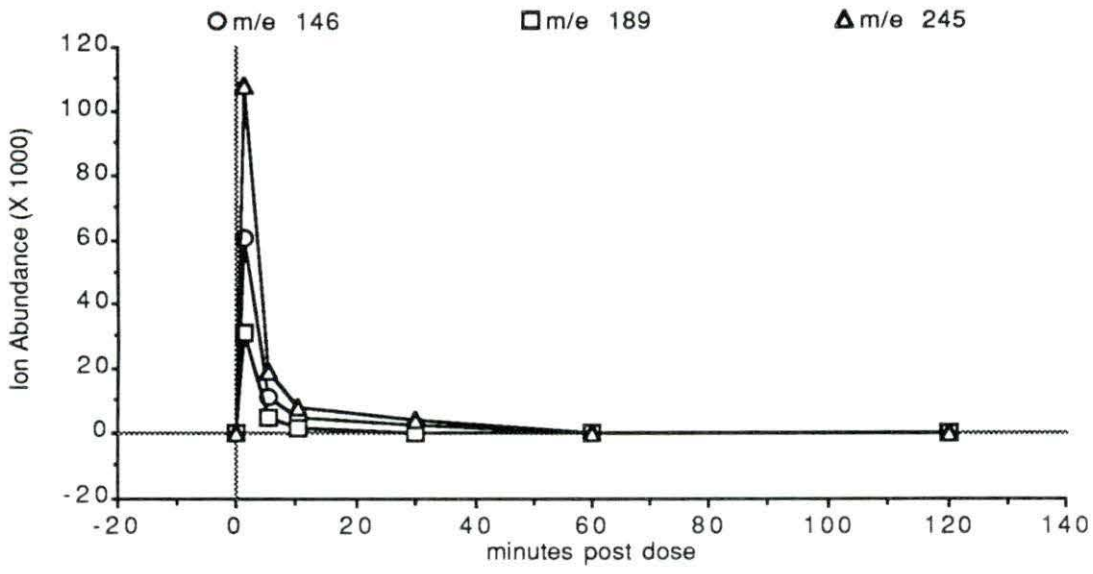


Figure 39. Mare #17 plasma profiles following administration of 500 μg fentanyl i.v. by GC/MS in SIMS mode, n = 3

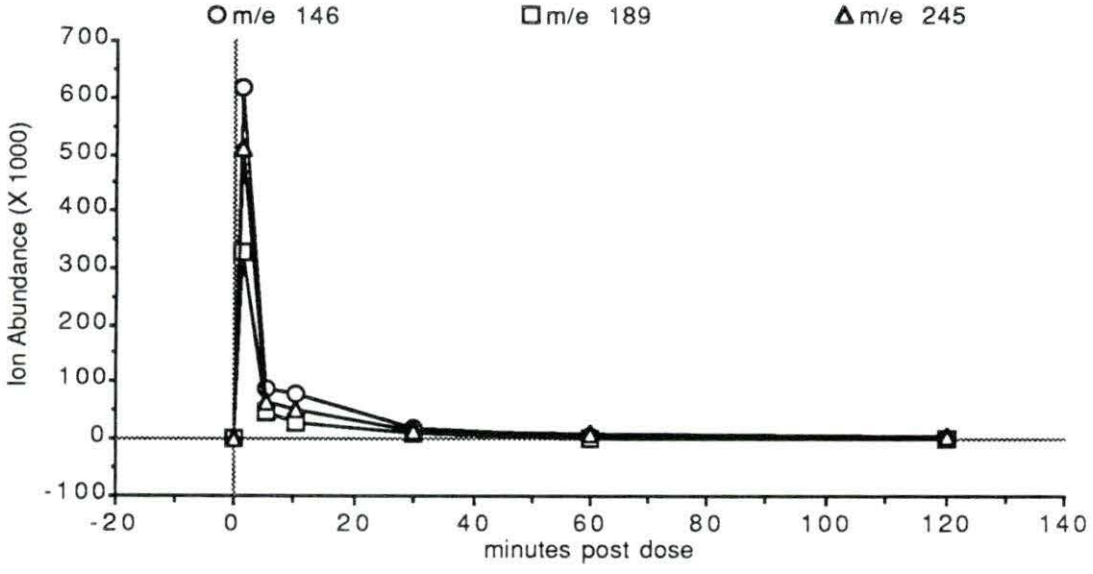


Figure 40. Mare #18 plasma profiles following administration of 500 μg fentanyl i.v. by GC/MS in SIMS mode, n = 3

Table 19. Average ion abundance (fentanyl) of plasma from mares #17 and #18 by GC/MS in single ion monitoring mode following base extraction (n = 3)

Mare #17

				m/e 146			
	0 Hr	1 Min	5 Min	10 Min	30 Min	1 Hr	2 Hr
Ion Abundance[a]	NDA[b]	60.3	11.6	5.0	2.4	NDA	NDA
% RSD[c]	N/A[d]	49.6	52.6	114.0	87.5	N/A	N/A

				m/e 189			
	0 Hr	1 Min	5 Min	10 Min	30 Min	1 Hr	2 Hr
Ion Abundance	NDA	31.4	5.1	2.0	NDA	NDA	NDA
% RSD	N/A	49.4	41.2	175.0	N/A	N/A	N/A

				m/e 245			
	0 Hr	1 Min	5 Min	10 Min	30 Min	1 Hr	2 Hr
Ion Abundance	NDA	108.1	19.2	8.2	4.1	NDA	NDA
% RSD	N/A	53.9	56.8	101.2	51.2	N/A	N/A

Mare #18

				m/e 146			
	0 Hr	1 Min	5 Min	10 Min	30 Min	1 Hr	2 Hr
Ion Abundance	NDA	618.4	86.6	80.0	19.1	7.0	NDA
% RSD	N/A	81.6	101.0	96.4	173.8	171.4	N/A

				m/e 189			
	0 Hr	1 Min	5 Min	10 Min	30 Min	1 Hr	2 Hr
Ion Abundance	NDA	326.5	46.5	30.2	9.1	NDA	NDA
% RSD	N/A	83.2	114.8	90.4	173.6	N/A	N/A
				m/e 245			
	0 Hr	1 Min	5 Min	10 Min	30 Min	1 Hr	2 Hr
Ion Abundance	NDA	509.5	64.4	49.9	13.9	8.1	NDA
% RSD	N/A	77.8	108.4	92.8	173.4	174.1	N/A

[a]Ion abundance X 100.

[b]No detectable amount.

[c]Relative standard deviation as % of the mean.

[d]Not available.

Table 16. Fentanyl detection limits and latest collection times post dose indicating original fentanyl administration (500 µg) in both urine and plasma from mares #17 and #18 by classical detection techniques

	Urine (mls)		Plasma (mls)		
Technique	#17	#18	#17	#18	Detection Limit
TLC	8 Hr (45)	8 Hr (45)	NDA[a] (6)	NDA (6)	1.0 µg
HPLC (UV)	NDA (27)	NDA (27)	NDA (4)	NDA (4)	0.5 µg
GC/NPD	24 Hr (27)	24 Hr (27)	5 Min (2)	5 Min (2)	2.0 ng
GC/MS (SCAN)	12 Hr (27)	12 Hr (27)	5 Min (2)	5 Min (2)	10 ng
GC/MS (SIMS)	24 Hr (27)	24 Hr (27)	10 Min (2)	30 Min (2)	2.0 ng

[a]NDA = No detectable amount.

Time and ease of use

All classical chromatographic techniques for screening and confirmation of analyte presence in biological samples require extraction of drug from sample, possibly followed by several cleanup steps prior to injection into chromatograph. These extraction steps are somewhat cumbersome, taking several hours from start to finish. When analyzing a large number of samples by thin-layer chromatography, laboratory personnel may spend an entire day extracting and plating samples for screening purposes. The acid hydrolysis procedure for fentanyl (McDonald and Ozog, 1978) entails rather laborious extraction steps, which may take an entire day or two to perform. Most sophisticated chromatographic instrumentation such as GC/NPD and GC/MS require some degree of training in order to fully operate the instrument to its capacity. The sophisticated instrumentation, however, allows for automation of a large number of samples in a single automation run.

SUMMARY

The purpose of this study was to determine the relative merits between the immunochemical techniques, and to compare the relative sensitivity of classical detection to immunoassay and instrumental methods in the screening and confirmation of fentanyl, in equine urine and plasma. It is already known within the forensic racing community that gas chromatography/ mass spectrometry is superior to the classical screening techniques as well as the new immunoassay methods in the overall confirmation of fentanyl in biological samples.

Of all the commercially available immunochemical assays analyzed, the IDS PCFIA assay attained the lowest sensitivity limit and lowest threshold value at < 1.0 pg and 80 pg total fentanyl, respectively. The IDS ELISA had a sensitivity limit and threshold value of 1.5 and 100 pg total fentanyl, respectively, approaching those values seen with the IDS PCFIA. Thus, on an overall sensitivity standpoint, the IDS PCFIA and IDS ELISA are extremely sensitive, with the PCFIA being more sensitive to the parent fentanyl molecule.

All the immunoassay methodologies investigated had good intra-assay reproducibility with standards and administration samples. The Janssen ^{3}H RIA was indicated as being superior in terms of inter-assay (overall day-to-day) reproducibility of results among the immunochemical methods examined. However, the major drawback of radioimmunoassays include the prospect of handling radioactive material, as well as their rather laborious execution, long time to completion, and low sensitivity levels in comparison to the ELISA and PCFIA procedures.

In terms of administration samples, the IDS ELISA detected presence of fentanyl equivalents at the farthest time post administration in both urine and plasma. The assay produced positive responses as far out as 24 hour and 1 hour post administration for urine and plasma, respectively. The IDS PCFIA methodology also detected fentanyl equivalents up to 24 hours post administration in urine, but only detected fentanyl

equivalents in plasma up to 5 minutes post administration, possibly due to endogenous fluorescent materials in equine plasma.

A major factor in selecting an immunochemical technique for the detection of exogenous compounds in the forensic chemistry community is time to completion of an assay. If large numbers of samples must be analyzed in a rapid fashion on a daily basis, assays allowing for these considerations must be utilized in the testing process. The IDS PCFIA is extremely rapid, requiring approximately 30 minutes to complete the assay and generate results with minimal intra-assay variability.

Among the classical screening techniques analyzed, the lowest detection limit of 2.0 ng on column, was accomplished by gas chromatography with nitrogen-phosphorus thermionic detection. The sensitivity of the IDS PCFIA is approximately 1000X greater in relation to the GC/NPD technique. The results generated by the classical screening methods on the administration urine and plasma, did not indicate fentanyl administration at a farther time post dose in relation to the immunoassay techniques.

The optimum laboratory practice for the screening and confirmation of biological samples would include initial screening by commercially available immunoassays, PCFIA preferably if available. Immunoassay methods are faster, have increased sensitivities, and require little instrumentation in comparison to the classical screening procedures (TLC, GC/NPD). When samples are deemed suspicious by immunoassay, samples could then be analyzed in triplicate by immunoassay to insure the initial positive response was not an anomaly. Lastly, gas chromatography/mass spectrometry as a confirmatory tool is a must for legal records and legal identification of analyte presence within a sample.

SUGGESTIONS FOR FUTURE WORK

Future work could include investigating and comparing cross-reactivity of the commercially available immunochemical techniques to fentanyl analogs and fentanyl-like compounds. It would be of interest to know which of the various immunochemical techniques reacts best with the various fentanyl-like narcotic analgesics, such as alfentanil, sufentanil, carfentanil, lofentanil, etc., as many of these fentanyl analogs are more potent than fentanyl itself in their pharmacological response.

Attainment and purification of equine fentanyl or fentanyl analog metabolites, either through dosing or chemical synthesis, would allow for the study of individual metabolite responses with immunochemical assays of interest. It could then be determined which metabolites respond to the commercially available immunoassays, and which ones do not.

In addition, administered urines and plasma (equine) could be analyzed with daily laboratory procedures to determine how well or poorly the routine screening practices, those performed on a day-to-day basis, can ascertain the presence of fentanyl equivalents in the samples.

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APPENDIX

Revision of Janssen ^{3}H RIA

Reagents Supplied in Kit

^{3}H-labelled fentanyl (1.1 µCi/vial)

Standard fentanyl (2.0 µg/ml)

Fentanyl antiserum

Dextran-charcoal Mixture

Phosphate buffer-BSA mixture

Materials Not Supplied

Distilled water

Methanol/distilled water (30:70,v/v)

1.5 ml microcentrifuge tubes

20 ml disposable scintillation vials

Scintillation cocktail

Preparation of Reagents

^{3}H-fentanyl-

Dilute stock solution with 4.0 ml methanol/distilled (30:70, v/v) to obtain a solution corresponding to 25,000 dpm/50 µl.

Fentanyl standards-

Dilute fentanyl standard solution (2.0 µg/ml) with methanol/distilled water (30:70, v/v) to acquire desired concentrations.

Fentanyl antiserum-

Reconstitute contents of vial with 20 ml distilled water and mix thoroughly.

Dextran-charcoal mixture-

Add 25 ml distilled water to dry dextran-charcoal mixture to obtain a 2% dextran-coated charcoal suspension.

Phosphate buffer- BSA mixture-

Dissolve vial contents into 50 ml distilled water to obtain a 0.05 M buffer solution at pH 7.5, containing 2% albumin.

Procedure

1. Prior to performing the assay, allow all reagents to reach room temperature.
2. Ultrasonicate samples for 5 minutes prior to use.
3. Pipet the following into 1.5 ml microcentrifuge tubes (volumes given in µls):

	Samples	Standards	Zero Standard	NSB	TC
^{3}H-Fentanyl	50	50	50	50	50
30% MeOH/water	50	NA[1]	50	50	50
Fentanyl Standards	NA	50	NA	NA	NA
Sample	50	NA	NA	NA	NA
PBS	450	450	500	500	500
Fentanyl antiserum	200	200	200	NA	NA
Distilled water	NA	NA	NA	200	200

4. Mix the contents of the tubes and incubate for 2 hours at room temperature under continuous rotation (25 rpm).
5. Add the following:

Dextran-charcoal	200	200	200	200	NA
PBS	NA	NA	NA	NA	200

6. Mix the contents of the tubes and incubate for 1 hour at room temperature under continous rotation (25 rpm).
7. Centrifuge the tubes at 8000 x g for 5-10 minutes.
8. Pipet the total supernatant into counting vials with 10 mls scintillation cocktail and determine the "total tritium activity" of the vials by scintillation counter.
9. Convert "total activity" (counts) to $\%B/B_0$ and interpret results.

[1]NA = No addition.

Cambridge Medical ^{125}I-RIA

Reagents Supplied in Kit

^{125}I-labelled fentanyl (1.0 μCi/vial)

Standard fentanyl (2.0 μg/ml)

Fentanyl antiserum

Precipitating reagent

Fentanyl assay buffer

Normal rabbit serum

Materials Not Supplied

1.5 ml microcentrifuge tubes

20 ml disposable scintillation vials

Scintillation cocktail

Preparation of Reagents

Normal rabbit serum-

Reconstitute with 11.0 ml assay buffer.

^{125}I-fentanyl-

Dilute vial with 11.0 ml of normal rabbit serum. Recap and swirl contents.

Fentanyl Standards-

Dilute fentanyl standard solution (2.0 μg/ml) with assay buffer to acquire desired concentrations.

Assay buffer-

Ready to use.

Fentanyl Antiserum-

Ready to use.

Precipitating Reagent-

Ready to use.

Procedure

1. Prior to performing the assay, allow all reagents to reach room temperature.
2. Ultrasonicate samples for 5 minutes prior to use.
3. Pipet the following into 1.5 ml microcentrifuge tubes (volumes given in µls):

	Samples	Standards	Zero Standard	NSB[1]	TC[2]
Assay Buffer	NA[3]	NA	100	NA	NA
Fentanyl Standards	NA	100	NA	NA	NA
Samples	100	NA	NA	NA	NA
^{125}I-Fentanyl	100	100	100	100	100
Fentanyl antiserum	200	200	200	NA	NA

4. Vortex gently and incubate for 90 minutes at room temperature.
5. Add the following:

Precipitating reagent	1000	1000	1000	1000	NA

6. Vortex and incubate at room temperature for 15 minutes.
7. Centrifuge the tubes at 1500 x g for 5-10 minutes.
8. Pipet 100 µl of supernatant into counting vials with 10 mls scintillation cocktail and determine the "total tritium activity" of the vials via a scintillation counter.
9. Convert "total activity" (counts) to $\%B/B_0$ and interpret results.

[1]NSB = Non-specific binding.
[2]TC = Total counts.
[3]NA = No addition.

IDS ELISA

Materials Supplied in Kit

Fentanyl microtitre strips (coated with antibody) and Holder

Substrate solution A and B

Fentanyl-enzyme conjugate

Enzyme diluent

ELISA wash solution (10X)

ELISA standard and sample diluent

Reagents Not Supplied

Fentanyl standard at 1 µg/µl (methanol)

Distilled water

Procedure

1. Prior to performing the assay, allow all reagents to reach room temperature.
2. Ultrasonicate samples for 5 minutes prior to use.
3. Add 20 µl of standard and/or sample extracts to appropriate wells of microtitre strips.
4. All 100 µl of enzyme solution (prepared by a 1:8000 dilution with diluent) to each well, and gently mix by shaking.
5. Let reaction proceed at room temperature for 30 minutes.
6. Dump wells and wash three times with wash solution (diluted 1:10 with distilled water), ensuring that the wells are overflowed with wash solution each time.
7. Tamp dry and remove the presence of any bubbles in the wells.
8. Add 150 µl of substrate to each well (substrate is made by mixing equal amounts of solution A and B).
9. Allow reaction to proceed at room temperature for 30 minutes.
10. Read optical density (absorbance) of each well at 650 nm, and interpret results.

Tri-Tec ELISA

Materials Supplied in Kit

1. One microtitre plate (coated with fentanyl antigen)
2. One vial HRP-Antibody to fentanyl (rabbit)
3. Two vials substrate buffer for OPD (o-phenylenediamine) containing hydrogen peroxide
4. One vial (2 tablets) OPD tablets (5 mg)
5. One vial stop solution
6. 100 ml wash concentrate containing phospho-buffered saline with Tween 20
7. Fentanyl Urine Standards-

   1.0 ng/ml, 2.0 ng/ml, 4.0 ng/ml, 10.0 ng/ml, and 20.0 ng/ml

Reagents Not Supplied

Distilled water

Procedure

1. Prior to performing the assay, allow all reagents to reach room temperature.
2. Ultrasonicate samples for 5 minutes prior to use.
3. Add 50 µl of standard/sample extracts to appropriate wells of microtitre plate.
4. Add 50 µl/well of fentanyl conjugate solution.
5. Shake gently to mix and let reaction proceed at room temperature for one hour.
6. At approximately 10 minutes prior to end of incubation period, prepare substrate. To 30 or 60 mls of substrate buffer, add 1 OPD tablet.
7. Dump wells and wash three times with wash solution (diluted 1:10 with distilled water), ensuring that the wells are overflowed with wash solution each time.
8. Tamp dry and remove the presence of any bubbles in the wells.
9. Add 200 µl of substrate to each well.
10. Allow reaction to proceed at room temperature for 15 minutes.
11. Stop reaction by adding 50 µl of stop solution to each well.
12. Read optical density (absorbance) of each well at 490 nm, and interpret results.

IDS PCFIA

Reagents Supplied in Kit

Fentanyl-BPE (beta-phycoerythrin) reagent

Fentanyl antibody reagent

Fentanyl latex particles (goat anti-rabbit) reagent

Wash solution (10X)

PCFIA standard and sample diluent

Reagents Not Supplied

Fentanyl standard at 1 µg/µl (methanol)

Distilled water

Procedure

1. Prior to performing the assay, allow all reagents to reach room temperature.
2. Ultrasonicate samples for 5 minutes prior to use.
3. Spin filter all urine samples for 5 minutes at 10,000 x g in Spin-X™ filters.
4. Add 20 µl of standard and/or sample extracts to appropriate wells in the ninety-six well vacuum filtration microtitre plate.
5. Add 20 µl of the drug-phycoerythrin conjugate to each well.
6. Add 20 µl of drug antisera to each well.
7. Allow the reaction to proceed at room temperature for 10 minutes.
8. Add 20 µl of latex particles to each microtiter well.
9. Gently mix by tapping the microtiter plate and allow reaction to proceed at room temperature for 10 minutes.
10. To all empty wells, add 20 µl distilled water.
11. Place plate in Fluorescence Concentration Analyzer (FCA) and vacuum filter plate at 20 mm Hg until all wells are completely aspirated.
12. Wash plate one time with wash solution (diluted 1:10 with distilled water).
13. When wells are completely dry, read fluorescence at an excitation/emission wavelength of 545/575 nm and interpret results.

Iodoplatinate Reagent

Ingredients

10% chloroplatinic acid solution (3 ml)

97 mls water

Aqueous 6% KI solution (100 ml)

Procedure

1. Add 3 mls 10% chloroplatinic acid to 97 mls water.
2. Add 100 mls aqueous 6% KI solution and mix.