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Development of an enzyme-linked immunosorbent assay for fentanyl and applications of fentanyl antibody-coated nanoparticles for sample preparation

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Abstract

A sensitive enzyme-linked immunosorbent assay (ELISA) was developed for the detection of fentanyl in serum and urine. The ELISA used an indirect competitive method produced by coating the plate with thyroglobulin conjugated with fentanyl hapten. Antibodies against fentanyl–hemocyanin were detected by a goat–anti-rabbit antibody conjugated with alkaline phosphatase. Calibration standard curves ranged from 0.5 ng/ml to 50 μg/ml (IC_{50} = 10 ng/ml), and the limits of detection were 0.5 and 1.0 ng/ml for serum and urine, respectively. The intra- and inter-assay variations were less than 8% and 10%, respectively. The antibody produced against fentanyl completely cross-reacted with *p*-fluorofentanyl, thienylfentanyl and 3-methylthienylfentanyl, cross-reacted highly with carfentanil (85%), but was considered non-cross-reactive with α-methylfentanyl (5%), sufentanil (<1%), alfentanil (<1%) and lofentanil (<1%). Nano-sized iron oxide magnetic particles coated with the developed fentanyl antibody were capable of specific binding and releasing of fentanyl from urine samples. This enabled the drug to be effectively pre-concentrated and decreased the limit of detection by approximately one order of magnitude. The analytical background noise was significantly reduced to enable fentanyl detection at concentrations originally below chromatographic limit of detection. The change of platform for antibody binding with nanoparticles demonstrated a novel use of antibodies for sample preparation and should facilitate drug screening by traditional ELISA.

Keywords: Fentanyl; Enzyme immunoassay; Antibody-coated magnetic nanoparticles; Capillary electrophoresis

1. Introduction

The fentanyls are a group of related narcotic analgesics of the 4-anilidopiperidine series. The parent drug, fentanyl (*N*-phenyl-*N*-[l-(2-phenylethyl)-4-piperidinyl] propanamide), is a synthetic opioid derivative of meperidine and a potent mu-receptor agonist with 80–150 times the potency of morphine [1,2]. Fentanyl has been used as an intravenous anesthetic/analgesic agent in the United States since 1968 [3]. The superior action for pain relief provided by fentanyl has made it a drug with high potential for abuse in humans. In animals, the dose-dependent CNS excitation and marked locomotor stimulation along with the analgesic effects [4,5], have promoted the illicit use of the fentanyls in athletic events, such as horse racing. Transdermal patches of fentanyl have been formulated to provide long-lasting continuous relief of moderate to severe post-operative or cancer pain in humans [6,7] and a number of domestic animals such as dogs, cats and horses [8,9]. Careful monitoring of fentanyl concentration was warranted since sudden death associated with fentanyl transdermal patch residues has recently been reported [10,11].

The pharmacokinetics of fentanyl is characterized by rapid (2–3 min) onset, large volume of distribution, low peak concentration (ng/ml range), short plasma half-life (30–120 min) and extensive biotransformation to active

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metabolites [12,13]. All of these characteristics pointed to a need for more sensitive detection methods. Biotransformed metabolites, along with common congeners of fentanyls (such as alfentanil, lofentanil, sufentanil and carfentanil) have the potential to be used for illicit purposes. In addition, the development of many "designer" fentanyl analogs such as α-methylfentanyl, 3-methylfentanyl, *p*-fluorofentanyl, α-methyl-acetylfentanyl, α-methyl-acrylfentanyl, thienylfentanyl, benzylfentanyl, 3-methyl-thienylfentanyl and β-hydroxy-(3-methyl)-thienylfentanyl has further complicated detection and screening and added to the potential for human abuse of fentanyl-related compounds [14].

In view of the wide diversity of fentanyl analogues available, the high potential for abuse and the importance to assure therapeutic safety, there is a need for rapid, inexpensive and sensitive methods that detect specific members of the fentanyl group. Various methods have been developed for detection of the fentanyls in different matrices, including immune-based radioimmunoassay (RIA) [15–17], enzyme-linked immunosorbent assay (ELISA) [18–21], and chromatographic analyses such as capillary electrophoresis (CE) and high performance liquid chromatography (HPLC) [22–25]. Gas chromatography or gas-liquid chromatography with a variety of detectors has also been reported [26–29]. While most methods shared similar degrees of sensitivities (1–10 ng/ml for HPLC, LC and <1 ng/ml for RIA and ELISA), chromatographic analyses were generally limited by expensive instrumentation. The RIA method had the disadvantages associated with handling radioactive materials and detecting only single fentanyl. The ELISA has the advantage of being simple, sensitive, inexpensive and capable of detecting metabolites. A sensitive ELISA is described here that differs in specificity from previously reported immunoassays. In view of the semi-quantitative nature of ELISA, it is imperative to facilitate the confirmation of positive samples screened by ELISA methods; therefore, we propose to utilize the developed antibody in combination with nano-sized particles to selectively extract and pre-concentrate fentanyl for subsequent chromatographic analysis. The concept of using nanoparticles for detection and purification purposes has been reported previously [30,31] but the applications were limited to column-packed cartridges. Synthesis was typically achieved by coating the nanoparticles with biocompatible molecules such as dextran, polyvinyl alcohol (PVA) and phosopholipids [30,32]. The use of nanoparticles for similar applications in biology or medicine was focused on the bio-detection, separation and purification of pathogens, cells or cellular components (proteins, nucleic acids, DNA and RNA) [33–35]. To the knowledge of the researchers, there were few attempts using antibody-coating for selective binding of drug molecules. Previous studies have shown that nanoparticles provided ubiquitous characteristics, such as minimal diffusional limitations. The extremely high surface area per unit mass and highly effective loading for proteins [36] made nanoparticles an attractive material for separation and purification purposes [34]. Therefore, the goal of the second part of this study was to investigate the application of nanoparticle-immobilized antibody in sample preparation to facilitate the detection and confirmation of minute concentrations of fentanyl in biological matrices.

2. Materials and methods

2.1. Drugs and chemicals

Fentanyl citrate, carfentanil, lofentanil, alfentanil, sufentanil, α-methylfentanyl, *p*-fluorofentanyl, 3-methylfentanyl and thienylfentanyls were purchased from Janssen Life Sciences (Piscataway, NJ, USA). Norfentanyl was purchased and carboxyfentanyl was synthesized by Bio-Mol Research Lab (Plymouth Meeting, PA, USA). *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) and other chemicals were purchased from Sigma (St. Louis, MO, USA). Acetonitrile (ACN), hexane, triethylamine and methanol were HPLC grade or better and were purchased from Fisher Scientific (Pittsburgh, PA). Sodium bicarbonate and sodium carbonate were purchased from Fisher Scientific. Formic acid (99%) was purchased from Acros Organics (Geel, Belgium). For liquid chromatography–mass spectrometry (LC–MS) analysis, fentanyl and deuterated fentanyl (d_5) was purchased from the Cerilliant Corporation (Round Rock, TX).

2.2. Preparation of immunogen and antibody production

To prepare for the immunogen, carboxyfentanyl was conjugated to hemocyanin by use of the carbodiimide method. A mixture solution of 50 mg carboxyfentanyl, 100 mg of hemocyanin and 200 mg of l-ethyl-3(3-dimethylaminopropyl) carbodiimide were prepared in 22 ml of deionized water. The solution was permitted to react for 48 h at 4 °C before it was dialyzed against 4 l of phosphate-buffered saline (PBS), pH 7.4, for 72 h at 4 °C. Lowry protein determination was performed on the product to confirm the presence of the conjugate. Two adult New Zealand White rabbits were immunized with 0.25 ml of carboxyfentanyl–hemocyanin conjugate in 1.0 ml of PBS and emulsified with an equal volume (1.25 ml) of Freund's complete adjuvant. Intradermal injections were given along both sides of the back and booster immunizations were carried out every 4–6 weeks. Blood samples were collected from the central ear artery 1 week after each booster injection and tested for the presence of antibodies to fentanyl by the Coat-A-Count fentanyl radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA, USA).

2.3. Fentanyl ELISA

To establish the assay, carboxylfentanyl–thymoglobulin conjugate was first prepared. Carboxylfentanyl (120 mg) was dissolved in 40 ml of dioxane containing 75 μl of tributylamine and 42 μl of isobutylchloroformate. The mixture was stirred for 30 min at 20 °C before a cooled (4 °C) thymoglobulin solution (260 mg in 100 ml deionized water, pH 8.5) was slowly added to the mixture. The resultant solution was left to react for 4 h at 20 °C. Thereafter, the final volume was reduced using pressure filtration (Amicon filtration/nitrogen gas) and dialyzed against 4 l of PBS for 4 days at 4 °C. Formation of carboxylfentanyl–thymoglobulin conjugate was confirmed by UV spectroscopy in combination with capillary electrophoresis.

Optimal antibody and antigen concentrations were determined by Checkerboard ELISA. In brief, a 96-well polystyrene microtiter plate (Nunc, Denmark) was coated with 200 μl/well of various dilutions of the prepared carboxylfentanyl–thyroglobulin across rows and kept at 4 °C overnight. Plates were then rinsed with PBS in an automatic microplate washer (Bio-Rad Model 1550). Antisera (200 μl/well) were added at various dilutions down columns and plates were incubated for 1 h at 37 °C. After washing the plates with PBS, GARGAP (goat–anti-rabbit IgG conjugated with alkaline phosphatase 1:5000, Pierce) was added at 200 μl/well and allowed to incubate at 37 °C for an additional hour. The plate was immediately washed and 200 μl of *p*-nitrophenyl phosphate substrate solution (1 mg/ml, Sigma) was added to each well to initiate a color change. The plate was read at 15–20 min after color initiation. It was determined that that was the optimal length of time to allow the color formation reaction to reach equilibrium, eliminating the need for an acid solution to stop the process. A microplate reader (Bio-Tek Instruments) was used to measure optical density at 405 nm. The antibody–antigen combination that resulted in absorbance of approximately 1.0 within 15 min was selected to be used in the construction of standard curves in subsequent experiments.

2.4. ELISA standard curve and cross-reactivity

To establish standard curves, microtiter plates were coated with carboxylfentanyl–thyroglobulin and washed as described above. Aliquots (10 μl/well) of fentanyl stock standards (10 ng/ml to 50 μg/ml diluted in PBS, normal horse urine or serum) were added to each well containing 90 μl of PBS and 100 μl of antisera. Plates were incubated at 37 °C for 1 h and washed, followed by addition of GARGAP and substrate as described previously for the checkerboard. The plate was read at 15 min. Immunologic cross-reactivity between fentanyl and 10 pharmacologically or structurally related analogs were assessed by the ability of the test compound to inhibit the reaction between the carboxylfentanyl–thyroglobulin and anti-fentanyl antibody. This was performed by substituting various concentrations of the test analogs for the fentanyl standards in the above ELISA procedure. In using the developed ELISA, a standard curve for fentanyl was generated along with serial dilutions of various analogs and the cross-reactivity was determined by comparing the calculated IC_{50} (concentration at which 50% fentanyl–thyroglobulin binding was inhibited). The sensitivity of assay was determined according to Hayashi et al. [37]. The lowest concentration in the standard curve that showed less than 30% variation was determined as the limit of detection (sensitivity) of this assay. All experiments were performed in triplicate for five plates.

2.5. Preparation of antibody-coated particle and interaction with fentanyl

To prepare antibody-coated nanoparticles (Ab-P), the antiserum was first purified by filtration through a Sephadex G25 column (Pharmacia, Piscataway, NJ, USA) equilibrated with 0.01 M PBS, followed by a Sepharose 4 gel column (Pharmacia). The binding and elution buffer were 0.02 M, pH 7.0 sodium phosphate and 0.1 M, pH 2.7 glycine–HCl, respectively. The eluant was immediately neutralized with 1 M Tris base (pH 8.0) and concentrated by ultrafiltration before it was lyophilized and stored at −70 °C. One milligram of ultra-small super paramagnetic iron oxide particles (USPIO-201, NH_2-surface modified, 6 nm, i.d., Taiwan Advance Nanotech Inc., Taoyuan, Taiwan) was mixed with 2 ml of purified fentanyl antibody (2 mg/ml protein, diluted 200-fold in the coating buffer) and 0.2 mg of EDC at 25 °C and reacted under constant shaking (100 rpm) for 4 h. This allowed the antibody to bind covalently to the particles. The coating buffer consisted of nine parts of saline solution (0.09% NaCl) and one part of 20 mM carbonate–bicarbonate solution (pH 9.6). The particles were then washed three times with coating buffer using the TANBead (TANBead, Taiwan Advance Nanotech Inc.) magnetic base system. This system is composed of strong magnets which are used to attract and separate magnetic particles in solution from the liquid components. To capture the drug, 1 mg of Ab-P was added to 5 ml of fentanyl standard at a concentration of 200 ng/ml in normal horse urine and kept under constant shaking (30 rpm at room temperature) for 1 h. Following the drug binding, the particles were precipitated with the TaNBead system and the supernatant decanted. In the final steps, the bound fentanyl was eluted by washing the particles with 1 ml of 100 mM glycine–HCl (pH 2.5) and subjected for CE analysis. Similar experiments were carried out in which 1 mg of Ab-P mass was added to 1 ml of fentanyl PBS solutions at 1 or 20 μg/ml to determine the binding capacity of Ab-P to fentanyl at these two concentrations and their subsequent release from the particles. The Ab-P was processed and washed as described above and eluted with 10 ml of 100 mM glycine–HCl (pH 2.5). The fentanyl–glycine solution was then dried under speed vacuum and reconstituted into 1 ml before CE analysis. Another experiment was used to estimate the accumulated binding of fentanyl. Ab-P mass was serially added at 1 mg increment into 1 ml of 20 μg/ml fentanyl in PBS. Twenty microliter of the supernatant was removed for CE analysis after each addition of Ab-P to estimate for the accumulated binding of fentanyl. All experiments were repeated three times.

2.6. Analysis of fentanyl by capillary electrophoresis and LC–MS/MS

Capillary zone electrophoresis (CZE) was used to determine fentanyl concentration. The detection was performed on a Beckman P/ACE System MDQ (Beckman Coulter, Fullerton, CA, USA) equipped with a UV/LIF detector and operated by 32 Karat Software (Beckman Coulter) for data acquisition. The analytical conditions were modified from analysis of pethidine reported by Quaglia [38]. Briefly, 35 mM acetate buffer (sodium acetate, pH 6.5) was used as run buffer. Separation was carried out in an uncoated fused-silica capillary (60 cm × 75 μm i.d.) thermoregulated at 25 °C, with an applied voltage of 20 kV. The sample was injected with hydrodynamic pressure (0.5 p.s.i.) for 2 s. UV wavelength was set at 200 nm. The limit of detection (S/N

ratio > 3) for fentanyl under these conditions was 500 ng/ml. Paired fentanyl standard solutions at four concentrations (from 10 to 1000 ng/ml) were tested on both the developed ELISA and CZE (concentrations below CE limit of detection were pre-concentrated) to evaluate the accuracy of the ELISA as a semi-quantitative method.

For LC–MS/MS analysis, working solutions of fentanyl were prepared by diluting the stock solution (0.1 mg/ml) in HPLC grade methanol to 1, 0.1 and 0.01 μg/ml. Deuterated fentanyl was prepared at 1 μg/ml. Solutions of drug and internal standards were stored in a 3 °C refrigerator when not in use. Calibration (0.05, 0.1, 0.5, 1.0, 3.0, 5.0, 10.0 and 20.0 ng/ml), positive (0.5 and 5.0 ng/ml) and negative control samples were prepared in drug free horse serum. A Finnigan LTQ linear ion trap mass spectrometer (Thermo Electron Corporation, San Jose, CA) equipped with a Surveyor Autosampler was used for fentanyl analyses. The analytical column was an Atlantis dC18 (2.1 × 150 mm × 3 μm particle size, Waters, Milford, MA, USA). The mobile phase consisted of a mixture of 0.1% (v/v) formic acid in deionized water and 1% (v/v) formic acid in ACN. Flow rate was 200 μl/min and the injection volume was 15 μl. Both the analyte of interest and the internal standard had a mean retention time of 8.4 min. The molecular ions for fentanyl and d_5-fentanyl were isolated, fragmented, and the resultant full MS/MS spectra were collected. Product ions at *m/z* 281.2, 216.2, 188.2 and 105.1 were used for the qualitative identification of fentanyl. Product ions at 286.2, 221.2, 188.2 and 105.1 were used for the qualitative identification of d_5-fentanyl. The *m/z* 216.2 and 221.2 product ions from fentanyl and d_5-fentanyl, respectively, were used for quantitation. Analysis of the raw data was carried out using the Xcalibur Qualitative Analysis software (Thermo Electron Corporation). Blood samples of sequential time points from a horse received fentanyl infusion were obtained from Florida racing laboratory and were analyzed by the LC–MS/MS method. The same serum samples were also analyzed by the developed ELISA and a commercialized ELISA (Neogen Corp. Lexington, KY, USA) following the manufacture's instruction.

3. Results

3.1. Sensitivity of assay and correlation to capillary electrophoresis

The optimal dilution of the antibody and fentanyl–thyroglobulin conjugate, as determined by the checkerboard, was 1:1000 and 1:5000, respectively. Inhibition binding of 50% (IC_{50}) was attained at fentanyl concentrations of approximately 10 ng/ml. The sensitivity of the assay was within the range of 5–10 pg/well (0.5–1 ng/ml) as illustrated by the standard curve (Fig. 1). Addition of normal horse serum and urine caused no interferences; however, addition of normal horse serum caused a slight leftward shift in the standard curve (Fig. 1). Because the slope of the standard curve was unaffected by drug free urine or serum, it was concluded that the assay was suitable for detection of fentanyl immunoreactive compounds in both urine and serum samples. The coefficient of correlation (R^2) between CZE and the developed ELISA in PBS, serum and urine were determined to be 0.998, 0.991 and 0.998, respectively (Fig. 2), indicating the ELISA could be a good candidate for quantitative analysis at this concentration range.

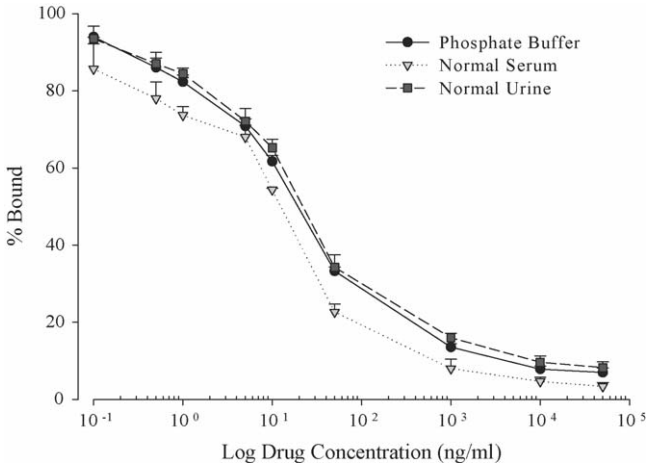


Fig. 1. Standard curves for fentanyl in phosphate-buffered saline (PBS) in the absence (circles) or presence of drug-free serum (triangles) or urine (squares). Data represented mean ± S.E.M. for five plates ($n = 5$).

3.2. Intra- and inter-assay variations

Mean intra- and inter-assay variations were determined through repeated analyses of five plates with triplicate wells containing fentanyl at three concentrations (200, 20 and 3 ng/ml) representing the 75%, 50% and 25% inhibition of binding in PBS and urine standard curves. For serum, 50 ng/ml (75%), 10 ng/ml (50%) and 1 ng/ml (25%) were used, respectively. The results indicated that for PBS and urine, the intra- and inter-assay variations were all less than 6% and 9%, respectively, while the intra- and inter-assay variations for serum were less than 8% and 10% (Table 1).

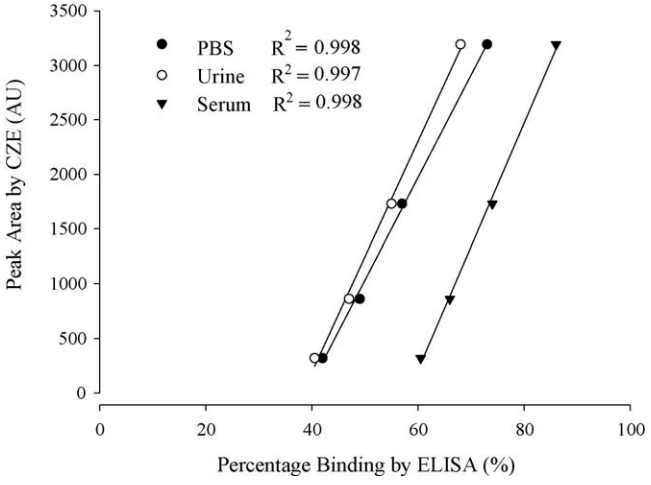


Fig. 2. Correlations between capillary zone electrophoresis (CZE) and ELISA at four concentrations (10, 50, 250 and 1000 ng/ml).

Table 1
Intra- and inter-assay variations for fentanyl analyzed by developed ELISA

Nominal concentration (ng/ml)	Intra-assay CV (%)	Inter-assay CV (%)
PBS		
200	5.3	7.2
20	4.5	5.9
3	5.6	7.8
Urine		
200	5.2	7.2
20	4.9	5.8
3	5.8	8.6
Serum		
50	7.6	8.4
10	6.2	7.6
1	8.0	9.8

Variations were tested on three concentrations representing the 75%, 50% and 25% inhibition of binding of fentanyl standard curves prepared in phosphate-buffered solution (PBS), normal horse urine and serum, respectively ($n = 5$).

3.3. Cross-reactivity

Cross-reactivity was calculated as 100 times the concentration ratio of a test drug that caused 50% inhibition of fentanyl–thyroglobulin binding over the fentanyl concentration required to achieve an equivalent inhibition ($100 \times IC_{50\,drug}/IC_{50\,fentanyl}$). Using this ELISA, it was determined that the anti-fentanyl antibody cross-reacted completely with thienylfentanyl, *p*-fluorofentanyl and 3-methylthienylfentanyl (150%, 165% and 200%, respectively), cross-reacted highly with carfentanil (85%), cross-reacted moderately with *N*-methylfantanyl (65%) and norfentanyl (50%), and cross-reacted very minimal with α-methylfentanyl (4.8%). Alfentanil, lofentanil and sufentanil showed almost no cross-reactivity (<1%) with the developed fentanyl antibody (Fig. 3). The chemical structures of fentanyl and its analogs and their cross-reactivities are summarized in Table 2.

3.4. Application of ELISA to fentanyl-containing samples

The use of the developed assay for clinical samples was validated by comparing the fentanyl concentration–time plot determined by the developed ELISA to those determined by a commercial ELISA and confirmed by LC–MS/MS. The results indicated that the ELISA readings were consistently higher than those determined by the LC–MS/MS, but the concentration–time plots changed in a near-parallel fashion indicating good correlations between the developed ELISA and the LC–MS/MS method (Fig. 4). The concentrations determined by LC–MS/MS method were expected to be lower because it detects only the parent drug.

3.5. Pre-concentration and sample cleaning by antibody-coated particles

Fig. 5 illustrates the capacity and efficiency of Ab-P to selectively bind fentanyl in an aqueous solution and released the drug in 100 mM glycine–HCl. Following 1 mg Ab-P particle treatment, the fentanyl concentration in the supernatant was reduced by 28.3 ± 3.6% from the original 20 μg/ml (Fig. 5, electropherogram A) to an estimated 14.3 ± 1.8 μg/ml (Fig. 5, electropherogram B), indicating that under the current conditions, 1 mg of Ab-P was able to bind a total mass of 5.7 ± 0.7 μg fentanyl. After reconstituting the particles with glycine–HCl to release the bound fentanyl, the fentanyl concentration was determined to be 5.6 ± 0.8 μg/ml (Fig. 5, electropherogram C), representing an extraction efficiency >90%. Overall, for 1 mg of immobilized Ab-P, it is possible to extract (binding and releasing) about 5.5 μg of fentanyl from a solution containing 20 μg/ml fentanyl. The fentanyl mass extraction capacity and efficiency for each mg of Ab-P at high (20 μg/ml), medium (1 μg/ml) and low (200 ng/ml) concentrations are summarized in Table 3. Fig. 6 shows the accumulated mass of fentanyl captured by the serial addition of 1 mg Ab-P. The total fentanyl mass bound to Ab-P

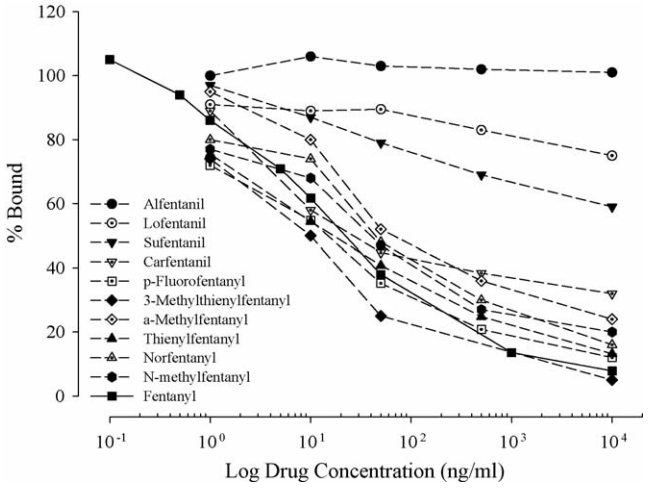


Fig. 3. Standard inhibition curves depicting cross-reactivity of fentanyl (solid line) and selected structural congeners of fentanyl (dashed lines), using conditions described in Section 2.

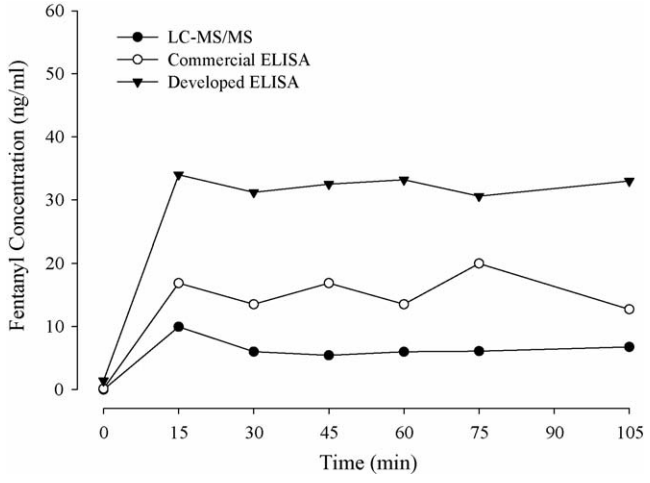
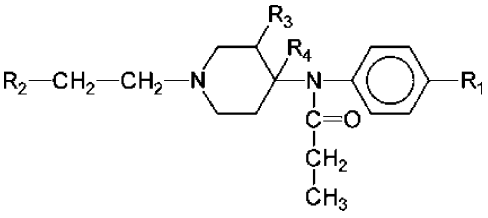


Fig. 4. Fentanyl concentrations analyzed by LC–MS/MS and the developed ELISA. Serum samples were determined for fentanyl concentrations by LC–MS/MS and a commercial ELISA to validate the results obtained from the developed fentanyl ELISA.

Table 2
Chemical structures of fentanyl and fentanyl analogs and their cross-reactivity to fentanyl

Drug	R_1	R_2	R_3	R_4	Cross-reactivity (%)
Fentanyl	H	Phenyl	H	H	100
p-Fluorofentanyl	F	Phenyl	H	H	150
Carfentanil	H	Phenyl	H	$COOCH_3$	85
α-Methylfentanyl	H	Phenyl	CH_3	H	5
Lofentanyl	H	Phenyl	CH_3	$COOCH_3$	<1
Sufentanil	H	2-Thienyl	H	CH_2OCH_3	<1
Thienylfentanyl	H	Thienyl	H	H	165
3-Methylthienylfentanyl	H	Thienyl	CH_3	H	200
Alfentanil	H	Oxo-tetrazol	H	CH_2OCH_3	<1
Norfentanyl	H	H[a]	H	H	50
N-Methylfentanyl	H	CH_3[a]	H	H	65



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[a] Indicated R_2 substitution is directly attached to the nitrogen atom.

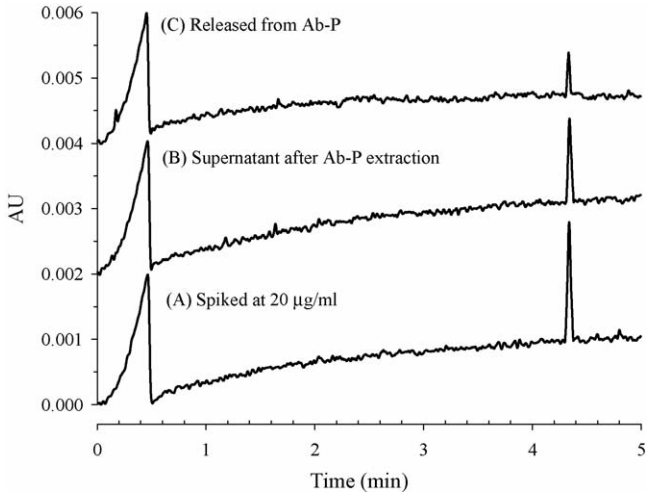

Fig. 5. Representative electropherograms of: (A) spiked 20 μg/ml fentanyl in phosphate-buffered saline and (B) supernatant of (A) after fentanyl extraction by antibody-particle. (C) Represented the reconstitution of extracted fentanyl. Buffer and run conditions: 35 mM sodium acetate; pH 6.5; voltage, 20 kV; injection, hydrodynamic for 2 s; wavelength, 200 nm.

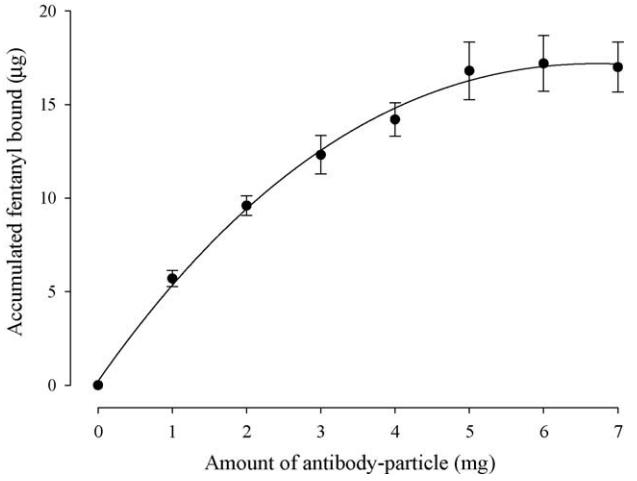

Fig. 6. Accumulated fentanyl mass bound to antibody-particle (Ab-P). Ab-P was added at 1 mg increment into 1 ml of 20 μg/ml fentanyl in PBS. Twenty microliter of the supernatant were taken serially for the determination of fentanyl concentration and estimation of total fentanyl mass bound to the Ab-P.

Table 3
The binding capacity and releasing efficiency of 1 mg fentanyl antibody-bound nanoparticles from urine and phosphate-buffered saline solutions

Starting concentration	Mass bound to Ab-P	Total mass recovered	Final concentration	Releasing efficiency (%)
200 ng/ml[a]	NA	200 ± 24 ng	1 ± 0.12 μg/ml	100 ± 12
1 μg/ml[a]	NA	0.97 ± 0.08 μg	0.95 ± 0.10 μg/ml	98 ± 11
20 μg/ml[b]	5.7 ± 0.7 μg	5.6 ± 0.8 μg	5.6 ± 0.8 μg/ml	100 ± 14

Values represent mean ± S.E.M. ($n = 3$). NA, not available due to low supernatant concentration.
[a] The drug was prepared in normal horse urine.
[b] The drug was prepared in phosphate-buffered saline.

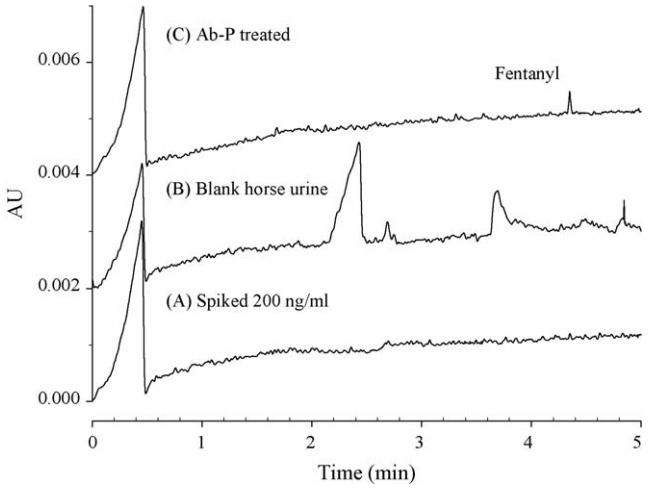

Fig. 7. Representative electropherograms of spiked 200 ng/ml fentanyl in: (A) phosphate-buffered saline and (B) blank normal horse urine. After antibody-particle (Ab-P) extraction and concentration, the fentanyl became detectable at 4.34 min with a calculated concentration of 1 μg/ml (C). Note that the background interferences were absent after Ab-P treatment. Buffer and run conditions refer to Fig. 4.

reached a plateau at around 17 μg (85% of 20 μg) after the addition of the 5th mg of Ab-P. Fig. 7 shows the ability of Ab-P to pre-concentrate samples for chromatographic analysis. At 200 ng/ml there was no detectable fentanyl peak when the drug was in PBS or in normal horse urine (Fig. 7, electropherograms A and B). This concentration was below the determined limit of detection for CZE. After Ab-P treatment and releasing of the drug in glycine–HCl solution, the peak became detectable at an average of 1.00 ± 0.12 μg/ml. The baseline noise is greatly reduced when compared to the normal urine sample (Fig. 7, electropherogram C). Overall, a five-fold increase in concentration (from 200 ng/ml to 1 μg/ml) was easily achieved after the Ab-P treatment process.

4. Discussion

Misuse of fentanyls in human medicine and racing industry has a long history and has been controlled only partially by regulatory efforts. Analytical testing for fentanyl in biological samples is challenging because of the high potency of fentanyls and the low dose required for this class of drugs. When a specific drug is found in large numbers in performance animals, the use of that drug declines and it starts to be replaced by new drugs. The appearance of new drugs in racing animals depends on the availability of new drugs and the analytical abilities of racing chemists. The development of a useful screening test is therefore a necessary first step to control the abuse. The incentive to develop an effective ELISA testing procedure is based in part on the speed, sensitivity and low cost of this technique. Although there are ELISA-based tests specifically designed for fentanyl detection [18–21], the ELISA test for fentanyls described here offered at least an equal or improved combination of sensitivity and selectivity compared with most previously published assays. Fentanyl concentrations as low as 5 pg/well could be detected in urine and serum samples. The broad spectrum of drugs detected and the high sensitivity makes this method particularly useful as a screening technique for fentanyls in biological fluids. The selectivity of an ELISA is characteristic of the specific antibody raised against the target compounds, and therefore is of great importance insofar as it reflects the nature of the substances that are able to be detected by ELISA tests. Based on our results, it appears that the developed fentanyl antibody recognized multiple epitopes on the fentanyl molecules (Table 2). The polyclonal nature of this antibody adds additional complexities to the specificity of the technique. For example, given the significant cross-reactivity drop with α-methylfentanyl and lofentanyl, it appeared that the R_3 position was a critical determinant for antibody recognition. On the other hand, R_4 position showed differential effects in cross-reactivity based on the substitution group. Carfentanyl, which differed only at R_4 substituent with fentanyl, shared 85% cross-reactivity with fentanyl, indicating R_4 recognition of $COOCH_3$ by this antibody was not significant. Support for this view also derived from comparison of the cross-reactivity between carfentanyl (85%) and lofentanyl (1%), which differed only at R_3 but had vastly different cross-reactivity. However, substitution of R_4 by CH_2OCH_3 (alfentanyl and sufentanyl) resulted in significantly reduced cross-reactivity (to only <1%) regardless of the identity of the R_2 group. These results pointed to a greater recognition of smaller R_3 and R_4 groups for this antibody. In addition to R_3 position, a phenyl structure at the R_2 position seemed to be the other determinant for antibody recognition. This was supported by the observation that replacement of the phenyl structure (alfentanyl, sufentanyl and thienylfentanyls) by different chemical functionalities could greatly change their cross-reactivity in the ELISA assay. The most significant replacement was the thienyl group as seen in thienylfentanyl, which has a cross-reactivity of 165%. 3-Methylthienylfentanyl with a thienyl group at R_2 position is another example of high cross-reactivity. Although 3-methylthienylfentanyl also contains a methyl group at R_3, which may affect the Ab recognition as previously discussed, the overall structural geometry and the presence of thienyl group apparently outweighed the influence of the methyl group at R_2. It should be noted that the overall cross-reactivity is the result of multiple Ab recognitions at multiple sites with different cross-reactive significances. Therefore, it may not be explained by substitutions at one or two sites. In addition to the substituents at R_2 and R_3 positions, it was clear that the interactions with fentanyl antibodies were also influenced by other additions of functional groups. Evidence included the R_1 addition of fluorine (*p*-fluorofentanyl, 150%), which significantly increased the cross-reactivity. The extent to which position would have the greatest effect on the cross-reactivity remains to be discussed. Usually the antibodies elicited against a hapten-carrier conjugate recognize a partial structure on the hapten molecule that is remote from the coupling position. The remote carrier is better than the structure near the coupling site because of better steric accessibility [39]. From the structural relationship it is plausible that carboxyfentanyl and hemocyanin conjugates, at or close to the R_4 position, render R_3 and R_4 less significant

to recognition by this antibody. These structural analyses are relevant to the ability of this fentanyl ELISA to detect other structural related fentanyl analogs as well as known or unknown fentanyl metabolites. Hammargren reported that oxidative *N*-dealkylation of fentanyl is the major metabolic pathway for fentanyl in human and most animals [3]. The pathway generates nor-metabolites (norfentanyl and *N*-methylfentanyl), whose activities are often an order of magnitude greater than the parent drug in urine samples. The ELISA described here was capable of detecting the above-mentioned metabolites, although at reduced sensitivity due to modification at the R_2 position. We expect that more fentanyl metabolites could be detected with the current method and the fentanyl ELISA assay would be more applicable to human samples since concentrations of the drug and metabolites present in the blood and urine of humans are likely to be much higher than those found in racing animals.

In the bioanalytical field, sample preparation is often considered to be the time-limiting step. The development of extraction supports, allowing the direct and selective binding and releasing of the target compound, is an attractive means to reduce the sample preparation time and increase sensitivity. Characterized by quantum size effect, surface little-size effect and grand quantum tunnel effect, nanoparticles have provided desirable features to the analysts [38]. At dimensions smaller or comparable to those of a virus (20–450 nm), a protein (5–50 nm), or a gene (2 nm wide and 10–100 nm long), nanoparticles exhibit many advantages over other solid materials such as greater specific surface area, greater density of surface active centers, higher surface activity, higher catalytic efficiency and stronger adsorbing ability [38]. In this study, the Ab-P was designed to specifically bind drugs for pre-concentration or cleaning of the sample by reducing the analytical background noise for subsequent analysis. This approach was intended to retain the advantages of solid-phase extraction, while achieving a higher degree of selectivity through the use of a specific antibody. As various antibodies are coated to these particles and bind specifically to their matching antigen, this approach provides a highly specific way to target a particular type of compounds. The concentration effect under the current ELISA conditions has allowed drug to become detectable from below the chromatographical limit of detection. The potential advantage of Ab-P over other concentration process (such as vacuum or freeze drying) is that other processes will inevitably concentrate all interference compounds in the background while the Ab-P method will be more selective in concentrating the specific drug (illustrated in Fig. 7, electropherogram B) and greatly eliminating the background noise. The selectivity gained by antibody–antigen interactions is generally expected to be higher than solid phase extraction (SPE) procedure where extraction is based on the differences in hydrophilicity (or hydrophobicity) between the analytes and micro-sized particles. While the purpose of the study precludes making comparisons between Ab-P and SPE, the ability of Ab-P to selectively bind and release drug for subsequent analysis presents a very attractive addition to the field of sample preparation. Similar results were obtained in a preliminary study in which amoxicillin and sulfamethazine

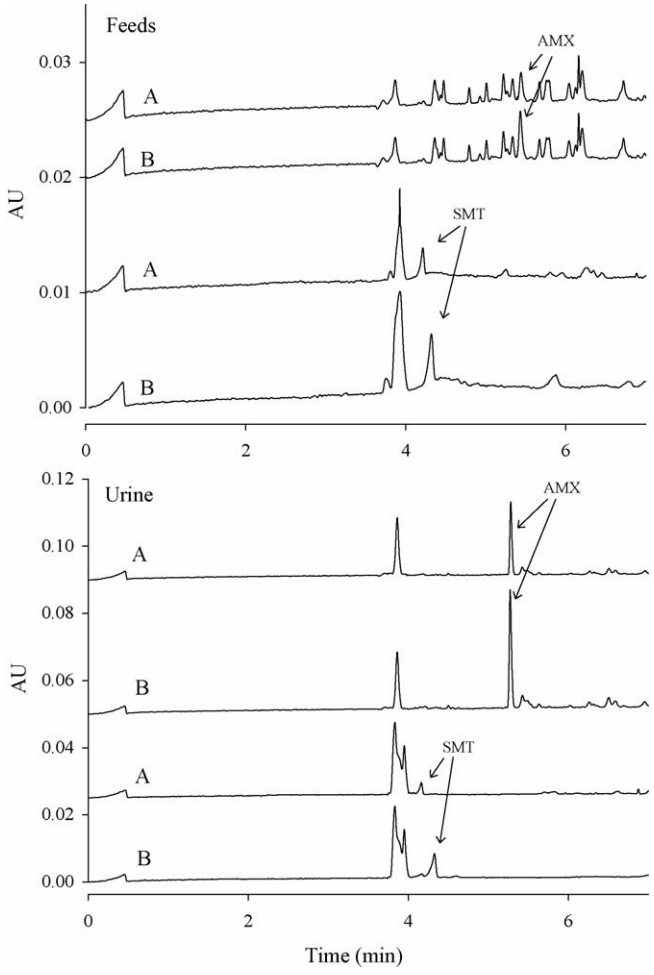

Fig. 8. Extractions of amoxicillin (AMX) and sulfamethazine (SMT) from feeds and urine by antibody-coated particles (Ab-P). AMX and SMT were each spiked at 20 μg/ml and extracted by 1 mg of Ab-P against the respective drugs. CZE electropherograms before (B) and after (A) Ab-P extraction in feeds and urine were shown in order.

were specifically extracted from urine and feeds by their respective Ab-P prepared similarly to the method described in this study for chromatographical analysis (Fig. 8), suggesting that this model was potentially applicable to the detection of other small drug molecules. Furthermore, in view of the high releasing efficiency (>90%) and the fact that 1 mg of Ab-P was capable of binding more than 5 μg of fentanyl, it is likely that the binding sites provided per mg of Ab-P was ample for most illegal adulterations or doping in the animals. This is consistent to most forensic situations in which the unknown drug concentration is usually very low. When Ab-P is applied at mg level, the amount of Ab-P binding sites (binding capacity) is expected to be in excess of the drug molecules. Therefore, good efficiency in releasing the Ab-P bound drugs becomes the deciding factor for the overall success of the method. On the three concentrations investigated in this study, the releasing efficiency was all above 90% (Table 3), suggesting a well dissociation of fentanyl from Ab-P. Whether or not applying more Ab-P would further increase the pre-concentrating factor

and the detection capacity this method could achieve remained to be studied. Based on the accumulation study in Fig. 6, it is likely that additional Ab-P could increase the total mass of extracted drug. The binding capacity of Ab-P exhibited a non-linear relationship in which the amount of fentanyl bound per mg of Ab-P reduced with the addition of more Ab-P. The curve reached a plateau when 85% of the drug was bound. Therefore, the binding of fentanyl to Ab-P was likely associated with drug concentration and/or Ab-P/drug ratios rather than directly proportion to the total Ab-P mass. Studies regarding the reusability of Ab-P, the efficiency of drug-releasing solution, and the extraction of (multiple) drugs from various matrices warrant further investigation. It should be noted that this method was not designed to replace SPE and other sample preparation procedures, but rather to provide an alternative for samples with definite drug targets in complex matrices. The specificity of Ab-P is advantageous in reducing analytical background noise and can be utilized along with traditional techniques as an additional cleanup step. This application may also be useful when unknown metabolites are implicated in the sample because the polyclonal antibody was capable of binding structurally related compounds. Use of magnetic separation allows the magnetic nanoparticles to be easily collected after sample washing. The cost of Ab-P might be one of the limitations for widespread adoption or routine application of this method. However, the cost could potentially be reduced by recycling the Ab-P, since the antibody is covalently bound to the particles. Our preliminary study suggested that the total efficiency (binding and releasing) was maintained well after three repeated uses of Ab-P (unpublished results).

In conclusion, a sensitive and highly reproducible method for fast screening of fentanyl and its analogs has been developed and validated for use in equine urine and serum samples, with potential application in monitoring illegal fentanyl uses in humans. This method is faster and less costly than conventional methods, which are based on a combination of HPLC, RIA and gas chromatography/MS analyses. The assay was also successfully applied to clinical samples collected from horses, exhibiting very similar concentration–time plot pattern to LC–MS/MS. Although the concentrations determined by ELISA are higher than those determined by LC–MS/MS, the assay suited well as a screening method and gave reasonable semi-quantitative data. The developed assay had been successfully used in conjunction with other methods for screening and confirming illegal doping of fentanyls in Florida racing tracks. An average of 150 suspected serum samples per year were tested in the past few years with no positive confirmations at this time. The utilization of the developed anti-fentanyl antibody to covalently bind to magnetic nanoparticles provided an attractive addition to the library of techniques used in extracting and pre-concentrating minute concentration of fentanyl before subsequent analysis. The limit of detection for fentanyl in biological matrices was improved through this process. The progress in nanotechnology, especially the surface-modification of nanoparticles, should facilitate further development of applications in pharmacology and biotechnology in the near future.

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