

Alfentanil: Detection and Confirmation

(A modified SPE Procedure For Equine Urine)

Developed By
The Oklahoma City Police Department Equine Testing Laboratory
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For

The Testing Integrity Program

Principles:

Alfentanil is a narcotic analgesic often administered to race horses. It is detected by ELISA using a generic Fentanyl test kit (Neogen Corp.) and confirmed by GC/MS after sample cleanup with Solid Phase Extraction (SPE) procedures. In our hands, we have found this method of Solid Phase Extraction to be useful in the extraction of Alfentanil, Fentanyl, Sufentanil, Carfentanil and Lofentanil from the equine urine.

Safety Precautions

General Good Laboratory Practices

Scope

The following SOP is proposed for detection by ELISA and GC/MS confirmation of Alfentanil in equine urine.

Definitions

GC/MS: Gas Chromatography with Mass Spectrometry

OCPDETL: Oklahoma City Police Department Equine Testing Laboratory

SOP: Standard Operating Procedure

SPE: Solid Phase Extraction

ELISA: Enzyme Linked Immuno-Sorbent Assay

PC: Positive Control

NC: Negative Control

Detection:-

By **ELISA** using Generic Fentanyl test kit (OCPDETL SOP) by Neogen Corp..

Reagent Concentrations:

Concentrated enzyme diluted 1:90 with enzyme diluent (EIA buffer for Neogen Corp.),

20 µl of each sample and control were analyzed

180 µl enzyme conjugate added to each well

150 µl of K-Blue substrate (Neogen Corp.) was added

Note: Initial (+)'s on the ELISA Fentanyl kit should be subjected to the additional screens detailed in the "Fentanyl ELISA Flow Scheme" to further predict the suspect analyte.

GC/MS Prep:

EH/SPE: 3x5 mL aliquots of each administration urine sample are subjected to enzyme hydrolysis (EH) followed by solid phase extraction (SPE)

Pretreatment of Samples: Enzyme Hydrolysis

1. Label five 16x125mm disposable extraction tubes three sample plus QA samples (normally one PC, 25 ng/ml of Alfentanil, and one NC, negative urine).
2. Label for each tube in step #1 a corresponding 16x100 mm culture tube for sample transfer.
3. To each extraction tube add 4 mL pH 4.5 phosphate buffer.
4. To each extraction tube add 1.2 mL enzyme solution .
5. Add 5 mL urine to corresponding extraction tube from each urine sample.
6. Cap each tube and rock tube back and forth once to mix contents by inversion.
7. Place extraction tubes in 65°C water bath for three hours.
8. Remove from water bath and cool by immersion in cold tap water in a sink.

Solid Phase Extraction Procedures (SPE)

- (1) Following the EH treatment, the Alfentanil administration aliquots were pooled and the pH of each group of samples was adjusted to 6.00 using 50% KOH or 10% HCl as necessary.
- (2) The pooled administration urine, the negative and positive control samples (all at pH 6.00) were then centrifuged at 3,000 rpm for 15 minutes.
- (3) The supernatant from each sample was recovered by decantation.

Please note: the volume of the sample will increase from 5.0 mL to well over 10 mL after enzyme hydrolysis and dilution with phosphate buffer.

note: if the urine sample is viscous, a dilution of at least 1:3 of the urine with potassium phosphate buffer (0.1 M; pH 6.00) or Methanol will be necessary before adjusting the final pH to 6.00. If filtration step is included, a 1:1 dilution of a "normal" consistency of the urine would still be necessary to minimize clogging of the filter in the SPE procedure.

The column used was **XTRACKT** (500 mg; Part #XRDAH515) by Worldwide Monitoring.

The column was sequentially conditioned with 4 mL each of methanol, water (HPLC Grade) and potassium phosphate buffer (0.1M; pH 6.00) and the sample was loaded

onto the column and collected to waste (loading rate should not exceed 2 ml/min). Thereafter, the column was sequentially rinsed with 3.0 mL water (HPLC Grade), 1.0 mL 0.1M acetic acid and 3.0 mL methanol (HPLC grade). The column was then dried under maximum vacuum for 300 seconds. The analyte was eluted with 3 mL of freshly prepared CH₂CL₂/IPA/NH₄OH (78/20/2) (note the eluate should be collected at approximately 1-2 mL/min).

Concentration: Label three 5.0 mL conical screw top tubes with the sample #, positive control and negative control. Transfer as much eluate as possible to the appropriate labeled tubes. Concentrate the eluate to dryness under a 10 mL/min stream of dry nitrogen in a 45 degree C water bath (note, keep adding the sample eluate until the entire sample is concentrated into one tube). Reconstitute the residue with 40 uL of ethyl acetate and place into a micro conical vial for GC/MS analysis.

Confirmation Of Alfentanil By GC-MS:

Limit = **10 nanograms/milliliter** of urine sample.

Derivatization: NONE

GC/MS Conditions:

Column: Capillary - 25mm, DB-5MS or HP-1

Head Pressure: 12 psi

Initial Temp: 80°C 1 min. hold

Program Rate: 20°C/min.

Final Temp: 285°C, 6.0 min. hold

GC-MS run was performed in full scan EI mode over the mass range of 45-500 amu (Base Peak = 289)

Note: The cleanliness of the GC/MS is mandatory for this analysis. A marginally clean instrument will produce unsatisfactory results.

Analysis Performance History:

5.0 mgs Alfentanil administered IV: post administration samples of 1.0, 2.0, 4.0 and 6.0 hours all produced acceptable full scan data utilizing 10 mL of sample.

1.5 mgs Alfentanil administered IV: post administration samples 1.0 thru 8.0 hours indicated positive results by Elisa. The 24 hour post administration did not demonstrate any reaction to the Elisa testing. Full scan GC/MS confirmation data was obtained on the 1.0 and 2.0 hour samples. Confirmation data using SIM was obtained on the 4.0 and 6.0 hour post administration samples.

Reagents for Alfentanil SOP

Enzyme Hydrolysis:

Formula #4. Ammonium Hydroxide (NH₄OH):DI H₂O (1:1) Reagent For Use in EH Urine Extraction

Procedure (perform under fume hood):

Combine 500 mL concentrated NH₄OH and 500 mL DI H₂O in an Oxford re-pipettor bottle.

Formula #6. Ascorbic Acid solution 10% for Use in Enzyme Hydrolysis Urine Extraction

Procedure for 1 Liter:

Dissolve 100 gm of L-Ascorbic Acid (Fisher) into 1 liter of DI H₂O.

Formula #32. β-Glucuronidase (*Patella vulgata*) Enzyme Used for Enzyme Hydrolysis Urine Extraction

Procedure: For a minimum final concentration of 5000 AU/mL

1. 1 vial 500,000 units in 88 mL DI H₂O.
2. 1 vial 1,000,000 units in 175 mL DI H₂O
3. 1 vial 2,000,000 units in 350 mL DI H₂O
4. Use 1 1/2 mL per sample.
5. Store at 4° C (Good for 4 to 6 days).

Formula #13. DCM:IPA (10:1) Reagent For Use in EH Urine Extraction. Procedure for 4000 mL:

1. From a fresh 4000 mL bottle of dichloromethane (DCM) remove 500 mL DCM (place 3500 mL DCM in DCM pipettor bottle).
2. Add 350 mL isopropanol (IPA) and **mix thoroughly**.

Formula #73. pH 4.5 Buffer For Use with Enzyme Hydrolysis Urine Extraction.

Procedure for 1 liter:

1. Prepare a saturated solution of monobasic potassium phosphate (KH₂PO₄) by adding KH₂PO₄ to 1 liter of DI H₂O while stirring until saturated (no more will go into solution) and a precipitate remains. The pH of this solution should be 4.5 if it is saturated.
2. Let stand a minimum of 12 hours and decant clear solution into a clean reagent bottle.

Formula #42. Sulfuric Acid (1N(0.5M) H₂SO₄) For Use in EH Urine Extraction

Procedure for 3600 mL. (Wear goggles.):

1. Pour 2000 mL of DI h₂O into a 4000 mL flask.
2. Slowly add 100 mL of Conc. H₂so₄ (36N)/mix thoroughly while you add.
3. Dilute to 3600 mL w/ DI h₂O.

Warning: Add acid to H₂O. **Never** add water to acid

Solid Phase Extraction:

- **Potassium Phosphate** 0.1M, pH 6 (1 Liter)

Weigh 13.61 g of KH₂PO₄ (MW 136.09) into a 1 Liter volumetric flask. Dissolve the KH₂PO₄ into 900 mL DI H₂O. Adjust pH to 6.0 with 1.0 M potassium hydroxide. Bring to 1 Liter volume. Good for 30 days.

- **MeOH (HPLC GRADE)**
- **Acetic Acid** 0.1 M
- **Water (HPLC GRADE)**
- **Elution Solvent: CH₂CL₂ / IPA / NH₄OH (78 /20 /2)**

10121**Addendum to TIP Alfentanil SOP**

These additional suggestions are offered to increase the ruggedness and sensitivity of the TIP Alfentanil SOP:

Solid Phase Extraction:

1. The Solid Phase prep is crucial to recovery and confirmation of low levels of Alfentanil
2. The same columns (XTRACKT, part #XRDAH515, 500 mg) by Worldwide Monitoring Should be used.
3. ALL SPE reagents must be fresh (i.e. no more than one week old)
4. Elution solvent (DCM/IPA/NH₄OH 78/20/2) **MUST** be **made fresh each day**
5. Centrifugation of the sample before SPE as per the SOP is also crucial to remove the sediment normally present in equine urine (for those without a high speed centrifuge, 3000 rpm works almost as well)
6. The pH values as detailed in the SOP are also crucial