

SCREENING AND CONFIRMATION OF OXYCODONE AND CLENBUTEROL IN EQUINE PLASMA BY ION TRAP LC/MS/MS

DEVELOPED BY PA EQUINE TOXICOLOGY & RESEARCH LABORATORY

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I. INTRODUCTION

Oxycodone (Oxycontin, Percodan, Percocet) is a semi-synthetic narcotic similar to morphine (Figure 1). Recently, abuse of prescription painkillers has reached epidemic proportions on a nationwide scale. From these overwhelming statistics, it became obvious that usage of oxycodone in racehorses may have increased and may have gone undetected during screening of urine only by ELISA. To address these concerns, plasma oxycodone screening and confirmation procedures were developed.

Clenbuterol is a β_2 -adrenergic bronchodilator effective in the treatment and management of acute and chronic respiratory disorder in horses (Figure 1). On May 11, 1998, the United States Food and Drug Administration approved Ventipulmin Oral Syrup formulation (Boehringer-Ingelheim) for veterinary use only.

To more efficiently manage resources, a single method was developed for simultaneous screening and confirmation of the presence or absence of clenbuterol and oxycodone in equine plasma. Clenbuterol- d_9 was used as the internal standard (IS).

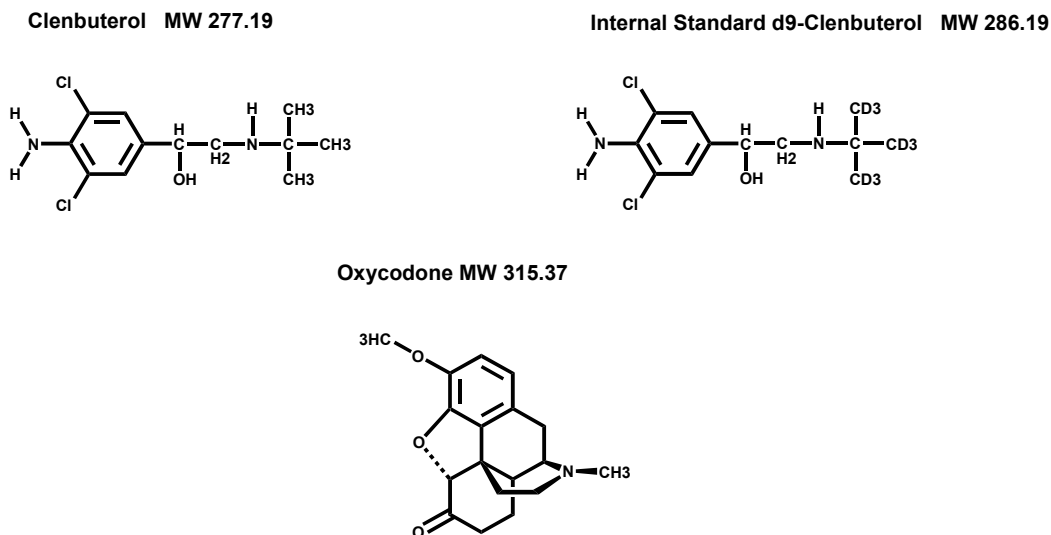


Figure 1. Chemical structures of clenbuterol, clenbuterol- d_9 (IS), and oxycodone

II. SCOPE

This standard operating procedure will be limited to the identification and confirmation procedures for the presence or absence of clenbuterol and/or oxycodone in equine plasma using the Deca XP Plus ion trap LC/MS/MS (ThermoFinnigan). Routine screening for clenbuterol and oxycodone in racehorse urine samples will continue to be conducted by Enzyme-Linked Immunosorbent Assay (ELISA). This SOP will only pertain to plasma samples collected from racehorses. It may however, be successfully applied to urine samples using 100 uL sample aliquots and tissue sample homogenate following deproteinization procedures.

III. PRIMARY DRUG AND INTERNAL STANDARD REFERENCE MATERIALS

- A. Clenbuterol hydrochloride salt, FW: 313.7; Salt Equivalent of Free clenbuterol: Salt MW/ free MW=1.13 (Cat. No. C-5423, Sigma).
- B. Clenbuterol-d₉ (internal standard), 200 µg/mL in methanol, Neogen (Lexington, Kentucky)
- C. Oxycodone, 1 mg/mL in methanol, obtained from Alltech –Applied Science Labs (Cat. No. 013543)

Obtain these materials from the pharmacy. Record accession of these materials on the pharmacy log sheet.

IV. PREPARATION OF PRIMARY REFERENCE STOCK SOLUTIONS

- A. Clenbuterol
Weigh between 5 and 10 mg (X.xx mg) clenbuterol salt into a glass bottle.
Dilute to volume using HPLC grade (or better) methanol (Volume Y.yy mL = X.xx mg/1.13).
Cap and mix until clenbuterol salt is completely dissolved in methanol.

The resulting concentration of clenbuterol is 1 mg/mL.
Store at approximately 4 °C.

Complete Balance Use Log and QA Primary Reference Standard Log for this process. Label the primary reference stock solutions with QA Primary Reference Log SR# (i.e. SR# 686 and Primary Reference Powder Designation (i.e. R-Clen-2).

V. PREPARATION OF WORKING STOCK SOLUTIONS

Materials Needed: Clenbuterol Primary Reference Stock (1 mg/mL) **(A)**
Oxycodone Primary Reference Stock (1 mg/mL) **(B)**

d9-Clenbuterol Primary Reference Stock (200 ug/mL) **(IS)**

Acetonitrile: Water: Formic Acid (50:50:1)

| Stock Label | Vol A | Vol B | Vol C | Vol D | mL 50:50:1 | Final Concentration |
|-------------|--------|--------|--------|---------|------------|---------------------|
| C | 100 ul | 100 uL | | | 9.8 mL | 10 ug/mL |
| D | | | 100 uL | | 9.9 mL | 100 ng/mL |
| E | | | | 1000 uL | 9 | 10 ng/mL |
| F | | | | 100 uL | 9.9 | 1 ng/mL |

Table 1. Preparation of Oxycodone-Clenbuterol Working Stock Dilutions (Stock Labels refer to the stock solution used for the respective dilution.)

| Stock Label | Vol IS | Vol ISA | mL Methanol | Final Concentration |
|-------------|--------|---------|-------------|---------------------|
| ISA | 50 ul | | 4.95 | 2 ug/mL |
| ISB | | 150 uL | 14.85 | 20 ng/mL |

Table 2. Preparation of d9-Clenbuterol Internal Standard Working Stock Dilutions (Stock Labels refer to the stock solution used for the respective dilution.)

VI. PREPARATION OF CALIBRATOR STOCK SOLUTIONS

Materials Needed: Oxycodone-Clenbuterol mixed working stocks D (100 ng/mL) and E (10 ng/mL)

Acetonitrile: Water: Formic Acid (50:50:1)

| Tube Label | Target Concentration | Volume Stock | Stock Used | Vol 50:50:1 | Used for Target Concentration pg/mL |
|------------|----------------------|--------------|------------|-------------|-------------------------------------|
| Cal1 | 0.4 ng/mL | 400 | EE | 9.6 | 20 |
| Cal2 | 1 ng/mL | 1000 | EE | 9 | 50 |
| Cal3 | 2 ng/mL | 2000 | EE | 8 | 100 |
| Cal4 | 5 ng/mL | 500 | DD | 9.5 | 250 |
| Cal5 | 10 ng/mL | 1000 | DD | 9 | 500 |
| Cal6 | 15 ng/mL | 1500 | DD | 8.5 | 750 |
| Cal7 | 20 ng/mL | 2000 | DD | 8 | 1000 |

Table 3. Preparation of Calibrator Stock Dilutions

VII. SAMPLE REQUIREMENTS FOR ANALYSIS

- A. Mobile phase blank
 - 1. Designate MB1.....MBn
- B. Negative (control) sample
 - 1. Designate plasma NC or plasma blank.
 - 2. Prepare negative (Control) sample from negative (Control) plasma.
- C. Positive control samples
 - 1. Designate plasma QC (or PC)1.....QC (or PC)n.
 - 2. Prepare positive control samples as described in Table 4.
- D. Calibrators
 - 1. Prepare a set of calibrators for analysis of plasma samples.
 - 2. Calibrator concentrations as designated in Table 3.
 - 3. Prepare plasma calibrators using negative (control) plasma and clenbuterol standard working solutions as described in Section VIII.
- E. Test samples
 - 1. Designated to use the date of which the sample is analyzed and
 *.raw data file designated to use sequence row number.

Table 4. Preparation of Analysis Samples. Controls, and Calibrators

| Tube Label | Target Concentration pg/mL | Volume Stock uL | Stock Used | Vol plasma | uL Internal Standard ISB |
|----------------------------------|----------------------------|-----------------|------------|------------|--------------------------|
| NC | 0 | 0 | na | 1 mL | 50 |
| QC1 | 50 | 50 | SCal2 | 1 mL | 50 |
| QC1 | 50 | 50 | SCal2 | 1 mL | 50 |
| QC2 | 100 | 50 | SCal3 | 1 mL | 50 |
| QC2 | 100 | 50 | SCal3 | 1 mL | 50 |
| QC3 | 500 | 50 | SCal5 | 1 mL | 50 |
| QC3 | 500 | 50 | SCal5 | 1 mL | 50 |
| S ₁ ...S _n | na | na | na | 1 mL | 50 |
| Cal1 | 20 | 50 | SCal1 | 1 mL | 50 |
| Cal2 | 50 | 50 | SCal2 | 1 mL | 50 |
| Cal3 | 100 | 50 | SCal3 | 1 mL | 50 |
| Cal4 | 250 | 50 | SCal4 | 1 mL | 50 |
| Cal5 | 500 | 50 | SCal5 | 1 mL | 50 |
| Cal6 | 750 | 50 | SCal6 | 1 mL | 50 |
| Cal7 | 1000 | 50 | SCal7 | 1 mL | 50 |

VIII. SEQUENCE OF SAMPLES FOR ANALYSIS

- A. The sequence order for screening and confirmation is the same, except for unknown samples. Screening samples are sequentially ordered by track and sample, with blanks bracketing the individual track sets. Confirmation is independent, repeat preparation of all QC and calibrators, with target samples prepared in triplicate, and triplicates bracketed by blanks.

1. Blank
2. 10 ng/mL oxycodone, clenbuterol, internal standard Column Test
3. Blank
4. Negative Control
5. Negative Control +Internal Standard
6. QC1
7. QC2
8. QC3
9. Blank
10. C1
11. C2
12. C3
13. C4
14. C5
15. C6
16. C7

- | |
|---------------------------------------------------------|
| 17. Blank 18. Track A, Samples 1 thru N 19. Blank |
|---------------------------------------------------------|

20. Repeat 17 thru 19 as needed

21. Blank
22. QC1
23. QC2
24. QC3
25. Blank
26. C1
27. C2
28. C3
29. C4
30. C5
31. C6
32. C7
33. Blank
34. Blank-Standby Method

| |
|----------------------------------------------------------------------------------------------------------------------|
| Blank Track A, Sample X ₁ Track A, Sample X ₂ Track A, Sample X ₃ Blank |
|----------------------------------------------------------------------------------------------------------------------|

| |
|-------------------------------------------------------------|
| Repeat as needed for the number of samples for confirmation |
|-------------------------------------------------------------|

Screening analysis uses no waste injection, to allow for repeat analysis in case of power failure, sequence error, retention drift, or other unforeseen need for reanalysis. Confirmation uses partial loop injection due to lower sample throughput and better repeatability statistics at these concentrations.

IX. Sample Preparation by Liquid-Liquid Extraction

Safety Requirements: Lab coat, fume hood, eye protection

1. 1 mL plasma (controls, calibrators, and samples) into labeled 16x125 mm screw cap culture tubes
2. Add 1 mL saturated sodium borate, pH 10
3. Add 50 uL calibrator stock dilutions to respective calibrator and QC tubes
4. Add 50 uL internal standard (**ISB**) to all tubes except NC1 (negative control no internal standard)
5. Gently vortex all tubes
6. Add 5 mL of methyl tert-butyl ether into each tube, cap all screw-top tubes tightly and rotorack for 5 minutes.
7. Centrifuge at 2,500 ~ 3,000 rpm (839 ~ 1,409 g) for 5 minutes.
8. Decant the (top) organic layer into a labeled fresh test tube for each sample.
9. Bring the extracts in test tubes to dryness in a fume hood at 40 °C under a steady stream of nitrogen or air.
10. Remove test tubes from the drying block, place in a rack, and allow to cool to room temperature.
11. Reconstitute the residues with 100 µL of 0.1% formic acid (aqueous)
12. Transfer the above solution into a 200 µL insert and load in the auto sampler vials. All the samples are now ready for LC/MS/MS analysis.

X. LIQUID CHROMATOGRAPHIC/MASS SPECTRAL IDENTIFICATION AND QUANTITATION OF OXYCODONE AND CLENBUTEROL

A. Instrumentation

1. ThermoFinnigan Deca XP Plus ion trap mass spectrometer with Xcalibur V1.3 for system control and data acquisition and processing
2. ThermoFinnigan Surveyor quaternary HPLC pump, autosampler, column compartment and on-line degasser. Autosampler uses no waste injection in screen mode and partial loop injection mode in confirmation mode.

B. HPLC conditions

1. Ace C8 Analytical Column, 2.1 x 50 mm, 5 micron particle size (Part # ACE-122-0502, Mac-Mod Analytical, 127 Commons Court, PO Box 2600, Chadds Ford, PA 19317)

Or

2. Ace C18 Analytical Column, 3.0 x 50 mm, 3 micron particle size (Part # ACE – 111-0503, Mac-Mod Analytical, 127 Commons Court, PO Box 2600, Chadds Ford, PA 19317)

3. Corresponding LC Guard Column

Type: Ace C8 (Part No.ACE-111-0103GD, Mac-Mod Analytical, 127 Commons Court, PO Box 2600, Chadds Ford, PA 19317)

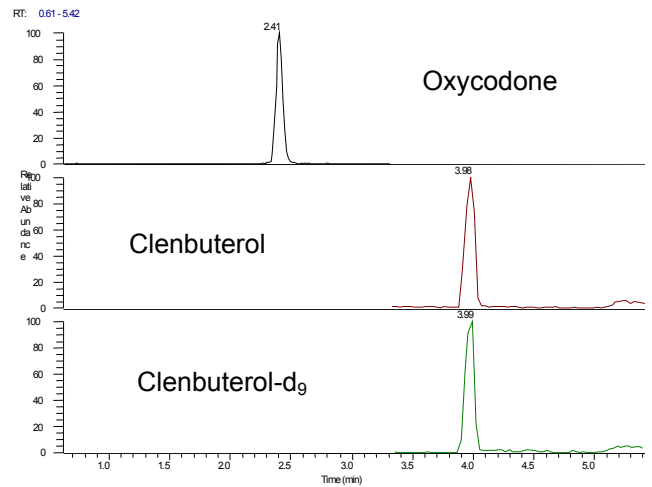
- a) Dimension: 2.1 × 12.5 mm
- b) Particle size: 5 micron
- c) Temperature: ambient

4. Pre-Column Filter Column-Saver (Part # MMCCS210 – Mac-Mod)

5. Mobile Phase Mobile phase A: 2.3 mM Formic Acid₄ (pH 3.0)
 Mobile phase B: Acetonitrile

3 micron C-18 Gradient

| Time | % A 2.3 mM Formic Acid | % B Acetonitrile | Flow uL/min |
|------|------------------------------|---------------------|----------------|
| 0 | 90 | 10 | 20 |
| 0.2 | 90 | 10 | 20 |
| 0.3 | 90 | 10 | 200 |
| 0.5 | 90 | 10 | 200 |
| 2.5 | 0 | 100 | 200 |
| 4 | 0 | 100 | 200 |
| 4.01 | 90 | 10 | 400 |
| 5.9 | 90 | 10 | 400 |
| 5.91 | 90 | 10 | 20 |
| 6.2 | 90 | 10 | 20 |



5 micron C-8 Gradient

| Time | % A 2.3 mM Formic Acid | % B Acetonitrile | Flow uL/min |
|------|------------------------------|---------------------|----------------|
| 0 | 90 | 10 | 20 |
| 0.2 | 90 | 10 | 20 |
| 0.3 | 90 | 10 | 200 |
| 0.5 | 90 | 10 | 200 |
| 3.85 | 10 | 90 | 200 |
| 4 | 10 | 90 | 200 |
| 4.01 | 90 | 10 | 400 |
| 5.9 | 90 | 10 | 400 |
| 5.91 | 90 | 10 | 20 |
| 6.2 | 90 | 10 | 20 |

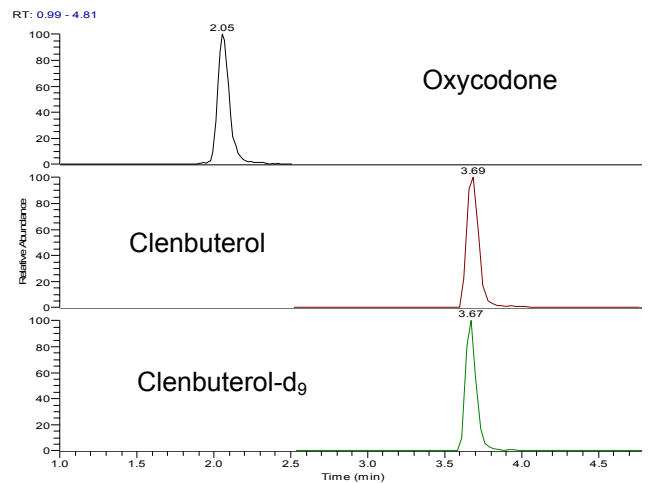


Figure 2. Gradient conditions and chromatograms for oxycodone, clenbuterol, and d9-clenbuterol

C. Mass Spectrometer Conditions

Figure 2. LC separation and conditions for oxycodone, clenbuterol, and clenbuterol-d₉

2. Deca XP Plus Acquisition Parameters (WB=Wideband Activation)

| | OxyCodone | Clenbuterol | d9-Clenbuterol |
|-----------------|--------------|--------------|----------------|
| Parent Ion | 316.2 | 277.2 | 286.2 |
| Collision % | 36 WB | 30 WB | 30 WB |
| Scan Range | 85-350 | 75-350 | 75-350 |
| Isolation Width | 1.2 | 1.2 | 1.2 |
| Q | 0.25 | 0.25 | 0.25 |
| Time | 30 | 30 | 30 |
| Segment Time | 0.5-2.85 min | 2.85-5.5 min | 2.85-5.5 min |

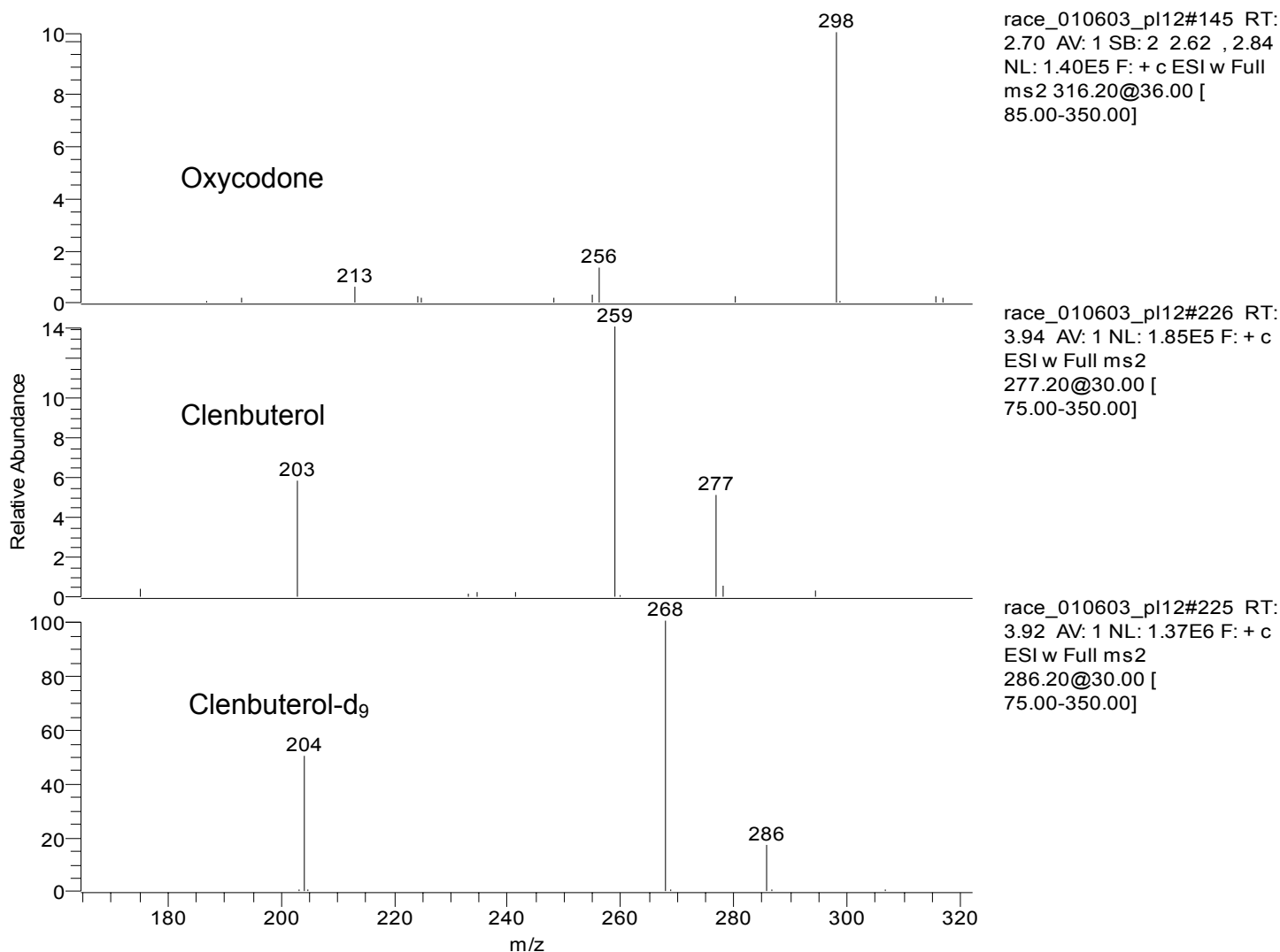


Figure 3. ESI+ Conditions and spectra for oxycodone, clenbuterol, and d9-clenbuterol

XI. METHOD STATISTICS

A. Instrument Repeatability

No Waste Injection Using Internal Standard Ratio

| n=4 x 4 | Clenbuterol | Oxycodone |
|---------|-------------|-----------|
| QC50 | 14.3-19.5% | 7.5-11.8% |
| QC100 | 6.6-10.2% | 4.0-10.8% |
| QC500 | 3.1-5.3% | 3.0-6.6% |

Using Analyte Area Alone

| n=4 x 4 | Clenbuterol | Oxycodone |
|---------|-------------|-----------|
| QC50 | 17.1-22.1% | 8.2-13.2% |
| QC100 | 7.6-15.5% | 3.1-10.6% |
| QC500 | 1.9-6.7% | 2.1-6.2% |

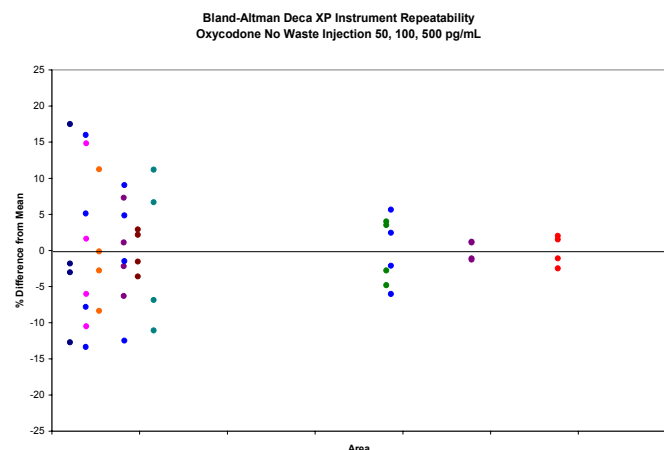
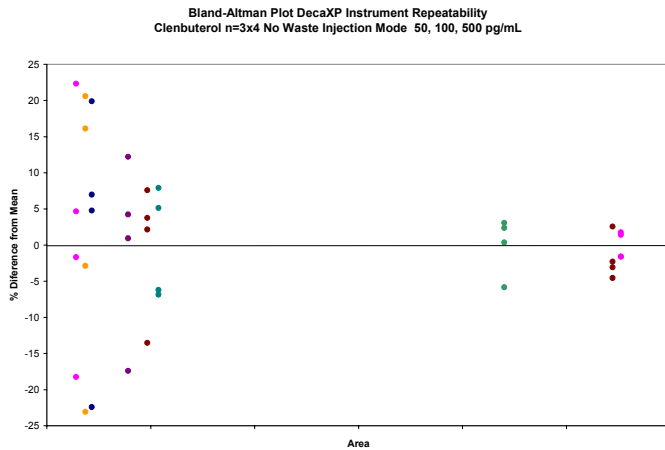


Figure 4. Instrument Repeatability of Decca XP Plus Ion Trap for clenbuterol (left) and oxycodone (right) using area of analyte only and no waste injection (ranges are 50, 100, and 500 pg/mL)

Partial Loop Injection Using Internal Standard Ratio

| n=4 x 4 | Clenbuterol | Oxycodone |
|---------|-------------|-----------|
| QC50 | 1.2-13.3% | 4.5-10.8% |
| QC100 | 5.1-10.0% | 0.3-6.7% |
| QC500 | 3.2-9.4% | 1.1-5.1% |

Using Analyte Area Alone

| n=4 x 4 | Clenbuterol | Oxycodone |
|---------|-------------|-----------|
| QC50 | 1.9-10.4% | 2.3-13% |
| QC100 | 5.5-14% | 1.8-7.3% |
| QC500 | 0.4-13.5% | 2.3-7.3% |

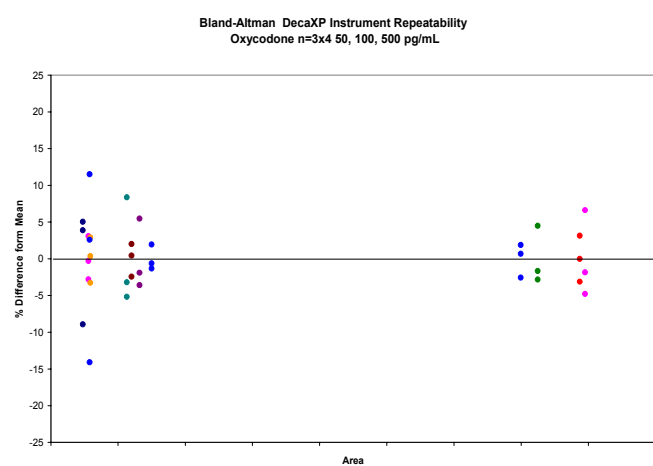
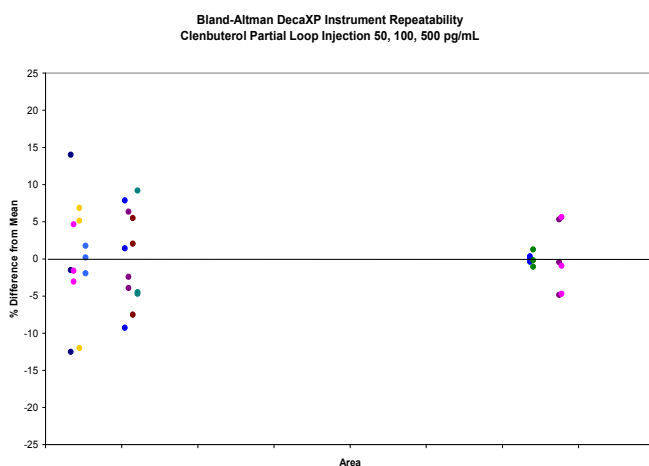


Figure 5. Instrument Repeatability of Decca XP Plus Ion Trap for clenbuterol (left) and oxycodone (right) using area of analyte only and partial loop injection (ranges are 50, 100, and 500 pg/mL)

B. Within Run Accuracy and Precision

| CLENBUTEROL | | |
|-------------|-------|--------|
| pg/mL | RSD% | AR% |
| 50 | 14.45 | 64.68 |
| 100 | 7.58 | 80.53 |
| 500 | 10.64 | 101.56 |

| OXYCODONE | |
|-----------|--------|
| RSD% | AR% |
| 6.78 | 84.63 |
| 6.16 | 91.81 |
| 2.86 | 123.26 |

n=4

$RSD\% = (\text{StdDev}/\text{Mean}) * 100$

$AR\% = (\text{Observed Value}/\text{Target Value}) * 100$

C. Between Run Accuracy and Precision

| CLENBUTEROL | | |
|-------------|-------|--------|
| pg/mL | RSD% | AR% |
| 50 | 20.40 | 100.25 |
| 100 | 5.68 | 91.63 |
| 500 | 3.45 | 98.14 |

| OXYCODONE | |
|-----------|-------|
| RSD% | AR% |
| 30.16 | 91.50 |
| 10.97 | 95.50 |
| 4.60 | 99.28 |

n=4

$RSD\% = (\text{StdDev}/\text{Mean}) * 100$

$AR\% = (\text{Observed Value}/\text{Target Value}) * 100$

Clenbuterol Youden Plot of Control Sample Within Run and Between Run Repeatability

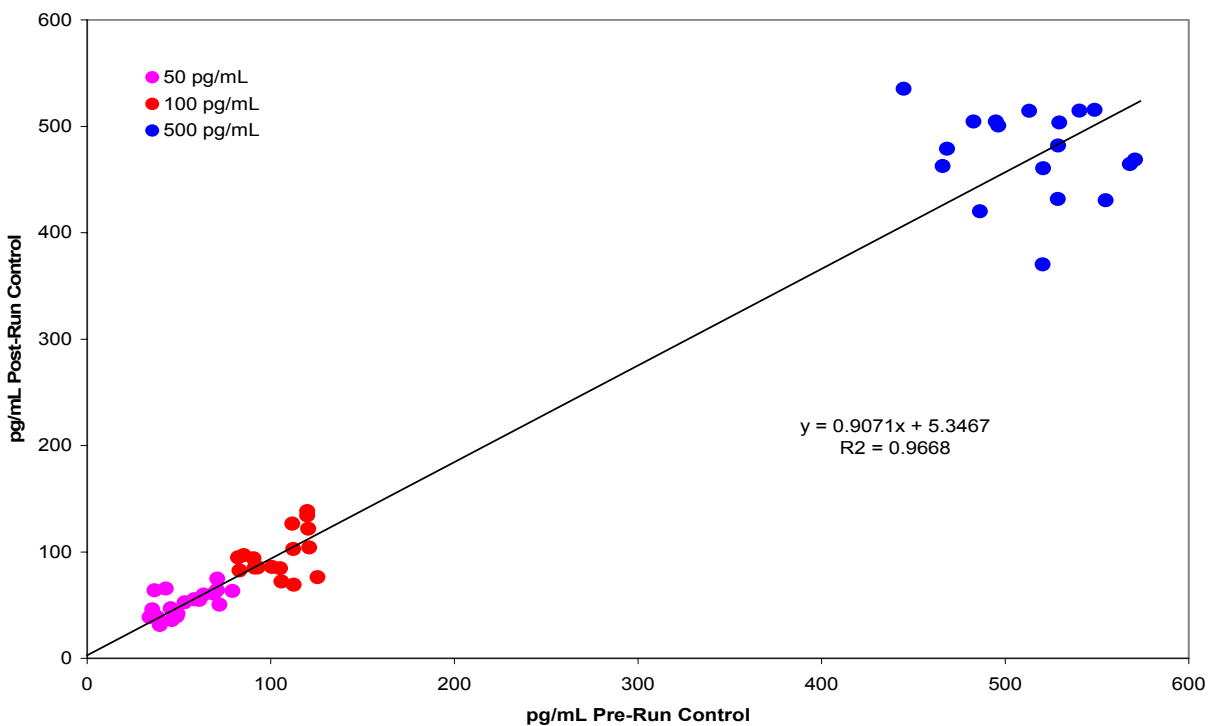


Figure 6. Youden Plot of duplicate control data from actual analyses of greater than 40 plasma injections (excluding calibrators, blanks and controls) indicating real-world dispersion of pre- and post- analysis values. Within analysis pairs are plotted as x,y pairs. Plot gives a representation of combined inter-assay and intra-assay variation (ranges are 50, 100, and 500 pg/mL).

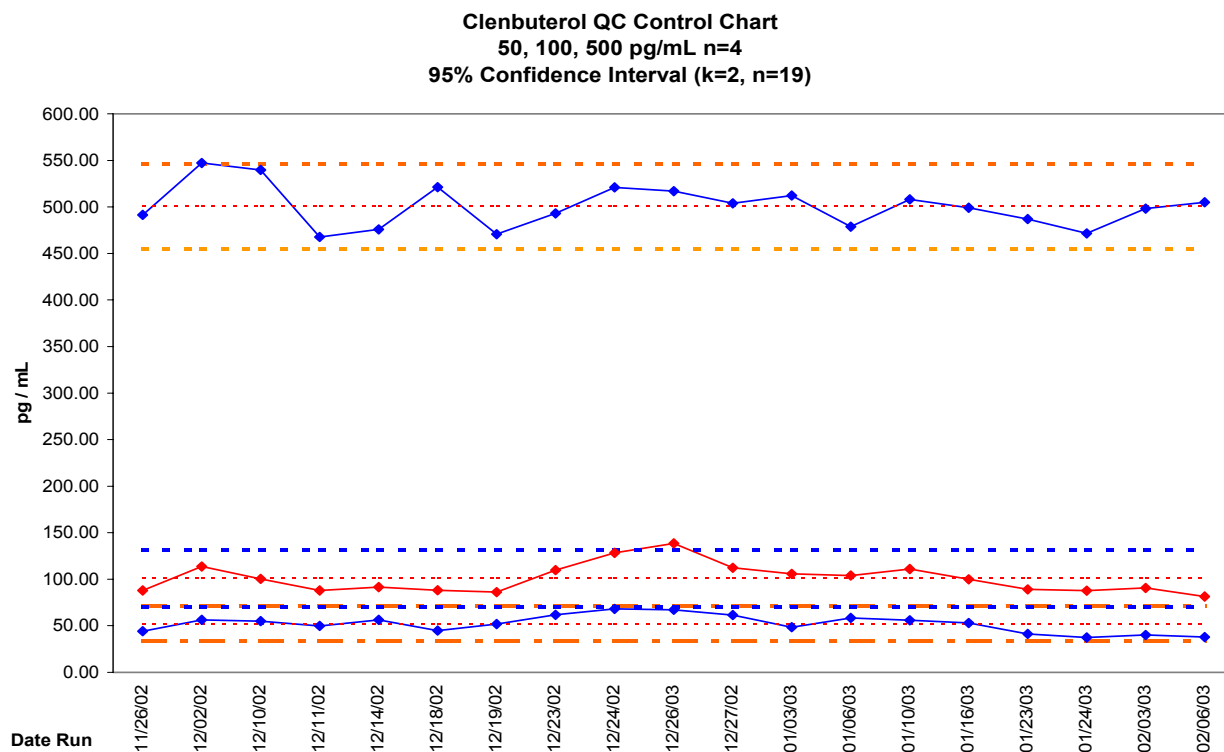


Figure 7. Control Plot of control data (n=4 mean of 2 calibrators, 2 controls) from actual analyses of greater than 40 plasma injections (excluding calibrators, blanks and controls). Dispersion is reduced due to averaging of replicates. Plot indicates a representation of Type A confidence (ranges are 50, 100, and 500 pg/mL).

This SOP is intended strictly for screening and confirmation purposes, but quantitative considerations are included to characterize method performance, deterioration of system responsiveness, and as value added estimates for positive results. Primary deviations from more formally quantitative aspects include pre-run small volume preparation of calibrators and controls (as compared to large volume batch preparation and validation), and choice of analytical instrument (compared to continuous throughput instruments such as quadrupole devices). It should be emphasized, that in the absence of urine positive data, the action threshold for this SOP is the limit of confirmation.

- D. Limit of detection for oxycodone and clenbuterol is 10-20 pg/mL. Limit of detection is defined as the ability of automatic integration to report estimated concentrations that represent a chromatographic signal to noise level greater than 5:1 for the respective quantifying ions (or sum of quantifying ions).
- E. Limit of quantitation is 20- 50 pg/mL for oxycodone and clenbuterol. Limit of quantitation is defined as +/- 50% residual for the respective calibrator. The low end calibrators primarily serve as an indicator of chromatographic and ion spray probe condition.
- F. Limit of confirmation for oxycodone and clenbuterol is 30 to 70 pg/mL. Limit of confirmation is defined as all qualifying and diagnostic ions present +/- 30% relative abundance, with no interfering ions > than 25%.

G. Suppression may be observed for clenbuterol-d9-clenbuterol due to elution of retained lipophilic compounds during the high organic segment of the gradient. Suppression of the internal standard is sometimes observed. If this rarely and randomly occurs, it usually indicates poor sample condition. If this systematically occurs, it usually indicates the need to change or regenerate the chromatographic column and guard column.

H. Extraction recovery percentages:

- i. oxycodone – 80%
- ii. clenbuterol – 89%

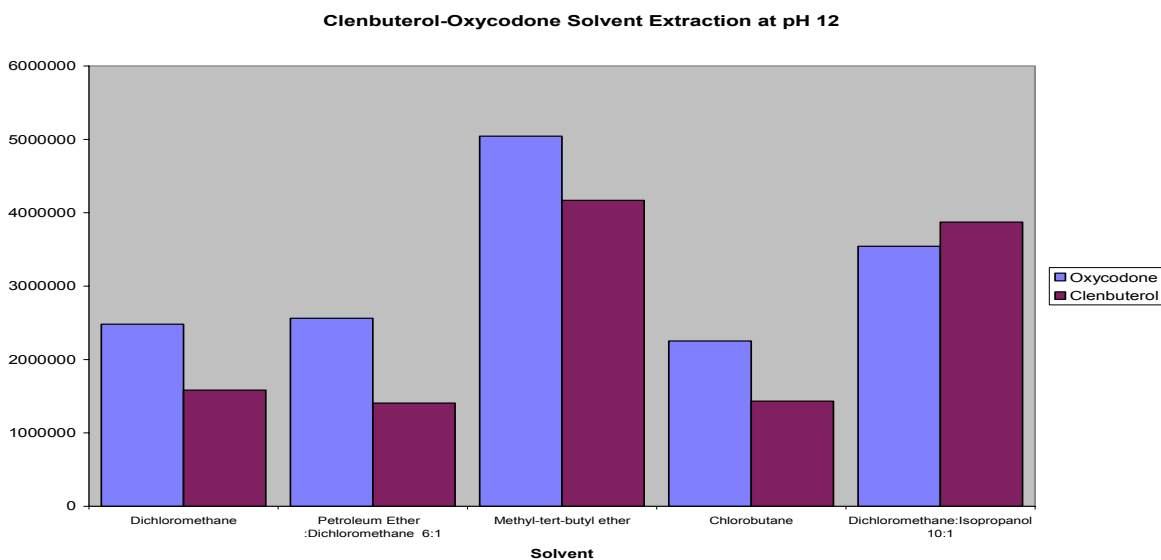
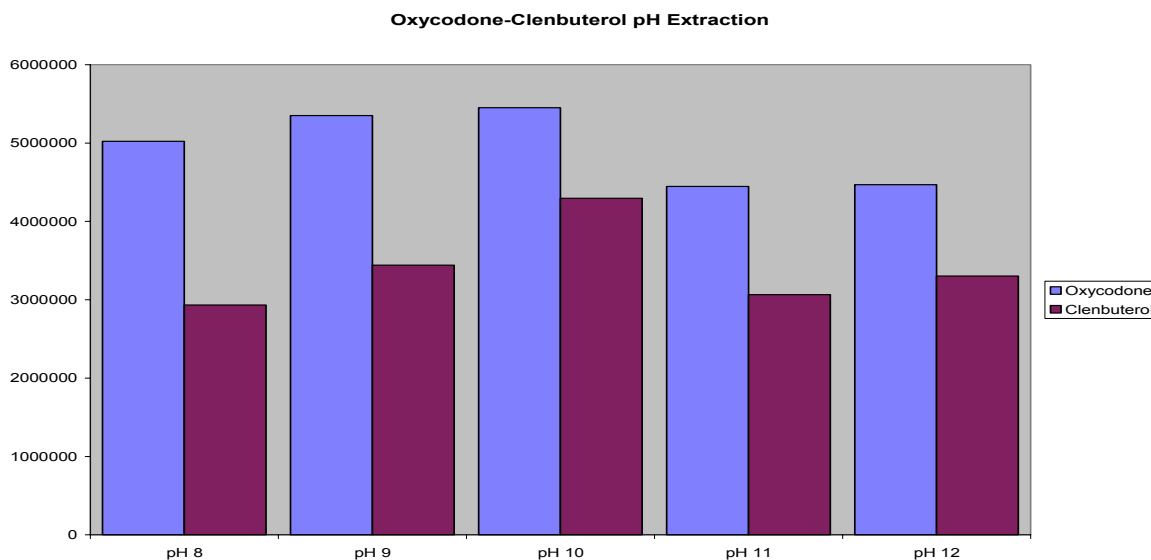


Figure 8. Extraction solvent (top panel) and extraction pH (bottom panel) for combined oxycodone and clenbuterol liquid/liquid extraction sample preparation



XII. Criteria for Identification of Oxycodone and Clenbuterol from Equine Plasma

Identification of oxycodone

The diagnostic ions for oxycodone are 298 m/z (BP) and 256 m/z and the retention time for the suspect sample, calibrator, and QC control agree to +/- 0.15 minutes.

Confirmation of oxycodone

All ions for oxycodone are present in the full scan MSMS +/- 30% of that ion compared with oxycodone calibrator and control spectra, with no interfering ions > than 20%. Spectra may be averaged and/or subtracted

Identification of clenbuterol

The diagnostic ions for clenbuterol are 259 m/z (BP) and 203 m/z and the retention time for the suspect sample, calibrator, and QC control agree to +/- 0.15 minutes.

Confirmation of clenbuterol

All ions for clenbuterol are present in the full scan MSMS spectrum +/- 30%, of that ion compared with clenbuterol calibrator and control spectra, with no interfering ions > than 20%. Spectra may be averaged and/or subtracted.

XIII. SEQUENCE OF POSITIVE SAMPLE DATA PACKET

- A. SAMPLE TRANSFER SHEET (WS#32)
- B. SAMPLE USAGE SHEET (FORM #7)
- C. SAMPLE LIST
- D. COLUMN TEST CHROMATOGRAM
- E. LC METHOD
- F. MS METHOD
- G. EXTRACTED ION CHROMATOGRAM COMPARISON
- H. SPECTRA COMPARISON
- I. CONFIDENCE REPORT
- J. QUANTIFICATION REPORT
- K. QUANTIFICATION CALIBRATION CURVE

Other Required Documentation

In addition to the positive data packet, the following documentation is required:

Sequence Sample list print-out is maintained in the Deca XP three ring binder
Instrument usage logbook is completed (and maintenance log if needed), indicating date and project.

Data packets for samples determined to be negative will contain the following elements:

- a. Sample Transfer Sheet (WS # 32)
- b. Sample Usage Sheet (Form #7)
- c. Confidence Determination Report
- d. Quantification Report

XV. REAGENTS

- A. Methanol, Optima grade (Cat. No. A 454-4, Fisher Scientific.)
- B. Acetonitrile, Optima grade (Cat. No. A 996-4, Fisher Scientific.)
- C. Water, Optima grade (Cat. No. W7-4, Fisher Scientific.)
- D. Methyl-tert-Butyl Ether, HPLC grade (Cat. No. E127-4, Fisher Scientific)
- E. Formic Acid, SupraPur (Cat. No. 11670-1, EM Science)
- F. Sodium hydroxide 50% (Cat. No. SO-S-410, Fisher Scientific)
- G. Sodium Tetraborate (Cat. No. S-248-3, Fisher Scientific)
- H. Water, HPLC grade (Cat. No. 4218-03, J.T. Baker)

XVI. FORMULAE

Safety Requirements: Lab coat, fume hood, gloves, safety glasses. CAUTION: Strong alkali solutions generate heat upon mixing.

- A. 0.1% Formic Acid: (Using E. and C. above), add 1000 uL Formic Acid (E.) to 1000 L of Water (C.). Mix thoroughly. Cap, label.
- B. 2.3 mM Formic Acid: (Using E. and C. above), add 400 uL Formic Acid (E.) to 1000 mL of Water (C.). Mix thoroughly. Check pH. Cap, label, and record pH.
- C. Saturated Sodium Borate, pH 10: (Using H., G., and F. above), add sufficient Sodium Borate (G.) to 1 L HPLC grade water (H.), till no more dissolves. Heat with stirring. Continue to add (G.) until no more dissolves. Cool mixture. Adjust to pH 10 with 50% Sodium Hydroxide (F.).

XVII. MATERIALS

- A. 16 × 100 mm test tubes.
- B. 16 × 125 mm screw-top test tubes.
- C. 16 × 150 mm screw-top test tubes
- D. Polypropylene Caps
- E. Test tube rack
- F. Pipettes and tips.
- G. Rotorack
- H. Centrifuge
- I. Vortex mixer (Scientific Industries, Inc.)
- J. Branson Ultrasonic Water Bath, 8510 (Fisher Scientific or equivalent)
- K. pH meter (IQ Scientific Instruments)
- L. Sample Concentrator (Dri-Block DB-3, Techne)
- M. IEC HN-SII Centrifuge (International Equipment Company)

- N. Rotorack (Speci-Mix, Thermolyne)
- O. 2 mL autosampler vials
- P. 200 uL Insert (Target PP Polyspring, National Scientific Company)

XVIII. Additional Information:

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Clenbuterol Elimination Profiles

Clenbuterol (3.2 µg/kg, po)

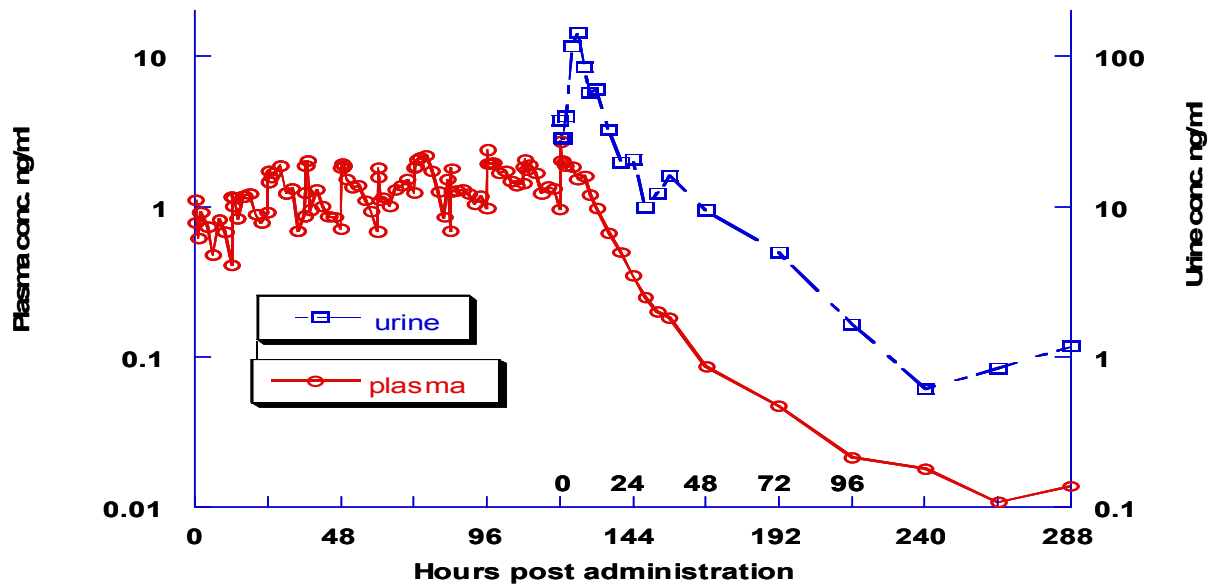


Figure 9: Clearance of clenbuterol following repeated approved usage. Urine monitoring by ELISA and GC/MS confirmation continues to maintain 1-10 ng/mL sensitivity corresponding to 24 to 48 hour withdrawal from therapeutic dose prior to racing. Plasma concentration of clenbuterol in the absence of a corresponding urine concentration is indicative of improper, non-therapeutic administration of clenbuterol to the horse.

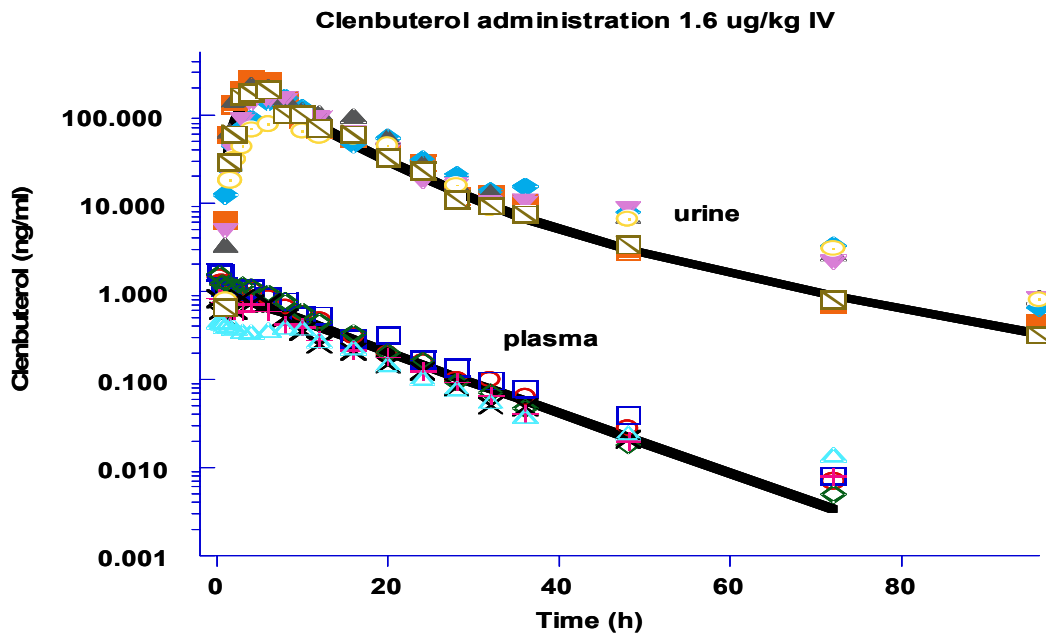


Figure 10: Clearance of clenbuterol following single IV administration. The ratio of clenbuterol in plasma to that in urine is 1: 100

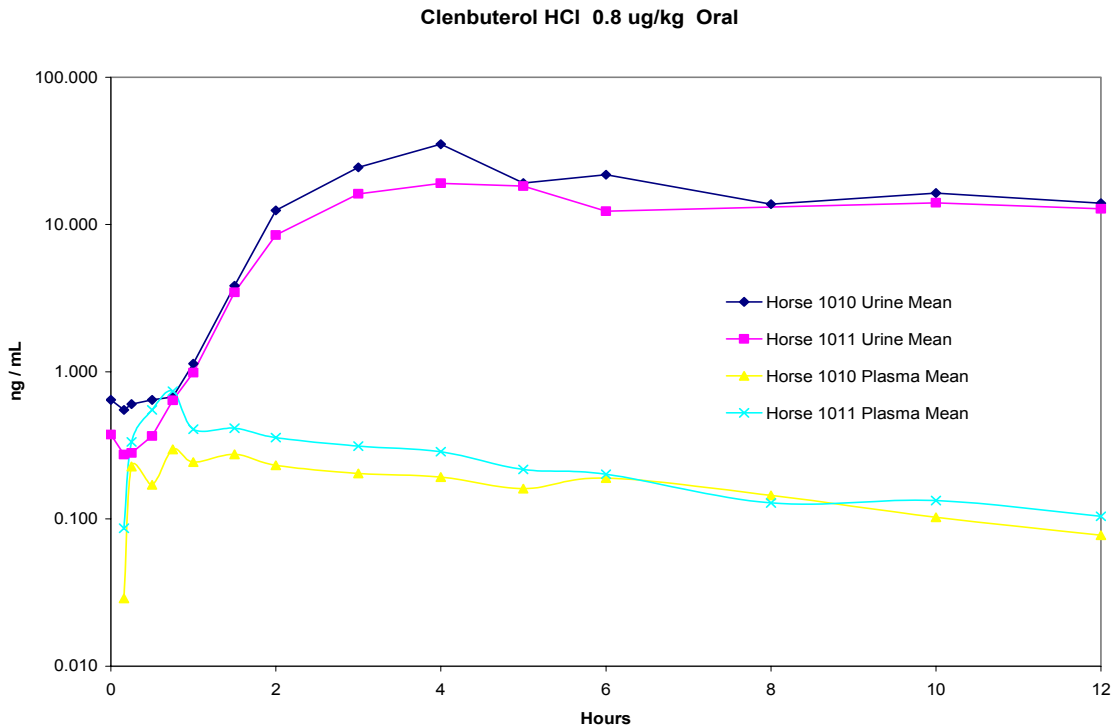


Figure 11. Examination of the IV and Oral studies indicates that only in the 0 to 4 hour time period post administration does the plasma concentration equal or exceed the urinary concentration of clenbuterol. After this time period, urine levels exceed plasma levels by greater than an order of magnitude. Consequently, plasma clenbuterol levels, with little or no detected urine clenbuterol concentrations indicate administration within four hours of racing in violation of prescribed administration policies.

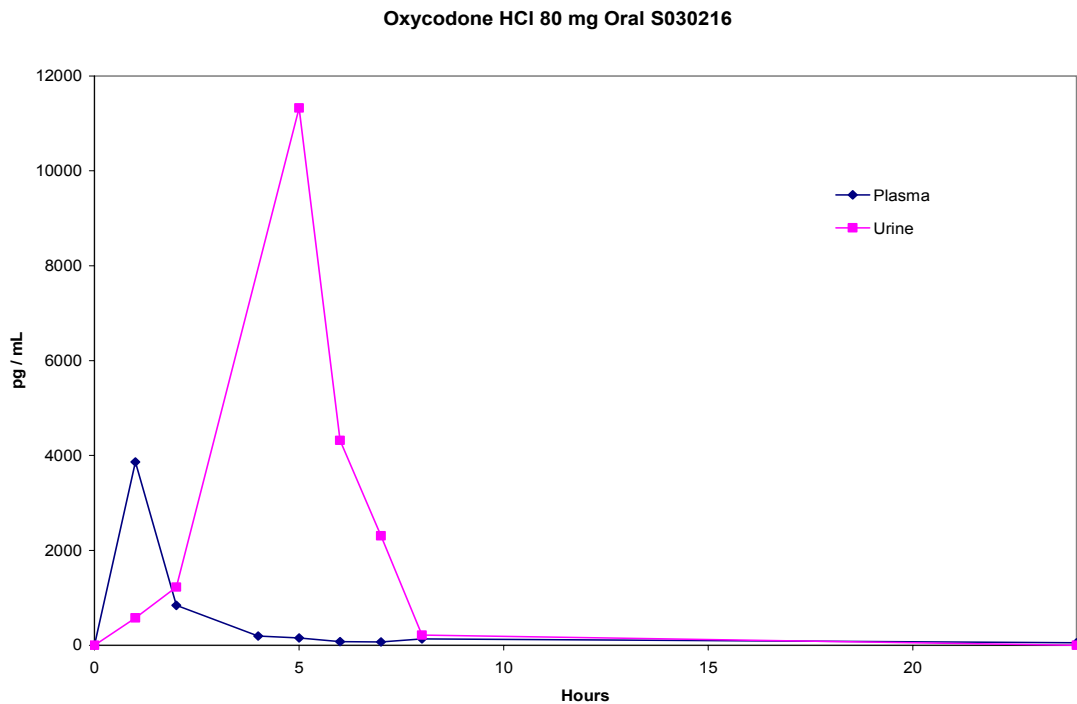


Figure 12: Oxycodone plasma and Urine concentrations following 80 mg Oral administration of oxycodone hydrochloride.

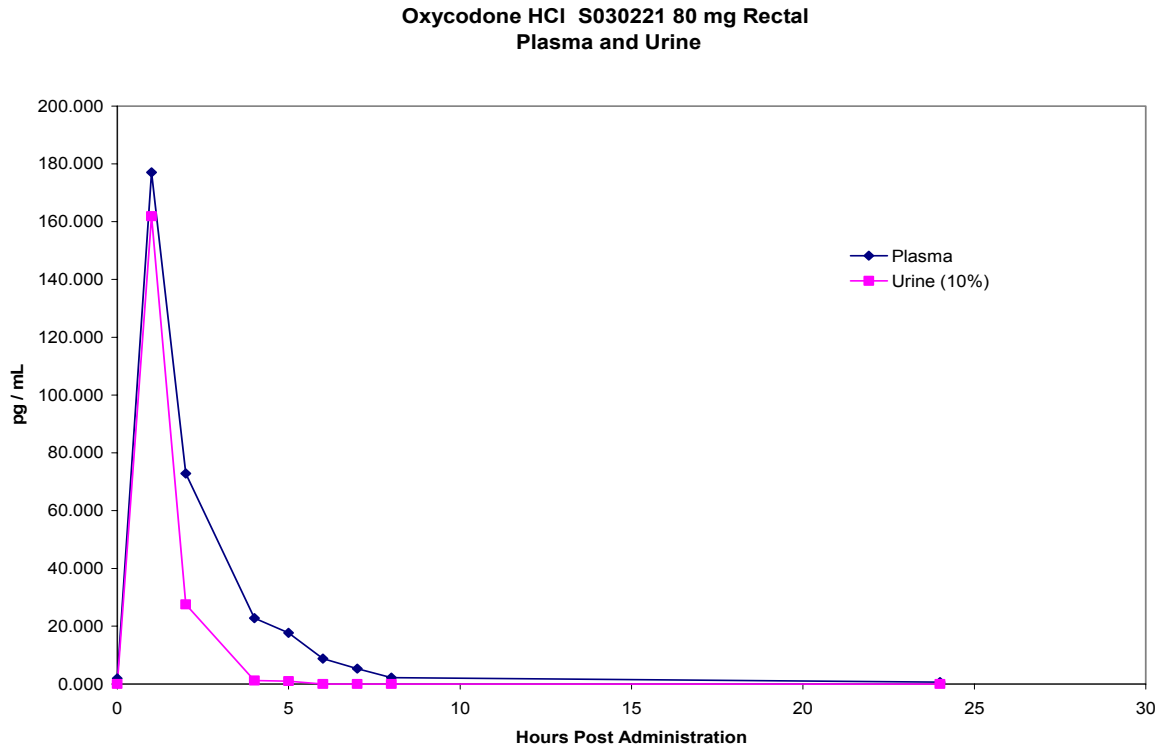


Figure 13: Plasma and urine concentrations of oxycodone following 80 mg rectal administration of oxycodone hydrochloride. Oral administration of oxycodone HCl is indicated if the plasma concentrations observed (50-500 pg/mL) have no detectable urinary concentrations (less than 100 pg/mL). OxyContin administration as a time-release formulation still needs to be investigated.

Stability Data

Oxycodone & Related Analyte Plasma Stability at Temperature (3 hours)

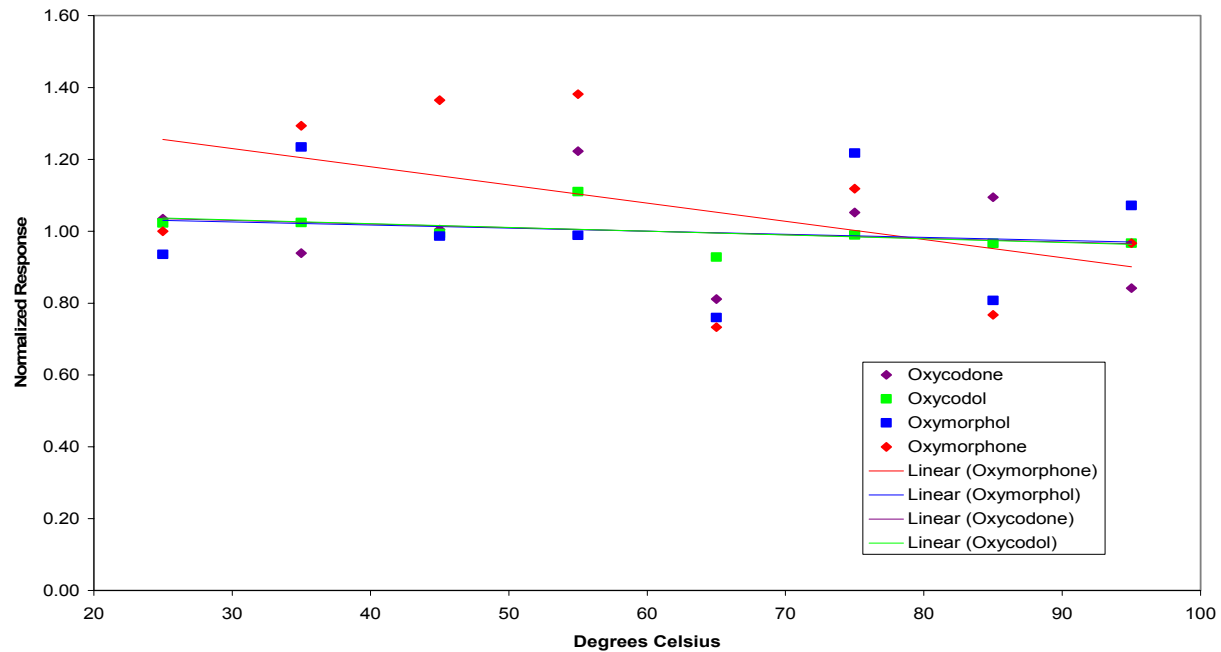


Figure 14. Stability of oxycodone (and related analytes) in pooled plasma, residence time of 3 hours at select temperature. While denaturation of plasma proteins occurs at 65°C, recovery was affected only to a minor extent. The apparent loss of Oxymorphone is not significant because the levels present were below 50 pg/mL.

Oxycodone Analyte Stability Response Normalized to Oxycodone
 3 hours at Temperature

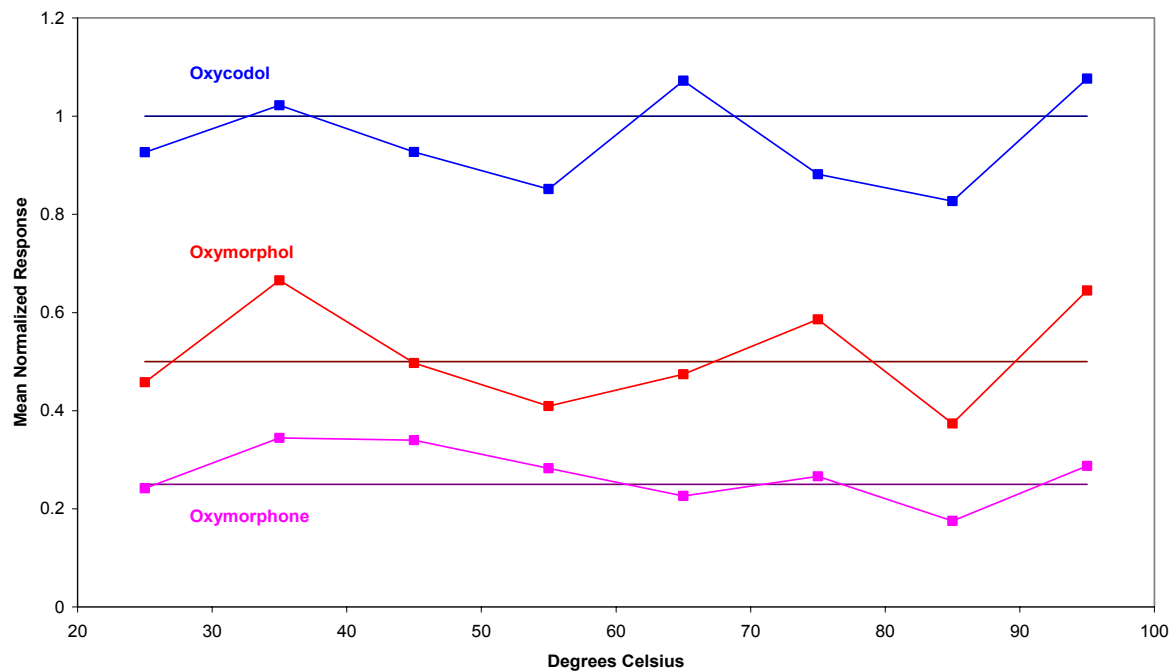


Figure 15. Stability of oxycodone metabolites relative to oxycodone indicating the absence of *ex vivo* conversion from oxycodone due to temperature effects..

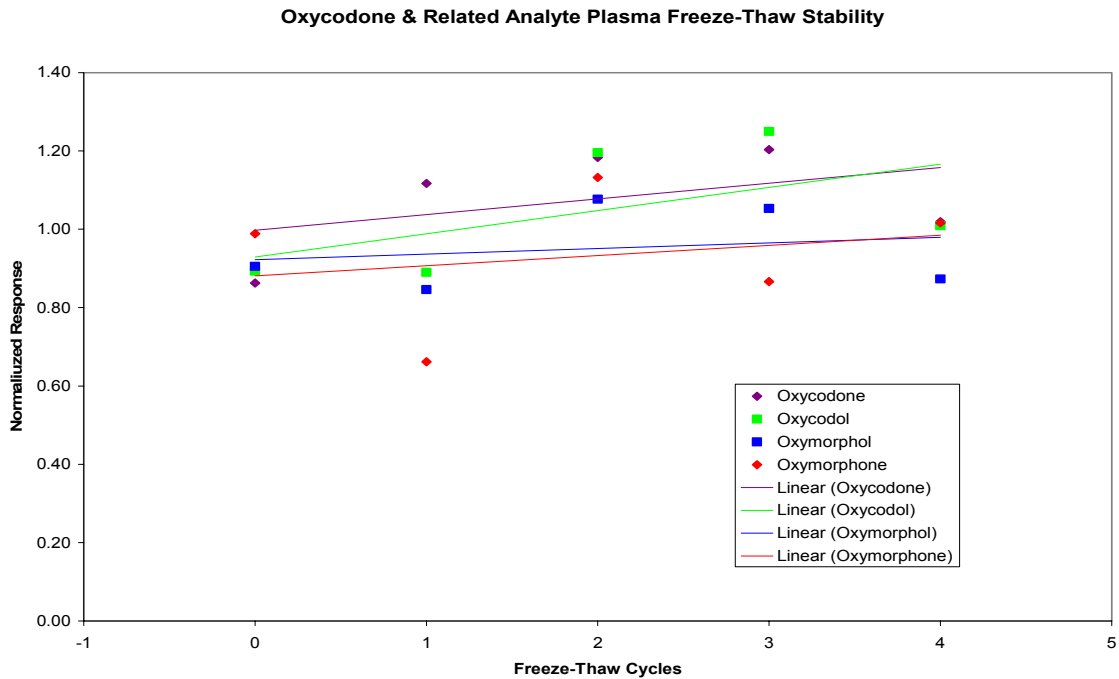


Figure 16: Stability of oxycodone (and related analytes in pooled administration plasma, through freeze-thaw cycles of -5°C to 35°C .

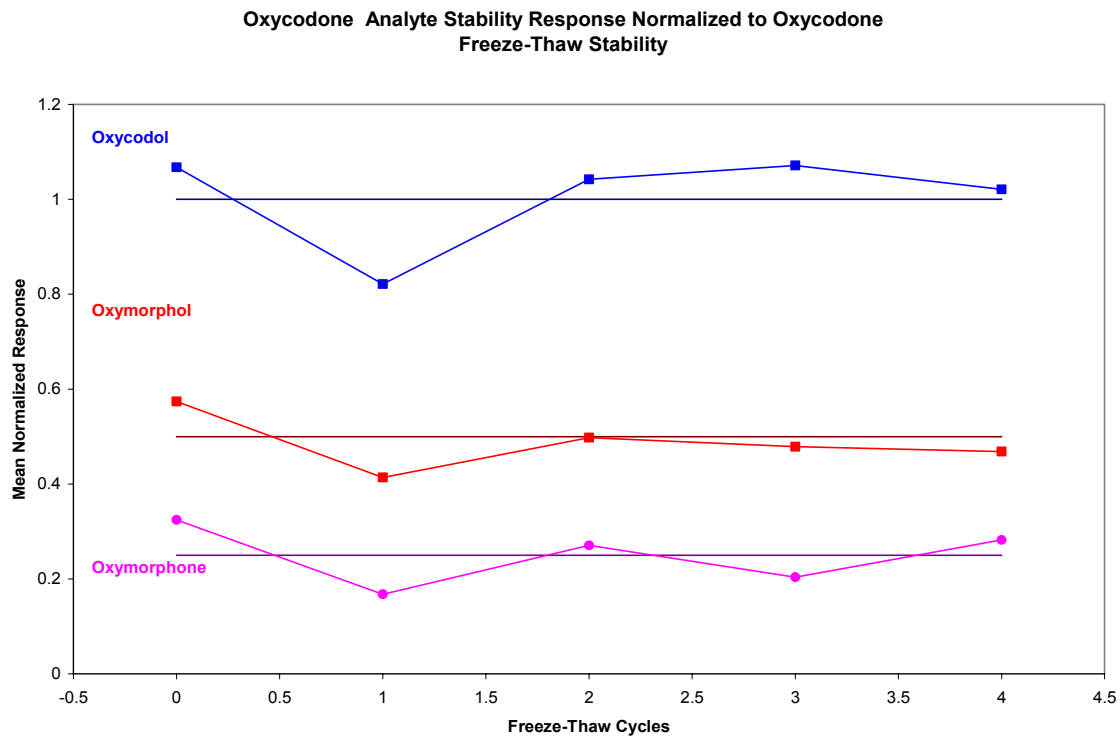


Figure 17: Freeze-Thaw cycle Stability of oxycodone metabolites relative to oxycodone indicating the absence of *ex vivo* conversion from oxycodone.

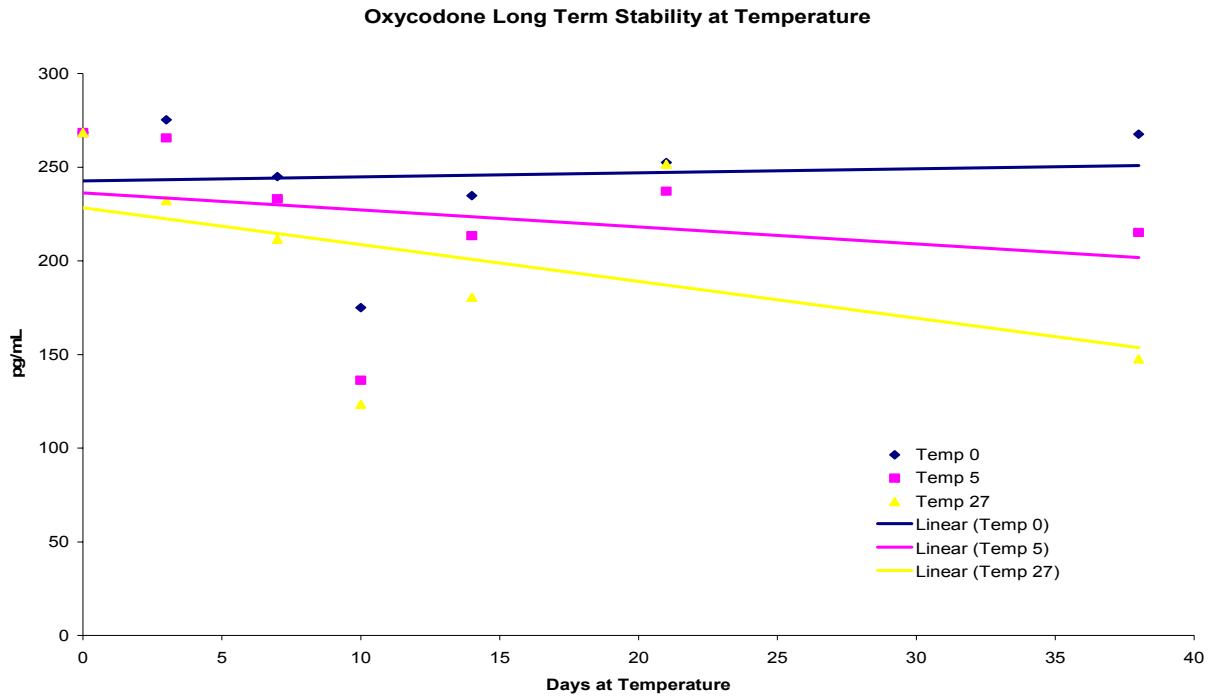


Figure 18: Long-term oxycodone stability at various temperatures (degrees Centigrade).

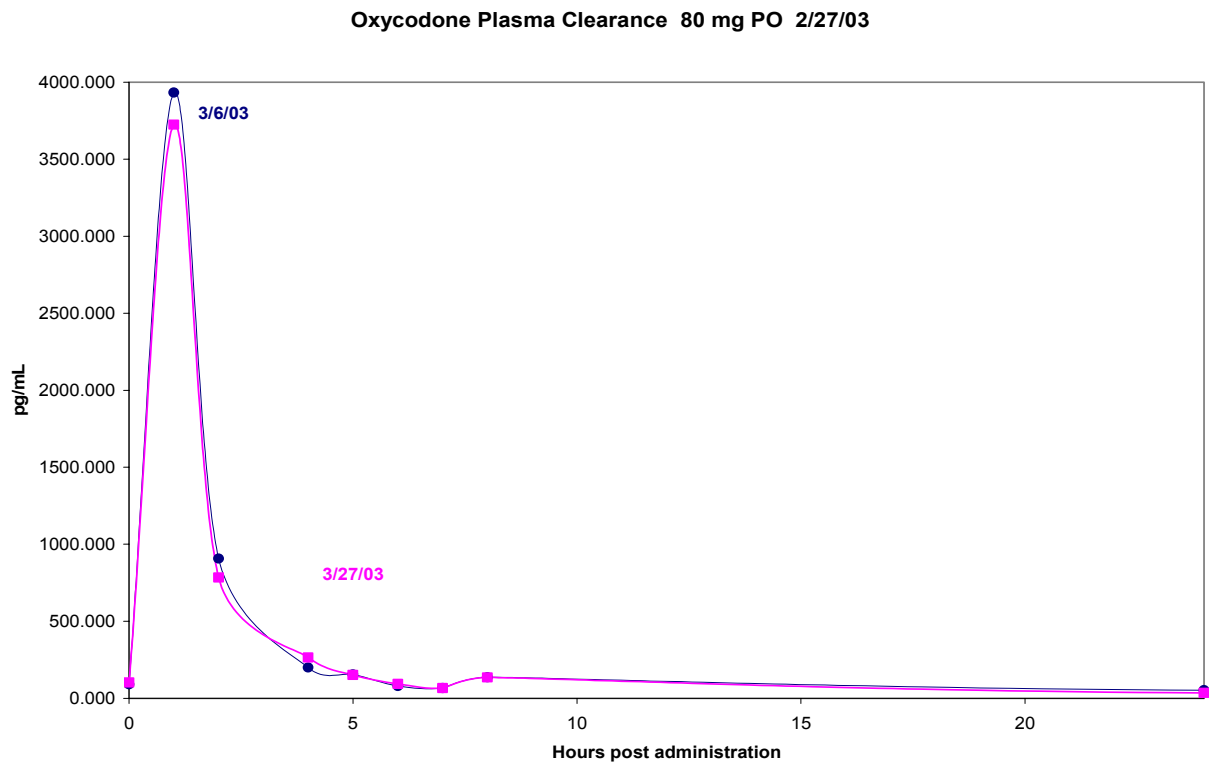


Figure 19: Multiple analysis of oxycodone administration (80 mg PO) plasma samples at 3 week interval indicating good repeatability of the analytical method.

Uncertainty Budget

While not a quantitative procedure, the following treatment illustrates assessment, assignment, and combination of type A (statistical) and Type B (general) uncertainties.

Clenbuterol Uncertainty

Sigma C-5423 Lot # 128H1480

Minimum 95 %

| Designation | Source of Uncertainty | Value | Units % | Distribution | Divisor | Standard Uncertainty | Deg of Freedom (n-1) | Type | Other |
|-----------------|-------------------------------------|---------|---------|--------------|---------|----------------------|----------------------|------|-------------------|
| U ₁ | Reference purity | 2.5 | | R | 1.5 | 1.667 | ∞ | B | Stated as minimum |
| U ₂ | Balance Accuracy @ 5 mg | 0.2 | | R | 1.5 | 0.133 | ∞ | B | |
| U ₃ | Balance Repeatability @ 5 mg | 0.2614 | | N | 1 | 0.261 | 4 | B | |
| U ₄ | Molecular Formula (mass defect) | 0.02054 | | R | 1.5 | 0.014 | ∞ | B | |
| U ₅ | Stock Pipette Accuracy | | | | | | | B | |
| U ₆ | Stock Pipette Repeatability | | | | | | | A | |
| U ₇ | Method Pipette Accuracy | | | | | | | B | |
| U ₈ | Method Pipette Repeatability | | | | | | | A | |
| U ₉ | Instrument Repeatability (L) | 20.91 | | N | 1 | 20.91 | 4 | A | |
| U ₁₀ | Instrument Repeatability (M) | 11.24 | | N | 1 | 11.24 | 4 | A | |
| U ₁₁ | Instrument Repeatability (H) | 9.67 | | N | 1 | 9.67 | 4 | A | |
| U ₁₂ | Method Instrument Repeatability (L) | 17.95 | | N | 1 | 17.95 | 20 | A | |
| U ₁₃ | Method Instrument Repeatability (M) | 15.31 | | N | 1 | 15.31 | 20 | A | |
| U ₁₄ | Method Instrument Repeatability (H) | 4.53 | | N | 1 | 4.53 | 20 | A | |
| U ₁₅ | Method Instrument Accuracy (L) | 4.104 | | R | 1.5 | 2.74 | 20 | A | |
| U ₁₆ | Method Instrument Accuracy (M) | 0.784 | | R | 1.5 | 0.52 | 20 | A | |
| U ₁₇ | Method Instrument Accuracy (H) | 0.094 | | R | 1.5 | 0.06 | 20 | A | |

| | | |
|--------------------------|--------|--------------------------------------------------------------------|
| Combined Uncertainty (L) | 18.236 | $SQR(u_1^2+u_2^2+u_3^2+u_4^2+u_5^2+u_6^2+u_7^2+u_{12}^2+u_{15}^2)$ |
| Combined Uncertainty (M) | 15.412 | $SQR(u_1^2+u_2^2+u_3^2+u_4^2+u_5^2+u_6^2+u_7^2+u_{13}^2+u_{16}^2)$ |
| Combined Uncertainty (H) | 4.836 | $SQR(u_1^2+u_2^2+u_3^2+u_4^2+u_5^2+u_6^2+u_7^2+u_{14}^2+u_{17}^2)$ |

| | | |
|--------------------------|--------|-----|
| Expanded Uncertainty (L) | 36.472 | k=2 |
| Expanded Uncertainty (M) | 30.824 | k=2 |
| Expanded Uncertainty (H) | 9.672 | k=2 |

It is noted that instrument repeatability (type A) and assumed reference purity (type B) are the largest contributors to overall uncertainty. While efforts have been made to identify all system contributions, many are included within another multivariate parameter, and are therefore excluded from combined uncertainty calculation. The usefulness of this budgetary listing is realized in root cause analysis of system control issues. For instance, a degraded performance of low concentration calibrators can be traced to:

1. Aging reference standards or stocks
2. Stock preparation pipettes
3. Method recovery
4. Ion trap tuning
5. Ion probe condition
6. Chromatographic conditions
7. Integration parameters

While many of these points do NOT, in fact, appear in the uncertainty budget, a stepwise systematic uncertainty analysis can shed light on appropriate areas for correction and control. It must be realized that the correction and control process, while parallel to, is not the same as estimation of process uncertainty.

While this process, for this example, depicts the major contributor to uncertainty to be instrument repeatability, it is not advised that this single parameter be generalized to be representative of uncertainty in every similar case.

Another drug using these same processes and procedures may have entirely different uncertainties associated with it due to such factors as chemical stability, detector response, chromatographic co-elution, and matrix effects, to suggest just a few.