

**IDENTIFICATION AND DETERMINATION OF ZOLPIDEM AND METABOLITE FROM
EQUINE URINE SAMPLES – LC/MS METHOD**

I. INTRODUCTION

Zolpidem is a hypnotic depressant drug, trade name Ambien. Zolpidem (Figure 1) is a Drug Enforcement Administration controlled substance. It has been classified by the Association of Racing Commissioners International, Inc. as a class 2 drug in horses. One of the primary metabolites in equine urine is carboxy-zolpidem.

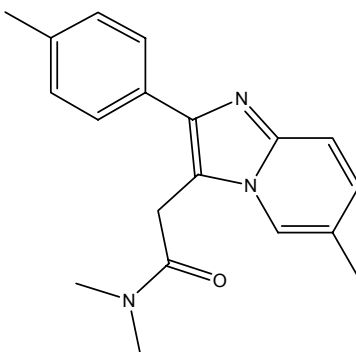


Figure 1. Structure of Zolpidem

II. SCOPE

This standard operating procedure is to be used for the identification of zolpidem and/or carboxy-zolpidem and quantitation of zolpidem in equine urine. The lower limit of quantitation for this method is approximately 0.02 ng/mL of urine.

III. PRINCIPLE OF METHOD

The urine sample is first treated with β -glucuronidase from *Patella vulgata* to hydrolyze glucuronide and sulfate conjugates of zolpidem and carboxy-zolpidem. Zolpidem and carboxy-zolpidem are isolated from buffered horse urine by solid phase extraction. The dried residue remaining after evaporation of the extract is redissolved and analyzed by liquid chromatography / mass spectrometry operated under electrospray conditions in the selected reaction monitoring mode. The concentration of zolpidem in the sample is determined by the internal standard method using the peak area ratio and linear regression analysis.

IDENTIFICATION AND DETERMINATION OF ZOLPIDEM AND METABOLITE FROM
EQUINE URINE SAMPLES – LC/MS METHOD

IV. REAGENTS

A. Water

Use double distilled water in any reagent or procedure requiring the use of water.

B. 1.6 M Acetate Buffer (pH 5.0)

1. Reagents

- a) sodium acetate trihydrate ($\text{NaCH}_3\text{COO} \cdot 3 \text{H}_2\text{O}$), reagent grade
- b) concentrated glacial acetic acid, reagent grade
- c) water

2. Procedure

- a) Dissolve 136 g of sodium acetate trihydrate in approximately 200 mL of water.
- b) Add 33 mL of concentrated glacial acetic acid.
- c) Dilute to 1000 mL with water and mix. Adjust the pH to 5.0 ± 0.1 with 1 N sodium hydroxide solution or concentrated glacial acetic acid, if necessary

3. Storage Requirements

- a) Store at approximately 4°C in a glass container.
- b) Discard 1 year after preparation.

C. 1.0 M Acetic Acid

1. Reagents

- a) glacial acetic acid, reagent grade
- b) 1 N potassium hydroxide solution
- c) water

2. Procedure

- a) Prepare under a fume hood.
- b) Add 28.6 mL of glacial acetic acid to 500 mL Graduate cylinder (TC)
- c) Dilute to 500 mL with water.

3. Storage Requirements

- a) Store at approximately 4°C in a glass container.
- b) Discard 1 year after preparation.

**IDENTIFICATION AND DETERMINATION OF ZOLPIDEM AND METABOLITE FROM
EQUINE URINE SAMPLES – LC/MS METHOD**

- D. β -Glucuronidase Reagent (10,000 units/mL in sodium acetate buffer)
1. Reagents
 - a) β -Glucuronidase from *Patella vulgata* (cat. no. G-8132, Sigma Chemical Co., St. Louis, MO 63178 or equivalent)
 - b) 1.6 M acetate buffer (pH 5.0)
 2. Procedure
 - a) Dilute the contents of one vial (2,000,000 units per vial) of β -glucuronidase reagent to 200 mL with 1.6 M acetate buffer. Mix.
 3. Storage Requirements
 - a) Store at approximately 4 °C in a glass container.
 - b) Discard 1 month after preparation.
- E. 1 N Hydrochloric Acid Solution
1. Reagents
 - a) Concentrated hydrochloric acid, reagent grade
 - b) Water
 2. Procedure
 - a) Prepare under a fume hood.
 - b) Add 40 mL of concentrated hydrochloric acid to approximately 400 mL of water and dilute to 480 mL with water. Mix.
 3. Storage Requirements
 - a) Store at room temperature in a glass container.
 - b) Discard 1 year after preparation.
- F. 1 N Sodium Hydroxide Solution

Preparation of this reagent generates heat.

1. Reagents
 - a) Sodium hydroxide pellets, reagent grade
 - b) Water
2. Procedure
 - a) Prepare under a fume hood.
 - b) Dissolve 20 g of sodium hydroxide pellets in sufficient water to produce 500 mL of solution. Mix.

**IDENTIFICATION AND DETERMINATION OF ZOLPIDEM AND METABOLITE FROM
EQUINE URINE SAMPLES – LC/MS METHOD**

3. Storage Requirements
 - a) Store at room temperature in a glass container.
 - b) Discard 1 year after preparation.
- G. Methanol (B&J, cat. no. 230-4, Muskegon, MI or equivalent)
- H. Elution solvent: Methylene Chloride: 2-Propanol:Ammonium Hydroxide (78:20:2; v/v/v)
 1. Reagents
 - a) Methylene Chloride, Optima grade (cat. no. D-151, Fisher Scientific, Pittsburgh, PA or equivalent)
 - b) 2-Propanol, Optima grade (cat. no. A4664, Fisher Scientific or equivalent)
 - c) Concentrated Ammonium Hydroxide, reagent grade
 2. Procedure
 - a) Prepare under a fume hood.
 - b) To a 100 mL graduate cylinder, add 20 mL of propanol, then 2 mL of ammonium hydroxide. Mix.
 - c) Dilute to 100 mL with Methylene Chloride
 3. Storage Requirements
 - a) Prepare the reagent fresh daily.
 - b) Store at room temperature in a glass container.
- I. 0.1 M Phosphate Buffer, pH 6.0 (1 L)
 1. Reagents
 - a) Dibasic sodium phosphate (Na_2HPO_4), reagent grade
 - b) Monobasic sodium phosphate monohydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$), reagent grade
 - c) Water
 2. Procedure
 - a) Weigh 1.7 g of Na_2HPO_4 and 12.1 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$. Add DI water to a final volume of 1 L in a mixing cylinder or volumetric flask and mix thoroughly.
 - b) Adjust pH to 6.0 ± 0.1 with saturated monobasic sodium phosphate (lowers pH) or saturated dibasic sodium phosphate (raises pH), if necessary.
 3. Storage Requirements

**IDENTIFICATION AND DETERMINATION OF ZOLPIDEM AND METABOLITE FROM
EQUINE URINE SAMPLES – LC/MS METHOD**

- a) Store at approximately 4°C in a glass container.
 - b) Discard 1 year after preparation.
- J. Formic Acid, Certified ACS Grade, (98%, cat. no. FX0440-11, EM Science, Gibbstown, N.J.).
- K. Acetonitrile, HPLC Grade (cat no. 015-4, Burdick & Jackson, Muskegan, MI).
- L. Water, HPLC Grade (cat no. AH365, Burdick & Jackson).
- M. HPLC Solvent A (Acetonitrile + 0.2% Formic Acid)
- 1. Reagents
 - a) Acetonitrile
 - b) Formic Acid
 - 2. Procedure
 - a) Add 1000 mL acetonitrile to a HPLC solvent reservoir bottle. Remove and discard 2 mL using a glass syringe.
 - b) Add 2 mL of Formic acid using a glass syringe. Cap and mix thoroughly before placing on HPLC.
 - 3. Storage Requirements
 - a) Prepare the reagent fresh monthly.
 - b) Store at room temperature in an amber glass container.
- N. HPLC Solvent B (Water + 0.2% Formic Acid)
- 1. Reagents
 - a) Water, HPLC Grade
 - b) Formic Acid
 - 2. Procedure
 - a) Add 1000 mL HPLC Grade Water to a HPLC solvent reservoir bottle. Remove and discard 2.0 mL using a glass syringe
 - b) Add 2.0 mL of Formic acid using a glass syringe. Cap and mix thoroughly before placing on HPLC.
 - 3. Storage Requirements
 - a) Prepare the reagent fresh weekly.
 - b) Store at room temperature in an amber glass container.
- O. Sample Re-dissolving Solvent
- 1. Reagents

**IDENTIFICATION AND DETERMINATION OF ZOLPIDEM AND METABOLITE FROM
EQUINE URINE SAMPLES – LC/MS METHOD**

- a) HPLC Solvent A
- b) HPLC Solvent B
2. Procedure
 - a) Mix 10 mL HPLC Solvent A with 90 mL of HPLC Solvent B, vortex.
3. Storage Requirements
 - a) Prepare the reagent fresh daily.
 - b) Store at room temperature.

V. MATERIALS

- A. 16 x 125 mm borosilicate glass disposable culture tubes with caps.
- B. 12 x 75 mm borosilicate glass disposable culture tubes with caps.
- C. Pipettes and tips.

Note: Use the following positive displacement pipettes to pipette the standard solutions and working standard solutions.

1. 0.1 - 10 μ L adjustable volume pipette (Eppendorf 2100, Brinkmann Instruments Inc., Westbury, NY 11590-0207).
 2. 2.0 - 20 μ L adjustable volume pipette (Eppendorf 2000, Brinkmann Instruments Inc., Westbury, NY 11590-0207).
 3. 10 - 100 μ L adjustable volume pipette (Eppendorf 2000, Brinkmann Instruments Inc., Westbury, NY 11590-0207).
 4. 20 - 200 μ L adjustable volume pipette (Eppendorf 2000, Brinkmann Instruments Inc., Westbury, NY 11590-0207).
 5. 100 - 1000 μ L adjustable volume pipette (Eppendorf 2000, Brinkmann Instruments Inc., Westbury, NY 11590-0207).
 6. 500 - 5000 μ L adjustable volume pipette (Eppendorf 2100, Brinkmann Instruments Inc., Westbury, NY 11590-0207).
- D. Vortex mixer (Glas-Col® Apparatus Co. Terre Haute, IN 47802 or equivalent).
 - E. pH meter (Corning 445, Fisher Scientific Co., Pittsburgh, PA 15219 or equivalent).
 - F. Branson Ultrasonic Water Bath, 5510 (Fisher Scientific Co., Pittsburgh PA 15219 or equivalent).
 - G. Centrifuge (Sorvall Super T21, Kendro Laboratory Products, Newtown, CT -6470 or equivalent).

**IDENTIFICATION AND DETERMINATION OF ZOLPIDEM AND METABOLITE FROM
EQUINE URINE SAMPLES – LC/MS METHOD**

- H. Rotorack (Glas-Col® Apparatus Co., Terre Haute, IN 47802 or equivalent).
- I. Cerex 24 or 48-place solid phase extraction apparatus (Cera Inc. Baldwin Park, CA 91706).
- J. Solid phase extraction columns (p/n CSDAU133 United Chemical Technologies, Bristol, PA 19007) or equivalent.
- K. Tissue paper wipers (e.g. Kimwipes®).
- L. Evaporator (TurboVap, Zymark, Cambridge, MA or equivalent).
- M. Nitrogen gas.
- N. Heating block (Techne Instruments Inc or equivalent).
- O. 2 mL autosampler vials with 100 µL inserts and 11 mm crimp caps (Hewlett-Packard Co., Palo Alto, CA 94304 or equivalent).
- P. Glass pasteur pipettes, disposable.
- Q. Bottle Top Dispensers (0.4-2 mL, 1-5 mL, 1-10 mL, 2-10mL) Brinkmann Dispensette Digital (Brinkmann Instruments Inc., Westbury, NY 11590-0207).

VI. TEST SUBSTANCE

horse urine

VII. VOLUME REQUIRED

1 mL or appropriate dilution. Refer to section XI.E for guide to dilution.

VIII. WORKING STANDARD SOLUTIONS

- A. Zolpidem working standard solution in methanol - 0.05 ng/µL (A)
 - 1. Reagents
 - a) Zolpidem (cat. no. Z-103, Sigma Chemical Co., St. Louis, MO 63178 or equivalent). Store the standard in a tightly closed container, at room temperature, and protected from the light.
 - b) Methanol

**IDENTIFICATION AND DETERMINATION OF ZOLPIDEM AND METABOLITE FROM
EQUINE URINE SAMPLES – LC/MS METHOD**

2. Procedure
 - a) Prepare a 1.0 µg/µL standard solution from the Zolpidem reference standard as per SOP B001.
 - b) Use the 1.0 µg/µL standard solutions to prepare **two** 0.05 ng/µL working standard solutions as described below. Use one working standard solution for the preparation of the calibrators and the standard mixture, and the other working standard solution for the preparation of the positive control samples.
 - c) To prepare 0.1 ng/µL working standard solution: dilute 50 µL of the 1.0 µg/µL standard solution to 5.0 mL with methanol (for a 10 ng/µL solution) followed by a further dilution of 50 µL of the 10 ng/µL standard solution to 10.0 mL with methanol for the desired 0.05 ng/µL working standard solution (A). Use volumetric flasks and vortex thoroughly upon each dilution.
 - d) Store the standard and working standard solutions at < 0° C.
- B. Zolpidem working standard solution in methanol - 0.005 ng/µL (B)
 1. Reagents
 - a) Zolpidem working standard solution in methanol - 0.05 ng/µL (A)
 - b) Methanol
 2. Procedure
 - a) To prepare 0.005 ng/µL (B) working standard solution: dilute 500µL of the 0.05 ng/µL (A) standard solution to 5.0 mL with methanol in a volumetric flask. Vortex thoroughly.
 - b) Store the standard and working standard solutions at < 0° C.
- C. Azaperone-IS working standard solution in methanol - 2.0 ng/µL
 1. Reagents
 - a) Azaperone (cat. no. 04575, U.S.P. or equivalent). Prepare a 1.0 µg/µL Azaperone-IS standard solution as per SOP B001.
 - b) Methanol.
 2. Procedure
 - a) Dilute 10 µL of the 1.00 µg/µL standard solution to 5.0 mL with methanol for a 2.0 ng/µL working standard solution.
 - b) Store the standard and working standard solutions at < 0° C.

IDENTIFICATION AND DETERMINATION OF ZOLPIDEM AND METABOLITE FROM
EQUINE URINE SAMPLES – LC/MS METHOD

IX. CONTROL SAMPLES

- A. Negative control horse urine - Horse urine sample negative for Zolpidem, carboxy-zolpidem and Azaperone-IS.
- B. Zolpidem positive control urine, 2.5 ng/mL - Add 50 μ L of the 0.05 ng/ μ L (A) working standard solution to tubes labeled **PC_a**, **PC_b**, and **PC_c** (see Table 1). Note: Prepare the positive control sample using a different working standard solution from the one used to prepare the calibrators.
- C. Administration Positive Control - Whenever possible include a zolpidem administration positive control sample. This control is necessary for identification of carboxy-zolpidem, for which a standard is not currently available.

X. SAMPLE REQUIREMENTS FOR ANALYSIS

Prepare the following samples and standards for each analysis:

- A. Calibrators designated **C₁**, **C₂**, **C₃**, **C₄**, **C₅**, **C₆** and **C₆**; prepare calibrators at concentrations of 0.02, 0.1, 0.25, 1.0, 2.5 and 10 ng/mL, respectively from negative control horse urine and the zolpidem working standard solution.
- B. System washes designated **SYS₁** and **SYS₂**; prepare system washes from sample redissolving solvent.
- C. Negative control sample designated **NC**; prepare negative control sample from negative control urine.
- D. Test sample(s) designated **TS_{1a...TS_{nb}}** where n is the total number of test samples; a and b are designations for sample replicates.
- E. Solvent blank(s) designated **SB_{1a...SB_{nb}}** where n is the total number of test samples; a and b are designations for sample replicates.
- F. Positive control samples designated **PC_a**, **PC_b**, and **PC_c** where a, b, and c are designations for sample replicates.
- G. Standard mixture designated **S₁**.

XI. CALIBRATOR AND SAMPLE PREPARATION

- A. Pipette 10.0 μ L of Azaperone-IS working standard solution into each labeled 12 x 75 mm test tube except those labeled **SYS₁**, **SYS₂**, **SB_{1a...SB_{nb}}** and **S₁**.

NOTE: Prepare **S₁** during step XIV.I and **SYS₁** and **SYS₂** during step XV.A.

- B. Pipette 0.05 ng/ μ L Zolpidem working standard solution into the calibrator tubes

IDENTIFICATION AND DETERMINATION OF ZOLPIDEM AND METABOLITE FROM EQUINE URINE SAMPLES – LC/MS METHOD

labeled **C₁**, **C₂**, **C₃**, **C₄**, **C₅**, and **C₆** as described in Table 1.

- C. Pipette 50 µL of the second 0.05 ng/µL (A) Zolpidem working standard solution (different working standard solution from the one used to prepare the calibrators) into tubes labeled **PC_a**, **PC_b**, and **PC_c**. See Table 1.
- D. Pipette 1 mL of negative control urine or diluted negative control urine into the tubes labeled **NC**, **PC_{a-c}**, **C₁**, **C₂**, **C₃**, **C₄**, **C₅**, and **C₆**.
- E. Pipette 1.0 mL of the test sample, in duplicate, into the tubes labeled **TS_{1a}** and **TS_{1b}** if the estimated concentration of zolpidem in the test sample is between 0.02 and 10 ng/mL. If the estimated concentration of zolpidem is greater than 10 ng/mL, prepare an appropriate dilution of an aliquot of the test sample with water and pipette duplicate 1.0-mL aliquots of the diluted sample into the tubes labeled **TS_{1a}** and **TS_{1b}**. Repeat this process for each test sample.
- F. Pipette 1.0 mL of water into each of the tubes labeled **SB_{1a}**..**SB_{nb}**.
- G. Vortex mix the contents of each tube for 5-10 seconds.

Table 1. Volumes of working standard solutions required to prepare calibrators, control samples and test samples.

TUBE NO.	Volume of Zolpidem Working Standard Solution*, µL	Volume of Azaperone-IS Working Standard Solution, µL	Equivalent to Zolpidem in the Urine, ng/mL	Equivalent to Azaperone-IS in the Urine, ng/mL
C ₁	4 of (B)	10	0.02	20
C ₂	20 of (B)	10	0.1	20
C ₃	50 of (B)	10	0.25	20
C ₄	20 of (A)	10	1.0	20
C ₅	50 of (A)	10	2.5	20
C ₆	200 of (A)	10	10	20
SYS ₁₋₂	0	0	na	na
NC	0	10	0	20
TS _{1a-1b}	0	10	unknown	20
SB _{1a-1b}	0	0	na	na
PC _{a-c}	50 of (A)	10	2.5	20
S ₁	50 of (A)	10	na	na

*(A) = 0.05 ng/µL; (B) = 0.005 ng/µL; na = not applicable

XII. ENZYME HYDROLYSIS OF CONJUGATES

**IDENTIFICATION AND DETERMINATION OF ZOLPIDEM AND METABOLITE FROM
EQUINE URINE SAMPLES – LC/MS METHOD**

- A. Pipette 0.4 mL of β -glucuronidase reagent into each tube.
- B. Vortex mix the contents of each tube for 5 -10 seconds.
- C. Adjust the contents of each tube to pH 4.5 - 5.5 with 1 *N* hydrochloric acid or 1 *N* sodium hydroxide solution.
- D. Place the tubes in a Bransonic Ultrasonic Cleaner and incubate at approximately 65°C for 2 hours with sonication for 99 minutes.
- E. Remove the tubes from the incubator and allow them to cool to room temperature.

XIII. SAMPLE PREPARATION FOR SOLID PHASE EXTRACTION

- A. Adjust the contents of each tube to pH 5.5 - 6.5, if necessary, with 1 *N* hydrochloric acid or 1 *N* sodium hydroxide sodium hydroxide solution.
- B. Centrifuge the tubes for 5 minutes at 3000 rpm to remove sediment, if necessary.

XIV. SOLID PHASE EXTRACTION PROCEDURE

- A. Wipe off the gasket with methanol soaked Kimwipe, then a dry Kimwipe.
- B. Place the solid phase columns on the manifold rack, and condition each solid phase column by applying a small amount of pressure (1-5 psi) and successively eluting to waste 3 mL of methanol, 3 mL of water and 2 mL of 0.1 *M* sodium phosphate buffer (pH 6). Stop the flow when a reagent reaches the top of the sorbent bed.
- C. Decant each solution into the corresponding column reservoir and adjust the flows so that the solutions flow through the columns in not less than 2 minutes.
- D. Rinse each column with 3 mL of water.
- E. Rinse each column with 2 mL of 1 *M* acetic acid solution.
- F. Rinse each column with 3 mL of methanol.
- G. Dry the columns under full vacuum for 2 minutes at 20 psi.

**IDENTIFICATION AND DETERMINATION OF ZOLPIDEM AND METABOLITE FROM
EQUINE URINE SAMPLES – LC/MS METHOD**

- H. Place labeled 12 x 75 mm tubes into position under the corresponding cartridges. Verify that the cartridge tips are positioned into the tubes. Elute to collect with 3 mL of the elution solvent (methylene chloride:propanol:ammonium hydroxide (78:20:2; v/v/v).
- I. Prepare the standard mixture by adding 50 µL of 0.05 ng/µL (A) Zolpidem working standard solution and 10.0 µL of Azaperone-IS working standard solution to a 12 x 75 mm tube labeled **S₁**.
- J. Evaporate the contents of each tube to dryness at 50 ± 5 °C under nitrogen.

XV. REDISSOLVING

- A. Prepare the system wash tubes by labeling two 12 × 75 mm test tubes **SYS₁** and **SYS₂**.
- B. Add 120 µL of Sample Re-dissolving Solvent (10% Acetonitrile in water with 0.2% formic acid) to each tube.
- C. Cap and vortex mix the contents of each tube for 30 seconds.
- D. Transfer to pre-labeled autosampler vials with inserts and submit for LC/MS analysis.

**XVI. LIQUID CHROMATOGRAPHIC/MASS SPECTRAL IDENTIFICATION OF
ZOLPIDEM AND CARBOXY-ZOLPIDEM**

- A. Liquid Chromatographic and Mass Spectrometer Operating Parameters
 - 1. Instrumentation:
Finnigan TSQ Quantum Triple Quadrupole Mass Spectrometer and Agilent Technologies Model 1100 HPLC pump, autosampler, column compartment, and degasser. Xcalibur™ software (ThermoQuest Inc., Santa Clara, CA) used for system control and data processing on a Microsoft Windows 2000 platform.
 - 2. LC column:
 - a) type: ACE C18 or equivalent
 - b) length: 100 mm
 - c) i.d.: 2 mm
 - d) particle size: 3 µm
 - e) temperature: 30 °C

**IDENTIFICATION AND DETERMINATION OF ZOLPIDEM AND METABOLITE FROM
EQUINE URINE SAMPLES – LC/MS METHOD**

3. Mobile Phase

Time (min)	% ACN + 0.2% Formic Acid	% Water + 0.2% Formic Acid
0.0	10	90
0.5	10	90
6	90	10
7	90	10
7.01	10	90
13	10	90

- a) Solvents: HPLC Grade, Burdick & Jackson
- b) flow rate: 0.40 mL/min

4. Injection volume: 40 μ L

5. Ionization and Detection Using TSQ Quantum:

Positive Ion Electrospray with Selected-Reaction Monitoring LC/MS/MS
Detection of the pseudomolecular ions of Zolpidem, Carboxy-Zolpidem
and Azaperone-IS

- a) Source CID Coll. energy: 15
- b) Scan Width: 0.7 dalton
- c) Peak Width: 0.7 dalton (Q1 and Q3)
- d) Q2 Argon Gas Pressure: 1.5 mTorr
- e) Segment 1:
 - (1) Azaperone-IS MS/MS Transitions followed: 328.4 amu
(collision energy 20V, scan time 0.25 sec) \rightarrow 121.4 amu;
(collision energy 16V, scan time 0.25 sec) \rightarrow 165.4 amu;
 - (2) Zolpidem MS/MS Transitions followed: 308.2 amu
(collision energy 42V, scan time 0.25 sec) \rightarrow 221.2 amu;
(collision energy 38V, scan time 0.25 sec) \rightarrow 235.2 amu;
(collision energy 28V, scan time 0.25 sec) \rightarrow 263.2 amu
 - (3) Carboxy-Zolpidem MS/MS Transitions followed: 338.2 amu
(collision energy 38V, scan time 0.25 sec) \rightarrow 221.2 amu;
(collision energy 38V, scan time 0.25 sec) \rightarrow 265.2 amu;
(collision energy 38V, scan time 0.25 sec) \rightarrow 293.2 amu;

**IDENTIFICATION AND DETERMINATION OF ZOLPIDEM AND METABOLITE FROM
EQUINE URINE SAMPLES – LC/MS METHOD**

6. Program:
Name: Zolpidem_Confirm
a) Initial divert time: ca. 3 minutes or as appropriate for the retention times of the compounds of interest
7. Perform analyses in the order and with the method specified in Table 2:

Table 2. Run number, vial number, acquisition method, and sample designation for LC / MS analysis for identification and determination of zolpidem and carboxy-zolpidem from horse urine.

Run #	Vial	Method	Sample
1-6	1-6	Zolpidem_Confirm	C₁, C₂, C₃, C₄, C₅, C₆
7-8	7-8	Zolpidem_Confirm	SYS₁, SYS₂
9	9	Zolpidem_Confirm	NC
10	10	Zolpidem_Confirm	TS_{1a}
13	13	Zolpidem_Confirm	SB_{1a}
14	14	Zolpidem_Confirm	TS_{1b}
15	15	Zolpidem_Confirm	SB_{1b}
16-18	16-18	Zolpidem_Confirm	PC_a, PC_b, and PC_c
19	19	Zolpidem_Confirm	S₁

* Analyze additional test sample extracts and solvent blanks by duplicating the specified sequence indicated in runs 10-15.

- B. Evaluation of Mass Spectral Data For Zolpidem and Carboxy-Zolpidem
- For each test sample, calibrator, and positive control sample, obtain the total ion chromatogram (TIC), and the integrated ion areas for qualifying and quantifying ions listed in Table 3.
 - Calculate the relative ion area ratio for zolpidem and/or carboxy-zolpidem by dividing the qualifying ion area by the ion area of the most abundant qualifying ion as indicated in Table 3 for each replicate of the test sample and the standard.

IDENTIFICATION AND DETERMINATION OF ZOLPIDEM AND METABOLITE FROM EQUINE URINE SAMPLES – LC/MS METHOD

Table 3. Qualifying and quantifying MS/MS product ions for analysis of zolpidem and carboxy-zolpidem in extracts of horse urine using TSQ Quantum; the most abundant qualifying ions are indicated in bold type and the least abundant qualifying ions are underlined.

Analyte	Qualifying Ions (amu)	Quantifying Ions (amu)
Zolpidem (308.2 amu parent mass)	<u>221.2</u> , 235.2 , 263.2	<u>221.2</u> + 235.2 + 263.2
Carboxy-zolpidem (338.2 amu parent mass)	221.2, 265.2 , <u>293.2</u>	221.2 + 265.2 + <u>293.2</u>
Azaperone-IS (328.4 amu parent mass)	121.4 , <u>165.4</u>	121.4 + <u>165.4</u>

3. Calculate the peak area ratio (PAR) for zolpidem by dividing quantifying ions at the retention time of zolpidem by quantifying ions at the retention time of the azaperone-IS peak for each calibrator, test sample, and control sample.
 4. Calculate the peak area ratio (PAR) for carboxy-zolpidem by dividing quantifying ions at the retention time of carboxy-zolpidem by quantifying ions at the retention time of the azaperone-IS peak for each calibrator, test sample, and control sample.
 5. Measure the signal-to-noise ratio for both replicates of each test sample for the least abundant qualifier ion at the retention time of zolpidem and carboxy-zolpidem.
- C. Criteria for Identification of Zolpidem and Carboxy-Zolpidem from Urine Extracts
1. The retention times of the qualifier ions (Table 3) for each replicate of the test sample must be within ± 0.10 minutes of the retention time of the same ions from the zolpidem standard. For carboxy-zolpidem, the retention times of the qualifier ions (Table 3) for each replicate of the test sample must be within ± 0.10 minutes of the retention time of the same ions from the carboxy-zolpidem peak of an administration positive control sample.
 2. For zolpidem, the ion area ratio of the qualifier ions (Table 3) from each replicate of the test sample must be within $\pm 25\%$ compared to the values of the same ions from the zolpidem standard. For carboxy-zolpidem, the ion area ratio of the qualifier ions (Table 3) from each replicate of the test sample must be within $\pm 25\%$ compared to the values of the same ions from the carboxy-zolpidem peak of an administration positive control sample.

IDENTIFICATION AND DETERMINATION OF ZOLPIDEM AND METABOLITE FROM
EQUINE URINE SAMPLES – LC/MS METHOD

3. The chromatographic peak shape must be approximately Gaussian, with a narrow base, with baseline separation from neighboring peaks, and with little evidence of tailing. The following criteria will define an acceptable peak:
 - a) The width of the peak at its base should be less than 0.60 minutes.
 - b) The peak should appear to be Gaussian, *i.e.*, symmetrical about the vertical mid-line.
 - c) There should be no interfering peaks. A neighboring peak is considered to be interfering if the height from the baseline to the lowest part of the valley between the peaks is greater than 10% of the height of the peak of interest.
 - d) There is no significant peak tailing. Unacceptable peak tailing is defined as the condition in which the ratio of *b* to *a* is greater than 1.5 at 15% of the peak height where *a* is the distance from the leading edge to the apex of the peak and *b* is the distance from the apex to the tailing edge.

- D. Determination of the Concentration of Zolpidem and/or Carboxy-Zolpidem (for estimation only) in Horse Urine
 1. Plot PAR for each calibrator versus the concentration of zolpidem in the calibrator. Perform linear regression analysis on these data to obtain the slope, intercept, and correlation coefficient of the standard curve.
 2. Calculate the concentration of zolpidem and/or carboxy-zolpidem (carboxy-zolpidem concentration IS estimated from the zolpidem calibration curve) in each test sample or diluted test sample and positive control sample from the PAR and the slope and intercept of the standard curve.
 3. Calculate the concentration of zolpidem and/or carboxy-zolpidem in each diluted test sample from the calculated concentration and the dilution factor used:
Concentration = Calculated concentration / dilution factor
 4. Determine the mean concentration for each test sample and positive control sample.
Mean concentration = $\frac{1}{2}$ (concentration **TS_{1a}** + concentration **TS_{1b}**)

**IDENTIFICATION AND DETERMINATION OF ZOLPIDEM AND METABOLITE FROM
EQUINE URINE SAMPLES – LC/MS METHOD**

XVII. CRITERIA FOR REPEATING THE ANALYSIS

Repeat the analysis of the test sample if any of the following conditions apply:

- A. The peak area ratio of any test sample replicate is greater than the peak area ratio of calibrator **C₆**. Repeat the analysis after diluting the urine sample with water as described in Section XI.E of this Standard Operating Procedure.
- B. The negative control sample or the solvent blanks contain zolpidem and/or carboxy-zolpidem peaks as evidenced by the presence of the characteristic ions and ion ratios within the expected retention time window.
- C. The standard curve for zolpidem has a correlation coefficient less than 0.98.
- D. The azaperone-IS ions are not detectable within the expected retention time window for any of the sample replicates.
- E. The average concentration of the positive control sample replicates is greater than 20% different from the nominal concentration.

XVIII. CRITERIA FOR REPORTING A POSITIVE SAMPLE

Report a test sample as positive when all of the following criteria are met:

- A. The test sample contains the analyte according to the criteria described in XVI.C.
- B. The average concentration of zolpidem and/or carboxy-zolpidem (estimated from zolpidem standard curve) in the sample is greater than the lowest calibrator.
- C. The signal-to-noise ratio of the least abundant qualifying ion of zolpidem and/or carboxy-zolpidem in each replicate of the test sample is greater than 3.
- D. The negative control sample and solvent blanks do not contain zolpidem and/or carboxy-zolpidem.
- E. The standard curve has a correlation coefficient greater than 0.98.
- F. The mean zolpidem concentration in the positive control samples are within 20% of the nominal concentration.

XIX. REFERENCES

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**IDENTIFICATION AND DETERMINATION OF ZOLPIDEM AND METABOLITE FROM
EQUINE URINE SAMPLES – LC/MS METHOD**

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XX. RESPONSIBLE PERSONS

- A. Confirmation section

XXI. FITNESS FOR USE

This Standard Operating Procedure for identification and determination of zolpidem and/or carboxy-zolpidem from horse urine samples by LC/MS was validated using in-house criteria for method evaluation and is fit for the intended use.

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