GUANABENZ

DETECTION AND CONFIRMATION IN PERFORMANCE ANIMALS BY LIQUID CHROMATOGRAPHY

A Method Developed
By

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For

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1.0 PURPOSE

1.1 This Standard Operating Procedure (SOP) describes the general guidelines for the detection and confirmation of guanabenz in performance animals by liquid chromatography.

2.0 APPARATUS

2.1 Test tubes, 15 ml, with screw cap and Teflon liner
2.2 Hamilton syringes, assorted sizes
2.3 Fixed volume pipettes, 1 to 5 ml, Eppendorf or equivalent
2.4 Class A volumetric pipettes and flasks, assorted sizes
2.5 Disposal glass pipettes
2.6 Centrifuge
2.7 Vortex mixer
2.8 Vacuum Manifold
2.9 Tube Rocker/Mixer or equivalent
2.10 Solid Phase Extraction (SPE) Columns (Varian Bond Elut Certify, 1 gram, Varian Part No. 1210-2085). Any mix-mode SPE with a non-polar C8 sorbent and a strong cation exchanger (SCX) will work (will retain basic compounds while non-polar, polar and anionic matrix contaminants are washed away).

3.0 REAGENTS

3.1 Water (Deionized or HPLC-grade)
3.2 Acetonitrile (HPLC-grade)
3.3 Phosphoric Acid, 85% (HPLC-grade)
3.4 Ethyl Acetate (HPLC-grade)
3.5 Acetic Acid, glacial (ACS Reagent grade)
3.6 Ammonium Hydroxide (ACS Reagent grade)
3.7 Formic Acid (HPLC-grade)

4.0 SOLUTIONS

4.1 0.1M Phosphate Buffer, pH 6

Weigh 0.17 g of Na_2HPO_4 and 1.21 g of NaH_2PO_4 into a 100-ml volumetric flask. Dissolve in approximately 80 ml of DIUF water. Bring to volume with water. If required, adjust the pH to 6.0 (+/- 0.1) with saturated monobasic sodium phosphate (lowers pH) or saturated dibasic sodium phosphate (raises pH). Stable for 1 month at refrigeration.

4.2 1.0M Sodium Hydroxide

Weigh 4.0 grams of sodium hydroxide into a 100-ml volumetric flask. Dissolve the sodium hydroxide in DIUF water and bring to volume. Stable for 3 months at room temperature.

4.3 1.0M Acetic Acid

Pipette 5.75 ml of glacial acetic acid into a 100-ml volumetric flask approximately half filled with DIUF water. Mix, bring to volume with DIUF water. Stable for 3 months at room temperature.

4.4 5% (v/v) Ammonium Hydroxide in Ethyl Acetate

Pipette 5 ml of concentrated ammonium hydroxide into a 100-ml volumetric flask. Bring to volume with ethyl acetate (HPLC-grade) and mix well. Prepare fresh daily.

4.5 0.1% (v/v) Phosphoric Acid in Water

Pipette 1 ml of 85% phosphoric acid into a 1-Liter volumetric flask and bring to volume with water (HPLC-grade). Stable at room temperature for 1 month.

4.6 0.1% (v/v) Phosphoric Acid in Acetonitrile

Pipette 1 ml of 85% phosphoric acid into a 1-Liter volumetric flask and bring to volume with acetonitrile (HPLC-grade). Stable at room temperature for 1 month.

4.7 0.1% (v/v) Formic Acid in Acetonitrile: Water 1:1 (v:v)

Pipette 0.1 ml of formic acid into a 100-ml volumetric flask and bring to volume with acetonitrile:water 1:1, v:v (HPLC-grade). Stable at room temperature for 1 month.

4.8 0.1% (v/v) Formic Acid in Water

Pipette 1 ml of formic acid into a 1-Liter volumetric flask and bring to volume with water (HPLC-grade). Stable at room temperature for 1 month.

4.9 0.1% (v/v) Formic Acid in Acetonitrile

Pipette 1 ml of formic acid into a 1-Liter volumetric flask and bring to volume with acetonitrile (HPLC-grade). Stable at room temperature for 1 month.

5.0 SAMPLE EXTRACTION PROCEDURE

5.1 Enzyme hydrolyze urine samples (5 ml) per SOP.

5.2 Adjust the pH of the cooled sample to 9.5 (+/- 0.5) with NaOH (4.2) and extract with ethyl acetate (5 ml). Add the ethyl acetate, put the sample on a tube mixer or equivalent for 10 minutes and centrifuge to separate phases. Decant the ethyl acetate fraction and concentrate to dryness under a gentle stream of nitrogen. Redissolve in 0.4 ml acetonitrile and vortex tube. Dilute further in 5 ml 0.1M phosphate buffer, pH 6 (4.1).

5.3 Prepare a SCX/C8 mix mode SPE column (Varian Bond Elut Certify or equivalent) by washing with 2 ml MeOH followed by 2 ml of 0.1M phosphate buffer, pH 6 (4.1)(Note: turn off vacuum as soon as the water reaches the top of the sorbent bed to prevent column drying!!!).

5.4 Pour sample onto the column and draw it through slowly (Note: at a rate of ~1ml/min). Rinse column with 2 ml 0.1M phosphate buffer, pH 6 (4.1) and 2 ml of 1.0M acetic acid (4.3).

5.5 Dry column under full vacuum for at least 5 minutes.

5.6 Rinse column with 5 ml chloroform:acetone (3:1, v:v).

5.7 Rinse column with 2 ml methanol.

5.8 Dry column under full vacuum for at least 2 minutes.

5.9 Elute guanabenz and its metabolites with 5 ml ethyl acetate containing 5% concentrated ammonium hydroxide (4.4). Collect eluent in a 15-ml test tube and concentrate to dryness under a gentle stream of nitrogen. Redissolve in 0.4 ml solvent (4.7), sonicate and vortex well. Split the sample in half by putting 200 µl in an insert for HPLC/DAD analysis and the other half in an insert for LC/MS analysis.

6.0 ANALYTICAL PROCEDURES

6.1 HPLC/DAD Screening Parameters:

Column:	Reverse-phase LC18, 5 µm particle size, 4.6 mm x 25 cm
Flow Rate:	1.0 ml/min
Injection volume:	25 µl
UV detection:	DAD 190-600 nm

Mobile Phase: A = 0.1% phosphoric acid in water (4.5)
B = 0.1% phosphoric acid in acetonitrile (4.6)

Gradient:

Time (min)	%A	%B
0	90	10
15	0	100
17	0	100
17.1	90	10
23	90	10

Retention Time: 10.1 minutes (Guanabenz).

6.2 LC/MS Confirmation Parameters:

Column: Altima C-18, 2.1 mm x 25 cm, 5 µm particle size
Flow Rate: 0.25 ml/min
Injection volume: 25 µl
UV detection: 220 nm
Mass Spec.: Electrospray, positive mode
SIM: Ions 231, 233, 235

Mobile Phase: A = 0.1% formic acid in water (4.8)
B = 0.1% formic acid in acetonitrile (4.9)

Gradient:

Time (min)	%A	%B
0	90	10
15	0	100
25	0	100
30	90	10
40	90	10

Retention Time: 10.9 minutes (Guanabenz).

```
Injection Date  : 09/26/2000 7:28:56 PM          Seq. Line :  10
Sample Name     : Pos. CTRL #3                       Vial :   8
Acq. Operator   : Sherri                             Inj :   1
Acq. Instrument : 1100 DAD                     Inj Volume : 0 µl
Different Inj Volume from Sequence !     Actual Inj Volume : 25 µl
Acq. Method     : C:\HPCHEM\1\METHODS\EQUINE2.M
Last changed    : 09/13/2000 2:43:52 PM by Sherri
Analysis Method : F:\HPCHEM\1\METHODS\EQUINE2.M
Last changed    : 10/25/2000 3:17:41 PM by Sherri
HPLC Screening Method
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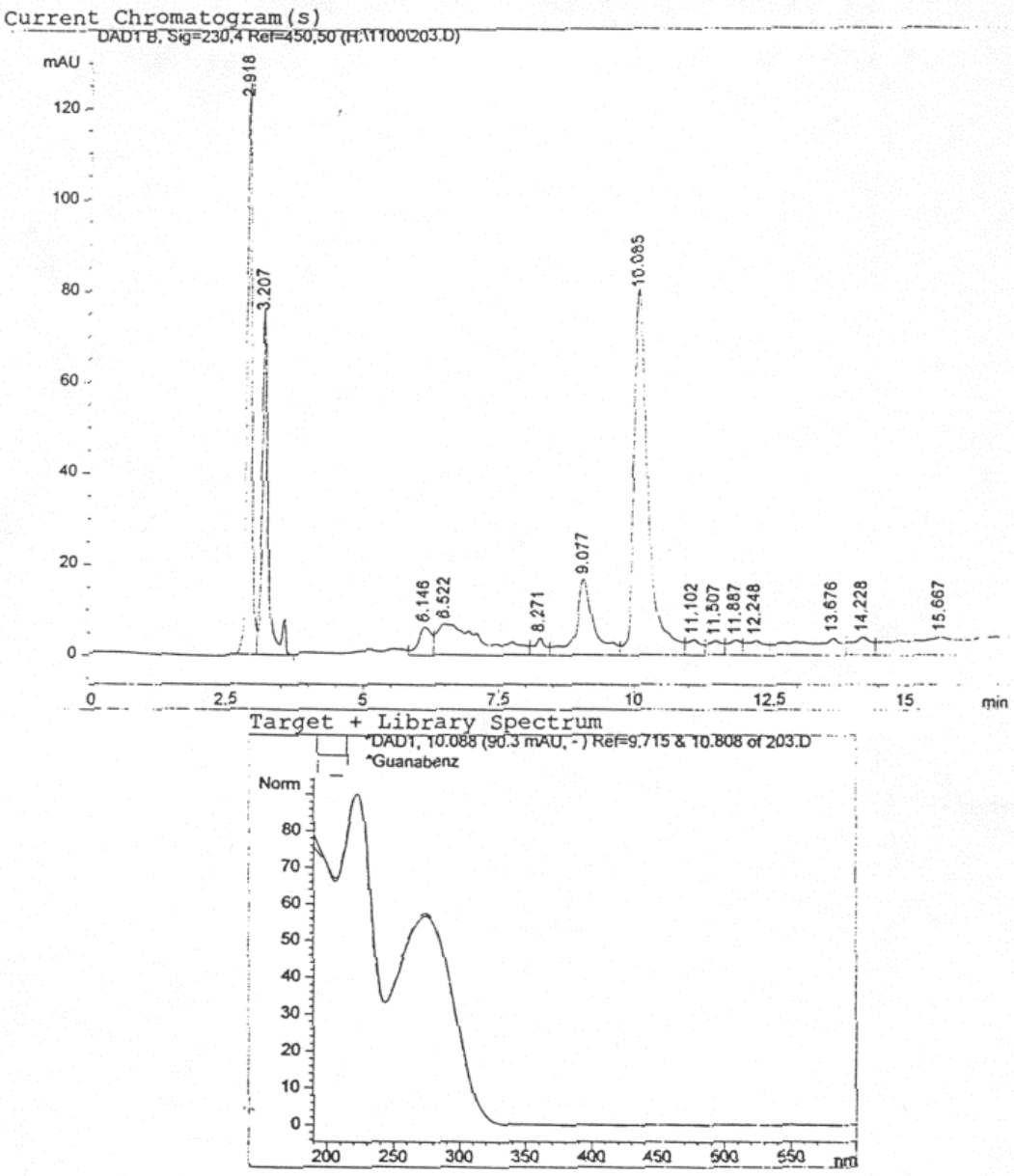
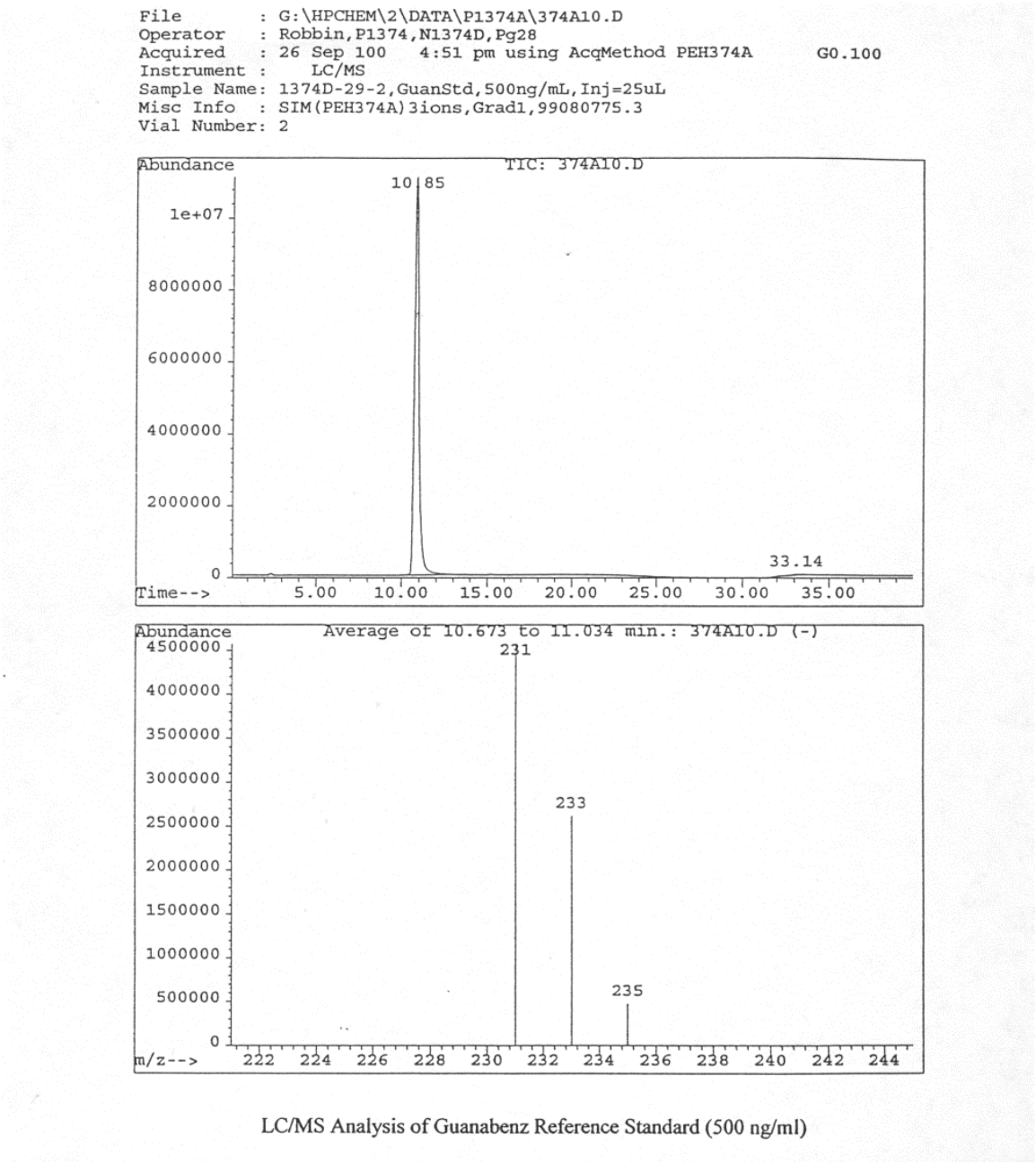


HPLC/DAD Analysis of Guanbenz Positive Control in Urine (500 ng/ml)



LC/MS Analysis of Guanabenz Reference Standard (500 ng/ml)

LC/MS Analysis of Guanabenz Reference Standard (500 ng/ml)

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Operator    : Robbin,P1374,N1374D,Pg28
Acquired    : 26 Sep 100  10:26 pm using AcqMethod PEH374A      G0.100
Instrument :    LC/MS
Sample Name: 1374D-30-2,PosContUrine50ng/mL,Inj=25uL
Misc Info   : SIM(PEH374A)3ions,Grad1,99080775.3
Vial Number: 7
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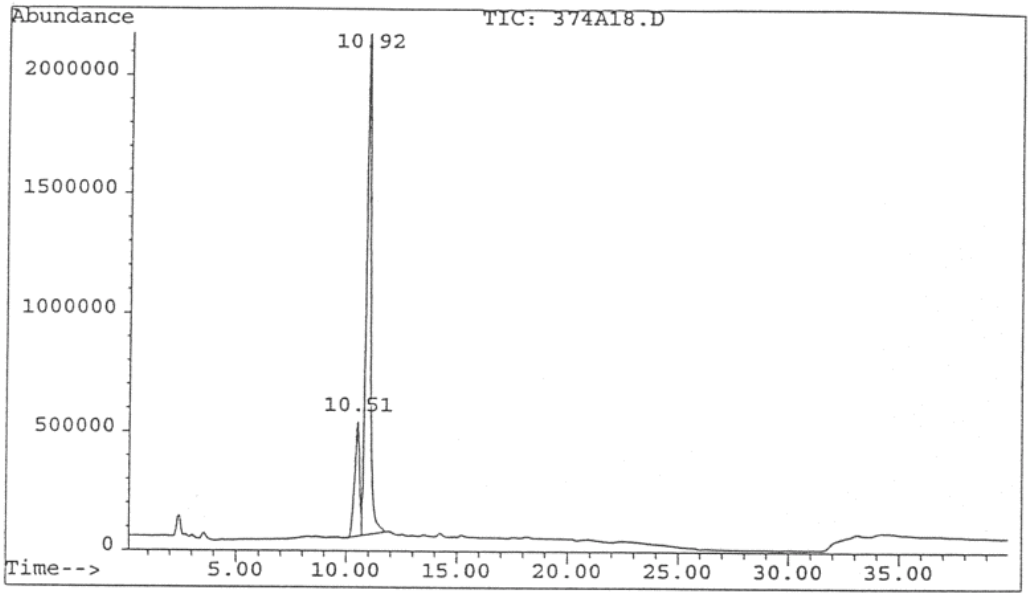

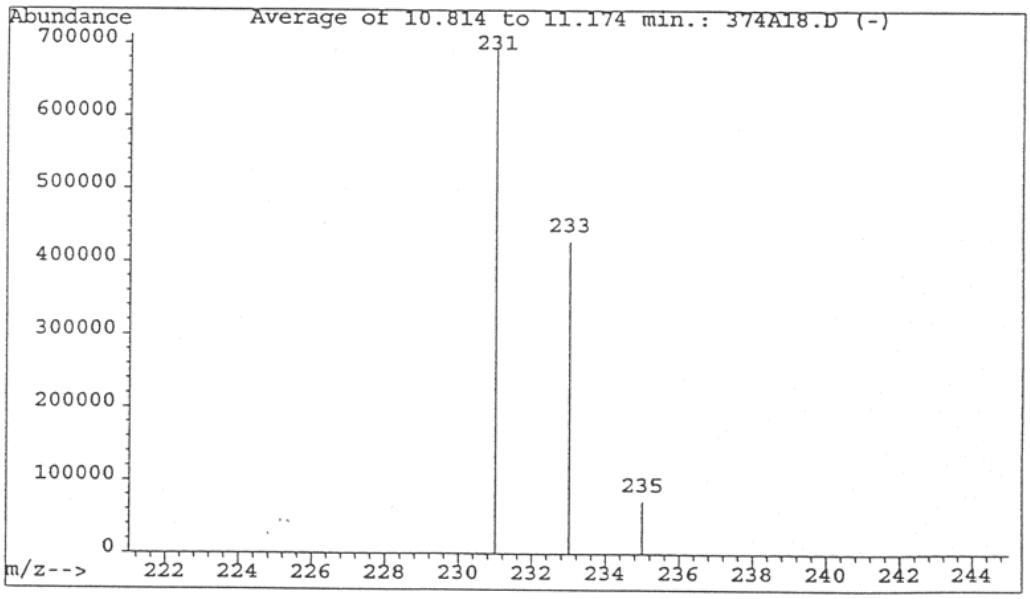

LC/MS Analysis of Guanbenz Positive Control in Urine (50 ng/ml)

LC/MS Analysis of Guanbenz Positive Control in Urine (50 ng/ml)

```
File        : G:\HPCHEM\2\DATA\P1374A\374A15.D
Operator    : Robbin,P1374,N1374D,Pg28
Acquired    : 26 Sep 100   8:21 pm using AcqMethod PEH374A      G0.100
Instrument :    LC/MS
Sample Name: 1374D-30-7,GuanAdmin(2HR),Inj=25uL
Misc Info   : SIM(PEH374A)3ions,Grad1,99080775.3
Vial Number: 5
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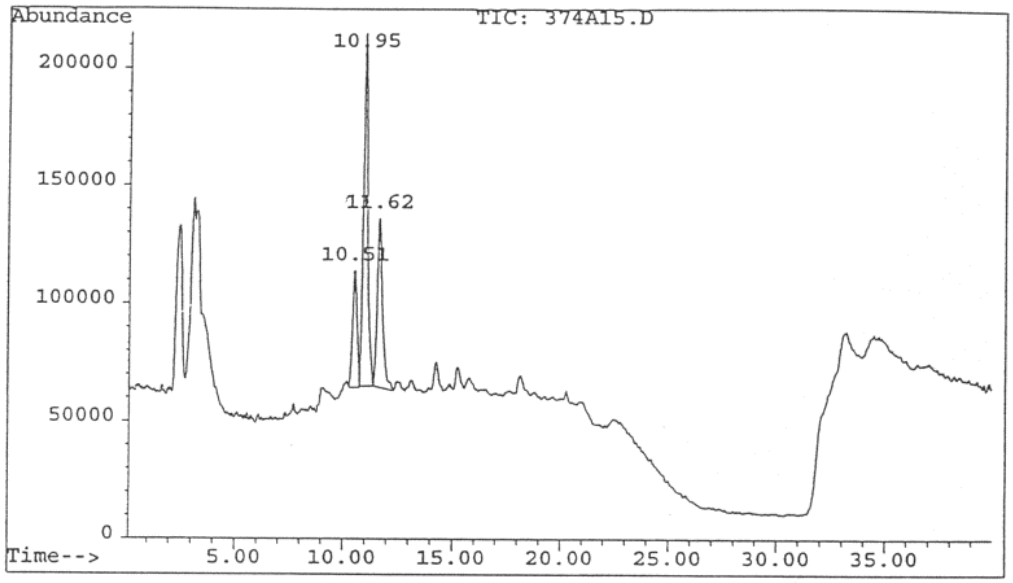

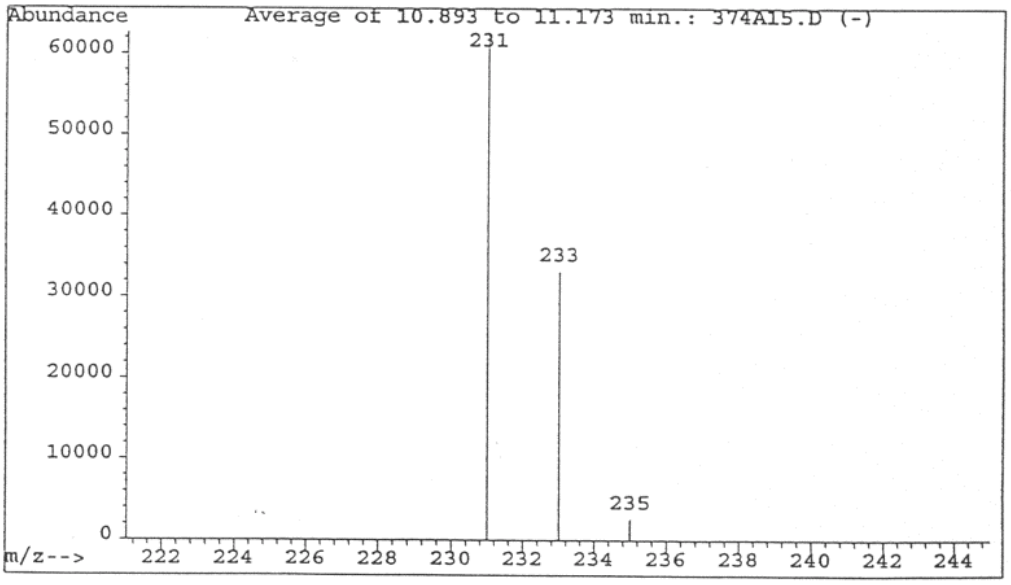

LC/MS Analysis of Guanabenz 2-Hour Administration

LC/MS Analysis of Guanabenz 2-Hour Administration