

---

## Liquid-Chromatography Mass Spectrometric Method For Screening and Confirmation of Sildenafil and metabolites in Equine Urine and Plasma

Pennsylvania Equine Toxicology and Research Laboratory  
Jeffrey Rudy, Mark Kahler

### Introduction

Sildenafil (Viagra®, 1-[4-ethoxy-3- (6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo [4,3-d] pyrimidine-5-yl) phenylsulfonyl-4-methyl piperazine) is a selective inhibitor of phosphodiesterase type 5 (PDE5) in the human *corpus cavernosum*. Sildenafil is effective in the treatment of impotence and male erectile dysfunction in humans. Most equine athletes suffer from exercise induced pulmonary hemorrhage (EIPH), and are treated with the loop diuretic, furosemide (Lasix®) prior to racing in most North American racing jurisdictions. Since the effectiveness of furosemide in managing EIPH is widely debated, alternative treatments for EIPH are constantly being sought. Active research on PDE5 inhibitors suggests that pulmonary pressure can be reduced by the site-specific relaxant properties of PDE5 inhibitors, thus eliminating one of the potential causes of EIPH. Without the availability of a lung-specific PDE5 inhibitor (such as E-4021; a “Viagra-like” investigational drug), attempts to use Sildenafil for the management of EIPH in racehorses are growing.

### Scope

This procedure describes the extraction of Sildenafil and metabolites from equine plasma or urine using liquid-liquid extraction at basic pH. Enzyme hydrolysis may be used to increase the yield of certain urinary hydroxylated metabolites, but it is not essential due to the aliphatic nature of the major hydroxylated metabolites that are detected by this method. Screening of urine samples is performed instrumentally by Liquid Chromatography-Mass Spectrometry product ion scanning (LC/MS/MS) using positive ion electrospray in batches (up to 90 samples per batch). Plasma and urine of presumptive findings are freshly prepared along with positive and negative Sildenafil administration controls (for urines) and calibrators (for plasma). The instrumental conditions contained within this Standard Operating Procedure are specific to the MicroMass Q-ToF (Series 1).

### Limitations

The mode and duration of action of Sildenafil in humans suggest rapid and extensive metabolism. This metabolic process was also noted in the equine. As a result of the unavailability of traceable reference standards of the proposed metabolites, they cannot be quantitatively determined, even though they form the basis of both the screening and confirmation methods presented in this SOP. While Sildenafil and desmethyl Sildenafil are both available as traceable reference standards (Pfizer), they are not readily detected in equine urine due to extensive metabolism. While confirmation and quantitation of these analytes are possible in equine plasma, rapid and extensive conversion to inactive metabolites results in low plasma concentrations of Sildenafil. Therefore, failure to confirm Sildenafil in plasma does not preclude the possibility of significant dosing of horses with Sildenafil within twelve hours prior to racing. Positive findings should be reported if the data are compared to those of equine urine samples obtained from administrations of traceable reference standard Sildenafil.

### Precautions

The hydroxylated urinary metabolites of Sildenafil are susceptible to dehydration (loss of water) in the electrospray and entrance regions of the instrument. Liquid chromatograph-mass spectrometer

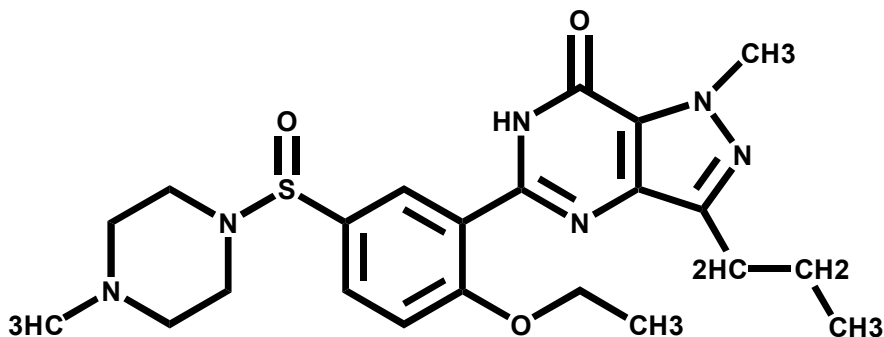
systems of differing manufacturer and configuration may require differing or additional settings to minimize or eliminate this effect.

## Philosophy

The Pennsylvania Equine Toxicology and Research Laboratory is a service provider, mandated under Pennsylvania Statute to provide service to: the Racing Commissions of Pennsylvania, the Pennsylvania Department of Agriculture, the taxpayers and racing patrons of the Commonwealth of Pennsylvania, and the racing industry at large. The nature of this service is often incorrectly perceived to be regulatory and enforcement. However, the laboratory neither makes the rules and regulations, nor enforces them. The laboratory exists primarily as an informational service provider. Within this context, the laboratory (and its associated research component) is sometimes called upon to investigate information relayed from any of the constituents that it serves. It is therefore, sometimes necessary to provide “as-is” current state-of-the-art information, regardless of deviations from otherwise standard procedures. Guided by sound scientific procedure and good laboratory practices, useful information is generated within the laboratory and presented as guidance to the bodies charged with the “regulation and enforcement” responsibility of the sport of racing in Pennsylvania. It is the responsibility of the information provider (PETRL) to be fair, impartial, and diligent with regard to this information it provides and its interpretation.

**Sildenafil**     $C_{22}H_{30}N_6O_4S$     **MW 474**

**Manufacturer - Pfizer (Viagra<sup>®</sup>)**



**Sildenafil Citrate**    **CAS # 121599-83-0**  
**Sildenafil**            **CAS # 139755-83-2**

**Figure 1. Sildenafil**

**Primary Reference Standards and Stock Standard Solutions**

Sildenafil and Desmethyl Sildenafil were acquired from Pfizer as certified analytical reference materials. Obtain these materials from the QAO. Record accession of these materials on the pharmacy log sheet.

Primary reference stock solutions (1 mg/mL ) are prepared by weighing between 5 and 10 mg primary reference stock powder (X.xx mg) and diluting to volume (Y.yy mL) with HPLC grade methanol to produce 1 mg/mL primary stock solutions (where X.xx=Y.yy). 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# 591; SR# 592) and Primary Reference Powder Designation (i.e. R-Sild-1; R-DesSild-1).

**Secondary Stock (Working) Solutions**

Prepare Secondary Stock Standard solutions from the primary reference stock solutions described above, according to the following table:

This preparation table will be entered into the respective laboratory secondary stock, standards, and calibrators' preparation logbook.

TABLE 1. Secondary Stock Preparation Table

<i>Reference</i>	<i>LABEL MMDDYY <u>SILD</u> (ug/mL)</i>	<i>Volume methanol mL</i>	<i>Volume Primary Reference Stock uL</i>	<i>Volume Secondary Stock (A) uL</i>
A	122001 <u>Sild</u> 10	9.9	100	*
B	122001 <u>Sild</u> 1	9	*	1000
<i>Reference</i>	<i>LABEL MMDDYY <u>DesSILD</u> (ug/mL)</i>	<i>Volume methanol mL</i>	<i>Volume Primary Reference Stock uL</i>	<i>Volume Secondary Stock (A) uL</i>
C	122001 <u>DesSild</u> 10	9.9	100	*
D	122001 <u>DesSild</u> 1	9	*	1000
<i>Reference</i>	<i>LABEL MMDDYY <u>SildCT</u> (ng/mL)</i>	<i>Volume acetonitrile mL</i>	<i>Volume Primary Reference Stock uL</i>	<i>Volume Secondary Stock (A,C) uL</i>
ColTest*	122001 <u>SildCT</u> 100	9.8	*	100,100

\* ColTest = Column Test solution

## **Calibrators**

The following calibrators are prepared in negative pooled equine plasma, demonstrated by this SOP to be negative for all 5 analytes described in the following sections (Sildenafil, Desmethyl Sildenafil, {a} Hydroxy Sildenafil, {p} Hydroxy Sildenafil, and {a} Hydroxy Desmethyl Sildenafil). Using negative control pooled plasma, 20 mL aliquots are prepared according to the following table:

**Table 2. Calibrator Preparation Table**

<i>ng/mL</i>	<i>LABEL</i> <i>MMDDYY <u>SDMS</u> (ng/mL)</i>	<i>ng</i> <i>Needed</i>	<i>uL B &amp; D</i> <i>Secondary</i> <i>Stocks</i>	<i>mL Negative</i> <i>Control</i> <i>Plasma</i>
0	122001 <u>SDMS</u> 0	0	0/0	20
1	122001 <u>SDMS</u> 1	20	20/20	19.96
5	122001 <u>SDMS</u> 5	100	100/100	19.8
10	122001 <u>SDMS</u> 10	200	200/200	19.6
25	122001 <u>SDMS</u> 25	500	500/500	19
50	122001 <u>SDMS</u> 50	1000	1000/1000	18
75	122001 <u>SDMS</u> 75	1500	1500/1500	17
100	122001 <u>SDMS</u> 100	2000	2000/2000	16
250	122001 <u>SDMS</u> 250	5000	500/500 (A&C)	19

- A. Batch preparation for a total of 20 mL per item  
LABEL (format MMDDYYSDMSNG/ML)  
Print using AVERY Template 5267 in MS Word) 9 of each
- B. Label 9 culture tubes (16x125 mm) according to the LABEL column of Table 2 and prepare 20 mL of each calibrator as described in Table 2.
- C. Label 81 screw cap culture tubes (16x125 mm) in 9 x 9 format, and aliquot 2 mL of the appropriate calibrator from step B into each respective tube. Cap and store at -70 °C.
- D. The zero calibrator doubles as the negative control, and the 250-ng/mL calibrator is not used in calibration, but as the positive control.
- E. Negative and Positive Control Urine samples collected from dosed research horses are used for urine control comparison of detected urine metabolites in suspected race horse samples. The Sildenafil administration must be traceable to Sildenafil primary reference material characterized by this SOP.

### **Sildenafil Base Extraction Procedure (urine and plasma)**

Liquid-liquid extraction is utilized to isolate the analytes for LC/MS screening and confirmation

1. To 2 mL urine or plasma, add 2 mL ammonium hydroxide: water (1:1)
2. Add 5 mL dichloromethane: isopropanol (10:1) and rotorack for 10 minutes
3. Centrifuge @ 2000 rpm and aspirate the top aqueous layer to waste
4. Transfer the organic layer to a clean-dry culture tube (16x125 mm) and evaporate at 65°C (hot block)
5. Add 100 uL acetonitrile injection solvent to each test tube
6. Mix the contents of the tubes by vortexing to ensure the sides are rinsed, then centrifuge @ 2000 rpm to drain all liquid to the bottom of the tube
7. Transfer injection solvent in step 6 to labeled, liquid chromatograph (LC) auto sampler vials fitted with limited volume inserts. Cap vials and place in auto sampler tray for analysis.

### **Sildenafil Enzyme Hydrolysis Procedure (optional – for urine only)**

1. To 2 mL urine, add 3 mL 1 M sodium acetate (pH 5), 2 mL  $\beta$ -glucuronidase (*patella vulgata* - 5000 units/mL)
2. Cap and incubate at 65°C for 3 hours
3. Cool tube and adjust to pH 9.0 with ammonium hydroxide: water (1:1)
4. Extract with 5 mL dichloromethane: isopropanol (10:1)
5. Centrifuge and aspirate aqueous layer to waste
6. Extract organic layer remaining from step 5 with 3 mL 0.1 N sulfuric acid
7. Centrifuge and aspirate organic layer to waste.
8. Adjust aqueous layer remaining from step 7 to pH 9.0 with ammonium hydroxide: water (1:1)
9. Extract with 5 mL dichloromethane: isopropanol (10:1)
10. Centrifuge and aspirate aqueous layer to waste
11. Proceed from step 4 of [Sildenafil Base Extraction Procedure (urine and plasma)] above.

### **Liquid Chromatography Conditions**

Instrument: Hewlett Packard 1100 LC  
Column: Hewlett Packard LC-MS Zorbax SB-CN 5 micron 2.1x 50 mm  
(Part number 860975-905)  
Mobile Phase: 80:20 acetonitrile: 50 mM aqueous ammonium acetate (pH 4)  
Flow rate: 0.2 mL/min  
Run Time: 3 minutes  
Injection Volume: 30  $\mu$ L

### **ESI<sup>+</sup> MS/MS Analysis Conditions**

Instrument: MicroMass Q-ToF  
Precursor Ions: Urine (491.1, 477.1)  
Plasma (475.1, 461.1)  
Cone Voltage: 21  
Scan Time: 0.5 sec  
Inter-Scan Time: 0.05 sec  
Acquisition Time: 0-5 minutes  
Focus Voltage: 112  
Capillary Voltage: 3000  
Desolvation: 220 °C

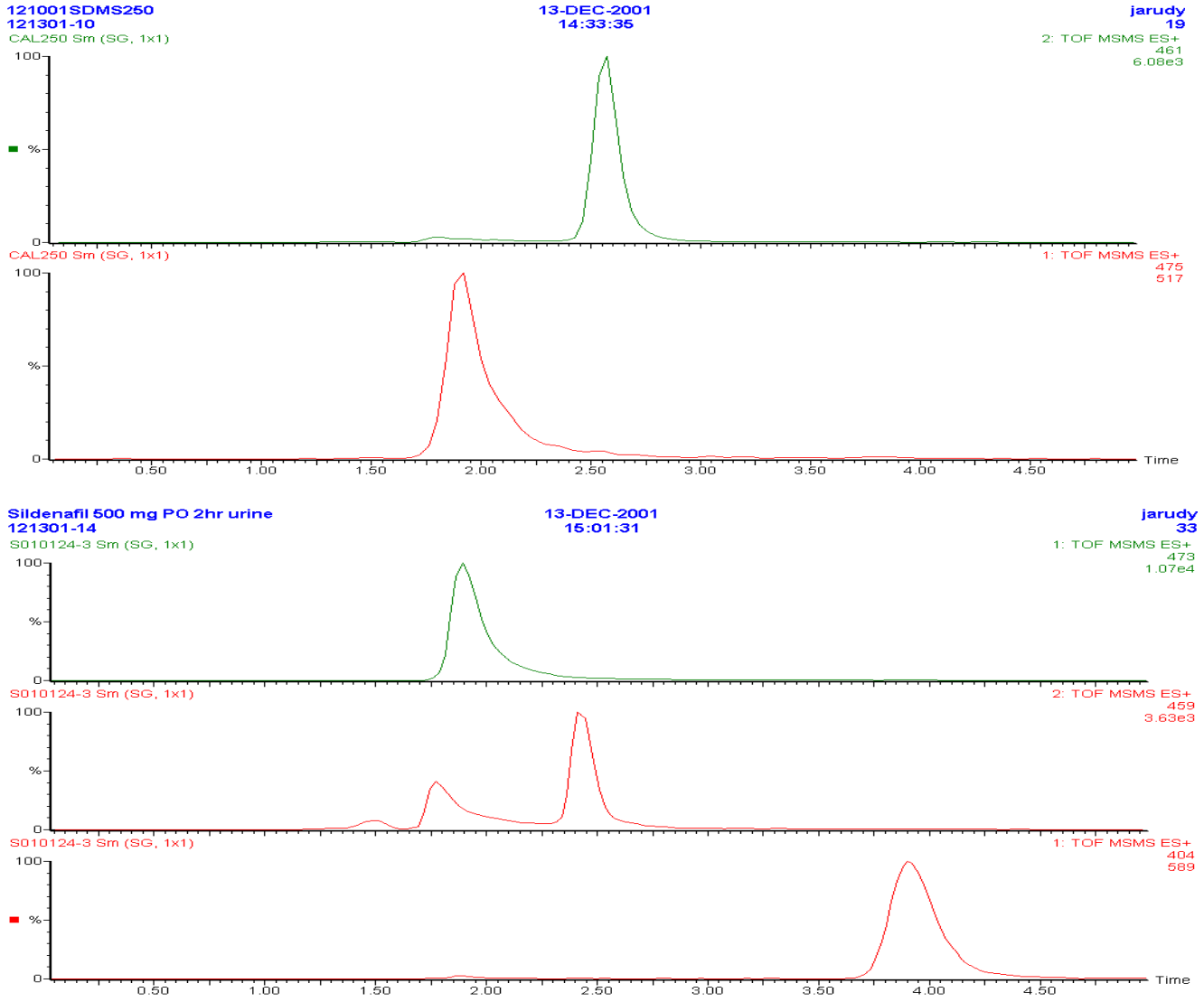


Figure 2. LCMSMS Extracted Ion Chromatograms for Plasma (Top) and Urine (Bottom) analytes

## LCMS Analysis Sequence

### Screening

Instrumental screening for Sildenafil and metabolites is performed on urine samples. The following injection sequence is followed for MSMS screening:

- A. Column Test mix
- B. Blank
- C. Negative Control Urine
- D. Blank
- E. Sample1,2,,,,-n
- F. Blank
- G. Positive Control Urine

The qualifying ions for extracted ion chromatographic data interrogation in urine are:

Desmethyl Hydroxy Sildenafil: 459.1 m/z  
Hydroxy Sildenafil: 477.1 m/z

Criteria for follow-up confirmation analysis:

1. The screening suspect sample must have qualifying extracted ion chromatographic retention times within +/- 20 % of the positive control.
2. The screening suspect sample MSMS spectrum must contain the following qualifying ions within +/- 20 % of the urine positive control:

<u>Desmethyl Hydroxy Sildenafil</u>	477.1, 459.1, 375.1, 309.1, 281.1
<u>Hydroxy Sildenafil</u>	491.1, 473.1, 309.1, 281.1

3. Ions not present in the positive control MSMS spectrum may be present in the suspect sample MSMS spectrum no greater than 20% (= S/N 5:1).

**Confirmation**

Suspect urine and plasma samples are freshly prepared, along with plasma calibrators and urine control samples, using the procedure described previously. The analysis order for MSMS screening is as follows:

- A. Column Test mix
- B. Blank
- C. Negative Control Urine
- D. Blank
- E. Sample 1
- F. Sample 2
- G. Blank
- H. Repeat E thru G for each additional sample
- I. Positive Control Urine
- J. Blank
- K. Plasma Calibrators ( 0 thru 100 ng/mL)
- L. Blank
- M. Sample Plasma1
- N. Sample Plasma2
- O. Blank
- P. Repeat M thru O for each additional sample
- Q. Plasma Control (250 ng/mL)

Urine Positive Criteria are as described above.

The qualifying ions for extracted ion chromatographic data interrogation in plasma are:

Desmethyl Sildenafil:	461.1 m/z
Sildenafil:	475.1 m/z

Criteria for plasma confirmation:

1. The suspect sample must have qualifying extracted ion chromatographic retention times within +/- 20 % of the positive control.
2. The suspect sample MSMS spectrum must contain the following qualifying ions within +/- 20 % of the plasma positive control:

<u>Desmethyl Sildenafil</u>	461.1, 377.1, 311.1, 283.1
<u>Sildenafil</u>	475.1, 377.1, 311.1, 283.1

3. Ions not present in the positive control MSMS spectrum may not be present in the suspect sample MSMS spectrum greater than 20% abundance.

### Spectra and Proposed Structures (matrix = urine)

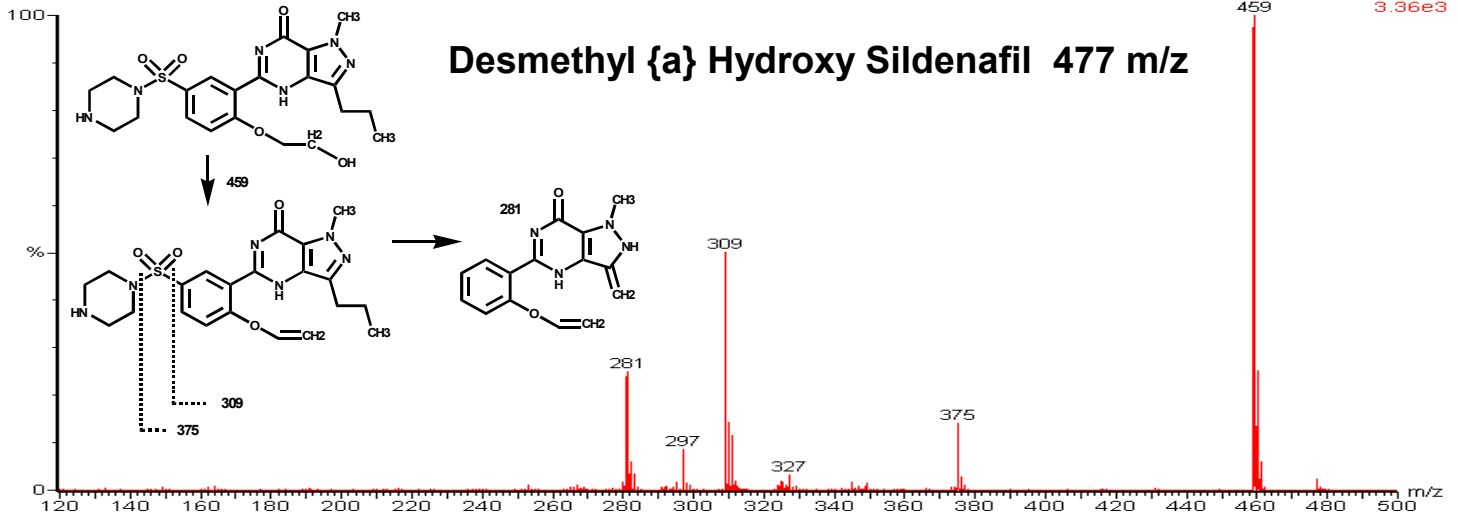
Sildenafil 500 mg PO 4 hr urine  
121301-15

13-DEC-2001  
15:12:30

jarudy  
34

S010124-4 92 (2.448) Cm (90:95-83:88)

2: TOF MSMS 477.20ES+  
3.36e3



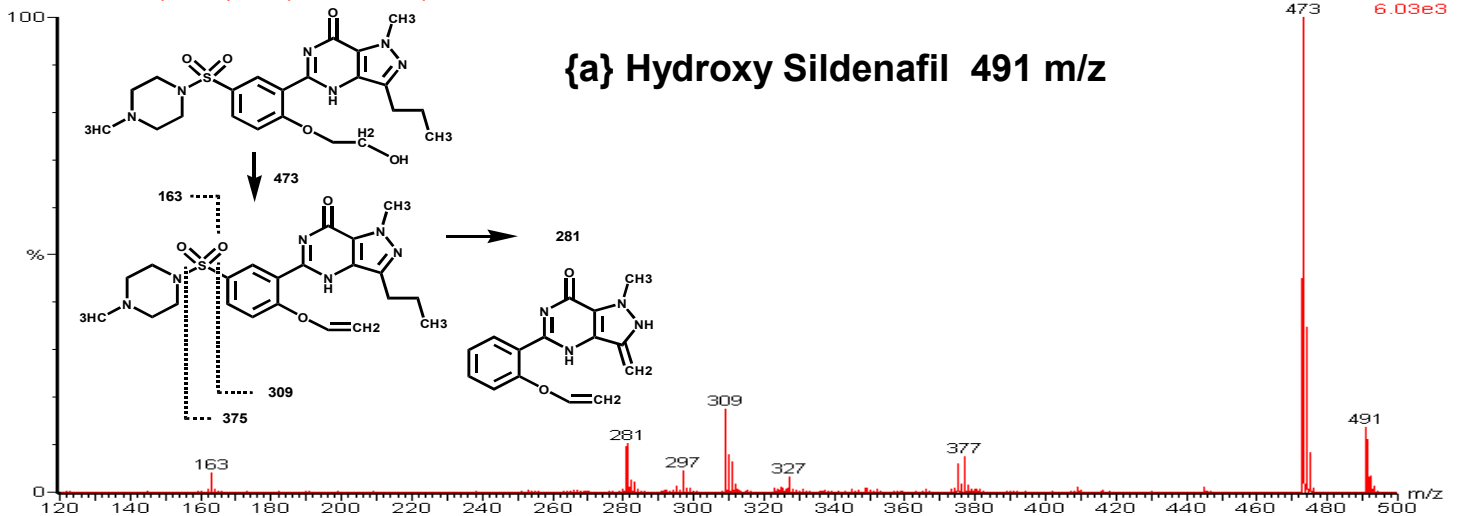
Sildenafil 500 mg PO 4 hr urine  
121301-15

13-DEC-2001  
15:12:30

jarudy  
34

S010124-4 71 (1.903) Cm (70:75-62:69)

1: TOF MSMS 491.20ES+  
6.03e3



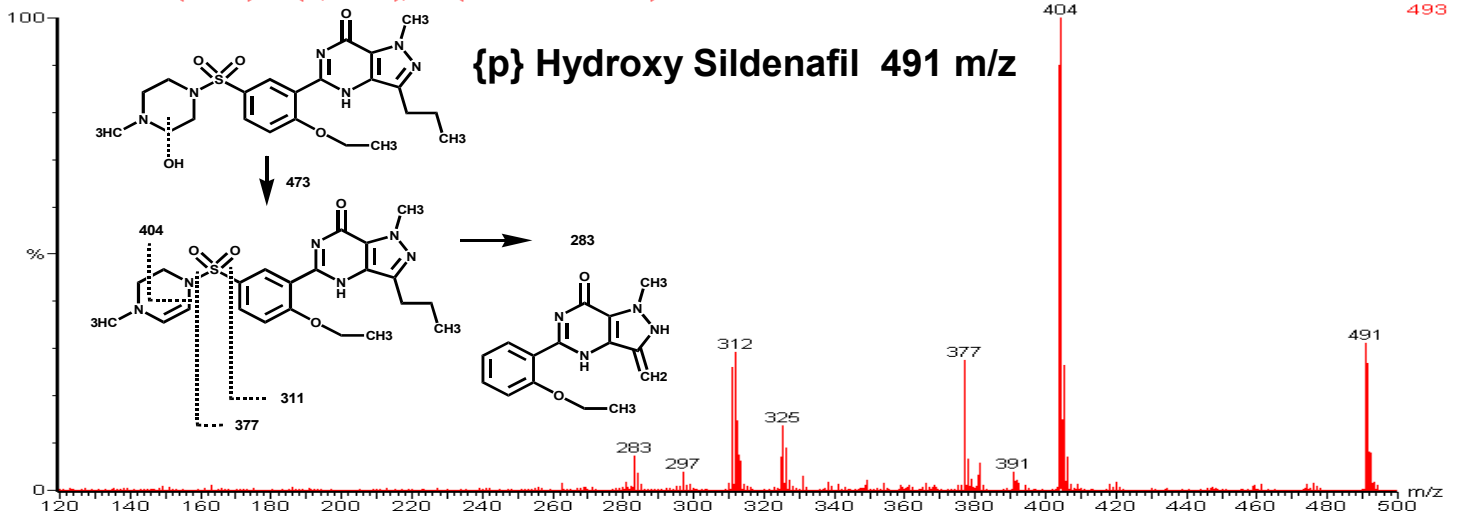
Sildenafil 500 mg PO 4 hr urine  
121301-15

13-DEC-2001  
15:12:30

jarudy  
34

S010124-4 158 (3.935) Sb (2,40.00); Cm (153:164-136:149)

1: TOF MSMS 491.20ES+  
493



### Spectra and Proposed Structures (matrix=plasma)

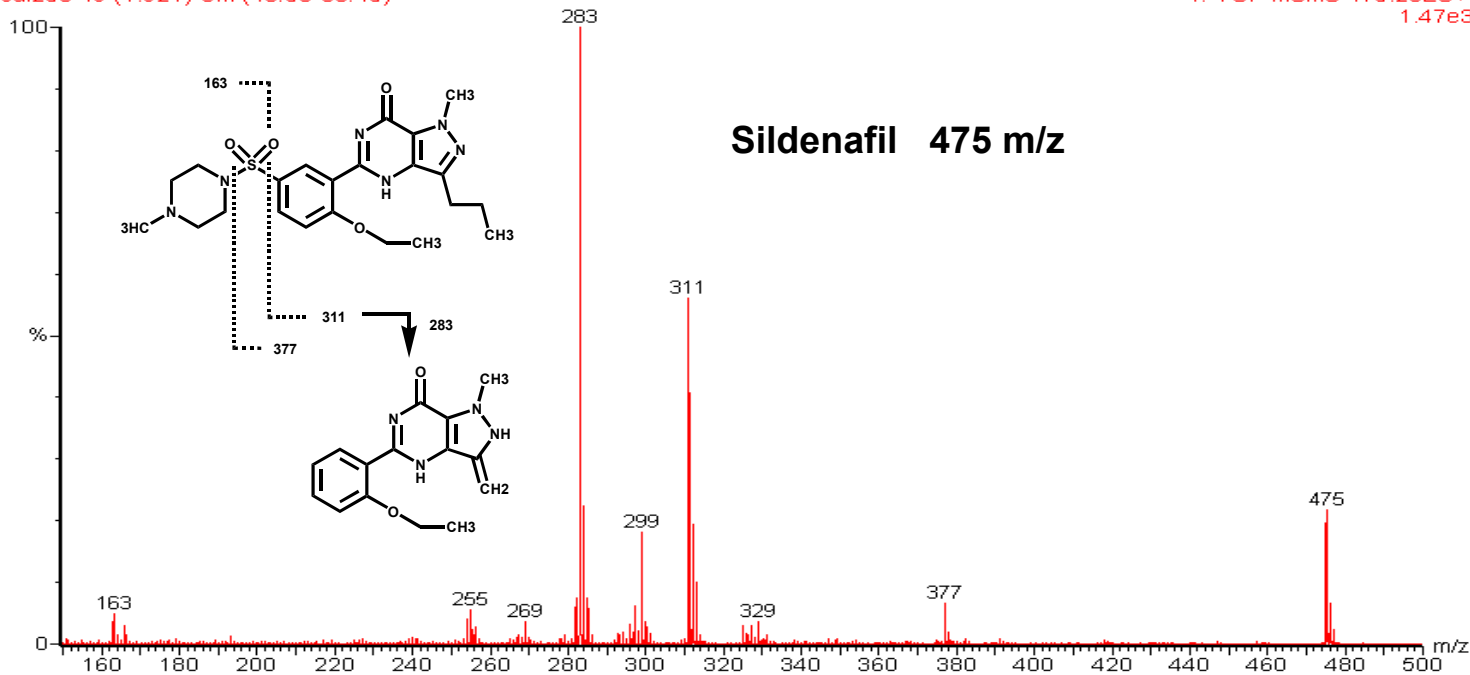
121001SDMS250  
121301-10

13-DEC-2001  
14:33:35

jarudy  
19

cal250 49 (1.921) Cm (46:53-38:45)

1: TOF MSMS 475.20ES+  
1.47e3



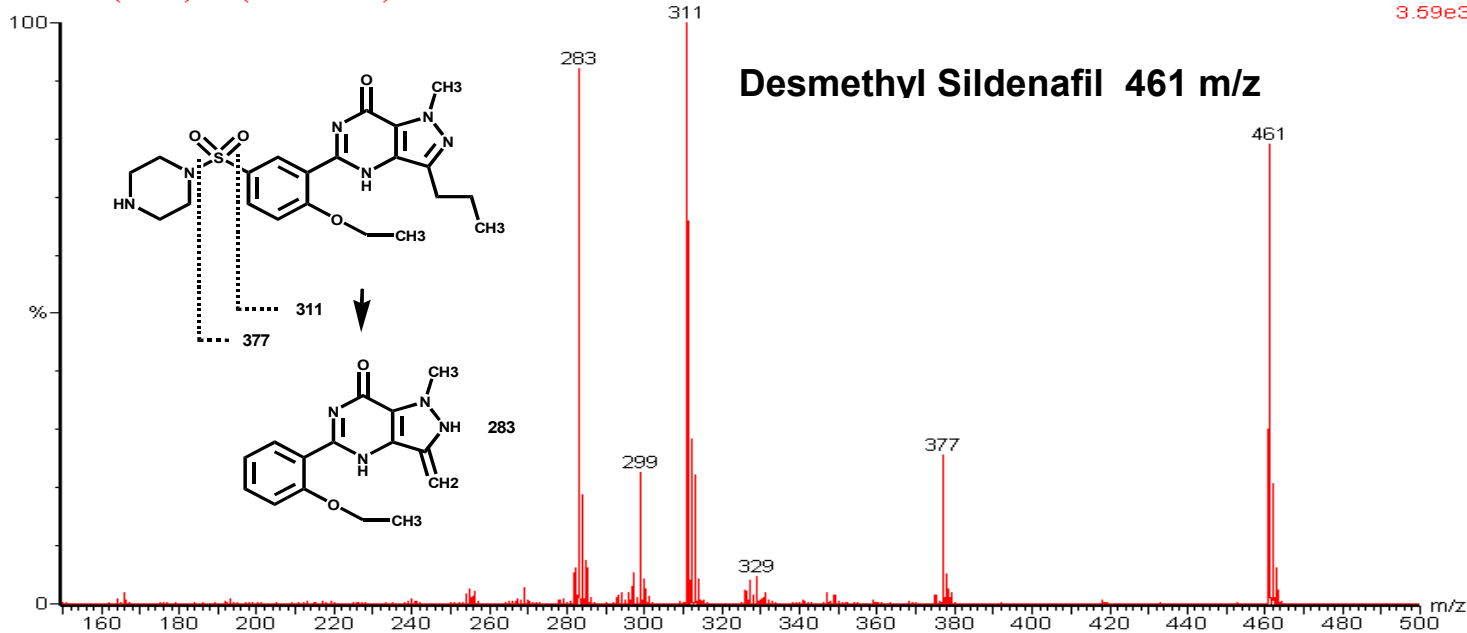
121001SDMS250  
121301-10

13-DEC-2001  
14:33:35

jarudy  
19

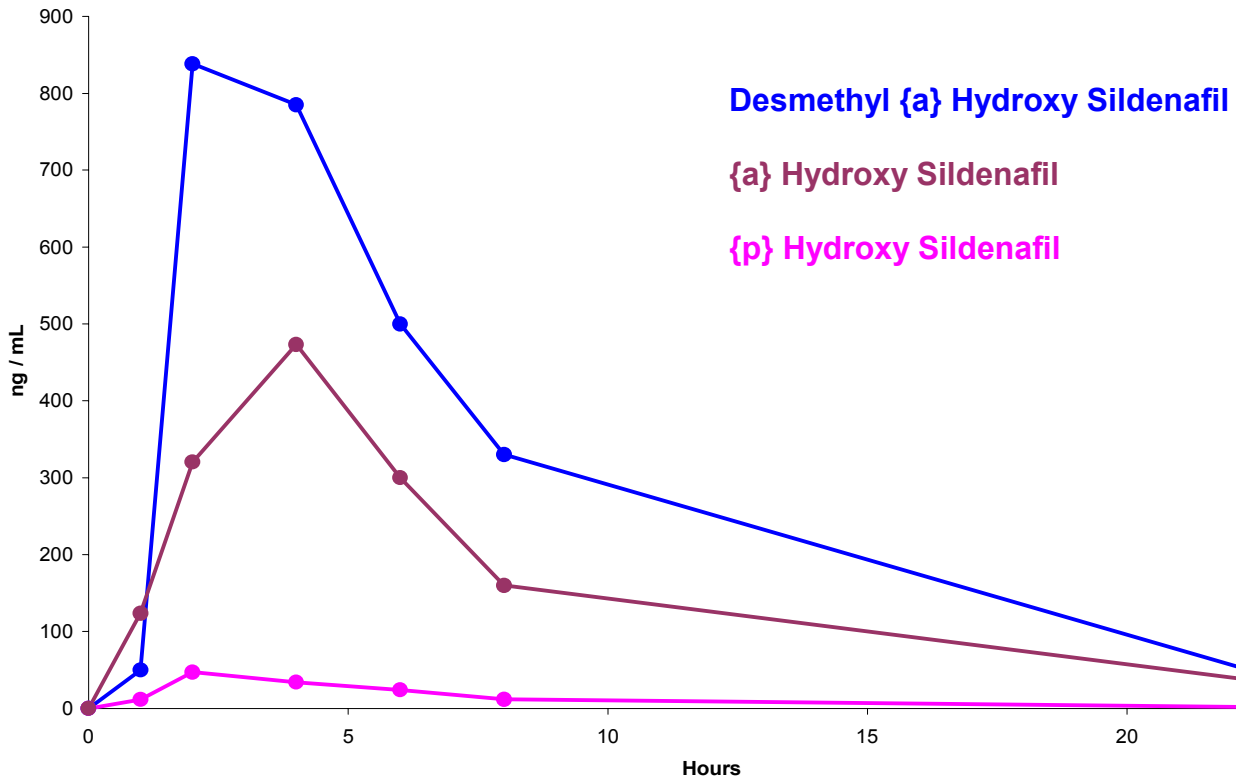
cal250 66 (2.574) Cm (64:68-56:62)

2: TOF MSMS 461.20ES+  
3.59e3

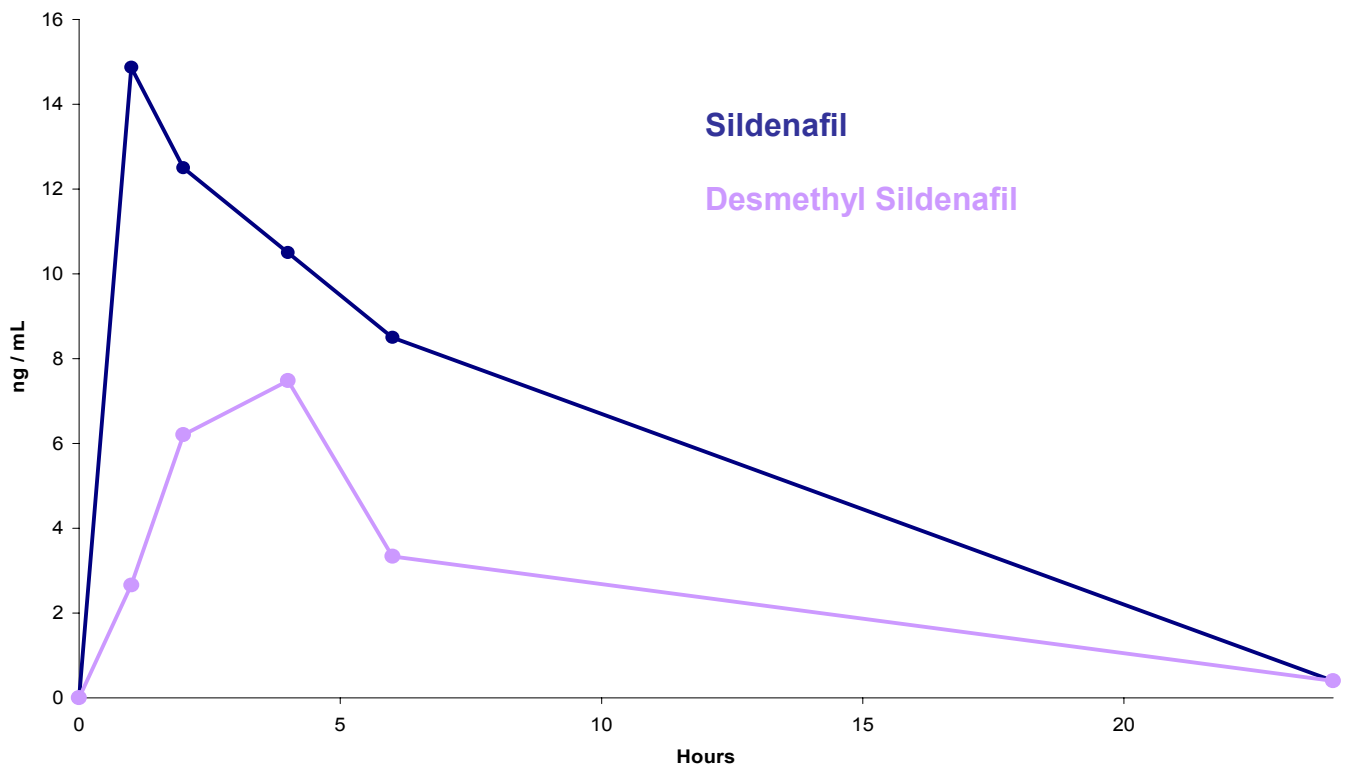


## Sildenafil and Metabolite Elimination Curves for Urine and Plasma ( 500 mg PO)

500 mg PO Sildenafil Urine



500 mg PO Sildenafil Plasma



## **MATERIALS**

- A. 16 x 100mm culture tubes.
- B. 16 x 150mm screw cap test tubes.
- C. Pipettes and tips.
- D. Vortex mixer ( Scientific Industries, Inc. )
- E. Branson Ultrasonic Water Bath, 8510 ( Fisher Scientific or equivalent )
- F. pH meter ( IQ Scientific Instruments )
- G. Sample Concentrator ( Dri-Block DB-3, Techne )
- H. IEC HN-SII Centrifuge ( International Equipment Company )
- I. Rotorack ( Speci-Mix, Thermolyne )
- J. Kimwipes
- K. 2 mL autosampler vials
- L. 200 uL Insert (Target PP Polyspring, National Scientific Company )
- M. 15 x 45 mm, 1 x 35 mm and 28 x 57 mm VWR brand vials
- N. Balance (Mettler AT 261 Delta range, Mettler-Toledo Inc.)

## **REAGENTS**

### **Ammonium Hydroxide (NH<sub>4</sub>OH)-DI-H<sub>2</sub>O (1:1) Reagent**

For Use in Enzyme Hydrolysis (EH) Urine Extraction and Special Base Urine (SBU) Extraction

Procedure for 4000 mL (under fume hood):

Combine 2 L concentrated NH<sub>4</sub>OH and 2 L DI-H<sub>2</sub>O in a 4 Liter reagent bottle.

### **β-Glucuronidase (*patella vulgata*) Enzyme**

Used for Enzyme Hydrolysis (EH) Urine Extraction

Considerations to Ensure Viability:

1. Maximum shelf life is 6 days if enzyme solution is stored at 4°C (less, if left at room temperature for several hours).
2. Make up only one weeks estimated volume.
3. Do not store close to freezer coils or compartments, do not allow freezing.
4. Enzyme loses potency every day when in solution.
5. Enzyme is characterized for our routine usage at 5000 FU/ mL.

Procedure For Enzyme Preparation:

1.  $(5000 \text{ FU/mL}) \times (\text{Total Volume mL}) = (\text{Bottle total units/Bottle total weight FU/mg}) \times (\text{Weight prepared mg})$
3. 1 vial 2,000,000 units in 350 mL DI H<sub>2</sub>O (~116 MG per 50 mL)
4. Use 1 1/2 mL per sample.
5. Store at 4°C.
6. Label solution with Reagent name, weight, volume, date, and initials.
8. Record in Reagent Prep Log Book lab lot #, formula #, date, and initials.

### **Sulfuric Acid 1N(0.5M) H<sub>2</sub>SO<sub>4</sub>**

For Use in Enzyme Hydrolysis (EH) Urine Extraction

Procedure for 3600 mL: **(Maximum personal protection reagent - Lab coat, gloves, and safety glasses required):**

1. Pour 2000 mL of DI-H<sub>2</sub>O into a 4000 mL volumetric flask.
2. Slowly add 100 mL of Conc.H<sub>2</sub>SO<sub>4</sub> (36N) mixing thoroughly while you add.
3. Dilute to 3600 mL using DI-H<sub>2</sub>O.

**Warning: Slowly add acid to H<sub>2</sub>O. Never add water to acid. High heats of mixing are produced. Sufficient heat is generated to burn skin and fracture glass containers.**

### **500 mM Ammonium Acetate pH 4**

Stock solution for LCMS mobile phase dilutions

Procedure for 1000 mL:

1. Dissolve 29 mL Glacial Acetic Acid in 800 mL HPLC grade (or better) water
2. Adjust to pH 4.0 with Ammonium Hydroxide
3. Bring to volume (1000 mL) with HPLC grade (or better) water
4. Dilute 1:10 to 1:100 with HPLC grade water for 0.05 to 0.005 M solutions

---

## **References**

1. In Vitro Biotransformation of Sildenafil (Viagra): Identification of Human Cytochromes and Potential Drug Interactions, J. Warrington, et al, **Drug Metabolism and Disposition** 28:4,(Apr 2000) pgs 392-397.
2. Pharmacokinetics and metabolism of Sildenafil in mouse, rat, dog, and man, D. Walker et al, **Xenobiotica** 29:3,(Mar 1999) pgs 297-310.
3. The cGMP-specific Phosphodiesterase Inhibitor E4021 Dilates the Pulmonary Circulation, R.Dukarm, et al, **Am. J Respir. Crit. Care Med.**, 160:3 (Sept. 1999) pgs 858-865.
4. Direct Sale of sildenafil (Viagra) to consumers over the Internet, D.deKieffer, **N Engl J Med**, 342:10(Mar 2000), pg 742.
5. Sildenafil as a selective pulmonary vasodilator in childhood primary pulmonary hypertension, D.Abrams, et al, **Heart (Br Card Soc Online)**, 84:2,(Aug 2000): E4
6. Sildenafil is a pulmonary vasodilator in awake lambs with acute pulmonary hypertension, J.Weimann, et al, **Anesthesiology**, 92:6(June 2000), pgs 1702-12.