Federal Aviation Administration

DOT/FAA/AM-06/3
Office of Aerospace Medicine
Washington, DC 20591

Identification of Sildenafil (Viagra®) and Its Metabolite (UK-103,320) in Six Aviation Fatalities

Robert D. Johnson
Russell J. Lewis
Civil Aerospace Medical Institute
Federal Aviation Administration
Oklahoma City, OK 73125

February 2006

Final Report

NOTICE

Technical Report Documentation Page

1. Report No.	2. Government Accession No.	3. Recipient's Catalog No.
DOT/FAA/AM-06/3		

4. Title and Subtitle	5. Report Date
Identification of Sildenafil (Viagra®) and Its Metabolite (UK-103,320) in Six Aviation Fatalities	February 2006
	6. Performing Organization Code

7. Author(s)	8. Performing Organization Report No.
Johnson RD, Lewis RJ	

9. Performing Organization Name and Address	10. Work Unit No. (TRAIS)
FAA Civil Aerospace Medical Institute P.O. Box 25082 Oklahoma City, OK 73125	11. Contract or Grant No.

12. Sponsoring Agency name and Address	13. Type of Report and Period Covered
Office of Aerospace Medicine Federal Aviation Administration 800 Independence Ave., S.W. Washington, DC 20591	14. Sponsoring Agency Code

15. Supplemental Notes

Work was accomplished under approved task AM-B-05-TOX-204.

16. Abstract

During the investigation of aviation accidents, postmortem samples from victims are submitted to the Federal Aviation Administration's Civil Aerospace Medical Institute for toxicological analysis. This report presents a rapid and reliable method for the identification and quantitation of sildenafil (Viagra®) and its active metabolite, UK-103,320. This procedure utilizes sildenafil-d_8 as an internal standard for more accurate and reliable quantitation. The method incorporates solid-phase extraction and LC/MS/MS and MS/MS/MS utilizing an atmospheric pressure chemical ionization ion trap mass spectrometer in the positive chemical ionization mode. Solid-phase extraction provided an efficient sample extraction yielding recoveries ranging from 79 – 88%. The limit of detection for sildenafil and UK-103,320 was 0.39 and 0.19 ng/mL, respectively. The linear dynamic range for both compounds was 0.78 – 800 ng/mL. The method was employed for the determination of sildenafil and UK-103,320 in postmortem fluid and tissue specimens collected from 6 fatal aviation accident victims. The current method proved to be simple, accurate, and robust for the identification and quantitation of sildenafil and UK-103,320 in postmortem fluids and tissues.

17. Key Words	18. Distribution Statement
Toxicology, Erectile Dysfunction, Sildenafil, Viagra, LC/MS/MS/MS, Aircraft Accident Investigation	Document is available to the public through the Defense Technical Information Center, Ft. Belvior, VA 22060; and the National Technical Information Service, Springfield, VA 22161

19. Security Classif. (of this report)	20. Security Classif. (of this page)	21. No. of Pages	22. Price
Unclassified	Unclassified	14	

Form DOT F 1700.7 (8-72) Reproduction of completed page authorized

Identification of Sildenafil (Viagra®) and Its Metabolite (UK-103,320) in 6 Aviation Fatalities.

INTRODUCTION

The Federal Aviation Administration's Civil Aerospace Medical Institute (CAMI) is responsible, under Department of Transportation Orders 8020.11B and 1100.2C, to "conduct toxicologic analysis on specimens from … aircraft accident fatalities" and "investigate … general aviation and air carrier accidents and search for biomedical and clinical causes of the accidents, including evidence of … chemical [use]." Therefore, following an aviation accident, samples are collected at autopsy and sent to CAMI's Forensic Toxicology Research Laboratory where toxicological analysis is conducted on various postmortem fluids and tissues.

Sildenafil (Viagra®), used for the treatment of erectile dysfunction, is one of the most popular and widely used drugs in the United States and Europe. Sildenafil is so popular that 6 million prescriptions for this agent were written during the first 6 months following its introduction in 1998.[1] Contrary to popular belief, sildenafil is not an aphrodisiac, does not work in the absence of sexual arousal, and does not make a potent man more virile.[2] Sildenafil, 1-[4-ethoxy-3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1*H*-pyrazolo-[4,3-d]pyrimidin-5-yl)phenylsulphonyl]-4-methylpiperazine, shown in Figure 1, is a potent inhibitor of the cGMP-specific phosphodiesterase type 5 enzyme (PDE5) found predominantly in the penile corpus cavernosum.[3-6] Cyclic guanosine monophosphate (cGMP), which is broken down by PDE5, is directly responsible for producing smooth muscle relaxation in the corpus cavernosum and allowing the inflow of blood. Thus, by inhibiting PDE5, sildenafil has the potential to improve male erectile function.[7, 8]

After oral administration, sildenafil is rapidly absorbed, reaching peak plasma concentrations in 30-120 min.[9] It is metabolized in the liver predominantly to the active desmethyl metabolite, UK-103,320. UK-103,320, 1-[4-ethoxy-3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1*H*-pyrazolo[4,3-d]pyrimidin-5-yl)phenyl-sulphonylpiperazine, also shown in Figure 1, exhibits approximately 50% of the potency of the parent drug and, hence, contributes to the observed pharmacological effects.[10] Under steady-state conditions, the plasma concentration of UK-103,320 is approximately 40% of that seen for Sildenafil.[11] Both sildenafil and its metabolite have an elimination half-life of approximately 2.5 hours.[12]

Sildenafil, when used properly, is relatively safe. There are, however, certain side effects that could create potential hazards. Sildenafil has been shown to potentiate the hypotensive effects of nitrates commonly employed in the treatment of certain heart conditions.[13] Moreover, while sildenafil inhibits PDE5, it also has a high affinity for phosphodiesterase type 6 (PDE6), which is a retinal enzyme involved in phototransduction.[9, 14] The inhibition of PDE6 can result in the inability to discriminate between blue and green colors, resulting in a condition known as "blue tinge."[15] Although only about 3% of patients report visual disturbances, this blue-green impairment could cause problems in the execution of certain tasks. For example, this impairment could lead to a problematic situation for a pilot relying upon instruments during night flights or adverse meteorological conditions.[16] Additionally, the more prevalent use of color video terminals in aviation is anticipated to increase the probability of an accident for a color-deficient pilot.

Because of its popularity, the presence of both sildenafil and its metabolite in aviation accident victims is becoming more common. This paper describes a method for the quantitation of both sildenafil and UK-103,320 in postmortem fluids and tissues utilizing solid phase extraction (SPE) and liquid chromatography (LC) with atmospheric pressure chemical ionization (APCI) ion trap mass spectrometry (MS). This technology allowed for MS/MS and MS/MS/MS detection in the positive ionization (PCI) mode. The internal standard utilized for these experiments (sildenafil-d_8) is of note, as the authors were unable to find any other published quantitative method that employed this analogue.[17, 18] Following method validation, fluid and tissue specimens from 6 aviation fatalities were examined that previously screened positive for either sildenafil and/or UK-103,320.

MATERIALS AND METHODS

Reagents, Standards and Supplies

All aqueous solutions were prepared using double deionized water (DDW), which was obtained using a Milli-QT_{plus} Ultra-Pure Reagent Water System (Millipore®, Continental Water Systems, El Paso, TX). All chemicals were purchased in the highest possible purity and used without any further purification. All solvents were of HPLC-grade and were obtained from Fisher

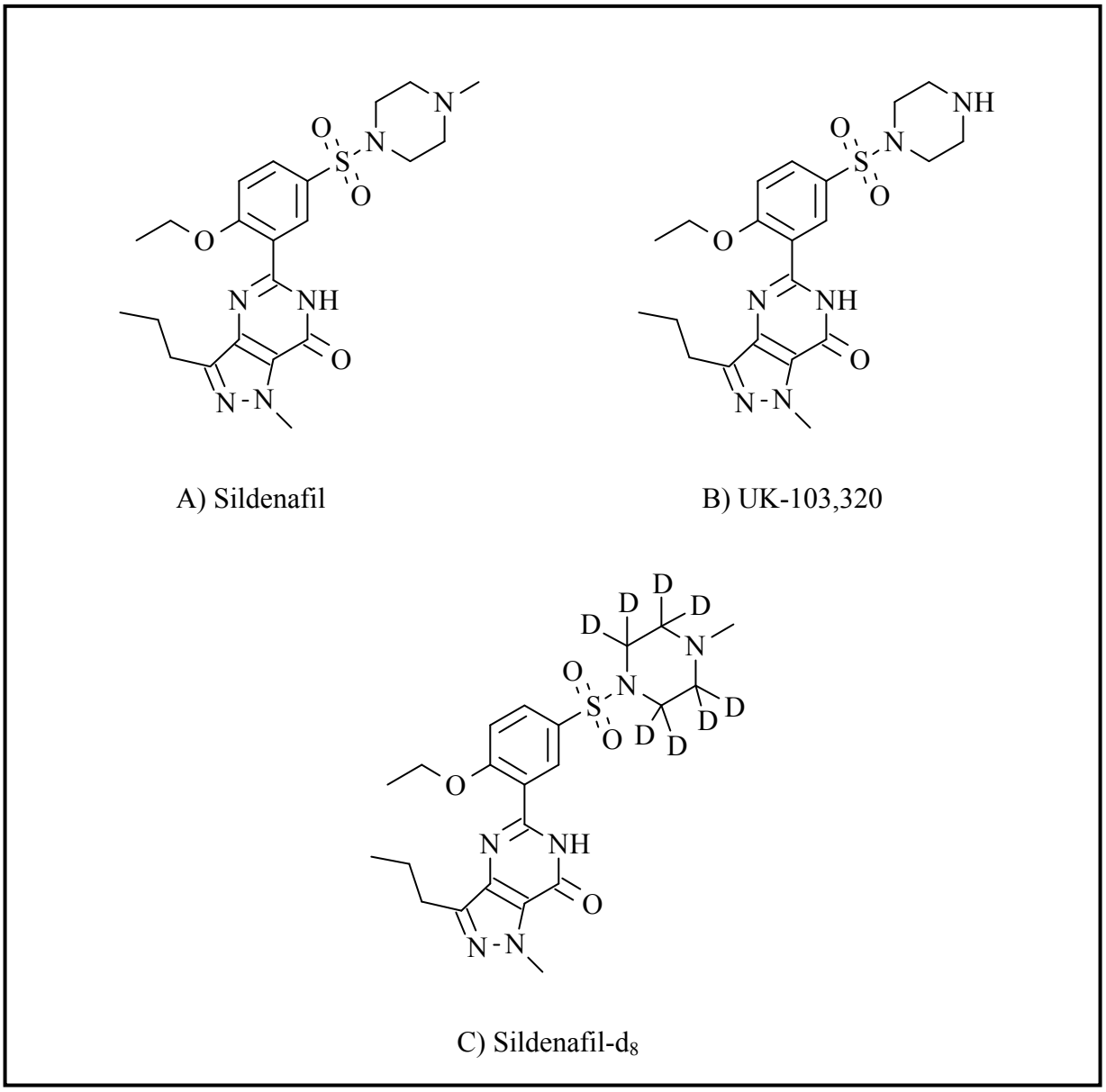

Figure 1. Chemical structures of sildenafil (a), UK-103,320 (b) and sildenafil-d_8 (c).

Scientific (Fischer Scientific Co., Fair Lawn, NJ). Formic acid (97%) was purchased from ICN (ICN Biomedicals, Inc., Irvine, CA). Sildenafil and UK-103,320 were obtained from Pfizer pharmaceutical company (Pfizer Ltd., UK). Sildenafil-d_8 was synthesized by and obtained from SynFine Research Inc. (Ontario, Canada).

Stock standards of both sildenafil and UK-103,320 were prepared at 1 mg/mL in methanol. A stock solution of the internal standard, sildenafil-d_8, was prepared at 100 µg/mL in methanol. The aqueous portion of the HPLC mobile phase was prepared with a concentration of 50.0 mM formic acid and adjusted to pH 5.00 with conc. ammonium hydroxide. The formic acid buffer was mixed with acetonitrile in a 98:2 (v:v) ratio, respectively, to help prevent the growth of microbes. This mixture was filtered through a vacuum filtering apparatus that incorporated a 0.45 µm GH polypro 47 mm hydrophilic, polypropylene membrane filter obtained from Pall Gelman laboratory (Pall Corp., East Hills, NY). The primary organic component of the mobile phase was HPLC grade acetonitrile, which was filtered prior to use through a vacuum filter apparatus that incorporated the same type of membrane filter as described above.

Instrumentation

Analyte separation was achieved using a Hewlett Packard 1100 HPLC (Hewlett Packard Co., Wilmington, DE) equipped with a Security Guard™ C-8 guard column (4.0 x 3.0 mm i.d., 3 µm particles) from Phenomenex® (Torrance, CA), followed immediately by an Atlantis™ LC-18 (150 x 4.6 mm i.d., 3 µm particles) analytical column obtained from Waters (Milford, MA). Samples were injected using a Hewlett Packard G1313A autosampler (Hewlett Packard Co., Wilmington, DE). Identification and quantitation were accomplished using a Thermo Finnigan model LCQ atmospheric pressure chemical ionization (APCI) ion trap mass spectrometer (Thermo Finnigan Corp., San Jose, CA), which utilized nitrogen as the sheath gas and helium as the reagent gas. Control of the HPLC system, integration of the chromatographic peaks, and communication with the mass spectrometer were accomplished using a Gateway 2000 E-4600-SE personal computer using Xcalibur™ LC/MS software (Thermo Finnigan Corp., San Jose, CA).

LC/MS/MS and MS/MS/MS Method

For all determinations, the HPLC was operated in an isocratic mode with a flow rate of 1.00 mL/min. The sample injection volume was 10 µL. The HPLC column was routinely equilibrated overnight prior to use. Following use, the column was washed and stored in 50:50, acetonitrile:H_2O. Working with standards of sildenafil, sildenafil-d_8, and UK-103,320, we began the initial investigation by observing the response received from these 3 compounds when injected directly into the LCQ. Initial ionization evaluation of these compounds indicated that positive chemical ionization (PCI), creating $[M+H]^+$ ions, was much more effective than negative chemical ionization (NCI), which formed $[M-H]^-$ ions. Initially, APCI-PCI-MS $[M+H]^+$ parent ions were identified for each of the three compounds by infusing the desired compound at a concentration of approximately 10 µg/mL, prepared by dilution from the stock solutions using methanol, into the LCQ at a constant rate of 25 µL/min. Following $[M+H]^+$ ion identification, ionization conditions were optimized by infusing each analyte directly into the mobile phase, which was then introduced into the mass spectrometer at a flow rate of 1.00 mL/min. Tuning the MS for the desired ions was then accomplished using the autotune feature of the Xcalibur™ software. As a result of preliminary APCI-PCI-MS investigations, each sample analysis was subsequently split into 2 unique data collection segments. Segment 1 collected data for UK-103,320 and segment 2 collected data for sildenafil and the internal standard sildenafil-d_8.

The operating conditions for segment 1, which was employed for the analysis of UK-103,320, were as follows: APCI capillary temperature, 250°C; APCI vaporizer temperature, 450°C; source voltage, 8.0 kV; source current, 5 µA; sheath gas flow (nitrogen), 80.0; auxiliary gas flow (nitrogen), 15.0; capillary voltage, 34.0 V; tube lens offset, 40.0 V; octapole 1 offset, -3.75 V; octapole 2 offset, -7.0 V; interoctapole lens voltage, -20 V; trap DC offset voltage, -10 V; multiplier voltage, 0.0 V; and 1 micro-scan having a maximum ion injection time of 200 msec. Segment 1 was further split into 3 separate scan events. Scan event 1 collected the UK-103,320 parent molecular ion at *m/z* 461.5. Scan event 2 collected the UK-103,320 daughter ion at *m/z* 313.1 following collision induced dissociation (CID) of the parent ion (*m/z* 461.5) using a collision energy of 40%. Scan event 3 collected the UK-103,320 granddaughter ion at *m/z* 285.1 following CID of the daughter ion (*m/z* 313.1) using a collision energy of 42%.

The operating conditions for segment 2, which was used for the analysis of both sildenafil and sildenafil-d_8, were as follows: APCI capillary temperature, 250°C; APCI vaporizer temperature, 450°C; source voltage, 8.0 kV; source current, 5 µA; sheath gas flow (nitrogen), 80.0; auxiliary gas flow (nitrogen), 20.0; capillary voltage, 16.0 V; tube lens offset, 5.0 V; octapole 1 offset, -2.50 V; octapole 2 offset, -6.0 V; interoctapole lens voltage, -22 V; trap DC offset voltage, -10 V; multiplier voltage, 0.0 V; and 3 micro-scans having a maximum

ion injection time of 200 msec. Segment 2 was further split into 3 separate scan events. Scan event 1 collected the sildenafil and sildenafil-d_8 parent ions at *m/z* 475.5 and at *m/z* 483.5, respectively. Scan event 2 collected the sildenafil daughter ion at *m/z* 311.1 following collision-induced dissociation (CID) of the parent ion (*m/z* 475.5) using a collision energy of 42%. Scan event 3 collected the sildenafil granddaughter ion at *m/z* 283.1 following CID of the daughter ion (*m/z* 311.1) using a collision energy of 42%.

Ions having the highest abundance in the MS/MS mode were used for quantitation of each analyte. The ion employed for the quantitation of sildenafil was at *m/z* 311.1. The ion employed for the quantitation of UK-103,320 was at *m/z* 313.1. The molecular ion of the internal standard, sildenafil-d_8, at *m/z* 483.5 was utilized for quantitation. The MS/MS and MS/MS/MS full spectra were used for analyte confirmation.

Calibrators and Controls

Calibration curves were prepared by serial dilution utilizing bovine whole blood as the diluent. Calibrators were prepared from one set of original stock standard solutions of both sildenafil and UK-103,320. Controls were prepared in a similar manner to calibrators, using the same bovine whole blood as diluent but employing a second set of original stock solutions. Calibration curves were routinely prepared at a concentration range of 0.78 – 800 ng/mL. A minimum of 7 calibrators was used to construct each calibration curve. Controls, used for the determination of accuracy, precision, and compound stability, were prepared at 50 and 200 ng/mL. Controls were prepared in pools large enough to provide samples for the entire study. A sildenafil-d_8 working standard was prepared at a final concentration of 400 ng/mL by dilution with DDW from the stock solution.

Quantitation of sildenafil and UK-103,320 in biological specimens was achieved via an internal standard calibration procedure. Response factors for both sildenafil and UK-103,320 were determined for each sample. The response factor was calculated by dividing the area of the analyte peak by the area of the internal standard peak. Calibration curves were prepared by plotting a linear regression of the analyte/internal standard response factor versus the analyte concentration for the calibrators and were used to determine the concentrations of sildenafil and UK-103,320 in controls and specimens.

Sample Extraction

Calibrators, controls, and postmortem fluid and tissue specimens were extracted in the following manner. Tissue specimens were homogenized using a PRO250 post-mounted homogenizer (PRO Scientific, Oxford, Ct) employing a 30.0 mm saw-toothed generator set to rotate at 22,000 RPM following a 2:1 dilution with 1.00% sodium fluoride (2 g 1.00% NaF:1 g wet tissue). Three mL aliquots of fluids, controls, calibrators, and 3 g of tissue homogenate were transferred to individual 15 mL screw-top vials. To each sample, 400 ng of internal standard was added (1.00 mL of the 400 ng/mL stock solution). The samples were vortexed and allowed to stand for 10 min. Nine mL ice-cold acetonitrile was added to each sample. The mixture was then placed on a rotary mixing wheel and mixed for 15 min by simple rotation of the wheel at 15 rpm. Centrifugation at 820 × *g* for 5 min provided removal of cellular debris and proteins. Following centrifugation, the supernatant was transferred to clean 15 x 125 mm culture tubes and evaporated in a water bath at 40°C under a stream of dry nitrogen to a volume less than 1 mL. Following acetonitrile evaporation, 4.00 mL 0.10 M phosphate buffer, pH 6.00 was added to each sample. The extracts were transferred to solid-phase extraction (SPE) columns, which had been pre-conditioned with 2.00 mL methanol, followed by 2.00 mL 0.10 M phosphate buffer, pH 6.00. Care was taken not to dry the column prior to extract addition. Bond Elute Certify® SPE columns employed for this study were obtained from Varian (Varian Co., Harbor City, CA). Column flow rates of 1 – 2 mL/min were maintained in each step using a Varian 24 port Cerex™ SPE processor (Varian Co., Harbor City, CA) with a nitrogen pressure of 3 psi. Once each sample had passed through its respective column, the columns were washed with 1.00 mL of 1.0 M acetic acid, then dried completely with 25 psi nitrogen for 5 min. The columns were then washed by adding 6.00 mL methanol and were again dried completely with 25 psi nitrogen for 5 min. The analytes were eluted from the columns with 4.00 mL of 2% ammonium hydroxide in ethyl acetate, which was prepared fresh daily. Eluents were evaporated to dryness in a water bath at 40°C under a stream of dry nitrogen, reconstituted in 50.0 µL acetonitrile, and transferred to LC sample vials for analysis. All specimens were analyzed at one time to avoid inter-assay variations. Specimens with analyte concentrations above the associated calibration curves were diluted by an appropriate factor and re-extracted. When specimen dilution was necessary, a control was diluted by the same factor to ensure dilution accuracy.

Recovery

The recovery of each analyte was determined using the following procedure.[19] Two groups, X and Y, of controls prepared using negative whole blood diluent were

extracted in the same manner as described immediately above. Group X was spiked with a precisely known amount of both sildenafil and UK-103,320 prior to extraction, and group Y was spiked with the same precisely known amount of sildenafil and UK-103,320 following the solid phase extraction step. Upon analysis, the average response factor obtained from group X was divided by the average response factor obtained from group Y to yield the percent recovery value (100 * X/Y = % recovery) for each of the compounds.

RESULTS AND DISCUSSION

Method Validation

The procedure described herein provides a rapid, reproducible, and accurate method for the determination of sildenafil and its biologically active metabolite, UK-103,320. This procedure incorporates SPE and LC/MS/MS/MS utilizing an APCI ion trap MS in the PCI mode. The use of SPE provided a cleaner extract and required less organic solvent than did an alternative liquid-liquid extraction procedure.

By incorporating APCI-MS in the PCI mode, MS spectra were produced consisting predominantly of the protonated $[M+H]^+$ ion. By utilizing an ion trap MS we were able to conduct MS/MS on these unique ions. The $[M+H]^+$ ions used to create the MS/MS spectra were *m/z* 475.5 and *m/z* 461.5 for sildenafil and UK-103,320, respectively. MS/MS/MS spectra were then obtained by conducting MS on the MS/MS daughter ions at *m/z* 311.1 for sildenafil and at *m/z* 313.1 for UK-103,320. The full scan MS/MS and MS/MS/MS spectra for sildenafil and UK-103,320 provided "fingerprints" used for analyte identification and confirmation. Full scan MS/MS and MS/MS/MS spectra for each analyte are shown in Figures 2 through 5.

Sildenafil and UK-103,320 peaks were completely resolved and experienced no interference from endogenous sample matrix components. Matrix interference was monitored by the use of whole-blood negative controls that were spiked only with sildenafil-d_8 prior to extraction. All analytes were eluted from the analytical column in less than 4 min. Figure 6 shows a representative LC/MS/MS chromatogram. LC retention times were used as one analyte acceptability criteria. Retention times for each analyte were required to be within 2.0% of the average calibrator retention time for that analyte. Typical retention times were 1.83, 2.98, and 2.81 min for UK-103,320, sildenafil, and sildenafil-d_8, respectively.

Quantitation was accomplished by collecting the highest abundance ion in the MS/MS mode for each analyte. The area of the MS/MS ion for sildenafil and UK-103,320, divided by the area of the molecular ion of the internal standard, sildenafil-d_8, resulted in a response factor that was used for quantitation. The linear dynamic range (LDR), limit of detection (LOD), and limit of quantitation (LOQ) were determined by analysis of calibrators ranging in concentration from 0.19 – 6400 ng/mL. The LDR for each compound was determined following this analysis. The LDR for both UK-103,320 and sildenafil was found to be 0.78 – 800 ng/mL. The correlation coefficient for both of these curves exceeded 0.99 when a weighting factor of 1/X was used. For both compounds, non-linearity was observed at concentrations greater than 800 ng/mL. The LOD and LOQ determined for sildenafil and UK-103,320 are listed in Table 1. The LOD was defined as the lowest concentration of analyte having a minimum signal-to-noise ratio (S/N) of 5, in addition to meeting the MS/MS and MS/MS/MS spectral "fingerprint" identification and retention time criteria. The LOQ was defined as meeting all LOD criteria plus having a S/N of 10 and an experimentally determined value within ± 20% of its prepared concentration. The LOD and LOQ for sildenafil were 0.39 ng/mL and 0.78 ng/mL, respectively. The LOD for UK-103,320 was determined to be 0.19 ng/mL and the LOQ 0.78 ng/mL.

Instrumental carryover from one sample to the next was not found to be a problem. It was, however, initially investigated and subsequently monitored by the use of solvent blank injections. An acetonitrile blank, initially injected following the highest calibrator, showed no carryover contamination. Subsequently, blanks were used throughout the sample sequence to verify that no sample-to-sample contamination occurred.

The extraction efficiency for sildenafil and UK-103,320 utilizing SPE exceeded our initial expectations, considering the simplicity of the extraction procedure. As can be seen in Table 1, the average recovery of sildenafil and its metabolite at a concentration of 50 ng/mL was 84 ± 6% and 79 ± 3%, respectively. The average recovery of sildenafil and its metabolite at a concentration of 200 ng/mL was 88 ± 4% and 85 ± 3%, respectively.

Intra-day (within day) and inter-day (between days) accuracy and precision were examined for this extraction. Accuracy was measured as the percent relative error between the experimentally determined and prepared concentrations of a sample. Precision was measured as the relative standard deviation (RSD) for the experimentally determined concentrations. Accuracy and precision studies were performed using whole blood controls at concentrations of 50 and 200 ng/mL. These values were chosen because they represent typical lower and upper therapeutic sildenafil concentrations.[20] These controls were prepared in 500 mL pools on Day 1 and stored in a refrigerator at 4°C until extracted.

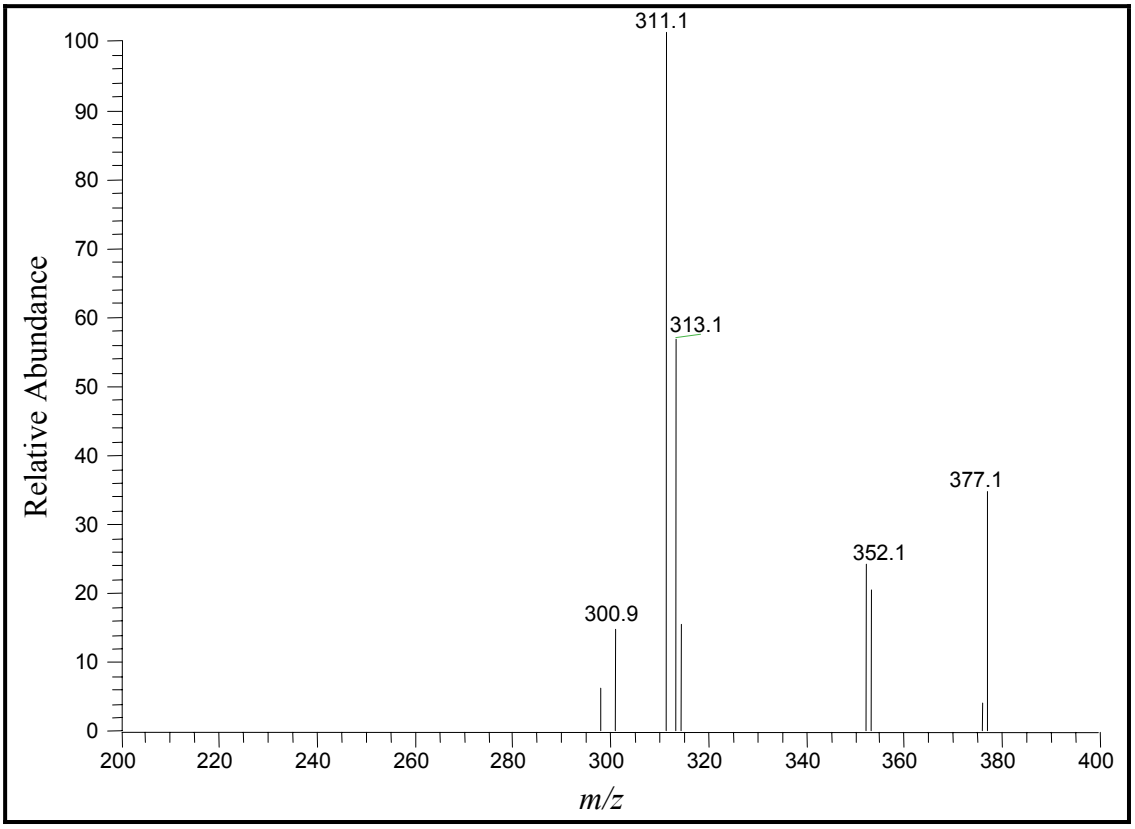

Figure 2. MS/MS spectrum of sildenafil (*m/z* 475.5 → spectrum).

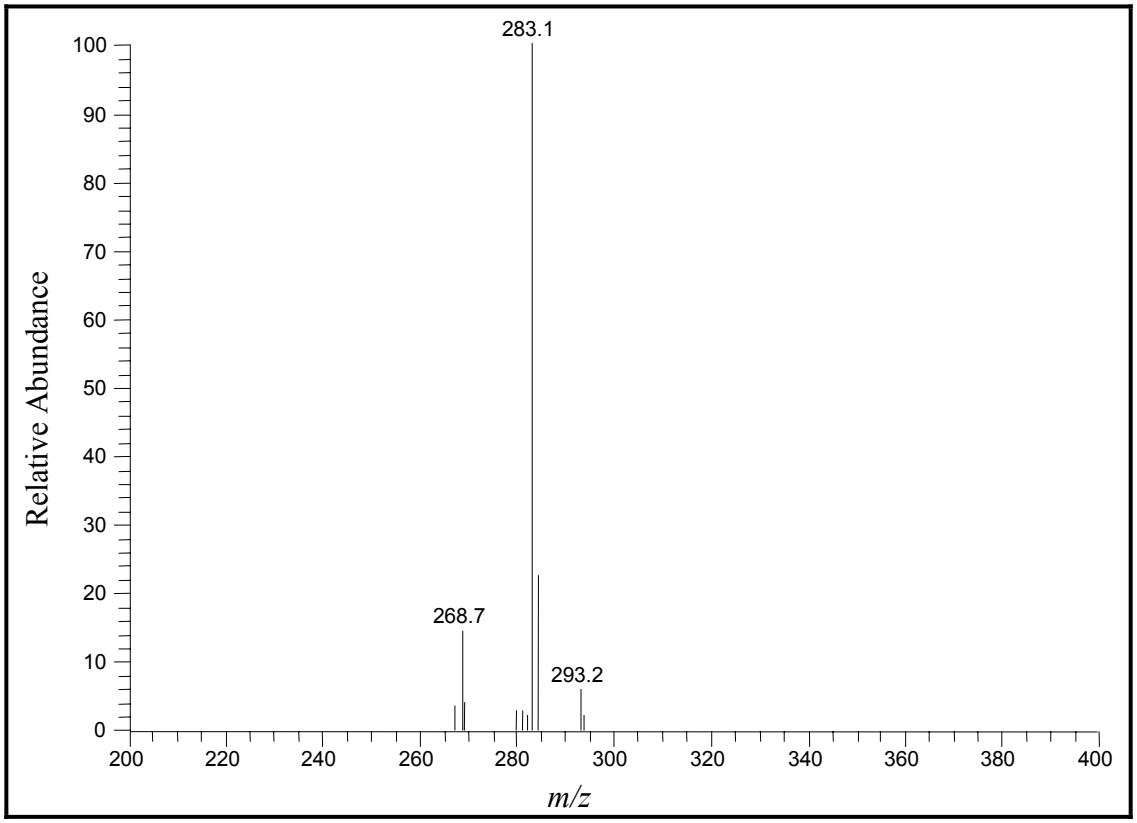

Figure 3. MS/MS/MS spectrum of sildenafil (*m/z* 475.5 → 311.1 → spectrum).

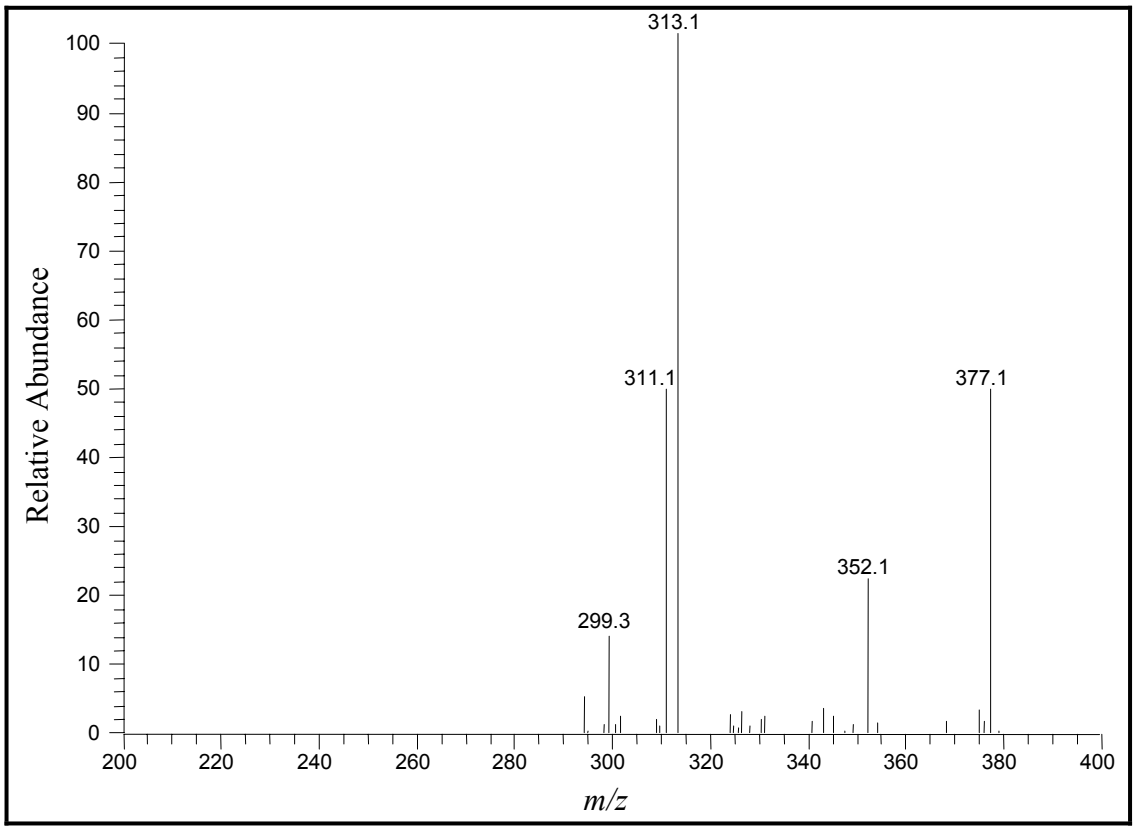

Figure 4. MS/MS spectrum of UK-103,320 (*m/z* 461.5 → spectrum).

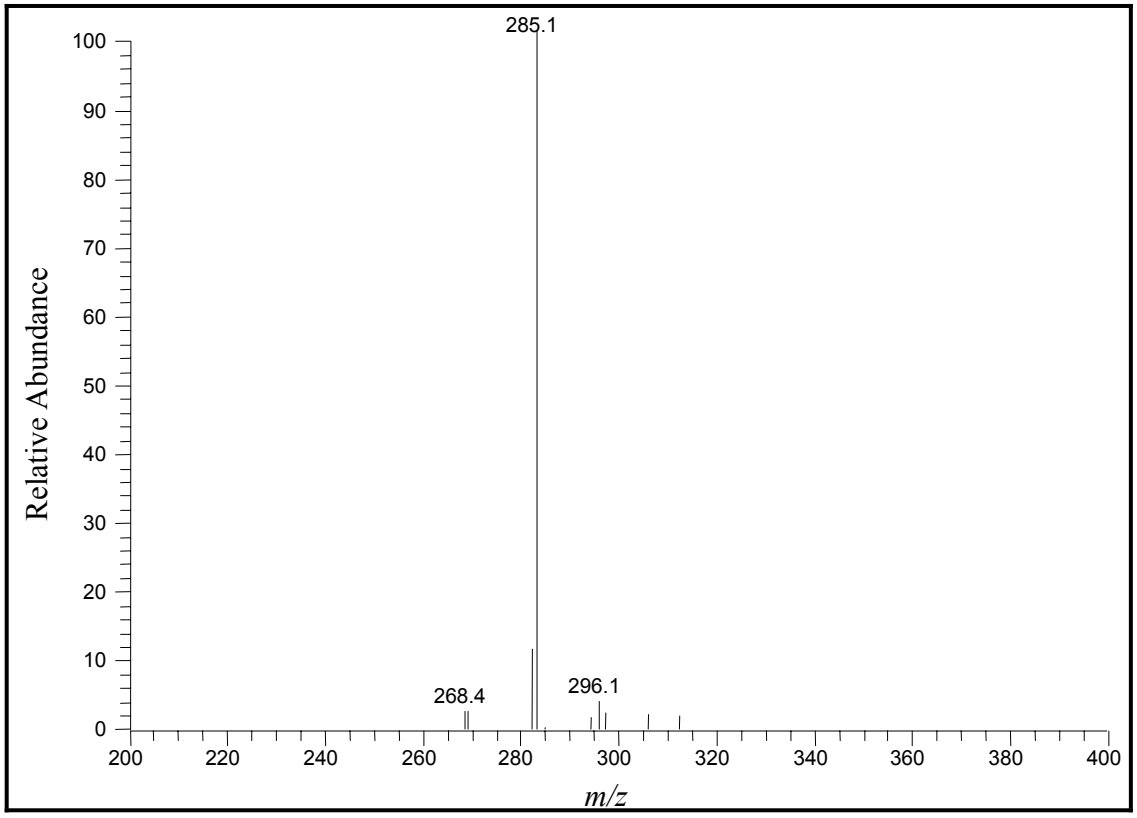

Figure 5. MS/MS/MS spectrum of UK-103,320 (*m/z* 461.5 → 313.1 → spectrum).

For intra-day analyses, a calibration curve was extracted along with 5 replicates of each control concentration on Day 1 of the experiment. Intra-day relative errors in the 50 ng/mL and 200 ng/mL control groups were ≤ 3% for both analytes. Furthermore, the intra-day RSD was ≤ 7% for both the 50 ng/mL and 200 ng/mL sildenafil and UK-103,320 controls. Intra-day results are summarized in Table 2.

Inter-day accuracy and precision were evaluated by extracting 5 replicates of each of the two control concentrations on Days 2, 3, and 7, and basing the quantitation on the calibration curve originally prepared on Day 1. The inter-day relative errors for these analytes at both control concentrations did not exceed 4%. The RSD of the 50 ng/mL sildenafil and UK-103,320 control were ≤ 7% over Days 2, 3, and 7. The 200 ng/mL control had RSD values ≤ 5% for both compounds over the same time period. The inter-day results show that this method is both accurate and precise over a 7-day period (Table 2).

The stability of sildenafil and UK-103,320 in whole blood was evaluated by looking at the control values obtained on Day 7 (Table 2). The analytes showed no apparent decrease in concentration after 1 week of storage at 4°C. In fact, all values were within 4% of their target concentrations. These results demonstrate that whole-blood specimens are stable for a minimum of 1 week when refrigerated. However, as good laboratory practice and to ensure the highest quality analytical data, we recommend that biological specimens be analyzed promptly after thawing.

Postmortem Specimen Analysis

In fatal aviation accidents, specimens from accident victims are routinely sent to the Federal Aviation Administration's Civil Aerospace Medical Institute for toxicological analysis. Postmortem fluid and tissue samples obtained from 6 separate aviation fatalities that had previously screened positive for sildenafil and/or UK-103,320 were re-examined using the current method. The fluid and tissue specimens examined from each victim, if available, included: blood, urine, vitreous humor, bile, liver, kidney, heart, lung, spleen, brain, and skeletal muscle. The results are presented in Table 3.

The pharmacokinetics and pharmacodynamics of sildenafil and its active metabolite are beyond the scope of this paper. These topics are, however, extensively covered elsewhere.[21] As previously stated, sildenafil-d_8 was used as the internal standard in this study. Therefore, the interpretation of quantitative data for UK-103,320 obtained from specimen types other than blood should be closely scrutinized due to possible variations in extraction efficiency between specimen types.

Blood concentrations for sildenafil and UK-103,320 ranged from 0 – 42 ng/mL and 0.85 – 18 ng/mL, while urine concentrations for sildenafil and UK-103,320 ranged from 0 – 103 ng/mL and 11 – 583 ng/mL, respectively. Bile concentrations for sildenafil and UK-103,320 ranged from 4.0 – 5083 ng/mL and 1181 – 18,950 ng/mL, respectively. Liver concentrations for sildenafil and UK-103,320 ranged from 0.98 – 278 ng/g and 5.9 – 328 ng/g, while kidney concentrations for sildenafil and UK-103,320 ranged from 0 – 99 ng/g and 0 – 34 ng/g, respectively. Skeletal muscle concentrations for sildenafil and UK-103,320 ranged from 0 – 39 ng/g and 0 – 35 ng/g, while heart concentrations for sildenafil and UK-103,320 ranged from 7.1 – 125 ng/g and 4.3 – 35 ng/g, respectively. Vitreous humor, lung, spleen, and brain were available in only 1 case and had concentrations for sildenafil of 9.5, 331, 87, and 12 ng/g, and UK-103,320 of 1.3, 145, 24, and 1.3 ng/g, respectively.

As can be seen from these data, no apparent correlation between sildenafil and UK-103,320 concentrations existed within any of the specimen types analyzed. Additionally, no consistent distribution patterns between cases were observed. The highest concentrations of sildenafil and UK-103,320 present in each victim was found in bile. This was expected, as the major excretion route for both analytes is in the feces.[22] Other than bile, no consistent trend was observed for the highest to lowest concentration of sildenafil or UK-103,320 between specimen types analyzed. These findings were not completely unexpected, as many unknown variables exist in these cases. These variables include: time between sildenafil consumption and death, amount of sildenafil consumed, age of the victim, and health of the victim, i.e., renal and hepatic function.

CONCLUSION

The use of sildenafil for the treatment of erectile dysfunction is widespread. Thus, the possible occurrence of undesirable side effects is of concern in the aviation community. With this in mind, a method for the identification and quantitation of sildenafil and its active metabolite, UK-103,320, has been developed that is rapid, reliable, and sensitive. By utilizing SPE, a clean extract was achieved with minimal solvent use. Additionally, the extraction provided excellent analyte recovery. The method described in this paper exemplifies the effectiveness of combining LC with MS for the determination of large, nonvolatile and/or thermally labile compounds. APCI-MS in the PCI mode is a "soft" ionization technique that yielded a simple spectrum consisting of a predominant protonated $[M+H]^+$ ion. The LCQ ion trap enhanced the specificity and sensitivity of the method by providing MS/MS and MS/MS/MS analyses of these unique $[M+H]^+$ ions. This methodology was demonstrated to be highly effective for the identification and quantitation of sildenafil and UK-103,320 in various postmortem fluid and tissue specimens.

Table 1. LDR, LOD, LOQ and recovery for sildenafil and UK-103,320.

Compound	LDR (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)	Recovery (%) ± sd*	
				50 ng/mL	200 ng/mL
Sildenafil	0.78 – 800	0.39	0.78	84 ± 6	88 ± 4
UK 103,320	0.78 – 800	0.19	0.78	79 ± 3	85 ± 3

* n = 5 for each recovery group.

Table 2. Intra-day accuracy and precision for repeated determinations over 7 days.*

	Sildenafil		UK-103,320	
	Day 1		Day 1	
Target Conc. (ng/mL)	50	200	50	200
Mean ± SD (ng/mL)	51 ± 2	201 ± 14	49 ± 3	206 ± 15
Relative Error	+2%	+1%	-1%	+3%
R.S.D.	5%	7%	5%	7%
	Day 2		Day 2	
Target Conc. (ng/mL)	50	200	50	200
Mean ± SD (ng/mL)	50 ± 4	198 ± 10	50 ± 3	201 ± 10
Relative Error	0%	-1%	0%	+1%
R.S.D.	7%	5%	6%	5%
	Day 3		Day 3	
Target Conc. (ng/mL)	50	200	50	200
Mean ± SD (ng/mL)	49 ± 2	197 ± 8	51 ± 2	204 ± 8
Relative Error	-2%	-2%	+2%	+2%
R.S.D.	4%	4%	3%	4%
	Day 7		Day 7	
Target Conc. (ng/mL)	50	200	50	200
Mean ± SD (ng/mL)	51 ± 2	196 ± 5	48 ± 3	200 ± 10
Relative Error	+2%	-2%	-4%	-0%
R.S.D.	5%	2%	6%	5%

*n = 5 at each concentration for each day, controls were run on Days 1, 2, 3, and 7.

Table 3. Concentrations of sildenafil and UK-103,320 found in the victims of 6 separate aviation accidents.

Specimen	Case 1		Case 2		Case 3		Case 4		Case 5		Case 6	
	Sildenafil	UK-103,320	Sildenafil	UK-103,320	Sildenafil	UK-103,320	Sildenafil	UK-103,320	Sildenafil	UK-103,320	Sildenafil	UK-103,320
Blood (ng/mL)	7.5	18	0	1.5	42	6.7	9.4	4.2	7.3	0.85	0.94	0.95
Urine (ng/mL)	13	583	0	11	–	–	77	58	103	243	30	87
Bile (ng/mL)	158	6632	4.0	1181	5083	18,950	–	–	–	–	–	–
VH* (ng/mL)	–	–	–	–	9.5	1.3	–	–	–	–	–	–
Liver (ng/g)	47	77	0.98	5.9	278	328	54	58	108	185	19	31
Kidney (ng/g)	24	24	0	0	99	34	26	11	31	18	5.4	5.3
Muscle (ng/g)	35	35	0	0	39	7.5	8.9	1.1	5.5	4.8	1.6	0.79
Heart (ng/g)	125	24	7.1	4.3	74	28	30	7.4	42	35	–	–
Lung (ng/g)	–	–	–	–	331	145	–	–	–	–	–	–
Spleen (ng/g)	–	–	–	–	87	24	–	–	–	–	–	–
Brain (ng/g)	–	–	–	–	12	1.3	–	–	–	–	–	–

*Vitreous humor
– Specimen type not available for analysis

REFERENCES

1. Kloner, R.A. and Zusman, R.M. Cardiovascular effects of sildenafil citrate and recommendations for its use. *Am J Cardiol*. 84: 11N-17N (1999).
2. de Mey, C. Opportunities for the treatment of erectile dysfunction by modulation of the NO axis-alternatives to sildenafil citrate. *Curr Med Res and Opinion*. 14: 187-202 (1998).
3. Henion, J., Brewer, E., and Rule, G. Sample preparation for LC/MS/MS: Analyzing biological and environmental samples. *Anal Chem*. 70: 650A-656A (1998).
4. Krenzelok, E.P. Sildenafil: Clinical toxicology profile. *J Toxicol Clin Toxicol*. 38: 645-51 (2000).
5. Moreland, R.B., Goldstein, I.I., Kim, N.N., and Traish, A. Sildenafil citrate, a selective phosphodiesterase type 5 inhibitor. *Trends Endocrinol Metab*. 10: 97-104 (1999).
6. Altiokka, G., Atkosar, Z., Sener, E., and Tuncel, M. FIA of sildenafil citrate using UV-detection. *J Pharm Biomed Anal*. 25: 339-42 (2001).
7. Langtry, H.D. and Markham, A. Sildenafil: A review of its use in erectile dysfunction. *Drugs*. 57: 967-89 (1999).
8. Berzas, J.J., Rodriquez, J., Villasenor, M.J., Contento, A.M., and Cabello, M.P. Validation of a capillary gas chromatographic method for the determination of sildenafil citrate in its pharmaceutical formulations (Viagra). Experimental design for evaluating the ruggedness of the method. *Chromatographia*. 55: 601-6 (2002).
9. Pfizer, I. U.S. prescribing information. *Viagra: Compound data sheet.* 1 (1999).
10. Jeong, C.K., Lee, H.Y., Jang, M.S., Kim, W.B., and Lee, H.S. Narrowbore high-performance liquid chromatography for the simultaneous determination of sildenafil and its metabolite UK-103,320 in human plasma using column switching. *J Chromatogr B Biomed Sci Appl*. 752: 141-7 (2001).
11. Cooper, J.D., Muirhead, D.C., Taylor, J.E., and Baker, P.R. Development of an assay for the simultaneous determination of sildenafil (Viagra) and its metabolite (UK-103,320) using automated sequential trace enrichment of dialysates and high-performance liquid chromatography. *J Chromatogr B Biomed Sci Appl*. 701: 87-95 (1997).
12. Baselt, R.C. *Disposition of toxic drugs and chemicals in man*, 6th ed., Foster City, CA; Biomedical Publications (2002).
13. Kloner, R.A. and Jarow, J.P. Erectile dysfunction and sildenafil citrate and cardiologists. *Am J Cardiol*. 83: 576-82, A7 (1999).
14. Fraunfelder, F.T. and Laties, A.M. Visual side effects possibly associated with Viagra. *J Toxicol - Cut & Ocular Toxicol.* 19: 21-25 (2000).
15. TOXI-NEWS. Sildenafil (Viagra). TOXI-LAB, Irvine, CA. 18: 1 (1999).
16. Borrillo, D.J. Dangers of Viagra use in pilots. *Federal Air Surgeon's Medical Bulletin*. 98-3: 1-10 (1998).
17. Dumestre-Toulet, V., Cirimele, V., Gromb, S., Beloussoff, T., Lavault, D., Ludes, B., and Kintz, P. Last performance with Viagra: Post-mortem identification of sildenafil and its metabolites in biological specimens including hair sample. *Forensic Sci Int*. 126: 71-6 (2002).
18. Weinmann, W., Bohnert, M., Wiedemann, A., Renz, M., Lehmann, N., and Pollak, S. Post-mortem detection and identification of sildenafil (Viagra) and its metabolites by LC/MS and LC/MS/MS. *Int J Legal Med*. 114: 252-8 (2001).
19. Johnson, R.D., Lewis, R.J., Canfield, D.V., and Blank, C.L. Accurate assignment of ethanol origin in postmortem urine: liquid chromatographic-mass spectrometric determination of serotonin metabolites. *J Chromatogr B Analyt Technol Biomed Life Sci* 805: 223-34 (2004).
20. Umrani, D.N. and Goyal, R.K. Pharmacology of sildenafil citrate. *Indian J Physiol Pharmacol*. 43: 160-4 (1999).
21. *Physicians' Desk Reference*, 57th ed. Thompson PDR, Montvale, NJ (2003).
22. Walker, D.K., Ackland, M.J., James, G.C., Muirhead, G.J., Rance, D.J., Wastall, P., and Wright, P.A. Pharmacokinetics and metabolism of sildenafil in mouse, rat, rabbit, dog and man. *Xenobiotica*. 29: 297-310 (1999).