K9 An Analytical Protocol for the Identification of Sildenafil (Viagra®) in Specimens Commonly Submitted to the Toxicology or Analytical Laboratory

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Attendance at this presentation will enable the participant to learn a new analytical protocol for the determination of sildenafil (Viagra®) in specimens commonly submitted to the toxicology or analytical laboratory such as clinical biological specimens or tablets suspected to contain this substance.

As licit and illicit sildenafil use is on the increase due the ease with which this substance may be purchased via the internet, it is important for laboratories to have methods for its detection in unknown tablets or in biological specimens of those suspected or known to be using it for recreational or therapeutic purposes. This paper's contribution to the forensic community is that it offers a variety of analytical protocols for the identification of sildenafil.

Our Forensic Service offers a screening and quantification toxicology service to most of Her Majesty's Coroners and Forensic Pathologists in London as well as various Police Forces and one branch of the Armed Forces. As a result, we are required to screen for a large number of prescribed and illicit drugs in anteand post-mortem specimens followed by quantification of those detected. All analyses must be completed and our final report must be submitted to the Courts within 15 business days of the arrival of the case at the Service. In addition, we provide a tablet and capsule identification service to national and international clients using the commercially available product, TICTAC (www.tictac.org).

The methodology developed involves the detection of sildenafil in unknown tablets confiscated by the Police, purchased on the Internet or voluntarily surrendered by the public. Methods used included thin layer chromatography (TLC), UV-Visible spectrophotometry, gas chromatography – mass spectrometry (GC-MS) and high performance liquid chromatography – tandem mass spectrometry (HPLC-MS-MS). Each unknown tablet was weighed and approximately 10mg were removed from its core, dissolved in 10mL of methanol or 10mL of deionized water and sonicated for 45 minutes in an appropriately-labeled glass tube. The methanolic tablet mixture was centrifuged and approximately 10µL of the supernatant were spotted onto a silica bonded TLC plate alongside a standard mixture containing five standards (amitryptiline, dextropropoxyphene, methaqualone, morphine and nicotine) and a known positive sildenafil control prepared using the pure analyte of interest generously provided by the drug manufacturer, Pfizer Ltd. The TLC mobile phase consisted of methanol and ammonia (v/v 100:0.5). The developed plates were visualized under UV illumination and spots including those of the known and suspected sildenafil fluoresced. After spraying the developed plate with neutral and acidic iodoplatinate reagent, sildenafil produced a visible strong purple and brown colored spot, respectively. The response factor, Rf, for sildenafil was 0.66 whereas those for methaqualone, dextropropoxyphene, nicotine, amitriptyline and morphine were 0.85, 0.69, 0.58, 0.48 and 0.36, respectively. Each aqueous tablet mixture was adjusted to acidic or alkaline pH and subjected to UV-Visible spectrophotometry from 400 to 190nm. Very characteristic spectra were produced with strong absorbances noted at 290nm and 210nm. 1mL of each methanolic tablet mixture was added to 1mL of methyl tert-butyl ether (MTBE) and 100µL of internal standard (quinoline, pyribenzamine, flurazepam, 1mg/mL, respectively), mixed, centrifuged and an aliquot of the resulting supernatant (1µL) was injected onto a GC-MS comprising of the HP 5890 GC coupled to an HP 5971 MS. The analytical column used was Solgel (30m x 0.25mm i.d., 0.25µm film thickness). The injector was maintained at 250°C, the detector was maintained at 280°C and the column temperature program started at 70°C for 4 min, ramped 40°C/min and held at 280°C for 50.75 minutes giving a total run time of 60 minutes. Each unknown tablet together with positive and negative controls was screened in full scan mode and sildenafil was identified by its retention time (R_t), which measured 48.5 minutes. Each methanolic tablet mixture was further diluted in 80% aqueous methanol to an approximate final concentration of 0.1mg/L and 20µL were injected onto an HPLC-MS-MS. The ions monitored were 472.9 and 281.9. The total run time per sample was 3.5 minutes with sildenafil eluting at 2.4 minutes. The analytical column used was a 15cm x 4.6mm i.d., Supercosil LC-18-DB (5µm particle size) ODS column maintained at 50ºC using a Perkin Elmer series 200 column oven. Isocratic solvent delivery was achieved using a Perkin Elmer series 200 pump set at 1mL/min. Sample injection, 20µL, was performed by a Perkin Elmer series 200 auto-injector. The mobile phase consisted of methanol/water (v/v 85:15) supplemented with ammonium acetate solution to achieve a final concentration of 2mmol/L. Detection was by tandem mass spectrometry (HPLC-MS-MS), using a Sciex API 2000 triple quadrupole mass spectrometer (Applied Biosystems). A turbo ion spray (heated electrospray) source heated to 300°C was used to introduce the sample into the mass spectrometer. A post-column splitter (10:1) was installed just before the ion spray interface. The mass spectrometer was operated in positive ionization, multiple reaction mode (MRM, MS-MS), with the resolution set to unit resolution (± 0.5m/z). High purity air was used as the nebulizer gas and high purity nitrogen as the collision gas. The Applied Biosystems Sciex Analyst software was used to control the HPLC-MS-MS, record the output from the detector, integrate and calculate peak areas. In assays requiring quantification, the Analyst software was used to calculate the peak area

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ratios, produce the calibration line using $1/x^2$ weighed through zero regression and to calculate the concentration of sildenafil.

Finally, the HPLC-MS-MS analytical protocol was successfully used to screen post-mortem human blood from cases in which sildenafil was suspected to be involved. To compensate for the lack of a suitable internal standard, analytical standards were prepared and analyzed in duplicate in deionized water and added to 100µL of sildenafil-free human blood. 100µL of the case blood specimen were added to 100µL of deionized water. 250µL of phosphate buffer (pH 7.0) and 1mL of MTBE were added to the case specimen and standards. The solutions were then mixed for 5 minutes and centrifuged at 3500rpm for 5 minutes. The supernatant for each tube was collected and evaporated to dryness using a Savant SpeedVac SC200 coupled to a Savant RT4104 refrigerated condensation trap. The residue was then reconstituted in 250µL of 80% aqueous methanol, vortex mixed for 30 seconds and injected onto the HPLC-MS-MS using the analytical protocol described above.

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