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A rapid, quantitative liquid chromatography-mass spectrometry screening method for 71 active and 11 natural erectile dysfunction ingredients present in potentially adulterated or counterfeit products

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ABSTRACT

A rapid LC-MS/MS method has been developed to simultaneously separate 71 erectile dysfunction (ED) drugs and 11 natural ingredients that are sometimes found alongside ED drugs, present in suspected adulterated or counterfeit samples. The separation was achieved in 10 min using 2.6 μm fused-core C_{18} particles in a 100 × 2.1 mm column coupled to an LTQ Orbitrap XL mass spectrometer operated in positive electrospray mode. Using a straightforward methanolic extraction procedure, recovery from real samples (tablets, capsules, oral liquids and herbal products) was 92–111% and the lower and upper limits of detection and quantification were in the sub ng/mL and the sub μg/mL ranges, respectively. The intra- and inter-assay precision were ≤3.2% and 10.4% respectively across three concentrations of standards (50, 250 and 1000 ng/mL) measured for 4 representative drugs spiked into a tablet-based matrix. This behavior was consistently observed for all the other compounds. The mass accuracy was less than 3 ppm. Moreover, an advantage of this method is that the full scan event in the acquisition method associated with the high resolution of the Orbitrap XL allows post-analysis identification, in an untargeted approach, of additional species in the complex matrices. Our LC-MS/MS method for ED drugs was successfully applied to 32 samples and the drug identifications were in 100% agreement with those obtained by the conventional methods HPLC-UV and GC-MS. Following the complete validation of the ED method, it has been introduced in the current counterfeit identification procedures at Health Canada.

1. Introduction

Phosphodiesterase type 5 (PDE-5) inhibitors, which are clinically indicated for treatment of erectile dysfunction (ED), are widely available on the illegal market and as undeclared adulterants [1–5]. Some suppliers deliberately adulterate herbal medicines or dietary supplements with these synthetic compounds, or related substances, claiming to enhance sexual abilities and vitality in a natural, safe, healthy way. Others try to replicate and thus infringe on the patented drugs and sell them at a lower price. Products from these unethical sources are often made in unsanitary environments, can contain more ED drugs than recommended and the active ingredients are often mislabelled; combinations of 3–5 compounds may be present [6–12]. Aside from the drugs sildenafil citrate (Viagra), tadalafil (Cialis) and vardenafil hydrochloride (Levitra), few formal studies have been performed on the many analogues of PDE 5 inhibitors. The assumption that such analogues have toxicity and clinical effect similar to the parent compound is very dangerous. Since Health Canada's mission is to maintain and improve the health of Canadians, it was essential to develop fast screening methods to analyze as many PDE-5 inhibitors and their analogues as possible.

Erectile dysfunction drugs encompass a range of compounds that include many closely related structures and the presence of isomers, therefore requiring the use of a powerful analytical separation method like LC-MS/MS. An excellent review article by Venhuis and de Kaste [13] puts this challenge into perspective and also addresses the history and health risks of PDE-5 inhibitor analogues in food supplements. Also present in natural (e.g., herbal) samples are natural ingredients such as flavonoids and isoflavonoids. Those from the *Epimedium* species (icariin, epimedin A, epimedin B, epimedin C, icaritin and baohuoside I) have beneficial properties (anxiolytic, antioxidant, etc) and are touted to improve sexual performance; icariin is known to have PDE-5 inhibitor-like activity [14,15]. Flavonoids from the *Scutellaria* species (scutellarin, baicalin, wogonin) also show anxiolytic activity [16]. Thus the

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identification of 11 natural products was also incorporated in the method development, not only because of their potential to have PDE-5 inhibitor-like activity but also because they are present in some herbal products sold to treat ED.

Dapoxetine, which is a short-acting selective serotonin reuptake inhibitor (SSRI) designed to treat depression, has been shown effective to treat premature ejaculation (PE) even though it is not typically labeled for this use [17,18]. Patients with ED and PE may be prescribed both dapoxetine and a PDE-5 inhibitor simultaneously whereas some products sold to enhance sexual performance already contain both, e.g., Tadapox (tadalafil and dapoxetine) and Snovitra (vardenafil and dapoxetine).

The original goal was to develop a fast screening method for identification and quantification of 10 ED drugs in suspected adulterated or counterfeit medications and dietary supplements by high resolution mass spectrometry, which was achieved in 5 min with high accuracy and reproducibility for sildenafil citrate, vardenafil HCl, tadalafil, nortadalafil, dimethylsildenafil, homosildenafil, norneosildenafil, pseudovardenafil, thiodimethylsildenafil and hydroxythiohomosildenafil. Even though these 10 compounds are the most often found in the adulterated samples, we realized that many other ED drugs were also present and thus a better and wider screening method was needed. Most reports describe the separation of only a small number of ED drugs and these required run times longer than 20 or even 30 min. In reports where identification was achieved in less than 5 min, the researchers were monitoring only 2–5 compounds [6–12]. To the best of our knowledge, no separation method has been reported for the simultaneous determination of 71 ED-active ingredients and 11 natural ingredients in just 10 min.

2. Materials and methods

2.1. Reagents and standards

LC-MS grade methanol and acetonitrile were purchased from J.T. Baker (Tekniscience, Canada) and LC-MS grade formic acid (98%) was bought from Fluka (Sigma–Aldrich, St. Louis, MO). HPLC grade water from a Milli-Q Reference A+ system (Fisher Scientific, Canada) was used to prepare all aqueous solutions and eluants.

The following 82 standards, whose chemical structures are shown in Fig. 1 and whose purity and supplier are provided in the Supporting Information, were used in this study: 2-(2-ethoxyphenyl)-5-methyl-7-propyl-3H-imidazo[5,1-f][1,2,4]triazin-4-one, acetil acid, acetildenafil (hongdenafil), acetylvardenafil, aminotadalafil, apixaban, avanafil, baicalin, baohuoside I, benzyl sildenafil, carbodenafil, chlorodenafil, chloropretadalafil, cinnamyldenafil, cyclopentynafil, daidzein, dapoxetine, demethylpiperazimyl sildenafil, desmethyl carbodenafil, dimethyl acetildenafil, dimethylsildenafil, dioxohongdenafil, dithio-desmethyl carbodenafil, epimedin A, epimedin B, epimedin C, gendenafil, genistein, homosildenafil, hydroxyacetildenafil, hydroxychlorodenafil, hydroxyhomosildenafil, hydroxythiohomosildenafil, hydroxythiovardenafil, hydroxyvardenafil, icariin, icaritin, imidazosagatriazinone (desulfovardenafil), N-butyl tadalafil, N-desmethyl acetildenafil, N-desmethyl sildenafil, N-desmethyl vardenafil, N-ethyl tadalafil, nitrodenafil, N-octyl-nortadalafil, nor-acetildenafil (desmethylacetildenafil), norneosildenafil, norneovardenafil, nortadalafil, O-desmethyl sildenafil sildenafil impurity C), oxohongdenafil, papaverine, Phentolamine mesylate, piperiacetildenafil, propoxyphenyl sildenafil, pseudovardenafil, pyrazole N-desmethyl sildenafil, scutellarin, sildenafil amine HCl, sildenafil analogue (propoxyphenyl-aildenafil), sildenafil Analogue I (propoxyphenyl-thiohydroxyhomosildenafil), sildenafil Analogue III (propoxyphenyl-thioaildenafil), sildenafil chlorosulfonyl, sildenafil citrate, sildenafil coupled, sildenafil-d_8 (internal standard), sildenafil dimer impurity, sildenafil 2nd step impurity, sildenafil impurity A (isobutyl sildenafil), tadalafil, tadalafil impurity A, tadalafil impurity B, tadalafil impurity C, tadalafil impurity D, thiodimethyl sildenafil (dimethylthiosildenafil), thiohomosildenafil, thiomethisosildenafil (thioaildenafil; sulfoaildenafil) sildenafil impurity Z, thiosildenafil, udenafil, vardenafil HCl, wogonin, yohimbine. Stock solutions of individual standards were prepared separately in 10 mL volumetric flasks at an approximate concentration of 100 μg/mL in methanol, with small quantities of acetonitrile added if required for solubilisation. Diluted stock solutions (100 ng/mL–1 μg/mL) were directly infused into the mass spectrometer for adjustment of the experimental parameters for each analyte as described in Section 2.3.

2.2. Samples and sample preparation

Viagra (25 mg) from Pfizer (Kirkland, Canada), Cialis from Eli Lilly (Toronto, Canada), Levitra from Bayer Health Care (Toronto, Canada) and 32 samples were analysed to determine the effectiveness and robustness of the LC-MS/MS method. Because some of the samples analysed during this project have not yet been released by Health Canada, only the dosage form and the detected analytes have been presented.

The samples analysed were present in different forms: tablets, conventional and liquid–gel capsules, oral liquids and herbal samples. Tablets and herbal products were finely ground. Aliquots of 3–5 mg of the resulting powder were transferred to 10 mL volumetric flasks and dissolved in a mixture of methanol:water:acetonitrile (70:20:10) containing 0.1–1% formic acid by treatment with vortex for 2 min, sonication for 10 min and vortex again for 3 min. The supernatant was filtered through a 0.45 μm pore polytetrafluoroethylene (PTFE) syringe filter (Phenomenex, Torrence, CA). The more complex herbal samples required an added centrifugation step at 3500 rpm for 10 min to reduce the possibility of mass overloading the syringe filter. Supernatants, which contain the analyte, were diluted from 10 to 100-fold in water:acetonitrile (75:25), the initial mobile phase, before injection to avoid detector saturation and to maintain mass accuracy.

In the case of liquid-based oral samples, a 100 μL aliquot was mixed with 1 mL of the same ternary solvent as above and then the previous procedures were applied, except centrifugation. For gel capsule-based samples, 20 mL of 0.1–1% formic acid in water was added to the whole capsule (the casing included) in a 50 mL volumetric flask and shaken vigorously on a mechanical shaker for 10 min followed by sonication until the capsule opened (10–20 min). The solution was then diluted to volume with methanol. A second ultrasonic treatment was applied for 5-10 min then the solution was filtered thought a 0.45 μm pore PTFE filter and finally diluted 100-fold in the initial mobile phase.

2.3. LC-MS/MS operating conditions

Data were acquired on an LTQ Orbitrap XL (Thermo Scientific, San Jose, CA) coupled to an Acella HPLC system (Thermo Scientific) equipped with an autosampler and Acella 600 pump. Xcalibur 2.1 and Thermo LTQ Tune Plus 2.5.5 software (Thermo Scientific) were used to control the system and process the data. External mass calibration was used for all studies.

Amongst the available reversed-phase columns in our laboratory, five were tested for separation of the 82 standards: an XTerra C_{18} (100 × 2.1 mm, 3.5 μm particles) from Waters (Mississauga, Canada); a Kinetex C_{18} (75 × 2.1 mm, 2.6 μm particles) from Phenomenex (Torrance, CA); a Halo C_{18} (100 × 3.0 mm, 2.7 μm particles) from Canadian Life Science (Montreal, Canada);