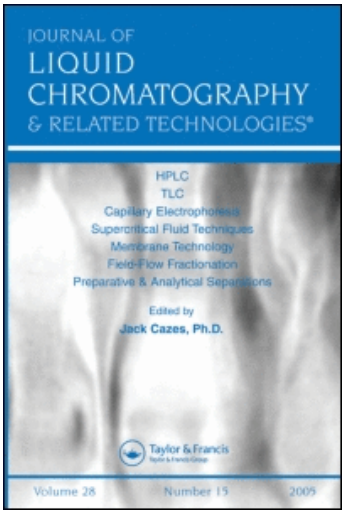

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M. E. Abdel-Hamid[a]
[a] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Kuwait University, Safat, Kuwait

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Determination of Sildenafil, Tadalafil, and Vardenafil in Tablets and Adulterated Herbal Products by ESI-MS-MS

M. E. Abdel-Hamid
Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Kuwait University, Safat, Kuwait

Abstract: An electrospray tandem mass spectrometry (ESI-MS-MS) method was developed for the analysis of selected phosphodiesterase type 5 (PDE-5) inhibitors, namely, sildenafil, tadalafil, and vardenafil in pharmaceutical preparations and adulterated herbal products. The method permits a fast (<3 min), selective detection and determination of these compounds in the presence of bulk powders of tablets or bulk herbal matrices of herbal products. Detection of sildenafil or tadalafil in adulterated herbal formulas was simply accomplished by recording the daughter scan of the product's extract and picking up significant fragment ions for the identification of PDE-5 inhibitors. Quantification of sildenafil, tadalafil, and vardenafil, was achieved using multiple reaction monitoring (MRM) ion chromatograms at m/z $475 > 100$, $390 > 268$, $489 > 169$, respectively. The concentration of each compound was determined from the calibration curve constructed by the internal standard calibration using phenylalanine (m/z $166 > 103$) as an internal standard. Validation data showed that the developed tandem mass procedure was linear (r: 0.99), accurate (%DEVs $\pm$ 10%), and reproducible (RSD%. $< 7.5\%$) with a LLOQ of 20.0 ng mL^{-1}. Recovery studies of pre-analyzed tablets spiked with known amounts of PDE-5 inhibitors showed average percents of 100.4 (sildenafil), 101.5 (tadalafil), and 101.0 (vardenafil). Application of a tandem mass procedure to the analysis of three marketed herbal products with sexual enhancement properties, revealed the presence of undeclared sildenafil or tadalafil compounds at concentrations 43.5 and 33.9 mg of sildenafil and 7.7 mg of tadalafil. The derived data suggested that the developed tandem mass spectrometric method can be adopted

Address correspondence to Dr. M. E. Abdel-Hamid, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Kuwait University, P.O. Box 24923, Safat 13110, Kuwait. E-mail: abdel-hamid@hsc.edu.kw

for routine analysis of either dosage forms or herbal products adulterated with of PDE-5 inhibitors.

Keywords: ESI-MS-MS, PDE-5 inhibitors, Sildenafil, Tadalafil, Vardenafil, Dosage forms, Herbal products

INTRODUCTION

Recently, chemically synthetic phosphodiesterase-5 (PDE-5) inhibitors have been extensively used to improve erectile dysfunction in men. Sildenafil (Viagra®) was the first PDE-5 inhibitor licensed for clinical use.[1]In the last 2 years, two other PDE-5 inhibitors, tadalafil (Cialis®) and vardenafil (Levitra®) have been marketed. Although, pharmaceutical products containing PDE-5 inhibitors should be dispensed under medical supervision, however, in many Arab countries, these products are dispensed without doctor's prescription. Recently, herbal medicines have been greatly dispensed as alternatives of chemically synthetic products. People have the belief that natural substances are safer and healthier than synthetic derivatives. In the Gulf Region, herbal products are frequently administered for management of various disorders such as erectile dysfunction, diabetes, hypertension, etc, and for solving problems related to over weight or under weight bodies. Clinical studies have indicated that concomitant administration of PDE-5 inhibitors and nitrates (as anti-anginal agents) can produce adverse effects on the blood pressure.[2] Recently, clinical studies have also reported eye blindness in one eye in some cases, as an adverse reaction of taking Viagra® tablets.[3] Therefore, administration of the herbal products, which might be adulterated with synthetic PDE-5 inhibitors can produce undesirable consequences. On the other hand, pharmaceutical products of PDE-5 inhibitors might be admixed with drug intermediates or degradation products that can produce toxic effects. For purposes of quality control and product safety, it is necessary to establish analytical procedures which can selectively detect and quantify PDE-5 inhibitors in dosage forms, as well as in adulterated herbal products. As shown in the literature, the majority of the reported methods were described for the analysis of sildenafil in dosage forms, dietary supplements, and biological fluids. Reversed-phase HPLC,[4] liquid chromatography mass spectrometry,[5] capillary gas chromatography,[6] and spectrometric[7] methods were reported. Recently, LC-ESI-MS procedure was described for the analysis of undeclared synthetic PDE-5 inhibitors in dietary supplements.[8] The present paper reports on the application of tandem mass spectrometry for screening and quantification of sildenafil, tadalafil, and vardenafil in commercial samples using daughter and MRM scanning modes, respectively. The chemical structure of molecular and fragment ions of the examined compounds according to MS-MS profiles were proposed. Validation studies of the developed tandem mass method

for routine analysis of PDE-5 inhibitors in pharmaceutical dosage forms and adulterated dietary products were elucidated.

EXPERIMENTAL

Materials

Pharmaceutical grades of sildenafil citrate, tadalafil, and vardenafil hydrochloride were used. Phenylalanine (IS) was purchased from (Sigma-Aldrich, USA). Methanol and acetonitrile (HPLC grade) were supplied from Fischer Scientific and formic acid (98–100%) was purchased from Surechem Products, UK. All other reagents were of analytical grade. Water was purified using Milli-Q device (Millipore, Bedford, MA, USA). Syringe filters (Millipore) were 20 mm diameter Nylon disc-filters with pore size of 0.45 μm.

Standard Solutions

Stock solutions of sildenafil citrate, tadalafil and vardenafil hydrochloride were separately prepared in acetonitrile/water 50:50 v/v at concentrations of 1 $\mu g\,\mu L^{-1}$ and were stored at 4°C. Working standard solutions were daily prepared in the same solvent at concentrations of 1 $ng\,\mu L^{-1}$. Calibrators were prepared in mobile phase (acetonitrile/water/formic acid 50:50:0.025 v/v/v) in a concentration range 20–100 $ng\,mL^{-1}$. The solutions were mixed with 40 μL of 0.05 $\mu g\,\mu L^{-1}$ of IS before final dilution to 1 mL volume. The calibrators were placed in the autosampler and an aliquot volume of 20 μL of each standard was directly injected into mass spectrometer without column separation. The mobile phase was delivered at flow rate of 0.1 $mL\,min^{-1}$ and the run-cycle time was ~2–3 min for all runs.

Mass Spectrometry

A triple quadrupole tandem mass spectrometer (Quattro LC, Micromass, Manchester, UK) fitted with Z-spray ion source and connected to a positive electrospray ionization probe was used. The system was coupled to Waters Alliance 2690 LC and Waters Autosampler (Waters Association, Milford, MA, USA). The mass spectrometer permits the running of different scanning modes including daughter (MS-MS) scan for screening and MRM scan for quantification studies. System operation and data acquisitions were controlled by MassLynx NT 3.5 software. Tuning parameters for MS-MS and MRM analysis of PDE-5 inhibitors and IS were adjusted by direct infusion of separate solutions of PDE-5 inhibitors and IS in the mobile phase to the ionization probe, at flow rate 10 $\mu L\,min^{-1}$, using a Harvard syringe pump. The ion source and desolvation temperatures were fixed at

100 and 250°C, respectively. A capillary voltage of 3.2 kV and cone voltage of 30 V were used for PDE-5 inhibitors and IS. The values of collision energy were 40 eV (sildenafil), 20 eV (tadalafil), 30 eV (vardenafil), and 15 eV (IS), respectively. Based on MS-MS data, the MRM scans for sildenafil, tadalafil, vardenafil, and IS were m/z $475 > 100$, $390 > 268$, $489 > 169$, and $166 > 103$, respectively.

Validation Study

The developed tandem mass method for the determination of PDE-5 inhibitors was validated. The linearity was evaluated by preparing and analyzing five calibrators of each compound in the concentration range 20–100 ng mL^{-1}, using the appropriate MRM transition. The slope, intercept, and regression coefficient were determined by the weighting method (1/x) of the least squares linear regression analysis. The lowest limit of quantification (LLOQ) was calculated on the basis of the lowest concentration of each compound that gives RSD% and %DEVs <20%. The precision and accuracy were evaluated by determining the RSD% and %DEVs for QC samples containing sildenafil or tadalafil or vardenafil at 40 ng mL^{-1} (low) and 100 ng mL^{-1} (high) concentrations.

Sample Analysis

Content Uniformity of PDE-5 Inhibitors (Sildenafil, Tadalafil, and Vardenafil) in Film-Coated Tablets

A film-coated tablet of either sildenafil or tadalafil or vardenafil dosage form was powdered and carefully transferred into a 100 mL volumetric flask. The powder was mixed with ~50 mL of mobile phase, sonicated for 15 min and then completed to volume with the same solvent. The mixture was filtered through a 0.45 μm nylon syringe filter and collected in a new flask. An appropriate volume of clear filtrate was mixed with IS solution and was appropriately diluted with mobile phase. A 20 μL aliquot was injected into the mass spectrometer and the MRM scans at m/z $475 > 100$, $390 > 268$, and $489 > 169$, respectively, were selected for quantification of sildenafil, tadalafil, and vardenafil, respectively, using the MRM transition at m/z $166 > 103$ for IS. The concentration of analyte was automatically computed from the regression equation of the calibration curve run simultaneously with the samples.

Detection and Quantification of PDE-5 Inhibitors (Sildenafil, Tadalafil) in Herbal Products

The content of an herbal product was carefully transferred into a 100 mL volumetric flask, mixed with ~50 mL of the mobile phase, sonicated for

15 min and then completed to volume with the same solvent. A portion of the extract was filtered through a 0.45 μm nylon syringe filter and was suitably diluted with mobile phase. A 20 μL aliquot was injected into the mass spectrometer and the MS/MS and MRM profiles were recorded. Identification of a PDE-5 inhibitor was done by matching the spectrum with standard spectra of PDE-5 inhibitors, whereas quantification was achieved by reference to the appropriate calibration curve using a MRM chromatographic scan.

Recovery Studies

Recovery experiments were performed to judge the reliability and suitability of the developed MS-MS for analysis of PDE-5 inhibitors in tablets and adulterated herbal products. This was achieved by adding known amounts of sildenafil or tadalafil or vardenafil authentic powder to the pre-analyzed powdered tablets. An appropriate sample of the mixed powder of each compound was treated as above. The percent recoveries were estimated by comparing the concentrations calculated from spiked samples with the nominal concentrations added.

RESULTS AND DISCUSSION

Recently, a positive LC-ESI-MS procedure has been described for the analysis of PDE-5 inhibitors in pharmaceutical preparations and adulterated dietary supplements.[8] According to this method, screening of PDE-5 inhibitors in dietary products was based on measuring of the MS profiles of analytes following a complete LC separation, whereas determination of PDE-5 inhibitors was based on UV detection. Since the chemical structure and chromatographic behavior of PDE-5 inhibitors were close, chromatographic separation was crucial to distinguish and determine the analytes and to eliminate matrix interference from bulk powder of tablets or herbal materials. Recently, tandem mass spectrometry (MS-MS) has been extensively used in our laboratories for screening and quantification of drugs and biomolecules in biological matrices, pharmaceutical formulations, and plant extracts.[9–13] The technique relies on measurement of the parent/daughter couple of an analyte in the mixture. This permits significant enhancement of selectivity and sensitivity for screening and quantification purposes using daughter (MS-MS) and MRM scans without a complete chromatographic resolution. Initially, the MS and MS-MS profiles for the analysis of sildenafil, tadalafil, and vardenafil via positive electrospray ionization (+ESI), were optimized by adjusting the major ESI parameters such as ion source and desolvation temperatures, capillary voltage, cone voltage, and collision energy.

Separate solutions of the analytes and IS were prepared in the mobile phase and were directly infused to the ionization probe. The ion source and desolvation temperatures were fixed at 100 and 250°C, respectively.

A capillary voltage of 3.2 kV and cone voltage of 30 V were used for PDE-5 inhibitors and IS. The values of collision energy were 40 eV (sildenafil), 20 eV (tadalafil), 30 eV (vardenafil), and 15 eV (IS), respectively. The composition of the mobile phase (acetonitrile/water/formic acid 50:50:0.025 v/v/v) was found to be the most appropriate in terms of ionization enhancement, analysis time, and detection of analytes. Under the selected liquid chromatography-mass spectrometry conditions, sildenafil, tadalafil, and vardenafil produced their molecular ions $[M + H]^+$ at m/z 476, 390, 489, respectively. These molecular ions were used in MS-MS experiments to

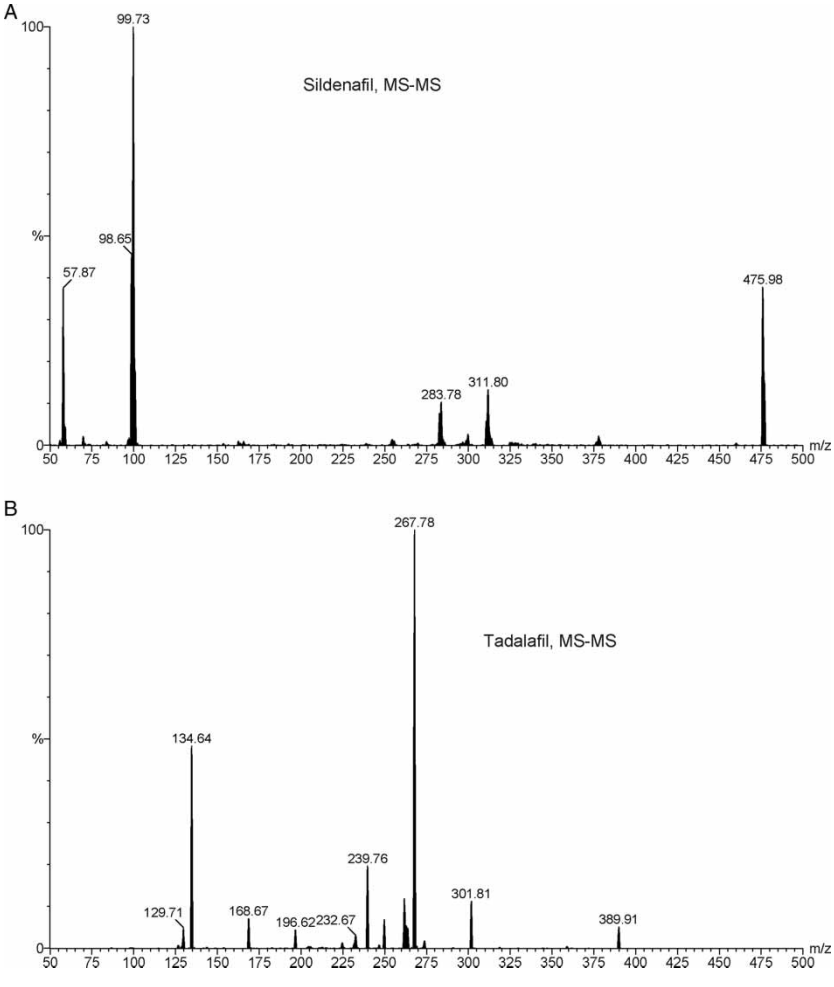

Figure 1. A. MS-MS of sildenafil. B. MS-MS of tadalafil. C. MS-MS of vardenafil. D. MS-MS of Phenylalanine (IS).

(*continued*)

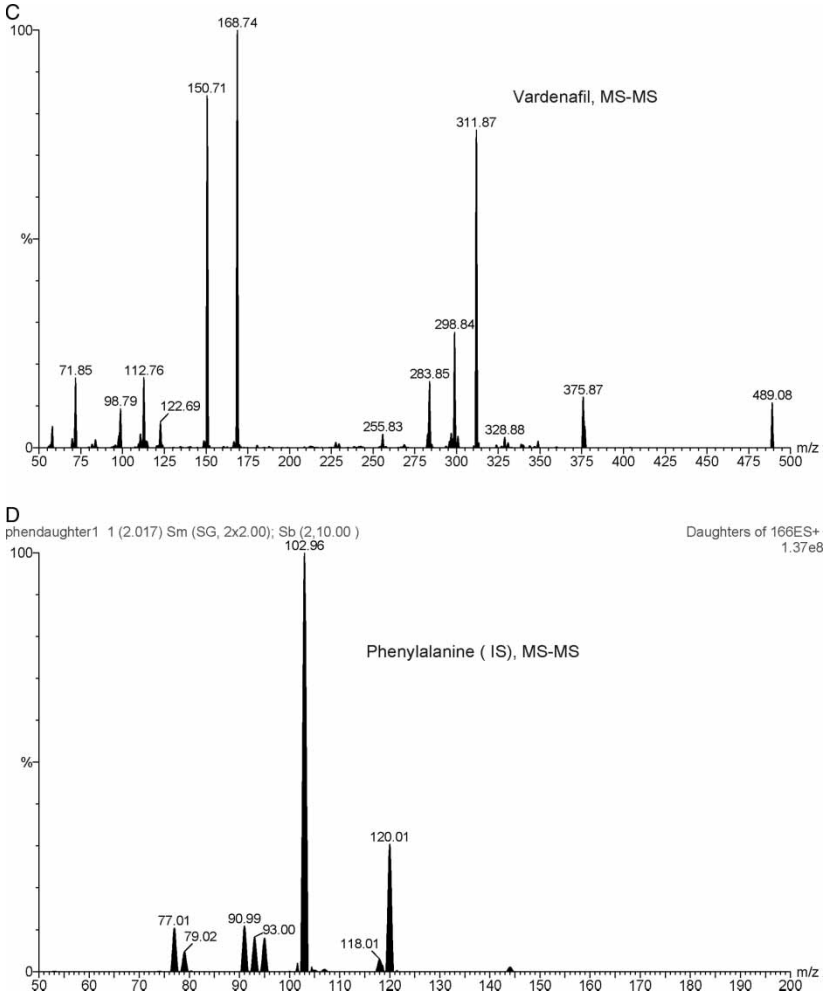

Figure 1. Continued.

generate the corresponding fragment ions. Figure 1A–D displayed MS-MS scans of sildenafil, tadalafil, vardenafil, and IS with the characteristic molecular and fragment ions. The proposed chemical structure of these ions are shown in Figure 2. As indicated, sildenafil exhibits a strong fragment ion at m/z 100, which was not found in the MS-MS profiles of either vardenafil or tadalafil. This signal was attributed to N-methylpiperazine. Although vardenafil has a chemical structure that is close to that of sildenafil (only one nitrogen atom difference), the fragmentation patterns are different. Vardenafil displayed characteristic signals at m/z 169 and 150 due to hydrated and anhydrous N- sulfonylpiperazine. These signals are completely absent in the

spectrum of sildenafil. Tadalafil was identified by a strong signal at m/z 268, which was attributed to indolepiperidindione moiety. The nominated fragments were selected for quantification of sildenafil, tadalafil, and vardenafil using MRM, at m/z 475 > 100, 390 > 268, and 489 > 169, respectively.

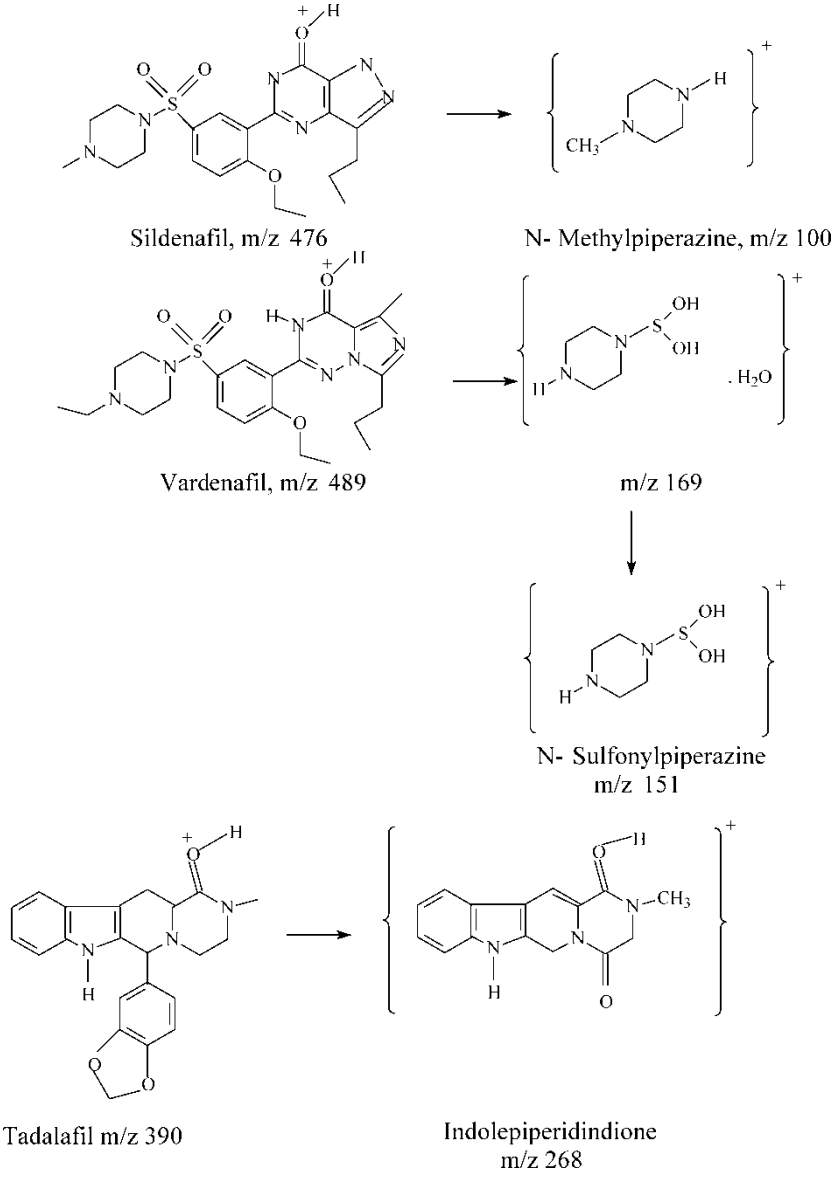

Figure 2. Molecular and fragment ions of sildenafil, tadalafil, and vardenafil determined by MS-MS.

The MRM scan at m/z 166 > 103 was selected for IS. The MRM scans were found to be highly specific for analysis of mixtures of sildenafil, tadalafil, and vardenafil without a preliminary chromatographic separation (Figure 3). The run-cycle time was ~2–3 min injection-to-injection, which allows high sample throughput for routine analysis. The combination of MS-MS spectral data along with MRM chromatographic data provides a fast and specific method for analysis of PDE-5 inhibitors.

Method validation was necessary before the application of the developed tandem mass procedure to commercial products. The linearity was established by plotting the peak area ratio of an individual compound to IS vs. concentration over the concentration range 20–100 ng mL^{-1}. Calculations were automatically derived by the quantifying program of the instrument software using weighting least squares linear regression analysis. Linear correlations of the peak area ratio and concentration of analyte was achieved (r: 0.99). Mean values of slope and regression coefficient for 10 calibration curves of each analyte are reported in Table 1. The LLOQ was found to be 20 ng mL^{-1} of the examined PDE-5 inhibitors (RSD% <12% and %DEVs <20%). The derived values indicate good reproducibility and sensitivity of the method. Quality control samples containing known concentrations of either sildenafil or tadalafil or vardenafil at 40 and 100 ng mL^{-1}, were analyzed by the developed method. The RSD% and %DEVs values were <7.5% and ±10%, respectively, indicating good precision and accuracy (Table 2).

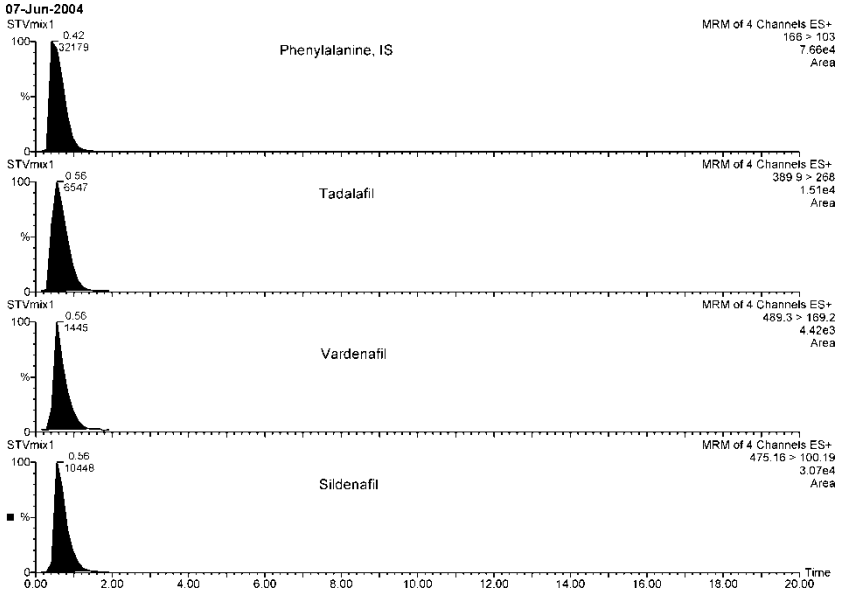

Figure 3. MRM chromatogram of a mixture of sildenafil (50 ng mL^{-1}), tadalafil (50 ng mL^{-1}), vardenafil (50 ng mL^{-1}) and IS (2 μg mL^{-1}).

Table 1. Linearity parameters for determination of sildenafil, tadalafil and vardenafil by tandem MS method

Conc. range ng mL^{-1}	Slope ± SD	RSD (%)	Correlation coefficient ± SD	RSD (%)
Calibration curves[a]				
Sildenafil				
20–100	0.0064 ± 0.0001	1.6	0.99 ± 0.005	0.51
Tadalafil				
20–100	0.0039 ± 0.0002	5.1	0.98 ± 0.008	0.82
Vardenafil				
20–100	0.0122 ± 0.001	8.2	0.99 ± 0.004	0.40
	Calculated Conc. ± SD	RSD (%)	DEV (%)[b]	
LLOQ[a]				
Sildenafil	19.3 ± 0.9	4.7	−9.3 to +6.1	
Tadalafil	19.9 ± 2.3	11.6	−7.0 to +17.0	
Vardenafil	20.8 ± 2.2	10.6	−8.4 to +10.5	

[a] n = 10.
[b] Nominal concentration 20 ng mL^{-1}.

The validation data encouraged the utility of the described tandem mass method in the analysis of tablets of sildenafil, tadalafil, and vardenafil after an extraction procedure with mobile phase. The data obtained are summarized in Table 3. Using the standard addition method, recovery experiments of the spiked amounts of analytes to pre-analyzed tables, were calculated. Recovery percents of 100.4–101.5% indicated good correlation of the

Table 2. Precision and accuracy of the tandem MS method for determination of sildenafil, tadalafil and vardenafil

Compound	Nominal concentration ± SD	Found[b] concentration ± SD	Precision[a] RSD (%)	Accuracy[a] DEV (%)
Sildenafil	40 ng mL^{-1}	40.7 ± 2.7	6.6	−6.0 to + 9.0%
	100 ng mL^{-1}	100.2 ± 1.9	1.9	−2.2 to + 2.9%
Tadalafil	40 ng mL^{-1}	41.7 ± 3.0	7.2	−6.3 to +8.0%
	100 ng mL^{-1}	101.4 ± 4.3	4.2	−4.1 to +5.8 %
Vardenafil	40 ng mL^{-1}	39.5 ± 1.9	4.8	−6.0 to +3.5%
	100 ng mL^{-1}	101.1 ± 2.8	2.8	−2.1 to +5.5%

[a] n = 10.
[b] Mean of 10 determinations.

Table 3. Assay results and recovery analysis of sildenafil, tadalafil and vardenafil in tablets and adulterated herbal products

	Tablets		
	Viagra®[a]	Cialis®[a]	Levitra®[a]
Labelled claim (mg/tablet)	50 mg	20 mg	10 mg
Mean found (mg) + SD	48.5 ± 1.4	18.6 ± 1.3	9.5 ± 0.6
RSD %	2.9	7.0	6.3
Added (mg)	50 mg	20 mg	10 mg
Recovered	50.2 ± 0.3	20.3 ± 0.5	10.1 ± 0.3
RSD (%) of recovery	0.6	2.5	3.0
Recovery (%)	100.4	101.5	101.0

Herbal products

Product form	Undeclared PDE-5	Found concentration (mg) ± SD[b]
Capsules	Sildenafil	43.5 ± 2.3
Tablets	Sildenafil	33.9 ± 2.8
Capsules	Tadalafil	7.7 ± 1.2

[a]Viagra® tablets labelled to contain 50 mg of sildenafil citrate, Cialis® tablets labelled to contain 20 mg of tadalafil, Levitra® labelled to contain 10 mg of vardenafil hydrochloride trihydrate.

[b]n = 5.

calculated and nominal concentrations added. Furthermore, no interference from tablet additives in the analysis of sildenafil, tadalafil, and vardenafil could be detected (Table 3).

The sensitivity and specificity of the developed MS-MS method for quality control purposes was evaluated by analyzing 3 herbal products marketed as herbal formulations with sexual enhancement properties. Analysis of the extracted products revealed the presence of tadalafil in one and sildenafil in two commercial products. The adulterants were qualitatively detected from the daughter profiles, which were matched with sildenafil or tadalafil reference spectra (Figures 1A–C). The concentration of adulterant was further determined from the calibration curves of sildenafil or tadalafil using MRM transitions at m/z 475 > 100 and m/z 390 > 268, respectively. Average contents of 43.5 and 33.9 mg of sildenafil and 7.7 mg tadalafil were determined (Table 3).

CONCLUSIONS

A direct tandem mass spectrometric method was described for screening and quantification of sildenafil, tadalafil, and vardenafil in tablets and adulterated

herbal products. The MS-MS profiles were more sensitive and specific than MS profiles for the detection of any undeclared PDE-5 inhibitors in herbal products. Furthermore, the method was accurate and reproducible for measurement of sildenafil, tadalafil, and vardenafil in commercial tablets. The high levels of sildenafil or tadalafil detected in the herbal products might be dangerous if these products were not properly tested by drug quality control laboratories, or if they were taken without medical prescriptions. The described method presents a highly reliable technique for rapid detection of drugs that are illegally added to herbal products. By using the technique of tandem mass spectrometry in our laboratory, it was possible to detect cyproheptadine in weight-gain herbal preparations and phenolphthalein, amphetamines in weight loss herbal products (unpublished data).

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