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Development of a Robust Method for Screening PDE-5 Inhibitor Additives in Dietary Supplements Using UPLC-MS/MS

Hua Wang [a*]

[a] *Instrumental Analysis Center, Yancheng Teachers University, Yancheng-224007, China.*

Author's contribution

The sole author designed, analyzed, interpreted and prepared the manuscript.

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ABSTRACT

A novel ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) method was developed to detect the illegal additive of phosphodiesterase type 5 (PDE-5) inhibitors in dietary supplements. With the optimized chromatographic program, vardenafil, sildenafil, and tadalafil were separated within 5 minutes. The MS^1, MS^2, and retention time of PDE-5 inhibitors were acquired simultaneously within the information dependent acquisition mass spectrometry mode. Quantification was achieved via the quantity ion current chromatogram that was extracted from the total ion current. The linear range of vardenafil and sildenafil was 0.5-48 mg/L while the range of tadalafil was 0.3-36 mg/L. The correlation coefficients of the calibration curves of three PDE-5 inhibitors were all greater than 99.9%. At three concentration levels, the RSD values of the five repeat tests were all better than 1.30%. Sildenafil was detected in one dietary supplement. The comprehensive method of this study is reliable and may be a powerful tool for routine PDE-5 inhibitor screening and determination.

**Corresponding author: Email: wangh@yctu.edu.cn;*

Keywords: PDE-5 inhibitor; illegal additive; dietary supplements; tandem mass spectrometry.

1. INTRODUCTION

Phosphodiesterase type 5 (PDE-5) is an enzyme responsible for the decomposition of cyclic guanosine monophosphate [1]. PDE-5 inhibitors are the first-line drugs for the treatment of male erectile dysfunction (ED) [2]. In addition, PDE-5 inhibitors have great potential in the treatment of neuroinflammation, neurodegeneration [3], cancer, diabetic peripheral neuropathy [4], and kidney protection [5]. However, some PED-5 inhibitors have obvious clinical side effects [6], such as headache, facial flushing, nasal congestion, visual impairment [7], and back pain. In addition, PDE-5 inhibitors may also lead to potential drug-drug interactions. Combined with nitrates or blockers, they can cause severe hypotension and syncope [8]. Therefore, it is dangerous for unwitting patients with heart problems to take both nitrates and PDE-5 inhibitors [9].

Driven by interests, the phenomenon of illegally adding PDE-5 inhibitors to dietary supplements is increasing [10]. Although seven compounds have been approved for PDE-5 inhibitors, more unapproved analogues have been illegally abused [11]. In 2013, Lee and coworkers reported the presence of more than 46 PDE-5 analogues in various forms of health foods in South Korea [12]. More recently, a study in the Czech market showed that more than 10 of the 64 natural herbal supplements for erectile dysfunction treatment were added with unregistered synthetic PDE-5 inhibitors [13]. Malaysian market research shows that 82% of unregistered products and 14% of registered products in the tested products are doped with PDE-5 inhibitors or their analogues [14].

Due to the large number of new synthetic PDE-5 enzyme inhibitor analogues, the regulation of PDE-5 inhibitors becomes complicated. Hence, there are no regulatory implications for detecting illegal PDE-5 inhibitors in dietary supplements [11]. Various techniques have been used for the analysis of approved inhibitors and novel analogues. One of the most used is high performance liquid chromatography (HPLC) [15]. Mass spectrometry (MS) [16], gas chromatography-mass spectrometry (GC-MS) [17], nuclear magnetic resonance spectroscopy (NMR) [18], ultra-high performance liquid chromatography (UHPLC) [19] are also reported.

The structural elucidation of new analogues of PDE-5 inhibitors such as piperidine, sildenafil, and acetyldenafil is generally achieved by MS [20] and its hyphenated techniques [21]. For example, the flow injection coupled with the collision-induced dissociation (CID) MS/MS method provides useful information for the structural elucidation of piperidine and acetyldenafil [22]. Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS), as an over-resolution mass spectrometer, is often used independently for the identification of sildenafil, hydroxyacetyldenafil, and piperidine acetyldenafil [23]. In some cases, the structure of analogues cannot be characterized only with liquid chromatography-mass spectrometry (LC-MS) [24]. Therefore, gas chromatography in tandem with mass spectrometry (GC-MS) can be used as a supplementary tool. The PDE-5 inhibitors and analogues always need derivatization or hydrolysis before analysis with GC-MS [17].

Ultra-high performance liquid chromatography (UHPLC) uses smaller fillers, which greatly improves the separation efficiency [25] and shortens the time required for analysis. For example, Sacré et al. reported a UHPLC-UV method that can isolate three licensed PDE-5 inhibitors and their five analogues in less than 5 minutes [26].

All the above-mentioned detection methods have their own deficiencies. When GC-MS is used to analyze sildenafil, vardenafil, and tadalafil, complex derivatization of the sample is required [27]. When analyzing PDE-5 inhibitors by NMR, high purity of the sample is required. Moreover, NMR cannot be used for quantitative analysis. UHPLC analysis alone cannot guarantee the specificity of the method.

The LC-MS technique is sensitive and selective for target substances in complex matrices without extensive sample preparation procedures [28]. LC-MS also has the advantage of combining online optical detectors to obtain the ultraviolet spectrum and mass spectrum simultaneously. In addition, depending mass spectrometer, LC-MS can also perform selective ion monitoring (SIM) [29], product ion scanning (PIS) [30], constant neutral loss scanning (CNLS) [31], selective reaction monitoring (SRM) [32], or multiple reaction monitoring (MRM) [33]. These scanning methods

could render structure information about the target compounds.

In this study, we intend to detect illegal additives of PDE-5 inhibitors in dietary supplements. The novel protocol employs ultra-performance liquid chromatography coupled with tandem mass spectrometry to analyze three PDE-5 inhibitors that have been approved for clinical use by FAD. The model PDE-5 inhibitors include sildenafil, vardenafil, and tadalafil.

The specificity of suspected molecules in unlabeled dietary supplements was checked with retention time, accurate molecular ion weight, and MS/MS spectrum. Thereby, the quick determination of illegal dope was achieved. This will contribute to the rapid detection of other illegal additions of PDE-5 inhibitor analogues.

2. MATERIALS AND METHODS

2.1 Reagents and Materials

Methanol and acetonitrile (HPLC-grade) were purchased from Merck (Germany). Anhydrous ethanol was purchased from Jiangsu Tongsheng Chemical Reagent Co., Ltd. (China). Acetonitrile (AR) was purchased from National Pharmaceutical Group Chemical Reagents Co., Ltd. (China). Formic acid （purity >98.0%） was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Deionized water (18.2 MΩ/cm) was prepared by the Milli-Q system (Molsheim, France).

The standards of vardenafil (purity >99.5%), tadalafil (>99.2%), and sildenafil (>99.8%) were purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Two dietary supplements were purchased on the internet. One was tablet candy branded in Ginseng Huangjing-lubian, which was numbered as s-1. Another is Ginseng Wubao tea, which was numbered as s-2.

2.2 Apparatus and Instrument

Centrifugal tubes used in sample preparation were purchased from Moen Technology Co., Ltd. (China). Weighing paper was purchased from Changde Beekman Biotechnology Co., Ltd. (China). The volumetric flasks were purchased from Changde Beekman Biotechnology Co., Ltd. (China).

The electronic balance (BSA124S-CW) was purchased from Sedoris Scientific Instruments Co., Ltd. (China). The ultrasonic cleaner (KQ-250DE) was purchased from Kunshan Ultrasonic Instrument Co., Ltd. (China). The volume flask was purchased from Hunan Xiangyi Laboratory I nstrument Development Co., Ltd. (China).

The Infinity Liquid Chromatograph System (1290 Infinity) was purchased from Agilent Technologies (USA). The ACQUITY UPLC BEH C18 column (50 mm × 2.1 mm, 1.7 μm) was purchased from Waters. The Triple TOF TM 5600^{+} mass spectrometer was purchased from ABSciex (USA).

2.3 Sample Preparation

Standards of vardenafil, tadalafil, and sildenafil were weighted precisely and dissolved into a methanol solution. The stock solutions of the three standards were all 2.0 mg/mL. Then the stock solution was diluted gradually or mixed to be the working solution.

For the concrete sample s-1, 10 tablets were weighed, mixed, and grinded. An aliquot of 30 mg of the powder was added to 10 mL of methanol. After a 30-minute ultrasonic bath, the sample was centrifuged at 3000 r/min for 10 minutes. The supernatant was isolated. This extraction process was repeated three times. The supernatant was combined. This sample solution was diluted 100 times and then filtered through a 0.22 μm membrane to be tested.

For sample s-2, five grams of content were ground. An aliquot of 30 mg of powder was dissolved in 10 mL of methanol. After a 30-minute ultrasonic bath, the sample was centrifuged at 3000 r/min for 10 minutes. The supernatant was isolated. This extraction process was repeated three times. The supernatant was combined. Without dilution, the sample solution was filtered through a 0.22 μm membrane to be tested.

2.4 Methods

Mobile phase A was a formic acid/water solution (0.1%, v/v). The mobile phase B was acetonitrile. The total flow rate was 0.2 mL/min. The column temperature was 30 °C. The sampling volume was 1 μL. The chromatography was performed on an ACQUITY UPLC BEH C18 (50 mm × 2.1 mm × 1.7 μm) column with a gradient program.

The mass spectrometer worked in positive mode. The electron spray ionization (ESI) voltage was +5.0 kV. The source temperature was set at 500 °C. The back blowing gas was 35 psi, the auxiliary heating gas was 50 psi, the sheath gas flow was 55 psi. The tandem mass spectrometry worked in TOF scan mode or information dependent acquisition (IDA) mode. The TOF scan range was 100-1000 Dalton.

3. RESULTS AND DISCUSSION

3.1 Method Optimization

In order to analyze the target PDE-5 inhibitors in a short time, an optimization for the chromatography process was conducted. The mixed standard sample was analyzed with an empirical LC program. The empirical time program is listed in Table 1. The mobile phase A is a 0.1% formic acid aqueous solution, and the mobile phase B is acetonitrile.

Table 1. Empirical gradient program with a total flow of 0.2 mL/min

Time (min)	phase A (%)	phase B (%)
1.0	80	20
3.5	65	35
4.5	55	45
6.0	40	60
6.1	80	20
8.0	80	20

With this empirical method, a model standards mixture was analyzed. In the mixture standard, sildenafil, vardenafil, and tadalafil were all 1.0 ug/mL. The total ion chromatogram (TIC) result is shown in Fig. 1. There, the peak at 3.52 min is assigned to vardenafil. The peak at 4.48 min is of sildenafil. The retention time of tadalafil is 5.69 min. It could be found that there are many interfere peaks and the total separation time is relatively long.

Based on the empirical method, the chromatographic program was improved. The optimized gradient program is shown in Table 2.

Table 2. Optimized gradient program with a total flow of 0.2 mL/min

Time (min)	phase A (%)	phase B (%)
1.0	80	20
2.8	65	35
4.8	35	65
4.9	80	20
7.0	80	20

After optimization, the result of the same standard mixture sample is shown in Fig. 2. It could be found that the retention times of vardenafil, sildenafil, and tadalafil were, respectively, 3.331 min, 4.038 min, and 4.895 min. Which saves 15% of the time and greatly improves the separation efficiency.

Since the PED-5 inhibitor molecule always contains conjugated *pi* systems, ultra-violet (UV) absorption is an alternative detector approach. In order to select the appropriate absorption wavelength for quantification, the contour spectrum was used. The contour spectrum was obtained by the diode array detector (DAD) along with the chromatography progress, as shown in Fig. 3.

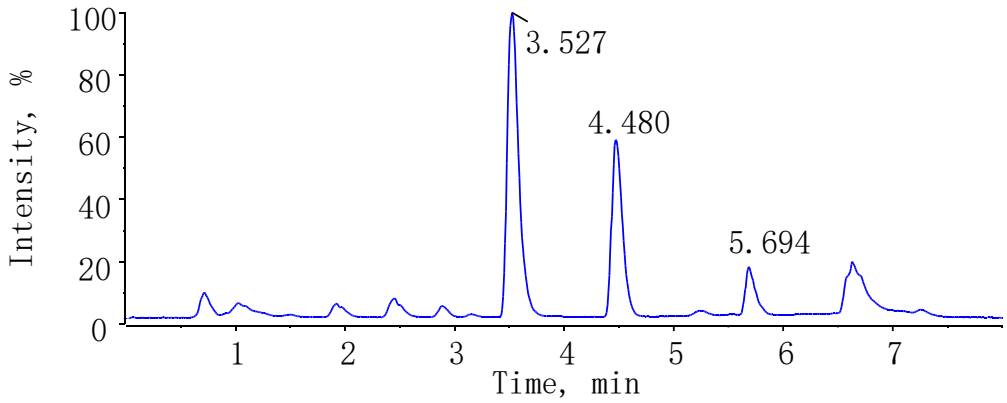

Fig. 1. The chromatogram of three mixed standard samples under empirical chromatographic conditions

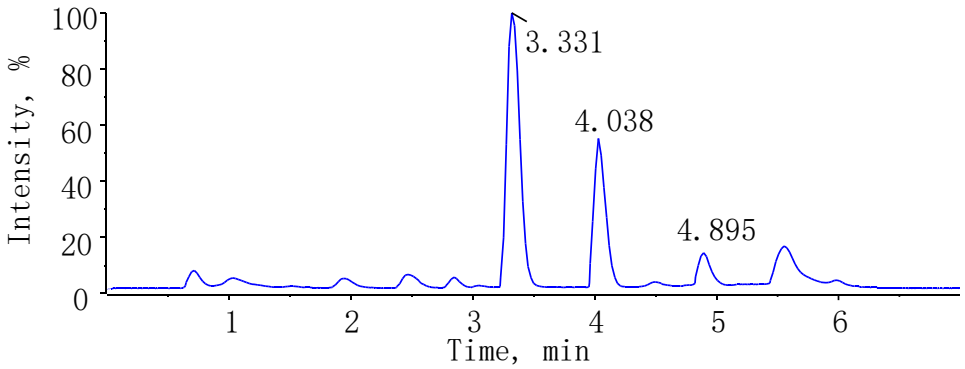

Fig. 2. The chromatograms of three mixed standard samples under optimized chromatographic conditions

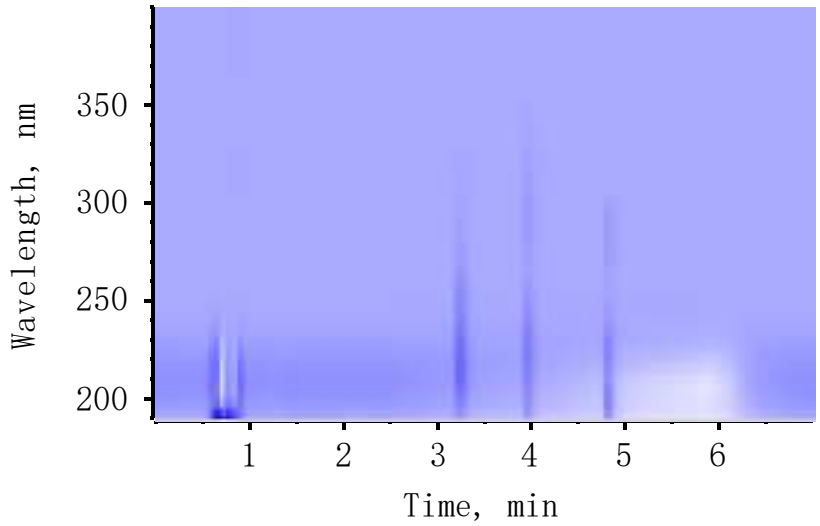

Fig. 3. Two-dimensional waterfall diagram of a diode array detector under chromatographic conditions

In Fig. 3, the spectral peaks of multiple groups of compounds can be observed below 260 nm. When the wavelength is less than 240 nm, there are many interference peaks that appeared between 0.6 and 0.9 min. It is the unpredicted solvent behavior. Therefore, the wavelength choice for PDE-5 inhibitor quantification is 254 nm. The UV chromatogram of the model mixture standard is shown in Fig. 4.

From Fig. 4, there are three peaks at 3.23, 3.95, and 4.79 min. They are vardenafil, sildenafil, and tadalafil, respectively. The separation resolution is excellent, and the peak shape is symmetrical.

The specificity is further investigated with individual mass spectrum results. The mass spectrometry behavior of each peak was investigated by the information dependent acquisition (IDA) mass spectrometry technique. With IDA, the first-stage mass spectra (MS^1) and second-stage mass spectra (MS^2) of the target analyte were recorded simultaneously.

The MS^1 spectra of vardenafil, sildenafil, and tadalafil were collected, as shown in Fig. 5.

In Fig. 5a, the maximum abundance ion is m/z 245.117, which is assigned to the double-charged ion $[M+2H]^{2+}$ of vardenafil. In Fig. 5b, two strong abundance ions, m/z 238.109 and m/z 475.212, are observed. The ion of m/z 475.212 is the molecular ion $[M+H]^+$ of sildenafil. The ion of m/z 238.109 is the double-charged ion of sildenafil. In Fig. 5c, the maximum abundance

ion is m/z 390.144, which belongs to the molecular ion of tadalafil. No double-charged ion was observed for tadalafil. The MS^1 behavior difference between the three analytes is attributed to their chemical structure characteristics.

Since in IDA mode, the MS^2 spectra were recorded, the specificity of target compounds could be checked in addition to the retention time and high-resolution MS^1 results.

The MS^2 spectra of vardenafil, sildenafil, and tadalafil are shown in Fig. 6.

It can be seen from the secondary spectrum that the fragment ion distribution of the three PDE-5 inhibitors is consistent with structural inference.

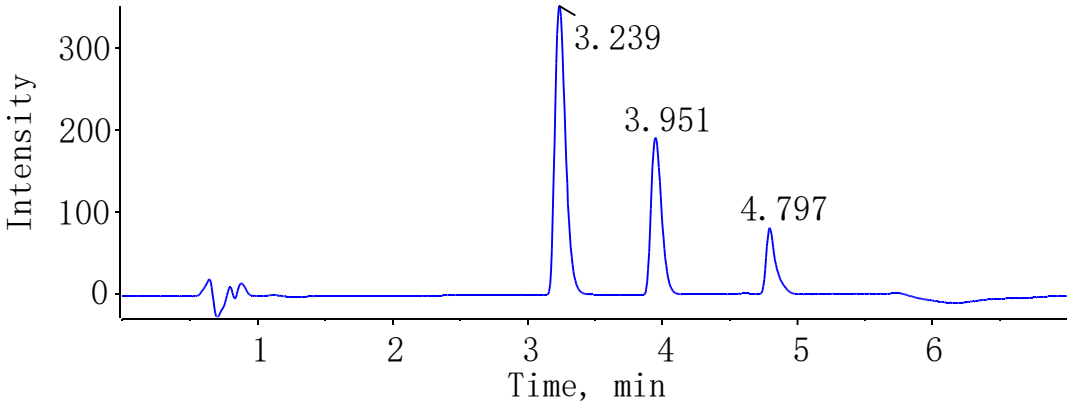

Fig. 4. Ultra-violet chromatogram of three mixed standard samples at 254 nm

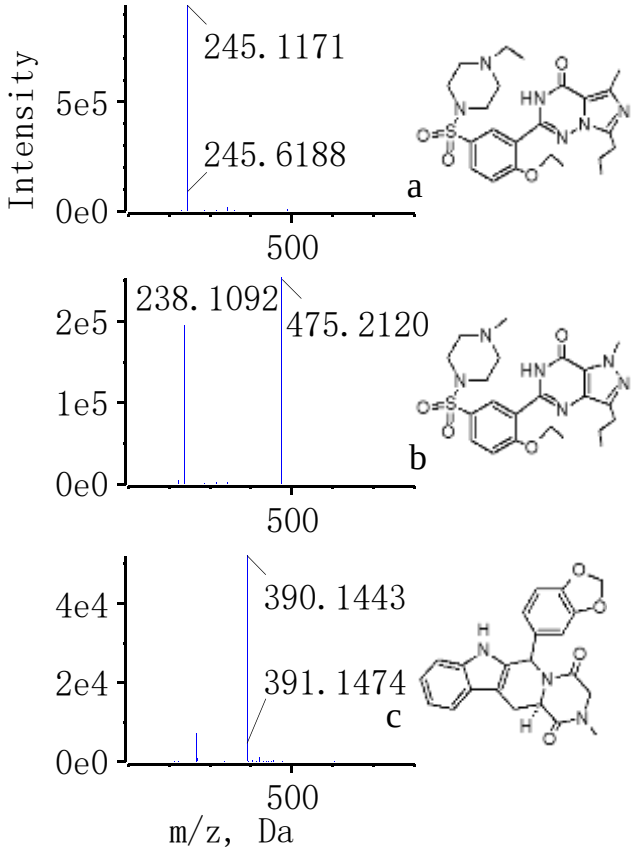

Fig. 5. MS^1 spectra of (a)vardenafil, (b)sildenafil, and (c)tadalafil

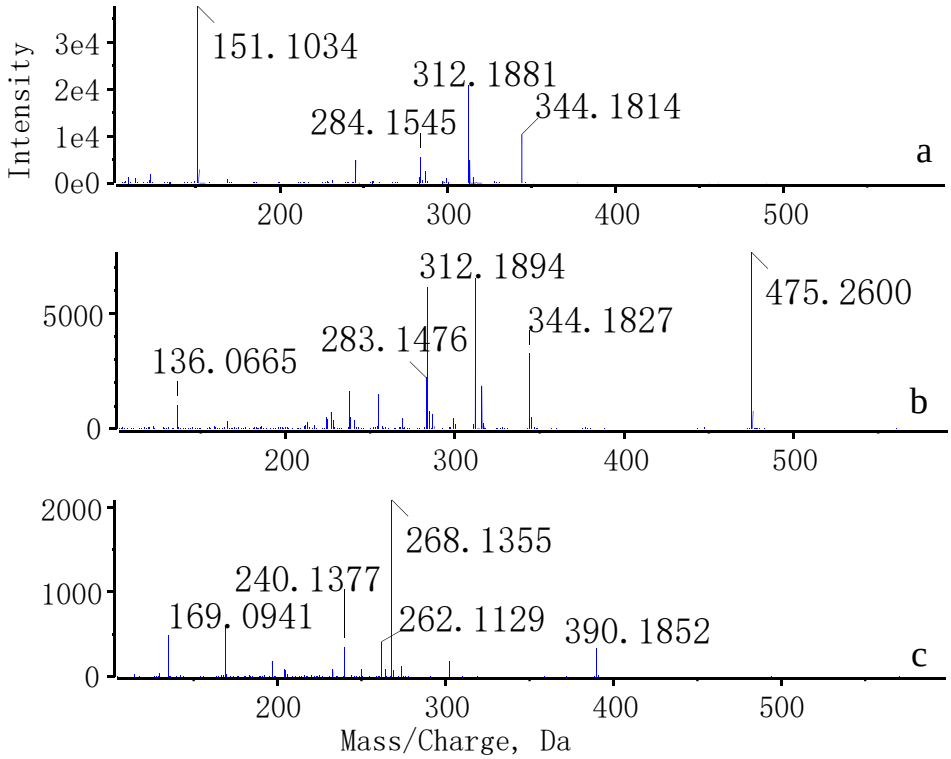

Fig. 6. MS² spectra of (a)vardenafil, (b)sildenafil, and (c)tadalafil obtained in IDA mode

3.2 Linear Range

In order to investigate the quantitative analysis of the three PDE-5 inhibitors, we extracted the respective quantity ion current chromatogram from the total ion current in MS¹. The quantity ions of vardenafil, sildenafil, and tadalafil were, respectively, m/z 245.1, m/z 475.2, and m/z 390.1. Typical extracted ion chromatograms (EIC) are shown in Fig. 7.

Contrary to the UV spectra (Fig. 4), the EIC chromatogram is better in terms of signal-to-noise ratio. It infers that mass spectrometry is more robust when the sample is more complicated or has a matrix effect.

The calibration curves were constructed based on the peak area y of the standards versus concentration. The mean peak areas were recorded in Table 3.

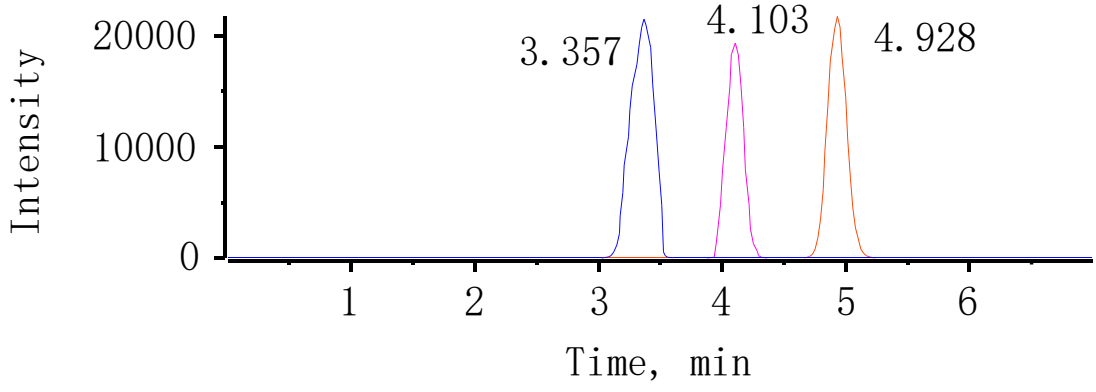

Fig. 7. Extracted ion chromatograms of vardenafil (3.357 min), sildenafil (4.103 min), and tadalafil (4.928 min)

Table 3. Concentration (mg/L) and mean peak area (n=3) of the standard substance

vardenafil		sildenafil		tadalafil	
Conc.	Area	Conc.	Area	Conc.	Area
0.5	221337	0.5	73090	0.3	22925
1	398522	1	114113	0.6	39759
8	2668999	8	688438	6	230439
16	5123333	16	1392911	12	458469
48	14816586	48	3954267	36	1388042

In the concentration range of 0.5-48 mg/L, the linear equation of vardenafil was y = 30656x + 139383, and the correlation coefficient R^2 was 0.9999. The linear equation of sildenafil was y = 81741x + 42965, and R^2 was 0.9998. In the range of 0.3-36 mg/L, the linear equation of tadalafil is y = 38221x + 8264, and R^2 was 0.9998. The correlation coefficients were all greater than 99.9%. It indicates that the method was linearly correlated in the concentration range investigated.

3.3 Precision

In order to investigate precision, five parallel tests were performed on three concentration levels. The relative standard deviation (RSD) was calculated. The results are shown in Table 4. The precision of the method was satisfied.

3.4 Concrete Samples

The concrete samples of s-1 and s-2 were analyzed with the developed UPLC-IDA-MS method. The typical IDA result of s-1 is shown in Fig. 8.

In the IDA dependents diagram (Fig. 8) of s-1, sildenafil (m/z 475.1 at 4.08 and 4.11 min) was recognized directly. The retention time is consistent with the MS^1 (Fig. 2) and UV (Fig. 4) results. The identification was further confirmed with the MS^2 spectra that were obtained simultaneously.

Table 4. RSD value of standard substance

analyte	Conc. (mg/L)	RSD(n=5)
vardenafil	0.5	1.30%
	8	0.89%
	16	0.07%
sildenafil	0.5	0.71%
	8	0.42%
	16	0.05%
tadalafil	0.3	0.82%
	6	0.10%
	36	0.09%

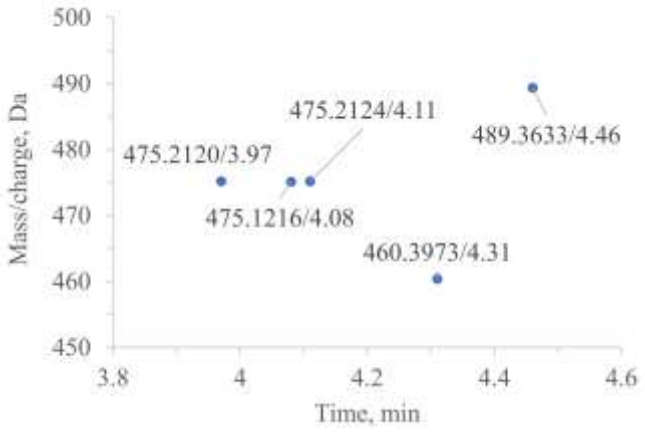

Fig. 8. Time versus precursor mass/charge of s-1 for IDA dependents

Since sildenafil was identified, its quantification was also investigated. Based on its peak area in the monitor ion (m/z 475.2) chromatogram, the concentration in the prepared s-1 solution was determined to be 3.21 mg/L. According to the sample pretreatment process, the content of sildenafil in the original raw s-1 was 321 mg/g.

As for sample s-2, no PDE-5 inhibitor was detected with the developed method.

4. CONCLUSION

In this paper, a novel ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) method was developed. With the optimized chromatographic program, vardenafil, sildenafil, and tadalafil were separated within 5 minutes. The chromatogram of the UV detector was compared with the MS results. With information dependent acquisition (IDA) mode, the MS provided MS^1 and MS^2 information about the target analyte simultaneously. The specificity of the PED-5 inhibitor was confirmed by the retention time, accurate MS^1, and accordingly MS^2 spectra. The MS^1, MS^2, and retention time were acquired simultaneously within the IDA mass spectrometry mode. The method validation of three typical PDE-5 inhibitors was carried out. Quantification was achieved with the quantity ion current chromatogram that were extracted from the total ion current. The quantity ions of vardenafil, sildenafil, and tadalafil were, respectively, m/z 245.1, m/z 475.2, and m/z 390.1. The linear range of vardenafil and sildenafil was 0.5-48 mg/L, while the range of tadalafil was 0.3-36 mg/L. The correlation coefficients of the calibration curves of three PDE-5 inhibitors were all greater than 99.9%. The method was precise and accurate. At three concentration levels, the RSD values of the five repeat tests were all better than 1.30%. Application of the method to two dietary supplements showed sildenafil was detected in one of the samples. The concentration of this unlabeled sildenafil is significant. The next work will be the application of this method to more real samples. The method may be a powerful tool for routine PDE-5 inhibitor screening and determination.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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