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Simultaneous determination of sildenafil, vardenafil and tadalafil as forbidden components in natural dietary supplements for male sexual potency by high-performance liquid chromatography–electrospray ionization mass spectrometry

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Abstract

A high-performance liquid chromatographic method coupled with ultraviolet detection and electrospray ionization mass spectrometry (HPLC–UV–ESI-MS) was developed for simultaneous determination of banned additives—sildenafil, vardenafil and tadalafil in dietary supplements for male sexual potency. The separation was achieved on a C_{18} column with acetonitrile and aqueous solution (20 mmol ammonium acetate, 0.2% formic acid) as mobile phase at a flow rate of 1 ml/min with a linear gradient program. UV detection was at 292 nm. Identification of drugs was accomplished using ESI-MS. Good linearity between response (peak area) and concentration was found over a concentration range of 0.8–80 μg/ml for sildenafil; 2.25–225 μg/ml for vardenafil; and 1.1–110 μg/ml for tadalafil, with regression coefficient is better than 0.999. The recovery of the method ranged from 93.3 to 106.1%, and the relative standard deviation varied from 2.0 to 5.6% ($n = 6$). The method has been successfully applied to the analysis of practical samples of natural dietary supplements.

Keywords: Sildenafil; Vardenafil; Tadalafil; Dietary supplements

1. Introduction

Sildenafil (Viagra), an inhibitor of phosphodiesterase type 5 (PDE5), which was used in the past to treat patients with pulmonary artery hypertension [1–3], was approved for the treatment of erectile dysfunction (ED) in man by the US Food and Drug Administration (FDA). Afterwards, vardenafil and tadalafil was also approved for the treatment of ED [4,5]. These drugs should be administrated under doctors' instruction because their over-dose might cause a series of side-effects. For example, there were reports that color discrimination error scores increased after taking sildenafil [6,7]. Tadalafil and vardenafil are safer than sildenafil, but they still can cause headache, dyspepsia and back pain [8].

A dietary supplement is a product taken by mouth that contains a "dietary ingredient" intended to supplement the diet. The "dietary ingredients" in these products may include: vitamins, minerals, herbs or other botanicals, amino acids, and substances such as enzymes, organ tissues, glandulars, and metabolites. The dietary supplement manufacturer is responsible for ensuring that a dietary supplement is safe before it is marketed. The FDA is responsible for taking action against any unsafe dietary supplement product after it reaches

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the market. In general, natural dietary supplements for male sexual potency consist of different herbal extracts such as ginseng root (*Panax ginseng* C.A. Mey), lychee seed (*Litchi chinensis* Sonn.), barbary wolfberry fruit (*Lycium barbarum* L.), longan aril (*Dimocarpus Longan* Lour.), aweto (*Cordyceps sinensis* (Berk.) Sacc.), common peony root (*Paeonia lactiflora* Pall.), Chinese magnoliavine fruit (*Schisandra chinensis* (Turcz.) Baill), Indian bread (*Poria cocos* (Schw.) Wolf), shorthorned epimedium root (*Epimedium brevicornum* Maxim.) and so on. These dietary supplements could improve male sexual potency without causing any danger, even when over dose occurs. However, in the Southeastern Asian market, for the sake of profit, illegal dealers add some drugs such as sildenafil, vardenafil and so on to their products. The illegal products may endanger people's health. To ensure the quality of this kind of dietary supplements and protect people's health, it is important to develop a method to determine these components.

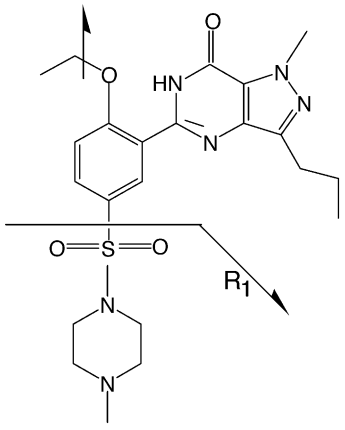
Concerning the analysis of these compounds, there are a few reports that introduced the strategy for the determination of sildenafil by the widely used HPLC technology [9–16]. Tracqui and Ludes developed an HPLC–MS method for the determination of sildenafil [17]; Li et al. reported a method for determining sildenafil with capillary electrophoresis [18]. While the strategy for the determination of vardenafil and tadalafil is seldom reported, simultaneous determination of these three analytes has been seldom reported up-to-date. The purpose of this study was to develop a method for determining sildenafil, vardenafil and tadalafil simultaneously in natural dietary supplements. The structures of these compounds are shown in Fig. 1. The developed method showed some merits such as specificity, sensitivity, and simplicity in sample preparation.

2. Experimental

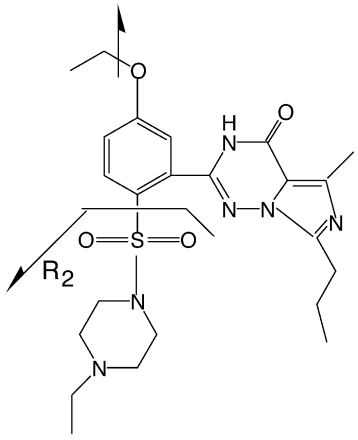
2.1. Materials and chemicals

The HPLC system used was a Waters (Milford, MA, USA) Alliance 2695 module, which was interfaced to a Waters 2487 dual absorbance detector. The mass spectrometer used was a Micromass ZQ 2000 (Manchester, UK) equipped with an ESI probe and quadrupole analyzer. The control of system and data acquiring was performanced with Masslynx$^{3.5}$ workstation (Waters).

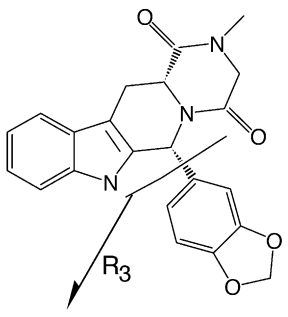
The standards of sildenafil and tadalafil were obtained from Hunan Chemicals and Reagent Corp. (Changsha, China). Vardenafil (>98%, HPLC) was prepared in this laboratory on Waters preparative liquid chromatography of Prep



sildenafil



vardenafil



tadalafil

Fig. 1. The structure of the investigated drugs.

Table 1
Main herbal constituents contained in samples

Sample	Plant sources
Oral liquid preparation 1	Barbary wolfberry fruit, ginseng root, Chinese magnoliavine fruit
Oral liquid preparation 2	Ginseng root, aweto, Indian bread
Oral liquid preparation 3	Barbary wolfberry fruit, common peony root, Indian bread
Oral liquid preparation 4	Barbary wolfberry fruit, aweto
Oral liquid preparation 5	Barbary wolfberry fruit, shorthorned epimedium root
Wine 1	Chinese magnoliavine fruit, barbary wolfberry fruit, ginseng root
Wine 2	Barbary wolfberry fruit, Indian bread
Wine 3	Ginseng root, Chinese magnoliavine fruit
Beverage	Lychee seed

LC 4000 module. Samples for examination were purchased from supermarket (Changsha, China). All of these products examined are natural dietary supplements for male sexual health, not for therapy of ED. The drugs are forbidden to be added in these products according to the Chinese law. And these products are also not sexual potency enhancing preparation. HPLC-grade acetonitrile and methanol were from Shanghai Ludu Chemical Plant (Shanghai, China). Ultrapure water was prepared using a Millipore Milli-Q purification system (Millipore, Bedford, MA, USA). Other reagents were of analytical grade, including ammonium acetate and formic acid, triethylamine. Mobiles used for HPLC were filtered (0.45 μm) and ultrasonically degassed before use.

2.2. Preparation of standards

Stock solutions of sildenafil, vardenafil and tadalafil were prepared in methanol. Their concentrations were 0.80, 2.25 and 1.10 mg/ml, respectively. One milliliter aliquots of each stock solution were transferred into a 10-ml volumetric flask, mixed and diluted to volume to yield a mixed standard solution. Then, 5, 2, 1, 0.5, and 0.1 ml of the mixed standard solution were transferred to five 10-ml volumetric flasks, and diluted to volume with methanol to yield a series of working solutions. All stocking solutions and working solutions were stored in a refrigerator and brought to room temperature before use.

2.3. Preparation of samples

Because the drugs have good solubility in water or methanol, they are often been added into fluid products such as wine, beverage, and oral liquid formulation, etc. Hence, eight liquid products for examination (five oral liquid formulation, two wines, and one beverage, their herbal constituents are listed in Table 1), was purchased from a supermarket. The oral liquid formulation sample was filtered through a 0.45 μm nylon membrane, and 1 ml of the sample transferred into 50-ml volumetric flask and diluted to volume with methanol. Then aliquot of the diluted solution was injected into the HPLC–MS system. The wine sample and beverage sample were just filtered off and injected into the HPLC–MS system without further pretreatment.

2.4. HPLC–MS analysis

The separation of the drugs was completed on a spherigel analytical column (Johnson, Dalian, China), which was packed with 5 μm C_{18} sillica. The mobile phase consisted of acetonitrile (A) and aqueous solution (B) containing 20 mmol/l ammonium acetate and 0.2% formic acid (v/v). The gradient elution was programmed as follows: A was maintained at 35% within the first 10 min, then linearly increased to 80% during the following 5 min, then A maintained at 80% for another 5 min. The column was washed with 100% acetonitrile for 5 min after gradient elution, and then equilibrated for 10 min with the initial mobile phase for the next injection. The flow rate was kept at 1 ml/min and the column temperature was maintained at 30 °C. Injection volume was 5 μl. The detection wavelength was set at 292 nm. The outlet of the UV detector was split, and only 0.2 ml/min portion of the column effluent was delivered into the ion source of MS.

Electrospray was operated in positive ion mode to generate protonated ions and sodiated ions. The voltage of capillary, extractor and RF lens was set at 3.2 kV, 4 and 0.5 V, respectively. The temperature was maintained at 105 and 200 °C for source and desolvation, respectively. The gas flow rate for desolvation and cone was set at 250 and 50 l/h, respectively. The full scan mass spectra was acquired over a range of *m*/*z* 160–600. The cone voltage was switched from 60 to 20 V in scan mode at the point of 10 min according to the electrical stability of the drugs. In selective ionization recording (SIR), the cone voltages for sildenafil, vardenafil and tadalafil were set at 50, 50, and 20 V, respectively.

2.5. Linearity, limit of detection, limit of quantification

The mixed standard solutions (the working solutions) at each concentration level were injected in triplicate, calibration curves were constructed by plotting the average peak areas of the standard compounds against the corresponding concentrations. The limit of detection (LOD) of UV detection and MS–SIR was evaluated as the mass giving a signal equal to three times of noise (S/N = 3), the limit of quantification (LOQ) was determined as the mass giving a signal equal to ten times of noise (S/N = 10).

3. Results and discussion

3.1. Mobile phase consideration

Firstly, methanol was applied to separate the tested compounds, however, sildenafil and vardenafil could not be separated under the use of a mixed methanol aqueous solution with any proportion of organic to aqueous phase. When acetonitrile was used, the two substances could be separated, their retention time and separation resolution mainly depended on the concentration of acetonitrile in the aqueous solution. A mobile phase consisting of acetonitrile–water programmed as described in experimental section provided the best compromise between the separation efficiency and the time duration of the analytical procedure.

The examined compounds in this work all contain several N atoms in their structure; it results in serious peak-tailing on RP-C_{18} column if no modifier was added to the mobile phase. In order to suppress peak-tailing, the effects of several additives and their concentration were investigated. In liquid chromatography, triethylamine was the most common additive used in analyzing compounds containing N atoms. In this work, 5, 10, 15, 25, and 50 mmol/l concentrations of triethylamine were tested. It was found that when 10 mmol/l triethylamine was employed, the peak is sharp and relatively symmetric. When higher concentrations of triethylamine were used, the resulted peak shape was not improved any more, however the baseline shifted greatly when gradient mobile phase was employed and the resolution of sildenafil and vardenafil decreased. And the ionization of all analytes was greatly suppressed, sildenafil and tadalafil gave no signal even in the SIR chromatogram, and the signal of vardenafil was very weak. In addition, the effect of ammonium acetate, as a modifier of the mobile phase, was also investigated. When 20 mmol/l ammonium acetate was used, the peak area RSD of three consecutive injections for each compound was less than 5% which is lower than in the case of triethylamine used as modifier. However, when 50 mmol/l ammonium acetate was used, the response of sildenafil; vardenafil and tadalafil decreased 10.5, 18.4, and 20.4%, respectively, compared to that when 20 mmol/l ammonium acetate was applied. So, high concentration of modifier was not recommended.

3.2. MS conditions

The MS parameters were optimized attentively by flow injection analysis (FIA). ESI is a soft ionization technique, while sildenafil and vardenafil have a relatively stable structure, so they can bear higher voltage. They gave little fragment ions under 50 V cone voltage, and produced only a few fragment ions under 60 V. Tadalafil is easier to be cracked down, the abundance of its molecular ion was still low even when the applied cone voltage is higher than 30 V. Therefore, as described in the previous experimental section, in SIR mode, the cone voltage for sildenafil and vardenafil was set at 50 V, while the cone voltage for tadalafil was set at 20 V; in scan mode, the cone voltage was set at 60 V in the previous 10 min to generate some fragment ions for identification of sildenafil and vardenafil, then switched to 20 V during the following 10 min.

3.3. HPLC–UV–MS analysis of standards

The examined analytes was baseline separated under the given chromatographic condition. Fig. 2 shows the chromatogram of mixed standards recorded with 292 nm and with SIR, the retention times for sildenafil, vardenafil, tadalafil are 7.9, 8.8, and 14.8 min, respectively. Fig. 3 displays the mass spectrum of the three compounds. Fig. 3A exhibites the intensive protonated molecule of sildenafil $[M+H]^+$ at *m/z* 475, *m/z* 497 is the sodiated molecule $[M+Na]^+$ of sildenafil, *m/z* 311 and 283 are the fragment ions of sildenafil. The assignment can be done as follows: *m/z* 311 is the fragment ion losing an $-R_1$ group, *m/z* 283 is the fragment ion losing an [R_1 + ethyl] group. The same results were obtained by Weinmann et al. [19] and Walker et al. [20]. The presence of *m/z* 489 in Fig. 3B, represents the molecular ion $[M+H]^+$ of vardenafil, *m/z* 311 and 283 are the fragment ions losing an $-R_2$ group and [R_2 + ethyl] group, respectively. And the ion at *m/z* 390 in Fig. 3C is the molecular ion $[M+H]^+$ of tadalafil, *m/z* 412 is the sodiated molecule $[M+Na]^+$, while *m/z* 268 is the result of losing an R_3 group. It can be seen from Fig. 3A and B that sildenafil and vardenafil produce the same fragment ions. This is because that they possess very similar structures and it can partly explain why the two substances cannot be separated with methanol as mobile phase.

3.4. Linearity, limit of detection, limit of quantification

Linearity of the three analytes was obtained over concentration range from 0.8 to 80 ppm, 2.25 to 225 ppm and 1.1 to 110 ppm, for sildenafil, vardenafil and tadalafil, respectively. Results are shown in Table 2. All these substances have con-

Table 2
Linearity, limit of detection (LOD), limit of quantification (LOQ) ($n = 3$)

Component	Regression equation[a]	r^2[a]	Linear range (ppm)[a]	LOD (ng)	LOQ (ng)
Sildenafil	$y = 204x - 48$	0.9998	0.8–80	0.80[a], 0.020[b]	3.00[a]
Vardenafil	$y = 152x - 30$	0.9998	2.25–225	1.12[a], 0.011[b]	4.48[a]
Tadalafil	$y = 263x + 71$	0.9999	1.1–110	0.55[a], 0.04[b]	1.65[a]

[a] Result with detection at 292 nm.
[b] Result with SIR.

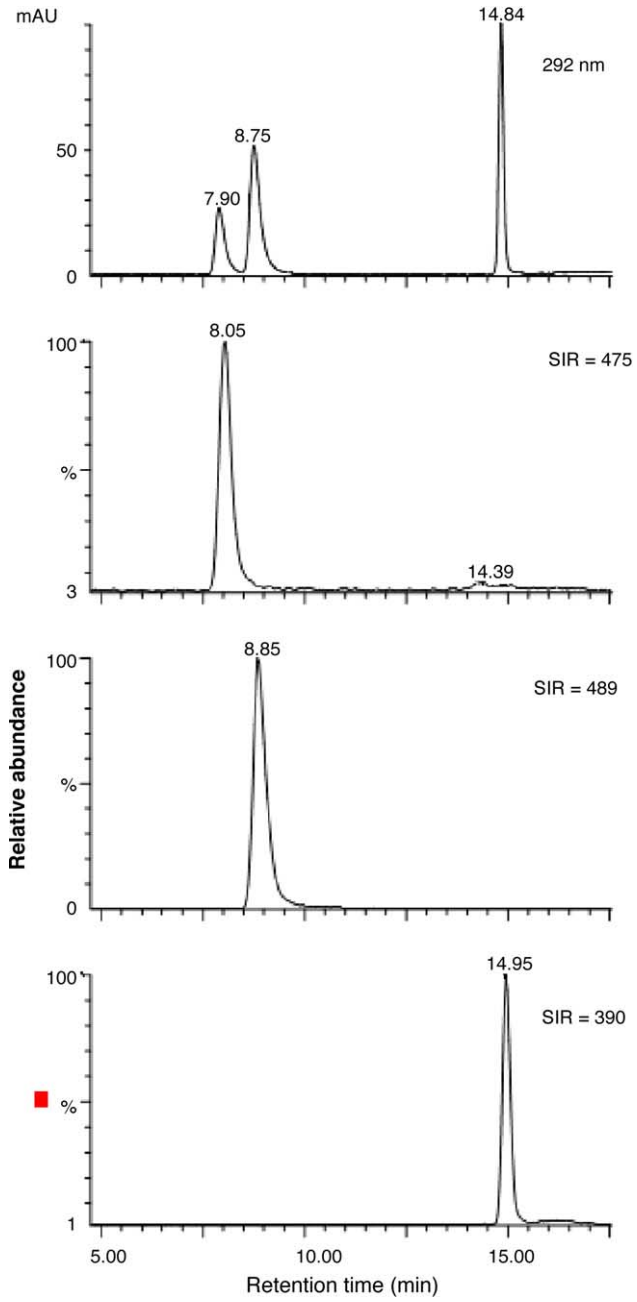


Fig. 2. The chromatogram of mixed standards. Peak identification: sildenafil (t_R = 7.9), vardenafil (t_R = 8.8) and tadalafil (t_R = 14.8). The concentration of the three compounds in the mixture was 16, 45, and 22 μg/ml, respectively.

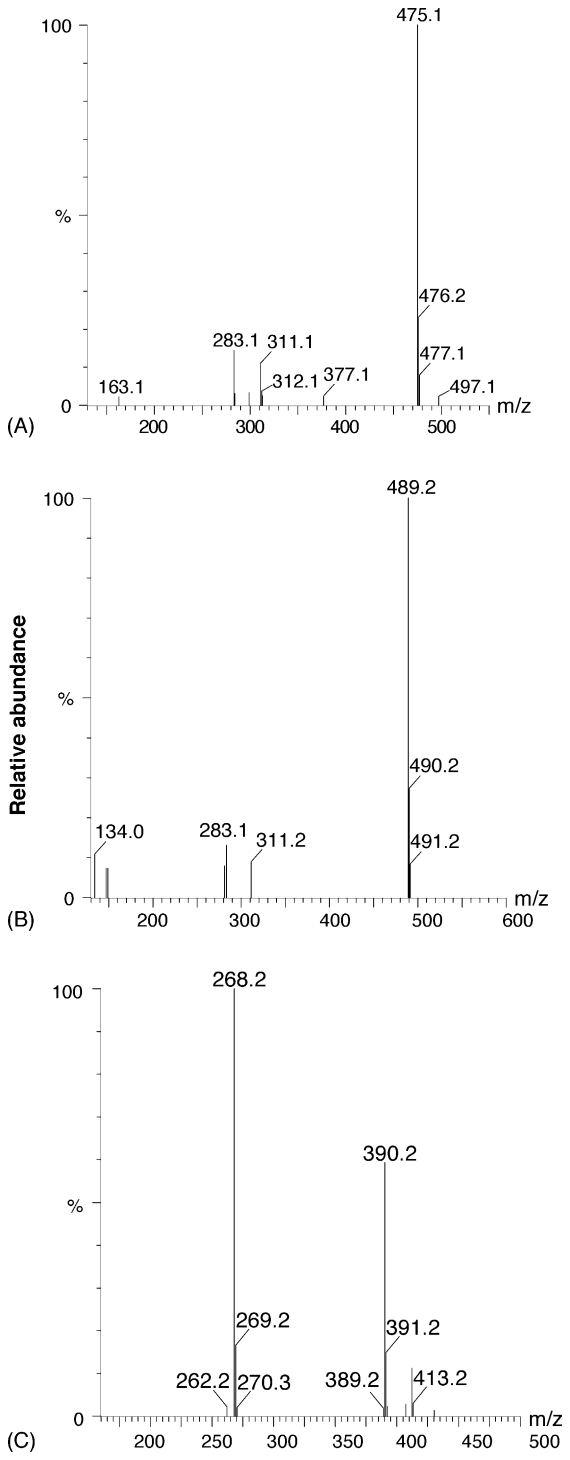


Fig. 3. The mass spectrum of examined analytes. (A) Sildenafil, (B) vardenafil and (C) tadalafil.

jugated structures and displayed intensive ultraviolet absorption, which resulted in quite a low LOD and LOQ with UV detection. Hence the UV detection method can be used for conventional analysis of these compounds even without mass spectrometry. However, the MS LOD of these compounds was found to be even much more lower. On line analysis displays that the proposed HPLC–ESI-MS method is advantageous in trace analysis of these compounds and can provide structure information for identification when no standards are available.

3.5. Precision and accuracy

Precision of the method was evaluated by six consecutive injections of the investigated samples, the resulting RSD varied from 2.6 to 4.7%.

The accuracy of the method was studied by calculating the mean recovery of the target compounds after adding stan-

Table 3
Precision and recoveries ($n = 3$)

Sample	Component	Low level			Medium level			High level		
		Added (mg)	Found (mg)	Recovery (%)	Added (mg)	Found (mg)	Recovery (%)	Added (mg)	Found (mg)	Recovery (%)
Sample A (oral liquid formulation)	Sildenafil	1.3	1.24 ± 0.04	95.4	10.8	10.4 ± 0.25	96.3	25.7	25.0 ± 0.61	97.3
	Vardenafil	1.0	0.94 ± 0.03	94.0	11.0	10.4 ± 0.26	94.6	24.9	24.8 ± 0.79	99.6
	Tadalafil	1.2	1.13 ± 0.04	94.2	10.5	10.0 ± 0.37	95.2	26.2	27.8 ± 0.59	106.1
Sample B (wine)	Sildenafil	1.4	1.33 ± 0.07	95.0	10.6	10.3 ± 0.32	97.2	25.5	26.5 ± 0.54	104.0
	Vardenafil	1.3	1.33 ± 0.06	102.3	10.9	10.4 ± 0.29	95.4	25.2	26.5 ± 0.63	105.2
	Tadalafil	1.5	1.44 ± 0.08	96.0	10.5	10.1 ± 0.35	96.2	26.2	27.6 ± 0.75	105.3
Sample C (beverage)	Sildenafil	1.2	1.24 ± 0.05	103.3	9.8	9.40 ± 0.33	95.9	25.0	25.8 ± 0.83	103.2
	Vardenafil	1.5	1.56 ± 0.06	103.3	10.5	10.8 ± 0.35	102.9	24.6	25.1 ± 0.76	102.0
	Tadalafil	1.2	1.26 ± 0.07	105.0	10.6	11.0 ± 0.40	103.8	25.9	27.4 ± 0.68	105.8

The result was obtained by employing UV detection at 292 nm.

dards to three blank samples (wine, beverage, oral liquid formulation) at low, medium and high levels. Each sample of the same concentration was injected at least three times. The results are summarized in Table 3. From this Table, it can be seen, that the mean recovery for all three drugs was 94.0–106.1%. These results about precision and accuracy met the acceptable criteria.

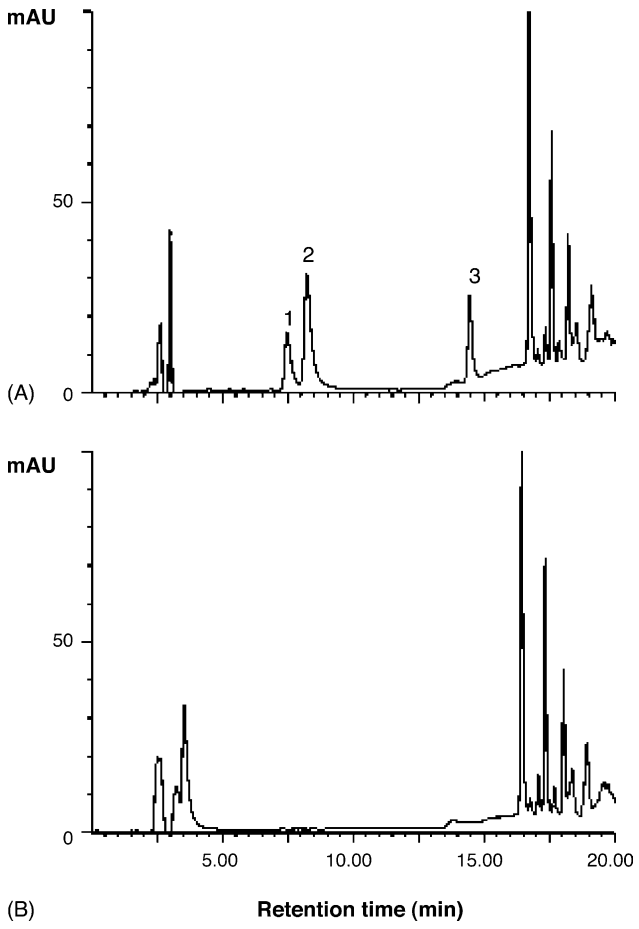


Fig. 4. The chromatogram of sample (oral liquid formulation 5) acquired with detection at 292 nm. (A) Chromatogram after adding three standards, (B) chromatogram of sample before adding standards. Peak identification: 1, sildenafil; 2, vardenafil and 3, tadalafil.

3.6. HPLC–UV–MS analysis of samples

Herbs are very complex because they contain many kinds of compounds. The samples examined in present work included barbary wolfberry fruit, ginseng root, Chinese magnoliavine fruit, aweto, Indian bread, common peony root, shorthorned epimedium root and lychee seed. The compositions of all these herbs are rather complicate. How-

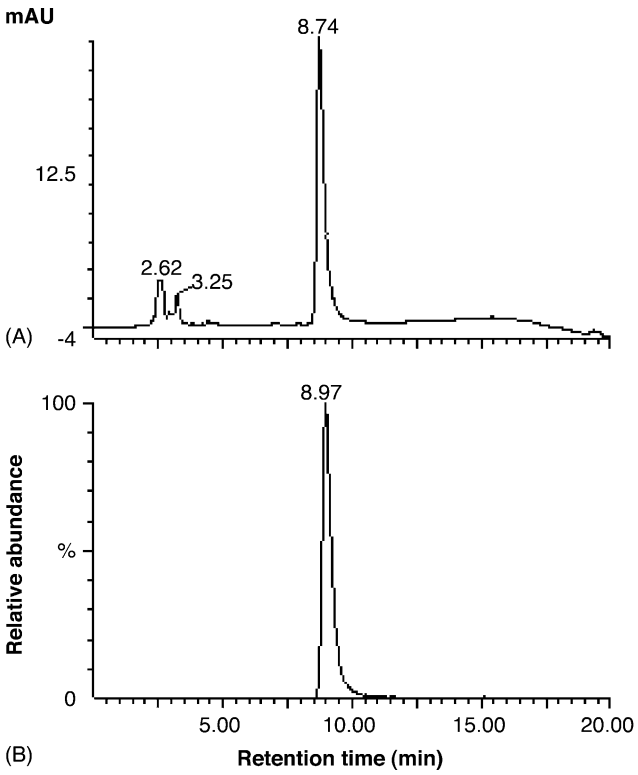


Fig. 5. The chromatogram of oral liquid preparation sample 2. (A) Recorded with detection at 292 nm; (B) recorded with SIR 489.

ever, under the above-given conditions, no interference from these herbs was observed. Fig. 4 shows the chromatogram of oral liquid formulation 5 after (A) and before (B) adding sildenafil, vardenafil and tadalafil standards. It can be seen that no interfering components were co-eluted with these three drugs simultaneously. Among the eight examined samples, one sample (oral liquid fomulation 2) was found to contain vardenafil, its concentration was 2.25 mg/ml (RSD = 1.7%, $n = 6$). The chromatogram of this sample is shown in Fig. 5.

4. Conclusion

With the improvement in production technology of sildenafil and its analogous compounds, the quantity of these compounds is becoming bigger and bigger and their prices are on decline, hence even more of these compounds are being added to dietary supplements by illegal businessmen. The method presented in this paper is useful for simultaneous determination of sildenafil, vardenafil, tadalafil. It can be employed to inspect those dietary supplements which may contain these substances to ensure people's safety, and the suggested method has the advantage of simplicity, rapidity and accuracy.

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