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Short communication

Screening of Indian aphrodisiac ayurvedic/herbal healthcare products for adulteration with sildenafil, tadalafil and/or vardenafil using LC/PDA and extracted ion LC–MS/TOF

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ABSTRACT

Ayurvedic/herbal healthcare products are considered safe under the impression that they are derived from natural products. But recently, there have been several reports worldwide on the adulteration of synthetic PDE-5 inhibitors in aphrodisiac herbal formulations. Therefore, the objective of the present study was to explore the presence of synthetic PDE-5 inhibitors (sildenafil, tadalafil and/or vardenafil) in ayurvedic/herbal healthcare products sold in Indian market for aphrodisiac/related uses. In total, 85 herbal formulations (HFs) were included in the study. The formulations were extracted with methanol and subjected to centrifugation. The supernatant was analysed by HPLC and LC–MS/TOF. Early detection of the presence of sildenafil, tadalafil and vardenafil in the herbal samples was done by the study of extracted ion mass chromatograms at the *m/z* values of respective parent ions, and two prominent fragments of each. In case of sildenafil and tadalafil, adulteration was also detected by comparing the relative retention times (RR_T) and UV spectra. Further substantiation was done through comparison of accurate mass spectra with those of the two available standards. Of the 85 HFs tested, only one was eventually found to be adulterated with sildenafil. The extent of adulterant in this sample was determined to the therapeutic dose in the formulation. The study thus indicates emergence of the problem of adulteration of Indian herbal products with PDE-5 inhibitors.

1. Introduction

Herbal formulations (HFs) are popular worldwide due to the belief that they are safer than synthetic drugs. However, nowadays, HFs have not remained trustworthy as lots of evidence are coming into literature about adulteration of these products with synthetic drugs or their congeners, in order to enhance the claims stated on the label. This poses a health threat to patients who unwittingly consume a compound which may be untested for safety [1–4].

The reports on adulteration of HF with synthetic phosphodiaesterase-5 (PDE-5) inhibitors have been most common in recent past. The three approved inhibitors for the treatment of penile erectile dysfunction (ED), *viz.*, sildenafil citrate (Viagra, Pfizer), tadalafil (Cialis, Elli Lilly) and vardenafil hydrochloride (Levitra, Bayer), have many documented side effects and are required to be used under medical supervision [5]. Around 60–70% cases of ED occur in patients with hypertension and ischemic heart disease, and unfortunately PDE-5 inhibitors show negative pharmacodynamic interactions with the drugs indicated for these diseases, *e.g.*, nitroglycerine, doxazosin and terazosin [6]. Hence these ED drugs are not advised for such patients. Unfortunately, those using HF may be at risk, if there is clandestine adulteration with synthetic PDE-5 inhibitors.

There are various reports from US, China, Taiwan, Singapore, Thailand, Korea, Hong Kong, etc. on adulteration of HFs (available in local markets or purchased covertly over the internet) with PDE-5 inhibitors [7–22]. India is a big pharmaceutical market and is also on rising graph in terms of sale and exports of HF. Most of the reports on Indian HFs mainly focus on excessive contents of toxic heavy metals [23,24], however, no study is yet known on the status of adulteration of Indian HFs with synthetic PDE-5 inhibitors. So the objective of the present study was to investigate the Indian produced HFs for the adulteration with sildenafil, tadalafil and/or vardenafil. A recently reported strategy involving LC/PDA and LC–MS studies [25] was employed, with additional emphasis on the study of extracted ion chromatograms (EICs). The results on 85 aphrodisiac HFs are discussed in this report.

2. Experimental

2.1. Materials

Sildenafil citrate and tadalafil were obtained as gratis samples from Orchid Chemicals and Pharmaceuticals Limited, Chennai,

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India. The marketed HFs were procured randomly. Of the total of 85 HFs, 28 were purchased from local chemist shops in the vicinity, while remaining were procured through contacts in other parts of India (18 from Gujarat, 16 from Andhra Pradesh, 10 from Himachal Pradesh, 5 from Haryana, 3 each from Madhya Pradesh and Delhi, and 2 from Maharashtra). HPLC grade methanol was procured from J.T. Baker (Phillipsburg, NJ, USA). Buffer salts and all other chemicals were of analytical reagent grade. Ultra pure water obtained from ELGA water purification unit (Wycombe, Bucks, England) was used throughout the studies.

2.2. Instruments

The HPLC system consisted of an on-line degasser (DGU-14A), low-pressure gradient flow control valve (FCV-10ALVP), solvent delivery module (LC-10ATVP), auto-injector (SIL-10ADVP), column oven (CTO-10ASVP), UV–visible dual-wavelength detector (SPD-10AVP), photo-diode-array (PDA) detector (SPD-M10AVP), system controller (SCL-10AVP) and a computer system loaded with CLASS-VP software (all from Shimadzu, Kyoto, Japan). LC–MS/TOF studies were carried out on a system in which LC part consisted of 1100 series HPLC from Agilent Technologies (Waldbronn, Germany) and MS part consisted of MicrOTOF-Q spectrometer (from Bruker Daltonik, Bremen, Germany). The LC part comprised of an on-line degasser (G1379A), binary pump (G131A), auto-injector (G1313A), column oven (G1316A) and diode-array detector (G1315B). The system was controlled by combination of Hyphenation Star (version 3.1) and MicrOTOF Control (version 2.0) software. Chromatographic separations were achieved on a C-18 column (250 mm × 4.6 mm i.d., particle size 5 μm, Supelco Discovery Inc., Bellefonte, PA, USA).

2.3. Sample preparation

Based on the solubility of the synthetic drugs, all the herbal formulations including capsules, tablets or herbal oils were extracted with methanol. The powder of crushed tablets, inner content of capsules and herbal oils were extracted using 5 ml methanol by sonication for 40 min. The extract was then centrifuged at 10,000 rpm for 10 min at 25 °C. The supernatant was directly injected to HPLC and LC–MS.

2.4. HPLC method development and validation

Initially, separation of available standards, *viz.*, sildenafil and tadalafil, was attempted by varying relative ratio of methanol (A) and 0.01 M potassium dihydrogen phosphate (B). The pH of buffer was also varied. The best separation of the two drugs and removal of herbal components from the column at the end of run were achieved by using a gradient method with buffer of pH 3.0. The injection volume was 10 μl. The data were acquired in the wavelength range of 200–400 nm using a PDA detector. UV signals were extracted at 230 and 292 nm.

The developed method was validated with respect to linearity, precision (intra-day, inter-day and intermediate precision), and accuracy. To establish linearity and range, solutions of sildenafil were prepared in the range of 10–1000 μg/ml, followed by their analysis in triplicate by the developed HPLC method (Section 2.4). The calibration curve was generated and regression parameters were established. The intra- and inter-day precision were investigated by analyzing 250, 500 and 750 μg/ml drug solutions three times on the same day and on three consecutive days, respectively. To determine intermediate precision, the brand of the column was changed and also whole experiment was performed on a different system. Accuracy was determined by spiking herbal formulation extract with the above given three known concentrations of sildenafil (in triplicate) and then determining the percent recovery of the added drug. For the determination of limit of detection (LOD) and quantification (LOQ) of method, the samples showing no signal at R_T of the standard were considered as blank. The concentration in the spiked samples that gave three times higher response than the noise in blank was taken as LOD, and the concentration producing ten times more response than the noise was determined to be LOQ [26].

2.5. LC–MS/TOF studies

First, MS/TOF parameters were optimized for analysis of sildenafil and tadalafil in ESI positive mode. The developed HPLC method was then transferred to LC–MS by replacing phosphate buffer with ammonium acetate buffer (0.010 M, pH 3). The calibration solution used was ES Tuning mix solution (Agilent Technologies, Waldbronn, Germany), diluted to a suitable concentration with mixture of ACN–water (95:5% v/v). All the masses were corrected by internal reference ions of *m/z* 322.0481 ($C_6H_{19}O_6N_3P_3$), 622.0290 ($C_{12}H_{19}O_6N_3P_3F_{12}$) and 922.0098 ($C_{18}H_{19}O_6N_3P_3F_{24}$) in positive ESI mode. All the formulations were then subjected to LC–MS/TOF study after appropriate sample preparation. LOD values of the available standards were established by determining the concentration of the spiked samples that gave three times higher signal than the noise observed for the blank [26].

2.6. Spiking studies with standards

To confirm the presence of synthetic adulterant, all the herbal samples were spiked with sildenafil and tadalafil, separately. Additionally, as per the published strategy [25], the spiking studies were also performed using a reported different method to confirm the adulteration in the suspected sample [8]. Because of the non-availability of vardenafil standard, it could not be included in the spiking studies.

2.7. Quantitative estimation of sildenafil in herbal formulation

The adulterant was quantitatively determined using the regression equation of the linearity plot of the HPLC method.

3. Results and discussion

3.1. HPLC method development

The mobile phase for the optimized method consisted of methanol (A) and 0.010 M potassium dihydrogen phosphate (B) (pH 3.0), which was used in a gradient mode at a flow rate of 1.0 ml/min. The gradients were: T_{min}/A:B (v/v); $T_{0.01-20}$/57:43 (v/v), T_{24}/60:40 (v/v), T_{26}/80:20 (v/v) and T_{30-35}/57:43 (v/v). Using the developed method, prominent peaks of sildenafil and tadalafil resolved at 10.19 and 19.29 min, respectively (Fig. 1). The UV/PDA spectra for the two drugs were also recorded, which are included in the figure.

3.2. EIC based screening of herbal formulations

The optimized parameters for LC–MS/TOF studies were: hexapole Rf, 200.0 Vpp; collision cell Rf, 150.0 Vpp; pre-pulse storage, 4.0 μs; collision energy, 20 eV; quadrupole ion energy, 8.0 eV; nebulizer gas pressure, 1.2 bar; dry gas flow rate, 6.0 l/min; and dry temperature, 200 °C. Under these conditions, EIC screening of all 85 herbal formulations was carried out using *m/z* values of all the three standards, *viz.*, sildenafil (*m/z* 475), tadalafil (*m/z* 390) and vardenafil (*m/z* 489) [25]. Based on known fragmentation patterns of these drugs [27–32], specificity was further established by extracting mass chromatograms using two prominent fragments of each

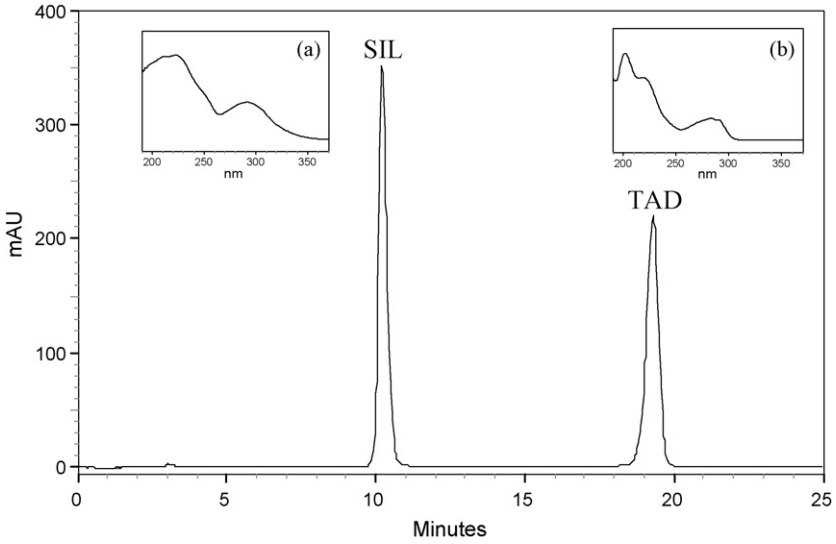

Fig. 1. Chromatogram of a mixture of sildenafil (SIL) and tadalafil (TAD). The UV-DAD spectra of the two drugs are shown in insets (a) and (b), respectively.

drug, *viz.*, *m*/*z* 311 and 283, 268 and 262, and 377 and 312, respectively. The LOD values of sildenafil and tadalafil, determined for the EIC method, were 52 and 35 ng/ml, respectively. Among the screened herbal formulations, only HF46 showed intense EIC peak corresponding to sildenafil (Fig. 2), while no other product had any peak corresponding to the three drugs.

3.3. Confirmation of the presence of sildenafil in herbal formulation

The product HF46 even showed HPLC peak at the same RR_T as pure sildenafil (Fig. 3(a)), the UV spectrum was also superimposable to that of the standard, and the presence of adulterant was proven through a spiking study (Fig. 3(b)). The adulteration of HF46 with sildenafil was confirmed by using the reported method [8], where a peak was obtained at R_T of the standard adulterant. Further, the accurate mass spectrum of HF46 showed similar pattern as that of sildenafil (Fig. 4).

3.4. Validation and quantitative study

A linear response was obtained for the drug concentration in the range of 10–1000 μg/ml ($Y = 44.47X - 462.5$, $R^2 = 0.998$). The percent R.S.D. for each concentration was less than 0.5%. Even, percent R.S.D. for intra- and inter-day precision studies at three different

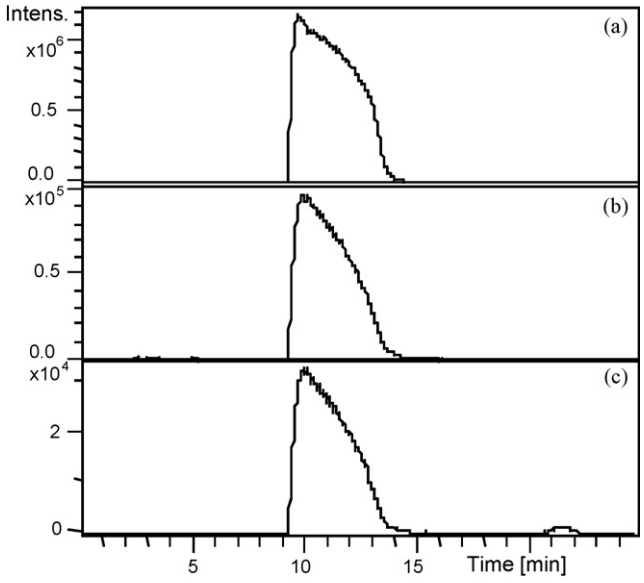

Fig. 2. Extracted ion chromatograms (EICs) of methanolic extract of HF46 at molecular ion peak of *m*/*z* 475 (a), and fragments of *m*/*z* 311 (b) and *m*/*z* 283 (c).

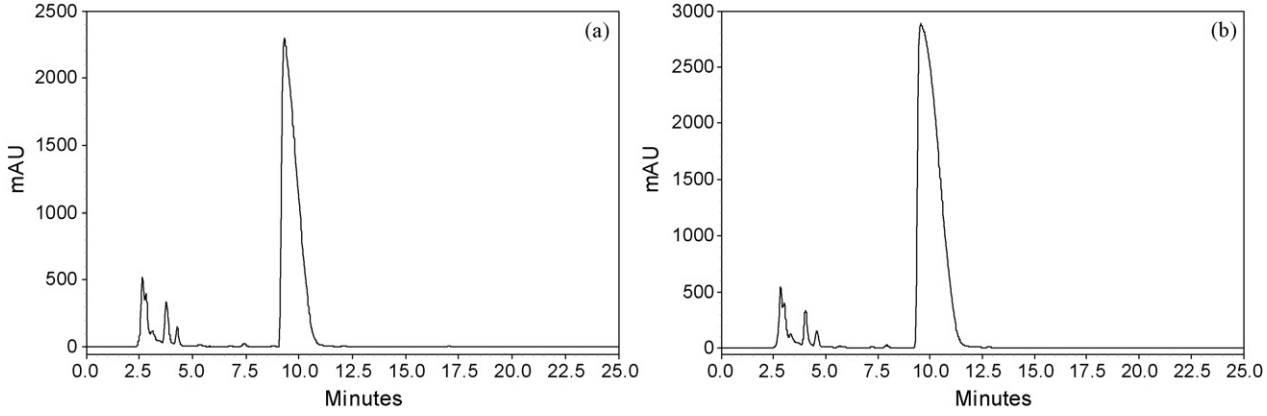

Fig. 3. HPLC chromatograms of methanolic extract of HF46 (a) and sildenafil spiked in HF46 (b).

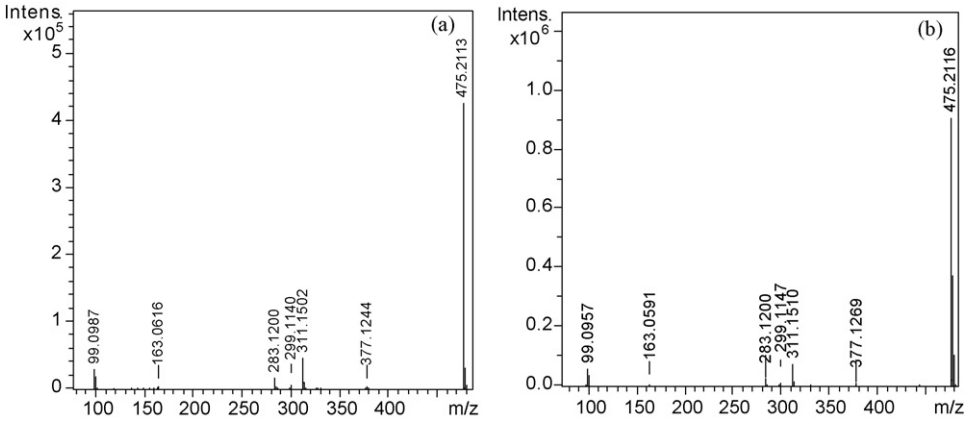

Fig. 4. Mass spectra of pure sildenafil (a) and methanolic extract of HF46 (b).

Table 1
Intra- and inter-day precision studies.

Concentration (μg/ml)	Intra-day precision measured concentration ± S.D. (μg/ml), R.S.D. (%)	Inter-day precision measured concentration ± S.D. (μg/ml), R.S.D. (%)
250	250.56 ± 8.15, 0.08	251.46 ± 25.45, 0.25
500	500.85 ± 10.54, 0.05	499.49 ± 32.97, 0.16
750	751.1244 ± 23.30, 0.06	751.98 ± 71.76, 0.21

Table 2
Recovery studies.

Spiked concentration (μg/ml)	Calculated spiked concentration ± S.D. (μg/ml), R.S.D. (%)	Recovery (%)
75	76.19 ± 5, 0.17	101.5867
350	344.14 ± 166.94, 1.12	98.32571
850	834.04 ± 100.77, 0.27	98.12235

concentrations, *viz.*, 250, 500 and 750 μg/ml was less than 0.5% (Table 1). A similar resolution behavior was observed on repeating the experiment on two different HPLC systems by two different analysts. Also, good recoveries were obtained when a herbal sample was spiked with 75, 350 and 850 μg/ml of sildenafil (mean recovery, 99.34%, Table 2). The precision values of RR_T for sildenafil and tadalafil were within ±0.05 and ±0.06 min, respectively. LOD and LOQ values of HPLC method for sildenafil were 0.37 and 1.2 μg/ml, and same for the tadalafil were 0.3 and 1.0 μg/ml, respectively. The amount of sildenafil in HF46 was determined to be ∼72.5 mg in a total capsule mass of 500 mg.

4. Conclusion

A study was carried out to explore Indian aphrodisiac herbal formulations for adulteration with PDE-5 inhibitor drugs. The LC-PDA and LC–MS based strategy was employed for the purpose. Of the total of 85 Indian aphrodisiac herbal formulations included in the study, only one was found to contain sildenafil. This shows initiation of the clandestine activity, though limited yet.

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