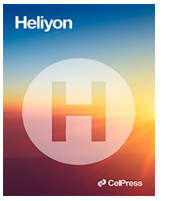
Heliyon 9 (2023) e15418

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Separation and structural elucidation of a novel vardenafil analogue as an adulterant in a natural health care dietary supplement

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ARTICLE INFO

Keywords:
Vardenafil analogue
PDE-5 inhibitor
Dietary supplement
Adulterants
Morpholine ring
HRMS
NMR

ABSTRACT

A novel vardenafil analogue was identified in dietary supplement as an adulterant in herbal formulations. The structure of this analogue was elucidated using HRMS, NMR after extraction from the pulverized powder. It was named morphardenafil as a morpholine ring has replaced the *N*-ethyl piperazine ring in vardenafil. A tablet of this dietary supplement contained about 50 mg of unspecified morphardenafil, which is 2.5 – 20-times the prescriptive dosage of Levetra, the commercial formulation of the vardenafil monohydrochloride salt in the market and probably places unwary consumers at risk for potentially serious adverse effects or drug-drug interaction (DDI).

1. Introduction

Since the first synthetic phosphodiesterase type-5 inhibitors (PDE-5i) sildenafil was approved to treat erectile dysfunction (ED) in 2003, two generations of seven different versions of PDE-5i, sildenafil citrate, tadalafil, vardenafil hydrochloride, avanafil, udenafil, mirodenafil hydrochloride and lodenafil carbonate have been developed and are in the market globally [1,2]. Accompanying with the great success of these prescription drugs, the various analogues flooded as adulterants in dietary supplement (DS) or counterfeit ED medicines [3,4]. In 2003, the first sildenafil analogue, homosildenafil, was identified in beverage, which had attracted much attention from the regulatory agencies soon after the launch of sildenafil [5]. Up to date, there are more than 80 analogues identified in DS or counterfeit drugs [3]. With patents of the original ED drugs expired and strict regulations on illicit adulterants implemented, the rapid growing trend of new adulterants from the modified chemical structure in designer analogues seems to be interrupted. However, these analogues were still found occasionally as adulterants in the dietary supplements. Among the adulterants, most of them are analogues of approved drugs deriving from the variant of the amine moiety of the molecule. Although the most frequent adulterants show difference geographically, vardenafil and its analogues are rarely adulterated according to a recent report, in which only two cases of the detected PDE-5i adulterants were ascribed to vardenafil among 92 samples [6]. Seven analogues of vardenafil, piperidino vardenafil [7], Desulfovardenafil [8], Hydroxyvardenafil [9], Norneovardenafil [1], *N*-Desethylvardenafil [1], Acetylvardenafil [10],

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https://doi.org/10.1016/j.heliyon.2023.e15418
Received 28 July 2022; Received in revised form 23 March 2023; Accepted 6 April 2023
Available online 12 April 2023

Hydroxythiovardenafil [11], were reported in past decades, accounting for 9% of PDE-5i analogues detected as adulterants in dietary supplements according to previous report [1].

Undoubtedly, these analogues, as common chemicals not medicines, pose high risk on public health, especially in long-term unwary administration on account of lacking of pharmaceutical and toxicological assessment based on the rigorous principles of drug development and good manufacture practice (GMP) for the quality control of the medicines. It is paramount of importance to distinguish the unspecified composition in the counterfeit medicines or adulterated DS for the regulation or safety purpose.

In the current study, a vardenafil analogue, 2-(2-ethoxy-5-morpholin-4-ylsulfonylphenyl)-5-methyl-7-propyl-3H-imidazo [5,1-f] [1,2,4]triazin-4-one (Fig. 1) is described and characterized as an adulterant in dietary supplements for the first time. The structure of this vardenafil analogue was tentatively established from the mass spectral fragmentation pattern referring to those of vardenafil (VAR) and desethylvardenafil (DEV). The substituted amine moiety was unequivocally elucidated, which was well supported by the typical chemical shifts, hydrolyzed free amine and neutral loss scan based on different techniques. The compound was given the name morphardenafil in accordance to the nomenclature used for such analogues, as the eighth vardenafil analogue identified as an adulterant in dietary supplements. Structure elucidation was carried out by UPLC-HRMS, UPLC-MS/MS and NMR.

2. Materials and methods

2.1. Chemicals and reagents

A sample with oval beige tablet form (200mg/tablet) was submitted for testing by a consumer who was skeptical on its claim of "pure nature". Vardenafil (VAR) and desethylvardenafil (DEV) as reference chemicals were purchased from Alta Scientific (Tianjin, China). LC-MS grade methanol, acetonitrile (ACN), formic acid, ammonium hydroxide (NH_4OH) and GC-grade dichloromethane (DCM) were purchased from Fisher Scientific (New Jersey, USA). Deuterated chloroform ($CDCl_3$) was purchased from Merck (Darmstadt, Germany). AR (analytical reagent)-grade chemicals and reagents were purchased from local commercial suppliers. The lab water (ultra-pure grade with 18.2 MΩ) was prepared by a Milli-Q IQ7005 water purification system (Millipore SAS, Molsheim, France).

2.2. Sample preparation

The tablets were crushed and grinded to be homogeneous fine powder. A potion of 200 mg powder was transferred into a 5 mL centrifuge tube with 2 mL of methanol/water (50:50, v/v) added into, vortexed, and then centrifuged for 5min at 3000 rpm. The supernatant was separated and filtered through a 0.22 μm nylon filter (Shimsen, Shimadzu). Whereafter, 100 μL of the filtrate was transferred for hydrolysis experiments (see Section 2.3), and another 10 μL of filtrate was transferred into 1 mL methanol/water (50:50, v/v) as stock solution for UPLC-HRMS/MS analysis (see Section 2.5). The rest part of the filtrate was placed under nitrogen flux to remove the methanol, replenished with 1 mL of water, and neutralized with 1 M NH_4 OH. With 2 mL of DCM added into, the sample was vigorously vortexed and then well-settled. The DCM fraction was separated and back-extracted with 1 mL water. The isolated DCM fraction was treated with anhydrous sodium sulfate to remove the residual moisture. Subsequently, the anhydrous DCM fraction was placed under the nitrogen flux to remove the solvent. The obtained yellowish residue was reconstituted in 0.5 mL of $CDCl_3$ for NMR analysis (see Section 2.6).

2.3. Hydrolysis

Hydrolytic experiments were conducted as well to provide unequivocal proof of the structure by confirming the existence of

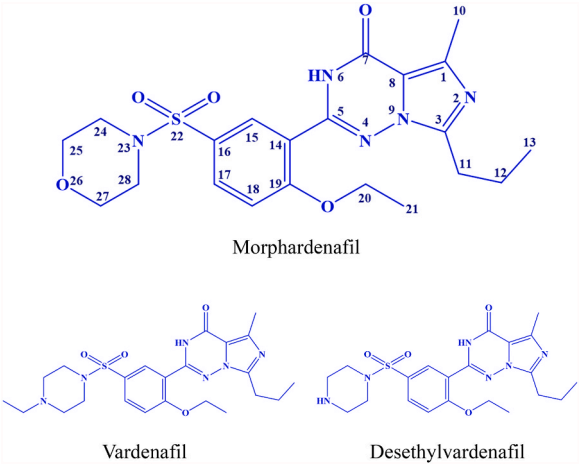

Fig. 1. Chemical structures of vardenafil, desethylvardenafil and the new analogue, morphardenafil.

morpholine amine in hydrolyzed sample. 100 μL of the filtrate obtained from Section 2.2 was added into 900 μL 6.1 M HCl, heated at 105–110 °C for 24 h as described [7]. After hydrolysis and neutralization, the samples were analyzed by UPLC-HRMS.

2.4. Calibration solution

A stock solution of reference chemical DEV was purchased at a concentration of 100 μg/mL in methanol and stored in the refrigerator. Working standards (2–500 ng/mL) were freshly prepared for the calibration experiments by diluting stock standards with methanol/water (50:50, v/v). A portion of the pulverized powder was weighed precisely and treated as Section 2.2. The supernatant was diluted further with methanol/water (50:50, v/v) prior to analysis.

2.5. UPLC-MS/MS instrument parameters

The sample was analyzed to acquire high-resolution mass spectrum (HRMS) with a Thermo Scientific Orbitrap 240 Fourier transform mass spectrometer (FTMS) hyphenated with a Vanquish A10 UPLC system via a H-ESI source (Thermo-Fisher, San Jose, CA, USA). Global parameters were set as: Spray voltages, 3200 V for positive ion scan and 2200 V for negative ion scan; Temperatures, 350 °C for Vaporizer and 320 °C for Ion Transfer Tube; Gas settings, 40 arbitrary units for Sheath Gas and 10 arbitrary units for Aux Gas. Scan parameters were set as: Collisional energy (CE) for higher-energy collisional dissociation (HCD) experiments was set to 30% with normalized type at fixed mode for full scan analysis to obtain the global information of the new analogue, or to be optimizable together with source fragmentation on for the tMS^2 analysis, from which rich-detailed mass spectra were acquired for structure elucidation. RF lens was set to 55%, and other parameters were set as default. Full scan experiments were carried out with scan ranges from m/z 80–1000 while tMS^2 experiments were conducted starting from m/z 50 at the resolution of 120,000 (FWHM at m/z 200). TraceFinder software (Version 5.1) was used for data acquisition and processing. A Thermo Hypersil GOLDTM VANQUISH column (2.1 mm × 100 mm, 1.9 μm) (Thermo-Fisher, San Jose, CA, USA) was applied.

MS experiments were also carried out on a Waters Xevo TQ-S mass spectrometer hyphenated with a Waters ACQUITY UPLC system via a ZprayTMESI source (Waters, Manchester, UK) to monitor the fragmentation reactions and neutral losses of the new analogue in the sample. The ESI source parameters were set as: capillary voltage, 3.2 kV; cone voltage, 120 V; source temperature, 150 °C; desolvation temperature, 600 °C; cone gas flow rate, 150 L/h; and desolvation gas flow rate, 600 L/h. The mass analyzer worked over a mass range of 50–600 Da. CE was set to 30 eV for multiple reaction monitoring (MRM) experiments. Masslyx software was used for data acquisition and processing. A Waters ACQUITY UPLC®BEH column (2.1 mm × 50 mm, 1.7 μm) (Waters, Milford, MA, USA) was applied.

The UPLC analysis was carried out with methanol/water (50:50, v/v) as mobile phase A and methanol as mobile phase B. The mobile phase composition B was initiated at 10%, then linearly ramped up from 50% to 95% over 3 min from 0.5min and kept at 95% for 1.5 min, then returned to 10% to stabilize the column for 2 min. The flow rate was set at 0.3 mL/min.

2.6. NMR

For NMR analysis, a Bruker Ascend™ 600 spectrometer (Bruker, Rheinstetten, Germany) with a 5 mm probe was used. The probe temperature was maintained at 298 K and standard 5 mm NMR tubes were used. ^{1}H NMR and ^{13}C NMR spectra were acquired from 0.5 mL of $CDCl_3$-reconstituted sample in Section 2.2, and the chemical shifts (δ_C: 77.0, δ_H: 7.26) were the residual solvent peaks. Coupling constants (J) were measured in hertz (Hz) and chemical shifts (δ) were recorded in parts per million (ppm), respectively. The data were processed with MestReNova (version 14) software.

3. Results and discussion

3.1. Sample preparation

3.1.1. For MS analysis

The exact mass of the quasi-molecular ions ($[M+H]^+$, $[M-H]^-$) were obtained, and the fragmentation pathway was studied to establish the chemical structure of the unknown adulterant based on the stock solution prepared as Section 2.2. The fractions for MS analysis were further diluted to proper concentration to avoid the mass spectra distorted and instrument contaminated due to the overloaded samples. The use of water/methanol (50:50, v/v) as extraction solvent assured good recovery of the analogue to perform the quantitative analysis since the pH value of the aqueous solution was tested as pH 4–5 before, which indicated that the analogue was probably present as salt form.

3.1.2. For NMR analysis

It could be anticipated that the interferences from the ingredients of the dietary supplements would not be eliminated completely. For NMR analysis, two specific treatments were devised for the sample to separate the new analogue from the impurities. Liquid-liquid extraction was applied to obtain sufficient amount of the analogue for NMR analysis. Solid phase extraction with C-18 cartridge (Waters, Milford, MA, USA) was also attempted to purify the analogue. However, the obtained amount of the analogue, though less impurities, is insufficient to acquire decent NMR spectra due to the low recovery in the solid phase extraction process.

3.1.3. For quantitative analysis

5.3 mg of the homogeneously pulverized powder was weighted and dissolved into 5.3 mL methanol/water (50:50, v/v), prepared as Section 2.2. The obtained fraction was further diluted for quantitative analysis to adapt the calibration curve ranged from 2 to 500 ng/mL established with reference analogue DEV. Hydrophilic solvent was preferred to extract the adulterant with good yield from the formulation of the natural dietary supplements. Meanwhile, from point of view, a kind of mild treatment as dissolution-filtration is sufficient to extract the adulterants from such samples, rather than the ways such as sonication, elixation etc., to avoid the interferences of the herbal ingredients.

3.2. MS analysis

3.2.1. High-resolution MS analysis

The mass spectrum of the new analogue found in the DS shows the exact mass at *m/z* 462.1782 in positive ion mode (Fig. 2A) and at 460.1657 in negative ion mode (Fig. 2B), which represent the quasi-molecular ions $[M+H]^+$ and $[M-H]^-$, respectively. The molecular formula were composed as $C_{21}H_{28}N_5O_5S$ and $C_{21}H_{26}N_5O_5S$ based on the measured exact masses with 2.5 ppm mass tolerance between the experimental and calculated values. Both the positive ion scan and negative ion scan shall be carried out for cross-verification of the quasi-molecular peaks, which is paramount to identify such analogues from the point of view. The adducts of $[M+Na]^+$ are rarely visible in the mass spectra, while the adducted ions $[2M+H]^+$ or $[2M-H]^-$ are evident as shown in Fig. 2.

3.2.2. Fragmentation pattern

Further HCD experiments were carried out to obtain the structure information combining with optional source induced dissociation (SID) to study the fragmentation pattern of the unknown component in the DS. The elucidation of the fragmentation pattern was primarily based on the rich information acquired in positive ion scan mode. Mass spectra of the parent ions at *m/z* 489, 461, and 462 (Integer values given for terse elucidation) for vardenafil, desethylvardenafil and the new analogue, morphardenafil in the DS, are given in Fig. 3, respectively，as well as the MS^3 spectra of their typical ions *m/z* 312 and *m/z* 299. Each precursor ion shows a common fragmentation pattern, which indicates the common pyrazolopyrimidine portion of the molecule. All the spectra of quasi molecular ions under HCD show the major fragments at *m/z* 312, 299, 284, 183, 169, and 151. Among them, *m/z* 312, 284 can be applied to tell the vardenafil analogues apart from sildenafil analogues with *m/z* 311, 283 [1]. Referring to the further fragmentation of *m/z* 434 (not presented), it demonstrates that the molecule of vardenafil family is fragmented as shown in Fig. 5 in ESI source. The compounds under HCD can be cleaved from the pyrazolopyrimidine portion of the molecule with two patterns of fragmentation of $[M+H]^+ \rightarrow$*m/z* 377 (negligible，loss of amine moiety)→*m/z* 312→*m/z* 284→*m/z* 183→*m/z* 169→*m/z* 151, and $[M+H]^+ \rightarrow [M+H\text{-}28]^+ \rightarrow$*m/z* 349 (negligible，loss of amine moiety) →*m/z* 299→*m/z* 270→*m/z* 169→*m/z* 151 (Fig. 5).

It is key important to identify the vardenafil analogues given that the amine moiety could be identified by the indicating fragments, such as *m/z* 113 and *m/z* 85 from the piperazine moieties of vardenafil (Fig. 3A) and DEV (Fig. 3B), respectively. However, the *m/z* 86, which may indicate the type of the amine moiety, is not convincing enough due to negligibility in the acquired spectra (Fig. 3C).

Be that as it may, this little ion makes a big difference because a key clue was offered that the elemental formular of this fragment was composed as C_4H_8NO by the measured exact mass with 2.5 ppm mass tolerance. And the structure of the amine moiety was tentatively deduced as morpholine ring. Therefore, the structure of this unknown compound, as shown in Fig. 1 (morphardenafil), was assigned to the adulterant in this “natural” dietary supplement. This compound is an analogue of vardenafil in which the *N*-ethyl piperazine ring of vardenafil has been replaced with a morpholine ring.

3.2.3. Neutral loss scan

The neutral loss of 28 was reported as common fragments in vardenafil analogue [7]. Further experiments were also carried out to verify such consistence between the new analogue and reference chemicals with a Waters Xeno TQ-S mass spectrometer. However, the

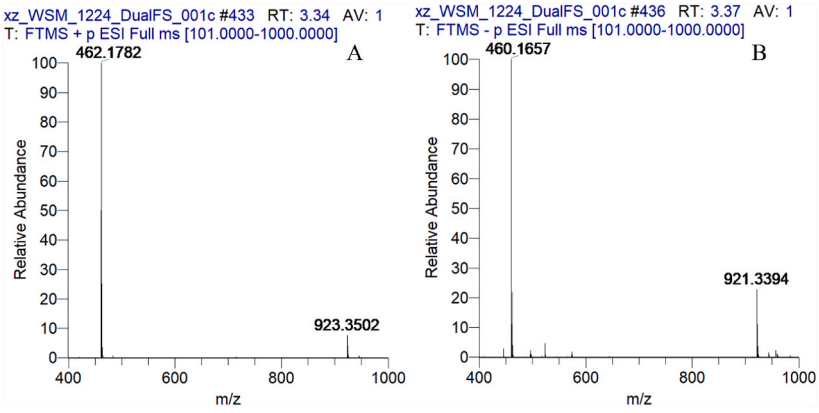

Fig. 2. High resolution mass spectra of the new vardenafil analogue, morphardenafil. A: MS in ESI + mode; B: MS in ESI- mode.

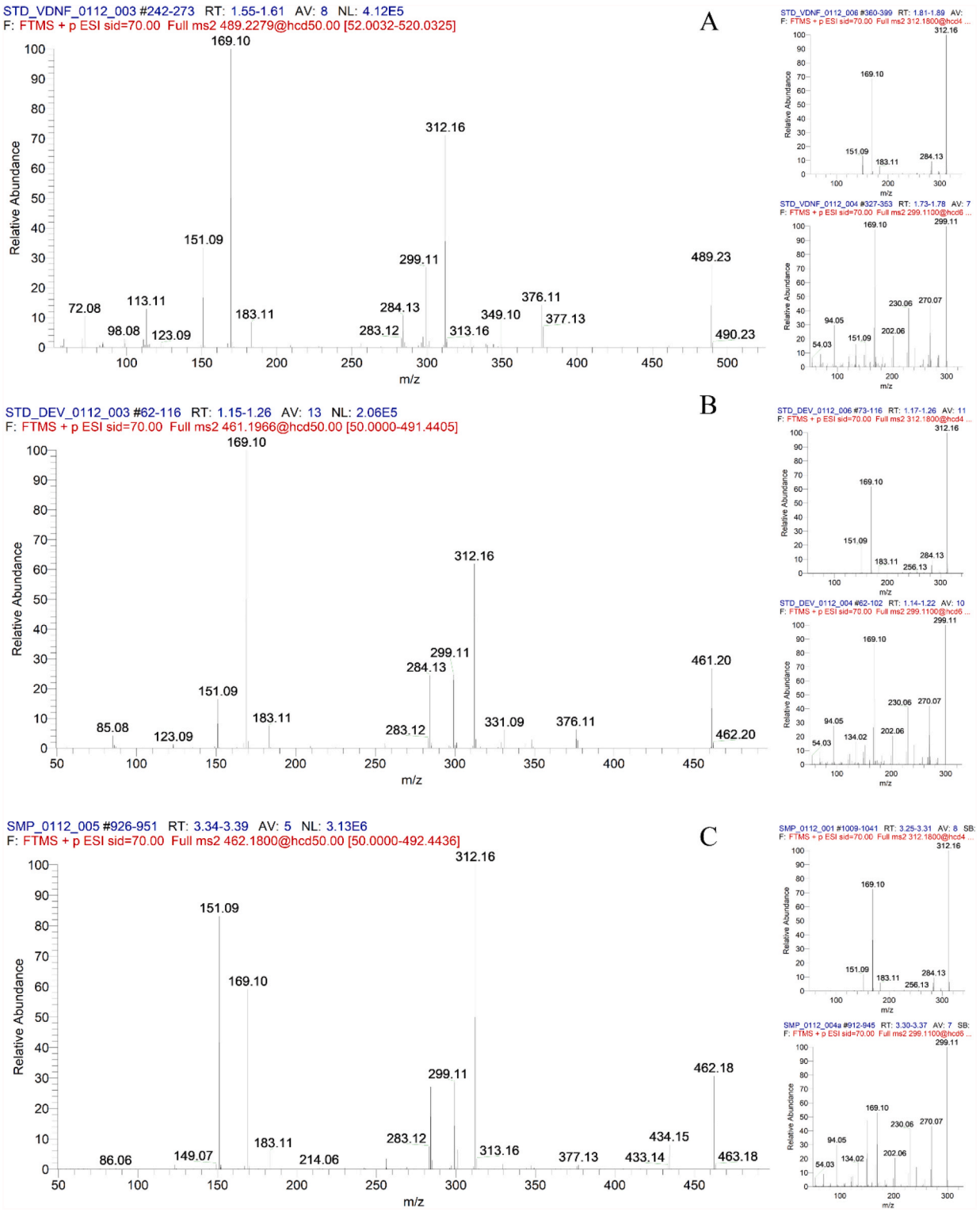

Fig. 3. MS fragmentation of vardenafil (A), desethylvardenafil (B) and the new vardenafil analogue, morphardenafil (C) with typical fragments at *m/z* 312 and *m/z* 299.

neutral loss of 28 was only monitored from the new analogue morphardenafil as shown in Fig. 4(A-B). It is identical to the mass spectra shown in Fig. 3 in which only the new analogue gave the desethyl fragment ion at *m/z* 434 at considerable intensity in H-ESI source. This indicates that this compound may have a potential stable *O*-desethyl analogue with the ethylene group removed.

Experiments of neutral loss were carried out to verify the real existence of morpholine amine moiety. As a result, neutral loss of 113, 85 and 86 were successfully monitored in vardenafil, DEV and the new analogue morphardenafil (Fig. 4C), respectively.

The morpholine as the substituted amine moiety of sildenafil analogue has been studied in the previous report [12], while we found that morphardenafil gave the fragments at *m/z* 86 with much less intensity, comparing to that of sildenafil analogue.

3.2.4. Quantification of the new analogue

The content of morphardenafil was measured in SRM mode by UPLC-HRMS with the calibration curve of DEV. It makes sense to use the analogue with similar structure as standard for quantitative analysis due to the lack of the authentic chemical, which has been discussed previously [7]. These two chemicals presented the adjacent retention time in the mobile phase gradient as Section 2.3 for the proximate chemical structure. The method to prepare the sample for quantification assured the good recovery of this new vardenafil analogue. The morphardenafil was amounted to approximately 50 mg/tablet. The amount of this analogue of vardenafil in the DS was 2.5–20 times prescriptive dosage of Levetra, the commercial formulation of the vardenafil monohydrochloride salt in the market. Thus, the "natural" DS may place its consumers at high risk for potentially serious adverse effects to take this unspecified chemical component with such high dosage. To our knowledge, this morpholine analogue was not subjected to the rigorous process of drug research and development up to now, and its use in DS was not approved by any regulatory authorities either.

3.3. Hydrolysis

A portion of sample was hydrolyzed as Section 2.3 and analyzed on UPLC-HRMS. The elemental formular of morpholine amine was successfully composed as $C_4H_{10}NO$ based on the measured exact mass of quasi molecular ion $[M+H]^+$ at *m/z* 88.0756 with an average mass error of −0.46 ppm in the hydrolyzed sample, which proved the existence of morpholine directly. Some other typical hydrolysis products of vardenafil family were also detected in the sample, which also evidenced the adulterant as an analogue of vardenafil [7].

3.4. NMR analysis

NMR has been used as a profiling method for detecting DS adulteration. The NMR profiles were obtained after the simple preparation as described in Section 2.6. With NMR spectra, the presence of morpholine amine moiety was clearly revealed, as shown in Fig. 6. The H10 was recorded clearly at δ2.82 ppm (Fig. 6A) meeting the reported data at δ 2.5–2.8 ppm in the vardenafil family which is differentiated to sildenafil [6]. The presence of characteristic aromatic signals of H15, H17 and H18 was also well-marked as reported at δ 8.38 ppm, δ 7.90 ppm and δ 7.19 ppm in the spectrum, which was considered as an indication of the adulteration from sildenafil or vardenafil families [6]. A pair of broad triplets at about δ 3.70 and δ 2.98 ppm (J_{HH} = 6 Hz), combining with δ 66 ppm and δ 46 ppm of the carbon signals in the ^{13}C NMR (Fig. 6B), demonstrates unambiguously the presence of the morpholine ring comparing to the reported data [12], which supported the previous deduction based on the evidences from both MS elucidation and other experiments. This finally ascertained the specific structure of the analogue, morphardenafil.

4. Conclusions

A synthetic analogue of vardenafil the PDE-5 inhibitor, named morphardenafil, was found in a natural dietary supplement for male erectile dysfunction. To our knowledge, this analogue, in which the *N*-ethyl piperazine ring had been replaced with morpholine amine, was firstly identified as an adulterant in dietary supplements. The chemical structure of this compound was established with structure elucidation by combined techniques of UPLC-HRMS, UPLC-MS/MS and NMR, and also supported by hydrolytic experiments. The

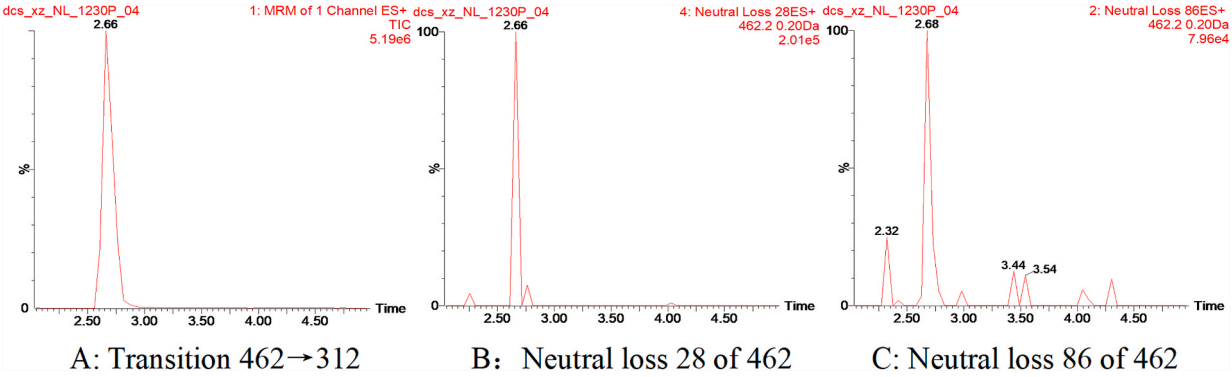

Fig. 4. Typical transition (A) and neutral losses (B, C) of the new vardenafil analogue, morphardenafil.

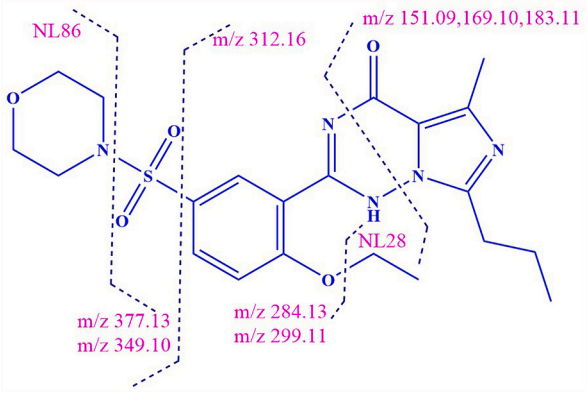

Fig. 5. Proposed fragmentation patterns of the new analogue, morphardenafil, identical to the vardenafil family.

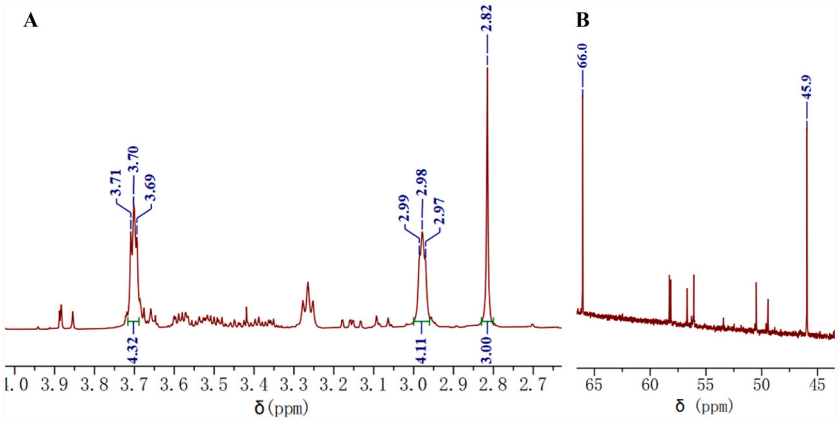

Fig. 6. Chemical shifts of ^{1}H (A) and ^{13}C (B) assigned to morpholine amine moiety of the new analogue, morphardenafil, as well as the typical H10. (Spectra were recorded in $CDCl_3$).

content of this analogue in the formulation was also quantified with an attainable reference analogue.

This finding shows that the new functional chemicals keep entering into commercial market in disguise, which demands that analytical methods on surveillance of the over-the-counter dietary supplements be versatile to cover the registered pharmaceuticals as well as their analogues to enhance the regulatory on illicit unspecified adulteration for the sake of people's health and safety.

Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Data availability statement

Data will be made available on request.

Additional information

Supplementary content related to this article has been published online at [URL].

Author contribution statement

Chunzheng Li: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote and revised the paper.

Xinzheng Wang, Shengming Wu, Junqing Zhao, Junjian Fang, Hui Li, Yingjie Zhu, Kang Zhang, Jing Peng, Jie Mao: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Weihua Li, Kun He: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Reviewed the paper.

Na Wang, Fangting Dong: Conceived and designed the experiments; Reviewed and revised the paper; Supervision; Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e15418.

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