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Isolation and structural elucidation of dapoxetine as an adulterant in a health supplement used for sexual performance enhancement

Lin Li[a], Min-Yong Low[a,b], Xiaowei Ge[b], Bosco C. Bloodworth[b], Hwee-Ling Koh[a,*]

[a] Department of Pharmacy, Faculty of Science, National University of Singapore, 18 Science Drive 4, Singapore 117543, Singapore
[b] Pharmaceutical Division, Applied Sciences Group, Health Sciences Authority, 11 Outram Road, Singapore 169078, Singapore

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ABSTRACT

A health supplement used for sexual performance enhancement was sent to Health Sciences Authority of Singapore for testing. An unknown compound was detected and isolated and its structure was elucidated using NMR, high-resolution MS, ESI-MS/MS, UV and IR. The compound, dapoxetine, is reported to be a selective serotonin reuptake inhibitor under investigation for the treatment of premature ejaculation.

1. Introduction

According to the World Health Organization (WHO), premature ejaculation (PE) is persistent or recurrent ejaculation with minimal stimulation before, on, or shortly after penetration, and before the person wishes it, over which the sufferer has little or no voluntary control, causing the sufferer and/or partner bother or distress [1]. Unlike erectile dysfunction (ED), PE affects men of all ages and is common among adolescents, young adults and men who lack sexual experience and frequency [2].

For the treatment of ED, synthetic phosphodiesterase Type 5 enzyme (PDE-5) inhibitors, such as sildenafil, tadalafil and vardenafil, are widely used. PE is usually treated by selective serotonin reuptake inhibitors (SSRIs) [3]. However, there is currently no FDA-approved therapy for PE. Dapoxetine is a short-acting SSRI. It was initially investigated as a potential drug for the treatment of depression, but it was clinically tested for the treatment of PE [4]. Currently it is being considered for approval by FDA for the treatment of PE in men [5–7].

It was reported that PDE-5 inhibitors can be used for the treatment of PE, either as a single agent or in combination with SSRI [8–10]. Wang et al. [8] investigated the safety and efficacy of sildenafil in the treatment of primary PE in 180 men. Vardenafil and tadalafil were also studied by some other groups for the treatment of PE [9,10]. In recent years, health supplements have become increasingly popular as they are believed to be "100% natural" and "have no side effects". But synthetic PDE-5 inhibitors as well as their analogues had been detected in some health supplements. Generalized strategies for characterizing PDE-5 inhibitors and their analogues in health supplements have been reported [11]. Our group has previously reported several analogues of PDE-5 inhibitors and isolated them from health supplements [12–14]. Adulteration of health supplements with PDE-5 inhibitors, their analogues and SSRIs is illegal and dangerous. Adverse events, such as nausea, dizziness, headache, diarrhoea, insomnia and upper respiratory tract infection [4,6–7] have been reported. In addition, prescription drugs should only be taken under a clinician's supervision. Consumers who use these kinds of so-called "natural" health supplements are at risk.

In this paper, we report the detection and isolation of dapoxetine from a health supplement used for sexual performance enhancement and its structural elucidation using MS, NMR and IR.

2. Materials and methods

2.1. Sample and chemicals

A health supplement, named MHD1, was sent to Health Sciences Authority (HSA) of Singapore for testing. It is a brown powder. Methanol (AR grade) and acetonitrile (HPLC grade) were supplied by Merck (Singapore). Methanol (HPLC grade) was supplied by Tedia (OH, USA). 0.45 μm nylon membrane filters were

* Corresponding author. Tel.: +65 6516 7962; fax: +65 6779 1554.
E-mail address: phakohhl@nus.edu.sg (H.-L. Koh).

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supplied by Whatman International Ltd. (Maidstone, UK). MilliQ water was obtained using a Synergy Purification System (Molsheim, France). $NaH_2PO_4{\cdot}H_2O$ was supplied by Merck (Darmstadt, Germany). Formic acid, methanol-d_4 used for NMR analysis and KBr powder used for IR analysis were purchased from Sigma–Aldrich (Steinheim, Germany).

2.2. Extraction of MHD1

2 g of MHD1 powder was ultrasonically extracted in 50 ml methanol (AR grade) for 30 min. The extract was filtered and the solvent was evaporated under vacuum. After filtration and solvent removal, the residue was reconstituted with 4 ml methanol (HPLC grade) and filtered through the 0.45 μm nylon membrane filter. The sample was further purified by preparative HPLC by successive injections of 50 μl of this sample.

2.3. Preparative HPLC

A Shimadzu HPLC system with two preparative pumps (LC-8A, Kyoto, Japan) and an automatic fraction collector (FRC-10A, Kyoto, Japan) were used. An Agilent ZORBAX SB-C18 reversed phase semi-preparative column (250 mm × 9.4 mm i.d., 5 μm) was used for the sample separation. The mobile phases were 0.1% formic acid in MilliQ water and 0.1% formic acid in methanol (HPLC grade). The gradient elution profile was as follows: 0.1% formic acid in methanol was increased from 10% to 90% in 7.5 min and maintained for 6 min. The flow rate of mobile phase was 4 ml/min and injection volume was 50 μl. The UV and visible spectra from 200 nm to 800 nm were recorded on-line during the chromatographic run. Fractions containing the target compound were collected by the automatic fraction collector based on the wavelength of 280 nm. The solvents were removed using a rotary evaporator, giving the compound of interest.

2.4. Melting point

The melting point (uncorrected) of the isolated compound was measured on a Gallenkamp melting point apparatus (Loughborough, UK).

2.5. LC-DAD

An Agilent 1100 series HPLC chromatograph with diode-array detector (Palo Alto, CA, USA) was employed. A ZORBAX Eclipse Plus C18 column (250 mm × 4.6 mm i.d., 5 μm) from Agilent Technologies (Wilmington, DE, USA) was used. The mobile phases were 25 mM $NaH_2PO_4{\cdot}H_2O$ (pH 3.2 ± 0.1) and acetonitrile (HPLC grade). The gradient elution profile was as follows: 0–30 min, acetonitrile rose from 10 to 70% (v/v), and maintained for 5 min. The flow rate of mobile phase was 1 ml/min. The injection volume was 10 μl. The UV spectra from 200 nm to 400 nm were recorded on-line during the chromatographic run. The chromatograms of both original methanol extract and purified dapoxetine were recorded at the wavelength 254 nm.

2.6. ESI-MS/MS and high-resolution MS analyses

The isolated compound was dissolved in methanol (HPLC grade) at a concentration of 1 μg/ml and was injected using an Agilent 1100 series HPLC chromatograph with diode-array detector (Palo Alto, CA, USA). An Agilent ZORBAX RX-C18 column (150 mm × 2.1 mm i.d., 5 μm) was used. The mobile phase was 0.1% formic acid in water and 0.1% formic acid in acetonitrile (HPLC grade). The gradient elution profile was as follows: 0–6.5 min, 0.1% formic acid in acetonitrile rose from 10 to 90% (v/v), and maintained for 3 min. The flow rate of the mobile phase was 300 μl/min. The injection volume was 5 μl. ESI-MS and MS/MS analyses were performed on an API 2000 mass spectrometer from Applied Biosystems (Foster City, CA, USA). The diode-array detector and the ESI-MS detector were operating in series. The $[M+H]^+$ was selected as a precursor ion and the ESI-MS/MS spectra were acquired. Collision energy (CE) was set at 35 V. Data acquisition and processing were performed using *Analyst* software (Version 1.4.1) from Applied Biosystems (Foster City, CA, US). The high-resolution MS spectrum was acquired in the positive ionization mode by direct infusion on a Finnigan MAT 95 XL-T mass spectrometer (Bremen, Germany) coupled with an electrospray ionization source.

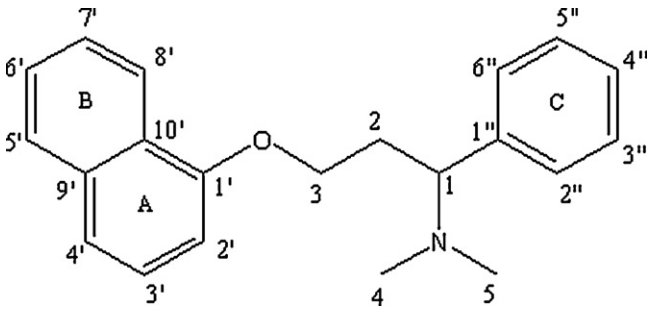

Fig. 1. Chemical structure of dapoxetine.

2.7. NMR and IR analyses

The isolated compound was dissolved in MeOD-d_4 for NMR analysis. DEPT spectra were recorded on a Bruker AVANCE300 spectrometer (^{1}H 300 MHz; ^{13}C 75 MHz). ^{1}H, ^{13}C, COSY, HMQC and HMBC spectra were recorded on a Bruker AMX500 spectrometer (^{1}H 500 MHz; ^{13}C 125 MHz). Chemical shifts were reported in ppm using the solvent peak as an internal standard. IR spectra in KBr disks were recorded on a Perkin Elmer Precisely Spectrum 100 FTIR spectrometer and recorded over the spectral range 4000–400 cm^{-1}.

3. Results and discussion

Approximately 15 mg of an off-white amorphous powder (melting point 175–178 °C, uncorrected) was isolated from 2 g of MHD1 powder. Fig. 1 shows its chemical structure. As shown in Fig. 2, the UV spectrum of isolated compound in methanol showed maximal absorbances at 210 nm, 230 nm and 293 nm, different from the UV spectra of the three approved PDE-5 inhibitors as well as their

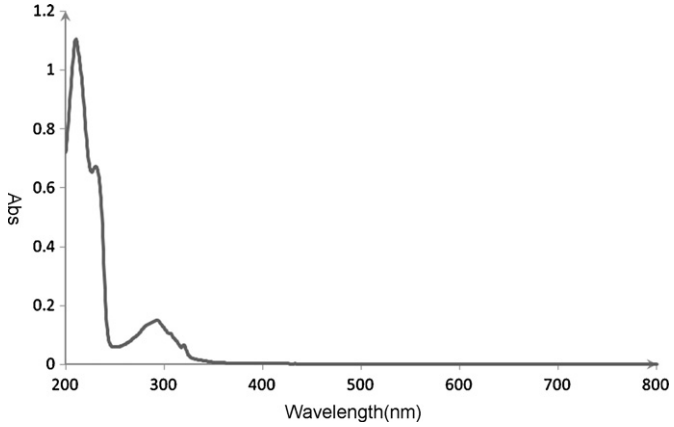

Fig. 2. UV–vis spectrum of dapoxetine in methanol.

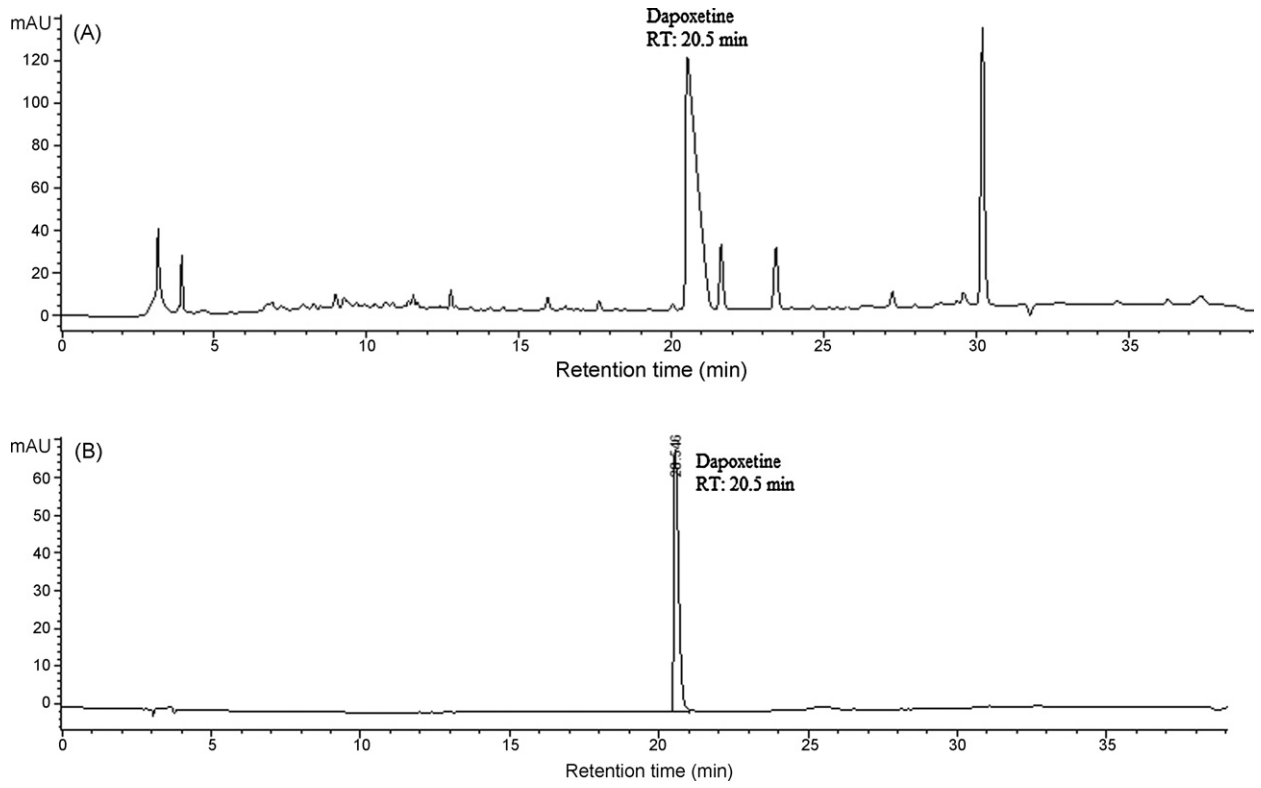

Fig. 3. HPLC chromatograms of (A) methanol extract of MHD1 and (B) purified dapoxetine at a wavelength 254 nm.

reported analogues. The retention times for the compound in both the extract and purified sample were 20.5 min under the current chromatographic conditions [Fig. 3(A) and (B)]. High-resolution ESI-MS spectrum (Fig. 4) of the compound revealed $[M+H]^+$ at *m*/*z* 306.1860, suggesting a molecular formula of $C_{21}H_{23}NO$. The error between observed mass and theoretical mass of $[M+H]^+$ was 2.34 ppm. This molecular formula was further supported by ^1H and ^{13}C NMR data which indicated the presence of 21 carbon atoms and 23 protons (Table 1).

The ^1H NMR spectrum revealed a sharp singlet at 2.84 ppm and two small neighboring peaks at 2.72 ppm and 2.88 ppm, integrating for a total of 8H (Table 1). The signals were attributed to six protons from two methyl (H-4/H-5) groups and two protons from one methylene (H-2) group at approximately the same chemical shift. The singlet revealed that the atom connected to the methyl groups was not attached to a proton. Besides, the chemical shift showed that the two methyl carbon atoms (C-4/C-5) were attached to a relatively electronegative atom. The electron withdrawing effect of this electronegative atom resulted in the deshielding of the methyl protons. Hence, it was speculated that the two methyl groups were attached to the nitrogen atom.

^{13}C NMR and DEPT data showed the signals of 2 methyl, 2 methylene, 13 methine and 4 quaternary carbon atoms. The four quaternary carbon signals at 155.2 ppm, 135.9 ppm, 133.0 ppm and 126.6 ppm were likely to be due to the carbon atoms in benzene rings. The quaternary carbon at 155.2 ppm was more deshielded compared to the other carbon atoms on the aromatic ring because it was attached to the oxygen atom. Hence, it was proposed that the oxygen atom was directly attached to the benzene ring A. Moreover, the COSY spectral data displayed the correlation between the protons of the methylene group with signals at 2.72 ppm/2.88 ppm (H-2) and the proton of methine group with a signal at 4.67 ppm (H-1) as well as protons of methylene group with signals at 4.17 ppm/3.81 ppm (H-3) respectively. Hence, these three groups were likely to be connected together.

In the ^{13}C NMR spectrum, the carbon atom (C-2′) giving rise to a signal at 105.8 ppm was in the *ortho* position of the carbon atom (C-1′) with a signal at 155.2 ppm because the –OR– group was

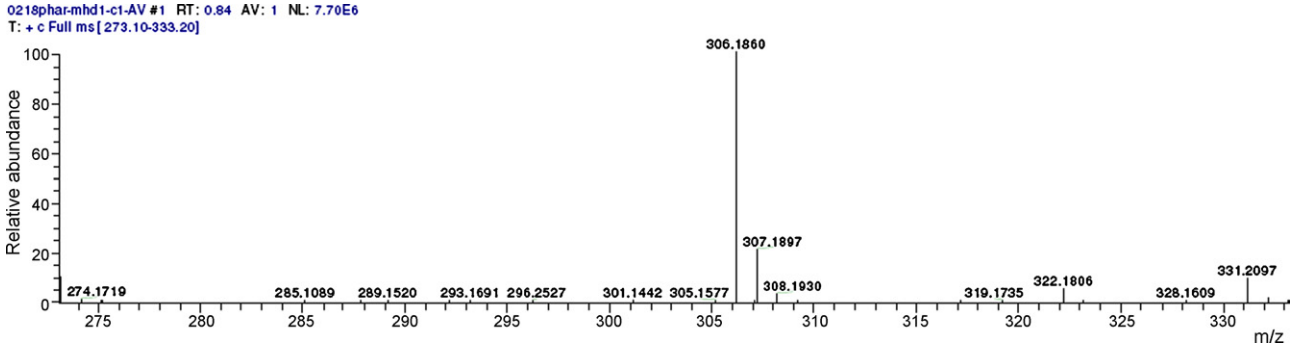

Fig. 4. High-resolution MS spectrum of dapoxetine.

Table 1
NMR data for dapoxetine.

No.	^{1}H (δ_H)	^{13}C (δ_C)	DEPT[a]	COSY	HMBC
1	4.67 (1H, dd, J = 4.0, 8.0)	70.0	1	H-2	C-3/C-4/C-5/C-2″/C-6″
2	2.72 (1H, m) 2.88 (1H, m)	31.1	2	H-1/H-3	C-1/C-3/C-1″
3	4.17 (1H, m) 3.81 (1H, m)	65.4	2	H-2	C-1/C-2/C-1′
4	2.84 (3H, s)	41.6	3	–	C-1/C-5
5	2.84 (3H, s)	41.6	3	–	C-1/C-4
1′	–	155.2	0	–	–
2′	6.64 (1H, d, J = 7.5)	105.8	1	H-3′	C-1′/C-3′/C-4′/C-10′
3′	7.26 (1H, t, J = 8.0)	126.9	1	H-2′/H-4′	C-1′/C-2′/C-9′
4′	7.38 (1H, d, J = 8.5)	121.6	1	H-3′	C-2′/C-5′/C-9′/C-10′
5′	7.76 (1H, d, J = 7.0)	128.5	1	H-6′	C-4′/C-7′/C-9′/C-10′
6′	7.47 (1H, m)	127.5	1	H-5′/H-7′	C-5′/C-8′/C-9′
7′	7.45 (1H, m)	126.3	1	H-6′/H-8′	C-5′/C-8′/C-10′
8′	8.05 (1H, d, J = 9.0)	122.7	1	H-7′	C-1′/C-6′/C-9′
9′	–	135.9	0	–	–
10′	–	126.6	0	–	–
1″	–	133.0	0	–	–
2″	7.48 (1H, d, J = 8.2)	130.6	1	H-3″	C-1/C-1″/C-3″/C-4″/C-6″
3″	7.53 (1H, m)	131.8	1	H-2″/H-4″	C-1″/C-2″/C-4″/C-5″
4″	7.43 (1H, m)	131.4	1	H-3″/H-5″	C-2″/C-3″/C-5″/C-6″
5″	7.53 (1H, m)	131.8	1	H-4″/H-6″	C-1″/C-3″/C-4″/C-6″
6″	7.48 (1H, d, J = 8.2)	130.6	1	H-5″	C-1/C-1″/C-2″/C-4″/C-5″

δ (ppm) in MeOD, J in Hz.
[a] Number in DEPT is the number of attached protons.

electron donating and hence the *ortho* position was more shielded. The quaternary carbon at position C-10′ giving rise to a signal at 126.6 ppm was long-range coupled with protons giving rise to signals at 6.64 ppm (H-2′), 7.38 ppm (H-4′), 7.45 ppm (H-7′) and 7.76 ppm (H-5′) in the HMBC spectrum. The HMBC spectral data also revealed that another quaternary carbon giving rise to a signal at 135.9 ppm (C-9′) was coupled to the protons giving rise to signals at 7.26 ppm (H-3′), 7.38 ppm (H-4′), 7.76 ppm (H-5′), 7.47 ppm (H-6′), 8.05 ppm (H-8′). Hence, it was proposed that two benzene rings (A and B) were connected together, sharing two quaternary carbons with signals at 126.6 ppm and 135.9 ppm to form a naphthalene group.

The methine carbon (C-1) giving rise to a signal at 70.0 ppm was long-range coupled to the protons giving rise to signals at 2.84 ppm (H-4/H-5), 3.81 ppm (H-3), 4.17 ppm (H-3), 7.48 ppm (H-2″/H-6″) in the HMBC spectrum, suggesting its connection with the benzene

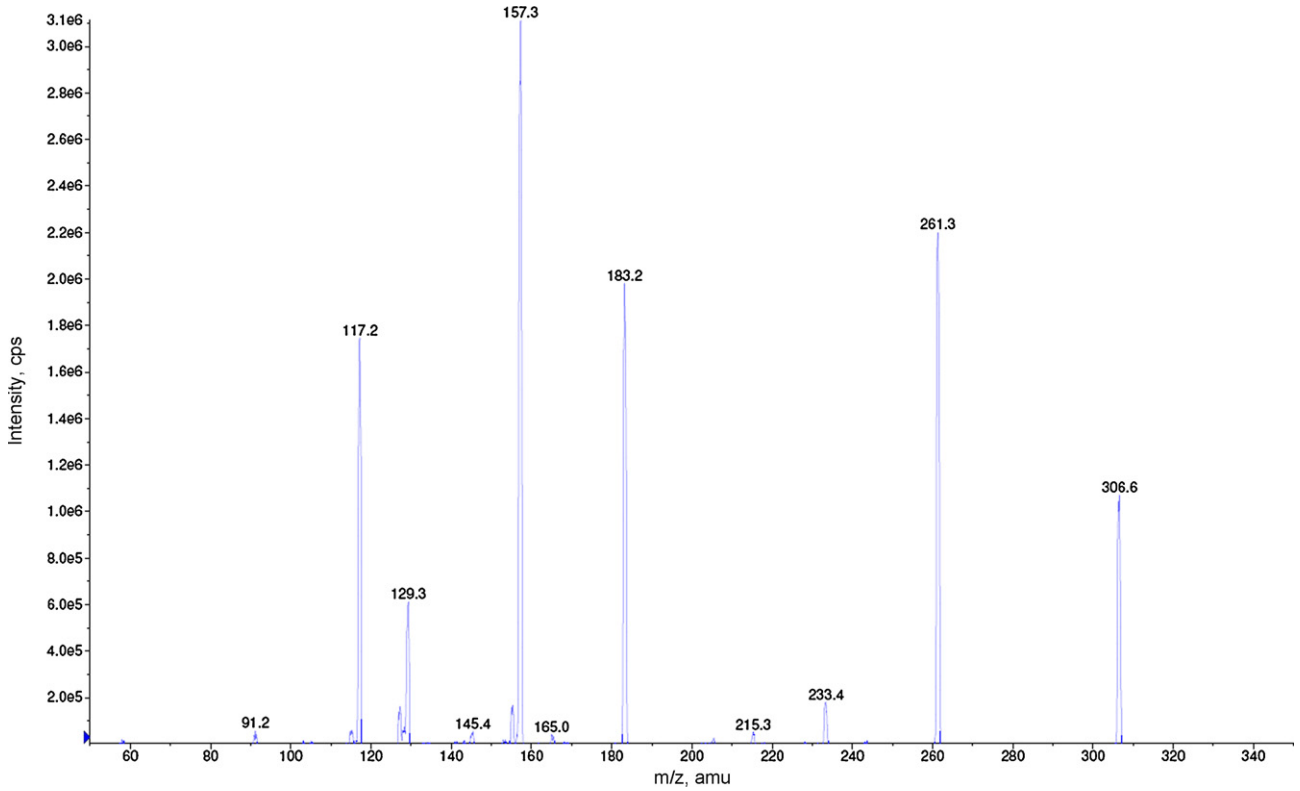

Fig. 5. ESI-MS/MS spectrum of dapoxetine.

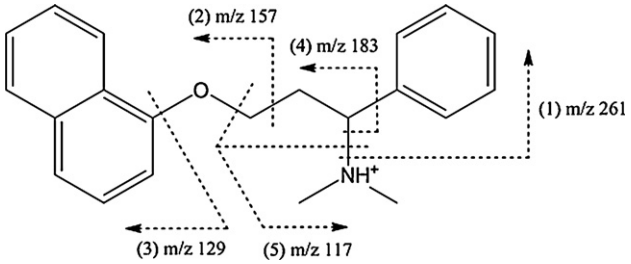

Fig. 6. Proposed ESI-MS/MS fragmentation of the protonated molecules of dapoxetine ([M+H]$^+$ *m*/*z* 306), further confirmed by Mass Frontier^{TM} 5.0.

ring C and a dimethylamino group. It was confirmed by the methyl carbons with a signal at 41.6 ppm (C-4/C-5) and the methine carbon atoms with a signal at 130.6 ppm (C-2″/C-6″) were long-range coupled with the proton giving rise to a signal at 4.67 ppm (H-1) in the HMBC spectrum. Furthermore, the methine carbon giving rise to a signal at 131.4 ppm (C-4″) was long-range coupled to the protons giving rise to signals at 7.48 ppm (H-2″/H-6″) and 7.53 ppm (H-3″/H-5″) in the HMBC spectrum. The methine carbons giving rise to a signal at 130.6 ppm (C-2″/C-6″) were long-range coupled to the protons giving rise to a signal at 7.43 ppm (H-4″) in the HMBC spectrum. Hence, it was deduced that benzene ring C was mono-substituted, with the quaternary carbon giving rise to a signal at 133.0 ppm (C-1″) and attached to the methine carbon which gave rise to a signal at 70.0 ppm (C-1).

Hence, the compound was determined to be N,N-dimethyl-3-(naphthalene-1-yloxy)-1-phenylpropane-1-amine [15] and also known as dapoxetine. It was previously clinically tested for the treatment of PE. This structure was further confirmed by ESI-MS/MS, IR and by comparing ^{1}H and ^{13}C NMR data with previous reports [15,16]. The MS^2 spectrum revealed several fragments of the parent ion [M+H]$^+$ 306.6 (Fig. 5). As shown in Fig. 6, the product ion at *m*/*z* 261 (fragmentation 1) was an alkene generated by the elimination of the dimethyl amine group, with a protonated oxygen atom. The product ion at *m*/*z* 157 (fragmentation 2) was formed by the cleavage of the bond between C-2 and C-3. The product ion at *m*/*z* 129 (fragmentation 3) was produced by the cleavage of the bond between the oxygen atom and the aromatic carbon. The product ion at *m*/*z* 183 (fragmentation 4) was formed by cleaving the benzene ring C from the group with *m*/*z* 261. The product ion at *m*/*z* 117 (fragmentation 5) was produced by cleaving the C-O bond between C-3 and O atom from the group with *m*/*z* 261. These fragments were further confirmed by Mass Frontier^{TM} 5.0 (HighChem, Ltd., Slovak Republic).

The IR spectrum of dapoxetine showed absorption bands of amine (ν_{C-N} 1267 cm^{-1}), aromatic ring (ν_{Ar-H} 3051 cm^{-1}, 3010 cm^{-1} and $\nu_{C=C}$ 1594 cm^{-1}, 1579 cm^{-1}, 1508 cm^{-1}, 1459 cm^{-1}), ether (ν_{C-O-C} 1135 cm^{-1}) and other bands at 2925 cm^{-1}, 2854 cm^{-1}, 2675 cm^{-1}, 2640 cm^{-1}.

4. Conclusion

In this study, a selective serotonin reuptake inhibitor, dapoxetine, was isolated from a health supplement and its chemical structure was elucidated using NMR, IR, high-resolution MS and ESI-MS/MS. The presence of dapoxetine in the health supplement is dangerous for consumers because it is not an approved drug. This is the first report of dapoxetine as an adulterant in a health supplement used for sexual performance enhancement.

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