Printed in the Republic of Korea
ANALYTICAL SCIENCE & TECHNOLOGY
Vol. 28 No. 4, 278-287, 2015
http://dx.doi.org/10.5806/AST.2015.28.4.278

Identification and evaluation of fragmentation pathways of PDE-5 inhibitor analogues using LC-QTOF-MS

Jung-Ah Do†, Eunyoung Noh†, Soon-Byung Yoon, Hyoung-Joon Park, Sooyeul Cho, Sung-Kwan Park and Chang-Yong Yoon★

Advanced Analysis Team, Toxicological Evaluation and Research Department, National Institute of Food and Drug Safety Evaluation, Ministry of Food and Drug Safety, Chungcheongbuk-do 28159, Korea
(Received June 22, 2015; Revised July 13, 2015; Accepted July 15, 2015)

LC-QTOF-MS를 이용한 발기부전치료제 유사물질의 fragmentation pathway 분석

도정아† · 노은영† · 윤순병 · 박형준 · 조수열 · 박성관 · 윤창용★

식품의약품안전처 식품의약품안전평가원 독성평가연구부 첨단분석팀
(2015. 6. 22. 접수, 2015. 7. 13. 수정, 2015. 7. 15. 승인)

Abstract: Phosphodiesterase type 5 inhibitors (PDE-5 inhibitors) are used in the treatment of erectile dysfunction. In recent years, a number of reports have been conducted on dietary supplements contaminated with PDE-5 analogues. In this study, 58 analogues of PDE-5 inhibitors were sorted into five groups: tadalafil, sildenafil, hongdenafil, vardenafil, and other analogues. These analogues were then evaluated using a liquid chromatography-quadrupole-time of flight mass spectrometry (LC-QTOF-MS) electrospray ionization mass method. Each compound has a unique fragmentation ion, which can be easily analyzed qualitatively. The fragmentation pathways of the analogues were elucidated based on the QTOF-MS and MS/MS data. Common ions were confirmed for each group by analyzing the structural characteristics and fragmentation pathways. Specifically, common ions were observed at *m/z* 169.08 and 135.04 (tadalafil analogues), *m/z* 311.15 and 283.12 (sildenafil analogues and hongdenafil analogues), and *m/z* 312.16 and 151.09 (vardenafil analogues). The advantage of this method is that the structure of unknown components can be determined by interpreting the product ions. Hence, the developed method can be used for the identification of unknown compounds. Fragmentation pathways may also aid in the detection and identification of PDE-5 inhibitor analogues.

Key words: PDE-5; LC–QTOF–MS; fragmentation pathway; identification; unknown compounds

★ Corresponding author
Phone : +82-(0)43-719-5305 Fax : +82-(0)43-719-5300
E-mail : jado@korea.kr
†The authors consider that the first two authors should be regarded as joint First Authors.

1. Introduction

Phosphodiesterase type 5 (PDE-5) inhibitors, which are clinically indicated for the treatment of erectile dysfunction (ED), are widely available on the illegal market and as undeclared adulterants.[1-5] Some products have been found to be adulterated with approved PDE-5 inhibitors as well as their unapproved synthetic analogues, which contain minor structural modifications compared to the approved compounds. The presence of PDE-5 inhibitors or their analogues in herbal supplements could pose a significant risk to the public health.[6] PDE-5 inhibitors can cause several adverse effects such as headaches, facial flushing, nasal congestion, dyspepsia, visual disorders, and back pain.[7-9] Furthermore, patients taking nitrate medications should not use PDE-5 inhibitors, as this combination may provoke potentially life-threatening hypotension.[10] However, the widespread use and popularity of PDE-5 inhibitors have led to an increase in the prevalence of illicit sexual performance enhancement products in many countries.[11-15] The presence of various analogues of PDE-5 inhibitors in dietary supplements has increased tenfold over the past decade, potentially posing a serious health risk to humans.

A number of analytical techniques have been developed for the detection and determination of PDE-5 inhibitors, such as immunoassays,[16] atomic emission spectrometry,[17] ion mobility spectrometry,[18,19] micro-Raman spectroscopy,[20] high-performance liquid chromatography (HPLC) with ultraviolet or fluorescence detection,[21-23] gas chromatography–mass spectrometry (GC-MS),[24,25] and liquid chromatography-mass spectrometry (LC-MS).[26-31] Liquid chromatography-quadrupole-time of flight mass spectrometry (LC-QTOF-MS) carried out in full scan mode can be used to elucidate the accurate mass of target and nontarget compounds, as well as that of unknown compounds, while the multiple reaction monitoring (MRM) mode can be used only to detect the mass of target compounds. Fragmentation patterns can be used to determine the structure of unknown components by interpreting the product ions. Accurate mass information is important for confirming the identities of product ions and it plays a key role in the elucidation of fragments.

In the present study, a convenient and economic method was established to detect the analogues of PDE-5 inhibitors. Fast and accurate analysis of these analogues was performed by evaluating the MS spectra and fragmentation pathway of 58 PDE-5 inhibitor analogues using LC-QTOF-MS. Explaining the fragmentation pathway would be very useful for monitoring and identifying the parents and their metabolites. Furthermore, to the best of our knowledge, no detailed fragmentation pathway research on cyclopentyltadalafil, homotadalafil, acetaminotadalafil, propoxyphenylthioaildenafil, propoxyphenylthiohomosildenafil, propoxyphenylthiohydroxyhomosildenafil and propoxyphenylthiosildenafil has been reported to date. The structural similarities and differences among the analogues are also presented.

2. Experimental

2.1. Reagents and standards

Dapoxetine and icariin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetaminotadalafil, aminotadalafil, chloropretadalafil, demethyltadalafil, *n*-butyltadalafil, *n*-octylnortadalafil, tadalafil, tadalafil E, acetyl acid, benzylsildenafil, carbodenafil, chlorodenafil, cinnamyldenafil, cyclopentynafil, dimethylsildenafil, dimethylacetildenafil, gendenafil, homosildenafil, hongdenafil, hydroxychlorodenafil, hydroxyhomosildenafil, hydroxythiohomosildenafil, mirodenafil, nitrodenafil, norneosildenafil, oxohongdenafil, thiohomosildenafil, thiosildenafil, udenafil, acetylvardenafil, hydroxyvardenafil, imidazosagatriazinone, norneovardenafil, pseudovardenafil, vardenafil, avanafil, desmethylcarbodenafil, and lodenafilcarbonate were purchased from TLC PharmaChem (Vaughan, On, Canada). Dimethylthiosildenafil, sildenafil, thioquinapiperifil, desulfovardenafil, yohimbin, xanthoanthrafil, dichlorodenafil, hydroxyhongdenafil, piperidinohongdenafil, dimethylhongdenafil, epi-aminotadalafil, methylhydroxyhomosildenafil (homothiomethylsildenafil), homotadalafil, propoxyphenylthiohomosildenafil, pro-

poxyphenylthiohydroxyhomosildenafil, propoxyphenylthiosildenafil, propoxyphenylthioaildenafil, and cyclopentyltadalafil were obtained from the Korea Ministry of Food and Drug Safety. To prepare the stock solutions, all standards were dissolved in methanol from Sigma-Aldrich (MeOH, HPLC grade) at a concentration of about 1 mg/mL. The stock solutions were stored in a refrigerator (4 °C) and diluted before analysis. Acetonitrile (ACN, HPLC grade), methanol (HPLC grade), and formic acid (analytical reagent grade) were supplied by Merck (Darmstadt, Germany), and Sigma-Aldrich. The water was purified with a Milli-Q system (18.2 mΩ) by Millipore (Billerica, MA, USA).

2.2. LC-QTOF-MS analysis

The mass of the PDE-5 inhibitor were accurately measured using HPLC combined with Agilent 6530 Accurate-Mass Quadrupole (QTOF-MS) equipped with ESI Jet Stream Technology (Agilent Technologies, Waldbronn, Germany). An Agilent XDB C_{18} column (150×2.1 mm^2 I.D., 3.5 μm) kept at 35°C in an oven was used, and the mobile phases consisted of DW (v/v, A) and ACN (v/v, B) containing 0.1% formic acid. The gradient elution profile was as follows: 0-3 min (A:B = 80:20%), 3-13 min (A; 80%-40%, B; 20%-60%), 13-16 min (A:B = 40:60%), 16-18 min (A; 40%-0%, B; 60%-100%), 18-21 min (A:B = 0:100%), 21-22 min (A; 60%-80%, B; 100%-20%), and 22-25 min (A:B = 80:20%). The flow rate of the mobile phase was 0.25 mL min^{-1}, and the injection volume was 3 μL. A Jet Stream ESI source was operated in the positive ionization mode; other acquisition conditions of QTOF-MS were as follows: gas temperature, 350°C; drying gas flow, 8 L min^{-1}; nebulizer, 35 psi; sheath gas temperature, 350°C; and sheath gas flow, 11 L min^{-1}. The scan source parameters were as follows: capillary voltage, 3500 V; nozzle voltage, 1000 V; and fragment voltage, 120 V. The collision energy was set at 15 V, and *m/z* 121.0506 and 922.0098 were selected as the reference masses to tune the QTOF-MS. The mass resolution was 10000-20000 at *m/z* 100-1000. The MS/MS spectra were generated in product ion scan mode at CID energies of 5, 10, 15, 20, 25, 30, 35, and 40 eV. Data acquisition and processing were conducted using Mass Hunter Workstation Software (Ver. B.02.01, Agilent Technologies).

2.3. Analysis of possible fragmentation pathway

The possible fragmentation pathways were reviewed and selected in accordance with the following criteria: (1) the tendency for the radical site to initiate a reaction in competition with the charge site generally parallels the radical site's tendency to donate electrons: N > S, O, π, R· > Cl, Br > H, where π signifies an unsaturated site and R· is an alkyl radical; (2) in *n*-alkanes, the most easily cleaved bonds (σ-bonds between secondary carbons) have nearly equivalent bond strength; and (3) a number of the most significant differences arise owing to the low ionization energies of sulfur compounds, which are approximately 1 eV below those of the corresponding oxygen compounds.[32]

3. Results and discussion

Fifty-eight PDE-5 inhibitors and their analogues were analyzed by QTOF–MS; the analogues were classified into five groups: tadalafil, sildenafil, hongdenafil, vardenafil, and other analogues. The results are summarized in *Table* 1. The fragmentation pathways of the five types of PDE-5 inhibitors were studied using a combination of QTOF-MS. The protonated $[M+H]^+$ molecule ions were selected as the precursor ions for the fragments in the MS/MS product ion spectra. To aid in the interpretation of the product ion spectra, accurate mass measurements of the product ions were conducted using LC-QTOF-MS. And the accurate mass measurement error for between the experimental and the theoretical mass of $[M+H]^+$ ion was lee than 10 μg/kg. Identification of the compounds was based on retention time, protonated molecular ions, and fragment ions at individually selected collision energies (CEs). The CEs were set to 5, 10, 15, 20, 25, 30, 35, and 40 eV, and the optimal CE was determined for each PDE-5 inhibitor.

For the MS/MS studies, the mass spectrometric

Table 1. Retention time, accurate mass, parent ion, collision energies, and product ion of 58 PDE-5 inhibitor-like components

Group		No.	Compound	RT*	Exact mass	Parent ion	Error (in ppm)	CE** (eV)	Product ion
Tadalafil (11)		1	Acetaminotadalafil	10.1	433.1506	433.1496	2.3	20	391.14, 311.11, 262.09, 169.08, 135.04
		2	Aminotadalafil	10.1	391.1401	391.1389	3.0	15	302.08, 269.10, 169.08, 135.04
		3	Chlrolopretadalafil	14.7	427.1055	427.1031	5.6	20	334.11, 302.08, 274 .08, 135.04
		4	Cyclopentyltadalafil	6.02	444.1918	444.1906	2.7	18	322.15, 197.06, 169.07,135.04
		5	Demethyltadalafil	10.3	376.1292	376.1264	7.4	20	302.08, 262.08, 254.09, 169.07, 135.04
		6	Epi-Aminotadalafil	10.3	391.1401	391.1401	0	20	302.08, 269.10, 169.08, 135.04
		7	Homotadalafil	5.1	404.1605	404.1647	-10.3	15	282.12, 197.07, 169.07,135.04
		8	*n*-Butyltadalafil	14.1	432.1918	432.1919	-0.2	20	310.15, 282.16, 197.07, 169.08, 135.04
		9	Octylnortadalafil	19.2	488.2544	488.2579	-7.1	20	366.22, 338.22, 262.07, 169.07, 135.04
		10	Tadalafil	11.1	390.1448	390.1433	3.8	20	302.08, 268.11, 240.11, 169.08, 135.05
		11	Tadalafil impurity E	11.2	390.1448	390.1409	9.9	20	302.08, 268.11, 240.11, 169.08, 135.05
Sildenafil	Sildenafil (9)	1	Benzylsildenafil	11.3	551.2435	551.2434	0.1	40	508.24, 459.18, 377.13, 312.15, 284.12, 134.20
		2	Cyclopentynafil	10.3	529.2591	529.2594	-0.3	40	461.19, 377.13, 311.15, 283.11, 153.14, 112.11
		3	Dimethylsildenafil	9.7	489.2279	489.2251	5.7	40	432.17, 377.12, 311.15, 283.12, 113.11
		4	Homosildenafil	9.4	489.2279	489.2252	5.5	40	461.19, 377.14, 311.15, 283.12, 166.10, 113.11
		5	Hydroxyhomosildenafil	9.1	505.2228	505.2220	1.5	40	487.21, 377.13, 311.15, 283.12, 129.10, 112.09
		6	Mirodenafil	11.5	532.2588	532.2597	-1.6	40	404.17, 338.18, 312.13, 296.14, 282.12, 129.10
		7	Norneosildenafil	15.8	460.2013	460.1956	-9.3	40	432.17, 377.12, 311.15, 299.11, 283.12, 256.10, 166.10, 136.05
		8	Sildenafil	9.1	475.2122	475.2094	5.8	40	377.14, 311.15, 283.12, 100.10
		9	Udenafil	10	517.2591	517.2626	-6.5	45	325.17, 313.13, 299.11, 283.12, 255.12, 112.11
	Thio sildenafil (9)	1	Thiohomosildenafil	13.1	505.2050	505.2011	7.7	40	341.13, 327.12, 299.09, 113.11
		2	Thiosildenafil	12.6	491.1894	491.1908	-2.8	40	341.14, 327.12, 299.09, 113.10
		3	Homothiomethyl sildenafil	9.2	519.2384	519.1935	1.7	45	475.17, 326.15, 297.10, 129.09, 112.09
		4	Dimethylthiosildenafil	13.3	505.2050	505.2020	5.9	40	448.15, 393.11, 327.13, 299.10, 271.10, 113.11
		5	Hydroxythiohomo sildenafil	12.3	521.1999	521.1993	1.1	40	503.21, 327.12, 299.10, 129.10, 112.09
		6	Propoxyphenyl thiohomosildenafil	12.5	519.2207	519.2071	1.1	40	327.11, 315.07, 299.09, 271.09, 228.03, 113.10
		7	Propoxyphenylthio hydroxyhomosildenafil	12.0	535.2156	535.2156	0	40	327.11, 299.09, 228.03, 271.10, 129.10, 112.09
		8	Propoxyphenylthio sildenafil	12.2	505.2050	505.1970	-3.3	40	341.14. 329.10, 315.09, 299.09, 112.09, 100.09
		9	Propoxyphenyl thioaildenafil	12.7	519.2207	519.2115	-1.5	40	327.08, 299.09, 271.09, 113.10
Hongdenafil (5)		1	Dimethylhongdenafil	7.5	453.2609	453.2591	3.9	40	435.26, 353.16, 325.13, 313.12, 297.13, 285.14, 113.10, 101.10
		2	Hongdenafil	7.3	467.2765	467.2740	5.3	40	369.20, 353.16, 311.15, 297.13, 285.13, 111.09
		3	Hydroxyhongdenafil	7.2	483.2714	483.2723	-1.8	40	439.24, 396.21, 341.16, 311.14, 297.14, 143.11, 127.09, 111.09
		4	Piperidinohongdenafil	8.7	438.2500	438.2523	-5.2	40	380.20, 341.16, 325.13, 313.12, 297.13, 285.13, 166.10
		5	Oxohongdenafil	8.2	481.2558	481.2547	2.2	20	453.25, 410.21, 396.20, 311.13, 297.13, 284.11

Table 1. Retention time, accurate mass, parent ion, collision energies, and product ion of 58 PDE-5 inhibitor-like components

Group	No.	Compound	RT*	Exact mass	Parent ion	Error (in ppm)	CE** (eV)	Product ion
Vardenafil (6)	1	Acetylvardenafil	3.7	467.2765	467.2831	7.2	40	341.16, 312.16, 297.13, 151.09
	2	Desulfovadenafil	10.6	313.1659	313.1628	9.8	30	284.13, 256.10, 213.09, 151.09, 123.09
	3	Hydroxyvardenafil	7.3	505.2228	505.2255	-5.3	40	376.11, 312.16, 151.09, 129.10
	4	Norneovardenafil	7.9	357.1552	357.1553	-0.2	35	329.12, 312.15 151.09, 123.09
	5	Pseudovardenafil	12.6	460.2013	460.2000	2.8	40	432.17, 312.16, 284.13, 256.10, 151.09, 123.10
	6	Vardenafil	7.2	489.2279	489.2263	3.2	40	461.21, 376.10, 312.16, 151.09
Other analogues (18)	1	Aceyl acid	10.7	357.1557	357.1547	2.7	30	329.12, 300.08, 285.13, 311.11, 216.09, 166.10
	2	Avanafil	9	484.1858	484.1849	1.8	30	375.12, 303.06, 221.10
	3	Carbodenafil	7.1	453.2609	453.2580	6.3	25	339.14, 311.11, 283.11
	4	Chlorodenafil	14	389.1375	389.1359	4.1	35	361.10, 311.11, 285.13, 256.10, 166.10
	5	Cinnamyldenafil	11.5	555.3078	555.3056	3.9	25	437.23, 355.17, 285.14, 117.07
	6	Dapoxetine	12.3	306.1852	306.1837	4.8	15	261.12, 233.10, 183.08, 157.06, 117.07
	7	Desmethylcarbodenafil	4.8	439.2452	439.2533	4.3	25	339.15, 311.12, 283.12, 166.10, 147.01
	8	Dichlorodenafil	19.5	407.1036	407.0949	6.6	35	379.07, 350.03, 280.09, 136.05
	9	Dimethylacetildenafil	7.7	467.2765	467.2839	5.5	40	410.23, 325.16, 297.14, 166.10, 127.12, 112.10
	10	Gendenafil	12.6	355.1765	355.1745	5.6	40	327.14, 311.10, 285.13, 256.10, 216.08, 166.10
	11	Hydroxychlorodenafil	12.5	391.1531	391.1521	2.5	35	363.12, 313.13, 285.13, 256.09, 166.10
	12	Icariin	8.5	677.2440	677.2431	1.3	15	531.19, 463.12, 369.14, 313.07, 129.06
	13	Imidazosagatriazinone	15.1	313.1659	313.1657	0.6	30	285.13, 256.10, 216.08, 166.10
	14	Lodenafilcarbonate	8.4	505.2228	505.2343	-2.9	35	461.19, 377.13, 283.12, 129.10
	15	Nitrodenafil	14.4	358.1510	358.1499	3.0	30	330.12, 312.15, 284.13, 256.09, 1356.05
	16	Thioquinapiperifil	5.8	449.2118	449.2184	0.8	25	258.08, 204.14, 120.08
	17	Xanthodenafil	14	390.1660	390.1677	-4.3	10	344.18, 223.07, 151.08
	18	Yohimbin	6.1	355.2016	355.2045	-8.1	30	326.18, 212.13, 144.08, 117.07

RT*: Retention time
CE**: Collision energy

parameters were optimized in order to obtain the best mass fragmentations. Accurate mass values were observed for the product ions in MS/MS, and the element compositions were derived from the measured *m/z* values and the general concept that fragmentation should involve logical neutral losses. Additionally, the product ion data were used to construct the fragmentation pathways.

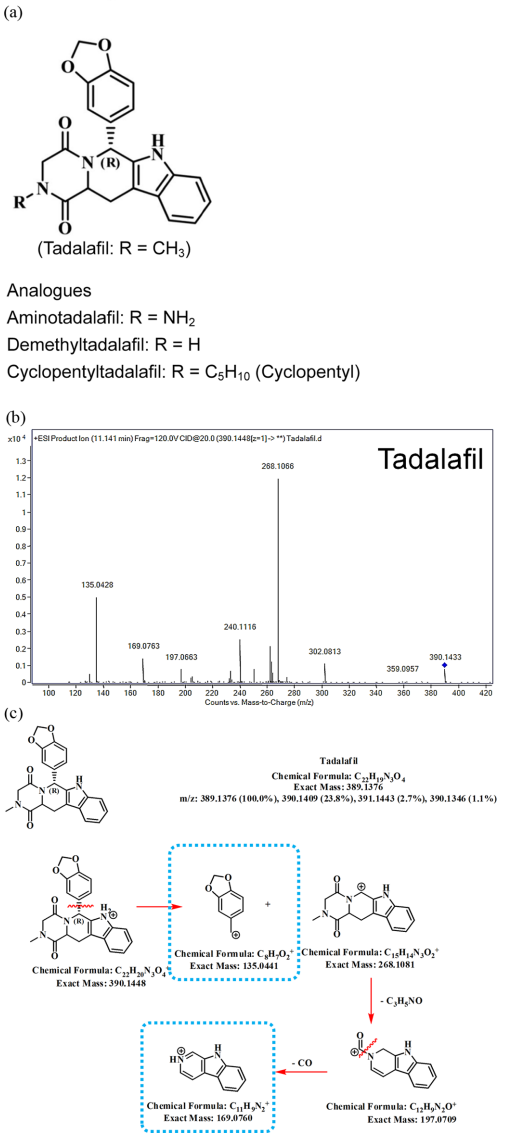

Fig. 1. (a) Chemical structures of tadalafil and tadalafil analogues, (b) typical spectra of tadalafil obtained by LC–QTOF–MS in positive electrospray mode, and (c) fragmentation pathway of $[M+H]^+$ from tadalafil in positive ion mode.

3.1. Fragmentation of tadalafil and tadalafil analogues

Tadalafil analogues contain different functional groups on the tadalafil backbone. For example, aminotadalafil contains an amino group in the R position, while demethyltadalafil lacks a methyl group in the R position, and cyclopentyltadalafil contains a cyclopentyl moiety in the R position. Tadalafil exhibits an *m/z* 390.14 as $[M+H]^+$, and fragment ions at *m/z* 268.11, 197.07, 169.07, and 135.04 were observed in the QTOF–MS/MS mode. Fragments of *m/z* 268.10 $[M+H\text{-}C_7H_6O_2]^+$ and *m/z* 135 $[M+H\text{-}C_{14}H_{14}N_3O_2]^+$ could be suggested from $[M+H]^+$ in CE value of 20 eV. A peak at *m/z* 197 was observed owing to the elimination of C_3H_5NO from *m/z* 268.10 $[M+H\text{-}C_7H_6O_2]^+$, and a peak at *m/z* 169.08 was observed due to the elimination of a carbonyl group (*Fig.* 1). Among the fragments and MS/MS spectra of the tadalafil analogues, common ions at *m/z* 169.08 and 135.05 were observed. Although tadalafil analogues have different molecular weights, they exhibit common ions at *m/z* 169.08 and 135.05, and show similar patterns in their MS/MS spectra and fragmentation pathways.

3.2. Fragmentation of sildenafil, sildenafil analogues, and hongdenafil analogues

Sildenafil analogues were sorted into 18 categories based on the functional group; several examples are shown in *Fig.* 2. The structures of sildenafil and hongdenafil were found to contain the sulfonyl group. Substituting an oxygen atom on R_2 with a sulfur atom in sildenafil results in the formation of thiosildenafil. Common product ions of sildenafil and sildenafil analogues were observed at *m/z* 311.15 and 283.12, respectively; major product ions of thiosildenafil analogues were found at *m/z* 327.12 and 299.09 in the QTOF-MS/MS mode. The peak at *m/z* 377.12 was attributed to a methyl piperazine-detached molecule from $[M+H]^+$, whereas the peak at *m/z* 311.15 was attributed to a methyl sulfonyl piperazine-detached molecule from $[M+H]^+$. Because

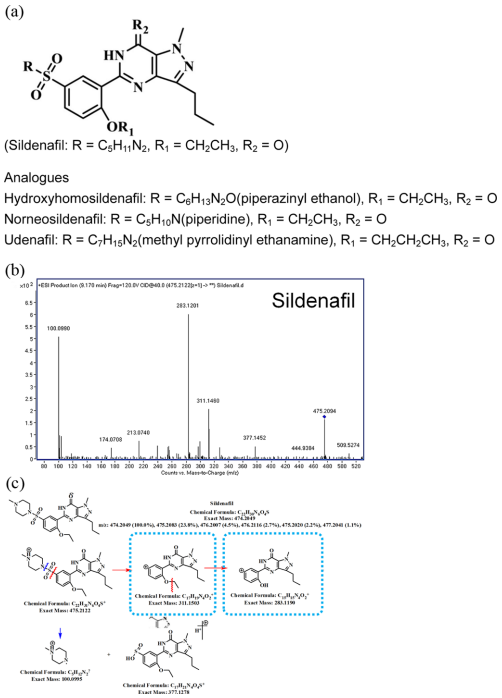

Fig. 2. (a) Chemical structures of sildenafil and sildenafil analogues, (b) typical spectra of sildenafil obtained by LC-QTOF–MS in positive electrospray mode, and c) fragmentation pathway of $[M+H]^+$ from sildenafil in positive ion mode.

of the fragmentation pathway, the peak at *m/z* 283.12 could be ascribed to the backbone structure of sildenafil and that at *m/z* 311.15 originated from the separation of the methylpiperazine-detached molecule from $[M+H]^+$. In contrast, hongdenafil analogues were synthesized from sildenafil by substituting the sulfonyl group with a carbonyl group. Hongdenafil analogues exhibited similar ions as sildenafil analogues at *m/z* 311.15 and *m/z* 297.13. The detailed pathways of the formation and structure of the compounds is shown in the *Fig*. 3. As with tadalafil analogues, sildenafil analogues also showed similar patterns in their MS/MS spectra and fragmentation pathways, even though their molecular weights varied due to the presence of different functional groups.

3.3. Fragmentation of vardenafil and vardenafil analogues

Vardenafil has six types of analogues; several

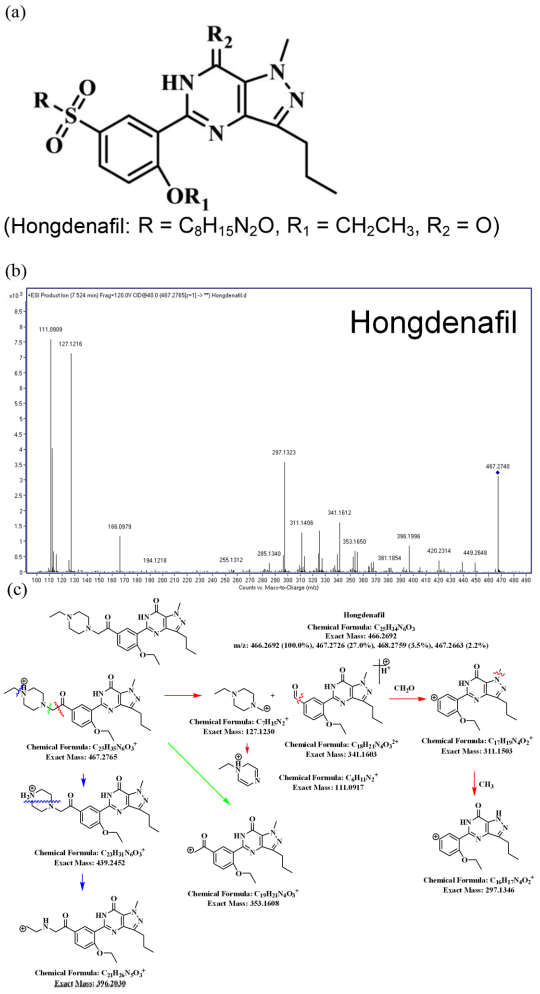

Fig. 3. (a) Chemical structures of hongdenafil, (b) typical spectra of hongdenafil obtained by LC-QTOF-MS in positive electrospray mode, and (c) fragmentation pathway of $[M+H]^+$ from hongdenafil in positive ion mode.

examples are shown in *Fig*. 4. The piperazinyl ethanol-substituted analogue is called hydroxyvardenafil and the piperidine-substituted analogue is called pseudo-vardenafil. In the mass scan, vardenafil showed a peak at *m/z* 489.22 as $[M+H]^+$, and fragment ions at *m/z* 376.12, 312.15, and 151.08 were observed. The peak at *m/z* 376.12 was attributed to the ethylpiperazine-detached structure from $[M+H]^+$, while that at *m/z* 312.15 originated from the detachment of the ethylpiperazine and sulfonyl groups. The peaks at *m/z* 312.16 and 151.09 are common ion molecules,

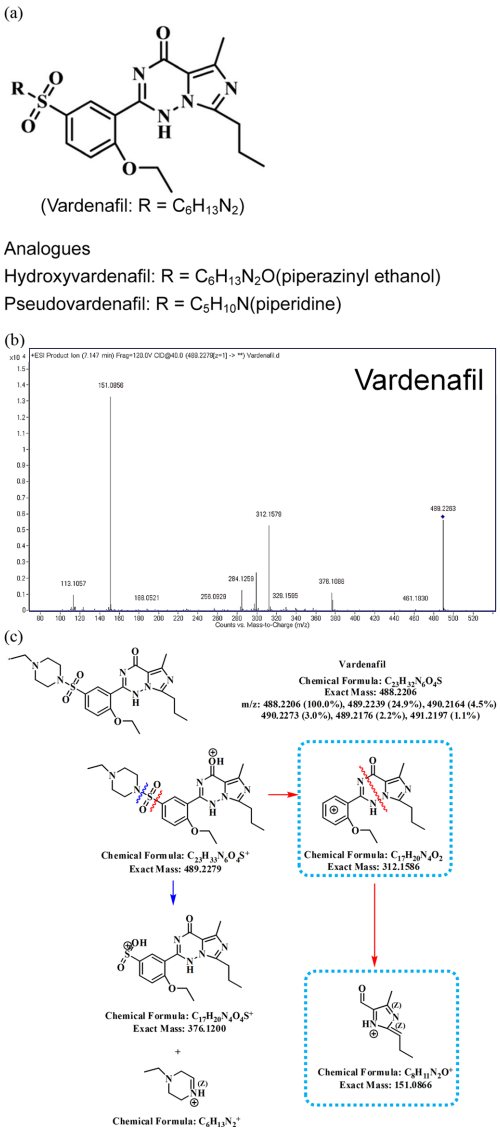

Fig. 4. (a) Chemical structures of vardenafil and vardenafil analogues, (b) typical spectra of vardenafil obtained by LC-QTOF-MS in positive electrospray mode, and (c) fragmentation pathway of $[M+H]^+$ from vardenafil in positive ion mode.

which were observed in all the six vardenafil analogues. The vardenafil analogues exhibited common ions at *m/z* 312.16 and 151.09 and showed similar patterns in their MS/MS spectra and fragmentation pathways.

3.4. Fragmentation of other analogues

Fig. 5 shows the QTOF-MS spectra and fragmentation pathways of other representative analogues. Among the 18 types of other analogues, similarities or common ion peaks could not be found, except for tadalafil, sildenafil, and vardenafil. For example, in the mass scan, avanafil exhibited a peak at *m/z* 484.18 as $[M+H]^+$, and fragment ions at *m/z* 375.12 and 221.10 were observed. Further, *m/z* 375.12 was observed due to the elimination of $C_5H_9N_3$ from *m/z* 484.18 $[M+H]^+$; *m/z* 221.10 was observed due to the elimination of C_8H_9ClO from *m/z* 375.12 $[M+H-C_5H_9N_3]^+$ (*Fig.* 5).

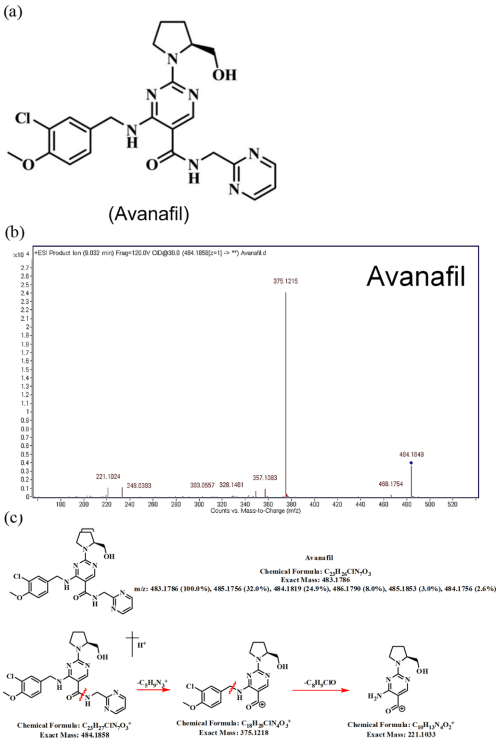

Fig. 5. (a) Chemical structure of avanafil, b) typical spectra of avanafil obtained by LC-QTOF-MS in positive electrospray mode, and (c) fragmentation pathway of $[M+H]^+$ from avanafil in positive ion mode.

3.5. Identification of unknown compounds: Potential application

A difference of 14 Da was observed between the peak at *m/z* 390.14 from $[M+H]^+$ of tadalafil and the peak at *m/z* 404.00 from $[M+H]^+$ of an unknown compound; therefore, the loss of a CH_2 group could

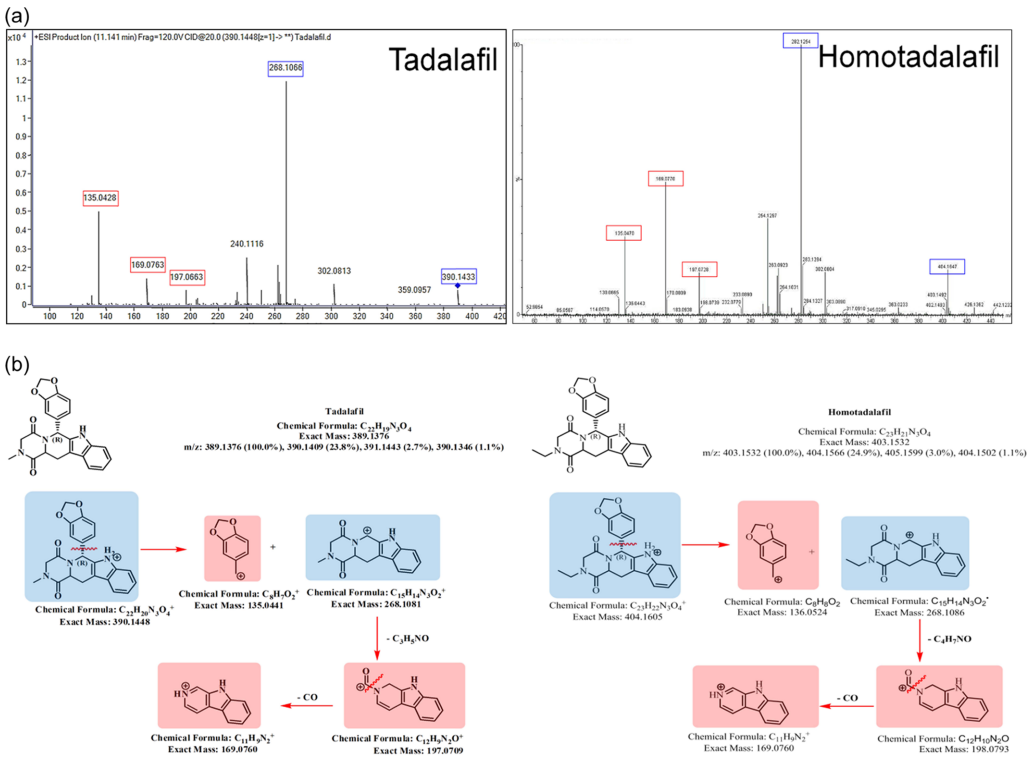

Fig. 6. (a) Typical spectra of tadalafil and homotadalafil obtained by LC-QTOF-MS in positive electrospray mode and (b) fragmentation pathway of $[M+H]^+$ from tadalafil and homotadalafil in positive ion mode.

be expected. The unknown compound was predicted to be a tadalafil analogue, as it exhibited similar characteristic ions as tadalafil at *m/z* 135.05, 169.08, and 197.07 in the MS/MS spectrum.[33] Furthermore, in regard to the fragmentation pathway, a difference of 14 Da between the peak at *m/z* 268.10 of tadalafil and that at *m/z* 282.12 of the unknown compound indicates that the attached group on the R position was an ethyl group, not a methyl group. The daughter ions generated from the parent ions help predict the fragmentation pattern of the molecule and are useful for confirming the target analytes. Therefore, the fragmentation pathway of homotadalafil could be represented as shown in *Fig.* 6.

4. Conclusion

Structural analysis of 58 PDE-5 inhibitor analogues was performed using LC-QTOF-MS. The 58 analogues were sorted into five groups: tadalafil, sildenafil, hongdenafil, vardenafil, and other analogues. By analyzing the structural characteristics and fragmentation pathways of the compounds, the common ion molecules were confirmed for each group. The developed analytical method could be utilized in the investigation and identification of new PDE-5 inhibitor analogues and can be very useful in the detection of adulteration. The developed method is expected to serve as a novel and facile method for the expeditious identification of unknown compounds.

Acknowledgments

This research was supported by a grant (13181MFDS724) from the Ministry of Food and Drug Safety issued in 2013.

References

1. B. J. Venhuis, G. Zomer, M. Hamzink, H. D. Meiring, Y. Aubin and D. de Kaste, *J. Pharm. Biomed. Anal.*, **54**(4), 735-741 (2011).
2. L. Blok-Tip, B. Zomer, F. Bakker, K. D. Hartog, M. Hamzink, J. Ten Hove, M. Vredenbregt, D. de Kaste, *Food Addit. Contam*., **21**(8), 737-748 (2004).
4. Q. Liang, J. Qu, G. Luo and Y. Wang, *J. Pharm. Biomed. An*al., **40**(2), 305-311 (2006).
5. S. R. Gratz, C. L. Flurer and K. A. Wolnik, *J. Pharm. Biomed. Anal*., **36**(3), 525-533 (2004).
6. M. H. Shin, M. K. Hong, W. S. Kim, Y. J. Lee and Y. C. Jeoung, *Food Addit. Contam*., **20**(9), 793-798 (2003).
7. R. Yuan and Y. Lin, *Pharmacol. Ther*., **86**(2), 191-198 (2000).
8. FDA approves Stendra for erectile dysfunction, http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm302140.htm/, Assessed 13 Jan 2001.
9. O. Shaeer. The Global Online Sexuality Survey (GOSS): The United States of America in 2011 Chapter II: phosphodiesterase inhibitors utilization among English speakers. *J. Sex. Med.*, **10**(2), 532-540 (2012).
10. A. W. Shindel. Update on phosphodiesterase type 5 inhibitor therapy part 2: updates on optimal utilization for sexual concerns and rare toxicities in this class. *J. Sex. Med*., **6**, 2352-2364 (2009).
11. C. G. Stief, S. Uckert, A. J. Becker, M. C. Truss and U. Jonas, *J. Urol.*, **159**(4), 1390-1393 (1998).
12. B. J. Venhuis and D. Kaste, *J. Pharm. Biomed. Anal.,* **69**, 196-208 (2012).
13. M. Y. Low, Y. Zeng, L. Li, X. W. Ge, R. Lee and B. C. Bloodworth, H. L. Koh, *Drug Saf*., **32**(12), 1141-1146 (2009).
13. Illicit erectile dysfunction products in the Netherlands http://www.rivm.nl/Documenten_en_publicaties/Wetenschappelijk/Rapporten/2011/september/Illicit_erectile_dysfunction_products_in_the_Netherlands_A_decade_of_trends_and_a_2007_2010_product_update/, Assessed 8 Sept 2011.
14. M. Sugita and M. Miyakawa, *Health Preventive Med*., **15**(4), 244-251 (2010).
15. S. Singh, B. Prasad, A. A. Savaliya, R. P. Shah and V. M. Gohil, A. Kaur, *Trends Anal. Chem*., **28**(1), 13-28 (2009).
16. J. B. Guo, Y. Xu, Z. B. Huang, Q. H. He, S. W. Liu, *Anal. Chim.,* **658**(2), 197-203 (2010).
17. S. M. Khalil, *Microchim.,* **158**, 233-238 (2007).
18. C. M. Gryniewicz, J. C. Reepmeyer, J. F. Kauffman and L. F. Buhse, *J. Pharm. Biomed. Anal*., **49**(3), 601-606 (2009).
19. D. J. Mans, R. J. Callahan, J. D. Dunn and C. M. Gryniewicz-Ruzicka, *J. Pharm. Biomed. Anal*., **75**(5), 153-157 (2013).
20. D. Z. Mao, X. X. Weng and Y. J. Yang, *J. Raman Spectrosc*., **43**(12), 1985-1990 (2012).
21. C. L. Cheng, G. J. Kang and C. H. Chou, *J. Chromatogr.,* **1154**(1-2), 222-229 (2007).
22. M. Park and S. Ahn, *J. Forensic Sci.,* **57**(6), 1637-1640 (2012).
23. P. Y. Sacre, E. Deconinck, P. Chiap, J. Crommen, F. Mansion, E. Rozet, P. Courselle and J. O. De Beer, *J. Chromatogr.,* **1218**(37), 6439-6447 (2011).
24. C. N. Man, N. M. Noor and R. Lajis, *J. Chromatogr.,* **1218**(39), 7055-7060 (2011).
25. P. Nikolaou, I. Papoutsis, S. Athanaselis, G. Alevisopoulos, A. Khraiwesh, C. Pistos and C. Spiliopoulou, *J. Pharm. Biomed. Anal*., **56**(3), 577-581 (2011).
26. R. Patterson, P. Mabe, E. N. Mitchell and W. Cory, *Forensic Sci. Int.,* **222**(1-3), 83-88 (2012).
27. C. S. Ng, T. Y. Law, Y. K. Cheung, P. C. Ng and K. K. Choi, *Anal. Methods.,* **2**, 890-896 (2010).
28. Y. Ren, C. S. Wu and J. L. Zhang, *J. Sep. Sci*., **35**(21), 2847-2857 (2012).
29. M. E. Hadwiger, M. L. Trehy, W. Ye, T. Moore, J. Allgire and B. Westenberger, *J. Chromatogr. A.,* **1217**(48), 7547-7555 (2010).
30. Y. Cai, T. G. Cai, Y. Shi, X. L. Cheng, L. Y. Ma, S. C. Ma, R. C. Lin and W. Feng, *J. Liq. Chromatogr. Rel. Technol.,* **33**, 1287-1306 (2010).
31. A. Lanzarotta, J. B. Crowe, M. Witkowski and B. M. Gamble, *J. Pharm. Biomed. Anal*., **67-68**, 22-27 (2012).
32. F. W. McLafferty. *Interpretation of Mass Spectra*, third edition. University Science Books, p 51-215, Mill Valley, California, 1980.
33. J. H. Lee, H. J. Kim, E. Noh, J. Y. Kim, S. H. Cho, J.-A Do, C.-Y. Yoon, S. Cho and W. S. Kim, *J. Pharm. Biomed. Anal.,* **103**, 80-84 (2015).