更多期刊、圖書與影音講座，請至【元照網路書店】www.angle.com.tw

Journal of Food and Drug Analysis, Vol. 14, No. 1, 2006, Pages 19-23 藥物食品分析 第十四卷 第一期

Isolation and Identification of a Sildenafil Analogue Illegally Added in Dietary Supplements

KUO-CHIH LAI, YI-CHU LIU, MU-CHANG TSENG AND JER-HUEI LIN*

Bureau of Food and Drug Analysis, Department of Health, Executive Yuan
161-2 Kunyang St., Nangang District, Taipei City 115, Taiwan, R.O.C.

(Received: June 3, 2005; Accepted: September 20, 2005)

ABSTRACT

A new sildenafil analogue was found to be added illegally to a dietary supplement marketed for erectile dysfunction. The structure was determined as 5-{2-ethoxy-5-[2-(4-ethylpiperazine-1-yl)-acetyl]phenyl}-1-methyl-3-*n*-propyl-1,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one. The sample was extracted with methanol and purified with a liquid-liquid partition method. The structure was identified with a series of 1-D and 2-D NMR techniques, infrared spectrum, high-resolution mass and LC/MS/MS. Having compared the structure with sildenafil, the results showed the methylpiperazine was switched with an ethylpiperazine and the sulfonyl group with an acetyl group. This new sildenafil analogue was isolated and identified for the first time in Taiwan. Since the structure was similar to that of sildenafil, side effects of sildenafil might associate with this new analogue. Therefore, it has been included in the inspection list of illegal health-related substances in Taiwan.

Key words: sildenafil analogue, isolated, identified, NMR, LC/MS/MS

INTRODUCTION

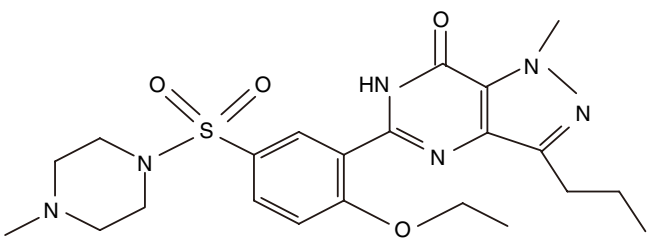
Foods using any illegal additive such as synthetic chemical compounds shall not be sold, manufactured, imported, processed, used, prepared, stored or transported.

Male erectile dysfunction, the persistent inability to achieve or maintain an erection for satisfactory sexual performance, is a common medical problem[1]. According to a random study, over half of men between 40 and 70 years age have experienced erectile dysfunction. It is also estimated that the number of men who have erectile dysfunction will double in the next 25 years, ultimately affecting more than 330 million men worldwide[2]. Recent development of sildenafil **1** (Figure 1) is used popularly as an orally effective drug in the treatment of male erectile dysfunction[3,4], in spite of its association with several serious side effects[5].

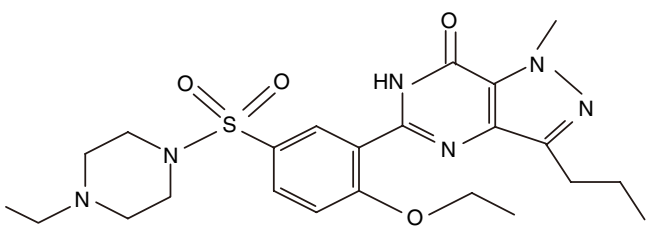
A substantial identification system for sildenafil in health foods was reported using three different analytical methods, i.e. TLC, TLC/MS and HPLC/PDA in Japan[6]. In our laboratory we have found sildenafil **1** (Figure 1) in a dietary supplement by LC/MS/MS[7].

One sildenefil analogue was found from a functional food marketed for penis erectile dysfunction in Korea in 2003[8], which was named homosildenafil **2** (Figure 1) with an ethylpiperazine replaced the methylpiperzine.

Another unknown suspicious compound related to sildenafil was found in a dietary supplement marketed for enhancing male sex ability in our laboratory. The unknown suspicious compound's molecular weight and UV spectra were different from those of sildenafil **1** (Figure 1)[7] and sildenafil analogue **2** (Figure 1)[8]. Hence, another new sildenafil analogue was inferred.



1: Sildenafil



2: Homosildenafil

Figure 1. Structures of sildenafil **1** and homosildenafil **2**.

MATERIALS AND METHODS

I. *Equipments*

The melting point was determined on a Jasco DIP-1000 Digital Polarimeter. The LC/MS/MS was performed using

* Author for correspondence. Tel: +886-2-26531239; Fax: +886-2-26531244; E-mail: linjerhuei@nlfd.gov.tw

更多期刊、圖書與影音講座，請至【元照網路書店】www.angle.com.tw

a Waters 2690 Alliance LC module, equipped with 996 photodiode array detector and Micromass Quattro Ultima tandem mass. The NMR spectra were recorded on a Varian UNITY INOVA 500 (500 MH_Z for ^1H, 125 MH_Z for ^{13}C) with dimethylsulfoxide-d_6 as solvent. The infrared spectrum was recorded in the 400~4000 cm^{-1} range using a Nicolet 510P FT-IR spectrometer and KBr pellets. The high-resolution mass was acquired on a Finnigan MAT 95S with an electron-impact ionization module. All chemicals used were of analytical grades.

II. *Extraction and Isolation*

Test samples were randomly obtained from local markets and the consumer services centers of the local health bureaus by the health bureau officers. Thirteen tablets of sample (6.4 g) were broken and extracted three times with methanol. The filtrate was concentrated under reduced pressure using a rotary evaporator at 40°C. The residue (616 mg) was diluted with dichloromethane to a total of 200 mL and acidified with 2% hydrochloric acid to pH 5~6, and then partitioned with water (200 mL) repeatedly, until the aqueous phase became clear. The combined water layers were concentrated to about 200 mL. This water layer was basified (pH 8~9) with ammonia water and extracted three times with equal volume of dichloromethane. The organic phase was collected and concentrated to about 200 mL at 40°C.

This acid-base partition steps were repeated until the desired compound was isolated. At last, the dichloromethane layers were combined and washed with water. The neutralized dichloromethane fraction was evaporated to dryness and crystallized with dichloromethane to yield the solid compound A (24 mg).

II. *NMR Correlation Data of Compound **A***

The isolated compound **A** was identified with a series of 1-D and 2-D NMR spectroscopic techniques, including ^1H, ^{13}C, DEPT, COSY, HMQC and HMBC. The data are showed in Table 1.

III. *Analysis Condition of LC/MS/MS*

The HPLC of LC/MS/MS was carried out on a column of Cosmosil 5C18-AR (4.6 × 150 mm, 5 μm) with methanol/acetonitrile/1% acetic acid (25:17:58) as mobile phase. The flow rate was 0.5 mL/min, the injection volume was 10 μL and the running time was 35 min. The eluate was monitored by a photo-diode array detector and scan range was 200~350 nm.

The analytical condition of tandem mass was as

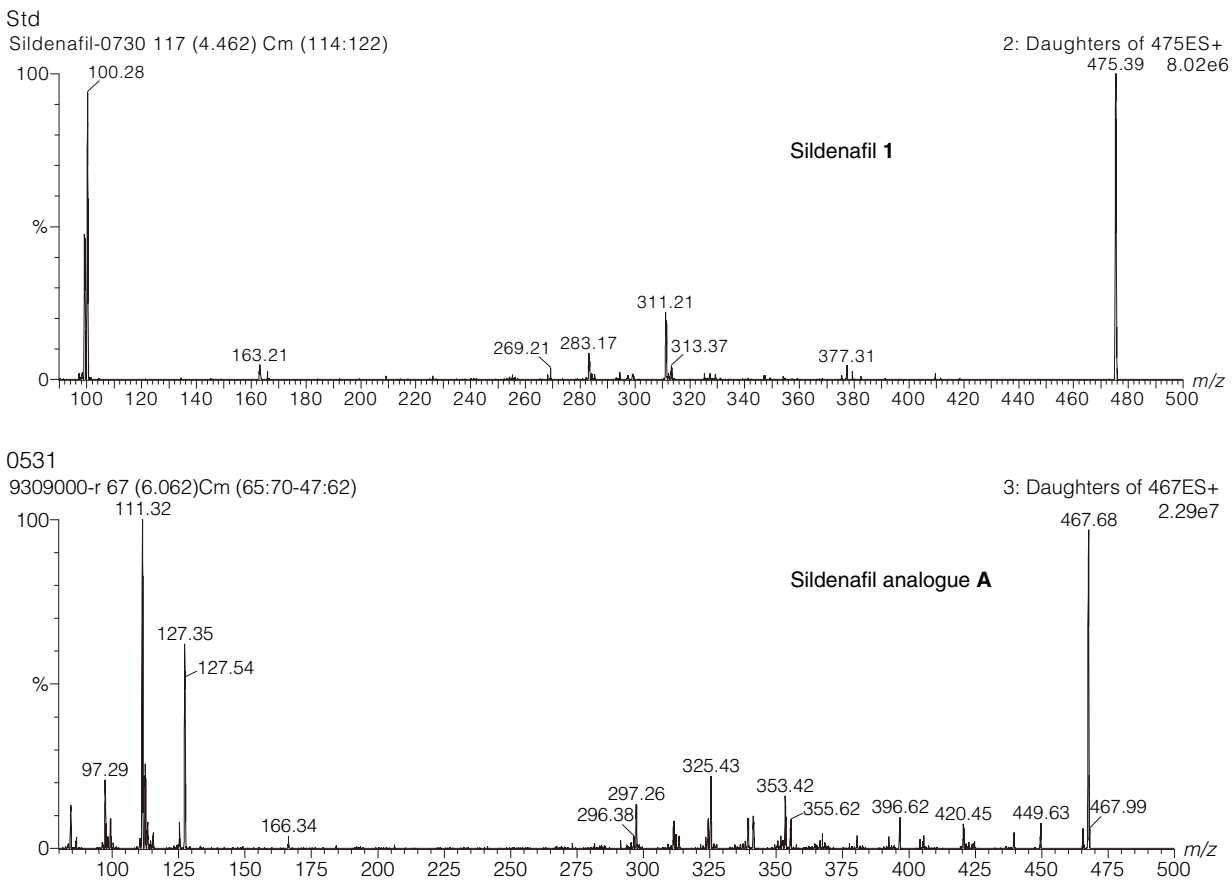


Figure 2. The LC/MS/MS fragmentation of sildenafil **1** and sildenafil analogue **A**.

更多期刊、圖書與影音講座，請至【元照網路書店】www.angle.com.tw

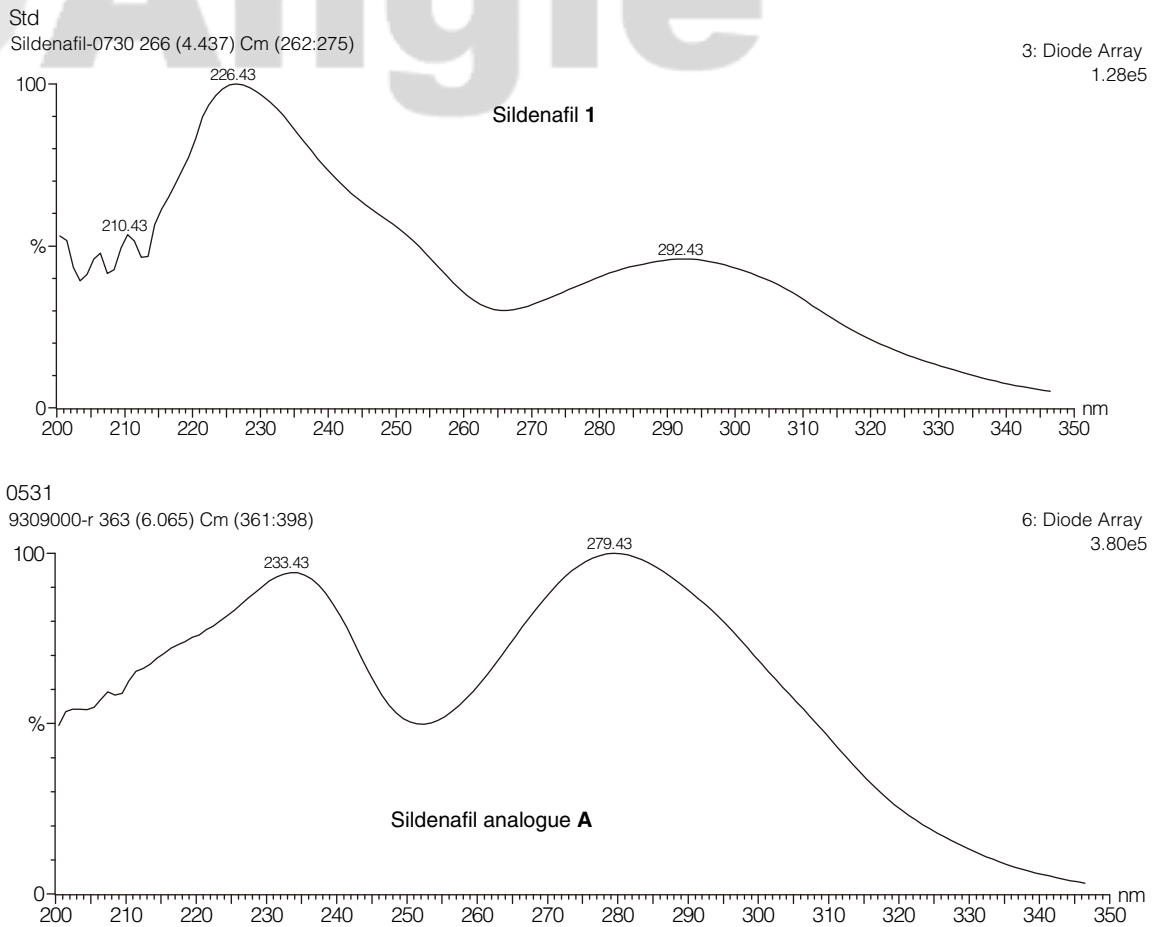


Figure 3. UV spectrum of sildenafil **1** and sildenafil analogue **A**.

follows: positive ion eletrospray (ES+) modes; daughters ion: 467; capillary voltage: 3.0 kV; cone voltage: 80 V; collision energy: 25 eV; source temperature: 100°C; desolvation temperature: 300°C.

RESULTS AND DISCUSSION

An acid-base partition technique was applied to the separation and purification of alkaloid compound **A** from one dietary supplement enchanting male sex ability. The isolated compound **A** was obtained as colorless powder from dichloromethane. The melting point was between 98 and 101°C. This new sildenafil analogue **A** was unstable in silical gel TLC and liable decomposition while exposed to light and air.

The HREIMS of compound **A** founded at *m/z* 466.2672, corresponding to the molecular formula $C_{25}H_{34}O_3N_6$. The fragmentation of compound **A** by LC/MS/MS is shown in Figure 2. The UV spectra of compound **A** by LC/MS/MS showed as λ_{max} at 233 and 279 nm (Figure 3). Both the molecular weight and the maximum and minimum absorption of UV spectra was different from those of sildenafil **1** by LC/MS/MS.

The IR spectrum showed absorption bands with the characteristics of an amine at 3314 cm^{-1}, an aromatic ring at 1580 and 1497 cm^{-1}, an αβ-unsaturated lactam at 1688 cm^{-1} and an ether group at 1249 and 1030 cm^{-1}.

Table 1 shows the ^{1}H-NMR, ^{13}C-NMR, DEPT, ^{1}H-^{1}H COSY and HMBC spectral data of isolated compound **A**, which were similar to that of sildenafil[6]. The ^{1}H-^{1}H COSY, HMQC and HMBC spectrums of sildenafil analogue **A** are shown in Figures 4, 5 and 6, respectively. The spectroscopic numbering used is given in Figure 7. All signals were assigned unequivocally according to the various NMR spectroscopic data.

The ^{1}H-NMR spectrum showed characteristics of an amide at δ_H 12.10 (1H, *br. s*), three aromatic protons at δ_H 8.25 (1H, s), 8.14 (1H, *d, J* = 9.0 H_Z), 7.23 (1H, *d, J* = 9.0 H_Z) and a singlet methyl attached to an aromatic amine at δ_H 4.15 (3H, *s*). Two multiple peaks at δ_H 2.50 and δ_H 2.32 were assigned as the piperazinyl methylene for H_4-25,29 and H_4-26,28, respectively. The ethyl protons attached to nitrogen of piperazinyl group were shown at δ_H 2.28 (2H, *q, J* = 7.5 H_Z) and 0.97 (3H, *t, J* = 7.5 H_Z). Another methylene's proton attached to the nitrogen of piperazinyl group was assigned as 3.72 ppm (2H, *s*).

The ^{13}C-NMR and DEPT spectra indicated four

更多期刊、圖書與影音講座，請至【元照網路書店】www.angle.com.tw

Table 1. NMR correlation of sildenafil analogue **A**

No.	^{13}C (δ_C)	^{1}H (δ_H)[a]	DEPT[b]	COSY	HMBC
1	144.8	—	0	—	H-11/H-12
4	153.7	—	0	—	—
5	—	12.10 (1H, *br. s*)	—	—	—
6	148.9	—	0	—	H-15
8	137.8	—	0	—	H-11
9	124.2	—	0	—	H-10
10	37.8	4.15 (3H, *s*)	3	—	-
11	27.1	2.78 (2H, *t*, J = 7.5 H_Z)	2	H-12	H-12/H-13
12	21.7	1.74 (2H, *m*)	2	H-11/H-13	H-11/H-13
13	13.8	0.94 (3H, *t*, J = 7.5 H_Z)	3	H-12	H-11/H-12
14	128.2	—	0	—	H-18
15	131.1	8.25 (1H, s)	1	—	H-17
16	122.7	—	0	—	H-18
17	132.4	8.14 (1H, *d*, J = 9.0 H_Z)	1	H-18	H-15
18	112.2	7.23 (1H, *d*, J = 9.0 H_Z)	1	H-17	—
19	160.1	—	0	—	H-15/H-17/H-18/H-20
20	64.5	4.20 (2H, *q*, J = 7.0 H_Z)	2	H-21	H-21
21	14.2	1.34 (3H, *t*, J = 7.0 H_Z)	3	H-20	H-20
22	195.2	—	0	—	H-15/H-17/H-23
23	64.1	3.72 (2H, *s*)	2	—	—
25,29	52.6	2.50 (4H, *br. m*)	2,2	H-26, H-28	H-23
26,28	52.2	2.32 (4H, *br. m*)	2,2	H-25, H-29	H-30
30	51.5	2.28 (2H, *q*, J = 7.5 H_Z)	2	H-31	H-31
31	11.8	0.97 (3H, *t*, J = 7.5 H_Z)	3	H-30	H-30

[a]δppm in DMSO-d_6, J in H_Z, 125 MH_Z for ^{13}C, 500 MH_Z for ^{1}H.
[b]DEPT is the number of attached protons.

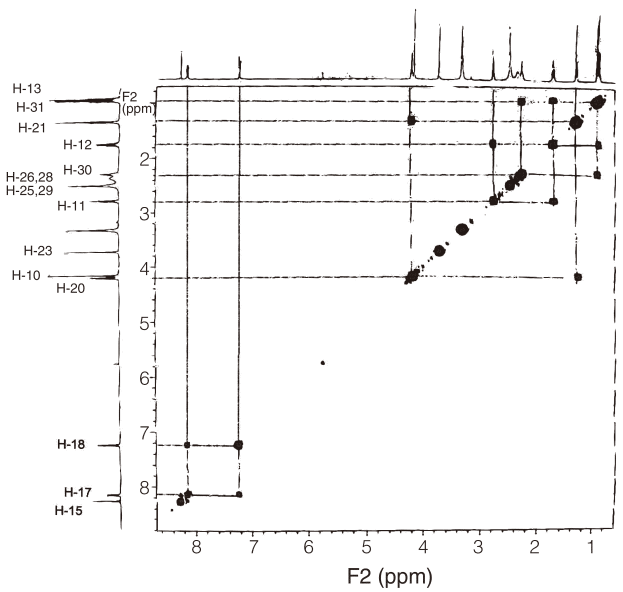


Figure 4. ^{1}H-^{1}H COSY spectrum of sildenafil analogue **A**.

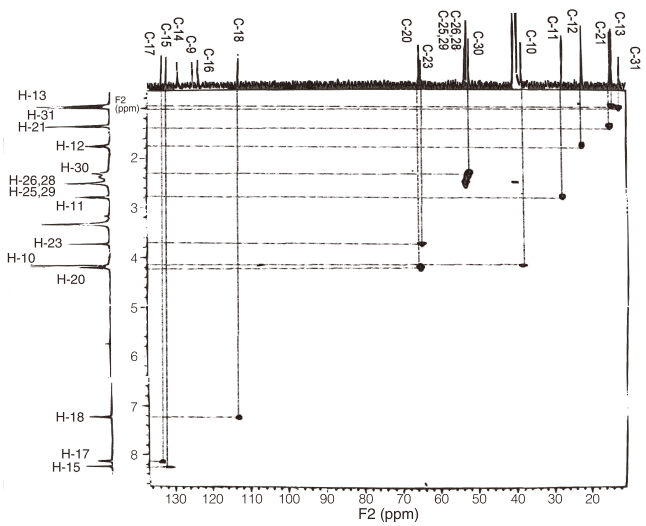


Figure 5. HMQC spectrum of sildenafil analogue **A**.

primary carbons, nine secondary carbons, three tertiary carbons and nine quaternary carbons. One carbon peak at δ_C 153.7 belonged to lactam. One carbonyl characteristic at δ_C 195.2, two methylene signal at δ_C 64.1 and δ_C 51.5 were obviously different from sildenafil **1**[(6)] (Figure 1).

In the HMBC spectrum (Figure 6), the correlation of H-30/C-26,28 indicated that an ethyl group (δ_H 2.28, 0.97) attached to piperazinyl nitrogen (N-27). The correlation of H-20/C-19 exhibited the attachment of the ethoxy group (δ_H 4.20, 1.34) to phenolic carbon (δ_C 160.1). The correlation of H-11, H-12/C-1 showed the linkage of the propyl group (δ_H 2.78, 1.74, 0.94) to pyrazolic carbon (δ_C 144.8). The correlation of H-10/C-9 exhibited the attachment of a methyl group (δ_H 4.15) to the pyrimidine ring (δ_C 124.2). The correlation between H-15(δ_H 8.25) and C-6 (δ_C 148.9) suggested that the pyrimidine ring linked to the phenolic ring.

更多期刊、圖書與影音講座，請至【元照網路書店】www.angle.com.tw

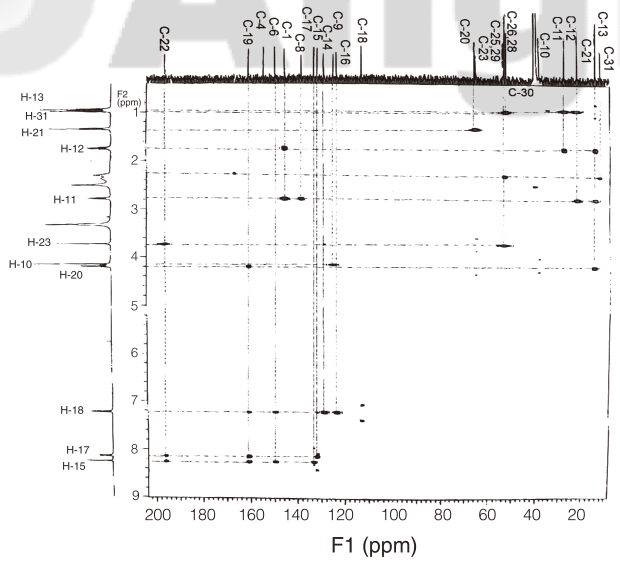


Figure 6. HMBC spectrum of sildenafil analogue **A**.

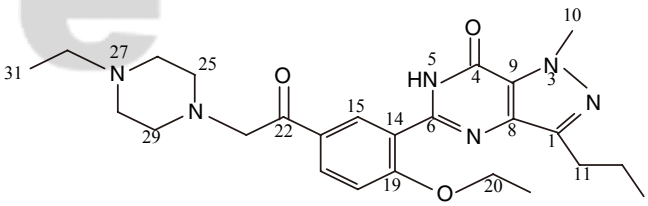


Figure 7. Structure of sildenafil analogue **A**.

Based on the high-resolution mass, infrared spectrum and NMR spectroscopic data, the structure of compound **A** was determined as 5-{2-ethoxy-5-[2-(4-ethylpiperazine-1-yl)-acetyl]phenyl}-1-methyl-3-*n*-propyl-1,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one. Compound **A** was a new sildenafil analogue (Figure 7), which compared with sildenafil, showed the methylpiperazine was switched with an ethylpiperazine and the sulfonyl group was switched with an acetyl group.

The structure of this new sildenafil analogue **A** in imported Chinese herbal drinks had been reported recently in Korea[9]. However, we have shown isolation procedure and established many data, i.e. a series of 1-D and 2-D NMR spectral data, the fragmentation of mass, the infrared spectrum, the absorption of UV spectra and the analytical condition of LC/MS/MS.

Having similar structure as sildenafil, therefore, it has been put on the inspection list for illegal health-related substances due to possible side effects.

ACKNOWLEDGEMENTS

The authors thank Dr. Yun-Lian Lin for her assistance of NMR analysis.

REFERENCES

1. Jeffecoate, W. J. 1991. The investigation of impotence. Br. J. Urol. 68: 449-453.
2. Goldstein, I. 2000. Male sexual circuitry. Sci. Am. 283: 56-61.
3. Terrett, N. K., Bell, A. K., Brown, D. and Ellis, P. 1996. Sildenafil (Viagra) a potent and selective inhibitor of type-5 cyclic GMP phosphodiesterase with utility for the treatment of male erectile dysfunction. Bioorg. Med. Chem. Lett. 6: 1819-1824.
4. Boolell, M., Allen, M. J., Ballard, S. A., Gepi-Attee, S., Muirhead, G. J., Naylor, A. M., Osterloh, I. H. and Gingell, C. 1996. Sildenafil: an orally active type-5 cyclic GMP specific phosphodiesterase inhibitor for treatment of penile erectile dysfunction. Int. J. Impot. Res. 8: 47-52.
5. Beavo, J. A. 1995. Cyclic nucleotide phosphodiesterases functional implication of multiple isoforms. Physiol. Rev. 75: 725-748.
6. Takako, M., Sutemi, S., Kiyoko, K., Fusako, I., Jyunichi, N., Hisashi, K. and Ichiro, Y. 2001. Identification system for sildenafil in health foods. Yakugaku Zasshi 121: 765-769.
7. Tseng, M. C. and Lin, J. H. 2002. Determination of sildenafil citrate adulterated in a dietary supplement capsule by LC/MS/MS. J. Food Drug Anal. 10: 112-119.
8. Shin, M. H., Hong, M. K., Kim, W. S., Lee, Y. J. and Jeoung, Y. C. 2003. Identification of a new analogue of sildenafil added illegally to a functional food marketed for penile erectile dysfunction. Food Addit. Contam. 20: 793-796.
9. Hong, M. K., Chio, D., Lim, M. H. and Park, G. S. 2004. Structure Determination of Sildenafil Analogues Found in Chinese Herb Drinks. 118th AOAC poster Abstracts. p. 154.