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시판 기능성식품으로부터의 실데나필 유도체 부정첨가물질의 분리 및 구조규명

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Isolation and Identification of an Unauthorized Sildenafil Analogue in a Commercial Functional Food

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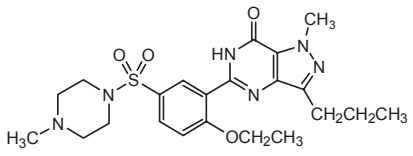
요약. 기능성식품으로 시판되는 건강음료를 HPLC 분석한 결과 발기부전치료제로 허가받은 비아그라®의 활성주성분인 실데나필과 구조적으로 유사한 부정첨가물질이 발견되었다. 이 부정첨가물질의 구조를 규명하기 위해 건강음료를 methylene chloride로 추출한 다음 그 추출물을 semipreparative HPLC로 정제하였다. 분리된 물질의 화학구조는 IR, LC/MS-ESI, NMR 분광학으로 규명하였는데 실데나필과 유사한 특징을 보였고 구조상의 유일한 차이는 실데나필의 methylpiperazine 부분구조를 부정첨가물질에서 hydroxyethylpiperazine으로 치환한 것이었다.
주제어: 기능성 식품, HPLC 분석, 부정첨가물질, 실데나필

ABSTRACT. HPLC analysis of a commercial herb drink marketed as a functional food revealed to contain an unauthorized substance similar to sildenafil, the active ingredient of the prescription drug Viagra® approved for the treatment of male erectile dysfunction. In order to identify the illegal additive, the herb drink was extracted with methylene chloride, and the extract was purified further using semipreparative HPLC. The chemical structure of the isolated substance was elucidated based on IR, LC/MS-ESI, and NMR spectroscopy, which showed the characteristics similar to sildenafil with minor modification. The only difference was the substitution of the methylpiperazine moiety of sildenafil to the hydroxyethylpiperazine group of the illegal additive.
Keywords: Functional food, HPLC analysis, Illegal additive, Sildenafil

INTRODUCTION

It is illegal to add unauthorized synthetic compounds to commercial functional foods with the intention of deceitfully advertizing their effectiveness as alternatives to conventional medicines.[1] One of the chemicals to adulterate the functional foods was sildenafil citrate, the active ingredient of Viagra® approved to Pfizer for the treatment of male erectile dysfunction. Sildenafil (**I**) is the inhibitor of phosphodiesterase-5 (PDE-5), the enzyme involved in the decomposition of c-GMP to contract smooth muscles inducing penile erectile dysfunction.[2]



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While this medicine is active against the erectile dysfunction, it is not safe for patients of diabetes or hypertension with nitrate medications (e.g., nitroglycerin, isosorbide dinitrate), and taking these drugs at the same time could cause the blood pressure to drop drastically, thus the sildenafil-containing drugs should not be obtained without doctor's prescription.[3] Therefore, in order to prevent the potential health risk caused by the misuse and overuse of sildenafil from the functional foods, it is necessary to check the presence of this pharmaceutical in commercial foods, and selective and simultaneous screening methods were developed by Korea Food and Drug Administration (KFDA) and others.[4,5]

Later, it was found that some food manufacturers and importers made alternative attempts to evade the screening system by adding other substances similar but not identical to sildenafil. The functional foods that contain these chemicals pose more serious health risk to consumers, since

the safety of the sildenafil analogs to human body has not been established yet. Actually, one commercial herb drink was found recently to contain the sildenafil-like substance which was detected in high-performance liquid chromatography (HPLC) analysis. In order to identify the unknown illegal additive, separation and purification were performed and its chemical structure was determined by spectroscopic analysis, and here we report the results.

EXPERIMENTAL SECTION

General Procedures

Melting points were determined on a Thomas-Hoover Unimelt apparatus and are uncorrected. UV spectra were obtained on a Varian CARY 100, and IR spectra were taken on a Bruker IFS48 spectrometer using a thin liquid film of solution in chloroform and the data are presented as cm^{-1} for important diagnostic absorptions. NMR spectra were obtained on a Bruker Avance 400 spectrometer (400 and 100 MHz for ^1H and ^{13}C, respectively) in DMSO-d_6, and chemical shifts are reported in δ scale in parts per million from tetramethylsilane as an internal standard. Electrospray ionization mass spectra (ESI-MS) were obtained from a VG Micromass, and data are reported in the form *m*/*z* (intensity relative to base peak = 100). Elemental microanalysis was performed on a CE Instrument EA1110. HPLC analysis data were obtained from a Waters 2690 separation module coupled to a 996 photodiode array detector. Thin-layer chromatography (TLC) was performed with E. Merck silica gel 60 F-254 precoated glass plates (0.25 mm). All solvents used for extraction and isolation were of HPLC grade purchased from Fisher Scientific.

Extraction and Isolation Procedures

The content of ten bottles of a commercial herb drink sample (30 mL/bottle) was mixed with 1% sodium bicarbonate solution (1 mL) and the mixture was extracted three times with methylene chloride (300 mL each). The organic layer was dried with anhydrous sodium sulfate and filtered, and the filtrate was concentrated *in vacuo* using a rotary evaporator. The residue was dissolved in methanol (15 mL), and after filtration through membrane filter, the solution was purified by semipreparative HPLC on μBondapak C_{18} column (7.8 × 300 mm, 5 μm) using acetonitrile-water-trifluoroacetic acid (90:10:0.1) as eluent with a flow rate of 5 mL/min. The collected fractions were freeze-dried to give 20 mg of the purified unknown compound.

Characterization of the Isolated Compound

Colorless crystal: mp 194 - 196 °C; UV λ_{max} (Ethanol) 220, 293 nm; IR ($CHCl_3$) 3314 (w), 2955 (w), 1696 (s), 1595 (w), 1490 (w), 1394 (w), 1350 (w), 1166 (m), 952 (w), 740 (w); ^1H and ^{13}C NMR, DEPT, COSY, and HMBC (*Table* 1); Mass *m*/*z* 505 (M+1, 100), 486 (23), 445 (21), 404 (31),

***Table* 1.** The NMR spectral data of the sildenafil analogue.

Assignment	^1H (δ)	^{13}C (δ)	DEPT	COSY	HMBC
1	-	145.0	C	-	C1/H12, C1/H11
4	-	153.8	C	-	-
5	12.23 (1H, br)	-	-	-	-
6	-	148.2	C	-	C6/H15
8	-	137.8	C	-	C8/H11
9	-	124.4	C	-	C9/H10
10	4.16 (3H, s)	37.9	CH_3	-	-
11	2.77 (2H, t, *J* = 7.5)	27.2	CH_2	H11/H12	C11/H13, C11/H12
12	1.74 (2H, m)	21.7	CH_2	H12/H13, H12/H11	C12/H13, C12/H11
13	0.94 (2H, t, *J* = 7.4)	13.9	CH_3	H13/H12	C13/H12, C13/H11
14	-	125.8	C	-	C14/H18
15	7.85 (1H, d, *J* = 2.3)	130.0	CH	H15/H17	C15/H17
16	-	123.7	C	-	C16/H18
17	7.82 (1H, dd, *J* = 8.6, 2.3)	131.6	CH	H17/H18, H17/H15	C17/H15
18	7.38 (1H, d, *J* = 8.6)	113.2	CH	H18/H17	-
19	-	159.9	C	-	C19/H15, C19/H17
20	4.21 (2H, q, *J* = 6.9)	64.9	CH_2	H20/H21	C20/H21
21	1.33 (3H, t, *J* = 6.9)	14.3	CH_3	H21/H20	C21/H20
24,28	2.88 (4H, br)	45.9	CH_2	H24,28/H25,27	-
25,27	2.48 (4H, br)	51.9	CH_2	H25,27/H24,28	C25,27/H29
29	2.36 (2H, t, *J* = 5.8)	58.4	CH_2	H29/H30	C29/H30
30	3.42 (2H, t, *J* = 5.8)	59.5	CH_2	H30/H29	C30/H29
OH	4.39 (1H, br)	-	-	-	-

377 (35), 312 (81), 283 (29), 129 (29), 99 (53).

Anal. Calcd for $C_{23}H_{32}N_6O_5S$: C, 54.75; H, 6.39; N, 16.65; S, 6.35. Found C, 54.74; H, 6.39; N, 16.76; S, 6.51.

RESULTS AND DISCUSSION

HPLC analysis was generally used to screen the illegal additives in commercial functional foods, and the solvent program for this investigation was a gradient system: "A", acetonitrile (95%), and "B", water with 0.1% sodium 1-hexanesulfonate and 0.1% phosphoric acid, with the ratio of "A" to "B" from 7:3 to 2:8.[6] According to this HPLC analysis of a commercial herb drink on a C_{18} reversed-phase column (4.6 × 250 mm, 5 μm), previously undeclared compound was detected at the retention time of 23.8 min, which was close to that of sildenafil (25.3 min). Further, after the isolation and purification of this compound, the UV spectrum in methanolic solution showed λ_{max} at 220 and 293 nm, which was identical to that of sildenafil. Thus, this compound was believed to be similar to sildenafil in chemical structure and physical property.

The chemical structure of this compound was determined by IR, MS, and NMR spectroscopy. IR spectrum showed absorption bands characteristic of hydroxyl (3314 cm^{-1}), amide (1696 cm^{-1}), aromatic (1595, 1490 cm^{-1}), and sulfonamide group (1350, 1166 cm^{-1}). The molecular structure of the unknown substance was further analyzed by LC/MS-ESI in the positive-ion mode and fragmentation patterns, which exhibit $[M+H]^+$ of 505 with fragments at *m*/*z* 404, 312, and 129 (*Fig*. 1). This mass spectrum was similar to that of sildenafil ($[M+H]^+$ of 475 with fragments at *m*/*z* 404, 312, and 99), and the only difference in MS between this unknown compound and sildenafil was 30 mass units in the protonated molecular ions of 505 *vs*. 475 and fragments 129 *vs*. 99. This 30 amu difference was believed to arise from the presence of hydroxyethyl ($HOCH_2CH_2$-) instead of methyl (CH_3-) group of sildenafil.

1D NMR (^{1}H-NMR, ^{13}C-NMR, and DEPT) and 2D NMR (COSY and HMBC) spectra of this compound were obtained and listed in *Table* 1, wherein the numbering system of the compound is given in *Fig*. 2. ^{1}H-NMR spectrum (*Fig*. 3) showed characteristics of amide at δ 12.23 (1H, br); methyl group attached to nitrogen at δ 4.16 (3H, s); propyl group at δ 2.77, 1.74, and 0.94; three aromatic protons with 1,2,4-orientation at δ 7.85 (1H, d), 7.82 (1H, dd), and 7.38 (1H, d); ethoxy group at δ 4.21 (2H, q) and 1.33 (3H, t); two methylene groups of the piperazine ring at δ 2.88 (4H, br) and 2.48 (4H, br); and hydroxyethyl group at δ 2.36 (2H, t), 3.42 (2H, t), and 4.39 (OH, br). There are 21 peaks in ^{13}C-NMR spectrum, and DEPT experiment shows three methyl groups, seven methylene groups, three methine groups, and eight quarternary carbons. Eleven peaks in the downfield region were assigned to carbons of benzene ring and pyrazolopyrimidinone ring. Six peaks in the range of δ 35 ~ 65 were assigned to carbons connected to oxygen or nitrogen as follows: methyl group attached to nitrogen at δ 37.9, methylene of the ethoxy group at δ 64.9, methylene carbons of the piperazine ring at δ 45.9 and 51.9, and hydroxyethyl group connected to the piperazine ring at δ 58.4 and 59.5. Four peaks in the upfield region were assigned to propyl carbons (δ 27.2, 21.7 and 13.9) and methyl carbon of the ethoxy group (δ 14.3). This peak assignment was further supported by 2D NMR techniques: ^{1}H-^{1}H homonuclear COSY and ^{13}C-^{1}H heteronuclear

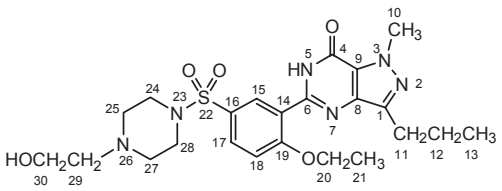

***Fig*. 2.** The chemical structure and numbering system of the sildenafil analogue.

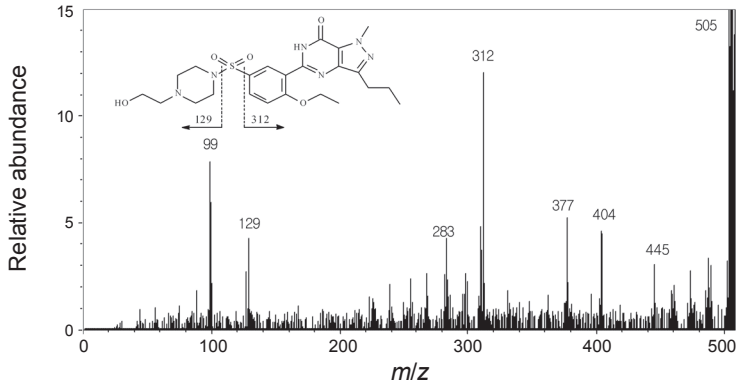

***Fig*. 1.** Mass spectrum of the sildenafil analogue. The fragment peaks at *m*/*z* 312 and 129 were assigned as indicated in the structure shown.

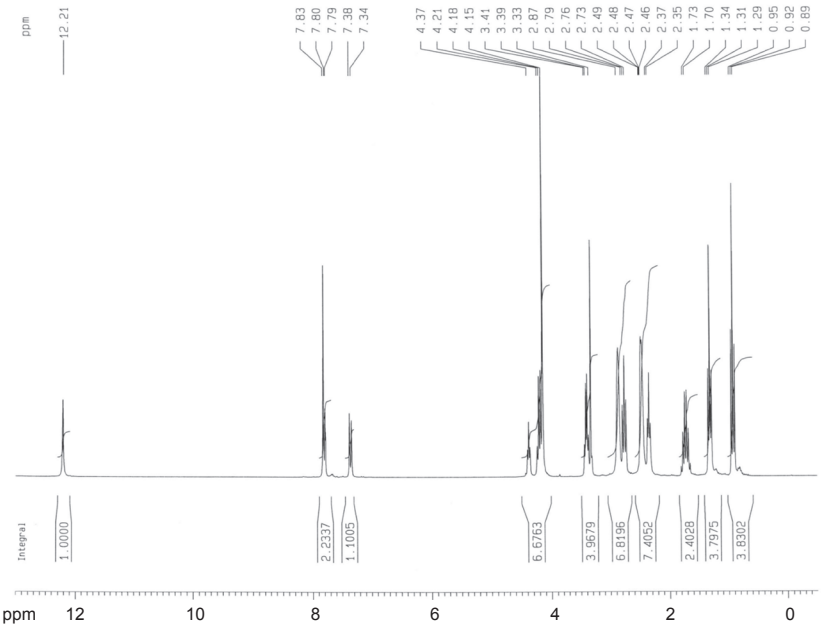

***Fig*. 3.** ^{1}H-NMR spectrum of the sildenafil analogue.

HMBC as indicated in *Table* 1.

These ^{1}H- and ^{13}C-NMR spectra were compared to those of sildenafil, and they were similar each other except hydroxyethyl group of the unknown compound *vs*. methyl group of sildenafil as follows: δ 2.36 (2H, t), 3.42 (2H, t) and 4.39 (1H, br) of hydroxyethyl *vs*. δ 2.12 (3H, s) of methyl group in ^{1}H-NMR; and δ 58.4 and 59.5 of hydroxyethyl *vs*. δ 44.3 of methyl group in ^{13}C-NMR.

In order to confirm the chemical structure of the isolated compound further, it was synthesized according to the literature,[7] and all the spectral data of the synthesized compound were completely identical to those of the purified substance from the herb drink. The synthesized compound is now used as a standard sample in detecting the presence of pharmaceuticals that are illegally added to commercial herb drinks and tablets.

CONCLUSION

In summary, previously undeclared compound was detected from the HPLC analysis of a commercial herb drink marketed as a functional food, and the unknown additive was isolated from the herb drink by extraction with methylene chloride and purification using semipreparative HPLC. The chemical structure of the isolated substance was identified from UV, IR, MS, and NMR spectroscopic analysis to be sildenafil analog with minor change of methyl group to hydroxyethyl group at the piperazine ring. The compound was synthesized in order to use as a standard sample in the inspection of the drinks and tablets sold as functional foods for the presence of illegal additives.

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