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Short communication

Structure elucidation of a novel synthetic thiono analogue of sildenafil detected in an alleged herbal aphrodisiac

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Abstract

A new analogue of sildenafil was detected in a herbal aphrodisiac. The structure of the compound was established using LC–MS, UV and IR spectroscopy, MS–MS, and NMR. The compound, named thio-homosildenafil is a synthetic *N*-ethylpiperazine analogue of sildenafil in which also the C=O moiety has been converted into a C=S group. This is the first time a sildenafil analogue modified at the chromophore was identified as an adulterant of a herbal aphrodisiac. Preliminary pharmacological analysis confirmed the erectogenic potency of thio-homosildenafil.

Keywords: Dietary supplements; Sildenafil analogue; Thio-homosildenafil; Liquid chromatography–mass spectrometry (LC–MS); Nuclear magnetic resonance (NMR)

1. Introduction

Since 1998 three drugs for the treatment of erectile dysfunction (ED) have been approved by the European Medicines Evaluation Agency (EMEA): sildenafil (Viagra®), tadalafil (Cialis®), and vardenafil (Levitra®). These ED-drugs were developed to inhibit the phosphodiesterase-5 (PDE5) enzyme, mediating the erectogenic effect [1,2].

Patents protecting the three ED-drugs also describe many of their analogues and their syntheses [3–5]. Because patents are publicly accessible over the internet, they are believed to have inspired chemists to synthesize the analogues that were not developed into licensed drugs. Out of the 13 analogues currently known [6], only amino-tadalafil is not described in patents on the three approved drugs.

Recently, Reepmeyer et al. reported the identification of a 3,5-dimethylpiperazine analogue of sildenafil [7], a yet unapproved ED-drug being developed in China under the name of aildenafil [8,9]. Because adulterators seem to follow current trends in drug development, our laboratory routinely screens for second generation ED-drugs [10] and analogues thereof.

In the current study, LC–DAD–MS^n, IR and NMR analyses were applied to the detection and structure elucidation of a new sildenafil analogue. The compound was identified as 5-[2-ethoxy-5-(4-ethylpiperazine-1-sulphonyl)-phenyl]-1-methyl-3-propyl-1,6-dihydro-pyrazolo[4,3-d]pyrimidine-7-thione. The structure of this new analogue, named thio-homosildenafil is given in Fig. 1.

2. Experimental

2.1. LC–DAD–MS^n

Materials and equipment used, including reference homosildenafil, were obtained as described earlier by our laboratory [11]. Reference thio-homosildenafil was obtained as described below.

White capsules, allegedly containing herbal products, contained an average of 240 mg of brown amorphous powder. A composite was prepared from the contents of three capsules. An amount of the capsule composite equivalent to half a dosage unit was extracted into 100 mL of MeOH in an ultrasonic bath for 20 min, centrifuged at 3200 rpm for 5 min. An aliquot (5.0 mL) of the supernatant liquid was diluted 100× using MeOH/acidified water 50:50), which solution was subsequently used for LC–DAD–MS analysis.

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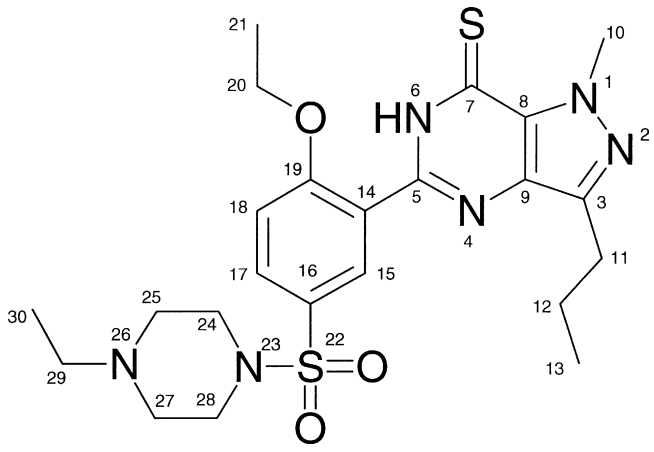

Fig. 1. Structure of thio-homosildenafil. The numbering system corresponds to the IUPAC systematic name.

For chromatographic separation and UV detection the described method was adapted to include a broader UV detection range (200–450 nm) and elongated elution under gradient conditions: solvent A – MeOH and solvent B – acidified water (v/v): 0–15 min isocratic (A/B = 50/50), 15–20 min gradient to A/B = 70/30, 20–25 min isocratic A/B = 70/30, 25–30 min: gradient to A/B = 50/50.

2.2. Isolation of a reference standard and IR analysis

The contents of 10 capsules was transferred to a glass-sintered dropping-funnel and was subsequently washed with Et_2O (2 × 25 mL), EtOAc (4 × 25 mL), and MeCN (7 × 50 mL). The combined washings of EtOAc and MeCN were evaporated to dryness in vacuo, yielding a yellow solid. The solid was dissolved in hot EtOAc (20 mL) that precipitated upon cooling yielding 623 mg of a faint yellow solid. A sample of the solid was used for IR analysis (ATR) in a Bruker IFS55 FT-IR spectrometer with a DTGS detector and OPUS software version 4.0. Sildenafil citrate was used as a reference.

2.3. NMR

NMR spectra were recorded on a JEOL JNM400 spectrometer. For qualitative analysis (^{1}H, ^{13}C and ^{1}H–^{13}C correlation) spectra were recorded of a sample of the solid isolated above dissolved in $CDCl_3$. For quantitative NMR analysis an accurately weighed sample was transferred to a pre-weighed glass tube, evaporated under a stream of nitrogen gas and weighed again. This material was dissolved in a mixture of acetone-D_6/D_2O (1/1) containing a known amount of trimethylsilyl-D_4-propanoate sodium salt (TMSP). ^{1}H NMR was recorded using a pulse delay of 12 s to allow for complete relaxation of the signals.

2.4. Pharmacological analysis

To demonstrate the potential efficacy of thio-homosildenafil, it was pharmacologically evaluated by Cerep (Celle l'Evescault, France) in an in vitro PDE5 and PDE6 assay [12]. Inhibition of the PDE5 enzyme is associated with erectogenic efficacy whereas inhibition of the PDE6 enzyme is associated with side effects, specifically, visual disturbances [2,13].

3. Results and discussion

3.1. LC–UV–MS^n

The ion chromatograms, UV and MS data for the herbal product and a mixture of sildenafil, tadalafil and vardenafil are shown in Fig. 2 and Table 1. The first eluting unknown component (7.38 min) was identified as homosildenafil (HS) which is an analogue previously identified in herbal aphrodisiacs [11]. The major and unknown component elutes much later than the three reference ED-drugs and generates a pseudo-molecular ion at *m/z* 505 being 30 units higher than sildenafil and 16 units higher than

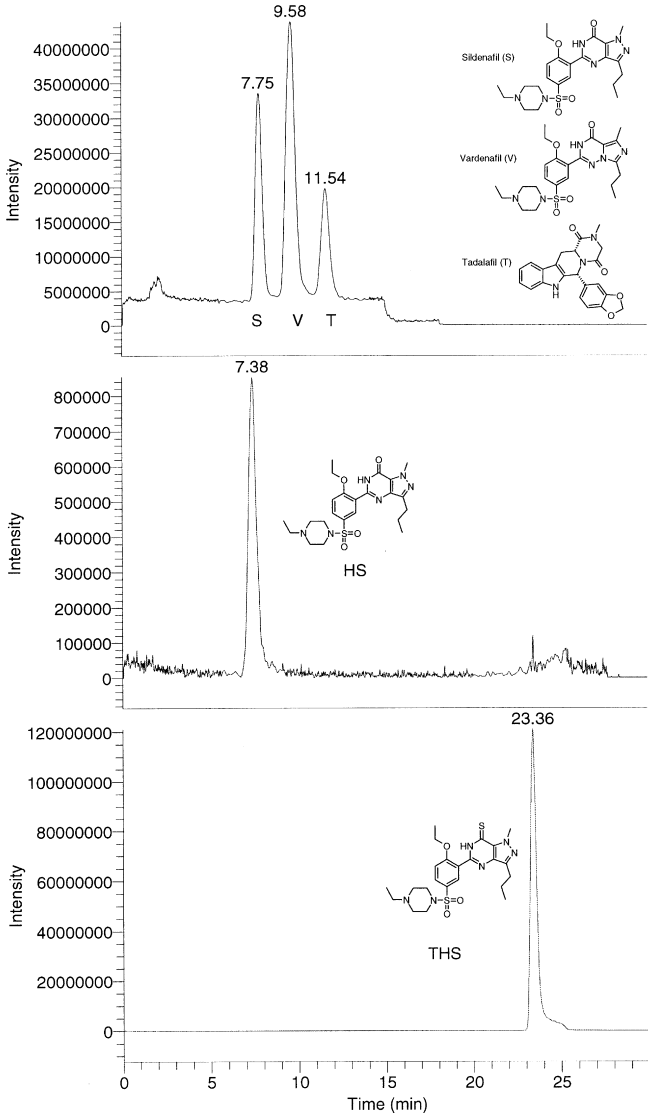

Fig. 2. The total ion chromatograms of sildenafil (S), vardenafil (V), and tadalafil (T) standards (top) and single ion chromatograms for homosildenafil (HS) and thio-homosildenafil (THS) in the same extract of the herbal erectogenic (below).

Table 1
LC–DAD–MS^n data for the reference standards (top three) and the herbal product

Compound	RRT	UV_{max} (nm)			MS^1	MS^2				
Sildenafil	1.00	227	291		475	377	313	311	283	
Vardenafil	1.24	226			489	461	377	375	312	299
Tadalafil	1.49	228	283		390	268	262	250		
Homosildenafil	0.95	226	292		489	461	377	313	311	283
Thio-homosildenafil	3.01	242	293	353	505	477	421	357	355	327

vardenafil. The UV absorption spectrum for the major unknown component markedly deviates from sildenafil, vardenafil and tadalafil.

The MS^2 profile for the unknown component (*m/z* 505) shows five major ions, four of which (*m/z* 327, 355, 357, and 421) match the MS^2 fragments observed for sildenafil with a difference in mass of 44 units (*m/z* 283, 311, 313, and 377). It was therefore assumed the unknown component was an analogue of sildenafil with, according to the *N*-rule, the same number of N-atoms. The observed MS^1 isotope ratio of *m/z* 505 (100%), 506 (29.27%), 507 (11.88%) and 508 (3.41%) suggests the presence of a second S-atom putting forward thio-homosildenafil as a candidate for being the unknown component [14].

Finding the combination of homosildenafil and thio-homosildenafil is not surprising because patent literature describes homosildenafil is converted into its thiono analogue upon treatment with P_2S_5 [15]. The presence of homosildenafil could be accounted for by poor purification procedures after synthesis or by hydrolysis of the product.

The larger and less partially charged S-atom may cause the decreased polarity of thio-homosildenafil by the shielding the neighbouring N–H bond from interaction with the column. Differences in partial charges in the chromophore are probably also reflected in the observed deviant UV spectrum.

3.2. IR spectroscopy

An ATR recording of the unknown component shows the absence of a strong C=O signal at 1695 cm^{-1} as is observed for sildenafil [16]. An additional strong signal is observed at 1240 cm^{-1} in agreement with a C=S moiety (Fig. 3).

3.3. NMR

^{13}C and ^1H NMR data for the unknown component are listed in Table 2. When compared to ^{13}C NMR-data of sildenafil and homosildenafil from our laboratory [11] and thio-sildenafil analogues in patent literature [15], the unknown component was positively identified as thio-homosildenafil. The C=S bond is indicated by the 20 ppm downfield shift of the C-7 signal, relative to the C-7 signal in homosildenafil. A signal unaccounted for in both the ^1H and ^{13}C NMR proved absent in NMR recordings after acid–base separation. The chemical shifts being typical for a methanesulphonate suggest the unknown was originally present in the capsules as a methanesulphonate salt [17].

The purity of the reference standard was >98% using the LC–DAD–MS method using standard sildenafil citrate as a comparator. To account for inorganic impurities ^1H NMR integral values for the internal standard (TMSP) and thio-homosildenafil signal at 8.54 ppm demonstrating a purity of about 75%. Homosildenafil was not observed in the isolated standard (NMR, LC–DAD–MS).

3.4. Pharmacological analysis

Thio-homosildenafil was found to be pharmacologically active on both the PDE5 enzyme (15 nM) and the PDE6 enzyme (66 nM). These figures are not corrected for the purity of the

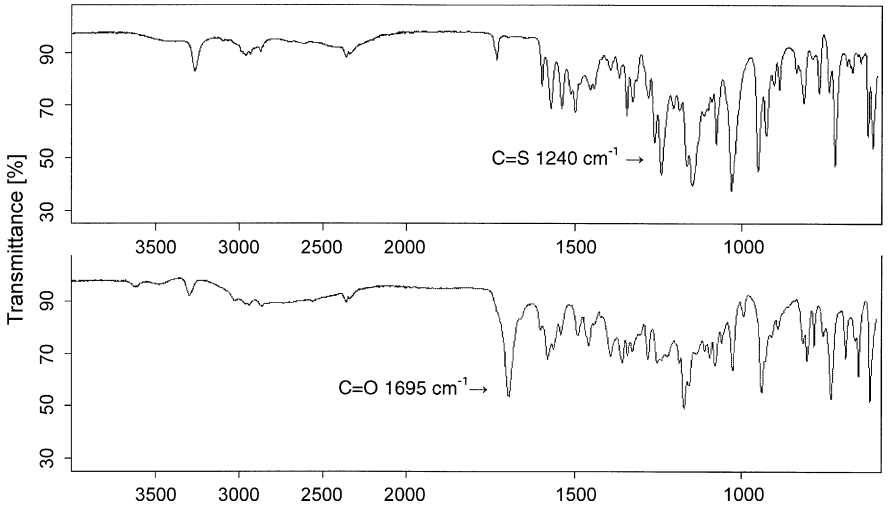

Fig. 3. The ATR spectra of the unknown component thio-homosildenafil methanesulphonate (top) and sildenafil citrate (bottom).

Table 2
NMR data for thio-homosildenafil methanesulphonate ($CDCl_3$)

Group	Atom #	δ (^{1}H, ppm)	Multiplicity	δ (^{13}C, ppm)
N–H	6	11.18	1H, br S	
N–CH_3	10	4.51	3H, s	39.36
$CH_2CH_2CH_3$	11	2.91	2H, t, J = 7.7 Hz	27.48
	12	1.83	2H, m, J = 7.5 Hz	22.24
	13	1.00	3H, t, J = 7.3 Hz	13.99
Benzene ring	14			130.67
	15			160.60
	16	7.19	1H, d, J = 9 Hz	113.61
	17	7.81	1H, dd, J = 9Hz, J = 2.4 Hz	132.53
	16	7.19	1H, d, J = 9 Hz	127.18
	19	8.54	1H, d, J = 2.4 Hz	131.71
OCH_2CH_3	20	4.34	3H, t, J = 7 Hz	66.29
	21	1.61	2H, q, J = 7 Hz	14.59
Piperazine	24ax, 28ax	2.93	2H, m	43.13 (C24, C28)
	24eq, 28eq	3.17	2H, bt, J = 13Hz	
	25eq, 27eq	3.57	2H, bd, J = 11.7 Hz	50.96 (C25, C27)
	25ax, 27ax	3.86	2H, bd, J = 13.1 Hz	
NCH_2CH_3	29	3.10	2H, bm, J = 6 Hz	52.61
	30	1.39	3H, t, J = 7.3 Hz	9.05
Pyrimidi-7-thione	3			146.30
	5			146.39
	7			172.21
	8			133.73
	9			121.63
$CH_3SO_3^-$		2.65	3H, s	39.43

sample. For sildenafil these figures are 7 nM and 21 nM, respectively. The content of thio-homosildenafil (base) was calculated to be at least 55 mg per capsule when corrected for purity of the standard. The content of homosildenafil was estimated at 2 mg. Therapeutic doses of sildenafil ranges from 25 to 100 mg.

4. Conclusions

For the first time an analogue has been identified in a herbal aphrodisiac, modified both at the piperazine moiety and at the chromophore. Preliminary pharmacological analysis showed thio-homosildenafil to be a potent PDE5 and PDE6 inhibitor in vitro, meaning the content of a capsule will presumably cause pharmacological effects.

Consumers are deceived into taking adulterated herbal aphrodisiacs and are being put at risk because these products contain untested drug substances of unknown purity and toxicity. The highest health risk is with consumers not expecting potentially serious drug–drug interactions.

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