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Isolation and structural characterization of a new tadalafil analog (chloropropanoylpretadalafil) found in a dietary supplement

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Abstract

A screen for known PDE-5 inhibitors in a dietary supplement product marketed for “enhanced sexual performance” detected a compound that structurally resembled chloropretadalafil, a known analog of tadalafil. The compound was isolated from the supplement matrix using high performance liquid chromatography with ultraviolet detection (HPLC-UV) and a fraction collector, and was further characterized using gas chromatography with Fourier Transform infrared detection and mass spectral detection (GC/FT-IR/MS), as well as high resolution mass spectrometry (HRMS). The analog had an accurate mass of m/z 441.1216 (error is 0.8706 ppm) for the protonated species $[M+H]^+$, corresponding to a molecular formula of $C_{23}H_{22}ClN_2O_5$. HRAM and GC/FT-IR/MS mass spectral fragmentation data suggested that the modification is a chloropropanoyl moiety extending from the nitrogen on the piperidine ring of chloropretadalafil. The proposed new analog has been named chloropropanoylpretadalafil.

Keywords

Phosphodiesterase type-5 (PDE-5); inhibitors; New tadalafil analog; LC-HRAM-MS; GC/FT-IR/MS

1. Introduction

Synthetic phosphodiesterase type-5 (PDE-5) inhibitors including sildenafil, vardenafil, and tadalafil are being detected with increasing regularity in products that are labeled as “all natural” herbal supplements that offer “sexual performance enhancement.” [1–11] The discovery of novel analogs of approved PDE-5 inhibitors is a common occurrence, and they are routinely added to the established screening methods typically run in regulatory agencies. [12–14] The analogs generally display minor structural alterations to their approved correlative drug compounds while retaining the active moiety. [15] The analogs are not declared on the labeling, and little to no information is available regarding their toxicological or pharmacological effects, which presents a danger to public health. Regulatory agencies are becoming increasingly more skilled at detecting and identifying PDE-5 inhibitor analogs in these products. A rapid and quantitative LC–MS/MS screening method for 71 known erectile dysfunction drugs was recently published [16] and new analogs are continuously being discovered in adulterated dietary supplements. [17–32]

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This article describes the structural characterization of chloropropanoylpretadalafil (“Compound 1”), an analog of chloropretadalafil (Fig. 1). Compound 1 was detected during a screening of a “sexual performance enhancing” dietary supplement. The compound was isolated from the supplement matrix using HPLC-UV coupled to an analytical scale fraction collector. Accurate mass and GC/FT-IR/MS were performed to elucidate the proposed structure of Compound 1. The synthesis of this analog was reported in a 2002 world patent from Lily Icos, LLC, where it was identified as “Intermediate 8” during the preparation of fused heterocyclic derivatives as PDE-5 inhibitors. [33]

2. Experimental

2.1. Sample and chemicals

A dietary supplement product was obtained during an undercover purchase from an online company that listed their manufacturing site as the United States and was submitted to the laboratory for analysis. The product was supplied in a paper board box containing twenty-four product packets, each containing a single capsule. The product was composited by combining the contents of ten capsules into a scintillation vial and using a vortex mixer to thoroughly mix the composite. The average capsule content weight was 0.37 g.

Chloropretadalafil was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Tadalafil (lot # 0469910) was purchased by Cayman Chemical (Ann Arbor, MI, USA). Sildenafil citrate (lot # F0K412) was purchased from US Pharmacopeia Convention (Rockville, MD, USA). The following HPLC grade solvents were purchased from Fisher Scientific (St. Louis, MO, USA): CH₃OH, CH₃CN. Formic acid, 0.1% (v/v) trifluoroacetic acid (TFA) in water, and 0.1% TFA in CH₃CN was also purchased from Fisher Scientific. The 18.2 MΩ cm deionized H₂O (DIW) was generated using a Milli-Q Academic system (Millipore, Bedford, MA, USA).

2.2. HPLC-UV analyses

Sample extracts were prepared by mixing 0.10–0.11 g of the supplement composite and 5 mL of a 50:50 (v/v) mixture of CH₃CN and DIW with shaking. Aliquots were filtered through 0.45 μm nylon syringe filters prior to injection.

Fraction collection experiments were conducted on an Agilent 1200 Series HPLC-UV. Separation of matrix components was accomplished using a Zorbax Eclipse XDB-C8, 4.6 mm × 150 mm, 5 μm column maintained at 25 °C and a flow rate of 1 mL/min. The mobile phase consisted of a 50:50 (v/v) mixture of CH₃CN and DIW with an isocratic elution. The spectrum of each peak in the chromatogram was compared to the spectrum obtained from a tadalafil reference standard, in order to determine which component was most closely related to tadalafil. The presence of two other tadalafil analogs in the supplement (nortadalafil and *N*-ethyl tadalafil) necessitated the use of retention time information from the LC-MS experiment to confirm that the correct compound was collected. Once the peak corresponding to the compound of interest was identified, the HPLC-UV was coupled to an Agilent 1200 Series analytical scale fraction collector. The injection volume was increased to 20 μL, and peak collection occurred using a time-based trigger. The collected fractions

were consolidated and taken to dryness at an ambient temperature under a stream of nitrogen supplied by a Reacti-Vap Evaporating Unit. The fraction was analyzed using HNMR; however, the data did not provide structural elucidation as the fraction was not pure or abundant enough. Further experiments were conducted utilizing the capsule-content composites as described below.

Assay experiments were conducted on an Agilent 1200 Series HPLC-UV. The column, column conditions, and flow rate were identical to that described for the fraction collection experiments. Solvent A consisted of DIW with 0.1% TFA and Solvent B consisted of CH₃CN with 0.1% TFA. The mobile phase was programmed with an initial composition of 25% B with a linear gradient to 75% B in 7 min, which was held for 5 min with a 5 min post-run equilibration at initial gradient conditions. The injection volume was 10 μ L with detection at 285 nm with spectral collection from 190 to 400 nm. The spectrum of each peak in the chromatogram was compared to the spectrum obtained from a tadalafil reference standard, in order to determine which component was most closely related to tadalafil, as it has been demonstrated to be spectrally similar to its analogs and reference standards are not always available for newly discovered analogs.

2.3. LC–MS accurate mass analyses

Samples were prepared for initial screening experiments by extracting 0.04 g composite in 5 mL of a 50:50 (v/v) mixture of CH₃CN and DIW and shaking. The extracts were filtered through 0.2 μ m PTFE syringe filters, and were diluted further, 10 μ L filtrate and 990 L extraction solvent, prior to analysis.

Accurate mass analyses were performed on a Thermo Dionex UltiMate 3000 liquid chromatograph (LC) equipped with an Agilent ZORBAX SB-C18, 1.8 μ m, 2.1 $\times$ 50 mm column, coupled to a Thermo Scientific Q Exactive mass spectrometer (MS). Data were acquired and analyzed using Xcalibur 2.2 software from Thermo Scientific. Mobile phase flowed at a constant rate of 0.350 mL/min. Gradient elution was performed with initial conditions of 95% A (DIW with 0.1% formic acid) and 5% B (HPLC-grade CH₃CN), ramped to 95% B in 3.5 min, and held for 3 min. Each injection was followed by a 3 min post equilibration at the initial conditions. The injection volume was 1.0 μ L and the column was held at 40 $^{\circ}$ C.

The instrument parameters for the mass spectrometer were as follows: ionization = positive, electrospray; sheath gas flow = 40 arbitrary units; auxiliary gas flow = 5 arbitrary units; sweep gas flow = 2 arbitrary units; spray voltage = 3.25 kV; capillary temperature = 275 $^{\circ}$ C; S-lens RF level = 60; resolution = 140,000 (full scan), 35,000 (MS/MS); automatic gain control (AGC) target = 1,000,000; scan range m/z 50–470 (full scan), data dependent (MS/MS). The MS/MS spectra were collected based on the observation of ions on the following precursor list in scan event 1, using an isolation window of 5.0 and a normalized collision energy (NCE) of 15.0 eV. The instrument was calibrated per manufacturer's specifications. The comparison of the m/z values of fragments from Compound 1 and chloropretadalafil are based on the elucidation of fragment ions in Mass Frontier version 5.1 spectral interpretation software (Thermo Fisher Scientific, San Jose, CA) or based on information available in mzCloud database (HighChem LLC, Slovakia) and scientific literature [16].

2.4. GC/FT-IR/MS analysis

Sample extracts were prepared by mixing approximately 600 mg of the capsule contents with 1 mL acetonitrile. The mixture was vortexed and then centrifuged. The supernatant was filtered with a 0.2 μm polytetrafluoroethylene syringe filter and dried in an oven at approximately 60 °C. The residue was reconstituted with 50 μL acetonitrile, and 1 L of which was injected into the instrument.

Separation and detection of the unknown and a chloropretadalafile standard reference material was conducted using a fully integrated GC/FT-IR/MS instrument.

Chromatography was conducted using an Agilent 7890 B Series GC outfitted with a G4567A Series autosampler and an Agilent HP-5 ms column consisting of (5%-Phenyl)-methylpolysiloxane and length, I.D. and film thickness dimensions of 30 m, 0.25 mm and 0.50 μm , respectively. Helium carrier gas was employed in constant flow mode using a flow rate of 2 mL/min. Injections were performed in splitless mode with an injection volume of 1.0 μL and an injector temperature of 250 °C. The method included a starting temperature of 75 °C with a hold time of 1.0 min and a ramp rate of 12 °C/min until a final temperature of 325 °C was reached. The final temperature was held for 15.0 min, which resulted in a total run time of 37 min. The terminus of the column was inserted into an inert capillary tee that splits approximately $\frac{3}{4}$ of the GC effluent to a transfer line connected to the IR interface and approximately $\frac{1}{4}$ of the GC effluent to a transfer line connected to the MS interface. The transfer line temperatures from the GC to the MSD and from the GC to the IRD were 280 °C and 300 °C, respectively.

Infrared detection was accomplished using a Dani Instruments DiscovIR FT-IR spectrometer. The terminus of one transfer line from the GC was inserted into the IR interface and positioned directly above the ZnSe disk. FT-IR spectral data were collected using a 100 $\mu\text{m} \times 100 \mu\text{m}$ MCT detector, 4000–700 cm^{-1} spectral range, 4 cm^{-1} resolution, 3 mm/min disk speed, 4.0 min solvent delay, 300 °C restrictor temperature, 300 °C oven temperature, 35 °C dewar cap temperature and –40 °C disk temperature. Instrument operations and data analysis were conducted using workbooks designed in Grams software version 9.2 by Dani Instruments.

Mass spectrometric detection was performed using an Agilent 5977A series mass selective detector. The terminus of the second transfer line from the GC was inserted into the MS and positioned directly in front of the electron ionization (EI) source. Mass spectral data were collected from 50 to 550 amu using full scan mode, a 5.0 min solvent delay, –100 relative voltage, a threshold of 10, quadrupole temperature of 150 °C and a source temperature of 230 °C. Data analysis was performed using Agilent MSD Chemstation software version F.01.03.2357.

3. Results and discussion

3.1. HPLC-UV analyses

The isolation of Compound 1 utilized retention time as the trigger for fraction collection, and the peaks were characterized by their UV spectra in comparison to the spectrum

obtained from tadalafil. The UV spectrum, compared to that of tadalafil, displayed almost identical characteristics (see Fig. 2).

Based on comparisons of the peak areas to those obtained from tadalafil standard solutions, Compound 1 was present at approximately 2 mg per capsule. The supplement was also found to contain 77 mg sildenafil per capsule (based on comparison to a sildenafil standard), estimated to contain 3 mg *N*-ethyl tadalafil per capsule, and estimated to contain less than 3 mg nortadalafil per capsule.

3.2. Mass spectrometry

The protonated molecular formula of Compound 1 was determined to be $C_{23}H_{22}ClN_2O_5$ (m/z 441.1216, error is 0.8706 ppm). The MS/MS spectrum of the $[M+H]^+$ ion at m/z 441.1216 resulted in product ions at m/z 409.0937, 334.1071, 262.086, and 135.0442 (Fig. 3a, b). Based on the isotope distribution pattern, a chlorine is present on the precursor ion (m/z 441.1216) and one of the product ions (m/z 409.0937). Chloropretadalafil has an observed $[M+H]^+$ ion at m/z 427.1068 and product ions of m/z 395.0786, 334.1074, 262.0860, and 135.0442 that have been previously reported (Figs. 4a, b). [16] The isotope distribution pattern indicates that there is a chlorine present on the precursor ion (m/z 409.0937) and one of the product ions (m/z 395.0786). Based on the shared fragment ions that occurred due to the loss of the $-C(O)CH_2Cl$ group from the nitrogen in the piperidine ring of chloropretadalafil, it is hypothesized that the substitution occurred on that moiety. It is likely that an additional methylene group was inserted between the chlorine and amide carbonyl in Compound 1.

3.3. GC/FT-IR/MS analysis

GC/FT-IR/MS analysis of the unknown yielded a peak at about 23.5 min for both the absorbance chromatogram (IR detector) and total ion chromatogram (MS detector); the infrared and mass spectra corresponding to these peaks are shown in Fig. 5a and b, respectively. Analysis of a reference standard of chloropretadalafil yielded a peak at 24.6 min for both the absorbance and total ion chromatograms; the infrared and mass spectra corresponding to these peaks are shown in Figs. 5c and 5d, respectively. The infrared spectrum of the Compound 1 (Fig. 5a) exhibited all of the same major infrared absorption bands as chloropretadalafil (Fig. 5c). For example, both spectra exhibited N–H stretching, ester C=O stretching, amide C=O stretching, aromatic C=C stretching, C–O antisymmetric stretching and C–O symmetric stretching vibrations around 3400, 1745, 1665, 1490, 1240, and 1040 cm^{-1} , respectively. These similarities indicate that the two molecules have similar structures. However, significant differences were observed in the region around 1400 cm^{-1} , which is where CH_2 deformation vibrations are found. Additionally, while all other major infrared absorption bands in the fingerprint region differ by only 1–2 cm^{-1} , a difference of 12 cm^{-1} was observed for the amide C=O stretching vibration. The frequency position of the amide C=O stretching vibration is sensitive to conjugation with neighboring double bonds and lone pair electrons; stronger conjugation shifts this band toward lower wavenumber values and weaker/no conjugation shifts this band toward higher wavenumber values. Since the unknown exhibited an amide C=O absorption shifted toward higher wavenumber values compared to chloropretadalafil, it is likely that the amide carbonyl in Compound 1 exhibits

less conjugation than that of chloropretadalafil. In the case of chloropretadalafil, the amide carbonyl exhibits conjugation with chlorine's lone pair electrons. As a result, the unknown molecule likely contains an amide C=O functional group that is either conjugated with a less electronegative atom or unconjugated. The latter is consistent with the proposed structure that has an additional methylene group inserted between the chlorine and amide carbonyl, in which case conjugation between the carbonyl and chlorine's lone pair electrons is eliminated.

Regarding the MS data, Compound 1 (Fig. 5b) and chloropretadalafil spectra (Fig. 5d) both exhibited fragment ions at m/z 169, 204, 289, and 349, which indicate that the two molecules share similar structures. Both spectra also exhibit a loss of 35 and an [M+2] isotope peak approximately 1/3 the intensity of the molecular ion [M+], which indicate that each molecule contains a chlorine. However, the chloropretadalafil spectrum contained an [M+] ion at m/z 426 and the unknown spectrum contained an [M+] at m/z 440, which is consistent with the addition of a methylene group to the chloropretadalafil molecule as indicated by the proposed structure. The isolated fraction was analyzed by NMR; however, the fraction was not pure or ample enough to collect usable data.

4. Conclusion

In the present study, a new chloropretadalafil analog was isolated from a dietary supplement and its structure was proposed using UV, GC/FT-IR/MS, and high-resolution accurate mass MS information. The data indicate that the difference in the structure is due to an addition of a methylene group on the amide carbonyl moiety of chloropretadalafil. The compound was given the name chloropropanoylpretadalafil. To our knowledge, this is the first identification of this analog in a dietary supplement.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jpba.2016.05.038>.

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Fig 1.
Structures of Compound 1 (chloropropanoylpretadalafil) and Chloropretadalafil.

Fig. 2. UV spectra of tadalafil and Compound 1. Spectra were obtained using the chromatographic conditions indicated in the text.

Fig. 3. Mass spectra of Compound 1: 3a: mass spectrum of Compound 1 showing the precursor ion and product ion that contain chlorine, 3b: mass spectrum of Compound 1 showing product ions and proposed structures.

Fig. 4. Mass spectra of chloropretadalafil: 4a: mass spectrum of chloropretadalafil showing the precursor ion and product ion that contain chlorine, 4b: mass spectrum of chloropretadalafil showing product ions and proposed structures.

Fig. 5. GC/FT-IR/MS analysis: infrared and mass spectra of the unknown (a,b) compared to those of ofchloropretadalafil (c,d), respectively.