Identifying *N*-Nitrosofenfluramine in a Nutrition Supplement

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Abstract

The analytical data for identifying an unknown substance that was found in a nutrition supplement is presented. The unknown substance is purified using thin-layer chromatography and then measured using high-resolution mass spectrometry (HRMS) giving the exact mass from which the structure of the unknown substance was proposed. The procedure for synthesizing *N*-nitrosofenfluramine from fenfluramine is described. The extracted, synthesized, and standard *N*-nitrosofenfluramine are compared using HRMS, high-performance liquid chromatography (HPLC)–MS, HPLC–UV, Fourier transform IR spectroscopy, gas chromatography–MS, TLC, and NMR (^{1}H NMR and ^{13}C NMR). All analytical data obtained confirm that the unknown peak in the nutrition supplement is *N*-nitrosofenfluramine and that the synthetic procedure described can easily provide the *N*-nitrosofenfluramine reference substance for identification.

Introduction

Recently, some cases of hepatic failure suspected to be associated with *N*-nitrosofenfluramine or some other unlicensed substances were reported (1–3). In these cases, *N*-nitrosofenfluramine was found in the nutrition supplements ingested. In our routine analysis, an unknown peak was found in one nutrition supplement. Further experiments were carried out to identify the unknown peak. Thin-layer chromatography (TLC) was used to isolate the unknown substance from the extracted matrix. With high-resolution mass spectrometry (HRMS) and high-performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) using electrospray ionization (ESI), the molecular ion and the elemental composition of the ions was obtained and the structure could therefore be proposed. *N*-Nitrosofenfluramine was then synthesized from fenfluramine. Some months later, a *N*-nitrosofenfluramine reference substance was obtained from a Japanese company for fine chemicals. Some analytical measurements, such as HRMS, gas chromatography (GC)–MS, HPLC–diode array detection (DAD)–ESI-MS, NMR, and Fourier transform infrared spectroscopy (FTIR) were used to compare the extracted, synthesized, and standard substances. All the results confirmed that the unknown peak found in this nutrition supplement was *N*-nitrosofenfluramine. The analytical data are discussed in this paper.

The nutrition supplement in question was suspended from the market in early 2002 because of the presence of fenfluramine. The supplement contained two plastic bottles, each with 60 capsules. One contained blue capsules and the other light yellow ones. Both capsules contained about 0.24 g powder content. Its label declared that the supplement was used for weight loss and made from medicinal and edible plants.

Experimental

Chemical reagents

Acetonitrile and tert-butyl-methyl ether (both HPLC grade) were purchased from Tedia (Fairfield, OH) and Dikma (Beijing, China), respectively. *N*-Nitrosofenfluramine was obtained from Wako Pure Chemical Industries (Osaka, Japan). The label of the standard *N*-nitrosofenfluramine described the standard as a mixture of isomers and for pharmacological use only. All other chemical reagents used were analytical grade and produced by different Chinese chemical factories.

Preparation of reagents

Water purification

The water for solution preparation was purified by a Barnstead water purifier. The resultant purity was approximately 16 Megohms/cm.

Distillation of tert-butyl-methyl ether

An amount of 0.1 g of calcium hydride was added to 1.0 L of tert-butyl-methyl ether and the mixture was distilled. The fraction at 55–56°C was collected and stored in the dark at –20°C.

Distillation of ethyl acetate

One hundred milliliters of acetic anhydride and 10 drops of concentrated sulfuric acid were added to 1.0 L of ethyl acetate. The mixture was refluxed for 4 h and shaken with 20–30 g of

anhydrous potassium carbonate, filtered, and redistilled. The redistilled ethyl acetate was stored in a glass container in the dark at –20°C.

Anhydrous sodium sulfate

The salt was washed with diethyl ether to remove trace amounts of organic contaminants (e.g., phthalate), dried at 200°C for 8 h, and stored in a dry glass container.

Internal standard for screening with GC–nitrogen-specific detection and GC–MS

For the standard solution of diphenylamine, 0.5 mg of diphenylamine was dissolved in 100 mL of freshly distilled tert-butyl-methyl ether. The solution was stored at –20°C.

Extraction procedure

The capsule was dissolved in 1 mL of distilled water containing three drops of methanol then mechanically shaken for 30 min. After centrifugation at 2500 rpm and separation, 0.5 mL of 5M potassium hydroxide, 3 g of sodium chloride, and 2 mL of tert-butyl-methyl ether containing 5.0 ng/mL diphenylamine as an internal standard were added to the water phase. The mixture was mechanically shaken for 10 min and centrifuged at 2500 rpm for 5 min. Then the organic phase of the mixture was transferred into a vial containing 0.1 g of anhydrous sodium sulfate. Two microliters of the solution was injected into the GC–MS.

Procedure for synthesizing *N*-nitrosofenfluramine

An amount of 0.5 g of fenfluramine standard was dissolved in 10 mL ethanol and cooled to 5°C. Sodium nitrite (0.3 g) was then added, and the mixture was agitated. At 5°C, 0.7 mL of concentrated hydrochloric acid was added, and the mixture was stored at room temperature for 28 h. After completion of the reaction, the mixture was poured into approximately 10 mL of ice water. The pH value of the mixture was adjusted to pH 8 with 10% aqueous sodium bicarbonate solution. *N*-Nitrosofenfluramine was extracted with 20 mL ether. The ether phase was washed with distilled water until neutral. All ether was removed by reduced pressure distillation. The final yellow oil was obtained upon vacuum drying.

Purification procedure with TLC

The extract and the reference substances (synthesized and commercially obtained *N*-nitrosofenfluramine) were applied to a silica 254 plate (20 × 20 cm). Methanol was used as the solvent for developing at room temperature. Under the UV light, the major area with the same retention factor (R_f) value as the reference substances was scratched from the silica plate. The silica residue was mixed with 1 mL methanol in an ultrasonic generator for 10 min. After centrifugation, the methanol phase was isolated for further analysis.

Instruments and working conditions

GC–nitrogen specific detection (NPD) was performed using the HP-5890A GC–NPD. A 17-m HP-5 column (0.2-mm i.d., 0.33-mm film thickness) was used (Hewlett-Packard, Palo Alto, CA). The carrier gas was helium at a flow rate of 2.0 mL/min (at 20°C). The injector was set at 250°C, and the injected volume was 2 µL. The split ratio was 10:1. The septum purge was set at 2.0 mL/min. The NPD operating conditions were as follows: temperature at 280°C, hydrogen at 3.5 mL/min, helium make-up at 27.0 mL/min, and air at 90.0 mL/min. The starting oven temperature program was 100°C (held for 1.0 min), then 10°C/min to 200°C, and then 20°C/min to 300°C (held for 6.0 min). GC–NPD was only used for screening of samples.

GC–MS was performed using the Agilent 6890A/HP5973 system (Agilent Technologies, Palo Alto, CA). A 25-m HP-5 column (0.2-mm i.d., 0.33-µm film thickness) was used with a head pressure of 100 Kpa. The oven temperature program was: 80°C (held for 1 min) then increased by 20°C/min to 240°C, then increased by 15°C /min to 280°C (held for 12 min). The injector temperature was 250°C and the transfer line 280°C. The split ratio was 10:1. Data were obtained in EI mode at 70 eV scanning from 50 to 500 amu in 0.25 s.

HRMS was performed using the Micromass ZabSpec HRMS at a resolution of 10,000 (Micromass, Manchester, U.K.). The scan range was *m/z* 50 to 600 with a scan rate of 1. The electron impact mode was set at 70 eV. The temperature of the probe was 100°C and the ion source was 200°C.

NMR was performed using the Varian Inova 600 MHz instrument (Varian, Palo Alto, CA). Deuterated chloroform was used as the solvent. Pulse sequence was a preset mode. Relaxation delay was 3.000 s and pulse 27.6 degrees. The acquisition time was 1.333 s.

HPLC–DAD–ESI-MS was performed using the Agilent 1100 HPLC/1000 ion trap mass selective detector in ESI (+) ionization mode. A Zorbax SB 18 column was used (Agilent). The isocratic elution solvent for the HPLC was water (containing 0.5% formic acid) and acetonitrile in a ratio of 40:60. The flow rate was 1 mL/min.

FTIR was performed using the Bio-Rad FTS-65A instrument at a resolution of 4 cm^{-1} (Bio-Rad Laboratories, Hercules, CA) The samples were scanned in liquid film.

Results and Discussion

A GC–NPD chromatogram of the nutrition supplement extract was obtained for screening of nitrogen-containing compounds. As shown in Figure 1, a large unknown peak was found with a retention time of 8.3 min and a relative retention time to the

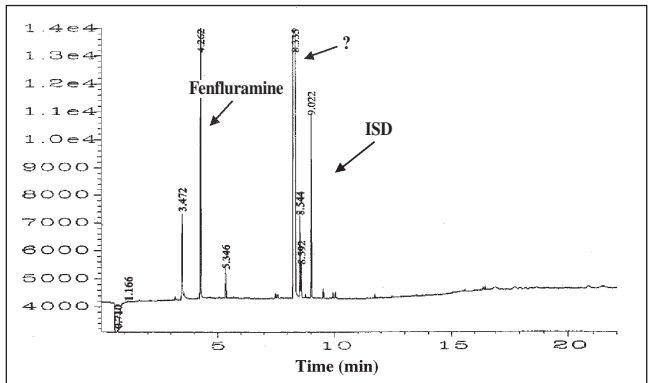


Figure 1. GC–NPD chromatogram of the extract.

internal standard (diphenylamine) of 0.92. Both the synthesized and standard *N*-nitrosofenfluramine showed the same retention data under these conditions.

The TLC R_f values found for the extracted, synthesized, and standard *N*-nitrosofenfluramine are summarized in Table I. The R_f values were determined with different solvent systems using TLC (4).

Figure 2 shows the total ion chromatogram (TIC) obtained with GC–MS. The fenfluramine was readily identified using routine procedures, and because of the presence of fenfluramine, this supplement was banned from the market before *N*-nitrosofenfluramine was identified in our laboratory. Comparing the peak heights in Figure 2, the concentration of the unknown peak (*N*-nitrosofenfluramine) was considerably higher than that of fenfluramine. In Figure 3 the mass spectrum of the unknown peak shows major fragments of *m/z* 260, 230, 211, 186, 159 (base peak), 101, 71, 56, and 42. Both the synthesized and standard *N*-nitrosofenfluramine showed retention times of 8.8 min and the same mass spectra under the same GC–MS conditions.

The HRMS spectrum of the extract purified using TLC provides the exact mass of the molecule and all its fragments (Table II). All the proposed structures of the fragments found in the EI mass spectra of the extract, synthesized and standard *N*-nitrosofenfluramine, agreed with the fragmentation pathway (Figure 4).

The molecular weight of the extracted *N*-nitrosofenfluramine was also confirmed by HPLC–ESI-MS. The high-pressure liquid chromatograms of the extract recorded with ESI-MS (ion trap, TIC) and UV detector (234.16 nm) are shown in Figure 5. The peaks with retention times of 8.7 and 9.4 min could be isomers of *N*-nitrosofenfluramine because they showed very similar mass spectra (ions at *m/z* 521, 262, 187, and 159) with ESI. Only the relative abundances of these ions in the spectra were slightly different.

Under the same HPLC–ESI-MS conditions, both the synthesized and the standard *N*-nitrosofenfluramine have two peaks with the same retention time of 8.7 and 9.4 min and the same mass spectral fragments. In HPLC–ESI-MS mass spectra, the ion *m/z* 261 is the protonated molecular ion, and ion *m/z* 283 is the

Table I. The R_f Values in TLC

TLC solvent system	R_f
Methanol	0.85
Cyclohexane–toluene–triethyl-amine (75:15:10)	0.77
Methanol–*n*-butanol (60:40)	0.83
Cyclohexane–toluene (80:20)	0.092
Cyclohexane–methanol (80:20)	0.50

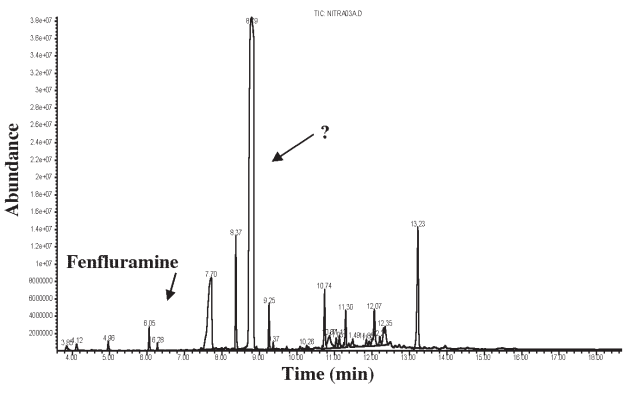


Figure 2. GC–MS TIC of the extract.

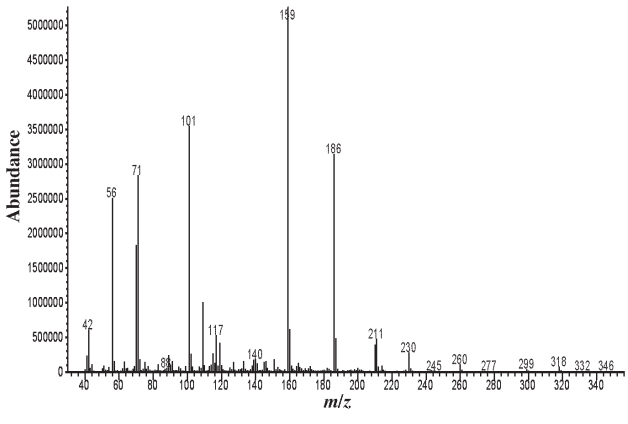
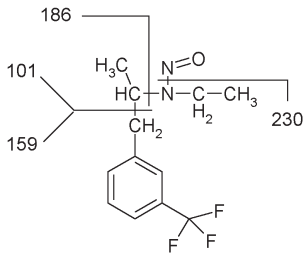


Figure 3. Mass spectrum of the unknown peak obtained with GC–MS.

Table II. The Exact Mass and Proposed Elements Composition

Exact mass	ppm	C	H	N	O	F
260.1136	0	12	15	2	1	3
230.1142	6.3	12	15	1	–	3
211.2285	–6.1	12	15	1	–	2
186.0655	0.6	10	9	–	–	3
159.0428	–4.1	8	6	–	–	3
101.0711	3.5	4	9	2	1	–
71.0701	–	4	9	1	–	–



***m/z* 260.1136: the molecular ion.**

***m/z* 211.2285: the lost of fluorine from the ion of *m/z* 230.1142.**

***m/z* 71.0701: the lost of NO from the ion of *m/z* 101.0711.**

Figure 4. Proposed fragmentation path.

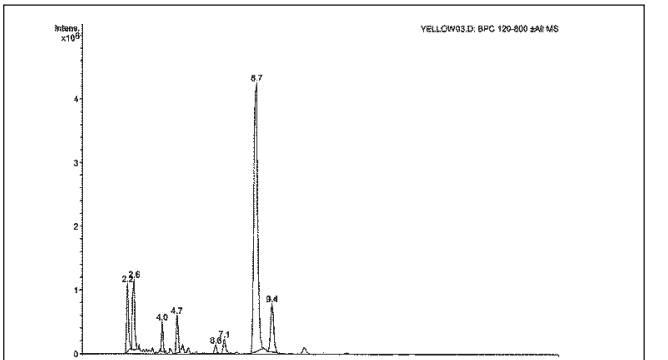


Figure 5. The high-pressure liquid chromatograms of the extract obtained with MS and UV detectors: (A) HPLC–MS-ESI and (B) HPLC–DAD.

molecule ion + Na^+. The highest mass of m/z 521 is the dimer of the protonated molecule.

It was previously published that *N*-nitrosofenfluramine could have trans- and cis-isomers with the *N*-nitroso group and also *S* and *R* optical isomers because of the chiral centers (5).

Figure 6 shows the UV spectra of the peak with a retention time of 8.6 min, and the symmetry of the peak in the high-pressure liquid chromatogram of the extract indicate high sample purity. The found purity factor was 987. The symmetric pattern can be seen in the lower picture of Figure 6. The UV spectra of the synthesized and the standard *N*-nitrosofenfluramine have the identical UV spectra as the extract shown in Figure 6 with an absorption maximum at 234 nm.

The ^1H NMR spectra data of the *N*-nitrosofenfluramine are presented in Table III. The ^{13}C NMR spectra for the *N*-nitrosofenfluramine exhibited chemical shifts of 138.551, 132.544, 129.102, 125.722, 125.701, 123.734, 123.710, 60.615, 41.751, 38.694, 19.971, and 11.656 ppm.

The FTIR spectra of *N*-nitrosofenfluramine gave signals at 2981.3, 2939.5, 1448.6, 1331.2, 1202.7, 1164.9, 1125.1, 1074.5, 973.8, 912.3, 802.7, 750.7, 704.7, and 659.1 cm^{-1} and was the same for all three samples.

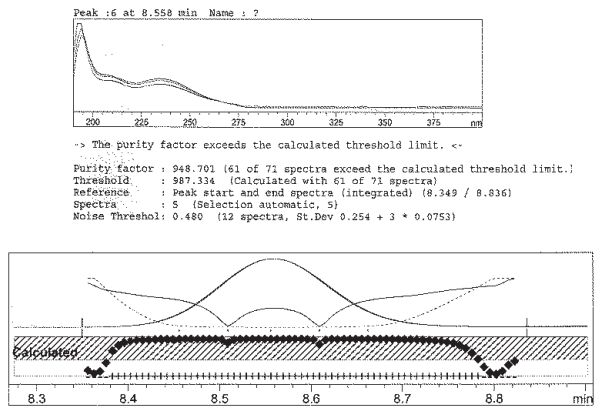


Figure 6. The UV spectrum and peak symmetry of the extract using HPLC–DAD.

Table III. The ^1H NMR Spectra (in $CDCl_3$) for *N*-Nitrosofenfluramine

Number of hydrogens	Chemical shift (ppm)	Peak shape	Coupling constant (Hz)
1H	7.49	doublet	7.8
1H	7.43	broad single	–
1H	7.40	doublet	8.4
1H	7.35	quartet	7.8/8.4
1H	4.61	multiplet	
1H	3.50	multiplet	
1H	3.46	multiplet	
1H	3.26	quartet	
1H	3.09	quartet	
3H	1.51	double	
3H	0.98	triplet	

Conclusion

Comparison of the mass (GC–MS, HRMS, and HPLC–ESI-MS), FTIR, and NMR (^1H-NMR and ^{13}C NMR) spectra shows the unknown compound in the nutrition supplement to be *N*-nitrosofenfluramine. The procedure for the synthesis of *N*-nitrosofenfluramine presented in this paper provides a *N*-nitrosofenfluramine reference. However, toxicological research with optical isomers of *N*-nitrosofenfluramine has to be carried out.

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