**Supplemental Online Material to:** The stimulant higenamine in weight loss and sports supplements

**Synthesis**

**Scheme 1**.



Synthesis of higenamine (a) and preparation of synthetic intermediates utilized in the synthesis of higenamine (b) have been previously described. Condensation of 4-Methoxyphenylacetic acid (**1**) and 3,4-dimethoxyphenethylamine (**2**) yielded N-(3', 4'-dimethoxyphenylethyl)-4-methoxyphenyl acetamide (**3**). Bischler-Napieralski cyclization of (**3**) with phosphorus oxychloride afforded 1-(4' -Methoxybenzyl)-6, 7-dimethoxy-3,4-dihydroisoquinoline (**4**). Reduction of the dihydroisoquinoline (4) with sodium borohydride yielded 1-(4'-Methoxybenzyl)-6, 7-dimethoxy-1, 2, 3, 4-tetrahydroisoquinoline (**5**). Refluxing (**5**) in hydroiodic acid afforded higenamine hydroiodide (**6**, 1-(4'-Hydroxybenzyl)-6, 7-dihydroxy-1, 2, 3, 4-tetrahydroisoquinoline hydroiodide).

1. Arch. Pharm. Res. 7 (2), 133-136 (1984)
2. European Journal of Medicinal Chemistry 45 (2010) 11–18

**Materials**

NSF obtained ammonium formate (ACS reagent grade) from Alfa Aesar (Ward Hill, MA); acetonitrile (HPLC grade), methanol (Ultra Resi Analyzed grade) and formic acid (ACS reagent grade), from JT Baker (Center Valley, PA); water (Optima, LC-MS grade), from Fisher Scientific (Fairlawn, NJ) and formic acid (LC-MS grade) from Sigma-Aldrich (St Louis, MO); and acetonitrile (LCMS grade), from EMD-Millipore (Billerica, MA).

RIVM obtained higenamine reference standards from Sigma-Aldrich (Zwijndrecht, NL). Formic acid (p.a.), acetonitrile (p.a.) and ammonium hydroxide (p.a.) were obtained from Merck (Darmstadt, Germany); water (ULC/MS), from Biosolve (Valkenswaard, NL); leucine enkephalin, from Waters (Ettenleur, NL); and sodium formate, from Sigma Aldrich (Zwijndrecht, NL).

**Instrumentation**

At the NSF International laboratory, products were analyzed for higenamine using a Xevo-TQS with an Acquity UPLC (Waters, Milford, MA) fitted with a BEH C18 column (2.1 mm ID x 50 mm, 1.7 μm particle size). The flow rate was 0.5 mL/min with a column temperature of 40oC. An injection volume of 5 μl was used. The mobile phase consisted of component A, 0.1% formic acid in de-ionized water and component B, acetonitrile. An isocratic elution was employed at 95% A for 2.5 min. The column was flushed with 90% B for 2.9 min and re-equilibrated at 95% A for 4.4 min between runs. Tandem mass spectrometry was performed using electrospray ionization in positive polarity under the following conditions: capillary of 3.5 kV, cone of 4 V, source offset of 50 V, source temperature of 150°C, desolvation temperature of 600°C, desolvation gas flow of 1000 L/hr and a collision gas flow of 0.11 mL/min. The precursor ion was m/z 272.35 and the product ions were m/z 107.09, 161.06 and 143.05 in multiple reaction monitoring mode at collision energies of 24 eV, 18 and 26 respectively. Quantitation was performed using the product ion m/z 107.09.

At the RIVM laboratory products were analyzed using a Waters Acquity™ ultra-performance liquid chromatography (UPLC) system fitted with an HSS C18 column (150 mm x 2.1 mm i.d., 1.8 μm; Waters Chromatography B.V., Etten-Leur, NL). Detection of the analytes was carried out using a Waters Synapt™ G2 quadrupole time of flight (QTOF) mass spectrometer (Waters Chromatography B.V., Etten-Leur, NL) with a Z-spray electrospray ionization (ESI) source operating in the positive ion mode. The instrument was tuned and calibrated in the mass range of 50–1200 Da using sodium formate in resolution mode (≥ 20000 FWHM). Exact mass measurements were based on the protonated molecules [M+H]+. Leucine enkephalin (1 μg/mL) was used as lock mass standard after instrument calibration. Chromatographic and mass data were acquired and analysed using Waters MassLynx v4.1 software.

The flow rate was 0.4 mL/min with a column temperature of 50oC. An injection volume of 10 μl was used. The mobile phase consisted of mixture of component A: 5 mM ammonium formate, adjusted to pH 3.0 using formic acid and component B: 0.1% formic acid in acetonitrile (87:13). A gradient elution was employed as follows: 0–0.50 min 13% B, 0.50–10.00 min 50% B, 10.75 min 95% B, 10.75–12.25 95% B, 12.25–12.50 min 13% B, 12.50–15.00 min 13% B.

Tandem mass spectrometry was performed using electrospray ionization in positive polarity under the following conditions: capillary of 3.0 kV, cone of 30 V, source temperature of 120oC, desolvation temperature of 500oC, desolvation gas flow of 800 L/hr and a collision energy of 4 eV and collision energy ramp of 15–50 eV. Precursor ion was observed at *m/z* 272.1287 for higenamine. Product ions were observed at *m/z* 107.0497, 161.0603 and 255.1021. Stock standards were prepared at 0.2 mg/mL in methanol and diluted to 0.5 μg/mL in a mixture of component A and B (87:13). The limit of detection was extrapolated to a minimum signal of 200 and found to be 10 ng/mL.

**Extraction method**

At NSF International, 1 gram of powdered bulk product or capsule content was extracted with 10 mL of methanol by shaking for 20 minutes followed by sonication for 20 minutes. The mixture was centrifuged at 5525 g for 20 minutes. The supernatant was reserved and filtered through a 0.45 μm polyvinylidene fluoride membrane (PVDF) syringe filter. The resulting solution was diluted 1:4 in 0.1% formic acid in de-ionized water, which was then serially diluted by factors of 10 from 1-10 to 1-106 with a mixture of 0.1% formic acid in deionized water and methanol (75:25).

At RIVM, products were prepared for qualitative analysis in duplicate. A serving size of homogenized powdered bulk product up to a maximum of 5 g. or the contents of 1 capsule were extracted with 50 mL of methanol by shaking for 5 minutes followed by sonication for 15 minutes. Methanol is a common solvent in pharmaceutical sample preparation as small drug molecules dissolve well as a free base or as a salt. The resultant mixture was centrifuged at 3000 g for 15 minutes. The resulting solution was diluted 100x with a mixture of component A: 5 mM ammonium formate, adjusted to pH 3.0 using formic acid and component B: 0.1% formic acid in acetonitrile (87:13). All samples and solutions were filtered before use over a 0.2 µm filter (Spartan 30, Whatman GmbH, Dassel, DE).

**Method validation**

The method validation study evaluated the method’s suitability for routine use and was performed at NSF International.

*Linearity, Range, Limits of Detection (LOD) and Limits of Quantification (LOQ)*

Linearity and range were evaluated by replicate analysis (n=12) of solutions of higenamine (0.20, 0.50, 1.0, 2.0, 5.0, 10, 20 and 50). The LOD (80 ng/g) was determined using the lowest point of the calibration range and calculated as a weight/weight concentration. The LOQ (5 µg/g) was determined empirically from replicate analysis (n=12) of a sample fortified at 5 µg/g.

*Extraction efficiency*

The effect of volume of methanol on extraction efficiency was assessed. Eight replicates of a product with incurred higenamine (58 mg/g) were measured. Four replicates used a 10 mL volume of methanol for the extraction and the other four used 20 mL of methanol. The volume of methanol did not impact the measured higenamine concentration (p = 0.98).

*Accuracy and precision*

The method accuracy and precision were assessed by fortifying a single product with known amounts of higenamine. Replicate analysis was performed on three different days at three fortification levels (5 µg/g, 200 µg/g and 790 µg/g). The accuracy was assessed by determining the average recovery as a percentage at each of the fortified concentrations. The precision was assessed by determining the coefficient of variation (%CV) at each fortified level. An additional product was assessed for precision using its incurred amount of higenamine (42 mg/g). The average recoveries and %CV at each fortification level are reported in Table S1.

Table S1. Method validation results

|  |  |  |
| --- | --- | --- |
|  | | **higenamine** |
| **Range** | | 0.2-50 ng/mL |
| **LOD** | | 0.8 ng/g |
| **LOQ** | | 5.0 µg/g |
| **Accuracy**  **(Recovery %)** | **5 µg/g** | 118.8 |
| **200 µg/g** | 118.3 |
| **790 µg/g** | 95.7 |
| **Intra-day precision**  **(coefficient of variation)** | **5 µg/g** | 4.7 |
| **200 µg/g** | 2.0 |
| **790 µg/g** | 2.3 |
| **42 mg/g** | 2.5 |
| **Inter-day precision**  **(coefficient of variation)** | **5 µg/g** | 0.30 |
| **200 µg/g** | 1.9 |
| **790 µg/g** | 1.7 |
| **42 mg/g** | 3.9 |
| **Total precision**  **(coefficient of variation)** | **5 µg/g** | 4.0 |
| **200 µg/g** | 2.3 |
| **790 µg/g** | 3.0 |
| **42 mg/g** | 4.9 |