Supporting Information

Linking Opiate Metabolites to Heroin through Gas Chromatography–Combustion–Isotope Ratio Mass Spectrometry

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Supporting Information I

Chemicals and reagents

Morphine (1 mg/mL in methanol), morphine-d_6 (1 mg/mL in methanol), sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$, ≧ 99.0 %, w/w), and disodium hydrogen orthophosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$, ≧ 99.0 %, w/w) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). N,O-bis(trimethylsilyl) trifluoroacetamide (99 %) from Sigma-Aldrich with the same batch and series number (SHBJ0606) were used through experiments. N-alkane mixture A7 and caffeine (USGS61 and USGS62) were purchased from Lab. of Arndt Schimmelmann at Indiana University. Pyrene (98 %) was purchased from Merck (Kenilworth, NJ, USA). Caffeine anhydrous (≧ 98.5 %) was purchased from SHOWA (Tokyo, Japan). Dichloromethane, methanol, ethyl ethanoate, ammonium hydroxide (NH_4OH, ≧ 28.0 %, w/w), and acetic acid glacial were purchased from J.T. Baker (Phillipsburg, NJ, USA). SPE cartridges were obtained from Agilent (Bond Elut, CA, USA). Ultrapure water (18.2 mΩ·cm) from a Milli-Q ultrapure water system (Millipore, Billerica, MA, USA) was used throughout the experiments.

Urine and heroin samples.

Blank urine samples. Urine samples from fifteen healthy volunteers aged 18-50 years were collected for evaluating matrix effect and amount-independent uncertainty measurements in the analysis of bis(trimethylsilyl)morphine. These samples were stored in the refrigerator at 4℃ before assays. These urine samples were analyzed through GC–MS to ensure no morphine contaminants were present.

Heroin abusers' urine and heroin samples. Heroin and urine samples from 15 actual convicted cases were collected. Heroin samples were found on the criminals in all cases.

Supporting Information II

Sample preparation.

Standard heroin solutions for obtaining a calibration curve using GC–MS analysis. Standard heroin solutions (0.02–0.2 mg/mL, 500 μL) were prepared by mixing separate aliquots (10, 20, 40, 60, 80, and 100 μL) of heroin stock solution (1mg/mL in methanol) with 100 μL of pyrene stock (250 μg/mL in methanol) and 390 to 300 μL of methanol for calibration by GC–MS analysis.

Heroin samples in actual cases for quantitation by GC–MS. Seized heroin samples (1mg) were separately dissolved in 1 mL of methanol and then subjected to quantitation using GC–MS with a calibration curve. A 0.12 mg/mL heroin solution was used for a QC sample. The concentration of QC sample after analyzing must be within of ±20 % bias.

Standard morphine solutions for obtaining a calibration curve using GC–MS analysis. Aliquots (10, 25, 50, 100, and 200 μL) of morphine stock solution (10 μg/mL in methanol) separately mixed with 50 μL of morphine-d_6 (10 μg/mL in methanol) were added into 1 mL of blank urine for obtaining a calibration curve. In addition, aliquots (0, 22.5, and 37.5 μL) of morphine stock solution (10 μg/mL in methanol) separately mixed with 50 μL of morphine-d_6 (10 μg/mL in methanol) were added into 1 mL of blank urine as QC samples. Next, each mixture was adjusted to pH 9.0 by using NH_4OH and then transferred onto an SPE column. The SPE column had been treated with 4 mL of methanol and 1 mL of phosphate buffer (pH 9.0, 100 mM). To remove unspecific adsorbed substrates, the column was washed subsequently with 4 mL of H_2O, 1 mL of acetic buffer (pH 4.0, 100 mM), and 4 mL of methanol. The retained components were eluted using 3 mL of dichloromethane/isopropyl alcohol/NH_4OH (40:10:1; v/v/v) under a vacuum. After evaporation to dryness under a gentle stream of nitrogen at 40 °C, 100 μL of BSTFA was added. The mixture was then heated at 80 °C for 1 h. After being evaporated to dryness under a gentle stream of nitrogen at 40 °C, the residue was dissolved in 100 μL of ethyl acetate for calibration using GC–MS analysis. The concentration of QC sample after analyzing must be within of ±20 % bias.

Heroin abusers' opiate metabolites in actual cases for quantitation using GC–MS. Fifteen heroin abusers' urine samples (1 mL), subjected to HCl hydrolysis (2 mL of 6 M HCl at 80 °C for 90 min) to obtain the free form of morphine, were mixed separately with 50 μL of morphine-d_6 (10 μg/mL in methanol). Each of the mixtures was adjusted to pH 9.0, and then purified and concentrated through SPE, subsequently subjected to a reaction with BSTFA. The residue dissolved in 100 μL of ethyl acetate was then analyzed using GC–MS. The concentrations of morphine in the urine samples were determined using a calibration curve.

GC–MS analysis

A GC–MS spectrometer with a DB-5 fused silica capillary column was employed for the analysis of bis(trimethylsilyl)morphine and heroin samples in this study. The peak area ratios of heroin at the *m/z* of 327 over pyrene at the *m/z* of 202 were then used to establish a calibration curve. The temperature of injection port was 260 °C. The column temperature was administrated as follows: the initial temperature of 200 °C was kept for 0.3 min, which was increased to 300 °C at a rate of 40 °C/min and finally kept for 4.2 min. The MS detector was operated at 70 eV with interface temperature at 230 °C. The carrier gas was helium at a flow rate of 1.0 mL/min. For quantitation of heroin abuser's urines, the condition of GC-MS was modified slightly to obtain better sensitivity and resolution. The column temperature was administrated as follows: the initial temperature of 150 °C was set for 5.0 min, which was increased to 280 °C at a rate of 10 °C/min and finally kept for 6.3 min. The MS detector with interface

temperature at 250 °C was administrated on a selected ion mode at *m/z* 429, 414 and 401 for bis(trimethylsilyl)morphine, and at *m/z* 435, 420 and 404 for bis(trimethylsilyl)morphine-d_6.

Supporting Information III

Sample preparation.

Morphine spiked in blank urine and ultrapure water for GC–C–IRMS analysis. Aliquots (50, 75, 100, and 125 μL) of morphine stock solution (1 mg/mL in methanol) were spiked separately into 10 mL of 15 volunteers' urine samples and 10 mL of ultrapure water. Each mixture was adjusted to pH 9.0, and then transferred for SPE purification and BSTFA reaction as per the standard morphine solutions. The residue dissolved in 250 μL of ethyl acetate was then analyzed using GC–C–IRMS for $\delta^{13}C$ measurement of bis(trimethylsilyl)morphine in the urine samples. For $\delta^{15}N$ measurement, aliquots (150, 200, 250, and 500 μL) of morphine stock solution (1 mg/mL in methanol) were spiked separately into 10 mL of 15 volunteers' urine samples and 10 mL of ultrapure water. The spiked samples were subjected to the same preparation, SPE purification, and BSTFA reaction as that used for the $\delta^{13}C$ measurement, but the residue was dissolved in 250 μL of ethyl acetate.

Samples for evaluating the effect of BSTFA. A total of 200 μL of morphine stock (1 mg/mL in methanol) was spiked in 10 mL of blank urine sample. The mixture was adjusted to pH 9.0, and then transferred for SPE purification and BSTFA reaction as per the standard morphine solutions. The residue dissolved in 200 μL of ethyl acetate was then analyzed using GC–C–IRMS for $\delta^{13}C$ and $\delta^{15}N$ measurements of bis(trimethylsilyl)morphine. On the other hand, a total of 200 μL of morphine stock (1 mg/mL in methanol) was spiked in 10 mL of blank urine sample. The mixture was adjusted to pH 9.0, and then transferred for SPE purification as per the standard morphine solutions but without conducting the BSTFA reaction. The residue dissolved in 100 μL of ethyl acetate was then analyzed using GC–C–IRMS for $\delta^{13}C$ and $\delta^{15}N$ measurement of morphine.

Samples for calculating $\delta^{13}C$ contribution from bis(trimethylsilyl) groups of BSTFA. A total of 100 μL of morphine stock (1 mg/mL in methanol) and 100 μL of methanol were mixed in a glass vial. After vortex mixing for 5 s, the solution was analyzed using GC–C–IRMS to acquire the $\delta^{13}C$ of morphine. In addition, 100 μL of morphine stock was evaporated to dryness under a nitrogen stream at 40°C and then 100 μL of BSTFA was added and heated at 80°C for 1 h. After being evaporated to dryness under a gentle stream of nitrogen at 40°C, the residue dissolved in 200 μL of ethyl acetate was then transferred to the glass vial for measurement of $\delta^{13}C$ of bis(trimethylsilyl)morphine.

Heroin samples in actual cases for GC–C–IRMS analysis. To acquire the free form of

morphine, 5 mg of the heroin sample was hydrolyzed with 2 mL of 6 M HCl at 80 °C for 90 min. After being cooled, NH_4OH was added into the mixture to adjust the pH to 9.0. Next, 1 mL of phosphate buffer (pH 9.0, 100 mM) was added. The mixture was transferred for SPE purification and BSTFA reaction as per the standard morphine solutions. Before GC–C–IRMS analysis, the residues were dissolved in a suitable amount of ethyl acetate to the concentrations of bis(trimethylsilyl)morphine within a signal linearity range of $\delta^{13}C$ and $\delta^{15}N$ measurements.

Abusers' opiate metabolites in actual cases for GC–C–IRMS analysis. Suitable amounts (1–20 mL) of 15 heroin abusers' urines were subjected to HCl hydrolysis (2 mL of 6 M HCl at 80 °C for 90 min) to obtain free form of morphine. The amounts were selected on the basis of the concentration of morphine in the urine sample determined through GC–MS. Next, each mixture was adjusted to pH 9.0, and then transferred for SPE purification and BSTFA reaction as per the standard morphine solutions. The residue dissolved in 100 μL of ethyl acetate was then analyzed for measurements of $\delta^{13}C$ and $\delta^{15}N$ of bis(trimethylsilyl)morphine through GC–C–IRMS.

GC–C–IRMS analysis.

Carbon isotope measurement. The IRMS was composed of an electron impact source held at 3.0 kV acceleration voltage, a magnet, and three Faraday collectors for monitoring ions at 44, 45, and 46 *m/z*. Helium was used as a carrier gas with a constant flow rate of 2.0 mL/min, and CO_2 was used as a reference. Gas flows were regulated using the Thermo Scientific Conflo IV interface. An aliquot (1 μL) of the sample was injected into the Thermo Trace GC 1310 Gas Chromatograph. The initial temperature of the GC oven was maintained at 150 °C for 1 min, and then the temperature was increased to 200 °C at a rate of 20 °C/min and maintained for 3 min; it was then increased to 250 °C at a rate of 20 °C/min and maintained for 5 min, and finally, increased to 290 °C at a rate of 30 °C/min and maintained for 25 min. The combustion furnace was set at 1000 °C and oxidized with O_2 before analyses.

Nitrogen isotope measurement. Three Faraday cups collected ions at 28, 29 and 30 *m/z* in IRMS. A cold trap between the GC Isolink and Conflo IV was used to trap the CO_2 produced from the analytes in the combustion furnace. An aliquot (3 μL) of the sample was injected into the Thermo Trace GC 1310 Gas Chromatography.

Supporting Information IV

Two-point calibration procedure was performed to calibrate the meatured isotope values to be true values onto an international scale by using reference materials of heptadecane, docosane, and triacontane in n-alkane mixture A7 for $\delta^{13}C$ and caffeine (USGS61 and USGS62) for $\delta^{15}N$.

GC–C–IRMS analysis.

Carbon isotope measurement. IRMS condition was according to that of Supporting Information III, but condition of gas chromatograph was modified as following: The initial temperature of the GC oven was maintained at 130 °C for 3 min, and then the temperature was increased to 310 °C at a rate of 5 °C/min and maintained for 10 min.

Nitrogen isotope measurement. IRMS and GC conditions were according to those of Supporting Information III.

Two-point calibration.

Two-point calibration curves of $\delta^{13}C$ and $\delta^{15}N$ were respectively shown in Figure S-IV (A) and (B). A linear relationship ($R^2 = 0.998$) of true $\delta^{13}C$ against measured $\delta^{13}C$ over the range -29.84 to -34.33 ‰ was acquired, and the relationship equation was fitting to $\delta^{13}C_{ture} = 1.0066\ \delta^{13}C_{measured} + 0.302$. On the other hand, a linear correlation ($R^2 = 1.000$) of true $\delta^{15}N$ against measured $\delta^{15}N$ over the range -2.87 to 20.17 ‰ was acquired, and the relationship equation was fitting to $\delta^{15}N_{ture} = 1.0382\ \delta^{15}N_{measured} - 0.430$

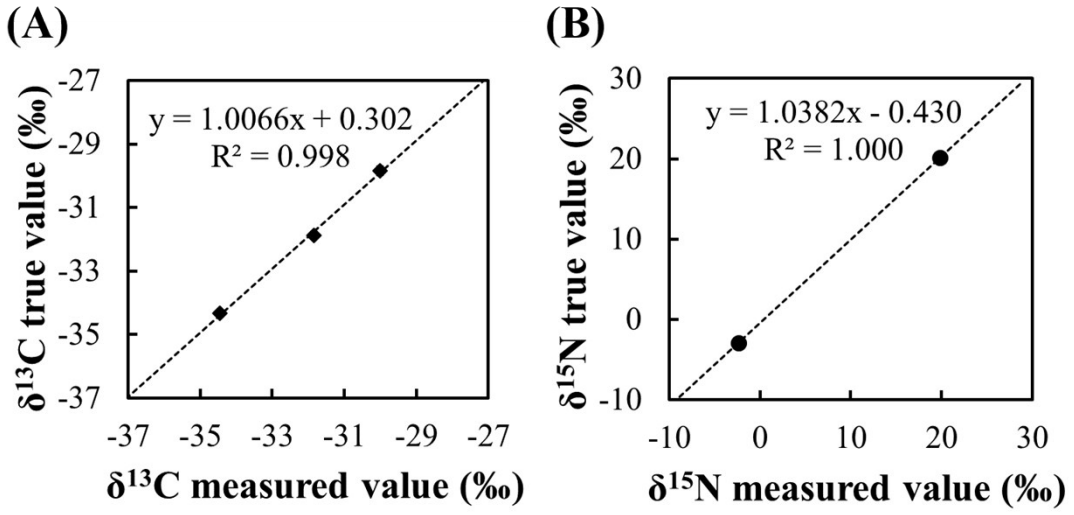

Figure S-IV. Two-point calibration curves of (A) $\delta^{13}C$ and (B) $\delta^{15}N$.

Supporting information V

Mass balance equation:

$$N_{[morphine]}\delta^{13}C_{[morphine]} + N_{[bis(trimethylsilyl)]}\ \delta^{13}C_{[bis(trimethylsilyl)]} =$$

$$N_{[bis(trimethylsilyl)morphine]}\delta^{13}C_{[(bis(trimethylsilyl)morphine]}$$

$N_{[morphine]}$ is the carbon number of morphine; $N_{[bis(trimethylsilyl)]}$ is the carbon number of bis(trimethylsilyl) groups; $N_{[bis(trimethylsilyl)morphine]}$ is the carbon number of bis(trimethylsilyl)morphine; $\delta^{13}C_{[morphine]}$ is the isotope ratio of carbon for morphine; $\delta^{13}C_{[bis(trimethylsilyl)]}$ is the isotope ratio of carbon for bis(trimethylsilyl) groups; and $\delta^{13}C_{[(bis(trimethylsilyl)morphine]}$ is the isotope ratio of carbon for bis(trimethylsilyl)morphine.

Supporting information VI

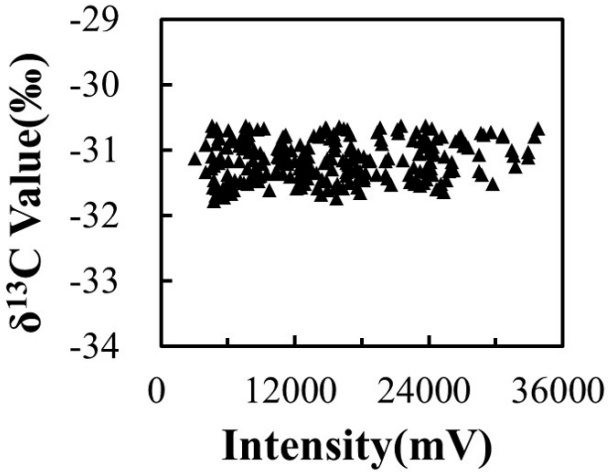

Figure 2. $\delta^{13}C$ of morphine acquired from $\delta^{13}C$ of bis(trimethylsilyl)morphine for evaluating matrix effect and amount-independent certainty in ultrapure water and 15 volunteers' urine samples based on the mass balance equation.