APPENDIX

A) *Quantitation of serum ethylamine concentration*

1. Chemicals and reagents

The reference standards of ethylamine hydrochloride (purity 99.8%) and ethyl-2,2,2-d3-amine hydrochloride (purity 99.3%) that were used as internal standards (IS) were purchased from Nacalai Tesque Inc. (Kyoto, Japan) and C/D/N Isotopes Inc. (Quebec, Canada), respectively. Methanol (LC/MS grade), acetonitrile (LC/MS grade), ammonium formate (analytical grade), sodium hydrogen carbonate (analytical grade), formic acid (analytical grade), and acetone (analytical grade) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Dansyl chloride (analytical grade) was purchased from Kanto Kagaku Co. (Tokyo). Ultrapure water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2. Instruments and conditions

All chromatographic measurements were performed using a Shimadzu Triple Quadrupole LCMS-8050 system (Shimadzu, Kyoto, Japan) equipped with a system controller (CBM-20A), two pumps (LC-30AD), a vacuum degasser (DGU-20A5R), an autosampler (SIL-30ACMP), and a column oven (CTO-20AC). The LabSolutions

LCMS version 5.82 SP1 software controlled the LC-MS/MS system and processed the data. Chromatographic separations were performed on a Waters ACQUITY UPLC HSS T3 column (50 mm×2.1 mm i.d., 1.8 µm) connected to a Waters ACQUITY UPLC HSS T3 VanGuard Pre-Column (5 mm×2.1 mm i.d., 1.8 µm) at a column temperature of 30 °C. The mobile phases consisted of (A) 10 mM ammonium formate in water and (B) 0.1% (v/v) formic acid in acetonitrile. The gradient elution was as follows: 0-2.20 min, 30-65% B; 2.20-2.21 min, 65-95% B; 2.21-2.70 min, 95% B; 2.70-2.71 min, 95-30% B; and 2.71-2.80 min, 30% B. The retention times of dansyl-ethylamine and dansyl-IS were both 2.0 min, using a flow rate of 0.7 mL/min. The autosampler tray was maintained at 4 °C. The electrospray ionization source was operated in the positive ion mode. Source parameters were as follows: nebulizer gas (nitrogen gas) flow, 3 L/min; heating gas (air) flow, 10 L/min; drying gas (nitrogen gas) flow, 10 L/min; interface temperature, 400 °C; heat block temperature, 400 °C; and desolvation line temperature, 250 °C. Argon gas was used for the collision-induced dissociation at 270 kPa. The mass transitions used for multiple reaction monitoring were *m/z* 279 > 156 for dansyl-ethylamine, and *m/z* 282 > 156 for dansyl-IS. The compound-dependent parameters of voltage potential Q1 PreBias, collision energy, and Q3 PreBias were −20, −38, and −28 V for dansyl-ethylamine and −21, −37, and −27 V for dansyl-IS. The

dwell time for each transition was 50 ms and the mass resolutions of Q1 and Q3 were set at high.

3. Preparation of standards

The stock solutions of ethylamine and IS (1 mg/mL) were prepared in water. The stock solution of ethylamine was diluted with water to yield working solutions with serial concentrations of 2, 5, 10, 25, 50, 125, 200, and 250 ng/mL for calibration standards and quality control samples. The IS working solution (20 ng/mL) was prepared in water from 1 mg/mL of the stock solution. All solutions were stored at 4 °C and brought to room temperature before use.

4. Preparation of calibration standards and quality control samples

The calibration standards and quality control samples were prepared by adding 10 μL of their respective solvent working solutions to 50 μL of water. Calibration standards were prepared at concentrations of 0.4, 1, 2, 5, 10, 25, 40, and 50 ng/mL. The peak area ratios of dansyl-ethylamine to the dansyl-IS (y) were plotted versus the concentrations of ethylamine (ng/mL) (x) with a weighting factor of $1/x^2$, and the obtained regression lines were used as the calibration curves. Quality control samples were prepared at 1

ng/mL (low quality control), 10 ng/mL (middle quality control), and 40 ng/mL (high quality control).

5. Sample preparation

A 50 μL aliquot of serum sample was transferred into a 1.5 mL Eppendorf tube, followed by the sequential addition of 10 μL water, 10 μL IS working solution, and 200 μL methanol. After vortex-mixing for 5 s, the mixtures were centrifuged at 20,000×g for 3 min at 4 °C. A 150 μL aliquot of the supernatant was transferred into a new Eppendorf tube, supplemented with 75 μL of 0.1 M aqueous sodium hydrogen carbonate solution, and derivatized with 150 μL of 2 mg/mL dansyl chloride acetone solution at 65 °C for 30 min. The supernatant was collected and transferred to an analytical vial after cooling to laboratory temperature. A 1 μL aliquot of the solution was injected into the LC-MS/MS system.