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Assay of plasma semicarbazide-sensitive amine oxidase and determination of its endogenous substrate methylamine by liquid chromatography

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Abstract

Semicarbazide-sensitive amine oxidase (SSAO) is present in plasma, as well as in other tissues. Previous studies indicated that SSAO is of important physiological and pathophysiological functions. HPLC methods were developed for the assay of SSAO in plasma, and for the determination of plasma methylamine, an SSAO's endogenous substrate. Benzylamine was used as artificial substrate for the enzyme activity assay of SSAO. A 0.2-ml aliquot of plasma was incubated with benzylamine at 37 °C for 30 min. Benzaldehyde, the enzymatic reaction product, was derivatized with 2,4-dinitrophenylhydrazine (DNPH), and analyzed with HPLC and UV detection. SSAO enzyme activity is defined as benzaldehyde (nmol) formed per ml plasma per hour. Recoveries of benzaldehyde spiked to plasma were between 63.5 and 68.2% with relative standard deviation less than 3%. To determine methylamine in plasma, a 0.1-ml aliquot of plasma was deproteinized by trichloroacetic acid and centrifugation. The supernatant was derivatized with dansyl chloride and analyzed by HPLC with fluorescence detection. Recoveries of spiked methylamine at ppb (ng/ml) level were between 93.7 and 97.6% with relative standard deviation less than 2.5%.

Keywords: Semicarbazide-sensitive amine oxide; Methylamine; Enzymes

1. Introduction

Semicarbazide-sensitive amine oxidase (SSAO, EC 1.4.3.6) is present in many tissues and also circulating in the blood plasma. SSAO is a group of copper and quinone-containing amine oxidases which catalyze oxidative deamination reaction of amine compounds and is sensitive to semicarbazide [1–3]. Methylamine, existing in plasma, is one of the endogenous substrate for SSAO and is deaminated and oxidized into formaldehyde, hydroperoxide and ammonia. These enzymatic products are potentially toxic and have been implicated to vascular disorders and diabetic vascular complications [4–15]. Studies also indicated SSAO is expressed in adipocytes and could be involved in the regulation of glucose uptake [16,17]. SSAO has also been found to be identical to another protein called vascular adhesion protein-1 (VAP-1) [1,18]. The expression of VAP-1 located on the plasma membrane of endothelial cells and other tissues might contribute to the recruitment of lymphocytes [19–22].

To further study the physiological and pathophysiological functions and mechanism of SSAO/VAP-1, researchers would be interested in methods for assay of the enzyme activity and for quantitative determination of plasma methylamine, an endogenous substrate of SSAO, and plasma formaldehyde, the oxidative deamination product of methylamine. Quantitative HPLC methods had been reported for the determination of plasma formaldehyde [23,24]. Radiometric procedure using ^{14}C-benzylamine as substrate has been reported for human serum SSAO activity assay [25]. HPLC methods with fluorescence detection have also been reported for human plasma monoamine oxidase (MAO) and SSAO activity assay [26,27]. An HPLC method for the SSAO activity toward aminoacetone-one of the SSAO's endogenous

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substrates has also been reported [28]. Very few methodologies are available for the quantitative determination of blood plasma methylamine [29]. In this article, an assay method for human plasma SSAO activity is described. In the method, benzylamine was used as the substrate, and benzaldehyde was reacted with 2,4-dinitrophenylhydrazine, and analyzed with HPLC and UV detection. A method for the quantitative determination of human plasma methylamine, an endogenous substrate of plasma SSAO with HPLC and fluorescence detection is also described.

2. Experimental

2.1. Materials

N-Methyl-*N*-propargyl-3-(2,4-dichloro-phenoxy)propylamine hydrochloride (clorgyline, an typical inhibitor for monoamine oxidase A, and at the higher concentration, also inhibiting monoamine oxidase B), benzylamine chloride, benzaldehyde, 2,4-dinitrophenylhydrazine (DNPH), dansyl chloride, methylamine chloride were purchased from Sigma (St. Louis, MO, USA). Acetonitrile and methanol were HPLC grade and purchased from Merck KGaA (Darmstadt, Germany), and water was Milli-Q deionized water. Trichloroacetic acid (TCA) and other reagents were analytical grade. Human blood plasma obtained from the local blood station was stored at −70 °C.

2.2. Preparation of reagents and standards

(a) Phosphate buffer solution (PBS) was prepared by dissolving 3.22 g Na_2HPO_4 and 0.136 g KH_2PO_4 in about 90 ml deionized water and adjusting pH to 7.8 with phosphoric acid and NaOH then calibrating to 100 ml with deionized water. (b) Benzylamine (1 mM) was prepared with PBS. (c) DNPH (3 mg/ml) was prepared with 2 N hydrochloric acid. (d) Clorgyline (1 mM) was prepared with PBS. (e) TCA (20%) was prepared with 5 mM $NaHSO_4$ water solution. (f) Formic acid (0.1%) was prepared with deionized water. (g) Benzaldehyde (8 mM) was prepared in methanol and stored at −20 °C, and diluted to target concentrations with methanol daily to prepare calibration working standards. (h) Methylamine (1 mg/ml) was prepared with deionized water and stored at 4 °C, and diluted to target concentrations with deionized water daily to prepare calibration working standards. (i) Dansyl chloride (2 mg/ml) was prepared with acetonitrile and stored at −20 °C.

2.3. Sample preparation procedure for SSAO enzyme activity assay

An aliquot of 0.2 ml human blood plasma was pipetted into a 1.5-ml eppendorf tube, to which 20 μl clorgyline (1 mM) was added. The tube was vortexed for 1 min and incubated at room temperature for 30 min. An aliquot of 180 μl benzylamine (1 mM) was added to the tube which was vortexed for 1 min, incubated in a 37 °C water bath for 1 h. Then 100 μl TCA (20%) was immediately added to the tube, which was vortexed for 1 min, centrifuged at 12,000 × *g* for 5 min. An aliquot of 300 μl of the supernatant was pipetted to a 5-ml glass test tube with cap, to which 50 μl DNPH (3 mg/ml) was added. The tube was vortexed for 1 min and incubated in a 40 °C water bath for 15 min. After cooling to room temperature, the content in the tube was extracted with 1 ml ethyl acetate twice. The combined ethyl acetate extract was evaporated in a dry heater at 45 °C to dryness with a stream of nitrogen. The residue was dissolved in 0.5 ml acetonitrile–0.1% formic acid (50:50, v/v), ready for HPLC analysis.

2.4. Sample preparation procedure for the determination of plasma methylamine

An aliquot of 0.1 ml human blood plasma was pipetted into a 1.5-ml eppendorf tube, to which 20 μl TCA (20%) was added while vortexing. The tube was vortexed for 1 min, centrifuged at 12,000 × *g* for 10 min. An aliquot of 60 μl of the supernatant was pipetted to a 1.5-ml eppendorf tube, to which 90 μl $Na_2B_4O_7$ (0.1 M) and 50 μl dansyl chloride (2 mg/ml) were added sequently. The tube was vortexed for 30 s and heated in a 50 °C water bath for 10 min. After cooling to room temperature, 100 μl mobile phase [acetonitrile–0.05 M ammonia acetate and 1% acetic acid in water (45:55, v/v)] was added to the tube which was then vortexed for 30 s, ready for HPLC analysis.

2.5. Liquid chromatographic analysis for SSAO enzyme activity assay

The high performance liquid chromatographic (HPLC) system was an HP 1100 HPLC (Hewlett Packard, USA), which consisted of a pump, an autosampler, a column chamber, a diode array (DAD), UV–vis detector, and an HP ChemStation for LC system. The column chamber temperature was set at 45 °C and the sample injection volume was set at 5 μl. The detection wavelength of the DAD detector was set at 382 nm. The mobile phase was acetonitrile–0.01 M formic acid in water (62:38, v/v) with a flow rate of 1 ml/min. The HPLC column was an HP Zorbax StableBondSB-C18, 5 μm, 150 mm × 4.6 mm column (Germany). The peak area was used for quantitative calculation.

2.6. Liquid chromatographic analysis for plasma methylamine

The same HPLC system was used. A fluorescence detector was used instead of the DAD detector. The column chamber temperature was set at 30 °C and the sample injection volume was set at 10 μl. The excitation wavelength and the emission wavelength of the fluorescence detector were set at 350 and 530 nm, respectively. The mobile phase was acetonitrile–0.05 M ammonia acetate and 1% acetic acid in

water (45:55, v/v) with a flow rate of 1 ml/min. After each sample, the column was washed with 95% methanol in water for 6 min, and reconditioned with mobile phase for 8 min before analyzing the next sample. The HPLC column was an HP Zorbax StableBondSB-C18, 5 μm, 150 mm × 4.6 mm column (Germany). The peak area was used for quantitative calculation.

2.7. Calibration for SSAO enzyme activity assay

To prepare a calibration curve, benzaldehyde stock standard solution (8 mM) was diluted with methanol to prepare a series calibration working standards, 5, 10, 20, 40, 80, 120, 160, and 200 nM, respectively. An aliquot of 50 μl calibration working standard, 300 μl PBS, and 50 μl DNPH (3 mg/ml) were sequently added to a 5-ml glass test tube with cap. The tube was vortexed for 1 min and incubated in a 40 °C water bath for 15 min. After cooling to room temperature, the content in the tube was extracted with 1 ml ethyl acetate twice. The combined ethyl acetate extract was evaporated in a dry heater at 45 °C to dryness with a stream of nitrogen. The residue was dissolved in 0.5 ml acetonitrile–0.1% formic acid (50:50, v/v), ready for HPLC analysis. For daily calibration, a one-point standard (80 nM) was run in duplicate and used for the calculation of the samples.

2.8. Validation of the procedure for the quantitative determination of benzaldehyde formed by the action of plasma SSAO

Aliquots of 0.2 ml human blood plasma were pipetted into 1.5-ml eppendorf tubes, to each tube 20 μl clorgyline (0.31 mg/ml) was added. The tubes were vortexed for 1 min and incubated at room temperature for 30 min. An aliquot of 130 μl PBS was added to each tube which was vortexed for 1 min, incubated in a 37 °C water bath for 1 h. After cooling to room temperature, aliquots of 50 μl benzaldehyde standard solutions with different concentration (0.02, 0.04, 0.08 mM) were spiked to the tubes, respectively. The tubes were vortexed for 1 min. An aliquot of 100 μl TCA (20%) was immediately added to each tube, which was vortexed for 1 min, centrifuged at 12,000 × *g* for 5 min. An aliquot of 300 μl of the supernatant was pipetted from each tube to 5-ml glass test tubes with caps, to each tube 50 μl DNPH (3 mg/ml) was added. The tubes were vortexed for 1 min and incubated in a 40 °C water bath for 15 min. After cooling to room temperature, the content of each tube was extracted with 1 ml ethyl acetate twice. The combined ethyl acetate extract was evaporated in a dry heater at 45 °C to dryness with a stream of nitrogen. The residue in each tube was dissolved in 0.5 ml acetonitrile–0.1% formic acid (50:50, v/v), ready for HPLC analysis. Recoveries of the spiked benzaldehyde was calculated based on the results determined by the HPLC method and the amounts of benzaldehyde actually spiked to the samples.

2.9. Calibration for determination of plasma methylamine

A series calibration working standards of methylamine, 10, 20, 50, 100, and 200 ng/ml were prepared by diluting the methylamine stock standard (1 mg/ml) with deionized water. An aliquot of 20 μl calibration working standard, 80 μl deionized water, 50 μl $Na_2B_4O_7$ (0.1 M), and 50 μl dansyl chloride (2 mg/ml) were sequently added to a 1.5-ml eppendorf tube. The tube was vortexed for 30 s and heated in a 50 °C water bath for 10 min. After cooling to room temperature, 100 μl mobile phase [acetonitrile–0.05 M ammonia acetate and 1% acetic acid in water (45:55, v/v)] was added to the tube which was then vortexed for 30 s, ready for HPLC analysis. For daily calibration, a three-point (20, 100, 200 ng/ml) standard calibration curve was prepared and used for the calculation of the samples.

2.10. Validation of the procedure for the quantitative determination of plasma methylamine

Aliquots of 950 μl human blood plasma were pipetted into 1.5-ml eppendorf tubes, and 50 μl deionized water, or 50 μl methylamine standard solutions with different concentration (1, 2, 4 μg/ml) were spiked to the tubes, respectively. After vortexing for 1 min, these samples were analyzed followed the above described procedure for the determination of methylamine in blood plasma. Recoveries of spiked methylamine were calculated based on the analytical results and the actual amounts of methylamine spiked into the blood plasma.

2.11. Confirmation of the identity of the methylamine dansyl chloride derivative

Fraction of chromatographic peak of the methylamine dansyl chloride derivative was collected from several injections of human blood plasma samples. Solvent was evaporated under vacuum and residue was dissolved in a small volume of solvent (50% methanol in water containing 0.1% ammonia acetate), ready for LC/ESI/MS/MS analysis. The LC/MS system was composed of an HP 1100 HPLC (Hewlett Packard, USA), linked to a Q Trap MS/MS Spectrometer (Applied Biosystem, Canada) equipped with an ESI ion source. A Q1 scan (single MS) was done to identify the molecular ion (+265.2), and after fragmentation a MS spectrum of the molecular ion was obtained by product ion scan. A methylamine standard derivative was used for comparison.

2.12. Application of the SSAO assay procedure and the methylamine analytical method to human plasma samples

Blood plasma samples were collected from more than 320 healthy human subjects and patients with cardiovascular diseases and/or mellitus diabetes from the local hospital. The

plasma SSAO activity was assayed and methylamine was analyzed with the above described methodologies.

3. Results and discussion

3.1. Sample preparation and HPLC analysis for SSAO enzyme activity assay

Benzylamine was used as an artificial substrate for the enzyme activity assay of SSAO. By the action of SSAO, benzylamine is deaminated and oxidized into benzaldehyde, hydroperoxide and ammonia. Plasma SSAO enzyme activity is defined as benzaldehyde (nmol) formed per ml plasma per hour. Clorgyline was added to plasma before adding benzylamine to inhibit the enzyme activity of monoamine oxidases A and B.

A typical chromatogram of an assay sample is depicted in Fig. 1A. The SSAO activity was inhibited by adding semicarbazide to the plasma as indicated in Fig. 1B. A calibration curve was prepared as described in Section 2.1 and its linearity was tested with a statistical software. The regression equation of the calibration curve was $y = a_1x + a_0$ with a linear regression coefficient (γ) of 0.9996, where x is the peak area count of the UV absorbance of the benzaldehyde DNPH derivative and y is the concentration (nM) of benzaldehyde standard solutions (Section 2.1) used for preparing the calibration curve. The coefficients a_1 and a_0 were 1.169 and −1.857, respectively. The confident interval ($\alpha = 0.05$) of the intercept (a_0) of the regression equation was calculated to be -1.857 ± 1.937, which includes the point of 0. Therefore, in routine analysis, a one-point calibration could be used instead of a calibration curve. The detection limit for the assay of plasma SSAO activity was estimated to be 0.1 nmol/ml/h based on three times noise level.

3.2. Validation of the procedure for the quantitative determination of benzaldehyde formed by the action of plasma SSAO

In order to validate the procedure for quantitative determination of benzaldehyde formed by the action of plasma SSAO, benzaldehyde standard solution was spiked to the enzymatic reaction mixture (without adding the SSAO's substrate benzylamine) and the recoveries of spiked benzaldehyde and the precision of the analytical procedure were determined. The results of the experiment are summarized in Table 1. Recoveries of benzaldehyde spiked to plasma were between 63.5 and 68.2% with relative standard deviation less than 2.9%. The range of the recoveries of spiked benzaldehyde was narrow although the recoveries were not high. But the precision of the method was very good and the analytical results were very producible as indicated by relatively low within-day and day-to-day variation values.

3.3. Application of the SSAO assay procedure to human plasma samples

The methodology was used to survey the blood plasma SSAO activity of more than 320 health human subjects and patients with cardiovascular diseases and/or mellitus diabetes. The SSAO activities ranged from 0.8

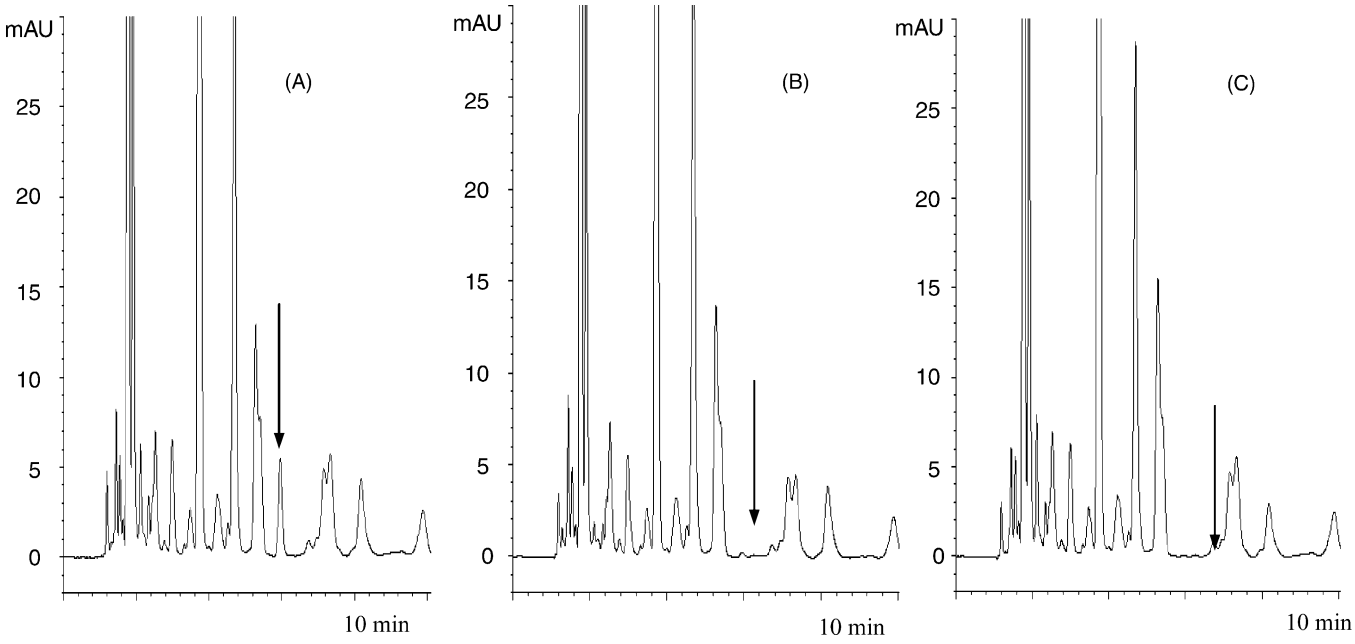

Fig. 1. The chromatogram (A) of a typical blood plasma sample indicated the peak with an arrow of the benzaldehyde DNPH derivative was well-separated from interfering peaks. In the SSAO assay procedure, benzylamine used as substrate was oxidatively deaminated catalyzed by human plasma SSAO to form benzaldehyde. When the specific inhibitor (semicarbazide, 0.3 mg/ml plasma) was added to serum, the SSAO activity was inhibited as indicated the chromatogram (B). The chromatogram (C) indicated the disappearance of the benzaldehyde derivative when the substrate-benzylamine was not added to the serum.

Table 1
Recoveries of benzaldehyde from the spiked enzymatic reaction mixtures

Spike level (mM, 50 μl)	Recovery (%)	*n*	R.S.D. (%)
Within-day			
0.02	68.2	3	0.4
0.04	65.9	3	2.9
0.08	63.5	3	2.5
Day-to-day			
0.02	68.2	3	0.4
0.04	66.6	3	0.02
0.08	65.3	3	0.9

to 61.9 nmol/ml/h. The averaged SSAO activity was 19.4 nmol/ml/h with a standard deviation of 9.2. The described SSAO enzyme activity assay methodology was performing well and reliable.

3.4. Sample preparation and HPLC analysis for methylamine in human blood plasma

Human blood plasma methylamine content was in low ppb (ng/ml) level. Many other amine compounds in human blood plasma render the quantitative determination of methylamine very difficult. Different reagents had been tried to precipitate and remove proteins in plasma and trichloroacetic acid was found suitable for good chromatographic separation and recovery of methylamine. A fluorescent derivative of methylamine was needed for good detection sensitivity. Dansyl chloride was used in this method to serve for the purpose. A typical chromatography of a human blood plasma sample is depicted in Fig. 2, which indicates a well separation of the methylamine dansyl chloride derivative from the complex of interfering peaks. The amount of $Na_2B_4O_7$ (0.1 M) was optimized in order to obtain a proper pH of the reaction mixture.

A calibration curve was prepared as described in Section 2.9 and its regression equation was $y = a_1x + a_0$ with a linear regression coefficient (γ) of 0.9999, where x is the peak area count of the fluorescent derivative of the methylamine and y is the concentration (ng/ml) of methylamine standard solutions (Section 2.9) used for preparing the calibration curve. The coefficients a_1 and a_0 were 16.08 and −2.26, respectively. The confident interval ($\alpha = 0.05$) of the intercept (a_0) of the regression equation was calculated to be -2.26 ± 1.21, which does not include the point of 0 and indicates that the calibration curve did not pass the origin point. In routine analysis, a three-point calibration curve, which covers the concentration range of the samples was prepared routinely and used for calculation. The detection limit was lower than 0.4 ng/ml of methylamine in human blood plasma based on three times noise level of the chromatographic signal.

3.5. Validation of the procedure for the quantitative determination of methylamine in human blood plasma

Control human blood plasma was spiked with methylamine standard solution at different levels and both control and spiked plasma samples were analyzed with the described procedure. Results of spiked recoveries and variations were summarized in Table 2. The data indicate that the analytical method for human plasma methylamine is accurate with good precision and reproducibility.

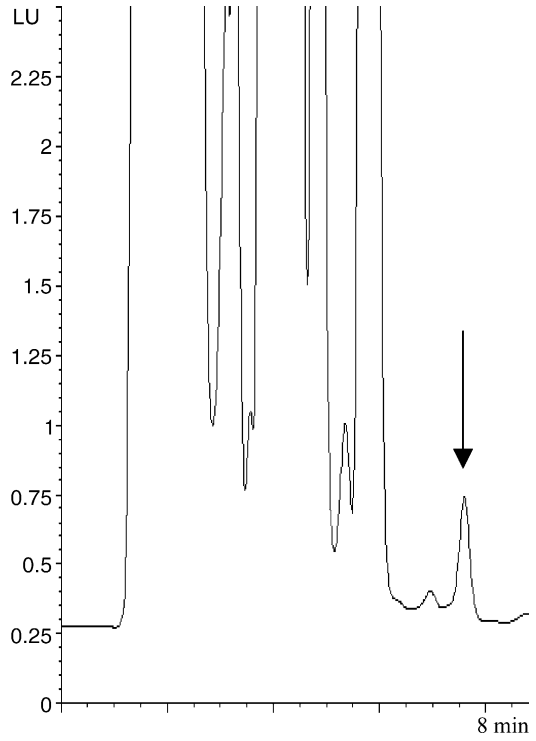

Fig. 2. The chromatogram of a typical blood plasma sample indicated the peak with an arrow of the methylamine dansyl chloride fluorescent derivative was well-separated from interfering peaks.

3.6. Confirmation of the identity of the methylamine dansyl chloride derivative

Methylamine is endogenously present in human blood plasma. Methylamine formed fluorescent derivative with dansyl chloride and analyzed by HPLC with fluorescence detection with very high sensitivity. In this research a tandem mass spectrometry method was used to confirm the identity of the methylamine derivative. The molecular weight of the derivative was 264.2 based on the chemical structure and the molecular ion had an *m*/*z* of 265.2. The molecular ion selected

Table 2
Recoveries of methylamine from the spiked human blood plasma

Spike level (μg/ml, 50 μl)	Recovery (%)	*n*	R.S.D. (%)
Within-day			
2	97.6	3	1.9
4	97.5	3	2.1
8	95.5	3	1.1
Day-to-day			
2	94.3	3	0.3
4	95.3	3	1.0
8	93.7	3	2.4

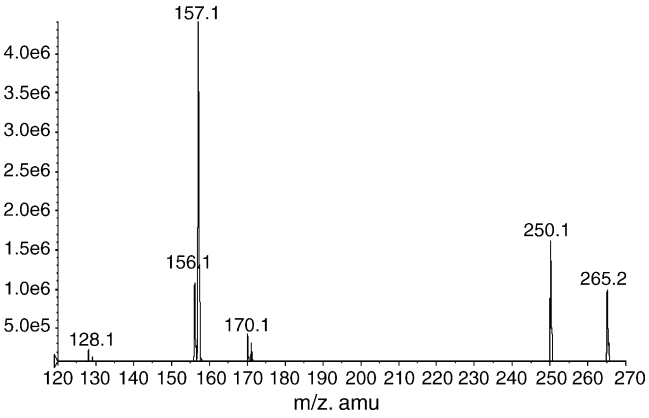

Fig. 3. The mass spectrum of the molecular ion (+265.2) of the methylamine dansyl chloride fluorescent derivative was used to confirm the identity of the endogenous human blood plasma methylamine.

by the Q1 mass analyzer of the tandem mass spectrometer was fragmented in the Q2 collision cell, and the mass spectrum of the fragments was scanned by the Q3 mass analyzer. The chromatographic peak collected from the human blood plasma sample showed the same characteristic mass spectrum with the derivative prepared from methylamine standard compound. The characteristic mass spectrum of the fragmented molecular ion (+265.2) is depicted in Fig. 3.

3.7. Application of the methylamine analytical method to human plasma samples

The methodology was used to analyze blood plasma methylamine of 320 health human subjects and patients with cardiovascular diseases and/or mellitus diabetes. The plasma methylamine content ranged from 11.5 to 123.4 ng/ml. The averaged plasma methylamine content was 31.8 ng/ml with a standard deviation of 14.8. These values of plasma methylamine agree well with those reported by Baba et al. [29]. The described methodology proved to be suitable for the quantitative determination of plasma methylamine with high throughput.

4. Conclusion

A well-optimized method for the assay of human blood plasma SSAO activity was developed, and a rapid and straightforward method was developed for the quantitative determination of endogenous methylamine in human blood plasma. Both methods had been successfully applied to a survey of healthy human subjects and patients with cardiovascular diseases and/or mellitus diabetes.

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