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〈Original Article〉

Development of an enzymatic assay for ethanolamine in plasma

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Summary Ethanolamine (Etn) contributes to the generation of phosphatidyl ethanolamine (PE) *in vivo*. However, its plasma concentration and clinical significance in humans are still unclear. Therefore, we developed a simple, rapid enzymatic method using an amine oxidase and copper from *Arthrobacter* sp. (AAO) (EC 1.4.3.6). We found a strong correlation between the values obtained with this method and those obtained with the HPLC (high performance liquid chromatography)-based method (r = 0.89). Thus, it is suitable for routine clinical use in the laboratory. Moreover, we would like to examine the clinical significance of Etn.

Key words: Ethanolamine, Amine oxidase, Enzymatic assay

1. Introduction

Ethanolamine (Etn) contributes to the generation of phosphatidyl ethanolamine (PE), a membrane phosphatide. A study reported that urine Etn was increased in newborns with Zellweger syndrome, a congenital metabolic disease.[1] And more, recently, a metabolomics study, using mass spectrometry, reported that the saliva Etn of pancreatic cancer patients was significantly increased compared to that of normal.[2] However, these have not yet become clear clinically.

Baba *et al.*[3] reported that the reference interval of Etn in healthy volunteer's serum is 11.84 ± 4.15 μmol/L. However, the clinical significance of Etn in adult blood was not clarified.

Etn is measured conventionally by high performance liquid chromatography (HPLC)[4] and capillary electrophoresis-mass spectrometry. These methods, however, have drawbacks including complicated preprocessing operations, and wasting of measuring time and introduction and maintenance costs. Therefore, the aim of this study was to develop a simple, rapid enzymatic method to measure Etn in the blood, using an amine oxidase and copper from *Arthrobacter* sp. (AAO) (EC 1.4.3.6).

2. Materials and Methods

2.1. Method principle

Figure 1 shows the reaction sequence of the

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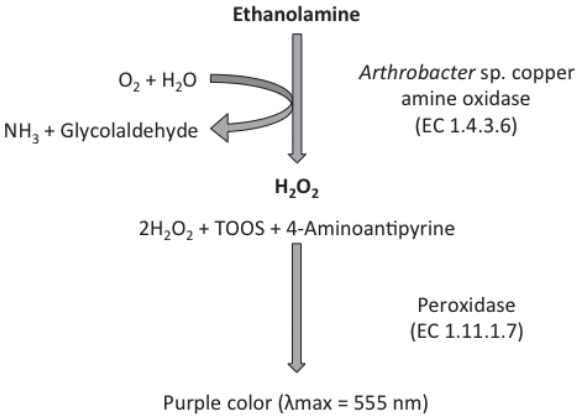

Fig. 1 Principles of the measurement method for plasma ethanolamine (Etn). Etn is acted on by AAO, and produced H_2O_2, TOOS and 4-AA generate a quinoneimine derivative by POD. The generation is measured at 555 nm as it is proportional to the plasma EA concentration.

present method. AAO acts on Etn, and produces H_2O_2, 4-aminoantipyrine (4-AA), and *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline (TOOS), which then reacts with peroxidase (POD) to generate a quinoneimine derivative. The colored product is measured at 555 nm, which is proportional to the concentration of Etn in the plasma.

2.2. Instrumentation and reagents

Instruments. For the assay, we used a Hitachi 7600 type automated analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan). For the HPLC method, we used the LaChrom Elite System (Hitachi High-Technologies Corporation, Tokyo, Japan) equipped with an auto-sampler (L-2200), a pump (L-2130), and a fluorescence detector (L-2480). The column used was a multi-mode type column Asahipak GS-220 HQ (7.5 mm ID × 300 mm, Showa Denko K.K., Tokyo, Japan).

Reagents. AAO (EC 1.4.3.6), choline oxidase (COD, EC 1.1.3.17), POD (EC 1.11.1.7), and ascorbic acid oxidase (ASOM, EC 1.10.3.3) were obtained from Asahi Kasei Pharma (Tokyo, Japan). 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) and TOOS were obtained from Doujin Laboratories (Kumamoto, Japan). TritonX-405 was obtained from Sigma-Aldrich Japan Co. (Tokyo, Japan). 4-AA and sodium azide were obtained from Nacalai Tesuque, Inc. (Kyoto, Japan). Other reagents were of analytical grade (Wako Pure Chemical Industries, Ltd., Osaka, Japan). A three-reagent system was used in the assay. Reagent 1 (R-1) contained 0.1 mol/L of HEPES buffer (pH 8.0 at 25 °C), 50 μmol/L of choline chloride, and 25 mmol/L of guanidine hydrochloride. Reagent 2 (R-2) contained 0.1 mol/L of HEPES buffer (pH 8.0 at 25 °C) 6.0 mmol/L of TOOS, 10 kU/L of POD, 20 kU/L of ASOM, 0.2% (v/v) TritonX-405, 8.0 kU/L of COD, and 1200 kU/L of catalase (EC 1.11.1.6). Reagent 3 (R-3) consisted of 0.1 mol/L of HEPES buffer (pH 8.0 at 25 °C), 0.30 mmol/L of 4-AA, 30 kU/L of AAO, 0.1% (v/v) TritonX-405, 90 μmol/L of potassium ferrocyranide, and 0.05% (w/v) NaN_3. The Etn standard solution for the calibrator (30 μmol/L) was diluted with deionized water. We arranged Laus's and Hokazono's HPLC method.[5, 6] For the assay condition in the HPLC method, the mobile phase contained an eluent (0.1 mol/L boric acid/sodium hydroxide buffer (pH 9.0 at 25 °C) and methanol (4:1, v/v)). The derivative solution contained 0.1 mol/L of boric acid/sodium hydroxide buffer (pH 9.0 at 25 °C) and methanol (1:1, v/v) that included 10.0 mmol/L of *o*-phthalaldehyde and 30.0 of mmol/L *N*-acetylcystein (NAC).

2.3. Procedure

Analytical conditions. The analytical conditions for the automated analyzer were as follows: a 30 μL specimen was mixed with 90 μL of R-1. After incubation for 1.5 min at 37 °C, 90 μL R-2 was added. After incubation for 3.5 min at 37 °C, 90 μL of R-3 was added. After another 5 min, the mixture was measured using a two-point end assay performed at 37 °C, with wavelengths of 800/546 nm (sub/main wavelength). Steps for the HPLC method were as follows: Etn in plasma was first isolated at a 1.0 mL/min flow rate, column temperature of 25 °C, and an injection volume of 40 μL (diluted 10-fold with saline). After separation, we changed the flow rate to 0.7 mL/min and the derivative solution was sent with another pump. The fluorescence of the

derivation compound was then detected at a wavelength of 350/450 nm (excitation/emission).

3. Results

3.1. Reproducibility

Table 1 shows the within-run (n = 20) and between-day (n = 10) variations of data for the two-level solutions (S1, S2; respectively), which were 3.13%, 1.38% and 5.47%, 1.43%, respectively.

3.2. Linearity studies

Figure 2 shows the linearity studies. Etn showed linearity at ranges of 0 – 50 μmol/L.

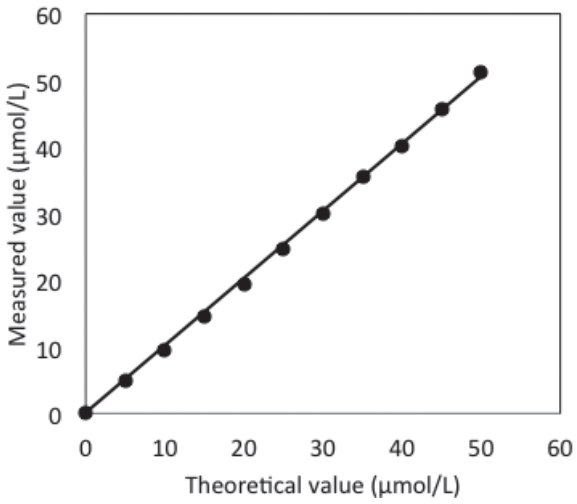

Fig. 2 Linearity of detection for Etn.

3.3. Limit of determination

The limit of detection was estimated by using Etn solutions that had been successively diluted with deionized water to 10 levels until reaching zero concentration. The detection limit of present method, defined as the Etn concentration for the zero concentration (limit of absence) of ± 3 standard deviations, was 1.5 μmol/L.

3.4. Interference studies

Figure 3 shows the interference studies. Various materials in plasma that might interfere with this assay were added to the pooled plasma (1:9 volume) and measured. For the interfering substances (conjugated and unconjugated bilirubin, hemoglobin and formazin), the Interference Check-A Plus kit (Sysmex Co., Kobe, Japan) was used. The grade of interference was estimated as a percentage of the interference rate: interference rate (%) = (Etn value of the added interference substances − Etn value in the reference sample [without interference materials])/Etn value in the reference sample × 100. In the samples added with 36.4 μmol/L of conjugated bilirubin and 32.3 μmol/L of unconjugated bilirubin, their rates were −13.1% and −8.2%, respectively. In the samples added with 0.5 g/L of hemoglobin and 1,410 FTU of turbidity, their rates were 15.2% and −4.8%, respectively.

3.5. Recovery test

Nine volumes of pooled EDTA plasma and one volume of 50 or 150 μmol/L Etn solution were mixed. An analytical recovery test was conducted using these samples with two different levels (approximately 5 or 15 μmol/L). The average analytical recovery of the triplicate assays was 91.1% and 92.2%, respectively.

Table 1. Within-run and between-day reproducibility

	Within-run (n=20)		Between-day (n=10)	
Sample	S1	S2	S1	S2
Mean* (μmol/L)	9.01	29.94	9.86	30.02
SD (μmol/L)	0.28	0.41	0.54	0.43
CV (%)	3.13	1.38	5.47	1.43

* Mean of triplicate measurements

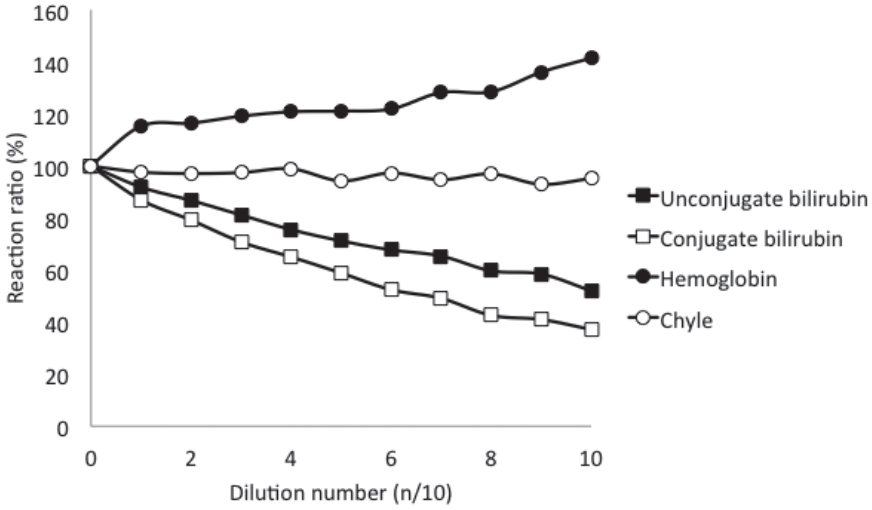

Fig. 3 Interference studies. Each level of maximum dilution number (10/10): Unconjugete bilirubin; 323 μmol/L, Conjugate bilirubin; 364 μmol/L, Hemoglobin; 5.0 g/L, Chyle; 1,410 FTU (formazine turbidity units)

3.6. Effect of having COD and choline for bilirubin

Figure 4 shows a difference by having preprocessing or without COD and choline. The present method was better than without COD and choline.

3.7. Correlation with the HPLC method

Correlation was examined between the present method (*y*) and the HPLC method (*x*) using plasma specimens (n = 50) collected from normal volunteers. In the regression analysis, the Deming method was adopted. The results (Fig. 5-a) indicate a good correlation with the present method. However, the Deming method has problems, such as fixed bias (shown in Fig. 5-b).

4. Discussion

Etn is part of phosphoethanolamine (P-Etn) and PE. In the pathway for PE synthesis in the liver, Etn is phosphorylated by ethanolamine kinase (EK) to produce P-Etn and is then converted into CDP-ethanolamine[7]. Finally, PE is produced from CDP-ethanolamine by ethanolaminephosphotransferase (EPT). Etn has an important role in cell proliferation because PE participates in the promotion of synthetic DNA. It has been reported that the concentration of Etn in the serum of healthy volunteers is, on average, 11.84 ± 4.15 μmol/L.[3] In addition, Sasaki and Kume *et al.* reported that Etn in rats is increased during the proliferative phase and regeneration phase of hepatocytes, and promotes hepatocellular genesis *in vivo*.[8, 9, 10] However, its clinical significance in human blood has not been clarified.

Ota *et al.* reported that a copper amine oxidase from *Arthrobacter* sp. reacted with Etn and produced H_2O_2[11]. Therefore, we developed a rapid, simple enzymatic assay to detect Etn in plasma using AAO.

The oxidative condensation reaction of TOOS, 4-AA and H_2O_2 in the presence of POD is easily affected by the reduction material because POD has an inferior substrate specificity.[12, 13] In fact, there are various reducing substances found in blood: ascorbic acid, bilirubin, and so on. Therefore, we tried to remove ascorbic acid up to 0.57 mmol/L by ascorbate oxidase. However, for bilirubin, we were not able to use bilirubin oxidase (BOD) to remove bilirubin. BOD influenced the measurement of Etn because it reacts with TOOS. Therefore, we reduced the influence of bilirubin using hydrogen peroxide, and added choline and choline oxidase (COD) so that 30 μmol/L of H_2O_2 was formed (at final concentration), in consideration of the quantity of blood bilirubin. From the results, the samples with 36.4

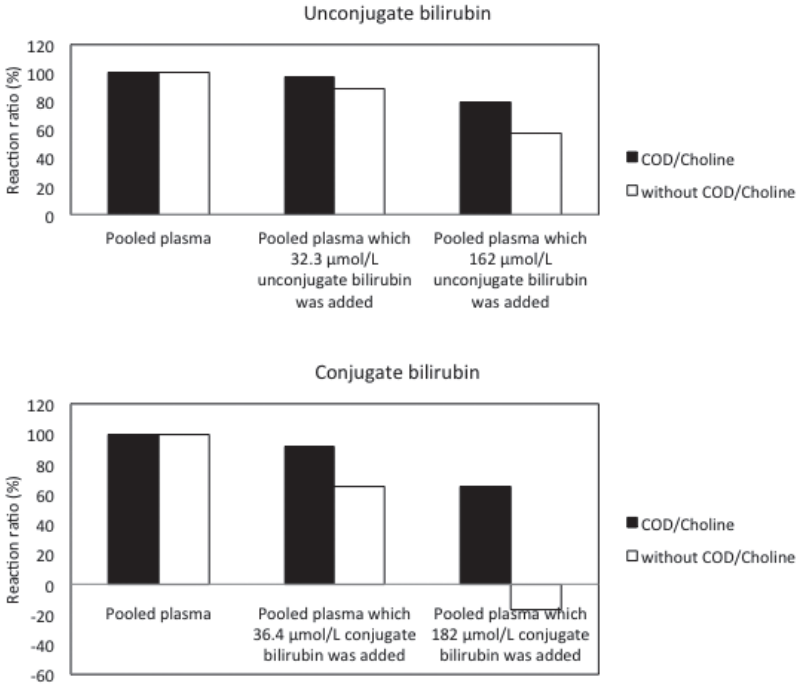

Fig. 4 Effect of having COD and choline for each bilirubin.

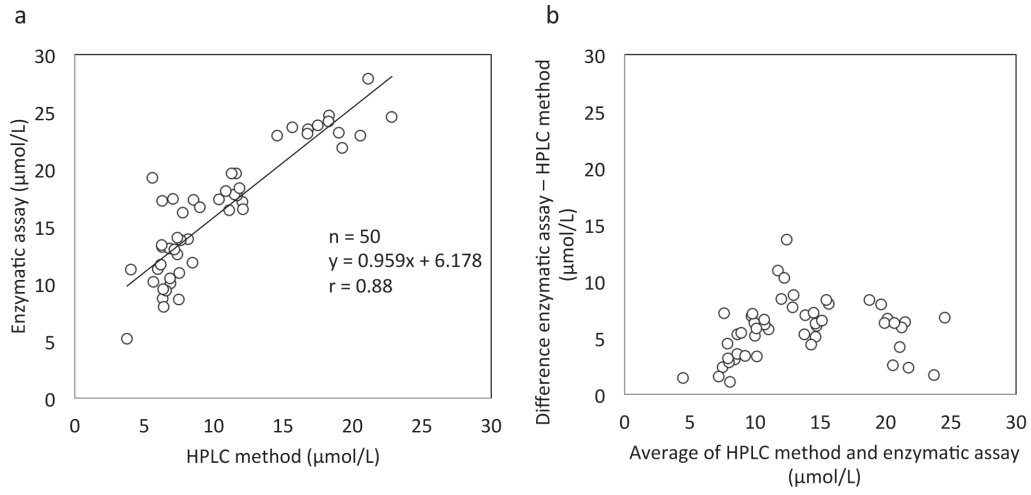

Fig. 5 Correlation study. a; Deming method between the present method (y) and the high performance liquid chromatography (HPLC) method (x) (y = 0.959x + 6.178, r = 0.88, n= 50). b; Bland-Altman graph

μmol/L of conjugated bilirubin and 32.3 μmol/L of unconjugated bilirubin added, had interference rates of −13.1% and −8.2%, respectively. We speculate that Etn is underestimated in a highly concentrated bilirubin sample. The level of bilirubin in a physically unimpaired person is lower than 17.1 μmol/L. Using the present method; the margin of error is approximately −5%. Therefore, in future research, we need to improve the methodology by avoiding the influence of bilirubin.

The validation of our method was good. It is reported that AAO, used in our method, also reacts with tyramine. Tyramine is found in food, but is inactivated by tyramine oxidase in the intestinal wall, and is rarely absorbed in the body. However, when an MAO-I is administered, which is commonly used to treat Parkinson's disease,[14] Etn may show a high plasma value because tyramine is absorbed in

the body. In addition, materials that are substrates of AAO, like tyramine, include dopamine and histamine; however, the amount of dopamine and histamine in the blood were less than 0.64 nmol/L and 67 nmol/L, respectively.[15, 16] Therefore it will estimate that the possibility that those materials affect our present method is extremely low.

We selected the HPLC method to separate and detect Etn directly. We used the post-label method, which labels a purpose ingredient after having separated it in a column. This method is able to remove materials of plasma molecular weight more than 3,000 Da by using a multi-mode column GS-220 HQ, and therefore deproteinization pretreatment is unnecessary.

Then, we measured the plasma processed in AAO to consider whether the peak was the single peak of Etn. Furthermore, there was a strong correlation between the values obtained with the present method and the HPLC method. However, the present method had regular bias of 6 μmol/L toward in the correlation of HPLC method and enzyme method. We examined plasma tyramine by HPLC method, but plasma tyramine was not detected. Therefore, we have to study this bias moreover.

In conclusion, this new accurate enzymatic method for detecting plasma Etn is suitable for routine clinical use in the laboratory. Future research should clarify the clinical significance of Etn.

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