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Enzymatic assay of phosphatidylethanolamine in serum using amine oxidase from *Arthrobacter* sp

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ABSTRACT

Background: In human serum, as for phospholipids not containing choline, phosphatidylethanolamine (PE) exists approximately 5% in a whole phospholipid. PE is well known as one of the main components of biological membranes, and also plays important roles that contribute to apoptosis and cell signaling. However, it could not measure PE with other phospholipids due to a lack of choline in them.
Methods: Using an amine oxidase (EC 1.4.3.6), from *Arthrobacter* species, a simple and rapid enzymatic assay for measurements of PE in serum was established. That assay used the Hitachi 7170 analyzer to evaluate the analytical performance.
Results: The average within-run CVs were 0.38–1.27% (n = 20) at 69–160 μmol/l. The correlation between values obtained with the present method (y) and the high-performance liquid chromatography (HPLC) method (x) was: $y = 0.944x + 9.441$ ($r = 0.977$, $S_{y|x} = 5.82$, n = 34). In addition, the reference interval of healthy subjects was 115 ± 45 μmol/l.
Conclusions: This new enzymatic method shows a high specificity for serum PE and can be easily applied to an automated analyzer. The present method is available as a novel marker of changes in the clinical condition of serum phospholipids.

1. Introduction

There are many kinds of phospholipids in human serum, e.g., phosphatidylcholine (PC), sphingomyelin (SM) and lysophosphatidylcholine (LysoPC); these three account for more than 95% of total phospholipids, and contain choline in their composition [1]. As for phospholipids without them, phosphatidylethanolamine (PE) exists [2] in approximately 5% of total phospholipids. PE is recognized as one of the main components of biological membranes, and also plays important roles in the biological contribution to apoptosis and cell signaling [3].

Most of the phospholipids that clinical laboratory usually measure as total phospholipids comprise the total sum of PC, SM and LysoPC. Phospholipase D (PLD) reacts to each phospholipid, and the choline included in their phospholipids is isolated, and is measured as a total phospholipid. However, PE is not usually included among the total phospholipid values since it has no choline in its composition. The conventional methods of analyzing PE are thin-layer chromatography [4–8] and HPLC [9–12]. When these methods are compared with an automated analyzer at the points of handling a large quantity of samples, they are not so suitable for routine clinical use in the laboratory since they need special costly devices and require preliminary treatment of samples.

Ota et al. previously detected ethanolamine (EA) oxidase activity in *Athrobacter* species, and purified *Arthrobacter* sp. copper amine oxidase (AAO; EC 1.4.3.6) [13]. Therefore, in this paper we report an original, sensitive and specific enzymatic method for serum PE, using AAO with no preliminary treatment. In addition we showed the reference interval for PE in serum.

2. Materials and methods

2.1. Method principle

Fig. 1 shows the reaction sequence in this method. PE was first hydrolyzed by phospholipase D (PLD), and EA was isolated. AAO acted on the isolated EA, and the produced H_2O_2, 4-aminoantipyrine (4-AA), *N*-ethyl-*N*- (2-hydroxy-3-sulfopropyl) -3-methylaniline (TOOS) generate a quinoneimine derivative by peroxidase (POD). The color product was measured at 555 nm as it is proportional to the concentration of PE in serum.

Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcoline; SM, sphingomyelin; LysoPC, lysophosphatidylcholine; PLD, phospholipase D; EA, ethanolamine; AAO, *Arthrobacter* sp. copper amine oxidase.

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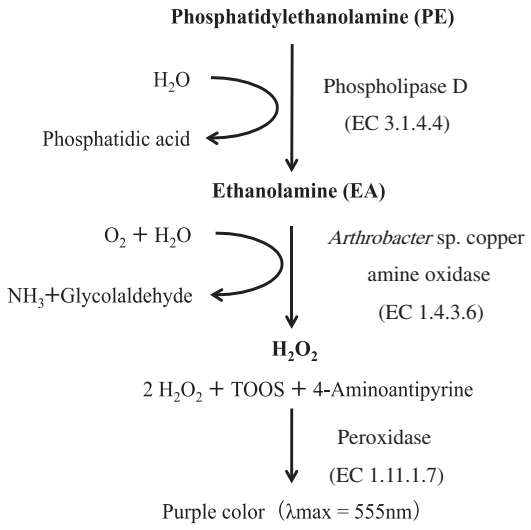

Fig. 1. Principles of the measurement method for serum phosphatidylethanolamine (PE). PE is hydrolyzed by PLD and EA is isolated. AAO acts on the isolated EA, and produced H_2O_2, 4-aminoantipyrine, and TOOS generate a quinoneimine derivative by peroxidase (POD). The generation is measured at 555 nm as it is proportional to the serum PE concentration.

2.2. Instrumentation and reagents

2.2.1. Instruments

The assay used a Hitachi 7170 type 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 detector (L-2140). The column used was a multi-mode type column Asahipak GS-320 HQ (7.5 mm ID × 300 mm l, Showa Denko K.K., Tokyo, Japan).

2.2.2. Reagents

AAO (EC 1.4.3.6), PLD (EC 3.1.4.4), choline oxidase (COD, EC 1.1.3.17) and ascorbic acid oxidase (ASOD, EC 1.10.3.3) were from Asahi Kasei Pharma (Tokyo, Japan). POD (EC 1.11.1.7) was from Oriental Yeast Co., Ltd. (Tokyo, Japan). 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) and TOOS were from Doujin Laboratories (Kumamoto, Japan). TritonX-100 and 2-mercaptethanol were from Sigma-Aldrich Japan Co. (Tokyo, Japan). Other reagents were of analytical grade (Wako Pure Chemical Industries, Ltd., Osaka, Japan). A 2-reagent system was used in this assay. Reagent 1 (R-1) was contained of 0.1 mol/l HEPES (pH 7.5 at 25 °C), 2.97 mmol/l TOOS, 5.0 kU/l POD, 39.6 kU/l AAO, 0.25%(w/v) TritonX-100, 1.15 mmol/l *N*-ethylmaleimide, 17.3 kU/l ASOD, and 7.69 mmol/l NaN_3. When the first reagent (R-1) was mixed with a specimen, endogenous EA in the serum is eliminated by AAO, TOOS and POD. Reagent 2 (R-2) consisted of 0.1 mol/l HEPES (pH 7.5 at 25 °C), 2.64 mmol/l 4-AA, 66.0 kU/l PLD, 0.25%(w/v) TritonX-100, 6.6 mmol/l $CaCl_2$, and 7.69 mmol/l NaN_3. PE standard solution for the calibrator (60 and 180 μmol/l) is prepared L-α-phosphatidylethanolamine added first dissolved in 5%(w/v) TritonX-100 aqueous solution by heating and stirring slowly. After completely dissolving, it was left to cool off naturally, and a 5%(w/v) TritonX-100 solution was added to the target volume. For the assay condition in HPLC method, the mobile phase contains an eluent (0.1 mol/l boric acid/sodium hydroxide buffer (pH 9.5 at 25 °C) and methanol (4:1, v/v)). The derivative solution contains 0.1 mol/l boric acid/sodium hydroxide buffer (pH 9.5 at 25 °C) and methanol (1:1, v/v) that included 20.0 mmol/l *o*-phthalaldehyde and 30.0 mmol/l 2-mercaptoethanol. The preliminary treatment solution was prepared for a measurement of the free EA derived from PE. That solution was contained 0.1 mol/l HEPES (pH 8.0 at 25 °C), 150 kU/l PLD, 3.0%(w/v) TritonX-100 and 20.0 mmol/l $CaCl_2$.

2.3. Preliminary treatment of samples

The samples were prepared for measurements of the free EA derived from PE as follows: a 50 μl preliminary treatment solution was mixed with a 450 μl serum sample, and after reacting for 40 min it was used for measurement sample.

2.4. Specimens

Serum specimens that were normal in biochemical test items (27 items) including the lipids item (Total cholesterol, HDL-cholesterol and LDL-cholesterol) were collected from inpatients and outpatients of the Kyushu University Hospital after receiving informed consent and approval from the institutional ethics committee in Kyushu University.

2.5. Procedure

2.5.1. Analytical conditions

The analytical conditions for the automated analyzer were as follows: a 30 μl specimen was mixed with 200 μl R-1; after incubation for 5 min at 37 °C, 100 μl R-2 was added; after another 5 min, the mixture was measured using a 2-point end assay performed at 37 °C, with wavelengths of 700/546 (sub/main wavelength). Steps for the HPLC method were as follows: EA in serum was first isolated at a 1.0 ml/min flow rate, column temperature of 25 °C, and an injection volume of 60 μl (direct injection). After separation, we changed the flow rate to 0.5 ml/min and the derivative solution was sent with another pump. The absorption of the derivation compound was then detected at a wavelength of 330 nm. The PE concentration was calculated by deducting the endogenous EA in serum from the whole of EA, including the free EA derived from PE.

3. Results

3.1. Reagent compositions, reaction time course and reagent stability

First we examined each enzymatic optimum activity at the following pH; AAO 6.5–7.5, PLD 7.5–8.5, POD 5.0–8.0, and decided to use the Tris–HCl buffer, which had a buffer capacity in the range of pH 7–9 (*p*Ka 8.20, 20 °C). Since it was reported [1] that the PE concentration in serum was about 10^{-5} mol/l, we needed to choose a highly sensitive combination of color reagents. Thus, a pair of color reagent of 4-AA and TOOS was the color reagent chosen (molar absorption coefficient (ε) was $3.92 \times 10^4\ l \cdot mol^{-1} \cdot cm^{-1}$ under the condition of being POD and H_2O_2, at 37 °C [14]), and further determined that the concentrations of 4-AA and TOOS were 0.8 mmol/l and 1.80 mmol/l, respectively. Since the three enzyme reaction steps that were shown in Fig. 1 must be completed within 5 min after R-2 was added, sufficient quantities of AAO, PLD and POD were included in R-2, i.e., 24.0 kU/l, 20.0 mmol/l and 20.0 kU/l in the final reaction mixture, respectively. The linearity with EA solution from step 2 to 3 in Fig. 1 was estimated, and it was found that the linearity of the method was severely strained at ≤200 μmol/l EA. Therefore, the linearity was improved by changing a buffer into HEPES from Tris–HCl (Fig. 2). Thus, the HEPES buffer was used instead of Tris–HCl in subsequent analyses without changing other reagent conditions. However, as the linearity of the low concentration remained slightly strained, two levels of calibrator (60 and 180 μmol/l) were set in consideration for the PE concentration estimated in normal serum. Fig. 3 shows the reaction time course for some samples in the present method. In the final reaction stage, their reactions accomplished to a plateau.