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An enzyme combination assay for serum sphingomyelin: Improved specificity through avoiding the interference with lysophosphatidylcholine

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ABSTRACT

Serum sphingomyelin (SM) has predictive value in the development of atherosclerosis. Furthermore, SM plays important roles in cell membrane structure, signal transduction pathways, and lipid raft formation. A convenient enzymatic method for SM is available for routine laboratory practice, but the enzyme specificity is not sufficient because of nonspecific reactions with lysophosphatidylcholine (LPC). Based on the differential specificity of selected enzymes toward choline-containing phospholipids, a two-step assay for measuring SM was constructed and its performance was evaluated using sera from healthy individuals on a Hitachi 7170 autoanalyzer. Results from this assay were highly correlated with theoretical serum SM concentrations estimated by subtracting phosphatidylcholine (PC) and LPC concentrations from that of total phospholipids determined using previously established methods. There was a good correlation between the results of SM assayed by the proposed method and the existing enzymatic method in sera from healthy individuals. Moreover, the proposed method was superior to the existing method in preventing nonspecific reactions with LPC present in sera. The proposed method does not require any pretreatment, uses 2.5 μl of serum samples, and requires only 10 min on an autoanalyzer. This high-throughput method can measure serum SM with sufficient specificity for clinical purposes and is applicable in routine laboratory practice.

Sphingomyelin (SM), an important lipid component of cell membranes and lipoproteins, consists of a ceramide moiety linked via a phosphodiester bond to phosphorylcholine. SM is believed to play significant roles in cell membranes, including maintenance of membrane structure, participation in signal transduction pathways, and lipid raft formation [1–4].

Clinical and experimental studies have suggested that SM plays an important role in the pathogenesis of coronary artery disease, in which SM accumulates in atheromas via plasma lipoprotein uptake [5]. The serum SM concentration and the ratio of SM to phosphatidylcholine (PC) in serum have been reported to be positively associated with cardiovascular diseases [6]. Plasma SM can be a marker of atherogenic remnant lipoprotein accumulation and an outcome predictor in acute coronary syndrome patients [7], and it is elevated in human familial hyperlipidemia [8] as well as in animal models of atherosclerosis [9].

Abbreviations used: SM, sphingomyelin; PC, phosphatidylcholine; LDL, low-density lipoprotein; SMase, sphingomyelinase; HPLC, high-performance liquid chromatography; LPC, lysophosphatidylcholine; PL, phospholipid; EMSE, *N*-ethyl-*N*-(3-methylphenyl-*N*′-succinylethylenediamine); Pipes, piperazine-*N*,*N*′-bis-(2-ethanesulfonic acid); SD, standard deviation; VLDL, very-low-density lipoprotein; HDL, high-density lipoprotein; CV, coefficient of variation.

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Low-density lipoprotein (LDL) extracted from human atherosclerotic lesions is much richer in SM than LDL from plasma [10]. The SM/PC ratio in lipoproteins appears to be an important factor in their susceptibility to sphingomyelinase (SMase) in vascular endothelium [11]. Thus, high serum SM concentrations and a high SM/PC ratio might be risk indicators for atherosclerosis. Accurate measurement of serum SM is crucial for research into lipid metabolic mechanisms and the diagnosis of atherosclerosis. Classically, serum and plasma SM concentrations have been measured by thin-layer chromatography [12], high-performance liquid chromatography (HPLC) [13], or matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy [14]. None of these methods, however, has been sufficiently validated for use in the clinical laboratory; thus, there is a need for a simple, rapid, automated method to quantify serum SM.

During recent years, an enzymatic assay using SMase from *Bacillus cereus* and alkaline phosphatase has been reported [5,15–18]. In general, enzymatic measurement of serum SM is carried out by the following procedure. In the first step, SMase hydrolyzes SM to *N*-acylsphingosine and phosphorylcholine, from which choline is generated by alkaline phosphatase. In the second step, the resulting choline is used to generate hydrogen peroxide in a reaction catalyzed by choline oxidase. Finally, with peroxidase as a catalyst, hydrogen peroxide is used together with chromogens to form a chromophore, which is quantitated spectrophotometrically. This method uses microtiter plates and plate readers and can test a large number of samples in a short time. However, the enzyme specificity is not sufficient, given that accurate quantitation is not achieved because of nonspecific reactions with lysophosphatidylcholine (LPC), and remains an issue to be resolved [16]. We have, therefore, attempted to establish a new, specific, and high-throughput enzymatic method for serum SM quantitation that can be carried out using a universal autoanalyzer without any sample pretreatment. Especially, the proposed method is superior to the existing method in preventing nonspecific reactions with LPC. Here, we describe such an assay for measuring serum SM using a combination of phospholipid (PL)-hydrolyzing enzymes.

Materials and methods

Materials

Phospholipase D (EC 3.1.4.4: 46 kDa, from *Streptomyces* species), phospholipase D (EC 3.1.4.4: 50 kDa, from *Streptomyces chromofuscus*), phospholipase C (EC 3.1.4.3: 20 kDa, from *B. cereus*), phospholipase A2 (EC 3.1.1.4: 15 kDa, from *Streptomyces violaceoruber*), glycerophosphorylcholine phosphodiesterase (EC 3.1.4.2: from *Gliocladium roseum*), and monoglycerolipase (lysophospholipase, EC 3.1.1.23: 20 kDa, from *Bacillus* species) were obtained from Asahi Kasei Pharma. Choline oxidase was obtained from Kyowa Hakko Kogyo. Horseradish peroxidase and lipoprotein lipase (EC 3.1.1.34: 134 kDa, from *Pseudomonas* species) were obtained from Toyobo, 4-aminoantipyrine was obtained from Saikyo Kasei, catalase was obtained from Kikkoman, and *N*-ethyl-*N*-(3-methylphenyl-*N'*-succinylethylenediamine) (EMSE) was obtained from Daito Chemix. Sphingomyelin, *sn*-phosphatidylcholine, and lysophosphatidylcholine for SM, PC, and LPC standards, respectively, and Triton X-100 were obtained from Sigma–Aldrich. Good's buffer piperazine-*N,N'*-bis-(2-ethanesulfonic acid) (Pipes) was obtained from Dojindo Laboratories. A commercially available enzymatic assay kit for SM (Sphingomyelin Colorimetric Assay Kit) was purchased from Cayman Chemical and used in the comparison study.

Human serum samples were obtained from 127 healthy individuals who had fasted for 12–14 h. The age of the enrolled individuals (82 men and 45 women) in this study was 32 ± 8 years (mean ± standard deviation [SD]), ranging from 20 to 55 years. The study was approved by the ethics committees at Kyowa Medex (no. 3145) and Kumamoto University (no. 760). All serum samples were immediately stored at −80 °C until use. Serum lipoprotein fractions were isolated by ultracentrifugation, yielding very-low-density lipoprotein (VLDL), LDL, and high-density lipoprotein (HDL) fractions (0.96–1.006, 1.019–1.063, and 1.063–1.021 kg/L, respectively) according to a previously described method [19–21]. The corresponding lipoprotein fractions were pooled and stored at 4 °C until use. All other materials and solvents were analytical reagent grade.

Determination of enzyme kinetic parameters

The kinetic parameters for PL-hydrolyzing enzymes were determined in 20 mM Pipes/NaOH (pH 7.0) in the presence of 1 mM $MgCl_2$ and 2 mM $CaCl_2$ at 37 °C for 5 min. Enzymatic hydrolysis was initiated by the addition of 2 μl of a PL stock solution, at different concentrations, in 20 mM Tris–HCl buffer containing Triton X-100 (10 g/L, pH 7.4, 37 °C) to 200 μl of buffer containing a certain amount of each enzyme. The reaction was terminated with 200 μl of 50 mM Pipes buffer (pH 8.0) containing 10 mM ethylenediaminetetraacetic acid (EDTA), Triton X-100 (5 g/L), 2.5 kU/L choline oxidase, 2.5 kU/L peroxidase, 1 mM 4-aminoantipyrine, and 2 mM EMSE. The mixture was incubated at 37 °C for 5 min to produce choline from the remaining PLs by the addition of 1.0 U/ml phospholipase D from *S. chromofuscus*. The kinetic parameters were determined from subsequent Lineweaver–Burk plots of the data.

Determination of total PL, PC, and LPC

The total PL concentration was determined enzymatically using a commercial kit (Kyowa Medex). PC and LPC concentrations were determined by previously described methods [15,18,22,23].

Analytical procedures

In the final reagent formulation used for the current assay, the first reagent contained EMSE (1.1 mM), phospholipase D from *Streptomyces* species (10 kU/L), monoglycerolipase from *Bacillus* species (1 kU/L), choline oxidase (10 kU/L), catalase (10 kU/L), 10 mM $CaCl_2$, and Triton X-100 (0.01 g/L) in Pipes buffer (50 mM, pH 7.5). The second reagent contained phospholipase D from *S. chromofuscus* (4 kU/L), peroxidase (25 kU/L), sodium azide (0.2 g/L) as a catalase inhibitor, and 4-aminoantipyrine (2.5 mM) in Pipes buffer (50 mM, pH 7.0). To 240 μl of the first reagent was added 2.5 μl of a standard solution or serum sample, and the mixture was incubated at 37 °C for 5 min. Next, 80 μl of the second reagent was added, and the mixture was incubated at 37 °C for an additional 5 min. The chromophore formed in a coupled reaction with peroxidase was measured spectrophotometrically at dual wavelengths of 600 nm (main) and 700 nm (subsidiary), and the end-point method was used for calculations. Standard SM solution was prepared by dissolving SM in 20 mM Tris–HCl buffer (pH 7.4) with Triton X-100 (10 g/L). The instruments used for this assay were a Hitachi 7170 autoanalyzer (Hitachi High Technologies) and an ultraviolet (UV) 160A spectrophotometer (Shimadzu).

Pooled serum samples from healthy volunteers were used in all interference studies. In the bilirubin interference study, pooled serum samples were mixed with increasing volumes of 2 g/L bilirubin F or C (Interference Check A Plus, Sysmex) dissolved in pooled serum to yield final bilirubin concentrations ranging up to 0.4 g/L. A control serum was made by adding the same volume of saline solution to pooled serum. In the hemolysis interference study, pooled serum samples were mixed with increasing volumes of 25 g/L hemoglobin (Interference Check A Plus, Sysmex) dissolved in pooled