〈Original Article〉

Turbidimetric measurement of γ -globulin in cerebrospinal fluid with use of sulfosalicylic acid and Triton X-100

Hiroshi Ihara[1)], Yoshikazu Morita[1)], Shunji Nanba[2)] and Yuji Suzuki[3)]

Summary We describe a simple, rapid, and cost-effective method using sulfosalicylic acid and Triton X-100 for turbidimetric measurement of γ -globulin in cerebrospinal fluid (CSF). Measurement of γ -globulin in CSF is an indicator of immunoglobulin production in the subarachinoid space. Immunoglobulin in CSF is assessed principally by expensive IgG assay or by semiquantitative Pandy or Nonne-Apelt tests. The method described requires 0.2 mL of CSF and 1.5 mL of precipitant, and turbidity formation measured at 660 nm completed within 15 min at 37℃. Reagent cost is approximately 1% of the cost of IgG by immunonephelometry. The turbidity was linear from 3 up to 100 mg/dL. The reagent reacted 100% with γ -globulin, 9% with α - and β -globulins, and 1% with albumin. The within- and between-day coefficient of variation for γ -globulin was better than 5.9%. Values of γ -globulin in CSF by our method were significantly correlated with IgG concentration by immunonephelometry (γ = 0.954, P< 0.001), and the reference interval for IgG (< 5 mg/dL) could also be adapted to our method.

Key words: Protein, Immunoglobulin G (IgG), CSF, Immunonepherometry, Pandy test, Nonne-Apelt test, Multiple sclerosis

1. Introduction

Compared to serum, the total protein concentration in cerebrospinal fluid (CSF) is approximately 1/200[1)], and the concentration is normally 10-40 mg/dL. It is increased in most pathological conditions due to the alternation in the permeability of the blood-brain barrier. CSF protein is composed of albumin and globulins, i.e., α -, β - and γ -globulin. γ -Globulin in CSF is assessed as an indicator of immunoglobulin production (especially, the production of immunoglobulin G: IgG) in the subarachinoid space. Measurement of γ -globulin or IgG provides valuable information in the diagnosis of multiple sclerosis[2)]. The method generally used to quantitate IgG in CSF is immunonephelometry or turbidimetric immunoassay, however these methods involve a relatively high reagent cost. For this reason laboratories unable to

[1)]Department of Laboratory Medicine, Toho University Ohashi Medical Center, 2-17-6 Ohashi, Meguro, Tokyo 1538515, Japan
[2)]Department of Laboratory Medicine, Toho University Omori Medical Center, Tokyo, Japan
[3)]Department of Health Sciences, School of Health and Social Services, Saitama Prefectural University, Saitama, Japan
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measure IgG in CSF used Pandy or Nonne-Apelt tests[3,4]. Since these two tests make a semiquantitative assessment of γ -globulin, we developed a cost-effective quantitative test for γ -globulin in CSF.

2. Materials and methods

1. Reagents

Sulfosalicylic acid (SSA: 5-sulfosalicylic acid dihydrate, cat. No. 194-04575) and Triton X-100 (cat. No. 807426) were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan and MP Biomedicals, Inc., Solon, OH, USA, respectively. Human serum albumin (essentially globulin free, lyophilized powder, approximately 99% purity by agarose electrophoresis, cat. No. A-8763), human globulins (Cohn fraction Ⅳ, predominantly α - and β -globulins, cat. No. G-3387), and human γ -globulin (prepared from Cohn fraction Ⅱ and Ⅲ, 99% purity by electrophoresis, cat. No. G-4386) were all purchased from Sigma-Aldrich Japan KK.

Precipitation reagent was prepared by dissolving 30 g of SSA in 1,000 mL of 60 g/L Triton X-100. This reagent was stable for at least four weeks at 4℃ in a brown bottle. Solutions of albumin and globulin (Cohn fraction Ⅳ) were prepared by dissolving 100 mg each in 100 mL of distilled water. Calibrant was prepared by dissolving 100 mg of γ -globulin (Cohn fraction Ⅱ and Ⅲ) in aliquot of 0.1 mol/L solution of hydrogen chloride, and diluted to 100 mL with distilled water. The concentration of γ -globulin calibrant was checked by turbidimetric immunoassay (using a Hitachi Labospect automated analyzer and Iatro-IgG, -IgA and -IgM reagents: Mitsubishi Chemical Medience), and its γ -globulin concentration was expressed as the sum of IgG, IgA and IgM. Immunoglobulin composition of γ -globulin calibrant was 96% for IgG, 3% for IgA and 1% for IgM.

2. Assays

For γ -globulin measurement in CSF, each 0.2 mL of CSF or γ -globulin calibrant was diluted with 0.3 mL of distilled water, and then 1.5 mL of the precipitation reagent was added. After vortex mixing, the mixture was allowed to stand at 37℃ for 15 min. γ -Globulin is precipitated as a fine white precipitate by the addition of precipitation reagent. The resulting turbidity was measured with a Shimadzu CL-770 clinical spectrophotometer at 660 nm against distilled water. SSA gives greater turbidity with albumin than with globulin, however, coexisting Triton X-100 completely inhibited precipitation of albumin and facilitated γ -globulin precipitation[5].

CSF-specimens were collected from 113 patients

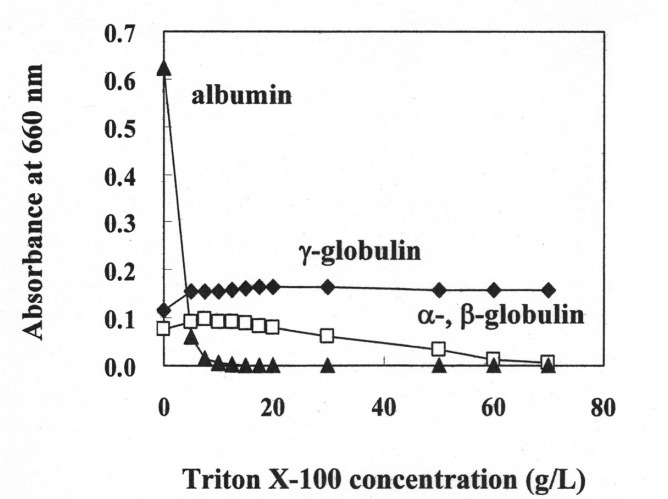

Fig. 1 Effect of Triton X-100 concentration on the inhibition of precipitation of albumin and α -, β -globulin by sulfosalicylic acid.

suspected of a variety of neurological diseases. Written informed consent was obtained from all patients; our study was in compliance with the rules for human experimentation in our institution.

3. Results

1. Specificity for γ -globulin

To investigate the specificity of our assay method for γ -globulin, 100 mg/dL solution of albumin, Cohn fraction Ⅳ, and γ -globulin was determined by the precipitation reagent as increasing Triton X-100 concentration from 0 to 70 g/L with a constant SSA concentration of 30 g/L (Fig. 1). Precipitation of albumin was 99% inhibited at a Triton X concentration greater than 15 g/L. Precipitation of γ -globulin was facilitated in the presence of Triton X-100. In the presence of Triton X-100, the turbidity (i.e., absorbance at 660 nm) of γ -globulin was increased to 135% as compared to the turbidity solely produced by SSA. In conjunction with the increasing concentration of Triton X-100, Cohn fraction Ⅳ precipitation was inhibited, although not completely even at a greater than 60 g/L concentration of Triton X-100. At Triton X-100 concentrations of 60 and 70 g/L, the 100 mg/dL solutions of Cohn fraction Ⅳ were measured as 9 and 6 mg/L of γ -globulin, respectively. However, due to abundant formation of bubbles in the precipitation reagent at the 70 g/L concentration of Triton X-100, we selected the concentration of a 60 g/L in our study.

2. Incubation temperature

We measured the influence of the incubation temperature on turbidity formation. Turbidity produced by the precipitation of γ -globulin increased and was accompanied by an elevation in the incubation temperature and the length of incubation period. Turbidity formed at 37℃ was 14% higher than that formed at 27℃. Turbidity formation was completed within 15 min at 37℃, but required 30 min at 27℃, and the turbidity was stable at least for 90 min.

3. Linearity

A series of human γ -globulin dilutions (prepared from Cohn fraction Ⅱ and Ⅲ, 99% purity by electrophoresis, cat. No. G-4386, Sigma-Aldrich Japan KK) in distilled water ranging from 0 to 100 mg/dL were assayed by our method. Our method gave a linear Beer's law response at 660 nm for γ -globulin concentration up to 100 mg/dL. We determined the slope of the line shown in Fig. 2 (from 0 to 25 mg/dL) at least ten times to get the mean and SD values. The numerical range of the mean±2SD values for the 3

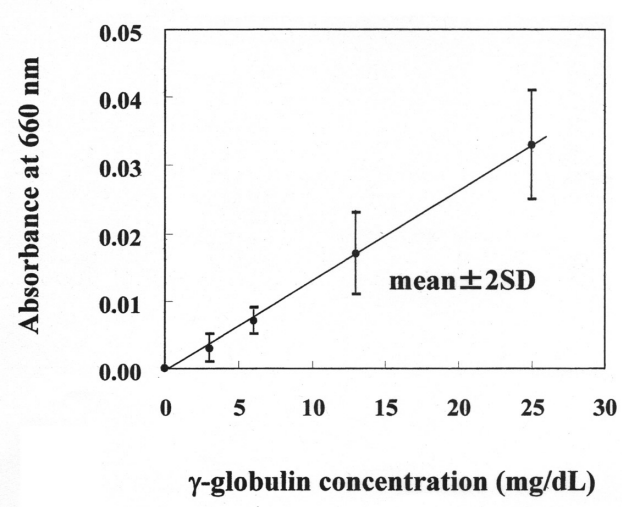

Fig. 2 Relation between the γ -globulin concentration and turbidity formation (absorbance at 660 nm) by our method. The detection limit was 3 mg/dL. Points are mean±2SD of ten measurements.

mg/dL solution did not overlap the same values for the 0 mg/dL solution. In our method, the 3 mg/dL γ -globulin solution was near the lower detection limit.

4. Within- and between-day precision

Two CSF pools were prepared: one with normal γ -globulin concentration and the other with elevated γ -globulin concentration. Aliquots of these two pools were frozen at -80℃ until analysis. The γ -globulin concentration was measured by our method on twenty consecutive replicates of these two pools.

The within-day assay with our method gave a mean±SD of 5.9±0.4 mg/dL for the normal pool and 19.4±0.8 mg/dL for the elevated pool. The between-day assays for the same pools gave a mean±SD of 5.8±0.4 mg/dL for the normal pool and 18.1±0.7 mg/dL for the elevated pool.

5. Analytical recovery

Analytical recoveries were performed by adding 0.2 mL of 11 and 33 mg/dL γ -globulin solution to 1.8 mL of two different CSF pools having endogenous γ -globulin concentrations of 9 and 16 mg/dL. We determined the γ -globulin concentration by our method. The analytical recoveries were acceptable at 100-102% and 102-103% in the CSF pool having endogenous γ -globulin concentrations of 9 and 16 mg/dL, respectively.

6. Effect of potential interferences

We investigated the effect of hemoglobin and bilirubin on the values of γ -globulin as determined by our method. Human hemoglobin (Sigma-Aldrich) at 100 mg/dL was measured as 14 mg/dL of γ -globulin. Unconjugated bilirubin up to 1.0 mg/dL had no effect on the measurement. Xanthochromia in CSF had no effect on spectrophotometry at 660 nm.

7. Correlation study

We compared the γ -globulin concentration determined by our method to the IgG determined by immunonephelometry (BN ProSpec Nephelometer System from Siemens Healthcare Diagnostics, Munich, Germany) on 113 CSF-specimens. The results of γ -globulin were near equal to IgG concentration observed; Y (γ -globulin concentration by our method)= 1.0X (Nephelometry) + 0.8, Sy/x= 1.9 mg/dL, r= 0.954, P< 0.001 (Fig. 3). Concentrations of γ -globulin and IgG in 44 patients with no abnormal neurological finding by the CSF tests were 2.9±1.0 mg/dL (upper 95th percentile, 5 mg/dL) and 2.4±1.4 mg/dL (upper 95th percentile, 5 mg/dL) with our

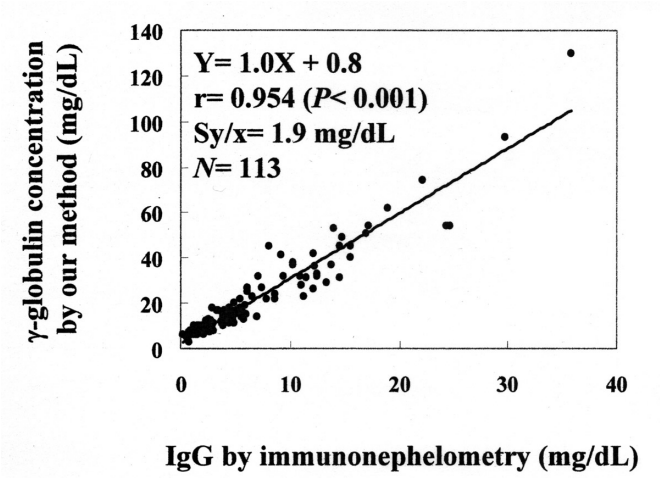

Fig. 3 Comparison between γ -globulin concentration by our method and IgG concentration by immunonephelometry (BN ProSpec Nephelometer System from Siemens Healthcare Diagnostics, Munich, Germany) in 113 CSF-specimens from patients suspected of a variety of neurological diseases.

method and BN ProSpec, respectively.

4. Discussion

Previous trials and improvement in the SSA test were to enhance the reactivity of γ -globulin to approach that of albumin, while in our study we aimed to decrease the reactivity of albumin to near zero. Triton X-100 completely inhibited the precipitation of albumin and facilitated γ -globulin precipitation in our SSA test. Thus, we can exclusively measure γ -globulin concentration in CSF, and this is the first SSA test that has improved for γ -globulin measurement. Although we selected 660 nm for measurement, some report measured turbidity at 430 nm[6]. Absorbance measured at 430 nm was three times higher than that measured at 660 nm, but the calibration equation measured at 430 nm was non-linear from 0 to 3 mg/dL. In addition, hemoglobin and the color of xanthochromia have absorptivity at 430 nm.

The concentration of γ -globulin in CSF was reported to be 3-12% of total protein[7]. If the upper limit of CSF-total protein is 40 mg/dL, the γ -globulin portion can be calculated at 4.8 mg/dL. Other reported reference intervals for IgG in CSF are 0.4 to 5.1 mg/dL[8]. Comparison study between our method (γ -globulin) and immunonephelometry (IgG) revealed that γ -globulin values by our method would be approximately equal to the IgG concentration in CSF measured by immunonephelometry, and the upper 95th percentile was 5 mg/dL. Thus the established reference interval for IgG (<5.1 mg/dL) was applicable to our method.

The key benefit of the present method is the low running cost; reagent costs are about 3 JPY (0.03 USD) per test and about 5 JPY (0.05 USD) per test including calibration. The cost of IgG assay in CSF is 550 JPY (5.7 USD) per test. Nevertheless, patients with neurologic diseases require assessment of IgG levels in CSF. Therefore, the simple, rapid, and cost-effective method proposed here would be a screening method substitute for the IgG assay in CSF.

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