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Competitive binding assay for quantitative determination of GM1 ganglioside in plasma and cerebrospinal fluid

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Abstract: A competitive binding assay for the quantitative determination of GM1 ganglioside is described. After extraction from biological fluids, GM1 was incubated with a known amount of cholera toxin B-subunit conjugated with horseradish peroxidase, and exposed to GM1 adsorbed onto polystyrene microwells. Since GM1 in solution blocks the binding of toxin B-subunit to GM1 adsorbed onto the solid phase, enzyme activity serves as a reciprocal measure of GM1 concentration in the sample. The assay was used to determine the basal level of GM1 in plasma and cerebrospinal fluid in different populations.

Keywords: *GM1; GM1 analysis; ganglioside; cerebrospinal fluid; competitive binding assay.*

Introduction

Gangliosides are located in the outer leaflet of the plasma membrane where they appear to function as components of receptors for toxins, glycoprotein hormones and viruses [1–3]. Gangliosides interact with proteins such as interferon and fibronectin [4, 5], may induce cell fusion [6], and modulate the immune response *in vitro* [7–11].

Current evidence suggests that neuronal death secondary to brain damage may be related either to deficits in the availability of neurotrophic factors [12, 13] or to the increased activity of neurotoxic agents, such as excitatory amino acids [14]. Treatment with monosialoganglioside GM1 (nomenclature according to Svennerholm [15]), has been reported to ameliorate the deficit following various types of experimental central nervous system injuries [16–19]. In human, the neuropharmacological effects of GM1 have been confirmed in controlled clinical trials in head and spinal cord injury and stroke [20].

Growing recognition of the importance of this ganglioside has created the need for a simple, fast and reliable method for its determination, one which also permits simultaneous analysis of a large number of samples. And since it is frequently necessary to determine the ganglioside content in small samples, specificity, sensitivity, and reproducibility are essential.

A variety of methods for the quantitation of gangliosides have been described. Most use thin-layer chromatography (TLC) with ligands such as ganglioside specific antibodies [21], the B-subunit of cholera toxin, or specific cholera toxin antisera, radiolabelled [22] or conjugated with enzymes such as alkaline phosphatase [23]; or TLC followed by the immunostaining technique of Magnani [24]. Another related method consists of transforming gangliosides into asialogangliosides: after TLC-separation, determination is by anti-asialoganglioside antibodies [25].

Other approaches include those of Ginns *et al.* [26] which measures the presence of GM1 ganglioside in cerebrospinal fluid via radioimmunoassay, and of Wu and Ledeen [27] who used an ELISA technique to determine the presence of gangliotetraose gangliosides after adsorption of the sample onto polystyrene microwells and treatment with cholera toxin B-subunit conjugated to horseradish peroxidase.

The aim of the present study was to combine the specificity of the biological reaction of the B-subunit of cholera toxin and GM1 [28, 29] with the analytical performances of the competitive binding assay. The resultant method

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was standardized as a commercially available diagnostic assay for use in an automated diagnostic machine.

A preliminary report of the methodology was presented at the XIV International Carbohydrate Symposium, in Stockholm, Sweden by Kirschner *et al.*

Experimental

Materials

The following substances were obtained from the sources indicated: cholera toxin, B-subunit, horseradish peroxidase conjugate (B-CT-HRP), from List Biological Laboratories (Campbell, CA, USA); bovine serum albumine (BSA) from Sigma (St Louis, MO, USA); hydrogen peroxide ortho-phenylendiamine (OPD) chromogen substrate from Sorin Biomedica S.p.A. (Vercelli, Italy); phosphate buffered salts (PBS) from Flow Laboratories (McLean, VA, USA); flat bottom polystyrene honeycomb microtitre plates and Auto-EIA-II Analyzer from Labsystems (Helsinki, Finland). The analyzer was equipped with an Olivetti M240 microcomputer (Ivrea, Italy).

Individual gangliosides, GM1, GD1a, GD1b and GT1b were prepared in our laboratories and characterized as previously described [30].

Sample preparation

Gangliosides were extracted from biological samples according to the method of Tettamanti *et al.* [31]. Briefly, 500 μl of sample was dispensed in a glass centrifuge tube, 2 ml of tetrahydrofuran was added, shaken well and centrifuged at about 6000 rpm for 1 min. The supernatant was removed and the pellet extracted twice with 0.5 ml of phosphate buffer (pH 6.8) 10 mM and 2 ml of tetrahydrofuran; 0.3 volumes of ethyl ether was added to the pooled supernatant, shaken well and centrifuged. The aqueous phase was collected and 0.1 volume of distilled water was added to the ether phase. The pooled aqueous–organic extracts were dried under vacuum centrifugation and diluted with water to an appropriate volume in order to be in the linearity range. Normal plasma and CSF samples are redissolved with 1.5 and 0.2 ml, respectively.

Seven different concentrations of GM1, between 10 and 750 nmol l^{-1} PBS, were prepared in order to obtain a standard reference curve.

GM1 plastic coating

Polystyrene plates were coated with 50 μl of a solution of 1.5 μg ml^{-1} of GM1 methanol–water (1:1, v/v) for 90 min at room temperature, washed three times with distilled water and maintained at 4°C until use.

Non-specific binding sites were blocked with a solution of 2% BSA in PBS for 90 min at room temperature.

Assay procedure

B-CT-HRP was dissolved in 100 μl of distilled water (stable for 1 month at 4°C), and immediately before use 5 μl of this solution was diluted with 25 ml PBS.

A 400 μl volume of the B-CT-HRP solution was added to 100 μl of each standard and sample and left to react for 45 min at room temperature. A 50 μl volume of each sample was added to at least three wells of the GM1-coated microtitre plate, again left to react for 45 min at room temperature, and drawn off. Wells were washed three times with PBS and twice with water. Finally, 50 μl of hydrogen peroxide–OPD chromogen substrate reagent was added to each well, and after 15 min the reaction was blocked with 1.25 M sulphuric acid. Absorbance was read at 492 nm.

GM1 content in the samples was calculated from the standard curve.

Automated procedure

The above described procedure for quantitative determination and standard curve preparation was transferred via a suitable working program to the Auto-EIA-II Analyzer System. Transfer allowed for full automation of sample dilution and dispensing, dispensing of the reagent, incubation, washing, measuring, calculation and printing of the results.

Absorbances were transmitted to the Olivetti M240 microcomputer. The mean blank absorbance was subtracted from each mean test absorbance, and results compared with the standard GM1 curve.

Results and Discussion

A competitive immunoenzymatic assay for the determination of GM1 was developed based on the knowledge that GM1 is a specific, high-affinity receptor for cholera toxin and that it adsorbs spontaneously to the hydrophobic portion of plastic surfaces such that the oligo-

saccharide portion is free to react with other molecules [32].

In the reported method, GM1 in solution inhibits the tracer, B-CT-HPR, from binding to GM1 absorbed onto the solid phase, and enzyme activity is measured as a reciprocal of the GM1 concentration in the sample. The assay requires neither constant binding of GM1 to the polystyrene wells [27], particularly difficult to obtain with plasma samples (data not shown), nor chromatographic separation and purification as required by all methods using the immunostaining technique. Also, unlike methods requiring the immunostaining technique, it is neither tedious nor unsuitable for a large number of samples.

The specificity of the reaction between B-CT-HPR and GM1 was confirmed by determining cross-reactivity with other gangliosides of the gangliotetraose series, i.e. GD1a, GD1b and GT1b. Incubation of from 0.050 to 1.000 nmol l^{-1} of single gangliosides yielded cross-reactions of 1.6% of GD1a, 2.4% for GD1b and 1.1% for GT1b, as shown in Fig. 1. The cross-reactivity of the cholera toxin B-subunit with other major gangliosides of the ganglio-tetraose series present in plasma and in CSF [32–33] is highly reduced with this method as compared to immunostaining on TLC plates and the direct ELISA method, where an average cross-reactivity with GD1a and GD1b of about 5 and 10%, respectively, is found (Fig. 1). This may be because ELISA and TLC methods require the reaction to be performed on a plastic or silica gel surface with gangliosides in a monomeric form, whereas with our method the reaction occurs in aqueous solution with gangliosides in micellar form.

The plot of absorbance versus GM1 was linear between 31.8 and 95.6 nmol l^{-1} (Fig. 2), based on 12 replications of the same sample (within-run RSD) and five complete analytical procedures (between-run RSD). Data are summarized in Table 1.

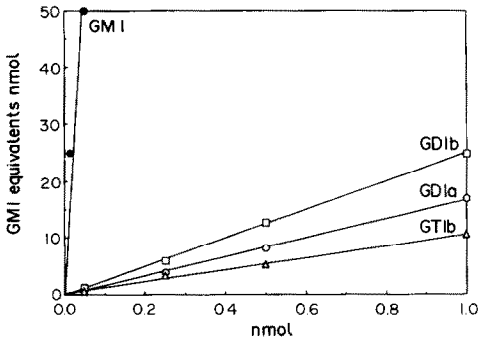

Figure 1
Cross-reactivity of different gangliosides with cholera toxin B-subunit. Data are expressed as nmol equivalents of GM1.

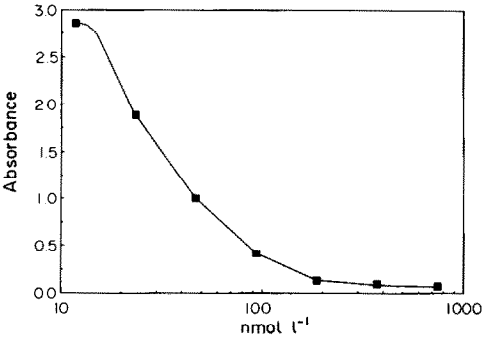

Figure 2
Calibration curve for the quantitative determination of GM1. Details are given under Experimental.

Table 1
Precision (RSD) of GM1 determination

		nmol l^{-1}		
	n	Mean	SD	RSD
Within-run				
plasma (basal)	12	151.1	7.7	5.1
plasma (treated)	5	2041.2	87.1	4.3
Between-run				
plasma	5	191.7	12.1	6.3

Table 2
GM1 content in normal plasma and CSF (in brackets — number of samples)

	Mean values (nmol l^{-1})	SD	Ranges (nmol l^{-1})
Normal healthy volunteers 18–40 years (350)	166	60	120–230
Elderly >70 years (21)	170	42	104–309
Pregnant (33)	172	71	93–331
Newborn, umbilical cord (33)	139	13	77–230
Cerebrospinal fluid (18)	13	4.5	5–23

To evaluate the possible influence of the biological substrate on recovery, amounts ranging from 1.49 to 14.96 μmol l^{-1} of GM1 were added to plasma. The recovery for 1.49, 7.59 and 14.96 μmol l^{-1} was 1.41 ± 0.11, 7.53 ± 0.38 and 14.54 ± 0.75 μmol l^{-1}, respectively.

The described technique was used to determine the basal plasma and cerebrospinal fluid (CSF) content of GM1 in a series of different populations as reported in Table 2. Treatment of a ganglioside mixture with neuraminidase, hydrolyses all gangliosides of the gangliotetraose series to GM1 [34], and permits the determination of their overall content in biological samples.

The transfer of the procedure to an automated analytical system makes possible large-scale screening of both normal and pathological populations, and an eventual evaluation of the relationship of plasma and/or CSF content of GM1 to disorders of the central nervous system. Moreover, it greatly facilitates pharmacokinetic studies of GM1, which are now in progress.

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