Glycoconjugate Journal (1994) **11**:97–104

An improved method for the measurement of total lipid-bound sialic acids after cleavage of α2,8 sialic acid linkage with *Vibrio cholerae* sialidase in the presence of cholic acid, SDS and Ca^{2+}*

MATHEW A. MALIAKAL, MEPUR H. RAVINDRANATH‡, REIKO F. IRIE and DONALD L. MORTON

Laboratory of Glycolipid Immunotherapy, John Wayne Cancer Institute, 2200 Santa Monica Blvd, Santa Monica, CA 90404, USA

Received 8 November 1993

In the measurement of total lipid-bound sialic acids involving periodic acid oxidation, as in the periodate-resorcinol assay, the inner sialic acids of disialoglycolipids (such as GD_3 and GD_2) are not involved because their α2,8 ketosidic linkages are resistant to periodic acid oxidation, even after acid/enzyme hydrolysis or alkali pretreatment. However, the sialic acids from these glycolipids can be recovered completely after cleavage of α2,8 linkages by *V. cholerae* sialidase in the presence of cholic acid, sodium dodecyl sulphate and calcium. Interestingly, removal of calcium or detergent(s) or both significantly minimizes the sialidase action on the disialyl residues of these gangliosides. Therefore, we recommend sialidase (*Vibrio cholerae*) pretreatment of the glycolipids in the presence of cholic acid, SDS and Ca^{2+} for complete recovery of sialic acids from di- and polysialogangliosides and for accurate measurement of total lipid-bound sialic acids by periodate-resorcinol assay.

Keywords: total lipid-bound sialic acid, LBSA; α2,8 ketosidic linkage; *Vibrio cholerae* sialidase; cholic acid; sodium dodecyl sulphate; SDS, calcium

Introduction

Lipid-bound sialic acids (Gangliosides) have been used as tumour markers in patients with malignant melanoma, neuroblastoma, neurofibrosarcoma, astrocytoma, glioma, meningioma, leukaemia and carcinoma of the thyroid, lung, prostate, ovary, breast and colon [1–14]. Lipid-bound sialic uacids (LBSA) are released from tumour cells and enter into the circulation [4, 15]. Serial measurements of serum LBSA have therefore been used to detect early metastasis or monitor the level of residual tumour after surgery. In addition, there are other diseases, such as hypercholesterolaemia with elevated levels of serum gangliosides [16], which require routine clinical measurement of lipid-bound sialic acids. The gangliosides in the circulation can suppress immune functions [17, 18].

Accurate measurement of the total LBSA in serum and tissues under normal and pathological conditions requires complete recovery of sialic acids from the glycolipids. The most commonly used method for estimating the sialic acids in glycoconjugates is Svennerholm's resorcinol assay [19]. In this assay, acid treatment produces furfural derivatives of sialic acids that oxidize resorcinol into a coloured product (quinone), the intensity of which is enhanced by the addition of $KuSO_4$. Using periodate oxidation prior to acid-resorcinol treatment increases the assay's specificity and sensitivity for sialic acids bound by α2,3 and α2,6 linkages to carbohydrate chains of glycoproteins [20, 21], but is not useful for measuring sialic acids bound to other sialic acids by α2,8 ketosidic linkages [22], because the ketosidic linkage is resistant to periodate oxidation. Therefore, neither the resorcinol assay nor any other assay involving periodate oxidation, such as the thiobarbituric acid assay, can be extended to gangliosides with α2,8 linkages. Nevertheless, the resorcinol assay, with or without periodate oxidation, has been extensively employed for measuring total LBSA in tumour tissues and sera containing a high level of gangliosides with α2,8 ketosidic linkages [4, 5, 16, 18, 23–25].

Thus the periodate-resorcinol assay can recover 1 mol of

* Presented at the Second International Glycobiology Symposium which was held in San Francisco, CA, USA (14 February 1994).

‡ To whom correspondence should be addressed.

Table 1. Structure of gangliosides commonly found in human normal and cancer tissues.

Glycolipids	*Structure*	*Molecular weight (AMU)*
GM_3	Galβ1,4Glcβ1,1Cer 3 \| 2αNeuAc	1236 (1182)
GM_2	GalNAcβ1,4Galβ1,4Glcβ1,1Cer 3 \| 2αNeuAc	1385
GD_3	Galβ1,4Glcβ1,1Cer 3 \| 2αNeuAc8,2αNeuAc	1545 (1473)
GD_2	GalNAcβ1,4Galβ1,4Glcβ1,1Cer 3 \| 2αNeuAc8,2αNeuAc	1694
GM_1	Galβ1,3GalNAcβ1,4Galβ1,4Glcβ1,1Cer 3 \| 2αNeuAc	1547
GD_{1a}	Galβ1,3GalNAcβ1,4Galβ1,4Glcβ1,1Cer 3 3 \| \| 2αNeuAc 2αNeuAc	1838
GD_{1b}	Galβ1,3GalNAcβ1,4Galβ1,4Glcβ1,1Cer 3 \| 2αNeuAc8,2αNeuAc	1838
GT_3	Galβ1,4Glcβ1,1Cer 3 \| 2αNeuAc8,2αNeuAc8,2αNeuAc	1854

Atomic mass unit (molecular weight) of a particular ganglioside will vary slightly due to the differences in the length of fatty acids, presence of double bonds and hydroxyl groups. The values presented here are based on the CalBiochem catalogue, 1992. The values given in parentheses are the molecular weights reported by Matreya, Inc. (Catalog No. 4 1993–1994).

sialic acid from 1 mol of monosialoganglioside but will not measure the 2 mol of sialic acids with α2,8 linkages to 1 mol of disialogangliosides. In the present investigation, we have developed strategies for complete recovery of sialic acids from di- or polysialogangliosides with α2,8 ketosidic linkages (Table 1).

Materials and methods

Materials

All solvents used were of high performance liquid chromatography (HPLC) grade. Ethanol, HCl, H_2SO_4, glacial acetic acid, resorcinol, NaCl and $CuSO_4$ were obtained from Aldrich (Milwaukee, WI, USA). Albumin (human, 25% solution) was obtained from Baxter Healthcare Corp., Glendale, CA, USA. Water was deionized and double-distilled. Borosilicate glass tubes (13 × 100 mm, Fisher) with polypropylene screw caps (Cat. 14-959-35C) were used for the assay. Purified gangliosides, *N*-acetylneuraminic acid, periodic acid, *t*-butyl alcohol, sodium acetate, $CaCl_2$ (anhydrous), ethylenediamine tetraacetate (tetrasodium salt) and all detergents used in this investigation were obtained from Sigma Chemical, St Louis, MO, USA. Bovine buttermilk GD_3 and human brain GD_3 were obtained from Sigma and Advanced Immunochemical Services, Inc., Long Beach CA, USA, respectively. Human melanoma GD_3 was purified (alkali-treated) from human melanoma cell line M14 following a protocol described earlier (see [31]). Neuraminidase (sialidase) from *Vibrio cholerae* was obtained from Boehringer Mannheim. The enzyme was suspended in sodium acetate, 50 $mmol^{-1}$; sodium chloride, 154 $mmol^{-1}$; calcium chloride, 9 $mmol^{-1}$; sodium azide, 0.005% (w:v); human serum albumin, 25 $mg\,l^{-1}$; pH 5.5.

Methods

The periodate-resorcinol assay was carried out following the protocol described by Jourdian *et al.* [21]. Throughout

this investigation, we have used commercially obtained gangliosides. The purity of commercially obtained gangliosides was tested by overloading thin layer chromatography. The accuracy of the amount of gangliosides supplied by commercial sources was confirmed by gravimetric measurement. For routine analysis bovine buttermilk GD_3 was used. The proton nuclear magnetic resonance and the fast atom bombardment mass spectrometric studies and other assays reveal the presence of two molecules of *N*-acetylneuraminic acid in the buttermilk GD_3 [26, 27]. For the chemical assays, the ganglioside was suspended in ethanol in glass tubes and evaporated to dryness over nitrogen before use. The assay was performed as follows: 500 µl of water or buffer was added to ganglioside, vortexed and sonicated before adding 100 µl of 0.05 M periodic acid solution. The solutions were thoroughly mixed and allowed to stand in an ice bath or at 37 °C for varying time intervals. After adding the resorcinol reagent (1.25 ml), the solutions were mixed, placed in an ice bath for 5 min, heated to 100 °C for 15 min, and cooled in tap water. 1.25 ml of *tert*-butanol was then added. A single-phase solution obtained after vigorous mixing was placed in a 37 °C bath for 3 min to stabilize the colour. After cooling at room temperature, the absorbance was measured at 630 nm in a Beckman DU spectrophotometer.

Calculation of percentage recovery of sialic acids

The percentage recovery of sialic acid from a ganglioside was calculated as follows:

$$\frac{\text{Absorbency of a ganglioside(s) tested} \times 100}{\text{Absorbance of } GM_3 \times \text{number of molecules of sialic acids in the ganglioside tested}}$$

Each experiment included one or more monosialogangliosides for comparison. In all experiments, the nanomole of gangliosides used for assay was kept constant. We optimized conditions to recover 100% of the sialic acid from each ganglioside. We observed that the yield of sialic acid from the monosialoganglioside GM_3 is always at maximum. However, the same trend was also seen for other monosialogangliosides such as GM_2 and GM_1.

Results

Prolonging the periodate-resorcinol assay

To investigate whether ganglioside susceptibility to periodic acid oxidation and resorcinol-HCl can be increased by prolonging the duration of periodic acid oxidation, we suspended 16.2 nm of each ganglioside in 500 µl of water and added 100 µl of 0.04 M periodic acid solution. After thorough mixing, the solution was incubated at 37 °C for varying intervals and its colour compared with a control solution containing free sialic acids. Whereas the control solution reached its maximum colour almost immediately,

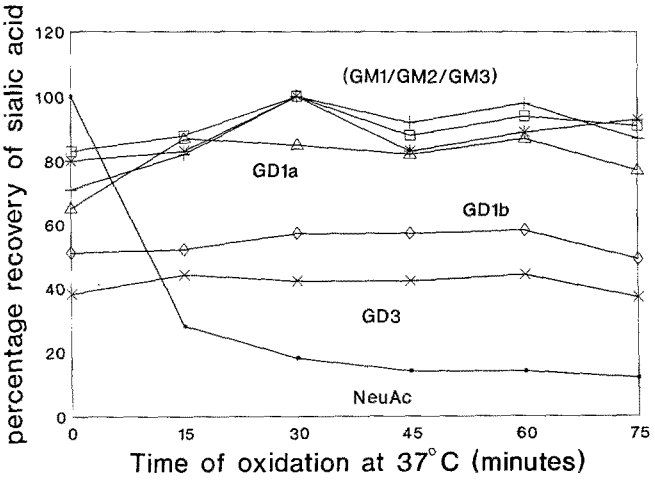

Figure 1. The influence of time of periodate oxidation at 37 °C on the percentage recovery of sialic acid. All gangliosides and *N*-acetyl neuraminic acid were tested at 16.2 nmol concentration. Note that recovery of sialic acids from $GM_1/GM_2/GM_3/GD_{1a}$ is 100%. In all these gangliosides sialic acids are linked to galactose by 2,3 linkage. GD_3 and GD_{1b} contain disialoresidues with sialic acid-α,2,8-sialic acid linkage. The recovery of sialic acids is not improved even after prolonged periodate oxidation.

gangliosides with α2,3 linkages (GM_1, GM_2, GM_3 and GD_{1a}: Table 1) required 30 min, and gangliosides with α2,8 linkages (GD_{1b} and GD_3) failed to reach the control maximum even after more than 60 min (Fig. 1).

We then conducted a parallel experiment to investigate whether ganglioside susceptibility to the periodate-resorcinol assay could be increased by prolonging the duration of resorcinol-HCl treatment. The results were inconclusive: colour yield decreased for solutions of GM_3 and GD_{1a}, remained unchanged for GD_3, and increased slightly for GD_{1b}, but did not reflect total recovery (Fig. 2).

Acid hydrolysis

Since sialic acids are released from glycosidic linkages by dilute acids, we treated each ganglioside with 0.0625 M sulfuric acid and heat (80 °C) for 1–2 h, following a modification recommended by Leeden and Yu [22] for thiobarbituric acid assay. The sialic acid α2,8 linkage in GD_3 was more resistant than that of GD_{1b} (Fig. 3). We recovered all sialic acids from GM_3, GD_{1a}, GD_{1b}, but only 60% of sialic acids from GD_3.

Enzymatic hydrolysis: alone or with base

Since acid hydrolysis was unsuccessful, we examined the alternative of enzyme hydrolysis. McGuire and Binkley [28] have observed that the release of sialic acid from bacterial colominic acid, a sialopolymer with α2,8 ketosidic linkage, is facilitated by saponification of the sialoconjugate prior to enzymatic hydrolysis. We, therefore, attempted to increase recovery of sialic acid from GD_3 by treating it with sialidase with or without a base. The results presented in

Table 2. Recovery of sialic acids after treatment with sialidase on untreated and base-treated (NH_4OH at 24 °C for 60 min) gangliosides. To 16.2 nmol of ganglioside, dried over N_2, 50 µl of NH_4OH (conc.) or water was added and incubated for 60 min. The ganglioside was dried over N_2 and washed with methanol and dried again. To the dried ganglioside, 20 mU of enzyme in 500 µl of buffer or 500 µl of buffer alone was added and incubated for 1 h at 37 °C. After enzymatic hydrolysis, the mixture was treated with periodate for 20 min on ice and assayed with resorcinol-HCl. The number of determinations or the sample size is given in parentheses.

Treatments	GM_3		GD_3		GD_{1b}	
	A630	% *recovery*	*A630*	% *recovery*	*A630*	% *recovery*
Untreated	0.082 ± 1.035^{-02} (5)	88	0.076 ± 4.868^{-03} (5)	41	0.133 ± 4.98^{-03} (5)	72
Base-treated	0.093 ± 1.845^{-02} (5)	100	0.093 ± 1.585^{-02} (5)	50	0.119 ± 0.011 (5)	64
Sialidase	0.089 ± 3.049^{-03} (5)	94	0.135 ± 4.098^{-03} (5)	73	0.174 ± 9.465^{-03} (4)	94
Base-treated + sialidase	0.087 ± 4.324^{-03} (5)	94	0.127 ± 7.701^{-03} (5)	68	0.169 ± 2.534^{-02} (5)	91

* We have used NH_4OH instead of NaOH because this base can be completely removed by N_2 gas, whereas NaOH, if used, often remains after drying and may affect the pH of the sialidase reaction mixture. Note that the absorbance for GM_3 has increased slightly after base treatment, suggesting that the pH of the sialidase reaction mixture is not affected sufficiently to reduce the efficiency of the enzyme.

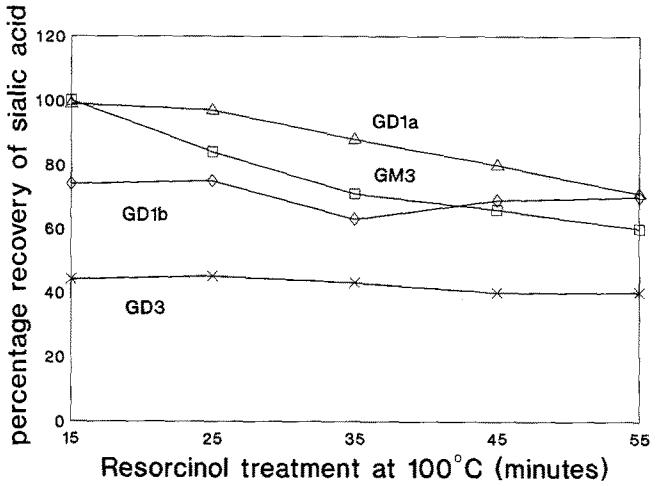

Figure 2. The influence of time of resorcinol-HCl treatment at 100 °C on the percentage recovery of sialic acid. Prolonged acid treatment destroys free sialic acids derived from GD_{1a} and GM_3. The recovery of sialic acid from GD_{1b} and GD_3 is not improved even after prolonged resorcinol-acid treatment.

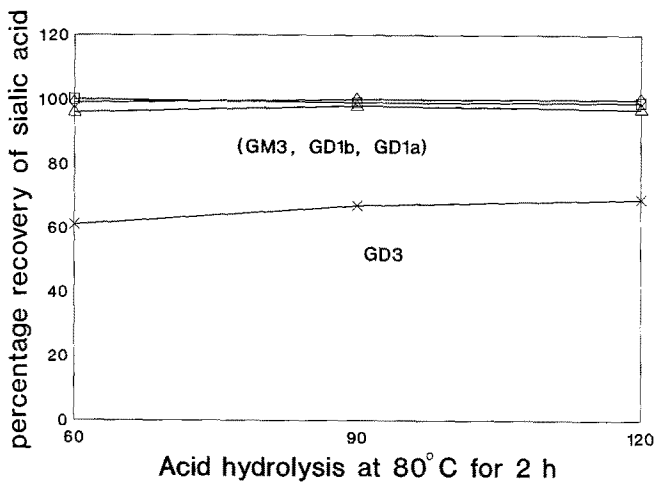

Figure 3. The influence of mild acid hydrolysis (0.0625 N H_2SO_4) on the percentage recovery of sialic acids. Sialic acids were recovered completely from GM_3, GD_{1a}, and GD_{1b}. Even prolonged acid treatment did not improve recovery of all sialic acids from GD_3.

Table 2 show that the percentage recovery of sialic acid did not exceed 73% for GD_3 and 94% for GD_{1b}. In fact, the base destroyed some of the free sialic acids formed by the enzymatic cleavage, resulting in lower values.

Enzymatic hydrolysis: with detergent(s) and calcium

Corfield *et al.* [29] reported that detergents may solubilize the gangliosides to enable the enzymatic cleavage of internal sialic acid. In order to maximize recovery of sialic acid from GD_3, we treated the ganglioside with *Vibrio cholerae* sialidase in the presence of a variety of detergents. No single detergent enabled better recovery of sialic acid from sialidase-treated GD_3 (Table 3). However, sialidase treatment in the presence of cholic acid or SDS increased recovery of sialic acid from GM_3 or GD_3, respectively. Sialidase treatment in the presence of cholic acid plus SDS (Table 4), further increased recovery of sialic acids from GD_3 (96%). In these experiments, we used a ganglioside-free mixture of the reagent as the blank to rule out the possible interference of other factors promoting the colour yield.

The recovery of sialic acid was best when the enzyme was suspended in 180 nmol of calcium.* A ten-fold increase in the calcium concentration of the reaction mixture did not enhance the colour yield. However, chelating calcium with an eight-fold excess of EDTA resulted in poor recovery of

* The enzyme was obtained from Boehringer-Mannheim and the calcium is part of the suspension provided by the manufacturer. Addition of calcium did not improve the assay, therefore no additional calcium is added. However, removal of calcium or introduction of factors that may chelate calcium may affect the performance of the method.

Table 3. Recovery of sialic acids after treatment with sialidase in the presence or absence of detergents. To 16.2 nmol of ganglioside dried over N_2, we added 200 µl of water or 200 µl of 1.6 µmol of detergent and 300 µl of 0.4 M sodium acetate buffer pH 5.5 or 20 mU of *Vibrio cholerae* sialidase in 280 µl of 0.4 M sodium acetate buffer, pH 5.5 and incubated at 37 °C for 2 h. After incubation, 100 µl of 0.04 M of periodic acid was added and incubated on ice for 20 min. Following which the resorcinol assay was carried out.

Treatments	GM_3		GD_3	
	A630	*% recovery*	*A630*	*% recovery*
Untreated	0.083 ± 3.055^{-03}	76	0.084 ± 2.000^{-03}	39
Sialidase alone	0.091 ± 7.024^{-03}	84	0.124 ± 4.509^{-03}	57
Sialase + PEG	0.095 ± 9.192^{-03}	87	0.118 ± 8.888^{-03}	54
Sialase + SDS	0.098 ± 3.125^{-03}	90	0.161 ± 7.071^{-03}	74
Sialase + Cholic	0.109 ± 2.081^{-03}	100	0.148 ± 6.557^{-03}	68
Sialase + Deoxycholic	0.087 ± 5.132^{-03}	80	0.140 ± 2.310^{-03}	64
Sialase + Taurocholic	0.107 ± 7.211^{-03}	98	0.148 ± 6.557^{-03}	68
Sialase + DeoxyTaurocholic	0.096 ± 5.774^{-04}	88	0.149 ± 3.215^{-03}	68
Sialase + Chapso	0.103 ± 4.163^{-03}	95	0.151 ± 2.646^{-03}	69
Sialase + Chaps	0.105 ± 4.933^{-03}	96	0.148 ± 5.567^{-03}	68

Table 4. Maximum recovery of sialic acids from disialoganglioside, GD_3, after treatment with *Vibrio cholerae* sialidase in the presence of detergent and optimal calcium concentration. To 16.2 nmol of ganglioside dried over N_2, 220 µl of water or 100 µl of 0.8 µmol of each detergent (SDS and cholic acid) with or without calcium or EDTA was added. Then either 280 µl of 0.4 M sodium acetate buffer, pH 5.5 or 20 mU of *Vibrio cholerae* neuraminidase in 280 µl of 0.4 M sodium acetate buffer pH 5.5 was added. The mixture was incubated and treated as described in Table 2. In this series of experiments, the blank used for each treatment is different in that it was the medium without ganglioside, but treated alike in all other respects. For example, the blank for the experiment involving the enzyme and detergent with EDTA, contained the same proportion of enzyme and detergent with EDTA but without the ganglioside.

Treatments	GM_3		GD_3	
	A630	*% recovery*	*A630*	*% recovery*
Untreated	0.0833 ± 1.078^{-02} (4)	100	0.0718 ± 9.946^{-03} (4)	43
Untreated + SDS + Cholic	0.0693 ± 2.045^{-02} (4)	83	0.0780 ± 6.928^{-03} (4)	47
Sialidase alone	0.0573 ± 4.856^{-03} (4)	68	0.1000 ± 8.286^{-03} (4)	60
Sialidase + SDS + Cholic + 0.18 µmol Ca^{2+}	0.0710 ± 2.000^{-03} (4)	86	0.1590 ± 2.449^{-03} (4)	96
Sialidase + SDS + Cholic + 1.80 µmol Ca^{2+}	0.0673 ± 3.304^{-03} (4)	71	0.1573 ± 2.987^{-03} (4)	95
Sialidase + SDS + Cholic + 1.0 µl EDTA	0.0590 ± 5.228^{-03} (4)	81	0.0835 ± 1.353^{-02} (4)	50

sialic acids. Therefore, it is obvious that two factors determine enzymatic cleavage of α2,8 ketosidic linkage: the combination of SDS and cholic acid and the presence of calcium (< 1.0 µmol). Results presented in Table 4 clearly demonstrate that α2,8 ketosidic linkage is resistant to *V. cholerae* sialidase in the absence of detergents and calcium.

Table 5 summarizes these results obtained with all other gangliosides under the conditions described in Table 4. These results indicate that the total LBSA for gangliosides with α2,3 and α2,8 ketosidic linkages can be measured by a periodate-resorcinol assay after treatment with *V. cholerae* sialidase in the presence of SDS, cholic acid and calcium.

Although our procedure recovered all sialic acids from di- and polysialogangliosides, Tables 3 and 4 show that there may be about 15% loss of GM_3. Such a loss may pose a problem for measuring gangliosides from tissues which express more than 75% GM_3, such as normal melanocytes [30]. Therefore, we have tested whether the loss can be minimized by lowering the duration of enzyme incubation from 2 to 1 h (Table 6). The results of time sequence study on enzyme incubation of gangliosides GM_3, GD_3, GD_2, and GD_{1b} reveal that 2 h treatment results in loss of sialic acids. This can be prevented easily by restricting the duration of enzyme treatment to 1 h, which is ideal for all gangliosides.

Table 7 shows the results obtained with GD_3 from bovine buttermilk, human brain and human melanoma cell line, M14. The results show that the recovery of sialic acids is significantly greater for GD_3 from all sources.

Table 5. Maximum recovery of sialic acids from different gangliosides after treatment with sialidase in the presence of detergents and optimal calcium concentrations. The experimental protocol is the same as in Table 3. 'Untreated' included water and buffer containing 0.180 μmol of calcium, whereas 'sialidase treated' included enzyme, buffer, 0.180 μmol of calcium and detergents.

Gangliosides (16.2 *nmol*)	*Untreated*		*Sialidase treated in the presence of detergents*	
	A630	*% recovery*	*A630*	*% recovery*
GM_3	0.083 ± 6.028^{-03}	100	0.068 ± 3.055^{-03}	82
GM_2	0.078 ± 2.000^{-03}	94	0.072 ± 2.517^{-03}	87
GM_1	0.083 ± 9.994^{-04}	100	0.083 ± 1.528^{-03}	100
GD_3	0.083 ± 5.033^{-03}	50	0.160 ± 6.083^{-03}	96
GD_{1b}	0.127 ± 1.528^{-03}	77	0.166 ± 4.582^{-03}	100

Table 6. Maximum recovery of sialic acids from different gangliosides after treatment with sialidase in the presence of detergents and optimal calcium concentration at varying incubation times. The experimental protocol is the same as in Table 3. 'Untreated' included detergent and buffer containing 0.180 μmol of calcium, whereas 'sialidase' treated include enzyme, buffer, 0.180 μmol of calcium and detergents. In all these experiments only gangliosides obtained from Sigma were used and the amount used is the same for all gangliosides (16.2 nmol). Each experiment was done in triplicate and mean ±SD are presented. The colour intensity was measured at A630.

Treatments	*GM_3*	*GD_3*	*GD_2*	*GD_{1b}*
Untreated + detergents (120 min)	0.057 ± 1.732^{-03}	0.055 ± 5.508^{-03}	0.081 ± 3.512^{-03}	0.105 ± 6.000^{-03}
Sialidase + detergents (30 min)	0.074 ± 3.464^{-03}	0.137 ± 2.309^{-03}	0.145 ± 4.000^{-03}	0.178 ± 4.041^{-03}
Sialidase + detergents (60 min)	**0.074 ± 6.506^{-03}**	**0.143 ± 5.033^{-03}**	**0.163 ± 4.000^{-03}**	**0.195 ± 2.645^{-03}**
Sialidase + detergents (90 min)	0.068 ± 5.774^{-04}	0.138 ± 4.933^{-03}	0.156 ± 1.526^{-03}	0.187 ± 4.933^{-03}
Sialidase + detergents (120 min)	0.062 ± 2.000^{-03}	0.132 ± 1.731^{-03}	0.151 ± 1.001^{-03}	0.179 ± 5.758^{-04}

Table 7. Maximum recovery of sialic acids from GD_3 obtained from different sources. The experimental protocol was carried out simultaneously with the experiments reported in Table 6.

Source of GD_3	*Amount used**	*Untreated + detergents (120 min)*	*Sialidase + detergents (120 min)*
Bovine buttermilk (Sigma)	25 μg	0.055 ± 5.508^{-03}	0.132 ± 1.731^{-03}
Human brain (Adv. Imm. Chem.)	40 μg	0.170 ± 8.622^{-03}	0.370 ± 9.001^{-03}
Human melanoma (M14-AL)	5.8 μg	0.016 ± 0	0.050 ± 3.464^{-03}

* The amount used is based on the amount indicated by respective manufacturer.

Discussion

Our findings confirm an earlier report [22] that α2,8 sialic acid linkages in glycolipids are resistant to periodic acid oxidation. We have shown that the α2,8 sialic acid linkage of GD_3 cannot be dissociated by prolonged treatment in resorcinol-HCl assay or by weak or strong acid hydrolysis. Interestingly, the disialyl residues of GD_{1b} were dissociated by weak (<0.1 N) acid hydrolysis, probably because of the orientation of the disialyl residues of GD_{1b} due to the attachment of Gal-GalNAc residues to the same galactose to which the disialyl residues are attached. Data obtained with reference to GD_2 (Table 6) also support the above contention. Based on our data, we interpret the different behaviour of GD_3, GD_2 and GD_{1b} could be due to the differences in the angle of orientation of the disialyl residues among these gangliosides. The angle of orientation may possibly be dependent on the *N*-acetylgalactosamine or Gal-GalNAc residues. We found that the α2,8 ketosidic linkages of the disialyl residues of both GD_3 and GD_{1b} were susceptible to *Vibrio cholerae* sialidase in the presence of calcium and detergents. Removal of calcium or detergent, or both, significantly affects sialidase action on the disialyl residues of glycolipids.

The combination of cholic acid and SDS detergents was superior to any single detergent. Possibly cholic acid solubilizes the gangliosides, exposing the hydrophilic sites for enzymatic recognition, while SDS, a protein detergent, may enhance the efficacy of the sialidase. It is interesting that removing Ca^{2+} from a reaction mixture arrested this function. The optimal concentration of Ca^{2+} in our assay system was 180 nm per 20 mU of enzyme. A ten-fold excess of calcium did not augment the release. With the modifications introduced in this investigation, we are confident that the periodate-resorcinol assay can be used for measuring total LBSA, particularly those containing high levels of gangliosides with α2,8 ketosidic linkages.

Only an assay that measures all sialic acids of the gangliosides, will provide the true picture of total LBSA. Recently it has been shown that the ratio GM_3:GD_3 varies markedly among tumour biopsies obtained from melanoma patients [31]. In some patients, the GD_3 level exceeds two- to five-fold that of GM_3. In analysis of the LBSA of these tissue samples, it is important to recover both sialic acids of GD_3.

Based on this investigation, we recommend the following protocol for measuring less than 20 nm of gangliosides in biological samples.

(1) Dissolve the extracted ganglioside or standard ganglioside in ethanol and dry over nitrogen.

(2) Add 100 µl each of 0.8 µM SDS and 0.8 µM cholic acid. Sonicate and vortex for 3 min.

(3) Add 20 µl of *Vibrio cholerae* sialidase (20 mU) suspended in 280 µl of 0.4 M sodium acetate buffer containing 50 mg human albumin and 300 mM NaCl, pH 5.5. This mixture contains 180 nmol of calcium. Vortex and incubate at 37 °C for 1 h on a shaker.

(4) Place the reagent mixture on ice, add 100 µl of ice-cold 0.04 M periodic acid, mix and incubate for 20 min.

(5) Add resorcinol-HCl reagent (1.25 ml), mix and place it on an ice bath for 5 min and then incubate at 100 °C for 15 min.

(6) Cool the tube at 37 °C for 5 min and add 1.25 ml of 95% *tert*-butanol and mix well to form a single phase; keep at 37 °C for 5 min to stabilize the colour.

(7) Measure the absorbance at 630 nm against a blank containing all the respective reagents except gangliosides. It is important to run standards of GM_3 and GD_3 separately and compare the values with the standards.

Using the above protocol, we are able to recover all sialic acids and measure total LBSA from the di- and polysialogangliosides in the sera of melanoma patients. However, the problem associated with determination of serum gangliosides is the separation of gangliosides from proteins and glycopeptides. We are now developing strategies to overcome this problem.

Acknowledgements

We wish to thank Miss Gwen Berry and Dr Amy Walsh for critically reading and editing the manuscript. We thank Ms Ann Tolles, Advanced Immunochemical Services, for the gift of human brain GD_3. This work is supported by munificent funds from The Concern Foundation for Cancer Research and The John Wayne Cancer Institute for Research and Treatment (MHR) and NCI PO1 grant CA 12582 (DLM & RFI).

References

1. Mabry EW, Carubelli R (1972) *Experientia* **28**:182–83.
2. Horgan IE (1982) *Clin Chim Acta* **118**:327–31.
3. Ravindranath MH, Irie RF (1988) In *Malignant Melanoma: Biology, Diagnosis, and Therapy* (Nathanson L, ed.) pp. 14–17. Boston: Kluwer Academic.
4. Ladisch S, Wu ZL, Feig S, Ulsh L, Schwartz E, Floutsis G, Wiley F, Lenarsky C, Seeger C (1987) *Int J Cancer* **39**:73–76.
5. Narasimhan R, Murray RK (1979) *Biochem J* **179**: 199–211.
6. Hogan-Ryan A, Fennelly JJ, Jones M, Cantwell B, Buffy MJ (1980) *Br J Cancer* **41**:587–92.
7. Goff B, Lee WMF, Westerick MA, Macher BA (1983) *Eur J Biochem* **130**:553–57.
8. Tsuchida T, Otsuka H, Niimura M, Inoue Y, Kukita A, Hashimoto Y, Seyama Y, Yamakawa T (1984) *J Dermatol* **11**:129–36.
9. Bouchon B, Portoukalian J, Bornet H (1985) *Biochem Int* **10**:531–38.
10. Fredman P, van Holst H, Collins VP, Ammar A, Delheden B, Wahren B, Granholm L, Svennerholm L (1986) *Neurol Res* **8**:123–26.
11. Berra B, Gaini SM, Ribino L (1985) *Int J Cancer* **36**:363–66.
12. Berra BM, Riboni L, De G, Gaini SM, Ragnotti G (1983) *J Neurochem* **40**:777–82.
13. Sunder-Plassman M, Bernheimer H (1974) *Acta Neuropathol (Berl)* **27**:289–94.
14. Westrick A, Lee WMF, Goff B, Macher BA (1983) *Biochim Biophys Acta* **750**:141–45.
15. Portoukalian J, Zwingelstein G, Abdul-Malak N, Dore JF (1978) *Biochem Biophys Res Commun* **85**:916–20.
16. Senn H, Orth M, Fitzke E, Koster W, Wieland H, Gerok W (1992) *Atherosclerosis* **94**:109–17.
17. Ravindranath MH, Morton DL (1991) *Intern Rev Immunol* **7**:303–29.
18. Grayson G, Ladisch S (1992) *Cell Immunol* **139**:18–29.
19. Schauer R (1987) *Method Enzymol* **138**:132–61.
20. Spiro RG (1964) *J Biol Chem* **239**:567–73.
21. Jourdian GW, Dean L, Roseman S (1971) *J Biol Chem* **246**:430–35.
22. Ledeen RW, Yu RK (1982) *Methods Enzymol* **83**:139–91.
23. Tsuchida T, Ravindranath MH, Saxton RE, Irie RF (1987) *Cancer Res* **47**:1278–81.
24. Tsuchida T, Saxton RE, Morton DL, Irie RF (1989) *Cancer* **63**:1166–74.
25. Senn H, Orth M, Fitzke E, Wieland H, Gerok W (1989) *Eur J Biochem* **181**:657–62.

26. Takamizawa K, Iwamori M, Mutai M, Nagai Y (1986) *J Biol Chem* **261**:5625–30.
27. Ren S, Scarsdale JN, Ariga T, Zhang Y, Klein RA, Hartmann R, Kushi Y, Egge H, Yu RK (1992) *J Biol Chem* **267**: 12632–38.
28. McGuire EJ, Binkley SB (1964) *Biochemistry* **3**:247–50.
29. Corfield AP, Schauer R, Dorland L, Vliegenthart JFG, Wiegandt H (1985) *J Biochem (Tokyo)* **97**:449–54.
30. Carubia JM, Yu RK, Macala LJ, Kirkwood JM, Varga JM *et al.* (1984) *Biochem Biophys Res Commun* **120**:500–4.
31. Ravindranath MH, Tsuchida T, Morton DL, Irie RF (1991) *Cancer* **67**:3029–35.