*Glycoconjugate Journal* (1996) **13:** 347–352

# Evaluation of the efficiency of an assay procedure for gangliosides in human serum

ELENA NEGRONI, VANNA CHIGORNO, GUIDO TETTAMANTI and SANDRO SONNINO*

*Department of Medical Chemistry and Biochemistry, The Medical School, University of Milan, Via Saldini. 50, 20133 Milano, Italy*

Received 30 June 1995, revised 27 October 1995

An efficiency assessment of a ganglioside assay procedure was carried out on human serum gangliosides from healthy subjects of different sex and age. The analysis of the gangliosides, extracted with chloroform/methanol and purified by lipid partitioning, ion exchange column chromatographic separation and desalting procedures as described by Senn *et al.* (1989) *Eur J Biochem* **181**: 657–62, was performed by HPTLC followed by densitometric quantification. The yield of the procedure, expressed as radioactivity recovery, was determined by adding GM3 ganglioside, tritium labelled at the sialic acid acetyl group and at the C3 position of sphingosine, to the lyophilized serum or by associating it with the serum lipoproteins. In spite of the fact that the extraction and purification procedures were performed exactly as described we found the radioactivity recovery to be variable (25–50%) and much lower than that proposed. Much of the radioactivity was found in the organic phase after lipid partitioning, whilst all the ganglioside purification steps led to some further loss. After the introduction of some modifications to the procedure the recovery improved, reaching 67–79%.

The analyses on 33 samples of 5 ml showed a human serum ganglioside content of about 10 nmol $ml^{-1}$ (as corrected for the recovery), and confirmed that GM3 ganglioside is the main component of the total serum ganglioside mixture.

*Keywords*: gangliosides, human serum, extraction, recovery

*Abbreviations*: Ganglioside nomenclature is in accordance with Svennerholm (1980) [37] and the IUPAC-IUB Recommendations (1977, 1982) [38]. GM3, $II^3$Neu5AcLacCer, α-Neu5Ac-(2-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer; Cer, ceramide; Neu5Ac, *N*-acetyl-neuraminic acid; *erythro*-GM3, GM3 containing *erythro*-sphingosine; *threo*-GM3, GM3 containing *threo*-sphingosine; *erythro*-C18 sphingosine, (2*S*,3*R*,4*E*)-2-amino-1,3-dihydroxy-octadecene; *erythro*-C20 sphingosine, (2*S*,3*R*,4*E*)-2-amino-1,3-dihydroxy-eicosene; *threo*-C18 sphingosine, (2*S*,3*S*,4*E*)-2-amino-1,3-dihydroxy-octadecene; *threo*-C20 sphingosine, (2*S*,3*S*,4*E*)-2-amino-1,3-dihydroxy-eicosene; DDQ, dichloro-dicyano-benzoquinone.

## Introduction

Gangliosides [2], sialic acid containing glycosphingolipids, are components of vertebrate cell plasma membranes. In malignant cells ganglioside structure and content have been shown to be modified, and it is now recognized that such modifications probably contribute to the aberrancy of tumour cells [3].

In human serum gangliosides are present in low amounts and are components of lipoproteins [1, 4–7]. During tumour development the concentration of total serum gangliosides (or of some ganglioside species) has been reported to increase, as a consequence of ganglioside shedding from tumour cell membrane [7–14]. In normal human serum the ganglioside content, as ganglioside-bound sialic acid has been reported, depending on the assay method and the laboratory, to be from 6 to 28 nmol $ml^{-1}$ [1, 7–20]. This variability makes a statistical assessment of the differences associated with tumour pathology difficult.

Considering that the procedures for ganglioside extraction, purification and quantification vary greatly from one laboratory to another this high variability is not surprising. Furthermore, no priority has been given to

*To whom correspondence should be addressed.

assessing the final yield. In this paper we show that ganglioside recovery using the methodology proposed by Senn *et al.* [1] for ganglioside serum extraction and purification is much lower than has been reported.

We evaluated the recovery of the serum ganglioside extraction/purification procedure by adding radioactive GM3 ganglioside, isotopically tritium labelled at the sialic acid acetyl group and at the 3 position of sphingosine to the serum, directly to the lyophilized serum or associating it with the serum lipoproteins.

## Materials and methods

### *Materials*

The commercial chemicals were the purest available, common solvents were distilled before use and deionized water was purified with the MilliQ system (Millipore).

Serum specimens from healthy donors were collected and frozen at −20 °C until assayed.

Ganglioside GM3 was extracted [21] from calf brain, purified and characterized [22]. [$^3$H-*Neu5Ac*]GM3, containing tritium at the sialic acid acetyl group, was prepared as previously reported [22–23]. [$^3$H-*Sphingosine*]GM3 containing tritium at position 3 of sphingosine was prepared by properly adapting the dichloro-dicyanobenzoquinone (DDQ)/sodium boro [$^3$H]hydride and reversed phase HPLC purification [24] procedures already applied to other gangliosides [25]. The main modifications introduced into the original procedures were: i) the 3-keto-GM3, obtained by oxidation of GM3 with DDQ, was purified by precipitation with acetonitrile instead of acetone; ii) the radioactive *erythro*-GM3 was separated from the unnatural *threo*-GM3, a by-product of the reduction reaction [24], by reversed phase HPLC performed at 45 °C instead of room temperature. The experimental chromatography conditions were: 2.5 cm × 25 cm LiChrophere reversed phase RP18-column (Merck, Germany) equilibrated and eluted with the solvent system acetonitrile: 5 mM phosphate buffer, pH 7.0, 60:40 by vol, at a flow rate of 10 ml min$^{-1}$; the elution profile was monitored by flow-through detection of both the UV absorbance at 195 nm and the radioactivity content.

### *Human serum lipid extraction and fractionation*

Human serum was subjected to ganglioside extraction and purification. We carried out two parallel procedures – one in strict accordance with the procedure proposed by Senn *et al.* [1], the other modified as follows. Five ml of human serum were lyophilized and the dried residue suspended in 1 ml of 10 mM potassium-phosphate buffer pH 6.8, vortexed and sonicated for 2 min. After addition of 4 ml of tetrahydrofuran and homogenization (5 min) the suspension was centrifuged at 10 000 × **g** for 10 min at 4 °C. The pellet was treated three more times with 0.5 ml of the same above buffer and 2 ml of tetrahydrofuran. The pooled supernatants were dried and the residue suspended in 3.6 ml of chloroform:methanol, 1:1 by vol, vortexed and sonicated for 6 min. The suspension was centrifuged at 2000 × **g** for 20 min at 4 °C, left overnight at 4 °C and centrifuged again at 10 000 × **g** for 20 min at 4 °C. The supernatant was dried and suspended in 5.6 ml of diisopropyl ether:butan-1-ol, 3:2 by vol, vortexed and sonicated for 5 min. After addition of 2.8 ml of 50 mM NaCl and vortexing for 10 min, the suspension was centrifuged at 2000 × **g** for 10 min at room temperature. The aqueous phase was washed with the same organic solvent system and the organic phase added to the previous one. The organic phase was sequentially washed with a half volume of 50 mM NaCl, 25 mM NaCl and water. The aqueous phases were collected and purified by the Sep-Pak C18 and DEAE-Sephadex procedures as reported in [1].

### *Addition of tritium labelled GM3 to the serum*

Two procedures were used to add GM3 to the serum. i) 20–50 pmol of radioactive GM3, in 20–50 μl of propan-1-ol:water, 7:3 by vol, were added to the lyophilized serum; ii) a solution of chloroform:methanol, 2:1 by vol, containing 8 pmol of radioactive GM3 and 352 pmol of egg phosphatidylcholine was dried and the lipid film suspended in 1 ml of serum by vortexing; the serum was sonicated for up to 15 min at 4 °C.

### *Analytical procedure*

HPTLC separation of the gangliosides contained in the serum extract was performed at room temperature on 10 × 10 cm silica gel plates with the solvent system chloroform:methanol:water, 110:40:6 by vol, and after plate drying with the solvent system chloroform:methanol:0.2% aqueous $CaCl_2$, 50:42:11 by vol. Gangliosides were detected using a *p*-dimethylaminobenzaldehyde spray reagent [26] and densitometrically quantified as reported [27]. Identification of serum gangliosides was accomplished using reference gangliosides of known structure prepared as reported [19].

Gangliosides were assayed as bound Neu5Ac by the resorcinol-HCl method [28–29], pure Neu5Ac being used as the reference standard.

HPTLC of radioactive GM3 was performed at room temperature on aluminium silica gel plates with the following solvent systems: chloroform:methanol:0.2% aqueous $CaCl_2$, 50:42:11 by vol, chloroform:methanol:water, 110:40:6 by vol, and chloroform:methanol, 2:1 by vol.

The GM3 associated radioactivity, detected by fluorography, was quantified by radiochromatoscanning (Digital Autoradiograph, Berthold, Germany).The lipid fraction

radioactivity was determined by liquid scintillation counting.

The association of radioactive GM3 ganglioside to serum lipoproteins was determined by cellulose acetate electrophoresis followed by radiochromatoscanning.

The GM3 ceramide composition was determined by gas-chromatography [24].

## Results

The purity of [$^3$H-*Neu5Ac*]GM3 and [$^3$H-*Sphingosine*]GM3 was over 99.5%, as determined by digital radiochromatoscanning of HPTLC plates developed in three solvent systems; specific radioactivity was 2.3 Ci mol$^{-1}$ and 2 Ci mmol$^{-1}$, respectively.

Due to the low solubility of GM3 in the acetonitrile used in HPLC purification we modified the original procedure [24–25] to prepare [$^3$H-*Sphingosine*]GM3. All the chromatographic procedural steps – sample preparation, loading into the injector-loop and chromatographic separation – were performed at 45 °C. Figure 1 shows the reversed phase HPLC separation of radiolabelled GM3. Four peaks, completely resolved, are present. The four peaks were identified, from the shorter to the longer retention times, as the molecular species containing the C18 2S,3R-sphingosine (*erythro*), the C18 2S,3S-sphingosine (*threo*), the C20 2S,3R-sphingosine (*erythro*) and the C20 2S,3S-sphingosine (*threo*). The four species contained mainly stearic acid (over 95% of the total fatty acid content). The molecular species containing *erythro*-C18 sphingosine was used in this work.

In normal human serum gangliosides are components of serum lipoproteins [1, 4–7] and GM3 is the main component. Therefore we chose radioactive GM3 to follow the ganglioside recovery throughout the extraction and purification procedures. A very minor amount of

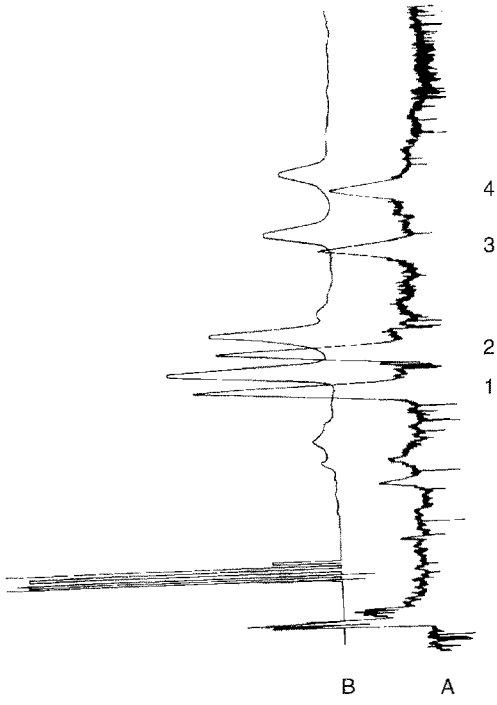


**Figure 1.** HPLC separation of the GM3 ganglioside molecular species tritium labelled at position 3 of long chain base. Chromatographic conditions: RP18 LiChrophere column 4 × 25 cm; flow rate 10 ml min$^{-1}$; solvent system: acetonitrile:5 mM sodium phosphate buffer, pH 7, 3:2 by vol; injector and column temperature: 45 °C; trace A: UV detection at 195 nm; trace B: radioactivity detection throughout solid scintillator cells (Berthold LB503). The four peaks were identified as the molecular species of GM3 containing stearic acid and: 1) *erythro*-C18 sphingosine; 2) *threo*-C18 sphingosine; 3) *erythro*-C20 sphingosine; 4) *threo*-C20 sphingosine. The UV absorption and the radioactivity counting elution profiles were automatically shifted by the recorder, and each one of the four peaks showed the same UV and radioactivity retention time.

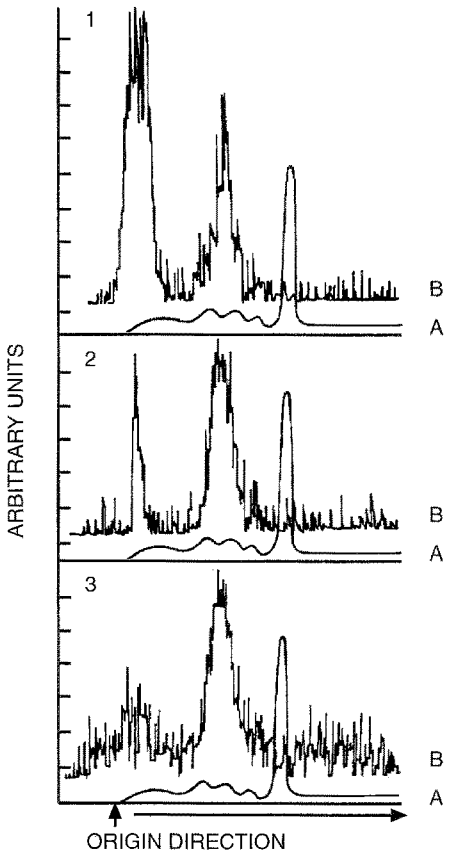


**Figure 2.** Cellulose acetate electrophoresis of human serum proteins after addition of [$^3$H-*Neu5Ac*]GM3 to the serum, as reported in the Methods section. 1) control, zero time of sonication; 2) after 10 min of sonication; 3) after 15 min of sonication. A) human serum protein pattern. B) radioactivity detection; the radioactive peak present at the origin corresponds to free GM3, the other one overlaps the serum lipoproteins.

radioactive GM3, usually <0.1–0.2% of the total endogenous serum ganglioside content, was added to either fresh or lyophilized serum.

Direct addition of GM3 to serum leads to only trace amounts of the ganglioside becoming associated with the serum lipoproteins, and the formation of stable complexes with albumin [30]. Therefore we developed a procedure to associate exogenous radioactive GM3 to serum lipoproteins (Fig. 2). When this procedure was followed the exogenous GM3 associated very rapidly with the lipoproteins.

It should be pointed out here that in [1] the authors added radioactive ganglioside to the total lipid extract whereas we added it to the serum prior to extraction. In this way we were able to monitor the ganglioside recovery throughout the extraction and purification procedures.

On assessing the radioactivity recovery in the total lipid extract from serum obtained according to [1] and to our modified procedure we found similar results, irrespective of the position of the GM3 radionuclide or of the way the radioactive GM3 was associated with the serum (Table 1). In [1] the extraction solvent system adopted is chloroform/methanol, whilst we used a tetrahydrofuran/phosphate buffer system. Both systems gave similar high variable yields (88–99%) (Table 1). In [1] the total radioactivity loss is stated to be 17%, leading one to consider a recovery of 83%. When we applied the same extraction/purification procedures the final recovery was much lower than 83% and varied greatly, ranging from 25–50% (Table 1).

An analysis of the purification revealed that most of the radioactivity had remained in the organic phase after partitioning and further losses occurred with each purification step. The organic phase was shown to contain only GM3 radioactivity, regardless of the type of labelling, thus excluding any GM3 degradation. The partition of GM3 into the organic phase could be related to a too high salt concentration of the partitioning system, as suggested by Ladisch and Gillard, 1987 [31].

After some modifications (see Materials and methods) the final recovery improved, but was still far from satisfactory, reaching 67–79%.

We applied our modified procedure to 33 human serum samples and found that the ganglioside content varied greatly, from 4 to 8.9 nmol $ml^{-1}$ serum (not corrected for the recovery). Tables 2 and 3 show the results grouped according to age and sex. GM3 was the main component, 51.4–69%, of the serum ganglioside mixture.

## Discussion

It has been suggested that an increased ganglioside content in human blood serum could be associated with

**Table 1.** Recovery of added [$^3$H]GM3 following extraction and purification of human serum gangliosides. In samples 6 and 7, and 10–15 the [$^3$H]GM3 was previously associated to serum lipoproteins; in the other samples [$^3$H]GM3 was added to the lyophilized serum. [$^3$H-*Neu5Ac*]GM3 was added to samples 1–3, and 8–13; [$^3$H-*Sphingosine*]GM3 was added to samples 4–7, and 14–15.

| *Sample (No)* | *Serum (ml)* | *[$^3$H]GM3 added (dpm)* | *Recovered radioactivity in the* | | | | *Total recovery (%)* |
|---|---|---|---|---|---|---|---|
| | | | *Lipid extract* | *Organic phase* | *Aqueous phase* | *Purified gangliosides* | |
| Extraction according to Senn *et al.* [1] | | | | | | | |
| 1 | 14 | 102700 | ND | 27700 | ND | 26070 | 25.4 |
| 2 | 14 | 102700 | ND | 27800 | ND | 31310 | 30.5 |
| 3 | 14 | 102700 | ND | 31900 | ND | 23770 | 23.1 |
| 4 | 14 | 149550 | 131660 | 56000 | 60940 | 53070 | 35.5 |
| 5 | 14 | 149550 | 140460 | 52300 | 83854 | 70730 | 47.3 |
| 6 | 14 | 323610 | 320130 | 127400 | 141700 | 137080 | 42.4 |
| 7 | 14 | 323610 | 320060 | 114500 | 185549 | 160100 | 49.5 |
| Extraction as reported in the Methods section | | | | | | | |
| 8 | 5 | 108000 | 103570 | 8740 | 74420 | 72750 | 67.4 |
| 9 | 5 | 108000 | 100245 | 6300 | 80280 | 73680 | 68.2 |
| 10 | 5 | 106378 | 104280 | 3240 | ND | 84900 | 79.8 |
| 11 | 5 | 106378 | 97580 | 3800 | 84960 | 76740 | 72.1 |
| 12 | 5 | 106378 | 102520 | 5200 | 85580 | 80790 | 75.9 |
| 13 | 5 | 106378 | 101880 | 4970 | 86590 | 80920 | 76.1 |
| 14 | 5 | 133000 | 127280 | 2130 | 114220 | 103140 | 77.5 |
| 15 | 5 | 133000 | 123130 | 2800 | 113800 | 98240 | 73.9 |

ND, not determined

**Table 2.** Ganglioside content in human serum. Gangliosides were extracted and purified from 5 ml of serum from human males, M, and females, F. Data are averages of *n* samples ± SD, and are grouped according to age and sex.

| *Age group (years)* | *Sex* | n | *Lipid-bound sialic acid (nmol ml$^{-1}$ serum)* |
|---|---|---|---|
| 20–30 | F | 5 | 6.8 ± 1.3 |
| 20–30 | M | 7 | 5.7 ± 1.8 |
| 31–40 | F | 5 | 7.2 ± 1.7 |
| 31–40 | M | 3 | 6.0 ± 1.1 |
| 41–50 | F | 3 | 7.2 ± 0.8 |
| 41–50 | M | 4 | 7.9 ± 1.0 |
| 51–60 | F | 3 | 7.8 ± 0.7 |
| 51–60 | M | 3 | 6.6 ± 1.4 |

cancer [7–20]. Thus the availability of reliable methods for an accurate determination of human serum gangliosides has become very important.

Ladisch and Gillard [15] reported a procedure for the isolation and quantification of gangliosides in the serum of healthy individuals. This procedure was then modified by Senn *et al.* [1] who added radioactive GM3 and GM1 to the total lipid extract to determine the final yield of the procedure. The method of Ladisch and Gillard [15], which is used in some laboratories [18, 32] was never tested by us. We tested the method of Senn *et al.* [1], and in spite of the fact that the procedure was applied exactly as described and with no modifications, it did not lead to reproducible results, the recovery being low and variable. To analyse the yield of the procedure we added, to the serum, highly purified radioactive GM3 ganglioside containing tritium on the sialic acid or sphingosine moieties. We also developed a procedure to associate GM3, added to the serum, to lipoproteins. All the experimental models we used led to similar results.

The contrast in the results could be explained by the following considerations.

Apart from the big difference we found in the radioactivity content of the organic phase after lipid partitioning, despite repeatedly washing the tubes, Sep-Pack C18 cartridges and the DEAE-Sephadex column, we could not recover all the added radioactivity; 2–5% of the total added radioactivity appeared to remain adsorbed or stuck to surfaces at each step of the procedure. This is not surprising as it must be remembered that gangliosides are amphiphilic compounds, and at very low concentration in aqueous solutions the concentration of free monomers becomes percentually higher, and thus more relevant, than the ganglioside aggregate concentration [33]. In organic solvents gangliosides are present only as monomers. Such monomers bind to surfaces like natural membranes [34], synthetic polymers [35] and the plastic and glass surfaces of laboratory equipment. As a further example note the ELISA experiments: microtitre plate wells are coated with gangliosides by simply adding ethanol solutions of gangliosides [36].

Moreover in [1] all the radioactivity data were expressed as *cpm*, and the measurements were made with a dual-label programme in the presence of $^{14}C$. *Cpm*, in contrast to *dpm*, are strictly related to the efficiency of the system, which depends on the components present in the final counting solution.

The radioactive GM3 used in [1] was prepared using a sialyltransferase-enriched Golgi membrane fraction, lactosylceramide and radioactive CMP-sialic acid. The specific radioactivity of CMP-sialic acid was quite high, but no data on the final specific radioactivity of the biosynthesized GM3 were reported. If this value was low or very low the amount of added ganglioside would be high and could, in part, explain a low loss of radioactivity. It is our opinion that the ganglioside recovery is mainly related to ganglioside concentration. This is further suggested by the higher ganglioside recovery obtained in the few experiments we carried out starting from a minimum 20 ml of serum (data not shown).

Our conclusion is that the described procedures are

**Table 3.** Ganglioside distribution in human serum. Gangliosides were extracted from the serum of human males, M, and females, F. Data, expressed as ganglioside-bound sialic acid % of the total, are averages of *n* samples ± SD, and are grouped according to age and sex.

| *Age years* | *Sex* | n | *GM3 (%)* | *GM2 + MG3 (%)* | *MG4 (%)* | *GD3 (%)* | *GD1a (%)* | *GD1b (%)* | *GT1b (%)* |
|---|---|---|---|---|---|---|---|---|---|
| 20–30 | F | 5 | 56.8 ± 4.3 | 6.1 ± 1.9 | 5.2 ± 1.7 | 19.6 ± 1.6 | 6.6 ± 0.8 | 1.5 ± 1.2 | 4.2 ± 0.5 |
| 20–30 | M | 7 | 62.3 ± 4.5 | 5.2 ± 0.2 | 4.0 ± 1.8 | 17.6 ± 1.3 | 7.1 ± 1.5 | 0.7 ± 0.5 | 3.2 ± 1.5 |
| 31–40 | F | 5 | 60.3 ± 7.3 | 4.6 ± 0.8 | 5.9 ± 4.5 | 18.4 ± 1.6 | 5.7 ± 0.9 | 1.1 ± 1.0 | 3.9 ± 0.8 |
| 31–40 | M | 3 | 64.1 ± 3.9 | 4.2 ± 0.8 | 3.9 ± 0.8 | 17.2 ± 1.4 | 5.8 ± 0.6 | 1.0 ± 1.5 | 3.6 ± 1.4 |
| 41–50 | F | 3 | 55.4 ± 3.2 | 5.8 ± 0.8 | 6.6 ± 1.0 | 21.3 ± 1.3 | 4.9 ± 0.2 | 1.5 ± 1.1 | 4.4 ± 1.1 |
| 41–50 | M | 4 | 63.9 ± 2.4 | 4.1 ± 0.2 | 3.8 ± 0.3 | 19.1 ± 0.8 | 6.1 ± 1.2 | 0.7 ± 0.5 | 3.4 ± 0.8 |
| 51–60 | F | 3 | 56.7 ± 5.3 | 6.0 ± 0.7 | 4.5 ± 0.5 | 20.8 ± 1.2 | 7.0 ± 2.4 | 0.9 ± 1.3 | 3.9 ± 0.8 |
| 51–60 | M | 3 | 66.6 ± 2.4 | 4.7 ± 0.5 | 4.0 ± 0.4 | 18.1 ± 0.4 | 4.3 ± 0.6 | 0.2 ± 0.3 | 2.1 ± 1.5 |

still far from satisfactory and, given the difficulty of the procedures, still not appropriate for routine use.

## Acknowledgements

This work was partially supported by grants from the Consiglio Nazionale delle Ricerche (CNR), Italy (Grant 93.02246.PF39 Target Project 'ACRO').

## References

1. Senn H-J, Orth M, Fitzke E, Wieland H, Gerok W (1989) *Eur J Biochem* **181**: 657–62.
2. Wiegandt H (1985) *New Compr Biochem* **10**: 199–266.
3. Hakomori S-I (1981) *Ann Rev Biochem* **50**: 733–64.
4. Dawson G, Kruski AW, Scanu AM (1976) *J Lipid Res* **17**: 125–31.
5. Clarke JTR (1981) *Canad J Biochem* **59**: 412–17.
6. Chatterjee S, Kwiterovich PO (1984) *Canad J Biochem Cell Biol* **62**: 385-97.
7. Valentino LA, Ladisch S (1992) *Cancer Res* **52**, 810–14.
8. Shaposhnikova GI, Prokazova NV, Buznikov GA, Zvezdina ND, Teplitz NA, Bergelson LD (1984) *Eur J Biochem* **140**: 567–70.
9. Valentino L, Moss T, Olson E, Wang HJ, Elashoff R, Ladish S (1990) *Blood* **75**: 1564–67.
10. Portoukalian J, David MJ, Shaen X, Richard M, Dubreuil C (1989) *Biochem Int* **18**: 759–65.
11. Bernhard H, Meyer zum Buschenfelde KH, Dippold WG (1989) *Int J Cancer* **44**: 155–60.
12. Kloppel TM, Keenan TW, Freeman MJ, Morre' DJ (1977) *Proc Nat Acad Sci USA* **74**: 3011–13.
13. Portoukalian J, David MJ, Gain P, Richard M (1993) *Int J Cancer* **53**: 948–51.
14. Schwartz PE, Chambers SK, Chambers JT, Gutmann J, Katopodid N, Foemmel R (1987) *Cancer* **60**: 353–61.
15. Ladisch S, Gillard B (1985) *Anal Biochem* **146**: 220–31.
16. Igashi H, Hirabayashi Y, Hirota M, Matsumoto M, Kato S (1987) *Jpn J Cancer Res* **78**: 1309–13.
17. Horgan IE (1982) *Clin Chim Acta* **118**: 327–31.
18. Ayabe M, Scishichijo S, Yokoyama MM (1989) *J Clin Lab Anal* **3**: 301–6.
19. Kundu SK, Diego I, Osovitz S, Marcus DM (1985) *Arch Biochem Biophys* **238**: 388–400.
20. Gornati R, Rapelli S, Montorfano G, Cattaneo C, Berra B (1991) *Int J Biol Markers* **6**: 91–98.
21. Tettamanti G, Bonali F, Marchesini S, Zambotti V (1973) *Biochim Biophys Acta* **296**: 160–70.
22. Sonnino S, Cantu' L, Acquotti D, Corti M, Tettamanti G (1990) *Chem Phys Lipids* **52**: 231–41.
23. Sonnino S, Kirschner G, Ghidoni R, Acquotti D, Tettamanti G (1985) *J Lipid Res* **26**: 248–57.
24. Sonnino S, Ghidoni R, Gazzotti G, Kirschner G, Galli G, Tettamanti G (1984) *J Lipid Res* **25**: 620–29.
25. Gazzotti G, Sonnino S, Ghidoni R, Orlando P, Tettamanti G (1984) *Glycoconjugate J* **1**: 111–21.
26. Chigorno V, Sonnino S, Ghidoni R, Tettamanti G (1982) *Neurochem Int* **5**: 397–403.
27. Sonnino S, Acquotti D, Riboni L, Giuliani A, Kirschner G, Tettamanti G (1986) *Chem Phys Lipids* **42**: 3–26.
28. Svennerholm L (1957) *Biochim Biophys Acta* **24**: 604–11.
29. Miettinen J, Takki-Lukkainen JT (1959) *Acta Chem Scand* **13**: 856–58.
30. Venerando B, Roberti S, Sonnino S, Fiorilli A, Tettamanti G (1982) *Biochim Biophys Acta* **692**: 18–26.
31. Ladisch S, Gillard B (1987) *Methods Enzymol* **138**: 300–6.
32. Sela B-A, Iliopoulos D, Guerry D, Helryn D, Coprowski H (1989) *J Natl Cancer Inst* **81**: 1489–92.
33. Sonnino S, Cantu' L, Corti M, Acquotti D, Venerando B (1994) *Chem Phys Lipids* **71**: 21–45.
34. Toffano G, Benvegnu' D, Bonetti AC, Leon A, Orlando P, Ghidoni R, Tettamanti G (1980) *J Neurochem* **35**: 861–66.
35. Taki T, Kasama T, Handa S, Ishikawa D (1994) *Anal Biochem* **223**: 232–38.
36. Marcus DM, Latov N, Hsi BP, Gillard BK (1989) *J Neuroimmunol* **25**: 255–59.
37. Svennerholm L (1980) *Adv Exp Biol Med* **125**: 11.
38. IUPAC-IUB Commission on Biochemical Nomenclature (1977) *Lipids* **12**: 455–68; (1982) *J Biol Chem* **257**: 3347–51.