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New method for quantitative analysis of GD2 ganglioside in plasma of neuroblastoma patients★

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Neuroblastoma, the most common extracranial solid tumour of childhood, is a malignancy of unknown origin and non-specific symptoms. One of the markers of the disease is GD2 ganglioside (disialoganglioside), which is abundantly expressed on the surface of neuroblastoma cells. Gangliosides are known to be shed by tumour cells and this phenomenon can be significant in cancer progression as they inhibit a number of immune responses both *in vitro* and *in vivo*. In search for novel markers useful in monitoring and prognosis of neuroblastoma, we developed and validated a new quantitative method of GD2 ganglioside analysis in human blood plasma. We evaluated the level of gangliosides in blood serum of 34 neuroblastoma patients using high-performance liquid chromatography. The technique was used to detect fluorescently labelled oligosaccharides derived from serum glycosphingolipids by enzymatic digestion with ceramide glycanase. The developed method allowed determination of GD2 concentrations at the picomole level and required only 40 μl of plasma, which should be particularly useful when the quantity of clinical material is limiting. Moreover, this method can be applied to study concentration of other gangliosides, as shown for GD3 ganglioside. Analysis of plasma samples from the 34 neuroblastoma patients did not reveal any correlations between the concentration of GD2 ganglioside and clinical parameters, including the results of therapy; it showed, however, that the concentration of GD2 ganglioside in the plasma of neuroblastoma patients decreased substantially in the course of treatment.

Keywords: gangliosides, neuroblastoma, human blood plasma, HPLC, quantitative analysis

INTRODUCTION

Neuroblastoma (NB) is a paediatric neoplasm that occurs mostly in children less than 5 years old. The disease develops from precursor cells of the peripheral nervous system and the primary tumour is usually located in adrenal glands, but may also occur in other sympathetic neuron sites. NB is the most common extracranial solid tumour of childhood, with 6–11 new cases each year per million children aged 0–15 years. In Poland, about 60 new cases of NB are diagnosed annually (Balwierz, 2004). Aetiology of the disease remains unknown. At diagnosis, the disease is usually spread to metastatic foci,

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Abbreviations: FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GU, glucose units; HPLC, high performance liquid chromatography; LDH, lactate dehydrogenase; MFI, mean fluorescence intensity; NB, neuroblastoma; NSE, neuro-specific enolase; PBS, phosphate-buffered saline.

primarily in the lymph nodes and bone marrow and bones. Nevertheless, NB has the highest rate of spontaneous remission among all human tumours. NB in infants may occur with a specific pattern of metastases to liver and skin, but in spite of this the disease has favourable prognosis and usually responds well to chemotherapy or spontaneously undergoes complete remission (Evans *et al.*, 1981). In contrast, the prognosis of metastatic disease in children over 1 year old is bad. The current treatment protocols of NB are based on chemotherapy combined with surgical removal of the tumour. Irradiation is used as a complementary therapy. In cases with bad prognosis, megachemotherapy followed by autologous bone marrow transplant is used to destroy residual cancer cells. Despite intensive treatment, the long-term survival in advanced NB is as low as 30% (Balwierz, 2004). The major challenge is the treatment of minimal residual disease associated with incomplete removal of tumour cells by chemo- and radiotherapy (Beiske *et al.*, 2005). New therapeutic options are under development, including: passive immunotherapy, radioimmunotherapy, tyrosine kinase inhibitors and anti-angiogenic therapies (Brodeur, 2003).

Prognostic factors in NB. The clinical course of NB can be very heterogeneous and a number of factors have been found relevant for prognosis and monitoring of the disease. Two most important factors are: patient's age at diagnosis and stage of the disease. The biological features of NB in infants are different than in older children (>1 y.o.). In most infants the metastatic form of the disease can be successfully treated with limited chemotherapy or solely with surgical procedure, while patients more than 1 year old require a much more aggressive treatment. Depending on the advancement of the disease, NB is staged from 1 to 4 according to the International Neuroblastoma Staging System. In some cases, particularly in infants, the tumour undergoes spontaneous remission (NB stage 4S), while in older children it may differentiate to a benign form of ganglioneuroma. Histology of the tumour is also of prognostic significance; in NB two histological types have been defined: favourable and unfavourable, depending on the degree of differentiation and the mitotic index (Shimada *et al.*, 1999). Serum levels of three biochemical markers — ferritin, lactate dehydrogenase (LDH) and neurospecific enolase (NSE) — seem to be significant in NB; elevated concentrations of those markers are associated with worse prognosis (Riley *et al.*, 2004). Several genetic markers are important prognostic factors in NB; those include *MYCN* oncogene amplification, genome ploidy and chromosomal aberrations in the tumour cells (Weinstein *et al.*, 2003).

Gangliosides of malignant cells. Gangliosides are a class of complex molecules belonging to glycosphingolipids. Their structure is formed by the ceramide core and a sugar chain of several carbohydrate residues, including at least one sialic acid. Ganglioside biosynthesis is a complex enzymatic pathway (d'Azzo *et al.*, 2006). Gangliosides are components of eukaryotic cell membranes — mainly in the central nervous system, but are also found on the surface of cancer cells. However, ganglioside expression in malignant cells is often altered. For example, the number and linkage of sialic acids can change, leading to production of atypical glycolipid molecules (Varki, 1999). Moreover, in many cancers such as melanoma, soft tissue sarcoma and small cell lung cancer the pathway of ganglioside synthesis is shortened, which is manifested by overexpression of simpler forms such as GM3, GD3, GM2 and GD2 (Hettmer *et al.*, 2003; nomenclature according to Svennerholm, 1980). This phenomenon is related to neoplastic transformation, e.g., GD3 ganglioside is abundant on melanoma cells, but only small quantities are present on normal melanocytes (Carubia *et al.*, 1984). Ganglioside overexpression may occur in more than 90% of cases of a particular type of cancer (Chang *et al.*, 1992). Differentiation of tumour cells induced *in vitro* leads to the restoration of a normal expression profile of gangliosides (Liour *et al.*, 2000).

Surface gangliosides can be shed from the cell membranes in a number of ways — gangliosides may be released in the form of micelles and aggregates (Kong *et al.*, 1998) as well as complexes with antibodies (Hakansson *et al.*, 1985) and plasma proteins (Valentino & Ladisch, 1992). Ganglioside shedding seems to have biological importance — there are reports that the circulating gangliosides are able to weaken cellular immunity and thereby accelerate tumour progression (Sietsma *et al.*, 1998). Several mechanisms of immunosuppression are possible: loss of antigen from the surface of cancer cells, blocking of specific antibodies and T cell receptors or direct suppression of patient's lymphocytes (Ladisch *et al.*, 1994). Some gangliosides may also inhibit the biological activity of cytokines, such as interleukin 2 (Ravindranath *et al.*, 2001).

GD2 ganglioside in neuroblastoma. GD2 ganglioside (disialoganglioside) is one of the antigens associated with NB. Chemically, GD2 is a derivative of cerebroside containing two sialic acid residues; the structure of GD2 is: GalNAcβ1 $\rightarrow$ 4 (Neu5Acα2 $\rightarrow$ 8) (Neu5Acα2 $\rightarrow$ 3) Galβ1 $\rightarrow$ 4Glcβ1 $\rightarrow$ 1'-ceramide (Fig. 1). The antigen is characteristic to cells of neuroectodermal origin and its normal distribution in the human organism is limited to neurons and peripheral nerve fibres (Varki, 1999). The GD2 content on NB cells is particularly high and may reach up to 30% of all gangliosides present on the cell surface (Wu *et al.*, 1986). In addition, the antigen is shed from the tumour cell and released into the bloodstream (Li

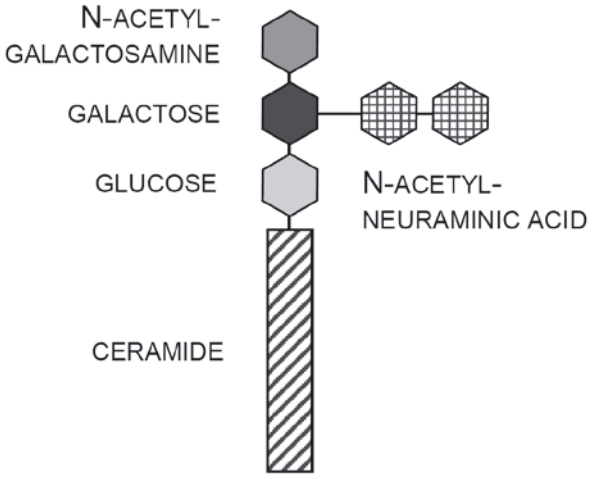

Figure 1. Schematic chemical structure of GD2 ganglioside.

& Ladisch, 1991), and its concentration in the blood may be correlated with progression of the disease (Ladisch *et al.*, 1987). It has been suggested that the increased shedding of GD2 ganglioside and *MYCN* amplification jointly characterise the aggressive type of NB cells (Valentino *et al.*, 1990).

MATERIALS AND METHODS

Cell lines. A hybridoma cell line producing 14G2a murine monoclonal antibody specific to GD2 ganglioside (Mujoo *et al.*, 1989) was a kind gift from Dr. R. A. Reisfeld from the Scripps Institute (La Jolla, CA, USA). HTLA-230 cell line was a kind gift from Professor Vito Pistoia from the Gaslini Institute (Genova, Italy). IMR-32, BE(2)-C, SH-SY5Y and SK-MEL-28 cell lines were obtained from ATCC.

Clinical samples. A total of 42 blood serum samples from 34 patients diagnosed with different stages of NB were collected between 2003 and 2008 at the Department of Paediatric Oncology and Haematology of the University Children's Hospital (Kraków, Poland). In six cases, two different samples were obtained from the same patient, and in one case — three samples from the same patient. Where possible, the samples were collected before treatment, after treatment and at a follow-up time point (14–33 months after treatment). Samples were also collected from seven patients with non-neuroblastoma disease; those samples were used as controls in HPLC analysis (non-NB). Collection of clinical samples was authorised by the Bioethical Commission of the Jagiellonian University (opinion no. KBET/311/B/2002, prolonged accordingly).

Characteristics of patients. Clinical parameters were gathered on the basis of available medical records. In the group of 34 patients, 17 were male (50%) and 17 female (50%). Age of patients at the time of diagnosis ranged from 39 days to 12 years, median 22.3 months. Twelve (35.3%) and 22 (64.7%) patients were in the age group below 1 year of age and above 1 year of age, respectively. NB was staged in all patients according to the International Neuroblastoma Staging System: the largest group — 19 (55.9%) patients — were patients with stage 4 NB; 1 (2.9%), 5 (14.7%) and 7 (20.6%) patients were diagnosed in stages 1, 2 and 3, respectively; 2 (5.9%) patients had NB stage 4S. *MYCN* gene amplification was observed in 4 (11.8%) patients, for another 4 (11.8%) *MYCN* analysis results were not available and in the remaining 26 patients (76.4%) normal copy number of *MYCN* was found. The histological type of the tumour, assessed according to the International Neuroblastoma Pathology Classification criteria, was found to be favourable in 13 (38.2%) patients and unfavourable in 14 (41.2%) patients; in other cases, histopathological evaluation was not possible. The patients exhibited high variability of biochemical parameters: the level of LDH in plasma varied in the range 393–5966 IU/l (mean 2049.4 IU/l), while NSE levels in plasma ranged from 0 to 1122.8 ng/ml, with an average value of 200.7 ng/ml. All patients with NB were treated according to the current European SIOPEN protocols with the following results: 29 patients survived (24 with no disease progression, 5 after progression or in relapse, in two cases — early stage treatment), 5 patients died (4 as a result of progression, 1 due to complications).

Isolation of blood plasma. Plasma was isolated by centrifugation in density gradient. Blood (5 ml) was layered on 5 ml of Ficoll (density 1.077 g/cm^3) in a 15-ml tube. The tube was then centrifuged for 30 min at 800×*g* at room temperature. After separation the plasma was collected as the upper phase.

Lipid extraction and isolation of gangliosides. Plasma samples (40 μl) or cell pellets (5×10^5) were extracted with 4 ml of a mixture of chloroform/methanol, 2:1 (v/v) and shaken vigorously for 15 s in a glass tube. The solution was sonicated for 15 min in an ultrasonic water bath at 22°C and then incubated on ice for 2 min. The samples were centrifuged for 10 min at 4°C and 3200×*g*. The supernatant was transferred to a clean tube and dried under a stream of nitrogen. Then, after dissolving in 200 μl of chloroform, the obtained mixture of lipids was applied to a column containing 0.1 g silicic acid in 1 ml of chloroform. Specific lipid fractions were sequentially eluted from the column with 5 ml of chloroform (neutral lipids), 1.8 ml of methanol/acetone mixture, 1:9 (v/v; neutral glycolipids) and 1.8 ml of methanol (gangliosides). The last fraction, containing gangliosides, was dried in a vacuum concentrator and then dissolved in 10 μl of buffer for enzyme di-

gestion, which consisted of 1 mg/ml sodium cholate dissolved in 50 mM sodium acetate, pH 5.0.

Enzymatic digestion of gangliosides. After complete dissolution of the sample, 10 μl of ceramide glycanase solution (Merck Biosciences) was added at a concentration of 5 U/ml, which gave the final activity of 0.05 U per sample. The enzymatic reaction lasted for 18 h and was conducted at 37°C.

Glycan purification. After completion of digestion, the glycans in the reaction mixture were separated from the ceramide, enzyme and other contaminants by column purification using an Oasis HLB 10 mg cartridge (Waters). The column resin was activated beforehand with 1 ml of methanol and equilibrated with 1 ml of HPLC grade water; 180 μl of HPLC grade water was added to the reaction mixture and the entire solution was transferred to the Oasis column. The reaction tube was washed with 50 μl of water which was then transferred to the column. The column was washed with 200 μl of 5% (v/v) methanol in HPLC grade water and the combined eluate was dried in a vacuum concentrator.

Fluorescent labelling of glycans. The purified glycan samples were labelled with 2-aminobenzamide using the GlycoProfile 2-AB Labeling Kit (Sigma-Aldrich) according to the manufacturer's instructions.

High performance liquid chromatography. Labelled glycan samples were diluted by adding 200 μl of HPLC grade water. A Shimadzu model 10AVP HPLC system was used for chromatography, equipped with a 105×4.6 mm Hypersil ODS(C18) 3 μm column (Phenomenex, Cat. No. 00F-0145-E0). Reversed-phase HPLC was performed in a two-phase system in which phase A was 50 mM formic acid brought to pH 5.0 with triethylamine, while phase B consisted of a 1:1 mixture of phase A and HPLC grade acetonitrile. The injection volume for each sample was 180 μl. A gradient with the following time programme was used: t = 0 min, flow 0.5 ml/min, 95% phase A; t = 30 min, 0.5 ml/min, 95% phase A; t = 95 min, 0.5 ml/min, 90% phase A; t = 100 min, 0.5 ml/min, 76% phase A; t = 101 min, 1.5 ml/min, 5% phase A; t = 107 min, 1.5 ml/min, 5% phase A; t = 108 min, 1.5 ml/min, 95% phase A; t = 112 min, 1.5 ml/min, 95% phase A; t = 113 min, 0.5 ml/min, 95% phase A; total time: 114 min. Following column separation, the labelled glycans were detected using a Shimadzu RF-10AXL fluorescence detector with excitation wavelength of 330 nm and the emitted signal was measured at 420 nm. Chromatograms were recorded and analyzed using Shimadzu Class VP software ver. 6.

Qualitative analysis of HPLC data. Qualitative analysis was based on a comparison of the retention times of detected components with the retention times of standards, which were glycans isolated from commercially available gangliosides GM1, GM3, GD2, GD3 (Merck Biosciences) and a mixture of gangliosides from bovine brain (Sigma-Aldrich). In order to confirm the validity of the method, the retention times obtained for the standards were compared with previously published values (Wing *et al.*, 2001). Due to the differences in methodology, the retention time was converted into glucose units (GU). Fluorescently labelled samples of arabinobiose (Megazyme) with GU=2 and arabinotriose (Megazyme) with GU=3 were used as reference samples. The values compared were in agreement with the published data, which provided evidence of good reproducibility of the method. GD2 ganglioside analysis by HPLC has not been published previously, so the value of 2.48 GU obtained for the GD2 retention time cannot be compared with any available studies (Table 1).

Quantitative analysis of HPLC data. On the basis of quantitative analysis of samples containing standards of known ganglioside content, a relationship between fluorescence signal and the amount of ganglioside was established. For GD2 ganglioside, a peak area of 1000000 corresponded to 16 pmol. For GD3 ganglioside, a peak area of 1000000 corresponded to 1.25 nmol. Those values were used to convert the fluorescence signal to the ganglioside concentration in the plasma samples.

GD2 ganglioside analysis by flow cytometry. For additional confirmation of the quantitative HPLC analysis, flow cytometry was used as a reference method, as it allows quantitative assessment of antigen expression. One hundred thousand cells of five selected human cell lines (IMR-32, HTLA-230, BE(2)-C, SH-SY5Y — neuroblastoma, SK-MEL-28 — melanoma) were suspended in phosphate-buffered saline (PBS) with 10% foetal bovine serum (FBS) and centrifuged at 800×*g*. Subsequently, the cells were suspended in a solution of 14G2a murine monoclonal antibody at a concentration of 1 μg/ml in PBS with 10% FBS. After a 45-min incubation on ice, samples were centrifuged again and washed twice with cold PBS with 10% FBS. 14G2a antibodies bound to cell surface were detected using $(Fab')_2$ antibody fragments coupled with fluorescein isothiocyanate (ICN, Cat. No. 55526). Secondary antibodies used were diluted 1:40 in PBS with 10% FBS for 45 min on ice. After washing the cells were suspended in PBS with 10% FBS and analysed with a FACScan cytometer (BD Biosciences) equipped with Cellquest software. The level of GD2 ganglioside expression was presented as the fluorescence index, according to the formula:

[fluorescence index]=([% positive cells]×MFI)/100%

Table 1. Comparison between qualitative results produced with the method described in this work and results published by Wing *et al.*, 2001.

Retention time was expressed in glucose units (GU); by definition, retention time equals 2.00 GU for arabinobiose and 3.00 GU for arabinotriose.

Standard sample	Retention time published by Wing *et al.*, 2001 [GU]	Retention time measured with the described method [GU]
GM1	1.91	2.01
Arabinobiose	2.00	2.00
GM3	2.04	2.11
GD1b	2.34	2.28
GD3	3.00	2.78
Arabinotriose	3.00	3.00
GD1a	3.26	3.14
GT1b	3.73	3.73
GD2	not measured	2.48

where MFI is mean fluorescence intensity of the sample. The values obtained for the five cell lines were then compared with the result of HPLC analysis performed on samples of the same cells.

Statistical analysis: Arithmetic mean was the estimator of the expected value. Student's *t*-test was used to compare the average values: *P*-value of 0.05 was the significance level cut-off, and for *P*-values in the range of 0.05–0.10 the results were interpreted as a trend indication. Correlation between data sets was assessed using the squared Pearson's correlation coefficient. The Excel spreadsheet, a part of Microsoft Office 2003 package, was used for statistical analysis.

RESULTS

Quantitative analysis of GD2 ganglioside

HPLC analysis was performed for all samples from the NB patients, as well as control non-NB samples. The concentration of GD2 ganglioside in serum samples of patients with NB ranged from 0 to 3940 pmol/ml, with an average value of 660.7 pmol/ml for all 42 samples analysed. The mean concentration for samples collected prior to treatment was 591.4 pmol/ml (31 samples), for those collected after treatment — 1156.1 pmol/ml (7 samples), and for follow-up samples — 330.3 pmol/ml (4 samples). The average GD2 content in serum of non-NB patients was 106.1 pmol/ml (7 samples). The results indicate a substantial difference in GD concentrations between serum samples from NB patients and age-matched non-NB patients. The concentration of GD2 in NB samples was on average 6.2 times higher than in non-NB samples (660.7 pmol/ml *versus* 106.1 pmol/ml), but the difference was not statistically significant.

Correlations with known prognostic factors and other parameters

The analysis also showed that among the 31 patients from whom serum samples were collected before treatment, 14 (45.2%) had elevated GD2 concentrations in plasma as compared to non-NB samples (over 106.1 pmol/ml), and the average value for this group was 1239 pmol/ml. Nevertheless, statistical analysis failed to reveal any direct correlations with known prognostic factors. In patients with favourable tumour histology, the average level of GD2 in plasma was 623.1 pmol/ml, while in the group with unfavourable histology — 511.0 pmol/ml. The mean value for patients younger than 1 year was 360.3 pmol/ml, while for patients older than 1 year — 718.6 pmol/ml. In patients with *MYCN* oncogene amplification in the tumour, GD2 concentration in plasma was 24.1 pmol/ml on average, while it was 729.5 pmol/ml for cases with no *MYCN* amplification. The differences between the compared groups were not statistically significant. The concentration of GD2 in the plasma of patients before treatment was 595.7 pmol/ml for patients with NB stages 1–3, while for patients in stage 4 (excluding 4S) it was 564.2 pmol/ml. There were no statistically significant differences between these groups. The average result for patients in stages 1–4 (excluding 4S) was 591.4 pmol/ml. The average content of GD2 ganglioside according to sex of patients was as follows: female — 707.9 pmol/ml, male — 467.2 pmol/ml. Also that difference was not statistically significant.

Changes of GD2 level during treatment

Analysis of chromatograms obtained for different samples from the same patient allowed tracking of changes in GD2 concentrations in the course of treatment (Fig. 2). The GD2 concentration in the plasma decreased substantially during treatment.

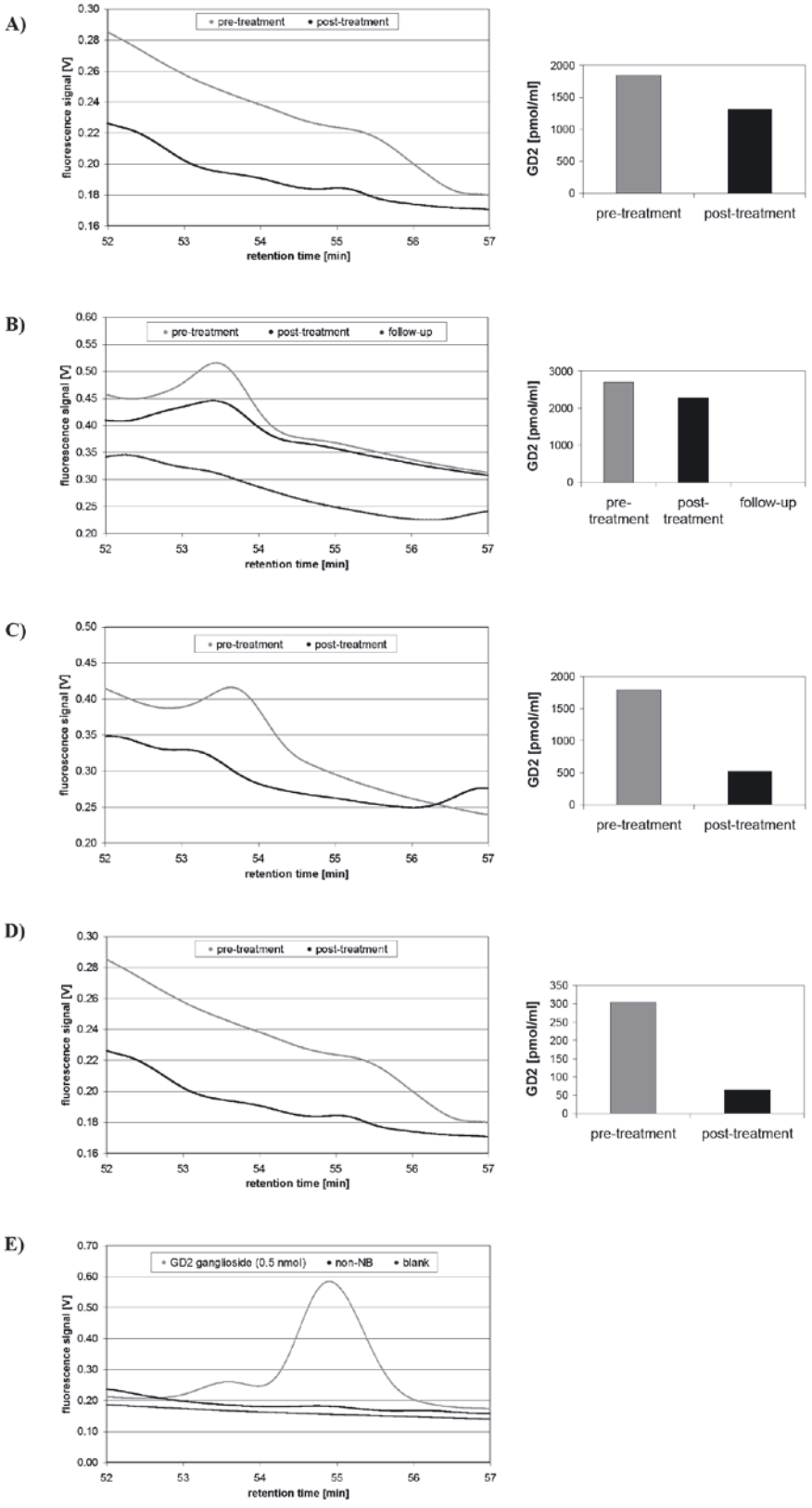

Figure 2. GD2 level in serum samples of four patients for whom both pre-treatment and post-treatment samples were available.

The following changes in GD2 level were detected throughout therapy: 29% decrease for patient A, 16% decrease followed by diminishing to undetectable level for patient B, 71% decrease for patient C and 88% decrease for patient D; panel E, reference samples (GD2 standard, non-NB sample, blank sample).

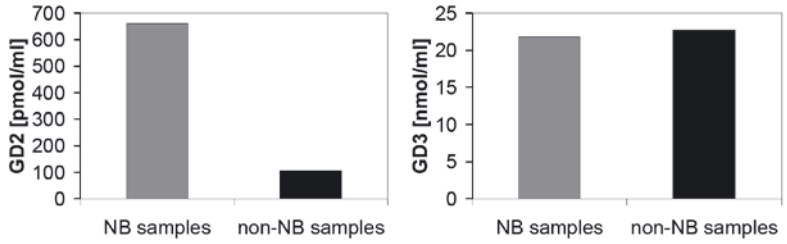

Figure 3. GD2 and GD3 ganglioside concentration in plasma samples.
Mean ganglioside concentration in plasma samples for GD2 (left) and GD3 (right) ganglioside; NB — patients with neuroblastoma, non-NB — other paediatric patients. The concentration of GD2 ganglioside in NB samples (n=42) ranged from 0 to 3940 pmol/ml with a mean value of 660.7 pmol/ml. The average GD2 content in serum of 7 non-NB patients was 106.1 pmol/ml.

The average GD2 level after treatment — calculated for the four patients from whom relevant samples were available — was 30% of the pre-treatment level. This may provide additional evidence that the level of GD2 ganglioside is correlated with the occurrence of active disease and the treatment leads to its decrease.

Analysis of gangliosides other than GD2

The method of glycolipid analysis with HPLC can be extended to other ganglioside species present in human plasma. With the use of appropriate standards, the analysis can be performed simultaneously for many types of gangliosides in the same plasma sample. In this work, GD3 ganglioside analysis was chosen for comparison with the GD2 concentration studies. The chemical structure of GD3 is similar to that of GD2, but GD3, unlike GD2, is present in normal blood plasma. The concentration of GD3 ganglioside was evaluated in all samples analysed for the content of GD2. The level of GD3 was approximately two magnitudes of order higher than the GD2 concentration. In plasma samples of NB patients GD3 concentration varied from 0 to 107.3 nmol/ml, with an average value of 21.8 nmol/ml for all 42 samples analysed. The average level of GD3 in non-NB plasma samples was similar — 22.7 nmol/ml (7 samples). A comparison between average concentrations of GD2 and GD3 in NB and non-NB plasma samples is shown in Fig. 3. In 26 serum samples from NB patients the GD3 concentration was below the average for all NB samples (21.8 nmol/ml), while in 16 samples the GD3 level exceed this value. During treatment (as followed in 4 patients) the level of GD3 decreased in three cases by 79–91%, and increased by about 74% in one case. There was no relation between the content of GD3 ganglioside and clinical factors such as sex, age, stage of disease or the outcome of treatment. The GD3 level showed no correlation with the level of GD2 measured in the same samples (Fig. 4).

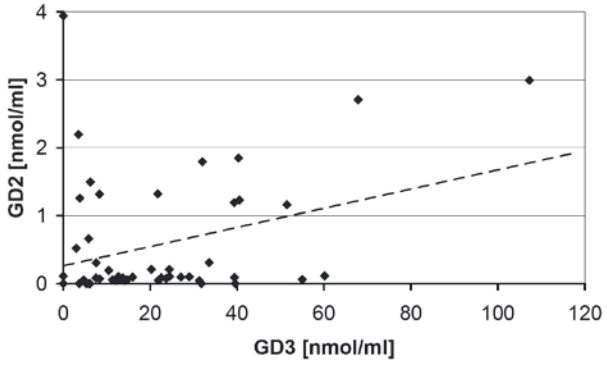

Figure 4. Correlation between GD2 and GD3 concentration in NB plasma samples.
The squared Pearson's correlation coefficient equals 0.1052.

Comparison between HPLC analysis and flow cytometry results

To confirm the quantitative nature of the chromatographic method, results of GD2 ganglioside analysis were compared with GD2 expression level estimated with a reference method of flow cytom-

Table 2. GD2 ganglioside analysis performed with HPLC and flow cytometry in five different cell lines.

HPLC results are expressed as fluorescence peak areas. Flow cytometry results are shown as fluorescence index. For flow cytometry, cells were stained with anti-GD2 murine monoclonal antibody and FITC-conjugated anti-mouse secondary antibodies. Cell lines: IMR-32, HTLA-230, BE(2)-C, SH-SY5Y — neuroblastoma; SK-MEL-28 — melanoma.

Cell line	HPLC signal	Flow cytometry
IMR-32	480 129	431.5
HTLA–230	574 594	425.8
BE(2)-C	316 726	345.2
SH-SY5Y	368 343	339.1
SK-MEL-28	0	4.2
Correlation coefficient	$R^2 = 0.9277$	

etry, which is commonly used in antigen expression studies. Five different human cell lines were analysed simultaneously with both methods (Table 2). The results of the comparison suggest that HPLC analysis is a sensitive and quantitative method for GD2 ganglioside analysis.

DISCUSSION

The HPLC method developed for the purpose of this study can be used to detect gangliosides in plasma samples at the picomole level with the sample volume of less than 50 μl. It should be noted that the previously described methods based on thin-layer chromatography require approximately 1 ml of plasma (Ladisch & Gillard, 1987). In the case of small children the amount of blood that can be collected is limited. In addition, the novel HPLC method has a number of advantages, discussed below.

The results of GD2 concentration analysis are consistent with previously published data (Ladisch *et al.*, 1987). Although no correlations between clinical data and the concentration of GD2 in the plasma of patients were found, it cannot be excluded that a longer period of observation following the therapy could lead to more definite conclusions. The search for new methods of prognosis is particularly important in the case of NB due to the minimal residual disease, which outlasts the treatment of advanced forms of this cancer and often leads to relapse.

The measured level of GD2 ganglioside is characterised by high variability among individual patients. Similar observations have already been published (Valentino *et al.*, 1990), but more recent works suggest that the phenomenon of ganglioside shedding may be highly complex (e.g. Kong *et al.*, 1998). The universal expression of GD2 ganglioside in NB tumours is a paradigm in NB biology (Wu *et al.*, 1986). However, it is not clear whether this assumption is fully justified. The level of GD2 expression in NB cells *in vitro* is varied (Chen *et al.*, 2000); moreover, there have been reports of NB tumours that completely lack GD2 expression (Schumacher-Kuckelkorn *et al.*, 2005) — a fact that can have significant clinical implications. It should be noted, however, that those publications have been much criticised by researchers in the field of NB diagnosis and therapy.

This HPLC method allows simultaneous analysis of a number of different species of plasma gangliosides. Potentially, it is possible to analyse serum samples from patients with other diseases. Ganglioside expression is altered in melanoma cells, in which the level of GM2, GD2 and GD3 gangliosides can be elevated (Hakomori, 2001). Other examples are: small cell lung cancer, astrocytoma, soft tissue sarcoma. Analysis of plasma gangliosides may be useful in prognosis of the disease (Perez *et al.*, 2002). Extension of the analysis to other ganglioside species requires as little as an appropriate qualitative and quantitative standardisation by using high purity ganglioside preparations.

Simultaneous analysis of different gangliosides may have an additional aspect related to the biosynthesis pathway of b-series gangliosides: GD3 → GD2 → GD1b → GT1b → GQ1b. NB cell lines exhibit low expression of complex gangliosides (GD1b, GT1b, GQ1b) of this pathway (Hettmer *et al.*, 2005). It seems that differences in the expression profile of b-series gangliosides may have a stronger clinical relevance than has the level of GD2 ganglioside alone (Hettmer *et al.*, 2003). Under the influence of 13-*cis*-retinoic acid *in vitro*, NB cells change the ganglioside expression pattern towards more complex structures (Hettmer *et al.*, 2004). 13-*cis*-retinoic acid is already used in the treatment of patients with NB, and a study of changes in the ganglioside synthesis during treatment is possible using the proposed analytical method, although it would require standardisation.

Reports published in recent years suggest that it is possible to further improve the sensitivity of the assay by using an alternative method of glycan labelling using 2-aminobenzoic acid and several further modifications (Neville *et al.*, 2004).

HPLC may also be useful for the analysis of other components of the ganglioside biosynthesis pathway, such as ceramide and sphingosine (He *et al.*, 2005). Studies confirm that high-performance liquid chromatography potentially has powerful applications in clinical practice, which offers hope for gradual improvement in diagnosis and prognosis of cancer on the basis of well-defined, measurable factors, in accordance with the concept of evidence-based medicine.

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