


Quantitative GSL-glycome analysis of human whole serum based on an EGCCase digestion and glycoblotting method^S

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Abstract Glycosphingolipids (GSLs) are lipid molecules linked to carbohydrate units that form the plasma membrane lipid raft, which is clustered with sphingolipids, sterols, and specific proteins, and thereby contributes to membrane physical properties and specific recognition sites for various biological events. These bioactive GSL molecules consequently affect the pathophysiology and pathogenesis of various diseases. Thus, altered expression of GSLs in various diseases may be of importance for disease-related biomarker discovery. However, analysis of GSLs in blood is particularly challenging because GSLs are present at extremely low concentrations in serum/plasma. In this study, we established absolute GSL-glycan analysis of human serum based on endoglycoceramidase digestion and glycoblotting purification. We established two sample preparation protocols, one with and the other without GSL extraction using chloroform/methanol. Similar amounts of GSL-glycans were recovered with the two protocols. Both protocols permitted absolute quantitation of GSL-glycans using as little as 20 μ l of serum. Using 10 healthy human serum samples, up to 42 signals corresponding to GSL-glycan compositions could be quantitatively detected, and the total serum GSL-glycan concentration was calculated to be 12.1–21.4 μ M. We further applied this method to TLC-prefractionated serum samples.  These findings will assist the discovery of disease-related biomarkers by serum GSL-glycomics.—Furukawa, J-i., S. Sakai, I. Yokota, K. Okada, H. Hanamatsu, T. Kobayashi, Y. Yoshida, K. Higashino, T. Tamura, Y. Igarashi, and Y. Shinohara. **Quantitative GSL-glycome analysis of human whole serum based on an EGCCase digestion and glycoblotting method.** *J. Lipid Res.* 2015. 56: 2399–2407.

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Glycolipids are lipid molecules linked to carbohydrate units and are classified as glycosphingolipids (GSLs) containing ceramide or glycolycerolipids containing glycerol or diacylglycerol. In mammals, GSLs are the predominant component of the plasma membrane lipid raft, which is clustered with sphingolipids, sterols, and specific proteins, and thereby contribute to membrane physical properties and specific recognition sites for various biological events (1). GSLs are often structurally classified according to their glycan structure [i.e., ganglio-, globo-, and (neo) lacto-series]. These bioactive GSL molecules consequently affect the pathophysiology and pathogenesis of various diseases, including various GSL lysosomal storage diseases (2), various types of cancer (3), infections, atherosclerosis (4), diabetes (5), and central nervous system diseases (6). For example, ganglioside profiles differ between malignant tumors and healthy tissues from which they originate (7, 8), and serum GSL profiles are altered in some patients as a result of the shedding of GSLs from tumors cells (9). Several anti-GSL antibodies have been found in the sera of patients with a variety of neurological disorders, such as Guillain-Barré syndrome (10), Parkinson's disease (11),

Abbreviations: aoWR, *N*^ε-((aminoxy)acetyl)tryptophanylarginine methyl ester; C/M, chloroform/methanol; EGCCase, endoglycoceramidase; Gb, globo; Gg, ganglio; GOD, glucose oxidase; GSL, glycosphingolipid; (n)Lc, (neo)lacto; SPE, solid phase extraction.

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and amyotrophic lateral sclerosis (12). Thus, altered expression of GSLs in various diseases may be of importance for disease-related biomarker discovery.

Given that serum/plasma GSLs originate from a variety of tissues and enter the circulation as a result of shedding processes (13), the serum/plasma GSL-glycome would be a good tool with which to identify and/or screen disease-related biomarkers. The presence of gangliosides in human serum was first described in 1963 (14). Kundu et al. (15) observed 25 GSLs consisting of 15 acidic gangliosides and 10 neutral GSLs in human plasma by TLC separation and gas-liquid chromatography analysis. Recent advances in MS analysis allowed quantitation of the intact forms of targeted GSLs using the multiple reaction monitoring mode (16–18). However, efforts to clarify the comprehensive GSL-glycomics of human serum have been limited since the characterization of serum GSLs using TLC and MS. It is also important to improve the sensitivity in order to reduce the amount of serum required for analysis and to simplify the analytical protocol in order to improve its throughput such that it is applicable to mass screening.

We recently established a procedure for quantitative and qualitative cellular glycomic analysis of GSLs based on rhodococcal endoglycoceramidase (EGCase)-assisted glycan cleavage, glycoblotting-assisted purification, and MALDI-TOF MS analysis (19). The aim of this study was to apply this method to quantitative analysis of GSL-glycans and to clarify the GSL-glycomic profile of human serum. We established two sample preparation protocols, one with and the other without GSL extraction using chloroform/methanol (C/M). Both protocols recovered a similar amount of GSL-glycans using as little as 20 μ l of serum. Our protocol permitted absolute quantitation of up to 42 signals corresponding to GSL-glycan compositions in sera of 10 healthy subjects. Validation of the quantitative nature of the method allowed absolute quantitation of these GSL-glycans, and the serum GSL-glycome of healthy subjects was revealed. This provides a firm basis for the discovery of disease-related biomarkers by serum GSL-glycomics.

MATERIALS AND METHODS

Materials

Human sera were purchased from BioPredic International (Saint-Gregoire, France). Rhodococcal EGCase I was prepared as described previously (20, 21). Glucose oxidase (GOD) was from Wako Chemical Company, Ltd. (Osaka, Japan). β 1,4-Galactosidase (*Streptococcus pneumoniae*) and β 1-2,3,4,6-*N*-acetylglucosaminidase (*S. pneumoniae*) were from Calbiochem (San Diego, CA). Glyco[®] α (1-2,3,4,6) fucosidase was from Prozyme (Hayward, CA). BlotGlyco[®] beads and *N*^ε-((aminooxy)acetyl)tryptophanylarginine methyl ester (aoWR) reagent were from Sumitomo Bakelite Company, Ltd. (Tokyo, Japan). The MultiScreen Solvint 0.45 μ m low-binding hydrophilic PTFE plate was from Merck Millipore (Darmstadt, Germany). Disialyloctasaccharide (A2GN1) was from Tokyo Chemical Industry (Tokyo, Japan). A2F glycan was from Ludger Ltd. (Oxford, UK). The MassPrep[™] hydrophilic interaction chromatography (HILIC) μ Elution plate was from Waters (Milford, MA). Other solvents and reagents were of the highest grade commercially available.

GOD digestion of serum

In total, 10 μ l of 1.52 M acetate buffer (pH 3.4) was added to 200 μ l of serum in order to attain a pH of about 5.6, followed by the addition of 10 μ l of GOD (2.1 U/ml prepared in 100 mM phosphate buffer, pH 7.0). Oxidization was performed at 37°C overnight. Oxidized serum was used for GSL-glycan analysis in both protocols.

Extraction of GSLs from serum (protocol 1)

The GSL extraction procedures were essentially the same as previously described (19). Briefly, the lipid fraction was extracted by adding C/M solution (2:1, v/v; 450 μ l) to oxidized human serum (1.4–44 μ l), followed by sonication using an ultrasonic homogenizer (Taitec Corp., Saitama, Japan) at room temperature. Methanol (150 μ l) was then added, yielding a C/M composition of 1:1 (v/v). Sonication was repeated in the same manner. Finally, methanol (300 μ l) was added (C/M = 1:2, v/v), and sonication was repeated once again. The resulting extracts were centrifuged at 15,000 rpm for 10 min. Supernatants containing GSLs were concentrated with a centrifugal evaporator and resuspended in 50 mM acetate buffer, pH 5.5 (48 μ l), containing 0.2% Triton X-100 (Sigma-Aldrich Japan, Tokyo, Japan) as a surfactant, followed by the addition of 2 μ l of EGCase I (25 mU) to release intact glycans from GSLs, as previously described (19). Enzymatic digestion was performed at 37°C for 16 h. To distinguish GSL-glycans from contaminating free oligosaccharides, crude gangliosides were also suspended in the absence of EGCases, which served as a negative control.

Direct deglycosylation with EGCase I (protocol 2)

For GSL-glycan analysis of untreated serum, 22 μ l of oxidized serum was diluted with 50 mM acetate buffer, pH 5.5 (50 μ l), containing 0.2% Triton X-100, followed by sonication with an ultrasonic homogenizer, as described above. After solubilization, EGCase I was added and the mixture was incubated at 37°C for 16 h. Finally, 5 μ l of pronase (10 mg/ml) was added, and the mixture was incubated at 37°C overnight, followed by heat denaturation at 90°C for 15 min.

GSL-glycan purification using a glycoblotting procedure

Released intact GSL-glycans were subjected to a glycoblotting procedure (22). In brief, the EGCase-digested sample (25 μ l) containing an internal standard of Neu5Ac2Gal2GlcNAc2 + Man3GlcNAc1 (A2GN1, 20 pmol) was directly applied to the well of a MultiScreen Solvint filter plate containing BlotGlyco[®] beads (5 mg). GSL-glycans were captured in 2% acetic acid prepared in MeCN (225 μ l) and incubated at 80°C for 60 min. Unreacted hydrazide groups on beads were capped with acetyl groups by treatment with 10% acetic anhydride in methanol for 30 min at room temperature. Next, on-bead methyl esterification of the carboxyl groups in glycan-derived sialic acid was performed by incubation with 100 mM 3-methyl-1-p-tolyltriazene prepared in dioxane at 60°C for 60 min. The trapped glycans with modifications on the beads were subjected to transamination by incubation with a mixture of 2% acetic acid prepared in MeCN (180 μ l) and 20 mM aoWR (20 μ l) at 80°C for 45 min. aoWR-labeled glycans were recovered in distilled water (100 μ l). The collected solution was purified using a HILIC μ Elution plate to remove excess reagents.

Acid hydrolysis and sequential exoglycosidase digestion

A2F and A2GN1 were sequentially digested by acid hydrolysis and various exoglycosidase digestions. For acid hydrolysis, A2F or A2GN1 (1200 pmol) was dissolved in 10 mM HCl aq (pH 2.0) and incubated at 90°C for 30 min. After hydrolysis, the reaction

mixture was neutralized with NaOH aq. For galactosidase treatment, NA2F or NA2GN1 (800 pmol) was dissolved in 50 mM acetate buffer (pH 5.5) and incubated at 37°C for 16 h in the presence of 9 mU of β -galactosidase (*Streptococcus*), followed by heat denaturation at 90°C for 15 min. For *N*-acetylglucosaminidase treatment, NGA2F or NGA2GN1 (400 pmol) was dissolved in 50 mM acetate buffer (pH 5.5) and incubated at 37°C for 18 h in the presence of 25 mU of β -*N*-acetylglucosaminidase (*Streptococcus*), followed by heat denaturation at 90°C for 30 min. For fucosidase treatment, a mixture of equal quantities of four fucosylated glycans (22.5 pmol of A2F, NA2F, NGA2F, and M3F) were dissolved in 20 mM sodium citrate phosphate buffer (pH 6.0) and incubated at 37°C for 16 h in the presence of 6.25 mU of fucosidase (bovine kidney), followed by heat denaturation at 90°C for 15 min. A mixture of equal quantities of 14 glycans, including Lac and GM3, was subjected to the glycoblotting procedure and analyzed by MALDI-TOF MS, as described above.

Tandem MALDI-TOF MS analysis

Purified GSL-glycan solutions were mixed with 2,5-dihydrobenzoic acid solution (10 mg/ml prepared in 30% acetonitrile) and subjected to MALDI-TOF MS analysis as previously described (19), with minor modifications. Briefly, all measurements were performed using an Ultraflex II TOF/TOF mass spectrometer equipped with a reflector and controlled by the FlexControl 3.0 software package (Bruker Daltonics, Bremen, Germany) according to general protocols. All spectra were obtained in reflectron mode with an acceleration voltage of 25 kV, a reflector voltage of 26.3 kV, and a pulsed ion extraction of 160 ns in positive ion mode. Masses were annotated using the FlexAnalysis 3.0 software package. SphinGOMAP (<http://www.sphingomap.org/>) online databases were used for structural identification of GSL-glycans. Absolute quantification was performed by comparative analyses between the areas of the MS signals derived from each GSL-glycan and a known amount of the internal standard (A2GN1).

Separation of serum GSLs by TLC for structural analysis

GSLs extracted from serum were subjected to acetone precipitation by adding a 5-fold volume of acetone. TLC of GSLs was performed on precoated silica gel 60 plates (E. Merck, Darmstadt, Germany) with C/M/water (60:35:8, v/v/v). Each serum GSL was deposited twice on a silica-TLC plate. GSLs were separated with R_f values of 0.05 and added to C/M solution (2:1, v/v). After centrifugation at 15,000 rpm for 10 min, the resultant supernatants containing GSLs were used for detailed GSL-glycan analysis as described above.

LC-MS/MS analysis

A Prominence UFLC system (Shimadzu, Kyoto, Japan) coupled to a TripleTOF 5600 system (Ab Sciex, Foster City, CA) was used for the structural analysis. Lipid extracts from the TLC fraction were injected onto an InertSustain column (5 μ m, 2.1 internal diameter \times 150 mm; GL Science, Tokyo, Japan). Mobile phase A was acetonitrile/methanol/formic acid (95:5:0.2, v/v) containing 5 mM ammonium formate and mobile phase B was methanol/formic acid (100:0.2, v/v) containing 5 mM ammonium formate. A sample was eluted at 0.2 ml/min through a 45 min gradient: mobile phase B, 0–5 min 0%, 5–10 min from 0 to 20%, 10–12 min hold 20%, 12–15 min from 20 to 50%, 15–22 min hold 50%, 22–27 min from 50 to 80%, 27–30 min hold, 30–45 min from 80 to 0%. TOF-MS and MS/MS analysis was run in the positive ion mode with the following instrument parameters: curtain gas of 10, ion spray voltage of 5,500, collision energy of 35, and temperature of 300. Data acquisition and analysis were performed using AnalystTF software version 1.7 (Ab Sciex).

A normal glucose concentration in human blood is reportedly about 4.4–6.1 mmol/l (23). Glycoblotting utilizes chemoselective ligation toward hemiacetal groups at the reducing ends of glycans released from glycoconjugates; therefore, a high blood glucose concentration is apparently undesirable for measurement of GSL-glycans in serum. Therefore, we studied the efficiency of GOD digestion. GOD is an enzyme that oxidizes D-glucose to form D-glucono- δ -lactone. As intended, the signal of glucose derivatized with aoWR was markedly decreased after oxidation (supplementary Fig. 1). Serum oxidized with GOD reduced the ion suppression effect of glucose and provided a better signal-to-noise ratio of GSL-glycans. This glucose-free serum was used for further optimization of the analytical protocol.

Total sphingolipids in serum were extracted using C/M (protocol 1), as is usually performed (24). Concentrated supernatants containing GSLs were digested with EGCCase I (25 mU) or a mixture of EGCCase I and II (both 25 mU). After enzymatic digestion, glycans were selectively captured by high-density hydrazide beads (BlotGlyco[®]) for highly efficient purification of oligosaccharides from complex biological samples. The captured oligosaccharides were subjected to on-bead methyl esterification using the 3-methyl-*p*-tolyltriazene reagent to stabilize sialic acid(s). Oligosaccharides were finally recovered as aoWR derivatives via imine exchange, which enables highly sensitive analysis by MALDI-TOF (25). Methyl esterification of the sialic acid carboxyl group used in this study is highly efficient, without any significant fragmentation of sialic acid(s), and allows simultaneous quantitative analysis of neutral and acidic glycans by MALDI-TOF MS with reflector and positive ion mode (26).

The MALDI-TOF MS spectrum of serum GSL-glycans of a healthy subject (number 1) using protocol 1, in which EGCCase I was used to liberate GSL-glycans, is shown in **Fig. 1A**. Up to 34 glycans were detected. Several glycan signals were observed even when the analysis was performed in the absence of EGCCase (Fig. 1B). These glycans are most probably present as free oligosaccharides in serum. Some free oligosaccharides have the same molecular mass as GSL-glycans; therefore, the quantitative values of serum GSL-glycans were corrected by subtraction of the values of free oligosaccharides. When the serum GSL-glycomic profiles were compared between the two EGCCase digestion conditions, one with a mixture of EGCCase I and II (both 25 mU) and the other with EGCCase I alone (25 mU), the resulting profiles were almost the same, with a correlation coefficient (r^2) of 0.99 (supplementary Fig. 2) and comparable amounts of recovered GSL-glycans. Therefore, we used only EGCCase I for further optimization.

Due to the high purification power of the glycoblotting procedure, the serum GSL-glycome was also analyzed when C/M extraction was omitted (protocol 2). GOD-treated serum was directly deglycosylated by EGCCase I, and the released GSL-glycans were analyzed as described above, except that the EGCCase I-treated sample was further

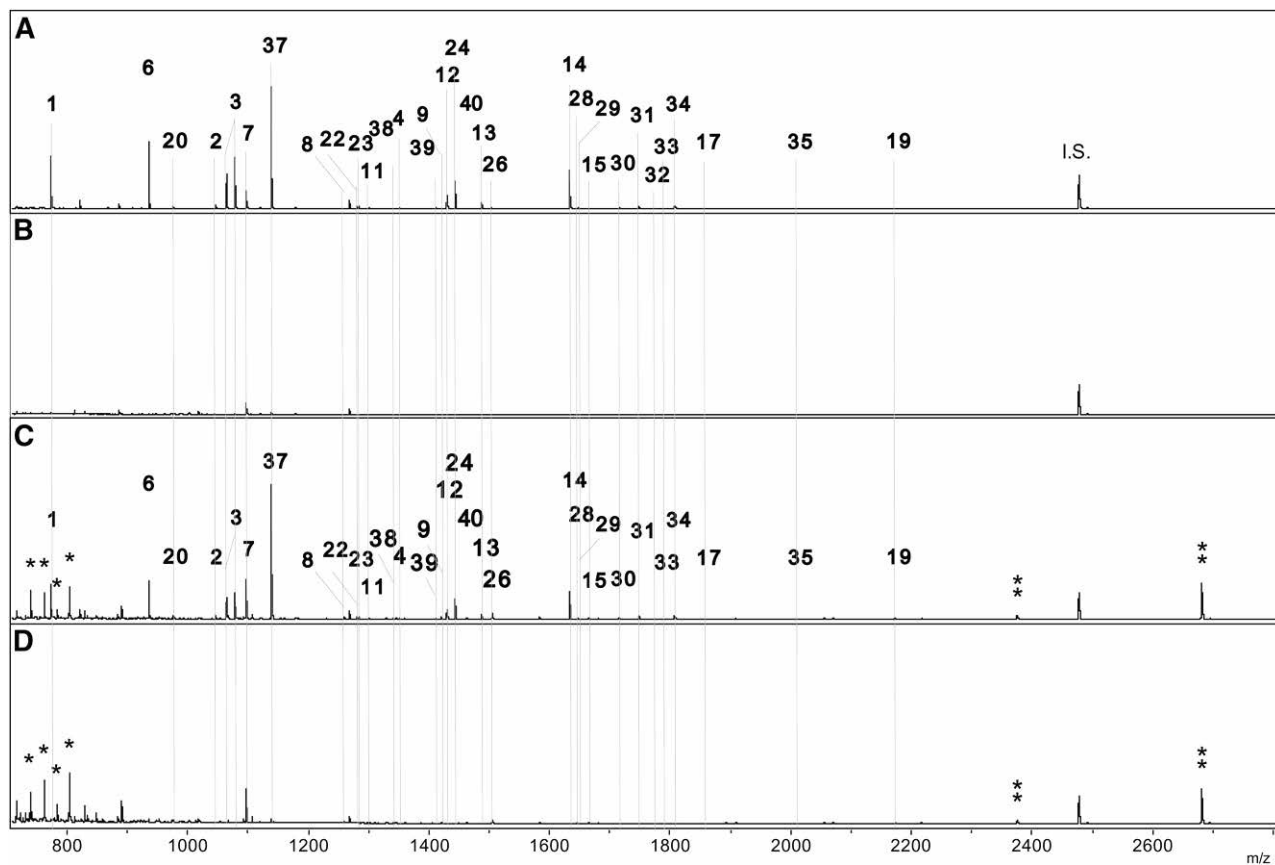


Fig. 1. MALDI-TOF MS spectra showing quantitative human GSL-glycan profiles. C/M-extracted serum in the presence of EGCase (A) and C/M-extracted serum in the absence of EGCase (B) (protocol 1). Nonextracted serum in the presence of EGCase (C) and nonextracted serum in the absence of EGCase (D) (protocol 2). The asterisks shown in (C) and (D) indicate peptide signals. The double asterisks indicate N-glycan signals.

digested with pronase prior to the glycoblotting procedure in order to reduce the viscosity of the solution (Fig. 2). Almost the same number of GSL-glycans were detected in both protocols, and the values of absolute quantitation were similar regardless of whether C/M extraction was performed (Fig. 1C, D). In protocol 2, several peptide signals were observed around m/z 800, which were most probably produced during pronase digestion. Signals derived from N-glycans were also observed at m/z 2,376 [(Neu5Ac)₁(Hex)₂(HexNAc)₂ + (Man)₃(GlcNAc)₂] and m/z 2,681 [(Neu5Ac)₂(Hex)₂(HexNAc)₂ + (Man)₃(GlcNAc)₂] (supplementary Fig. 3). Thus, protocol 2 has a great advantage of simplifying the sample preparation procedure and not using toxic reagents such as chloroform. However, we employed protocol 1 to clarify the human serum GSL-glycome by preventing contamination of peptides and N-glycans.

To evaluate the quantitative nature of the method, we performed GSL-glycan analysis using serially diluted serum samples (corresponding to 1.25, 2.5, 5, 10, and 20 μ l of serum). We detected 34 GSL-glycan signals in a healthy subject (number 1) when 5 μ l of serum, or more, was analyzed. When the starting amount of serum was reduced to 2.5 μ l, we still detected \sim 25 GSL-glycans. We realized that the signal strengths of glycans of LacCer and GM3 are too small, although they are reportedly the most abundant

GSLs in human serum (27). We thought that low molecular weight glycans might be lost during the solid phase extraction (SPE) process to remove excess reagents. The recovery ratio of aoWR-labeled GSL-glycans by SPE was measured by comparing the amount of each glycan before and after SPE. The quantitative values of most GSL glycans were not affected by the SPE process. However, those of LacCer, Gb3, and GM3 were significantly reduced after the SPE process (supplementary Fig. 4), which may be attributable to their weak hydrophilic interactions with the sorbent due to the relatively low number of hydroxyl groups. The analysis was fairly reproducible; therefore, absolute/relative abundance was estimated by applying correction factors (supplementary Table 1). To further verify the quantitative nature of the employed method, a mixture of equal quantities of 14 glycans (supplementary Table 2), with molecular weights ranging from m/z 772 to m/z 2,827, was subjected to the glycoblotting procedure, and their signal strengths were compared by MALDI-TOF MS in triplicate (Fig. 3). The signal strengths of the various aoWR-labeled glycans were similar, indicating that the structure and molecular weight have no significant effect on the signal strength. Naven and Harvey (28) also showed that oligosaccharides with masses greater than 1,000 Da exhibit similar signal strengths, irrespective of their structure, when examined by MALDI-TOF MS.

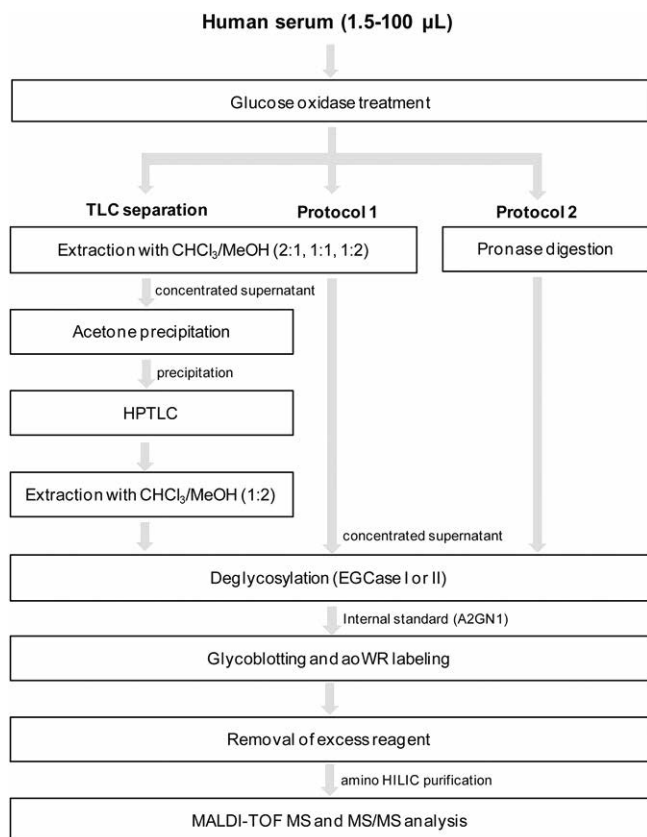


Fig. 2. Streamlined protocol for the analysis of GSL-glycans obtained from human serum.

There was a linear relationship between the amounts of various GSL-glycans and the amount of serum (**Fig. 4**). The reproducibility of protocol 1 was also good, with triplicate analyses giving similar results (supplementary Fig. 5).

Using the optimized and validated method, we performed absolute quantitation of GSL-glycans from individual serum samples (20 µl) of 10 healthy subjects with blood type A (numbers 1–10). The GSL-glycan profile of each serum sample is illustrated as a pie chart (**Fig. 5**). GSL-glycans were categorized into three series {ganglio (Gg), globo (Gb), and (neo)lacto [(n)Lc]}, according to their internal core carbohydrate sequence by tandem TOF MS analysis (supplementary Fig. 6). Some isomers were very difficult to distinguish even after tandem TOF analysis; thus, they were categorized as Gg/(n)Lc, (n)Lc/Gb, or Gg/(n)Lc/Gb isomers. The size of each circle and its constituent colors reflect the absolute glycan quantity (picomoles per 100 µl of serum) and the glycan substructures, respectively. The GSL-glycan profiles had similar patterns among the individual serum samples of healthy subjects, with a high degree of correlation [$r^2 = 0.81$ – 1.00 , mean = 0.98] (supplementary Table 3). Up to 42 signals corresponding to GSL-glycan compositions were observed in total, which is greater than the number detected by Kundu et al. (15), who detected 25 GSLs by HPLC and TLC coupled with gas-liquid chromatography. In all human serum samples, sialyl lactose $(\text{Hex})_2(\text{Neu5Ac})_1$ of GM3 was the most abundant GSL-glycan. The four next most abundant GSL-glycans were neutral species [$(\text{Hex})_2$, $(\text{Hex})_3$, and $(\text{Hex})_3(\text{HexNAc})_1$], and acidic ganglioside type sugars of $(\text{Hex})_3(\text{HexNAc})_1(\text{Neu5Ac})_1$. Tetraosyl glycan $(\text{Hex})_3(\text{HexNAc})_1$ agrees well with the previous observation that Gb4 $(\text{Gal}\beta 1-4\text{GlcNAc}\beta 1-3\text{Gal}\beta 1-4\text{Glc})$ is the major plasma GSL-glycan. Although $(\text{Hex})_3(\text{HexNAc})_1$ can be either Gb4 or Lc4, the level of Gb4 was about 50-fold higher than that of Lc4 (supplementary Fig. 7). Di-fucosylated glycans of Le^b [$(\text{Hex})_3(\text{HexNAc})_1(\text{Fuc})_2$] and ALe^b [$(\text{Hex})_3(\text{HexNAc})_2(\text{Fuc})_2$] were observed at high levels except in subject number 2. The estimated composition/

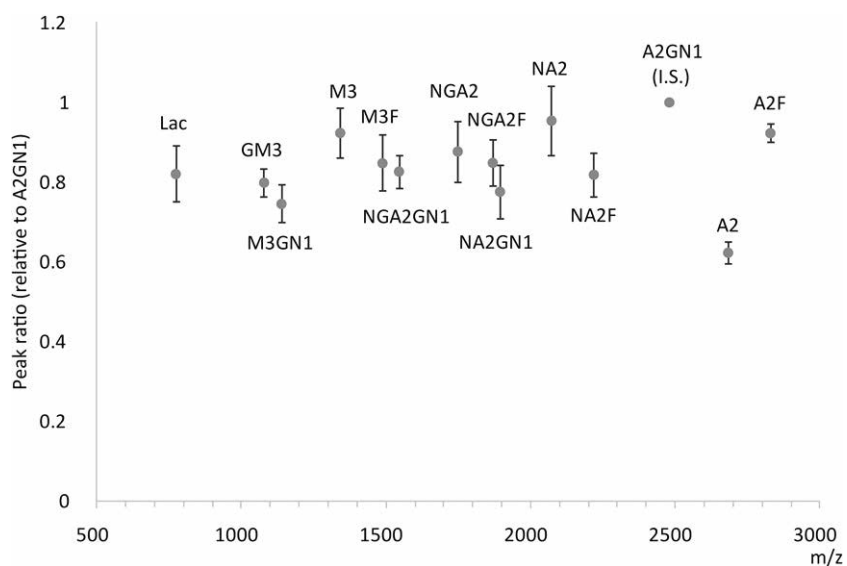


Fig. 3. Ionization efficiency of various aoWR-labeled glycans in MALDI-TOF MS. Fourteen glycans labeled with aoWR were analyzed by MALDI-TOF MS in triplicate. The peak areas of the glycans relative to those of an internal standard (A2GN1) are plotted (mean \pm SD). Equimolar amounts of glycans were prepared by acid hydrolysis and sequential enzymatic digestions of A2, A2F, and A2GN1. The prepared glycan structures are listed in supplementary Table 2.

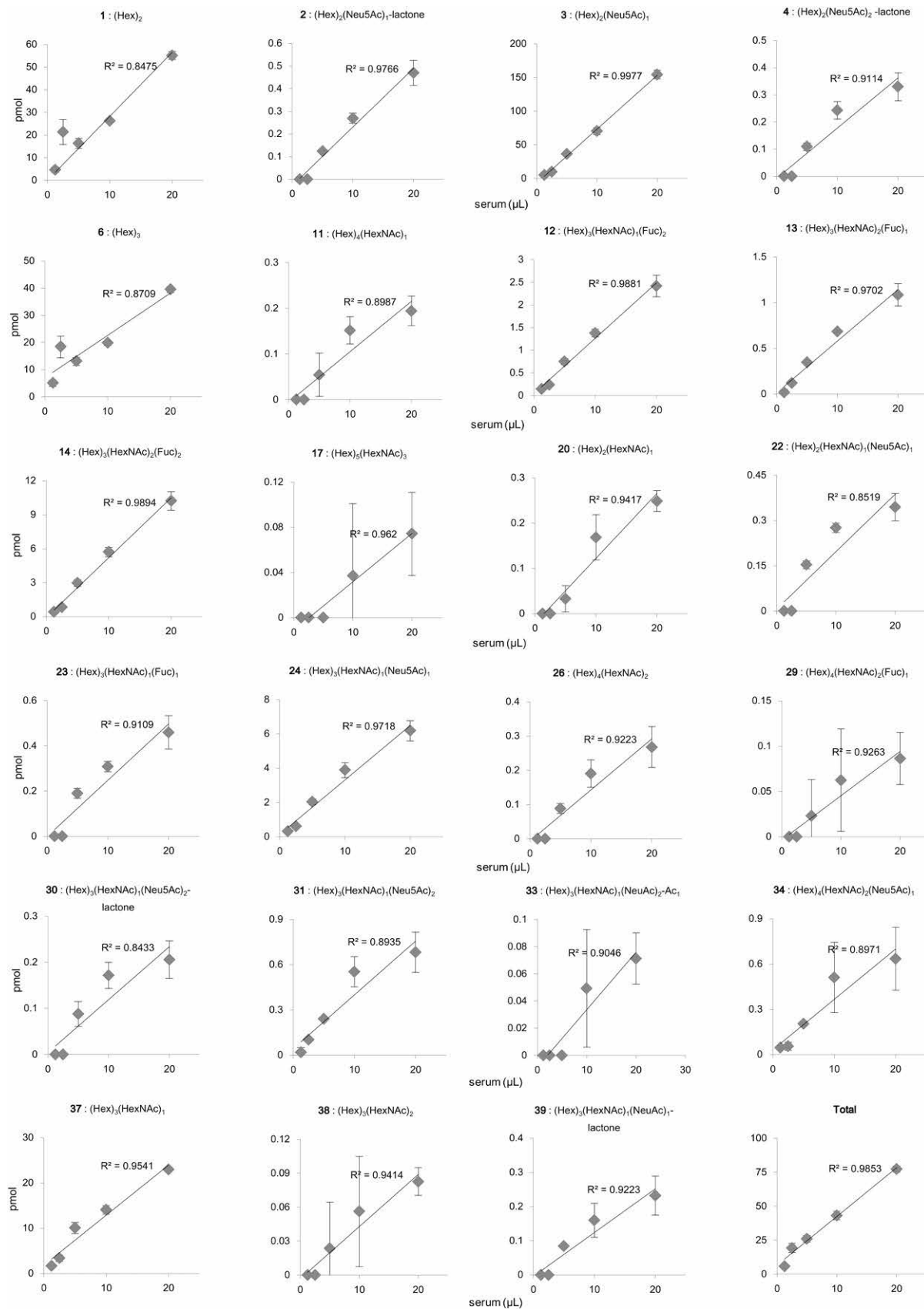


Fig. 4. Linear dynamic ranges of quantification of various GSL-glycans using protocol 1. The ion peak areas of GSL-glycans in MALDI-TOF MS were normalized to those of 20 pmol of A2GN1 as an internal standard.

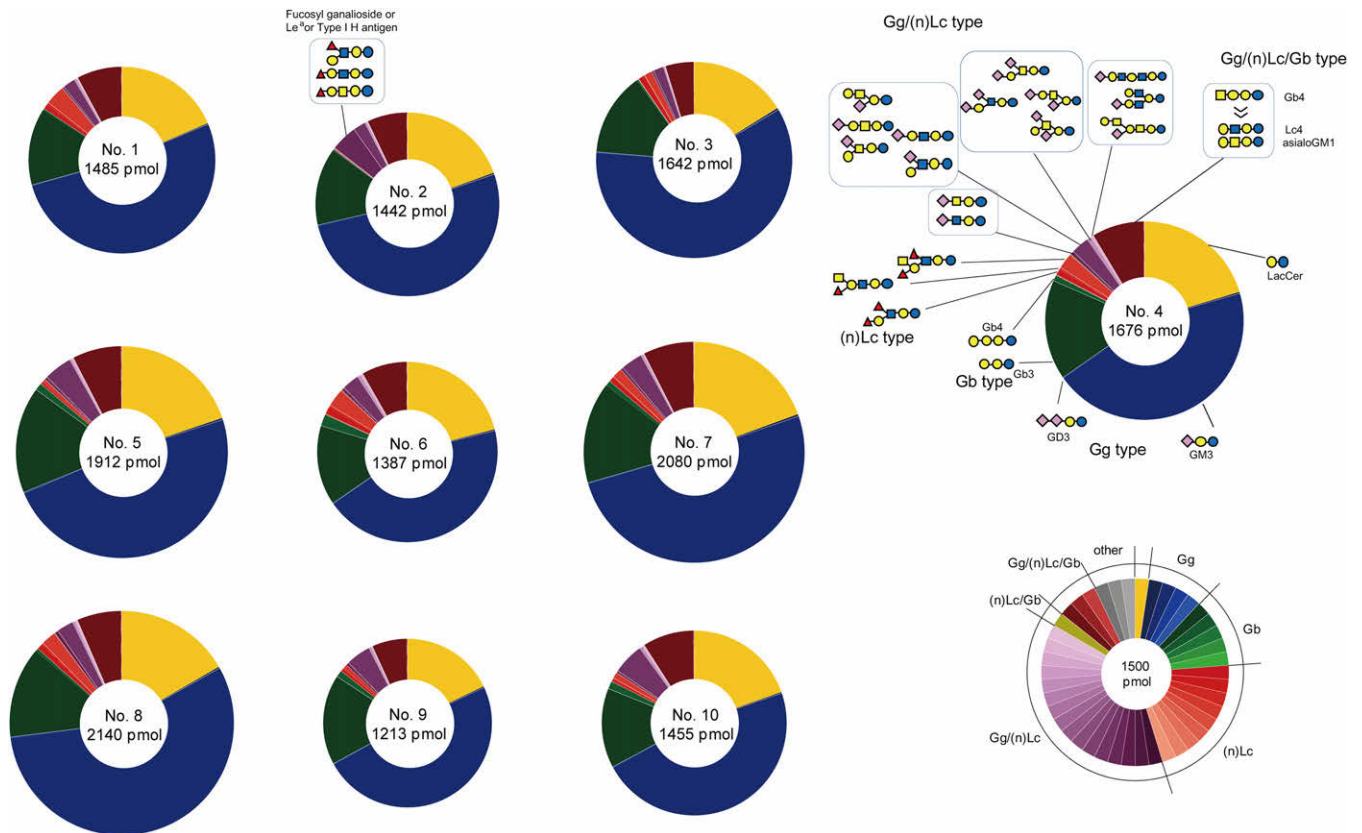


Fig. 5. Quantitative GSL-glycan analysis of healthy human serum. Both the absolute quantity of GSL-glycans (i.e., the size of the glycome) and the relative GSL-glycan composition are represented for each serum sample from healthy subjects. The areas of the circles represent the amount of total GSL-glycans per 100 μl of serum. The signal numbers correspond to those described in supplementary Table 4.

structure and quantitative values of human serum GSL-glycans are summarized in supplementary Table 4. These analyses revealed that the total concentration of serum GSL-glycans was 12.1–21.4 μM .

We further analyzed serum GSL-glycans after serum was fractionated by TLC. When the extracted lipid fraction of serum was subjected to TLC, only a few bands were stained with orcinol (supplementary Table 5). The TLC plate was cut into pieces with a width corresponding to a fixed R_f value of 0.05, and each piece was subjected to C/M extraction, EGCCase digestion, glycoblotting, and MALDI-TOF MS analysis. We detected 25 GSL-glycans. The merit of using TLC is not only its ability to separate isomers, but also to characterize the lipid moieties of GSLs whose glycan moieties have a unique expression profile. As an example, when fraction 5, which contained LacCer, was analyzed by LC-MS/MS, the majority of the sphingoid base and fatty acids of LacCer were sphingosine (d18:1) and palmitic acid (C16:0)/myristic acid (C14:0)/nervonic acid (C24:1), respectively (supplementary Fig. 8).

DISCUSSION

In the present study, we established two protocols for serum GSL-glycan analysis by applying recently established

cellular GSL-glycomics analysis based on rhodococcal EGCCase-assisted glycan cleavage, glycoblotting-assisted purification, and MALDI-TOF MS analysis. GSLs could be directly subjected to EGCCase digestion without further fractionation, such as traditional Folch separation. Likewise, it was not necessary to separate GSLs into acidic and neutral fractions by ion exchange chromatography, because the standard glycoblotting protocol includes methyl esterification of sialic acid to render sialylated oligosaccharides chemically equivalent to neutral oligosaccharides (27). In protocol 2, we demonstrated that even the extraction of total sphingolipids from serum could be omitted and that serum could be directly subjected to EGCCase digestion. Avoidance of the prefractionation of GSLs prior to glycan release and glycoblotting not only simplifies the analysis but also avoids the risk of sample loss. The amounts of GSL-glycans recovered were similar between protocols 1 and 2, demonstrating the quantitative nature of both methods.

Based on the optimized analytical technique, the serum GSL-glycome of 10 healthy subjects was analyzed qualitatively and quantitatively. We detected ~ 42 GSL-glycan signals, which is the highest number of GSLs detected in human serum, to our knowledge. Although the total amount of GSL-glycans differed by up to 1.8-fold among individual healthy human subjects, the GSL-glycan profiles exhibited a high correlation, except for that of healthy

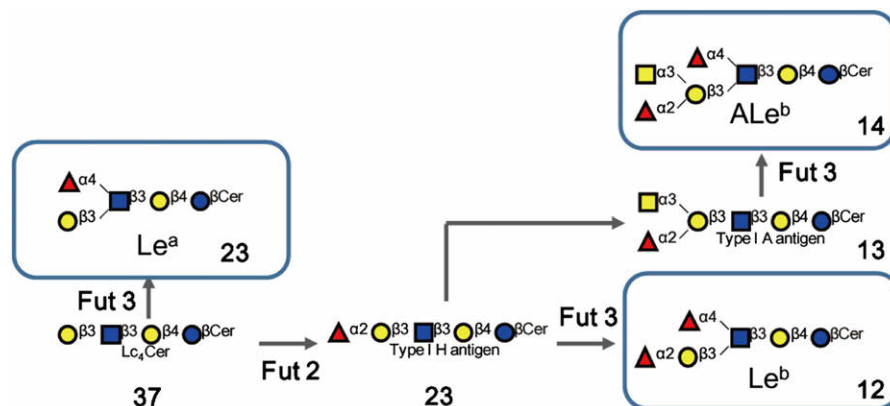


Fig. 6. Biosynthesis pathway of Le^a/type I H antigen, Le^b, and ALe^b.

subject number 2. In the GSL-glycan profile of subject number 2, expression of (Hex)₃(HexNAc)₁(Fuc)₂ and (Hex)₃(HexNAc)₂(Fuc)₂ was absent or extremely low, while that of (Hex)₃(HexNAc)₁(Fuc)₁ was extremely high. MS/MS analysis suggests that (Hex)₃(HexNAc)₁(Fuc)₂, (Hex)₃(HexNAc)₂(Fuc)₂, and (Hex)₃(HexNAc)₁(Fuc)₁ are Fucα1-2Galβ1-3(Fucα1-2)GlcNAcβ1-3Galβ1-4Glc (Le^b), Fucα1-2(GalNAcα1-3)Galβ1-3(Fucα1-2)GlcNAcβ1-3Galβ1-4Glc (ALe^b), and Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc (type I H antigen)/Galβ1-3(Fucα1-2)GlcNAcβ1-3Galβ1-4Glc (Le^a), respectively, which is supported by a plausible biosynthetic pathway and the fact that all serum samples were from healthy subjects (Fig. 6). This phenotype (Le^{a+}, Le^{b-}) is typical of nonsecretor individuals who lack the Se gene (FUT2). These results explain the marked differences in the GSL-glycomic profile of healthy subject number 2. Thus, it is extremely important to be aware of the marked differences in expression of GSL-glycans between secretors and nonsecretors for biomarker discovery studies. By omitting these three GSL-glycans, the *r*² values of the GSL-glycan profiles were increased to more than 0.96, which suggests the usefulness of human serum GSLs as a source of for biomarker discovery. Note that all the serum samples used in this study were from healthy subjects with blood group A. Detailed GSL-glycomic analysis of people with other blood groups is an important task for the future.

To the best of our knowledge, the absolute concentration of GSL-glycans in whole human serum (12.1–21.4 μM) was determined for the first time with estimation of the variability among several healthy subjects. We previously calculated the total N-glycan concentration in whole human serum to be 700–850 μM, indicating that the serum GSL concentration accounts for 1.4–3.1% of the total serum N-glycan concentration. In protocol 2, we detected two signals of N-glycans in the absence of EGCase, while no signals were observed when protocol 1 was used (Fig. 1B, D). Therefore, it is unlikely that these N-glycans are present as free oligosaccharides in serum; instead, they are artificially generated from glycoproteins during sample preparation. Indeed, these two N-glycans are the two major N-glycans among serum glycoproteins.

Most GSLs, as well as sphingolipids, circulating in blood are associated with lipoproteins (28). Protocols 1 and 2 gave similar serum GSL-glycomic results, both quantitatively and qualitatively, which confirms the quantitative extraction of GSLs by the traditional C/M extraction method.

The protocol established in the current study was quantitative, and GSL-glycomic profiles were fairly constant among healthy individuals when differences in GSL expression between secretors and nonsecretors were taken into consideration. This suggests that comprehensive human GSL-glycomic analysis is a promising approach for biomarker discovery. Furthermore, we demonstrated a glycoform-focused lipidomic approach to clarify the structure of lipid moieties by isolating GSLs of interest by TLC.¹⁶

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