Fluorometric detection of glycosphingolipids on thin-layer chromatographic plates

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Abstract A microdetection system for glycosphingolipid analysis has been developed using 5-hydroxy-1-tetralone as the fluorescent labeling reagent. The reagents in H_2SO_4 permit the fluorometric detection of acidic and neutral glycosphingolipids both in test tube and on thin-layer chromatographic plates. Glycosphingolipids can be detected at concentrations as low as 5 pmol on the thin-layer chromatographic plate. The method is a rapid and simple, and feasible for determination of glycosphingolipid from small amounts of biological samples.—**Watanabe, K., and M. Mizuta.** Fluorometric detection of glycosphingolipids on thin-layer chromatographic plates. *J. Lipid Res.* 1995. **36:** 1848–1855.

Supplementary key words fluorescent labeling • 5-hydroxy-1-tetralone • TLC • glycolipid • skeletal muscle • house musk shrew

It has become apparent that the carbohydrate moieties of glycoconjugates are involved in numerous important biological processes (1, 2). A number of chemical and immunological tools have been developed for carbohydrate determinations. Analyses of glycoconjugates from biological specimens were limited. Thus, to increase sensitivity of carbohydrate determination, a fluorometric method was used. A number of fluorogenic reagents have been proposed for determination of carbohydrates. The reagents are classified into two groups according to their reaction conditions. One group includes fluorescent probes for alcohol derivatization such as *m*-dansylaminophenylboronic acid and 5-(4,6-dichlorotriazinyl) aminofluoresein (3). The reagents of the other group are amino-containing reactive probes for aldehydes and ketones including hydrazine derivatives such as aromatic amines, aromatic diamine, Lucifer Yellow, and dansylhydrazine (3). Nonreducing carbohydrate chains on glycoproteins were selectively oxidized with $NaIO_4$ and a fluorescent label via a Schiff base type reaction with amino- or hydrazine-containing probes. Many phenolic amine derivatives have been introduced for derivatization of carbohydrates such as 5-hydroxytetralone (4, 5) and N-(1-naphyl) ethylenediamine (6). Glycosphingolipids (GSLs) are constituents of cell membranes. To elucidate the physiological roles of GSLs, much effort has been spent on isolation and characterization. High-performance thin-layer chromatography (HPTLC) is one of the major techniques for the determination of GSLs. The visualization of GSLs on TLC plates is most often performed with α-naphthol–H_2SO_4 (7) or resorcinol–HCl (8) reagents. The sensitivities of the reagents, however, were too low for the determination of limited amount GSLs from biological specimens such as biopsy samples, blood plasma, lymphocytes, and culture cells. For the fluorescent labeling of GSLs on TLC plates, we developed a method that involves periodate oxidation followed by reductive amination with aminomethylcoumarin (9). However, it was time-consuming and two reactions (oxidation and fluorescent labeling) were required. We have now developed a more rapid and simple method for determination of GSLs. 5-Hydroxy-1-tetralone has been used for the determination of blood sugar by Momose and Ohkura (5). We used the reagent for the specific fluorescent labeling of GSLs on TLC plate. Described herein is a simple and rapid fluorescent labeling technique designed to be generally applicable to subpicomolar determination of GSLs on TLC plates.

MATERIALS AND METHODS

Glycolipids and chemicals

Ganglioside standards were purchased from Iatron Lab. Inc. (Tokyo, Japan). SM4s, GM4, and neutral GSLs

Abbreviations: GSLs, glycosphingolipids; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; HPTLC, high performance thin-layer chromatography; HOT, 5-hydroxy-1-tetralone; THF, tetrahydrofuran. The designation of glycosphingolipids follows the IUPAC-IUB recommendations (27). Gangliosides have been designated according to Svennerholm (28). Schematic structures of neutral and acidic GSLs are shown in Table 2.

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were purified in this laboratory. 5-Hydroxy-1-tetralone (HOT) was obtained from Sigma Chemical Co. (St. Louis, MO). QAE-Sephadex A-25 was supplied by Pharmacia (Uppsala, Sweden). All other organic solvents and chemicals used were of the highest grade.

Apparatus

For determination of fluorescent-labeled GSLs on TLC plates, a Shimadzu dual-wavelength flying-spot scanner CS-9000 equipped with a fluorescence detector system with a xenon lamp was used. Fluorescent spectra were measured with a Hitachi Fluorescence photometer 650-10S in 10 × 10 mm quartz cells; spectral bandwidths of 5 nm were used in both the excitation and emission.

High performance thin-layer chromatography (HPTLC)

HPTLC precoated silica gel plates (10 × 10 cm, glass-backed) were purchased from J. T. Baker Inc. (Phillipsburg, NJ). Precoated plastic sheets (Polygram sil G) were purchased from Macherey-Nagel (Posfach, Germany). The following solvents were used for determination of GSLs: solvent A, chloroform–methanol–0.2% $CaCl_2$ 60:35:8; solvent B, chloroform–methanol–0.2% $CaCl_2$ 55:45:10.

Analytical procedure for carbohydrates and GSLs in test tube

To portions (100 µl) of carbohydrate or GSL (1 mM solution) in a test tube, 1 ml of 0.1% HOT in 80% H_2SO_4 was added. The mixture was heated at 120°C for 10 min, and then cooled in ice-water. The reagent blank was prepared in the same way. The fluorescent intensity was measured at 530 nm emission against 475 nm excitation.

Standard assay procedure for quantitation of GSLs on TLC plate by fluorescent reagent

GSLs (5–100 pmol) were spotted on a precoated TLC plate (Baker) and developed with solvent A for neutral GSL or solvent B for gangliosides. After TLC, GSLs on the plate were visualized by spraying with 0.1% HOT in 80% H_2SO_4. Then the plate was heated at 120°C for 10 min. The fluorescent intensities of GSLs on the plate were determined at an excitation wavelength of 470 nm with an optical cut-off filter (500 nm). GSLs on the TLC plate can be detected as yellow spots under UV light (365 nm). Phospholipids were also labeled by HOT reagent and appeared as a light blue color under UV light. The spots were easily distinguishable from yellow spots of GSLs.

Excitation and emission spectra of lactose, lactosylceramide, and GM3 ganglioside

Lactose, lactosylceramide, and GM3 ganglioside (100 µg each) were dissolved in 100 µl water. To this solution 1 ml of 0.1% HOT in 80% H_2SO_4 was added, and the mixture was incubated at 80°C for 40 min. After cooling, 5 ml of water was added. Fluorescent spectra were measured in 10 × 10 mm quartz cell.

Applications for determination of GSLs from house musk shrew and rat skeletal muscle

Because of their low concentration, there have been relatively few studies on GSLs of skeletal muscles. Quantitative studies on gangliosides in various tissues showed that the ganglioside concentration in skeletal muscles was 1/15 of that in brain and 1/3–1/4 of that in other organs (10). Thus GSLs from rat and house musk shrew were determined by the HOT reagent. GSLs were extracted from temporal and thigh muscle of a Wistar rat (16-week-old male, 1.0 g of temporal muscle and 35 g of thigh muscle) and a Jic:SUN strain house musk shrew (*Suncus murinus,* 10-week-old male, Japan Clea Co., Tokyo, 7.8 g of temporal muscle and 23.5 g of thigh muscle) by the THF method described by Tettermanti et al. (11). GSLs were separated to acidic and neutral GSL fractions by QAE-Sephadex column chromatography (12). GSL compositions of both fractions were determined by HPTLC with the HOT reagent. Total neutral and acidic GSL fractions were dissolved in 0.1 ml chloroform–methanol 2:1 per 1 g wet tissue. An aliquot (5 µl of neutral GSL and 10 µl of acidic GSLs) was spotted on HPTLC plate (Baker). The plate was developed with solvent A and GSLs were detected by the HOT reagent.

RESULTS

Optimal conditions

Optimal conditions for fluorescent labeling were examined so as to formulate an analytical procedure. The results obtained were assessed from the peak area in the chromatogram after fluorescent derivatization.

Concentrations of 5-hydroxy-1-tetralone and H_2SO_4

Fluorescent intensities for GbOse4Cer and GM1 ganglioside increased with increasing concentration of reagent. Maximum intensities were at 0.1–0.2% for GM1 and 0.5% for GbOse4Cer, and decreased thereafter (**Fig. 1A**). Influence of H_2SO_4 concentration between 97 and 60% was minimum for GbOse4Cer. However, fluorescent intensity of GM1 decreased rapidly with decreasing H_2SO_4 concentration. The fluorescent intensity in 60% H_2SO_4 was one-half that in 80% H_2SO_4 (data not shown).

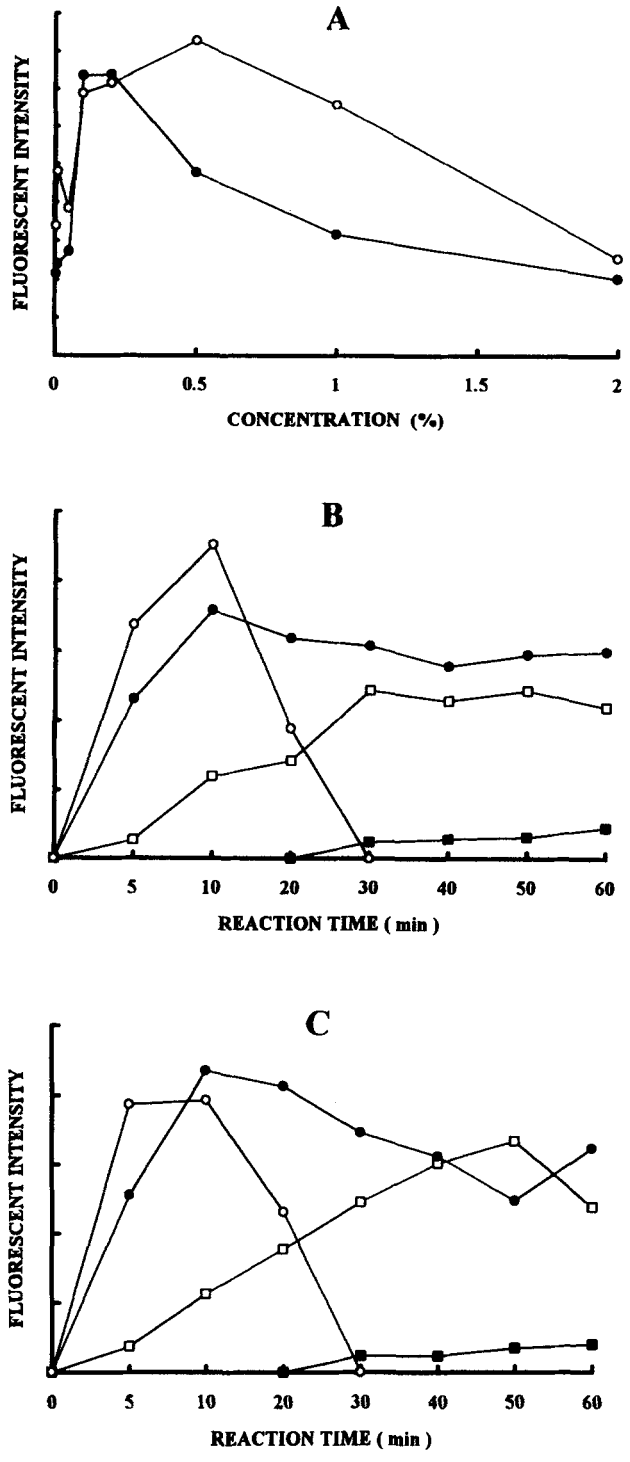

Fig. 1. Effects of the concentrations of 5-hydroxy-1-tetralone, reaction temperature, and time on fluorescent intensity. Panel A: Portions (100 pmol each) of GbOse4Cer (○) and GM1 ganglioside (●) were spotted on TLC plates and developed with solvent A. GSLs were reacted with various concentrations of HOT in 80% H_2SO_4 and heated at 120°C for 10 min. The fluorescent intensities of GSLs were determined at an excitation wavelength of 470 nm with an optical cut-off filter (500 nm). Panels B and C: GbOse4Cer (B) and GM1 ganglioside (C) on the TLC plate were reacted with 0.1% HOT reagent under various reaction times and temperatures; ○, 140°C; ●, 120°C; □, 100°C; ■, 80°C.

Reaction time and temperature

When the reaction temperature was set at 80°C, the fluorescent intensities of both GbOse4Cer and GM1 remained at a low level (Fig. 1B and 1C). On the other hand, the intensity increased rapidly and the maximum was attained within 5–10 min at 140°C and dropped rapidly thereafter. Over a 30-min incubation at 140°C, no fluorescence was detected. At 100°C, the intensity increased continuously to 30–50 min. At 120°C, the intensity increased rapidly; the maximum occurred at 10 min and gradually decreased thereafter. Consequently, to obtain an intense fluorescence, the reaction conditions for the analytical procedure were selected as 10 min for reaction time and 120°C for reaction temperature.

Fluorescent spectra of GSL

The fluorescent spectra of lactose, lactosylceramide, and GM3 ganglioside are shown in **Fig. 2.** The excitation and emission spectra produced from lactosylceramide and GM3 were similar in shape and maximum to the spectra for the product from lactose. The fluorescent spectra data indicated that reaction product of lactose,

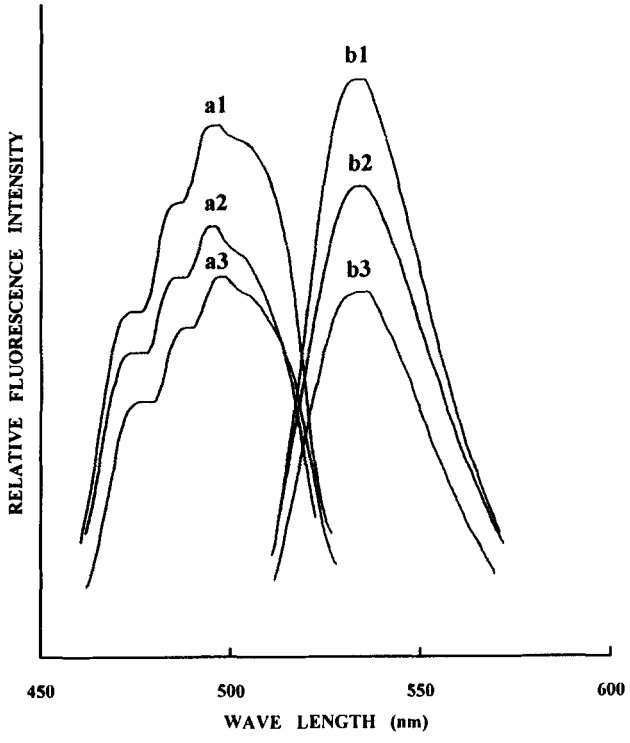

Fig. 2. Excitation and emission spectra of lactose, lactosylceramide, and GM3 ganglioside. Emission and excitation spectra of 100 μg of lactose, lactosylceramide, and GM3 ganglioside were analyzed after fluorescent labeling with HOT reagent; a, excitation spectra; b, emission spectra; a1 and b1, lactose; a2 and b2, lactosylceramide; a3 and b3, GM3 ganglioside.

lactosylceramide, and GM3 ganglioside might be the same fluorescent compound.

Fluorescent labeling of carbohydrates and GSLs

Hexoses, lactose, and GSLs reacted with the HOT reagent in the test tube and gave the fluorescence under the standard labeling condition. Their relative fluorescent intensities are listed in **Table 1.** The reagent did not provide fluorescence for N-acetylhexosamines, fucose, xylose, and N-acetylneuraminic acid. On equimolar bases, galactose and mannose showed 30–40% less fluorescent intensity than glucose. Glucosylceramide and galactosylceramide showed fluorescent intensities similar to that of corresponding hexoses. Lactosylceramide, however, gave a 1.45-fold higher intensity than lactose.

Relative fluorescent intensities of neutral and acidic GSLs listed in **Table 2** reflected varying yields of the total number of fluorescent-positive sugars (Glc and Gal). Sulfated glycolipid (SM4s) was also labeled by this method. We have tested HPTLC plates made by five different manufacturers including HPTLC Kiesel gel 60 plates (E. Merck, Darmstadt, Germany). Fluorescence of GSLs on TLC plates was detected only when a Si-HPF HPLC plate (J. T. Baker Inc.) or a Polygram Sil G plastic sheet (Macherey-Nagel) was used.

Calibration curves and sensitivity

The calibration graphs for GSLs listed in Table 2 were prepared and were linear over the concentration range 10 to 100 pmol. **Figure 3** shows the typical calibration curves for neutral (GalCer, LacCer, GbOse3Cer, GbOse4Cer, and IV^{3}GalNAcα-GbOse4Cer) and acidic GSLs (SM4s, GM3, GM1, and GD1a), although each GSL shows a different slope. The lower limits of detection of GSLs were 2–5 pmol. The coefficiency of variation from plate to plate, however, was 1.6–22%; thus, at least one GSL standard was needed for each plate. Fluorescent labeling patterns of standard neutral and acidic GSLs are seen in **Fig. 4,** lane 1 for neutral GSLs and lane 6 for acidic GSLs.

TABLE 1. Fluorescent intensities of various carbohydrates and glycolipids

Carbohydrate and Glycolipid	R.F.I.[a]
D-Glucose	100
α-Methyl-D-glucoside	139
D-Galactose	68
α-Methyl-D-galactoside	63
D-Mannose	57
D-Xylose	NF[b]
L-Fucose	NF
N-Acetylglucosamine	NF
N-Acetylgalactosamine	NF
N-Acetylneuraminic acid	NF
Lactose	146
Glucosylceramide	111
Galactosylceramide	64
Lactosylceramide	212

A portion (100 μl) of 1 mM solution of each compound in a test tube was treated with HOT reagent according to the procedure described in the text. The relative fluorescent intensity obtained by the reaction of D-glucose was taken as 100.

[a]Relative fluorescent intensity.

[b]No fluorescence was detected.

Determination of GSLs from rat and house musk shrew muscle

Acidic and neutral GSLs from rat and house musk shrew muscle were extracted and determined by HPTLC with HOT reagent (Fig. 4). The GSLs (two spots each) migrated to the positions nearby GM3 and were detected as the major component in acidic GSL fraction from both house musk shrew and rat skeletal muscle. Many minor spots (four to seven spots) that migrated under GM3 were detected. These minor GSL spots of house musk shrew muscle were different from those of the rat. Two heavy spots of monohexosylceramide and two spots of dihexosylceramide were detected in the neutral GSL fraction in both animals. Two additional spots that migrated near GbOse4Cer and nLcOse4Cer were detected in rat temporal and thigh muscle and these spots were absent in house musk shrew. GSLs with a long carbohydrate chain were also detected as minor spots especially in rat temporal muscle (lane 5 in Fig. 4).

DISCUSSION

Recently, with accumulating knowledge of biological functions of GSLs, a number of methods for micro determination of GSL from tissue, blood sample, and culture cells have been developed: isotope labeling (13), TLC-immunostaining with lectins (14) or monoclonal antibodies (15), and HPLC after derivatization with a UV absorbent tag (16–22). Described herein, a new procedure for glycosphingolipid detection on a TLC plate using 5-hydroxy-1-tetralone as a fluorescent reagent is introduced. The method is rapid, simple, and sensitive. Carbohydrates and GSLs on the TLC plate or in a test tube are fluorescently labeled by this method (except pentoses, methylpentose, hexosamines and sialic acid). GbOse3Cer, nLcOse4Cer, GbOse4Cer, and GgOse4Cer showed same the fluorescent intensity because these GSLs contain the same number of fluorescent-positive sugars (Glc and Gal). In the case of IV^3 GalNAc α-GbOse4Cer (Forssman GSL) the additional hexosamine–hexosamine unit is attached to the nonreducing terminal of the carbohydrate chain of GbOse3Cer, and fluorescent intensity is decreased. Substitution at the C3 position of Gal (sialic acid or sulfate attached to Gal) affected fluorescent intensity. The dif-

TABLE 2. Fluorescent intensities and schematic structure of neutral and acidic glycolipids

Glycolipid	Structure	R.F.I.[a]	F.P.S.[b]
Glucosylceramide	Glc-Cer	100	1
Galactosylceramide	Gal-Cer	59(1)	1
Lactosylceramide	Gal-Glc-Cer	179(9)	2
GbOse3Cer	Gal-Gal-Glc-Cer	176(19)	3
nLcOse4Cer	Gal-GlcNAc-Gal-Glc-Cer	186(17)	3
GbOse4Cer	GalNAc-Gal-Gal-Glc-Cer	192(13)	3
GgOse4Cer	Gal-GalNAc-Gal-Glc-Cer	189(10)	3
IV^{3}GalNAc α-GbOse4Cer	GalNAc-GalNAc-Gal-Gal-Glc-Cer	164(4)	3
nLcOse6Cer	Gal-GlcNAc-Gal-GlcNAc-Gal-Glc-Cer	258(24)	4
SM4s	Gal-Cer | SO3H	126(9)	1
GM4	Gal-Cer | NeuAc	115(12)	1
GM3	Gal-Glc-Cer | NeuAc	231(28)	2
GD3	Gal-Glc-Cer | NeuAc-NeuAc	182(12)	2
GM2	GalNAc-Gal-Glc-Cer | NeuAc	141(28)	2
GM1	Gal-GalNAc-Gal-Glc-Cer | NeuAc	195(43)	3
SPG	Gal-GlcNAc-Gal-Glc-Cer | NeuAc	206(3)	3
GD1a	Gal-GalNAc-Gal-Glc-Cer | | NeuAc NeuAc	229(40)	3
GD1b	Gal-GalNAc-Gal-Glc-Cer | NeuAc-NeuAc	229(36)	3
GT1b	Gal-GalNAc-Gal-Glc-Cer | | NeuAc NeuAc-NeuAc	152(16)	3
GQ1b	Gal-GalNAc-Gal-Glc-Cer | | NeuAc-NeuAc NeuAc-NeuAc	137(18)	3

A portion (100 pmol) of each GSL was spotted on an HPTLC plate and developed with solvent A for neutral GSLs and with solvent B for acidic GSLs. GSLs on TLC plates were labeled with HOT reagent according to the procedure described in the text. The relative fluorescence intensity obtained by the reaction of glucosylceramide with HOT reagent was taken as 100. Average values and standard deviation (in parentheses) were calculated from triplicate experimental data.

[a]Relative fluorescence intensity.

[b]Number of fluorescent positive sugars (Gal and Glc) in the carbohydrate chain of GSL.

ference of intensity is seen in the case of GM4 and SM4s compared to galactosylceramide. The intensity was also affected by the presence of external and internal sialic acid on the carbohydrate chain of the ganglioside. As seen in GM4, GM3, and SPG, the presence of external sialic acid increased fluorescent intensity as compared to the corresponding asialo type neutral GSLs, galactosylceramide, lactosylceramide, and nLcOse4Cer, respectively. The presence of the NeuAc α2-8NeuAc units on the carbohydrate chain of the ganglioside decreased its intensity as seen in GD3 compared to GM3, and GT1b and GQ1b compared to GM1 and GD1a, respectively. Fluorescent spectra data indicated that hexoses and GSLs gave the same fluorescent product. According to Momose and Ohkura (23, 24), the fluorescent compound might be a quinone formed by the addition of three carbons to HOT skeleton.

The method described here offers a simple and sensitive technique for the detection of both neutral and acidic GSLs. The method might be helpful for determination of GSLs from limited amounts of biological specimens such as biopsy materials, blood samples of

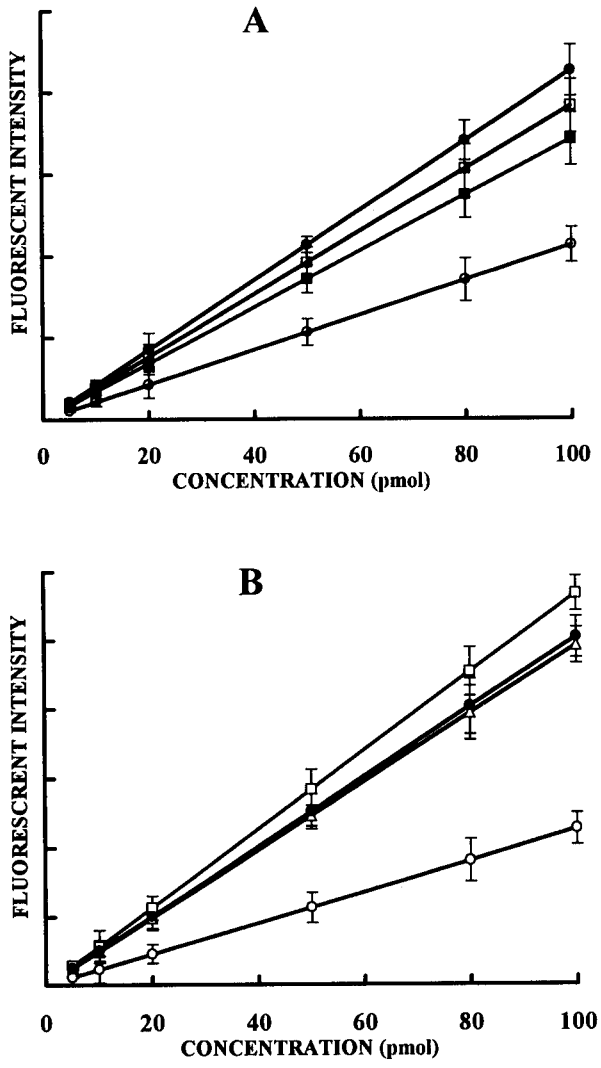

Fig. 3. Calibration curves for neutral (A) and acidic (B) GSLs. GSLs in various concentrations were spotted on HPTLC plate and developed with solvent A for neutral GSLs and with solvent B for acidic GSLs. GSLs on TLC plate were labeled with HOT reagent according to the procedure described in the text. Each calibration curve with standard deviations was calculated from five determinations. Panel A: ○, GalCer; □, LacCer; △, GbOse3Cer; ●, GbOse4Cer; ■, IV^{3}GalNAcα-GbOse4Cer; panel B: ○, SM4s; □, GM3; △, GM1; ●, GD1a. Calibration curves of LacCer and GbOse3Cer overlapped each other.

patients with various diseases, and culture cells. As shown in Fig. 4, differences of skeletal muscle GSL pattern of house musk shrew and rat have been detected by this method. Müthing et al. (25) described the differential distribution of GSLs in mouse and rabbit skeletal muscle. We found in this study that the neutral and acidic GSL pattern of rat muscle was similar to that of mouse. Mono- and dihexosylceramide were found as main compounds in house musk shrew. GSLs that migrated to the position of GbOse4Cer and nLcOse4Cer in rat muscle were not detected in house musk shrew muscle. The TLC pattern of gangliosides that migrated under GM3 was different between rat and house musk shrew. Quantitative determination and structural characterization of GSLs from house musk shrew muscle are the next subject of interest for future studies.

Recently, Müthing and Heitman (26) introduced the lipophilic fluorochromes for the detection of gangliosides. The reagent was used for non-destructive visualization with preparative TLC. The method, however, can be applied only to gangliosides and the detection limit was in range of 0.5–1 μg. The advantage of the HOT reagent is rapidity of the reaction over other fluorogenic reagents for the determination of GSLs on TLC plates. Thus, the reagent can also be used as a detection system for monitoring GSL elution from an HPLC column (K. Watanabe, unpublished observation). For the purification of GSLs, the GSL elution pattern from the HPLC column was monitored by absorption at 190–215 nm or by TLC analysis of the effluent fractions. When the GSL fraction contained neutral lipids and phospholipids, the UV monitoring system was not effective because non-glycolipid contaminants have much stronger UV absorption near 200 nm than GSLs. When peaks are detected, it does not imply the presence of GSLs. Thus, a UV monitoring system is not secure for monitoring GSL elution. A reliable detection system for GSLs eluting from an HPLC column is detection of GSLs after TLC. However, colorimetric detection required a large amount of sample (the minimum amount is in the range of 1–5 μg). For HOT reagent detection as compared to orcinol or resorcinol reagent detection, only 1/100–1/1000 of the amount of GSLs is required. Thus, the present method can effectively be used for the monitoring of GSL elution by HPLC with limited amounts of biological samples. The main advantages of our method are: *1)* it is specific for GSL detection without radioactive isotope labeling; *2)* it is simple, rapid, and sensitive; and *3)* it can be used for the HPLC monitoring of GSL elution. One disadvantage of the method is that GSLs are destroyed during the reaction, thus the method cannot be used for preparative purposes. It must be noted that GSLs can be fluorescently labeled only on the Si-HPF plate (HPTLC precoated silica gel plate, glass-backed, J. T. Baker Inc.) and Polygram Sil G (precoated plastic sheet, Marchery-Nagel). Each manufacturer uses a different binder for the preparation of silica gel TLC precoated to glass, plastic plates, and aluminum sheets. We suppose that must be the reason why GSLs can only be labeled by the HOT reagent on a particular TLC plate.

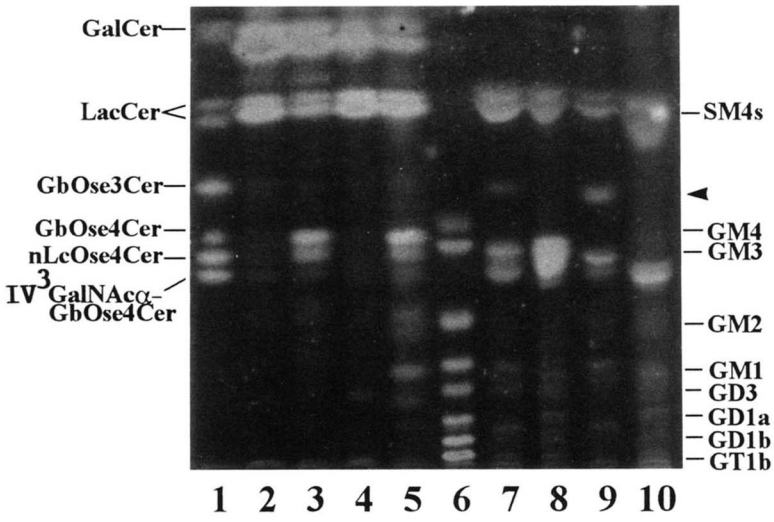

Fig. 4. Thin-layer chromatogram of GSLs from house musk shrew and rat skeletal muscle. GSL was dissolved in chloroform–methanol (2:1 v/v, 1 g wet tissue/0.1 ml) and a portion (5 μl of neutral fraction and 10 μl of acidic fraction) of GSL was spotted on HPTLC plate (Baker). TLC plate was developed with solvent A and GSLs were visualized by spray with HOT reagent. The fluorescent GSL spots were photographed under 365 nm UV lamp. Lane 1, standard neutral GSLs (50 pmol each); from the top: GalCer, LacCer (two spots), GbOse3Cer, GbOse4Cer, nLcOse4Cer, IV^{3}GalNAcα-GbOse4Cer; lane 6, standard gangliosides (50 pmol each), from the top, GM4, GM3, GM2, GM1, GD3, GD1a, GD1b and GT1b; lanes 2–5, neutral GSL fraction; lanes 7–10, acidic GSL fraction; lanes 2, 4, 7 and 9, house musk shrew muscle; lanes 3, 5, 8 and 10 rat muscle; lanes 2, 3, 7 and 8, thigh muscle; lanes 4, 5, 9 and 10, temporal muscle. Arrow head indicates the position of non-GSL spot (light blue) on lanes 7 and 9.

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