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A Quantitative Method for Separating Reaction Components of CMP-Sialic Acid:Lactosylceramide Sialyltransferase Using Sep Pak C_{18} Cartridges

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A rapid procedure is described for the separation of CMP-sialic acid:lactosylceramide sialyltransferase reaction components using Sep Pak C_{18} cartridges. The quantitative separation of the more polar nucleotide sugar, CMP-sialic acid, and its free acid from the less polar G_{M3}-ganglioside is simple and rapid relative to previously described methods. Recovery of G_{M3} is optimized by the addition of phosphatidylcholine to the reaction mixture prior to the chromatographic step. Using rat liver Golgi membranes as a source of CMP-sialic acid:lactosylceramide sialyltransferase activity (G_{M3} synthase; ST-1), the transfer of [^{14}C]sialic acid from CMP-[^{14}C]sialic acid to lactosylceramide can be quantified by this assay. The procedure is reliable and may be applicable to the isolation of ganglioside products in other *in vitro* glycosyltransferase assays.

The cell surface has been postulated to play an important role in the regulation of cell growth and differentiation. Gangliosides, ubiquitous sialic acid-containing glycosphingolipids of plasma membrane, have been implicated in these phenomena. Changes in ganglioside composition and metabolism have been associated with oncogenic transformation [1]; during which there is often an accumulation of less complex gangliosides than in the non-transformed cells, due to a block or impairment of the

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Abbreviations: G_{M3}, G_{M3}-ganglioside, II^{3}NeuAc-LacCer, NeuAcα2-3Galβ1-4Glcβ1-1Cer; G_{D1a}, G_{D1a}-ganglioside, IV^{3}NeuAc,II^{3}NeuAc-GgOse$_4$Cer, NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1Cer; G_{D3}, G_{D3}-ganglioside, II^3(NeuAc)$_2$LacCer, NeuAcα2-8NeuAcα2-3Galβ1-4Glcβ1-1Cer; GgOse$_4$Cer, asialo-G_{M1}, Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer; FucG_{M1}, fucosyl-G_{M1}-ganglioside, Fucα1-2Galβ1-3GalNAcβ1-4Galβ1-4 Glcβ1-1Cer; ST-1, G_{M3} synthase, CMP-sialic acid:lactosylceramide sialyltransferase; LacCer, lactosylceramide, Galβ1-4Glcβ1-1Cer; CMP-NeuAc, cytidine 5'-monophospho-*N*-acetylneuraminic acid; PC, phosphatidylcholine; PMSF, phenylmethylsulfonyl fluoride.

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ganglioside biosynthetic pathway. Alternatively, there may be synthesis of novel gangliosides due to activation of specific quiescent embryonic or fetal glycosyltransferases [2].

Several lines of evidence suggest that synthesis of G_{M3}-ganglioside, catalyzed by CMP-sialic acid:lactosylceramide sialyltransferase (ST-1), may be critical in the regulation of ganglioside biosynthesis and cell growth. ST-1 catalyzes the first committed step in the synthesis of gangliosides of the ganglio type. An especially important finding was the inhibition of cell growth via platelet dependent growth factor- and epidermal growth factor-mediated mitogenesis by the G_{M3}-ganglioside incorporated into the plasma membrane [3, 4].

The present assay was developed for use in the purification and characterization of ST-1 to homogeneity from Golgi membranes and for studies of the mechanisms involved in the regulation of G_{M3} synthesis by this enzyme. Like other glycosyltransferases, ST-1 is relatively unstable in solubilized preparations and its specificity for lactosylceramide (LacCer) and the sugar nucleotide is low [5]. *In vivo* sialyltransferase activity may be dependent upon the composition of the surrounding membrane, leading to difficulties in assays of its activity in partially purified preparations. Separation and analysis of the labeled G_{M3} product from its labeled precursors by most chromatographic methods [6-13] is time-consuming. For example, determination of G_{M3} synthase activity in our laboratory by the paper chromatographic method described by Basu and coworkers [10] requires two to three days [14]. On the other hand, employing Sep Pak C_{18} cartridges (Waters Associates) for the analysis requires only 2-3 h and gives high yields of labeled G_{M3} that is completely separated from the labeled sugar nucleotide precursor and free acid.

Materials and Methods

Materials

Sep Pak C_{18} cartridges were obtained from Waters Associates (Milford, MA, USA); Luer-lok tip syringes from Becton-Dickinson (Rutherford, NJ, USA); reagent grade solvents from Fisher Scientific (Pittsburgh, PA, USA); cytidine 5'-monophosphate [4-^{14}C]sialic acid, (NEC-636, 1.8 mCi/mmol) from New England Nuclear (Boston, MA, USA); [4,5,6,7,8,9-^{14}C]sialic acid (20 mCi/mmol) from ICN Pharmaceuticals (Irvine, CA, USA); phosphatidylcholine type III-L (from bovine liver) and type V-E (from frozen egg yolk), sodium cacodylate, cytidine 5'-monophosphate sialic acid (CMP-sialic acid) and Triton CF-54 from Sigma Chemical Co. (St. Louis, MO, USA); and HPTLC silica gel 60 from EM Science (Cherry Hill, NJ, USA).

Three sources of lactosylceramide were employed: a product from human erythrocytes prepared according to the method of Kundu [13], *N*-palmitoyl-DL-dihydro-lactocerebroside purchased from Sigma Chemical Co, and synthetic lactosylceramide prepared in our laboratory[15]. The labeled substrates [^{3}H]-G_{M3}, [^{3}H]-LacCer, [^{3}H]-GD_{1a}, [^{3}H]-G_{D3}, [^{3}H]-FucG_{M1} and [^{3}H]-GgOse$_4$Cer ([^{3}H]-asialo-G_{M1}) were prepared according to the potassium [^{3}H]borohydride reduction method of Schwarzmann [16]. The FucG_{M1} was a gift from Dr. S. Hakomori; asialo-G_{M1} was derived by mild acid hydrolysis of G_{M1}.

Rat Liver Golgi Membranes

CMP-sialic acid:lactosylceramide sialyltransferase (ST-1) was partially purified from rat liver Golgi membranes. The Golgi microsomal fraction was prepared by a method similar to those previously described by Morre *et al.* [17], Fleischer and Kervina [18] and Carey and Hirschberg [19]. Briefly, livers were removed from 176-200 g male CD rats (Charles River, Wilmington, MA, USA) and homogenized in 5 vol of 25 mM sodium cacodylate buffer (pH 6.5) containing 0.25 M sucrose and 1 mM phenylmethylsulfonyl fluoride (PMSF). Golgi microsomes were collected by differential centrifugation followed by sucrose density gradient centrifugation. Protein determinations were by the method of Peterson [20].

CMP-Sialic Acid:lactosylceramide Sialyltransferase Assay and Sep Pak C_{18} Separation Strategy

Assays of ST-1 activity were performed as previously described [5, 21]. The reaction was terminated by addition of 1000 μg phosphatidylcholine in 0.4 ml 100 mM KCl and frozen. This reaction mixture, containing LacCer, [^{14}C]G_{M3}, CMP-[^{14}C]sialic acid and, possibly, free [^{14}C]sialic acid formed by the hydrolysis of the sugar nucleotide, was transferred to a Sep Pak C_{18} cartridge (pre-conditioned as suggested by the manufacturer). Typically, the cartridges were attached to the end of a 5 ml Luer trip syringe, and washed with 5 ml methanol followed by 10 ml water. The sample was applied repeatedly with slight pressure until the eluate, after loading the sample mixture, was clear. Typically 2-3 times was adequate. However, when analyzing ST-1 activity in crude preparations, the assay mixture may need to be applied to more than one cartridge or the same cartridge may be reutilized. The cartridges are re-usable after repeating the washing procedure, three times for assays containing crude homogenates and about eight times in assays with Golgi vesicles. The radiolabeled nucleotide sugar, CMP-[^{14}C]sialic acid, and free [^{14}C]sialic acid were eluted from the cartridge with 10 ml water. Chloroform/methanol, 2/1 by vol, 0.5 ml, was added to change the solvent system in the cartridge. This step reduced the amount of time required to evaporate the organic solvent. The G_{M3}-ganglioside was subsequently eluted with 5.0 ml of chloroform/methanol, 2/1 by vol, and collected directly into a glass scintillation vial. The organic solvent was evaporated to dryness (traces of chloroform quench the radioactive signal) and the amount of [^{14}C]-G_{M3} was determined by liquid scintillation spectrometry in a Packard Tri-Carb 460C in 5 ml Flo Scint^{TM} II (Radiomatic Instruments and Chemical Co., Tampa, FL, USA).

Quantitative Recovery of ST-1 Reaction Components from Sep Pak C_{18} Cartridges

Solutions containing 40 nmol CMP-sialic acid (donor substrate), 100 nmol LacCer (acceptor substrate), 5 nmol G_{M3} (product) and 5 nmol sialic acid (free acid) in a final volume of 500 μl (see above) were applied to the reverse-phase cartridge. The elution profiles of the various reaction components were ascertained for reaction mixtures containing either CMP-[^{14}C]NeuAc, [^{14}C]NeuAc, or [^{3}H]G_{M3}. The radiobiologicals were eluted with sequential applications of 0.5 ml aliquots of various aqueous and organic solvents and processed as described above.

Recovery of each reaction component was also examined by GLC for sialic acid content. A modification of the method using trimethylsilyl (TMS) ethers, first developed by Sweeley and coworkers [23, 24] and later amended by Ledeen and Yu [24] was employed. The reaction mixtures for these studies contained only one sialic acid-containing component per assay. All other conditions were as described above. All samples were taken to dryness, TMS derivatized, and fractions analyzed. Methanol was added to aid in evaporation of the solvent.

Qualitative Recovery of the [^{14}C]G_{M3} Product

Identification of the ST-1 product was verified by TLC. The ST-1 activity assay was performed and G_{M3} product recovered as described above. The assay was repeated ten times and the organic phases pooled. The sample was taken to dryness and the phosphatidylcholine and [^{14}C]G_{M3} product were separated according to a published procedure [25]. The [^{14}C]G_{M3} was chromatographed on HPTLC silica gel 60 plates in chloroform/methanol/water, 60/30/5 by vol. The TLC plate was scraped in 0.5 cm segments, transferred to glass scintillation vials containing 1.0 ml water and sonicated. The amount of radioactivity was quantified by liquid scintillation spectrometry in a Packard Tri-Carb 460C in 5 ml Flo Scint^{TM}.

Results

Separation of ST-1 Reaction Components

Conditions for optimal recovery of the G_{M3}-ganglioside from the ST-1 assay mixture were determined from experiments employing a variety of eluting solvents. Under the conditions of glycosyltransferase assays, in addition to the expected transfer of sialic acid to the LacCer acceptor substrate to form G_{M3}, hydrolysis of the radiolabeled sugar nucleotide to radiolabeled free sugar and unlabeled nucleotide may occur if phosphodiesterases or phosphatases are present in crude preparations [26]. Therefore, it was necessary to compare recovery of CMP-[^{14}C]sialic acid, [^{14}C]sialic acid and [^{14}C]G_{M3} from our applied sample.

The separation of the radiolabeled reactants and the G_{M3} product of the ST-1 reaction mixture was performed as described in the Materials and Methods section. The recoveries of CMP-[^{14}C]sialic acid, [^{14}C]sialic acid and [^{3}H]G_{M3} were determined in H_2O, 100 mM KCl, phosphate-buffered saline (PBS; 137 mM NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4, 8.06 mM NaH_2PO_4) and 25 mM sodium cacodylate (pH 6.5): methanol, chloroform/methanol, 1/1 by vol; chloroform/methanol, 2/1 by vol; and chloroform/methanol/water, 60/30/5 by vol; and in the absence and presence of phosphatidylcholine. Employing the strategy of Williams and McCluer [27], application of G_{M3}-gangliosides to the cartridge in 100 mM KCl gave recoveries of only 39-53% with the aqueous solvents surveyed and 47-61% with the organic solvents from the ST-1 reaction mixture. This method has been reported to give 94% recovery of rat brain gangliosides from the theoretical upper phase containing 100 mM KCl [27]. Our inability to recover the G_{M3} completely was attributed to the presence of 0.3% Triton CF-54 in our ST-1 assay buffer. To enhance the recovery of the [^{3}H]G_{M3} by increasing the size of the

Table 1. Recovery of [^{3}H]-G$_{M3}$ from Sep Pak C$_{18}$ cartridges. The effects of phosphatidylcholine on recovery.

Sample no.	100 mM KCl (µl)	PC[a] (µg)	H$_2$O (µl)	[^{3}H]-G$_{M3}$ cpm Recovered	cpm Average	% Recovered
1	—	—	—		209490 ± 6060	100.0
2	0.4	—	—	122270	129340 ± 7070	61.7
3	0.4	—	—	136400		
4	—	10	0.4	148960	144080 ± 4880	68.8
5	—	10	0.4	139210		
6	—	100	0.4	181020	182930 ± 1920	87.3
7	—	100	0.4	184850		
8	—	1000	0.4	198010	206340 ± 8330	98.5
9	—	1000	0.4	214670		
10	0.4	10	—	156870	165890 ± 9020	79.2
11	0.4	10	—	174900		
12	0.4	100	—	175000	18000 ± 5000	85.9
13	0.4	100	—	185000		
14	0.4	1000	—	210850	210850	101.0
15	0.4	1000	—	228920[b]	(219890 ± 9030)	(106.0)[b]
16	—	10	0.4	186400	174810 ± 11590	83.4
17	—	10	0.4	163220		
18	—	100	0.4	173690	184290 ± 10600	88.0
19	—	100	0.4	194890		
20	—	1000	0.4	209080	200730 ± 9000	95.8
21	—	1000	0.4	191730		
22	0.4	10	—	125970	144280 ± 18320	68.9
23	0.4	10	—	162600		
24	0.4	100	—	184290	181360 ± 2930	86.6
25	0.4	100	—	178430		
26	0.4	1000	—	201560	207045 ± 5500	98.8
27	0.4	1000	—	212530		

[a] For samples 2-15, the PC was added to the 0.4 ml additive solution. For samples 16-27, the PC was added to the ST-1 reaction matrix.

[b] Value outside confidence limits of standard.

lipid-detergent-lipid micelles, phosphatidylcholine was added. PC enhances ST-1 activity and is probably a membrane-associated lipid [14]. PC from two different sources (bovine liver and egg yolk) was examined and both gave similar results. Other phospholipids were not assayed. The recovery of G$_{M3}$ was dependent upon the amount of PC added (10-1000 µg). Formation of the PC/Triton CF-54/G$_{M3}$ micelles gave recoveries of 70-100% of the ganglioside from the ST-1 reaction mixture in the organic wash (Table 1). Complete recovery of G$_{M3}$ was accomplished with the addition of 1000 µg PC in 100 mM KCl. Further, the detergent served to reduce the interaction of the nucleotide sugar with the cartridge. Williams and McCluer [27] reported that 0.39% of added CMP-NeuAc

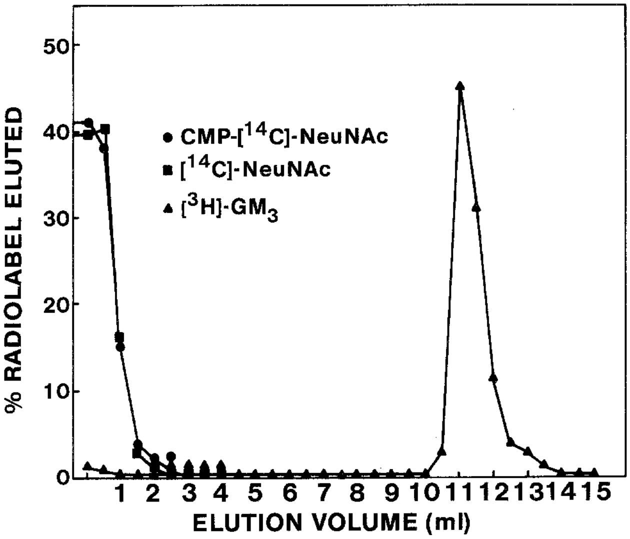

Figure 1. Elution profile of radiolabeled ST-1 reaction components on Sep Pak C_{18} cartridges. Chromatography on Sep Pak C_{18} was performed on complete reaction mixtures composed of 100 μl of a protein fraction containing ST-1, and 400 μl 100 mM KCl containing 1000 μg PC (sonicated before addition to the reaction mixture). The final concentrations of components contained in the ST-1 reaction mixture were 1 mM LacCer, 0.4 mM CMP-NeuAc, 0.05 mM G_{M3}, 0.05 mM NeuAc, 112.5 mM sodium cacodylate (pH 6.5), 15 mM $MnCl_2$, 0.125 mM sucrose, 0.05 mM PMSF and 0.3% (w/v) Triton CF-54. CMP-[^{14}C]NeuAc (●), [^{14}C]NeuAc (■) or [^{3}H]-G_{M3} (▲) were individually substituted into the ST-1 reaction mixture and their recovery monitored by collecting 0.5 ml fractions from cartridges.

was absorbed on Sep Pak C_{18} cartridges and that with extensive washing of the cartridge with water, retention of nucleotide sugars could be reduced. In our experiments, the nucleotide sugar and free sialic acid were recovered in good yield (99-100%) from the cartridge regardless of the aqueous solvent used. The recovery of CMP-sialic acid and sialic acid was completed within 4-5 ml aqueous solvent; however, 10 ml was typically employed. Negligible amounts (0.01 -0.04%) were recovered in the organic solvent and as shown by GLC analy!sis for sialic acid content, the anomeric mixture of sialic acid recovered from the CMP-sialic acid after methanolysis was different from that obtained from G_{M3}. Thus, critical differentiation can be made between the amount of product formed and any possible contamination from nucleotide sugars. Additionally, the recovery of the ganglioside correlated with the degree of polarity of the solvent employed. With the exception of methanol (92% recovery of G_{M3}), all the organic solvents examined gave complete recovery of the G_{M3}-ganglioside. The most reproducible results were obtained when chloroform/methanol, 2/1 by vol, was the solvent. A typical elution profile for the recovery of the G_{M3}-ganglioside according to our separation scheme, as described in the Materials and Methods section, is shown in Fig. 1.

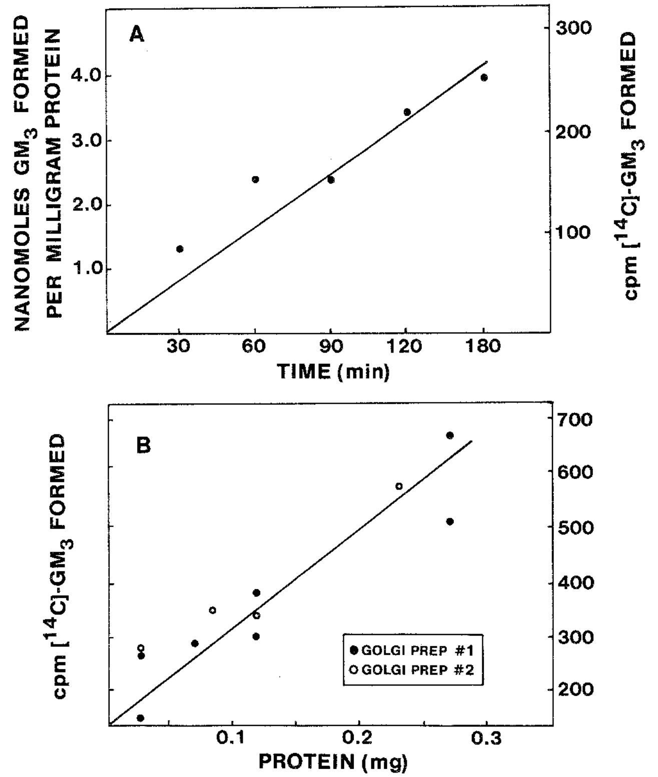

Figure 2. Enzymatic synthesis of [^{14}C]-G_{M3} from CMP-[^{14}C]NeuAc and LacCer as a function of time and protein. A. Reaction mixtures with 2.28×10^{-2} mg Golgi-enriched microsomal protein were incubated for the indicated period of time at 37°C and the [^{14}C]-G_{M3} product was recovered as described in the Materials and Methods section.

B. Reaction mixtures containing the indicated amount of Golgi-enriched microsomal protein were incubated for 155 min at 37°C and processed as described in the Materials and Methods section. These data were collected from two different Golgi membrane preparations from rat liver.

The recovery of the ST-1 reaction components, as investigated by GLC analysis of both the polar and non-polar eluants for sialic acid content, gave similar results for the devised separation scheme of: 1000 μg PC in 100 mM KCl, water, and $CHCl_3$/MeOH, 2/1 by vol (applied volume, aqueous solvent, and organic solvent, respectively). The sugar nucleotide and free sialic acid eluted with the aqueous solvent and the G_{M3}-ganglioside was recovered with the organic solvent. Methanolysis of G_{M3} gave the methyl ketal of *N*-acetylneuraminic acid in the β-anomeric configuration which was recovered in the organic solvent without contamination from other reaction components. The free sialic acid liberated from the other reaction components was primarily in the α configuration and recoverable in the aqueous solvent. Some traces of α-NeuAc (0.05 ± 0.02 μg) were detected in the organic fraction. However, these amounts did not significantly increase the background in blanks for the analysis of the G_{M3} by radioactivity.

Table 2: Recovery of other gangliosides from ST-1 reaction mixture from Sep Pak C_{18} cartridges.

Glycosphingolipid	Amount added (cpm)	cpm [^{3}H]-GSL recovered above background	% Recovered
[^{3}H]-LacCer	300 000	297460 ± 8680	99.2
[^{3}H]-G_{M3}	184 000	188580 ± 5250	102.5
[^{3}H]-fucosyl G_{M1}	1600	1970 ± 110	123.1
[^{3}H]-$GgOse_4Cer$ (asialo-G_{M1})	20 000	17350 ± 750	86.8
[^{3}H]-G_{D3}	9 800	8380 ± 60	84.9
[^{3}H]-G_{D1a}	5 700	4900 ± 610	85.9

The capacity of the Sep Pak C_{18} cartridge for G_{M3} was examined by analysis of radioactivity and by gas chromatographic analysis. In analysis of radioactivity, application of up to 50 nmol of [^{3}H]G_{M3} was completely recoverable. Gas chromatographic analyses indicated that application of G_{M3} equivalent to 25 μg of sialic acid (81 nmol) overloaded the Sep Pak C_{18} cartridge whereas 13.6 μg of the G_{M3} sialic acid (44 nmol) was retained by the cartridge. To determine the maximum capacity of the cartridge with optimal recovery, the recovery of 0-15 μg of G_{M3} sialic acid was examined. For amounts of G_{M3} not exceeding 2.5 μg (8 nmol) of sialic acid, 96% of the G_{M3} was recovered. This is in contrast to the 5 μmol reported by Williams and McCluer for ganglioside sialic acid recovery from theoretical upper phase [27]. When 5-15 μg (16-49 nmol) of G_{M3} sialic acid was applied to the cartridge using our ST-1 system, the recovery was reduced to 75-87%.

Application of ST-1/Sep Pak C_{18} Strategy in Determining ST-1 Activity

The transfer of [^{14}C]NeuAc from CMP-[^{14}C]NeuAc to LacCer to form [^{14}C]-G_{M3} by ST-1 was examined using the Sep Pak C_{18} separation strategy described in the Materials and Methods section. The enzymatic synthesis of G_{M3} in rat liver Golgi was studied as a function of time and Golgi microsomal protein (Fig. 2). The synthesis of G_{M3} proceeded linearly for at least 180 min and the synthesis of the product was proportional to the amount of protein in the reaction mixture up to 0.25 mg of microsomal protein. The cartridges were saturated by excess protein in the crude enzyme preparation, resulting in non-linearity of the curve beyond 0.3 mg of Golgi fraction.

The acidic glycosphingolipid formed from LacCer and CMP-sialic acid was identified as G_{M3}-ganglioside by TLC. The ST-1 activity assays were set up with two different formats. Pooled eluates of glycosphingolipid were obtained from 10 assays employing CMP-[^{14}C]sialic acid and LacCer while separate products were pooled from a second set of 10 assays employing CMP-sialic acid and [^{3}H]LacCer. The organic eluants from each set were dried under nitrogen and the phosphatidylcholine and salts removed by the procedure described by Ladisch and Gillard [25]. The radiolabeled products comigrated with a reference sample of [^{3}H]-G_{M3} on HPLTC plates (data not shown).

The applicability of this reverse-phase chromatography strategy on Sep Pak C_{18} cartridges for the recovery of other glycosyltransferase products was briefly investigated.

Table 2 shows that while the conditions have been optimized for the recovery of G_{M3}; other glycosphingolipids such as LacCer, Fuc-G_{M1}, $GgOse_4Cer$, G_{D3} and G_{D1a} can be recovered from the detergent-containing ST-1 reaction mixture by this procedure. The recoveries for these glycosphingolipids ranged from 85% to 123%. Thus, this system for recovery and analysis of ST-1 sialyltransferase with slight modifications may be equally effective for assays of other glycosyltransferase activities.

Discussion

The major purpose of this investigation was to simplify the analysis of CMP-sialic acid:lactosylceramide sialyltransferase (ST-1) activity. We have developed a rapid and convenient system for the separation and analysis of G_{M3}-ganglioside from crude reaction mixtures. Sep Pak C_{18} cartridges (octadecylsilane bonded-phase packings) are useful for the separation of problem sample preparations containing components of differing polarities in complex aqueous solvents. A common problem in studies of ganglioside synthesis and regulation is the need for complete separation of radiolabeled sugar nucleotides from the radiolabeled ganglioside product. Several methods have been developed for removal of non-ganglioside reactants [6-13].

Williams and McCluer [27] have previously reported that Sep Pak C_{18} reverse-phase cartridges can be employed for the recovery of gangliosides from theoretical upper phases of Folch-extracted tissues with the addition of 100 mM KCl to the sample matrix. However, their modification is not applicable for the recovery and analysis of gangliosides synthesized from detergent-solubilized Golgi membrane preparations. With detergent in our ST-1 assay mixtures, the recovery of G_{M3} was 39-62%, which is not acceptable for enzymatic analyses. Since purification and characterization of membrane-bound ST-1 requires detergent solubilization for *in vitro* analysis of enzyme activity, we found it necessary to modify the Sep Pak C_{18} procedure to accommodate the composition of our reaction mixtures. The Sep Pak C_{18} cartridges provide a rapid, convenient and reliable system for determining ST-1 activity during its purification and characterization. ST-1 activity can be quantified accurately up to the formation of 8 nmol of G_{M3}. Enzyme activity can be determined by the recovery of radiolabeled G_{M3} or by GLC analysis of sialic acid recovered from the G_{M3} by methanolysis. This method can be applied to quantitative analyses of other glycosyltransferase activities involved in the biosynthesis of gangliosides. The described procedure has been modified in our laboratory for the analysis of G_{D3} synthase activity. The only problem we have encountered is that some detergents, e.g., Triton X-100 ($>0.5\%$, by vol), completely solubilize 1000 μg PC in the sample matrix. Therefore, depending on the nature of the detergent employed and the nature of the lipid environment of the glycosyltransferase, a different amount or type of phospholipid may have to be used to obtain optimal recovery of the ganglioside product.

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