Isolation and characterization of human liver hematoside

Thomas N. Seyfried, Susumu Ando, and Robert K. Yu[1]

Department of Neurology, Yale University School of Medicine, New Haven, CT 06510

Abstract The monosialoganglioside hematoside (G_{M3}) is an important precursor in the synthesis of the more complex gangliosides. To obtain large quantities of G_{M3} for use in ganglioside biosynthetic experiments, we have devised a column chromatographic procedure for the isolation and purification of G_{M3} from human liver. A total ganglioside mixture was obtained from a 550 g sample of normal human liver. Quantitative analysis by gas–liquid chromatography indicated about 66 μg of lipid-bound *N*-acetylneuraminic acid per gram of fresh tissue. Hematoside appeared as double bands on thin-layer plates and represented 86.4% of the total sialic acid content. Additional ganglioside species, notably G_{D3}, were also seen. Hematoside was separated from the other gangliosides by gradient elution on an Iatrobeads column. The total G_{M3} obtained was 120 mg, which represented a 90% recovery. Although the long-chain base and sugar compositions of the upper and lower G_{M3} fractions were similar, striking fatty acid differences were detected. The upper fraction contained predominantly unsubstituted fatty acids, while the lower fraction showed a preponderance of α-hydroxy fatty acids. The unsubstituted fatty acid and hydroxy fatty acid composition of the total G_{M3} fraction was calculated to be 56.9% and 43.1%, respectively.

Supplementary key words gangliosides · long-chain bases · fatty acids

The term hematoside was first introduced by Yamakawa and Suzuki (1) to describe a substance that they had isolated from horse erythrocyte stroma which contained fatty acid, sphingosine, hexose, and sialic acid in the molar ratio of 1:1:2:1.[2] The structure of hematoside (G_{M3}) was subsequently characterized as *N*-acylneuraminyl(2-3)galactosyl(1-4)glucosyl(1-1)-*N*-acylsphingosine (2, 3). Two major types of hematosides (G_{M3}) that contain either *N*-acetyl- or *N*-glycolylneuraminic acid (NeuAc or NeuGc) have been isolated from various sources (4–7). A third type of hematoside that contains an additional *O*-acetyl group on the NeuGc moiety was found in equine erythrocyte membranes (8).

Hematoside usually occurs in brain tissues as a minor ganglioside species (9, 10) whereas a preponderance of this species is generally found among gangliosides in extraneural tissues and body fluids (11). Structural variations in the fatty acid and long-chain base (LCB) compositions of hematosides isolated from different sources have also been observed. In general, brain G_{M3} contains predominantly stearic acid with smaller amounts of the relatively longer chain fatty acids (C22–C24) (12, 13). The major LCB is sphingosine (d 18:1) but *trans*-4-icosasphingenine (d 20:1) is also present (10–15%) (13). The latter is characteristic of brain gangliosides (14, 15). However, G_{M3} isolated from extraneural tissues usually contains more of the relatively longer chain fatty acids in addition to large amounts of palmitic and stearic acids (16–18). The LCB consists primarily of the C18 variety while *trans*-4-icosasphingenine is generally absent.

Hematoside is known to be a key intermediate in the synthesis of the more complex gangliosides (19–23). It is therefore desirable to isolate large quantities of G_{M3} for use in studies of ganglioside biosynthesis. The present paper describes a simple column chromatographic procedure for the bulk isolation and purification of G_{M3} from human liver. Human liver is easily available and is also known to contain G_{M3} as the predominant ganglioside species (24, 25). In addition, we have found unusually large amounts of α-hydroxy fatty acids in human liver G_{M3}. The presence of these fatty acids has only recently been recognized in human kidney gangliosides (26). A preliminary account of our work has been presented (27).

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; LCB, long-chain base; d 16:1, *trans*-4-hexadecasphingenine; d 18:1, sphingosine; d 18:0, sphinganine; EDTA, ethylenediamine tetraacetate.

[1] To whom reprint requests should be sent. Department of Neurology, Yale University School of Medicine, 333 Cedar St., New Haven, CT 06510.

[2] The term hematoside has frequently been used to describe the species of gangliosides that lack hexosamine. It is used in this paper as a trivial name for G_{M3}.

MATERIALS AND METHODS

A scaled-up modification of the Ledeen, Yu, and Eng (28) procedure was used to obtain a total ganglioside mixture from a 550 g sample of normal human liver obtained at autopsy (84 yr, female). The tissue was homogenized in a mechanical blender with 10 vol/g of chloroform–methanol 1:1. The homogenate was first filtered through a Buchner funnel and then through a sintered glass funnel. The residue was washed once with chloroform–methanol 1:1 and the combined lipid extract was readjusted to 9 l of chloroform–methanol–water 30:60:8 (solvent A). A column containing 200 g of DEAE–Sephadex (acetate form) was prepared as follows. The DEAE–Sephadex resin was first washed three times with chloroform–methanol–0.8 M sodium acetate 30:60:8 (solvent B) followed by equilibration with the same solvent overnight. The resin was washed with solvent A until neutral and then poured into the column (5 cm ID × 53 cm). After slow application of the sample, the neutral lipids were eluted by the addition of 2 l of solvent A.

Gangliosides and other acidic lipids were then eluted with 6 l of solvent B and this fraction was evaporated to dryness. The dried residue was treated with 100 ml of 0.1 N sodium hydroxide in methanol at 37°C for 1 hr. The methanolic solution was concentrated to about 20 ml at 20°C followed by the addition of 5 ml of aqueous 0.5 M EDTA (tetrasodium salt) and 30 ml of distilled water. The sample was dialyzed at 4°C against deionized water for 2 days with frequent changes of water. The retentate was finally lyophilized. The residue was then dissolved in 100 ml of chloroform–methanol 80:20. The solution was next applied to a 100-g Unisil (200–325 mesh, Clarkson Co., Williamsport, PA) column (2.6 cm ID × 37 cm) prepared with chloroform. Sulfatides and fatty acids were eluted from the column with 900 ml of chloroform–methanol 80:20. The gangliosides were recovered by elution with 2.9 l of chloroform–methanol 2:3. The solvents were evaporated and the ganglioside extract was dissolved in 100 ml of chloroform–methanol 1:1.

A 1-ml aliquot was taken from the above solution for quantitative determination of ganglioside sialic acid and for TLC analysis. The concentration of ganglioside sialic acid was determined by the GLC method of Yu and Ledeen (29). High-performance thin-layer chromatography (HPTLC) plates (silica gel 60, E. Merck, Darmstadt, W. Germany) were used for qualitative examination of the gangliosides. The plates were developed by one ascending run in either chloroform–methanol–water 60:40:9 (containing 0.02% $CaCl_2 \cdot 2H_2O$) or chloroform–methanol–2.5 N ammonia 60:40:9. The ganglioside bands were visualized by spraying the plates with the resorcinol reagent. The percentage distribution of the ganglioside sialic acid was determined by scanning the plates with a scanning densitometer (Transidyne, Ann Arbor, MI).[3] Normal human white matter gangliosides prepared by the method of Ledeen et al. (28) and human brain hematoside isolated by the method of Ando and Yu (30) were used as standards.

Hematoside was separated from the other gangliosides by gradient elution between 1000 ml of chloroform–methanol–water 75:23:2 and 1100 ml of chloroform–methanol–water 50:47:3 on a 150 g Iatrobeads (Iatron Lab., Inc., Tokyo, Japan) (31) column (1.9 cm ID × 114 cm) (**Fig. 1**). Fractions of 16-ml eluants were collected and the concentration of sialic acid in each tube was determined by the resorcinol method of Svennerholm (32) as modified by Miettinen and Takki-Luukkainen (33) and Suzuki (34).

The total hematoside, which was partially resolved into two peaks, was collected between fractions 66 and 110. Thin-layer chromatographic analysis revealed that fractions 66–83, 84–93, and 94–110 corresponded to the upper, intermediate, and lower hematoside bands, respectively. Each fraction represented about one-third of the total G_{M3}. Long-chain base analysis was performed by the method of Sweeley and Moscatelli (35), while the fatty acids were analyzed as their methyl esters by GLC using two different types of columns (6 ft × ⅛ in ID): 10% SP 222 PS and 3% OV-1 (Supelco). Identification of the fatty acids was achieved by employing authentic fatty acid standards and by comparing the retention times on semi-logarithmic plots. The sugar components were analyzed by GLC as their *N*,*O*-trifluoroacetyl derivatives (36).

RESULTS

The total concentration of gangliosides in human liver was 65.9 μg of lipid-bound sialic acid per gram of wet tissue and the total G_{M3} obtained was 120 mg, which represented a recovery of about 90%. In two independent small-scale isolations from two different normal human livers, the concentration of gangliosides was found to be 60 and 79.3

[3] Ando, S., N. C. Chang, and R. K. Yu. *Anal. Biochem.* (In press)

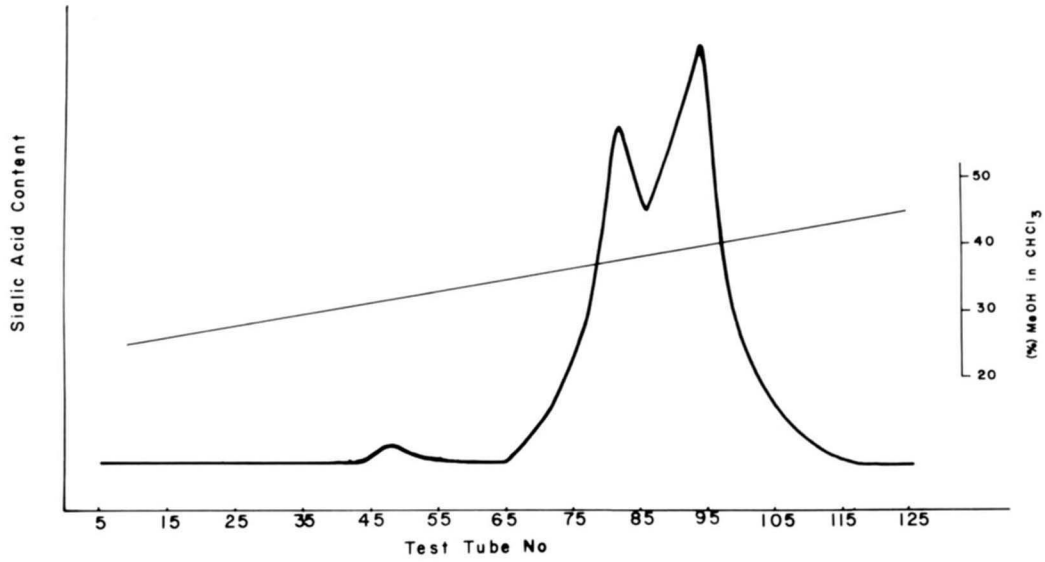

Fig. 1. Linear gradient elution of hematoside with increasing concentration of methanol in chloroform–methanol–water. See text for experimental details.

μg of lipid-bound sialic acid per gram wet weight. Only NeuAc was detected in human liver. Hematoside appeared as double bands on TLC plates (**Figs. 2** and **3**) and its sialic acid content represented 86.4% (39.2% upper band and 47.2% lower band) of the total ganglioside sialic acid as determined by densitometric measurement. Small amounts of other liver ganglioside species, notably, G_{D3} (6.6%) and G_{M2} (3.8%) were also seen.

The molar ratios of glucose, galactose, NeuAc, and LCB in the upper and lower G_{M3} fractions were 1.00, 0.94, 1.08, 1.16; and 1.00, 1.03, 1.00, 0.91, respectively. The LCB composition was also similar in

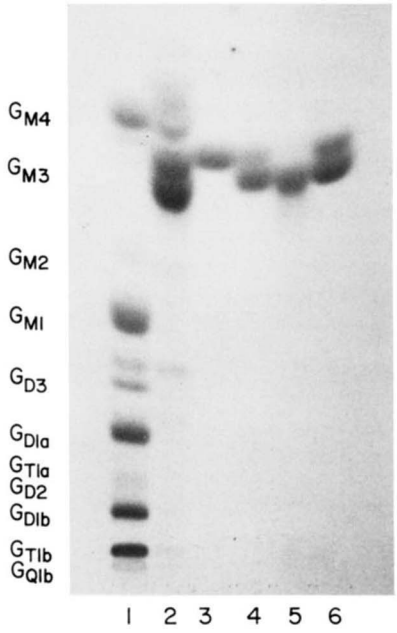

Fig. 2. Thin-layer chromatogram of human liver gangliosides. Lane 1, normal human white matter; 2, total liver ganglioside; 3, liver G_{M3} upper; 4, liver G_{M3} intermediate; 5, liver G_{M3} lower; 6, human brain G_{M3}. HPTLC (Merck silica gel) plates were used. The plate was developed by one ascending run in chloroform–methanol–water 60:40:9 (v/v/v) containing 0.02% $CaCl_2 \cdot 2H_2O$. The bands were visualized by the resorcinol reagent and the ganglioside species were named according to the system of Svennerholm (9).

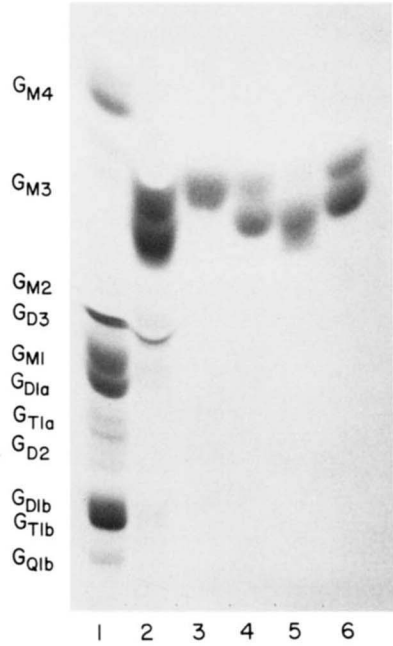

Fig. 3. Thin-layer chromatogram of human liver gangliosides. All lanes are the same as shown in Fig. 2. The plate was developed by one ascending run in chloroform–methanol–2.5 N ammonia 60:40:9 (v/v/v).

the upper and lower G_{M3} fractions (**Table 1**). However, striking fatty acid differences were detected between the two fractions (**Table 2**). The upper fraction contained only unsubstituted fatty acids that were mostly of the relatively long-chain variety. Interestingly, the lower fraction contained large amounts of α-hydroxy fatty acids with chain lengths shorter than those of the upper fraction. The lower fraction also contained small amounts of shorter chain unsubstituted fatty acids. The unsubstituted fatty acid composition of the total G_{M3} fraction was calculated to be 56.9% and 43.1%, respectively; this was quite similar to the composition of the intermediate fraction (Table 2). In contrast to brain gangliosides, where both *trans*-4-icosasphingenine (d 20:1) and sphingosine (d 18:1) are present, we found no *trans*-4-icosasphingenine in human liver hematoside.

A minor sialic acid-positive band was observed that migrated in front of the major G_{M3} upper band on the TLC plate developed with the solvent system containing $CaCl_2$ (Fig. 2), and was also eluted before the upper G_{M3} fraction on the Iatrobeads column (tubes 41–6, Fig. 1). The oligosaccharide chain composition of the minor fraction and the two major G_{M3} fractions were similar. Because the fraction accounted for only 0.5% of the total G_{M3} fraction, not enough material was available for analyses of the long-chain base and fatty acid compositions.

DISCUSSION

The concentration of human liver gangliosides that we found (65.9 μg of sialic acid/g wet tissue) was higher than the value (48 μg/g wet tissue) obtained by Kwiterovich, Sloan, and Frederickson (24). This difference probably results from the different ganglioside methodologies employed. Kwiterovich et al. (24) used a solvent partition step which is known to cause some of the less polar gangliosides to remain in the lower phase (17, 24). We feel that our system is especially suited for the bulk isolation of G_{M3} from human liver for the following reasons. (*a*) Hematoside constitutes almost 90% of the total ganglioside distribution of human liver. Hence human liver can serve as an easily accessible source for this ganglioside. (*b*) Quantitative recovery of G_{M3} is assured by the use of DEAE–Sephadex and Unisil columns as an alternative to solvent partitioning. (*c*) The high resolution of the Iatrobeads column facilitates the efficient and rapid separation of G_{M3} from other ganglioside species. Partial separation of hematoside bands could also be achieved.

TABLE 1. Long-chain base composition of human liver G_{M3} (% distribution)

	d 16:1	d 18:1	d 18:0	Unknown
Upper	1.1	97.7	1.2	0
Lower	4.0	89.1	0.3	6.6

Our most interesting finding was the presence of rather large amounts (82%) of α-hydroxy fatty acids in the lower hematoside band, while the α-hydroxy fatty acid content of the total hematoside fractions was 43.1%. Because the sugar and LCB compositions of the upper and lower G_{M3} fractions are similar, the double-band appearance of liver G_{M3} on TLC appears to result from differences in the fatty acid composition. The faster mobility of the upper band results from the preponderance of long-chain unsubstituted fatty acids, while the slower mobility of the lower band results from the preponderance of α-hydroxy fatty acids and small amounts of fatty acids with shorter chain lengths. It is well known that such fatty acid differences are responsible for the double-band appearance of brain cerebroside and sulfatide. However, the double-band appearance of brain G_{M3} is due largely to differences in fatty acid chain length rather than

TABLE 2. Fatty acid composition of human liver G_{M3} (% distribution)

	16:0	18:0	18:1	20:0	22:0	22:1	23:0	24:0	24:1	Other	Total %
Upper											
n FA[a]	3.9	3.0	1.0	4.8	34.5	3.0	11.1	21.5	17.2	0.2	100
Lower											
n FA	8.3	3.3	0.2	0.9	3.4			1.1	0.5		17.7
h FA[b]	14.3	14.6		7.3	23.7	1.1	7.3	7.3	5.2	1.8	82.3
Intermediate											
n FA	24.4	5.8	0.1	2.7	9.1	0.5	2.2	4.5	3.8		53.1
h FA				0.4	10.4	1.3	8.3	15.6	11.0		46.9

[a] Unsubstituted fatty acids.
[b] α-Hydroxy fatty acids.

to differences in normal and α-hydroxy fatty acids.[4] The minor sialic acid-positive band that migrated in front of the major G_{M3} upper band may represent yet another fatty acid variant of G_{M3}, since the sugar composition of this band was similar to the sugar composition of the two major G_{M3} fractions.

Finally, our observation that α-hydroxy fatty acids are present in human liver hematoside is consistent with the work of Rauvala (26) who also noted the presence of these fatty acids in human kidney gangliosides where they comprised 29% of the hematoside fraction. It would be interesting to examine the distribution of α-hydroxy fatty acids in the hematoside fraction of other non-neural tissues.

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