notes on methodology

An improved assay of gangliosides separated by thin-layer chromatography

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Summary There were no differences in the recoveries of ganglioside sialic acid from silica gel G thin-layer chromatograms when they were sprayed with resorcinol reagent varying in normality between 3 and 10. However, both the temperature at and the time for which the plates were heated after spraying did affect the recoveries, which can reach 100% when the plates are heated for 6 min at 135°C.

Supplementary key words ganglioside · sialic acid · glycolipid

The gangliosides compose a group of sialic acid-containing glycosphingolipids that are found in a variety of biological tissues of both neural and nonneural origins (1–4). Currently there is no universally accepted system of nomenclature for these compounds (5, 6). However, different subtypes have been distinguished from one another either on the basis of their carbohydrate components, when these are known, or on the basis of differences in R_f values following thin-layer chromatography when information concerning their chemical structures is insufficient. Indeed, the carbohydrate components are primarily responsible for differences in R_f on silica gel G plates using certain solvent systems. This fact was exploited by Suzuki (7) who developed a method for quantitating individual ganglioside species separated by TLC. MacMillan and Wherrett (8) modified this procedure, making it both more sensitive and specific by detecting the gangliosides on the thin-layer plate by first spraying it with a resorcinol reagent and then heating it at 150°C until the resorcinol-positive (i.e., sialic acid-containing) spots became visible. These were then scraped into test tubes and sialic acid was quantitated by the method of Svennerholm (9) as modified by Miettinen and Takki-Luukkainen (10). Although they obtained recoveries of approximately 100% from some of their plates, this was not consistent and they noted some as low as 60%. In subsequent studies employing this method, we obtained recoveries usually in the range of 80–90% but seldom higher and occasionally lower (11). We have improved these recoveries to 98.9 ± 3.0% by studying the effects on ganglioside sialic acid recoveries of (*a*) normality of the resorcinol spray, (*b*) temperature, and (*c*) length of time of heating the thin-layer plate after spraying it with the resorcinol spray.

Abbreviation: TLC, thin-layer chromatography.

Materials

All solvents and acids were of ACS grade. Solvents were redistilled and the normality of hydrochloric acid was determined by titration prior to use. Both *N*-acetylneuraminic acid type VI and resorcinol were purchased from Sigma Chemical Co., St. Louis, MO. Thin-layer glass plates (20 × 20 cm) precoated with 0.5 mm silica gel G were purchased from Analtech Inc., Newark, DE (Catalogue # 1012).

Methods

A mixture of gangliosides was prepared by the method of Suzuki (7) from normal human cerebral cortex obtained at the time of autopsy. Aliquots of this sample, each containing 15 μg of sialic acid, were spotted along 1-cm lines on silica gel G plates. Each plate containing three separately spotted aliquots was developed once in chloroform–methanol–water–concentrated ammonium hydroxide 60:35:7:1 and then air dried. The plate was sprayed lightly with the resorcinol reagent of Svennerholm (9), which was prepared in titrated hydrochloric acid of different normalities (1 N, 3 N, 5 N, 8 N, 10 N). It was immediately covered with a glass plate and heated at different temperatures for various times on a sand bath inside an oven. The temperature of the sand was checked with a thermometer and maintained constant to within ±2°C. At the end of the heating interval the cover plate was removed and the sample was cooled to room temperature.

Typical patterns of human brain gangliosides were seen on the thin-layer plates with four large and two smaller resorcinol-positive bands. These have been named according to Korey and Gonatas (12). Each band was scraped with a cellulose strip into a separate test tube. Gel blanks that corresponded in size and vertical position to each resorcinol-positive band were also separately scraped from each plate. The sialic

acid content of each was assayed by the resorcinol method as described by MacMillan and Wherrett (8) with the time interval between spraying the plate and spectrophotometric reading never exceeded 2 hr. Absorbancies were measured at both 470 and 580 nm; those at 580 nm were corrected for galactose interference. This correction reduced the apparent percentage recovery of sialic acid by an average of 3.7% on 57 separate lanes. Reagent blanks, *N*-acetyl neuraminic acid standards, and aliquots of human cortex gangliosides were processed in the absence of silica gel with each resorcinol reaction.

Results and discussion

Two plates containing chromatographed samples were sprayed with a solution made to 1 N but, even after 15 min of heating at 150°C, no purple color was obtained and the ganglioside-containing regions had charred, producing brown spots. However, the mean recoveries of gangliosides from plates sprayed with solutions varying from 3 to 10 N and heated for 6 min at 150°C differed by less than 5% and no significant differences were found among them when these values were tested with a one-way analysis of variance. Therefore, it is concluded that, between 3 and 10 N, the concentration of HCl has no effect on recovery of ganglioside sialic acid.

The recoveries of gangliosides from plates sprayed with resorcinol reagent prepared in 8 N aqueous HCl and heated at 150°C for different time periods are shown in **Table 1.** The difference between mean recoveries from plates heated 5 min and those heated 7 min was less than 2%; after 8 min of heating the recovery decreased only slightly from these values to 84.9%. However, one min more of heating caused the mean recovery to drop by 16%, and a further decrease in recovery of 21% occurred with heating for 12 min. The results were analyzed using Tukey's post-hoc test for multiple comparisons.

No significant differences were found between the recoveries at 5, 6, 7, and 8 min, but the recoveries at 9, 10, and 12 min were all significantly lower at the 1% level. Furthermore, the differences between the recoveries at 9, 10, and 12 min were also significantly different at the 1% level. It is thus obvious that the duration of heating is critical for acceptable recoveries to be obtained. Six min of heating has been chosen by us for two reasons. First, the resorcinol-positive spots are easily seen and, second, there is left sufficient time prior to the rapid fall-off in recoveries for the thin-layer plate to cool to room temperature.

The effects of heating the thin-layer plates at different temperatures for 6 min were also studied. The results are shown in **Table 2.** One plate was heated at 130°C, but the purple resorcinol-positive bands did not develop sufficiently to reliably identify all spots. While recoveries of over 90% were obtained at 135, 140 and 145°C, the highest mean recovery (98.9 ± 3.0%) was at 135°C.

The proportion of sialic acid within each band as a percentage of the total recovered from the lane in which it was located was calculated for each experiment. The results indicate that the differences in recoveries noted under the different heating conditions examined are not associated with preferential degradations of any one or more bands (**Tables 3,** *a* and *b*). Nor are there differences in the proportions of sialic acid in these bands after spraying the plates with resorcinol reagents prepared in acid concentrations varying between 3 N and 10 N (Table 3 *c*). Differences that are seen are undoubtedly due to slight variations in the resolutions of individual bands on the thin-layer gel.

TABLE 1. Effect of heating time on recovery of ganglioside sialic acid from thin-layer chromatograms[a]

Heating Times	Recovery[b]
min	*% ± SD*
5	89.0 ± 3.72
6	87.4 ± 4.01
7	89.0 ± 3.99
8	84.9 ± 8.03
9	68.9 ± 2.80
10	61.2 ± 3.37
12	47.5 ± 2.08

[a] Each plate was sprayed with resorcinol reagent prepared in 8 N HCl and heated at 150°C.

[b] Values shown are the means and standard deviations of the percentage recoveries from three separate lanes, expressing the total sialic acid recovered from a lane as the percentage of the total sialic acid spotted in that lane.

TABLE 2. Effect of temperature on recoveries of ganglioside sialic acid from thin-layer chromatograms[a]

Temperature	Recovery[b]
°C	*% ± SD*
135	98.9 ± 2.95
140	94.9 ± 5.58
145	95.5 ± 1.01
150	87.4 ± 4.01
155	89.1 ± 4.16
160	73.7 ± 7.50
170	56.0 ± 2.69

[a] Plates were sprayed with resorcinol solution prepared in 8 N HCl and heated for 6 min.

[b] Values are as in Table 1.

TABLE 3. Percentage distributions of sialic acid in resorcinol-positive bands separated on thin-layer chromatograms[a]

A. With Variable Times of Heating[b]

	Heating Times (min)						
	5	6	7	8	9	10	12
G-5	2.8 ± 3.3	1.0 ± 1.4	2.9 ± 0.2	1.8 ± 0.2	0.7 ± 0.9	1.6 ± 2.5	3.1 ± 1.1
G-4	13.2 ± 0.8	14.0 ± 0.8	13.7 ± 0.4	10.7 ± 1.8	15.7 ± 0.6	9.9 ± 1.5	11.5 ± 0.7
G-3	27.8 ± 1.7	29.3 ± 0.5	29.2 ± 1.6	29.2 ± 0.6	28.7 ± 1.3	28.6 ± 0.9	26.4 ± 0.5
G-2	23.4 ± 0.9	24.4 ± 1.4	23.7 ± 1.0	26.2 ± 0.6	20.9 ± 0.6	26.4 ± 1.5	22.3 ± 0.6
G-1	28.4 ± 1.0	27.5 ± 1.6	26.0 ± 1.2	26.3 ± 3.0	25.4 ± 0.3	26.6 ± 1.6	26.0 ± 0.6
G-0	3.3 ± 0.7	3.5 ± 1.7	4.0 ± 0.9	5.4 ± 0.8	5.0 ± 1.6	5.0 ± 1.3	6.1 ± 1.1
S	1.3 ± 1.1	0.5 ± 1.3	0.4 ± 0.5	0.3 ± 0.7	3.7 ± 0.6	1.8 ± 1.6	4.5 ± 0.8

B. With Variable Temperatures[c]

	Temperature (°C)					
	135	140	145	150	155	160
G-5	2.1 ± 0.1	0.9 ± 0.8	1.9 ± 0.2	0.8 ± 0.7	1.0 ± 1.0	0.8 ± 1.2
G-4	12.7 ± 0.2	12.4 ± 0.3	12.2 ± 0.8	12.1 ± 0.5	12.2 ± 0.5	14.3 ± 0.3
G-3	22.7 ± 1.0	28.9 ± 1.5	30.2 ± 0.5	32.3 ± 0.6	29.6 ± 0.8	30.9 ± 1.3
G-2	30.4 ± 1.1	26.6 ± 0.8	24.4 ± 0.7	20.6 ± 1.1	24.1 ± 0.5	24.3 ± 1.0
G-1	23.4 ± 0.8	26.1 ± 2.3	26.8 ± 1.3	26.8 ± 0.5	25.4 ± 0.3	26.8 ± 1.6
G-0	7.4 ± 0.4	3.5 ± 0.3	3.0 ± 2.0	5.8 ± 0.5	5.4 ± 0.6	2.9 ± 0.7
S	1.7 ± 0.4	1.6 ± 0.2	1.5 ± 0.2	1.7 ± 0.5	2.3 ± 0.6	1.7 ± 0.4

C. With Variable Acid Concentrations of Resorcinol Spray[d]

	Normality of Spray				
	3 N	4 N	5 N	8 N	10 N
G-5	2.1 ± 0.5	1.4 ± 1.4	1.1 ± 0.2	1.0 ± 1.4	0.8 ± 0.7
G-4	11.9 ± 0.4	11.5 ± 0.3	14.0 ± 1.1	14.0 ± 0.8	12.1 ± 0.5
G-3	23.1 ± 0.6	29.1 ± 0.7	24.6 ± 2.7	29.3 ± 0.5	32.3 ± 0.6
G-2	29.9 ± 1.3	25.4 ± 0.8	25.3 ± 0.9	24.4 ± 1.4	20.6 ± 1.1
G-1	26.8 ± 1.1	26.7 ± 0.8	26.4 ± 1.4	27.5 ± 1.6	26.8 ± 0.5
G-0	4.6 ± 2.2	4.6 ± 0.6	4.3 ± 0.2	3.5 ± 1.7	5.8 ± 0.5
S	1.6 ± 0.2	1.2 ± 1.3	4.3 ± 2.8	0.5 ± 1.3	1.7 ± 0.5

[a] % ± SD.
[b] Spraying and heating conditions are as in Table 1.
[c] Conditions are as in Table 2.
[d] Plates were sprayed with resorcinol solution prepared in HCl at normalities indicated and heated at 150°C for 6 min.

In a previous study (11) an attempt was made to improve the recoveries by mixing the resorcinol spray in a 50% ethanol solution. In the present investigation we sprayed two plates (total of six lanes) with a 5 N resorcinol solution mixed in 50% ethanol and heated them at 150°C for 6 min. The mean recovery was 85.9% which is no improvement over that of the plate sprayed with an aqueous 5 N solution and heated under similar conditions (84.0%).

MacMillan and Wherrett (8) prepared their resorcinol spray in 4 N HCl, but equal recoveries can be expected when it is within the range of 3–10 N. However, because of the volatile nature of HCl, the normality of the solution will decrease with time when unstoppered, and it is therefore suggested that the resorcinol spray reagent be prepared in the range of 5–8 N. With such a spray we recovered 98.9 ± 3.0% of spotted ganglioside sialic acid from thin-layer plates coated with silica gel G after heating them for 6 min at 135°C.

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