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Note

A densitometric method for the determination of gangliosides after their separation by thin-layer chromatography and detection with resorcinol reagent

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As gangliosides are the only glycolipids that contain sialic acid, the determination of their total content has usually been based on the colorimetric determination of this sugar. The two most widely used reagents are resorcinol[1,2] and thiobarbituric acid[3]. Other methods have been used less frequently for the determination of gangliosides[4–7].

For the determination of individual gangliosides after their thin-layer chromatographic (TLC) separation, the most widely used method is the resorcinol method[8,9]. The gangliosides, separated on the TLC plate, are located with iodine vapour and, after the complete sublimation of the iodine, the areas of the individual compounds are scraped off into centrifuge tubes. The gangliosides are then determined by assaying the sialic acid by applying the resorcinol method to a mixture of silica gel G and the gangliosides.

Another method for the determination of individual gangliosides is by their densitometric measurement on a plate after their non-specific detection by charring with a 2% solution of sulphuric acid in glacial acetic acid[10].

This paper describes a new densitometric method for determining gangliosides after their detection with the specific resorcinol reagent.

MATERIALS AND METHODS

Silica gel G and resorcinol (analytical-reagent grade) were obtained from E. Merck (Darmstadt, G.F.R.). Other reagents, all of analytical-reagent grade, were purchased from Lachema (Brno, Czechoslovakia).

Gangliosides of bovine brain (Koch-Light, Colnbrook, Great Britain) were used as standards. Gangliosides from the frontal tip of human brain were prepared according to the standard double extraction procedure described by Suzuki[8].

Thin-layer plates (20 × 20 cm) were prepared by slurrying 30 g of silica gel G in 60 ml of water and spreading the slurry as a 250 μm thick layer using an adjustable applicator. The plates were allowed to dry in air overnight and then heated for 20 min at 120° just before use. Immediately after heating, part of the chromatogram above the start was covered with a clean glass plate so as to prevent the deactivation of the adsorbent layer during cooling and application of the sample. In this way, we were able to reduce the time and temperature of activation recommended previously by Penick *et al.*[11] (90 min at 130–135°).

The ganglioside samples were dissolved in chloroform–methanol–water (10:10:2) to give a concentration of *ca.* 2 mg in 0.5 ml. A 20–25-μl volume of this solution was spotted in 1-cm streaks on the start line, 1.5 cm from the bottom edge of the plate. Two different solvent systems were used: (1) propanol–water (7:3), and (2) chloroform–methanol–2.5 *N* ammonia solution (60:35:8). The chromatograms were developed in a saturated chamber, dried in air and sprayed lightly with resorcinol–HCl reagent[1]. They were covered with a clean glass plate and then heated at 120° for 20–25 min.

Substances containing neuraminic acid appeared as purple spots on white background.

The chromatograms were scanned using an ERI-65 densitometer (Carl Zeiss, Jena, G.D.R.) in reflected light at a wavelength of 560 nm, and areas under the curves were measured by planimetry. The data were expressed as the percentage distribution of the N-acetylneuraminic acid (NANA) content in individual gangliosides. The results were compared with those obtained by the colorimetric method described by Suzuki[8,9].

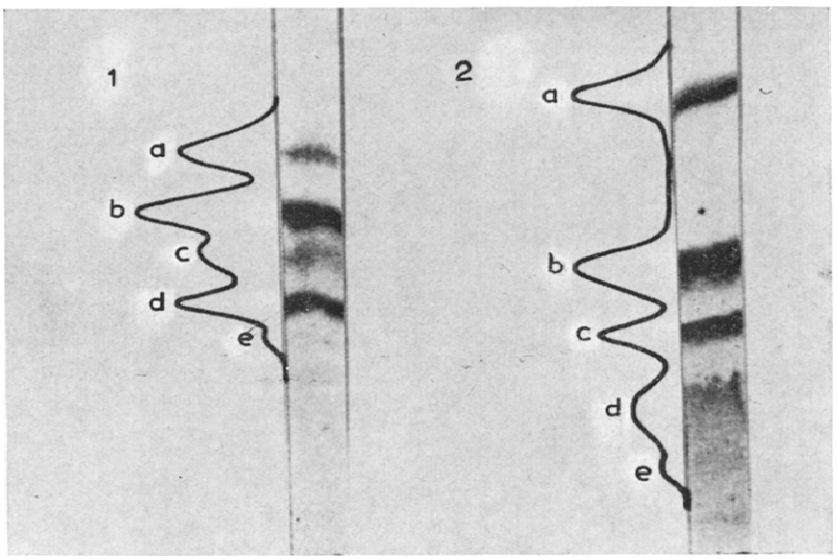

Fig. 1. Separation of individual gangliosides on a TLC plate in the solvent systems (1) propanol–water (7:3) and (2) chloroform–methanol–2.5 *N* ammonia solution (60:35:8). Detection: resorcinol–HCl.

Notation of gangliosides:	a	b	c	d	e
Svennerholm system:	G_{M1}	G_{D1a}	G_{D1b}	G_{T1}	—
Korey system:	G_4	G_3	G_2	G_1	G_0

TABLE I

QUADRUPLICATE DETERMINATIONS OF THE FOUR MAIN GANGLIOSIDES BY DENSITOMETRY AND PHOTOMETRY
Each result is the mean of four determinations (A–D).

Method	*Ganglioside*	*Propanol–water (7:3)*						*Chloroform–methanol–2.5 N ammonia solution (60:35:8)*					
		NANA (%)						*NANA (%)*					
		A	*B*	*C*	*D*	*Average*	*Standard deviation*	*A*	*B*	*C*	*D*	*Average*	*Standard deviation*
Colorimetry	G_{M1}	19.0	20.2	20.5	22.0	20.4	1.23	22.8	21.1	20.5	22.0	21.6	1.01
	G_{D1a}	37.8	38.0	36.0	35.6	36.9	1.23	35.4	36.8	38.8	35.6	36.6	1.57
	G_{D1b}	21.6	19.0	20.5	20.4	20.4	0.93	20.2	18.4	20.2	19.2	19.5	0.87
	G_{T1}	21.6	22.8	23.0	22.0	22.4	0.66	21.6	23.7	20.5	23.2	22.4	1.48
Densitometry	G_{M1}	23.4	24.3	22.7	24.9	23.8	0.97	23.1	23.4	23.8	20.8	22.7	1.35
	G_{D1a}	34.6	36.4	37.9	35.1	36.0	1.48	35.4	33.3	34.0	33.4	34.0	0.97
	G_{D1b}	19.8	17.9	19.0	20.0	19.2	1.01	19.0	20.0	20.2	20.8	20.0	0.83
	G_{T1}	22.2	21.4	20.4	20.0	21.0	0.99	22.5	23.3	22.0	25.0	23.2	1.32

RESULTS AND DISCUSSION

Fig. 1 shows the separation of the gangliosides in the two solvent systems and the respective densitometric curves.

The results obtained by the densitometric and colorimetric methods after chromatography are expressed as the percentage distribution of NANA in the four main brain gangliosides, G_{M1}, G_{D1a}, G_{D1b} and G_{T1} (Table I). On comparing our method with the widely used colorimetric method, it can be seen that both give satisfactory results. A statistical evaluation of results obtained shows that the standard deviation is less than 2 % in all instances.

The factors that affected the results of the densitometric determination include the following.

(1) The quality of separation, which depends mainly on the activity of the adsorbent layer. Poor separation or a great variation of R_F values are the most frequent sources of errors.

(2) Detection processes. We investigated some factors that can affect the quality of detection:

(a) Stability of the spray solution. Resorcinol reagent is stable for 1 week in a refrigerator, and we found no deviation in the percentage distribution of NANA when using fresh reagent and 7-day-old reagent.

(b) Temperature and time of heating. Wherrett and Cumings[12] heated the plate for 8–10 min at 140° and stated that heavy spraying and prolonged heating resulted in the browning of the resorcinol-positive spots, as well as the charring of other lipid compounds. We avoided this disadvantage by reducing the heating temperature to 120°; the time was prolonged to 20–25 min, and gangliosides were then visible as purple spots.

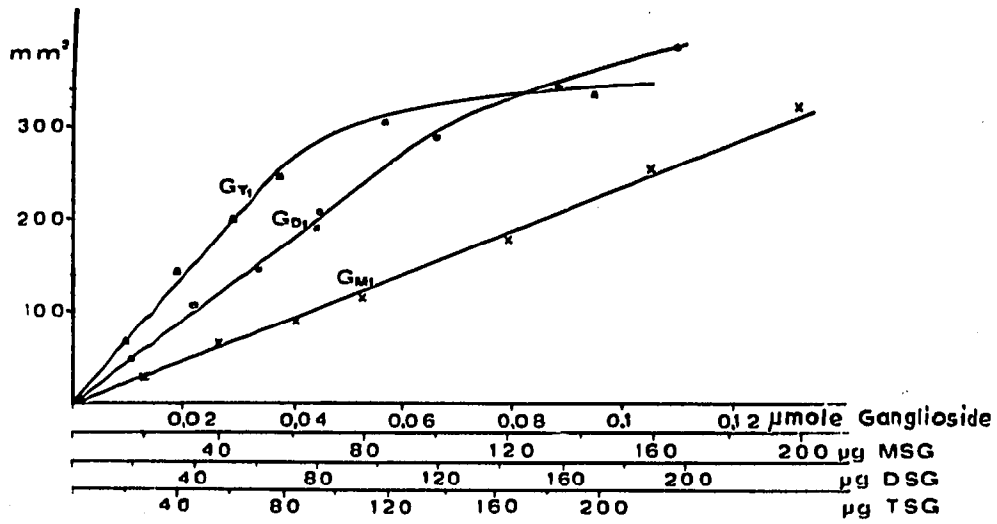

Fig. 2. Calibration graph for mono-(MSG), di-(DSG) and tri-(TSG)sialogangliosides. The concentration, expressed in µmole or µg of a particular compound, is plotted against the corresponding peak area in mm².

(c) Intensity of spraying. Very heavy spraying caused a 5–10 % deviation from the percentage distribution obtained with normal, light spraying.

(3) The sample size giving the best results is 0.02–0.04 μmole (40–80 μg) of individual gangliosides. As shown in Fig. 2, this calibration graph is linear in the range 0.00–0.04 μmole (0.0–80 μg). It can be seen that equal amounts of mono-, di- and trisialogangliosides give densitometric areas in the ratio 1:2:3 according to the content of NANA in the molecule.

Saifer and Feldman[7] quantified mono-, di- and trisialogangliosides partly on the basis of the presence of an equimolar amount of sphingosine, and partly by using the resorcinol method to determine NANA. The NANA:sphingosine ratios were *ca.* 1:1, 2:1 and 3:1, respectively, which confirms the good agreement between the densitometric and photometric results in our work.

The fact that the calibration graph curves first for trisialogangliosides is again influenced by the high content of NANA in the molecule of these gangliosides.

In their study of rat hepatocyte and hepatoma gangliosides, Brady *et al.*[13] referred briefly to the determination of these compounds by using densitometry after resorcinol detection. They also found good agreement with the photometric results, but further procedural details were not specified.

A great advantage of the described method is the considerable saving in time and labour. The method is useful for the analysis of large numbers of small samples.

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