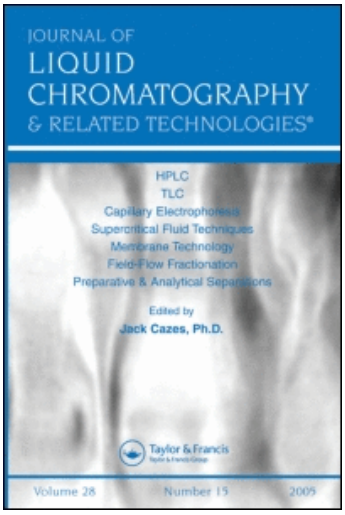

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A METHOD FOR DETERMINATION OF LIPID-BOUND SIALIC ACID AFTER CHROMATOGRAPHIC ISOLATION OF BRAIN GANGLIOSIDES

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ABSTRACT

The lipid-bound sialic acid determination gives an indirect idea of the ganglioside composition. An attempt at adapting the 'resorcinol method' of Svennerholm using thin-layer chromatography is described in this article.An aliquot of a lipid extract from bovine brain was chromatographed and the ganglioside carrying area was visualised. The sorbent was scrapped and eluted. The sialic acid was hydrolysed with a mixture of resorcinol/hydrochloric acid and the color product formed was extracted with butanol/butylacetate. The color intensity was measured at 580 nm. It was found that the lipid-bound sialic acid in bovine brain was about 780 μg/g fresh tissue.The recovery data calculated by means of a standart solution were 97-102%. This method is particularly useful for some routine diagnostic studies.

INTRODUCTION

Gangliosides are presented in relatively small amounts in brain and other tissues. Chromatographic methods are widely used for their qantitation. Another indirect method is based on defining the sialic acid (N-acetylneuraminic acid) content as a specific molecular component. Two colorimetric procedures are mainly in use: the method of Svennerholm [1,2] with some modifications [3] and the 'thiobarbituric method' [4,5]. By the former the free sialic acid is treated with resorcinol/hydrochloric acid at high temperature while the latter approach rests on an oxidising cleavage of the side chain of the free sialic acid after reaction with thiobarbituric acid. The color compounds formed by both methods are extracted with organic solvents and their intensity measured spectrophotometrically. Some authors claim that the 'thiobarbituric method' is more sensitive while other prefer the Svennerholm's procedure because of its rapidity, facility and cheapness.

The ganglioside isolation from a total lipid extract is a preliminary procedure and various techniques such as liquid-liquid partition, ion exchange chromatography, gel filtration could be applied. A method for preparative thin-layer chromatographic isolation of gangliosides was published earlier [6]. This approach was adapted for quantitative determination of gangliosides according to their sialic acid content.

MATERIAL AND METHODS

A sample of whole bovine brain was used. The extraction procedure was similar to the one published earlier [6]. The tissue was subjected to three successive extractions with: a) cyclohexane (1:5 w/v) ; b) chloroform/methanol = 1:3 (1:15 w/v) mixture ; c) chloroform/methanol = 1:1 (1:10 w/v) mixture. The combined extracts were evaporated at 35-40^0 C using a rotary vacuum evaporator. The residue formed was dispersed by ultrasonication after addition of portions of a chloroform/methanol = 1:1 mixture to form a brain tissue weight solvent volume ratio 1:5 or 1:7. The lipid solution thus obtained was divided in vials (0.5-1.0 ml) and stored frozen for the next procedures.

Preparative Thin-Layer Chromatography

The sample was submitted to preparative TLC fractionation (10x10 cm glass plates; 0.75 mm silica gel G layer thickness (Merck, Darmstadt,Germany)). The solution from one vial was applied bandwise by hand or automatically (Camag Linomat IV, Muttenz, Switzerland). Mobile phase: chloroform/methanol/0.3% KCl = 30:18:4 (v/v/v). After 8 cm run distance the plate was dried and the zones were detected by spraying with reagent, prepared by dissolving of 0.5 g 3,5-dihydroxytoluene in 100 ml of water followed by addition of 12 ml concentrated sulfuric acid. A small path at the edge of the plate was sprayed followed by local heating until color spots on a white background could be seen on the plate. The sorbent of the corresponding ganglioside carrying zones was scrapped (Fig. 1) and placed into a centrifuge tube. The whole ganglioside carrying area scrapped area ratio was measured (Q = 1.90-1.95). Five ml of a mixture of 96 ml methanol/4 ml concentrated hydrochloric acid was added and, after shaking, was centrifuged at 4000 min^{-1} for 10 min. Two ml supernatant were transferred into a distillation flask and the solvent was evaporated at 40°C under nitrogen. Two ml bidistilled water and 2 ml reagent (a mixture of 1 ml 3% resorcine, 8 ml hydrochloric acid, 0.03 ml 0.1M copper sulfate) were added to the residue. After 20-25 min heating in a boiling water bath the flask was cooled to 5-10°C and the chromogen was extracted with 3 ml of butanol/butylacetate = 15:85 (v/v). The solvent mixture was then centrifuged and the absorbance of the organic phase was read at 580 nm (Carl Zeiss Jena VSU-2P spectrophotometer, 1 cm pathlength). Standard and blank samples were prepared separately.

The sialic acid content was calculated according to the following equations:

$$R = \frac{A.St}{A_{st}} \cdot \frac{V_t.Q.V}{V_1.Vp.g} \; [\,\mu g/g \text{ fresh tissue}\,] \qquad (1)$$

where: A - absorbance at 580 nm (sample)

St - sialic acid content (μg) in the standard sample

A_{st} - absorbance of the standard sample at 580 nm

V_t - total volume of eluate obtained from the scrapped zones

Vp - volume of eluate taken for analysis

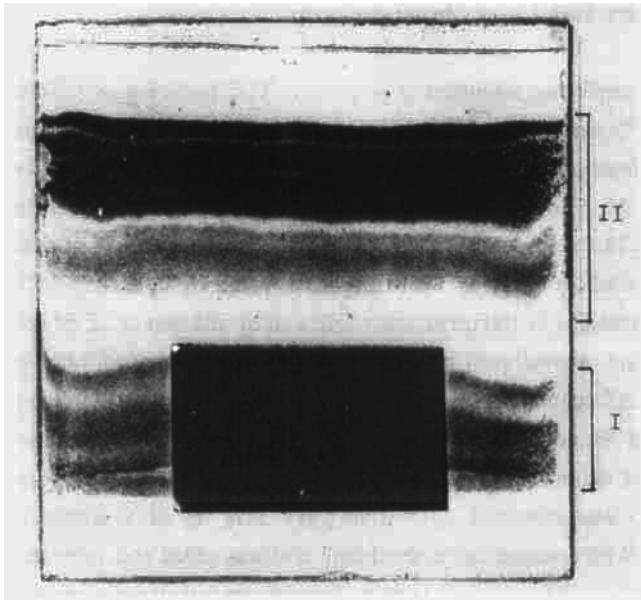

Figure 1. Preparative thin-layer chromatogram of total brain lipids (conditions - see text). I - gangliosides; II - other lipid fractions; III - scrapped sorbent area

Q - whole ganglioside carrying area scrapped area ratio (see Fig. 1)

V - total lipid extract volume

V_1 - volume applied on the TLC plate

g - sample weight in grams

$$R_1 = \frac{R}{0.01.d} \; [\; \mu g/g \text{ dry tissue }] \quad (2)$$

where: d - dry tissue in %

$$R_2 = \frac{R_1}{M_W} \; [\; \mu mol/g \text{ dry tissue}] \quad (3)$$

where: M_W - sialic acid molecular weight (309.28)

RESULTS AND DISCUSSION

A standard curve obtained by a series of dilutions of a water solution of sialic acid (Sigma, St. Louis, Missouri, U.S.A.) is given in Figure 2. The average St / A_{St} ratio (see equation 1) is 68±6 (n=19) using 13620 as a molar extinction coefficient.

We have accomplished an additional experiment in order to determine the overall recovery. Aliquot volumes containing (30 μg) standard solution of pure sialic acid were processed as already described. The calculated recovery data were 97.5-102.4% (n=5). Fifteen samples of brain tissue have been analysed by this procedure and the following values were found:

$R = 780 \pm 68$ μg sialic acid/g fresh tissue

$R_1 = 3760 \pm 334$ μg sialic acid/g dry tissue

$R_2 = 12.2 \pm 1.08$ μmol sialic acid/g dry tissue

Using these data the sialic acid distribution for the main ganglioside fractions could be presented according to the corresponding rel.% from the densitogram according to the following equation (Table 1):

$$S_{Gi} = \frac{R_2}{\dfrac{(Pi.Mw)}{\Sigma \dfrac{}{Mw_{Gi}}}} \cdot \frac{Pi.Mw}{Mw_{Gi}} \quad [\mu mol] \qquad (4)$$

where:

- S_{Gi} - sialic acid content (μmol)
- R_2 - μmol sialic acid/g dry tissue (12.2) (see text)
- Mw - sialic acid molecular weight (309.28).(1, 2, ...)
- Pi - ganglioside part from the densitogram in rel. % (see Table 1) (densitometry conditions are described in [6])
- Mw_{Gi} - ganglioside molecular weight (monosialo - 1545; disialo - 1836; trisialo - 2127)

The ganglioside fractions are colorless (both in UV and VIS region) and should be visualised by spraying with reagents. The mixture of resorcinol/HCl reacts with the lipid-bound sialic acid and the intensity of the color compound formed is proportional to its quantity and could be expressed in rel. % from the densitogram.

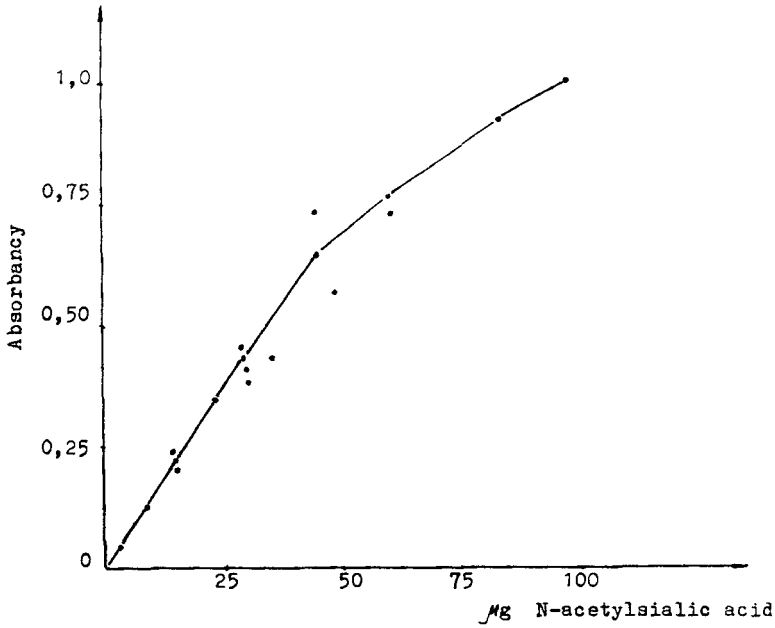

Figure 2. Standard curve of a series of dilutions of pure sialic acid

TABLE 1

Sialic Acid Distribution

Gangliosides	GT1b	GD1b	GD1a	GM1
part from the densitogram (rel. %) n=9	16.1±3.7	12.3±2.4	38.2±3.6	33.4± 2.6
sialic acid distribution (R2) (µmol)	2.80	1.64	5.10	2.67

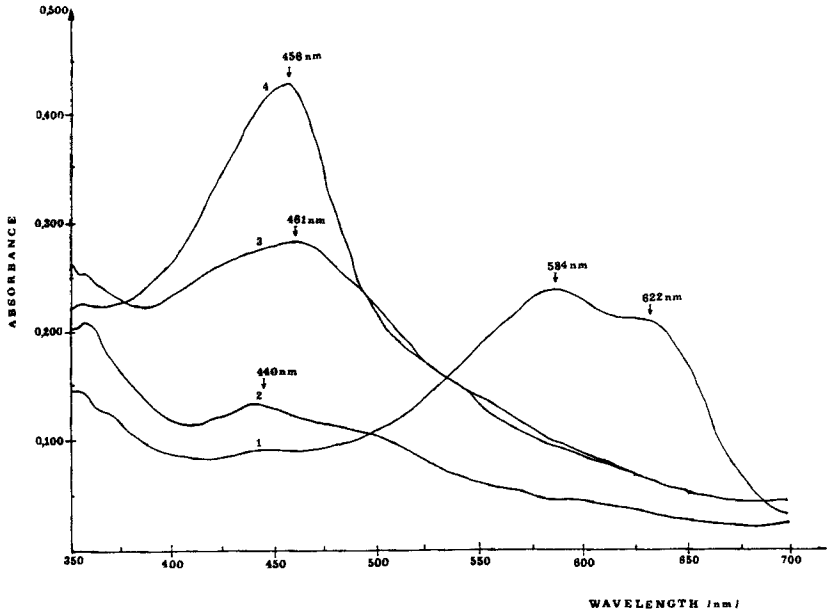

Figure 3. Absorption spectra: I-pure sialic acid; II- phospholipids and cholesterol (eluate-see Fig. 1-II); III- pure glucose; IV- pure fructose

TABLE 2

Comparative Data

Human and mammalian brain	Grey matter	White matter	Whole brain	References
µmol/g fresh tissue	2.85 - 3.55	0.90 - 1.57		[8]
µg/g fresh tissue	992	205		[9]
Our data:				
µmol/g fresh tissue	-	-	2.52	
µg/g fresh tissue	-	-	780	

There is a linear part in the standard curve covering concentrations from 0 to 50 mg sialic acid (Fig. 2). Experimental conditions ensuring hit this part could be chosen.

Some authors [7] suggest the color intensity to be measured at the second absorption maximum (620 nm) in order to avoid interference of the impurities (carbohydrates, phospholipids). Absorption spectra of such a possible impurities (ca. 100 μg of each) are given in Figure 3. In the course of time their color is changing with a shift to the lower wavelengths. The amount of impurities could affect the results and precautions (preliminary purification of the ganglioside fractions) should be taken into account. The sensitivity of the method proposed in this article has a lower limit of 3 nmol. Comparisons of data for sialic acid content in bovine brain presented by other authors [8, 9] are shown in Table 2. They correlate well with those described in this article.

ACKNOWLEDGEMENTS

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