notes on methodology

Determination of long-chain base in glycosphingolipids with fluorescamine

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Summary A method is described for determining the long-chain base content of glycosphingolipids after acid hydrolysis, using the new reagent fluorescamine. The reaction is sensitive and can be used to characterize or measure glycosphingolipids in quantities routinely separated by thin-layer chromatography.

Supplementary key words fluorescence · sphingosine · galactosyl ceramide · cytolipin H · cytolipin K · cytolipin R · ganglioside · ceramide trisaccharide

The recent introduction of fluorescamine (1) for measurement of compounds containing amino groups by fluorescence led us to examine its usefulness for determination of long-chain base (sphingosine) in glycosphingolipids. We were particularly interested in three characteristics that might offer an improvement over existing methods: sensitivity, absence of interference from adsorbents used for thin-layer chromatographic separation of glycosphingolipids, and convenience. In all three respects, fluorescamine proved to be an excellent reagent.

Materials

All solvents were redistilled except for ethyl acetate, which was "Spectranalyzed" grade (Fisher Scientific Co.). Fluorescamine was obtained from Roche Diagnostics (Nutley, N.J.). Glycosphingolipids were isolated from natural sources and were homogeneous when tested by thin-layer chromatography using charring or iodine vapor for detection. Galactocerebroside (galactosyl ceramide) was isolated from bovine spinal cord (2) (hexose, 21.6%). Cytolipin H[1] was isolated from bovine spleen (3) (hexose, 38.2%). Cytolipin R[1] was isolated from rat lymphosarcoma (4) (long-chain base content [5], 23.8%). Cytolipin K[1] was isolated from human kidney (6) (long-chain base content [5], 22.2%). Ceramide trisaccharide (long-chain base content [5], 27.9%) was isolated from human kidney during the preparation of cytolipin K. G $_{M1}$ ganglioside (G_4) was isolated by column chromatography on Anasil S (7) (Analabs, Inc., North Haven, Conn.) of the product obtained by the action of neuraminidase on mixed gangliosides from beef brain (long-chain base content [5], 18.7%).

[1] The chemical structures of cytolipins are as follows. Cytolipin H: gal(β1 → 4)glc ceramide; cytolipin K: galNAc(β1 → 3)gal(α1 → 4)gal(β1 → 4)glc ceramide; cytolipin R: galNAc(β1 → 3)gal(α1 → 3)gal(β1 → 4)glc ceramide.

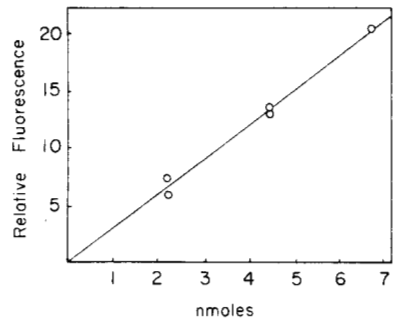

Fig. 1. Standard curve obtained with pure galactosyl ceramide after hydrolysis with 3 N HCl for 2 hr at 98–100°C and evaporation to dryness.

Procedure

Hydrolysis. Samples of glycosphingolipid were hydrolyzed with 0.3 ml of 3 N HCl for 2 hr in a boiling water bath in glass-stoppered centrifuge tubes. Hydrochloric acid was then removed in vacuo in a desiccator over sodium hydroxide pellets.

Fluorescamine reaction. To the dry residue, 4 ml of ethyl acetate and 2.5 ml of buffer solution (0.1 M acetate, pH 3.7) were added. For some studies the buffer was 0.2 M boric acid adjusted to pH 7.5 with 10 N NaOH. The mixture was shaken and then centrifuged at low speed to remove buffer solution from the walls of the tube. To each tube 0.5 ml of an acetone solution of fluorescamine (6–8 mg in 25 ml) was added, and the mixture was immediately shaken vigorously *by hand.* The tubes were then centrifuged again, and fluorescence in the ethyl acetate layer was determined with an Aminco-Bowman spectrophotofluorometer, using an excitation wavelength of 410 nm and an emission wavelength of 490 nm (400 nm and 480 nm with borate buffer).

Results

A standard curve obtained with galactosyl ceramide (Fig. 1) served as a basis for determining the long-chain base content of the other glycosphingolipids. The molar fluorescence of these glycosphingolipids relative to that of galactosyl ceramide is shown in Table 1 for determinations carried out in borate and acetate buffers. With borate buffer, high values were obtained with two of the three compounds known to contain amino sugar among the products of hydrolysis. With acetate buffer the discrepancy was not significant.

TABLE 1. Measurement of long-chain base in various pure glycosphingolipids with fluorescamine

Glycosphingolipid	Assumed Molecular Weight	Molar Fluorescence Relative to Galactosyl Ceramide	
		Borate Buffer	Acetate Buffer
Galactosyl ceramide	828	1.00	1.00
Cytolipin H	972	1.06	1.07
Ceramide trisaccharide	1134	1.00	1.03
Cytolipin K	1300	1.01	1.07
Cytolipin R	1300	1.22	0.99
G_{M1} ganglioside	1600	1.20	1.08

TABLE 2. Comparison of long-chain base analysis on impure glycosphingolipid fractions by methyl orange method and fluorescamine method

Fraction	Long-chain Base Analysis		
	Methyl Orange	Fluorescamine	
		Borate Buffer	Acetate Buffer
	%	%	%
1	22.5	25.1	21.6
2	20.8	26.0	20.7

To test the suitability of the method for measuring long-chain base in glycosphingolipids recovered from a thin-layer plate, galactosyl ceramide and cytolipin H in amounts of 1.5–5 μg were applied to a plate of silica gel G (Brinkmann Instruments, Westbury, N.Y.) 0.25 mm thick. The plate was not developed. Areas containing the lipid were scraped and eluted twice with 2-ml volumes of chloroform–methanol 1:1. Recoveries of long-chain base in the hydrolyzed samples of galactosyl ceramide and cytolipin H were 104 and 111%, respectively. (Recovery of [^{3}H]dihydrocytolipin H based on scintillation counting was 98.9%: 20,830 dpm applied; 20,600 dpm recovered.) Fractions of tissue glycosphingolipids obtained by chromatography on silicic acid (8) and probably contaminated with phosphoglycerides gave high values with fluorescamine compared with the methyl orange method (5) when borate buffer was used, but the results were in agreement using acetate buffer (Table 2). Presumably, nitrogen-containing phosphatides can interfere under basic conditions, but their hydrolysis products are not extracted into ethyl acetate at low pH.

Discussion

Although we have not had extensive experience with fluorescence measurements of long-chain base using 1-naphthylamino-4-sulfonic acid (9) because of interferences encountered in the various laboratory reagents, it is our impression that the sensitivity of the fluorescamine reagent is greater. This technique appears to be less susceptible to interference from materials contained in the adsorbents used in thin-layer chromatography. The method should be serviceable in measuring quantities of sphingolipids by systematic elution of regions of thin-layer plates when these lipids are present in quantities below the limits of detection by conventional techniques. This method will probably be most useful in establishing the radiopurity of labeled glycosphingolipids and in studying changes in concentration of these substances in very small amounts of tissue samples.

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