notes on methodology

Fluorimetric determination of sphingosine and its application to natural mixtures of glycosphingolipids

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SUMMARY A sensitive estimation of sphingosine, by measurement of the fluorescence of a complex formed with 1-naphthylamino-4-sulfonic acid, is described. The practical range is 5–35 nmoles sphingosine. The method is used to estimate, in terms of sphingosine, amounts of ceramide and glycosphingolipids. The isolation of microamounts (5–30 μg) of individual glycosphingolipids from a mixture, and their quantitative estimation is described. The percentage composition of a glycosphingolipid mixture from the kidneys of adult C57/BL male mice is given.

SUPPLEMENTARY KEY WORDS glycosyl ceramides · sulfatide · thin-layer chromatography · mouse kidney

The method of Lauter and Trams (1) for the determination of sphingosine depends on its extraction into ethyl acetate and colorimetric estimation of the complex formed between the basic-NH_2 group of sphingosine and the anionic dye methyl orange (sodium *p*-dimethylamino-azobenzene sulfonate). The method is sensitive over the range 10–100 nmoles of sphingosine but is most reliable in the range 30–100 nmoles.

In determining the quantitative composition of the glycosphingolipids[1] in small amounts of mouse tissues, we needed a more sensitive method. Very small amounts of fluorescent compounds can be detected by fluorimetry. Accordingly, complex formation between sphingosine and some fluorescent sulfonic acids was investigated. The estimation of sphingosine using 1-naphthylamino-4-sulfonic acid is described.

Materials

All solvents were freshly distilled to avoid contamination from fluorescent impurities. Ethyl acetate was distilled from a 1% solution of octadecanol (2) and was checked in the fluorimeter before use.

Buffer Solution. Sodium acetate–acetic acid, pH 3.65, 0.05 M, washed with ethyl acetate.

Solution A. Freshly prepared 0.3% aqueous sodium 1-naphthylamino-4-sulfonate (purified reagent; Hopkin and Williams Ltd., Essex, England) washed twice with ethyl acetate.

Standards. (a) SPHINGOSINE Sphingosine sulfate was obtained from Koch–Light Laboratories, Ltd., Colnbrook, Bucks, England. The sphingosine, which contained small amounts of dihydrosphingosine and *O*-methyl sphingosines, was converted to the free base by treatment with alkali, and then purified by chromatography on silicic acid and converted to its hydrochloride. After precipitation of the sphingosine hydrochloride with acetone, its purity was checked by thin-layer chromatography and by gas–liquid chromatography of the *N*-acetyl,trimethylsilyl derivative (3). Only traces of dihydrosphingosine were present.

(b) CERAMIDE Ceramide was prepared by treating sphingomyelin (from pig lung) with phospholipase C (4). Its purification and sphingosine and fatty acid content have been reported (4).

Fluorimetric measurements were made with a Hilger and Watts fluorimeter (primary filter Woods glass, transmission band 300–400 nm, secondary filter Kodak 558/6, filter transmission 10%, 396 nm) which was set to read 100 with a solution of quinine sulfate (0.05 μg/ml, in 0.1 N sulfuric acid).

Methods

Estimation of Sphingosine. Samples of sphingosine hydrochloride were evaporated to dryness in stoppered tubes (10 ml) and were redissolved in 0.01 N HCl (0.4 ml). Water (1 ml) was added, and the solution was made alkaline by addition of 0.1 N NaOH (0.6 ml). The free sphingosine was extracted into ethyl acetate (7 ml), and the organic phase was washed with 2 ml of water. Buffer (2 ml) and solution A (0.2 ml) were added, the tubes were shaken for 1 min, and the fluorescence in ethyl acetate was measured.

Methanolysis. Samples (1–35 nmoles) of ceramide and glycosphingolipids were subjected to methanolysis with 0.5 N methanolic HCl (0.2 ml) in a sealed tube at 75°C for 16 hr. Examination of the methanolysis products of *O*-β-D-glucosyl-(1 → 1)-ceramide by thin-layer chromatography on Silica Gel H using chloroform–methanol–water 65:25:4 showed that under these conditions, the methanolysis was complete. The products included both sphingosine and *O*-methyl sphingosines (3).

O-Methyl Sphingosines. The methanolysis products of a sample (50 mg) of ceramide were separated on a silicic acid column, and the fraction containing the total long-

[1] In this paper the term glycosphingolipid excludes glycosphingolipids that contain sialic acid, although the method for sphingosine estimation is equally applicable to these substances.

chain bases was eluted with chloroform–methanol 2:1 (v/v). Thin-layer chromatography showed it to be a mixture of sphingosine and *O*-methyl sphingosines. The molar ratio, sphingosine–*O*-methyl sphingosines, as estimated by gas chromatography (3) was 60:40. The fluorescence of a known quantity of this mixture was measured and compared with that of the same quantity of pure sphingosine.

The Estimation of Sphingosine in Mixtures of Glycosphingolipids.

GLYCOSPHINGOLIPIDS A mixture containing known amounts of monoglycosyl-, diglycosyl-, triglycosyl-, and tetraglycosyl ceramide (aminoglycolipid) and sulfatide, was prepared from pure compounds isolated from pig lung (6) and human brain (sulfatide).

GLYCOSPHINGOLIPIDS FROM TISSUES Glycosphingolipids were isolated from two pairs of kidneys (wet weight 0.8 g) of male adult C57/BL mice. The procedure was essentially that described by Gray (7) except that hydrolysis of the alkali-labile phospholipids at 37°C for 90 min was carried out before chromatography on Silica Gel H (Merck and Co., Inc., Rahway, N.J.) with the tetrahydrofuran solvent systems. A blank (B1) was carried through from the extraction step.

SEPARATION OF SULFATIDE FROM THE NEUTRAL GLYCOSYL CERAMIDES Sulfatide was separated by small scale application of the method of Svennerholm and Thorin (8). DEAE-cellulose (Whatman DE-52) was washed with acetone and dried. Approximately 0.2 μmoles of the glycosphingolipid mixture was applied to a column containing 80 mg of the DEAE-cellulose which was previously equilibrated with chloroform–methanol 4:1 (v/v). Neutral glycosyl ceramides were eluted with this solvent (2 ml), and sulfatide was recovered with chloroform–methanol 2:1 (v/v) containing 5% of aqueous 0.7 M lithium chloride (2 ml). Lithium chloride was removed from the eluate by washing with an upper phase solvent system prepared according to the directions of Folch, Lees, and Sloane Stanley (9). Over-all recovery was greater than 90%. The blank (B1) from the extraction procedure was similarly treated to give a neutral glycosyl ceramide blank (B2) and a sulfatide blank (B3).

SEPARATION OF NEUTRAL GLYCOSYL CERAMIDES The neutral glycosyl ceramides were separated by thin-layer chromatography on plates (8.2 × 8.2 cm) of Silica Gel H (Merck) (7). The solvent chloroform–methanol–water 40:10:1 was allowed to ascend the plate four times in order to obtain the optimal separation of the lipid components Compounds were located by reference to standard glycosyl ceramides chromatographed on the same plate and made visible with iodine vapour. A band of silica gel containing each component was scraped from the plate, and the lipids were recovered with chloroform–methanol 1:4 (v/v). A blank (B 4) for the procedure was obtained by extracting an equivalent amount of silica gel scraped from an unmarked part of the plate.

The recovered glycosphingolipid samples, together with the appropriate blanks, were subjected to methanolysis, and the resulting sphingosine was determined as above. Known amounts of ceramide (2–35 nmoles) and a blank sample (B 5) were similarly treated with methanolic HCl, and a standard curve was obtained by plotting fluorimeter readings (sample − B5) against sphingosine concentration. The amount of sphingosine in a methanolysed sample of isolated glycosphingolipid was determined by reference to the standard curve after correcting the fluorimeter reading for the appropriate blank value (B1, B2, B3, or B4).

The proportion of sulfatide was calculated as a percentage of the total lipid recovered from the DEAE-cellulose chromatography. The proportions of the neutral glycosphingolipids were calculated as a percentage of the total lipid recovered from thin-layer chromatography.

Results and Discussion

A consistent relationship was found between sphingosine concentration and fluorscence in the ethyl acetate phase. Whereas the plot of concentration versus fluoresence is linear in the range of 5–35 nmoles of sphingosine, at very low amounts of base (0–5 nmoles), it is curved (Figs. 1 and 2). The curvature may be due to an initial concentration of molecules of the complex at the interface between ethyl acetate and aqueous buffer, thus lowering the concentration in the bulk phase. However, with suitable calibration curves the method is applicable over the range of 2–35 nmoles of sphingosine. Estimation of the mixture of sphingosine and *O*-methyl sphingosines re-

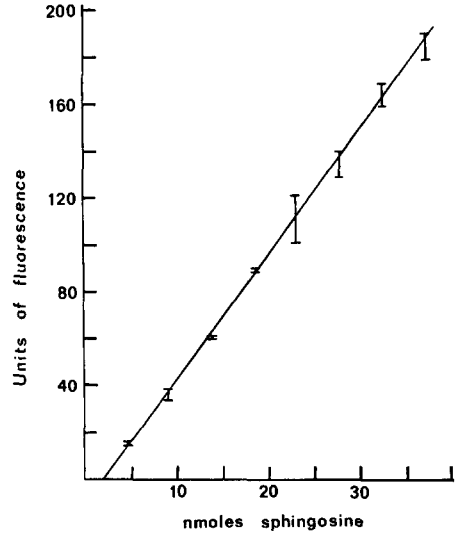

FIG. 1. Relationship between sphingosine concentration (5–35 nmoles) and fluorescence in the ethyl acetate phase. The ends of each vertical bar represent the values of duplicate samples.

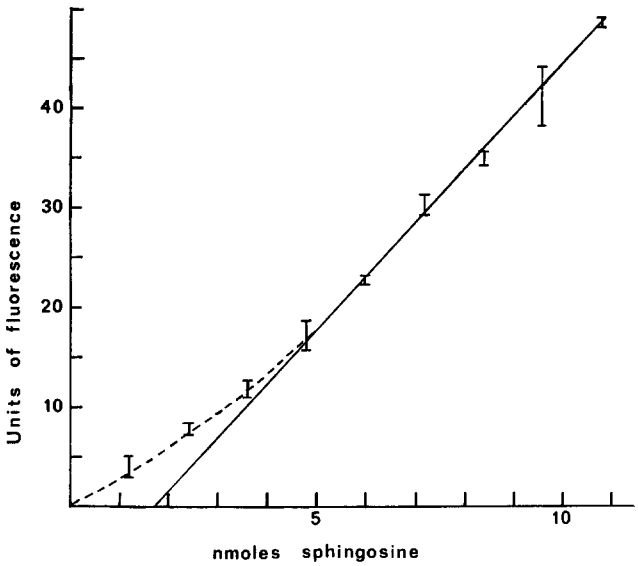

FIG. 2. Relationship between sphingosine concentration (0–10 nmoles) and fluorescence in the ethyl acetate phase.

leased from ceramide and glycosphingolipids (2–35 nmoles) by methanolysis gave similar results. The fluorescence values for the total long-chain bases (sphingosine and *O*-methyl sphingosines) from ceramide was not less than 95% of the value for an equivalent amount of pure sphingosine.

Since this method for the determination o sphingosine depends on measurement of a fluorescent complex at very low concentrations, traces of fluorescent impurities introduce an appreciable error. Glycosphingolipid samples may be contaminated from the accumulation of impurities from solvents and adsorbents used in the isolation procedure, and it is therefore essential that all sample readings be corrected by subtraction of the appropriate blank values. The error involved in using uncorrected values is in the region of 5% (10 units in a fluorimeter reading of 200 units) for large amounts of sphingosine (30 nmoles), but increases proportionally as the amount of sphingosine decreases.

TABLE 1 EVALUATION OF THE PROCEDURE FOR ISOLATING AND ESTIMATING AMOUNTS OF INDIVIDUAL GLYCOSPHINGOLIPIDS BY USING A GLYCOSPHINGOLIPID MIXTURE OF KNOWN COMPOSITION

	Estimation of Glycosphingolipids		Percentage of Total Glycosphingolipids	
	Sample 1	Sample 2	Found	Calculated
	nmoles of sphingosine			
Sulfatide	18.4	13.4	12.7 ± 1.7	12
Netural glycosphingolipids	110	108	87.3 ± 1.7	88
Monoglycosyl ceramide	30.6	28.1	38 ± 2.1	40.5
Diglycosyl ceramide	29.2	21.1	32.5 ± 2.1	31.5
Triglycosyl ceramide	6.1	6.5	8.3 ± 1.1	7.5
Tetraglycosyl ceramide (Aminoglycolipid)	8.6	5.6	9.1 ± 1.1	8.5

TABLE 2 COMPOSITION OF GLYCOSPHINGOLIPIDS IN KIDNEYS OF MALE ADULT C57/BL MICE (WET WEIGHT, 0.8 g; TOTAL GLYCOSPHINGOLIPID, 0.298 μMOLES)

	Percentage of Total Glycosphingolipid
Sulfatide	27.0
Neutral glycosyl ceramides	73.0
Monoglycosyl ceramide	25.5
Diglycosyl ceramide	29.0
Triglycosyl ceramide	9.5
Tetraglycosyl ceramide	9.0

Recovery of glycosphingolipids from DEAE-cellulose was greater than 90%. However, recovery from thin-layer chromatography of glycosyl ceramides was consistently between 70 and 80% and could not be increased by extracting with other solvents (cf reference 10). Since the recoveries of the individual glycosyl ceramides were found to be approximately the same, calculation of the amount of each component present as a percentage of the total recovered after chromatography was deemed to be valid.

Results of the determinations of two samples from a glycosphingolipid mixture of known composition, each containing approximately 200 nmoles, are given in Table 1. The average percentage composition found for the mixture agrees well with the calculated values.

Table 2 shows the results of an estimation of the glycosphingolipids in the kidneys of C57/BL adult male mice. A determination of the total glycosphingolipid could be carried out several times on a single kidney (150–200 mg wet weight).

The support of this work by the Medical Research Council is gratefully acknowledged.

Manuscript received 14 August 1969; accepted 1 December 1969.

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