Simplified fluorometric assay for sphingosine bases

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Summary A simplified procedure for fluorometric determination of sphingosine bases is presented. Nitrogenous bases are reacted with fluorescamine and then extracted into a lower phase solvent, obviating the need to transfer the reaction products prior to quantitation. Consequently, the entire procedure may be carried out in a single set of screw-capped reaction tubes. Chloroform was found to be the best solvent of those tested for the maximal extraction of derivatized sphingosine bases with negligible extraction of nitrogenous contaminants derived from phospholipids and amino sugars. The simplified method retains excellent sensitivity and selectivity and allows for simultaneous processing of large numbers of samples. Furthermore, quantitation of glycolipid-derived hexosamine can be obtained by measuring the fluorescence of the aqueous phase after partitioning.—**Higgins, T. J.** Simplified fluorometric assay for sphingosine bases. *J. Lipid Res.* 1984. **25:** 1007–1009.

Supplementary key words fluorescence • lower phase partitioning • single tube assay

Every mole of glycosphingolipid contains one mole of sphingosine base (1). Consequently, quantitation of sphingosine bases has commonly been used to estimate the glycosphingolipid content of lipid extracts. Several procedures have been used to measure sphingosine bases including gas–liquid chromatography (2), colorimetry using methyl orange (3) or trinitrobenzenesulfonic acid (4), fluorometry using 1-naphthylamino-4-sulfonate (5) or fluorescamine (6, 7), and more recently high-performance liquid chromatography (8, 9). The fluorometric assay using fluorescamine is currently one of the more popular methods since it offers excellent sensitivity and allows a large number of samples to be processed easily. The major drawback to the currently available fluorometric assays is the need to perform the hydrolysis, derivatization, and extraction of the base in one tube followed by transfer of the derivatized base to a second vessel for fluorometric quantitation. Many if not all fluorometers, however, will accommodate 13-mm test tubes as cuvettes. Consequently, by extracting the fluorescent sphingosine derivatives into a suitable lower phase solvent, a single screw-cap test tube can be used throughout the procedure obviating the need to transfer the reaction product. By using a single tube throughout the procedure the risk of sample loss is minimized and, when processing large numbers of samples, considerable time is saved. The procedure reported here used chloroform as the extracting solvent and offers excellent sensitivity and selectivity.

Abbreviations: F.I.U., fluorescence intensity unit; GD_{1a}, disialo ganglioside.

MATERIALS

All solvents were either reagent or HPLC grade and were obtained from Fisher Scientific Co. (King of Prussia, PA). Globotetraose (globoside; cat. #4-6058); IV^3 Neu NAc, II^3 Neu NAc-$GgOse_4$ (GD_{1a}; cat. #4-6034) and phosphatidylserine (cat. #4-6004) were purchased from Supelco, Inc. (Bellefonte, PA). Phosphatidylethanolamine (cat. #P-3511), cerebrosides (cat. #C1516), and ceramide (cat. #C2512) were purchased from Sigma Chemical Co. (St. Louis, MO). All lipids were used without additional purification. Fluorescamine (Fluram; Roche Diagnostics) and 13 × 100 mm screw-cap test tubes (Kimax) with Teflon-faced caps were purchased from Fisher Scientific Co. In converting to nmoles the following molecular weights were used; globotetraose, 1300; GD_{1a}, 1836; phosphatidylserine, 788; phosphatidylethanolamine, 744; cerebroside, 700; and ceramide, 548.

PROCEDURE

Screw-capped test tubes were originally selected by adding 1 ml of borate buffer and reading the background fluorescence at the wavelength used for the assay but at a sensitivity setting 10× that used in the regular assay. Each tube was then rotated 90° and read again and tubes giving a reading of 15 ± 2 (scale of 0–145) on the meter were retained as assay tubes. Approximately 30% of the tubes tested were found suitable. The assay tubes were not re-matched when used for measuring the fluorescence of derivatized bases in chloroform, but the consistently good duplicate and triplicate values observed indicated that the tubes remained acceptably matched for performance of the assay using this modification.

Samples are dried in 13 × 100 mm screw-cap tubes at 35–40°C under a gentle stream of nitrogen and then hydrolyzed in 0.3 ml of 3 N HCl for 2 hr at 98–100°C. The hydrolysate is neutralized with 0.3 ml of 3 N NaOH and buffered with 1.0 ml of 0.5 N borate buffer, pH 9.0. Fluorescent derivatives are generated by adding 0.5 ml of 0.02% fluorescamine in acetonitrile while agitating on a vortex mixer. Following derivatization, 1.0 ml of chloroform is added to each tube, the mixture is shaken vigorously by hand several times, and the

phases are separated by centrifugation at 400 *g* for 5 min at 22°C. Fluorescence was measured with a Perkin-Elmer model 650 spectrofluorometer using excitation and emission wavelengths of 394 nm and 474 nm, respectively. The excitation bandwidth was set at 20 nm while the emission bandwidth was set at either 15 or 20 nm as noted in the legend to Fig. 1. Results are expressed as fluorescence intensity units (F.I.U.) arrived at by dividing the percent emission by the sensitivity setting. During the course of these experiments it was observed that if chloroform was added prior to the fluorescamine reaction a considerable amount of amino sugar contaminants partitioned into the chloroform phase. Consequently, it is imperative that the chloroform be added after the fluorescamine reaction has taken place.

RESULTS

After establishing optimal hydrolysis conditions for the release of sphingosine bases from ceramide and glycolipids, several solvents were tested for their ability to extract derivatized bases from the aqueous solution. The efficiency of derivatized base extraction from a ceramide hydrolysate by three common heavier than water solvents was compared to ethyl acetate (7) and the results are shown in **Table 1.** For these experiments, the fluorescence in the ethyl acetate phase was measured after transfer to additional 13 × 100 mm screw-capped tubes, while the fluorescent derivatives extracted into the lower phase by the denser solvents were measured in the original reaction tube. The results of the experiment shown in Table 1, as well as additional experiments, indicated that the lower phase partitioning solvents were at least equally as efficient as ethyl acetate for the quantitation of derivatized bases in the reaction mixture. Chloroform was generally more efficient than methylene chloride and 1, 2-dichloroethane in these experiments and therefore was used for the remainder of the study.

This assay is intended for use with crude glycolipid extracts as well as purified glycosphingolipids. Consequently, it was necessary to determine whether chloroform would also extract undesirable fluorescent contaminants derived from phospholipids and amino sugars which are present in gangliosides and many glycolipids. In these experiments, the fluorescence found in both aqueous and organic layers was quantitated and the results of one such experiment are shown in **Table 2.** It is obvious from the data presented in this table that either insignificant amounts of fluorescent derivatives of amino sugars or amino-containing head groups of phospholipids partition into the chloroform layer or, if they do, that their quantum yield of fluorescence in this solvent is negligible. These results indicate that chloroform provides the selectivity required for this assay. From these data it is also apparent that the aqueous-soluble fluorescent derivatives can be quantitated by transferring this layer to additional cuvettes and determining its fluorescence.

To further demonstrate the selectivity and sensitivity of the simplified method, the fluorescence obtained from hydrolysates of various amounts of cerebroside and an amino sugar containing glycolipid, globotetraose, were compared. The results of these experiments are shown in **Fig. 1.** Expressed as nmol, the values obtained with various amounts of the two compounds fall about a common line, indicating that fluorescamine derivatives of amino sugars are not extracted into the chloroform phase and the values obtained in this assay are truly representative of the long chain nitrogenous base content of the hydrolysate. Furthermore, a linear response is obtained between 0.2 nmol and 20 nmol of lipid, showing that the simplified method retains excellent sensitivity. In order to keep the readings of the higher concentration samples on scale, it was necessary to reduce the emission slit width to 15 nm, which probably accounts for the difference in slopes of the curves in Fig. 1 (a) and (b). Finally, the fluorescence of the

TABLE 1. Solvent extraction of fluorescent sphingosine bases

Solvent	Fluorescence Intensity Units
Ethyl acetate	647 ± 22[a]
Chloroform	767 ± 31
Methylene chloride	643 ± 42
1,2-Dichloroethane	570 ± 47

Triplicate 5-nmol samples of ceramide were hydrolyzed and extracted with 1 ml of each solvent listed.

[a] The mean fluorescence intensity ± the standard error of the mean.

TABLE 2. Partitioning of fluorescamine derivatives of sphingosine and amino-bearing contaminants by chloroform

Lipid	Fluorescence Intensity Units	
	Aqueous Phase	Organic Phase
Expt. 1		
Ceramide	24 ± 5[a]	535 ± 28[a]
GD_{1a}	344 ± 14	485 ± 5
Phosphatidylserine	243 ± 24	0
Phosphatidylethanolamine	209 ± 5	5 ± 0
Expt. 2		
Ceramide	60 ± 20	555 ± 25
GD_{1a}	465 ± 45	560 ± 0
Globoside	562 ± 42	533 ± 33
Cerebroside	59 ± 25	576 ± 0

Duplicate or triplicate 5-nmol samples of each lipid were used for assay.

[a] Mean fluorescence intensity ± SEM for both aqueous and organic (chloroform) phases.

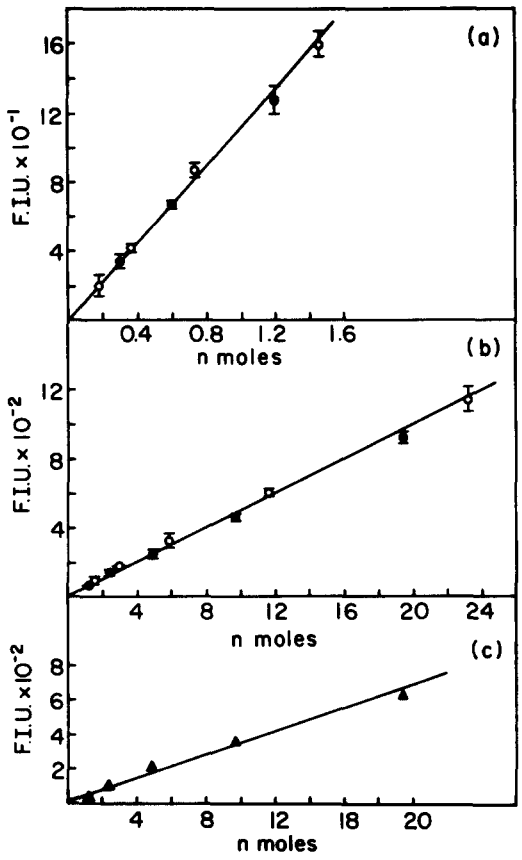

Fig. 1. Comparison of fluorescence obtained from hydrolysates of various amounts of cerebroside (○) and globoside (●) ±SEM of triplicate samples. (a), Excitation and emission bandwidth both set at 20 nm; (b), excitation bandwidth at 20 nm and emission bandwidth at 15 nm; (c), aqueous layer (hexosamine) from globoside hydrolysate shown in (b) measured under same conditions as for (b). Error bars have not been included in panel (c) since these values were less than the height of the symbols.

aqueous layer of the globoside hydrolysate was also measured and found to be linear [Fig. 1(c)] indicating that the amino sugar content of the hydrolysates could be measured if desired.

DISCUSSION

Fluorometric quantitation is a very sensitive method for measuring the sphingosine content of lipid extracts which lends itself to the simultaneous processing of large numbers of samples. Previous procedures partitioned the derivatized bases into upper phase solvents (6, 7) which necessitated transfer of the organic phase to a second cuvette before quantitating fluorescence. The transfer step becomes tedious for large numbers of sample and can lead to error from sample loss. This note describes a simplified procedure which obviates the need to transfer the fluorescent bases by partitioning into a lower phase solvent. Therefore, by using screw-capped test tubes which have been previously matched for the fluorometer at the wavelengths used in the assay, the entire procedure can be carried out in one set of tubes.

Chloroform was found to be the most suitable solvent for this procedure by virtue of its ability to provide efficient extraction and quantum yield of fluorescence from derivatized sphingosine bases without significant interference from amino-containing contaminants likely to be found in glycolipid hydrolysates. A linear response was observed using chloroform partitioning between 20 and 0.2 nmol of sample but no attempt was made to increase the sensitivity beyond 0.2 nmol. Since only a relatively low fluorometer sensitivity setting was required to measure 0.2 nmol of ceramide, considerably smaller quantities of materials should be measurable if required. Furthermore, performed as described, this method can also be used to quantitate amino sugars in glycolipid hydrolysates by measuring the fluorescence in the aqueous phase following its transfer to a suitable cuvette. The hexosamine-derived fluorescence in the aqueous layer, however, became variable and nonlinear below 0.5 nmol of either globoside or GD_{1a} under the assay conditions described herein.

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