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SPHINGOSINE DETERMINATION AT THE PICOMOLE LEVEL USING DIMETHYLAMINOAZOBENZENE SULPHONYL CHLORIDE

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SUMMARY

A sensitive method (lower detection limit 5 picomoles) is proposed for the determination of sphingoid bases liberated from mammalian glycosphingolipids by acid hydrolysis. The azo dye 4-dimethylaminoazobenzene-4'-sulfonyl chloride reacts with the primary amino group of the sphingosine bases, forming a stable derivative. Excess reagent, which is degraded during the derivatization, and free amino sugars as common hydrolysis products of glycosphingolipids are completely separated by reversed-phase high-performance liquid chromatography. This method was applied to the determination of the glycosphingolipid content of mouse spleen and thymus.

INTRODUCTION

Sphingoid bases are backbone constituents of sphingolipids, which occur in the form of three major classes: the sphingomyelins, and neutral and acidic glycosphingolipids. The quantitative determination of these amino alcohols may be achieved by spectrometric or fluorospectrometric methods after coupling a chromophore to the primary amino group [1—6]. Alternatively, gas—liquid chromatography of either the trimethylsilyl derivative of sphingosine bases [7] or of the fragments obtained after cleavage of the 4-sphingenine double bond may be used [8]. Because of their relatively low sensitivity levels (nanomole range), these methods are not applicable to the determination of the glycosphingolipid content of small amounts of murine lymphoid tissues and isolated lymphocytes.

Recently, high-performance liquid chromatographic (HPLC) separation and determination of amino acids and aliphatic amines at the picomole level has been attained by their precolumn derivatization with the azo dye 4-dimethylaminoazobenzene-4'-sulfonyl chloride (dabsyl-Cl) [9, 10]. Dabsyl-Cl,

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like dansyl chloride, only reacts with primary and secondary amino groups, thiols, imidazoles and phenols under the dabsylation conditions described in this paper. Here, we describe a sensitive method (lower detection limit 5 pmoles) for the determination of sphingoid bases liberated from mammalian glycosphingolipids by acid hydrolysis. Excess reagent, which is degraded during the derivatization, and free amino sugars as common hydrolysis products of glycosphingolipids are completely separated by reversed-phase HPLC. This method was applied to the determination of the glycosphingolipid content of mouse spleen and thymus.

EXPERIMENTAL

Materials

Dabsyl-Cl was purchased from Fluka (Buchs, Switzerland). 4-Spingenine*, sphinganine*, cerebrosides and GD_{1a} ganglioside, all from bovine brain, were obtained from Supelco (Crans, Switzerland). Synthetic sphingoid bases were kindly provided by Prof. E. Jenny (Ciba-Geigy Ltd., Basle, Switzerland).

Mice

AKR and CBA/J mice (4—6 weeks old) were purchased from Bomholtgard (Ry, Denmark); BALB/c and C57BL/6 strains were bred at Ciba-Geigy animal facilities (Sisseln, Switzerland).

HPLC system

The components of the HPLC system were: a Zorbax CN column, particle size 10 μm (25 cm × 4.6 mm I.D.; DuPont Instruments, Wilmington, DE, U.S.A.), a Model U6K injector (Waters Assoc., Milford, MA, U.S.A.), 2 Model 110A pumps (Altex, Berkeley, CA, U.S.A.), an Altex Model 420 programmer, a Kontron Uvikon LCD 725 variable-wavelength detector (Kontron Analytic, Zürich, Switzerland), an SP 4020 interface and an SP 4000 integrator (Spectra-Physics, Darmstadt, G.F.R.), and a W+W Model 314 recorded (W+W Electronics, Basle, Switzerland).

A 5—10-μl sample volume was injected. The flow-rate was 1.0 ml/min. The solvent program was as follows: 60% acetonitrile, 40% 0.0175 *M* sodium acetate, pH 6.0. Isocratic conditions were maintained for 10 min, followed by a linear increase of the acetonitrile concentration up to 90% within 5 min, a linear decrease to initial conditions in 5 min, and isocratic conditions for another 10 min. The detector wavelength was 430 nm, and the sensitivity range was 0.01 a.u.f.s. The integrator was programmed to calculate area per cent.

Mass spectrometry

A ZAB fast atom bombardment mass spectrometer (Vacuum Generators, Altrincham, Great Britain) was used. The sample was dissolved in glycerol—methanol and bombarded by a 8 kV xenon source.

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*Abbreviations: 4-sphingenine = D-*erythro*-2-amino-4-*trans*-1,3-dihydroxy-octadecane; 4-sphinganine = D-*erythro*-2-amino-1,3-dihydroxy-octadecane.

Preparation of mouse spleen and thymus glycosphingolipids

The spleen and thymus from 25 mice were excised and homogenized in 50 ml of cold chloroform—methanol (2:1, v/v) using a Sorvall Omnimixer (DuPont Instruments). The tissue extract was passed through glass filter discs. This procedure was repeated twice. The combined extracts were dried in a rotary evaporator. The further fractionation was done as described by Saito and Hakomori [11]. Briefly, gangliosides were obtained from the upper phase of a modified Folch partition. The lower phase lipids were acetylated and fractionated on a Florisil column. Thus, sphingomyelin and neutral glycosphingolipids were completely separated. The glycosphingolipid fractions were checked for purity by high-performance thin-layer chromatography on silica gel plates [12]. Gangliosides were stained with resorcinol, and neutral glycosphingolipids with orcinol [13].

Hydrolysis of glycosphingolipids

Sphingoid bases were liberated by hydrolysis in 150 μl of methanol—water—conc. hydrochloric acid (29:4:3, v/v) in 200-μl Reactivials (Pierce, Rockford, IL, U.S.A.) at 79°C for 18 h [14]. Samples were dried in vacuo over KOH pellets, and used directly for dabsylation.

Dabsylation

Hydrolyzed glycosphingolipids were added with 20 μl of 0.2 *M* $NaHCO_3$—NaOH solution (pH 8.75) and 40 μl of dabsyl-Cl solution (5 nmoles/μl in acetone). The sample was heated at 70°C for 10 min with constant shaking. The dabsylated sample was diluted, if necessary, with methanol and injected directly for HPLC analysis.

RESULTS AND DISCUSSION

The proposed structure of dabsyl-sphingosine (DABS-Sph) is shown in Fig. 1. Fast atomic bombardment mass spectrometry showed the prominent molecular ion MH^+ at *m/e* = 587. There was no evidence of mass fragments indicating the reaction of the dabsyl-Cl with free sphingosine hydroxyl groups.

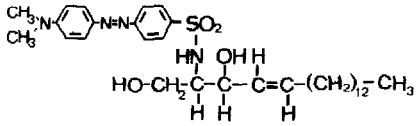

Fig. 1. Structure of dabsyl-sphingosine (dabsyl derivative of D-*erythro*-2-amino-4-*trans*-1,3-dihydroxy-octadecene).

Dabsylation of sphingosine was carried out in acetone—sodium bicarbonate, pH 8.75 (2:1, v/v) solution. The extent of dabsylation is dependent upon the initial concentration of dabsyl-Cl. However, like amino acid analysis using dabsyl-Cl [9], at any fixed concentration of dabsyl-Cl, there is a linear relationship between the peak response of DABS-Sph and the amount of sphingosine subjected to dabsylation (Fig. 2). This linearity comprises the basic and most important requisite for a quantitative analytical method using pre-

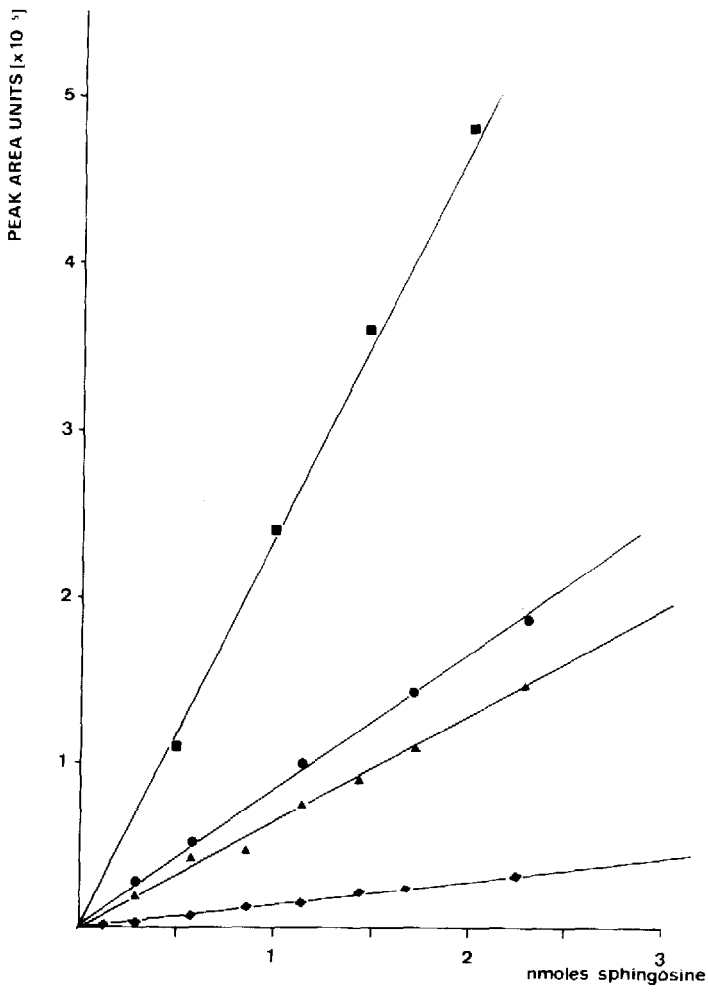

Fig. 2. Linear relationship of the peak response of dabsyl-sphingosine as a function of amount of sphingosine subjected to dabsylation at different dabsylation concentrations: (♦) 0.67 m*M*, (▲) 1.67 m*M*, (●) 3.33 m*M*, (■) 16.7 m*M*. For other experimental details see text.

column derivatization technique. The concentration 3.33 nmoles of dabsyl-Cl per μl of reaction volume (i.e. 5 nmoles dabsyl-Cl per μl of acetone) has been selected throughout this study on the basis that it gave the optimal sensitivity at tolerable level of excess reagent.

Fig. 3 shows the HPLC separation of DABS-Sph (Fig. 3b). The DABS-Sph which clearly separated from the excess reagent and by-products was eluted at 7 min. With the detector sensitivity set at 0.01 a.u.f.s., the integrator gave 930,000 area units per nanomole of DABS-Sph. Since 3000 area units can be easily recognized, the detection limit of dabsyl-Cl is thus around 5 pmoles.

Generally, in glycosphingolipids from distinct mammalian tissues only a few sphingosine bases occur at substantial levels. In most cases 4-sphingenine is predominant, but also sphinganine, isocasphingosine and 4-hydroxy-sphinganine are found [14]. In order to establish the contribution of the chemi-

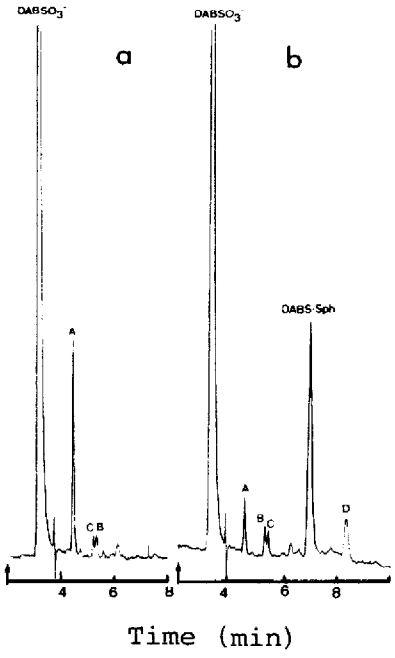

Fig. 3. (a) Blank run (dabsyl-Cl heated for 10 min at 70°C). (b) HPLC separation of dabsyl-sphingosine (Supelco). Peaks A, B and C represent reagent-derived by-products. Peak D is probably C20-4-sphingenine present in Supelco bovine brain sphingosine. For experimental details see text.

cal structure of sphingosines (i.e. number of carbon atoms or chain length, presence of double bonds and of additional hydroxyl groups) to the retention behaviour upon reversed-phase HPLC, a number of naturally occurring and synthetic sphingosines were dabsylated and submitted to the system.

Table I summarizes the sphingoid bases chromatographed and the retention times relative to 4-sphingenine. It is obvious that the separation behaviour of sphingosines is primarily according to their carbon atom chain length. A one C-atom difference will result in complete separation. Saturation of double bonds does not significantly change retention times. Thus, the sphinganine peak chromatographs as a shoulder of the 4-sphingenine peak. The configuration at asymmetric C-atoms, i.e. *erythro-* or *threo*-configuration and *cis-* or *trans*-isomerism of the double bond, does not contribute essentially to the separation on this column. Separation of isomers remains incomplete. With the exception of isocasphingenine which overlaps with a minor peak there is no interference of sphingoid base retention times with peaks of reagent-derived origin. Preliminary results indicate that also the 4-hydroxy-sphinganine (relative retention time = 0.84) is clearly separated from 4-sphingenine in our HPLC system.

Next this method was applied to the determination of the glycosphingolipid content of purified lipid fractions from mouse lymphoid organs. The average total lipid weight of a mouse spleen containing in the order of 10^8 lymphocytes is about 3 mg (G. Rosenfelder, unpublished results).

From human and pig lymphocytes it is known that glycosphingolipids

TABLE I

RETENTION TIMES OF SYNTHETIC AND NATURALLY OCCURRING SPHINGOID BASES SEPARATED ON A ZORBAX CN REVERSED-PHASE HPLC COLUMN

Sphingoid base (systematic denomination)	Origin	Relative retention time* (*T*)
D-*erythro*-2-Amino-4-*trans*-1,3-dihydroxy-octadecene	Bovine brain**	1.00
D-*erythro*-2-Amino-1,3-dihydroxy-octadecane	Bovine brain**	1.04
DL-*threo*-2-Amino-1,3-dihydroxy-octadecane	Synthetic***	1.04
DL-*erythro*-2-Amino-4-*cis*-dihydroxy-nonadecene	Synthetic***	1.10
DL-*threo*-2-Amino-4-*cis*-1,3-dihydroxy-nonadecene	Synthetic***	1.11
DL-*threo*-2-Amino-4-*trans*-1,3-dihydroxy-nonadecene	Synthetic***	1.07
DL-*threo*-2-Amino-1,3-dihydroxy-nonadecane	Synthetic***	1.14
D-*erythreo*-2-Amino-4-*trans*-1,3-dihydroxy-eicosene	Bovine brain ganglioside GD_{1a}**	1.17
DL-*threo*-2-Amino-1,3-dihydroxy-tricosane	Synthetic***	1.71

*Relative to the major bovine brain sphingosine 4-sphingenine = 1.00.
**Supelco.
***Synthesized by Prof. E. Jenny, Ciba-Geigy Ltd.

constitute up to 10% of total lipids [15, 16]. Therefore, a content of 30—300 μg of glycosphingolipids was expected in one mouse spleen. This equals 30—300 nmoles if an average molecular weight of 1000 is assumed. Our results (Table II) show a range of 60—166 nmoles of glycosphingolipids per spleen and of 24—62.4 nmoles per thymus, depending on the inbred strain that was used.

The ganglioside concentration is very low compared to the neutral glycosphingolipid concentration, with the lowest value (1.1. nmoles) in the C57BL/6 thymus.

TABLE II

GLYCOSPHINGOLIPID CONTENT OF SPLEEN AND THYMUS FROM MICE OF DIFFERENT INBRED STRAINS

Mouse strain	Glycosphingolipid content per mouse				Relative ganglioside content***	
	Gangliosides*		Neutral glycosphingolipids**			
	Spleen (nmoles)	Thymus (nmoles)	Spleen (nmoles)	Thymus (nmoles)	Spleen (%)	Thymus (%)
BALB/c	13.5 ± 0.48	1.7 ± 0.1	166.0 ± 7.2	26.4 ± 2.3	7.5	6.1
C57BL/6	7.2 ± 0.36	1.1 ± 0.1	60.9 ± 3.0	62.4 ± 4.0	10.6	1.7
AKR	13.4 ± 0.37	4.6 ± 0.3	63.3 ± 3.9	49.6 ± 1.8	1.8	8.5
CBA/J	6.0 ± 0.42	1.8 ± 0.2	60.0 ± 4.2	24.0 ± 1.4	9.1	7.0

*Dialyzed Folch upper phase lipids.
**Obtained by peracetylation and Florisil chromatography of Folch lower phase lipids [11].
***Gangliosides + neutral glycosphingolipids = 100%.

These low concentrations stress the necessity to use a quantitative glycosphingolipid determination method working at the picomole level if fractions of glycosphingolipids from separated lymphoid cell populations are studied in the mouse system. The dabsylation method described can be applied to the study of 10^5 or less lymphoid cells, i.e. of minor lymphocyte subpopulations. Moreover, it may be useful to study the total glycosphingolipid content of other extraneural cells where low concentrations of glycosphingolipids may play an important role in physiological processes like cell—cell recognition, receptor function or malignant transformation [17].

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