

High performance liquid chromatography of ceramides: application to analysis in human tissues and demonstration of ceramide excess in Farber's disease

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Abstract Conditions have been determined for the benzylation of ceramides containing nonhydroxy and hydroxy fatty acids, and a high performance liquid chromatography system for the separation and measurement of these derivatives has been devised that is capable of good resolution and high sensitivity. These methods have been used to determine quantitatively the levels of ceramides in human tissues, and in serum and urine, and to demonstrate elevated amounts of ceramide in Farber's disease urine and tissues.

Supplementary key words fatty acids · benzylation

Ceramides play a central role in sphingolipid metabolism. They are the basic lipid moiety of the sphingolipids and may be the precursors of sphingomyelin, cerebrosides, sulfatides, and gangliosides (1). Present at various levels in different tissues, ceramide has usually been determined using a colorimetric assay of the sphingosine (2) or fatty acid content (3) or by GLC and GLC-mass spectrometry (4-6). Two laboratories (2, 5, 7, 8) have reported finding increased amounts of both hydroxy and nonhydroxy fatty acid ceramides in several tissues of patients with Farber's lipogranulomatosis, a lipid storage disease. Recently, Evans and McCluer (9) have reported a convenient method for determining benzyolated neutral glycosphingolipid patterns using high performance liquid chromatography (HPLC). In this report, we describe our studies on the preparation of benzyolated hydroxy and nonhydroxy fatty acid ceramides and the separation and measurement of these derivatives by use of HPLC.

MATERIALS AND METHODS

Reagents

Benzoic anhydride was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. Nonhydroxy fatty acid

(NFA) ceramide standard was obtained from Supelco, Inc., Bellefonte, Pa. Hydroxy fatty acid (HFA) ceramide standard was purchased from Sigma Chemical Co., St. Louis, Mo. All solvents were of reagent grade and were used as supplied by the company (Fisher Scientific Co., Fair Lawn, N.J.) with the exceptions mentioned below. Chloroform, methanol, *n*-hexane, and ethyl acetate were redistilled; pyridine was refluxed with pellets of KOH and then distilled and stored over pellets of KOH and molecular sieve (type 5A, Fisher Scientific).

Preparation of derivatives

Preparation of benzyolated standard ceramides was carried out as follows. Ceramides (about 100 μ g) were dissolved in 0.5 ml of 10% benzoic anhydride in pyridine and sealed in a screw-cap tube with a Teflon-lined cap. The reaction was allowed to proceed for 3 hr at 110°C, and the mixture was then dried under a stream of nitrogen at 40°C. The residue was dissolved in 1 ml of 95% methanol and extracted two times with 2 ml of *n*-hexane. The *n*-hexane was evaporated, and the residue was dissolved in 4 ml of methanol and heated for 1 hr at 75°C in a screw-cap tube in order to destroy excess reagent. After evaporation of methanol, the residue was dissolved in 4 ml of *n*-hexane and washed successively with 1 ml each of 95% methanol that had been equilibrated with excess dry Na₂CO₃, 95% methanol, 0.6 N HCl in 95% methanol, and 95% methanol. The *n*-hexane layer was dried under a stream of nitrogen.

High performance liquid chromatography

HPLC analysis was performed on a Perkin-Elmer model 1220 high pressure liquid chromatograph equipped

Abbreviations: HPLC, high performance liquid chromatography; NFA, nonhydroxy fatty acid; HFA, hydroxy fatty acid; TLC, thin-layer chromatography; GLC, gas-liquid chromatography.

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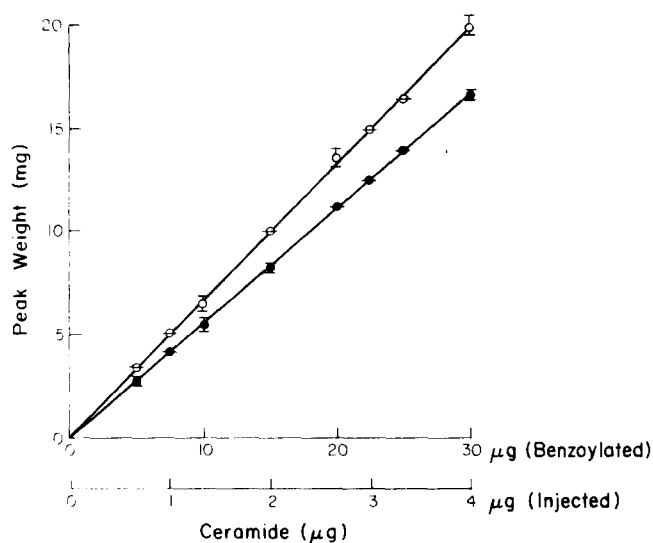


Fig. 1. Relation of peak weight to amount of benzoylated ceramide injected. After benzoylation of varying amounts of HFA and NFA ceramides, the derivatives were dissolved in 30 μ l of CCl_4 each, and 4- μ l aliquots of each sample were injected. O, HFA ceramide; ●, NFA ceramide.

with a Laboratory Data Control (Riviera Beach, Fla.) model 1285 UV monitor (254 nm). The analytical column (2 mm \times 50 cm, stainless steel tubing) was packed with Zipax, a controlled surface porosity support, average particle size 27 μ m (Instrument Products Division, E. I. duPont de Nemours & Co., Inc., Wilmington, Del.). As an elution solvent, 0.05% methanol in *n*-pentane was usually used, but it was occasionally found necessary to vary slightly the concentration of methanol in order to improve resolution of the peaks representing benzoylated ceramides. Alternatively, elution has been performed with 2.5% ethyl acetate in hexane, which provides satisfactory resolution and more stable adsorbent activity. All data presented in this report were obtained using the methanol-pentane system. Benzoylated ceramide samples were dissolved in carbon tetrachloride and a 2–10- μ l aliquot containing 1–20 μ g of ceramide was injected, and elution was performed at a flow rate of 1 ml/min. Column inlet pressure was approximately 200 psi.

Preparation of samples

The control tissues were from a 3-yr-old male patient who had died of pneumonia; the case of Farber's disease has been fully described (7). Serum and urine samples were obtained both from residents of W. E. Fernald State School (mentally retarded) and from normal controls.

Tissues (0.25–0.50 g wet wt) were extracted with 20 vol of chloroform-methanol 2:1 (v/v). Serum (1–5 ml) was lyophilized and the residue was extracted twice with 3-ml volumes of chloroform-methanol 2:1 (v/v). Urine (50–100 ml) was extracted twice with equal volumes of chloroform, and the lower phase, including the emulsified layer, was taken to near dryness with the aid of a small

amount of methanol on a rotary evaporator at 40°C. This residue was extracted twice with 5-ml volumes of chloroform-methanol 2:1 (v/v).

Each extract was then partitioned according to Folch, Lees, and Sloane Stanley (10). 0.2 vol of 0.74% KCl was mixed with the chloroform-methanol extracts, and the upper phases were discarded. The chloroform phases were then washed twice with "theoretical" upper phase. The final chloroform layer was subjected to mild alkaline hydrolysis to remove glycerolipids, according to the method of Hübscher, Hawthorne, and Kemp (11). The alkali-stable lipids obtained were fractionated by Unisil (Clarkson Chemical Co., Williamsport, Pa.) column chromatography (Unisil, 1 g; column, 1 \times 10 cm). Elution was carried out successively with (A) 25 ml of benzene, (B) 25 ml of benzene-ethyl acetate 9:1 (v/v), and (C) 50 ml of ethyl acetate. The fraction containing both HFA and NFA ceramides (C) was benzoylated with benzoic anhydride as described above.

Urine creatinine determinations (kindly performed by Miss Marjorie Scott) were run on a Technicon AutoAnalyzer using the picric acid method essentially according to Hawk, Oser, and Summerson (12).

RESULTS

Studies of recovery, preparation of derivatives, and separation by HPLC

Studies using *N*-[1- 14 C]palmitoyl sphingosine and *N*- α -[1- 14 C]hydroxypalmitoyl sphingosine, synthesized according to Kopaczyk and Radin (13), revealed that recovery of added ceramides from tissue homogenates and from urine after mild alkaline hydrolysis was 97% for the NFA ceramide and 96% for the HFA ceramide. Recovery of these ceramides after extraction, hydrolysis, and Unisil column chromatography was found to be 92 and 93%, respectively, and recovery of each from the benzoylation step itself was 87%. The overall recovery of both types of ceramide through the entire procedure (extraction, hydrolysis, Unisil column chromatography, and benzoylation) was 80%. The effect of varying the length of time of the benzoylation reaction was examined, and it was found that the reaction reached completion in 3 hr; after this time the amount of derivative produced was constant for as long as 18 hr, and after the reaction and destruction of excess reagent the derivative was stable for up to 48 hr. Using a sample of serum NFA ceramide, benzoylated as described above, it was shown that elution of the sample from the HPLC column followed by mild alkaline hydrolysis and TLC in chloroform-methanol-acetic acid 94:1:5 (v/v/v) resulted in recovery of NFA ceramide with an R_f identical with that of standard NFA ceramide.

Inspection of Fig. 1 shows that the ratio of recorder peak weight (area) to amount of benzoylated ceramide in-

jected is linear, indicating that this method is satisfactory for quantitative determination of this sphingolipid. The theoretical relative response of HFA ceramide to NFA ceramide is 3 to 2 (3 vs. 2 hydroxyl groups); the observed deviation from this ratio is unexplained but may result from differences in the fatty acid compositions of the standards, since for convenience the results are expressed on a weight rather than molar basis. Fig. 2 illustrates the clear separation of benzoylated NFA and HFA ceramides achieved by use of HPLC. The reproducibility of the method was checked by determining ceramide levels in six aliquots (5 ml) of pooled control serum. It was found that NFA ceramide amounted to $5.66 \pm 0.166 \mu\text{g/ml}$ of serum (mean \pm SD; range, 5.35–5.81).

Studies of tissue and body fluid ceramide levels

Table 1 shows the amount of ceramides found in human tissues by the HPLC method. These values are not corrected for loss because, as noted earlier, the recovery from the extraction, hydrolysis, and column chromatographic separation is high, and after this step weighed standard samples were subjected to the same derivatization procedures and used for calibration of recorder response. It should be noted that the ceramide content of some tissues examined here varies by as much as 200% from values reported earlier (14). This is most likely due to localized differences in ceramide concentration and pathological involvement within a given organ, in addition to the methodological differences alluded to in the introductory remarks.

Using this method to determine levels of ceramide in blood serum, it was found that total ceramide amounted to (mean) $4.70 \mu\text{g/ml}$ of serum (range of 12 controls, 2.42–6.33). Only trace amounts of HFA ceramide were observed. This figure is in good agreement with the value reported by Samuelsson (4), who found $5.64 \mu\text{g/ml}$ by GLC–mass spectrometry. Values of $9.5 \mu\text{g}$ of NFA ceramide and $4.0 \mu\text{g}$ of HFA ceramide per ml of serum were found for the Farber's disease patient whose tissues were examined (Table 1), somewhat elevated above control levels but not of the magnitude observed in such tissues as kidney or lung.

Urine from the patient (Table 1) with Farber's disease was found to contain $1.0 \mu\text{g}$ of NFA ceramide and $0.2 \mu\text{g}$

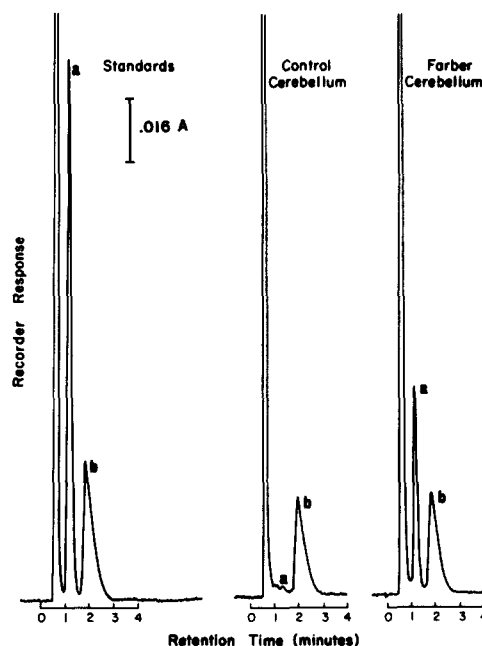


Fig. 2. High pressure liquid chromatographic analysis of benzoylated ceramides. The benzoylated derivatives of standard HFA and NFA ceramides and of ceramides isolated from control cerebellum and Farber's disease cerebellum were prepared as described in the text. Aliquots injected represented $10 \mu\text{g}$ of standard HFA ceramide and $5 \mu\text{g}$ of NFA ceramide, one-tenth of the ceramide extracted from 0.63 g (wet wt) of control cerebellum, and one-tenth of the ceramide obtained from 0.33 g of Farber's disease cerebellum. a, HFA ceramide; b, NFA ceramide. A, absorbance units.

of HFA ceramide per mg of creatinine. No ceramide was demonstrated in 28 control urines, which were obtained from patients with metachromatic leukodystrophy, mucopolysaccharidoses, Down's syndrome, phenylketonuria, and mental retardation of unknown etiology. The creatinine content of these controls was comparable to the Farber's disease sample. Since the urine extracted and benzoylated in each case was that volume which contained 50 mg of creatinine, and since the minimum detectable amount of ceramide was $0.3 \mu\text{g}$, amounts greater than $0.006 \mu\text{g}$ of ceramide/mg of creatinine ($0.3 \mu\text{g}/50 \text{ mg}$) would have been detected in the control samples. Hence, the Farber's disease urine contained at least 200 times ($1.2 \mu\text{g}$) as much ceramide per milligram of creatinine as the controls.


TABLE 1. Ceramide concentrations in control and Farber's disease tissues

	Kidney		Cerebellum		Lung		Heart		Stomach	
	C ^a	F ^a	C	F	C	F	C	F	C	F
	<i>μg/g wet tissue</i>									
NFA ceramide	94.7	408.7	54.0	103.0	14.1	131.5	15.9	40.0	11.3	102.3
HFA ceramide	<1.7	106.5	<1.2	63.6	<1.7	31.5	<1.2	<1.2	1.9	48.8
Total ceramide	94.7	515.2	54.0	166.6	14.1	163.0	15.9	40.0	13.2	151.1

^a C, control; F, Farber's disease.

DISCUSSION

The HPLC analysis of benzoylated HFA and NFA ceramides has the advantages of short analysis time, high sensitivity, and nondestructive measurement. As illustrated in Fig. 2, chromatographic elution time is less than 5 min, which allows the rapid processing of samples. The instrumentation employed in these studies allows the detection of less than 1 μg of ceramide. Sensitivity could be increased approximately sixfold by detection at 230 nm, the $\lambda_{\text{max}}^{\text{pentane}}$ for the benzoylated derivatives. Studies on the benzoylation of cerebroside (15) have shown that derivatization with benzoic anhydride leads only to *O*-acylation. Derivatization with benzoyl chloride leads to *N*-acylation in addition to the normal *O*-acylation. The ceramide derivatives formed by the procedure described in the present work are only *O*-acyl derivatives, and the original ceramides can be recovered by treatment with mild alkali as mentioned in Results.

The HPLC analysis of ceramide in the urine of a Farber's disease patient may prove to be the simplest procedure for detecting the metabolic disturbance in this disorder. Since the urine sample had been frozen for long periods, we could no longer determine whether the excess ceramide was initially contained in the urinary sediment. This has been shown to be the case for the excess urinary lipids in the other sphingolipidoses and has led Desnick et al. (16) to suggest that study of urinary sediment is in fact equivalent to a nonsurgical kidney biopsy. The high ceramide levels in Farber's disease kidney suggest that this is true for Farber's disease also. 

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