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ANALYSIS OF CERAMIDE AND MONOHEXAOSYL GLYCOLIPID DERIVATIVES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ITS APPLICATION TO THE DETERMINATION OF THE MOLECULAR SPECIES IN TISSUES

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SUMMARY

Several molecular species of monohexaosyl glycolipids and ceramides were analyzed as their benzoyl derivatives by high-performance liquid chromatography on a silica column with mixtures of dioxane and hexane as eluents. Monohexaosyl glycolipid derivatives were classified according to their hydrophobic structures, and ceramides were separated based on the fatty acid groups and the long-chain bases. The method allowed us to observe the heterogeneities in the molecular species of cerebrosides and ceramides from the epidermis and dermis of the guinea pig, and clear differences in the molecular species were observed between the different regions.

INTRODUCTION

Recently, considerable attention has been paid to the possible involvement of glycosphingolipids in various cellular functions such as differentiation antigens and tumor-associated antigens, toxin and lymphokine receptors, cell-surface markers, etc.[1,2]. Glycosphingolipids are composed of long-chain bases, fatty acids, and carbohydrates and are mainly classified on the basis of their carbohydrate structure. However, individual glycosphingolipids are always heterogeneous due to their hydrophobic chains, which are thought to participate in regulating the antigenicity or reactivity of the carbohydrate portion[3–6]. Therefore, the qualitative and quantitative determination of glycosphinolipids in the smallest possible samples, taking into account the heterogeneity in the hydrophobic chains, has become an important objective. High-performance liquid chromatography (HPLC) was recognized as the most promising method for this purpose. Although a number of methods for the analysis of glycosphingolipids and ceramides have been reported[7–12], HPLC of glycolipids, including the analysis of the heterogeneous hydrophobic chains, has not been reported so far. We have attempted to establish a convenient procedure for the analysis of molecular species of glycosphingolipids and to apply this procedure to the determination of the ceramide and glycolipid profiles of guinea pig epidermis and dermis,

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in which the glycosphingolipid composition is quite unique and is thought to play a modulating role in keratinocyte differentiation.

EXPERIMENTAL

Materials and instrumentation

All standard monohexaosyl glycolipids used in this work were prepared in our laboratory. Ceramides were synthesized from sphingosine (prepared from bovine brain cerebrosides by the method of Karlsson[13]) or from phytosphingosine (purchased from Sigma, St. Louis, MO, U.S.A.) and fatty acids (Sigma) by the method of Kopaczyk and Radin[14]. The ceramides were purified by chromatography on Silica gel (Iatrobeads RS8060, Iatron, Tokyo, Japan) with a linear gradient from 20 to 50% (v/v) ethyl acetate in hexane. The other reagents were purchased from Wako (Osaka, Japan). The HPLC equipment was a Model 344 CRT-based gradient liquid chromatography system (Beckman, Berkeley, CA, U.S.A.), equipped with a Model 164 variable-wavelength detector and a Chromatopac CR-3A chromatographic processor (Shimadzu, Kyoto, Japan). The chromatographic column used was 5-μm Ultrasphere Si column (250 × 4.6 mm I.D., Beckman).

Preparation of lipids from guinea-pig skin

All lipids were extracted from the epidermis and dermis of guinea-pig footpads with chloroform–methanol–water (20:10:1 and 10:20:1, v/v/v) and with chloroform–methanol (1:1, v/v) at 45°C. The lipids were fractionated into neutral and acidic lipids by anion-exchange chromatography (DEAE-Sephadex A-25 in the acetate form) according to the method described previously[15].

Benzoylation

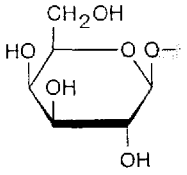
Samples containing 5–100 μg of monohexaosyl glycolipids or ceramides were placed in screw-capped tubes, evaporated to dryness under nitrogen, and dried further *in vacuo*. To the dried sample, 0.5 ml of benzoyl chloride–pyridine (1:9, v/v) was added, and reaction was allowed for 2 h at 70°C. After reaction, 1 ml of methanol was added, and the mixture was incubated at 70°C for 30 min to convert excess benzoyl chloride to volatile methyl benzoate. The solution was evaporated under a stream of nitrogen, and the products were recovered by partitioning between 1 ml of a saturated solution of sodium carbonate in methanol and 1 ml of hexane. The hexane layer was collected, and the methanol layer was extracted twice with 1 ml of hexane. The hexane layers were combined and used for thin-layer chromatography (TLC) and HPLC. The benzoylated ceramides and cerebrosides were developed on TLC with hexane–diethyl ether (70:30 and 50:50, v/v, respectively) and the spots were located with cupric acetate for ceramides[16] and with orcinol reagent for cerebrosides[17].

HPLC analysis

A volume of 5 μl of the benzoylated monohexaosyl glycolipid was injected into an Ultrasphere Si column, which had previously been equilibrated with dioxane–hexane (8:92, v/v). The derivatives were eluted with dioxane–hexane (8:92) for 5 min, and then with a linear gradient from 8 to 23% of dioxane in hexane at a rate of

TABLE I

STRUCTURES OF SEVERAL MONOHEXAOSYL GLYCOLIPIDS



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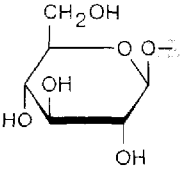

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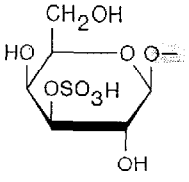

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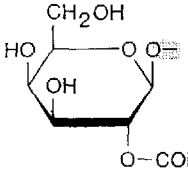
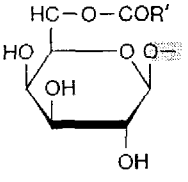
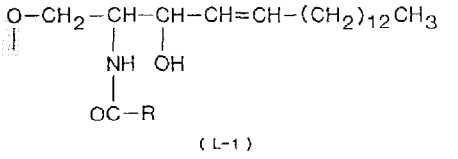


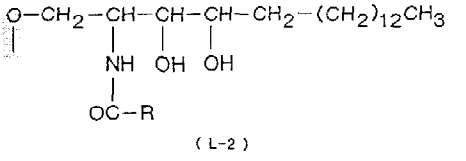
(S-4)



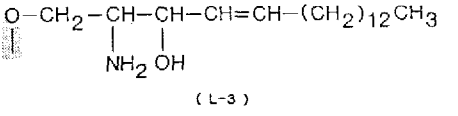
(S-5)



(L-1)



(L-2)



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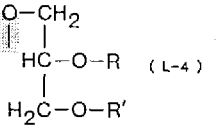


(L-4)

NFA = non-hydroxy fatty acid; HFA = hydroxy fatty acid.

No.	*Name*	*Components*		
		Sugar	*Lipid*	*R, R'*
G-1	2-O'-Acyl-β-D-galactopyranosyl-N-Acylsphingosine	S-4	L-1	NFA
G-2	6-O'-Acyl-β-D-galactopyranosyl-N-Acetylsphingosine	S-5	L-1	HFA
G-3	β-D-Galactopyranosyl-1-alkyl-2-diacyl-sn-glycerol	S-1	L-4	Alkyl and acyl
G-4	β-D-Glucopyranosyl-N-acyl-sphingosine	S-2	L-1	NFA or HFA
G-5	β-D-Galactopyranosyl-N-acyl-phytosphingosine	S-1	L-2	NFA
G-6	β-D-Galactopyranosyl-N-acyl-sphingosine	S-1	L-1	NFA or HFA
G-7	β-D-Galactopyranosyl-1-alkyl-glycerol	S-1	L-4	One is H, other is alkyl
G-8	β-D-Galactopyranosyl-N-acetyl-sphingosine	S-1	L-1	
G-9	3-O'-Sulpho-β-D-galactopyranosyl-1-alkyl-2-acyl-sn-glycerol	S-3	L-4	Alkyl and acyl
G-10	3-O'-Sulpho-O-β-D-galactopyranosyl-N-acylsphingosine	S-3	L-1	NFA or HFA
G-11	3-O'-Sulpho-β-D-galactopyranosyl-1-alkyl-sn-glycerol	S-3	L-4	One is H, other is alkyl
G-12	β-D-Galactopyranosyl-sphingosine	S-1	L-3	

0.25% per min, and finally with dioxane–hexane (23:77, v/v) for 10 min. The flow-rate was kept at 1 ml/min. The benzoylated ceramides were analyzed on the same column as above, but the elution was isocratic with 3% dioxane in hexane at a flow-rate of 1 ml/min. The benzoylated derivatives were monitored at 228 nm.

RESULTS AND DISCUSSION

Benzoylation of glycolipids

Table I shows the structures of the standard monohexaosyl glycolipids used. The TLC patterns of the glycolipids before (A) and after benzoylation (B) are shown in Fig. 1. After benzoylation, all of the samples became less polar, but the mobility of the derivatives on TLC could not be correlated with that of the original glycolipids. The recovery of the derivatives in the total hexane extract was estimated by densitometry to be about 75%. In a report by other workers[8], the hexane layer was washed with a saturated solution of sodium carbonate in methanol, but we found that this did not cause a significant difference in the chromatograms. The structures of the ceramides used as standards are shown in Table II, and the TLC patterns of the ceramides before (A) and after benzoylation (B) are shown in Fig. 2. The separation of ceramides or of their benzoyl derivatives on TLC, is based on differences in chain length, and therefore the patterns were essentially similar.

HPLC analysis of monohexaosyl glycolipids as their benzoyl derivatives

The benzoylated glycolipids obtained as described above were analyzed under the conditions described in the Experimental section. Because the polarity of all derivatives was quite similar after benzoylation (Fig. 1), complete separation of all glycolipids was not achieved. However, we were able to separate them as follows due to differences in their basic structures: sphingo- and glycerolipids, NFA- and HFA-containing glycolipids, sphingosine- and phytosphingosine-containing glycolipids, cerebrosides with and without acylester, and galactosyl mono- and diglycerides (Fig. 3). In addition, by using N-acetyl psycosine as an internal standard, >10 pmole of monohexaosyl glycolipids could be determined quantitatively and the calibration curve was linear from 10 to 1000 pmol. The procedure was then applied to the analysis of cerebrosides from the epidermis and the dermis. As shown in Fig. 4, the cere-

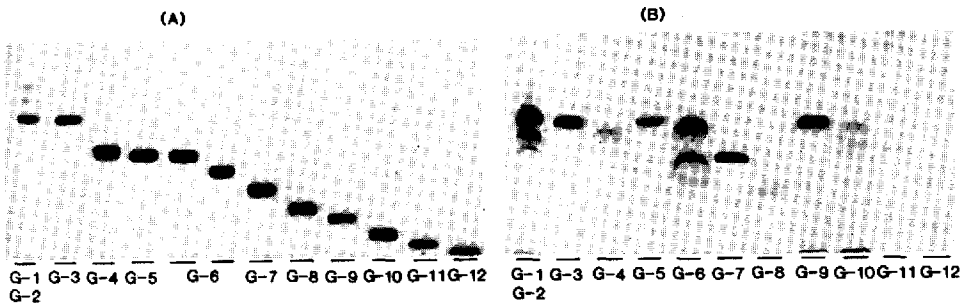
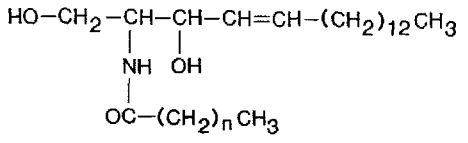


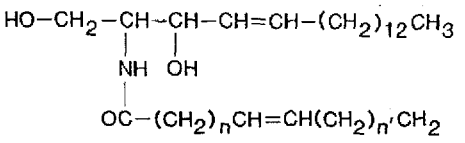
Fig. 1. TLC chromatogram of several monohexaosyl glycolipids before (A) and after (B) benzoylation. Developing solvents: (A) chloroform–methanol–water (40:10:1, v/v/v); (B) hexane–diethyl ether (50:50, v/v). Spots were located with the orcinol reagent. Numbers as in Table I.

Table II

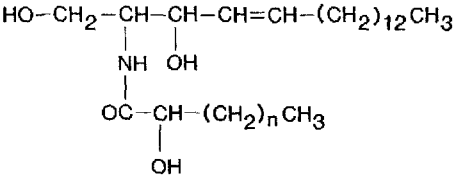
STRUCTURES OF SYNTHETIC CERAMIDES



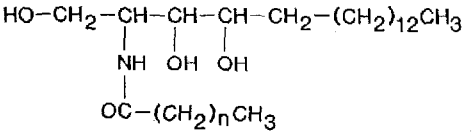
(F-1)



(F-2)



(F-3)



(F-4)

No.	*Name*	*Structure*	*n*
C-1	N-Lignoceroylsphingosine	F-1	22
C-2	N-Nervonylsphingosine	F-2	$n=13$ $n'=7$
C-3	N-Behenoylsphingosine	F-1	20
C-4	N-Oleoylsphingosine	F-2	$n=7$ $n'=7$
C-5	N-Palmitoylsphingosine	F-1	14
C-6	N-Decanoylsphingosine	F-1	8
C-7	N-Butylsphingosine	F-1	2
C-8	N-(1-Hydroxy-lignoceroyl)sphingosine	F-3	21
C-9	N-Palmitoylphytosphingosine	F-4	14

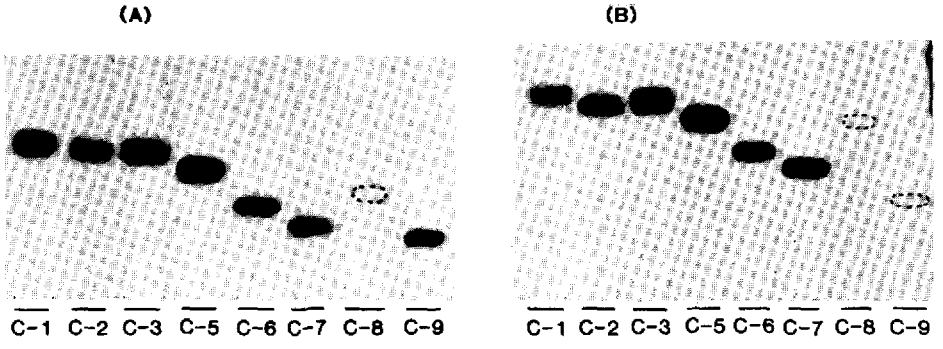


Fig. 2. TLC chromatogram of synthetic ceramides before (A) and after benzoylation (B). Developing solvents: (A) chloroform–methanol (95:5, v/v); (B) hexane–diethyl ether (70:30, v/v). Spots were located with the cupric acetate reagent. Numbers as in Table II.

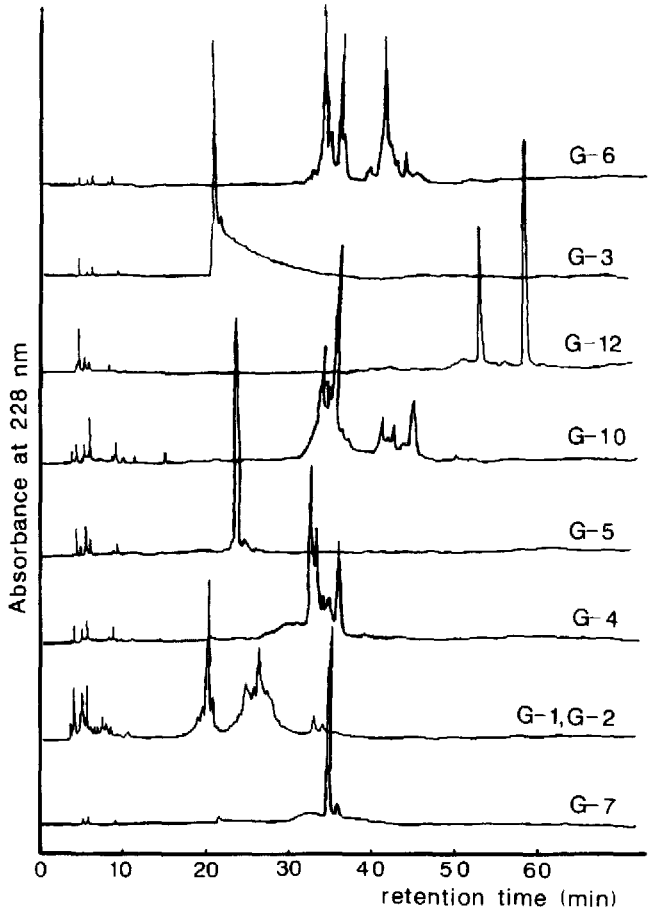


Fig. 3. HPLC chromatograms of benzoylated monohexaosyl glycolipids. Experimental conditions as described in the text. Numbers as in Table I.

brosides from both tissues were quite heterogeneous, but the two compositions were clearly different. It is clear that the largest signal for the epidermis corresponds to monohexaosyl ceramides containing acylesters, followed by NFA-, phytosphingosine- and HFA-containing monohexaosyl ceramides. In contrast, NFA-containing monohexaosyl ceramides are the major component in the dermis, and HFA- and phytosphingosine-containing monohexaosyl ceramides are present in smaller concentrations. After isolation of each peak, the composition was further analyzed by GLC for peak identification[18].

HPLC analysis of ceramides as the benzoyl derivatives

Benzoylated ceramides were analyzed in a similar way. Fig. 5 shows the chromatograms of the benzoyl derivatives of synthetic ceramides. In contrast with the elution order of cerebrosides, where the HFA-cerebrosides were eluted faster than the NFA-cerebrosides, the NFA-ceramides were eluted before the HFA-ceramides.

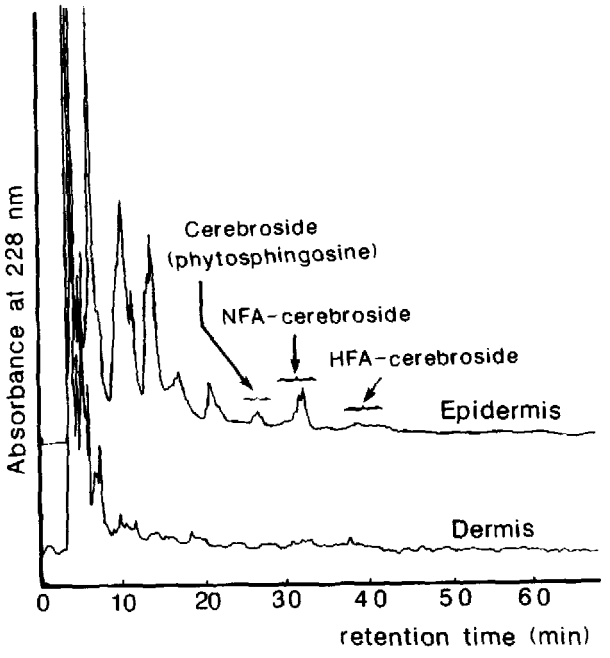


Fig. 4. HPLC chromatograms of benzoylated cerebrosides from the epidermis and dermis of guinea-pig footpad. Experimental conditions as described in the text.

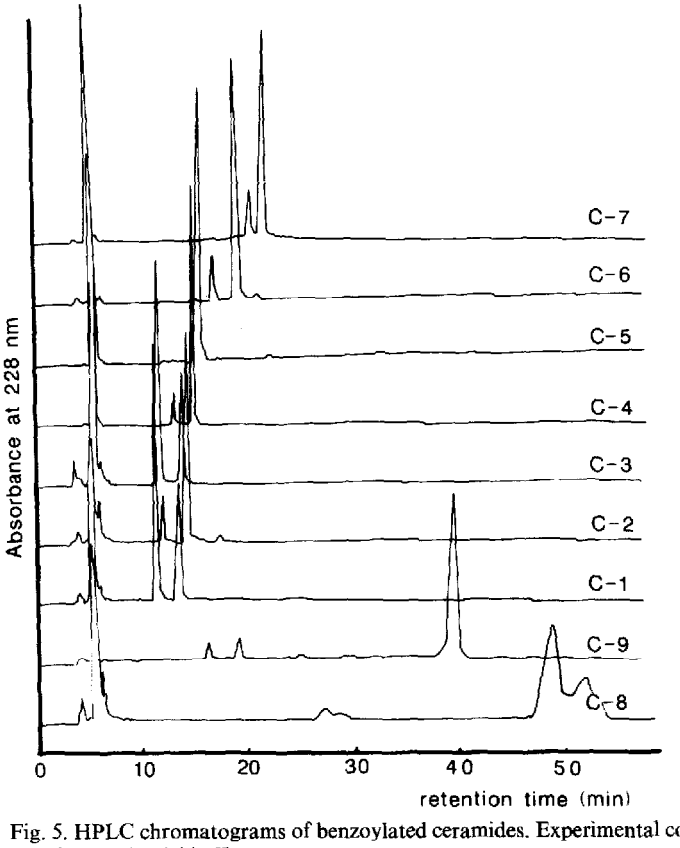


Fig. 5. HPLC chromatograms of benzoylated ceramides. Experimental conditions as described in the text. Numbers as in Table II.

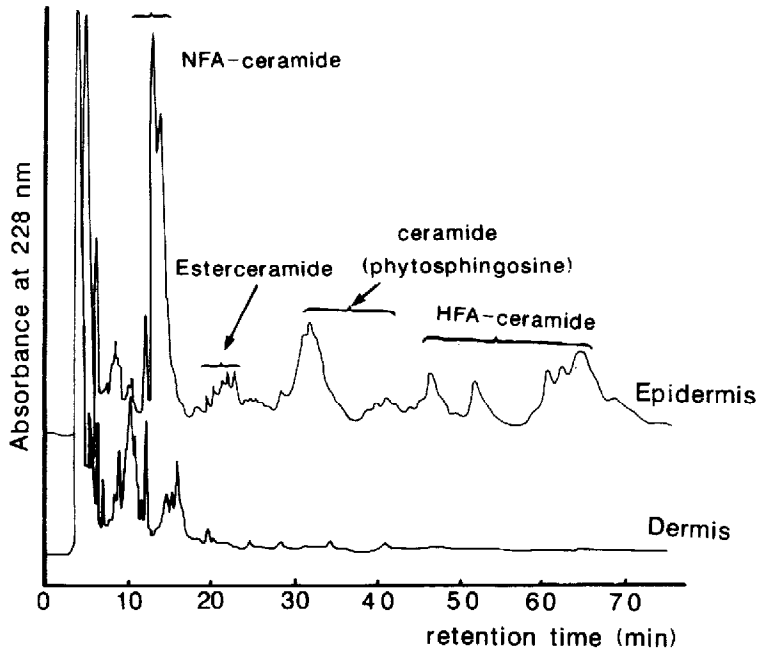


Fig. 6. HPLC chromatograms of benzoylated ceramides from the epidermis and dermis of guinea-pig foot-pad. Experimental conditions as described in the text.

Also, the retention time of the phytosphingosine-containing ceramide was longer than that of the sphingosine-containing ceramide (Fig. 5). As shown in Fig. 6, the ceramide fraction from guinea-pig skin was also found to be heterogeneous. The epidermis contained the faster-moving ceramides, which were probably composed of long-chain fatty acids, as the major component, and further NFA-, phytosphingosine-, and HFA-containing ceramides. Each fraction consisted of peaks distributed over a wide region, indicating a heterogeneous composition due to different chain lengths. The ceramides from the dermis were mainly composed of NFA-ceramides. Each peak was collected and its composition was analyzed by gas–liquid chromatography for peak identification[18].

As indicated by these experiments, the cerebrosides and ceramides from the epidermis and dermis of the guinea pig comprised highly heterogeneous molecular species[18–22]. Each region revealed a characteristic composition of cerebrosides and ceramides. A high concentration of esterified cerebrosides and ceramides in the epidermis distinghuished it from the dermis. The uniqueness in the hydrophobic chain of sphingolipids may be related to functions of skin, such as that of physical barrier, viscoelasticity, and intracellular adhesion. To clarify the functional significance of the molecular heterogeneity of ceramides and cerebrosides, it is necessary to analyse the molecular species in detail. The procedure described in this communication should be very useful for further investigations in our laboratory.

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