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In vivo distribution of free long-chain sphingoid bases in the human stratum corneum by high-performance liquid chromatographic analysis of strippings

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Abstract

Conditions were established for the *in vivo* determination of free long-chain bases (phytosphingosine, sphingosine and sphinganine) in human stratum corneum layers by reversed-phase HPLC analysis of adhesive-tape strippings. Long-chain bases were first extracted from individual strippings with pure methanol in an ultrasound bath, then *o*-phthalaldehyde derivatives were separated on a C_{18} column with a gradient from methanol–water (80:20, v/v) to pure methanol. By performing a second series of strippings for protein determination it was possible to express the amounts of free long-chain bases per milligram of protein for each individual stratum corneum layer. Co-elution of endogenous phytosphingosine, sphingosine and sphinganine was demonstrated in separate experiments by addition of standards to typical strips. From a study of three human volunteers, average concentrations of free long-chain bases in the stratum corneum were found to be 2.8, 1.2 and 0.5 nmol/mg of protein for phytosphingosine, sphingosine and sphinganine, respectively.

1. Introduction

Long-chain sphingoid bases (phytosphingosine, sphingosine, sphinganine and homologues, Fig. 1) constitute the backbone moiety of sphingolipids, an important class of lipids to which belong the epidermal ceramides. Free long-chain bases (FLCB) may occur as sphingolipid breakdown products by the action of hydrolytic enzymes [1,2]. In fact, relatively high concentrations of FLCB have already been reported in porcine and human epidermis [3,4]. This presence is of a great interest as numerous studies demonstrated that these molecules were potent inhibitors of protein kinase C both *in vitro* and *in vivo* [5–7], and as a consequence may be involved in the differentiation of epidermal keratinocytes [8].

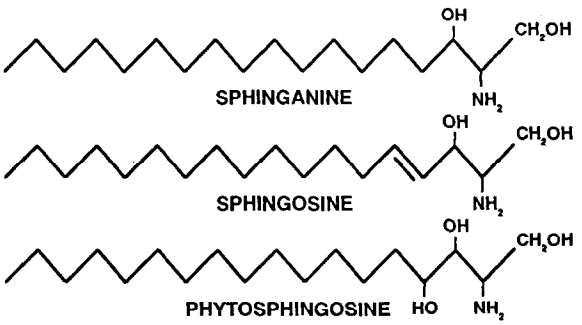

Fig. 1. Long-chain sphingoid bases.

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Although several workers have reported the determination of sphingosine in biological tissues [3,4,9–11], little has been done concerning the simultaneous determination of phytosphingosine, sphingosine and sphinganine. In this respect, the results of Wertz and Downing [3] are questionable, as they found no phytosphingosine in the stratum corneum, although this long-chain base is involved in several epidermal ceramides [12]. This paper describes the precise conditions for the simultaneous determination of phytosphingosine, sphingosine and sphinganine in human stratum corneum. Further, using highly sensitive *o*-phthalaldehyde (OPA) derivatization, it has been possible to measure the distribution of the three long-chain bases within the stratum corneum depth by HPLC analysis of individual tape strippings on the forearm of three human volunteers.

2. Experimental

2.1. Chemicals and reagents

D-Sphingosine (D-*erythro*-1,3-dihydroxy-2-amino-4-*trans*-octadecene) from bovine brain cerebrosides (99%), D-phytosphingosine (D-1,3,4-trihydroxy-2-aminooctadecane) from yeast as hydrochloride (97%) and synthetic DL-sphinganine (DL-1,3-dihydroxy-2-aminooctadecane) (99% with 66% *erythro* and 33% *threo*) were purchased from Sigma (St.-Quentin Fallavier, France).

Tape strippings were performed using 3M adhesive tape (Scotch Magic, 3M, ref. 810) in a ribbon of width 1.9 cm. Fluoraldehyde was purchased from Pierce (Oud-Beijerland, Netherlands) as a ready-for-use *o*-phthalaldehyde reagent.

Solvents used for the mobile phase were spectroscopic-grade methanol (Carlo Erba, Rueil Malmaison, France) and HPLC-grade water (J.T. Baker, Deventer, Netherlands). Solvent A was methanol–water (80:20, v/v) and solvent B was pure methanol for the HPLC gradient.

A chromogenic solution of 1% Bright Xylene Cyanin G in 7% aqueous acetic acid was used for protein determination. Elution of the fixed chromogenic agent was achieved with methanol–water–ammonia solution (330:170:5, v/v/v). Bovine serum albumin from Merck (Darmstadt, Germany) was used as a protein standard.

2.2. Biological samples

Stratum corneum layers for FLCB analysis were removed by successive strippings with pieces of 3M adhesive tape (1.9 × 4.7 cm) from the ventral forearm of three male healthy volunteers (50, 40 and 51 years old for subjects 1, 2 and 3, respectively).

A second series of strippings (1.9 × 3.1 cm) were also performed on the same day, on each subject and on a site next to the first one for a protein determination in each stratum corneum layer. Both series of strippings were carried out by the same physician and the same materials according to the same protocol.

2.3. Extraction of lipids and o-phthalaldehyde derivatization

Each strip was cut with a scalpel into three pieces (for a better extraction) and extracted with 1 ml of pure methanol in a 20-ml flask. Extraction was carried out in an ultrasonic bath at 20°C for 15 min. A second extraction was carried out using the same conditions with an additional 30-min stirring on a magnetic stirrer. The extracts were collected in a 5-ml glass vial, rinsed with pure methanol and evaporated at 20°C under a gentle stream of nitrogen. They were stored, protected from light, at −20°C until analysis.

The samples were then taken up with 100 μl of pure methanol and allowed to react for 10 min at 20°C with 100 μl of the OPA reagent. After dilution to 1 ml with methanol–water (90:10, v/v), the samples were filtered through an HV 0.5-μm membrane (Millipore, Bedford, MA, USA).

2.4. Liquid chromatography

HPLC analyses were carried out using two Waters Model 6000 A pumps, a Valco (SFCC, Neuilly-Plaisance, France) manual injector with

a 20-μl injection loop, a Waters Model 470 spectrofluorimeter and a Merck D 2500 integrator for the first apparatus. The second apparatus used was equipped with a Merck gradient pump, a Rheodyne manual injector with a 20-μl loop, a Hitachi (Tokyo, Japan) F 2000 spectrofluorimeter and a Merck D 2500 integrator.

Separations of FLCB were achieved on a LiChrosorb C_{18} (5-μm) column (250 × 4 mm I.D.) (Merck), equipped with a C_{18} guard column. Elution of the OPA derivatives of FLCB was performed at a flow-rate of 1.0 ml/min using solvent A–solvent B gradients as follows: first a 2-min gradient from 100% to 40% A, then a 25-min isocratic segment (during which the three free bases were eluted), followed by a second gradient of 2 min to 100% B, a second isocratic segment of 7 min, and a final gradient of 2 min to 100% A.

The fluorescence intensity was measured using an emission wavelength of 433 nm and an excitation wavelength of 344 nm.

2.5. Protein analysis

A modified method of Bramhall *et al.* [13] was used for protein determination. Each strip was first treated with 2 ml of the chromogenic solution (Bright Xylene Cyanin G) for 15 min at 50°C, then the strip was washed with 7% aqueous acetic acid until complete discoloration of the medium was observed. The protein-fixed chromogenic agent was eluted with 5 ml of methanol–water–ammonia solution (330:170:5, v/v/v) and the absorbance was measured at 578 nm against a blank with adhesive tape. The protein content was determined by using a calibration graph established with bovine serum albumin.

3. Results and discussion

3.1. Analysis of long-chain bases as o-phthalaldehyde derivatives

A methanolic solution containing 100 pmol of phytosphingosine, sphingosine and sphinganine was deposited on the adhesive side of a 1.9 × 4.7 cm piece of adhesive tape. After drying, the three bases were extracted and derivatized as described previously. The elution profiles for the OPA derivatives are shown in Fig. 2. A blank chromatogram obtained by the extraction and derivatization of an identical piece of adhesive tape is shown for comparison in Fig. 3. The detection limit measured at a signal-to-noise ratio of 3 was estimated to be 30 fmol for each injected standard. The fluorescence intensity was linear between 0.1 and 10 pmol of each free base injected.

As shown on the representative chromatogram in Fig. 4, it was not possible to use an internal standard because of the presence of numerous

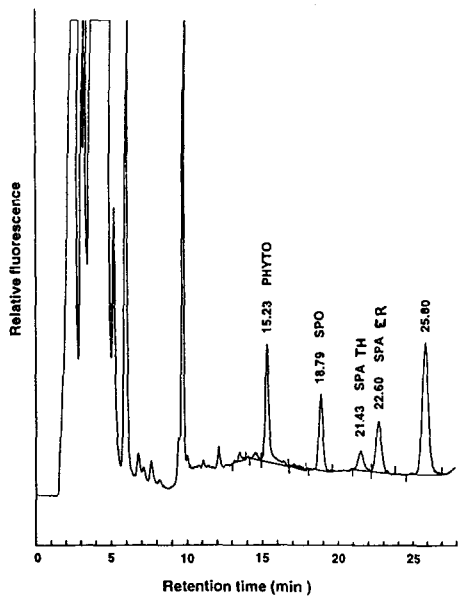

Fig. 2. Chromatographic profile of OPA derivatives obtained from standard compounds deposited on a piece of adhesive tape. A 20-μl volume of a methanolic solution containing 100 pmol of each standard free base was deposited on a 1.9 × 4.7 cm piece of adhesive tape. After evaporation and extraction with methanol, the concentrate was derivatized with OPA and diluted to 1 ml with methanol–water (90:10, v/v). A 20-μl aliquot (corresponding to 2 pmol of each added standard) was injected on to the LiChrosorb C_{18} (5-μm) column (250 × 4 mm I.D.). OPA derivatives were separated using a gradient from methanol–water (80:20, v/v) to methanol and detected with a spectrofluorimeter (λ_{ex} = 344 nm, λ_{em} = 433 nm). Peaks: PHYTO = phytosphingosine; SPO = sphingosine; SPA TH = sphinganine (*threo* isomer); SPA ER = sphinganine (*erythro* isomer).

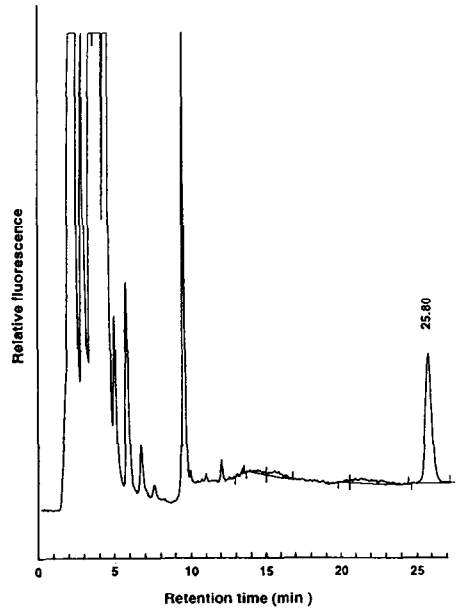

Fig. 3. Blank chromatogram obtained after methanolic extraction and OPA derivatization of a 1.9 × 4.7 cm piece of adhesive tape under the same conditions as in Fig. 2.

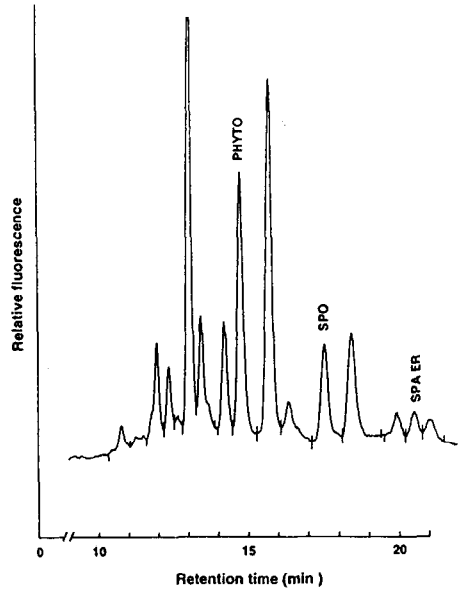

Fig. 4. FLCB pattern obtained from subject 1; chromatographic profiles of OPA derivatives of endogenous FLCB extracted from strip 14 and analysed under the same chromatographic conditions as in Fig. 2.

endogenous peaks. Therefore, the extraction recoveries were evaluated from the deposition of 100 pmol of each standard on calibrated pieces of adhesive tape. The extraction yields obtained from the calibration graph were 72.7 ± 2.3%, 76.8 ± 1.3% and 72.5 ± 2.0% ($n = 5$) for phytosphingosine, sphingosine and sphinganine, respectively.

A strong indication of the identity of endogenous phytosphingosine, sphingosine and sphinganine was obtained from two other experiments. Five successive strippings were performed on the ventral forearm of a human volunteer using 1.9 × 8 cm pieces of 3M adhesive tape. The fourth strip was cut into three identical pieces of 1.9 × 2.5 cm, then 30 pmol of each standard free base were deposited on the left and right pieces from their methanolic solution and analysed after drying with the remaining piece of strip according to the above-described procedure. Coelution of endogenous phytosphingosine and sphingosine with their respective standards was obvious. As expected, *threo*-sphinganine, not being a naturally occurring isomer, was not coeluted with an endogenous peak, unlike the natural *erythro* isomer.

OPA derivatives from adhesive-tape strippings and spiked samples with each standard were also analysed under different chromatographic conditions using a Zorbax phenyl column (250 × 4.6 mm I.D.) (DuPont, Wilmington, DE, USA) and a gradient from methanol–water (50:50, v/v) to methanol. Although complete separation of endogenous peaks was not achieved as with a C_{18} column, the elution profile was similar and coelution of phytosphingosine, sphingosine and sphinganine with their respective standards was also observed.

3.2. Distribution of FLCB in the human stratum corneum

On the first subject, twenty successive strippings were performed on both areas of the forearm as described above. From the first to the twentieth strip, the HPLC profiles of the OPA derivatives show a similar general pattern (see as an example the chromatogram from strip No. 14

Table 1
Determination of phytosphingosine, sphingosine and sphinganine in the stratum corneum of subject 1

Strip No.	Protein ($\mu g/cm^2$)	Phytosphingosine (nmol/mg protein)	Sphingosine (nmol/mg protein)	Sphinganine (nmol/mg protein)
1	4.700	1.068	0.561	0.306
2	4.000	1.321	0.611	0.206
3	4.500	1.989	0.716	0.286
4	4.200	1.923	0.721	0.196
5	3.800	3.883	1.247	0.473
6	3.700	2.726	0.845	0.361
7	4.100	3.748	1.037	0.376
8	3.700	3.033	1.201	NA[a]
9	3.800	2.608	0.861	0.298
10	3.500	3.181	1.047	0.191
11	3.700	2.714	0.792	0.278
12	3.000	3.231	1.010	0.411
13	2.700	3.234	0.977	0.419
14	2.600	4.232	1.428	0.534
15	3.100	2.704	0.867	0.249
16	3.800	2.872	1.106	0.162
17	3.200	2.838	1.160	0.386
18	2.600	3.426	1.597	0.164
19	2.900	3.041	1.028	0.411
20	2.900	2.665	1.044	0.211
Mean	3.525	2.822	0.993	0.311
S.D.	0.140	0.176	0.058	0.025

[a] Not available.

Table 2
Determination of phytosphingosine, sphingosine and sphinganine in the stratum corneum of subject 2

Strip No.	Protein ($\mu g/cm^2$)	Phytosphingosine (nmol/mg protein)	Sphingosine (nmol/mg protein)	Sphinganine (nmol/mg protein)
1	4.100	0.692	0.500	0.476
2				
3	3.600	1.953	1.086	0.728
4				
5	3.400	1.490	0.805	0.484
6				
7	2.800	1.622	0.803	0.422
8				
9	2.700	2.296	1.013	0.800
10				
11	3.500	1.522	0.586	0.426
12				
13	3.300	2.104	0.562	0.592
14				
15	3.100	1.591	0.867	0.431
Mean	3.313	1.659	0.778	0.545
S.D.	0.160	0.173	0.076	0.052

in Fig. 4). The FLCB were quantified from peak integration per unit area of strip and expressed by using the corresponding protein content in nmol/mg of protein. As shown in Table 1, a high proportion of phytosphingosine was encountered although a significant decrease in this free base was observed in the outer layers of stratum corneum. On the two other volunteers, fifteen strippings were performed and only odd strips were analysed. The HPLC profiles show the same general pattern and the distributions of FLCB within the stratum corneum depth were not very different from those for the first subject (see Tables 2 and 3). Average levels of FLCB estimated from the three subjects were 2.8, 1.2 and 0.5 nmol/mg of protein for phytosphingosine, sphingosine and sphinganine, respectively.

This method developed using non-invasive tape-stripping sampling and sensitive OPA derivatization seems to be very suitable for the careful *in vivo* determination of FLCB, including phytosphingosine, within the human stratum corneum. Further investigations under physiopathological conditions such as aged skin, dry skin or UV-exposed skin, in which FLCB are supposed to play a key role, would be of interest.

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Table 3
Determination of phytosphingosine, sphingosine and sphinganine in the stratum corneum of subject 3

Strip No.	Protein ($\mu g/cm^2$)	Phytosphingosine (nmol/mg protein)	Sphingosine (nmol/mg protein)	Sphinganine (nmol/mg protein)
1	2.500	2.480	1.915	1.213
2				
3	2.200	3.830	2.043	1.098
4				
5	2.100	5.447	2.419	1.199
6				
7	2.400	3.911	2.035	0.878
8				
9	2.500	3.894	1.934	0.740
10				
11	2.300	4.423	1.890	0.581
12				
13	1.900	5.905	2.211	0.785
14				
15	2.900	3.282	1.297	0.532
Mean	2.350	4.147	1.968	0.878
S.D.	0.107	0.392	0.114	0.094

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