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Analysis of all stratum corneum lipids by automated multiple development high-performance thin-layer chromatography

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Abstract

An optimized gradient enabling the separation of all stratum corneum lipids by automated multiple development on HPTLC plates is presented. An initial isocratic step separates sebum lipids. This is followed by a 25-step development using a gradient with a polarity range of methanol–water to hexane. Application to in-vivo extracted and isolated stratum corneum lipids demonstrates the possible quantification of the lipid classes with a "one-experiment" separation.

1. Introduction

The outermost layer of the skin, called the stratum corneum, consists of corneocytes embedded in a lipid-rich matrix [1]. Stratum corneum lipids, excreted during differentiation of the epidermis, are the main determinants of skin permeability and also prevent water loss [2–4]. Depending on the body region, the composition of these lipids may slightly vary [5]. Stratum corneum lipids are a complex mixture of polar and non-polar lipids, i.e phospholipids, cholesterol sulfate, cholesterol, glucosylceramides, ceramides and sterol esters. Sebaceous lipids, which are widespread at the skin surface, are mainly composed of triglycerides, wax esters, squalene and free fatty acids [6,7]. Standards corresponding to these classes have been chosen to determine the migration zones of the different classes and their quantification. Up to now, bidimensional or unidimensional high-performance thin-layer chromatography (HPTLC) using consecutive solvent systems have been used for lipid analysis ex vivo or in vitro [8–12].

In this paper, we present an optimized automated multiple development (AMD) technique applied to HPTLC of stratum corneum lipids. The main advantages of this recent technique are that it is fully automated and it avoids band broadening during migration due to a band reconcentration effect [13]. Optimization of the AMD-HPTLC experimental conditions for stratum corneum lipids led to their "one-experiment" separation with high resolution. The method has been used on isolated stratum corneum and on in-vivo extracted lipids.

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2. Experimental

2.1. Standards

Sphingomyelin (SM), phosphatidylcholine (PC), glucosylceramides (Glc-CER), cholesterol sulfate (CS), Type IV ceramide (CER IV), type III ceramide (CER III), cholesterol (CH), triolein (TG), oleic acid (FFA), oleylstearate (WE), cholesteryl oleate (SE) and squalene (SQ) were all purchased from Sigma (St. Quentin Fallavier, France). SM, PC, CER IV and CER III are of bovine origin; Glc-CER of human origin and the others of synthetic origin. Unless otherwise stated, all other chemicals are from Merck (Nogent, France) and are of the highest purity available. Standards were prepared in hexane–methanol (2:3, v/v) at 1 mg ml^{-1}.

2.2. Chromatographic conditions

Chromatography was performed on silica gel HPTLC plates (60F254 ref. 5642, Merck), 10 × 20 cm. All solvents were HPLC quality (Merck). An automated TLC sampler III ATS3 (Camag, Basel, Switzerland) was used to apply standards as 8-mm bands, 8 mm from the bottom side of the plate using approximately 0.2, 0.4, 0.5, or 1 μg of each standard, band velocity 10 mm/s, dosing speed 200 nl/s. These application parameters were identical for all analyses performed.

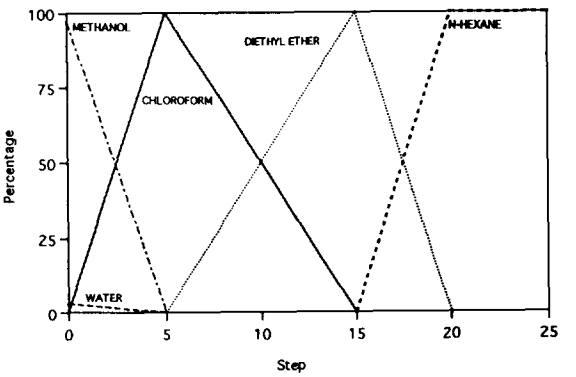

Fig. 1. Optimized gradient solvent composition for each step.

Plates were developed using a Camag AMD instrument with the solvent system described below which was developed for these biological studies. Prior to development, plates were activated at 110°C for 24 h then prewashed twice with chloroform–methanol (2:1, v/v) before use.

A initial isocratic step was used to separate sebum lipids from stratum corneum-specific lipids. The solvent was then purged using the "empty mixer" function. A gradient solvent system and 25 steps were used for each development (Table 1 and Fig. 1). For each step, the migration chamber was preconditioned once with air for 15 s. The development times for each step determining the migration distance are described in Fig. 2.

Table 1
Chromatographic conditions for AMD separation describing the composition of each bottle for each step

Starting with step No.	Isocratic step[a]	1	2	6	11	16	20
Use bottle No.	1	2	3	4	5	6	6
Toluene	100						
Methanol-water (97:3)		100					
Chloroform			100	50			
Diethyl ether				50	100		
n-Hexane						100	100
Acetic acid glacial			1	1	1		
Drying time (min)	20	4	4	4	4	4	4
Wash bottle	Air	Air	Air	Air	Air	Air	Air

[a] This is followed by "empty mixer".

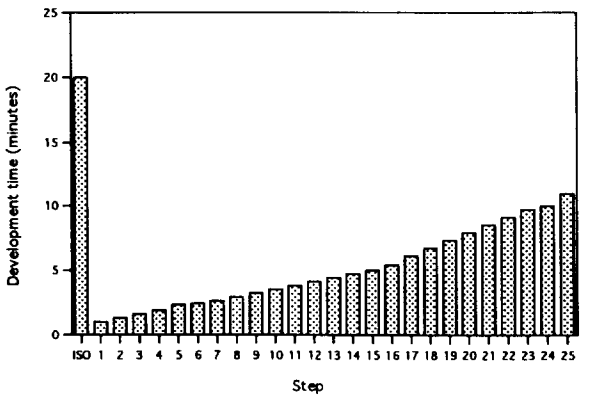

Fig. 2. Development time determining the running distance for each step.

2.3. Detection of each lipid class

After chromatography, the plates were sprayed with 10% (w/v) cupric sulfate pentahydrate in an 8% (v/v) *o*-phosphoric acid solution and heated for 10 min at 110°C.

2.4. Quantification

After spraying, the lipids on the HPTLC plates were quantified with a TLC2 scanner and Camag TLC-software 3 (Camag, Basel, Switzerland) under the following conditions: slit width 0.4×8 mm, wavelength 450 nm, photomode reflection, scan mode. The sensitivity of the photomultiplier was 208 (arbitrary units, Camag) and the attenuation 7.

2.5. Preparation of biological extracts

Isolated stratum corneum

Skin samples from plastic surgery (mammary, $n = 4$, mean age 25 ± 10) were rinsed with PBS and cut into 1.5×4.5 cm fragments, the dermis being removed by microdissection. After 72 h of incubation in 0.25% (w/v) trypsin solution, the epidermis was removed. Isolated stratum corneum was extensively rinsed with PBS, transferred onto a glass plate and dried to constant weight at 24°C. Dried pooled mammary stratum corneum (3–5 mg dry weight) was extracted three times with 5 ml of hexane–methanol (2:3, v/v) in an ultrasonic bath for 15 min. Supernatants were filtered through a 0.45-μm Millex SR filter (Millipore, St. Quentin, France) and dried under nitrogen. Lipids were dissolved in 50 μl of hexane–methanol (2:3, v/v), and 2 μl were applied to HPTLC plates.

In-vivo extraction of stratum corneum lipids

Healthy volunteers (caucasians males, $n = 7$, mean age 35.4 ± 6) were selected for the study. As already described, since a seasonal influence was possible, all extractions were performed on untanned skin during the same week of May [14].

Before extraction, the surface of the inner forearm was cleaned with a paper towel soaked in 30% ethanol. A glass adaptor, open at both ends, surface 3.14 cm^2, was pressed on the skin and filled with 5 ml of non-irritating solvent to extract stratum corneum lipids. After 1 min of contact and gentle stirring, the solvent was transferred to a glass tube. The extraction was done three times at three close surfaces on the inner forearm and the extracts (9 per volunteer) were combined and dried under a stream of nitrogen (technique modified from Serizawa et al. [11]). Lipids were dissolved in 50 μl of hexane–methanol (2:3, v/v) and 5 μl were applied on HPTLC plates.

Choice of solvent for in-vivo lipid extraction

Hexane–methanol (2:3, v/v) was determined to be the most suitable solvent for extraction of stratum corneum lipids, especially for palm or flexor upper arm. However exhaustive lipid extraction in vivo with this solvent mixture was impossible on the inner forearm due to strong irritation, as previously observed by Serizawa et al. [11]. A non-irritating solvent mixture consisting of cyclohexane–ethanol (2:8, v/v) was proposed, which gave similar results without irritation (data not shown), allowing three successive in-vivo extractions.

Table 2
Linear regressions for all standards

Lipid	Range (μg)	Equation	r
Sphingomyelin	0–0.3	$y = 106.81x - 1.38$	0.9945
Phosphatidylcholine	0–1	$y = 187.52x + 19.74$	0.9936
Glycosylceramides	0–1	$y = 85.16x + 4.69$	0.9949
Cholesterol 3 sulfate	0–0.5	$y = 88.46x + 6.61$	0.9845
Type IV ceramide	0–1	$y = 71.31x + 0.29$	0.9918
Type III ceramide	0–1	$y = 56.56x + 17.42$	0.9929
Cholesterol	0–0.5	$y = 87.26x + 20.01$	0.986
Triglycerides	0–0.5	$y = 78.18x + 12.19$	0.9809
Free fatty acids	0–0.3	$y = 852x + 69.3$	0.9965
Wax esters	0–1	$y = 87.74x + 72.28$	0.9933
Sterol esters	0–0.5	$y = 898.14x + 57.88$	0.9977
Squalene	0–1	$y = 555.81x + 246.66$	0.9928

y: area (mV × mm); x: applied quantity of lipid on the plate (μg); r: correlation coefficient; $n = 5$.

3. Results and discussion

The lipids of the sebum and stratum corneum, ranging from phospholipids to squalene (an hydrocarbon), exhibit a very wide range of polarity. In this paper we present the application of an AMD-HPTLC procedure for the analysis of these compounds.

The separation of all standards representative of the different classes of skin lipids is demonstrated by cochromatography of these lipids separately and as a mixture. A linearity study was performed on all the standards to evaluate the possibility to quantify the lipids. For all these compounds a linear relationship was observed between the integrated area and the amount of compound applied over the range 0–300 ng (SM, FFA), 0–500 ng (CS, CH, TG, SE), and 0–1000 ng (PC, Glc-CER, CER IV, CER III, WE, SQ). The regression equations of the calibration curves are shown in Table 2. The equation that gave the closest fit under the conditions employed for the standards is of the type $y = ax + b$. When the lipids were scanned repeatedly, the variation coefficients (reproducibility of applications and detection) for all these compounds

Table 3
In-vivo and isolated mammary stratum corneum lipid composition

Lipid	Mammary		In-vivo stratum corneum	
	μg/mg isolated stratum corneum	% total lipids	μg/cm²	% total lipids
Phospholipids	19.3	12.6	4.6	7
Glycosylceramides	ND	ND	ND	ND
Cholesterol 3 sulfate	1.9	1.2	1.1	1.7
Ceramides	29.8	19.5	11.6	17.9
Cholesterol	20.7	13.5	3.8	5.9
Triglycerides/free fatty acids	24.7	16.1	4.4	6.8
Wax esters	16.8	11	30.7	47.3
Sterol esters	12.4	8.1	4.2	6.5
Squalene	27.6	18	4.5	6.9

ND: none detected

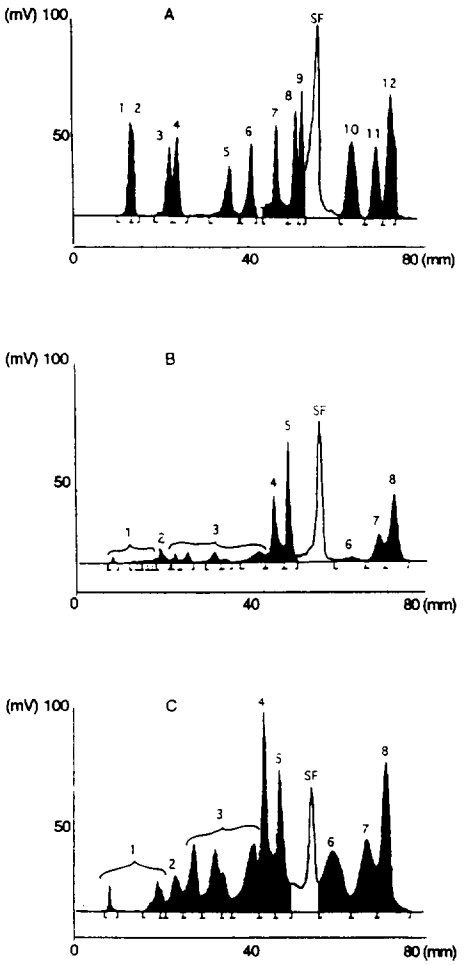

Fig. 3. Scanning profiles of the lipid standards mixture, isolated mammary and in-vivo extracted lipids from stratum corneum. SF = final solvent front. (A) lipid standards mixture; 1 = sphingomyelin, 2 = phosphatidylcholine, 3 = glycosylceramides, 4 = cholesterol 3-sulfate, 5 = type IV ceramide, 6 = type III ceramide, 7 = cholesterol, 8 = oleic acid, 9 = triolein, 10 = oleyl stearate, 11 = cholesteryl oleate, 12 = squalene. (B) Isolated mammary stratum corneum lipids, 1 = phospholipids, 2 = cholesterol 3 sulfate, 3 = ceramides, 4 = cholesterol, 5 = free fatty acids - triglycerides, 6 = wax esters, 7 = sterol esters, 8 = squalene, (C) In-vivo stratum corneum lipids, peak identification as for (B).

were between 1.8 and 10%. As shown in Fig. 3, AMD gradient optimization was carried out for in-vivo extracted and isolated stratum corneum lipids of mammary origin. The solvent gradient used separates the different lipid classes with high resolution. Previously, de la Vigne et al. [15] described the separation of phospholipids by AMD using a methanol–methylene chloride–hexane gradient under alkaline conditions allowing densitometric quantification. The six-solvent gradient system associated with air conditioning described here permits the separation of lipids with a wide range of polarity, together with efficient separation and densitometric quantification of the phospholipid classes. One important feature of the method presented here is the use of a separation process based on a gradient-development mode instead of a time-consuming and less accurate multi-development mode, as classically described [8–12]. Table 3 gives the lipid contents for in-vivo extracted and isolated stratum corneum lipids. All classes usually found were extracted with the non-irritating extraction solvent system proposed for the in-vivo experiments.

Furthermore, when expressed as percentage of the total lipids, the results are of the same order of magnitude as those described in the literature, keeping in mind that the values are always related to a human test-group with specific lifestyle and environmental conditions. One should also bear in mind that age, body site, gender, steroids and UV have been described as possible factors influencing the composition of skin lipids [16,17].

In conclusion, the AMD approach and the non-irritating extraction solvent mixture proposed are suitable for the analysis of surface and horny layer lipids in vivo or in vitro. Moreover this method enhances the in-vivo screening possibilities of chemicals or environmental stress on skin-lipid variations.

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