Journal of Chromatography A, 1378 (2015) 8–18

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Analysis of 1,2-diol diesters in vernix caseosa by high-performance liquid chromatography – atmospheric pressure chemical ionization mass spectrometry

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ARTICLE INFO

Article history:
Received 9 September 2014
Received in revised form 4 November 2014
Accepted 27 November 2014
Available online 4 December 2014

Keywords:
Skin lipids
Neutral lipids
Lipidomics
Mass spectrometry
Double-bond position

ABSTRACT

Fatty acid diesters of long-chain 1,2-diols (1,2-DDE), or type II wax diesters, were analyzed in the vernix caseosa of a newborn girl. 1,2-DDE were isolated from the total lipid extract by the semipreparative TLC using plates coated with silica gel. Chromatographic separation of the 1,2-DDE molecular species was achieved on the non-aqueous reversed-phase HPLC with two Nova-Pak C18 columns connected in series (a total length of 45 cm) and using an acetonitrile–ethyl acetate gradient. 1,2-DDE eluted from the column in the order of their equivalent chain number. The analytes were detected as ammonium adducts by an ion-trap mass spectrometer equipped with an atmospheric pressure chemical ionization source. Their structures were elucidated using tandem mass spectrometry with MS, MS^2 and MS^3 steps in a data-dependent mode. More than two thousand molecular species of 1,2-DDE were identified in 141 chromatographic peaks. The most abundant 1,2-DDE were monounsaturated lipids consisting of a C22 diol and a C18:1 fatty acid together with C16:0, C14:0 or C15:0 fatty acids. The positions of double bonds were characterized by the fragmentation of $[M+C_3H_5N]^{+\bullet}$ formed in the ion source.

1. Introduction

Fatty acid diesters of long-chain 1,2-diols (1,2-DDE), or type II wax diesters, are condensation products of two fatty acid molecules with a long-chain 1,2-diol. These lipids are widely associated with the skin of mammals; they are frequently found in sebum, a species-specific mixture of relatively neutral lipids synthesized de novo by sebaceous glands [1]. The first reports on 1,2-DDE appeared in the literature in the 1960s [2–5], about a decade after the structural characterization of 1,2-diols in wool wax [6,7]. Since then, 1,2-DDE have been identified in the skin surface lipids of many mammalian species. 1,2-DDE have been detected in the skin of rodents such as the mouse, rat, guinea pig, golden Syrian hamster or gerbil [2,5,8–10], as an abundant lipid class forming 14–61% of the total lipids. The ventral gland secretion of a male dwarf hamster was found to contain 1,2-diols and their monopentanoates [11]. An analysis of the rat 1,2-DDE has shown that 1,2-diols (having the chiral center on carbon 2) exist in the D form [12]. Further experiments with the lipase hydrolysis of 1,2-DDE from the golden Syrian hamster and the mouse have revealed that fatty acids in the positions 1 and 2 are not distributed randomly [13]. Canine skin lipids were reported to comprise 32% of the 1,2-DDE constituting branched diols esterified with long-chain fatty acid and isovaleric acid [14]. The skin of cows was found to produce 8% of diol diesters [15]. In primates, 1,2-DDE formed 21% of the skin lipids of the baboon [8]. Two types of 1,2-DDE were found in the skin lipids of the macaque: the less abundant type (17%) with two long-chain fatty acids and the second type (40%) with short branched-chain acids (mostly isovaleric acid) in the position 1 of the diol [16]. Concerning the human species, 1,2-DDE are almost missing in the adult skin [17], but they are produced in the early stages of skin development. 1,2-DDE are present in vernix caseosa,

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http://dx.doi.org/10.1016/j.chroma.2014.11.075
0021-9673/

a uniquely human proteolipid film coating the skin of the fetus in the last trimester of pregnancy and protecting the embryonic skin from amniotic water. Vernix caseosa has remarkable hydration, waterproofing, anti-infective, antioxidant and wound-healing properties [18]. 1,2-DDE constitute 3–9% of the total lipids in vernix caseosa [3,19]. Although vernix caseosa diols have been found to be mostly methyl-branched (*iso*, *anteiso*) and saturated straight-chain 1,2-diols with 20–25 carbons [3,4], shorter chains (C14–C19) have been detected as well [5]. Fatty acyls have been identified as saturated straight-chain or methyl-branched (*iso*, *anteiso*) and unsaturated straight-chain, mostly monoenic. The most abundant saturated chains appeared to contain 16 carbons (both straight and *iso*-methyl branched chains) and the predominant unsaturated acids were C18:1*n*-7 and C18:1*n*-9 [19]. The biosynthesis of human 1,2-DDE was found to involve catalysis by acyl CoA:diacylglycerol acyltansferase DGAT1, an enzyme highly expressed in the skin [20]. 1,2-DDE are rare in other biotas. They have been reported in uropygial (preen) gland secretions of some birds [21–23], where isomeric 2,3-DDE are usually found. In the plant kingdom, long-chain 1,2-diols have been discovered in the skin wax of apples [24] and, together with their monoacetates, in the cuticular wax of Mexican aster petals [25]. Alkane-1,2-diol-based glycolipids have been found in hot spring microbial mats [26,27].

1,2-Diols and 1,2-DDE are utilized in cosmetic and pharmaceutical products. Non-esterified 1,2-diols are frequently used as skin and hair conditioning agents, viscosity- and foam-increasing agents [28–30]. They have bacteriostatic activities against a broad range of strains, which makes them useful as a treatment of skin diseases caused by bacteria, such as acne [31–33], and they are also suggested as a prophylaxis and/or treatment of fungal skin infections [34]. Recently, 1,2-diols have been patented as antispasmodics to relieve the spasms associated with pain [35], and they can also serve for controlling underarm and foot odor [31]. Esters of 1,2-diols are used less frequently. Monoesters of 1,2-diols can be used for the treatment of acne or seborrheic dermatitis [36]. 1,2-DDE have been suggested as edible fat with substantially fewer calories than commonly used triacylglycerols [37]. Mixtures of mono- and diesters of propane-1,2-diol are utilized as emulsifiers and aerating agents for bakery products (food additive E477).

Taking into account the large variability of the fatty acids biosynthesized by the mammal skin, the total number of 1,2-DDE might be enormous. The theoretical number of the 1,2-DDE that can be formed from N diols and n fatty acids equals $N \times n^2$. We have reported previously that vernix caseosa lipids contain at least 167 different fatty acids [38], which, even for a low number of diols, gives 10^4–10^5 possible combinations. The complexity of 1,2-DDE thus compares with extremely rich mixtures of triacylglycerols (where the number of theoretically formed species equals n^3). Not surprisingly, a comprehensive characterization of 1,2-DDE mixtures at the level of intact molecular species has not been published so far. 1,2-DDE with very short-chain diols were earlier analyzed by electron ionization MS [39], but the method is not applicable for mixtures and lipids with long-chain diols. Previously published methods for skin 1,2-DDE mostly relied on saponification or transesterification, i.e. procedures that release fatty acids and alcohols. Fatty acids and diols released from 1,2-DDE were investigated using GC, often preceded by various chemical derivatizations including the formation of trimethylsilyl, acetyl, isopropylidene or acetonide derivatives, hydrogenation or oxidation [4,19,40]. Although these approaches have enabled the structural characterization of fatty-acid and diol building blocks, the structures of intact 1,2-DDE have not been disclosed yet.

In this work, we analyzed the 1,2-DDE of vernix caseosa using non-aqueous reversed-phase HPLC/APCI-tandem MS. The method was carefully optimized to achieve good chromatographic resolution and obtain reliable information on the molecular species structure. More than 2000 molecular species have been identified and their retention behavior has been studied.

2. Experimental

2.1. Sample collection

The vernix caseosa sample (1.0 g) was collected from the skin of a full-term healthy female neonate immediately after spontaneous vaginal delivery and stored at −25 °C in an amber glass vial. The sample was collected with informed parental consent and the work was approved by the Ethics Committee of the General University Hospital, Prague (910/09 S-IV); the study was performed according to the Declaration of Helsinki.

2.2. The isolation of 1,2-DDE

The sample was suspended in 50 mL of chloroform:methanol (2:1, v/v) with 0.05% of butylated hydroxytoluene (2,6-bis(1,1-dimethylethyl)-4-methylphenol; an antioxidant). The suspension was cleared of epithelial cells by filtration through a column containing purified cotton wool and silica gel (60–120 μm, ca 0.2 g). Anhydrous magnesium sulfate (ca 5 g) was added to absorb water, and the suspension was filtered again. The solvents were removed by a rotary evaporator (35 °C, 170 mbar) and a stream of argon, yielding 78 mg of the total lipid extract. The extract (ca 20 mg) was separated on 9 cm × 12 cm glass TLC plates coated with silica gel using hexane:diethyl ether (93:7, v/v) as a mobile phase. Each plate was developed twice to focus the zones (in the first step to 3/4 of the plate height and then, after air-drying, to the top). The zones were visualized under UV light after being sprayed with rhodamine 6G (0.05% in ethanol). The zone corresponding to 1,2-DDE (R_F = 0.46–0.52) was scraped off the plate into a small glass column with purified cotton wool and silica gel; lipids were eluted with diethyl ether. The solvent was evaporated under a stream of argon; the residues were dissolved in chloroform:methanol (2:1, v/v; 10 mg/mL) and stored at −25 °C in the dark. Prior to HPLC analysis, the sample was diluted by acetonitrile:chloroform (10:9, v/v) to a concentration of 1 mg/mL (compound identification) or concentrated to a concentration of 25 mg/mL (the localization of double bonds).

2.3. Chemical synthesis of standards

Fatty acid chloride (or an equimolar mixture of fatty acid chlorides) was added dropwise to a stirred solution of alkane-1,2-diol in 10 mL of anhydrous pyridine at 0 °C. After stirring for 72 h at ambient temperature, the mixture was diluted with diethyl ether (100 mL), washed with 10% hydrochloric acid (2× 100 mL), a saturated solution of sodium hydrogen carbonate (2× 100 mL), brine (100 mL), and dried over sodium sulfate. The solvent was evaporated in vacuo and the oily residue was purified by flash chromatography (150 g of Merck Kiesegel 60; the mobile phase hexane:ethyl acetate (95:5, v/v)) to give 1,2-DDE. The reactant weights and the reaction yields are specified in the Supplementary Information.

2.4. Chemicals

Acetonitrile, ethyl acetate and methanol (purity: for MS, Sigma–Aldrich, St. Louis, MO, USA) were used as received; the other solvents (chloroform, hexane, dichloromethane; all from Penta, Czech Republic) were distilled in glass from analytical-grade solvents. Ammonium formate (Fluka, Buchs, Switzerland), magnesium sulfate (Sigma–Aldrich), rhodamine 6G (Sigma–Aldrich) and