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Short communication

Enzymatic diagnosis of Sjögren-Larsson syndrome using electrospray ionization mass spectrometry

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ABSTRACT

Background: Sjögren-Larsson syndrome is a metabolic disorder characterized by accumulation of long-chain fatty alcohols in plasma of patients due to mutations in the *ALDH3A2* gene, that codes for a microsomal fatty aldehyde dehydrogenase (FALDH). Recent studies have demonstrated that FALDH is involved in the last step of the conversion of 22-hydroxy-C22:0 into the dicarboxylic acid of C22:0 (C22:0-DCA).
Methods: FALDH activity was determined by incubating fibroblast homogenates with ω-hydroxy-C22:0 in the presence of NAD^+. Electrospray ionization mass spectrometry (ESI-MS) was used to quantify the amounts of C22:0-DCA produced.
Results: All SLS patients were deficient in C22:0-DCA productions with activities ranging from 3.2–26.3% of mean control.
Conclusions: The new assay described in this paper has substantial advantages over previous assays, and allows for the easy, reliable and rapid diagnosis of SLS.

1. Introduction

Sjögren-Larsson syndrome (SLS; MIM#270200) is an autosomal recessive metabolic disorder that is characterized by congenital ichthyosis, mental retardation and spastic diplegia or tetraplegia [1,2]. SLS is caused by mutations in the *ALDH3A2* gene that encodes the fatty aldehyde dehydrogenase (FALDH; EC 1.2.1.48), which is part of the microsomal alcohol NAD^+-oxidoreductase complex (FAO) [3–5]. At present, more than 70 mutations have been reported in SLS patients, including deletions, insertions, missense mutations, splicing defects and complex gene rearrangements [6]. The FALDH protein is a member of a large aldehyde dehydrogenase family encoded by 19 distinct genes in man [7]. FALDH catalyzes the NAD^+-dependent oxidation of saturated and unsaturated aliphatic aldehydes ranging from 6 to 24 carbons [8]. Furthermore, FALDH is also involved in the oxidation of leukotriene B4, fatty aldehydes derived from glycerolipids, and branched chain aliphatic aldehydes, including 2-methyl-undecanal, 3,7,11-trimethyl-dodecanal as well as phytal and phytenal [8–10]. A deficiency of FALDH in SLS patients leads to the accumulation of long-chain aliphatic alcohols in plasma [2,11]. It remains unresolved why a deficiency in the fatty aldehyde dehydrogenase leads to an accumulation of the fatty alcohol instead of its corresponding fatty aldehyde. Possible explanations for this are: instability of the fatty alcohol dehydrogenase in the absence of the fatty aldehyde dehydrogenase, or inhibition of the fatty alcohol dehydrogenase due to accumulation of fatty aldehydes either directly or via a feedback mechanism [12]. SLS patients also excrete high levels of leukotriene B4, a pro-inflammatory cytokine, that may play a role in the characteristic ichthyosis that is observed in these patients [10].

Our previous studies demonstrated that (very-)long-chain fatty acids can be converted into ω-hydroxy fatty acids by cytochrome P450 enzymes in the endoplasmic reticulum [13,14]. In humans, these products can be oxidized further into dicarboxylic acids via microsomal alcohol- and aldehyde dehydrogenases and/or by one or more cytochrome P450 enzymes [15,16]. We also identified FALDH as the major enzyme involved in the omega-oxidation of (V)LCFAs [16]. Indeed, when fibroblast homogenates from control subjects were incubated with 22-hydroxy-docosanoic acid (ω-hydroxy-C22:0), the corresponding dicarboxylic acid of C22:0 (C22:0-DCA) was readily produced, whereas it was deficient in fibroblast homogenates from patients with SLS (Fig. 1). These observations led us to develop a novel diagnostic assay

Abbreviations: ω-hydroxy-C22:0, 22-hydroxy-docosanoic acid; ω-oxo-C22:0, 22-oxo-docosanoic acid; C22:0-DCA, docosanedioic acid; FALDH, fatty aldehyde dehydrogenase; SLS, Sjögren-Larsson syndrome.

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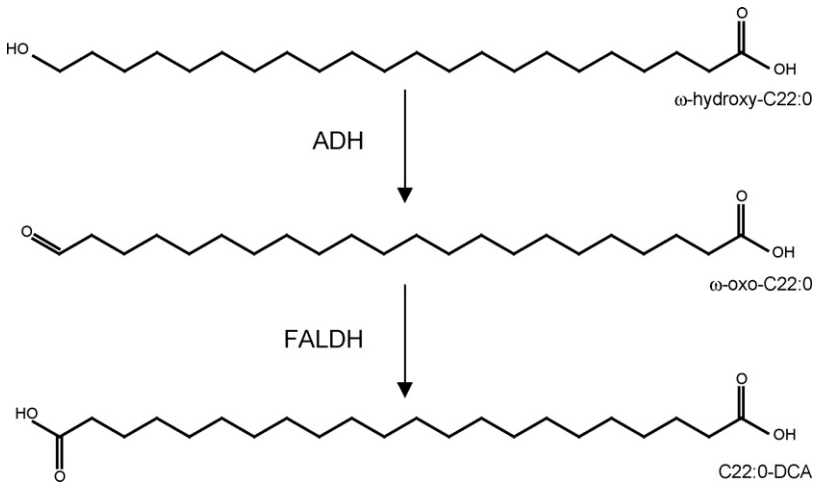

Fig. 1. Scheme of the enzymatic conversion of ω-hydroxy-C22:0 to C22:0-DCA. The first step in which ω-hydroxy-C22:0 is oxidized to ω-oxo-C22:0 involves a yet unknown alcohol dehydrogenase (ADH). Fatty aldehyde dehydrogenase (FALDH) catalyzes the conversion of ω-oxo-C22:0 to C22:0-DCA in the second reaction step.

for Sjögren-Larsson syndrome on the basis of a deficiency of ω-hydroxy-C22:0 degradation, as described in this paper. The assay was optimized for human skin fibroblast homogenates, which were incubated with ω-hydroxy-C22:0 in the presence of NAD^+, and activity was assessed by quantification of the amount of C22:0-DCA produced in the reaction mixture using electrospray ionization mass spectrometry (ESI-MS). The assay proved to be sensitive, reliable and rapid for diagnosis of SLS patients. This is of interest since previously described assays have several drawbacks, including non-commercially available or radioactive substrates, high residual activities in SLS patients with nonsense mutations in the *ALDH3A2* gene and time-consuming sample preparation and analysis by GC–MS [4,17,18].

2. Materials and methods

2.1. Materials

HEPES, calf serum, penicillin, streptomycin and Nutrient Ham's F-10 were purchased from Gibco (Invitrogen, Merelbeke, Belgium). 22-Hydroxy-docosanoic acid (ω-hydroxy-C22:0) and hexacosanedioic acid were obtained from Larodan Fine Chemicals (Malmö, Sweden). $^2H_4$-C22:0 was purchased from Dr. H.J. ten Brink, Free University Hospital, Amsterdam, The Netherlands. NAD was obtained from Roche (Roche Applied Science, The Netherlands). All other chemicals used were of analytical grade.

2.2. Cultured skin fibroblasts

Fibroblasts were cultured in Nutrient Ham's F-10 supplemented with 10% fetal calf serum, L-glutamine (2 mmol/L), penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37 °C under 5% CO_2. Primary human skin fibroblast cell lines were obtained from healthy subjects and patients with SLS. Cells were harvested using trypsin, washed twice with phosphate buffered saline (PBS), once with 0.9% NaCl, and stored as cell pellets at −80 °C. The FALDH-deficient fibroblasts were from SLS patients as concluded from the clinical history, biochemical and molecular methods including the identification of mutations in the *ALDH3A2* gene [2]. All cell lines used were with passage number below 20.

2.3. FALDH activity measurement including the analysis of the reaction products by ESI-MS

Fibroblast pellets were resuspended in phosphate buffered saline (PBS) and homogenized by sonication (two cycles of 10 s at 8 W) on ice. Protein concentration was determined with the bicinchoninic acid assay using bovine serum albumin as standard [19].

The reaction mixture contained 40 μg protein, 100 mmol/L HEPES, 100 mmol/L glycine, 1 mmol/L NAD^+, 1 mg/mL α-cyclodextrin to solubilize ω-hydroxy-C22:0 [12] and 100 μmol/L ω-hydroxy-C22:0 in a total volume of 200 μL at a final pH of 9.7. Incubations were performed at 37 °C and initiated by addition of the substrate. After 60 min, reactions were terminated by addition of 1 mL hydrochloric acid to a final concentration of 1.7 mol/L. Next, 100 μL internal standard solution was added that contained 65 μmol/L $^2H_4$-C22:0 in toluene. After addition of 2 mL hexane, the samples were vortex-mixed thoroughly for 30 s and about 1.5 mL of the upper phase was transferred to a glass tube and hexane was evaporated at 37 °C under a constant stream of nitrogen. Finally, the residue was dissolved in 100 μL chloroform–methanol–water (50:45:5, v/v/v) containing 0.01% of a 25% aqueous ammonia solution and transferred to autosampler vials (Gilson, Middleton, WI). The samples were analyzed on a Quattro II triple-quadrupole mass spectrometer (Micromass, Manchester, United Kingdom), in negative ESI mode following the method described by Valianpour et al. [20]. The *m/z* for ω-hydroxy-C22:0, ω-oxo-C22:0, and C22:0-DCA are 355.5, 353.5, and 369,5, respectively. For calculation of C22:0-DCA concentrations, a five-point calibration curve was made for hexacosanedioic acid (C26:0-DCA, *m/z* 425.5) using $^2H_4$-C22:0 as internal standard.

2.4. Validation

To determine the variation coefficients of the FALDH enzyme assay, pellets from a pool of 10 different control cell lines were used. The individual primary human skin fibroblasts were cultured under the same conditions as described in the 'Cultured skin fibroblasts' section above. At the final wash step with 0.9% NaCl, cells were pooled, and stored as cell pellets at −80 °C. The intra-assay (within-day) variation was calculated from 10 independent experiments in triplicate at optimal enzyme assay conditions within the same day. To determine the inter-assay variation, FALDH activity was measured in 10 independent experiments at optimal assay conditions

performed on different days using the same pool of fibroblasts stored at −80 °C.

3. Results

3.1. Optimization of the assay in human skin fibroblasts

In order to study the conversion of ω-hydroxy-C22:0 into C22:0-DCA in human fibroblasts, fibroblast homogenates were incubated in a buffered reaction medium supplemented with NAD^+ and α-cyclodextrin at 37 °C. Reactions were started by adding ω-hydroxy-C22:0 and the metabolites were analyzed by ESI-MS. The effect of pH on the product formation was determined using a combined buffer system that contained 100 mmol/L HEPES and 100 mmol/L glycine to cover the pH range 6.6–10.5. Formation of C22:0-DCA was optimal at pH 9.7 (Fig. 2A). At this pH, production of C22:0-DCA was linear with protein up to 250 μg/mL, and linear with time up to 60 min (Fig. 2B and C). Enzyme activity using different concentrations of ω-hydroxy-C22:0 showed first order kinetics (Fig. 2D). The kinetic constants were calculated from the Michaelis–Menten plot (Fig. 2D insert), resulting in a Km of 4 μmol/L for ω-hydroxy-C22:0 and V_{max} of 65 pmol min^{-1} mg^{-1}. Based on the results obtained, the following standard protocol was used to measure the activity of FALDH in fibroblasts homogenates: incubations were carried out for 60 min at 37 °C in a total volume of 200 μL containing 40 μg protein, 100 mmol/L HEPES–glycine buffer (pH 9.7), 1 mmol/L NAD^+, 1 mg/mL α-cyclodextrin and 100 μmol/L ω-hydroxy-C22:0.

The intra-assay coefficient of variation (CV) was calculated from the activity of FALDH measured, i.e. production of C22:0-DCA, using the optimal conditions as defined above, in pellets derived from a pool of 10 fibroblast homogenates on a single day. The specific FALDH activity was 48.2 ± 4.2 pmol min^{-1} mg^{-1} (mean ± SD; range 41.4–53.7) which corresponds to an intra-assay CV value of 8.7%. The inter-assay (between day) CV was determined by measuring the activity of FALDH at optimal conditions in pellets derived from the same pool of cultured human skin fibroblast homogenates in 10 separate experiments performed on 10 subsequent days. A specific FALDH activity of 55.5 ± 8.5 pmol min^{-1} mg^{-1} (mean ± SD; range 47.3–74.0) was calculated, which corresponds to an inter-assay CV value of 15.3%.

Mass spectrometric analysis of the reaction mixture derived from incubations in which control fibroblasts homogenates were incubated with ω-hydroxy-C22:0, led to the identification of a peak of *m/z* 353 (data not shown), which corresponds to the ω-aldehyde intermediate of C22:0 (ω-oxo-C22:0, Fig. 1). Definitive characterization of this compound turned out to be difficult since ω-oxo-C22:0 is not commercially available. Limited formation of ω-oxo-C22:0 was observed at pH values up to pH 9.7 (Fig. 2A). However, at higher pH values C22:0-DCA formation decreased rapidly, whereas ω-oxo-C22:0 production was inversely increased.

3.2. Dehydrogenation activity in control fibroblasts and fibroblasts from SLS patients

The formation of C22:0-DCA from ω-hydroxy-C22:0 was measured in homogenates of 12 different control fibroblast cell lines and a specific activity of 62.5 ± 16.5 pmol min^{-1} mg^{-1} (mean ± SD; range 32.1–84.1) was calculated (Table 1). In fibroblast homogenates from SLS patients a specific activity of 7.8 pmol min^{-1} mg^{-1} (mean ± SD; range 2.0–16.4) was determined. An overview of the results of the individual SLS patients including the results of mutation analysis is listed in Table 1. All SLS patients were deficient in C22:0-DCA formation with residual FALDH activities ranging from 3.2 to 26.3% of mean control. The production of the presumed ω-oxo-C22:0 intermediate could be

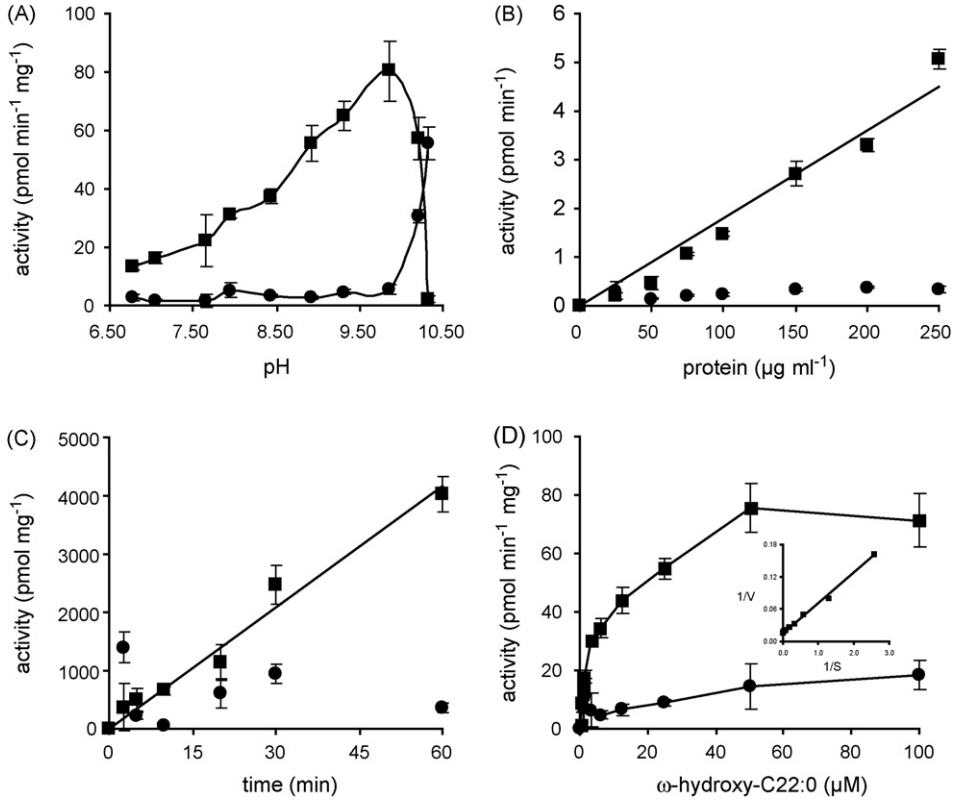

Fig. 2. Optimization of the FALDH assay for the conversion of ω-hydroxy-C22:0 to ω-oxo-C22:0 (●) and C22:0-DCA (■). Production of C22:0-DCA was measured as a function of pH (A), protein (B), and time (C), and ω-hydroxy-C22:0 concentration (D). Each data point represents the mean of three different experiments.

Table 1
ω-Hydroxy-C22:0 dehydrogenation activity in human skin fibroblast. The activity was measured in fibroblast homogenates from control subjects ($n = 12$) and patients with SLS ($n = 10$) as described.

	n	ω-Oxo-C22:0 (pmol min^{-1} mg^{-1})	C22:0-DCA (pmol min^{-1} mg^{-1})	*ALDH3A2* mutation			
				Allele 1		Allele 2	
Control	12	12.8 ± 5.1	62.5 ± 16.5[†]				
SLS patient 1		8.2	6.4	No detectable mRNA		No detectable mRNA	
SLS patient 2		33.0	7.2	c.80T > C	p.Leu27Pro	c.80T > C	p.Leu27Pro
SLS patient 3		13.4	4.0	c.738_39delTA	p.Tyr246fs	c.1297_1298delGA	p.Arg432fs
SLS patient 4		15.7	11.2	c.292C > T	p.Gln98X	c.551C > T	p.Thr184Met
SLS patient 5		11.1	2.0	c.1297_1298delGA	p.Arg432fs	c.943C > T	p.Pro315Ser
SLS patient 6		10.5	3.3	c.943C > T	p.Pro315Ser	c.25-50del	p.Val8fs
SLS patient 7		12.2	3.8	c.906delT	p.Ala301fs	c.906delT	p.Ala301fs
SLS patient 8		3.6	16.4	c.1384_87delGAAA	p.Lys461fs	c.1384_87delGAAA	p.Lys461fs
SLS patient 9		4.1	15.4	c.1297_1298delGA	p.Arg432fs	c.1297_1298delGA	p.Arg432fs
SLS patient 10		32.3	8.3	c.943C > T	p.Pro315Ser	c.943C > T	p.Pro315Ser
SLS average	10	14.4 ± 10.3	7.8 ± 5.1[†]				
SLS parent 1		10.1 ± 3.1	29 ± 2.6	c.1297_1298delGA	p.Arg432fs	-	
SLS parent 2		15.2 ± 3.0	27 ± 2.7	c.906delT	p.Ala301fs	-	

[†] $P < 0.001$, controls versus SLS patients.

detected in the reaction mixture of incubations with control and SLS fibroblasts. Formation of ω-oxo-C22:0 in control cell lines was 12.8 ± 5.1 pmol min^{-1} mg^{-1} (mean ± SD; range 6.2–21.5) and in SLS fibroblasts 14.4 ± 10.33 pmol min^{-1} mg^{-1} (mean ± SD; range 3.6–33.0).

Production of ω-oxo-C22:0 in fibroblast homogenates from SLS patients did not significantly differ from controls. However, the specific FALDH activity, i.e. formation of C22:0-DCA, in SLS fibroblast homogenates were significantly reduced compared to the activity in control fibroblast homogenates ($P < 0.001$).

In addition, enzyme activity was determined in fibroblasts from two parents from SLS patients thus being heterozygous for SLS (Table 1). The production of C22:0-DCA was considerably lowered compared to controls (approximately 45% of mean control).

4. Discussion

SLS is diagnosed by measuring FALDH enzyme activity in cultured fibroblast homogenates using fluorometric [4,8] or gas chromatography–mass spectrometry assays [18]. Alternatively, the diagnosis can also be established by histochemical staining for FAO activity in a fresh skin biopsy [21]. SLS can also be diagnosed using molecular methods by screening for common mutations in selected populations or by sequencing the entire gene. Due to the variety of different mutations in SLS, molecular analysis is usually only performed after initial enzymatic verification of SLS by demonstrating a deficient FALDH activity [6]. In all patients studied so far bona fide mutations have been identified in the *ALDH3A2* gene and no patients have been described with a deficiency of the alcohol dehydrogenase (ADH) enzyme. Since it is believed that FALDH might form a complex with the, as yet, unidentified ADH, identification of the latter enzyme may lead to insights in its possible role in SLS. Rational therapeutic approaches for treatment of SLS patients are focused on the metabolic abnormalities in SLS. Some therapeutic options could be beneficial for patients with SLS, including inhibition of LTB4 synthesis by Zileuton, which improves the disturbing pruritus, and stimulation of *ALDH3A2* gene transcription by bezafibrate, a PPAR agonist, to increase residual FALDH activity in fibroblasts from these patients with certain missense mutations [22,23].

The results of our previous experiments [16] in which it was established that FALDH is involved in the degradation of ω-hydroxy-C22:0 led to the generation of a novel assay for the biochemical diagnosis of SLS patients as described in the paper. As depicted in Fig. 1, degradation of ω-hydroxy-C22:0 is mediated via a two step mechanism. Conversion of ω-hydroxy-C22:0 into ω-oxo-C22:0, the aldehyde intermediate, is catalyzed by an as yet unknown alcohol dehydrogenase [4]. Subsequent oxidation of ω-oxo-C22:0 into C22:0-DCA is catalyzed predominantly by FALDH [16]. Although different assays for measuring FALDH activity have been described previously, our new assay has some significant advantages since it does not require radiolabeled- or custom synthesized substrates [4,17]. Moreover, reaction products are measured directly with ESI-MS and do not require derivatization prior to analysis [18]. The assay is based on the degradation of ω-hydroxy-C22:0 which allows easy discrimination between controls and Sjögren Larsson patients with residual FALDH activities in SLS fibroblasts. All SLS patients were deficient in C22:0-DCA formation with residual FALDH activities ranging from 3.2–26.3% of mean control. In contrast to the previously developed assays, the ESI-MS technique was not limited to the measurement of the final reaction step. The presumed ω-oxo-C22:0 intermediate could also be determined and therefore, our novel assay may be used to identify new patients with clinical features of SLS, which may be caused by a deficiency in the alcohol dehydrogenase component of the FAO complex without deficient FALDH activity.

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