Sjögren–Larsson syndrome: accumulation of free fatty alcohols in cultured fibroblasts and plasma

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Abstract **Sjögren–Larsson syndrome (SLS) is an inherited disorder associated with deficient oxidation of long-chain aliphatic alcohols. Previous studies have reported modest elevations in total (free + esterified) fatty alcohols in SLS, but free fatty alcohols have not been selectively measured, in part because of their low concentrations in most tissues and the presence of trace fatty alcohol contaminants in some solvents used for their analysis. We adapted methods to measure free fatty alcohols in cultured cells and plasma that minimize exogenous alcohol contamination. Fatty alcohols were analyzed as acetate derivatives, using capillary column gas chromatography. By this method, cultured skin fibroblasts from SLS patients were found to have 7- and 8-fold elevations in the mean content of hexadecanol (16:0-OH) and octadecanol (18:0-OH), respectively. The mean plasma 16:0-OH and 18:0-OH concentrations in SLS patients (n = 11) were 9- and 22-fold higher than in normal controls, respectively. In SLS fibroblasts, most of the fatty alcohol (59%) that accumulated was free rather than esterified alcohol, whereas free alcohol accounted for 23% of the total alcohol in normal cells. These results indicate that elevations in free fatty alcohols provide a sensitive marker for the enzymatic defect in SLS. The ability to measure free fatty alcohols in cultured cells and plasma should prove useful for investigations of normal fatty alcohol metabolism and the deranged metabolism in SLS.**—Rizzo, W. B., and D. A. Craft. **Sjörgren–Larsson syndrome: accumulation of free fatty alcohols in cultured fibroblasts and plasma.** ***J. Lipid Res.*** **2000.** 41: **1077–1081.**

Supplementary key words metabolic disease • ichthyosis • mental retardation • spasticity • wax esters • ether lipids

Aliphatic alcohols that are 16–20 carbons long have been detected in a variety of mammalian tissues (1). They constitute a minor portion of the lipid composition, typically accounting for less than 0.01% of the total lipid mass (2–4). Because of their hydrophobic nature, fatty alcohols readily partition into biological membranes (5, 6) and serve as precursors for the biosynthesis of wax ester lipids and ether glycerolipids (1).

The medical importance of long-chain alcohols has been heightened by the identification of several inborn errors of fatty alcohol metabolism in humans (7). One of these, Sjögren–Larsson syndrome (SLS), is characterized by a genetic block in the oxidation of fatty alcohol to fatty acid because of deficient activity of fatty aldehyde dehydrogenase (FALDH) (8), a component of the fatty alcohol:NAD oxidoreductase enzyme complex (9). SLS patients have debilitating neurologic and cutaneous symptoms, including mental retardation, spasticity, and ichthyosis. Other diseases are associated with defects in the incorporation of fatty alcohol into ether glycerolipids (7).

To investigate normal fatty alcohol metabolism and understand the biochemical abnormality in SLS, it is essential to monitor the free fatty alcohol content of cultured cells and affected tissues. However, measurement of free fatty alcohols in small tissue or cell samples is complicated by the low concentration of these lipids and the presence of trace fatty alcohol contaminants in some solvents used for their analysis. Several studies have reported that total (free + esterified) hexadecanol (16:0-OH) and octadecanol (18:0-OH) were increased 2- to 3-fold in plasma of SLS patients (10) and in FALDH-deficient cells (9, 11), but free fatty alcohols were not determined and the possible contribution of alcohol contaminants was not considered.

We describe a method to reliably measure the free fatty alcohol content of small biological samples, and apply this method to demonstrate fatty alcohol accumulation in cultured cells and plasma from SLS patients.

MATERIALS AND METHODS

Chemicals and supplies

All solvents and water used for fatty alcohol measurements were high-performance liquid chromatography (HPLC) grade (Mallinkrodt, St. Louis, MO) except as noted. Reagent-grade diethyl ether (99%) was obtained from Aldrich (Milwaukee, WI). Pyridine and acetic anhydride were analytical-grade reagents (Mallinkrodt). Channeled silica gel thin-layer chromatography

Abbreviations: FALDH, fatty aldehyde dehydrogenase; SLS, Sjögren–Larsson syndrome; 14:0-OH, tetradecanol; 15:0-OH, pentadecanol; 16:0-OH, hexadecanol; 18:0-OH, octadecanol; 18:1-OH, octadecenol.

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(TLC) plates (LK6D) with a preadsorbent layer were obtained from Whatman (Clifton, NJ). Three-milliliter disposable silica gel columns (Bakerbond) containing 1 mL of packed silica gel were made by J. T. Baker (Phillipsburg, NJ). Pentadecanol and fatty alcohol standards were obtained from Sigma (St. Louis, MO). [1-^{14}C]stearic acid (56 mCi/mmol) was obtained from Amersham (Arlington Heights, IL) and used to synthesize radioactive octadecanol as described (12).

Fibroblast and plasma specimens

Cultured skin fibroblasts were grown in 75-cm^2 culture flasks with Dulbecco's minimal essential medium containing 10% fetal bovine serum, penicillin, and streptomycin at 37°C in an atmosphere of 5% CO_2. The cells were collected by trypsinization and washed twice with phosphate-buffered saline. Cell pellets were analyzed immediately or stored at –70°C for up to 1 week before analysis. Blood was drawn in heparinized tubes and centrifuged at 2,000 *g* for 5 min to obtain plasma. Plasma specimens from both normal controls and SLS patients were frozen at –70°C for up to 1 year before analysis.

Human subjects

All patients had the typical clinical features of SLS and were confirmed to have deficient fibroblast FALDH activity. For measuring plasma fatty alcohols, SLS patients (8 males and 3 females) who ranged from 3 to 35 years of age were studied. These patients differed from those who provided fibroblast cultures. Normal subjects (5 males and 14 females) whose ages ranged from 4 to 52 years served as controls. This research was approved by the Committee on the Conduct of Human Research at the Medical College of Virginia (Richmond, VA).

Measurement of free fatty alcohols in cultured cells

To minimize fatty alcohol contamination, all glassware was rinsed with hexane before use, and solvents and reagents were dedicated to fatty alcohol measurements only. TLC plates were predeveloped with hexane–diethyl ether 1:1 before use. Solvents were checked for fatty alcohol contamination by drying 2–3 mL under nitrogen, resuspending any residue in hexane, and analyzing by gas chromatography (see below). Water was extracted with hexane before use. Only fatty alcohol-free solvents were used in the following procedure.

Cultured fibroblasts from one or two 75-cm^2 flasks were harvested by trypsinization and washed twice with phosphate-buffered saline. The cell pellet was resuspended in 1 mL of water and homogenized by 15 strokes in a motor-driven glass–Teflon homogenizer. An aliquot (100 μL) of the cell homogenate was removed for protein determination (13). The remaining cell homogenate (0.90 mL) was placed in a 16 × 125 mm screw-cap glass test tube. One microgram of pentadecanol (15:0-OH) in 10 μL of hexane was added to each sample as internal standard. Two milliliters of methanol was added and the tubes were vigorously mixed by vortexing for 1 min without capping. Caps were subsequently placed on the tubes and samples were allowed to extract for 1 h. After removing the caps, 2 mL of hexane was added to each tube and the alcohols were extracted by vortexing the tubes for 1 min. The tubes were centrifuged at 2,500 *g* for 5 min in a swinging-bucket rotor to facilitate phase separation. While avoiding the lipid interphase, the upper hexane layer in each tube was carefully removed with a glass Pasteur pipette and transferred to a new tube. The cell samples were extracted a second time with 3 mL of hexane and the hexane layers were combined with the first hexane extracts. The combined hexane extracts were dried under a stream of N_2 in a 37°C water bath. The sides of each test tube were washed down with 0.5 mL of hexane and dried again. Alcohol acetate derivatives were made by adding 0.5 mL of acetic anhydride and 1 mL of pyridine to each tube, which were then capped tightly and incubated for 1 h at 80°C or overnight at room temperature. Three milliliters of H_2O was added to each tube and fatty alcohol acetate derivatives were extracted with 4 mL of petroleum ether by vortexing the tubes without caps for 1 min as described previously. The upper ether phases were transferred to new 13 × 100 mm test tubes, using Pasteur pipettes, and evaporated under N_2 at 37°C. The dried lipids were washed down from the sides of the tubes with 0.5 mL of hexane and the hexane was then evaporated. The dried lipids were dissolved in 10–15 drops of hexane and spotted onto a prewashed, channeled silica gel TLC plate. After skipping a lane, 25 μg of pentadecanol acetate was spotted as standard. The TLC plate was developed with hexane–diethyl ether 95:5 and the lipids were visualized under UV light after spraying the plate with rhodamine G (0.1 mg/mL in methanol). The alcohol acetates (R_f = 0.8) migrated slightly ahead of cholesterol in this solvent system. The alcohol regions (together with the leading edge of cholesterol) were collected by scraping and transferred to clean 13 × 100 mm test tubes. Alcohol acetates were extracted from the silica gel with 4 mL of hexane–diethyl ether 3:1 by vortexing, centrifuging, and transferring the solvent to a new 13 × 100 mm test tube. The hexane–ether was evaporated under N_2, the sides of the tubes were washed down with 100 μL of hexane, and fatty alcohol acetates were transferred to 6 × 50 mm glass culture tubes (Kimble). After drying the hexane solvent, the alcohol acetates were dissolved in 10 μL of hexane.

Approximately 5 μL of each sample was injected on a Hewlett–Packard (Palo Alto, CA) HP5890 gas chromatograph equipped with an HP-1 capillary column (50 m length × 0.32 mm i.d.) and flame-ionization detector. Samples were injected in splitless mode. The injection temperature was 250°C, the detector temperature was 320°C, and the initial oven temperature was 100°C. After 1 min, the oven temperature was raised at 20°C/min to 175°C, followed by 2°C/min to 225°C, and a final rapid increase at 20°C/min to 300°C, which was maintained for 10 min. Alcohol acetates were identified according to retention time, using appropriate standards. The peak areas were integrated and the amounts of the alcohol acetates were calculated by comparison with the internal standard, using the response factor for pentadecanol acetate. To detect and correct for any fatty alcohol contamination introduced in the method, duplicate hexane-extracted water blanks were simultaneously processed along with the cell samples. The amount of fatty alcohol detected in the water blanks was subtracted from that measured in the cell samples to arrive at the cell-specific fatty alcohol content. The cell content of fatty alcohol was expressed as nanograms per milligram of cell protein.

Measurement of plasma free fatty alcohol concentration

The method described above for measuring fatty alcohols in cultured cells was modified for plasma samples, which have a large amount of lipid that interferes with TLC. Plasma samples (1 mL) were processed exactly according to the method for cells, up to the TLC step. The dried lipids containing alcohol acetates were dissolved in 2 mL of hexane and applied to 3-mL disposable silica gel columns, which were previously washed in a vacuum filtration apparatus under mild vacuum with 5 bed volumes of hexane, followed by hexane–diethyl ether 1:1 and finally hexane. After application of the alcohol acetate lipids in hexane, the columns were washed five times with 2 mL of hexane, and the alcohol acetates were collected by eluting the columns with 8 mL of hexane–diethyl ether 95:5. Samples were dried under N_2. Each sample was applied to four lanes of a channeled silica gel TLC plate and developed as described above. The purified alcohol acetates were recovered and analyzed by gas chromatogra-

phy. The fatty alcohol concentrations were expressed as nanograms of fatty alcohol per milliliter of plasma.

Measurement of total (free + esterified) fatty alcohols in fibroblasts

To measure the total (free + esterified) fatty alcohol concentrations in fibroblasts of SLS patients and normal controls, cell lipid extracts were subjected to alkaline hydrolysis before formation of acetate derivatives. Briefly, cell homogenates were extracted with methanol and hexane as described above for measurement of free alcohols. After drying the hexane extracts, the cell lipids were resuspended in 2 mL of 0.3 N NaOH in 95% ethanol, capped with a Teflon-lined screw cap, and heated for 2 h at 80°C. Tubes were cooled to room temperature. Two milliliters of water was added to each tube and fatty alcohols were extracted with 4 mL of hexane. Hexane extracts were dried under nitrogen, treated with acetic anhydride, and purified according to the method described above for free alcohols.

RESULTS

The method for measuring fatty alcohols reduced, but did not entirely eliminate, exogenous fatty alcohol contaminants, and the use of water controls (lacking cells) was necessary to detect and correct for this contamination. The fatty alcohol contamination was fairly consistent and its relative contribution to the alcohol measured in the cells was greater when fewer cells were analyzed. When one 75-cm^2 flask of normal cultured fibroblasts (1–2 mg of cell protein) was extracted, contaminating 16:0-OH and 18:0-OH accounted for 24 ± 6% (n = 6) and 34 ± 3%, respectively, of the fatty alcohols measured. When two or more flasks of cells were extracted, the relative contribution of contaminating fatty alcohol to the apparent cellular alcohol content diminished accordingly. This contaminating fatty alcohol was subtracted from that measured in cell samples to arrive at the true fatty alcohol content of the cells.

Using the fatty alcohol purification method for cultured cells, the recovery of radioactive tracer 18:0-OH was 67% ± 6% (n = 5). No single step in the procedure accounted for the bulk of alcohol loss. The addition of an internal standard (15:0-OH) to each specimen at the time of initial extraction permitted a means for correcting for fatty alcohol recovery. With 100 ng of nonradioactive 16:0-OH and 18:0-OH, the intraassay coefficients of variation were 5.6 and 12.0%, respectively.

The chromatograms of free fatty alcohols in cultured skin fibroblasts from a normal control subject and a patient with SLS are shown in **Fig. 1**. The major fatty alcohols identified were 16:0-OH and 18:0-OH. Most SLS fibroblasts also showed a small peak of octadecenol (18:1-OH), but tetradecanol (14:0-OH) and fatty alcohols longer than 18 carbons were not detected. The column chromatographic conditions separated these aliphatic alcohols from a large contaminating peak of cholesterol that came off the column at the end of the run.

As shown in **Fig. 2**, the mean 16:0-OH content of SLS fibroblasts was elevated 7-fold compared with normal cells, and the mean 18:0-OH content was 8-fold above normal. There was no overlap in the free fatty alcohol content of normal cells and SLS cells. In contrast, 18:1-OH was usually undetectable in normal cells and appeared more variable with no consistent elevation in the SLS cells (not shown). The free fatty alcohol content of 16:0-OH + 18:0-OH seemed to discriminate SLS cells from normal cells better than the individual fatty alcohols (Fig. 2). In SLS fibroblasts, the 16:0-OH content tended to be greater than the 18:0-OH content ($P = 0.02$).

Similar to cultured fibroblasts, SLS patients had increased concentrations of free fatty alcohol in plasma (**Fig. 3**). The relative accumulation of free 18:0-OH in plasma from SLS patients was increased 22-fold (range, 9- to 42-fold) above the mean concentration in normal controls, and mean 16:0-OH in SLS was elevated 9-fold. Measurement of the free 16:0-OH + 18:0-OH concentration was elevated 14-fold above normal and, like the skin fibroblasts, more effectively discriminated SLS from normal subjects than did the individual alcohols. In contrast to SLS fibroblasts, which tended to accumulate more 16:0-OH than 18:0-OH, plasma from SLS patients had a higher mean concentration of 18:0-OH than 16:0-OH ($P = 0.004$). Plasma fatty al-

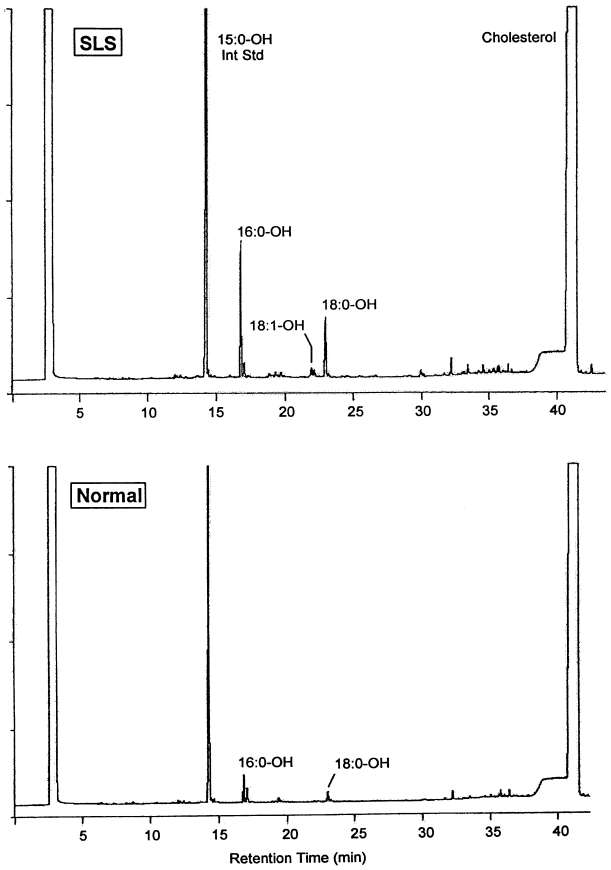

Fig. 1. Gas chromatogram of fibroblast free fatty alcohols. Fatty alcohols were isolated from one 75-cm^2 flask of cells, and the alcohol acetates were analyzed by gas chromatography. Top: SLS fibroblasts. Bottom: normal fibroblasts. 15:0-OH, Pentadecanol acetate internal standard; 16:0-OH, hexadecanol; 18:0-OH, octadecanol; 18:1-OH, octadecenol. Note the elevations in 16:0-OH and 18:0-OH in the SLS cells and a large peak of cholesterol eluting at the end of the chromatogram.

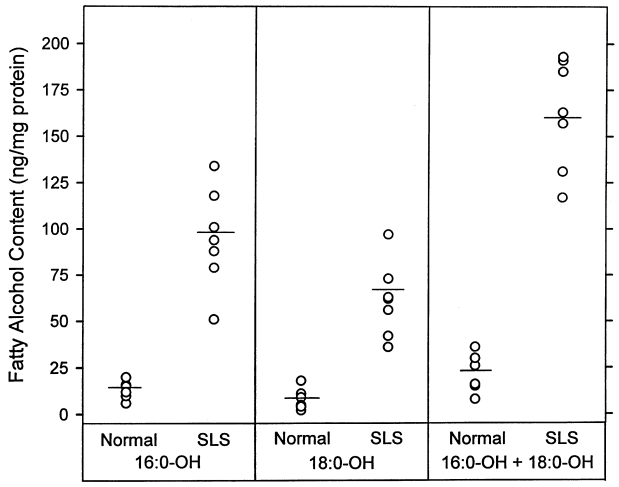

Fig. 2. Free fatty alcohol content of normal (n = 6) and SLS fibroblasts (n = 7). Horizontal lines indicate the mean fatty alcohol content. The mean fatty alcohol content (ng/mg protein ± SD) of normal and SLS cells were as follows: 16:0-OH (normal, 13 ± 5; SLS, 95 ± 26), 18:0-OH (normal, 8 ± 6; SLS, 61 ± 20), and 16:0-OH + 18:0-OH (normal, 22 ± 10; SLS, 162 ± 29).

cohols did not differ between males and females in SLS patients or normal controls.

There was little or no correlation between 16:0-OH and 18:0-OH levels in fibroblasts (r = 0.06) or in plasma (r = 0.001) from SLS patients. Both alcohols were elevated in SLS samples, but some patients showed greater accumulation of 16:0-OH and others tended to accumulate more 18:0-OH.

We compared the free fatty alcohol content of fibroblasts with the total (free + esterified) content, which was measured after alkali hydrolysis of the cell lipids. In normal cells, free alcohols (16:0-OH + 18:0-OH) accounted for 23% of the total fatty alcohol content, whereas free alcohols comprised 59% of the total alcohols in fibroblasts from SLS subjects.

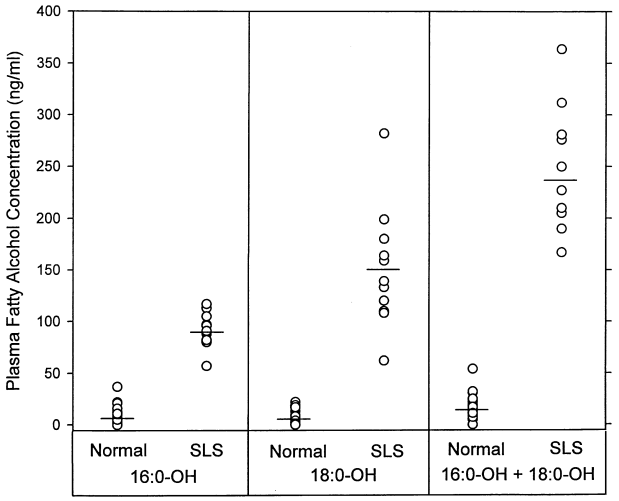

Fig. 3. Plasma free fatty alcohol concentrations in normal subjects (n = 19) and SLS patients (n = 11). Horizontal lines indicate the mean fatty alcohol concentrations. The mean plasma fatty alcohol concentrations (ng/mL ± SD) were as follows: 16:0-OH (normal, 10 ± 8; SLS, 92 ± 16), 18:0-OH (normal, 7 ± 7; SLS, 150 ± 57), and 16:0-OH + 18:0-OH (normal, 17 ± 12; SLS, 242 ± 60).

DISCUSSION

We developed a method for measuring free fatty alcohols that minimizes alcohol contamination arising from various sources, including Teflon-lined caps and certain solvents (chloroform, benzene) that are often used in lipid analyses. The extraction of cells and plasma with methanol, rather than a chloroform–methanol mixture, eliminated a major source of fatty alcohol contamination, and formation of alcohol acetate derivatives before further purification by TLC bypassed concern about subsequent contamination of the samples with free alcohols. An important control in the method was the inclusion of water blanks to detect and correct for any fatty alcohol contamination that may be introduced, especially when measuring samples that have low levels of free alcohols, such as plasma and fibroblasts from normal subjects. The alcohol contamination was less of a problem with biological samples containing greater amounts of endogenous fatty alcohols, such as SLS specimens, and could be further minimized by increasing the number of cells processed. Previous studies that analyzed fatty alcohols in specimens that are rich in these lipids, such as meibomian glands (14), skin surface lipids (15), and cultured MCF-7 breast cancer cells (16), may not have appreciated the existence of trace alcohol contaminants. The method described here is also suitable for measuring the fatty alcohol content of small tissue samples (50 mg wet weight) including liver and brain (W. B. Rizzo and D. A. Craft, unpublished observations). Although gas chromatography-negative ion chemical ionization mass spectrometry (17) is much more sensitive than capillary gas chromatography for detecting fatty alcohols, it has not been applied to the measurement of free alcohols in small biological samples.

Our results suggest that free fatty alcohols are a more sensitive marker for the metabolic defect in SLS than total (free + esterified) alcohols. Free long-chain alcohols were increased 7- to 22-fold in fibroblasts and plasma from SLS patients, but previous reports of total fatty alcohols found more modest 2- to 3-fold elevations (9, 10). In fibroblasts, this difference may be explained in part by the finding that most fatty alcohol that accumulated in SLS was free rather than esterified alcohol, whereas normal cells showed the opposite pattern. Although we did not determine the relative contribution of free versus esterified fatty alcohols in plasma, SLS patients may show a similar trend toward accumulating more free alcohol than esterified alcohol compared with normal subjects. The mean free 18:0-OH concentration in the plasma of our SLS patients was 81% of the mean concentration of total 18:0-OH reported previously (10), whereas our normal subjects had a mean plasma free 18:0-OH concentration that was only 10% of the total 18:0-OH (10). Thus, in striking contrast to total fatty alcohols, differences between normal and SLS samples were magnified by measuring free fatty alcohols. Cultured fibroblasts and plasma, however, have relatively

low levels of fatty alcohol and it is possible that SLS patients accumulate more esterified alcohol in certain tissues that actively synthesize wax esters, such as the epidermis (18).

The oxidative defect in SLS is more profound with 18-carbon than 16-carbon substrates (8), and it might be expected that patients would accumulate more of the longer chain alcohol. However, there was no consistent pattern between the levels of 16:0-OH and 18:0-OH in SLS fibroblasts and plasma. In fibroblasts, the free 16:0-OH content tended to be higher than 18:0-OH, whereas the opposite trend was seen in SLS plasma. It is unclear whether these differences arise from environmental or genetic variation among subjects, because the fibroblast cultures and plasma specimens were derived from different patients. The amounts of specific alcohols in tissues are probably determined in part by the substrate specificity of acyl-CoA reductase, which catalyzes fatty alcohol biosynthesis (19, 20), and the availability of acyl-CoA substrates derived from fatty acids. Furthermore, dietary or cell media sources may contribute to the tissue or cell content of specific fatty alcohols.

The ability to measure free fatty alcohols in small biological samples should prove useful for the study of SLS and other inborn errors of fatty alcohol metabolism. Determination of fatty alcohol levels in tissues and cultured cells from patients is critical for understanding the pathogenesis of these diseases and investigating potential therapeutic agents that may influence fatty alcohol metabolic pathways.

This research was supported in part by National Institutes of Health grant R01-AR44552 and General Clinical Research Center grant RR00065. We thank Dr. C. Jakobs for initially drawing our attention to the problem of trace alcohol contaminants.

Manuscript received 16 November 1999 and in revised form 14 March 2000.

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