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We describe a rapid and sensitive method involving time-of-flight secondary-ion mass spectrometry (TOF-SIMS) for specific laboratory diagnosis of the Smith–Lemli–Opitz syndrome, which is characterized by massive (~1000-fold) accumulation of the biosynthetic cholesterol precursor 7-dehydrocholesterol. Minute amounts of blood (1–50 μL) were extracted with n-hexane, and aliquots were analyzed by TOF-SIMS. 7-Dehydrocholesterol and its isomers were detected at 491.3 mass units ([M + 107Ag]+) and cholesterol at 495.3 mass units ([M + 109Ag]+). Quantitation of 7-dehydrocholesterol and cholesterol was achieved after saponification and addition of stigmasterol as internal standard. Whereas 7-dehydrocholesterol and isomeric dehydrocholesterol were not detectable in controls, the patients revealed concentrations ranging between 0.84 and 1.25 mmol/L. Comparison with results obtained by gas chromatography indicated that quantitation by TOF-SIMS yielded the sum of 7-dehydrocholesterol, isomeric dehydrocholesterol II, and sterol III, the latter two also being increased in the patients. Consistent with quantitation by gas chromatography, the cholesterol concentrations in the patients ranged between 1.54 and 2.12 mmol/L (controls: 6.10 ± 1.37 mmol/L).

Indexing Terms: heritable disorders/cholesterol synthesis/7-dehydrocholesterol/stigmasterol/silver isotopic labeling

Smith–Lemli–Opitz (SLO) syndrome is one of the most common autosomal recessive genetic disorders in the North American white population (1 in 20 000 newborns) (1). The syndrome is characterized by microcephaly, mental and growth retardation, facial dysmorphy, and syndactyly and (or) polydactyly. Frequently observed other abnormalities include cataracts and malformations of the cardiovascular and urogenital system. Recently, Tint et al. (2) reported that defective cholesterol biosynthesis at the level of conversion of 7-dehydrocholesterol (7-DHC) to cholesterol catalyzed by sterol Δ7-reductase was associated with SLO syndrome. Diagnosis relies on the clinical phenotype and the detection of usually as much as 1000-fold increased concentrations of the cholesterol precursor 7-DHC. In addition to 7-DHC, two other unusual non-cholesterol sterols, isomeric dehydrocholesterol II (cholesterol,5,6-dien-3β-ol) and sterol III (structure yet unknown), are increased in most patients (2). Three methods are currently used for laboratory diagnosis of SLO syndrome: gas chromatography–mass spectrometry (GC-MS) after derivatization with trimethylsilyl ether (2), ultraviolet spectral analysis of plasma samples extracted with n-hexane (3), and HPLC of plasma neutral sterols (Seedorf et al., ms. in preparation). GC-MS is highly specific but requires long processing times because of the necessity of sample derivatization. Ultraviolet spectral analysis is extremely simple but its specificity may not be as good (3). HPLC analysis, although relatively specific and reasonably quantitative, also requires relatively long processing times. A major disadvantage of all three methods is that they require relatively large blood samples not easily available from newborn patients. Because 7-DHC differs from cholesterol by only two mass units, we have employed time-of-flight secondary-ion mass spectrometry (TOF-SIMS) for specific laboratory diagnosis of SLO syndrome.

Materials and Methods

Reagents, blood samples, patients. Cholesterol, 7-DHC, and stigmasterol were obtained from Sigma Chemical Co. (St. Louis, MO). Ethanol, KOH, and n-hexane were obtained from Merck (Darmstadt, Germany). Blood samples were collected into EDTA-treated tubes. Three samples were from patients with SLO syndrome, as verified by 7-DHC concentrations ranging between 73 and 158 mg/L determined by GC-MS, ultraviolet spectral analysis, and HPLC. Thirteen samples were from pediatric patients with partial or complete phenocopies of the SLO syndrome but without increased 7-DHC concentrations (unpublished observation). The remaining 10 samples were obtained from healthy volunteers. The studies of human subjects were performed in accordance with the Helsinki Declaration of 1975, as revised in 1983. Informed consent with regard to the quantitative study was received only for the two patients appearing in Table 1. Sample preparation and TOF-SIMS. Mass spectrometry was carried out with a TOF-SIMS II (IONTOF, Münster, Germany) (4). The best results were obtained with the following procedure. One droplet

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Nonstandard abbreviations: DHC, dehydrocholesterol; GC, gas chromatography; MS, mass spectrometry; SLO, Smith–Lemli–Opitz; and TOF-SIMS, time-of-flight secondary-ion mass spectrometry.

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548 CLINICAL CHEMISTRY, Vol. 41, No. 4, 1995
(1-50 μL) of whole blood was collected into a glass centrifuge vial. Then, 50 μL of freshly prepared ethanolic KOH (150 g/L) was added, followed by brief vortex-mixing. We extracted neutral sterols once with 100 μL of n-hexane for 30 s (7-DHC yield: 97.5%), then applied directly 1 μL of the upper organic phase to a silver target (pre-etched for 2 min with 20% HNO₃). After evaporating the solvent under a stream of N₂ at 30°C for 15 min, we bombarded the resulting submonolayer with ⁴⁰Ar⁺, 10¹¹ ions/cm² (11 keV). Measurements were performed with typical acquisition times of 100 s. The approximate mass resolution was M/ΔM = 10 000, where M is the target ion mass and ΔM the resolved mass difference at half-width full-peak mode.

Quantitation of 7-DHC and cholesterol. For quantitative analyses, 1 g/L stigmastanol was added to the samples, which were then saponified at 37°C for 3 h. TOF-SIMS analyses were performed as described above, except that the extracts were diluted 50-fold with n-hexane before TOF-SIMS analysis.

Quantitation was carried out by determining relative sensitivity factors (or Sᵣ), calculated from the slopes of linear calibration curves. When analyte and internal standard are present in a sample at concentrations c(A) and c(S), respectively, their peak height ratio I(A)/I(S) in the SIMS spectrum can be expressed by the "fundamental" SIMS formula (5-7):

\[
\frac{I(A)}{I(S)} = \frac{c(A) Y_{tot} \alpha(A) a(A) T(A)}{c(S) Y_{tot} \alpha(S) a(S) T(S)} \tag{1}
\]

where \( Y_{tot} \) is the total secondary ion yield, \( \alpha(A) \) and \( \alpha(S) \) are the degrees of ionization, \( a(A) \) and \( a(S) \) are the isotopic dependence factors, and \( T(A) \) and \( T(S) \) are the transmission of the analyzer system for analyte and standard molecules, respectively. The last four factors in Eq. 1 are combined into the "relative sensitivity factor" of the analyte with respect to the internal standard Sᵣ:

\[
Sᵣ(A) = \frac{\alpha(A) a(A) T(A)}{\alpha(S) a(S) T(S)} \tag{2}
\]

To determine an unknown amount of an analyte \( m(A)_x \) in a sample, we used the formula in the form

\[
m(A)_x = \frac{m(S) [I(A)]}{Sᵣ(A) [I(S)]_x} \tag{3}
\]

where m(S) is the known amount of the internal standard. \( I(A)_{chol} \) was obtained from the peak height at 495.3 Da; stigmastanol was quantified accordingly at 521.3 Da ([M + ¹⁰⁹Ag⁺]). In samples containing cholesterol, I(A) for 7-DHC was corrected according to the equation \( I(A)_{7-DHC} = I(A)_{491.3} - 0.1417 I(A)_{chol} \) where \( I(A)_{491.3} \) is the peak height at 491.3 Da.

The calibration curve for cholesterol was measured with calibrators from 0 to 3 g/L at a fixed concentration of 1 g/L stigmastanol. For 7-DHC, the concentrations were 0–1 g/L (and stigmastanol was 1 g/L). Because its concentration was negligible in controls, calibration with 7-DHC was performed after adding the compound to control plasma and then extracting with n-hexane.

Results and Discussion

Analysis for Cholesterol and 7-DHC

Mass spectra obtained for cholesterol and 7-DHC are shown in Fig. 1. Major cholesterol-derived peaks were seen at 493.3 Da ([M + ¹⁰⁹Ag⁺]) and 495.3 Da ([M + ¹⁰⁹Ag⁺]). Minor peaks were seen at 386.3 Da ([M⁻]) and a 369.3 Da ([M – OH⁺]) (Fig. 1A). Analysis of 7-DHC revealed major peaks at 367.3 Da ([M – OH⁺]), 384.3 Da ([M⁺]), 491.3 Da ([M⁻ + ¹⁰⁹Ag⁺]), and 493.3 Da ([M⁺ + ¹⁰⁹Ag⁺]), indicating a mass difference of 2 Da attributable to the Δ₁ double bond in 7-DHC (Fig. 1B). The distinctive quantitative and qualitative differences in the two compounds, seen between mass units 365 to 370 and 384.3 to 386.3 (Fig. 1, A and B), indicated that 7-DHC underwent more extensive fragmentation and had less tendency to complex with Ag than did cholesterol. However, the most specific difference concerned the 7-DHC-derived peak detected at 491.3 mass units ([7-DHC + ¹⁰⁹Ag⁺]), which was almost completely absent from the cholesterol spectrum, and the cholesterol-derived peak at 495.3 Da (cholesterol + ¹⁰⁹Ag⁺), which is very small in the 7-DHC spectrum (Fig. 2). The mean height ratio of the peaks at 491.3 and 495.3 mass units measured for cholesterol in a concentration range of 0.5 to 3 g/L was 0.1417 ± 0.023 (n = 9). Because we could...
The conditions were as described for Fig. 1; y-axis represents intensity (counts/channel × 10⁻⁴).

The spectra of Plasma Samples Extracted with n-Hexane
A representative mass spectrum obtained with a normal sample and a sample from a patient with verified SLO syndrome is shown in Fig. 3. The main peaks in the spectrum from the control consisted of cholesterol ([M + ¹⁰⁷Ag]⁺ (493.3 Da) and [M + ¹⁰⁹Ag]⁺ (495.3 Da). In addition, a cholesterol-derived fragment was seen at [M − OH]⁻ (369.3 Da). The mass accuracy from spectrum to spectrum was ± 0.05 Da. The spectrum derived from a patient with verified SLO syndrome (Fig. 3B) revealed additional main peaks that were characteristic for 7-DHC, as indicated by comparison with the spectrum obtained for the freshly prepared 7-DHC calibrator (Fig. 1B). The main peak of interest was at 491.3 mass units [M + ¹⁰⁷Ag]⁺ (Fig. 4). Also, the peak height at 493.3 mass units was consistently greater in the samples from SLO patients than in the controls (Fig. 4). The most likely explanation for this result was superimposition of the [7-DHC − ¹⁰⁶Ag]⁺ with the [chol − ¹⁰⁷Ag]⁺ ion. Prominent fractionation products of 7-DHC that were hardly present in the controls were seen at mass units of 365.3, 366.3, and 367.3—most probably corresponding to [M − H₂O − H]⁺, [M − H₂O]⁺, and [M − OH]⁺.

To investigate the potential of the method, we analyzed 26 samples from patients (see Materials and Methods). By using the peak height ratios at 495.3/491.3 mass units as discriminating values, we classified correctly the three SLO samples with no false positives or false negatives. The peak height ratios in the SLO patients ranged between 1.0 and 1.3, whereas the lowest ratio found for the controls was 5.7 (mean:...
6.6 ± 0.89; n = 23), indicating a >5-fold difference between the two groups. In the controls, the mean height ratio of the peaks at 491.3 and 495.3 mass units was 0.150 ± 0.021, not significantly different from the respective ratio obtained with the cholesterol calibrator (0.1417 ± 0.023); this suggests that the signal at 491.3 mass units was almost entirely derived from cholesterol. Therefore, we think it likely that 7-DHC was in fact not detectable in controls by TOF-SIMS analysis.

Quantitation of 7-DHC and Cholesterol

To confirm this result and to compare the sterol concentrations of various patients, or to monitor the effects of dietary and (or) drug treatment on cholesterol and 7-DHC concentrations in SLO patients, requires quantitation of cholesterol and 7-DHC. We used stigmasterol as an internal standard to correct for any variations due to variable yields after extraction or to variable counting efficiencies in TOF-SIMS analysis. Stigmasterol seems ideal; its TOF-SIMS spectrum has no possibly interfering signals within the mass range of interest (491.3–495.3 mass units) (Figs. 1C, 2C). Moreover, no contaminating cholesterol or 7-DHC was contained in this calibration. In addition, the stigmasterol concentration was negligible in the samples from patients and controls, so that extraction with n-hexane provided identical yields for the three sterols (data not shown). However, because stigmasterol is increased in certain patients with sitosterolemia (8), it should not be used as internal standard in these rare patients.

To quantify 7-DHC in samples containing cholesterol, we corrected the peak height measured at 491.3 Da [I(A)491.3] according to the equation I(I)7-DHC = I(A)491.3 - 0.1417 I(Chol)495.3 derived earlier, thus correcting for cholesterol interference at 491.3 Da. Under appropriate conditions, we obtained linear relationships between the molar ratios for 7-DHC/stigmasterol or cholesterol/stigmasterol and the respective corrected peak heights (at 491.3 Da for 7-DHC, 495.3 Da for cholesterol, and 521.3 Da for stigmasterol) (Fig. 5). Because the linearity of the calibration curves was restricted to concentrations well below the theoretical monolayer particle concentration of the silver target, the quantitative analyses were performed with rather dilute solutions (1 μL of blood or plasma volume after dilution by 5000- to 100-fold during organic extraction).

To evaluate the accuracy of cholesterol quantitation with the TOF-SIMS method, we studied two standard plasma pools with total cholesterol concentrations of 5.13 mmol/L (1980 mg/L, serum 1) and 7.25 mmol/L (2800 mg/L, serum 2), as determined enzymatically with the CHOD-PAP method and an automated analyzer system (Hitachi–Boehringer Mannheim, Mannheim, Germany). The performance of this method in our laboratory has been under routine verification by analyses of reference sera supplied by the national German INSTAND proficiency testing program and the international quality assurance program of the US Centers for Disease Control and Prevention, with satisfactory results for several years. As shown in Table 1, quantitation with TOF-SIMS after saponification of cholesteryl esters yielded results almost identical to those of the CHOD-PAP method; this suggests that the accuracy of the method was good.

Concentrations in Subjects

Quantitation of cholesterol in SLO patients by TOF-SIMS provided lower values than the CHOD-PAP procedure did (Table 1). This result was expected because the enzymatic procedure does not discriminate between 7-DHC and its isomers or cholesterol and thus can overestimate the true cholesterol concentration in these patients. In contrast, comparison of the values provided by quantitative TOF-SIMS with those obtained from cholesterol determination by GC showed a reasonably good agreement (Table 1). The values of 0.84 and 1.25 mmol/L obtained for 7-DHC by quantitative TOF-SIMS were higher than those obtained by GC (Table 1)—again, as expected, since quantitation by TOF-SIMS is based on relative signal intensities at ion mass of 491.3 Da and thus does not discriminate between 7-DHC and isomeric dehydrocholesterol II or sterol III (two unusual sterols that were also increased in two patients). Isomeric dehydrocholesterol II most probably is cholest-5,8-dien-3β-ol; the mass spec-
trum for sterol III was very similar to that of 7-DHC, although we could not determine the precise identity of the compound (2). Adding the concentrations of isomeric DHC and sterol III to 7-DHC in Table 1 resulted in a reasonably good agreement between GC and quantitative TOF-SIMS. Consistent with these results, Tint et al. (2) reported values ranging from 0.81 to 1.72 mmol/L for the sum of 7-DHC, isomeric DHC, and sterol III in five SLO patients. Thus, the values obtained by quantitative TOF-SIMS were consistent with the expected finding that all three sterols that were increased in the two SLO patients would be measured simultaneously. Because differentiation between 7-DHC, isomeric DHC, and sterol III does not appear to be of specific diagnostic value (2), we think that simultaneous determination does not interfere with appropriate diagnosis of the syndrome. However, whenever differentiation is desired, samples must be analyzed for neutral sterols by GC.

In contrast, neither 7-DHC nor its isomers were detectable in samples from normal individuals. The median value for 7-DHC in normal controls is 0.14 μmol/L, the upper quintile value being 0.20 μmol/L (9). In addition, human plasma contains small amounts of other noncholesterol sterols, e.g., desmosterol (24-dehydrocholesterol) (10, 11), which should also produce a signal at 491.3 mass units by TOF-SIMS analysis. Compared with 7-DHC, cholesterol produced a relatively minor signal at 491.3 mass units; however, because the concentration of cholesterol in normal plasma exceeds the concentration of the noncholesterol sterols by roughly 3 to 4 orders of magnitude, one should not expect TOF-SIMS analysis to be specific enough to quantify such a minor component without prefractionation. This deficiency of the method does not differ in principle from other methods, because GC, HPLC, and thin-layer chromatography also require extensive prefractionation and enrichment to be able to accurately quantify 7-DHC in normal controls (9).

We conclude that TOF-SIMS provides a simple, rapid, and extremely sensitive tool for quickly determining whether or not 7-DHC and (or) isomeric dehydrocholesterol II and sterol III are increased in a patient with suspected SLO syndrome. The method is particularly useful whenever collecting enough sample for conventional laboratory diagnosis is difficult. The procedure also yields characteristic fragmentation products of 7-DHC that may facilitate its unequivocal identification.

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