Measurement of 3β-hydroxysteroid Δ^7-reductase activity in cultured skin fibroblasts utilizing ergosterol as a substrate: a new method for the diagnosis of the Smith-Lemli-Opitz syndrome

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Abstract A new sensitive and specific method for the evaluation of 3β-hydroxysteroid Δ^7-reductase activity, the defective enzyme in the Smith-Lemli-Opitz (SLO) syndrome, is described. The assay is based on the use of gas chromatography–mass spectrometry with selected-ion monitoring to measure the mass of brassicasterol (ergosta-5,22-dien-3β-ol) produced by the incubation of ergosterol (ergosta-5,7,22-trien-3β-ol) with cultured human skin fibroblasts. Although the conversion of ergosterol to brassicasterol was slower than the transformation of [^{3}H]7-dehydrocholesterol to [^{3}H]cholesterol, cells from control subjects produced brassicasterol efficiently. In contrast, cells from SLO patients produced very little brassicasterol ($P < 0.0001$, patients vs. parents or vs. controls). These results indicate that the reduction of ergosterol can be used as an assay for 3β-hydroxysteroid Δ^7-reductase in human skin fibroblasts, which avoids the many problems caused by the instability and lack of availability of radiolabeled 7-dehydrocholesterol. The present method made it possible to diagnose the SLO syndrome with high sensitivity and reliability using a commercially available compound.—**Honda, M., G. S. Tint, A. Honda, A. K. Batta, T. S. Chen, S. Shefer, and G. Salen.** Measurement of 3β-hydroxysteroid Δ^7-reductase activity in cultured skin fibroblasts utilizing ergosterol as a substrate: a new method for the diagnosis of the Smith-Lemli-Opitz syndrome. *J. Lipid Res.* 1996. **37:** 2433–2438.

Supplementary key words brassicasterol • cholesterol • 7-dehydrocholesterol • gas chromatography–mass spectrometry

The Smith-Lemli-Opitz syndrome (1) is a common autosomal recessive disorder caused by the markedly reduced activity of 3β-hydroxysteroid Δ^7-reductase (7-dehydrocholesterol Δ^7-reductase) (2, 3), the final enzyme in the cholesterol biosynthetic pathway. Patients are characterized clinically by severe mental retardation, failure to thrive, and multiple congenital anomalies (1, 4–7), and biochemically by abnormally low plasma cholesterol concentrations and the accumulation of the final cholesterol precursor, 7-dehydrocholesterol (8, 9).

Plasma sterol analysis is the most convenient and useful way to screen for the syndrome. However, it is important to develop more sensitive and accurate methods because occasional atypical cases exhibit normal plasma cholesterol levels with concentrations of 7-dehydrocholesterol that are considerably below that reported for most individuals with the syndrome (10, 11). In addition, increasing numbers of new metabolic and genetic studies of the syndrome require the measurement of 7-dehydrocholesterol Δ^7-reductase activity. Procedures used up until now made use of the conversion of [^{3}H]lathosterol (the precursor of 7-dehydrocholesterol) or of [^{3}H]7-dehydrocholesterol to cholesterol. Although these substrates have been used successfully to demonstrate the enzyme defect (2, 3, 12), neither of these radiolabeled compounds is available commercially and [^{3}H]7-dehydrocholesterol is especially unstable and difficult to work with.

This report describes a new method for the evaluation of 3β-hydroxysteroid Δ^7-reductase activity in cultured human skin fibroblasts by gas chromatography–

Abbreviations: brassicasterol, ergosta-5,22-dien-3β-ol; cholesterol, cholest-5-en-3β-ol; 7-dehydrocholesterol, cholesta-5,7-dien-3β-ol; ergosterol, ergosta-5,7,22-trien-3β-ol; GC–MS, gas chromatography–mass spectrometry; lathosterol, cholest-7-en-3β-ol; SIM, selected-ion monitoring; SLO syndrome, Smith-Lemli-Opitz syndrome; stigmasterol, stigmasta-5,22-dien-3β-ol; TMS, trimethylsilyl.

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mass spectrometry (GC–MS) with selected-ion monitoring (SIM), using non-radioactive ergosterol as a substrate (**Fig. 1**).

MATERIALS AND METHODS

Chemicals

Ergosterol was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI) and purified by recrystallization. Brassicasterol and stigmasterol were obtained from Research Plus, Inc. (Denville, NJ) and Supelco, Inc. (Bellefonte, PA), respectively. [1,2-^{3}H]7-dehydrocholesterol was synthesized as described previously (3, 13, 14).

Fibroblast culture

Skin biopsies were obtained from ten patients and from six parents. Five of the patients were among the least clinically affected and were assigned to group I. The other five patients assigned to group II were the most profoundly affected type II phenotypes (four of the children had died by the age of 6 months) (5). One of the group I patients was an atypical case who had a normal plasma cholesterol level and a 7-dehydrocholesterol concentration of only 0.15 mg/dl. Controls were patients from other metabolic studies without abnormalities in cholesterol metabolism. Fibroblasts were grown and maintained as monolayers in Dulbecco's Modified Eagle Medium (D-MEM) (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum. On day 1, 25-cm^2 tissue culture flasks were seeded with 3×10^5 cells/flask. On day 7, when the cells were nearly confluent (approximately 1×10^6 cells/flask), the culture was used for the following 3β-hydroxysteroid Δ^7-reductase assays. All cells were used before the 20th passage.

Ergosterol conversion assay

The original growth medium was removed, the attached cells were washed twice with phosphate-buffered saline (PBS), and the medium was replaced with 2 ml of fresh D-MEM containing 5% delipidized calf serum (Sigma Chemical Co., St. Louis, MO). Ergosterol (4 μg) was then added in 20 μl ethanol and incubated for 24 h at 37°C, after which the cells were scraped from the flasks and washed twice with PBS. One ml of 1 N ethanolic NaOH was added to the cells, and saponification was carried out by heating at 70°C for 1 h. In the time course experiment, 1 μg of stigmasterol was added as an internal recovery standard before saponification. After adding 0.5 ml water, sterols were extracted twice with 2 ml of n-hexane and converted into TMS ether

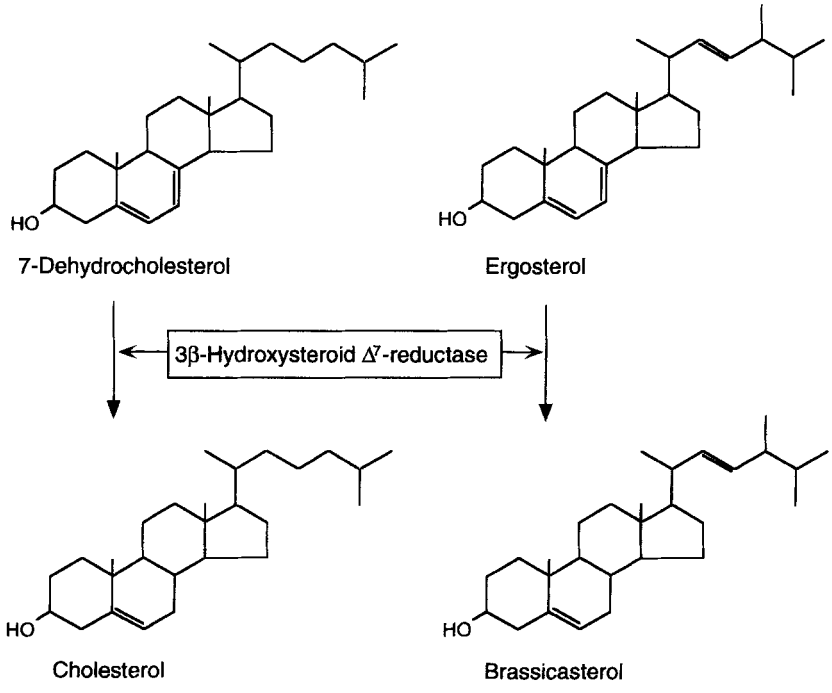

Fig. 1. Structures of 7-dehydrocholesterol, cholesterol, ergosterol and brassicasterol. 7-Dehydrocholesterol and ergosterol are converted to cholesterol and brassicasterol, respectively, by 3β-hydroxysteroid Δ^7-reductase in human skin fibroblasts.

derivatives (15) before analysis by GC–MS with selected-ion monitoring (SIM) using a Hewlett-Packard model 5988 mass spectrometer. A nonpolar CP-Sil 5CB (25 m × 0.25 mm ID) capillary column (Chrompack, Raritan, NJ) was used with a flow-rate of helium carrier gas of 1.0 ml/min. The column oven was programmed to change from 100° to 290°C at 35°C/min, after a 1-min delay from the start time. The mass spectral resolution was about 1000. The multiple ion detector was focused on m/z 363 for ergosterol, m/z 380 for brassicasterol (Table 1) and m/z 484 for stigmasterol.

[^{3}H]7-dehydrocholesterol conversion assay

The original growth medium was removed, the attached cells were washed twice with PBS, and the medium was replaced with 2 ml of fresh D-MEM containing 5% delipidized calf serum. [^{3}H]7-dehydrocholesterol (4 μg, 2.0 × 10^4 dpm) was then added in 20 μl ethanol and incubated at 37°C for 24 h after which the cells were harvested with trypsin and washed twice in PBS. After the addition of 4,000 dpm of [^{14}C]cholesterol as an internal recovery standard, lipids were extracted from the cells with chloroform–methanol 2:1 (16). The extracts were applied to argentation TLC plates (20 cm × 20 cm × 0.25 mm) (17, 18), developed in chloroform–acetone 9:1, and the radioactivities from the 7-dehydrocholesterol band (R_f = 0.25) and the cholesterol band (R_f = 0.55) were determined by liquid scintillation counting.

Statistics

Data are reported here as the mean ± SEM. The statistical significance of differences between the results in the different groups was evaluated with the Student's two-tailed t test or the Bonferroni multiple comparisons test. Significance was accepted at the level of $P < 0.05$.

RESULTS

TMS ether derivatives of ergosterol and brassicasterol (retention times, 11.2 and 10.8 min, respectively) were completely separated by capillary gas chromatography. The major fragments generated by GC–MS in the electron ionization (EI) mode are listed in **Table 1.** The calibration curve for the weight ratio of brassicasterol and ergosterol was established by focusing the ion detector on m/z 363 for ergosterol and m/z 380 for brassicasterol (**Fig. 2**). The linearity was excellent for weight ratios between 0.001 and 1.0. **Figure 3** shows a representative SIM chromatogram obtained by the analysis of ergosterol-treated fibroblasts from a control subject (Fig. 3a) and a patient (Fig. 3b). The peaks of ergosterol and brassicasterol in the control SIM (Fig. 3a) corresponded to ca. 40 ng and 20 ng, respectively. In contrast, only a very low intensity background ion fragment corresponding to 0.06 ng of brassicasterol was detected in the patient. This latter result was expected because ergosterol and brassicasterol are fungal sterols and are not normally present in mammalian cells. For evaluation of recovery, known amounts of ergosterol and brassicasterol were added to untreated cell pellets. These samples were subjected to saponification and extraction, and SIM was carried out after the addition of stigmasterol as an internal standard. The recoveries of ergosterol and brassicasterol were found to be 98 ± 1% (n = 3) and 95 ± 3% (n = 3), respectively.

TABLE 1. Mass spectral data for the TMS ether derivatives of ergosterol and brassicasterol

Ion Species	Ergosterol m/z (% Int)[a]	Brassicasterol m/z (% Int)
M^+	468 (24)	470 (27)
$M-(CH_3)_3SiOH$	378 (22)	380 (32)[c]
$M-(CH_3)_3SiOH-CH_3$	363 (79)[c]	365 (13)
M–A-ring fragment[b]	337 (53)	341 (20)
$M-(CH_3)_3SiOH$–side chain	253 (48)	255 (57)
A-ring fragment[b]	131 (48)	129 (80)
$(CH_3)_3Si$	73 (100)	73 (100)

[a] % Relative intensities.
[b] $(CH_3)_3SiO^+{=}CH{-}CH_2{-}CH_3$ (m/z 131) and $(CH_3)_3SiO^+{=}CH{-}CH{=}CH_2$ (m/z 129) for ergosterol and brassicasterol, respectively.
[c] Ions used for ergosterol and brassicasterol SIM assay.

Figure 4 illustrates the effects of incubation time on the relative amount of ergosterol taken up by the cells (approximately 1 × 10^6 cells/flask) and then converted

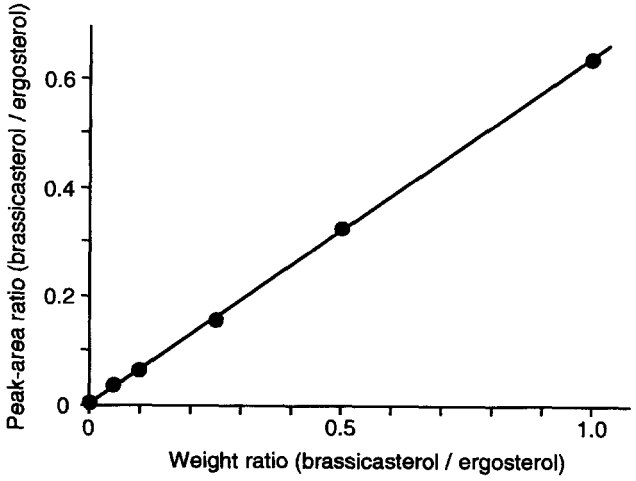

Fig. 2. Calibration curve for the weight ratio of brassicasterol to ergosterol using selected-ion monitoring. The linearity was checked by simple linear regression, and the line satisfies the relationship y = 0.632x + 0.002 (n = 6; r = 0.9999; $P < 0.0001$).

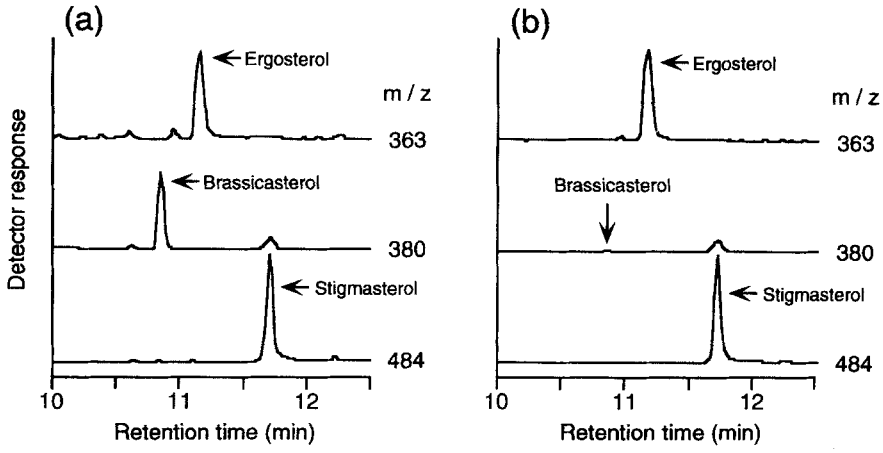

Fig. 3. Selected-ion monitoring of the TMS ether derivatives of ergosterol, brassicasterol, and stigmasterol (internal standard) in extracts from fibroblasts from a control (a) and an SLO patient (b) incubated with 4 μg of ergosterol for 24 h.

to brassicasterol in a control and a typical (group II) patient. The conversion of ergosterol to brassicasterol was calculated as the percentage of brassicasterol formed compared to the mass of brassicasterol + ergosterol extracted from the cells. The plots of the uptake of ergosterol versus incubation time in the patient's cells were very similar to those of control cells. However, the conversion of ergosterol to brassicasterol in the patient's cells was very different from that in the controls. In fibroblasts from the patient, little brassicasterol could be detected while increasing amounts of brassicasterol were observed in control cells.

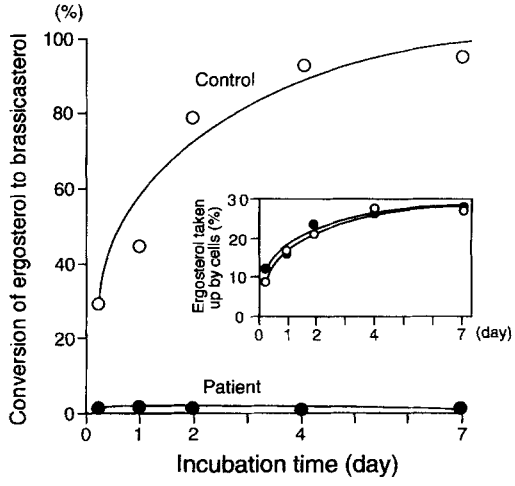

Fig. 4. Conversion of ergosterol to brassicasterol in fibroblasts from a control subject (○) and a patient (●) as a function of time of incubation. The inset plot depicts the time course of ergosterol uptake by cells calculated as 100 × (mass of ergosterol + brassicasterol extracted from cells) divided by the mass of ergosterol added to the medium.

Table 2 summarizes the results of the transformation of ergosterol to brassicasterol after a 24-h incubation compared to the conversion of [^{3}H]7-dehydrocholesterol to [^{3}H]cholesterol. Approximately 16% (16.3%, 15.6%; n = 2) of the added ergosterol and 15 ± 1% (n = 7) of radiolabeled 7-dehydrocholesterol were taken up by the washed fibroblasts after a 24-h incubation. In parents, the conversion of ergosterol to brassicasterol was significantly lower than that in controls ($P < 0.01$). However, due to the wide variation, it was not possible to discriminate completely between fibroblasts from controls and from carriers. In contrast, cells from patients, including the atypical case, made very little brassicasterol (0.4% in the atypical case) so that cells from

TABLE 2. Conversion of ergosterol to brassicasterol in cultured fibroblasts from patients, parents, and controls, and comparison to [^{3}H]7-dehydrocholesterol as a substrate for 3β-hydroxysteroid Δ^7-reductase

Subject	Conversion to Δ^7-Reduced Form (%)[a]	
	Ergosterol	[^{3}H]7-Dehydrocholesterol
	Mean ± SEM [range] (n)	
Group I patients	1.1 ± 0.4[d] [0.3–2.6] (5)	0.6 ± 0.2[e] [0.1–1.0] (4)
Group II patients	0.1 ± 0.1[b] [0.0–0.3] (5)	0.2 ± 0.1[e] [0.0–0.6] (4)
Parents	16 ± 1[c] [12–21] (6)	ND[f]
Controls	24 ± 2[d] [13–33] (10)	81 ± 6 [64–90] (4)

[a]Calculated as brassicasterol divided by (brassicasterol + ergosterol) × 100% or [^{3}H]cholesterol divided by ([^{3}H]cholesterol + [^{3}H]7-dehydrocholesterol) × 100%. Ergosterol or [^{3}H]7-dehydrocholesterol was incubated with cultured fibroblasts for 24 h.

[b]$P < 0.001$, significantly different from controls and parents.

[c]$P < 0.01$, significantly different from controls.

[d]$P < 0.0001$, significantly different from controls of [^{3}H]7-dehydrocholesterol assay.

[e]$P < 0.001$, significantly different from controls.

[f]Not determined.

affected individuals were completely distinguishable from control and parents' cells ($P < 0.001$). In control subjects after a 24-h incubation, the conversion of ergosterol to brassicasterol took place at about 30% of the rate at which 7-dehydrocholesterol was transformed to cholesterol ($P < 0.0001$). After a 4-day incubation, however, the transformation of ergosterol to brassicasterol increased to 82 ± 4% (n = 4) in controls and 78 ± 3% (n = 4) in parents, values that are similar to the conversions observed for the 7-dehydrocholesterol assay.

DISCUSSION

Ergosterol is fungal sterol, having the same ring structure as 7-dehydrocholesterol, but differing by the addition of a methyl group on the side chain at C-24 and the presence of a double bond at C-22 (Fig. 1). Our results indicate that human skin fibroblasts can catalyze the reduction of the C-7 double bond of ergosterol to make brassicasterol. Although it is not known whether this reaction is catalyzed by the same enzyme that transforms 7-dehydrocholesterol to cholesterol, the markedly reduced conversion of ergosterol to brassicasterol in SLO patients strongly suggests that a single enzyme (3β-hydroxysteroid Δ^7-reductase) catalyzes both reactions. The yeast Δ^7-reductase has recently been cloned and was found to bear considerable homology to yeast Δ^{14} and $\Delta^{24(28)}$ reductases as well as to the human lamin B receptor (19). The human gene has not yet been identified but is believed to reside on chromosome 7 (20).

Although almost the same amounts of added substrates (ergosterol or [^{3}H]7-dehydrocholesterol) were taken up by cells, the rate of conversion of ergosterol to brassicasterol was about 30% of that of 7-dehydrocholesterol to cholesterol. This means that the rate of reduction of the ergosterol C-7 double bound was about one-third the rate of reduction of the 7-dehydrocholesterol C-7 double bound. In rat liver, the 3β-hydroxysteroid Δ^7-reductase activity does not seem to be influenced by side chain double bonds at C-22 or C-24 (21). Therefore, the reduced availability of ergosterol as a substrate seems to be caused by the methyl group at C-24 and may relate to the slower passage of side chain-substituted sterols in and out of membranes (22).

Because ergosterol is a fungal compound, neither the sterol itself nor its Δ^7-reducted product, brassicasterol, is detected in untreated human fibroblasts. Therefore, radioisotope- or stable isotope-labeled substrates that are not commercially available are not required for assaying the enzyme activity. Although ergosterol was less effectively converted to the Δ^7-reduced form (brassicasterol) compared to 7-dehydrocholesterol, the 3β-hydroxysteroid Δ^7-reductase activity was easily evaluated by use of ergosterol because the activity is usually very high (3, 23). In addition, although we incubated cells with ergosterol for only 24 h, the conversions could be increased to the same levels as those obtained with [^{3}H]7-dehydrocholesterol by incubating for a longer time (Fig. 4).

The ergosterol conversion assay is a sensitive and accurate method for making a diagnosis of the SLO syndrome. This procedure also will be very useful for measuring the regulation of the enzyme in experimental models (24, 25). The syndrome can usually be diagnosed from the biochemical abnormalities, reduced plasma cholesterol, and markedly elevated 7-dehydrocholesterol concentrations (8, 9). However, a few atypical patients have been identified who exhibit normal plasma cholesterol levels with only moderately increased concentrations of 7-dehydrocholesterol that are within the range found in non-SLO patients (26). In addition, it is possible that high cholesterol diets may raise plasma cholesterol levels and reduce plasma 7-dehydrocholesterol concentrations (27). Therefore, it is important to establish more reliable and useful methods for the definitive diagnosis of the syndrome. The present method as well as [^{3}H]7-dehydrocholesterol and [^{3}H]lathosterol conversion assays appear to be able to detect atypical cases and not to be influenced by any treatment because all of these methods directly evaluate the deficient enzyme activity in the syndrome.

In summary, we have established a useful and reliable method for the evaluation of 3β-hydroxysteroid Δ^7-reductase activity in human skin fibroblasts by use of ergosterol as a substrate. This method made it possible to make a definitive diagnosis of the SLO syndrome using a commercially available substrate and can be used to track the regulation of the enzyme in vivo and in vitro studies.

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