Brazilian Journal of Medical and Biological Research (2003) 36: 1327-1332
ISSN 0100-879X

Diagnosis of Smith-Lemli-Opitz syndrome by ultraviolet spectrophotometry

F.B. Scalco[1], V.M. Cruzes[2], R.C. Vendramini[2], I.L. Brunetti[2] and D. Moretti-Ferreira[1]

[1]Serviço de Aconselhamento Genético, Instituto de Biociências, Universidade Estadual Paulista, Botucatu, SP, Brasil
[2]Departamento de Análises Clínicas, Faculdade de Ciências Farmacêuticas, Universidade Estadual Paulista, Araraquara, SP, Brasil

Abstract

Smith-Lemli-Opitz syndrome (SLOS) is an autosomal recessive disorder due to an inborn error of cholesterol metabolism, characterized by congenital malformations, dysmorphism of multiple organs, mental retardation and delayed neuropsychomotor development resulting from cholesterol biosynthesis deficiency. A defect in 3ß-hydroxysteroid-Δ^7-reductase (Δ^7-sterol-reductase), responsible for the conversion of 7-dehydrocholesterol (7-DHC) to cholesterol, causes an increase in 7-DHC and frequently reduces plasma cholesterol levels. The clinical diagnosis of SLOS cannot always be conclusive because of the remarkable variability of clinical expression of the disorder. Thus, confirmation by the measurement of plasma 7-DHC levels is needed. In the present study, we used a simple, fast, and selective method based on ultraviolet spectrophotometry to measure 7-DHC in order to diagnose SLOS. 7-DHC was extracted serially from 200 µl plasma with ethanol and n-hexane and the absorbance at 234 and 282 nm was determined. The method was applied to negative control plasma samples from 23 normal individuals and from 6 cases of suspected SLOS. The method was adequate and reliable and 2 SLOS cases were diagnosed.

Correspondence
D. Moretti-Ferreira
Serviço de Aconselhamento Genético
Instituto de Biociências, UNESP
Caixa Postal, 529
18618-000 Botucatu, SP
Brasil
E-mail: sag@fmb.unesp.br

Research supported by CAPES, Fundação Lucentis and NAC-FCF/UNESP.

Publication supported by FAPESP.

Received May 14, 2002
Accepted August 4, 2003

Key words
- Smith-Lemli-Opitz
- 3ß-Hydroxysteroid-delta-7-reductase
- Cholesterol
- 7-Dehydrocholesterol
- Ultraviolet spectrophotometry

Introduction

Smith-Lemli-Opitz syndrome (SLOS) is an autosomal recessive disorder due to an inborn error of cholesterol metabolism. The syndrome was first described in 1964 by Smith et al. (1) in a report of three patients who had common clinical features which included microcephaly, dysmorphic facies, limb anomalies, genital malformations, global developmental delay, and others. This syndrome was first named "RSH", an acronym of the first letters of the original patient's surnames, by Opitz et al. (2)

Irons et al. (3) reported marked elevation of 7-dehydrocholesterol (7-DHC) and low plasma cholesterol levels in two patients with SLOS, suggesting a defect in the Kandutsch-Russel cholesterol biosynthesis pathway. In 1994, Natowicz and Evans (4) and Tint et al. (5) also demonstrated abnormal plasma steroid levels in SLOS patients, confirming that

the fundamental biochemical abnormality in SLOS is elevated plasma 7-DHC levels. Shefer et al. (6) demonstrated a marked deficiency of 3ß-hydroxysteroid-Δ^7-reductase (Δ^7-sterol-reductase), the enzyme that converts 7-DHC to cholesterol.

It has been recently shown that proteins of the developmental gene *Shh* (Shh proteins) must be covalently linked to cholesterol to be activated (7). Shh proteins are essential for the development of the brain, limbs and face. Thus, there is a relationship between Shh dysfunction and the malformations observed in SLOS. Cell interactions that occur in the cardiovascular, respiratory and renal systems may be explained, at least partially, by irregular cell migration, while the changes observed in the urogenital system are directly related to the synthesis of steroid hormones, whose main precursor is cholesterol (7-9).

The clinical diagnosis of SLOS cannot always be conclusive because of the remarkable variability of clinical expression. Until 1993, when the cause of SLOS was still unknown, its diagnosis could not be confirmed biochemically.

Table 1. Clinical characteristics of 6 patients with suspected Smith-Lemli-Opitz syndrome.

	Patient number					
	1	2	3	4	5	6
Sex	Female	Male	Female	Male	Male	Male
Age (years)	5	1	4	4	2	6
Microcephaly	+	+	–	–	–	+
Narrow forehead	+	+	–	+	+	–
Rotated ears	?	+	+	+	–	–
Ptosis	?	+	+	–	–	+
Anteverted nares	?	+	+	+	+	+
Micrognathia	+	+	–	–	+	–
High palate	+	–	–	–	–	+
Podal syndactyly between 2nd and 3rd toes	+	+	–	+	+	+
Postaxial polydactyly of feet	–	–	+	–	–	–
Abnormal male genitalia	–	+	–	+	+	+
Mental retardation	+	+	+	+	+	+
Renal problems	+	–	–	–	–	–

? = not informed; + = present; – = absent.

The earliest estimate of the incidence of SLOS, based on clinical diagnosis, was reported to be 1:20,000 to 1:40,000 (10). These rates have changed since 1993 with the advent of biochemical testing. The incidence of SLOS, in similar populations diagnosed biochemically by gas chromatography and mass spectrometry, seems to be much lower. It is reported to be 1:60,000 in England and the United States (11,12) and 1:80,000 to 1:100,000 in the Netherlands (12).

Today, the biochemical diagnosis is based on the measurement of plasma 7-DHC or the activity of Δ^7-sterol-reductase in fibroblast cultures. However, even though SLOS is frequently associated with low plasma cholesterol levels, these cholesterol levels cannot be used separately to confirm or exclude the diagnosis because 10% of the affected patients show normal cholesterol levels (12, 13).

7-DHC shows characteristic ultraviolet (UV) absorbance at 271, 282, and 294 nm. Honda et al. (14) suggested the possibility that UV spectrophotometry could be useful for the detection of plasma 7-DHC since it is available in most clinical laboratories, provides a faster and cost-effective screening, and is as reliable as sophisticated techniques such as mass spectrometry and gas chromatography that may also be used to quantify 7-DHC.

In the present study, six patients with clinical features consistent with SLOS were selected to test the UV spectrophotometric measurement of 7-DHC as a screening test for SLOS.

Material and Methods

Patients

The study group comprised six patients who were referred to the Serviço de Aconselhamento Genético, Departamento de Genética, Universidade Estadual Paulista

(UNESP), Botucatu, SP, Brazil, because they presented clinical features consistent with SLOS (Table 1). The control group included 23 normal individuals. Blood samples were collected into vacuum tubes containing EDTA (3 ml). Following centrifugation at 800 *g*, plasma was separated and stored at -20ºC until analysis.

The study was approved by the research Ethics Committee of the Hospital de Clínicas, UNESP, Botucatu, SP, Brazil, and Comissão Nacional de Ética em Pesquisa (CONEP), Brasília, DF, Brazil. All participants in the study signed an informed consent form authorizing the use of their blood samples in laboratory assays and the publication of the results.

Reagents and equipment

7-DHC was purchased from Sigma (D4429; St. Louis, MO, USA), spectral quality n-hexane was purchased from Mallinckrodt (Chron AR-HPLC UN1208, New York, NY, USA) and ethanol from Merck (Rio de Janeiro, RJ, Brazil). Cholesterol was analyzed with the Bayer Sera-Pak® Cholesterol kit (Fast color; New York, NY, USA).

Cholesterol was determined with a RAX Bayer autoanalyzer and UV spectra were determined with a Hitachi spectrophotometer (U-3000; Tokyo, Japan) slit 2.0 nm.

Cholesterol

Total cholesterol was determined by a method of enzyme coupling with cholesterol ester hydrolase, cholesterol oxidase and peroxidase (15).

UV determination of 7-DHC

A calibration curve was obtained for 7-DHC after adding known amounts of an ethanolic solution of 7-DHC (1 to 40 mg/dl) to plasma. Absorbance at 282 nm was corrected by a modified Honda equation (14; see below). 7-DHC in the ethanolic stock solution was determined using $\varepsilon 293\ nm^{EtOH} = 6.74 \times 10^3\ M^{-1}\ cm^{-1}$ (16).

A 200-µl aliquot of plasma was transferred to a glass tube which was vortexed for 10 s after the addition of 200 µl ethanol. One milliliter of n-hexane was then added and the mixture vortexed for another 20 s. After centrifugation at 400 *g* for 1 min, the clear n-hexane layer was collected in a 1-ml quartz cuvette and used for UV measurement. The UV spectrum was obtained using n-hexane as blank in the reference beam, and absorbance at 271, 282 and 294 nm was used for the qualitative detection of 7-DHC in plasma samples, while absorbance at 234 and 282 nm was used for quantitative analysis as proposed by Honda et al. (14), where absorbance at 282 nm was corrected by the equation described below.

The absorbance at 282 nm ($A_{282\,nm}$) is the sum of the absorbance of 7-DHC (B) plus background absorbance (C). $A_{282\,nm} = B + C$.

The absorbance at 234 nm ($A_{234\,nm}$) is the sum of the absorbance of 7-DHC (D) plus background absorbance (E). $A_{234\,nm} = D + E$.

In an experiment using standard 7-DHC, the absorbance of 7-DHC at 234 nm (D) was estimated as being 14.7% of the absorbance at 282 nm (B). $D = 0.147B$.

By means of the correlation between absorbances at 282 nm and 234 nm in control plasma, the background absorbance was estimated at 282 nm. $C = 0.01042E^2 + 0.05901E + 0.02366$.

Resolving the equation system above, the following equation is obtained:

$$B = \frac{12.18 \times A_{234} - 3525.49 + \sqrt{(-12.18 \times A_{234} + 3525.49)^2 - 4 \times 1 \times [(210.07 \times A_{234} + 37.10) \times A_{234} + 84.23 - 3559.99 \times A_{282}]}}{2}$$

where B = 7-DHC absorbance at 282 nm, A_{234} = value of absorbance at 234 nm, and A_{282} = value of absorbance at 282 nm.

EDTA/plasma samples from six patients suspected of having SLOS and 23 negative controls were submitted to 7-DHC extraction. The extracts obtained were analyzed by UV spectrophotometry.

Results

UV spectrophotometric 7-DHC detection was standardized using plasma from 23 normal individuals (negative control group) whose 7-DHC levels could not be quantitated and 7-DHC spiked samples. The negative control samples did not present characteristic 7-DHC absorption peaks such as those observed in poisoned samples.

The diagnosis of SLOS was confirmed by the observation of characteristic absorption spectra of 7-DHC identical to those in 7-DHC spiked samples and plasma with high 7-DHC levels in two of the six patients presenting clinical features consistent with SLOS (Figure 1 and Table 1). Table 2 also shows the plasma cholesterol levels observed in both patients and controls. It is noteworthy that these cholesterol levels of the SLOS-positive cases should be low due to interference of 7-DHC with the cholesterol measurement by the enzymatic method of Allain et al. (15).

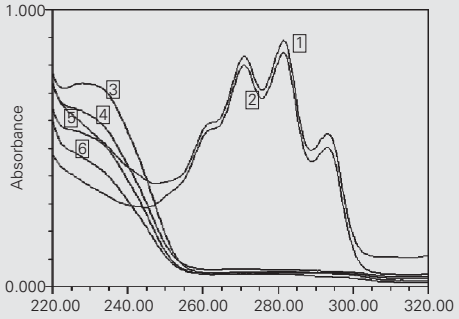

Figure 1. Ultraviolet spectra of plasma extracts from 6 patients with clinical signs consistent with Smith-Lemli-Opitz syndrome (SLOS). SLOS diagnosis was confirmed in patients 1 and 2.

Plasma from patient number 1 was sent to the Kennedy Krieger Institute (USA) and the diagnosis was also confirmed by mass spectrometry.

Discussion

The measurement of 7-DHC by UV spectrophotometry is a useful and sensitive biochemical test for SLOS diagnosis (17,18). In our study group, two of the six suspected SLOS patients presented characteristic 7-DHC absorbance peaks at 271, 282 and 294 nm which were not seen in the other patients (Figure 1) and were the same as observed in 7-DHC spiked samples.

The quantitative analysis of our controls and negative patients showed undetectable plasma 7-DHC levels, whereas other authors have reported mean plasma 7-DHC values of 0.0052 mg/dl (18) in adult controls. These values are very close to those obtained by us in the control group of children.

Guzzetta et al. (17) also used spectropho-

Table 2. Cholesterol and 7-dehydrocholesterol (7-DHC) plasma concentration in 6 suspected Smith-Lemli-Opitz syndrome patients and 23 negative controls.

	Patient number						Negative controls (N = 23)
	1	2	3	4	5	6	
Cholesterol (mg/dl)	99.5[a]	67.5	165	137	163	151	111-308
7-DHC (mg/dl)	12.34	11.83	NQ	NQ	NQ	NQ	NQ
Corrected cholesterol (mg/dl)[b]	87.16	55.67	165	137	163	151	–
Correlation 7-DHC/cholesterol	0.1416	0.2125	–	–	–	–	–

[a]During treatment with a high cholesterol diet. [b]Cholesterol values were corrected by subtracting 7-DHC values (Ref. 19). NQ = not quantitated.

tometry and compared it with mass spectrometry. They demonstrated that the detection of plasma 7-DHC by UV spectrophotometry is highly specific and sensitive in the detection of biochemical anomalies in patients with SLOS, showing, therefore, a good correlation with mass spectrometry analysis. For diagnostic purposes, the analysis of other sterols such as 8-dehydrocholesterol and the 7-DHC/cholesterol ratio has also been used. The problem in using cholesterol for diagnostic purposes is that the literature reports low to baseline cholesterol values but warns against problems in the measurement of plasma cholesterol in patients with SLOS. Jira et al. (19) demonstrated the interference of high concentrations of 7-DHC and 8-DHC in the determination of cholesterol by enzymatic methods, with results showing high plasma cholesterol levels (19). These investigators analyzed the steroid/cholesterol relationship using gas chromatography and obtained values lower than 0.004 for control groups and values higher than 0.3 for SLOS patients. Our results with UV spectrophotometry showed values of 0.1-0.2 for SLOS patients and lower values for the control group and other patients (Table 2).

Most individuals with SLOS present elevated plasma 7-DHC levels in addition to the phenotype characteristics of the syndrome. Atypical cases may include patients displaying very few clinical features and increased 7-DHC levels as well as individuals with the "complete" phenotype of the syndrome and normal 7-DHC levels (14,20-22). Elevated 7-DHC levels may also be observed in patients showing some form of hypercholesterolemia and cerebrotendinous xanthomatosis (12). Moreover, mildly to moderately increased 7-DHC levels were observed in three patients while they were on haloperidol. When haloperidol therapy was discontinued, 7-DHC levels decreased back to normal (12,23).

SLOS is likely to be underdiagnosed in Brazil due to the lack of simple detection tests. Our study demonstrates that the spectrophotometric method is fast and accurate for the diagnosis of SLOS. The treatment includes dietary cholesterol replacement and we offer genetic counseling to the parents of SLOS children.

Acknowledgments

We thank Drs. Salmo Raskin (Laboratory Genetika, Curitiba, PR, Brazil), Chong Ae Kim (Instituto da Criança, São Paulo, SP, Brazil), Agnes Fett-Conte (Faculdade de Medicina de Ribeirão Preto, Ribeirão Preto, SP, Brazil) and Raquel de Camargo (Universidade Estadual Paulista, Botucatu, SP, Brazil) for allowing access to patients and their medical records.

References

1. Smith DW, Lemli L & Opitz JM (1964). A newly recognized syndrome of multiple congenital anomalies. *Journal of Pediatrics*, 64: 210-217.
2. Opitz JM, Zelwerguer H & Shannon WR (1969). The RSH syndrome. *Birth Defects*, V: 43-52.
3. Irons M, Elias ER, Salen G, Tint GS & Batta AK (1993). Defective cholesterol biosynthesis in Smith-Lemli-Opitz syndrome. *Lancet*, 341: 1414.
4. Natowicz MR & Evans J (1994). Abnormal bile acids in the Smith-Lemli-Opitz syndrome. *American Journal of Medical Genetics*, 50: 364-367.
5. Tint GS, Irons M, Elias ER, Batta AK, Frieden R, Chen TS & Salen G (1994). Defective cholesterol biosynthesis associated with the Smith-Lemli-Opitz syndrome. *New England Journal of Medicine*, 330: 107-113.
6. Shefer S, Salen G, Batta AK, Honda A, Tint GS, Irons M, Elias ER, Chen TC & Holick M (1995). Markedly inhibited 7-dehydrocholesterol-Δ^7-reductase activity in liver microsomes from Smith-Lemli-Opitz homozygotes. *Journal of Clinical Investigation*, 96: 1779-1785.
7. Roux C, Wolf C, Mulliez N, Gaoua W, Cormier V, Chevy F & Citadelle D (2000). Role of cholesterol in embryonic development. *American Journal of Clinical Nutrition*, 71: 1270-1279.
8. Porter JA, Young RF & Beachy PA (1996). Cholesterol modification of hedgehog signaling proteins in animal development. *Science*, 274: 255-259.
9. Tabin CJ & McMahon AP (1997). Recent advances in hedgehog

signaling. *Trends in Cell Biology*, 7: 442-446.
10. Lowry RB & Yong SL (1980). Borderline normal intelligence in the Smith-Lemli-Opitz (RSH) syndrome. *American Journal of Medical Genetics*, 5: 137-143.
11. Ryan AK, Bartlett K, Clayton P, Eaton S, Mills L, Donnai D, Winter RM & Burn J (1999). Smith-Lemli-Opitz syndrome: a variable clinical and biochemical phenotype. *Journal of Medical Genetics*, 35: 558-565.
12. Kelley RI & Hennekam RCM (2000). The Smith-Lemli-Opitz syndrome. *Journal of Medical Genetics*, 37: 321-335.
13. Nowaczyk MJM, Whelan DT, Heshka TW & Hill RE (1999). Smith-Lemli-Opitz syndrome: a treatable inherited error of metabolism causing mental retardation. *Canadian Medical Association Journal*, 161: 165-170.
14. Honda A, Batta AK, Salen G, Tint S, Chen T & Shefer S (1997). Screening for abnormal cholesterol biosynthesis in the Smith-Lemli-Opitz syndrome: rapid determination of plasma 7-dehydrocholesterol by ultraviolet spectrometry. *American Journal of Medical Genetics*, 68: 288-293.
15. Allain CC, Poon LS, Chan CSG, Richmond W & Fu P (1974). Enzymatic determination of total serum cholesterol. *Clinical Chemistry*, 20: 470-475.
16. Nair PP (1966). Quantitative methods for the study of vitamin D. *Advances in Lipid Research*, 4: 227-256.
17. Guzzetta V, De Fabiani E, Galli G, Colombo C, Corso G, Lecora M, Parenti G, Strisciuglio P & Andria G (1996). Clinical and biochemical screening for Smith-Lemli-Opitz syndrome. Italian SLOS Collaborative Group. *Acta Paediatrica*, 85: 937-942.
18. Axelson M (1991). Occurrence of isomeric dehydrocholesterols in human plasma. *Journal of Lipid Research*, 32: 1441-1448.
19. Jira PE, Jong JGN, Jansen-Zijlstra FSM, Wendel U & Wevers RA (1999). Pitfalls in measuring plasma cholesterol in the Smith-Lemli-Opitz syndrome (1997). *Clinical Chemistry*, 43: 129-133.
20. Angle B, Tint GS, Yacoub AO & Clark AL (1998). Atypical case of Smith-Lemli-Opitz syndrome: implications for diagnosis. *American Journal of Medical Genetics*, 78: 322-326.
21. Anderson AJ, Stephan MJ, Walker WO & Kelley RI (1998). Variant RSH/Smith-Lemli-Opitz syndrome with atypical sterol metabolism. *American Journal of Medical Genetics*, 78: 413-414.
22. Nowaczyk MJM, Whelan DT & Hill RE (1998). Smith-Lemli-Opitz syndrome: phenotypic extreme with minimal clinical findings. *American Journal of Medical Genetics*, 78: 419-423.
23. Nowaczyk MJM & Waye JS (2001). The Smith-Lemli-Opitz syndrome: a novel metabolic way of understanding developmental biology, embryogenesis and dysmorphology. *Clinical Genetics*, 59: 375-386.