Diagnosis of Smith–Lemli–Opitz syndrome from stored filter paper blood specimens

L Starck, A Lövgren

Abstract

Background—Smith–Lemli–Opitz (SLO) syndrome is a recessively inheritable metabolic disease with deficiency of cholesterol and accumulation of dehydrocholesterols, caused by a defect in the last step of cholesterol biosynthesis. Biochemical methods for identification of affected individuals, even prenatally, have been developed. Reliable genetic counselling is now possible.

Aim—To find a method of proving or disproving whether a child in whom SLO syndrome had been suspected but not confirmed during lifetime had in fact died of the SLO syndrome.

Methods—Lipid extracts of stored filter paper blood specimens collected at the national neonatal metabolic screening were used. The ratio of dehydrocholesterols to cholesterol was measured by combined gas chromatography–mass spectrometry.

Results—The ratio of 8-dehydrocholesterol to cholesterol in stored filter paper specimens clearly distinguished affected infants from normal infants. SLO syndrome was thus proven in two children who had died more than seven years earlier.

Conclusion—It is possible to diagnose SLO syndrome from dried paper specimens, even when the samples were collected more than a decade ago. Genetic counselling is available for families of affected children who died before the discovery of the defect in cholesterol synthesis.

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Keywords: Smith–Lemli–Opitz syndrome; filter paper blood specimens; inborn errors of metabolism; cholesterol; dehydrocholesterols

Neonatal screening programmes for metabolic diseases are used worldwide. The dried filter paper blood which is not used for routine analyses can be stored, and in Sweden samples from most infants since 1965 are available. Methods of using these for diagnosis of rare metabolic diseases have been developed. In 1987 the diagnosis of Zellweger syndrome was obtained by analysis of bile acids and plasmalogens from such specimens.¹

The autosomal recessive disorder Smith–Lemli–Opitz (SLO) syndrome was formerly diagnosed from certain stigmata such as microcephaly, dysmorphic facial features, genital abnormalities, and minor anomalies of the limbs.² Malformations of internal organs are common and the patients are mentally retarded and have impaired growth, severe failure to

thrive, photosensitivity, neurological problems, and an increased susceptibility to infections. The phenotype shows great variability and diagnosis is difficult from the clinical appearance.^{3–4} The discovery of a defect in the synthesis of cholesterol in the SLO syndrome⁵ therefore offers a most valuable tool for identifying patients even prenatally.⁶ A reduced activity of the enzyme catalysing the last step in the synthesis of cholesterol, 3 β -hydroxysterol Δ^7 -reductase, leads to a decrease in cholesterol and a notable increase in the precursor 7-dehydrocholesterol (7-DHC) and its epimer 8-dehydrocholesterol (8-DHC).⁷ The accumulation of dehydrocholesterols is detected in serum by gas chromatography–mass spectrometry. Before biochemical testing was available, the incidence of SLO in the United States was estimated to be 1/20 000–1/40 000.⁸ The true incidence is not known. The most severely affected individuals die prenatally or during the first year, but there is also some mortality during the toddler and preschool years. In surviving patients, beneficial effects of dietary supplementation with bile acids and cholesterol have been seen.^{9–10}

In the present work, we have investigated the possibility that the defect in the cholesterol synthesis found in SLO can be established by analysing sterols in stored filter blood samples collected at the neonatal screening. If it is possible to prove or disprove the diagnosis several years after a child has died without diagnosis, this would be of value, especially in connection with genetic counselling.

Materials and methods

SUBJECTS AND BIOLOGICAL MATERIAL

We have examined blood specimens on filter paper from six Swedish children with SLO. The samples were collected after the age of 72 hours and had been stored at a temperature of 4°C for 5–14 years at the time of analysis. We also analysed a sample from a boy born in 1985 who died in 1991 with strong clinical suspicion of this syndrome, and another sample from a sister of a girl with verified SLO who had clinical signs of SLO and died at the age of 5 weeks. As controls, we analysed 2–3 week old samples from 20 presumably non-affected newborn infants.

PROCEDURE

The paper discs (filter paper no. 2992, Schleicher and Schull, Dussel, Germany) containing between 12.5 and 15 μ l blood were cut in small pieces and sonicated for five seconds in a solution containing 0.2 ml 6 M aqueous KOH and 1 ml ethanol. The esters were hydrolysed in this mixture for one hour at room

Karolinska Institutet,
Sachs' Children's
Hospital, S-118 95
Stockholm, Sweden
L Starck

Division of Clinical
Chemistry, Huddinge
University Hospital,
Huddinge, Sweden
A Lövgren

Correspondence to:
Dr Starck
email: lena.starck@sos.sll.se

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temperature. After centrifugation, the mixture was extracted with chloroform/methanol (2:1, vol/vol) and applied to a C18 column in methanol/water (4:1, vol). The steroids were eluted with methanol and converted into trimethylsilyl ether.¹¹ Gas chromatographic analysis was performed on an HP 5972 MSD instrument equipped with an HP-5MS column (30 m × 0.25 mm × 0.25 µm) containing 5% Ph Me Silicone phase. Helium gas was used as the carrier, 0.8 ml/min. The temperature programme was as follows: initially 180°C for one minute, followed by an increase of 20°C per minute to 250°C, followed by 5°C per minute to 300°C. Finally, a fixed temperature of 300°C was used for 5.5 minutes. 7- and 8-DHCs were quantified by monitoring the molecular ion at *m/z* 456 and use of the peak area calculated by the computer (fig 1); cholesterol was quantified by monitoring the molecular ion at *m/z* 458 in the same way (chromatograms not shown).

Figure 1 Selected ion mass chromatography (*m/z* 456) of a silylated extract of filter paper from an SLO case and from a control infant. Under the chromatographic conditions employed, 8-DHC and 7-DHC have retention times of about 14.15 and 14.49 min, respectively. The compound occurring at a retention time between 14.03 and 14.12 min is mainly cholesterol, which has a very small isotope peak at *m/z* 456 (the molecular weight of the trimethylsilyl ether of *m/z* cholesterol is 458). Owing to the great overload of this compound on the column, the retention time is less well defined. The compound occurring with a retention time of 14.40 (X) has not been identified and may be a contaminant from the analytical procedure.

Table 1 Sterol to cholesterol ratio in stored dried blood samples from eight patients with SLO syndrome

Patient	Age of sample (y)	8-DHC:cholesterol	7-DHC:cholesterol
1	5	0.0189	0.0101
2*	5	0.0269	0.0209
3	7	0.0110	0.0091
4	9	0.0168	0.0073
5	14	0.0160	0.0144
6	14	0.0095	0.0051
7†	9	0.0093	0.0082
8‡	14	0.0257	0.0159

Died at the age of 2.5 years*, 6 weeks†, and 6 years‡. The tentative clinical SLO diagnosis was confirmed for the latter two cases.

Table 2 Sterol to cholesterol ratio in stored dried blood samples from 20 normal newborns

Ratio	8-DHC:cholesterol (number of samples)	7-DHC:cholesterol (number of samples)
0.0000	17	7
0.0001	3	1
0.0002		6
0.0003		1
0.0004		3
0.0005		1
0.0006		1

Results

In the chromatograms obtained in the analysis of derivatised material from children with SLO, there were significant peaks with retention times corresponding to those of 7- and 8-DHC which were not seen in the controls (fig 1).

In patients with SLO, the ratios of 8-DHC and 7-DHC to cholesterol were 0.0093–0.0269 and 0.0051–0.209, respectively (table 1). For children without SLO, the corresponding ratios were not higher than 0.0001 and 0.0006 (table 2).

The analyses of the nine and 14 year old filter paper blood specimens from the two deceased children showed ratios of 0.0093 and 0.0257 for 8-DHC to cholesterol and 0.0082 and 0.0159 for 7-DHC to cholesterol, thus confirming that they both had SLO syndrome.

Discussion

The measurement of the ratios between dehydrocholesterols and cholesterol in stored filter paper specimens in this investigation unambiguously distinguished patients with SLO syndrome from non-affected children. The 8-DHC:cholesterol ratio was a somewhat better discriminator than the 7-DHC:cholesterol ratio.

It should be emphasised that the concentrations of 7-DHC obtained by analyses of freshly collected specimens from SLO patients are more than one magnitude higher than in this 5–14 year old material.

7-DHC is known to be unstable and easily oxidised, and this is most probably also the case with 8-DHC. According to our experience, both 7- and 8-DHC are relatively stable in plasma. When they are present in dried plasma absorbed to a filter paper and exposed to air during long periods of time, a substantial auto-oxidation would be expected. From the limited material analysed here and with lack of information about the plasma concentrations of the different sterols at the time of collection

of the samples, it was not possible to evaluate whether there is a correlation between the time of storage and degree of degradation of 7- and 8-DHC.

The possibility of a falsely negative result cannot be completely excluded when analysing very old samples of the present type. Falsely positive results are unlikely however, as 7- and 8-DHC cannot be formed by auto-oxidation of cholesterol.

The present report is not the first concerning the use of filter paper blood specimens for the diagnosis of SLO syndrome. In 1997 Zimmerman *et al* described direct time of flight secondary ion mass spectrometry (TOF-SIMS) of filter paper analysed without hydrolysis, extraction, or separation.¹² In their study also, ratios of fragment ions for cholesterol:dehydrocholesterol were used. The oldest sample in the investigation was five years old. There was a clear difference between affected and normal subjects. This difference was more than 30-fold when the samples had been stored at -20°C but only about 3 to 10-fold in samples stored at room temperature. In our study with samples up to 14 years old, the difference between SLO and unaffected individuals was two orders of magnitude or more when the 8-DHC:cholesterol ratio was used. The higher discriminative power in our investigation may be because of a lower background and the fact that Zimmermann *et al* did not separate 7- and 8-DHC in their study. In addition, they only analysed the free fraction of the steroids. The degree to which storage at room temperature affects the stability of 7-DHC compared with storage at 4°C is not clear. However, the diagnosis of metabolic diseases from stored dried blood in patients no longer living is mainly limited by decomposition of compounds with time. In the study of Zellweger syndrome,¹ no plasmalogens were found in patient or controls in 13 year old samples.

In the present study, the diagnosis of SLO was obtained by analysis of stored filter blood

specimens. According to our results, the ratio between 8-DHC and cholesterol is the best discriminator by this procedure.

The SLO syndrome can thus be confirmed in deceased children in which it has been a tentative diagnosis and where no other material is available. With reliable prenatal diagnostic methods, this will have an impact on genetic counselling. Furthermore, information about the diagnosis is itself valuable to parents, no matter how much time has passed.

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