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Pitfalls in measuring plasma cholesterol in the Smith–Lemli–Opitz syndrome

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Correct quantitative results for plasma cholesterol, 7-dehydrocholesterol (7-DHC), and 8-dehydrocholesterol (8-DHC) are invaluable for making the correct diagnosis in patients with the Smith–Lemli–Opitz syndrome (SLO) and for biochemical monitoring of these patients during therapy. The enzymatic method for cholesterol measurement based on cholesterol oxidase gives falsely high values for plasma cholesterol in samples from patients with SLO. Both 7-DHC and 8-DHC contribute substantially to the test result, given that they are accepted substrates of cholesterol oxidase. All cholesterol methods making use of this enzyme are expected to give unreliable results with plasma samples from SLO patients. Cholesterol values found with these methods may be low-normal in individual cases with SLO. Therefore, other techniques for measuring cholesterol, 7-DHC, and 8-DHC, e.g., gas chromatography, should be used for diagnosing these patients and for follow-up during therapy. However, a normal value for plasma cholesterol, as obtained by gas chromatography, does not exclude SLO. The diagnosis should always be confirmed or excluded by testing for the presence of high concentrations of 7-DHC and 8-DHC in plasma. We found that one patient with a severe form of the disease had a plasma cholesterol concentration of 20 μmol/L—to our knowledge, the lowest value ever recorded in a human being.

INDEXING TERMS: cholesterol oxidase • inborn errors of metabolism • gas chromatography • dehydrocholesterol • enzymatic assays • analytical error

In 1964, Smith et al. *[1]* described a syndrome, now generally referred to as the Smith–Lemli–Opitz syndrome (SLO), characterized by a number of birth defects affecting nearly every organ system.[3] The patients are mentally retarded, have a growth disorder, and show failure to thrive. Both dysmorphic facial signs (microcephaly, palatoschizis, cataracts, ptosis, micrognathia) and limb abnormalities (syndactyly of the second and third toe, polydactyly) occur in the patients. Genital disorders, hypospadia, and cryptorchidism have been described. The syndrome is estimated to be among the most common autosomal recessive disorders among Caucasians, its prevalence being ~1:20 000 births *[2]*. A possible abnormality of steroid secretion was first postulated by Chesalow et al. *[3]*. Since then, Irons et al. have shown that SLO is caused by a defect in the cholesterol biosynthesis pathway *[4, 5]*. Patients have decreased concentrations of cholesterol in plasma and increased concentrations of the precursor 7-dehydrocholesterol (cholesta-5,7-dien-3β-ol; 7-DHC) and its isomer 8-dehydrocholesterol (cholesta-5,8-dien-3β-ol; 8-DHC) in plasma, erythrocytes, cultured skin fibroblasts, amniotic fluid, and various tissues *[4–8]*—findings that suggest a block in reduction of the C-7 double bond *[4, 5]*. Recently, those researchers confirmed that the enzyme defect involved the 7-dehydrocholesterol-Δ^7-reductase in liver microsomes *[9]*.

The aim of our study was to evaluate the reliability of the standard enzymatic assay (based on cholesterol oxidase) for measuring plasma cholesterol in samples from SLO patients. Because the technique for measuring 7-DHC in plasma is not widely available, clinicians may wish to use the presence of low concentrations of plasma cholesterol in patients with SLO as a first step towards confirming the diagnosis. Furthermore, measurement of plasma cholesterol will play a role in the follow-up of therapy strategies with high-cholesterol diets. We also compared the plasma cholesterol results obtained with

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[3] Nonstandard abbreviations: DHC, 7-dehydrocholesterol; 8-DHC, 8-dehydrocholesterol; GC, gas chromatography; and SLO, Smith–Lemli–Opitz syndrome.

the cholesterol oxidase method with those of a gas-chromatographic (GC) technique.

Materials and Methods

Patients. We analyzed plasma from eight patients (ages 2 weeks to 33 years) who had the characteristic clinical signs and symptoms of SLO. Forty-eight plasma samples, included as a diseased control group, had been sent to our laboratory for metabolic screening; however, the patients in this control group did not show clinical signs and symptoms characteristic for SLO, and some were under special clinical conditions (e.g., feeding problems, metabolic crises, special dietary formulas). This group provides the background against which metabolic screening laboratories have to diagnose patients with SLO. Because this study was not devised to obtain reference ranges for cholesterol in various age groups, the data presented later (in Table 1) should not be considered as such.

Enzymatic assays. Plasma cholesterol was measured at 30 °C with an enzymatic test on a Hitachi 747 analyzer (Boehringer Mannheim, Mannheim, Germany) according to the instructions of the manufacturer and with use of Boehringer reagents (CHOD-PAP test; SYS-3:1127578/1489704). In this test, cholesterol and cholesterol esters are converted by the sequential action of cholesterol esterase and cholesterol oxidase. The H_2O_2 formed in the latter reaction is determined quantitatively in the last step by using a peroxidase that converts phenol and 4-aminophenazone into 4-(*p*-benzoquinone-monoimino)-phenazone.

GC assays. For GC determination of cholesterol and its precursors (7-DHC and 8-DHC), we combined 60 μL of plasma with 7.58 nmol of 5β-cholestane-3α-ol as internal standard (no. C5050; Steraloids, Wilton, NH) in 1 mL of a solution of 0.32 mol/L KOH in 95% ethanol. The CFAS-calibrator for cholesterol was used (no. 759350; Boehringer Mannheim) in combination with two control sera (Precinorm and Precipath; also from Boehringer Mannheim). After an incubation of 15 min at 55 °C, 1 mL of H_2O and 4 mL of pentane were added and mixed for 5 min. The steroids, which were extracted into the pentane layer, were taken up by pipetting after centrifugation (5000*g* · min). The pentane was then evaporated with nitrogen at ~50 °C. To derivatize the steroids, we added 100 μL of an equivolume solution of *N,O*-bis(trimethylsilyl)trifluoroacetamide (no. 15238; Fluka, Buchs, Switzerland) and pyridine and incubated at 60 °C for 30 min. GC analysis was performed with a Hewlett-Packard (Amstelveen, The Netherlands) Model 5890 GC and a 25 m × 0.25 mm (i.d.) CP-Sil-19 CB column (film thickness 0.2 μm; Chrompack, Bergen op Zoom, The Netherlands). The temperature program was started at 240 °C, increased to 300 °C at 5 °C/min, and held for 3 min at 300 °C. Temperatures of the injector and detector were 280 and 300 °C, respectively. Pure 7-DHC for calibration purposes was purchased from Sigma Chemical Co., St. Louis, MO; no. D-3625); to quantify 8-DHC, we used the calibration curve for 7-DHC because 8-DHC for calibration was not available commercially.

Table 1. Sterol concentrations (μmol/L[a]) in plasma from patients with Smith–Lemli–Opitz syndrome and from diseased controls.

	Sex, age	Enzymatic cholesterol	Gas chromatography				
			Cholesterol	7-DHC	8-DHC	Total[b]	Ratio[c]
SLO patient							
1	F, 11 d	700	20	430	266	716	34.8
2	M, 7 m	1000	260	300	385	945	2.6
3	M, 7 y	1820	975	280	264	1519	0.6
4	M, 26 y	1560	1118	182	127	1427	0.3
5	M, 27 y	1300	884	143	107	1134	0.3
6	M, 28 y	1700	1170	220	284	1674	0.4
7	F, 29 y	2080	1261	442	308	2011	0.6
8	F, 33 y	3800	2030	848	555	3433	0.7
Range, diseased control group	<1 y (12)[d]	2400–4900	2523–4805	<7	<5		<0.004
	1–5 y (12)	2100–5300	2139–5447	<5	<5		<0.003
	6–15 y (10)	3200–5700	3138–5775	<5	<5		<0.002
	>15 y (14)	2900–8600	3174–8226	<8	<10		<0.003
Reference intervals for healthy children[e]	1–3 y	1150–4700					
	4–6 y	2800–4800					
	7–9 y	2900–6400					

[a] To convert cholesterol from μmol/L to mg/dL: 1000 μmol/L = 38.67 mg/dL.
[b] Cholesterol (GC) + 7-DHC (GC) + 8-DHC (GC).
[c] [7-DHC (GC) + 8-DHC (GC)]/cholesterol (GC).
[d] n for each group is listed in parentheses.
[e] 0.025–0.975 fractiles, according to Lockitch et al. *[13]*.

GC-MS. Mass spectra from peaks separated by GC were obtained by using a VG Trio 2 quadrupole mass spectrometer (Micromass, Altrincham, Cheshire, UK) coupled to the Hewlett-Packard 5890 GC. The cholesterol peak was characterized by fragments at *m/z* 329, 353, and 458. 7-DHC and 8-DHC gave identical spectra, with characteristic fragments at *m/z* 351 and 456.

Recovery studies. To test whether 7-DHC contributes to the result of the enzymatic cholesterol assay, we performed recovery experiments for cholesterol and 7-DHC in a human serum matrix and in human serum albumin solution (Sigma, no. A 16535; 50 g/L in 0.15 mol/L NaCl). The sterols, which had been dissolved to 33 mmol/L solution in ethanol, were added to both matrices to yield final concentrations of 1, 2, and 3 mmol/L. Complete solubilization was obtained by adding Nonidet P-40 (no. N6507; Sigma) to a final concentration of 100 mL/L.

Results

Enzymatic assay for plasma cholesterol. Table 1 shows the plasma cholesterol concentrations obtained with the cholesterol oxidase method for our diseased control group and the SLO patients. For the group of SLO patients as a whole, the cholesterol concentrations generally were below the concentration range found in the control group. However, there was an overlap between values for the two groups. One SLO patient (case 8) had a plasma cholesterol concentration of 3.8 mmol/L with this test, which would be interpreted as a low but normal value for an adult. The values found for the SLO children (cases 1–3) were close to the range found in our diseased control group; their diagnosis could easily have been missed if no further data had been available.

GC analysis of cholesterol, 7-DHC, and 8-DHC. The gas chromatograms of the plasma samples from a patient in the diseased control group and from two patients with SLO all showed peaks for the internal standard, cholesterol, 8-DHC, and 7-DHC (Fig. 1). Identification of the compounds was based on their retention times and on mass spectra (not shown). In agreement with the data from Axelson *[10]*, we observed that 7-DHC occurs in trace amounts in normal human plasma. Using calibration curves for cholesterol, 7-DHC, and the internal standard, we confirmed the linearity of the method for the concentration range used in this study (data not shown).

Plasma concentrations of cholesterol measured with GC vary considerably among SLO patients (20–2030 μmol/L) and also show an overlap in concentrations between the SLO group and the diseased control group, similar to the overlap observed in the enzymatic cholesterol assay. In all patients with SLO, the concentrations of 7-DHC and 8-DHC were clearly increased (GC results, Table 1). These data lead to a straightforward diagnosis for all SLO cases in this study. The ratio of (7-DHC + 8-DHC)/cholesterol may correlate with the severity of the disease. Patients 1 and 2, who had a severe form of the disease (type 1), gave higher values for this ratio than did the rest of the SLO group.

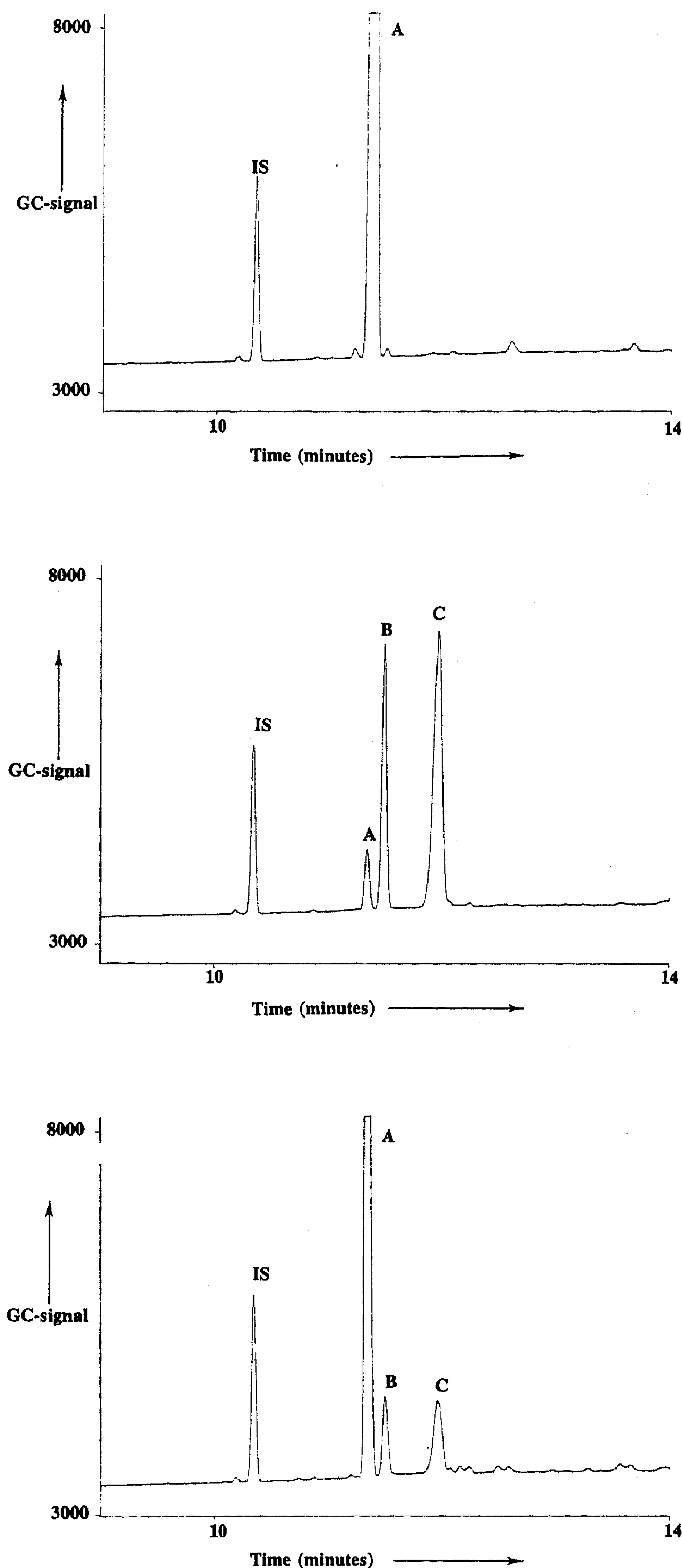

Fig. 1. Capillary-column gas chromatograms of control plasma (*top panel*) and plasma from two SLO patients (the *middle panel* shows plasma from patient 1 and the *bottom panel* plasma from patient 5, Table 1).

Peaks: *IS*, internal standard (5β-cholestane-3α-ol); *A*, cholesterol; *B*, 8-DHC; and *C*, 7-DHC.

The correlation between the enzymatic assay and the

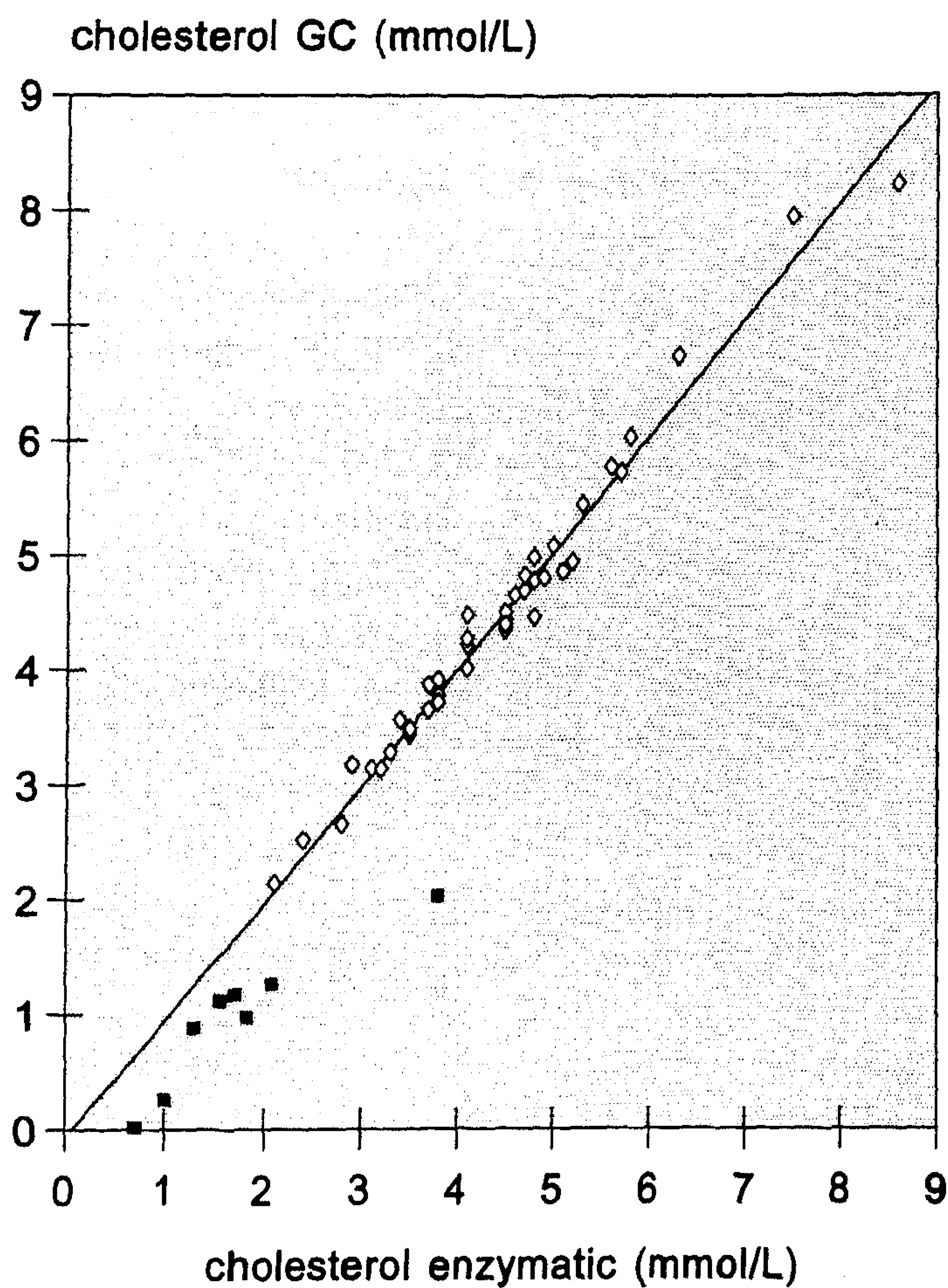

Fig. 2. Correlation between enzymatic cholesterol oxidase results and GC results for plasma cholesterol in SLO patients (■) and diseased control patients (◇).

GC assay results for plasma cholesterol was good in the diseased control group (Fig. 2), the Passing and Bablok (*[11]*) regression line (and 95% confidence intervals) being $y = 1.02$ (0.95–1.08) $x - 0.080$ (−0.31 to 0.20). In the SLO group, the results of both methods for the plasma samples all deviated from that correlation line, to yield $y = 0.68$ (0.37–1.15) $x - 0.211$ (−0.84 to 0.44). Because the cholesterol concentrations found for the SLO group were systematically higher by the enzymatic test than by GC (Table 1), we hypothesized that 7-DHC and 8-DHC might contribute to the results of the enzymatic cholesterol assay; consequently, we performed recovery experiments with cholesterol and 7-DHC.

Recovery experiments. Cholesterol recovery in the enzymatic assay was complete, the mean recovery in the serum matrix being 108% (n = 9, range 100–120%) and the mean in the albumin matrix being 94% (n = 9, range 90–100%). Most of the added 7-DHC was also measured: mean in serum matrix, 61% (n = 6, range 5–70%); mean in albumin matrix, 71% (n = 6, range 70–75%). Possibly, the known instability of 7-DHC contributed to the partial recovery of this compound.

Discussion

Reliable quantitative results for cholesterol, 7-DHC, and 8-DHC are invaluable to confirm the diagnosis in patients clinically suspected to have SLO, and for follow-up of SLO patients being treated with high-cholesterol diets. Until now, studies on reference values for plasma cholesterol have focused on the upper limit of the reference range: Very few data are available for the lower limit of the cholesterol reference range in plasma from children of various age groups. Lane and McConathy have studied changes in serum cholesterol in the first month of life using GC *[12]*. Lockitch et al. *[13]* determined age-related reference values for serum cholesterol in 450 healthy children, ages 1 to 19 years. Plasma cholesterol data in our diseased control group are in line with the data of Lockitch et al. *[13]*. The 0.025 fractile those authors found for the age groups older than 6 years is similar to the lowest value found in our diseased control group. For the age group between 1 and 5 years, Lockitch et al. found a 0.025 fractile value of 1.15 mmol/L, whereas the lowest value in our diseased control group was 1.67 mmol/L.

Because patients with SLO are often severely affected at birth, reference values for plasma cholesterol in the first weeks of life are of special interest. Lane and McConathy *[12]* observed that cholesterol at day 28 postpartum ordinarily is in the lower 5th percentile of the adult population values; at day 3 postpartum, values were even lower *[12]*. Age-related reference values are thus invaluable for the correct interpretation of plasma cholesterol concentrations in children. As Table 1 makes clear, SLO patients measured with the enzymatic assay may give plasma cholesterol values close to the lower limit of the range of the diseased control group or even within this range. In one of our adult patients (case 8), the plasma cholesterol concentration of 3.8 mmol/L would be interpreted as normal. We were able to better discriminate between both groups by assessing the cholesterol values measured by GC. However, even with this assay, the result for cholesterol could easily be misinterpreted. We conclude that the diagnosis of SLO cannot be excluded definitely on the basis of the plasma cholesterol results.

The control samples gave results by both the enzymatic and the GC method for cholesterol that correlated well (Table 1 and Fig. 1). For SLO patients, however, obvious differences between the techniques were apparent, the cholesterol concentrations measured with the enzymatic assay being invariably higher than the GC results. As Table 1 shows, the summed GC data for cholesterol, 7-DHC, and 8-DHC correlated well with the enzymatically determined cholesterol concentration. This suggests that 7-DHC and 8-DHC may contribute to the plasma cholesterol results measured by the enzymatic cholesterol assay. We partly confirmed this by adding 7-DHC to solutions of albumin and to plasma samples. The majority (61–71%) of 7-DHC added was measured as cholesterol by the enzymatic cholesterol test, in both the serum matrix and the human albumin matrix. The results for cholesterol measured by GC were not influenced by these additions (data not shown). Apparently the enzymatic cholesterol test cannot discriminate between the various steroids. This may be due to aspecific conversion of 7-DHC and 8-DHC by cholesterol oxidase. Using a sterol monolayer

system, Slotte has described that cholesterol oxidase from *Streptomyces cinnamomeus* oxidizes 7-DHC at a rate 5.1-fold slower than it oxidizes cholesterol *[14]*. We expect that similar results will be found for other commercially available reagents for plasma cholesterol determinations that make use of cholesterol oxidase. Our results indicate that the enzymatic test for measuring plasma cholesterol gives falsely high results for SLO plasma samples.

The plasma cholesterol concentration in our youngest patient with SLO was 20 μmol/L, whereas the (7-DHC + 8-DHC)/cholesterol ratio for this patient was by far the greatest we saw. This is, to our knowledge, the lowest plasma cholesterol ever reported in humans. Clinically, the affected girl had a very severe form of the disease (type I), with major malformations; the patient died at age 5 weeks. As also described by Tint et al. *[15]*, we observed that the plasma cholesterol concentration in older SLO patients is generally higher than in young, more severely affected patients. In our study the lowest and the highest cholesterol values we saw differed by 20-fold. Those for 7-DHC differ by only 6-fold, and the concentrations of 7-DHC and 8-DHC are not significantly different between younger and older patients in our study. The (7-DHC + 8-DHC)/cholesterol ratio, however, is clearly higher in the young patients (cases 1 and 2 in Table 1).

The differences in plasma cholesterol concentrations of SLO patients at different ages might result from a higher dietary intake of cholesterol in older, clinically more mildly affected patients. Kelley et al. have suggested *[16]* that higher cholesterol concentrations correlate more with the length of survival and the amount of dietary cholesterol than with clinical severity. Another explanation for higher cholesterol concentrations in older SLO patients could be the residual activity of the cholesterol biosynthesis pathway in older patients, as was suggested by Tint et al. *[15]*. This would also explain the milder course of the disease in this group. At present, it is unknown whether the shortage of cholesterol and the abundance of cholesterol precursors both contribute to the development of the clinical signs and symptoms of SLO. As with cholesterol oxidase, other enzymes involved in cholesterol-converting pathways may also accept a 7-dehydro or 8-dehydro variant of their normal substrates. This would give rise to unexpected intermediates with unpredictable functional characteristics. This concept may give further impetus to attempts to understand the importance of cholesterol precursors in the development of the clinical picture.

Summarizing, we conclude that the enzymatic test for measuring plasma cholesterol gives unreliable results in SLO patients. For diagnosis of patients clinically suspected to have the disease, measurements of plasma cholesterol (by any method) should not be used. Obviously, quantification of plasma 7-DHC and 8-DHC is the method of choice here *[4, 5, 15]*. For follow-up studies of SLO patients receiving high-cholesterol dietary treatment, the enzymatic test for measuring plasma cholesterol is also not suitable. Instead, the method of choice for this purpose may be GC, which provides reliable quantitative data for plasma cholesterol, 7-DHC, and 8-DHC.

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