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Short communication

Effects of sample storage on 7- and 8-dehydrocholesterol levels analysed on whole blood spots by gas chromatography–mass spectrometry-selected ion monitoring

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Abstract

Smith–Lemli–Opitz syndrome (SLOS) patients have increased 7- and 8-dehydrocholesterol (DHC) concentrations. Using gas chromatography–mass spectrometry with selected ion monitoring we investigated whether storage time (24 h, 7 and 30 days, and 22 months at room temperature or at 4°C) affected DHC concentrations in whole blood spots (WBSs) from SLOS patients and normal controls. Our results suggest that WBS sterol analysis can be used for SLOS screening and possibly related inborn errors of sterol metabolism with a 100% sensitivity and specificity on specimens stored for up to 30 days, either at room temperature or 4°C. After 22 months of storage at both temperature SLOS samples can be indistinguishable from control samples. Therefore, great caution should be used to exclude SLOS by sterol analysis of WBSs stored for a long time.

Keywords: Dehydrocholesterol

1. Introduction

Smith–Lemli–Opitz syndrome (SLOS) is a multiple congenital anomalies-mental retardation syndrome, characterised by delayed psychomotor development, failure to thrive, typical facies, limb malformations, incomplete development of male genitalia and possible association of internal organ anomalies [1]. These signs are variably associated and the disease phenotype represents a clinical continuum from the severe ''type 2'' SLOS to the milder ''type 1'' form [2].

SLOS is a dysmorphic syndrome characterized by major malformations due to a single biochemical defect. In 1994 Tint et al. [3] demonstrated that SLOS results from deficiency of Δ^7-dehydrocholesterol reductase (DHCR), the enzyme that catalyses the last step of cholesterol biosynthesis. SLOS patients typically have low cholesterol levels and increased 7- and 8-dehydrocholesterol (DHC) concentrations. Recently, the DHCR-encoding gene has been cloned and several mutations have been identified in SLOS patients [4–6]. Interestingly, high levels of 8-DHC have been reported in Conradi–

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Hunermann–Happle syndrome (CDPX2) [7] and in the CHILD syndrome [8].

The incidence of SLOS is controversial. An incidence of 1:40 000 was initially suggested on clinical grounds. When a biochemical diagnosis became available, the incidence rose to 1:20 000 [9], although this value might be underestimated in some ethnic groups [10,11]. Since ''type 2''-SLOS patients often die shortly after birth, before a precise diagnosis can be made [10], neonatal screening of multiple malformed newborn babies for SLOS would lead to a more accurate definition of the prevalence in different ethnic groups. Neonatal screening would also result in timely cholesterol supplementation.

Recently dehydrocholesterols have been evaluated as a ratio versus total cholesterol in whole blood spots (WBSs) [12,13]. It was suggested that DHC concentrations on WBSs decrease with time [13] but nothing is known about the relation between storage time and degree of DHC degradation. Consequently, we used quantitative gas chromatography–mass spectrometry with selected ion monitoring (GC–MS-SIM) analysis to investigate changes in DHC concentrations in WBSs after different storage times.

2. Experimental

2.1. Patients

Five SLOS type 1 patients and 20 normal controls were recruited for the study. Four SLOS patients showed typical clinical features, enhanced serum levels of 7- and 8-DHC, and mutations of both alleles of the DHCR gene (patients 1, 2, 3 and 4, in Ref. [5]). Patient 5 also showed the typical clinical and biochemical phenotype (patient 6 in Ref. [14]). SLOS patients followed a normal diet for age supplemented with 50–100 mg/kg/day of pure cholesterol. Control subjects were on normal diets. Blood was obtained after an overnight fast. The total serum cholesterol levels of SLOS patients, detected with an enzymatic method, ranged between 530 and 1280 mg/l.

2.2. Chemicals

All chemicals were purchased from Sigma (St. Louis, MO, USA).

2.3. Blood samples

Blood samples from control subjects were collected with vacutainer tubes (EDTA-K3). The tubes were returned to the laboratory and several WBSs for each sample were obtained by pipetting 20 μl of well-mixed blood onto two Guthrie cards (Schlicher and Schuell, type no. 2992). When the spots were dry, they were cut and analyzed in duplicate. The remaining spots were stored at room temperature or at 4°C, protected from light, for analysis after 7 and 30 days and 22 months. Whole blood spots from SLOS patient 5 and from negative controls (his parents) were punched off and analysed after 5 years of storage at room temperature protected from light.

2.4. Total sterol analysis

Total sterols were hydrolysed directly from WBSs by an alkaline solution. A fixed amount (2.5 μg) of 5α-cholestane (internal standard) and 3 ml of 1 *M* KOH in 90% alcohol were added and the solution was incubated for 1 h at 80°C. After cooling, the samples were diluted with 3 ml of water and extracted three times with 3 ml of hexane. The upper organic phases were pooled and evaporated under a gentle stream of nitrogen. The dry residue was derivatized with 100 μl of a mixture of bis-trimethylsilyltrifluoroacetamide–pyridine (1:1) for 20 min at 80°C, to obtain trimethylsilyl (TMS) ether derivatives. After cooling, 1 μl of this solution was injected into a GC–MS apparatus (Model GC 5890, MSD 5970, Hewlett-Packard, Palo Alto, CA, USA).

2.5. Analysis conditions

A HP1 fused-silica capillary column (25 m×0.2 mm I.D., 0.33 μm thick, Hewlett-Packard) was used to separate sample sterols. An injector, operating in the splitless mode (0.5 min), and transfer line temperatures were fixed at 280°C. Helium (1 ppm impurity) was used as carrier gas with a flow-rate of 45 cm/s. The oven temperature was maintained at 200°C for 1 min, then increased to 260°C at 20°C/min, and finally to 300°C at 3°C/min. The retention times and mass spectra of chromatographic peaks obtained from blood spots as TMS sterol derivatives were compared with those obtained from authentic compound (7-DHC standard from Sigma). The SIM

mode was employed to quantify sterols and internal standard:three ions at m/z 372, 357 and 217 were used to monitor 5α-cholestane from 12 to 16 min, and the ions at m/z 456, 351 and 325 were used to detect 7-DHC and 8-DHC from 16 to 25 min.

Sterol concentrations (mg/l) were obtained from the peak area ratios of each compound compared with the internal standard, multiplied for relative response factors (RRFs) previously calculated from calibration curves obtained with variable amounts of 7-DHC standard and fixed amounts of internal standard. A plasma pool spiked with 7-DHC standard (450 mg/l) was used for the calibration curves. This solution was diluted with a pool of negative plasma to prepare different points of the calibration curve. The method was linear from 3.5 to 450 mg/l, a typical equation of calibration curve was $y=0.00365x+0.0239$ ($n=3$; $r^2=0.9863$; SE of slope=0.000092; SE of intercept=0.0167). Within-day and day-to-day imprecisions of 7-DHC at a level of 60 mg/l were 6.6 and 7.3%, respectively. Using this method the lower limit of detection (LLD) was 0.67 mg/l with a 20 μl sample. To quantify 8-DHC, we used the RRF of the calibration curve for 7-DHC because 8-DHC authentic standard was not available as already reported [15].

3. Results

Fig. 1 shows typical ion chromatograms of the WBS sterol analysis of an SLOS patient (stored for

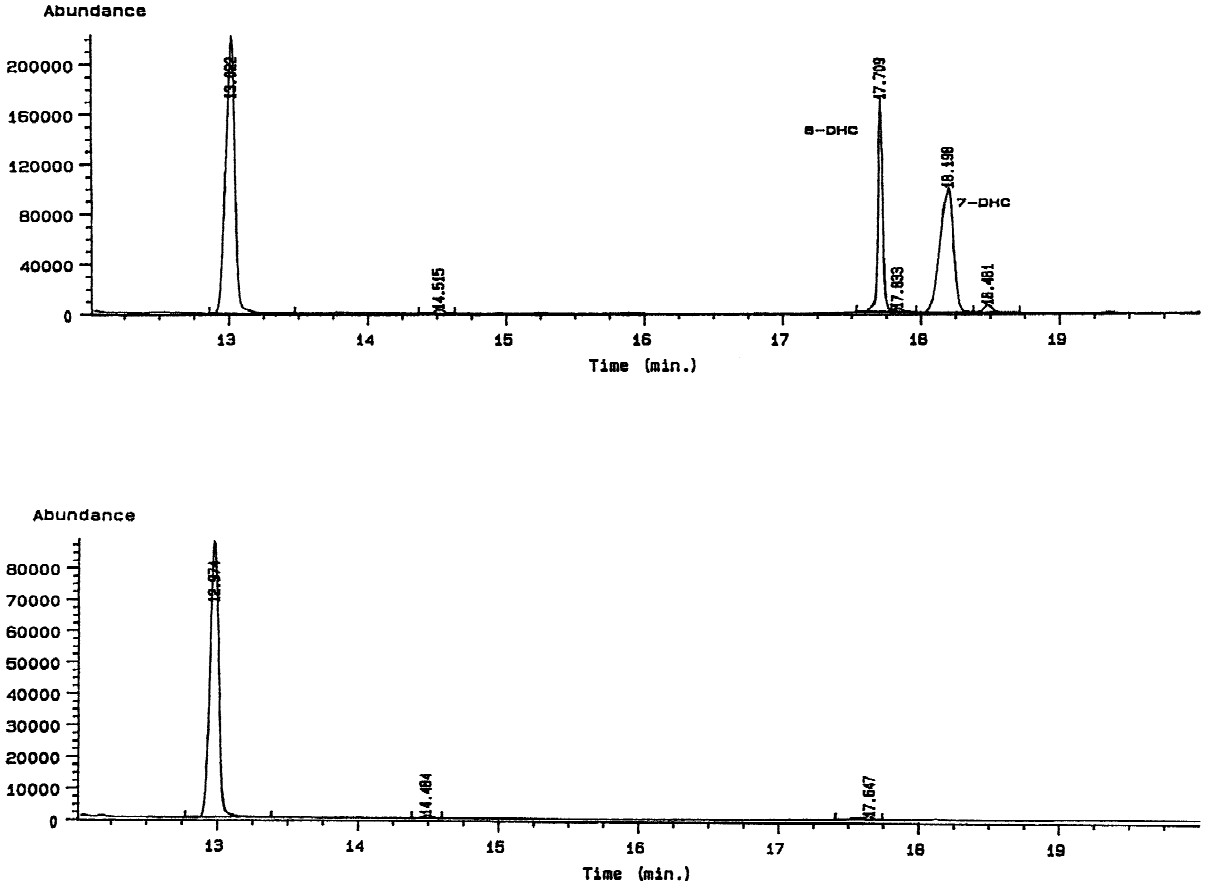

Fig. 1. Typical selected ion chromatogram obtained from WBS sterol analysis of an SLOS patient stored at 4°C for 30 days (upper panel) and normal control not stored (lower panel). The peaks identified as 7-DHC (185 mg/l) and 8-DHC (131 mg/l) are well resolved and consistent, in the chromatogram of the SLOS patient compared to the control. The peak of 7-DHC is broader than that of 8-DHC as previously reported by Axelson [16].

30 days at 4°C) and a control sample (not stored). Three mass/charge ions were monitored for 7- and 8-DHC (m/z 456, 351, 325) and the chromatogram was derived from the total ion current. The peaks identified as 7-DHC (eluted at 18.198 min) and 8-DHC (eluted at 17.709 min) were well resolved and more consistent in the SLOS chromatogram (Fig. 1, upper panel) compared with the control (Fig. 1, lower panel). The peaks at 12.974 and 13.022 min represent the internal standard 5α-cholestane. All normal samples showed a short and broad peak of unknown origin that eluted at the same retention time (17.647 min) as 8-DHC; no additional peak was found at the retention time of 7-DHC.

GC–MS-SIM analysis of WBSs was performed on SLOS samples within the first 24 h of withdrawal, after 7 and 30 days and after 22 months of storage at room temperature or 4°C. Up to 30 days of storage, all patients were identified by the WBS GC–MS-SIM analysis (Table 1 and Fig. 2). After 7 and 30 days, irrespective of storage temperature, DHC concentration decreased, although not significantly, in SLOS specimens; this effect was more pronounced in samples stored at room temperature (RT). This finding can be attributed to the previously reported instability of DHC [12]. After 22 months of storage the mean concentration of DHC in SLOS samples was significantly lower than basal values (Table 1 and Fig. 2). After 30 days, DHC was significantly increased on control specimens. This finding can be due either to the instability of cholesterol or to the interference from decomposition products eluted at the retention times of DHC (Table 1).

4. Discussion

We evaluated the correlation between storage time and DHC levels on WBSs using the procedure recommended for the classical plasma/serum sterol analysis for the diagnosis of SLOS [14]. The sensitivity and specificity of WBS sterol analysis by GC–MS-SIM is 100%; reproducibility and accuracy is good, and the method is fairly fast (about 2 h and 20 min). Only a small blood sample is required, which is important given the low amount of blood available during the neonatal period.

In a time-of-flight ion mass spectrometry (TOF-SIM) method [12], DHC concentrations are identified from only one peak (365.31 Da) and the DHC/cholesterol ratio is used to distinguish SLO patients from normal individuals. Although TOF-SIM is a faster procedure, GC–MS is less expensive and is more widely used. Second, unlike TOF-SIM that measures the cholesterol/DHC ratio, GC–MS is a direct quantitative method. Third, GC–MS-SIM sterol analysis is more specific because it is based on both gas chromatography and mass spectrometry, and not single mass spectrometry like TOF-SIM.

In a recently described qualitative DHC analysis

Table 1
Comparison between WBS DHC levels detected by GC–MS-SIM in SLOS patients and controls

	Time of storage			
	First 24 h	7 days	30 days	660 days
SLOS patients ($n=4$)				
7-DHC (RT)	366±174	139±88	114±54	1.01[b]
7-DHC (4°C)	332±155	209±75	147±83	23±18
8-DHC (RT)	215±42	102±62	83±33	1.24[b]
8-DHC (4°C)	200±42	110±59	126±48	14±7.9
Controls ($n=20$)				
7-DHC (4°C)	0.65±1.02	0.93±0.83[a]	3.4±2.01[a]	ND
8-DHC (4°C)	3.53±2.41	2.21±1.57[a]	3.63±1.96[a]	ND

All values are reported as mean±standard deviation (SD) (mg/l).
ND: Not determined.
[a] These values are overestimated by the presence of unknown metabolites eluting at the same retention time of the DCHs (see the text).
[b] Value obtained from patient 2.

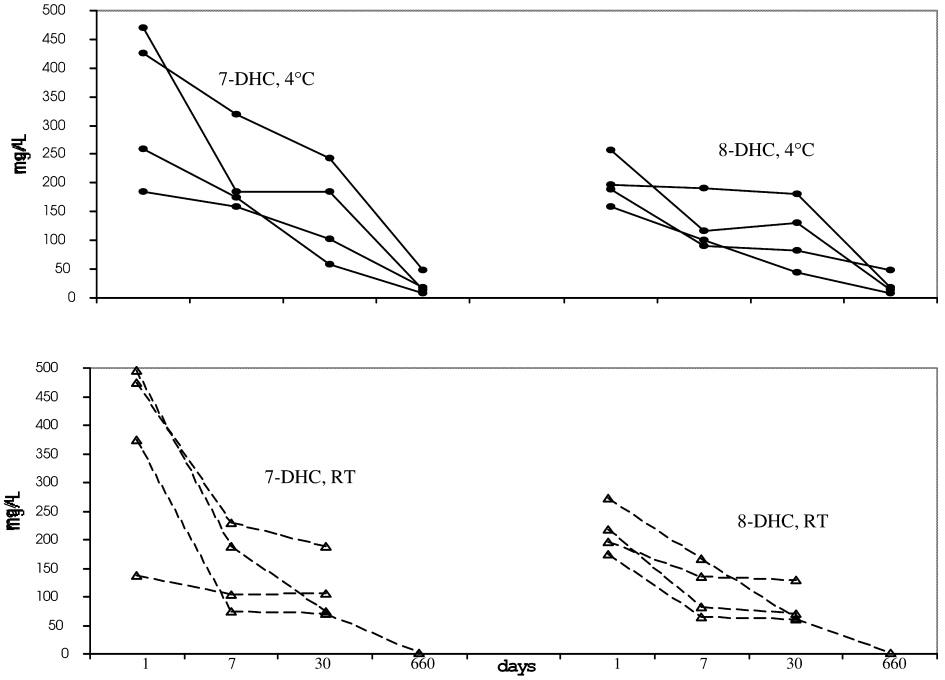

Fig. 2. Time course of 7-DHC and 8-DHC obtained from WBS sterol analysis of SLOS patients ($n=4$) after storage at room temperature (RT) and refrigerated (4°C).

by GC–MS on WBSs [13], DHC was identified by monitoring the molecular ion at m/z 456. Patients were distinguished from controls by the 8-DHC/cholesterol ratio. However, 7-DHC is considered the diagnostic metabolite of SLOS, whereas 8-DHC has been also found in other syndromes due to defects of other steps of cholesterol biosynthesis [7,8]. If only the molecular ion is monitored, as in the two methods described above [12,13], it cannot be excluded that the procedures are liable to false positives, since unknown metabolites, may result from sterol decomposition and they would not be distinguished from DHC. In the method described herein, three mass/charge ions for 7- and 8-DHC are monitored.

We analysed DHC for WBSs stored at room temperature and at 4°C within 24 h of sampling, after 7 and 30 days, and after 22 months. Up to 1 month of storage GC–MS-SIM significantly distinguished between controls and patients. Levels of 7- and 8-DHC lower than 3.5 and 7 mg/l, respectively were considered negative results. Levels of 7- and 8-DHC between 3.5 and 8 mg/l and between 7 and 10 mg/l, respectively, were considered borderline. Borderline DHC values, especially when obtained on WBS samples stored for 30 days, should be confirmed by fresh plasma GC–MS sterol analysis. At 22 months and after 5 years of storage at room temperature, the procedure did not distinguish SLOS patients from controls. Consequently, caution must be exercised in excluding SLOS on the basis of WBS sterol analysis of specimens stored for long periods. Because the concentration of DHC in SLOS WBS decreases with time, particularly in samples stored at room temperature, it is reasonable to store the spots at 4°C until analysis.

In conclusion, our data indicate a decrease of DHC in WBSs probably due to their instability. All patients were correctly diagnosed by the WBS GC–MS-SIM sterol analysis when the WBS were stored for 7 and 30 days at room temperature or 4°C. False negatives cannot be excluded in the sample stored for years. This simple quantitative method for 7- and 8-DHC analysis of WBSs by GC–MS-SIM could be

a valid method to screen multiple congenital anomaly syndromes for SLOS and, possibly, for other related sterol disorders, particularly in the neonatal age using WBSs stored at RT or 4°C for up to 30 days.

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