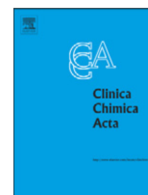
Clinica Chimica Acta 413 (2012) 525–526

Contents lists available at SciVerse ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim



Letter to the editor

Stability study of dehydrocholesterols in dried spot of blood from patients with Smith–Lemli–Opitz syndrome, using filter-paper treated with butylated hydroxytoluene

Smith–Lemli–Opitz syndrome (SLOS; MIM 270400) is an autosomal recessive disease caused by a defect in 7-dehydrocholesterol (7-DHC) reductase (EC 1.3.1.21), the enzyme that catalyzes the last step of cholesterol (CHOL) biosynthesis. Patients affected by this disorder show a large and variable clinical spectrum with multiple morphogenic and congenital anomalies including internal organ, skeletal and/or skin abnormalities, growth and mental retardation and behavioral problems. Typically, patients affected by SLOS show an accumulation of 7-DHC and 8-dehydrocholesterol (8-DHC) in blood, and carry disease-causing mutations in the gene encoding the implicated enzyme [1–3]. Clinical severity correlates negatively with the CHOL concentration, and positively with the 7-DHC concentration and the sum of dehydrocholesterols (DHC; 7-DHC + 8-DHC) [2,4]. The DHC fraction (DHC/total sterols) and DHC/CHOL ratio better express the systemic sterol abnormality than absolute blood sterol concentrations [2].

The SLOS incidence is variable and difficult to estimate accurately, and its exact value is unknown. As recently reported, the SLOS incidence is estimated at one in 15,000–60,000 births in the European population [4–6] and at one in 60,000 births in the United States [2]. Based on the allele frequencies and the proportion of the most frequently occurring mutation (IVS8-1 G > C) observed in various patient populations, the expected incidence of SLOS in those populations was calculated and reported to range from 1:1,590 to 1:17,000. The discrepancy between the expected incidence and prevalence can be explained only in part by the neonatal and infancy deaths of the most severely affected children with SLOS and the under-assessment of mild and atypical cases [7].

Until now, there has been no screening test that could be applied routinely to the newborn filter paper specimen. Techniques such as routine biochemical assays and tandem mass spectrometry have not been employed successfully. Time-consuming and impractical methods for high-throughput screening, such as gas chromatography (GC) of plasma sterol profiles, currently are preferred for diagnosis. In addition, the instability of 7-DHC and 8-DHC is particularly elevated in dried blood spot (DBS) specimens, which present significant preanalytical variability, influencing the accuracy of results [8–10]. Considering the clinical importance of implementing a screening method for CHOL and DHC in DBS, this study aims to standardize the preanalytical variability of DHC in DBS for detecting SLOS by using filter paper impregnated with BHT as an antioxidant.

Samples from SLOS patients (n = 5) were obtained during follow-up of sterol profiles. Whole blood was collected into Vacutainer tubes containing EDTA through venipuncture at pediatric units, and was sent to our laboratory. All samples analyzed in this study were reserve materials that were not needed for further diagnostic investigations, which prevented the need to take extra materials or a greater sample volume from patients. The DBS were prepared by spotting 20 μL of whole blood onto filter paper (Whatmann 3MM) with and without BHT. Spots were dried for 3 h, while being protected from direct light, and stored in a desiccant sealed plastic bag at 4 °C until analysis. Filter paper treatment with BHT was performed with strips of 6 × 10.5 cm, which were horizontally immersed in 2.5 mL of $CHCl_3$ solution containing 0.1 mg/mL BHT. The strip was soaked in this solution for ≤1 min. After complete absorption of the BHT solution (about 3.97 μg of BHT per cm^2 of filter paper), the strip was air dried at room temperature for 30 min while being protected from direct light. For each sample, at least 16 spots were prepared on both filter papers (with and without BHT). A 6-mm disc was punched from the DBS and mixed with 1 mL of 1 N potassium hydroxide in 90% ethanol and 25 μL of cholestane as internal standard (IS, 0.4 mg/mL). The sterols were hydrolyzed for 60 min at 80 °C. After, the sample was diluted with 1 mL of distilled water and extracted three times with 1 mL of hexane. The upper organic phases were pooled and evaporated under N_2 flow at 40 °C. The dried residue was dissolved in 50 μL of CH_2Cl_2, and 1 μL of this solution was injected into a GC instrument. All samples were analyzed in duplicate. Sterols were fractionated with a capillary column (SAC-5, 30 m length, 0.25 mm I.D., 0.25 μm film thickness; Supelco, Germany) by a GC system equipped with a flame ionization detector (GC-FID, HP-5890, Agilent Laboratories, CA, USA). The injector and detector temperatures were fixed at 300 °C, and the oven temperature was maintained at 290 °C. The total run time was 10 min. Sterol concentrations (mg/dL) were obtained by interpolation of the analyte/IS peak-area ratio on calibration curves. The relative retention times of chromatographic peaks of CHOL, 8-DHC, and 7-DHC, compared to IS, were 1.43, 1.46, and 1.52, respectively. The imprecision of method (CV%), performed on two levels of the positive plasma pool, for CHOL (24.1 and 40 mg/dL) was 3.9 and 4.3, for 8-DHC (4.4 and 7.6 mg/dL) was 4.2 and 5.1, and for 7-DHC (11.1 and 18 mg/dL) was 5.5 and 5.6, respectively.

In SLOS patients, the DBS concentration values at baseline (analyzed within 1 d) ranged, for CHOL, from 10.5 to 56.3 mg/dL (0.27 to 1.46 mmol/L) without BHT and from 9.4 to 56.3 mg/dL (0.24 to 1.46 mmol/L) with BHT; for 8-DHC, from 3.6 to 7.4 mg/dL (0.095 to 0.19 mmol/L) without BHT and from 3.8 to 8.4 mg/dL (0.1 to 0.22 mmol/L) with BHT; and for 7-DHC, from 5.6 to 15.2 mg/dL (0.15 to 0.4 mmol/L) without BHT and from 5.9 to 19.1 mg/dL (0.15 to 0.5 mmol/L) with BHT. The DHC/CHOL ratio ranged from 0.23 to 2.1 without BHT and from 0.24 to 2.2 with BHT.

Table 1 reports the average sterol levels and DHC/CHOL ratios in DBS without and with BHT obtained after sample storage at 4 °C over 1 (baseline), 3, 7, 14, 28, or 56 d. In DBS without BHT, a statistically significant decrease from baseline values (based on ANOVA analysis with Bonferroni posttest correction) was observed, for 7-DHC, after storage for 21 d (−72.4%), 28 d (−75.5%), or 56 d (−81.2%); for 8-DHC, after storage for 7 d (−49.6%), 14 d (−54.3%), 21 d (−63.1%), 28 d (−67.4%), or 56 d (−72.7%). No significant changes from baseline values were observed in the 7-DHC and 8-DHC levels in DBS with BHT. Although the DHC/CHOL ratio values in DBS without BHT after 56 d of storage revealed a marked

0009-8981/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.cca.2011.11.008

Table 1
Sterol levels and DHC/CHOL ratios in SLOS patients obtained from DBS without and with BHT, the values are reported as averages and standard deviations (SD).

SLOS DBS ($n = 5$)	Storage days						
	1	3	7	14	21	28	56
	(mg/dL)[a]						
CHOL no BHT	30.6 (17.6)	29.3 (17.0)	28.5 (15.1)	27.0 (11.4)	25.9 (12.6)	26.8 (12.9)	29.9 (20.6)
CHOL with BHT	30.1 (17.8)	31.3 (18.5)	34.3 (20.5)	31.7 (18.7)	29.2 (17.7)	31.1 (18.7)	29.9 (17.9)
7-DHC no BHT	10.8 (4.4)	7.1 (4.3)	4.8 (4.5)	3.9 (3.8)	3.1 (2.5)[b]	2.7 (2.0)[b]	2.0 (1.5)[b]
7-DHC with BHT	11.4 (5.3)	11.9 (5.2)	12.5 (5.8)	11.6 (5.3)	10.5 (5.1)	11.0 (4.8)	9.3 (3.6)
8-DHC no BHT	5.8 (1.5)	4.3 (1.2)	2.8 (1.2)[c]	2.5 (1.0)[c]	2.1 (0.6)[c]	1.8 (0.5)[c]	1.5 (0.3)[c]
8-DHC with BHT	6.2 (1.8)	6.2 (1.7)	6.6 (2.0)	5.9 (1.9)	5.4 (2.0)	5.3 (1.8)	4.5 (1.2)
DHC/CHOL no BHT	0.80 (0.75)	0.58 (0.53)	0.35 (0.28)	0.30 (0.24)	0.28 (0.24)	0.24 (0.22)	0.19 (0.18)
DHC/CHOL with BHT	0.85 (0.78)	0.86 (0.80)	0.82 (0.75)	0.81 (0.76)	0.79 (0.73)	0.78 (0.73)	0.73 (0.72)

[a] Conversion factors to SI units (mg/dL × Factor = mmol/L) are: 0.0259 for Cholesterol; 0.0260 for 7- and 8-DHC.
[b] Significantly different from storage day 1: $p<0.05$.
[c] Significantly different from storage day 1: $p<0.001$.

decrease from baseline (−76%) compared to values obtained in DBS with BHT (−17.1%), the results were not statistically significant (Fig. 1 in Supplementary file). For CHOL, after 56 d of storage, no significant changes of values compared to baseline were found in DBS without (−2.1%) or with BHT (−4.8%). Furthermore, the DHC/CHOL ratio in DBS showed a significant negative logarithmic correlation with the storage time at 4 °C without BHT [$y = -0.1482 \ln(x)$; $r = 0.955$], and the decreasing rate of the DHC/CHOL ratio was 5-fold faster than that observed in DBS with BHT [$y = -0.0296 \ln(x)$; $r = 0.935$].

These data confirm that DHC in DBS are highly unstable and easily oxidizable. From our experience and according to others, DHC in plasma are relatively stable, but substantial auto-oxidation would be expected for DHC in plasma adsorbed onto filter paper and exposed to air for long periods of time [9,10]. Recently, we have also demonstrated that the oxidation rate of DHC in erythrocyte membranes from SLOS patients was at least 2-fold higher than that in plasma [11]. Based on our results, the antioxidant BHT added to filter paper for the preparation of DBS stabilizes the concentration of DHC at least for two months of storage. This procedure could be adopted to prevent the potential occurrence of false negative results for the analysis of sterols in DBS using gas chromatography or other methods.

In summary, considering that SLOS is the most frequent CHOL biosynthesis defect, and its prevalence has not yet been accurately determined, DBS treated with BHT could be recommended for the measurement of DHC in DBS by using a screening method.

Supplementary materials related to this article can be found online at doi:10.1016/j.cca.2011.11.008.

Acknowledgments

We are grateful to Dr. Oceania D'Apolito (University of Foggia) for her helpful suggestions.

References

[1] Porter FD. Smith–Lemli–Opitz syndrome: pathogenesis, diagnosis and management. Eur J Hum Genet 2008;16:535–41.
[2] Kelley RI, Hennekam RC. The Smith–Lemli–Opitz syndrome. J Med Genet 2000;37:321–35.
[3] Tint GS, Irons M, Elias ER, et al. Defective cholesterol biosynthesis associated with the Smith–Lemli–Opitz syndrome. N Engl J Med 1994;330:107–13.
[4] Cunniff C, Kratz LE, Moser A, et al. Clinical and biochemical spectrum of patients with RSH/Smith–Lemli–Opitz syndrome and abnormal cholesterol metabolism. Am J Med Genet 1997;68:263–9.
[5] Witsch-Baumgartner M, Schwentner I, Gruber M, et al. Age and origin of major Smith–Lemli–Opitz syndrome (SLOS) mutations in European populations. J Med Genet 2008;45:200–9.
[6] Jezela-Stanek A, Ciara E, Małunowicz E, et al. Differences between predicted and established diagnoses of Smith–Lemli–Opitz syndrome in the Polish population: underdiagnosis or loss of affected fetuses? J Inherit Metab Dis 2010, doi:10.1007/s10545-010-9132-4 [Electronic publication ahead of print].
[7] Nowaczyk MJ, Waye JS, Douketis JD. DHCR7 mutation carrier rates and prevalence of the RSH/Smith–Lemli–Opitz syndrome: where are the patients? Am J Med Genet A 2006;140:2057–62.
[8] Zimmerman PA, Hercules DM, Naylor EW. Direct analysis of filter paper blood specimens for identification of Smith–Lemli–Opitz syndrome using time-of-flight secondary ion mass-spectrometry. Am J Med Genet 1997;68:300–4.
[9] Starck L, Lovgren A. Diagnosis of Smith–Lemli–Opitz syndrome from stored filter paper blood specimens. Arch Dis Child 2000;82:490–2.
[10] Corso G, Rossi M, De Brasi D, et al. Effects of sample storage on 7- and 8-dehydrocholesterol concentrations analyzed on whole blood spots by gas chromatography–mass spectrometry-selected ion monitoring. J Chromatogr B Analyt Technol Biomed Life Sci 2002;766:365–70.
[11] Corso G, Gelzo M, Barone R, et al. Sterol profiles in plasma and erythrocyte membranes in patients with Smith–Lemli–Opitz syndrome: a six-year experience. Clin Chem Lab Med 2011;49(12):2039–46.

Monica Gelzo
Dipartimento di Biochimica e Biotecnologie Mediche, Università di Napoli Federico II, Via S. Pansini, 5-80131 Napoli, Italy

Antonio Dello Russo
Dipartimento di Biochimica e Biotecnologie Mediche, Università di Napoli Federico II, Via S. Pansini, 5-80131 Napoli, Italy

Gaetano Corso
Dipartimento di Biochimica e Biotecnologie Mediche, Università di Napoli Federico II, Via S. Pansini, 5-80131 Napoli, Italy
Dipartimento di Scienze Biomediche, Università di Foggia, Via L. Pinto 1, 71122 Foggia, Italy
Corresponding author at: Dipartimento di Scienze Biomediche, Università di Foggia, Via L. Pinto 1, 71122, Foggia, Italy.
E-mail address: g.corso@unifg.it.

4 November 2011