Interference of 7-Dehydrocholesterol in α-Tocopherol Determination by High-Performance Liquid Chromatography: A Possible Screening Test for the Smith-Lemli-Opitz Syndrome

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ABSTRACT: 7-Dehydrocholesterol (7-DHC), a normal cholesterol precursor, accumulates in plasma and tissues of patients affected by the Smith-Lemli-Opitz (SLO) syndrome, a recessive autosomic disease characterized by mental retardation and physical malformations. In these patients, we suspected that 7-DHC interfered with α-tocopherol (α-T) determination because this steroid could be coeluted with α-tocopherol acetate (α-TA), an internal standard commonly used for α-T determination, and because the conjugated dienic structure of 7-DHC strongly absorbs at 292 nm. The aim of this work was (i) to characterize the interfering material and (ii) to evaluate the possibility of a rapid screening test for SLO syndrome identification from 7-DHC determination by reverse-phase chromatography high-performance liquid chromatography (HPLC). Human plasmas (control and SLO syndrome) were extracted with hexane after ethanol protein precipitation and addition of ascorbic acid as protective agent plus α-TA or δ-tocopherol (δ-T) as internal standards. The dry hexane extract was used for HPLC, and trimethylsilyl ethers derivatives of the extracts were submitted to gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS). The material interfering in the α-T determination was unambiguously identified to 7-DHC by retention time (RT) relative to standard, RT relative to δ-T, and analysis of the eluted material by GC and by GC–MS. In α-T determination, the interference can be eliminated by using δ-T as internal standard instead of α-TA. Rapid detection and evaluation of 7-DHC in plasma appear to be possible by HPLC under the conditions described; comparison with the GC reference method suggests that the rapid HPLC determination of 7-DHC in plasma can be used within the 1–40 mg/dL range, values commonly found in SLO syndrome.
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KEYWORDS: 7-Dehydrocholesterol, GC–MS, HPLC, interference, Smith-Lemli-Opitz Syndrome, α-tocopherol.

7-Dehydrocholesterol (5,7-cholestadien-3β-ol) (7-DHC) is a normal component of human plasma. The plasma concentration of this cholesterol (5-cholesten-3 β-ol) precursor usually ranges between 0.005 and 0.01 mg/dL (1,2) in normal subjects. Dramatically higher concentrations (16 ± 10 mg/dL) have been reported in plasma from patients affected by Smith-Lemli-Opitz syndrome (SLO). This autosomal recessive metabolic disorder is characterized by multiple morphological abnormalities, severe mental retardation, and failure to grow (3). It was recently demonstrated (4) that an important deficiency of 7-dehydrocholesterol-Δ7-reductase is involved in both accumulation of 7-DHC (the substrate) and drastic deprivation of cholesterol (the product) in plasma as well as in tissues.

In these patients, we suspected that 7-DHC interfered with α-tocopherol (α-T) determination. Indeed, this sterol can be coeluted from reverse-phase high-performance liquid chromatography (HPLC) with α-tocopherol acetate (α-TA), an internal standard widely used for α-T determination. The conjugated dienic structure of 7-DHC strongly absorbs between 260–300 nm (5), a zone of ultraviolet spectrum largely overlapping the wavelength used for α-T determination (292 nm).

The aim of this work was to characterize the interfering material in α-T determination in plasma from patients affected by SLO syndrome and to evaluate the possibility of a rapid screening test for SLO syndrome identification from 7-DHC determination by reverse-phase HPLC.

MATERIALS AND METHODS

HPLC analysis of vitamin E. Human plasma from normal subjects (100 µL) or from patients affected by the SLO syndrome was added to 500 µL absolute ethanol (Merck, Darmstadt, Germany), and 10 µL of internal standard solution that contained 0.1 mg/mL of δ-tocopherol (Merck) or α-TA (Fluka, St. Quentin Fallavier, France). After mixing, the sample was extracted with 1.5 mL *n*-hexane (HPLC SDS, Peypin, France). The aqueous phase was discarded, and the hexane extract was evaporated to dryness under a nitrogen stream. The residue was dissolved in 100 µL methanol (HPLC; BDH Laboratories Supply, Poole, England), and 20-µL aliquots

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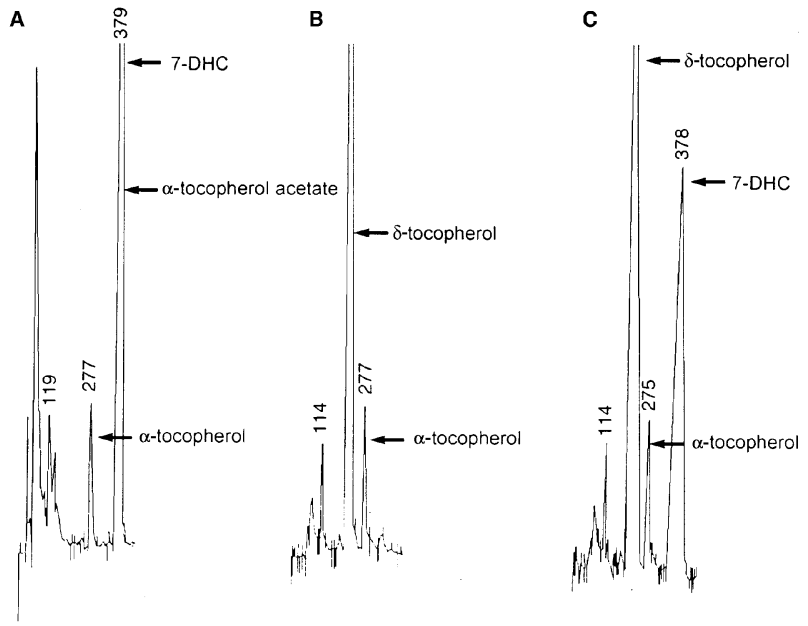

FIG. 1. Reverse-phase high-performance liquid chromatography (HPLC) profiles of plasma extracts from normal subjects and from patients affected with Smith-Lemli-Opitz (SLO) syndrome with either α-tocopherol acetate or δ-tocopherol as internal standards: normal plasma plus α-tocopherol acetate and 7-dehydrocholesterol (DHC) overloading (A); normal plasma plus δ-tocopherol as internal standard (B); plasma from SLO syndrome plus δ-tocopherol as internal standard (7-DHC plasma concentration determined by gas chromatography (GC) was 1 mg/dL) (C). Other experimental details are described in text.

were injected on a Nova-Pack-C18 column (3.9 × 150 mm; Waters, St. Quentin en Yvelines, France) (6). A methanol flow rate of 1 mL/min was maintained throughout the column with a Millipore-Waters HPLC device, equipped with a variable wavelength detector (model 480) set at 292 nm.

Gas-chromatographic analysis of 7-DHC. The internal standard epicoprostanol 5 β-cholestan-3 α-ol (Sigma Chemical Co., St. Louis, MO) was layered at the bottom of an assay tube as 50 µL of a 200 mg/L solution in ethanol and evaporated to dryness under a nitrogen stream. After adding 50 µL

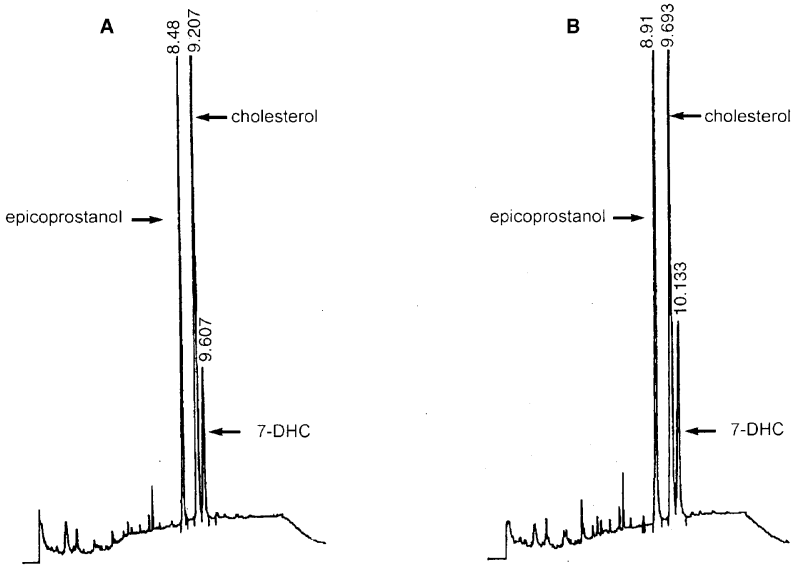

FIG. 2. Gas-chromatographic analysis of plasma extracts from patients affected with SLO syndrome. Epicoprostanol was used as internal standard (for other experimental details, see text). 7-DHC plasma concentration was 9 mg/dL (A), same extract overloaded with reference 7-DHC (B). See Figure 1 for abbreviations.

of plasma sample and 1 mL potassium hydroxide (Merck) in ethanol (60 mL of 33% KOH in 940 mL of 95% ethanol), the mixture was agitated at 56°C for 15 min and diluted by addition of 1 mL water. Then, the sample was extracted twice with 2 mL hexane. The combined hexane extracts were evaporated to dryness under a nitrogen stream. The sterols of the extract were derivatized by silylation with 100 µL Tri-Sil-TBT (Pierce Chemical Co., Rockford, IL) at 80°C for 30 min. After acidification with 2 mL HCl (0.1 N; Merck) the products were extracted with 2 mL hexane. The hexane extract was washed with 2 mL water. Two µL aliquots of the trimethyl silyl ether-derivatives were injected on an SE 54 capillary column (25 m × 0.32 mm, 0.2 µm) (Chrompack, Les Ulis, France) (7). Helium was used as the carrier gas (flow rate 3.75 mL/min) in a Carlo Erba 4100 gas chromatograph (Palo Alto, CA), equipped with a flame-ionization detector and a needle injector (Ross). Injector and detector temperatures were 290°C. The oven temperature was kept at 180°C for 1 min, then programmed at 20°C/min until a final temperature of 275°C, which was maintained for 10 min.

Gas chromatography–mass spectrometry (GC–MS) analysis. Plasma samples were prepared as described above for GC. Aliquots of 1 µL were injected on a CP-Sil 5 CB column (25 m × 0.32 min, i.d. 0.2 µm) (Chrompack) under the operating conditions described above. The interface temperature of the GC–MS coupling was 280°C with electronic ionization set at 70 eV and positive ion detection in a Nermag R10-10H (France).

RESULTS AND DISCUSSION

We recently observed abnormally high signals from α-TA when we used this compound as internal standard for vitamin E determination in patients affected with SLO syndrome. In

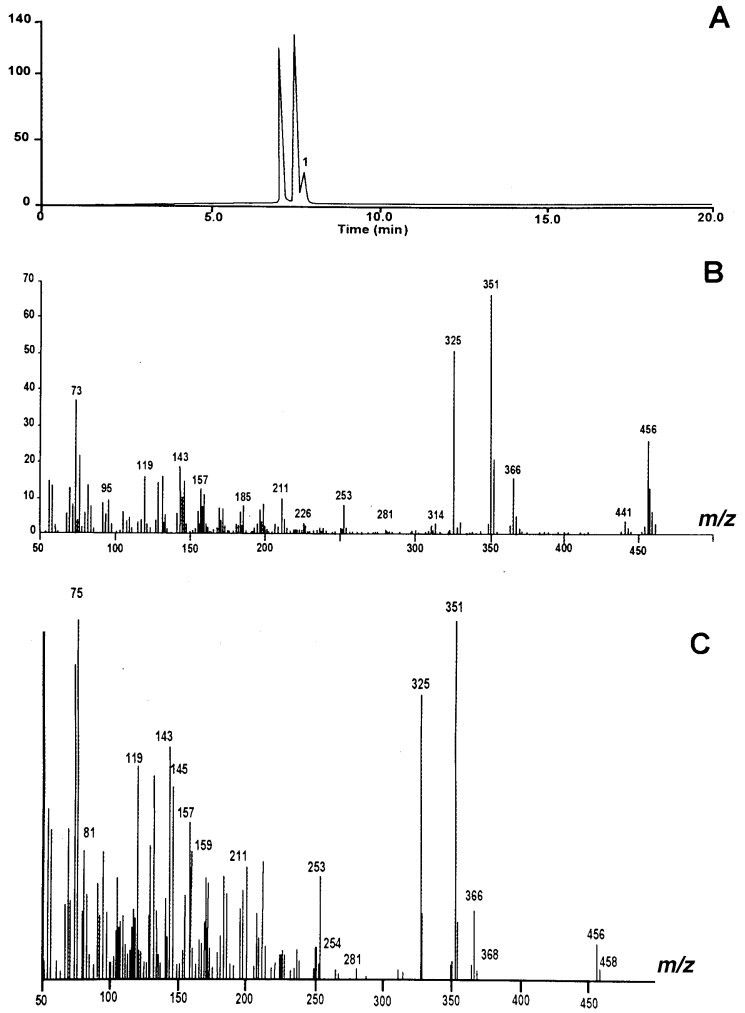

FIG. 3. GC–mass spectrometry (MS) analysis of a plasma extract from patient affected with SLO syndrome: (A) total ionic current, peak 1 is presupposed 7-DHC; (B) mass spectrum (electronic impact) from peak 1 (molecular ion at *m/z* = 456, characteristic ions at *m/z* = 366, 351, 325); (C) mass spectrum (electron impact) from a reference sample of 7-DHC (same characteristic ions as in B). For other experimental details, see text. See Figure 1 for other abbreviations.

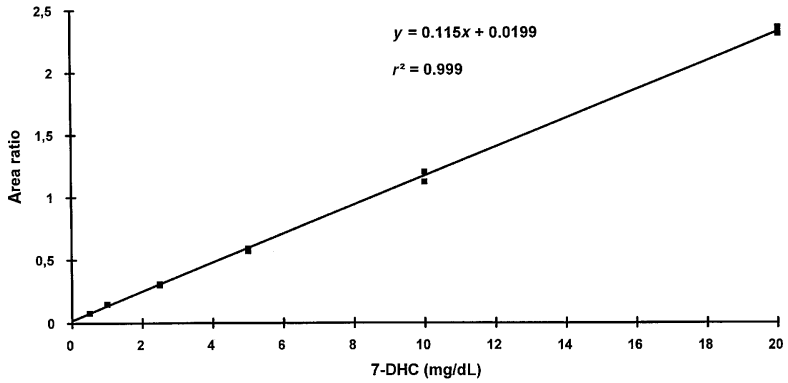

FIG. 4. Reverse-phase HPLC analysis of 7-DHC: area ratio (7-DHC signal area/δ-tocopherol signal area) was plotted as a function of 7-DHC concentration in the sample. Ultraviolet detection at 292 nm. See Figure 1 for abbreviations.

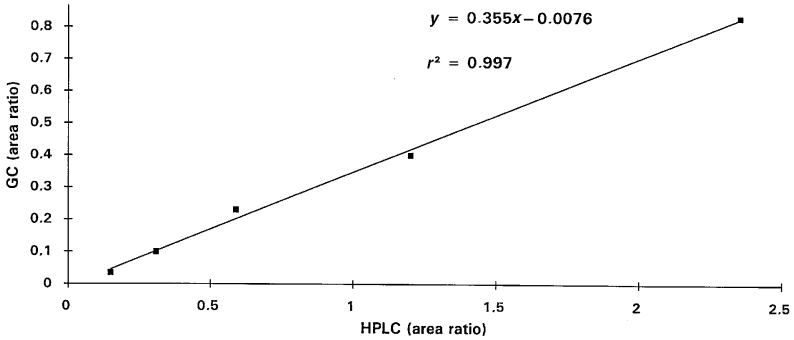

FIG. 5. Correlation between GC and HPLC analysis of samples with increasing concentrations of 7-DHC. See Figure 1 for abbreviations.

these patients, plasma 7-DHC concentrations higher than 1 mg/dL are commonly found. Because the conjugated dienic structure of this sterol strongly absorbs at 292 nm, the wavelength used for tocopherol detection, we looked for possible interference from 7-DHC during vitamin E determination. Figure 1 (A) shows the HPLC profiles obtained when a normal plasma extract, supplemented with α-TA as internal standard and with 7-DHC, was injected on a C18 Nova-Pack column under the conditions described in the methods. The signal of α-TA and 7-DHC are superimposed, indicating that both compounds are coeluted from the column. Figure 1A showed the elution profiles observed when plasma extracts obtained from a normal subject (Fig. 1B), and from a patient affected with SLO syndrome (Fig. 1C), were supplemented with δ-TA as internal standard. It clearly appeared that δ-TA can easily be substituted for α-TA for vitamin E determination either in plasma or in tissues because, in SLO syndrome, concentrations of 7-DHC are commonly higher in tissues than in plasma.

To characterize further the material interfering with vitamin E determination in SLO syndrome, plasma extracts from these patients were analyzed by GC and GC–MS under the conditions described in the methods. Figure 2A shows the elution profiles obtained with plasma extracts from a patient affected with SLO syndrome before and after overloading with reference 7-DHC (Fig. 2B). The peak of reference 7-DHC and the peak of the presupposed 7-DHC from the patient affected by SLO syndrome are exactly superimposed. In Figure 3, the mass spectrum obtained by GC–MS of the peak of 7-DHC from SLO syndrome is compared to the GC–MS spectrum of a reference sample of 7-DHC. The profile obtained from total ionic current is shown in Figure 3A, where 1 is the peak of presupposed 7-DHC. The mass spectrum of peak 1 obtained after electronic impact is shown in Figure 3B and compared to reference 7-DHC mass spectrum (Fig. 3C). The same molecular ion was observed at $m/z = 456$, and the same profile was observed, especially the main characteristic ions at $m/z = 366, 351, 325, 253, 211$, which identify unambiguously the interfering material in plasma from SLO as 7-DHC.

It was recently suggested that the incidence of SLO syndrome might be underestimated at 1 of 900 births, whereas 1 of 50 individuals could be a heterozygote carrying the defective gene (8). A rapid screening method may be useful for SLO syndrome detection, either in newborns or in amniotic fluid for prenatal diagnosis (9). The ultraviolet absorption

spectrum of 7-DHC in extracts from plasma was recently proposed as a tool for screening for SLO syndrome (5). However, owing to possible interference by other ultraviolet-absorbing material, HPLC fractionation of the plasma extract was previously proposed (10). Our data suggest that the extraction and HPLC procedures described in the present study could be applied to determination of both α-T and 7-DHC. To investigate further this possibility, we used δ-T as internal standard and compared it with the results of a reference method, the GC procedure. Figure 4 shows that a good linear relationship was obtained with this analytical procedure between 0.2 and 20 mg/dL of 7-DHC. Figure 5 shows the correlation between the HPLC and the GC methods. It appears that between 0.2 and 20 mg/dL of 7-DHC the reverse-phase HPLC procedure can be used for 7-DHC determination. Because in SLO syndrome 7-DHC plasma concentrations ranging between 1 and 25 mg/dL are commonly observed, this method could be useful for SLO syndrome screening. Finally, owing to the interference of 7-DHC with α-TA, use of the latter compound as in internal standard should be avoided.

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