Cerebrotendinous xanthomatosis: reduced serum 26-hydroxycholesterol

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Abstract Serum 26-hydroxycholesterol was quantitated by isotope dilution–mass spectrometry in normal individuals and in patients with cerebrotendinous xanthomatosis. In the normal individuals, the concentration of 26-hydroxycholesterol in serum ranged from 4.3 to 13.0 μg/100 ml. In five patients with CTX, 26-hydroxycholesterol in serum ranged from 0 to 0.6 μg/100 ml. The findings can be explained by reduced or absent mitochondrial C_{27} steroid 26-hydroxylase activity. The method is useful for detection of CTX and perhaps other disturbances of sterol and bile acid metabolism.—**Javitt, N. B., E. Kok, B. Cohen, and S. Burstein.** Cerebrotendinous xanthomatosis: reduced serum 26-hydroxycholesterol. *J. Lipid Res.* 1982. **23:** 627–630.

Supplementary key words isotope dilution–mass spectrometry

Oftebro and co-workers (1) reported that mitochondrial preparations from the liver of a patient with cerebrotendinous xanthomatosis (CTX) were completely devoid of C_{27}-steroid 26-hydroxylase. It has also been shown that although microsomal and mitochondrial steroid 26-hydroxylases exist, only the mitochondrial enzyme can oxidize the side chain of cholesterol to yield 26-hydroxycholesterol (2, 3). From these findings it is reasonable to conclude that the rate of production in vivo of 26-hydroxycholesterol is dependent on the activity of the mitochondrial enzyme and might be reflected in circulating plasma levels. Because of our interest in the metabolism of 26-hydroxycholesterol (4, 5), we determined the amount of this sterol in the serum of normal individuals and in three patients with cerebrotendinous xanthomatosis.

METHODS

Quantitation of 26-hydroxycholesterol in serum was done by isotope dilution–mass spectrometry following the principles outlined by Biemann (6). Deuterated 26-hydroxycholesterol prepared by Clemmensen reduction (7, 8) was used as an internal standard.

The complete spectrum of the deuterated 26-hydroxycholesterols and its usefulness in tracer studies has been published in detail (9). In brief, the use of D_2O and DCl in the Clemmensen reduction step introduces deuterium at the C_{16} and C_{22} keto groups of kryptogenin and to a variable extent at adjacent carbon atoms giving a range of deuterated isotopes of 26-hydroxycholesterol that are 5 to 9 mass units greater than the naturally abundant compound (10). The most abundant mass was found to be 410 or 8 mass units greater than the protium compound (mol wt 402). Therefore, for quantitation a known amount, 5 μg, of the deuterated analog containing a constant fraction of the D_8 species was added to 2.5 ml of serum.

The proteins were precipitated by the addition of 2,2-dimethoxypropane (Aldrich Biochem) and 0.12 ml of 12 N HCl (11). The filtrate was taken to dryness and saponified in ethanolic KOH using a procedure established for saponification of cholesterol (12). In preliminary studies, it was established that omission of a saponification step reduced the amount of 26-hydroxycholesterol by approximately 60% implying that, in common with cholesterol, it was present in plasma as both the free and esterified sterol. Following saponification, the sterol fraction was obtained by partitioning between ether and aqueous alkali and the neutralized ether fraction was taken to dryness, redissolved in 1.6 ml of hexane–ethyl acetate 9:1, and applied to a 25-g column of Glycophase G on controlled pore glass 80–100 mesh (Pierce Biochemical). Use of this packing has been described in detail previously (13). Cholesterol was removed from the column in 160 ml of hexane–ethyl acetate 9:1 at a flow rate of 1.8 ml/min. An additional volume of 100 ml of hexane–ethyl acetate 3:1 removed 26-hydroxycholesterol. Both 7α and 7β hydroxycholesterol were retained on the column (**Fig. 1**). The eluate was taken to dryness and silylated with Sil-prep (Pierce Biochemical) and injected on to a 4-ft column of SP-2550 at 260°C using a Hewlett-Packard Model #5592 B GLC–Mass Spectrometer. It was determined that 25- and 26-hydroxycholesterol had retention times of 14 and 16.3 min, respectively, and

Abbreviations: CTX, cerebrotendinous xanthomatosis; GLC–MS, gas–liquid chromatography–mass spectrometry.

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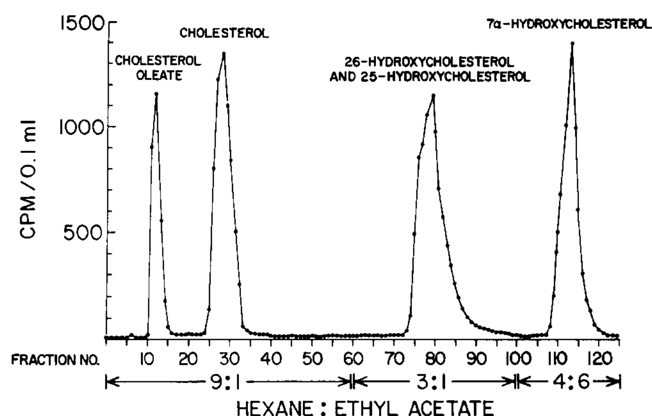

Fig. 1. Separation of hydroxysterols by column chromatography using Glycophase G on controlled pore glass. The residue, following protein precipitation and saponification, is dissolved in hexane-ethyl acetate 9:1 and applied to a 25-g column of Glycophase G. Complete separation of 25- and 26-hydroxysterols from cholesterol, cholesteryl ester, and 7α-hydroxycholesterol is obtained. Complete separation of 25- from 26-hydroxycholesterol occurs during gas-liquid chromatography.

therefore were completely separated. In addition, as reported by Pedersen (14), the complete spectrum of 25-hydroxycholesterol is distinctly different from 26-hydroxycholesterol. The latter compound has a high peak at m/z 129 and a diminutive peak at m/z 131, in contrast to 25-hydroxycholesterol where the reverse relationship obtains. A complete spectrum of the fraction isolated from serum contained all the peaks present in an authentic standard of 26-hydroxycholesterol.

Analysis of a serum from a patient with CTX failed to give a molecular ion characteristic of the di-trimethylsilyl ethyl of 26-hydroxycholesterol (m/z 546). To insure that recovery from the Glycophase column was the same in normal individuals and CTX patients, 10,000 dpm of [16,22-^{3}H]-26-hydroxycholesterol (sp act 3.48×10^5 dpm/μg) prepared as described previously (8) was added to each serum in addition to the 5 μg of deuterated 26-hydroxycholesterol. An aliquot of each eluate for Glycophase G was counted and it was established that recovery ranged from 63 to 85% with no significant difference between normal and CTX sera. This dual internal standard gave an m/z 554/546 ratio of 23. No change in the ratio of the internal standard occurred where it was added to solutions of cholesterol and cholesteryl oleate which were subjected to solvolysis, saponification, and column separation.

Calibration standards were prepared by adding different weighed amounts of 26-hydroxycholesterol to 5-μg aliquots of the deuterated analog. The m/z 554/546 ratio did not change when these standards were carried through the entire procedure. A graph was constructed by comparing the mass ratio of the calibration standards (calculated) to the observed mass ratio using electronic integration to obtain the total area of each mass (**Table 1**). Both the m/z 554/546 and m/z 464/456 area ratios were used. The latter ratio represents the respective prominent M−90 (minus one silyl ether) peaks. No major concentration differences were found and the m/z 554/546 ratio was used routinely.

RESULTS

A typical GLC–MS pattern using the simultaneous ion monitoring program is shown in **Fig. 2.** The left section shows the peak areas of the calibration standard at 16.3 min. M/z 554, 553, 552 are from the deuterated analog and m/z 546 is from the natural 26-hydroxycholesterol. M/z 464 and 456 are the respective M−90 peaks. The middle section shows a normal serum and the right section shows serum from a patient with CTX. The trace of m/z 554 in the CTX patient is identical to the peak obtained from the standard added to validate recovery for the column. The normal serum shows larger m/z 546 and m/z 456 peaks than observed for the CTX patients. Note also that early in the tracings there are other m/z 456 peaks that are much more prominent in the CTX patients and probably represent residual monohydroxy sterols that were eluted with 26-hydroxycholesterol.

Table 1 summarizes the data. In six normal individuals the observed m/z 554–546 ratios were within the range of the calibration standards and calculated to a total 26-hydroxycholesterol concentration ranging from 4.3 to 13.9 μg/100 ml. In five patients with CTX, the concentration of 26-hydroxycholesterol ranged from 0 to 0.6 μg/100 ml.

DISCUSSION

The markedly reduced concentration of 26-hydroxycholesterol in the serum of patients with CTX is consistent with the report that there is a decrease in mitochondrial C_{27} steroid 26-hydroxylase activity in an individual with this genetically determined abnormality in sterol and bile acid metabolism (1). Since it is known that only the mitochondrial enzyme can oxidize cholesterol to 26-hydroxycholesterol (2, 3), it is reasonable to assume that the circulating level is a reflection, in part, of the activity of the enzyme. Nonenzymatic origin is excluded by the absence of 25-hydroxycholesterol in these sera. Van Lier and Smith (15) have established that if autooxidation does occur considerably more 25- than 26-hydroxycholesterol is generated. Moreover, neither 25- nor 26-hydroxycholesterol were found after analysis of standards of cholesterol and cholesteryl oleate that were exposed to all the procedures used. Thus the circulating

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TABLE 1. Concentration of 26-hydroxycholesterol in serum

Specimen	26-hydroxycholesterol Deuterated μg	Protium μg	Ratio D/P	M/Z 554 Area[b]	M/Z 546 Area[b]	Ratio 554/546	Concentration[a] μg/100 ml Serum
Standards							
#1	5	1.25	4	43502	18126	2.4	
#2	5	0.625	8	56712	14927	3.8	
#3	5	0.156	32	49582	3673	13.5	
Int. std.[c]	5	0.086	58	67560	2937	23	
Normal							
N.J.	5	0.397[d]	12.6	88398	15241	5.8	12.5
E.K.	5	0.410[d]	12.2	57433	9253	6.2	13.0
C.E.	5	0.231[d]	21.7	51460	5416	9.5	5.8
G.B.	5	0.192[d]	26	69985	6193	11.3	4.3
D.F.	5	0.278[d]	18	31611	3436	9.2	7.7
S.K.	5	0.385[d]	13	98806	13723	7.2	12.0
CTX							
G.A.	5	.086[d]	58	67715	2944	23	0.0
B.D.	5	0.100[d]	50	45089	2254	20	0.6
B.H.	5	0.096[d]	52	56645	2697	21	0.45
W.S.	5	0.091	56	73436	3338	22	0.20
R.S.	5	0.091	56.8	82676	3758	22	0.20

[a] Calculated from μg protium compound in serum minus the internal standard (0.086) × 40.
[b] Total area of each peak in relative numbers given by simultaneous ion monitoring program of Hewlett-Packard 5992B mass spectrometer.
[c] Amount added to each serum sample.
[d] Obtained from graph of relationship of D/P ratio to 554/546 ratio of standards.

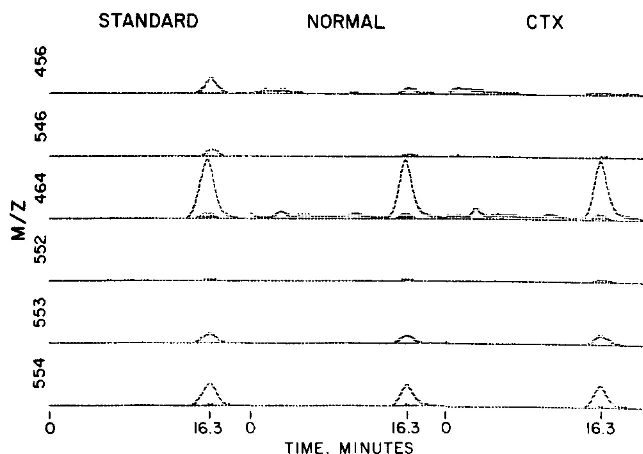

Fig. 2. GLC-MS analysis of 26-hydroxycholesterol. The GLC-MS instrument (HP 5992B) was programmed for monitoring m/z 554, 553, 552, 546, 464, and 456 simultaneously. M/z 554 and 546 represent the molecular ions of the di-trimethyl silyl ethers of a deuterated analog and the natural 26-hydroxycholesterol. M/z 464 and 456 are the respective M−90 (minus one silyl group) peaks. M/z 553 and 552 represent other deuterated analogs. The panel on the left is a calibration standard and shows peaks of the respective ions at 16.3 min. The center panel is the tracing from normal serum and the panel on the right is for a patient with CTX. The slight m/z 546 and 456 peaks at 16.3 min in the CTX patient are no greater than those obtained for the internal standard consisting of 5 μg of deuterated analog and 10,000 dpm of [^{3}H]-26-hydroxycholesterol. Also note other m/z 546 peaks early in the tracing which probably represent residual amounts of sterols present in the serum of CTX patients in greater than normal amounts.

levels represent, in part, the activity of the enzyme. Isotope ratio mass spectrometry used in this and previous studies of the sterol composition of biologic fluids (1) provides a sensitivity and precision that is difficult to achieve with other methods of quantitative analysis. Although radioactive isotopes may be determined with more sensitivity, their use for quantitative purposes requires an independent estimate of mass that may be less sensitive and accurate. In contrast, using a stable isotope of the compound, the area of each mass is simultaneously determined and provides a ratio from which the abundance of the natural compound is precisely determined from the known amount of isotope initially added to the specimen. Although slight separation of isotopes can occur during isolation procedures, they are easily recognized and prevented. Those losses which usually occur during sample preparations do not affect the area ratios and therefore have no effect on quantitation. In this sense, the stable isotope functions as an internal standard which more closely resembles the naturally abundant compound than conventional internal standards of different chemical composition and structure. As stable isotopes become more available, it seems likely that isotope ratio mass spectrometry will permit detection and quantitation of a variety of compounds present in relatively small amounts in biologic fluids.

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