26-Hydroxycholesterol

IDENTIFICATION AND QUANTITATION IN HUMAN SERUM*

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Using isotope dilution mass spectrometry, 26-hydroxycholesterol was identified in the serum of normal adults. Total values ranged from 9.2 to 25.6 μg/100 ml of which 31–35% was free sterol. Density gradient ultracentrifugation indicates that the steroid is distributed among the low and high density lipoproteins.

Cholest-5-ene-3β, 26-diol (26-hydroxycholesterol) is a major sterol component of human meconium (1). Its presence in biological fluids after neonatal life has not been established. A number of recent findings (2–6) imply that hydroxysterols have an important biologic role. Thus, using cell culture techniques, hydroxysterols have been shown to reduce cholesterol synthesis by inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase (7), stimulate esterification of cholesterol in liver cells (2), and inhibit thymidine incorporation into DNA in lymphocytes (3, 6). In the latter study (6), a fraction isolated from normal human low density lipoprotein, referred to as LDL′-In, was also shown to have similar activity. For these reasons and because of our interest in the metabolism of 26-hydroxycholesterol (7, 8), we have developed an isotope dilution mass spectrometric method for the quantitation of free and esterified 26-hydroxycholesterol.

MATERIALS AND METHODS

Cholest-5-ene-3β, 26-diol—The compound and its tritiated and deuterated analogs were prepared from kryptogenin using minor modifications of the synthesis reported by Scheer et al. (9) and described previously in detail in this journal (7). Use of D,O and DC1 during the Clemmensen reduction step introduced deuterium at the C-16 position (10, 9). The fragmentation pattern of 25-hydroxycholesterol has a prominent m/z-129 peak which is the opposite of the fragmentation pattern of 26-hydroxycholesterol (14) and readily distinguishes the two compounds. In preliminary studies, recrystallized cholesterol and cholesterol oleate in amounts 10-fold greater than present in 2.5 ml of serum reacted with deuterium at C-16. 2.2-dimethoxypropane (Aldrich) and 0.12 ml of 12 N KOH in 94% ethanol were added and heated in a sealed tube at 50–55°C for 24 hr. The volume was then reduced to approximately 2.0 ml and 2.0 ml of water was added. After extraction with diethyl ether and backwashing to neutrality, the sterol fraction was taken to dryness and then dissolved in 1.6 ml of hexane/ethyl acetate, 91, and applied to the Glycophase G column. Utilizing [16-22-3H]26-hydroxycholesterol prepared from kryptogenin (7) in tracer amounts added to serum, it was found that recovery after protein precipitation was complete and after column chromatography ranged from 68 to 83%. In contrast, it was found that the diisulfate of [16-22-3H2]26-hydroxycholesterol precipitated with proteins and could be recovered as the free sterol by resuspension of the precipitate in tetrahydrofuran (97.5%) containing 0.04 ml of 70% perchloric acid and allowing to remain at room temperature for 72 h. In applying this procedure to the precipitate from serum, we failed to detect additional 26-hydroxycholesterol.

The eluate from the Glycophase G column was silylated and injected on a 4-ft column (2 mm internal diameter) of SP2560 (Supelco Inc., Bellefonte, PA) at 260°C. At this temperature, 25-hydroxycholesterol and 26-hydroxycholesterol have retention times of 14.0 and 16.3 min, respectively. GLC-MS analysis of a serum to which no deuterated or tritiated 26-hydroxycholesterol was added is depicted in Fig. 2. As shown in the upper panel, monitoring for m/z-546 shows no masses until a peak appears at 15.9 min. A complete spectrum taken at 16.3 min (lower panel) shows all the fragment ions characteristic of 26-hydroxycholesterol. Since Glycophase G column chromatography does not separate 25- and 26-hydroxycholesterol, the absence of a peak at 14 min indicates little or no compound in plasma. Also, the fragmentation pattern of 25-hydroxycholesterol has a prominent m/z-131 peak, representing cleavage between C-24 and C-25 and a diminutive m/z-129 peak which is opposite of the fragmentation pattern of 26-hydroxycholesterol (14) and readily distinguishes the two compounds. In preliminary studies, recrystallized cholesterol and cholesterol oleate in amounts 10-fold greater than present in 2.5 ml of serum were subjected to protein precipitation, saponification, column chromatography and GLC-MS analysis. Neither 25 nor 26-hydroxycholesterol could be detected indicating they were not derived from the starting compounds.

For quantitative analysis of 26-hydroxycholesterol, the mass spectrometer was programmed to simultaneously monitor m/z-554, -553, -552, -546, -464, and -456. The m/z-554 and -464 and -546 represent the molecular ion and M-90 peak (silyl ether) of the deuterated and natural compound respectively. The M-90 peaks are proportionally greater than the molecular ion and in the correct proportion further verifies the identity of the molecular ion. The results in these studies were calculated from both the ratio of the total areas of m/z-554/546 and m/z-464/456. Identical concentrations of 26-hydroxycholesterol were obtained.

For quantitative determination of 26-hydroxycholesterol in serum, a series of weighed standards was prepared by adding 1.25, 0.625, 0.3125, 0.15625, 0.078125, 0.0390625, 0.01953125, 0.009765625, 0.0048828125, 0.00244140625, 0.001220703125, 0.0006103515625, 0.00030517578125, 0.000152587890625, 0.0000762939453125, 0.00003814697265625, and 0.0000019073486328125 μg of [16-22-3H]26-hydroxycholesterol to 2 ml of serum. Each of these standards were analyzed in duplicate.
Serum 26-Hydroxycholesterol

of known amount of the deuterated analog and determination of the alteration by Clemmensen reduction provides a number of enriched m/z-554/546 ratios. All the peaks characteristic of 26-hydroxycholesterol at 26° contain m/z-129 characteristic of 3β,Δ5 structure. The amount of 26-hydroxycholesterol in biological fluids can be determined by addition of di-trimethylsilyl ether and injected onto a 4-foot column of TMS, trimethylsilyl.

A 2.5-ml aliquot of serum (from P. M., Table I) was fractionated on a deuterated analog after protein precipitation, saponification, and ether extraction. The eluate from the column was converted to the di-trimethylsilyl ether and injected onto a 4-foot column of 3% SP2250 at 260 °C. The instrument was set to monitor the m/z-546 (molecular ion). As shown at top, a single peak was obtained with a retention time of 16.3 min. A complete spectrum obtained at this time contained all the peaks characteristic of 26-hydroxycholesterol (bottom).

Identification of 26-Hydroxycholesterol in Human Serum

Table 1

| Individual | m/z-554/546 ratio | Serum | VLDL Free | Total | LDL* Free | Ester | HDL* Free | Ester |
|------------|-------------------|-------|-----------|-------|-----------|-------|-----------|-------|
| N. B. J.   | 13.0              | 4.3   | 13.9      | N.D.*| 22        | 34    | 22        | 22    |
| P. M.      | 13.1              | 4.3   | 12.9      |       | 61        |       | 39        |       |
| E. K.      | 12.7              | 5.5   |           |       |           |       |           |       |
| C. E.      | 12.7              | 5.2   |           |       |           |       |           |       |
| P. S.      | 6.6               | 9.2   |           |       |           |       |           |       |
| B. C.      | 4.5               | 25    |           |       |           |       |           |       |
| P. V.      | 3.8               | 22.2  |           |       |           |       |           |       |
| C. R.      | 6.6               | 9.2   |           |       |           |       |           |       |

* Percentage of total serum 26-hydroxycholesterol distributed in lipoprotein fractions.

† Analysis in duplicate values were 4.9, 4.9, and 5.1.

‡ None detected.

Results

As shown in Table 1, the total 26-hydroxycholesterol in serum in 8 normal individuals ranged from 9.2 to 25.6 µg/100 ml. In 3 individuals, the free sterol ranged from 31 to 35%. No 26-hydroxycholesterol was found in the very low density lipoproteins. Both free and esterified 26-hydroxycholesterol are found in the low density and high density lipoproteins.

The findings establish that 26-hydroxycholesterol is present throughout life. Although it is present as the disulfate in meconium (1), it can also be metabolized to both chenodeoxycholic and cholic acids (8) and is the likely source of 3β-hydroxy-5-cholenoic acid, also present in meconium (16) and urine as a sulfate (17).

Frederickson and Ono initially identified both 25- and 26-hydroxycholesterol as metabolites of cholesterol after incubation with mouse liver mitochondria (18). Danielsson (19) confirmed the enzyme origin of 26-hydroxycholesterol but considered 25-hydroxycholesterol to be an autoxidation product.

Extensive studies by Van Lier and Smith (20, 21) established that 25-hydroxycholesterol is an autoxidation product and that 26-hydroxycholesterol is an unlikely autoxidation product. In the present studies as part of the development of the serum method, 12.5 mg of cholesterol were carried through the entire procedure without any detectable 26-hydroxycholesterol being found by mass spectrometry. Also, the VLDL separated by ultracentrifugation and carried through the entire procedure had no detectable 25- or 26-hydroxycholesterol. The absence of 26-hydroxycholesterol in our processed samples, which would have been detected at 14.0 min as an m/z-546 peak,

0.313, and 0.156 µg of 26-hydroxycholesterol to 5 µg of the deuterated compound to give known m/z-554/546 ratios of 4, 8, 16, and 32, respectively. Analysis of these standards by GLC-MS yielded observed m/z-554/546 ratios of 1.96 ± 0.40 S.D., 3.49 ± 0.98, 8.95 ± 2.12, and 18.6 ± 2.3 uncorrected for proportion of D₂ enrichment in the deuterated analog. A complete set of standards was run with each group of serum samples and a standard curve was constructed from the weighed and observed m/z-554/546 ratios as described by Biehn (15) from which the serum values could be calculated. Addition of 26-hydroxycholesterol to 2.5 ml of serum ranging in amounts from 0.115 to 2.14 µg gave recovery of 84% ± 0.9 S.D. Monitoring of m/z-554/553, 554/552, and 553/552 ratios established that no deuterium was lost during the processing of the serum.

Fig. 1. Mass spectra of 26-hydroxycholesterol (top) and the deuterated analog (bottom) as the di-trimethylsilyl ethers. Deuteration by Clemmensen reduction provides a number of enriched m/z-554 and 546 of 456. None of the deuterated compounds contain m/z-546 and -545 that characteristic of 26-hydroxycholesterol. Both compounds contain m/z-129 characteristic of 3β,Δ5 structure. The amount of 26-hydroxycholesterol in biological fluids can be determined by addition of m/z-546/544 ratio. TMS, trimethylsilyl.

Fig. 2. GLC-MS analysis of 26-hydroxycholesterol in serum. A 2.5-ml aliquot of serum (from P. M., Table I) was fractionated on a previously standardized Glycolc phase G column without the addition of a deuterated analog after protein precipitation, saponification, and ether extraction. The eluate from the column was converted to the di-trimethylsilyl ether and injected onto a 4-foot column of 3% SP2250 at 260 °C. The instrument was set to monitor the m/z-546 (molecular ion). As shown at top, a single peak was obtained with a retention time of 16.3 min. A complete spectrum obtained at this time contained all the peaks characteristic of 26-hydroxycholesterol (bottom).
implies that the 26-hydroxycholesterol found in serum was not generated by the methods used. We concur therefore, with the view of Van Lier and Smith that the 26-hydroxycholesterol present in vitro is of enzymic origin and that our more sensitive isotope dilution mass spectrometric method establishes its presence in serum. This interpretation is fully in keeping with the recent report that the 7α,9α-cholesterol 26-hydroxylase in human liver mitochondria is the major determinant of normal side chain oxidation of cholesterol to 7α,9α bile acids (23).

In vitro studies thus far have utilized mostly 25-hydroxycholesterol because it is more readily available. However, Dr. Donald McNamara has found that 26-hydroxycholesterol prepared as described (7) is at least as active as 25-hydroxycholesterol in the inhibition of cholesterol synthesis in vitro. In addition, the presence of 26-hydroxycholesterol in LDL raises the possibility that it may be the mediator of its inhibitory effects in cell culture.

Brown et al. (24) developed the concept that the normal regulation of cholesterol synthesis via the activity of hydroxymethylglutaryl-Co A reductase is dependent on the internalization of cholesterol obtained from the binding of LDL to a receptor on the cell surface. Kandutsch et al. (25) propose that the actual mediator is an oxygenated sterol rather than cholesterol. Our studies establish that 26-hydroxycholesterol is present in biologic fluids and therefore could play a role in the regulation of cholesterol metabolism. With the methods that have been developed, this possibility can be explored rigorously.

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