Resolution of desmosterol, cholesterol, and other sterol intermediates by reverse-phase high-pressure liquid chromatography

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Abstract A highly efficient technique has been developed for the resolution of several sterols that are intermediates in the biosynthesis of cholesterol and that differ only by one carbon–carbon double bond or by one methyl group. The technique described utilizes reverse-phase high-pressure liquid chromatography on a μBondapak-C18 column with acetonitrile as eluting solvent. This procedure is capable of measuring the enzymatic conversion of desmosterol to cholesterol. This chromatographic separation can be conducted by reverse-phase high-pressure liquid chromatography in approximately 10 min, whereas other procedures can require several days.

Supplementary key words cholesterol biosynthesis · sterols · microsomal sterol Δ⁵-reductase · lanosterol · dihydrolanosterol · Δ⁴-cholestadienol · Δ⁴,4-dimethyl-Δ⁸-cholestenol · desmosterol

Previous investigations from this laboratory have focused upon the enzymatic conversions of squalene and various sterols to cholesterol (1–5). While rather simple techniques have been developed to assay some of these reactions, e.g., squalene to sterol (1) and 7-dehydrocholesterol to cholesterol (2), certain reactions in cholesterol biosynthesis have required laborious procedures. An example of such a reaction is the enzymatic conversion of desmosterol to cholesterol. This reaction is catalyzed by microsomal sterol-Δ⁵-reductase, i.e., the Δ⁴-bond of desmosterol is reduced by NADPH to yield cholesterol. The only structural difference between desmosterol and cholesterol is the presence in desmosterol of one additional carbon–carbon double bond at C-24–25. While these compounds can be separated by silicic acid column chromatography (6–8), it takes approximately 3 days per sample. GLC and silver nitrate-impregnated silica gel TLC procedures require the formation of derivatives and it can be difficult to achieve quantitative assay of radioactive compounds.

The present article describes a rapid procedure for the resolution of several sterols that are intermediates in the biosynthesis of cholesterol and that differ only by one carbon–carbon double bond or by one methyl group. This method has also been applied to the assay of the enzymatic conversion of desmosterol to cholesterol. The technique employed utilizes reverse-phase high-pressure liquid chromatography on a μBondapak-C18 column.

MATERIALS AND METHODS

Materials

[¹⁴C]4,4-Dimethyl-Δ⁸-cholestenol and [³H]7-dehydrocholesterol were prepared as previously described (3, 5) except that [5-¹⁴C]mevalonate was the precursor for the 4,4-dimethyl sterol. Cholesterol was purified through the dibromide (9) and recrystallized from acetone. [4-¹⁴C]Cholesterol and [26-¹⁴C]desmosterol were obtained from New England Nuclear, Boston, MA. Unlabeled desmosterol was from Organon, West Orange, NJ. Dihydrolanosterol was from Schwarz-Mann, Orangeburg, NY, and was recrystallized from chloroform–methanol. Lanosterol and NADPH were from Sigma, St. Louis, MO. Acetonitrile, chloroform, and cyclohexane, Spectrograde, were obtained from Burdick and Jackson Laboratories, Muskegon, MI. Toluene (reagent grade) was from MCB, Norwood, OH, and was dried over a molecular sieve (Linde, Type 3A).

Chromatography

High-pressure liquid chromatography was carried out on a Waters liquid chromatograph Model ALC/...
GPC that was equipped with a refractive index detector, a Houston recorder, and a Buchler fraction collector. The column employed was a μBondapak-C_{18} reverse-phase column (3.9 mm × 30 cm) purchased from Waters Associates.

Samples to be chromatographed were dissolved in acetone (Spectrograde) and filtered through a sample clarification apparatus (Waters Associates) containing a 0.45 nm fluoropore filter and an organic prefilter. The filters were from Millipore Corp. Aliquots of approximately 100 μg of sterol in acetone (10 μl) were injected on to the column. The chromatographic conditions were: μBondapak-C_{18} column eluted with acetonitrile at a flow rate of 2.5 ml/min, chart speed 2.5 cm/min, attenuator 4×. Unlabeled samples were detected by the change in refractive index, using the refractive index detector. Radioactive samples were detected by collecting 15-sec fractions (0.63 ml) on a fraction collector and by measuring radioactivity in a scintillation spectrometer (Packard Model 3375) after the addition of 2,5-diphenyloxazole (0.3%) in toluene (10 ml) to each fraction. The retention values were measured from the point of injection to the apex of the peak. The recorded peak of an unlabeled sample detected by the change in refractive index occurred 0.8 min earlier than the peak as measured by detection of radioactivity. This difference was produced by the degree of physical separation between the refractive index detector and the fraction collector. This factor of 0.8 min was determined in the following manner. Cholesterol and desmosterol were each prepared as mixtures of labeled and unlabeled compounds. Separations were then conducted and each sterol was detected by change in refractive index and by radioactivity. The difference obtained was a factor of 0.8 min. This factor was constant in all experiments. The retention times for the radioactive samples shown in Table 1 have been corrected by this amount to correspond to the values obtained for unlabeled sterol by the refractive index detector.

**Preparation of microsomes and soluble supernatant**

Sprague-Dawley male rats (250–300 g) were maintained ad libitum on a diet of Wayne Lab-Blox and tap water. Rat liver microsomes and soluble supernatant were prepared as previously described (5) except that ultracentrifugation was carried out at 303,000 g for 40 min in a Beckman 50.2 Ti rotor. The microsomes were washed three times with buffer and also centrifuged at 303,000 g for 25 min each time. The buffer employed was potassium phosphate (0.02 M, pH 7.4) containing EDTA (0.1 mM).

**Enzymatic conversion of desmosterol to cholesterol**

The enzymatic conversion of [14C]desmosterol (I) to cholesterol (II) in the absence (A) and in the presence (B) of rat liver S_{303} was determined using HPLC (see Fig. 1). Both incubations contained NADPH (1.2 mM), rat liver microsomes (2 mg protein) and [14C]-desmosterol (9900 cpm, 19.8 nmol). The desmosterol substrate was added to the incubations as a solution in propylene glycol (10 μl). Reaction B contained in addition 0.5 ml of rat liver S_{303} (15.5 mg protein). The total incubation volume for each incubation was 3 ml. Incubation was conducted in an atmosphere of nitrogen for 2 hr at 37°C in a Dubnoff shaker. The reactions were stopped by the addition of 3 ml of 15% ethanolic KOH to each flask, followed by saponification at 60°C for 2 hr. After cooling, the samples were extracted twice with 6-ml portions of petroleum ether as previously described (1), the extracts were filtered through a fluoropore filter with pre-filter, the

### Table 1. Separation of sterols by reverse-phase high-pressure liquid chromatography

<table>
<thead>
<tr>
<th>Sterols with One Double Bond</th>
<th>Sterols with Two Double Bonds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention Time</td>
<td>% Band Width at 50% Peak Height</td>
</tr>
<tr>
<td>min</td>
<td>min</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>7.48</td>
</tr>
<tr>
<td>4,4-Dimethyl-Δ^{4}-cholestenol</td>
<td>7.60</td>
</tr>
<tr>
<td>Δ^{7}-Cholestenol</td>
<td>7.68</td>
</tr>
<tr>
<td>Dihydrolanosterol</td>
<td>7.86</td>
</tr>
</tbody>
</table>

* Higher % band width values were observed with radioactive samples compared with the corresponding unlabeled compounds. This was the result of peak diffusion occurring because of the distance between the fraction collector and the refractive index detector. 

* The retention time of the sterol with two double bonds was subtracted from the retention time of the corresponding sterol with one double bond.
Fig. 1. Enzymatic conversion of \([^{14}C]\) desmosterol (I) to cholesterol (II) in the absence (A) and in the presence (B) of rat liver S30. Incubation conditions are described in Methods. Radioactive sterols were separated by HPLC as described in Methods. The graph is uncorrected for a lag of 0.8 min between the refractive index detector and the fraction collector. The fraction collector was turned on after the solvent front had passed. Mic, microsomes.

petroleum ether was removed under a stream of nitrogen, and the samples were redissolved in acetone (17 µl, Spectrograde) for high-pressure liquid chromatography. Chromatography was conducted as described above.

Fig. 2. Separation of lanosterol (III) and dihydrolanosterol (IV) by reverse-phase high-pressure liquid chromatography. The samples were lanosterol, Peak III, (a commercial preparation), and dihydrolanosterol, Peak IV, (after recrystallization of a commercial preparation from chloroform–methanol). Two sequential chromatographic separations were employed, one for each sample, under identical conditions. An aliquot of approximately 100 µg of the appropriate sample (dissolved in 10 µl of acetone) was used for each run. The chromatographic conditions were: µBondapak-C18 column eluted with acetonitrile at 2.5 ml/min, chart speed 2.5 cm/min. Ten ml of 2,5-diphenyloxazole in toluene (0.3%) was added to each fraction for radioactivity measurements. The graph is uncorrected for a lag of 0.8 min between the refractive index detector and the fraction collector. The fraction collector was turned on after the solvent front had passed. Mic, microsomes.

RESULTS

In the first experiment, reverse-phase HPLC was tested for its ability to serve as an assay for the enzymatic conversion of desmosterol to cholesterol. Fig. 1 shows the results of two incubations after chromatography of the reaction products by reverse-phase high-pressure liquid chromatography. When microsomes alone were incubated with desmosterol (I) (incubation A) 4 nmol of cholesterol (II) were formed. When microsomes plus S30 were incubated with desmosterol (I) (incubation B), 15.1 nmol of cholesterol (II) were formed. These results show that the assay technique works well over a wide range of enzymatic activities. These results also show a significant increase in cholesterol formation in the presence of the soluble fraction from rat liver.

Fig. 2 shows the results of experiments conducted with lanosterol and dihydrolanosterol. In these experiments unlabeled sterols were utilized and they were detected by measuring the changes in refractive index in the eluting solvent (acetonitrile). In the first experiment a commercial sample of lanosterol was chromatographed. The major peak, lanosterol (III), was followed by a smaller peak, an impurity which is dihydrolanosterol. In the immediately following experiment, authentic dihydrolanosterol (IV) (which had been recrystallized from chloroform–methanol) was chromatographed. A single major peak was observed with the same chromatographic mobility as the impurity in
the commercial lanosterol. Fig. 2 thus demonstrates the rapid and complete separation between lanosterol and dihydrolanosterol.

Fig. 3 shows the results of two experiments in which \(^{[3H]}\)-dehydrocholesterol (V) and \([4-14C]\)cholesterol (VI) were chromatographed under identical conditions. Complete separation was obtained in 10 min.

Table 1 summarizes the retention times for eight sterols studied by HPLC on \(\mu\)Bondapak-C\(_{18}\). The left-hand column contains sterols with one carbon–carbon double bond, while the right-hand column contains sterols with two carbon–carbon double bonds. The addition of a carbon–carbon double bond, e.g., at C-24 or at C-7, results in an average decrease in the retention time of 1.69 min. The one-half bandwidth at 50% of the peak height varied from 0.18 to 0.34 min for the eight compounds studied.

It is interesting to compare the values in the table with that of the hydrocarbon squalene which, although not a sterol, is a sterol precursor. This compound has six double bonds and has a retention time of 5.16 min. Squalene-2,3-oxide has a retention time of 3.80 min.

**DISCUSSION**

Reversed-phase high-pressure chromatography on \(\mu\)Bondapak-C\(_{18}\), as described in the present article, has made possible the efficient separation of certain sterol mixtures in 10 min. Previously these separations have been tedious and time-consuming. For example, the separation of desmosterol and cholesterol has been achieved previously by other techniques (6–8, 10–14), but these procedures have several disadvantages. Some required the preparation of derivatives of the sterols in order to improve resolution (10, 13, 14). A problem with TLC methods using silver nitrate-impregnated silica gel plates (11, 13) is that it can be necessary to scrape the radioactive spots from the plates into counting vials for radioactive assay. A long-column (100 cm) silicic acid technique that separated desmosterol from cholesterol was developed by Frantz (6), but it took approximately 3 days.

The highest grades of lanosterol available from commercial sources are only 70–80% pure; the major impurity is dihydrolanosterol. By the use of conditions as described in Fig. 2, the rapid purification of milligram (or larger) quantities of lanosterol and dihydrolanosterol is now feasible.

Another difficult purification accomplished by this system was that of \([4\text{-}14C]\)4,4-dimethyl-\(\Delta^2\)-cholestadienol, since evidence for the presence of this material has been noted earlier (3).

Reproducibility is enhanced by the use of 100% acetonitrile rather than solvent mixtures which are unstable over long periods of time and with varying temperatures.

The HPLC system described in the present article has the advantages of speed, reproducibility, and simplicity, and it does not require the preparation of derivatives. It should facilitate studies on the enzymatic synthesis of cholesterol.

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**REFERENCES**


