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Note

Determination of 7-dehydrocholesterol in rat skin and liver by high-performance liquid chromatography

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7-Dehydrocholesterol (7-DHC) is not only an intermediate of cholesterol biogenesis but is also a photosynthetic precursor of vitamin D_3, *i.e.*, provitamin D_3 (ref. 1).

Conventional methods used for the determination of 7-DHC in animal tissues are spectrophotometric methods, including fractionation of unsaponifiable matter by means of column chromatography[2] or thin-layer chromatography[3]. However, the methods so far reported were neither accurate nor applicable to liver, owing to irrelevant UV-absorbing impurities and low sensitivity. In order to investigate the metabolism of provitamin D_3 in detail, a simple, precise and sensitive method for the determination of 7-DHC in body tissues was required. This paper reports a method for the determination of 7-DHC in rat skin and liver by high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Chemicals

Crystalline 7-DHC was purchased from Fluka (Buchs, Switzerland) and was recrystallized from diethyl ether (m.p. 150–151°C). Its purity was checked by HPLC.

All solvents were purified according to conventional methods in our laboratory and were redistilled before use. Other reagents were of analytical-reagent grade.

Apparatus

Two HPLC systems were used. The system used for the pre-purification of unsaponifiable matter obtained from rat liver consisted of a Shimadzu–DuPont LC 841 high-performance liquid chromatograph, equipped with a UV detector (254 nm) (Shimadzu, Kyoto, Japan), a 25 cm × 6.2 mm I.D. Zorbax SIL column (DuPont, Wilmington, DE, U.S.A.) and a Model SIL-1A LC injector (Shimadzu). The other system, used for the determination of 7-DHC in the unsaponifiable matter of rat skin or in the pre-purified 7-DHC fraction of rat liver, consisted of a Shimadzu LC 3A high-performance liquid chromatograph, a 25 cm × 4.6 mm I.D. Zorbax SIL column, and a Model UVD-2 UV detector (254 nm; maximum sensitivity $1 \cdot 10^{-3}$ absorbance unit) (Shimadzu).

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A Gilson Micro Fractionator (Gilson Instrument Co., Middleton, WI, U.S.A.) was connected to the first chromatography system to fractionate the eluate.

UV absorption spectra were recorded with a Hitachi 323 automatic spectrophotometer (Hitachi, Tokyo, Japan).

Assay of 7-DHC in skin

A piece of dorsal rat skin (1 g) was cut into small pieces and saponified by refluxing for 30 min with a mixture of 25 ml of ethanol, 10 ml of 20% pyrogallol solution in ethanol and 4 ml of 85% (w/v) potassium hydroxide solution. The unsaponifiable matter was extracted with 50 ml of benzene according to the method of Mulder and Vries[4]. After the separated benzene layer had been filtered through a Whatman 1PS phase-separating paper, 2 ml of the filtrate were dried under a stream of nitrogen and the residue was dissolved in 500 μl of *n*-hexane. A 50-μl volume of the solution was applied to a Zorbax SIL column (25 cm × 4.6 mm I.D.), and was eluted at a flow-rate of 1.8 ml/min with 0.5% 2-propanol in *n*-hexane. The sensitivity of the UV detector was set at $32 \cdot 10^{-3}$ absorbance units full-scale (a.u.f.s.).

Assay of 7-DHC in liver

Rat liver (5–10 g) was cut into small pieces, saponified and extracted as above, but with double the amounts of reagents. The residue was dissolved in 100 μl of *n*-hexane and 50 μl of the solution were subjected to HPLC for the pre-purification of the unsaponifiable matter, using a Zorbax SIL column (25 cm × 6.2 mm I.D.) with a flow-rate of 1.6 ml/min of 1.0% 2-propanol in *n*-hexane at a pressure of 60–80 kg/cm^2. The eluate corresponding to the 7-DHC fraction (20–24 min) was collected with the fraction collector and was subsequently applied to analytical HPLC on a Zorbax SIL column (25 cm × 4.6 mm I.D.) under the same conditions as for the assay of 7-DHC in skin.

As the content of 7-DHC in the liver is much smaller than that in the skin, the sensitivity of the UV detector was set to the maximum ($1 \cdot 10^{-3}$ a.u.f.s.).

RESULTS AND DISCUSSION

A piece of dorsal skin obtained from an adult male Wistar rat was treated according to the method described above and a typical chromatogram of the unsaponifiable matter is illustrated in Fig. 1. The 7-DHC peak was clearly separated from peaks of unknown material. The chromatogram shows that 7-DHC is a major UV-absorbing substance in the unsaponifiable matter of rat skin.

Peak identification was carried out on the eluate fraction corresponding to the peak of 7-DHC by means of reversed-phase HPLC on a Nucleosil $5C_{18}$ column (30 cm × 7.5 mm I.D.) eluted with methanol–2-propanol–water (4:2:1) at a flow-rate of 1.7 ml/min and UV spectrophotometry.

The overall recovery of 100 μg of 7-DHC added to the sample was 107.3 ± 3.4% [mean ± standard deviation (S.D.), $n = 6$].

Application of the same procedure to the determination of 7-DHC in liver was not successful, owing to extensive peak overlap with impurities. The content of 7-DHC in rat liver was too small to quantify directly from the crude unsaponifiable matter. In order to eliminate the UV-absorbing impurities, the unsaponifiable matter

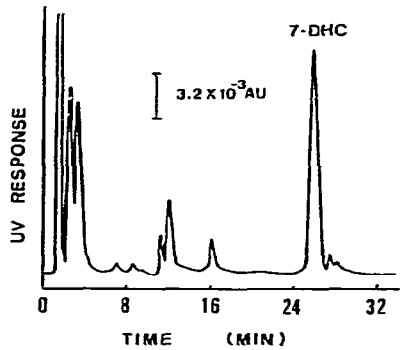

Fig. 1. Analysis of unsaponifiable matter obtained from rat skin by HPLC on a Zorbax SIL column (25 cm × 4.6 mm I.D.) with a flow-rate of 1.8 ml/min of 0.5% 2-propanol in *n*-hexane.

was first subjected to semi-preparative HPLC on a Zorbax SIL column (25 cm × 6.2 mm I.D.). Fig. 2a shows a typical chromatogram of the unsaponifiable matter obtained from rat liver. The eluate corresponding to the 7-DHC fraction (represented by arrows) was subsequently subjected to analytical HPLC under the same conditions as for the assay of 7-DHC in skin. Although baseline separation from unknown peaks could not be obtained, an adequate and reproducible separation was achieved (Fig. 2b). The overall recovery of 1 μg of 7-DHC added to the sample was 95.0 $\pm$ 5.6% (mean $\pm$ S.D., $n = 6$).

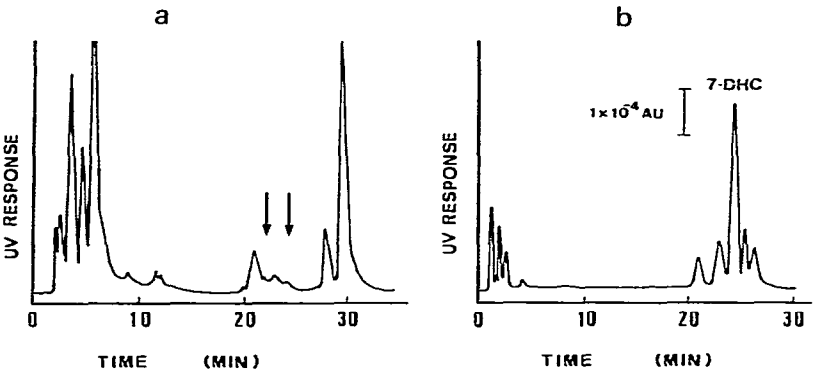

Fig. 2. (a) Semi-preparative HPLC of unsaponifiable matter obtained from rat liver on a Zorbax SIL column (25 cm × 6.2 mm I.D.) with a flow-rate of 1.6 ml/min of 1.0% 2-propanol in *n*-hexane. Arrows indicate the beginning and end of elution of the 7-DHC fraction. (b) Analytical HPLC of the pre-purified 7-DHC fraction obtained by semi-preparative HPLC. The conditions were identical with those for the analysis of skin unsaponifiable matter.

The peak corresponding to 7-DHC was identified as follows. A large amount of pre-purified 7-DHC fraction was collected from the other parts of the unsaponifiable matter and was subjected once more to semi-preparative HPLC. The 7-DHC fraction obtained was further purified by subjecting it to analytical HPLC. The eluate corresponding to the 7-DHC peak was collected and then the UV absorption spectrum was recorded. As Fig. 3 shows, the spectrum gave the characteristic four absor-

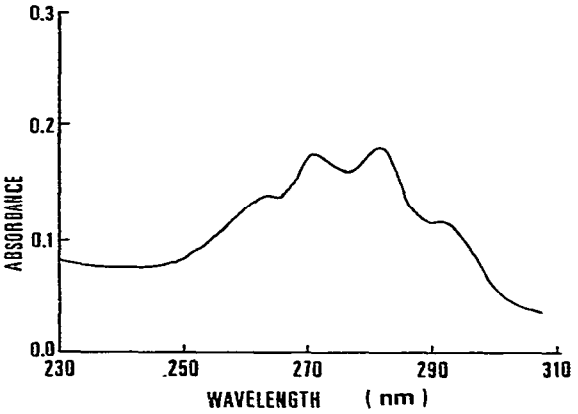

Fig. 3. Ultraviolet spectrum of purified 7-DHC fraction obtained from rat liver.

TABLE I

COMPARISON OF 7-DHC CONTENTS IN RAT SKIN AND LIVER

Sample No.	*7-DHC content (μg/g)*	
	Skin	*Liver*
1	274	1.88
2	207	1.10
3	256	0.64
4	262	0.74
5	280	1.25
6	235	0.96
7	210	1.17
8	256	0.79
Mean ± S.D.	248 ± 26	1.07 ± 0.37

bance maxima for a 5,7-diene sterol such as 7-DHC. The isolation and identification of 7-DHC from rat liver have not been accomplished before by HPLC.

These methods were then applied to a comparative study of the levels of 7-DHC in rat skin and liver. Table I shows the 7-DHC levels found in the skins and livers of adult male Wistar rats (8 weeks old). As suggested before[2], the 7-DHC contents of the liver were very small in comparison with the skin contents (*ca.* 1:250). The high level of 7-DHC in the skin would be adequate for the photosynthesis of vitamin D_3.

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