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Note

Ultramicro determination of 7-dehydrocholesterol in rat skin by high-performance liquid chromatography with fluorescence detection

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7-Dehydrocholesterol (7-DHC; 5,7-cholestadien-3β-ol) is present as free and esterified forms in skin and is converted photochemically into vitamin D_3 via previtamin D_3 by UV radiation. The studies on the photochemical conversion have been made predominantly by using rat skin [1–6].

Gas chromatographic (GC) [1], GC–mass fragmentographic [2] and high-performance liquid chromatographic (HPLC) [3,4] methods with spectrophotometric detection have been reported for the determination of 7-DHC in rat skin.

We have previously introduced 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxoquinoxaline-2-carbonyl azide (DMEQ-CON_3) as a highly sensitive and reactive fluorescence derivatization reagent for the measurement of alcoholic compounds by HPLC [7]. Recently, we found that DMEQ-CON_3 reacts with 7-DHC to form a highly fluorescent derivative, and from this we have developed a method for the determination of free and total (the sum of free and esteri-

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fied) 7-DHC in minute amounts of rat skin by HPLC with fluorescence detection.

EXPERIMENTAL

Reagents and solution

All chemicals and solvents were of reagent grade, unless stated otherwise. Deionized and distilled water was used. 7-DHC was purchased from Sigma (St. Louis, MO, U.S.A.). DMEQ-CON_3 was prepared as described previously [7]. DMEQ-CON_3 solution (2.0 mM) was prepared in benzene and could be used for two days when stored in a refrigerator at 4°C. Pyrogallol (antioxidant) solution (0.46 M) was prepared in ethanol. Male Wistar rats (ten weeks old, 210–230 g) were used.

Instrumentation

IR spectra were recorded with a Shimadzu 430 IR spectrophotometer (Kyoto, Japan) in potassium bromide pellets. ^1H NMR spectra were obtained with a Hitachi R-90H spectrometer (Tokyo, Japan) at 90 MHz using ca. 5% solution in [^2H]chloroform containing tetramethylsilane as an internal standard. Splitting patterns were designated as follows; s, singlet; d, doublet; m, multiplet. Field desorption mass spectra were obtained with a JEOL DX-300 spectrometer (Tokyo, Japan).

A Waters 510 high-performance liquid chromatograph (Japan Waters, Tokyo, Japan) equipped with a U6K universal injector (20-μl loop), and a Hitachi F1100 fluorescence spectromonitor equipped with a 12-μl flow-cell operated at an excitation wavelength of 360 nm and an emission wavelength of 440 nm, were used. The column was a TSK gel ODS-120T (250 mm×4.6 mm I.D., particle size 5 μm; Tosoh, Tokyo, Japan). The mobile phase was acetonitrile–methanol–water (80:12:8, v/v). The flow-rate was 1.0 ml/min.

When uncorrected fluorescence excitation and emission spectra of the eluates needed to be measured, a Hitachi 650-60 fluorescence spectrophotometer fitted with a 20-μl flow-cell was used; the spectral bandwidths were 5 nm for both the excitation and emission wavelengths.

Fluorescent derivative of 7-DHC

DMEQ-CON_3 (100 mg, 0.35 mmol) and 7-DHC (150 mg, 0.39 mmol) were dissolved in 40 ml of benzene and the solution was refluxed for 60 min. The reaction mixture was evaporated to dryness in vacuo. The residue, dissolved in 5 ml of chloroform, was chromatographed on a silica gel 60 (ca. 25 g, 70–230 mesh; Japan Merck, Tokyo, Japan) column (18 cm×2 cm I.D.) using hexane–ethyl acetate (2:1, v/v) as the solvent system. A fraction containing the fluorescent material was evaporated to dryness, and the residue was recrystallized from ethyl acetate as colourless needles (m.p. 175–176°C; yield 40 mg). The

spectral data were as follows: IR ν_{max} (cm^{-1}): 1750 (N–C=O); 1647 (C=O); 1619 (C=C and/or C=N); 2950 (NH); ^1H NMR (C^2HCl_3) δ (ppm): 0.63–2.64 (43H, m, $C_{27}H_{43}$); 3.74 (3H, s, N–CH_3); 3.93 and 3.98 (3H each, s each, O–CH_3); 4.60–4.90 (1H, m, COOCH); 5.39 and 5.59 and (2H each, d each, HC=CH); 6.69 and 7.26 (1H each, s each, aromatic H); 8.53 (1H, s, NH). Analysis calculated for $C_{39}H_{55}N_3O_5$: C, 72.53; H, 8.58; N, 6.51; found: C, 71.92; H, 8.61; N, 6.45. Mass spectra, $m/z=645$ (M^+).

Procedure

A portion (ca. 50 mg wet mass) of rat skin was minced and homogenized with a Polytron homogenizer (Kinematica, Switzerland) in 3.5 ml of the pyrogallol solution. For the determination of total 7-DHC, the homogenate was saponified by heating for 30 min at 80 °C in 0.4 ml of 15 *M* potassium hydroxide previously [4,5]. After cooling, the mixture was extracted with 5 ml of benzene, and the organic layer (2 ml) was washed once with 3 ml of 0.34 *M* sodium chloride and three times with 3 ml of water. The benzene layer (0.5 ml) was transferred into a PTFE screw-capped 10-ml vial and evaporated to dryness under a nitrogen stream. The residue was dissolved in 0.2 ml of the DMEQ-CON_3 solution and the mixture was heated for 60 min at 120 °C. After cooling, the reaction mixture was diluted with 1.8 ml of methanol, and a 10-μl portion of the resulting solution was subjected to HPLC. For the determination of free 7-DHC, the same procedure was carried out except that saponification was omitted.

The calibration graphs for the determination of free and total 7-DHC were prepared according to the procedure without and with alkaline hydrolysis, respectively, except that 3.5 ml of the pyrogallol solution was replaced with 3.5 ml of the solution containing 7-DHC (36.0 pmol to 65.1 nmol or 13.8 ng to 25 μ per 3.5 ml).

RESULTS AND DISCUSSION

Derivatization conditions

Among the solvents examined for the derivatization reaction (benzene toluene, acetone, acetonitrile, ethyl acetate, diethyl ether, chloroform, dimethyl sulphoxide and N,N-dimethylformamide), the use of benzene resulted in the most intense peaks in chromatogram. Although benzene is prohibited in many laboratories because of its toxicity, we found no other suitable solvent for extraction and derivatization of 7-DHC. Therefore, benzene was used in the procedure.

A chromatogram obtained with a standard solution of 7-DHC is shown in Fig. 1. Since DMEQ-CON_3 gave the most intense peak for 7-DHC at concentrations greater than ca. 1.0 m*M* in the solution, a concentration of 2.0 m*M* was chosen. The derivatization reaction occurred at moderate temperatures,

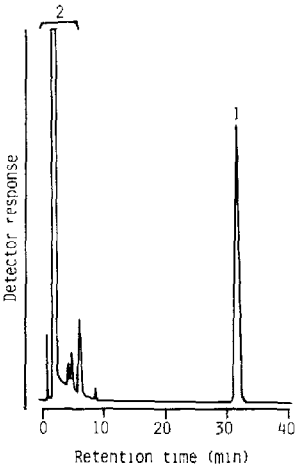

Fig. 1. Chromatogram of the DMEQ derivative of 7-DHC. A portion (3.5 ml) of a standard solution of 7-DHC (13.0 nmol or 5 μg per 3.5 ml) in ethanol was treated according to the procedure. Peaks: 1 = 7-DHC; 2 = the reagent blank.

but proceeded more rapidly at higher temperatures. At 120–140°C, the peak height reached almost maximum and remained constant after heating for 30–60 min; heating at 120°C for 40 min was employed in the procedure.

The efficiency of conversion of 7-DHC into the DMEQ derivative was examined by comparing the peak height obtained under the conditions of the procedure with that given by the reaction product; the extent of conversion (7-DHC, 13.0 nmol or 5.0 μg per 3.5 ml) was 67.2 ± 2.8% (mean ± S.D., $n=8$). The extent of conversion of 7-DHC in rat skin was almost the same as for the standard. The DMEQ derivative of 7-DHC in the final solution was stable for at least 24 h in the dark at room temperature.

Fluorescent derivative in the determination of 7-DHC

The fluorescent product from 7-DHC was identified as the corresponding carbamic ester by the analytical data described in Experimental. The fluorescence excitation (maximum, 360 nm) and emission (maximum, 440 nm) spectra of the product in acetonitrile–methanol–water (80:12:8, v/v) were identical with those of the peak 1 in Fig. 1.

Determination of 7-DHC in rat skin

When the rat skin homogenate was treated according to the procedure, many fluorescent peaks were observed in the chromatogram. The separation of the peak for 7-DHC from the other peaks was achieved on a reversed-phase column, TSK gel ODS-120T. The best separation was achieved using acetonitrile–methanol–water (80:12:8, v/v). Typical chromatograms obtained with

rat skin homogenates before and after saponification are shown in Fig. 2. The peak for 7-DHC (peak 1) was characterized on the basis of the retention time and fluorescence excitation and emission spectra, in comparison with the standard compound, and co-chromatography with the standard using aqueous 80–100% (v/v) methanol as mobile phase. When the residue obtained from the benzene extract was treated with 3-chloroperbenzoic acid (to oxidize the double bounds of 7-DHC) under the conditions of epoxidation of cholesterol [8], peak 1 and peak 2 (Fig. 2) disappeared. The eluates of peak 1 in Fig. 2A and B were collected and lyophilyzed, and the residues were subjected to field desorption mass spectrometry. The spectra were identical with those of the fluorescent product. These observations indicate that peak 1 in Fig. 2A and B is a single component, the DMEQ derivative of 7-DHC.

A linear relationship was observed between the peak heights and the amounts of 7-DHC added to rat skin (50 mg), up to at least 65.1 nmol (25 μg). The detection limit was 36.0 pmol (13.8 ng) per 50 mg rat skin (corresponding to 18 fmol on 6.9 pg in a 10-μl injection volume) at a signal-to-noise ratio of 3. Recovery tests were carried out by adding known amounts of 7-DHC (3.13 nmol or 1.2 μg and 13.0 nmol or 5 μg for the determination of free and total 7-DHC, respectively) to rat skin (50 mg), and the mixtures were left standing for 30 min at 4 °C; the recoveries were 99.8 ± 0.32 and 98.9 ± 0.28% (mean ± S.D., $n = 10$), respectively. The within-day precision was determined from repeated analyses ($n = 10$) of a rat skin containing 62.5 nmol (24 μg) and 260 nmol (100

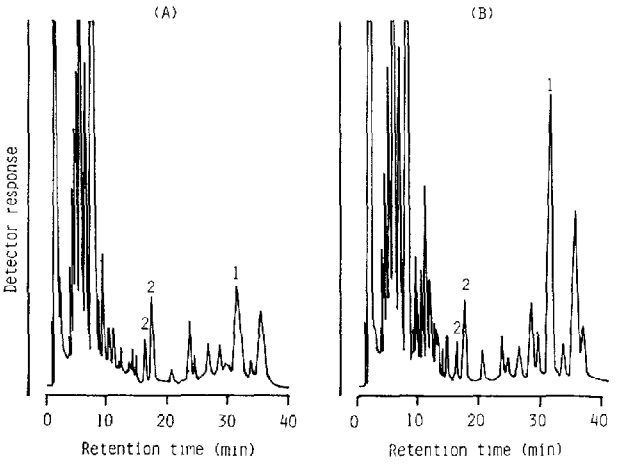

Fig. 2. Chromatograms obtained with rat skin homogenate (A) before and (B) after saponification. Experimental details are described in the text. Peaks: 1 = 7-DHC, disappeared on oxidation; 2 = unidentified, disappeared on oxidation; others = the reagent blank and endogenous substances in rat skin. Concentrations of free and total 7-DHC: 59.9 nmol (23.0 μg) and 260 nmol (100 μg) per g skin [equivalent to 30.0 pmol (11.5 ng) and 130 pmol (50 ng) per 10-μl injection volume], respectively.

TABLE I

CONCENTRATIONS OF FREE AND TOTAL 7-DEHYDROCHOLESTEROL AND THEIR RATIOS IN RAT SKIN

Sample	Concentration				Free/total [(mol/mol)×100]
	Free 7-DHC		Total 7-DMC		
	nmol/g of skin	μg/g of skin	nmol/g of skin	μg/g of skin	
1	63.7	16.5	265	102	25.0
2	59.8	23.0	260	100	23.0
3	84.2	32.4	224	86	37.7
4	52.5	20.2	213	82	24.6
5	62.4	24.0	213	82	29.4
6	55.6	21.4	166	64	33.4
Mean	61.8	21.4	207	80	23.5
S.D.	2.0	0.8	53	20	0.5

μg) per g of free and total 7-DHC, respectively. The relative standard deviations were 4.7 and 5.2% for free and total 7-DHC, respectively.

The concentrations of free and total 7-DHC in skin of six animals determined by this method and their ratios are shown in Table I. The mean values are in good agreement with the published data [3].

This study is the first reported HPLC method with fluorescence detection for the determination of 7-DHC in rat skin. The method is highly sensitive and rapid, and thus should be useful for biomedical investigation of 7-DHC, particularly in very small amounts of skin.

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