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Determination of 7-dehydrocholesterol in human skin by high-performance liquid chromatography

JOHN P. MOODY*, CARL A. HUMPHRIES, STELLA M. ALLAN and COLIN R. PATERSON

Department of Biochemical Medicine, Ninewells Hospital and Medical School, Dundee DD1 9SY, Scotland (U.K.)

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ABSTRACT

A two-stage chromatographic procedure has been devised for the measurement of 7-dehydrocholesterol in human skin. Extracts containing ergosterol as internal standard underwent preparative chromatography on a Spherisorb S5W column using hexane–1% isopropanol as solvent, and an eluted fraction was analysed with an Ultrasphere 5-μm ODS column with methanol–tetrahydrofuran 17.5 m*M* KH_2PO_4 (95:1:4, v/v) as solvent and using an amperometric detector at 1.7 V. 7-Dehydrocholesterol could be reliably assayed in human skin samples as small as 5 mm in diameter. In hospital patients skin 7-dehydrocholesterol concentrations ranged from 12 to 81 μg/g dry weight.

INTRODUCTION

The photoconversion of 7-dehydrocholesterol (7-DHC) to cholecalciferol in man is a vital physiological process for the maintenance of intestinal calcium absorption; failure to synthesise vitamin D leads to osteomalacia or rickets if dietary sources are also inadequate [1]. Appreciable quantities of 7-DHC are found in human skin, especially in the stratum spinosum, stratum basale and dermis [2]. Little is known concerning the role of reduced levels of 7-DHC in the skin as a risk factor for osteomalacia and rickets. In recent years, however, epidermal concentrations have been shown to decrease with advancing age [3], and the capacity of the skin to produce vitamin D3 is reduced.

Several methods, based on high-performance liquid chromatography (HPLC), have been devised for measuring 7-DHC in skin [2,4–7]. The concentrations found in animal skins after saponification were sufficiently large to allow quantitation on normal-phase columns with UV detection. Concentrations of 7-DHC in human skin measured by chromatographic techniques are much lower than those found in animals and considerably lower than the levels reported by earlier workers [8]. We found that a single-stage isocratic HPLC procedure was not sufficiently sensitive to provide accurate and precise measurements [9] and have devised a two-stage method of analysis using an amperometric detector with improved sensitivity. This allows the use of smaller skin samples.

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EXPERIMENTAL

Chemicals

Cholesterol, 7-DHC and ergosterol were purchased from BDH Biochemicals, and lathosterol, desmosterol and stigmasterol acetate were obtained from Sigma (St. Louis, MO. U.S.A.). All other chemicals and solvents were either Analar or HPLC grade. When kept under refrigeration solid 7-DHC slowly deteriorated with loss of UV spectral integrity; 100-mg aliquots of newly purchased 7-DHC were therefore vacuum-sealed in glass ampoules and stored at −70°C until needed.

Glassware

Because of the light sensitivity of 7-DHC, amber glass tubes were used in sample preparation. In addition 10-ml conical tubes were silanised prior to use. All tubes were rinsed with methanol before each experiment.

Skin samples

The larger specimens were supplied by surgeons at Ninewells Hospital. Excess subcutaneous fat was removed with a scalpel, after which each specimen was cut into small pieces (*ca.* 5 mm × 5 mm). Smaller specimens were obtained from healthy outpatients of the Department of Dermatology. All the patients were Caucasian. The samples were weighed and dried in a dessicator under vacuum at 4°C for at least seven days.

High-performance liquid chromatography

Two chromatographic systems were used. For sample preparation a Gilson isocratic high-performance liquid chromatograph (Gilson France, Villiers le Bel, France) was employed, comprising a Model 302 pump and Model 802 manometric module and a Holochrome UV–visible detector set at 286 nm with a Servoscribe chart recorder set at 100 mV and a paper speed of 120 mm/h. A Rheodyne Model 7125 injector was fitted with a 100-μl loop, and a 25 cm × 0.5 cm I.D. stainless-steel column packed with Spherisorb S5W (Phase Separations, Clwyd, U.K.) and a guard column were used. The guard column was repacked after every fifteen to twenty injections. The solvent was hexane–isopropanol (99:1) at a flow-rate of 1.5 ml/min.

For analytical chromatography the detector system was replaced with a Bioanalytical Systems LC3A amperometric detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.) set at 1.7 V, LC17 transducer with glassy carbon and an Ag/AgCl electrode. The chart recorder was set at 10 mV. The injector was fitted with a 20-μl loop and a 25 cm × 0.5 cm I.D. stainless-steel column packed with 5-μm Ultrasphere ODS (Scotlab Instrument Sales, Bellshill, U.K.) and a guard column. The solvent was methanol–tetrahydrofuran (THF)–17.5 m*M* KH_2PO_4 (95:1:4, v/v) at 1.5 ml/min. All chromatography was carried out at ambient temperature.

Analytical procedure

The analytical procedure was developed from the method of Takada *et al.* [5].

Standards

Stock solutions, containing 1 mg/ml 7-DHC and ergosterol in ethanol, were prepared fresh at fortnightly intervals and stored at 4°C. The purity of each stock solution was checked by UV spectrophotometry and HPLC. These solutions were diluted 1:50 with methanol prior to use. 7-DHC was further diluted with methanol to provide calibrators containing 4, 8, 12, 16 and 20 μg/ml which were mixed with equal volumes of diluted ergosterol (internal standard).

Specimens

Dried skin (10–20 mg) was added to a 20-ml stoppered tube containing 1 ml of 10% pyrogallol and 100 μl of internal standard. A 1-ml volume of 85% potassium hydroxide was added and the tube was lightly stoppered and heated in a boiling water bath for 60 min. On cooling, 7 ml of hexane-3% amyl alcohol were added and the tube was stoppered, mechanically shaken (5 min) and centrifuged. The lower layer was aspirated; 4 ml of 0.2 *M* hydrochloric acid were added and the tube was stoppered, shaken (2 min) and centifruged.

Two 100-μl aliquots of the hexane layer were added to 10-ml stoppered conical tubes and evaporated to dryness (45°C, nitrogen) for cholesterol determination. The remainder was transferred to a silanised 10-ml stoppered conical tube using a silanised glass Pasteur pipette and evaporated to 100 μl (45°C, nitrogen). The complete extract was chromatographed using a normal-phase column of Spherisorb S5W and hexane–1% isopropanol, collecting the fraction eluting between 9 and 12.5 min, corresponding to 7-DHC and ergosterol, in a silanised 10-ml stoppered conical tube. The solvent was evaporated to dryness (45°C, nitrogen). Tubes could be stored overnight at 4°C at this point.

Residues were redissolved in 30 μl of chromatographic solvent and subjected to analytical HPLC with an Ultrasphere ODS column and a mixture of methanol–THF–17.5 m*M* KH_2PO_4 (95:1:4, v/v) as solvent.

Cholesterol determination

Cholesterol was determined as the trimethylsilyl derivative by gas chromatography. A 50-μl aliquot of a 1 mg/ml solution of stigmasterol acetate in ethanol was added to each tube and dried down at 60°C in nitrogen. Acetonitrile (30 μl) and bis(trimethylsilyl)trifluoroacetamide (BSTFA) (10 μl) were added, and the tubes were stoppered and incubated at 70°C for 15 min. Aliquots of 1 μl were chromatographed with a Pye Unicam 104 gas chromatograph–flame ionisation detector with appropriate standards using a 1.52 m × 0.4 mm I.D. silanised glass column packed with 3% SE30 on Chromosorb W HP (80–100 mesh). The carrier gas was nitrogen at 40 ml/min. The temperatures were: inlet, 300°C; column, 270°C; detector, 290°C.

TABLE I

RESOLUTION OF 7-DHC AND ERGOSTEROL BY NORMAL- AND REVERSED-PHASE HPLC

System	Mobile phase	Capacity factor (k')		Resolution (R_s)
		7-DHC	Ergosterol	
Spherisorb S5W	Hexane–isopropanol mixtures			
	0.5% Isopropanol	11.82	11.69	0.256
	1.0% Isopropanol	5.06	5.24	0.694
	2.0% Isopropanol	2.20	2.21	0.069
	3.0% Isopropanol	1.21	1.23	0.179
5-μm Ultrasphere ODS	Methanol–THF– 17.5 m*M* KH_2PO_4 (95:1:4, v/v)	15.06	14.19	0.979

RESULTS

Hexane–isopropanol mixtures do not separate 7-DHC and ergosterol on normal-phase chromatography using a 25-cm-long column of Spherisorb S5W (Table I). However, these sterols can be resolved sufficiently well for analysis using reversed-phase chromatography with a 25-cm-long column of 5-μm Ultrasphere ODS using methanol–THF–17.5 m*M* KH_2PO_4 (95:1:4, v/v) as solvent (Fig. 1).

7-DHC and ergosterol have similar UV absorption spectra and similar absorptions [9]; quantities as small as 7.5 ng may be detected on-column by normal-phase chromatography, using a UV–visible detector (signal = 2 × background noise). With the reversed-phase system and amperometric detection 7-DHC and

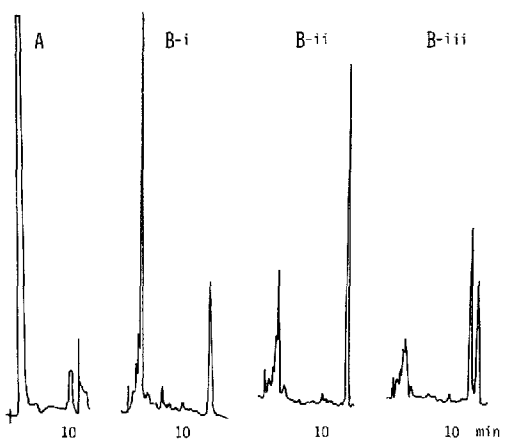

Fig. 1. HPLC of skin extract. (A) Normal-phase chromatography with UV detector. (B) Reversed-phase chromatography with amperometric detection: (i) skin extract; (ii) skin extract plus 100 ng 7-DHC; (iii) skin extract plus 100 ng ergosterol.

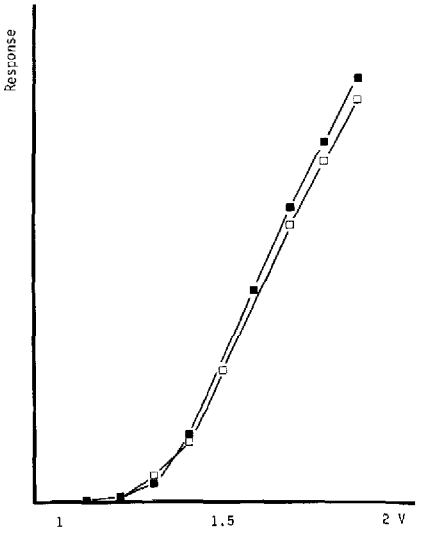

Fig. 2. Signal current–potential difference curve for 7-DHC and ergosterol with the amperometric detector. Sequential 1-μg aliquots of 7-DHC and ergosterol were injected into the chromatograph using the reversed-phase system and amperometric detector. Sensitivity was set to minimum and electrode potentials were varied between 1.0 and 1.9 V. Solvent: methanol–THF–10 m*M* potassium chloride (95:1:4 v/v).

ergosterol gave identical signal–electrode potential curves with voltage maxima in excess of the instrument capacity (Fig. 2). Furthermore, peak heights were proportional to the chromatographic load over the range 40–2000 ng on-column. The detection limit was 1.5 ng. Cholesterol, lathosterol and desmosterol were not detected at column loads of 100 ng.

Reversed-phase separation of skin extract with amperometric detection yield-

TABLE II

RECOVERY OF 7-DHC ADDED TO SKIN HOMOGENATES

Added (μg/g)	*n*	Found (μg/g)		Recovery (%)	
		Mean	Range	Mean	Range
1	4	1.1	1.0–1.3	108	95–130
2	4	2.1	1.8–2.4	103	88–120
3	4	2.9	2.8–2.9	95	93–97
4	4	3.8	3.7–3.9	94	91–98
5	4	4.7	4.5–5.0	93	89–100
10	3	9.7	8.6–10.3	97	86–103
20	3	19.2	17.9–20.2	96	90–101
30	3	29.2	27.4–30.2	97	91–101
40	3	38.9	36.9–40.3	97	92–101
50	3	49.1	46.6–51.1	98	93–102

TABLE III

7-DEHYDROCHOLESTEROL AND CHOLESTEROL CONTENT OF HUMAN SKIN SAMPLES

Subject		Age (years)	7-Dehydrocholesterol (μg/g wet weight)	7-Dehydrocholesterol (μg/g dry weight)	Cholesterol (mg/g dry weight)	7-Dehydrocholesterol (mg/g of cholesterol)
Surgical		73	15.2 ± 1.9	52.8 ± 5.1	7.8 ± 1.0	6.9 ± 0.9
patients		43	10.9 ± 2.3	34.8 ± 7.3	4.4 ± 0.7	8.0 ± 1.1
		20	6.1 ± 2.4	20.0 ± 7.8	3.9 ± 0.4	5.1 ± 1.6
		18	8.5 ± 4.4	23.9 ± 3.8	3.3 ± 0.4	7.2 ± 0.4
All	Mean	41	–	35.7	4.3	9.2
males	S.D.	22	–	18.7	1.4	4.3
	n	9	–	9	9	9
All	Mean	37	–	44.4	4.3	10.6
females	S.D.	19	–	14.8	0.9	3.8
	n	15	–	15	15	15

ed a chromatographic peak which did not separate from 7-DHC (Fig. 1) and which possessed identical signal current–potential difference characteristics. In addition, a similar peak was absent from a reagent blank.

The results of recovery experiments in which 0.1–5.0 μg of 7-DHC were added to 1 ml of skin homogenate (100 mg wet weight in 0.1 *M* phosphate buffer pH 7.4) are summarised in Table II. 7-DHC could be quantitatively recovered with relative efficiencies of 93–108%, and absolute recoveries, estimated by comparing experimental and standard peak heights, ranged from 30 to 75%. Within-batch imprecision for 20 μg/g standard addition was 2.1% and between-batch imprecision ranged from 12.9 to 4.6% over the concentration range. Overall imprecision was 7.3%.

Skin concentrations of cholesterol and 7-DHC in surgical inpatients and dermatology outpatients are given in Table III. Throughout the group 7-DHC concentrations ranged from 12 to 81 μg/g dry weight or 4 to 16 μg/mg cholesterol. In four surgical patients we had sufficiently large pieces of skin that multiple samples could be taken. Table III indicates the variation seen between samples.

There was a tendency for 7-DHC concentrations in males to be lower than in females but the differences were not statistically significant.

Within the whole group there was a statistically significant correlation between age and skin cholesterol content ($r = 0.621$, $t = 3.7$ $n = 24$, $p < 0.005$). Skin 7-DHC and skin cholesterol were not significantly correlated ($r = 0.397$) and there was no correlation between age and skin 7-DHC content expressed as μg/g dry weight ($r = 0.311$) or between age and skin 7-DHC expressed as μg/mg cholesterol ($r = 0.036$).

DISCUSSION

Vitamin D is an essential precursor of the hormone calcitriol needed for active calcium absorption in the intestine. The photoconversion of 7-DHC to cholecalciferol by sunlight is the major source of vitamin D in man and prevents the development of osteomalacia and rickets. It is not known whether the lack of precursor ever contributes to the development of vitamin D deficiency. 7-DHC is not detectable in blood [10] but is present in skin and some major internal organs in significant amounts [5,11]. Skin biopsy provides the only possible material for investigating 7-DHC status.

A major drawback of the current methods for the analysis of 7-DHC in skin is the absence of a suitable internal standard which can be extracted from the biological matrix. Yasamura *et al.* [4] used naphthol as internal standard after the extraction of skin sterols, but this substance cannot be extracted from saponified material. Ergosterol coelutes with 7-DHC on normal-phase columns with hexane–1% isopropanol but the substances are adequately separated on a reversed-phase column. These properties have been exploited in a two-stage chromatographic procedure of high specificity which also permits the use of an internal standard throughout.

The relatively small quantities of 7-DHC in human skin in the presence of large amounts of unsaponified lipid makes normal-phase chromatography of skin extracts difficult; the peaks are mis-shapen and not easy to measure. Furthermore, the sensitivity of the UV detector is limited, especially in the reversed-phase mode (minimum detectable amount 25 ng). The use of an amperometric detector with an electrode potential of 1.7 V in conjunction with the reversed-phase system was appreciably more sensitive, as little as 1.5 ng being detectable on-column. For effective amperometric detection KH_2PO_4 was included in the reversed-phase solvent system at a total concentration of 0.7 m*M*. Optimum sensitivity is in excess of 1.9 V where interferences from other organic substances can be anticipated though this has not occurred on our chromatograms to date. For practical purposes a lower voltage has been used to extend the life of the glassy carbon electrode. The amperometric signal seems to be specific for sterols with conjugated 5,7-dien structures in the B ring as found in 7-DHC and ergosterol. Cholesterol and sterols with the single 5-en or 7-en configuration were not measured. At present nothing is known about the charged species generated by the electrochemical interaction.

The method has been in regular use in this laboratory for twelve months and has proved robust and reliable. Contamination of the normal-phase column was a problem in the early stages of development, but was overcome by regular repacking of the guard column after twelve to fifteen injections.

There is little published information on skin concentrations of 7-DHC in man. Maclaughlin and Holick [3] reported epidermal concentrations ranging from 2.4 to 11.7 μg/g wet weight in a small group of subjects. These values are similar to our own figures but considerably lower than the values reported by Reinertson and Wheatley [8] using less specific techniques. In a group of 24 subjects we observed 7-DHC contents ranging from 12.1 to 80.6 μg/g dry weight, a seven-fold variation in concentration.

Little is known about the mechanisms regulating the 7-DHC content of skin. It has been suggested that skin 7-DHC synthesis is under a positive feedback influence from calcitriol levels in the circulation [12].

Thus subjects of low vitamin D status may be more at risk from poor availability of 7-DHC for photoconversion to cholecalciferol. Further work is planned to test the hypothesis that low skin levels of 7-DHC contribute to the pathogenesis of vitamin D deficiency.

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