Journal of Chromatography, 353 (1986) 169–173
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 769

MEASUREMENT OF BLOOD LEVELS OF 7-DEHYDROCHOLESTEROL IN MAN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

M. S. DEVGUN*
Department of Biochemistry, Law Hospital, Carluke, Lanarkshire ML8 5ER (U.K.)
and
C. R. PATERSON
Department of Biochemical Medicine, Ninewells Hospital, Dundee (U.K.)

SUMMARY

A high-performance liquid chromatography procedure was established to measure 7-dehydrocholesterol (7DHC) in the blood. Interference studies were performed using 20 similar compounds likely to give analytical errors. 7DHC was extracted with a methanol chloroform mixture. Extraction recoveries were reproducible and acceptable, with low methodological variations from batch-to-batch. Results show that 7DHC is not detectable in the blood, contrary to the previous reports. Possible explanations for its absence in the blood are: (1) it is confined to the skin and is not present in the blood in this form; (2) because the molecule is labile, it is destroyed by the methods applied; (3) previous reports on 7DHC levels were based on non-specific methods.

INTRODUCTION

7-Dehydrocholesterol (7DHC) is the precursor of vitamin D_3 (cholecalciferol) and is found in the skin[1,2]. Transformation of 7DHC results from the action of ultraviolet radiation of 280–320 nm wavelength[3]. The newly synthesised vitamin D_3 is then transported into the blood, bound to vitamin D binding globulins; in the liver, it is hydroxylated to form 25-hydroxy-vitamin D_3 (25-hydroxy-cholecalciferol)[4]. A further hydroxylation occurs in the kidneys to form 1,25-dihydroxy-vitamin D_3 (1,25-dihydroxy-cholecalciferol)[5], the active form which has been shown to be a hormone[6].

Deficiency of vitamin D causes rickets in children and osteomalacia in adults[7]. Relatively large amounts of 25-hydroxy-vitamin D are found in plasma and therefore, easily measured. Measurements can be helpful in identifying vitamin D deficiency, for example, in house bound elderly patients[8]. However, assays of plasma 25-hydroxy-vitamin D provide no information about the cause of vitamin D deficiency. In patients with intestinal malabsorption vitamin D deficiency occurs despite apparently adequate exposure to sunlight. It seems possible that such patients lack not only vitamin D but also the precursor 7DHC. Such a hypothesis might be confirmed by

0021-9673/86/$03.50

demonstrating low 7DHC levels in plasma in these patients. This paper reports the establishment of an assay for 7DHC based on high-performance liquid chromatography (HPLC) and attempts to determine the levels of 7DHC in human serum.

MATERIALS AND METHODS

A Du Pont (Wilmington, DE, U.S.A.) HPLC system was used for measuring 7DHC. It comprised a pump (Model 848), detector (Model 837) and an elution gradient accessory. Zorbax-Sil (25 × 4.6 cm, 5 μm packing) was used. All analytical-grade reagents and other chemicals were purchased from Koch-Light (Edinburgh, U.K.) or Sigma (Poole, U.K.).

Using the gradient-elution accessory, hexane–dichloromethane–methanol (80:20:0.7) was shown to elute 7DHC from a Zorbax-Sil column. This isocratic phase was used at a pressure of 1000 p.s.i. to detect 7DHC at 282 nm (wavelength with maximal response) at a retention time of approximately 11–12 min. Many substances were examined as possible internal standards; these included lanosterol, stigmasterol, thymol, ergosterol, and sitosterol. Repeat injections of 7DHC were made on the same day and on consecutive days to determine the variations in retention time and in detector responses. The sensitivity of the detector was checked by injecting decreasing quantities of standard 7DHC prepared in the mobile phase.

7DHC was extracted from serum from 15 normal subjects (age range 20–34 years) and from six patients with hypercholesterolaemia using the Bligh and Dyer procedure[9]. Prior to extraction, 0.5 ml serum or plasma was mixed with 0.5 ml distilled water and allowed to equilibrate for 5–6 min. This was followed by the addition of 1.25 ml chloroform and 2.5 ml methanol and mixing. The mixture was allowed to stand for 30 min. The extraction was then continued by adding 1 ml distilled water and 1.25 ml chloroform. After mixing, the tubes were centrifuged for 15 min at 2000 *g* at 4°C. The supernatant was aspirated and two further extractions were carried out on the residue, using the 2.5-ml portions of chloroform.

The combined chloroform fractions were dried in a rotary evaporator (37°C), and the residue was dissolved in 50 μl mobile phase. This sample was then purified on a silicic acid micro-column (3 × 0.7 cm, mesh 325) before injecting it into a HPLC column. In order to eliminate phospholipids present in the extract, 10 ml diethyl ether was passed through the column, which was then eluted as stated above.

RESULTS

The peak height was a linear function of the 7DHC concentration up to 60 ng/ml with a sensitivity of less than 5 ng/ml at 0.001 a.u.f.s. Extraction recoveries of spiked serum samples ranged from 70 to 85 percent. Table I shows that the method was able to distinguish free sterols and vitamin D from its precursor. It is also clear that compounds likely to interfere with 7DHC were adequately resolved. The only exception was found to be ergosterol. Although tymol does not resemble 7DHC chemically, it was chosen as an internal standard because its extaction properties and retention time were similar to those of 7DHC and, more importantly, it was so considered because it is not found in the body and is unlikely to be prescribed as a drug. This last qualification is very important in any new assay; it is highlighted by

TABLE I

RELATIVE RETENTION TIME OF VARIOUS COMPOUNDS

For chromatographic conditions, see text.

Compound	*Retention time (min)*	*Comments*
Orcinol	–	No peak
Phloroglucinol	–	No peak
Cholesterol	–	No peak
Oestradiol	–	No peak
Stilboesterol	–	No peak
Mestranol	–	No peak
Cholestenol	–	No peak
Sitosterol	–	No peak
Stigmasterol	–	No peak
Cholestan-3β-ol	–	No peak
20α-Hydroxycholesterol	3.42	–
Lanosterol (isocholesterol)	2.87 and 2.88	Probably impure
25-Hydroxycholecalciferol	5.76	–
Ergocalciferol	7.96	–
Cholecalciferol	7.80	–
Calciferol	7.62	–
Ergosterol (provitamin D)	8.72 and 11.76	Probably impure
1α,25-Dihydroxycholecalciferol	5.91	–
7-Dehydrocholesterol	11.65	–
Thymol	6.07	–
Naphthol	11.37	–
Naphthol + 7DHC	11.20	No resolution

a recent report describing the falsely elevated levels of 25-hydroxy-vitamin D_3 in an HPLC method resulting from interference by a prescribed medication[10].

The intra- and inter-assay coefficients of variation were less than 6% when hormone-free serum was spiked with 7DHC at a concentration of 30 ng/ml. The identity of the 7DHC peak was confirmed by conversion to vitamin D_3 after exposure of serum samples, in quartz tubes, to ultraviolet irradiation for 10 min, using an Ultraphil HP.3114 ultraviolet and infrared lamp (Philips Electrical Co.). These lamps had previously been shown to be effective in emitting radiation of 280–320 nm which is necessary to transform 7DHC into vitamin D_3[11].

Surprisingly, none of the samples analysed showed any detectable 7DHC. 7DHC was successfully eluted and estimated when repeat duplicate analysis were performed on all samples in the presence of spiked 7DHC standard at a concentration of 30 ng/ml (Fig. 1). Fig. 1 also shows that an unidentified substance (peak A) was eluted immediately after the 7DHC peak; we were unable to identify this compound, because of the small amount eluted. However, we were able to confirm that it was not 7DHC by recovering it from serum samples by HPLC analysis, after exposure to ultraviolet irradiation.

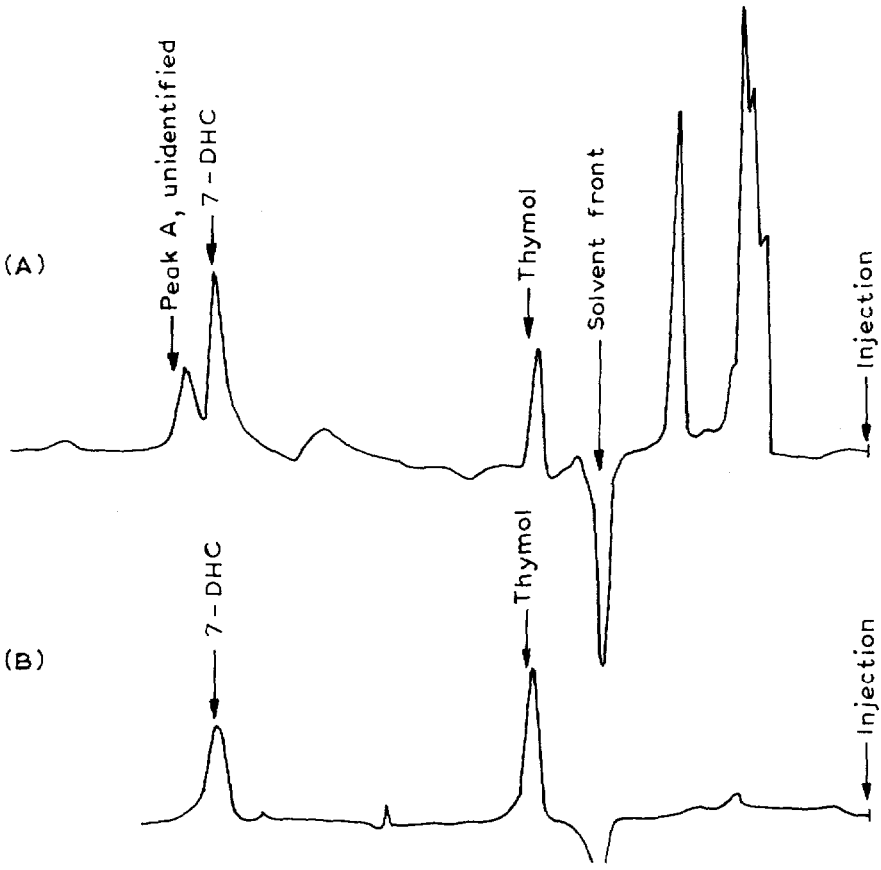

Fig. 1. (A) Chromatogram of serum spiked with 7-dehydrocholesterol and with thymol as an internal standard. (B) Chromatogram of a standard solution containing internal standard and 7-dehydrocholesterol.

DISCUSSION

Our method was able to isolate 7DHC without interference of the compounds tested. Only ergosterol was eluted with the 7DHC peak. Ergosterol is the corresponding 5,7-diene of vitamin D_2: our method is therefore unable to resolve compounds with similar B-ring structure. Since it is unlikely that ergosterol would be present in blood no modification to the assay was made.

Results of our HPLC method show that 7DHC is not detectable in normal or hypercholesterolaemic serum or plasma in man. The latter was so chosen because 7DHC is an intermediate in the biosynthesis of cholesterol as well as of vitamin D_3. Data from our study are consistent with the observations of Gaylor[12] but not with those of others[13,14]. Koehler and Hill[13] found that 7DHC is present in appreciable quantities (2–7 mg/100 ml), both in normal and in patients with pathological conditions, but their observations were made by the use of a non-specific colorimetric method. Such methods also measure fast-acting sterols, including Δ^7-sterols that may be present in human serum[15].

Possible explanations for the absence of 7DHC in plasma are that it is confined to the skin and not released into the blood. On the other hand, it is possible that 7DHC is present in the blood, but only as a conjugated compound. If this were the case, it would be necessary to perform further experiments to hydrolyse the sample before repeating the assay. Lastly, it is possible that the B-ring of the 7DHC molecule is so unstable under the conditions employed in our method, that the molecule breaks down into various possible photoisomeric products, as observed in in-vitro experiments. However, this is unlikely to be the case, since we were able to recover the spiked 7DHC standards by using the same procedure.

REFERENCES

1 A. Windaus, F. Schenck and F. Von Werder, *Z. Physiol. Chem.*, 241 (1936) 100.
2 J. W. M. Bunker, R. S. Harris and L. M. Mosher, *J. Am. Chem. Soc.*, 62 (1940) 508.
3 E. Havinga, *Experientia*, 29 (1973) 1181.
4 M. H. Bhattacharyya and H. F. DeLuca, *Arch. Biochem. Biophys.*, 160 (1974) 58.
5 K. Saarem, J. I. Pedersen and S. Tollersrud, *Comp. Biochem. Physiol.*, 61B (1978) 485.
6 M. F. Holick and H. F. DeLuca, in D. E. M. Lawson (Editor), *Vitamin D*, Academic Press, London, 1978, p. 51.
7 C. R. Paterson, *Metabolic Disorders of Bone*, Blackwell, Oxford, 1974, p. 165.
8 M. S. Devgun, C. R. Paterson, C. Cohen and B. E. Johnson, *Age Ageing*, 9 (1980) 117.
9 E. G. Bligh and W. J. Dyer, *Chem. J. Biochem. Physiol.*, 37 (1959) 911.
10 S. H. Majors, J. C. Howe and G. R. Beecher, *J. Clin. Endocrinol. Metabol.*, 60 (1985) 706.
11 M. S. Devgun, A. J. M. Cruickshank, B. E. Johnson and C. R. Paterson, *Postgrad. Med. J.*, 57 (1981) 159.
12 J. L. Gaylor, *NY State J. Med.*, 62 (1962) 251.
13 E. Koehler and E. Hill, *Fed. Proc.*, 12 (1953) 232.
14 R. J. Henry, in R. J. Henry (Editor), *Clinical Chemistry —Principles and Technics*, Harper & Row, New York, 1964, p. 832.
15 R. Schoenheimer and E. A. Evans, *J. Biol. Chem.*, 114 (1936) 572.