J. steroid Biochem. Vol. 36, No. 4, pp. 351–355, 1990
Printed in Great Britain.

0022-4731/90 $3.00 + 0.00

ESTIMATION OF 26-HYDROXYCHOLESTEROL IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ITS MEASUREMENT IN PATIENTS WITH ATHEROSCLEROSIS

RAIDA HARIK-KHAN and ROSS P. HOLMES[1]*

Department of Food Science, University of Illinois, Urbana, IL 61801 and [1]Department of Urology, The Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, NC 27103, U.S.A.

(*Received* 5 *October* 1989)

Summary—A method for analysing 26-hydroxycholesterol (26OHC) in serum and tissue samples using solid phase extraction and high-performance liquid chromatography is reported. This procedure was used to measure the levels of 26OHC in the sera of apparently healthy subjects and of 18 patients with angiographically proven atherosclerosis. Sixteen of the patients had levels within or below the range detected in the apparently healthy subjects (125–294 ng/ml), indicating that high 26OHC levels cannot be a major factor in the development of atherosclerosis. However, when the patients and the normal subjects were combined in a group, there was a significant positive correlation ($r = 0.54$, $P < 0.01$) between serum cholesterol and serum 26OHC, and that correlation approached significance for each of the individual groups ($P = 0.06$ for each group). These results suggest that there is an association between cholesterol and 26OHC levels in human serum.

INTRODUCTION

26-Hydroxycholesterol (26OHC) is synthesised in the liver by a cytochrome *P*-450-linked monooxygenase localized in the inner mitochondrial membrane [1]. Other organs may also synthesize 26OHC, because mRNA for the 26-hydroxylase also has been detected in the duodenum, adrenal gland and lung [2]. The preferred substrates of 26OHC appear to be diols or triols that are bile acid intermediates, although cholesterol itself can also be hydroxylated [1, 3]. Significant amounts of 26OHC, both free and esterified with fatty acids, are released into the circulation. One laboratory reports the normal circulating concentration of 26OHC to range from 92 to 256 ng/ml serum [4], while another reports a range of 30–129 ng/ml [5]. Although the biological function of this circulating 26OHC has not been ascertained, other oxygenated sterols are known to be potent modifiers of cellular metabolism [6], with concentrations as low as 20 ng/ml in the medium being capable of affecting cholesterol biosynthesis [7]. 26OHC is one of the most potent of the oxysterols, and its circulating concentration is well above that known to affect cellular metabolism. However, the bulk of the sterol is esterified [4]. Esterification decreases the potency of 25-hydroxycholesterol [8], an autoxidation product of cholesterol, and may have a similar effect on 26OHC.

Because of their pathological effects on arterial smooth muscle cells *in vitro* and *in vivo*, oxysterols have been postulated to play a role in the development of atherosclerosis [9–11]. One study has disputed these claims by showing that large dietary doses of oxysterols are not atherogenic in an animal model [12], although both the experimental conditions and the method of assessing atherosclerosis differed from those in the earlier studies. Much attention has been focused on dietary sources of the oxysterols that may arise from oxidation of cholesterol-containing foods, but Javitt *et al.*[4], for example, failed to detect any 25-hydroxycholesterol in normal human sera. In view of the potency of 26OHC *in vitro*, its endogenous source, and its high circulating concentration, a study of the association of 26OHC with atherosclerosis appeared warranted. Previous studies have shown that 26OHC is one of the major oxysterols in human atherosclerotic plaques [13, 14].

In this study we developed a method for the analysis of serum 26OHC, and used that method to measure the concentration of 26OHC in atherosclerotic patients and to assess its correlation with serum cholesterol.

*To whom correspondence should be addressed.

EXPERIMENTAL

Materials

Kryptogenin (cholest-5-ene-3β,26-diol-16,22-dione) was kindly provided by Syntex Research, Palo Alto, Calif. All other chemicals were of analytical grade. 26OHC was synthesised from kryptogenin by the method of Scheer *et al.*[15], as modified by Wachtel *et al.*[16].

Subjects

Plasma samples were obtained from 18 patients with angiographically proven atherosclerosis from the Urbana-Champaign (Ill.) area, who ranged in age from 39 to 79 yr; two-thirds were men. For comparison, plasma samples were obtained from 4 female and 3 male laboratory personnel in good health, who ranged in age from 25 to 63 yr.

Synthesis of [^{3}H]26-hydroxycholesterol

[^{3}H]26OHC prepared by New England Nuclear, Boston, Mass., using catalytic exchange was not satisfactory for this project due to excessive tritium–hydrogen exchange in solvents. Instead, the isotope-labeled compound was synthesised by an *in vitro* enzymatic procedure using rat liver mitochondria as the source of 26-hydroxylase to synthesise [^{3}H]26OHC from [^{3}H]cholesterol [1, 17]. Mitochondria were isolated by the method of Saarem *et al.*[17]. For the isotope labeling, 10 μg cholesterol and 0.1 μCi of [1,2-^{3}H(N)]cholesterol (30 Ci/mmol, New England Nuclear, Boston, Mass.) were dissolved in 10 μl of ethanol and added to 1 mg of mitochondrial protein in 1 ml of 0.25 M sucrose, 25 mM MOPS (3-[*N*-morpholino]propanesulfonic acid), 10 mM $MgCl_2$, 2.7 mM ATP and 3 mM potassium malate. The incubation mixture was flushed with 100% oxygen for 45 s. After 1 h incubation at 37°C, the reaction was terminated by adding 5 ml of chloroform/methanol (1:2, v/v). The organic phase was withdrawn and the aqueous phase was extracted three more times with 5 ml of chloroform. The lipid extract was dried under N_2, redissolved in acetonitrile/water (1:1, v/v) and loaded on a pre-washed, C_{18} Sep-Pak (Waters) cartridge. The cartridge was washed with 3 ml of 70% methanol, and the fraction containing 26OHC was eluted with 4 ml of acetonitrile. This fraction was dried and injected on an Altex 5-μ silica column (Beckman) with 2.5% isopropanol in hexane as the mobile phase and a flow rate of 1.8 ml/min. The fraction coeluting with a 26OHC standard was collected, dried and reinjected on a Radial Pak C_{18}, 5-μ Resolve column (Waters) with a mobile phase of 10% H_2O in methanol running at 1.2 ml/min. The final purification of the 26OHC fraction was obtained by injecting it on a Radial Pak 10-μ CN column (Waters) with a mobile phase of 2.5% isopropanol in hexane at a constant flow rate of 2.0 ml/min. The specific activity of [^{3}H]26OHC thus obtained was 931 mCi/mmol. The dilution in specific activity may have been due to the presence of cholesterol and 26OHC in the mitochondria used.

Assay of 26OHC

Plasma samples, 0.5 ml in volume, containing 1000 dpm of [^{3}H]26OHC, were saponified by the addition of 15 ml of methanol/3 M NaOH (9:1, v/v), and incubation overnight at 37°C in a shaking water bath. Sterols were extracted by adding 15 ml H_2O, 30 ml of hexane, mixing and harvesting the upper hexane layer. The hexane extraction was repeated twice, and the pooled hexane extractions were dried by rotary evaporation. The extract, dissolved in 1 ml of acetonitrile and water (1:1, v/v), was loaded onto a washed C_{18} Sep Pak cartridge, then washed with 3 ml methanol/H_2O (7:3, v/v) and eluted with 4 ml of acetonitrile.

The 26OHC fraction was dried, dissolved in methanol, and loaded onto a radial compression C_{18} Resolve column. A mobile phase of H_2O/methanol (15:85, v/v) at a flow rate of 1.2 ml/min was used, and the peak coeluting with authentic 26OHC at approximately 17 min was collected.

26OHC was quantified by injecting the fraction from reversed phase high-performance liquid chromatography (HPLC) onto a 5-μ Altex silica column. The mobile phase, 2.5% isopropanol in hexane at a flow rate of 1.8 ml/min, gave a retention time of approximately 11 min. The 26OHC peak was collected, dried and its radioactivity estimated. Concentrations of 26OHC in plasma were calculated using a standard curve after correcting for losses during the assay procedure.

Mass spectroscopy

The gas chromatograph/mass spectrometry instrument consisted of a Ribermag R 10-10 C Quadrupole (Nermag, Houston, Tex.), a Girdel gas chromatograph (Nermag, Houston, Tex.), and a microcomputer that uses software by Teknivent (St Louis, Mo.). Samples were injected on a 7 m DB-1 column (J&W Scientific, Folsom, Calif.), using an oven temperature program of 250–300°C at 10°C/min.

RESULTS

An initial solid phase extraction of the nonsaponifiable lipids from sera produced a significant clean-up of the lipid fraction, which we found superior to that of the glass bead column used by Javitt *et al.*[4]. With the HPLC steps, better resolution was obtained by using the Resolve reversed phase column followed by the normal phase column, rather than the reverse sequence. Three types of radial compression ODS columns offered by Waters Associates were tested. The μ-Bondapak column gave an inadequate resolution from other contaminants, and with the Novapak column, we had difficulty obtaining consistent results. A typical chromatogram of the final quantitative HPLC step is shown in Fig. 1. for a sample of normal

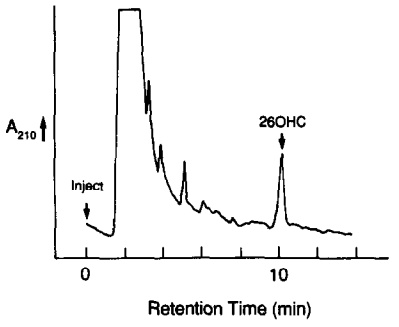

Fig. 1. HPLC profile of the final analytical step in the quantitation of 26OHC in human serum. The extract from 0.5 ml of normal serum was injected on an Altex 5-μ silica column with a mobile phase of 2.5% isopropanol in hexane and a flow rate of 1.8 ml/min. AUFS was 0.01.

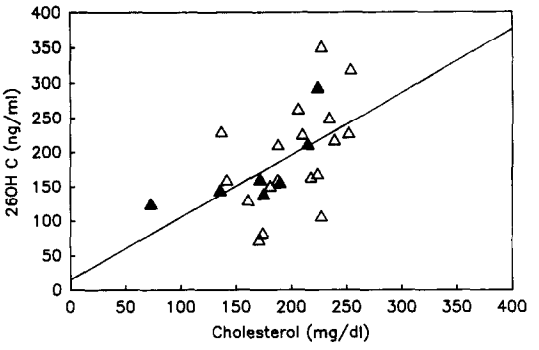

Fig. 2. Correlation of serum 26OHC concentration with the serum cholesterol concentration. The line of best fit was drawn by a computerised linear regression analysis: △ = patients and ▲ = controls.

human serum. The 26OHC peak is well resolved from other peaks. The identity and purity of the peak in three different samples was confirmed by electron impact mass spectrometry and a comparison of the spectra with authentic 26OHC. A molecular ion was detected at m/z 402 and major fragments at m/z 384 and 369. The absence of any molecular ions greater than m/z 402 and the similarity of the fragmentation patterns of the standard and the samples confirmed the purity of the peak. The reproducibility of the method was confirmed by repetitive analysis of a pooled blood bank serum sample which, on analysis, yielded 156 ± 14 (SD) ng/ml ($n = 9$) with a coefficient of variation of 8.9%. The recovery of added radioisotope was 59.2 ± 4.6%. An analysis of sera from seven normal subjects yielded a mean of 176 ± 59 ng/ml with a range of 125–294 ng/ml. This is consistent with the range of 92–286 ng/ml ($n = 8$) detected by Javitt *et al.*[4], but higher than the 30–129 ng/ml ($n = 40$) reported by Koopman *et al.*[5]. The assumption is made that the added [^{3}H]26OHC behaves similarly to endogenous 26OHC and the 26OHC produced from the saponification of 26OHC esters.

An analysis of the total 26OHC content of sera collected from patients with angiographically-confirmed atherosclerosis at the time of coronary bypass surgery revealed that levels in 16 of 18 patients were within the range of normal values (patient range 73–263 ng/ml). Two patients had elevated levels of 319 and 351 ng/ml. Values of all patient sera and control sera were compared when possible with serum cholesterol concentrations measured by an automated chemical analyser on fasted samples obtained within 1 month of the day of surgery. A positive correlation for the relationship between serum 26OHC content and serum cholesterol content was detected (Fig. 2). The correlation coefficient (r) was 0.54 and was significant at $P < 0.01$ for pooled patient and normal values, but was not significant ($P = 0.06$) for each group analysed separately.

DISCUSSION

Recent methods for the identification and quantitation of 26OHC in serum have utilised gas chromatography coupled with mass detection [4], a combination of gas chromatography and HPLC [5], or HPLC followed by CI mass spectrometry [18]. This report describes using HPLC procedures in conjunction with an initial solid phase extraction for the quantitation of 26OHC in serum. That this method is also applicable to the analysis of 26OHC in other tissues is illustrated by our synthesis of [^{3}H]26OHC using rat liver mitochondria as a source of hydroxylation enzymes, because 26OHC was isolated to purity. For complete purification from this source, however, an extra HPLC step was required. This extra step was not unexpected, given the involvement of the liver in both sterol and bile acid synthesis and its resulting high sterol and oxysterol content.

Evidence for a role of oxysterols in atherogenesis has been derived from several sources. (1) In dietary experiments with rabbits, oxysterol contaminants in the USP-grade cholesterol normally used in dietary studies caused atherosclerosis, whereas purified cholesterol had no effect [9]. Contradictory evidence was obtained by Higley *et al.*[12] using a different source of oxysterols with different compositions. A further argument against dietary-derived oxysterols being a significant factor in the development of atherosclerosis is that several studies show no autoxidation products of cholesterol [4, 5] or very low levels of those products [18] in normal human sera. Furthermore, we failed to detect 25-hydroxycholesterol, an autoxidation product of cholesterol, in a range of cholesterol-containing foods, suggesting it is not widely encountered in the food chain [19]. (2) Cultured aortic smooth muscle cells show detrimental changes following incubation with oxysterols in the culture media [20]. However, the concentrations required for a toxic response are high, generally > 1 μg/ml. Cellular metabolism is modified at lower concentrations, affecting HMG-CoA reductase activity at concentrations as low as 20 ng/ml [7].

Oxysterols also modify permeability in artificial membranes [21] and in cultured cells [22]. Recent studies in our laboratory showed that a short incubation of platelets with 250 ng 26OHC/ml increased the cytoplasmic Ca^{2+} concentration, but lower concentrations of 26OHC had no effect (Kou I.-L. and Holmes R. P., unpublished results). (3) Significant amounts of oxysterols have been detected in human atheromata [13, 14, 18]. The principal sterol is 26OHC in diesterified form (225 μg/g aortic tissue); 25-hydroxycholesterol is present in lower concentrations [18].

Comparisons of the measurements of cholesterol and 26OHC in serum indicate that there is a significant correlation between the two sterols. This association warrants further testing in a population with a much wider range in serum cholesterol concentrations. Such an association could be expected, in that hepatic cholesterol increases with increasing serum cholesterol [23] and thus more cholesterol would be available for 26-hydroxylation. The accelerated influx of lipoproteins into the arterial wall that occurs in atherosclerosis may produce the observed accumulation of 26OHC in atheroma [13, 14, 18], because 26OHC, like cholesterol, is predominantly carried by lipoproteins [4]. Whether this accumulation of 26OHC influences the development of atherosclerosis must still be ascertained.

Whether 26OHC has a normal physiological role is unknown, but a role in the regulation of cholesterol synthesis has been proposed [24]. The method that we have developed for the analysis of 26OHC should facilitate research directed toward increasing our understanding of the physiological role of 26OHC, determining factors that influence circulating 26OHC concentration, and investigating the relationship between serum 26OHC and cholesterol.

Acknowledgements—This study was supported in part by the National Dairy Council and by the National Livestock and Meat Board. We would like to acknowledge the capable technical assistance of the late Randy Moore and to thank Dr I.-Ling Kou for her advice concerning the development of the method. We are indebted to Michael Samuel for the GC–MS analyses.

REFERENCES

1. Pedersen J. I., Björkhem I. and Gustafsson J.: 26-Hydroxylation of C_{27}-steroids by soluble liver mitochondrial cytochrome P-450. *J. Biol. Chem.* **254** (1979) 6464–6469.
2. Andersson S., Davis D. L., Dahlbäck H., Jörnvall H. and Russell D. W.: Cloning, structure and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. *J. Biol. Chem.* **264** (1989) 8222–8229.
3. Gustafsson J.: On the heterogeneity of the mitochondrial C_{27}-steroid 26-hydroxylase system. *J. Lipid Res.* **17** (1976) 366–372.
4. Javitt N. B., Kok E., Burstein S., Cohen B. and Kutscher J.: 26-Hydroxycholesterol. Identification and quantitation in human serum. *J. Biol. Chem.* **256** (1981) 12644–12646.
5. Koopman B. J., Van Der Molen J. C., Wolthers B. G. and Vanderpas J. B.: Determination of some hydroxycholesterols in human serum samples. *J. Chromat.* **416** (1987) 1–13.
6. Kandutsch A. A., Chen, H. W. and Heiniger H. J.: Biological activity of some oxygenated sterols. *Science* **201** (1978) 498–501.
7. Taylor F. R., Saucier S. E., Shown E. P., Parish E. J. and Kandutsch A. A.: Correlation between oxysterol binding to a cytosolic binding protein and potency in the repression of hydroxymethylglutaryl coenzyme A reductase. *J. Biol. Chem.* **259** (1984) 12382–12387.
8. Krieger M., Goldstein J. L. and Brown M. S.: Receptor-mediated uptake of low density lipoprotein reconstituted with 25-hydroxycholesterol oleate suppresses 3-hydroxy-3-methylglutaryl-coenzyme A reductase and inhibits growth of human fibroblasts. *Proc. Natn. Acad. Sci. U.S.A.* **75** (1978) 5052–5056.
9. Imai H., Werthessen N. T., Taylor C. B. and Lee K. T.: Angiotoxicity and arteriosclerosis due to contaminants of USP-grade cholesterol. *Arch. Path. Lab. Med.* **100** (1976) 565–572.
10. Imai H., Werthessen N. T., Subramanyam V., LeQuesne P. W., Soloway A. H. and Kanisawa M.: Angiotoxicity of oxygenated sterols and possible precursors. *Science* **207** (1980) 651–653.
11. Peng S.-K., Taylor C. B., Mosbach E. H., Huang W. Y., Hill J. and Mikkelson B.: Distribution of 25-hydroxycholesterol in plasma lipoproteins and its role in atherogenesis. A study in squirrel monkeys. *Atherosclerosis* **41** (1982) 395–402.
12. Higley N. A., Beery J. T., Taylor S. L., Porter J. W., Dziuba J. A. and Lalich J. J.: Comparative atherogenic effects of cholesterol and cholesterol oxides. *Atherosclerosis* **62** (1986) 91–104.
13. van Lier J. E. and Smith L. L.: Sterol metabolism. I. 26-hydroxycholesterol in the human aorta. *Biochemistry* **6** (1967) 3269–3278.
14. Gilbert J. D., Brooks C. J. W. and Harland W. A.: Lipids of human atheroma. The isolation of diesters of cholest-5-ene-3β,26-diol from extracts of advanced atherosclerotic lesions of human aorta. *Biochim. Biophys. Acta* **270** (1972) 149–155.
15. Scheer I., Thompson M. J. and Mosettig E.: 5-Cholestene-3β,26-diol. *J. Am. Chem. Soc.* **78** (1956) 4733–4736.
16. Wachtel N., Emerman S. and Javitt N. B.: Metabolism of cholest-5-ene-3β,26-diol in the rat and hamster. *J. Biol. Chem.* **243** (1968) 5207–5212.
17. Saarem K., Bergseth S., Oftebro H. and Pedersen J. I.: Subcellular localization of vitamin-D_3 25-hydroxylase in human liver. *J. Biol. Chem.* **259** (1984) 10936–10940.
18. Smith L. L., Teng J. I., Lin Y. Y., Seitz P. K. and McGehee M. F.: Sterol metabolism—XLVII. Oxidized cholesterol esters in human tissues. *J. Steroid Biochem.* **14** (1981) 889–900.
19. Kou I.-L. and Holmes R. P. The analysis of 25-hydroxycholesterol in plasma and cholesterol-containing foods by high performance liquid chromatography. *J. Chromat.* **330** (1985) 339–346.
20. Peng S.-K., Tham P., Taylor C. B. and Mikkelson B.: Cytotoxicity of oxidation derivatives of cholesterol on cultured aortic smooth muscle cells and their effect on cholesterol biosynthesis. *Am. J. Clin. Nutr.* **32** (1979) 1033–1042.
21. Holmes R. P. and Yoss N. L: 25-hydroxysterols increase the permeability of liposomes to Ca^{2+} and other cations. *Biochim. Biophys. Acta* **770** (1984) 15–21.

22. Holmes R. P. and Kummerow F. A.: Membrane perturbations in atherosclerosis. In *Membrane Fluidity in Biology. Vol. 3—Disease Processes* (Edited by R. C. Aloia and J. M. Boggs). Academic Press, Orlando, Fla. (1985) pp. 281–305.
23. Quintão E. C. R., Brumer S. and Stechhahn K.: Tissue storage and control of cholesterol metabolism in man on high cholesterol diets. *Atherosclerosis* **26** (1977) 297–310.
24. Esterman A. L., Baum H., Javitt N. B. and Darlington G. J.: 26-hydroxycholesterol: regulation of hydroxymethylglutaryl-CoA reductase activity in Chinese hamster ovary cell culture. *J. Lipid Res.* **24** (1983) 1304–1309.