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THE ANALYSIS OF 25-HYDROXYCHOLESTEROL IN PLASMA AND CHOLESTEROL-CONTAINING FOODS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method for the analysis of 25-hydroxycholesterol by high-performance liquid chromatography (HPLC) in a wide range of materials is described. Lipid extracts were initially purified on octadecyl silicic acid cartridges and by reversed-phase HPLC before quantitation by HPLC on a silicic acid column. The reproducibility of the method was confirmed by the analysis of plasma from rats fed 25-hydroxycholesterol. 25-Hydroxycholesterol was not detected in lard, cream, fresh egg yolk or spray-dried egg yolk powder. It was detected in egg yolk powder after heating at 110°C for 4 days and its authenticity was confirmed by mass spectrometry.

INTRODUCTION

25-Hydroxycholesterol is the major auto-oxidation product of crystalline cholesterol[1,2]. Small amounts are toxic to cultured cells and experimental animals[3–5]. In rabbits, when 5 mg 25-hydroxycholesterol per kg body weight were injected intravenously on each of 3 days, severe atherosclerosis was detected in pulmonary arteries 24 h after the final injection[5]. Necrosis of smooth muscle cells occurs within 24 h when they are cultured in medium containing 10 μg 25-hydroxycholesterol per ml[4]. Lower concentrations inhibit HMG-CoA reductase activity in cultured cells thereby suppressing cholesterol biosynthesis[3]. This inhibitory action has been used as a tool to study the regulation of the enzyme and to determine the functional significance of fluctuations in the cellular levels of cholesterol and other lipids that result from the activity of the enzyme[6,7].

Because of these properties of 25-hydroxycholesterol, concern has been expressed that substantial amounts may form in cholesterol-containing foods during storage or processing. It has been reported, for instance, that 25-hydroxycholesterol is produced in butter when it is moderately heated for 5 min[8]. The ingestion and absorption of 25-hydroxycholesterol and other oxygenated sterols resulting from the oxidation of cholesterol could perturb the metabolic functioning of many different types of cells. A reproducible, sensitive method for the identification and quantitation

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of 25-hydroxycholesterol is required to determine its content in a variety of cholesterol-containing foods and to study its metabolism and deposition in tissue cells.

The principal method used to date to measure hydroxysterols in biological materials is gas–liquid chromatography–mass spectrometry[9,10]. Mass detection, whilst being the best detection method available, requires expensive instrumentation and is not routinely available to most investigators. High-performance liquid chromatography (HPLC) is a more accessible, less expensive technique and is the approach we adopted. Tsai and Hudson[11] demonstrated that HPLC could resolve a variety of oxygenated derivatives of cholesterol with polar groups on various carbon atoms of the isoprenoid side-chain. The method proposed by Csiky[8] did not give a good baseline resolution of 25-hydroxycholesterol as it appeared as a shoulder peak on a large UV absorbing peak. For the materials we wished to examine, this procedure did not give sufficient resolution from contaminants. We describe here a method which we have found to be sensitive, reproducible and applicable to a wide range of materials.

EXPERIMENTAL

Materials

Cholest-5-ene-3β,25-diol (25-hydroxycholesterol) was obtained from Steraloids (Wilton, NH, U.S.A.) and was found to be >99% pure using thin-layer chromatography and both normal and reversed-phase HPLC. [^{3}H]25-Hydroxycholesterol with a specific activity of 87 Ci/mmol was purchased from New England Nuclear (Boston, MA, U.S.A.). Sephadex LH-20 was obtained from Pharmacia (Piscataway, NJ, U.S.A.), Lipidex-5000 from Packard (Downer's Grove, IL, U.S.A.), and silicic acid Sep-Pak and C_{18}-bonded silicic acid cartridges from Waters Assoc. (Milford, MA, U.S.A.). Eggs and cream were purchased as fresh items from a local supermarket. Lard was a gift of Best Foods/CPC International (Union, NJ, U.S.A.) and the egg yolk powder from Henningsen Food Inc. (Omaha, NE, U.S.A.).

Dietary experiments

Male, albino Sprague Dawley rats (200–400 g) from Holtzman Co. (Madison, WI, U.S.A.) and raised on chow diets were used in experiments. 25-Hydroxycholesterol was dissolved in 1 ml corn oil and fed to rats by gavage. Blood was drawn into a heparinized tube after 48 h by heart puncture. Plasma was collected by centrifugation at 1000 *g* for 10 min at 4°C.

Column chromatography

For Sephadex LH-20 chromatography, extracts were loaded on a 30 × 1 cm glass column and eluted with a mobile phase of hexane–chloroform–methanol (9:1;1, v/v/v) and a flow-rate of approximately 1 ml/min. 25-Hydroxycholesterol was obtained in the fractions collected between 23 and 32 ml under these conditions. Using a 30 × 1 cm Lipidex-5000 column and a mobile phase of hexane–chloroform (9:1, v/v), 25-hydroxycholesterol was obtained in the fractions collected between 72 and 123 ml.

High-performance liquid chromatography

The equipment used included a Rheodyne 7125 injector, a Waters M-45 solvent delivery system, a Waters 480 variable-wavelength detector and a Hewlett-Packard 3390A integrator. A Waters Radial Compression RCM 100 module containing an 8-cm, 5-μm C_{18} column was used for reversed-phase chromatography. For normal phase, a 250 × 4.6 mm, 5-μm silicic acid column (Altex) was used.

Mass spectrometry

Samples were introduced into a Hewlett-Packard 5985 mass spectrometer using a direct probe and the molecular ion and fragments analyzed following electron impact.

RESULTS

Development of the method

(a) *Saponification.* The saponification of triglyceride-rich material required a minimum 100 ml of alcoholic sodium hydroxide per gram to obtain >90% recovery of tracer [^{3}H]25-hydroxycholesterol added to the material. With serum and non-fatty tissues 30 ml of alcoholic sodium hydroxide per gram gave a good recovery of the added radioactive tracer. The use of HPLC-grade hexane for the extraction of non-saponifiable material prevented complications arising from UV-absorbing contaminants present in solvents of a lower quality, such as occurs with the use of light petroleum. Saponification overnight at 37°C avoided the occurrence of contaminants that appeared when fluxing for 1 h was used.

(b) *Lipid extraction.* In the examination of biological tissues, knowledge of the free and esterified 25-hydroxycholesterol content is often required. This can be obtained from a total lipid extract where esterified sterols will chromatograph with neutral sterols and the free 25-hydroxycholesterol can be estimated. An acetonitrile

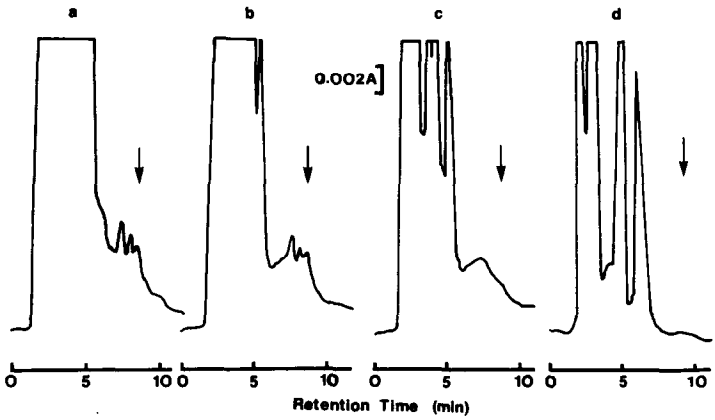

Fig. 1. A comparison of procedures for purifying an acetonitrile extract of rat plasma. An acetonitrile extract from 1 ml of plasma is shown in (a) when injected directly on a silicic acid HPLC column. In (b) the extract was first purified by Sephadex LH-20 chromatography before injection. In (c) silicic acid cartridge purification was used and in (d) C_{18} cartridge purification. Extracts were injected on a silica column with a mobile phase of 2.5% 2-propanol in hexane and a flow-rate of 1.7 ml/min. Arrows indicate where a 25-hydroxycholesterol standard elutes.

extraction was compared with a chloroform–methanol extraction. The former gave a slightly better recovery from plasma (100%) than the chloroform–methanol extraction (97%), used less solvent and was less tedious.

(c) *Sep-Pak fractionation.* The use of cartridges of silicic acid was time-saving and used less solvents than column chromatography for removal of neutral lipids, including cholesterol, from the extract. By examining the UV-absorbing profiles of fractions collected, Sep-Pak cartridges were also found to remove more contaminating material than chromatography on either Sephadex LH-20 (Fig. 1) or Lipidex-5000. C_{18} Sep-Pak cartridges were superior to silica Sep-Pak cartridges in purifying the extract (Fig. 1).

(d) *HPLC.* The use of two columns was found to be necessary to obtain consistent baseline resolution of 25-hydroxycholesterol from other contaminating peaks. Initial purification was achieved using a C_{18} reversed-phase column, followed by quantitation on a silicic acid normal phase column, using detection at 205 nm. Using the columns in the reverse way was complicated by the appearance of a contaminating peak which appeared in the fraction collected with 25-hydroxycholesterol on the final reversed-phase step. This material was apparently a trace contaminant in the solvents used.

Method adopted

For estimation of total 25-hydroxycholesterol 0.1–5 g of material were saponified overnight at 37°C in a solution containing 10 000 dpm of [^{3}H]25-hydroxycholesterol, 3 ml of 0.3 *M* sodium hydroxide and 27 ml of methanol. The non-saponifiable material was extracted by the addition of one volume of water and two volumes of hexane. After emulsification, phases were separated in a separatory funnel. The methanolic aqueous phase was re-extracted twice more with the same volume of hexane. The combined hexane extracts were taken to dryness by rotary evaporation. The lipid was redissolved in 1 ml of acetonitrile.

For estimation of free 25-hydroxycholesterol, radioactive tracer was added to the sample and it was extracted with a minimum of four volumes of acetonitrile. After vortexing for 1 min, samples were maintained in an ice-bath for 1 h for complete extraction. The acetonitrile extract was obtained by centrifugation at 1000 *g* for 10 min at 4°C. The extract was evaporated to a 1-ml volume under a stream of oxygen-free nitrogen.

The acetonitrile extracts were loaded on C_{18}-silicic acid Sep-Pak cartridges prewashed with acetonitrile and water. The cartridge was washed with 10 ml of 75% methanol in water and the 25-hydroxycholesterol fraction was eluted with 4 ml of acetonitrile.

After taking the fraction to dryness and redissolving in methanol, it was injected on a 5-μm C_{18} radial compression column and eluted at a flow-rate of 1 ml/min with a mobile phase of 5% water in methanol. The fraction corresponding to 25-hydroxycholesterol was collected, taken to dryness, redissolved in 2.5% 2-propanol in hexane and injected on a 5-μm silicic acid column with a flow-rate of 1.7 ml/min and a mobile phase of 2.5% 2-propanol in hexane. The 25-hydroxycholesterol peak was collected, taken to dryness and the radioactivity estimated to determine the recovery of added [^{3}H]25-hydroxycholesterol. Areas of peaks were measured by electronic integration and the amount of 25-hydroxycholesterol determined by compar-

ison to a standard curve. The amount of 25-hydroxycholesterol in the sample was calculated by accounting for the loss in recovery of radioactivity.

Recovery of added tracer

The recovery of [^{3}H]25-hydroxycholesterol added to samples was monitored after each chromatographic or extraction step. The stepwise and cumulative recoveries associated with the method described above for the estimation of free 25-hydroxycholesterol in plasma are shown in Table I. With a saponification of plasma, 97.9 ± 5.8% of the added tracer was recovered in the non-saponifiable fraction. Recoveries for all steps were similar to plasma when muscle and liver tissue and egg yolk powder were analyzed. For triglyceride rich samples such as adipose tissue and vegetable oil, recoveries after saponification and three extractions with hexane were 80–85% when using 1 g of sample and 100 ml of methanolic sodium hydroxide. The recovery was improved using greater volumes of the saponifying solvent but required larger and unwieldy volumes of extraction solvent.

TABLE I

THE RECOVERY OF 25-HYDROXYCHOLESTEROL FROM RAT PLASMA FOLLOWING EXTRACTION AND CHROMATOGRAPHY

Step	*Stepwise recovery (mean % ± S.D.)*	*Cumulative recovery (mean %)*
Acetonitrile extraction	100.8 ± 9.0	100.8
C_{18} Sep-Pak	93.1 ± 6.5	93.8
C_{18} HPLC	80.2 ± 5.2	75.3
Silica HPLC	77.6 ± 2.8	58.4

Reproducibility of the method

Reproducibility of the method was tested by repetitive analysis of plasma from a rat fed 25-hydroxycholesterol in corn oil. The mean of five measurements for total 25-hydroxycholesterol concentration was 306.7 ng/ml with a standard deviation of 19.5 ng/ml. The coefficient of variation was thus 6.4%. The mean recovery of added [^{3}H]25-hydroxycholesterol was 51.9 ± 4.8% (S.D.).

Plasma analysis

An analysis of normal rat plasma (5 ml) indicated that it did not contain any detectable 25-hydroxycholesterol (< 10 ng/ml) and that no other contaminating lipids co-eluted with 25-hydroxycholesterol on the analytical column (Fig. 2). 25-Hydroxycholesterol was detected, however, in the plasma of rats 48 h after feeding 10 mg of 25-hydroxycholesterol dissolved in corn oil (Fig. 2). In plasma from one rat, the total level was 630 ng/ml and the free level, 270 ng/ml. In a second rat, the total level was 1180 ng/ml and the free level, 470 ng/ml. These results indicate that approximately 60% of the circulating 25-hydroxycholesterol in rats is esterified 48 h after ingestion.

Food analysis

We tested several cholesterol-containing foods for their content of 25-hydroxycholesterol. They were lard, which contains 2 mg cholesterol per g; fresh egg yolk,

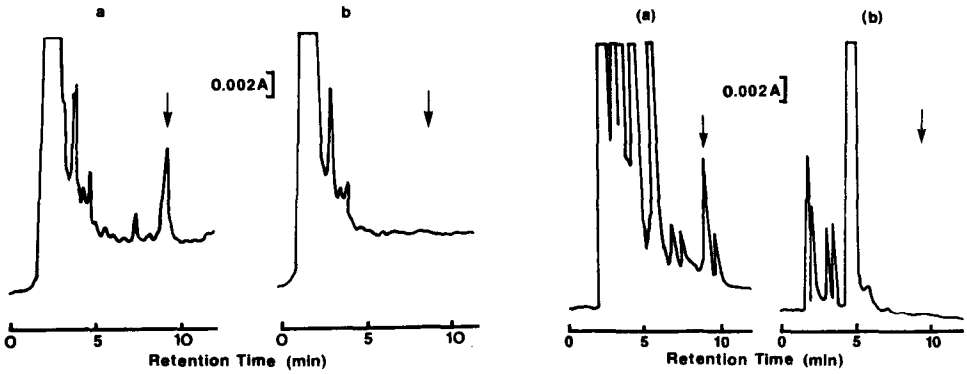

Fig. 2. Chromatograms of plasma from rats fed 25-hydroxycholesterol (a) and normal plasma (b). The method described in the text was used, and the profile obtained on the final silica HPLC column is shown. Arrows indicate where a 25-hydroxycholesterol standard elutes.

Fig. 3. Chromatograms of heated (a) and unheated (b) egg yolk powder. The powder in (a) was spread as a thin layer and heated at 110°C for 4 days. The method described in the text was used, and the profile obtained on the final silica HPLC column is shown. Arrows indicate where a 25-hydroxycholesterol standard elutes.

which contains 13 mg/g; egg yolk powder, which contains 29 mg/g; and cream, which contains 1.1 mg/g. 25-Hydroxycholesterol was not detected in 1 g of lard, fresh egg yolk or egg yolk powder, and not in 2 g of fresh cream. This indicated that the extent of oxidation of the cholesterol in these products to 25-hydroxycholesterol was $<0.005\%$.

25-Hydroxycholesterol was detected in egg yolk powder when it was spread as a thin layer and heated in an oven at 110°C for 4 days but not when heated for only 1 day. Profiles before and after incubating 0.1 g of powder are illustrated in Fig. 3. Confirmation that the peak co-eluting with a 25-hydroxycholesterol standard was 25-hydroxycholesterol was obtained by mass spectrometry. Direct probe analysis revealed a molecular ion species, m/z 402 and major fragments at m/z 384 and 369. The spectrum was identical to that of a 25-hydroxycholesterol standard. The concentration of 25-hydroxycholesterol was 26.4 μg per g egg yolk powder. This represented the oxidation of 0.09% of its cholesterol content to 25-hydroxycholesterol.

DISCUSSION

We have described a method for the analysis of 25-hydroxycholesterol which should be applicable to a wide range of biological materials. The method works equally well irrespective of whether samples are saponified or not, enabling the estimation of both free and esterified forms of the sterol. It is rapid, as after an overnight saponification at 37°C, the estimated analysis time for one sample is 5–6 h with access to two HPLC systems, and 2–3 h longer if only one system is available. The use of C_{18}-bonded silicic acid-packed cartridges as the initial purification step aided in significantly reducing the analysis time. Whereas the recovery of added [^{3}H]25-hydroxycholesterol was quantitative with either solvent extraction or saponification of samples, significant losses were apparent during chromatographic steps.

In the method described by Javitt *et al.*[9] using mass detection, complete recoveries were not reported as the ratio of 25-hydroxycholesterol or 26-hydroxycholesterol detected to the amount of deuterated 26-hydroxycholesterol added was used to compensate for losses during analysis. They did report a recovery of 68–85% of added [^{3}H]26-hydroxycholesterol after extraction of serum and chromatography on glass beads, which is consistent with the recoveries reported in this study. A loss of sterol during chromatography has also been reported with the analysis of the seco-sterols, cholecalciferol (vitamin D_3) and calcifediol (25-hydroxyvitamin D_3)[12–14]. Cumulative recoveries ranged from 45–55%. We have observed that losses are greater using silicic acid or bonded silicic acid as the chromatographic support rather than with the Sephadex-based supports, Sephadex LH-20 and Lipidex-5000. This loss on silicic acid chromatography suggests that a small portion of the sterol molecules binds tightly to the chromatographic support and does not elute with the bulk of the sterol. This may be related to the pronounced peak tailing of the dihydroxy derivatives of vitamin D_3 studied by Jones[15]. The recoveries of 25-hydroxycholesterol we observed during chromatography indicate the importance of using a radioactive tracer or an internal standard to assess losses during analysis of this and related sterols.

The method was capable of detecting 50 ng of 25-hydroxycholesterol. The sensitivity could probably be increased by derivatization but this would not appear to be necessary given the range of concentrations at which it most probably exerts a biological effect. The inhibition of HMG-CoA reductase activity in cultured cells occurs at concentrations of 25-hydroxycholesterol in the medium as low as 20 ng/ml[6]. We did not detect significant interference with contaminating lipids when 5 ml of serum were analyzed, indicating that for serum our detection limit was 10 ng/ml. With cholesterol-containing foods, this method permits the detection in 1-g samples of 0.005% oxidation of the cholesterol to 25-hydroxycholesterol in lard, 0.001% in fresh egg yolk, 0.0005% in egg yolk powder and 0.01% in whipping cream. The method has advantages over those utilizing mass detection in that the instrumentation is less expensive and routinely available in most laboratories. [^{3}H]25-Hydroxycholesterol is commercially available to determine HPLC losses during HPLC analyses whereas deuterated 25-hydroxycholesterol, the best internal standard for mass detection, is not.

Our results indicate that 25-hydroxycholesterol does not occur in significant amounts in cholesterol-containing foods. This includes egg yolk powder which was exposed to pro-oxidant conditions during preparation by spray-drying and had been stored in our cold room for 2 years. It would seem that either the antioxidants present in the foods, butylated hydroxytoluene in the lard and tocopherols in the other foods, limited oxidation or the conditions the foods were exposed to did not promote cholesterol oxidation. It is possible that heating of foods under certain conditions could produce oxidation as claimed by Csiky[8] in the heating of butter. However, we found that extensive heating, 110°C for 4 days, was required to induce significant oxidation of egg yolk powder. The method we have developed should enable a more rigorous determination of whether cholesterol oxidation to 25-hydroxycholesterol occurs in significant amounts in heated fats or in the processing of cholesterol-containing foods.

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