Determination of 6-Ketocholestanol in Unirradiated and Irradiated Chicken Meats

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A method to detect 6-ketocholestanol in unirradiated and irradiated chicken meats was developed by means of chloroform-methanol-water extraction, adsorption chromatographic column separation and gas chromatography. This method is able to measure cholesterol oxidation products at levels that are much lower than those of previous methods. The new procedure was used to detect 6-ketocholestanol in fresh, unirradiated chicken and measured more than 97% of the test compound added to chicken below the ppm level. Irradiation of the chicken meats to a dose of 10 kGy increased the concentration of this compound to about four times the level of unirradiated meats.

KEY WORDS: Chicken, cholesterol, irradiation, 6-ketocholestanol, meat.

As part of a search for a diagnostic test capable of detecting whether meats have been previously irradiated, cholesterol oxidation products have been investigated in our laboratory. In model systems, the production of the most common cholesterol autoxidation products, such as 7-ketocholesterol (3β-hydroxycholest-5-en-7-one), cholesterol-5β,6β-epoxide (5,6β-epoxy-5α-cholestan-3β-ol; β-epoxide) and cholesterol-5α,6α-epoxide (5,6α-epoxy-5α-cholestan-3β-ol; α-epoxide), has shown a different pattern than that produced by γ-radiation (1,2). A-ring oxidation products (4-cholesten-3-one, 4,6-cholestadien-3-one and 4-cholestene-3,6-dione), generated in trace amounts by autoxidation, were found to be produced in much larger amounts from cholesterol in liposomes exposed to γ-radiation (3). The A-ring oxidation products were also found in irradiated meats in higher concentrations than in control samples. However, neither the prominent autoxidation products nor the A-ring oxides were entirely satisfactory for the detection of irradiated meat and poultry (4), because the distinction between irradiated and unirradiated samples was not always clear.

Methods to detect cholesterol derivatives in meat systems have been reported (4–13). Some of these methods have limited sensitivity and are not adequate for the measurement of even the most prominent cholesterol oxidation products in fresh meats. While the concentrations of the common cholesterol oxidation products in meats are 0.1–1 ppm, it will be shown in this paper that 6-ketocholestanol (3β-hydroxy-5α-cholestan-6-one) occurs in concentrations of 1–10 ppb. Its lower background level has the potential of providing greater sensitivity in the detection of its formation by irradiation. Therefore, there was a need to develop a methodology consistently capable of measuring this compound in the complex systems of meat tissues and of detecting the changes of the concentration of the compound as a result of γ-radiation.

The objective of the present study was to develop a method for detecting 6-ketocholestanol in chicken meats. The goal was an uncomplicated procedure that can produce consistent results and that could perhaps be used to discriminate irradiated chicken from unirradiated.

EXPERIMENTAL METHODS

Materials and reagents. Cholesterol (5-cholesten-3β-ol), α-epoxide (5,6α-epoxy-5α-cholestan-3β-ol), 6-ketocholestanol (3β-hydroxy-5α-cholestan-6-one) and 5α-cholestane were purchased from Sigma Chemical Co. (St. Louis, MO); β-epoxide (5,6β-epoxy-5α-cholestan-3β-ol) and 7α-hydroxycholesterol (5-cholestene-3β,7α-diol) from Research Plus, Inc. (Bayonne, NJ); and 7-ketocholesterol (3β-hydroxycholest-5-en-7-one), 7β-hydroxycholesterol (5-cholestene-3β,7β-diol) and desmosterol (5,24-cholestadien-3β-ol) from Steraloids Inc. (Wilton, NH). Solid-phase extraction (SPE) columns (B & J SPE Inert Silica 500 mg) and bulk SPE inert silica packing material were purchased from Baxter Diagnostic Inc. (McGaw Park, IL). Thin-layer chromatography (TLC) plates (scored silica gel GHL 250 μm and silica gel G 500 μm) were purchased from Analtech, Inc. (Newark, DE). Meats, including deboned, skinless chicken breast, were obtained from a local retail store. Water used was double-deionized and glass-distilled. All solvents used were "distilled in glass" grade, and chemicals were reagent-grade quality.

Method I. The content of 6-ketocholestanol in various meats was measured in 10-g samples by the method used for epoxides and 7-ketocholesterol in the previous report (4). In this procedure, separation by TLC is an important step.

Method II. This method was developed to measure 6-ketocholestanol in 50-g samples of chicken meat without the use of TLC. The method follows:

Sample preparation and irradiation. Visible fat was removed from the chicken breast. The meat (about 150 g) was put into an oxygen-permeable (2500 mL/100 in^2/24 h) meat and poultry bag (Mobile Chemical Co., Macedon, NY), which was then fastened. The bag with the meat was irradiated in a Cs-137 γ-radiation source at 0–4°C to a dose of 10 kGy (0.114 kGy/min). Subsequently, the irradiated or unirradiated (control) samples were cut into smaller pieces. The dose rate was established with National Physical Laboratory (Middlesex, United Kingdom) dosimeters. Samples were maintained at the desired temperature during irradiation by the injection of liquid nitrogen into the irradiation chamber.

Fat extraction. A modification of the Bligh and Dyer method (14) was used for fat extraction. Chloroform/methanol (50 mL, 1:1, vol/vol) was added to 50 g of meat sample in a 250-mL Erlenmeyer flask. The content was homogenized with a Tekmar SDT-1810 motor and an SDT-182EN shaft (Tekmar Co., Cincinnati, OH) for 2 min. The homogenate was filtered through Whatman No. 2 filter paper (7 cm) (Whatman International Ltd., Maidstone, England) on a Buchner funnel and a vacuum flask connected to mild vacuum. The sample container was rinsed with 20 mL of chloroform/methanol (1:1), and the rinse

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was passed through the filtering system. The filtrate was transferred to a 500-mL separatory funnel. The sample residue and filter paper were transferred to a 250-mL Erlenmeyer flask, 100 mL of chloroform/methanol (1:1) was added and the suspension was homogenized for 1 min. The homogenate was filtered, and the filtrate was added to the separatory funnel. The homogenizing container and the homogenizer were washed with 30 mL of chloroform/methanol (1:1), and the wash was passed through the filter. The filtrate was added to the separatory funnel. Forty mL of 0.88% KCl solution were added to the solvent extract in the separatory funnel, and the content was shaken vigorously. The extract was allowed to settle for 1 h, and the lower layer was collected in a 500-mL round-bottomed flask. Chloroform (20 mL) was added to the upper layer in the separatory funnel, and the content was vigorously shaken and allowed to settle for 1 h. The lower layer was collected and added to the round-bottomed flask. The combined chloroform extracts were evaporated under vacuum at 35°C. When the residue became viscous, the vacuum was released, and the residue was dried under a nitrogen stream in a warm-water bath until it had solidified. The residue was dissolved with 30 mL of hexane/ethyl acetate (9:1) and dried over 20 g of Na_2SO_4 (anhyd.).

Silica column separation. Silica (10 g, B & J SPE Inert) was packed in a glass column [22 mm i.d. × 300 (or 500) mm h] by gently tamping the column on a rubber stopper 20 times, and 20 g of Na_2SO_4 (anhyd.) were placed on top of the silica bed. The column was prewashed consecutively with 100 mL hexane, 100 mL of hexane/ethyl acetate (4:6), 100 mL ethyl acetate and 100 mL hexane prior to sample application. The sample, dissolved in hexane/ethyl acetate (9:1), was decanted to the prewashed column, and sufficient nitrogen pressure was applied for the solvent to elute at a rate of about 2–3 drops per second. The sample container was rinsed twice with 30 mL of hexane/ethyl acetate (9:1) and once with 50 mL of the same solvent mixture, and each rinse was applied to the column. Additional hexane/ethyl acetate (9:1) (50 mL) was applied onto the column. The total eluate was discarded. Hexane/ethyl acetate (8:2) (100 mL) and hexane/ethyl acetate (4:6) (30 mL) were applied to the column, and the eluates were discarded. Additional hexane/ethyl acetate (4:6) (30 mL) was applied to the column, and the eluate was collected in a 100-mL round-bottomed flask. The collected eluate was freed of solvent in a vacuum evaporator at 35°C. The residue in the 100-mL flask was dissolved in 1 mL ethyl acetate and transferred to a 10-mL conical test tube. The round-bottomed flask was washed three times with 1 mL ethyl acetate each time, and the washes were added to the sample in the test tube. The sample was dried under a nitrogen stream and reconstituted with 200 μL ethyl acetate containing 10.6 μg of desmosterol as an internal standard for gas chromatography (GC) analysis.

GC analysis. Samples were analyzed on a Varian 3600 Gas Chromatograph (Varian Associates, Sugar Land, TX) equipped with a flame-ionization detector and an on-column capillary injector as described previously (4). A GC trace of a mixture of standards of cholesterol and its derivatives is shown in Figure 1.

GC-mass spectrometry (GC-MS). GC-MS work was carried out under the conditions reported earlier (15) by the Center for Advanced Food Technology, Cook College, Rutgers University (New Brunswick, NJ).

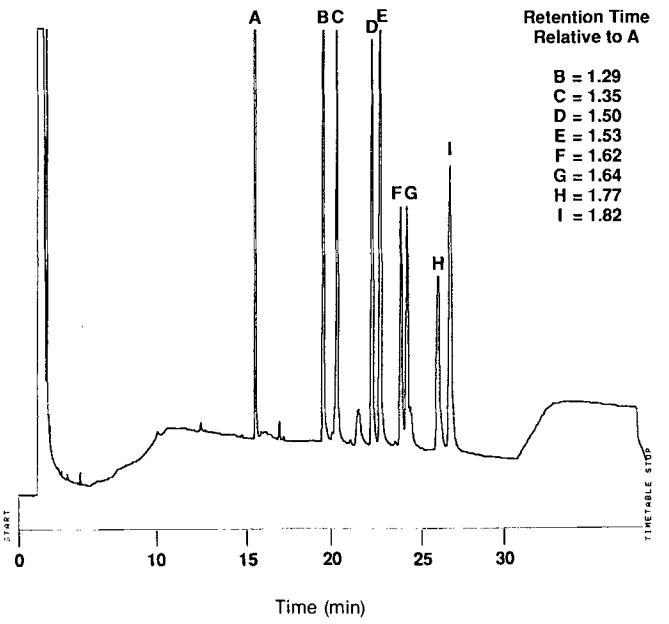

FIG. 1. Gas chromatography of cholesterol and its derivatives. A: 5α-cholestane; B: cholesterol; C: desmosterol; D: β-epoxide; E: α-epoxide; F: 7α-hydroxycholesterol; G: 7β-hydroxycholesterol; H: 6-ketocholestanol; I: 7-ketocholesterol.

Statistical analysis. The experiments were carried out in duplicate or triplicate as indicated in Tables 1–4. Data were analyzed by the *t*-test ($\alpha = 0.05$) or by Tukey's multiple range test, comparing the values by the use of SAS (16).

RESULTS AND DISCUSSION

6-Ketocholestanol in meats. 6-Ketocholestanol had been reported to be present in pig's spleen (17,18) and in some processed meats (6); however, these reports have not been confirmed. Because this compound is not formed in the autoxidation of cholesterol, it has been used as an internal standard for GC analysis of cholesterol autoxidation products (19–21). In our laboratory, it was discovered, however, that 6-ketocholestanol may be among the cholesterol oxidation products present in muscle tissue. Its origin in unirradiated, freshmeat is not known. The detected levels of 6-ketocholestanol in various meat samples are shown in Table 1. These data were obtained by use of the previously reported method (Method I as described above) (4), in which 6-ketocholestanol was isolated together with the major cholesterol oxidation products from 10-g samples of substrate. 6-Ketocholestanol was detected in most of the fresh red meats, and it increased as a result of irradiation (Table 1). Because in most cases the meats analyzed consisted of single samples, the data of Table 1 are only indicative and cannot be used to evaluate the possibility that irradiated meat can be distinguished from unirradiated. The levels of 6-ketocholestanol in fresh red meats were much lower than those of 7-ketocholesterol, reported in the previous study to be on the order of μg/10 g

TABLE 1

6-Ketocholestanol in Unirradiated and Irradiated Meats (ng/10 g)[a]

Type	Unirradiated	Irraddiated
Beef (bottom round)	210 ± 46[a]	313 ± 44[b]
Lamb (loin chop) I	117 ± 4[c]	116 ± 48[c]
Lamb (loin chop) II	128 ± 64[d]	321 ± 167[d]
Veal (loin chop)	58 ± 12[c]	301 ± 125[f]
Shrimp	182 ± 69[g]	515 ± 33[h]
Chicken breast I (tender loin)	ND	174 ± 49
Chicken breast II (thin slice)	ND	96 ± 6
Chicken drumsticks I	ND	270 ± 41
Chicken drumstick II	ND	152 ± 17
Chicken drumstick III		
0 d	ND	255 ± 65[i]
2 wk	—	269 ± 1[i]
3 wk	—	346 ± 5[i]

[a]Value ± SD; triplicate; determination by Method I; ND, not detected; 2 wk and 3 wk are stored at 0–4°C in contact with air; same superscript letters a–h in a row denote no significant difference by the *t*-test ($\alpha = 0.05$); i, no significant difference in the column by Tukey's multiple range test ($\alpha = 0.05$).

TABLE 2

Recovery of Major Cholesterol Oxidation Products and 6-Ketocholestanol (silica column step of Method II only)

	Added amount (μg)	Detected amount (μg)	Recovery (%)
β-Epoxide	3.12	2.90	93.0
α-Epoxide	4.24	3.87	91.2
7-Ketocholesterol	3.26	3.05	93.5
6-Ketocholestanol	2.17	2.09	96.6

of wet tissue (4). There was insufficient 6-ketocholestanol in fresh chicken for detection by Method I, but it clearly was present in irradiated chicken. When the irradiated chicken was stored from 2 or 3 wk, its levels of 6-ketocholestanol did not change significantly. Because the amounts of the 6-ketone present in unirradiated, fresh chicken were too low to be measured by Method I, there was a need to modify the method, so that larger chicken samples could be analyzed with greater sensitivity.

Isolation and measurement of 6-ketocholestanol from chicken meat. Measurement of cholesterol oxidation products present in muscle tissue requires that small amounts of the oxides be isolated from substantial amounts of neutral lipids, polar lipids and unchanged, endogenous cholesterol. In Method I, the isolation problem required that about 3 μg of each of the principal cholesterol oxides be separated from the usual amounts of lipids present in 10 g of meat: 0.5–1.0 g of neutral lipids, about 80 mg of polar lipids and about 7 mg of cholesterol (13).

To measure the much lower levels of 6-ketocholestanol in unirradiated chicken, it became necessary to increase the sample size, and hence the amounts of undesired lipids, by a factor of five (Method II). This forced abandonment of the TLC isolation step of Method I. Solvent usage for the fat-extraction step was reduced in the new procedure to about one-half the quantity that would have been used if Method I had been employed for a 50-g sample. In the present procedure, the sample extracts were allowed to settle for a sufficient time (about an hour) to reduce the size of the interphase. The fat extract was dried over Na_2SO_4 (anhyd.) and applied to the silica column. Because the prepacked SPE silica columns (B&J, 500 or 1000 mg) had insufficient capacity for the larger amount of lipid sample, a column (10 g) of this special silica packing material was prepared in the laboratory (see Experimental Methods). The column was not tightly packed and was kept wet with solvents during sample separation. With use of sufficient amounts of less polar solvent mixtures, most of the nonpolar lipids and cholesterol were removed, while cholesterol oxides and polar lipids and cholesterol were removed, while cholesterol oxides and polar lipids were retained on the column. Cholesterol oxides were then eluted in a minimum volume of solvent to reduce contamination with more polar lipids. Thus, the sample, collected from the silica column could be injected directly onto the GC, and a fairly stable GC baseline was obtained.

When a mixture of standards was applied to the silica column, more than 95% of 6-ketocholestanol and more than 90% of other major cholesterol oxidation products were recovered (Table 2). Thus, this simplified method can be employed for measuring the major cholesterol oxidation products as well as 6-ketocholestanol. More than 95% of 6-ketocholestanol added to chicken meats was recovered by use of Method II (Table 3). Moreover, low levels of 6-ketocholestanol were determined to be present in fresh chicken, and its identity was confirmed by GC–MS analysis (Table 4).

Generation of 6-ketocholestanol in chicken meat by the effect of γ-radiation. According to our previous study (4), the three major cholesterol oxidation products, *i.e.*, β-epoxide, α-epoxide and 7-ketocholesterol, increased substantially when meat was irradiated (10 kGy). However, these compounds also increased during storage (0–4°C) of the meat in the presence of air. From the small number of samples analyzed, it was not apparent that irradiated meat could be distinguished clearly from autoxidized meat in all cases by measurement of the major cholesterol oxidation products. Similarly, cholesterol A-ring oxidation products, *e.g.*, 4-cholesten-3-one and 4,6-cholestadien-3-one, did not permit a clear distinction to be made in meat as they did in model systems (4).

Irradiation of cholesterol in model systems as well as in meat gives rise to substantial amounts of the two isomeric cholesterol epoxides (4,15). Furthermore, irradiation of the two epoxides has been shown to generate 6-ketocholestanol (15). Hence it was logical to search for 6-ketocholestanol in irradiated meat and chicken. That this search was successful is demonstrated by the data of Table 1.

The previously unreported presence of 6-ketocholestanol in unirradiated, fresh meat and chicken (Tables 1, 3 and 4) was unanticipated. The biochemical pathway by which the ketone is formed in fresh muscle tissue is apparently unknown and requires further investigation. Only a few samples of each meat have been analyzed, and further study is required to establish the normal levels of 6-ketocholestanol in each fresh muscle tissue.

γ-Radiation seems to increase the level of 6-ketocholestanol in some tissues. For example, in chicken this increase may be on the order of 4–6 times (Table 4). Further studies are needed to establish the types of meat and poultry

TABLE 3

Recovery of 6-Ketocholestanol from Chicken Breast by Method II

	Added amount (μg/50 g)	Detected amount[a] (μg/50 g)		Recovery (%)[a]
		Control[b]	Spiked	
Chicken breast I	14.65	0.20 ± 0.06	14.87 ± 0.49	99.0 ± 2.9
Chicken breast II	13.12	0.06 ± 0.01	12.84 ± 0.28	97.5 ± 2.1

[a]Value ± SD; duplicate.
[b]Without addition of 6-ketocholestanol in chicken samples.

TABLE 4

6-Ketocholestanol in Unirradiated and Irradiated (10 kGy) chicken (ng/50 g)[a]

	Unirradiated	Irradiated
Method II + TLC	77 ± 8[a]	462 ± 15[b]
Method II	108 ± 1[c]	513 ± 57[d]

[a]Value ± SD; duplicate; additional thin-layer chromatography (TLC) analysis before gas chromatography analysis (in Method II + TLC); same superscript letters a–d in a row denote no significant difference by the *t*-test ($\alpha = 0.05$).

(and perhaps seafood) for which 6-ketocholestanol may be a suitable indicator of past irradiation.

The main purpose of the current study, to isolate and quantitate 6-ketocholestanol as a possible indicator of prior irradiation of chicken meats, was achieved by the development of a simplified, sensitive and reproducible method. In the current experiment, however, the meats were irradiated to a dose of 10 kGy, which is the maximum dose internationally recommended for food irradiation. This dose level is above the maximum dose (3 kGy) permitted for poultry in the United States. In addition to the future studies suggested above, further investigations must be carried out to determine the lowest dose at which the indicator compound is useful. In addition, the effects of radiation temperature and of post-irradiation storage temperature and time on 6-ketocholestanol detection should be established. These are some of the parameters which will make this method useful and reliable in the detection of meat and poultry irradiation.

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[Received May 10, 1993; accepted June 18, 1993]