Oxidation of Cholesterol in Commercially Processed Cow’s Milk

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(Received for publication March 26, 1987)

ABSTRACT

Pasteurized whole milk, ultra-high temperature heated milk, canned evaporated milk, skim milk and instant nonfat dry milk were analyzed for presence of oxidized cholesterol compounds. Effects of heating whole milk and storage of whole milk lipid extracts were also examined. Analytical thin-layer chromatography data indicate that cholesterol in liquid milk was stable during commercial pasteurization, sterilization and evaporation. However, instant non-fat dry milk contained 7-hydroxy-cholesterol. Heating whole milk for 12 h at 85°C did not produce oxysterols, but GC-MS analysis indicate that storage of whole milk lipids may have produced steroidal ketones.

The role of cholesterol in health and disease is being scrutinized and questioned as investigators bring new evidence to light. Of recent concern are the conditions under which cholesterol oxidizes and the effect that these oxidized products have on diet and health. Over 60 reaction products of cholesterol oxidation have been isolated and identified and a number of these products have demonstrated biological activity in various cell and organ cultures and intact animal tissues (1,4,5,9,10,12,13,17,22). The most common oxidized cholesterol compounds are products from A or B ring or side chain reactions (20).

In recent years, researchers have reported the presence of oxidized cholesterol compounds in some foods, notably spray-dried egg products (2,8,15), beef tallow (16,19), French fried potatoes (14,16) and milkfat (3). To date only one group has reported cholesterol ketones in stored milk (6), and no reports have appeared in which milk was studied as purchased from the supermarket.

Various types of processing such as sterilization, spray drying or evaporation are done to provide milk in convenient forms for the consumer. The purpose of this study was to investigate the presence of certain oxidized cholesterol products in four types of milk and to study the effects on cholesterol after heating whole milk or after storage of isolated milk sterol fractions.

MATERIALS AND METHODS

Sample

Whole pasteurized milk, ultra-high temperature (UHT) heated whole milk, canned evaporated whole milk, skim milk and instant nonfat dry milk (NDM) were purchased from local supermarkets and analyzed the same day. NDM was hydrated at 50% with distilled water and refrigerated 30 min before lipid extraction. Fresh raw milk, taken from a Guernsey cow on a local farm, was collected into a clean glass-stoppered flask which had been flushed with nitrogen and placed on dry ice. The flask with freshly drawn milk was immediately stoppered and kept on dry ice. Lipids were extracted within 15 min after the milk was drawn from the cow.

Whole milk and rehydrated NDM were used for determination of the influence of heating on cholesterol oxide formation. The milks were heated at 85°C for 12 h uncoverd with continuous stirring. To study the effect of storage on development of oxidized cholesterol compounds, whole milk lipid extracts that had been stored in the dark at 5°C for 2 months before analysis were examined.

Extraction of lipids

A modified Radin method (18) was used for extracting lipids. A 5-ml milk sample was added to 12 ml of HPLC glass-distilled grade isopropanol/hexane (Burdick & Jackson, Muskegon, MI) (2:1, v/v) mixture in a glass centrifuge tube with teflon lined cap, mixed for 60 sec and allowed to stand 20 min. An additional 8 ml of hexane was added and the mixture was centrifuged for 15 min at 2500 x g at 20°C. The lipid portion was transferred quantitatively to an evaporation flask and the solvent was evaporated under vacuum. The lipid extract was transferred to a glass test tube with hexane/isopropanol (10:1, v/v) solvent and dried under nitrogen.

Thin-layer chromatography

High performance analytical silica gel SI-HPF, 200-μm thin-layer chromatography (TLC) plates (J. T. Baker Chemicals, Phillipsburg, NJ) were used. Extracts from the whole milk samples were dissolved in 300 μl chloroform and a 2-μl sample was applied 2 cm from the bottom of the plate. Nonfat dry milk extractions were dissolved in 100 μl of chloroform and 2 μl were applied to the chromatoplate. Standards including cholesterol, cholest-4-en-3-one, cholest-4,6-dien-3-one, 20α-hydroxy-cholesterol, cholest-5α,6α-epoxide and 7-ketocholesterol (Sigma, St. Louis, MO) and cholesterol-3,5-dien-7-one, 7β-hydroxycholesterol, 25-hydroxycholesterol and cholestane-3β,5,6-triol (Steraloids, Wilton, NH) were applied for identification purposes. Plates were developed first in ethylene dichloride (17 cm) to separate sterol esters, triglycerides, fatty acids and phospholipids from free sterols and second in ethyl acetate (5 cm) for further separation of the sterols. Plates were viewed under UV light at 266 and 254 nm.
TABLE 1. TLC data on some oxidized cholesterol compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>UV (254nm)</th>
<th>H₂SO₄ (color)</th>
<th>Mobilities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholest-4-en-3-one</td>
<td>+</td>
<td>light pink</td>
<td>4.0</td>
</tr>
<tr>
<td>Cholest-4,6-dien-3-one</td>
<td>+</td>
<td>pink</td>
<td>3.9</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>weak</td>
<td>magenta</td>
<td>3.8</td>
</tr>
<tr>
<td>Cholest-3,5-dien-7-one</td>
<td></td>
<td>beige</td>
<td>3.75</td>
</tr>
<tr>
<td>20α-hydroxycholesterol</td>
<td></td>
<td>gray-green</td>
<td>3.25</td>
</tr>
<tr>
<td>25-hydroxycholesterol</td>
<td></td>
<td>blue-gray</td>
<td>2.95</td>
</tr>
<tr>
<td>Cholest-5α,6α-epoxide</td>
<td>+</td>
<td>yellow</td>
<td>2.85</td>
</tr>
<tr>
<td>7-Ketocholesterol</td>
<td></td>
<td></td>
<td>2.8</td>
</tr>
<tr>
<td>7β-hydroxycholesterol</td>
<td></td>
<td>blue</td>
<td>2.6</td>
</tr>
<tr>
<td>7α-hydroxycholesterol</td>
<td></td>
<td>blue</td>
<td>2.5</td>
</tr>
<tr>
<td>Cholestan-3β,5,6β-triol</td>
<td></td>
<td>light yellow</td>
<td>1.5</td>
</tr>
</tbody>
</table>

then sprayed with 50% aqueous H₂SO₄, air-dried, and heated at 110°C up to 5 min for color development. The coloration and Rₑₒ values for the sterols are given in Table 1. Plates were heated an additional 30 min for complete charring of the compounds.

Preparative PK6F silica gel 500-μm TLC plates (Whatman, Hillsboro, OR) were used for isolation of cholesterol and oxidized cholesterol compounds. Two bands were marked, scraped and eluted for further analysis. The first band, Fraction 1, between 3.5 and 4.5 cm included cholesterol, cholest-3, 5-dien-7-one, cholest-4-en-3-one and cholest-4,6-dien-3-one. Some of these compounds have similar Rₑ values but were separated with gas chromatography. Fraction 2, between 2.2 and 3.5 cm from the sample application, contained oxycholesterols 7α- and 7β-hydroxycholesterols, 20α-hydroxycholesterol, 25-hydroxycholesterol, 7-ketocholesterol and cholesta-5,6-epoxide. Although Rₑ values were similar for the 7-ketone and the epoxide in this group, the ketone was detected under UV light but did not give a color reaction to sulfuric acid. The epoxide, on the other hand, was not detected under UV light but displayed a yellow color after spraying with H₂SO₄ and then heating.

Gas chromatography-mass spectrometry

Samples were analyzed on a Varian 3740 gas chromatograph (Varian Associates, Palo Alto, CA) equipped with a flame ionization detector and methyl silicone capillary column, SP-2100 (Supelco, Inc., Bellefonte, PA) 30 m long. Column temperature was programmed to start at 220°C, increasing 4°C/min to 260°C. Injection port and detector temperatures were 200°C and 330°C, respectively. Nitrogen was the carrier gas.

Capillary column gas chromatography-mass spectrometry (GC-MS) was performed with a Finnigan 4510 GC mass spectrometer equipped with an INCOS data reduction system. A 30 m DB-5 fused silica capillary column (J & W Scientific, Inc., Cordova, CA) which was passed directly through a vacuum interlock into the ion source was used. The electron energy was 70 eV and the ion source pressure was less than 5 × 10⁻⁶ torr for the electron ionization experiments.

RESULTS AND DISCUSSION

Liquid whole milk

The major sterol in milkfat is cholesterol (11). The major TLC spot (spot 2, Chromatograms C through F, Fig. 1) for raw, UHT, evaporated and whole milk, re-
respectively, displayed color and Rf value of cholesterol. The spot above cholesterol in the chromatograms corresponds with Rf values for a sterol ketone. However, the spot was not detected with UV light as would be expected for a ketone nor did it give the color reaction of a ketone, as indicated in Table 1, when the chromatoplate was sprayed with H2SO4 and heated.

Oxidized cholesterol compounds which are more polar than the parent sterol can be separated, detected and tentatively determined by analytical TLC (7). Chromatograms for the fresh raw milk and commercial liquid milks did not indicate presence of oxidized compounds when compared with Rf values and color reactions of the standard compounds. Chromatograms C, D, E and F (Fig. 1) for raw, UHT, evaporated and whole milk samples respectively displayed a beige spot with similar Rf values for 7-hydroxycholesterol in Chromatogram A (spot 6 in Fig. 1), which is a mixture of oxidized sterols. However, spot 4 from the liquid milk samples (Fig. 1, Chromatograms C, D, E, F) did not display the immediate distinct blue color of the diol after H2SO4 treatment and heating. The TLC analysis showed consistent results in 5 different samples of each type of milk studied.

GC chromatograms for the TLC sterol fractions were similar for all types of liquid milk. The major component of this fraction was cholesterol. Some minor peaks which represented compounds eluted after cholesterol did not agree with retention times of oxidized cholesterol nor were they identified by MS analysis as cholesterol ketones or other common cholesterol derivatives. Flanagan et al. (6) analyzed anhydrous milkfat after it had been stored for 18 months at 5°C and isolated two steroidal ketones, cholest-4-en-3-one and cholest-3,5-dien-7-one, by GC-MS. In the current study, these ketones were not identified in raw milk or milk purchased from the supermarket. To determine the effect of storage of the sterol fraction of these milks, the whole milk extract was stored in the dark for 2 months at 5°C in a glass vial with teflon cap and then analyzed in the same manner as the other samples. Mass spectra of two substances (Fig. 2, 3) were in excellent agreement with spectra of authentic cholest-4-en-3-one and cholest-3,5-dien-7-one. Figure 2 shows the high resolution mass spectral measurements of cholest-4-en-3-one with a molecular weight of 384.3. This is consistent with the elemental composition D27H44O. The intense ion at m/e 124 (base peak) and one at 342 are indicators of a 4 ene, 3 keto moiety (6). The mass spectral measurements of the compound identified as cholest-3,5-dien-7-one (Fig. 3) show a molecular weight of 382.3 consistent with the elemental composition C27H42O. The rearrangement ion at m/e 174 (base peak) and those at m/e 187 and m/e 161 are consistent for the spectrum of the standard compound, cholest-3,5-dien-7-one (6).

It is suggested from this investigation that the sterol ketones were formed during storage of the isolated sterols. Analysis of raw milk and commercial milk samples did not indicate the presence of these ketones, which suggests that they are not a natural constituent of milk nor are they formed under normal processing procedures for commercial sales. Both of these ketones have been identified among products of cholesterol autoxidation (21,24) and cholest-4-en-3-one is an oxygen-independent isomerization product of cholest-5-en-one, an initial reaction product of cholesterol dehydration (20).

Nonfat milk

The nonfat dry milk chromatogram B (Fig 1) demonstrated a small blue spot (spot 6) below the cholesterol band after spraying with 50% aqueous H2SO4. This spot
was identical to the Rf value of the 7β-hydroxycholesterol standard compound. The blue color on chromatogram B for NDM appeared within 1 min of heating. No other spots on the chromatograms corresponded with the standard oxysterol compounds in Chromatogram A. Since only the nonfat dry milk displayed possible 7-hydroxycholesterol, comparison was made with the lipid extract of skim milk. No compounds yielding blue coloration were observed upon reaction with H2SO4. Analysis by GC yielded inadequate resolution of the diol. The diol, in both trimethylsilylated and non-trimethylsilylated samples, was not detected by GC-MS analysis.

The presence of 7-hydroxycholesterol in instant nonfat dry milk would agree with reports of its presence in spray-dried egg foods such as scrambled egg mix (15,23) some of which contain 30% dried milk. By comparison, absence of this compound in UHT and evaporated milk, which are heated to high temperatures during processing, lends evidence to the suggestion that the spray drying process rather than heating the food itself may induce cholesterol oxidation (23).

Heated milk

Figure 4 shows chromatograms for both heated and unheated whole milk and liquid skim milk. While a more complex pattern is seen for the heated milk samples than for corresponding unheated samples, treatment with H2SO4 reagent and UV light analyses did not indicate presence of the oxysterols investigated in this study. The heated milk sample chromatograms (Fig. 4) displayed unidentified compounds more polar than cholestane-3β,5,6β-triol (Fig. 4, spot 7, Chromatogram A). In addition, the substance with Rf similar to cholestanetriol was detected under UV 254 nm light, which is contrary to the standard triol. Heating the chromatoplates gave only faint detection of the material in the samples, whereas the standard cholestanetriol displayed a light yellow color (Table 1) after heating and gradually darkened with further heating of the chromatoplate.

CONCLUSIONS

No evidence was found of oxidized sterols in whole pasteurized milk, UHT milk, canned evaporated milk and skim milk as purchased from the supermarket. After heating whole milk or liquid milk at 85°C for 12 h cholesterol oxides were not detected. However, storage of thesterol fraction of whole milk at 5°C for 2 months produced cholest-4-en-3-one and cholest-3,5-dien-7-one. TLC chromatograms indicated presence of 7-hydroxycholesterol in spray dried instant nonfat milk. The spray drying process, which introduces oxides in addition to heat to the dry milk particles, may catalyze cholesterol oxidation.

ACKNOWLEDGMENTS

Research was supported by a Sigma Xi Grant in Aid of Research and the Florida State University E. N. Whitney Fund.

REFERENCES

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