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Stability of Cholesterol; Cholesterol-5β,6β-Epoxide; Cholesta-3,5-Dien-7-One, and 5α-Cholestane in Solution During Short-Term Storage

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Abstract

Because cholesterol and its oxide products (COPs) are used as standards during analysis, their stability and autoxidation were assessed under typical storage conditions. Individual toluene solutions of cholesterol; cholesterol-5β, 6β-epoxide; cholesta-3,5-dien-7-one; and 5α-cholestane were prepared and aliquots were stored at 23 ± 2°C, 4°C, and -20°C for 7 days. Samples were removed from storage, dried under nitrogen, derivatized and analyzed using gas chromatography-flame ionization detection and gas chromatography-mass spectrometry. β-sistosterol and 5-colest-ene-7-one were found to be a suitable recovery internal standard and an instrument internal standard, respectively. All compounds in solution had no detectable artifact generation during 7 days of storage; thus, they were stable. Storage temperatures of 23 ± 2°C, 4°C, and -20°C did not affect stability. Chromatograms revealed contaminants and other peaks likely associated with incomplete derivatization of some compounds; potential causes for the unusual lack of derivitization are discussed.

Keywords: Oxysterols; Artifacts, Artifact generation; Autoxidation; Injector liner; Liner effects; Derivatization effects

Introduction

Cholesterol is susceptible to oxidation, including enzymatic oxidation, photooxidation, and autoxidation with complex breakdown pathways leading to many cholesterol oxide products (COPs) [1-3]. During autoxidation, cholesterol undergoes a series of reactions, initially producing various cholesterol (cholesteryl) hydroperoxides that yield epoxy-cholesterols, cholesterol-diols and keto-cholesterols. These products can, in turn, be further altered by epimerizations and dehydration. COPs are found in the body and in processed foods of animal origin. They have been implicated in the etiology of several diseases and are studied to investigate their presence and role in metabolism, diseases and diet.

Cholesterol and COPs present in samples from metabolic studies related to diseases or in food products from various processing conditions can be oxidized or further degraded during analysis resulting in artifacts. COP artifacts are defined as compounds generated from cholesterol and other COPs by conditions or processes during isolation, detection, and quantification. Results may show an artificial increase in nascent COPs or their deterioration with concomitant development of derived artifactual products. Thus, it is difficult to accurately quantify, identify and distinguish naturally occurring COPs that are in samples due to metabolism, possible disease conditions or food processing conditions from the same COPs that might be generated during analysis.

Several studies have investigated causes and prevention of artifact generation and artifact monitoring [4-11]. Common measures taken to limit artifact generation are low light conditions, nitrogen flushing, and silanization of glassware. Additionally, there has been much discussion about saponification and the generation of artifacts in heat and strong alkaline conditions [8]. Most researchers recognize the importance of cold (room temperature) saponification in limiting artifact generation. Effective concentrations for saponification most likely depend on the matrix investigated. Park et al. found artifact generation with KOH concentrations greater than 1M [8]. While complete prevention of artifact generation during analysis may not be possible; it is important to

continue to investigate potential sources. Few studies have investigated stored solutions of cholesterol and COPs over time to determine if solutions used in model studies, standard spiking, or for quantification generate artifacts that could compromise results.

An extensive investigation was undertaken to determine if solutions of COPs remain stable over a 7 day period and whether these same solutions of cholesterol; 7-ketocholesterol; cholesterol-5β,6β-epoxide; cholestane-3β-5α,6β-triol' cholesta-3,5-dien-7-one; 5α-cholestane; and 19-hydroxycholesterol contribute artifacts. Information on the stability of 7-ketocholesterol; 19-hydroxycholesterol; and cholestane-3β-5α,6β-triol was presented previously [12]. In this paper, we focused on the stability of cholesterol; cholesterol-5β,6β-epoxide; cholesta-3,5-dien-7-one; and 5α-cholestane. Solutions consisting of a single compound were stored at 23 ± 2°C, 4°C, and -20°C to determine the most suitable storage conditions.

Experimental Procedures

Cholesterol, 5-cholestene, 5α-cholestane (5αC), cholesterol-5β,6β-epoxide (β-epoxide) and β sistosterol (βS) were purchased from Sigma (St. Louis, MO). Cholesta-3,5-dien-7-one (3,5-7-one); and cholest-5-ene-7-one (5-ene-7-one) were from Steraloids (Newport, RI). Toluene (Omni-Solv, EMD Chemicals, Gibbstown, NJ); anhydrous pyridine (Aldrich, Milwaukee, WI); Sylon BTZ (Supelco, Bellafonte, PA); and

silanized inserts and amber vials (National Scientific, Duluth, Georgia) were purchased also.

The stability of 2 standards (cholesterol and 5αC) and 2 COPs (β-epoxide and 3,5-7-one) were evaluated. Sampling times were day 0, 1, 3, 5, and 7; storage temperatures were 23°C ± 2°C, 4°C, and -20°C. In order to monitor artifact generation, antioxidants were not added to standard solutions. No antioxidants were added to standard solutions. All conditions and procedures for gas chromatography-flame ionization detection (GC-FID, Varian 3400, Walnut Creek, CA, USA) and gas chromatography-mass spectrometry (GC-MS, Thermo Finnigan GCQ Plus Ion Trap, Thermo Electron Corp., Waltham, MA, USA) were fully delineated in the work of Busch et al. [12].

Cholesterol and β-epoxide were stored during the same week. Silanized GC vial inserts, inadvertently not placed in GC vials for runs on days 0 and 1, were inserted and samples for day 0 and 1 were rerun and inserts were used on subsequent days. On day 7, after a new injector liner was installed, samples were re-run. On day 7, samples were re-run after a new injector liner was installed. 5αC and 3,5-7-one were stored at the same time and silanized GC vial inserts were used throughout the study for these compounds. After analyses on day 3 and prior to day 5 analyses, the injector liner was replaced. Compounds were identified by GC-MS at > 90% match.

Explanations for construction of models and validity for all assumptions for statistical analysis of data are delineated in Busch et al. [12]. Factors of day, temperature, vial insert, injection number (covariate), and day by temperature were included in the general linear models for cholesterol and β-epoxide [13] For 5αC and 3,5-7-one, factors of temperature, injection number, liner by temperature, liner, and day nested within liner were included in the general linear models.

Least squares means (LS means), instead of arithmetic means, allowed for adjustment of potentially confounding variables like injection number. A Tukey Kramer adjustment was used to compare *Post hoc* LS means. Significance was determined at *p*<0.05.

Results and Discussion

Terminology

In Table 1, derivatized standards and contaminants are identified. Actual COP standards are denoted as "standard" and the name of contaminants are preceded by the name of the parent compound. For instance, β-epoxide triol denotes triol which was initially present in the β-epoxide standard as a contaminant. Unknowns which are associated with the presence of a standard compound are referred to as "related" to the standard, for example a β-epoxide related peak.

Cholesterol

Table 2 presents LS means relative to day 0 for all compounds.

Stored Standard	Observed Peaks				
	Standard Peaks		Contaminant Peaks		
	Derivatized	Underivatized	Derivatized	Underivatized	Unknown
Cholesterol	Cholesterol tms ether	Cholesterol			
β-epoxide	β-epoxide tms ether	β-epoxide	β-epoxide triol tms ether		β-epoxide triol Related
3,5-7-one		3,5-7-one			
5αC		5αC			

Table 1: Peaks observed from the original standards after storage and derivatization.

Standard	Associated Compound	Day 0	Day 1	Day 3	Day 5	Day 7
Cholesterol	Cholesterol tms ether	100.0	83.6	69.5	89.7	103.4
	Cholesterol[a]	100.0	-96.4	-113.1	-111.5	-113.6
	Total Cholesterol	100.0	92.1	93.2	98.1	107.2
β-epoxide	β-epoxide tms ether	100.0	211.8	198.1	228.9	242.4
	β-epoxide	100.0	129.0	61.2	67.9	57.8
	Total β-epoxide	100.0	180.2	154.4	176.8	179.7
	β-epoxide Triol tms ether	100.0	179.5	176.7	205.5	225.6
	β-epoxide Triol Related	100.0	63.1	46.2	48.1	42.5
	7-keto 3,5-7-one	100.0	99.4	112.2	90.6	101.2
3,5-7-one	3,5-7-one	100.0	120.3	139.9	105.1	127.9
5αC	5αC	100.0	126.3	147.8	58.7	96.4

LS means for each temperature were averaged and standardized to day 0. aDay 0 LS means for cholesterol was negative while subsequent days were positive, resulting in negative percent LS means relative to day 0.

Table 2: Stability of COPs standards over time: percent least squares means (LS means) relative to day 0.
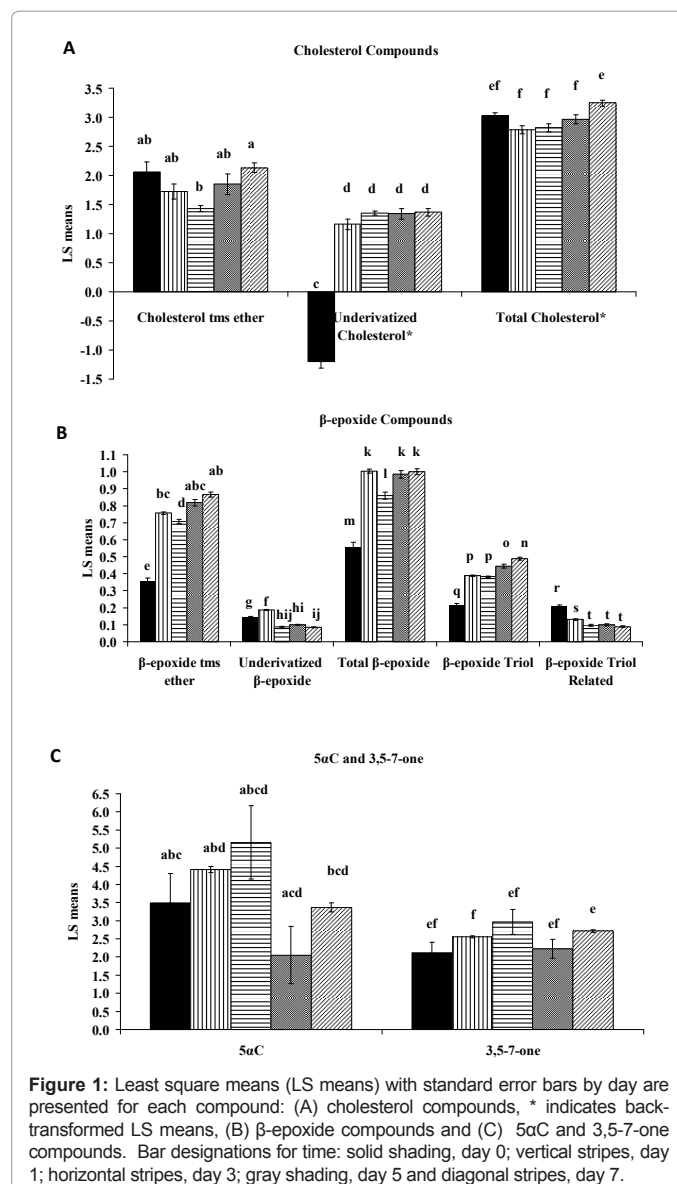

Figure 1: Least square means (LS means) with standard error bars by day are presented for each compound: (A) cholesterol compounds, * indicates back-transformed LS means, (B) β-epoxide compounds and (C) 5αC and 3,5-7-one compounds. Bar designations for time: solid shading, day 0; vertical stripes, day 1; horizontal stripes, day 3; gray shading, day 5 and diagonal stripes, day 7.

LS means for cholesterol tms ether were significantly different by day (F=7.23, p=0.016) due to a downward trend from day 0 to day 3 and an upward trend for days 5 and 7 (Figure 1a). However, there was no significant difference between day 0 and day 7. Cholesterol tms ether was not significantly affected by storage temperature (F=0.13, p=0.8803). Insert was not significant (F=0.91, p=0.3481); injection number was significant (F=35.34, p<0.0001). A second peak was observed in all cholesterol chromatograms.

Cholesterol related peak: This second peak may have been underivatized cholesterol or a contaminant that did not significantly change over time (F=0.00, p=0.9640, Table 1). This data was generated using the best fit model which utilized temperature and insert as categorical effects. However, in order to graph day effects, the statistical model utilizing temperature, insert, and day was used. In this model, several transformations were required and day was significant (F=13.39, p<0.0001) with day 0 significantly lower than subsequent days, which were not significantly different from each other. As a result of the transformations, day 0 LS means for the cholesterol related compound was a negative number, indicating that the response was very low and that the adjustment for the change in injector liner over time was an over-adjustment. This resulted in a negative value for day 0 (Figure 1a). Using the best fit model, underivatized cholesterol was not significantly affected by storage temperatures (F=0.76, p=0.4760) or insert (F=0.27, p=0.6064), but injection number was significant (F=35.61, p<0.0001).

Total Cholesterol: Total cholesterol (cholesterol tms ether plus cholesterol related compound) was significantly affected by day (F=56.23, p<0.0001), but there was no significant difference between day 0 and day 7. Total cholesterol (F=0.12, p=0.8845) was not significantly affected by varying storage temperatures. There was a significant insert effect only for total cholesterol (F=33.19, p<0.0001). Injection number was significant also (F=16.04, p=0.0004). Overall, cholesterol was stable for 7 days. Stability was not affected by differences in storage conditions at 23 ± 2°C, 4°C, and -20°C.

β-epoxide

β-epoxide is a commonly reported COPs. LS means for β-epoxide tms ether significantly increased by day (F=235.26, p<0.0001). Day 0 was substantially lower than subsequent days (Figure 1b). β-epoxide tms ether was not significantly affected by storage temperature (F=2.93, p=0.0743). The presence of inserts was significant (F=364.06, p<0.0001) as was injection number (F=169.21, p<0.0001).

β-epoxide related peak: In every β-epoxide chromatogram, several peaks were present: β-epoxide related peak, β-epoxide triol peak and β-epoxide triol related peak. The β-epoxide related peak was most likely underivatized β-epoxide, which significantly decreased (F=318.96, p<0.0001) over time. LS means increased from day 0 to day 1, decreased on day 3 and then remained relatively constant through day 7. Underivatized β-epoxide was not significantly affected by storage temperature (F=2.74, p=0.0904). The insert effect (F=1.04, p=0.3213) was not significant. Injection number was significant (F=180.00, p<0.0001).

Total β-epoxide: Total β-epoxide (derivatized plus underivatized) were similar to results of β-epoxide tms ether with statistically significant effects for day (F=56.23, p<0.001), insert (F=33.19, p<0.0001), and injection number (F=16.04, p=0.0004). For day effect, day 0 was not only significantly lower than subsequent days, but also showed much lower numerical LS means. Day 3 was significantly lower than days 1, 5, and 7 which were statistically similar. Total β-epoxide was not significantly affected by storage temperature (F=2.66, p=0.0920).

β-epoxide triol: β-epoxide triol was initially present in the β-epoxide standard as a contaminant on day 0, but triol can also be generated as an autoxidation artifact of β-epoxide. The LS means for β-epoxide triol tms ether significantly increased by day (F=233.12, p<0.0001). As with β-epoxide, day 0 was significantly less than all other days. β-epoxide triol tms ether was not significantly affected by storage temperature (F=1.10, p=0.3493). There were significant effects for insert (F=99.52, p<0.0001) and injection number (F=63.69, p<0.0001).

β-epoxide triol related peak: A β-epoxide triol related peak was initially present at day 0 and LS means significantly decreased over time (F=24.0, p<0.0001). However, day 0 was significantly elevated compared to the other days. The β-epoxide triol related peak was not significantly affected by temperature (F=0.75, p=0.4828). Insert effect was significant (F=55.06, p <0.0001) whereas injection number was not (F=0.2, p=0.6565).

β-epoxide stability was difficult to determine because there were several compounds present. Stability of this compounds was assumed based on (1) the increase in β-epoxide tms ether and β-epoxide total increased over time, (2) no loss of β-epoxide over time, and (3) no effect by differences in storage conditions.

5α-cholestane

LS means increased for days 0 through 3, then decreased on day 5 and increased on day 7 (Figure 1c). Days 0 and 7 were statistically similar. Temperature was not significant (F=0.42, p=0.6634). While injection number was not significant (F=0.21, p=0.6530) overall, it might have affected results observed at sampling times. The injector liner was changed after analysis of day 3 samples and prior to analysis of day 5 samples; liner had a significant effect (F=7.84, p=0.0123). Day within liner was significant (F=13.22, p=0.0001) as well. There were no other peaks of interest found in the 5αC chromatograms. 5αC was stable when stored in solution for up to 7 days.

3,5-7-one

3,5-7-one exists solely as an artifact of 7-keto and is rarely identified. It was investigated in order to determine its stability without the influence of 7-keto. Standard 3,5-7-one demonstrated a trend similar to 5αC (Figure 1c). The LS means for days 0 through 3 increased, decreased on day 5, and increased on day 7. Days 0 and 7 were not statistically different. Storage temperature (F=1.95, p=0.1714) was insignificant as were injection number (F=0.42, p=0.5246) and liner (F=0.17, p=0.6814). There was a significant interaction between temperature and liner (F=3.62, p=0.0477) for day within liner (F=6.21, p=0.0089). 3,5-7-one was stable for the 7 days of storage, regardless of temperature. No other significant peaks were found in 3,5-7-one chromatograms.

Peak identification

Cholesterol and β-epoxide standards each contained an additional peak on day 0. Peaks which were observed in the original standards are noted in Table 1. These additional peaks represent contaminants (possibly a similar COP) initially present in the standards or products of incomplete derivatization. The cholesterol related peak may have been a contaminant. It did not exhibit a similar trend of increased derivatized compound and decreased underivatized compound over time.

Overall, β-epoxide and β-epoxide triol showed similar derivatization trends. The increase of derivatized compound indicates no loss. Insert

presence was significant. It appears that day 0 LS means were elevated for underivatized compounds and reduced for derivatized compounds compared to subsequent days due to an insert effect. Injection number was significant indicating that there was an association between liner changes experienced with increased injection number and increased derivatized compound or decreased underivatized compounds

For the β-epoxide related compound and β-epoxide triol related compound, LS means of initial unknowns decreased as LS means of the standard compound increased over time and no loss of COP was observed. Increase of the standard (as a tms ether) in conjunction with corresponding loss of the initial unknown as well as mass spectra of compounds, indicated derivatization issues rather than artifact generation.

All compounds investigated were stable over 7 days of storage and generated no artifacts. The stability of 5αC is particularly important because it is used as an internal standard. If an internal standard experiences loss of stability during storage or artifact generation during analysis, quantification of COPs would be greatly affected.

Derivatization

In addition to temporal and temperature effects, the other main observation for this study was derivatization effects. Unlike the outcome of previous work, incomplete derivatization was apparent in our samples. Derivatization of COPs is commonly performed to increase volatility and thermal stability for improved GC analysis. For COPs, the most common derivatization reaction is silanization of hydroxyl groups to tms ethers. Epoxide groups are not silanize; 5αC, 3,5-7-one; and 5-ene-7-one do not have functional groups that are silanized.

As discussed by Busch et al [12], incomplete derivatization may have many causes, including variance in consistency of Sylon BTZ and sub-optimal ratio of silylating reagent to hydroxyl groups [14-16]. Other investigators have reported multiple GC peaks from derivatized COPs [14,17,18], however, none have been reported for compounds used in this or our previous study [12]. Because consistent trends of increased derivatized and decreased underivatized compounds over time were observed, it was postulated that incomplete derivitazation was potentially due to changes in the injection liner, injection bias or continued derivatization of underivatized polar compounds as they transferred onto the column.

Use of new internal standards

Because the stability of 5αC was investigated, an alternative internal standard was required. Two internal standards were needed: one as a recovery internal standard and one as an instrument internal standard. βS, a phytosterol that has been used as an internal standard for COPs, was chosen as a recovery internal standard. βS was added immediately after samples were removed from storage prior to drying and derivatization. 5-ene-7-one was investigated as an instrument internal standard because it does not co-elute with COPs. 5-ene-7-one lacks functional groups that can be derivatized by Sylon BTZ or that can undergo tautomerization in the presence of excess derivatization agents as the conjugated double bond at position 5 stabilizes the keto group at position 7 [17]. Due to its polarity, it may not be best suited as a recovery internal standard for all COPs. 5-cholestene co-eluted with 5αC, so it was not an appropriate internal standard for this study. Several compounds, other than 5αC, 19-OH, and βS, have been investigated by others as internal standards for COPs, but are not suitable for use due to tautomerization during derivatization [8,17,19] .

Conclusions

To prevent or counter incomplete derivatization in the future, excess silanization reagents could be evaporated using a stream of nitrogen. However, after complete COP analysis usually including lipid extraction, saponification followed by extractions, solid phase extraction, and derivatization with multiple evaporation/liquid reduction steps, evaporation of silanization reagents would be yet another step in the complex and lengthy analysis of COPs in terms of recovery. Injection liners should be changed often, but results from multiple liners should be investigated to determine if the different liners are behaving similarly. In this study, we identified challenges associated with incomplete derivatization because the matrix was simple, consisting of single standard solutions. For quantification of COPs, complete derivatization is particularly important in light of multiple compounds, complex matrices, and potential artifacts.

Cholesterol, β-epoxide; 3,5-7-one; and 5αC are stable in toluene solutions when flushed with nitrogen and stored in aluminum foil covered vials at 23°C ± 2°C, 4°C and -20°C for up to 7 days. These COPs did not generated artifacts when stored under these conditions. 5-ene-7-one may be effectively used as an instrument internal standard for COPs analysis. Further investigation of the stability of these COPs in solution when stored for longer time periods is needed.

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