Cholesterol quantitation by GLC: artifactual formation of short-chain steryl esters

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Summary A simple and rapid method for the quantitation of total cholesterol in lipid extracts using gas–liquid chromatography is presented here as a modification of an earlier saponification procedure (Ishikawa, T. T., J. MacGee, J. A. Morrison, and C. J. Glueck. 1974. Quantitative analysis of cholesterol in 5 to 20 µl of plasma. *J. Lipid Res.* **15:** 286–291). Using the original method, as well as a slightly modified version, we found a systematic loss of cholesterol measured as total cholesterol that was attributable to the formation of a byproduct during the procedure. Depending on the nature of the solvent mixture used for extraction after saponification, different byproducts were produced that had longer retention times than cholesterol. The byproducts were identified as cholesteryl butyrate (produced when methyl butyrate was included in the solvent mix) and cholesteryl propionate (with ethyl propionate in the solvent mix) by comparison to authentic standards using gas chromatography–mass spectroscopy. Using mixtures of cholesterol standards, we compared several solvents in lieu of the solvent mixture used in the original extraction procedure to identify those that eliminate the formation of the byproducts. Our optimized microsaponification procedure uses a single solvent, tetrachloroethylene, to extract lipids after the saponification reaction, and improves the accuracy of the cholesterol determination.—**Klansek, J. J., P. Yancey, R. W. St. Clair, R. T. Fischer, W. J. Johnson, and J. M. Glick.** Cholesterol quantitation by GLC: artifactual formation of short-chain steryl esters. *J. Lipid Res.* 1995. **36:** 2261–2266.

Supplementary key words foam cells • cholesteryl esters • lipid quantitation

For studies of cholesterol metabolism in cultured cells, precise methods to measure the free and total cholesterol contents of small samples are often required. The procedure described by Ishikawa et al. (1) for sample preparation for gas–liquid chromatographic quantitation, which consists of saponification with tetramethyl ammonium hydroxide in isopropanol followed by extraction with a mixture of tetrachloroethylene and methyl butyrate, is widely used (2–6). In the course of using this method, we observed a high degree of variability in determinations of total cholesterol. An examination of chromatograms revealed that many had an additional peak with a greater retention time than that of cholesterol, and the size of the additional peak varied widely, occasionally comprising up to 10% of the mass. Adding the mass in this anomalous peak to that of the cholesterol improved the reproducibility of replicate determinations of total cholesterol, suggesting that this peak was derived from cholesterol during the preparation of saponified samples. This paper describes the identification of this product, as well as modifications to the original method that eliminate the anomalous peak and improve the quantitation of cholesterol by gas–liquid chromatography.

EXPERIMENTAL PROCEDURES

Materials

Unesterified cholesterol, cholesteryl oleate, and 5-cholesten-3β-ol 3-methyl ether (cholesteryl methyl ether) were purchased from Sigma Chemical Company (St. Louis, MO). Reagent grade organic solvents were obtained from Fisher Scientific (Pittsburgh, PA). Tetrachloroethylene, ethyl propionate, methyl butyrate, and tetramethylammonium hydroxide (24% in methanol), were purchased from Eastman Kodak Company (Rochester, NY). Glass centrifuge tubes (screw-top conical tubes (Kimble No. 73785, 5 ml, 13 mm × 110 mm)) for saponification and back extraction were obtained from VWR Scientific (Bridgeport, NJ). Teflon-lined caps (Kimble No. 73802) for these tubes were obtained from Fisher Scientific (Pittsburgh, PA).

Equipment

The analyses were done on a Hewlett-Packard Model 5890 gas chromatograph equipped with a hydrogen flame ionization detector and a splitless inlet system. The automatic injector was a Hewlett-Packard Automatic Sampler 7673A. Studies were conducted using a DB-17 column, obtained from J&W Scientific, which was a fused silica capillary made up of 50% phenyl, 50% methyl silicone (15 meters, 0.53 mm ID, megabore). A precolumn (3 meters, 0.53 mm ID, megabore) of deactivated fused silica (Alltech) was used in addition to the capillary column, functioning as a deposition site for low volatiles, which helped to preserve the non-polar column (7).

Abbreviations: Chol, cholesterol; CME, cholesteryl methyl ester; CB, cholesteryl butyrate; CO, cholesteryl oleate; CP, cholesteryl propionate; EP, ethyl propionate; GLC, gas–liquid chromatography; MB, methyl butyrate; MC, methylene chloride; rt, retention time; pk, peak area %; std ratio, standard ratio; TCE, tetrachloroethylene.

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GLC conditions

Operating conditions were as follows. Injector and detector temperatures were 275°C and 260°C, respectively. The oven temperature was kept at 245°C throughout the analysis. For all studies, ultra-high purity nitrogen was used as the carrier gas at a flow rate of approximately 10–20 ml/min. Additional gases used as support included air (zero grade, 400 ml/min) and hydrogen (ultra-high grade, 30 ml/min).

GLC/MS analysis

Comparison of saponified cell samples to authentic standards was performed on a Hewlett-Packard Model 5890 Series II gas chromatograph equipped with a 5971A mass selective detector. For electron impact analysis the voltage was set at 70 v. Samples were separated using a DB-17 (20 m, 0.25 mm ID, 25 μM film thickness) column (J & W Scientific). The injector temperature was 300°C, the interface temperature was 280°C, and the separation was isothermic at 265°C. Total ion chromatograms were run in the ion range of 50–500 atomic mass units. All studies were analyzed using computer software ChemStation (Ver B.00.02 © Hewlett-Packard 1989, 1990, 1991).

Sample preparation

Lipid standards were prepared at a concentration of 250 ng per μl and stored in toluene. Mixtures of lipid standards were as follows. For the mixture of cholesterol, cholesteryl oleate, and CME, the ratio of cholesterol to cholesteryl oleate was 1:1 (mol:mol) and the ratio of cholesterol to CME was 1:1 (mass:mass). After saponification this mixture gave a cholesterol to CME ratio of 2:1 (mass:mass). Lipid standards or cell extracts were initially dried under nitrogen in conical tubes. For saponification, samples were dried in tapered glass tubes, which facilitated phase separation and recovery of small volumes as described below. For the studies using lipid standards, 100 μl of a given mixture was subjected to either the saponification or back extraction procedures.

Free cholesterol. Our procedure for analyzing unesterified cholesterol included a back-extraction method to remove any extraneous protein or other contaminants derived from the extraction of cellular lipids from cells on plastic culture dishes. Although there is no demonstrable effect of this procedure on individual lipid determinations, it has been our experience that the lifetime of the GLC columns has been substantially extended by routine use of the following procedure. Dried samples were resolubilized in 50 μl of tetrachloroethylene and vortexed for 10 sec. To this mixture, we added 200 μl of deionized water and the samples were again mixed for 10 sec. The cloudy samples were centrifuged at room temperature for 10 min at 2000 rpm. Approximately 40 μl of the clear bottom phase (tetrachloroethylene) was removed, transferred to an autosampler vial, and dried to completion under nitrogen. Samples were resolubilized in 40 μl of hexane prior to injection of 1 μl into the gas chromatograph.

Total cholesterol. Samples were saponified to quantitate total cholesterol. Our original method followed the saponification procedure of Ishikawa et al. (1). Samples in tapered glass tubes were dried under nitrogen and were solubilized in 100 μl of a tetramethylammonium hydroxide–isopropanol 1:3 mixture. The sample tubes were sealed with Teflon-lined caps, mixed on a vortex mixer, and then heated for 15 min at 80°C. After removing the samples from the heating block, they were allowed to cool for 30 sec, and 50 μl of methyl butyrate–tetrachloroethylene 3:1 mixture was added. The samples were mixed for 30 sec on a vortex mixer, and 200 μl of deionized water was added to the tubes. With the tubes capped, the samples were exposed to vigorous manual shaking for 5 sec. The cloudy mixture was centrifuged for 10 min at 2000 rpm, and the bottom phase was isolated, dried, and resolubilized in hexane prior to injection. Because of the offensive odor of methyl butyrate, we modified the above procedure by substituting ethyl propionate. These two solvents have the same molecular weight, similar chemical and solvent properties, and very similar densities.

Cell extracts

Lipid extracts were prepared from CHO 25-RA cells, a mutant cell line that contains large amounts of cholesteryl ester due to lack of regulation of cholesterol homeostasis (8). The cells were grown as monolayers in T75 Falcon flasks in Hams F12 medium with 10% fetal bovine serum at 37°C. At confluency, growth medium was removed and the monolayers were rinsed three times with phosphate-buffered saline. Isopropanol was added to the flasks to extract the lipids. The extracts from two flasks were pooled and dried to completion under nitrogen. A known amount of cholesteryl methyl ether (CME) was added to the extract which was then divided into 12 equal aliquots.

Calculations

The cholesterol concentrations of the standards and unknown samples (cell extracts) were determined by comparing the cholesterol and cholesterol methyl ether (CME) peak areas of the samples as seen by the equation below:

$$\frac{\left(\frac{\text{chol}_{pk}}{\text{CME}_{pk}}\right)}{\text{std ratio}} \times \text{CME } \mu g = \text{chol } \mu g \qquad Eq.\ 1)$$

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TABLE 1. Gas chromatographic analysis of CHO 25-RA cellular lipid extracts: A, nonsaponified samples; B, saponified samples extracted in the presence of ethyl propionate

Type	n	CME_{pk}[a]	$Chol_{pk}$	$Extra_{pk}$	Chol[b]	$Chol_{corr}$[c]	Esterified Chol[d]	Esterified $Chol_{corr}$[e]
					μg	μg	μg	μg
A	10	54.44 ± 0.67	41.16 ± 0.78	—	3.95 ± 0.08	—		
B	12	48.28 ± 0.74	48.32 ± 0.79	1.90 ± 0.77	10.51 ± 0.32[f]	10.92 ± 0.30[f]	6.52 ± 0.34[g]	6.98 ± 0.37[g]

[a]Area % is defined as the area of the peak as a percentage of the total area accumulated during the run, as determined by integrator HP 3396A.
[b]Calculation of concentration of cholesterol in sample using only the Chol and CME area percentages as defined in Methods.
[c]Calculation of the concentration of cholesterol in sample by summing the Extra and Chol peak area percentages as related to the CME area %.
[d]Calculation of the concentration of esterified cholesterol in sample by subtracting the free cholesterol from the total cholesterol.
[e]Calculation of the concentration of esterified cholesterol as calculated ([d]), using the corrected value of total cholesterol ([c]), which takes into account the additional peak.
[f]Very significant, $P = 0.0067$ as determined by Student's unpaired t test.
[g]Very significant, $P = 0.0092$ as determined by Student's unpaired t test.

in which CME_{pk} and $chol_{pk}$ are the peak area percentages (AREA %). The ratio of cholesterol to CME (std ratio) in a standard solution of cholesterol–CME 1:1 (in hexane) is calculated daily from injecting a series of samples of the standard until the ratio is constant. The ratio is calculated as:

$$\frac{chol_{pk}}{CME_{pk}} = \text{std ratio.} \quad \textit{Eq. 2)}$$

This correction accounts for day-to-day variations in the instrument.

Statistical analysis

Statistical comparisons were made using a standard two-tailed, paired Student's t-test (GraphPad Instat t_m, Graphpad Software V2.02).

RESULTS AND DISCUSSION

In the following studies, lipid extracts from cultured cells were used to demonstrate the formation of a byproduct during the Ishikawa procedure that produced an anomalous third peak when the sample was analyzed by GLC. Using cholesterol methyl ether (CME) as a standard to quantitate the cholesterol concentrations in cell extracts (9), we analyzed 12 separate aliquots of the same extract to demonstrate the variability seen in total cholesterol contents which were saponified following the method of Ishikawa et al. (1). Shown in **Table 1** are the cholesterol mass determinations for the samples analyzed. Shown in **Fig. 1A** is a representative chromatogram from a cell lipid extract that was saponified and gave rise to a third peak, which had a retention time of 14.6 min. The chromatogram in Fig. 1B is from a replicate sample that did not undergo saponification and did not have an extra peak, supporting the hypothesis that the extra peak is generated during the saponification procedure. The importance of the third peak is demonstrated by comparing the concentrations of cholesterol calculated by summing the areas of the third peak and the cholesterol peak amounts to that based on the cholesterol peak alone (Table 1). By assuming that the third peak comprises a part of that which makes up total cholesterol, we estimated a significantly higher mass amount of total cholesterol.

To determine the origin of the extra peak, we next analyzed known mixtures of cholesterol, cholesteryl oleate, and cholesteryl methyl ether. The first set of samples analyzed in **Table 2** were those that were in-

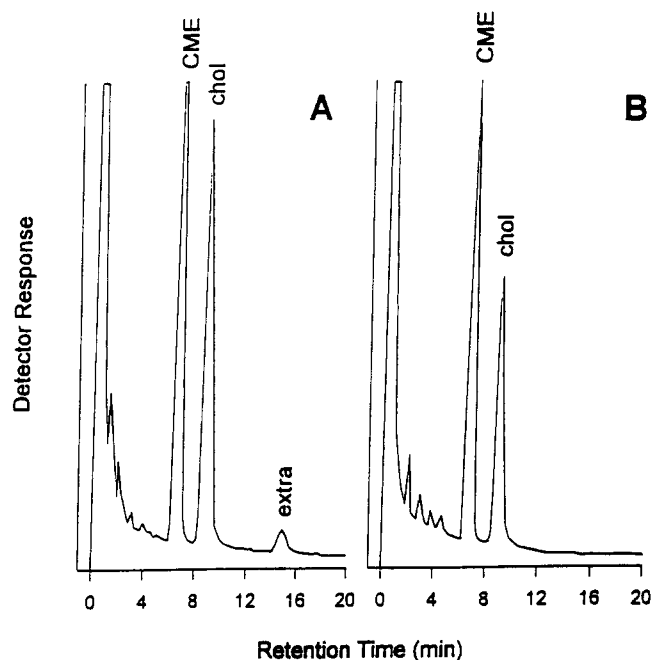

Fig. 1. GLC profiles of CHO 25-RA cellular lipid extracts. (A) Saponified sample, using ethyl propionate and (B) sample not subjected to saponification. Peak identification on figure: CME, cholesteryl methyl ether; chol, cholesterol; extra, unidentified compound. GLC conditions were column: fused-silica capillary (15 m × 0.53 mm ID) coated with 50% phenyl, 50% methyl silicone (DB-17); constant temperature: 245°C; cholesterol retained at 8.7 min. Carrier gas and other GLC instrumentation conditions as given in text. Sample: 1 μl in hexane.

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TABLE 2. Gas chromatographic analysis of lipid standard extracts (CME–chol–CO 1:1:1): comparison of extraction of saponified lipids with various solvents

Treatment	Ratio[a]	Extra Peak?[b]	Retention Time Extra	$Ratio_{corr}$[c]
		% area	*min*	
Direct injection[d]	0.9775 ± 0.0025	–	–	–
Back extracted[e]	0.9997 ± 0.0065	–	–	–
Saponification with EP[f]	1.8570 ± 0.0156	1.80 ± 0.29	14.25 ± 0.03	1.9150 ± 0.0160
Saponification with MB[g]	1.9150 ± 0.0095	1.54 ± 0.07	18.27 ± 0.01	1.9610 ± 0.0080
Saponification with toluene[f]	1.8876 ± 0.1010	–	–	–
Saponification with MC[f]	1.8987 ± 0.0280	–	–	–
Saponification with TCE alone[f]	1.9479 ± 0.0110	–	–	–

For all treatments, n = 4. Data are representative of several experiments, mean ± SD.
[a]Calculated standard ratio of chol:CME as defined in Methods, also represents the amount of cholesterol in the sample.
[b]Percentage of extra peak as determined by integrator HP 3396A.
[c]Corrected calculation of concentration of cholesterol in sample by summing the extra peak and chol peak area %s as related to the CME area %.
[d]Direct sample injection were those samples that were dried, resolubilized in hexane, and directly injected into GLC.
[e]Back extracted samples were those samples that underwent the procedure described in Methods. Samples are not saponified.
[f]Saponification as described in Methods, except ethyl propionate (EP), toluene, methylene chloride (MC), or tetrachloroethylene alone (TCE) were substituted for methyl butyrate (MB) in extraction mixture.
[g]Saponification as described in Methods using methyl butyrate (MB) in extraction mixture.

jected directly into the GLC without any treatment. These data are within range of the values we obtained on a daily basis as the column standard ratio (see Methods for calculation). The second set of data represents those samples that underwent the back extraction procedure. The next two sets of data are derived from the similar samples that were saponified by either the original Ishikawa procedure (using methyl butyrate in extraction mixture) or the modified version of the same procedure (using ethyl propionate in place of methyl butyrate). In the saponified lipid samples we see an additional peak which comprises 1–2% of the sample. Interestingly, the retention time of the third peak ($extra_{pk}$) changed depending on whether the post-saponification extraction was performed with methyl butyrate (rt = 18 min) or ethyl propionate (rt = 14 min). The data from this experiment also show that, when cholesterol mass is calculated without considering the third peak, there appears to be a small fractional loss of cholesterol. Calculations that take the third peak into consideration ($ratio_{corr}$) are significantly closer to the ratio of 2 (in saponifying, the CME–chol–CO mixture should give a ratio of 2:1 total cholesterol:CME). These data supported our conclusions that the third peak was a byproduct of the saponification procedure, and thus should be included when calculating total cholesterol mass.

Based on the observed differences in retention times of the third peaks obtained when the post-saponification samples were extracted with either methyl butyrate or ethyl propionate, we hypothesized that the peaks were cholesteryl butyrate and cholesteryl propionate, formed by transesterification with the butyrate and propionate ester solvents, respectively. Thus, standard solutions of these cholesteryl esters were subjected to GLC. Chromatographic analysis of a standard containing CME–chol–cholesteryl propionate (1:1:1) (mass/mass/mass) demonstrated a retention time for cholesteryl propionate to be 14.175 min., identical to the retention time of the third peak obtained when ethyl propionate was used (data not shown). Chromatographic analysis for the standard comprised of CME–chol–cholesteryl butyrate (1:1:1) (mass/mass/mass), showed the retention time of cholesteryl butyrate to be 18.168 min, identical to that observed for the third peak when methyl butyrate was used (data not shown).

To verify that the byproducts formed during the microsaponification procedure were cholesteryl butyrate and cholesterol propionate, we performed GC/MS analysis on authentic standards and saponified cell extracts. **Figure 2A** shows the total ion chromatogram for a standard mixture of cholesterol–CME–cholesteryl propionate (1:1:1) (mass/mass/mass). The ion scan for cholesteryl propionate, which resolved at approximately 20 min, is shown on the right, demonstrating the ion fragmentation pattern. We find an absence of the parent ion (M+), which is sometimes characteristic of electron impact GC/MS when analyzing sterol compounds (10). The most abundant ion fragment found when analyzing the standard was found to be 57 (100%; $CH_3CH_2CO^+$), which is most likely derived from the fragmentation of the propionic ester side chain. Another prominent ion, 368 (93%; M + $-CH_3CH_2CH_2COOH$) can be explained by the cleavage of the ester side chain as well as the removal of oxygen at the 3β-position on the A ring of the cholesterol molecule. Shown in Fig. 2B is the total ion chromatogram for the saponified CHO 25-RA lipid extract after extraction with the ethyl propionate mixture. The additional peak, or byproduct of the extraction mixture, resolved at a similar time as cholesteryl propionate in Fig. 2A, approximately 20 min. The mass spectrum behavior of the cellular saponified lipid source was

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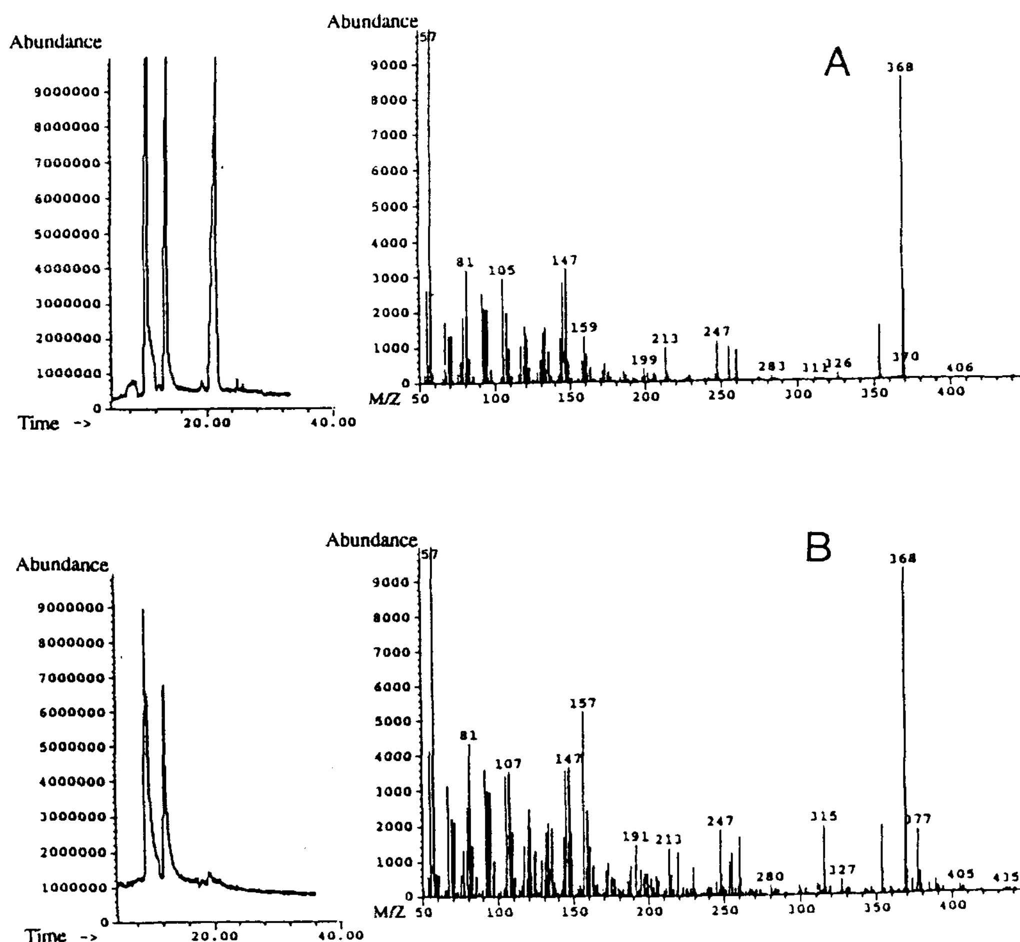

Fig. 2. GC/MS total ion chromatogram and ion scan of (A) standard containing CME–chol–cholesteryl propionate 1:1:1 and (B) cellular saponified lipid byproduct formed during extraction with ethyl propionate. Samples were analyzed by GC/MS as described in Experimental Procedures. For electron impact analysis, performed on a Hewlett-Packard Model 5890 Series II GC equipped with a 5971A mass selective detector, the voltage was set at 70 v. Conditions were column: fused silica (20 m × 0.25 mm ID) coated with 50% phenyl, 50% methyl silicone (DB-17); injector temperature: 300°C; interface temperature: 280°C; and constant temperature for separation: 265°C. Total ion chromatograms were run in the ion range of 50–500 atomic mass units and mass spectra depicted are for (A) cholesteryl propionate and (B) byproduct formed during extraction using ethyl propionate.

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found to be nearly identical to the cholesteryl propionate. Ion extraction of 315 *m/z* and 377 *m/z* across cellular chromatogram did not indicate abundance above baseline. Statistical analysis comparing the ten most prominent ions found the two samples (Fig. 2A and B) to match at 90%. These data demonstrate that the additional peak produced when extracting with ethyl propionate is a byproduct and can be identified as cholesteryl propionate. Similar studies were done analyzing cholesteryl butyrate standard and saponified cellular extract samples after extraction with methyl butyrate. In these studies, no parent ion was detected. The most abundant ion fragment was 368 (100%; M + $-CH_3CH_2CH_2COOH$), where the second most abundant fragment was found to be 71 (54%; $CH_3CH_2CH_2CHO^+$), representing the butryl side chain (data not shown). Comparison demonstrated that the cellular saponified lipid sample byproduct matched the cholesteryl butyrate standard within 93%. From these data, we concluded that the byproduct formed during the saponification procedure that used methyl butyrate as the extracting solvent was cholesteryl butyrate.

In the interest of eliminating the formation of these byproducts, we compared the outcomes of substituting three different solvents: toluene, methylene chloride, or tetrachloroethylene to that obtained with ethyl propionate (control) in the extraction mixture. As seen in Table 2, the artifactual third peak was eliminated in all cases. However, with both toluene and methylene chloride, considerable variation in the peak ratios was observed. In addition, the use of toluene led to a large solvent front which affected the reliability of peak integration (data not shown). Use of tetrachloroethylene alone gave highly reproducible ratios of cholesterol to CME and

more accurate determinations of cholesterol mass.

As the generation of an artifactual third peak is a common occurrence in our hands, using either methyl butyrate or ethyl propionate in the post-saponification extraction, one might question why this was not seen in the original work of Ishikawa et al. (1). There are several procedural differences. Their experiments used packed columns exclusively, although in our hands, extra peaks were observed using a packed column (data not shown). The duration of the chromatographic analysis also appears to be different. Chromatograms presented in that paper are extended only to 10 min, which is not sufficient to reveal the cholesteryl butyrate peak at 18 min. This could potentially introduce another source of error, as the additional mass might be eluted in subsequent GLC runs. Another possible difference relates to the mass of cholesterol in the samples. In the plasma samples studied by Ishikawa et al. much larger masses of cholesterol are subjected to the saponification procedure. In our case with cell extracts, extremely small masses of cholesterol are used. Although we have not made a systematic comparison, it is possible that the formation of a small amount of byproduct might not have been sufficient to affect the outcome of the determination of total cholesterol content in samples containing relatively large amounts of cholesterol.

Classical methods for saponification and extraction of cholesterol samples for GLC do not lead to the production of artifactual byproducts. However, these methods are time consuming, requiring multiple extractions, are not well suited to accurate analysis of samples containing very small amounts of cholesterol, and often use large amounts of highly flammable solvents. The method of Ishikawa et al. (1) provided an important improvement in this regard and has been widely used for analysis of samples from cultured cells. The modification of the Ishikawa procedure presented here improves the quantitation of the free and esterified cholesterol contents of extracts from cultured cells. Using only tetrachloroethylene for extraction after saponification eliminates the potential for formation of byproducts and the necessity for including the peak areas generated by these byproducts in the calculation of total cholesterol mass. Because esterified cholesterol mass is a calculated value derived by subtracting the mass of cholesterol determined in a sample that was not saponified from the mass of cholesterol in an identical sample that was saponified, formation of a byproduct is likely to have the most impact in samples that have small amounts of esterified cholesterol. Thus, elimination of the byproduct was particularly important for our studies of cellular cholesterol metabolism, which often include analysis of very small amounts of cellular lipid that contain highly variable amounts of esterified cholesterol.

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