Measurement of Cholesterol in Serum by Gas Chromatography/Mass Spectrometry at Moderate Mass Resolution, with a Nonendogenous Cholesterol Isomer as Internal Standard

Michael Kinter,1 David A. Herold,1 Judy Hundley,1 Michael R. Willis,1,2 and John Savory1,2

We describe a gas chromatography/mass spectrometry method for the quantitative analysis of cholesterol in serum. A structural isomer of cholesterol, 7,(5a)-cholesten-3β-ol, is used as an internal standard, its primary advantage being its lesser cost relative to that of a stable-isotope-labeled analog. Analysis of the National Bureau of Standards Certified Reference Serum (SRM 909) was used to validate the method. The results show this method to be highly accurate (bias = -0.6%) and precise (CV = 1.6% between-run, 1.2% within-run). The performance of this method is, therefore, sufficiently good to allow its use as a reference method for determinations of cholesterol in serum.

The concentration of cholesterol in serum is currently recognized as an important risk factor in atherosclerosis and coronary heart disease. A Consensus Development Conference of the National Heart, Lung, and Blood Institute and the National Institutes of Health has recommended intensive dietary treatment of individuals whose cholesterol concentrations exceed the 75th percentile (defined as moderate risk) of that individual's age and sex cohort, with individuals whose concentrations exceed the 90th percentile (defined as high risk) receiving both diet and drug therapy (1). The cholesterol values used in assigning these age-dependent risk groups were obtained by using Lipid Research Center methodologies based on the Abell–Kendall colorimetric method of measuring cholesterol in serum (2, 3).

However, values determined by the enzymatic methods generally used in routine clinical assays do not always compare well with values determined by the more rigorous Abell–Kendall method (4, 5). For example, in a recent survey by the College of American Pathologists, serum specimens with cholesterol concentrations of 208 mg/dL and 3057 mg/dL, as determined by the Clinical Chemistry Standardization Section of the Centers for Disease Control (Atlanta, GA), using Lipid Research Center methods, were sent to >4500 laboratories. The average concentrations reported, determined by enzymatic methods and grouped by instrument manufacturer, ranged from 2468 mg/dL (n = 117, SD = 138 mg/dL) to 2841 mg/dL (n = 37, SD = 185 mg/dL) for the former sample and from 2876 mg/dL (n = 117, SD = 37, SD = 185 mg/dL) to 3259 mg/dL (n = 37, SD = 208 mg/dL) for the latter (6). Interestingly, some experts attribute such interlaboratory variation not to the instruments or the methods themselves, but rather to the standards and controls used for calibration and quality assurance (5). Some of these materials have values assigned by methods not traceable to the methods of the Lipid Research Centers. Obviously, it is important to be able to standardize the methods used routinely in clinical chemistry laboratories to the methods of the Lipid Research Centers with which the treatment criteria were set.

In response to this problem, the Laboratory Standardization Panel of the National Cholesterol Education Program of the National Institutes of Health recommends, as a national goal, that clinical laboratories achieve an overall precision and accuracy of cholesterol quantification consistent with a CV and bias of ≤5% initially, and that a CV and bias of ≤3% be achieved within five years. In addition, the National Committee for Clinical Laboratory Standards, which manages a national reference system (7), has established guidelines for the development and acceptance of definitive and reference methods (8, 9). By these criteria, an assay based on isotope-dilution/mass-spectrometry has been accepted as the Definitive Method for cholesterol quantification and a modified Abell–Kendall assay has been accepted as the Reference Method for measurements of cholesterol in serum (10, 11). There is, however, a continued need for the widespread availability of reference methods, particularly in light of questions concerning calibration and quality-control materials. Ideally, such methods would provide for local monitoring of assay performance through correlation experiments. The exact approaches used for these methods might vary from laboratory to laboratory, depending on the resources and abilities of a given laboratory, but methods based on physical rather than biochemical properties are preferred.

We have developed a gas-chromatographic/mass-spectrometric (GC/MS) procedure for the measurement of cholesterol in serum with a structural isomer, 7,(5a)-cholesten-3β-ol (7-cholesterol), as the internal standard.4 We chose such a standard to overcome the cost of stable-isotope-labeled internal standards. In addition, the method we report here involves GC/MS measurements made at moderately high mass resolution (R = 5000); this enhances selectivity and ensures an interference-free assay.

Materials and Methods

Reagents and standards. The cholesterol Standard Reference Material (SRM 911a) and Certified Human Serum (SRM 909) were obtained from the National Bureau of Standards (NBS, Gaithersburg, MD). 7,(5a)-Cholesterol-3β-ol was obtained from Steraloids, Inc., Wilson, NH. Reagents used in the hydrolysis, extraction, and derivatization are as follows: absolute ethyl alcohol; "HPLC-grade" hexane, methanol, and methylene chloride; and "reagent-grade" potassium hydroxide (all from Aldrich Chemical Co., Milwaukee, WI) and N,O-bis(trimethylsilyl) trifluoroacetamide containing trimethylchlorosilane, 10 g/L (Pierce Chemical Co., Rockford, IL).

Preparation of Certified Serum. The lyophilized SRM 909 was reconstituted according to NBS procedure A. The pool used has a NBS-assigned concentration of 1431 mg/L.

4 Nonstandard abbreviations: GC/MS, gas chromatography/mass spectrometry; SRM, Standard Reference Material; 7-cholesterol, 7,(5a)-cholesten-3β-ol; NBS, National Bureau of Standards.
Preparation of standards. SRM cholesterol and 7-cholesterol were dried thoroughly in a desiccator, under reduced pressure. We prepared standard solutions by dissolving 105.7 mg of cholesterol in ethanol in a calibrated 50.00-mL volumetric flask and 204.9 mg of 7-cholesterol in ethanol in a 100.0-mL volumetric flask. The standards were then stored at 4 °C until use.

Sample preparation. For each sample, pipet 500 μL of serum and 500 μL of the internal standard solution, with an automatic pipettor, into a centrifuge tube and hydrolyze the sample in alcoholic KOH (0.6 mL of 8.9 mol/L KOH in 5 mL of ethanol) at 37 °C for 3 h. Then extract the sample with 10 mL of hexane, evaporate the hexane, and reconstitute the residue in 5 mL of methanol. Transfer 100 μL of the methanol solution into a reaction vial and evaporate for derivatization. Add 100 μL of the N,O-bis(trimethylsilyl)trifluoroacetamide containing trimethylchlorosilane, and 100 μL of methylene chloride to the residue and silylate the sample for 0.5 h at 60 °C. Again evaporate the solvents and reconstitute with 500 μL of tetradeacne. Also prepare a 2114 mg/L standard for each analysis set by pipetting 500 μL of the standard cholesterol solution and 500 μL of the internal standard solution into a centrifuge tube, evaporate, and reconstitute the standard in 5 mL of methanol; from this, remove 100 μL for derivatization.

GC/MS. We used a double-focusing, reverse-geometry mass spectrometer (Model 8230; Finnigan MAT, San Jose, CA) with a SpectroSystem 300 data system. The instrument was operated in the electron-ionization mode with 100-eV electrons. Resolution, defined by M/ΔM at 10% valley, was 5000. Data were acquired by selected-ion monitoring of m/z 458.394, at a rate of 4 Hz, yielding approximately 25 data points per chromatographic peak. The DB-1 bonded-phase fused silica capillary column (J & W Scientific, Folsom, CA) was 30 m × 0.32 mm. One microliter of the sample was injected onto the column at 200 °C, after which the temperature was increased at 20 °C/min to 300 °C.

Calculations. We analyzed, in triplicate, duplicate preparations of the Certified Human Serum and the 2114 mg/L cholesterol standard. Ratios were determined by integrating the areas of the cholesterol and 7-cholesterol peaks. We calculated a response factor from the average of the three ratio determinations for the 2114 mg/L standard, then used this response factor to calculate the cholesterol concentration for the individual serum analyses. No determinations were excluded at this stage of the analysis; the following reported concentrations in serum for each serum preparation are thus the average of those three determinations.

Results

The 100-eV electron-ionization mass spectra of cholesterol and 7-cholesterol are shown in Figure 1. As can be seen, a molecular ion is observed for each compound; therefore, we chose to monitor this ion. The chromatographic separation of these compounds is shown in Figure 2, a typical chromatogram obtained in the analysis of the Certified Human Serum. This figure also shows that the intensity of the signal obtained under these conditions is large enough to provide good counting statistics; the fact that no additional chromatographic peaks are observed indicates good selectivity. As shown in Figure 3, the area ratios vary linearly with

Fig. 1. 100-eV electron ionization mass spectra and structures of (a) cholesterol and (b) 7,5a1-cholesten-3α-ol (7-cholesterol)

Fig. 2. A typical selected-ion-monitoring chromatogram for the analysis for cholesterol in serum

Fig. 3. Calibration curve for the GC/MS analysis of serum cholesterol, with 7-cholesterol as an internal standard
cholesterol concentration over a wide range of values (0–10,000 mg/L). This linearity allows calculation of the concentration in a sample by using a response factor calculated from the assay of a single standard.

Table 1 lists concentration values for the Certified Human Serum as determined with this method in eight consecutive analyses. Accuracy of the method appears to be excellent, the average concentration of 1413 mg/L indicating a bias of −1.3%. Between-run precision (SD = 37 mg/L, CV = 2.6%) approaches the target precision of 2.5% specified for a Reference Method for serum cholesterol (7). Within-run precision, estimated from the average of the ranges for the duplicate determinations, is also acceptable: SD = 20 mg/L, CV = 1.5%. Excluding data set number 7 as an outlier improves the performance statistics considerably: the average concentration of 1423 mg/L indicates a bias of −0.6%, and between-run precision (SD = 22 mg/L, CV = 1.6%) and within-run precision (SD = 17 mg/L, CV = 1.2%) are improved.

Discussion

As Figure 1 shows, 7-cholestenol differs structurally from cholesterol only in the position of the double bond, which is the 7 position in 7-cholestenol and in the 5 position in cholesterol. The chemical behavior of these compounds through the rather simple hydrolysis, extraction, and derivatization procedures would, therefore, be nearly identical, yet the chromatographic separation in the measurement step is easily obtained (Figure 2).

Although 7-cholestenol is a metabolic precursor of cholesterol, we detected no endogenous 7-cholestenol in the several blood specimens we examined (n = 10). However, an extremely rare metabolic dysfunction, cerebroretinoid xanthomatosis, is characterized by the accumulation of cholesterol precursors in tissue, bile, and, presumably, in blood (12). This disorder is rare, so the utility of the present method should not be lessened. As a phototransformation product of cholesterol, 7-cholestenol may be present in some commercially available cholesterol (13, 14). It is not, however, present in the NBS Standard Reference Material SRM 911a, which should be the primary standard for reference-type determinations of cholesterol.

The primary advantage of using this isomer rather than an isotopically labeled cholesterol analog for an internal standard is cost. (13)C3)Cholesterol, only recently available, currently costs about $25,000/g. Because precision is best when the standard is added at a concentration approximately equal to that of the analyte, one would need to add 1 mg of the labeled standard to a 0.5-mL serum sample, at a cost of $25 per sample. Smaller sample volumes could be used, but the precision and accuracy of sampling such small volumes would be questionable. The 7-cholestenol isomer, on the other hand, costs approximately $370 per g; the cost of adding 1 mg to a 0.5-mL sample is thus $0.37 per sample. In addition, the initial cost of purchasing even a modest amount of standard and the cost of wasted, outdated, or contaminated standard are much higher for the isotopically labeled compound than for the isomer. The high cost of the stable isotopically labeled standard is easily justified in a Definitive Method; however, to be of any use, a Reference Method should be more frequently applied and more cost effective.

Other investigators have used other compounds as internal standards in GC-based cholesterol assays (15). 7-Cholesterol offers two primary advantages over those compounds for GC/MS analyses. The similarity in structure and chemistry between cholesterol 7-cholestenol effectively minimizes differences in extraction and derivatization efficiency. Moreover, because the analyte and the internal standard have exactly the same mass, there is no need for extraneous switching between masses in the data acquisition.

The performance characteristics of this assay are within the goals established for a serum cholesterol reference method (7, 9). In fact, with the exclusion of the outlier, the accuracy and precision data are excellent. Good accuracy was to be expected because of the high molecular specificity of gas-chromatography/high-resolution mass-spectrometry. This specificity ensures an interference-free assay and eliminates biological matrix interferences as a limitation to accuracy. The CV of approximately 1.5% would appear to approach the lower limit of precision for this type of assay, i.e., a GC/MS assay in which the analyte and the standard are chromatographically separated. It very nearly represents the sum of the sampling precision (separation and standard, 0.3–0.5%) and measurement precision (replicate injections of the same sample, 1.0–1.5%).

In conclusion: A GC/MS method for the measurement of cholesterol in serum, with a structural isomer as an internal standard, has been developed. It has the accuracy and precision characteristics of a Reference Method, it is cost effective, and it is specific. This assay should allow establishment of reference assays of cholesterol in laboratories with high-resolution mass spectrometry capabilities and could, therefore, lead to the wider availability of Reference Methods for cholesterol.

Funding for the purchase of the high-resolution mass spectrometer was obtained from the National Institutes of Health, Division of Research Resources Shared Instrumentation Grant Program, grant no. 1-S10-RR-2418-01. Additional funding supporting this research, from the John Lee Pratt Fund of the University of Virginia, is also gratefully acknowledged.

References