Serum Cholesterol Determined by Liquid Chromatography with 6-Chlorostigmasterol as Internal Standard

Wen Xiang Chen,1 Pei Ying Li, Shu Wang, Jun Dong, and Jian Zhai Li

We describe an accurate and precise method for determining serum cholesterol by high-performance liquid chromatography (HPLC). After addition of 6-chlorostigmasterol as internal standard, serum is treated with alcoholic potassium hydroxide. Subsequently the cholesterol and internal standard are extracted from the mixture into n-hexane and then derivatized to phenylurethanes for measurement by HPLC with ultraviolet detection. The effective chromatographic separation and the use of an appropriate internal standard make this procedure free from interferences by other sterols and precise. The mean cholesterol concentration in Standard Reference Material (SRM) 909 (human serum) assayed by this procedure (4.346 mmol·L⁻¹) agreed well with the value assigned by the National Institute of Standards and Technology (4.359 mmol·L⁻¹). Within-run and total CVs were 0.56% and 0.78%, respectively. Therefore the performance of this procedure is sufficiently good to allow its use as a candidate reference method for serum cholesterol determination.

Indexing Terms: candidate reference method • noncholesterol sterols • hexane-saturated mobile phase • standardization

Increased concentrations of serum cholesterol are recognized as an important risk factor for athero-sclerotic cardiovascular diseases. Although hypercholesterolemia and coronary heart disease are not as prevalent in China as in the Western countries, steady increases in serum cholesterol and the incidence of coronary heart disease have been observed in the last two decades (1, 2). Because there is not yet a national standardization and quality-control program for serum cholesterol determination in China, the performances of serum cholesterol measurements in clinical laboratories are inadequate to meet the present demands in collaborative populational studies and in clinical evaluations. In response to this problem, we began a study on the standardization of serum cholesterol determination. Part of the study was to establish an accurate and precise method that could be easily transferred to reference (or standardization) laboratories for the evaluation and monitoring of routine cholesterol methods and the assignment of the target value of cholesterol reference sera.

The most widely accepted Reference Method for serum cholesterol measurement is the optimized Abell–Levy–Brodie–Kendall (ALBK) method, as described by the US Centers for Disease Control (CDC) (3, 4). This procedure has been used as the Reference Method in our laboratory and a few other laboratories in China for many years. However, this colorimetric method has some inherent problems. It needs to be performed very carefully with high-quality liquid-delivery systems, because most of the deliveries of liquid volumes and the manipulations are critical to the precision of the results. Many laboratories in China can hardly perform this method exactly according to the procedure proposed by CDC, owing to technological and instrumental limitations. In addition, this method may be susceptible to interferences by noncholesterol sterols in serum, though these interferences are generally small and relatively constant, regardless of the serum matrix (4, 5).

Several methods for measuring serum cholesterol by gas chromatography/mass spectrometry have been published (6–10), in addition to the Definitive Method (isotope dilution/gas chromatography/mass spectrometry method) of the National Institute of Standards and Technology (NIST) (11, 12). Their accuracy and precision are sufficient to allow their use as Reference Methods. However, they require expensive instrumentation and, in most cases, stable-isotope-labeled cholesterol as internal standard. High-performance liquid chromatography (HPLC) is relatively inexpensive and convenient and has also been used for serum cholesterol measurement (13–16); however, these HPLC methods were developed mostly for purposes other than use as Reference Methods and their performances could not meet our requirements.

Here we describe a novel HPLC method for determining serum cholesterol. It is free of interferences, performs with good precision, uses widely available equipment, and is low in cost.

Materials and Methods

Reagents. Cholesterol Standard Reference Material (SRM) 911b obtained from NIST (Gaithersburg, MD) was used as the standard. Its purity was certified to be 99.8% ± 0.1%. Desmosterol, ergosterol, 7-dehydrocholesterol, lathosterol, campesterol, epicoprostanol, cholesterol, and β-sitosterol used in the optimization of chromatographic conditions and the estimation of noncholesterol sterols in serum were obtained from Sigma Chemical Co. (St. Louis, MO). The derivatization agent

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phenylisocyanate (purity >98%) was obtained from Aldrich Chemical Co. (Milwaukee, WI) and stored at 4 °C in ampouled aliquots for everyday use. Other chemicals used in the internal standard synthesis, hydrolysis, extraction, derivatization, and chromatography were as follows: stigmasterol (Merck, Darmstadt, Germany); iso-dobenzene (Shanghai Chemical Reagent Purchase and Supply Station, Shanghai, P.R. China); HPLC-grade acetonitrile (Huangyan Experimental Factory of Chemicals, Zhejiang, P.R. China); and AR-grade anhydrous ethanol, n-hexane, potassium hydroxide, pyridine, chloroform, carbon tetrachloride, manganese dioxide, hydrochloric acid, and benzyl chloride (Beijing Chemical Plant, Beijing, P.R. China).

Serum samples. Lyophilized human serum SRM 909 was obtained from NIST. Other samples used in this study were pooled fresh sera from clinical laboratories.

Preparation of calibrators. We dissolved 2.000 g of dry SRM 911b in ethanol by gentle warming and added ethanol to a final volume of 200.0 mL at 20 °C. This solution was diluted with ethanol for the preparation of working calibrator solutions of 1.293 (500), 2.586 (1000), 5.172 (2000), 7.758 (3000), 10.34 (4000), and 12.93 (5000) mmol/L (mg/L). These calibrators were stored in ampoules at 4 °C.

Synthesis of 6-chlorostigmasterol and preparation of internal standard solution. 6-Chlorostigmasterol was synthesized from stigmasterol as described by Berg and Wallis (17) and Barton and Miller (18). We passed chloroform, which was prepared by reacting manganese dioxide with hydrochloric acid, into iso-dobenzene in dry chloroform until iso-dobenzene dichloride precipitated (19). Equivalent moles of iso-dobenzene dichloride and stigmasterol benzoate (prepared by mixing stigmasterol and benzyl chloride in pyridine) were mixed in dry carbon tetrachloride. This mixture was then refluxed for 20 min and the carbon tetrachloride was evaporated. We obtained 6-chlorostigmasterol by hydrolyzing the 5α, 6α-dichlorostigmasteryl benzoate formed with excess alcoholic potassium hydroxide, and further purified it by recrystallization in acetonitrile/chloroform. Such substitutional derivatives of other sterols with Δ5-configuration could be prepared in a similar manner.

We dissolved 232 mg of 6-chlorostigmasterol in 100 mL of ethanol and further diluted this solution with ethanol to prepare an internal standard solution of 0.52 mmol/L (232 mg/L).

Sample preparation. Pipet 0.50 mL of serum samples or standard solutions and 5.0 mL of internal standard solution to a series of 30-mL stopped tubes with calibrated pipets. Add 0.6 mL of 8.9 mol/L aqueous potassium hydroxide solution to each tube and mix gently. Incubate the tubes at 37 °C for 3 h. Add 5 mL of water and 10 mL of n-hexane to each tube and vortex-mix 3 times for 10 s each. Wait until the phases separate. Pipet 0.5 mL of the n-hexane layer into a 5-mL stopped tube and evaporate to dryness under a nitrogen stream.

Add 20 μL of pyridine and 20 μL of phenylisocyanate to each of the above residues and mix. Allow the tubes to stay at room temperature for 10 min. Add 1 mL of ethanol and 1 mL of n-hexane and mix. Add 1 mL of water and vortex-mix 3 times, 10 s each. Allow the phases to separate, then transfer 50 μL of the n-hexane layer to a 5 × 50 mm tube and evaporate to dryness under reduced pressure. Dissolve the residue in 100 μL of acetonitrile for HPLC measurement.

HPLC measurement and calculation. The HPLC system used in this study consisted of a Model 510 pump, a U6K injector, a Pico-Tag column (3.9 × 150 mm), a Model 490 ultraviolet detector operating at 235 nm and 0.05 A full-scale, and a Model 740 data module with an attenuation set at 128 mV (Waters Chromatography Div., Millipore Corp., Milford, MA). The mobile phase was acetonitrile/water (88/12 by vol) saturated with n-hexane. We performed our analysis with a flow rate of 1 mL/min at room temperature. The preparation of the mobile phase was as follows. Acetonitrile and water in the indicated proportion were mixed and warmed to room temperature. The mixed solvent was shaken with excess n-hexane and filtered through a 0.45-μm pore-size filter. After the complete separation of the phases, the n-hexane layer was removed and the bottom layer was used as mobile phase. The column was first flushed with pure acetonitrile for 10 min and then equilibrated with the mobile phase until a steady baseline appeared.

We injected 10 μL of the derivatized samples or calibrators onto the column. Peak area ratios of cholesterol to internal standard for the six calibrators were linearly regressed on the corresponding cholesterol concentrations (1.293–12.93 mmol/L) and the resulting regression equation was used to calculate the serum cholesterol concentrations.

Results

HPLC separation of cholesterol. Small amounts of noncholesterol sterols, mostly desmosterol, lathosterol, campesterol, cholestanol, and β-sitosterol, have been demonstrated in human serum (20, 21). Figure 1A shows the separation of cholesterol and the internal standard from these sterols (as their phenylurethanes). Table 1 summarizes the retention times of 11 sterols. None had a retention time identical with cholesterol or the internal standard. To check the profile of serum sterols under our chromatographic conditions, we prepared a serum sample without adding internal standard and injected it onto the column. The injected amount was much larger than that for ordinary analysis. As shown in Figure 1B, the small peaks evident (nos. 1, 4, 6, 7, and 8) had retention times identical with those of the major serum noncholesterol sterols mentioned above, respectively, and were well resolved from cholesterol. No detectable serum constituent had a retention time identical with that of the internal standard.

Validity of the internal standard selection. The appropriate chromatographic behavior of the internal standard has been illustrated in Figure 1. It was completely separated from cholesterol and other substances in serum, and had a retention time slightly shorter than cholesterol. Aside from a proper chromatographic behavior, an internal standard should have physical and
chemical properties very similar to cholesterol, so that its behaviors in the rather simple extraction and derivatization procedures would be nearly identical with that of cholesterol. To investigate this, we prepared an equimolar mixture of cholesterol and 6-chlorostigmasterol in ethanol. Ten equal portions of the mixture were subjected to the whole sample preparation procedure. Each of the 10 specimens was injected onto the column and their peak-area ratios were measured. The CV of the 10 peak-area ratios was 0.41%. Deducing the contribution of the variation of the HPLC measurements, which was obtained by injecting one specimen repeatedly 10 times, we determined that the variation derived from the sample preparation was 0.27% (Table 2). These results suggest that 6-chlorostigmasterol behaves satisfactorily as an internal standard in cholesterol analysis.

**Linearity.** The linear correlation coefficients between cholesterol concentrations (1.293–12.93 mmol/L) and peak-area ratios of cholesterol to internal standard in eight independent runs were 0.99995–0.99998. The mean slope and intercept of the regression equations were 0.1485 and 0.0215, respectively, with respective CVs of 0.92% and 15.3%.

**Precision.** We analyzed three serum pools, each of different cholesterol concentrations, to study the reproducibility of the HPLC method. Each pool was analyzed six times in three or four replicates. Within-run and total CVs were 0.56% and 0.78%, respectively (Table 3).

**Analytical recovery.** We added 0.50 mL of the cholesterol calibrators 2.586 mmol/L (1000 mg/L) and 5.172 mmol/L (2000 mg/L) to 0.50-mL aliquots of serum pool C (n = 4 for each of the two cholesterol standards). Cholesterol concentrations of the supplemented serum aliquots were analyzed with the HPLC method. The analytical recoveries of added cholesterol at the two concentrations were 99.96% and 99.73%, respectively.

**Analysis of SRM 909.** Two vials of SRM 909 were reconstituted by weight according to procedure A in the SRM insert. To minimize errors caused by sampling of the sera and standard solutions, we took aliquots of them by weighing. The densities of the sera and calibrators were determined by weighing aliquots of them (n = 6 for each) transferred with calibrated 1.0-mL glass pipets. Three aliquots from each SRM vial were taken

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**Table 1. Retention Times of Sterols under the Selected Chromatographic Conditions**

<table>
<thead>
<tr>
<th>Sterol</th>
<th>Retention time, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desmosterol</td>
<td>6.2</td>
</tr>
<tr>
<td>Ergosterol</td>
<td>6.8</td>
</tr>
<tr>
<td>7-Dehydrocholesterol</td>
<td>7.1</td>
</tr>
<tr>
<td>6-Chlorostigmasterol</td>
<td>8.8</td>
</tr>
<tr>
<td>Lathosterol</td>
<td>10.8</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>11.7</td>
</tr>
<tr>
<td>Campesterol</td>
<td>14.0</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td>14.4</td>
</tr>
<tr>
<td>Epiprostanolet</td>
<td>15.3</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>16.3</td>
</tr>
<tr>
<td>β-Sitosterol</td>
<td>18.2</td>
</tr>
</tbody>
</table>

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**Table 2. Behavioral Similarity (Peak-Area Ratios) of Cholesterol and 6-Chlorostigmasterol during Sample Preparation**

<table>
<thead>
<tr>
<th>Manipulations</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Extractions, derivatization, and HPLC measurement</td>
<td>0.8867</td>
<td>0.0036</td>
<td>0.41</td>
</tr>
<tr>
<td>b. HPLC measurement</td>
<td>0.8807</td>
<td>0.0027</td>
<td>0.31</td>
</tr>
<tr>
<td>c. Extractions and derivatization</td>
<td>0.0024*</td>
<td>0.27</td>
<td></td>
</tr>
</tbody>
</table>

* (SD)c = √[(SD)a2 - (SD)b2].

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**Table 3. Reproducibility of HPLC Determination of Serum Cholesterol**

<table>
<thead>
<tr>
<th>Cholesterol conc, mmol/L</th>
<th>Pool A</th>
<th>Pool B</th>
<th>Pool C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.863</td>
<td>5.433</td>
<td>3.325</td>
</tr>
<tr>
<td>SD (and CV, %)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Within-run</td>
<td>0.030 (0.44)</td>
<td>0.030 (0.55)</td>
<td>0.023 (0.70)</td>
</tr>
<tr>
<td>b. Between-run</td>
<td>0.032 (0.47)</td>
<td>0.042 (0.77)</td>
<td>0.010 (0.30)</td>
</tr>
<tr>
<td>c. Total</td>
<td>0.044 (0.64)</td>
<td>0.052 (0.95)</td>
<td>0.025 (0.75)</td>
</tr>
</tbody>
</table>

* Calculated as follows:

(SD)a = √[(SD)b2 - (SD)c2]

(SD)b = √[(SD)a2 - (SD)c2], n = 4 or 3

(SD)c = √[(SD)a2 + (SD)b2]

where (SD) and (SD)x are standard deviations of the replicates in each run and the means of the six runs, respectively.
for analysis as a set, and three such sets were analyzed independently. Variance analysis showed that within-
run errors were the main component of variation. Neither vial-to-vial nor run-to-run variation contributed signifi-
cantly to overall variation. Therefore, we pooled all the results and calculated the standard deviation as shown in Table 4. The mean value determined by our method (4.346 mmol·g⁻¹·L⁻¹) was very close to the value assigned by NIST (4.359 mmol·g⁻¹·L⁻¹).

**Comparison with the ALBK method.** Thirteen serum pools of different cholesterol concentrations were analyzed in triplicate by both the HPLC method and the ALBK method. The performance of the CDC ALBK method in our laboratory had been verified by analysis of reference sera from CDC. Values obtained by the HPLC method averaged 1.3% lower than those by the ALBK method. Linear regression analysis with the ALBK method results (range, 2.736 to 8.591 mmol/L) as independent variables gave a regression equation of
\[ y = 1.010x - 0.108 \] 
\( r = 0.9996 \), standard error of estimate = 0.042 mmol/L.

**Discussion**

**Method Optimization**

Most of the highly accurate determinations of serum cholesterol are at present performed with isotope dilu-
tion gas chromatography/mass spectrometry. In this technique, most of the sources of imprecision are mini-
mized by using as the internal standard isotopelabeled cholesterol, which has the same physical, chemical, and even chromatographic (under given conditions) character-
istics as cholesterol, the analytical specificity is du-
yally guaranteed by gas chromatography and mass spec-
 trometry. The major problems of the previous HPLC

methods for serum cholesterol determination are: (a) insufficient resolution of cholesterol from other serum constituents in an acceptable time, (b) lack of internal standard or use of internal standards that are insufficiently close to cholesterol in structures and properties, and, consequently, (c) inadequate accuracy and precision. In the development of our HPLC method, we made several attempts to overcome the inherent limitations of HPLC with ultraviolet detection so as to make the

<table>
<thead>
<tr>
<th>Vial</th>
<th>Aliquot</th>
<th>Set 1</th>
<th>Set 2</th>
<th>Set 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>4.345</td>
<td>4.350</td>
<td>4.356</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>4.337</td>
<td>4.347</td>
<td>4.351</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>4.330</td>
<td>4.334</td>
<td>4.339</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>4.341</td>
<td>4.344</td>
<td>4.357</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>4.317</td>
<td>4.366</td>
<td>4.345</td>
</tr>
<tr>
<td>Overall mean</td>
<td>4.358</td>
<td>4.345</td>
<td>4.369</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>0.013</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CV, %</td>
<td>0.29</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Derivatization.** We initially tried an analysis of un-
derivatized serum cholesterol by HPLC with stigmas-
terol as internal standard. However, this procedure had two problems. One was the coelution of cholesterol with lathosterol and of stigmasterol with campesterol; the other was the imprecision of the results. Assuming that the imprecision might be attributed to the low detection wavelength (210 nm) and wanting to improve the reso-

lution of sterols, we incorporated the derivatization into our method and successfully achieved our goals as discussed below.

**Separation of cholesterol.** The addition of n-hexane to the solvent system differentially shortened the retention times of different sterols, depending on their molecular configurations. The sterols were completely sepa-
rated in a relatively short time. It was difficult to achieve such a resolution with ordinary mobile phases consisting of miscible solvents such as tetrahydrofuran, methanol, acetonitrile, water, etc.

We also tested the separation of the sterols on differ-
ent reversed-phase columns with n-hexane-saturated mobile phases and again obtained satisfactory results (Figure 2). This further demonstrates the efficiency of such mobile phases, and adds flexibility to column choices for serum cholesterol measurement. With this

![Figure 2](image-url)

**Fig. 2.** HPLC separations of steryl phenylurethanes on Resolve C₁₈
(5 μm; 3.9 × 150 mm; Waters) (A) and µ-Bondapak C₁₈ (10 μm; 3.9
× 300 mm; Waters) (B) columns

The mobile phases were hexane-saturated acetonitrile/water (93/7, by vol) (A) and acetonitrile/isopropanol (99/1, by vol) (B). Peak 9 is 6-chloro-β-altosteryl phenylurethane. Other chromatographic conditions and peak identities are the same as for Fig. 1.
mobile phase, we have been able to use the Pico-Tag column frequently for >2 years without loss of efficiency.

Selection of internal standard. In chromatographic analysis with detection techniques other than mass spectrometry, an internal standard should be: (a) absent in the sample, (b) similar to the analyte in structure and properties, (c) separated from the analyte and other constituents of the sample, and (d) eluted with a retention time near, but preferably shorter than, that of the analyte. Epicoeprostanol may be one of the few choices among commercially available sterols, because most others could be present in serum. We used epicoeprostanol as internal standard for a long time; unfortunately, however, quite significant differences were observed between epicoeprostanol and cholesterol regarding reactivity with several derivatization agents, and thus the derivatization condition became critical to the precision of the results. Then we synthesized a series of sterol derivatives and evaluated their behaviors under our conditions. We found that the 6-chloro-substituted derivatives of some sterols (stigmasterol, campesterol, δ-sitosterol, and cholesterol) were very useful. They are similar to cholesterol in structure (3β-hydroxyl and 5-ene) and properties (as shown in Table 2) and are stable (vinyl chlorides). Different 6-chlorosterols can be selected for different conditions. For example, 6-chlorostigmasterol was used satisfactorily in the present study, and 6-chloro-δ-sitosterol may be the choice for use with the μ-Bondapak C18 column (Figure 2B). These 6-chlorosterols may also be useful in serum sterol determinations by gas chromatography.

Precision and Accuracy of the HPLC Method

For reasons detailed above, the HPLC method performed with good accuracy and precision. The within-run and total CVs of serum analyses were 0.56% and 0.78%, respectively. Reasonably, when sampling was performed by weighing, as in the analysis of SRM 909, the total CV was reduced to 0.29%.

Imprecision obviously affects accuracy, but good precision does not guarantee accuracy. One of the evidences for the accuracy of the HPLC method is the good analytical recovery. Obviously, other sources of inaccuracy will not be revealed by analytical recovery, including incomplete hydrolysis of serum cholesteryl esters and chromatographic interferences by serum constituents coeluted with cholesterol and the internal standard. However, the first source of inaccuracy can be eliminated, because we used the same hydrolysis condition as in the Definitive Method (10). The second was reduced to a minimum in our method by the complete separation of cholesterol and the internal standard from the predominant noncholesterol sterols in serum as shown in Figure 1. Other noncholesterol sterols are possibly present in human serum, e.g., coprostanol, campesterol, δ-sitosterol, and oxidation products of cholesterol, but at concentrations generally much lower than those of the major sterols mentioned above. Further, the molecular configurations of these minor sterols make them unlikely to be coeluted with cholesterol and the internal standard.

Further evidence of accuracy was obtained by analyzing the NIST SRM 909. Our result (4.346 mmol·L⁻¹) differed by only −0.2% from that of NIST (4.369 mmol·L⁻¹).

Comparison with the ALBK Method

As we expected, when we compared our method with the ALBK method, our results differed by about −1.3%. This may be related to the complete separation of cholesterol and the internal standard from noncholesterol sterols in serum in our method. These sterols have similar structures and properties to cholesterol. They therefore cannot be eliminated in the hydrolysis and extraction procedures and may interfere with cholesterol determination in the ALBK method. To investigate this, we estimated the serum contents of four predominant noncholesterol sterols—lathosterol, campesterol, cholestanol, and δ-sitosterol—by HPLC and studied their responses to Liebermann–Burchard reagents. Mean concentrations of the above sterols in the samples used in the comparison were 4.65, 8.98, 7.30, and 7.47 μmol/L, respectively. Their sum was about 0.6% of the cholesterol concentration. Converted to responses in Liebermann–Burchard reaction, they would cause a positive bias of about 0.5% in the ALBK method. Obviously, there are still more interfering substances in serum extracts. As demonstrated recently by CDC (5), another significant source of interferences in the ALBK method is oxidation products of cholesterol. These oxidation products do not interfere with the cholesterol determination in our method, as discussed above.

In conclusion, a precise and accurate HPLC method for determining serum cholesterol has been developed. It can be easily performed with low cost and less technical efforts in laboratories that have HPLC capabilities. Therefore, a wider application of this method for routine method evaluation and reference serum certification in cholesterol measurements seems justified.

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