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Jones oxidation and high performance liquid chromatographic analysis of cholesterol in biological samples

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Abstract

A simple pre-column derivatization procedure for HPLC analysis of cholesterol in biological samples was developed. Cholesterol was treated with chromic acid and sulfuric acid in acetone (the Jones oxidation) and cholest-4-en-3,6-dione was formed. The reaction was finished in 5 min at room temperature and the product showed a strong UV absorbance at 250 nm that enabled an HPLC detection limit of 0.2 pmol. With stigmasterol as an internal standard, the reaction was applied to the analysis of total and free cholesterol in serum and high-density lipoproteins and the analysis showed a within-run and total coefficient of variation of about 0.2% and 0.5%, respectively.

Keywords: Cholesterol; Cholest-4-en-3,6-dione; Stigmasterol; Chromic acid; HPLC

1. Introduction

Precise analysis of cholesterol is often needed in lipid and lipoprotein studies. HPLC with ultraviolet detection is a simple and precise analytical technique and has been used for the analysis of cholesterol [1–5]. Though cholesterol can be analyzed directly by HPLC [1,2], pre-column derivatizations or conversions are needed for more effective separation and more sensitive detection of cholesterol [3–5]. Chemical acylation [3,4] and enzymatic oxidation [5] have been reported for the analysis of cholesterol in biological sample by HPLC with UV detection.

Cholesterol can also be oxidized by chemical oxidants to give ultraviolet-absorbing products. In organic synthesis, cholesterol has been oxidized by pyridinium dichromate or pyridinium chlorochromate [6–8] to cholest-4-en-3,6-dione that has a maximum absorption wavelength of 250 nm and molar extinction coefficient of about 1.3×10^4. But these reactions take long-time (6–24 h). Another chemical oxidation of alcohols is the Jones oxidation [9] that uses chromic acid, sulfuric acid in acetone as its reagent. This reaction is not only simple but also proceeds rapidly and has also been used for the oxidation of cholesterol to prepare cholest-4-en-3-one and/or cholest-4-en-3,6-dione [10,11].

Prompted by the simplicity of the Jones oxidation, we investigated the applicability of the reaction to HPLC analysis of cholesterol. Our investigation led to the development of a simple procedure for the conversion of cholesterol to cholest-4-en-3,6-dione and subsequently the establishment of a highly precise and sensitive HPLC method for the analysis of cholesterol in biological samples.

2. Experimental

2.1. Materials

Cholesterol (Sigma Grade, standard for chromatography) and stigmasterol were obtained from Sigma (St. Louis, MO, USA). Chromic acid (CrO_3), sulfuric acid and acetone (all analytical grade) were obtained from Beijing Chemical Works (Beijing, China). HPLC-grade acetonitrile, isopropanol and *n*-hexane were products of Fisher Scientific (Pittsburgh, PA, USA).

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A commercial enzymatic reagent for serum total cholesterol (Biosino Biotech, Beijing, China) was used for the enzymatic oxidation of cholesterol.

Serum high-density lipoprotein (HDL) fractions were prepared from pooled serum aliquots by dextrin sulfate–magnesium chloride precipitation [12].

2.2. Equipment

All the HPLC analyses were performed on an HP 1100 HPLC system (Hewlett Packard, Waldbronn, Germany) consisting of an isocratic pump, an autosampler and an ultraviolet detector controlled by the ChemStation. An Agilent 5975/6890N GC/MS system (Agilent Technologies, USA) was used for the structure analysis of the major cholesterol oxidation products, and A MicroLab 500 automatic dilutor (Hamilton, USA) was used for the sampling and the addition of internal standard in the serum and HDL cholesterol analyses.

2.3. Characterization of Jones oxidation of cholesterol

2.3.1. Jones oxidation of cholesterol

A cholesterol solution (5.17 mmol/L) was prepared by dissolving cholesterol in ethanol. Aliquots of 20 μL of the solution were dried under vacuum at 50 °C and the residues were dissolved in 0.4 mL of acetone.

Chromic acid solutions of 4, 2 and 1 mol/L and sulfuric acid solutions of 8 and 4 mol/L were prepared by dissolving the acids in water and equal volumes of the solutions were mixed for the preparation of mixtures with final chromic acid and sulfuric acid concentrations of 2 and 4, 1 and 4, 0.5 and 4, 2 and 2, 1 and 2, and 0.5 and 2 mol/L.

Various amounts (10–40 μL) of the chromic acid–sulfuric acid solutions were added to the cholesterol in acetone solutions and the mixtures were allowed to stand at room temperature for various times (0.5–32 min). After the addition of 0.6 mL of water, the mixtures were extracted with 1 mL of *n*-hexane. The hexane phase was subjected to HPLC or GC/MS analyses.

2.3.2. Enzymatic oxidation of cholesterol

An aqueous cholesterol solution was prepared. Cholesterol (200 mg) was first dissolved in 2 mL of 1,4-dioxane and mixed with 3 g of Triton X-100, and then water was added to a final volume of 100 mL (5.17 mmol/L).

Aliquots of 20 μL of the solution were mixed with 1 mL of the enzymatic cholesterol reagent. The mixture was incubated at 37 °C for 10 min. After the addition of 1 mL of ethanol, the mixture was extracted with 1 mL of *n*-hexane by shaking on a mechanical shaker for 10 min. The hexane phase was used for HPLC analysis or other tests.

2.3.3. HPLC and GC/MS analysis

A Novapak C18 column (5 μm, 3.9 mm × 150 mm) (Waters, USA) was used for the HPLC analysis. The mobile phase was acetonitrile–isopropanol (90:10) and the flow-rate was 1 mL/min. The detection wavelength was either 240 or 250 nm. An aliquot of 0.2 mL of the hexane phase was dried and reconstituted with 0.4 mL of the mobile phase. An aliquot of 10 μL of the solution was injected.

The GC/MS analysis was performed with a HP-5 MS column (30 m × 0.25 mm i.d., 0.25-μm film thickness) (Agilent Technologies) with helium as the carrier gas. The injection port, oven and transfer line temperatures were all 290 °C. An electron impact ion source was used and the electron energy was 70 eV. An aliquot of 1 μL of hexane phase was injected onto the column in splitless mode and the mass spectrometry detection was performed in scan mode with a mass range of *m/z* 100–400.

2.4. Analysis of total and free cholesterol in serum and HDL

2.4.1. Preparation of calibrators and internal standards

A stock solution of 25.86 mmol/L (1000 mg/dL) was prepared by dissolving cholesterol in ethanol. Calibrators of 0.647, 1.293, 2.586, 5.172 and 7.759 mmol/L (25–300 mg/dL) for the analysis of serum and HDL total cholesterol and calibrators of 0.129, 0.259, 0.517, 1.035 and 1.552 mmol/L (5–60 mg/dL) for free cholesterol were prepared by diluting the stock solution with ethanol.

Stigmasterol was used as the internal standard. Internal standards of 5 mmol/L for total cholesterol and 1 mmol/L for free cholesterol were prepared by dissolving stigmasterol in ethanol.

2.4.2. Sample preparation for total cholesterol

The hydrolysis of cholesteryl esters and extraction of cholesterol were performed with conditions similar to those described previously [3]. Briefly, cholesteryl esters were hydrolyzed with alcoholic potassium hydroxide and, after the addition of water, the cholesterol was extracted with hexane. In the present study, however, a higher hydrolysis temperature (50 °C vs. 37 °C) and a shorter time (2 h vs. 3 h) were used, and the sampling and the addition of the internal standard were performed with an automatic dilutor in place of manual pipettes.

With the automatic dilutor, an aliquot 50 μL of serum, HDL or the calibrators was washed into a 2-mL screw-capped vial with 0.5 mL of 8.9 mol/L potassium hydroxide–ethanol (10:90). The mixture was incubated at 50 °C for 2 h and 0.5 mL of water was then added. Also with the dilutor, an aliquot of 50 μL of the internal standard was washed into the vial with 1 mL of hexane. The vial was capped and shaken on a mechanical shaker for 10 min. An aliquot of 0.2 mL of the hexane phase was transferred to another vial and dried under vacuum at 50 °C. The residue was dissolved in 0.4 mL of acetone and 20 μL of a chromic acid–sulfuric acid solution (both 2 mol/L) were added. The mixture was allowed to stand at room temperature for 5 min and then 1 mL of hexane and 0.6 mL of water were added. The vial was shaken for 10 min. An aliquot of 0.2 mL of the supernatant was dried for HPLC analysis.

2.4.3. Sample preparation for free cholesterol analysis

For the extraction of free cholesterol, serum or HDL samples were first mixed with a dilute solution of potassium hydroxide in water–ethanol (50:50) and then extracted with hexane. The

potassium hydroxide water–ethanol solution served as a lipoprotein disrupting agent and a washout solvent in the sampling with the automatic dilutor. Potassium hydroxide was present to make the solution more efficient for the both purposes. Its concentration was much lower, as compared to that used in the total cholesterol analysis, to avoid cholesteryl ester hydrolysis.

With the automatic dilutor, an aliquot of 50 μL of serum, HDL or the calibrators was washed with 0.5 mL of 0.1 mol/L potassium hydroxide–ethanol (50:50), and then 50 μL of the internal standard with 0.5 mL of hexane into a 2-mL vial. The vial was shaken for 10 min. An aliquot of 0.2 mL of the hexane phase was dried and oxidized as for total cholesterol analysis. An aliquot of 0.4 mL of the final hexane phase was dried for HPLC analysis.

2.4.4. HPLC analysis and calculation

The HPLC analysis was the same as described in Section 2.3.3 except that the detection wavelength was 250 nm. Peak area ratios of cholesterol to stigmasterol for the calibrators were linearly regressed on the corresponding cholesterol concentrations and the resulting equation was used to calculate cholesterol concentrations of serum or HDL samples.

2.4.5. Comparison with the Abell-Kendall method

The present HPLC method for total cholesterol was compared with the Abell-Kendall method on 38 patient serum samples. The Abell-Kendall method was performed as by the United States Centers for Disease Control and Prevention (CDC) Cholesterol Reference Method Laboratory Network (CRMLN) [13]. Results obtained with two methods were linearly regressed and biases were calculated.

3. Results and discussion

3.1. Jones oxidation of cholesterol and its applicability to HPLC analysis

3.1.1. Jones oxidation of cholesterol and its products

To test the Jones oxidation of cholesterol, cholesterol in acetone was treated with various chromic acid–sulfuric acid solutions for various times at room temperature. In most cases, at the beginning of the reaction, two major oxidation products (i and ii) were observed as shown in Fig. 1A, and as the reaction proceeded, product (ii) dropped and product (i) rose, then product (i) became the only major product as shown in Fig. 1B.

To identify the two products, the samples of Fig. 1A and B were analyzed by GC/MS. The total ion chromatogram and the mass spectra of two major products in the sample of Fig. 1A are shown in Fig. 2. Spectrum searches were performed with the NIST MS Search 2.0 Program. Peak 1 matched cholest-4-en-3-one with a Match factor of about 850 and a Probability value of about 70. The next probable compound was cholest-5-en-3-one with Match and Probability values of about 750 and 20. Peak 2 matched cholest-4-en-3,6-dione with a Match factor of about 750 and a Probability value of about 85. GC/MS analysis of the sample of Fig. 1B showed a single peak (Peak 2 in Fig. 2A) and its spectrum matched cholest-4-en-3,6-dione with Match and

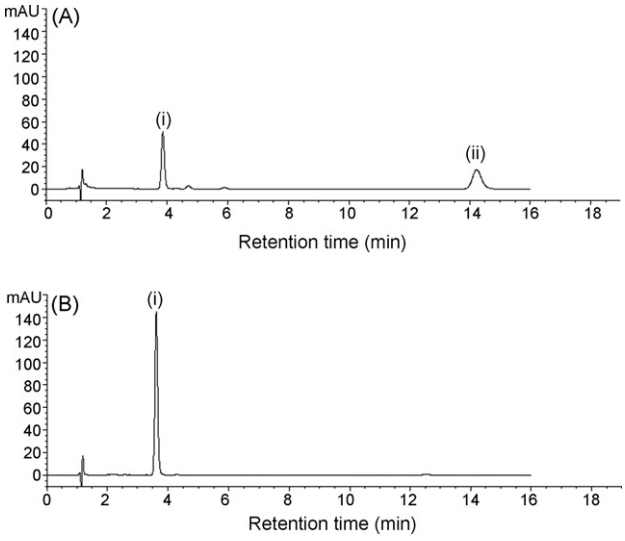

Fig. 1. HPLC chromatograms of cholesterol oxidation products obtained with different oxidation times. About 100 nmol of cholesterol was dissolved in 0.4 mL of acetone and treated with 20 μL of a chromic acid–sulfuric acid (2 and 4 mol/L) solution for 0.5 (A) or 2 (B) min at room temperature. The oxidation products were extracted and chromatographed by HPLC. Chromatographic conditions were as follows: column, NOVA-PAK C18 (5 μm, 3.9 mm × 150 mm); mobile phase, acetonitrile–isopropanol (90:10); flow-rate, 1 mL/min; detection, ultraviolet absorbance at 250 nm. Peaks (i) and (ii) are unidentified cholesterol oxidation products.

Probability values of about 830 and 90 (chromatogram and mass spectrum not shown). HPLC of the two products with different detection wavelengths showed maximum absorption wavelength of 250 nm for product (i) and 240 nm for product (ii). These results would justify identifications of products (i) and (ii) as cholest-4-en-3,6-dione and cholest-4-en-3-one.

HPLC peak areas of cholest-4-en-3,6-dione (detected at 250 nm) and cholest-4-en-3-one (240 nm) obtained with different chromic acid and sulfuric acid concentrations and reaction times are shown in Fig. 3. Both products seemed to undergo first formation and then decomposition during the reaction, but the change for cholest-4-en-3,6-dione was slower. Increases in either chromic acid or sulfuric acid concentrations increased the reaction rate. It was also noted that when chromic acid and sulfuric acid concentrations were both 2 mol/L, little cholest-4-en-3-one was observed.

3.1.2. Formation of the products

As shown in Fig. 3, increases of cholest-4-en-3,6-dione seemed to be accompanied by decreases of cholest-4-en-3-one. This would suggest that cholest-4-en-3-one might be an intermediate via which cholest-4-en-3,6-dione was formed. This mechanism was also proposed by Hector et al. [6] when they oxidized steroidal 5-en-3β-ols to 4-en-3,6-dione with pyridinium dichromate. But Nangia and Anthony [7] and Li and Li [8] suggested that the 4-en-3,6-dione was formed through the oxidation of the 5-en-3-one rather than the 4-en-3-one in the oxidations of the sterols with pyridinium dichromate or pyridinium chlorochromate as the oxidants.

We tried our Jones oxidation on cholest-4-en-3-one, which was prepared by the enzymatic oxidation of cholesterol, and

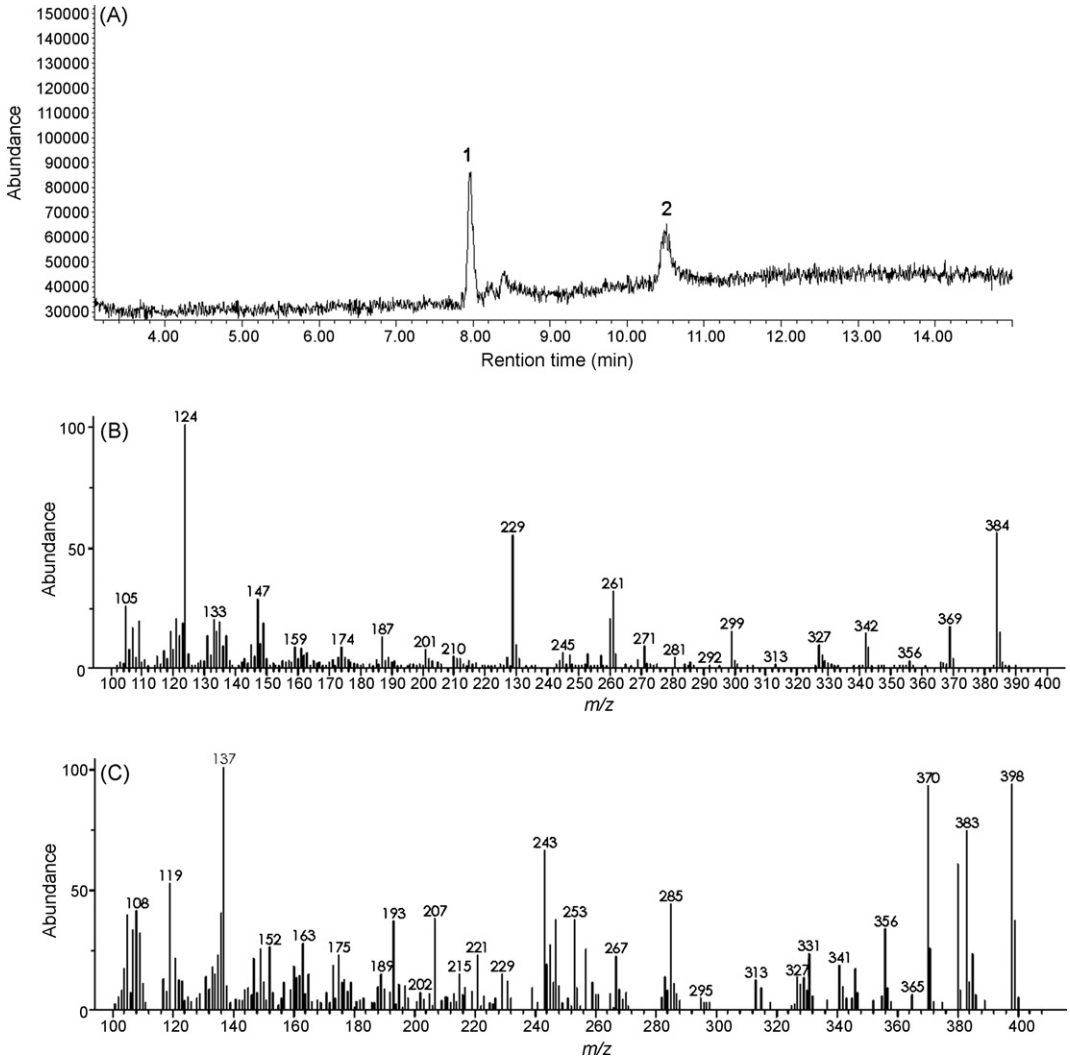

Fig. 2. Gas chromatography/mass spectrometry chromatogram and mass spectra of cholesterol oxidation products. The cholesterol oxidation products of Fig. 1A were analyzed by GC/MS. The chromatography was performed on an HP-5 MS column with helium as the carrier gas. The injection port, oven and transfer line temperatures were all 290 °C. An electron impact ion source was used and the electron energy was 70 eV. The detection was performed in the scan mode with a mass range of *m/z* 100–400. (A) Total ion chromatogram of the cholesterol oxidation products. Peaks (1) and (2) are unidentified cholesterol oxidation products. (B) Mass spectrum of peak (1) of (A). (C) Mass spectrum of peak (2) of (A).

no cholest-4-en-3,6-dione was detected and cholest-4-en-3-one remained almost unchanged.

The Jones oxidation is supposed to convert secondary alcohols to ketones and not to disturb isolated double bonds [9]. Thus the first oxidation product would be cholest-5-en-3-one. As reported by Li and Li [8], cholest-4-en-3-one would obviously be a product of isomerization and cholest-4-en-3,6-dione a product of oxidation of cholest-5-en-3-one; the reaction would be in favor of the formation of cholest-4-en-3,6-dione and cholest-4-en-3-one only be formed during the following extraction, or otherwise the decrease and near disappearance of cholest-4-en-3-one in Fig. 3 would not be observed. The isomerization would also require an acidic condition, as observed by Parish et al. [14] in their preparation of steroidal 5-en-3-ones, because little cholest-4-en-3-one was observed when chromium trioxide and sulfuric acid were both 2 mol/L. A possible mechanism for the formation of cholest-4-en-3-one and cholest-4-en3,6-dione during the Jones oxidation is given in Fig. 4.

3.1.3. Selection of product and oxidation conditions for HPLC analysis

Both products would have the potential to be used for HPLC detection of cholesterol, but the formation of cholest-4-en-3,6-dione would be more manageable as shown in Fig. 3 and discussed above.

Cholest-4-en-3,6-dione could be formed with various chromic acid and sulfuric acid concentrations (Fig. 3), but when the concentration ratio of chromic acid to sulfuric acid was 1:2 and lower, which is the case in traditional Jones oxidations, cholest-4-en-3-one was present in a magnitude of 1–3% of cholest-4-en-3,6-dione at a reaction time when cholest-4-en-3,6-dione was highest (Fig. 5A). Presence of cholest-4-en-3-one will cause a longer HPLC analysis time. As discussed above,

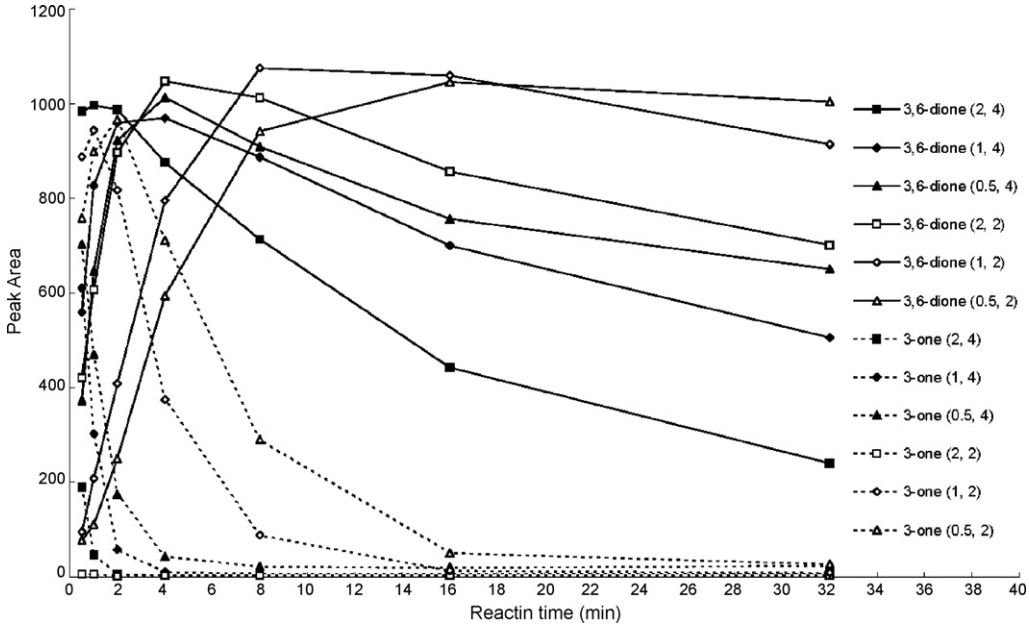

Fig. 3. Kinetic changes of HPLC peak areas of cholest-4-en-3,6-dione and cholest-4-en-3-one. About 100 nmol of cholesterol was dissolved in 0.4 mL of acetone and treated with 20 μL of chromic acid–sulfuric acid solutions of varied concentrations for different times at room temperature. The oxidation products were extracted and chromatographed by HPLC. Chromatographic conditions were as described for Fig. 1, except that the detection wavelength was 240 nm for cholest-4-en-3-one. The "3,6-dione" and "3-one" in the legend area represent cholest-4-en-3,6-dione and cholest-4-en-3-one and the numbers in parenthesis are chromic acid and sulfuric acid concentrations in mol/L, respectively.

when chromic acid and sulfuric acid were both 2 mol/L, little cholest-4-en-3-one (<0.2% of cholest-4-en-3,6-dione) was observed (Fig. 5B).

3.1.4. Reaction yield and detection limit

It is normally believed that chemical oxidation products of cholesterol are multiple and complex. Indeed, multiple products were observed as shown in Fig. 5, but with the chosen oxidation and chromatographic conditions, normalized peak areas of 95–98% for cholest-4-en-3,6-dione were typically obtained (Fig. 5B).

To estimate the actual reaction yield, cholest-4-en-3,6-dione from known amount of cholesterol was chromatographed and the UV absorbance at 250 nm of the dione fraction was measured. Based on the reported molar extinction coefficient, the reaction yield was estimated to be 85–90%.

Cholest-4-en-3,6-dione from known amount of cholesterol was chromatographed for the estimation of the detection limit. The detection limit (defined as six times the standard deviation of the baseline) for cholesterol was about 0.2 pmol.

3.1.5. Selection and validation of the internal standard

The nature of the oxidation and precise HPLC analysis necessitate the use of an internal standard. All steroidal 5-en-3β-ols could potentially be used as an internal standard. Stigmasterol was chosen as a candidate because of its absence in biological samples and its common availability. To test its suitability, a mixture of cholesterol and stigmasterol was prepared and oxidized with various amount (10–40 μL)

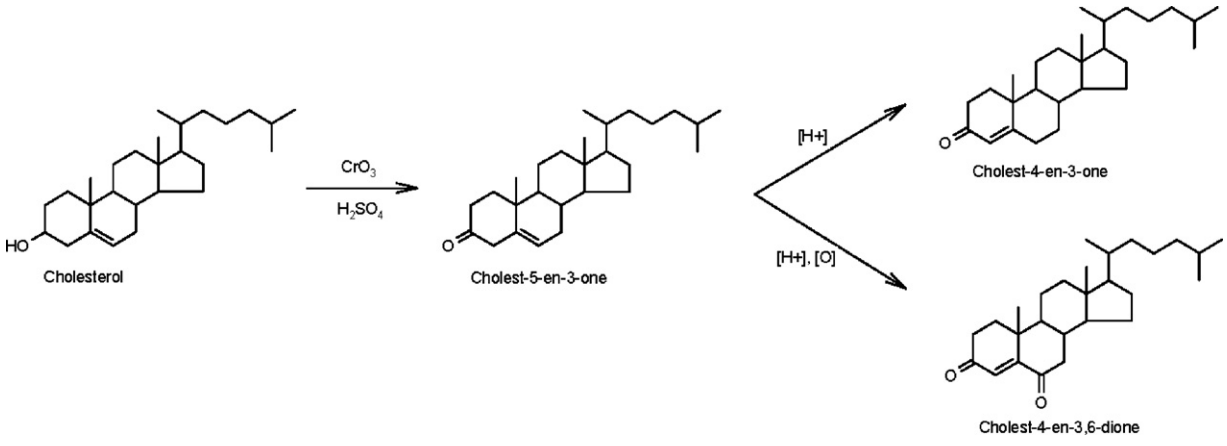

Fig. 4. Postulated formation of cholest-4-en-3-one and cholest-4-en-3,6-dione during Jones oxidation of cholesterol.

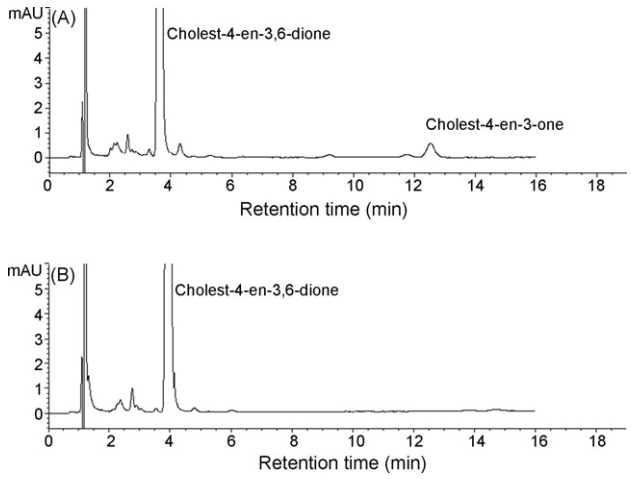

Fig. 5. HPLC chromatograms of cholesterol oxidation products obtained with different chromic acid and sulfuric acid concentrations. About 100 nmol of cholesterol was dissolved in 0.4 mL of acetone and treated with 20 μL of a chromic acid–sulfuric acid solution of either 2 and 4 mol/L (A) or 2 and 2 mol/L (B). The oxidation products were extracted and chromatographed by HPLC. Chromatographic conditions were as described for Fig. 1.

of chromic acid–sulfuric acid (both 2 mol/L) solution for 2.5–15 min. The absolute peak areas varied up to twofold but the peak area ratios showed a CV of 0.3% ($n = 24$), suggesting that stigmasterol has very similar properties to cholesterol during the oxidation. A typical chromatogram of cholesterol and stigmasterol in the cholesterol analysis is shown in Fig. 6.

3.2. Analysis of total and free cholesterol in serum and HDL

3.2.1. Linearity

The linear correlation between cholesterol concentration (x) (the five calibrators, each in duplicate) and the peak area ratio (y) of cholesterol to stigmasterol in 11 analytical runs were assessed by linear regression analysis. The slopes, intercepts and standard errors of the y estimate, slopes and intercepts are shown in Table 1.

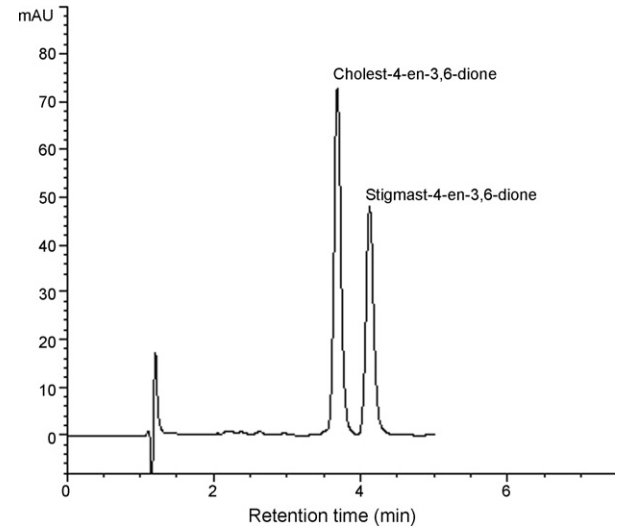

Fig. 6. HPLC chromatogram of cholesterol analysis.

Table 1
Linearity between cholesterol concentration and peak height ratio of cholesterol to the internal standard

	Mean	Range
Slope	0.36875	0.37560–0.36512
Intercept	0.00113	0.00581 to −0.00293
Standard error of y estimate	0.00438	0.00898–0.00190
Standard error of slope	0.00048	0.00108–0.00023
Standard error of intercept	0.00245	0.00471–0.00099
r^2	0.99998	1.00000–0.99993

3.2.2. Precision

To estimate the precision of the method, frozen serum pools (stored at −70 °C) were repeatedly analyzed. Serum total cholesterol was analyzed in duplicate in 12 runs and serum free cholesterol, HDL total cholesterol and HDL free cholesterol were analyzed in triplicate in five runs. The cholesterol levels and the within-run and total CVs are presented in Table 2. All the within-run CVs were about 0.2% and the total CVs ranged from 0.34% to 1.45%. The total CVs for serum free cholesterol, HDL total cholesterol and HDL free cholesterol would include variations caused by instabilities of free cholesterol (some serum enzyme activities) and and/or variations from the precipitation procedure for the preparation of HDL, and the total CV for the cholesterol analysis *per se* would be represented by the total CV of serum total cholesterol which was typically less than 0.5%. This precision for serum total cholesterol was also observed on our quality control samples. Results on a control serum (averaged 4.72 mmol/L) obtained in more than 50 runs during a period of about 16 months showed a total CV of 0.5%.

3.2.3. Analytical recovery

Serum total cholesterol and HDL free cholesterol analyses were selected as representatives to be tested for analytical recovery. Known amounts of cholesterol were added to a pooled serum or an HDL sample and the cholesterol concentrations of the samples with and without added cholesterol were analyzed in triplicate. The observed analytical recoveries averaged 99.8% for total cholesterol and 100% for HDL free cholesterol.

Table 2
Precision of HPLC analysis of serum and HDL total and free cholesterol

	Serum pools	Mean (mmol/L)	CV (%)	
			Within-run	Total
Serum total cholesterol	Pool 1	7.124	0.22	0.48
	Pool 2	4.721	0.17	0.40
HDL total cholesterol	Pool 1	1.936	0.19	0.63
	Pool 2	1.376	0.23	0.34
Serum free cholesterol	Pool 1	1.433	0.09	0.76
	Pool 2	0.915	0.18	1.22
HDL free cholesterol	Pool 1	0.304	0.16	1.45
	Pool 2	0.215	0.19	1.17

3.2.4. Specificity

There are some non-cholesterol sterols in human serum [3,15]. Coelution of the sterols with cholesterol or the internal standard will cause interferences to the cholesterol analysis. Possible interfering sterols would be lathosterol, campesterol, sitosterol and cholestanol [3,15]. The Jones oxidation was applied to these sterols and the products were analyzed by GC/MS and HPLC. Lathosterol (cholest-7-en-3-ol) and cholestanol gave products of cholest-7-en-3-one and cholestanone, respectively, as characterized by a GC/MS analyses and showed no absorption at 250 nm when chromatographed. Campesterol and sitosterol, which have the 5-en-3-ol structure, were oxidized to 4-en-3,6-diones. Sitosterol (as its oxidation product) was well resolved from the internal standard with a longer retention time under the present chromatographic conditions, but campesterol showed almost the same retention time as the internal standard, and thus would interfere with the cholesterol analysis. The concentration of campesterol in human serum is normally about 0.2% of that of cholesterol [3,15] and this interference would be negligible for most applications of the method.

3.2.5. Comparison with the Abell-Kendall method

The CDC Abell-Kendall method has been the National Cholesterol Education Program (NCEP) reference method for serum cholesterol. The NCEP medical decision points for serum cholesterol were derived from cholesterol assays standardized to this method. To compare the present HPLC method for total cholesterol with the Abell-Kendall method, 38 serum samples from individual patients were analyzed with the two methods. As a CRMLN member, the laboratory performs the Abell-Kendall method with <1% CVs and <1% biases as required by CDC. The cholesterol concentrations of the samples ranged from 130.3 to 302.3 mg/dL as measured by the Abell-Kendall method. Results obtained with the two methods and the biases of the HPLC results are listed in Table 3. Regression analysis showed a correlation of the HPLC results (y) with the Abell-Kendall results (x) of $y = 0.983x + 1.1$ (in mg/dL) with a correlation coefficient of 0.999. The HPLC results showed an averaged bias of −1.1% (range 0.1% to −2.5%). The biases may be explained by the observations that the Abell-Kendall method measures some non-cholesterol sterols in serum [15] and by the interferences to the HPLC method as discussed above.

3.3. Other potential applications

The above serum and HDL cholesterol analyses have successfully been used in our lipoprotein stability studies and are given here as examples of the application of the HPLC method based on a pre-column Jones oxidation of cholesterol. In this application, the calibration ranges were much higher than the detection limit (0.2 pmol of cholesterol) (Section 3.1.4). If expressed in relation to the sample matrix and in comparable unit, the detection limit would be about 4 μmol/L for total cholesterol and 1 μmol/L for free cholesterol with the present sample preparation formats. These detection limits of the method would allow its application to other biological samples, such as cell cultures.

Table 3
Comparison of Abell-Kendall and HPLC total cholesterol results

Serum number	Abell-Kendall (mg/dL)	HPLC (mg/dL)	HPLC bias (%)
1	242.7	240.4	−0.95
2	147.5	146.7	−0.52
3	207.7	203.4	−2.11
4	184.6	184.7	0.08
5	218.0	215.5	−1.18
6	207.9	207.6	−0.14
7	222.8	221.2	−0.72
8	227.9	227.8	−0.05
9	230.6	226.3	−1.88
10	199.0	196.1	−1.50
11	188.9	185.0	−2.08
12	130.3	128.5	−1.34
13	275.9	273.6	−0.84
14	199.8	197.8	−1.00
15	152.8	151.5	−0.82
16	188.0	184.5	−1.86
17	163.5	162.1	−0.86
18	225.2	224.6	−0.27
19	194.0	190.9	−1.60
20	165.6	162.9	−1.68
21	163.4	162.6	−0.46
22	236.5	232.5	−1.71
23	153.2	153.0	−0.12
24	234.9	232.7	−0.93
25	201.3	199.2	−1.01
26	147.3	145.0	−1.55
27	183.6	183.5	−0.05
28	204.5	200.8	−1.81
29	248.6	246.7	−0.75
30	280.1	275.6	−1.61
31	248.0	244.0	−1.62
32	290.2	288.1	−0.71
33	274.6	271.2	−1.22
34	246.6	240.5	−2.46
35	272.5	266.1	−2.37
36	302.3	299.3	−0.99
37	228.4	225.5	−1.27
38	234.7	231.3	−1.45

4. Conclusion

An HPLC method based on a pre-column Jones oxidation and ultraviolet detection for the measurement of total and free cholesterol in biological samples was established. The method is highly precise, sensitive, simple and economical and can be used in research or clinical laboratories where precise and specific cholesterol analyses are needed.

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