ORIGINAL ARTICLE

Proficiency testing of total serum cholesterol assay by the ATAC 8000® random access chemistry auto analyzer at the Komfo Anokye teaching hospital

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Cardiovascular disease has become a leading cause of death with hypercholesterolaeemia being one of the most important and implicated modifiable risk factor in both developed and developing countries. Analysis of cholesterol is thus important and many analytical techniques have been developed. Different methods for determining total cholesterol can produce varying laboratory results, thus illustrating the importance of quality control. The present study, therefore, aims at comparing the ATAC 8000 Random Access Chemistry Autoanalyzer used at the KATH, the manual enzymatic method and the WHO recommended manual method with reference to total cholesterol assay. A 20 day replication run was conducted utilizing stabilized human control serum. Methods comparison was then performed on 90 patient samples using the ATAC 8000 autoanalyzer adopted method for estimating cholesterol (enzymatic end point method) as the test method, the manual enzymatic end point method and the WHO recommended Liebermann-Burchard method as comparative methods, to analyse total serum cholesterol. ATAC 8000 gave the lowest CV of 3.0% and total error (TE) of 6.5%, followed by the Liebermann-Burchard method (CV of 4.6%; TE of 9.0%) and the manual enzymatic method (CV of 7.0%; TE of 13.7%). The recommended CV ranges from 3-5% with TE being ≤ 8.9%. The autoanalyzer consistently generated results that were higher than the other methods with good precision and accuracy. Based on the capability index (Cp), ATAC 8000 had the highest Cp of 2.5 followed by Liebermann-Burchard method with a Cp of 1.8. The higher the Cp, the lower the risk of jumping the tolerance limits and therefore the higher the quality. The Bland-Altman analysis showed good agreement between the ATAC 8000 and other methods. Total analytical error, capability index and CV produced by the method adapted for cholesterol estimation by ATAC 8000 Random Access Chemistry autoanalyzer is acceptable since all are within the recommended and set ranges for total cholesterol.

Keywords: ATAC 8000, Liebermann-Burchard Method, Enzymatic endpoint method, Total Cholesterol

INTRODUCTION

Cardiovascular disease (CVD) is said to be a major cause of morbidity and mortality worldwide (Murray and Lopez, 1997). Even though mortality associated with CVD has declined in economically developed countries, the epidemic of CVD in recent times has been observed in developing countries (Reddy and Yusuf, 1998). This observed trend has resulted, in a large part, from the economic growth and associated socio-demographic changes that have occurred over recent decades. Notwithstanding the declines in illnesses from infectious diseases, changes in lifestyle and diet have led to increased burden of CVD and other chronic diseases with an overall resultant fall in life expectancy (He et al., 2004; Reddy and Yusuf, 1998).

With high blood cholesterol being one of the most
important modifiable risk factor for CVD and its associated mortality (He et al., 2004; LaRosa et al., 1999), there is, however, paucity of data on the population levels of serum cholesterol in developing countries (He et al., 2004; Hughes et al., 1997). There are indications of people being quite well aware of their blood pressure levels and possible hypertension in many countries. The same can however not be said for cholesterol levels and general awareness of hypercholesterolaemia. The prevalence of hypercholesterolaemia varies considerably between countries (Cutter et al., 2001; Tolonen et al., 2005; Ulmer et al., 2001), within countries, between different areas and population groups as well as the method of estimation employed (Cirera et al., 1998; Polednak, 1992; Ulmer et al., 2001).

Several methods for the estimation of serum cholesterol have been outlined for which some are still being evaluated for their precision, accuracy and recovery. Differing methods of determining total cholesterol can produce varying laboratory results, thus illustrating the importance of quality control in the measurement of total cholesterol. Quality control plays a vital role in the harmonization of results for total cholesterol. Accurate analysis of cholesterol is therefore imperative given the fact that high serum cholesterol is a well-noted health hazard (Thompson and Wood, 1995).

This study therefore aims at comparing the ATAC 8000 Random Access Chemistry autoanalyzer used at the Clinical Chemistry Department of the Komfo Anokye Teaching Hospital (KATH), the manual enzymatic method, and the WHO recommended manual method (Liebermann-Burchard method).

Pooled human serum stabilized with 15% ethylene glycol was used in the replication study in order to obtain unbiased observations concerning the day-to-day performance of the assay. The stabilized human sera were prepared as recommended by the WHO (WHO-LAB/86.4). Residual serum samples from patients visiting the clinical laboratory were pooled in a 120 ml portion and stored at 4°C until assayed. Sera with apparent turbidity, excessive bilirubin, or haemolysis were excluded from the pool. After pooling, the sera were centrifuged in 15-mL volumes at 3000 g for 30 minutes, after which the chylomicrons at the meniscus were removed by manual aspiration. Before distribution, the subpools were verified to be free of human immunodeficiency virus (HIV) and hepatitis, although necessary precautionary measures were implemented during the entire process.

**Standard Concentration**

The study was specifically designed to have a short turnaround time so that the laboratory could be monitored closely. A reference curve was constructed from known total serum cholesterol standard for kit calibration and linearity determination of
the spectrophotometer used and the comparative analysis of the manual method and the autoanalyzer (ATAC 8000). The results of total serum cholesterol using the manual method was used as the ‘target’ values i.e. the best result the reference laboratory could obtain for the analyte within that sample.

**Principle for the determination of total serum cholesterol (ATAC 8000 and the Manual Enzymatic Method)**

Cholesterol esters are converted into their fatty acid and cholesterol components by cholesterol esterase. The cholesterol produced is converted to cholest-4-en-3-one and hydrogen peroxide by cholesterol oxidase in the presence of oxygen. The peroxide reacts with hydroxybenzoic acid (HBA) and 4-aminoantipyrine by the action of peroxidase to form the red colour-producing quinoneimine. The intensity of the red color produced is directly proportional to the total cholesterol in the sample when read at 500 nm.

**WHO recommended Liebermann-Burchard Method**

Cholesterol reacts with a strong acid medium of the combined reagent containing sulphosalicylic acid-glacial acetic acid, acetic anhydride and concentrated sulphuric acid to form a blue-green chromophore whose absorbance is measured at 600 nm.

**Statistical Analysis**

The results were presented as means. Methods comparison was done using Bland-Altman plot. Bland-Altman analysis estimates the difference in measurement values obtained by two methods on the same subject (Altman and Bland, 1986). The mean of such differences in a sample of subjects is the estimated bias (difference between methods) and the standard deviation (SD) of the differences measures random fluctuations around this mean. If the “limits of agreement” (mean difference ± 2SD) between two methods are not clinically important, one can use the two methods interchangeably. All statistical analyses and methods comparison were done using GraphPad Prism version 5.00 for windows (GraphPad software, San Diego California USA, [www.graphpad.com](http://www.graphpad.com)) and MultiQC version 5.3.2.2.

**RESULTS**

Using the 20-day replication run on the pooled human serum, the obtained CVs for the various methods are as shown in Table 1. The manual enzymatic method could not meet the WHO recommended allowable CV of 3.5% for total cholesterol. The CV gives an indication of the precision of the method with high CV indicating greater degree of imprecision. The ATAC 8000 autoanalyzer gave the highest capability index (Cp) of 2.5, followed by that of the WHO recommended Liebermann-Burchard manual method (Cp = 1.8) and the manual enzymatic method (Cp = 1.2).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ATAC 8000</th>
<th>Enzymatic</th>
<th>Liebermann-Burchard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range (mmol L⁻¹)</td>
<td>4.4–4.8</td>
<td>3.6–4.4</td>
<td>3.5–3.9</td>
</tr>
<tr>
<td>Mean (mmol L⁻¹)</td>
<td>4.55</td>
<td>4.00</td>
<td>3.73</td>
</tr>
<tr>
<td>SD (mmol L⁻¹)</td>
<td>0.15</td>
<td>0.28</td>
<td>0.17</td>
</tr>
<tr>
<td>CV (%)</td>
<td>3.30</td>
<td>7.00</td>
<td>4.60</td>
</tr>
<tr>
<td>Cp</td>
<td>2.50</td>
<td>1.20</td>
<td>1.80</td>
</tr>
</tbody>
</table>

*SD – standard deviation; CV – coefficient of variation; Cp – capability index*

The exponentially weighted moving average (EWMA) was calculated daily to detect the presence of process shift or bias. The EMWA is a cumulative score that weighs the earlier observations slightly lower than the subsequent observations in such a way as to automatically phase out distant observations almost entirely. The EWMA deviations at the end of the 20-day replication study was 0.3%, 3.8% and -2.7% for ATAC 8000 autoanalyzer, the manual enzymatic kit and the Liebermann-Burchard methods respectively. The manual enzymatic kit method yielded the highest EMWA of the three methods.
The total errors calculated were 6.5%, 13.7% and 9.0% for the ATAC 8000, the manual enzymatic and the Liebermann Burchard methods respectively. The performance index (Pp) for the ATAC 8000, the manual enzymatic method and the WHO reference method using control serum for 20 replicate runs yielded 1.1, 0.7 and 1.1 respectively as shown in Table 2.

Table 2: Performance studies for the various analytical methods used in estimating total cholesterol

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ATAC 8000 (N = 20)</th>
<th>Enzymatic (N = 20)</th>
<th>Liebermann-Burchard (N = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TE</td>
<td>6.5</td>
<td>13.7</td>
<td>9</td>
</tr>
<tr>
<td>Pp</td>
<td>1.1</td>
<td>0.7</td>
<td>1.1</td>
</tr>
</tbody>
</table>

**TE** – total error [% bias + 1.96 (CV)]; **Pp** – performance index;

Comparison of the ATAC 8000 autoanalyzer and the manual enzymatic method with the WHO reference method was conducted using 90 patient samples by the Bland–Altman Plot. ATAC 8000 autoanalyzer and manual enzymatic turn out mean values that are about the same as compared to the WHO recommended Liebermann-Burchard manual methods. The general characteristics of the three methods are as shown in Tables 3 and 4.

Table 3. Parameter estimates for total cholesterol as determined with the three comparative methods for 90 patient samples

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Liebermann-Burchard (N = 90)</th>
<th>Enzymatic (N = 90)</th>
<th>ATAC 8000 (N = 90)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>4.75</td>
<td>5.94</td>
<td>6.03</td>
</tr>
<tr>
<td>SD</td>
<td>2.01</td>
<td>2.16</td>
<td>1.36</td>
</tr>
<tr>
<td>Minimum</td>
<td>1.3</td>
<td>2.1</td>
<td>2.9</td>
</tr>
<tr>
<td>Maximum</td>
<td>12.4</td>
<td>12.1</td>
<td>9.2</td>
</tr>
</tbody>
</table>

**SD** – standard deviation

**DISCUSSION**

This study aimed at quantifying total serum cholesterol using the ATAC 8000 autoanalyzer, the manual enzymatic kit method and the Liebermann-Burchard method with the aim of determining the quality and comparability of results generated by each of the methods. In assessing the quality of the determination of total serum cholesterol, factors of major consideration are imprecision, which has to be low and accuracy.

Accuracy, represents the reliability in performance evaluation of methods and is most frequently hampered by the fact that the control sera used in such evaluations are not comparable with the fresh human serum matrix (Thompson and Wood, 1995). Such existing variations thereby induce serious method, reagent, and analyzer-dependent differences that obscure assessment of the real laborato-
Figure 1: Bland-Altman plots for total cholesterol as determined for 90 patient samples. (A = agreement between manual enzymatic method and ATAC 8000; B = agreement between WHO recommended Liebermann-Burchard manual method and ATAC 8000; C = agreement between the manual enzymatic method and the WHO recommended Liebermann-Burchard method. The 95% confidence limits of the bias are shown as two dotted lines).

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ery performance with patient specimens (Ross et al., 1993). This setback is especially true in analyses of total serum cholesterol (Baadenhuijisen et al., 1995). In this approach, the replication experiment was thus, carried out using serum pooled from 350 patient samples since that was likely to have a similar matrix to human serum than bovine or lyophilised serum which for total serum cholesterol could have yielded higher readings due to matrix bias.

To eliminate the bias of comparing two methods which have different principles of operation, the manual enzymatic method, employing the same principle for total cholesterol estimation as the ATAC 8000 autoanalyzer was included in the study. The WHO recommended acceptable limits for CV is 3 – 5% and the National Cholesterol Education Programme (NCEP) Laboratory Standardization Panel recommends that laboratories perform cholesterol analyses with a bias ≤ 3.0% from the true value (reference method). The ATAC 8000® autoanalyzer generated results that were within the recommended CV using the two criteria. The WHO recommended Liebermann-Burchard manual method generated results with CV within the WHO recommended value but above that of NCEP recommended value. The CV for the manual enzymatic method was above the recommended CVs using the two criteria.

The capability index (Cp) which is the ratio of the allowed tolerance to the expanded uncertainty of the assay is highest with ATAC 8000 autoanalyzer as compared to other methods. The higher the Cp, the lower is the risk of jumping the tolerance limits and therefore the higher the quality. The capability index, 1.2 of the manual enzymatic kit method despite the methods poor CV reduces the tendency of the method to fall out of the tolerance. The implication is that the contributing factors, which affect the reproducibility of total cholesterol, were minimised to produce reliable results from the patient samples. Thus, good handling techniques such as accurate pipetting, homogeneity and accurate timing, use of clean cuvettes (specifically glass cuvettes as the acidic nature of the reagent could lead to cloudiness in the plastic cuvettes which would lead
to inaccurate readings), or reading of samples without air bubbles, use of clean glass wares and pipettes et cetera were practiced.

ATAC 8000 furthermore yielded a higher mean value from method comparison using the 90 patient samples, indicating the tendency that the ATAC 8000 would yield consistently higher readings relative to that of the two manual methods. The incidence of interferences with direct cholesterol determinations have been documented (Fasce and Vanderlinde, 1972; Lolekha et al., 2004; Moline and Barron, 1969) and the earlier reports of this method were careful to point out the lack of effects traceable to elevated bilirubin, haemolysis or \( \gamma \)-globulins. Though, the manual method reportedly has considerable serum-clearing effects on lipaemic sera and results are said to correlate very well with that of the Abell method for highly lipaemic sera (Parekh and Jung, 1970). Previous reports have mentioned the depressed cholesterol values displayed by highly lipaemic sera analyzed by a direct manual method (Moline and Barron, 1969).

Performance indices (Pp) which denote the capabilities on one side of the distribution, the side for which the larger proportion of non-conformers will result indicates that, the ATAC 8000 and the WHO reference method are theoretically capable but are practically prone to the slightest drifts or shifts, thus indicating an insufficient quality of the two methods under consideration. The manual enzymatic method however with its performance index of 0.7 shows that the quality of the method is bad and needs to be improved.

The total error for the manual enzymatic method was above that recommended by the NCEP. This observation combined with the bias and the CV indicates the poor precision and accuracy of the manual enzymatic method in this study. The NCEP recommends that clinical laboratories achieve total error \( \leq 8.9\% \) on patient specimens (NCEP, 2001). Precision can be improved by adherence to accepted principles of good laboratory practice and quality assurance. Accuracy can be improved by establishment of traceability to the National Reference System for Cholesterol (NRS/CHOL) through a fresh sample comparison with one of the Cholesterol Reference Method Laboratory Network (CRMLN) laboratories.

The study further compared the ATAC 8000\( ^\circ \) autoanalyzer with the enzymatic method for total serum cholesterol and the Liebermann-Burchard method on 90 patient samples covering a wide range of cholesterol concentrations. The results indicate good agreement between the ATAC 8000\( ^\circ \) vs. the manual enzymatic method and ATAC 8000\( ^\circ \) vs. Liebermann-Burchard method. This study found a poor agreement between the manual enzymatic methods and the Liebermann-Burchard manual method which finding is however, contrary to previous work (Lie et al., 1976) which indicate good agreement.

**CONCLUSION**

The study showed that the ATAC 8000\( ^\circ \) was suitable for clinical studies involving the estimation of total cholesterol judging from the results obtained for the CV, Pp and Cp. There was also significant agreement between the result generated by the ATAC\( ^\circ \) 8000 and the WHO reference method. The manual enzymatic method is inappropriate for quality control work although it employs the same principle as the ATAC 8000, as can be seen from its poor performance index. The Liebermann-Burchard WHO reference method although employing a dissimilar principle of operation with reference to that utilized by the ATAC 8000 autoanalyzer is suitable for quality control analysis as per the results from this study.

**COMPETING INTERESTS**

The authors declare that they have no competing interests.

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