Bioscene

Bioscene
Volume- 22 Number- 01
ISSN: 1539-2422 (P) 2055-1583 (O)
www.explorebioscene.com

A Novel Micro Method for Rapid Quantification of Serum Cholesterol in Swiss Albino Mice Using Zak's Approach

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Abstract
Cholesterol estimation is a crucial biochemical parameter in toxicological and metabolic studies. Conventional quantification methods require large serum volumes, limiting their functionality for small animal models like Swiss albino mice. This study presents a novel micromethod for rapid serum cholesterol quantification using a modified Zlatkis and Zak approach, requiring only 10 µl of serum. The method is based on the colorimetric reaction of cholesterol with ferric chloride and sulphuric acid, forming a stable reddish-purple complex measurable at 540 nm. Assessment of the modified micromethod against the conventional technique showed a high correlation ($R^2 > 0.99$), with statistically insignificant differences in cholesterol values ($t = 0.908$, $p > 0.05$). The mean cholesterol concentration obtained using the micromethod was 124.93 ± 6.11 mg/100 ml, closely matching the traditional method's result of 123.92 ± 6.09 mg/100 ml. This technique significantly reduces the required sample volume while maintaining accuracy, making it an efficient, ethical and minimally invasive approach for biochemical assessments in small animal models. The modified micromethod facilitates longitudinal studies and multiple biochemical evaluations, thereby improving research efficiency in toxicology and metabolic disorders.

Keywords: Cholesterol, Biochemistry, Micromethod, Colorimetry, Serum

1. Introduction

Cholesterol is a fundamental component of cell membranes, a precursor for steroid hormones, and plays a critical role in lipid metabolism (Ikonen, 2008). The regulation of cholesterol levels is essential for maintaining cellular homeostasis and abnormalities in its metabolism are linked to various diseases, including cardiovascular disorders, metabolic syndrome and liver dysfunction (Maxfield and Tabas, 2005). In toxicological assessments, cholesterol levels serve as an important biomarker for evaluating the effects of xenobiotics, including pesticides, heavy metals and pharmaceuticals, on lipid metabolism and liver function (Gallaher et al., 1993).

In experimental research, cholesterol estimation is a vital parameter for studying the impact of toxicants and potential therapeutic interventions (Gonzalez et al., 2011). A colorimetric reaction with ferric chloride and sulphuric acid to measure

cholesterol concentration was given by Zlatkis and Zak (1953). However, conventional methods for cholesterol estimation require significant serum volumes, limiting their feasibility for simultaneous biochemical analyses in small animals. Since mice have a restricted blood volume, excessive sampling can cause physiological stress and mortality, necessitating the development of microquantity estimation techniques (Parasuraman et al., 2010). The modification of the Zlatkis and Zak method to require only 0.01 ml (10 µl) of serum addresses this limitation while ensuring accurate and reproducible results.

By reducing the required sample volume, this study facilitates comprehensive biochemical evaluations in toxicology and metabolic research while adhering to ethical considerations in animal experimentation. This method enhances the feasibility of longitudinal studies, where multiple biochemical parameters need to be assessed over time without compromising the health of the test subjects.

2. Materials and Methods

2.1 Chemicals

Standard cholesterol and all other chemicals used in the present study were of analytical grade and were purchased from Merck, Sigma-Aldrich, Himedia and Loba (India) chemical manufacturers.

2.2 Model Animal and Sample Collection

Healthy adult, Swiss albino mice (*Mus musculus*) weighing 30.0 ±5.0g were procured from the University Department of Zoology, T. M. Bhagalpur University, Bhagalpur. Animals were housed in polypropylene cages maintained with 12h light-dark cycle at 22 ±4°C and 45-60% relative humidity. Food and water were provided *ad libitum*. Experimental protocol was conducted following the guidelines of Institutional Animal Ethics Committee (IAEC) and Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA), India. The study was reviewed and approved by T. M. Bhagalpur University under the registration number 795/2022. Proper care and handling of animals were ensured to minimize any potential distress, following the ethical principles outlined by CPCSEA. Blood samples from the lateral caudal vein of mice were collected in Serum Separator Tubes and were centrifuged at 3000rpm for 10min to obtain serum.

2.3 Principle

The colorimetric assessment of cholesterol in Zak's method is basedon the oxidation of cholesterol and formation of a coloured complex. Sulphuric acid acts as a dehydrating agent, facilitating the conversion of cholesterol into its reactive form i.e., cholestadienyl cation (Abell et al., 1952). Ferric chloride serves as a colour developer by forming a complex with the oxidised cholesterol, resulting in a stable reddish-purple colour proportional to the cholesterol concentration (Zak et al., 1954). The intensity of the developed colour is measured at 540 nm

(green filter) using a colorimeter or spectrophotometer, and cholesterol concentration is determined based on the standard calibration curve.

2.4 Reagents

(1) 10% of stock ferric chloride reagent is prepared in glacial acetic acid (GAA) i.e., 1.0g $FeCl_3$ in 10ml GAA. (2) 0.1% Working ferric chloride reagent is prepared by pipetting 1ml of previously prepared stock ferric chloride reagent and making up to 100ml using GAA. (3) 0.4% of standard cholesterol solution is prepared in GAA i.e., 200mg of standard cholesterol dissolved in 50ml of GAA, giving the final concentration of 4mg/ml.

2.5 Procedure

The standard calibration curve for the colorimetric assay is prepared by setting up series of test-tubes or cuvette marked A to F, in which tube A receives 10µl of standard cholesterol solution, tube B receives 20µl, tube C receives 30µl, tube D receives 40µl, tube E receives 50µl and the last tube F receives no standard solution which serves as Blank. Each tube is made up to1500µlusing working ferric chloride reagent using micropipette i.e., tube A receives 1490µl and so on whereas tube F which serves as Blank, receives 1500µl of working ferric chloride reagent only.

To each tubes including Blank, 500µl of concentrated sulphuric acid is added, mixed well and allowed to stand for 10min. After cooling the tubes at room temperature, absorbance is recorded at 540nm to plot the standard calibration curve. The final concentration of standard cholesterol in the tubes A to E are 0.02mg/ml, 0.04mg/ml, 0.06mgml, 0.08mg/ml, 0.1mg/ml respectively, considering the dilution by working ferric chloride and concentrated sulphuric acid whereas concentration of tube F is 0.0mg/ml (Blank).

For the assessment of cholesterol concentration in serum samples, add 10µl of serum in a test-tube or cuvette and add 1490µl of working ferric chloride reagent and 500µl of concentrated sulphuric acid, allow the tubes to cool down at room temperature and record the absorbance after 10-15min.

2.6 Calculation

The concentration of cholesterol in blood serum is obtained by solving the linear equation ($y = mx + c$) obtained by the preparation of calibration curve in which 'y' serves as the absorbance recorded and 'x' is the concentration of the unknown (Fig. – 1). Since, the dilution factor was 200 (considering working ferric chloride reagent and concentrated sulphuric acid), the obtained value is multiplied by 200, which gives the cholesterol concentration in terms of mg/ml. Therefore, to obtain the concentration of cholesterol in terms of mg/100ml, the obtained value is further multiplied by 100.

2.7 Statistical analysis

Data were analysed statistically using t-test to compare results obtained from the modified method and the traditional technique on SPSS and Microsoft Excel to plot the curve.

3. Result and Discussion

To check the reproducibility of the modified method, four replicate samples of the same mice were divided into two groups. The first group was analysed using the modified micromethod using 10µl aliquot whereas the other group was analysed by traditional method using 100µl aliquot. In each case the colour developed successfully and the standard calibration curve was plotted for the modified micromethod (Fig. – 1) as well as the traditional method (Fig. –2). The optical density of each sample and standard was measured at 540nm exactly 10-15min after the addition of concentrated sulphuric acid (Table – 1). Several hundred samples were routinely analysed by both methods. The mean serum cholesterol recorded using the modified micro method was 124.93 ±6.11 mg/100ml, whereas the mean obtained from the traditional method was 123.92 ±6.09 mg/100ml. Statistical analysis using the t-test indicated that the difference between the two methods was statistically insignificant ($t = 0.908$, $p > 0.05$). The precision and accuracy of this micro-quantity method were validated through repeated trials, showing minimal variance. The modified method demonstrated a high correlation with the conventional method ($R^2 > 0.99$), indicating its reliability. The minor difference in cholesterol readings between the two methods may be attributed to variations in sample handling, reagent interaction and instrument sensitivity. In the modified method, the sample volume was reduced to 10µl, which may slightly influence the reaction kinetics due to changes in surface-area-to-volume ratio and reagent diffusion (Parasuraman et al., 2010). However, since the same principle of cholesterol oxidation and colorimetric detection was applied, the overall consistency of the results remained unaffected.

Additionally, the preparation of reagents and sample dilution could introduce minor variations in absorbance readings. Factors such as pipetting precision, colorimetric calibrations and differences in colorimetric-reading time may contribute to subtle deviations in measured cholesterol values (Allain et al., 1974). Despite these minor differences, the modified method effectively maintains accuracy while significantly reducing the serum volume required for cholesterol estimation.

This modification is particularly advantageous in small laboratory animals like Swiss albino mice, where total blood volume is limited and excessive sampling can lead to physiological stress or mortality (Parasuraman et al., 2010). As this modified method requires only 10µl of serum, this technique allows for simultaneous biochemical assessments, improving efficiency in toxicological and metabolic studies. Furthermore, the micro quantity method aligns with the ethical

considerations in animal research by minimizing blood collection and reducing the number of animals required for experiments (Gonzalez et al., 2011).

Overall, this study validates the modified micro method as a practical and efficient approach for cholesterol estimation in small animal models. Future studies may further optimize reagent compositions and incubation conditions to enhance sensitivity and reduce variability. The implementation of this method can contribute to broader applications in biochemical and toxicological research, ensuring accurate lipid profile assessments with minimal sample volumes.

Table – 1: Cholesterol concentration (mg/ml) in different serum samples and their mean ± S.E. recorded using modified micro method and traditional method.

Serum sample	Modified micro method (540nm)	Traditional method (540nm)
1	114.72	112.99
2	122.72	121.02
3	130.68	130.15
4	162.54	161.06
5	130.78	127.02
6	128.12	129.34
7	90.64	90.12
8	116.34	116.66
9	110.44	108.86
10	142.28	141.95
Mean± S.E.	124.93 ± 6.11	123.92 ± 6.09

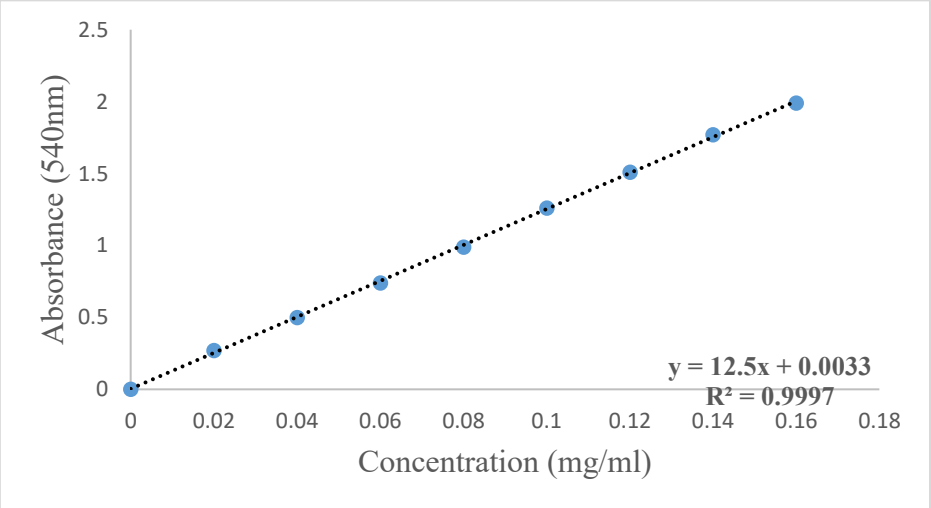

Fig. – 1: Standard calibration curve using the modified micromethod. Absorbance recorded at 540nm (y-axis) and concentration of cholesterol (mg/ml) is represented of x-axis.

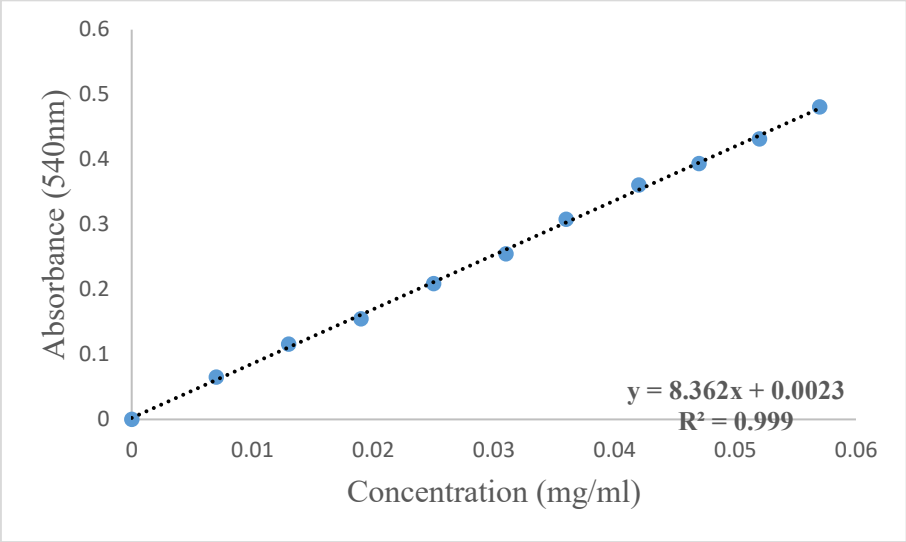

Fig. – 2: Standard calibration curve using the traditional method. Absorbance recorded at 540nm (y-axis) and concentration of cholesterol (mg/ml) is represented on x-axis.

Acknowledgement

I sincerely express my gratitude to the University Department of Zoology, T. M. Bhagalpur University for providing the essential laboratory facilities required to conduct this research. Their support and infrastructure were instrumental in carrying out the research work successfully. I am deeply grateful to the late Prof. (Dr.) Prabhat Kumar Roy for providing essential knowledge that greatly contributed to the study. I also extend my gratitude to Prof. (Dr.) Md. Equbal Ahmad for his valuable guidance on statistical analysis.

Funding

The author declare that this research work was conducted without any external funding or financial support. All expenses related to study were personally borne by the author.

Ethical approval

All experimental procedures in the study were conducted in accordance with the guidelines of the Institutional Animal Ethics Committee (IAEC) and the Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA), India. The study was reviewed and approved by T. M. Bhagalpur University under the registration number 795/2022. Proper care and handling of animals were ensured to minimize any potential distress, following the ethical principles outlined by CPCSEA.

Conflict of interest

The author declares that there is no conflict of interest.

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