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ABSTRACT

A sensitive spectrophotometric method for the determination of cholesterol in serum samples was described. 4-Hydroxybenzaldehyde was used for spectrophotometric determination of cholesterol. The method based on the formation of coloured compound showed maximum absorbance at 525 nm. Beer’s law was obeyed in the concentration range 1.0 – 30.0 µg/ml with detection limit 0.14 µg/ml. The method was applied for the analyses of cholesterol in human serum samples. The results were compared with biolabo kit and biolis automatic instrument as a standard method which shows good agreement between them.

1. INTRODUCTION

Cholesterol is the most prominent member of the steroid family of lipids (Page, 1981). Is probably the best known steroid because of its association with atherosclerosis. However biochemically it is also of significance because it is precursor of large number of equally important steroids which include the bile acids, adrenocortical hormones, sex hormones, D vitamins, cardiac glycoside, sitosterol of the plank kingdom and some alkaloids (Murray et al. 1993, Karlson 1975). Cholesterol is an essential structural component of extra and intra cell membranes and it is a significant constituent of many tissues is widely distributed in all cells of the body but particularly in nervous tissue ((Murray et al. 1993, Metwally et al. 2009). Cholesterol is present in tissues and plasma lipoproteins either as free cholesterol or combined with a long chain fatty acid as cholesterol ester(Jove et al. 2010, Warnick et al. 2008).

In humans, cholesterol is obtained directly from diet and also biosynthesized from acetate via squalene in the liver (Page, 1981). Although all tissues can make cholesterol e.g adrenal glands, ovaries, testes, skin and intestine (Mckee and Mckee 1996). The total amount of cholesterol in the blood depends to large extent on diet, age and sex. A normal level is less than 200 mg/dl of blood, but in order people it can increase to 250 mg/dl or higher (Page, 1981). This paper describes UV/visible spectrophotometric method and optimum conditions for the direct determination of cholesterol in human serum using 4-hydroxybenzaldehyde reagent.
2 Materials and Methods

2.1 Apparatus

Spectral and absorbance measurements were carried out on a BIO-TIK UV/Visible spectrophotometer model: J643002, sn 1025, by using 1-cm quartz cells Centrifuge. PLC series model PLC 02. TLC aluminum sheets 20 X 20 cm, silica gel 60 F 254.

2.2 Reagents

Chemicals used are of the highest purity available.

Standard cholesterol stock solution (1000 µg/ml). The solution was prepared by dissolving 0.1000 g standard cholesterol (Scharlau) in absolute ethanol and diluting to 100 ml. Working solutions were prepared by appropriate dilution with absolute ethanol.

4-Hydroxybenzaldehyde (0.082 M). Was prepared fresh on day by dissolving 0.2504 g of 4-hydroxybenzaldehyde (Yacoo) in glacial acetic acid and diluted to 25.0 ml with glacial acetic acid.

Concentrated sulphuric acid (H₂SO₄ 95 – 98%) (Scharlau).

Absolute ethanol (99.9%) (Scharlau).

Potassium hydroxide (85%) (BDH).

2.3 Sample collection

Human blood samples were collected. Twenty samples were used throughout the present work. The samples included different ages (20.0 – 60.0) years. All samples were taken from Hawler hospital and Khanzad private laboratory in Hawler.

2.4 Extraction and separation

Cholesterol was extracted with mixture of chloroform – methanol. 0.5 ml of normal human serum was added to 5.0 ml of chloroform – methanol 2:1 (V/V). The mixture was agitated manually for 20 second and centrifuged at 2500 rounds per min for 10.0 min. After centrifugation the aqueous phase was collected (Ferraz et al. 2004). The extracts were taken for saponification with 2% alcoholic KOH and separated by thin layer chromatography (TLC) using petroleum ether (boiling point 40 - 80 °C), diethyl ether and acetic acid : 90 : 10 : 1 as a mobile phase for separation by TLC on aluminum sheets silica gel plates under stream of nitrogen.

2.5 Recommended procedure for spectrophotometric determination of cholesterol using 4-hydroxybenzaldehyde

A known volume of solution contains (1.0 – 30.0) µg/ml of standard cholesterol was charged with 4.1× 10⁻³ M of 4-hydroxybenzaldehyde and 2.5 ml concentrated sulfuric acid diluted with absolute ethanol in 10.0 ml volumetric flask. The reagent blank was prepared as the same way in the absence of cholesterol. The absorbance was measured at 525 nm.

2.6 Spectrophotometric determination cholesterol using biolabo kit

Sets of tubes were prepared according to the procedure of biolabo kit shown in Table (1).

Table (1): Recommended procedure for determination of cholesterol with biolabo kit.
The results were calculated using the following expression:

\[
\text{Result} = \frac{\text{absorbance assay}}{\text{absorbance standard}} \times 2000
\]

Note:

\[
R_1 = (\text{Phosphate buffer} + \text{chloro-4-phenol} + \text{sodium cholate} + \text{Triton} \times 100 \text{ preservative})
\]

\[
R_2 = (\text{Cholesterol oxidase} + \text{cholesterol esterase} + \text{peroxydase} + 4-\text{amino-antipyrine})
\]

### 3. Results and Discussion

Spectrophotometric method was described for determination of cholesterol depending upon the reaction with 4-hydroxybenzaldehyde in acidic medium to form violet colored compound at 525 nm. Whereas, the blank solution has no significant absorbance at this region (Figure 1).

![Absorption spectra](image)

Figure 1: Absorption spectra of (a) 10.0 µg/ml of cholesterol (b) blank against absolute ethanol according to the preliminary work.

**General scheme reaction for cholesterol:**

![General scheme](image)

**Suggested mechanism:**

**Step one:**

![Step one](image)

**Step two:**

![Step two](image)
Step three:

\[ \text{Hemiacetal} \]

Step four:

\[ \text{Violet coloured compound} \]

3.1 Effect of 4-hydroxybenzaldehyde concentration

The effect of different concentrations of 4-hydroxybenzaldehyde solution on the absorbance of the coloured product was studied. The results indicated that 4.1×10^{-3} M of 4-hydroxybenzaldehyde solution gave maximum absorbance for cholesterol. Therefore, this concentration was used in the subsequent works.

3.2 Effect of concentrated sulfuric acid

The effect of different volumes of concentrated sulfuric acid solution on the absorbance of the coloured product was studied. The results showed that 2.5 ml of concentrated sulfuric acid solution gave maximum absorbance for cholesterol. Therefore, this volume was used in the subsequent works.

3.3 Effect of time (stability)

The stability of the coloured compound was studied at the maximum wavelength by measuring the absorbance of the coloured solution against the time. It seems that the absorption measurements were nearly constant within 20.0 min as shown in Table (2). The subsequent experiments were carried within 6.0 min after dilution.

Table (2): Stability of coloured compound against time for cholesterol.

| Time (min) | Absorbance of cholesterol |
|------------|---------------------------|
| Immediately | 0.449                     |
| 1          | 0.472                     |
| 2          | 0.488                     |
| 3          | 0.496                     |
| 4          | 0.500                     |
| 5          | 0.500                     |
| 6          | 0.505                     |
| 8          | 0.505                     |
| 10         | 0.505                     |
| 15         | 0.505                     |
| 20         | 0.505                     |
| 30         | 0.501                     |
| 45         | 0.490                     |
| 60         | 0.467                     |

3.4 Calibration curve

Applying the recommended procedure shown in Table (3) straight line of calibration curve for cholesterol which show a good linearity over the concentration rang 1.0 – 30.0 µg/ml of cholesterol, with detection limits 0.14 µg/ml.

Table (3): Optical characteristics of the determination of cholesterol.
The precision and accuracy of the method was investigated by determining the percentage relative standard deviation (RSD %) and relative error percentage (Error %) respectively from ten analyses of a series of solutions, containing 1.0, 15.0, and 30.0 µg/ml of standard cholesterol by applying the recommended procedure. The results obtained are shown in Table (4).

### Table (4): Precision and accuracy of the determination of cholesterol.

| Parameter                        | Results for cholesterol |
|----------------------------------|-------------------------|
| Colour                           | Violet                  |
| $\lambda$ max (nm)               | 525                     |
| Linear range (µg/ml)             | 1.0 – 30.0              |
| Detection limit (µg/ml)          | 0.14                    |
| Molar absorptivity (L mol$^{-1}$ cm$^{-1}$) | $1.6472 \times 10^4$ |
| Correlation coefficient ($r$)    | 0.9983                  |
| Sandell index (µg/cm$^2$)        | 0.0234                  |

### 3.5 Precision and accuracy

The precision and accuracy of the method was investigated by determining the percentage relative standard deviation (RSD %) and relative error percentage (Error %) respectively from ten analyses of a series of solutions, containing 1.0, 15.0, and 30.0 µg/ml of standard cholesterol by applying the recommended procedure. The results obtained are shown in Table (4).

### Table (5): Effect of interfering ions on the determination of 15.0 µg/ml of cholesterol.

| Interfering species | Amount add (µg/ml) | Error % |
|---------------------|--------------------|---------|
| Triglyceride        | 3.0                | + 3.50  |
| Glucose             | 62.0               | - 4.74  |
| Urea                | 68.0               | + 4.46  |
| Ascorbic acid       | 73.0               | + 4.96  |
| Aceton              | 85.0               | - 4.63  |
| Calcium (II)        | 97.0               | + 4.65  |
| Potassium           | 94.0               | - 4.43  |
| Sodium              | 96.0               | + 3.30  |
| Chloride            | 91.0               | + 4.95  |
| Iron (II)           | 24.0               | + 3.80  |
| Iron (III)          | 8.0                | - 3.10  |

### 3.6 Effect of interferences

In order to study the effect of some common foreign species (interferences) that may be present with cholesterol in the human serum samples. Separate synthetic solutions were prepared, containing 15.0 µg/ml of cholesterol with different amounts of the interferences. Table (5) shows the maximum amount of the interfering ions that caused errors ≤ ± 5.0.

### Table (6): Recoveries of the method and analyses of cholesterol in some human serum samples.

| No. | Cholesterol (µg/ml) | Biolis (A) | Biola recovery % | Recovery % |
|-----|---------------------|------------|------------------|------------|
| Cholesterol | 1.0 | 0.47 | 2.67 | 1.06 |
| 15.0 | 0.034 | +1.63 | |
| 30.0 | 0.018 | +1.06 | |
5. CONCLUSIONS

In the present work sensitive spectrophotometric method for determination of cholesterol in serum samples have been proposed based on the formation of coloured compounds. The method was applicable over the range of concentration with good precision and accuracy.

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