The Comparison between Spectrophotometer and Reflotron Method to Measure Serum Lipid Profile

Mohammed Ahmed Wsoo Jawameer Rasool Hama
University of Raparin
Chemistry Department

Abstract
Nowadays, all clinical laboratories use a technique to determine the lipid profile such as total cholesterol (TC), triglycerides (TG) and high density lipoproteins – cholesterol (HDL-C). We obtained blood from ten patients from Rania General Hospital in Rania – Kurdistan and each parameters of lipid profile were analyzed in triplicate. In the present study the techniques of spectrophotometer (wet enzymatic method) and reflotron (dry enzymatic method) were used to determine the lipid profile. There were significant differences in the means of TC, TG and HDL-C by the two methods. The significant differences for TC, TG and HDL-C were $p<0.0001$, $p<0.0045$ and $p<0.0024$, respectively. The statistical analysis, paired t-test and Pearson's correlation were used, by adopting a significance level for a value of $p<0.01$. The results of total cholesterol and triglycerides by spectrophotometer were significatly higher than reflotron, however the result of HDL-C by reflotron was higher than spectrophotometer. The results of the study show that lipid profile measurement by spectrophotometer is more accurate than by reflotron.
Keywords: Comparison analysis; Lipid profile; enzymatic method; Spectrophotometer; Reflotron
Email: mohammed1981@raparinuni.org, phone: +9647511923621

Introduction

Lipids have low polarity and limited solubility in water. They are strongly associated with specific proteins. The lipid components of lipoproteins are total cholesterol (TC), triglycerides and phospholipids. The cholesterol is an amphipatic lipid, component of the lipoprotein particles. Triglycerides (TG) are another form of fat in the body. It's comprised by linking three fatty acids molecules with a glycerol molecule. The high density lipoproteins - cholesterol (HDL-C) is lipoproteins are composed of protein and fat (1).

The TC and low density lipoprotein - cholesterol (LDL-C) levels are positively related to coronary heart disease (CHD), however HDL-C levels are negatively related to CHD. Lipid metabolism disorders are an extremely relevant factor of screening diagnosis with point of care devices (2). The determination of the lipid panel is essential for primary and secondary prevention of CHD (2, 3). Determination of serum TG level is important in patients with secondary hypertrigliceridemia and with occlusion of peripheral arteries. It is widely accepted today that a combined determination of TC and TG in serum is the simplest and most reliable way to detect hyperlipidemia. Determination of HDL-C is important for patients of young ages with vascular diseases, with xanthomas, and secondary hypercholesterolemias (2, 4).

Cardiovascular Disease (CVD) accounts for the greatest number of deaths of adult individuals worldwide (5). It is the world's leading cause of death, and as such, represents a serious global health problem. It is caused by the natural ageing process and the excesses of a western lifestyle. The major risk factors for CVD are high blood pressure, hypercholesterolemia, unhealthy diet, tobacco use, diabetes, obesity, advancing age, genetic disposition, excess alcohol consumption and sedentary lifestyle (6, 7, 8).

In the present study, we describe enzymatic methods for determination of lipid profile such as (TC, TG and HDL-C). Researchers found that enzymatic methods for determination of serum lipid profile are more specific and accurate than chemical methods (19). On the basis of these findings, the lipid profile was determined by two different enzymatic methods, which are spectrophotometer (wet method) and reflotron (dry method). Part of this study is to investigate the accuracy of the methods. Accuracy was assessed by establishing the correlation between spectrophotometer and reflotron.

Materials and Methods

All the chemicals and reagents used were of analytical reagent grade, supplied by (BIOLABO SA, France). Single beam visible Spectrophotometer (Jen way 6310) and Reflotron analyser were used for lipid profile analysis.

Subject: University of Raparin ethical committee approved the study. Any cases with chronic heart disease, chronic renal disease and chronic liver diseases were excluded from the present study.

Sample collection: Ten patients from General Rania Hospital in Rania - Kurdistan were selected as a sample. 4-5 ml of blood samples after 9-12 hours of fasting were obtained from healthy adults by vein puncture into plain tube. Samples were allowed

to clot at room temperature for 15 minutes and then the serum was separated from the clotted blood by centrifugation at 4000 rpm for 10 minutes, then decanted into clean and sterile plain tubes and then lipid profile were analyzed on the day it was drawn.

Standards

Stock Cholesterol Standard: A stock solution of cholesterol (200 mg/dL) was used, that purchased from the manufacturer. The spectrophotometric method performed to measure absorbance of standards. The absorbance is converted into concentrations based on the standard calibration curve. Calibration curve of cholesterol was obtained from six different known of standard cholesterol solutions, as (0 mg/dL, 25 mg/dL, 50 mg/dL, 100 mg/dL, 150 mg/dL and 200 mg/dL). The cholesterol standards were analysed in triplicate. The R^2 indicates that the calibration curve is accurate enough to be used, which was 0.9963.

Triglyceride Standard: A stock solution of TG (200 mg/dL) was used, that purchased from the manufacturer. The spectrophotometric method performed to measure absorbance of standards. The absorbance is converted into concentrations based on the standard calibration curve. Calibration curve of TG was obtained from six different known of standard TG solutions, as (0 mg/dL, 25 mg/dL, 50 mg/dL, 100 mg/dL, 150 mg/dL and 200 mg/dL). The TG standards were analysed in triplicate. The R^2 indicates that the calibration curve is accurate enough to be used, which was 0.9997.

HDL-C Standard: A stock solution of (100 mg/dL) HDL-C was used, that purchased from the manufacturer. The spectrophotometric method performed to measure absorbance of standards. The absorbance is converted into concentrations based on the standard calibration curve. Calibration curve of HDL-C was obtained from six different known of standard HDL-C solutions, as (0 mg/dL, 20 mg/dL, 40 mg/dL, 60 mg/dL, 80 mg/dL and 100 mg/dL). The HDL-C standards were analysed in triplicate. The R^2 indicates that the calibration curve is accurate enough to be used, which was 0.9985.

Determination Lipid Profile by Spectrophotometer (Wet Enzymatic Method)

Principle of Estimation TC: The serum TC was determined by spectrophotometer, using a BIOLABO-Reagent/France kit (9, 10). Cholesterol esters are hydrolyzed to free cholesterol by cholesterol ester hydrolase (EC 3.1.1.13). The free cholesterol is oxidized to cholest-4-en-3-one with the simultaneous production of hydrogen peroxide, which oxidatively couples with 4-aminoantipyrine and phenol in the presence of peroxidase to yield a chromogen with maximum absorption at 500 nm. The relative cholesterol content can be calculated by using the formula [y = 0.0024x + 0.0003], which is obtained from the calibration curve

Principle of Estimation TG: The serum TG was determined by spectrophotometer, using a BIOLABO-Reagent/France kit (11, 12). TG was hydrolysed by enzyme lipase into glycerol and free fatty acids and then liberated glycerol convert to glycerol-3-

phosphate by glycerol kinase. Glycerol-3-phosphate oxidized by glycerol-3-phosphate oxidase into dihydroxyacetone phosphate and hydrogen peroxide. Finally, the hydrogen peroxide is monitored in the presence of horseradish peroxidase with 3,5-dichloro-2-hydroxybenzenesulfonic acid/4-aminophenazone as the chromogenic system. The high absorbance of this chromogen was at 500 nm. The relative TG content can be calculated using the formula [y = 0.0013x + 0.0146], which is obtained from the calibration curve.

Principle of estimation HDL-C: In serum, the precipitation method (by phosphotungstic acid with magnesium chloride ($MgCl_2$)) was used to precipitate LDL-C, Very Low density lipoproteins and chylomicrons from a fasting serum sample, leaving HDL-C in the supernatant. HDL-C obtained in supernatant after centrifugation is then measured by spectrophotometer with TC reagent (13, 14). The relative HDL-C content can be calculated using the formula [y = 0.0053x + 0.0308], which is obtained from the calibration curve.

Determination Lipid Profile By Reflotron (Dry Enzymatic Method)

TC: The serum TC measurement was done by reflotron, using the reflotron analyzer (15).

TG: The serum TG measurement was done by reflotron, using the reflotron analyzer (16).

HDL-C: In serum, HDL-C estimation was done by reflotron, using the reflotron analyzer (17, 18). In the method, dextran sulfate and $MgC1_2$ precipitate chylomicron, VLDL, LDL and lipoprotein on the test strip, and HDL-C remains in the sample. The HDL-C content is then measured by the reflotron analyzer.

Statistical Analysis

All Results and measurements were in three independent measurements, mean values ± standard deviations were reported for each case. Analysis of variance was performed on statistical analysis system at a level of $P<0.01$ to evaluate the significance of differences between mean values, using pair T-test of the Graphpod Prism, version (6.01).

Results and Discussion

The mean and standard error of serum lipid profile by spectrophotometer and Reflotron are shown in table (1). TC and TG concentration were significantly (p value <0.001, 0.0045, respectively) higher by spectrophotometer when compared to reflotron. Whereas HDL-C concentration was significantly ($P<0.024$) higher by reflotron than spectrophotometer.

Table 1. The mean and standard error of mean of lipid profile parameters were obtained by spectrophotometer and reflotron methods.

parameters	spectrophotometer (mg/dL)		Reflotron (mg/dL)		P-Value
	Mean	±SE	Mean	±SE	
TC	208.2	9.99	182.2	9.04	< 0.0001
TG	198.6	29.8	183.4	25.3	0.0045
HDL-C	25.72	0.95	30.33	1.72	0.0024

Data regarding spectrophotometer and reflotron methods of TC measurement are summarized in Table 2. During the measurement, the result of spectrophotometer was significantly different than the result of reflotron, their difference were about 26.06 mg/L. The difference is significantly high, which may change doctor's decision regarding to medical treatment. Significantly positive correlation is found for TC measurement by spectrophotometer and reflotron, as shown in figure (1). The serum TC profile significantly differed ($p<0.0001$) between spectrophotometer and reflotron method.

Table 2. Statistical summary of serum TC concentration (mg/dL) obtained by spectrophotometer and reflotron.

Parameters	spectrophotometer	reflotron	reflotron-spectrophotometer
Minimum value	125.13	111	-47.63
Maximum value	284.71	260	-9.71
Mean	208.22	182.16	-26.06
Median	238.04	205	-27.04
Std. Deviation	55.66	50.35	8.82
Std. Error of Mean	9.99	9.04	1.58
P-value	< 0.0001		

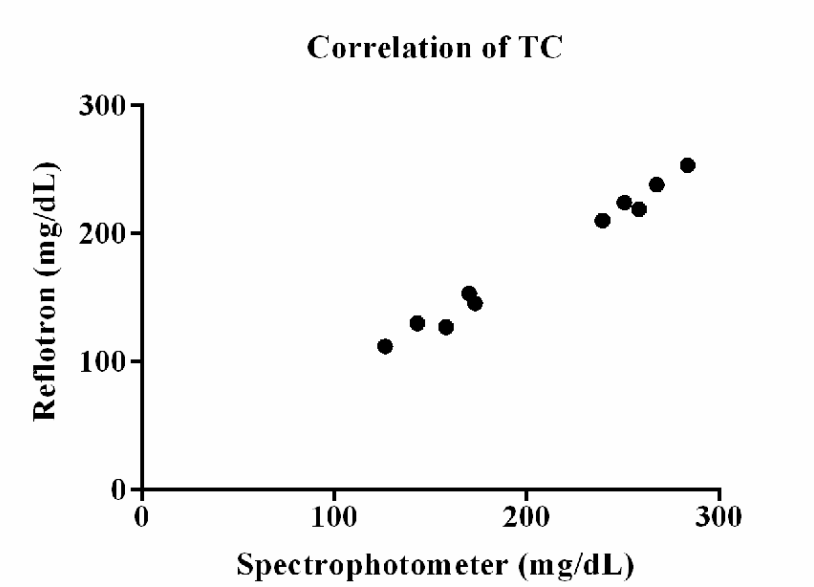

Figure (1): Comparison of results for TC between the spectrophotometer and reflotron method.

The data for TG by spectrophotometer and reflotron methods are shown in Table 3. The result of spectrophotometer was significantly higher than the result of reflotron, by 15.16 mg/L, these dates which may change doctor's decision regarding to medical treatment. The correlation (figure 2) between spectrophotometer and reflotron was found to be significant to determine TG. There were significant differences in the means TG levels between two methods, which was ($p<0.0045$). Other disadvantage of reflotron method for TG measutment related with detection limit high than spectrophotometer (70, 10) respectively.

Table 3. Statistical summary of serum TG concentration (mg/dL) obtained by spectrophotometer and reflotron.

Parameters	spectrophotometer	reflotron	reflotron-spectrophotometer
Minimum	58	70	-98
Maximum	628	545	16.54
Mean	198.6	183.4	-15.16
Median	133.8	138.5	-8.04
Std. Deviation	163.2	139	26.97
Std. Error of Mean	29.8	25.37	4.93
P-value	0.0045		

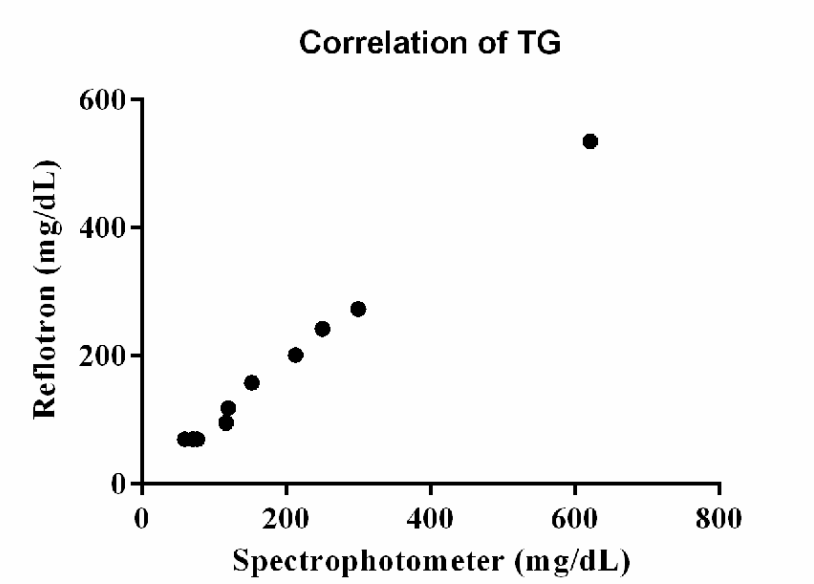

Figure (2): Comparison of results for TG between the spectrophotometer and reflotron method.

Statistical summary of serum HDL-C are given in Table (4), the results of HDL-C by reflotron were higher than spectrophotometer methods. Whereas the TC and TG results of spectrophotometer were higher than the reflotron. Significantly positive correlation was found for HDL-C determination by reflotron and spectrophotometer, p value was 0.0024, as shown in figure (3).

Table 4. Statistical summary of serum HDL-C concentration (mg/dL) as obtained by spectrophotometer and reflotron methods.

Parameters	spectrophotometer	reflotron	reflotron-spectrophotometer
Minimum	17.77	17	-11.15
Maximum	35.32	48.7	18.24
Mean	25.72	30.33	4.61
Median	23.72	25.6	4.70
Std. Deviation	5.36	9.73	7.91
Std. Error of Mean	0.95	1.72	1.40
P value	0.0024		

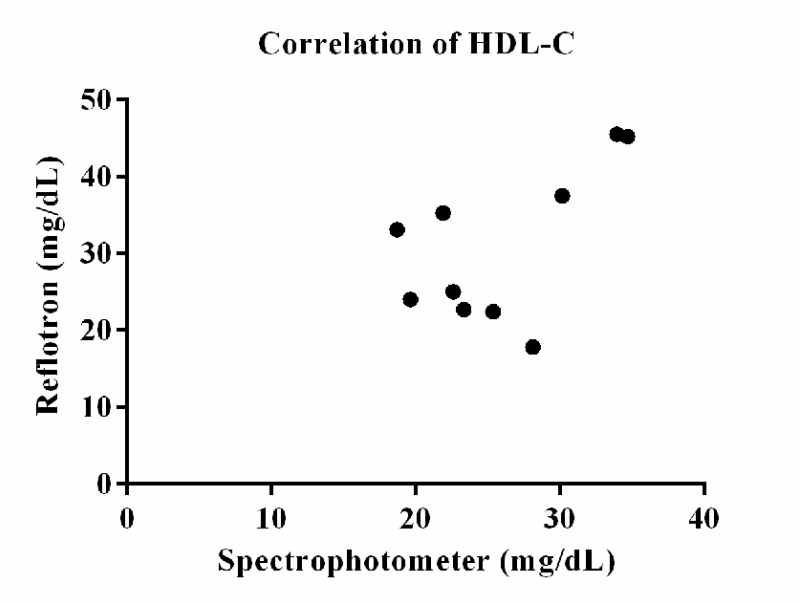

Figure (3): Comparison of results for HDL-C between the spectrophotometer and reflotron method.

Conclusions

In conclusion, the results of the study show that significant differences were found between the two methods during the test for TC, TG and HDL-C. The TC levels obtained by spectrophotometer were 208.2±9.99 mg/dL and by reflotron were 182.2±9.04 mg/dL, ($p<0.0001$), the TC concentration was always higher by spectrophotometer. The TG levels obtained by the two methods were 198.6±29.8 mg/dL and 183.4±25.3 mg/dL for spectrophotometer and reflotron, respectively, ($p<0.0045$). The TG concentration was statistically higher by spectrophotometer. Reflotron was not be able to determine a low concentration of TG below 70 mg/dL, on the other hand spectrophotometer could determine even lower than 10 mg/dL. The HDL-C levels obtained by spectrophotometer were 25.72±0.95 mg/dL and by reflotron were 30.33±1.72 mg/dL, ($p<0.0024$). However the HDL-C concentration was obtained by reflotron significantly higher when compared to spectrophotometer method. From the results it was found that spectrophotometer is more accurate than reflotron for lipid profile measurement.

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Conflict of Interest: None

Research Ethics: All participants agreed to have their lipid profile measured by the study investigator as part of their management and for the data to be used in the study. There were no refusals to participate. The study was approved by the ethics committees in Chemistry Department at University of Raparin in Kurdistan Region - Iraq.

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پوختە

بەراوردکردنی ڕێگای سپێکترۆفۆتۆمیتر (Spectrophotometer) و ڕفلۆترۆن (Reflotron) بۆ پێوانە کردنی چەوریەکانی خوێن.

لە ئێستادا لە زۆربەی تاقیگەکانی شیکاری نەخۆشیەکان، تەکنیکی جۆراو جۆر بەکاردیت بۆ پێوانە کردنی چەوریەکانی خوێن. لەم توێژینەوەدا خوێن لە (10) دە نەخۆش وەرگیراوە لە نەخۆشخانەی گشتی ڕانیە لە ڕانیە، بە مەبەستی پێوانە کردنی چەوریەکانی خوێن لە وانە کۆلیسترۆلی گشتی (total cholesterol)، چەوری سیانی (triglyceride) وە چەوریە پرۆتینی چڕی بەرز (HDL-C).لەمڕۆدا پێوانە کردنی چەوریەکانی خوێن یەکێکە لە شیکاریە ڕۆتینیەکان کە ئەنجام دەدرێت بۆ زۆربەی نەخۆشەکان.

لەم توێژینەوەدا تەکنیکی سپێکترۆفۆتۆمیتر (ڕێگای گیراوەی ئەنزیمی) وە ڕفلۆترۆن (ڕێگای ئەنزیمی وشك بە شێوەی ستریپ) بەکارهاتووە بۆ پێوانە کردنی چەوریەکانی خوێن.

ئەنجامی توێژینەوەکە دەریدەخات کە جیاوازی بەرچاو هەیە لە تێکرای بڕی کۆلیسترۆلی گشتی (total cholesterol)، چەوری سیانی (triglyceride) وە چەوریە پرۆتینی چڕی بەرز (HDL-C) لە نێوان ئەو دوو ڕێگایەی کە بەکار هاتوون بە شێوەیەك کە جیاوازی نێوانیان بۆ کۆلیسترۆلی گشتی ($P < 0.0001$)، چەوری سیانی ($P<0.0045$) وە چەوریە پرۆتینی چڕی بەرز ($P < 0.0024$). ئەو ڕێگایانەی بەکار هاتوون بۆ ئاماری شیکاری بریتین لە (Pearson's correlation, paired T - Test) بە گونجاندنی لە گەڵ ئاستی بەرچاوی نرخی ($P < 0.01$). هەروەها لەم توێژینەوەدا بۆمان دەرکەوت کە ئەنجامی شیکاری کۆلیسترۆلی گشتی (total cholesterol)، چەوری سیانی (triglyceride) کە بە ڕێگای سپێکترۆفۆتۆمیتر (ڕێگای گیراوەی ئەنزیمی) پێوانە کرابوو بەرزترە لە ڕێگای ڕفلۆترۆن (ڕێگای ئەنزیمی وشك بە شێوەی ستریپ) وە بە پێچەوانەوە بۆ چەوریە پرۆتینی چڕی بەرز (HDL-C)، ئەمەش کاریگەری دەبێت لە سەر بڕیارە پزیشکیکان.