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Spectrophotometric determination of Cholesterol by using procaine as coupling reagent

Muthana Saleh Mashkour[1], Naser Abd Alhassan – Almatori[1], Azhar Mohammed Brbber[1]

Chemistry Department / Faculty of Science / Kufa University, Iraq

Abstract : A new, simple, sensitive and rapid spectrophotometric method is proposed for the determination of cholesterol. The method is based on the diazotization reaction of procaine hydrochloride with sodium nitrite in hydrochloric acid medium to form diazonium salt, which is coupled with cholesterol in alkaline medium to form soluble products. The reaction product with cholesterol was azoxy which showed maximum absorption at 428nm. After optimization, the calibration curves were constructed, and subsequently. Result was found that Beer's law obeyed within concentration ranges of 1–15 µg.mL^{-1}of cholesterol. The molar absorptivity was 1.824×104 L.mol^{-1}.cm^{-1}, Sandell's sensitivity was0.0213 µg.cm^{-2}, R.S.D% was 0.578% , with a correlation coefficient of 0.9798 for cholesterol . The interferences were studied, they didn't effect on concentration determination of cholesterol. The method was successfully applied for determination of cholesterol in serum blood and milk.
Keywords: cholesterol, spectrophotometry, milk, serum blood.

Introduction

The reaction between a diazo species and a coupling agent results in the formation of azo compounds. The diazo species is obtained by the diazotization of an aromatic primary amine using nitrous acid1. Azoxa compound can be prepared in several ways, including the reduction of nitro compounds or coupling reaction of diazonium salts with phenols orwith aliphatic compounds containing hydroxyl groups[2]. Cholesterol firstly isolated from gallstones close to the turn of the 19th century and found it not just in human gallstones, but in the blood, brain, and liver, too. Cholesterol was also widely distributed in the organs and tissues of animals. Pioneering work by chemists Otto Diels, Heinrich Wieland and Adolf Windaus led to the discovery, in1932[3]. Cholesterol is a sterol (Sterols is steroid alcohols), not dissolve in water but soluble in organic solvents, found in the cell membranes of all body tissues, and transported in the blood plasma of all animals, lesser amounts of cholesterol are also found in plant membranes[4].

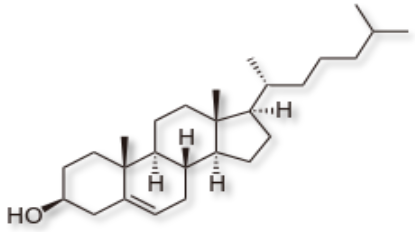

Figure 1-1: Structure of Cholesterol

At present , several techniques for determination cholesterol such as Chromatographic[5], Fluoremetric[6], Chemiluminscence[7], Spectrophotometric[8], Voltammetric[9], Amperometric[10], Coulometric[11], Flow injection[12], and several techniques for determination tyrosine such as Chromatographic[13], Chemiluminscence[14], Spectrophotometric[15], Flow injection[16], Coulometric[17], Fluoremetric18, show good sensitivity but is limited because of expensive instrumentation and high cost for routine analysis . The aim of the study was to develop and validate a simple method for determination of cholesterol concentration. The analytical procedure was characterized to ensure its selectivity, accuracy, and precision for the analysis Cholesterol content in milk sample was determine and serum blood to validate the accuracy of this method.

Experimental

Solutions used in procedure for the selecting maximum wavelength of colure product.

1. Standard solution of cholesterol 200 ug.ml^{-1}

Precisely 0.002g of cholesterol was weighed and dissolved in ethanol and the volume made up to mark of 10 mL volumetric flask.

2. Diazotized Procaine hydrochloride solution

Precisely 0.116 g of procaine hydrochloride was weighed and dissolved with less amount of distilled water in 100 mL beaker, then 3 mL of 0.1 M NaNO2 solution and 3 ml of 1 M HCl solution were added to the beaker. The solution was well mixed and permitted to remain for 5 min at(5-10) ^{0} C, then the solution was transferred into 25 mL volumetric flask and the volume was made up to mark with distilled water where the temperature at(5-10) ^{0} C was kept.

3. Diazotized procaine hydrochloride

Precisely weight of 0.0818 g of procaine hydrochloride was dissolved with less amount of distilled water, then 3 mL of NaNO2 0.1M and 3 mL of HCl1 M were added to the beaker. The solution was allowed to stand for 5 min at (5-10) ^{0} C, the solution was transferred into 50 mL volumetric flask and the volume was made up to mark with distilled water where temperature at (5-10) ^{0} C was kept.

Procedure of selecting maximum wavelength of colure product

1 ml of standard solution of Cholesterol(200 µg.mL^{-1}) was added into 10 mL volumetric flask. 1 ml of the diazotized procaine hydrochloride solution and 1 mL of sodium hydroxide solution (2M) was added to the volumetric flask. The solution was mixed thoroughly, the volume was made to mark with distilled water and the solution allowed standing for 10 min. The solution was scanned in the range of 190 – 1100 nm against reagent blank.

Solutions used in optimization

1. Cholesterol, 100 µg.mL-1

Cholesterol solution was prepared by dissolving 0.05 g in ethanol and the volume was made up in 500 mL volumetric flask. Solutions of further dilute concentrations were prepared from this working standard solution.

2. Diazotized procaine hydrochloride

Precisely weight of 0.0818 g of procaine hydrochloride was dissolved with less amount of distilled water, then 3 mL of NaNO2 0.1M and 3 mL of HCl1 M were added to the beaker. The solution was allowed to stand for 5 min at (5-10) ^{0} C, the solution was transferred into 50 mL volumetric flask and the volume was made up to mark with distilled water where temperature at (5-10) ^{0} C was kept.

Procedure for formation of the colored product and measuring absorbance

1 mLof cholesterol solution (100 µg.mL^{-1})was added into 10 mL volumetric flask. 2 mL of the diazotized procaine hydrochloride solution and 2 mL of 2M NaOH were added to the volumetric flask. The solution was mixed thoroughly, the volume was made up to mark with distilled water and the solution allowed to stand for 5 min. Absorbance of a colored product was measured at 428 nm in case of cholesterol against reagent blank

Apparatus

Spectrophotometric measurement were made with Shimadzu UV – visible – 1800 double beam spectrophotometer) using 1.00 cm glass cells. Infra red spectra were recorded on FTIR – spectrophotometer AL-PHA , BRUKER , Germany.

Results and Discussion

Absorption spectra

When the colorless cholesterol solution is added to the cold solution, also colorless, of diazotized procaine hydrochloride in basic medium, a yellow solution is the result which indicates that there is a reaction. To emphasize the reaction, the yellow colored product as well as to the reactants are scanned in uv-vis.

Region within range of 190-800 nm. Figures 1 show spectra of the colored product, aqueous solution of pure cholesterol and the blank solution, (prepared on addition of sodium hydroxide to this diazonium salt of procaine hydrochloride). From these figures, it's obvious that the yellow colored product which has maximum absorption at 428 nm is significantly different from maximum absorptions of both reactants. The utility of this red shift for the product can be used as a suitable assay procedure for cholesterol determination.

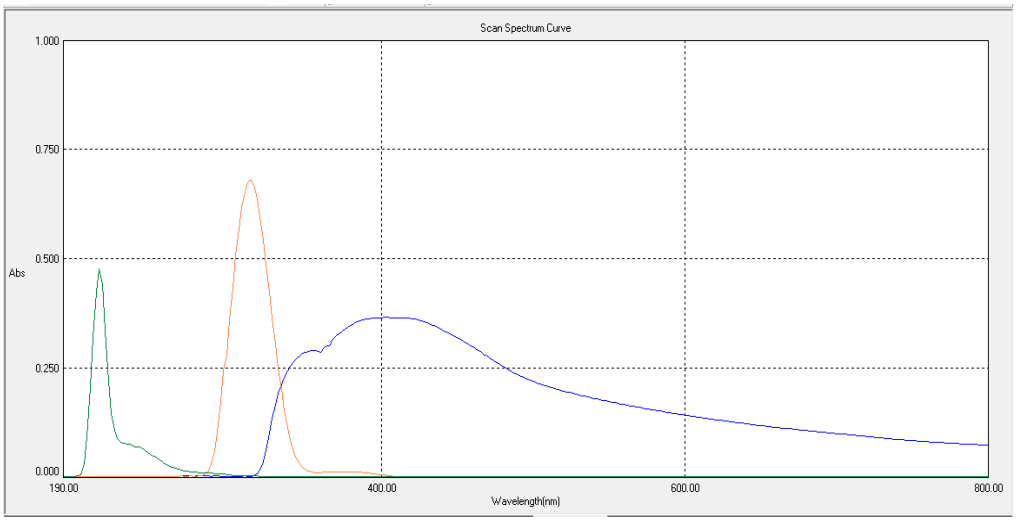

Figure 1 Absorption spectrum of the cholesterol , blank and colored product.

FTIR spectra

FTIR spectrum of product and cholesterol in the Figures (2, 3) show the disappearance of the hydroxyl group in the spectrum of the product as well as the appearance of a band in the region 1601 cm-1,1312 cm-1 dating back to C-N, N-O bonds respectively .

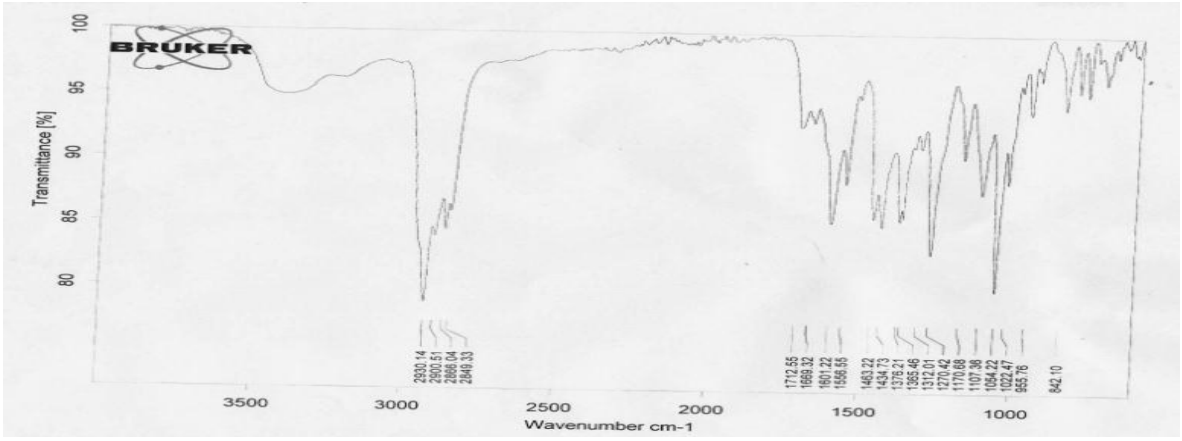

Figure 2:FTIR spectrum of the cholesterol colored product.

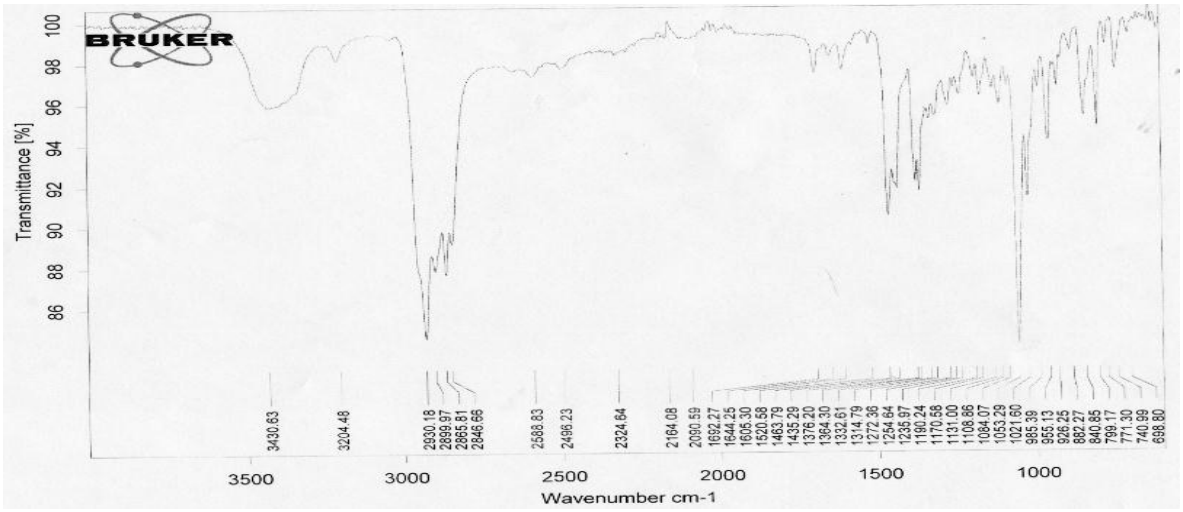

Figure 3: FTIR spectrum of the cholesterol.

Optimization of the experimental condition:

Effect of acids

Acids of different types are used to examine their effect on formation of the colored product. Table 1 shows effect of acid on the absorbance of the colored product. Higher absorbance obtained when HCl used for optimization.

Table 1: Effect type of acid on absorbance of the product

Acids (1 M)	**H_2SO_4**	**HCl**	**HNO_3**	**CH_3COOH**
Abs	0.124	0.164	0.109	0.121

After the best acid is selected, its volume also optimized. The experiment performed in the range of 1 to 6 ml of 1M HCl, Figure 4. The maximum absorbance reached when 2 mL is added. Therefore 2 ml of hydrochloric acid (1M) is adopted for this method.

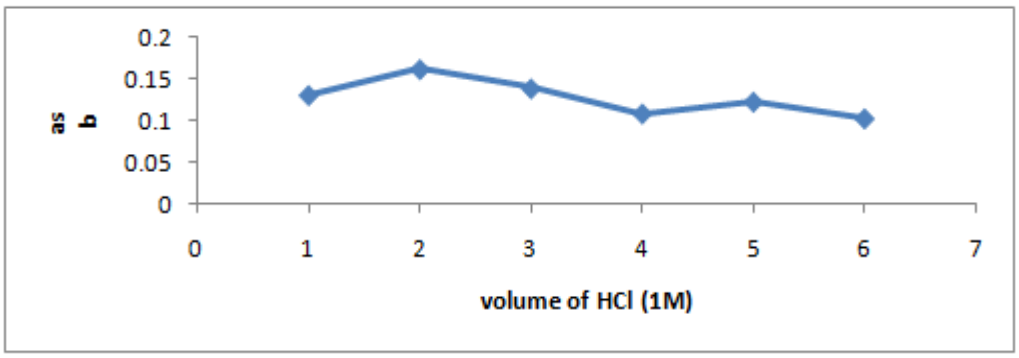

Figure 4 Effect of volume of acid on absorbance of the product.

Effect of sodium nitrite

The amount of sodium nitrite required to obtain maximum absorbance is investigated. Figure 5 shows effect of volume of $NaNO_2$ on absorbance of the product. Higher absorbance obtained when 2 ml of 0.1M of sodium nitrite solution is used, after that absorbance is depressed.

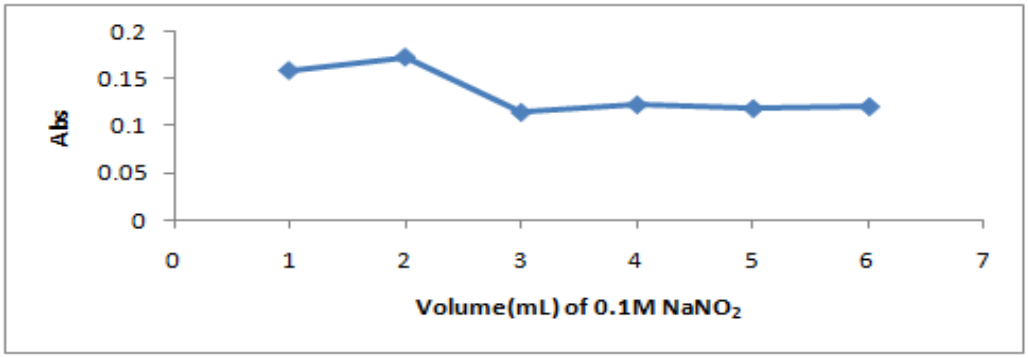

Figure 5 Effect of volume of $NaNO_2$ on absorbance of the product.

Effect of Bases

The effect of different types of bases on enhancement intensity of color for the product is studied. Results are demonstrated in Table 2. Low absorbance is obtained in case for NH_4OH and Na_2CO_3, therefore these two bases are excluded and NaOH is chosen.

Table 2: Effect type of bases on absorbance of the product

Bases(1M)	NH_4OH	Na_2CO_3	KOH	NaOH
Abs	0.105	0.130	0.250	0.160

Figure 6 shows effect of volume of NaOH (2M) on absorbance of the product. From this figure, 2 mL of the base gives highest absorbance and volumes of less than this value insufficient for color development.

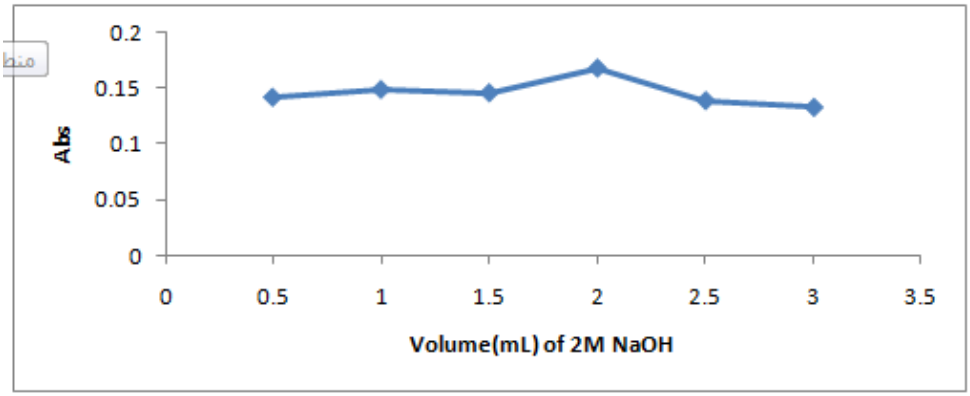

Figure 6 Effect of volume of NaOH on absorbance of the product.

Sequence of addition

Different orders of addition of reagents are examined and it is found from table 3 that the order of addition of reagents by mixing cholesterol, then diazotized procaine and then sodium hydroxide (Ch + P + B)gave the highest absorbance. This sequence gives the best formation of the product and it used in all subsequent experiments.

Table 3: effect of sequence of addition on the absorbance of product

Sequence	Ch + P +B	B + Ch + P	P +B +Ch
Abs	0.162	0.103	0.070

Ch: Cholesterol ; P: Procaine ; B: Base

Stability of the colored product

The color of the formed product reached the full intensity at10 minutes. The results show little stability of formed product with progress in time. Therefore, 10 min development time was selected as optimum in the general procedure, Figure (7).

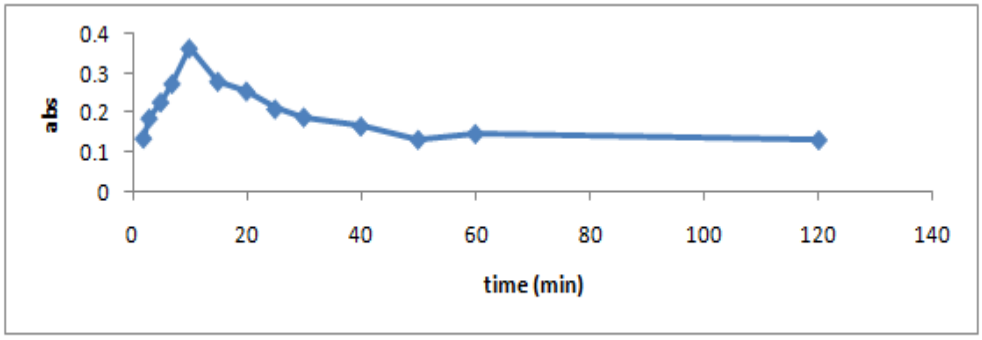

Figure 7: Stability of the colored product with time.

Temperature effect on the color product formed

The effect of temperature on coupling rate was studied at different temperature ranges. Figure 8 shows effect of temperature on color product stability. Coupling rate remained stable at highest values of absorbencies between (20-25) ^{o}C, below this range the stability decreased due to decomposition of the product.

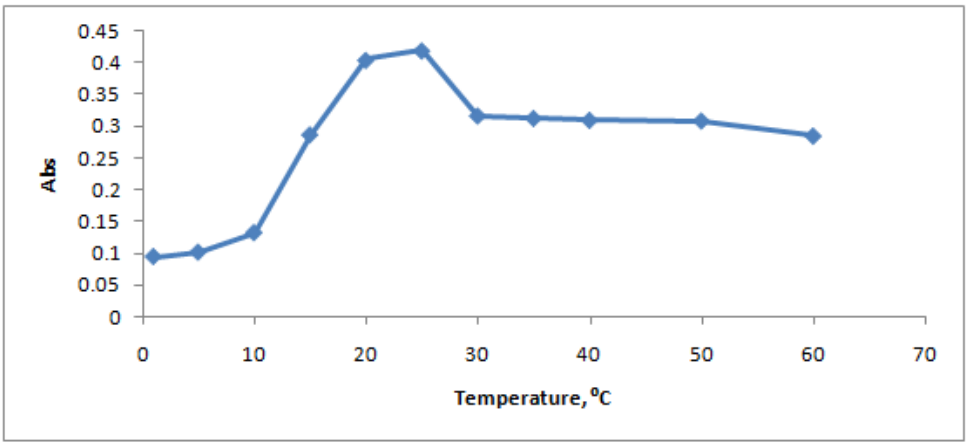

Figure 8: Effect of temperature on absorbance of the product

Calibration curve

Under the optimum conditions which studied above, standard calibration curve has been constructed for the colored product. Figure (9) shows calibration curve for cholesterol which obey to Beer's law within range of concentration (0.5-12)µg.mL^{-1} at 428 nm with correlation coefficient 0.9789.

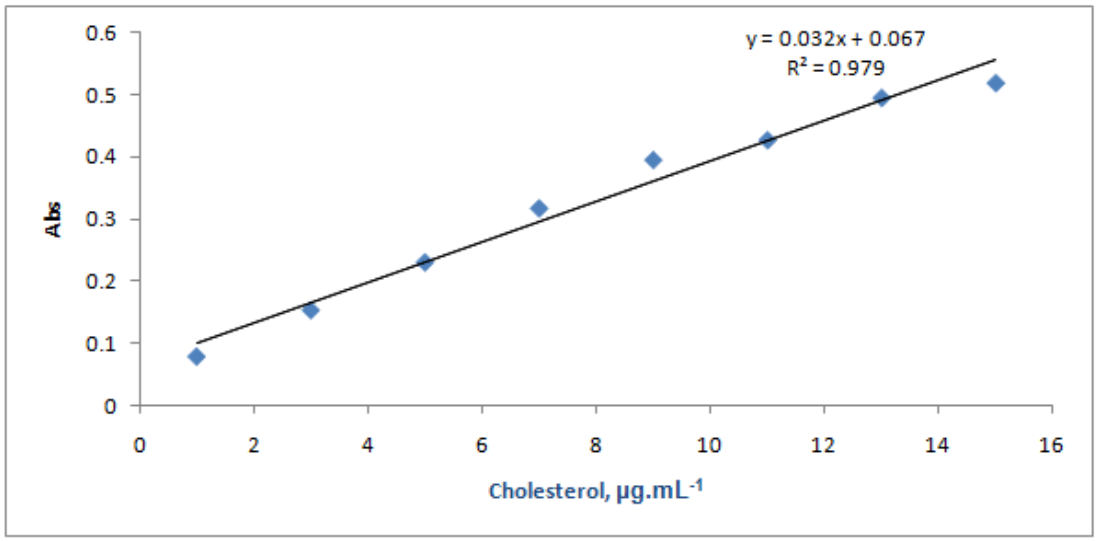

Figure 9: Calibration curve of Cholesterol

Table 4: Analytical parameter for cholesterol determination Estimation the composition of the product

parameter Value	
Beer's law limit (µg.mL^{-1})	1 – 15
Molar absorptivity (L mol^{-1} cm^{-1})	1.824×10^{4}
Sandell's sensitivity(µg.cm^{-2})[19]	0.0213
Detection limit (µg.mL^{-1}) [20]	0.069
LOQ (µg.mL^{-1}) [21]	0.207
Correlation coefficient (r)	0.9798
Determination coefficient(r^2)	0.9600
Slope(b)	0.0675

Figure (10) shows mole ratio plot for the cholesterol product where the two lines are crossed at mole fraction 1.1, also Figure (11) shows continuous variation plot for same product which gives maxima at volume fraction of 0.5. The results refer to that the ratio of cholesterol:procaine is 1:1.

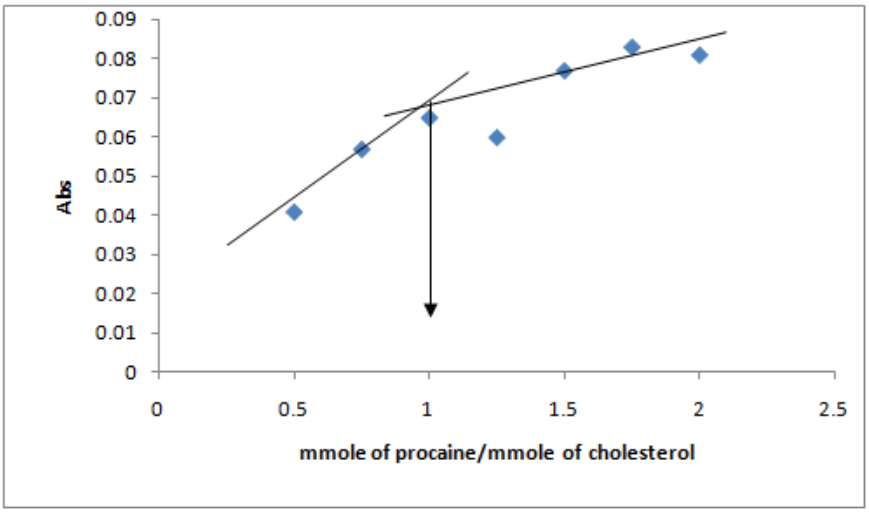

Figure 10: Mole ratio plot (cholesterol : procaine)

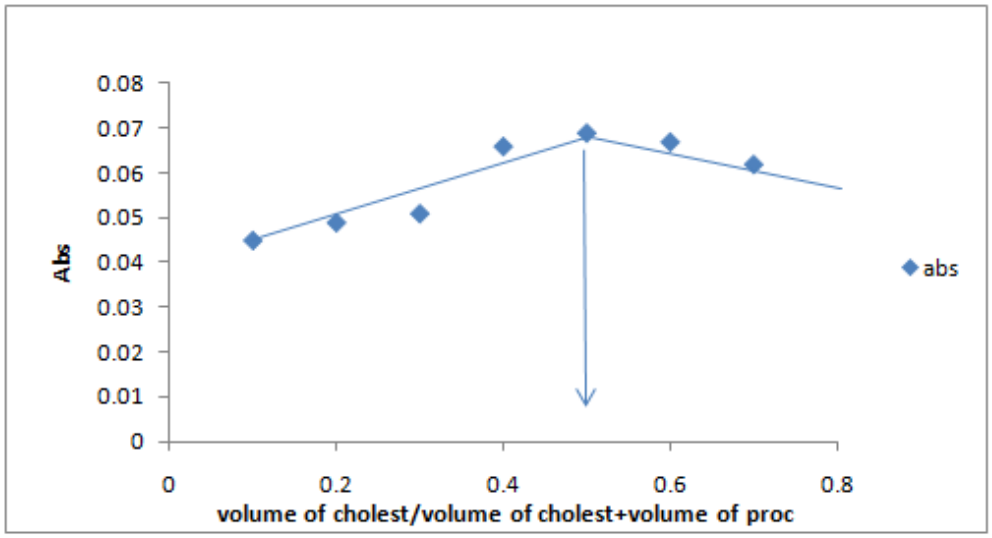

Figure 11: Continuous variation plot (cholesterol : procaine

Estimation of stability constants of product

After it was determine the proportion of complex components (then be prepared solutions containing equal concentrations of the cholesterol and diazotized procaine hydrochloride , and measured absorbance of the solution formed at 428 nm, and expresses this absorbance value of Es.

As well as the preparation of solutions containing the same amount of cholesterol and diazotized procaine hydrochloride, but there is an increase of diazotized procaine hydrochloride, measured absorbance at 428 nm and expresses this absorbance value of E_m and using the equation following can be obtained on the degree of dissociation.

Interferences

In order to assess the possible analytical application of the spectrophotometric method described above, the effect of interferes on the determination of cholesterol in real samples was studied by analyzing synthetic sample solution that contain cholesterol and various excess amount of interferes, table (5).

Table 5: Effect of interference on the determination of cholesterol (10 ug.ml^{-1})

Interferent	Found	Erel %	Recovery %
Perodxine	10.055	-3.188	96.812
Folic acid	10.175	-2.031	97.969
Vitamin D	10.446	0.577	100.577
Vitamin A	10.567	1.742	101.742

Application of the method

Determination of cholesterol in food samples and serum blood

To test the applicability of the present method, it has been applied to the determination of cholesterol in samples above. The reliability of the method to analyze this real sample is checked by recovery experiment with method of Kit as standard method for cholesterol, table (6). The recovery is close to 100% and indicates by applying the proposed procedure, good recovery is obtained.

Table 6: Comparison of suggested method with kit method

Sample	Present µg . mL^{-1}	Suggested method µg . mL^{-1}	Recovery %	Present µg . mL^{-1}	kit method mg/dl	Recovery %
Al-mudhish milk	140	144.063	102.9	140	138.1	98.64
Kala milk	150	132.778	88.5	150	146.3	97.53
Serum blood 8	1910.2	1930.89	102.08	1910.2	1897.9	99.35
KDD milk	10	10.251	102.5	10	9.82	98.2

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