Square Wave Voltammetric Method as a Tool for Determination of Cholesterol Quantity in Human Blood Serum

Amer Th. Al-Taee & Ramiz S. Kassir
Department of Chemistry, College of Science, University of Mosul
Mosul, IRAQ.

(NJC)

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Abstract

A new method for the determination of Cholesterol in human blood serum based on Square Wave Voltammetry (SWV) has been described. This method is relay on the decrease in the reduction peak of 4-aminoantipyrine appeared at (+0.004 V) vs. (Ag/AgC1,3M KCl) as a reference electrode using Phosphate buffer (pH 7.0) as supporting electrolyte, the method is sensitive and rapid. The procedure was successfully applied to the determination of cholesterol in various blood samples represent different cases such as: Hypothyroidism, Nephrotic syndrome, Obstructive Jaundice & Diabetes Mellitus. The results have been compared with those obtained from the spectrophotometric method, a good agreement between the two methods has been obtained with correlation coefficient (r = 0.9986).

الخلاصة

يتضمن البحث طريقة جديدة لتقدير كمية الكوليستيسرول (Cholesterol) في مصل الدم البشري باستخدام تقنية فولتامتري الموجة المربعة (SWV). حيث تعتمد على قياس الانخفاض في القمة البولاروغرافية لاختزال مادة 4-امينو انتي بايرين (4-aminoantipyrine) المستهلك خلال التفاعل الانزيمي للكوليستيرول والتي تظهر عند جهد (+0.004) فولت ضد قطب المرجع (فضة / كلوريد الفضة، 3 مولاري كلوريد البوتاسيوم) باستخدام محلول الفوسفات المنظم (pH=7.0). وقد تم تطبيق الطريقة بنجاح لقياس كمية الكوليستيرول في عينات مصل الدم لاشخاص اصحاء وكذلك المصابين في حالات مرضية مختلفة مثل : قلة افراز الغدة الدرقية ، التهاب الكلية الحاد ، مرض الانسداد اليرقاني وكذلك مرضى داء السكري. وكذلك تم مقارنة النتائج المستحصلة مع الطريقة اللونية وكانت العلاقة خطية بمعامل ارتباط (r = 0.9986).

Introduction

Cholesterol is a fatty substance found only in animal and human cells. Cholesterol is essential for the proper functioning of your body. It is used to produce new cells and certain hormones. About 75% of the cholesterol in your body is made in the liver [(1)]. The Chemical Structure of Cholesterol as show in **[Fig. (1)]**.

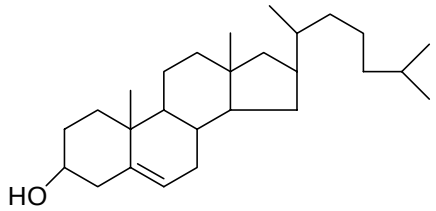

Fig. (1): Show the chemical structure of Cholesterol. [2]

Everyone's body contains several proteins that attach themselves to cholesterol. One is called L.D.L. (Low density lipoprotein), which is particularly *harmful*. It is believed to collect cholesterol and deposit it in the cells. The good cholesterol is called H.D.L. (High density lipoprotein), which is thought to pick up excess cholesterol and help the body to eliminate it. Those who have high HDL in their blood seem to a have lower incidence of coronary heart disease than others [3,4]

At present it is recommended to have a blood cholesterol below 200 mg/dl (5.2 mmol/l), a blood level of cholesterol between 200-240 mg/dl (5.2-6.2 mmol/l)is called borderline-high. If your blood cholesterol is above 240mg/dl (6.2 mmol/l) this is considered high. It is worth remembering that the higher the level of bad cholesterol, the higher the risk of having a heart attack. [4] [9,10]

Too much cholesterol in the body is thought to be associated with heart disease, so that it is worth to find a new and simple method for monitoring level in human blood serum. Square-wave voltammetry has several advantages. Among these are its excellent sensitivity and the rejection of background currents. Another is the speed (for example, its ability to scan the voltage range over one drop during polarography with the Dropping Mercury Electrode (DME)). This speed, coupled with computer control and signal averaging, allows for experiments to be performed repetitively and increases the signalto-noise ratio. Applications of square-wave voltammetry include the study of electrode kinetics with regard to preceding, following, or catalytic homogeneous chemical reactions, determination of some species at trace levels, and its use with electrochemical detection in HPLC. [11,12]

Several methods are available for determination of cholesterol. The colorimetric method is based on the flowing reaction: [6-8]

$$Cholesterolester \xrightarrow{Cholesterolesterase} Cholesterol + Fattyacids$$

$$Cholesterol \xrightarrow{Cholesteroloxidase} Cholest-4-en-3-one + H_2O_2$$

$$2H_2O_2 + Phenol + 4-a\min oantipyrine \xrightarrow{Peroxidase} Quinonei\min e + 4H_2O$$

This method needs many different reagent for colour development and its less sensitive than electrometric method.

The Differential Pulse Polarography (DPP) and Differential Pulse Anodic Stripping Voltammetry (DPASV) have been applied to measure the activity of some enzymes such as: Alcohol dehydrogenase (ADH)[13,14], Lactate dehydrogenase (LDH), Isocitrate dehydrogenase (ICDH) [15-18],

In the present work, the Square

Wave Voltammetry (SWV) is successfully applied for determination of cholesterol quantity in human blood serum.

Experimental

Reagents:

1. ***Reagent 1 (Buffer)*** from ***Cholesterol Enzymatique PAP (Ref. 61224) (bioMerieux):***
   Phosphate buffer (0.1 mol/l), phenol (15 mmol/l) & sodium cholate surfactant (3.74 mmol/l) in a total volume (100 ml).
2. ***Reagent 2 (Enzymes)*** from ***Cholesterol Enzymatique PAP (Ref. 61224) (bioMerieux):***
   4-aminoantipyrine(0.5mmol/l), peroxidase (≥1000U/l),cholesterol oxidase (≥ 200 U/l) & cholesterol esterase (≥ 125 U/l)
3. **Standard solution:** Calibrator 5.17 mmol/l (200 mg/100 ml) (2 g/l) ***(Ref.62473)***
4. ***Phosphate Buffer*** **(0.2 M)** ***at*** **(pH 7.0).**
   Freshly prepared by dissolving (3.4836 gm) of K_2HPO_4 and (2.7218 gm) of KH_2PO_4 in a total volume (100 ml) of Distill water.
5. ***(PRECISION MULTI-SERA LOW HUMAN)(Cat.No.UL2701,)(RANDOX,):***
   Reconstitute each vial of lypophilised serum with exactly (5 ml) of distilled water. then stand for (30 min.) out of bright light before use.
6. ***(PRECISION MULTI-SERA NORMAL HUMAN)(Cat. No. UN 1557) (RANDOX,):***
   Reconstitute each vial of lypophilised serum with exactly (5 ml) of distilled water. then stand for (30 min.) out of bright light before use.
7. ***(PRECISION MULTI-SERA ELEVATED HUMAN)(Cat. No. UE 1558) (RANDOX,):***
   Reconstitute each vial of lypophilised serum with exactly (5 ml) of distilled water. then stand for (30 min.) out of bright light before use.

Specimen Collection and Preparation

Samples of human serum were obtained from routine clinical assays. Serum samples were prepared and assayed within (1 hr), otherwise the serum should be kept frozen.

Apparatus:

All the voltammetric measurements were performed using (Computerized Polarographic Analyzer) from (***EG&G***) company which include (HMDE) model (303A) and (Digital Plotter) model (DMP 04-44). The (HMDE) unit consists three electrodes: The Working electrode was a Hanging Mercury Dropping Electrode (HMDE). The Reference Electrode was (Ag/AgCl,3M KCl) and a Pt wire as a Counter Electrode.

Temperature control was perform using thermostatic water bath type radiometer VTS 13 (±0.1 ^{0}C).

But the colorimetric measurements were performed by using (Cecil Spectrophotometer) model (CE 10211 Ultra Violet & Visible Spectrohotometer) from Cecil Instruments Limited. Temperature control was perform using thermostatic water bath model (SB 10) from British Grant Instruments Limited.

Procedure:

The optimum conditions were examined using (SWV) are shown in **Tables (1-5)** as: Deposition time (35 s), Conditioning time (15 s), Equilibrium time (5 s), Scan Rate (100 mV/s) & Frequency (120 Hz). The voltammetric cell was thermostated at (37^{o}C). The solution was deaerted by passing a slow stream of purified N_2 gas through it for (5 min.) to remove the dissolved oxygen.

Table (1): Effect of Deposition Time (Dep. Time) on the reduction peak of 4-aminoantipyrine at Ep = + 0.004 V

Dep. Time (sec.)	Ip (nA)
5	6140
10	6535
15	6860
20	7340
25	7950
30	8100
35	**8540**
40	8750
45	8750
50	8850
55	9050
60	9090

Table (2): Effect of Conditioning Time (Cond. Time) on the reduction peak of 4-aminoantipyrine at Ep = + 0.004 V

Cond. Time (sec.)	Ip (nA)
0	8410
5	8440
10	8460
15	**8800**
20	8690
25	8630
30	8510

Table (3): Effect of Equilibrium Time (Equi. Time) on the reduction peak of 4-aminoantipyrine at Ep = + 0.004 V

Equi. Time (sec.)	Ip (nA)
0	8740
5	**8850**
10	8300
15	8190

Table (4): Effect of Scan Rate on the reduction peak of 4-aminoantipyrine at Ep = + 0.004 V

Scan Rate (mV/sec)	Ip (nA)
100	**8910**
200	8550
300	7960
400	8210

Table (5): Effect of Frequency on the reduction peak of 4-aminoantipyrine at Ep = + 0.004 V

Frequency (Hz)	Ip (nA)
50	3500
100	8850
120	**11600**

For Voltammetric measurement, the sample cuvette contained (5ml) of (0.2 M) Phosphate Buffer at (pH 7.0) and (0.1 ml) Working solution. The Square Wave voltammogram of 4-aminoantipyrine was recorded between (+0.2 V to -0.2 V), the reaction was initiated by addition of (5 µl) of Serum, then voltammogram was again recorded.

The concentration of Inorganic Phosphorus $= \frac{\mathbf{Ip}_{\mathbf{Blank}} - \mathbf{Ip}_{\mathbf{Sample}}}{\mathbf{Ip}_{\mathbf{Blank}} - \mathbf{Ip}_{\mathbf{Standard}}} \mathbf{x\ n}$ **,** which in :-

$\mathbf{Ip}_{\mathbf{Blank}}$	is the value of diffusion current of 4-aminoantipyrine before serum addition, the unit of $\mathbf{Ip}_{\mathbf{Blank}}$ is nanoAmper (nA).
$\mathbf{Ip}_{\mathbf{Sample}}$	is the value of diffusion current of 4-aminoantipyrine after serum addition, the unit of $\mathbf{Ip}_{\mathbf{Sample}}$ is (nA).
$\mathbf{Ip}_{\mathbf{Standard}}$	is the value of diffusion current of the standard, the experimental value of $\mathbf{Ip}_{\mathbf{Standard}}$ equal to **8530 nA**, which in the value of $\mathbf{Ip}_{\mathbf{Blank}}$ for standard equal to **11150 nA**
n	is the value of the standard concentration equal to **5.17 mmol/l**.

For colorimetric determinations, we prepared **Working solution** by prepared by reconstitute the contents of one vial of (***Reagent 2***) with the contents of one vial of (***Reagent 1***), mix the solution by inverting and store it in the Reagent 1 bottle, this solution is stable for about three months at (2-8^{o}C). The wavelength that used in colorimetric method was 500 nm. We used Distilled water as Reagent blank to made Zero adjustment. The procedure that used in colorimetric method could be shown in the following list :-

	Reagent blank	Standard	Sample
Standard	----------	10 µl	----------
Sample	----------	----------	10 µl
Working solution	1.0 ml	1.0 ml	1.0 ml
Distilled Water	10 µl	----------	----------

After prepared three tubes for each sample as shown in above, we were mixing these tubes and measured the absorbance of each sample after incubation at 37° C in water bath for (5) minutes. The color intensity was stable about (30) minutes. The calculation of colorimetric method were show in the flowing equation:-

The concentration of Inorganic Phosphorus = $\frac{\mathbf{A_{Sample}}}{\mathbf{A_{S\,tan\,dard}}}$ **x n ,** which in :-

$\mathbf{A_{Sample}}$	is the value of the absorbance for each sample.
$\mathbf{A_{Standard}}$	is the value of the absorbance standard, the experimental value of $\mathbf{A_{standard}}$ equal to **0.356**
n	is the value of the standard concentration equal to **5.17 mmol/l**.

Results and Discussion

In the enzymatic reaction of Cholesterol from *4 - aminoantipyrine* → *Quinoniemine* direction, continuous under regular consumption of 4-aminoantipyrine with time could be quantitatively followed through the Square Wave Voltammetry.

Recently, we found that 4-aminoantipyrine gave a well known defined peak at (+ 0.004V) vs. (Ag/AgCl, 3M KCl) in phosphate buffer at (pH 7.0) **[Fig. 2(a)]**

After addition of serum, we saw decrease (Ip) of 4-aminoantipyrine, with increasing addition of serum due to the consumption of 4-aminoantipyrine during the enzymatic reaction. **[Fig. 2(b)]**

Optimization of Conditions:

Effect of Working Solution

In order to determine the optimum concentration of 4-aminoantipyrine, a series of experiments were carried out in which working solution added in a range between (0.01-0.1ml) to a voltammographic cell containing (5ml) of phosphate buffer at (pH 7.0). The result indicates that (0.1 ml) of working solution is suitable to determine the cholesterol quantity in clinical applications due to the maximum diffusion current (Ip) obtained **(Table 6)**

Effect of Standard

The effect of standard amount was examined by addition of increasing amount of standard in the range (1.0-5.0 µl) to a voltammographic cell containing (5 ml) of phosphate buffer (pH 7.0), and (0.1ml) of working solution, The result shows that (5 µl) of standard represent the optimum amount for determination, because it gives the maximum value of diffusion current (Ip) **(Table 7).**

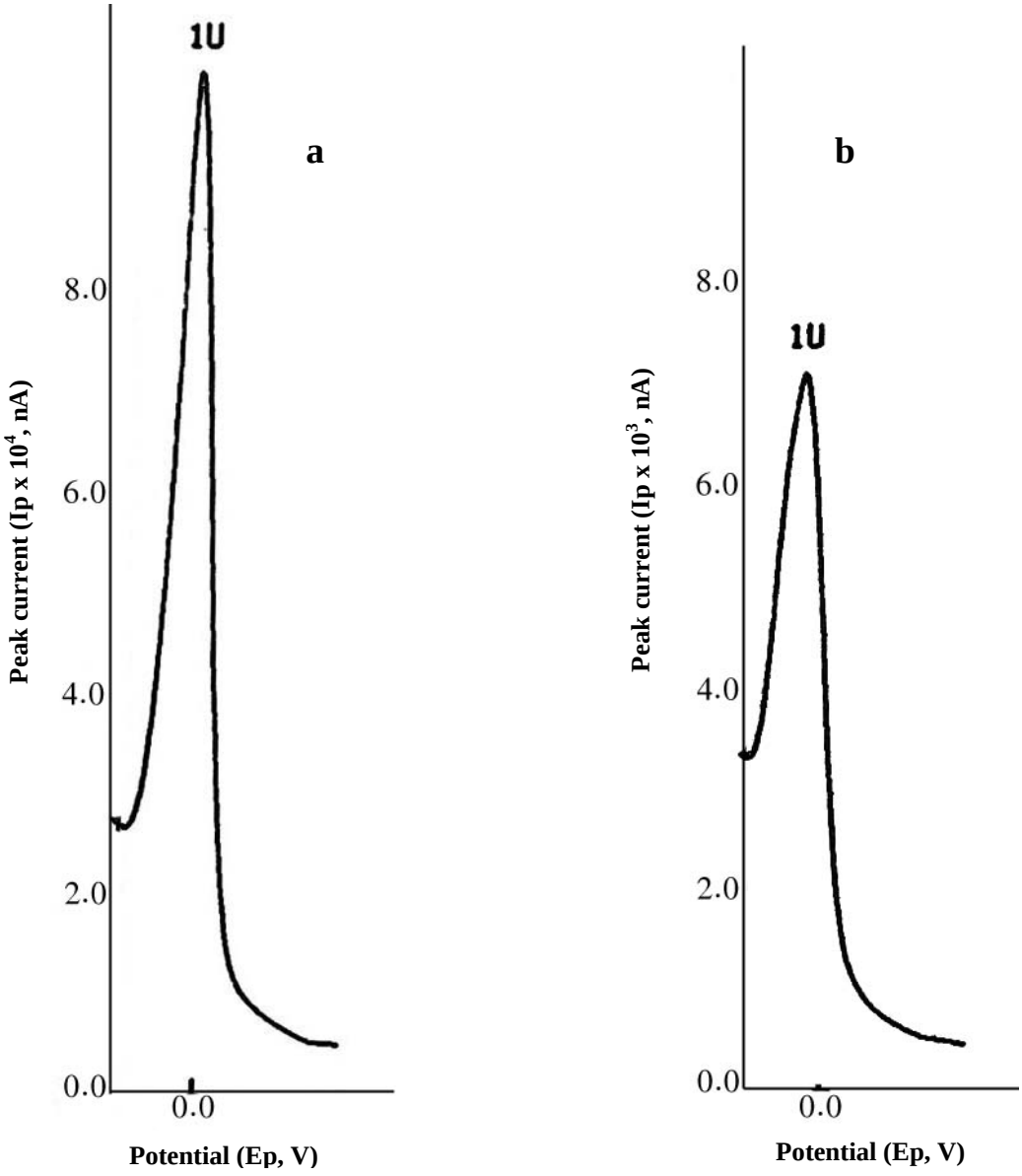

Fig. (2) : Square-wave Voltammograms of 4-aminoantipyrine in Phosphate buffer pH 7.0 :-
(a) Before the addition of human blood serum.
(b) After the addition of human blood serum.

Table (6): Effect of Working Solution amount on the reduction peak of 4-aminoantipyrine at Ep = + 0.004 V

Addition of Working Solution (ml)	Ip (nA)
0.01	525
0.02	7990
0.03	5200
0.04	7360
0.05	8380
0.06	9140
0.07	9450
0.08	9730
0.09	10009
0.10	**10045**

Table (7): Effect of Standard amount on the reduction peak of 4-aminoantipyrine at Ep = + 0.004 V

Addition of Standard (µl)	Ip (nA)
1.0	10000
1.5	7850
2.0	8210
2.5	6900
3.0	6830
3.5	6660
4.0	6540
4.5	6390
5.0	**6150**

A Comparison Between SWV and Colorimetric Methods :

A comparison between the two methods were carried out using (26 Samples) represent a normal cases patients suffered from different diseases such as: Hypothyroidism, Diabetes Mellitus, Nephritic Syndrome & Obstructive Jaundice. The results obtained are shown in **(Table 9).**

The plot of concentration measured by SWV method versus that measured by colorimetric method that seen in **[Fig. (3)]** gives a straight line with correlation coefficient (r = 0.9986) , (RSQ = 0.9971) that's indicates the good agreement between the two methods and the relation between them can be represented by the following equation:

Conc. by SWV method = -1.7601 + [1.5019 * Conc. by Colorimetric method]

Quality Control

To examine the accuracy and reproducibility control for the proposed method multi-sera low, normal and elevated were assayed, the results shows good agreement between the two methods **(Table 8).**

Table (8): A Quality Control of Cholesterol quantity by the two methods: [(SWV method) and (Colorimetric method)].

Quality Control Standards	Range (mmol/l)	Colorimetric Method (mmol/l)	SWV Method (mmol/l)
Low Human Sera	3.5-4.8	4.226	4.519
Normal Human Sera	3.7-4.7	4.589	5.078
Elevated Human Sera	6.9-8.45	7.408	9.458

Table (9): The obtained results for determination of Cholesterol using two methods: SWV method and Colorimetric method in Normal & Abnormal cases.

Voltammetric Method (SWV method)				*Colorimetric Method*	
Sample No.	**Ip $_{Blank}$ (nA)**	**Ip $_{Sample}$ (nA)**	**Conc. (mmol/l)**	**A $_{Sample}$**	**Conc. (mmol/l)**
1	11030	6357	**9.221**	0.499	**7.247**
2	11530	7823	**7.315**	0.416	**6.041**
3	11780	8367	**6.735**	0.375	**5.446**
4	11530	5364	**12.167**	0.633	**9.193**
5	11940	10250	**3.335**	0.221	**3.209**
6	11460	8760	**5.328**	0.321	**4.662**
7	11910	9643	**4.473**	0.280	**4.066**
8	11340	7803	**6.980**	0.401	**5.824**
9	11540	6494	**9.957**	0.548	**7.958**
10	10640	8084	**5.044**	0.310	**4.502**
11	11880	8853	**5.973**	0.356	**5.170**
12	11860	8901	**5.839**	0.350	**5.083**
13	13080	9951	**6.174**	0.365	**5.301**
14	12640	11449	**2.350**	0.194	**2.817**
15	10530	7583	**5.815**	0.349	**5.068**
16	9910	4967	**9.754**	0.551	**8.002**
17	10110	5496	**9.105**	0.496	**7.203**
18	10640	8316	**4.586**	0.294	**4.270**
19	11244	6982	**8.410**	0.465	**6.753**
20	11244	6483	**9.395**	0.509	**7.392**
21	11244	9589	**3.266**	0.235	**3.413**
22	11244	6993	**8.388**	0.464	**6.738**
23	11244	6242	**9.870**	0.549	**7.973**
24	11244	8342	**5.726**	0.345	**5.010**
25	10740	6024	**9.306**	0.505	**7.334**
26	11260	2136	**18.004**	0.894	**12.983**

Which in:-
Conc. is the Concentration of Cholesterol in human blood serum measured by two methods: SWV method and Colorimetric method.

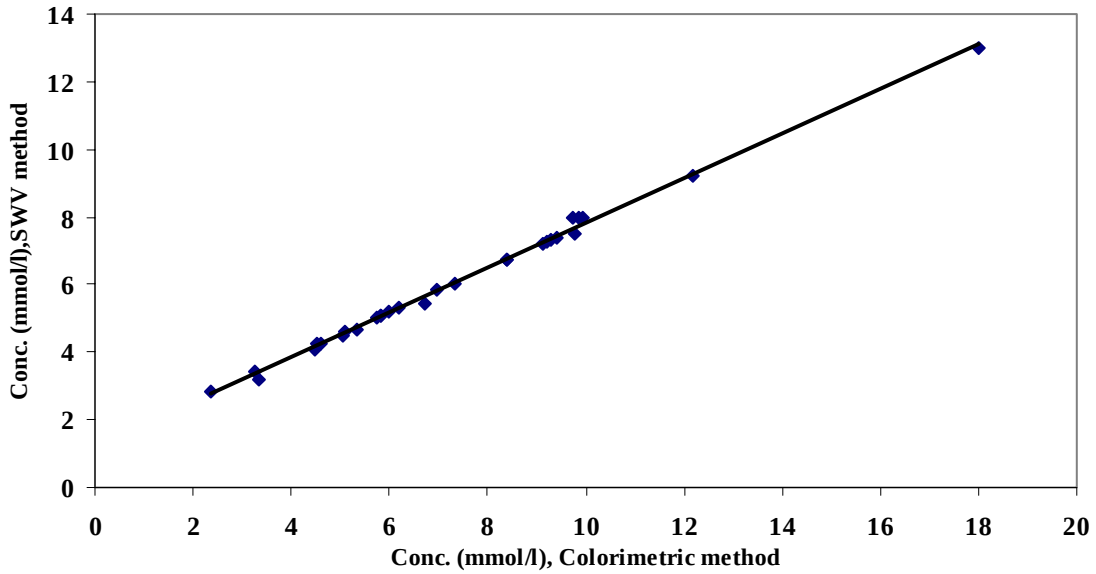

Fig. (3): The relation between Concentrations of Cholesterol measured by SWV method and Colorimetric method for Normal & Abnormal cases.

References

1. N. W. Tietz, "Fundamentals of clinical chemistry," 2nd ed.,W. B. Saunders company, Philadelphia, (1976), p. 1056-1058.
2. J. F. Zilva, and P. R. Pannall, "Clinical chemistry in diagnosis and treatment," 5th ed., Lloyd-Luke (Medical Books) LTD, London, (1984), p.232-233.
3. K. Talaro, and A. Talaro, "Foundations In MICROBILOLOGY," 2nd ed., Wm.C.Brown Publishers, Chicago, (1996), p. 220-223.
4. Hassan Chamsi Pasha & Dr. Fawaz Akhras, " Patient Guidelines to Heart Disease: Questions and Answers ", (1999), Dar Almanara – Jeddah, p.100-104.
5. T. S. AL- Najafi, "Cell Biology," 1st ed., Mosul University Press, (l994)' p. 49-51.
6. RICHMAN W.- ***Clin. Chem***., 1973, **19**, 1350.
7. FLEGG H. M. – ***Clin. Biochem.***, 1973, **10**, 79.
8. ALLAIN C. C. et al. – ***Clin. Chem.***, 1974, **20**, 470.
9. Study Group, Eurpean Atherosclersis society, ***European Heart Journal***, 1988, **9**, 571.
10. ARCOL, ***ISB,*** 1989, **15,** 121-124.
11. W. M. Peterson, and R. V. Wong, Fundamentals of stripping voltammetry, EG&G PRINUE TON APPLIED RESEARCH Electrochemisuy Product Group, (1981).
12. www.yahoo.com/Science/Chemistry/Electrochemistry/VoltammetricTechniques.pdf, Yahoo!Inc.,(2004)
13. S. T. Sulaiman and M. M. N. Saleen, Freserius Z., ***Anal. Chem.***, 1984, **317**, 750.
14. S. T. Sulaiman and M. M. N. Saleen, ***Analyst***, 1985, **110**, 1193.
15. S. T. Sulaiman and T. S. AI-Najafi and H. S. Hamdoon, ***Analyst***,1994, **119**, 2199.
16. S. T. Sulaiman and T. S. AI-Najafi and H. S. Hamdoon, "Polarographic measurement of lactate dehydrogenase activity in human serum, clinical application". Accepted for publication in Raf. J. Science (2001).

17. S. T. Sulaiman and T. S. AI-Najafi and H. S. Hamdoon, "Differential-pulse polarographic behavior of a-ketoglutarate application to the measurement of Isocitrate Dehydrogenase Activity in serum", submitted for publication (2002).

18. S. T. Sulaiman, H. A. Al-wahab & R. S. Kassir, ***National Journal of Chemistry***, 2003, **11**, 377.