Determination of Inorganic Phosphorus in Human Blood Serum by use Square Wave Voltammetry (SWV)

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Abstract

A square wave voltammetric method is described for the determination of Inorganic Phosphorus in human blood serum. This method is based on the decrease on the reduction peak of ferrous ammonium sulphate $Fe(NH_4)_2(SO_4)_2.6H_2O$ appeared at (-0.2 V) vs. (Ag/AgCl,3M KCl) as a reference electrode using (Sodium acetate, acetic acid) buffer adjusted by (1.0 N) NaOH to (pH 7.0) as supporting electrolyte due to its consumption via the enzymatic reaction of Inorganic Phosphorus. The method was successfully applied to measure the Inorganic Phosphorus in various blood samples represent different cases such as: hyperparathyroidism, rickets (in children), osteomalacia (in adults), chronic renal failure, Addison's disease and diabetic ketoacidosis.

The results have been compared with those obtained from the spectrophotometric method and show a good agreement between the two methods with correlation coefficient (r = 0.9996).

الخلاصة

تتضمن الطريقة تقدير كمية الفوسفات اللاعضوية (Inorganic Phosphorus) في مصل الدم البشري باستخدام طريقة فولتامتري الموجة المربعة (SWV). حيث تعتمد على قياس الانخفاض في القمة البولاروغرافية لاختزال مادة كبريتات الامونيوم الحديدوزي (ferrous ammonium sulphate) المستهلك خلال التفاعل الانزيمي للفوسفات اللاعضوية والتي تظهر عند جهد (0.2-) فولت ضد قطب المرجع (فضة / كلوريد الفضة، 3 مولاري كلوريد البوتاسيوم) باستخدام محلول (خلات الصوديوم، حامض الخليك) المنظم مع بعض القطرات من (1 عياري) هيدروكسيد الصوديوم للوصول الى الرقم الهيدروجيني (pH=7.0) كعامل مساعد . حيث تم تطبيق الطريقة بنجاح لقياس كمية الفوسفات اللاعضوية في عينات مصل الدم لاشخاص اصحاء وكذلك المصابين في حالات مرضية مختلفة مثل : زيادة افراز الغدة الدرقية، مرض الكساح (لدى الاطفال)، مرض نخر العظام (لدى الكبار)، التهاب الكلية المزمن، مرض اديسون و مرض السكري وكذلك تم مقارنة النتائج المستحصلة مع الطريقة اللونية وكانت العلاقة خطية بمعامل ارتباط (r = 0.9996).

Introduction

Phosphorus is a group 5 element of the periodic table and has an atomic weight of 30.97. It is most commonly found in nature in its pentavalent form in combination with oxygen, as phosphate (PO_4^{3-}). Phosphorus is widely found in many food groups, largely as phosphate(s). Dietary sources that are rich in phosphorus include red meats (1600 mg/kg), dairy products (> 900 mg/kg), fish (4000 mg/kg), poultry (2100 mg/kg) and bread and other cereal products (> 900 mg/kg). A number of phosphate salts are used in foods and soft drinks as additives [(1)]. Phosphorus is also used in food supplements (at levels up to a daily dose of 1100 mg/day) and licensed medicines, in the form of inorganic phosphate salts and sodium acid phosphate, respectively. [(2)]

Phosphorus is abundant in the body with largest amounts found in bone. It is also found in all soft tissues including muscle, liver, heart and kidneys. Approximately 80% of the body phosphorus is present in the skeleton and the remainder is distributed in soft tissues and extracellular fluid. About 70% of the phosphorus in blood is as a constituent of phospholipids; the remainder is present as inorganic phosphate (HPO_4^{-2}), about 85% free and 15% protein-bound [(3,4)]. It is a constituent of all major classes of biochemical compounds. Structurally, phosphorus occurs as phospholipids, which are a major constituent of most biological membranes, and as nucleotides and nucleic acids. Phosphorus plays an important role in carbohydrate, fat and protein metabolism and is essential for optimum bone health.[(5,6)]

The Normal levels of serum phosphorus range 2.5-4.5 mg/dl (0.81-1.45 mmol/l). Increased levels of serum phosphorus are seen in renal disease, hypoparathyroidism and excessive Vitamin D intake. Decreased levels are seen in rickets, osteomalacia (adult rickets), hyperparathyroidism and diabetic coma [(7-10)], Lastly, Phosphorus is primarily excreted in the urine. The regulation of phosphorus excretion is apparent from early infancy. In infants, as in adults, the major site for the regulation of the amount of phosphorus retained by the body is the kidney. [(11)]

Square Wave Voltammetry (SWV) is a pulsed voltammetry technique that uses a potential waveform as shown in **[Fig.(1)]**. The advantage of square wave voltammetry is that the entire scan can be performed on a single mercury drop in about 10 seconds, as opposed to about 5 minutes for the techniques described previously. SWV saves time, reduces the amount of mercury used per scan by a factor of 100. If used with a pre-reduction step, detection limits of 1-10 ppb can be achieved.

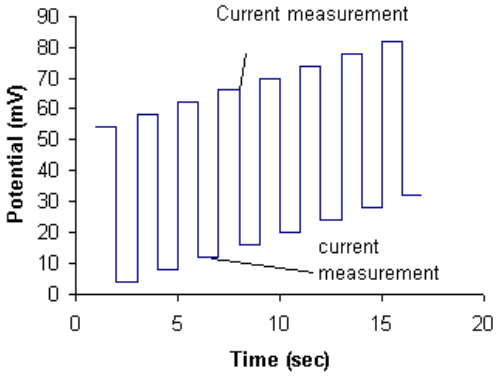

Fig.(1): The Relationship between Potential & Time for Square Wave Voltammetry (SWV) [12]

The data for SWV appear much the same as data for Differential Pulse Polarography (DPP), although the height and width of the wave depends on the exact combination of experimental parameters (i.e. scan rate and pulse height), unlike DPP. This feature makes the running of standards important, as with any analytical technique. Like DPP, the current at the beginning of a pulse is subtracted from the current at the end of a pulse. [13-16]

Many methods are available for determination of Inorganic Phosphorus in human blood serum[17,18] . The colorimetric method (UV Phosphomolybdate method) is based on the formation of *phosphor - inorganic molybdate complex* as shown [19]:

$$\textit{Ammonium molybdate} + \textit{sulfuric acid} \xrightarrow{\textit{Phosphorus}} \textit{phosphor - inorganic molybdate complex}$$

This method required different reagents for color development and its sensitive too much lower than electrometric method.

The Differential Pulse Anodic Stripping Voltammetry (DPASV) had been applied to measure the activity of Lactate Dehydrogenase (LDH) enzyme in human blood serum. [20]

In the present work, SWV is used for determination of Inorganic Phosphorus quantity in human blood serum as described in the following procedure.

Experimental

Reagents

1. ***Reagent 1 (Standard)*** from ***P-Kit (Ref. 61 599) (bioMerieux)*** containing **:**
   Phosphorus (1.61 mmol/l) (5 mg/100ml – 50 mg/l) and Sodium azide (1 g/l) in a total volume (5 ml).

2. ***Reagent 2 (Reducting agent)*** from ***P-Kit (Ref. 61 599) (bioMerieux)*** containing **:**
   Sulfuric acid (1.06 N), ferrous ammonium sulphate (100 g/l) and ferrous nitrate (2g/l) in a total volume (50 ml).

Reagent 3(Color Reagent) from ***Ref. (61 599) (bioMerieux)*** containing**:**
Sulfuric acid (1.05 N) and ammonium hepta-molybdate (4.5 g/l) in a total volume (50 ml).

3. ***(Sodium acetate, acetic acid) Buffer*** **(0.2 M)** ***at*** **(pH 7.0).**
Freshly prepared by mixing (9.5 ml) of CH_3COONa and (0.5 ml) of CH_3COOH diluted to (100 ml) by Distill water (pH = 5.9), Then the pH adjusted to (7.0) by adding few drops of (1.0 N) NaOH solution.[21]

4. ***Sodium Hydroxide*** **NaOH (4.0 N) from** ***(Cat. No. AS 147,)(Randox)*** containing **:**
Sodium Hydroxide (4.0 N) in a total volume (100 ml).

5. ***Ferrous Ammonium Sulphate*** **(10^{-3} M) from** ***(Fluka):***
Freshly prepared by dissolving (0.0098 gm) of $Fe(NH_4)_2(SO_4)_2.6H_2O$ in (25 ml) of distilled water in a volumetric flask.

6. ***(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.

7. ***(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.

8. ***(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 voltammetric cell 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 auxiliary electrode. Temperature control made by using radiometer VTS 13 water thermostat ($\pm 0.1\ ^0C$).

But the colorimetric measurements were performed by using (Cecil Spectrophotometer) model (CE 10211 Ultra Violet & Visible Spectrohotometer) from Cecil Instruments Limited.

Procedure

The optimum conditions were examined using (SWV) were show in **Tables (1-4)** as: Deposition time (5 s), Conditioning time (0 s), Equilibrium time (5 s), Scan Rate (100 mV/s). The voltammetric cell was thermostated at (37^oC). The solution was deaerted by passing a slow stream of pure N_2 gas through it for (4 min.) to remove the dissolved oxygen.

For voltammetric measurement, the sample cuvette contained (5ml) of

(0.2 M) (Sodium acetate, acetic acid) Buffer (pH 7.0) and (100µl) ***Reagent 2***. The square wave voltammogram of ferrous ammonium sulphate was recorded between (0.0 V to -0.4 V).and the reaction was initiated by addition of (5µl) of serum. The voltammogram was again recorded. The calculation of voltammetric method (SWV) were show in the flowing equation:-

The concentration of Inorganic Phosphorus $= \frac{\mathbf{Ip - Ip_1}}{\mathbf{Ip_{Standard}}} \mathbf{x\ n}$ **,** which in :-

Ip: is the value of diffusion current of ferrous ammonium sulphate before serum addition, the unit of **Ip** is nanoAmper (nA).

Ip_1: is the value of diffusion current of ferrous ammonium sulphate after serum addition, the unit of **Ip_1** is (nA).

Ip $_{standard}$: is the value of diffusion current of the standard, the experimental value of **Ip $_{standard}$** equal to **1000 nA**.

N: is the value of the standard concentration equal to **1.61 mmol/l**.

For colorimetric determinations, we prepared **working solution** by mixing 1 volume of ***Reagent 2*** with 1 volume of ***Reagent 3***, the stability of working solution is one month at 2-8 ^{o}C in a dark bottle. The wavelength that used in colorimetric method was 690 nm (Hg 691). 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
Sample	----------	----------	100 µl
Reagent 1 (standard)	----------	100 µl	----------
Distilled water	100 µl	----------	----------
Working solution	2.5 ml	2.5 ml	2.5 ml

After prepared three tubes for each sample as shown in the above, we were mixing these tubes and waiting for 10 minutes then we measured the absorbance for each sample. The color intensity was stable about one hour. The calculation of colorimetric method were show in the flowing equation:-

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

A $_{Sample}$: is the value of the sample absorbance.

A $_{Standard:}$ is the value of the standard absorbance, the experimental value of **A $_{standard}$** equal to **0.195**

N: is the value of the standard concentration eq ual to **1.61 mmol/l**.

Results and Discussion

Colorimetric determination, without deproteinization of serum phosphrous using a single reagent which forms a phosphomolybdate complex in the presence of a reducing agent (ferrous sulphate).[19]

In voltammetric method, the principle of SWV method is based on the reduction process of ferrous ammonium

sulphate on the surface of HMDE when combining with inorganic phosphorus in serum to form the complex between them as shown in the following equation:-

$$Fe(NH_4)_2(SO_4)_2.6H_2O + HPO_4^{-2} \longrightarrow Fe(NH_4)_2(HPO_4).6H_2O + SO_2 + \tfrac{1}{2} O_2 - 2e^-$$

Ferrous ammonium sulphate — **Inorganic Phosphorus** — **Complex**

Table (1): Effect of Deposition Time (Dep. Time) on the reduction peak of ferrous ammonium sulphate at E_p = -0.2 V

Dep. Time (sec.)	0	**5**	10	15	20
Ip (nA)	6275	**8120**	7261	5439	5274

Table (2): Effect of Conditioning Time (Cond. Time) on the reduction peak of ferrous ammonium sulphate at E_p = -0.2 V

Cond. Time (sec.)	**0**	5	10	15
Ip (nA)	**8425**	7375	7329	6045

Table (3): Effect of Equilibrium Time (Equi. Time) on the reduction peak of ferrous ammonium sulphate at E_p = -0.2 V

Equi. Time (sec.)	**5**	10	15	20
Ip (nA)	**8546**	8035	7817	7514

Table (4): Effect of Scan Rate on the reduction peak of ferrous ammonium sulphate at E_p = -0.2 V

Scan Rate (mV/sec)	**100**	200	300	400
Ip (nA)	**8945**	7920	6811	5787

From the above equation, Fe(II) is reduce to Fe(0) on the surface of HMDE then forming the complex. Therefore after addition of (100µl) ***Reagent 2*** to the cuvatte sample which contain (5 ml) of (sodium acetate, acetic acid) Buffer at (pH 7.0), we found that ferrous ammonium sulphate gives a well known defined peak at (-0.2 V) vs. Ag/AgCl, 3M KCl in (Sodium acetate, acetic acid) Buffer at (pH 7.0) **[Fig. 2(b)]** and can be used for indirect determination of Inorganic Phosphorus by measuring the decrease in this peak after addition of (5 µl) of serum as shown in **[Fig. 2(c)]**.

Optimization of Conditions:

Effect of **(10^{-3}M)** *Volume of Ferrous Ammonium Sulphate Solution:*

In order to determine the reduction peak of ferrous ammonium sulphate that appeared at the reduction potential (-0.2 V), a series of experiments were carried out in which **(10^{-3}M)** ***Ferrous Ammonium Sulphate*** solution added in a range between

(10-100µl) to a voltammographic cell containing (5 ml) of (sodium acetate, acetic acid) Buffer at (pH 7.0). The results shows increasing in the diffusion current (Ip) of the $FeSO_4(NH_4)_2SO_4.6H_2O$ at (-0.2 V). therefore the result for this experiment proved that the reduction peak is related to ferrous ammonium sulphate. **(Table 5)**

Table (5): Effect of (10^{-3}M) volume of Ferrous Ammonium Sulphate solution on the reduction peak of ferrous ammonium sulphate at E_p = -0.2 V

Addition of (10^{-3}M) *Ferrous- Ammonium Sulphate solution* (µl)	**Ip (nA)**
10	3650
20	3970
30	4217
40	4495
50	5360
60	5718
70	6030
80	6235
90	6785
100	**7120**

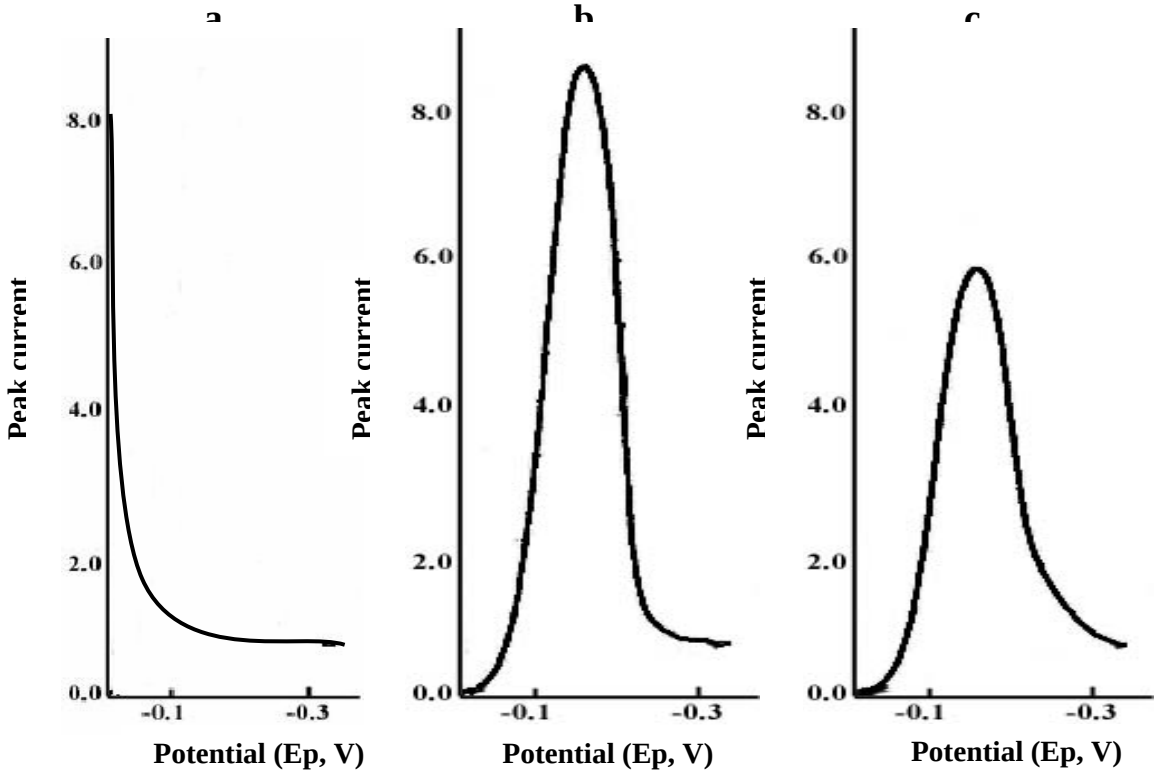

Fig.(2): Square-wave Voltammograms :-

(a) For the supporting electrolyte only (Sodium acetate, acetic acid) buffer at pH=7.0
(b) For the Ferrous Ammonium Sulphate before the addition of human blood serum.
(c) For the Ferrous Ammonium Sulphate after the addition of human blood

serum,(shows the decrease in the reduction peak).

Effect of Reagent 2 (Reducting agent)

In order to determine the optimum concentration of ferrous ammonium sulphate, a series of experiments was carried out in which ***Reagent 2*** solution added in a range between (10-100µl) to a voltammographic cell containing (5 ml) of (Sodium acetate, acetic acid) Buffer at (pH 7.0). The result shows that (100µl) of ***Reagent 2*** solution is suitable to determine the inorganic phosphorus quantity in clinical application due to the maximum diffusion current (Ip). **(Table 6)**

Effect of Reagent 1 (Standard)

The effect of ***Reagent 1*** examined by adding increasing amount of standard in a range between (1-5µl) to a voltammographic cell containing (5 ml) of (Sodium acetate, acetic acid) Buffer at (pH 7.0), and (100µl) of ***Reagent 2*** solution. The result shows that (5µl) of standard represent the optimum amount for determination because giving the maximum value of diffusion current (Ip). **(Table 7)**

Effect of Serum

The effect of ***Serum*** examined by increasing amount of normal human serum in a range between (1-5µl) to a voltammographic cell containing (5 ml) of (Sodium acetate, acetic acid) Buffer at (pH 7.0), and (100µl) of ***Reagent 2*** solution. The result shows that (5µl) of serum represent the optimum amount for determination because it gives the maximum value of diffusion current (Ip). **(Table 8)**

Clinical Applications

A comparison between SWV method and spectrophotometric method was carried out using (36 Samples) represent a normal cases and patients suffered from different diseases such as: hyperparathyroidism, rickets (in children), osteomalacia (in adults), chronic renal failure, Addison's disease and diabetic ketoacidosis. The results obtained are shown in **Table (11) .**

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 = 0.9996) and (RSQ = 0.9992) 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* = 0.0084 + [1.2209 * *Conc. by Colorimetric method*]**

Quality Control

For accuracy and reproducibility control, we assayed Multi-Sera Low, Normal and Elevated by the two methods: SWV and Colorimetric methods. The results shown in **(Table 9)**.

For ensure the accuracy of our purposed voltammetric method (SWV method), we calculated each of : Related Standard Deviation (RSD%), Relative Error (Error%) and (Recovery%) to increase the accuracy of our purposed method, the results shown in **(Table 10)**.

Table (6): Effect of *Reagent 2* amount on the reduction peak of ferrous ammonium sulphate at E_p = -0.2 V

Addition of *Reagent 2* (µl)	Ip (nA)
10	6990
20	7030
30	7114
40	7800
50	7970
60	8250
70	8545
80	8820
90	9180
100	**9390**

Table (7): Effect of *Reagent 1* on the reduction peak of ferrous ammonium sulphate at E_p = -0.2 V

Addition of *Reagent 1* (µl)	1	2	3	4	**5**
Ip (nA)	9000	8360	7980	6990	**6260**

Table (8): Effect of *Serum* on the reduction peak of ferrous ammonium sulphate at E_p = -0.2 V

Addition of *Serum* (µl)	1	2	3	4	**5**
Ip (nA)	8975	8250	7815	7120	**6760**

Table (9): A Quality Control of Inorganic phosphorus 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	0.50-0.74	0.687	0.850
Normal Human Sera	1.20-1.80	1.510	1.869
Elevated Human Sera	1.75-2.60	2.502	3.096

Table (10): The statistical results for accuracy of purposed voltammetric method (SWV method).

	RSD %	Error %	Recovery %
Low Human Sera	0.153416	0.94118	99.05882
Normal Human Sera	0.091658	0.32103	99.67897
Elevated Human Sera	0.041787	0.22610	99.77390

Table (11): The results obtained for determination of Inorganic Phosphorus in Normal and Abnormal cases using two methods: SWV method and Colorimetric method.

Voltammetric Method (SWV method)					*Colorimetric Method*	
Sample No.	**Ip (cm)**	**Ip_1 (cm)**	**$Ip-Ip_1$ (cm)**	**Conc. (mmole/l)**	**A_{Sample}**	**Conc. (mmole/l)**
1	8370	7209	1161	**1.869**	0.183	**1.510**
2	8370	7982	388	**0.625**	0.065	**0.538**
3	8370	7909	461	**0.742**	0.076	**0.624**
4	8369	7881	488	**0.785**	0.078	**0.648**
5	8370	7827	543	**0.875**	0.083	**0.687**
6	8368	7809	559	**0.900**	0.090	**0.741**
7	8371	7768	603	**0.970**	0.095	**0.784**
8	8370	7735	635	**1.022**	0.100	**0.826**
9	8372	7731	641	**1.032**	0.102	**0.842**
10	8371	7654	717	**1.155**	0.113	**0.933**
11	8370	7618	752	**1.210**	0.117	**0.966**
12	8371	7597	774	**1.247**	0.122	**1.007**
13	8369	7537	832	**1.339**	0.131	**1.082**
14	8370	7507	863	**1.390**	0.136	**1.123**
15	8371	7476	895	**1.441**	0.141	**1.164**
16	8370	7431	939	**1.512**	0.148	**1.222**
17	8368	7297	1071	**1.725**	0.170	**1.404**
18	8369	7252	1117	**1.799**	0.176	**1.453**
19	8370	7223	1147	**1.847**	0.181	**1.492**
20	8368	8098	270	**0.434**	0.043	**0.351**
21	8370	7209	1161	**1.869**	0.192	**1.585**
22	8370	7145	1225	**1.972**	0.193	**1.593**
23	8369	7036	1333	**2.146**	0.210	**1.734**
24	8366	6841	1525	**2.456**	0.241	**1.990**
25	8368	6807	1561	**2.514**	0.246	**2.031**
26	8370	6754	1616	**2.602**	0.255	**2.102**
27	8371	6740	1631	**2.626**	0.257	**2.122**
28	8372	6497	1875	**3.018**	0.303	**2.502**
29	8370	6171	2199	**3.540**	0.366	**3.022**
30	8369	5671	2698	**4.343**	0.425	**3.509**
31	8369	5531	2838	**4.569**	0.447	**3.694**
32	8368	5179	3189	**5.135**	0.524	**4.327**
33	8369	4959	3410	**5.490**	0.548	**4.523**
34	8370	4822	3548	**5.712**	0.560	**4.624**
35	8371	4808	3563	**5.736**	0.564	**4.658**
36	8370	4379	3991	**6.425**	0.630	**5.202**

Which in:-

Conc. is the Concentration of Inorganic Phosphorus in human blood serum measured by two methods: SWV method and Colorimetric method.

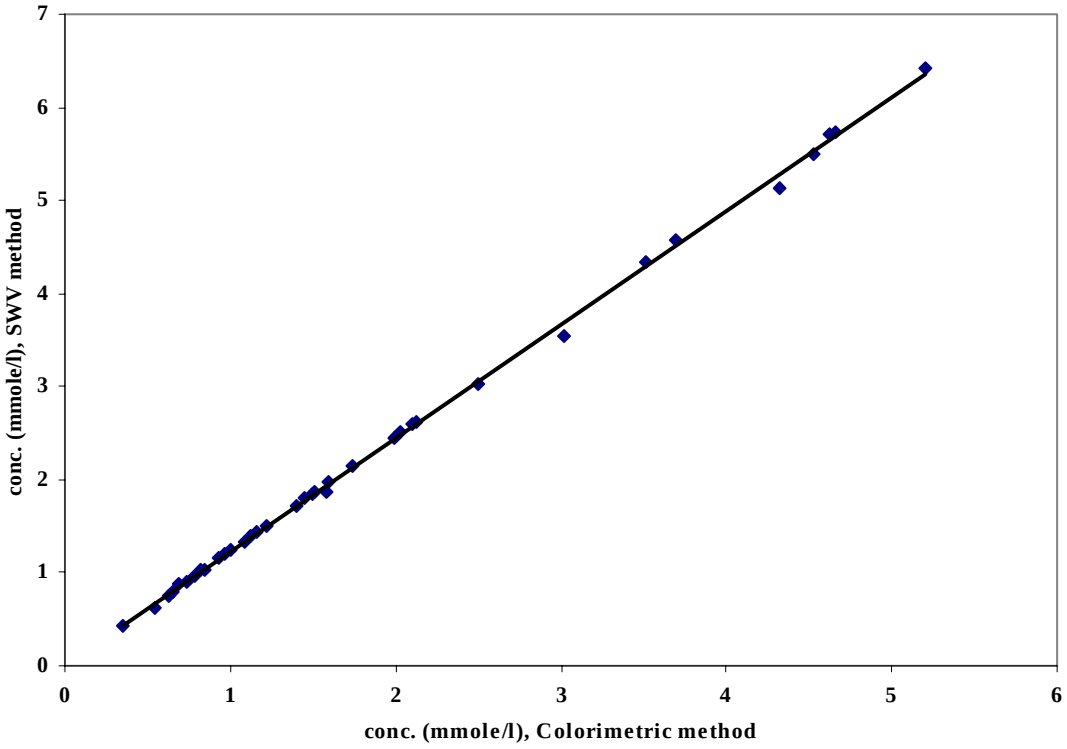

Fig.(3) : The relation between Concentrations of Inorganic Phosphorus measured by SWV method and Colorimetric method in Normal & Abnormal cases.

References

1. JECFA-Summary of evaluations performed by the joint FAO/WHO Expert Committee on Food Additives, ILSI Press (1994).
2. COMA, Dietary Reference Values for Food Energy and Nutrients for the United Kingdom. Report of the Panel on Dietary Reference Values, Committee on Medical Aspects of Food and Nutrition Policy. HMSO, London, (1991).
3. Goldsmith, R.S., Jowsey, J., Dube, W.J., Riggs, B.L., Arnaud, C.D. Kelly (1976) Effects of phosphorus supplementation on serum parathyroid hormone and bone morphology in osteoporosis., ***Journal of Clinical Endocrinology and Metabolism,*** 1976, **43**, 523.
4. Bablok W. et al. A General Regression Procedure for Method Transformation., ***J Clin Chem Clin Biochem,*** 1988; **26**, 783.
5. Berner YN, Shike M: Consequences of phosphate imbalance. ***Annu Rev Nutr***, 1988, **8**, 48
6. Fauci A, Braunwald E, et al, eds: Harrison's Principles of Internal Medicine. NY: McGraw-Hill; 1998:1510, 2217-8, 2243, 2245, 2262.
7. Thomas L. Clinical Laboratory Diagnostics. 1st ed. Frankfurt: TH-Books Verlagsgesellschaft; 1998. p. 241-7.
8. Endres DB, Rude RK. Mineral and bone metabolism. In: Burtis CA, Ashwood ER, editors. Tietz Textbook of Clinical Chemistry. 3rd ed. Philadelphia: W.B Saunders Company; 1999. p. 1395-1457.
9. Burtis CA, Ashwood ER, editors. Tietz Textbook of Clinical Chemistry. 3rd ed. Philadelphia: W.B Saunders Company; 1999. p. 1829.
10. P. D. Mayne and I. Z. Kovar, ***Ann Clin***

Biochem, 1991, **28**, 131.

11. A. Whybro, H. Jagger, M. Barker and R. Eastell, ***European Journal of Clinical Nutrition,*** 1998, **52**, 29.
12. M. Ozsoz, A. Erdem, P. Kara, K. Kerman and D. Ozkan, "Electroanalysis",2003, **15**, 613.
13. V. Mirceski, B. Jordanoski and S. Komorsky-Lovric, ***Portugal. Electrochem. Acta***,1998, **16**, 43.
14. S. Komorsky-Lovric, V. Mirceski and F. Scholz, ***Mikrochim. Acta***,1999, **132**, 67.
15. Valentin Mirceski, Rubin Gulaboski, Simka Petrovska-Jovanovic and Kornelija Stojanova, ***Portugal. Electrochim. Acta,*** 2001, **19**, 25.
16. V. Mirceski and R. Gulaboski, ***Croatica Chim. Acta,*** 2003, **76**, 37.
17. D. S. Young, D. W. Thomas, R. D. Friedman and L. G. Pestaner, ***Clin. Chem.***, 1972, **18(10),**10.
18. N. Weissman and V. J. Pileggi, *Clin. Chem.*, "Principle and Techniques", Harper and Row, Hagerstown, MD, 1974, p. 726.
19. J. A. Daly and G. Erthinghsausen, ***Clin. Chem.***, 1972, **18**, 263.
20. S. T. Sulaiman, H. A. Al-Wahab & R. S. Kassir, ***National Journal of Chemistry***, 2003, **11**, 377.
21. D. D. Perrien & Boyd Dempsey, "Buffers for pH and Metal Ion Control", Australian National University, Canberra, 1st ed., 1974, p. 137-138.