Kinetic Studies and electrophoretic separation of Alkaline Phosphatase Isoenzyme from Cancerous patients

Al-jebory M. Abdulhussien*

Received 24/4/2005 ; accepted 26/7/2005

الخلاصة

استخدمت الطريقة اللونية في تقدير فعالية إنزيم الفوسفاتيز القاعدي في مصل 303 مريضا مصابا بالسرطان و 42 شخصا سليما تم اعتبارهم مجموعة السيطرة. لوحظ من خلال النتائج أن فعالية الإنزيم تزداد بصورة عامة في جميع مرضى السرطان. ولكن كانت هناك زيادة معنوية فقط في مرضى سرطانات العظام , الكبد والبنكرياس. إن هذه الزيادة يمكن اعتبارها دليلا أو مؤشرا لتشخيص سرطانات هذه الأعضاء الثلاثة. ولم يكن هناك تأثير واضح للجنس على تغير فعالية الإنزيم في هذه الحالات.

أجريت دراسة حركية الإنزيم المفصول من مصل دم مجموعة السيطرة وكانت القيمة المثلى لتركيز المادة الأساس هي 10 ملي مول ودرجة الحرارة 37 درجة مئوية والأس الهيدروجيني 10. وكانت قيمة ثابت ميكالس –منتن بحدود 207 ملي مول. استخدمت طريقة الهجرة الكهربائية باستخدام تقنية الهلامي العمودية لفصل متناظرات الإنزيم لكل من مجموعة السيطرة والمجاميع المرضية المصابة بالسرطان وأوضحت النتائج إن هنالك حزمة واحدة فقط تظهر للأصحاء في حين أكثر من حزمة وصلت إلى خمسة حزم في مرضى السرطان اعتمادا على نوع النسيج المفصول منه الإنزيم ولوحظ أن هنالك اختلاف في شدة اللون للحزمة اعتمادا على نوع المرض وعمر المريض وتقدم الحالة المرضية.

ABSTRACT

The activity of sALP was determined by the calorimetric method King_Armistrong in serum of 303 cancerous patients and 42 normal healthy controls. The results show that the ALP activity was increased in all types of cancer tissues, however ALP activity show a significant increase only in bone, liver and pancreas tissues. The increase of ALP activity could be used as tumor marker for bone, liver and pancreas tissues.

The increase in sALP activity could be used as tumor marker for bon and liver cancer and to detect metastasis to the organ. No significant differences of sALP activity were found between males and females. The activity was increase with the stage of development of the disease.

The kinetic studies for normal healthy human were measured at a substrate concentration 10 mM, Km 2.7 mM, temperature 370c and pH 10. The electrophoretic studies show that one band of ALP in serum of normal subjects, as compared to cancerous patients where several bands were detected in cancer patients; the intensity of band color varied with type; age and stage of disease.

INTRODUCTION:

Alkaline phosphatases (ALP) are a group of enzymes, which hydrolyzed phosphate esters at alkaline pH. They are present in most tissues, but are particularly high in the estoblastes of bones, the hepatobiliary tract, intestinal wall, the renal tubules and the placenta[(1)]. The Alkaline Phosphates (ALP) in serum of normal adults comes primarily from the liver or biliary tract; elevated levels of ALP are seen in primary or secondary liver cancer. Its level may be helped in evaluating metastases cancer with bone or liver involvement; greatest elevations are seen in patients with osteoblastic lesion such as breast cancer with bone metastases[(2)].

* Department of Associated Basic Sciences, College of Pharmacy, Almustansiriya University, Baghdad – Iraq .

In liver metastases the serum ALP level correlates with extent of liver involvement better than determines of liver function. To differentiate the origin of elevated levels, tests of other liver enzymes may be performed such as that for 5-nucleotidase or glutamyl transferase. the liver isoenzyme thermally more stable than the bone isoenzyme, serum sALP was first identified as the Regan isoenzyme[3,4], and recognized as of the first.
Oncodevelopemental markers[5]. sALP isoenzyme of bone had been observed to be elevated in patients with bone disorders[6], while the intestinal sALP isoenzyme is seldom encountered in large amounts[7]. Many physical and catalytic differences among ALP isoenzymes have been exploited in order to measure the concentration of these isoenzymes using different techniques for such purpose, such as electrophoresis; which include paper[8], starch-block[9]; agar-gel[10] cellulose acetate[11]; cellulose polyacetate[12]; starch gel[13] and poly acryl amide gel[14] electrophoresis. The best separation of sALP isoenzymes could be obtained with polyacrylamide gel; where as the poorest separation is obtained by using cellulose acetate[15].
sALP is rapidly denatured at 560c but is relatively stable at lower temperature[16], sera kept at room temperature usually show a slight but real increase in activity, which varies from 1% over a6 hours period to 3-6% over al to 4-days period. Even when sera stored at refrigetor temperature, the activity increase slowly by 2 units/day[17].
Our aim in this studies are to separate ALP isoenzyme using a horizontal gel electrophoreses from different cancerous patients and healthy human, and established the effects of each of substrate concentration , pH ,and temperature on the enzyme activity.

MATERIALS AND METHODS:

The blood samples were taken from 303 cancerous patients of the hospital of Radiology and Nuclear Medicine, and 42 normal healthy control after taken all information about the selected patients, the blood was drawn by venipunture. the samples were left at room temperature for about half an hour then serum was separated by spinning for 15 minutes at 3500 rpm in a centrifuge, at room temperature. The unseparated specimens (serum mixed with hemoglobin) were discarding.
The activity of sALP was measured according to procedure of phosphate-kit which obtained from Bionerieux Company, France, kinetic studies were performed including:

1- Effect of substrate concentration:

Disodium phenyl phosphate (DPP) solution 16mM was prepared as stock solution, from which the following concentrations (14, 12, 11, 10, 9, 8, 6 and 4mM) were prepared by serial dilution with distilled water. The activity at each concentration was measured and the rate of reaction was plotted versus DPP concentration.

2- Effect of temperature:

The effect of temperature on the rate of reaction was studied by measuring the activity at different temperature s (15, 25, 30, 37, 45, 55, 65 and 70°c) using optimum substrate concentration 1×10^2 mol/L and pH10; rate of reaction was plotted versus temperature.

3- Effect of pH:

The activity of ALP was measured using the optimum concentration (1×10^2 mol/L) and 37ºc, at different pH values (8, 9, 9.5, 10, 10.5, 11 and 12). The rate of reaction was plotted versus pH values.

4- Determination of Michaels-Mentin constant (Km):

K_m values were determined using the final concentration of DPP at optimum pH and temperature. K_m values were calculated according to Line Weaver-Burk plot of 1/v against 1/[S], where [S] substrate conc. And (v) the velocity of reaction rate at any time.

Conversional polyacrylamide gel electrophoresis:

A developed method was used to estimates the molecular weight of partially purified ALP from serum[18] using 30gm acryl amide and 0.8gm bisacrylamide dissolved in 50ml deionized water, when the acryl amide was completely dissolved, water then was added to final volume of 100ml, the solution was then filtered under vacuum and stored at 4ºC in dark bottle for not more than one month (solution-1). Standard protein solutions of low molecular weight were used as calibration kit as in the table.

Protein	**Mwt**
Phosphorelase	94×10^3
albumin	67×10^3
ovalbumin	43×10^3
Carbonic anhydrase	30×10^3
Trypsin inhibitor	20×10^3
α-lactalbumin	14×10^3

Procedure:

(1) The monomer solution for the appropriate resolutions gel was prepared by combining the following compounds:

Compounds	Volume(ml)
Solution 1	5.00
Solution 2	3.75
Deionized water	5.25
Ammonium persulphate 1.5%	0.005
Tetra methyl ethylene diamine	0.01

The prepared monomer solution, mixed well, this solution was with drown by syringe then added to the instrument tubes, the addition should be continuously to avoid any air bubble formation. The monomer solution was immediately over lied with few drops of butanol and the gel was allowed to stand until polymerization was essentially completed.

(2) Staking gel monomer solution:

Compounds	Volume(ml)
Solution 1	2.6
Tris-HCl solution pH8.8	6.0
Deionized water	9.0
Ammonium persulphate 1.5%	0.006
Tetra methyl ethylene diamine	0.01

The butanol on the top of the resolving gel was with drowning and the stacking gel was poured on top of each tube. The monomer was immediately overlie with butanol and stored at 4°c overnight.
(3) 25µl of serum in 100ml of sample buffer solution {which prepared from dissolving 40gm sucrose and 1.25gm Tris base in 40ml deionized water, then 2ml of
2- Mercaptoethanol and 1ml of 0.25% bromo phenol blue were added} was prepared.
(4) The upper and lower reservoirs were filled with electrode buffer Tris-glycine, pH8.3, and then were connected to the power supply.
(5) 50µl of serum solution prepared above was loaded by Hamilton into the well in the staking gel by layering them under electrode buffer.
(6) Electrophoresis process was started immediately after the sample was loaded and continued until the bromophenol blue tracking dye has reached the bottom of the gel (about 3-4hours).
(7) Permanent fixation was obtained by incubating gels in fixing solution (ethanol 50%, 10% glacial acetic acid and 40ml deionized water) for 2 hours before immersion in the staining solution (0.05gm brilliant blue R-250 in fixing solution), all steps were performed at room temperature (25 ^{o}c) and the gel was stained for 2.5 hours.
(8) The staining gel was incubated in distaining solution (5% ethanol, 7% acetic acid and 88% deionized water) the solution was changed several time until the tack round has been satisfactory removed.
(9) When the bands were observed, the gel was incubated in solution containing 7% glacial acetic acid and 93% water.

Standard solution:

The standard protein of low molecular weight was placed in a test tube and treated with same steps as in the sample.

Determination of Proteins Molecular weight:

The relative mobility of migration of standard proteins was determined according to the equation below. (Measurement were achieved using the center of each died band and the sharp front of bromophenol blue).

$$\text{Relative mobility (Rm)} = \frac{\text{Distance of protein migration}}{\text{Distance migrated by bromophenol blue}} \times 100$$

A straight line should be obtained when the log of the molecular weight of the each of the standard protein was plotted against the relative mobility, samples molecular weight could be estimated by interpretation of its Rm% on the standard curve.

RESULTS AND DISCUSSION:

The results obtained show that ALP activity in cancer patients is affected by several variables such as; type of cancer, sex and age, table-1 show the effect of type of cancer on in different tissues on ALP activity.

Table 1 . ALP level for different types of cancer cases compared to the control

Site of cancer	number	ALP(K.A.)mean	Standard deviation	Standard error
Normal(control)	42	07.1690	02.1010	00.3242
Bile	16	40.9167	32.0662	13.0910
Leukaemia	13	11.1154	04.5742	01.2686
cervix	12	36.6429	14.8849	05.6206
stomach	14	15.2222	06.9016	02.3005
lung	18	23.3611	06.9065	01.6279
pancreas	12	44.1417	47.6026	13.7417
bone	21	28.8286	11.4850	02.5062
liver	23	37.0154	21.0919	05.8499
Urinary bladder	14	17.8714	08.5935	03.2481
Toxic goitre	12	16.6625	06.8529	02.4229
colon	27	14.4148	11.1777	02.1512
breast	36	23.7361	19.0973	03.1829
thyroid	13	22.3333	15.8140	09.1302
prostate	15	17.5000	05.5902	02.5000
ovary	16	22.0933	09.1441	03.7331
meningioma	06	12.5000	03.1623	01.2910
Testicular ca	05	21.7250	11.4354	05.7177
spleen	06	16.6250	03.6372	01.8186
kidney	09	30.9000	13.0403	05.8318
Squemous ca tonsil	15	10.3200	01.7570	00.7857
total	345	20.7979	18.5554	01.1575

However a significant increase was occurred only in bone, liver and pancreas tissues as shown in table-2. These results are in agreement with many publishers[19-20]. Zilva[21] also concluded that the increase of plasma ALP activity in patients with carcinoma is usually due to osteoblastic secondary deposits in bone or hepatic metastases giving rise to cholestasis.

Table 2 . The difference of ALP activity between cancer tissue and normal subject

Control(A)	Site of cancer(B)	Mean(K.A.),B-A	Standard error	significant
	Bile	-33.7676	6.959	0.117
	leukaemia	-03.9463	5.061	0.436
	cervix	-29.7438	6.509	0.213
	Stomach	-08.0532	5.857	0.170
	lung	-16.1921	4.492	0.661
	pancreas	-36.9726*	5.219	0.027
	Squamous Ca tonsil	-03.1510	7.543	0.677
	bone	-21.6595*	4.261	0.000
	liver	-29.8463*	5.061	0.000
	bladder	-10.7024	6.096	0.101
NORMAL	Toxic goitre	-09.4935	6.151	0.124
	colon	-07.2458	3.933	0.999
	breast	-16.5671	3.622	0.197
	thyroid	-15.1643	9.529	0.113
	prostate	-10.3310	7.543	0.172
	ovary	-14.9243	6.959	0.129
	meningioma	-05.3310	6.959	0.444
	Testicular carcinoma	-14.5560	8.343	0.386
	spleen	-09.4560	8.343	0.258
	kidney	-23.7310	7.543	0.218

*significant

The effect of substrate concentration:

The activity of SALP was increased with increasing DPP concentration, at a fixed enzyme concentration, the reaction rate order is first order with respect to the substrate low concentration, but at high substrate concentration the reaction is zero order. At low substrate concentration, only a fraction of the enzyme is associated with substrate and the rate observed reflects the low concentration of the enzyme–complex. At very high substrate concentration, all enzymes are bound to substrate, and a much higher rate of reaction is obtained. Moreover, because all the enzyme is now present in the form of the complex, no further increase in complex concentration and no further increment in reaction rat are possible .the maximum possible velocity for the reaction has been reached[(22)].The maximum velocity reached at DPP concentration of 1×10^{-2} mol/L at 37°c and pH 10 as shown in fig-1.

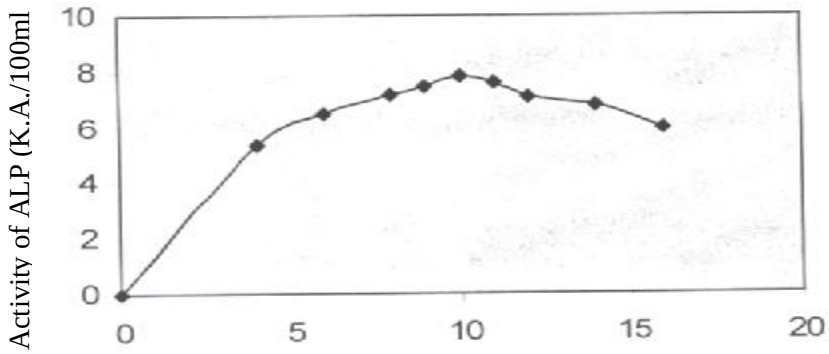

FIG 1 . EFFECT OF SUBSTRATE CONCENTRATION ON REACTION RATE OF ALP FROM HEALTHY HUMAN SERUM

Effect of temperature on reaction rate:

In general the enzyme reaction velocity increase with increasing incubation temperature until the optimum temperature is reached. The reaction between the rate of chemical; reaction and the absolute temperature is given by the empirical equation of Arrhenius; the initial rate of reaction measured instantaneously goes on increasing with rising temperature, at least in theory. In practice, however a finite time is needed in all methods to allow the components of the reaction mixture including the enzyme solution to reach temperature equilibrium and to permit the formation of a measurable amount of Products[(24)].

The velocity will be reduced stepwise due to protein denaturation at a high temperature, fig–2 show the effect of incubation temperature on the enzyme activity. The optimum temperature is 37ºC at a substrate concentration of 1×10^{-2} mol/L and pH 10, there is remaining activity until 65ºC, this may be due to the Regan isoenzyme only whereas the other isoenzyme were denatured at high temperature.

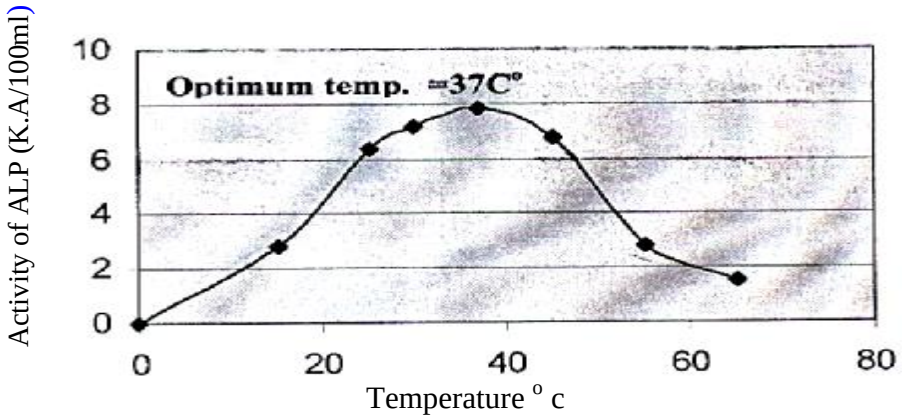

FIG 2 . EFFECT OF TEMPERATURE ON REACTION RATE OF ALP ACTIVITY FROM HEALTHY HUMAN SERUM

Effect of pH:

It seems that the optimum pH was 10 at a substrate concentration of 1×10^{-2} mol/L and temperature is 37ºC, fig- 3, the rate of reaction was low below this value which may be due to the presence of H+ in the solution which act as a competitors with [EH+] the active sites of the enzyme toward the attachment to the substrate ion so according to the competitive inhibition the enzyme activity was reduced, or it may be due to the change in the protein folding performed from the H- bond or other interactions in the tertiary structure of protein which affect the location of the active site in the surface of the enzyme. Lowering the activity at high pH above 10 caused by the presence of OH^- ions[25].

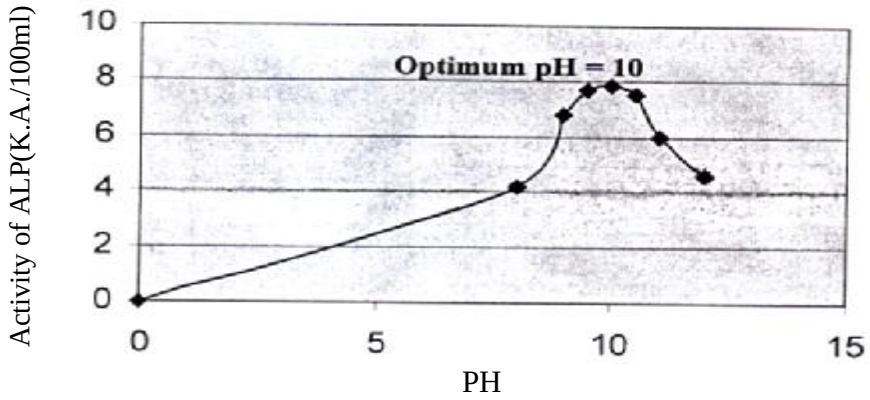

FIG 3 . EFFECT OF PH ON REACTION RATE OF ALP ACTIVITY FROM HEALTHY HUMAN SERUM

Determination of Michealis-Menten constant:

The numerical value of K_m is of interest for several reasons[23].

a-The K_m establishes an approximate value for the intercellular level of the substrate .it is unlike that this level would be significantly grater or lower than K_m .

If $[S]_{intracellular} << K_m$, V would be very sensitive to changes in [S] but must of the catalytic potential of the enzyme would be wasted since V would be $<< V_{max}$.

b- Since K_m is a constant for a given enzyme , its numerical value provides a means of comparing enzyme from different organisms or from different tissues of the organisms ,or from the same tissues at different stages of development .

c- A ligand induced change in the effective value of Km is one mole of regulating the activity of an enzyme.

d- If we know Km, we can adjust the assay condition so that $[S] >> K_m$ and there by determine Vmax, which is a measure of E concentration.

e- K_m indicates the relative suitability of alternate substrates of a particular enzyme. The constant can be calculated from the equation when velocity of the reaction is equal to half the maximum velocity thus Km equal to substrate when:

$r = ½V_{max}$, so $K_m = [S]$

K_m was 2.7 mmol/L when $½ V_{max}$ = 3.9 K.A. ; as shown in fig-4.

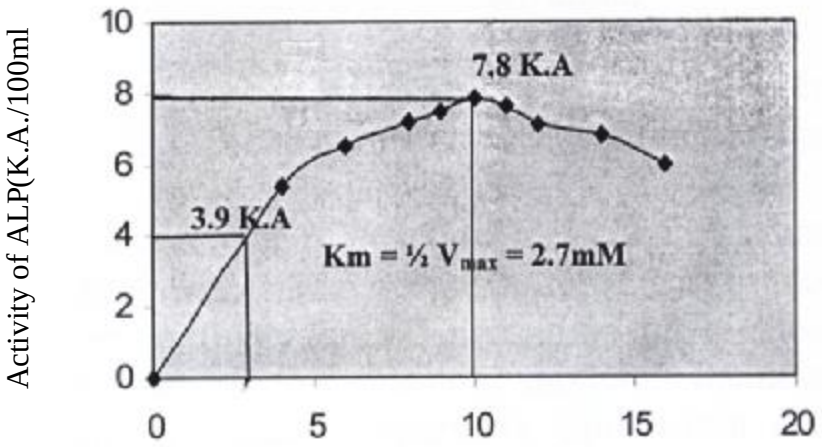

FIG 4 . THE RELATIONSHIP BETWEEN KM AND [S] OF ALP FROM HEALTHY HUMAN SERUM

Poly acryl amide gel electrophoresis (PAGE) of ALP isoenzymes:

Partially purified ALP forms were subjected to PAGE gel electrophoresis for Mwt determination, pattern of sALP isoenzymes were stained with Brilliant Blue R-250, one to three to detect various bands. The molecular weights of the possible isoenzymes were determined by plotting Mwt of the standard protein vs. Rm values obtained from each isoenzyme after extrapolation on the curve.

To differentiate among the sample, the following system of comparison was used.

1- Effect of type of cancer: The effect of type of cancer was shown in the PAGE figs numerate as samples:- (1,7);(7,8);(11,16);(21,24);(27,28);(29,30);(33,34);(35,36).

2- Effect of age: The effect of age was shown in the PAGE figs numerate as samples:- (7,8);(11,13);(17,20);(21,32);(24,25);(29,30)(34,36).

3- Effect of stage of diseases: The effect of disease on ALP was shown in the PAGE figs numerate as samples:- (1,2);(4,5);(6,7);(13,14);(18,20);(21,23);(27,30);(34,36).

In poly acryl amide gel electrophoreses, serum ALP isoenzyme were separated into 1-3 band, in general there is one band only in all samples of normal human, while there are more than 3 bands in all types of cancer patients as in the slabs.

To show the effects of type, age and stage of the disease on the sALP activity ,by the differences in the color density degree among samples in the same band, the sample which have low color density degree have a low ALP activity ,and the sample which have high color density degree have ALP activity, as in the slabs.

Total sALP in normal consisted of isoenzyme contributed by liver, bone, and in some individuals intestine[25,26], typically, only liver and bone isoenzyme are detected in electrophoretic analysis of ALP isoenzyme employing polyacrylamide gel. While the intestinal isoenzyme is not normally detected[26].

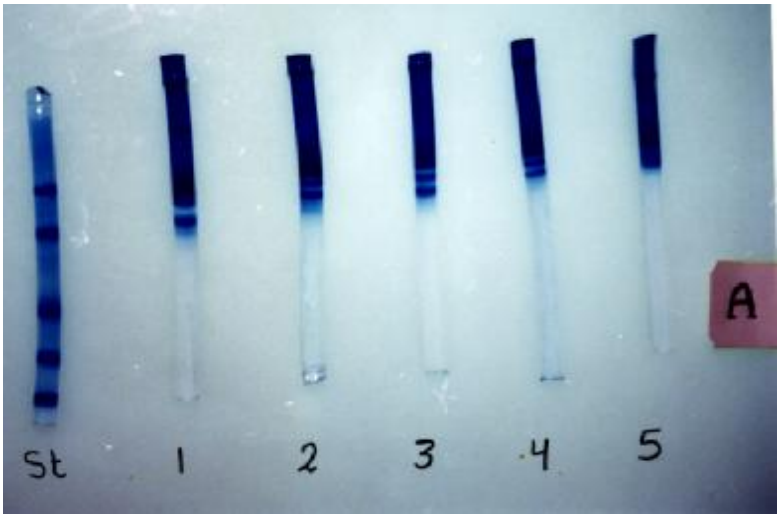

FIG 5 . PAGE FOR DIFFERENT BONE CANCER

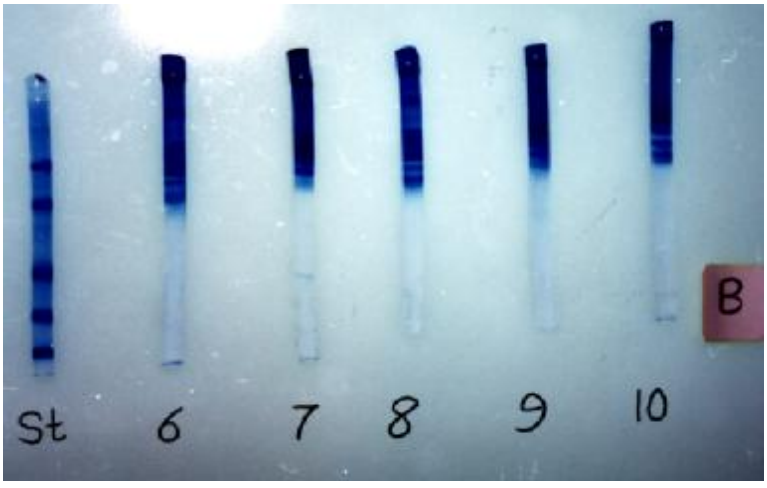

FIG 6 . PGAE FOR CANCER IN DIFFERENT BODY TISSUE. LIVER (6,7); BREAST (8, 9,10)

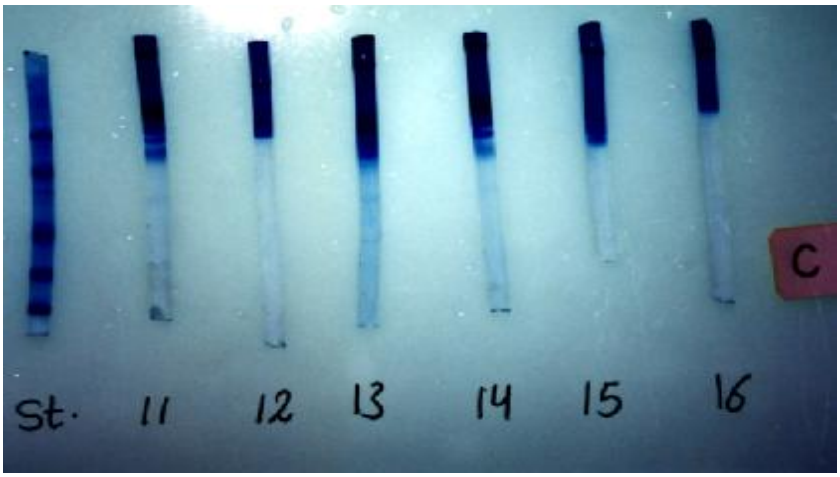

FIG 7 . PAGE FOR CANCER IN DIFFERENT BODY TISSUES. CERVIX (11,12), BLADDER (13,14,15), LEUKEMIA (16)

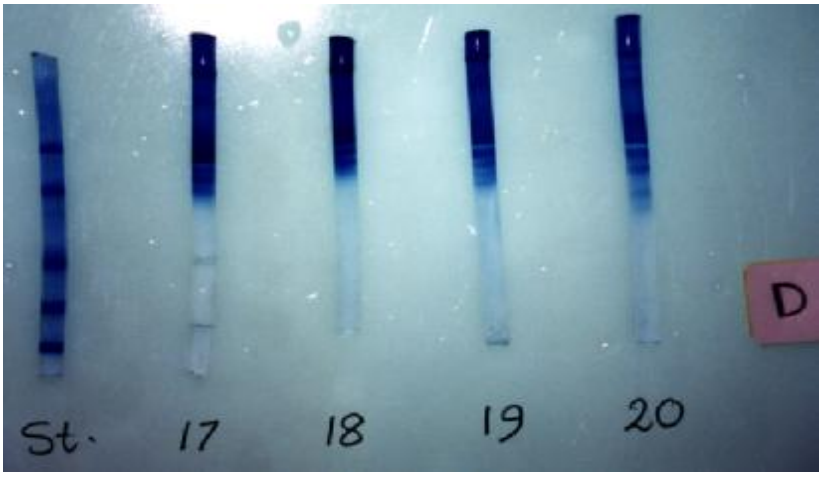

FIG 8 . PAGE FOR CANCER IN DIFFERENT BODY TISSUES. COLON (17,18,19), AND SKIN (20).

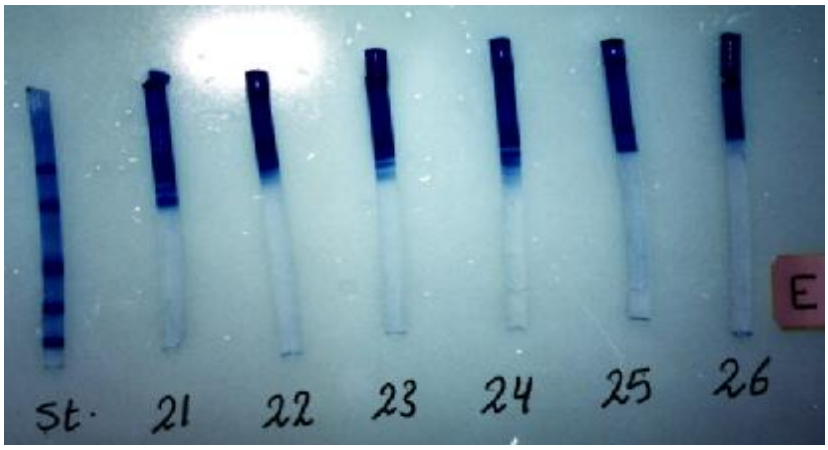

FIG 9 . PAGE FOR CANCER IN DIFFERENT BODY TISSUES. LUNG (21,22,23), SPLEEN (24,25,26).

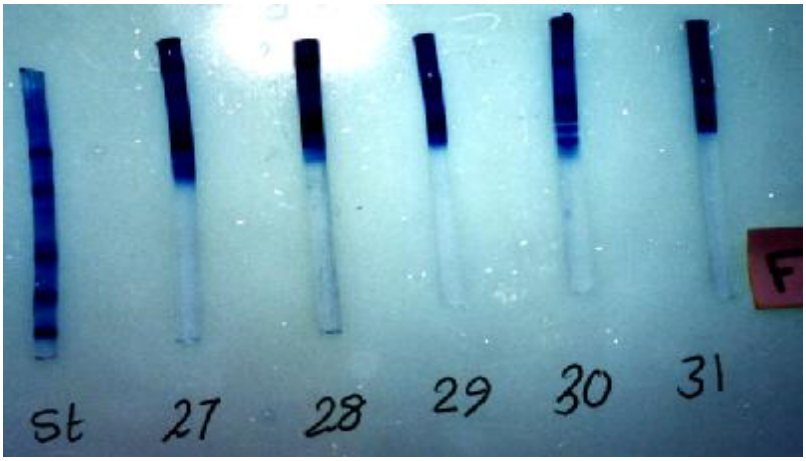

FIG 10 . PAGE FOR CANCER IN DIFFERENT BODY TISSUES. SQUEMOUS CA TONSIL (27) STOMACH (28,29) AND THYROID (30,31).

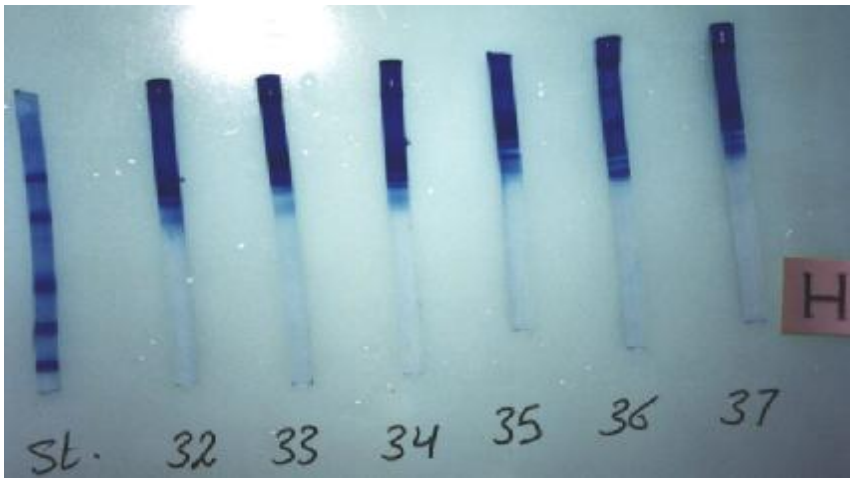

FIG 11 . PAGE FOR CANCER IN DIFFERENT BODY TISSUES.
TESTICULAR (33), PANCREAS (34), PROSTATE (35), MENINGIOMA (36) AND CERVIX (37)

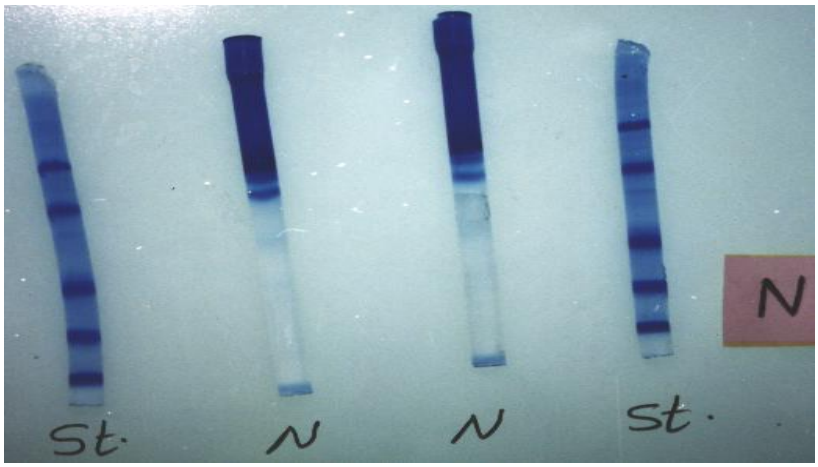

FIG 12 . PAGE FOR NORMAL HUMAN.

REFERENCE:

1. Cadeau B. J., Back Slein M. E., Department of clinical biochemistry University of Toronto, Canada M 4N 3M 5 (1972).
2. Stigbr T. and Wahren B.; Alkaline phosphate as tumor Marker, in serological cancer markers (S.Sell ed.)Totowa, NJ, the human press.pp135-1219(1992).
3. Fishman ,W.H., Inglis, NR., Stolbach L.I.; Cancer Res., 1968,28;150-154.
4. Devlin M.T.; Text book of Biochemistry, chapter 14 (1986).
5. Schwartz, M. K. Enzyme test in cancer, Clin. Lab. Med., (1982),2; 479.
6. Moss D. W. and Ejward R.K. Clin. Chem.Acta; (1984),143;177.
7. Enstrom L. Biochem. Biophys. Acta, (1964),92;71.
8. Baker, R.and Pellegrino C.;J Clin. Lab. Inverest; (1994),6;94.
9. Mathews, K.c.&Van Hold K.E.; Biochemistry; the Benjamin/Cummings publishing company ' inc.(1996).
10. CarlA.B.&Edward R.A.; Tietz Textbook of clinical chemistry;W.B.Sanders company;(2002).
11. Viot, M. & Jouline, Cc; Biomedicine (1979);31;74.
12. Evan D. M.D.; Special Test &Their Meanings, faber & Faber London & BOSTON;(2001)
13. Costa B.& Arisi M.; Brazilian J. Med. Biol. Res. (1994);27;449.
14. Stigbr t.&Warhan B.; Alkaline phosphatase as tumor marker. In Seriological CancerMarkers (S.Sell ed.)Totowa;NJ,the Human Press.Pp135(1992).
15. Kalan A.Clinical chemistry,theory analysis&correlation,the C.V .Mosby Co.2nded.Pp904 (1989).
16. Bergerger H., Bergeyer J. & Gra B., Methods enzymatic analysis 3rd vol. Federal republic of Germany (1983).
17. Norbort W. Tietz, Fundamentals of clinical chemistry, (2000).
18. Practical manual for course Z303; Principles of physiology, univ. of Kuwait pp13 (1986).
19. Whitby. C., smith, F.&Bechkett, J., Lecture notes on clinical chemistry .Black well Scientific Publications ,4th ed, p 123,(1988).
20. Loot, J.A. and Wolf, P.L., clinical enzymology; acaseoriented approach, Chicago,1986,Year Book Medical Publishers pp57-74.
21. Zilfa J.F.5th ed. Clinical chemistry Diagnosis and treatment (1995).
22. Segal I. Enzyme kinetic ; Behaviour and Analysis of Rapid equilibrium and study state enzyme system, 100-907, John Willy and Sion, New York (1975).
23. Murray K. R. and Granner K. D.; Harpers Illustrated Biochemistry 26th ed.; McGraw-Hill Companies, Inc.,(2003).
24. Al-Sharify K. J., M.Sc. Theses University of Tikret (2000).
25. Varleys, Practical Clinical Biochemistry, Gowenlock Ah. Editor; 6th ed. Pp540 (1988).
26. Henry J.; Clinical diagnosis and management; W. B. Saunders Comp.; 17th ed Pp260 (1988).