UNDER PEER REVIEW

The Role of Inhibitors in Ascertaining the Type of Isoenzyme of Alkaline Phosphatase (ALP) and its Clinical Correlations

ABSTRACT

AIMS: To estimate the level of Alkaline Phosphatase (ALP) in the serum and to evaluate the role of inhibitors in ascertaining the type of isoenzyme of ALP and further correlation with clinical diagnosis and other biochemical parameters of the patients.

STUDY DESIGN: Cross Sectional with Diagnostic Accuracy Type

PLACE AND DURATION OF STUDY: Department of Biochemistry, Guru Gobind Singh Medical College, Faridkot, October 2017 to October 2018.

METHDOLOGY: 250 Patients (143 women and 107 men; age range 19-85 years) with elevated ALP level were enrolled. ALP activity was measured in the serum and isoenzymes were ascertained by using chemical inhibitors i.e. Urea, L-Phenylalanine, Guanidine Hydrochloride, and heat inactivation which inhibit ALP activity. For heat inactivation, serum was incubated at 65°C for 10 minutes. Various other biochemical parameters were estimated for the correlation.

RESULTS: The Mean±SD of ALP level was 373±274 U/L. The percentage of inhibition by Urea, L-Phenylalanine, Guanidine hydrochloride and heat inactivation was 49.5%, 40.5%, 48.6%, and 94.0% respectively in liver disorders, 56.6%, 38.1%, 45.8%, and 89.0% in bone disorders and 29%, 41.6%, 42.8% and 95% in intestinal disorders. The percentage of inhibition in third-trimester pregnant females was 44.4%, 36.8%, 47.1%, and 36.8%, and 51.1%, 47.0%, 50.7%, and 51.2% in preeclampsia. The percentage of inhibition of the Isoenzyme of ALP was according to the type of carcinoma/lymphoma. The correlation of ascertained ALP isoenzyme with AST and ALT is highly significant ($P<0.001$) in patients of liver diseases. In the patients of Bone disorders the correlation of ALP isoenzyme with Calcium and Phosphorous is also significant ($P=0.01$ and $P=0.01$ respectively).

CONCLUSION: The sensitivity and specificity of this inhibition method to ascertain isoenzymes was 50.85% and 69.57% respectively. But, sensitivity and specificity are 96.6% and 98.4% when this method was to be correlated with other biochemical parameters.

Key Words: Alkaline Phosphatase, Isoenzyme, Inhibition, Urea, L-Phenylalanine, Guanidine Hydrochloride, Heat Inactivation.

1. INTRODUCTION

The catalysis of the cleavage of the phosphoric ester bond is carried by the enzyme called alkaline phosphatase (ALP), which is a dimeric [1]. This membrane-bound glycoprotein shows their activity at basic pH [2]. Mammalian alkaline phosphatase (ALP) is metalloenzymes that contain two molecules of zinc and one of magnesium, in their active site which is essential for enzymatic activity [3].

1.1. Isoenzymes of ALP: Four tissue-specific forms or isoenzymes of ALP are identified mainly according to the specificity of the tissue to be expressed, termed as placental alkaline phosphatase (PALP or Regan isoenzyme), Intestinal alkaline phosphatase (IALP), liver/bone/kidney alkaline phosphatase (L/B/K ALP), and germ cell alkaline phosphatase (GCAP or NAGAO) [4]. Each isoenzyme is encoded by different gene loci [5]. The heat-labile isoenzyme represents the liver/bone/kidney or tissue-nonspecific (TNSALP) forms and is expressed in many tissues and especially abundant in hepatic, skeletal, and renal tissue [6]. IALP is ubiquitously expressed by enterocytes in the duodenum [7], and thereafter to a much lesser extent in the jejunum, ileum, and colon and is largely absent in the stomach [8]. Placental ALP is a thermostable glycoprotein of human trophoblast membranes [9], secreted by the placenta in the third trimester of pregnancy [10]. The gene encoded for placental ALP can be re-expressed by cancer cells as the Regan isoenzyme [11]. GCAP is a heat-stable isoenzyme present at low levels in germ cells [6], embryonal, and some neoplastic tissues [12].

1.2. Clinical Significance of ALP: Increased TNSALP is seen in bile duct obstruction and also in conditions with increased osteoblastic activity (Paget’s disease) or a disease that affects the blood calcium level (hyperparathyroidism), vitamin D deficiency, or damaged liver cells [2]. IALP Levels are also elevated in Celiac Disease [13] and also in patients with metastatic colorectal cancer [14]. The increased expression of the IALP gene had been seen in many hepatocellular carcinomas [15]. Placental ALP is secret by Syncytiotrophoblast cell of the placenta and a variety of tumors with Unknown function. PALP is also a marker of cancer of the ovary, testis, lung, and gastrointestinal tract [16]. Germ cell alkaline phosphatase (GCAP) secreted from Testis, malignant trophoblast, and testicular cancer with unknown function [11].

1.3. Inhibitors of ALP: Mammalian ALPs are inhibited by L-amino acids and peptides through an uncompetitive mechanism [17]. Selective inhibition by urea was used by other workers to differentiate the Isoenzymes of alkaline phosphatase [18]. A protein denaturant, Guanidine hydrochloride can be used for selective inactivation of alkaline phosphatase Isoenzymes [19]. The IALP and TNSALP are rapidly inactivated at a temperature of 65°C as compared to PALP, Regan, and GCAP which are remarkably thermostable [20]. Therefore this study was aimed to ascertain the type of Isoenzyme of ALP with the use of inhibitors and the correlation of isoenzyme of ALP with clinical diagnosis or/and lab investigations of the patients.

2. METRIAL AND METHODS

2.1. **Subjects-** This study was conducted in the department of biochemistry, Guru Gobind Singh Medical College and Hospital, Faridkot, Punjab, India from October 2017 to October 2018. The study was a cross-sectional with diagnostic accuracy type. A patient who had increased serum ALP level and wants to participate in the study were enrolled. The patients with normal serum ALP level, suffering from Hypothyroidism, using drugs like D-Penicillamine, Acyclovir, Azithromycin, Vitamin D supplements, and those who did not want to participate in the study were excluded from the study. The study was conducted on the patients of age more than 18 years of either sex visiting the central lab of Guru Gobind Singh Hospital, Faridkot for the biochemical investigations i.e. blood Urea, serum creatinine, ALT, AST, ALP, total bilirubin, calcium, and phosphorus The plan of the study was approved by the Ethical Committee of Guru Gobind Singh Medical College, Faridkot, and issued an ethical approval letter. The sample was taken from the patients after proper informed consent.

2.2. **Methods: -** 5 ml of the venous blood sample of the patient with increased ALP level was drawn under aseptic conditions. After clotting, the sample was centrifuged at 3000 rpm for 10 minutes to separate serum. The serum was used for the required investigation. All required investigations i.e. blood Urea, serum creatinine, ALT, AST, ALP, total bilirubin, calcium, and phosphorus was done on the fully auto analyzer Beckman Coulter AU480 (Beckman Coulter Inc., California, USA). For isoenzyme ascertaining Urea, L-Phenylalanine, and Guanidine hydrochloride were used as a chemical inhibitor of ALP isoenzymes. Urea extra pure AR 99.5% (86854, SRL Chem., Mumbai, India), L- Phenylalanine extra pure CHR 99% (85081, SRL Chem., Mumbai, India) and Guanidine hydrochloride extra pure for biochemistry (25722, SRL Chem., Mumbai, India) were used in the study. The urea, L-Phenylalanine, Guanidine Hydrochloride in the concentration of 1.3 mol/L (78g/L), 10 mmol/L (1.6 g/L), and 0.3 mmol/L (28.6 g/L) respectively were used as chemical inhibitors. First, ALP activity was measured without inhibitors, then 100 µl of inhibitor was added to 400 µl of serum and again ALP activity was measured after 5 minutes. For the heat inactivation, the serum was incubated at 65° C for 20 minutes after that ALP activity was measured.

2.3. Statistical analysis*: -* The sample size for the study was calculated by utilizing the proportion (P) of raised ALP as 0.6875 at a 5% margin of error and a 95% confidence level with the following formula:

$$X = Z_{a/2}^2 \quad *p*(1-p) / e^2$$

Or

$$1.96*1.96*p*(1-p)\ e^2$$

Where $Z_{a/2}$ is 1.96, e is the margin of error (5%) and p is the sample proportion (0.6875). The derived sample size was 232. So, a sample size of 250 was taken for the study.

The percentage of inhibition was calculated by the following formula:-

Percentage of inhibition = $\frac{V-Vi}{V} \times 100$

Where, V = Activity of ALP without inhibitor, V*i* = Activity of ALP with inhibitor

Other Statistical analysis was done on SPSS[20] software.

RESULTS

A total of 250 patients with increased ALP levels were enrolled, out of which 107 were males and 143 were females. The patients were grouped according to their clinical diagnosis or disorders (Figure 1). The Mean±SD of the age of patients was 54.7±29.2 years (range 19-85 years). The Mean±SD of the ALP level in 250 patients was 373±274 U/L (173-2523 U/L), which was higher than the reference range of 60-140 U/L (Table 1). Serum ALT, AST, and total bilirubin levels were elevated in patients with liver disorders. Serum calcium and phosphorous levels were deranged in patients with bone disorders. All the required biochemical parameters were within the normal limits in patients with pregnancy and intestinal disorders. Serum AST and ALT activity were deranged in patients with preeclampsia but, in patients of tuberculosis and other disorders along with serum ALT, AST activity; blood urea, and serum creatinine levels were also deranged (Table 2). The results of inhibition of ALP activity by chemical inhibitors and heat inactivation are variable according to disorder (Table 3). The percentage of inhibition in patients with liver disorders by urea, L-Phenylalanine, Guanidine Hydrochloride and heat inactivation was 49.5%, 40.5%, 48.6%, and 94.0% respectively and in patients with bone disorders, the percentage of inhibition by urea, L-Phenylalanine, Guanidine hydrochloride and heat inactivation was 56.6%, 38.1%, 45.8%, and 89.0% respectively. The percentage of inhibition by Urea, L-Phenylalanine, Guanidine Hydrochloride, and heat inactivation was 29%, 41.6%, 42.8%, and 95% respectively in patients with intestinal related disorders.

The percentage of inhibition in third-trimester pregnant females by Urea, L-Phenylalanine, Guanidine Hydrochloride, and heat inactivation was 44.4%, 36.8%, 47.1%, and 36.8%, respectively whereas, the percentage of inhibition in preeclampsia was 51.1%, 47.0%, 50.7%, and 51.2%. 90 patients of carcinoma and their secondary metastasis were subdivided into 8 groups based on the type of carcinoma and their secondary metastasis (Figure 2). Their biochemical parameters were investigated which are abnormal concerning the type of carcinoma. ALP levels were very high in patients with liver and bone involvement and patients involving the hepatobiliary tract (Table 4).

The percentage of inhibition (Table 5) of patients with carcinoma of liver and the biliary tract was 47%, 46%,48%, and 96% by Urea, L-Phenylalanine, Guanidine Hydrochloride, and heat inactivation respectively as compared to 36%, 44%, 45% and 94% in patients with carcinoma of the gastrointestinal tract. The percentage of inhibition of patients with carcinoma involving bones was 58%, 42%, 49%, and 86% by urea, L-Phenylalanine, guanidine hydrochloride, and heat inactivation respectively. In patients with leukemia and lymphomas, the percentage of inhibition was 41%, 38%, 31%, and 94% by Urea, L-Phenylalanine, Guanidine Hydrochloride, and heat inactivation respectively as comparable to 46%, 39%, 46%, and 96% in patients with carcinoma of the unknown primary site.

The percentage of inhibition in patients with carcinoma involving both bone and liver by Urea, L-Phenylalanine, Guanidine Hydrochloride, and heat inactivation was 41%, 40%, 41%, and 96% respectively. In patients with carcinoma of lungs, the percentage of inhibition was 50%, 39%, 44%, and 69% by Urea, L-Phenylalanine, Guanidine Hydrochloride, and heat inactivation respectively. In 10 out of 17 patients with tuberculosis, the pattern of inhibition by Urea, L-Phenylalanine, Guanidine Hydrochloride, and heat inactivation was similar to patients with liver diseases i.e. 46-55% by Urea and Guanidine Hydrochloride, ≤45% by L-Phenylalanine, and 90-95% by heat inactivation. In 6 patients with septic shock the percentage of inhibition by Urea was ≥46% and by Guanidine Hydrochloride was ≥42%, which indicated liver isoenzyme which further confirmed by their serum AST, ALT, and total bilirubin levels.

DISCUSSION

We drew an algorithm to find the type of isoenzyme of ALP (Figure 3). The 94.0% inhibition by heat inactivation ruled out the placental fraction of ALP in patients with liver disorders. The 49.5% inhibition by Urea and 48.6% inhibition by Guanidine Hydrochloride, which is approximately 50%, indicating the liver or bone isoenzyme of ALP which is a concordance to the study which was conducted by Shephard MD and Peake MJ [19], and 53% by Guanidine Hydrochloride according to another study [21]. In the patient of bone disorders of our study, 89% inhibition by heat inactivation indicated the absence of placental ALP and the percentage of inhibition of bone isoenzyme by Urea is more than that of the liver isoenzyme. The percentage of inhibition by Guanidine Hydrochloride is not comparable to previously conducted studies. This can be because of the inhibitory effects of a wide range of Guanidine Hydrochloride concentrations ranged from 0.025 mol/L to 1.2 mol/L. At the extremes of these concentration ranges inhibition was found to be either too small or too great [19]. Overlapping of percentage inhibition by Urea, L-phenylalanine, and Guanidine Hydrochloride for liver and bone isoenzymes, the sensitivity and specificity of inhibitors to ascertain the type of isoenzymes was low (50.85% and 52.3% respectively). But when the concentration of ALP after inhibitors was correlated with ALT and AST in liver disorders then it was very significant with $P<0.0001$ and in bone disorders correlation with serum calcium and phosphorous is also significant with $P=0.01$ and $P=0.01$ (Table 6). After correlating the percentage of inhibition with these biochemical parameters, the sensitivity and specificity increased to 96.6% and 98.4% respectively.

In patients of intestinal disorders, the placental fraction was ruled out because of 95% inhibition by heat inactivation. 41.6% inhibition by L-Phenylalanine was not able to confirm the presence of intestinal ALP. Therefore, it was easy to identify intestinal fractions by Urea inhibition, which inhibits 29% of the ALP activity in patients of intestinal disorders as compared to 49.5% in the liver and 56.6% in bone disorders. The percentage of inhibition of intestinal isoenzyme by Urea (33%) and heat inactivation is quite comparable to another study, but the percentage of inhibition by Guanidine Hydrochloride and L-Phenylalanine is not comparable but, it was suggested that inhibition of intestinal isoenzymes by Urea is more potent than Guanidine Hydrochloride [19].

The 37% inhibition in females of third-trimester pregnancy by heat inactivation indicated the type of the isoenzyme, which was placenta because the only placental fraction is heat stable. By this percentage of inhibition by heat inactivation, we identified the type of isoenzyme in 41 pregnant females. In preeclampsia, the percentage of inhibition by heat inactivation was 51.2%. This 51.2% inhibition by heat inactivation indicated that the type of isoenzyme was not the only placenta which was then confirmed by the percentage of inhibition of Urea and Guanidine Hydrochloride which was 51.1% and 50.7% respectively and this percentage indicated the other type of ALP isoenzyme was liver because Urea and guanidine hydrochloride inhibited ALP activity in the same pattern as in patients of liver disorders. The further correlation with serum ALT and AST level confirmed the type of isoenzyme of ALP. In the study, it was found that the placenta was the only isoenzyme which was more stable after heat inactivation as compare to other isoenzymes. The percentage of inhibition of placental isoenzyme by heat inactivation was 36.8% which is comparable to other studies [22, 23]. Placental isoenzyme being heat resistant, the sensitivity and specificity of this method to identify placental isoenzyme by heat inactivation were very good (94%, 92%).

The pattern of the percentage of inhibition in patients with liver carcinoma was quite similar to the percentage of inhibition of patients with liver disorders. The 36% inhibition by Urea in patients with carcinoma of the gastrointestinal tract was similar to 29% inhibition in intestinal disorders.

In cases of carcinoma of the genitourinary tract, the percentage of inhibition by heat inactivation was only 62%, while the percentage of inhibition by Urea, L-Phenylalanine, and Guanidine Hydrochloride was 45%, 32%, and 38% respectively (Table 8). This was because of the Regan like isoenzyme, a heat-stable ALP, which was first observed in a patient with metastatic bronchogenic carcinoma [24]. But it is significantly associated with tumors of the female breast and genitourinary tract and secreted by uterine cervical reserve and endometrial luminal surface lining cells [25]. The 86% inhibition by heat inactivation in

patients with carcinoma involving bones indicated that isoenzyme was not the placenta. 42% and 49% inhibition by L-Phenylalanine was not significant because it didn't indicate the type of isoenzyme, but 58% inhibition by urea was significant to identify bone isoenzyme because it was similar to the percentage of inhibition by urea (>55%) in bone disorders, which was further confirmed by correlation with increased serum calcium and phosphorus level. This hypercalcemia had been attributed to osteolytic bone metastases because of the production of various cytokines such as transforming growth factor-a (TGF a), tumor necrosis factor-α (TNF- α), interleukin-1, and interleukin-2 lead to increasing bone osteolysis [26].

In patients with leukemia and lymphomas, the 94% inhibition by heat inactivation assured that isoenzyme was neither placenta nor Regan. The inhibition pattern was nonspecific in these patients. Increased level of ALP, especially PLAP was seen in a previously conducted study on patients with leukemia, out of which ALP raised insignificantly, whereas PALP was raised significantly ($P < 0.001$) in leukemia patients [27].

In the patients with carcinoma involving both liver and bone, the placenta and Regan type of isoenzyme were absent because of 96% inhibition by heat inactivation. The percentage of inhibition by Urea and Guanidine Hydrochloride was 41% for both, which was less than the percentage of inhibition in liver disorders i.e. around 50%, but the percentage of inhibition by urea was more than that of inhibition in bone disorders. This pattern of inhibition indicated the involvement of both liver and bone isoenzyme. The correlation with other biochemical parameters was made in which serum ALT, AST, calcium, and phosphorus were elevated. In patients with Carcinoma of the unknown primary site, the 96% inhibition by heat inactivation ruled out the placental fraction of ALP, but 46% inhibition by Urea and 46% inhibition by Guanidine Hydrochloride, which was quite similar to the percentage of inhibition of patients with liver disorders.

In patients with carcinoma of the lungs, the 69% inhibition by heat inactivation assured that isoenzyme was either placenta or Regan. The percentage of inhibition by heat inactivation was strong than inhibition in pregnancy but not as strong as in the liver, bones, and intestinal disorders, which indicated the Regan type of isoenzyme. By further correlation with other biochemical parameters and clinical diagnosis, it was confirmed that the type of Isoenzyme was Regan because all the patients are non-pregnant and with normal biochemical parameters. Regan isoenzyme was found to be strongly associated with several cancers such as lung, gastric, uterine cervical, endometrial, ovarian, testicular and prostate, germ cell, medullary thyroid, as well as hematopoietic tumors [28].

In 10 out of 17 patients of tuberculosis, the pattern of inhibition by Urea, L-Phenylalanine, Guanidine Hydrochloride, and heat inactivation was similar to patients with liver disease and ALT and AST levels were elevated in these patients. This was because of tuberculosis infection of the liver, which is an extrapulmonary manifestation of active infection [29]. But, in 7 patients the patterns of inhibition were different. In these patients ALT and AST levels were normal but blood urea and serum creatinine levels were elevated. This was because of Urogenital tuberculosis, represents 27% of extrapulmonary cases [30]. In 6 patients of septic shock, the percentage of inhibition by Urea was ≥46% and Guanidine Hydrochloride was ≥42%, which indicated liver isoenzyme which further confirmed by correlation with increased AST, ALT, and total bilirubin levels. In the other 2 patients of septic shock, the percentage of inhibition by Urea was ≤25% and Guanidine Hydrochloride was ≤35% which indicated the involvement of the liver and intestine in these patients.

TABLES AND FIGURES

Table No 1: Serum Alkaline Phosphatase Activity.

No. of Patients	Mean±SD of Serum ALP (U/L)	Range of ALP (U/L)	Normal Range (U/L)
250	373±274	173- 2523	60- 140

Mean±SD of Raised ALP level, Range and Normal range of ALP in 250 patients.

Table 2: Biochemical Parameters in Patients of Various Disorders.

	Mean±SD of Level of							
	ALP (IU/L)	ALT (IU/L)	AST (IU/L)	Total Bilirubin (µmol/L)	Blood urea (mmol/L)	Serum Creatinine (µmol/L)	Serum Calcium (mmol/L)	Serum Phosphorus (mmol/L)
Liver	346±191	124±60.9	157±51.4	66.6±47.8	6.5±2.7	70.7±35.3	2.4±0.8	1.0±0.2
Bone	268±70.6	35.3±11.7	37.8±13.2	15.3±5.1	5.3±1.6	53.0±17.6	1.8±0.3	0.9±0.3
Intestine	235±18.8	21.8±6.2	32.4±4.9	11.9±3.4	4.6±0.8	61.8±17.6	2.2±0.2	1.2±0.1
Pregnancy	269±56.5	23.0±8.7	36.0±12.0	11.9±5.1	4.9±2.5	53.0±17.6	2.3±0.3	1.1±0.1
Preeclampsia	290±76.9	101±45.5	126±65.1	18.8±10.2	4.6±2.2	53.0±35.3	2.4±0.2	1.1±0.1
Tuberculosis	308±127	78.0±60.0	98.4±86.6	17.1±10.2	14.9±6.8	194.5±176.8	2.0±0.2	1.0±0.2
Others	676±475	156±118	380±399	87.2±64.9	27.3±16.9	353.6±221.8	2.2±0.4	1.1±0.2

Mean±SD of serum ALT, AST, Total Bilirubin, Blood Urea, Serum Creatinine, Serum calcium, and Serum Phosphorus levels in patients having liver, bone, intestinal disorders, pregnant females, preeclampsia, tuberculosis infection, and other disorders.

Table 3: Percentage of inhibition of ALP activity by various inhibitors.

	Percentage of Inhibition			
	Urea (1.3 Mol/L)	**L-Phenylalanine (10 mmol/ L)**	**Guanidine Hydrochloride (0.3 Mol/L)**	**Heat Inactivation (65°C)**
Liver	49.5%	40.5%	48.6%	94.0%
Bone	56.6%	38.1%	45.8%	89.0%
Intestine	29.0%	41.6%	42.8%	95.0%
Pregnancy	44.4%	36.8%	47.1%	36.8%
Preeclampsia	51.1%	47.0%	50.7%	51.2%
Carcinoma & Secondary Metastasis	46.5%	41.2%	45.9%	86.6%
Tuberculosis	46.6%	37.5%	48.7%	91.7%
Others	44.6%	35.8%	43.5%	95.0%

The percentage of inhibition of ALP activity by Urea (1.3 mol/L), L-Phenylalanine (10 mmol/L), Guanidine Hydrochloride (0.3 mol/L), and Heat Inactivation (65°C)

Table 4: Levels of various biochemical parameters in patients with Carcinoma and their secondary metastasis.

	Mean±SD level of							
	ALP (U/L)	ALT (U/L)	AST (U/L)	Total Bilirubin (μmol/L)	Blood Urea (mmol/L)	Serum Creatinine (μmol/L)	Serum Calcium (mmol/L)	Serum Phosphorus (mmol/L)
Liver & Biliary tract	687±630	207±224	208±220	88.9±70.1	8.6±6.7	176.8±123.7	2.2±0.2	1.0±0.2
Gastrointestinal Tract	307±120	37.2±14.2	44.0±17.4	15.3±6.8	7.8±3.0	88.4±35.3	2.3±0.4	1.3±0.3
Genitourinary Tract	341±140	38.8±29.8	46.9±31.8	10.2±1.7	5.9±2.2	70.7±44.2	2.3±0.3	1.3±0.4
Involving Bones	435±201	51.5±33.4	58.9±40.5	11.9±3.4	7.8±4.1	167.9±106.1	3.2±0.2	2.0±0.1
Leukemia & Lymphomas	213±68.7	58.0±30.4	41.7±13.2	10.2±1.7	5.1±1.3	70.7±17.6	2.3±0.3	1.4±0.3
Bone & Liver	927±437	64±13	136±84.9	18.8±3.4	4.3±1.5	44.2±17.6	3.4±0.1	2.1±0.2
Unknown Primary Site	334±216	52±21	51.4±19.3	11.9±3.4	4.1±1.6	61.8±8.8	2.4±0.2	1.3±0.2
Lung	560±379	28±18	38.5±17.4	8.5±1.7	4.9±1.3	53.0±26.5	2.2±0.1	1.2±0.1

Mean±SD of ALP activity and levels other biochemical parameters in patients with a different type of Carcinoma and their secondary metastasis. ALP levels were very high in patients with liver and bone involvement and patients involving the hepatobiliary tract.

Table 5: Percentage of inhibition of ALP activity by various inhibitors in patients with carcinoma and their secondary metastasis.

	Percentage of Inhibition			
	Urea (1.3 Mol/L)	L-Phenylalanine (10 mmol/ L)	Guanidine Hydrochloride (0.3 Mol/L)	Heat Inactivation (65°C)
Liver & Biliary tract	47%	46%	48%	96%
Gastrointestinal Tract	36%	44%	45%	94%
Genitourinary Tract	45%	32%	38%	62%
Involving Bones	58%	42%	49%	86%
Leukemia & Lymphomas	41%	38%	31%	94%
Bone & Liver	41%	40%	45%	96%
Unknown Primary Site	46%	39%	46%	96%
Lung	50%	39%	44%	69%

The percentage of inhibition by Urea (1.3 mol/L), L-Phenylalanine (10 mmol/L), Guanidine Hydrochloride (0.3 mol/L), and Heat Inactivation (65°C) in patients of cancer and their secondary metastasis.

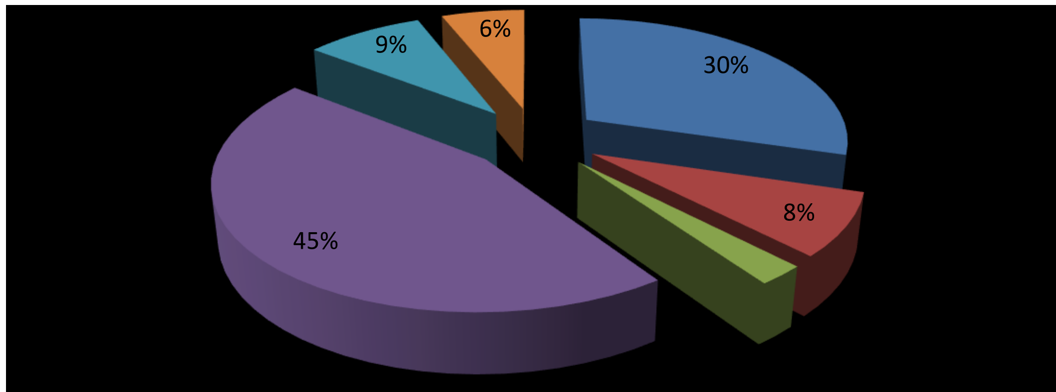
Table 7: Correlation between ALP level after Inhibition and biochemical parameters of patients with liver and bone disorders.

Parameters	Mean±SD in		P Value	95% Confidence Interval	Significance Level
	Liver	Bone			
ALT (IU)	124±60.9	35.3±11.7	<0.0001	-119.3397 to -58.0603	Highly Significant
AST (IU)	157±51.4	37.8±13.2	<0.0001	-145.1561 to -93.2439	Highly Significant
T.Bil. (µmol/L)	66.6±47.8	15.3±5.1	<0.0058	-5.1045 to -0.8955	Significant
Calcium (mmol/L)	2.4±0.8	1.8±0.3.	0.01	-4.0375 to -0.3625	Significant
Phosphorus (mmol/L)	1.0±0.2	0.9±0.3	0.01	-8.879 to -0.1221	Significant

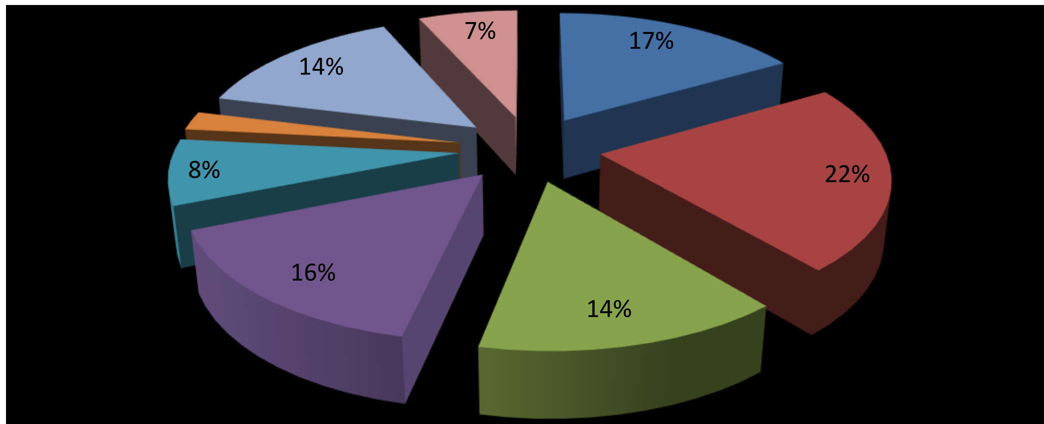
Significance difference between biochemical parameters of patients with liver and bone disorders. AST, ALT and Total Bilirubin were significantly increased in patients with liver disease as compared to bone disorders. Calcium and phosphorus were significantly decreased in patients with bone disorders

Figure:1 Distribution of Patients

Liver Disorders= 59
Bone Disorders= 16
Intestinal Disorders= 05
Cancer & Secondary Metastasis= 90
Tuberculosis= 17
Others= 12

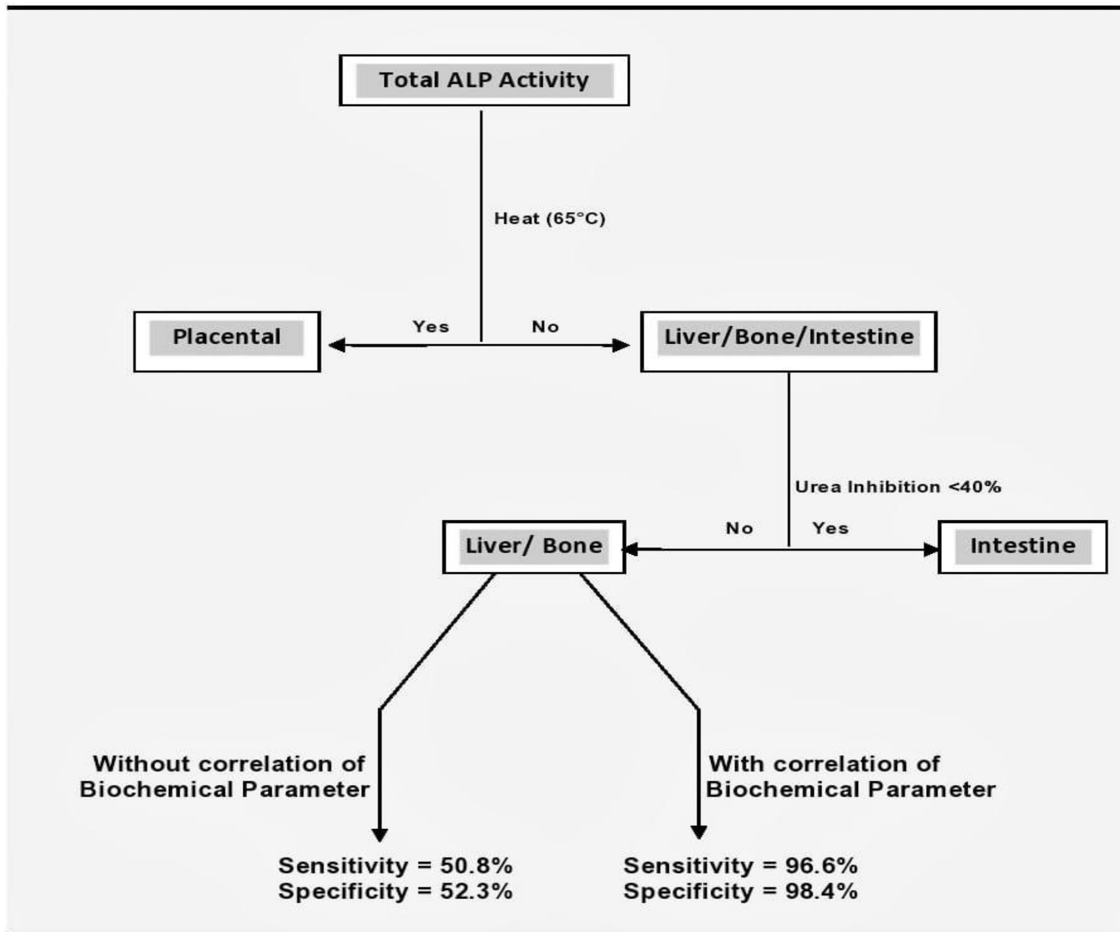


Distribution of 250 patients according to their known clinical diagnosis



Distribution of 90 patients with carcinoma and their secondary metastasis according to organ involvement

Figure: 3 Algorithm



Algorithm, Drew to find the type of isoenzyme based on the percentage of inhibition of ALP Activity by various inhibitors

CONCLUSION

Our study concluded that ascertaining of an isoenzyme of ALP by inhibition method is not specific and sensitive enough to be used alone. But chemical inhibition by urea, L-phenylalanine and Guanidine hydrochloride provides 96.6% sensitivity and 98.4% specificity when correlated with other biochemical parameters and clinical diagnosis. Ascertaining of PLAP and Regan ALP by heat inactivation is more sensitive and specific, which can be used to differentiate various carcinoma involving germ cells, various gynecological malignancies such as endometrial and uterine cancer, prostate and testicular cancer.

REFERENCES

1. Sergienko E, Su Y, Garcia X, Brown B, Hurder A, NarisawaS, et al. Identification and Characterization of Novel Tissue Nonspecific Alkaline Phosphatase Inhibitors with Diverse Modes of Action. J Biomol Screen. 2009; 14(7): 824-37.
2. Sharma U, Pal D, Prasad R. Alkaline Phosphatase an Overview. Ind J Clin Biochem. 2014; 29(3): 269-78.
3. Hoylaerts MF, Manes T, Millan JL. Mammalian alkaline phosphatases are allosteric enzymes. J Biol Chem. 1997; 272(36): 22781-7.
4. Sligbrand T. Present status and future trends of human alkaline phosphatases. Prog Clin Biol Res. 1984; 166: 3-14.
5. Weiss MJ, Ray K, Henthorn PS, Lamb B, Kadesch T, Harris H. Structure of the human liver bone kidney alkaline phosphatase gene. J Biol Chem. 1988; 263(24): 12002-10.
6. Tsai LC, Hung MW, Chen YH, Su WC, Chang GG, Chang TC. Expression and regulation of alkaline phosphatases in human breast cancer MCF 7 cells. Eur J Biochem. 2000; 267(5): 1330-9.
7. Narisawa S, Huang L, Iwasaki A, Hasegawa H, Alpers DH, Millan JL. Accelerated fat absorption in intestinal alkaline phosphatase knockout mice. Mol Cell Biol. 2003; 23(21): 7525–30
8. Molnar K, Vannay A, Szebeni B, Banki NF, Sziksz E, Cseh A, et al. Intestinal alkaline phosphatase in the colonic mucosa of children with inflammatory bowel disease. World J Gastroenterol. 2012; 18(25): 3254-9.
9. Wei SC and Doellgast GJ. Immunochemical studies of human placental-type variants of alkaline phosphatase. Structural differences between the Nagao isoenzyme and the placental D variant. Eur J Biochem. 1981; 118(1): 39-45.
10. Vergote IB, Abeler VM, Bormer OP, Stigbrand T, Trope C, Nustad K. CA125, and placental alkaline phosphatase as serum tumor markers in epithelial ovarian carcinoma. Tumour Biol. 1992; 13(3): 168-74.
11. Fishman WH, Inglis NR, Green S, Anstiss CL, Gosh NK, Reif AE, et al. Immunology and biochemistry of Regan isoenzyme of alkaline phosphatase in human cancer. Nature. 1968; 219(5155): 697-9.
12. Hofmann MC, Jeltsch W, Brecher J, Walt H. Alkaline phosphatase isozymes in human testicular germ cell tumors, precancerous stage, and three related cell lines. Cancer Res. 1989; 49(17): 4696-700.
13. Preussner HT. Detecting celiac disease in your patients. Am Fam Physician. 1998; 57(5): 1023-34.
14. Saif MW, Alexander D, Wicox CM. Serum Alkaline Phosphatase Level as a Prognostic Tool in Colorectal Cancer A Study of 105 patients. J Appl Res. 2005; 5(1): 88-95.
15. Higashino K, Otani R, Kudo S, Yamamura Y. Fetal intestinal-type alkaline phosphatase in hepatocellular carcinoma tissue. Clin Chem. 1977; 23(9): 1615-23.
16. Le Du MH and Millan JL. Structural evidence of functional divergence in human alkaline phosphatases. J Biol Chem. 2002; 277(51): 49808-14.
17. Millan JL. Alkaline Phosphatases Structure, substrate specificity, and functional relatedness to other members of a large superfamily of enzymes. Purinergic Signal. 2006; 2(2): 335-41.
18. Buttery JE, Milner CR, Nenadovic P, Pannal PR. Detection of alkaline phosphatase immunoglobulin complexes. Clin Chem. 1980; 26(11): 1620-1.
19. Shephard MD and Peake MJ. A quantitative method for determining serum alkaline phosphatase isoenzyme activity I Guanidine hydrochloride new reagent for selectively inhibiting major serum isoenzymes of alkaline phosphatase. J Clin Pathol. 1986; 39(9): 1025-30.
20. Harris H. The human alkaline phosphatases what we know and what we don’t know. Clin Chim Acta. 1990 Jan 15; 186(2): 133-50.

21. Shephard MD, Peake MJ, Walmsley RN. A quantitative method for determining serum alkaline phosphatase isoenzyme activity II. Development and clinical application of the method for measuring four serum alkaline phosphatase isoenzymes. J Clin Pathol. 1986; 39(9): 1031-8.
22. Mahjaoub S and Raudari JM. Comparison of heat inactivation and urea inhibition method for the determination of bone and liver alkaline phosphatase isoenzymes. J Babol Univ Med Sci. 2006; 8(1): 34-9.
23. Mahjoub S and Roudsari JM. Quantification of Liver Alkaline Phosphatase Isoenzyme Activity Using Heat Inactivation and Phenylalanine Inhibition Techniques Comparison of Two Methods. World Applied Sciences Journal. 2012; 17 (8): 941-6.
24. Fishman WH, Inglis NI, Stolbach LL, Krant MJ. A serum alkaline phosphatase isoenzyme of human neoplastic cell origin. Cancer Res.1968; 28(1): 150-4.
25. Cadeau BJ, Blackstein ME, Malkin A. Increased incidence of placenta-like alkaline phosphatase activity in breast and genitourinary cancer. Cancer Res. 1974; 34(4): 729-32.
26. Esbrit P. Hypercalcemia of malignancy new insights into an old syndrome. Clin Lab. 2001; 47(1-2): 67- 71.
27. Patel PS, Adhvaryu SG, Balar DB. Clinical significance of serum total and heat-stable alkaline phosphatase in leukemia patients. Tumori. 1993; 79(5): 352-6.
28. Watanabe S, Aihara Y, Kikuno A, Sato T, Komoda T, Kubo O, et al. A highly sensitive and specific chemiluminescent enzyme immunoassay for placental alkaline phosphatase in the cerebrospinal fluid of patients with intracranial germinomas. Pediatr Neurosurg 2012; 48(3): 141-5.
29. Hickey AJ, Gounder L, Moosa MY, Drain PK.A systematic review of hepatic tuberculosis with considerations in human immunodeficiency virus co-infection. BMC Infect Dis. 2015;15: 209
30. Daher Ede F, da Silva GB Jr, Barros EJ. Renal Tuberculosis in the Modern Era. Am J Trop Med Hyg. 2013; 88(1): 54–64.