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

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Authors' contributions

This work was carried out in collaboration between both authors. This work was carried out by the corresponding author under the guidance of a co-author. Author GSG designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author GG managed the analysis of the study and the literature searches. Both authors read and approved the final manuscript.

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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-

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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 in raised ALP level was 82.45% and 66.13% respectively. The area under the ROC curve (AUC) is 0.744 which is significant (P<0.0001).

Keywords: Alkaline phosphatase; isoenzyme; inhibition; urea; l-phenylalanine; guanidine hydrochloride; heat inactivation.

1. INTRODUCTION

The catalysis of the cleavage of the phosphoric ester bond are carried by the enzyme called alkaline phosphatase (ALP), which is a dimeric [1]. This membrane-bound glycoprotein shows its 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 is especially abundant in hepatic, skeletal, and renal tissue [6]. IALP is ubiquitously expressed by interests 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 encoding 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 cells of the placenta and a variety of tumors with Unknown functions. 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 an isoenzyme of the ALP with clinical diagnosis or/and lab investigations of the patients.

2. MATERIALS 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 cross-sectional with diagnostic accuracy type. Since we aim to identify the type of isoenzyme in raising ALP level and its correlation with clinical diagnosis. So, we enrolled all the Patients aged more than 18 years who had increased serum ALP level and wanted to participate in the study. The patients under the age of 18 years were excluded because of the high level of ALP due to high osteoblastic activity during that period of age. The Patients with normal serum ALP levels, 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 either sex visiting the central lab of the Guru Gobind Singh Hospital, Faridkot for biochemical investigations i.e. Blood Urea, serum creatinine, ALT, AST, ALP, total bilirubin, calcium, and phosphorus.

2.2 Methods

5 ml of the venous blood sample of the patient with an 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 mmol/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 raising ALP as 0.6875 at a 5% margin of error and a 95% confidence level with the following formula:

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

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:-

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

Where, V = Activity of ALP without inhibitor, V*i* = Activity of ALP with inhibitor
Another Statistical analysis was done on SPSS[20] software.

3. RESULTS

A total of 250 patients with increased ALP levels were enrolled, out of which 107 were males and 143 were females. Patients were categorized according to their clinical diagnosis or condition (Fig. 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). Patients with liver disorders were classified based on a variety of liver conditions. Serum ALT, AST and T.Bil. Levels were high in patients with liver disease (Table 2). 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 3). The results of inhibition of ALP activity by chemical inhibitors and heat inactivation are variable according to the disorder (Table 4). 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 with carcinoma and their secondary metastasis were subdivided into 8 groups based on the type of carcinoma and their secondary metastasis (Fig. 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 5).

The percentage of inhibition (Table 6) of patients with carcinoma of the 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% of 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 the 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 was further confirmed by their serum AST, ALT, and total bilirubin levels.

Out of 250 patients, the type of isoenzyme was determined in 172 patients, while isoenzyme was not identified in 78 patients (Table 8).

4. DISCUSSION

We drew an algorithm to find the type of an isoenzyme of ALP (Fig. 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 with 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 ranging from 0.025 mmol/L to 1.2 mmol/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 disorder correlation with

serum calcium and phosphorous is also significant with *P=0.01* and *P=0.01* (Table 7).

In patients with 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 with 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 an isoenzyme of ALP. In the study, it was found that the placenta was the only isoenzyme that was more stable after heat inactivation as compared 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 identify placental isoenzyme by heat inactivation were very good.

The pattern of the percentage of inhibition in patients with liver carcinoma was quite similar to the percentage of inhibition in 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 6). 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 increased bone osteolysis [26].

In patients with leukemia and lymphomas, the 94% inhibition by heat inactivation assured that the 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 the 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 stronger than inhibition during 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 urogenital tuberculosis represent 27% of extrapulmonary cases [30]. In 6 patients with septic shock, the percentage of inhibition by Urea was ≥46% and Guanidine Hydrochloride was ≥42%, which indicated liver isoenzyme which was further confirmed by correlation with increased AST, ALT, and total bilirubin levels. In the other 2 patients with 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.

The 250 patients were assigned to 10 groups based on the range of ALP levels. It was observed that the percentage of identification of ALP isoenzyme is around 80% in patients who have increased ALP level ranged up to 400-450 IU/L. But the percentage of identification declines when the ALP level exceeds 400 IU/L (Table 8).

The sensitivity and specificity of this method were 82.45 and 66.13 respectively. AUC was 0.744 with a significance level P≤0.0001 (Fig. 4).

Table 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. Classification of Patients with Hepatobiliary disorders

Hepatobiliary Disorders	Number of patients	Mean±SD			
		ALP (IU/L)	ALT (IU/L)	AST (IU/L)	T. Bill. (mg/dl)
Acute Viral Hepatitis	04	264±80	80±12	85±4.0	0.8±0.2
Alcoholic Cirrhosis	07	296±28	40±14	76±24	1.5±0.8
Cholelithiasis	07	550±306	37±26	48±21	23±5.0
Chronic Liver Disease	14	326±151	64±37	88±22	1.0±0.5
Hepatomegaly	03	598±343	401±190	245±99	2.4±0.7
Hepatosplenomegaly	06	406±221	57±24	80±36	0.9±0.3
Liver Laceration	05	253±14	99±48	61±18	0.4±0.2
Obstructive Jaundice	02	373±105	28±9.0	39±4.0	5.0±1.3
Portal Hypertension	02	240±68	25±5.0	37±7.0	0.5±0.4
Primary Biliary Cirrhosis	09	239±47	30±12	48±26	2.0±1.8

Mean±SD of Liver Function Tests in patients with various Hepatobiliary disorders which are used to correlate Liver isoenzyme with the clinical diagnosis of patients

Table 3. 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 4. Percentage of inhibition of ALP activity of 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 mmol/L), L-Phenylalanine (10 mmol/L), Guanidine Hydrochloride (0.3 mmol/L), and Heat Inactivation (65°C)

Table 5. Levels of various biochemical parameters in patients with Carcinoma and their secondary metastasis

	Mean±SD level off							
	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 of other biochemical parameters in patients with different types of carcinomas and their secondary metastases. ALP levels were very high in patients with liver and involvement

Table 6. Percentage of inhibition of ALP activity of 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 mmol/L), L-Phenylalanine (10 mmol/L), Guanidine Hydrochloride (0.3 mmol/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. Bill. (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

Significant difference in biochemical endpoints in patients with liver and bone problems. Levels of AST, ALT and total bilirubin were significantly higher in patients with liver disease compared to bone disorders. Calcium and phosphorus were significantly decreased in patients with bone disorders

Table 8. Number of patients with different ranges of ALP and percentage of identification of Isoenzyme

Range of ALP level (IU/L)	No. of Patients	Isoenzyme Identified in	Isoenzyme unidentified in	Percentage of identification
170-200	31	25	6	81%
200-300	116	93	23	80%
300-400	46	36	10	78%
400-500	14	10	4	71%
500-600	12	7	5	58%
600-700	10	3	7	30%
700-800	8	2	6	25%
800-900	6	1	5	16%
900-1000	4	1	3	25%
≥1000	3	0	3	0%

Showing patient groups, based on a range of ALP concentration in which isoenzyme has been identified or not identified

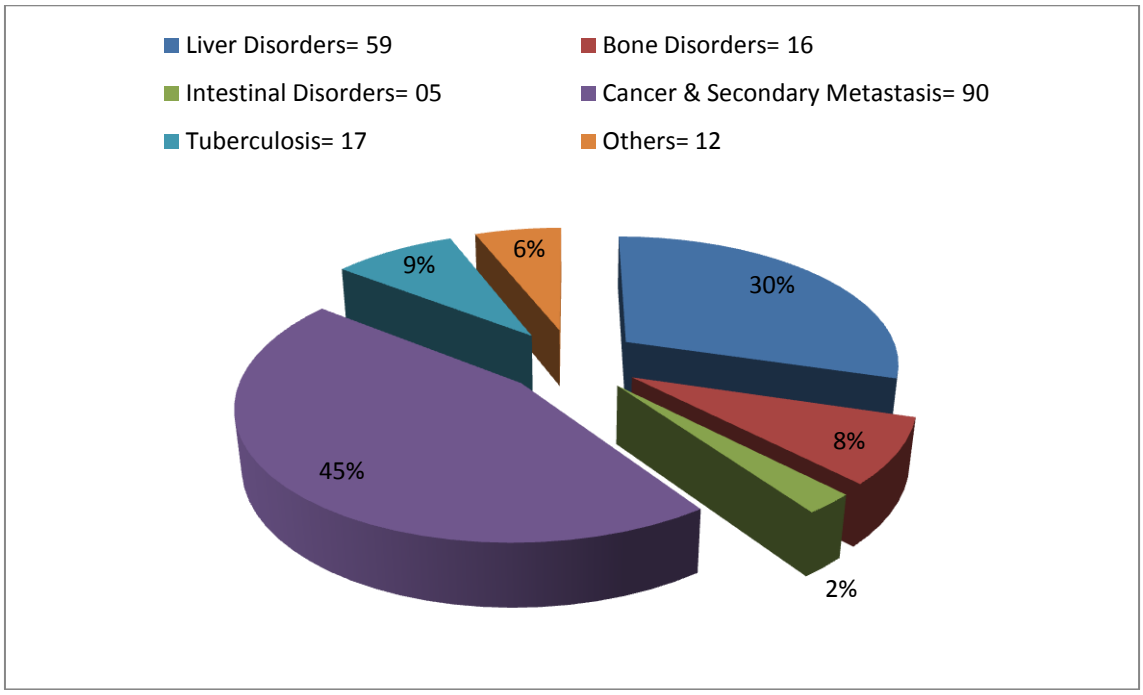

Fig. 1. Distribution of Patients
Distribution of 250 patients based on their identified clinical diagnosis

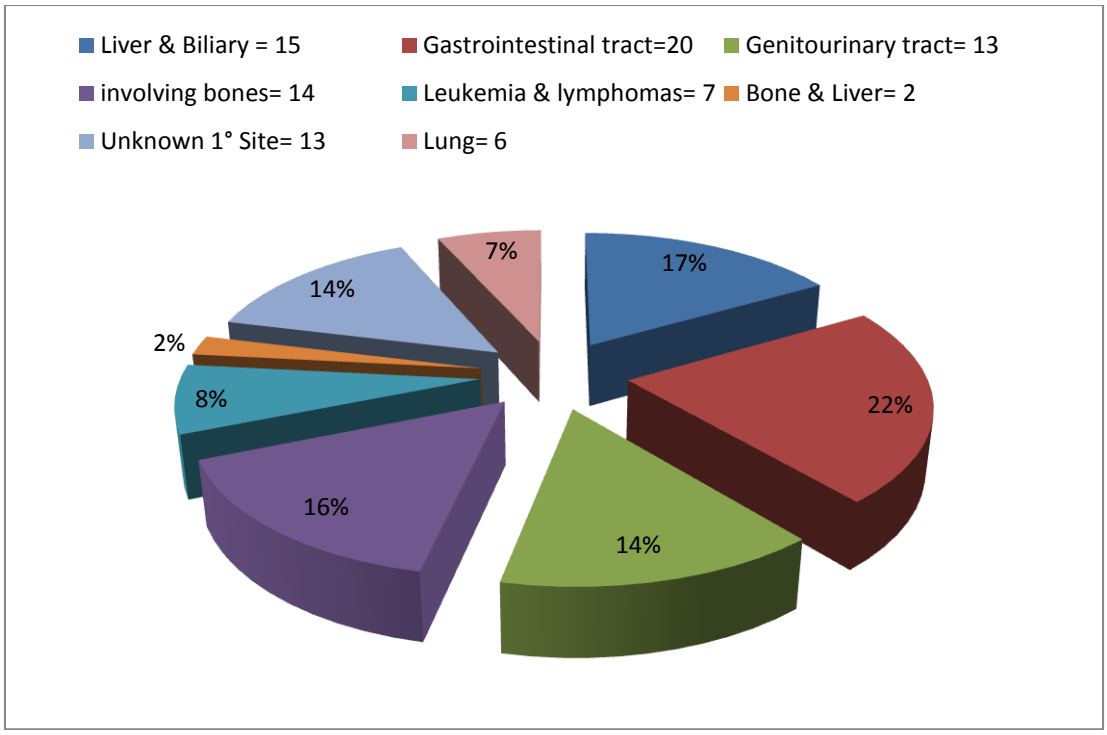

Fig. 2. Distribution of cancer patients
Breakdown of 90 patients with carcinoma and their secondary metastases by organ involvement

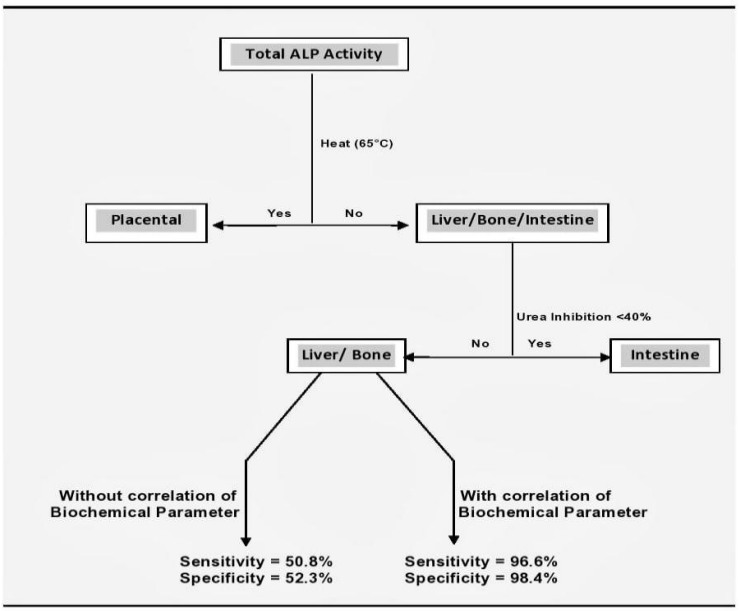

Fig. 3. Algorithms

A Drew algorithm for finding the type of isoenzyme based on the percentage inhibition of ALP activity by various inhibitors

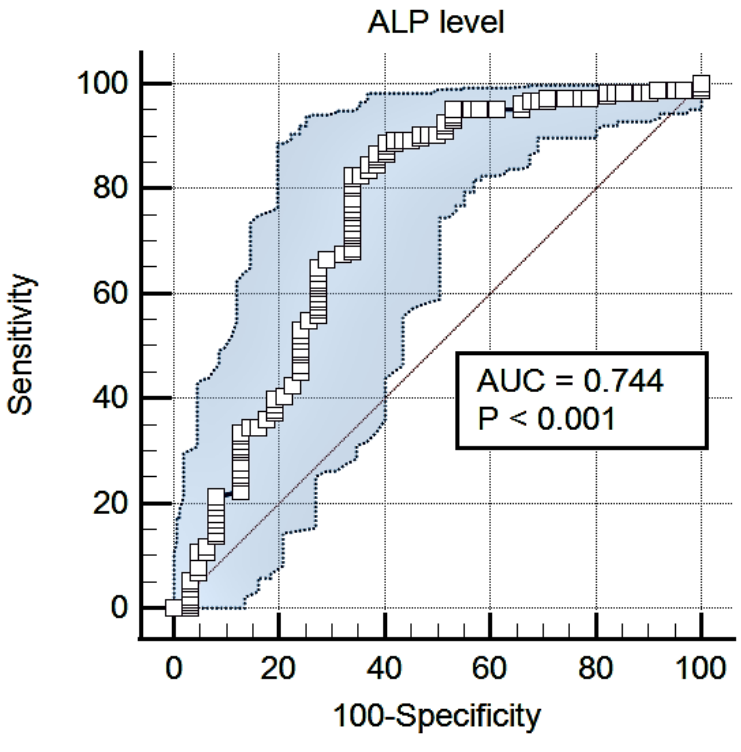

Fig. 4. ROC Curve

The ROC curve shows the sensitivity and specificity of this inhibition method for identifying the type of isoenzyme at various ranges of ALP levels

5. CONCLUSION

Our study concluded that ascertaining an isoenzyme of ALP by inhibition method can play a pivotal role in the identification of isoenzyme in patients who have raised the ALP level to a range of 400-450 IU/L. This method has a sensitivity and specificity of 82.45 and 66.13 respectively. However, for patients with ALP levels above 450 IU/L, this method is unreliable when used alone. Ascertaining of PLAP and Regan ALP by heat inactivation is more sensitive and specific, which can be used to differentiate various carcinomas involving germ cells, various gynecological malignancies such as endometrial and uterine cancer, prostate and testicular cancer. This method can be used for the detection of various diseases in which ALP levels are being raised, but to replace the golden standard for detection of each disease furthermore, studies should be conducted with more samples and more advanced techniques such as electrophoresis.

CONSENT AND ETHICAL APPROVAL

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 writtent consent.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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