[Chem. Pharm. Bull. 36(5)1814—1818(1988)]

Assay of Alkaline Phosphatase Isoenzymes by a Convenient Precipitation and Inhibition Methodology

TOSHIO MAZDA*,[a] and WILLIAM L. GYURE[b]

Japanese Red Cross Tokyo Metropolitan Blood Center,[a] 1–26–1, Kyonan-cho, Musashino-shi, Tokyo 180, Japan and Seton Hall University,[b] South Orange, New Jersey, 07079, U.S.A.

(Received September 2, 1987)

By combining the theophylline inhibition assay for placental isoenzyme analysis with the lectin precipitation procedure for bone isoenzyme detection, a new more complete method for alkaline phosphatase isoenzyme analysis was devised. Moreover, the method employs common clinical laboratory instruments and is easy to perform.

After an examination of the method and its verification by electrophoretic methods with commercial control samples and clinical samples, a careful study was performed to determine the normal ranges. These results were then used as guidelines for placing patients in clinical categories. Verification of these categories by other clinical data on the patients confirmed the validity of the method.

Keywords——alkaline phosphatase; isoenzyme analysis; lectin precipitation; theophylline inhibition

Introduction

Increases in serum alkaline phosphatase activity are signs of liver, bone, intestinal and placental disorders and possibly of several types of tumors.[1] Because of this and because easily performed substitutes for bone activity monitoring are not available, physicians continue to request alkaline phosphatase isoenzyme analysis. Electrophoretic procedures have been most successful in the past in performing this assay, but because of poor separations between the two most important isoenzymes, *i.e.* bone and liver, this and other methods are still being improved. Several different procedures for some isoenzymes have been recently published which reliably identify activity from several of the isoenzymes, while being fast and convenient enough for the common hospital laboratory.[2,3] Neither of these methods alone detects or documents all the enzyme fractions of interest. Therefore studies were conducted in our laboratories to combine these methods into a more universal procedure and to adapt it to existing laboratory instruments. We present here a report of our studies with a proposed general procedure and its application to hospital samples.

Methods

Samples, Chemicals and Instruments——Serum samples were prepared from blood and stored at 4 °C until assay (within 3 d).

Theophylline (catalog number T-1633) and lectin from *Triticum vulgaris* (wheat germ) (catalog number L-1005) were both obtained from Sigma Chemical Company, St. Louis, Missouri. Triton X-405 wetting agent was obtained from the Alpkem Corporation, Clackamas, Oregon (catalog number A21-0302-33). Alkaline phosphatase reagent (catalog number 81184), was obtained from the Sclavo Diagnostics Company, Wayne, N. J., and was prepared exactly as directed by the firm. The alkaline phosphatase reagent consisted of a *p*-nitrophenylphosphate substrate in alkaline buffer. *p*-Nitrophenol released by enzymatic action colors the solution yellow at alkaline pH values and its concentration is read at 405 nm.

An Electronucleonics Gemeni centrifugal analyzer was used for all enzyme analyses following the company's directions; 15 μl of sample and 700 μl of reagent were routinely used in all tests for the enzymes.

Reagents——Theophylline inhibition reagent was prepared by dissolving 125 mg of theophylline in 100 ml of alkaline phosphatase reagent. This solution was stable at 4 °C for the same length of time as the original alkaline phosphatase reagent. Lectin precipitating reagent was prepared by dissolving 25 mg of lectin in 5 ml of deionized water. This solution was stable at 4 °C for at least 60 d. Triton X-405 reagent was prepared by adding 10 g of detergent to 50 ml of deionized water.[4] This solution was stable at 4 °C for at least 60 d.

Protocol for the Assay——Extended Assay: A 100 μl aliquot of serum was mixed with 10 μl of Triton X-405 reagent in test tubes and allowed to remain at 37 °C for 30 min. After the incubation period, an aliquot was removed for total alkaline phosphatase analysis using the alkaline phosphatase reagent. Another aliquot was removed for alkaline phosphatase analysis using the theophylline inhibition reagent. Another 50 μl aliquot was removed and mixed with 50 μl of lectin reagent and allowed to remain at 37 °C for 30 min, followed by centrifugation at 2500 × g for 15 min. An aliquot of supernatant solution was removed for analysis using the alkaline phosphatase reagent. The precipitate was dissolved in 100 μl of 0.9 g/dl saline solution and analyzed with the alkaline phosphatase reagent.

Rapid Assay: Omit analysis of the precipitate from the above protocol. It was convenient with the rapid assay to use microcentrifuge tubes with clot activator to trap and remove all precipitate during centrifugation.

Calculations: 1. Total alkaline phosphatase activity = instrument results × 1.1. 2. Placental alkaline phosphatase activity = results from theophylline inhibition assay × 2.45. 3. Liver and intestinal activity = supernatant solution analysis × 2.2 − the placental fraction activity from step 2. 4. Bone fraction activity = redissolved precipitate analysis × 2.75. If the rapid assay is used, then the bone fraction is the quantity obtained when the values obtained in calculations 2 and 3 above are subtracted from the total enzyme activity 1.

Electrophoresis——Electrophoresis was carried out in 1% agarose gel using a commercial kit (Al-Phos Isozyme-Test, Wako Pure Chemical Industries Ltd., Osaka).

Results

Normal Ranges

Sera from twenty adult males and twenty adult non-pregnant females were assayed to determine normal ranges for the various enzyme fractions. Clinical records and serum gamma-glutamyl transferase (γ-GT) values of each patient were studied so that only healthy subjects were chosen. The results of this study using the described protocol are presented in Table I.

Identification and Recovery of Isoenzymes

Identification of placental isoenzyme in serum samples by theophylline inhibition was studied in several ways. First, the isoenzyme was prepared from a pool of serum samples obtained from females during the last trimester of pregnancy. The pool was heated at 65 °C for 30 min to inactivate all but the placental isoenzyme.[5] When the preparation was assayed by the described protocol, 95—100% of the activity was recovered and correctly identified as placental. Second, aliquots of the preparation were added to sera from normal subjects, and liver disease patients, and to saline solutions: 90 to 119% of the added placental activity was recovered and identified as placental in origin. Third, samples from healthy women during the last trimester of pregnancy were examined and the activities obtained are listed in Table I.

Identification of bone isoenzyme by lectin precipitation was made by first examining sera from healthy growing children free of disease as judged by normal γ-GT values and absence of clinical signs of disease. These samples contained high total alkaline phosphatase activity and large amounts of bone fraction activity as shown in Table I. No placental alkaline phosphatase was detected in the precipitated fraction when assayed by the theophylline inhibition method. When samples with large amounts of bone isoenzyme activity were mixed with sera from normal subjects, from pregnant women, and from patients with liver disease, and with saline solutions, 95—114% of the added activity was recovered and identified as being of bone origin.

Identification of the liver fraction was studied in adult patients' sera with elevated levels of alkaline phosphatase and γ-GT activity. In all these samples large amounts of liver

TABLE I. Alkaline Phosphatase Isoenzyme Analysis

Sample	Number of samples		Placental fraction		Bone fraction	
			I.U./l	% of total	I.U./l	% of total
Normal males	20	$\bar{x}\pm$S.D.	1.9±2.2	3.2±3.7	22.6±11.7	38.7±17.8
		Range	0—7	0—13	3—47	4—68
Normal non-pregnant females	20	$\bar{x}\pm$S.D.	3.4±3.8	6.0±6.6	19.4±8.8	34.3±15.3
		Range	0—12	0—18	3—39	4—65
Females, last trimester of pregnancy	17	$\bar{x}\pm$S.D.	45.3±20.5	33.1±8.0	46.1±30.7	32.4±10.6
		Range	22—92	23—50	8—149	8—54
Children 2 months to 16 years	18	$\bar{x}\pm$S.D.	10.3±12.0	3.4±3.0	119.8±94.4	73.3±18.2
		Range	0—41	0—10	83—443	35—97
Liver disease patients	14	$\bar{x}\pm$S.D.	11.2±7.1	6.2±4.5	55.6±21.2	30.1±13.1
		Range	0—21	0—16	19—94	10—55

Sample	Number of samples		Liver fraction		Total activity I.U./l
			I.U./l	% of total	
Normal males	20	$\bar{x}\pm$S.D.	39.7±14.3	67.6±17.2	58.8±10.9
		Range	23—74	40—99	40—80
Normal non-pregnant females	20	$\bar{x}\pm$S.D.	39.8±13.1	68.8±12.9	57.1±14.3
		Range	17—68	46—98	35—83
Females, last trimester of pregnancy	17	$\bar{x}\pm$S.D.	47.5±18.3	34.8±8.4	135.5±49.4
		Range	23—87	20—47	83—278
Children 2 months to 16 years	18	$\bar{x}\pm$S.D.	79.5±120.2	24.2±19.4	293.4±179.1
		Range	0—430	0—59	109—750
Liver disease patients	14	$\bar{x}\pm$S.D.	148.4±73.0	72.1±13.3	202.2±78.1
		Range	78—309	46—88	111—397

The normal range for alkaline phosphatase total activity in this laboratory is 20—105 I.U./l and that for γ-GT activity is 0—50 I.U./l. γ-GT activities for liver disease patients ranged from 97—1000 I.U./l.

TABLE II. Precision Studies on Pooled Sera (Intraassay Variation)

($n=10$)

	Mean±S.D. (I.U./l)	C.V. (%)	Mean±S.D. (I.U./l)	C.V. (%)
Total alkaline phosphatase	149.2±3.0	2.0	215.8±6.6	3.1
Placental alkaline phosphatase	69.8±2.8	4.0	9.3±2.4	25.8
Bone alkaline phosphatase	39.8±2.0	5.0	167.3±3.0	1.8
Liver alkaline phosphatase	41.1±8.0	8.0	38.2±3.3	8.6
Serum	Pregnant females' pool		Pediatric patients' pool	

isoenzyme activity were detected by the procedure (Table I). When the liver fraction preparations were mixed with sera from children and pregnant females, 106—108% of the activity was recovered and identified as liver fraction.

The procedure is reproducible, giving in our hands recoveries on pooled sera as shown in Table II. Figure 1 shows electrophoretic patterns of samples. Electrophoretic migrations of placental, bone and liver isoenzyme varied slightly. It is clear that bone isoenzyme was precipitated by lectin during the procedure, while liver isoenzyme was not precipitated by

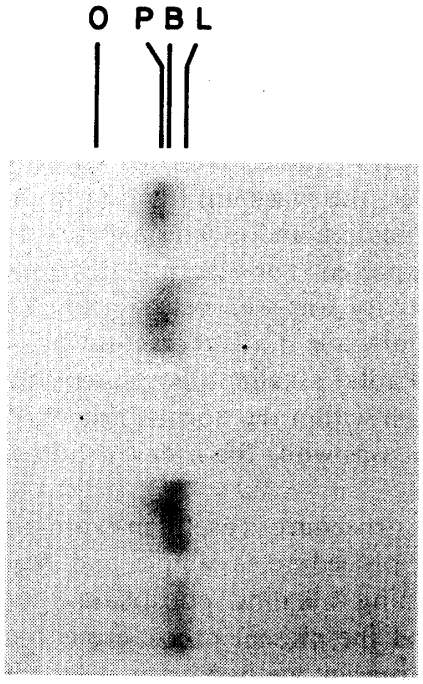

Fig. 1. Comparison of Electrophoretic Sample Patterns

The anode is to the right. O, origin; P, placenta; B, bone; L, liver. Lane 1, authentic placental isoenzyme; lane 2, elevated bone isoenzyme sample (16-year-old male); lane 3, supernatant from lectin precipitation of serum of lane 2; lane 4, liver disease patient; lane 5, supernatant from lectin precipitation of serum of lane 4.

lectin. Human liver and placental control (AP-Isotrol, Sigma, No. A0140) was electrophoretically tested and verified as pure by the manufacturer. Our method found only consistent liver and placental values using this same control serum (data not shown).

Discussion

Normal subjects had 0—12 I.U./l of placental alkaline phosphatase activity in this study. Because 1 to 2% of each non-placental alkaline phosphatase isoenzyme remains uninhibited by theophylline,[3] and because of reagent, instrument and pipetting errors, this cumulative range of activity represents the assay baseline value for placental activity. Values in excess of 12 I.U./l also occurred in samples from non-pregnant patients when the total alkaline phosphatase level was grossly elevated. In these cases the percent placental isoenzyme was less than 10%, the same percentage as found in normal subjects. In the last trimester of pregnancy the values for the placental fraction increase both in terms of absolute units of activity and in percentage of total activity. Efficient recovery of the placental fraction after its addition to samples with high levels of other isoenzyme activity and its identification at high levels during the last trimester of pregnancy validate the use of this procedure for detection of this fraction.

Bone fractions of alkaline phosphatase are at least 80% exclusively precipitated by lectin.[2] Theophylline inhibition studies indicate that no contamination by placental fractions occurs in the precipitate. Normal sera examined by this procedure contain 3—47 I.U./l of bone alkaline phosphatase activity, 4—68% of the total activity. During childhood bone growth, activity found in this study for the bone fraction was from 83 to 443 I.U./l or 35 to 97% of total activity. Addition and recovery studies showed that bone activity added to other pathologic sera samples was 95 to 114% recovered. These findings support the validity of the method for identification of bone isoenzyme.

The liver fraction was studied using a commercially prepared purified human liver and placental control (AP-Isotrol, Sigma, No. A0140). Triton X-405 was used in this work and in the general procedure after its use was validated by studies on samples from liver disease patients as in the original reference.[2] Repeated use of the procedure on this control sample resulted in consistent liver and placental values only, and 100% of these isoenzymes was

recovered after admixing with other serum samples. Normal subjects showed from 17 to 74 I.U./l of liver activity, *i.e.* 40—99% of the total. Fourteen liver disease patients on the other hand showed from 78 to 309 I.U./l of activity, *i.e.* 46—88% of the total. In all these studies, the small contribution of intestinal activity was ignored.

With these normal ranges in I.U./l and in percent of total, abnormal sera can be easily identified. In the 17 last-trimester pregnancy samples, the placental fraction in units and in percent of total exceeded that found in normals, in children and in liver patients. In children, total alkaline phosphatase activity was elevated as expected. The placental fraction range was 0—41 I.U./l, 0—10% of the total. The percent activity was well below normal. The bone fraction was elevated in activity and in percent of total while the liver fraction was elevated in only 3 of 18 cases. For liver patients with elevated total alkaline phosphatase activity and elevated γ-GT activity, the placental fraction was well within the normal range in percent of total activity. Bone activity was elevated in 6 of 14 cases while the liver fraction was greatly increased in activity.

From these studies it can be concluded that this procedure is useful in examining sera for the major fractions of alkaline phosphatase. The procedure is simple and requires little technical skill beyond that required for performing alkaline phosphatase analysis. A comparison between the electrophoretic method and the present procedure is found in our data and references 2, 4 and 6. The originality of the present method lies in its combination of two separate methodologies and its employment of common laboratory instruments. Its clinical utility has been verified by and with clinical samples. Using the above normal ranges, these additional criteria were found useful in interpretation of abnormal sera. Placental fraction abnormalities are shown by a percent value higher than in normals. Bone and liver fraction abnormalities are shown by examining the relative activities of these two fractions. The greater the elevation of activity over the normal activity range, the greater the emphasis laid on that abnormal fraction. It must be noted that some samples may show elevations in both bone and liver activities.[6)]

References

1) W. H. Fishman, *Am. J. Med.*, **56**, 617 (1974).
2) S. B. Rosalki and A. Y. Foo, *Clin. Chem.*, **30**, 1182 (1984).
3) A. Ansari and F. A. Salem, *Clin. Chim. Acta*, **118**, 135 (1982).
4) S. B. Rosalki and A. Y. Foo, *Clin. Chem.*, **32**, 2118 (1986).
5) F. A. Aleem, *Clin. Chim. Acta*, **33**, 125 (1971).
6) W. E. Schreiber and L. Whitta, *Clin. Chem.*, **32**, 1570 (1986).