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Note

Diagnostic aspects of alkaline phosphatase: separation of isoenzymes in normal and pathological human serum by high-performance liquid chromatography

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Alkaline phosphatases (AP) are a group of similar enzymes, coded for by at least three distinct genetic loci, that hydrolyse monophosphate esters with an alkaline pH optimum. They occur in different tissues and are conveniently named after the tissue in which they are found. The placental isoenzyme is highly polymorphic in most human populations and is characterized by its heat stability and susceptibility to inhibition by L-phenylalanine. Intestinal isoenzyme is also inhibited by L-phenylalanine but is heat-labile. The liver/bone/kidney isoenzyme is heat-labile and resistant to phenylalanine, but markedly inhibited by L-homoarginine.

By using a combination of heat denaturation and L-phenylalanine or L-homoarginine methods, it is possible to assign the AP activity of any mixture of isoenzymes, such as those found in serum, to three distinct categories: placental; liver, bone or kidney; and intestinal. The disadvantage of these methods is that they are tedious or, if electrophoresis is used, they allow mostly only a visual assessment or semiquantitative estimates of the relative proportions of the isoenzymes. Furthermore, the bone isoenzyme is not well separated from liver and merges with the liver band. With the advent of high-performance liquid chromatography (HPLC), it has become possible to perform rapid and automatic analysis of various enzymes [1,2]. Recently, various authors have used HPLC for the separation of AP isoenzymes, but with conflicting results [3,4].

In order to contribute to the study of AP isoenzymes, this paper describes an HPLC method based on ion-exchange chromatography for a rapid and reproducible separation of serum AP isoenzymes.

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EXPERIMENTAL

Serum samples

Sera were obtained from 30 healthy subjects as controls, from 35 patients with hepatic diseases grouped according to the clinical diagnosis and the histopathological response (Table I), and from 10 patients affected by bone diseases.

HPLC system

HPLC analyses were carried out using a Perkin-Elmer Series 3B system, equipped with dual pumps capable of generating a gradient elution, and a Spherogel TSK IEX DEAE 540 column, 30 cm × 0.5 cm I.D. (Beckman Instruments) with sphere diameter of 10 μm and an exchange capacity of more than 0.3 mequiv./g.

Chromatograms were recorded by monitoring the absorbance at 405 nm using an LC 85 UV detector (Perkin-Elmer).

Reagents

All chemicals used were from Sigma (St. Louis, MO, U.S.A.). All HPLC reagents and buffers were made up in glass-bidistilled water, degassed and filtered through a 0.45-μm pore size filter (Millipore).

The AP substrate was 5 m*M* 4-nitrophenyl phosphate in 0.5 *M* diethanolamine hydrochloride (pH 10.4) containing 2.0 mol of MgCl. Intestinal liver and bone AP isoenzymes were purchased from Sigma (Poole, U.K.).

TABLE I

VALUES OF TOTAL AP AND THE AP ISOENZYMATIC PATTERN IN HPLC AND ELECTROPHORETIC ANALYSIS

The AP total values are those before HPLC. The recovery after HPLC ranged from 89 to 95%.

	n	AP total (mean ± S.D.) (U/l)	Isoenzymes electrophoresis	Isoenzymes HPLC
Normal subjects	30	87 ± 32	ALP2	AP1
				AP2
				AP3
				AP4
Chronic hepatitis	20	358 ± 107	AP2 large	AP1
				AP3[a]
				AP4[a]
Obstructive jaundice	15	957 ± 189	AP2 large	AP2
				AP3
				AP4
				AP5
Bone diseases	10	738 ± 275	ALP2	AP2[a]

[a] Increased.

HPLC procedures

Serum samples were analysed without delay. Before analysis, the samples were diluted two- to tenfold with 10 m*M* Tris–HCl (pH 7.5), depending on the total activity of AP, and a 100-μl sample was injected into the column. The column was equilibrated with 20 ml of 10 m*M* Tris–HCl (pH 7.5) buffer at a flow-rate of 0.8 ml/min.

After the sample had been applied the isoenzymes were eluted with a 10-min linear gradient form 0 to 0.15 *M* KCl in the 10 m*M* Tris–HCl buffer. This composition was held for 10 min, and then a linear gradient from 0.15 to 0.4 m*M* KCl in 10 m*M* Tris–HCl buffer was applied. Then any remaining protein was removed from the column with 2 ml of 1 *M* NaCl.

Rather than measuring enzyme activity in effluent fractions separated by HPLC, the column eluate was mixed with buffered substrate pumped at a flow-rate of 0.7 ml/min.

The mixture was directed through a 1-m PTFE capillary incubation coil at 37°C to the detector, and the isoenzyme activity was detected in the same manner as the total AP.

Quantitation of the isoenzymes was based on peak-area integration.

Measurement of total AP

Total AP activity was measured at 37°C using 4-nitrophenyl phosphate substrate and diethanolamine buffer, according to the Association of Clinical Biochemists proposed method [5]. The liberated 4-nitrophenol was measured at 410 nm with a spectrophotometer DU 50 with temperature control (Beckman Analytical). One unit (U) of AP catalyses the reaction of 1 μmol of substrate per minute under the given conditions.

Electrophoresis of AP isoenzymes

The peaks collected from HPLC were evaporated under a nitrogen stream, the dried residue was resuspended in 50 μl of distilled water, and a 5-μl sample was submitted to electrophoresis using reagents furnished by Beckman Analytical (Agarose, 1% sodium barbital, 0.1% sodium azide) at 150 V for 30 min at 25°C with 360 m*M* Tris–borate buffer (pH 9.5) with Paragon system (Beckman Instruments, Palo Alto, CA, U.S.A.). We demonstrated AP activity after electrophoresis by incubating the gel with 5-bromo-4-chloro-4-indolyl-3-phosphate in 2-amino-2-methylpropanol buffer (pH 10.6) for 1 h at 45°C. This reaction produced an insoluble blue colour at the side of each isoenzyme band. The stained gel was soaked for 15 min in aqueous solutions, then the gel was dried and isoenzyme bands were scanned in an automatic recording and integrating densitometer (Ultroscan XL, LKB, Pharmacia, Uppsala, Sweden).

Statistical methods

We used logarithmic transformation to normalize skewed data for calculation

of the reference ranges. Means and standard deviations were calculated from the non-transformed data.

RESULTS

Fig. 1 shows a representative chromatogram of normal serum. The mean ± S.D. values of total AP activity in controls were 87.9 ± 32.4 (n = 40, range 49–122). Under the described assay conditions four distinct AP isoenzyme peaks were observed. Each peak was collected, concentrated and subjected to electrophoresis with marker solutions containing liver, bone and intestinal isoenzymes (Fig. 2). By these means, it was shown that the first peak (AP1) corresponded to intestinal isoenzymes, the second (AP2) to the bone isoenzyme and the third and fourth (AP3 and AP4) to liver isoenzyme. The first peak, not present in all subjects, migrates with β-globulin. All other peaks migrated with α_2-globulin.

The representative chromatograms of AP isoenzymes in patients are illustrated in Fig. 3. We analysed sera from fifteen patients with obstructive jaundice having a total mean AP activity of 957 ± 159 U/l, from twenty patients with chronic hepatitis (total AP 358 ± 107 U/l) and from ten patients with bone diseases (total AP 738 ± 275 U/l). AP2, AP3 and AP4 isoenzymes were present in all patients with obstructive jaundice. Furthermore, these patients showed a new peak (AP5), which migrated with α-1-globulin. Patients with chronic hepatitis showed increased AP3 and AP4 (liver isoenzymes), and in four cases an increment in the AP1 fraction. Increased AP2 was observed in patients with bone diseases. Total AP was increased in 70% of the patients with chronic hepatitis and in all cases of obstructive jaundice.

The values (mean ± S.D.) of the total AP and the isoenzymatic pattern (eval-

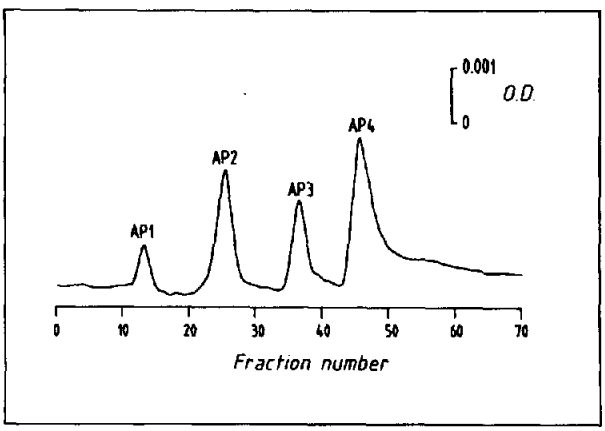

Fig. 1. Elution profile of AP isoenzymes of normal serum sample analysed as described in the text. Peaks: AP1 = intestinal; AP2 = bone; AP3 and AP4 = liver isoenzymes.

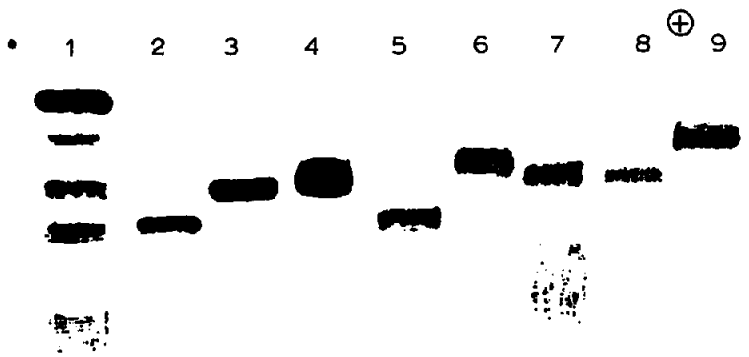

Fig. 2. Electrophoretic pattern of AP isoenzymic fractions separated by HPLC and marker solutions. Lanes: 1 = serum proteins (from anode to cathode: albumin, and α_1-, α_2-, β- and γ-globulins); 2, 3 and 4 = marker solutions of intestinal, liver and bone AP isoenzymes, respectively; 5, 6, 7, 8 and 9 = AP1, AP2, AP3, AP4 and AP5 HPLC peaks, respectively.

uated on the basis of the presence or absence of AP bands) in chromatography and electrophoresis are given in Table 1.

We established the linearity of the method by using the isoenzyme preparations and different pathological sera over the range 0–350 U/l for each of the

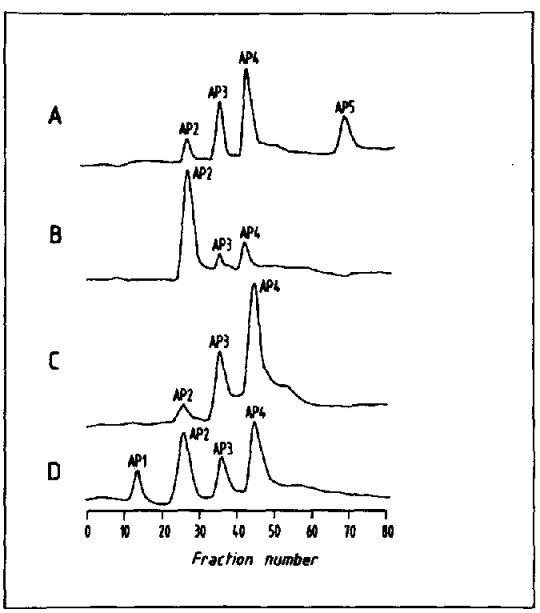

Fig. 3. Representative chromatograms of normal and pathological serum samples. The range of the LC 85 UV detector was set to 0.01 a.u.f.s. for chromatograms A, B and C, and to 0.001 a.u.f.s. for chromatogram D. (A) Patient with obstructive jaundice, total AP activity = 580 U/l; (B) patients with bone diseases, total AP = 410 U/l; (C) patients with chronic hepatitis, total AP = 404 U/l; (D) normal, total AP = 122 U/l.

isoenzymes. The detection limit at a signal-to-noise ratio higher than 2 was 1 U/l for AP1 and AP2, and 2 U/l for AP3 and AP4. The between-assay coefficient of variation determined from repeated analyses ($n = 10$) on consecutive days was less than 7.8% for each of the AP isoenzymes.

The recovery for each isoenzyme ranged from 89% to 95%.

DISCUSSION

Identification and measurement of bone and liver phosphatases is numerically the most frequent application of AP isoenzymes in clinical chemistry. However, the liver and bone forms of AP resemble each other so closely that, despite some differences in their structural properties, which have been noted and ascribed to amino acid or carbohydrate composition [6], current methods of analysis have failed in safely discriminating between them [7,8].

Our HPLC procedure has the advantage of distinguishing clearly enough between the bone and liver forms. The separation method overcomes most of the resolution problems encountered with electrophoretic, inhibition or immunological methods. Our results are in general agreement with the findings of Schonau *et al.* [3] and Parvianen *et al.* [9], who also identified the intestinal (AP1), bone (AP2), liver (AP3 and AP4) and bile (AP5) isoenzymes. In addition to this pattern they found an early-eluting bone isoenzyme that overlapped with intestinal isoenzyme.

The present method is sensitive enough to quantify the low activities in healthy persons. The AP5 isoenzyme is present only in obstructive jaundice. The possible mechanisms of formation of AP5, the abnormal anodal band constantly present in biliary obstruction, have been widely debated. Experimental work [10] in the rat suggests that it depends on enzyme induction due to cholestasis. In another study [11], a significant correlation was observed between the presence of this fraction, migrating with α_1-globulin, and the increase of α-glutamyltransferase activity in biliary obstruction.

We conclude that the Spherogel TSK column provides an efficient and sensitive method for the determination of AP isoenzymes.

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