

## High-Molecular-Mass Alkaline Phosphatase: Simplified and Highly Sensitive Determination by Liquid Chromatography

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This simplified HPLC method for measurement of high-molecular-mass alkaline phosphatase (high- $M_r$  AP; EC 3.1.3.1) in serum and bile is rapid (time for column preparation and separation 30 min), reproducible (CV 4.2%), and highly sensitive (detects high- $M_r$  AP in healthy controls at 1-3% of total AP activity in serum), and is suitable for processing small batches of sample. We characterized high- $M_r$  AP in serum and bile by incubating samples with L-phenylalanine, neuraminidase, 1-butanol, or wheat-germ lectin, and by determining stability to heat. High- $M_r$  AP activity was determined in sera of patients with various liver diseases (4-32% of total AP serum activity) and results were compared with those by electrophoresis on agarose.

**Additional Keyphrases:** *isoenzymes · chromatography, ion-exchange · fluorescence · agarose electrophoresis compared · liver diseases · cancer · cystic fibrosis*

Liver, bone, and intestinal alkaline phosphatase (AP, EC 3.1.3.1) are the isoenzymes of AP most commonly found in serum. Their relative molecular mass ( $M_r$ ) is about 200 000. However, in patients with cholestasis, intrahepatic or extrahepatic AP may be present in serum, for which numerous defining adjectives have been proposed: cholestatic, biliary, pathological biliary, high molecular mass, particulate, R-type, koinoenzyme, origin, stationary, slow liver, fast liver, liver-2, and alpha-1-isoenzyme (1).

Association of this "isoenzyme" with lipoprotein-X (LP-X), nucleotide pyrophosphatase, 5-nucleotidase (2), L-leucyl-beta-naphthylamidase, and gamma-glutamyltransferase (3) has been described. The "isoenzyme" is excluded from the gel matrix on Sephadex G200 chromatography, and it remains at or near the origin on electrophoresis on starch gel or polyacrylamide gel (4).

In sera of cancer patients, as many as four high-molecular-mass isoenzymes may be detected (5). The molecular mass determined by gel filtration is at least 669 000 Da. This high-molecular-mass AP (high- $M_r$  AP) behaves like a plasma membrane fragment in isopycnic density-gradient ultracentrifugation (6). Electron-microscopic examination reveals a triple-layered vesicle that retains AP activity (6).

Here we report a modification of our previous liquid-chromatographic method for determining isoenzymes of AP (7). It is a quick, sensitive, and simple method for detecting high- $M_r$  AP.

### Materials and Methods

#### Materials

**Apparatus.** We used two Gilson Model 302 pumps (Abimed, Düsseldorf, F.R.G.), a 5 × 50 mm column of Mono

Q HR 5/5 ion-exchange resin (Pharmacia, Uppsala, Sweden), a Shimadzu 3 F-530 fluorescence detector, and an LKB-2210 two-channel recorder and a Model 11300 Ultragrad gradient mixer, both from LKB, Bromma, Sweden.

**Reagents.** Diethanolamine was from Fluka, Buchs, Switzerland. Tris HCl, LiCl, L-phenylalanine, 1-butanol (E. Merck, Darmstadt, F.R.G.), wheat-germ lectin (Sigma, Deisenhofen, F.R.G.), and neuraminidase (sialidase, EC 3.2.1.18) from *Vibrio cholerae* (Behring, Marburg, F.R.G.) were also used.

#### Methods

**Sample preparation.** Serum samples from 10 patients with liver diseases and from 10 healthy control subjects were stored at 4 °C and examined within a week. Specimens of bile were obtained from patients who were undergoing T-tube drainage of the common bile duct after cholecystectomy. Just before assay, we centrifuged the serum and bile samples for 5 min at 13 000 × *g*, then diluted 400 μL of the supernatant liquid to 1 mL with Tris HCl buffer (20 mmol/L, pH 8.2).

**Separation.** After equilibrating the column as formerly described (7) and applying the sample, we modified the elution procedure with a two-step gradient of LiCl solution, as follows: 10 min at 175 mmol/L, then 5 min of a linearly increasing concentration up to 500 mmol/L, at a flow rate of 1 mL/min.

**Detection of isoenzyme activity.** The eluate was mixed at a flow rate of 0.05 mL/min with the substrate, 4-methylumbelliferyl phosphate (final concentration, 1 mmol/L) in diethanolamine HCl solution (0.5 mol/L, pH 9.8) containing 0.5 mmol of MgCl<sub>2</sub> per liter. Incubation time was 1.5 min at 37 °C.

**Heat inactivation.** The samples were heated for 15 min at 56 °C, then quickly cooled under running tap water.

**L-Phenylalanine inhibition.** We added 10 mmol of L-phenylalanine per liter to the eluent and the substrate solution.

**Precipitation with lectin.** We mixed 400 μL of an aqueous 5 g/L solution of wheat-germ lectin with 400 μL of serum and let the mixture stand for 30 min at 37 °C. After centrifugation (15 min, 2000 × *g*) we applied 400 μL of the supernate to the column for isoenzyme separation (8).

**1-Butanol.** We incubated 400 μL of serum with 200 μL of 1-butanol overnight, then applied 400 μL of the aqueous phase to the column for isoenzyme separation.

**Neuraminidase digestion.** We mixed 400 μL of serum with neuraminidase (100 mU of enzyme in 200 μL of acetate buffer, 50 mmol/L, pH 5.5) and let the mixture stand for 2 h.

**Electrophoresis on agarose gel.** We used a diagnostic kit from Sigma Chemical Co., Taufkirchen, F.R.G. (procedure no. 710-EP), with 5-bromo-4-chloro-3-indolyl phosphate as substrate.

#### Results

Figure 1 shows the improved isoenzyme elution pattern obtained with the modified method. With the two-step

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gradient, bone and liver isoenzymes were eluted together at 175 mmol LiCl per liter, the high- $M_r$  AP appearing afterwards as a sharp peak eluted at the higher LiCl concentration.

Figure 2 shows some selected examples of elution patterns of sera from patients with various liver diseases. The fraction of the liver-bone isoenzyme could not be depicted completely, because the peaks were off scale. Figure 3 demonstrates the elution pattern for sera from two pediatric patients with cystic fibrosis.

Table 1 summarizes the clinical data on control subjects and patients with cystic fibrosis or liver disease. The activity of high- $M_r$  AP in sera of 10 healthy adult control subjects was ~1–3% of the total activity of AP in serum (specific data not shown). Only subjects 5 and 9 showed above-normal activity of AP in serum. Subjects 4, 5, 7, and 8 had pathologic increases of the aminotransferases. The concentrations of analytes used for detecting liver dysfunction were within normal limits in subjects 1, 2, 3, 6, and 10; only high- $M_r$  AP was increased.

The elution pattern for isoenzymes in bile was identical

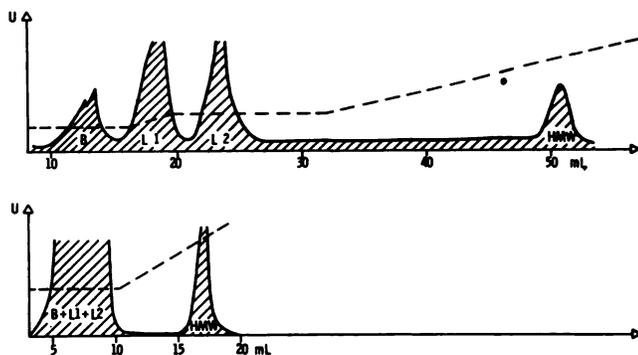


Fig. 1. Dependence of AP isoenzyme elution pattern on elution time and salt gradient (interrupted line)

In Figs. 1–4, because the flow rate was 1 mL/min, the numbers on the abscissa also indicate the elution time (min) after sample application. B, bone isoenzyme; L1, L2, liver isoenzymes; "U", arbitrary fluorescence units

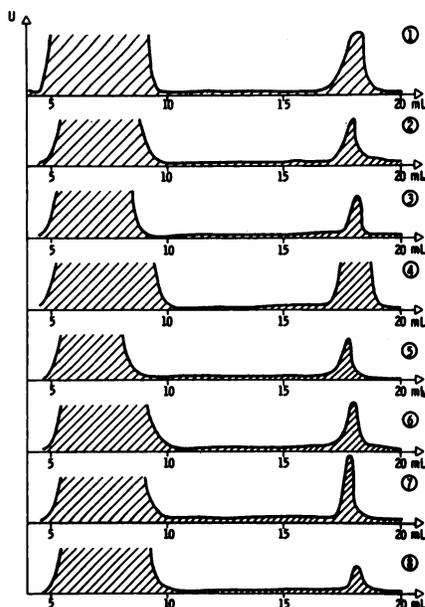


Fig. 2. Measurement of high- $M_r$  AP in several liver diseases. Numbers correspond to those in Table 1

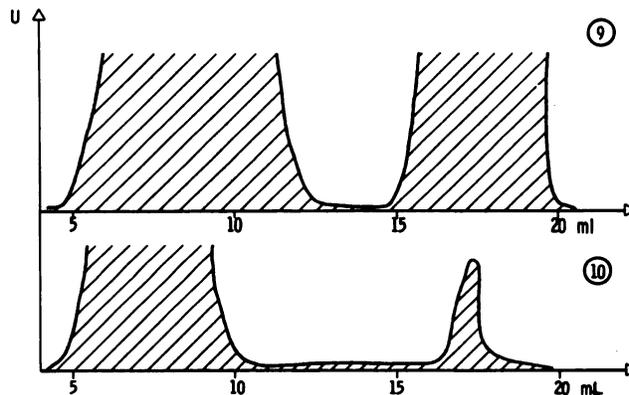


Fig. 3. High- $M_r$  AP in two patients with cystic fibrosis

with the pattern in serum (Figure 4, AP activity in bile 1400 U/L). Table 2 shows results for serum and bile enzyme inhibition and digestion by several methods. Serum high- $M_r$  AP and bile high- $M_r$  AP did not show the same decrease in enzyme activity after heat, lectin, and neuraminidase, or after incubation with L-phenylalanine, but the treatment with 1-butanol resulted in nearly identical behavior.

Figure 5 illustrates the isoenzyme pattern of sera as it appears on electrophoresis on agarose. In contrast to results obtained by two-step gradient chromatography, high- $M_r$  AP could be electrophoretically recognized only if a high activity of this isoenzyme was present.

In healthy controls, high- $M_r$  AP was never detected electrophoretically.

**Precision studies.** For 10 replicate analyses of the same serum, the CV for the activity of high- $M_r$  AP was 4.2%.

## Discussion

Crofton et al. (4) first described an ion-exchange assay for high- $M_r$  AP. In 1986 we reported a high-performance liquid-chromatographic method for measuring several isoenzymes of AP in serum (7). The aim of this two-step gradient method was to obtain a simple, highly-sensitive method for measuring high- $M_r$  AP. This was achieved by decreasing the elution volume from 60 to 15 mL. The absorbance interference at 405 nm of bilirubin—which shows similar elution behavior to high- $M_r$  AP—with 4-nitrophenyl phosphate as

Table 1. Data on Some Serum Analytes Used for Detection of Liver Diseases

Subject no.	Disease	AP	GGT	AST	ALT	Bilirubin, mg/L	High- $M_r$ AP, % of total AP
		Acty. concn, U/L					
1.	Liver tumor	144	24	6	10	3	5.8
2.	Liver metas.	73	28	12	19	4	8.2
3.	Liver metas.	112	20	11	11	5	4.7
4.	Liver tumor	152	31	18	13	14	12.7
5.	Liver tumor	175	117	26	37	15	5.4
6.	Liver metas.	106	5	7	8	5	4.0
7.	Liver metas.	91	44	20	20	7	4.0
8.	Liver metas.	93	43	16	21	9	4.7
9.	Cystic fib.	1400	258	54	75	—	32.5
10.	Cystic fib.	471	11	16	24	1	9.5
11.–20.	Controls	170	28	19	23	10	3

Reference intervals for normal (7): AP, children 110–700 U/L, adults 55–175 U/L; GGT, newborns 2–91 U/L, adults 4–28 U/L; AST, newborns 7–27 U/L, adults 1–19 U/L; ALT, newborns 6–36 U/L, adults 1–23 U/L; total bilirubin, adults up to 11 mg/L.

Abbreviations: GGT, gamma-glutamyltransferase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; AP, alkaline phosphatase.

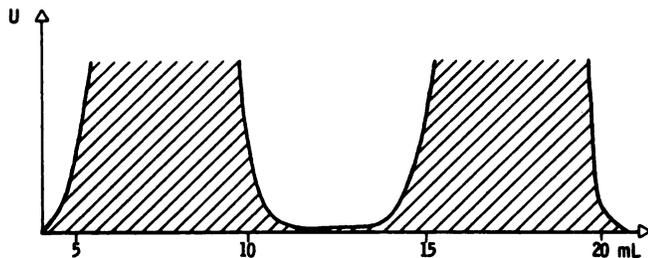


Fig. 4. Elution pattern of high- $M_r$  AP in bile

substrate (7) was avoided by using the fluorescent substrate 4-methylumbelliferyl phosphate.

The necessary gradient was formed by a simple two-chamber system. Time-consuming column preparation and equilibration, overnight dialysis of samples, and measurement of AP activity in a series of fractions was thus avoided (4, 10, 11). We separated high- $M_r$  AP from sera of healthy controls and from patients with various types of liver diseases but with mostly normal values for AP in serum. Even small amounts of high- $M_r$  AP activity were measured in selected samples. In contrast, only large amounts of high- $M_r$  AP activity produced a visible and detectable band in electrophoresis on agarose. However, Crofton and Smith (10) report that, in 24 sera shown by electrophoresis to contain undetectable concentrations of high- $M_r$  AP, activity measured by two-step ion-exchange ranged from 1 to 4%.

We found that quantification by electrophoresis on cellulose acetate strips followed by densitometry yielded poor protein separation, diffusion of staining dye, and variable background staining (12, 13). A problem common to nearly all electrophoresis methods is the possibility that the staining-reaction product influences isoenzyme kinetics to varying degrees.

There are several reports in the literature regarding the origin of high- $M_r$  AP in serum. Brocklehurst et al. (14) suggest the existence of two forms of high- $M_r$  AP, as part of

Table 2. Characterization of High- $M_r$  AP in Serum and Bile

Treatment	Percentage of activity remaining after treatment			
	Serum		Bile	
	Fraction 1	Fraction 2	Fraction 1	Fraction 2
L-Phenylalanine	50.7	54.5	21.3	10.5
Heat	13.5	27.2	79.4	42.1
1-Butanol	84.7	13.3	69.6	19.8
Lectin	84.0	51.1	140.0	61.5
Neuraminidase	65.0	103.0	109.0	41.3

Fraction 1: liver, bone, and intestinal isoenzymes of AP.

Fraction 2: high- $M_r$  AP.

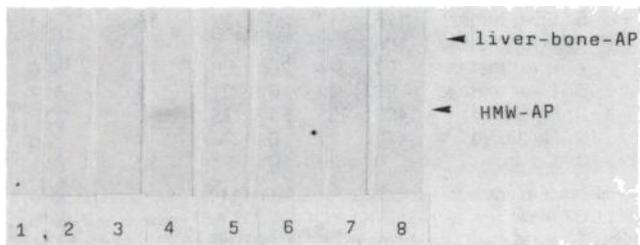


Fig. 5. Electrophoresis of sera from patients with liver diseases on agarose

Numbers are the same as in Fig. 2 and Table 1

a multi-enzyme plasma membrane complex and as soluble liver enzyme associated with lipoprotein-X. The multi-enzyme complex appears to develop from shedding of plasma membrane and from a more rapid turnover of plasma cell membrane. LP-X-liver isoenzyme complex occurs as a result of regurgitation of bile components from the canalicular to the sinusoidal surface of the hepatocyte via the tight junctions or via the hepatocyte, a regurgitation that takes place when there is sufficient obstruction of the bile secretory passage. Weijers (17) also discusses two high- $M_r$  AP forms: bile isoenzyme and AP-LP-X complex. The complex of membrane enzymes and AP is seen frequently in hepatic malignancy, in contrast to higher degrees of AP-LP-X seen in patients with cholestasis. Both mechanisms may operate simultaneously, giving rise to the appearance of several bands (1). Fritsche et al. (5) describe up to four high- $M_r$  AP isoenzymes resolved by cellulose acetate electrophoresis of sera from cancer patients. They suggest that the "pre-liver" isoenzyme of biliary origin and the three slower high- $M_r$  AP fractions are variants of the intestinal isoenzyme. De Broe et al. (6) demonstrated by electron microscopic examination that high- $M_r$  AP is in the form of a triple-layered vesicle, which retains alkaline phosphatase activity. Using antiserum against purified high- $M_r$  AP, they showed that plasma membrane fragments in serum are not of biliary origin.

Our results with a two-step gradient show that high- $M_r$  APs in serum and bile have identical elution behavior. After incubation with 1-butanol, the two high- $M_r$  APs have an almost similar decrease in activity. Jennings et al. (15) report a similar decrease in enzyme activity after treatment with 1-butanol. After extraction with butanol, the electrophoretic mobility corresponds to that of the normal liver isoenzyme.

In contrast to Crofton and Smith (10), we found that high- $M_r$  AP in bile is more heat-stable than the high- $M_r$  AP in serum. We suggest that characterizing by heat stability is of little value because of the many factors that influence the stability (e.g., serum albumin content) of proteins. The results of inhibition by L-phenylalanine show values for high- $M_r$  AP in bile and the first fraction comparable with those for intestinal alkaline phosphatase. In comparison, Warnes et al. (16) report evidence of enterohepatic circulation of intestinal AP and its appearance in bile. The inhibition rates for serum and bile high- $M_r$  AP were not identical.

The percentage of activity remaining after treatment with L-phenylalanine and neuraminidase suggests to us that the first chromatographic fraction of bile (Figure 4) may be of intestinal origin. The cause of increasing activity of the first bile fraction after lectin binding is unclear. It may be that components of bile were bound by lectin, which had an inhibitory effect on the activity of the first bile fraction.

After incubation with neuraminidase, serum high- $M_r$  AP showed no decrease in activity as compared with that of the first fraction. This may be explained by the high net charge of high- $M_r$  AP, which does not decrease significantly after removal of sialic acids. In contrast, isoenzymes from liver and bone were eluted earlier. High- $M_r$  AP in bile showed a greater sensitivity to neuraminidase. The binding rates to lectin were nearly similar.

Serum and high- $M_r$  AP in bile show identical elution behavior, but structural identity cannot be demonstrated by the reported methods. Isolation of these proteins would be

necessary to solve this problem.

Results of high-*M<sub>r</sub>* AP measurement in the selected sera lead us to conclude that our simple gradient method is sensitive in detecting hepatic disease, and the method can even be used in laboratories that lack experience in liquid chromatography.

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## Physiological Changes in Human Erythrocyte Cholinesterase as Measured with the "pH-stat"

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Cholinesterase activity in human erythrocytes was determined in 1903 blood samples by the "pH-stat" method. Differences in activity were examined as a function of sex, age, and pregnancy. Reliability intervals for the population average and approval or normality intervals for individual values were established. Sex- and age-related differences were very significant.

**Additional Keyphrases:** *sex- and age-related differences · reference interval · pregnancy · organophosphate pesticide toxicity*

The main function of acetylcholinesterase (EC 3.1.1.7; AChE) is to inactivate the neurotransmitter, acetylcholine, thereby making possible the transmission of the nerve impulse. This enzyme is found in nerves, neuromuscular junctions, and erythrocytes (1), in erythrocytes as a glycoprotein with a carbohydrate/protein ratio of 0.16. Glucose, galactose, mannose, glucosamine, and sialic acid are the main constituent sugars and phosphatidylserine and cholesterol the main lipid constituents (2). Its physiological function in erythrocytes is unknown, although it has been pointed out (3) that it takes part in the ionic exchange between erythrocytes and the surrounding medium.

Acetylcholinesterase activity in erythrocytes has not been

studied extensively, although it is the best indicator available to assess the degree of exposure to organophosphate pesticides (4) used in agriculture. Our aim here is to present data on normal reference intervals for AChE, and on some physiological variables affecting them.

#### Materials and Methods

We analyzed 1903 samples of venous blood, selected at random from collections made during a year in different laboratories in the province of Granada, Spain. Blood was collected, by venipuncture, into tubes that contained EDTA as anticoagulant, and the specimens were taken to the laboratory in refrigerators at 4 °C and prepared for assay within 48 h.

To determine AChE activity in the same volume of erythrocytes in all samples (from the hematocrit value as determined by the microhematocrit method), we washed a given volume of blood three times with isotonic saline and diluted the cells to 2 mL with isotonic saline to obtain a hematocrit of 45% for each sample.

To release the enzyme from the stroma of the erythrocytes, we treated samples with ultrasound (MSE 1300 sonicator, 3/4-inch titanium probe) at a frequency of 45 to 60 cycles per second, applying three 30-s ultrasonic bursts at 1-min intervals. During this sonication, the vials containing the samples were kept in an ice bath. Then, 0.5-mL samples were lyophilized and stored at 4 °C. At the time of analysis, each sample was rehydrated with 0.5 mL of distilled water and diluted 10-fold with 4.5 mL of isotonic saline.

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