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Determination of alkaline phosphatase isoenzymes in serum by high-performance liquid chromatography with post-column reaction detection

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ABSTRACT

A weak anion-exchange high-performance liquid chromatographic procedure with post-column reaction detection for simultaneous determination of alkaline phosphatase (EC 3.1.3.1, ALP) isoenzymes is described. We identified six peaks with ALP isoenzyme activity in normal serum. The peaks were, in order of elution, one intestinal/bone, two bone and three liver ALP isoenzymes. This new assay with automatic injection, on-line post-column reaction detection and powerful integration data system could be of significant value in the routine clinical biochemistry laboratory. The advantages include improved sensitivity and selectivity over previous methods for the determination of ALP isoenzymes.

INTRODUCTION

Alkaline phosphatase [EC 3.1.3.1, orthophosphoric-monoester phosphohydrolase (alkaline optimum); ALP] catalyses the hydrolysis of a wide range of phosphomonoesters at an alkaline pH and is present in practically all tissues in the body, anchored to cell membrane lipid bilayers [1].

The term "isoenzyme" is used to designate different molecular forms of proteins with similar enzymic specificity but differences in primary structure, *i.e.* encoded by separate structural gene loci. According to this, it is likely that there are at least four human ALP isoenzymes with four gene loci [2]. The four gene loci are the "tissue non-specific", placental, germ cell and small intestinal locus. ALP from the "tissue non-specific" locus is expressed in tissues such as bone, liver and kidney, and has nevertheless some "tissue specific" variations. These variations are introduced during the expression of this gene locus in different cells by posttranslational modification in the carbohydrate composition [3]. Despite this, the terminology bone ALP isoenzyme, liver ALP isoenzyme, etc. will be used here to maintain continuity with the literature and established practice in clinical chemistry.

Numerous techniques have been reported for the determination of ALP isoenzymes, including

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electrophoresis on various supporting media, such as cellulose acetate [4] and agarose gel [5]. Various chemical inhibitors, such as L-phenylalanine, L-tryptophan, L-homoarginine [6], urea [7], guanidine hydrochloride [8], L-*p*-bromotetramisole [9] and wheat-germ lectin [10], are also frequently applied. Other techniques, such as heat inactivation at 56°C [11], isoelectric focusing (IEF) [12,13] and different immunological methods [14,15] have also been used.

Ever since the late 1960s, when the high-performance liquid chromatographic (HPLC) technology became available, applications for the determination of isoenzymes have been reported [16,17]. Measurement of ALP isoenzymes by anion-exchange HPLC has been described by several investigators [18–22] and, quite recently, Anderson *et al.* [23] reported a high-performance affinity chromatographic (HPAC) method.

The need for a better differentiation of ALP isoenzymes in many clinical conditions was recently pointed out [24]. However, the many techniques and applications reported indicate the complexity of the ALP isoenzymes and the lack of an ideal method. Here we report a new sensitive and specific HPLC method for ALP isoenzyme identification and determination that could be of significant value in the routine clinical biochemistry laboratory.

EXPERIMENTAL

Apparatus

Fig. 1 shows a schematic diagram of the set-up for our HPLC system with post-column reactor. Two Jasco Model 880-PU Intelligent HPLC pumps and a Jasco Model 880-30 Solvent mixing module (Japan Spectroscopic, Tokyo, Japan) were used to form the mobile phase with a gradient of acetate. An autoinjector, Model Promis (Spark Holland, Emmen, Netherlands), with a 100-μl loop, was used for sample injections. The analytical column was a SynChropak AX300 (250 mm × 4.6 mm I.D.) (SynChrom, Lafayette, IN, USA) packed with polyethyleneimine-coated 6.5-μm porous silica, a weak anion-exchanger. The analytical column temperature was controlled at 30°C with a column water jacket (Skandinaviska GeneTec, Kungsbacka, Sweden) connected to a water thermostat. A pre-column, P/N

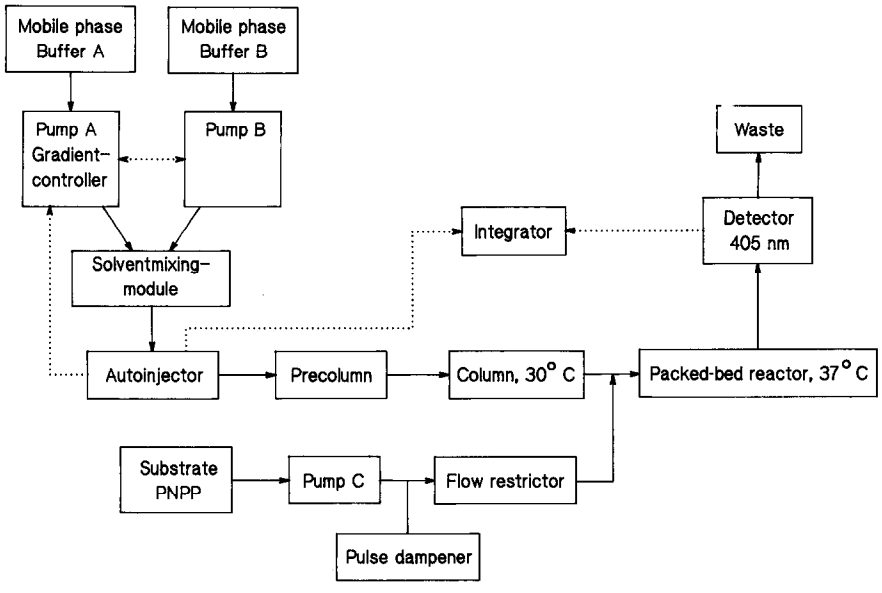

Fig. 1. Schematic diagram of the HPLC post-column system for separation and quantitation of ALP isoenzymes. Dotted lines indicate electrical connections and solid lines indicate fluid connections.

84550 (23 mm × 3.9 mm I.D.), (Waters Assoc., Milford, MA, USA) filled with 10-μm anion-exchange material, Cat. No. 301SC, (Vydac, Hesperia, CA, USA), was placed before the analytical column. The activities of the separated ALP isoenzymes were detected on-line by mixing the column effluent in a tee, into which the substrate was pumped at 0.95 ml/min by a ConstaMetric IIG HPLC pump (LDC/Milton Roy, Riviera Beach, FL, USA). A pulse dampener was placed in series with a flow restrictor after the substrate pump to allow an even supply of substrate to the reactor. The isoenzyme–substrate complex was directed on-line into a packed-bed post-column reactor, where the ensuing reaction proceeded. This reactor was made of a stainless-steel tube (300 mm × 4.6 mm I.D.) filled with non-porous glass beads, particle size 60–70 μm (Tillquist Analys, Stockholm, Sweden), and controlled at 37°C with a column water jacket (Skandinaviska GeneTec) connected to a water thermostat. The *p*-nitrophenol formed was monitored at 405 nm with a SpectroMonitor III Model 1204 A (LDC/Milton Roy).

Calculation of isoenzyme activity

The areas under each peak were integrated by a Barspec data system (BarSpec, Rehovot, Israel), and the value of the total ALP activity was used to calculate the relative activity of each of the ALP isoenzymes present. The total ALP activity was measured at 37°C with a Hitachi 717 analyser (Boehringer Mannheim, Mannheim, Germany) [25]. One unit (U) of ALP hydrolyses 1.0 μmol of *p*-nitrophenyl phosphate (PNPP) per min at 37°C.

Chemicals and reagents

Mobile phase. Buffer A consisted of 20 m*M* Tris–acetate, adjusted to pH 7.8, and buffer B of 20 m*M* Tris–acetate and 0.6 *M* sodium acetate, adjusted to pH 7.8.

Substrate. The substrate solution (pH 10.1) contained 1.8 m*M* PNPP, 1.0 m*M* magnesium chloride (Sigma, St. Louis, MO, USA), 0.25 *M* diethanolamine (J. T. Baker, Deventer, Netherlands) and 6 g/l Triton X-405 (Technicon Instruments, Tarrytown, NY, USA).

All chemicals were reagent grade. Water used for reagent preparation was from a Milli-Q system (Millipore, Molsheim, France), and all solutions were filtered and degassed under vacuum through 0.45-μm HA-type filters (Millipore).

Chromatographic conditions

A gradient profile is presented in Fig. 2. The chromatograph was operated at a flow-rate of 0.8 ml/min by a linear gradient from the initial condition of 82% buffer A and 18% buffer B, to 55% A and 45% B for 7 min, and then by a linear gradient to 5% A and 95% B for 7 min. It was then run isocratically (5% A, 95% B) for 10 min, and then for 6 min to re-establish the initial column conditions (82% A, 18% B). The column must be thoroughly washed with the initial mobile phase composition for 50 min to remove excessive salt before the next sample is injected.

Characterization of the peaks

To verify that the observed peaks were truly ALP and to identify the different ALP isoenzyme peaks we used different approaches, such as human ALP standards, heat inactivation at 56°C for 15 min and inhibition by 10 m*M* L-phenylalanine (Sigma). The standards were human bone

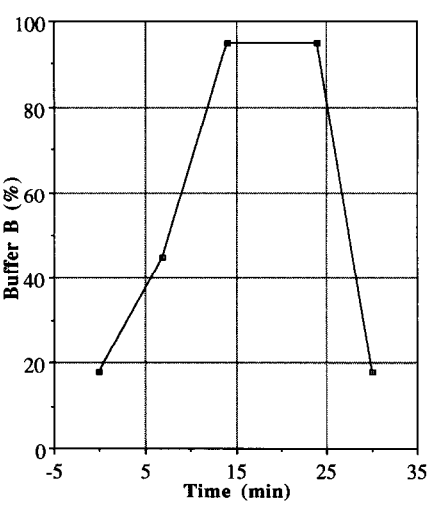

Fig. 2. Gradient profile for the separation of ALP isoenzymes in serum.

ALP (8.4 U/mg), Cat. No. 124A0001, and human intestinal ALP (1.1 U/mg), (Cat. No. 155A0001 (Calzyme Labs., San Luis Obispo, CA, USA).

Other procedures for method validation

The intra-assay coefficient of variation (C.V.) was evaluated by analysing pooled serum (n = 10) from healthy individuals. To evaluate the inter-assay C.V., we analysed pooled serum on ten consecutive days. The analytical recovery was determined by adding known activities of the respective ALP isoenzyme of pathological serums or standards to serum from healthy individuals. The linearity was determined by analysing a series of ALP isoenzyme preparations from pathological serums up to 4200 U/l of total ALP. Interference effects of lipemia, hemolysis and bilirubinemia were studied. Carry-over was determined by injecting serum samples with extremely high activities of ALP, followed by pure isotonic saline.

Samples

Venous blood samples were collected into Vacutainer Tubes without additives (Becton Dickinson, Stockholm, Sweden). Serum did not require any kind of pretreatment with heat or any inhibitor before injection except for a 1:3 dilution with isotonic saline when the samples had normal activities of total ALP. The upper reference limit for total ALP is 276 U/l (4.6 μkat/l) in our laboratory. All samples were filtered through low-protein-binding Cameo IIS (0.45-μm pore size) filter units (Micron Separations, Westborough, MA, USA) before analysis to remove particulate materials.

RESULTS

We were able to identify six peaks with ALP activity in normal serum. Fig. 3 shows a chromatographic profile of normal serum, total ALP 162 U/l. The peaks were, in order of elution: one intestinal/bone ALP isoenzyme (I/B), 5 U/l; two bone ALP isoenzymes (B1, B2), 17 and 62 U/l; three liver ALP isoenzymes (L1, L2, L3), 53, 17 and 8 U/l. The resolution (R_s) between B2 and L1 is 0.8 [$R_s = 1.18\,(t_2 - t_1)/(W_{h1} + W_{h2})$. Here, t_1 and t_2 refer to the retention times of the two adjacent peaks, and W_{h1} and W_{h2} are their peak widths at half height.]

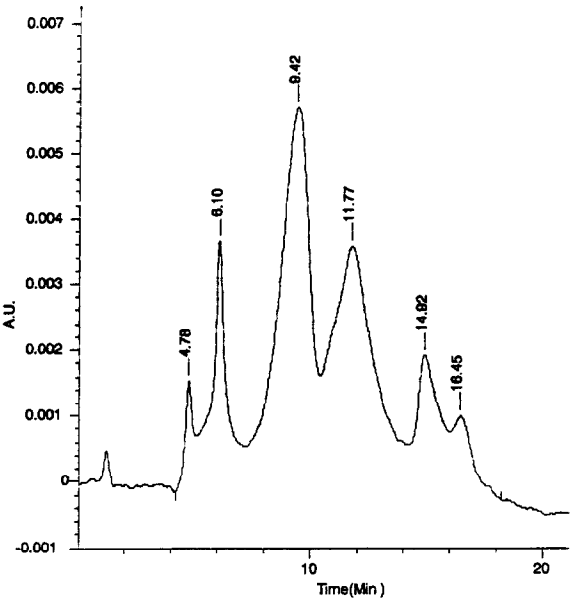

Fig. 3. Chromatogram of a normal serum sample: total ALP, 162 U/l. Peaks and retention times (min) are: intestinal/bone, 4.78; bone 1, 6.10; bone 2, 9.42; liver 1, 11.77; liver 2, 14.92; liver 3, 16.45.

Fig. 4 shows a chromatographic profile from a patient with hypophosphatasia, total ALP 30 U/l. All ALP isoenzyme activities were decreased compared with normal serum samples, especially the bone ALP isoenzymes, and B2 was not detectable at all.

Fig. 5 shows a chromatographic profile from a patient with hypophosphatemic vitamin D-resistant rickets, total ALP 1500 U/l, and the bone ALP isoenzyme activities are clearly increased, which is in agreement with the patient's pathology.

The detected peaks were characterized by human ALP standards, heat inactivation and L-phenylalanine inhibition. The human bone ALP standard showed three peaks with the same retention times as I/B, B1 and B2, and the human intestinal ALP standard had the same retention time as the peak I/B. The remaining ALP isoen-

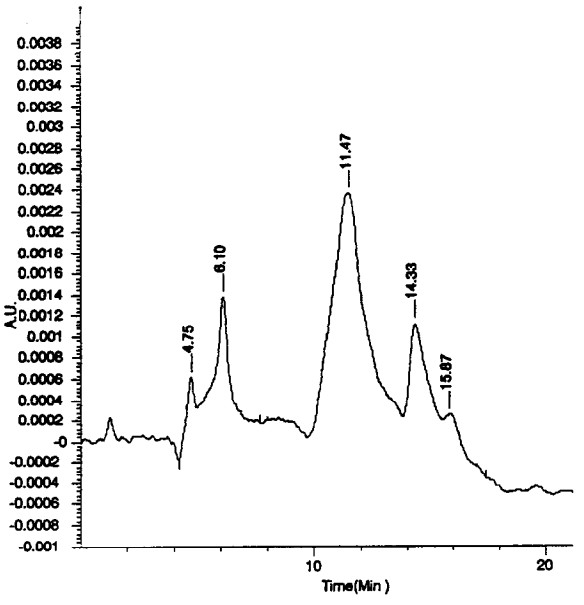

Fig. 4. Chromatogram of serum from a patient with hypophosphatasia: total ALP, 30 U/l.

zyme activities for the observed peaks after heat inactivation and L-phenylalanine inhibition are presented in Table I.

The intra- and inter-assay C.V. were less than

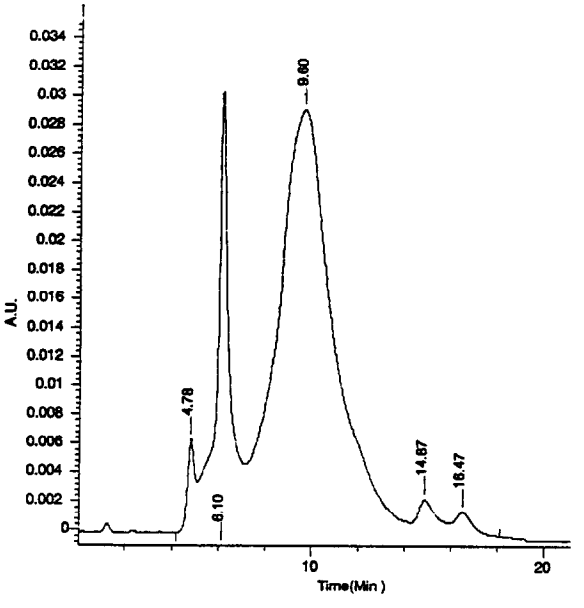

Fig. 5. Chromatogram of serum from a patient with hypophosphatemic vitamin D-resistant rickets: total ALP, 1500 U/l.

TABLE I

EFFECTS OF HEAT INACTIVATION AND L-PHENYLALANINE INHIBITION OF ALP ISOENZYME ACTIVITIES

ALP isoenzyme	Remaining activity (%)	
	Heat (56°C, 15 min)	10 m*M* L-Phenylalanine
Intestinal/bone	33	48
Bone-1	14	68
Bone-2	5	62
Liver-1	64	79
Liver-2	50	73
Liver-3	71	84

4.5% and less than 5.8%, respectively, for each of the ALP isoenzymes at isoenzymic activities from 5 to 70 U/l. The analytical recovery for the bone and liver ALP isoenzymes ranged from 94 to 99% and from 94 to 97%, respectively. The linearity was examined up to 103 U/l (I/B), 283 U/l (B1), 572 U/l (B2), 195 U/l (L1), 302 U/l (L2) and 159 U/l (L3), for the respective ALP isoenzymes. Linear regression equations for the ALP isoenzymes were as follows: $y = 4.06 \cdot 10^{-4}x - 2.13 \cdot 10^{-4}$ (I/B), $y = 2.33 \cdot 10^{-4}x + 8.00 \cdot 10^{-4}$ (B1), $y = 1.94 \cdot 10^{-4}x - 8.03 \cdot 10^{-4}$ (B2), $y = 1.78 \cdot 10^{-4}x - 2.44 \cdot 10^{-4}$ (L1), $y = 3.38 \cdot 10^{-4}x - 9.00 \cdot 10^{-4}$ (L2) and $y = 2.65 \cdot 10^{-4}x - 3.92 \cdot 10^{-4}$ (L3). Correlation coefficients were ≥ 0.999, and Student's t-tests for intercept $\neq 0$ in the linearity ranges were non-significant for all the ALP isoenzymes. No interference was observed from lipids (up to triglyceride 25 m*M*). Visible hemolysis, above 150 mg/l hemoglobin, increased L1. The other ALP isoenzymes were less sensitive to hemoglobin, interference occurred only above 500 mg/l hemoglobin. Bilirubin concentrations above 100 μM increased L2. No signs of carry-over were detectable. The detection limit, defined by a signal-to-noise ratio ≥ 2, were as follows for the ALP isoenzymes: 0.5 U/l (I/B), 1 U/l (B1, L3), 2 U/l (B2, L2) and 3 U/l (L1).

DISCUSSION

Of all community hospital admissions, 4–8%

have an unexplained elevation of total ALP at the time of admission [26]. This indicates the need for an exact determination of the source of increased ALP activity. De Broe *et al.* [24] recently emphasized the need for an improvement of both "clinical and biochemical knowledge" about ALP isoenzyme patterns to increase their clinical usefulness. In this respect, our HPLC method might contribute to a better understanding of the ALP isoenzymes. To our knowledge, no other HPLC application can separate and quantify six peaks with ALP isoenzyme activity in non-placental serum with normal levels of total ALP activity. The described HPLC assay includes an autoinjection system, and thus it is possible to perform determinations on eighteen samples within 24 h, which makes the method suitable for routine use in clinical biochemistry laboratories.

Lower flow-rates for the mobile phase lead to improved resolution, especially between peaks I/B and B1. However, this also means extended analysing time. Therefore we maintained the flow-rate at 0.8 ml/min as a practical compromise.

Our weak anion-exchange column, SynChropak AX300, has previously been used by other investigators to separate isoenzyme fractions for several enzymes: ALP [21], lactate dehydrogenase (EC 1.1.1.27) [27], creatine kinase (EC 2.7.3.2) [28] and glutathione S-transferase (EC 2.5.1.18) [29]. These reports, together with our results, show that SynChropak AX300 is a suitable column for the separation of isoenzymes in serum. However, to achieve good precision and reproducible results, the temperature for the analytical column must be strictly controlled. The performance of the analysis, especially retention times and peak heights, is affected by changes in the ambient temperature of as little as 1–2°C. For this reason we used a column water-jacket connected to a water thermostat.

It is generally recommended that a packed bed is used rather than open-tubular post-column reactors when reaction times between 0.5–2 min are involved [30]. The enzymic reaction procedure described here takes *ca.* 1 min and does not cause significant post-column band broadening.

The addition of detergent Triton X-405 to the substrate is another factor that diminishes the band broadening. Triton X-405 also prevents adsorption of proteins in the tubings and post-column reactor without interfering with the enzymic reaction [31].

The ALP isoenzyme activity was measured at 37°C because of the apparent increase in activity compared with 25 and 30°C. This yields a method more sensitive to small differences in the activities of the different ALP isoenzymes in a sample [32]. Another factor in favour of 37°C as reaction temperature is that a shorter reaction time can be used in the post-column reactor, and consequently a shorter post-column reactor with decreased band broadening.

Our findings on the inhibitory effects of ALP isoenzymes (Table I) demonstrate that the peak I/B is the peak most sensitive to L-phenylalanine, which is in agreement with the intestinal ALP isoenzyme [6]. The peaks B1 and B2 are the most heat-sensitive peaks, which is in agreement with earlier descriptions of bone ALP isoenzymes [11]. According to analysis of human bone and intestinal ALP standards, and to the inhibitory effects of ALP isoenzymes shown in Table I, we suggest that the first peak (I/B) consists of both intestinal and bone ALP isoenzymes. The bone ALP isoenzyme is probably the dominant ALP isoenzyme in this peak, partly because it has a greater affinity for PNPP and diethanolamine than the intestinal ALP isoenzyme [26]. The three peaks with bone ALP isoenzyme activity, I/B, B1 and B2, were all increased in different bone metabolic disorders. These findings support our identification of the bone ALP isoenzymes. Multiple isoforms of the bone ALP isoenzymes have been reported previously [13,20,21,23]. Root *et al.* [33] described as many as four distinct IEF bands of the bone ALP isoenzyme in Paget's disease.

The three liver ALP isoenzymes detected here, L1, L2 and L3, were all increased in various hepatobiliary disorders. Further characterization and validation of these isoenzymes are required before any conclusions can be drawn concerning their hepatobiliary origin.

Methods based on various chemical inhibitors

affect all ALP isoenzymes to a varying extent. Heat-inactivation techniques require very precise procedural control for even moderate reproducibility, since the rates of heat denaturation change markedly with small variations in temperature [34]. When inhibition and inactivation techniques are used, it is also recommended that the results be subjected to algorithmic or multi-component analysis, together with thorough error analysis, to take account of the imprecision of the isoenzyme estimations [35].

Electrophoresis gives limited information of the distinction between a bone and a liver source of increased total ALP activity, and is mostly used for qualitative identification of the predominant ALP isoenzyme. A major disadvantage with most electrophoretic procedures is incomplete separation between the bone and liver ALP isoenzymes, and often only one bone and two liver ALP isoenzymes can be identified in normal serum samples from adults.

Another electrophoretic procedure that is being used increasingly, because of its high resolving power, is IEF. However, many such reports present blurred or diffuse bands [36]. A more promising technique is the use of IEF in immobilized pH gradients (IPG) [13]. However, reports presenting IEF methods and ALP isoenzyme reference intervals and applications for the diagnosis of human diseases are rare. Thus, this might indicate that it is difficult to achieve accurate quantification with any IEF-related technique. It seems that IEF and IPG techniques can best be used to characterize purified ALP isoenzymes if the multiple bands are correctly identified.

Immunoassays with monoclonal antibodies give accurate results if the aim is to quantify ALP isoenzymes from one of the three gene loci of the placenta, germ cells or small intestine. If the aim is to separate and quantify bone and liver ALP isoenzymes from the "tissue non-specific" gene locus, these methods often show cross-reactivity. This can be explained by the fact that different carbohydrate side-chains in bone and liver ALP isoenzymes probably consist of components so widely distributed in different human cells that they do not act as antigenic determinants. There are, however, two reports [37,38] on specific antibodies without cross-reactivity for bone and liver ALP isoenzymes. The reasons for these exceptions are still not clarified [14,39].

One of the earlier reports on ALP isoenzyme analysis with HPLC [18] presented no results concerning normal human serum, but only from the analysis of beef liver ALP and calf intestinal ALP. Hsu and Chen [19] reported no results concerning normal human serum, but only results from the analysis of bovine intestinal ALP and AP Isotrol, a commercial electrophoretic marker derived from human liver and placenta. According to our observations and those of Hua *et al.* [40], bovine ALP is different from human ALP; results based on organs from species other than human cannot be used to interpret the analysis of human serum. The HPLC method described by Schoenau *et al.* [20] can detect four peaks with ALP activity in normal serum samples. However, their method has been questioned by other workers [41–43] because of its inability to separate bone from liver ALP isoenzyme. Parviainen *et al.* [21] reported an HPLC method using the same type of analytical column as ours. These authors described two peaks of bone ALP isoenzymes and two peaks containing liver ALP isoenzymes in normal serum samples. Severini *et al.* [22] recently reported an HPLC method with fine resolution between the bone and liver ALP isoenzymes. However, they could not detect more than four peaks with ALP activity in normal serum samples: one intestinal, one bone and two liver ALP isoenzymes. In pathological serum samples from patients with obstructive jaundice they also observed an additional peak with ALP activity that probably corresponds to our L3 peak. The HPAC method described by Anderson *et al.* [23] detected one bone and one liver ALP isoenzyme in normal adult serum.

In conclusion, the presented HPLC method for the identification and determination of ALP isoenzymes represents an improvement over previously published methods.

Our method may contribute to a better understanding of different ALP isoenzyme patterns and thus be of significant value in the routine

clinical biochemistry laboratory for the diagnosis and monitoring of treatment of metabolic bone and liver diseases.

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