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Comparison of two commercially available systems for the electrophoretic separation of alkaline phosphatase isoenzymes

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ABSTRACT

Two commercially available electrophoretic methods for the separation of the human serum enzyme alkaline phosphatase (ALP, EC 3.1.3.1) were evaluated: the Isopal system (Beckman, Brea, CA, USA) was compared with the Iso-PAL system (Sebia, Issy-les-Moulineux, France). Both use agarose as supporting medium to separate ALP into its clinically relevant isoenzymes: bone, liver, high-M_r (or "fast liver"), intestinal and placental ALP. With both methods, additional fractions for bone, liver and intestinal ALP were found, true isoforms that were called "variant" fractions. The migration pattern differed considerably between the systems, owing to the use of different detergents. Bone and liver ALP were partially separated with both methods. However, when bone ALP exceeded 50% of the total ALP activity, sample treatment was necessary, either with neuraminidase (Beckman) or by applying the sample on a second gel containing wheat-germ lectin to precipitate bone ALP (Sebia). The within- and between-gel reproducibilities of both systems were comparable and remained between 2 and 6% for normal isoenzyme activities. Both systems correlated well, except for high M_r ALP. The Sebia system was more sensitive for detecting intestinal ALP, whereas higher liver and bone variant ALP activities were detected with the Beckman system. It is concluded that both methods are convenient for routine use in the clinical laboratory.

INTRODUCTION

Alkaline phosphatase (ALP, EC 3.1.3.1) is a membrane-bound metalloenzyme consisting of a group of isoenzymes, all glycoproteins, encoded for by four different gene loci [1–3]. ALP can be resolved into tissue non-specific (liver, bone and kidney type), placental and intestinal ALP. Although its physiological role is largely unknown, the enzyme is relatively familiar to clinicians, who have long relied upon the analysis of serum total ALP activity as an indicator of bone and/or liver disease. Several methods have been proposed to differentiate between bone and liver ALP. Thermodenaturation and specific inhibition by amino acids are among the oldest [4–8]. For the electrophoretic separation of the ALP isoenzymes, various supporting media have been used, reviewed by Moss [9] and Meyer-Sabellek

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[10]. Some techniques are too cumbersome for routine use, and in others a poor resolution of the liver- and bone-derived isoenzymes, an absent or poor visualization of high-M_r ALP and a lack of sensitivity and reproducibility made interpretation of the isoenzyme patterns difficult.

In a search for a reliable method, applicable in a routine clinical chemistry laboratory, we evaluated two commercially available electrophoretic techniques. The Isopal system was developed by Analis (Namur, Belgium) in 1985 and commercialized by Beckman (Brea, CA, USA). The Sebia (Issy-les-Mouligneux, France) system was commercialized in 1988. Both are agarose electrophoretic systems. Sebia added a complementary gel containing wheat-germ lectin for the precipitation of bone ALP.

EXPERIMENTAL

Total ALP activity

Measurement of total ALP activity was performed on a Hitachi (Boehringer, Mannheim, Germany) automatic chemical analyzer Model 705, according to the method recommended by the Scandinavian Society for Clinical Chemistry and Clinical Physiology [11], but at 25°C. Reagents for ALP were commercially available as a kit using *p*-nitrophenyl phosphate as substrate and diethanolamine as buffer (J.T. Baker, Deventer, Netherlands). The between-run R.S.D. was 4.4% for an ALP activity of 49 U/l and 1.3% for an ALP activity of 237 U/l.

Electrophoresis

Electrophoresis was performed in Beckman and Sebia K20 electrophoresis chambers. Agarose gels were Paragon SPE gels from Beckman (1% agarose in 1.2% barbital buffer and 0.1% sodium azide) and Hydragel Iso-AP gels from Sebia (0.9% agarose either with or without 0.05 g/l of wheat germ lectin). The substrates were 5-bromo-4-chloro-3-indolyl phosphate in 2-amino-2-methyl-1-propanol buffer, with a final concentration of 1.89 mmol/l (pH 10.4) (Beckman), and indolyl phosphate, $MgCl_2$ and nitro blue tetrazolium chloride monohydrate in ethanolamine buffer (pH 9.8) (Sebia). They were used with a 0.4 *M* Tris–borate buffer (pH 9.45) (Beckman) and a Tris–barbital buffer (5.75 g/l Tris, 1.47 g/l barbital, 8.24 g/l sodium barbital and 0.08 g/l sodium azide, pH 9.1) (Sebia). The equilibration buffer for the Beckman system consisted of electrophoresis buffer to which the organic surfactants alkaryl polyoxyethylene glycol and alkyl sulphosuccinate (<4%) and 0.1% sodium azide were added.

Quantification of the ALP isoenzymes

Total ALP activities of the samples were introduced in a computerized densitometer (Appraise; Beckman) and the gels were scanned at 600 nm. To quantify the ALP activity of the scanned surfaces, the inflection point between two ALP peaks on the tracing was automatically determined by the scanner (and eventually visually corrected), and a vertical separation line was drawn automatically to the base of the scan. The area under the curve and the corresponding isoenzyme activities were computed by the densitometer.

Sample treatment

For treating the samples with neuraminidase [12], 5 μl of a 2 kU/l neuraminidase solution (from *Clostridium perfringens*, EC 3.2.1.18; Analis, Namur, Belgium) were added to 25 μl of serum and the mixture was incubated either for 30 min at 37°C (to separate bone and liver ALP) or overnight at 37°C (to identify the intestinal ALP fractions). Phosphatidylinositol-specific phospholipase-C (PI-PLC, from *Bacillus cereus*, EC 3.1.4.3; Sigma, St. Louis, MO, USA) treatment consisted in the addition of 5 μl of an aqueous PI-PLC solution (60 or 120 kU/l, depending on the batch) to 25 μl of serum and incubating the mixture for 30 min at 37°C. Incubation with polyclonal and monoclonal antibodies was performed by adding 5 μl of the antibody solution to 25 μl of serum and letting the mixture incubate for 5 min (polyclonal anti-placental antiserum from Analis), 1 h (monoclonal anti-intestinal ALP anti-serum "250") [13] or overnight (monoclonal anti-placental antiserum "17E3") [14] at 37°C.

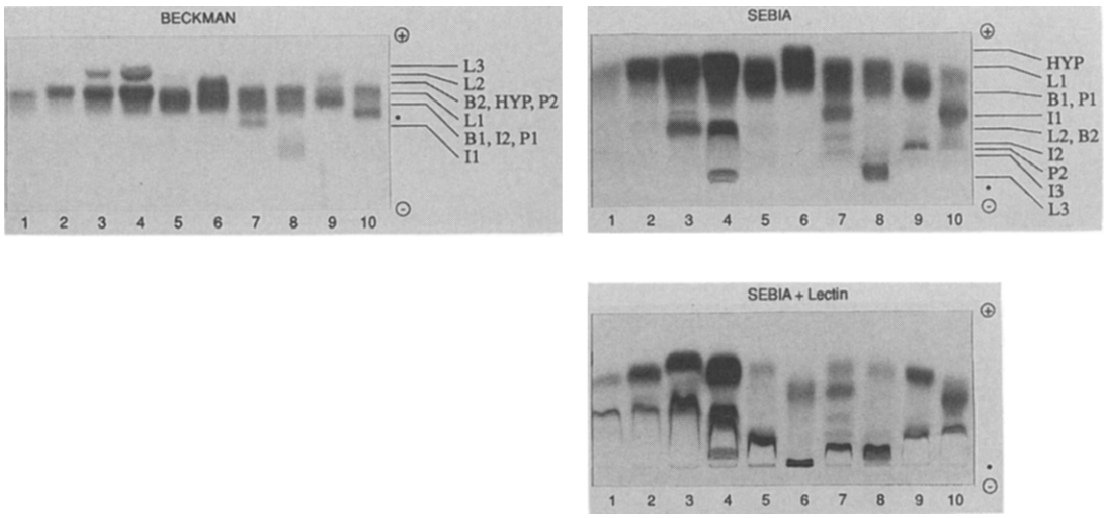

Fig. 1. Typical alkaline phosphatase isoenzyme patterns obtained with the Beckman and Sebia systems. Lanes: 1 = normal pattern (serum from a healthy 51-year-old woman); 2 = high liver ALP + trace of high-M_r ALP (serum from a 40-year-old woman with rheumatoid arthritis and drug-induced hepatocellular disease); 3 = high liver + high-M_r ALP (serum from a 60-year-old man with intrahepatic cholestatic disease); 4 = high liver + high-M_r + lipoprotein-bound ALP (serum from a 66-year-old woman with severe extrahepatic cholestatic disease); 5 = high bone + bone variant ALP (serum from a 70-year-old man with Paget's disease of bone); 6 = transient hyperphosphatasaemia (serum from a healthy, 7-month-old infant); 7 = high intestinal ALP + trace of intestinal variant ALP (serum from a 66-year-old man with hepatic cirrhosis); 8 = sample from lane 7 treated with polyclonal anti-intestinal antiserum; 9 = high placental ALP (serum from a 29-year-old pregnant woman at 30 weeks of gestation); 10 = sample from lane 9 incubated with monoclonal anti-placental antiserum. Note: the symbols indicate ALP fractions of untreated samples. L1 = main liver ALP; L2 = membrane-bound liver ALP ("high-M_r"); L3 = lipoprotein-bound liver ALP ("ultra-fast"); I1 = main intestinal ALP; I2 and I3 = intestinal variant ALP; HYP = fraction of transient hyperphosphatasaemia; B1 = main bone ALP; B2 = bone variant ALP; P1 = main placental ALP; P2 = placental variant ALP.

RESULTS

Migration patterns

As can be seen in Fig. 1, the migration patterns differed considerably between the electrophoretic systems.

Liver and bone ALP. With both systems liver ALP migrated slightly ahead of bone ALP (Fig. 1, upper left and upper right gels). Although these two isoenzymes were only partially separated, their activity could be reproducibly quantified on the densitometric scan as long as the bone ALP activity remained below 50% of the total ALP activity. When it exceeded this value, as was the case for the serum in lane 5 of Fig. 1, sample treatment was necessary, either with neutraminidase (Beckman) (Fig. 2) or by applying the sample on a second gel containing wheat germ lectin to precipitate bone ALP (Sebia) (Fig. 1, lower right gel).

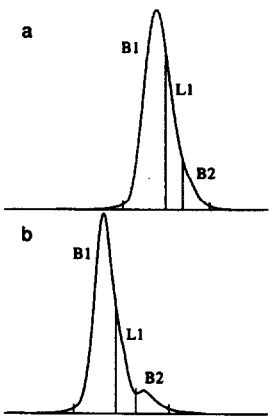

Fig. 2. Quantification of bone, liver and bone variant ALP in a sample with high bone ALP activity with the Beckman system. Densitometric scanning of the serum from a healthy child, (a) untreated and (b) following neuraminidase treatment for 30 min. Note: the symbols indicate ALP fractions of untreated samples. L1 = main liver ALP; B1 = main bone ALP; B2 = bone variant ALP.

Bone variant ALP. An additional fraction, migrating slightly faster than liver ALP, was detected with the Beckman system in >99% of the sera from healthy children [15] (Fig. 3, lanes 5–8). This fraction was also present in sera from adults suffering from bone diseases characterized by a high osteoblastic activity (Fig. 1, lane 5, and Fig. 3, lanes 1–4). The bone variant was inactivated by heat treatment at 56°C for 10 min in the same way as the major bone fraction. Although the activity of this bone variant ALP fraction was much lower than that of the major bone fraction, it could be clearly separated from the latter and from liver ALP by treatment with neuraminidase for 30 min (Fig. 2). With the Sebia system, bone variant ALP co-migrated with high-M_r ALP (present as a trace in Fig. 1, upper right gel, lane 5, and very clearly in Fig. 3).

High-M_r ALP. High-M_r ALP, also called "bile" ALP [16], "koinozyme" [17] or "fast liver" ALP [18], migrated ahead of liver ALP with the Beckman system (Fig. 1, upper left gel, present as a trace in lane 2, and very clearly in lanes 3 and 4), whereas with the sebia system it migrated slower than liver and bone ALP (Fig. 1, upper right gel, present as a trace in lane 2, and very clearly in lanes 3 and 4). As high-M_r ALP co-migrated with the bone variant in the Sebia system, sample treatment with PI-PLC was sometimes required to differentiate between these fractions: high-M_r ALP is converted into liver ALP by PI-PLC [15], whereas bone variant ALP is resistant to this treatment (Fig. 3, lanes 3 and 7).

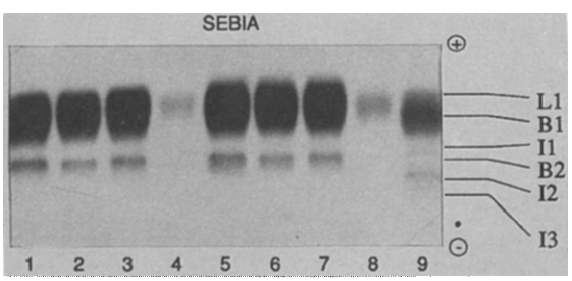

Fig. 3. Migration of bone variant ALP with the Sebia system. Serum sample from a 73-year-old man with Paget's disease of bone (lanes 1–4) and from a 3-year-old healthy child (lanes 5–8). Lanes: 1 and 5 = untreated serum; 2 and 6 = serum treated with polyclonal anti-intestinal antibody; 3 and 7 = serum treated with phospholipase-C; 4 and 8 serum treated at 56°C for 10 min; 9 = serum with high intestinal (I1) and intestinal variant ALP (I2 and I3) activity. Note: the symbols indicate ALP fractions of untreated samples. L1 = main liver ALP; I1 = main intestinal ALP; I2 and I3 = intestinal variant ALP; B1 = main bone ALP; B2 = bone variant ALP.

Ultra-fast ALP. Very icteric samples sometimes showed an additional fraction migrating well ahead (anodally) of the high-M_r fraction with the Beckman system (Fig. 1, upper left gel, lane 4) and remaining at the application point with the Sebia technique (Fig. 1, upper right gel, lane 4). This fraction was previously identified as lipoprotein-bound ALP (lipopotein-X) [19].

Intestinal ALP. By treating sera with polyclonal and monoclonal anti-intestinal antibodies and with neuraminidase (removes sialic acid residues from all but the non-sialylated intestinal ALP isoenzyme, thus influencing the migration pattern of the sialylated isoenzymes only), two "intestinal" fractions could be identified with the Beckman system: intestinal ALP, corresponding to the soluble, dimeric form of the isoenzyme, and intestinal variant ALP, a hydrophobic entity with a higher molecular mass than intestinal ALP [19–23]. With the Beckman system, intestinal ALP migrated slightly towards the cathode. The intestinal variant, on the other hand, co-migrated with bone ALP, making treatment of the samples with a polyclonal antibody directed against both intestinal and placental ALP necessary to correctly quantify bone ALP (Fig. 1, upper left gel, lanes 7 and 8, and Fig. 4). With the Sebia system, three intestinal ALP fractions were identified: I1, migrating between bone and high-M_r ALP and corresponding to the soluble, dimeric form of the isoenzyme, and two other fractions, I2 and I3, migrating between high-M_r ALP and the application point, corresponding to intestinal variant ALP (Fig. 1, upper right gel, lanes 7 and 8).

Placental ALP. The major placental ALP fraction co-migrated with bone ALP in both systems (Fig. 1, upper left and right gels, lane 9), so treatment with a monoclonal anti-placental ALP antibody was necessary to differentiate it from both bone and intestinal variant ALP (Fig. 1, upper left and right gels, lane 10). A second placental ALP fraction was sometimes present,

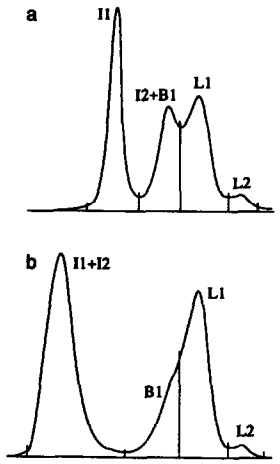

Fig. 4. Quantification of intestinal, intestinal variant, bone, liver and high-M_r ALP in a sample with high intestinal and intestinal variant ALP activity with the Beckman system. Densitometric scanning of the serum from a dialysed patient with chronic renal failure, (a) untreated and (b) following treatment with a polyclonal anti-intestinal antiserum for 5 min. Note: the symbols indicate ALP fractions of untreated samples. L1 = main liver ALP; L2 = membrane-bound liver ALP ("high-M_r"); I1 = main intestinal ALP; I2 = intestinal variant ALP; B1 = main bone ALP.

migrating between liver and high-M_r ALP with the Beckman system (Fig. 1, upper left gel, lane 9). This was also shown by heat treatment for 10 min at 65°C (Fig. 5, lane 4). With the Sebia

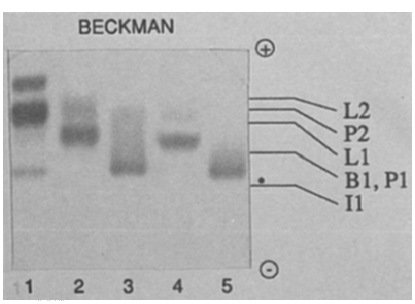

Fig. 5. Beckman electrophoresis of placental ALP. Lanes: 1 = serum containing high-M_r, liver, bone and intestinal ALP; 2–5 = serum sample from a 30-year-old pregnant woman at 37 weeks of gestation; 2 = untreated serum; 3 = serum treated with monoclonal anti-placental antiserum; 4 = serum heated for 10 min at 65°C; 5 = serum heated for 10 min at 65°C and treated with monoclonal anti-placental antiserum (lane 5). Note: the symbols indicate ALP fractions of untreated samples. L1 = main liver ALP; L2 = membrane-bound liver ALP ("high-M_r"); I1 = main intestinal ALP; Bd1 = main bone ALP; P1 = main placental ALP; P2 = placental variant ALP.

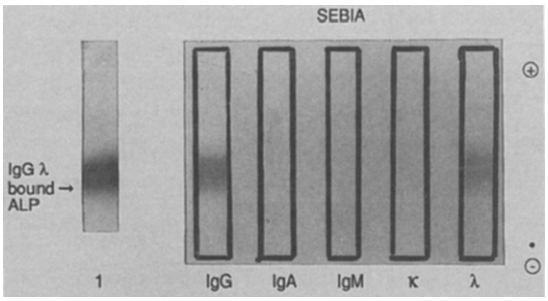

Fig. 6. Sebia electrophoresis of a serum containing immunoglobulin-bound ALP. Untreated serum (lane 1) and immunofixation of the same sample to identify the immunoglobulin type of the complex (IgG–λ).

system, two placental fractions were also identified, the major one co-migrating with bone ALP and the other migrating between the two intestinal fractions I2 and I3 (Fig. 1, upper right gel, lanes 9 and 10).

Immunoglobulin-bound ALP. ALP can form complexes with immunoglobulins. Usually, these fractions were easily identified by both systems because of their peculiar migration patterns (Fig. 6).

Transient hyperphosphatasaemia. Occasionally, a transient rise of total ALP activity towards extremely high values, accompanied by an ALP fraction of unknown origin, was encountered in young children (0–6 years old) [24–26]. This phenomenon is extremely rare in adults [27]. In these sera, bone ALP activity is grossly elevated, and an additional fraction, migrating slightly anodal of liver ALP, was found with both systems (Fig. 1, upper left and right gels, lane 6). With the Beckman system, it had to be differentiated from bone variant ALP. The activity of the fraction of transient hyperphosphatasaemia was generally much higher than that of the variant bone fraction, whereas the latter was less sensitive to neuraminidase treatment and more sensitive to heat treatment at 56°C.

The different migration patterns are represented schematically in Fig. 7.

Reproducibility

Area under the curve. The within- and between-gel reproducibilities of the scanned areas under the curve are summarized in Table I.

Relative mobility. With the Beckman system,

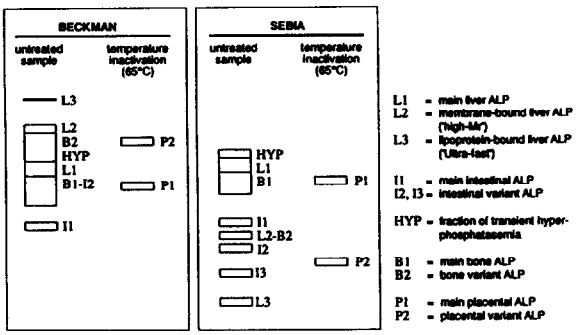

Fig. 7. Schematic representation of the alkaline phosphatase isoenzyme migration patterns obtained with the Beckman and Sebia system.

within-gel differences in relative mobility (distance relative to the application point) were 1–2% for liver, bone and high-M_r ALP; between-gel differences were 5% for liver and bone and 6% for high-M_r ALP ($n = 10$).

With the Sebia system, within-gel differences in relative mobility were 3% for liver ALP, 2% for bone ALP, 3% for high-M_r ALP and I1 and 0% for I2 and I3 ($n = 10$).

Sensitivity and detection limit

Provided that the total ALP activity was between 50 and 200 U/l, the lowest activity that

TABLE I

REPRODUCIBILITY OF THE BECKMAN AND SEBIA SYSTEMS FOR THE ELECTROPHORETIC SEPARATION OF ALKALINE PHOSPHATASE ISOENZYMES

System	ALP fraction	Within-gel ($n = 10$)			Between-gel ($n = 10$)		
		x (U/l)	S.D. (U/l)	R.S.D. (%)	x (U/l)	S.D. (U/l)	R.S.D. (%)
Beckman	Intestinal	3	0.7	24	5	2.1	42
	Bone	37	0.6	2	29	2.0	7
	Liver	90	1.8	2	60	1.8	3
	High-M_r	9	1.7	18	7	1.0	14
Sebia	Intestinal:						
	I1	8	0.4	5	9	0.8	9
	I2	9	0.8	9	8	1.8	22
	I3	18	0.7	4	17	1.8	11
	Bone	20	1.3	6	22	1.2	6
	Liver	34	0.7	2	35	1.2	4
	High-M_r	4	0.6	16	4	0.8	19

TABLE II

COMPARISON OF THE BECKMAN AND SEBIA SYSTEMS FOR THE DETERMINATION OF THE ALKALINE PHOSPHATASE ISOENZYMES IN 34 SERUM SAMPLES FROM DIALYSED PATIENTS

See also Fig. 8.

Parameter[a]	Bone ALP	Liver ALP	High-M_r ALP	Intestinal ALP	Intestinal variant ALP	Bone variant ALP
r	0.95	0.84	0.76	0.89	0.84	0.83
a	1.03	0.75	0.98	1.52	1.35	0.39
b	−0.04	1.55	−0.18	−1.28	−0.01	0.01

[a] r = Correlation coefficient. $y = ax + b$, where x = Beckman and y = Sebia and b, x and y are expressed in U/l.

could be reproducibly measured was 1–2 U/l and the detection limit was 2 U/l for both systems.

Correlation between the two systems

The correlation between the activities of the different isoenzymes obtained with the two systems was studied in a group of patients with chronic renal failure treated with haemodialysis. These particular sera were chosen as they represented a broad variety of isoenzyme patterns. The results are summarized in Table II and Fig. 8. A good correlation was obtained for bone ALP and the correlation between the other isoenzymes was satisfactory, except for high-M_r ALP. As high-M_r ALP migrates in the close proximity of I1 with the Sebia system, a minor high-M_r or I1 fraction was sometimes missed when the activity of the other fraction was high. The absolute activities of bone and high-M_r ALP were comparable for both systems. Liver ALP activity was higher when determined with the Isopal system, while intestinal and intestinal variant ALP activities were much higher with the Sebia system. The Beckman system, on the other hand, was definitely more sensitive for the detection of bone variant ALP.

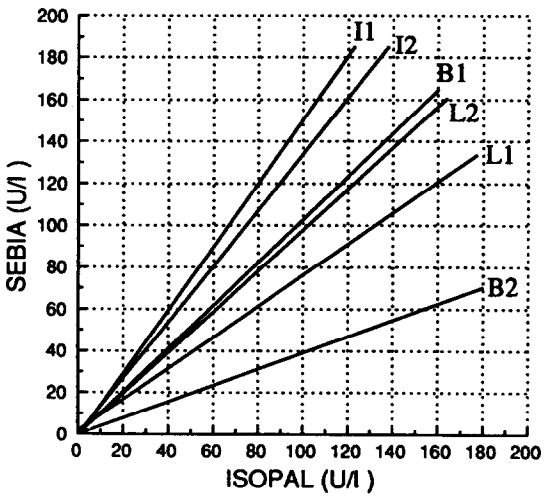

Fig. 8. Graphical representation of the comparison of the Beckman and Sebia systems for the determination of alkaline phosphatase isoenzymes in 34 serum samples from dialysed patients (see also Table II). L1 = main liver ALP; L2 = membrane-bound liver ALP ("high-M_r"); I1 = main intestinal ALP; I2 = intestinal variant ALP (I2 + I3 for the Sebia system); B1 = main bone ALP; B2 = bone variant ALP.

Aspecific precipitation of high-M_r ALP by wheat-germ lectin, as was described previously by Rosalki and Foo [28], was not frequently observed, and it did not interfere with the correct quantification of liver ALP in these samples. Exceptionally, however, varying amounts of liver ALP appeared to be precipitated by the lectin. As a consequence, liver ALP activities determined with the native Sebia gel correlated better with those from the Beckman system than the liver ALP activities obtained with the lectin-containing Sebia gel.

DISCUSSION

Both the Beckman and the Sebia systems are readily applicable in any routine laboratory as they pose no major technical problems. On drying, the transparent gels can easily be scanned and stored. The sensitivity and reproducibility were comparable and satisfactory. As the variations in relative mobility were very small, no marker sera were needed for most purposes. A marker serum containing the two intestinal fractions I2 and I3 proved useful for identifying the second placental ALP fraction with the Sebia system.

Owing to the addition of detergents, the migration patterns differed considerably.

In this way, the Sebia system offered the advantage that intestinal variant ALP (I2 and I3) was well separated from bone ALP, allowing the latter fraction to be quantified without sample treatment. As intestinal variant ALP is present in more than 30% of a healthy reference population [15], treatment of all the samples with a polyclonal anti-intestinal antiserum is to be advised with the Beckman system. On the other hand, the migration of high-M_r ALP near to I1 with the Sebia system caused problems when the activity of I1 was considerably higher than that of high-M_r ALP or *vice versa*. This is clinically important, *e.g.*, in cases of hepatic cirrhosis where both intestinal ALP and high-M_r ALP can be present. High-M_r ALP consists of liver membrane fragments shed by the hepatocyte [29]. Its presence suggests cholestasis. As intestinal ALP

is normally cleared by the hepatocyte, a rise in intestinal ALP suggests a decreased liver function.

Bone variant ALP migrated like high-M_r ALP with the Sebia system. This may lead to an erroneous interpretation when both are present, *e.g.*, in sick children (bone variant ALP is present in most growing children, but the presence of high-M_r ALP in a child's serum is always pathological) or in metastasis of malignant tumours (bone variant ALP in osteoblastic bone metastasis, high-M_r ALP in liver metastasis). "Ultra-fast" ALP, a fraction that indicates severe extrahepatic or generalized intraphepatic cholestasis, was readily identified by both systems.

Recognizing the electrophoretic pattern of transient hyperphosphatasaemia is also clinically relevant, as in children this benign and transient condition, which needs no treatment, has to be differentiated from rickets, also causing grossly elevated total ALP activities, and a condition that needs prompt treatment with vitamin D. It is also advantageous to recognize the (very) rare adult cases of transient hyperphosphatasemia, as this saves the patient from tedious, costly and sometimes invasive examinations. For the same reason, recognition of immunoglobulin-bound ALP fractions, a phenomenon that is more common amongst hospitalized patients, but to which no particular disease state can be attributed, can be beneficial for the patient.

Owing to the finding of additional fractions in pathological conditions, some experience is needed with both systems to decide whether or not sample treatment(s) is (are) required and which treatment(s) to choose. Once this knowledge is acquired, however, electrophoresis allows one to identify correctly and quantify most of the ALP isoenzymes.

In all instances it is advisable to scan the patterns properly, and not to rely solely on visual inspection. Moreover, quantification of the isoenzymes is necessary when the isoenzyme pattern is to be used for follow-up of the patient. Because of the differences between the two systems, reference values for one method are not readily applicable to the other.

We conclude that separating ALP into its various isoenzymes by these two commercially available methods is a clinically relevant test which is reproducible, sensitive, relatively simple and therefore applicable in routine clinical laboratories.

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