PAPERS AND ORIGINALS

Comparative study of alkaline phosphatase isoenzymes, bone histology, and skeletal radiography in dialysis bone disease

R B NAIK, P GOSLING, C P PRICE

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Summary

Liver, intestinal, and bone alkaline phosphatase isoenzymes were measured using heat stability and L-phenylalanine inhibition techniques in 78 patients on intermittent haemodialysis. Fifty-five patients had abnormalities in one or more of the isoenzymes. Changes in bone and intestinal alkaline phosphatase activities seemed to be related and raised liver isoenzyme activity was associated with the development of liver disease. Abnormal histological and radiological findings were better correlated with bone alkaline phosphatase levels than with total alkaline phosphatase, and serial estimations of bone isoenzyme activity were useful in assessing the response of renal osteodystrophy to treatment with a vitamin D analogue. Serum alkaline phosphatase isoenzyme measurement provides another useful and non-invasive index for monitoring metabolic bone disease in patients with chronic renal failure.

Introduction

Metabolic bone disease is a common complication of chronic renal failure and may be progressive in patients on regular dialysis.[1] In such patients the total activity of serum alkaline phosphatase may be used as an indirect index of the severity and progression of bone disease,[2] but its use has been questioned because of the presence in the blood of several alkaline phosphatase isoenzymes of non-skeletal origin. Many techniques are available for fractionating the isoenzymes of alkaline phosphatase. We describe here the application of a systematic procedure for performing this fractionation in patients with chronic renal failure undergoing regular haemodialysis.

Departments of Renal Medicine and Clinical Chemistry, East Birmingham Hospital, Birmingham B9 5ST

R B NAIK, MRCP, honorary senior registrar (now senior registrar, Renal Unit, St Mary's Hospital, Portsmouth)

P GOSLING, PHD, biochemist

C P PRICE, PHD, principal biochemist (now top-grade biochemist, Division of Chemical Pathology, Southampton General Hospital, Southampton)

Patients and methods

Alkaline phosphatase isoenzyme fractions were measured in blood collected immediately before dialysis from 78 patients with chronic renal failure. Eight of these patients were treated with 1-α-hydroxycholecalciferol for metabolic bone disease during the study, and serial blood samples were taken every six weeks. A reference range for serum alkaline phosphatase isoenzymes was established by measuring the values in 46 staff aged over 20 from the laboratory and renal unit.

Bone histology—Transiliac bone biopsies were performed under local anaesthesia in 32 patients. After the specimen had been fixed in formalin 5-μm undecalcified sections were cut and examined histologically after staining with haematoxylin and eosin, toluidine blue, and von Kossa stain. A diagnosis of osteitis fibrosa was based on the presence of marrow fibrosis. Osteomalacia was defined as the presence of widened osteoid seams (more than 10 μm) without features of osteitis fibrosa.

Skeletal radiology—Skeletal surveys were carried out on all patients when the blood samples were taken and at six- to 12-month intervals in the patients treated with 1-α-hydroxycholecalciferol. The presence of subperiosteal erosions in the phalanges and abnormal mottling in the skull vault was considered to indicate secondary hyperparathyroidism, and rib fractures and Looser's zones were indicative of osteomalacia.

Alkaline phosphatase isoenzymes—Total serum alkaline phosphatase activity was determined on an LKB 8600 Reaction Rate Analyser at 37°C, using α-napthylphosphate as substrate.[3] Alkaline phosphatase isoenzymes were separated by polyacrylamide gel electrophoresis,[4] and liver, bone, and intestinal isoenzymes were found to be the only alkaline phosphatase isoenzymes in serum. The bone fraction is inactivated by heat, and alkaline phosphatase derived from the intestine is strongly inhibited in the presence of L-phenylalanine.[5] Each isoenzyme fraction was therefore measured as follows: (*a*) the heat-stable activity was determined using 0·2 ml of serum that had been heated in a thin-walled glass tube at 56±0·2°C for exactly 10 minutes and then cooled by immersion in iced water; (*b*) The phenylalanine-

stable activity (predominantly liver and bone fraction) was determined by measuring enzyme activity in the presence of 0·005-M phenylalanine. Preliminary work had shown that these conditions gave the maximal differentiation between the isoenzyme fractions. Stability data for the three isoenzymes (table I) was obtained by observing the inhibitory effects of heat and L-phenylalanine on homogenates of liver and intestine taken at necropsy from normal human tissue and on the diluted serum of patients with Paget's disease. Using these stability data and a simple computer programme,[6] the activities of bone and intestinal isoenzymes were calculated and the activity due to the liver fraction was obtained by subtracting the sum of bone and intestinal levels from the total alkaline phosphatase level.

TABLE I—*Stability of main alkaline phosphatase isoenzymes after heat inactivation and in presence of* L-*phenylalanine*

Isoenzyme	Proportion of activity after heat inactivation (%)	Proportion of activity L-phenylalanine stable (%)
Liver	35·9	81·4
Bone	5·4	82·1
Intestine	40·1	30·4

EVALUATION OF ISOENZYME TECHNIQUE

The within-batch precision of the procedure was determined from 20 replicate analyses of a pooled serum. The coefficient of variation was 1·7% for total activity (mean activity 66 IU/l), 2·0% for the L-phenylalanine-stable fraction (mean activity 39 IU/l), and 4·3% for the heat-stable fraction (mean activity 19 IU/l). The between-batch precision was determined on 13 paired samples with a mean percentage difference between each pair of values of 5·7% (0-10%) for the bone isoenzyme (mean 46·4 IU/l), 9·8% (0-20%) for the liver isoenzyme (mean 32·3 IU/l), and 4·8% (0-15%) for the intestinal isoenzyme (mean 3·4 IU/l).

Varying proportions of the isoenzyme preparations, diluted to 100 IU/l with heat-inactivated serum, were mixed and then measured to assess the recovery of isoenzyme activity. The mean recovery was 105% of expected for bone (range 100-108%), 101% for the liver (range 94-115%), and 93% for intestine (range 82-100%).

The distribution of values for bone and liver isoenzymes determined on laboratory staff was Gaussian (assessed by linearity of frequency distribution on probability plot). The distribution for the intestinal fraction was non-Gaussian, 95% of the values lying between 0-18 IU/l.

The reference range for each isoenzyme activity was:

Bone 0-30 IU/l (mean 14·0; SD 8·0 IU/l)
Liver 0-25 IU/l (mean 11·0; SD 7·0 IU/l)
Intestine 0-18 IU/l.

Results

Not all three isoenzyme fractions were detected in all patients: a liver isoenzyme was found in 85%, an intestinal isoenzyme in 71%, and a bone isoenzyme in 94% of patients. Fifty-five (70%) patients had abnormalities in one or more of the alkaline phosphatase isoenzymes (table II). Twelve of the 19 patients with raised liver isoenzyme activity also had a raised bone isoenzyme but the association was not significant ($P > 0·1$). Ten of the patients with raised intestinal alkaline phosphatase also showed increased bone isoenzyme activities ($0·05 > P > 0·1$), but there was no significant correlation between the presence of a bone fraction (irrespective of whether it was increased) and the presence of an intestinal fraction ($P > 0·01$).

Fig 1 shows that bone alkaline phosphatase activity correlated well with total alkaline phosphatase ($r = 0·89$). Thirteen patients had a normal total alkaline phosphatase and a raised bone fraction, and eight of these patients had proved bone disease. Although higher activities of bone alkaline phosphatase were more common in patients with osteitis fibrosa than in those with osteomalacia, this difference was not statistically significant using Student's t test. The four patients with high total and normal bone alkaline phosphatase had raised liver isoenzyme fractions and proved liver disease. The remaining patients with raised liver fractions had no other evidence of liver disease.

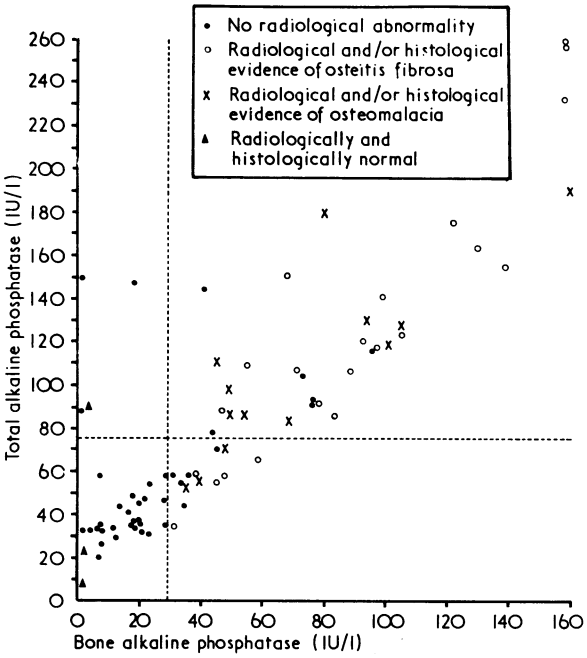

FIG 1—Total alkaline phosphatase activity compared with bone alkaline phosphatase activity. Dotted lines indicate upper limits of normal.

Radiographic abnormalities correlated better with bone alkaline phosphatase activity ($\chi^2 = 23·3$; $P < 0·01$) than with total alkaline phosphatase activity ($\chi^2 = 7·9$; $P < 0·01$) (table III). Of the 21 patients with normal radiographs but increased bone alkaline phosphatase activity eight underwent biopsy and all had histological evidence of osteomalacia. Similarly, abnormal histological findings were better correlated with bone alkaline phosphatase activity ($P < 0·01$) than with total alkaline phosphatase activity ($P < 0·02$) (table III). In one patient with normal histological features the bone alkaline phosphatase fraction was just raised (38 IU/l), although total alkaline phosphatase activity was normal (57 IU/l). There were subperiosteal erosions of the phalanges on a previous skeletal survey but a repeat radiograph

TABLE II—*Distribution of abnormal isoenzyme fractions in 78 patients on haemodialysis*

Isoenzyme abnormality	No (%) of observations
No abnormality	23 (29·5)
Raised bone only	26 (33·3)
Raised liver only	7 (9·0)
Raised intestine	1 (1·3)
Raised bone and liver	11 (14·1)
Raised bone and intestine	9 (11·5)
Raised liver and intestine	0
Raised bone, liver, and intestine	1 (1·3)

TABLE III—*Number of patients with increased isoenzymes and total alkaline phosphatase activity in relation to bone histological and skeletal radiological appearances*

	No of patients studied	No of patients with increased isoenzymes and total alkaline phosphatase			
		Bone	Intestine	Liver	Total
Radiological diagnosis					
Osteitis fibrosa	22	22	6	4	17
Osteomalacia	4	4	0	2	2
No abnormality	52	21	5	13	19
Histological diagnosis					
Osteitis fibrosa	16	16	4	4	14
Osteomalacia	12	12	2	6	9
Normal histology	4	1	0	1	1
No biopsy	46	18	5	8	14

of the hand at the time of bone biopsy showed no abnormality. The total alkaline phosphatase activity was raised in another patient with normal bone histology, and the increase was found to be due entirely to a raised liver fraction.

Eight patients received 1-α-hydroxycholecalciferol for metabolic bone disease and five showed a significant fall in bone isoenzyme (fig 2) and improvement of bone radiographs. The three remaining patients showed no significant change in the level of bone isoenzyme and no improvement of bone disease. Four of the eight patients underwent biopsy after six months of treatment and two showed improved histological features of bone (decreases in osteoid volume and the maximum number of birefringent lamellae, a decrease in the abnormal osteoid coverage, and a decrease in bone fibrosis) and a fall in plasma levels of parathyroid hormone.[7] Changes in bone alkaline phosphatase activity during treatment were associated with changes in intestinal alkaline phosphatase activity (figs 2 and 3) but not with changes in liver alkaline phosphatase activity.

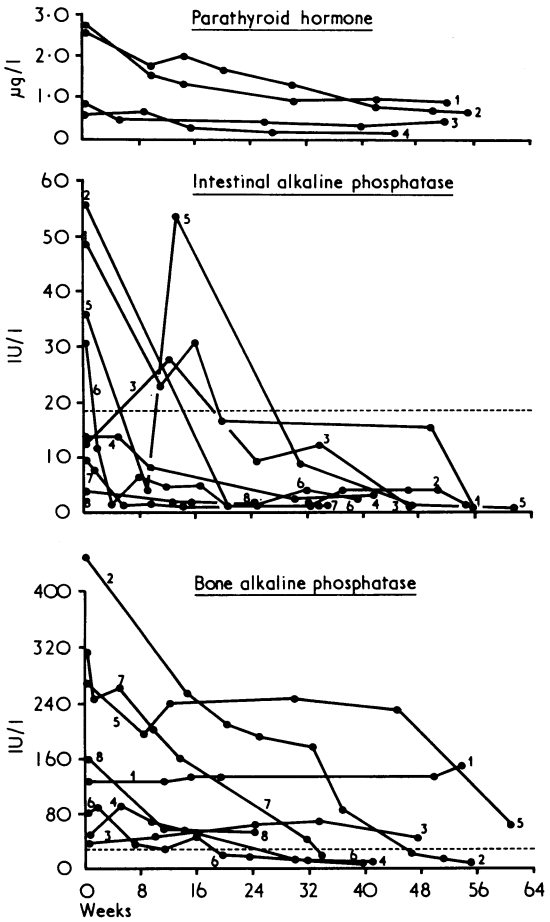

FIG 2—Bone and intestinal alkaline phosphatase activities in eight patients treated with 1-α-hydroxycholecalciferol (1-2 μg/day) and plasma parathyroid hormone levels in four patients. Upper limits of normal for isoenzymes are represented by dotted lines; that for parathyroid hormone is <0·2 μg/l.

Discussion

Increases in the serum alkaline phosphatase activity of patients with bone disease may reflect increased osteoblastic activity. The heterogeneity of serum alkaline phosphatase suggests, however, that an estimate of the bone isoenzyme rather than that of total enzyme activity might be more helpful. Although they indicate the heterogeneous nature of the isoenzyme pattern electrophoretic techniques for separating alkaline phosphatase isoenzymes do not allow accurate and reproducible measurement of the isoenzyme fractions. The procedure we used does allow such measurement and can be used to diagnose metabolic bone disease and monitor its response to treatment.

The limitations of the value of total serum alkaline phosphatase activity have been recognised. In a comparison of alkaline phosphatase and serum hydroxyproline levels Bishop *et al*[8] and Moorhead *et al*[9] concluded that the serum hydroxyproline value was a more sensitive index of osteitis fibrosa. Sagar *et al*,[10] using the heat sensitivity of the bone isoenzyme, found a significant correlation between this measure of bone alkaline phosphatase activity and serum hydroxyproline concentrations. Heat sensitivity alone does not, however, reflect abnormalities that might occur in other alkaline phosphatase isoenzymes. By measuring all three isoenzymes, the bone fraction can be measured. Unlike hydroxyproline, the bone fraction may be a sensitive indicator of osteoblastic function. Simpson *et al*[11] showed that only with the more severe grades of osteitis fibrosa was the association with subperiosteal erosions significant; similarly, Looser's zones appear only late in osteomalacia. Our results show that radiological bone disease is clearly associated with increased bone alkaline phosphatase activity, but bone alkaline phosphatase abnormalities are also seen without radiological abnormalities. In the cases where bone biopsy specimens were taken these showed histological evidence of osteomalacia. Alkaline phosphatase activity assessed in this manner is therefore a more sensitive marker than radiographs. The measurement of bone alkaline phosphatase activity provides a better discrimination of patients with normal or abnormal radiological features than the use of total alkaline phosphatase activity, and, similarly, abnormal histological findings are better correlated with bone alkaline phosphatase activity than with total alkaline phosphatase. We have also shown that serial estimations of bone alkaline phosphatase can be useful in assessing the response of renal osteodystrophy to treatment with vitamin D analogues.

Serum total alkaline phosphatase activity lacks specificity as an index of bone disease and may reflect, for example, liver abnormalities. Four of our patients had increased total alkaline phosphatase activity (fig 1) due solely to increases in the liver fraction. They showed no radiographic abnormalities, bone histology was normal in the one patient who underwent biopsy, and they all had clinical evidence of liver disease. Some patients on haemodialysis do develop a chronic type of liver disease,[12 13] and there may also be an increased incidence of malignancy in patients with chronic renal failure.[14] The liver alkaline phosphatase isoenzyme may therefore contribute significantly to the total enzyme activity.

Eleven patients had a raised intestinal isoenzyme level. This is a lower incidence than that found by other workers who have used slightly different techniques.[15–17] The intestinal isoenzyme shows a much wider variation in activity in the presence of different substrates than the liver and bone isoenzymes,[18] and this may partly explain the variation in results observed by different workers.

It has been suggested that intestinal tissue alkaline phosphatase activity may be related to calcium absorption. Intestinal alkaline phosphatases are thought to be a heterogeneous group of enzymes,[19 20] and there is evidence to suggest that uptake of calcium at the mucosal surface may be mediated through a brush-border phosphatase whose synthesis is vitamin-D dependent,[21] the hormone stimulating the production of the brush-border enzyme. A further phosphatase of the mucosal cell has been isolated from the basal lateral surface, and synthesis of this isoenzyme seems to be stimulated by parathyroid hormone.[22] It is interesting to note that 10 of the 11 patients with raised intestinal alkaline phosphatase also showed increased bone isoenzyme activities. In addition there was a reduction in intestinal alkaline phosphatase in all patients treated with 1-α-hydroxycholecalciferol with a concomitant fall in parathyroid hormone in those patients in whom it was measured. The increased activity of the intestinal isoenzyme in serum may reflect disordered vitamin D metabolism and hyperparathyroidism, and the two intestinal alkaline phosphatases should be

investigated in relation to their dependence on active vitamin D and parathyroid hormone.

Measurement of serum alkaline phosphatase isoenzymes provides another useful and non-invasive index with which to monitor metabolic bone disease in patients with chronic renal failure. It seems to be more sensitive than the total alkaline phosphatase value, which can be normal even in patients with symptomatic bone complications. Measuring the fractions may also indicate the development of liver disease, but the full significance of raised levels of serum intestinal alkaline phosphatase is not yet understood.

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Requests for reprints should be addressed to Dr R B Naik, Renal Unit, St Mary's Hospital, Milton Road, Portsmouth.

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25-Hydroxycholecalciferol absorption in steatorrhoea and postgastrectomy osteomalacia

J M GERTNER, MARGARET LILBURN, MERCEDES DOMENECH

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Summary

Post-absorption levels of 25-hydroxy vitamin D (25-OHD) after oral administration of 25-hydroxycholecalciferol (25-OHD$_3$) were measured in 11 subjects. Five had presented with steatorrhoea of various causes while six had post-gastrectomy osteomalacia.

Post-absorption levels of 25-OHD were low in four of the patients with steatorrhoea but normal in five of those with post-gastrectomy osteomalacia. There was a significant inverse correlation between peak post-absorption 25-OHD levels and faecal fat excretion.

All patients with active post-gastrectomy osteomalacia had subnormal baseline plasma 25-OHD levels, which indicates that the condition is due to a deficiency of vitamin D. Only two of the patients with osteomalacia had estimated dietary vitamin D intakes over 1·75 μg/day.

These findings suggest that an oral 25-OHD absorption test may be a valuable measure of small intestinal function and that poor dietary vitamin D intake rather than impaired absorption of the vitamin may be the major cause of post-gastrectomy osteomalacia.

Department of Human Metabolism, University College Hospital Medical School, London WC1

J M GERTNER, MB, MRCP, lecturer (now instructor in paediatrics, Yale University School of Medicine, New Haven, Connecticut 06510, USA)
MARGARET LILBURN, dietitian
MERCEDES DOMENECH, MD, research assistant (present address: Catedra de Pediatria y Puericultura, Universidad de Valencia, Valencia, Spain)

Introduction

The availability of a sensitive assay for 25-hydroxy vitamin D (25-OHD) has allowed workers to assess gastrointestinal absorption of this sterol without having to administer radioactive substances to human subjects.[1] Under physiological conditions 25-OHD is the major circulating vitamin D metabolite in man[2] and other animals. Some animal tissues eaten by man—for example, mammalian liver and muscle—contain more 25-OHD than cholecalciferol (vitamin D$_3$).[3] 25-OHD is closely related to vitamin D in structure, polarity, and physiochemical behaviour, and it is therefore likely to be absorbed from the gastrointestinal tract in the same way as vitamin D—namely, by inclusion in fat micelles formed with the help of bile salts.

We have explored the usefulness of a 25-OHD absorption test in patients presenting with steatorrhoea and in another group presenting with post-gastrectomy osteomalacia. Our first objective was to discover whether there was any relation between the degree of fat malabsorption and the post-absorption rise in plasma 25-OHD levels after an oral load of 25-OHD. Our second aim was to attempt to assess the relative contributions of malabsorption and inadequate vitamin D nutrition (dietary and cutaneous) in causing post-gastrectomy osteomalacia by studying 25-OHD absorption in patients with this condition.

Patients and methods

Details of the five patients who presented with steatorrhoea are given in the table. Only case 7 showed evidence of vitamin D de-