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# Quantitative method for determining serum alkaline phosphatase isoenzyme activity: estimation of intestinal component

M J PEAKE, M PEJAKOVIC, G H WHITE

*From the Department of Biochemistry and Chemical Pathology, Flinders Medical Centre, Bedford Park, South Australia*

SUMMARY Intestinal alkaline phosphatase activity was measured using levamisole inhibition, and results were compared with a previously reported method using L-phenylalanine. Sixty two per cent intestinal, 39% placental, and 1·3% of either bone or liver alkaline phosphatase activity remained when alkaline phosphatase activity was inhibited in a 2-amino-2-methyl-l-propanol (AMP) buffer reagent system with 10 mmol/l levamisole (final assay concentration 8·1 mmol/l). The assay imprecision (SD) was 0·6 U/l compared with 3·9 U/l using L-phenylalanine for specimens with total alkaline phosphatase activity less than 250 U/l (reference range 30–120 U/l). In serum pools with raised total alkaline phosphatase activity errors in recovered intestinal activity were small (usually less than 3 U/l) when intestinal alkaline phosphatase was added. Much larger errors and many underestimated results were found using L-phenylalanine. For non-haemolysed specimens it is concluded that an assay based on levamisole inhibition provides a better measure of intestinal alkaline phosphatase activity than L-phenylalanine.

A raised serum alkaline phosphatase activity [EC 3.1.3.1] is a common finding in the clinical chemistry laboratory. In a proportion of such cases either the tissue source of the enzyme activity is not identifiable by the clinician, or the contribution from one or more pathological processes is masked.[1] A method for separately quantifying bone, liver, intestinal and placental alkaline phosphatase isoenzyme activities has, however, recently been described.[2]

This method determines the residual serum alkaline phosphatase activity following various inhibitory treatments, with individual isoenzymes being affected in a predictable manner. Each isoenzyme activity present in the original specimen is then quantified by insertion of the residual activities into an appropriate algorithm. Because four separate activity measurements must be made, it is important that the precision and accuracy of each quantitative step is optimised so that the cumulative error on the calculated isoenzyme activities is minimised. One of the inhibitors used is L-phenylalanine, a non-competitive inhibitor of intestinal alkaline phosphatase. The activity of the intestinal component is computed from activities remaining in the presence and absence of L-phenylalanine, with a correction being made for its partial inhibition of the other isoenzymes. In routine use, however, we have noticed that the reported method may underestimate intestinal alkaline phosphatase activity when total alkaline phosphatase activity is very high.

Levamisole, a phenylimidazothiazole compound, has been reported to act in the opposite manner to L-phenylalanine by inhibiting non-intestinal alkaline phosphatase isoenzyme activity, while leaving that of the intestinal component largely unaffected.[3] We therefore investigated whether levamisole and a similar compound, bromotetramisole,[3] may be better reagents than L-phenylalanine for measuring intestinal alkaline phosphatase activity by permitting a more direct measurement of the activity of the desired component.

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## Material and methods

Total, placental, and intestinal alkaline phosphatase activities (using L-phenylalanine) were measured as previously reported.[2] Levamisole (Sigma, molecular weight 240·8 daltons) was accurately weighed out and dissolved in 2-amino-2-methyl-l-propanol (AMP) buffer from single phial Monotest alkaline phos-

phatase kits (Boehringer, West Germany) at concentrations of 5, 10, and 20 mmol/l. Bromotetramisole (Janssen Pharmaceutica Belgium molecular weight 372·2 daltons and Sigma molecular weight 373·2 daltons) was dissolved in AMP buffer at concentrations of 0·27 mmol/l (0·01%) and 1·34 mmol/l (0·05%). Assays were performed on a Cobas Bio centrifugal analyser (Roche Instruments, Basel, Switzerland) using the instrument settings shown in table 1. Extracts of intestinal alkaline phosphatase activity were prepared and measured as previously described.[4]

## Results

### LEVAMISOLE OR BROMOTETRAMISOLE

Inhibition of alkaline phosphatase activity by levamisole and bromotetramisole has previously been reported in assay systems incorporating N-ethylaminoethanol(NEAE) buffer—the placental and intestinal isoenzymes being the most resistant to inhibition.[3] We tested these reagents in our assay system, using AMP buffer, at reagent concentrations designed to inhibit maximally bone and liver activity while minimally reducing intestinal activity. The maximum solubility of bromotetramisole was about 0·27 mmol/l (0·01%); the Sigma preparation was slightly more soluble. Bromotetramisole was dissolved in glass tubes because the reagent partially adhered to plastic. Following inhibition with 0·27 mmol/l bromotetramisole about 90% of intestinal activity remained, and 3–5% of bone and liver alkaline phosphatase activity. Unfortunately, an attempted five-fold increase in bromotetramisole concentration minimally increased the inhibition of the bone and liver isoenzyme activity, due mainly to its low solubility. In contrast, levamisole was readily soluble to at least 20 mmol/l, the highest concentration used, and inhibited more bone and liver activity (table 2). With specimens containing bone or liver alkaline phosphatase activity as high as 1000 U/l, only 2·0%, 1·3%, and 0·7% of activity remained after treatment with 5, 10, and 20 mmol/l levamisole, respectively. In contrast, 75%, 62%, and 47% of intestinal activity remained under the same conditions.

The stability of placental alkaline phosphatase towards levamisole was investigated using serum from

Table 1 *Reaction conditions on Cobas Bio for measuring intestinal alkaline phosphatase isoenzyme activity using levamisole*

| *Instrument settings* | | |
|---|---|---|
| 1 | Units | U/l |
| 2 | Calculation factor | 2688 |
| 3 | Standard 1 concentration | 0 |
| 4 | Standard 2 concentration | 0 |
| 5 | Standard 3 concentration | 0 |
| 6 | Limit | 1·5 |
| 7 | Temperature (°C) | 37 |
| 8 | Type of analysis | 2 |
| 9 | Wavelength (nm) | 405 |
| 10 | Sample volume (µl) | 5 |
| 11 | Diluent volume (µl) | 30 |
| 12 | Reagent volume (µl) | 150 |
| 13 | Incubation time (seconds) | 120 |
| 14 | Start reagent volume (µl) | 0 |
| 15 | Time of first reading (seconds) | 30 |
| 16 | Time interval (seconds) | 10 |
| 17 | Number of readings | 20 |
| 18 | Blanking mode | 1 |
| 19 | Printout mode | 1 |

Table 2 *Percentage activity remaining for each isoenzyme after inhibition with levamisole or bromotetramisole*

| | *Percentage activity remaining* | | | |
|---|---|---|---|---|
| | *Levamisole* | | | *Bromotetramisole* |
| *Isoenzyme* | *5 mmol/l* | *10 mmol/l* | *20 mmol/l* | *0·27 mmol/l* |
| Bone | 2·0 | 1·3 | 0·7 | ~ 4 |
| Liver | 2·0 | 1·3 | 0·7 | ~ 4 |
| Intestinal | 75 | 62 | 47 | ~90 |
| Placental | 53 | 39 | 25 | ~90 |

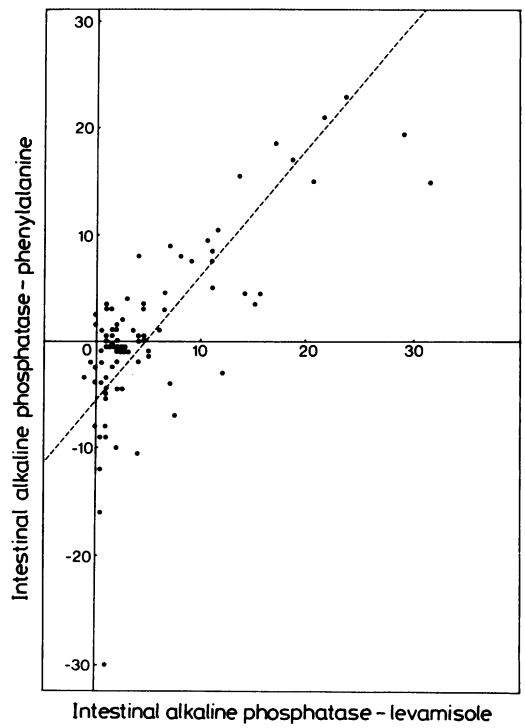


Fig 1 *Comparison of intestinal alkaline phosphatase activity in patient specimens. L-phenylalanine or levamisole as inhibitor (total alkaline phosphatase ⩽250 U/l, n = 88, r = 0·922, y = 1·185x − 5·5). Points not shown: x, y; 97, 95; 53, 56.*

Table 3 *Comparison of intestinal alkaline phosphatase (AP) activity in patient specimens using L-phenylalanine (y) or levamisole (x) as inhibitor*

| | *Total AP ⩽ 250 U/l* | | *Total AP > 250 U/l* | |
|---|---|---|---|---|
| | *L-Phenylalanine* | *Levamisole* | *L-Phenylalanine* | *Levamisole* |
| Mean intestinal activity (U/l) | 2·6 | 6·8 | −17·6 | 3·3 |
| Assay imprecision (SD) from duplicates (U/l)* | 3·9 | 0·6 | 17·5 | 0·6 |
| Regression equation | $y = 1{\cdot}185x - 5{\cdot}5$ | | $y = 3{\cdot}826x - 32{\cdot}4$ | |
| | $r = 0{\cdot}922$ | | $r = 0{\cdot}666$ | |
| | $Sy.x = 4{\cdot}991$ | | $Sy.x = 8{\cdot}129$ | |
| | $n = 88$ | | $n = 26$ | |

*$S = \sqrt{\frac{\sum d^2}{2n}}$

a patient in the 34th week of pregnancy with a total alkaline phosphatase of 940 U/l (885 U/l placental alkaline phosphatase, heat stable for 10 minutes at 65°C); 54%, 39% and 26% of activity remained after treatment with 5, 10, and 20 mmol/l levamisole, respectively. Similar results were found for 15 other sera with placental activity ranging from 50–100 U/l.

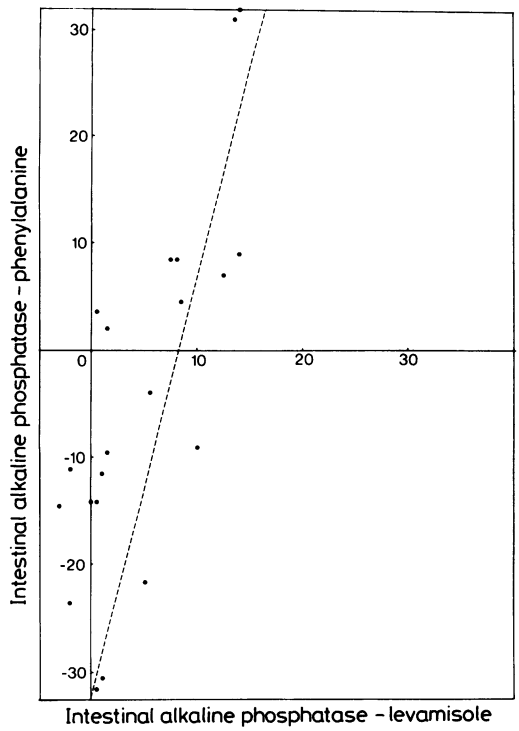


Fig 2 *Comparison of intestinal alkaline phosphatase activity in patient specimens using L-phenylalanine or levamisole as inhibitor (total alkaline phosphatase > 250 U/l, n = 26, r = 0·666, y = 3·826x − 32·4. Points not shown: x, y; 51, 41; −1, −54; 3, −55; 1, −56; −4, −67.*

Following these experiments levamisole was used for further studies because of its better solubility, ease of preparation, and greater inhibition of non-intestinal components.

## LEVAMISOLE COMPARED WITH L- PHENYLALANINE

Intestinal alkaline phosphatase activity was measured in 114 selected specimens, inhibiting with either 10 mmol/l levamisole or 12·3 mmol/l L-phenylalanine (final concentrations in Cobas rotor 8·1 mmol/l and 10 mmol/l, respectively). Total alkaline phosphatase activity exceeded 250 U/l in 26 specimens and 750 U/l in five of these specimens. The highest activity was 1850 U/l in a patient with biliary obstruction. All assays were performed in duplicate in separate analytical runs. With L-phenylalanine, intestinal alkaline phosphatase activity was calculated as previously described.[2] With levamisole, intestinal alkaline phosphatase activity was calculated using an algorithm derived from the data shown in table 2.

$$I = 1{\cdot}6474L - 0{\cdot}0214T - 0{\cdot}6210\,P$$

where I = intestinal activity; P = placental activity; T = total activity; L = activity remaining after levamisole inhibition.

Figs 1 and 2 compare results, which are summarised in table 3.

For specimens with total alkaline phosphatase activity of less than 250 U/l, the mean intestinal activity was 2·6 U/l using L-phenylalanine as inhibitor, and 6·8 U/l using levamisole. Imprecision calculated from duplicate assays was significantly less using levamisole (SD = 0·6 U/l) than with L-phenylalanine (SD = 3·9 U/l). For specimens with total activity greater than 250 U/l, many negative results were generated using L-phenylalanine as inhibitor. Additionally, the reproducibility of some of the duplicate assays was poor. In contrast, the standard deviation for intestinal activity calculated from duplicate assays using levamisole was similar to that obtained when total activity was less than 250 U/l.

Table 4 *Specimens with intestinal alkaline phosphatase (AP) exceeding 20 U/l (duplicate analysis)*

| *Total AP (U/l)* | *Intestinal AP (U/l) Levamisole* | *Intestinal AP (U/l) L-Phenylalanine* | *Clinical notes* |
|---|---|---|---|
| 248/239 | 99(95) | 95(95) | Poorly controlled (non)insulin-dependent diabetes mellitus with metastatic carcinoma of prostate |
| 156/157 | 53(53) | 53(59) | Congestive cardiac failure, chronic renal failure, multiple drug treatment |
| 463/448 | 51(50) | 47(34) | Congestive cardiac failure, analgesic nephropathy, multiple drug treatment |
| 161/155 | 31(32) | 18(12) | Chronic renal failure on continuous ambulatory peritoneal dialysis |
| 105/103 | 29(29) | 19(20) | Insulin-dependent diabetes mellitus + renal transplant + multiple drug treatment |
| 228/221 | 24(23) | 21(25) | Non-Hodgkin's lymphoma on chemotherapy |
| 79/ 78 | 21(22) | 21(21) | Chronic renal failure |
| 77/ 77 | 21(20) | 14(16) | Presumed acute severe multiple sclerosis |

Table 5 *Comparison of intestinal alkaline phosphatase (AP) activity in serum pools supplemented with intestinal extract using levamisole or L-phenylalanine as inhibitor*

| | | *Measured intestinal AP (U/l)* (duplicate analysis)* | | | | | |
|---|---|---|---|---|---|---|---|
| | *Added intestinal AP* | *Pool 1 Total AP (79 U/l)* | *Pool 2 Placental AP (179 U/l)* | *Pool 3 Liver AP (456 U/l)* | *Pool 4 Liver AP (896 U/l)* | *Pool 5 Bone AP (361 U/l)* | *Pool 6 Bone AP (877 U/l)* |
| Levamisole | 176 | 174(173) | 174(172) | 184(179) | 178(174) | 183(185) | 188(187) |
| Phenylalanine | | 162(166) | 185(180) | 162(147) | 178(198) | 176(167) | 152(209) |
| Levamisole | 88 | 83( 81) | 94( 91) | 88( 88) | 87( 86) | 92( 92) | |
| Phenylalanine | | 81( 76) | 93( 94) | 74( 88) | 115( 59) | 100( 97) | |
| Levamisole | 44 | 45( 46) | 44( 41) | 43( 42) | 44( 45) | 44( 44) | |
| Phenylalanine | | 36( 43) | 51( 45) | 46( 42) | 21( 32) | 47( 40) | |
| Levamisole | 22 | 23( 23) | 22( 21) | 22( 21) | 19( 21) | 23( 21) | |
| Phenylalanine | | 21( 20) | 27( 20) | 8( 12) | −9 ( 2) | 7( 14) | |
| Levamisole | 11 | 12( 12) | 11( 7) | 9( 10) | 9( 11) | 11( 11) | |
| Phenylalanine | | 9( 10) | 13( 13) | 5(−7) | −12(−4) | 13( 15) | |
| Levamisole | 0 | 2( 2) | 1( 3) | 0( 1) | −2(−2) | −1(−1) | 0(−1) |
| Phenylalanine | | −1( 2) | 2( 6) | 3(−8) | −8(−39) | −6(−4) | −2(−3) |

*In sera 2 → 6, stated isoenzyme activity represents at least 90% of total activity.

Of the 114 specimens analysed using levamisole, only eight had intestinal alkaline phosphatase above 20 U/l. The highest activity was 97 U/l in a poorly controlled diabetic with a metastatic carcinoma of the prostate. Results from other patients in this category are summarised in table 4.

### RECOVERY OF INTESTINAL ALKALINE PHOSPHATASE

To investigate the recovery of intestinal alkaline phosphatase activity intestinal extracts with alkaline phosphatase activities of 11, 22, 44, 88 and 176 U/l were added to each of six pools of serum. One of these pools was a normal serum with a total alkaline phosphatase activity of 80 U/l; two further pools contained liver alkaline phosphatase activities of 455 and 900 U/l; another two pools had bone activities of 360 and 880 U/l; the remaining pool had a placental activity of 190 U/l. The intestinal activity recovered from these 30 samples, using either levamisole or L-phenylalanine as inhibitor, is shown in table 5. Duplicate results obtained for these prepared pools using levamisole had less imprecision than those determined using L-phenylalanine, as was also the case when estimating patient specimens. The latter inhibitor also generated a number of negative results in samples containing low intestinal activities. The errors found using levamisole were in all cases less than 3 U/l when the added intestinal activity was below 22 U/l, and less than 6 U/l when the added intestinal activity fell between 22 and 88 U/l.

### EFFECT OF HAEMOLYSIS

When levamisole was used as the inhibitor it was noticed that the reaction rates of some haemolysed sera were less than that for the reagent blank. To investigate this, 10 serum samples with total alkaline phosphatase activity ranging from 40 to 325 U/l were supplemented with 300 mg/l of haemoglobin (visual haemolysis). The total alkaline phosphatase activity of each sample uniformly fell by about 8 U/l in the presence of haemoglobin. An identical fall was evident when L-phenylalanine was used to inhibit intestinal alkaline phosphatase in these specimens, although the

impact on calculated intestinal activity was small. With levamisole, however, the amount of haemoglobin added was sufficient to render the measured residual activity unreliable, values often being less than that of the reagent blank.

DETECTION LIMIT

After levamisole inhibition residual serum alkaline phosphatase activity is often less than 20 U/l, even when total activity is high. Depending on the analytical performance of the available equipment, it may be desirable to increase the reaction rate by adjusting sample volume. Using 15 sera with total activity ranging from 75 to 820 U/l, the algorithm produced similar results for sample volumes of 5, 10, and 25 µl. Thus sample volume can be increased without affecting the calculation of intestinal alkaline phosphatase activity.

## Discussion

Increased intestinal alkaline phosphatase activity has been reported in certain diseases of the digestive tract,[5] cirrhosis of the liver,[6] and in patients receiving maintenance haemodialysis.[7] Another study found that a raised serum intestinal alkaline phosphatase activity in a jaundiced patient suggested an intrahepatic cause.[8] It has also been reported that decreased amniotic fluid intestinal alkaline phosphatase activity may be indicative of cystic fibrosis.[9]

The contribution to serum total alkaline phosphatase activity by the intestinal isoenzyme is relatively small, even when the enzyme is pathologically increased. It is therefore important that such values are estimated with reasonable accuracy and precision. Use of L-phenylalanine as previously described[2] resulted in poor duplicate results, and a high incidence of negative values for intestinal alkaline phosphatase activity when total activity was high. The inhibitor levamisole, however, permitted a more direct measurement of intestinal alkaline phosphatase activity and produced positive values with acceptable precision. Levamisole therefore has advantages as an inhibitor for determining intestinal alkaline phosphatase activity in non-haemolysed serum, and we conclude that this is a useful reagent for the estimation of intestinal alkaline phosphatase activity.

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Requests for reprints to: Dr GH White, Department of Biochemistry and Chemical Pathology, Flinders Medical Centre, Bedford Park, South Australia, 5042, Australia.