Journal of Chromatography, 163 (1979)19—28
Biomedical Applications

CHROMBIO. 311

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR ACID AND ALKALINE PHOSPHATASE IN SERUM

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(Received October 23rd, 1978)

SUMMARY

High-performance liquid chromatography was used to assay serum acid and alkaline phosphatase. Samples were incubated with adenosine-5'-monophosphoric acid (AMP) in a buffer of required pH, 5'-nucleotidase was inhibited with Ni^{2+} ions, and the phosphatase activity was determined by measuring the concentration of the reaction product, adenosine. The analysis time, after the incubation is terminated, is short (7 min), and the assay is quantitative and reproducible. Complete separation of the reaction product from the substrate and the naturally occurring serum constituents and the high sensitivity of the ultraviolet detection system eliminate some of the problems commonly encountered in spectrophotometric assays.

INTRODUCTION

Enzymatic assays play a prominent role in medicine and they have been recognized as routine tests to assist in interpreting pathological conditions [1, 2]. Serum enzymes have been increasingly used for these purposes in recent years and there is a great demand for simple assay for measuring enzyme activities accurately and rapidly in the clinical laboratory. This is particularly important since certain enzymes are used as early indicators of neoplasia and as management aids in following the progression of a disease.

Phosphatases are organ-specific enzymes which catalyze the hydrolytic cleavage to their pH optima. Alkaline phosphatase (EC, 3.1.3.1, orthophosphoric acid monoester phosphohydrolase, AP), with optimal activity at a pH of about 10, occurs in practically all tissues of the body, especially in the cell membranes, intestinal epithelium, kidney tubules, osteoblasts, liver and placenta [3].

Determination of serum alkaline phosphatase activity is of interest in several disease states. Elevated levels of alkaline phosphatase have been observed in patients with osteogenic sarcoma [4], parathyroid adenoma or carcinoma [5] and cancer metastatic to the bone [6]. Abnormal levels of alkaline phosphatase have also been found in serum of patients with viral hepatitis or cirrhosis [7] and in diseases which do not involve the liver or bone such as stage I or II Hodgkin's disease [8], myeloid metaplasia [9], congestive heart failure [9] and intra-abdominal infections [9].

Acid phosphatase (EC 3.1.3.2, orthophosphoric acid monoester phosphohydrolase, AP) includes a group of similar or related enzymes with optimal activity below a pH of 7.0.

Serum acid phosphatase elevations have been observed in a variety of diseases, such as carcinoma of the prostate [10], Gaucher's disease [11] and breast carcinoma [12]. Because of the great clinical significance and potential use of serum acid and alkaline phosphatase assays, chemists are constantly searching for better, more rapid methods for the determination of the activity of these enzymes.

Because of the presence of 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5., 5′-N) in serum, its activity must be inhibited prior to the assay for acid and alkaline phosphatase. This can be achieved by selective inhibition with Ni^{2+} ions [13]. Methods generally used for assaying phosphatases monitor as an index of activity the amount of phosphate liberated from a substrate or the absorbance of 4-nitrophenol liberated in alkaline solutions at a wavelength of 400—420 nm [14, 15]. We investigated the use of high-performance liquid chromatography (HPLC) for determining concentrations of alkaline and acid phosphatase in biological samples because the method is more selective than spectral techniques and quicker and easier to carry out than radiochemical assays.

EXPERIMENTAL

Reagents

Adenosine-5′-monophosphoric acid (AMP), adenosine, alkaline phosphatase (EC 3.1.3.1, Type I), acid phosphatase (EC 3.1.3.2, Type I), 5′-nucleotidase (EC 3.1.3.5, Grade V), and tris(hydroxymethyl)aminomethane (Trizma base) were purchased from Sigma (St. Louis, Mo., U.S.A.); reagent-grade sodium citrate and diethanolamine were from J.T. Baker (Phillipsburg, N.J., U.S.A.); methanol distilled in glass was from Burdick and Jackson (Muskegon, Mich., U.S.A.); and potassium dihydrogen phosphate was from Mallinckrodt (St. Louis, Mo., U.S.A.). Water used for the preparation of eluents and standard solutions was distilled—deionized and filtered through Millipore membrane filters, pore size 0.45 μm (Millipore, Bedford, Mass., U.S.A.).

Instrumentation

A Waters Assoc. (Milford, Mass., U.S.A.) Model 6000 liquid chromatograph, equipped with solvent delivery system and a Model U6K universal injector were used in all determinations. An SF 770 Spectroflow monitor with a deuterium lamp, SF 339 wavelength drive and MM 700 memory module (Kratos,

Schoeffel Instrument Division, Westwood, N.J., U.S.A.) were used for the detection and stopped-flow ultraviolet (UV) scanning. A prepacked RP 8 Knauer column, particle size 7.0 μm (4.6 mm I.D. × 10.0 mm O.D., 250 mm long), was obtained from Unimetrics (Anaheim, Calif., U.S.A.).

Separation conditions

Samples were analyzed using an isocratic elution mode of reversed-phase partition HPLC. The eluent was anhydrous methanol—0.02 *M* KH_2PO_4 (pH 5.5) (1:9, v/v). Solvents were filtered through Millipore membrane filters, Type HA, pore size 0.45 μm, and degassed under vacuum. The flow-rate was 1.5 ml/min, and the temperature was ambient for all determinations.

Sample preparation

Serum samples were obtained from freshly drawn blood collected in tubes without anticoagulant. The blood was allowed to clot spontaneously for 10—15 min at room temperature. The supernatant fluid was then spun down at 630 *g* for 10 min, the liquid withdrawn and filtered through Millipore membrane filters (pore size 0.22 μm).

Enzyme assays

For the assay of serum acid and alkaline phosphatase, 0.1 ml of a 0.1 *M* solution of nickel chloride was added to 0.3 ml of serum to inhibit the activity of 5′-nucleotidase, and the mixture was incubated for 5 min at 25°. Then 0.8 ml of either 0.1 *M* diethanolamine buffer (pH 9.8), or sodium citrate buffer (pH 4.8) was added. The mixture was then incubated with 1.0 ml of 0.1 *M* AMP solution for 25 min at 25°. The reaction was stopped by heating the sample in boiling water until the protein coagulated. Samples were then spun down and the supernatant liquid was withdrawn and filtered through a Millipore filter, pore size 0.22 μm. The pH of the incubated samples was adjusted to 7.0 with solid Trizma base prior to chromatography.

Peak identification and confirmation of purity

In order to confirm the identity and the purity of the adenosine peak in incubated samples, UV spectra of the peaks were obtained using a stopped-flow UV scanning technique [16]. These spectra were scanned from 220 to 300 nm. To correct for the spectral background arising from changing optical properties of flow-cells and the monochromator, a blank spectrum was scanned over the same wavelength range and stored in the memory unit. This background was later subtracted from the scan of the compound under investigation. Next, the samples were chromatographed, the flow stopped at the top of the peak with retention time of adenosine, and the corrected spectrum scanned. These spectra were compared with the spectrum of standard adenosine. Good agreement between the spectra was taken as an indication of the purity of the peak.

RESULTS AND DISCUSSION

Because of the presence of adenosine deaminase in serum, adenosine is usually not found in detectable quantities. In order to ensure this, a blank serum sample was always chromatographed prior to the enzymatic assay. For the analysis of adenosine, a rapid, reversed-phase HPLC analysis developed by Hartwick and Brown [17] was used. This isocratic elution mode provides a fast separation (6—7 min) of adenosine from all the naturally occurring serum constituents.

Because of the presence of adenosine deaminase in serum, the adenosine which is produced from the AMP may be converted to inosine. Thus the levels of adenosine may be decreased by catalysis by adenosine deaminase. Therefore, experiments were conducted to determine the activity of adenosine deaminase at the two pH levels used in the assay of acid and alkaline phosphatase. Due to the low activity of adenosine deaminase in serum at pH 4.8 and 9.8 and the unfavorable kinetics at low concentrations of adenosine, it was found that the levels of inosine formed were insignificant. Therefore, no corrections for the adenosine deaminase activity were necessary.

Because of the presence of 5′-N in serum, an enzyme which specifically catalyzes the dephosphorylation of nucleotides having phosphate groups attached to the C_5 position on the ribose ring, Ni^{2+} ions were added to inhibit activity of 5′-N. Prior to the assay of acid and alkaline phosphatase, experiments were carried out to determine whether the presence of Ni^{2+} ions would have any effect on the activities. Therefore, solutions of 5′-N and acid and alkaline phosphatase were incubated with AMP in the absence and presence of Ni^{2+}, under the conditions of the assay. Incubation of 5′-N with AMP at a pH of 4.8 (sodium citrate buffer) in the absence (Fig. 1a) and in the presence of Ni^{2+} (Fig. 1b) indicated that this enzyme is completely inhibited in the presence of Ni^{2+}. At the same time, acid phosphatase assays in the absence (Fig. 1c) and in the presence of Ni^{2+} (Fig. 1d) showed no loss in the acid phosphatase activity in the presence of Ni^{2+} inhibitor.

The influence of Ni^{2+} ions on the enzymes under study was also tested at a pH of 9.8 (diethanolamine buffer). Results showed that 5′-N is completely inhibited by Ni^{2+} at pH 9.8. It should be noted that whereas the order of addition of reagents in the assay is not critical at pH 4.8, under alkaline conditions Ni^{2+} must be added to serum before the addition of diethanolamine buffer (pH 9.8), otherwise the Ni^{2+} ions would be removed in the form $Ni(OH)_2$. Under the same conditions at pH 9.8, incubation of alkaline phosphatase with AMP in the absence and presence of Ni^{2+} indicated no loss in the activity of this enzyme upon addition of the inhibitor of 5′-N.

To ensure that Ni^{2+} ions would have the same effect on serum enzymes, experiments were also conducted on serum samples. In testing the procedure, first a blank serum sample (no AMP added) was incubated for 25 min at 25°, the protein precipitated by heating, and the filtered sample chromatographed under the standard conditions (Fig. 2a). This procedure also indicated whether the sample itself contained any adenosine or any other compound with a similar retention time. Another blank mixture containing AMP and distilled water instead of serum was incubated under the same conditions. Fig. 2b

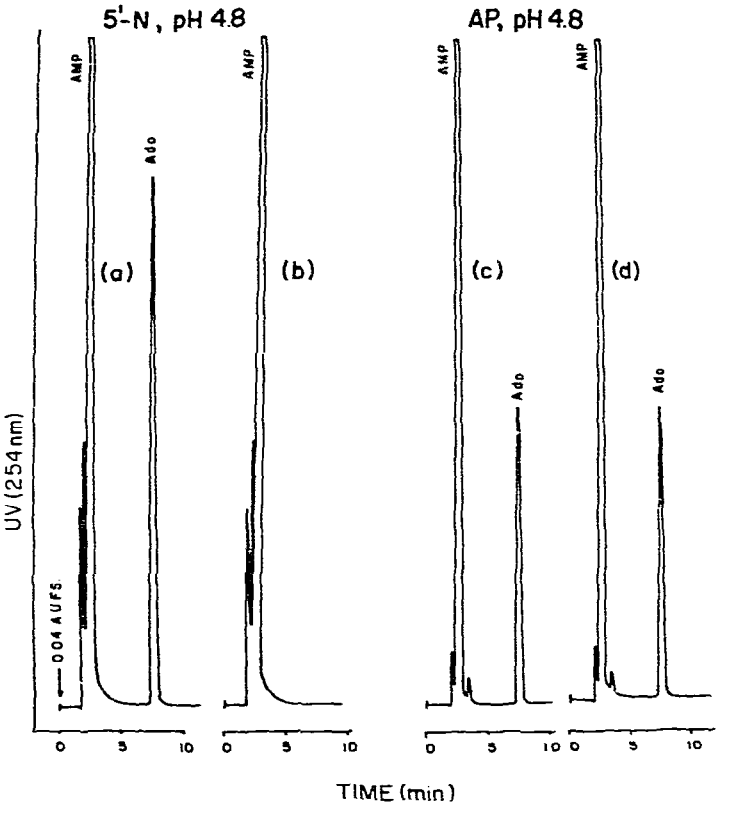

Fig. 1. Chromatograms of a mixture of AMP buffered to pH 4.8 and incubated (a) with 5′-N in the absence of Ni^{2+}, (b) in the presence of Ni^{2+}, (c) with AP in the absence of Ni^{2+}, and (d) in the presence of Ni^{2+}. Chromatographic conditions: mobile phase, anhydrous methanol—0.02 *M* KH_2PO_4 (pH 5.5); flow-rate, 1.5 ml/min; temperature, ambient. Sample volume, 75 µl.

shows a chromatogram of the mixture of AMP and a diethanolamine buffer (pH 9.8). The concentration of adenosine resulting from the hydrolysis of AMP under the alkaline conditions is negligible compared to the adenosine liberated from the alkaline phosphatase cleavage. Fig. 2c and d show the chromatograms of serum samples incubated with AMP in the absence and presence of Ni^{2+}, respectively. Little or no difference in the adenosine content of the two samples reflects the lack of 5′-N activity.

To confirm the spectral purity of adenosine resulting from the enzymatic cleavage of AMP, UV spectra of the adenosine peak in the incubated samples were obtained using the stopped-flow UV scanning technique. Fig. 3 shows a comparison of a UV spectrum of adenosine standard with that of the adenosine peak in serum sample incubated with AMP. Close agreement between the spectra confirms the identity and the purity of the peak in serum.

For the determination of serum acid and alkaline phosphatase, sera from seven healthy, normal subjects were incubated with AMP in buffer solutions of pH 4.8 and 9.8, respectively. Ni^{2+} ions were added to ensure the inhibition of the 5′-N activity. The activities were calculated using the formula

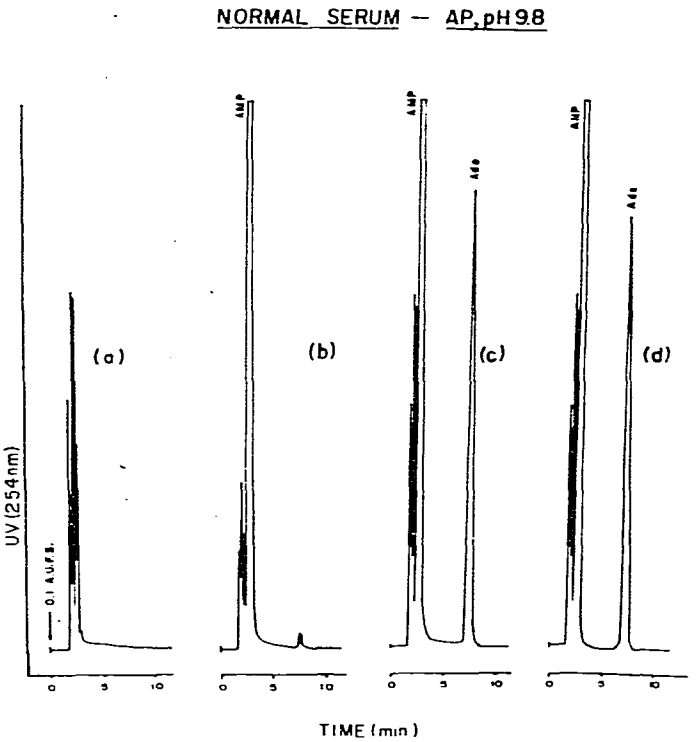

Fig. 2. Chromatograms of (a) normal serum sample blank, (b) AMP solution buffered to pH 9.8 and incubated for 25 min at 25° with AMP, (c) normal serum sample buffered to pH 9.8 and incubated with AMP in the absence of Ni^{2+} and (d) normal serum sample buffered to pH 9.8 and incubated with AMP in the presence of Ni^{2+}. Chromatographic conditions are the same as in Fig. 1; sample volume 25 μl in each case.

$$\text{activity (U/l)} = \frac{(\text{Ado}_{(s)} - \text{Ado}_{(b)}) \times \text{sample volume (ml)} \times \text{volume injected } (\mu\text{l})}{\text{serum volume (ml)} \times \text{response factor}_{(\text{Ado})}}$$

where $\text{Ado}_{(s)}$ and $\text{Ado}_{(b)}$ are the areas of adenosine peaks in the incubated serum sample and the blank, respectively, the blank containing AMP, buffer and water instead of serum; the response factor is area per μg of adenosine standard. The sample volume is the total volume of the incubation mixture, and serum volume is the volume of serum incubated.

Using the described procedure, the average of the seven normal sera gave consistent results of 5.11 ± 0.202 U/l for the acid phosphatase, and 150.16 ± 1.50 U/l for the alkaline phosphatase (Table I). The values compare well to the normal values obtained by other methods [18, 19]. Fig. 4 is a comparison of the chromatograms of three serum samples incubated with AMP in the presence of Ni^{2+} at pH 4.8; (a) is a chromatogram of a normal serum sample, (b) is of a serum sample from a patient suffering from cirrhosis, and (c) is of a serum sample from a patient with hepatitis. As can be seen from Fig. 4, there are small but definite differences in the activity of acid phosphatase in the three serum samples. Larger differences were noticed in the alkaline phosphatase activity in the same three samples. Fig. 5 shows the chromatograms

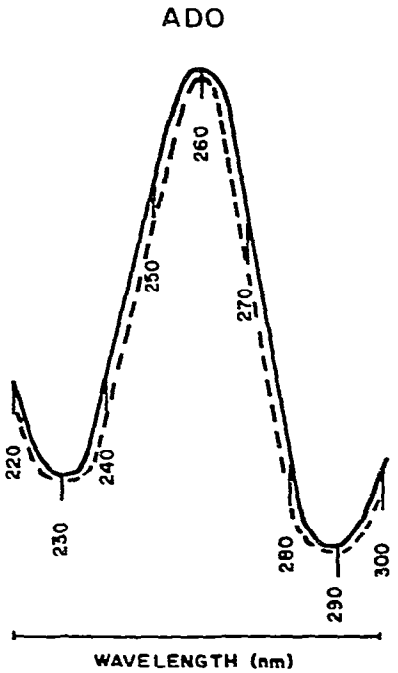

Fig. 3. Comparison of the corrected UV spectra of (——) adenosine standard and (— — —) adenosine peak in serum sample incubated with AMP, obtained using the stopped-flow UV scanning technique. Scanning rate, 100 nm/min; UV range, 0.1 a.u.f.s.

TABLE I

SERUM ACID AND ALKALINE PHOSPHATASE ACTIVITIES OF SEVEN HEALTHY SUBJECTS

Sample No.	Acid phosphatase activity (U/l)	Alkaline phosphatase activity (U/l)
1	4.92	150.1
2	5.01	151.5
3	5.20	148.9
4	5.02	150.5
5	5.20	148.6
6	5.19	151.2
7	5.20	150.3
Mean ± S.D.	5.11 ± 0.102	150.16 ± 1.50

of the three serum samples at pH 9.8, indicating alkaline phosphatase activity an order of magnitude higher in the serum from patients suffering from cirrhosis and hepatitis compared to the normal serum.

CONCLUSIONS

Results presented in this paper illustrate the utility of HPLC as a tool for the study of enzyme-catalyzed reactions. This assay has several advantages: first,

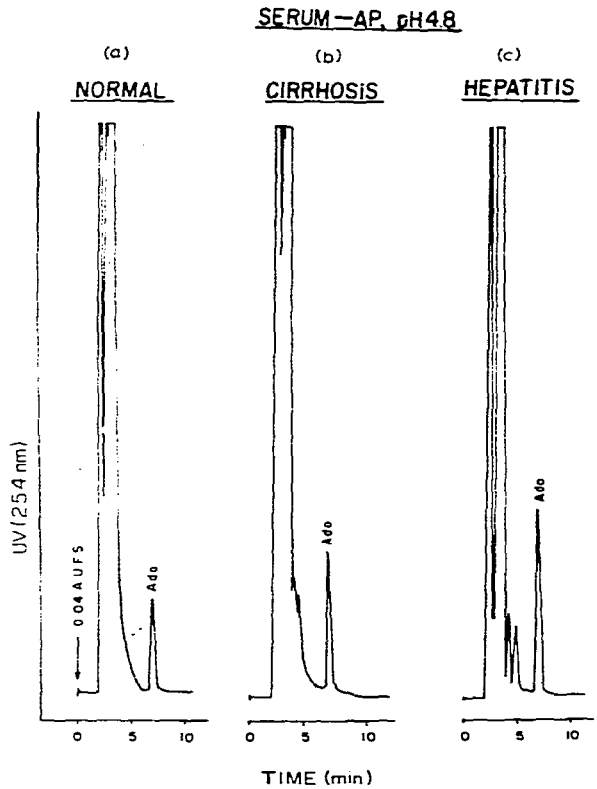

Fig. 4. Comparison of acid phosphatase activity in (a) normal serum, (b) serum sample from a patient with cirrhosis, and (c) serum sample from a patient with hepatitis. All three samples were incubated with AMP in the presence of Ni^{2+}. Chromatographic conditions are the same as in Fig. 1; sample volume, 75 μl in each case.

it is sufficiently sensitive to detect adenosine at the picomole level; secondly, the results are available within 6—7 min after the incubation and protein precipitation steps; thirdly, the reaction product, adenosine, is completely separated from the substrate or UV-absorbing serum constituents, thereby eliminating interferences often encountered in spectrophotometric or any other assays.

ACKNOWLEDGEMENTS

The authors thank Waters Associates Inc., and Kratos Inc., Schoeffel Instrument Division, for the use of their instruments, Unimetrics Corporation for their RP 8 column, Klaus Lohse for his valuable comments and Marion Barry for the preparation of the manuscript. This work was supported by Grant No. RO1 CA17603 from the National Cancer Institute of the United States Public Health Service.

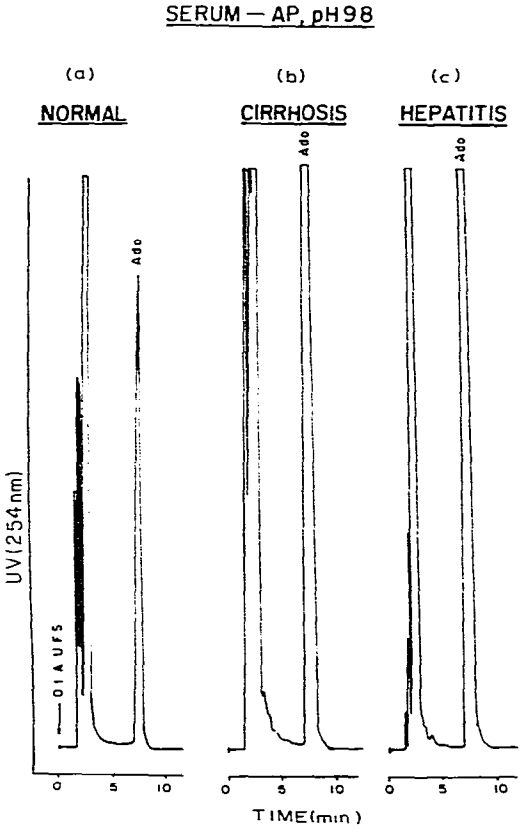

Fig. 5. Comparison of alkaline phosphatase activity in (a) normal serum sample, (b) serum sample from a patient with cirrhosis, and (c) serum sample from a patient with hepatitis. All samples were incubated with AMP in the presence of Ni^{2+}. Chromatographic conditions are the same as in Fig. 1; sample volume, 25 μl in each case.

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