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Note

High-performance liquid chromatographic determination of acid and alkaline phosphatases in urine

HIROYOSHI MATSUKURA*, YOSHIFUMI SUZUKI and TOSHIO OKADA

Department of Pediatrics, Faculty of Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-01 (Japan)

and

SABURO NAIKI, SHIRO HAYASHI and YUKO YAMAJI

Department of Central Laboratory, Faculty of Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-01 (Japan)

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The measurement of urinary enzymes can be used as a possible index of renal injury. In the last decade, enzymes (alkaline phosphatase, aspartate aminotransferase, γ-glutamyltransferase, N-acetyl-β-glucosaminidase, and lactate dehydrogenase) have been most extensively studied [1–3]. These enzymes are primarily located within the renal tubular cells and therefore an increase in excretion of urinary enzymes may be related to tubular injury. Acid phosphatases are a group of enzymes capable of hydrolysing phosphate esters in acid environment. They are found in large amounts in human kidney, prostate, semen and erythrocytes, and are present throughout the nephron [4,5]. Since the highest activity of these enzymes within the human kidney is found in glomeruli [4,6–8], glomerular damage may cause considerable excretion of the enzymes into urine [9]. The determination of urinary acid phosphatase, together with brush border enzymes in urine, seems to be a more sensitive marker of renal disease. In previous papers, we described an automated system for the separation of urinary enzymes using ion-exchange chromatography [10]. This chromatographic method succeeded in separating urinary isoenzymes without preconcentration of urine specimens. The present investigation was undertaken to see if it is possible to use ion-exchange chromatography for the simultaneous assay of acid phosphatase (ACP; orthophosphoric monoester phosphohydrolase, EC 3.1.3.2.) and alkaline phosphatase (AP; orthophosphoric monoester phosphohydrolase, EC 3.1.3.1.).

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EXPERIMENTAL

Subjects

We studied 60 patients (age range 2–20 years). Of these, 33 patients had IgA nephropathy and 27 patients had nephrotic syndrome. Control urine samples were obtained from 51 healthy people (age range 2–24 years), who had no history or evidence of urinary tract infection or pre-existing renal disease. A normal urinalysis was a prerequisite. As male subjects after puberty excrete large amounts of prostatic ACP into urine, all male subjects over 13 years were excluded from the study.

Random urine samples of 10 ml were centrifuged at 3000 *g* for 5 min. A fraction of the supernatant (2.5 ml) was filtered through a PD-10 column (Pharmacia, Uppsala, Sweden) with 3.5 ml of 10 m*M* Bis-Tris propane buffer (pH 7.4).

Apparatus

A fast protein liquid chromatography (FPLC) system from Pharmacia was used throughout this study. The apparatus consisted of two P-500 high-pressure pumps, an LCC-500 gradient controller and an MV-7 sample injector. A Pharmacia Mono Q (HR5/5, 50×5 mm I.D.) anion-exchange column was equilibrated with buffer 1 (10 m*M* Bis-Tris propane, pH 7.4), and the sample (500 µl total volume) was loaded onto the column at a flow-rate of 1.5 ml/min. The column was then washed with 3 ml of buffer 1 at the same flow-rate, followed by a 30-ml increasing linear gradient of sodium chloride (0–0.35 *M*) in 10 m*M* Bis-Tris propane (pH 7.4) at room temperature. All buffers were filtered through a Advantec 0.2-µl filter unit (Toyokagaku, Tokyo, Japan) and degassed prior to use. The continuous column effluent was then assayed for enzyme activity using a Technicon® Autoanalyzer™ II system (Technicon Instruments, Tarrytown, NY, U.S.A.). A heating-bath II-G coil (37°C) was used for the incubation

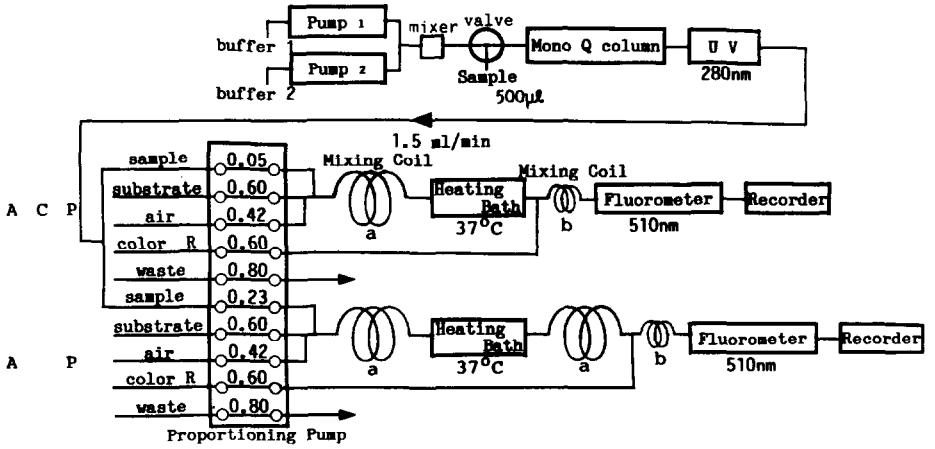

Fig. 1. Flow diagram of the FPLC system: a = mixing coil, 40 turns P/N 190-0051-10; b = manifold coil assembly, 20 turns P/N 157-B095-01. The numbers of proportioning pump represent the flow-rates of pump tubes in ml/min. ACP = acid phosphatase; AP = alkaline phosphatase.

(Technicon). The flow diagram is shown in Fig. 1. Peak areas were calculated and integrated by a Model X-Plan 360 area curve meter (Ushikata, Tokyo, Japan).

Enzyme assays

ACP was determined by the method of Gutman and Gutman [11], except that the colour reagent contained 357 m*M* sodium bicarbonate, 162 m*M* boric acid, 150 m*M* sodium hydroxide, and 11 m*M* potassium ferricyanide. Production of red quinone was monitored at 510 nm with an Autoanalyzer II single-channel colorimeter. AP was measured according to a modified method of Kind and King [12]. The substrate solution was prepared in 60 m*M* carbonic acid buffer containing 10 m*M* disodium phenylphosphate, 5 m*M* 4-aminoantipyrine, 5 m*M* magnesium chloride and 1 ml/l Brij-35, adjusted to pH 10.0 at 37°C. Daitest ALP colour reagent was used unless stated otherwise. Production of red quinone was monitored at 510 nm with the Autoanalyzer II single-channel colorimeter. The sample blanks of both phosphates were measured, as disodium phenylphosphate was excluded from the substrate solution. Enzyme activity was related to creatinine concentration to correct differences in urinary volume output and expressed as U/g creatinine (U/cr). Urinary creatinine concentration was determined with an RA-1000 (Technicon).

To make a comparison between an established method and our high-performance liquid chromatographic (HPLC) method, phosphatases were assayed using a Cica PHOS acid kit and a Daitest ALP kit with a Shimazu CL-720 spectrophotometric detector (Shimazu, Kyoto, Japan).

Reagents

Cica PHOS acid kit reagent was from Chugai (Tokyo, Japan). Daitest ALP kit reagents, substrate (Cat. No. 001883) and colour reagent (Cat. No. 001007) were obtained from Daiichi (Tokyo, Japan). AcP LIN-TROL® (Cat. No. A1538) and Bis-Tris propane were from Sigma (St. Louis, MO, U.S.A.). Brij-35 (30%) was from Technicon (Orcq-Tournai, Belgium). Other reagents used for the enzyme assay were of the highest commercially available purity. A bottle of AcP LIN-TROL dissolved in 2 ml of buffer 1 was used as a standard solution throughout the study.

RESULTS AND DISCUSSION

The standard plots of peak area, y, against enzyme activity, x, were linear from 0.94 to 30.0 I.U./l for ACP and from 0.29 to 9.24 I.U./l for AP. The correlation coefficient (r) was 0.999 in both studies, and the regression equations were as follows: $y=0.48x-0.17$ for ACP and $y=0.11x+0.16$ for AP. Within-day precision was determined from quadruplicate injections of two different standard solutions with low and high enzyme activities. Low standard solution with low enzyme activity and high standard solution with high enzyme activity were prepared by diluting 32-fold and 4-fold with buffer 1, respectively. Between-day precision was assayed by injections of low standard solution on fourteen consecutive working days. Results of these studies are summarized in Table I. Excellent pre-

TABLE I

PRECISION OF ASSAYS FOR ACID PHOSPHATASE (ACP) AND ALKALINE PHOSPHATASE (AP) ACTIVITIES

Enzyme	Activity (low) (mean ± S.D.) (I.U./l)	C.V. (%)	Activity (high) (mean ± S.D.) (I.U./l)	C.V. (%)
Within-day (n=4)				
ACP	1.06 ± 0.034	3.2	9.24 ± 0.087	1.0
AP	3.73 ± 0.065	1.7	29.7 ± 0.39	1.3
Between-day (n=28)				
ACP	1.00 ± 0.060	6.0		
AP	3.96 ± 0.22	5.6		

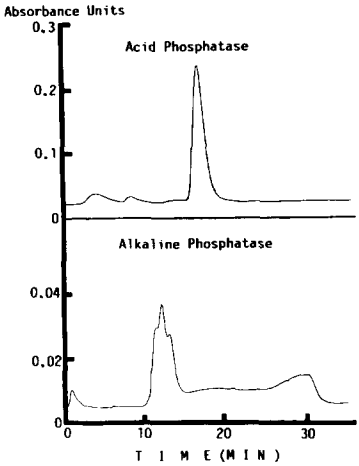

Fig. 2. Elution pattern of acid and alkaline phosphatases in urine from a healthy child.

cision was noted in both studies and the coefficients of variations (C.V.) were less then 6.0%. The lower detection limits of enzyme activity were 0.024 I.U./l for ACP and 0.008 I.U./l for AP.

The correlation between results of the manual method of ACP (x) and the HPLC assay (y) was determined with 28 urine samples containing ACP activity from 1.2 to 28.2 I.U./l: $y=0.99x+0.67$, $r=0.987$, $n=28$. To compare the HPLC assay (y) with the manual method of AP (x), we analysed 24 urine samples with AP activity from 0.1 to 5.9 I.U./l: $y=1.01x+0.13$, $r=0.991$, $n=24$.

Chromatographic profiles of urinary ACP and AP in a healthy child are shown in Fig. 2. Urinary enzymes of ACP and AP were separated into three components. Urinary ACP showed a large peak towards the anionic site compared with the peak of urinary AP. Enzyme activities (mean ± S.D.) of ACP and AP in urine from healthy children were 53.9 ± 22.1 U/cr and 3.5 ± 2.1 U/cr, respectively. Patients with IgA nephropathy and nephrotic syndrome had elevated enzyme activity of ACP (107.7 ± 97.2 and 146.7 ± 112.4 U/cr). Urinary AP activity in patients

with IgA nephropathy was 5.7±4.6 U/cr. Patients with nephrotic syndrome showed a significant increase in urinary AP activity (21.6±18.3 U/cr).

Increases in urinary ACP activity have been reported in various conditions, including renal tubular acidosis, pyelonephritis, nephrocalcinosis and hypokalemia [9]. Kramer and Gonick [9] reported that urinary excretion of ACP actually increased in active glomerulonephritis, but did not increase in inactive glomerulonephritis. If this is so, the determination of urinary ACP activity may be useful in following up patients with chronic glomerulonephritis. Urinary AP activity, as well as urinary ACP activity, increases in renal diseases [1,3]. More recently the determination of urinary AP activity was found to be valuable for early diagnosis of rejection episodes following renal transplantation [13]. Chromatograms of both phosphatases showed multiple peaks. Pla et al. [14] showed that extracts from rat kidney contained three components of ACP on electrophoresis. There was a single component of AP and occasionally other components were seen in normal urine [15]. Hodson and Latner [15] reported that some urine samples from patients with renal disease showed three components of AP. These results are in good agreement with ours.

Since the post-column reaction system is very sensitive, there is no need to concentrate urine samples. The post-column reaction system is directly connected to the HPLC system, and we can see chromatographic profiles of urinary enzymes in real time. Enzyme activities in urine from patients with renal disease show such a wide distribution that it is sometimes really difficult to distinguish them from normal controls by those activities. In such cases, patients with renal disease probably show a different elution pattern of urinary enzymes from that of normal controls.

In conclusion, the simultaneous determination of ACP and AP in urine using ion-exchange chromatography offers excellent precision, linear response and high sensitivity. The measurement of urinary enzymes in particular with a preferential location within the human kidney may be useful in diagnosing renal conditions.

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