Journal of Chromatography, 414 (1987) 47–54
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3434

AUTOMATED SEPARATION AND MEASUREMENT OF URINARY ISOENZYMES AND PROTEIN BY ION-EXCHANGE LIQUID CHROMATOGRAPHY

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

SABUROU NAIKI and NOBUO SAKURAGAWA

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

(First received May 28th, 1986; revised manuscript received October 6th, 1986)

SUMMARY

This paper deals with a totally automated detection system for the assay of urinary isoenzymes and protein using high-performance liquid chromatography with a continuous post-column detection system. We attempted to determine the distribution of three enzymes in urine samples from a healthy child and in tissue extracts of rabbits. Alkaline phosphatase, γ-glutamyltransferase and lactate dehydrogenase isoenzymes were each separated into six peaks. In comparison with the previous methods, this procedure provides better precision and accuracy, and it is sufficiently sensitive to allow the analysis without preconcentration of urine samples.

INTRODUCTION

During the course of an investigation of urinary enzymes, it became desirable to develop a simple method for separating and measuring the activity of urinary isoenzymes. The separation and identification of urinary isoenzymes will certainly become an important tool for diagnosis and follow-up, especially in renal disorders. As the activity of urinary enzymes is low and many physical inhibitors are present in urine, urine samples must be concentrated by dialysis against water or gel chromatography before the enzyme assay. After preconcentration, urinary isoenzymes were analysed by electrophoresis [1], gel permeation [2], ultracentrifugation [3] and immunological technique [4,5]. These methods were diffi-

0378-4347/87/$03.50 © 1987 Elsevier Science Publishers B.V.

cult to carry out and time-consuming. Because the activity of isoenzymes decreased at different rates, reliable results were difficult to obtain.

The purpose of the work reported here was to describe the separation of urinary isoenzymes and protein, and their determination. We present the chromatographic profiles of urinary protein and the three enzymes of alkaline phosphatase (AP; EC 3.1.3.1), γ-glutamlytransferase (γ-GT; EC 2.3.2.2) and lactate dehydrogenase (LD; EC 1.1.1.27).

EXPERIMENTAL

Apparatus

High-performance ion-exchange chromatography was performed with a fast protein liquid chromatography (FPLC) system from Pharmacia (Pharmacia Fine Chemicals, Uppsala, Sweden). The system consisted of a GP-250 gradient programmer, two P-500 reciprocating pumps, a V-7 injection valve with a 500-μl loop, an ACT-100 autosampler, a UV-1 monitor with a HR-10 flow cell, and an REC-482 chart recorder. The column used was a prepacked Mono Q HR 5/5 (50$\times$5 mm I.D.) with a bed volume of 1 ml (Pharmacia). The post-column enzyme detection system used was a Technicon® AutoAnalyzer™ II system (Technicon Instruments, Tarrytown, New York, NY, U.S.A.).

Chromatographic conditions

The salt gradient was made by mixing buffers A and B using two pumps controlled by a programmer. Buffer A was 10 m*M* Bis–Tris propane chloride (Sigma, St. Louis, MO, U.S.A.), pH 7.5. Buffer B was 10 m*M* Bis–Tris propane chloride containing 0.35 *M* sodium chloride, pH 9.5. All buffers were filtered through 0.2-μm membrane filters and degassed by a Model US-200S ultrasonic cleaning bath (Sakura Seiki, Tokyo, Japan). For all chromatographic analyses, the flow-rate was 1.5 ml/min, and the gradient was started automatically 3 min after sample injection. A 30-min linear gradient from 100% buffer A to 100% buffer B was used. The column was kept at room temperature.

Reagents

Daitest ALP kit reagent (substrate, Cat. No. 001883; colour reagent, Cat. No. 001007), 716 Daitest LDH-L kit reagent (substrate, Cat. No. 001307; coenzyme, Cat. No. 001314), magnesium chloride and disodium phenylphosphate were obtained from Daiichi (Tokyo, Japan). γ-GT new kit reagent (Cat. No. 125954) was from Boehringer Mannheim Japan. Benzethonium chloride hydrate, tetrasodium ethylenediaminetetraacetate tetrahydrate, L-phenylalanine and L-homoarginine were from Wako (Osaka, Japan). Brij-35 (30%) was from Technicon (Orcq-Tournai, Belgium). Other chemicals were of reagent grade.

Sample preparation

Serum samples were obtained from the clinical laboratory at our university hospital. A pooled serum sample and a fresh serum sample were prepared. The pooled serum sample was frozen after collection and stored at $-20\,^{\circ}$C until anal-

ysis. The fresh serum sample, with high enzyme activity from hospitalized patients, was stored at 4 °C and was analysed within 24 h after it was obtained. Both serum samples were diluted 40-fold with buffer A unless otherwise specified, filtered through 0.2-μm membrane filters and used as reference materials. We obtained serum sample with low enzyme activity by diluting the pooled serum sample and serum sample with high enzyme activity by diluting the fresh serum sample. Urine samples from healthy children in our Department of Pediatrics were obtained. Samples (10 ml) of urine were centrifuged at 3000 *g* for 5 min, 2.5 ml of the supernatant were filtered through the Sephadex G-25M column (Pharmacia) and 0.5 ml of the filtrate were injected onto the FPLC column.

Enzyme assays

The post-column detection system of AP activity was performed according to a modified method of Kind and King [6]. 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 of Brij-35, adjusted to pH 10.0 at 37°C. The colour reagent contained 10 m*M* potassium periodade and 13 g/l boric acid. Production of red quinone was monitored at 510 nm with an AutoAnalyzer II single-channel colorimeter (Technicon). A sample blank was measured as described above except that disodium phenylphosphate was excluded from the substrate solution. Sample blank measurement was found to be unnecessary in this test and blank measurements were not done in the following experiments unless otherwise specified.

The post-column detection of γ-GT activity was assayed by a colorimetric method [7] with the kit reagent unless otherwise specified. The kit reagent was prepared according to the manufacturer's instructions by adding 1 ml/l Brij-35 and adjusted to pH 8.25 at 37°C. The formation of 5-amino-2-nitrobenzoate was monitored at 410 nm with an AutoAnalyzer II single-channel colorimeter. The assay solution was stable for five days at 2–8°C and for two days at 15–25°C.

The post-column detection of LD activity was evaluated spectrophotometrically [8]. Activity was calculated from the rate of production of NADH at 340 nm with an AutoAnalyzer II single-channel colorimeter. LD activity was measured with the kit reagent unless otherwise specified. The kit reagent, prepared according to the manufacturer's instructions, contained 1 ml/l Brij-35, adjusted to pH 9.0 at 37°C. A sample blank was measured by substituting buffer (Daiichi) for NAD as a sample blank and was deducted from overall LD activity.

The protein concentration was measured by a modified benzethonium chloride method [9]. The assay solution contained the following: 800 m*M* NaOH, 24 m*M* Na_4-EDTA, 0.4 g/l benzethonium chloride and 1 ml/l Brij-35. After incubation at 37°C, the absorbance was measured at 580 nm with an AutoAnalyzer II single-channel colorimeter.

The temperature was controlled by means of a heating bath II-G coil 37°C (Technicon).

Total enzyme activity

Urine samples were injected directly into the post-column detection system and total enzyme activity was calculated from the peak heights using a 100-fold

diluted pooled serum sample with buffer A as reference materials. The total enzyme activity of the serum sample were determined by a Model 716 discrete analyzer (Hitachi, Tokyo, Japan).

Urinary protein concentration was measured according to the procedure described earlier [9].

Calculations

All chromatograms were copied, and the net areas for peaks were cut out and their weights determined using a Model ED-200-10 electronic reading balance (Shimazu, Kyoto, Japan).

Tissue preparations

Female New Zealand rabbits (2 kg of body weight) were killed and then the lungs, heart, liver, small intestine, kidneys, bladder and femoral muscle were rapidly removed. These organs were rinsed and homogenized in three volumes of buffer A. Homogenates were centrifuged for 10 min at 13 000 *g*. The decanted supernatants were filtered through 0.2-μm membrane filters and injected onto the column.

Effect of L-phenylalanine and L-homoarginine

Inhibition studies were performed using the method described elsewhere [10,11]. Both L-phenylalanine and L-homoarginine concentrations in the buffered substrate were 10 m*M*.

System for chromatographic resolution by FPLC

The coupling of an FPLC column with an automated post-column detection system is shown in Fig. 1. The flow-rate was 1.5 ml/min. The STD cal dial of an AutoAnalyzer II single-channel colorimeter was fixed at 500, and the output signal was conducted through connector pin of Telemetry 5V to a Model U-228-2P-00 Unicorder (Nippon Densi Kagaku, Tokyo, Japan). The DC voltage range unit of the recorder was set at 2 V. The optical path length was set at 50 mm. The column effluent was monitored for enzyme activity and protein concentration by the continuous-flow analyser. The assay reagents were continuously added to the column effluent and the combined solutions passed through the mixing coils, where the reaction occurred.

RESULTS AND DISCUSSION

Dynamic range

The dynamic range for the measurement of enzyme activity and protein concentration was determined by injecting a processed pooled serum sample. The configuration is shown in Fig. 1 without the column. The enzyme activities of these solutions ranged from 0.011 to 2.9 I.U./l for AP, from 0.0076 to 2.0 I.U./l for γ-GT and from 0.022 to 5.6 I.U./l for LD. Protein concentration ranged from 0.016 to 4.1 g/l.

The linear dynamic ranges of the system were 0.011–2.9 I.U./l for AP, 0.0076–2.0

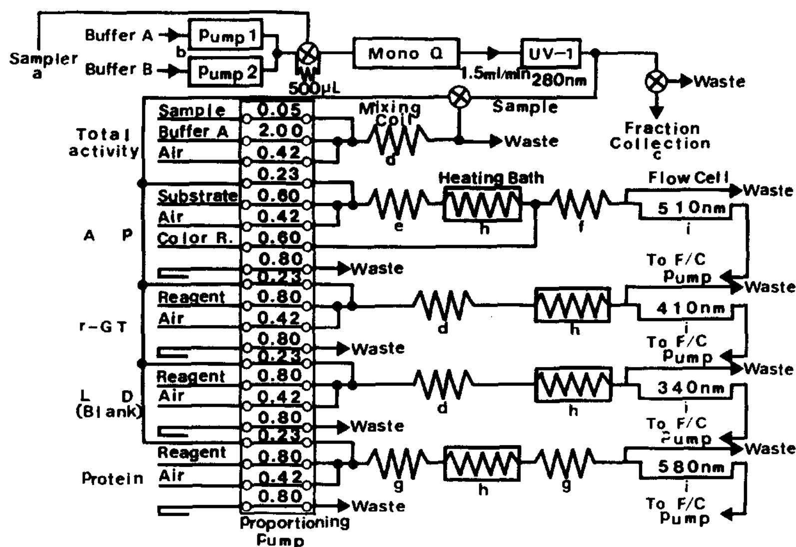

Fig. 1. Flow diagram. a = ACT-100 autosampler; b = P-500 reciprocating pump; c = FRAC-100 fraction collector (Pharmacia); d = mixing coil, six turns P/N 190-0051-07; e = mixing coil, 28 turns P/N 190-0051-03; g = mixing coil, 40 turns P/N 190-0051-10; f = manifold coil assembly, 20 turns P/N 157-B095-01 (Technicon); h = heating bath II-G coil 37°C; i = flow cell, 50×1.5 mm I.D., P/N 199-B007-01 (Technicon). The number of proportioning pump shows the flow-rate of pump tubes in ml/min.

I.U./l for γ-GT, 0.022–5.6 I.U./l for LD and 0.016–0.13 g/l for protein. The correlations between the enzyme activity or protein concentration (x) and the peak height (y) can be expressed by the following equations: AP, $y=0.028x+5.2\cdot10^{-5}$ ($r=0.99$); γ-GT, $y=0.15x+2.2\cdot10^{-4}$ ($r=0.99$); LD, $y=0.68x+1.4\cdot10^{-1}$ ($r=0.99$); protein, $y=1.6x-5.0\cdot10^{-3}$ ($r=0.99$) ($n=26$).

Precision and analytical recovery

Within-day precision was evaluated from quadruplicate injections of processed serum samples with low and high enzyme activity. Pooled serum samples were diluted 20-fold with buffer A for enzyme assay and 100-fold with buffer A for protein determination. Between-day precision was evaluated from two injections of the diluted serum sample on fourteen consecutive working days. Results of these studies are shown in Table I. The coefficients of variation (C.V.) for the corresponding peak area were found to be 1.5–1.8% for AP, 2.2–11.4% for γ-GT, 2.3–4.9% for LD, 2.8–8.7% for protein (UV) and 6.1–6.8% for protein (BC).

Analytical recovery was evaluated by using a mixture of serum samples containing low or high enzyme activities. The serum sample with low enzyme activity (solution I) and the serum sample with high enzyme activity (solution II) were prepared as described above. A mixture of 8 ml of solution I and 2 ml of solution II (solution III) and another mixture of 2 ml of solution I and 8 ml of solution II (solution IV) were prepared. Both solutions were analysed in quadruplicate. The theoretical values of solution III and solution IV were calculated from the results

TABLE I

PRECISION AND ANALYTICAL RECOVERY

Enzyme/ protein	Within-day ($n=4$)				Between-day ($n=14$)		Recovery ($n=4$) (%)	
	Low enzyme activity (L)		High enzyme activity (H)		I.U. (mean ± S.D.)	C.V. (%)	L/H = 4:1	L/H = 1:4
	I.U. (mean ± S.D.)	C.V. (%)	I.U. (mean ± S.D.)	C.V. (%)				
AP	2.3 ± 0.034	1.5	2.7 ± 0.044	1.6	2.6 ± 0.048	1.8	100–103	99–101
γ-GT	1.9 ± 0.053	2.8	4.0 ± 0.086	2.2	3.5 ± 0.40	11.4	97–102	106–119
LD	4.3 ± 0.19	4.4	5.6 ± 0.13	2.3	4.9 ± 0.24	4.9	103–105	100–101
Protein (UV)	1.1 ± 0.031★	2.8	1.7 ± 0.079★	4.8	0.67 ± 0.058★	8.7	96–102	100–105
Protein (BC)	1.1 ± 0.68★	6.8	1.7 ± 0.071★	6.1	0.67 ± 0.041★	6.1	96–102	101–107

★Protein concentration in g/l.

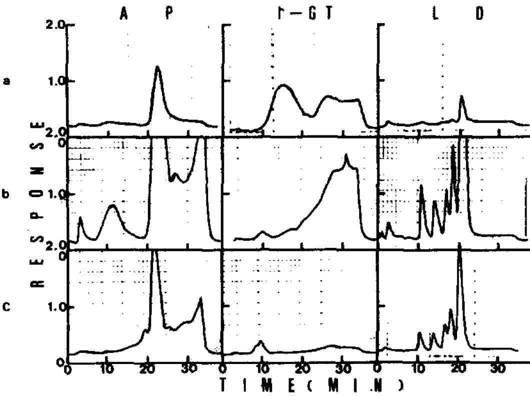

Fig. 2. Distribution of three enzymes in the New Zealand rabbit. (a) Urine; (b) kidney cortex; (c) kidney medulla. AP = alkaline phosphatase; γ-GT = γ-glutamyltransferase, LD = lactate dehydrogenase.

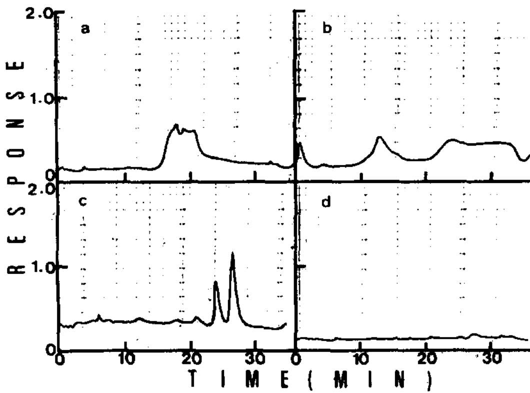

Fig. 3. Chromatograms of urine from a healthy child. (a) AP, (b) γ-GT, (c) LD, (d) protein.

of within-day precision studies. Results of these studies are summarized in Table I. The average recovery was found to be 100.5±1.3% for AP, 108.5±7.3% for γ-GT, 102.3±2.3% for LD, 101.1±2.8% for protein (UV) and 100.5±2.7% for protein (BC).

Detection limits

The sensitivity of the method was limited by the amount of baseline noise, which was usually attributed to the 10 mV of output voltage in an AutoAnalyzer II single-channel colorimeter. The lower detection limits of each enzyme and protein were found to be 0.003 I.U./l for AP, 0.007 I.U./l for γ-GT, 0.01 I.U./l for LD and 0.001 g/l of protein at a signal-to-noise ratio of 4.

Distribution of enzymes

The distribution of the three enzymes in urine and kidney extracts in the New Zealand rabbits is shown in Fig. 2. AP isoenzymes were separated into six peaks. Hodson et al. [1] detected up to five components of AP in an individual kidney and showed that the enzyme was unequally distributed between kidney cortex and medulla. This was in good agreement with our experiments on tissue extracts, and we found a characteristic peak in kidney cortex that was not found in medulla. γ-GT isoenzymes were separated into six peaks. Rambabu et al. [3] observed that the urinary γ-GT enzymes were separated into two fractions, which they found not to be of clinical significance. Multiple LD-3 peaks have also been observed previously [12–14]. LD isoenzymes exhibited similar chromatographic profiles except for those of the heart and muscle.

Inhibition studies on L-phenylalanine and L-homoarginine were performed to confirm the source of alkaline phosphatase. AP activity in urine, liver, intestine, kidney cortex and medulla were inhibited to the extent of 97, 47, 18, 41 and 52% by L-phenylalanine, and 95, 5, 1, 19 and 51% by L-homoarginine, respectively.

Urinary AP was completely inhibited by either L-phenylalanine or L-homoarginine. In an earlier paper [10,11], it was shown that intestinal AP was more susceptible to L-phenylalanine but resistant to the effect of L-homoarginine, and liver AP was contrary to intestinal AP. In this study, intestinal AP was insignificantly inhibited by L-phenylalanine, however, liver AP was distinctly inhibited by L-phenylalanine. It was not well known that these results were in poor agreement with those in the literature [10,11].

Chromatographic profiles of urine samples from a healthy child are shown in Fig. 3. These demonstrate that the separations of urinary isoenzymes in normal urine are also possible by the new procedure.

CONCLUSIONS

The described procedure circumvents troublesome sample treatments described previously and enables simultaneous separations of multiple isoenzymes and protein in urine. In future work, we will investigate the relationship between the various forms of urinary isoenzymes and renal disorders.

ACKNOWLEDGEMENTS

The authors thank Prof. T. Tanimura for help with the preparation of this manuscript and Mr. Anahara (Pharmacia) and Mr. Takao (Technicon) for their technical assistance.

REFERENCES

1 A.W. Hodson and A.L. Latner, Clin. Chim. Acta, 61 (1975) 53.
2 M. Werner, D. Maruhn and M. Atoba, J. Chromatogr., 40 (1969) 254.
3 K. Rambabu, R.T. Jacob and T.N. Pattabiraman, Clin. Chim. Acta, 109 (1981) 257.
4 A. Cornell, A.W. Hodson and A.L. Latner, Clin. Chim. Acta, 97 (1979) 217.
5 G. Pfleiderer, M. Baier, A.W. Mondorf, T. Stefanescu, J.E. Scherberich and H. Muller, Kidney Int., 17 (1980) 242.
6 P.R.N. Kind and E.J. King, Clin. Pathol., 7 (1954) 322.
7 J.P. Persijin and W. van der Slik, J. Clin. Chem. Clin. Biochem., 14 (1976) 421.
8 E. Amador, L.E. Dorfman and W.E.C. Wacker, Clin. Chem., 9 (1963) 391.
9 J. Iwata and O. Nishikaze, Clin. Chem., 25 (1979) 1317.
10 W.H. Fishman, S. Green and N.I. Inglis, Nature, 198 (1963) 685.
11 W.H. Fishman and H.G. Sie, Clin. Chim. Acta, 29 (1970) 339.
12 P.J. Kudirka, R.R. Schroeder, T.E. Hewitt and E.C. Toren, Jr., Clin. Chem., 22 (1976) 471.
13 T.D. Schlabach, S.H. Chang, K.M. Gooding and F.E. Regnier, J. Chromatogr., 134 (1977) 91.
14 J.A. Fulton, T.D. Schlabach, J.E. Kerl and E.C. Toren, Jr., J. Chromatogr., 175 (1979) 283.