Journal of Chromatography, 383 (1986) 229—231
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3318

Letter to the Editor

Assay of enzyme activities and low-molecular-mass constituents in serum using stopped-flow technique coupled with gel-permeation chromatography

Sir,

Determinations of enzyme activities and of low-molecular-mass constituents in serum are important for diagnoses of certain diseases. Enzymatic techniques are useful for clinical assays because of their high specificities. In the assay of serum enzymes by enzymatic techniques, however, endogeneous serum constituents may interfere with the enzyme reactions by acting as enzyme inhibitors or activators. In addition, the enzymatic reactions sometimes occur naturally in serum. Thus the removal of low-molecular-mass compounds from serum is necessary for the reliable estimation of serum enzymes. Similarly, in the assay of low-molecular-mass compounds in blood, macromolecules such as proteins, polynucleotides and polysaccharides often interfere and their removal is often an essential part of the pretreatment of biological samples.

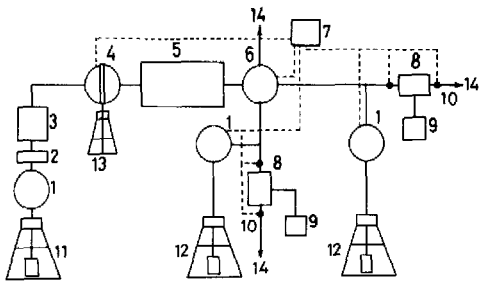

Fig. 1. Schematic diagram of the apparatus: 1 = pump; 2 = filter; 3 = damper; 4 = automatic sample injector; 5 = GPC column; 6 = automatic multiway valve; 7 = microprocessor; 8 = UV detector; 9 = recorder; 10 = electromagnetic valve; 11 = buffer solution; 12 = reaction solution; 13 = sample solution; 14 = waste.

To solve this problem, we carried out the assays for enzymes and low-molecular-mass serum constituents by the stopped-flow technique coupled with gel-permeation chromatography (GPC) (Fig. 1). In this technique, a small amount (normally 10 μl) of biological sample was first injected into a simple GPC apparatus (Milton-Roy SF-0396 pump and hydrophilic polymer gel column, 50 × 4 mm I.D., Micropearl SFW-214 from Sekisui, Osaka, Japan)

0378-4347/86/$03.50

using an automatic sample injector (EA-25, Kyoto Chromato, Kyoto, Japan). Phosphate buffer (0.03 *M*, pH 8.0) was used as mobile phase at a flow-rate of 0.5 ml/min. Compounds of high molecular mass and those of low molecular mass were roughly separated and directed into different flow-lines via an automatic multiway valve (MV-1, Kyoto Chromato) controlled by a computer (PC 2001, NEC, Tokyo, Japan). The elution times of the compounds under study (enzymes and low-molecular-mass constituents) were stored beforehand in the computer memory. Both fractions were mixed with the reaction solutions (flow-rate 0.2 ml/min) before the UV monitor (S-310A II, Soma Optics, Tokyo, Japan), and when the objective molecules arrived at the detection cells (volume 8 μl), the flow of the solution was stopped automatically by small magnetic valves (MTV-31-M6, Takasago Electric, Nagoya, Japan) at the inlet and outlet of the detection cells. The estimation was then carried out by a rate-assay method. After several tens of seconds, the flow of mobile phase through the cell was initiated to elute the sample solution already estimated. By this method the samples can be assayed with much less loss than by the conventional method, because enzymes and low-molecular-mass molecules in the same sample solution can be assayed at the same time.

We first examined the efficiency of the GPC apparatus using bovine serum albumin (MW 66 000) and uric acid (MW 168). The GPC column (50 × 4 mm I.D.) was suitable for the separation of the protein fraction from that of low-molecular-mass compound so that both fractions could be assayed.

As the model reaction system, we examined the estimation of a representative small molecule, glucose, in serum and a typical serum enzyme, alkaline phosphatase (ALP) or glutamic-oxalacetic transaminase (GOT). For the estimation of ALP, the solution of the substrate, *p*-nitrophenyl phosphate disodium salt, was mixed with the fraction of macromolecules, and the reaction was followed by the increase in absorption at 430 nm due to the product, *p*-nitrophenoxide anion [1, 2]. (Although the maximum of absorption of the *p*-nitrophenoxide anion is at 400 nm, the substrate also absorbed at this wavelength so the reaction was followed at 430 nm.) The plot of the change in absorbance at 430 nm versus the concentration of ALP in the serum (Hyland control serum I) shows a linear relationship (Fig. 2) (1.5—9.5 U/l; correlation coefficient r = 0.997).

We also used the method to examine the estimation of equal number units of ALP from bovine intestine dissolved in phosphate buffer (pH 8.0). The results obtained were the same as those from serum, which shows the validity of this

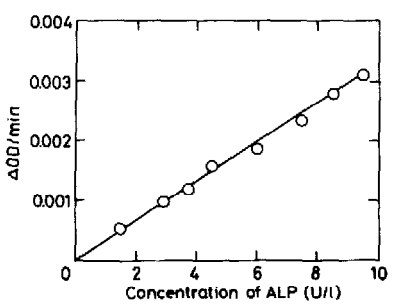

Fig. 2. Quantitative analysis of serum ALP at 430 nm. Mobile phase, 0.03 *M* phosphate buffer (pH 8.0); flow-rate, 0.5 ml/min; substrate concentration, 1.2 m*M*.

method. In the case of GOT, the GOT—malate dehydrogenase (MDH) system was used (concentrations of aspartate, α-ketoglutarate, NADH and MDH were 5 m*M*, 2 m*M*, 0.2 m*M* and 43 U/ml, respectively), and the reaction was followed by the decrease in absorption at 340 nm due to the consumption of NADH [3]. The results also showed a linear relationship between the decrease in absorbance at 340 nm and the concentration of GOT in serum (5—30 U/l; r = 0.999). A similar relationship was observed in the GOT (pig heart)—MDH system, which again shows the validity of the method.

The estimation of glucose in serum was carried out using the glucose oxidase (GOD)—peroxidase (POD) system [4, 5]. As a substrate of peroxidase, we used 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (concentrations of POD, GOD and ABTS were 2 U/ml, 12 U/ml and 2 m*M*, respectively). In the estimation of glucose by the enzymatic method, certain types of protein tend to act as acceptors of the hydrogen peroxide produced by the reaction of glucose with GOD [6]. In addition, it has been reported that catalase from erythrocytes may leak into serum during the removal of blood cells and consume hydrogen peroxide [7]. These proteins and enzymes lead to large uncertainties in the results of assays [6]. By using the GPC method, such interfering compounds of high molecular mass can be removed before the enzymatic reaction is carried out. Using this apparatus we were able to perform assays of glucose in hundreds of serum samples continuously (50—200 mg/l, r = 0.998). The serum sample gave the same peak area as that of a glucose solution of the same concentration. Arvidsson et al. [8] examined the precolumn venting system, where the protein fraction was drained using a three-way valve after passage through a precolumn made of silica gel (LiChrosorb), and only small molecules passed through the analytical column. Our system represents an improvement, in that both protein fractions and small molecules can be analysed simultaneously.

ACKNOWLEDGEMENT

We thank the Sekisui Chemicals Co., Osaka, Japan, for kind donation of polymer beads Micropearl SFW-214 for GPC.

Department of Polymer Chemistry,
Kyoto University,
Kyoto (Japan)

HIROMI KITANO*
YOUHEI HIRAI
KATSUNORI NAKAMURA
NORIO ISE

1 T.W. Reid and I.B. Wilson, in P.D. Boyer (Editor), The Enzymes, Vol. 4, Academic Press, New York, 3rd ed., 1971, p. 373.
2 H.N. Fernley, in P.D. Boyer (Editor), The Enzymes, Vol. 4, Academic Press, New York, 3rd ed., 1971, p. 417.
3 A.E. Branstein, in P.D. Boyer (Editor), The Enzymes, Vol. 9, Academic Press, New York, 3rd ed., 1973, p. 379.
4 A.S. Keston, Abstracts of the 129th Meeting of the American Chemical Society, 1956, p. 31.
5 J.D. Teller, Abstracts of the 130th Meeting of the American Chemical Society, 1956, p. 69C.
6 H.S. Mason, Adv. Enzymol., 19 (1957) 79.
7 D. Herbert and J. Pincent, Biochem. J., 43 (1948) 203.
8 T. Arvidsson, K.-G. Wahlund and N. Daoud, J. Chromatogr., 317 (1984) 213.

(First received April 29th, 1986; revised manuscript received July 11th, 1986)