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Continuous Fluorescence Detection of Creatine Phosphokinase Isoenzymes by the Use of a Stream-Switching Valve

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We describe a new flow injection system with a stream-switching valve for continuous monitoring of the isoenzyme activities of creatine phosphokinase (EC 2.7.3.2) after liquid chromatographic separation. Serum, injected into a mini-column (15 mm × 2 mm) of DEAE-Sepharose, was eluted stepwise with Tris-buffered sodium chloride (30, 145 and 300 mM). The separated isoenzymes were subsequently mixed with the enzyme reagents and immediately passed to the fluorescence detector to measure serum background before the beginning of the enzyme reactions. After the first measurement of background fluorescence, the mixture, which was passed to the delay coil by the stream-switching valve, underwent a series of enzyme reactions, ultimately resulting in the formation of fluorescent nicotinamide adenine dinucleotide, reduced form (NADH). The fluorescence intensity of NADH was measured again with the same detector. Isoenzyme activity was determined by subtracting the area of serum background from the area of fluorescent NADH. A major advantage of this detection system is the ability to carry out continuous monitoring of the isoenzyme activities with removal of the serum background. Fluorometric detection of the separated isoenzymes permits sensitive and unambiguous detection of three isoenzymes.

Keywords——isoenzyme separation; continuous isoenzyme monitoring; stream-switching valve; fluorometry; myocardial infarction; NADH

Isoenzymes are different proteins that catalyze the same reaction. Creatine phosphokinase (CPK) (adenosine 5′-triphosphate (ATP); creatine *N*-phosphotransferase; EC 2.7.3.2) occurs as three dimeric isoenzymes composed of the M and B subunits. MM is predominant in skeletal muscle, MB in heart and BB in brain. MB and BB are detectable in serum of patients with certain disease states producing tissue damage. The MB isoenzyme (CPK–MB) appears in the serum of patients with myocardial infarction. Its activity rises sharply within 4—6 h after myocardial infarction, peaks within 12—24 h and generally returns to normal levels within 48 h, but CPK–MB activity is always under 30% of total CPK activity even at 12—24 h after myocardial infarction.[1] CPK–BB appears in the serum of patients with brain tissue injury,[2] renal damage[3] and certain carcinomas.[4] It was pointed out[1] that CPK–BB activity is less than that of CPK–MB, in accordance with data obtained by electrophoresis.

Thus, the determination of CPK isoenzymes is very important for diagnosis and for identifying damaged tissues or organs. The most common technique for the separation and the measurement of isoenzyme activity is electrophoresis. In this method, a small sample of about 10 μl is streaked on the medium. However, the method is semiquantitative and slow. In recent years, immunoassays[5] have become popular for assay of specific isoenzymes, notably CPK–MB. These methods can be automated, but one cannot always be certain that the antibody to CPK–MB will not crossreact with CPK–BB.

Recently, disposable mini-column ion-exchange techniques have been developed mainly to measure the heart fraction of CPK. Mercer[6] originally described an anion-exchange

column (diethylaminoethylcellulose) made with a Pasteur pipet. The column was eluted with a series of buffers to obtain the MM, MB and BB fractions of CPK. These chromatographic methods are generally more sensitive, quantitative and faster than the other methods discussed above with the exception of radioimmunoassay, which shows very high sensitivity. However, manual methods are extremely labor-intensive and a continuous detection system is required. Schlabach *et al.*[7] reported a continuous detection apparatus for monitoring the activity of isoenzyme eluates. With relatively pure samples, few problems with continuous flow detection were noted. However, with human sera and tissue extracts significant interferences, high reagent blanks and drifting base line were observed, so an additional detector and computer were required.[8]

In this paper, we describe a method similar to that of Mercer, but modified to increase the sensitivity with one fluorescence detector and incorporating a stream-switching valve to eliminate the serum background by on-line monitoring. Although CPK isoenzymes are increased in serum as a consequence of certain disease states or tissue injury, the activities of CPK–MB and –BB are comparatively low even in such situations. Fluorometric detection of the separated isoenzymes permits sensitive and unambiguous detection of CPK–MB and –BB in the presence of a preponderance of CPK–MM. The detector drifts due to gradient effects, *etc.* are constant for the detection of nicotinamide adenine dinucleotide, reduced form (NADH) as the reaction product of enzyme assay.

Experimental

Enzymes——Hexokinase (HK) (ATP; D-hexose-6-phosphotransferase; EC 2.7.1.1) (from yeast, about 40 U/mg, lyoph.) and glucose-6-phosphate-dehydrogenase (G-6-PDH) (D-glucose-6-phosphate; nicotinamide adenine dinucleotide phosphate (NADP) oxidoreductase; EC 1.1.1.49) (from yeast, about 15 U/mg, lyoph.) were obtained from Boehringer Mannheim.

Samples——Sera from patients for CPK isoenzyme determination were provided by Ueno civic hospital. Control serum (from Hyland) was used as a reference sample. It was previously confirmed by electrophoresis on cellulose acetate sheets that the control serum contained all three CPK isoenzymes. Sera (5 μl aliquots were injected from the injection port into the flow system without further pretreatment.

Reagents——Creatine phosphate (5 mM), nicotineamide adenine dinucleotide (NAD) (0.4 mM), adenosine 5′-diphosphate (ADP) (0.25 mM), adenosine 5′-monophosphate (AMP) (0.92 mM), glucose (2.8 mM), G-6-PDH ($\geq$0.8 U/ml), HK ($\geq$0.8 U/ml), magnesium acetate (1.25 mM) and mercaptoethanol (2 mM) were simultaneously dissolved in pH 7.5 50 mM Tris-(hydroxymethyl)methylamine buffer containing 300 mM sodium chloride and this solution was designated as CPK reagent. The CPK reagent was prepared to give suitable concentrations of substrate based on the optimum conditions described by Szasz.[9]

Column——CPK isoenzyme separations were performed on a glass column (15 mm × 2 mm) with a small glass-wool plug fitted at the downstream end to retain the Sepharose and with short Teflon inlet and outlet tubes. Diethylaminoethyl Sepharose (Pharmacia, DEAE-Sepharose, CL-6B) was slurry-packed by the use of buffer containing 30 mM sodium chloride.

Elution——CPK isoenzymes were separated by stepwise elution with Tris buffer which contained sodium chloride at 30 mM (MM eluent), 145 mM (MB eluent) or 300 mM (BB eluent). After each CPK isoenzyme determination, the whole system was thoroughly washed with MM eluent for 15 min.

Determination of CPK Isoenzyme——The detection system was based on the enzymatic conversion of the CPK reagent by CPK isoenzymes to intermediate products and finally to the monitored product, NADH as shown below

$$\text{creatine phosphate} + \text{ADP} \overset{\text{CPK}}{\rightleftharpoons} \text{creatine} + \text{ATP}$$

$$\text{ATP} + \text{D-glucose} \overset{\text{HK}}{\rightleftharpoons} \text{ADP} + \text{glucose-6-phosphate}$$

$$\text{glucose-6-phosphate} + \text{NAD} \overset{\text{G-6-PDH}}{\rightleftharpoons} \text{gluconolactone-6-phosphate} + \text{NADH}$$

NADH which was produced in the enzymatic reaction was proportional to the creatine phosphate and could be monitored by measuring its native fluorescence (λ_{ex}: 360 nm λ_{em}: 460 nm). CPK reagent containing substrate was mixed with CPK isoenzymes in the column effluent by means of a tee-connector.

Instrumentation for Determination of CPK Isoenzymes——A schematic diagram for the determination of CPK

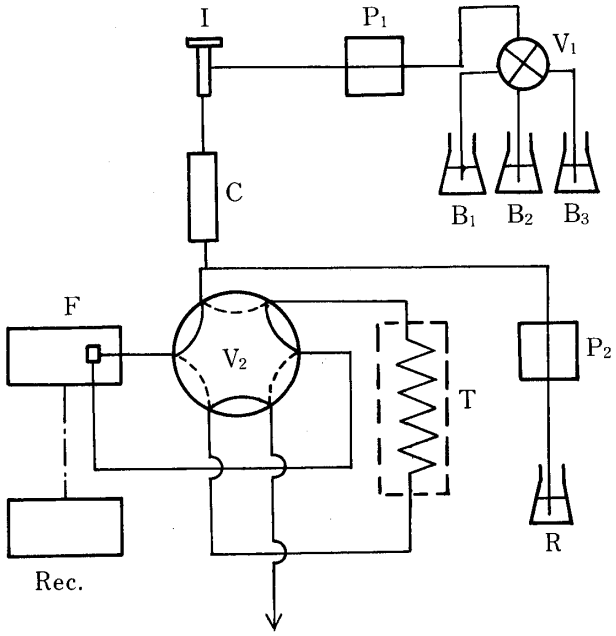

Fig. 1. Schematic Diagram of the Apparatus for the Determination of CPK Isoenzymes

B_1, B_2 and B_3: 50 mM Tris buffer (pH 7.5) containing sodium chloride at 30 mM (MM eluent), 145 mM (MB eluent) or 300 mM (BB eluent), respectively.
R, CPK reagent containing the substrate and cofactors; P_1 and P_2, peristaltic pump; V_1, four-port valve; V_2, six-port valve for stream-switching; I, injection port; C, DEAE-Sepharose column (15 mm × 2 mm); F, fluorescence detector for HPLC; Rec., recorder; T, water bath circulator.

isoenzymes is shown in Fig. 1. Three buffer reservoirs were controlled manually and a peristaltic pump P_1 (Atto Co., Ltd., type SJ-1215) delivered the step gradient at a flow rate of 0.1 ml/min. All tubings and the delay coil used were made of stainless steel (0.8 mm i.d.), except for the silicon tubes of the pumps. With the stainless steel six-port valve V_2 (Gaschro Kogyo Co., Ltd., type HPV-6) in the position shown by solid lines, each buffer was drawn through injection port I into the DEAE-Sepharose column C. CPK reagent was mixed in a tee-connector with column effluent between the column outlet and the six-port valve. The column effluent plus 0.1 ml/min CPK reagent flow made up the total 0.2 ml/min system flow. The outlet of the tee-connector was connected to a 2 cm length of tubing, which was connected to the six-port valve. The outlet of the six-port valve was connected to a 10 cm length of tubing, which was connected to a fluorescence detector F for high-performance liquid chromatography (HPLC) (Shimadzu Seisakusho, type FLD-1) equipped with a coated low-pressure mercury lamp emitting light at 300—400 nm (maximum intensity at 360 nm) and an EM-3 filter which cuts off light at shorter wavelength than 405 nm. The outlet of the flow cell was connected to a 10 cm length of tubing through the six-port valve with a delay coil. The 2 m delay coil was maintained at 37 °C with a water bath circulator and its outlet was connected to the six-port valve again. The fluorescence intensity was recorded with a recorder (Shimadzu Seisakusho, type R-11M).

Determination Procedure——The whole system was flushed with the MM eluent in both positions (solid and broken lines) of the six-port valve, then serum (5 μl) was injected from the injection port into the flow system in the position of the solid line. This allowed CPK–MM to transfer to the fluorescence flow cell with mixed CPK reagent. At about 1 min after the injection of serum, a elution peak was detected on the recorder. Because the eluted CPK–MM did not react significantly with CPK reagent in such a short time, the peak gives the background of serum eluted with MM eluent. When the peak returned to the base line, the mixture of the eluent and CPK reagent was transferred to the delay coil (solid lines). Then valve V_2 was switched to the position indicated by the broken lines. In the delay line, NADH was produced by enzyme reaction of the CPK reagent. At about 8 min after switching, the produced NADH passed to the fluorescence detector again and a peak of NADH (containing serum background) was detected. When NADH was completely eluted from the flow cell, valve V_2 was returned to its initial position and valve V_1 was switched to the next MB eluent. The same procedures, except for the first substitution with the MM eluent and the sample injection, were repeated for the determination of CPK–MB and CPK–BB. The DEAE-Sepharose column was disposed of after the determination of the three isoenzymes. The total analysis time for the three isoenzymes was about 30 min. At the end of the day, the fluorescence cell and valve system were flushed with water and then with methanol. This treatment eluted the impurities that had been adsorbed on the wall.

Electrophoresis——Electrophoresis was performed on cellulose acetate sheets by a modification of the method of Heinbokel *et al.*[10] (Helena Electrophoresis System, U.S.A.). The sheets were first activated in 25 mM Tris buffer, pH 6.9, containing 60 mM *N*-acetylcysteine, for 20 min. The same buffer was used as the bridge buffer. Electrophoresis was carried out at 300 V for 10 min. The sheets were then stained by a modification of the method of Rosalki.[11] The reaction mixture contained 30 mM creatine phosphate, 100 mM D-glucose, 2 mM ADP, 2 mM AMP, 4 mM NADP, 4 mM Mg-acetate, 2000 U/l HK, 2000 U/l G-6-PDH and 40 mM dithiothreitol, which were dissolved in sucrose–bridge buffer solution (1 : 5, w/v). A 1 ml aliquot of this staining mixture was spread over another activated cellulose acetate sheet. After a 1 min incubation, the paper carrying the sample was laid face to face on top of it. This sandwich was then incubated at 37 °C for 40 min. The sheets were dried and the fluorescence intensities of CPK isoenzyme bands

were determined with a Helena densitometer under ultraviolet (UV)-light.

Results and Discussion

Background of CPK Reagent

An unsuitable CPK reagent which contained the substrate and cofactors in Tris buffer but not sodium chloride was prepared first. We examined the reagent blanks according to the normal step-gradient method without sample injection. As shown in Fig. 2A, the fluorescence intensity of the reagent blanks increased with increase in the salt concentration of the eluents, and the fluorescence blanks showed some peaks each time the switching valve. Thus, the effects of mixing the CPK reagent with the three eluents as well as the effect of the switching valve must be considered. It appeared that the fluorescence background was influenced by the variation of ionic strength, because the CPK reagent itself gave a fluorescence background. With respect to some peaks seen each time the switching valve, we presumed that the previous mixture (for example, CPK reagent and MM eluent) which remained in the six-port valve (both solid and broken lines), fluorescence cell and remaining parts of the system was replaced progressively with the next mixture (for example, the reagent and MB eluent) in the process of step-gradient elution. The fluorescence background can be easily compensated for, because it was constant and reproducible, unlike the variable fluorescence background of sera. This was done by using a new CPK reagent in which the substrate and cofactors were dissolved in Tris buffer containing 300 mM sodium chloride, that is, at high ionic strength. Fluorescence peaks seen each time the switching valve also became negligibly small, as shown in Fig. 2B.

Fluorescence Background of Serum

Fluorescence materials which interfere with the detection of enzyme activity in serum

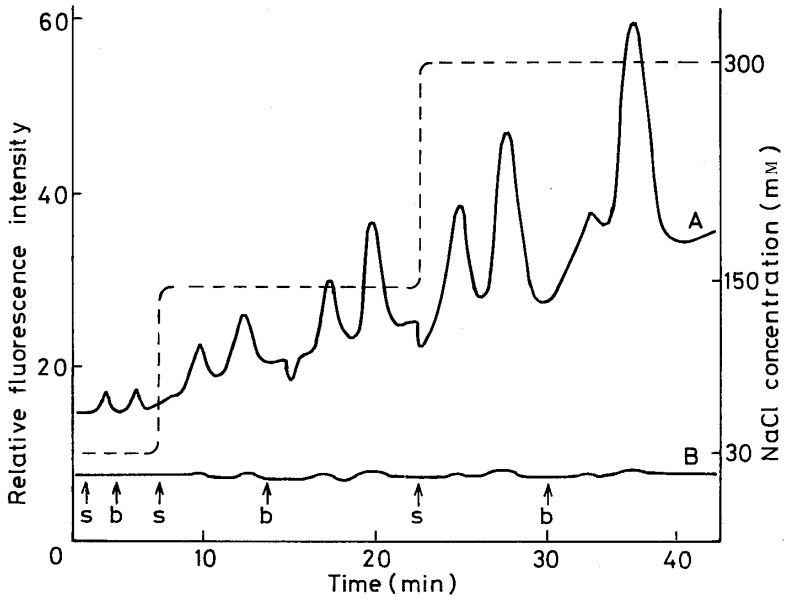

Fig. 2. Fluorescence Background Arising from the Switching Valve and the Mixing of CPK Reagent with the Three Eluents

A: CPK reagent dissolved in Tris buffer without sodium chloride was mixed with the three step-gradient eluents.

B: CPK reagent dissolved in Tris buffer containing 300 mM sodium chloride was mixed with the three eluents.

Letters s and b show the time of switching to the positions of the solid and broken lines in Fig. 1, respectively.

Fluorescence detector range, 4.

----, sodium chloride concentration of stepwise gradient eluents.

have been observed by Mckenzie.[12] Similarly, Denton *et al.*[13] reported that three chromatographic peaks of CPK isoenzyme were detected by fluorometry in an immobilized enzyme reactor, but three peaks were also observed in human control serum without the addition of the enzyme reagent. They designed a dual-beam photometric detection system to compensate for the background even in the ultraviolet region. However, in the determination of trace-level CPK–BB, a highly sensitive detector such as a fluorometer is necessary. Thus, we considered that it was necessary to fabricate a fluorescence detection system with a six-port valve for background correction and to provide well-defined peak identification in the presence of complicating background fluorescence. We examined the background of serum according to the usual determination procedure (no mixing with the CPK reagent, but with a normal step-gradient). The result is shown in Fig. 3. In Fig. 3, a fluorescent constituent of serum is eluted as two fluorescence peaks because it passes through the flow cell of the detector twice as a result of switching of the valve from the solid to the broken line position in Fig. 1.

Several difficulties arise in this determination system. As can be seen in Fig. 5, peaks of S_1', S_2' and S_3' are incorporated in the peaks of CPK–MM, CPK–MB and CPK–BB, respectively. To obtain corrected chromatograms of CPK isoenzyme, the peak areas of S_1, S_2

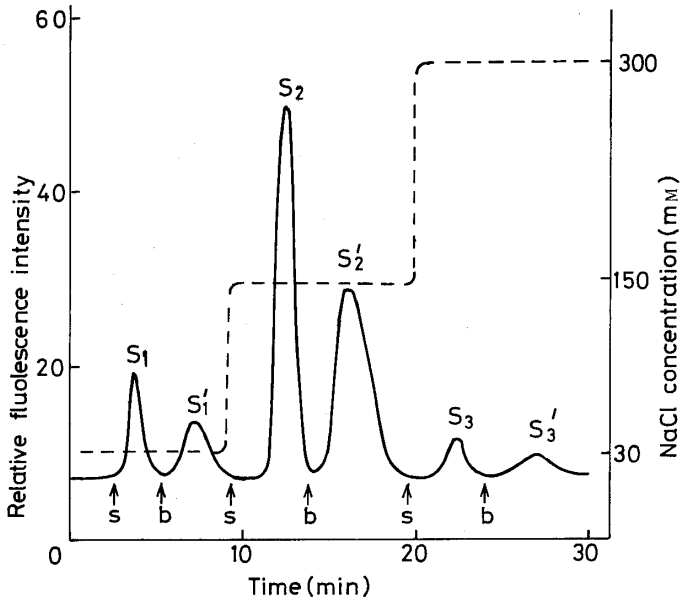

Fig. 3. Fluorescence Background of Serum as Affected by Step-Gradient and Switching Valve

S_1, S_2 and S_3, peak areas eluted with MM, MB and BB eluents in the solid line position of the six-port valve, respectively. S_1', S_2' and S_3', peak areas in the broken line position.

TABLE I. Empirical Parameter K for Correction of Serum Background Caused by the Switching Valve

	Ratio of fluorescence peak area of serum		
	MM eluent S_1'/S_1	MB eluent S_2'/S_2	BB eluent S_3'/S_3
K	0.993	0.988	0.990
S.D.	0.048	0.068	0.051
C.V.	4.8	6.9	5.2

K values were obtained as the mean of fifteen cases of human sera.

and S_3 were utilized. The two peak areas (S_1 and S_1', S_2 and S_2', S_3 and S_3' appearing at the solid and broken line positions of the six-port valve) should be identical because the same fluorescent constituent passes through the flow cell twice, but the two areas were not in good agreement according to the calculations. It is likely that the peaks were transformed in shape as a result of the effects of tubing dimensions, flow rate, viscosity and diffusion coefficient. However, this problem was solved by using an empirical parameter, K. K values were determined from the results with fifteen cases of human sera. The results are listed in Table I. From the relationship of S_1, S_2, S_3 and K values, the peak areas of S_1', S_2' and S_3', which are incorporated in peaks of CPK–MM, CPK–MB and CPK–BB, can be calculated.

Column Separation

We checked the resolution of a mini-column packed with DEAE-Sepharose using CPK isoenzyme control serum (Ortho Diagnostics Inc.). CPK control serum (5 μl) was injected into the flow system and samples eluted from the mini-column with MM, MB and BB eluents were successively collected as fractions MM, MB and BB, respectively. Three 500 μl fractions were concentrated for 1 h in a concentrator (Amicon Corporation; Minicon-B 15) and electrophoresis was performed on cellulose acetate sheets. The results of electrophoresis were analyzed with a densitometer (Beckman; microzone integrated densitometer) and the protein patterns are shown in Fig. 4. In Fig. 4, β- and γ-globulin fractionated from fraction MM, α-globulin and albumin from fraction MB and albumin from BB fraction can be seen. We concluded that the three isoenzymes were separated satisfactorily by the mini-column.

Fluorescence Chromatogram of CPK Isoenzymes

The fluorescence chromatogram of CPK control serum containing all three isoenzymes (Fig. 5) shows high resolution of the isoenzymes in less than 30 min. The chromatogram was obtained by DEAE-Sepharose chromatography involving stepwise gradient elution with MM, MB and BB eluents, with the use of the switching valve. Although the serum background in control serum was comparatively small, the peak areas of eluted MM, MB and BB were corrected by applying the empirical constants and the areas of serum background. The relative proportions of the corrected areas agreed well with the values reported by the manufacturer. The three CPK isoenzyme activities were estimated from three calibration

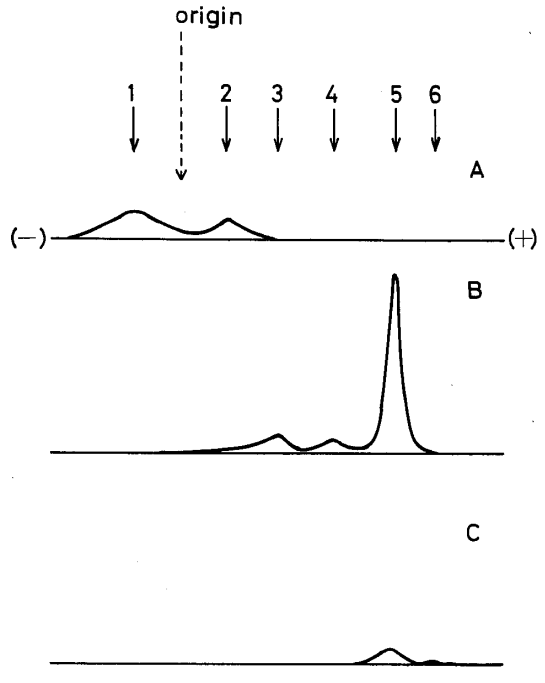

Fig. 4. Protein Pattern in Fractions Obtained during CPK Isoenzyme Separation

A, fraction MM eluted with MM eluent; B, fraction MB eluted with MB eluent; C, fraction BB eluted with BB eluent.

1, γ-globulin region; 2, β-globulin region; 3, α_2-globulin region; 4, α_1-globulin region; 5, albumin region; 6, pre-albumin region.

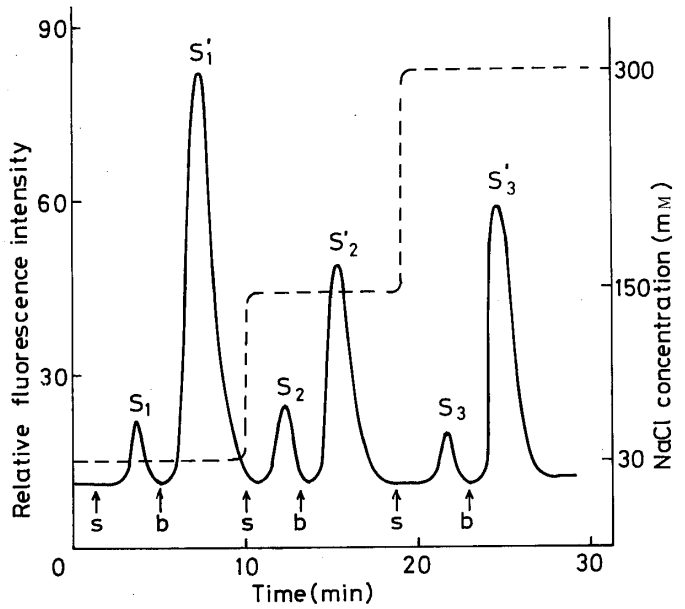

Fig. 5. Chromatographic Separation and Fluorescence Monitoring of Control CPK Isoenzyme Sample

Sample, control CPK isoenzyme (5 μl); column, 15 mm × 2 mm, DEAE-Sepharose; stepwise gradient, MM, MB and BB eluents; flow rate, 0.2 ml/min.

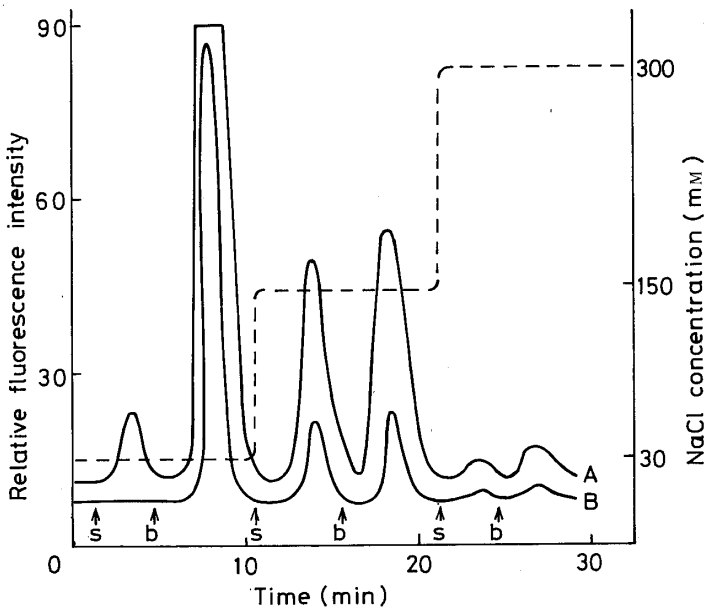

Fig. 6. Fluorescence Chromatogram of Serum from a Patient with Myocardial Infarction

Column conditions were the same as in Fig. 5.
A, fluorescence detector range, 4; B, fluorescence detector range, 16.

curves obtained with NADH standards by the same determination procedure.

Figure 6 shows a fluorescence chromatogram of a serum sample from a patient with myocardial infarction. The increased CPK–MB, previously also found by electrophoresis, is readily apparent in the chromatogram. The total CPK activity of the sample, 800 U/l (MM 712 U/l, MB 76 U/l and BB 13 U/l from the measurement of fluorescence), was also verified by means of spectrophotometry. Each isoenzyme activity was calculated by use of the equation

as shown below.

$$U/l = \frac{C \cdot 10^3 \cdot 10^3}{m}$$

Where C is the NADH concentration obtained from the calibration curves and m is the time of the peak appearance. The coefficient of variation was 2.7% for CPK–MM, 4.3% for MB and 7.5% for BB ($n=6$).

With the present system, efficient isoenzyme separations have been achieved on a very small column packed with readily available DEAE-Sepharose. The major advantage of the system is the detection of the serum fluorescence background by the use of the six-port valve, which is useful for correcting the false peaks that appear with isoenzymes in complex matrixes. The method consequently allows isoenzyme separation and detection in clinical samples without pretreatment of the serum. As a result, the low-activity isoenzyme, CPK–BB, which is not detectable by absorbance measurement in the UV region, can be sensitively determined with a fluorescence detector.

Conclusion

The combination of chromatographic separation, sensitive fluorescence detection and a six-port switching valve allows clear and unambiguous identification of all three CPK isoenzymes, MM, MB and BB. This approach should be useful for research on the role of CPK–BB in disease states. Recently, sensitive and specific double-antibody immuno techniques have been developed for assaying CPK–BB with the use of radioactivity[14] or bioluminescence detection.[15] These techniques require more extensive sample pretreatment and more expensive reagents than our proposed method.

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