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Assay of Adenosine Deaminase in Serum by Flow-Injection Analysis with Fluorescence Detection

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A sensitive assay method for adenosine deaminase activity in human serum is described based on the flow-injection determination of inosine formed enzymatically from substrate adenosine. Serum (10 μ l) is incubated with adenosine in the presence of urate oxidase and catalase, and the resulting mixture, after deproteinization with perchloric acid, is introduced into a flow-injection system in which immobilized enzyme columns of purine nucleoside phosphorylase, xanthine oxidase, urate oxidase and horseradish peroxidase are connected in series in that order in the flow line. Hydrogen peroxide formed in the enzymatic conversion of inosine is measured fluorimetrically by reaction with 3-(*p*-hydroxyphenyl)propionic acid in the system. The lower determinable limit of adenosine deaminase activity is 0.17 U/l serum.

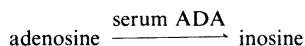
Keywords—adenosine deaminase activity; flow-injection analysis; inosine determination; immobilized enzyme; hydrogen peroxide; horseradish peroxidase; 3-(*p*-hydroxyphenyl)propionic acid; fluorescence detection

Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4, ADA), one of the enzymes on the purine salvage pathway, catalyzes the hydrolytic deamination of adenosine to inosine and ammonia. ADA activity in human serum is usually increased in patients with hepatic disorder^{1,2}; in particular, abnormally high ADA activity in sera was found for acute hepatic disorder,³ liver cirrhosis, chronic hepatitis and hepato-cellular carcinoma.^{3,4} On the other hand, ADA deficiency has been found in patients with severe combined immunodeficiency.^{5,6}

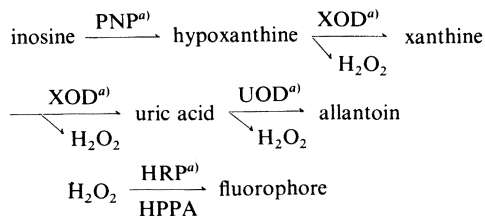
ADA activity has been assayed by spectrophotometric methods,⁷⁻¹⁰ though they are usually insensitive. A highly sensitive radiochemical method¹¹ requires radioactive substrate and tedious separation of the enzyme reaction product from the substrate by a column chromatographic or electrophoretic technique.

We have previously developed a sensitive fluorimetric flow-injection analysis (FIA) of

reaction in a test tube



reaction in the FIA system



a) immobilized enzyme

Chart 1

hydrogen peroxide,¹²⁾ based on an immobilized horseradish peroxidase (HRP)-mediated reaction with 3-(*p*-hydroxyphenyl)propionic acid (HPPA), an efficient fluorogenic substrate for HRP.¹³⁾ The FIA system has since been successfully applied to the flow-injection determinations of adenosine¹⁴⁾ and inosine^{14,15)} in human blood plasma, serum guanase activity,¹⁶⁾ erythrocyte purine nucleoside phosphorylase (PNP) activity¹⁷⁾ and plasma lactic acid,¹⁸⁾ by using suitable immobilized enzyme columns connected in series in the FIA systems.

This paper describes the establishment of a highly sensitive FIA method for the assay of ADA in human serum on the basis of immobilized enzyme column techniques. Inosine formed enzymatically from the substrate adenosine is degraded to form hydrogen peroxide during passage through columns of immobilized PNP, xanthine oxidase (XOD) and urate oxidase (UOD) and the hydrogen peroxide is quantified by the above-mentioned FIA technique (Chart 1).

Experimental

Chemicals—Deionized water was filtered through a Milli-QII system (Japan Millipore Ltd., Tokyo). HPPA was obtained from Dojindo Laboratories (Kumamoto, Japan). Tris(hydroxymethyl)aminomethane (Tris, Ultrol grade), HRP (285 purpurogallin units/mg, Type VI) and UOD (4.0 U/mg, from *Candida* sp., Grade II) were from Calbiochem (San Diego, CA), Sigma Chemical Co. (St. Louis, MO) and Toyobo Biochemicals (Osaka), respectively. Adenosine and inosine were from Kohjin Co., Ltd. (Tokyo). XOD (0.4 U/mg, from cow's milk, in 3.2 M ammonium sulfate suspension, 10 mM in disodium ethylenediaminetetraacetate (EDTA·2Na)) and catalase (260000 U/ml, from bovine liver, in 30% (v/v) glycerol solution containing 10% (v/v) ethanol) were obtained from Boehringer Mannheim Yamanouchi (Tokyo). Unless otherwise noted, all other chemicals were of reagent grade.

Solutions—The substrate solution for the ADA reaction was 21.3 mM adenosine in 50 mM Na phosphate buffer (pH 6.5, buffer A). The catalase solution (0.75 U/ml) in buffer A was prepared from 100 U/ml stock catalase solution. The UOD solution (12.5 mU/ml) in buffer A was prepared from 4.0 mU/ml stock UOD 50% (v/v) glycerol solution. These working solutions were prepared when required.

Serum—Normal sera were obtained from healthy volunteers in this laboratory. Sera with abnormally high glutamate-oxalate transaminase were supplied by Chidoribashi Hospital (Fukuoka, Japan).

Enzyme Reaction and Sample Solution for FIA—A mixture of the substrate solution (or buffer A for the blank) (750 μ l), the UOD solution (20 μ l) and the catalase solution (20 μ l) was pre-incubated at 37 °C for *ca.* 2 min and then incubated at 37 °C for exactly 30 min after addition of 10 μ l of serum. The reaction was stopped by the addition of 400 μ l of 2 M HClO₄. The resulting mixture was mixed with 800 μ l of 1 M K₂CO₃ to remove the perchlorate ion and centrifuged for 10 min at 1000 *g*. An aliquot (20 μ l) of the supernatant (sample solution for the test or blank) was injected into the FIA system.

FIA System and Assay Procedure for Inosine—A schematic flow-diagram of the FIA system is shown in Fig. 1. The immobilized PNP, XOD, UOD and HRP columns were prepared as previously reported^{14,15)} and connected in series in that order in the flow line using polytetrafluoroethylene tubing (4.0 \times 0.86 mm i.d., 1.46 mm o.d.; Sanko Plastic Co., Osaka). The reagent solution, which was 5 mM HPPA in 0.1 M Tris-HCl buffer (pH 8.0), 0.15 M in NaCl and 10 mM in EDTA·2Na, and carrier solution, which was the same as the reagent solution but without HPPA, were pumped separately with a Sanuki DM2M-1026 pump; the flow rates were both 0.25 ml/min.

The sample solution for the test or blank was injected through a Rheodyne 7125 syringe-loading sample injection valve (20- μ l loop) into the carrier stream. The fluorescence intensities were measured at an excitation wavelength of 305 nm and an emission wavelength of 405 nm with a Shimadzu RF-530 fluorescence spectromonitor equipped with a

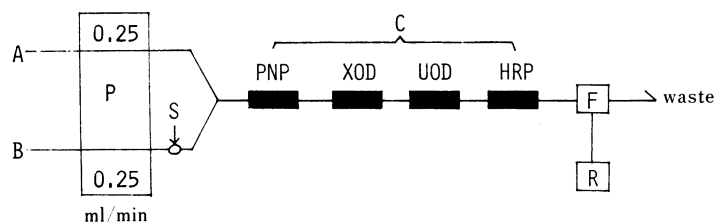


Fig. 1. Schematic Diagram of the FIA System

A, HPPA solution; B, carrier solution; C, immobilized enzyme columns; F, fluorescence detector; P, pump; S, sample injector; R, recorder.

12- μ l flow-cell and a Rikadenki R-21 chart recorder (chart speed; 2.5 mm/min). To prepare a calibration curve, inosine standard solutions (3–360 pmol/20 μ l, corresponding to 1–120 U/l serum ADA) in buffer A were injected into the FIA system. The amount of inosine formed enzymatically in the 20 μ l injection volume (A pmol) was read on the calibration curve using net peak height. ADA activity is calculated as $A/3$ U/l serum.

Results and Discussion

The reaction conditions of the immobilized enzymes in the FIA system were optimum, and essentially the same as described previously.^{14,15} ADA activity in serum can be obtained by subtracting the peak height of the blank from that of the test.

When the concentration of uric acid is sufficient to inhibit the HRP-catalyzed fluorescence reaction of HPPA, uric acid should be decomposed by adding urate oxidase to the enzyme reaction mixture; the hydrogen peroxide formed was destroyed by using catalase during the enzyme reaction. UOD and catalase that remained in the enzyme reaction mixture were removed by deproteinization with perchloric acid. A small amount of uric acid was also produced from xanthine in the sample solution. The resulting uric acid slightly inhibited the

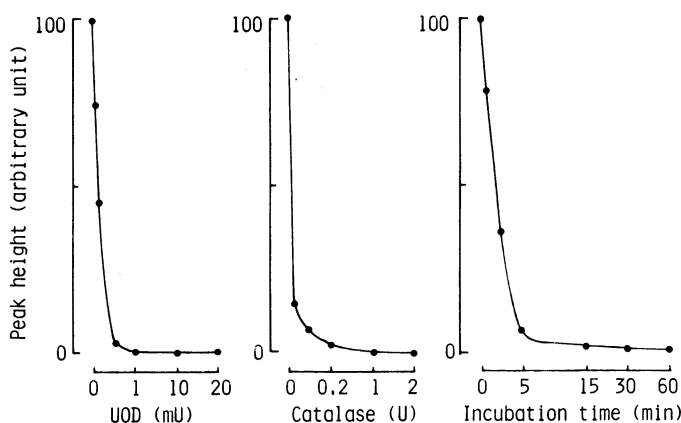


Fig. 2. Effect of the Amounts of UOD and Catalase and Incubation Time on the Peak Height from Uric Acid in the Serum Sample

PNP and XOD columns were removed from the FIA system. The concentration of uric acid was adjusted to 10 mg/ml by adding uric acid to the enzyme reaction mixture.

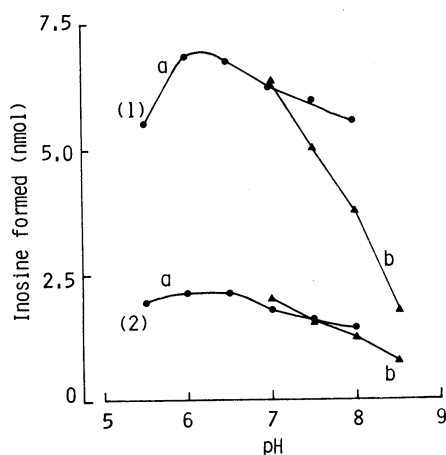


Fig. 3. Effect of pH on the Amount of Inosine Formed

a, 50 mM Na-phosphate buffer; b, 50 mM Tris-HCl buffer. ADA activities (U/l serum): (1) 22.5; (2) 7.0.

fluorescence reaction, but in fact this was prevented by installing an immobilized UOD column in the flow line. For complete destruction of uric acid in sample serum in the ADA reaction, 10 mU of UOD, 0.6 U of catalase and the reaction time of 30 min were used as optima (Fig. 2a, b and c).

ADA in human serum was most active at pH 6.0–6.5 in 50 mM Na phosphate buffer (Fig. 3); pH 6.5 was selected, since this has been used in a spectrophotometric assay method for serum ADA activity, where the phosphate concentration in the incubation mixture was 47.6 mM.⁹⁾

Two K_m values for adenosine were obtained as 0.061 and 0.68 mM from Lineweaver–Burk plots, and as 0.062 and 0.72 mM from Hans–Wilkinson plots when a serum with an ADA activity of 6.7 was used (Fig. 4). The K_m values for the two purified human liver ADA isozymes, ADA₁ and ADA₂, have been reported to be 0.050 mM and 2.0–2.8 mM, respectively.¹⁹⁾ The value for ADA₁ is close to those obtained with the serum sample at low adenosine concentrations, though the value for ADA₂ is different from those obtained at high adenosine concentrations. This might be due to the fact that the K_m values obtained at low adenosine concentrations were not very much influenced by ADA₂ but those at high adenosine concentrations were greatly influenced by ADA₁; 21.3 mM adenosine was recom-

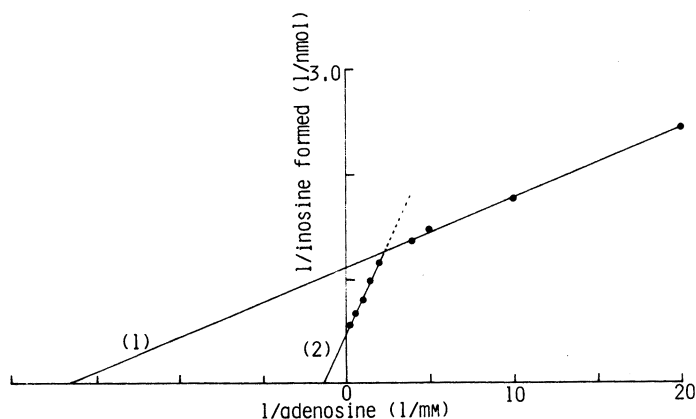


Fig. 4. Lineweaver–Burk Plots of the Dependency of ADA Activity on Adenosine Concentration

ADA activity (U/l serum): 6.7.

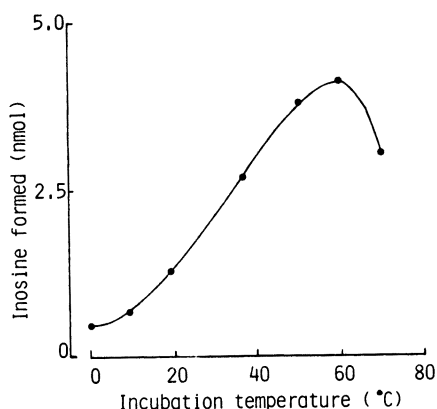


Fig. 5. Effect of Incubation Temperature on the Amount of Inosine Formed

ADA activity (U/l): 9.0.

mended for serum samples, which corresponded to 20 mM in the enzyme reaction mixture. This concentration was the same as that used in the spectrophotometric method for serum ADA.⁹⁾ When ADA₁ and ADA₂ activities are to be assayed, 0.5 and 20 mM adenosine in the enzyme reaction mixtures should be used, respectively, as described in the spectrophotometric method.³⁾

The enzyme reaction rate was constant for at least 90 min at 37°C for sera with ADA activities of 4.4, 14.0 and 30.8 U/l serum. The produced amount of inosine was proportional to serum sample size up to at least 20 μ l.

The enzymatic production of inosine was strongly influenced by the reaction temperature, and the maximum production was observed around 60°C (Fig. 5); 37°C was employed for convenience.

A calibration curve was prepared by subjecting inosine standard solutions directly to the FIA. The relationship between the concentrations of inosine and the peak heights was linear up to at least 600 pmol per 20- μ l injection volume, which corresponds to an ADA activity of 200 U/l serum, and passed through the origin. The lower determinable limit (signal-to-noise ratio = 5) for inosine was 0.5 pmol, which corresponds to an ADA activity of 0.17 U/l serum. This limit is one-tenth of that of a spectrophotometric method (sample serum size of 50 μ l).⁹⁾

Typical flow-injection peaks obtained with 50–300 pmol of inosine in 20 μ l injection volume (corresponding to ADA activities of 16.7–100 U/l serum) and with sample sera for the test and blank are shown in Fig. 6. A sampling rate of 40 injections/h could be achieved.

A comparison with a spectrophotometric method⁹⁾ based on the measurement of ammonia was made on normal and pathological sera. The correlation coefficient was 0.978 ($n=40$), and the linear regression equation for the present method (X) against the spectropho-

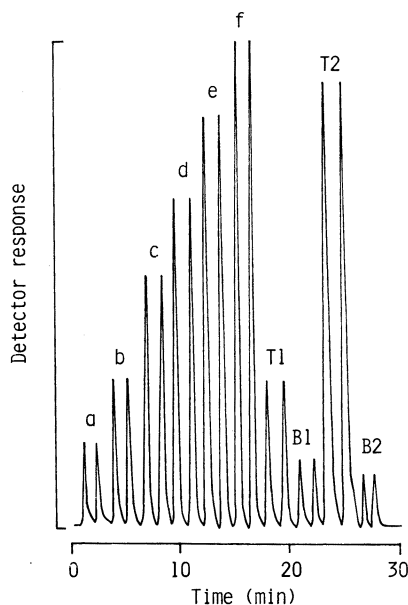


Fig. 6. Flow-Injection Peaks for Standard and Serum Samples

ADA activities (U/l serum); 15.0 (obtained from T1 and B1) and 80.0 (obtained from T2 and B2). Amounts of inosine (pmol): a, 50; b, 100; c, 150; d, 200; e, 250; f, 300. T and B: test and blank, respectively. T1 and B1 = normal serum. T2 and B2 = serum with high ADA activity.

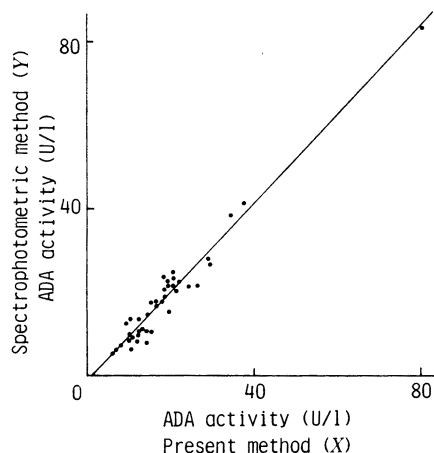


Fig. 7. Correlation between the Values of Serum ADA Activity Obtained by the Present Method and the Spectrophotometric Method

tometric method (Y) was $Y=1.06X-1.33$ (Fig. 7). The within-day precision of the present method was examined by using sera with mean ADA activities of 3.5, 13.9 and 53.3 U/l serum. The relative standard deviations were 11.1, 3.29 and 3.25% ($n=10$ each), respectively.

Bilirubin in serum at concentrations less than 5 mg/dl did not affect the determined activity of ADA but higher concentrations caused an apparent decrease in ADA activity. Ascorbic acid and glucose added to serum at concentrations of less than 4 and 300 mg/dl serum, respectively, had no effect on the determined ADA activity.

The FIA system was most successfully used when the immobilized PNP and XOD columns were renewed every 3 and 4 months, respectively. This study has provided the first fluorimetric method for the assay of ADA. This method is highly sensitive and precise, and should be useful for biological investigations and clinical use.

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