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A New Photometric Method for the Determination of Serum Glutamate Pyruvate Transaminase Activity using Pyruvate and Glutamate as Substrates

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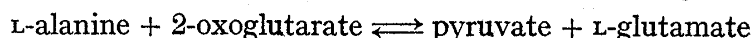
A new photometric method is presented for the assay of serum glutamate pyruvate transaminase activity on the basis of the determination of 2-oxoglutaric acid produced in the enzyme reaction under its optimal conditions by means of the previously established method for selective determination of the acid with diazotized sulfamethizole. The method gives reliable results and is simply performed with a small amount of sample with a wide range of activity.

Glutamate pyruvate transaminase (GPT)[2] catalyzes the following reaction.



L-alanine + 2-oxoglutarate ⇄ pyruvate + L-glutamate

The equilibrium in the reaction lies to the left[3] and the initial rate in the forward reaction is almost identical to that in the reverse reaction,[4] and therefore the reverse reaction is considered to be more favorable for the assay of GPT activity. However, the practical methods so far proposed for the assay of serum GPT activity are based on the determination of pyruvate or glutamate formed in the forward reaction,[5-9] and no method is presented which measures 2-oxoglutaric acid (2-OG) produced in the reverse reaction because of the lack of selective method for the determination of the acid.

In the previous paper,[10] we presented a selective microphotometric method for the determination of 2-OG on the basis of a color reaction with diazotized sulfamethizole in a sodium hydroxide solution in the presence of sodium sulfite and sodium hypophosphite. This paper describes a new method for the assay of serum GPT activity, which measures the amount of 2-OG formed in the reverse enzyme reaction under its optimal conditions by means of the method for the determination of 2-OG. The method is precise enough to use practically and easily performed using low cost of reagents with a small amount of serum over a wide range of the activity.

Experimental[11]

Reagents[12]

Reagents for the Enzyme Reaction——Tris-(hydroxymethyl)aminomethane (Tris) buffer solution: Dissolve 2.4 g of Tris in about 80 ml of H_2O, adjust the pH to 7.5 at 37° with diluted HCl and then dilute the

1) Location: *Maidashi, Higashi-ku, Fukuoka*; *a*) To whom all inquires should be addressed.
2) L-alanine: 2-oxoglutarate aminotransferase, EC 2.6.1.2.
3) An equilibrium constant of 1.6 was reported at pH 7.3 and 37° in the direction of 2-OG and alanine formation in the reaction with GPT obtained from the supernatant fraction of rat liver homogenate: H.L. Segal, D.S. Beattie, and S. Hopper, *J. Biol. Chem.*, **237**, 1914 (1962).
4) S. Hopper and H.L. Segal, *J. Biol. Chem.*, **237**, 3189 (1962).
5) S. Reitman and S. Frankel, *Am. J. Clin. Pathol.*, **28**, 56 (1957).
6) K.S. Henly and H.M. Pollard, *J. Lab. Clin. Med.*, **46**, 785 (1955).
7) A. Karmen, *J. Clin. Invest.*, **34**, 131 (1955).
8) M. Kageura and Y. Ohkura, *Japan J. Clin. Chem.*, **3**, 221 (1974).
9) U. Lippi and G. Guidi, *Clin. Chem. Acta*, **28**, 431 (1970).
10) Y. Ohkura, K. Matsumura, H. Hamada, and T. Momose, *Bunsekikagaku*, **20**, 480 (1971).
11) The absorbance and pH were measured by a Hitachi 124 Spectrophotometer with a cell of 10 mm optical path length and a Hitachi-Horiba M-7 pH meter, respectively.
12) All reagents used were Reagent Grade unless otherwise stated.

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resulting solution to 100 ml with H_2O (0.2 M). This solution is usable for several months when stored in a refrigerator.

Glutamate Solution: Dissolve 22 g of L-glutamic acid in 50 ml of 25% KOH,[13] adjust the pH to 7.5 at 37° with diluted HCl and then dilute to 100 ml with H_2O (1.5 M). Add one drop of $CHCl_3$ as a preservative. This solution is usable for several weeks when stored in a refrigerator.

Pyruvate Solution: Dissolve 300 mg of sodium pyruvate (purity, 99.7%[14]) in 200 ml of H_2O (13.2 mM). Add one drop of $CHCl_3$. This solution is usable for several months when stored in a refrigerator.

Substrate Solution: Mix Tris buffer solution, glutamate solution and pyruvate solution in the volume ratio of 3 : 3 : 4 (the concentrations of Tris, glutamate and pyruvate are 59, 449, and 5.45 mM, respectively). This solution is usable for at least 3 weeks when stored in a refrigerator.

Reagents for the Color Development——Sodium nitrite solution: Freshly prepare 2.5% aqueous solution.

Diazotized sulfamethizole solution: Dissolve 0.9 g sulfamethizole[15] in 17 ml of concentrated HCl and then dilute to 100 ml with H_2O (the final concentration of HCl, 6%). To 100 ml of the solution, add 10 ml of sodium nitrite solution at about 10°, and shake the mixture occasionally during 10 min. The solution is stable for 5 hr at room temperature (about 25°) and remains usable for two days in a refrigerator.

Alkaline Solution: Dissolve 90 g of NaOH in about 600 ml of H_2O. After cooling, add a solution prepared by dissolving 10 g of Na_2SO_3 and 12.5 g of $NaH_2PO_2 \cdot H_2O$ in about 300 ml of H_2O and dilute to 1000 ml with H_2O. The solution is stable when stored in a tightly closed container.

2-Oxoglutaric Acid Standard Solutions——Prepare first 105 μg/ml solution by dissolving 105 mg of 2-OG in 1000 ml of H_2O (0.72 μmol/ml solution). Using this solution, prepare 8.8, 17.5, 35 and 70 μg/ml solutions (corresponded to 0.06, 0.12, 0.24, and 0.48 μmol/ml, respectively). Add one drop of $CHCl_3$ per 100 ml of each solution and store the solutions in a refrigerator. The solutions are stable for at least 3 months.

Procedure

Pipet 0.5 ml of the substrate solution in a test tube and pre-heat in a water bath at 37° for 5 min. At zero time, add 20 μl of serum, mix well, cover the tube with Parafilm and incubate at 37° for exactly 30 min. Add immediately 1.0 ml of diazotized sulfamethizole solution under cooling in an ice-water bath, mix well, and then add 8.0 ml of the alkaline solution. Then warm at 37° for 60 min to develop the color and cool in running water. Prepare a serum blank in the same way as described above omitting the incubation. Within 30 min after the end of the reaction period, read the absorbance at 540 nm against the serum blank.

Calibration Curve and Calculation of Units

To 0.5 ml of each 2-OG standard solution, add 1.0 ml of diazotized sulfamethizole solution under cooling, and then treat the mixture in the same way as described in the procedure to develop the color. Read the absorbance of the reaction mixture against the reagent blank which is prepared by treating 0.5 ml of H_2O instead of 2-OG standard solution. The calibration curve thus drawn up is a straight line which passes through the origin.

The units of GPT activity are defined as the μmol of 2-OG formed by the enzyme in 1000 ml of serum per min at 37°, similarly to the manner of representation of the international units. Therefore, the units are calculated by the following equation.

$$\text{Units} = A \times \frac{K}{146.10} \times \frac{1}{30} \times \frac{1000}{0.02}$$

Where, A, 146.10, 30, and 0.02 are the absorbance, the molecular weight of 2-OG, the incubation time (min) and the amount of serum (ml), respectively. K is the ratio of 2-OG concentration (μg/0.5 ml) to the value of A given by this concentration of the acid in the preparation of calibration curve. For example, 61.4 was obtained in the present experiment, and the units were calculated by $0.7 \times 1000 \times A$.

Results and Discussion

The visible absorption spectrum of the color developed in the procedure had a maximum at 540 nm, and the shape of the spectrum was entirely identical to that observed in the determination of 2-OG.[10]

The each individual substance which was concerned with the enzyme reaction was first investigated as the effect on the color development of 2-OG under the conditions of procedure. Tris gave no absorbance and did not interfere with the color development. L-Glutamic acid and sodium pyruvate showed the absorbances of 0.038 and 0.216 at the prescribed concentra-

13) When NaOH solution was used instead of KOH solution, a 3% decrease of the enzyme activity was observed in the procedure.

14) T. Momose, S. Moritani, and Y. Ohkura, *Bunsekikagaku*, **21**, 5 (1972).

15) Purchased from Eisai Co., Tokyo.

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tions in the substrate solution, respectively, but did not interfere with the color development of 2-OG. Serum (20 μl) usually showed the absorbance of 0.02.

An almost constant absorbance of the serum blank, 0.254±0.005 (mean value±standard deviation) was observed on 100 different sera.

A complete recovery of 2-OG, 99±2%, was observed when the acid was added to the enzyme reaction mixture at a concentration of 8.8, 17.5, 35, 70 or 105 μg/ml and then treated as described in the procedure without the incubation.

The stability of pyruvic acid under the conditions of procedure was investigated by adding sodium pyruvate in the prescribed concentration in the substrate solution to the buffer solution or the enzyme reaction mixture where serum was replaced by 5% albumin solution[16] and then incubating at 37° for several periods. The remaining pyruvic acid was determined by the selective assay method of the acid with *p*-dimethylaminobenzaldehyde.[17] Only a slight loss of pyruvic acid, below 1%, was observed for the incubation time of 60 min.

In order to estimate the consumption of glutamic acid by enzymes or other unknown factors present in serum under the conditions of procedure, glutamic acid in the prescribed concentration was incubated with serum in the buffer solution and then treated as in the procedure to develop the color. The absorbance of resulting mixture was compared with that of the mixture obtained without the incubation. A maximum increase in the absorbance by the incubation, 0.008, was observed on 30 sera with various GPT activities, indicating that the consumption of glutamic acid in the absence of pyruvic acid was negligible. On the other hand, when sodium pyruvate was treated in the same way as glutamic acid, the change of absorbance by the incubation in the absence of glutamic acid was not observed.

A phosphate buffer has been commonly used in the assay of GPT activity, though phosphate slightly inhibits GPT.[18] In fact, under the conditions of procedure, a phosphate buffer (67 mM, pH 7.5) gave 8% lower value of the activity than a Tris buffer (57 mM, pH 7.5) whose concentration gave the maximum activity in the procedure. A triethanolamine buffer (50mM, pH 7.5) gave an identical GPT activity to that given by the Tris buffer, but showed an absorbance when treated under the conditions of the procedure. Thus, the Tris buffer was employed in the present procedure. The effect of pH on the enzyme activity was studied in a range of 6.5 to 9.3, and a maximum activity was obtained in the range of 7.0 to 8.0. Therefore, pH 7.5 was selected as the optimum.

The measured GPT activity did not largely change over a concentration range of sodium pyruvate of 4.0—7.2 mM with sera of low and high activities, and the prescribed concentration, 5.45 mM, was selected as a sufficient concentration for fairly high activity. The Michaelis constant (K_m) for sodium pyruvate was obtained as 0.7 mM when determined with 5 different sera of various GPT activities, and therefore the prescribed concentration of sodium pyruvate was to be about 7.5 times the K_m value. The concentration of glutamic acid did not largely affect the determined activity in a range of 150—570 mM and a more concentrated glutamic acid inhibited GPT when examined with sera of low and high activities. The prescribed concentration of the acid, 449 mM, was employed to obtain the maximum value of the activity.

When the prescribed concentrations of the substrates were used, a linear relationship between the incubation time and the amount of 2-OG formed was observed throughout at least 40 min incubation even on serum with a fairly high activity, as shown in Fig. 1, suggesting that the present procedure might permit the assay of GPT activity up to about 500 units for the prescribed incubation time of 30 min. The range might be extended to a very high activity by carrying out the procedure for shorter incubation time.

16) Bovine serum albumin (fraction V) with no GPT activity was used.

17) M. Kageura, Y. Ohkura, and T. Momose, *Chem. Pharm. Bull.* (Tokyo), **19**, 2241 (1971).
18) T. Kawanishi, Y. Takegawa, U. Ozaki, K. Kurahashi, and A. Ishihara, *Rinshobyori*, **22**, Supplement, 51 (1974).

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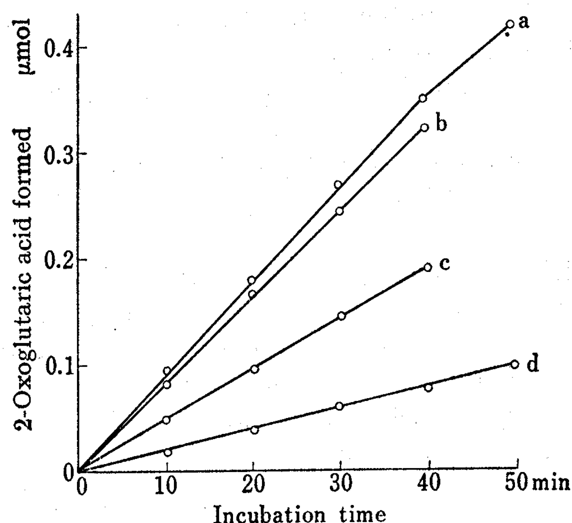

Fig. 1. Relationship between the Incubation Time and the Amount of 2-Oxoglutaric Acid Formed

a, 444; b, 406; c, 239; d, 97 units serum

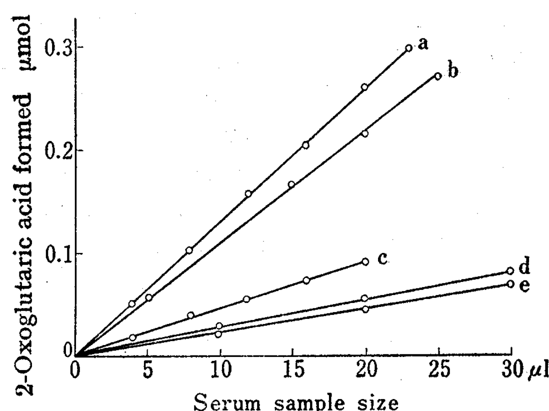

Fig. 2. Relationship between Serum Sample Size and the Amount of 2-Oxoglutaric Acid Formed

a, 463; b, 362; c, 157; d, 95; e, 76 units serum

Under the conditions of procedure, the amount of 2-OG formed was proportional to the serum sample size until the amount reached about 0.3 μmol which corresponded to the activity of about 500 units, as shown in Fig. 2. The prescribed sample size, 20 μl, was employed in the procedure to obtain a reliable result in the assay of serum with the activity of borderline between normal and abnormal ones.

Interfering substances were examined in regard to their colorations and their effects on the color development of 2-OG. D-Glucose in serum gave no absorbance, but reduced the absorbance from 2-OG by 9% at the concentration of 600 mg/100 ml, which became negligible in the concentration of 150 mg/100 ml. Cortisone and thiamine caused increase in absorbances of 0.011 and 0.015 at the concentration of 13 mg/100 ml in serum, respectively, which seemed to be non-interfering substances even when occurred in unusually high concentrations in serum. Urea and cholesterol did not show any absorbance and any effect on the color development at the concentration of 260 mg/100 ml, respectively. Other substances tested gave no absorbance and did not influence the color development of 2-OG even if present in serum at a concentration of 25 mg/100 ml. These include bilirubin, acetoacetic acid, oxalacetic acid, acetone, 3-hydroxybutyric acid, formaldehyde, acetaldehyde, L-ascorbic acid, D-xylose, D-glucuronolactone, inositol, citrulline, androsterone, dehydroepiandrosterone, uric acid, alloxan, allantoin, creatine, creatinine, glutathione, histamine and 20 different α-amino acids.

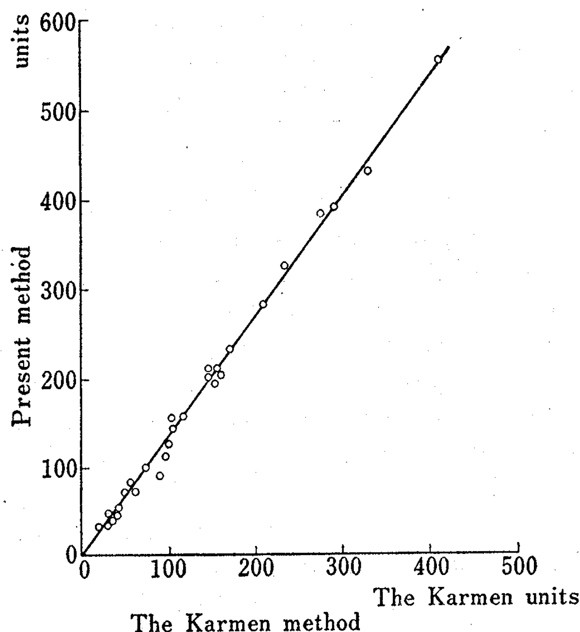

Fig. 3. Correlation between the Values obtained by the Present Method and the Karmen Method

Parallel tests with the Karmen method,[6] which has been widely accepted as a standard method for the assay of GPT activity, were carried out with 27 different sera. The results

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are shown in Fig. 3. A satisfactory coefficient of correlation for both method, 0.996, was obtained. The units obtained by the present method (Y) could be converted to the Karmen units (X) by the regression equation of $Y=1.290X-4$. The ratio of the enzyme reaction rate in the present method to that in the Karmen method at 37° was observed to be 1.2, suggesting that the rate in the reverse reaction was almost identical to that in the forward reaction.

The precision of the present method was studied as to repeatability, which was obtained by performing 20 determinations on 3 sera with the mean activities of 33, 98 and 280 units. The standard deviations were 5.3, 5.7 and 5.0, respectively (the coefficient of variation, 16, 6 and 2%, respectively).

Thirty determinations of the activity were carried out on sera of healthy individuals. The values obtained did not exceed 18 units, and the mean value was 9 units.

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