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Determination of aspartate aminotransferase activity by high-performance liquid chromatography

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Abstract

A sensitive and reproducible assay of aspartate aminotransferase activity based on UV detection of the reaction products after their separation by HPLC is described. The main advantage is the direct measurement of the enzyme activity as micromoles of product (glutamate) formed within a known period of time without any coupled reaction. Further, with the chromatographic method, all components of the reaction mixture are identified, allowing the reaction course to be controlled and the possible presence of side-reactions to be monitored.

1. Introduction

In recent years we have been involved in the development of improved methods for enzyme activity determination [1–3]. For this purpose we have employed HPLC, which offers some advantages over conventional methods such as the simultaneous determination of substrate and products and the possibility of evidencing side-reactions [4–6].

The determination of the activity of the aspartate aminotransferase (EC 2.6.1.1) (AST), an enzyme of great clinical interest, is of importance. The activity of this enzyme is usually determined spectrophotometrically [7,8], through the following reactions:

$$\text{oxoglutarate} + \text{asparate} \overset{\text{AST}}{\rightleftharpoons} \text{glutamate} + \text{oxaloacetate} \quad (1)$$

$$\text{oxaloacetate} + \text{NADH} \overset{\text{MDH}}{\rightleftharpoons} \text{malate} + \text{NAD}^+ \quad (2)$$

where MDH = malate dehydrogenase. The application of the spectrophotometric method has some drawbacks, mainly due to the necessity for a coupled enzymatic reaction, which may be subject to interfering reactions. For instance, glutamate dehydrogenase, which may be present in large amounts in serum samples, could cause NADH oxidation. The spontaneous degradation of NADH is also possible [9]. An assay based on the direct analysis of the enzymatic reaction mixture is thus feasible. For these reasons, a sensitive and reproducible assay for AST activity based on direct UV detection of the products after their separation by HPLC was developed.

2. Experimental

2.1. Apparatus and chromatographic conditions

The chromatographic system consisted of a Perkin-Elmer Series 3B liquid chromatograph with a Rheodyne Model 71055 injection valve

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connected to a Varian Model 2550 variable-wavelength UV–visible detector. UV absorption was measured at 220 nm. A precolumn of 5-μm Supelcosil LC-18 (20 × 4 mm I.D.) and a 5-μm Supelcosil LC-SAX column (250 × 4.6 mm I.D.) from Supelco (Bellefonte, PA, USA) were used.

2.2. Materials

All chemicals were of commercial grade. Aspartate aminotransferase (EC 2.6.1.1), pyridoxal-5-phosphate and lyophilized reference serum (bovine) were obtained from Sigma (St. Louis, MO, USA).

Water was deionized and distilled, and phosphate buffer was filtered through Millipore (Bedford, MA, USA) membrane filters (pore size 0.45 μm).

2.3. Enzymatic reaction procedure

The experimental assay conditions [type and concentration of buffer, pH, substrates and pyridoxal-5-phosphate (coenzyme) concentrations and temperature] were chosen in accordance with the optimized values reported in the literature [10,11].

Reagent A

A 0.303-g amount of tris(hydroxymethyl) aminomethane (Tris) and 3.462 g of aspartic acid (Asp) were dissolved in 50 ml of water and the pH was adjusted to 7.5 with 1 *M* HCl. Then 3.1 mg of pyridoxal-5-phosphate (P5P) were added and the pH was adjusted to 7.5 again. The solution was diluted to 100.0 ml with water (final concentrations 200 m*M* Asp, 25 m*M* Tris and 125 μM P5P). It remained stable for 6 weeks at 4°C.

Reagent B

This consisted of AST solution (0.25 mg/l) in 25 m*M* Tris buffer (pH 7.5) or reference serum (AST activity = 22–38 U/l).

Reagent C

A 2.19-g amount of oxoglutarate was dissolved in 80 ml of water and the pH was adjusted to 7.5 with 1 *M* NaOH. The solution was then diluted to 100.0 ml with water (final concentration 15 m*M*) and remained stable for 6 weeks at 4°C.

Assay conditions

To 2.0 ml of reagent A were added different amounts (0.1–0.3 ml) of reagent B. The resulting mixture was then diluted to 2.3 ml with 25 m*M* Tris (pH 7.5). After preincubation at 37°C for 5 min, the reaction was started by addition of 0.2 ml of reagent C (total volume 2.5 ml). Immediately after mixing and every 20 min thereafter, 20-μl aliquots of the reaction mixture were analysed by the chromatographic method.

The reaction rate was measured by monitoring the increase in the amount of glutamate produced. Glutamate was identified by its retention time and the concentration was determined by integration of peak areas detected at 220 nm and comparison with a calibration graph. One unit of enzyme was defined as the amount forming 1 μmol of glutamate per minute.

3. Results

3.1. Chromatographic conditions

The main problem encountered in the optimization of the chromatographic conditions was the complete resolution of glutamate (Glu) and aspartate (Asp), which differ only slightly in both hydrophobicity and charge. The problem is enhanced by a concentration of substrate 10 000 times greater than that of the reaction product. In order to obtain a good separation, different chromatographic retention mechanisms were tested. Reversed-phase chromatography, carried out with C_{18}, C_8 and C_1 columns (Supelco), did not give good results. Glu and Asp were retained to a slight degree, and poorly separated. No improvement in the separation was achieved when an ion-pair mechanism (on C_{18} and C_8 columns with tetrabutylammonium as ion-pair agent) was used.

A baseline separation of all components of the reaction mixture was obtained with an ion-exchange mechanism employing a LC-SAX

(Supelco) column and eluting with phosphate buffer. In order to optimize the chromatographic conditions, the pH of the eluent was varied over the range 3–7, the buffer concentration (phosphate 0.01 *M*) and flow-rate (1.0 ml/min) being kept constant (Fig. 1). The results obtained showed that the pH of the mobile phase, as expected, is very important for peak resolution. According to the pK_a values, the separation between Glu ($pK_{a_2} = 4.2$) and Asp ($pK_{a_2} = 3.7$) was achieved within the pH range 3.5–4.5. The best resolution was obtained at pH 4.2.

In order to study the influence of phosphate buffer concentration (pH 4.2), the concentration range between $1.5 \cdot 10^{-3}$ and 0.1 *M* was examined (Fig. 2). It is evident that ion concentrations above 0.04 *M* do not permit the separation of Glu and Asp. On the basis of the data obtained, the following chromatographic conditions were chosen.

After 10 min of isocratic elution with a mobile phase composed of 99.9% of $1.07 \cdot 10^{-2}$ *M* phosphate buffer (pH 4.2) (eluent A) and 0.1% of 0.2 *M* phosphate buffer (pH 4.2) (eluent B) at a flow-rate of 1.2 ml/min, with which the separation of Glu and Asp was obtained, a concentration gradient was started (Fig. 3). The gradient profile is represented by the following equation:

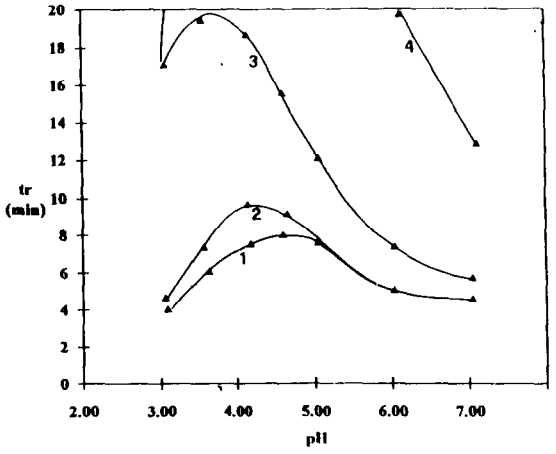

Fig. 1. Effect of pH of the mobile phase on retention times. Conditions: eluent, 0.1 *M* sodium phosphate; flow-rate, 1.0 ml/min; detection at 220 nm. 1 = Glutamate; 2 = aspartate; 3 = oxaloacetate; 4 = oxoglutarate.

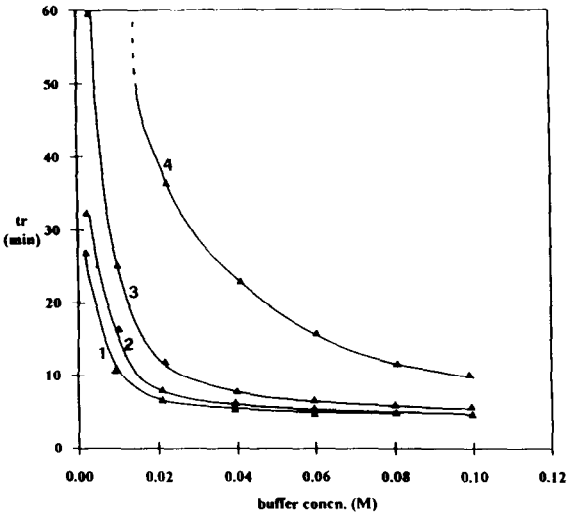

Fig. 2. Effect of buffer concentration in the mobile phase on retention times. Conditions: eluent, sodium phosphate (pH 4.2); flow-rate, 1.0 ml/min; detection at 220 nm. 1 = Glutamate; 2 = aspartate; 3 = oxaloacetate; 4 = oxoglutarate.

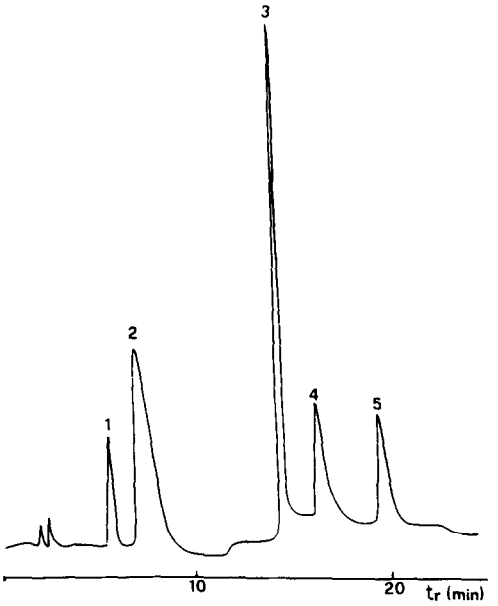

Fig. 3. Chromatogram of a standard mixture of (1) glutamate, (2) aspartate, (3) oxaloacetate, (4) pyridoxal-5-phosphate and (5) oxoglutarate. Column, SAX; eluent composition as reported in the text; detection at 220 nm.

$$P(t) = P(\mathrm{i}) + [P(\mathrm{f}) - P(\mathrm{i})](t/T)^n \quad (1)$$

where $P(t)$ = eluent A percentage as a function of time, n = gradient curvature (0.5), $P(\mathrm{i})$ = initial eluent A percentage (99.9%), $P(\mathrm{f})$ = final eluent A percentage (50%), t = elapsed time (min) and T = length of the time segment (8 min). Simultaneously, a gradient flow-rate represented by Eq. 1, where $P(t)$ = flow-rate, $P(\mathrm{i})$ = 1.2 ml/min and $P(\mathrm{f})$ = 1.5 ml/min, was also used in order to shorten the retention time of oxoglutarate. The equilibrium time was 12 min. The retention times (Fig. 3) for the products were 6.1 min for glutamate and 14.8 min for oxaloacetate. They resulted in baseline separation from the substrates (7.5 min for aspartate and 20.2 min for oxoglutarate).

Glutamate determination was preferred to oxaloacetate determination because the latter is chemically unstable and is subject to enolization and isomerization equilibria [12]. The quantitative evaluation of the peak areas was performed by integration. The system was calibrated by injecting different known amounts of glutamate ranging from 0.1 to 20 nmol in 25 m*M* Tris buffer (pH 7.5) and measuring the areas under the peaks. The resulting calibration graph was linear over the concentration range investigated.

3.2. Enzymatic assay

The proposed method was applied to the determination of AST activity in reference serum. Time-course experiments showed that the rate of glutamate formation proceeded linearly up to 150 min at 37°C.

In Fig. 4, AST activity is shown as a function of amount of serum. Excellent linearity was observed between 0.03 and 0.3 ml of serum. The within-day reproducibility of the method (determined on ten replicate samples) was 3.0% at an enzyme activity of 30 U/l.

AST activity in serum was also determined by the spectrophotometric method employing a Sigma diagnostic kit; the within-day reproducibility was 5.5%. In addition, the activity values obtained by HPLC were in each instance 11% higher than those obtained by the spectrophotometric method. This can probably be ascribed to the inhibition caused by the phosphate buffer [13] used in the spectrophotometric method.

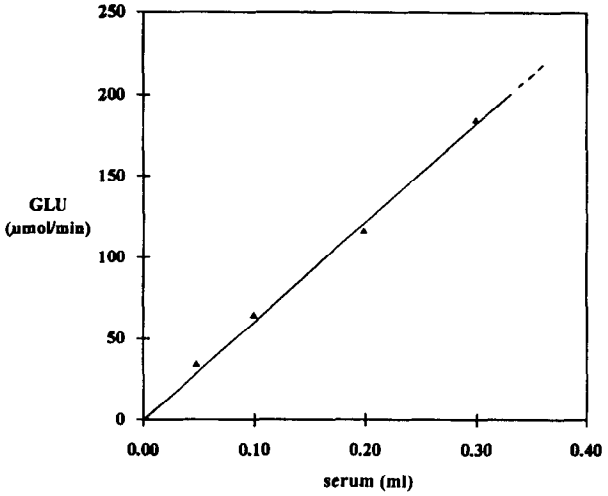

Fig. 4. Glutamate formed after incubation for 30 min at 37°C as a function of amount of serum. Chromatographic conditions as reported in the text.

4. Conclusions

In conclusion, the main advantage of the proposed method is the direct measurement of the enzyme activity as micromoles of product (glutamate) formed in a known time, without the use of any coupled reaction. Further, in the chromatographic method, all components of the reaction mixture are monitored, allowing the reaction course to be controlled and the possible presence of side-reactions to be detected. In addition, the limit of detection of the HPLC method, which permits the determination of 3 U/l with a 20-μl of injection volume and a 30-min incubation time, renders the method more suitable for the measurement of lower levels of AST activity than the spectrophotometric method (limit of detection = 9 U/l).

5. Acknowledgement

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6. References

[1] V. Carunchio, A.M. Girelli, M. Sinibaldi and A.M. Tarola, *Chromatographia*, 25 (1988) 870.

[2] S. Canepari, V. Carunchio, A.M. Girelli and A. Messina, *Chromatographia*, 32 (1991) 23.

[3] S. Canepari, V. Carunchio, A.M. Girelli and A. Messina, *J. Chromatogr.*, 616 (1993) 25.

[4] E. Rossomando, *J. Chromatogr.*, 566 (1991) 275.

[5] P.L. Mauri, P.G. Pietta and M. Pace, *J. Chromatogr.*, 566 (1991) 327.

[6] T. Nagatsu, *J. Chromatogr.*, 566 (1991) 287.

[7] S. Ohmori, Y. Tanaka, M. Ikeda and K. Hirota, *Anal. Biochem.*, 112 (1981) 204.

[8] S.F. Velick and J. Vavra, in P.D. Boyer (Editor), *The Enzymes*, Vol. 6, Academic Press, New York, 2nd ed., 1973, Ch. 15.

[9] M. Horder and R. Rej, in H.U. Bergmeyer (Editor), *Methods of Enzymatic Analysis*, Vol. 3, Verlag Chemie, Weinheim, 3rd ed., 1983, Ch. VI.

[10] K. Jung and K.D. Grutzmann, *Clin. Chim. Acta*, 81 (1977) 299.

[11] Committee of the Scandinavian Society for Clinical Chemistry and Clinical Physiology, *Scand. J. Clin. Lab. Invest.*, 33 (1974) 291.

[12] C.I. Pogson and R.G. Wolfe, *Biochem. Biophys. Res. Commun.*, 46 (1972) 1048.

[13] H.U. Bergmeyer, P. Scheibe and W. Wahlefeld, *Clin. Chem.*, 24 (1978) 58.