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Simultaneous assay for aspartate aminotransferase and guanase in human serum by high-performance liquid chromatography

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Abstract

A simple high-performance liquid chromatography (HPLC) assay for the simultaneous determination of guanase and aspartate aminotransferase (AST) activities in a single serum sample is described. The method is based on direct detection of enzymatically formed products xanthine and glutamate, respectively. The procedure is sensitive, precise (C.V. below 2% for guanase and 3% for AST), suitable for routine purposes and requires only 100 μl of sample. Kinetic measurements have shown the guanase activity to have an apparent Michaelis constant of 24.5 μ*M* and the AST activity of 11.1 and 0.18 m*M* for aspartate and oxoglutarate, respectively, at 37°C in Tris–HCl buffer (pH 7.5).

Keywords: Aspartate aminotransferase; Guanase; Enzymes; Xanthine; Glutamate; Oxoglutamate

1. Introduction

The liver is a rich source both of aspartate aminotransferase (AST) and guanase [1,2], and their determination is of great importance in the diagnosis of liver disorders. In particular, guanase is potentially a more specific indicator of liver damage than AST, as its value remains normal in patients with a variety of non-liver-related diseases, including acute myocardial infarction without liver damage [3].

AST and guanase are present in normal serum at low concentrations. However, when tissue cells are injured, the enzymes diffuse into the bloodstream where a temporarily high degree of activity occurs. The degree of activity depends principally on the extent of the tissue damage and on the time course following the tissue injury. We have observed that, although AST is a more sensitive measure than guanase, the latter returns to the normal value more rapidly than aminotransferases, thus providing an early indicator of remission [4].

As the comparison between AST and guanase activity values is relevant for diagnostic purposes [4,5], a simultaneous determination is needed.

HPLC is a suitable technique for this purpose as it allows the resolution of the reaction mixture components. Furthermore, previous studies [6–9] have provided evidence that HPLC methods for enzyme assays allow us to overcome the main drawbacks and interferences encountered in conventional spectrophotometric methods.

The aim of this paper is the optimization of a HPLC method for the determination of both enzymes in a single serum sample.

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2. Experimental

2.1. Chemicals and reagents

Guanine (gua), aspartate (asp), oxoglutarate (oxoglu), xanthine (xa), glutamate (glu), oxolacetate (oxoac), lyophilized control serum and the diagnostic kit of AST were obtained from Sigma (St. Louis, MO, USA). Distilled water and phosphate buffer were filtered through Millipore membrane filters (pore size 0.45 μm). All other chemicals used were of analytical grade.

2.2. Biological materials

The investigations were carried out on normal and pathological human sera. Serum samples were stored at −20°C.

2.3. Apparatus and chromatographic conditions

The apparatus used was a Kontron Series 432 chromatograph with a Rheodyne 7125 injection valve connected to a Kontron 422 variable-wavelength UV–Vis detector. The refillable pellicular C_{18} guard column was from Alltech Italia (Sedriano, Milano), and the 5-μm Supelcosil LC-18 (250×4.6 mm I.D.) column was from Supelco (Bellefonte, PA, USA). The elution with 5 m*M* phosphate buffer was performed at room temperature using a pH gradient from 3.0 to 4.9 containing 1-hexanesulphonate (5 m*M*). The flow-rate was 1.2 ml/min. The absorbance values of the effluent were monitored at 210 nm.

2.4. Assay conditions

The reaction mixture contained in its final volume of 2.7 ml: 100 m*M* L-aspartate, 4.44 m*M* 2-oxoglutarate, 122 μ*M* guanine and 18.5 m*M* Tris–HCl. This assay mixture was preincubated for 2 min at 37°C, and the reaction was then initiated by adding 100 μl of serum to the mixture. Immediately after mixing, 40 min later, 20-μl aliquots of the reaction mixture were analyzed by the chromatographic method.

The reaction rates were measured by monitoring the increase in the amount of glutamate and xanthine produced. The concentrations of both analytes were determined by integration of peak areas detected at 210 nm and by comparison with the calibration curves.

3. Results

3.1. Chromatographic conditions

The chromatographic separation of the reaction mixture was more complex than that previously obtained for the AST [8] and guanase [9] assays, because of the presence of compounds with very similar chromatographic behaviour (glu, asp, oxoglu and oxoac, and purines such as xanthine and guanine) with hydrophobicity and charge very different from the above-mentioned compounds. A baseline separation of all components of the reaction mixture was obtained with an ion pair mechanism employing an LC-18 (Supelco) column and by elution with a phosphate buffer (5 m*M*) containing 1-hexanesulphonate (5 m*M*) as counter ion. In order to optimize the chromatographic conditions, the pH of the eluent was varied from 2.7 to 7 (Fig. 1). According to the p*K* values, the best separation of the acidic compounds was achieved at pH 2.7. The retention of xanthine is not influenced by pH while a reasonable retention of guanine was achieved at pH>4.8. On the basis of the data obtained, the following chromatographic conditions were chosen.

After 8 min of isocratic elution with a mobile phase composed of 99.9% of 5 m*M* phosphate buffer (pH 2.7) (eluent A) and 0.1% of phosphate buffer (pH 4.9) (eluent B) containing 5 m*M* 1-heptanesulphonate at a flow-rate of 1.2 ml/min, a pH linear gradient (2.7–4.9) is started and 99.9% of eluent B is reached in 9 min. The return to initial conditions (pH 4.9–2.7) starts at 17 min and takes 2 min. The equilibration time is 12 min.

Glutamate and xanthine were completely resolved from the other components of serum samples, as shown by the elution profile in Fig. 2.

3.2. Enzymatic assays

In order to define the optimal standard conditions of enzymatic reactions, the effect of several parameters such as pH, [asp], [oxo] and [gua] concentrations was examined using a control serum.

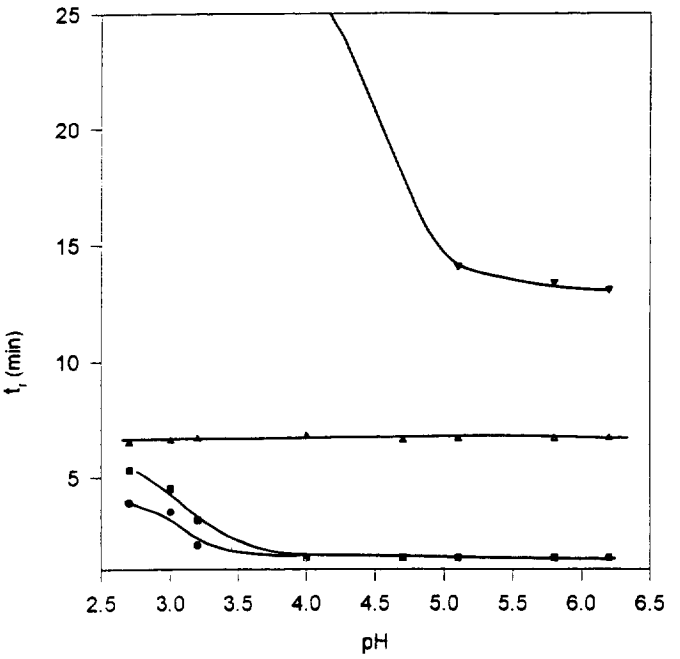

Fig. 1. Effect of pH of the mobile phase on retention times. Chromatographic conditions: eluent, 5 m*M* sodium phosphate containing 5 m*M* 1-hexanesulphonate; flow-rate, 1.2 ml/min; detection at 210 nm. (●) Aspartate, (■) glutamate, (▲) xanthine, (▼) guanine.

The optimum pH was 7.5 for AST and 8.0 for guanase. Due to the lower sensitivity of the AST assay compared to that of guanase, a pH of 7.5 was chosen.

The optimal substrate concentrations for the AST assay were determined by measuring the dependence of v on [oxo] by keeping [asp] constant, and vice versa (Figs. 3 and 4). The concentrations of 100 m*M* L-aspartate and 4.4 m*M* 2-oxoglutarate are a good compromise for the AST determination. The chosen conditions are the most convenient and economical, without an inconveniently high concentration of amino acid (solubility, price) and an impractically high concentration of 2-oxoglutarate (inhibitory effect).

A mathematical treatment of data according to bisubstrate reaction [10] has allowed us to view the "ping pong bi–bi" mechanism, as seen in Fig. 5: the dependencies of $1/v$ on $1/[\text{oxo}]$ at various constant asp concentrations gives parallel straight lines according to the following equation:

$$\frac{1}{v} = \frac{K_{m_{oxo}}}{V}\frac{1}{[\text{oxo}]} + \frac{1}{V}\left(1 + \frac{K_{m_{asp}}}{[\text{asp}]}\right) \quad (1)$$

In addition, the K_m values of L-aspartate (11.1 m*M*) and of 2-oxoglutarate (0.18 m*M*), and a maximal velocity (V) value of 74.9 pmol/min have been determined.

An optimal guanine concentration of 122 μ*M* has been fixed for the guanase assay. The mathematical treatment of Lineweaver–Burk yields a Michaelis constant (K_m) of 23.5 μ*M* in Tris–HCl buffer at 7.5. This value appears to be slightly higher compared to the value of 13.3 obtained at pH 8.0 in our previous work [9]. The discrepancy is probably due to the different pH, reconfirming the fact that guanine has a higher affinity for the enzyme at pH 8.0 than at pH 7.5.

The rate of glutamate and xanthine formation proceeded linearly up to 70 min at 37°C. We selected an incubation time of 40 min as a standard assay condition.

AST and guanase activities as a function of enzyme concentrations are shown in Fig. 6 (r^2 = 0.993 and 0.992, respectively).

The reproducibility of ten assays of the same samples was 2.7% for an AST activity of 45 U/l and 2% for a guanase activity of 1 U/l.

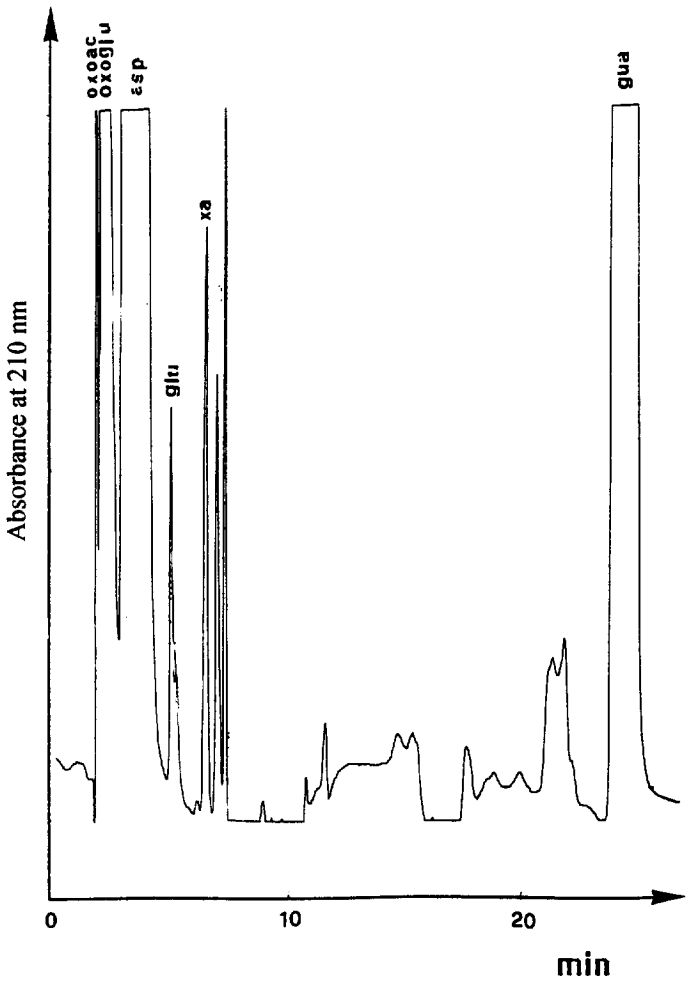

Fig. 2. Chromatogram of a human serum sample. Column, RP-18, eluent composition as reported in the text; detection at 210 nm; $s=1$, atten. = 2.

3.3. Inter-method comparison

The accuracy of the proposed HPLC method was evaluated by comparison of the results obtained for normal and pathological sera with the spectrophotometric [11] and HPLC methods [9] for AST and guanase assays, respectively. The correlations, reported in Figs. 7 and 8, are good in both cases ($r^2=0.986$ and 0.991 for AST and guanase, respectively).

The AST activity values obtained by the spectrophotometric method (y) are in each instance higher than those obtained by the present one (x), as demonstrated by the regression equation $y=0.79x+5.29$. This discrepancy is not observed with the AST standard solution and can probably be ascribed to the presence of positive interferences in sera (i.e. glutamate dehydrogenase that could cause NADH oxidation and the spontaneous degradation of NADH) which probably cause a higher $\Delta A/\text{min}$ value than expected.

A linear regression of $y=1.0966x$ was obtained by comparison of the present method (x) with our previous HPLC guanase assay (y). The deviation

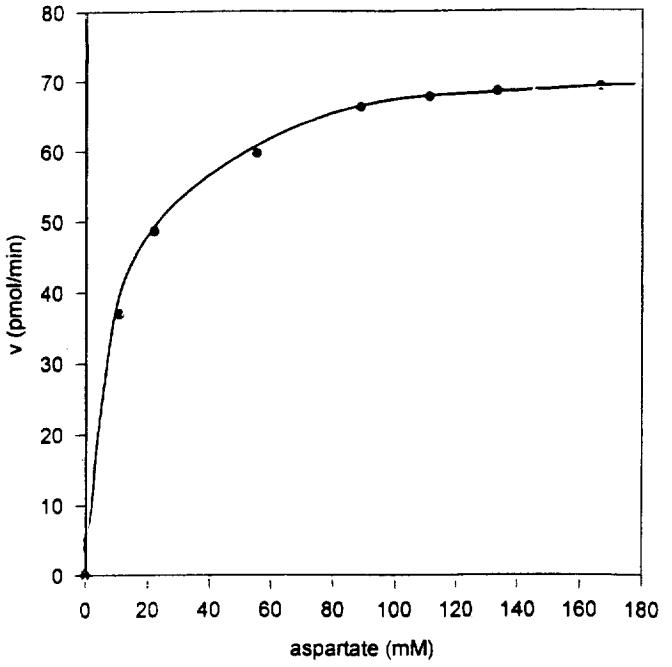

Fig. 3. Effect of aspartate concentration on the rate of AST reaction at 8.33 m*M* 2-oxoglutarate concentration. Assays were performed as described in Section 2.

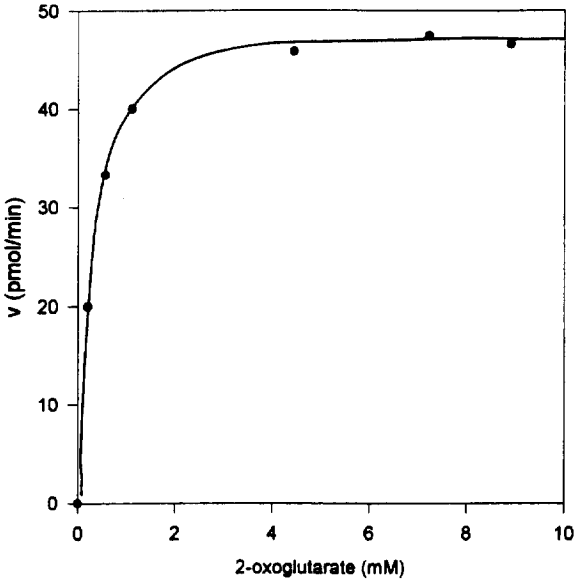

Fig. 4. Effect of 2-oxoglutarate concentration on the rate of AST reaction at 100 m*M* aspartate concentration. Assays were performed as described in Section 2.

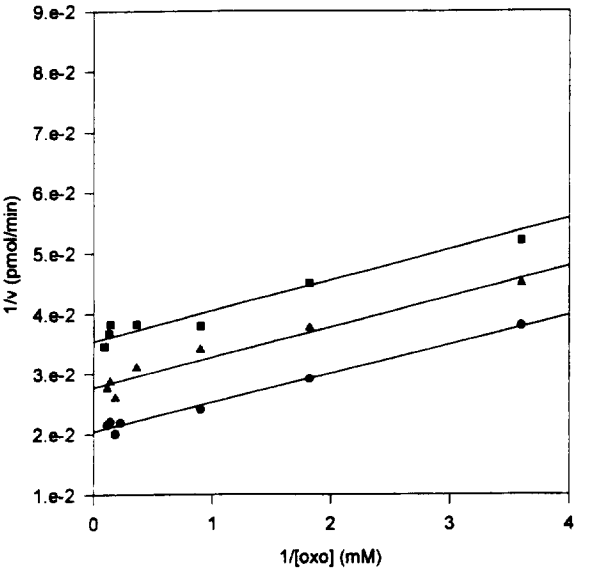

Fig. 5. Reciprocal plots: $1/v$ vs. 1/[oxo] at different aspartate concentrations: (●) 100 m*M*, (▲) 77.6 m*M*, (■) 50 m*M*.

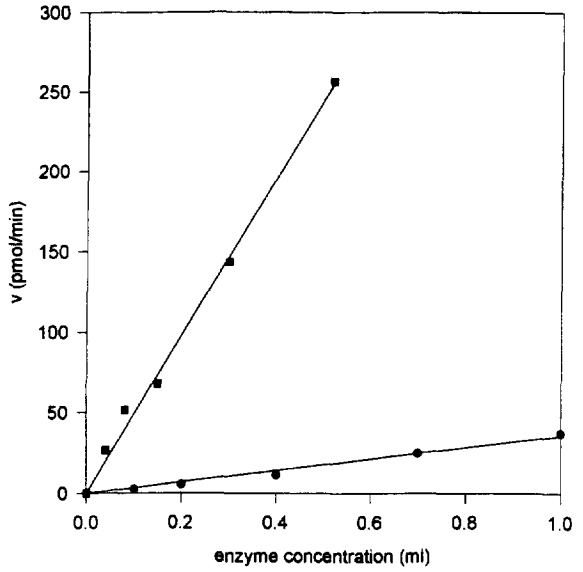

Fig. 6. AST and guanase activity, expressed as the amount of glutamate (■) and xanthine (●) formed in 1 min, respectively, at 37°C, as a function of enzyme concentration. The AST and guanase concentrations were changed by using different volumes of enzyme stock solutions (AST, 70 U/l; guanase, 5 U/l). Assays were performed as described in Section 2.

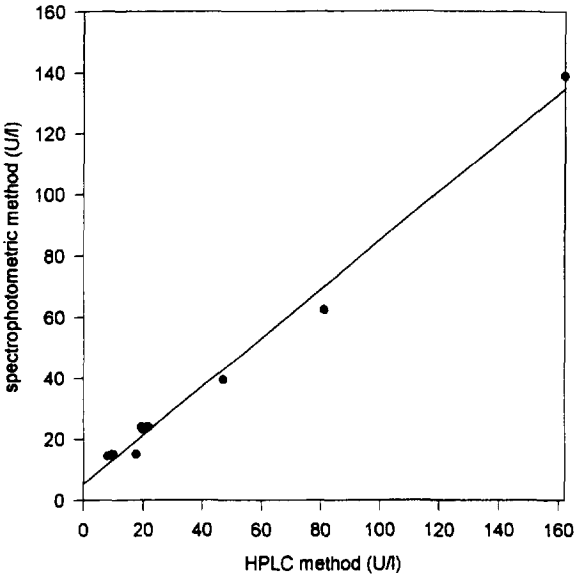

Fig. 7. Correlation of AST activity values determined by the spectrophotometric and HPLC methods.

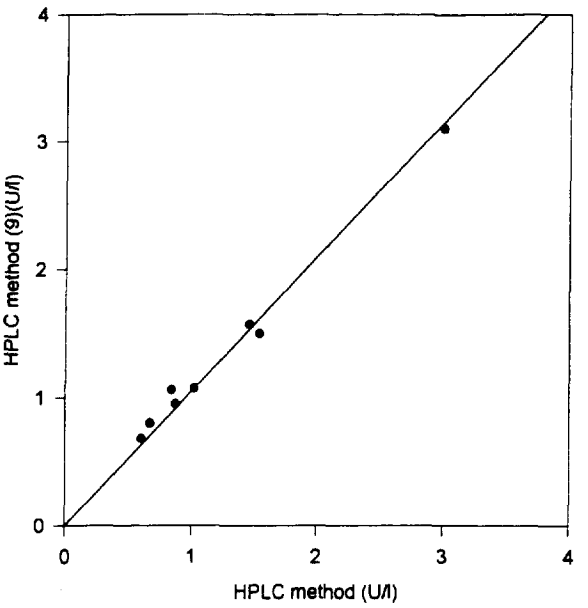

Fig. 8. Correlation of guanase activity values determined by the HPLC method reported in the literature [9] and the one proposed in this paper.

from the theoretical curve ($y=x$) is due to the difference in pH (7.5 in the present method vs. 8.0 in the previous one).

4. Conclusion

We have developed a method for a simultaneous assay for AST and guanase activities in human serum using HPLC. The method is sensitive enough to detect picomolar amounts of xanthine and glutamate released into the reaction mixture, and can simultaneously and accurately measure guanase and AST activity as low as 0.1 and 5 U/l, respectively.

Moreover, only 0.1 ml of serum is required in the present method for both analyses, while 0.5 ml of serum for spectrophotometric assay of AST [11] and 0.2 ml for guanase activity HPLC detection [9] are required.

The absence of any coupled reaction has permitted a high accuracy and reproducibility for both assays.

In conclusion, the proposed method should prove very useful for the diagnosis of liver disease because the simultaneous assay of two enzyme activities

improves the information received for a single serum. Besides, considering that the sensitivities and specificities of these markers vary considerably, their simultaneous determination may be very useful for differentiation of liver diseases, as well as for assessing liver damage.

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