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New method for guanase activity measurement by high-performance liquid chromatography

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ABSTRACT

A rapid isocratic high-performance liquid chromatographic method for the determination of guanase (EC 3.5.4.3) activity is proposed. The method is highly reproducible, with a coefficient of variation of less than 1%, and requires only *ca.* 10 min for a complete chromatographic separation of the enzyme reaction mixture. The method allows the detection of nanomolar changes in the concentrations of both the substrate and the product, and does not require additional reactions or sample pretreatment. Kinetic studies with the proposed method showed the guanase activity to have an apparent Michaelis constant of 13.3 and 8.5 μM, and a maximum rate of 1.95 and 3.84 pmol/min per mg protein at 37°C, in Tris–HCl and phosphate buffer, respectively.

INTRODUCTION

Guanase (guanine aminohydrolase, EC 3.5.4.3) catalyses the hydrolytic deamination of guanine to xanthine and ammonia. Its activity is very low (less than 5 U/l) in normal serum, and increases by up to ten-fold or more in patients with liver disease, especially acute hepatitis and metastatic carcinoma of the liver [1–3]. The measurement of guanase activity could represent a safer test for the evaluation of cell damage than aspartate-L-aminotransferase (AST) and alanine-L-aminotransferase (ALT) [4–7]. Furthermore, in the case of banked blood, this value can be useful for preventing hepatic injury in cases of blood transfusion [8,9]. Nevertheless, the application of this assay for diagnostic purposes is currently limited by the lack of an analytical method that is accurate and rapid enough for routine use.

In recent years, several methods have been proposed for the assay of guanase, such as the spectrophotometric measurement of the decrease in substrate concentration [10,11], the spectrophotometric determination of uric acid [12,13] or hydrogen peroxide [14–17] formed in the presence of xanthine oxidase and the determination of the ammonia produced either by a kinetic method based on an NADH-linked reaction [18] or by colorimetric phenol–hypochlorite reaction [19–21]. A new method [6] has been proposed, based on the detection of guanase activity by flow-injection analysis with fluorescence detection: xanthine, formed enzymatically from the substrate, is degraded to hydrogen peroxide by immobilized xanthine oxidase, uricase and horseradish peroxidase in columns connected in sequence in the flow-line. All these methods require the deproteination of the sample and/or involve additional reactions.

The reliability of chromatographic techniques in the development of improved methods for enzymic assay [22,23] has led us to examine the application of high-performance liquid chromatography (HPLC) to the measurement of guanase

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activity. Special attention has been paid to the achievement of conditions for the direct determination of both substrate and product without the use of any additional reaction, as well as to the applicability of the proposed method to routine diagnostic assays.

EXPERIMENTAL

Chemicals and reagents

Guanine, xanthine, guanase and lyophilized reference serum (bovine) were obtained from Sigma (St. Louis, MO, USA). Deionized and distilled water and acetate buffer were filtered through Millipore membrane filters (pore size 0.45 μm). All other chemicals used were of analytical grade.

Apparatus and chromatographic conditions

A Perkin-Elmer chromatograph Series 3B with a Rheodyne 71055 injection valve, connected to a Varian 2550 variable-wavelength UV–VIS detector, was used. The absorbance values of the effluent were monitored at 270 nm, which was the best wavelength for the determination of both xanthine and guanine.

A guard-column of Supelcosil LC-18 (5 μm) (20 mm × 4.0 mm I.D.) and a Supelcosil LC-18 (5 μm) (250 mm × 4.6 mm I.D.) column were from Supelco (Bellefonte, PA, USA). The elution was performed at room temperature with a 0.05 *M* acetate buffer (pH 6.0) as eluent. The flow-rate was 1.2 ml/min.

Enzymic assay conditions

Substrate solution. Guanine (25 mg) was dissolved in *ca.* 10 ml of 0.1 *M* NaOH, and the solution was made up to 100.0 ml with water (final concentration 1.65 m*M*). It was stable for six days at 4°C.

Buffer solution. Tris(hydroxymethyl)aminomethane (3.03 g) was dissolved in *ca.* 900 ml of water, and the pH was adjusted to 8.0 with 1 *M* HCl. The solution was then made up to 1 l with water (final concentration 0.025 *M*).

Enzyme solution. A 0.1-ml volume of a solution containing 0.2 mg of enzyme was diluted to 1 ml with 0.025 *M* Tris–HCl buffer (pH 8.0).

Serum sample. A 0.1-ml volume of a solution containing 0.2 mg of enzyme was added to 0.9 ml of reference serum in order to obtain an enzyme activity of the same order of magnitude as that detected in pathological serum.

The assay for the enzymic activity of guanase was performed as follows: to 200 μl of substrate solution were added 3 ml of Tris–HCl buffer and 20 μl of enzyme solution or serum sample. The solution was then made up to 5 ml of with water. The mixture was stored at 37°C for 20 min, and the reaction was stopped with 30 μl of 70% perchloric acid in an ice-bath. Aliquots of 20 μl were injected into the HPLC system.

RESULTS

Fig. 1 shows a representative elution profile of a mixture of guanine and xanthine. The compounds were well separated under the described chromatographic conditions within 10 min. The detector response was found to be linear in the range 1–500 pmol for xanthine and 2–1000 pmol for guanine. Elution profiles of the enzyme reaction mixture are presented in Fig. 2. The conversion of guanine into xanthine by guanase is evidenced by a concomitant decrease in the guanine concentration and with the appearance of a xanthine peak. The enzymic activity can then be

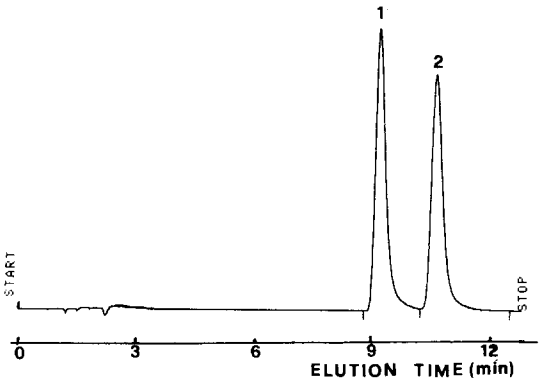

Fig. 1. Chromatogram of a standard mixture of guanine (1) and xanthine (2). Separation was achieved on a 5-μm Supelcosil LC-18 column (250 mm × 4.6 mm I.D.) eluted isocratically with 0.05 *M* acetate buffer (pH 6.0) at a flow-rate of 1.2 ml/min. The effluent was monitored at 270 nm.

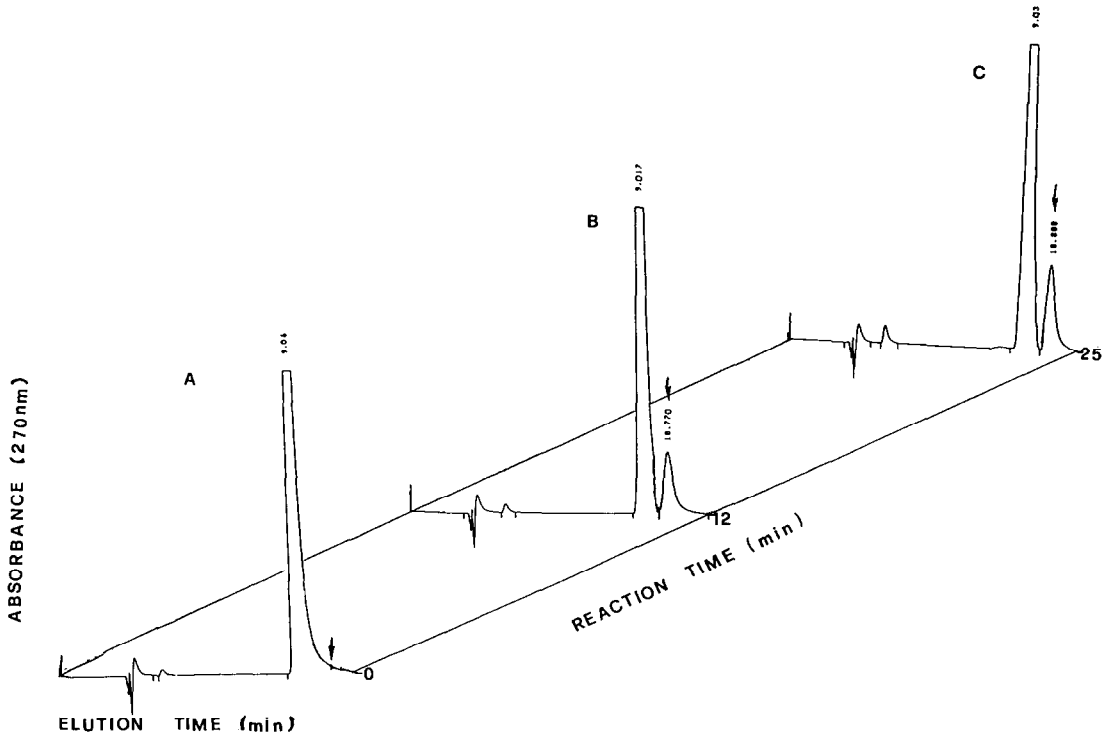

Fig. 2. Time-course of formation of xanthine from guanine as measured by guanase activity. The reaction mixture contained, in a final volume of 5 ml, 0.45 mmol of Tris–HCl (pH 8.0), 0.33 μmol of guanine and 20 μl of guanase solution (4 μg). A 20-μl aliquot of sample was injected. Chromatograms were obtained after incubation for (A) 0 min, (B) 12 min, and (C) 25 min. The arrows indicate the elution time for the reaction product (xanthine). Chromatographic conditions as in Fig. 1.

determined simultaneously from the rate of disappearance of the substrate and from the appearance of the product. No extraneous peaks appear in the chromatogram, which means that no secondary reactions occur.

The enzyme reaction was found to be linearly related to time for *ca.* 1 h at 37°C (Fig. 3). The enzymic activity was then routinely obtained from a one-point determination by stopping the reaction with 30 μl of 70% perchloric acid after 20-min incubation, in order to achieve the best compromise between analysis time and method precision.

In order to study the effect of the reaction medium, the kinetic parameters for the enzymic hydrolysis of guanine were determined in a set of experiments employing various substrate concentrations of both Tris–HCl and phosphate buffers (Fig. 4). The measurements were carried out at pH 8.0, which is considered the optimum condition by many investigators [6,7,20] and is close to the physiological value. The reaction rate is linear at low values of substrate concentration, and reaches a plateau at values greater than 40 and 80 μM, respectively, for the above-mentioned buffers. The Lineweaver–Burk analysis of these data (Fig. 4) yields a Michaelis constant (K_m) of 13.3 μM in Tris–HCl buffer and 7.84 μM in phosphate buffer, and maximum rate (V_{max}) values of 1.95 and 3.84 pmol/min, respectively.

From the reported data it is evident that in Tris–HCl buffer the reaction is slower than in phosphate, but a lower guanine concentration (66 μM) can be employed to allow optimization of chromatographic conditions. Thus the Tris–HCl buffer was chosen as the reaction medium.

As shown in Fig. 5, an excellent linearity was observed when the guanase activity, expressed as

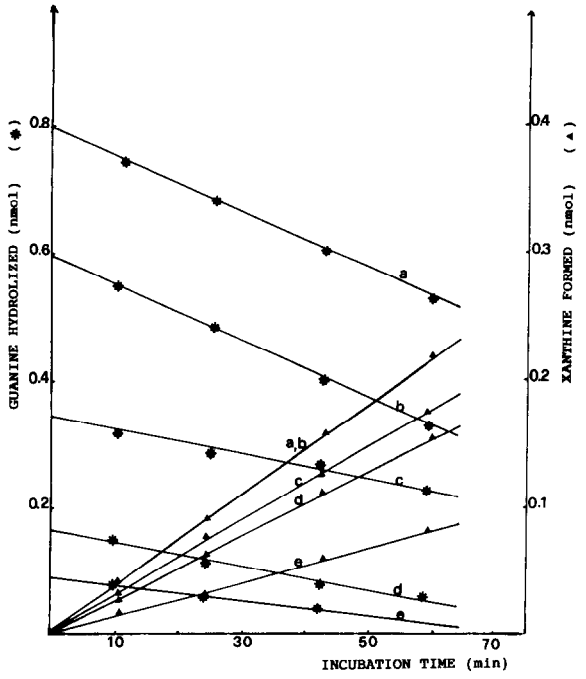

Fig. 3. Guanine (*) and xanthine (▲) amounts in the injected volume (20 μl) as a function of incubation time at different initial guanine concentrations: (a) 72.4 μ*M*; (b) 49.5 μ*M*; (c) 33 μ*M*; (d) 16.5 μ*M*; (e) 10 m*M*. Operating conditions as in Fig. 2.

the amount of xanthine formed enzymically, was plotted as a function of enzyme concentration in the absence (a) and the presence (b) of reference serum. It is evident that, in the presence of reference serum, an activation of 3.8% is achieved, which could be ascribed to the interaction of the enzyme with the metal ions in the serum sample.

In addition, the proposed HPLC method was compared with the test procedure described by Roush and Norris [10] and Demetriov *et al.* [24], based on absorbance changes at 245 nm. For this purpose ten replicate measurements of the enzymic assay systems were compared. The data obtained are listed in Table I, together with the coefficients of variation (C.V.). It is evident that the mean values of the guanase activities measured by the two methods were in close agreement, but the C.V. were very different: 0.7% for our method and 8.0% for the spectrophotometric one. The repeatability of the HPLC method is very high, even compared with other methods reported in literature (7.95% for xanthine oxidase dehydrogenase [11]; 3.17% for NADH [18]; 2.96% for NH_3-phenol [21] and 1.2% for the flow analysis method [6]).

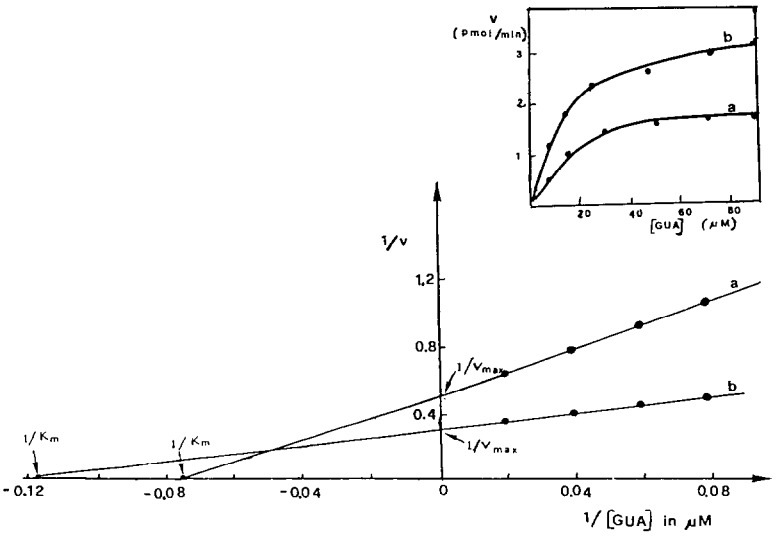

Fig. 4. Lineweaver–Burk and Michaelis–Menten plots for the evaluation of the kinetic parameters of guanase in Tris–HCl (a) and phosphate (b) buffers. The main panel shows the Lineweaver–Burk plot and the inset panel shows the Michaelis–Menten plot. The standard incubation mixture contained 10 μl (2 μg of protein) of the enzyme, and incubation was carried out for 20 min at 37°C with different concentrations or guanine.

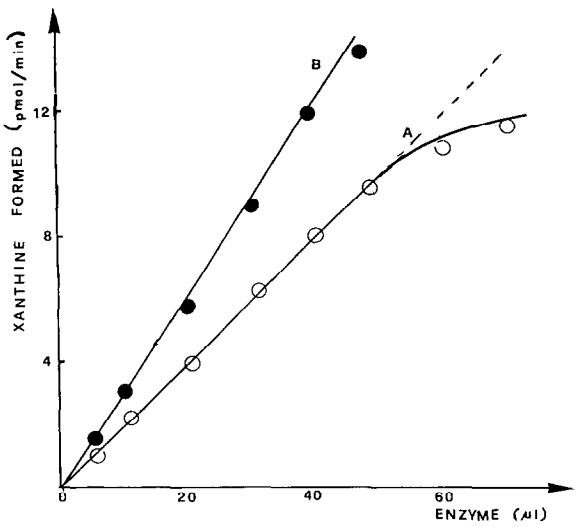

Fig. 5. Guanase activity in the standard incubation mixture in the absence (A) and presence (B) of reference serum, as a function of enzyme concentration. Chromatographic conditions as in Fig. 1.

TABLE I

COMPARISON BETWEEN HPLC AND SPECTROPHOTOMETRIC RESULTS FOR MEASUREMENTS OF GUANASE ACTIVITY IN REFERENCE SERUM

Sample No.	Guanase activity (U/l)	
	HPLC	Spectrophotometry
1	72.98	65.23
2	72.06	72.63
3	72.56	76.92
4	73.12	79.78
5	72.75	78.94
6	72.15	68.03
7	71.83	70.32
8	72.00	66.19
9	73.40	77.60
10	72.95	63.80
Mean value	72.58	71.94
C.V. (%)	0.71	7.99

CONCLUSION

The results clearly illustrate the advantages of HPLC in the determination of guanase activity, namely: (1) high sensitivity, which allows variations both of the substrate and the product to be determined at the nanomole level; (2) the absence of auxiliary reactions, which are generally required in the measurement of enzymic activity; (3) simplicity, ascribed to the possibility of direct analysis of the reaction mixtures by HPLC without any deproteination; (4) excellent specificity, owing to the use of a separation technique (HPLC) rather than a spectrophotometric method; (5) accuracy and reproducibility, attested by the low C.V. (1%), probably owing to the absence of interfering substances; (6) rapidity; (7) the possibility of simultaneously monitoring reagents and products present in the mixture, thus highlighting anomalous behaviour or secondary reactions.

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