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Journal of Chromatography B, 780 (2002) 13–19

JOURNAL OF CHROMATOGRAPHY B

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Capillary electrophoresis assay of alanine:glyoxylate aminotransferase activity in rat liver

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Received 11 March 2002; received in revised form 10 June 2002; accepted 10 June 2002

Abstract

We measured hepatic alanine:glyoxylate aminotransferase (AGT) activity using capillary electrophoresis. After rat liver homogenate was incubated in the presence of substrates and pyridoxal 5′-phosphate, the pyruvate and glycine produced by AGT were measured. The AGT activity was 10.02±0.31 μmol pyruvate/h/mg protein and 10.21±0.15 μmol glycine/h/mg protein. This method is relatively simple and shows superior sensitivity, allowing the measurement of enzyme activity in 5 μg of protein. Therefore, it appears to be suitable for laboratory use and may also have advantages for measuring AGT activity in liver biopsy specimens.

Keywords: Alanine:glyoxylate aminotransferase; Enzymes

1. Introduction

Urinary oxalate plays an important role in the formation of calcium oxalate stones, and an increase of urinary oxalate excretion precipitates calcium oxalate crystallization into stones in the urinary tract. The principal source of urinary oxalate is endogenous biosynthesis and its immediate precursors are ascorbate and glyoxylate [1]. Under physiological conditions, approximately 50–60% of urinary oxalate is derived from the endogenous metabolism of glyoxylate [2,3]. Glyoxylate is related to the metabolism of glycine and glycolate, and is enzymatically interconvertible with these substances as well as being partly transformable into oxalate [3–5].

Alanine:glyoxylate aminotransferase (AGT) is quantitatively the most important enzyme catalyzing transamination between alanine (an amino acid substrate) and glyoxylate (an alpha-keto acid substrate) to form pyruvate and glycine. There are reports of the measurement of AGT activity and which expressed the rate of pyruvate production (unit: μmol pyruvate/h/mg protein) from substrates (alanine and glyoxylate) incubated with liver extract [6,7]. In an earlier procedure, LDH/NADH-mediated colorimetry was used for the determination of pyruvate [8–10], and, in another procedure, incubation with L-[^{14}C]-alanine followed by high-voltage paper electrophoretic separation of the radioactive pyruvate was used [11]. However, these procedures are less sensitive and more time-consuming. Recently, a high-performance liquid chromatographic procedure

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1570-0232/02/$ – see front matter
PII: S1570-0232(02)00407-5

with high sensitivity was also used for measurement of AGT activity [7,12], but it requires a large quantity of specimen and buffer for analysis.

Capillary electrophoresis is a powerful separation technique that can achieve high resolution. Various capillary electrophoresis methods have been developed for the analysis of organic acids and amino acids [13–15]. In our previous studies, we performed oxalate and glyoxylate determination in urine and liver specimens using capillary electrophoresis [16,17]. The simplicity, high sensitivity, rapidity, and the small quantity of specimen required of the capillary method prompted us to employ it to measure hepatic AGT activity. In the present study, we examined the suitability of the capillary electrophoresis technique for determining AGT activity in rat liver.

2. Experimental

2.1. Reagents

Analytical reagent grade chemicals were used throughout these experiments. Glyoxylic acid monohydrate, sodium pyruvate, L-alanine, glycine, sucrose, and 1 N aqueous KH_2PO_4 solution were purchased from Wako (Japan), and pyridoxal 5′-phosphate was obtained from Sigma–Aldrich (Japan). Potassium phosphate buffer (pH 8.0, 0.2 mol/l) was prepared weekly. Glyoxylate, pyruvate, alanine, and glycine were dissolved in water to make stock standard solutions (100 mmol/l) that were stored at −20 °C, and working solutions were prepared daily. Deionized-purified water from a Milli-Q water purification system (Nihon Millipore, Japan) was used for dilution.

2.2. Liver specimens

Six male Wistar rats weighing 250–300 g were fed standard laboratory chow and had free access to tap water. The liver was removed from each rat under anesthesia with diethyl ether, and a liver specimen (10 mg) was immediately homogenized at 4 °C in 400 μl of a buffer that contained 100 mmol/l potassium phosphate (pH 8.0) and 250 mmol/l sucrose. The homogenate was centrifuged at 4 °C for 10 min at 11 000 r.p.m., and protein in the supernatant was quantified using an ultraviolet (UV) spectrophotometer by reading the absorbance at 280 nm with bovine serum albumin as the standard.

2.3. Enzyme reaction

The reaction solution for measuring AGT activity was prepared according to Petrarulo et al. [7] with some modifications. A 25 μl aliquot of homogenate supernatant was incubated at 37 °C in the presence of 10.0 mmol/l glyoxylate, 80.0 mmol/l alanine, 100 mmol/l potassium phosphate (pH 8.0), and 100 μmol/l pyridoxal 5′-phosphate in a final volume of 281 μl. The enzymatic reaction was initiated by adding alanine after prewarming the solutions at 37 °C for 5 min. After 30 min, AGT activity was arrested by heating at 95 °C for 10 min and the pyruvate and glycine levels generated in the reaction solution were measured. Enzyme and substrate blanks were prepared for each specimen by replacing either glyoxylate, alanine, or liver extract with an equal volume of water in the reaction mixture.

2.4. Capillary electrophoresis

The reaction solution was deproteinized using an Ultrafree C3 THK filter, and was diluted 20-fold with water. Specimens (~20 nl) were injected at 50 mbar for 4.0–6.0 s. Pyruvate and glyoxylate were separated using an organic buffer containing 5 m*M* pyridinedicarboxylic acid and 0.5 m*M* cetyltrimethylammonium bromide (pH 5.6; Agilent Technologies, Germany) and a capillary electrophoresis system (Hewlett-Packard ^{3D}CE, Germany) running at a constant voltage of −30 kV at 30 °C. The capillary tube was coated internally with polyimide fused silica and had a total length of 80.5 cm (72 cm effective length) with an internal diameter of 75 μm (Yokogawa Analytical Systems, Japan). In contrast, glycine and alanine were separated using a basic anion buffer containing 20 m*M* 2,6-pyridinedicarboxylic acid and 0.5 m*M* hexadesyl trimethylammonium hydroxide (pH 12.1; Agilent Technologies) and a capillary tube of 120.5 cm total length (104 cm effective length). The target substances were detected by indirect UV detection using a diode-array detector with the signal at 350 nm and

the reference at 200 nm. Each determination required about 14 min, followed by washing the capillary tube with the same buffer at 50 mbar for 4 min. Standard solutions of pyruvate ranging from 11.4 μmol/l to 22.7 mmol/l (11 different concentrations) and solutions of glycine ranging from 66.6 μmol/l to 26.7 mmol/l (10 different concentrations) were measured in triplicate, and the standard curve constructed from the results was used for the analysis of liver specimens.

2.5. Statistical analysis

Results are reported as the mean±standard deviation (SD). Student's unpaired *t*-test was used for statistical analysis, with significance being set at $P<0.05$.

3. Results

A standard mixture containing pyruvate, glyoxylate, glycine, and alanine (1 mmol/l each) was injected and separated by capillary electrophoresis. The migration time was 4.5 min for pyruvate and 4.7 min for glyoxylate in the organic buffer (pH 5.6), while it was 11.5 min for glycine and 12.5 min for alanine in the basic anion buffer (pH 12.1) (Fig. 1A and B). Standard curves for pyruvate and glycine (*Y*: absorbance of the peak area, versus *X*: concentration) were plotted over the range from 11.4 μmol/l to 22.7 mmol/l of pyruvate and from 66.6 μmol/l to 26.7 mmol/l of glycine. The regression lines for these plots were linear and each had a significant regression coefficient (pyruvate, $Y = 1.06X$, $r = 0.999$, $P<0.01$; glycine, $Y = 0.18X$, $r = 0.999$, $P< 0.01$). The minimum detection limit for pyruvate and glycine was 22.7 and 133.3 μmol/l, respectively, in either standard solution or liver specimen (signal-to-noise ratio 2).

In electropherograms of liver specimens separated using the organic buffer (pH 5.6), the pyruvate peak was sharp and it was considered to be specific for pyruvate because there were no other peaks close enough to cause interference. Also, the peak increased in height after the addition of standard

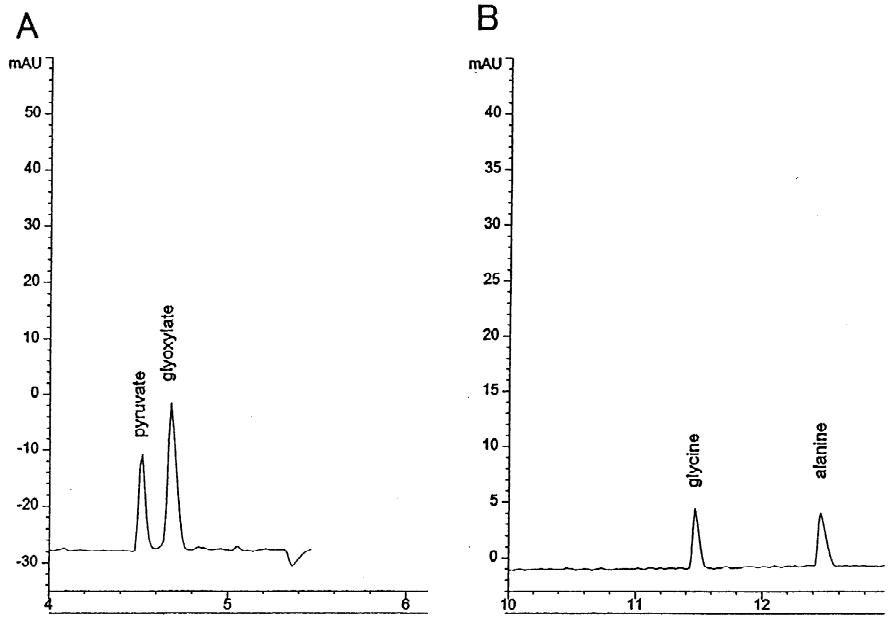

Fig. 1. Electropherograms of standard solutions. (A) Pyruvate and glyoxylate peaks obtained using the organic buffer containing 5 m*M* pyridinedicarboxylic acid and 0.5 m*M* cetyltrimethylammonium bromide (pH 5.6). (B) Glycine and alanine peaks obtained using the basic anion buffer containing 20 m*M* 2,6-pyridinedicarboxylic acid and 0.5 m*M* hexadesyl trimethylammonium hydroxide (pH 12.1).

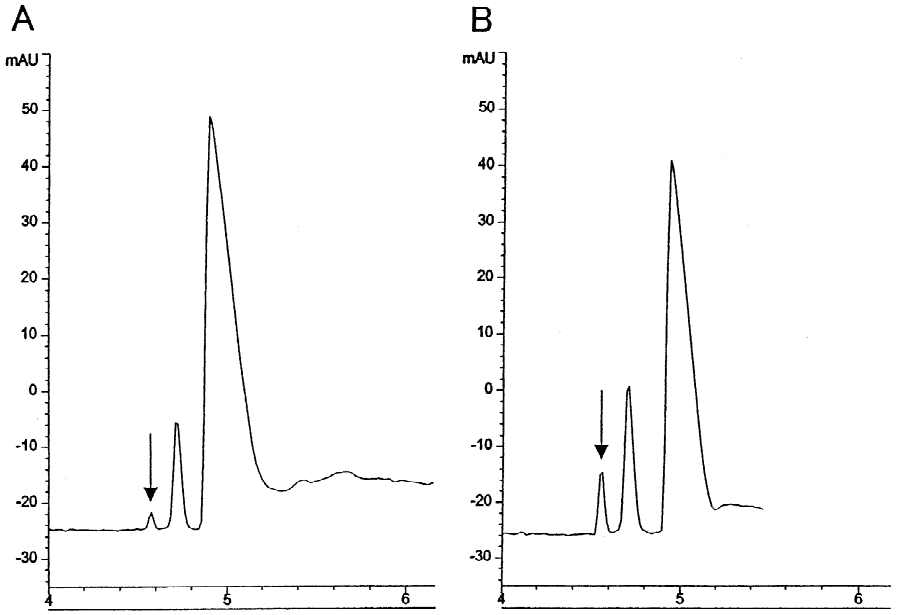

Fig. 2. Electropherograms of a liver specimen separated using the organic buffer containing 5 m*M* pyridinedicarboxylic acid and 0.5 m*M* cetyltrimethylammonium bromide (pH 5.6). (A) Electropherogram of the reaction mixture. (B) Electropherogram of the reaction mixture spiked with standard pyruvate solution (14.4 μmol). The peaks indicated by arrows are specific for pyruvate.

pyruvate (Fig. 2). The mean recovery of pyruvate (14.4 μmol) spiked into six liver specimens after 30 min of incubation was 99.55±2.68% (Table 1).

In electropherograms of liver specimens separated using the basic anion buffer (pH 12.1), the glycine peak was sharp and it was considered to be specific for glycine because there were no other peaks close enough to cause interference. Similarly, the peak

Table 1
Recovery of pyruvate and glycine spiked into six liver specimens from normal rats

	Initial amount (μmol/30 min/mg protein)	Additional amount (μmol)	Final glyoxylate (μmol/30 min/mg protein)	Recovery rate (%)
Pyruvate	6.35	14.4	21.56	103.90
	6.77	14.4	21.16	99.99
	6.87	14.4	21.25	99.91
	6.69	14.4	20.78	98.53
	6.87	14.4	21.15	99.41
	6.51	14.4	19.98	95.59
Mean±SD	6.68±0.21		20.98±0.55	99.55±2.68
Glycine	6.91	4.1	11.32	101.94
	6.73	4.1	11.66	106.69
	6.70	4.1	11.72	107.55
	6.74	4.1	10.95	100.03
	6.84	4.1	11.06	100.15
	6.94	4.1	11.62	104.31
Mean±SD	6.81±0.10		11.39±0.33	103.44±3.25

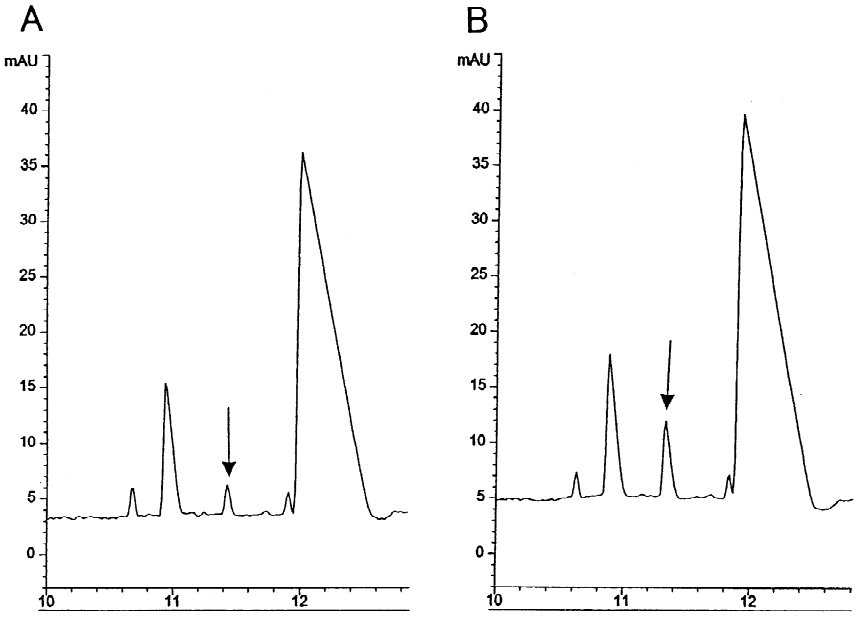

Fig. 3. Electropherograms of a liver specimen separated using the basic anion buffer containing 20 m*M* 2,6-pyridinedicarboxylic acid and 0.5 m*M* hexadesyl trimethylammonium hydroxide (pH 12.1). (A) Electropherogram of the reaction mixture. (B) Electropherogram of the reaction mixture spiked with standard glycine solution (4.1 μmol). The peaks indicated by arrows are specific for glycine.

increased in height after the addition of standard glycine (Fig. 3). The mean recovery of glycine (4.1 μmol) spiked into six liver specimens after 30 min of incubation was 103.44±3.25% (Table 1).

During the measurement of AGT activity, pyruvate and glycine levels increased as a function of both the incubation time (Fig. 4) and the amount of liver protein (Fig. 5). Therefore, it was possible to measure enzyme activity even in specimens containing only 5 μg of protein. AGT activity of liver specimens in the presence of substrates and pyridoxal 5′-phosphate was 10.02±0.31 μmol pyruvate/h/mg protein and 10.21±0.15 μmol glycine/h/mg protein, respectively. The generation of pyruvate and

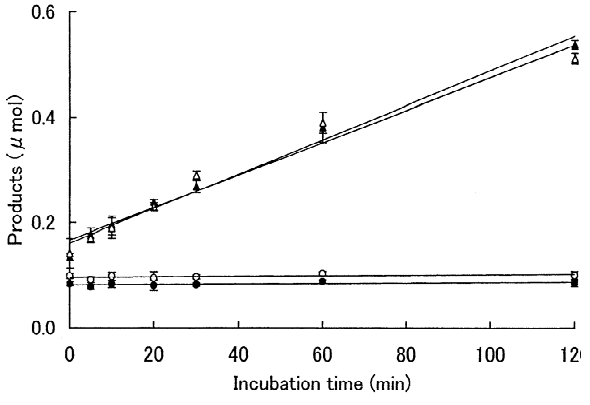

Fig. 4. Relationship between the incubation time and production of pyruvate and glycine in the presence (▲, pyruvate; △, glycine) or absence (●, pyruvate; ○, glycine) of AGT.

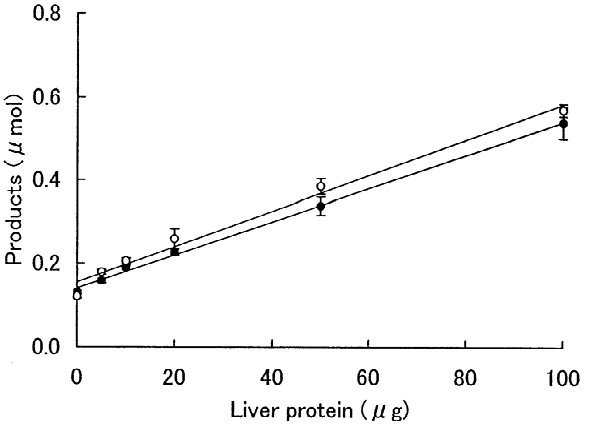

Fig. 5. AGT activity (protein level) in relation to the production of pyruvate (●) and glycine (○).

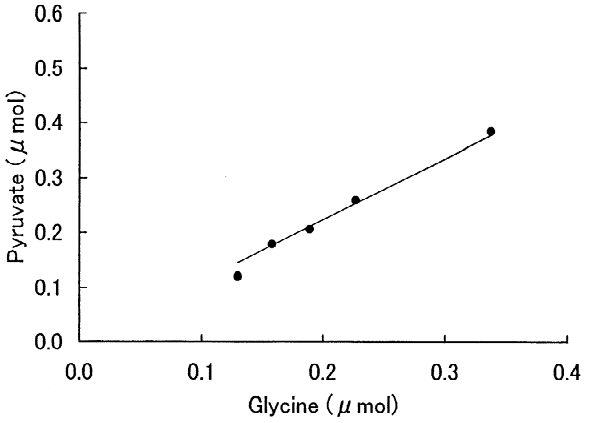

Fig. 6. Relationship between the generation of pyruvate and glycine at different levels of AGT activity (liver protein).

glycine after incubation of specimens containing each amount of liver protein showed a significant correlation ($Y = 1.12X$, $r = 0.991$, $P < 0.01$) (Fig. 6). No pyruvate or glycine peaks were detected when liver specimens were incubated without either alanine or glyoxylate. In addition, pyruvate and glycine were generated at a lower level in media with both alanine and glyoxylate incubated without any liver specimen, although their levels were negligible and stayed constant without being influenced by incubation time (Fig. 4).

4. Discussion

Capillary electrophoresis is a powerful separation technique that can achieve high resolution. Various capillary electrophoresis methods have been developed for the analysis of organic acids and amino acids. Since both organic acids and amino acids have little or no UV absorbance, indirect UV detection is generally utilized for their determination by capillary electrophoresis [14]. In the process of capillary electrophoresis, a specimen injected into the anode pole is moved towards the cathode pole, and substances are separated according to their different migration times. The electrolytes used for the separation and detection of organic acids and amino acids contain cetyltrimethylammonium bromide (CTAB) or cetyltrimethylammonium hydroxide (CTAH) to reverse the electroosmotic flow and to ensure the elution of anions through the detector. In particular, for the detection of amino acids, a high pH is used to ensure that anions are formed by all compounds and to promote migration toward the anode. At pH >12, amino acids are all anionic, since their isoelectric points range from 2.98 to 10.76. With the indirect UV detection method, displacement of a highly UV-absorbing electrolyte by the target anion creates a negative peak. All wavelengths from 190 to 600 nm can be detected by diode-array detection, and conversion of the negative peaks to positive peaks is achieved by switching the absorbances measured at the signal and reference wavelengths. The concentration of UV-absorbing substance in the electrolyte and the detection wavelength are selected to maximize the UV absorbance and to minimize noise. The advantage of indirect detection is that it is a universal method, which allows the identification of ions without any specific absorbance. When special buffers were used in the present study, there were no other substances close enough to interfere with the pyruvate or glycine peaks in the electropherograms [14,15], so the respective peaks were considered to be specific for each substance. Mass spectrometric confirmation of the peaks may be needed for definitive determination, but this method appears to be applicable for routine laboratory use.

Using capillary electrophoresis, AGT activity detected in normal rat liver (10.02 ± 0.31 μmol pyruvate/h/mg protein) was comparable to that in normal human liver reported by Petrarulo et al. (10.9 ± 2.0 μmol pyruvate/h/mg protein) [7]. AGT activity has been defined as the rate of pyruvate production during incubation of a mixture of tissue extract and substrates [6–11]. However, AGT catalyzes transamination between alanine and glyoxylate, and glycine is also subsequently produced by transamination between them. Therefore, glycine production also reflects the net AGT activity in the liver. In the present study, both pyruvate and glycine produced by AGT were measured and were shown to increase in parallel with each other, so the measurement of either substance can be used to assess AGT activity.

In the absence of AGT (liver specimen), pyruvate and glycine were still detected at low levels. Rowsell

et al. [9] observed that a mixture of alanine and glutamate (both at 24 mmol/l) in 60 mmol/l of phosphate buffer (pH 7.4) promoted the formation of α-ketoglutarate at a rate of 22–25 μmol/l/h at 37 °C. Spontaneous transamination between alanine and glyoxylate was also reported to occur at a low level in the presence of higher substrate concentrations. Therefore, these findings might be caused by a highly favorable driving force for the amination of glyoxylate [18].

AGT activity has been detected by various techniques [6–11]. Compared with other methods, capillary electrophoresis has high sensitivity and requires a very small amount of specimen and electrolyte volume for analysis. In the present study, capillary electrophoresis allowed the measurement of the enzyme activity even in 5 μg of protein within 30 min of incubation, while at least 10 μg of protein or more is required for analysis by the enzyme spectrophotometric method or the high-performance liquid chromatographic method [7,10]. Moreover, this procedure has superior simplicity, in that specimens can be analyzed with only deproteinization and dilution [9,14], and it provides excellent reproducibility and good linearity. Therefore, capillary electrophoresis may be useful for measuring AGT activity when the amount of tissue is limited such as with small human liver biopsy specimens, and it seems to be suitable for laboratory use.

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