

Assay of alanine:glyoxylate aminotransferase in human liver by its serine:glyoxylate aminotransferase activity

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ABSTRACT

We have examined assay methods and conditions for human liver alanine:glyoxylate aminotransferase (AGT). This enzyme shows not only the AGT activity but also serine:pyruvate and serine:glyoxylate aminotransferase (SPT and SGT) activities. In the assay of AGT activity using crude enzyme preparations, there is the complication that glutamate:glyoxylate aminotransferase (GGT) also contributes to AGT activity, but at present no other enzyme is known to catalyze transamination between L-serine and glyoxylate or pyruvate. Therefore, an assay for AGT using its SGT activity was investigated in which hydroxypyruvate formed from L-serine in the enzymic reaction with glyoxylate was determined by lactate dehydrogenase (LDH) in the presence of tris(hydroxymethyl)aminomethane (Tris) at pH 8.4. A possible obstacle to this assay is that pyruvate formed from L-serine by serine dehydratase (SDH) interferes with SGT assay as an additional substrate of LDH and AGT. However, the SDH activity in human liver is very low, and by performing the SGT reaction in the presence and absence of glyoxylate the SGT activity was represented as the glyoxylate-dependent hydroxypyruvate formation from L-serine. There was a combined good correlation between the AGT, SGT and SPT activities, and the activity ratio, AGT : SGT : SPT was about 1.0 : 0.17 : 0.13.

Alanine:glyoxylate aminotransferase (AGT) in the liver plays a pivotal role in glyoxylate metabolism as evidenced by the fact that primary hyperoxaluria type 1 (PH1) is caused by functional deficiency of this enzyme (3). PH1 is an inherited disease characterized by increased oxalate production from accumulated glyoxylate, urolithiasis, nephrocalcinosis and in most severe forms systemic oxalosis, and death due to complications of renal failure (1, 5). In addition, AGT substantially contributes to gluconeogenesis from L-serine in the livers of rabbits, humans and dogs (18), and this enzyme in the liver

of most animal species, including man, shows not only the AGT activity but also serine:pyruvate and serine:glyoxylate aminotransferase (SPT and SGT) activities.

At present, definitive diagnosis of PH1 requires measurement of AGT catalytic activity in a liver biopsy, and in some cases, immunolocalization to detect mitochondrial mistargeting of the enzyme (2).

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The abbreviations used are: AGT, alanine:glyoxylate aminotransferase (EC 2.6.1.44/2.6.1.51); SPT, serine:pyruvate aminotransferase; SGT, serine:glyoxylate aminotransferase; GGT, glutamate:glyoxylate aminotransferase (EC 2.6.1.4); SDH, serine dehydratase (EC 4.2.1.13); LDH, lactate dehydrogenase (EC 1.1.1.27); GIDH, glutamate dehydrogenase (EC 1.4.1.3); pyridoxal-5-P, pyridoxal 5'-phosphate; Tris, tris(hydroxymethyl)aminomethane; E64C, trans-epoxysuccinyl-L-leucylamido(3-methyl)butane; PH1, primary hyperoxaluria type 1.

In the studies of Danpure and Jennings who first demonstrated the deficiency of the AGT activity in PH1, glutamate:glyoxylate aminotransferase (GGT) activity was also assayed and the measured AGT activity was corrected for a 66% crossover from GGT (3, 4). This was based on the findings by Thompson and Richardson (15) that partially purified human liver GGT also catalyzed transamination between L-alanine and glyoxylate at a rate of 66% of its original GGT activity. The GGT activity detected by Danpure and Jennings in control human liver sonicate was about 13% (mean value) of the AGT activity (4). However, Rumsby, Weir and Samuelli (12) later showed that the GGT activity in human liver sonicate was low enough to allow use of measured AGT activity for tissue diagnosis of PH1 without correction. On the other hand, we have assayed AGT (SPT) mainly by its SPT activity, because we were first interested in this enzyme from studies on serine metabolism in the liver, and when enzyme activity was determined for diagnosis of PH1, to avoid complexity and less accuracy caused by the increase in measurement items and by the correction of the measured activity (6, 14, 17–19). In this SPT assay where hydroxypyruvate formed from L-serine was determined spectrophotometrically using spinach glyoxylate reductase, little activity was detected in the livers of three PH1 cases including a case with a S205P substitution (6, 8). This suggests that the SPT reaction is almost solely catalyzed by AGT (SPT) in the human liver. Unfortunately, the commercial supply of spinach glyoxylate reductase ceased recently. In the present study, therefore, we have investigated an assay of AGT in human livers using its SGT activity. The assay conditions of AGT activity were also reevaluated.

In the assay of AGT activity, pyruvate formed from L-alanine in the enzymic reaction with glyoxylate has been determined by lactate dehydrogenase (LDH) and NADH in the presence of a large excess of tris(hydroxymethyl)aminomethane (Tris) at pH 8.4 (10, 16). Under the above alkaline conditions glyoxylate forms a Schiff base with Tris and is no longer a substrate for LDH (16). This principle of the AGT assay can also be applied to the assay of SGT activity, because hydroxypyruvate formed from L-serine is also a substrate of LDH whose reactivity is not affected by Tris. The only possible obstacle to this SGT assay is that pyruvate formed from L-serine by serine dehydratase (SDH) will interfere with the SGT assay as an additional substrate of LDH and AGT (Fig. 1). However, SDH activity in the liver has been shown to decrease drastically as the body

size of animals increases (11), and in human liver it was barely detectable (18). Therefore, AGT in human livers could be reasonably assayed by its SGT activity.

MATERIALS AND METHODS

Materials. Small pieces of human liver were obtained at the time of surgical operation (hepatic left lobectomy) on eight patients who suffered from liver metastasis of a carcinoma. Other liver specimens were obtained at pathological anatomy performed approximately 5 h post mortem. Most liver samples thus obtained were immediately frozen and stored at -80°C until use. The use of liver samples from human subjects in this study was permitted by the Ethical Committee of Hamamatsu University School of Medicine, and the patients or the bereaved families consented to the use of the resected specimens. LDH from hog muscle and glutamate dehydrogenase (GIDH) from bovine liver were obtained from Roche and Sigma-Aldrich, respectively. Spinach glyoxylate reductase was purified as described by Sallach (13)

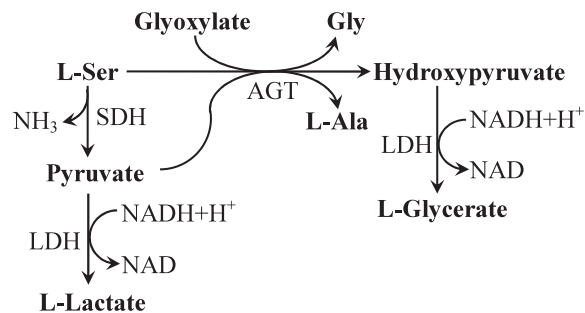


Fig. 1 Assay for alanine:glyoxylate aminotransferase by its serine:glyoxylate aminotransferase activity. In the assay of AGT using its SGT activity proposed in the present study, the SGT reaction is once stopped, and then hydroxypyruvate in assay samples is measured by LDH as described under "Materials and Methods". When the enzyme sample contains SDH, serine is converted to pyruvate which is also a substrate of LDH and causes oxidation of NADH. Therefore, the SGT reaction is carried out in the presence and absence of glyoxylate, and the difference in the formation of LDH substrates is regarded as SGT activity. However, when SDH activity is very high as in the cases of rat and mouse livers the SGT assay becomes inaccurate due to the high background level. In addition, pyruvate is also a good keto-acid substrate of AGT, and when a portion of pyruvate supplied from L-serine is used by AGT as a keto-acid substrate, hydroxypyruvate is formed at the expense of an equimolar quantity of pyruvate. In this case, therefore, SGT activity may be underestimated by the amount of pyruvate used by AGT. Thus the assay of AGT with its SGT activity is applicable only when SDH activity is low enough as in the case of human liver.

up to the second ammonium sulfate fractionation. The partially purified glyoxylate reductase was free of LDH activity. E64C, a thiol-protease inhibitor, was a kind gift from Dr. K. Hanada, Research Laboratories, Taisho Pharmaceutical Co., Ltd. All other chemicals were of analytical grade.

Preparation of sonicated liver extract. Frozen liver specimens were thawed, minced with scissors and homogenized with a Potter-Elvehjem type homogenizer in 9.2 vol (v/w) of 0.25 M sucrose (pH 7.2) containing 3 mM imidazole/HCl, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride and 10 µg/mL E64C. Usually, the homogenizing medium also contained 50 µM pyridoxal 5'-phosphate (pyridoxal-5-P). The homogenate was sonicated on ice with an ultrasonic disintegrator (CHO-ONPA KOGYO Co., Tokyo), using five cycles of alternate 15 s bursts at a maximum power and 30 s rests. The sonicated homogenate was passed through two layers of cheesecloth and then subjected to centrifugation at $1,000 \times g$ for 10 min at 4°C.

Assay of enzymes. The standard reaction mixture (400 µL) for the assay of AGT or SGT activity contained 100 mM Tricine/NaOH (pH 8.0), 1 mM EDTA, 0.1% Triton X-100, 50 µM pyridoxal-5-P, 6 mM glyoxylate, 125 mM L-alanine or L-serine and enzyme sample. In the assay of SGT activity control incubation contained no glyoxylate. The mixture was first preincubated at 37°C for 5–6 min without amino acid substrate in a total volume of 350 µL, followed by initiation of the reaction by the addition of 50 µL of 1 M L-alanine (pH 8.0) or L-serine (pH 8.0). The reaction was carried out for 10 min (for AGT activity) or 15 min (for SGT activity) at 37°C and stopped with 80 µL of 20% perchloric acid (HClO₄). Then 30 µL of 0.2 mg/mL phenol red was added and the mixture was neutralized on ice with 1 M KOH until the orange color of phenol red (pH 6.5–7) was displayed. The volume of the mixture was adjusted with water to 800 µL, and K-perchlorate precipitate formed was removed together with denatured protein by centrifugation at 3000 rpm for 10 min at 4°C. The amount of pyruvate (AGT assay) or hydroxypyruvate (SGT assay) formed was then determined in the second reaction in which 200 µL of the HClO₄-supernatant was incubated with an excess of LDH (10 µg) and NADH (0.2 mM) in 200 mM Tris/HCl (pH 8.4) in a total volume of 1 mL and the reduction in the absorbance at 340 nm was followed until end-point. The standard assay conditions for SPT activity were almost

the same as those for the SGT assay, except that 6 mM glyoxylate was replaced by 10 mM pyruvate and hydroxypyruvate formed from L-serine was determined in the second reaction with 60–70 milliunits of glyoxylate reductase in 200 mM Tris/HCl (pH 7.6). The activity of GGT was determined in this study at pH 8.0 and pH 7.4, and the standard assay conditions for GGT activity at pH 8.0 was also almost the same as those for SGT activity, except that 125 mM L-serine was replaced by 125 mM L-glutamate and α-ketoglutarate formed was determined by incubation with about 2 units of GIDH in the presence of 40 mM NH₄⁺ and 0.2 mM NADH in 150 mM K-phosphate buffer (pH 7.4). The assay conditions for GGT at pH 7.4 were similar to those used by Thompson and Richarson (15) for determination of the substrate specificity of partially purified human liver GGT. The composition of the reaction mixture (400 µL) was essentially the same as that for the GGT assay at pH 8.0, except that 100 mM Tricine/NaOH (pH 8.0), 6 mM glyoxylate and 125 mM L-glutamate (pH 8.0) were replaced by 50 µM K-phosphate (pH 7.4), 33 mM glyoxylate and 33 mM L-glutamate (pH 7.4), respectively, and the reaction was carried out for 20 min at 37°C. Then, α-ketoglutarate formed was determined using GIDH as above. For the assay of serine dehydratase (SDH), the SDH reaction was carried out essentially under conditions described by Ishikawa *et al.* (7), and after the reaction was stopped by perchloric acid, pyruvate formed was determined by LDH. In the present study, assay of all the enzymes and enzyme activities were performed under conditions in which reactions proceeded linearly over time and the rate of reaction was proportional to the amount of enzyme. Where indicated, the enzyme activity was expressed, by a rule-of-three sum, as µmol of product formed/min/mL of sonicated liver extract.

RESULTS AND DISCUSSION

Assay of AGT with its SGT activity

In order that the proposed assay of AGT by its SGT activity might be practicable, the SDH-catalyzed formation of pyruvate from L-serine should have been negligible as written above (Fig. 1). We have confirmed in this study the very low activity of SDH in human livers, and in the SGT assay using a sonicated human liver extract the formation of the substrate of LDH (pyruvate + hydroxypyruvate) from L-serine in the absence of glyoxylate was far less than that in the presence of glyoxylate (Fig. 2A). The SGT activity thus measured as the difference

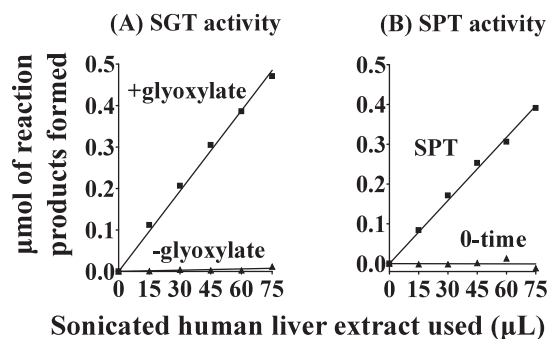


Fig. 2 Serine:glyoxylate and serine:pyruvate aminotransferase activities in a human liver specimen. The SGT and SPT reactions were carried out under respective standard assay conditions with 0, 15, 30, 45, 60 and 75 μL of a sonicated human liver extract. In the case of the SGT assay (A), the difference between the formation of LDH substrate in the presence and absence of glyoxylate was regarded as SGT activity, as described in the text. In the assay of SPT activity (B), 0-time incubation (HClO_4 was added before L-serine) was carried out as a control.

between the formation of LDH substrate in the presence and absence of glyoxylate was the same level as the SPT activity which was determined in parallel using the same enzyme sample (Fig. 2B).

Effect of sonication in the preparation of enzyme samples

In the present study, enzyme samples were prepared by subjecting liver homogenate to sonication with 5 cycles of alternate 15 s bursts and 30 s rests on ice, followed by 10 min centrifugation of the 5th sonicate at $1000 \times g$. The purpose of the centrifugation was primarily to remove non-suspended fibrous material. In order to ensure that enzyme samples were properly prepared, the SGT activity was determined through the enzyme preparation procedure. As shown in Fig. 3, the SGT activity of the homogenate was kept fairly constant in the sonicates through the 5 cycles of sonication. When the homogenate was centrifuged at $1000 \times g$, about 60% of the activity was recovered in the supernatant. AGT is known to locate in peroxisomes in human liver (9), but this organelle is also known to be fairly fragile. Therefore, partial destruction of peroxisomes may have occurred during freeze-thawing and homogenization of the liver. The activity recovered in the supernatant increased by repeated sonication, but even after the 5th cycle of sonication the recovery in the supernatant was about 87%. In this study, the $1000 \times g$ supernatant of the 5th sonicate was used as the enzyme sample throughout, but when the total activity in the liver was to be determined it may be bet-

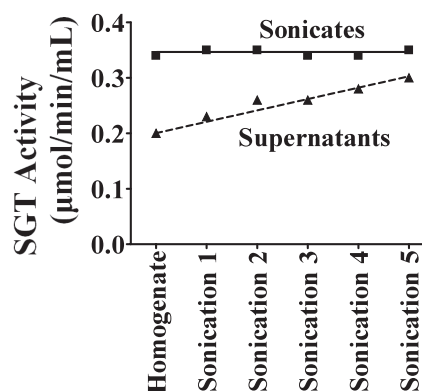


Fig. 3 The SGT activity in homogenate, sonicates and their supernatants. A human liver homogenate was subjected to 5 cycles of sonication as described under "Materials and Methods", except that a portion of homogenate and the sonicates at each cycle were subjected to centrifugation at $1000 \times g$ for 10 min at 4°C . Then the SGT activity of homogenate, sonicates and supernatants were determined under standard assay conditions.

ter to use homogenate or sonicate.

Effect of pyridoxal 5'-phosphate

Since every aminotransferase requires pyridoxal-5-P as a prosthetic group for their activity, effect of this cofactor was examined. As shown in Fig. 4, addition of 50 μM pyridoxal-5-P into the reaction mixture augmented about 1.8-fold the SGT activity of an enzyme sample (a $1000 \times g$ supernatant from the 5th sonicate) prepared without addition of pyridoxal-5-P. Preincubation for 1 h of the enzyme sample with 50 μM pyridoxal-5-P in an ice-bath or at 37°C did not cause further increase in the SGT activity, suggesting that apo-AGT, if present in the enzyme sample, quickly binds the pyridoxal-5-P cofactor under the conditions used. However, since there was no indication of any undesirable effect of the cofactor at this concentration, 50 μM pyridoxal-5-P was usually included in the homogenizing medium.

Optimal concentration of glyoxylate

We have previously observed that SPT activity of a purified rat liver AGT (SPT) was inhibited by high concentrations of pyruvate and the substrate inhibition by pyruvate was pH-dependent (19). Thus the inhibition was marked at pH 6.8 and 7.4, considerably less at pH 8.0, and only barely detectable at pH 8.8 (19). The substrate inhibition by pyruvate was also dependent on the concentration of the counterpart amino acid substrate, being more marked when the concentration of L-serine was low.

A similar inhibition by the keto-acid substrates

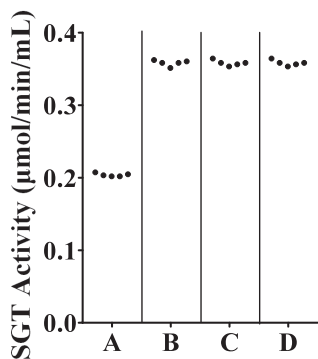


Fig. 4 Effect of pyridoxal 5'-phosphate. In this experiment, the enzyme sample ($1000 \times g$ supernatant from the 5th sonicate) was prepared from human liver as described under "Materials and Methods", except that pyridoxal-5-P was omitted from the homogenizing medium. A portion of the enzyme sample was preincubated in an ice-bath (C) and another portion at 37°C (D) for 1 h with $50 \mu\text{M}$ pyridoxal-5-P. Then, the enzyme sample prepared in the absence of pyridoxal-5-P was subjected to SGT assay in the absence (A) and presence (B) of $50 \mu\text{M}$ pyridoxal-5-P. The SGT activity of the enzyme samples preincubated with $50 \mu\text{M}$ pyridoxal-5-P was determined under standard assay conditions, *i.e.*, in the presence of $50 \mu\text{M}$ pyridoxal-5-P. Five assays were carried out in each group.

has also been observed in human liver AGT (4, 12). In the present study, therefore, the substrate inhibition by keto-acid substrates was reexamined under standard conditions for determination of SGT, AGT and SPT activities. As shown in Fig. 5, substrate inhibition was observed in the SGT assay with glyoxylate above 6 mM. The effect of glyoxylate concentration on AGT activity was almost the same as that on SGT activity, and substrate inhibition by pyruvate in the SPT assay was milder than that by glyoxylate in the SGT assay, the peak activity being observed at 10 mM pyruvate (data not shown).

Evaluation of the glutamate:glyoxylate aminotransferase activity

The assay conditions used by Thompson and Richardson (15) to examine the substrate specificity of partially purified human liver GGT was pH 7.3, 33.3 mM glyoxylate and 33.3 mM amino acids (reported pH optimum: 7.3, apparent K_m for glyoxylate and glutamate: 2 mM). For correction of the AGT activity, however, GGT activity should be determined under the same conditions as those for the AGT assay. In the present study, GGT activity measured at pH 8.0 and 6 mM glyoxylate was 55–60% of the activity at pH 7.4 and 33.3 mM glyoxylate. Then the GGT activity determined at pH 8.0 and 6 mM glyoxylate was compared with the activity of

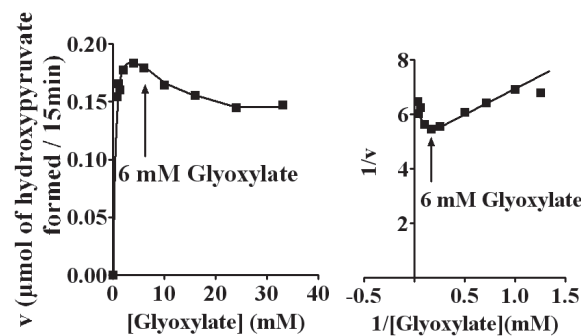


Fig. 5 Substrate inhibition by glyoxylate. The assay of SGT activity was performed under standard conditions with $25 \mu\text{L}$ of sonicated liver extract, except that the concentration of glyoxylate varied as indicated.

AGT. As shown in Table 1, the GGT activity measured in this study was only several per cent or at most 14% of the simultaneously measured AGT activity, and thus the correction with 66% of the measured GGT activity did not cause a big change in AGT activity. In this sense, the proposal by Rumsby *et al.* (12) that the measured AGT activity can be used for tissue diagnosis of PH1 without correction was understandable. Before concluding, however, we should know more about human liver GGT, for example, how stable this enzyme is during the freeze-storage of liver specimens, whether the AGT activity is 66% of the GGT activity even at pH 8.0 and the glyoxylate concentration of 6 mM, and so on. As to the stability of GGT during the freeze-storage, liver F with the lowest GGT activity was a specimen which had been stored at -80°C for as long as 16 months, but liver E which was obtained fresh and subjected to the assay the same day showed only a moderate GGT activity (cf. Table 1).

Correlation between the AGT, SGT and SPT activities

As shown in Fig. 6, SGT activity showed good correlation with both AGT and SPT activities. Although not shown in the figure, there was also a good correlation between the AGT and SPT activities. The activity ratio of AGT : SGT : SPT was about 1.0 : 0.17 : 0.13.

In conclusion, the activity of AGT in human liver can be determined by either AGT, SGT or SPT activity. In most assays for tissue diagnosis of PH1, AGT activity determination may recognize abnormal activity of AGT without correction for any crossover from GGT. However, for discrimination of the presence or absence of very small activity, AGT as-

Table 1 Alanine:glyoxylate aminotransferase and glutamate:glyoxylate aminotransferase activities in human livers

Livers	AGT activity		GGT activity	
	measured	corrected		
	$\mu\text{mol}/\text{min}/\text{mL}$	$\mu\text{mol}/\text{min}/\text{mL}$	$\mu\text{mol}/\text{min}/\text{mL}$	% in AGT activity
A	1.87	1.76	0.16	8.6
B	3.40	3.27	0.20	5.9
C	1.07	0.97	0.15	14.0
D	3.64	3.53	0.17	4.7
E	2.69	2.59	0.15	5.6
F	4.12	4.07	0.07	1.7
G	2.71	2.61	0.15	5.6
H	1.71	1.64	0.10	5.8
I	3.03	2.96	0.11	3.6

Liver F was a fresh liver used without storage at -80°C .

AGT and GGT activities were determined in parallel under respective standard assay conditions at pH 8.0, and the results were expressed as μmol of pyruvate or α -ketoglutarate formed/min/mL of the sonicated liver extract. The denominator of the “% of GGT activity in the AGT activity” is the AGT activity measured, i.e., before correction. Correction of the AGT activity was made assuming that the crossover of the AGT activity from GGT was 66% of its GGT activity at pH 8.0.

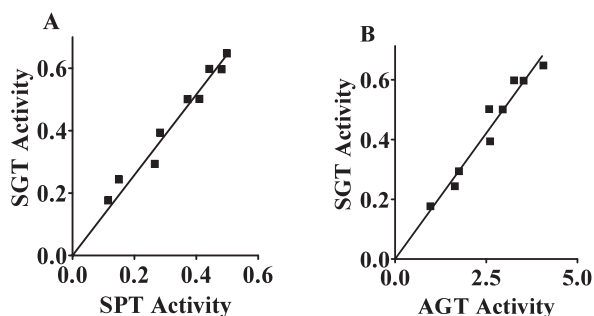


Fig. 6 Correlation between the alanine:glyoxylate, serine:glyoxylate, and serine:pyruvate aminotransferase activities. In this experiment, AGT, SPT and SGT activities in a sonicated liver extract were determined in parallel under respective standard assay conditions. GGT activity at pH 8.0 and 6 mM glyoxylate was also determined in parallel and the measured AGT activity was corrected for 66% of the GGT activity. In this way, the assay of AGT, SGT and SPT activities was extended to cover sonicated extracts from 9 human liver specimens. Activities are expressed as $\mu\text{mol}/\text{min}/\text{mL}$ of sonicated liver extract. (A) SGT activity vs. SPT activity, (B) SGT activity vs. AGT activity.

say with correction or SGT assay may be the proper method to be used. In the SGT assay, control incubation without glyoxylate should be run simultaneously. However, the “minus glyoxylate control” appears to be more simple and accurate than the separate assay for GGT and the use of a correction factor.

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