

AN IN-HOUSE ASSAY FOR SERUM DEOXYTHYMIDINE KINASE

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Summary

An in-house method which utilizes ^{14}C -thymidine as a substrate was used to assay deoxythymidine kinase in serum. The method is sensitive enough to detect normal levels of serum deoxythymidine kinase and the assay procedure also enables rapid handling of multiple samples. With a total reaction volume of 60 μl , the enzyme reaction was found to be linear with concentrations for up to 650 U/L of TK activity. On studying serum deoxythymidine kinase (s-TK) activity with incubation time, there was a proportional increase in activity with the length of incubation time. "Within-batch" precision showed a coefficient of variation (CV) of 4.7% for serum with extremely high s-TK levels and a CV of 8.8% for serum with normal s-TK levels. S-TK showed a CV of less than 16.0% in its activity when stored at -8°C and at -20°C . The normal reference range obtained for s-TK activity was 8.6 ± 7.5 U/L.

Keywords: Deoxythymidine kinase, enzyme assay.

INTRODUCTION

Deoxythymidine kinase (EC 2.7.1.21) catalyses the conversion of deoxythymidine to deoxythymidine monophosphate (dTMP) in the presence of ATP. Deoxythymidine monophosphate is then progressively converted to the triphosphate form (dTTP) which is subsequently used in the synthesis of DNA.¹ The growth rate of different tumours correlates in many cases with cellular thymidine kinase (TK) activity.² Three different cellular TK isozymes have been found in mammalian cells. The TK1 activity is found mainly in dividing cells in stages G1 to S and is more or less absent in resting cells.³ High levels of the enzyme are detected in the serum of patients with some neoplastic disorders and in connection with certain virus infections.⁴ The second isozyme, TK2, is found in the mitochondrial matrix and levels remain stable and low throughout the cell cycle.⁵ A third minor isozyme, TK-B, has been detected in the inner membrane fraction of mitochondria in some cell lines.⁶ Traditionally, TK is assayed by incubating a suspension of cells or a tissue section with a source of radioactive thymidine (labelled with either ^{14}C or ^3H). The thymidine incorporated is then quantified by liquid scintillation counting or by autoradiography. These techniques often require tumour biopsies which cannot be taken repeatedly. In addition, the traditional techniques only measures TK activity within that biopsy and do not reflect activity in other tumour sites. Thus, serum TK activity might be a better assay than the above techniques as it reflects total proliferation within the body. This study evaluates an in-house assay for s-TK and its reference range in normal subjects.

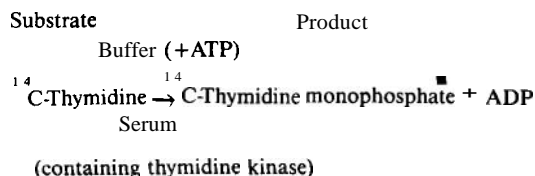
MATERIALS AND METHOD

Substrate

^{14}C -Thymidine of concentration 50 $\mu\text{Ci}/\text{ml}$ and specific activity 50-53 mCi/mmol was bought from Amersham International Plc.

Assay for s-TK activity

The principle of assay is depicted below:



Method of assay

The assay mixture consisted of 0.1 M *HEPES, 17 mM MgCl₂, 20 mM KCl, 1.2 mM NaF, 4.6 mM ATP, 2.7 mM dithiothreitol, 0.33 mg/ml bovine albumin and 6.6% glycerol.⁴ The pH of the assay mixture was adjusted to 7.5. The assay was performed at 37°C and all components were pre-warmed before mixing. The background control wells had the volume equivalent of serum made up with assay buffer. Inactivated sera (incubated at 60°C for 30 min) were used as negative controls. The reaction was terminated by pipetting the entire reaction mixture onto 2.3 cm Whatman DE-81 discs kept at $90-100^\circ\text{C}$.⁷ The ^{14}C -thymidine monophosphate formed was bound to the disc. The discs were washed once in methanol, thrice in 3 mM ammonium formate and subsequently dehydrated twice in methanol. The discs were dried at 60°C for 2 hr. and counted in a liquid scintillation counter. The

* N-2 hydroxyethylpiperazine N'-2-ethanesulfonic acid.

retention of unused substrate was less than 0.2% of total substrate added, normally giving background values between 300 and 450 cpm. All test counts were corrected for background.

One unit (U) of thymidine kinase is defined as the phosphorylation of one nmol of thymidine in one min. under the above described conditions. One U/L of TK activity is thus the phosphorylation of one nmol of thymidine in one min. per litre of serum under the above described conditions.

RESULTS

1. *Linearity*

Varying volumes of serum (1.0, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, 20.0 and 22.5 ul) were pre-mixed with 6.0 ul of substrate and made up to 60.0 ul with the rest of the reaction components. The reaction mixture was incubated at 37°C for one hr. Fig. 1 shows that the reaction was linear with enzyme concentrations for up to approximately 650 U/L of s-TK activity.

2. *Incubation time*

5.0 ul of normal serum was pre-mixed with 6.0 ul of radioactive substrate and made up to 60.0 ul with the reaction mixture. Fig. 2 illustrates the relationship between s-TK activity and the duration of incubation at 37°C. There was a proportional increase in activity up to 60 min. but beyond 80 min. the activity fluctuated and showed no definite trend.

3. *Substrate concentrations*

Varying concentrations of substrate (2.0-20.0 ul of 50 uCi/ml 14C-Thymidine) were used. 5.0 ul of serum was added in a final reaction mixture of 60 ul. The mixture was incubated at 37°C for one hr. The enzyme showed Michaelis-Menten kinetics and the Km value for serum TK was found to be $1.53 \times 10^{-6} M$. Fig. 3 shows a Lineweaver Burk plot of the inverse reaction velocity (1/v) against the inverse substrate concentration (1/S).

4. *Within-batch precision*

A normal sample was assayed 12 times simultaneously. A "high" sample (taken from a pretreated acute leukaemia patient) was assayed 14 times simultaneously. The mean, standard deviation (S.D.) and coefficient of variation (CV) were calculated and are shown in Table 1.

5. *Stability*

Sera kept at 4°C, -8°C, and -20°C were analyzed repeatedly over more than four months. The enzyme showed a CV of less than 16.0% in its activity when stored at -8°C and at -20°C. At 4°C, the s-TK activity varied by more than 15.0%. The results are tabulated in Table 2. However, when left at room temperature (26°C), the s-TK activity deteriorated after the fifth day.

6. *S-TK levels in healthy subjects*

In order to determine the normal s-TK range, 36 sera from healthy adults (ranging in age from 24 to 45 years) were

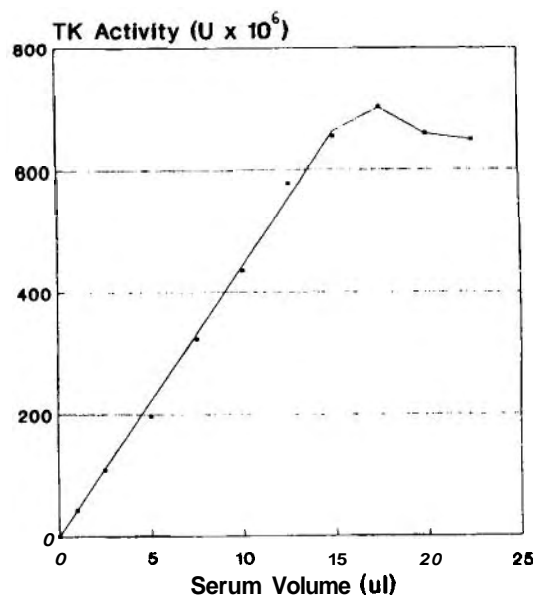


FIG. 1: S-TK activity against enzyme concentration.

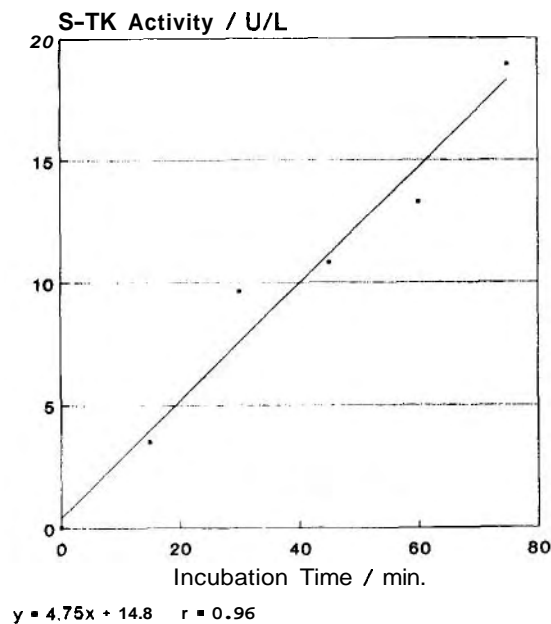


FIG. 2: S-TK activity with incubation time.

TABLE 1
PRECISION OF THE IN-HOUSE S-TK ASSAY

	No. of observations	Mean (U/L)	S.D. (U/L)	C.V. (%)
<i>Within-batch</i>				
a. Normal sample	12	18.3	1.6	8.8
b. High sample	14	691.7	32.2	4.7

analyzed. A normal s-TK range of 8.6 ± 7.5 U/L was obtained. There is no published range for s-TK utilizing ^{14}C -thymidine as substrate.

In a study of 20 patients with pretreated acute leukaemia, the mean s-TK level was 91.7 U/L. In addition, 14 patients with pretreated non-Hodgkin's lymphoma were studied and the mean s-TK level was 38.2 U/L; for five other cases of pretreated Hodgkin's lymphoma, the mean s-TK level obtained was 18.3 U/L.

DISCUSSION

From the above experiments, we advocate a working sample volume of 5.0 ul to accommodate samples with a much higher s-TK activity, as the linearity study was done on samples with a normal activity. Smaller working volumes are not recommended to avoid pipetting errors. The enzyme activity is linear with the length of incubation time up to 80 min. This is consistent with the findings of Gronowitz et al.⁴ It is therefore critical that

a fixed assay time is adhered to. An assay time longer than 80 min. is not recommended as the s-TK activity tends to fluctuate then.

Various radiolabelled substrates have been used for TK assay. Gronowitz and Kallander³ described the detection of TK using ^{125}I -deoxyuridine. Taylor et al used ^3H -deoxythymidine to assay TK. Our study showed that ^{14}C -thymidine is an adequate substrate to assay serum TK. We chose this substrate because of its long half-life, so as to avoid wastage through radioactive decay, especially since isotopes are expensive and shipment takes time.

For the purified human TK1 isozyme, the reported K_m values are $2.6 \times 10^{-6}\text{M}$ for deoxythymidine (dThd) and $2.4 \times 10^{-6}\text{M}$ for ^{125}I -5-iododeoxyuridine (IdUrd).¹⁰ The human TK2 have K_m values of $5.2 \times 10^{-6}\text{M}$ for dThd and $8.2 \times 10^{-6}\text{M}$ for IdUrd.¹⁰ The K_m value for s-TK derived in our study is not in the same range as the enzyme preparations were not purified and coupled with a different substrate.

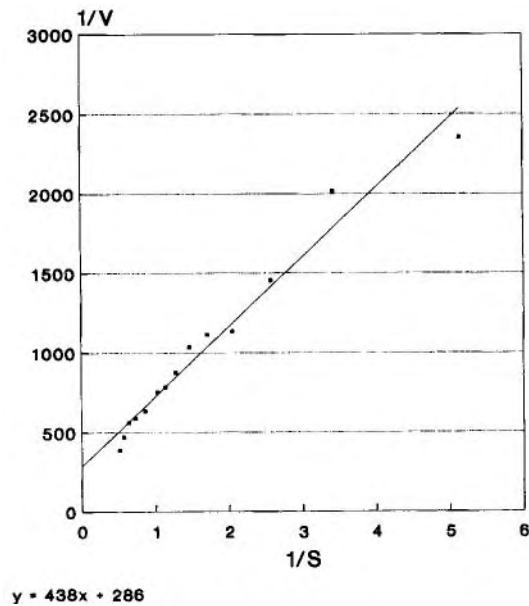


FIG. 3: Lineweaver Burk plot (1/v) against (1/s).

TABLE 2
STABILITY OF S-TK

Sample type	Storage time (days)	Temperature (°C)	Mean (U/L)	S.D. (U/L)	C.V. (%)
High s-TK	139	4°C	34.9	5.4	15.6
		-8°C	36.6	4.8	13.0
		-20°C	37.4	3.8	10.3

The observed stability of TK agrees with that documented by Gronowitz et al⁴ where the enzyme activity showed a CV of less than 10.0% when analyzed repeatedly over more than six months.

The method which is presented in this communication offers several interesting clinical applications in relation to malignant disease. Utilizing ¹⁴C-thymidine as substrate, our assay procedure is sensitive enough to detect normal levels of s-TK. An advantage with the in-house TK assay, besides its sensitivity, is the possible rapid handling of multiple samples. Only minute quantities of isotope are required in each assay and 40-50 samples can be processed at the same time, with no sophisticated instruments required other than a scintillation counter.

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