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Determination of thymidine phosphorylase activity by a non-radiochemical assay using reversed-phase high-performance liquid chromatography

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Abstract

Thymidine phosphorylase (TP) catalyses the conversion of thymidine into thymine. A non-radiochemical assay procedure for TP was developed in which thymine was detected at 265 nm after separation with reversed-phase HPLC. A complete separation of thymidine and thymine was achieved in 6 min and the minimum amount of thymine that could be detected was 0.8 pmol. The assay was linear with reaction times, up to at least 4 h, and protein concentrations up to at least 65 μg/ml. Population analysis showed no differences in TP activity between man and women or with increasing age.

Keywords: Thymidine phosphorylase; MNGIE; HPLC

1. Introduction

Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is an autosomal recessive disease which is caused by a thymidine phosphorylase deficiency [1,2]. Clinically, MNGIE is characterised by ptosis, progressive external ophthalmoplegia, severe gastrointestinal dysmotility, cachexia, peripheral neuropathy and skeletal myopathy [2]. Thymidine phosphorylase (TP) catalyses the first step in the degradation of the pyrimidine deoxynucleosides thymidine and deoxyuridine [3]. In patients with MNGIE, no or a severely reduced TP activity was detected in leukocytes which was accompanied by the presence of strongly elevated levels of thymidine and deoxyuridine in plasma [3–5]. In contrast, carriers for mutations in the TP gene, resulting in a substantially reduced activity of TP, did not show the elevated levels of thymidine and deoxyuridine [5]. Recently, a diagnostic algorithm has been developed based on the analysis of thymidine and deoxyuridine in plasma and the determination of the TP activity in leukocytes, to ensure the proper diagnosis of patients suffering from MNGIE [5].

To date, various radiolabeled or spectrophotometric methods have been described for the measurement of the TP activity [5–10]. The radiochemical methods include the separation of radiolabeled thymidine from radiolabeled thymine by chromatography on paper [8] or silica gel plates [9,10]. In the continuous spectrophotometric assays thymidine analogues are being used [7]. However, the activity of TP is generally measured by quantification of the amount of thymine, formed from thymidine in the presence of sodium arsenate, by using the difference in UV absorbance that exists between thymine and thymidine at alkaline pH [5,6,11]. A serious drawback of the spectrophotometric assay is the fact that the non-specific

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absorbance of interfering substances of crude tissue extracts hampers the accurate determination of the TP activity [6].

Therefore, we have developed a sensitive and fast assay of the TP activity which is based on the separation of thymine and thymidine using reversed-phase HPLC. In addition, we have established reference values for the TP activity in the Dutch population.

2. Experimental

2.1. Chemicals

All chemicals were of analytical grade. Thymidine and thymine were obtained from Sigma–Aldrich Inc. (St. Louis, MO, USA). Dithiothreitol was obtained from Boehringer Mannheim GmbH (Mannheim, Germany). Acetic acid, acetonitrile, perchloric acid and potassium phosphate were obtained from Merck (Darmstadt, Germany).

2.2. Control population

The control population consisted of patients admitted to the hospital with clinical and biochemical findings not indicative of inborn errors in the purine and pyrimidine metabolism. Samples were obtained according to the "Code for proper use of human tissue" as formulated by the Dutch Federation of Medical Scientific Societies.

2.3. Isolation of leukocytes

EDTA-anticoagulated blood (4 ml) was centrifuged at $450 \times g$ at room temperature for 10 min and the buffy coat, containing the leukocytes, was collected. To lyse the contaminating erythrocytes, three volumes of ice-cold ammonium chloride solution (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA) was added to the buffy coat and kept on ice for 10 min. After centrifugation ($250 \times g$, 4 °C, 10 min) the cell pellet was resuspended in approximately 10 ml phosphate buffered saline (9.2 mM Na_2HPO_4, 1.3 mM NaH_2PO_4 and 140 mM NaCl, pH 7.4). The cell pellet, obtained after centrifugation ($250 \times g$, 4 °C, 10 min), was resuspended in approximately 2.5 ml phosphate buffered saline and an aliquot was used for cell counting using a Coulter Counter Z1000 (Coulter Electronics Ltd., Buckinghamshire, UK). Subsequently, a suspension containing 10 million cells was centrifuged at $13{,}000 \times g$ for 30 s. The supernatant was discarded and the pellet was frozen in liquid nitrogen and stored at −80 °C until further analysis.

2.4. Standard TP assay procedure

The frozen cell pellet of leukocytes was suspended in 300 μl ice cold solution of 35 mM potassium phosphate (pH 7.4) and sonicated six times at 4 W (Vibra-cell Sonificator, output control 20%) for 5 s with intervals of 5 s under constant cooling in ice-water. After centrifugation ($11{,}000 \times g$ at 4 °C for 20 min) an aliquot of the supernatant was used for determination of the protein concentration and the remaining supernatant was stored at −80 °C, until further analysis. Protein concentration in the supernatant was determined by the copper-reduction method using bicinchoninic acid, essentially as described by Smith et al. [12].

The reaction mixture contained an aliquot of cell sample (2.5–32 μg), 35 mM potassium phosphate (pH 7.4), 1 mM dithiothreitol and 2 mM thymidine in a total volume of 0.5 ml. The reaction solution and the sample were equilibrated separately at 37 °C for 5 min and the reaction was started by the addition of the sample. After 15, 30, 45 and 60 min, 100 μl was removed from the reaction mixture and the reaction catalysed by TP was terminated by the addition of 11 μl of ice-cold 8 M $HClO_4$ and kept on ice for 10 min. After centrifugation ($11{,}000 \times g$ at 4 °C for 5 min) the resulting supernatants were removed and saved for analysis by reversed-phase HPLC.

2.5. HPLC analysis

The supernatant (75 μl) was injected into the HPLC system consisting of a Perkin-Elmer Series 410 LC pump (Perkin-Elmer, Norwalk, CT, USA) including a Gilson 200C diluter and a Gilson 231 XL autosampler (Gilson Medical Electronics, Villiers Le Bel, France). The detector was a Gynkotek UVD 340U photodiode array detector (Gynkotek GmbH, Munich, Germany). Separation of thymine from thymidine was performed isocratically [0.2% (w/v) acetic acid and 7% (v/v) acetonitrile] at a flow rate of 1 ml/min by HPLC on a reversed-phase column (Alltima C18 rocket, 250 mm × 4.6 mm, 3 μm particle size, Alltech Associates Inc., Deerfield IL) and a guard column (Alltima C18 5 μm particle size) with online UV detection at 265 nm. Metabolites were identified by comparing the retention times and spectra with those of pure standards.

2.6. Calculation of the TP activity

Quantification of the amounts of thymidine and thymine was performed by comparison with external standards. To determine the specific activity of TP, the amount of thymine formed (y) was plotted against time (x). The slope of the graph (nmol product/h) was calculated by linear regression analysis. The slope was then divided by the amount of protein (mg) in the assay to obtain the specific activity of TP (nmol/mg/h).

2.7. Assay validation

The intra-assay variation of the procedure was assessed by determining the TP activity in 10 replicates of a human leukocyte sample on the same day. The inter-assay variation was determined by analyzing the TP activity of a human lymphocyte sample on 10 different days. The reproducibility of the assay is expressed as the coefficient of variation (CV). The detection limit of the HPLC procedure is

defined as the baseline noise plus three times the standard deviation.

2.8. Kinetic properties of TP

The steady-state kinetics of human TP was performed using the cytosolic fraction obtained after centrifugation of a leukocyte homogenate. The initial reaction rates were determined in 35 mM potassium phosphate (pH 7.4) at 37 °C at various concentrations of thymidine (0.9–860 μM). For determination of the initial reaction rates at various concentrations of phosphate (20 μM–12.5 mM), the reaction was performed in 35 mM Tris-Mops (pH 7.4) at 37 °C. Under all conditions, no more than 10% of the substrate was consumed. Calculation of apparent K_m and V_{max} values were performed by fitting the data to the Michaelis–Menten equation.

2.9. Statistical analysis

Analysis to determine whether the TP activity followed a normal distribution pattern was performed using the Kolmogorov–Smirnov test. Differences in TP activity between two or more groups were analyzed with the two sample *t*-test and one-way ANOVA, respectively. The correlation between the TP activity and age was studied by the determination of the Pearson's correlation coefficients and linear regression. The level of significance was set a priori at $P \leq 0.05$. Analyses were performed with the Statistical Package for the Social Sciences (SPSS Inc., IL, USA).

3. Results

3.1. HPLC procedure

The TP activity was measured using thymidine followed by separation of thymidine and thymine by reversed-phase HPLC. The analyses of a stock solution of thymidine by reversed-phase HPLC showed that the thymidine standard was essentially pure (99.8%). By comparing spectra and retention times of the various peaks in the chromatogram with those of pure standards, the identity of some of the impurities present in the reaction mixture could be established and ascribed to that of thymine, TMP, TDP and TTP (Fig. 1). Fig. 1 shows that a complete baseline separation was obtained within 6 min for thymine and the other compounds present in the assay mixture. The retention times of thymine and thymidine were 2.5 and 3.6 min, respectively and the amount of thymine produced by TP from leukocytes was readily detectable (Fig. 1B). The detection limit of thymine and thymidine in the HPLC system, defined as three times the value of the baseline noise, was approximately 0.8 and 0.7 pmol, respectively.

3.2. Reaction conditions

The specific activity of TP proved to be two-fold higher when the preparation of the homogenates and the measurement of the activity were carried out at pH 6.5 instead of pH 7.4. However, this increase in specific activity was not due to an increased activity of TP itself but due to the slow precipitation of cytosolic (non TP) proteins in the leukocyte homogenates, a phenomenon not observed at pH 7.4. This would be in line with the fact that identical activities were observed for TP purified from human platelets, at pH 6.5 and 7.4 [13]. Therefore, all assays were performed at pH 7.4 to ensure a high reproducibility and accuracy.

Fig. 2 shows that the amount of thymine produced by TP from leukocytes increased linearly with the amount of protein in the assay up to at least 65 μg/ml. With respect to the time dependence of the reaction catalysed by TP, a linear increase in product formation was observed between 15 min and 4 h.

3.3. Kinetic properties

The steady-state kinetics of TP with thymidine and phosphate are shown in Fig. 3. Under these conditions, linear Lineweaver–Burk plots were obtained with apparent K_m values of 43 μM and 0.64 mM for thymidine and phosphate, respectively.

3.4. Assay validation

The intra-assay CV and the inter-assay CV for the complete assay, HPLC detection and protein determination, were

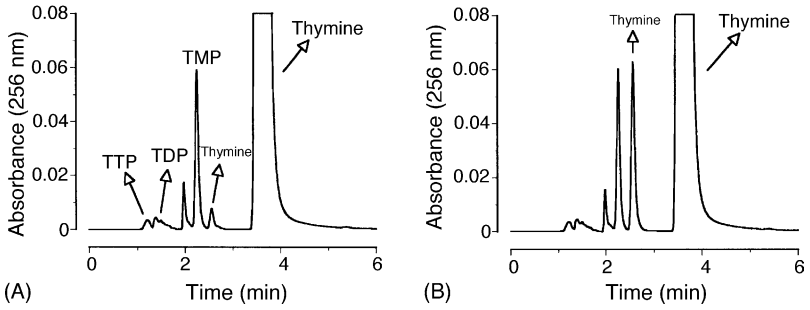

Fig. 1. HPLC elution profile of a reaction mixture. Panel A shows the elution profile of a reaction mixture prior to the start of the incubation. Panel B shows the elution profile of the same reaction mixture obtained after incubation at 37 °C for 1 h.

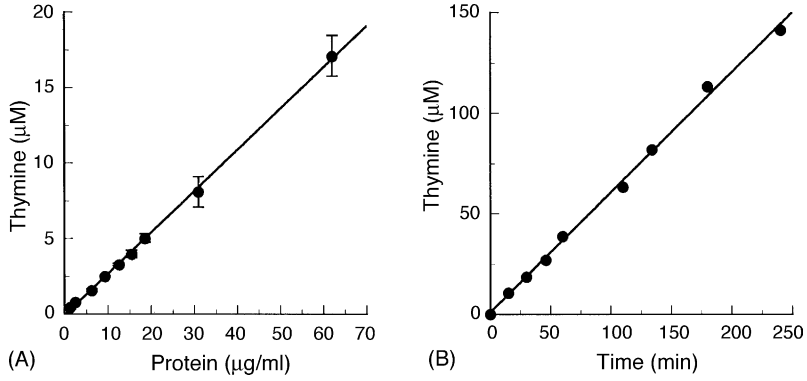

Fig. 2. Protein dependence and time dependence of the TP reaction. Panel A shows the amount of thymine produced by TP at various protein concentrations in the assay. The reaction was allowed to proceed for 1 h at 37 °C. Each data point represents the mean of three experiments ± S.D. Panel B shows the amount of thymine produced by TP at various time points. The TP activity was measured at a protein concentration of 55 μg/ml.

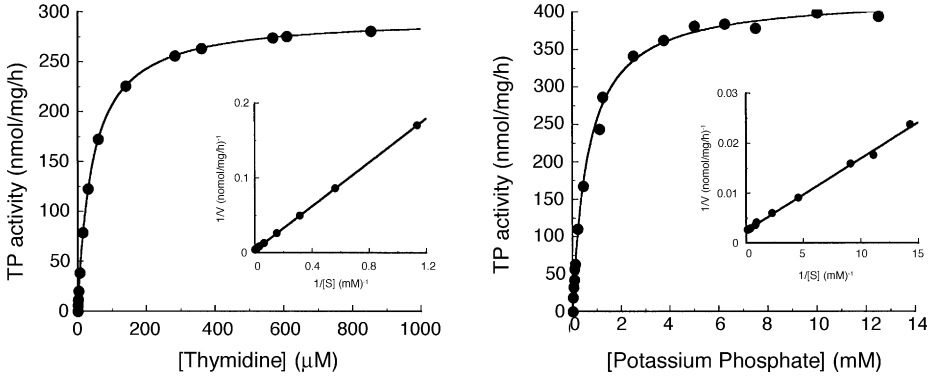

Fig. 3. Steady-state kinetics of human TP. Panel A shows the steady-state kinetics of TP with thymidine (0.9–860 μM). The data points were fitted according to the Michaelis–Menten equation. The insert shows the double-reciprocal plot of the reaction velocity versus the concentration of thymidine (0.9–610 μM). Panel B shows the steady-state kinetics of TP with phosphate (20 μM–12.5 mM). The data points were fitted according to the Michaelis–Menten equation and the insert shows the double-reciprocal plot of the reaction velocity versus the concentration of phosphate (70 μM–7.5 mM).

5.1% ($n = 10$) and 11% ($n = 10$), respectively. Comparable TP activities were detected in leukocytes isolated from EDTA–blood samples ($n = 3$) which had been stored at room temperature for 4, 24 and 48 h (CV < 4.7%). An amount of 1 ml of EDTA–blood was already sufficient to determine the specific activity of TP.

3.5. Population distribution of the TP activity

A normal Gaussian distribution was observed for the TP activity in leukocytes from controls. The TP activity did not differ with increasing age (Table 1). The mean TP activity in this population was 316 ± 85 nmol/mg/h and was identical in man and women.

4. Discussion

TP or platelet-derived endothelial-cell growth factor plays an essential role in the homeostasis of thymidine and the process of angiogenesis [14]. Furthermore, the activity of TP is increased in a variety of solid tumours when compared to that observed in normal tissues [11]. This observation underlies the enhanced tumour selectivity of the chemotherapeutic

Table 1
Population distribution of the TP activity in leukocytes

	Total group ($n = 103$)	Man ($n = 43$)	Women ($n = 60$)	0–20 year ($n = 10$)	20–40 year ($n = 31$)	40–60 year ($n = 26$)	>60 year ($n = 36$)
TP activity (nmol/mg/h)							
Mean ± S.D.	316 ± 85	316 ± 83	316 ± 87	305 ± 57	305 ± 68	351 ± 96	302 ± 91
Range	135–705	141–578	135–705	211–424	202–501	233–704	135–578
Age (years)							
Mean ± S.D.	47 ± 22	52 ± 20	43 ± 23	12 ± 7	29 ± 5	46 ± 5	73 ± 8
Range	0.6–91	11–91	0.6–90	0.6–19	22–37	40–57	61–91

agent capecitabine, a precursor of 5′-deoxy-5-fluorouridine which is preferentially converted to the active compound 5-fluorouracil in malignant tissues via TP [15]. In addition, inhibitors of TP are of potential use in cancer chemotherapy since they suppress tumor growth by inducing apoptosis and suppressing angiogenesis [16,17].

MNGIE is an autosomal recessive disease which is caused by loss of function mutations in the gene encoding TP [1]. Since these patients present with no or a severely reduced activity of TP in leukocytes, the availability of an accurate assay to measure the TP activity is, therefore, of paramount importance. To date, the most frequently used assay of the TP activity is that in which the amount of thymine is measured spectrophotometrically at alkaline pH [5,6,11]. Unfortunately, the presence of interfering substances in the crude tissue extracts has been shown to hamper the accurate determination of the TP activity [6]. In addition, the spectrometric assay was not linear over time which prevented the analysis of the steady-state kinetics of mutant TP enzymes [2].

In this study, we developed an accurate assay procedure for TP, in which a complete separation of thymine from thymidine and other interfering metabolites was achieved in 6 min, using reversed-phase HPLC. Furthermore, the assay proved to be highly sensitive with a minimum amount of thymine that could be detected of 0.8 pmol. Due to this high sensitivity, 2.5–32 μg of protein was sufficient to measure the TP activity at four different time points whereas the fixed time point spectrophotometric assay required 200–500 μg of protein to measure the TP activity [1,5].

It has been shown that the enzymatic arsenolysis of thymidine by TP is inhibited by thymine, the product of the reaction. Thus, the high protein concentration in the spectrophotometric assay, which is necessary to produce a sufficient amount of thymine to allow detection, might be responsible for the observed non linear kinetics [2,6]. In contrast, our assay proved to be linear with time and protein concentrations under all conditions studied. Furthermore, the K_m values for thymidine and phosphate are comparable to those observed for the purified enzyme from human platelets [13].

The onset of clinical symptoms of patients suffering from MNGIE occurred, on average, at 18.5 years of age, ranging from 5 months to 43 years [2]. Until now, no reference values have been reported for TP in the normal population and it was not known whether the TP activity is influenced by gender or age. Our results demonstrated that the activity of TP was identical in man and women and that there was no correlation of the TP activity with age.

The mean activity of TP in our control population was approximately 50% lower than the mean TP activity in leukocytes obtained from 20 controls [5]. A conceivable explanation for this apparent discrepancy is the fact that the TP activity in the latter study has been measured using an assay mixture containing arsenate. It has been shown that the conversion of thymidine into thymine occurred more rapidly in the presence of arsenate then with phosphate [6]. However, the amount of thymine produced from thymidine by TP in the presence of phosphate was readily detectable with our assay procedure and therefore, the highly toxic arsenate could be omitted in the reaction mixture.

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