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Application of HPLC to study the kinetics of a branched bi-enzyme system consisting of hypoxanthine-guanine phosphoribosyltransferase and xanthine oxidase—an important biochemical system to evaluate the efficiency of the anticancer drug 6-mercaptopurine in ALL cell line

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Abstract

The thiopurine antimetabolite 6-mercaptopurine (6MP) is an important chemotherapeutic drug in the conventional treatment of childhood acute lymphoblastic leukemia (ALL). 6MP is mainly catabolized by both hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and xanthine oxidase (XOD) to form thioinosinic monophosphate (TIMP) (therapeutically active metabolite) and 6-thiouric acid (6TUA) (inactive metabolite), respectively. The activity of both the enzymes varies among ALL patients governing the active and the inactive metabolite profile within the immature lymphocytes. Therefore, an attempt was made to study the kinetic nature of the branched bi-enzyme system acting on 6MP and to quantitate TIMP and 6TUA formed when the two enzymes are present in equal and variable ratios. The quantification of the branched kinetics using spectrophotometric method presents problem due to the closely apposed λ_{max} of the substrates and products. Hence, employing an HPLC method, the quantification of the products was done with the progress of time. The limit of quantification (LOQ) of substrate was found to be 10 nM and for products as 50 nM. The limit of detection (LOD) was found to be 1 nM for the substrate and the products. The method exhibited linearity in the range of 0.01–100 μM for 6MP and 0.05–100 μM for both 6TUA and TIMP. The amount of TIMP formed was higher than that of 6TUA in the bi-enzyme system when both the enzymes were present in equivalent enzymatic ratio. It was further found that enzymatic ratios play an important role in determining the amounts of TIMP and 6TUA. This method was further validated using actively growing T-ALL cell line (Jurkat) to study the branched kinetics, wherein it was observed that treatment of 50 μM 6MP led to the generation of 12 μM TIMP and 0.8 μM 6TUA in 6 h at 37 °C.

Keywords: Reverse-phase HPLC; Branched bi-enzyme system; Acute lymphoblastic leukemia; 6-Mercaptopurine; 6-Thiouric acid; Thioinosine monophosphate

1. Introduction

6MP, an analog of hypoxanthine is an antileukemic agent widely used to treat ALL, the most common malignancy affecting children and other leukemias [1]. 6MP is a prodrug that undergoes extensive metabolism by intracellular enzymes in order to exert its cytotoxic effect [2]. Biotransformation of 6MP mainly occurs via three competing pathways catalysed by HGPRT, XOD and thiopurine methyltransferase [3]. XOD (EC 1.1.3.22) [4] mediated pathway includes the oxidative hydroxylation of 6MP leading to the formation of 6TUA, an inactive metabolite. The other route generates the active metabolite TIMP by the action of the cytoplasmic enzyme HGPRT (E.C.2.4.2.8) [5]. XOD activity varies from tissue to tissue and is known to be overexpressed in tumors and other pathophysiological conditions [6–8]. The overexpression and hyper reactivity of

Abbreviations: XOD, xanthine oxidase; 6MP, 6-mercaptopurine; 6TUA, 6-thiouric acid; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; RP-HPLC, reverse-phase HPLC; ALL, acute lymphoblastic leukemia; TIMP, thioinosine monophosphate; PRPP, phosphoribosyl pyrophosphate

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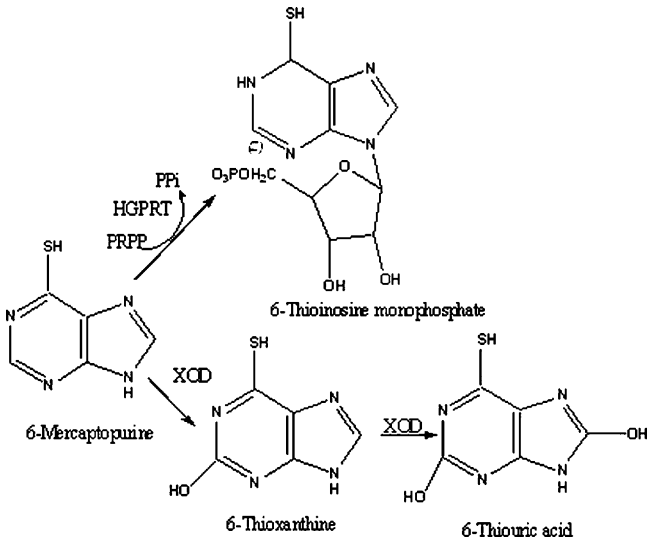

Fig. 1. Scheme demonstrating the action of HGPRT and XOD on 6MP showing the generation of TIMP and 6TUA.

XOD initiates a metabolic elimination process of the anticancer drug 6MP, as 6MP-binding to its target HGPRT is reduced due to the metabolic branching (Fig. 1). Thus, the action of XOD reduces the amount of parent drug available and therefore, the efficacy of 6MP therapy. So, to circumvent a relapse, higher doses of drug may be required adding to toxicity of the drug. Differences in the rate of extracellular catabolism of 6MP by XOD into 6TUA contribute to a large patient variation in the plasma 6MP concentration achieved following a uniform oral dose [9,10]. Hence, in the present context, it is very important to study the kinetic nature of this branched pathway consisting of XOD and HGPRT acting on 6MP under variable enzymatic ratios and in ALL cells.

Spectrophotometric quantification of this important branched pathway failed due to the interference and overlapping of monitoring wavelengths and nonspecific contribution in cellular system. The branched kinetic studies of 6MP was difficult to be evaluated using spectrophotometric method, as the substrate (6MP, λ_{max} 320 nm) and the products (TIMP, λ_{max} 320 nm and 6TUA, λ_{max} 350 nm) (Fig. 2 inset A curves a, b and c) share almost identical λ_{max}. Therefore, proper monitoring of substrate degradation with concomitant product formation is difficult to measure quantitatively (Fig. 2 inset B: at 320 nm and C: at 350 nm).

HPLC method has been used to separate and quantify purine nucleotides in body fluids [11,12], but to the best of our knowledge, this branched kinetics has not been reported in ALL cells earlier. Keeping in view the discrepancies of the spectrophotometric method, the purpose of this work was to develop a simple and efficient method to study the distribution of 6MP in the bi-enzyme system *in vitro* and also extend this method to the determination of the branched system in ALL cell lysate. Many HPLC methods have been used to separate and quantify purine nucleotides and 6MP nucleosides or nucleotides in the body fluids [13–17]. Although some researchers have used red blood cells (RBC) as surrogate tissue to measure antileukemic action of 6MP [11,12,18–22], RBCs are not the target tissue for 6MP.

Although human malignant lymphoblasts are the targets of 6MP, biotransformation studies with respect to its quantification are scarcely available [23]. Among the HPLC methods that have been described for the measurement of 6MP metabolites in lymphoid cells [24–26], one of the methods measures these nucleotides indirectly after hydrolysis to their bases and thus, requires an additional measurement of (methyl) thiopurine nucleosides and bases before hydrolysis to establish which part of the base originates from the hydrolyzed nucleotides.

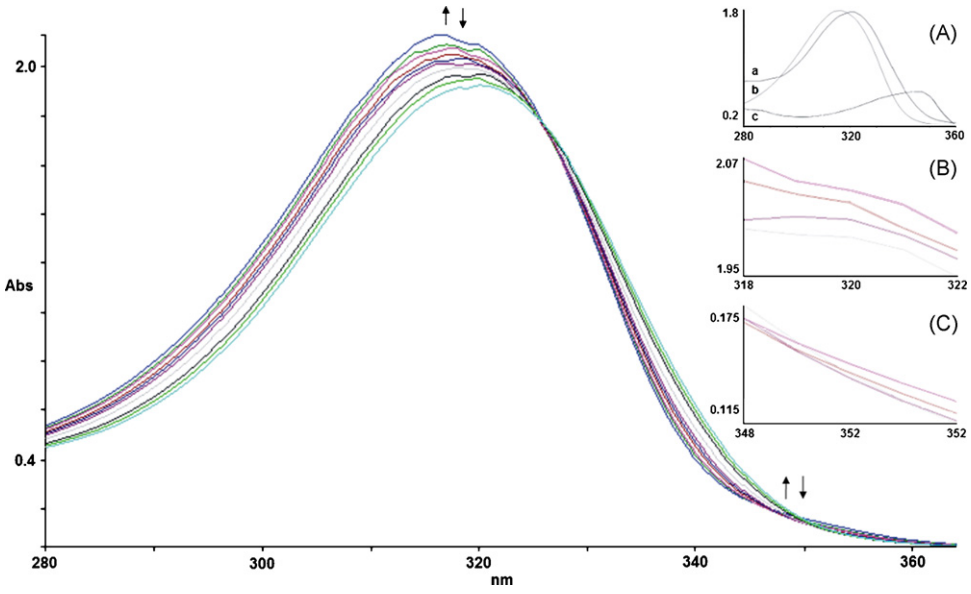

Fig. 2. Spectrometric analyses of the enzymatic conversion of 6MP showing the enzymatic reaction of 100 μM 6MP in the presence of 1 U/ml HGPRT and 0.1 U/ml XOD in presence of PRPP with progress in time. Inset figures show: (A) overlapping absorption spectra of 90 μM TIMP (curve b), 25 μM 6TUA (curve c) and 100 μM 6MP (curve a); (B) magnified view of reaction at 320 nm with time; (C) magnified view of reaction at 350 nm with time.

Other papers have used laborious procedures following either the ion-pairing HPLC assay that enables the measurement of thioguanosine monophosphate and TlMP [25] or mercuric cellulose resin [11] for the detection of 6MP metabolites. Rowland et al. [27] studied the primary metabolism of 6MP in the human liver cytosol [27]. The cytosol-6MP interaction has been studied *ex vivo* at 5 and 48 h, and the condition of the *ex vivo* liver sample cannot be predicted bearing in mind the inevitable degradation by cellular proteases. As the 6MP is being used for treatment of ALL, the effective dose of the active drug accumulated in lymphoblasts is of interest.

Till date, TIMP and 6TUA together have never been quantitated simultaneously *in vitro* as well in T-cell ALL. Therefore, the aim was two fold, one to develop a convenient and an accurate method to determine the stiochiometric distribution of these enzymatic products using variable enzyme ratios of HGPRT and XOD *in vitro*, and the other to study the extent of 6MP degradation by these two enzymes in the lymphoblasts.

2. Experimental

2.1. Materials

Bovine milk XOD was procured from Calbiochem (La Jolla, CA, USA). Hypoxanthine and 6MP were procured from Sigma (St. Louis. MO, USA). Disodium hydrogen phosphate and sodium dihydrogen phosphate were purchased from Himedia, India. Mobile phase component, namely, methanol for HPLC was purchased from Merck Inc. Ltd. (Germany). Nylon membrane filters were procured from Millipore, USA.

2.1.1. Purification of HGPRT

Human HGPRT gene cloned in pET23d vector and expressed in *E. coli* BL21DE3 strain was used to purify human HGPRT following the method of Subbayya et al. [28].

2.1.2. Cell line and Culture

Jurkat, a T-ALL cell line, originally derived from peripheral blood of a 14-year-old boy was purchased from National Centre for Cell Sciences (Pune, India) and was maintained in RPMI-1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS; Gibco-BRL, Grand Island, NY, USA) at 37 °C in an environment of 5% CO_2 and 95% humidity [30].

2.2. Instrumentation

A Shimadzu Inc. (Japan) HPLC system attached with an ultra violet detector (of a single channel wavelength in the present study) was used for our experiments. The chromatographic separations were carried out on a 10 μm Supelco C18 RP chemically bonded stationary phase (250 mm × 4.6 mm i.d.) equipped with security guard column. The spectrophotometric assays were performed using a temperature controlled, double beam Perkin Elmer Lambda 25 spectrophotometer.

2.3. Sample preparation

Purified enzyme was diluted accordingly for the *in vitro* assays.

2.3.1. Preparation of cell lysate

For the studies regarding cell lines, approximately 3×10^7 Jurkat cells were harvested and lysed using a Polytron homogenizer at 2000 rpm (40 cycles) in buffer composed of 250 mM sucrose, 20 mM HEPES buffer, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA and 1 mM EGTA, pH 7.4. The cell lysate was then subjected to centrifugation at $2500 \times g$ for 5 min to pellet down the cell debris and the supernatant was used for the spectrophotometric and HPLC assay [28].

2.4. Enzyme activity assay

Assay mixture for HGPRT contained 1 ml of 100 mM Tris, pH 7.4, 12 mM $MgCl_2$, 1 mM PRPP and 100 μM 6MP as the substrate [29]. The assay mixture for XOD contained 1 ml of 100 mM Tris, pH 7.4, 12 mM $MgCl_2$ buffer and 100 μM 6MP. One unit of HGPRT or XOD is defined as the amount of the respective enzymes that produces 5.9 μmol of their products per minute, as evaluated by the HPLC analysis. Enzyme units were also calculated for the single enzymes spectroscopically at 320 and 350 nm, but extrapolating the same in the bi-enzyme system raised a lot of complexity. In all the cases, the reaction was initiated with the addition of the enzyme(s).

2.5. HPLC analysis

A linear buffer-methanol gradient elution profile was obtained by employing a 100% A (sodium phosphate (0.02 M, pH 5.8) to 40% B (Methanol:water 60%, v/v) in 35 min at a flow rate of 1.5 ml/min at an ambient temperature. The mobile phase solvents were filtered through 0.22 μm nylon membrane and further degassed before use. The injection volume was 20 μl; total run time was 15 min. The HPLC procedure was performed at room temperature and a single monitoring wavelength was chosen as 320 nm.

Kinetic measurements for HGPRT were carried out by the addition of 100 μM of 6MP and 1 mM PRPP in 1 ml assay buffer consisting of 100 mM Tris, 12 mM $MgCl_2$ buffer (pH 7.4). These components were mixed and preincubated at 37 °C for 5 min followed by the addition of 1 U of purified HGPRT. After further incubation at 37 °C for different time intervals (*in vitro* as well as with the cell lysates), chilled EDTA (25 μl from 10 mM stock) was added and immediately kept in ice to terminate the enzyme reaction followed by protein precipitation mediated by perchloric acid (PCA) and potassium carbonate [26]. The mixture was then centrifuged at $10{,}000 \times g$ for 10 min. The supernatant was then withdrawn and filtered through 0.22 μm nylon membrane. This filtered sample was injected directly into the HPLC system for analysis. Similar treatment was given to the samples of XOD and XOD-HGPRT system.

2.5.1. Preparation of 6TUA and TIMP

For the preparation of 6TUA, 1 ml of assay buffer (Section 2.5) consisting of 15 mg 6MP and 50 U XOD was incubated for 48 h at 37 °C. The aforementioned reaction mixture sample was dried and redissolved in alkaline solution. This sample was taken for spectroscopic and HPLC analysis. Similarly, 50 mM PRPP was added to the 1 ml assay buffer consisting of 15 mg 6MP and 50 U HGPRT incubated at 37 °C for 10 h. The aforementioned reaction mixture was dried and redissolved in water, and the sample was used for spectroscopic and HPLC analysis.

2.6. Stock solutions

One millimolar stock solutions of 6MP and 6TUA were prepared in alkaline solution by adding 20 μl of 1 N NaOH to an appropriate amount of weighed standard in water. The calibration solutions were prepared in water. For TIMP, both the stock and the calibration solutions were prepared in water. The stock solutions of these three compounds were diluted in water accordingly in order to prepare calibration solutions of concentrations ranging from 0.001 to 100 μM. The individual stock standards and the calibration standards were stable at least for 6 months at −20 °C protected from light.

2.7. Validation of HPLC method

2.7.1. Linearity and range

The solutions were injected in triplicate for obtaining a representative result of the linearity and range.

2.7.2. Precision

Three different concentrations (0.01, 2 and 100 μM) of 6MP, 6TUA and TIMP were injected. Intraday analysis was performed with 10 replicates, whereas interday evaluation was assessed with 3 replicates on 3 different days. Precision was determined in terms of the coefficient of variation (CV).

2.7.3. Accuracy

The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. The accuracy studies were carried out with three concentrations of the product (0.01/0.05, 2 and 100 μM 6MP/6TUA/TIMP).

3. Results and discussion

3.1. Spectrophotometric analysis of kinetics of HGPRT and XOD

By using the spectrophotometric method, kinetics were observed for XOD and HGPRT individually and the two enzymes together continuously for 60 min with the time interval of 1 min using 100 μM 6MP as the substrate. Bi-enzyme reaction kinetics with 6MP as a substrate in the presence of 1 U HGPRT and 1 U XOD mixture (ratio of HGPRT:XOD is 1:1) and 1 U HGPRT and 0.1 U XOD (ratio of HGPRT:XOD is 10:1) led to the generation of the TIMP and 6TUA together in the same reaction mixture (data not shown). But the amount of the two products generated in the reaction mixture could not be quantitated because of the overlapping λ_{max} (6MP and TIMP, λ_{max} 320 nm and 6TUA λ_{max} 350 nm) (Fig. 2 inset A curve a, b and c, respectively). Then, in order to simulate the pathophysiological conditions of the body where the XOD level is increased, we performed the reaction in the presence of 0.1 U HGPRT and 1 U XOD (ratio of HGPRT:XOD is 1:10). Again, no clear results in terms of the products could be obtained (data not shown).

Looking at the spectroscopic analysis of the kinetics of formation of 6MP metabolites as depicted in Fig. 2, the exact quantification of the biotransformation of 6MP into TIMP and 6TUA could not be done accurately due to the problem of overlapping spectra of the substrate and products. Fig. 2 inset (B) and (C) depict the magnified view of the reaction scans at 320 and 350 nm with time. Thus, the HPLC method for the precise quantification of the two products was employed for the same purpose in which the molecules are separated based on the inherent polarity difference as reflected in their retention time (R_t) values.

3.2. HPLC analysis of kinetics of HGPRT and XOD

Using the RP-HPLC method, we quantitated the two products with single and bi-enzyme system. Various protein precipitation methods were tried, e.g. with trichloroacetic acid, thermal denaturation (heating at 60 °C), methanol and PCA (data not shown) but the PCA treatment gave the best results. Initial attempts were made using the mobile phase like Tris-HCl buffer and varying the pH of the mobile phase (data not shown), but the best results were obtained when a linear sodium phosphate buffer and methanol:water (60:40) gradient was employed. The concentration of 6MP was also standardized and 100 μM 6MP was used for the *in vitro* experiments. The R_t values of the substrate (6MP, $R_t = 8.5$ min) and the products (6TUA, $R_t = 3.19$ min) (TIMP, $R_t = 4.5$ min) (Fig. 3 insets A, B and C) were found to be reasonably different and the corresponding peaks were well separated in the chromatogram. The progress in the reaction catalysed by single enzyme HGPRT and bi-enzyme was studied for 60 min after standardizing the reaction time. The peak corresponding to EDTA was subtracted from each of the experimental chromatograms and individual integrated chromatograms were obtained.

3.3. Validation of HPLC method

3.3.1. Linearity and range

With the methanol–water and sodium phosphate mobile phase, the response of 6MP, 6TUA and TIMP were linear in the concentration range between 0.01 and 100 μM and of 6TUA and TIMP in the range between 0.05 and 100 μM. The mean values of slope, intercept and correlation coefficient were calculated for 6MP, 6TUA and TIMP as described in Table 1.

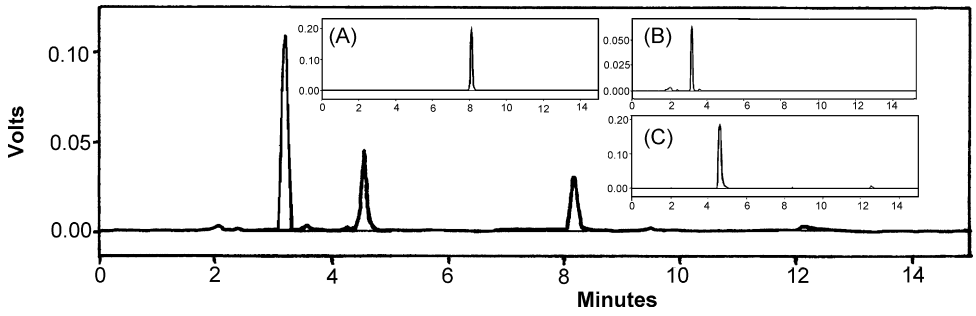

Fig. 3. HPLC chromatogram showing the retention time of 6MP (R_t = 8.5 min) 6TUA (R_t = 3.19 min) and TIMP (R_t = 4.5 min) in the same reaction mixture. Inset figures show individual retention time: (A) 100 μM of 6MP (R_t = 8.5 min); (B) 100 μM 6TUA (R_t = 3.19 min); and (C) 100 μM TIMP (R_t = 4.5 min).

Table 1
Slope, intercept and the correlation coefficient values for the three metabolites 6MP, 6TUA and TIMP in buffered medium as determined by RP-HPLC

Metabolite	Slope (mean ± SD)	*y*-intercept (mean ± SD)	Correlation coefficient (R^2) (mean ± SD)
6MP	39690 ± 51.39	649.47 ± 44.97	0.9955 ± 0.0037
6TUA	33476 ± 55.43	997.09 ± 173.63	0.9963 ± 0.0015
TIMP	29664 ± 4.72	573.48 ± 71.37	0.9920 ± 0.0026

3.3.2. Precision

The CVs for 6MP, 6TUA and TIMP for the intra-day precision ranged from 0.4 to 8.5%, 1.3 to 5.8% and 2.7 to 5.9%, respectively, and for the inter-day precision, they ranged from 2.3 to 5.4%, 2.0 to 10.0% and 2.5 to 7.8%, respectively (Table 2).

3.3.3. Accuracy

Mean intra-day accuracy efficiencies were 101.9%, 102.3% and 97.7% for 6MP, 6TUA and TIMP, respectively. Mean inter-day accuracy efficiencies were 101.7%, 99.5% and 97.4% for 6MP, 6TUA and TIMP, respectively (Table 2).

3.3.4. Limit of detection and quantification

LOD and LOQ values were determined by calculating signal-to-noise ratio. Signal-to-noise ratio of approximately 3:1 and 10:1 were used for estimating LOD and LOQ, respectively of the method. The LOQ value for 6MP was found to be 10 nM, and for 6TUA and TIMP it was 50 nM. LOD values for 6MP, 6TUA and TIMP were found to 1 nM.

3.4. Quantification of metabolites using HPLC results

6TUA and TIMP were finally quantitated under both single and bi-enzyme system by employing the HPLC method. A representative chromatogram showing all the three compounds 6MP, 6TUA and TIMP present in the same reaction mixture, well separated with good resolution is shown in Fig. 3. The amounts of the residual 6MP and the products formed are described in Table 3. In the time dependent enzymatic reaction, we observed a decrease in the area under the peak of 6MP (R_t 8.5 min) and a gradual increase in the area under the peak of 6TUA (R_t 3.2 min). Analyzing the XOD-mediated hydroxylation product of 6MP, we found that 95.672 μM of 6TUA was formed in 60 min. The results of HGPRT-mediated reaction of 6MP indicated the generation of 97.439 μM of TIMP in 60 min. The progress in the reaction of 6MP with equivalent units of HGPRT and XOD (ratio of HGPRT:XOD is 1:1 and in the presence of 1 mM PRPP) has depicted that 54.328 μM of TIMP and 45.668 μM of 6TUA were formed in 60 min. Therefore, when HGPRT and XOD are present in 1:1 ratio, TIMP is formed in a little higher concentra-

Table 2
Intra- and Inter-day precision and accuracy of 6MP, 6TUA and TIMP

Metabolite	Intra-day precision (n = 10)				Inter-day precision (n = 3)			
	Target concentration (μM)	Mean measured (μM)	% CV	Accuracy (%)	Target concentration (μM)	Mean measured (μM)	% CV	Accuracy (%)
6MP	0.01	0.010	8.521	101.405	0.01	0.009	5.470	98.775
	2	2.036	0.427	101.819	2	2.071	3.217	103.597
	100	102.526	2.756	102.526	100	102.736	2.319	102.736
6TUA	0.05	0.052	5.899	104.877	0.05	0.049	10.058	98.770
	2	2.033	1.333	101.668	2	1.970	3.674	98.503
	100	100.537	2.747	100.537	100	101.486	2.027	101.486
TIMP	0.05	0.048	5.963	95.639	0.05	0.047	7.886	94.034
	2	1.892	2.747	94.595	2	1.924	2.583	96.211
	100	103.050	3.244	103.050	100	101.958	2.616	101.958

Table 3
Amounts of TIMP and 6TUA generated and residual 6MP after the reaction of 100 μM 6MP with the mentioned enzymatic ratios with the progress of time as determined by RP-HPLC

S. No.	Enzyme ratio HGPRT:XOD	Time (min)	TIMP (μM) R_t = 4.5 min	6TUA (μM) R_t = 3.2 min	6MP (μM) R_t = 8.5 min
1	0:1	10	–	61.21	38.78
		60	–	95.672	4.328
2	1:0	10	56.78	–	43.22
		60	97.439	–	2.56
3	1:1	10	41.96	31.044	26.99
		60	54.328	45.668	0.004
4	10:1	10	75.037	3.815	21.14
		60	91.474	6.084	2.44
5	1:10	10	10.43	58.278	31.28
		60	17.784	80.046	2.17

tion than 6TUA. A minor peak with R_t value of about 12.5 min was observed in some chromatograms, where HGPRT reaction was carried out. This may be because of the *in situ* formation of some 6MP riboside, a hydrolysis product of TIMP. The formation of the riboside may be due to contaminating phosphatases in the enzyme preparation.

In the bi-enzyme system where 0.1 U XOD and 1 U HGPRT (ratio of HGPRT:XOD is 10:1) are present, the distribution pattern of 6MP into its respective enzymatic products showed that 91.474 μM TIMP and 6.084 μM 6TUA are formed in 60 min. Certainly, HGPRT is overpowering the XOD action on 6MP. In order to simulate the pathophysiological conditions where XOD is known to be overexpressed, the distribution pattern of 6MP into its corresponding products was studied by treating 6MP with 1 U XOD and 0.1 U HGPRT (ratio of HGPRT:XOD is 1:10). The results indicated that 80.046 μM 6TUA and 17.784 μM TIMP were enzymatically formed in 60 min (Table 3).

3.5. Extension of the HPLC method to Jurkat (T-ALL) cell line

Childhood acute lymphoblastic leukemia cells are very sensitive to 6 MP. Therefore, we planned to study the distribution profile of HGPRT and XOD in a widely used T-ALL cell line (Jurkat) derived from a 14-year old boy having resistant T-lymphoblast cancer [31,32]. Jurkat cells (3×10^7) were treated with a drug range of 25–200 μM 6MP (data not shown) but the best results were obtained with 50 μM 6MP. Time dependent studies for the optimum 6MP incubation time of Jurkat cells were also performed using a time frame of 1, 3, 6, 9 and 12 h (data not shown) but the best results were obtained in the time period of 6 h. Cell viability and cell counting was determined by haemocytometer using trypan blue and approximately 95% viable cells were used for each experiment [30].

3.5.1. Detection of XOD and HGPRT in ALL cell line

Assays for the presence of the enzymes in cell line lysate using spectrophotometric method presented more problems of nonspecific contribution. When the 6MP degradation was studied spectroscopically, there was a wavelength independent increase observed in absorbance in the range from 200 to 400 nm. Thus, we analyzed the product formation profile in Jurkat cell line lysates using the HPLC-based method. Unlike the *in vitro* system, here the ratio of the two enzymes was fixed.

The analytes, namely, 6MP, 6TUA and TIMP were added (50 μM concentrations) to the control cell extract (Section 2.3.1) (Fig. 4A). 3×10^7 Jurkat cells were harvested and treated with a concentration of 50 μM 6MP for 6 h at 37 °C and the cell lysate (as mentioned in Section 2.3.1) was prepared for HPLC analysis. From the results, we observed the presence of both the enzymes as indicated by 6MP utilization (shown by the decrease in AUC of the peak corresponding to 6MP at R_t 8.5 min) and the dual

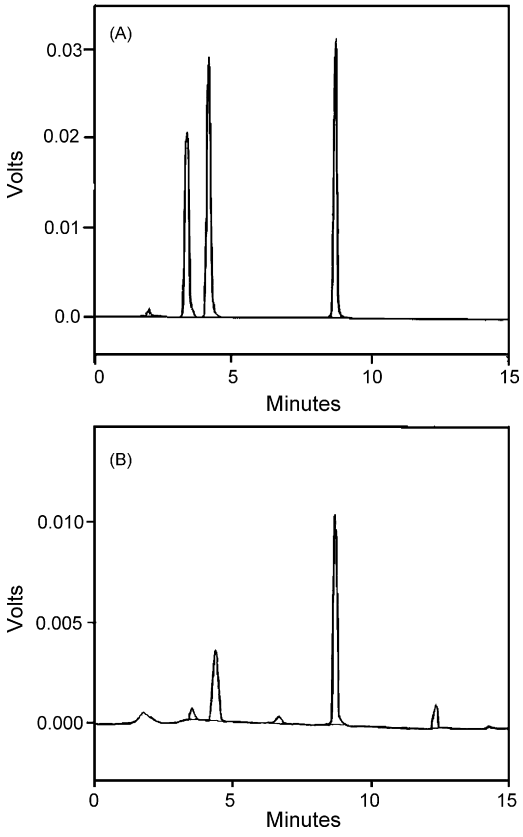

Fig. 4. HPLC chromatograms showing (A) Jurkat cell extract after addition of 50 μM 6MP, TIMP and 6TUA each; (B) Jurkat cell extract after 6MP treatment for 6 h. Fifty micromolar 6MP treatment was given to the cells for 6 h at 37 °C.

Table 4
Slope, intercept and the correlation coefficient values for the three metabolites 6MP, 6TUA and TIMP in cell lysates as determined by RP-HPLC

Metabolite	Slope (mean ± SD)	*y*-intercept (mean ± SD)	Correlation coefficient (R^2) (mean ± SD)
6MP	40493.33 ± 24.826	579.96 ± 128.50	0.9981 ± 0.0009
6TUA	36396.33 ± 23.07	3430.56 ± 216.19	0.9978 ± 0.0024
TIMP	30248 ± 39.50	563.04 ± 135.22	0.9950 ± 0.0040

Table 5
Intra- and Inter-day precision and accuracy of 6MP, 6TUA and TIMP in cell lysate

Metabolite	Intra-day precision ($n = 10$)				Inter-day precision ($n = 3$)			
	Target concentration (μM)	Mean measured (μM)	% CV	Accuracy (%)	Target concentration (μM)	Mean measured (μM)	% CV	Accuracy (%)
6MP	0.01	0.011	3.667	107.233	0.01	0.010	9.212	102.901
	2	2.023	2.781	101.155	2	2.011	1.615	100.575
	100	102.093	1.448	102.093	100	100.956	2.035	100.956
6TUA	0.05	0.055	10.845	109.596	0.05	0.049	5.791	97.399
	2	1.926	6.343	96.276	2	1.903	4.931	95.131
	100	99.030	2.183	99.030	100	100.123	1.170	100.123
TIMP	0.05	0.048	4.794	95.802	0.05	0.048	5.468	95.520
	2	1.989	1.758	99.439	2	1.996	1.444	99.795
	100	98.908	1.381	98.908	100	100.267	2.069	100.267

product generation (shown by the appearance of peaks corresponding to TIMP and 6TUA, R_t 4.5 and 3.2 min, respectively) (Fig. 4B) in comparison to the control cell extract.

3.5.2. Characterization of branched pathway in ALL cell line using HPLC method

After 6MP treatment of ALL cells, we obtained the peaks corresponding to the two products of 6MP metabolism, 6TUA and TIMP. 6TUA and TIMP were quantified and found to be present in concentrations equivalent to 0.8 μM and 12 μM, respectively (Fig. 4B).

3.5.3. Validation of HPLC method in cell lysate

The inter- and intra-day precision and accuracy were determined in order to validate the method in the cell lysate. The linearity and range values were 0.05–100 μM for 6TUA and TIMP while for 6MP, it was found to be 0.01–100 μM. The LOQ values for 6MP were calculated to be 10 nM and for 6TUA and TIMP as 50 nM. The LOD values of 6MP, 6TUA and TIMP are found to be 0.001 μM. The results have been shown in Tables 4 and 5.

4. Conclusion

The current HPLC method based on widely available C_{18} column was employed over spectroscopy in order to understand the fate of 6MP in the presence of the bi-enzyme system, consisting of HGPRT and XOD in equivalent and variable ratios both *in vitro* and *ex vivo*. This HPLC method provides a simple, sensitive, precise and accurate tool to characterize the branched enzymatic pathway and is devoid of non specific cellular interferences. Using the HPLC-based analysis, we realized that when the two enzymes, HGPRT and XOD were present in equivalent ratio, a considerable amount of 6MP was being degraded by XOD to form 6TUA leaving behind approximately 50% of the drug for the action of HGPRT. While in the case where HGPRT present was 10 times higher than XOD, almost 15 times more TIMP was formed as compared with 6TUA. On the other hand, when the activity of XOD was 10 times higher than HGPRT, 80% of 6TUA and 18% of TIMP were formed. Thus, this unique *in vitro* HPLC quantification of the stoichiometric distribution of the branched pathway simulates the *in vivo* condition of varied enzyme ratios depending on the type of cancer and its stage. The method was further validated with cell lysate of T-ALL cell line (Jurkat) and thus can meet the demand of analysis of a large number of clinical samples. Thus, from the stoichiometeric values of both the products (12 μM TIMP and 0.8 μM 6TUA) obtained in Jurkat cell line, it was clear that the ratios of these enzyme concentrations in various types (depending on age, stage and other factors) of ALL might play a vital role in determining the efficacy of 6MP in specific cases.

Thus, we have developed an HPLC method to study the branched bi-enzyme aspect of drug metabolism, which is interesting from the perspective that enzyme concentrations may vary during the physiological or pathophysiological conditions (like age, and stage of disease) and can be extended to patient lymphoblasts. Hence, the HPLC method has direct clinical application in cancer therapy.

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