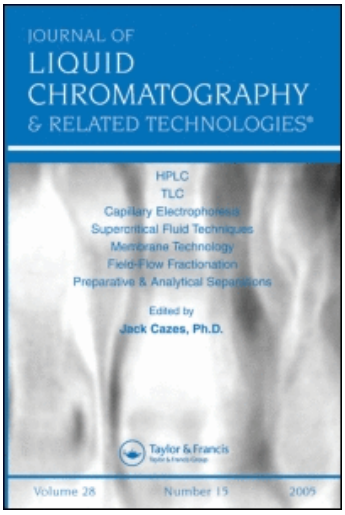

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M. L. Umrethia[a]; P. K. Ghosh[a]; R. J. Majithiya[a]; R. S. R. Murthy[a]
[a] Drug Delivery Research Laboratory, Pharmacy Department, The M.S. University of Baroda, Vadodara, Gujarat, India

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New RP-HPLC Method for the Estimation of 6-Mercaptopurine in Rat Plasma and Various Tissue Homogenates

M. L. Umrethia, P. K. Ghosh, R. J. Majithiya, and R. S. R. Murthy
Drug Delivery Research Laboratory, Pharmacy Department, The M.S. University of Baroda, Vadodara, Gujarat, India

Abstract: A reversed-phase high performance liquid chromatography (HPLC) method was developed to determine 6-mercaptopurine (6-MP) in plasma and tissue homogenates of rats in the present study. A known quantity of the drug and internal standard (metronidazole) was spiked in rat plasma and tissue extracts in a range of 50–1000 ng/mL and 100–1000 ng/mL. The spiked plasma samples were deproteinized using a methanol and acetonitrile mixture (1 : 1, v/v) and centrifuged. The supernatant was collected and analyzed for the drug content. A Shimadzu system with Thermosil® C_{18} (5 μm, 25 cm × 4.6 mm i.d.) column was used for the analysis. A mixture of 0.01 M KH_2PO_4 buffer : acetonitrile (80 : 20, v/v) was used as a mobile phase at a flow rate of 1 mL/min. Each sample containing 20 μL was injected through a Rheodyne injector, and the effluent was monitored at 325 nm.

The method was applied to determine pharmacokinetic parameters and biodistribution of a drug in tissues. The retention times were 4.0 min and 6.2 min for drug and internal standard, respectively. The plot of ratio of area of 6-MP and IS vs. concentration of 6-MP in ng/mL was found linear in the range of 50–1000 ng/mL in plasma and 100–1000 ng/mL in all tissues. High correlation coefficients were observed with plasma (0.998), liver (0.999), lung (0.999), kidney (0.999), heart (0.993), and spleen (0.990). The limit of detection in plasma was 38 ng/mL and 66–89 ng/mL in various tissues. The performed "t" test for the estimated concentration in recovery studies indicate no significant difference between the added and estimated concentration proving the accuracy and low % RSD values indicate the precision of the method. On the basis of total 6-MP plasma concentrations as determined by HPLC, the drug showed the AUC of 2098.76 ± 351.2 ng · h/mL, C_{max} of

Address correspondence to M. L. Umrethia, Drug Delivery Research Laboratory, GH Patel Pharmacy Building, Donor's Plaza, Opposite to M.S. University Office, Fatehgunj, Vadodara-390002, Gujarat, India. E-mail: manishumrethia@lycos.com

1437.27 ± 214.84 ng/mL with T_{max} of 30 min, Vss of 0.27 mL/kg, and total body clearance of 2.32 mL/h/kg in pharmacokinetic studies, by treating rats with 6-MP at a dose of 5 mg/kg intravenously. The biodistribution studies showed that the drug was more concentrated in the liver and kidney than other organs, with a general rank order of liver > kidney > heart > spleen > lung. The above study showed great potential of the reported method to estimate 6-MP in plasma and tissues of rats and may be a substitute for other methods, which are complex, time consuming, and consume high quantities of organic solvents for estimation.

Keywords: 6-Mercaptopurine, HPLC method, Pharmacokinetic and biodistribution studies

INTRODUCTION

6-Mercaptopurine (6-MP) is a purine analogue and has been used in cancer chemotherapy, primarily in childhood leukemia.[1] The bioavailability of 6-MP is low and highly variable, with only 16% of an orally administered dose of 6-MP gaining access to the systemic circulation,[2] that has been suggested as one of the possible causes of a relapse in children with leukemia receiving maintenance chemotherapy.[3–7] The importance of optimizing 6-MP therapy and achieving high and predictable systemic drug exposure has encouraged the use of i.v.6-MP in patients with ALL.[8] The half-life of 6-MP in plasma is short, ranging from 1–3 h. It is usually used in combination with other anticancer drugs and has interfered with the synthesis of adenine and guanine ribonucleosides, which are important precursors of DNA and RNA. 6-MP is cytotoxic because it acts predominantly on rapidly dividing cells such as the T lymphocytes.[1] A number of different analytical studies have been done to determine the drug content in drug products. 6-MP can be estimated by the UV spectroscopic method.[9] The HPLC methods are useful in determination of drug in pharmaceutical dosage form and biological samples. 6-MP and its metabolites have been determined by high performance liquid chromatography.[10–12] There are various HPLC methods published for the estimation of 6-MP in human erythrocytes, plasma, and serum.[13,14] These procedures showed long retention times, do not give results rapidly, need more use of expensive solvents, tedious extraction procedure, use of organic modifiers viz. triethylamine and glacial acetic acid in the mobile phase composition, and maintenance of pH,[15] which finally limits column life. Metabolism of 6-mercaptopurine in the erythrocytes, liver, and kidney of rats during multiple-dose regimens is also reported[16] in which 6-MP was administered i.p. The objective of the present study is to develop a sensitive, precise, selective, specific, reproducible, fully validated, easy to perform, and low cost routine reverse phase HPLC UV detection method for estimation of 6-MP in rat plasma and in different tissues of rats, without any time consuming sample preparation steps and any interferences

from endogenous substances from biological samples for routine analysis, as well as to determine pharmacokinetic parameters and accumulation of 6-MP in various tissues after intravenous administration of 6-MP in rats.

EXPERIMENTAL

Materials

6-Mercaptopurine was kindly supplied as a gift sample by Dabur Therapeutics Ltd., India. HPLC grade methanol, acetonitrile, potassium di-hydrogen phosphate, and water were purchased from S.D. Fine Chemicals Pvt. Ltd., India. All solvents were then combined in appropriate proportions and filtered (0.45 μm HA membrane filter, Millipore, Ireland) to prepare the mobile phase.

HPLC System and Chromatographic Conditions

The HPLC system consisted of a Rheodyne Isocratic pump (Model-LC-10 Avp, Shimadzu Corp., Kyoto, Japan) a model 2250 pump (Bischoff, Germany), and a diode-array detector (Model-SPD Avp, Shimadzu Corp., Kyoto, Japan) set at a wavelength of 325 nm (λ_{max}). The samples were chromatographed on a reverse phase Thermosil® C_{18} column (5 μm, 25 cm × 4.6 mm i.d., Thermo electron company, Bellefonte, North America) preceded by a guard column (40 × 4 mm) of the same material. A mixture of 0.01 M KH_2PO_4 buffer : Acetonitrile (80 : 20, v/v), mobile phase, was filtered through a 0.45 μm Millipore filter and degassed under vacuum before use. It was pumped at a flow rate of 1 mL/min for the run time of 10 min under these experimental conditions with an injection volume of 20 μL. The column was thermostated at an ambient temperature. Metronidazole was used as an internal standard (IS).

Animals

Wistar-Albino rats of both sexes, weighing 200–300 gm were obtained from Zydus Research Center, Gujarat, India. The animals were housed in a departmental animal house under natural light conditions, fed a standard pellet diet and water ad libidium. Their care and handling were in accordance with the provisions of Social Justice and Empowerment Committee, as recognized and adopted by the Ministry of Government of India, New Delhi.

Preparation of Stock and Standard Solutions

A stock solution (100 μg/mL) was prepared by dissolving 10 mg of 6-MP in 10 mL of methanol and then volume was made up to 100 mL with mobile

phase in a 100 mL volumetric flask. A second stock solution was also prepared by diluting the first stock solution in mobile phase to yield the concentration of 2 μg/mL. Working-standard solutions were prepared in mobile phase from the second stock solution. The concentration range of 6-MP for the standard curve samples was between 50–1000 ng/mL. On the other hand, 4 mg of IS was dissolved in 100 mL of mobile phase to obtain a stock solution of 40 μg/mL. Standard curve samples were prepared by adding 0.1 mL of IS to working standard solutions of 6-MP, and volume was made up to 2 mL a using mobile phase. The calibration curve for HPLC analysis was constructed by plotting the ratio of peak area of drug to that of IS against the drug concentration in ng/mL.

Blood Collection and Tissue Preparation

Albino rats of either sex weighing 200–250 gm were anaesthetized with chloroform, and blood was collected from retro orbital plexus using a sterile heparinized glass capillary tube in a glass tube containing Sodium Citrate (3.3% w/v) solution as anticoagulant. The rats were sacrificed by cervical dislocation and dissected to collect tissues such as liver, lung, heart, kidney, and spleen. The organs were blotted using a filter paper, weighed separately, and homogenized (Ultra-Turrax, T25, Germany) to a concentration of 10% w/v in methanol and stored at −20°C till further use.

Calibration of 6-MP in Plasma

Blood, 0.5 mL, was collected in a glass tube containing Sodium Citrate (3.3% w/v) solution for each sample and centrifuged at 5000 RPM for 15 min at 4°C to separate plasma. The plasma samples, 0.2 mL, were deproteinized with 2 mL of methanol and acetonitrile mixture (1 : 1, v/v), vortexed for 5 min, centrifuged at 6000 RPM for 15 min, and supernatants were collected. The supernatants were spiked with an appropriate volume of suitably diluted stock solutions of 6-MP and internal standard, giving final concentrations of 50–1000 ng/mL. Each sample containing 20 μL was injected through a Rheodyne injector and the effluent was monitored at 325 nm. The above procedure was repeated five times and the plot of ratio of area of 6-MP and IS vs. concentration of 6-MP in ng/mL was plotted in the range of 50–1000 ng/mL in plasma.

Calibration of 6-MP in Various Tissues

To 0.5 mL of tissue homogenate, the corresponding quantity of stock solution of 6-MP, 0.25 mL of IS, 0.5 mL of methanol was added as a protein

precipitant, and volume was made up to 5 mL using a mobile phase in a range of 100–1000 ng/mL. All solutions were vortexed on a cyclomixer for 30 s and centrifuged at 8000 RPM for 15 min. The supernatant was collected and 20 μL of each solution were injected for HPLC analysis. The above procedure was repeated five times and the plot of ratio of area of 6-MP and IS vs. concentration of 6-MP in ng/mL was plotted in the range of 100–1000 ng/mL in various tissues (lung, liver, kidney, heart, and spleen).

Application of the Method

Pharmacokinetic Study of 6-MP in Rats

Plasma levels of 6-MP were examined in Wistar-Albino Rats of both sexes weighing 200–300 gm. The animals were treated with intravenous injection of 6-MP solutions (5 mg of 6-MP dissolved in 5 mL of mixture of polyethylene glycol-400, ethanol, dimethyl sulphoxide, and 0.9% w/v saline, 0.75 : 0.75 : 0.15 : 3.35, v/v/v/v) via the tail vein at doses of 5 mg/kg. At various times after drug administration (30 min, 1, 2, 4, 6, and 8 h), three animals were anesthetized by diethyl ether and blood samples (0.5 mL) were collected from retro orbital plexus, and plasma was immediately separated by centrifugation and stored at −20°C until analysis; 6-MP was extracted and assayed as described above. One compartmental analysis was used to calculate the pharmacokinetic parameters from mean plasma concentration-time data. The AUC between the first and last sampling times, Cmax and Tmax were calculated by using NCSS® Software. The elimination half-life ($T_{1/2}$) was estimated by linear regression analysis of the terminal phase of the plasma concentration-time profile. Total body clearance (Clt), volume of distribution at steady-state (Vss), and Kel were also calculated using QUICKCALC® Software. AUC_{∞}, $AUMC_{\infty}$, and MRT were calculated from standard formulas.[18]

Biodistribution Study of 6-MP in Rats

Injecting the same dose of drug performed the biodistribution studies. The animals were anaesthetized, sacrificed (3/time point) after different time intervals (1, 4, 8, and 24 h), and different tissues (lung, liver, kidney, heart, and spleen) were collected. These time points were selected with an aim to identifying peak tissue concentrations, as well as to measure the rapid elimination of 6-MP. The organs were washed twice with 0.9% w/v NaCl, wiped, and the weight of each tissue was recorded. Approximately 500 mg of organ slices were excised, minced, homogenized with 5 mL of methanol, and centrifuged at 6000 RPM for 20 min. The drug content was determined in supernatant by the HPLC method. AUC, Cmax and Tmax of 6-MP in each drug were also recorded using NCSS® Software.

RESULTS AND DISCUSSION

In this study, the conditions were influenced by physicochemical properties of 6-MP like solubility, UV absorption, and interference from the endogenous substances. The most important target was, therefore, to exclude interference between endogenous substances in biological samples and 6-MP.

HPLC Method Development

For HPLC methods, precision and accuracy can often be enhanced by the use of an appropriate internal standard, which also serves to correct for fluctuations in the detector response. The IS should be a different compound from the analyte but one that is well resolved in the separation. The chemical structure of metronidazole is not similar to 6-MP. However, it was chosen as the internal standard because it not only gave the best peak shape but also gave better resolution and shorter retention time compared to other internal standards in rat plasma and tissue homogenates. Various mobile phase systems were prepared and used to get an appropriate chromatographic separation, but the proposed mobile phase consisting of 0.01 M KH_2PO_4 : acetonitrile (80 : 20, v/v) gave better resolution and sensitivity of 6-MP and IS. The optimum wavelength for detection was 325 nm at which much better detector response for 6-MP was obtained. The flow rate was obtained as 1 mL/min with these conditions and this mobile phase composition, best results were obtained in terms of shape of peak sensitivity and retention time for plasma samples and tissue homogenates.

Estimation of 6-MP in Plasma

For extraction of 6-MP in plasma, a mixture of methanol : acetonitrile (1 : 1, v/v) was used because of its low volatility, toxicity, and easy handling compared to other solvents. After addition of this mixture, the contents were vortexed to solubilize the drug adsorbed on precipitated proteins and then centrifuged. Hence, this method was expected to aid in maximum drug extraction from plasma, and avoids the problems of incomplete drug extraction encountered in procedures involving non-polar organic solvents. Typical chromatograms of (a) drug-free rat plasma, (b) rat plasma spiked with 800 ng/mL of 6-MP are shown in Fig. 1. A calibration plot was constructed in plasma after addition of 6-MP and correlation co-efficient of 0.999 was obtained, indicating a strong linear relationship between the ratio of area of 6-MP and IS and its concentration. Linearity was obeyed in the range of 50–1000 ng/mL, and limit of detection was found to be 38 ng/mL. The experimental data and regression analysis are shown in Table 1. The regression equation obtained is:

$$y = 0.00288\ (\pm 0.0004768) \times C + 0.029\ (\pm 0.03299)\ \text{for plasma} \qquad (1)$$

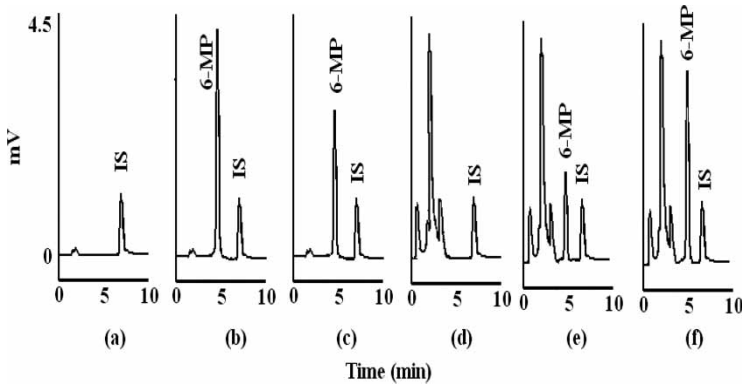

Figure 1. Typical chromatograms of (a) drug-free rat plasma, (b) rat plasma spiked with 800 ng/mL of 6-MP, (c) rat plasma collected at 1 h after intravenous injection of 5 mg/kg of 6-MP, (d) drug free rat spleen homogenate, (e) rat spleen homogenate spiked with 500 ng/mL of 6-MP, and (f) rat spleen homogenate collected at 4 h after intravenous injection of 5 mg/kg of 6-MP.

The variance of intercept, S_a^2 calculated was 7.3E−04. The variance of slope, S_b^2 was obtained as 5.8E−08. The test of intercept was used to study the interference of blanks with the measurements. The t-value obtained was 0.875 for the intercept of the curve. The calculated "t" value required, at a 5% confidence level, at 4 degrees of freedom was 2.78. This shows the intercept is not significantly different from zero, indicating no interference of solvent in the estimation.

Accuracy and precision of the method was investigated by subjecting a known amount of 6-MP for recovery studies, after addition of the drug in the plasma. Table 1 represents the results obtained. Accuracy of the method was evaluated by using the "t" test at each level of estimation. The t-values obtained for 100, 400, and 800 ng/mL were 0.450, 0.814, and 0.169, respectively. The t value required for confidence at a 5% level at 5 degrees of freedom is 2.57, and the obtained t-values were well below this value. Thus, no significant difference was observed between drug added and recovered. Precision of the method was ascertained by the % RSD and % coefficient of variance (% CV) (Table 1). The low % RSD and % CV indicate the precision of the method.

Estimation of 6-MP in Various Tissues

The tissues selected are lung, liver, kidney, heart, and spleen. Typical chromatographs of (d) drug free rat spleen homogenate, (e) rat spleen homogenate spiked with 500 ng/mL of 6-MP, and (f) rat spleen homogenate collected at 4 h after intravenous injections of 5 mg/kg of 6-MP are shown in Fig. 1.

Table 1. Validation results of the bioanalytical HPLC method for 6-MP

Tissues/concentrations (ng/mL)	r^2	Regression equation	Mean ± SD	RSD (%)	Accuracy (%)	t-value	CV (%)
Plasma							
100	0.999	$y = 0.00288x + 0.029$	100.42 ± 1.40	1.39	100.42	0.45	1.58
400			402.46 ± 1.97	0.48	100.33	0.81	3.12
800			800.3 ± 1.99	0.24	100.02	0.17	3.17
Lung							
100	0.999	$y = 0.0011x + 0.0014$	99.66 ± 0.96	0.97	99.67	0.44	0.93
400			398.11 ± 2.85	0.72	99.53	0.17	8.16
800			799.62 ± 2.96	0.37	99.95	0.77	8.79
Liver							
100	0.999	$y = 0.0011x + 0.0002$	100.28 ± 1.75	1.75	100.29	0.53	3.07
400			400.65 ± 1.55	0.39	100.16	0.36	2.42
800			801.13 ± 2.11	0.26	100.14	0.18	4.49
Kidney							
100	0.999	$y = 0.001x - 0.0022$	100.66 ± 1.46	1.45	100.67	0.31	2.14
400			400.33 ± 0.92	0.23	100.08	0.42	0.85
800			800.65 ± 1.056	0.13	100.08	0.19	1.12
Heart							
100	0.993	$y = 0.001x + 0.0267$	101.25 ± 1.06	1.06	101.26	0.03	1.14
400			400.07 ± 2.18	0.55	100.02	0.94	4.78
800			800.74 ± 1.86	0.23	100.09	0.37	3.49
Spleen							
100	0.990	$y = 0.0014x - 0.03$	98.98 ± 2.38	2.41	98.98	0.34	5.68
400			399.54 ± 1.86	0.47	99.89	0.58	3.47
800			801.22 ± 2.92	0.37	100.15	0.35	8.56

The Correlation co-efficients (r^2) obtained from calibration curves plotted for lung, liver, kidney, heart, and spleen were 0.999, 0.999, 0.999, 0.993, and 0.990, respectively, indicating a good relationship between ratio of area of drug to IS and its concentration. The linearity was found in the range of 100–1000 ng/mL for all tissues. The data of calibration curves are shown in Table 1. The variance of intercept, S_a^2, calculated were 4E−04, 3.69E−04, 4.2E−04, 1.57E−0.4, and 3.64E−04, as well as the variance of slope, S_b^2, were 2.7E−08, 3.6E−08, 1.69E−08, 3.4E−08, and 4.7E−08 for lung, liver, kidney, heart, and spleen, respectively.

Accuracy and precision of the method was determined by performing recovery studies of 6-MP in tissues in triplicate. The results are shown in Table 1. Low SD values indicate low variability between each data point of analysis. Accuracy and precision of the method was determined by applying the "t" test at each level of analysis. The "t" values obtained at each data points for each tissue are shown in Table 1. The tabulated "t" value required for confidence at a 5% level at 2 degrees of freedom is 4.3. The obtained "t" values are well below the required "t" value, indicating no significant difference between the amount of drug added and recovered. Precision of the method was ascertained using % RSD values and % CV values. The low values of % CV and % RSD indicate the precision of the method.

Pharmacokinetic Studies in Rats

Figure 2 shows the plasma concentration-time profile of 6-MP given at 5-mg/kg doses intravenously. After i.v. administration, 6-MP was rapidly

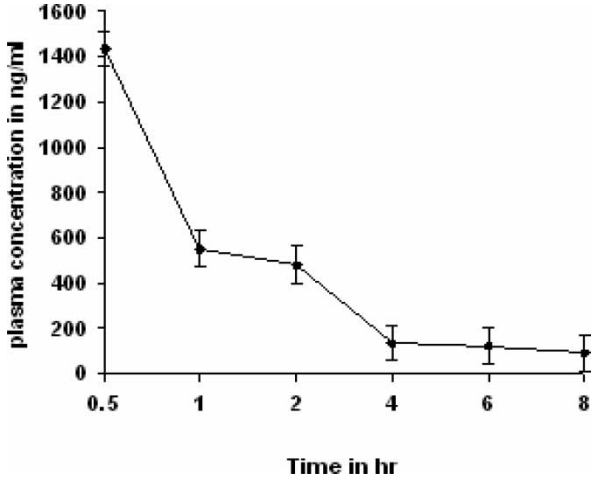

Figure 2. The plasma concentration-time profile of 6-MP given at a dose of 5-mg/kg intravenously.

cleared from the blood with an elimination half-life of 0.688 h at a dose of 5 mg/kg. The Cmax value (1437.27 ± 214.84 ng/mL) obtained following treatment with 6-MP was reached after T_{max} of 30 min of dosing, with the AUC_{0-8} h of 2098.76 ± 351.2 hr · ng/mL. The administration of 6-MP orally showed C_{max} of 158.1 ± 27.6 ng/mL and AUC_{0-4} h of 147.4 ± 24.3 h · ng/mL.[2] This limited bioavailability is the result of first-pass hepatic metabolism and variable absorption and presystemic metabolism of the drug by the intestine into the inactive metabolite 6-thiouric acid,[19] so the importance of optimizing 6-MP therapy and achieving high systemic drug exposure has encouraged the use of i.v.6-MP in patients with ALL. However, the antileukemic and cytotoxic effects of 6-MP have been related to the generation of intracellular nucleotides derived from 6-MP rather than to plasma 6-MP concentration.[20–22] After the first-pass of metabolism through the liver, the remaining 6-MP is taken up by blood cells from plasma and converted by two major pathways into its active metabolites, therefore, a higher plasma concentration of drug is advantageous to get more antileukemic effects. Table 2 represents plasma pharmacokinetic parameters of 6-MP administered intravenously in rats at doses of 5-mg/kg.

Biodistribution Studies in Rats

Selected tissues (liver, heart, kidney, lung, and spleen) were collected and analyzed for the accumulation of 6-MP at different time intervals after injecting doses of 5-mg/kg in rats intravenously. Extensive tissue distribution was observed (Table 3) after i.v. administration of 5 mg/kg/day of 6-MP to rats. The tissue distribution time kinetics of 6-MP clearly indicates that 6-MP is quickly absorbed and has good bioavailability, at least in the tissues examined in the present study. At this dose level, based on

Table 2. Plasma pharmacokinetic parameters (mean ± SD) of 6-MP administered i.v. in rats at dose of 5-mg/kg (n = 3)

Pharmacokinetic parameters	6-MP
$AUC_{0\text{-}8}$ h (h · ng/mL)	2098.767 ± 351.2
C_{max} (ng/mL)	1437.278 ± 214.84
T_{max} (h)	0.5
Kel	1.0135
AUC_{∞}	3031.716
$AUMC_{\infty}$	7519.059
MRT	2.48
Elimination $t_{1/2}$ h	0.6844
Vss mL/kg	0.27
Total body clearance (mL/min/kg)	2.32

Table 3. Biodistribution parameters (mean ± SD) of 6-MP administered i.v. in rats at a dose of 5-mg/kg (n = 3)

	Biodistribution parameters		
	AUC_{0-24} (h · μg/mL)	C_{max} (μg/mL)	T_{max} (h)
Liver	338.44 ± 21.3	27.55 ± 5.7	4
Heart	277.45 ± 18.45	17.72 ± 3.9	8
Kidney	257.95 ± 17.62	14.13 ± 3.62	8
Lung	107.04 ± 10.6	10.90 ± 2.84	4
Spleen	160.19 ± 13.81	12.28 ± 3.05	4

AUC_{tissue}, the rank order of tissue distribution was liver > heart > kidney > spleen > lung. On the basis of observed Tmax values, heart and kidney showed the slowest rates of 6-MP accumulation with a value of 8 h with C_{max} values of 17.72 ± 3.9 and 14.13 ± 3.62 μg/mL, respectively. The rate of distribution and clearance of the drug from tissues seemed to be tissue dependent. After their peak levels, 6-MP was eliminated from these tissues in an exponential manner (Fig. 3), with $t_{1/2}$ values ranging from 4–8 h.

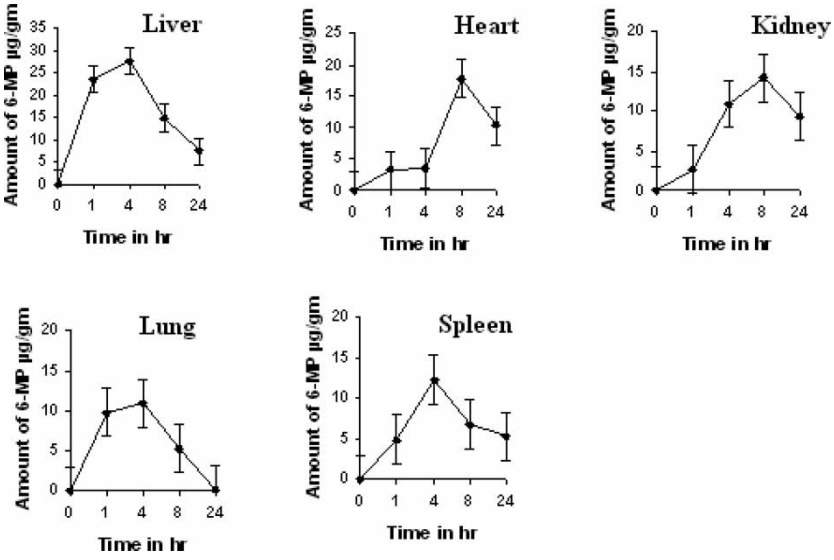

Figure 3. Distribution of 6-MP in various tissues at different time intervals after i.v. administration at a dose of 5-mg/kg (n = 3).

CONCLUSION

The present study explores the possibility of simple and sensitive estimation of 6-MP in plasma and tissues. The analytical parameters calculated from calibration plots were found to be satisfactory. This method proposes a simple, accurate, and precise determination of 6-MP in rat plasma and tissue homogenates. A column shorter than 25 cm can speed up the analysis further. The proposed method is suitable for the quality control laboratories, where economy and time are essential. Therefore, the HPLC UV method presented here can be considered of real interest for the rapid and reliable clinical, pharmacokinetic, and biodistribution studies of 6-MP in the rats.

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