Research Article

DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR ESTIMATION OF 6-MERCAPTOPURINE IN RABBIT PLASMA

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ABSTRACT

A HPLC method was developed and validated for the rapid determination of 6-Mercaptopurine in plasma of rabbit. The mobile phase consisted of 0.01 M phosphate buffer pH 6.8 and acetonitrile (80:20, v/v) and was pumped at a flow rate of 1mL/min and was monitored by UV-Visible detector at 322 nm. Metronidazole was used as internal standard for the study. The developed method was linear over the concentration range studied with a correlation coefficient >0.997 in rabbit plasma. Stability data, precision and accuracy degree of the proposed method was very high. The method described is selective and sensitive enough to analyse 6-Mercaptopurine in a single run and can be successfully applied for investigating the different pharmacokinetics parameters in rabbit as animal model.

Keywords: 6-Mercaptopurine, HPLC method, Analytical method development and validation.

INTRODUCTION

Analytical method development and validation are two key elements of any pharmaceutical development program. It is often considered routinely and much attention is paid with regard to its potential to contribute to the overall process development. Analytical method development and validation are continuous and inter-dependent task which is associated with the research and development, quality control and quality assurance process. Analytical procedures play a critical role in determining equivalence, risk assessment and management. It ensures establishment of product-specific acceptance criteria and reproducibility of the results. Method validation should demonstrate suitability of the analytical procedure for its intended purpose. Design of an experiment is a powerful tool for the method characterization and validation. An effective analytical method development and validation ensure significant improvements in precision and reduces bias and errors. It further helps to avoid costly and time consuming exercises.

Analytical method can be spectral, chromatographic or electrochemical. It is a process of selecting an accurate assay procedure to determine the composition of various formulations. It is a process of proving that an analytical method is acceptable for its intended use in laboratory to measure the concentration of different samples. The analytical methods should be used within good manufacturing practise (GMP) and good laboratory practise (GLP) environments and must be developed using the protocols and acceptance criteria set out in the ICH guidelines¹⁻⁴.

MATERIALS AND METHODS

6-MP was purchased from Tocris Biosciences, UK and Metronidazole (MNZ) was obtained as gift sample from RPG Life Sciences Pvt. Ltd, Mumbai, India. HPLC grade methanol and acetonitrile were procured from Rankem, Mumbai, India,

Potassium dihydrogen phosphate (KH₂PO₄), Sodium hydroxide (NaOH) and Sodium citrate were procured from Merck India Pvt. Ltd. through local supplier. HPLC grade water was prepared by Arium, 611UF, Sartorius, Germany water purifier. All other chemicals and reagents used were of analytical grade and procured from local suppliers. Wister albino rabbits of weight between 1.5 to 2.0 kg were obtained from M/s Ghosh Enterprise, Kolkara, India. The animals were housed in the Departmental animal house under natural light conditions, fed a standard pellet diet and water *ad libitum*. They were cared and handled in accordance with CPCSEA guidelines.

HPLC System and Chromatographic conditions

Normal phase chromatography is a technique that can be used for the separation of non-ionic and non-polar substances whereas reverse-phase chromatography technique can be used for the separation of non-ionic as well as ionic non-polar to semi-polar substances. HPLC system (Shimadzu, Japan), consisting of Isocratic pump (Model M 515, Shimadzu, Japan), Rheodyne manual injector (Model 7725i, Shimadzu, Japan) and UV-VIS detector (Model M 2489, Shimadzu, Japan) were used for analysis. The samples were processed by using Micro pipette (Model V233, Mettler Toledo, Ohio, USA), vortexed using vortex mixer (REMI, Mumbai) for 5 min, centrifuged using laboratory centrifuge (REMI, Mumbai) at 6000 rpm, sonicated using sonicator (REMI, Mumbai) for 5 min and evaporated with nitrogen gas evaporator. HPLC analysis was performed by using a Hypersil ODS C18 (average particle size 5µm), column (250 mm x 4.6 mm), protected with a guard column (40 mm x 4 mm), packed with the same material. The mobile phase was consisting of 80 parts of 0.01 M phosphate buffer pH 6.8 and 20 parts of acetonitrile (80:20, v/v). The 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 1mL/min with an injection volume of 20 µL. The column was thermostated at an ambient temperature 30±2 °C throughout the study. The effluent was

monitored with the UV-Visible detector at 322 nm. MNZ was used as an IS.

Preparation of 0.01 M Phosphate buffer solution (PBS)

Weighed quantity of phosphate (1.36 gm) was dissolved in de-ionized water and the volume was made up to 1000 mL to obtain 10 mM solution. The pH of the solution was adjusted to 6.8 with 0.1 N NaOH solution. The solution was filtered through a 0.45 µm Nylon filter and degassed under vacuum and sonicated for 5 min before use.

Preparation of working solutions for sample analysis

A mixture of 0.01 M potassium dihydrogen phosphate buffer (pH 6.8) : acetonitrile (80:20 v/v), was mixed in a reagent bottle, filtered and sonicated; and the same was used for the preparation of calibration curve of the drug in blank plasma and for sample analysis.

Preparation of Internal standard (IS) stock solutions

An accurately weighed quantity of MNZ (25 mg) was dissolved in 10 mL of methanol to obtain 2500 µg/mL of stock solution. The stock solution (200 µL) was diluted with methanol upto 10 mL in a volumetric flask to obtain a solution of 50 µg/mL of MNZ.

Preparation of stock solution of 6-MP

An accurately weighed 40 mg of 6-MP was transferred to a 100 mL volumetric flask containing 30 mL of 0.1N NaOH. The drug was allowed to dissolve and the volume was made up to 100 mL with 0.1 N NaOH. The above solution was diluted with mobile phase to obtain a working standard solution of 40 µg/mL. The stock solution was stored in refrigerator (2 to 8° C).

Preparation of working standard (WS) solutions of 6-MP

The working solutions were prepared by serial dilution of the measured volume of stock concentration of 6-MP with the working solvent i.e. mixture of 0.01 M phosphate buffer (pH 6.8) : Acetonitrile (80:20 v/v).

Preparation of standard calibration curve of 6-MP in rabbit Plasma

Blood, 2.0 mL, was collected from four rabbits (0.5 mL from each) in a glass tube containing sodium citrate (3.0% w/v) solution and centrifuged at 5000 rpm for 15 min at 4 °C to separate plasma. The calibration curve standards were prepared in fresh by spiking 20 µL of working solutions of 6-MP into the control blank plasma (180 µL) to give a series of concentrations from 2000 ng/mL to 12000 ng/mL. The study protocol was approved by the Institutional Animal Ethical Committee of Dibrugarh University, Assam vide approval No. **IAEC/DU/100 dated 06/08/2015**.

Preparation of quality control standards:

Standard quality control samples of the drug 6-MP in Rabbit plasma were prepared for selection of the solvent for extraction and recovery of drug from plasma sample and for validation of the analytical method in biological fluid. The quality control standards were prepared at low, medium and high concentration levels which were 2000, 6000 and 12000 ng/mL respectively and stored at -20 °C till analysis.

Estimation of 6-MP in rabbit plasma

To each of the 200 µL calibration curve standard and quality control standard samples, 100 µL of MNZ solution, IS (50 µg/mL) was added to yield the concentration 5 µg/mL of IS in each sample. For extraction of 6-MP and IS in plasma, a 1.5 mL mixture of methanol : acetonitrile (1:1, v/v) was used because of its low volatility, toxicity, and easy handling compared to other solvents. The contents were vortexed for 5 min to solubilize the

drug adsorbed on precipitated proteins and then centrifuged at 6000 rpm for 15 min and supernatant was collected. The supernatant was decanted into a clean centrifuge tube and evaporated to dryness under a gentle nitrogen stream at 40 °C. The residue was reconstituted in 100 µL of mobile phase, and the sample was filtered through 0.45 µm membrane filter using syringe filter holder. An aliquot of 20 µL of the sample was injected in to the injector of the HPLC system for analysis. Hence, this method was expected to extract maximum drug from plasma, and thus avoids the problems of incomplete drug extraction encountered in procedures involving non-polar organic solvents.

Validation of method

Validity of an analytical technique is a procedure aimed at obtaining experimentally justified evidence of the ability of this technique to give results characterized by the required accuracy and precision. The analytical method developed was validated following ICH Guidelines^{5,6} for the determination of specificity, linearity, precision, recovery and stability. The following parameters were studied under the validation programme.

Specificity

Specificity criterion indicates that the result of the developed method is not affected by the presence of small amount of interferences. Thus it clearly indicates that the compound of interest elutes without interfering with other compounds and components of plasma⁵⁻⁷. The specificity of the developed method was estimated by comparing chromatograms obtained from working standards of 6-MP and IS with those obtained from blank plasma. The blank plasma samples collected from each rabbit were processed in presence of IS. Another set of five samples collected were processed with 6-MP and IS to evaluate the presence of interference around the peak of 6-MP. The solution each containing 6000 ng/mL was injected into the column under optimized chromatographic conditions to obtain the chromatographic peaks of 6-MP in plasma in presence of IS so as to differentiate them from the interfering peaks of plasma components.

Selection of solvent for recovery of drug

Different extraction solvents (acetonitrile, methanol and mixture of acetonitrile and methanol) were tried in the experiment to recover 6-MP from plasma samples. The quality control samples in each solvent were prepared in triplicate, at three levels of concentration 2000 ng/mL, 6000 ng/mL and 12000 ng/mL of 6-MP and 100 µL of IS stock solution (50 µg/mL), which was then assayed by HPLC method. The extraction efficiency of 6-MP was determined by comparing the peak areas obtained from extracted quality control samples with that of the peak areas obtained from working solutions containing same concentration of 6-MP at three levels.

Linearity range

Quantitative analytical results are highly influenced by the quality of the calibration curve. Linearity was determined to confirm the ability of the method to elicit test results that were directly proportional to the concentration of analyte. Six different concentrations of 6-MP were processed and calibration curve was constructed in the specified concentration 2000 ng/mL to 12000 ng/mL. The calibration curve was plotted between the ratio of peak areas of 6-MP to IS against concentration of 6-MP from 2000 ng/mL to 12000 ng/mL by replicate analysis (n=6) in the concentration range of 2000 to 12000 ng/mL in plasma. The linear relationship was obtained using the least square method with Microsoft Excel program.

Precision and accuracy

Precision explains the degree of agreement among individual test results when procedure/method is applied to a homogeneous sample usually expressed as SD/RSD. It is a measure of degree of repeatability and reproducibility of the experimental result under normal conditions. Both repeatability (intra-day precision) and reproducibility (inter-day precision) were determined for three quality control samples. Five injections of specified quality control samples, at three levels, were injected into the HPLC column for analysis purpose within the same day for determining repeatability, and over a period of five days for determining reproducibility. A reference standard curve was created from the mean peak heights for each predicted concentration. Mean and relative standard deviation were calculated and used to predict the precision of the method. To study the accuracy of the proposed method, recovery studies were carried out by standard addition method at three different levels. A known amount of drug was added to pre-analysed drug sample and percentage recovery were calculated. Accuracy was calculated as the percentage of 6-MP

found in the intra-day and inter-day samples to that of the actual value.

Stability study^{1, 5, 7}

The stability of 6-MP at storage temperature (-20 °C) was determined by spiking of blank plasma containing three concentrations of 6-MP (2000 ng/mL, 6000 ng/mL and 12000 ng/mL). Fresh plasma samples were analyzed at time zero to determine the absorption peak of plasma constituents if any. One set of five samples was placed in polypropylene tubes and subjected to freeze-thaw cycles each at -20 °C for 24 h. Second set of five samples was kept at room temperature for 24 h. The third set of five samples was kept at room temperature for one month duration. Each and every samples were analyzed at standard chromatographic conditions to determine their peak areas. The samples were considered to be stable, when final assay values of the samples were found to be similar to that of the initial assay value of the drug.

Table-1 Preparation of working standard solution of 6-MP

Sample code	Stock Conc. (ng/mL)	Stock solution volume (mL)	Working solvent volume (mL)	Total volume (mL)	Working solution concentration (ng /mL)
WS1	100,000	1	9	10	10000
WS2	200,000	2	8	10	20000
WS3	300,000	3	7	10	30000
WS4	400,000	4	6	10	40000
WS5	500,000	5	5	10	50000
WS6	600,000	6	4	10	60000

Table-2 Preparation of calibration curve standards of 6-MP in Rabbit plasma

Sample code	Working solution (μL)	Plasma volume (μL)	Concentration of Std. 6-MP (ng/mL)	Peak area of 6-MP
STD1	20	180	2000	3196871
STD2	20	180	4000	4088406
STD3	20	180	6000	4904860
STD4	20	180	8000	5686182
STD5	20	180	10000	6208425
STD6	20	180	12000	6649908

Table-3 Peak area of chromatogram of 6-MP and IS at different concentration of 6-MP

Concentration of 6-MP (ng/mL)	Peak area of the chromatogram		Ratio of peak area
	6-MP	IS (MNZ)	
2000	3196871	17953490	5.61
4000	4088406	16904786	4.13
6000	4904860	15661962	3.19
8000	5686182	15578196	2.74
10000	6208425	14425637	2.32
12000	6649908	12718755	1.91

Table-4 Results of precision and accuracy studies

Amount of 6-MP (μg/mL)	Intra-day precision and accuracy			Inter-day precision and accuracy		
	Mean (μg/mL), (n=5)	RSD (%)	Accuracy (%)	Mean (μg/mL), (n=5)	RSD (%)	Accuracy (%)
30	29.92	0.501	99.73	29.80	0.268	99.33
40	39.80	0.275	99.50	39.75	0.150	99.37
50	49.75	0.305	99.50	49.82	0.160	99.64

Table-5 Results of stability of 6-MP in rabbit plasma

Stability, (n=5)	Concentration (ng/mL) of 6-MP in quality control samples			
Freeze-thaw stability				
Initial Mean, (ng/mL)	2000	6000	12000	
Final Mean, (ng/mL)	1988.20	5986.15	11978.10	
Deviation (%)	1.001	1.002	1.002	
RSD (±)	5.10	3.15	4.20	
Accuracy (%)	99.41	99.77	99.81	
24 h stability				
Final Mean, (ng/mL)	1984.40	5982.20	11982.40	
Deviation (%)	1.001	1.002	1.002	
RSD (±)	4.10	4.25	5.20	
Accuracy (%)	99.22	99.70	99.85	
1 month stability				
Final Mean, (ng/mL)	1978.40	5978.50	11960.50	
Deviation (%)	1.001	1.002	1.002	
RSD (±)	6.40	5.50	6.60	
Accuracy (%)	98.92	99.64	99.67

Figure-1 Standard calibration curve plot of 6-MP in rabbit plasma

Figure-2 Standard calibration curve of 6-MP vs peak area of 6-MP with IS in rabbit plasma

Figure-3 Chromatogram of the mixture of 6-MP and IS (MNZ) in mobile phase

Figure-4 Chromatogram of blank plasma of Rabbit

Figure-5 Chromatogram of low level quality control sample of 6-MP (2000 ng/mL) in rabbit plasma.

Figure-6 Chromatogram of medium level quality control sample of 6-MP (6000 ng/mL) in rabbit plasma

Figure-7 Chromatogram of high level quality control sample of 6-MP (12000 ng/mL) in rabbit plasma.

RESULTS AND DISCUSSION

Selection and development of Chromatographic method

Various mobile phase systems were prepared and used to get an appropriate chromatographic separation, but the proposed mobile phase consisting of 0.01M phosphate buffer (pH 6.8) : acetonitrile (80 : 20, v/v), gave better resolution and sensitivity of 6-MP and IS. The optimum wavelength for detection was 322 nm at which much better detector response for 6-MP was obtained. The flow rate was maintained as 1mL/min at these conditions and mobile phase composition. The best results were obtained in terms of peak sensitivity and retention time for plasma samples. The IS should be a different compound from the analyte but one that is well resolved in the separation. The chemical structure of MNZ is not similar to 6-MP. However, it was chosen as the internal standard because it not only gave the best peak intensity but also better resolution and shorter retention time compared to other IS in rabbit plasma.

Preparation of working standard solutions of 6-MP

The working solutions were prepared by serial dilution (WS1 to WS6) of the measured volume of stock concentration of 6-MP with the working solvent i.e. mixture of 0.01 M phosphate buffer (pH 6.8) : acetonitrile (80 : 20 v/v) as shown in Table-1.

Preparation of standard calibration curve of 6-MP in rabbit plasma

The calibration curve standards were prepared in fresh by spiking 20 µL of working solutions of 6-MP into the control blank plasma (180 µL) to give the concentration range from 2000 to 12000 ng/mL as shown in the Table-2. Each sample containing 20 µL was injected through a Rheodyne injector and the effluent was monitored at 322 nm. The above procedure was repeated for remaining concentration of 6-MP and the plot of area of 6-MP vs. concentration of 6-MP in µg/mL was plotted in the range of 2000 to 12000 ng/mL in plasma to construct the calibration curve. The area of various concentrations of 6-MP is presented in Table-2 and Figure-1. From the figure it is evident that linearity was obeyed between the ratio of peak area of 6-MP to its concentration range because, the correlation co-efficient was found to be 0.997.

Preparation of quality control standards

The quality control standards of 6-MP were prepared at low, medium and high concentration levels, i.e. at 2000, 6000 and 12000 ng/mL respectively and used for selection of extraction solvent for 6-MP and IS from plasma samples.

Estimation of 6-MP in rabbit plasma

The results of the peak area of chromatogram of 6-MP and IS at different concentrations of 6-MP are presented in Table-3. The calibration curve plot was constructed in plasma after addition of IS in each sample of calibration curve standard of 6-MP from which the correlation co-efficient was found to be 0.997, indicating a strong linear relationship between the ratio of peak area of 6-MP and IS and its concentration (Figure-2).

Validation of method

Typical chromatogram of mixture of 6-MP and IS (Figure-3) revealed that they are well separated under HPLC conditions adopted. A chromatogram of blank plasma is shown in Figure-4. There were no interfering peaks in the blank plasma. In the chromatogram (Figure-3) the retention time was found to be 4.075 min for 6-MP and 3.57 for IS and for blank plasma (Figure-4) it was only 1.8 min. The chromatogram of low level (2000 ng/mL), medium level (6000 ng/mL) and high level (12000 ng/mL) quality control samples of 6-MP showed good resolution peaks for 6-MP and IS which were well differentiated from the peaks of plasma components. The chromatogram of the low level, medium level and high level quality control sample of 6-MP are shown in Figure-5, 6 and 7 respectively.

Precision, accuracy, linearity specificity, robustness and stability Parameters

The precision, accuracy, linearity specificity, robustness and system suitability parameters were studied systematically to validate the proposed RP-HPLC method for the estimation of 6-MP. Solutions containing 30, 40, 50 µg/ml of 6-MP were subjected to intra-day and inter-day precision. The results were furnished in Table-4. The accuracy of the method was also determined by performing accuracy studies. The result of accuracy studies are furnished in Table-4.

The relative standard deviation (RSD) of intra-day assay of the drug ranged from 0.275 % to 0.501 % and for the inter-day assay of drug ranged from 0.150 % to 0.258 %. Accuracy data ranged from 99.50 % to 99.73 % for intra-day assay of drug and for inter-day assay of drug ranged from 99.33 % to 99.64 %, indicating that there was no interference from endogenous plasma components. The results of the study revealed that the precision and accuracy degree of the proposed method is very high.

For the linearity study the ratio of peak area of 6-MP to IS at various concentration of 6-MP in plasma was shown in Table-3. A calibration curve was plotted between peak area of 6-MP to IS versus concentration of 6-MP in plasma was presented in Figure-2. The chromatographic responses (ratio of peak area of 6-MP to IS versus concentration of 6-MP) were found to be linear over

an analytical range of 2000 to 12000 ng/mL with regression coefficient (r^2) value, 0.988 which showed reproducibility.

The result of stability validation is furnished in **Table-5**. The result of stability study indicated a deviation of 1.001, 1.002 and 1.002 % with an accuracy degree of 99.41, 99.77 and 99.81 % from the initial concentration of 2000, 4000 and 12000 ng/mL respectively for the samples storing at freeze- thaw cycle.

The study for stability of 6-MP in rabbit plasma was repeated after 24 h, the result indicated that a deviation of 1.001, 1.002 and 1.002 % with an accuracy degree of 99.22, 99.70 and 99.85 % from the initial concentration of 2000, 4000 and 12000 ng/mL, respectively.

The study for stability of 6-MP in rabbit plasma was repeated again after 1 month and the result indicated that a deviation of 1.001, 1.002 and 1.002 % with an accuracy degree of 98.92, 99.64 and 99.67 % from the initial concentration of 2000, 4000 and 12000 ng/mL, respectively.

CONCLUSION

These results revealed that 6-MP remained stable in the rabbit plasma up to 1 month period with up to 1.001 % loss of drug in case of 2000ng/mL initial concentration, whereas, the loss of drug was up to 1.002 % in case of 6000ng/mL and 12000 ng/mL initial concentration of 6-MP in rabbit plasma.

Hence, the adopted method is well validated in terms of stability parameter of drug for the estimation of 6-Mercaptopurine in rabbit plasma.

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