PART II

METHOD DEVELOPMENT, PREPARATION AND COMPARATIVE EVALUATION OF STEALTH LIPOSOMES AND MICROBUBBLES FOR THERAPEUTIC PURPOSE

Chapter 4

Analytical Methods

A new reverse phase HPLC method for determination of flutamide in liposomes, rat blood plasma and tablet dosage form, **Ars Pharmaceutica, 2005,** Vol 46, 74-82.

And

A new reverse-phase HPLC method for estimation of 6-mercaptopurine in plasma and tissues of rat, **Journal of Liquid Chromatography and Related Techniques** (In Press)

INTRODUCTION

The analytical methods used in studies of drug-containing liposomes and microbubbles should, in addition to possessing the desired characteristics of accuracy, precision, reproducibility, ruggedness, robustness etc., should also possess the ability to be used in conjunction with techniques common to liposomal adjuvants. The methods used should be stability indicating which would, when used, draw attention to any potential incompatibility between the various components of the liposomes and microbubbles.

4.1 ESTIMATION OF PHOSPHATIDYLCHOLINE IN LIPOSOMES

The Stewart assay (Stewart, 1980) was used for estimating phosphatidylcholine in liposomes. This method utilizes the ability of phospholipids to form a complex with ammonium ferrothiocyanate in organic solution.

4.1.1 Solutions

1. Ammonium ferrothiocyanate solution (0.1M) was prepared by dissolving 27.03% of ferric chloride hexahydrate and 30.4g of ammonium thiocyanate in double distilled water and making up the volume of the resulting solution to 1 litre.
2. Stock solution of phosphatidylcholine in chloroform (0.5 mg/ml) was prepared by dissolving 50 mg of phosphatidylcholine in 10 ml of chloroform. 1 ml of this solution was diluted 10 times to yield a solution of the required concentration.

4.1.2 Procedure for calibration curve

Suitable aliquots (0.4-2.8 ml) of the stock solution of phosphatidylcholine were transferred to 10 ml centrifuge tubes. Appropriate quantities of chloroform were then added such that the total volume of the contents of the tubes was 5 ml. To each tube, 2 ml of ammonium ferrothiocyanate solution (0.1M) was then added. The contents of each tube were mixed by vigorous vortexing on a cyclomixer for 15 sec. The tubes were then spun for 5 min at 1800 rpm in a tabletop centrifuge. The lower, organic

colored layer was then removed using a syringe and long needle (18 gauge) and transferred to a test tube. The absorbance of these solutions was measured at 485 nm on a Shimadzu 160I UV-Visible spectrophotometer with glass cells of 10 mm path length using a blank prepared in the same manner omitting the phospholipids.
The above procedure was repeated five times. Mean absorbance values along with the standard deviations, the regression equation obtained and the optical characteristics of the colored solutions are shown in Table 4.1.

4.1.3 Estimation of phosphatidylcholine from liposomes/supernatant

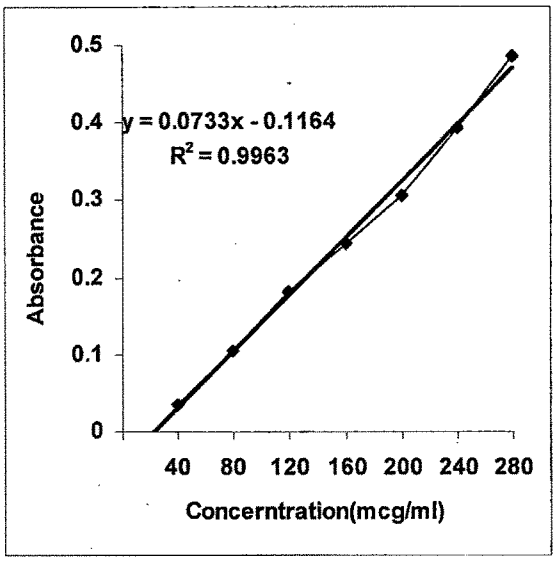
The Bligh-Dyer two-phase extraction method (New 1990) was modified for estimating phosphatidylcholine from liposomes. Briefly, 0.1 ml of liposomal dispersion or 0.5 ml of the supernatant was taken in a centrifuge tube and to this 1 ml of saturated sodium chloride solution was added 2 ml of chloroform was then added to the contents followed by vigorous vortexing on a cyclomixer for 30 sec and centrifugation at 1800 rpm for 5 min in a tabletop centrifuge. The lower chloroform layer was separated using a syringe and needle (18 gauge) and passed over a bed of anhydrous sodium sulphate into a 10 ml volumetric flask. The process was repeated with a further 2 ml and 1 ml of chloroform. The chloroform layers were then pooled and the column made up to 10 ml with chloroform. To 0.5 ml of this chloroform extract in a centrifuge tube, 2.5 ml of chloroform and 2 ml of 0.1 M ammonium ferrothiocyanate solution was added. The contents were then subjected to the same procedure as detailed above for the standards. Duplicate estimations were performed and the mean absorbance was used to determine the amount of phosphatidylcholine in the liposomes or supernatant using the regression equation.

4.1.4 Stability and selectivity

Stability of the colored solutions, prepared above for the calibration curve of phosphatidylcholine, was ascertained by observing the changes in absorbance of the solutions over a period of 4h. The selectivity of the method for phosphatidylcholine was investigated by carrying out the procedure detailed above in the presence of potential interferences such as cholesterol, flutamide and 6-Mercaptopurine, etc at the levels at which these materials were included in the liposomes.

Table 4.1: Calibration curve for estimation of phosphatidylcholine at 485 nm

Concentration (µg/ml)	Absorbance (Mean ± SD)
40	0.05±0.002
80	0.105±0.0021 6
120	0.18±0.0036
160	0.243±0.0021 6
200	0.307±0.0019 2
240	0.394±0.0015 8
280	0.484±0.0025



Absorption maxima (nm)	485
Linearity range (µg/ml)	40-280
Intercept	-0.1164
Slope	0.0733
Regression Co-efficient (R^2)	0.9963
Apparent molar Absorptivity (l/mol/cm)	4947.15

4.2 ESTIMATION OF CHOLESTEROL IN LIPOSOMES

The Zlatkis, Zak and Boyle's method was used for estimating cholesterol in liposomes. This method utilizes the ability of cholesterol in acetic acid to form a complex with ferric chloride and sulphuric acid (Zatkis et al 1953)

4.2.1 Solutions

1. Ferric chloride solutions: A 0.05% w/v solution of ferric chloride hexahydrate in glacial acetic acid was prepared by dissolving 50 mg of ferric chloride hexahydrate in 100 ml glacial acetic acid.
2. Stock solution of cholesterol: A 0.5 mg/ml solution of cholesterol in glacial acetic acid was prepared by dissolving 25 mg of cholesterol in 50 ml of glacial acetic acid.

4.2.3 Procedure for calibration curve

Suitable aliquots of the stock solution of cholesterol (0.1 to 1 ml) were transferred accurately into 10 ml volumetric flasks. To each flask, 4 ml of ferric chloride solution and 4 ml of concentrated sulphuric acid was added. The contents were mixed, made up to the volume with glacial acetic acid and allowed to stand for 30 min. The absorbance of the resulting colored solutions of the complex, formed between cholesterol, ferric chloride and sulphuric acid was measured at 550 nm using Shimadzu 1601 UV-Visible spectrophotometer against a blank prepared in the same manner as the standard solutions except cholesterol. The above procedure was repeated five times. The experimental data along with the results of the statistical evaluation of the data and the optical characteristics for the above solutions of the complex formed are shown in Table 4.2.

4.2.4 Estimation of cholesterol from liposomes/supernatant:

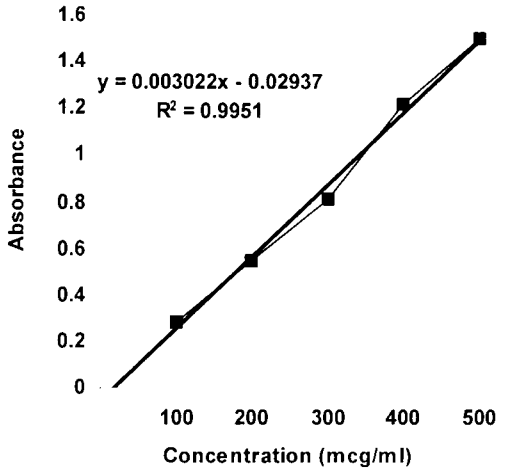
1 ml of the chloroform extract, obtained as described before in the estimation of phosphatidylcholine from liposomes or supernatant (Section 4.1.3) was taken in a 10 ml volumetric flask and evaporated to dryness by heating at 90°C in a thermostatically controlled, electrically heated water bath. The dried contents were then subjected to the same procedure as discussed previously (Section 4.1.3). The amount of cholesterol in the liposomes or supernatant was then obtained using the regression equation of the calibration curve.

4.2.5 Stability and selectivity

Stability of the solutions of the complex, prepared for obtained the calibration curve of cholesterol, was ascertained by observing the changes in their absorbance over a period of 24 h. Cholesterol was estimated in the presence of phosphatidylcholine, flutamide, 6-MP etc at the same concentrations at which these materials were included in the liposomes to ascertain the selectivity of the method.

Table 4.2: Calibration curve for estimation of cholesterol at 550 nm

Concentration (µg/ml)	Absorbance (Mean ± SD)
100	0.279±0.003
200	0.5502±0.0028
300	0.8096±0.0023
400	1.2166±0.0027
500	1.501±0.0047



Absorption maxima (nm)	550
Linearity range (µg/ml)	100-500
Intercept	-0.02937
Slope	0.003022
Regression Co-efficient (R^2)	0.9951
Apparent molar Absorptivity (l/mol/cm)	6007.8

4.3 ESTIMATION OF POLYETHYLENE GLYCOL DERIVATIVES IN LIPOSOMES

The estimation of polyethylene glycol derivatives methoxy polyethylene glycol 2000 activated with cyanuric chloride-phosphatidylethanolamine conjugate ($mPEG_{2000}$-CC-PE) was done using the method reported by Shimada et al (2000). This method is based on the spectrophotometric determination of complexes of polyethylene glycols with sodium ions after their extraction as picrates, into 1,2-dichloromethane, and provided a sensitive tool for determination of PEG-lipids.

4.3.1 Solutions

1. To a solution containing 0.1M sodium hydroxide, 280g of sodium nitrate (3.3 mol) and 4.58 g of picric acid (0.02M) were added and the final volume of the solution

was adjusted to 1000 ml. After sonication, the solution was filtered off and stored in a refrigerator at 5°C.

2. Stock solution of polyethylene glycol derivatives ($mPEG_{2000}$-CC-PE) (0.5 mg/ml) was prepared by dissolving 50 mg of polyethylene glycol ($mPEG_{2000}$-CC-PE) in 100 ml of distilled water.

4.3.2 Procedure for calibration curve

Suitable aliquots (0.2 – 2 ml) of the stock solution of polyethylene glycol derivatives were pipetted into 10 ml volumetric flasks and the volume was made up to 10 ml with distilled water to give final concentrations of 10, 20, 40, 60, 80 and 100 μg/ml. To the 10 ml of above sample solution, 10 ml of sodium nitrate picrate solution and 5 ml of dichloromethane was added. The solution was shaken well and the absorbance was measured at 378 nm on a Shimadzu 1601 UV-Visible spectrophotometer. The above procedure was repeated five times. Tables 3.19 tabulated the raw data and the optical characteristics for the solutions of polyethylene glycol derivatives in dichloromethane.

4.3.3 Estimation of polyethylene glycol derivatives from liposomes/supernatant

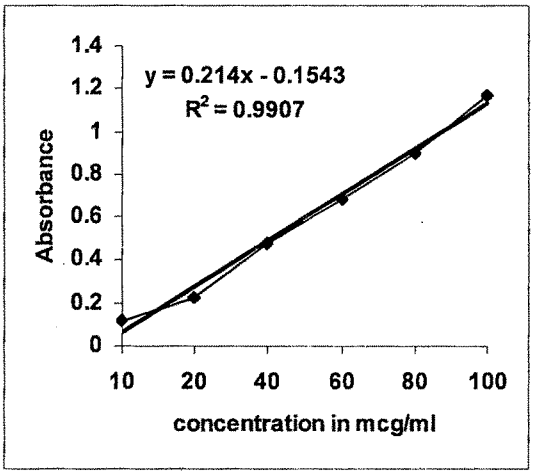
0.1 ml of liposomal suspension or 0.5 ml of supernatant was diluted to 5 ml with distilled water and was mixed with 10 ml of sodium nitrate picrate solution. To the mixture, 5 ml of 1, 2-dichloromethane was added. After vigorous shaking, the solution was centrifuged at 1500 rpm for 10 min and the organic layer was collected and spectrophotometrically measured at 378 nm.

4.3.4 Stability and selectivity

Stability of the solutions of polyethylene glycol derivatives, used for preparing the calibration curve, was ascertained by observing the changes in their absorbance at the analytical wavelength, over a period of 24 hr at room temperature. The above method for estimating polyethylene glycol derivatives was carried out in the presence of flutamide, 6-Mercaptopurine, phosphatidylcholine, cholesterol, α-tocopherol and other components of the liposomes to ascertain the selectivity of the method.

Table 4.3: Calibration curve for estimation of polyethylene glycol derivatives at 378 nm

Concentration (µg/ml)	Absorbance (Mean ± SD)
10	0.115 ± 0.0026
20	0.226 ± 0.0035
40	0.476 ± 0.0061
60	0.685 ± 0.0043
80	0.898 ± 0.018
100	1.168 ± 0.013



Absorption maxima (nm)	378
Linearity range (µg/ml)	10-100
Intercept	-0.1543
Slope	0.214
Regression Co-efficient (R^2)	0.9907
Apparent molar Absorptivity (l/mol/cm)	32677

4.4 ESTIMATION OF FLUTAMIDE

4.4.1 Spectrophotometric estimation of flutamide

4.4.1.1 Chemicals and Reagents

Flutamide was kindly supplied as a gift sample by Coral Drugs Pvt. Ltd., New Delhi; India. Phosphotidylcholine, cholesterol and α-tocopherol were purchased from Sigma Chemical Co., St. Louis, M.O.; HPLC grade Methanol and Water were purchased from Spectrochem Pvt. Ltd. Both solvents were then combined and filtered (0.45 μm HA membrane filter, Millipore, Ireland) to prepare the mobile phase

4.4.1.2 Calibration curve

Stock solution of flutamide (250 μg/ml) was prepared by dissolving 25 mg of flutamide in 100 ml of methanol. Suitable aliquots (0.2 to 2 ml) of the stock solution of flutamide were pipetted in to 5 ml of volumetric flasks and the volume was made up to 10 ml with methanol to give final concentrations of 10, 20, 40, 60, 80 and 100 μg/ml. The solutions were shaken well and their absorbance measured at 295 nm wavelength using methanol as blank on a Shimadzu 1601 UV-Visible spectrophotometer. The above procedure was repeated five times.

Figure 4.1: Absorptivity scan of flutamide in methanol over the UV wavelength range from 200 to 440 nm

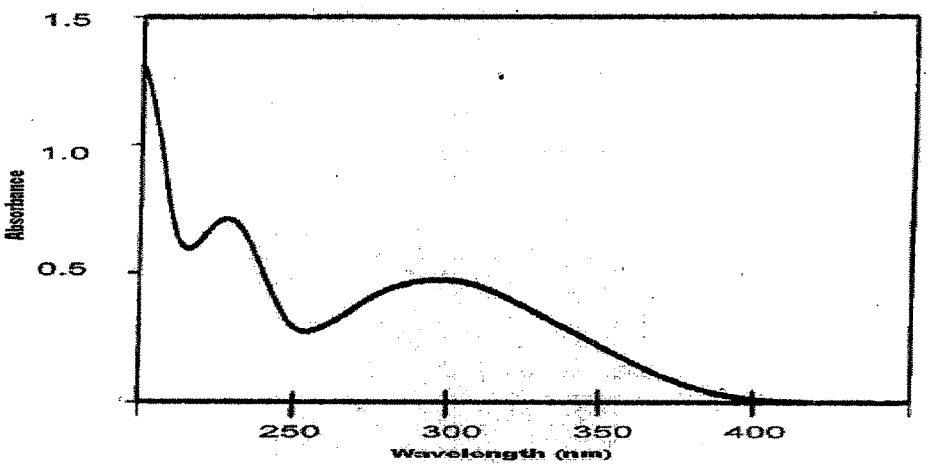
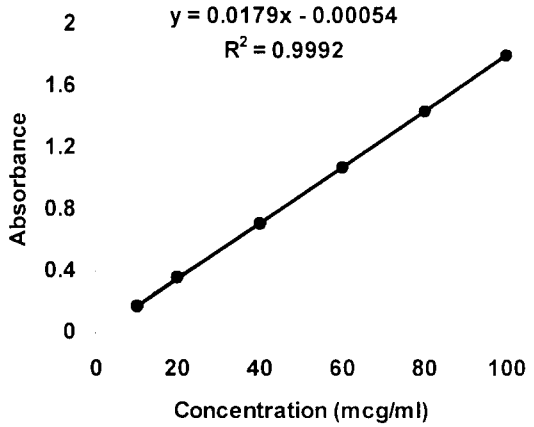


Table 4.4: Calibration curve of flutamide at 295 nm in methanol

Concentration (μg/ml)	Absorbance (Mean ± SD)
10	0.179 ± 0.0015
20	0.357 ± 0.0021
40	0.714 ± 0.0043
60	1.074 ± 0.0064
80	1.432 ± 0.0051
100	1.802 ± 0.0088



Absorption maxima (nm)	295
Linearity range (μg/ml)	10-100
Intercept	0.00054
Slope	0.0179
Regression Co-efficient (R^2)	0.9992
Apparent molar Absorptivity (l/mol/cm)	4947.15

Table 4.4 shows calibration curve and the optical characteristics for the solution of flutamide in methanol. Absorptivity scan over the UV wavelength range from 200 to 400 nm is shown in Figure 4.1. The above method for estimating flutamide was carried out in the presence of phosphotidylcholine, cholesterol, α-tocopherol and other substances of the liposomes to ascertain the selectivity of the method.

4.4.1.3 Estimation of flutamide from liposomes and supernatant

0.2 ml of liposomes or supernatant was taken in to 10 ml of volumetric flask and the volume was made up to 10 ml with methanol to break liposomes. The absorbance was measured at 295 nm wavelength against a blank liposomes diluted with methanol in the similar manner. Each estimation was done in triplicate and mean absorbance was determined. The amount of flutamide in the liposomes or supernatant was estimated using regression equation.

4.4.2 High-performance liquid chromatographic estimation of flutamide

4.4.2.1 Introduction

The HPLC methods are useful in determination of drug in pharmaceutical dosage form and biological samples. A number of different analytical studies have been done to determine drug content in drug products. FLT and its hydroxylated metabolites have been determined by high performance liquid chromatography. The chromatographic determination of FLT in presence of its hydrolyzed product (4-nitro-3-trifluoro methyl aniline) also has been described. Related to the determination of FLT in pharmaceutical forms a gas chromatographic method has been reported (265). The stability study of FLT in solid state and in aqueous solution was also studied by HPLC method (266). These procedures were shown long retention time, do not give results rapidly, more use of expensive solvents, use of organic modifiers viz. tri ethylamine and glacial acetic acid in the mobile phase composition and maintenance of pH (267), which can leads to the precipitation of lipids present in liposomes which finally limit column life. Only one HPLC UV detection method has been described in the literature for determination of FLT from pharmaceutical dosage form (268). This reported procedure has high determination limit and linearity range data. The objective of the present study is to develop sensitive, precise, selective, specific, reproducible, fully validated, easy to perform and low cost routine reverse phase HPLC UV detection method for determination of FLT in raw material, rat plasma and liposomes without any time consuming sample preparation steps and any interferences form endogenous substances from plasma samples.

4.4.2.2 Experimental

4.4.2.2.1 Apparatus

The instrument was equipped with Rheodyne Isocratic pump (Model-LC-10 Avp, Shimadzu Corp., Kyoto, Japan) and diode-array detector (Model-SPD Avp, Shimadzu Corp., Kyoto, Japan) set at a wavelength of 295 nm (λ_{max}).

4.4.2.2.2 Chromatographic conditions

The samples were chromatographed on reverse phase C_{18} Thermo Hypersil® (250 × 4.6 mm i.d., 5 µm particle size) column (Thermo electron company, Bellefonte, North America) preceded by a guard column (40 × 4 mm) of the same material. The mobile phase, methanol and water (80:20, v/v), was filtered through 0.45-µm

Millipore filter and degassed under vacuum before use. It was pumped at a flow rate of 1 ml /min. The column was thermostated at an ambient temperature. The run time was 8 min under these experimental conditions with an injection volume of 20 µl. 6-Mercaptopurine was used as an internal standard (IS).

4.4.2.2.3 Preparation of standards

A stock solution (2 mg/ml) was prepared by dissolving 20 mg of FLT in 10 ml of mobile phase in 10 ml volumetric flask. A second stock solution was also prepared by diluting the first stock solution in mobile phase four times to yield the concentration of 500µg/ml. Working-standard solutions were prepared in mobile phase from the second stock solution. The concentration range of FLT for the standard curve samples was between 20 and 1000 ng/ml. On the other hand, 5 mg of IS was dissolved in 10 ml of mobile phase to obtain a stock solution of 500 µg/ml. Standard curve samples were prepared by adding 0.3 ml of the IS to working standard solutions of FLT and volume was made up to 5 ml using the 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. Unknown concentration computed from the linear regression equation of the peak area ratio against concentration of FLT.

4.4.2.2.4 Blood collection

Albino rats of either sex weighing 200-250 gm were anaesthetized with chloroform and blood was collected from retro orbital plexus using sterile heparinized glass capillary tube in glass tube containing Na-Citrate (3.3% w/v) solution as anticoagulant.

4.4.2.2.5 Validation procedures

Accuracy of the assay was defined as the percentage of the agreement between the measured value and the true value as follows (269):

$$\text{Accuracy} = \frac{\text{True value - Measured value} \times 100}{\text{True value}} \qquad \text{(Equation 1)}$$

Accuracy values in intra-day and inter day variation studied at low, medium and high FLT concentrations. In this work, precision of the method was tested on both the inter-day and intra-day reproducibility in the assay. Intra-day variability of the assay method was determined by repeated analysis of three quality control samples at low, medium and high concentrations on the same day. Similarly, Inter-day variability was determined by repeated analysis of three quality control samples at low, medium and high concentrations on three different days. The quality control samples were prepared as a single batch on the same day at each concentration, and then divided into aliquots that were stored at 2-8°C until analysis (269). For the robustness study, different analytical columns (Thermosil® ODS and Spherisorb® C_{18}) and guard columns were used. Effect of temperature of the column, the change in percentage of methanol in mobile phase and variation in the flow-rate were also studied (269). Recovery studies of FLT in spiked rat plasma were performed by adding the known amount of pure drug to pre-analyzed samples rat plasma. The percentage recovery was calculated by comparing the concentration obtained from spiked samples with actual added concentration. After five repeated experiments the recovery was calculated.

4.4.2.2.6 Determination of FLT in rat plasma samples

Drug free plasma samples, obtained from rats, were stored frozen until assay. Thawed plasma samples of 1 ml were spiked with 2 mg/ml of FLT (dissolved in mobile phase) and 3 ml methanol (for denaturation and precipitation of plasma proteins). The tubes were tightly capped, vortexed for 10 min and centrifuged for 10 min at 5000 g. The upper layer was transferred in to clean tube. The different concentrations of FLT solutions were prepared in the range of 50 ng/ml to 1000 ng/ml. The concentration of IS was maintained at constant level of 30 µg/ml. These plasma samples of 20 µl were injected and standard curve was constructed for FLT in plasma using the ratio of observed peak area for FLT and IS. Unknown concentrations were computed from linear regression equation of the peak area ratio against the concentration of FLT (Table 4.5).

4.4.2.3 Results and Discussion

4.4.2.3.1 Characteristics of the chromatographic peak

Figure 4.2: Chromatograph of blank plasma

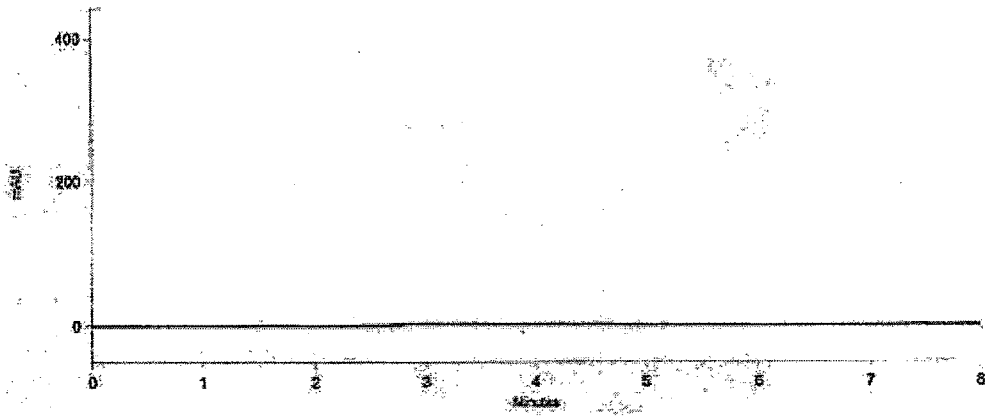


Figure 4.3: Chromatograph of plasma spiked with Internal Standard

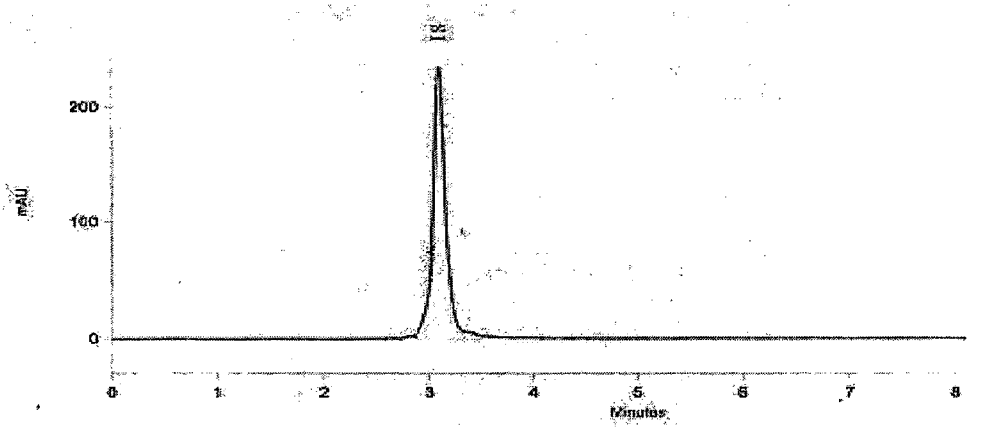
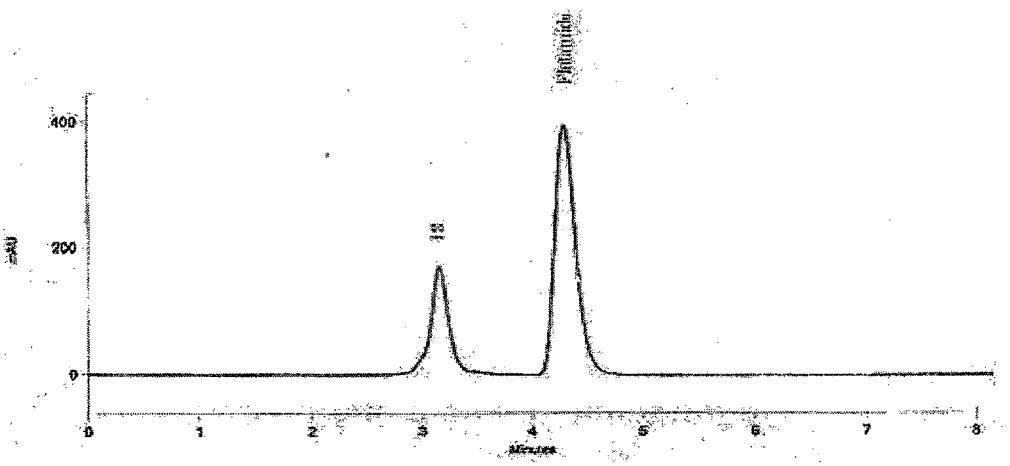


Figure 4.4: Chromatograph of plasma spiked with Internal Standard and FLT



According to the conditions described, the retention times for FLT were about 4.02 min and 5.1 min for IS and FLT in rat plasma, respectively. Typical chromatograms of blank rat plasma, plasma with IS, plasma with IS and FLT are shown in Fig. 4.2 to 4.4.

4.4.2.3.2 Linearity

Table 4.5: Analytical data of the calibration graphs obtained by the HPLC UV method for FLT

Medium	Linearity range (µg/ml)	Calibration Equation[a]	r	Sr
Mobile phase	0.02-1	y = 0.00348 (±0.0000561)*C – 5.7E-04 (±0.000105)	0.9998	0.0101
Plasma	0.05-1	y = 0.003324 (±0.0000939)*C – 3.28E-04 (±0.000111)	0.9959	0.0129

[a] Ratio of the peak area of the FLT to that of the internal standard, y, vs. concentration of FLT, C_{FLT}, in µg/ml; eight standards. (n=5)

[b]Correlation Co-efficient.

[c]Standard error of the estimate.

4.4.2.3.3 Accuracy, Precision and Robustness

Intra-day and inter-day precision and accuracy of method were assessed by FLT in mobile phase and analyzing spiking plasma samples at low (50 ng/ml), medium (100ng/ml) and high (500ng/ml) concentration. Five replicate of each concentration were analyzed and results are given in Table 4.6 and were found in acceptable range. In robustness study, if the temperature of column was increased to 40°C, the retention time were not modified. A high percentage of methanol (5% v/v more) in the mobile phase decreased 6-MP and FLT retention times (3.036 min and 4.1 min from 4.02 and 5.1 min, respectively) eluting both peaks more closely. Variation in flow-rate resulted in the change in retention times, prolonging the chromatogram time. Nevertheless, no single parameter, extended to specific limits, resulted in a dramatic adverse effect on the system suitability.

Table 4.6: accuracy and precision data of FLT HPLC method

Nominal concentration (ng/ml)	Mean ± SD		RSD (%)[a]		Er (%)[b]	
Intra-day (n=5)						
	MP*	**Plasma**	**MP**	**Plasma**	**MP**	**Plasma**
50	50.08 (±0.35)	49.78 (±0.39)	0.69	0.78	0.16	-0.44
100	100.42 (± 1.40)	99.63(±0.18)	1.39	0.180	0.42	-0.37
500	499.67 (± 1.20)	497.45 (± 1.14)	0.24	0.22	-0.06	-0.51
Inter-day (n=5)						
50	49.19 (± 0.49)	49.0 (± 0.53)	0.99	1.08	-1.62	-2
100	101.1(±1.5)	98.67(±1.42)	1.13	1.43	1.1	-1.33
500	499.24 (± 1.3)	500.42 (± 1.4)	0.26	0.27	-0.15	0.08

*MP: Mobile phase

4.4.2.3.4 Application to rat plasma samples

The related validation parameters and obtained recovery results of plasma samples are given in Tables 4.6 and 4.7, respectively. The estimation of drug in plasma requires time-consuming sample preparation, use of expensive organic solvents and other chemicals. Here, the plasma proteins are precipitated in mobile phase (methanol: water 80:20, v/v) and the mixture is vortexed and centrifuged at 5000 g for 10 min. The supernatant was taken carefully and diluted with mobile phase and directly inject to column and analyzed. Figure 4.2 to 4.4 shows the chromatogram of blank plasma, plasma with IS and plasma with FLT and IS, respectively. It shows that no interference from the endogenous substances present in plasma. Good recovery of FLT was achieved from rat plasma by this method as per Table 4.7.

Table 4.7: Determination and recovery study of FLT in rat plasma

FLT added (n=5)	0.5 µg/ml
FLT recovered (n=5)	0.496 µg/ml
Recovery %	99.2
RSD % of recovery	1.209677419

4.4.2.4 Summary and Conclusion

The method involved less time-consuming sample preparation steps. The internal standard (IS) for the assay procedure was 6-Mercaptopurine. The samples were injected on to reverse-phase (Thermosil® C_{18}) column. The mobile phase, Methanol: Water (80:20, v/v) was run at a flow rate of 1 ml/min for 8 min. The FLT was detected by UV detector at 295 nm wavelength. The retention times for IS and FLT were 3.03 and 4.02 min, respectively. The method was linear over the concentration range 20-1000 ng/ml and 50-1000 µg/ml in mobile phase and rat blood plasma, respectively. The method was validated for accuracy, precision, robustness and recovery. The limit of detection was 0.099µg/ml and 0.106µg/ml in mobile phase and rat blood plasma, respectively. The method was shown to be highly reproducible and it seems to be adequate for routine therapeutic drug monitoring. It could be used without any interference from lipids, tablet excipients and endogenous substances from the plasma samples.

4.5 ESTIMATION OF 6-MERCAPTOPURINE

4.5.1 Spectrophotometric estimation of 6-Mercaptopurine

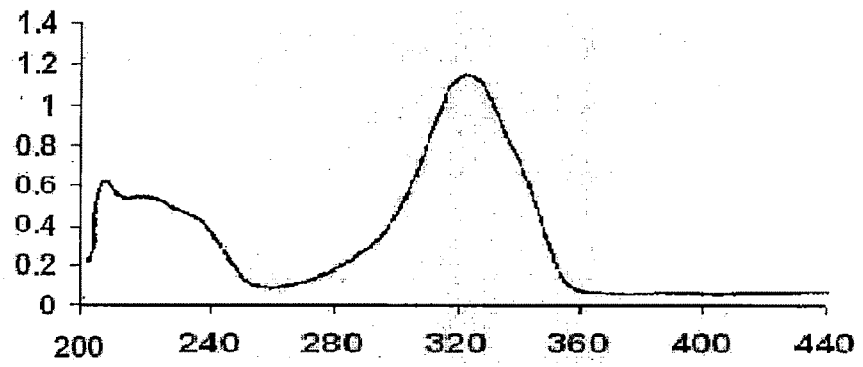
4.5.1.1 Chemicals and Reagents

6-Mercaptopurine (6-MP) was kindly supplied as a gift sample by Dabur Therapeutics Ltd., India. Hydrogenated soya phosphotidylcholine, cholesterol and α-tocopherol were purchased from Sigma Chemical Co., St. Louis, M.O. HPLC grade methanol, acetonitrile, potassium di-hydrogen phosphate and water were purchased from S.D. Fine chemicals Pvt. Ltd., India.

4.5.1.2 Calibration curve

Stock solution of 6-MP (100 μg/ml) was prepared by dissolving 10 mg of 6-MP in 100 ml of methanol. Suitable aliquots (0.2 to 1.5 ml) of the stock solution of 6-MP were pipetted in to 10 ml of volumetric flasks and the volume was made up to 10 ml with methanol to give final concentrations of 2, 4, 6, 8, 10 and 15 μg/ml. The solutions were shaken well and their absorbance measured at 325 nm wavelength using methanol as blank on a Shimadzu 1601 UV-Visible spectrophotometer. The above procedure was repeated five times. Table 4.8 shows calibration curve and contains the optical characteristics for the solution of –6-MP in methanol. Absorptivity scan over the UV wavelength range from 200 to 400 nm is shown in Figure 4.5.

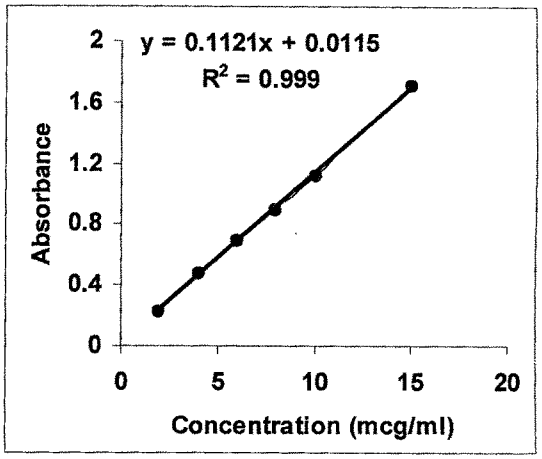
Figure 4.5: Absorptivity scan of 6-Mercaptopurine in methanol over the UV wavelength range from 200 to 440 nm



The above method for estimating 6-MP was carried out in the presence of phosphotidylcholine, cholesterol, α-tocopherol and other substances of the liposomes to ascertain the selectivity of the method.

Table 4.8: Calibration curve of 6-MP at 325 nm in methanol

Concentration (µg/ml)	Absorbance (Mean ± SD)
2	0.229±0.00241
4	0.480±0.00518
6	0.692±0.00336
8	0.889±0.01233
10	1.115±0.00270
15	1.707±0.00241



Absorption maxima (nm)	325
Linearity range (µg/ml)	2-15
Intercept	0.0115
Slope	0.1121
Regression Co-efficient (R^2)	0.999
Apparent molar Absorptivity (l/mol/cm)	19571.85

4.5.1.3 Estimation of 6-MP from liposomes and supernatant

0.2 ml of liposomes or supernatant was taken in to 10 ml of volumetric flask. 0.5 ml of chloroform was added to break liposomes and the volume was made up to 10 ml with methanol. The absorbance was measured at 325 nm wavelength against a blank liposomes diluted with chloroform and methanol in the similar manner. Each estimation was done in triplicate and mean absorbance was determined. The amount of 6-MP in the liposomes or supernatant was determined using regression equation.

4.5.2 High-performance liquid chromatographic estimation of 6-Mercaptopurine

4.5.2.1 Introduction

The HPLC methods are useful in the determination of drug in pharmaceutical dosage form and biological samples. 6-MP and its metabolites have been

determined by high performance liquid chromatography (270-272). There are various HPLC methods reported for the estimation of 6-MP in human erythrocytes, plasma and serum (273-274). These procedures showed drawback of long retention time, do not give results rapidly, 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 (275), which finally limits column life. Metabolism of 6-mercaptopurine in the erythrocytes, liver, and kidney of rats during multiple-dose regimens is also reported (276) in which 6-MP was administered i.p. The objective of the present study was to develop sensitive, precise, selective, specific, reproducible, fully validated, easy to perform and low cost routine reverse phase HPLC UV detection method for the estimation of 6-MP in rat plasma and in different tissues of rats without any time consuming sample preparation steps and any interferences form 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 on 6-MP in rats.

4.5.2.2 Experimental

4.5.2.2.1 Preparation of Stock and Standard Solutions

A stock solution of 6-MP (100 µg/ml) was prepared by dissolving 10 mg in 10 ml of methanol and then volume was made up to 100 ml with mobile phase in 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 (Metronidazole) was dissolved in 100 ml of mobile phase to obtain a stock solution of 400 µg/ml. Standard curve samples were prepared by adding 0.1 ml of IS to working standard solutions of 6-MP and

volume was make up to 2 ml 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.

4.5.2.2.2 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 sterile heparinized glass capillary tube in glass tube containing Na-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 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.

4.5.2.2.3 Calibration of 6-MP in plasma

0.5 ml of blood was collected in glass tube containing Na-Citrate (3.3% w/v) solution for each sample and centrifuged at 5000 RPM for 15 min at 4°C to separate plasma. 0.2 ml of the plasma samples were deproteinised with 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 variously 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 Rheodyne injector and 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.

4.5.2.2.4 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 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).

4.5.2.2.5 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 reverse phase Thermosil® C_{18} column (5µm, 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 0.01M KH_2PO_4 buffer: Acetonitrile (80:20, v/v), mobile phase, was filtered through 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).

4.5.2.3 Results and Discussion

4.5.2.3.1 HPLC method development

For HPLC method, precision and accuracy can often be enhanced by the use of an appropriate internal standard, which also serves to correct fluctuations in the detector response. The IS should be 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 0.01M 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.

4.5.2.3.2 Estimation of 6-MP in plasma

For extraction of 6-MP in plasma, 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 and (b) rat plasma spiked with 800 ng/ml of 6-MP are shown in Figure 4.6.

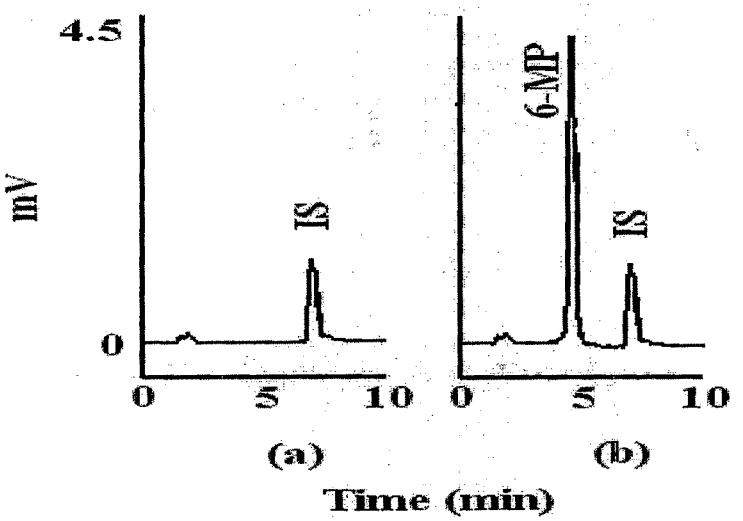


Figure 4.6: Typical chromatograms of (a) drug-free rat plasma and (b) rat plasma spiked with 800 ng/ml of 6-MP

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 4.9. The regression equation obtained is:

$y = 0.00288\ (\pm 0.0004768)*C + 0.029\ (\pm 0.03299)$ for plasma (Equation 1)

The variance of intercept, S^2_a calculated was 7.3E-04. The variance of slope, S^2_b was obtained as 5.8E-08. The test of intercept was used to study the interference of blank with the measurements. The t value obtained was 0.875 for intercept of the curve. The calculated 't' value required at 5% significance level at 4 degree of freedom (2.78). This shows the intercept is not significantly different from zero, indicating no interference of solvent in the estimation.

Table 4.9: Validation results of the bioanalytical HPLC method for 6-MP

Tissues/ Concentrations in ng/ml	r^2	Regression Equation	Mean ±SD	(%) RSD	Accuracy (%)	t-value	% CV
Plasma							
100			100.42 ±1.40	1.39	100.42	0.45	1.58
400	0.999	y=0.00288x +0.029	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			99.66±0.96	0.97	99.67	0.44	0.93
400	0.999	y = 0.0011x + 0.0014	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			100.28±1.75	1.75	100.29	0.53	3.07
400	0.999	y = 0.0011x + 0.0002	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			100.66±1.46	1.45	100.67	0.31	2.14
400	0.999	y = 0.001x - 0.0022	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			101.25±1.06	1.06	101.26	0.03	1.14
400	0.993	y = 0.001x + 0.0267	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			98.98±2.38	2.41	98.98	0.34	5.68
400	0.990	y = 0.0014x - 0.03	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

Accuracy and precision of the method was investigated by subjecting known amount of 6-MP for recovery studies, after addition of drug in the plasma. Table 4.9 represents the results obtained. Accuracy of the method was evaluated by using '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 significance at 5 % level at 5 degree 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 4.9). The low % RSD and % CV indicate the precision of the method.

4.5.2.3.3 Estimation of 6-MP in various tissues

The tissues selected are lung, liver, kidney, heart and spleen. Typical chromatograph of (d) drug free rat spleen homogenate and (e) rat spleen homogenate spiked with 500 ng/ml of 6-MP are shown in Figure 4.7. Correlation co-efficient (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 (Table 4.9).

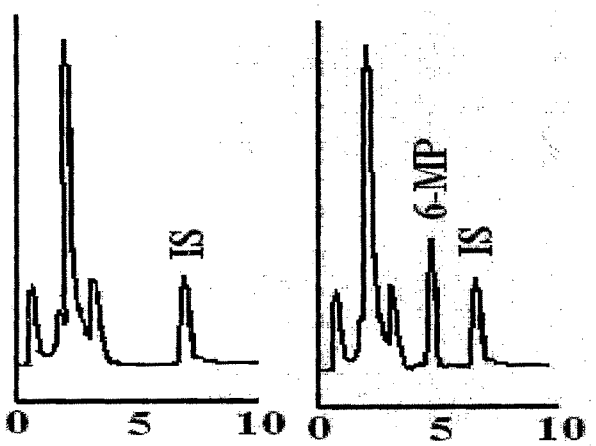


Figure 4.7: Typical chromatograph of drug free rat spleen homogenate and rat spleen homogenate spiked with 500 ng/ml of 6-MP

The linearity was found in the range of 100-1000 ng/ml for all tissues. The data of calibration curves are shown in Table 4.9. The variance of intercept, $S^2_{a,}$ calculated were 4E-04, 3.69 E–04, 4.2E-04, 1.57E-0.4 and 3.64E-04 as well as the variance of slope, $S^2_{b,}$ 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 4.9. Low SD values indicate low variability between each data point of analysis. Accuracy and precision of the method was determined by applying 't' test at each level of

analysis. The 't' values obtained at each data points for each tissue are shown in Table 4.9. The tabulated 't' value required for significance at 5 % level at 2 degree 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.

4.5.2.4 Summary and Conclusion

A reversed-phase high-performance liquid chromatography (HPLC) method was developed to determine 6-mercaptopurine (6-MP) in plasma and tissue homogenates of rat 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 deproteinised using methanol and acetonitrile mixture (1:1, v/v) and centrifuged. The supernatant was collected and analyzed for the drug content. Shimadzu system with Thermosil® C_{18} (5µm, 25 cm × 4.6 mm i.d) column was used for the analysis. A mixture of 0.01M 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 Rheodyne injector and effluent was monitored at 325 nm. 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. The above study shows great potential of estimation of 6-MP in plasma and tissues of rat and may substitute other methods, which are complex, time consuming and consumption of high quantities of organic solvents for estimation.