Chapter 3
Analytical Method

3.1 MATERIALS:

5-Fluorouracil (Dabur, Sahibabad) and Methotrexate (Sun Pharma, Mumbai) were received as gift samples. 5-bromouracil (Loba Chemie, Mumbai), p-Aminoacetophenone (Loba Chemie, Mumbai), Ethyl acetate, HPLC grade (E. Merck India Ltd., Mumbai), Acetonitrile, HPLC grade (SD Fine Chemicals, Mumbai), Methanol, HPLC grade (SD Fine Chemicals, Mumbai) were purchased from respective companies.

Trichloroacetic acid, Hydrochloric acid, Potassium dihydrogen phosphate, Sodium hydroxide were procured from SD Fine chemicals, Mumbai while TRIS(tris-hydroxymethyl aminomethane) was procured from Himedia labs, Mumbai. Double distilled water (DDW) was purified by passing through 0.45μ Millipore filters (Millipore, Bangalore).

3.2 ESTIMATION OF FU BY ULTRAVIOLET SPECTROPHOTOMETRY (UV)

3.2.1 Need and basis of analytical method development

As there is no analytical method reported for UV spectrophotometric analysis of FU at pH 6.8, the method was developed for the same.

3.2.2 Calibration Curve

Stock solution of FU was prepared by dissolving 10 mg of drug in 100 ml of pH 6.8 phosphate buffer. Scanning of standard solution of FU was performed (Scanning range; 200-400nm) to determine its λ_{max}(Fig. 3.1).

Suitable aliquots of the stock solution of FU were pipetted out into 10 ml volumetric flasks and the volume was made upto 10ml with phosphate buffer pH 6.8 to give final concentrations ranging from 1-20 μg/ml (Table 3.1). The solutions were mixed using vortex mixture and their absorbances measured at λ_{max} using phosphate buffer pH 6.8 as blank on Shimadzu 1601 UV-Visible Spectrophotometer and calibration curve was plotted (Table 3.1; Fig 3.2). The above procedure was repeated three times.

3.2.3 Analytical method validation:

The method was validated for accuracy, precision and linearity.

3.2.3.1 Linearity

The linearity of an analytical method is its ability within a definite range to obtain results directly proportional to the concentrations (quantities) of the analyte in the sample (Hubert et al., 1999; Hubert et al., 2003). Linearity of a light absorption determination should be examined to ensure that Beer's law operates over the range of interest.

The calibration curve was built by plotting the drug concentrations versus their respective absorbance. The concentrations for unknown samples and validation samples were obtained by using linear regression of the calibration curves.

3.2.3.2 Accuracy

Accuracy refers to the closeness of an individual observation or mean to true value (Bolton, 1990). The "true" value is the result which would be observed in absence of error. Accuracy of the assay is defined as the percentage of the agreement between the measured value and the true value as follows (Merodia et al, 2000):

$$Accuracy = \frac{True\ \ value\ \ -Measured\ \ value}{True\ \ value} x\ \ 100 \qquad \text{----(3.1)}$$

3.2.3.3 Precision

It refers to the extent of variability of a group of measurements observed under similar conditions. Precision provides an indication of random errors and is generally subdivided into two cases: repeatability and reproducibility, which were determined by calculating RSD (Relative standard deviation) or CV (Coefficient of variation) of inter-day and intra-day determinations. One of the common way of expressing the variability, which takes into account its relative magnitude is the ratio of the standard deviation to the mean, SD/Mean. This ratio, often expressed as a percentage, is called the *Coefficient of Variation* abbreviated as CV or RSD, the *relative standard deviation*.In biological data, the CV is often between 20 -50%,

and one would not be surprised to see an occasional CV as high as 100% or more. The relatively large CV observed in biological experiments is due mostly to "biological variation", the lack of reproducibility in living material. On the other hand, the variability in chemical and instrumental analysis of drugs is usually relatively small. Thus, it is not unusual to find a CV of less than 1% for some analytical procedures. (Bolton, 1990).

In order to determine precision and accuracy of the methods, solutions containing known amounts of pure drug (5-20 µg) were prepared and analyzed in three replicates. Intraday precision was determined by measuring absorbance of samples on the same day while for interday precision absorbance value were determined for different days (over 3 consecutive days).

3.2.4 Results and Discussion

FU in phosphate buffer (pH 6.8) yields a characteristic spectrum when scanned in the ultraviolet range between 200 and 400 nm. The scan (Fig. 3.1) shows absorption maximum at 267 nm and this wavelength was chosen as the analytical wavelength. Correlation coefficient for developed method was found to be 0.9978 signifying that a linear relationship existed between absorbance and concentration of the drug.Beer's law was obeyed between 2 and 20 µg ml^{-1}. Regression analysis was performed on the experimental data. The raw data along with the results of the regression analysis are shown in table 3.1, respectively. Regression equation for standard curve was y = 0.0506x + 0.0093 (Fig.3.2). Parameters for the developed UV spectrometric method of analysis for FU are shown in table 3.2.

Table 3.3 and 3.4 show intraday and interday precision and accuracy for the FU assay by UV spectroscopy. The low % CV values indicate precision of the method. No significant difference between the amount of drug added (actual) and observed concentration was noticed indicating accuracy of the method (Boulangeret al., 2003; Guidance for industry, 2001.).

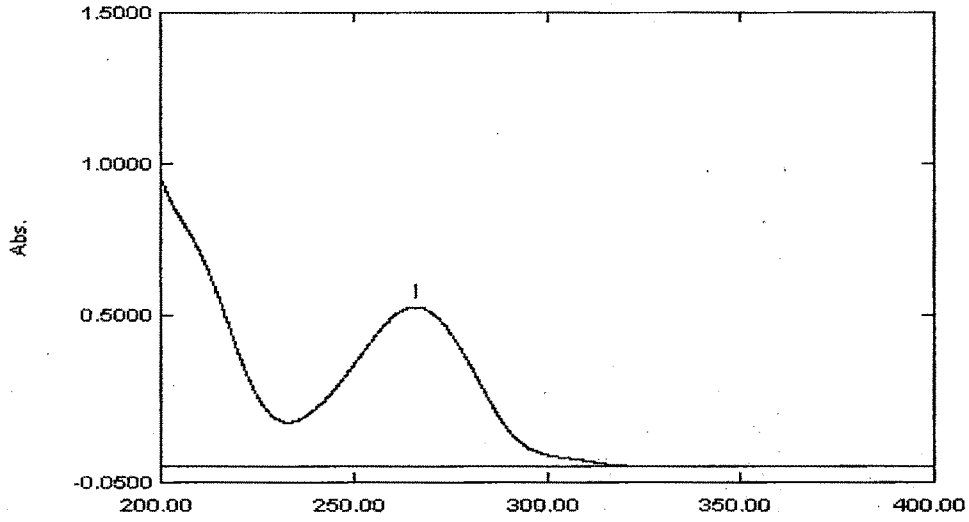

Fig. 3.1 Wavelength scan of FU in pH 6.8 phosphate buffer.

Table 3.1. Calibration curve for FU in phosphate buffer (pH 6.8)

Concentration (µg/ml)	Mean Absorbance* ± S.E.M.	Regressed value**
1	0.048 ± 0.004	0.0599
2	0.100 ± 0.003	0.1105
4	0.216 ±0.006	0.2117
8	0.428 ±0.005	0.4141
10	0.532 ±0.004	0.5153
12	0.626 ±0.008	0.6165
16	0.829±0.006	0.8189
20	0.987±0.005	1.0213

*= Average of 6 determinations

** Using regression equation y = mx + c; y = 0.0506x + 0.0093, Correlation Coefficient (R^2) =0.9978

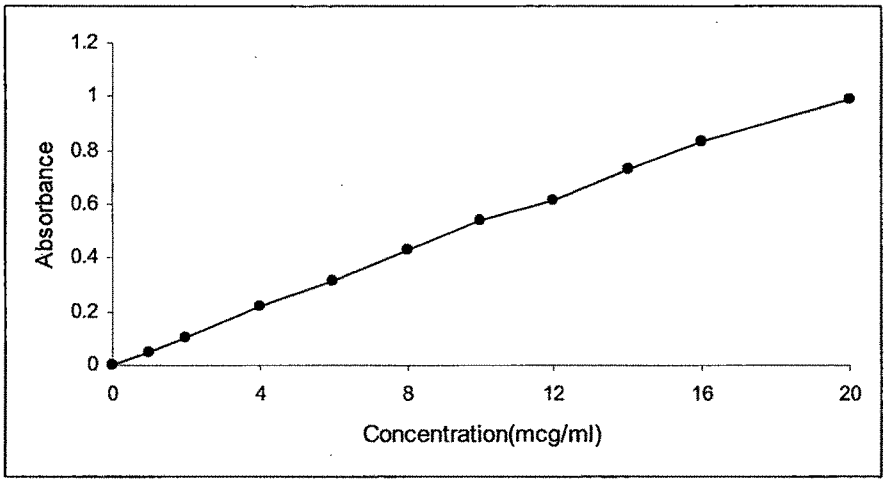

Fig. 3.2 Standard Curve of FU in pH 6.8 Buffer

Table 3.2 Parameters for UV spectrometric method of analysis for FU.

Parameters	Results
λ_{max}	267 nm
Linearity range	2-20 µg/ml
Regression equation	y = 0.0506x +0.0093
Correlation coefficient	0.9978

Table 3.3 Intraday precision and accuracy for the FU assay by UV spectroscopy.

Standard concentration (µg/ml)		Precision(%)[a]	Accuracy(%)[b]
Actual	**Observed**		
5	5.03 ±0.002	0.3976	100.6
10	9.87 ±0.004	0.6079	98.7
15	14.90 ±0.008	0.5369	99.33
20	20.19 ±0.007	0.6438	100.95

a Expressed as relative standard deviation,
[RSD] = (standard deviation/mean concentration) x 100
b Expressed as[(mean observed concentration/actual concentration) × 100]

Table 3.4 Interday variability of FU assay by UV spectroscopy over 3 consecutive days.

Standard concentration (µg/ml)		Precision(%)[a]	Accuracy(%)[b]
Actual	**Observed**		
5	5.08 ±0.003	0.7874	101.6
10	9.91 ± 0.006	1.009	99.1
15	15.14 ± 0.009	0.3963	100.93
20	19.78 ± 0.004	1.011	98.9

a Expressed as Relative Standard Deviation;
[RSD] = (Standard deviation/Mean concentration) x 100
b Expressed as[(Mean observed concentration/Actual concentration) × 100]

3.3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) METHOD FOR ESTIMATION OF FU IN PLASMA

3.3.1 Method

A modified method of that described by Nassim et al was used (2002). In this reported method, solid phase extraction was used to extract FU from plasma, but we have performed FU extraction by liquid extraction method using ethyl acetate. Dionex isocratic HPLC with a UV-visible detector was used for HPLC analysis. The mobile phase, consisting of potassium dihydrogen phosphate [KH_2PO_4: pH 6.8], was prepared at a 50 mM concentration with distilled deionised water (DDW) followed by degassing of the solution and filtering through a 0.45 μ inorganic filter. BDS Hypersil C_{18} column (Thermo Hypersil Keystone), 150 X 4.6 mm with 5 μ particles was used. The required parameters were programmed using Chromeleon software.

Blank human plasma samples (0.5ml) were spiked with FU stock solution (100-2000 ng/ml) and centrifuged at 2500 rpm (15 min). FU (10μg/ml) and Internal standard (15μg/ml) were added to plasma and processed in the same way. Proteins were precipitated by addition of acetonitrile (1ml) and subsequent centrifugation at 2500 rpm (20 min). The drug was extracted three times by using ethyl acetate (1ml) followed by centrifugation at 2500 rpm (10 min).The dried extract was reconstituted with 1 ml DDW and loaded into the injector using syringe. Calibration curves were drawn by plotting peak area of FU vs. drug concentration. Program parameters were: Flow rate-0.5 ml/min, Detection wavelength- 265nm, Run time-18 min. The column was equilibrated by passing at least 150-200 ml of mobile phase. 20μl of sample was loaded using syringe through rheodyne injector then standard curve was plotted.

3.3.2 Validation

Analytical method was validated for specificity, linearity, precision, accuracy and sensitivity (as described in section 3.1.3).

3.3.3 Results and Discussions

Retention time for FU is 6.3 min while for internal standard (5-Bromouracil) is 14.0 min (Fig. 3.3). Standard plot is shown in Fig. 3.4 and Table 3.5. Parameters for the developed HPLC method for estimation of plasma are shown in Table 3.6.

3.3.3.1 Specificity:

Three different lots of drug free rabbit plasma were tested before spiking to ensure that there was no endogenous interference at retention times of FU and 5-Bromouracil (internal standard). Typical chromatograms obtained after analysis of blank plasma, and plasma samples containing FU are illustrated in Fig. 3.3 a, b and c.

3.3.3.2 Linearity:

The data for calibration plot of FU in plasma by HPLC (Table 3.5) was fitted into a linear equation ($y=0.0015x + 0.0536$) with correlation coefficient of $R^2 = 0.9992$, which indicated the linearity of the plot.

3.3.3.3 Sensitivity:

The limit of detection (LOD) and the limit of quantitation (LOQ) were determined as defined by IUPAC (Winefordner et al, 1983) and ICH Topic Q2B, (ICH Topic Q2B, 1996). using the slope (m) of the calibration curve and the standard deviation (sd) of the intercept (c) by the following equation:

LOD = 3.3 X sd/m

LOQ = 10 X sd/m

The LOD was found to be 30 ng/ml and LOQ was found to be 92 ng/ml in rabbit plasma.

3.3.3.4 Precision:

Precision of the method was assessed by analyzing the plasma samples spiked with FU at different concentrations (100, 200, 300, 500, 1000 and 2000ng/ml). Three replicate of each concentration were analyzed and results are given in table 3.7 and

3.8. To evaluate precision, the mean values and the % RSD values were calculated for each concentration.

The % RSD values for intraday and interday assay precision are presented in table 3.7 and 3.8. The low % CV values indicate precision of the method.

3.3.3.5 Accuracy:

The accuracy is expressed as % bias or % relative error (difference from added concentration) and it takes into account the total error, i.e. systematic and random errors, related to the test result (Hubert et al, 2003; Hubert et al, 1999). The tolerance limits for intraday assay and interday assay samples are presented in Table 3.7 and 3.8 as a function of the introduced concentrations. No significant difference between the amount of drug added (actual) and observed concentration at all the concentration levels tested was noticed indicating accuracy of the method (Boulanger et al, 2003; Guidance for industry, 2001).

Table 3.5. Calibration curve for FU in plasma by HPLC

Concentration (ng/ml)	mAU* ± S.E.M.	Regressed value**
100	0.1745 ± 0.003	0.2036
200	0.349 ± 0.006	0.3536
300	0.502 ± 0.009	0.5036
500	0.868 ± 0.008	0.8036
1000	1.62 ± 0.002	1.5536
2000	3.1 ± 0.008	3.0536

*= Average of 6 determinations.
** Using regression equation y = mx + c
Intercept =0.0536, Slope =0.0015;
Equation of line : $y = 0.0015x + 0.0536$; $R^2 = 0.9992$

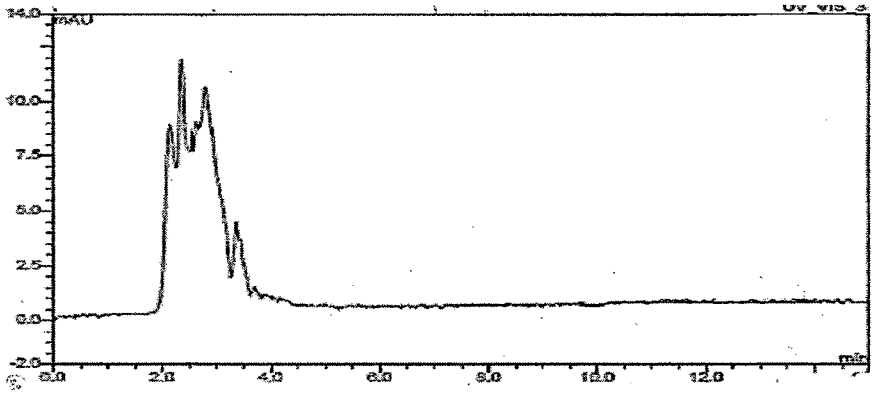

Fig 3.3a. Representative chromatogram of blank plasma

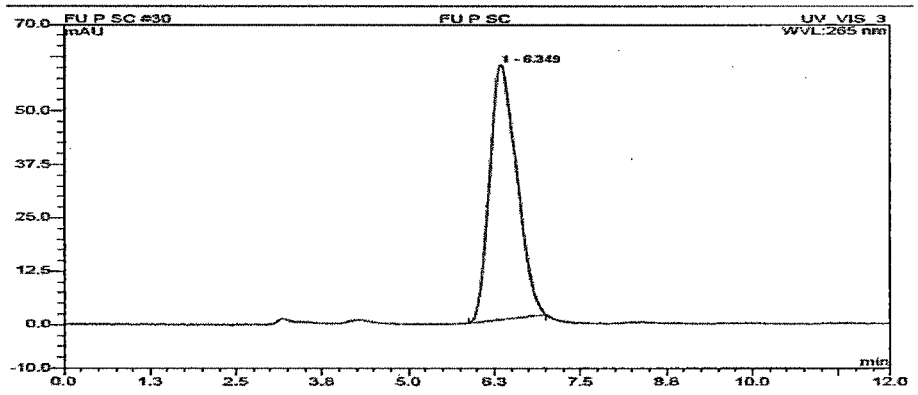

Fig 3.3b. Representative chromatogram of FU in plasma

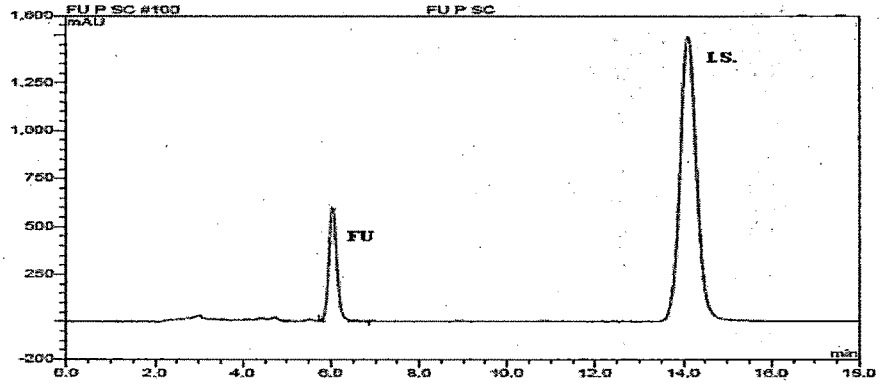

Fig 3.3c. Representative chromatogram of FU and Internal standard (I.S.) in plasma; I.S. - 5 - Bromouracil

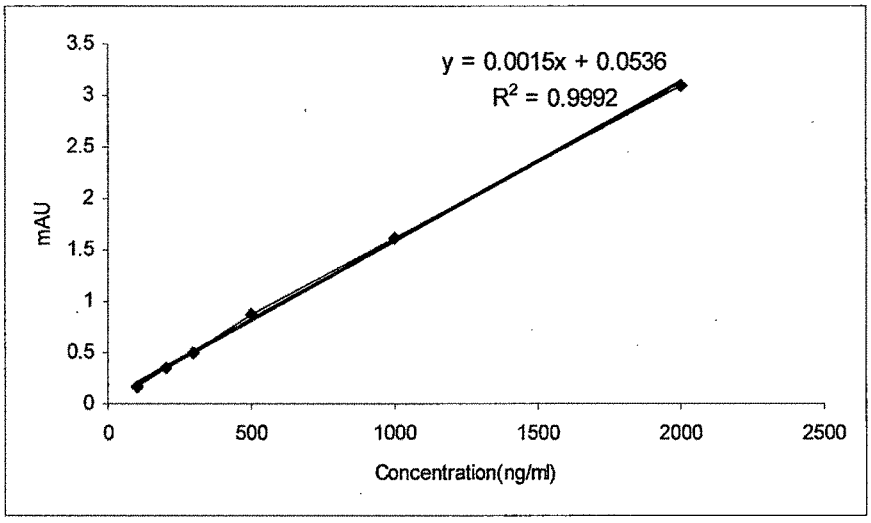

Fig. 3.4. Standard plot for FU in plasma by HPLC.

Table 3.6 Parameters for HPLC method for estimation of FU in plasma.

Parameters	Results
λ_{max}	265 nm
Linearity range	100-2000 ng/ml
Regression equation	y = 0.0015x + 0.0536
Correlation coefficient	0.9992
LOD	30 ng/ml
LOQ	92 ng/ml
Retention time	6.3 min

Table 3.7 Intraday precision and accuracy for FU plasma samples.

Standard concentration (ng/ml)		Precision(%)[a]	Accuracy(%)[b]	%RE
Actual	Observed #			
100	98.2	1.43	98.2	-1.8
200	204.8	1.95	102.4	2.4
300	312.4	2.56	104.1	4.13
500	519.1	2.70	103.8	3.82
1000	975.7	4.099	108.4	-2.43
2000	1979.5	2.66	99.47	-1.03

a Expressed as relative standard deviation
[RSD] = (standard deviation/mean concentration) x 100.

b Expressed as[(mean observed concentration/actual concentration) × 100]
% RE= (Mean Concentration-Nominal Concentration)/Nominal concentration x 100;
# n=6

Table 3.8. Interday precision and accuracy of FU assay by HPLC over 3 consecutive days.

Standard concentration (ng/ml)		Precision(%)[a]	Accuracy(%)[b]	%RE
Actual	Observed #			
100	94.1	4.25	94.1	-5.90
200	209.8	4.77	104.9	4.90
300	320.4	1.56	106.8	6.8
500	509.6	2.55	101.9	1.92
1000	964.7	2.07	96.47	-3.53
2000	1963.5	1.01	98.175	-1.83

a Expressed as Relative Standard Deviation;
[RSD] = (Standard deviation/Mean concentration) x 100

b Expressed as[(Mean observed concentration/Actual concentration) × 100]
% Relative Error (RE) = (Mean Concentration-Nominal Concentration)/Nominal concentration x 100
# n=6

3.3.3.6 Absolute recovery:

The absolute recovery of FU at three concentration levels was determined by comparing the peak areas measured after analysis of spiked plasma samples (containing FU= 200, 500 and 2000 ng/ml) according to the procedure discussed in 3.2.1 with those found after direct injection into the chromatographic system of non-biological samples at the same concentration levels. As shown in Table 3.9, the analyte recoveries were close to 100% and the extraction efficiency satisfactorily ranged from 88.6% to 94.9% for plasma samples. Recovery of internal standard was found to be 96.8%.

Table 3.9 Extraction efficiency of FU from rabbit plasma at various concentrations.

Concentration (ng/ml)	Extraction Efficiency ± S.E.M.
200	88.64±2.9
500	92.13±5.4
2000	94.85±4.8
Average extraction efficiency	91.87±4.4

Mean ± S.E.M.; n=3.

3.4 ESTIMATION OF MTX BY ULTRAVIOLET SPECTROPHOTOMETRY (UV)

3.4.1 Need and basis of analytical method development

As there is no analytical method reported for UV spectrophotometric analysis of MTX at pH 6.8, the method was developed for the same.

3.4.2 Calibration Curve

Stock solution of MTX was prepared by dissolving 10 mg of drug in 100 ml of pH 6.8 phosphate buffer. Scanning of a standard solution of MTX was performed (Scanning range; 200-400nm) to determine its λ_{max}.

Suitable aliquots of the stock solution of MTX were pipetted out into 10 ml volumetric flasks and the volume was made upto 10ml with phosphate buffer pH 6.8 to give final concentration of 1, 5, 10, 15 and 20µg/ml. The solutions were mixed well and their absorbances measured at λ_{max} using phosphate buffer pH 6.8 as blank on Shimadzu 1601 UV-Visible Spectrophotometer and calibration curve was plotted. The above procedure was repeated three times.

3.4.3 Analytical method validation:

The method was validated for accuracy, precision and linearity as per the methodology followed earlier in section 3.1.3.

3.4.4 Results and Discussion:

MTX in phosphate buffer (pH 6.8) yielded a characteristic spectrum when scanned in the ultraviolet range between 200 and 400 nm. The scan (Fig 3.5 shows absorption maximum at 303 nm and this wavelength was chosen as the analytical wavelength (Chamberlin et al 1976; Subramanian. et al., 2003). Correlation coefficient for the developed method was found to be 0.9996 signifying that a linear relationship existed between absorbance and concentration of the drug. Calibration plot is shown in Fig.3.6.

Beer's law was obeyed between 5 and 20 µg ml^{-1} for the method. Regression analysis was performed on the experimental data. The raw data along with the results of the regression analysis are shown in Table 3.10. Regression equations for standard curve was $y = 0.0491x + 0.0052$. Table 3.11 gives parameters for developed UV spectrometric method for analysis of MTX. Table 3.12 and 3.13 give the accuracy and precision values for the developed method. All R.S.D. values for interday assay and intraday assay were lower than the acceptance limit of 15%, illustrating good precision of the proposed method.

As can be seen from the results, the lower % CV values indicate precision of the method. No significant difference between the amount of drug added (actual) and observed concentration was noticed indicating accuracy of the method (Boulanger, B. et al, 2003; Guidance for industry, 2001).

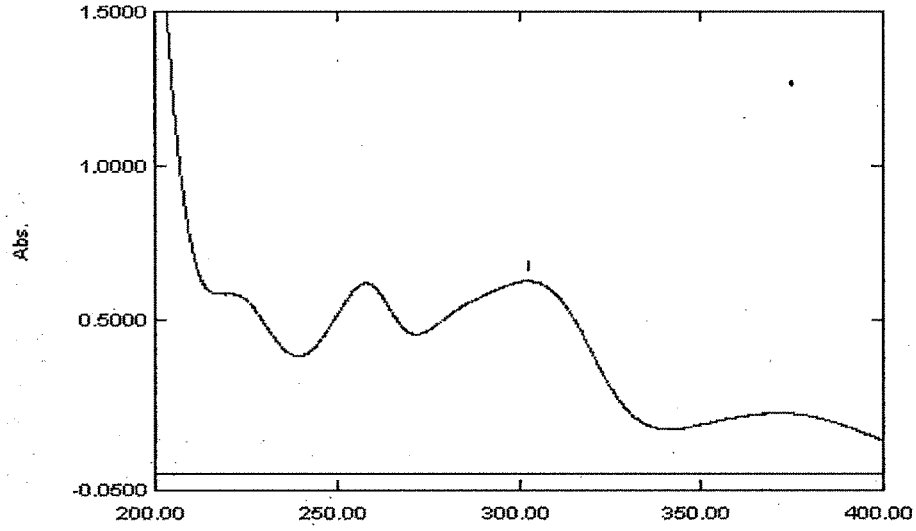

Fig.3.5 Wavelength scan of MTX in pH 6.8 phosphate buffer.

Table 3.10. Calibration curve values for MTX in phosphate buffer pH 6.8

Concentration (µg/ml)	Mean Absorbance* ± S.E.M.	Regressed value**
1	0.053 ± 0.002	0.0543
5	0.260 ± 0.003	0.2507
10	0.490 ±0.008	0.4962
15	0.753 ±0.004	0.7417
20	0.980 ±0.006	0.9872

*= Average of 6 determinations

** Using regression equation y = mx + c

Regression equation, y = 0.0491x + 0.0052

Intercept = 0.0052, Slope = 0.0491, Correlation Coefficient (R^2) = 0.9996

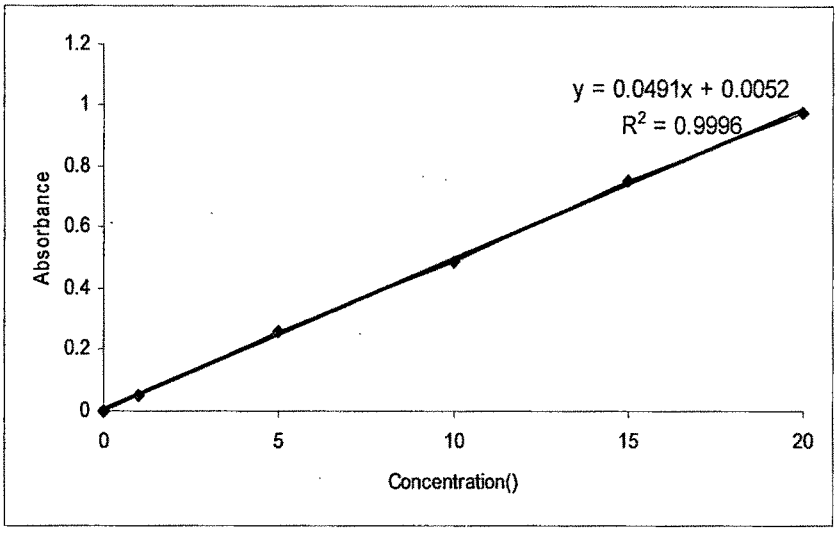

Fig. 3.6. MTX standard curve in pH 6.8 phosphate buffer

Table 3.11. Parameters for UV spectrometric method for analysis of MTX.

Parameters	Results
λ_{max}	303 nm
Linearity range	2-20 µg/ml
Regression equation	y = 0.0491x + 0.0052
Correlation coefficient	0.9996

Table 3.12 Intraday precision and accuracy for the MTX assay by UV spectroscopy.

Standard concentration (µg/ml)		Precision(%)[a]	Accuracy(%)[b]	% R.E.
Actual	Observed			
5	5.13	0.974	102.6	2.6
10	10.12	0.988	101.2	1.2
15	14.96	1.33	99.7	-0.27
20	20.12	0.4970	100.6	0.6

a Expressed as relative standard deviation
[RSD] = (standard deviation/mean concentration) x 100.

b Expressed as[(mean observed concentration/actual concentration) × 100]
% Relative Error (R.E.) = (Mean Concentration-Nominal Concentration)/Nominal concentration x 100

Table 3.13. Interday variability of MTX assay by UV spectroscopy over 3 consecutive days.

Standard concentration (µg/ml)		Precision(%) [a]	Accuracy(%) [b]	% RE
Actual	Observed#			
5	5.10	0.99	102	2
10	9.90	1.51	99	-1
15	15.19	1.76	101.27	1.27
20	19.72	1.42	98.6	-1.4

a Expressed as Relative Standard Deviation;
[RSD] =(Standard deviation/Mean concentration) x 100

b Expressed as[(Mean observed concentration/Actual concentration) × 100]
% Relative Error (R.E.)= (Mean Concentration-Nominal Concentration)/Nominal concentration x 100
# n=6

3.5 METHOD FOR ESTIMATION OF MTX IN PLASMA

The method described by Moghbel et al was used (2003). Dionex isocratic HPLC with a UV-visible detector was used for HPLC. The chromatography column was a 250 X 4.6 (i.d) millimeters, Spherisorb ODS 2 with 5-micron particles. The mobile phase consisted of phosphate –TRIS buffer (0.1 M dihydrogen phosphate and 0.01 M TRIS; pH 5.7): methanol: acetonitrile, with the ratio of 82:11:7, respectively. The mobile phase was filtered through 0.2μ membrane filter. Octadecyl silica (Waters) column was connected and required parameters were programmed using Chromeleon software.

3.5.1 Standards Solutions

A stock standard solution of methotrexate (200 μM) was prepared using TRIS-HCl buffer (pH 8): acetonitrile, 9:1, respectively. Stock solutions of internal standard {I.S.; (p-aminoacetophenone)} were also prepared in the same solvent at the concentrations of 15 and 50 μg/lit.

Blank human plasma samples were spiked with MTX stock solution with I.S. and centrifuged at 2500 rpm (15min). Proteins were precipitated by addition of 2 M trichloroacetic acid in ethanol (1 ml) and subsequent centrifugation at 3000 rpm (20min). 20 μL aliquots of the supernatant was directly injected into the chromatography column. Calibration curves were drawn by plotting peak area of MTX vs. drug concentration. Program parameters were as follows: Flow rate-1.1 ml/min, Detection wavelength- 313 nm, Run time-20 min. The column was equilibrated by passing at least 150-200 ml of mobile phase. 20μl of sample was loaded using syringe through rheodyne injector then standard calibration curve was plotted.

3.5.2 Results and Discussion:

HPLC chromatograms are shown in Fig 3.7. Table 3.14 and Fig. 3.8 show calibration curve of MTX in plasma. Table 3.15 shows parameters for HPLC method for the estimation of MTX in plasma.

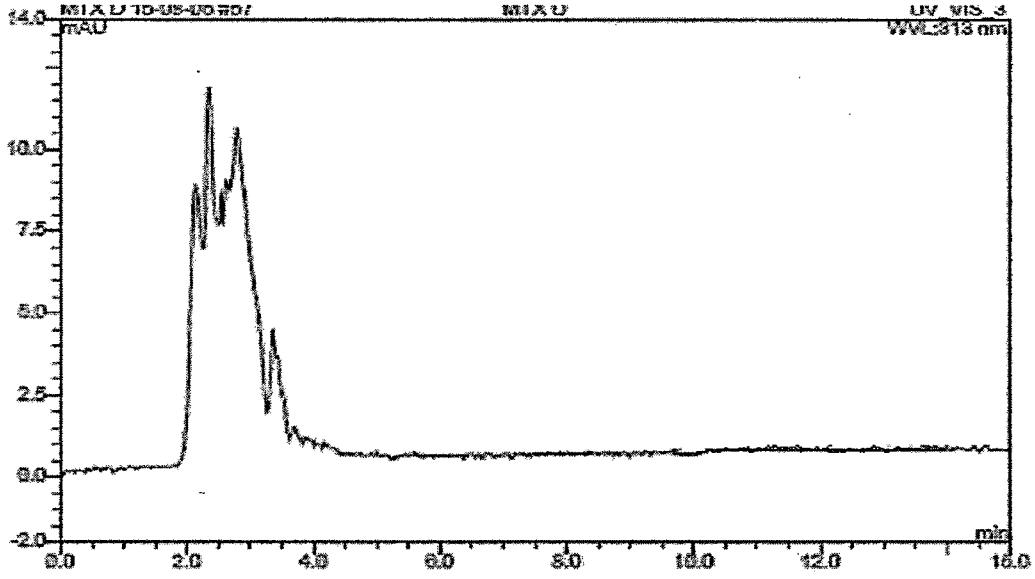

Fig 3.7a. Representative chromatogram of blank plasma

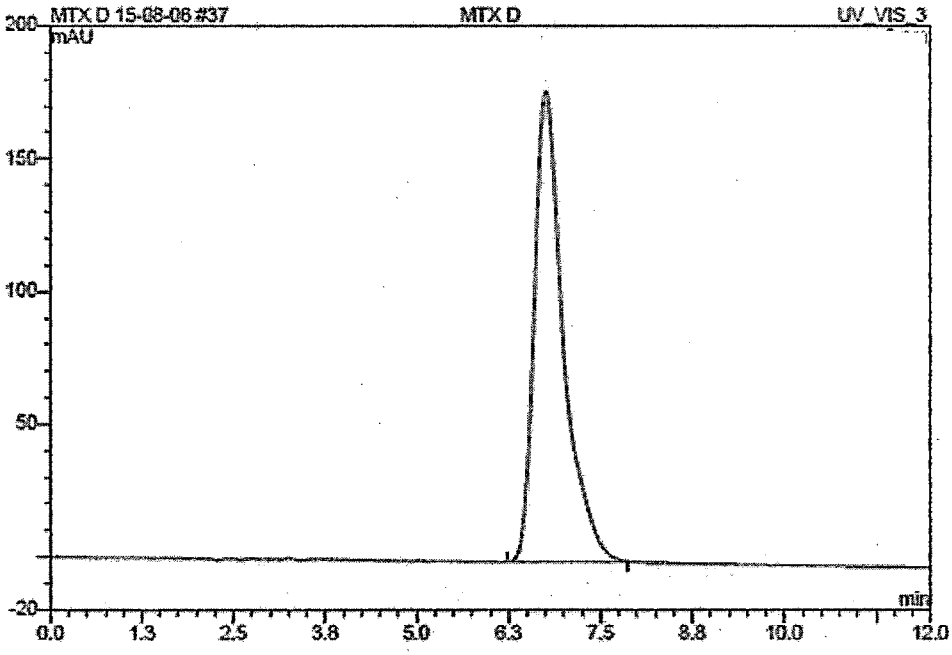

Fig 3.7b. Representative chromatogram of MTX in plasma

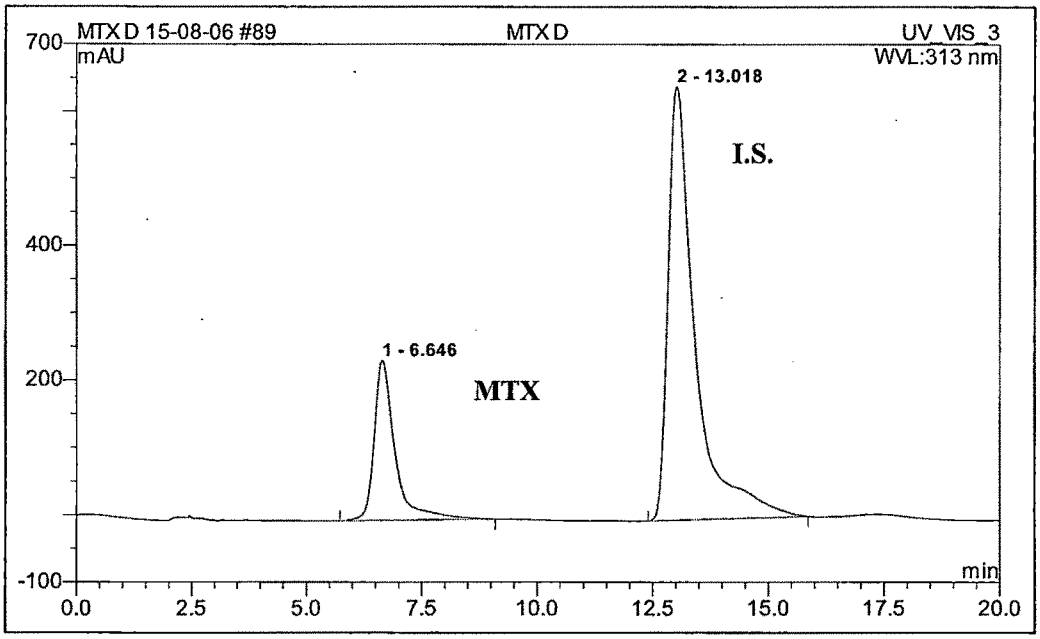

Fig 3.7c. **Representative chromatogram of MTX in plasma**
MTX – Methotrexate ; I.S. – Internal Standard(p-aminoacetophenone)

Table 3.14. Calibration curve of MTX in plasma by HPLC.

Concentration (ng/ml)	mAU*	Regressed value**
100	0.041 ±0.002	0.0579
200	0.09052 ±0.005	0.0979
500	0.2102 ±0.008	0.2179
1000	0.4236 ±0.014	0.4179
2000	0.7595 ±0.019	0.8179

* Average of 6 determinations
** Using regression equation y = mx + c.
Regression equation, y = 0.0004x + 0.0179.
Intercept = 0.0052, Slope = 0.0491, Correlation Coefficient (R^2) = 0.9965.

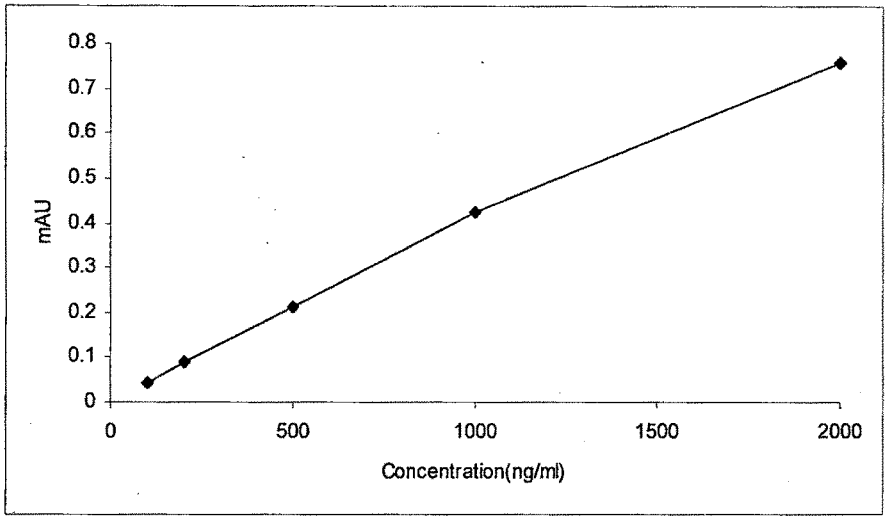

Fig. 3.8. Standard curve of MTX in plasma by HPLC.

Table 3.15. Parameters for HPLC method for estimation of MTX in plasma.

Parameters	Results
λ_{max}	313 nm
Linearity range	100-2000 ng/ml
Regression equation	y = 0.0004x + 0.0179
Correlation coefficient	0.9965
LOD	35ng/ml
LOQ	110ng/ml
Retention time	6.6 min

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