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Development and Optimization of RP-HPLC Method for analysis of 5-FU in Human and Rabbit Plasma Samples: Identification and Quantification

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ABSTRACT

In vitro and *in vivo* experiments revealed that drug loaded cross linked chitosan nanoparticles showed; a sustained release profile of 5-fluorouracil compared to the oral solution of 5-FU due to which Area Under Curve (AUC) was increased two times more folds, as promising site specific targeted site of 5-FU to the colon. A simple, accurate, precise, cost effective and sensitive Reversed Phase HPLC (RP-HPLC) method has been developed and validated then, for determination of 5-FU in human and rabbit plasmas. The current RP-HPLC chromatography system is an isocratic of Agilent technologies series 1200 consisted of a pump and variables. Data processing software chem station used with a Wave Length Detector (WLD) for assay of prepared plasma samples. Mobile phase composition was *i.e*. acetonitrile: water (10:90) at pH 6 and 1.0 mL/min flow rate for 3-4 minutes (retention time). 5-Fluorouracil was detected using a Waters 2996 photodiode array detector at a 260 nm wavelength. The calibration curve was linear over the concentration range of 2-100 ng/ml. This method was specific and co-relation coefficient (r^2) is less than or equal to 0.998. It is concluded that reproducible method may be employed for the analysis of 5-Fluorouracil (5-FU) is a broad spectrum anticancer and it is widely used in the treatment of various types of solid cancers. But due to its narrow therapeutic window, plasma concentration is very essential to determine at clinical setups, pharmacokinetic parameters, in rabbit and human plasma samples as well as nanoparticles formulations.

Keywords: RP-HPLC; Limit of Detection (LOD); Limit of Quantification (LOQ); Quantity Concentration (QC)

INTRODUCTION

Chemotherapy is considered as a part of treatment in conventional therapy during some stages of cancer and frequently, it is used to administer again after surgery as adjuvant chemotherapy [1,2]. 5-Fluorouracil (5-FU) was found to be used among the basic chemotherapeutic agents. 5-Fluorouracil (5-FU), an anticancer, introduced in 1958, higher therapeutic efficacy have produced in solid types tumors like colon, rectum and breast cancers [3,4]. Its oral conventional brand is showed erratic absorption through GIT. As it shows rapid gastrointestinal absorption, after oral administration its

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yielding peak blood levels between 15 to 60 minutes [5,6]. Additionally, 5-FU has nonspecific toxicity in normal healthy cells, speedy renal clearance as well as metabolism by Dihydro-Pyrimidine Dehydrogenase (DPD) enzyme and high digestive distress inhibits it application in management of cancer. 80% orally administered 5-FU, has reported to metabolize in liver and kidney [7,8]. It has detoxified and excreted as F-ß-alanine by urine. However due to high rate of metabolism in blood, it has shorter half-life also (8-20 min). Intravenous administration of 5-FU for solid types of cancers, demonstrates severe cytotoxic effects by a huge number of previous studies in the literature. As IV administration of 5-FU linked with disturbed microbial flora of GIT track so, accurately designed oral formulations of 5-FU using different biocompatible, biodegradable, smart pH sensitive polysaccharides have employed for sustained, controlled delivery of therapeutic agent *i.e*. depending upon selection of polymer and networking pattern on tumor cells by most of researchers in the literature as well. Cross linking can also influence drug loading and entrapment efficiency from NPs which affect therapeutic diffusion and tunable physiochemical properties of Nano carrier or nanoparticles facilitate sustained or controlled release of drug on targeted site of tumor without harming to the other body tissues [9,10]. Reversed Phase High Performance Liquid Chromatography (RP-HPLC) is most common chromatographic method that has been used for pharmacokinetic analysis. Less expensive and commonly available method could facilitate in terms of expense and time in analysis but other process parameters could not be missed in success of easy and inexpensive analysis [11,12]. There are several procedure parameters in relevance to each technique, like complex composition of mobile phase, long run time and particularly sample preparation (extraction of drug from biological fluid) can even make the HPLC analysis more complex. Previous studies have reported simple eluent but extraction process remained an important step to be studied for improvement fast and involvement of fewer solvents. 5-Flourouracil (5-FU) a pyrimidine analogue is widely used in various solid types of cancers particularly including head and neck, colorectal, and breast cancers. Several analytical procedures have been reported to quantify 5-FU in biological, intestinal and mucosal fluids. 5-FU assay has been developed using normal phase HPLC, reversed phase HPLC Pharmacokinetics of 5-FU has been reported previously. Pharmacokinetic variability is main reason for inter patient plasma drug concentration. This instability in the drug concentration even after administration of same dose under the same conditions enforces individualization of dose in patients. Simple and rapid detection of 5-FU with accuracy and precision become an important issue in its therapy. 5-FU has been administered mostly by bolus or continuous infusion or by combination of both for several days [13,14]. This currently used route of administration need plasma concentration analysis. Research studies are also being conducted for oral delivery of this anticancer drug to avoid the excessive adverse effects especially in the gastrointestinal tract cancers. This novel oral drug delivery system must be evaluated on animals first and then on humans [15]. This study main focus was to develop and validate reversed phase High Performance Liquid Chromatography (HPLC) method using mobile phase having simple composition and fast samples preparation procedure. The method has been developed in both human and rabbit's plasmas. The studied method could applied in Therapeutic Drug Monitoring (TDM), pharmacokinetic studies and evaluation of new oral dosage forms of 5-FU in humans as well as in other animals.

MATERIALS AND METHODS

Chemicals and Reagents

5-FU powder was received as a kind gift from Paramedic Laboratories (Pvt.) Ltd. Pakistan. Perchloric acid was purchased from Sigma Aldrich. HPLC grade water was prepared in our pharmaceutical technology laboratory. Human blood plasma sample was obtained from civil hospital, Bahawalpur. Rabbit plasma was obtained from animal house of our pharmacy department the Islamia university of Bahawalpur, Pakistan.

Chromatographic Requirements

The separation was carried out at 25°C (room temperature) on ODS hypersil C18 column having 4.6 mm x 250 mm, particle size of 5 µ (thermo electron, USA). HPLC chromatography system an isocratic of Agilent technologies series 1200 consisted of a pump and variables. Data processing software chemstation used with a wave length detector (VWD) for assay of prepared plasma samples. Mobile phase composition was *i.e*. acetonitrile: water (10:90) at pH 6 as well as 1.0 mL/min flow rate for 3-4 minutes (retention time). 5-Fluorouracil was detected using a Waters 2996 photodiode array detector at a 260 nm wavelength. This method was specific and co relation coefficient (r^2) is less than or equal to 0.998. Degassing of mobile phase by sonication (Elma D-78224 Singe/Htw, Germany), vacuum filtration *via* 0.45 µm Millipore filters papers (Merck, Germany). Injection of mobile phase as well as its flow rate was maintained at 1 ml/min and 10 µl of prepared plasma sample was injected and then elate was monitored at 260 nm wave length by Agilent VWD (Agilent Technologies, USA).

Stock Solution Preparation

5-Fluorouracil primary stock solution of (1000 ng/ml) was prepared at room temperature by dissolving 1 mg of pure 5-Fluorouracil in 1000 ml of Deionized Distilled (DD) water. Form this primary stock solution standard solutions of 100,50,40,30, 20, 10, 5,4,3 and 2 ng/ml were prepared by serial dilutions. Before construction of calibration curve these dilutions were normalized to room temperature.

Animal Plasma Sample

Rabbit drug free plasma sample was obtained from animal house of the faculty of pharmacy and alternative medicine, the Islamia university of Bahawalpur. Pakistan. It was then stored at -70°C and used before HPLC method development and validation. Study was performed with prior approval of

Pharmacy and Research Ethics Committee (PREC), the Islamia University of Bahawalpur, Pakistan.

Spiked Plasma and Preparation

For spiking of rabbit plasma drug free sample, was mixed with a proper quantity of standard working dilutions *i.e.,* 100,50,40,30,20,10,5,4,3, and 2 ng/ml of 5-FU. Serial dilutions were prepared by putting 50 µl of standard dilutions as wel as 50 µl of drug free plasma sample as well as 500 µl of 10% v/v perchloric acid and then for 10 minutes vortexes it on a vortex mixer. Perchloric acid as protein precipitating agent was added for precipitation of plasma proteins present into this mixture. Centrifugation of precipitated samples was performed on centrifuge machine by EBA-20, Hettich-Germany at 3500 rpm for 15 minutes. Then with the help of with micropipette, supernatant layer was removed in clean as wel as a dried labeled amber glass vials. This extract of drug solution (20 µl) was directly injected into HPLC system and then in the similar way, intra day (one day b/w batch) and inter day (six consecutive days) spiked plasma samples were prepared.

Standard Calibration Curve and Calculations

Eleven points 2-100 mg/ml in rabbit plasma was used for preparation and validation of analytical method. Firstly drug solutions were injected for identification of drug peak and rabbit plasma samples were analyzed. To confirm accuracy and precision, each step was repeated and each calibration curve was constructed in triplicate. For identification of interfering peaks blank plasma samples (without drug) were used.

Estimation of Recovery and Matrix Effect

Evaluation of 5-Fluorouracil recovery from human and rabbit plasma samples has been done by comparison and matrix effect. Relative % age recovery of 5-Fluorouracil from human and rabbit plasma samples were assessed by comparing measured concentration (extracted samples of both plasma samples) with spiking levels. Absolute matrix effect on 5-Fluorouracil in plasma of human and rabbit was calculated by peak area of extracted samples with corresponding peak area obtained at the same concentration from direct injection of aqueous spiked solution. It was noted that difference in plasma compositions of human as well as rabbit plasma affected differently.

RESULTS

Method Validation

Limit of Detection (LOD) and Limit of Quanti ication (LOQ): According to International Conference on Harmonization (ICH), Limit of Detection (LOD), in an analytical method is least concentration of the analyte present in sample which can be detected but not quantified as an exact value. While limit of quantification (LOQ) in an analytical process is minimum quantity of analyte present in given sample which can be determined quantitatively with suitable accuracy and precision?

Analysis Time

It determines total run time or total analysis time, *i.e.* indirectly effect on consumption of chemicals or solvents and overall cost of analytical method. For a standard analytical procedure run time 5-10 minutes was considered optimum but it may change from analyte to analyte. Optimization of proposed method was 10 minutes as total run time of analysis. Additional decreasing in run time compromised precision and accuracy of this method [16,17].

Linearity

Linearity is directly proportional to the concentration of analyte in sample. It was proposed method by analyzing all concentration ranges as mentioned above and repeatedly injected (n=3). Peak areas were plotted against estimated drug concentrations and time. Calculation of correlation coefficient, slope and intercept parameters was performed by least squares linear regression analysis [18-20].

Evaluation of Accuracy and Precision

According to ISO/IEC accuracy is defined as “closeness of agreement between a true quantity value and measured quantity value of an analyte”. It is the qualitative measurement that cannot be shown as a numerical value. Quality control sample concentration data for intra as well as inter day precision and accuracy were estimated by analyzing three to six different QC samples. Intra day (within batch precision or % repeatability and accuracy were calculated by analyzing six replicates of QC samples on same day, while inter day (one day b/w batch precision and accuracy)were evaluated by assessing each sample on six different days. Relative Standard Deviation (RSD) was calculation to evaluations of precision and accuracy within acceptable limits and it must be equal to or lower than 15 %.

Selectivity

5-Fluorouracil resolution peak was assessed from interfering peaks of plasma proteins and other components of mobile phase as these components used to get maximum resolution of analyte. Various protein precipitants, pH stabilizers were processed with plasma samples and resolution greater than 2 was proposed for this method.

Robustness

To investigate effect on separation of 5-Flurouracil, HPLC chromatographic conditions were consciously changed *i.e.* flow rate was altered by 0.2 units (1.0 ± 0.2). PH effect was observed by changing 0.5 units from 3.2 real pH values. Column age effect was also assessed by keeping other conditions constant and comparing of fresh column responses with that of 1 to 3 months old column.

5-Fluorouracil Stability in Rabbit Plasma

It was estimated by analyzing the replicates (n=6) of four dilution levels in rabbit plasma. Degradation effect in biological fluids by quality freeze thaw hours (0 hours, 2 hours, 4 hours and 6 hours) was analyzed as well as all plasma samples. To protect them from photo oxidation they were stored at -20°C and the thawed at room temperature in dark.

System Suitability

Parameters like Theoretical plates (Tp), Asymmetry factor (As), Capacity factor (K), Resolution (Rs), Retention Time (RT) and Tailing Factor (Tf) were reported in European pharmacopoeia as well as calculated by LC solution software. HPLC system was equilibrated with initial mobile phase composition and then followed by six injections of the same standard.

Optimization of HPLC Method

The HPLC analytical method development is a multistep process to ascertain the final chromatographic conditions as illustrated in **Tables 1-3**. During method development following conditions of mobile phases and stationary phases were selected and optimized as per ICH guidelines.

Table 1: Mobile phases used during HPLC method development.

Mobile phases	Proportion	pH	Peak symmetry
Methanol: Water	50:50:00	4.2	Peak spiting
Water: Methanol	40 : 65	5	Peak broader
Methanol: Water	60:40:00	6	Broader peak
Methanol: Water	40 : 60	6.5	Splitting
Acetonitrile: 0.05 M Na_2HPO_4 buffer	50:50:00	7	Dissociated peaks
Acetonitrile: Water	90:10:00	6.5	Sharp peak low resolution
Acetonitrile: Water	70:30:00	7	Dissociated overlapping peak
Acetonitrile: Water*	0.479167	6	Sharp peak good resolution
Acetonitile: 0.01 M Na_2HPO_4: 5 Mm	60:40:00	6.5	Sharp peak low resolution high retention time
Methanol: Acetonitile	50:50:00	6.5	Sharp peak low resolution and tailing
Acetonitle: Methanol	40:60	6.5	Sharp peak with tailing
Acetonitle: Methanol	60:40:00	6.5	Sharp peak with low resolution to plasma peak

Note: *selected mobile phase for the analysis.

Table 2: 5-Fluorouracil linearity in human plasma and its LOD and LOQ through standard deviation method.

Conc (mg. ml^{-1})	Area (m.Au)
100	305.374
50	143.893
40	120.114
30	90.553
20	70.114
10	48.754

5	35.346
4	28.901
3	20.023
2	15

Tablet 3: 5-Fluorouracil linearity in rabbit plasma and its LOD and LOQ through standard deviation method.

Conc. (mg/ml)	Area (m.Au)
100	312.11
50	155.102
40	115.664
30	95.513
20	65.869
10	51.773
5	38.885
4	24.77
3	17.023
2	13.551

Selection of Stationary Phase of HPLC Method

According to USP approximately 78 different types of stationary phases are available in the market. The column selection is dependent on the nature and type of analyte. ODS column with dimensions 250 mm x 4.6 mm, the particle size of 5 µm selected for our analytical purpose.

Selection of Optimized Mobile Phase (MP) of HPLC Method

Optimization of Mobile Phase (MP) of HPLC is an essential variable. The interaction between the mobile phase and the stationary phase directly affects the elution of the analyse concerning symmetry and retention time.

Retention Time

The short retention time (3.5 min to 4.3) was noted due to the hydrophobicity of 5-FU as the hydrophilic mobile phase reduces the affinity of the analyte for the column. Due to the presence of a high amount of water content poor peak symmetry was observed for 5-FU. The addition of acetonitrile in the aqueous phase at pH 6.0 resulted in an improvement in peak symmetry. Therefore, different proportions of acetonitrile and water were tested in isocratic mode ranging from 10:90 (v/v) to 70:30 (v/v). A broad and tailed peak of 5-FU was observed when the ratio of water is low than acetonitrile in the mobile phase. When the water and acetonitrile were present in proportion 10:90 respectively then peak for 5-FU peak was observed between 4.3 to 4.5 min retention times.

Method Validation

Linearity and range: Good linearity was observed for both human and rabbit plasma (2-100 ng.ml^{-1}) such as $r^2 \geq 0.998$ and $r \geq 0.998$ respectively (**Figures 1 and 2**).

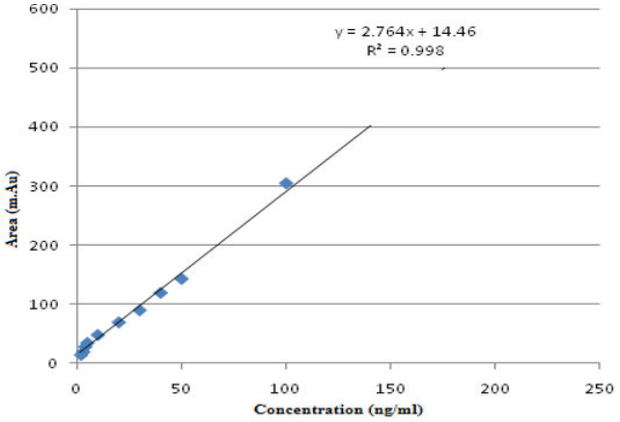

Figure 1: The linearity of the calibration curve in human plasma.

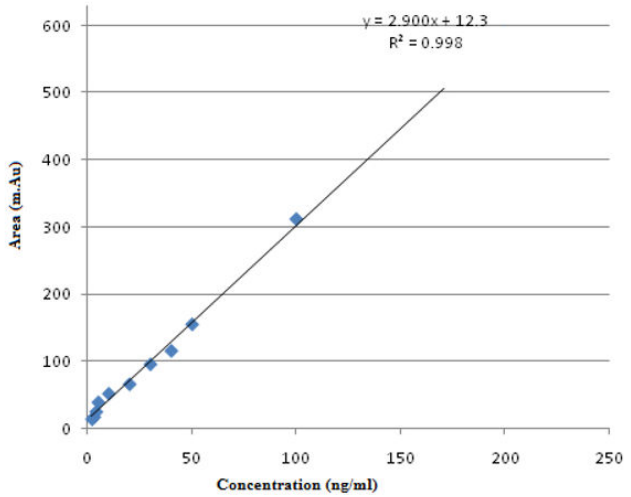

Figure 2: The linearity of the calibration curve in rabbit plasma.

LOD and LOQ

LOD and LOQ values were calculated through the regression equation using the data analysis tool excel 2016 as per ICH guidelines. LOD for human spiked plasma for 5-Fluorouracil calculated as 3.358 ng.ml^{-1} and LOD value for rabbit spiked plasma for 5-Fluorouracil calculated as 3.450 ng.ml^{-1}. LOQ of 5-Fluorouracil in human spiked plasma was determined as 10.176 ng.ml^{-1} and LOQ of 5-Fluorouracil in rabbit spiked plasma was calculated as 10.454 ng.ml^{-1}.

Accuracy and Precision

Intra day and inter day, precision and accuracy for the reverse micelle method were shown in **Tables 4-6**. The intraday percent coefficient of variance in human spiked plasma for Low Quantity Concentration (LQC), Medium Quantity Concentration (MQC), and high quantity concentration was calculated as 0.685, 1.0144 and 0.4377%, respectively, and the inter day percent coefficient of variance in human spiked plasma for Low Quantity Concentration (LQC), Medium Quantity Concentration (MQC) and High quantity concentration was calculated as 1.560, 1.11 and 1.29% respectively. The intraday percent coefficient of variance in rabbit spiked plasma for Low Quantity Concentration (LQC), Medium Quantity Concentration (MQC), and high quantity concentration was calculated as 2.254, 1.652 and 1.5146, respectively. The Inter day percent coefficient of variance in human spiked plasma for Low Quantity Concentration (LQC), Medium Quantity Concentration (MQC), and high quantity concentration was calculated as 1.786, 1.547 and 1.888%.

Table 4: Determination of LOD and LOQ for rabbit spiked plasma.

Description	Values
SD	2.813
SLOPE	2.765
LOD	3.358 ng.ml^{-1}
LOQ	10.176 ng.ml^{-1}

Table 5: Determination of LOD and LOQ for human spiked plasma.

Description	Values
SD	3.032
SLOPE	2.9
LOD	3.450 ng.ml^{-1}
LOQ	10.454 ng.ml^{-1}

Table 6: Intra day data (precision and accuracy in human spiked plasma).

Curve code	5-FU LQC 20 ng.ml^{-1}	5-FU MQC 50 ng.ml^{-1}	5-FU HQC 100 ng.ml^{-1}
Batch-01	20.236	50.078	99.987
	20.287	49.986	100.234

	20.154	49.487	99.737
Batch-02	20.124	50.145	99.88
	20.16	50.17	100.345
	19.85	50.643	100.361
Batch-03	19.978	49.234	99.965
	20.109	50.165	101.097
	20.243	49.098	100.75
Mean	20.1271	49.889	100.261
S.D	0.138	0.5061	0.4388
N	9	9	9
Spiked amount	20	50	100
% CV	0.6855	1.0144	0.4377
% Bias	0.6357	-0.2216	0.2612
% Accuracy	100.64	99.78	100.26

Accuracy

According to percent accuracy values inter day and intraday for low, medium, and high concentrations of 5-Fluorouracil in human spiked plasma were calculated as 100.64, 99.78, 100 % (inter day) and 100.38, 99.35, 98.65 (intra day), respectively. Percent accuracy values during inter day and intra day for low, medium, and high concentrations of 5-Fluorouracil in rabbit spiked plasma were calculated as 99.19, 100.17, 97.77% (inter day) and 99.22, 99.81, 100.25% (intra day), respectively (**Tables 7-9**).

Table 7: Inter day data (precision and accuracy in human spiked plasma).

Curve code	5-FU LQC 20 ng.ml^{-1}	5-FU MQC 50 ng.ml^{-1}	5-FU HQC 100 ng.ml^{-1}
1	20.131	50.25	97.098
2	20.167	49.89	99.895
3	19.878	49.976	98.458
4	20.634	48.723	99.234
5	19.865	49.324	97.213
6	19.786	49.897	99.987
Mean	20.0768	49.677	98.648
S.D	0.3132	0.5561	1.2798
N	6	6	6
Nominal	20	50	100
%CV	1.56	1.1194	1.2973
%Bias	0.3842	-0.6467	-1.3525
%Accuracy	100.38	99.35	98.65

Table 8: Intra day data (precision and accuracy in rabbit spiked plasma).

Curve code	5-FU LQC 20 ng.ml^{-1}	5-FU MQC 50 ng.ml^{-1}	5-FU HQC 100 ng.ml^{-1}
Batch-01	19.981	50.78646	102.647
	18.9177	51.20913	99.957
	19.987	49.98784	99.891
Batch-02	20.198	50.12	98.85
	20.54	48.798	99.998
	19.899	49.568	101.456
Batch-03	19.786	48.908	101.876
	19.645	49.356	99.844
	19.634	50.43	97.76
Mean	19.8434	49.907	100.253
S.D	0.4474	0.8248	1.5185
N	9	9	9
Nominal	20	50	100
%CV	2.2546	1.6528	1.5146
%Bias	-0.7829	-0.1855	0.253
%Accuracy	99.22	99.81	100.25

Table 9: Inter day data (precision and accuracy in rabbit spiked plasma).

Curve code	5-FU LQC 20 ng.ml^{-1}	5-FU MQC 50 ng.ml^{-1}	5-FU HQC 100 ng.ml^{-1}
1	20.169	50.532	98.987
2	19.789	51.254	96.098
3	19.856	49.897	96.365
4	19.936	49.654	95.876
5	19.176	50.187	99.287
6	20.098	48.987	99.984
Mean	19.8373	50.085	97.766
S.D	0.3543	0.775	1.8461
N	6	6	6
Nominal	20	50	100
%CV	1.7862	1.5475	1.8883
%Bias	-0.8133	0.1703	-2.2338
% Accuracy	99.19	100.17	97.77

Percept Recovery

5-FU was extracted from human and rabbit plasma protein by adopting a simple method using 10% v/v per chloric acid as a protein precipitating. Percent recovery of 5-Fluorouracil from human plasma (92% to 98%) was comparable with % recovery from rabbit plasma (90% to 95%). Percent recovery of 5-Fluorouracil from both plasma is summarized in **Table 10**.

Table 10: 5-Fluorouracil percentage recovered in human spiked plasma.

Concentration (ng.ml^{-1})	Amount recovered (ng.ml^{-1})	5-FU area standard (m.Au)	Spiked 5-FU area in plasma (m.Au)	Percentage recovered (%)
100	95.72672	266.774	255.37	95.8
50	45.39007	138.679	125.89	90.8
40	39.33132	120.297	118.29	98.3
30	27.69025	99.803	92.119	92.3
20	18.578	70	65.023	92.8
10	10.08854	55.34	55.83	100.8
5	4.673771	45.98	42.98	92.1
4	3.844422	39.08	37.56	96.2
3	2.831933	28.56	26.96	94.4
2	1.826934	17.45	15.94	91.5

Chromatographic Specificity

The aqueous dispersion of nano particles under gone ultracentrifugation (35000 rpm at 4°C for 15 min) and injected into HPLC and its chromatogram is represented in **Figure 3**. It was compared with HPLC chromatograph of 5-FU standard solution (supernatant layer of 5-FU) as shown in **Figure 4**. A very small peak of aqueous layer of nano particles at a retention time of 5-FU but it did not interfere with quantitative analysis of 5-FU in the formulation.

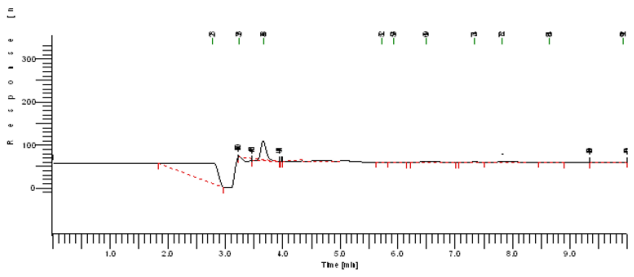

Figure 3: HPLC chromatograms of 1% (w/v) aqueous dispersion.

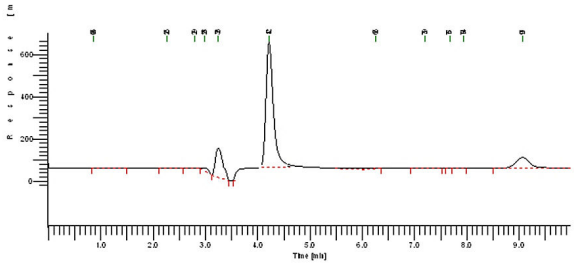

Figure 4: HPLC chromatograms of 5-FU–NPs.

DISCUSSION

Specificity

Successful separation of 5-Fluorouracil has been done from both plasma samples of human and rabbit and run time was not more than 10 minutes and 4-5 minutes was retention time of 5-FU. HPLC chromatograms analyzed 5-Fluorouracil in both human and rabbit plasma with excellent separation under chromatographic conditions as described above. HPLC chromatograms of the aqueous dispersion and spiked human and rabbit plasma have been revealed in **Figures 5 and 6**.

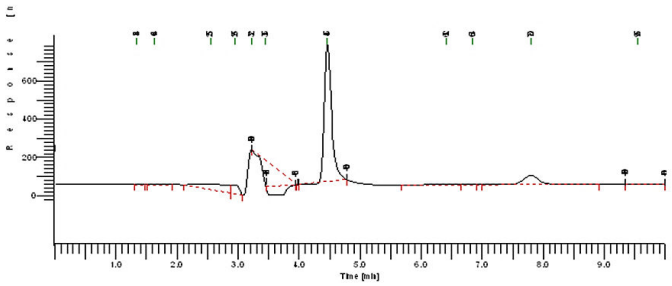

Figure 5: HPLC chromatograms of 5-FU–NPs spiked in human plasma.

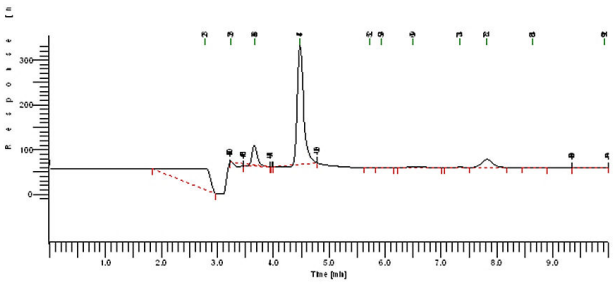

Figure 6: HPLC chromatograms of 5-FU-NPs spiked in rabbit plasma.

Robustness

The 5% intended changes in the parameters such as the flow rate, temperature, and composition of the mobile phase were made and no significant difference was observed. This indicated that the method developed is robust.

Drug Stability

Factors related to drug stability are like

- Storage environment.
- Physicochemical properties of the therapeutic agent.
- Container composition.
- Effects of another environmental stimulus (temperature, light, humidity, sunlight, etc.).

Bench top Stability Study of 5-Fluorouracil

The stability parameters are estimated through two methods *i.e*. bench top stability and long term stability. In this stability study, a freshly prepared stock solution of 5-Fluorouracil was stored at room temperature for at least six (6 hrs) hours. The spiked human and rabbit plasma aliquots such as low and high quantity concentrations were injected separately as per guidelines of bench top stability and long term stability studies. The change in quantification at LQC and HQC as % CV was calculated for both plasma samples concerning stability studies, and it was observed that the % CV of both the plasma for two concentration levels at different time intervals was less than 3% have been mentioned in **Tables 11-15**.

Table 11: 5-Fluorouracil percentage recovered in rabbit spiked plasma.

Concentration ($ng.ml^{-1}$)	Amount recovered ($ng.ml^{-1}$)	Area of 5-FU standard solution m.Au	Area of Spiked 5-FU in plasma m.Au	% Recovery
100	96.378	266.774	257.113	96.8
50	46.31	138.679	128.445	92.6
40	39.057	120.297	117.464	97.6
30	28.591	99.803	95.116	95.3
20	19.492	70.323	68.223	97.4
10	9.727	55.34	53.831	97.8
5	4.736	45.98	43.557	94.7
4	3.832	39.08	37.445	95.8
3	2.721	28.56	25.905	90.7
2	1.939	17.45	16.923	96.9

Table 12: 5-Fluorouracil bench top stability in human plasma.

Curve code	0 h		2 h		4 h		6 h	
	LQC $ng.ml^{-1}$	HQC $ng.ml^{-1}$	LQC $ng.ml^{-1}$	HQC $ng.ml^{-1}$	LQC $ng.ml^{-1}$	HQC $ng.ml^{-1}$	LQC $ng.ml^{-1}$	HQC $ng.ml^{-1}$
5-FU	20.301	202.67	19.987	201.65	20.654	199.87	19.89644	199.54
5-FU	19.965	200.67	20.135	202.15	20.498	198.99	20.2323	202.23
5-FU	19.876	201.8	20.476	200.25	20.365	201.35	20.12388	199.77
Mean	20.04	201.7	20.19	201.3	20.5	200.07	20.0842	200.89

S.D	0.2241	1.003	0.2508	0.9849	0.1447	1.1954	0.1714	1.8958
N	3	3	3	3	3	3	3	3
%CV	1.1181	0.4972	1.2415	0.4891	0.7054	0.5975	0.8535	0.9437
Nominal	20	200	20	200	20	200	20	200
% Difference	-	-	0.76	-0.18	2.29	-0.81	0.18	-0.41

Table 13: 5-Fluorouracil long term stability study in human spiked plasma.

5-FU	Week 1	Week 2	Week 3	Week 4	Mean	S.D.	%CV
LQC ng.ml^{-1}	20.03	19.976	19.998	20.709	20.17	0.36	1.78
HQC ng.ml^{-1}	199.497	200.688	200.523	198.176	199.721	1.16	0.58

Table 14: 5-Fluorouracil bench top stability in rabbit plasma.

Curve code	0 h		2 h		4 h		6 h	
	LQC ng.ml^{-1}	HQC ng.ml^{-1}	LQC ng.ml^{-1}	HQC ng.ml^{-1}	LQC ng.ml^{-1}	HQC ng.ml^{-1}	LQC ng.ml^{-1}	HQC ng.ml^{-1}
5-FU	20.134	198.87	20.487	199.798	19.984	199.531	19.86745	198.856
5-FU	19.978	197.387	20.665	202.876	20.561	201.287	20.376	198.098
5-FU	19.798	201.223	19.916	199.678	20.376	201.054	19.769	199.832
Mean	19.97	199.16	20.356	200.784	20.307	200.624	20.0042	198.477
S.D.	0.1681	1.9344	0.3914	1.8127	0.2946	0.9537	0.3258	0.536
N	3	3	3	3	3	3	3	2
%CV	0.842	0.9713	1.9225	0.9028	1.4508	0.4754	1.6285	0.27
Nominal	20	200	20	200	20	200	20	200
%Difference	-	-	1.93	0.82	1.69	0.74	0.17	-0.34

Table 15: 5-Fluorouracil long term stability in rabbit spiked plasma.

5-FU	Amount	Week 1	Week 2	Week 3	Week 4	Mean	S.D.	%CV
LQC	ng.ml^{-1}	19.47	20.054	19.376	19.807	19.67675	0.31	1.59
HQC	ng.ml^{-1}	198.107	199.43	199.87	198.69	199.0243	0.78	0.39

CONCLUSION

The precise and accurate RPH-HPLC method has been developed and validated which was found successfully applicable for quantification of 5-FU in spiked human and rabbit plasma, aqueous, and dispersion phase. The developed method can be employed for pharmacokinetic and Therapeutic Drug Monitoring (TDM) purposes. It can also be employed in pharmaceutical industries for quality control assurance between and within batches of pharmaceutical products or formulations as it is a simple, reliable, reproducible, and stable method.

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CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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