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RESEARCH ARTICLE

Development and Optimization of Fast and New Reversed-Phase HPLC Method for Analysis of 5-Fluorouracil in Human and Rabbit Plasma

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ABSTRACT

The objective was to develop and compare an accurate and reproducible HPLC method for determination of 5-fluouracil (5-FU) in rabbit and human plasma. A simple, rapid, accurate, precise and sensitive high pressure liquid chromatography method has been developed and subsequently validated for determination of 5-FU. The current method has been developed with simple mobile phase of de-ionized distilled water, pH adjusted at 3.2 with perchloric acid. An isocratic HPLC system (Agilent Technologies, 1200 series, USA) was used with variable wavelength detector and data processing software, ChemStation. Flow rate was 0.8 ml/min and 20 µl sample was injected and eluate was monitored at 260 nm wavelength. The developed method showed excellent linearity, reproducibility and sensitivity. The studied method was found robust for analysis of 5-FU in both, rabbit and human plasma samples. Run time was short (10 min) and simple extraction procedure with good response even at low drug concentration made this method suitable for pharmacokinetic and other applications. A rapid, accurate and precise high pressure liquid chromatography method was developed and validated. It is concluded that developed method is simple, cost effective, fast and reproducible for the analysis of drug concentrations, in rabbit and human plasma.

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INTRODUCTION

5-Flourouracil (5-FU) a pyrimidine analogue is widely used in various malignancies particularly in solid tumors including head and neck, colorectal, and breast cancers (Machover, 1997; Satta and Franchi, 1997). Reversed phase high performance liquid chromatography is the most common chromatographic technique that has been employed for its analysis. Less expensive and commonly available technique could facilitate in terms of expense and time in the analysis but other process parameters could not be neglected in the success of easy and inexpensive analysis.

High variability in pharmacokinetics of 5-FU has been reported previously (Desoize *et al.,* 1998; Young *et al.,* 1999). Pharmacokinetic variability is main reason for inter-patient plasma drug concentration differences. This instability in the drug concentration even after the administration of same dose under the same conditions necessitates 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 (Machover, 1997). This currently used route of administration need plasma concentration analysis. Research studies are also being conducted for oral delivery of this agent to avoid the excessive adverse effects especially in the gastrointestinal tract cancers. These novel oral drug delivery system must be evaluated on animals first and then on humans. Various liquid chromatography methods for 5-FU have been developed and validated in drug delivery systems and biological fluids (Alsarra and Alarifi, 2004; Ciccolini *et al.,* 2004; Gu *et al.,* 2010; Liu *et al.,* 2010; Lai and Guo, 2011; Li *et al.,* 2011; Rejinold *et al.,* 2011a; 2011b; Zhang *et al*., 2011; Mattos *et al.,* 2013).

In conclusion, the study focus was to develop and validate reversed-phase high performance liquid chromatography method using mobile phase having simple composition and fast sample preparation procedure. The method has been developed in human and in rabbit's plasma. The studied method could be applied

in therapeutic drug monitoring (TDM), pharmacokinetic studies and evaluation of new oral dosage forms of 5-FU in humans as well as in animals.

MATERIALS AND METHODS

Instruments and chromatographic conditions: The separation was carried out at room temperature on column of BDS hypersil C_{18} 4.6 mm x 250 mm, particle size of 5.0 µm (Thermo Electron, USA). An isocratic chromatography system of Agilent technologies series 1200 consisted of a pump and Variable Wavelength Detector (VWD) with data processing software ChemStation employed for assay. Deionized distilled water (DD Water) was used as mobile phase and pH was adjusted to 3.2 with perchloric acid solution, degassed by sonication (Elma® D-78224 Singen/Htw, Germany) and filtered. Flow rate was 0.8 ml/min. Sample of 20 µl was injected and monitored at 260 nm.

Sample preparation and analysis: The blank plasma was stored at -20°C and used for method development and validation. The study was conducted with the approval of Pharmacy Research Ethics Committee (PREC), the Islamia University of Bahawalpur. A series of drug (5-FU) solution was prepared in DD water and 500µl plasma was spiked with 50 µl drug solutions to prepare the specific concentration range. To the spiked plasma sample, 500µl of 10% v/v perchloric acid solution was added and vortexed for 10 minutes. The precipitated samples were centrifuged (centrifuge machine by EBA-20, Hettich-Germany) at 3500 rpm for 15 minutes. The clear supernatant layer was separated in neat and dried amber glass vials. The extracted drug solution (20 µl) was injected directly into the HPLC system. Two calibration curves were prepared to develop and validate the method both in human and rabbit plasma. Calibration curve prepared in human plasma from 0.05-100 µg/ml and in rabbit plasma from 0.1-100 µg/ml.

Method validation: Relative recovery of 5-FU from human and rabbit plasma was assessed by comparing the measured concentration (extracted samples) with the spiking levels. Absolute matrix effect on 5-FU in plasma of human and rabbit was calculated.

The limit of detection (LOD) was estimated as the amount of 5-FU which caused a signal that was three times the noise. The LOD was calculated using the equation;

LOD = (3N/S) x amount found (Barrett *et al.,* 2007).

Linearity, accuracy and precision: Linearity of proposed method was established by analyzing all concentration ranges as mentioned above and repeatedly injected (n=3). The peak areas were plotted against the anticipated drug concentrations. The intraday precision and accuracy were calculated by analyzing six replicates of QC samples on the same day, while inter-day precision and accuracy were evaluated by assessing each sample on six different days. Relative standard deviation (R.S.D.) was calculated to evaluate the precision and accuracy within acceptable limits. The R.S.D. for precision must be equal to or lower than 15%.

Robustness and stability of 5-FU in plasma: Chromatographic conditions were deliberately altered to investigate the effect on separation of 5-FU. Flow rate, pH of mobile phase and column age were studied to assess their effects. The stability of 5-FU was assessed by analyzing the replicates (n=6) of four dilution levels in human and rabbit plasma. The degradation effect of both biological matrixes by freeze-thaw cycles (cycle 0, cycle 1, cycle 2 and cycle 3) was analysed and all samples were stored at -20°C and thawed at room temperature in dark to protect from photo-oxidation.

RESULTS AND DISCUSSION

Optimization of method: The prerequisite of chromatographic method is to get separation of analyte of interest with good resolution, sensitivity, precision, accuracy and reproducibility. 5-fluorouracil can be assayed conveniently with HPLC-UV method (Alsarra and Alarifi, 2004). The maximum wavelength for 5-FU separation was confirmed by UV-spectrometric (UV-spectrometer 1601 Shimadzu, Japan) scan analysis of 10 µg/ml solution of 5-FU in DD water. The UV-visible scan was carried out from 500-200 nm and peak response was recorded. The maximum absorbance was recorded at 260 nm and λ_{max} was then confirmed on HPLC by analysing at reported λ_{max} values of 254 (Sinha *et al.,* 2009), 260 nm (Alsarra and Alarifi, 2004; Escoriaza *et al.,* 1999) and 268 nm (Ackland *et al.,* 1997).

In present work, analyte was eluted using various C_{18} stationary phases like hypersil ODS, hypersil BDS and Nucleosil C_{18} with different mobile phases. Mobile phases were investigated by changing ratio of components (aqueous and organic) or by using various buffers like phosphate, acetate and sulphate in pH range of 2-8. Various organic modifiers like acetonitrile, methanol and ethanol were investigated for their effect on separation of 5-FU in plasma of human and rabbit. Several mobile phases were evaluated to achieve good separation 5-FU in plasma samples. The critical problem with 5-FU chromatographic analysis was early elution of 5-FU (short retention time of 2 to 4 minutes) that caused the merger of peaks or poor resolution when spiked in human or rabbit plasma. The short retention time has been shown in some representative chromatograms as given in Figures 1 and 2. The mobile phase of DD water, pH adjusted with perchloric acid to 3.2 using column BDS hypersil C_{18} 4.6 mm x 250 mm, particle size of 5.0 µm eluted 5-FU after 6 minutes (retention time was 6.2±0.1 min). Initially the conditions were optimized on standard solution of 5-FU, simple DD water showed good response as compared to methanol: buffer and acetonitrile: buffer. The pH of DD water was adjusted with orthophosphoric acid (pH 3.2) but good resolution was obtained when pH of DD water was adjusted with perchloric acid.

Previously many researchers have developed HPLC method with mobile phases of various compositions. Sinha *et al.* (2009) developed HPLC method for 5-FU using 50mM KH_2PO_4 (pH 5.0) as mobile phase and detected at 254 nm. Wattanatorn and *et al.* (1997) reported HPLC method development of 5-FU, using 50mM KH_2PO_4 pH 3.0 (adjusted with orthophosphoric acid) at 270 nm, Escoriaza *et al.* (1999) used same mobile phase

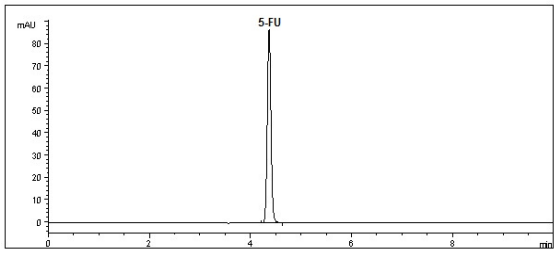

Fig. 1: Chromatogram of pure 5-FU solution in mobile phase of methanol:water (10:90) at pH 3.2 adjusted with phosphoric acid), shows the short retention time.

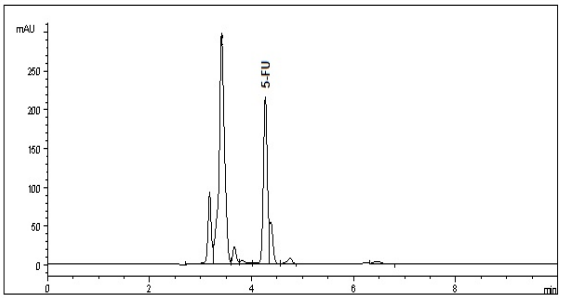

Fig. 2: Chromatogram of 5-FU of processed human plasma samples obtained by selecting the mobile phase for better resolution, short retention time and low resolution was obtained by methanol:water (10:90) pH 3.2 adjusted with phosphoric acid

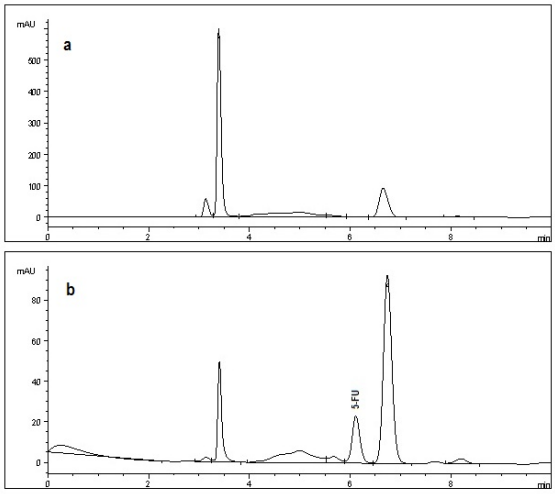

Fig. 3: Representative Chromatogram of 5-FU in human plasma (a) without drug, (b) spiked with drug (5µg/ml)

composition but detection wavelength was 266nm. Alsarra and Alarifi (2004) quantified 5-FU in human plasma by HPLC using methanol: water (10:90 v/v) at pH 3.2 (adjusted with perchloric acid) and analyzed on C_{18} column at 260 nm. A simultaneous separation of dihydrofluorouracil and 5-fluorouracil is carried out in patient's plasma samples using 1.5 mM potassium phosphate mobile phase of pH 8.0 at 268 nm (Ackland *et al.*, 1997). Recently, Mattos *et al.* (2013) developed a HPLC method for analysis of 5-FU in polymeric nanoparticles. Most of the researchers are using previously developed methods for detection of 5-FU in biological fluids and dosage forms.

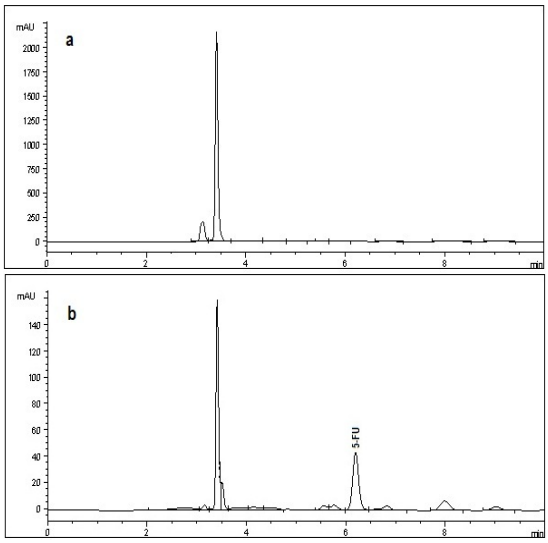

Fig. 4: Representative Chromatogram of 5-FU in rabbit plasma (a) without drug, (b) spiked with drug (5µg/ml)

Table 1: Percent recovery of 5-fluorouracil and matrix effect in human and rabbit plasma

Spiked drug conc. (µg/ml)	Recovered drug conc. (µg/ml)	% Recovery (Mean)	% Matrix effect
Human Plasma			
100	99.208	99.21	-6.222
10	9.187	91.87	-6.907
1.0	1.038	103.77	-9.179
0.25	0.236	94.44	-8.020
Rabbit Plasma			
100	98.899	98.899	-54.5883
10	9.286	92.86	-52.0647
1.0	0.923	92.30	-41.9928
0.25	0.233	93.20	-7.71826

Table 2: Calibration curves parameters of 5-FU in human and rabbit plasma

Run number	Equation Form: Y=BX+A		Correlation coefficient (r^2)
	B	A	
Human Plasma			
1	112.2	21.32	0.999
2	112.1	31.56	0.998
3	113.3	22.09	0.999
N	3	3	3
Mean±SD	112.533±0.6658	24.99±5.7028	0.9987±0.0006
Rabbit Plasma			
1	51.96	36.44	0.999
2	70.84	35.86	0.997
3	40.34	49.63	0.998
N	3	3	3
Mean±SD	54.380±15.3933	40.643±7.7881	0.998±0.0010

Table 3: LOD calculations in human and rabbit plasma

Sample code	Signal/ noise (S/N)	Drug amount Found (ng/ml)	LOD (ng/ml)
Human Plasma			
LOD-H1	8.332	70.201	25.276
LOD-H2	7.515	70.201	28.024
LOD-H3	7.112	70.201	29.612
LOD-H4	8.912	70.201	23.631
LOD-H5	9.221	70.201	22.839
LOD-H6	10.828	70.201	19.450
	Mean LOD Value		24.806±3.677
Rabbit Plasma			
LOD-R1	5.332	110.61	62.234
LOD-R2	6.515	110.61	50.933
LOD-R3	6.112	110.61	54.292
LOD-R4	7.912	110.61	41.940
LOD-R5	6.221	110.61	53.340
LOD-R6	8.828	110.61	37.588
	Mean LOD Value		50.055±8.934

Table 4: Intra-day and inter-day accuracy and precision

Theoretical Conc.	Intra-day			Inter-day		
	Conc. found (µg/ml)	Accuracy (%)	R.S.D. (%)	Conc. Found (µg/ml)	Accuracy (%)	R.S.D. (%)
Human Plasma						
100	99.221±1.021	99.22	1.029	98.907±1.078	98.91	1.0899
10	9.527±0.107	95.27	1.123	9.434±0.118	94.34	1.251
1.0	0.911±0.0118	91.10	1.295	0.916±0.012	91.60	1.310
0.25	0.228±0.004	91.20	1.754	0.231±0.005	92.40	2.164
Rabbit Plasma						
100	97.818±1.101	97.82	1.125	96.982±1.121	96.98	1.156
10	9.383±0.112	93.83	1.194	9.212±0.148	92.12	1.606
1.0	0.922±0.013	92.20	1.409	0.908±0.019	90.80	2.092
0.25	0.233±0.003	93.20	1.287	0.229±0.007	91.60	3.057

n=6 (Intra-day), n=36 (Inter-day). Values are mean±SD.

Table 5: Freeze-thaw stability of 5-FU in human and rabbit plasma

Freeze-thaw cycles	Stability in Human Plasma(thawed the samples after 48hrs)			
	Drug conc. spiked	Drug conc. found (mean±SD)	% RSD	% Difference
Cycle 0	100	99.311±1.033	1.040	0.00
	10	9.618±0.103	1.071	0.00
	1.0	0.942±0.0121	1.284	0.00
	0.25	0.236±0.003	1.271	0.00
Cycle 1	100	98.899±1.112	1.124	-0.412
	10	9.588±0.109	1.137	-0.030
	1.0	0.948±0.0127	1.339	-0.006
	0.25	0.228±0.004	1.754	-0.008
Cycle 2	100	98.549±1.122	1.138	-0.762
	10	9.501±0.113	1.189	-0.117
	1.0	0.917±0.0133	1.450	-0.025
	0.25	0.222±0.004	1.802	-0.014
Cycle 3	100	98.445±1.135	1.153	-0.866
	10	9.471±0.138	1.457	-0.147
	1.0	0.910±0.021	2.308	-0.032
	0.25	0.216±0.005	2.315	-0.020
		Stability in Rabbit Plasma		
Cycle 0	100	98.118±1.112	1.133	0.00
	10	9.412±0.108	1.147	0.00
	1.0	0.931±0.012	1.288	0.00
	0.25	0.237±0.003	1.266	0.00
Cycle 1	100	97.818±1.217	1.244	-0.300
	10	9.399±0.127	1.351	-0.013
	1.0	0.922±0.014	1.518	-0.009
	0.25	0.228±0.005	2.193	-0.012
Cycle 2	100	97.119±1.331	1.370	-0.999
	10	9.312±0.141	1.514	-0.100
	1.0	0.917±0.021	2.290	-0.014
	0.25	0.223±0.007	3.139	-0.014
Cycle 3	100	96.886±1.661	1.714	-1.232
	10	9.221±0.160	1.735	-0.191
	1.0	0.903±0.033	3.654	-0.028
	0.25	0.215±0.009	4.186	-0.022

Recovery and matrix effect: The extraction of 5-FUfrom plasma samples was optimized keeping in view the minimum processing steps, simple and reproducible. The extraction was carried out simply with perchloric acid as precipitant and investigated for column pressure, accuracy and recovery. Recovery of 5-FU from human and rabbit plasma has been compared to evaluate its effect on response. Recovery from human plasma (91 to 103%) was comparable with recovery from rabbit plasma (92 to 98%). Absolute matrix effect was assessed for comparative analysis. Human plasma decreased peak area of 5-FU up to -8.020 % and rabbit plasma effect the peak area up to -54.588 %. Rabbit plasma showed a significant decrease in response. Difference in plasma compositions of human and rabbit affected the response differently. 5-FU recovery and matrix effect are summarized in Table 1. Several studies on HPLC method and validation of 5-fluorouracil in various biological fluids have reported different extracting agents like trichloroacetic acid and ethyl acetate at pH 8.0 (Ackland *et al.,* 1997), ammonium sulphate (plasma protein precipitant) with isopropanol: ethyl acetate mixture (15:85) (Peer *et al.,* 2012), ethyl acetate (Alsarra and Alarifi, 2004), n-propanol diethyl ether (Escoriaza *et al.,* 1999). The developed method was robust for quantification of 5-FU in both biological fluids (human and rabbit plasma). The representative chromatograms of blank and spiked plasma of human and rabbit have been shown in Figures 3 and 4.

Linearity, LODs and LOQs: Calibration curves of human and rabbit plasma showed good linearity ($r^2 \geq 0.9987$ and $r^2 \geq 0.998$, respectively) over the specified concentration range. The regression correlation equations for calibration curves and their regression coefficients are summarized in Table 2. The LOD was established at 24.8 ng/ml for 5-FU in human plasma and 50 ng/ml in rabbit plasma. While LOQ values of 5-FU were found 40 ng/ml in human and 80 ng/ml in rabbit plasma with acceptable accuracy. LOD calculations are presented in Table 3.

Accuracy and precision: The % RSD of 5-FU as precision of the studied bio-analytical method was 1.029 to 1.754% (Intra-day) and 1.089 to 2.164% (Inter-day) in human plasma while 1.125 to 1.287% (Intra-day) and 1.156 to 3.057% (Inter-day) was calculated in rabbit plasma. The values confirmed the good precision of developed method both in human and rabbit plasma matrix systems, values presented in Table 4. More than 90% accuracy was found in both biological fluids, shown in Table 4. Good accuracy of current method in same and different days confirmed reproducibility and authenticated the method attributes in biological samples.

Stability: The stability findings in both biological fluids revealed that no significant degradation occurred in freeze-thaw cycles (-20°C to room temperature). After third cycle -0.866% degradation in human plasma and -1.232% decrease in rabbit plasma was observed at 100 µg/ml 5-FU concentration, decrease in concentration at various levels have been summarized in Table 5. The stability of 5-FU in the developed method has been found in agreement with previously reported stability studies. Alsarra and Alarifi, (2004) performed stability studies by freeze-thaw cycles of 72 hours at -20°C and less than 7.0% change in 5-FU concentration has been reported. Ackland *et al.* (1997) conducted stability studies on dihydrofluorouracil and 5-fluorouracil in various mediums and observed that 5-fluorourcil was stable when stored at -20°C. Peer *et al.* (2012) assessed autosampler stability of

5-FU at 4°C after 24 hours and found only 1% change in concentration. Jung *et al.* (1997) studies also confirm the stability of 5-FU, results showed that 5-FU remained stable for 2-3 weeks and can be stored at 4°C.

Conclusion: In present study, simple processing (simple mobile phase and sample preparation method) and validation in human as well as in rabbit plasma would enhance applicability of developed method in routine analysis of 5-FU and preclinical/clinical analysis (Pharmacokinetic and drug monitoring) in humans and animals as well.

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Author's contribution: All the authors have substantial contribution in completion of this study. MUM developed the method, validated and wrote the manuscript. MA supervised the whole study and critically reviewed the manuscript. MS and FS assisted in sampling and analysis.

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