DEVELOPMENT AND VALIDATION OF RAPID AND SENSITIVE HPLC METHOD FOR THE DETERMINATION OF 5-FLUOROURACIL IN HUMAN SERUM

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Summary

This study describes a simple and fast high-performance liquid chromatography method for the determination of 5-Fluorouracil in serum. Samples were collected from adult cancer patients receiving high dose 5-Fluorouracil at Mahathma Gandhi Memorial hospital (Warangal, AP.India) at various time intervals after the end of each infusion. Serum was deproteinized with trichloroacetic acid and the supernatant was injected into a 250×4.6 mm octadecylsilane column. Mobile phase was methanol: water (10:90) with a flow rate of 1ml/min. Ultraviolet detection was done at 313 nm and at ambient temperature. Para aminoacetophenone was used as internal standard. 5-Fu and internal standard [thymine] retention times were 4.6 and 9.5 minutes, respectively. Results showed that reproducibility (precision) of method within a day was 98 to 99.8 percent and between days was 95.6 to 99.7 percent. The recovery of the method was between 88.6 and 99.7 percent. The quantitation limit of the method for 5-Fu was 10µM. This method is suitable for quantitation of 5-Fu after infusion of high doses of this drug and has good accuracy, precision and quantitation limit.

Key Words: 5-Fu; HPLC; Serum Concentration.

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Introduction

5-Fluorouracil (5-Fu) is a commonly used antineoplastic agent and frequently used for the treatment of Colon cancer [1, 2]. Several analytical methods have been previously reported for the assay of 5-Fu in biological fluids, including those methods that were based on solid phase extraction [3], gas chromatography (GC) [4], gas chromatography combined with mass spectroscopy (GC–MS) [5], liquid–liquid extraction using four serial columns [6], and LC–MS/MS [7]. These methods required high sophisticated equipment and are not amenable to rapid and routine clinical assay [8]. The described techniques needed a relatively large plasma volume (2.0 ml) which may not be always available, and involving tedious extraction and derivatization steps. A successful analytical technique should be cost saving and easily available in most laboratories with minimal tedious work. Therefore, the aim of this present study was to describe a simple, fast, accurate and precise method for the determination of 5-fu in serum for pharmacokinetic studies and routine therapeutic drug monitoring in high-dose intravenous infusion of this drug.

Materials & Methods

Chemicals: 5-Fu (purity > 99%) was a kind gift sample of Dabur pharmaceuticals ltd [Delhi]. Thymine used as the internal standard purchased from SD Fine chemicals ltd [Mumbai]. Methanol and ethyl acetate were of HPLC grade and were obtained from Merck Laboratory Supplies (Germany). Distilled and deionized water was obtained by passage through ELGA® (a trade name of Vivendi Water Systems Ltd.,Wycombe, Bucks, UK).Stock solutions were prepared by dissolving the compounds in water. The standard solutions were prepared every day, stored in the dark and refrigerated.

Chromatographic conditions: A Schimadzu liquid chromatography system equipped with a LT 10AT VP pump, a SPD 10A VP variable wavelength UVvisible spectrophotometric detector and a Rheodyne 20 microliter loop injector system was used(Schimadzu, Kyoto, Japan). INERTSIL ODS-3V C-18, 4.6x250mm [GL sciences Inc, Japan] chromatography column was used for analysis.

The mobile phase consisted methanol: water, with the ratio of 10:90, respectively. The flow rate was 1ml/minute and the eluent was monitored spectrophotometrically at 260nm at room temperature.

Solutions of external and internal standards: A stock standard solution of 5-Fu (10000µM/ml) was prepared using water. A stock solution of internal standard (Thymine) was also prepared in the same solvent at the concentrations of 10µg/ml. Further dilution of 5-Fu stock solution was done

with drug free plasma to prepare different concentrations of 5-fu (10, 50,100,200,400 and 500µM/ml).

Sample collection and preparation: Received 5-fu at doses of 500mg/m2 as 4 or 6 hours small infusions, as part of protocols for the treatment of various cancer diseases at Mahathma Gandhi memorial [MGM] Hospital (Kakatiya medical college, Warangal, India). Blood samples were collected at various times after the end of each infusion. Different concentrations of 5-Fu were first added to 50µl of thawed serum samples which were vortex-mixed for 2 min. Then, 50 µl of 10 µg/ml internal standard was added. A solution of 1ml of ethyl acetate, as an extracted solvent, was added to the serum samples. Samples were vortex-mixed for 7 min and then centrifuged (4000 g, 10 min) (Jouan, GR 412, Saint Mazaire Cedex, France). The supernatant was collected and the organic extraction process was repeated collecting organic supernatant into the same glass tube. Samples were evaporated by heating on water bath and reconstituted in 200µl of water, vertex mixed. 10 or 20µL aliquots of the supernatant was directly injected into the chromatography column. Each sample was analyzed in duplicate. All samples or standard solutions were stored at –80ºC until analyzed.

Recovery and precision: The recovery was studied by preparation of the various amounts of 5-Fu in blank plasma (spiked blank). 5-Fu was determined according to the described method. The recovery was calculated by comparison of the found amounts with the added ones.

Results

Under the conditions used for the chromatography, the retention times for 5-fu and the internal standard were 5.4±0.03 and 7.5±0.98 minutes, respectively. Figure 1and 2 shows the chromatograms of human blank serum used for preparation of different concentrations of 5-fu standard solution (2) and patients samples (1 and 9) withdrawn from patients who received 500mg/sqm of 5-fu as a short infusion.. The chromatographic condition employed was quite specific for 5-fu and thymine [Fig 3 & 4]. Other drugs that might be administered concomitantly with 5-fu such as, cyclophosphamide, and methotrexate could not interfere with 5-fu Peaks because either they have no significant absorption at 260 nm or their retention times are quite different. This fact was proved in this study by adding each of these drugs to plasma samples containing methotrexate and analyzed by chromatography.

Fig 1 Human Blank serum

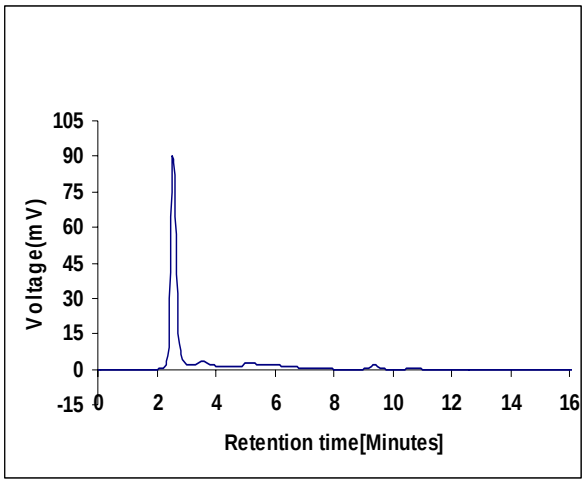


Fig 2 Serum sample spiked with 50 µM/ml 5-Fu and Thymine [Internal standard]

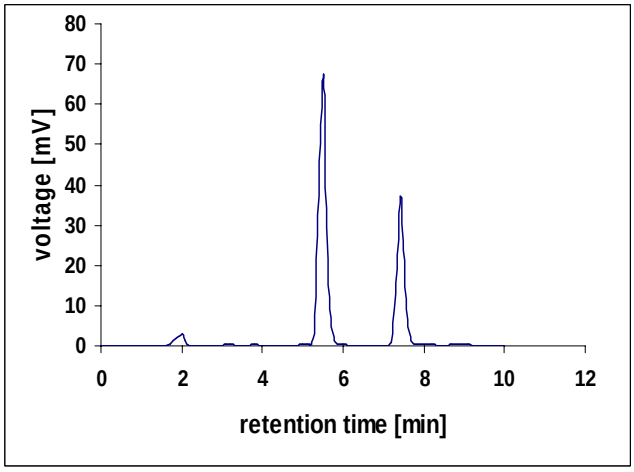


Fig 3 5-Fu and Thymine in patient serum

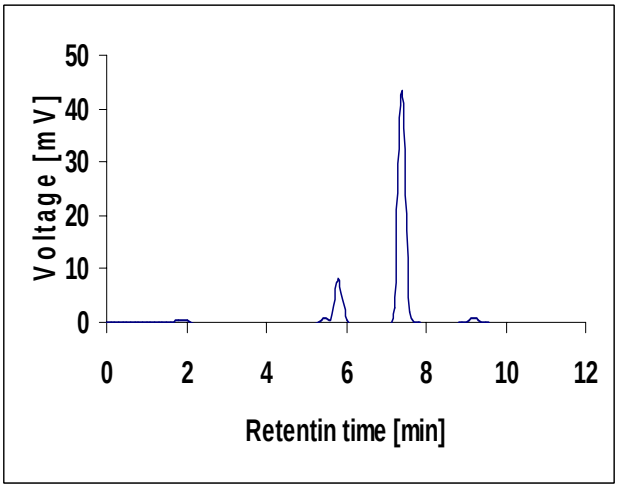


Fig 4 5-Fu and Thymine in patient serum sample, at 30 min after 4h infusion sample, at 10 min after 4h infusion

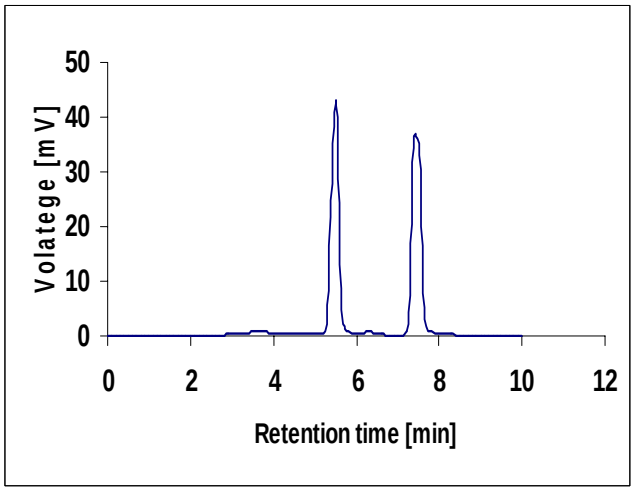
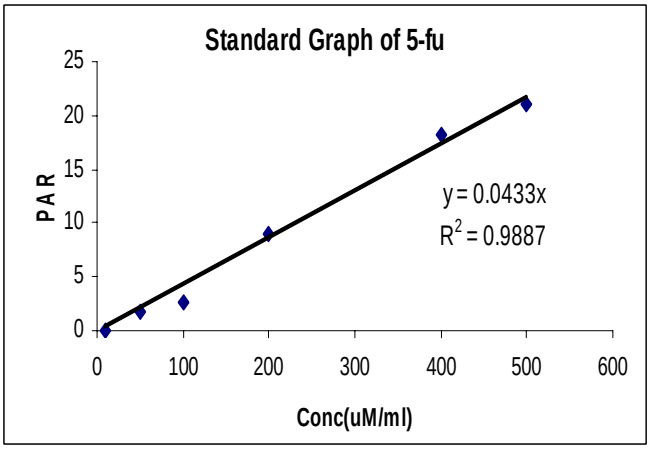


Fig 5 Linear standard curve determination of 5-Fu in serum (concentration range 5-70 µM/ml)



In order to determine plasma concentration of 5-fu, internal standardization method was used. After preparation of various concentrations of 5-fu and analyzing chromatography each standard solution, two standard curves were prepared by plotting the ratio of peak area of 5-fu to internal standard (thymine) versus concentration of 5-fu. A good linearity was seen in the standard curve of 5-fu (Figure 5). To assess the accuracy of the method, recovery of 5-fu from serum samples with known concentrations was compared with the solutions of 5-fu at the same concentrations as shown in Table 1. For the assessment of method precision, reproducibility of the results obtained for different concentrations of 5-fu was determined at 5 different days and 5 times in one day. The results of reproducibility study are shown in Table 2 as recovery and accuracy determination.

The limit of quantitation of 5-fu in serum with the above sample pretreatment method was 10µM. The precision of method within a day was 98 to 99.8 percent and between days was 95.6 to 99.7 percent. The recovery of the method was between 88.6 and 99.7 percent.

Table 1

Spiked concentration (µM/ml)	Day	Measured Concentration		
		Mean(µM/ml)	SD	RSD
Inter-day variation				
10	1	7.56	0.376	4.973545
	2	9.88	0.100	1.012146
	3	8.23	0.096	1.166464
	4	9.79	0.030	0.306435
100	1	98.40	0.650	0.660569
	2	99.31	0.120	0.120834
	3	99.04	0.050	0.050485
	4	97.97	0.960	0.979892
500	1	497.74	0.960	0.192872
	2	499.05	0.069	0.013826
	3	498.75	0.870	0.174436
	4	498.65	0.050	0.010027
Intra-day variation				
10		9.80	0.15	1.530612
100		99.18	0.97	0.97802
500		499.14	0.12	0.024041

SD: Standard deviation, RSD: Relative standard deviation and mean value of 4 determinations

Table 2 Assessment of Recovery and Accuracy determination of 5-Fu in human serum

Substance	Concentration	Recovery (%) Mean±SD	Accuracy (%) Mean±SD
5-FU	10(µM)	88.6±11.5	100.06±1.9
5-FU	100(µM)	98.6±0.6	97.7±1.07
5-FU	500(µM)	99.7±0.11	99.8±.77
Internal Standard	10(µM/ml)	95.95±1.52	96.05±1.09

SD: Standard deviation and mean value of 5 determinations

Discussion

Various methods of high-performance liquid chromatography for the determination of 5-fu in biological fluids have been described so far which differ in chromatography type (reverse phase or ion-pair chromatography) or detection system (UV or fluorescence) (6-8). Reverse-phase high performance liquid chromatography with UV detection has been most recommended. (10, 11). But, an important point is that most of them are tedious and expensive because they use more material and have many stages of experiment. Besides, they are not suitable for a routine and quick therapeutic drug monitoring (TDM) test which is necessary for a child cancer patient in a high dose infusion therapy at hospital, or reference laboratories.

In this article a simple and fast method for the determination of 5-fu in serum is described that has equal precision and accuracy to other similar methods (3-5,7-8). A full chromatography takes 10 minutes. To include sample preparation time it may need 25 minutes for the whole of each analysis, which is comparatively a short time. The short duration of assay time is of quite importance in routine monitoring of the drug in serum to predict and prevent future toxicity in high-dose 5-fu intravenous infusion. On the other hand this method has a satisfactory quantitation limit that makes it ideal for pharmacokinetic studies and therapeutic drug monitoring of 5-fu after the administration of high doses of this drug. To improve the quantitation limit further we could use solid phase extraction technique along with fluorescence detection after post column derivitization of the 5-fu to fluorescent compounds so that the method become suitable for determination of 5-fu after the administration of lower doses of the drug but such methods are more tedious, time consuming and expensive.

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