Original Article

TIPS Trends in Pharmaceutical Sciences 2017: 3(1): 49-54.

A reversed-phase high performance liquid chromatography approach for analysis of 5-Fluorouracil

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Abstract

A reproducible, sensitive, accurate and selective high performance liquid chromatographic (HPLC) method with ultraviolet absorbance detection has been developed and validated to quantitate 5-Fluorouracil (5-FU), an antimetabolite chemotherapeutic agent used in treatment of colorectal cancer as a drug of choice. Chromatographic separations were performed on a C18 column (Eurosphar 100-5, 150 mm × 4.6 mm) as the reversed stationary phase, eluted with a mobile phase composed of deionized water and methanol (95:5) with the flow rate of 1.0 mL/min. The UV wavelength was set at 261nm. The method produced linear responses throughout 5-fluorouracil range of 25-500 ng/ml with a correlation coefficient of 0.998. A limit of quantitation (LOQ) was established at 25 ng/ml. The within-day and between-day precision and accuracy were both in acceptable ranges. The outcomes of these tests indicate a proper separation efficacy as well as accuracy and sensivity of method which is a potential tool in many pharmaceutical studies such as drug delivery of this anti-cancer agent.

Keywords: 5-fluorouracil, Analysis, High performance liquid chromatography (HPLC).

1. Introduction

5-fluorouracil (5-Fu) [C4H3FN2O2/ 5-fluoro-2,4(1H,3H)-Pyrimidinedione] (1) is a chemotherapeutic agent classified as an "antimetabolite". Antimetabolites are similar enough to a normal biomolecule to be recognized by cells but different enough to interfere with the normal division and cell's function. 5-Fu belongs to category of pyrimidine analogue which inhibits thymidylate synthase, the rate-limiting enzyme in the pyrimidine nucleotide synthesis. It is a prodrug and would be subjected to a complex series of biotransformation reactions to ribosyl and deoxyribosyl nucleotide metabolites. One of these metabolites, 5-fluorodeoxyuridine monophosphate (FdMTP), binds to thymidylate synthase to form a covalently bound ternary complex in presence of reduced folate (5,10N-methylenetetrahydrofolate) as a methyl donor. This action inhibits the formation of thymidylate from uracil, results in less DNA replication. In addition, other metabolites, 5-fluorodeoxyuridine triphosphate (FdUTP) and 5- fluorouridine triphosphate (FUTP) can be incorporated into DNA and RNA molecules respectively in place of correct nucleotide produces defected DNA and RNA molecules leading to irregular nucleic acid function and protein synthesis (2, 3). 5-Fu has been widely used in the treatment of many disorders and still remains as an essential regimen in various malignancies including colorectal cancer, breast cancer, cancer of head and neck, ovarian cancer and pancreatic cancer (4-6). The pharmacokinetics and metabolism of 5-FU have been extensively investigated. Fluorouracil is usually given intravenously and has a short metabolic half-life of 15 minutes (2). Although, the major portion is degraded in the liver, to inactive metabolites, it continues to be

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Recieved: 14/02/2017; Accepted: 25/02/2017

the drug of choice in treatment of colorectal cancer (1) and has been administrated in high dose, to increase the concentration at targeted site (5). The oral administration is not recommended because the presence of high levels of the catabolic enzyme dihydropyrimidine dehydrogenase in the gut mucosa causes erratic bioavailability (2). High dose IV infusion of this antimetabolite caused different and undesirable side effects including anorexia, nausea, vomiting, diarrhea, GI ulcer and bleeding, leukopenia (principally granulocytopenia), thrombocytopenia, alopecia, dermatitis (principally pruritic maculopapular rash) (1, 5). In recent years the novel drug delivery systems have significantly been developed not only to minimize systemic side effects but also would provide a promising colorectal cancer therapy with reduced dose and duration of treatment (7). By reduction in dose and for more reliable pharmacokinetic studies, development of a selective as well as sensitive analysis method for drug assay in biological medium is a crucial step. In the present study a reproducible, sensitive, selective and accurate high performance liquid chromatography with ultraviolet detector (HPLC-UV) method has been developed and evaluated for detection and assay of 5-FU.

2. Materials and methods

2.1. Materials

5-fluorouracil (Ebeve, Austria) donated by Kavosh Gostar Darou Co. (Tehran, Iran), was purchased as injectable form (50 mg/ml). All other chemicals, solvents, and reagents with chemical or analytical grade were obtained locally. All water used in the study was filtered and deionized by Milli-Q-UF system (Millipore, Milford,MA,USA).

2.2. HPLC apparatus and conditions

The high performance liquid chromatography system consists of pump-controller unit (Knauer, Wellchrom®, model k-1001, Berlin, Germany) and a Rheodyne injector (Rheodyne, Model 7725, USA) equipped with a 100 µl loop for solvent delivery and sample injection respectively. Separation was achieved by isocratic elution with a mobile phase of water: methanol (95:5 v/v), delivered at a flow-rate of 1.0 ml/min through a C18 column (Eurospher 100-5 C18, 150 mm × 4.6 mm, Knauer, Germany) as the stationary phase. Chromatographic pattern was recorded by UV detector (Knauer, model k-2600, Berlin, Germany) at the wavelength of 261nm. The chromatograms were analyzed using compatible software (EZChrom Elite®, Germany). A complete series of analytical method validation tests were carried out via the developed HPLC method.

2.3. Standard preparation

The standard solutions were prepared with the following concentration of 10, 25, 50, 100, 200, 300, 400, 500 ng/ml of 5-FU in deionized water. After 5 minutes' vortex mixing, 100 µl of sample was injected to chromatography system.

2.4. System suitability tests

System suitability tests are used to verify the acceptable performance of chromatography method. The following parameters are documented as system suitability test:

Number of theoretical plates (N) indicates the column efficiency and is calculated by:

$$5.54\left(t_R / W_{h/2}\right)^2 \qquad \text{(Eq. 1)}$$

Peak symmetry or tailing factor is calculated by:

$$W / 2f \qquad \text{(Eq. 2)}$$

Retainability (k') also known as capacity factor is calculated by:

$$\left(t_R / t_a\right) - 1 \qquad \text{(Eq. 3)}$$

Where, t_R stands for the peak retention time, W is the peak width at 0.05 peak height, W h/2 is the peak width at 0.5 peak height, f indicates the front half-width of the peak at 0.05 peak height and ta is considered as the retention time of non-retained peak (solvent peak) (8).

2.5. Analysis validation tests

During the method development, typical characteristics including selectivity, linearity, limits of detection (LOD) and limits of quantitation (LOQ), range, accuracy, and precision should be evaluated to provide a high degree assurance of method (9).

2.5.1. Selectivity

To determine the selectivity of method different samples containing target analyte are analyzed to discover the method power in analyte separation and any interferences from possible degraded ingredients (10).

2.5.2. Limits of detection and quantitation

Limit of quantitation is the lowest 5-Fu concentration can be quantitatively determined with acceptable precision and accuracy (20% and 80-120%). Limit of detection is the lowest 5-Fu concentration can be detected but not quantitate as an exact value. It is determined based on Signal-to-Noise ratio of 3(11).

2.5.3. Linearity

Standard solutions of 5-Fu ranging from 25ng/ml to 500ng/ml were prepared as previously described. Separate samples at each concentration were investigated in triplicate. Linear regression was analyzed based on drug peak AUC versus related concentration.

2.5.4 Precision

2.5.4.1. Within-day variations

Within day variations were based on analysis of three replicates of low, medium and high concentration of 5-Fu in same day. The result were expressed as coefficient of variations (CV %) measurement for each case.

2.5.4.2. Between-day variations

Three replicates of each concentration of upper, medium and lower range of calibration curve were prepared and assayed on three different days. The CV% was calculated for each case.

2.5.5 Absolute recovery (accuracy)

For calculation of absolute recoveries of samples investigated in within and between day variations, 5-Fu concentration of each sample was measured by linear regression obtained from the analytical standard curve. Then the absolute recovery was determined by comparing the measured concentration with the nominal standard ones.

3. Results and Discussion

3.1. Drug assay

The aim of this study was developed a simple, reliable and applicable reversed phase HPLC for determination of 5-FU throughout the further researches. The HPLC detection of 5-Fu was isocratically examined by using Eurospher C18 analytical column with the mobile phase of water: methanol (95:5 v/v). Retention time was 5 minutes approximately, at the wavelength of 261 nm.

3.1.1. System suitability tests

The results of system suitability studies verified the chromatographic suitability by proper values of tailing factor, column efficiency and peak resolution. The number of theoretical plates (N), peak symmetry, and retentability (k') of the method were 4937, 0.92 and 2.62 respectively.

3.1.2. Analysis validation tests

Analysis validation tests are a series of processes demonstrating that an analytical determination is valid enough for its intended purpose. The assay was validated in terms of selectivity, linearity, LOD and LOQ, precision and absolute recovery. The linearity of the method was determined at concentration levels ranging from 25 to 500 ng/ml. The within and between day variations produced an acceptable degree of reproducibility for the method.

3.1.2.1. Specificity and selectivity

Selectivity of this analytical method was evaluated for aqueous 5-Fu solutions. The selectivity of a method indicates its ability to identify the analyte of interest from all other chemical interferences. As shown in figure 1, chromatogram produced acceptable resolution and no significant interferences with possible degraded ingredient at used wavelength.

3.1.2.2. Limit of detection and quantitation

The limits of detection (LOD) and quantitation (LOQ) of the method were determined as value of 10 ng/ml and 25 ng/ml, respectively.

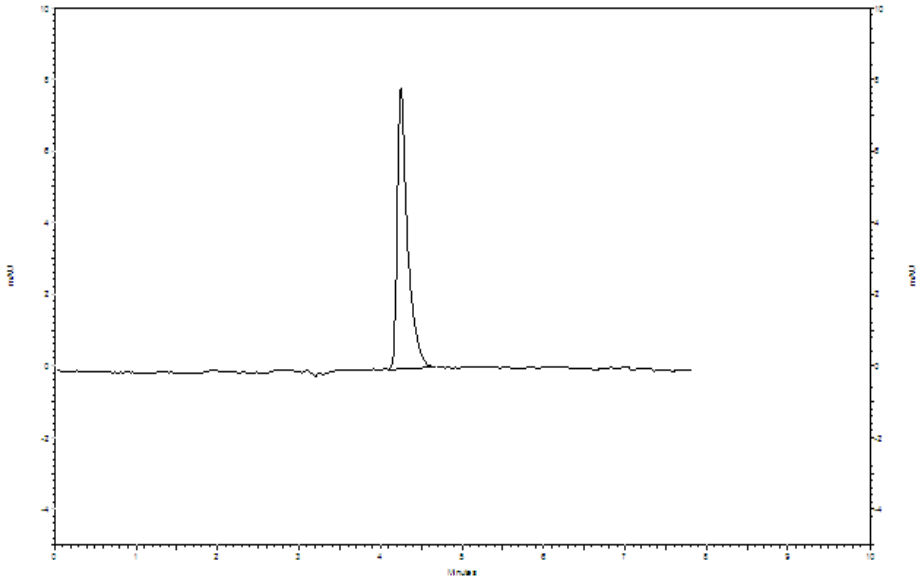

Figure 1. A typical HPLC chromatogram of 5-fluorouracil in concentration of 500 ng/ml.

3.1.2.3. Linearity

Linear response was observed by regression analysis obtained from plotting drug concentration against the peak area ratio over the concentrations ranging from 25 to 500 ng/ml. The regression coefficient was 0.998 and standard curve is given in Figure 2.

3.1.2.4. Precision

The values obtained during the within and between day validation summarized in Table 1.

3.1.2.5. Absolute recovery (Accuracy)

The within- and between-day absolute recovery values of the method are shown in Table 1.

4. Conclusion

Accurate and precise analysis of 5-Fu concentration is a vital step in follow up the colorectal cancer treatment. The present study has validated an accurate, precise, reproducible and Linear reversed high performance liquid chromatographic method for determination of 5-fu concentration. This validated technique selectively identifies 5-Fu in short run time and without any medium interferences and the sensitivity of assay is sufficient enough to detect the low concentration.

It seems that the described separation has

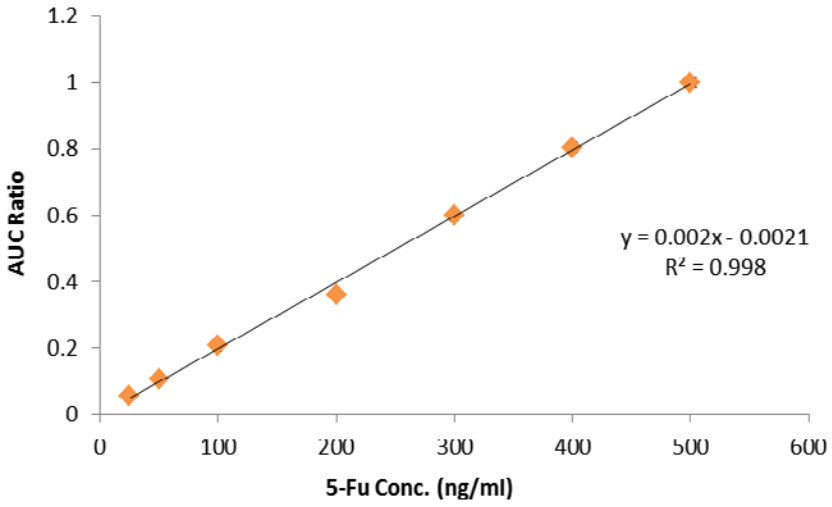

Figure 2. Calibration curves of aqueous solutions of 5-Fu (n=3).

Table 1. Within and between day variations of the assay method for quantitation of 5-fluorouracil (n=3).

5-Fu concentration (ng/ml)	Within day variations		Between day variations	
	CV%	Accuracy% Mean±SD	CV%	Accuracy Mean±SD
25	1.52	102.49±1.56	3.63	105.59±3.83
50	0.87	113.85±0.99	1.63	112.15±1.82
100	2.66	104.18±2.77	1.15	101.83±1.17
200	1.84	100.99±1.86	5.41	108.72±5.88
300	5.60	101.62±5.69	4.47	99.78±4.46
400	1.86	100.75±1.88	3.14	101.70±3.19
500	4.77	104.83±5.00	6.55	101.42±6.64

an optimum potency to be used for further pharmaceutical and pharmacokinetics study.

Acknowledgements

This study was part of a Pharm D thesis supported by Shiraz University of Medical Sciences. (SUMS; Grant no. 93-01-05-8066).

Conflict of Interest

None declared.

5. References

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