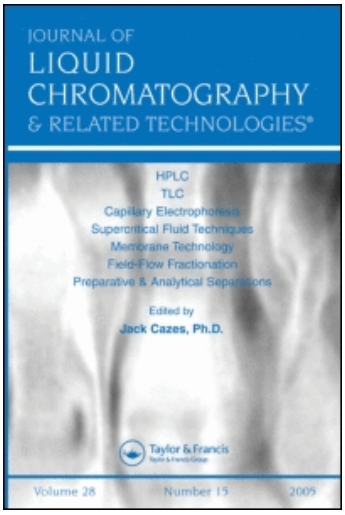

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HPLC ANALYSIS AND VALIDATION OF 5-FLUOROURACIL AND ITS METABOLITES IN RAT PLASMA

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ABSTRACT

An isocratic, reverse phase HPLC method, for the simultaneous determination of 5-fluorouracil (5-Fu), 5-fluoro-5,6-dihydrouracil (FuDH), and 5-fluorouridine (Furd), in rat plasma has been developed. The method utilizes a Spherisorb® column and a mobile phase composition of 100 μM phosphate buffer at pH 3.0. The extraction procedure from the plasma samples has been optimized to give maximum recovery of the analytes. The method has been validated as per USP guidelines in terms of linearity, accuracy and precision. The limit of quantitation for 5-Fu, FuDH, and Furd was found to be 0.2 μg/mL, 0.3 μg/mL and 0.8 μg/mL respectively.

INTRODUCTION

5-Fluorouracil (5-Fu) is an antimetabolite used in the treatment of carcinomas of the gastrointestinal tract and breast.[1,2] The biochemical importance of 5-Fu and its nucleosides has been demonstrated by Heidelberger.[3] Analysis of 5-Fu and its metabolites in biological fluid is, therefore, an imperative parameter not only in pharmacokinetic and pharmacodynamic studies but also during the development of conventional as well as targeted dosage forms of the drug. Reverse phase,[4,5] reverse phase ion-pair[6-8] and normal phase[9] HPLC systems were described for the analysis of 5-Fu. However, few workers have reported the simultaneous analysis of 5-Fu and its metabolites.

The present investigation deals with the development of an HPLC assay procedure for the simultaneous analysis of 5-Fu and its metabolites, 5-fluoro-5,6-dihydrouracil (FuDH) and 5-fluorouridine (Furd), in rat plasma. Optimization of any extraction procedure, in an analytical method that involves quantitation of a number of analytes, is an important step. As 5-Fu and its metabolites were required to be extracted simultaneously, an optimized procedure that would give maximum recovery of the compounds of interest became an obvious necessity in the present study. In order to achieve maximum recovery of the analytes, an ideal extraction procedure was developed by investigating the choice of a suitable extractant system, pH of the redissolving buffer solution and nature of the pH adjusting system required to minimize the co-extraction of plasma matrix components. Precision, accuracy and linearity were the validation parameters addressed for the proposed method and were in accordance with the requirements of Current Good Manufacturing Practice (cGMP) regulations [21 CFR 211.1949a)].[10] Both, Category I and II assays, defined by USP XXII, have been adhered to for compendial compatibility.

MATERIALS AND METHODS

Chemicals and Reagents

5-Fluorouracil (5-Fu), 5-fluoro-5,6-dihydrouracil (FuDH), 5-fluorouridine (Furd) and 5-fluorocytosine (5-Fc) were purchased from Sigma (St. Louis, MO, USA). Ammonium dihydrogen phosphate, HPLC grade water, n-propanol and ether were obtained from Loba Chemie (Bombay, India). All chemicals and reagents were either analytical or spectroanalytical grade.

Apparatus and Chromatographic Conditions

The chromatographic set-up used was a Perkin Elmer Series 410 quaternary solvent delivery system, Rheodyne Model 7125 injector and a Perkin Elmer Model LC-135 diode array UV-Vis detector. Data was recorded and processed on a Perkin Elmer Omega Data System.

Separation was performed on a Spherisorb® ODS 5 μ column (Phase Separation Inc., Norwak, CT, USA, 250 mm x 4.6 mm) using a 100 μM phosphate buffer, pH 3.0, as the mobile phase. The flow rate was kept constant at 1 mL/min and the volume of injection was 20 μL. The wavelength of spectrophotometric detection was 210 nm.

Experimental Animals and Intravenous Administration

Male Wistar rats (200 - 250 g), that were fed a balanced diet and, *ad libitum*, were used during the study. Each experimental station was done in quintuplicate. Rats were dosed with 15 mg per kg body weight through the right femoral vein. Blood samples were withdrawn at regular intervals of time and the concentration of 5-Fu and its metabolites recorded.

To prepare a standard working curve with spiked amounts of 5-Fu and its metabolites, the plasma of untreated rats was used. Plasma was collected in tubes containing sodium heparin, centrifuged at 3000 x g for 15 min and frozen at -20°C until further analysis.

Sample Extraction

Studies were conducted to optimize the extraction of 5-Fu and its metabolites from spiked samples of plasma. Co-solvent systems, as extractant, have been reported e.g. ethyl acetate:methanol,[11] acetonitrile:water[12] and light n-propanol:ether.[13] Preliminary investigations indicated that n-propanol:ether mixture gave good recoveries for 5-Fu. Therefore to optimize the ratio of co-solvents, recoveries of 100 μg each, of 5-Fu, FuDH and Furd, in 1 mL of plasma were determined by extracting with 15 mL of n-propanol:ether mixture (in the ratios of 10:90 through 90:10 v/v) and compared to that of an aqueous standard solution of the same. After ascertaining the optimum ratio of n-propanol:ether mixture, the following procedure was carried out.

Pooled plasma containing spiked amounts of 100 μg/mL each of 5-Fu and its metabolites was mixed with propanol:ether in an appropriate ratio and sonicated for 5 min at 4°C. The solution was then centrifuged at 3000 x g for 15 min at 4°C. The organic phase was separated and evaporated to dryness under a stream of nitrogen. The residue was redissolved in 1 mL of 50 μM ammonium dihydrogen phosphate and adjusted to varying pH values between 2 to 10. The solution was vortexed for 10 min at ambient temperature and filtered through 0.45 μm filter (Millipore® HV4, Millipore, France) prior to filling in autosampler vials.

A parallel study was carried out to study the effect of various pH adjusting systems in minimizing the interference of analyte peaks with that of co-eluents from the plasma matrix. The pH adjusting systems used were 0.1 mL of 1N sulfuric acid or 0.1 mL of 1N sodium hydroxide or 0.1 mL of 100 μM Tris buffer. These solutions were added, separately, to spiked samples of plasma containing 5-Fu and its metabolites, prior to carrying out the extraction procedure discussed above.

Linearity Studies

Two sets of working standard solutions were prepared; one in de-ionized water and the other in pooled plasma. The standard solutions in plasma were prepared by spiking the biological fluid with known concentrations of 5-Fu, FuDH and Furd in separate volumetric flasks. The concentration range for 5-Fu and its metabolites was adjusted between 2 - 200 μg/mL in de-ionized water and 10 - 100 μg/mL in plasma. 5-Fc was used as an internal standard at a concentration of 100 μg/mL. Acceptance criteria of r^2 not less than 0.98 was followed.

Precision and Accuracy Studies

Precision and accuracy of the assay was determined by making replicate injections of known amounts of 5-Fu and its metabolites and assessing the per cent co-efficient of variation (% C.V.) and analytical recovery respectively. A coefficient of variation not exceeding 4% was considered to be an acceptable one.[14]

Plasma samples were spiked with a standard solution of 50 μg/mL each of 5-Fu, FuDH and Furd in separate tubes. Ten replicate injections of these were made on the same day to assess the with-in day precision and accuracy.

To assess the between-day variability, a working standard series of 50 μg/mL each of 5-Fu, FuDH and Furd in plasma was analyzed for 10 replicate injections on three consecutive days. 5-Fc in a concentration of 50 μg/mL was used as an internal standard.

Specificity Studies

The purpose of this study was to ascertain if 5-Fu and its metabolites could be analyzed simultaneously in the presence of other plasma matrix components. This was done by spiking the plasma sample with a standard solution containing a mixture of 50 μg/mL each of 5-Fu and its metabolites. 5-Fc in a concentration of 50 μg/mL was used as an internal standard.

RESULTS AND DISCUSSION

Analysis of sample solutions was done in quintuplicate unless otherwise mentioned. All statistical evaluations were done using Student's t-test (double-sided) at $p < 0.05$ level of significance.

The variables that affected the extraction yield were n-propanol:ether ratio, pH of redissolving buffer solution and nature of pH adjusting system.

The extractant ratio of n-propanol:ether was varied from 10:90 though 90:10 v/v. It was observed that a high ratio of n-propanol resulted in extraction of plasma components as well. Therefore, the peaks for analytes could not be well resolved and the % recovery could not be determined. A high ether ratio minimized interference of plasma components with that of 5-Fu and its metabolites but decreased the % recovery. The optimum ratio of n-propanol:ether mixture, observed for 5- Fu, Furd and FuDH, was found to be 80:20, 85:15 and 80:20 respectively.

When the pH of the redissolving buffer solution was varied from 2 to 10, the % recoveries for 5- Fu, Furd and FuDH decreased gradually upto pH 6.0. After this pH value, the recoveries were not found to alter significantly.

Figure 1 shows the variation of % recovery of 5-Fu as a function of pH of redissolving buffer solution. Therefore, the pH of redissolving buffer solution was kept constant at 2.0 for maximum recovery of 5-Fu and its metabolites.

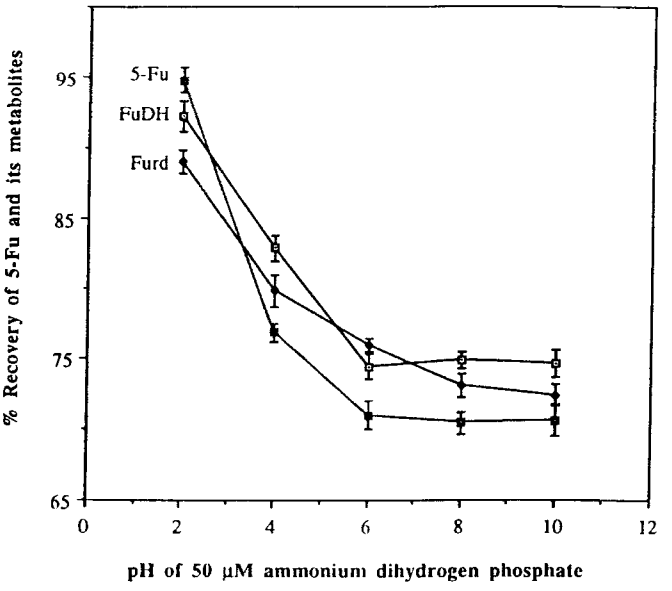

Figure 1. Percent recovery of 5-fluorouracil as a function of pH of 50 µM ammonium dihydrogen phosphate.

Amongst the various pH adjusting systems investigated to minimize the interference of plasma co-eluents with that of analytes, 0.1 mL of 1N sulfuric acid was found to give clear chromatograms. The analysis of samples treated with sodium hydroxide and Tris-buffer gave substantial interference between the analytes and co-eluents of the plasma matrix.

In the light of the above experiments, the extraction procedure established for the simultaneous analysis of 5-Fu and its metabolites requires a n-propanol:ether ratio of 80:20. After the sonication and centrifugation steps as discussed previously, the organic phase was evaporated. The residue was redissolved in 1 mL of 50 µM ammonium dihydrogen phosphate at a pH of 2.0, adjusted using 0.1 mL of 1N sulfuric acid.

A linear relationship was observed between the peak area ratio of the 5-Fu to that of the internal standard. The mathematical expression satisfying the linear regression equation are given in equations (1) and (2).

Table 1

Precision and Accuracy of 5-Fu, FuDH and Furd in Plasma

	5-Fu	FuDH	Furd
Within-day			
Mean concentration (μg/mL)	49.1	48.3	48.1
Standard deviation	0.5	0.7	1.1
% C.V.	1.0	1.4	2.3
% Recovery	98.2	96.7	96.2
Between-day			
Mean concentration (μg/mL)	49.6	48.1	48.5
Standard deviation	0.6	1.0	1.0
% C.V.	1.2	2.0	2.0
% Recovery	99.1	96.2	97.0

$y = 0.047\ x + 0.013$, $r^2 = 0.99$ in de-ionized water (1)

$y = 0.046\ x + 0.033$, $r^2 = 0.99$ in plasma (2)

The limit of quantitation for 5-Fu, FuDH and Furd in plasma was found to be 0.2 μg/mL, 0.3 μg/mL and 0.8 μg/mL respectively.

The with-in day coefficient of variation for 5-Fu, FuDH and Furd was found to be 1.0%, 1.4% and 2.3% respectively. The between-day coefficient of variation for 5-Fu, FuDH and Furd was found to be 1.2%, 2.0% and 2.0% respectively. The accuracy of the assay for 5-Fu and its metabolites was between 96.2% and 99.1%. The results are presented in Table 1.

The method was also found to be specific, as 5-Fu and its metabolites could be analyzed simultaneously with a good degree of precision and accuracy. The retention times of 5-Fc, FuDH, 5-Fu, and Furd were 3.8 min, 4.8 min, 5.5 min and 12.6 min respectively as shown in Figure 2.

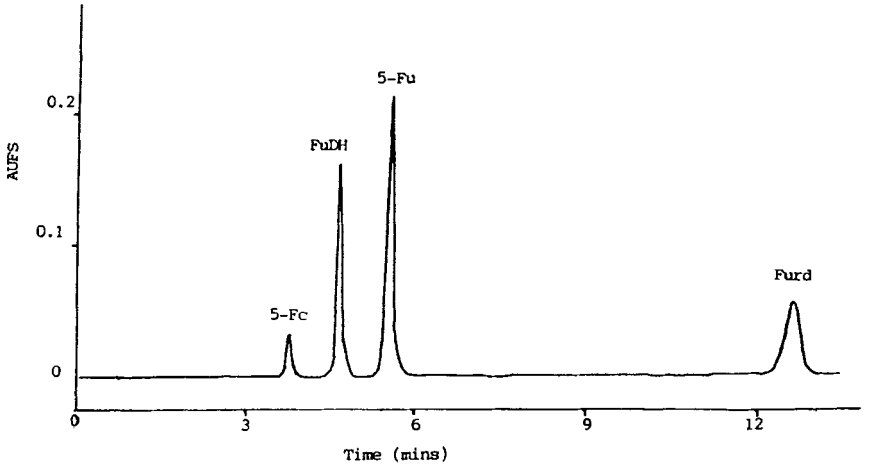

Figure 2. Representative HPLC chromatogram of 5-fluorouracil and its metabolites, 50 μg/mL each.

Table 2

Concentration of 5-Fu and FuDH in Plasma Samples After Intravenous Administration

Time (min)	Concentration in μg/mL	
	5-Fu	FuDH
1	89.4 ± 6.1	2.9 ± 1.3
10	25.8 ± 5.4	3.1 ± 1.0
20	29.6 ± 3.3	4.1 ± 0.8
30	12 ± 3.2	4.3 ± 0.9
40	11.1 ± 2.7	5.8 ± 0.5
50	08.5 ± 2.1	4.2 ± 1.1
60	07.6 ± 1.8	3.2 ± 1.0
120	03.1 ± 0.4	2.5 ± 0.9
180		2.2 ± 1.0

± indicates standard deviaton values for a mean of 5 determinations.

Upon intravenous administration of 15 mg of 5-Fu per kg body weight of rats, the concentration of the active drug reached its peak within 1 min. The results are presented in Table 2. FuDH could also be quantitated at this time period of 1 min. Afterwards, 5-Fu concentration gradually decreased over a period of time and could not be detected after 2 h. The concentration of FuDH increased initially upto 5.84% but after 40 min started decreasing. Surprisingly, no traces of Furd could be detected throughout this experiment.

CONCLUSION

The present study demonstrates the importance of an optimized extraction procedure for maximum recovery of 5-Fu and its metabolites. Evaluation of parameters that are necessary for achieving maximum recovery of 5-Fu and its metabolites have been investigated and optimized. The proposed method also confirms the suitability of this assay procedure for the analysis of 5-Fu and its metabolites in plasma samples with a good degree of precision and accuracy. The method has been validated as per compendial standards and can be routinely used for the estimation of 5-fluorouracil and its metabolites during pharmacokinetic experiments using plasma as the biological fluid.

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