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REVERSE-PHASE HPLC METHOD DEVELOPMENT TO FIND THE ORDER OF ELUTION OF 5-FLUOROURACIL, CAFFEINE, TEGAFUR

Harikrishna Rajasekhara Kurup[*1] and L. V. Vigneshwaran[2]

[1*]Queen's University Belfast, Northern Ireland, United Kingdom.
[2]Department of Pharmaceutics, Sree Abirami College of Pharmacy, Coimbatore, Tamil Nadu, India.

ABSTRACT

This study presents the method development of a Reverse-p*h*ase high-performance chromatographic method for separation and identification of three pharmaceutical compounds caffeine, 5- fluorouracil, and Tegafur. Caffeine is a natural central nervous system stimulant present in beverages like tea, coffee, and various energy drinks. 5-fluorouracil is a well-known anti-tumor drug used for the treatment of cancer. Tegafur is an antineoplasticpro drug of 5-fluorouracil[1]. It is a chemotherapeutic agent which is inactive in a normal state and becomes active when it reaches the body due to an enzymatic reaction. An HPLC method was developed to separate the three compounds using Agilent Infinity LC II apparatus equipped with Diode Array Detector. Agilent Zorbax Eclipse plus C18 column, which is nonpolar was used for the analysis of the samples. The method development started with a scouting run from which mode of elution for HPLC was selected. The experimental parameter from the scouting run was continuously changed through several trials to develop an optimized method for the separation of Pharmaceutical samples.

KEYWORDS

Reverse-phase, 5- fluorouracil and Tegafur.

Author for Correspondence:

Harikrishna Rajasekhara Kurup,
Queen's University Belfast,
Northern Ireland, United Kingdom.

Email: vigneshwaran85@gmail.com

INTRODUCTION

Cancer is one of the prime causes of death worldwide. It is a group of diseases, where cells in a specific part of the body grow and reproduce uncontrollably. These cells will invade the nearby cells and destroy the function of healthy tissue. The causative agents that result in cancer are, chemical or toxic compound (carcinogens) exposures, exposure to radiation, smoking, obesity, chronic inflammations, etc[2]. These agents induce various

unintended mutations to DNA within the cell and result in cancer. 5-fluorouracil (anti-metabolite) and Tegafur (pyrimidine analog) are anticancer drugs that can suppress uncontrollable cell division by inhibiting DNA synthesis[3]. Prodrugs are biologically inactive compounds and are activated into drugs when metabolized by the body. Tegafur, a prodrug of 5-fluorouracil is used in this present study[4].

Caffeine is a stimulant that stimulates the brain and nervous system. It is one of the most common drugs present in many beverages like coffee, tea, soft drinks, and energy drinks. In typical doses, caffeine shows antagonism at adenosine receptors (A_1- and A_{2A}-receptors). The result of this action is central inhibition of adenosine-mediated reduction of activity of the dopaminergic and ascending arousal system. In small doses caffeine help to feel refreshed and focused, but in a large dose, it can change sleep pattern and can cause anxiety. Caffeine improves, attention, focus, mainly in sleep-deprived individuals. Moreover, the benefit of caffeine is also reflected in sports in providing endurance during training and lower pain perception.

High-performance liquid chromatography is a widely used technique in the pharmaceutical industry to identify, quantify, separate, and purify individual compounds from their mixture. The Reverse phase chromatography technique was employed to analyze the pharmaceutical compounds. The Reverse phase chromatography used the nonpolar stationary phase and polar mobile phase for separation. The stationary phase uses 8-carbon to 18- carbon chains bonded with silica. If the analyte is nonpolar, then the attraction between the stationary phase will be high and retention time will be higher. More polar analytes are readily eluted due to less interaction with the stationary phase. The mechanism of separation is based on the distribution of the analyte molecule between the stationary phase (nonpolar) and a mobile phase (polar)[5]. The affinity of an analyte molecule dissolved in the mobile phase to the stationary phase depends on the polarity of the analyte, mobile phase, and stationary phase. The stronger the interaction of the analyte with the stationary phase, the higher will be the retention time. Agilent 1220 Infinity II LC system is used to study the order of elution of the desired pharmaceutical compounds. The column used is Agilent Zorbax Eclipse plus C18 4.61×50nm 1.8µm, 245 bar and the mobile phase employed is solvent A – Water and solvent B – Methanol. The actual order of elution of the compounds is compared with the predicted order of elution. The analysis of the sample should be performed under accurate experimental conditions or setup. Proper set-up of experimental parameters and conditions is necessary to get an accurate result.

Aim

To develop an optimized HPLC method for the separation of Pharmaceutical compounds caffeine, 5-fluorouracil, and Tegafur.

Objective

To predict the order of elution of given Pharmaceutical samples.

To perform a scouting run to choose appropriate HPLC elution mode for separation of compounds and decide an optimum wavelength for the experiment.

To analyze individual compounds using the optimized method.

To compare the predicted order of elution with the experimental order of elution.

To justify the choice of optimum method for separation of the pharmaceutical compounds from chromatogram of each attempted HPLC method.

PREDICTION ON ORDER OF ELUTION

The predicted order of elution was:

5-fluorouracil < Tegafur < caffeine

5-fluorouracil

5-fluorouracil may elute first as it is highly polar, the oxygen and nitrogen atom pulls electrons increasing the positive charge on the carbon atom. When compared to another sample it is having fewer carbon atoms in structure and also the presence of C=O and N-H bond and highly electronegative fluorine atoms make it more polar[6].

Tegafur

Tegafur may elute the second as it is having more carbon atoms when compared to 5-fluorouracil and

the presence of electronegative fluorine atom, amino bond, carbonyl bond makes it even more polar than caffeine[6-8].

Caffeine

Caffeine is a polar molecule but due to less number of C=O bonds and due to the presence of electronegative fluorine atoms in Tegafur, caffeine is having less polarity than Tegafur and may elute the third. But when compared with 5-fluorouracil and tegafur, it is having less polarity. This is because, in tegafur, a fluorine atom is present which is highly electronegative. Fluorine atom along with amino group results in tegafur being more polar than caffeine. So caffeine elutes the third.

MATERIAL AND METHODS

Apparatus

The analytical HPLC system used in this study was AGILENT 1220 INFINITY LC II. The hardware components are flexible and one can configure on his choice. The configuration includes an isocratic or gradient pump, manual injector for sample injection, auto-sampler to gather a sample from the vials, column oven to maintain a steady temperature, and a diode array detector. The detection limit is very low in Agilent 1220 Infinity LC II. This is achieved by using a variable wavelength detector up to 80 Hz. Maintain stable temperatures with the optional column oven. Agilent ZORBAX Eclipse plus 4.6 x 150mm, 5µm was the column used in the analysis. A 25-cm long column was used in this study, and the maximum temperature the oven maintains is 60°C. The flow rate of the gradient pump is from 0.2 to 10.0mL/min. It also has a low-pressure mixing unit, and an integrated degassing unit to avoid the formation of bubbles. The range at which auto-sampler operates is 0.1 to 100µL and can hold 2ml 100 vials[9].

Materials

Sample

Preparation of 5-fluorouracil solution

Two milligrams of 5-FU were accurately weighed and dissolved in Me-OH to obtain the stock solution with a concentration of 1000.0μg/Ml.

Preparation of caffeine solutions

The standard stock solution of Caffeine was prepared by weighing 30mg of the standard substances, respectively, and dissolving in 10mL water, pH 8 adjusted with 0.1M NaOH. The solution was stable for approximately 3 days under refrigeration (4°C).

Preparation of sample mix

A mixture of three pharmaceutical compounds, caffeine, Tegafur, and 5-fluorouracil is prepared by mixing the solvent together in a conical flask.

Chemicals

All chemicals were of pure analytical grade. Deionized water, Methanol was of HPLC grade.

Chromatographic condition

The stationary phase used was Agilent ZORBAX Eclipse plus 4.6 x 150mm, 5µm. Two solvent was employed as mobile phase, Water, and Methanol (95:5 ratio). The column was operated at 25°C. The flow rate at 1.0mL/min. A diode array detector was used to detect the presence of pharmaceutical samples, caffeine, Tegafur, and 5- fluorouracil in the sample.

Procedures

Scouting run

A scouting run will an idea of the complexity of the sample. It is an efficient way to achieve a fine-tuning stage of development faster.

A scouting run was performed for 10min by using the pre-installed C18 column, the stationary phase, and mobile phase A: 100% Water and B: 100% Methanol.

The parameters for scouting run is mentioned below:

SELECTION OF HPLC ELUTION MODE

There are two modes of elution:

Gradient elution:

Isocratic elution

Gradient elution

A type of elution in which the mobile phase characteristics are varied. There can be changes in the composition of the mobile phase which result in variation in the concentration of the mobile phase.

Isocratic elution

In isocratic elution, the mobile phase characteristics like concentration, composition are not changed. The same solvent is used for the whole analysis.

The equation to find a mode of elution:

ΔtR/tG= Retention time of final peak– Retention time of initial peak/ tG

Where,

ΔtR is the difference in retention time between the final peak and initial peak.

tG is the gradient time.

The mode of elution is selected based on:

If $\Delta tR/tG \leq 0.25$ use isocratic elution. (tG= gradient duration)

If $\Delta tR/tG \geq 0.4$ use gradient elution.

If isocratic elution is opted for: $\%B \approx 9.5 \times [(tR)av - tD] - 2$

Trialand error method

Developing an Efficient method for separation requires a quantitative understanding of how experimental conditions affect the quality of separation. An optimized method for the separation of the given pharmaceutical compounds can be developed using the trial and error method. In the trial and error method, the HPLC method parameters are changed to get better separation and well-resolved peaks. Minor alterations in experimental conditions are made to obtain a refined method for the separation of samples.

Analysis of individual compounds using optimized method

The optimized method obtained from the analysis of the unknown mixture was used to perform the individual sample analysis of given pharmaceutical sample caffeine, Tegafur, and 5-fluorouracil.

The retention time obtained from the chromatogram of both individual sample and unknown sample mixture was compared to identify the order of elution.

If the retention time of both individual samples and unknown mixture are similar, then it corresponds to the accuracy of the developed HPLC method.

The experimental order is obtained after comparing the retention time of both the sample mixture and the individual compound.

RESULTS AND DISCUSSION

Scouting run and selection of HPLC mode of elution

The below chromatogram shows the retention time of the sample mixture.

The peaks present in the above chromatogram show the retention time of the pharmaceutical sample, 5-fluorouracil, caffeine, and Tegafur.

From the chromatogram, the retention time of the final peak and initial peak can be taken for finding the mode of elution.

5.861min is the retention time of the final peak.

1.789min is the retention time of the final peak

tG= 10 min

Applying in above values in the formula,

ΔtR/tG= Retention time of final peak – retention time of initial peak/ TG

ΔtR/tG= 5.861-1.789/10 = 0.42

Here the ΔtR/tG is greater than 0.4, so Gradient elution was preferred.

Optimisation of the method

The optimization of the method from the scouting run was carried out using an unknown sample mixture.

Trial and error method-1

The peaks obtained from the scouting run were not fully resolved.

So changes were made in the experimental parameters to get better separation and resolved peaks.

The injection volume was set at 5µL.

The wavelength was brought down from 260nm to 230nm for better quantification of the sample.

Since there is 3 sample, the wavelength at which each sample absorb proportionally should be considered.

Mobile phase concentration was kept unchanged.

The total run time was 10min and the gradient time was set at 8 min.

The oven temperature was controlled at 25°C.

Discussion on chromatogram of method 1

The above chromatogram gives peaks that were not fully resolved.

Peak tailing and broadening were observed between two peaks having retention times 1.940min and 2.360min.

The peak tailing occurs due to sampling volume overload. The runtime of the whole process was longer. Since no peak overlapping was observed, the run time can be reduced to get better separation in a short duration.

Since Method-1 failed to give an accurate chromatogram, Method-2 for optimization must be carried out.

Trial and errormethod-2

The main changes or alterations brought in method-2 were in injection volume and run time. The injection volume was reduced to 3µL, to avoid peak tailing.

The total run time was brought down to 8 min to shorten the duration of the process.

The method parameters for Method-2 given below,

Discussion on chromatogram of method-2

When compared to the previous trial, in method 3 the peak tailing has decreased between 2 to 2.5 min. A well-resolved peak is obtained.

A small peak-like projection at time 2.367 is due to impurity or any variation in concentration. But still the overall run time and gradient time is high. So a trial 4 is conducted to reduce the overall time of the instrument.

The chromatogram for method 2 shows great improvement in the separation of the sample mixture.

Peak tailing observed in method 1 was not observed in the chromatogram of method 2. A small peak-like projection at retention time 2.367 may be due to the impurity present in the sample.

Since the run time and gradient, time was longer and there was no peak overlapping observed, Method-3 was considered to reduce the process duration.

Trial and error method method-3

In Method-3 the changes were made in run time and gradient time.

Every other parameter such as mobile phase concentration, wavelength, column temperature injection volume was kept constant.

The method parameters for Method-3 are shown below.

Discussion on chromatogram of method-3

An optimized method for the separation of unknown samples was achieved in Method-3.

The peaks were fully resolved and peak tailing was not observed.

Analysis of individual compounds

5-fluorouracil, Tegafur, Caffeine were analyzed, individually to verify the order of elution. The retention time of peaks obtained from the chromatogram of individual compounds was compared with the chromatogram of the unknown mixture and thus the order of elution was verified. The experimental parameters of the optimized method were used in individual analysis.

Identifying and assigning peak label for chromatogram of unknown sample

Based on the retention time obtained from the individual compound analysis, the peak labels were assigned for the unknown sample mixture.

Comparison between predicted order of elution and experimental order of elution

The above result shows that the predicted order of elution and experimental order of elution were the same.

The experimental order of elution is based on the retention time of analyte molecules and the order of elution was predicted based on the structure of the compounds.

The order of elution of compounds is:

5-fluorouracil<Tegafur<caffeine

5-fluorouracil will be the first compound to get eluted and has a retention time of 1.874 min. The structure of 5-fluorouracil has the oxygen and nitrogen atom pulls electrons giving the carbon a slightly positive charge. When compared to another sample it is having fewer carbon atoms in structure and also the presence of C=O and N-H bond and highly electronegative fluorine atoms make it more polar.

The second to elute will be Tegafur. It has got a retention time of 3.938 min. when considering the structure, Tegafur has an oxolane cyclic ring which further makes it polar than caffeine and so elutes the second.

Caffeine elutes the third among the compounds and has a retention time of 4.254 min. Caffeine is polar

and has a high solubility in water, when compared to other compounds caffeine is less polar so it elutes the third. The presence of oxolane ring in Tegafur induces more polarity in Tegafur than in caffeine. So caffeine elutes third among the given pharmaceutical sample.

Table No.1: Scouting Run Parameters

S.No	Injection Volume	Stationary Phase	Mobile Phase	Oven Temperature	Run Time	Flow Rate	Wavelength
1	5µL	ZORBAX Eclipse plus C18	A: Water 100% B: Methanol 100%	25°C oven temperature	10mins	1mL/min	260nm

Table No.2: Shows the experimental parameters

S.No	Experimental parameters
1	Injection volume
2	wavelength
3	Oven temperature
4	Mobile phase concentration
5	Column
6	Run time
7	Gradient time
8	Flow rate

Table No.3: Shows the experimental parameter for Method-1

S.No	Parameters	Values
1	Injection volume	5µL
2	Column temperature	25°C.
3	Total run time	10mins
4	Gradient time	8 mins
5	Flow rate	1µL
6	Mobile phase concentration	A: Water 95% B: Methanol 5%
7	Wavelength	230nm wavelength

Table No.4: Experimental parameters for method-2

S.No	Parameters	Values
1	Injection volume	3µL
2	Column temperature	25°C.
3	Run time	8min
4	Gradient time	8 min
5	Flow rate	1µL
6	Mobile phase	A: Water 95% B: Methanol 5%
7	Wavelength	230nm

Table No.5: Shows the experimental parameters for Method-2

S.No	Parameters	Values
1	Injection volume	3µL
2	Column temperature	25°C.
3	Run time	7min
4	Gradient time	4min
5	Flow rate	1µL
6	Mobile phase	A: Water 95% B: Methanol 5%
7	Wavelength	230nm

Table No.6: Experimental parameters for Individual solvent analysis

S.No	Parameters	Values
1	Injection volume	3µL
2	Column temperature	25°C.
3	Run time	7min
4	Gradient time	4min
5	Flow rate	1µL
6	Mobile phase	A: Water 95% B: Methanol 5%
7	Wavelength	230nm

Table No.7: Shows that the retention time of the individual compounds and sample mixture

S.No	Individual compounds	Retention time of individual compounds (min)	Sample mixture	Retention time of sample mixture (min)
1	Caffeine	4.254 min	Caffeine	4.254 min
2	Tegafur	3.938 min	Tegafur	3.938 min
3	5-fluorouracil	1.874 min	5-fluorouracil	1.875 min

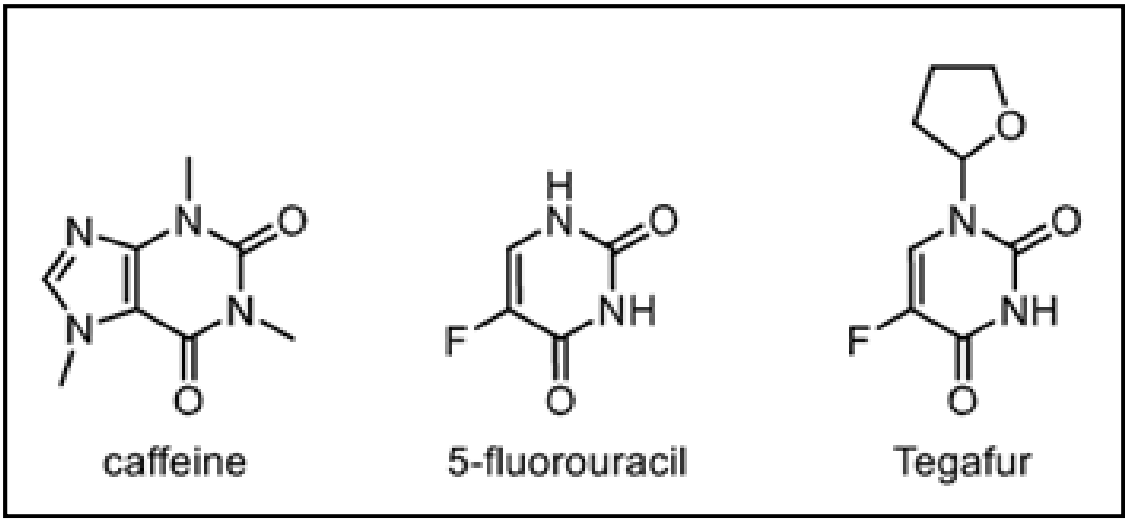

Figure No.1: Shows the structure of given compounds

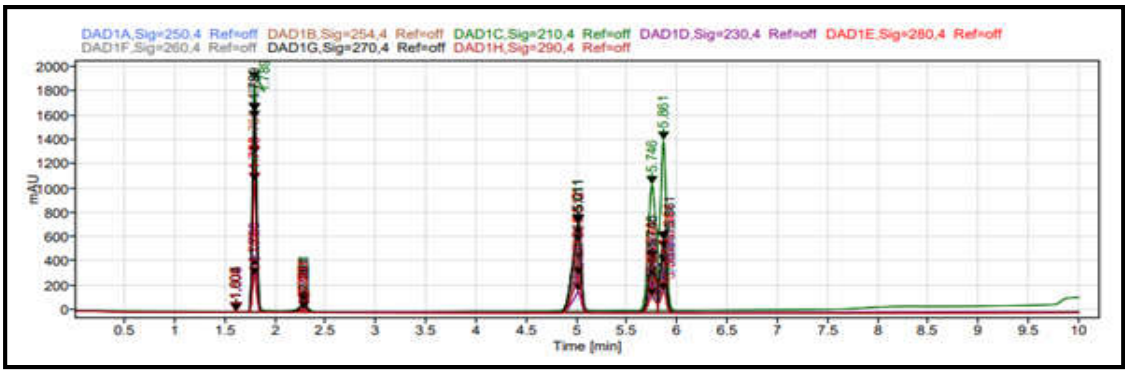

Figure No.2: Shows the chromatogram for a scouting run

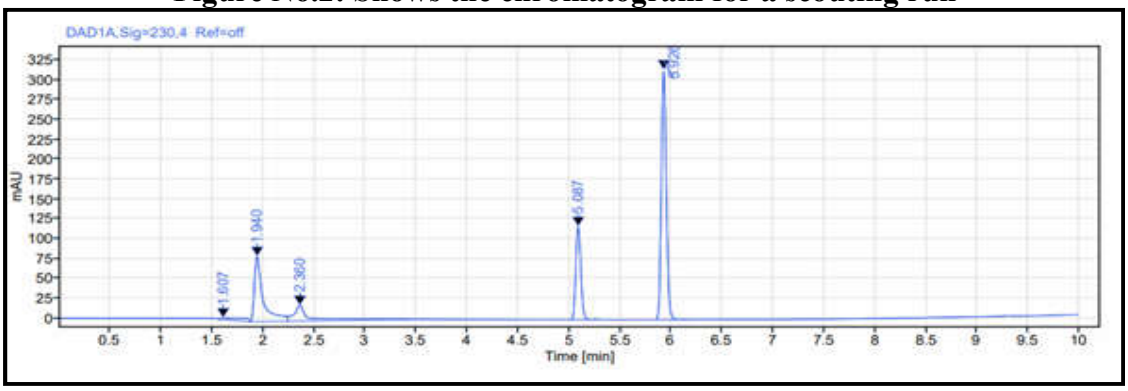

Figure No.3: Shows the chromatogram obtained for Method-1 optimization

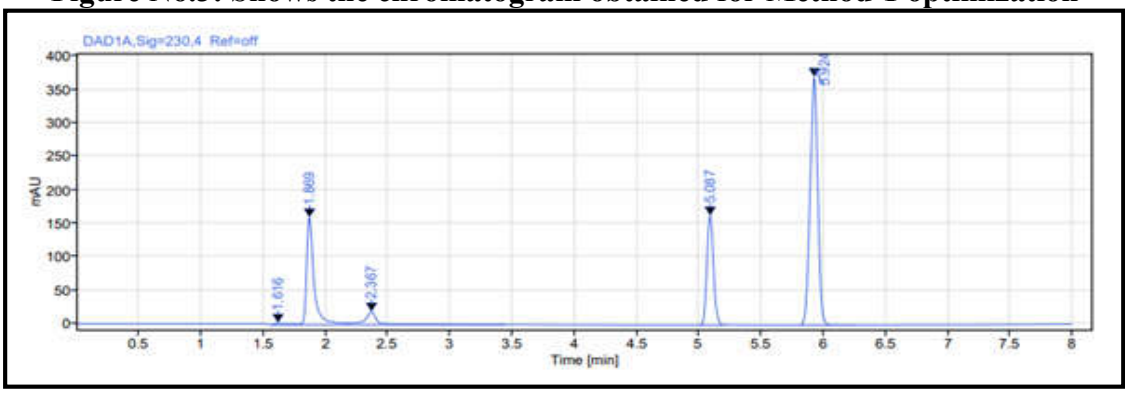

Figure No.4: Shows the chromatogram for Method-2

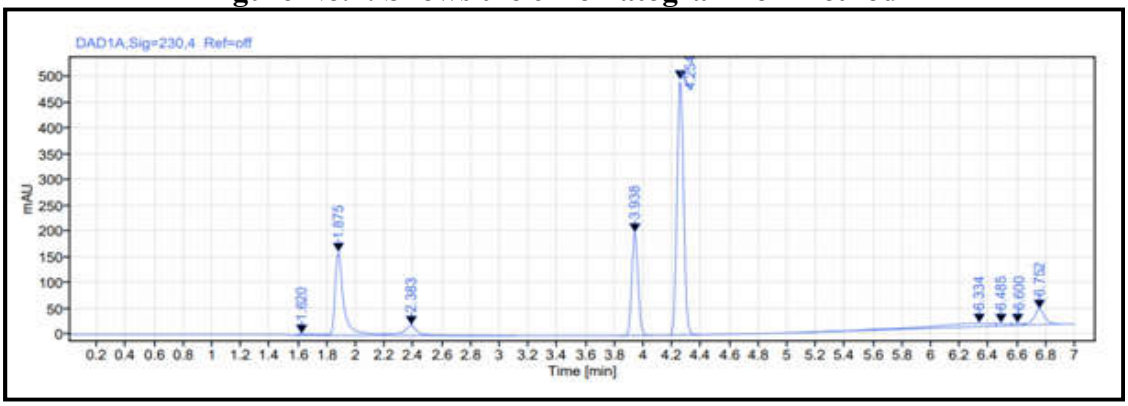

Figure No.5: Shows the chromatogram of Method-3

Caffeine

The chromatogram obtained from the analysis of caffeine is given below:

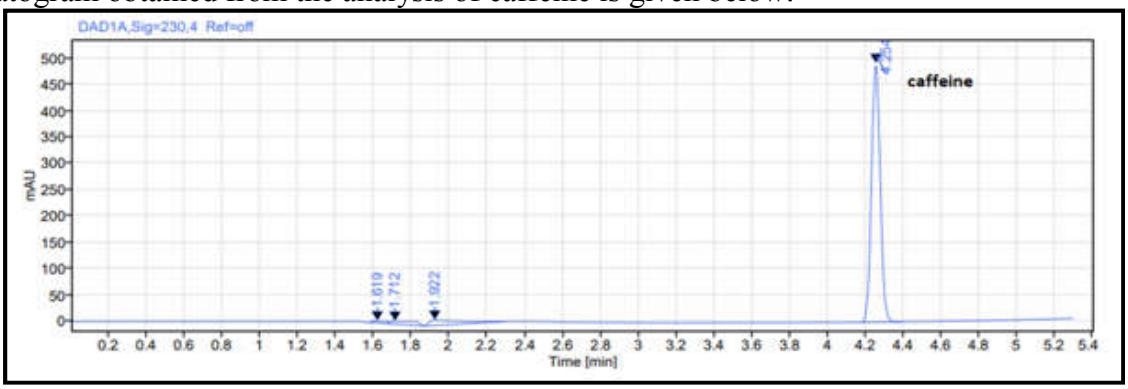

Figure No.6: Shows the retention time of caffeine. Caffeine was analyzed individually using the experimental parameters of the optimized method. On analysis, caffeine showed a retention time of 4.254min

Tegafur

The chromatogram obtained from the analysis of Tegafur is given below:

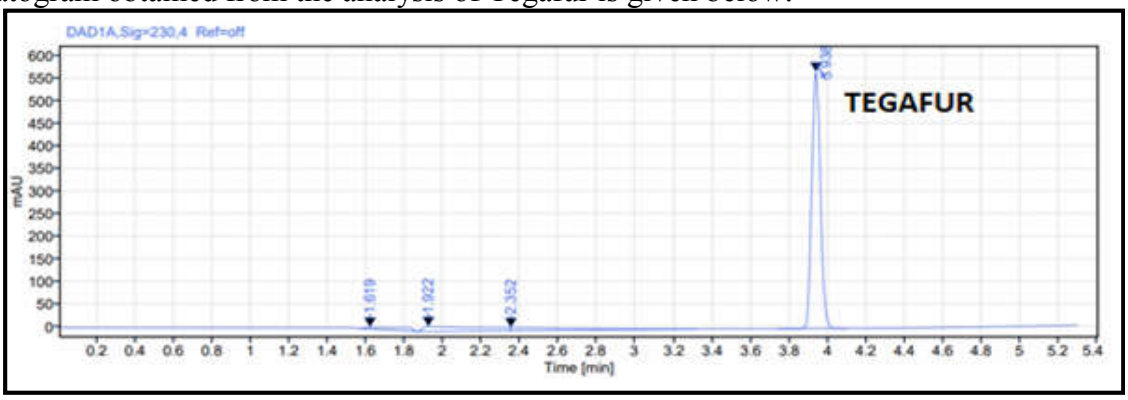

Figure No.7: Shows the retention time of Tegafur. Tegafur was analyzed individually using the experimental parameters of the optimized method. Based on results obtained from the chromatogram, the retention time of tegafur was found to be 3.939 min

5-fluorouracil

The chromatogram obtained from the analysis of 5-fluorouracil is given below:

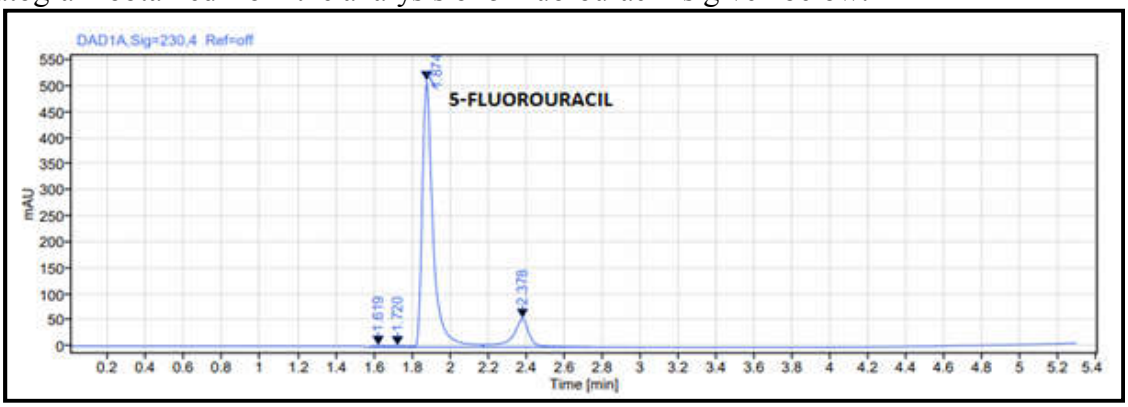

Figure No.8: Shows the retention time of 5-fluorouracil. It showed a retention time of 1.874 mins when analyzed individually

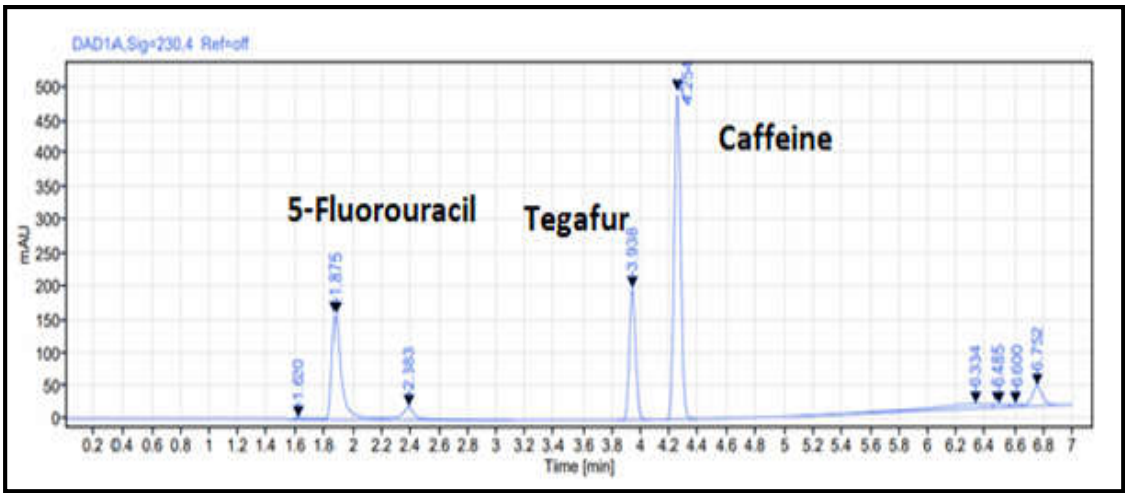

Figure No.9: Chromatogram shows the retention time and order of elution of given samples in an unknown mixture

CONCLUSION

The reported optimized HPLC method for separation of 5-fluorouracil, Tegafur, and Caffeine was simple, precise, accurate, sensitive, and reproducible. The method was optimized by altering different chromatographic parameters. The method parameters and other chromatographic conditions were selected based on trials. The analysis of individual compounds was carried out using the optimized method. Peak labels were assigned for sample mixture based on the retention time obtained from the chromatogram of individual sample analysis. The retention time of 5-fluorouracil (1875 min), Tegafur (3.938 min), and caffeine (4.254min) were reported. The experimental order of elution was compared with the predicted order of elution based on retention time, structure, and polarity of the analyte molecule and stationary phase.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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