Journal of Research in Pharmacy

Research Article

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Simultaneous Determination of Flurbiprofen and Thiocolchicoside in Pharmaceutical Preparations by a Validated HPLC Method

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Received: 10 December 2021 / Revised: 7 April 2022 / Accepted: 8 April 2022

ABSTRACT: In this study, a new high performance liquid chromatography (HPLC) analysis method was developed for the simultaneous determination of flurbiprofen (F) and thiocolchicoside (T) in pharmaceutical products. A C18 column (Agilent Poroshell 120 EC-C18) was used as the stationary phase. Elution of the analytes was achieved by using gradient elution system. Mobile phase A was an acetate buffer (100 mM, pH 4.8) and mobile phase B was acetonitrile. The flow rate for the method developed was 0.5 ml/min. The temperature of the column compartment was set at 40°C and the injection volume was 5 µl. Monitoring of the analytes was carried out at 248 nm. The method was found linear in the concentration range between 5-100 ppm. The correlation coefficient value was calculated as 0.999 for F and 0.999 for T. The method was found suitable in terms of accuracy, precision, specificity, linearity, and robustness ruggedness. Furthermore, it was applied successfully for the analysis of commercial tablets and gel samples that contains F and T without any time-consuming pre-procedure. The recovery values for Fand T were found as 99.5% and 97.0% in tablet formulations and 98.6% and 99.3% in gel formulations, respectively. All results were acceptable and this confirmed that the method is suitable for its intended use in routine quality control and an assay of drugs.

KEYWORDS: Flurbiprofen, Thiocolchicosoide, HPLC, analysis, validation, commercial dosage forms.

1. INTRODUCTION

Flurbiprofen (F) is a non-steroidal anti-inflammatory agent (NSAID), which has significant anti-inflammatory, analgesic, and antipyretic properties (Figure 1-(A)). It is used for the treatment of rheumatoid arthritis, degenerative joint disease, osteoarthritis, ankylosing spondylitis, acute musculoskeletal disorders, low back pain, and allied conditions [1-4].

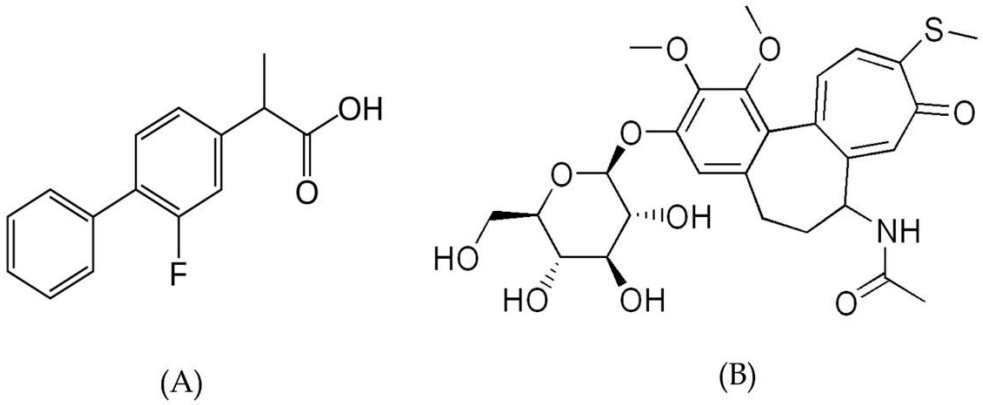
Thiocolchicoside (T) is a semisynthetic derivative of cholchicoside (natural compound) which is obtained from the seeds of *Gloriosa superb* and *Colchicum autumnale*. It is an analogue of colchicines, as they have the same benzo (alpha) heptalenic moiety [5]. Antiinflammatory and analgesic effects of this drug have also been reported in animal models [6]. It is used as muscle relaxant for the treatment of painful muscle contractions in acute and chronic rheumatic conditions, in traumatology and in patients with acute low back pain [7]. The chemical structure of T is shown in (Figure 1-(B)).

F and T are used as binary preparation for treatment of number of illnesses. Thus, monitoring of these drugs simultaneously by using an analytical method is important. When the literature was investigated it was seen that there are various methods published for the determination of F and T individually. Some of these methods achieved analysis of F which are spectrofluorometry, spectrophotometry [8-10], high performance liquid chromatography (HPLC) [11-15], liquid chromatography [16], gas chromatography (GC) [17], capillary electrophoresis (CE) [18] methods. T was analyzed by spectrophotometry [19-21], HPLC [22-28], thin layer chromatography (TLC) [29]. All these methods analyzed F and T separately. When these materials were applied in a binary formulation, usage of only one method will be needed. Usage of different methods for analysis of both of these materials causes consuming of more time, money and efficient. As the literature search was completed, it was understood that there is no method analyzing these materials in a single dosage

Aswaissi H, Türköz Acar E. Simultaneous Determination of Flurbiprofen and Thiocolchicoside in Pharmaceutical Preparations by a Validated HPLC Method. J Res Pharm. 2022; 26(3): 663-674.

http://dx.doi.org/10.29228/jrp.163
ISSN: 2630-6344

formulation simultaneously. The aim of this study is to present a simple validated HPLC method for the simultaneous determination of F and T in binary preparations.



(A) (B)

Figure 1. (A) Chemical structure of flurbiprofen (B) Chemical structure of thiocolchicoside

2. RESULTS

2.1. Optimum Method Conditions

Optimization of the method parameters were studied after developing of the HPLC method for simultaneous determination of F and T. The evaluated parameters were wavelength, flow rate, injection volume, concentration of the buffer solution, pH of the buffer solution, gradient profile of the mobile phase and, temperature. Each parameter was investigated by keeping of the other parameters constant. The optimum parameters were decided according to the peak shapes, peak area values and peak parameters like number of theoretical plates, capacity factor, tailing and resolution. Development of the methods started based on a previous method [12]. Phosphate buffer solution was selected as the mobile phase at pH 2.5 value. Also, higher pH values (pH 6.0-7.0 range) were tried for the separation. But F and T peaks could not be separated from each other well. Furthermore, the T peak was not appropriate for the analysis. Addition of organic modifiers like acetonitrile and methanol was also controlled but, the best separation of the peaks was not achieved. After this experiment, pH 4.0 levels were investigated by using acetate buffer solution. It was understood that this buffer solution provided more success for separation of F and T peaks. Different pH and concentration values were tried for the analysis conditions and it is decided that 100 mM pH 4.8 acetate buffer is the most appropriate. Optimization process also covers evaluation of the columns. Different C18 and C8 columns were tried like Agilent Poroshell 120 EC-C18 (3x150 mm, 2.7 µm) and phenyl by Inertsil Ph-3 (3µm 4.6x100mm) and ZORBAX Eclipse XDP-C8 (4,6x150mm 5-Micron) for the separation. But, only Agilent Poroshell 120 EC-C18 (3x150 mm, 2.7 µm) column provided the best separation. The other parameters were controlled after selection of the buffer and column. Gradient scheme was studied according to the time and acetonitrile ratio. Temperature of the column compartment was changed as starting from normal laboratory conditions and obtained results for the separated peaks were controlled. 40 ^{o}C temperature value was selected for the method. Flow rate and injection volume values were also evaluated by the same way. 0.5 mL/min and 5 µL were selected for flow rate and injection volume respectively

As a result of optimization studies, the chromatographic conditions of the developed method were as follow: column,Agilent Poroshell 120 EC-C18 (3x150 mm, 2.7 µm); mobile phase A, 100.0 mM pH 4.8 sodium acetate buffer; mobile phase B, Acetonitrile; injection volume, 5.0 µl; column temperature, 40^{o}C; detection wavelength, 248 nm; flow rate, 0.5 mL/min. Applied gradient system was like 0 min, 15% B; 5 min, 45% B; 6 min, 80% B; 12 min, 80% B; 13 min, 15% B; 18 min, 15% B. A chromatogram for the standard mixture of F and T was given in Figure 2. After completing optimization process, the method was validated according to the USP guidelines [30].

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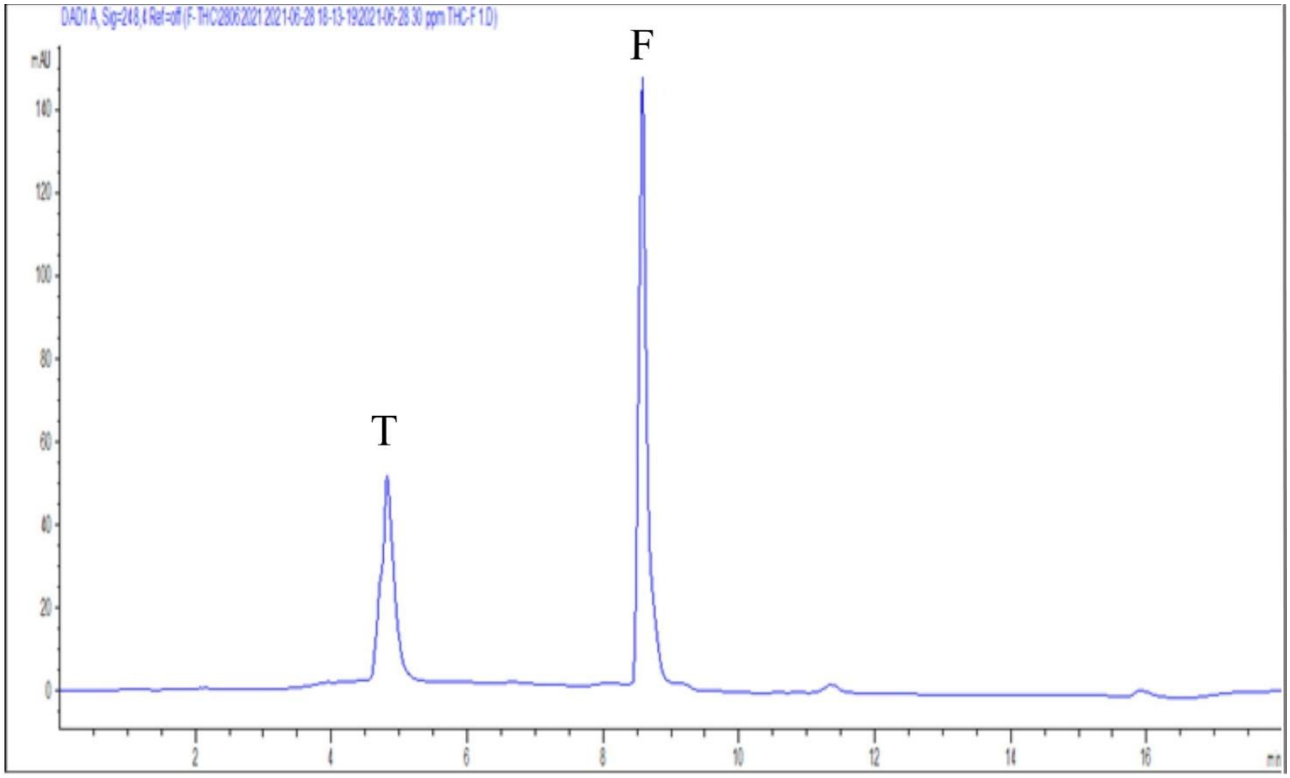

Figure 2. Obtained chromatogram for 30 ppm F and T under optimized conditions: Agilent Poroshell 120 EC-C18 (3x150 mm, 2.7 µm) column, 100 mM pH 4.8 acetate buffer and ACN, 5.0 µl injection volume, 0.5 ml/min flow rate, 40°C, 248 nm

2.2. Validation of the Method

2.2.1. System suitability test

System suitability test showing the appropriateness of the instrument and method parameters to the analysis was applied for the optimized method. System suitability parameters were investigated by applying six repetitive analysis of 30 ppm standard solution and obtained results were presented in Table 1. According to the results, the method and system were found to be suitable for the analysis of F and T, all system suitability parameters were provided necessary criterias. The difference between tailing values for F and T was remarkable during investigation of the system suitability result. The reason of the higher tailing factor for F can be explained by stronger adsorption of F then T onto the column surface.

Table 1. System suitability parameters and comparison of them with related criteria (n=6)

Parameter	Recommendation	F	T
Capacity Factor (k')	k' > 2	6.19	3.05
Resolution (Rs)	Rs > 2	18.78	21.31
Theoretical Plates (N)	N > 2000	6603	7917
RSD (Peak Area)	RSD ≤ 2	0.754	1.37
Tailing Factor (T)	T ≤ 2	1.60	1.09

2.2.2. Stability

Stability parameter used to detect how much time the analyte was stable. The stability was setting by determining auto sample QC concentration during 72h and compared to basis original concentration standards that was freshly prepared, recovery values was calculated. Result was demonstrated in Table 2.

Stability of stock solutions were investigated for the developed method. Stock solution of T was prepared in methanol (MeOH) and stock solution of F was prepared in acetonitrile (ACN). These stock

http://dx.doi.org/10.29228/jrp.163
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solutions were kept in refrigerator and their concentration changes were monitored according to the freshly prepared solutions. As the results of the monitoring, it was seem that their concentrations were stable for 10 days.

Table 2. Obtained recovery results for the stability study on QC samples for 72 h

Concentration (ppm)	Recovery%		Change%	
	F	T	F	T
8.0	94.3	108.2	3.8	2.0
15.0	92.4	93.6	6.5	6.9
30.0	94.2	101.0	2.8	4.0
70.0	92.8	100.6	3.7	1.0

2.2.3. Specificity

Specificity of the method was evaluated by applying forced degradation studies. For this purpose, 1.0 mL mixture containing 70 ppm F and T was added into separate tubes containing 0.1 M hydrochloric acid (A), 0.1 M sodium hydroxide (B) and 10% hydrogen peroxide (C). As the next step, all tubes were inserted in a boling water bath for an hour. After cooling of the solutions to the room temperature (25°C), they were filtered using 0.45 µm membrane filter and transferred to amber vials to keep them from the light. Then they were injected to HPLC system for the analysis. The chromatograms obtained for these solutions were shown in Figure 3.

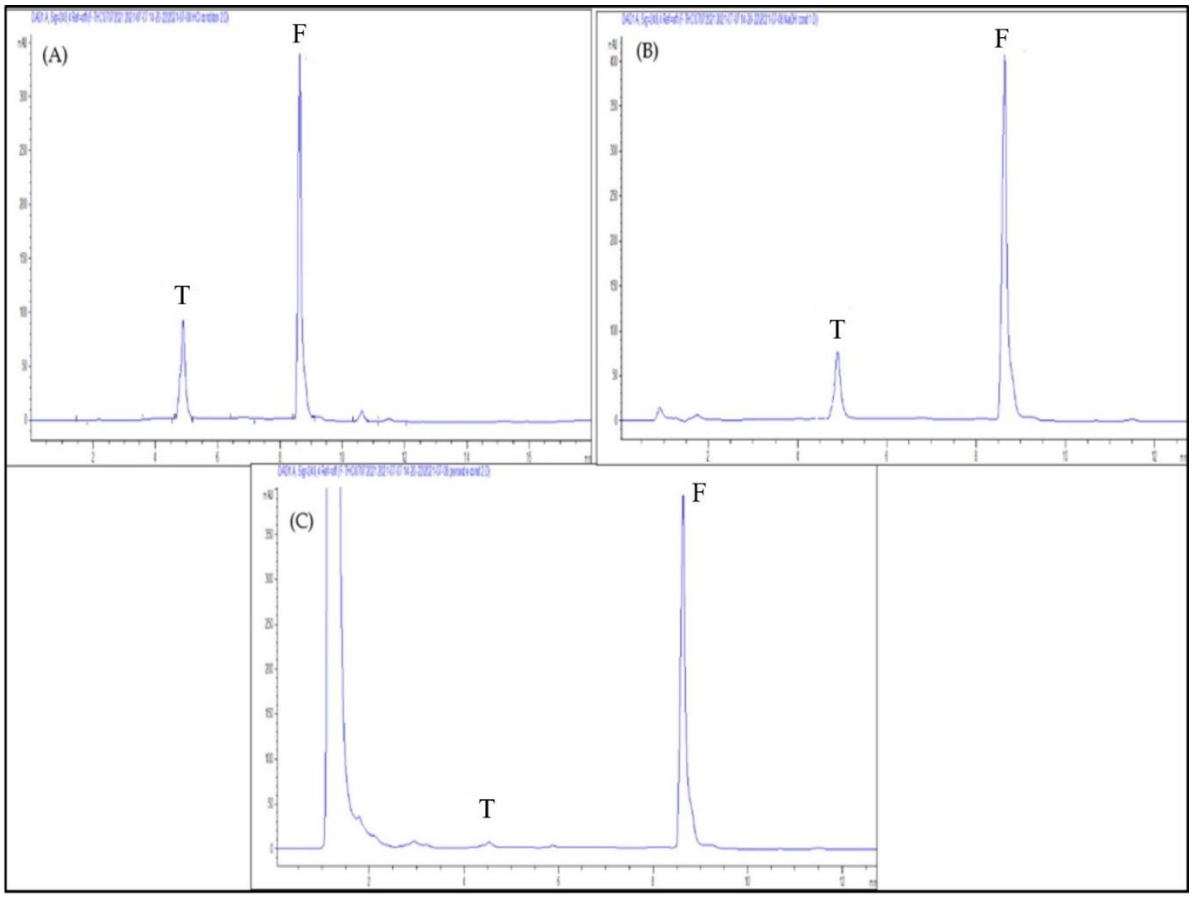

Figure 3. Obtained chromatograms for forced degradation studies in (A) HCl (B) NaOH (C) H_2O_2

According to the chromatograms obtained there was no interfering peaks. The peaks of the analytes were highly clear. This situation shows the specificity of the method.

2.2.4. Linearity and range

Linearity of the developed method was studied by standard solution mixture of F and T. In order to evaluate the linearity, standard solutions of F and T mixture at 5.0, 10.0, 20.0, 50.0, 80.0 and 100.0 ppm

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concentration values were used. The solutions were injected triplicate together with QC standard solutions. Calibration curves were obtained by plotting the peak areas versus concentrations. Calibration curve data and related parameters were presented in Table 3.

Table 3. Calibration curve parameters of developed method for F and T

Parameter	F	T
Linearity range(ppm)	5.0-100.0	5.0-100.0
R^2	0.999	0.999
Intercept	40.72	1.80
Slope	55.447	19.07
LOD	0.95	1.57
LOQ	3.18	5.23

On the other hand, limit of detection (LOD) and limit of quantification (LOQ) values were determined during this study. Obtained chromatograms of F and T at LOQ value were presented in Figure 4 and Figure 5.

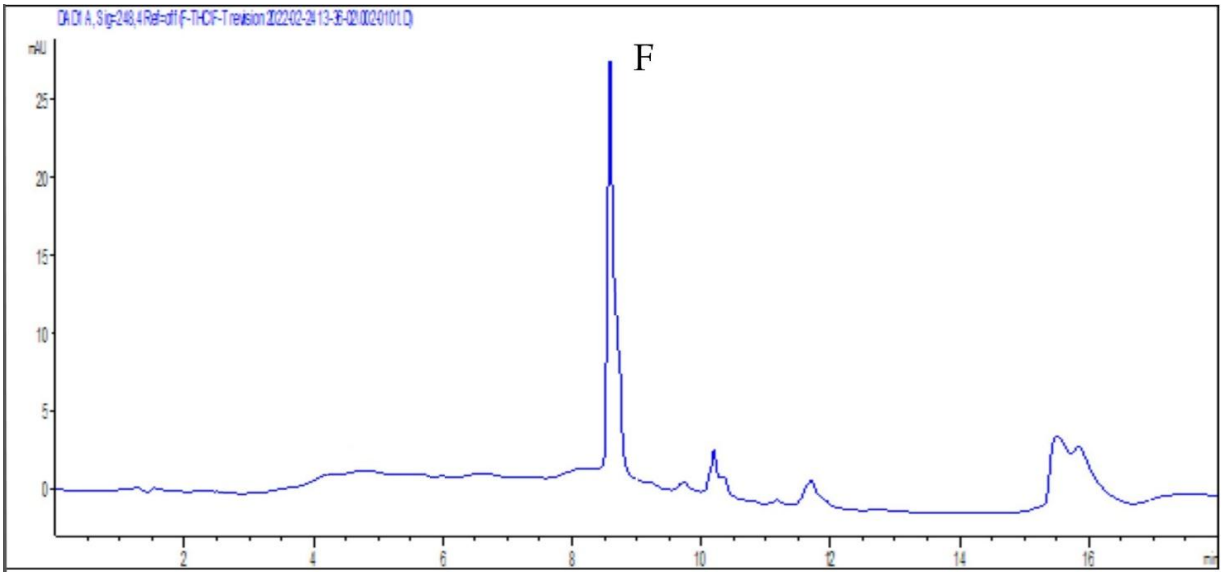

Figure 4. Chromatogram of F at LOQ level (3 ppm) under optimized conditions

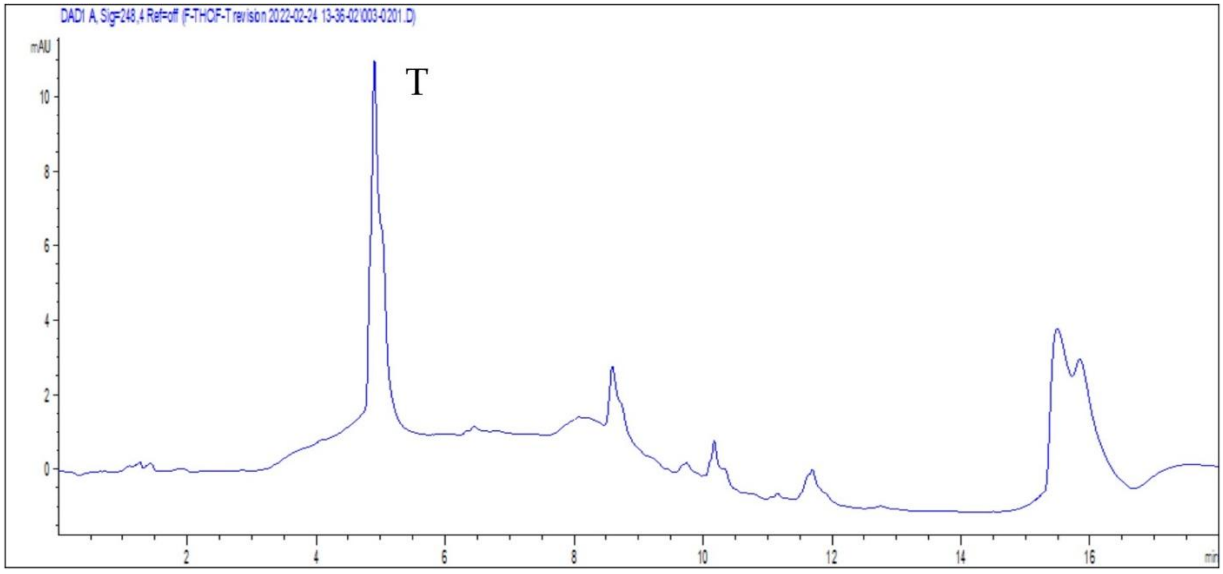

Figure 5. Chromatogram of T at LOQ level (5 ppm) under optimized conditions

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2.2.5. Precision and accuracy

Precision and accuracy of the method were evaluated by using triplicate analysis of QC samples of which concentration were 8.0, 15.0, 30.0 and 70.0 ppm. Their signal values were recorded and concentration values were calculated for intraday and interday studies. Recovery values were used to evaluate to mention accuracy, RSD values of the results were used for the precision. All results for accuracy and precision studies were presented in Table 4.

Table 4. Intraday and interday results of the accuracy and precision studies for F and T

Concentration (ppm)		Intraday (n=3)		Interday (n=9)	
		Recovery%	RSD	Recovery%	RSD
F	8.0	97.4	0.61	101.03	1.15
	15.0	95,7	0.25	99.64	1.14
	30.0	98.8	0.98	104.04	0.98
	70.0	101.2	0.13	100.88	0.38
T	8.0	100.6	1.27	100.01	0.99
	15.0	97.7	1.17	100.60	1.18
	30.0	100.3	1.20	102.60	1.05
	70.0	100.4	1.26	99.88	1.06

Recovery values for intraday studies and interday studies of the method ranged from 95.7- 101.2 %.and99.6 to 104.0% for F; 97.7.to 101.6 and 99.8.to 102.6% for T. The maximum relative standard deviation value for intraday and interday experiments were calculated as 0.98 and 1.15 for F; 1.27 and 1.18 for T, respectively. According to these results recovery and RSD values were in the accepted limit. Thus, it can be said that the developed method is accurate and precise.

Another recovery study was made on tablet and gel pharmaceutical preparations. The recovery values of added amounts of F and T on these preparations was evaluated. For this purpose, sample solutions were prepared as two sets and known amount of F and T were added onto these samples. After analyzing of the solutions, the amounts of F and T in the samples were calculated from the calibration curve by its equation. Added amounts and founded amounts for F and T were compared and then recovery values were calculated (Table 5).

Table 5. Results of recovery studies on spiked sample solution

Samples	F			T		
	Added amount (ppm)	Founded amount (ppm)	Recovery	Added amount (ppm)	Founded amount (ppm)	Recovery
Gel sample	10	9.86	98.6 %	25	24.8	99.3 %
Tablet sample	20	19.9	99.5 %	17	16.5	97.0%

The recovery value for F and T were found as 99.5 and 97.0% in tablet (mean, n = 3) and, 98.6 and 99.3% (mean, n = 3) in gel formulation, respectively.

2.2.6. Robustness

The robustness of the method was evaluated by appliying deliberately small variations on method parameters. Selected parameters for this step were temperature, pH of the mobile phase and, wavelength.

For this purpose, the quality control solutions were prepared as triplicate and each solution was analyzed by using developed method. Recovery values of the quality control solutions were determined using the equations of the calibration curve, as accuracy and precision studies. The results of the robustness study are shown in Table 6.

Robustness of the method for the analysis of F and T were tested by small variations in the method parameters. It was observed that the variations in pH, temperature and wavelength have no significant effect on the results.

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Table 6. Obtained results for the robustness study

Parameters		Concentration (ppm)	Recovery T	Recovery F
Temperature	38°C	8.0	93.3	90.1
		15.0	84.6	84.9
		30.0	96.1	98.7
		70.0	102.9	105.6
	42°C	8.0	102.4	109.0
		15.0	83.5	89.4
		30.0	108.6	98.7
		70.0	96.5	97.5
Buffer pH	pH 4.7	8.0	106.8	203.8
		15.0	92.9	93.2
		30.0	91.7	98.0
		70.0	93.1	100.6
	pH 4.9	8.0	94.0	90.4
		15.0	102.9	95.4
		30.0	92.0	95.3
		70.0	95.0	101.3
Wavelength	240 nm	8.0	106.3	97.4
		15.0	94.2	92.1
		30.0	100.0	97.1
		70.0	104.0	105.3
	256 nm	8.0	103.0	100.0
		15.0	93.5	93.8
		30.0	101.6	98.0
		70.0	106.8	104.0

2.3. Sample Analysis

The newly suggested method was applied for the analysis of tablet, and gel samples containing F and T in combination. The amounts of F and T in the pharmaceutical samples were calculated by using the calibration curve. The amounts of F and T in the samples was calculated according to the dilution process and obtained results were compared with claimed values. The results of analysis are demonstrated in Table 7. A chromatogram showing the analysis of F and T in tablet sample solution are shown in Figure 6.

Table 7. Analysis results on commercial pharmaceutical products.

Sample	F		T	
	Claimed amount	Founded amount	Claimed amount	Founded amount
Gel sample	5%	4.49 ±0.06	0.25 %	0.23 ±0.04
Tablet sample	100 mg/tb	108.71 ±1.06	8 mg/tb	7.05 ±0.05

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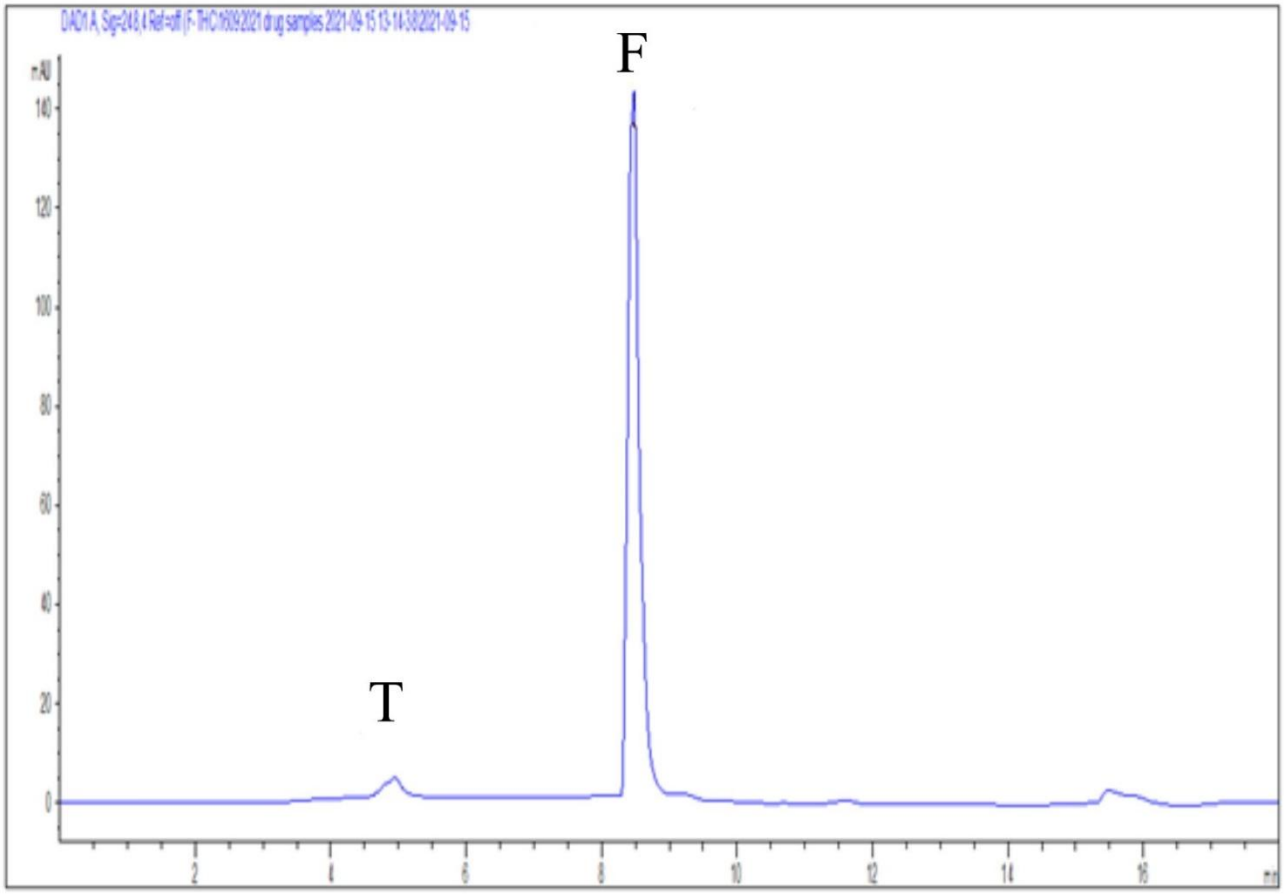

Figure 6. Obtained chromatogram of 40 ppm F and 16 ppm T prepared from tablet formulation under optimized conditions.

3. DISCUSSION

In this study, a new HPLC method was developed and applied for the simultaneous analysis of F and T without any interference. The method was found as precise and accurate, as indicated by the recovery studies and RSD values of obtained data which are not more than 2. The evaluation of statistical data shows that the values of the validation parameters are acceptable. Hence, the method is appropriate for the analysis of F and T. The most important point of this method is to achieve the analysis of F and T simultaneously. Because, there is no method in the literature declaring the analysis of these drugs together. In addition, the method needs no pre-procedure such as cleaning, precipitation etc. Hence, the newly developed method is simple and valid, and the usage of this method is suggested for the routine quality control analysis of F and T in gel and tablet pharmaceutical products.

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4. CONCLUSION

A simple, rapid, and robust stability indicating analysis method was developed for the simultaneous determination of flurbiprofen (F) and thiocolchicoside (T) by using HPLC. The method was found suitable in terms of accuracy, precision, specificity, linearity and robustness ruggeddness. The analysis of commercial tablets and gel samples containing F and T was achieved without any additional pre-procedure. All results were appropriate to analysis criterias and it is shown that the method can be used for quality control and assay of drugs.

5. MATERIALS AND METHODS

5.1. Materials

5.1.1. Chemicals and Reagents

F and T materials were kindly provided from Abdi İbrahim İlaç. Methanol and acetonitrile (J.T. Baker, Norway) were HPLC Gradient grade purity. Sodium acetate (Fluka, Steinheim, Germany) and acetic acid (Sigma-Aldrich, Steinheim, Germany) were of analytical grade. Commercial dosage forms were bought from local pharmacies.

5.1.2. Instruments

The Agilent 1260 Infinity HPLC system (Agilent Technologies, USA) consisting of quaternary pump, autosampler, thermostatted column compartment and diode array detector units were used for the chromatography experiments. The chromatographic data were recorded and evaluated by using Agilent ChemStation software. The separation of the F and T was carried out on the Agilent Poroshell 120 EC-C18 (3x150 mm, 2.7 µm) column. Ultrapure water used for aqueous mobile phase was produced by a Type I water purification system which is Millipore Simplicity (Darmstadt, Germany)

5.2. Methods

5.2.1. Preparation of mobile phase

Mobile phase A was the pH 4.8 100 mM acetate buffer solution which prepared by acetic acid and sodium acetate. After pH adjustments and completing volume, the buffer solution was filtered through 0.45 µm membrane filter and then degassed in sonicator for 15 minutes. Mobile phase B was acetonitrile. The gradient profile (time, %B) system was as given follow: 0 min, 15% B; 5 min, 45% B; 10 min, 80% B; 12 min, 80% B; 5 min, 80% B; 15 min, 15% B; 18 min, 15% B.

5.2.2. Preparation of standard solutions

Standard stock solutions of F and T were prepared by dissolving of 10.0 mg of standard material in 10.0 ml volumetric flask F with ACN and T with methanol at concentration of 1000.0 ppm. A 500 ppm mixture of F and T was prepared by mixing equal volume of stock solutions. Standard solutions were prepared at concentrations of 5.0, 10.0, 20.0, 50.0, 80.0 and 100.0 ppm. Quality control (QC) standard solutions were chosen as the solutions with concentrations of 8.0, 15.0, 30.0 and 70.0 ppm, A mixture of mobile phase A: Mobile phase B (85:15) was used for dilution process of standard solutions.

5.2.3. Preparation of sample solutions

Preparation of tablet samples:

Tablets were taken and weighed. After dissolving in acetonitrile:methanol (50:50) mixture in ultrasonic device for 5 min, the volume was completed and the solution was filtered with 0.45 µm membrane filter. Two different dilution procedure was applied to this solution due to concentration differences of F and T in samples. Prepared solutions were injected and analyzed under optimum conditions.

Preparation of gel samples:

To prepare sample solution from gel formulation, 1 g of gel was taken and dissolved in 100 mL volumetric flask with acetonitrile: methanol (50:50) mixture. After filtering of the solution, it was used directly injected to analyze T, F.

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5.3. Development of the method

During the optimization procedure, different buffers, pH, injection volumes and tempreture values were investigated. Peak shape, area/ height values of the peak, number of theorethical plates, capacity factor, resolution, tailing factors were examined to select the optimum conditions. Acetate buffer solution gave good results for the simultaneous determination of F and T. After determining buffer type, pH value was studied and pH 4.8 value was selected as the mobile phase pH. The following procedure was determination of instrumental parameters. Laboratory temperature (25 ^{o}C) was selected as the starting point. Obtained chromatograms showed that increasing of the temperature was crucial for the better separation, peak shape and related parameters. Flow rate and injection volume values were also evaluated by investigating same parameters.

5.4. Validation of the method

Applied validation procedure according to the USP guideline [30] included system suitability test, stability, and the investigation of the validation parameters involving linearity, accuracy, precision, specificity and, the robustness of the method.

5.4.1. System suitability

System suitability test (SST) was applied to HPLC system to indicate that the presented method and the used system are suitable and acceptable according to the SST criteria. SST was performed by investigating relative standard deviation of the peak areas, capacity factor, number of theoretical plates, resolution and, tailing factor. For this purpose, solution of F and T was injected six times and the results were evaluated.

5.4.2. Stability

Stability of the presented method was detected by analyzing quality control samples kept in autosampler. Amount of the T and F in these samples were calculated by obtained signal values. Then the calculated amounts of T and F were compared to amounts for freshly prepared solutions. Calculated recovery values were investigated for this investigation. Decreasing of the recovery value showed the end of the the stability of the solution.

5.4.3. Specificity

Specificity of the method was presented to shows the ability of method to produce specific and accurate present of analyte by exposure the analyte to different stress conditions. For this purpose, standard solutions containing equal molar F and T was kept in 0.1 M hydrochloric acid, 0.1 M sodium hydroxide and 10% hydrogen peroxide solutions in boiling water bath. After cooling and filtering of the solutions, they were injected for analysis. Obtained chromatograms were evaluated to control whether there was an interference or not.

5.4.4. Linearity

Linearity of the method was evaluated at six concentration levels (5.0, 10.0, .20, 50.0, 80.0, 100.0 ppm) were analyzed triplicate. The peak areas versus concentration were used for the construction of the calibration curve. Linearity of the method was presented by the term of correlation coefficient. Calculation of the limit of detection (LOD) and the limit of qualification (LOQ) also was involved.

5.4.5. Accuracy and Precision

Accuracy studies of the presented method was performed by calculation of recovery values of QC samples. For this purpose, QC samples were analyzed as three repetitive triplicate samples.
Precision studies of the presented method were performed by calculation RSD and recovery values of QC samples as intraday (n=3) and interday (n=9) precision. The solutions were analyzed as three repetitive triplicate system.

5.4.6. Robustness

Robustness of an analytical method is evaluated by applying small and deliberate changes on analysis parameters. Selected analytical method parameters were pH value of buffer solution, temperature and wavelength. All parameters were changed and results were compared with the optimized method results.

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Author contributions: Concept – E.T.A.; Design – E.T.A.; Supervision – E.T.A.; Resources – E.T.A, H.A.; Materials – E.T.A.; Data Collection and/or Processing – E.T.A., H.A.; Analysis and/or Interpretation – E.T.A., H.A.; Literature Search – H.A.; Writing – E.T.A., H.A.; Critical Reviews – E.T.A., H.A.

Conflict of interest statement: Fill in this section according to the signed conflict of interest statement when submitting your article. If there is no conflict of interest to be declared by any of the authors, write "The authors declared no conflict of interest" in the manuscript.

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