HAJER ASWAISI PHARMACUTICAL CHRMISTRY MASTERTHESIS 2022

TC.
YEDİTEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
DEPARTMENT OF PHARMACEUTICAL CHEMISTRY

SIMULTANEOUS DETERMINATION OF FLURBIPROFEN AND THIOCOLCHICOSIDE IN PHARMACEUTICAL PREPARATION BY A VALIDATED HPLC METHOD

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This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by the Administrative Board of Institute with the decision dated ..... and numbered.....

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Director of Institute of Health Science

DECLARATION

I declare that this thesis is my original work and that it contains neither material previously published or authored by any person, nor material that has been accepted for the award of any other degree, save where appropriate acknowledgment has been made in the text.

22.02/2022

Hajer Aswaisi

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LIST OF SYMBOLS AND ABBREVIATIONS

ACN	Acetonitrile
BSD	Base Deactivated Silica
C18	Carbon 18
DAD	Diode Array Detector
EP	European Pharmacopoeia
EDTA	Ethylenediaminetetraaceticacid
FBP	Flurbiprofen
FDA	US Food and Drug Administration
g	Gram
GC	Gas Chromatography
H_2O_2	Hydrogen peroxide
HCL	Hydrochloric acid
HPLC	High Performance Liquid chromatography
ICH	International Conference on Harmonization
k'	Capacity Factor
Kv	Kilowatt
LC	Liquid Chromatography
mg	Microgram
µl	Microliter
µm	Micrometer
mg	Miligram
ml	Mililiter
mM	Milimolar
min	Minute
N	Theoretical Plates
NaOH	Sodium hydroxide
nm	Nanometer
ng	Nanogram
NSAID	Non-steroidal Anti-inflammatory Drug
pH	Potential of Hyddrogen
Ppm	Parts Per Million
QC	Quality Control

R^2	Correlation Coefficient
RSD	Relative Standard Deviation
Rs	Resolution
S	Slope
SST	System Suitability Test
T	Tailing Factor
THC	Thiocolchicoside
TLC	Thin-layer chromatography
USP	ited States Pharmacopoeia
UV	Ultraviolet
v/v	Volume/Volume

ABSTRACT

Aswaisi H. [2022]. Simultaneous Determination of Flurbiprofen and Thiocolchicoside in Pharmaceutical Preparations by a Validated HPLC method. Yeditepe University, Institute of Health Sciences, Department of Pharmaceutical Chemistry Master Thesis, Istanbul.

Simple, rapid, and robust analytical method was presented by using high-performance liquid chromatography (HPLC) for the simultaneous determination of flurbiprofen (FBP) and thiocolchicoside (THC). The stationary phase used for the analysis was an Agilent Poroshell 120 EC-C18 column. An acetate buffer solution (100 mM, pH 4.8) and acetonitrile were used as the mobile phase A and B, respectively The injection volume was 5 µl. The flow rate was adjusted as 0.5 ml/min and column compartment temperature was set up to 40°C. Analytes were monitored at 248 nm by using a diode array detector (DAD). The validation procedure was applied according to the United States Pharmacopoeia. The developed method was found to be linear with a concentration range between 5-100 ppm. The correlation coefficient was calculated as 0.9993 for FBP and 0.9997 for THC. Other validation parameters evaluated were accuracy, precision, linearity, specificity, and robustness-ruggedness. The developed and validated method was successfully applied for HPLC analysis of commercial tablets and gel samples containing FBP and THC without any prior extraction process. The recoveries for FBP and THC were 99.5% and 97.0% in the tablet formulations and 98.6% and 99.3% in the gel formulations, respectively. All the results are acceptable and advanced that the method is appropriate for the proposed use in the assay of binary commercial dosage form and routine quality control.

Key words: Flurbiprofen, Thiocolchicoside, HPLC, analysis, validation.

ÖZET

Aswaisi H. [2022]. Flurbiprofen ve Tiyokolşikosidin Farmasötik PReparatta Valide edilmiş Bir HPLC Yöntemi ile Eş Zamanlı Tayini. Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Farmasötik Kimya Anabilim Dalı Yüksek Lisans Tezi, İstanbul.

Bu çalışmada, flurbiprofen (FBP) ve tiyokolşikosidin (THC) yüksek performanslı sıvı kromatografisi ile eş zamanlı analizi için basit, hızlı ve güvenilir bir yöntem sunulmuştur. Analizde kullanılan sabit faz Agilent Poroshell 120 EC-C18 kolon olup mobil faz A için 100 mM pH 4.8 asetat tampon çözeltisi ve mobil faz B için asetonitril kullanılmıştır. Enjeksiyon hacmi 5 µl, akış hızı 0.5 ml/min ve kolon bölmesi sıcaklığı 40 °C olarak belirlenmiştir. Analitler bir diyod array detektörü (DAD) kullanılarak 248 nm de izlenmiştir. Yöntem geliştirildikten sonar United States Pharmacopeia klavuzuna göre valide edilmiştir. Geliştirilen yöntem için 5- 100 ppm derişim aralığında doğrusal değişim gözlenmiş olup korelasyon katsayıları FBP için 0.9993 THC için 0.9997 dir. İncelenen diğer validasyon paramatereleri doğruluk, kesinlik, doğrusallık, spesifiklik ve yöntemin sağlamlığı ve güçlülüğüdür. Geliştirilen ve valide edilen yöntem FBP ve THC içeren farmasötik dozaj preparatlarında ön ekstraksiyon aşamasına gerek duyulmadan başarı ile uygulanmıştır. Geri kazanım değerleri tablet formülasyonunda FBP ve THC için % 99,5 ve % 97,0, jel formülasyonunda ise % 98,6 ve % 99,3 olarak belirlenmiştir. Tüm sonuçlar değerlendirme kriterlerine uygundur ve geliştirilen yöntemin FBP ve THC içeren dozaj formlarında tayin ve rutin kalite kontrol çalışmaları için kullanılabileceğini göstermektedir.

Anahtar Kelimeler: Flurbiprofen, Tiyokolşikosid, HPLC, analiz, validasyon.

1. INTRODUCTION

Flurbiprofen (FBP) is a non-steroidal anti-inflammatory drug (NSAID), which has important analgesic, and antipyretic anti-inflammatory properties, clinically 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 (THC) is a semisynthetic derivative of cholchicoside (natural compound) obtained from the seeds of *Gloriosa superb* and *Colchicum autumnale*. It is an analog of colchicines, as they have the same benzo (alpha) heptalenic moiety [5]. Analgesic and anti-inflammatory effects of this drug have also been reported in animal models [6]. According to its properties, it has been used as a muscle relaxant to treat painful muscle contractions in acute and chronic rheumatic conditions, in traumatology, and in patients with acute low back pain [7].

FBP and THC are used as binary preparations to treat osteoarthritis (calcification), painful syndromes of the vertebral column (neck, back, lumbar region), joint rheumatism, symptomatic treatment of painful muscle contractions, trauma and post-operative conditions. Therefore, simultaneous monitoring of these drugs by using an analytical method is important. According to the previous studies, different methods were illustrated for the detection of FBP and THC separately. Some of these methods achieved the analysis of FBP, which are spectrofluorometry, spectrophotometry [8-10], high-performance liquid chromatography (HPLC) [11-16], liquid chromatography [17], gas chromatography (GC) [18], capillary electrophoresis (CE) [19] methods. THC was analyzed by spectrophotometry [20-22], HPLC [23-29] and thin-layer chromatography (TLC) [30]. All these methods analyzed FBP and THC, separately. When these materials were applied in a binary formulation, the use of only one method is required. The use of different methods for the analysis of both of these two substances is time-consuming, costly and very efficient. During this literature review, it was found that no method can be used to analyze these materials simultaneously to analyze them in a combined dosage formulation. The aim of the study is to develop a validated HPLC method for the simultaneous determination of FBP and THC in binary preparations. The proposed method was optimized and validated according to the USP guidelines [31].

2. LITERATURE REVIEW

2.1. Flurobiprofen

Flurbiprofen (FBP) is a non-steroidal anti-inflammatory drug (NSAID) with significant analgesic, antipyretic, and anti-inflammatory properties. Clinically, it is used to treat rheumatoid arthritis, degenerative joint disease, osteoarthritis, ankylosing spondylitis, acute musculoskeletal disorders, low back pain and allied conditions [1-4].

FBP Chemical Name: 2-[3-fluoro-4-phenylphenyl] propionic acid. It appearance is as a white or almost white crystalline powder. It is freely soluble in ethanol (96 percent) and in methylene chloride, practically insoluble in water. It is soluble in aqueous solutions of alkali hydroxides and carbonates. The melting point at 114 °C to 117 °C. [32]. The chemical structure is shown in (Figure 2.1).

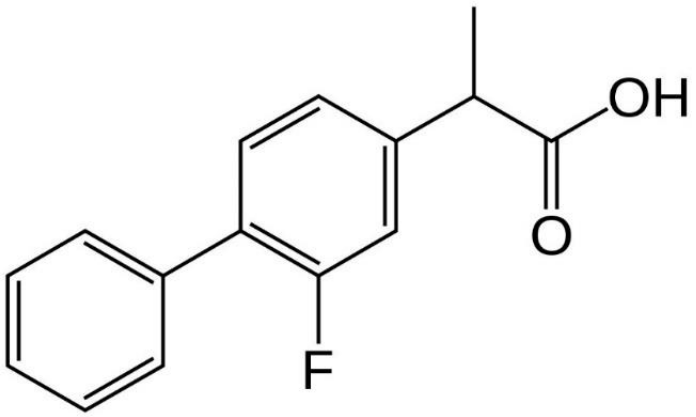

Figure 2.1. Chemical structure of FBP

FBP also was indicated for post-operative ocular inflammation, herpetic stromal keratitis, excimer laser photorefractive keratectomy, and dental gingivitis [33]. FBP previous research shows that it has also been suggested potential topical and systemic use of FBP in radio-protection, colon tumor inhibition, post-irradiation myelosuppression, pain management after foot surgery, and periodontal surgery [33].

2.1.1. Analytical Methods for Flurbiprofen

Several studies investigated analytical procedures for the analysis of FBP alone or in combination with other drugs. These procedures include using spectrofluorometry and spectrophotometry, HPLC, gas chromatography and capillary electrophoresis techniques. These analytical procedures are described below.

2.1.1.1. Spectrophotometric and Spectrofluorometric Methods

Spectrophotometric and spectrofluorometric analysis for the detection of medication can be used when HPLC or GC is not available in the laboratory due to their expensive cost. In contrast to other methods , the main benefit is referred to their low cost, less time, and is easy to do [8]. Numerous studies have attempted to explain spectrofluorometric and spectrophotometric methods.

The analysis of FBP in commercial pharmaceutical preparation and pure sample by spectrofluorometric and ultraviolet (UV) spectrophotometric methods were studied by Yılmaz and Alkan. In both methods, the solvent that was used is acetonitrile, in both methods no extraction process was needed. Optimization parameters were UV-visible spectrophotometer performed the wavelengths in the range190-320 nm, and fluorescence measurements were λ_{ex}=248 nm and λ_{em}=308 nm. It was found to be a linear relationship over a concentration range of 50- 350 ng/ml for spectrofluorometry and 1-14 µg/ml. Relative stander deviation (RSD)values of FBP for spectrofluorometry were less than 3.80% and UV spectrophotometry methods was 3.20%. The previous method was evidenced to be appropriate for analysis of commercial drug dosage form [8].

In another study, was performed by Mathew et.al, shows the usage of spectrophotometric methods for the determination of FBP in combination with other drug dosage forms. In this study, the analysis of FBP and gatifloxacin in commercial pharmaceutical preparation and a pure sample was analyzed by the spectrofluorimetric method using 10 mM sodium lauryl sulfate as a solvent with micellar solubilization to give an enhanced fluorescence. While using the RF-5301 spectrofluorophotometer, excitation wavelengths were found at 259 nm and 294 nm, respectively. The wavelengths of emissions were 315 nm and 470 nm respectively. . The percentage recovery was 98.2-100.1% and 98.6-102% respectively. It was found a linear relationship over a concentration range of 0.005-0.35 µg/mL and 0.005-2.5 µg/mL, respectively, for all validation parameter RSD values, was less than 2. The previous method was evidenced to be appropriate for the analysis of commercial drug dosage form [9].

In the study of Chandran et.al performed, FBP and celecoxib analysis in commercial pharmaceutical preparation and pure sample by spectrofluorimetric method. The solvent used was methanol: 0.1N sulphuric acid (1:1) for FBP and water for celecoxib. Jasco model FP-777, spectrofluorophotometer was used. The excitation wavelengths were found 403 nm and 256 nm respectively. The wavelengths of emission were 314 nm and 250 nm respectively. The percentage recovery was 99.45 to 101.65% and 99.76 to 102.13% respectively. A linear relationship was found in the concentration range of 0-300 ng/mL and l0-1000 ng/ml. The RSD 0.45% and 0.52%, respectively. The past technique was proven to be suitable for the examination of commercial drug forms [10].

2.1.1.2. Chromatographic Methods

2.1.1.2.A. High-Performance Liquid Chromatographic Method

HPLC techniques are widely used due to its high separation effect even at a low concentration level of analyte. They were used for qualitative and quantitative studies for different biological and chemical samples [8, 10]. According to the European Pharmacopeia recommendation, the analysis method for the detection of FBP active drug and in tablet needs using column size (15 × 3.9 cm), a stationary phase of octadecylsilyl silica gel for chromatography with a glacial acetic acid , acetonitrile , water (5:35:60) as mobile phase. Injection volume: 10 µL, and flow rate at 1ml/min [32].

Akhlaq *et.al* evaluated the analysis of FBP by HPLC in commercial pharmaceutical preparation and pure sample. The chromatographic column was Gemini C18 column. Disodium hydrogen phosphate solution (30 mM pH 7.0) and acetonitrile (50:50) was used as the mobile phase. Detection was made at 247 nm. The injection volume was 5 µl, and flow rate of 1.0 mL/min was used. A linear relationship was found in 5-50 µm/mL concentration range. The correlation coefficient was 0.9996. The detection and quantitation limits were estimated to be 0.173 µg/mlL and 0.578 µg/mL respectively. The previous method was appropriate for the analysis of commercial drug dosage forms [11].

In another study showing HPLC methods to determine FBP in combination with other drug dosage forms, Işık and Türköz Acar developed the HPLC method to analyze FBP and Chlorhexidine gluconate simultaneously. The chromatographic column was a stationary phase of Agilent Poroshell 120 EC-C18 column was used with a guard column having the same properties. Sodium phosphate buffer solution (100 mM, pH 2.5) and acetonitrile were used as mobile phase. The injection volume was 20.0µl, and flow rate of 0.5ml/min was

used. Diode array detector at 248 nm was used as the detector of this method. A linear relationship was found to be over a concentration range of 1-25µm/mL. The correlation coefficient was 0.9999. FBP and Chlorhexidine gluconate limit of detection were estimated to be 0.020µg/mL 0.033 and µg/mL, respectively, and limit of quantitation were estimated to be 0.061µg/mL and 0.121µg/mL, respectively. They evaluated the developed method on commercial pharmaceutical preparations [12].

In another study, Aminu *et.al* developed a method to determine FBP and triclosan simultaneously. The chromatographic column was an Agilent ZORBAX SB-C18 column as the stationary phase. A mixture of acetonitrile and 8×10^{-4} mol/L citric acid, pH 3.26 (90:10, v/v) component was used as mobile phase. The injection volume was10.0 µl, and flow rate of 0.3ml/min were used, UV detector was used in this method at 242 nm. A linear relationship was found to be over a concentration range of 15–300 µg/mL and10–200 µg for FBP and triclosan respectively. The correlation coefficient was 0.9991 for FBP and 1for triclosan. The detection limit was estimated to be 0.01µg/mL 0.02 and µg/ml, respectively, and the quantitation limit was estimated to be 0.03µg/mL and 0.06µg/ml, respectively. The previous method was appropriate for the analysis of commercial drug dosage forms [13].

HPLC method to determine FBP in plasma of six healthy Turkish volunteers who had been given 100mg of FBP, described by Yilmaz and Erdem. The optimum chromatographic condition was obtained with Ace C18 column as stationary phase. A combination of acetonitrile –0.05 M potassium dihydrogen phosphate solution (60:40, v/v) pH 3.5 with phosphoric acid was used as the mobile phase. The flow rate was 1 ml/min. UV detector measured at 245 nm. Blood samples were collected into the tubes containing disodium ethylenediaminetetraacetic acid (EDTA) and centrifuged at 4,500 g for 10 min. The extraction process was done by using liquid-liquid extraction. The main advantage of this is that only one step was required to extract the process, which could minimize the process time. A linear relationship was found to be over a concentration range of 0.10 –5.0 mg/mL. The detection limit and quantification limit of FBP were 0.03 and 0.10 mg/mL, respectively. The method analyzed FBP in plasma samples, which can be an advantage of the efficacy of this method in detecting FBP in large numbers of plasma samples. It also was mentioned that the method is suitable for pharmacokinetic and bioequivalence studies [14].

Ünal *et al.* performed HPLC method to detect FBP in plasma too. The optimum chromatographic condition was reversed-phase nucleosil C18 as stationary phase. A combination of 0.1 M sodium acetate and acetonitrile (65:35; v/v) and the pH of the

mobile phase 6.3 was used as mobile phase. The flow rate was 1 ml/min. injection volume was 25µL. The UV detector measured at 248 nm. The extraction process had been done by using liquid-liquid extraction. A linear relationship was found to be over a concentration range 100-40000 ng/mL. The limits of detection and limit of quantification of FBP within-day and between days precision were less than 7.3 and 12.0, respectively. They declared that the method is suitable for pharmacokinetic and bioequivalence studies [15].

Hassan and Alshana described HPLC method to detect FBP and other NSAID's which were ketoprofen, ibuprofen, and etodolac in body fluids, include milk, saliva, and urine. Human milk samples were collected from a 30-year old healthy volunteer who had breastfed for 20 months. Researchers asked volunteers to use the drugs, and the biological fluids were collected. Saliva and urine samples were obtained from a 25-year old healthy male volunteer. The optimum chromatographic condition was reversed-phase C18 as stationary phase. A combination of acetonitrile and 1.0% triethylamine (40:60v/v) and the pH of the mobile phase 1.4 was used as the mobile phase. The flow rate was 0.8 ml/min. The injection volume was 20µl. Diode array detector was used as the detector in this study at 230 nm. The extraction process had been done by salting-out extraction combined with switchable-hydrophilicity solvent liquid-liquid microextraction. The detection limit were in range between 0.04 and 0.18 g/mL, and quantitation limit were in range between 0.13 and 0.61 g/mL [16].

Mei *et al.* developed a method to determine FBP in human plasma by liquid chromatography-tandem mass spectrometry. The chromatographic condition was the Ultimate C18 column. The mobile phase was a combination of water-formic acid [99.9:0.1], and mobile phase B was acetonitrile-formic acid (99.9:0.1). The flow rate was 0.4 mL/min. The injection volume was 5µL. A negative ionization electrospray mass spectrometer was used on multi-reaction monitoring signals. The linearity was over the concentration range 40.0–10000 µg/L. Precision and accuracy intra-day recovery value was 0.2–2.2 %, the relative standard deviation was 3.2–8.4 %. Inter-day recovery value was 0.5–3.4 %, the relative standard deviation was 5.4–8.7 %. The method was suitable for large size of samples. It was also mentioned that the method is suitable for in vivo pharmacokinetics and was then used for bioequivalence studies [17].

2.1.1.2.B. Gas Chromatographic Methods

Yılmaz and Alkan performed a method to analyze FBP in pharmaceutical preparations by gas chromatography-mass spectrometry. The optimum

chromatographic condition was obtained by column HP-5 MS. Helium was set as the carrier gas. Flow rate of 1 mL/min. The derivatization reagent was N-methyl 6 (trimethylsilyl) trifluoroacetamide. There was no extraction process. A linear relationship was found to be over a concentration range of 0.25-5.0 μg/mL. The detection limit and quantitation limit were 0.05 and 0.15 μg/mL, respectively. The previous method was appropriate for the analysis of commercial drug dosage forms without any interference with the pharmaceutical excipient [18].

2.1.1.2.C. Capillary Electrophoresis Method

Hamoudova and Pospisilova applied a different method to detect FBP and Ibuprofen by capillary zone electrophoresis with UV detector. A constant voltage of 30 kV was applied. The UV detector was set at 232 nm. The optimum condition of electrolyte was set with 20 mM N-[2-acetamido]-2-aminoethanesulfonic acid (ACES) buffer and 20 mM imidazole and 10 mM alpha-cyclodextrin of pH 7.3. 2-Naphthoxyacetic acid was used as an internal standard. The linear calibration curve was between 1-60 mg/L for FBP and between 2-500 mg/L. The time of analysis of a single sample was less than 5 min. RSD value was 1.29% for FBP and 1.53 for ibuprofen. The method had lower sensitivity due to the high detection limit of 0.1 mg/L. The method was valid and applied for 10 commercial dosage forms, including tablet, gel, syrup, and cream [19].

2.2.Thiocolchicoside

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

THC Chemical name: N-[(7S)-1,2-dimethoxy-10-methylsulfanyl-9-oxo-3-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-6,7-dihydro-5H-benzo[a]heptalen-7-yl]acetamide, sulfur derivative of cochicosid. Contain 96.0 percent to 102.0 percent (anhydrous substance). Appearance is like yellow crystalline powder and slightly hygroscopic. Sparingly soluble in water, slightly soluble in ethanol (96 per cent), practically insoluble in acetone. [34].The chemical structure of THC is shown in (Figure 2.2).

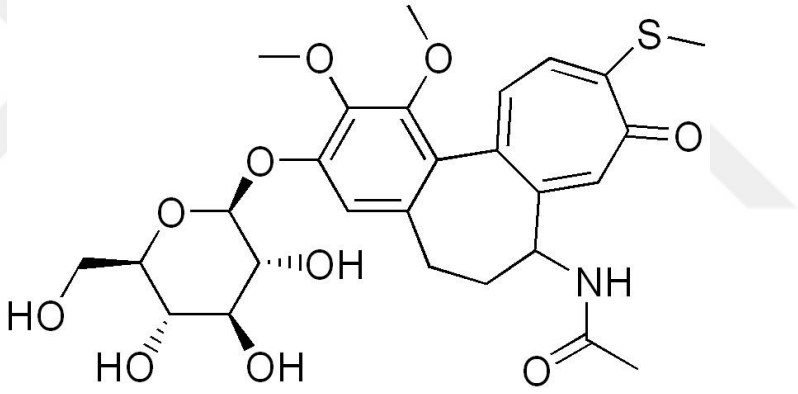

Figure 2.2. The chemical structure of THC.

The mechanism of action of THC is similar to glycine receptor and competitive antagonist properties of the gamma-amino butyric acid (GABA) receptor with a lower effect on nicotinic acetylcholine receptors [20].THC can also be used to treat acute and chronic lumbar and sciatic pain, post-traumatic and post-operative pain, cervicobrachial neuralgia, and persistent torticollis [20].

2.2.1. Analytical Method of Thiocolchicosoid

Several studies investigated analytical procedures for determining THC alone or in combination with other drugs. These procedures include using spectrophotometry, HPLC, liquid chromatography, gas chromatography, and capillary electrophoresis techniques. These analytical procedures are described in the next chapter.

2.2.1.1. Spectrophotometric Methods

It is one of the widely used techniques as spectrophotometric due to their low-cost, and easy to do. Acharjya *et al*. set four simple, precise, and cost-effective methods to determine THC in bulk and commercial pharmaceutical preparation by spectrophotometric methods. Using a double beam UV-Visible spectrophotometer (UV-1800) was selected as the instrument for all methods. The drug follows Beer-Lambert's law in the concentration range of 2.5 – 50.0 µg/mL for all the methods. THC shows with (first method) λmax at 259.0 nm in zero spectrum, (second method) 252.0 nm in the first spectrum, and (third method) 260.0 nm in second spectrum. (Fourth method) is based on the calculation of area under the curve (AUC) for analysis of THC in the wavelength range of 254.0 – 264.0 nm. The fourth method showed no significant difference when applied to a pharmaceutical preparation. The study also shows that all four methods were simple, sensitive, accurate, and precise. Also, they can be used for routine determination of THC in drug dosage forms [21].

Another study presented by Choudhar *et.al.* shows two spectrophotometric methods for the simultaneous determination of THC in combination with diclofenac potassium. The first method depended on absorption, and the second method depended on AUC spectrophotometry. A UV-Visible double beam spectrophotometer (Varian Cary 100) was used. The method that depended on the absorption of THC and diclofenac potassium exhibit λmax at 373.84 nm and 264.99 nm, respectively in methanol. The quantitative estimation of THC was carried out by subtracting the absorption due to diclofenac potassium 333.74 nm using an experimentally calculated absorption factor. The wavelength ranges of 278.51-285.53 nm and 252.56-260.59 nm were selected to detect both THC and diclofenac potassium by AUC respectively in method combined formulations. The drug follows Beer's law in the concentration 4-12 µg/mL for THC and range of 25-75µg/mL for diclofenac potassium for both methods. The percentage assay for the commercial formulation was found to be in the range 98.91–101.72 % for THC and 99.01–100.89 % for diclofenac potassium according to both previously presented methods. Both methods were reported as

simple, accurate, economical, and precise. The previous method was appropriate for the routine analysis of commercial drug dosage forms [22].

Shukla et.al. developed a method that THC and ketorolac were simultaneously analyze used (Shimadzu-1800) UV-Visible spectrophotometer. The absorption maxima were set at 259 nm and 323 nm respectively. A linear relationship was found to be over a range 5–25 mg/mL with a correlation coefficient value of 0.997. The accuracy results of the study of the standard drug in the range of 98.84–100.67% and 98.20–101.69% for ketorolac. The standard deviation value was 0.92 for THC and 1.96 for ketorolac. The detection limit and quantification limit for THC were 1.11 and 3.390 mg/ml, respectively. The ketorolac's detection limit and quantification limit were 0.986and 2.99 mg/ml, respectively. The method has been reported specific, accurate, sensitive enough, and precise. The previous method was appropriate for the routine analysis of commercial drug dosage form [20].

2.2.1.2. Chromatographic Methods

2.2.1.2.A. High-Performance Liquid Chromatographic Method

The method conditions for the determination of THC in active drug and tablet according to the European Pharmacopeia were: stationary phase column size (10 x 2.1 cm) of end-capped ethylene-bridged phenylsilyl silica gel for chromatography (hybrid material), the mobile phase could be mobile phase A: dissolve 0.22 g of ammonium formate in 350 mL of water for chromatography and mix with 25 mL of tetrahydrofuran and mobile phase B: dilute 45 µL of anhydrous formic acid in 900 mL of acetonitrile and mix with 100 mL of tetrahydrofuran. Injection volume: 1.0 µL, with flow rate at 0.4 mL/min and detection value at 370 nm spectrophotometer [34].

Aprile *et.al* developed a method by HPLC-UV that perform simultaneous determination of THC, and its main degradation products are thiocolchicoside S-oxide and 3-O demethylthiocolchicine. According to previous studies, the main degradation products of THC under chemical conditions of acid/base-catalyzed hydrolysis and oxidation are 3-O dimethyl thiocolchicine and thiocolchicoside S-oxide respectively. The separation and quantification of the analyte has been carried out by SynergiTM 4 m Polar-RP 80Å column 150 × 4.6 mm (Phenomenex) as a stationary phase. A combination of A: 20 mM sodium acetate buffer (pH 5.0) and B: methanol: acetonitrile (20:80) were used as a mobile phase. Gradient elution was carried out during this method. Detection was measured at 254 nm with the use of UV detector. The flow rate was 1 mL/min. The injection volume was 20 µL. The linearity was found to be in a range of THC assay in the 5–15g /ml, and for unknown THC

degradation product and known degradation products assay thiocolchicoside S-oxide and 3-O demethylthiocolchicine, in the 0.5–10μg/ml range. The correlation coefficients were greater than 0.999. RSD values of all analytes were less than 2. The method has been found as acute, precise, highly sensitive, specific, and simple to simultaneous analysis of THC and its main degradation products, thiocolchicoside S-oxide and 3-O demethylthiocolchicine. Also, the method has been successfully applied on liquid and solid pharmaceutical dosage forms [23].

It was presented in another study of Jadhav *et.al* that it was preformed simultaneous determination of THC and diclofenac potassium. The optimum chromatographic conditions were: a (Zorbax SB CN 250 mm × 4.6 mm, 5 μm) column was used as the stationary phase. A combination of 5mM sodium dihydrogen phosphate (pH 2.5) as line and methanol as B line was used as the mobile phase. Gradient elution was used during this analysis method. Detection was measured at 258nm using UV detector. The flow rate was 1 mL/min. Injection volume was 20 μL. The method showed linearity in the range of 0.4–13.19 μg/mL of THC and 2.7–82.50 μg/ml of diclofenac potassium respectively. The method has been found simple. Robust, rapid and stable for the simultaneous analysis of THC and diclofenac potassium. The method has been successfully applied to tablet pharmaceutical dosage forms [24].

Sahoo *et.al* developed a method with RP-HPLC using a PDA detector for the determination of THC and Lornoxicam simultaneously. The chromatographic column was Waters Symmetry C18 250 mm × 4.6 mm, 5.0 μm. A mixture consisting of methanol: tetrahydrofuran: acetate buffer (60: 10: 30 v/v); pH (5.5) with glacial acetic acid was used as the mobile phase. The detector wavelength was 382 nm. The flow rate was 0.75 mL/min. The injection volume was 20 μL. A linearity relationship was found 0.1- 40 μg/ml of THC and 0.2-80 μg/mL of Lornoxicam, respectively. The correlation coefficient values were than 0.999. The limit of detection and limit of quantification were 0.007 μg /mL- , 0.021 μg /mL and 0.008 μg /mL, 0.024 μg /ml for THC and Lornoxicam, respectively. The method has been reported as the method having a fast analysis time of less than 5 min. The method has been found very simple, economical, and rapid. Also, the preparation of the sample was easy, with no complicated steps. The method has been successfully applied on pharmaceutical dosage forms with high recovery value and without any interference with excipient that presented in pharmaceutical preparation [25].

Walash *et.al* provided a method for simultaneous determination of THC and Glafenine and THC and floctafenine. The chromatographic column was Waters Symmetry

C18 250 mm × 4.6 mm i.d., 5 µm particle size. The mobile phase was (50:50, v/v) methanol, 0.035 M pH 4.5 phosphate solution for the first mixture and for the second mixture were (70:30, v/v) methanol, 0.03 M pH 4 phosphate solution with detector wavelength at 400nm. The flow rate was 1 mL/min of both methods. The method showed linearity in the range of 0.2–2 µg/ THC in both cases and 20–200 µg/mL for both glafenine and floctefenine, respectively. The correlation coefficient values were 0.997 for THC and 0999 for glafenine in the first mixture and 0.999 for THC and 0.999 for floctefenine in the second mixture, respectively. The detection limit of THC and glafenine were 0.05 and 0.62 µg/mL, and the quantitation limit were 0.14 and 1.87 µg/mL, respectively. The detection limit of THC and floctefenine were 0.02 and 0.70 µg/mL, and the quantitation limit was 0.07 and 2.12 µg/mL, respectively. The method has been found simple, accurate, and precise. The method was mentioned to be suitable and appropriate for routine quality control analysis depending on the good result of the validation process. The method has been successfully applied on pharmaceutical dosage forms with high recovery value and without any interference with an excipient that presented in pharmaceutical preparation [26].

Kumar *et.al* set up a method for the determination of THC and etoricoxib simultaneously. The chromatographic condition was BDS Hypersil C-18 column 250 mm × 4.6 mm, 5-µm particle size as stationary phase. The mobile phase were trifluoroacetic acid buffer(pH 2.6) and acetonitrile (75:25). The detection was carried out at 220nm. The analysis method was carried on for 10 min with a 1.5 mL/min flow rate and the injection volume was 20 µL. A linearity relationship was found to be in a range of 2 to 16 ppm of THC and of 20 to 160 ppm for etoricoxib respectively. The correlation coefficient value of THC was 0.9994 and, 0.9918 for etoricoxib respectively. The Limit of detection of THC and for etoricoxib were 0.1531ppm, 1.1067 ppm respectively. The Limit of quantitation of THC and for etoricoxib was 0.4639 ppm, 3.3537 ppm respectively. The method has been found simple and accurate to simultaneous analysis of THC and etoricoxib. The method has been successfully applied on pharmaceutical dosage forms [27].

In another study, Dhiware et.al presented a method for the determination of THC and etodolac simultaneously. The chromatographic column was C18 HS column (250 × 4.6 mm i.d.) as the stationary phase. The detector was UV/VIS detector at 257nm. The mobile phase was acetonitrile: 20 mM potassium dihydrogen phosphate buffer (65:35). The flow rate was 1 mL/min. The injection volume was 5mL. A linearity relationship was found in the range of 0.5 –10µg/mL of THC and 25–150µg/mL for etodolac, respectively. The correlation coefficient value of THC was 0.999 and, 0.994 for etodolac respectively. The detection limit

of THC and etodolac were 0.095 µg/mL and 0.433 µg/mL, the quantitation limits were 0.29µg/mL and 1.31µg/mL, respectively. The method has been found simple, precise, robust, and accurate to simultaneous analysis of THC and etodolac. The method has been mentioned to be suitable for application to commercial combination tablets dosage form [28].

Wankhede et.al presented a method for the analytical determination of THC and ketoprofen simultaneously. The chromatographic condition used thermo was C18, (250×4.6 mm i.d.) column as stationary phase. A combination of acetonitrile: water: 0.025M phosphate buffer (pH 3.0) (60:30:10) were used as the mobile phase. The detection was carried out at 260nm. 1 mL/min flow rate was used. A linearity relationship was carried out over a range of 4 –20µg/mL of THC and 20–100µg/mL for ketoprofen respectively. The correlation coefficient value of THC was 0.9950 and, 0.9999 for ketoprofen. The detection limit of THC and for etodolac was 0.0379µg/mL and 0.2116µg/mL respectively. The quantitation limit of THC and ketoprofen was 0.1149µg/mL and 0.6412 µg/mL respectively. The method was reported as simple, precise, rapid, selective, and accurate for the simultaneous analysis of THC and ketoprofen. RSD value of all parameters was less than 2. The method has been mentioned to be appropriate for application on a combination pharmaceutical dosage form [29].

2.2.1.2.B. Thin-layer Chromatography Method

El-Ragehy et al. developed a TLC method to determine THC-glafenine and THC-floctafenine in their binary combination drugs form. Chromatographic separation was carried out on silica gel plates GF254 (20-20 cm^2), with usıng of solvent system ethyl acetate:methanol:acetic acid (84:13:3). Analytes were monitored by densitometry measurement at 375 nm. Calibration was performed using a third-order polynomial equation. It was found superior to first-order for quantification range (0.25-25 mg per spot), correlation coefficient, and standard error of estimation. The method has been found precise, and accurate to simultaneous analysis of THC detection of flocatfenien and glafenine in their combination drug dosage forms. The method has been mentioned to be appropriate for application on a combination tablets dosage form [30].

2.3. High-Performance Liquid Chromatography

Chromatography was discovered as a powerful separation technique by the Russian botanist Mikhail Tswett. He investigated numerous plant pigmentation by the simple form of liquid-solid chromatography [35]. Chromatography is the most powerful and general applied technique of separation, determination, and identification of complex component mixture. All types are composed basically of fixed stationary phase, which can be planar or column and mobile phase which differ according to the type of chromatography: gas or liquid. As in case of gas chromatography and in case of liquid chromatography, respectively. The technique of separation depends on different flows of the sample across the stationary phase (fixed phase) by the mobile phase. The interaction of each component mixture and stationary phase will happen at different times, which leads to the separation of each component from each other [36] as showen in Figure 2.3.

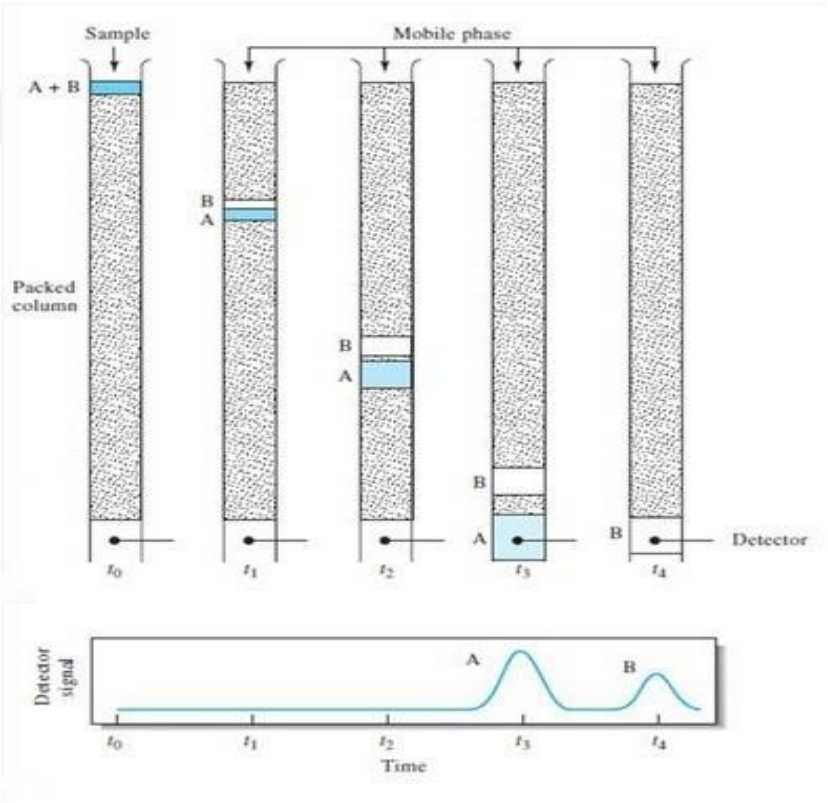

Figure 2.3. Represent the movement of two component through the column and the detector signal of these component

HPLC technique applications are widely used in different fields of life, including analytical separation of organic, inorganic, and biological samples. The technique also

applied in biochemistry science, food sciences, toxicology, pharmaceutical chemistry and environmental science [36]. According to its separation mechanism, HPLC has been classified into different categories base on the type of stationary phase [36].

2.3.1. High-Performance Liquid Chromatography Types:

2.3.1.1. Normal Phase Chromatography

The separation process depends basically on polarity where the stationary phase is polar composed in general from silica and eluted with chloroform, hexane, diethyl ether, methylene chloride and mixtures of these as mobile phase in general. According to this structure, the analyte mixture that is composed of species that have more polarity than others will pass slowly through the column than less polarity, which will pass firstly, that was according to the strong interaction between the surface of the column and more polarity species [37]. The main interaction forces in this type are hydrogen bond [polar interaction] and dipole-dipole bond [38]. The greatest disadvantage point related to this type is the ease of contaminating the polar phase due to the retained amount of analyte sample binding it. This problem is easily solved by binding silanol (Si-OH) groups with the polar functional groups cyano amino group [39].

2.3.1.2. Reversed Phase Chromatography

Reversed-phase HPLC type the separation process depends basically on the motion coefficient between the non-polar stationary phase and mobile phase. Instead of the normal phase HPLC type, the stationary phase here is coated with hydrophobic groups, such as octadecyl C18 bonded groups on a silica base. The mobile phase generally is used for acetonitrile, methanol, tetrahydrofuran, and water [36]. According to this structure, the more polar species move faster than the low polar ones remain in connection with the hydrophobic C18 group of the stationary phase. The main interaction forces in this type are hydrophobic force interactions [38] that reversed the mechanism of normal phase chromatography. Reversed HPLC has become a commonly used technique in more than 70% of all HPLC analyses due to its suitability to separate different species of chemical compounds [39]. In this study, the type of reversed-phase of HPLC is used.

2.3.2. Instrumentation of High-Performance Liquid Chromatography System

HPLC instrument have main parts that include: Mobile phase reservoir, degassing, pump, column, injector, detector, data collection, these part are illustrated in fallowing Figure 2.4.

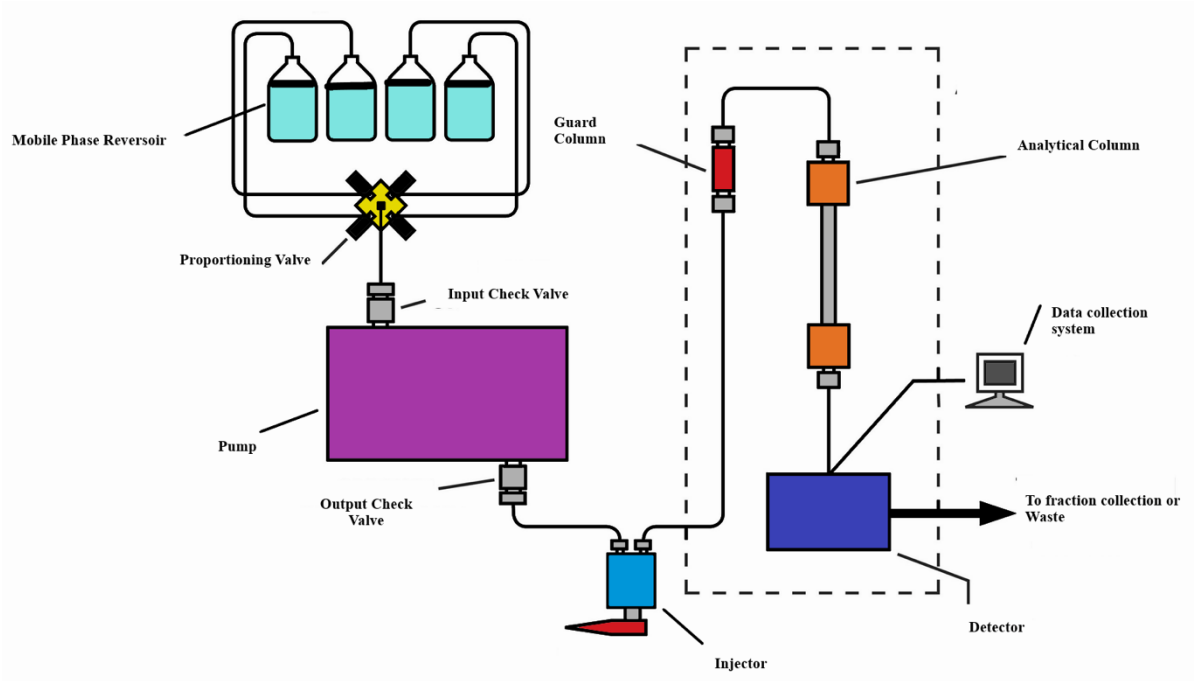

Figure 2.4. Represent HPLC instrumentation**.** The dashed line represent the columns and detector may be thermostatic [41].

2.3.2.1. Mobile Phase Reservoir

Mobile phase reservoir is usually a glass bottle that holds the mobile phase, it must be with a size that can hold enough amount of mobile phase. Generally, these bottles are connected with Teflon tubing called the "inlet line" that provides the delivery and connection of the mobile phase reservoir and the pump. The end of this tube is a sinker which is commonly glass stainless, which also acts as filtration, usually a10µm filter. According to the number line that is available in instrumentation can be recognized as isocratic when one line is available, binary when two lines are available, ternary when three lines are available. These lines are generally important in gradient profiles that are used with reversed HPLC technique in the separation of more than one organic component [37,40].

2.3.2.2. Degassing

Degassing the mobile phase's solvent is an extremely important step before using the device. It allows removing entrapped air bubbles that may cause variation in pressure, incorrect peak, and incorrect detection [40].

2.3.2.3. Pump

The pump is considered one of the most important parts of the HPLC system, which ensures the delivery of the mobile phase through the system at a specific flow rate. The pump has a function to extend constant mobile phase composition isocratic or increasing mobile phase composition gradient [36].

2.3.2.4. Column

The column is an important part of HPLC system. It's the part that supports the stationary phase and allows to connection between the injector and detector of the system. The most important requirement of HPLC column packing material is good stability, holding the pressure that generated during the analysis and the ability to defined particle size with a narrow distribution of particle size [41]. Columns are usually made of polished stainless steel [42].

2.3.2.5. Detector

The detector is the part of an HPLC system that translates the analyte concentration that under goes changes in HPLC column to electrical signal [41]. They are several types of HPLC detectors and, each of them has advantages and disadvantages. The most common types are: Ultraviolet, Refractive index, Evaporative light-scattering, charged aerosol, Electrochemical, Fluorescence, Conductivity, Mass spectrometry, Fourier transform infrared [43]. The most common type of detector is the ultraviolet detector used in pharmaceutical analysis [42]. In this study diode array detectors are used is a type of ultraviolet detector that provides a high quality of detection with full-scale absorbance in the range 0.000 5 to 3 absorbance unit, and near to1% noise level for full scale [43].

2.3.2.6. Data Collection System

In this section, all data information is received from the detector as electrical signal recorded and saved to the computer-based system. [42]. Thus, the system allows the detection of various properties of the whole analysis process like mobile phase volume, injection volume, flow rate, temperature, etc.

2.4. Validation

Validation is a fundamental demand to approve the quality and ability to produce accepted results of any analytical procedure, ensuring the presented analytical process is go for its intended use. There are different guidelines to determine the validation process of HPLC technique, which include: The International Conference on the Harmonization (ICH), which has the significant application of determination the quality and the requirement of validation process of the analytical procedure [44], US Food and Drug Administration (FDA) and US Pharmacopeia (USP). These documents approved that the presented method is suitable for the purpose of the whole of the validation process and obtain it's aim [45]. The typical characteristics of validation of analytical method were: Accuracy, Precision, Specificity, Detection Limit, Quantitation Limit, Linearity, Range, and Robustness.

According to the USP there are four different categories for analysis [46]:

Category I: Deals with measurement quantitation of presented analytical method of active drug substance and their preservatives in completed pharmaceutical preparation and bulk drug substance.

Category II: Deals with measurement impurities of presented analytical method of degradation compound of completed pharmaceutical preparation, and bulk drugs.

Category III: Deals with measurement performance characters of the presented analytical method, such as dissolution and drug release.

Category IV: Deals with identification tests of the presented analytical method.

The validation requirement of each category was illustrated in the fallowing Table 2.1.

Table 2.1 Validation parameters required according to USP[47]

alidation arameter	ategory I	ategory II uantitative limit		ategory III	ategory V
ccuracy	√	√	*	*	X
ercision	√	√	X	√	X
etection limit	X	X	√	*	X
uantitationlimit	X	√	X	*	X
inearity	√	√	X	*	X
ange	√	√	*	*	X
specificity	√	√	√	*	√

*May be required depend on nature of test.

√ required.

X not required.

In addition to previous parameter suitability of system, it is part of the validity of the realistic method. System suitability tests applied to detect the ability of the equipment, electronics device, process of analytical method and detection of sample analysis during the method constitute an integral system. System suitability test parameters usually involve: Resolution (Rs), RSD peak area, Theoretical Plates (N), Tailing factor (T), and Capacity factor (k'). The test is usually performed before or durging analysis and compare its parameter result with accepted limits of criteria [46].

2.4.1. Accuracy

The analytical method accuracy means the nearness of results obtained by that analytical process to the true value. The establishing of accuracy should across be its range. The accuracy can be determined by three different methods. The first method is by applying analytical with known analytic purity (stander material). The second method is by comparing the analytical method result with the method that decides and states as accurate analytical method. The third method applied a synthetic mixture of drug components to analyse specified kown amount within the range of analytical methods. ICH document recommended to measure accuracy can be determine in term of recovery value which applied for three times at minimum three concentration level from covered range of analytical method , usually at each concentration level should be in range 80–120%, and 95% to 105% at each concentration levels of analytical method that performed to the formulated products, individually [46,47].

2.4.2. Precision

The analytical process precision deals with the calculation of agreement degree through results individually when the method is repeatedly applied to multiple samplings of a homogeneous sample. The standard deviation or relative standard deviation RSD is usually used to express precision in the analytical method. Precision can be detected by measurement reproducibility on the variation of laboratories on different days or different equipment. It can be also performed by measuring the variation of different analytics from the same lab. Repeatability can be used for detection precision too by measurement through different periods of time of same laboratory analytic and equipment of the analytical method under

normal circumstances. ICH documents recommended measuring precision in terms of RSD value which has been applied for three-times at a minimum of three concentration levels from a covered range of analytical methods or at 100% six determinations of the test concentration. Precision (repeatability) criteria of analytical method for bulk active pharmaceutical ingredient are that the repeatability should be not more than 1.0%, and not more than 2.0% for formulated products [46, 47].

2.4.3. Specificity

The analytical process stability is used to show the ability of analyte response in the presence of all expected sample ingredients as the starting materials, intermediates, inactive ingredients in the formulated products, and the degradation products [46]. In case of low specificity, a particular analytical method may be compensated by other supporting analytical procedures [47].

The specificity of the assay for an analytical method can be determined by spiked drug substance or product with an appropriate level of impurities, and it is shown that assay result is unaffected with this material [47]. The degradation product can be applied by store the analyte of interest under stress conditions with a 90% sufficient purity degree. For bulk pharmaceutical preparation, typical stress condition was set as heating 50°C, 60°C, ultraviolet light, acidic condition 0.1 mol/L of HCL solution, alkaline condition 0.l mol/L of NaOH solution and oxidant in 3% H_2O_2 solution [47]. For formulated substances, different factors such as heat, light, and humidity are used. Resulting matrix should be analyzed and the peak of our interest should be evaluated with the nearest elution peak and detect its purity and resolution. Ultraviolet/diode array detector was recently used to detect the purity of peak and decide the presences of more than one component [46]. Specificity example of accepted criteria for an impurity that the resolution of all expected component, not more than 1.2 that was produced by stress conditions with level 0.1%, And for analytical assay 1.5 resolution between impurities and peak of interest is desirable [46].

2.4.4. Linearity and Range

The linearity of analytical process is mainly used for obtain results which are proportional to concentration of analyte samples with a given range. Linearity can be detected with the defination in mathematics transformation from synthetic mixture of product or by dilution standard material of active ingredient, for at least five concentration over the range of presented analytical method. Linearity evaluated by statistical calculation

of regression line was recommended, as intercept, correlation coefficient, slope, and residual sum of squares different parameter must be determinate during detection of the analytical process [44, 45].

ICH documents recommended to establish linearity with minimum concentration and with minimum specific range: for analysis of drug or a finished product from 80% -120% of concentration, for impurities 50% -120% of accepted criteria, and for dissolution ±20% over the specified range [46]. The correlation of analytical assay method is with more than 0.999 is considred as a accepted clue and appropriate data of regression line, for impurities more than 0.99 value of correlation were accepted [46].

The range of analytical processes is used to show the integral among the highest and lower concentrations of the analyte. The concentration that was used to determine accuracy, precision and linarite must be included in the range. The unit of range in analytical method defined by percent or part per million as the results of presented analytical method. Validation of the range is estimated when the linearity, precision and accuracy produce accepted results at the same concentration level of the analytic used [47].

2.4.5. Robustness

The analytical process robustness shows the capacity of the presented method to be unchanged and produce a reliable result with small change (variation) in different parameters. Parameter that can show the robustness of the presented method such as flow rate column temperature, injection volume, composition of mobile phase, and wavelength [46].

The robustness of the analytical method also may estimated system suitability under normal conditions. Also, it could be evaluated during the determination and development of the presented analytical method [46].

2.4.6. Limit of Detection

The analytical process limit of detection LOD measures the lowest concentration value that can be detected qualitatively (not quantitatively). The unit of range in the analytical method is presented by percent or part per billion as the concentration of analytic at presented analytical method. The limit of detection can be according to the non-instrumental and instrumental methods. Detection in the non-instrumental method could be made with the help of analyzing the sample known concentration and deciding the minimum level of analytic reliably detected. ICH document recommendation for the instrumental

method was declared that limit of detection would be accepted with signal-to-noise ratios are 2:1 or 3:1. The slope of the curve equation and the standard deviation of responses also can be used for the determination of the detection limit of the analytical process [47]. limit of detection expressed as the fallow [46]:

$$\text{Limit of detection} = 3.3\times (\sigma / S)$$

Where σ = the standard deviation of the response.

S = the slope of the calibration curve.

Calculation of standard deviation can be done with two ways the first one by analyzing suitable sample in the number of standard and calculating their standard deviation, the second one by residual standard deviation or standard deviation of y−intercepts of regression lines could be used as stander deviation. According to the previous method used, the LOD had to be considerable validation of the presented method, and the anlayte sample should be prepared with that limit or known to be close to detection limit [46].

2.4.7. Limit of Quantitation

The analytical process limit of quantitation LOQ is to measure the low level of the compound as degradation product or impurities in the sample matrix. “The lowest amount of analyte sample that can be determined with acceptable precision and accuracy under the stated experimental conditions”. The unit of range in the analytical method presented by percent or part per billion as the concentration of analytic at presented analytical methods. The limit of quantitation can be determined according to non-instrumental and instrumental methods. The non-instrumental method could be detected by analyzing the sample with analytic known concentration and deciding the minimum analyte level, which was produced acceptable accuracy and precision. For instrumental method ICH guideline document that limit of detection can be accepted with signal-to-noise ratios are 10.1It also could be defined by determination of the slope of the curve equation and the standard deviation of responses [47].limit of quantitation expressed as the fallow [46]:

$$\text{Limit of quantitation} = 10\times (\sigma / S)$$

Where σ = the standard deviation of the response.

S = the slope of the calibration curve.

Calculation of standard deviation can be done in two ways the first one by analysing suitable number of standard sample and calculate their standard deviation, the second one by the residual standard deviation or standard deviation of y−intercepts of regression lines could be used as stander deviation [46]

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals and Reagents

FBP and THC 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.

3.1.2. Instruments

The Agilent 1260 Infinity HPLC system (Agilent Technologies, USA) was used for the studies. The system consisted of a G1311B quaternary pump, a G1329B standard autosampler, a G1316A thermostated column compartment, and a G4212B diode array detector. The chromatographic separation of data signal was presented by Agilent ChemStation software [Rev. B. 04.03-SP2 (105). Chromatographic separation was performed on an Agilent Poroshell 120 EC -C18 (3x 150 mm, 2.7 μm) column. Ultrapure water was obtained using the Millipore Simplicity water purification system (Darmstadt, Germany).

3.2. Methods

3.2.1. Mobile Phase Preparation

The mobile phase was an acetate buffer solution with a pH 4.8 100 mM . Mobile phase prepared by acetic acid and sodium acetate and confirmed as A mobile phase. After adjusting the pH and completing the volume, the buffer solution was filtered through a 0.45 μm membrane filter and then degassed in a bath sonicator for 15 minutes. Acetonitrile was confirmed as B mobile phase. The gradient profile (time, %B) was set as follows: 0 min, 15% B; 5 min, 45% B; 6 min, 80% B; 12 min, 80% B; 13 min, 15% B; 18 min, 15% B.

3.2.2. Standard Solutions Preparation

Standard stock solution with a concentration of 1000 ppm was prepared by dissolving 10.0 mg of standard material in a 10 mL volumetric flask. FBP was dissolved in ACN, whereas THC was dissolved in methanol. A mixture of FBP and THC 500 ppm was prepared by mixing equal volumes of the stock solutions. The standard solutions were prepared at

concentrations of 5.0, 10.0, 20.0, 50.0, 80.0 and 100.0 ppm. Standard solutions with concentrations of 8.0, 15.0, 30.0, and 70.0 ppm were selected as quality control (QC). A mixture of mobile phase A acetate buffer: and B acetonitrile (85:15) was used to dilute the standard solutions.

3.2.3. Sample Solutions Preparation

3.2.3.1. Tablet Samples Preparation

Five 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 procedures were applied to this solution due to FBP and THC concentration differences in samples. Prepared solutions were injected and analyzed under optimum conditions.

3.2.3.2. Gel Samples Preparation

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

3.2.4. Development of the Method

The main objective of this research was to create an HPLC method for the determination of FBP and THC simultaneously. The optimum condition of this study was selected depending on the examination of different parameters, including wavelength, pH of buffer solution, temperature, and gradient profile of the mobile phase, flow rate, and injection volume. The best peak shape, peak area value with the best peak resolution was evaluated as the optimum condition of the method.

3.2.5. Validation of the Method

Validation of the analytical method was presented according to the USP guideline [31] of following different parameters.

3.2.5.1. System Suitability

System suitability test (SST) was applied to indicate that the presented method and the used system are suitable and acceptable according to the SST criteria. SST was performed by investigating capacity factor, resolution, number of theoretical plates, the

relative standard deviation of the peak areas, and tailing factor. For this purpose, a sample solution of FBP and THC was injected six times, and the results were evaluated.

3.2.5.2. Stability

The method stability was presented to determine until how much time the analyte can be stable and produce recovery value with the accepted range. Stability was detected by analyzing quality control samples kept in an autosampler for 72h. Results were presented in terms of recovery value and compared with freshly prepared solutions.

3.2.5.3. Specificity

The specificity of the method was evaluated to understand the ability of separation power of the method against some stress conditions. For this purpose, standard solutions containing equal molar FBP and THC were kept in 0.1 M hydrochloric acid (A), 0.1 M sodium hydroxide (B) and 10% hydrogen peroxide (C) solutions in a boiling water bath. After cooling and filtering of the solutions, they were injected for analysis. Obtained chromatograms were evaluated to control whether there was interference or not.

3.2.5.4. Linearity

The method linearity was calculated with six levels of concentration (5.0, 10.0, .20, 50.0, 80.0, 100.0 ppm). The peak areas were recorded, and the calibration curve was plotted for peak area concentration. Linearity of the method usually estimates the term of the correlation coefficient. LOD and LOQ calculation also was involved.

3.2.5.5. Precision and Accuracy

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

3.2.5.6. Robustness

The analytical process robustness is evaluated by making small and deliberate changes on analysis parameters and observing the effect of this parameter. Selected analytical method parameters were the pH value of buffer solution, temperature, and

wavelength. All parameters were changed, and results were compared with the optimized method results.

4. RESULTS

4.1. Optimum Method Conditions

During the optimization procedure, different buffers, pH, injection volumes and temperatures values were investigated. Peak shape, area/ height values of the peak, number of theoretical plates, capacity factor, resolution, tailing factors were examined to select the optimum conditions. Phosphate buffer solutions at different pH values were evaluated as an aqueous mobile phase system. The reason for this selection was previous study for the FBP [12]. But, the peak of THC were not appropriate for the analysis. Hence, it was tried with a different buffer solution. Acetate buffer solution gave good results for the simultaneous determination of FBP and THC. After determining buffer type, pH value was studied and pH 4.8 value was chosen as the mobile phase pH.

The determination of instrumental parameters was done by the following procedure. Laboratory temperature (25ºC) was selected as the starting point. Obtained chromatograms showed that increasing of the temperature was crucial for better separation, peak shape, and related parameters. 40 ºC temperature value was selected for the method. Flow rate and injection volume values were also evaluated by investigating the same parameters. 0.5 mL/min and 5 µL were selected for flow rate and injection volume respectively. The standard stock solutions of 100 ppm of FBP and THC separately were firstly examined under optimum conditions of the method to determine their retention time. As a result of optimization studies, the chromatographic conditions of the developed method was showed in Table 4.1. A chromatogram for the standard stock solution of 100 ppm THC, the standard stock solution of 100 ppm FBP, and the standard mixture of FBP and THC was illustrated in Figures 4.1, 4.2 and 4.3, respectively.

Table 4.1. Optimum method conditions for the simultaneous analysis of FBP and THC by HPLC-UV method.

Column	Agilent Poroshell 120 EC-C18 (3x150 mm, 2.7 µm)						
Mobile Phase	100.0 mM, pH 4.8 Sodium Acetate buffer and ACN						
Injection volume	5.0 µL						
Wavelength	UV/DAD: 248 nm						
Gradient Profile	Time(mıin)	0	5	6	12	13	18
	ACN(%)	15	45	80	80	15	15
Flow Rate	0.5ml/min						

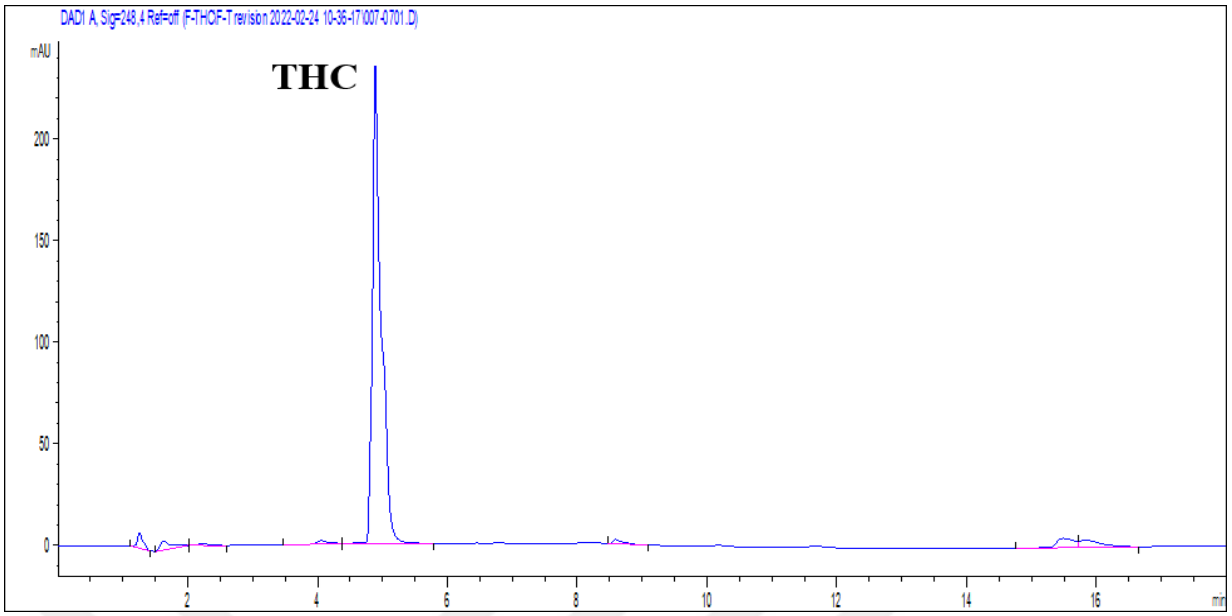

Figure 4.1. Obtained chromatogram for 100 ppm THC under optimized conditions.

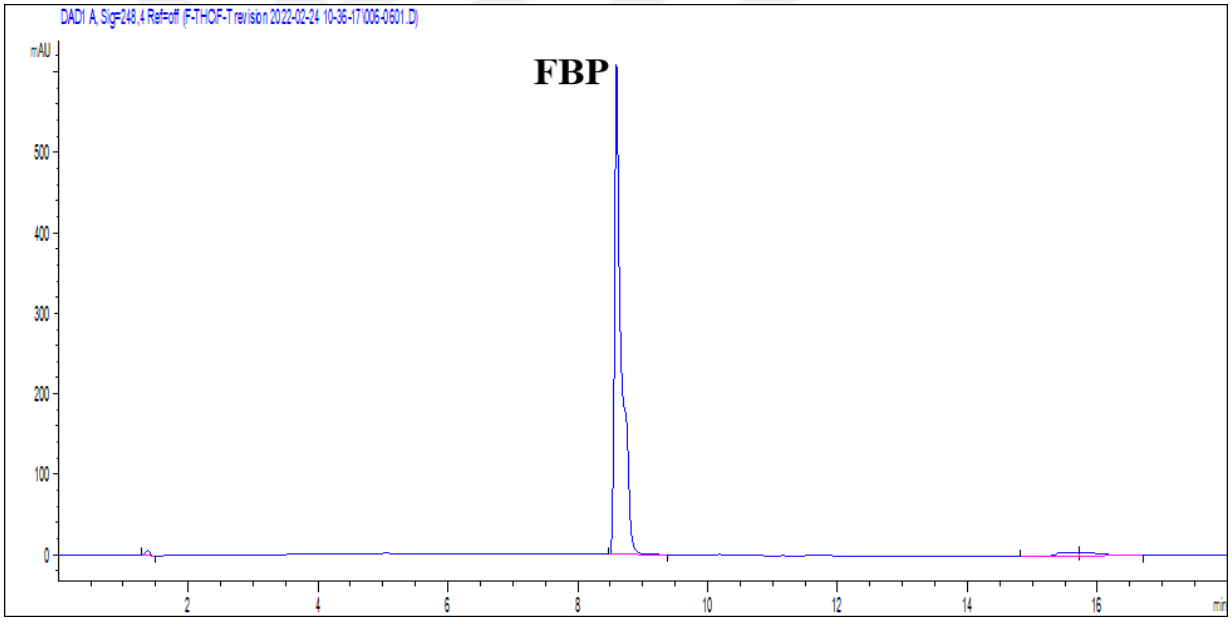

Figure 4.2. Obtained chromatogram for 100 ppm FBP under optimized conditions.

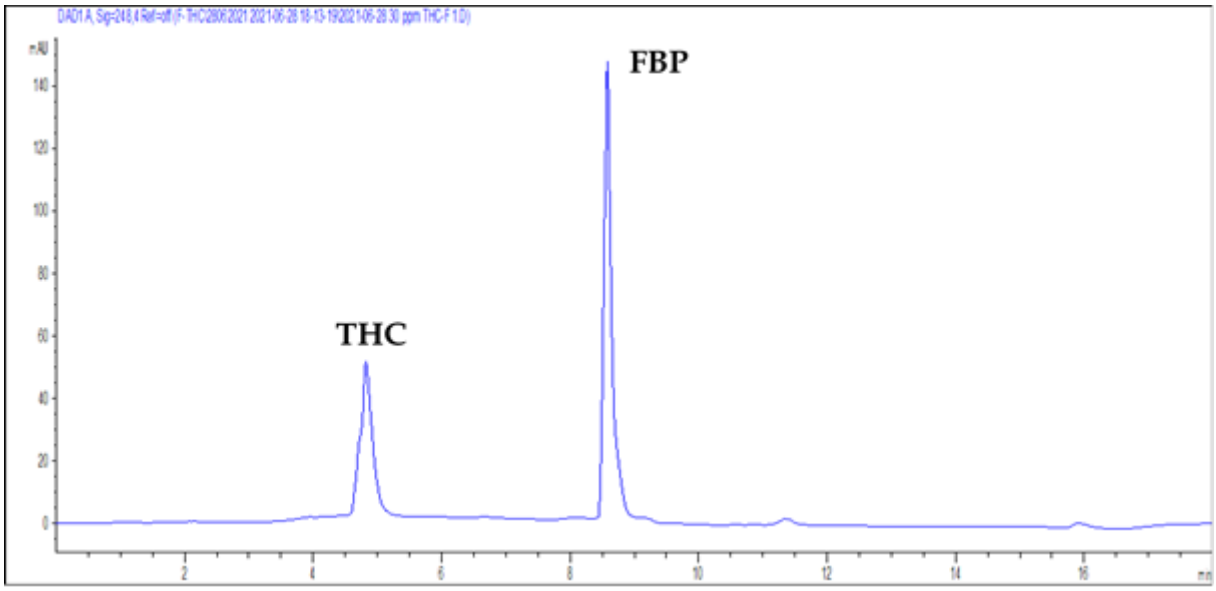

Figure 4.3. Obtained chromatogram for 30 ppm FBP and THC under optimized conditions: Agilent Poroshell 120 EC-C18 (3x150 mm, 2.7 µm) column, 100 mM pH 4.8 acetate buffer and ACN 5.0 µl injection volume, 0.5 ml/min flow rate, 40ºC, 248 nm

4.2. Method Validation

4.2.1. System Suitability Test

The suitability test of the system was utilized for the optimized method. Suitability parameters were defined by applying six repetitive analysis of 30 ppm standard solution, and obtained results were presented in Table 4.2. Depending on the following result, the system and analytical process were found to be appropriate for the analysis of FBP and THC, all suitability parameters of the system were within the recommended limits.

Table 4.2. System suitability parameters and comparison of them with related criteria (n=6)

Parameter	Recommendation	FBP	THC
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

4.2.2. Stability

The stability parameter was used to detect how much time the analyte was stable. The stability was set by calculation recovery value of auto sample QC concentration during 72h, and compared to the recovery value of QC sample that was freshly prepared. Results are demonstrated in Table 4.3.

Table 4.3. Obtained recovery results for the stability study on QC samples for 72 h

Concentration [ppm]	Recovery%		Change%	
	FBP	THC	FBP	THC
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

4.2.3. Linearity and Range

The analytical process linearity was evaluated at six concentration levels (5.0, 10.0, .20.0, 50.0, 80.0, 100.0 ppm). The calibration curve was plotted for peak area versus concentration. LOD and LOQ calculation also was involved. The calibration curves of FBP and THC are shown in Figure 4.4, and calibration curves parameters are illustrated in Table 4.4.

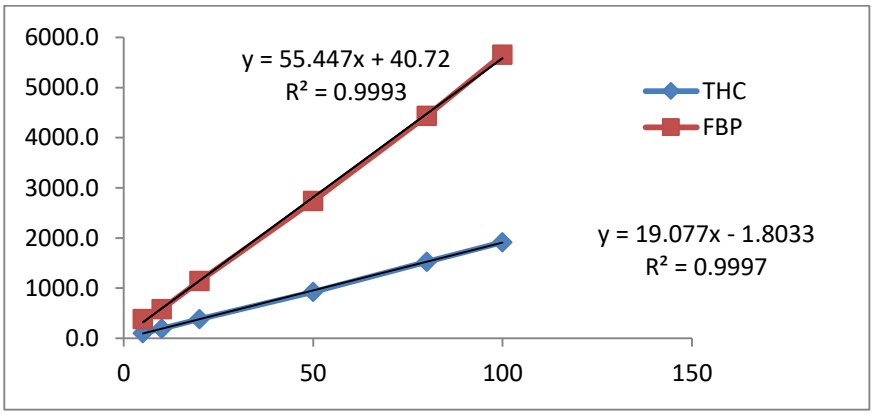

Figure 4.4. Linearity data for calibration curves of FBP and THC

The correlation coefficient (R^2) was found to be 0.9993 for FBP 0.9997 for THC,

respectively. A linearity relationship was found to be over the concentration range of 5-100 ppm of FBP and THC.

LOD and LOQ of the presented method were calculated by uses of calibration carve, which was investigated through three different days of FBP and THC individually. The formula that employed was 3.3 x (σ /S) for LOD and 10 x (σ /S) for LOQ in which σ represents the standard deviation of recovery values of standard mixture and S presents slope of formed calibration. The results of the linearity study are listed in Table 4.4.

Table 4.4. Calibration curve parameters of the developed method for FBP and THC

Parameter	FBP	THC
Linearity range[ppm]	5.0-100.0	5.0-100.0
R^2	0.9993	0.9997
Intercept	40.72	1.80
Slope	55.447	19.07
LOD	0.95	1.57
LOQ	3.18	5.23

4.2.4. Specificity

To evaluate the specificity of the analytical method, the analyte was presented under various stress conditions. For this purpose, 1.0 mL of 70 ppm standard solution was added to three different tubes containing 0.1 M hydrochloric acid (A), 0.1 M sodium hydroxide (B) and 10% hydrogen peroxide(C), separately. Then all the tubes were placed in a boiling water bath for an hour, then the solutions were cooled to room temperature (25ºC). The prepared solutions were filtered by a membrane filter with a 0.45 μm filter and then injected into the HPLC system after being transferred to their vial.

The chromatograms obtained from forced degradation studies are shown in Figures 4.5, 4.6 and 4.7, respectively. According to the chromatograms obtained, there were no interferences. The peaks of the analytes were shown clearly. This situation shows the specificity of the method.

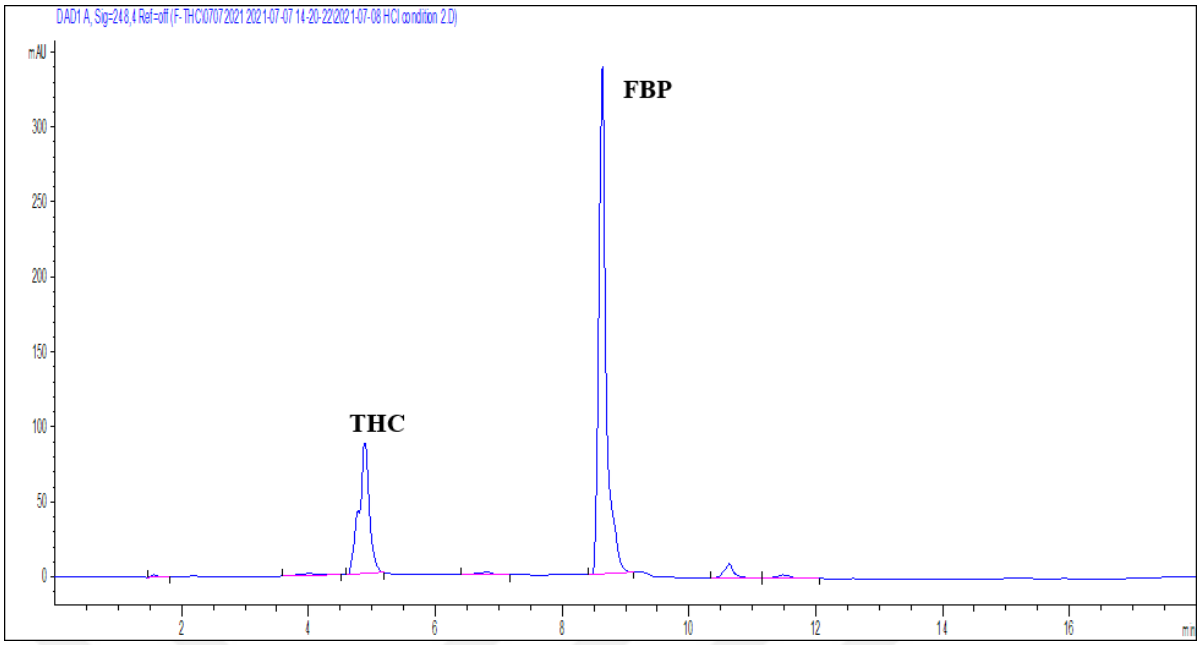

Figure 4.5. The obtained chromatogram of 70 ppm FBP-THC mixture after interatciton with 0.1 M HCl solution in boiling water

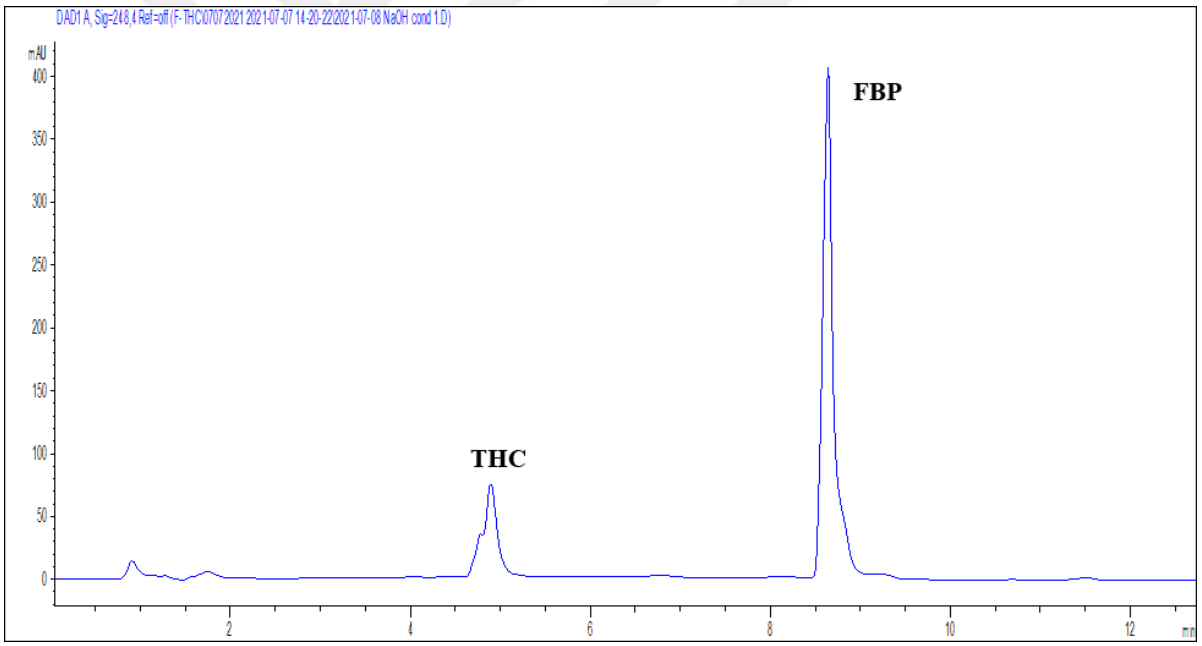

Figure 4.6. The obtained chromatogram of 70 ppm FBP-THC mixture after interatciton with 0.1 M NaOH solution in boiling water

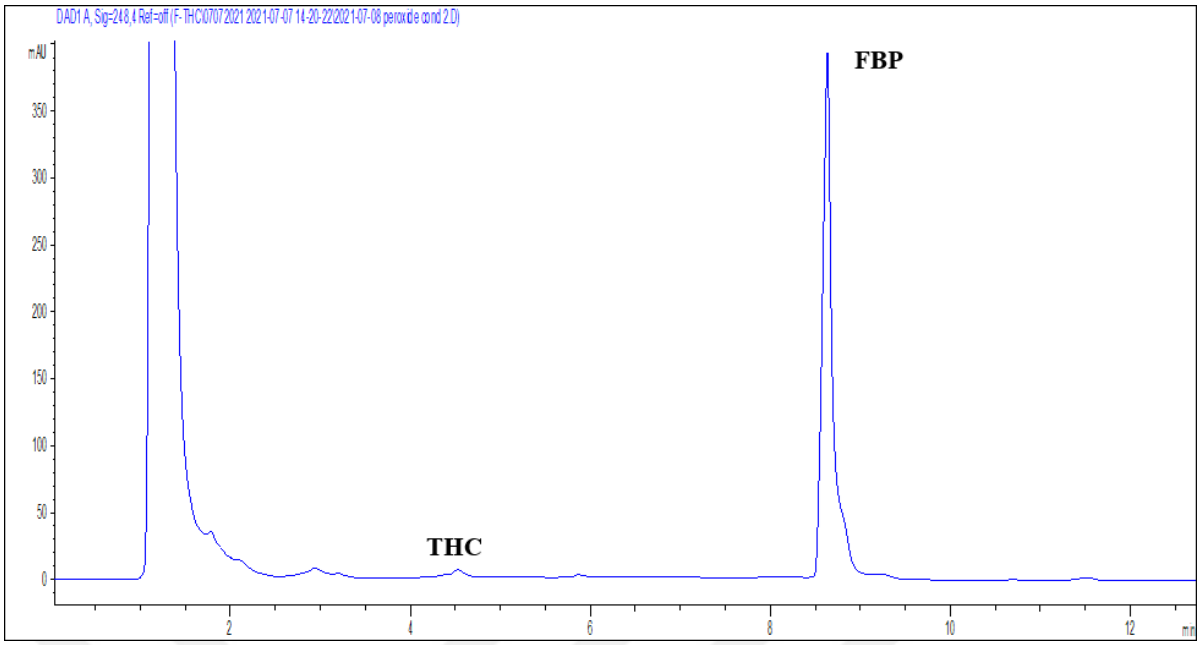

Figure 4.7. The obtained chromatogram of 70 ppm FBP-THC mixture after interatciton with 10% H_2O_2 solution in boiling water

4.2.5. Accuracy and Precision

The accuracy and precision of the method were evaluated by using triplicate analysis of QC samples of which concentrations 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 the accuracy, RSD values of the results were used for the precision. All results for accuracy and precision studies were presented in Table 4.5.

Recovery values for within-day and between-day studies ranged from 95.7 to 101.2% and 99.6 to 104.0% for FBP; 97.7 to 101.6 and 99.8 to 102.6% for THC. The RSD value was calculated as the highest value 0.98 and 1.15 for FBP and 1.27 and 1.18 for THC for within-day and between-day. According to these results, the recovery and RSD values were within the accepted limits. According to these results, it can be said that the developed method is accurate and precise.

The recovery values of added amounts of FBP and THC in pharmaceutical preparations, including tablets and gel forms, were calculated. For this purpose, sample solutions were prepared as two sets, and known amounts of FBP and THC were added and analyzed. The analytical results were calculated using the calibration curve equation. The amounts of FBP and THC in the commercial samples were calculated using the calibration curve equation and then the recovery values were calculated for an added amount of FBP

and THC (Table 4.6).

Table 4.5. Within-day and between-days results of the accuracy and precision studies for FBP and THC.

Concentration [ppm]		Within-day (=3)		Between days (n=9)	
		Recovery%	RSD	Recovery%	RSD
FBP	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
THC	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

Table 4.6. Results of recovery studies on spiked sample solution.

Samples	FBP			THC		
	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 values for FBP and THC were 99.5 and 97.0%, respectively, in the tablet (mean, n = 3) and 98.6 and 99.3% (mean, n = 3), respectively, in the gel formulation.

4.2.6. Robustness

The robustness of the analytical process was evaluated by making small variations of the method parameters. The parameters selected for this step were temperature (± 2ºC), mobile phase pH (± 0.1), and wavelength (± 8). Three-time the standard QC solution was prepared and introduced to the system for analyzing simultaneously with the standard calibration solutions. The QC standard solutions recovery values were determined using the equations of the calibration curve, as in the accuracy and precision studies.

The results of the robustness study are shown in Table 4.7. It was found that the changes in pH, temperature, and wavelength had no significant effect on the results.

Table 4.7. Obtained results for the robustness study

Parameters		**Concentration [ppm]**	**Recovery**	
			THC	**FBP**
Temperature	**38ºC**	8.0	93.3	90.1
		15.0	84.6	84.9
		30.0	96.1	98.7
		70.0	102.9	105.6
	42ºC	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

4.3. Sample Analysis

The analysis of tablet and gel samples containing FBP and THC in binary commercial

samples was presented by the valid and developed method. Calculating the amounts of FBP and THC in the commercial samples was done using calibration carve and its equation. Results were obtained and compared with claimed values. These results are presented in Table 4.8. A chromatogram showing the analysis of FBP and THC in the tablet solution is given in Figure 4.8.

Table 4.8. Results of the analysis on commercial pharmaceutical preparation.

Sample	**FBP**		**THC**	
	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

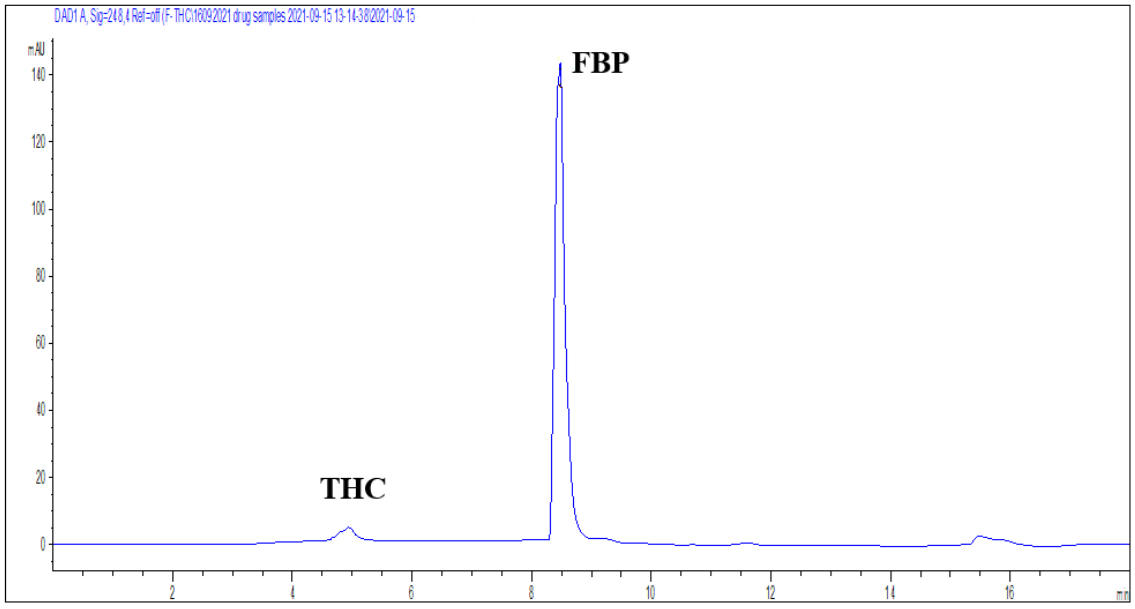

Figure 4.8. Obtained chromatogram of 40 ppm FBP and 16 ppm THC prepared from tablet formulation under optimized conditions.

5. DISCUSSION

In this study, a new HPLC method was created for application for the analysis of FBP and THC simultaneously without any interference. FBP and THC are used as binary preparations to treat osteoarthritis, joint rheumatism, muscle contractions, trauma and post-operative conditions. The method was proved to be accurate precise, the recovery value of the method was within the accepted limits and the RSD values were not more than 2. The analysis process shows that the validation parameters' values are appropriate and accepted. The optimum method condition that was detected according to the best resolution, best shape of peak, and spectrum. UV/DAD detector was applied at 248 nm wavelength, with 0.5mL/min flow rate, the column temperature was set at 40ºC, with 5.0µl injection volume was chosen, the mobile phase was decided to be used (85:15) sodium acetate pH (4.8) and ACN for A and B line respectively. ACN was chosen as organic solvent according to the potential power of separation. Gradient profile (time, %B) set was as follows: : 0 min, 15% B; 5 min, 45% B; 6 min, 80% B; 12 min, 80% B; 13 min, 15% B; 18 min, 15% B. The correlation coefficient was found 0.999 for FBP and THC, and a linearity relationship was found to be over a range of concentration 5-100 µg/mL. FBP and THC, according to the developed method, were applied for the assay of commercially binary dosage form, for tablets and gel dosage forms that contain FBP and THC.

The recovery % of tablets was found to be 99.5 and 97.2 for FBP and THC (mean, n = 3). The recovery % of gel was found to be 98.6 and 99.3 for FBP and THC (mean, n = 3). For accuracy and precision recovery, values for within-day and between-day studies ranged from 95.7 to101.2% and 99.6 to 104.0% for FBP; and 97.7 to 101.6 and 99.8 to 102.6% for THC. The RSD value for within-day and between-day studies was calculated highest value as 0.98 and 1.15 for FBP and 1.27 and 1.18 for THC, respectively. According to previous results, the % RSD values calculated from the method was not more than 2, which indicated the accuracy and precision of the presented analytical method. Specificity of the method was by investigation chromatograms of the solutions prepared under stress conditions according to the chromatograms obtained, there were no interferences. The peaks of the analytes were shown clearly. The robustness of the analytical process was investigated by making a few changes in the parameters of the method. The parameter that had been investigated was pH, temperature, and wavelength, these small change has not any significant effect on the results.

According to previous results of all validation test that was applied to the method

was found that presented analytical method is appropriate for the determination of FBP and THC. The most important point of this method is the simultaneous analysis of FBP and THC tablet and gel form. According to previous research, no method determines these two drugs simultaneously. In addition, the method does not require any prior extraction process. Therefore, it is simple and valid and can be used for the routine quality control of FBP and THC in gel and tablet form.

CONCLUSION

A simple, rapid, and robust stability analysis method was presented by HPLC for the simultaneous determination of FBP and THC in perpetrated form. The method was found to be suitable in terms of accuracy, precision, specificity, linearity, and robustness ruggedness. It was successfully applied to HPLC analysis of commercial tablets and gel samples containing FBP and THC without any prior extraction process. All results were appropriate and accepted; therefore, the method was approved to be suitable for the purpose of use in routine quality control and an assay of drugs.

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