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YEDİTEPE UNIVERSITY
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DEPARTMENT OF PHARMACEUTICAL CHEMISTRY

HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS DETERMINATION OF FLURBIPROFEN AND CHLORHEXIDINE GLUCONATE

MASTER'S THESIS

BERİL DENİZ IŞIK

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SUPERVISOR

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THESIS APPROVAL FORM

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APPROVAL

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 Administrative Board of Institute with decision dated ~~29.12.2017~~ and numbered 2017/27-02

Prof. Dr. Bayram Yılmaz
Director of Institute of Health Sciences

DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

Beril Deniz IŞIK

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Işık, B. D. (2017). HPLC Method Development and Validation for the Simultaneous Determination of Flurbiprofen and Chlorhexidine Gluconate. Yeditepe University Institute of Health Sciences, Thesis on Pharmaceutical Chemistry Master of Science Degree Programme, Istanbul.

Flurbiprofen (FBP) is a strong nonsteroidal antiinflammatory drug, which has analgesic, antipyretic and antiinflammatory effects. Chlorhexidine gluconate (CHG) is a potent antibacterial agent. Nowadays, FBP and CHG in combination are used in some commercial preparations. Thereby, there is a need for a method that analyzes these two drugs together. In this study, an analysis method was developed for simultaneous determination of FBP and CHG by using high performance liquid chromatography (HPLC) technique. During analyses, Agilent Poroshell 120 EC-C18 column was used as stationary phase. Phosphate buffer solution (100 mM, pH 2.5) and acetonitrile were used as mobile phase. The chromatography was performed by gradient elution at a flow rate of 0.5 ml/min. The column temperature was set at 30˚C and the injection volume was 20 µl. Analytes were detected at 248 nm with using a diode array detector (DAD). The developed method was validated according to United States Pharmacopoeia guideline. The method was found to be linear in the concentrations range between 1–25 ppm. Correlation coefficient value was calculated as 0.9999 for FBP and CHG. The method was found suitable in terms of specificty, linearity, accuracy, and precision. It was applied successfully for HPLC analysis of commercial samples that includes FBP and CHG.

Key words: Flurbiprofen, chlorhexidine gluconate, HPLC, analysis, validation

ÖZET

Işık, B. D. (2017). Flurbiprofen ve Klorheksidin Glukonatın Eşzamanlı Tayini İçin HPLC Yöntemi Geliştirilmesi ve Validasyonu. Yeditepe Üniversitesi Sağlık Bilimleri Enstitüsü, Farmasötik Kimya Yüksek Lisans Tezi, İstanbul.

Flurbiprofen (FBP), analjezik, antipiretik ve antienflamatuar etkilere sahip olan, kuvvetli bir nonsteroidal antienflamatuar ilaçtır. Klorheksidin glukonat (CHG), güçlü bir antibakteriyel ajandır. Günümüzde, FBP ve CHG kombine halinde bazı ticari preparatlarda kullanılmaktadır. Dolayısıyla, bu iki ilacın bir arada analiz edilmesini sağlayan bir yönteme ihtiyaç vardır. Bu çalışmada, FBP ve CHG eşzamanlı tayini için yüksek performanslı sıvı kromatografisi (HPLC) tekniği kullanılarak analiz yöntemi geliştirilmiştir. Yöntem sırasında sabit faz olarak Agilent Poroshell 120 EC-C18 kolonu kullanılmıştır. Hareketli faz olarak sodyum fosfat tampon çözeltisi (100 mM, pH 2.5) ve asetonitril kullanılmıştır. Kromatografi 0.5 ml/min akış hızında gradyan elüsyon ile uygulanmıştır. Kolon sıcaklığı 30˚C ve enjeksiyon hacmi 20 µl olarak ayarlanmıştır. Analitler 248 nm'de diyot dizisi dedektörü (DAD) kullanılarak saptanmıştır. Geliştirilen yöntem, Amerikan Farmakopesi'ne göre valide edilmiştir. Yöntem, 1–25 ppm konsantrasyon aralığında doğrusal bulunmuştur. FBP ve CHG için korelasyon katsayıları 0.9999 olarak hesaplanmıştır. Yöntem spesifiklik, doğrusallık, doğruluk ve kesinlik parametreleri yönünden uygun bulunmuştur. Yöntem, FBP ve CHG içeren ticari örneklerde HPLC analizi için başarıyla uygulanmıştır.

Anahtar kelimeler: Flurbiprofen, klorheksidin glukonat, HPLC, analiz, validasyon

LIST OF SYMBOLS AND ABBREVIATIONS

ACN	Acetonitrile
C18	Carbon 18
CE	Capillary Electrophoresis
CHG	Chlorhexidine Gluconate
DAD	Diode Array Detector
ECD	Electrochemical Detector
EP	European Pharmacopoeia
FBP	Flurbiprofen
FD	Fluorescence Detector
FDA	US Food and Drug Administration
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
ICH	International Conference on Harmonization
k'	Capacity Factor
kV	Kilowatt
LC	Liquid Chromatography
µl	Microliter
µm	Micrometer
mg	Milligram
ml	Milliliter
mM	Millimolar
min	Minute
MSD	Mass Detector
N	Theoretical Plates
nm	Nanometer
NSAID	Nonsteroidal Antiinflammatory Drug
ppm	Parts Per Million
QC	Quality Control
R^2	Correlation Coefficient
RID	Refractive Index Detector
RSD	Relative Standard Deviation
R_s	Resolution

S	Slope
SD	Standard Deviation
SST	System Suitability Test
T	Tailing Factor
USP	United States Pharmacopoeia
UV	Ultraviolet
v/v	Volume/Volume
w/v	Weight/Volume

1. INTRODUCTION AND AIM

Flurbiprofen (FBP) is a nonsteroidal antiinflammatory drug with excellent antipyretic, analgesic and antiinflammatory properties. It provides a pain relief rapidly as well as a strong antiinflammatory treatment for the painful inflammatory conditions of the mouth and throat (1-3). Chlorhexidine gluconate (CHG) is a potent antiseptic from biguanides. It is used to treat the swelling and bleeding gums associated with gingivitis. It is bactericidal, fungicidal and virucidal, causing cell wall decomposition which leads to the loss of the components of the cell (4-6). FBP and CHG in combination are applied for the treatment of dental illnesses, throat and mouth infections.

Drug analysis is very important in guaranteeing the quality and reliability of drugs. For this purpose, high performance liquid chromatography (HPLC) is the most common technique used through the pharmaceutical industry to confirm the identity of a drug and to acquire quantitative results. That is because HPLC provides high separation with highly accurate results. In addition, it is quick and customizable (7).

According to literature, various methods have been reported for the determination of FBP and CHG individually. These include spectrofluorometry (8, 9), spectrophotometry (8), liquid chromatography (LC) (10-17), gas chromatography (GC) (18, 19) and capillary electrophoresis (CE) (20) for the determination of FBP. CHG analyses are mostly applied by HPLC (21-25). The others include titrimetry (26), spectrophotometry (25, 26) and capillary electrophoresis (CE) (27). During our literature survey it was seen that, no analysis method for the simultaneous determination of FBP and CHG was reported. There was a need for an analysis method of these two drugs in a single dosage formulation.

The aim of this study is to develop an HPLC method for the simultaneous determination of FBP and CHG in binary preparations, to validate this method according to the United States Pharmacopoeia guideline and finally to apply the method for HPLC analysis of commercial samples containing FBP and CHG.

2. FLURBIPROFEN

FBP is a nonsteroidal antiinflammatory drug (NSAID) mainly used to treat osteoarthritis and rheumatoid arthritis (12, 28). It has excellent antiinflammatory, analgesic and antipyretic properties (1). Its pharmacological effect is based upon the inhibition of prostaglandin synthesis (20).

The appearance of FBP is white and it is a crystalline powder. It is soluble in alcohol as well as in methylene chloride and insoluble in water. It dissolves in aqueous solutions of carbonates and alkali hydroxides (11). Chemical formula of FBP is $C_{15}H_{13}FO_2$, and its molecular weight is 244.261 g/mol. Figure 2.1 shows the molecular structure of FBP. The (S)-enantiomer of FBP exhibits a stronger anti-inflammatory activity; however, FBP is presently produced as a racemic mixture of (S)- and (R)-enantiomers (29).

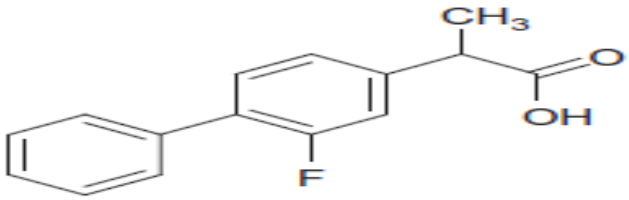

Figure 2.1. The molecular structure of FBP (29)

FBP exhibits comparable efficacy to other NSAIDs, e.g. ibuprofen, indomethacin, naproxen, aspirin, diclofenac. FBP is effective even at concentrations under the steady state plasma levels, which occur after therapeutic doses (30). Like other NSAIDs, the most common side effect in association with FBP therapy is gastrointestinal irritation (1).

FBP is also applied for the treatments of soft tissue injuries (bursitis, tendinitis), post-operative ocular inflammation and vernal keratoconjunctivitis. Due to antiinflammatory effects of FBP, it is also used for peridontal treatments (12, 13).

2.1. Flurbiprofen Analysis Methods

Various methods have been reported in the literature for the determination of FBP. These are obtained by using spectrofluorometry, spectrophotometry, liquid chromatography, gas chromatography and capillary electrophoresis techniques. The methods are presented in this section according to the techniques used.

2.1.1. Spectrofluorometric and spectrophotometric methods

Spectrofluorometric and spectrophotometric methods for the determination of drugs can be used in laboratories where expensive instruments such as required for HPLC or gas chromatography are not available. They have advantages like being easy, less time consuming and less expensive (8).

Yılmaz and Alkan developed a spectrofluorometric and ultraviolet (UV) spectrophotometric methods for the determination of FBP in pure and pharmaceutical preparations. The method was easy, however, the precision values for spectrofluorometry and UV spectrophotometry methods were 3.80% and 3.20% (8).

Chandran *et. al* determined FBP and celecoxib in pure and pharmaceutical forms by a spectrofluorometric method. The detection limits and quantitation limit of FBP were 0.00099 and 0.003 ppm. These limits of the method at nanogram level were lower than the earlier reported spectroscopic methods for the two drugs. The method was efficiently applied for the analysis of two drug formulations (9).

2.1.2. Chromatographic methods

2.1.2.1. Liquid chromatographic methods

HPLC methods are widely used as analytical techniques at qualitative and quantitative studies with its high separation power (7). The HPLC methods reported in the literature are applied for the determination of FBP in pharmaceuticals, plasma and urine samples.

The United States Pharmacopoeia (USP) and the European Pharmacopoeia (EP) recommended an HPLC method for the analysis of related substances in FBP (10, 11).

Sajeev *et. al* developed an HPLC method for determination of FBP in pharmaceutical formulations. The advantages of the method were low detection and quantitation limits at 0.015 and 0.05 ppm, respectively. The method was successfully

employed for the determination of FBP in two commercial ophthalmic drops (12).

In another study, Quayyum *et. al* determined FBP in human plasma by an HPLC method. The method was linear within the range of 0.25–25 ppm. It was applied successfully on twenty two people's blood samples The importance of the method was that it was the first study for investigation of pharmacokinetics of FBP in Pakistani subjects (13).

Hanif *et. al* performed an HPLC method for the simultaneous determination of FBP and famotidine in pharmaceutical preparations. The advantage of the method was to analyze FBP and famotidine at the same time, however, the linearity range was between 10-100 ppm with the detection and quantitation limits at 10 and 50 ppm, which indicates low sensitivity of the method (14).

Hutzler *et. al* described an HPLC method for the simultaneous determination of FBP and its major metabolite, 4'-hydroxyflurbiprofen, both in human urine and plasma The quantitation limits of FBP was 0.25 ppm in urine and in plasma (15).

Ünal *et. al* determined FBP in human plasma by an HPLC method. The method was linear between 0.1-40 ppm. The detection limit was 0.1 ppm. Intraday and interday precision were less than 7.3% and 12.0%, respectively. It was concluded that the method was sensitive, and it was suitable for pharmacokinetic and bioequivalence studies (16).

Mei *et. al* determined FBP in human plasma by liquid chromatography combined with tandem mass spectrometry. The relative standard deviations varied between 3.2-8.4% and 5.4-8.7%, respectively. The method was then used for a bioequivalence study of a FBP formulation. It was found to be suitable for the analysis and bioequivalence studies of FBP (17).

2.1.2.3. Gas chromatographic methods

In Yılmaz and Alkan's study, FBP in pharmaceutical preparations are determined by gas chromatography with mass spectrometry as a detector. It is reported that this technique is not sensitive enough to determinate FBP in solution medium. Therefore, derivatization reagent is used to increase the sensitivity in this technique. The detection and quantitation limit were 0.05 and 0.15 ppm. The relative standard deviations were less than 3.64% (18).

In another study, Yılmaz *et. al* determined FBP in human plasma by gas chromatography with mass spectrometry. The detection and quantitation limit were 0.03

and 0.10 ppm. The interday precision values were less than 5.49%. The method was also effective for analyzing plasma samples, which were obtained for pharmacokinetic study (19).

2.1.2.4. Capillary electrophoresis method

Hamoudova and Pospisilova determined FBP and ibuprofen in pharmaceuticals by capillary zone electrophesis. The method was linear between 1-60 ppm. The precision value for FBP was 1.29%. It was reported that the lower sensitivity of the capillary electrophoresis method compared to HPLC methods is sufficient for the analysis of FBP in pharmaceutical preparations (20).

3. CHLORHEXIDINE GLUCONATE

Chlorhexidine is a great cationic antiseptic which belongs to a class of biguanide drugs (4). Chlorhexidine is a strong base. Its salts including gluconate, acetate and hydrochloride are the most stable form of chlorhexidine (6, 31). But the gluconate form is the most soluble form in water (4). Therefore, CHG is the most frequent formulation used among its salts (31).

CHG can not be presented as a solid, therefore it is only available as an aqueous solution (27). The appearance of CHG is colourless or pale yellow liquid. CHG is soluble in ethanol, water and acetone (22). The chemical formula of CHG is $C_{22}H_{30}Cl_2N_{10}.2C_6H_{12}O_7$, and its molecular weight is 897,77 g/mol (31). Figure 3.1 shows molecular structure of CHG.

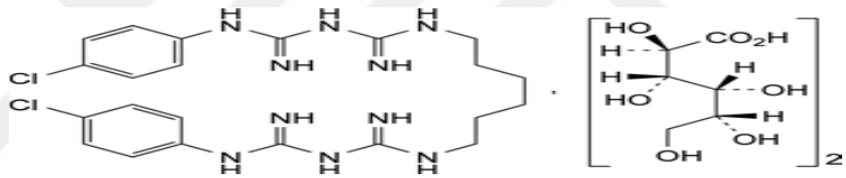

Figure 3.1. The molecular structure of CHG (22)

CHG is an excellent antibacterial agent. It is effective against Gram-negative and Gram-positive microorganisms, fungi and some types of viruses (5, 32). Its efficacy is based upon its cationic nature (33). The positive charge of the molecule and negatively charged phosphate groups on the bacterial cell wall allows the chlorhexidine molecule to penetrate into the bacteria with toxic effects (34).

CHG is included in many products e.g. soaps, gargles, sprays, toothpastes, eye drops and disinfectant solutions. It has an excellent binding potential which results in effectiveness (5, 32). Adverse effects associated with CHG are oral sensitivity, discoloration of the teeth and a bitter taste for several hours following the use of mouthwash. In addition, contact with the solution may cause irritation of eye and skin (5).

3.1. Chlorhexidine Gluconate Analysis Methods

There are several methods for the determination of CHG in the literature. These are obtained by using titrimetry, spectrophotometry, HPLC and capillary electrophoresis techniques. The methods were presented in this section according to the techniques used.

3.1.1. Titrimetric and spectrophotometric methods

Borissova and Mandjukova developed titrimetric and spectrophotometric methods for the determination of CHG in tooth pastes. The quantitative extraction of CHG from the tooth paste is proved by the fact that the results are identical both when analysing a 5 g (titrimetric method) or a 0.1 g (spectrophotometric method) sample (26).

Doğan and Başçı reported a UV spectrophotometric method for the simultaneous determination of CHG and benzydamine hydrochloride in spray and gargle preparations. Linearity range was within 1-50 ppm. Interday and intraday precisions were between 0.82-1.25% and 0.41-0.80%, respectively. It was reported that the method has advantages like simplicity, low cost and rapidity (25).

3.1.2. Chromatographic methods

3.1.2.1. High performance liquid chromatographic method

The physicochemical properties of CHG demonstrate that HPLC with UV detection must be the analytical technique to determine CHG. Therefore, HPLC is the most used technique for determination of CHG in various preparations (5).

The USP and the EP recommended an HPLC method for the analysis of related substances of CHG (21, 22).

Xu and Wond developed an HPLC method for the simultaneous determination of CHG, lignocaine hydrochloride and triamcinolone acetonide in suspension. Recovery studies showed good results (99.20% - 100.52%) and RSD values were ranged from 0.28% to 1.19% (23).

Havlikova *et. al* determined CHG and p-chloroaniline in topical ointment by an HPLC method. The linearity range for CHG was between 0.05 and 0.018 ppm. The significant advantages of the method are the fast (5.50 min) and simultaneous determination of the substances (24).

In another study, Doğan and Başçı developed an HPLC method for the

simultaneous determination of CHG and benzydamine hydrochloride in spray and gargle preparations. Hydrochlorothiazide was used as internal standard. Linearity range was obtained as 1-60 ppm. Interday and intraday precisions were between 0.06-0.16% and 0.03-0.15%, respectively. The importance of the method was to analyze both drugs at the same time (25).

3.1.2.2 Capillary electrophoresis method

Abad-Villar *et. al* determined CHG and polyhexamethylene biguanide in eye drops by capillary electrophoresis combined with contactless conductivity detection. The detection limit was determined to be 0.4 ppm for CHG. It was reported that capillary electrophoresis is useful in ophthalmic drug penetration studies because of its sensitivity, limited requirement on sample volume, simplicity and high tolerance to salt background of the sample (27).

4. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Chromatography is a technique in which the components of a mixture are separated based upon differences in the rates at which they are carried through a stationary phase by a liquid or gas mobile phase (35). The component of interest in the mixture is called the analyte and the rest is called the matrix. For chromatographic separation, the analyte is injected to the mobile phase which flows along a stationary phase. Because the interaction of the different species in the mixture with the stationary phase will be different, these species will leave the stationary phase at different times, and therefore, the compounds forming the mixture will be separated from each other. If the mobile phase is liquid, it is called liquid chromatography (LC); if the mobile phase is a gas, then it is called gas chromatography (GC) (36). Figure 4.1 demonstrates a representation of the separation with this technique.

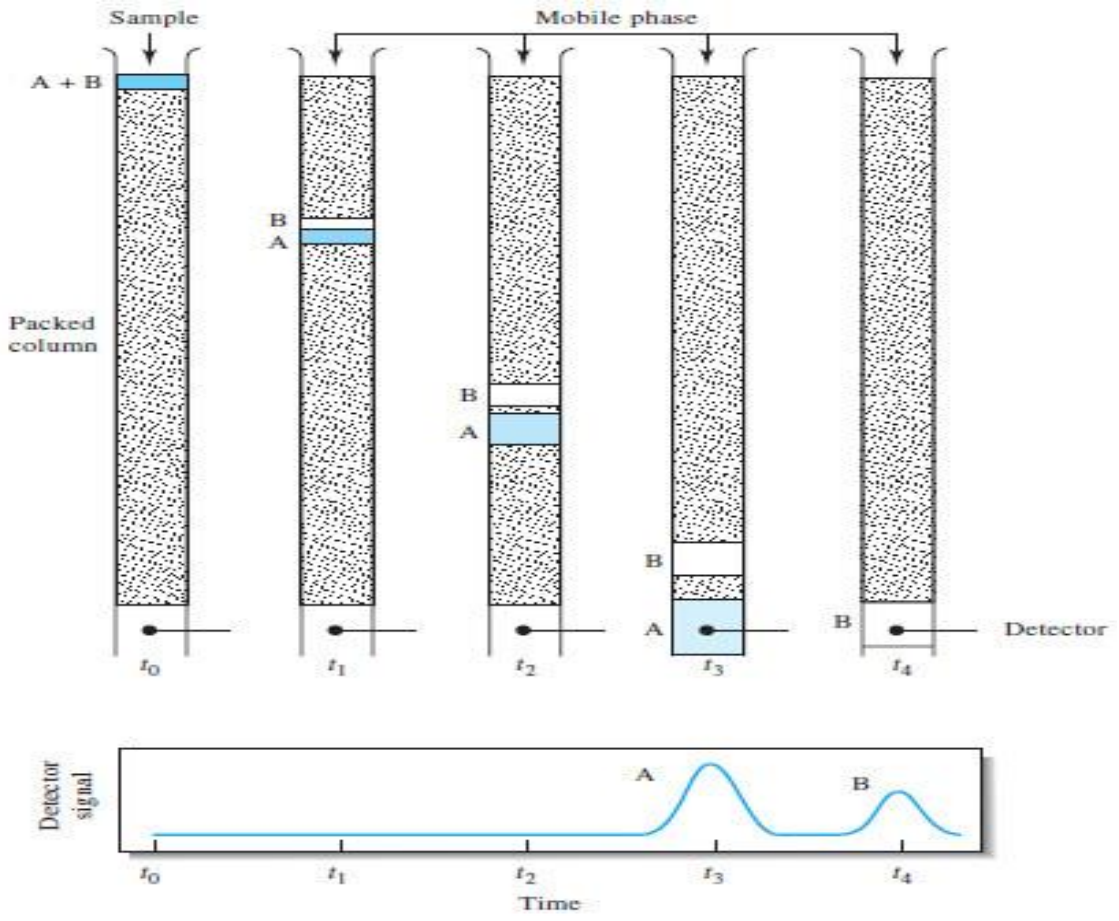

Figure 4.1. A representation of the separation of two compounds (35)

The HPLC technique, as the name suggests, uses a liquid mobile phase to separate compounds in the mixture. The analytes in the mixture are first dissolved in a solvent, introduced into the mobile phase by an autoinjector, and then the analytes go along the stationary phase that is under high pressure by means of the mobile phase. The chromatographic separation occurs by mass transfer of the analytes between mobile phase and stationary phase (37).

HPLC provides high resolution, in addition to that, the analysis time is short. It is capable of quantifying, separating and identifying the compounds which are present in the sample. Thereby, it is one of the most effective tools in analytical chemistry, and it is mostly used in analysis of drugs, foods and agrochemicals. It is the most accurate technique generally applied for the qualitative and quantitative analysis of drugs as well as stability determination of drugs (38).

4.1. Normal and Reversed Phase High Performance Liquid Chromatography

HPLC can be divided into two broad categories. These are normal phase HPLC and reversed phase HPLC.

4.1.1. Normal phase high performance liquid chromatography

Normal phase HPLC is a type in which the stationary phase is polar and the mobile phase is apolar. The analytes in a mixture that have a higher polarity retains in the column more than the analytes that have a lower polarity. This is based upon the desorption or adsorption of the analytes on the polar stationary phase (usually silica or alumina). The polar analytes move more slowly through the column due to the strong interaction between these analytes and cylanol groups on the stationary phase. Therefore, the least polar compound elutes first (39). The attractive forces in normal phase HPLC are mainly hydrogen bonds (polar) and dipole-dipole interactions (40).

The most significant drawback of this type is that the polar surfaces can easily be contaminated with the sample components. This drawback is reduced by adding cyano and amino functional groups to cylanol groups (39).

4.1.2. Reversed phase high performance liquid chromatography

It is an HPLC type in which the stationary phase is apolar and the mobile phase is polar solvent (such as acetonitrile, methanol, water). Octadecyl (C18) groups are used as

the stationary phase (36, 40). In reversed phase HPLC, firstly polar analytes elute from the stationary phase (from the column) as apolar analytes interact more strongly with the hydrophobic C18 groups (40). Due to the fact that the polar compounds separate first, as the opposite of the separation in normal phase HPLC, this type is called reversed phase HPLC. The attractive forces in reversed phase HPLC are mostly hydrophobic interactions (36).

This type is the most common HPLC type because of its ability to seperate a large number of organic compounds. It is used in more than 90% of HPLC analyzes (41). HPLC type used in this study is also reversed phase HPLC.

4.2. High Performance Liquid Chromatography System

The main units of an HPLC system include degasser, pump, injector, column, detector and data system. A flow scheme for the system is shown in Figure 4.2.

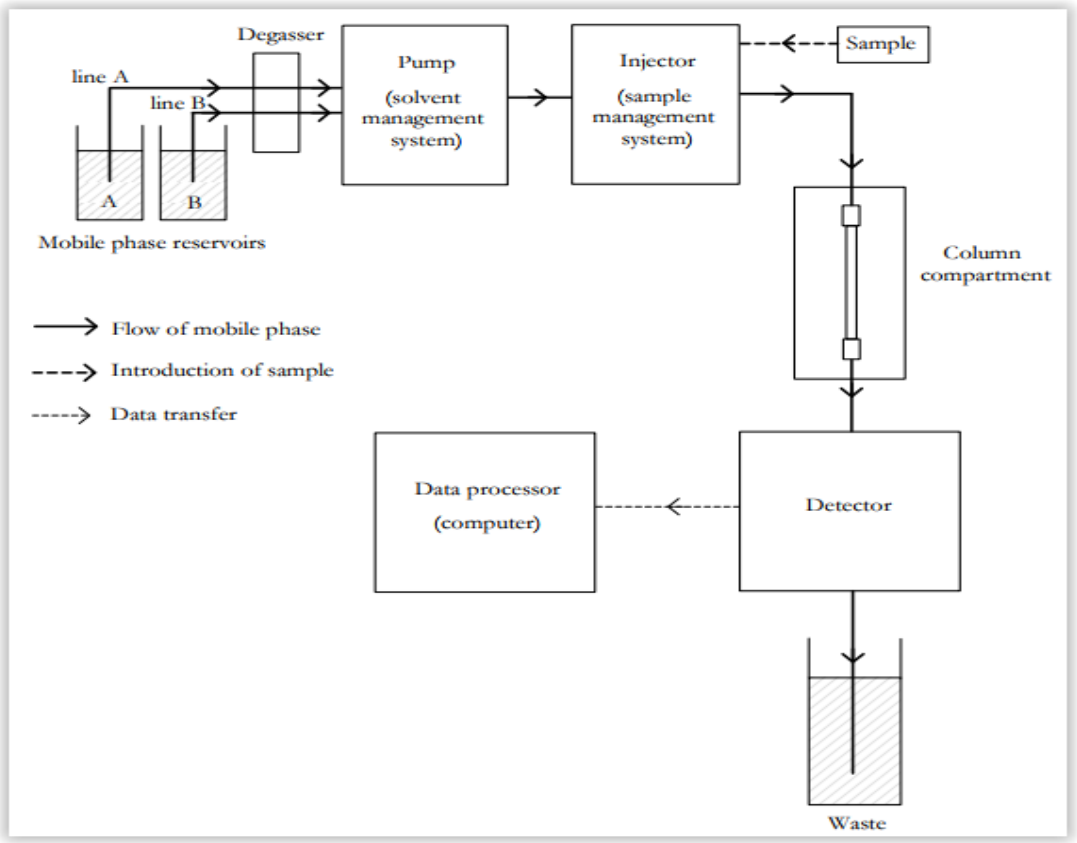

Figure 4.2. A flow scheme for HPLC system (40)

4.2.1. Degasser

Before using the system, a mobile phase always should be degassed first. HPLC system itself also provides a degassser that eliminates the dissolved gas from the flowing mobile phase. It is an important unit of HPLC system because any small gas bubbles may cause variations in the pressure (36).

4.2.2. Pump

Pump provides the mobile phase to be flowed through the system at a specific rate. During the experiment, the pump is able to deliver the constant mobile phase composition which is called isocratic or it can deliver the variable mobile phase composition which is called gradient (35).

4.2.3. Injector

The role of injector is to inject the analytes mixture into the mobile phase before it reaches to the column. Automatic version of injectors are autosamplers, which provides user to analyze many samples automatically instead of manual injection (41).

4.2.4. Column

Column is the most important part of an HPLC system. It allows the separation of the analytes which are in composition. It is the part where the mobile phase is in contact with the stationary phase, composing an interface with its surface (41). Columns are generally made of stainless steel. Stationary phase is placed in the columns (42).

4.2.5. Detector

Detector is a device to detect the molecules which elute the column. It provides continuous registration of the absorbances at a specific wavelength. When the analyte absorbs more than the mobile phase, the positive signal is obtained. There are several detectors used in HPLC systems such as ultraviolet (UV) detectors, fluorescence detectors (FD), refractive index detectors (RID), mass detectors (MSD) and electrochemical detectors (ECD) (43).

According to the literature research (10-16, 21-25), the HPLC analyses of CHG and FBP were applied by using either florescence detector or UV detector. However, the

most common detector applied in pharmaceutical analysis is UV detector (41).

In this study, diode array detector (DAD) kind of UV detection is used. The major advantages of the UV/DAD are that they are able to measure several wavelenghts at the same time and they have a higher signal to noise ratio (44).

4.2.6 Data system

A computer-based data system is required for the HPLC systems to control the instument parameters such as mobile phase composition, temperature, injection volume etc. It acquires the outputs from the detector. The electronic signals of the compounds in the mixture are recorded on the computer as a chromatographic peak (41).

5. VALIDATION

Validation is required to show that an analytical method measures the correct amounts of the correct substance and in the proper range for the intended samples. It is completed to guarantee that the method is specific, accurate and reproducible (45). For HPLC methods in pharmaceutical industry, USP, the US Food and Drug Administration (FDA) and the International Conference on Harmonization (ICH) provide guidelines for performing validation studies (46).

The USP defines 'validation of an analytical procedure' as the study which is employed by laboratory studies, so the performance characteristics of the procedure meet the requirements for the proposed analytical applications (47). Typical analytical performance characteristics that should be evaluated in the validation studies are shown in Table 5.1.

Table 5.1. Typical analytical characteristics used in method validation (47)

Accuracy
Precision
Specificity
Detection Limit
Quantitation Limit
Linearity
Range
Robustness

However, there is no final guideline for analytical method validation. It is customized by selecting necessary tests and acceptance criteria for the proposed method. Therefore, the comprehensiveness of validation is based upon the type of method and the requirements of it (45). The USP separates analytical methods into four categories as

described below (47).

Category I: Quantitation of main components of drug compounds or active agents in finished pharmaceutical products.

Category II: Impurity determination in drug compounds or determination of degradation compounds in finished pharmaceutical products. These methods involve limit tests and quantitative assays.

Category III: Determination of performance characteristics such as drug release, dissolution, etc.

Category IV: Identification tests.

Each of these categories requires different validation studies. Analytical performance characteristics that are necessary for each of the categories are listed in Table 5.2.

Table 5.2. USP characteristics required for validation (47)

Validation Characteristics	Category I	Category II		Category III	Category IV
		Quantitative	Limit Tests		
Accuracy	Yes	Yes	*	*	No
Precision	Yes	Yes	No	Yes	No
Specificity	Yes	Yes	Yes	*	Yes
Detection Limit	No	No	Yes	*	No
Quantitation Limit	No	Yes	No	*	No
Linearity	Yes	Yes	No	*	No
Range	Yes	Yes	*	*	No

*May be required, depending on the nature of the specific test.

In addition to the characteristics mentioned above, system suitability test has to be performed and the stability of the solutions should be assessed (45).

System suitability test should be employed before performing validation studies

to confirm that the method and the system are able to provide data with acceptable quality (47). System suitability parameters should be determined and compared with the limits as shown in Table 5.3.

Table 5.3. System suitability parameters and their recommendation limits (45)

Parameter	Recommendation
Capacity factor (k')	$k' > 2$
Tailing Factor (T)	$T \leq 2$
Theoretical Plates (N)	$N > 2000$
Resolution (R_s)	$R_s > 2$
Repeatability	$RSD \leq 1$

To obtain reliable test results, the stability of the analytes in the solutions should be determined before the validation studies. In most cases, samples are analyzed overnight by using HPLC systems which are equipped with autosampler. For the assay methods, the analytes in the standard and sample solutions should be stable for at least 24 hours under the storage conditions (45, 46).

5.1. Accuracy

Accuracy is the closeness of method results acquired by that method to the true value. The accuracy of an analytical method should be calculated across its range (47).

The true value for accuracy assignation can be determined in various ways. One option is to compare results of the method with results from an established reference method that is known to be accurate. Secondly, it can be determined by analyzing a sample with known concentrations and comparing the measured value with the true value. The third option is based upon recovery of known amounts of analyte. It is applied by spiking analyte in blank matrices (45).

Accuracy is determined as the percentage of recovery. Recovery can be calculated as the difference between the accepted true value and the mean value. The ICH guideline recommends that accuracy should be determined by using a minimum of nine measurements for a minimum of three concentrations which cover the specified range (47). Accuracy criteria for an assay method is that the recovery have to be between 95% and 105% at each concentration levels (46).

5.2 Precision

The precision is the degree of agreement between individual test results when the method is performed replicate to multiple samplings of a homogeneous sample. The precision is usually calculated as the relative standard deviation (RSD) of multiple measurements. Precision can be a determination of the degree of repeatability, intermediate precision or reproducibility of the analytical method under method operating conditions (47).

Repeatability refers the precision under the method operating conditions over a short interval of time. It is also entitled as intra assay precision. Intermediate precision refers variations within laboratories, such as different days, or with different analysts or different equipment. Reproducibility refers the precision between laboratories as in a collaborative study (46).

The precision is assessed from sufficient number of aliquots of a homogeneous sample to allow calculating statistically valid estimates of RSD. In this case, assays are independent analyses of samples, so that have been carried through the complete analytical method from sample preparation to final method result. It is recommended that precision should be evaluated by minimum of nine measurements which cover the specified range for the method (such as triplicate measurements for each of three concentration level). It can also be determined by measuring at least six determinations at 100% of the test concentration (47).

For assay methods, precision criteria is that the results of repeatability studies will be $\leq$1%, the intra assay precision and reproducibility will be $\leq$ 2% (45).

7.3 Specificity

Specificity is the capability to evaluate the analyte unequivocally in the presence of compounds that may be expected, such as degradation products, impurities and matrix

components. If the method is lack of specificity, a compensation can be made by other supporting analytical methods (47).

Specificity can be assessed by analyzing the samples involving impurities or other components spiked into the analytes. It is not required to spike potential interfering substances which do not exist in the test samples reasonably. The degradation products can be generated by exposing the analyte to the stress conditions which is sufficient to degrade it to around 80-90% purity. Typical stress conditions to produce the degradation products for bulk active pharmaceutical reagents are UV light, heat (50ºC-60ºC), alkaline condition (0.1 M sodium hydroxide solution), acidic condition (0.1 M hydrochloric acid solution), and oxidant (3% hydrogen peroxide solution). For formulated products, light, heat and humidity are the factors of severe conditions. The resulting mixtures should be analyzed, and the analyte peak should be assessed for resolution from the closest eluting peak and peak purity. An example for specificity criteria of an assay method to determine resolution is that the peak of the analyte will have a baseline resolution of minimum of 1.5 from all the other sample components (45, 46).

5.4 Linearity

The linearity of an analytical methodology is its capability to obtain test results which are proportional to the concentrations of analytes in samples in a specified range. Therefore, it is about the linearity of the relationships of concentrations and assay measurements (47).

Linearity is evaluated over the range of an analytical method. For assay methods, study of linearity is performed by preparing standards at five concentration levels which are from 80% to 120% of the target concentration. It is preliminarily obtained by visual examination of signals' plot like a function of analyte concentration of sample. If the relationship between results and concentrations is linear, method results should be acquired by proper statistical methods such as calculating a regression line from the approach of least squares. Data of the regression line itself is helpful to obtain mathematical determinations of the degree of linearity. Slope of the regression line, y-intercept and correlation coefficient should be determined (46, 47)

Acceptability of linearity study is generally judged by investigating y-intercept, correlation coefficient and residual sum of squares. For an assay method, the correlation coefficient higher than 0.999 is considered like a proof for acceptable fit of the data of

the regression line (46).

5.5. Range

The range of an analytical methodology is the interval among the lower and upper levels of analyte (involving these levels) which have been indicated to be established with an admissible level of linearity, precision and accuracy by performing the method as written. It is stated in the same units as method results (e.g. parts per million) acquired by the method. Therefore, the suitable range for the method is defined as the concentration interval across which precision, linearity and accuracy are acceptable (47).

5.6 Detection Limit

The detection limit is necessary especially for the limit tests. It is the lowest amount of analyte in a sample which can be detected (not quantitated) under the prescribed experimental conditions. Therefore, limit tests only demonstrates that the analyte amount is below or above an exact level. The detection limit is generally stated as the concentration of analyte (e.g. parts per billion) in the sample (47).

The ICH quidelines recognize a common approach, which is to make a comparison the measured signals from samples with known low concentrations of analyte with blank samples. The acceptable signal to noise ratios to establish detection limit are 2:1 or 3:1 (47).

There are also two other options to determine detection limit: calculation from an equation and visual non-instrumental methods. Detection limit is calculated based upon the standard deviation (SD) of the the slope (S) of the calibration curves and the response at the levels around the detection limit by using the formula: Detection Limit = 3.3 x (SD/S). The SD of the response is determined based upon the SD of y-intercepts of the regression lines, SD of the blank, or the residual SD of the regression line. For visual non-instrumental methods, detection limit is established by techniques like thin layer chromatography. The approach employed to establish detection limit must be documented and supported. A proper number of samples must be analyzed at that limit to validate the level (45).

5.7 Quantitation Limit

The quantitation limit is necessary for quantitative assays, as for determination of

low levels of substances in sample matrices, such as degradation products in finished pharmaceutical products and impurities in bulk drug compounds. It is the lowest amount of analyte in a sample which can be determined with admissible accuracy and precision under the expressed operational conditions. The quantitation limit is usually stated as the concentration of analyte (e.g. parts per billion) in the sample (47).

A signal to noise ratio of 10:1 can be used to establish quantitation limit. It should be noted that determination of quantitation limit is a harmony between the required precision/accuracy and the concentration. Therefore, the precision increases if the quantitation limit decreases (45).

As the determination of detection limit, ICH has reported the 10:1 signal to noise ratio as typical, and suggests the same additional approaches that can be considered to calculate quantitation limit: calculation from an equation and visual non-instrumental methods. The calculation method is again based upon the SD of the the slope (S) of the calibration curves and the response by using the formula: Quantitation limit = 10 x (SD/S). Again, the SD values can be determined based upon the residual SD of the regression line, the SD of y-intercept of the regression line, or the SD of the blank. In addition to that, the approach used to establish quantitation limit must be documented and supported. A proper number of samples must be analyzed at that limit to validate the level (45).

5.8 Robustness

The robustness is a measure of the method's capability to stay unaffected by deliberate and small variations in method parameters. It provides an evidence about the suitability during normal use. It may be considered during development process of the analytical method (47).

For the determination of the robustness, several chromatographic parameters such as flow rate, mobile phase composition, injection volume, detection wavelength and column temperature are varied in a range and then quantitative effect of the variables is determined. If the effect of variations is within a specified limits, the parameter is considered to be in the method's robustness range. Obtaining data on these variations allows the analysts to know whether a method should be revalidated when these parameters are changed. Crucial parameters should be reported during the method development, and thereby, these crucial parameters can be investigated for robustness

(45).

An example for robustness criteria is that the influence of the following changes in method's conditions will be evaluated: column temperature adjusted by (±1 to 5ºC), organic solvent content in mobile phase adjusted by (±2%) and mobile phase pH adjusted by (up to ±0.5 pH units). If the small changes are in the limits which produce acceptable chromatography, then they will be included in the method procedure (45).

6. MATERIALS AND METHODS

6.1. Materials

6.1.1. Chemicals

FBP and CHG were kindly supplied from Abdi İbrahim Pharmaceuticals (İstanbul, Turkey). Ortophosphoric acid was obtained from Merck (Darmstadt, Germany). Monosodium phosphate and HPLC grade acetonitrile (ACN) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Commercial pharmaceutical samples were bought from local pharmacy stores.

6.1.2. Instruments

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

6.2. Methods

6.2.1. Preparation of mobile phase

100.0 mM phosphate buffer solution of pH 2.5 was prepared with 2115.0 μl of ortophosphoric acid (H_3PO_4) and 8.2347 g of monosodium phosphate (NaH_2PO_4) as 1 liter. The solution was filtered through 0.45 μm membrane filter and then it was degassed in sonicator for 15 minutes. The prepared buffer solution was placed in HPLC and pumped together with ACN by gradient elution. The gradient profile (time, %B) set was as follows: 0 min, 30% B; 5 min, 80% B; 10 min, 80% B; 15 min, 30% B.

6.2.2. Preparation of standard solutions

Stock solutions of FBP and CHG were prepared by dissolving of 10.0 mg of standard material in 10.0 ml volumetric flask at a concentration of 1000.0 ppm. To dissolve FBP, ACN was used while ACN:water (50:50) mixture was used for CHG due

to its poor dissolution in ACN.

Working solutions for the experiments were prepared by diluting stock solutions with mobile phase. At the beginning, a 100.0 ppm standard mixture of FBP and CHG was prepared by diluting stock solutions of both standard materials. 2.5 ml of each stock solution was transferred to 25.0 ml volumetric flask and the volume was made with mobile phase. Standard solutions were prepared at concentrations of 1.0, 2.0, 5.0, 8.0, 10.0, 13.0, 15.0, 20.0, 22.0 and 25.0 ppm from 100.0 ppm standard mixture by suitable dilution in 10.0 ml volumetric flasks. Quality control (QC) standard solutions were chosen as the solutions with concentrations of 2.0, 8.0, 13.0 and 22.0 ppm, while the other standard solutions were used for the purpose of calibration curve in the studies.

6.2.3. Preparation of sample solutions

The developed method was applied on 10.0 ppm sample solutions. For gargle and spray samples, the analysis was performed by diluting products directly with mobile phase. The solutions were then transferred into vials by filtering through 0.45 μm membrane filters. For tablet analysis, the average tablet mass was calculated from ten tablets. The tablets were ground, homogenized and then proper amount of the powder that corresponds to 100.0 mg FBP was weighed, transferred into 100.0 ml volumetric flask and diluted to scale with ACN. The prepared mixture was sonicated for 10 minutes and filtered through 0.45 μm membrane filter. The solution was diluted further with mobile phase to obtain a solution of 10.0 ppm FBP and then transferred into a vial.

6.2.4. Development of the method

Optimization studies were carried out to develop the HPLC method for simultaneous determination of FBP and CHG. In this case, various parameters were investigated including wavelength, pH and concentration of the buffer solution, temperature, gradient profile of the mobile phase, flow rate and injection volume. The optimum parameters were selected based on the peak shapes and peak area values.

6.2.4.1. Determination of mobile phase

Different mobile phases were tested in the development stage of the method. According to the literature, ACN was the most common organic solvent for the determination of FBP and CHG, individually. In addition to that, ACN has ability to

produce better peak shapes compared to methanol. Therefore, ACN was used as organic solvent, and pH adjustments were made by using different phosphate and acetate buffer solutions.

To prepare phosphate buffer solutions at pH 2.0 and 2.5, pKa value of 2.16 was used. The solutions were prepared with orthophosphoric acid (H_3PO_4) and monosodium phosphate (NaH_2PO_4).

To prepare acetate buffer solutions at pH 3.0 and 4.0, pKa value of 4.76 was used. The solutions were prepared with acetic acid (CH_3COOH) and sodium acetate (CH_3COONa).

To prepare phosphate buffer solutions at pH 6.0 and 7.0, pKa value of 7.2 was used. The solutions were prepared with monosodium phosphate (NaH_2PO_4) and disodium phosphate (Na_2HPO_4).

The final mobile phase was determined based on optimization studies of pH and concentration of buffer solutions, and gradient profiles.

6.2.4.2. Optimization of wavelength

As a first step of the method development**,** wavelenghts which have been studied for the active materials in literature were examined. For this purpose, UV detector was set to 230 nm, 248 nm and 265 nm to investigate spectra of the analytes. Optimum wavelength was chosen.

6.2.4.3. Optimization of mobile phase pH

Secondly, pH optimization studies were performed. Different buffer solutions were tried at pH 2.0, 2.5, 3.0, 4.0, 6.0 and 7.0 to adjust the pH of the mobile phase. Peak shapes and peak area values were investigated. Optimum pH value was chosen.

6.2.4.4. Optimization of buffer concentration

After optimization of the mobile phase pH, the buffer concentration was investigated by testing the buffer solutions of 20.0 mM, 50.0 mM, 100.0 mM and 150.0 mM concentrations. Peak shapes and peak area values were investigated. Optimum concentration value was chosen.

6.2.4.5. Optimization of temperature

To determine the temperature, different temperatures of 25ºC, 30ºC, 35ºC, 40ºC and 45ºC were investigated. Peak shapes and peak area values were investigated. Optimum temperature was chosen.

6.2.4.6. Optimization of gradient profile

Different gradient profiles were examined. Peak shapes and peak area values were investigated. Optimum gradient profile was chosen.

6.2.4.7. Optimization of injection volume

For optimization of injection volume, a study was performed by investigating the peak shapes and peak area values for the injection volumes of 5.0 µl, 10.0 µl, 15.0 µl and 20.0 µl. Optimum injection volume was chosen..

6.2.4.8. Optimization of flow rate

For optimization of flow rate, a study was performed by investigating the peak shapes and peak area values at flow rate of 0.4 ml, 0.5 ml and 0.6 ml. Optimum flow rate was chosen.

6.2.4.9. Determination of diluent

To determine the diluent, the study was performed by using water-ACN (50:50) and phosphate buffer-ACN (70:30) as diluents. Peak shapes and peak area values were investigated and the best diluent was chosen

6.2.5. Validation of the method

The developed method was validated according to USP guideline (47). Method validation included system suitability test, stability, and the validation parameters involving specificity, linearity, accuracy, precision and robustness.

6.2.5.1. System suitability test

Before performing validation experiments, system suitability test (SST) has to be applied to indicate that HPLC system and method are capable of providing data with

admissible quality. SST was performed by investigating capacity factor, tailing factor, theoretical plates number, resolution and also RSD of the peak areas.

6.2.5.2. Stability

Stability was assessed by analyzing QC standard solutions after keeping them at room temperature for 48 hours. Obtained results were investigated as recovery values and compared to freshly prepared solutions.

6.2.5.3. Specificity

Specificity was performed by exposing the analytes to stress conditions (105 ºC, 0.1 M HCl, 0.1 M NaOH and 3% H_2O_2) and then analyzing the resulting mixtures. Specificity was also applied by analyzing tablet matrix. Peaks of analytes were examined for peak purity and resolution from the closest eluting peak.

6.2.5.4. Linearity

To investigate the linearity, standard solutions at six concentration levels (1.0, 5.0, 10.0, 15.0, 20.0, 25.0 ppm) were analyzed triplicate to plot calibration curves according to peak areas. Linearity of the method was evaluated by the term of correlation coefficient. In addition, LOD and LOQ values were calculated.

6.2.5.5. Accuracy

Accuracy was calculated as recovery of the QC standard solutions. For this purpose, the QC standard solutions were analyzed as three repetitive triplicate system.

6.2.5.6. Precision

Precision studies were performed as intraday and interday (n=3) precision. Precision of the method was investigated as RSD of the recovery values of the QC standard solutions. The solutions were analyzed as three repetitive triplicate system.

6.2.5.7. Robustness

Robustness of the method was performed to document whether the method was susceptible to variations in method parameters or not. For this purpose, some small changes were applied deliberately on temperature, flow rate and mobile phase pH and

recovery and RSD of the recovery values were recorded.

6.2.6. Sample analysis

The developed and validated method was applied to commercial pharmaceutical products that are binary mixtures of CHG and FBP, as well as products that contain only CHG or FBP. The study was performed by analyzing gargle, spray and tablet samples. The sample solutions were prepared in duplicate and each was analyzed in triplicate.

7. RESULTS

7.1. Development of the Method

7.1.1. Optimization of wavelength

The wavelenghts of 230 nm, 248 nm and 265 nm were investigated. The study was performed with 100.0 ppm standard mixture solution which was diluted from stock solutions with water-ACN (50:50). The mobile phase was 50.0 mM pH 2.5 phosphate buffer solution (A) and ACN (B) at a flow rate of 0.5 ml/min. The gradient profile (time, %B) set was as follows: 0 min, 40% B; 10 min, 60% B; 15 min, 60% B; 20 min, 40% B. The injection volume was 10.0 µl. The column temperature was set at 25ºC.

By taking literature into consideration and due to the spectra of the analytes, 248 nm was chosen as the optimum wavelength. This wavelength was applied for the following studies.

7.1.2. Optimization of mobile phase pH

For optimization of mobile phase pH, buffer solutions with different pH values at 2.0, 2.5, 3.0, 4.0, 6.0 and 7.0 were investigated. The buffer solutions were prepared as described in section number 6.2.4.1. The study was performed with 50.0 ppm standard mixture solutions which were diluted from stock solutions with water-ACN (50:50). The mobile phase was 50.0 mM buffer solutions (A) and ACN (B) at a flow rate of 0.5 ml/min. The gradient profile (time, %B) set was as follows: 0 min, 20% B; 10 min, 60% B; 15 min, 60% B; 20 min, 20% B. The injection volume was 10.0 µl. The wavelength was 248 nm, the column temperature was 30ºC. The chromatograms obtained are presented in Figure 7.1.

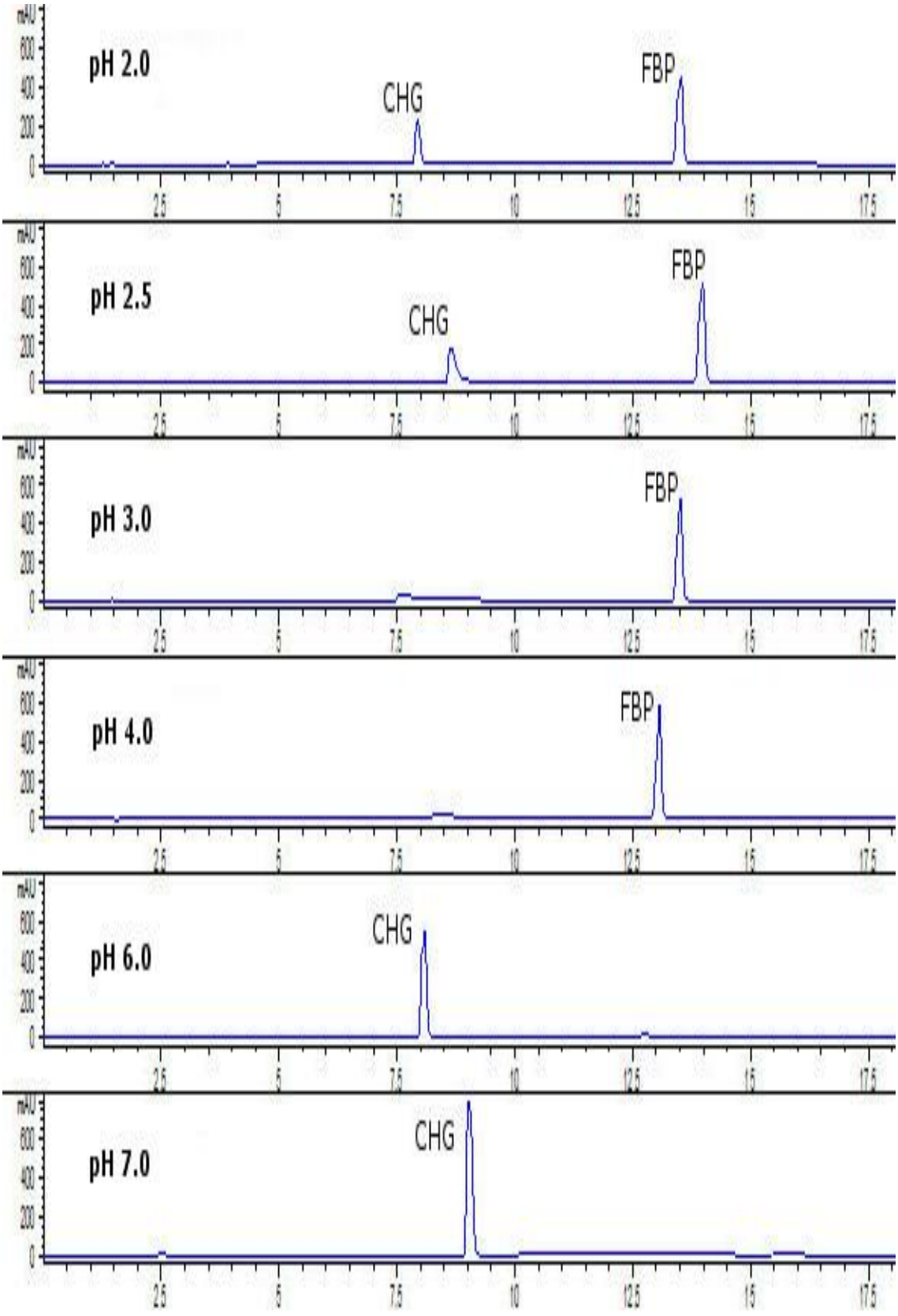

Figure 7.1. The chromotograms obtained by using 50.0 mM buffer solutions and ACN as a mobile phase at a flow rate of 0.5 ml/min, 10.0 µl injection volume, 248 nm wavelength and 30°C column temperature for optimization of mobile phase pH

Effect of the mobile phase pH on acquired peak area values are shown in Table 7.1 and in Figure 7.2 as a graph.

Table 7.1. Effect of the mobile phase pH in the range of 2.0 – 7.0 on acquired peak area values

pH	CHG Peak Area	FBP Peak Area
2.0	1788.3	4283.5
2.5	2521.5	4759.2
3.0	1716.7	4236.5
4.0	1867.3	4588.4
6.0	4224.3	-
7.0	6513.6	-

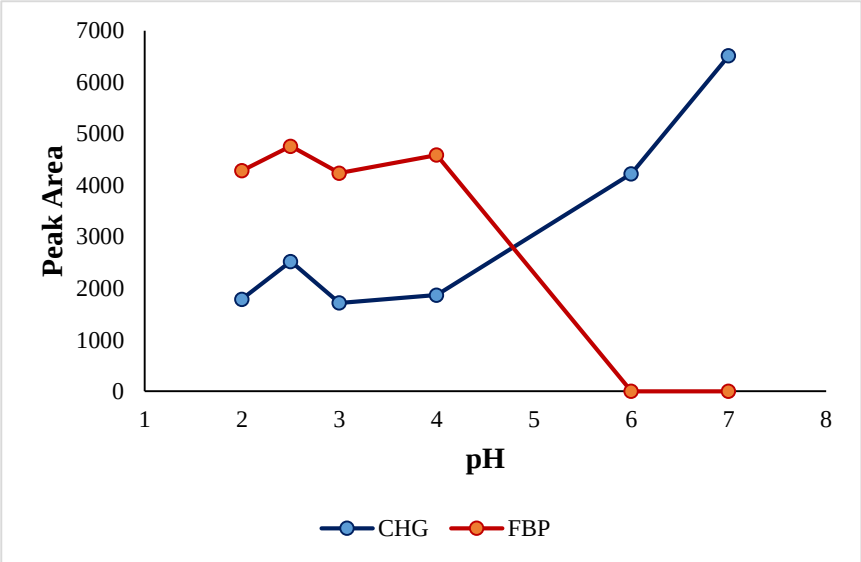

Figure 7.2. Effect of the mobile phase pH in the range of 2.0 – 7.0 on acquired peak area values

Both CHG and FBP are polar and ionizable compounds. FBP has acidic properties, and CHG has basic properties. It was seen that the mobile phase had to be acidic for FBP to be in ion-suppressed form and absorbed by the column. That is because pKa value of FBP is 4.42, and it ionizes at higher pH values. Therefore, FBP showed improved retention at low pH values, but no peak was observed at pH 6.0 and pH 7.0. On the other hand, CHG is adequately retained at low pH (2.0 and 2.5). However, it showed greater retention at pH 6.0 and pH 7.0. That is because pKa values of CHG are 3.39 and 10.52, and it is in ion-suppressed form at these pH values. Chlorhexidine cations interacts with acetic acid to form chlorhexidine acetate salts. For this reason, hardly visible CHG elution were noticed with big tailing at pH 3.0 and pH 4.0 due to the presence of acetic acid in the mobile phase. Therefore, optimum pH value was chosen as 2.5 based on the highest peak area values of the analytes together. The mobile phase with this pH value was applied for the following studies.

7.1.3. Optimization of buffer concentration

After optimization of the mobile phase pH, buffer concentration was investigated by testing the phosphate buffer solutions (pH 2.5) at concentrations of 20.0 mM, 50.0 mM, 100.0 mM and 150.0 mM. The study was performed with 50.0 ppm standard mixture solutions which were diluted from stock solutions with a mixture of water and ACN (50:50). The mobile phase was pH 2.5 phosphate buffer solution (A) and ACN (B) at a flow rate of 0.5 ml/min. The gradient profile (time, %B) set was as follows: 0 min, 20% B; 10 min, 60% B; 15 min, 60% B; 20 min, 20% B. The injection volume was 10.0 µl. The wavelength was 248 nm, the column temperature was 30ºC. The chromatograms obtained are presented in Figure 7.3.

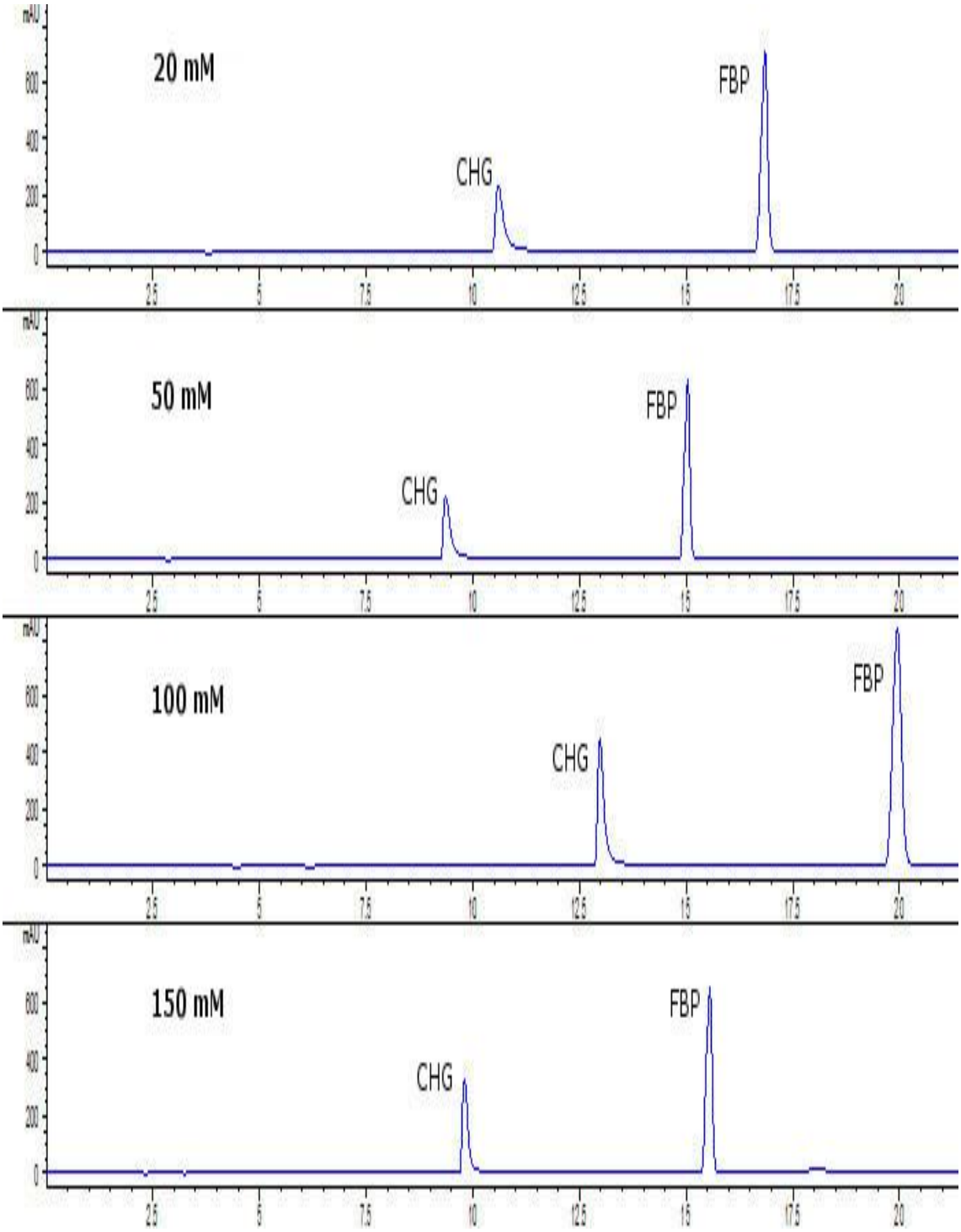

Figure 7.3. The chromotograms obtained by using pH 2.5 phosphate buffer solution and ACN as a mobile phase at a flow rate of 0.5 ml/min, 10.0 µl injection volume, 248 nm wavelength and 30°C column temperature for optimization of buffer concentration

Effect of the buffer concentration on acquired peak area values are shown in Table 7.2 and in Figure 7.4 as a graph.

Table 7.2. Effect of the buffer concentration in the range of 20.0 – 150.0 mM on acquired peak area values

Buffer Concentration (mM)	CHG Peak Area	FBP Peak Area
20.0	3807.1	7663.7
50.0	2899.3	5681.7
100.0	5146.1	12160.6
150.0	3297	6321.8

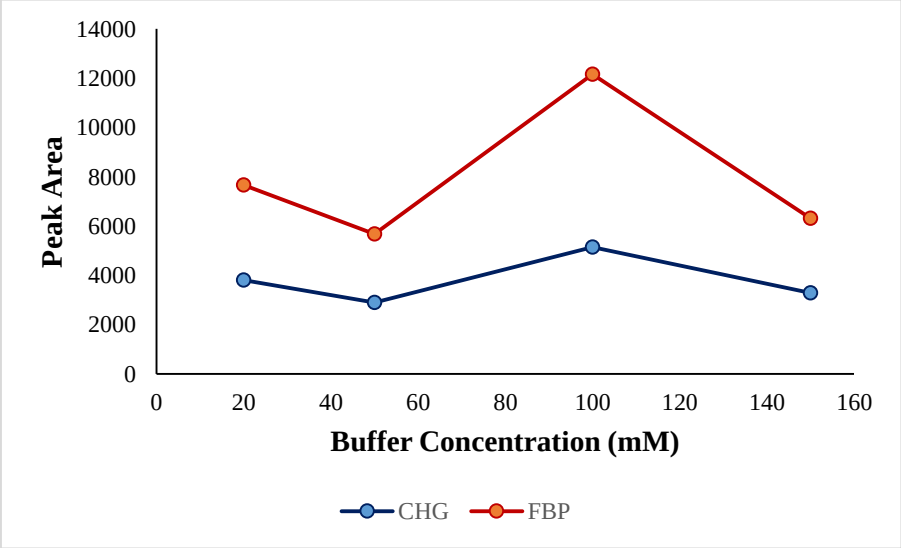

Figure 7.4. Effect of the buffer concentration in the range of 20.0 – 150.0 mM on acquired peak area values

The highest peak area values were obtained when using 100.0 mM phosphate buffer solution. For this reason, optimum buffer concentration was chosen as 100.0 mM. The buffer solutions with this concentration were applied for the following studies.

7.1.4. Optimization of temperature

For optimization of temperature, 25ºC, 30ºC, 35ºC, 40ºC and 45ºC were investigated. The study was performed with 50.0 ppm standard mixture solutions which were diluted from stock solutions with a mixture of water and ACN (50:50). The mobile phase was 100.0 mM pH 2.5 phosphate buffer solution (A) and ACN (B) at a flow rate of 0.5 ml/min. The gradient profile (time, %B) set was as follows: 0 min, 20% B; 10 min, 60% B; 15 min, 60% B; 20 min, 20% B. The injection volume was 10.0 µl. The wavelength was 248 nm. The chromatograms obtained are presented in Figure 7.5.

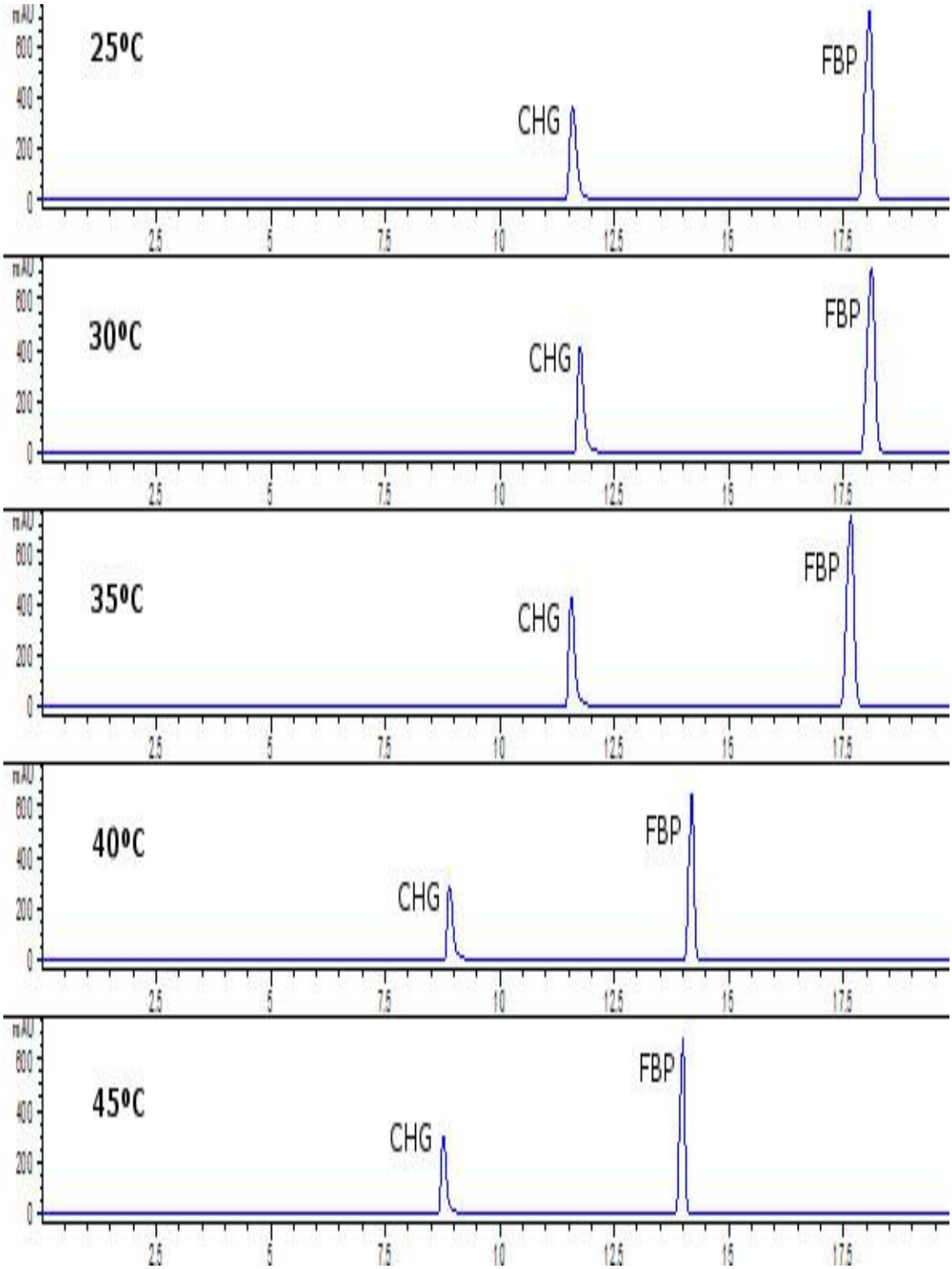

Figure 7.5. The chromotograms obtained by using 100.0 mM pH 2.5 phosphate buffer solution and ACN as a mobile phase at a flow rate of 0.5 ml/min, 10.0 µl injection volume, 248 nm wavelength for optimization of temperature

Effect of the temperature on acquired peak area values are shown in Table 7.3 and in Figure 7.6 as a graph.

Table 7.3. Effect of the temperature in the range of 25 – 45ºC on acquired peak area values

Temperature (ºC)	CHG Peak Area	FBP Peak Area
25	3949.2	8186.7
30	4153.6	8546.5
35	4089.8	8249.4
40	2847.5	5195.0
45	2560.3	5076.7

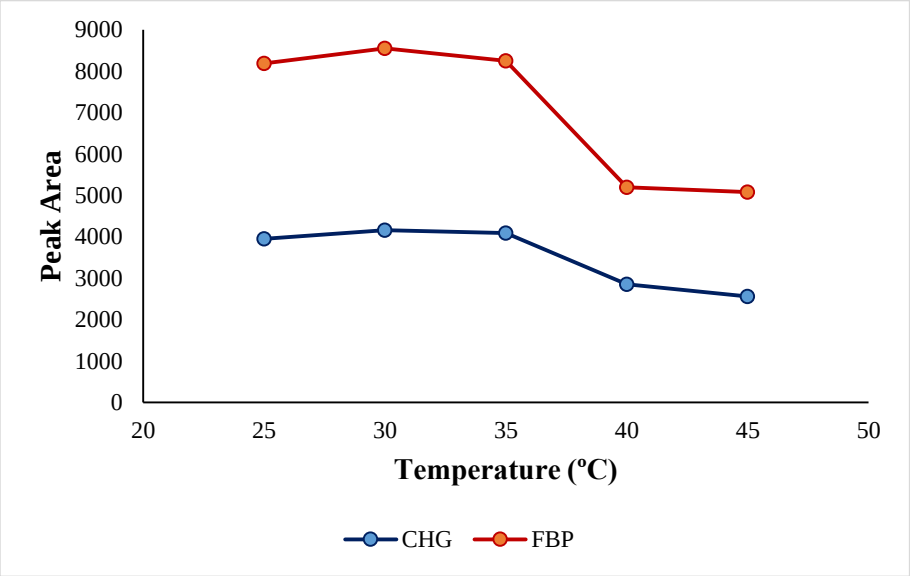

Figure 7.6. Effect of the temperature in the range of 25 – 45ºC on acquired peak area values

It was seen that the peak area values over 30ºC decreased with the increasing temperature, and the peak splitting was observed over 40ºC. However, increased temperature caused shorter retention times. Optimum temperature was chosen as 30ºC due to the highest peak area values. This temperature was applied for the following studies.

7.1.5. Optimization of gradient profile

To evaluate the effect of gradient, different gradient profiles of A, B, C, D and E were examined (Table 7.4). The study was performed with 50.0 ppm standard mixture solutions which were diluted from stock solutions with a mixture of water and ACN (50:50). The The mobile phase was 100.0 mM pH 2.5 phosphate buffer solution (A) and ACN (B) at a flow rate of 0.5 ml/min. The injection volume was 10.0 µl. The wavelength was 248 nm. The column temperature was 30ºC. The chromatograms obtained are presented in Figure 7.7. Properties of the applied gradient profiles are illustrated in Table 7.4, and effect of the gradient profiles on acquired peak area values are shown in Table 7.5.

Table 7.4. Properties of applied gradient profiles

Gradient Profile	Profile Properties				
A	Time (min)	0	10	15	20
	ACN%	20	60	60	20
B	Time (min)	0	5	10	15
	ACN%	30	60	60	30
C	Time (min)	0	5	10	15
	ACN%	30	70	70	30
D	Time (min)	0	5	10	15
	ACN%	30	80	80	30
E	Time (min)	0	5	10	15
	ACN%	30	90	90	30

Table 7.5. Effect of the gradient profile on acquired peak area values

ACN Ratio (%)	CHG Peak Area	FBP Peak Area
20 (Gradient A)	3959.7	8796.7
30 (Gradient B)	4252.4	9165.3
30 (Gradient C)	3726.9	7047.2
30 (Gradient D)	3594.9	6382.6
30 (Gradient E)	3478.8	5548.2

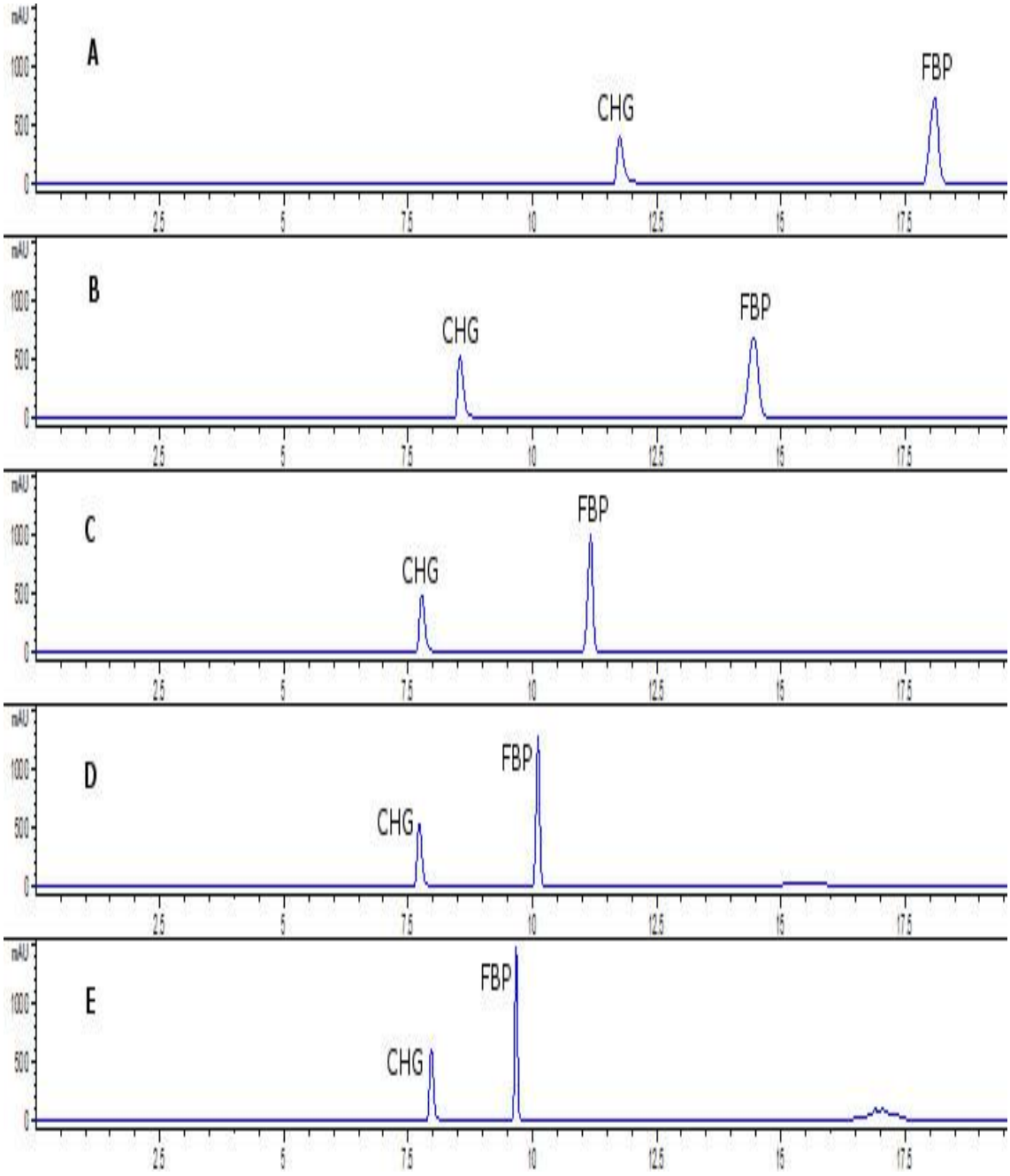

Figure 7.7. The chromotograms obtained by using 100.0 mM pH 2.5 phosphate buffer solution and ACN as a mobile phase at a flow rate of 0.5 ml/min, 10.0 µl injection volume, 248 nm wavelength and 30°C column temperature for gradient profiles A, B, C, D, E

The purpose of the optimization study for gradient profile was to obtain peaks without tailing. Peak tailing was not observed with the gradient profiles D and E. In addition, retention times were shorter. Optimum gradient profile was chosen as D due to higher peak area values compared to gradient profile E. This gradient profile was applied for the following studies.

7.1.6. Optimization of injection volume

Injection volumes of 5.0 µl, 10.0 µl, 15.0 µl and 20.0 µl were investigated. The study was performed with 50.0 ppm standard mixture solutions which were diluted from stock solutions with a mixture of water and ACN (50:50). The mobile phase was 100.0 mM pH 2.5 phosphate buffer solution (A) and ACN (B) at a flow rate of 0.5 ml/min. The gradient profile (time, %B) set was as follows: 0 min, 30% B; 5 min, 80% B; 10 min, 80% B; 15 min, 30% B. The wavelength was 248 nm. The column temperature was 30ºC. The chromatograms obtained are presented in Figure 7.8.

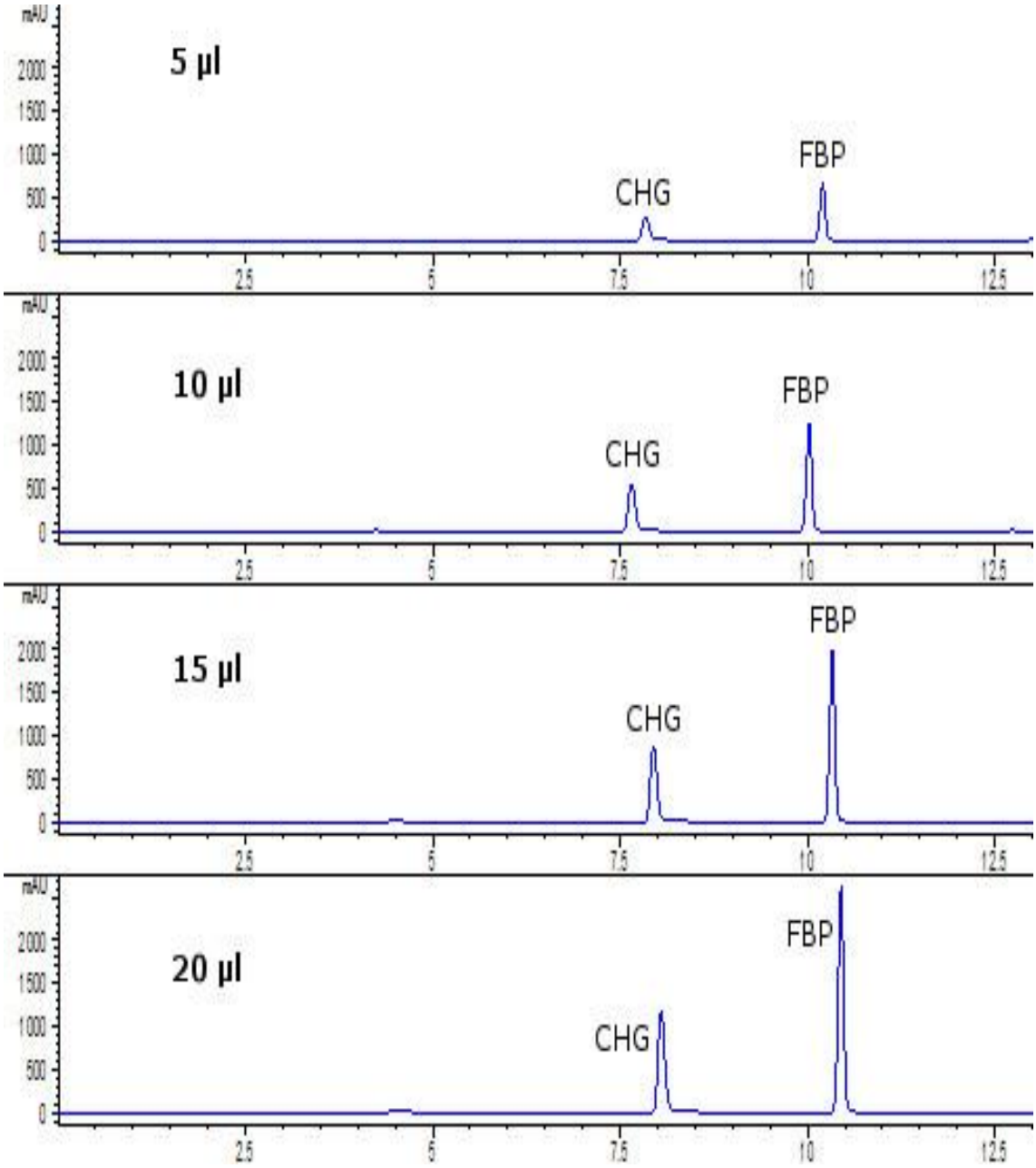

Figure 7.8. The chromotograms obtained by using 100.0 mM pH 2.5 phosphate buffer solution and ACN as a mobile phase at a flow rate of 0.5 ml/min, 248 nm wavelength and 30°C column temperature for optimization of injection volume

Effect of the injection volume on acquired peak area values are shown in Table 7.6 and in Figure 7.9 as a graph.

Table 7.6. Effect of the injection volume in the range of 5.0 – 20.0 µl on acquired peak area values

Injection Volume (µl)	CHG Peak Area	FBP Peak Area
5.0	1809.9	3273.3
10.0	3542.6	6239.2
15.0	5506.5	9876.6
20.0	7446.2	13303.1

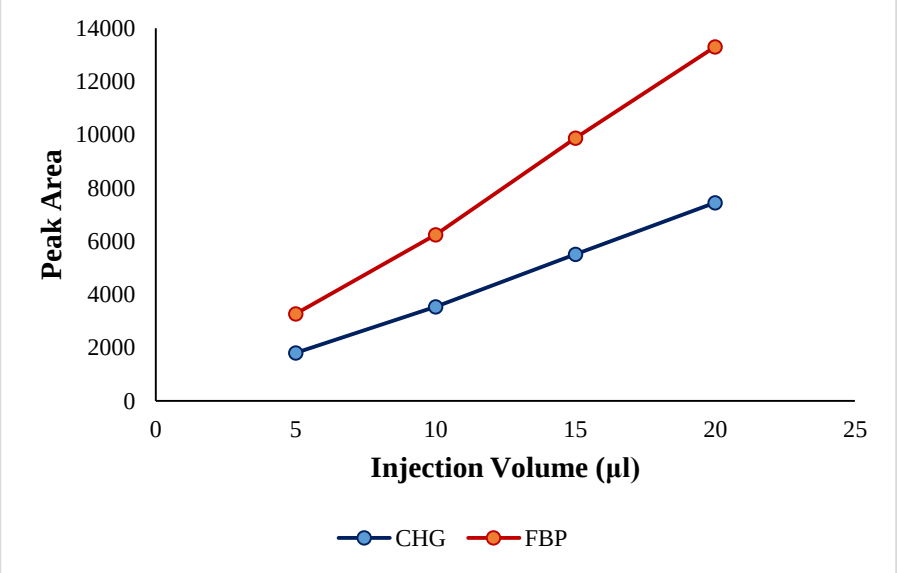

Figure 7.9. Effect of the injection volume in the range of 5.0 – 20.0 µl on acquired peak area values

Peak area values increased with the increasing injection volume. The peak shapes obtained at 20.0 µl injection volume were desirable, and therefore it was the highest injection volume investigated in this study due to the properties of the column used. For this reason, 20.0 µl was found to be the optimum injection volume. It was applied for the following studies.

7.1.7. Optimization of flow rate

Flow rates of 0.4 ml/min, 0.5 ml/min and 0.6 ml/min were investigated. The study was performed with 5.0 ppm standard mixture solutions which were diluted from stock solutions with a mixture of water and ACN (50:50). The mobile phase was 100.0 mM pH 2.5 phosphate buffer solution (A) and ACN (B) at a flow rate of 0.5 ml/min. The gradient profile (time, %B) set was as follows: 0 min, 30% B; 5 min, 80% B; 10 min, 80% B; 15 min, 30% B. The injection volume was 20.0 µl. The wavelength was 248 nm. The column temperature was 30ºC. The chromatograms obtained are presented in Figure 7.10.

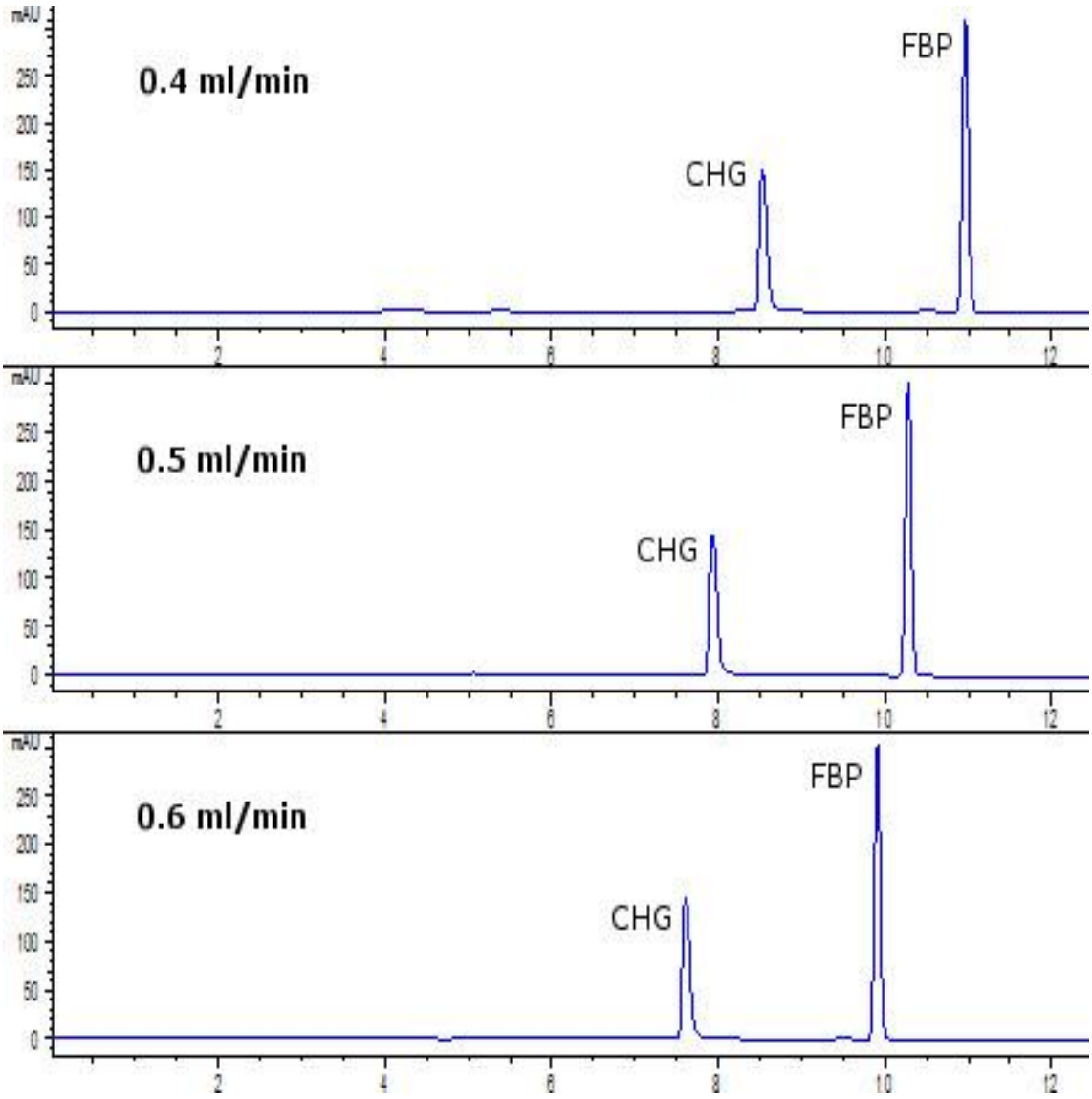

Figure 7.10. The chromotograms obtained by using 100.0 mM pH 2.5 phosphate buffer solution and ACN as a mobile phase, 10.0 µl injection volume, 248 nm wavelength and 30°C column temperature for optimization of flow rate

Effect of the flow rate on acquired peak area values are shown in Table 7.7 and in Figure 7.11 as a graph.

Table 7.7. Effect of the flow rate on acquired peak area values

Flow Rate (ml/min)	CHG Peak Area	FBP Peak Area
0.4	979.6	1592.6
0.5	934.5	1503.4
0.6	912.9	1456.6

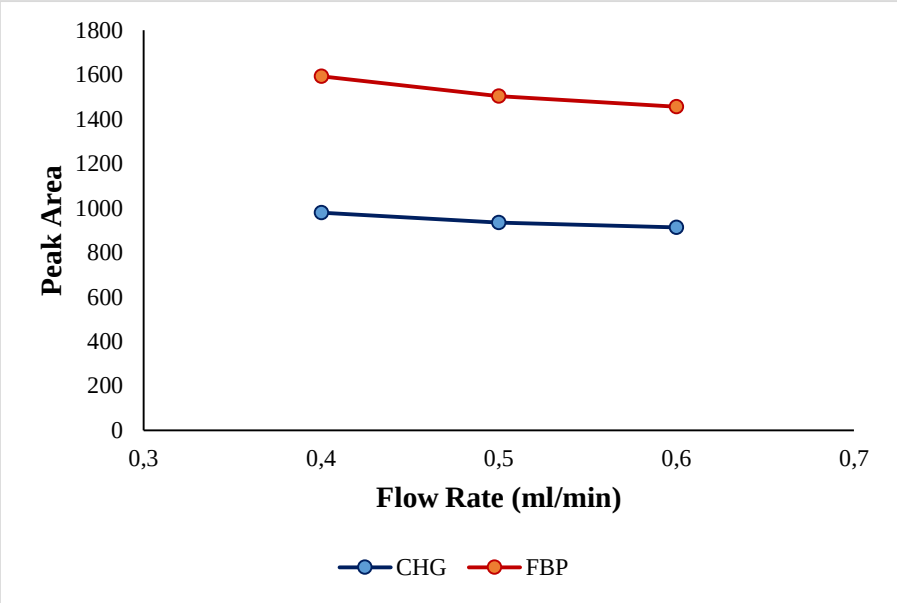

Figure 7.11. Effect of the flow rate in the range of 0.4 – 0.6 ml/min on acquired peak area values

There was no significant difference on the obtained peak area values. The highest peak area values were obtained with the flow rate of 0.4 ml/min, however, the peak tailing was a little bit more compared to other flow rates. Due to the fact that the increase on the flow rate caused higher column pressure, the flow rates higher than 0.6 ml/min were not evaluated and the lowest possible flow rate was preferred. For this purpose, the flow rate of 0.5 ml/min was chosen as the flow rate of the method. This flow rate was applied for the following studies.

7.1.8. Determination of diluent

To determine the diluent, water-ACN (50:50) and phosphate buffer-ACN (70:30) were investigated. The study was performed with 5.0 ppm standard mixture solutions which were diluted from stock solutions. The mobile phase was 100.0 mM pH 2.5 phosphate buffer (A) and ACN (B) at a flow rate of 0.5 ml/min. The gradient profile (time, %B) set was as follows: 0 min, 30% B; 5 min, 80% B; 10 min, 80% B; 15 min, 30% B. The injection volume was 20.0 µl. The wavelength was 248 nm. The column temperature was 30ºC. The chromatograms obtained are presented in Figure 7.12. Effect of the diluent on acquired peak area values are shown in Table 7.8.

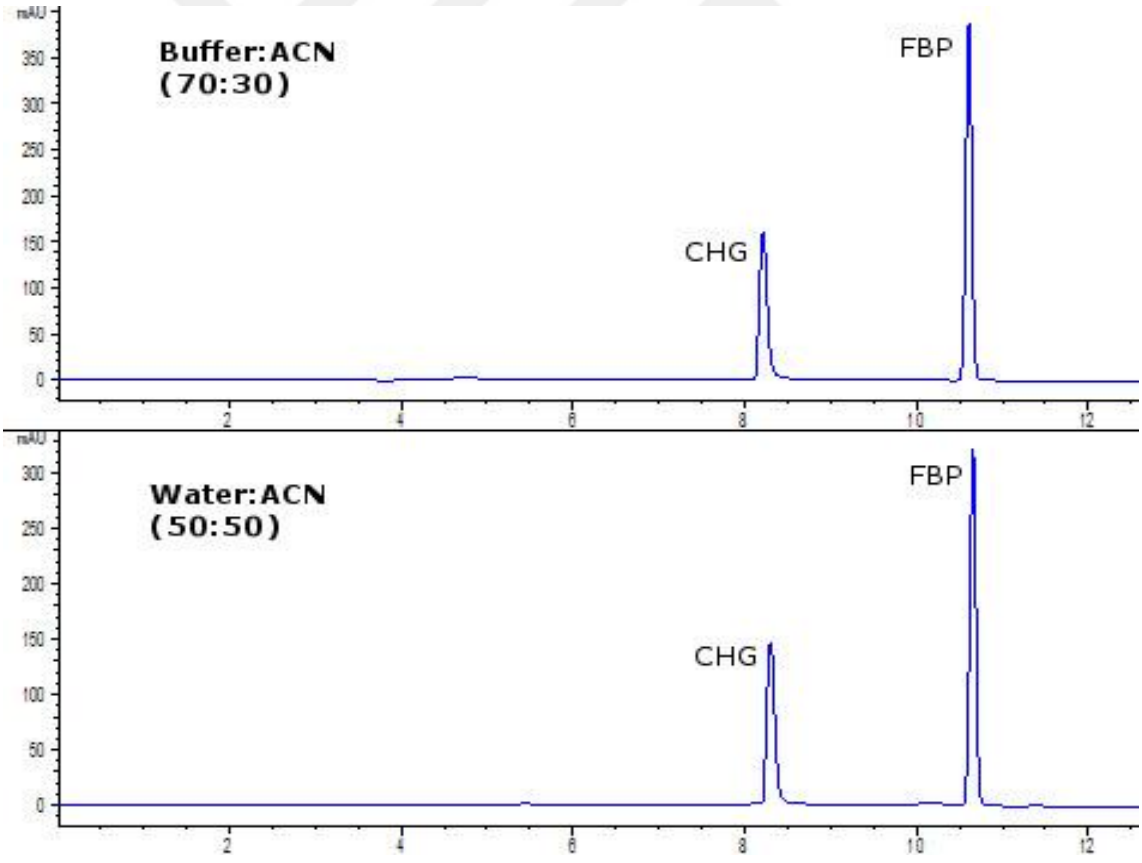

Figure 7.12. The chromotograms obtained by using 100.0 mM pH 2.5 phosphate buffer and ACN as a mobile phase at a flow rate of 0.5 ml/min, 10.0 µl injection volume, 248 nm wavelength and 30°C column temperature for determination of diluent

Table 7.8. Effect of the diluting solvent on acquired peak area values

Diluent	CHG Peak Area	FBP Peak Area
Buffer-ACN (70:30)	1040.8	1902.3
Water-ACN (50:50)	963	1611.8

The diluent was chosen as buffer-ACN (mobile phase) due to the peak area values and peak shapes. This diluent was applied for the following studies.

7.2. Optimum Method Conditions

As a result of optimization studies, the chromatographic conditions of the developed method were summarized in Table 7.9. Figure 7.13 illustrates the chromatogram obtained at optimum conditions.

Table 7.9. Optimum method conditions

Column	Agilent Poroshell 120 EC-C18 (3x150 mm, 2.7 µm)				
Mobile Phase	100.0 mM, pH 2.5 Phosphate buffer and ACN				
Gradient Profile	Time (min)	0	5	10	15
	ACN (%)	30	80	80	30
Injection Volume	20.0 µl				
Flow Rate	0.5 ml/min				
Column Temperature	30ºC				
Wavelength	UV/DAD: 248 nm				

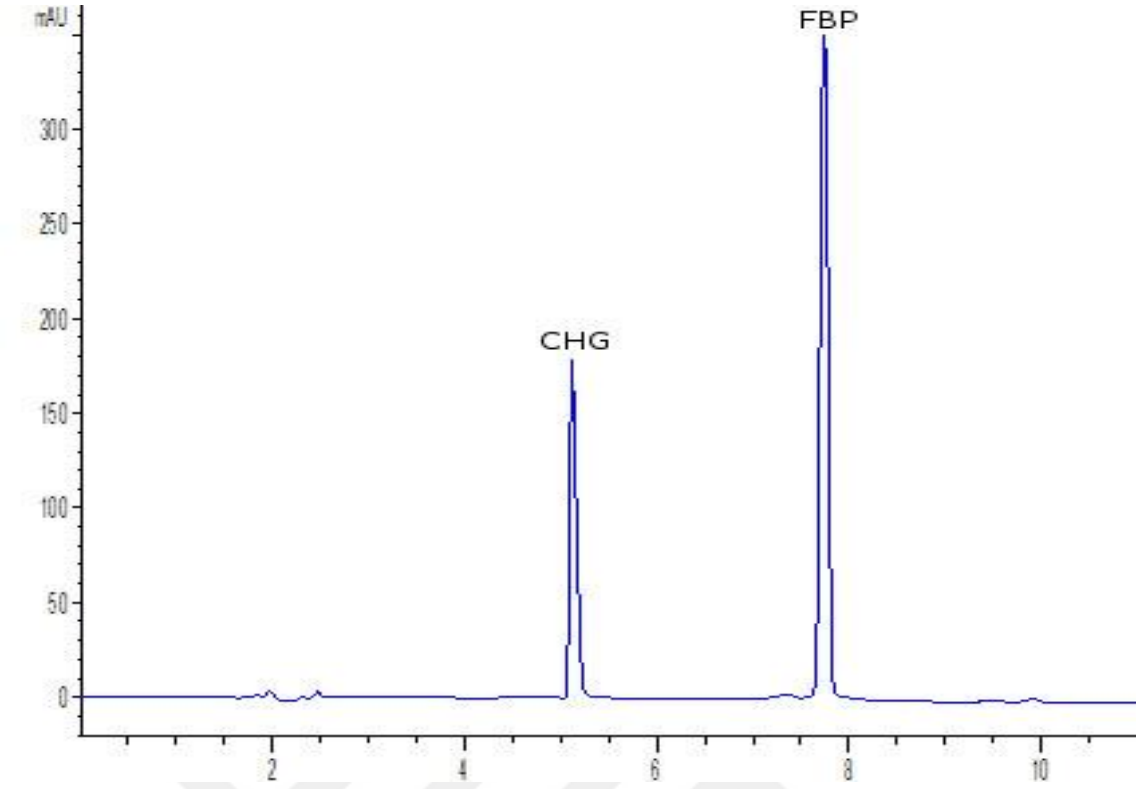

Figure 7.13. Typal chromotogram obtained at optimum conditions

7.3. Validation of the Method

7.3.1. System suitability test

After setting the optimum conditions, system suitability parameters for the developed method were determined and compared with recommended limits. The parameters stated in USP were applied. To determine the parameters, the study was performed with 13.0 ppm standard mixture solution and the results were acquired from six injections. Results of system suitability test are demonstrated in Table 7.10.

Table 7.10. Results of system suitability test (n=6)

Parameter	Recommendation	CHG	FBP
Capacity factor (k')	k' > 2	3.735	4.958
Tailing Factor (T)	T ≤ 2	1.402	0.992
Theoretical Plates (N)	N > 2000	31499	91832
Resolution (R_s)	R_s > 2	12.93	12,93
RSD (Peak Area)	RSD ≤ 1	0.28	0.12

According to the obtained results, all sytem suitability parameters were within the recommended limits and the method was found to be suitable for the analysis.

7.3.2. Stability

Stability of the method was studied on autosamples stability. For this purpose, QC standard solutions were investigated during 48h. The stability was evaluated by determining concentrations on the basis of original calibration standards.

The concentrations of QC standard solutions were determined from regression equations of the calibration curves, and recovery values were obtained according to the following formula:

Recovery = (Found Amount / Known Amount) x 100

At the end, obtained recovery values were compared with the recovery values of freshly prepared QC solutions. Table 7.11 shows the results of stability study.

Table 7.11. The results of autosamples stability of QC solutions during 48h

Concentration	Recovery%		Change%	
(ppm)	CHG	FBP	CHG	FBP
2.0	97.21	97.58	2.47	2.57
8.0	95.41	95.49	5.37	4.90
13.0	95.00	95.11	6.19	6.05
22.0	94.01	95.02	6.30	5.39

7.3.4. Specificity

Several studies were performed to assess specificity. To generate degradation products, the analytes were exposed to stress conditions. For this purpose, 100 ppm standard mixture solution was added into three different tubes containing 0.1 M HCl (A), 0.1 M NaOH (B) and 3% H_2O_2 (C), separately.

2.0 ml of the standard solution was transferred to each tube. The solution in tube A was mixed with 2.0 ml of 0.1 M HCl, the solution in tube B was mixed with 2.0 ml of 0.1 M NaOH and the solution in tube C was mixed with 2.0 ml of 3% H_2O_2. Afterwards, all tubes were placed in boling water bath (at 105ºC) for an hour and then the solutions were cooled to room temperature (25ºC). After cooling, tube A and B were neutralized with sufficient amount of base or acid by dripping technique. The prepared solutions were filtered using 0.45 μm membrane filter and then transferred to a vial and enjected to HPLC system.

At the same time, blank solutions were prepared by using distilled water instead of standard solution. 2.0 ml of the distilled water was transferred to each tube (tube D, tube E and tube F). The water in tube D was mixed with 2.0 ml of 0.1 M HCl, the water in tube E mixed with 2.0 ml of 0.1 M NaOH and the water in tube F was mixed with 2.0 ml of 3**%** H_2O_2. In the same way, base or acid was dripped into tube A and tube B. The prepared solutions were filtered and transferred to a vial and enjected to HPLC system.

The chromatograms obtained from solutions under stress conditions were shown in Figure 7.14, 7.15 and 7.16, respectively.

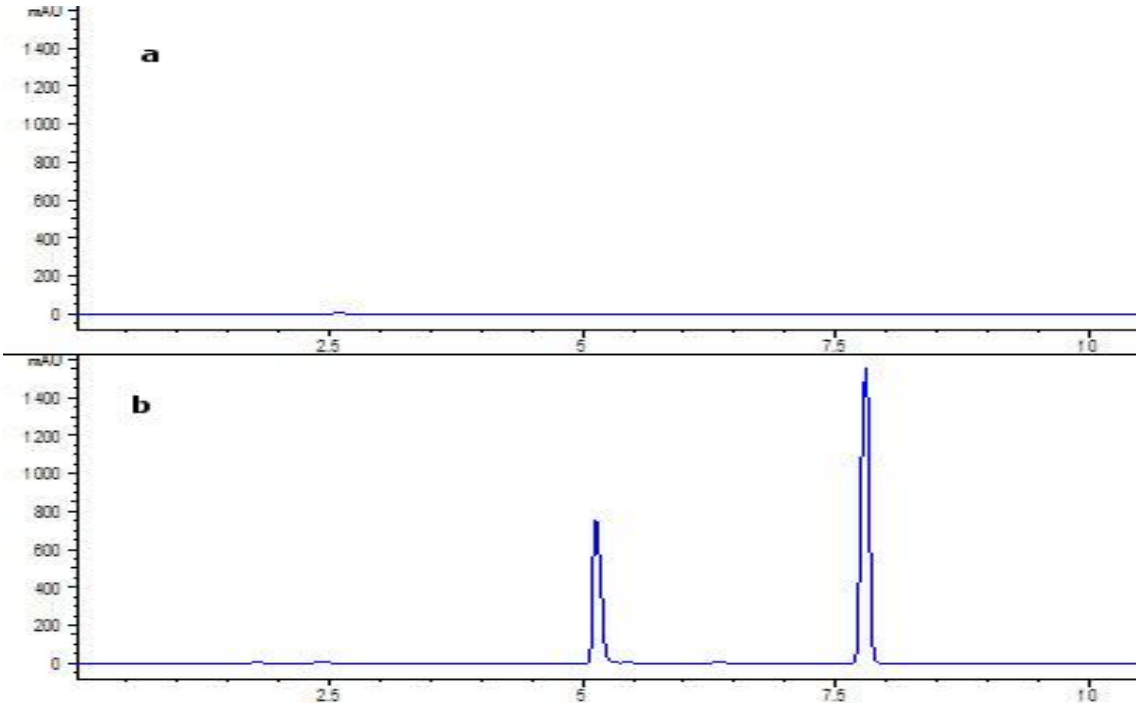

Figure 7.14. a) The chromatogram of the blank prepared with HCl b) The chromatogram of standard solution with HCl

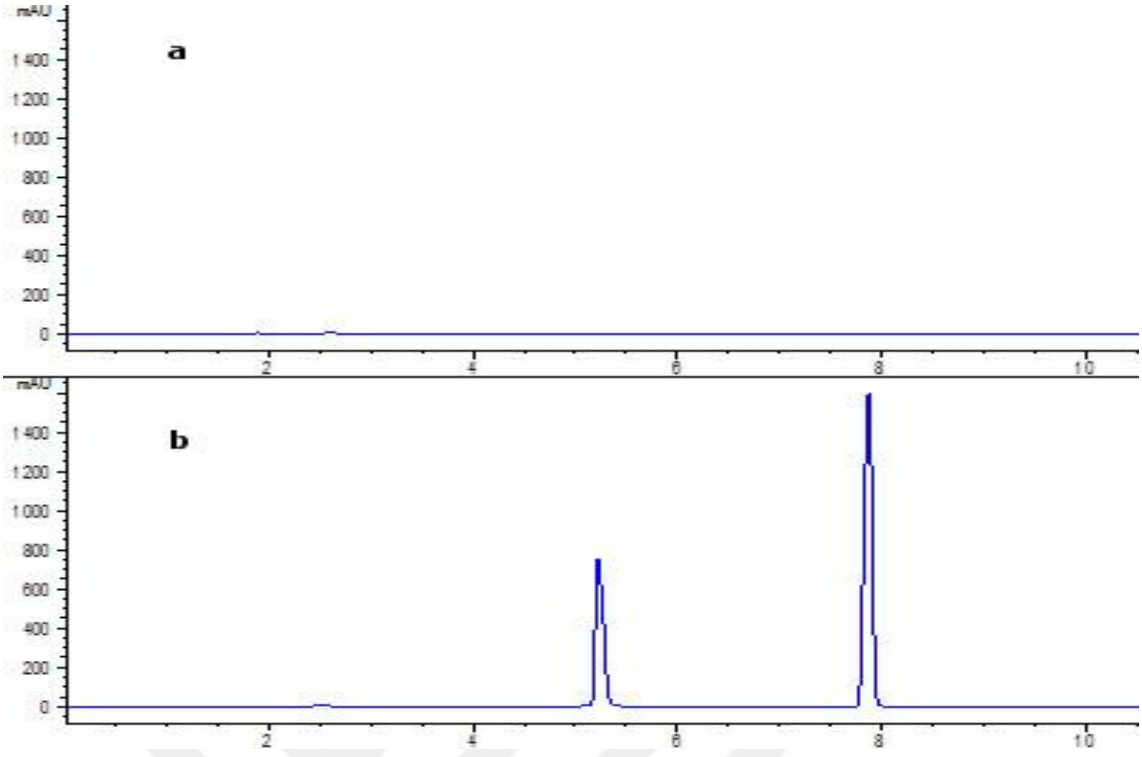

Figure 7.15. a) The chromatogram of the blank prepared with NaOH b) The chromatogram of standard solution with NaOH

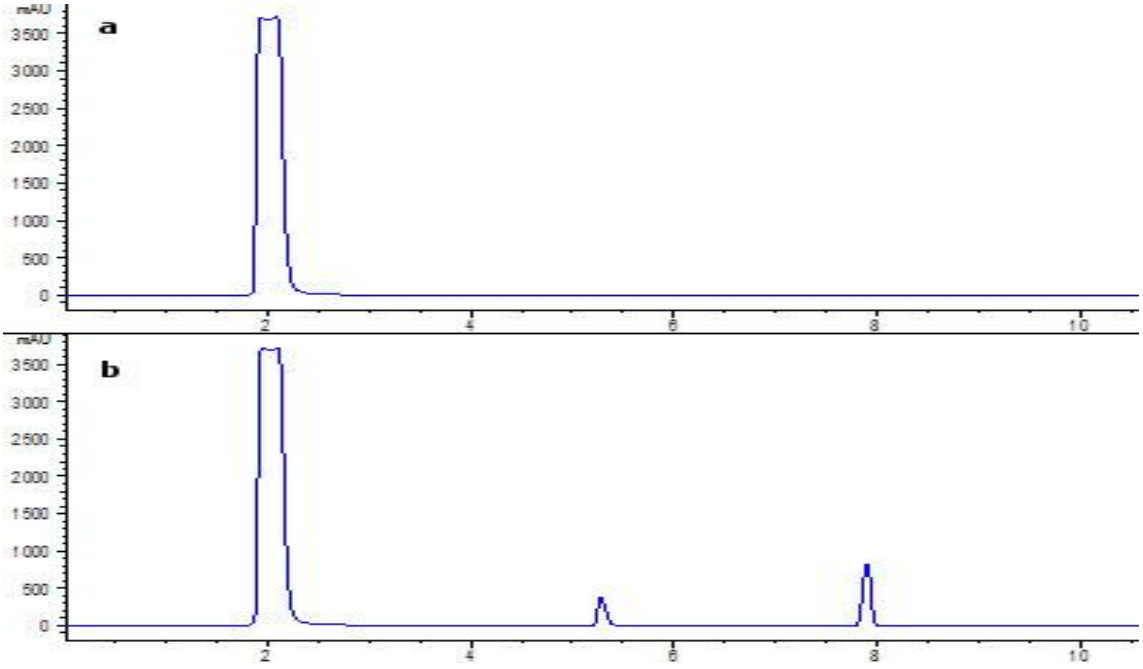

Figure 7.16. a) The chromatogram of the blank prepared with H_2O_2 b) The chromatogram of standard solution with H_2O_2

According to the chromatograms obtained there was no any interference. The peaks of the analytes were clear.

Another study was performed for the tablet sample. For that purpose, the excipients of the tablet was investigated and a matrix medium was prepared from the materials that were available at the university. The percentages of the excipients per tablet were given from pharmaceutical technologists. Table 7.12 shows the excipients and their amounts in the prepared matrix medium.

Table 7.12. The excipients and their amounts in the matrix medium

Excipient	Amount (mg)
Lactose	90.0
Microcrystalline cellulose	90.0
Croscarmellose sodium	6.0
Colloidal silica	1.0
Magnesium stearate	2.0
Titanium dioxide	0.75

The excipients were weighed accurately and then transferred to a mortar and homogenized. The prepared mixture was first diluted to scale with ACN in 10.0 ml volumetric flask, and then it was diluted with mobile phase. The solution was injected into the system after filtration. The chromatogram obtained was shown in Figure 7.17. There were no peaks observed which illustrates the specificity of the method.

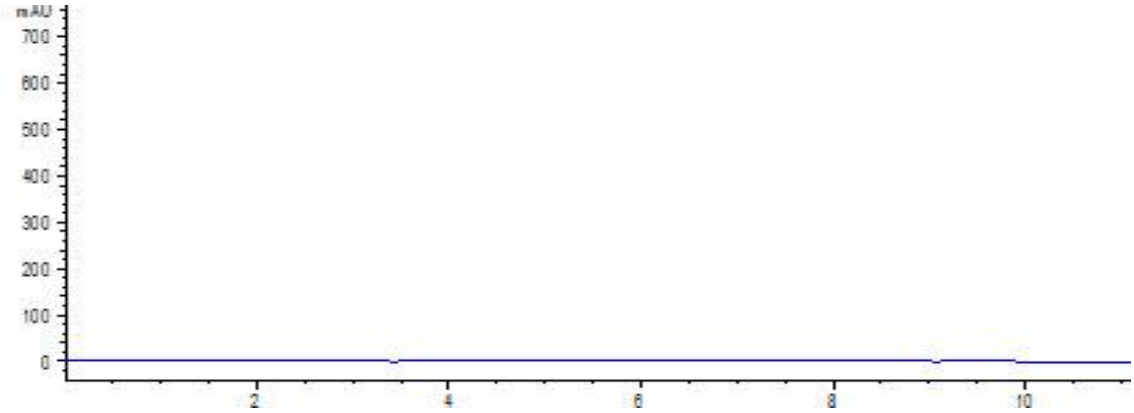

Figure 7.17. The chromatogram obtained from tablet matrix

7.3.5. Linearity and range

Linearity study was applied with standard mixture solutions at concentrations of 1.0, 5.0, 10.0, 15.0, 20.0 and 25.0 ppm. The solutions were injected triplicate together with QC standard solutions. Calibration curves were obtained by plotting the peak areas against concentrations. The calibration curves of CHG and FBP are shown in Figure 7.18 and 7.19, respectively.

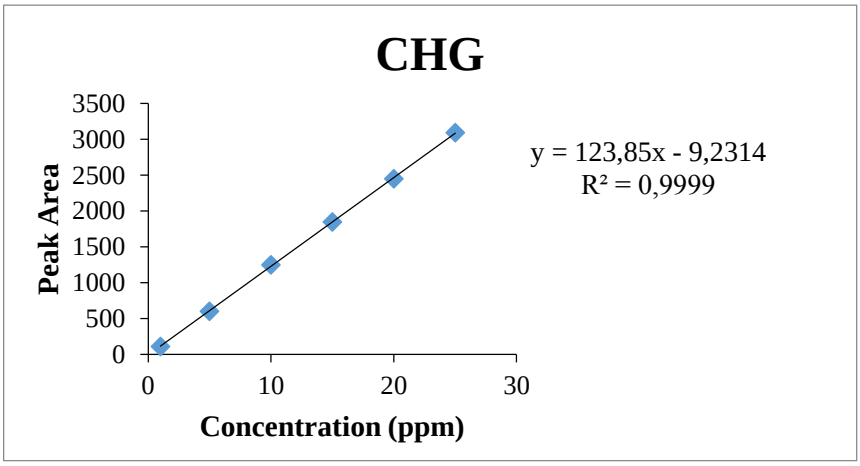

Figure 7.18. The calibration curve of CHG

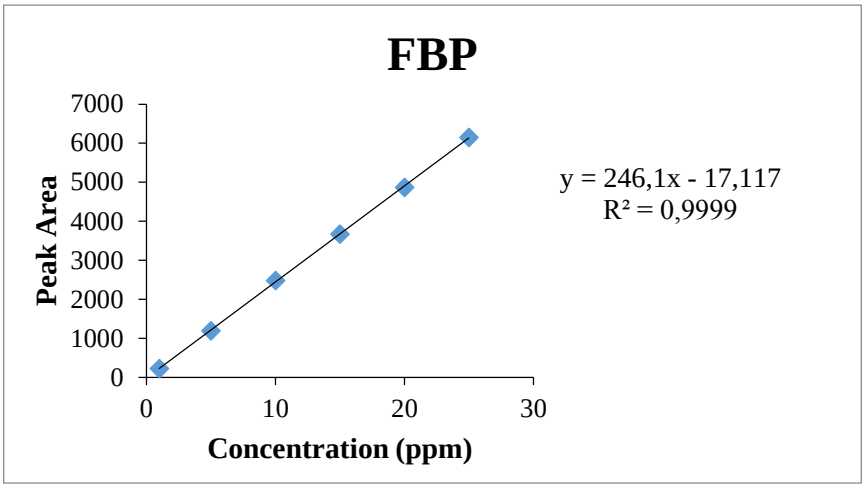

Figure 7.19. The calibration curve of FBP

The calibration curves were evaluated by correlation coefficient. The correlation coefficient (R^2) of the calibration curves was 0.999. Therefore, the calibration curves for CHG and FBP were found to be linear within the range of 1.0 – 25.0 ppm concentrations. The regression equations were calculated from the calibration curves and recovery values were determined using the regression equations.

LOD values were calculated from the formula 3.3 x (SD/S) and LOQ values were calculated from the formula 10 x (SD/S) in which SD represents standard deviation of recovery values of 2.0 ppm standard mixture and S presents slope of the calibration curve. The results of the linearity study are listed in Table 7.13.

Table 7.13. Linearity data for calibration curves of CHG and FBP

Parameter	CHG	FBP
Linearity Range (ppm)	1.0 – 25.0	1.0 – 25.0
R^2	0.9999	0.9999
Intercept	9.2314	17.117
S	123.85	246.1
SD *	1.261	1.508
LOD (ppm)	0.033	0.020
LOQ (ppm)	0.102	0.061

* SD: Standard deviation of recovery values of 2.0 ppm standard mixture

7.3.6. Accuracy and precision

Accuracy and precision studies were made by intraday and interday experiments at four concentration levels in the linearity range. For this purpose, the studies were applied with QC standard solutions of 2.0, 8.0, 13.0 and 22.0 ppm concentrations. The solutions were prepared triplicate and each solution was injected triplicate together with calibration standard solutions. For interday determinations, the study was carried out in 3 different days.

To determine the accuracy, the concentrations of QC standard solutions were obtained from regression equations of the calibration curves and then recovery values were calculated.

To determine the precision, RSD values of the recovery values were calculated according to the following formula:

RSD = (Standard Deviation / Average) x 100

The results of the accuracy and precision studies for CHG and FBP are presented in Table 7.14.

Table 7.14. Results of the accuracy and precision studies for CHG and FBP

Analyte	Concentration (ppm)	Intraday		Interday	
		Recovery%	RSD	Recovery%	RSD
CHG	2.0	98.47	0.63	98.49	1.16
	8.0	101.37	0.19	101.25	1.07
	13.0	100.46	0.33	100.63	1.33
	22.0	100.06	0.92	99.56	1.32
FBP	2.0	99.77	0.74	99.41	1.98
	8.0	100.67	0.28	101.92	1.94
	13.0	99.76	0.79	100.28	1.52
	22.0	99.31	0.28	99.39	1.18

The recovery results of the intraday and interday studies were ranged between 98.47-101.37% and 98.49-101.25% for CHG; 99.31-100.67% and 99.39-101.92% for FBP, respectively. The highest RSD value for intraday and interday studies were calculated as 0.92% and 1.33% for CHG, 0.79% and 1.98% for FBP, respectively. The results confirmed the accuracy and presicion of the developed method.

7.3.7. Robustness

Robustness study was performed by making small variations in method parameters to assess whether the response is influenced by the small changes. The method paratemers investigated in this study were temperature (± 3ºC), the flow rate (± 0.05 ml/min) and pH of the mobile phase (± 0.1). Parameters investigated are listed in Table 7.15.

Table 7.15. Robustness parameters

Parameter	**The Lower Value**	**The Higher Value**
Temperature	27ºC	33ºC
Flow rate	0.45 ml/min	0.55 ml/min
Mobile phase pH	2.4	2.6

As at the accuracy and precision studies, the QC standard solutions were prepared triplicate and each solution was injected to system triplicate together with calibration standard solutions. Recovery values of the QC standard solutions were determined using the regression equations of the calibration curves. The results of the robustness study are shown in Table 7.16.

Table 7.16. Results of the robustness study for CHG and FBP

Parameter	Concentration (ppm)	Recovery%	
		CHG	FBP
Temperature: 27ºC	2.0	102.06	101.63
	8.0	105.57	105.06
	13.0	99.24	99.29
	22.0	101.09	101.08
Temperature: 33ºC	2.0	103.13	102.39
	8.0	102.57	102.96
	13.0	97.57	96.94
	22.0	96.8	96.36
Flow rate: 0.45 ml/min	2.0	100.1	100.09
	8.0	98.61	98.61
	13.0	98.16	98.16
	22.0	97.99	97.99
Flow rate: 0.55 ml/min	2.0	98.78	101.01
	8.0	98.12	99.20
	13.0	98.01	98.08
	22.0	97.68	97.92
Mobile phase pH: 2.4	2.0	104.01	103.41
	8.0	102.50	101.26
	13.0	97.78	97.75
	22.0	98.72	99.52
Mobile phase pH: 2.6	2.0	102.53	101.03
	8.0	99.11	98.51
	13.0	97.20	97.45
	22.0	98.26	97.35

Robustness study showed that variations in flow rate and mobile phase pH do not have an effect on the analyte response. However, the changes in temperature made a little

effect on the results.

7.4. Sample Analysis

The developed and validated method was applied for the analysis of gargle, spray and tablet samples containing CHG and FBP in combination or individually. Sample solutions were prepared as described in section number 6.2.3. Each sample solution was prepared as two sets and each of them was analyzed in triplicate together with calibration standard solutions. Analysis results were evaluated by using a calibration curve. The amounts of analytes in the samples were calculated from the regression equation of the calibration curve, and then recovery and RSD values were determined. The results of the analyses are given in Table 7.17.

Table 7.17. Analysis results of pharmaceutical products

Sample	Claimed Amount (ppm)		Found Amount (ppm) (Mean ± SD)		Recovery%	
	CHG	FBP	CHG	FBP	CHG	FBP
Sample 1[a]	4.8	10.0	4.92 ± 0.02	10.18 ± 0.68	102.70	101.89
Sample 2[b]	4.8	10.0	4.94 ± 0.75	10.28 ± 0.11	102.98	102.80
Sample 3[c]	10.0	-	10.14 ± 0.08	-	101.42	-
Sample 4[d]	-	10.0	-	9.92 ± 0.34	-	99.23

[a] Gargle sample that contains 0.12% CHG and 0.25% FBP (w/v)
[b] Spray sample that contains 0.12% CHG and 0.25% FBP (w/v)
[c] Gargle sample that contains 0.12% CHG and 0.15% benzydamine hydrochloride (w/v)
[d] Tablet sample that contains 100 mg FBP

The recovery values of CHG and FBP were between 101.42-102.98% and 99.23-102.80%, respectively. The chromatograms obtained from the analysis studies are presented in Figures 7.20, 7.21, 7.22, and 7.23.

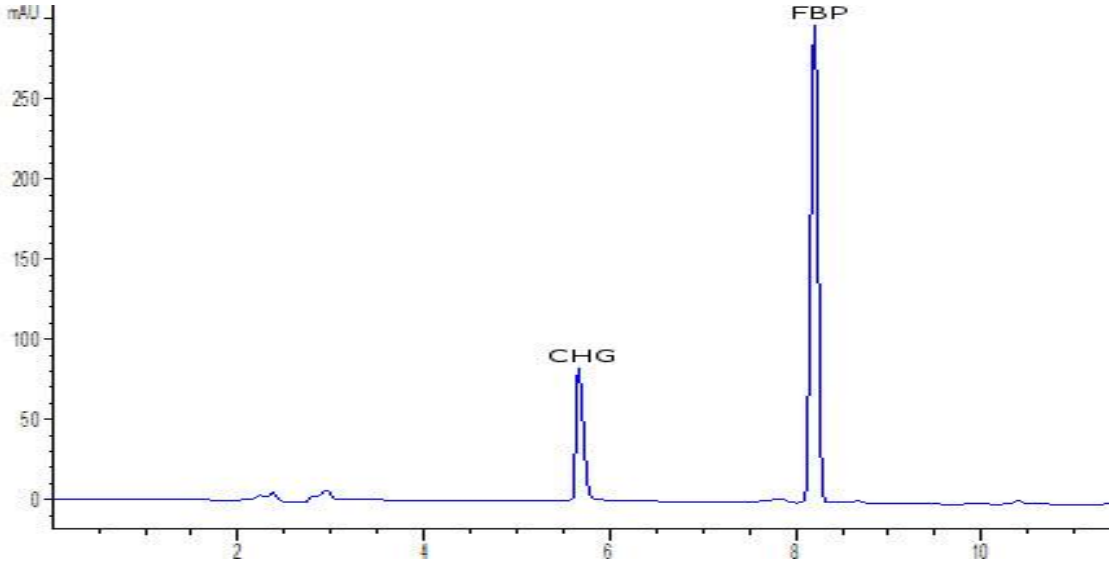

Figure 7.20. The chromatogram obtained from the analysis study of sample 1

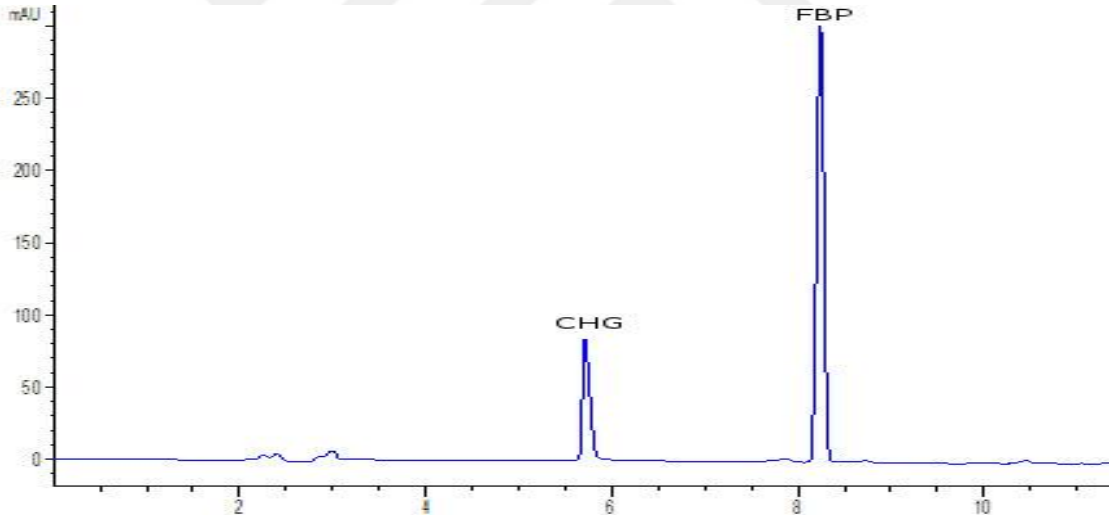

Figure 7.21. The chromatogram obtained from the analysis study of sample 2

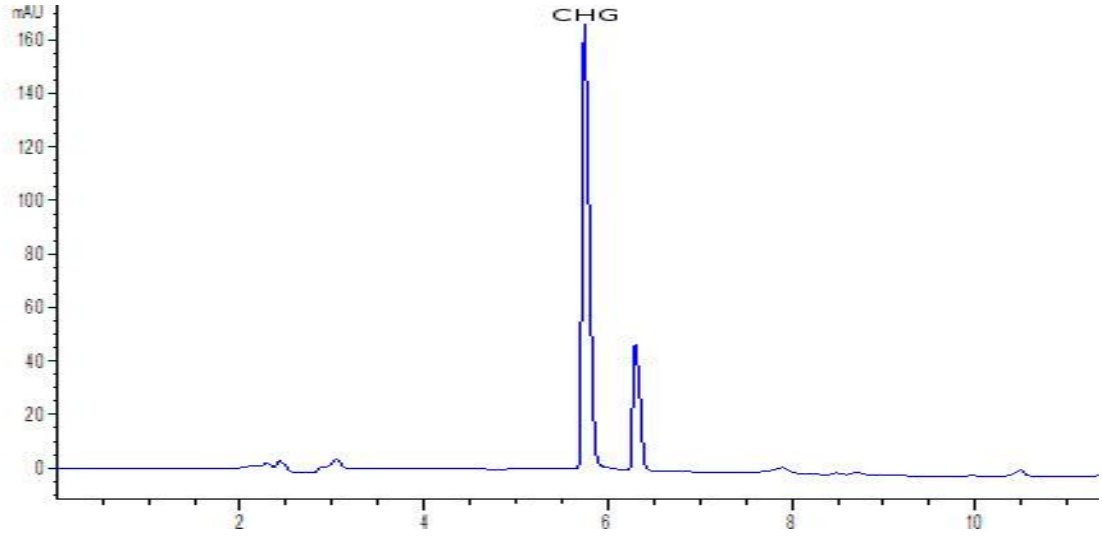

Figure 7.22. The chromatogram obtained from the analysis study of sample 3

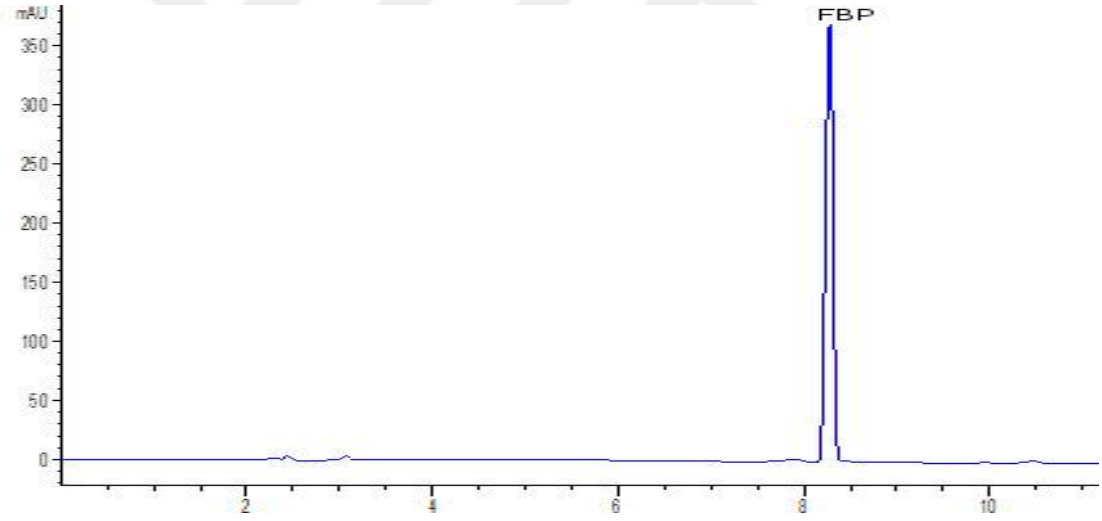

Figure 7.23. The chromatogram obtained from the analysis study of sample 4

The recovery values were in good agreement with the label claims. It was concluded that the method can be applied successfully for the analysis of CHG and FBP in commercial samples.

8. DISCUSSION AND CONCLUSIONS

In this study, an HPLC method for the simultaneous determination of CHG and FBP was developed, validated according to the USP guideline and applied to commercial gargle, spray and tablet products.

UV/DAD was used as a detector in the study. Based on the literature, various wavelengths of 230 nm, 248 nm and 265 nm were investigated. 248 nm was chosen based upon spectra of the analytes.

Different mobile phases were tested in the development stages of the method. ACN was chosen as the organic solvent because of its excellent separation potential. To adjust pH of the mobile phase, different buffer solutions and concentrations of acetic acid and orthophosphoric acid were investigated. To obtain better peak shapes with less tailing, different gradient profiles were investigated. As a result, 100.0 mM pH 2.5 phosphate buffer and ACN mixture was determined as the mobile phase, and the gradient profile (time, %B) was established as follows: 0 min, 30% B; 5 min, 80% B; 10 min, 80% B; 15 min, 30% B.

To determine the optimum temperature, 25ºC, 30ºC, 35ºC, 40ºC and 45ºC were tested. It was concluded that an increase of temperature over 30ºC caused a decrase of the peak area values. In addition, peak splitting was observed over 40ºC. Optimum temperature was chosen as 30ºC because of the highest peak area values with greater peak shapes.

The optimum injection volume was investigated by taking column properties into consideration. Injection volumes of 5.0 µl, 10.0 µl, 15.0 µl and 20.0 µl were investigated. Peak area values increased with the increasing injection volume. 20.0 µl was found to be the optimum injection volume.

To determine the optimum flow rate, flow rates of 0.4 ml/min, 0.5 ml/min and 0.6 ml/min were tested. The obtained peak area values were close to each other. In this case, the peak shapes and the fact that increasing flow rate causes higher column pressure were the factors for consideration of optimum flow rate. Hence, the flow rate of 0.5 ml/min was chosen as the optimum flow rate.

The developed method provided the requirements of system suitability test. Therefore, it was found to be suitable for the analysis of the compounds.

The stability of CHG and FBP standard solutions were investigated during 48 hours. The highest change in the recovery values were calculated as 6.30% for CHG and

6.05% for FBP.

Specificity studies illustrated that CHG and FBP were separated reasonably from each other. There was no interference observed, which indicated the specificity of the developed method.

The calibration curves for CHG and FBP were found to be linear within the concentration ranges of 1.0 – 25.0 ppm. The correlation coefficient was 0.9999 for both analytes. The LOD and LOQ values were calculated as 0.033 and 0.102 ppm for CHG, 0.020 and 0.061 ppm for FBP. In this case, the method was found to be much more sensitive compared to the method of Hanif *et. al* (14) which gave LOD at 10 ppm and LOQ at 50 ppm for FBP. The method was also found the be more sensitive than the methods of Hutzler *et. al* (14), Ünal *et. al* (16), Yılmaz *et. al* (19) and Abbad-Villar *et. al* (27) based on the LOD and LOQ values.

Accuracy and precision of the method were investigated by intraday and interday determinations of CHG and FBP. The recovery results of the intraday determinations were ranged between 98.47-101.37% for CHG, and 99.31-100.67% for FBP. Intraday recovery values were ranged between 98.49-101.25% for CHG, and 99.39-101.92% for FBP. The method was found to be accurate. In addition, it gave better results compared to the method of Hanif *et. al* (14) which had recovery results for FBP between 74.09-102.421%.

The highest RSD values in intraday and interday studies were calculated as 0.92% and 1.98%, respectively. The results were found to be low, and the method was precise. Additionally, the precision results were found to be lower compared with the methods of Yılmaz and Alkan (8, 18), Ünal et. al (16) and Mei et. al (17), which had RSD values for FBP higher than 3.20%. On the other hand, Doğan and Başçı (25) obtained RSD values less than 0.16% for CHG, however, their method proposed to use an internal standard which makes the method more time consuming compared to our method.

Robustness of the method for the analysis of CHG and FBP were tested by small variations in the method parameters. The variations in flow rate and pH of the mobile phase did not have an effect on the results, however, the variations in temperature effected the results slightly.

The method was successfully applied for the analysis of commercial gargle, spray and tablet products. The recovery values were ranged between 99.23 and 102.98. The chromatograms obtained were clear, and the recovery values were in good agreement with the label claims.

The developed method is rapid, and the mobile phase flow rate is very less, only 0.5 ml/min in comparison to 1 ml/min for the USP (10, 21) and EP (11, 22) methods.

In summary, the developed HPLC method is specific, linear, accurate and precise. The results of statistical analysis demonstrate that the values of the validation parameters are acceptable, and therefore, the method is suitable for the qualitative and quantitative determination of CHG and FBP. The most important advantage of this method is to analyze CHG and FBP at the same time as there are no methods in the literature for the simultaneous determination of these drugs. In addition to that, the method does not involve any pre-procedure such as extraction. The method is simple and reliable, and it can be used for the routine analysis of CHG and FBP in pharmaceutical products.

9. REFERENCES

1) Chao SH, Ho HT, Chen FA, Lin PY, Yu YC, Wu AB. Identification of flurbiprofen and its photoproducts in methanol by gas chromatography-mass spectrometry. *Biomed Chromatogr*. 2007; 21(5): 527-533.

2) Schachtel B, Aspley S, Berry P, et al. Efficacy of a novel (lozenge) delivery of flurbiprofen over 24 hours. *J Pain* [serial online] 2012, 13(4): S74. Erişim 11.10.2017, http://www.jpain.org/article/S1526-5900(12)00339-2/fulltext.

3) Russo M, Bloch M, Looze F, Morris C, Shephard A. Flurbiprofen microgranules for relief of sore throat: a randomised, double-blind trial. *Brit J Gen Pract*. 2013; 63: 149-155.

4) Másquio Fiorentino FA, Corrêa MA, Nunes Salgado HR. Development and validation of a microbiological assay for determination of chlorhexidine digluconate in aqueous solution. *Braz J Pharm Sci.* 2013; 49: 351-358.

5) Másquio Fiorentino FA, Corrêa MA, Nunes Salgado HR. Analytical methods for the determination of chlorhexidine: a review. *Crit Rew Anal Chem*. 2010; 40(2): 89-101.

6) Maha MY, Maan MN, Amer AT. An in-vitro evaluation of the antibacterial activity of experimental chlorhexidine gluconate solution using agar diffusion test. *Tikrit J for Dent Sci.* 2013; 1: 94-100.

7) Nikolin B, Imamović B, Medanhodžić-Vuk S, Šober M. High perfomance liquid chromatography in pharmaceutical analyses. *Bosn J Basic Med Sci.* 2004; 4(2): 5-9.

8) Yılmaz B, Alkan E. Spectrofluorometric and UV spectrophotometric methods for the determination of flurbiprofen in pharmaceutical preparations. *Res Rev Pharm Anal*. 2015; 4(4).

9) Chandran S, Jadhav PR, Kharwade PB, Saha RN. Rapid and sensitive spectrofluorimetric method for the estimation of celecoxib and flurbiprofen. *Indian J Pharm Sci.* 2006; 68 (1): 20-25.

10) United States Pharmacopoeial Convention, *Flurbiprofen,* in United States Pharmacopoeia, United States Pharmacopoeial Convention: Rockville. 2000. p. 955.

11) Council of Europe, *Flurbiprofen*, in European Pharmacopeia, Council of Europe: Strasbourg. 2005. p. 1623-1624.

12) Sajeev C, Jadhav PR, Ravishankar D, Saha RN. Determination of flurbiprofen in pharmaceutical formulations by uv spectrophotometry and liquid chromatography. *Anal Chim Acta.* 2002; 463: 207-217.

13) Qayyum A, Najmi MH, Farooqi Z. Determination of pharmacokinetics of flurbiprofen in Pakistani population using modified HPLC method. *J Chromatogr Sci*. 2011; 49(2): 108-13.

14) Hanif M, Nazer N, Chaurasiya V, Zia U. Simultaneous determination of famotidine and flurbiprofen by high performance liquid chromatography. *Trop J Pharm Res.* 2016; 15(3): 605-611.

15) Hutzler JM, Fryeb RF, Tracya TS. Sensitive and specific high-performance liquid chromatographic assay for 49-hydroxyflurbiprofen and flurbiprofen in human urine and plasma. *J Chromatogr B*. 2000; 749: 119-125.

16) Ünal DÖ, Güler S, Erol DD. Development and validation of bioanalytical method for determination of flurbiprofen from human plasma by liquid chromatography. *Hacettepe University J Fac. Pharm.* 2009; 29(1): 25-35.

17) Mei C, Li B, Yin Q, et al. Liquid chromatography-tandem mass spectrometry for the quantification of flurbiprofen in human plasma and its application in a study of bioequivalence. *J Chromatogr B.* 2015; 993-994: 69-74.

18) Yılmaz B, Alkan E. Determination of flurbiprofen in pharmaceutical preparations by gc-ms. *Arab J Chem.* In Press.

19) Yılmaz B, Sahin H, Akba V, Erdem AF. Determination of flurbiprofen in human plasma by gas chromatography with mass spectrometry and its pharmacokinetics. *J Aoac Int.* 2014; 97: 1061-1066.

20) Hamoudová R, Pospísilová M. Determination of ibuprofen and flurbiprofen in pharmaceuticals by capillary zone electrophoresis. *J Pharm Biomed Anal*. 2006; 41: 1463-1467.

21) United States Pharmacopoeial Convention, *Chlorhexidine Gluconate Solution*, in United States Pharmacopoeia, United States Pharmacopoeial Convention: Rockville. 2014. p. 477.

22) Council of Europe, *Chlorhexidine Digluconate Solution*, in European Pharmacopeia, Council of Europe: Strasbourg. 2005. p. 1258-1259.

23) Xu Y, Wong GY. Simultaneous determination of lignocaine hydrochloride, chlorhexidine gluconate, and triamcinolone acetonide in suspension by reversed-phase hplc. *J Liq Chrom Rel Technol.* 1999; 22(13): 2071-2091.

24) Havlíková L, Matysová L, Nováková L, Hájková R, Solich P. HPLC determination of chlorhexidine gluconate and p-chloroaniline in topical ointment. *J Pharm Biomed Anal.* 2007; 43(3): 1169-1173.

25) Dogan A, Bascı NE. Development and validation of RP-HPLC and ultraviolet spectrophotometric methods of analysis for the quantitative determination of chlorhexidine gluconate and benzydamine hydrochloride in pharmaceutical dosage forms. *Curr Pharm Anal.* 2011; 7: 167-175.

26) Borissova R, Mandjukova S. Titrimetric and spectrophotometric determination of chlorhexidine digluconate in tooth pastes. *Fresenius J Anal Chem.* 1997; 357: 977-980.

27) Abad-Villar EM , Etter SF, Thiel MA, Hauser PC. Determination of chlorhexidine digluconate and polyhexamethylene biguanide in eye drops by capillary electrophoresis with contactless conductivity detection. *Anal Chim Acta,* 2006; 561: 133-137.

28) Paradkar A, Maheshwari M, Tyagi AK, Chauhan B, Kadam SS. Preparation and characterization of flurbiprofen beads by melt solidification technique. *AAPS Pharm Sci Tech.* 2003; 4(4): 1-9.

29) Abdel Aziz A, Al Badr AA, Hafez GA., *Flurbiprofen*, in Profiles of Drug Substances, Excipients and Related Methodology. Elsevier: San Diego. 2012. p. 114-177.

30) Buchanan WW, Kassam YB. European experience with flurbiprofen: a new analgesic/anti-inflammatory agent. *Am J Med.* 1986; 80: 145-152.

31) Câmara De Bem SH, Estrela C, Guedes DF, Sousa-Neto MD, Pécora JD. Determination of chemical components derived from 2% chlorhexidine gel degradation using gas chromatography-mass spectrometry. *Acta Odontol Scand.* 2014; 72(8): 630-638.

32) Sousa CP, Oliveira RC, Freirea TM, et al. Chlorhexidine digluconate on chitosan-magnetic iron oxidenanoparticles modified electrode: electroanalysis and mechanisticinsights by computational simulations. *Sensor Actuat B-Chem.* 2017; 240: 417-425.

33) Estrela C, Ribeiro RG, Estrela CR, Pécora JD, Sousa-Neto MD. Antimicrobial effect of 2% sodium hypochlorite and 2% chlorhexidine tested by different methods. *Braz Dent J*. 2003; 14(1): 58-62.

34) Gomes BP, Souza SF, Ferraz CC, et al. Effectiveness of 2% chlorhexidine gel and calcium hydroxide against Enterococcus faecalis in bovine root dentine in vitro. *Int Endod J*. 2003; 36: 267-275.

35) Skoog DA, West DM, Holler FJ, Crouch SR., *Fundamentals of Analytical Chemistry*. Belmont, CA: Brooks Cole; 2014.

36) Moldoveanu SC, David V., *Essentials in Modern HPLC Separations.* New York: Elsevier; 2013.

37) Stuart B, Prichard E, Bedson B., *Practical Laboratory Skills Training Guides: High Performance Liquid Chromatography.* Cambridge, UK: RSC; 2003.

38) Bhardwaj SK, Dwivedi K, Agarwal DD. A review: HPLC method development and validation. *Int J Anal Chem Bioanal Chem.* 2015; 5(4): 76-81.

39) Sneha Lakshmi RP. A review on chromatography with high performance liquid chromatography (HPLC) and its functions. *RRJPA.* 2015; 4: 1-15.

40) Mcpolin O., *An Introduction to HPLC for Phamaceutical Analysis*. Newry, UK: Mourne Training Services; 2009.

41) Kazakevich Y, Lobrutto R., ed. *HPLC for Pharmaceutical Scientists*. 1st ed. HPLC for Pharmaceutical Scientists. John Wiley & Sons: Hoboken, NJ; 2007.

42) World Health Organization, *High Performance Liquid Chromatography*, in The International Pharmacopoeia, World Health Organization: Geneva. 2016. p. 1-9.

43) Kumar V, Bharadwaj R, Gupta G, Kumar S. An overview on HPLC method development, optimization and validation process for drug analysis. *Pharm Chem J.* 2015; 2(2): 30-40.

44) Harris DC, *Quantitative Chemical Analysis.* New York: W. H. Freeman and Company; 2007.

45) Shabir GA. Validation of high-performance liquid chromatography methods for pharmaceutical analysis: understanding the differences and similarities between validation requirements of the us food and drug administration, the us pharmacopeia and the international conference on harmonization. *J Chromatogr.* 2003; 987: 57-66.

46) Kazusaki M, Ueda S, Takeuchi N, Ohgami Y. Validation of analytical procedures by high-performance liquid chromatography for pharmaceutical analysis. *Chromatogr.* 2012; 33: 65-73.

47) United States Pharmacopoeial Convention, *Validation of Compendial Procedures*, in United States Pharmacopoeia, United States Pharmacopoeial Convention: Rockville. 2016. p. 1640-1645.

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