DETERMINATION OF SAPROPTERIN DIHYDROCHLORIDE IN SOLID DOSAGE FORMS BY VISIBLE SPECTROSCOPY

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ABSTRACT

DETERMINATION OF SAPROPTERIN DIHYDROCHLORIDE IN SOLID DOSAGE FORMS BY VISIBLE SPECTROSCOPY

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Sapropterin Dihydrochloride is the synthetic form of tetrahydrobiopterin (BH4) which is cofactor of phenylalanine hydroxylase (PAH) enzyme. For people with phenylketonuria (PKU), oral administration of sapropterin dihydrochloride decrease phenylalanine level in blood by converting it to tyrosine. The purpose of this study is to develop an analytical method for the determination of this active ingredient in order to follow the production of the generic drug development of Kuvan®. Fourier-Transform infrared spectroscopy (FT-IR), thermogravimetric analysis (TGA), X-Ray Diffraction (XRD), inductively coupled plasma/mass spectrometry (ICP-MS), chloride content and high-performance liquid chromatography with UV detector HPLC/UV analysis were used in order to investigate purity and to ensure specifications of Sapropterin Dihydrochloride (SAP). Because of high selectivity and sensitivity of HPLC/UV analysis were used as a reference method for the quantitative analysis of SAP. The study continued with UV/Visible Spectroscopy (UV/VIS) analysis. A method from the literature with Folin-Coicalteu (FC) reagent was studied and compatible results were obtained with respect to HPLC/UV method. By using cyclic voltammetry, oxidation and reduction potentials of SAP was calculated and with this oxidation potential $CuSO_4$ was selected as coloring agent for UV/$CuSO_4$

method of quantitative analysis. This method worked very well for the determination of sapropterin concentration for 5 to 65 ppm. without having any interference from the other ingredients present in the drug. Square wave voltammetry also used as an assay method at the potential value of 0.27 V.

In this thesis, reliable, precise and easy-to-use methods were successfully developed and validated for the assay investigation of Sapropterin dihydrochloride.

An analysis method with paper sensor[48] was developed and linearity of method was studied but further investigation was needed to use this method for pharmaceutical dosage forms.

Keywords: Sapropterin Dihydrochloride Assay; Tetrahydrobiopterin; Pharmaceutical Analysis; Paper Sensor

ÖZ

GÖRÜNÜR BÖLGE SPEKTROKOPİSİ KULLANILARAK KATI DOZAJ FORMUNDAKİ SAPROPTERİN DİHİDROKLORÜRÜN TAYİNİ

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Sapropterin Dihidroklorür, fenilalanin hidroksilaz (PAH) enziminin kofaktörü olan tetrahidrobiyopterin (BH4) 'ün sentetik formudur. Fenilketonüri (PKU) olan kişiler için, oral sapropterin dihidroklorür uygulaması, kandaki fenilalanin seviyesini tirozine dönüştürerek azaltır. Bu çalışmanın amacı, Kuvan®'ın jenerik ilaç gelişiminin üretimini takip etmek için bu aktif bileşenin belirlenmesi için analitik bir yöntem geliştirmektir. Fourier-Transform kızılötesi spektroskopisi (FT-IR), termogravimetrik analiz (TGA), X-Işını Kırınımı (XRD), endüktif olarak bağlanmış plazma / kütle spektrometresi (ICP-MS), klorür içeriği ve UV dedektörü HPLC / ile yüksek performanslı sıvı kromatografisi Saflığı araştırmak ve Sapropterin Dihidroklorür (SAP) spesifikasyonlarını sağlamak için UV analizi kullanılmıştır. HPLC / UV analizinin yüksek seçiciliği ve hassasiyeti nedeniyle SAP'nin kantitatif analizi için bir referans yöntem olarak kullanılmıştır. Çalışma UV / Görünür Spektroskopi (UV / VIS) analizi ile devam etti. Aynı sonuçları elde etmek için literatürden Folin-Coicalteu (FC) reaktifi ile bir yöntem kullanılmıştır. Siklik voltametri kullanılarak SAP'nin oksidasyon ve indirgeme potansiyelleri hesaplandı ve bu oksidasyon potansiyeli ile UV / CuSO4 kantitatif analiz yöntemi için renklendirici madde olarak CuSO4 seçildi. Bu yöntem, 5 ila 65 ppm sapropterin konsantrasyonunun belirlenmesi için çok iyi

çalıştı. Ürün içerisinde ki diğer bileşenlerden herhangi bir girişim olmamıştır. Kare dalga voltametrisi ayrıca 0.27 V potansiyel değerinde bir tahlil yöntemi olarak kullanılmıştır.

Bu tezde, Sapropterin dihidroklorürün tahlil araştırması için güvenilir, kesin ve kullanımı kolay yöntemler geliştirilmiş ve doğrulanmıştır.

Kağıt sensörü[48] ile bir analiz yöntemi geliştirildi ve yöntemin doğrusallığı araştırıldı, ancak daha fazla araştırmaya ihtiyaç duyuldu.

Bu çalışmada, farklı yöntemlerle, Sapropterin dihidroklorürün tayin çalışması için güvenilir, kesin ve kullanımı kolay yöntemler başarıyla geliştirilmiştir ve valide edilmiştir.

Anahtar Kelimeler: Sapropterin Dihidroklorür Deneyi; tetrahidrobiopterin; Farmasötik Analiz; Kağıt Sensör

To My Beloved Family,

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LIST OF ABBREVIATIONS

SAP: Sapropterin Dihydrochloride

HPLC/UV: High Performance Liquid Chromatography with Ultraviolet Detector

ICP/MS: Inductively Couple Plasma with Mass Spectrometer

FT-IR: Fourier Transform – Infrared Spectroscopy

PKU: Phenylketonuria

SWV: Square-Wave-Voltammetry

CV: Cyclic Voltammetry

FC: Folin Coicalteu

HPLC: High Performance Liquid Chromatography

GC: Gas Chromatography

WS: Working Standard

COA: Certificate of Analysis

DMF: Drug Master File

CHAPTER 1

INTRODUCTION

1.1. Phenylketonuria

Phenylketonuria (PKU) is an inherited disease which results in high blood phenylalanine levels. Phenylalanine is taken into body with diet. Phenylalanine exists in proteins and some artificial sweeteners. The reason behind the PKU is a mutation in the phenylalanine hydroxylase (PAH) gene. This gene is responsible for the production of PAH which turns phenylalanine to tyrosine which is an essential amino acid in human body as shown in Figure 1.1. When the activity of phenylalanine hydroxylase is reduced or absent, phenylalanine levels increase to toxic levels. Nerve cells in the brain specifically sensitive for phenylalanine levels can be affected from excessive amounts of phenylalanine which cause brain damage[1].

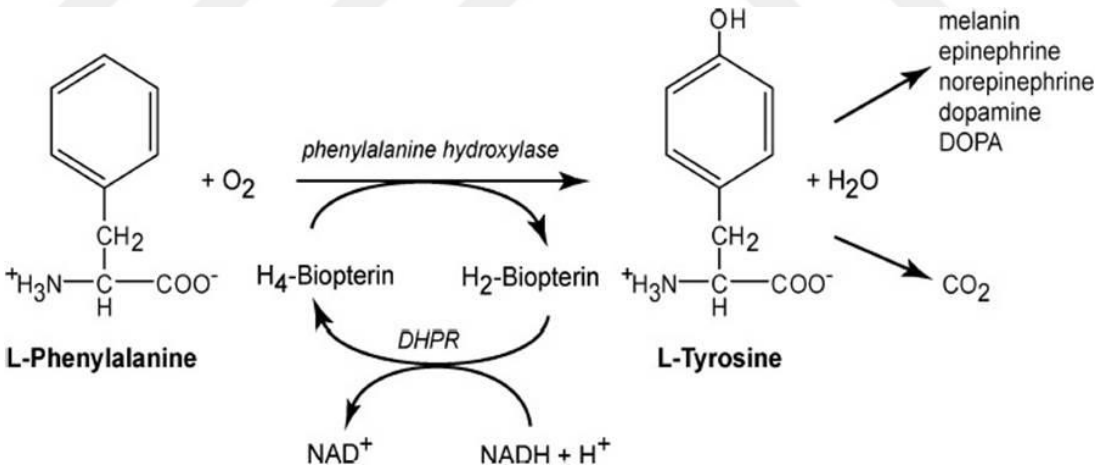


Figure 1.1. Phenylalanine to Tyrosine conversion catalyzed by PAH enzyme[2].

Appearance of phenylketonuria can vary depending on geographical region and ethnical groups. In Turkey, phenylketonuria occurs in 4 in 1000 newborn which is the maximum ratio in the world. This ratio is 1 in 15,000 in USA and 1 in 10,000 to 12,000 in Europe. The reason behind the high risk of PKU in Turkey is consanguineous marriage and lack of awareness of this disease[3].

1.2. Treatment of Phenylketonuria

To reduce symptoms of PKU, the most used treatment is to follow a well-controlled diet by limiting the food including phenylalanine. Beside from this controlled diet, it is necessary to take medication in order to reduce risks of phenylketonuria. Nowadays, there is only one medication is available in market to reduce phenylalanine levels in phenylketonuria which is KUVAN®[4].

1.2.1. Dietary Treatment

For a well physical and mental healthy person in terms of PKU level should follow a controlled diet from birth to elder life. It is very important to start early to PKU diet since it affects mental development of newborn. The only way to treat or prevent from mental disorder is to decrease phenylalanine intake just before to birth of the baby. The diet for phenylketonuria should be designed individually by a healthcare professional since the amount of safe phenylalanine level for patients can vary from one another [5].

1.2.2. Phenylketonuria Formula

Low protein diet is not enough for treatment of PKU since for a healthy mental and physical progress there are many essential amino acids which should be taken from proteins. Someone can take with PKU including supplements which are specifically produced amino acids without phenylalanine. It is very important to take PKU formula daily since low phenylalanine causes inadequate nutrition to have a healthy life.

For babies it is important to be fed with breast milk since it contains high amounts of phenylalanine. Amount of breast milk or ordinary infant formula which will be added

to phenylalanine-free formula should be calculated carefully by a dietitian. Adults and teenagers should also consume PKU formula to support their protein intake[6].

1.2.3. Medicinal Treatment

In our body, 5,6,7,8-Tetrahydrobiopterin (BH_4) exists as a cofactor of PAH enzyme[7]. In the presence of BH_4, PAH enzyme converts phenylalanine to L-Tyrosine. There is pharmaceutical form of BH_4 called as Sapropterin Dihydrochloride (Brand name as Kuvan®). Sapropterin Dihydrochloride treatment helps people to increase their level of PKU besides well-supported diet. Kuvan is not enough by itself to reduce phenylalanine levels to healthy limit[8].

1.2.3.1. Sapropterin Dihydrochloride

The structure of sapropterin is shown in Figure 1.2. Sapropterin Dihydrochloride is a synthetic (produced by a chemical reaction) form of 5,6,7,8-Tetrahydrobiopterin (BH_4). It is also used as a cofactor in synthesis of nitric oxide. It is a small molecule with molecular weight of 277.09 g/mol. As a raw material it should be kept around 2 to 8°C. Additionally, it is easily oxidized at +0.27 V oxidation potential and reduced at -0.16 V, [9]

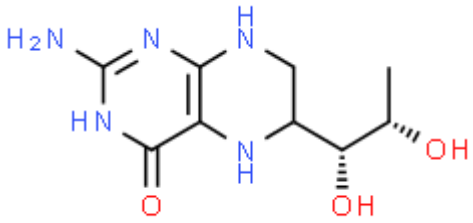


Figure 1.2 Sapropterin Molecular Structure[10].

1.3. The Importance of Analysis in Drug Industry

In drug industry, characterization of finished products or raw materials requires multidisciplinary work including chemistry, biology, toxicology, statistics, and physics to develop new methods to produce and characterize constituents of drug. To study bioavailability and to understand pharmacokinetics effects of drugs, it is very important to develop sensitive and selective analytical methods. All analytical methods used in drug industry should be carefully developed and validate with respect to guidelines of accepted regulations in this area. Analytical methods to analyze a drug dosage form can be categorized in four main categories. These categorizes are[11];

- Description of Dosage Form
- Identification of Ingredients
- Assay Analysis
    - In Vitro Dissolution Testing
- Impurity/Related Substances Analysis[11]

Also, quality specifications or limits of pharmaceutical substances are described in pharmacopeias.

1.3.1. Definition of the Pharmacopeia

Pharmacopoeia derives from the ancient Greek word of φαρμακοποιΐα (pharmakopoiia), from φαρμακο- (pharmako-) ″drug″ which is followed by the verb-stem ποι- (poi-) ″make″ and lastly the abstract noun ending -ια (-ia). These three elements from Greek origin together can be combined as ″drug-mak-ing″ or more sensibly ″to make a drug″[12].

A pharmacopeia is a legal collection of quality specifications and standards for pharmaceutical products/standard in a country or region. It includes pharmaceutical excipients, starting materials, intermediates and finished pharmaceutical products (FPPs). General requirements may also be given on medicinal product or chemicals'

quality, such as analytical methods and their limits, microbiological purity, dissolution/in-vitro testing, or stability of drug/starting materials[13].

Latest version of Turkish Pharmacopeia was published on December 2016[14]. This version was adopted from European Pharmacopeia and it is accepted as legal pharmacopeia for Turkish Ministry of Health. For pharmaceutical analysis below pharmacopeias also was used commonly in pharmaceutical industry.

- United States Pharmacopeia (USP) – Current version: USP43 – NF38,
- European Pharmacopeia (EP) – Current version: 10th version,
- British Pharmacopeia (BP) – Current Version: 2018 Edition.

1.3.2. Description of Dosage Form

There are almost 401 different dosage forms in European Medicinal Agency (EMA). There are only two different types of dosage forms which are tablets and powder packets for KUVAN®. In this thesis, tablet dosage form was studied. For the analysis of different dosage forms, there are different procedures. For tablet dosage forms, in-vitro dissolution test is performed to rate and extent of drug absorption in gastrointestinal fluid by simulate it in a well-controlled vessel[15]. The first step of the analysis is the description of dosage form for the certification of drug product. This description is a qualitative statement for the dosage form which can be size, color, shape, physical state (liquid, solid, suspension, solution, etc.) of the drug. If any of these parameters changes during the shelf life of drug, necessary precautions has to be taken immediately[16].

1.3.3. Identification of Ingredients

For the identification of active ingredients, FTIR, HPLC-MS, HPLC with Diode Array Detector (DAD), GC-MS[17] are used to identify active ingredients. If active ingredient is a salt, all the ions can be identified to determine the exact component.

If the active ingredient is an optically active compound, it can be characterized with chiral assay method. It is possible to identify chiral drugs with chiral HPLC columns.[11]

1.3.4. Assay Analysis

In pharmaceutical assay analysis, active ingredient(s) is/are determined in drug dosage form. It is very important to develop a selective and sensitive analytical method for assay analysis since it is very crucial for production process, releasement, shelf-life, bioavailability and pharmacokinetic of drug substance. To develop an assay method, there should be no interference due to impurities of active ingredient and placebo of drug dosage form. There should be no interference between increasing degradation products and active ingredient during the shelf life of pharmaceutical product. Degradation products are usually followed beside active ingredient of pharmaceutical dosage form during the shelf life of the drug. This study would be first step before the impurity/related substances analysis[16].

1.3.4.1. In Vitro Dissolution Testing

Dissolution testing is the one of the most important analysis in drug industry since it is important to show effectiveness of active substance in drug matrix. Dissolution media should be carefully selected to show in vivo – in vitro relationship. In vitro dissolution test shows and stimulates extraction of active ingredient in our body[18].

Dissolution tests are not enough by itself to see pharmacokinetics of active ingredient in human body.

After proper conditions are selected for dissolution testing, the next step will be quantitative analysis of dissolution media to see dissolution profile of pharmaceutical active ingredient. This analytical method could be same or different with assay method of drug substance.

An applicable and useful dissolution method should be quick and sensitive as far as possible. In general, HPLC/UPLC methods with short analysis time or simple UV/VIS methods are preferred to analyze active ingredient after the dissolution tests. Dissolution apparatus I and II are shown in Figure 1.3. These two different apparatuses are selected by considering dosage forms[19].

The assembly of apparatus I consists of a metal shaft connected to a motor for circular motion and a cylindrical basket. In apparatus II, assembly is almost same rather than there is a blade shape paddle insist of basket. All materials for such dissolution system should be selected as inert material for drug substance or dissolution media (in general stainless steel)[19].

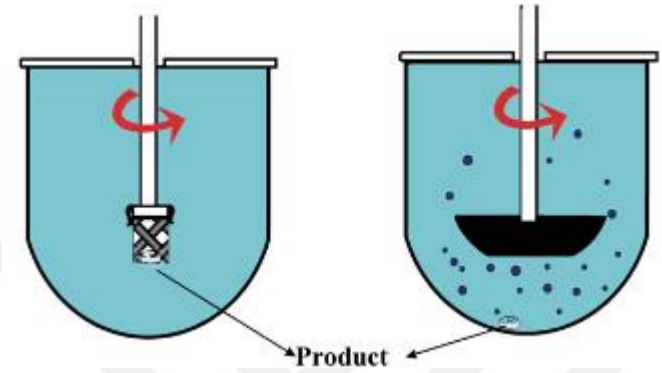


Figure 1.3. Dissolution apparatus of USP apparatus I (basket) and apparatus II (paddle)[20].

1.3.5. Impurity/Related Substances Analysis

There are mainly two types of impurities in pharmaceutical ingredients which are process related impurities and degradation products. Both should be analyzed during the analysis of raw material. After the production of drug dosage form only analysis of degradation products is mandatory. These methods should be carefully developed to identify all degradation impurities. It is important to for an analytical method that method should be sensitive enough to detect impurities at relatively low concentrations. These limits were considered by using general guidelines (ICH, Pharmacopeias, etc) or toxicology study of purified impurity itself. Unknown impurity limits for new drug products are given in Table 1.1[21]. In this table, maximum daily dose is meant that by considering dosage and maximum daily intake of these dosage form it is calculated and this maximum daily dose is used to calculate impurity limit for unknown impurities[21]. By using this table, one should be careful about LOQ (limit of quantification) of analysis since the method should be sensitive at given thresholds. (Table 1.1). In order to use Table 1.1, first daily dose of drug should be carefully

calculated by considering the literature about this medicine[22]. By using maximum daily dose appropriate threshold for each case (reporting, identification, qualification) found in table and limit is decided.

Table 1.1. Thresholds for Degradation Products in New Drug Substances[21].

Reporting Thresholds	
Maximum Daily Dose	Threshold
≤ 1 g	0.1 %
> 1 g	0.05 %
Identification Thresholds	
Maximum Daily Dose	Threshold
< 1 mg	1.0 % or 5 µg TDI, whichever is lower*
1 mg – 10 mg	0.5 % or 20 µg TDI, whichever is lower*
> 10 mg – 2 g	0.2 % or 2 mg TDI, whichever is lower*
> 2 g	0.10 %
Qualification Thresholds	
Maximum Daily Dose	Threshold
< 10 mg	1.0 % or 50 µg TDI, whichever is lower*
1 mg – 10 mg	0.5 % or 200 µg TDI, whichever is lower*
> 10 mg – 2 g	0.2 % or 3 mg TDI, whichever is lower*
> 2 g	0.15%

*whichever is lower means that stated amount and percent of dosage of drug should be compared and lower one should be stated as above limit.

Reporting threshold in Table 1.1 means that impurities higher than this limit should be reported in analysis certificate of substance.

Identification thresholds means impurities higher than this limit should be identified and carefully monitored during analysis.

Finally, qualification thresholds mean that impurities higher than this limit should be qualified by using other qualitative techniques and their safety should be guaranteed

to give higher limits. In general, LC-MS[23] is used in drug industry for the identification of these impurities.

There are also degradation and process related impurities named as process impurities in the certification analysis or pharmacopeia which is an official publication containing a list of medicinal drugs with their effects and directions for their use. Afterwards, it becomes a degradation product with the formulation of drug dosage form. During the method development studies it should be monitored whether there is an increase in the percentage of such process impurities. In total impurity evolution process of finished product, impurities which are not degradation product could not be summed as total impurity.

In some cases, there are also inorganic constituents such as metals and solvents (ethanol, isopropanol etc.). These residual solvents or inorganic materials should also be followed to verify for the compliance with certificate of analysis (COA) of the active ingredient of drug dosage form. To illustrate, before pharmaceutical production of a drug, active ingredient should be analyzed by Quality Control (QC) department to ensure it satisfies these limits even it had a COA.

1.4. Analytical Techniques Used for Drug Analysis

Classical methods and instrumental techniques used for drug analysis are listed below:

- HPLC coupled to ultraviolet (UV) / Electrochemical-Pulsed amperometric detector (ECD/PAD) / Refractive Index Detector (RID) / Florescence Detector (FD) / Mass Spectroscopy (MS)
- Potentiometric titrimetry.
- UV spectrophotometer,
- Cyclic/Square Wave Voltammetry,
- Quantitative NMR spectroscopy,
- TLC,

- Gas chromatography Flame Ionization detector (FID), Electron capture detector (ECD), Mass spectroscopy (MS), Thermal conductivity detector (TCD), Nitrogen phosphorous detector (NPD),
- ICPOES, ICPMS,
- AAS, AES.

There are also other methods such as gravimetric methods, microbiological assays (specifically for antimicrobial drugs).

There are mainly four steps for the validation of the analytical method:

- Identification tests.
- Quantitative analysis of impurities.
- Limit test for the control of impurities.
- Quantitative analysis of active ingredient or other ingredients present in drug dosage form (preservative, residual solvent etc.).

Identification tests are performed using HPLC by comparing reference and drug ingredients.

Impurity analysis could be both quantitative and limit test. Different characteristics are used to validate quantitative and limit test for impurities which are listed in Table 1.2. This table shows the requirement for the validation of different type of drug analysis. “+” means the parameter should be studied for the above analysis.

The following Figures of merit are followed for the validation of the new analytical method:

- Specificity
- Repeatability
- Intermediate Precision
- Accuracy
- Detection Limit

- Quantification Limit
- Linearity and Range
- Robustness

If the synthesis of active ingredient or the formulation of finished product are changed, then the method should be revalidated, or another method should be developed to be used in drug analysis.

Table 1.2 Validation Requirements for Different Analytical Analysis [21].

Type of Analytical Procedure	Identification	Testing for Impurities		Assay Dissolution Content/Potency
		Quantitative Analysis	Limit Test	
Accuracy	-	+	-	+
Repeatability	-	+	-	+
Intermediate Precision	-	+	-	+
Specificity	+	+	+	+
Detection Limit	-	-	+	-
Quantification Limit	-	+	-	-
Linearity and Range	-	+	-	+

These analytical validation characteristics should be described in detail to show their analytical features and importance for the drug industry.

1.4.1. Specificity

Specificity is the ability of an analytical method unequivocally analyze of studied material (active ingredient or preservative). There could be no interference from impurities/degradation products or matrix.

During the validation studies first specificity of the method should be established. The procedures for the specificity can be changed with the intended objective of analytical procedure.

Sometimes it is not possible to show that all analytical procedures are specific for the analyte. In such case combination of analytical procedures can be used to show the level of separation from matrix of drug dosage form[11].

In order to show the specificity of the method the following steps are usually followed:

For identification tests, HPLC-DAD, FTIR and NMR techniques are used[11]. In pharmacopeia, identification of active ingredients is generally done by HPLC by comparing with retention time of certified working standards. FTIR spectroscopy is also common method by using similarity index in its software which compares two spectra between certified standard and raw material. NMR is not very common but also needed in some case such as to identify bigger molecules with different alkyl groups. Simple chemical reactions which are specific for a salt or metal also can be used to identify different ions in active ingredients. There is a guideline in US Pharmacopeia <191> for the identification tests of different salts and metals by using specific chemical reactions[17]. Peak purity analysis is carried out using HPLC-DAD. When resolution (USP resolution[24]) is below 0.3 it is not possible to resolve the peak into its components. As an example, chromatograph, coelution of a peak is shown in Figure 1.4. Three components can be resolved using different columns, mobile phase with different polarity or pH and wavelength[25].

- Because of the coelution of such different compounds there are no shoulders and/or valleys are shown.
- There is no tailing which above the limit.

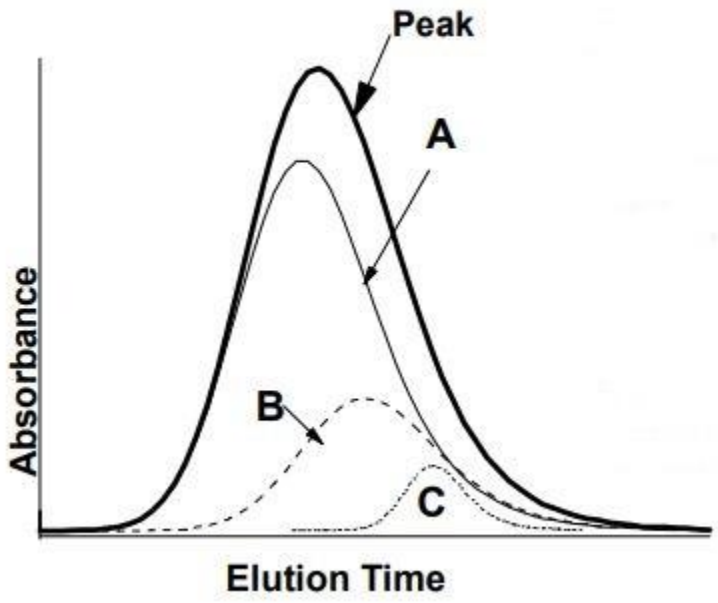


Figure 1.4 Coelution of A,B and C peaks in HPLC [25]

PDA/DAD homogeneity or purity is helped chromatographic method development and it could be indicating that a peak might not be a single compound. [25]

It is shown in Figure 1.5 that each spectrum is analyzed by Empower® 3[26] software for the analysis of peak purity across the chromatographic peak.

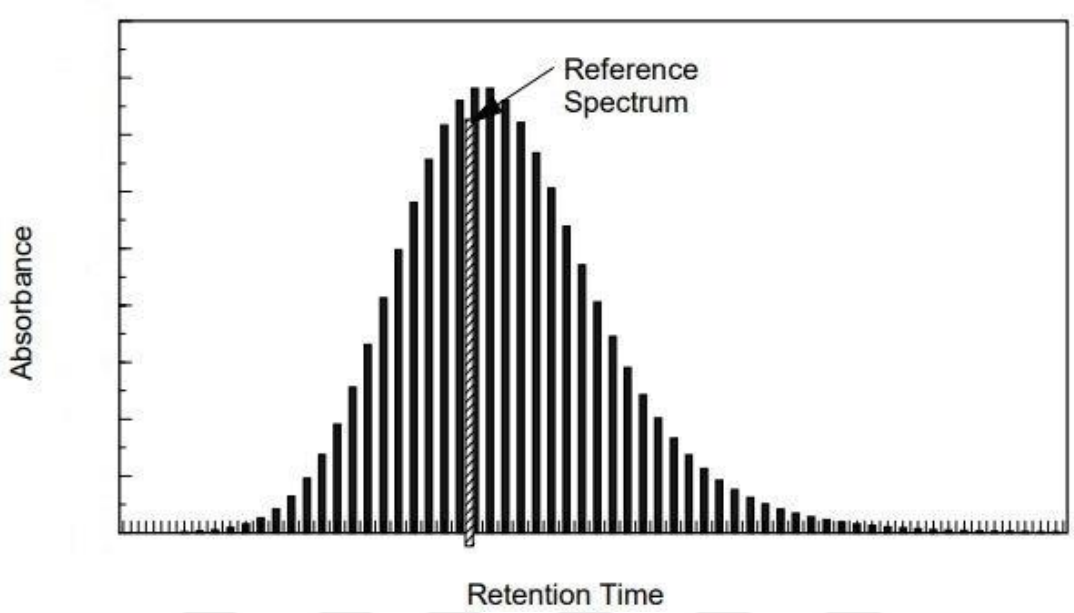


Figure 1.5 Peak purity calculation across the peak [25]

Result of two peak purity analysis is shown in Figures 1.6 and 1.7[27]. Figure 1.6 shows an example for pure peak without any coeluting substances.

Peak purity depends on the idea of impurities are spectrally different from the peak of the analyte. This spectral analysis is based on vector algorithms. When the two or more spectra are similar, the value gets closer to 0 degrees; when there are spectral differences, the value increase up to maximum 90 degrees. If there is no spectral difference, it can be concluded that there is spectral homogeneity and peak might be pure and assume that it is a single component. [27]

The peak shown in Figure 1.6 is an example for a chemically pure analyte and it is shown that there is no shoulder or valley across the peak. When there is low absorbance baseline noise can be contributing to spectral differences which could be sometimes an interference for peak purity. [27]

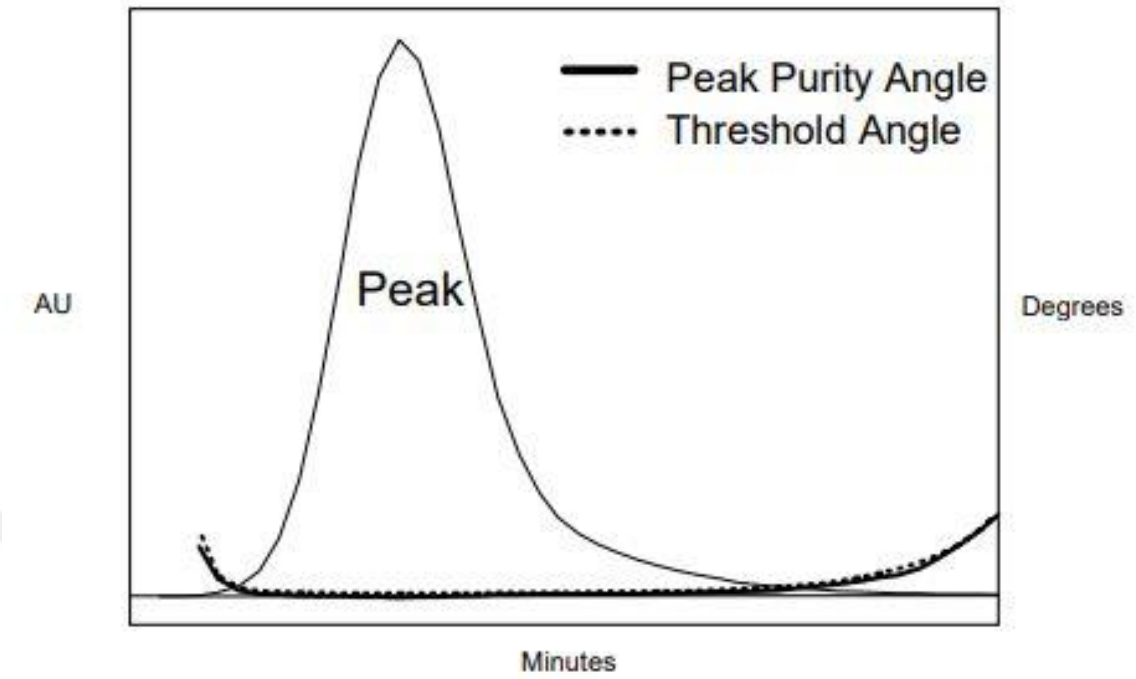


Figure 1.6 Peak purity threshold [27]

When an impurity detected across the desired chromatographic peak the purity plot increases the above of the threshold line. This implies that there are two or more components under the HPLC peak. Figure 1.7 shows the coeluting peaks with poor resolution. In this example, coeluting peak is the shoulder of the main peak[27].

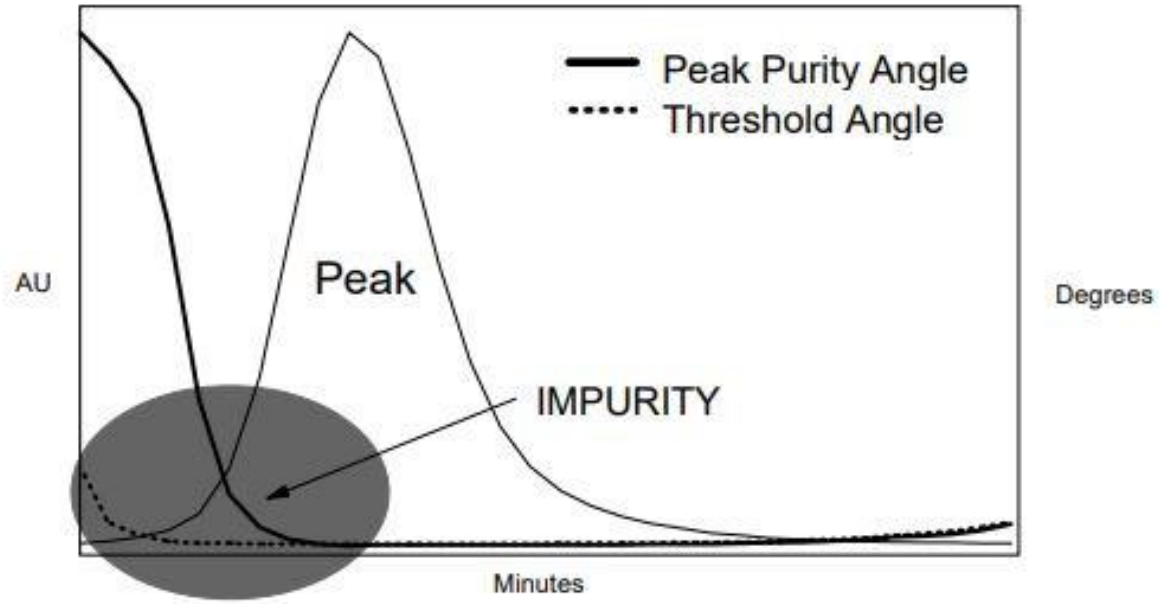


Figure 1.7. Coeluting Peaks with poor resolution [27]

1.4.1.1. Stress Testing

Stress testing is an important part of the specificity test since it is crucial to show degradation products and their resolution/coelution with active ingredient and also, to decide analysis time (should be longer than latest eluted degradation product).

Stress testing known as forced degradation studies can be applied for both drug and pharmaceutical product. It is used to show degradation pathways and possible degradation products during shelf-life of drug product. These studies will help the development of formulation and analytical method for the drug products[28].

Since stability studies can be performed within 6-12 months to see degradation product of substances, it is very helpful to check such impurities with accelerated stress testing. It is possible to generate degradants in a few weeks with stress testing[28].

It is important to select appropriate stress conditions for forced degradation studies. Paths for forced degradation study is shown in Figure 1.8. In this figure, different

stress conditions for different drug substances and drug products are shown as a list. These stress conditions are photolytic, thermal, thermal/humidity, acid/base hydrolysis and oxidative degradation of active ingredient or drug product[28].

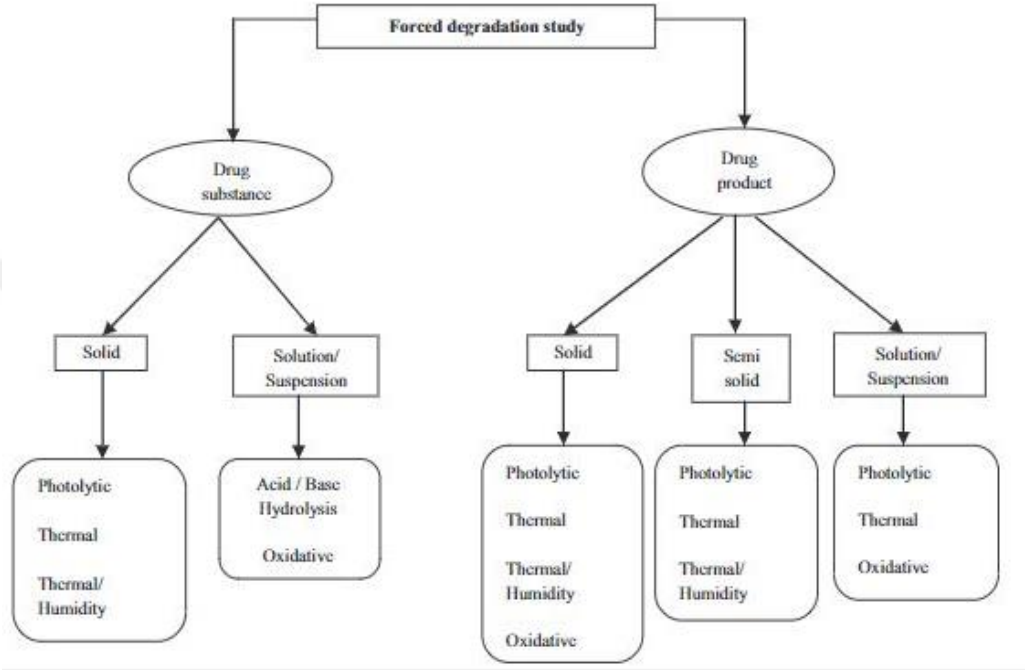


Figure 1.8 Paths for Forced Degradation Study[28].

1.4.2. Repeatability

Repeatability is a measure of precision under the same working conditions over a short time interval. Repeatability is also called as intra-assay precision[11]. A minimum of 6 test products at 100% concentration is studied in repeatability analysis[11].

1.4.3. Intermediate Precision

Intermediate precision expresses the variations in a laboratory such, different days, different equipment's, different analysts[11]. It is generally designed as repeatability and includes change in analysts, equipment and different days. It is not necessary to check the effect of changes individually. The experimental design can be used to check the effect of these changes[11].

1.4.4. Accuracy

Accuracy known as trueness of analytical procedure defines the closeness between accepted value as a conventional true value or the value which is accepted reference and experimental value[11]. After studying precision, linearity and specificity parameters of analytical validation, accuracy can be inferred[11].

1.4.5. Linearity and Range

The linearity for an analytical method to show test results is directly proportional with the concentration of the analyte in the sample[11].

Test results for the linearity study should be investigated statistically, for example, by calculation of a regression^2, y-intercept, correlation coefficient and residual sum of squares[11].

The establishment of linearity is needed minimum of 5 concentrations. Any other approaches should be justified to be accepted[11].

The range is the upper (LOL, limit of linearity) and lower concentration (LOQ, limit of quantitation) of analytes for the desired interval. It depends on the intended application of the procedure[11].

Following ranges should be satisfied for each case:

- Assay of a pharmaceutical product or an active substance should be in the range of 80 to 120 percent in general. The range could be wider to include content uniformity test which has wider range like 70 to 130 %.
- For dissolution testing ±20 percent of the specified dissolution range should be studied.
- For impurity testing range should be cover between reporting threshold to the 120 percent of the specification limit.

- In some cases, impurity and assay method can perform simultaneously, such linearity should cover the range between reporting threshold to the 120 percent of the assay specification[11].

1.4.6. Limit of Quantitation (LOQ)

For any individual analytical method, the limit of quantitation is the lowest possible sample which can be analyzed quantitatively with appropriate precision and accuracy. In pharmaceutical analysis, it is especially important for the determination of impurities, degradation products, residual solvents etc. It could be also important for low levels of compounds in quantitative assays[29].

1.4.6.1. Visual Evaluation

Visual evaluation can be used for instrumental analysis, but it is mainly used for non-instrumental analysis. Limit of quantitation is determined by analyzing samples with known concentration of analyte and by calculating at the minimum level which analyte can be calculated with high precision and accuracy.

1.4.6.2. Signal to Noise Approach

To use this approach analytical method should exhibit a baseline noise. To determine the signal-to-noise ratio, signals from low concentration samples are compared with blank samples. A typical S/N ratio is 10/1 for pharmaceutical procedures. This approach is mainly used in chromatography in pharmaceutical industry. In European Pharmacopoeia the limits for LOQ generally defined as Signal/Noise ratio of chromatographic peaks[30].

1.4.6.3. Standard Deviation of the Response and the Slope

The formula for the quantitation limit can be expressed as:

$$QL = \frac{10\sigma}{S}$$

Where; σ = standard deviation of the response of the analyte, S = the slope which is obtained from calibration curve of the analyte.

There are two ways to calculate the standard deviation:

- Appropriate number of the blank is analyzed, and their magnitude of analytical response is used to calculate standard deviation.
- For the second method a specific calibration curve is studied which includes LOQ limit in it. Standard deviation of y-intercepts or the residual standard deviation of regression curve is used as the standard deviation.

1.4.7. Limit of Detection (LOD)

For an analytical procedure limit of detection is the lowest possible concentration of analyte which can be detected.

For the limit of detection, a typical S/N ratio is between 3-2/1 for pharmaceutical procedures.

The formula for the detection limit can be expressed as:

$$QL = \frac{3\sigma}{S}$$

Where; σ = standard deviation of the response of the analyte, S = the slope which is taken from calibration curve of the analyte.

1.4.8. Robustness

Robustness parameter for analytical validation shows the reliability of an analytical analysis with changes in different method parameters. These changes and evaluation of them depends on the type of analytical method.

If the measurements are affected with variations in analytical parameters, the conditions for the analytical method should be appropriately controlled or in the procedure, there should be precautionary statement. One example for this statement could be this one; Before every analysis system suitability parameter should be checked (resolution, S/N, tailing factor) to ensure analytical method.

Typical variations for analytical methods are listed below:

- Solution Stability
- Time for the extraction

In the case of liquid chromatography:

- Change in pH of a mobile phase.
- Change in composition of mobile phase (different ratios of organic solvent and salt amount)
- Column type (different suppliers and/or lots)
- Column or sampler temperature
- Flow rate of the mobile phase

In the case of gas chromatography:

- Column type (different suppliers and/or lots)
- Column oven temperature
- Flow rate of the mobile phase

1.5. Aim of the Study

In this study, simple and easy-to-use methods were developed to analyze sapropterin dihydrochloride. It is very important to develop analytical method during development of drug dosage from. This drug is very crucial since there is no generic drug for Kuvan® and the price for it quite high in our country. There is no analytical method for sapropterin dihydrochloride in European or US pharmacopeias. There are few methods in literature comparable with validated method in this study. The method with folin-coicalteu[31] reagent was used to compare with the method developed using $CuSO_4$ in this study.

Experiments started with the characterization of pharmaceutical active substance (sapropterin dihydrochloride). For the characterization part, different analytical methods were used to identify physical and some chemical properties of sapropterin dihydrochloride. After active ingredient satisfies all the specifications of sapropterin dihydrochloride, the studies continued with the development of HPLC and UV methods for the assay and impurity/related substance analysis of active ingredient. HPLC methods were developed as a reference method to compare with other studies in this thesis. After analytical validation of all HPLC methods, the study continued with the UV[32] method which will help to analyze sapropterin dihydrochloride in a simple and less time-consuming way. A reference method from the literature selected and tried as a reference method for the assay analysis[31]. An UV method was studied with Copper (II) by considering oxidation potential of sapropterin and Cu (II). The purpose of this method is to develop a simple and sensitive analysis of dissolution tests of sapropterin dihydrochloride as tablet dosage form. After this study another study with electrochemical quantification of sapropterin is worked and the method is validated for the simple assay analysis of sapropterin[9]. This electrochemical study can be continued for further investigation with HPLC/PAD or HPLC/ECD systems. The main aim of these studies is to develop simple analytical methods for the dissolution tests and assay of active ingredient with UV spectrometry and Cyclic voltammetry used in this study. These methods are all validated with respect to guidelines of The

International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) and compatible with the regulations of Turkish Ministry of Health.

CHAPTER 2

EXPERIMENTAL

2.1. Characterization Analysis

This study was started with the characterization part of Sapropterin Dihydrochloride (SAP). In this part of study, Fourier-Transform infrared spectroscopy (FTIR), thermogravimetric analysis (TGA), X-Ray Diffraction (XRD), Inductively Coupled Plasma Mass Spectrometry (ICP-MS), chloride content and high-performance liquid chromatography with UV absorption detector HPLC-UV analysis were used to analyze purity of the samples and to check the specifications of Sapropterin Dihydrochloride (SAP). Because of high selectivity and sensitivity of HPLC-UV analysis, it was used as a reference method for the quantitative analysis of SAP.

2.1.1. Fourier-Transform Infrared (FT-IR) Spectroscopy

Sapropterin Dihydrochloride was analyzed against working-standard by using Agilent FTIR Cary 630 instrument. Spectra of working-standard and reference were compared with software and their similarity is reported[33].

2.1.2. Thermogravimetric Analysis (TGA)

In order to investigate water content of sapropterin dihydrochloride TGA analysis were performed.

Experimental parameters for TGA analysis are listed below Table 2.1

Table 2.1 TGA conditions for Sapropterin Dihydrochloride

Proactive Gas N2		40 mL/min	
Purge Gas N2		60 mL/min	
Crucible		Alumina 70µL	
Sample Weight		10-15 mg	
Initial Temperature (°C)	Temperature rate (°C/min)	Final Temperature (°C)	Hold time final temperature (min)
30	10	110	30
110	10	150	-

2.1.3. X-Ray Diffraction (XRD)

Since there are different mesomorphs for sapropterin dihydrochloride, XRD was used to identify type of mesomorphs present in the sample. For drug industry it is very important to check X-Ray diffractograms since different mesomorphs can show different pharmacokinetic effects.

Experimental parameters for XRD analysis are listed in Table 2.2.

Table 2.2 XRD Parameters for Sapropterin Dihydrochloride

X-Ray Tube	
Target	Copper (Cu)
Voltage	40.0 kV
Current	30.0 mA
Slits	
Divergence Slit	0.6 mm
Scattering Slit	6.0 mm
Receiving Slit	0.02 mm
Scanning Parameters	
Drive Axis	Theta – 2Theta
Scan Mode	Continuous Scan
Scan Range	3.0 – 40.0 (deg)
Step Size	0.029 (deg)
Step Time	0.5 sec

2.1.4. Inductively Coupled Plasma/Mass Spectroscopy (ICP/MS)

Platinum was used in the synthesis of sapropterin dihydrochloride as a catalyst so that it is very important to check it is amount in order to check residue of Pt in active ingredient. Samples were analyzed in MERLAB using ICPMS (Perkin Elmer DRC II) and compared with limits for the sapropterin dihydrochloride.

2.1.5. Chloride Content

Chloride in SAP was determined titrimetrically to see whether it satisfy its specification limit or not. Sapropterin dihydrochloride solution is titrated against 0.1000 M silver nitrate solution.

Mettler Toledo T50 potentiometric titrator was used for this analysis. Standard solution of 0.1000 M silver nitrate solution was bought from Merck.

Approximately 0.100 mg of Sapropterin dihydrochloride was weighed and 2.0 mL of nitric acid was added into a 100 mL beaker. 50.0 mL Milli Q water was added and titrated against 0.1000 M $AgNO_3$ solution potentiometrically by using Mettler Toledo T50® potentiometric titrator.

2.1.6. High Performance Liquid Chromatography (HPLC/UV)

HPLC was used as a reference method for assay and related substances analysis of SAP. By using developed and validated four different HPLC/UV method sapropterin dihydrochloride was analyzed for its potency and impurities in raw material. The experimental parameters are listed in Table 2.3. Gradient elution parameters are given in Table 2.4.

2.1.7. Cyclic Voltammetry (CV)

Oxidation potential of SAP was determined experimentally by Cyclic Voltammetry. By using this method, quantitative analysis of active ingredient was also shown. Square-wave voltammetry was used as an assay method to analyze sapropterin dihydrochloride[34]. In this method Cyclic voltammetry firstly used to find appropriate oxidation reagent (which was decided as Cu (II)) for UV analysis of SAP. It was very crucial to select proper oxidation reagent since it is important that it should not be oxidized any other chemical in the drug which can cause interference for analysis.

2.2. Development of HPLC/UV Assay Method

Studies started with HPLC analysis since it has highest accuracy with the help of autosampler and more sensitive detection. In assay method, the method should be fast since SAP can be easily oxidized during the analysis. In our method, it could not be possible to have running less time than 25 minutes (with current instrumentation) because of gradient elution and resolve main peak from their impurities. To stabilize sapropterin sampler with cooling was used.

It could be possible to have less analysis time with UPLC systems which also have their own disadvantages.

Agilent Infinity II 1260 HPLC/UV system was used to analyze SAP. YMC Pack ODS-AQ column (250 x 4.6) mm, with 3µ stationary phase diameter was used to optimize peak shapes.

Sapropterin Dihydrochloride was purchased from Strides Shasun (Lot: D2ES28041702). Working standard (Reference Standard) for SAP was also supplied from Strides Shasun (Lot: D2ES28041700). All reference standards for impurities are also provided from Strides Shasun. Potassium dihydrogen phosphate, hydrochloric acid %37 and methanol were purchased from Merck. Water was purified using Merck Millipore system (18 MΩ).

Chromatographic conditions were started with column selection for best separation. YMC Pack ODS-AQ column was selected because of its compatible with mobile phases with highly aqueous eluents (since SAP is freely soluble in water[35]).

Wavelength was selected as 220 nm by considering maximum absorbance of SAP (measured by using diode array detector).

Mobile phase A-B and flow rate selected for the best separation and gradient elution was used to reduce analyze time and optimize the separation of peaks from degradation products and main component.

Injection volume was 5 µL throughout the analysis.

Ideal solvent for sapropterin was selected as 0.1 M HCl since it gets slower the reduction of SAP and also this diluent was the official dissolution media for the SAP in ICH dissolution database[36].

Chromatographic conditions and gradient program for assay method was shown in below Tables 2.3, 2.4.

Table 2.3. HPLC Assay Chromatographic Conditions

Column	YMC Pack ODS-AQ (250 x 4.6)mm, 3 µ
Mobile Phase A	20 mM KH_2PO_4, pH 3.0
Mobile Phase B	Methanol
Solvent	0.1 M HCl
Column Temperature	35°C
Wavelength	220 nm
Flow Rate	0.8 mL/min
Injection Volume	5 µL
Sample Temperature	5°C

Table 2.4. Gradient Program

Time (min)	Mobile Phase %A	Mobile Phase %B
0	100	0
10	100	0
15	10	90
17	100	0
25	100	0

2.72 g of potassium dihydrogen phosphate dissolved in deionized water and diluted up to 1000 mL and pH adjusted to 3.0 with ortho-phosphoric acid. This solution filtered through 0.45 µ nylon membrane filter and degassed in ultrasonic bath.

2.2.1. Preparation of Standard Solutions

100 mg of SAP reference standard was dissolved in 0.1 M HCl and 5.0 mL of this solution was diluted to 100 mL with same diluent. (50 µg/mL Sapropterin Dihydrochloride)

2.2.2. Preparation of Test Solution

10 SAP tablet (Kuvan®) was weighed and grinded. Tablet powder equivalent to 100 mg of SA was weighed into 100 mL volumetric flask and dissolved in 0.1 M HCl. 5.0 mL of this solution was diluted to 100 mL with same diluent. (50 µg/mL Sapropterin Dihydrochloride).

2.3. HPLC Analysis for the Impurity/Degradation Products

Three HPLC methods were used to analyze impurities in sapropterin dihydrochloride. These methods are listed below Table 2.5.

For the impurities A, B, C, D, E, G, H same principle with the assay method was used since their maximum absorbance wavelength (correction factor ~1) is almost same with sapropterin. Few modifications were made in mobile phases with gradient elution to optimize resolution between impurities and main component. For impurity F Peak I-II different method was needed since it has maximum absorbance around 275nm. In this method Waters Symmetry C18 column was used since it has superior peak shapes when compared with column with similar specifications. All gradient programs are listed in Tables 2.6, Table 2.7 and Table 2.8.

Table 2.5. HPLC Method Parameters for Impurity Analysis

	Method I	**Method II**	**Method III**
Column	YMC Pack ODS-AQ (250 x 4.6) mm, 3µ	Waters Symmetry C18 (150 x 3.0) mm, 3.5µ	YMC Pack ODS-AQ (250 x 4.6) mm, 3µ
Mobile Phase A	20mM KH_2PO_4, pH 3.0	20mM KH_2PO_4	20mM KH_2PO_4, pH 3.0
Mobile Phase B	Methanol	Methanol: Acetonitrile (1:1) (v/v)	Acetonitrile
Solvent	0.1 M HCl	Water: Acetonitrile (20:80) (v/v)	Mobile Phase A
Column Temperature	30°C	30°C	35°C
Wavelength	220 nm	275 nm	220 nm
Flow Rate	0.8 mL/min	0.6 mL/min	0.8 mL/min
Injection Volume	5µL	10µL	5µL
Sample Temperature	5°C	5°C	5°C
Run Time	40 min	40 min	25 min
Elution Type	Gradient Elution	Gradient Elution	Gradient Elution
Impurities	A, H, G, B, C, D	Impurity F Peak I, Impurity F Peak II	E, H

Table 2.6 Method I Gradient Program

Time (minutes)	Mobile Phase (%A)	Mobile Phase (%B)
0	98	2
15	90	10
30	50	50
32	98	2
40	98	2

Table 2.7 Method II Gradient Program

Time (minutes)	Mobile Phase (%A)	Mobile Phase (%B)
0	95	5
15	50	50
20	30	70
30	30	70
32	95	5
40	95	5

Table 2.8 Method III Gradient Program

Time (minutes)	Mobile Phase (%A)	Mobile Phase (%B)
0	100	0
10	100	0
15	10	90
17	100	10
25	100	10

2.3.1. Standard and Test Solutions Preparation for Method I

2.3.1.1. Standard Preparation

- Impurity stock solution: 5.0 mg of each impurity from Impurity-A, Impurity-B, Impurity-C, Impurity-D and Impurity-G reference standards were transferred into 100 mL volumetric flask and dissolved and diluted to volume with the diluent.
- API standard stock preparation: 5.0 mg of Sapropterin dihydrochloride reference standard was weighed into 100 mL volumetric flask and dissolved and diluted to volume with the diluent

  1.0 mL of API standard stock solution and 3.0 mL of impurity stock solution were diluted to 100 mL with the diluent.

2.3.1.2. Test Preparation

Tablet powder which is equal to 50.0 mg of sapropterin dihydrochloride was dissolved and then diluted to 50 mL with the diluent.

Both standard and test solutions were filtered through 0.45 µm hydrophilic PTFE syringe filter before injection to the HPLC column.

2.3.2. Standard and Test Solutions Preparation for Method II

2.3.2.1. Standard Preparation

5.0 mg of Impurity-F reference standard was dissolved and diluted to 100 mL with diluent. 3.0 mL of this solution was taken and diluted to 100 mL with the same diluent.

2.3.2.2. Test Solution

Tablet powder which is equal to 50 mg of sapropterin dihydrochloride was dissolved and diluted to 50 mL with the diluent.

Both standard and test solutions were filtered through 0.45µm hydrophilic PTFE syringe filter before injection to the HPLC column.

2.3.3. Standard and Test Solutions Preparation for Method III

2.3.3.1. Standard Preparation

5.0 mg of each Impurity-E and Impurity-H reference standards were dissolved and diluted to 100 mL with the diluent. 3.0 mL from this solution was taken and diluted to 100 mL with the same diluent.

2.3.3.2. Test Preparation

Tablet powder which is equal to 50 mg of sapropterin dihydrochloride was dissolved and diluted to 50 mL with the diluent.

Both standard and test solutions were filtered through 0.45 µm hydrophilic PTFE syringe filter before injection to the HPLC column.

2.4. UV Analysis by Using Folin Coicalteu (FC) Reagent

Agilent Carry 60 UV was used for UV analysis.

This method from literature[37] and used to compare UV method with the method which was developed in this study. In this method water was used as a diluent but it is not very suitable since sapropterin is easily oxidized in water. In our study we made some changes to optimize this method for solution stability and suitability to use for dissolution analysis.

Since we already have a HPLC method for assay analysis our primary focus is to develop easy-to-use dissolution analysis since it needs much time to analyze in HPLC. This method was based on the principle reaction of SAP with FC reagent, which was resulted with color change, yellowish-to-blue. Chemical formula of FC reagent is given as;

$3H_2O.P_2O_5.13WO_3.5MoO_3.10H_2O$,

$3H_2O.P_2O_5.14WO_3.4MoO_3.10H_2O$[38]

In this molecule, sapropterin dihydrochloride probably reduced oxygen/oxygens atoms from molybdate/tungstate groups (phosphomolybdo tungstate), result of this reaction characteristic intense blue color occurs[31].

2.4.1. Materials

Folin Coicalteu reagent, 37% by w/w hydrochloric acid and Na_2SO_3 were bought from Merck.

2.4.2. Standard Stock Solution Preparation

25.0 mg of Sapropterin working standard was transferred into 25 mL volumetric flask and dissolved in 0.1 M hydrochloric acid and diluted to volume with same solvent. This solution should be stored at 4°C[31].

2.4.3. UV Scan for Maximum Wavelength

50 µg/mL solution was prepared from standard stock solution. 3.0 mL from this solution was taken and added to 25 mL volumetric flask. 2.5 mL of FC reagent was added to this solution. After 5 minutes, 7.0 mL of %10 Na_2CO_3 solution was added and this solution was diluted to volume with 0.1 M hydrochloric acid. All solutions were prepared in 0.1 M hydrochloric acid solution[31].

2.4.4. Calibration Standard Solutions

50 µg/mL solution was prepared from standard stock solution. This solution was used as calibration stock solution. Then, seven calibration solutions were prepared by taken 1.0 to 4.0 mL from stock solution to 25 mL volumetric flask. 2.5 mL FC reagent was added to each solution then, 7.0 mL of %10 Na_2CO_3 was added and then diluted to volume with 0.1 M hydrochloric acid. The absorbance was measured at maximum wavelength which was 770 nm measured previous step[31].

2.4.5. Preparation of Test Solution

10 tablets were granulized and granulated powder which is equal to 100 mg Sapropterin HCl was weighed into 100 volumetric flask. Then it was dissolved with 0.1 M HCl solution and diluted to volume with same solvent. This solution was filtered with 0.45 µm hydrophilic PTFE syringe filter and 1.0 mL was taken from this solution and diluted to 20 mL with 0.1 M HCl solution. 3.0 mL of this solution mixed with 2.5 mL FC reagent, 7.0 mL of %10 Na_2CO_3 and then diluted to 25 mL with 0.1 M HCl[31].

2.5. UV Analysis by Using Copper (II) Sulfate Solution

Agilent Carry 60 UV is used for this analysis.

Copper (II) was selected because of its high oxidation potential compared to SAP which is 0.34 V[39]. This potential prevents placebo from oxidation and there was not any interference from placebo of drug.

CV measurements were used to measure oxidation potential of sapropterin and compared and verified with the values in literature.

In this method, color development instantly occurs this gives advantage for instant analysis during in-vitro analysis which is important for pre-formulation studies and analysis of quality control batches.

In ICH dissolution database, sampling point for sapropterin dihydrochloride is assigned as 2.5, 5, 7.5, 10 and 15[36] minutes so that it is important to analyze each point instantly. In HPLC, total analyze time for 6 samples (minimum requirement for drug release test with regarding to current pharmacopeias) is around 12.5 hours for only sample without standard injections so that fast UV method is a crucial advantage for such case.

2.5.1. Materials

Copper (II) Sulfate pentahydrate bought from Merck and was used as an oxidizing agent. All the solution was dissolved in 0.1 M HCl which was prepared from %37 (v/v) HCl (Merck) stock solution.

2.5.2. Preparation of Copper (II) Sulfate Solution

50 mM Copper (II) Sulfate solution was prepared by weighing about 1250 mg of Copper sulfate pentahydrate into 100 volumetric flask and dissolved and diluted with 0.1 M HCl solution.

2.5.3. Preparation of Sapropterin Dihydrochloride Standard Stock Solution

10.0 mg Sapropterin Dihydrochloride was weighed into 20 mL volumetric flask and dissolved and diluted up to volume with 0.1 M HCl solution.

2.5.4. Preparation of Standard Solution

1.0 mL of SAP stock solution was taken and then added to 10 mL volumetric flask. Since the volume is fixed to 900 mL for this method which regarding to ICH. For this method concentration was 111 ppm.

2.5.4.1. Standard Solution Preparation for In-Vitro Dissolution Testing

111 mg Sapropterin Dihydrochloride was taken and dissolved in 100 mL volumetric flask in 0.1 M HCl solution. 5.0 mL was taken from this solution and diluted to 50 mL with the same diluent.

2.5.4.2. Test Solution Preparation for In-Vitro Dissolution Testing

One tablet having 100 mg sapropterin dihydrochloride was dissolved in 900 mL dissolution media (0.1 M HCl). After 1-hour analysis with 50 rpm Apparatus II, analyte was taken from this solution.

2.6. Assay Analysis with Square-wave Voltammetry

All electrochemical measurement for sapropterin dihydrochloride was conducted at ambient temperature with a three-electrode system which was including GCE (glassy carbon electrode) as a working electrode, Ag/AgCl as reference electrode and a Pt wire was used as counter electrode of the system. As an electrolyte and diluent solution 0.1 M potassium phosphate (KPi) solution was used. Gamry PCI4/300 potentiostat–galvanostat was used to collect electrochemical measurements (Before electrochemical measurements GCE electrodes were cleaned with alumina polishing suspension to minimize interferences from the system). For all measurements results are converted versus reversible hydrogen electrode (RHE) by adding (0.197 + 0.059 pH) V[40].

2.6.1. Materials

Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, potassium chloride reagents were supplied from Merck. Milli Q 18 MΩ deionized water also used for the preparation of the all solutions.

2.6.2. Preparation of Electrolyte Solution

61.5 mL of 1.0 mM K_2HPO_4 and 38.5 mL of 1.0 mM KH_2PO_4 were taken and mixed in 1000 mL volumetric flask. After that 67.1 g KCl was added and solution was diluted to 1000 mL with deionized water. [41]

2.6.3. Preparation of Standard Solution

25.0 mg of Sapropterin Dihydrochloride solution was diluted with the electrolyte solution (1000 ppm) to 50 mL. 5.0 mL of this solution was transferred into CV cell and used as a standard solution.

2.6.4. Preparation of Test Solution

About 10 tablets were granulized and granule having 100 mg of SAP was weighed into 100 mL volumetric flask and dissolved and diluted with electrolyte solution (1000 ppm). This solution filtered through 0.45µ Hydrophilic PTFE syringe filter and 5.0 mL of it transferred into CV cell.

2.6.5. Preparation of Placebo Solution

Weight of active ingredient is subtracted from average tablet weight, weighed into 100 mL volumetric flask and prepared with the same procedure described in Section 2.6.4

2.6.6. Method Validation and Preparation of Solutions

This method was validated using ICH Guidelines[11]. Specificity, linearity-range, accuracy, repeatability, intermediate precision and robustness (solution stability) were studied during this validation.

2.6.6.1. Specificity

To show specificity of this method blank (electrolyte solution), placebo, standard and test solution were analyzed by using square-wave voltammetry [42].

2.6.6.2. Linearity and Range

For the linearity study, a calibration curve was prepared using stock solution for the sapropterin dihydrochloride.

Calibration stock solution: 40.0 mg of sapropterin dihydrochloride was weighed into 20 mL volumetric flask, dissolved and diluted with the electrolyte solution.

2.6.6.3. Accuracy

Three different concentrations for the accuracy study were prepared as 400, 1000 and 1300 μg/mL and results were compared with the ICH limits[11].

2.6.6.4. Repeatability

Six samples of sapropterin dihydrochloride tablet was analyzed against a working standard.

2.6.6.5. Intermediate Precision

This study was same with the repeatability except within different days were analyzed and results were compared statistically with the repeatability results.

2.6.6.6. Solution Stability

Sapropterin dihydrochloride in electrolyte solution was tested for its solution stability with continuous measurement of SWV. This study showed the stability of SAP in the diluent/electrolyte solution.

CHAPTER 3

RESULTS AND DISCUSSIONS

3.1. Results and Comparisons of Characterization Analysis

Results of characterization tests were compared with the certificate of analysis (COA) of the active ingredient. These tests are crucial to use such raw material as an active ingredient in a pharmaceutical dosage form. Each test has its own limit and pharmaceutical substance should comply with each limit stated in COA. Otherwise, this excipient can not be used as an active ingredient in a medicine.

3.1.1. Result of FTIR Analysis

The analysis is started with the FTIR comparison between working standard and raw material as shown in Figures 3.1 and 3.2.

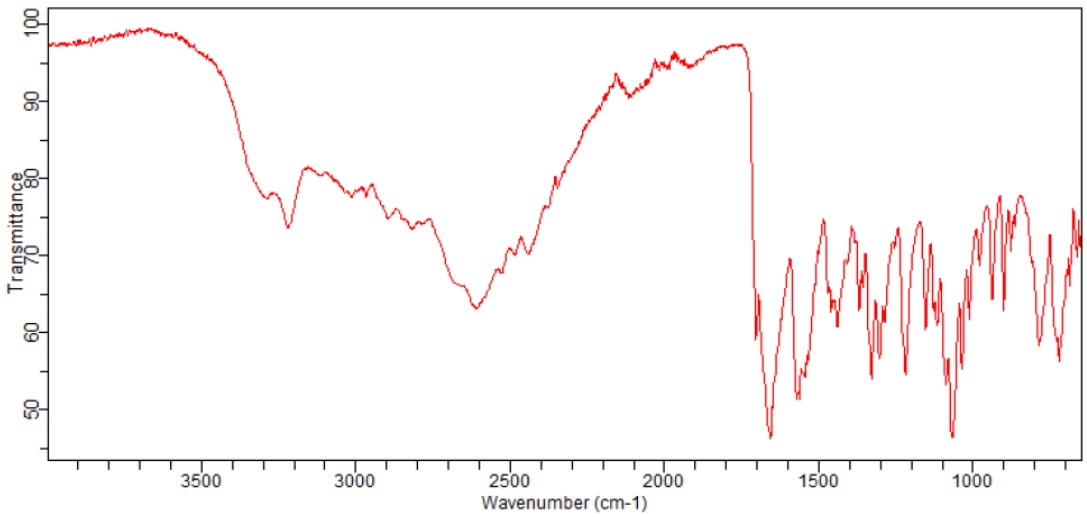


Figure 3.1 FTIR Spectrum of Sapropterin Dihydrochloride Working Standard

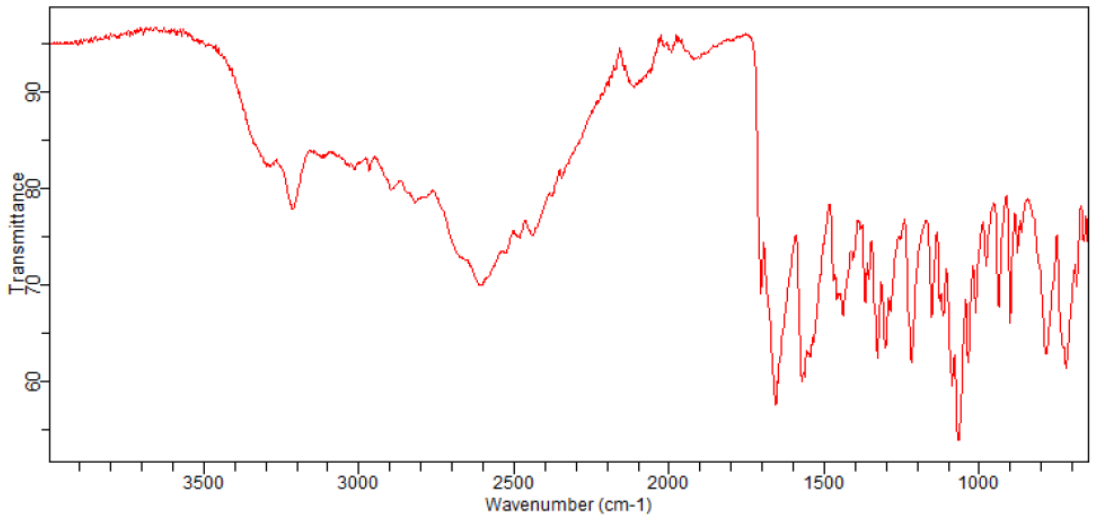


Figure 3.2 FTIR Spectrum of Sapropterin Dihydrochloride Raw Material

After the comparison of these two-spectra similarities between them was calculated by software (Micro Lab-PC by Agilent) and found as 99.8 percent. ICH limit for such comparison is ≥95[43] so that this raw material is suitable to use as excipient grade[44] and complies with COA.

In this spectrum also it is shown that each spectrum of different group of sapropterin such as O-H, N-H stretching around 3400 – 3300 cm^{-1}, around 2600 cm^{-1} stretching from C-H groups and around 1600-1650 amide I and amide II stretching's of amide groups are shown and matched with the molecular structure of sapropterin dihydrochloride.

3.1.2. Water Content Analysis by Using Thermogravimetric Analysis (TGA)

It is crucial to detect residual water in pharmaceutical excipients[45]. For this raw material since it is expensive and sensitive product for water TGA was proposed by supplier of the material. The result of TGA analysis is shown in Figure 3.3 and the total water content was found as 1.7 percent which is complied with COA of the sapropterin dihydrochloride.

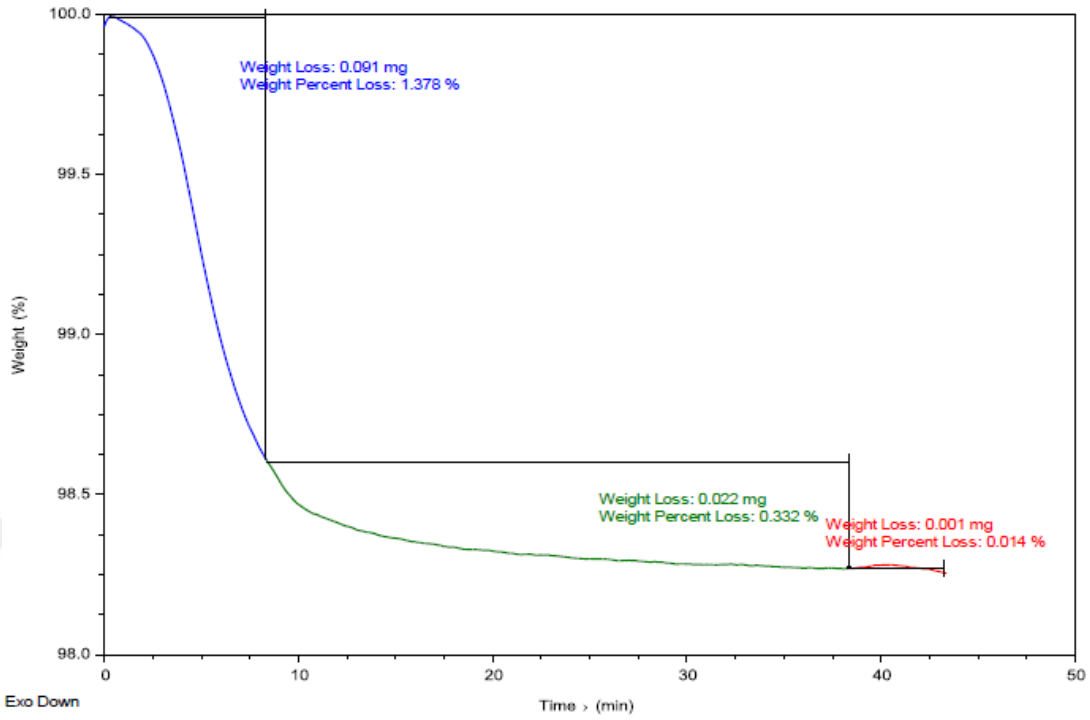


Figure 3.3 TGA Result of Sapropterin Dihydrochloride Raw Material

3.1.3. XRD analysis of Sapropterin Dihydrochloride

It is important to analyze crystal structure of pharmaceutical product before use it as an excipient because different mesomorphs of active ingredients can have different therapeutic effects which can be dangerous for health in some cases. In this method XRD was used to compare two diffractograms of sapropterin dihydrochloride taken from drug master file (DMF) and experimental result of raw material. Result of diffractogram is shown in Figure 3.4. Reference diffractogram is shown in appendix.

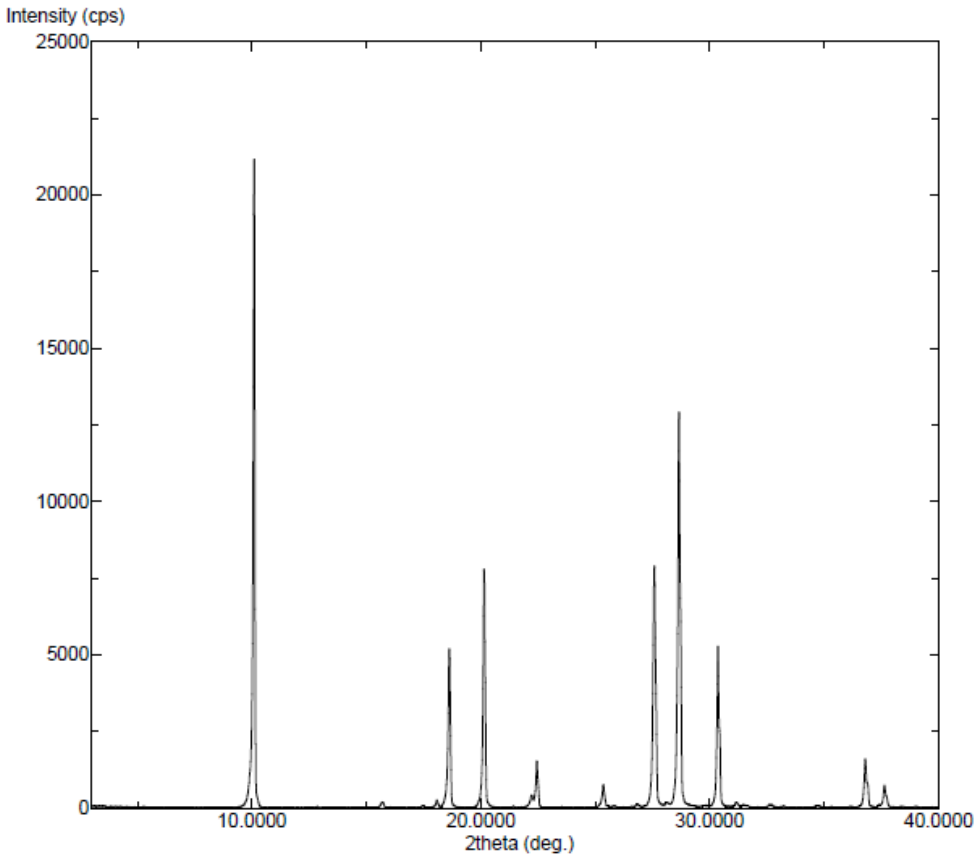


Figure 3.4 X-Ray Diffractogram of Sapropterin Dihydrochloride Raw Material

When two diffractograms are compared, it is shown that most 2theta values of these peaks agrees with each other. This result showed that raw materials' crystal structure complies with the reference material. The reference XRD is shown in Appendix as Figure 5.1.

3.1.4. Platinum Analysis with ICP/MS

Platinum is used in the synthesis of sapropterin dihydrochloride as a catalyst[46]. To determine the concentration of remaining platinum ICPMS was used in Merlab, METU. The result was reported as 0.96 ppm below the limit for Pt specie which is specified as 10 ppm.

3.1.5. Titrimetric Chloride Content Analysis

Results and preparations for chloride content are listed in Table 3.1.

Table 3.1 Chloride Content for SAP

Added 0.1 N AgCl as titrate (V)	6.178 mL
Water Content (by TGA)	1.7%
W of sample SAP (W_{smp})	100.0 mg
Molarity of Titrant (M)	0.1000 mol/L
Formula	$\frac{\text{V x N x 0.03545 x 100 x 100}}{\text{W}_{\text{smp}} \text{ x } (100 - \text{Water Content})}$
Chloride Content	22.3%
Limit 20-25%	Complies

This analysis is used to measure the concentration of chloride in sapropterin dihydrochloride to ensure its chloride content matches with 2:1 ratio chloride:sapropterin. The result for chloride test is in the limit of 20-25 % and complies with the certificate of analysis.

3.1.6. Peak identification using HPLC

Reference standard (Working standard) and drug substance (SAP) were injected to HPLC system and the chromatograms with blank (placebo) injection were overlaid as shown in Figure 3.5.

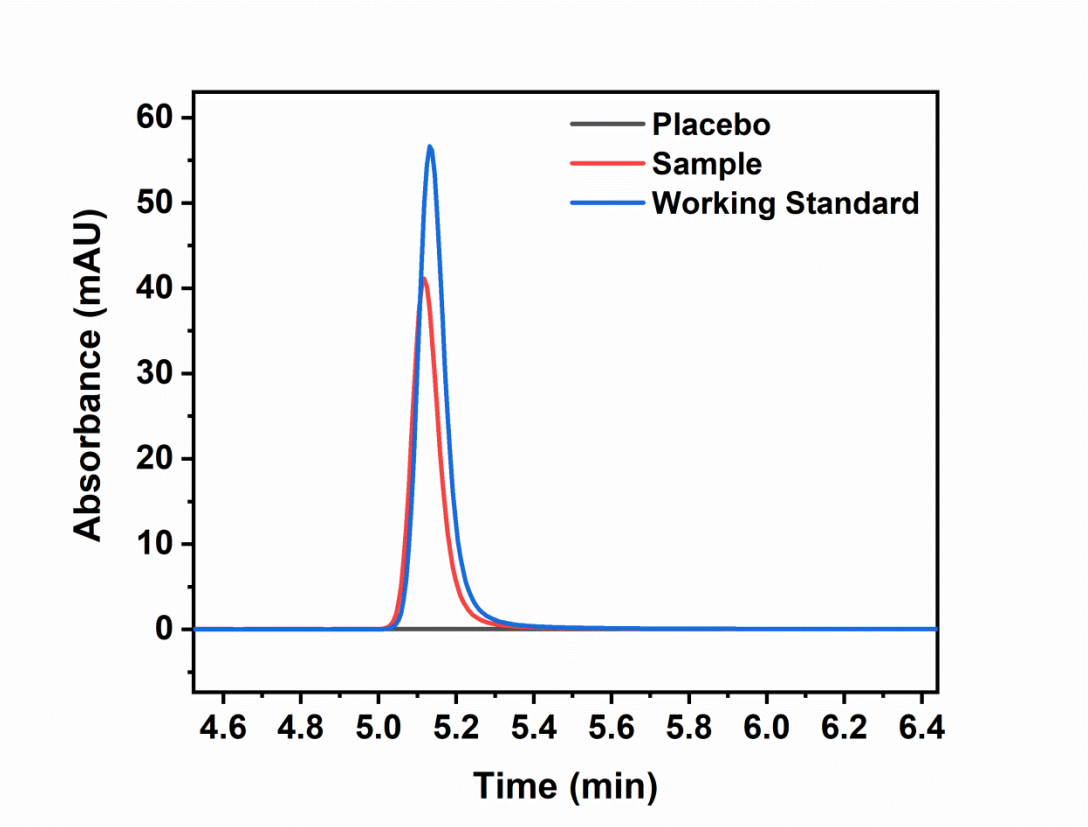


Figure 3.5 Peaks from reference standard (working standard) and sample (raw material) were overlaid.

In Figure, 3.5 it is shown that there is no interference from placebo since there is no peak due to placebo around 5.1 min. Additionally, sample (active ingredient) and reference standard peaks were aligned at the same retention time (around 5.1 min). This result is assumed as these two peaks are resulted from same compound by means of HPLC. This information is not enough by itself for the identification of compound.

3.1.7. Comparison of Identification Results

The results for identification tests were compared in Table 3.2 with certificate of analysis of SAP and their limits. The limits in the COA also compared with the latest EP/USP monographs to ensure for pharmaceutical substance. As shown in Table 3.2 the results are complies with the COA by means of identification tests.

Table 3.2 Comparison Table for COA of SAP

Analysis	Limit	Results in COA	Experimental Results
Appearance	Whitish, Pale yellow powder	Complies	Complies
FTIR	Spectrum of working standard and raw material should be compatible	Complies	Complies
XRD	Reference spectrum and sample spectrum should be compatible	Complies	Complies
HPLC	Retention time of working standard and sample injections should be compatible.	Complies	Complies
Water Content	≤ 3.0%	0.65 %	1.72 %
Chloride Content	20 – 25 %	22.5%	22.3%
Platin Content	≤ 10 ppm	0.1 ppm	0.96 ppm

3.2. Result of HPLC Assay and Related Substances Analysis

Four methods were developed to assay and different impurities of SAP. All the methods were validated with respect to ICH.

These analyses were used as a reference method since HPLC has autosampler and more sensitive detector. Also, by using HPLC, chromatographic separation from impurities and placebo were ensured.

3.2.1. Results of Assay Validation with HPLC

To show the specificity, in Figure 3.6 below SAP peaks (around 5.1 minutes) from sample (Kuvan®) and reference standard (working standard) of sapropterin were overlapped and there is no interference from placebo. In this chromatogram peaks after the 15 minutes are not taken into consideration since they are resulted from the change in gradient flow of HPLC system.

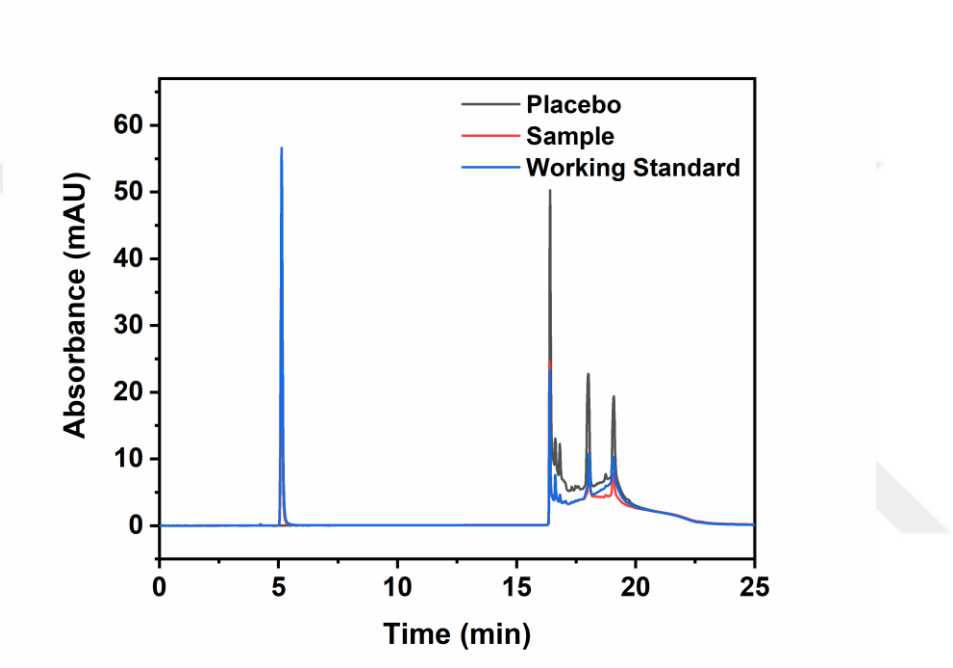


Figure 3.6 There is no interference from Placebo and peaks from Sample and Standard were overlaid.

Chromatographic peak purity of SAP was shown in Figure 3.7. Purity factor was calculated by software and found as 999.972 (36 of 36 spectra in chromatogram within the threshold limit which is 950.000)

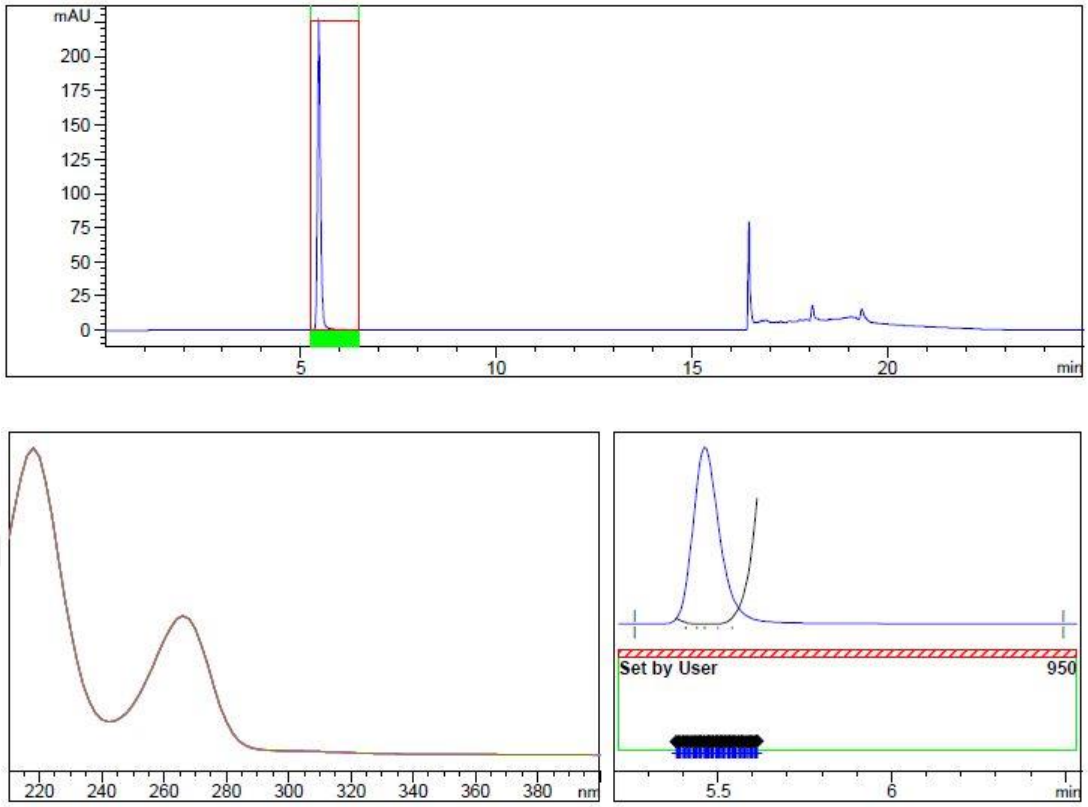


Figure 3.7 Peak purity analysis of Sapropterin Dihydrochloride in Sample

In Table 3.3, results were shown for the validation and the results are complies with the limits in ICH guidelines[11]. This method can be used as a reference method for UV and CV methods since it has well compatibility with ICH requirements.

Table 3.3 Results of Assay Validation of SAP

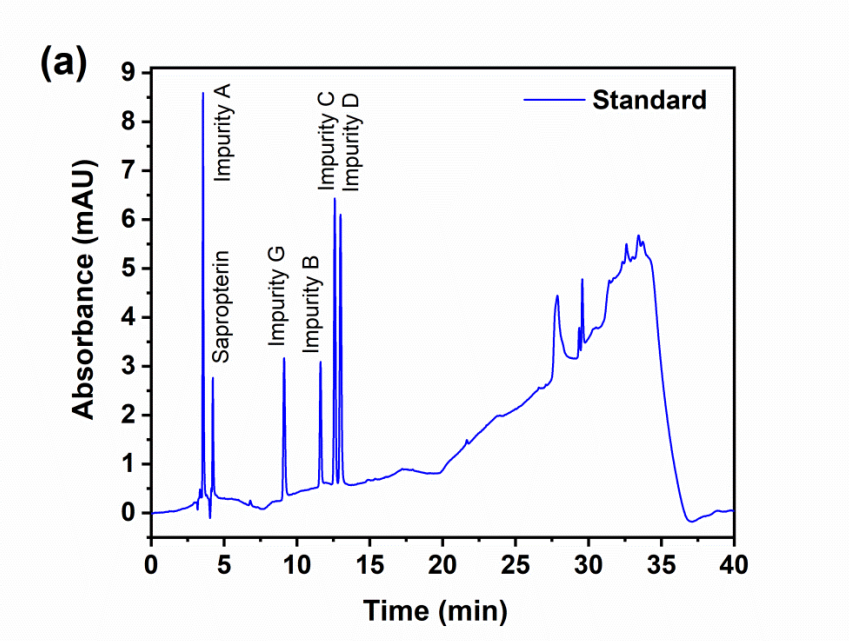
Parameters	**Results**	**Limit (ICH)**[11]
Specificity	Results are shown in below Figure 3.6. There is not interference from blank	Complies
Linearity	$R^2 = 0.9999$ 7 point was studied as; 35 ppm, 40 ppm, 45 ppm, 50 ppm, 55 ppm, 60 ppm, 70 ppm.	≥ 0.98
Range	Linear in the range of 35 ppm to 70 ppm (Working concentration for pharmaceutical product is 45 ppm – 55 ppm)	complies

Accuracy	RSD of each point is 35 ppm - 0.41, 50 ppm - 0.24, 65 ppm - 0.36	RSD ≤ 2
	Percent Recovery of each point is 35 ppm - 99.7, 50 ppm - 100.2, 65 ppm - 99.9	Conc. between 98-102 percent
Repeatability	RSD of six different Sapropterin tablet (Kuvan®) is 1.1%	RSD ≤ 2
Intermediate Precision	RSD of six different Sapropterin tablet (Kuvan®)1.6% (analysis was done in different day, also with different column having the same column material)	RSD ≤ 2
	RSD between Repeatability and Intermediate Precision is 1.0%	RSD ≤ 2
Limit of Quantification	0.5 ppm (much smaller from working concentration which is 50ppm) LOQ was calculated experimentally by using chromatogram from HPLC (s/N approach)	Complies
Robustness	Small changes of chromatographic parameters have no effect on analytical procedure. (calculated by using experimental design)	Complies

3.2.2. Result of Impurity Analysis with Method I

Impurities A, B, C, D and G (as specified in COA) were detected with this method I. This method was also used to identify unknown impurities. To analyze unknown impurities, SAP itself will be used in the impurity standard solution. In repeatability and intermediate precision analysis for impurity validation percent RSD of detected impurity should be reported. The only impurity in this sample was Impurity B which was used for these parameters. Each parameter for validation was carefully studied for details. LOQ of the analytical method is below the disregard limit of impurities. Disregard limit was calculated with regarding to ICH guideline[21] and maximum daily dose of sapropterin dihydrochloride is above 1 g (20 mg/kg/day for average person with 70 kg it was calculated as 1.4 g/day)[8] with this information reporting thresholds was calculated from Table 1.1 and found as 0.05%. Therefore, unknown impurities below this limit did not calculated and added to total impurities of sample.

In Figure 3.8, chromatograms of impurity standard solution and test solution was shown. Impurity B was the only Impurity in sample and other known or unknown impurities were not detected since they were lower than limit of detection of the system. Impurity B was also lower than disregard threshold, but it was measured to calculate repeatability and intermediate precision.



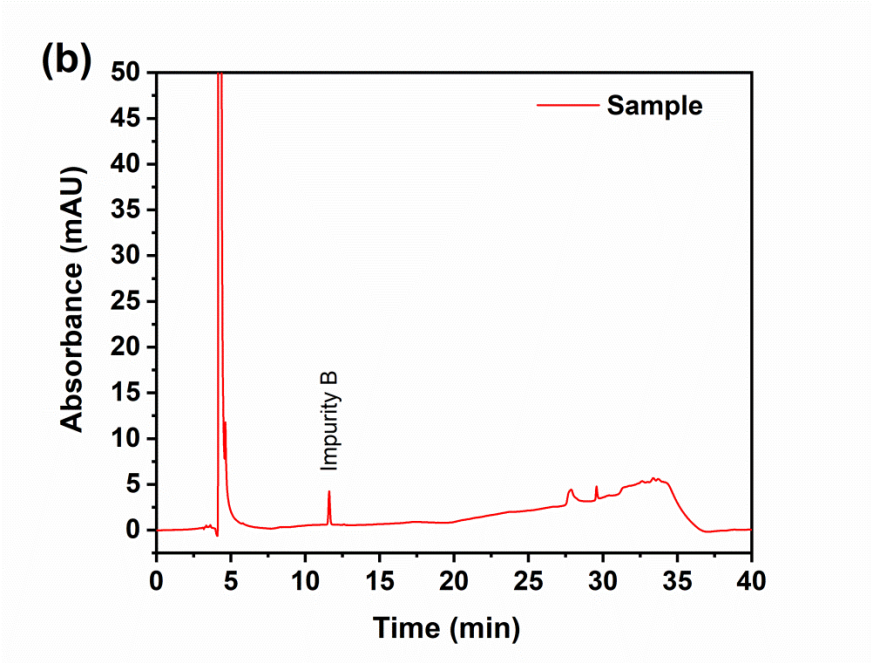


Figure 3.8 In the first chromatogram (a) injection of standard solution is shown. In the second chromatogram (b) impurity in the sample chromatogram is shown and it was reported in table 3.7.

Retention times of impurities in chromatogram (a) were 3.55 min for Impurity A, 4.22 min for Sapropterin, 9.14 for Impurity G, 11.59 for Impurity B, 12.56 for Impurity C, 12.96 min for Impurity D. For both sample and standard injections peaks after 20 minutes were resulted from gradient change of HPLC system and did not recorded.

Validation parameters and their results were listed in Table 3.4.

Table 3.4 Result of Validation of Impurity Method I

Parameters	**Results**	**Limit (ICH)**[11]
Specificity	There is no interference from blank	Complies
Linearity	$R^2 = 0.9997$ 6 point was studied as; 0.5 ppm (LOQ), 1 ppm (unknown impurity limit), 1.5 ppm (known Impurity Limit), 3 ppm, 5 ppm, 10 ppm	≥ 0.98

Range	Linear in the range of LOQ (0.5 ppm) to 10 ppm	complies
Accuracy	RSD of each point is below 10 0.5 ppm (LOQ) – 3.7, 1.5 ppm – 2.1, 10 ppm – 1.2	RSD ≤ 10
Repeatability	the range of concentration 90-110 percent. 0.5 ppm (LOQ) – 97.1, 1.5 ppm – 98.4, 10 ppm – 100.2 RSD of six different Sapropterin Tablet (Kuvan®) is 2.8% for Impurity B	Conc. between 90-110 percent RSD ≤ 10
Intermediate Precision	RSD of six different Sapropterin Tablet (Kuvan®) is 4.1% for Impurity B	RSD ≤ 10
	RSD between Repeatability and Intermediate Precision is 3.8% for Impurity B	RSD ≤ 10
Limit of Quantification	0.5 ppm (Limit for known impurities is 1.5 ppm and for unknown impurities 1.0 ppm) LOQ was calculated experimentally by using chromatogram from HPLC (s/N approach)	Complies
Limit of Detection	0.1 ppm LOD was calculated experimentally by using chromatogram from HPLC (s/N approach)	Complies
Robustness	Small changes of chromatographic parameters have no effect on analytical procedure. (calculated by using experimental design)	Complies

3.2.3. Result of Validation of Impurity Analysis with Method II

In impurity method II, impurity F (Peak-I and Peak-II) was studied since it needs a different wavelength and different chromatographic column for appropriate resolution and quantitation. In sample Impurity F (Peak-I and Peak-II) was not detected and to check repeatability and intermediate precision of system impurity standard was spiked into sample. LOQ of analytical method is calculated and it is below the disregard threshold, 0.05% as described in Impurity Method I.

Chromatogram for standard and sample injections were given in Figure 3.9.

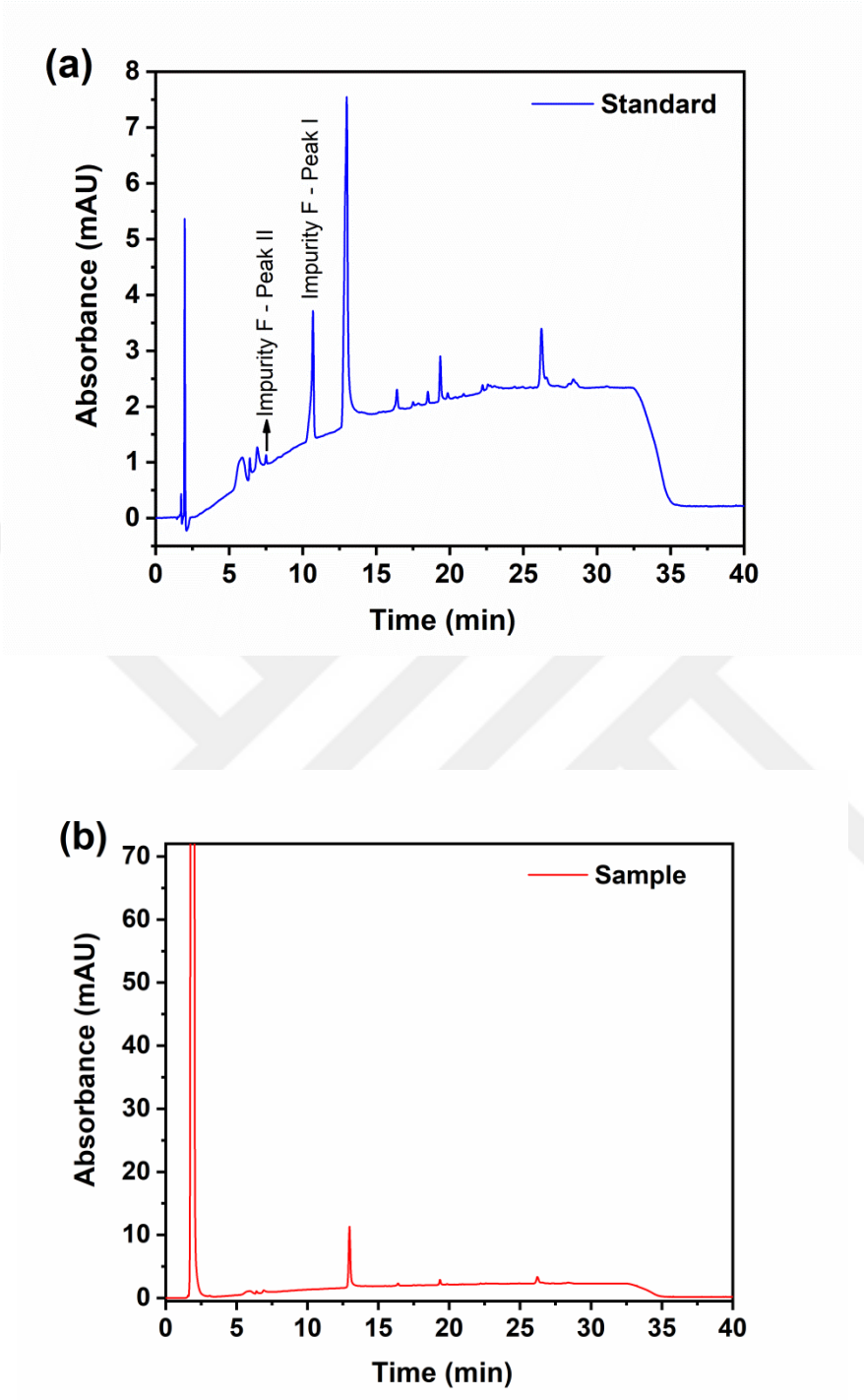


Figure 3.9 In the first chromatogram (a) standard injection for impurity F is shown. In the second chromatogram sample injection for impurity method II is shown and in this chromatogram Impurity F Peak I and Peak II not detected.

Retention time of Impurity F Peak-I and Peak II were 10.7 min and 7.5 min, respectively. The Peak at 12.6 min was resulted from blank solution and did not integrated.

Validation parameters and their results were listed in Table 3.5.

Table 3.5 Result of Validation of Impurity Method II

Parameters	**Results**	**Limit (ICH)**[11]
Specificity	There is not interference from blank	Complies
Linearity	$R^2 = 0.9998$ 6 point was studied as; 0.5 ppm (LOQ), 1 ppm (unknown impurity limit), 1.5 ppm (known Impurity Limit), 3 ppm, 5 ppm, 10 ppm	≥ 0.98
Range	Linear in the range of LOQ (0.5 ppm) to 10 ppm	complies
Accuracy	RSD of each point is below 10 0.5 ppm (LOQ) – 2.9, 1.5 ppm – 4.1, 10 ppm – 1.1	RSD ≤ 10
	the range of concentration 90-110 percent. 0.5 ppm (LOQ) – 101.2, 1.5 ppm – 100.6, 10 ppm – 99.7	Conc. between 90-110 percent
Repeatability	RSD of six different Sapropterin Tablet (Kuvan®) is 3.7% for spiked impurity	RSD ≤ 10
Intermediate Precision	RSD of six different Sapropterin Tablet (Kuvan®) is 3.5% for spiked impurity	RSD ≤ 10
	RSD between Repeatability and Intermediate Precision is 3.5% for spiked impurity	RSD ≤ 10
Limit of Quantification	0.42 ppm (Limit for known impurities is 1.5 ppm and for unknown impurities 1.0 ppm) LOQ was calculated experimentally by using chromatogram from HPLC (s/N approach)	Complies
Limit of Detection	0.15 ppm LOD was calculated experimentally by using chromatogram from HPLC (s/N approach)	Complies

Robustness	Small changes of chromatographic parameters have no effect on analytical procedure. (calculated by using experimental design)	Complies

3.2.4. Result of Validation of Impurity Analysis with Method III

This method was developed to separate impurities; impurity E and impurity H from sapropterin peak. Impurity H was detected in sample and reported. This impurity in sample was used in repeatability and intermediate precision analysis. LOQ of this analytical method is also appropriate for impurity analysis since it is lower than disregard limit which is 0.05%.

Standard and sample injections were shown in Figure 3.10. Only Impurity H was detected in sample and other known impurity E was not detected since it was lower than limit of detection of the system. Impurity H was higher than disregard threshold. Therefore, it was used to calculate repeatability and intermediate precision. Also, this impurity was compared with the results in COA.

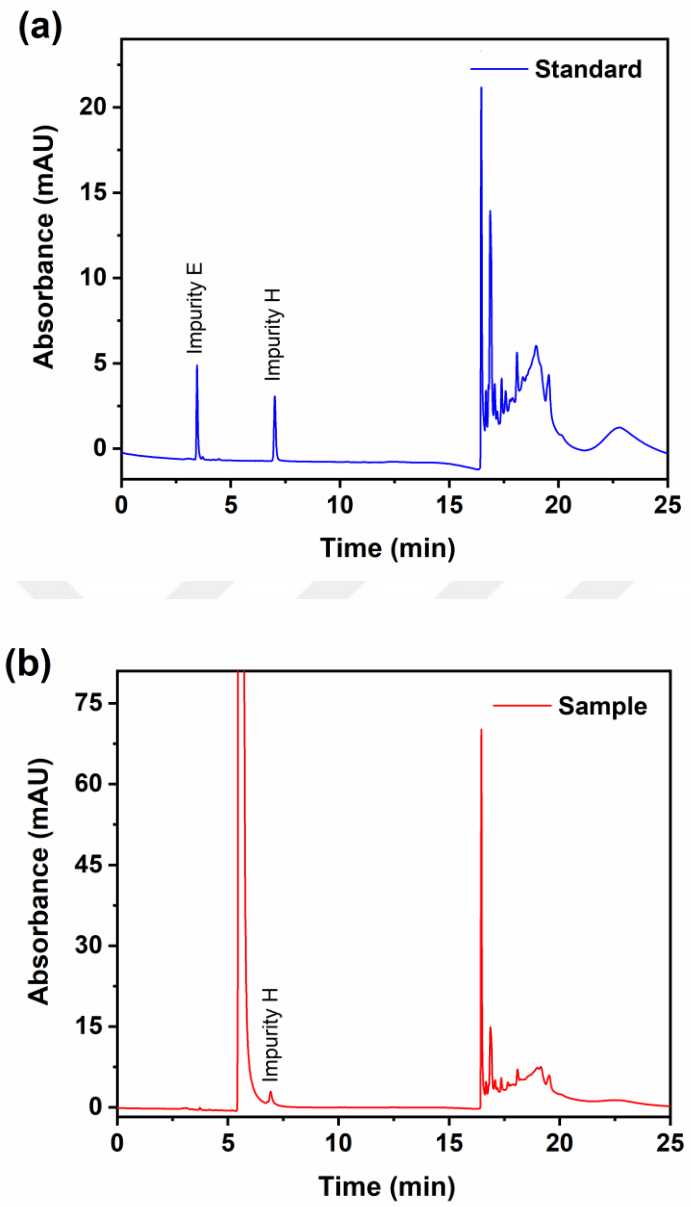


Figure 3.10 In the first chromatogram standard injection for impurity peaks is shown. In the second chromatogram, Impurity H was detected at sample chromatogram and reported at part 3.2.5.

Retention times of impurities in chromatogram (a) were 3.47 min for Impurity E, 7.05 min for Impurity H. For both sample and standard injections peaks after 15 minutes were resulted from gradient change of HPLC system and did not recorded.

Validation parameters and their results were listed in Table 3.6.

Table 3.6 Result of Validation of Impurity Method III

Parameters	**Results**	**Limit (ICH)**[11]
Specificity	Results are shown in below Figure 3.5. There is not interference from blank	Complies
Linearity	$R^2 = 0.9994$ 6 point was studied as; 0.5 ppm (LOQ), 1 ppm (unknown impurity limit), 1.5 ppm (known Impurity Limit), 3 ppm, 5 ppm, 10 ppm	≥ 0.98
Range	Linear in the range of LOQ (0.5 ppm) to 10 ppm	complies
Accuracy	RSD of each point is below 10 0.5 ppm (LOQ) – 3.3, 1.5 ppm – 2.4, 10 ppm – 1.6 the range of concentration 90-110 percent. 0.5 ppm (LOQ) – 100.9, 1.5 ppm – 99.5, 10 ppm – 102.1	RSD ≤ 10 Conc. between 90-110 percent
Repeatability	RSD of six different Sapropterin Tablet (Kuvan®) is 1.1 for Impurity H	RSD ≤ 10
Intermediate Precision	RSD of six different Sapropterin Tablet (Kuvan®) is 1.6% for Impurity H	RSD ≤ 10
	RSD between Repeatability and Intermediate Precision is 1.7% for Impurity H	RSD ≤ 10
Limit of Quantification	0.25 ppm (Limit for known impurities is 1.5 ppm) LOQ was calculated experimentally by using chromatogram from HPLC (s/N approach)	Complies
Limit of Detection	0.11 ppm LOD was calculated experimentally by using chromatogram from HPLC (s/N approach)	Complies
Robustness	Small changes of chromatographic parameters have no effect on analytical procedure. (calculated by using experimental design)	Complies

3.2.5. Impurity and Assay Results of Sapropterin Dihydrochloride

Table 3.7 Results of Analysis with HPLC/UV Methods

Sample	Assay (against WS)	Impurity Method I	Impurity Method II	Impurity Method III
Sapropterin Dihydrochloride Active ingredient LOT: D2ES28041702	99.4 % %RSD: 0.7	%0.01 Impurity B	Not Detected	%0.09 Impurity H
Kuvan® Tablet	99.0 % %RSD: 0.5	%0.04 Impurity B	Not Detected	%0.12 Impurity H
In-House Lab Scale production of SAP tablet	99.8 % %RSD: 1.1	%0.03 Impurity B	Not Detected	%0.10 Impurity H

These assay results shown in Table 3.7 used as a reference for the development of UV and CV methods.

With assay method percent assay of active ingredient found as 99.4% (dry basis) and it is suitable and complies with COA of the substance. Kuvan® tablet and In-house lab scale production of SAP also studied with assay and results were found 99.0% and 99.8% respectively. Since shelf-life limit for this drug is 90-110%, these results are suitable for drug product. For each analysis only impurity B and impurity H was detected and reported. Even impurity B is below the reporting threshold, it is reported for informative purposes. There are no unknown impurities for any of these samples.

3.3. Results for Square-Wave Voltammetry Method of Sapropterin Dihydrochloride

By using cyclic voltammetry oxidation and reduction potentials of sapropterin dihydrochloride were measured. These values are used to select appropriate oxidation reagent for sapropterin. Then, it was decided to use Oxidation A as a quantitative analysis method[9].

The oxidation A was used for quantitative analysis by using square wave voltammogram since it gives us more repeatable and precise results when compared with cyclic voltammetry (CV). SWV scan of sapropterin dihydrochloride is shown in Figure 3.11.

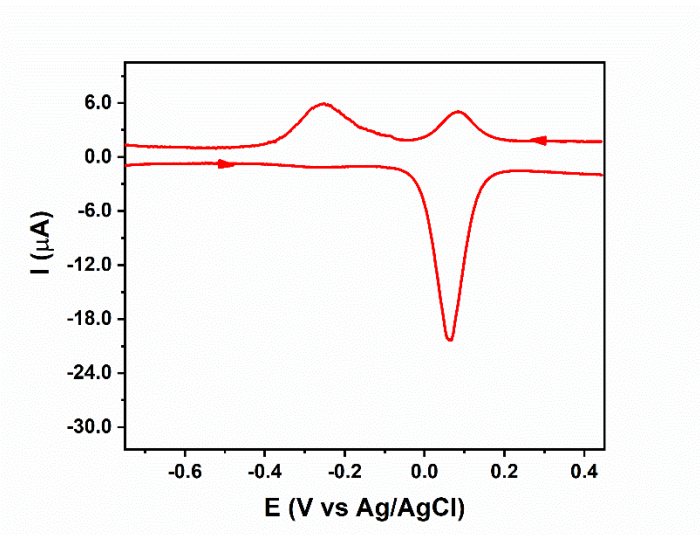


Figure 3.11 Voltammogram for Square Wave analysis of Sapropterin Dihydrochloride. Oxidation A at potential 0.06 V (V vs Ag/AgCl)

By converting these values for standard hydrogen reference electrode, results were calculated for sapropterin dihydrochloride as shown in In Table 3.8[47].

Table 3.8 CV oxidation and reduction potentials of SAP (mV vs SHE – Ag/AgCl)[9]

Electrode	Oxidation/ Reduction A	Reduction B
SHE	±0.27 V	-0.16 V
Ag/AgCl	±0.06 V	-0.26 V

Linearity study by using oxidation potential of 0.27 V (V vs SHE) was shown in Figure 3.12 in the range of 500-1300 ppm.

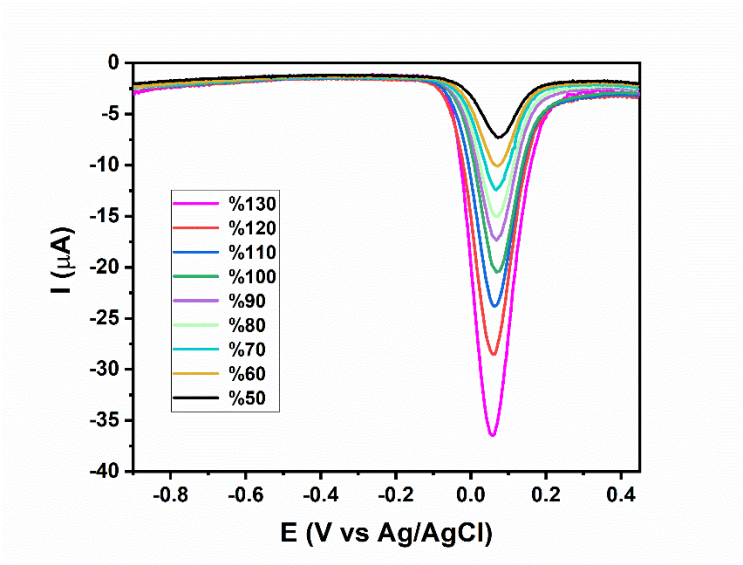


Figure 3.12 Linearity study of SAP with SWV in the concentration range of 500-1300 ppm. At potential 0.06 V (V vs Ag/AgCl)

Calibration plot (Figure 3.13) was drawn in the range of 500-1300 ppm versus current of oxidation A (0.06 V vs Ag/AgCl).

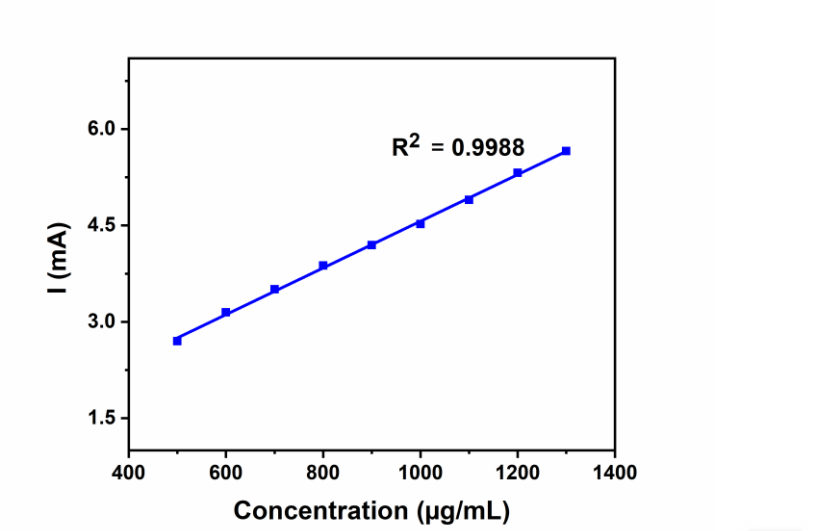


Figure 3.13 Linearity of SWV for quantitative analysis (500-1300 ppm).

Analysis of sapropterin with SWV method has a limitation for concentration of sample because of high quantitation limit which inhibits the quantitation of concentration below 500 ppm. With current instrumentation in laboratory used for these study, it is not possible to use such method for in-vitro/dissolution studies since working concentration of dissolution analysis should be 111 ppm (100 mg Sapropterin in one Kuvan tablet and ICH dissolution media[36] is 900 mL for this dosage form). It is possible to use more sensitive instrumentation to analyze in these concentrations. Also, this method can give ideas about usage of electrochemical detector with HPLC systems.

Validation Parameters for SWV analysis is shown in Table 3.9.

Table 3.9 Results of Validation for SWV method for SAP

Parameters	**Results**	**Limit (ICH)**[11]
Specificity	There is no signal from placebo and electrolyte solution.	Complies
Linearity	R^2 = 0.9988 (average of three different linearity study) Linearity Figure 3.15 was shown below	≥ 0.98

Range	Linear in the range of %50 to 130%	complies
Accuracy	RSD of each point is below 2 and in the range of concentration 98-102 percent.	RSD ≤ 3
Repeatability	RSD of six different samples is 1.45%	Conc. between 90-110 percent RSD ≤ 3
Intermediate Precision	RSD of six different samples is 1.68%	RSD ≤ 3
	RSD between Repeatability and Intermediate Precision is 1.80%	RSD ≤ 3
Limit of Quantification	500 ppm (calculated by theoretically by using slope of regression and standard deviation of blank solution)	Complies
Robustness	Solution Stability (stable for 12 hours)	Complies

3.4. Development of Quantitative UV Analysis of Sapropterin Dihydrochloride using $CuSO_4$

Results from Voltammetry was used to select appropriate oxidizing agent for sapropterin dihydrochloride. UV methods for sapropterin in literature[31] was based on oxidation-reduction reactions of Sapropterin dihydrochloride. In this sense, oxidation potential of SAP which is 0.27 V was used and Copper (II) was selected (0.34 V oxidation potential) to oxidize sapropterin dihydrochloride. Since Cu (II) has slightly higher oxidation potential than SAP, copper (II) sulfate was selected to react only SAP not the matrix of drug. Oxidizing agent with higher potentials than Cu (II) can cause oxidizing of placebo of drug and there would be interference in analysis. The results were complied with this estimation and there was not any interference from placebo/matrix of drug dosage form.

By using this UV scan, maximum wavelength for this UV method was selected as 336 nm. At this wavelength, there is no interference effect coming from placebo of drug. Spectrum of 50 ppm SAP treated with $CuSO_4$ was shown in Figure 3.14.

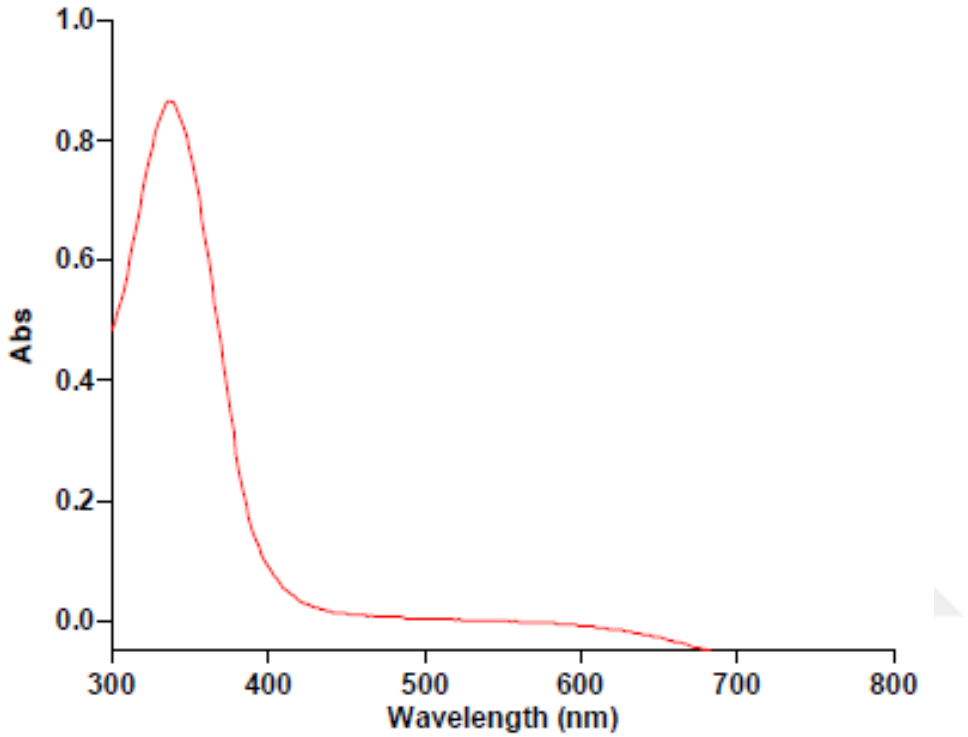


Figure 3.14 UV spectrum of Sapropterin Dihydrochloride with $CuSO_4$

Three different linearity stock solution and nine sets of diluted calibration solutions of sapropterin dihydrochloride were prepared and their absorbances were measured. The results show that all R^2 values for each calibration plot was above 0.98 which is limit of ICH guideline. Average of these three trendlines was used in analytical validation. These results were shown in Figure 3.15.

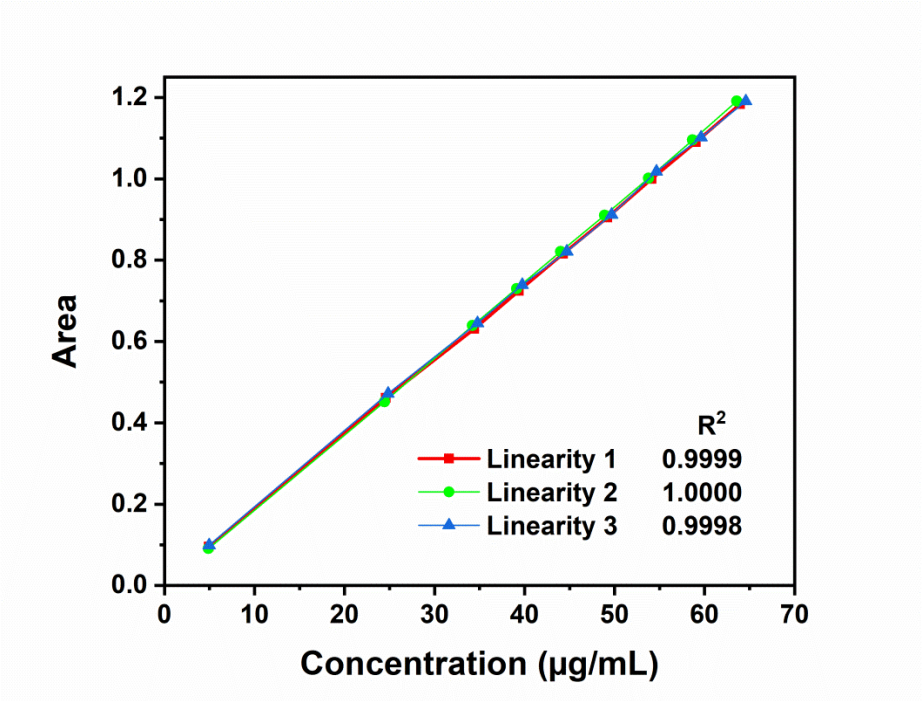


Figure 3.15 Linearity of Sapropterin UV analysis with $CuSO_4$

For this method, validation according to the ICH guidelines was done. The results for different validation parameters were shown in Table 3.10

Table 3.10 Validation Results of UV/$CuSO_4$ Method

Parameters	**Results**	**Limit (ICH)**[11]
Specificity	There is not interference from blank. UV Scan of Sapropterin Dihydrochloride with $CuSO_4$ was shown in above Figure 3.11	Complies
Linearity	R^2 = 0.9999 (average of three different linearity study) Linearity shows in Figure 3.12 above The concentration points for linearity study were; 5 ppm, 25 ppm, 35 ppm, 40 ppm, 45 ppm, 50 ppm, 55 ppm, 60 ppm, 65 ppm	≥ 0.98
Range	Linear in the range of 5 ppm to 65 ppm	complies
Accuracy	RSD of each point is given below 5 ppm – 1.75, 50 ppm – 0.74, 65 ppm – 0.42 and in the range of concentration 98-102 percent.	RSD ≤ 3

	5 ppm – 101.0%, 50 ppm – 100.1%, 65ppm – 100.4%	Conc. between 90-110 percent
Repeatability	RSD of six Sapropterin tablet (Kuvan®) samples is 0.41%	RSD ≤ 3
Intermediate Precision	RSD of six Sapropterin tablet (Kuvan®) samples is 0.55%	RSD ≤ 3
	RSD between Repeatability and Intermediate Precision is 0.49%	RSD ≤ 3
Limit of Quantification	0.11 ppm (calculated theoretically by using slope of regression and standard deviation of blank solution)	Complies
Limit of Detection	0.05 ppm (calculated theoretically by using slope of regression and standard deviation of blank solution)	Complies
Robustness	Solution Stability (stable for 10 hours)	Complies

3.5. Result of UV-VIS Assay with Folin Coicalteu (FC) Reagent

After detection of oxidation and reduction potentials of SAP, redox reaction followed by UV analysis was thought as simple and precise analysis methods and then UV/$CuSO_4$ method with redox reaction of SAP was selected to develop a simple and precise analytical method. This method with FC reagent was also used this principle for the analysis of active ingredient[31]. This method was selected since it has intense blue color after the reaction between sapropterin and FC reagent. This color can help to develop a paper based sensor[48] for the analysis of sapropterin dihydrochloride. For future work this method will be optimized and validated according needs of current guidelines for pharmaceutical analysis.

This method used for quantitative analysis method for UV assay[31]. The main disadvantage of this method is to wait around 30 minutes for complete color

development. This method was used to analyze commercial Kuvan® tablet and lab scale production of sapropterin tablet as a generic product of Kuvan®.

UV scan of Sapropterin/FC reagent complex was shown in Figure 3.14 and by using this spectrogram maximum wavelength of complex was found as 770 nm as described in literature[31].

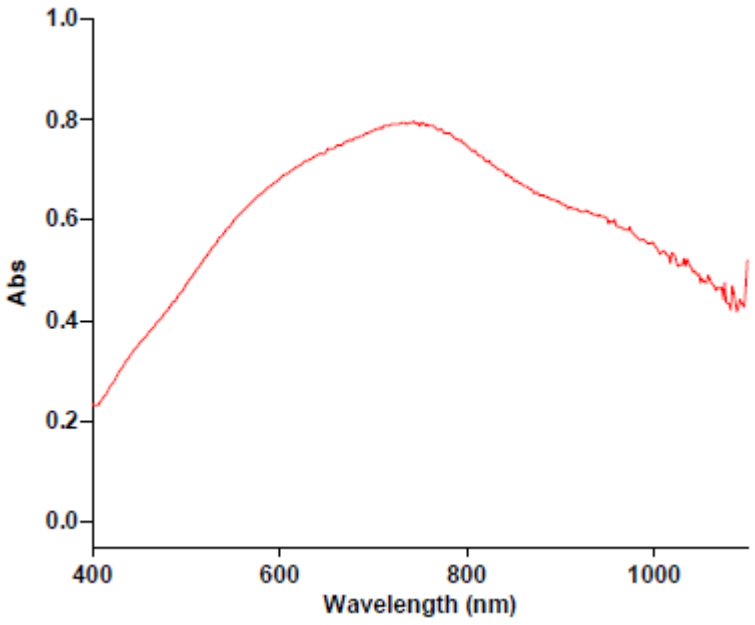


Figure 3.16 UV spectrum of Sapropterin Dihydrochloride with Folin Coicalteu Reagent.

Same scan also used to analyze placebo and blank solution and there is not interference around selected wavelength at 770 nm.

Linearity of this method studied in the range of 1-6 ppm with five different concentrations (1, 2, 3, 4, 5, 6 ppm). R^2 values of this line was calculated as 0.9999.

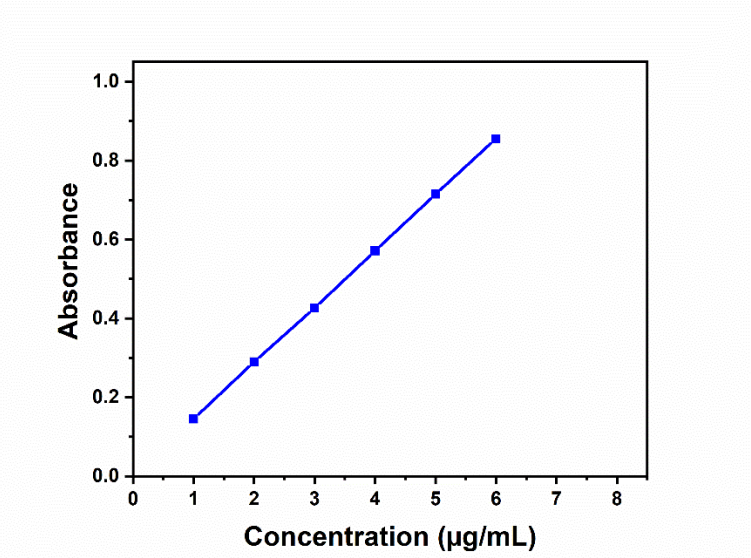


Figure 3.17 Linearity study for UV method with FC Reagent (1-6 ppm).

To calculate percent of sapropterin in analyzed dosage forms granulated powder from 20 tablets were used. Dissolution analysis were done by using Agilent 850-DS instrument. Dissolution parameters were decided using "FDA Dissolution Database" and found as 900 mL of 0.1 M HCl as a dissolution media, dissolution apparatus II (paddle) and analyze time as 15 minutes.

Results of Kuvan® and In-house production of sapropterin tablet was listed in Table 3.11 using UV/FC method.

Table 3.11 Results of Analysis with UV/FC Method

Sample	**%Amount of Sapropterin (w/w)**	**Dissolution Results % (w/w)**	**Solution Stability**	**Linear Range of the Method**
Kuvan® Tablet	99.1%	98.9%	Stable up to 4 hours	1-6 ppm
In-House Lab Scale production of SAP tablet	99.7%	99.4%	Stable up to 4 hours	1-6 ppm

3.6. Comparison of UV assay methods with HPLC/UV

UV assay results were compared with reference HPLC with UV detector method for the assay analysis of SAP. Table 3.12 listed differences between these three methods in different aspects.

Table 3.12 Comparison table for three methods

Method Name	HPLC-UV	UV/FC Reagent	UV/$CuSO_4$
%Amount	99.8	99.7	99.7
%RSD of Six Sample	0.41	1.3	0.85
Concentration Range	65 – 5 ppm	1 - 6 ppm	65 – 5 ppm
Sample Preparation Time	~15 min	~35 min	~5 min
Analysis Time	25 min	instant	instant
Solution Stability (h)	24 h	4 h	12 h
Instrumentation	complex	Simple	Simple
Cost	high	low	low

In this comparison HPLC/UV method was used as a reference method because of its higher sensitivity. When two UV methods are compared, the method using $CuSO_4$ is simpler in preparation because of in the other method the complete color developments is necessary[49]. Additionally, $CuSO_4$ method has longer solution stability and broader range for concentration. In general, UV method requires simpler instrumentation and less analyze time compared to HPLC method. These advantages provide faster analysis time which is important for the pharmaceutical industry.

3.7. Comparison of Voltammetry with UV/VIS Analyze Methods and HPLC method

Methods developed in this study are compared in Table 3.13 by their %amount, RSD for six samples, concentration range important for dissolution analysis (concentration for dissolution in-vitro analysis is fixed to 111 ppm), analysis and sample preparation times, solution stability, type of instrumentation and cost of instrumentation.

Table 3.13. *Comparison Table for Methods*

Method Name	**HPLC/UV**	**UV/$CuSO_4$**	**SWV**
%Amount	99.8	99.7	100.1
%RSD of Six Sample	0.4	0.8	1.9
Dynamic Range	65 – 5 ppm	65 – 5 ppm	500 – 1300 ppm
Sample Preparation Time	~15 min	~5 min	~5 min
Analysis Time	25 min	instant	1-2 minutes
Solution Stability (h)	24 h	12 h	6 h
Instrumentation	complex	Simple	Simple
Cost	high	low	low

CHAPTER 4

CONCLUSIONS

Sapropterin Dihydrochloride (Brand name as Kuvan®) is a synthetic drug used for the people with phenylketonuria Sapropterin Dihydrochloride treatment helps people to decrease their level of phenylalanine in blood for by converting phenylalanine to tyrosine. There are only few methods for the determination of sapropterin and most of them are based on chromatographic techniques. Therefore, the motivation of this study was to introduce a simple and reliable methods for sapropterin determination. Sapropterin is a reducing reagent and, accordingly, the newly developed methods for sapropterin determination were using this feature of sapropterin.

Method development studies were started with characterization analysis of active ingredients of sapropterin that was bought from Chine, utilizing FTIR, TGA, ICP/MS, HPLC/UV, XRD techniques All these analyses showed that specifications of active ingredients were matching with that of active ingredients of sapropterin provided by the pharmaceutical chemical company.

HPLC/Uv-Vis technique is considered as a reference method for drug analyses. Therefore, four different HPLC methods, namely quantitative analysis method, Impurity method I, Impurity method II, Impurity method III were investigated for the determination of degradation products and sapropterin dihydrochloride itself.

CV method was applied for the quantitative determination of sapropterin and to understand the redox behavior of sapropterin dihydrochloride. Later on, CV method was replaced with SWV voltammetry. The results were validated according to the ICH guidelines. The performances of established electrochemical method were compared with the performances of HPLC/DAD methods. Spectroscopic methods are much more convenient for rapid analysis of the species. Therefore, colorimetric

determination of oxidation, reduction products were decided to be used. Oxidation potential obtained from CV measurements was used to find a suitable redox couple for sapropterin. There are many strong reducing reagents that can be used for this purpose. However, strong reagents can also oxidize the ingredients in the drug matrix, therefore, due to its mild reduction property $CuSO_4$ was selected as an oxidizing agent. The $CuSO_4$ method yielded successful results in the 5-65 ppm sapropterin concentration range without any interference from other components present in the drug. One of the objectives of this study was to develop a paper sensor for rapid detection of the sapropterin. Therefore, we investigated an alternative oxidizing reagent, Folin-Coicalteu reagent that changes color due to redox reaction with sapropterin, in addition to CuSO4. The performance of FC method was compared with HPLC method and developed $CuSO_4$-UV method. It was found that $CuSO_4$-UV method has many advantages over electrochemical method and FC reagent method in different aspects such as percent relative standard deviation, concentration range, sample preparation time, analysis time, solution stability and cost.

In the quantitative analysis with the paper sensor prepared using FC reagent, a calibration line was obtained in the sapropterin dihydrochloride concentration range of 5-65 ppm. But, further investigation of this method is necessary to validate it according to the ICH guidelines.

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APPENDICES

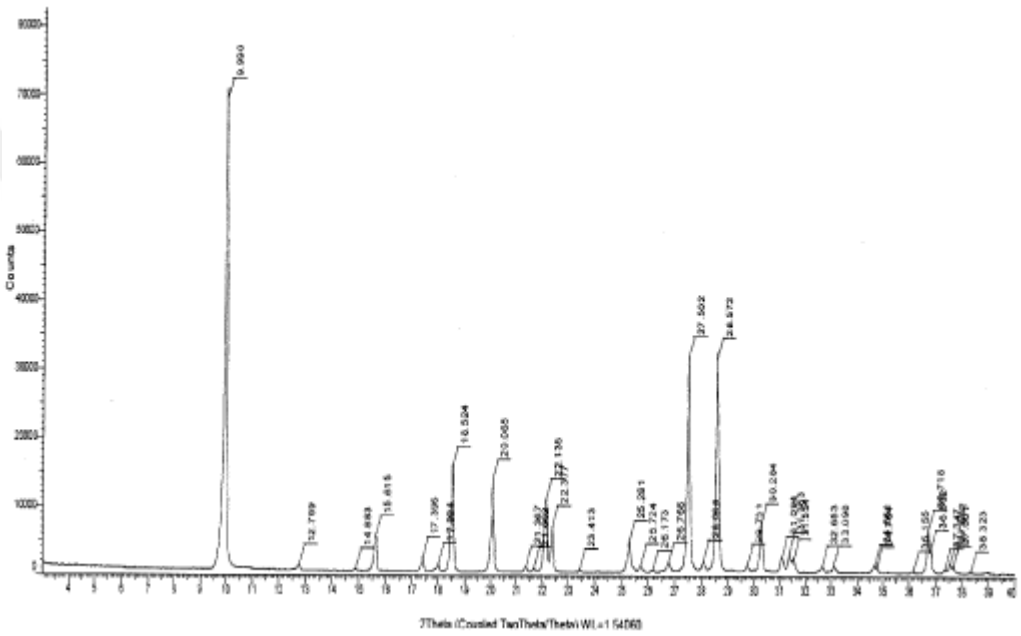


Figure 5.1 Reference Diffractogram of Sapropterin Dihydrochloride