REPUBLIC OF TURKEY
ANKARA UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

QUANTITATIVE ANALYSIS OF SEVERAL ANTIHYPERTENSIVE DRUGS USING DIRECT UV-SPECTROPOTOMETRIC AND DERIVATIVE SPECTROPHOTOMETRIC METHODS

Nezaket BAŞARAN

DEPARTMENT OF ANALYTICAL CHEMISTRY

MASTER'S THESIS

ADVISOR

Associated Prof. Özgür ÜSTÜNDAĞ

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2024

ETHICAL STATEMENT

Ankara University

Directorate of Health Sciences Institute,

The thesis titled “Quantitative analysis of several antihypertensive drugs using direct uv-spectropotometric and derivative spectrophotometric methods”, which I prepared and presented as a master's thesis, was written by me in accordance with scientific ethics and values. The idea/hypothesis of my thesis belongs entirely to my thesis advisor and me. The experimental study/research in the thesis was done by me, and all sentences and comments belong to me.

I declare that the above mentioned matters are true.

Name and Surname of the Student : Nezaket Başaran

Date:

Signature:

ACCEPTANCE AND APPROVAL

Ankara University Health Sciences Institute

Analytical Chemistry Department

Prepared by Nezaket Başaran

Named ‘Quantitative analysis of several antihypertensive drugs using direct uv-spectropotometric and derivative spectrophotometric methods’

thesis study has been UNANIMOUSLY/~~MAJORITYLY~~ accepted/~~rejected~~ as a MASTER'S THESIS by the following jury.

Thesis Defense Date: 18.10.2024

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The jury's decision regarding the thesis was approved by the Ankara University Health Sciences Institute Board of Directors.

Signature

Prof. Dr. Fügen Aktan

Director of the Institute of Health Sciences

ÖZET

Quantitative Analysis of Several Antihypertensive Drugs Using Direct Uv-Spectrophotometric and Derivative Spectrophotometric Methods

Tansiyon ilaçları olarak da bilinen antihipertansif ilaçlar, yüksek tansiyonun (hipertansiyon) yönetilmesinde çok önemli bir rol oynar. Kan basıncını düşürmek ve sağlığınız üzerindeki zararlı etkilerini önlemek için çeşitli şekillerde çalışırlar. Antihipertansiflerin nasıl çalıştığını anlamak, durumunuzu etkili bir şekilde yönetmenizi sağlayabilir. Diüretikler: Bu ilaçlar böbreklerinize etki ederek idrar çıkışını artırır, kan dolaşımındaki sıvı hacminin azalmasına ve dolayısıyla kan basıncının düşmesine neden olur. Örnekler arasında spironolakton bulunur. Beta blokerler: Bu ilaçlar adrenalinin (epinefrin) kalbiniz üzerindeki etkisini bloke ederek kalp atış hızınızı yavaşlatır ve kalp kasılmalarınızın gücünü azaltır; her ikisi de kan basıncını düşürmeye yardımcı olur. Örnekler arasında atenolol ve propranolol bulunur. Anjiyotensin dönüştürücü enzim (ACE) inhibitörleri: ACE, kan damarlarının daralmasında rol oynayan bir enzimdir. ACE inhibitörleri bu enzimin üretimini engelleyerek kan damarlarının genişlemesine ve kan basıncının düşmesine neden olur. Örnekler arasında lisinopril ve enalapril bulunur. Anjiyotensin II reseptör blokerleri (ARB'ler): Bu ilaçlar, kan damarlarını daraltan bir hormon olan anjiyotensin II'nin etkisini doğrudan bloke eder. Bu, kan damarlarının gevşemesine ve genişlemesine neden olarak kan basıncını düşürür. Örnekler arasında losartan ve valsartan bulunur. Kalsiyum kanal blokerleri: Bu ilaçlar kalsiyum iyonlarının kalp ve kan damarı hücrelerine hareketini bloke ederek kasları gevşetir ve kan damarlarını genişletir. Bunun sonucunda kan basıncı düşer. Örnekler arasında verapamil ve nifedipin bulunur.

Antihipertansif ilaçların miktar tayini genellikle yüksek performanslı sıvı kromatografisi (HPLC) ve kombine yöntemler gibi pahalı ve zaman alıcı kromatografik yöntemlerle gerçekleştirilir. İki veya daha fazla aktif bileşik ve numune matriksi içeren karmaşık problemleri çözmek için ekonomik, hızlı, kolay uygulanabilir ve doğru sonuçlar verebilen yeni analitik tekniklere ve yöntemlere ihtiyaç olduğu açıktır. Ayrıca analiz süreçlerinde klasik ve yüksek teknolojili kombine analitik yöntemlerin dezavantajlarını azaltmak veya ortadan kaldırmak, daha doğru ve daha hassas analitik sonuçlar elde etmek amacıyla yeni sinyal işleme ve çok değişkenli kalibrasyon algoritmaları klasik analitik yöntemlerle birlikte kullanılmaktadır.

Hazırlamış olduğumuz bu yüksek lisans tezinin esas amacı spironolakton içeren ticari farmasötik preparatlardaki etkin maddelerin hiçbir kimyasal ön ayırma işlemi gerektirmeden miktar tayinleri için sinyal işleme yöntemleri geliştirmektir. Bu kapsamda, spironolakton içeren preparatların analizi direkt UV spektrofotometrik ve türev spektrofotometrik yöntemleri kullanılarak yapılmış ve oldukça başarılı sonuçlar elde edilmiştir.

Anahtar Sözcükler : Antihipertansif, Spektrofotometri, Türev, Kantitatif, Tayin

SUMMARY

Quantitative Analysis of Several Antihypertensive Drugs Using Direct Uv-Spectrophotometric and Derivative Spectrophotometric Methods

Antihypertensive drugs, also known as blood pressure medications, play a crucial role in managing high blood pressure (hypertension). They work in various ways to lower blood pressure and prevent its harmful effects on your health. Understanding how antihypertensives work can empower you to manage your condition effectively. Diuretics: These drugs act on your kidneys to increase urine output, leading to reduced fluid volume in the bloodstream and consequently lower blood pressure. Examples include thiazides and loop diuretics. Beta blockers: These drugs block the action of adrenaline (epinephrine) on your heart, slowing down your heart rate and reducing the force of your heart contractions, both of which help to lower blood pressure. Examples include atenolol and propranolol. Angiotensin-converting enzyme (ACE) inhibitors: ACE is an enzyme involved in constricting blood vessels. ACE inhibitors prevent the production of this enzyme, leading to wider blood vessels and lower blood pressure. Examples include lisinopril and enalapril. Angiotensin II receptor blockers (ARBs): These drugs directly block the action of angiotensin II, a hormone that constricts blood vessels. This causes blood vessels to relax and widen, lowering blood pressure. Examples include losartan and valsartan. Calcium channel blockers: These drugs block the movement of calcium ions into heart and blood vessel cells, thereby relaxing the muscles and widening the blood vessels. This results in lower blood pressure. Examples include verapamil and nifedipine.

Quantification of antihypertensive drugs is usually performed by expensive and time-consuming chromatographic methods, such as high-performance liquid chromatography (HPLC) and combined methods. It is clear that new analytical techniques and methods that are economical, fast, easily applicable and can provide accurate results are needed to solve complex problems involving two or more active compounds and sample matrix. In addition, in order to reduce or eliminate the disadvantages of classical and high-tech combined analytical methods in analysis processes and to achieve more accurate and more precise analytical results, new signal processing and multivariate calibration algorithms are used in combination with classical analytical methods.

The main objective of this master's thesis is to develop signal processing methods for the quantification of active ingredients in commercial pharmaceutical preparations containing spironolactone without any chemical pre-separation. In this context, spironolactone-containing preparations were analyzed using direct UV spectrophotometric and derivative spectrophotometric methods and very successful results were obtained.

Keywords: Antihypertensive, Spectrophotometric, Derivative, Quantitative, Determination.

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PREFACE

This master's thesis study has been successfully carried out quantitative analysis of various antihypertensive drugs using direct uv-spectropotometric and derivative spectrophotometric methods. Spironolactone was used as the main ingredient. It has been demonstrated by the analytical validation of the methods and analysis results that the developed methods can be applied to quality control and routine analysis of spironolactone. In this respect, the thesis has achieved its purpose.

I would like to thank and express my respects to my advisor, Assoc. Professor Özgür ÜSTÜNDAĞ, who showed his close interest and support in the planning, execution and completion of my master's thesis studies and who did not spare his knowledge and experience. I also would like to thank and express my endless gratitude and respect to Prof. Dr. Erdal DİNÇ and especially I would like to thank Assistant Professor Asiye ÜÇER, who contributed a lot during my thesis studies and helped me in my experiments. Also I would like to thank other professors of the department.

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Finally, I would like to thank my most precious people, my beloved husband Oğuzhan BAŞARAN and my dear daughter Zeynep Ahsen BAŞARAN, by expressing my endless love.

ABBREVIATIONS

SPI	Spironolactone
ACE	Angiotensin-converting enzyme
ARB	Angiotensin II receptor blockers
CE-MS	Electrophoresis–mass spectrometry
DS	Derivative spectrophotometry
HPLC	High-Performance Liquid Chromatography
GC	Gass Chromatography
GC-MS	Gas chromatography–mass spectrometry
LC-DAD	Liquid chromatography-diode array detector system
LC-MS	Liquid chromatography-mass spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
SEC	Standard Error of Calibration
SEP	Standard Error of Prediction
UV	Ultraviolet
Vis	Visible

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1 INTRODUCTION

Analytical chemistry is a discipline that encompasses the development of methods and techniques for analysis in the food, clinical, environmental, agricultural, forensic, pharmaceutical industries and other branches of chemistry. Especially in the pharmaceutical industry for human health, routine analysis and quality control of components in commercial products before and after formulation are required by law and directives, so the techniques and methods of analytical chemistry are needed. More generally, analytical chemistry has an important place as a discipline in the analysis and control of natural and all industrial products.

As explained above, the methods and techniques of analytical chemistry are of indispensable and vital importance in analysis processes. Spectrophotometry, liquid chromatography, capillary electrophoresis and electrochemical methods are used in analysis processes. The main problems of analysis processes in the application of these methods are the easy applicability of the analysis method, analysis cost, analysis speed, precision and accuracy of the results. In order to overcome the difficulties in analysis processes, liquid chromatography-diode array detector system (LC-DAD), liquid chromatography-mass spectrometry (LC-MS), capillary electrophoresis-mass spectrometry (capillary). High-tech analytical devices or analytical methods with expensive and complex components such as electrophoresis–mass spectrometry→ CE-MS) and gas chromatography–mass spectrometry (gas chromatography–mass spectrometry→ GC MS) are used. These complex analytical methods require the use of pre-separation processes such as derivatization and extraction in the analysis processes. In the pharmaceutical industry, routine quality control laboratories and research laboratories, methods based on high pressure liquid chromatography (HPLC) or high-tech separation techniques are used in the analysis of mixtures containing more than one active compound. These methods consist of economically expensive and complex components, and optimization of experimental conditions during analysis is tedious and time-consuming. The longer the experiments, the higher the consumption of chemicals used. In some cases, the desired quality results cannot be obtained. To put it clearly, it is clear that new analytical techniques and methods that are economical, fast, easily applicable and can provide accurate results are needed to solve complex problems involving two or more active compounds and sample matrix. In addition, in order to reduce or eliminate the disadvantages of classical and high-tech combined analytical methods in analysis processes and to achieve more accurate and more precise analytical results, new signal processing and multivariate calibration algorithms are used in combination with classical analytical methods.

The general purpose of this study is to develop signal processing and multivariate calibration methods for the quantitative analysis of active compounds in combined pharmaceutical preparations as an alternative to separation methods that require expensive devices with complex components and high analysis costs.

In this master's thesis study, it was aimed to carry out quantitative analysis of a pharmaceutical drug containing spironolactone as the main ingredient, which is an antihypertensive drug, using direct UV spectrophotometric and derivative spectrophotometric methods.

1.1 Spironolactone

1.1.1 Chemical and physical properties

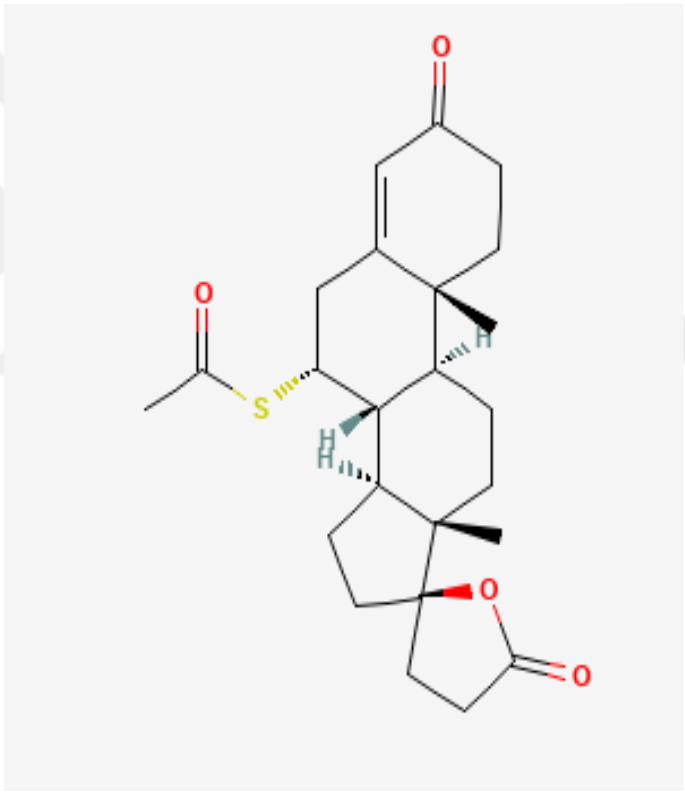

Figure1.1 Molecular formula for spironolactone

- Molecular Formula : $C_{24}H_{32}O_4S$
- Molecular Weight : 416.6 g/mol
- Iupac Name : *S*-[(7*R*,8*R*,9*S*,10*R*,13*S*,14*S*,17*R*)-10,13-dimethyl-3,5'-dioxospiro[2,6,7,8,9,11,12,14,15,16-decahydro-1*H*-cyclopenta[a]phenanthrene-17,2'-oxolane]-7-yl] ethanethioate
- Boiling point : 134 °C

1.1.2 Pharmacological Properties

Spironolactone was discovered in 1957, and was introduced in 1959. It is on the World Health Organization's List of Essential Medicines. It is avalible as a generic medication. In 2021, it was the 54th most commonly prescribed medication in the United States, with more than 12 million prescriptions. Spironolactone is taken by mouth.

Spironolactone has a potassium-sparing diuretic effect. It promotes sodium and water excretion and potassium retention. It increases renin and aldosterone levels. Spironolactone is a mineralocorticoid receptor antagonist and has a low affinity for the glucocorticoid receptor. It also exhibits progestogenic and anti-androgenic actions as it binds to the androgen receptor and, to a lesser extent, estrogen and progesterone receptors. Spironolactone exhibits anti-inflammatory effects.

Spironolactone is a potassium-sparing diuretic and this group of diuretics acts on the collecting tubules. It is frequently used in combination with thiazide group in the treatment of hypertension. Spironolactone is a synthetic aldosterone antagonist that competes with aldosterone for binding to intracellular cytoplasmic receptors. In many conditions that cause edema, blood aldosterone levels are high and are responsible for sodium retention. When spironolactone is given to a patient with high aldosterone levels, the effect of aldosterone is antagonized and potassium is retained and sodium is excreted. In cases where there is not enough aldosterone in the circulation, such as Addison's disease (primary adrenal insufficiency), the drug has no diuretic effect.

Although spironolactone provides poor sodium excretion, its potassium-sparing effect is important. Because of this effect, spironolactone is used in combination with thiazide diuretics to prevent hypokalemia.

Since spironolactone is similar in structure to sex steroids, it also has a weak hormonal effect. It may cause gynecomastia in men and menstrual irregularities in women. Therefore, it should not be used for a long time and in high doses. Hyperkalemia, nausea, lethargy and confusion may also be observed.

Spironolactone is used to treat heart failure, high blood pressure (hypertension), or hypokalemia (low potassium levels in the blood).

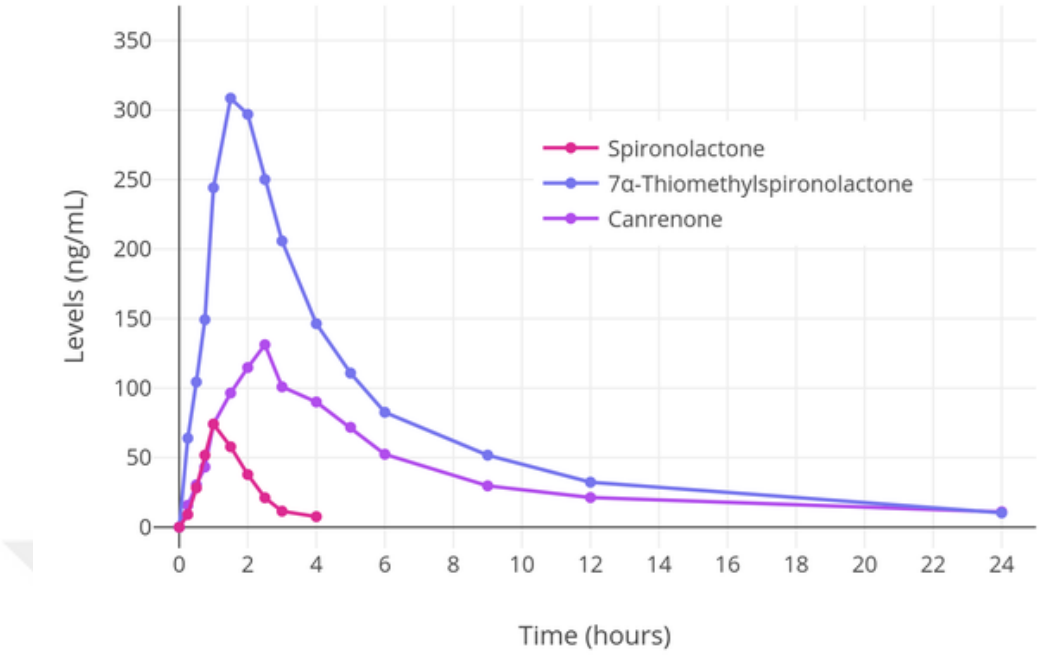

Figure 1.2 Levels of spironolactone and its major active metabolites after a single oral dose of 100 mg spironolactone in humans

1.2 Methods applied for analysis of spironolactone

Panchagnula et al., (2011) analysed the Frusemide and Spironolactone Mixtures using first-derivative spectrophotometry. The aim of the present study was to demonstrate the feasibility and the possibility of derivative UV spectrophotometry for routine analysis of frusemide and spironolactone in combination formulations, simultaneously, to circumvent the problems of overlapping spectral bands. The method was applied to formulations available in the local market and compared with HPLC analysis of the two compounds to evaluate sensitivity, accuracy, precision and day-to-day variation. The proposed method was successfully applied for determination of the drugs in solid dosage forms. The method was rapid and cost-effective when compared with HPLC.

Chandarana et al., (2018), studied on quantification of spironolactone by Fourier transform infrared spectrophotometry in bulk and tablet dosageform. A simple, specific, sensitive, accurate, precise and economical Fourier transform infrared spectrophotometric method was developed for the quantitation of Spironolactone in bulk and tablet dosage form. The method was based on the determination of Spironolactone by the measurement of absorption of radiation at absorption band corresponding to C=O stretch of cyclic ester centered at 1768

cm^{-1}, which is typically in the range of 1802–1748 cm^{-1} because those absorption bands did not occur in excipients present inpharmaceutical preparation. The proposed method was validated as per ICH guidelines. The linearity of spironolactone was obtained in the concentration range of 1–7 mg/g with correlation coefficient value (R^2) 0.995. The recovery studies confirmed by the accuracy of the proposed method and low values of standard deviation confirmed precision of the method.

Prajapati et al., (2021) studied about quantification of spironolactone by first and second order UV Derivative Spectrophotometry in bulk and tablet dosage form. For determination of Spironolactone in pharmaceutical formulation, Simple, fast and reliable first and second order derivative UV spectrophotometric methods were developed. Spectrophotometrically, Spironolactone was determined by measuring the 1D and 2D-values at 226 nm and 262 nm using methanol as background solvent. The Calibration curves were shown linear within a concentration range from 5–35 µg/ml. The developed and validated method was easily applied to the analysis of the pharmaceutical tablet preparations. The percentage recoveries were found to be 100% for given methods. The excipients did not interfere in the analysis. The results showed that this method can be used for rapid determination of Spironolactone in pharmaceutical tablet with specificity and accuracy.

Tekerek et al., (2008), studied on quantitative determination of hydrochlorothiazide and spironolactone in tablets by spectrophotometric and HPLC methods. In this study, simple, sensitive and reliable spectroscopic methods (absorbance ratio and Vierordt) were compared with HPLC method developed for quantitative determination of spironolactone (SPL) and hydrochlorothiazide (HCT) in commercial tablets. 260 nm was chosen as the isosbestic point in the absorbance ratio method. For Vierordt method; A/ values were calculated at 242 and 269 nm for both substances and used for quantitative analysis of HCT and SPL in its binary mixture. Linearity ranges for HCT and SPL was 2-15 jUg/mL and 2-12 jUg/mL respectively for both methods. The relative standard deviations for absorbance ratio and Vierordt-methods were found to be 1.58 % and 1.32 % for HCT, 1.26 % and 1.52 % for SPL respectively. In HPLC method, HCT, SPL and Mefrusid (MFS) as an internal standard were determined by isocratic system using water-methanol-phosphate buffer (pH 3.0 ± 0.1) (71:25:4 v/v/v) as mobile phase and with Luna.C18 column. Linear concentration range was 5-25 jUg/mL, 2-15 jUg/mL for HCT and SPL respectively, and the relative standard deviations were found to be 1.39 % for HCT and 1.44 % for SPL respectively. Therefore, it is concluded that two

spectroscopic methods and HPLC method can be used in routine simultaneous quantitative analyses of HCT-SPL in commercial tablets.

Millership et al., (2005), studied about ratio spectra derivative spectrophotometry for the determination of furosemide and spironolactone in a capsule formulation. The determination of furosemide and spironolactone in a capsule formulation has been investigated using techniques such as Vierordt's method and derivative spectroscopy $dA/d\lambda$ and $d^2A/d\lambda^2$ applying the zero-crossing technique following reported methods. In our hands, using standard mixtures, these methods gave unreliable results. We have therefore investigated the use of ratio spectra derivative spectrophotometry for this determination. The technique of ratio spectra derivative spectrophotometry was developed in 1990, and has recently been used for a number of analyses of co-formulated products. The method was applied to the analysis of standard mixtures of the two drugs and the combined contents of 20 capsules resulting in values (mean ± standard deviation) of 102.1 ± 1.9% and 101.4 ± 4.0% of the stated content for furosemide and spironolactone, respectively. Similarly, the analysis of individual capsules resulted in values of 101.5 ± 1.6% and 102.2 ± 1.4% of the stated content for furosemide and spironolactone, respectively.

Lotfy et al., (2022), studied on smart spectrophotometric methods for concurrent determination of furosemide and spironolactone mixture in their pharmaceutical dosage forms. Simple, precise, accurate and specific spectrophotometric methods are progressed and validated for concurrent analysis of Furosemide (FUR) and Spironolactone (SPR) in their combined dosage form depend on spectral analysis procedures. Furosemide (FUR) in the binary mixture could be analyzed at its $\lambda_{max\ 274}$ nm using its recovered zero order absorption spectrum using constant multiplication method (CM). Spironolactone (SPR) in the mixture could be analyzed at its λ_{max} 238 nm by ratio subtraction method (RS). Concurrent determination for FUR and SPR in their mixture could be applied by amplitude modulation method (AM), absorbance subtraction method (AS) and ratio difference (RD). Linearity ranges of FUR and SPR were (2.0µg/mL-22.0 µg/mL) and (3.0µg/mL-30.0 µg/mL), respectively. Specificity of the proposed spectrophotometric methods was examined by analyzing the prepared mixtures in laboratory and was applied successfully for pharmaceutical dosage form analysis which have the cited drugs without additives contribution. The proposed spectrophotometric methods were also validated as per as the guidelines of ICH. Statistical comparison was performed between the obtained results with those from the official methods of the cited drugs, using one-way

ANOVA, F-test and student t-test. The results are exhibiting insignificant difference concerning precision and accuracy.

Rohankumar et al., (2019) studied on degradation study of spironolactone by UV-Visible Spectrophotometry method in bulk form. Spironolactone is most commonly used anti-diuretic agent in clinical practices. Spironolactone site of action is intracellular aldosterone receptors in the distal tubule cells. This increases the excretion of water and sodium and decreases the excretion of potassium. The degradation is most commonly found problem in most of products. The ICH guidelines states the parameters for forced degradation studies which includes the Acid/base stress testing, heat or humidity, photo degradation, pH variation. official assay limilight for 30 min the drug shows extremely decreased availability.

Chaudhary et al., (2012) studied on the development and validation of ratio derivative spectrophotometric for simultaneous estimation of metolazone and spironolactone in pharmaceutical dosage form. In the present work for the simultaneous estimation of Metolazone (METO) and Spironolactone (SPI) Ratio Derivative Spectrophotometric method have been developed. In this method the overlapping spectra of Metolazone and Spironolactone were well resolved by making use of the first-derivative of the ratios of their direct absorption spectra. Methanol:water (1:4) was used as solvent for the method. Amplitude at 241.0nm and 263.0 nm were selected in the ratio derivative spectra to determine METO and SPI, respectively. Beer's law was obeyed in the concentration ranges of 1-5µg/mL and 5-25 µg/ mL METO and SPI, respectively in the method. The % assay for commercial formulation was found to be in the range 98.00% – 102.00 % for METO and 99.00% – 102.60 % for SPI by the proposed method. Recovery was found in the range of 99.74 – 100.76 % for METO and SPI in the Formulations. The results of analysis have been validated statistically and recovery studies confirmed the accuracy and reproducibility of the proposed methods which were carried out by following ICH guidelines.

Moussa et al., (1985) studied on colorimetric analysis of some diuretic drugs: hydrochlorothiazide and spironolactone. Two convenient spectrophotometric methods were developed for the determination of hydrochlorothiazide and spironolactone. A specific colour reaction for the determination of hydrochlorothiazide is reported. One method is based on the reaction of hydrochlorothiazide with*n*-butylamine and cobaltous chloride in anhydrous methanol. A blue-violet colour is produced and is measured at 570 nm. A highly selective

colorimetric method was adopted for the analysis of spironolactone by reacting with isoniazid forming a coloured hydrazone derivative and subsequent measurement of the coloured product at 405 nm.

Martín et al., (1999) studied on simultaneous kinetic spectrophotometric determination of spironolactone and canrenone in urine using partial least-squares regression. A method is proposed for the simultaneous determination of spironolactone and canrenone in urine based on the different rates at which they react with sulphuric acid to yield a trienone. Kinetic spectrophotometric data are processed by partial least-squares (PLS) regression. The optimum sulphuric acid concentration and temperature are determined from response surfaces, using PLS methodology to relate both variables to the relative square error of prediction (RSEP, the parameter to be minimized). The relative errors made in the quantitation of each diuretic by the proposed method are less than 5% and the overall error, as RSEP, ranges from 1.06 and 1.44%.

Resende et al., (2016) studied on the analysis of spironolactone polymorphs in active pharmaceutical ingredients and their effect on tablet dissolution profiles. Spironolactone (SPR) is a steroidal drug administered as a potassium-sparing diuretic for high blood pressure treatment. The drug shows incomplete gastrointestinal absorption due to its poor aqueous solubility. The physicochemical properties of SPR in crystal forms I and II suggest that differences in their aqueous solubility may lead to a lack of bioequivalence between solid-state formulations. In this study, SPR polymorphs in five batches of active pharmaceutical ingredients (APIs) from three manufacturers were characterized using powder X-ray diffraction, infrared spectroscopy, thermal analysis, and solubility measurements. SPR tablets (50 mg) were manufactured in our laboratory using API in pure form II, and API in form II contaminated with form I, which was found in a commercial batch. Physicochemical quality evaluations of the manufactured tablets, along with five SPR tablets marketed in Brazil, were performed, and results indicated differences in their dissolution profiles. In the manufactured tablets, differences were associated with the increased solubility of API in form II contaminated with form I compared to API in pure form II. In the marketed SPR tablets, the formulation composition demonstrated an important role in the dissolution rate of the drug, leading to lack of pharmaceutical equivalence among the drug products.

Israt et al., (2016), studied on simultaneous determination of furosemide and spironolactone in pharmaceutical formulations by spectrophotometric method using principal component regression. The combination of furosemide (furo) and spironolactone (spiro) is very effective in the treatment of heart failure. In that case, maintaining good quality of these drugs in commercial tablets is must. Therefore, a simple, economical, precise and accurate method, i.e; chemometric assisted UV spectroscopy, for simultaneous determination of furosemide and spironolactone in combined dosage form has been developed. In this study, principal component regression (PCR) has been reported for this purpose. A calibration set of 36 mixture solutions containing furosemide and spironolactone in methanol in the concentration range of 2.0-12.0 μg/ml and 5.0-30.0 μg/ml respectively has been prepared by means of an orthogonal experimental design. The absorbance data for the concentration set have been obtained by direct measurement in UV spectrophotometer at 101 wavelength points in the spectral region of 200-300 nm for the zero order spectra. The chemometric technique is also successfully applied to available pharmaceutical formulations, tablets, with no interference from excipients. The analytical performances of principal component regression are characterized by relative prediction errors and recoveries (%). The good recoveries obtained in this case proved that the proposed chemometric technique could be applied efficiently in the quality control of the studied drugs simultaneously in their mixture as well as in the commercial dosage form with satisfactory precision and accuracy as alternative analysis tools.

Kundu et al., (2016), studied on the analysis of carvedilol and spironolactone in pharmaceutical dosage form and dissolution samples by simultaneous equation method and derivative method. Two spectroscopic methods such as simultaneous equation method and third order derivative spectroscopic method for the simultaneous estimation of carvedilol (CARV) and spironolactone (SPRN) in pharmaceutical dosage form and dissolution samples were developed. The methods were validated as per the ICH guidelines for different parameters such as linearity, .specificity, accuracy, precission, limit of detection and limit of quantification.

Chavan et al., (2021), studied on the method development and validation of spectrophotometric and RP-HPLC methods for simultaneous estimation of spironolactone and furosemide in bulk and combined tablet dosage form. The intent of the present work was to develop a simple, sensitive, accurate, precise, rapid and economical UV- spectrophotometric and reverse phase high pressure liquid chromatographic method for the simultaneous

estimation of Spironolactone and Furosemide in bulk and combined tablet dosage forms. UVSpectrophotometry was carried out by simultaneous equation method using 0.02 M potassium dihydrogen phosphate buffer pH 3.5: Acetonitrile (50:50) v/v as a solvent. The linearity range was 2-14 µg mL−1 for Spironolactone and Furosemide with a correlation coefficient > 0.99. The chromatographic separation was achieved on 250 mm × 4.6 mm, hypersil BDS C18 column with particle size 5 µm, by using an isocratic mixture of 0.02 M potassium dihydrogen phosphate buffer pH 3.5: Acetonitrile: tert butyl methyl ether (49:50:1) v/v/v as a solvent at a flow rate of 1 mL min−1 and UV detection was carried out at 254 nm. The retention time were observed to be 3.666 and 6.661 minutes for Furosemide and Spironolactone respectively. The two developed methods were validated according to the ICH guidelines for accuracy, precision, linearity, LOD, LOQ and were found to be within the limits. It can be concluded that these two methods could be successfully used for the simultaneous estimation of Spironolactone and Furosemide in bulk and combined tablet dosage forms.

Emam et al., (2018) studied about successive ratio subtraction as a novel manipulation of ratio spectra for quantitative determination of a mixture of furosemide, spironolactone and canrenone. Furosemide and spironolactone are commonly prescribed antihypertensive drugs. Canrenone is the main degradation product and main metabolite of spironolactone. Ratio subtraction and extended ratio subtraction spectrophotometric methods were previously applied for quantitation of only binary mixtures. An extension of the above mentioned methods; successive ratio subtraction, is introduced in the presented work for quantitative determination of ternary mixtures exemplified by furosemide, spironolactone and canrenone. Manipulating the ratio spectra of the ternary mixture allowed their determination at 273.6 nm, 285 nm and 240 nm and in the concentration ranges of (2–16 µg mL− 1), (4–32 µg mL− 1) and (1–18 µg mL− 1) for furosemide, spironolactone and canrenone, respectively. Method specificity was ensured by the application to laboratory prepared mixtures. The introduced method was ensured to be accurate and precise. Validation of the developed method was done with respect to ICH guidelines and its validity was further ensured by the application to the pharmaceutical formulation. Statistical comparison between the obtained results and those obtained from the reported HPLC method was achieved concerning student's t-test and F ratio test where no significant difference was observed.

Sora et al., (2010), studied on the analytical issues in HPLC/MS/MS simultaneous assay of furosemide, spironolactone and canrenone in human plasma samples. A new sensitive

HPLC/MS/MS method for simultaneous determination of furosemide, spironolactone and canrenone in human plasma samples is presented. Electrospray ionization source (ESI) has been used. The tandem MS detection was performed under MRM conditions, in the negative ion mode for furosemide and indapamide (internal standard 1) and in the positive ion mode for spironolactone, canrenone and nitrazepam (internal standard 2). A simple plasma protein precipitation with acetonitrile was used as sample preparation technique. The chromatographic separation was carried out under the reversed phase mechanism, on a 250 mm length column packed with octadecyl modified silicagel and thermostated at 35 °C. The column was operated under isocratic conditions (3:7 aqueous 0.1% formic acid and methanol, v/v) at a flow rate of 0.8 mL/min. Quantitation intervals of 20–1600 ng/mL for furosemide and 2–100 ng/mL for spironolactone and canrenone have been concluded through validation. Precision and accuracy were situated within the accepted thresholds (maximum 15% relative standard deviation and ±15% percentage bias). The most sensitive aspects relating to the analytical method development and validation were highlighted and critically assessed in order to reach an objective opinion about the real performances and inherent applicability of the method in bioanalysis.

Hegazy et al., (2011) studied on validated chromatographic methods for determination of hydrochlorothiazide and spironolactone in pharmaceutical formulation in presence of ımpurities and degradants. Two specific, sensitive, and precise stability indicating chromatographic methods have been developed, optimized, and validated for Hydrochlorothiazide (HCT) and Spironolactone (SPR) determination in their mixtures and in presence of their impurities and degradation products. The first method was based on thin layer chromatographic (TLC) combined with densitometric determination of the separated spots. The separation was achieved using silica gel 60 F_{254} TLC plates and ethyl acetate-chloroform-formic acid-triethyl amine (7:3:0.1:0.1, by volume) as a developing system. Good correlations were obtained between the integrated peak area of the studied drugs and their corresponding concentrations in different ranges. The second method was based on the high-performance liquid chromatography with ultraviolet detection, by which the proposed components were separated on a reversed phase C18 analytical column using gradient elution system with deionized water-acetonitrile (97:3, v/v) for 8 min. Then acetonitrile was successively increased to 35% in the next 2 min, and kept constant in the following 10 min, finally 3% acetonitrile was regained again to stabilize the chromatographic system. The flow rate was maintained at 2 mL/min and the detection wavelength was at 230 nm. Linear regressions were obtained in

the range of 4.0–50 µg/mL and 5.0–50 µg/mL for both HCT and SPR, respectively. Different parameters affecting the suggested methods were optimized for maximum separation of the cited components. System suitability parameters of the two developed methods were also tested. The suggested methods were validated in compliance with the ICH guidelines and were successfully applied for determination of HCT and SPR in their commercial tablets. Both methods were also statistically compared to each other and to the reported method with no significant difference in performance.

Dinç et al., (2006), studied about an approach to quantitative two-component analysis of a mixture containing hydrochlorothiazide and spironolactone in tablets by One-Dimensional Continuous Daubechies and biorthogonal wavelet analysis of UV-Spectra. In this study a new method of quantitative two-component analysis of a mixture of hydrochlorothiazide (**HCT**) and spironolactone (**SP**) in the presence of strongly overlapping signals was achieved by using one-dimensional continuous wavelet analysis. This new method was built on the simultaneous use of both continuous wavelet transform and the zero crossing technique for the quantitative resolution of this binary mixture. A series of solutions, in the concentration range of 2–22 mg/ml for both compounds, in methanol and 0.2 M sodium acetate buffer, pH = 5 (20:80), were considered. The absorption spectra of the standard solutions were recorded in the wavelength range of 215–330 nm. To apply our methods we selected 400 points from the absorption spectra and we subjected the corresponding signal to Daubechies2 (**DAUB2**) and Biorthogonal1.5 (**BIOR1.5**) one-dimensional wavelet transform. In the transformed signals, the amplitude of **HCT** was measured at the position of a zero crossing point of SP and vice versa. Thus simple linear regression analysis can be applied to establish calibrations for both components. These calibrations were validated with synthetic mixtures of HCT and **SP**. *MATLAB 6.5* software was used for one-dimensional wavelet analysis, and the basic concepts about wavelet method are briefly explained. The method developed in this paper is rapid, easy to apply, inexpensive and it is suitable for analysis of the overlapping signals of compounds in their mixtures without any chemical pre-treatment.

Maślanka et al., (2009), studied on simultaneous analysis of hydrochlorothiazide, triamterene, furosemide, and spironolactone by densitometric TLC. A new chromatographic and densitometric method has been established for identification and quantitative analysis of hydrochloro-thiazide, triamterene, furosemide, and spironolactone, which are present,

together, in complex drugs used to treat hypertension. For separation, silica gel F_{254} plates were used with hexane-ethyl acetate-methanol-water-acetic acid 8.4:8:3:0.4:0.2 (v/v) as mobile phase. Densitometric measurements were performed at 264 nm selected for all of the constituents. The method is specific for the analyte constituents examined, and characterized by high sensitivity; LOD is from 0.022 to 0.150 μg per band, LOQ from 0.068 to 0.450 μg per band, recovery from 97.10 to 101.02%, and linear range from 0.060 to 2.650 μg per band. The method is characterized by good precision with RSD from 0.66 to 0.96%.

Zaid et al., (2024), studied on review on bioanalytical and analytical method development of two potassium-sparing diuretics, namely eplerenone and spironolactone. Eplerenone (EPL) and spironolactone (SPR) belong to the class of potassium-sparing diuretics. Both drugs are prescribed for the treatment of hypertension and heart failure. Spironolactone is structurally like progesterone and binds to progesterone, androgen and mineralocorticoid receptors. Eplerenone is a selective mineralocorticoid receptor antagonist, so it lacks the anti-androgenic side effects of spironolactone. Using a thorough computer assisted literature survey; this review article touches upon the reported analytical methods for the quantification of both drugs in raw materials, pharmaceutical dosage forms and biological fluids. Bioanalytical methods are widely used for quantitative estimation of drugs and their metabolites in biological matrices. Various analytical methods such as spectrophotometry, spectrofluorimetry, HPLC, TLC, and UPLC have been used in laboratories for the quantitative analysis of these drugs in biological samples and pharmaceutical preparations. Therefore, the aim of this review is to give a summary of the current analytical methods used for the assay of both drugs. The review represents a guide for the QC labs and the bioequivalence centers to analyze the studied drugs.

Patyi et al., (2010), studied on thermal and spectroscopic analysis of inclusion complex of spironolactone prepared by evaporation and hot melt methods. Solvent and melt techniques were used to obtain molecular dispersion of the poorly soluble spironolactone (SPIR) model drug enhancing its dissolution rate. DSC study of the interaction between SPIR and hydroxypropyl-β-cyclodextrin confirmed the need for molecular dispersion if their complexation is required. Solvent-free twin-screw extrusion was suitable for forming inclusion complex significantly below the melting temperature of the SPIR. According to DSC, Raman and XRPD results fine dispersion of both components was achieved in a hydrophilic polymer. The molecules of the active ingredient are separated from each other in the polymer and the lack of the lattice energy causes faster dissolution.

Brandão et al., (2008), studied on physical-chemical characterization and quality control of spironolactone raw material samples. It is well established that solid-state properties such as solubility, particle size, and morphology are critical factors in the development of pharmaceutical formulations. Thus, the evaluation of the physical-chemical properties of the substances that will be used must be the primary step for quality control in the pharmacy industry. The aim of this report is to characterize and to establish the quality of four spironolactone raw samples, derived from distinct laboratories, through thermal analysis (DSC and TG), infrared spectroscopy (IV), solubility assay, scanning electron microscopy, and digital image analysis. Capsules of spironolactone were also prepared with these different drug samples and dissolution profiles determined. The IR and the DSC assays confirmed the identity of the samples as spironolactone. Morphological differences relating to shape, size, and particle size distribution were found and can be directly related to the varied dissolution profiles presented by the different formulations.

Voicu et al., (2011), studied on incurred sample reanalysis that is different evaluation approaches on data obtained for spironolactone and its active metabolite canrenone. The inherent reproducibility of a bioanalytical approach is usually sustained through incurred sample reanalysis (ISR). Questions relating to the number of ISRs, the right moment for performing reanalysis, the way of performing an appropriate statistical refinement of experimental data and actions to be taken in the case of failure are frequently raised. Data resulting from ISR following a bioequivalence study for spironolactone formulations are discussed. Reanalysis of samples was carried out twice: immediately after the end of the study and after a period that overcame the long-term stability study achieved during method validation. The Bland–Altman approach was used to assess experimental results. ISR was successful over the short reanalysis period for both compounds. Data produced through reanalysis after the long-term period indicated a systematic positive bias for the metabolite canrenone (although results supported reproducibility). The results obtained for spironolactone were affected by a strong negative systematic bias and failed to support reproducibility. The explanation deals with the continuous conversion of spironolactone to canrenone in plasma samples. However, reproducibility of the method may be sustained by comparing original and repeated differences between concentration values in samples by means of a paired t-test, Wilcoxon sign rank-sum test and linear regression. Different statistical approaches for making data comparisons are discussed and may be successfully applied during

reanalysis of samples from a bioequivalence study. Results of the evaluations may differ in accordance with the statistical procedure being applied, thus a definitive conclusion requires consideration of all specific experimental circumstances arising during production of the processed data.

El-Dalsh et al., (2008) studied on spironolactone polymorphic forms. The phenomenon of polymorphism of spironolactone was studied using melting point and aqueous solubility determinations, infrared spectroscopy, differential thermal analysis, X-ray powder diffraction and powder dissolution. The results showed that spironolactone crystals obtained from ethyl acetate had the lowest melting range, while those obtained from acetonitrile had the highest range. The aqueous solubility data indicated the presence of different forms of spironolactone, each with a specific solubility, although practically all of these forms could be considered to be water-insoluble. The infrared spectra were not useful in clearly distinguishing between the different forms. Differential thermal analysis curves indicated that some of the forms obtained by treating spironolactone with different solvents were somewhat similar, others were variable, but all of them were different from the original form of the drug. Moreover, the thermal study proved the absence of any solvates. X-ray patterns were different in spacing (d) values and intensities of radiation absorption, confirming the presence of four different forms of spironolactone. The form obtained from ethyl acetate had the highest dissolution rate, while that obtained from acetonitrile had the lowest rate. Spironolactone is known to crystallize out from different solvents in different polymorphic forms and to undergo structural rearrangements on heating.1, 2 The study was mainly concerned with the infrared identification of steroids, and the results showed that sample preparation techniques have a profound effect on the spectra of these steroids. The purpose of the present work is to carry out studies to specify the number of polymorphs of spironolactone under the crystallization conditions.

Abdelhamid et al., (2019) studied on determination of antihypertensive drugs by using ratio difference spectrophotometric method. An accurate, sensitive and time saving spectrophotometric method has been developed and validated for the determination of two antihypertensive drug mixtures. Mixture 1 contains spironolactone (SPIR), furosemide (FUR) and anthranilic acid (ANTH) (impurity of furosemide) and mixture 2 contains triamterene (TRI), hydrochlorothiazide (HCZ) and chlorothiazide (CZ) (impurity of hydrochlorothiazide). In mixture 1, the determination of drugs depends on dividing the spectrum of ternary mixture

by the spectrum of 10 µg/mL of standard furosemide and then spironolactone and anthranilic acid were determined using the difference in amplitude between 242.3 and 254.6 nm, and between 250.8 and 242.4 nm in the ratio spectrum, respectively. On the other hand, furosemide could be determined by dividing the spectrum of ternary mixture by the spectrum of 10 µg/mL of standard spironolactone and then it was determined using the difference in amplitude between 244.8 and 229.7 nm in the ratio spectrum. In mixture 2, the determination of drugs depends on dividing the spectrum of ternary mixture by spectrum of 10 µg/mL of standard triamterene and then hydrochlorothiazide and chlorothiazide were determined using the difference in amplitude between 268.9 and 232.8 nm, and between 292.9 and 250.7 nm in the ratio spectrum, respectively. On the other hand, triamterene could be determined by dividing spectrum of ternary mixture by spectrum of 10 µg/mL of standard hydrochlorothiazide and then triamterene was determined using the difference in amplitude between 230.1 and 244 nm in the ratio spectrum. The developed analytical methods were validated regarding good accuracy and precision according to The International Conference on Harmonisation guidelines, and they were applied to pharmaceutical preparations in addition to laboratory prepared mixtures successfully. Statistically the results were compared with those obtained by reported method and no significant difference was found.

2.MATERIAL AND METHOD

2.1 Spectrophotometry

Spectroscopic methods; It is a broad group of analytical methods based on atomic and molecular spectroscopy. Spectroscopic methods are frequently used in qualitative and quantitative analysis of inorganic and organic compounds.

Spectroscopy is a general term for the branch of science that studies the interaction of various types of rays with matter. The measurement and interpretation of electromagnetic radiation absorbed or emitted during the transition of atoms, molecules or ions in a sample from one energy level to another is called "spectroscopy".

Rotation, vibration and electronic energy as a result of the interaction of an atom, molecule or ion with electromagnetic radiation.

Changes in levels form the basis of spectroscopy. In the past, only the interactions between electromagnetic radiation and matter were concerned; However, today the scope of spectroscopy has been expanded to include interactions between matter and other forms of energy.

Ray or electromagnetic radiation is a form of energy that moves through space at great speed. The most common types of electromagnetic radiation are visible light and heat, which we perceive with the eye, radio waves, and infrared radiation, which we perceive as x-rays.

The movement of the beam in space occurs in waves. Gamma rays, ultraviolet rays, microwaves, and radio frequency rays are rays whose existence is more difficult to understand. It has been proven that electromagnetic radiation behaves like waves and particles. The wave model has failed to explain the phenomena related to the absorption and emission of radiant energy. A particle model has been developed to understand these phenomena; In this model, the electromagnetic beam is seen to consist of particles or wave packets called photons, whose energies are proportional to the beam frequency. This dual perception of ray as particles and waves should be taken as complementary rather than mutually exclusive concepts. Indeed, the wave-particle pair property has been used to explain the behavior of electrons, protons, and other elementary particles and is fully accepted by wave mechanics.

Spectrophotometry is based on the absorption by matter of the energy carried by electromagnetic radiation.

2.1.1 Electromagnetic Radiation

Electromagnetic radiation consists of two fields that are electric and magnetic fields perpendicular to each other, propagating linearly at a constant speed in space and in the form of sinusoidal oscillations along the propagation axis. Electromagnetic radiation (light) is a form of energy whose behavior is described by wave and particle properties.

1) Wave character: It has electric and magnetic vectors that spread sinusoidally (with wave movements) in space. Electric vector is used in spectrophotometry.

Electromagnetic radiation is characterized by various parameters such as velocity (c=3.0x1010 cm/s), amplitude, frequency (υ) and wavelength (λ).

2) Particle character: An electromagnetic radiation beam consists of many particles and its energy is given by the following mathematical expression:

$E = h.\nu$ (1) or $E = h.c/\lambda$ (2)

It is expressed with the formulas (h=plank constant=$6.62x10^{-27}$erg.sec and c=$3x10^{10}$cm/sec).

According to these equations, as the wavelength of light increases, its energy decreases, but as the wavelength decreases, its energy increases.

The polarization of light ;

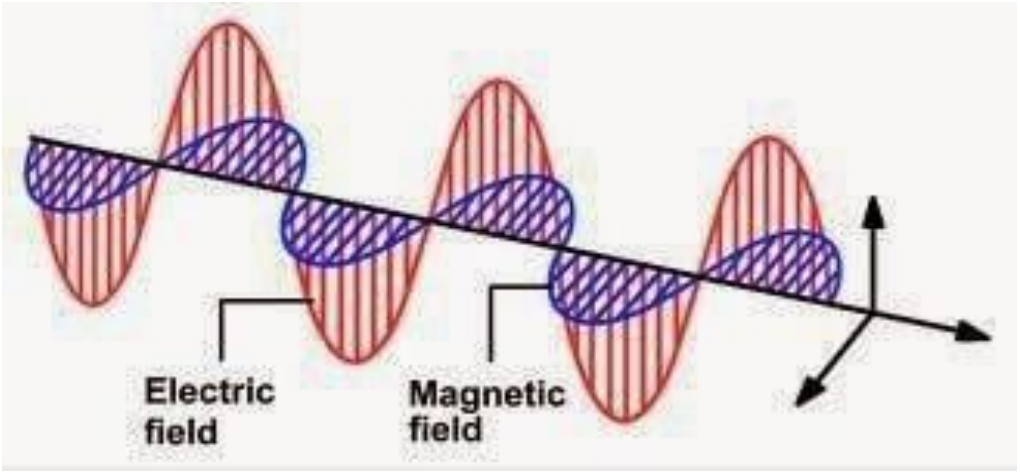

Figure 2.1 A plane polarized beam

In Figure 2.1 shows a plane polarized beam. By plane-polarized expression, it is meant that electric and magnetic fields vibrate only in one plane, vibrations in other directions are damped.

Visible light waves correspond to the part of the electromagnetic wave that is visible only to the naked eye. We can see these waves as colors formed in the rainbow. Each color here corresponds to a different wavelength. The wave corresponding to the color red corresponds to the longest wavelength of the visible region, while purple corresponds to the shortest wavelengths. When all the waves in the visible region are observed together, they form white light. The opposite is true. So if we pass white light through a prism, we observe all the waves in the visible region.

If the absorption or emission of electromagnetic radiation by matter (atom or molecule) is examined, it is called absorption or emission spectroscopy, respectively. The absorption of light by molecules depends on the type and arrangement of atoms in the molecule, their shape, size, etc. Because it's connected. Spectroscopic methods from molecules are applied in a wide variety of fields, such as finding the structures and stereochemical properties of substances, recognition and purity control. Since it is not possible to make a single device that will give all wavelengths in spectroscopic studies and detect which wavelengths are absorbed, devices that work between certain wavelengths have been developed.

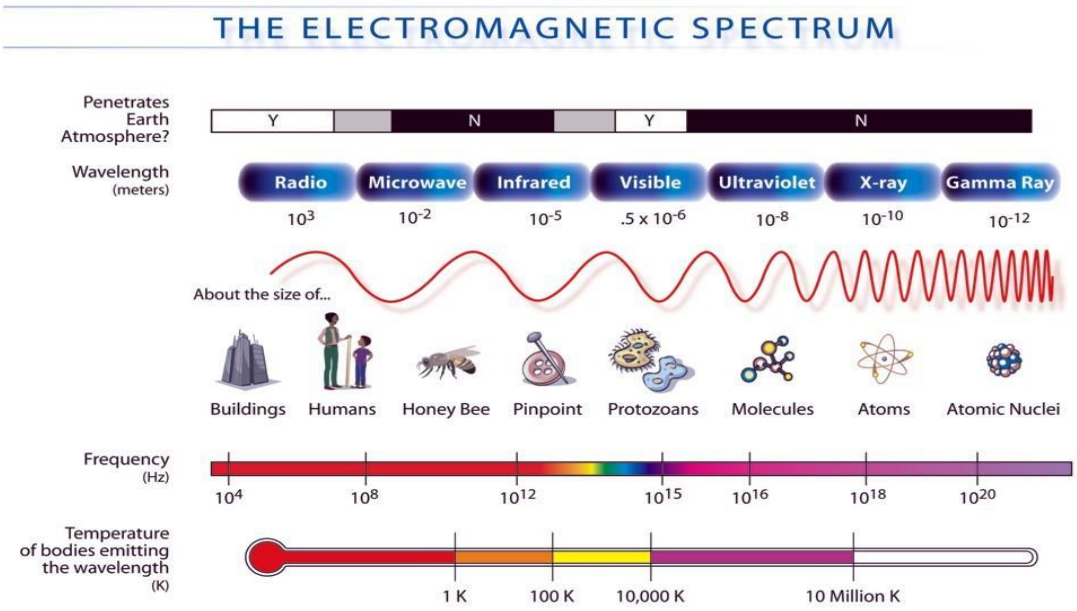

Figure 2.2 Electromagnetic Spectrum.

Devices operating with rays at wavelengths of 110-800 nm are called ultraviolet and visible field devices, devices operating at wavelengths of 0.78-1000 μm are called infrared devices, and devices operating with radio waves with wavelengths ranging up to hundreds of meters

are also called nuclear magnetic resonance devices. The spectroscopy methods to which these devices are applicable are called Ultraviolet-Visible (UV-Visible), Infrared (IR vibration) and Nuclear Magnetic Resonance (NMR) spectroscopies, respectively. The diagram showing the electromagnetic radiation spectrum according to energy for analytical purposes is shown in Fig.1.2.it is given in.

2.1.2 Absorption of the Beam

Light can be absorbed by atoms and molecules. The absorption of UV and visible light is related to the bonding electrons of the atoms that make up the molecule.

If a beam containing rays of various wavelengths is passed through a transparent and transparent medium, it is observed that some wavelengths disappear through it. This is called the absorption of the beam. By absorption, the beam energy is transferred to ions, atoms or molecules of matter. Thus, ions, atoms or molecules switch to an excited state.

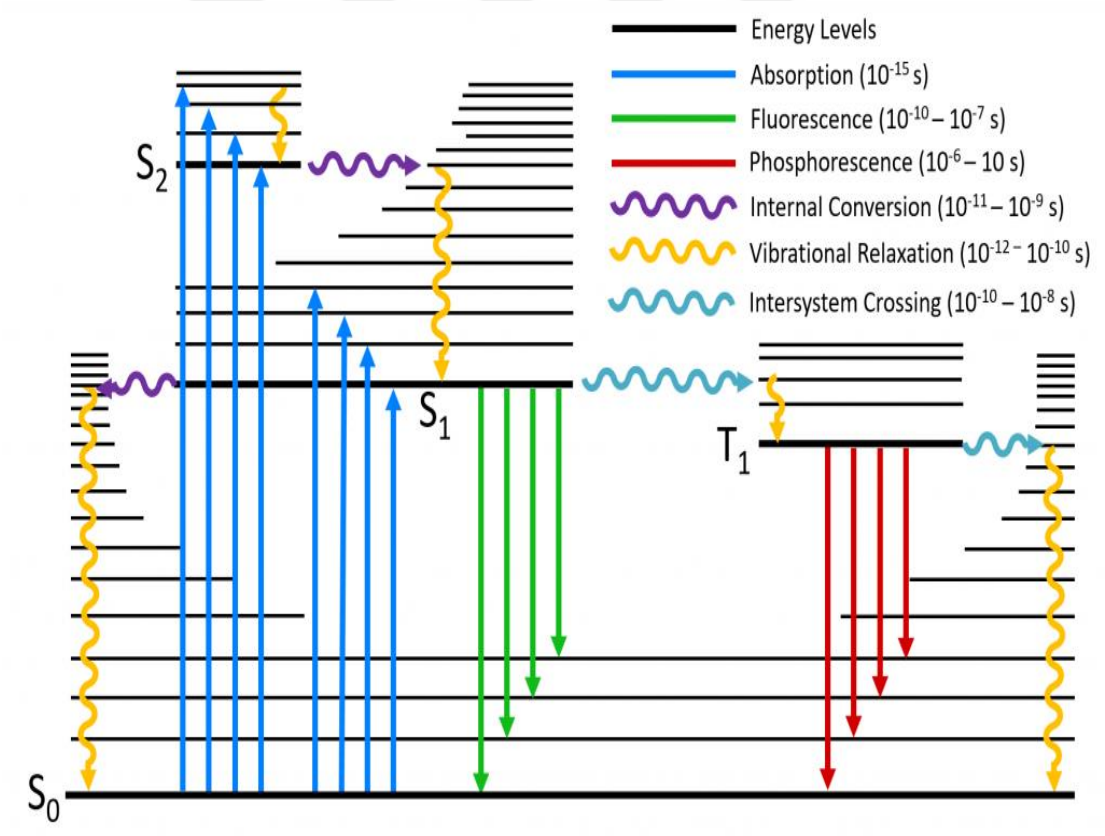

Figure 2.3 Jablonski Diagram

$\mathbf{S_0}$ is the singlet ground state of the molecule

$\mathbf{S_1}$ is the first excited singlet state and $\mathbf{S_n}$ is the n^{th} excited singlet state

$\mathbf{T_1}$ is the first excited triplet state and $\mathbf{T_n}$ is the n^{th} excited triplet state

An excited atom or molecule can live up to 10-8 seconds. Then, by giving back the absorbed ray energy, it returns to its old or basic state again. By article, the return of the absorbed beam energy is usually in the form of heat. In some substances, on the other hand, the absorbed beam energy is emitted as rays of a longer wavelength. This is called the photoluminescence event. The very short-term of this event is called fluorescence, the longer-term one is called phosphorescence. The fluorescence singlet is from the excited state while the phosphorescence is from the triplet excited state and the electronic singlet is from the basic state which represents transitions. Singlet and triplet excited states are different in terms of electron spin. The spin of an electron in the singlet excited state is the same according to the singlet fundamental state that is directional. The spin of the electron in the triplet excited state is in the opposite direction with the singlet ground state. These are shown in figure 1.3.

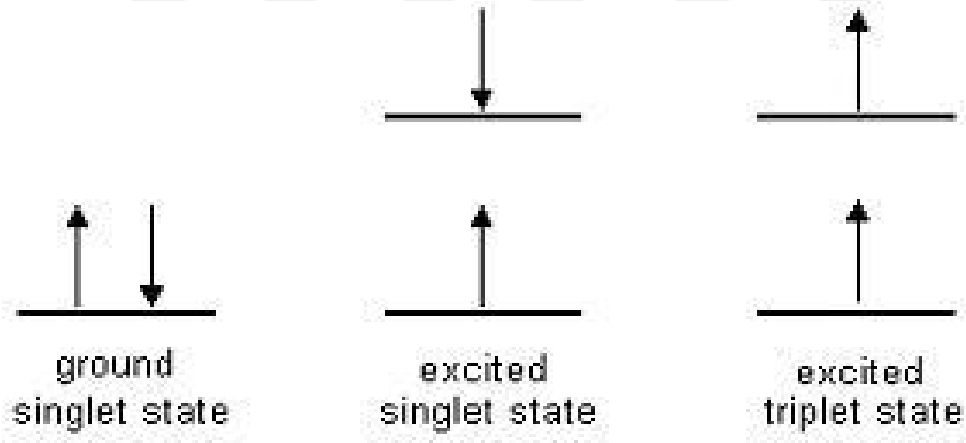

Figure 2.4. Energy states

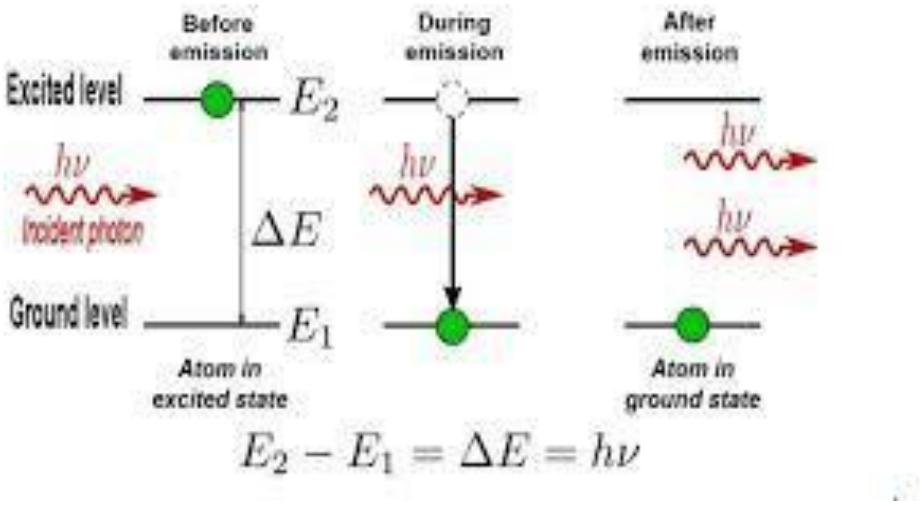

Figure 2.5 Difference between energy states

It causes electrons to move from lower energy orbitals (ground state) to higher energy orbitals (excited state).But not all energy can be absorbed; Only energy equal to the difference between the two energy levels can be absorbed.

Since the energy differences between the ground and excited states of a substance are different from those of another substance, each substance has a unique absorption spectrum. Absorption spectra are generally divided into two parts.

- Atomic Absorption Spectra

  If the polychromatic UV or visible region beam is passed through a monatomic medium such as mercury or sodium gas, it is seen that some rays disappear from the beam, for example the yellow ray from sodium vapor. This situation can be explained by the fact that an electron at the 3s energy level in sodium atoms absorbs the yellow ray and rises to the 3p energy level.

  In atoms, the outermost layer electrons are excited by ultraviolet-visible rays, and the inner layer electrons are excited by X-rays. X-rays are thousands of times more energetic than visible rays.

  The branch of spectroscopy based on the excitation of the outermost layer electrons of atoms is called atomic absorption, and the branch of spectroscopy based on the excitation of the inner layer electrons is called X-ray spectroscopy.
- Molecular Absorption Spectra

When molecules are excited with UV-visible and infrared rays,there are three types of quantized transitions. These are electronic, vibration and rotational transitions.

Etotal = Eelectronic + Evibration + Erotation + Etranslation

Each electronic energy level consists of many vibrational and rotational energy levels. Electronic energy is greater than other energies. Therefore, during electronic stimulation, vibrational and rotational energy levels also change. Vibrational and rotational energies are related to the vibration of atoms in the molecule and the rotation of the molecule. Translation energy is related to the translational movement of the entire molecule and is the lowest energy movement.

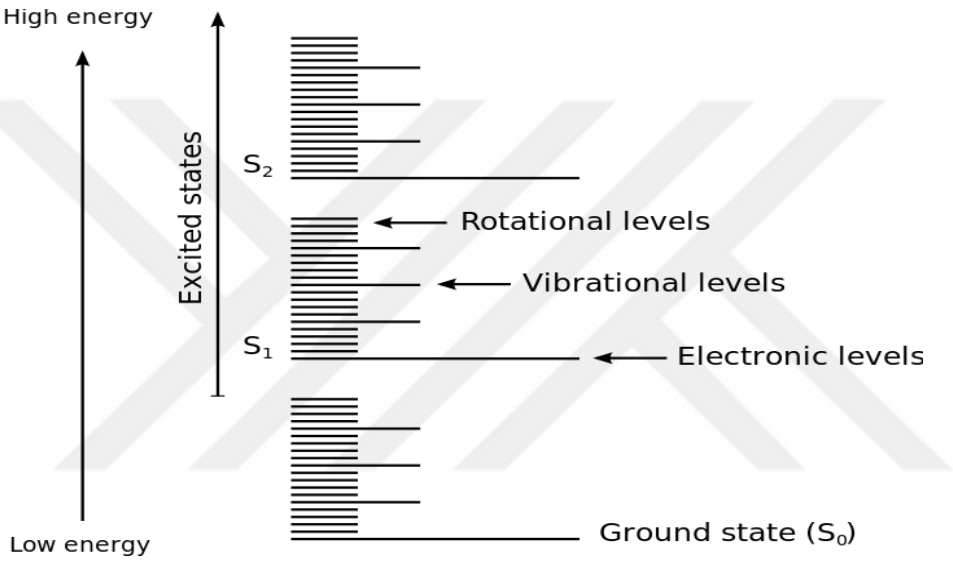

Figure 2.6. Energy levels in the molecule

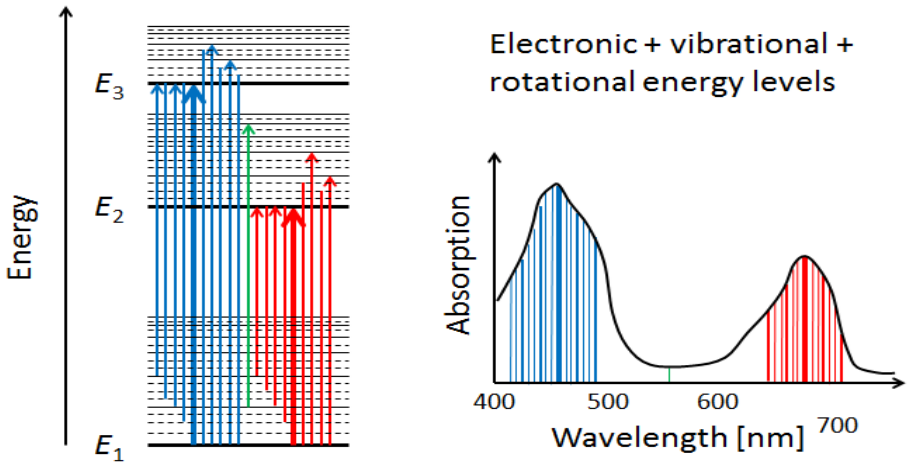

Figure 2.7 Continuous spectrum-Absorption spectrum (UV visible region)

Light absorption is measured in spectrophotometers. This measurement is the measurement of the difference between the intensity of the light sent to the atom, ion or molecule (Io) and the intensity of the light passing through (I).

The Beer-Lambert system (also called the Beer's law) is the relationship between the attenuation of light through a substance and its properties. This paper detailed first the definitions of the transmittance and absorbance of radiation by a substance and then an explanation of the Beer-Lambert law.

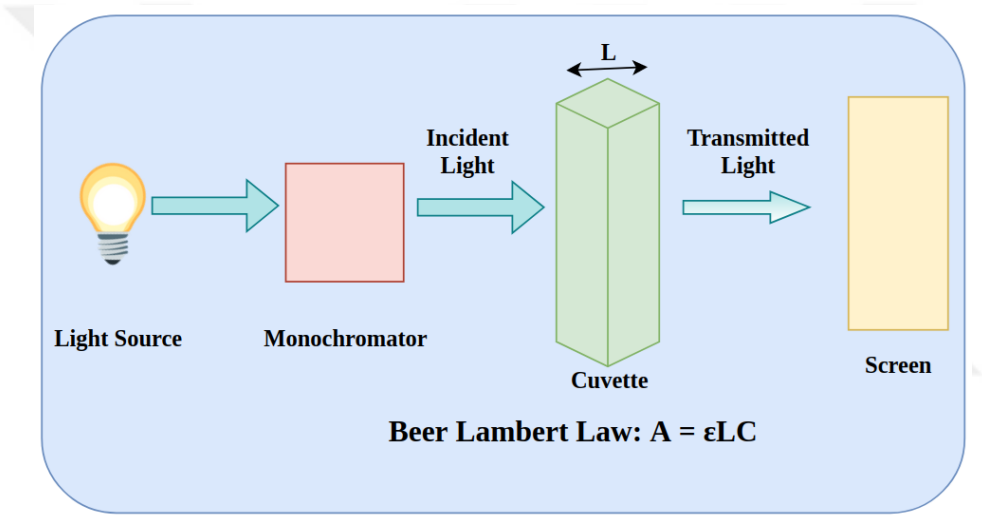

Figure2.8 Schematic representation of spectrophotometer

$$\log_{10}(I_0/I) = A = \varepsilon \ell c$$

where;

A is the absorbance

ε is the molar attenuation coefficient or absorptivity of the attenuating species

ℓ is the optical path length

c is the concentration of the attenuating species

I = Intensity of light leaving the sample

Io = Intensity of light sent on the sample

2.1.3. UV-Visible Spectrophotometry

Ultraviolet-visible (UV-Vis) spectrophotometry is a technique used to measure light absorption throughout the ultraviolet and visible ranges of the electromagnetic spectrum. When incident light hits matter, it can be absorbed, reflected or transmitted. Absorption of radiation in the UV-Vis range causes atomic excitation, which refers to the transition of molecules from a low-energy ground state to an excited state.

Visible light is a very low fraction of the total electromagnetic radiation (400–800 nm) and is seen as color by the human eye. There is a purple color at the lower end of 400 nm, and when it reaches the upper limit of 800 nm, following the rainbow colors, a red color appears. The region below visible light (100–400 nm) is called the "UV" region, and the region above it (800 nm–25μ) is called the "Infrared" region.

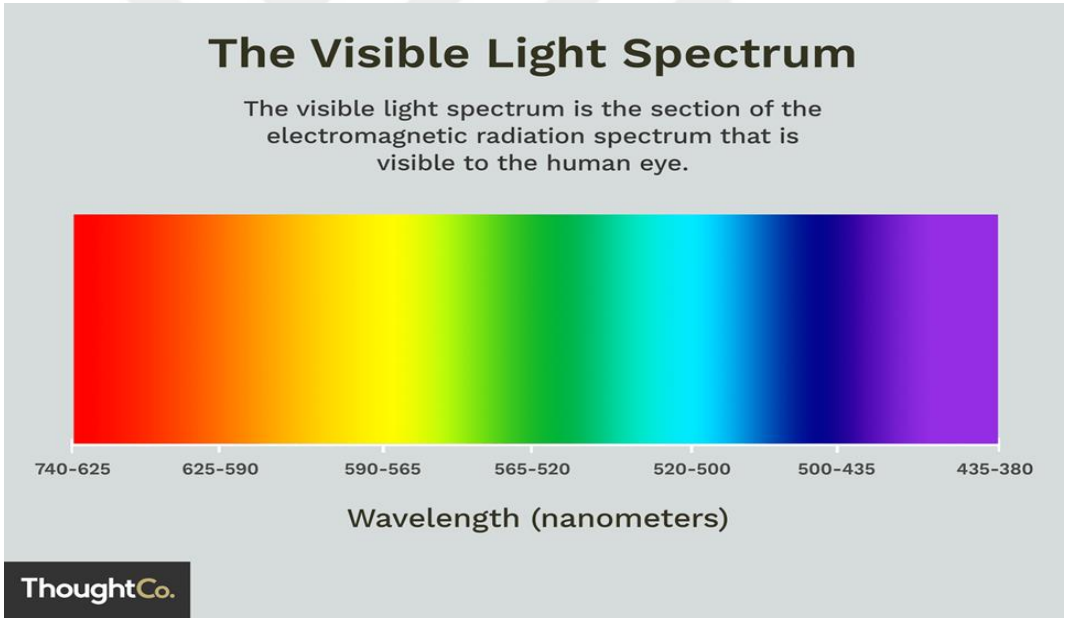

Fig. 2.9 The Visible Light Spectrum

UV–Visible spectroscopy is one of the most widely used methods for quantitative purposes. The fact that it can be used in the determination of both organic and inorganic substances and that it is sensitive up to around 10-6–10-7 M with recent developments makes this method attractive. At the same time, it is possible to achieve good precision and accuracy with this method.

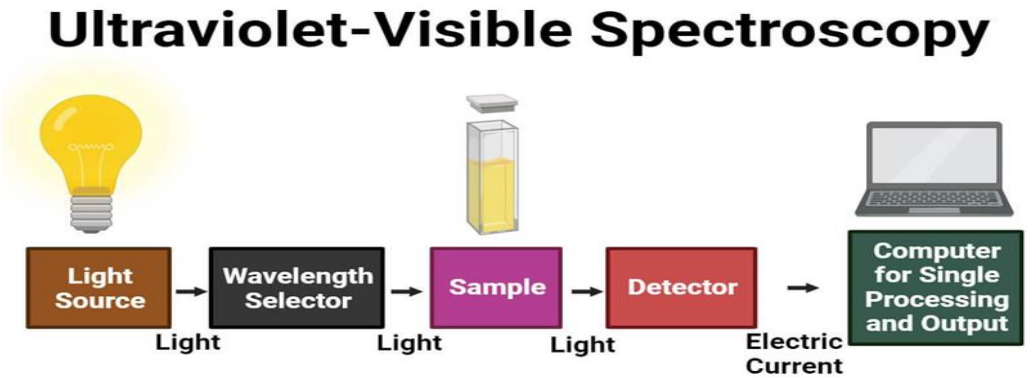

Figure 2.10 Instrumentation of UV-Vis Spectrophotometer.

The main components of UV- Vis spectrophotometer are light source, wavelength selector, sample, detector, computer for single processing and output.

2.1.4 Quantitative Analyse

Ultraviolet-Visible absorption spectrophotometry is one of the techniques that chemists use most in quantitative analysis. In quantitative analysis, first the wavelength at which the substance to be analyzed has the best absorbance is determined. The spectra of a series of solutions prepared at increasing concentrations at the specified wavelength are taken and their absorbances are read. The calibration curve is obtained by plotting absorbance against concentration. Then the concentration of the unknown substance is determined with the help of the calibration curve.

2.1.5 Derivative Spectrophotometry

Compared with conventional spectrophotometric determinations, derivative spectrophometry has proved to be a great value in eliminating the interference from excipients and coformulated drugs. The derivative spectrophotometry (DS) represents an elegant approach to the problem of resolving spectral overlap in pharmaceutical analysis. DS permits the simple, rapid, sensitive and direct determination of mixtures of drugs having closely overlapping spectra.
In pharmaceutical application, DS has led to significant developments in the analysis of drugs in the presence of their degradation products or in multi-component mixtures.

Derivative Absorption Spectrophotometry;

It is the 1, 2, 3....., n derivative of the absorbance versus wavelength as a function of wavelength.

As a function of F (λ) dA/dλ, d 2A/dλ2 , d 3A/dλ3 , ..., d nA/dλn

The following equations show the differences obtained in the Lambert-Beer law:

Zero order derivation : A= ε x b x C

First order derivation : dA/dλ = b x C x dε/d λ

Second order derivation : d2A/dλ2 = b x C x d 2 ε/d λ2

…

n th derivation : d nA/dλn = b x C x d n ε/d λn

Derivative absorption spectrophotometry; It is extremely sensitive to any change in the slopes of the absorption spectrum and is a very suitable method for the analysis of absorbance bands with overlapping peaks. With derivative absorption spectra, it is possible to quantify some substances among others without the need for any separation in the mixture. This means especially mixed substances; It makes the method a very effective and easy method of quantification for the analysis of pharmaceutical preparations containing them as active ingredients.

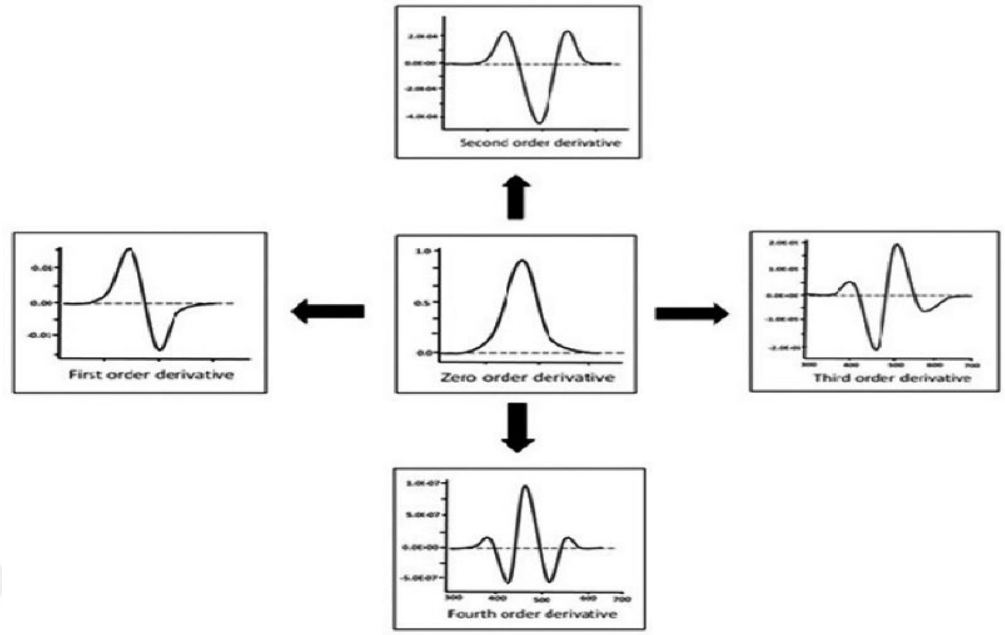

Figure 2.11 Derivative spectrums

The derivative of the absorbance is directly proportional to the concentration. The ordinate changes in the 1st, 2nd and 3rd order derivative spectra are related to the slope change, not to the magnitude of the ordinate value of the original spectrum. For this reason, the position of the derivative curve in the abscissa and the opposite positions of the turning points are characteristic.

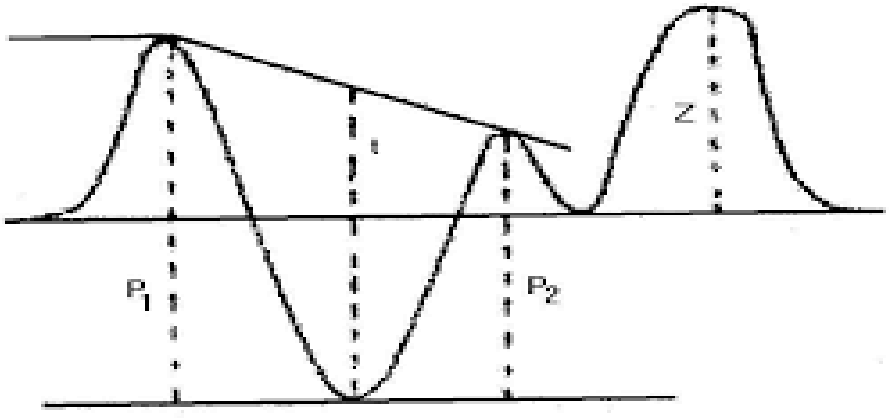

Figure 2.12 Evaluation of derivative absorption spectra, P1:peak to peak measurement, z measurement of peak to zero, tangent measurement.

In figure 2.12, There are three methods for evaluating derivative spectra. These are peak to peak measurement, peak to zero measurement and tangent measurement.

Advantages of Derivative UV-Spectrophotometry

Derivative UV-Spectrophotometry has increased sensitivity and selectivity. It has multiple advantages,; single component analysis and simultaneous determination of several components in a mixture, determination of traces in matrix, protein and amino acid analysis, environmental analysis, identification of organic and inorganic compounds.

Specific benefits of derivative spectral analysis includes;

Even in small wavelength range, in presence of two or more overlapped peaks, absorbance bands can be identified.

In presence of strong and sharp absorbance peak, weak and small absorbance peak can be identified.

Broad absorbance spectrum gives clear idea about the particular wavelength at that maximum spectrum.

Even in presence of existed background absorption, the quantitative analysis can studied as there is linear relationship between the derivative values and the concentration levels

2.2 Materials

2.2.1 Devices and Equipment

- UV-VIS Spectrophotometer (UV-2550 SHIMADZU)
- Precision scale: Sartorius Entris 224-1S
- Magnetic stirrer: Ikamak RH Jonke & Kunkel IKA (Labortechnik)
- Magnet
- pH-meter (WTW Model)
- Quartz bathtub
- Pipette (1-10 mL)
- Sartorius Minisart disposable membrane filter (Pore diameter = 0.45 μm)
- Balloon Volumetric flask (10-50ml)

2.2.2Chemicals

- Methanol (Merck)
- Spironolactone
- Ultra pure water

2.2.3 Commercial pre-products of the analyzed compounds

ALDACTONE®-A 25 mg (Tablet) Servier:

Spironolactone : …… 25 mg

3.EXPERIMENTAL

3.1 Method

3.1.1 Preparation of Stock Solution

In order to analyse in spectrophotometer, stock solution was prepared by dissolving 5 mg spironolactone in 50 ml methanol. Then, 0.6 ml, 1.0 ml, 1.4 ml, 1.8 ml, 2.0 ml were taken from the stock solution and diluted to 10 ml methanol and prepared for calibration. For recovery, 0.6 ml, 1.4 ml, 2.0 ml were taken from stock solution and diluted to the 10 ml methanol.

3.1.2 Preparation of Standard Solution

Aldactone-A 25 mg tablet, the drug used for analysis in the spectrophotometer. The main ingredient it contains is spironolactone. Methanol was used to prepare a standard solution using spironolactone, whose solution was prepared at different concentrations. Aldactone contains 10 tablets. Each tablet contains 25 mg. 25mg contains 0.2649 g of spironolactone. In 1 tablet, i.e. 2.5 mg, 0.0265g was weighed on a precision scale for the experiment. A solution of 0.0265 g of aldactone, that is, spirinolactone, in 25 ml of methanol required for analysis was prepared in this way.

3.1.3 Preparation of the Calibration Set

The 5-solution set was prepared from stock solution for calibration. The 3-solution set was prepared for recovery. From the standard solution, 12 sets were prepared by the standard addition method.

3.1.4. Derivative Spectroscopy

Derivative spectrophotometry is based on the analysis of mixtures by taking the derivatives of the original absorption curves and obtaining calibration equations calculated by measuring the derivative absorbance value of the other compound against the zero cut-off point of one of the compounds. In spectrophotometric studies, derivative curves from one to four are used in quantitative studies. x being the independent variable and y being the dependent variable, a

function its derivative at any point is dx/dy. Absorption spectra promise the derivative will be dA/dλ. At each point of the spectrum derivative values are calculated and plotted as a function of wavelength. When the first derivative of the peak is taken ascending regions form positive peaks and descending regions form negative peaks, turning extrema occur at the wavelengths where the original derivative curve at wavelengths corresponding to the extrema in the peak passes through zero. The nth derivative versus a peak in the original spectrum n + 1 peaks occur in the spectrum, and as the degree of derivative increases, the peaks sharpens and narrows. Absorption in the original spectrum a wavelength in the 2nd derivative spectrum at the wavelength where the maximum minimum, a maximum appears in the 4th derivative spectrum, while the 1st and 3rd derivative spectra pass through zero at this wavelength. Derivative sharpening and narrowing of the peaks as the degree of separation increases. This is important for the illumination of spectral details is utilized, which is important for purity tests and diagnostics. Original shoulders found in the spectrum of odd-numbered derivative spectra extrema. This is due to the fact that the overlapping peaks is utilized in the analysis. Single order derivative spectra from this shoulder extremes are quantitative changes in both substances without the influence of the other to determine the nature of the artifacts. This is important in artifact analysis.

3.1.5. Standard Solutions

a) Stock solutions SPI were prepared in methanol with a concentration of 5 mg/50 mL.

b) Calibration solutions were prepared in the concentration range 0.6-20 µg/mL of SPI.

3.1.6. Calculation of Calibration Equations

In the application of the derivative method, the spectra of the calibration solutions prepared in the linear working range of 0.6-20 µg/mL for SPI in the wavelength range of 200-300 nm were taken and stored in the computer memory. Figure 3.1. shows the original absorption spectra. The derivative spectra of the absorption spectra of the analyzed SPI were calculated using smoothing functions with different Δλ ranges from one to four, different scale factors and different Δλ ranges. The same spectral procedures were performed for artificial and pharmaceutical preparations.

Table 3.1. Statistical results of linear regression analysis

Parameter	Zero-order spectrophotometry	First derivative spectrophotometry
m	0.0417	0.0515
n	0.0382	0.0381
r	0.9996	0.9997
SE(m)	7.13×10^{-4}	7.85×10^{-4}
SE(n)	1.04×10^{-2}	1.14×10^{-2}
SE(r)	8.17×10^{-3}	7.85×10^{-4}
LOD	0.75	0.66
LOQ	2.48	2.22

m: Slope of regression equation

n: Intercept of regression equation

r: Correlation coefficient

SE (m): Standard error of slope

SE (n): Standard error of intercept

SE (r): Standard error of correlation coefficient

LOD: Limit of detection (μg/mL)

LOQ: Limit of quantification (μg/mL)

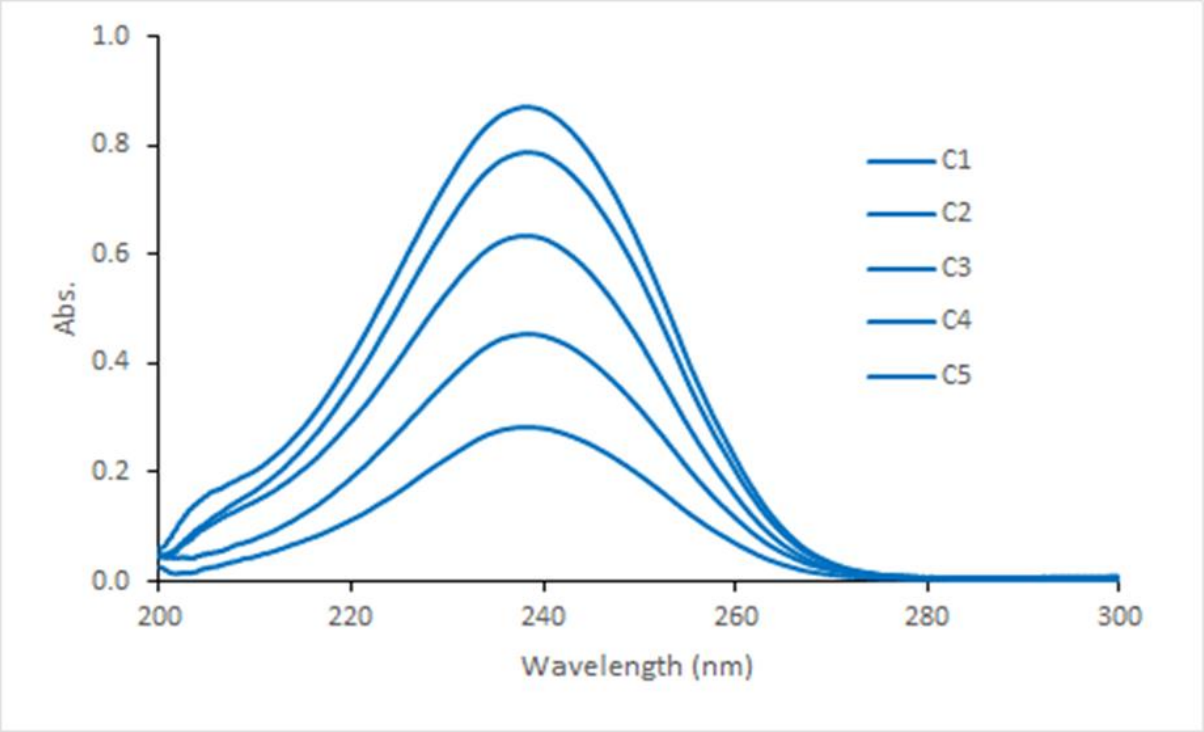

Fig 3.1 Original absorption spectra of SPI (0.6-20.0 µg/mL) in methanol.

After these experiments, it was decided that the first order derivatives of the absorption spectra of both compounds at Δλ=16 nm intervals and scale factor=10 conditions were suitable for analysis based on the high recovery values obtained. These first derivative spectra were smoothed at 24 points with Δλ=5 nm intervals.

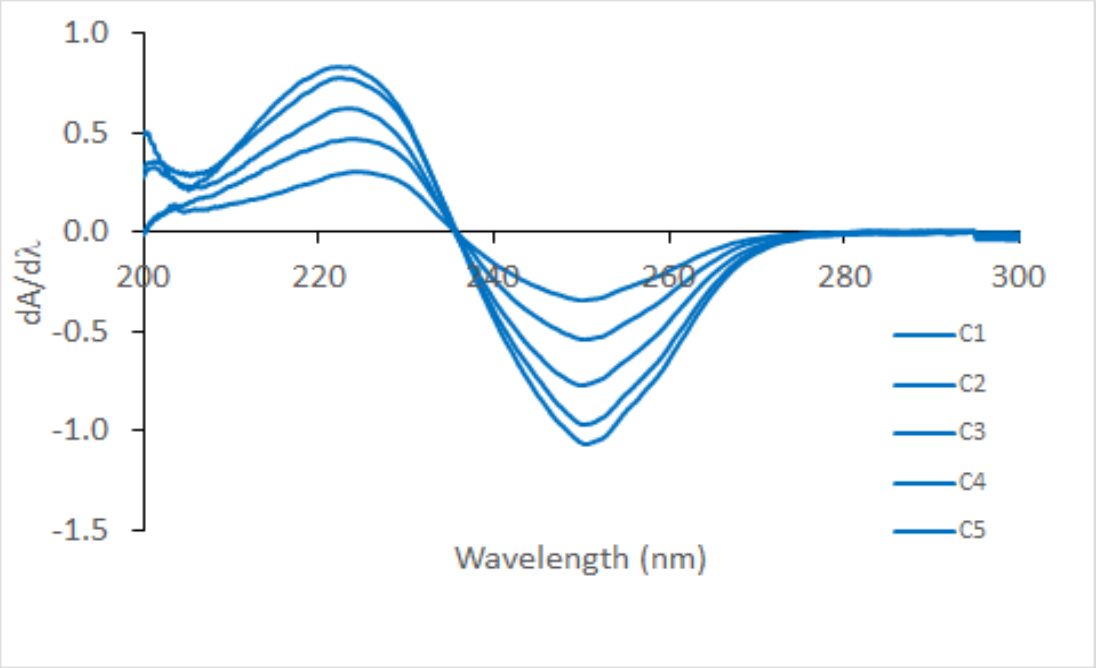

Fig 3.2. Fist derivative spectra of SPI (0.6-20.0 µg/mL) in methanol.

3.1.7. Validation of the Derivative Method

For the validation of the derivative method, a validation set consisting of 10 artificial mixture solutions in methanol at different concentrations within the linear working range for SPI. The precision and accuracy of the derivative method calibration established using this validation set was tested. The results obtained by applying the derivative method calibration method to the artificial mixtures are shown in Table 3.2.

Table 3.2. Recovery studies and results obtained by applying the methods to independent test samples

Experimental results were calculated from the average of three assays for each concentration level

Added	**Found (µg/mL)**		**Recovery (%)**	
(µg/mL)	**Zero-order spectrophotometry**	**First derivative spectrophotometry**	**Zero-order spectrophotometry**	**First derivative spectrophotometry**
	239 nm	250.4 nm		
6	5.85	5.94	97.5	99.0
14	14.11	14.06	100.8	100.4
20	19.98	19.99	99.9	99.9
		Mean	99.40	99.77
		SD	1.71	0.70
		RSD	1.72	0.71

SD: Standard deviation
RSD: Relative standard deviation

Before applying the direct spectrophotometric method and the derivative spectrophotometric method developed in this thesis to real commercial pharmaceutical preparations, the interference effects of tablet excipients were tested. For this, the standard addition technique was used. A solution in methanol of the contents corresponding to one tablet of the pharmaceutical preparation was prepared. After the necessary dilutions were made from this preparation solution, standards were added to the sample content according to the labeled amounts on the preparation from the stock solutions at 4.0, 8.0 and 12.0 µg/mL SPI. This procedure was applied to Aldactone® Tablet. At the end of the analysis, the amount of SPI from the preparation was deducted and recovery and other calculations were made for the SPI in the added standards. These experiments were performed in five replicates at three different concentration levels. The results obtained are presented in Table 3.3.

Table 3.3. Recovery results obtained by applying the methods to standard addition samples

	Added (µg/mL)	**Found (µg/mL)**		**Recovery (%)**		**RSD (%)**	
Sample	Pure SPI	**Zero-order spectrophotometry**	**First derivative spectrophotometry**	**Zero-order spectrophotometry**	**First derivative spectrophotometry**	**Zero-order spectrophotometry**	**First derivative spectrophotometry**
		239 nm	250.4 nm				
Tablet	4	3.98	3.86	99.5	96.5	0.96	0.84
Tablet	8	8.18	8.10	102.2	101.2	2.26	2.63
Tablet	12	12.09	12.03	100.7	100.2	1.11	1.05

RSD: Relative standard deviation
Tablet which contains spironolactone at a constant amount (approximately, 0.6 µg/mL)

3.2. Application of Derivative Method to Pharmaceutical Preparations

In the application of the comparison derivative method to pharmaceutical preparations, 20 tablets were accurately weighed and thoroughly powdered in a mortar and pestle and then the amount of tablets corresponding to one tablet was dissolved in a solution of methanol in a 50 mL balloonjug. The contents of the flask were stirred with a mechanical stirrer for 30 min and filtered through a membrane filter (Sartorius Minisart = 0.45 μm). Absorption spectra of these analysis solutions were plotted in the wavelength range of 200-300 nm. In the first derivative spectrum of the absorption spectra, SPI at 253 nm were calculated using the calibration functions. This process was repeated 10 times.

The tablet analysis procedure described above was applied to the Servier:preparation ALDACTONE®-A 25 mg (Tablet). The results of ALDACTONE®-A 25 mg (Tablet) Servier: are presented in Table 3.3.

Table 3.4. Determination results of spironolactone in tablet samples using Zero-order and First derivative spectrophotometry methods

Experiment Number	mg/ tablet	
	Zero-order spectrophotometry	**First derivative spectrophotometry**
1	24.87	24.91
2	25.09	25.23
3	24.65	24.66
4	24.73	24.83
5	24.77	24.97
Mean	24.82	24.92
SD	0.17	0.21
RSD	0.68	0.84

SD: Standard deviation
RSD: Relative standard deviation
Claimed label: 25 mg/per tablet

3.3. Statistical Analysis for Comparison of Methods

The t-test and F-test were applied to compare the means and variances of the results obtained by applying the developed direct spectrophotometric and derivative spectrophotometric methods to commercial pharmaceutical preparations. According to the t-test and F-test statistics, the calculated t-test value < t-table and the calculated F-test value < F-table, indicating that there was no significant difference between the methods. The t-test and F-test statistical analysis process in the comparison process is summarized in Table 3.5.

Table 3.5. Results of statistical analysis for SPI in the analysis of tablets

	SPI		
	Zero-order spectrophotometry		**First derivative spectrophotometry**
$\overline{X}$	24.82		24.92
SD	0.17		0.21
t-cal		0.91	
F-cal		0.66	

$\overline{X}$ = Mean
SD: Standard deviation
For t-test; critical value =2.306, $p < 0.05$
For F-test; critical value =0.700, $p < 0.05$

4.DISCUSSION

The development of methods for the determination of the active ingredient content of pharmaceutical formulations is of great importance for routine controls to be carried out after production as well as during the production phase. Spectrophotometric, HPLC and graphical methods are used in the analysis of pharmaceutical formulations. However, analysis by these methods requires a long preliminary preparation and the cost of the analysis is quite high. However, many pharmaceutical formulations can be analyzed in less time and at less cost by using signal processing methods.

In this master's thesis, zero-order spectrophotometric and first derivative spectrophotometric methods were developed for the quantification of SPI in an antihypertensive pharmaceutical preparation.

Calibrations were calculated by applying algorithms to original and derivative absorbance measurements for quantitative analysis of SPI in pharmaceutical preparation.

Optimal experimental and spectral conditions were determined for the application of developed calibration methods. The concentration set SPI and the original absorption spectra of the samples in the wavelength range of 200-300 nm were recorded.

The recovery results and statistical tests performed for the validation of the developed methods (Tables 3.1, 3.2, 3.3) showed that the methods have satisfactory accuracy and precision for SPI analysis with high recovery values and no influence of tablet excipients on the analysis. The developed methods were successfully applied to the quantification of SPI in tablets without any separation process, with highly satisfactory results.

The results of this thesis show that the developed direct spectrophotometric and derivative spectrophotometric methods can be used for routine analysis and quality control of commercial pharmaceutical preparations containing SPI.

5. CONCLUSIONS AND RECOMMENDATIONS

As it is known, methods such as chromatography and capillary electrophoresis, which use separation techniques that require expensive and complex devices, are tedious due to the difficulties in optimization of experimental conditions in analysis processes and long analysis times, and in some cases they do not give successful results, pushing analytical chemists to search for faster, more easily applicable and more economical methods.

The direct spectrophotometric and derivative spectrophotometric methods developed in this thesis make this thesis original for the analysis of SPI from pharmaceutical preparations because they are more easily applicable, fast and economical, and provide repeatable, precise, accurate and reliable results.

The direct spectrophotometric and derivative spectrophotometric methods developed within the scope of the thesis and successfully applied to the analysis of tablets containing SPI have yielded highly successful results. These calibration methods are extremely fast, easy to apply and economical in the analysis process.

These methods developed in this thesis for SPI quantification were successfully applied for the quantitative analysis of main compound. The developed methods have been optimized in this thesis make this thesis original in that they can be used for SPI analysis in further scientific research and industry.

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RESUME

1. **Educational Status**

Middle East Technical University Chemistry Department (2019)

2. **Titles**

3. **Professional Experience**

4. **Publications**