Chapter one

1. Introduction

1.1 Validation of analytical methods

Validation is defined as finding or testing the truth of something. The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose (Walash et al., 2013).

Analytical method validation is an important regulatory requirement in pharmaceutical analysis. Method validation provides documented evidence, and high degree of assurance that an analytical method employed for a specific test is suitable for intended use. Over recent years regulatory authorities have become increasingly aware of the necessity of ensuring that the data submitted have been acquired for marketing authorization using validated analytical methodology. The international conference on harmonization (ICH) has introduced guideline for analytical methods validation (ICH, 2005).

Regulatory analytical procedures are two types compendial and noncompendial. The non compendial analytical method procedures in the United States pharmacopeia (USP) are those legally recognized as regulatory procedure in food drug and cosmetic act. When used USP analytical methods, the guidance recommends that the information be provides for the following characteristics: specifity of the method, stability of analytical sample, and intermediate precision. Compendial analytical methods may not be stability indication, and this concern must be addressed when developing analytical procedure specification because formulation have interference may not be considered in the monograph specification. Additional analytical test for impurity may be necessary to support the qualities of the drug substance or drug product. Non compendial analytical

method must be fully validated. The most applied validation characteristics are: accuracy, precision (repeatability precision, intermediate precision and reproducibility), specificity, detection limit, quantitation limit, linearity and range of linearity.

The parameters that required for validation and approach adopted for each particular? are dependent on the type and applications of analytical method (ICH, 2005).

1.2 Types of validated analytical procedure:

There are three common types of analytical procedure that can be validated:

Identification tests are intended to ensure the purity of an analyte in a sample. This is normally achieved by comparison of a property of a sample (e.g. Spectrum, chromatogram behavior, chemical reactivity, etc) to that of reference standard.

Testing for impurities can be either a quantitative test or a limit test for the impurity in sample. Either test is intended to accurately reflect the purity characteristics of the sample. Different validation characteristics are required for a quantitative test than limit test.

Assay procedures are intended to measure the analyte present in a given sample. The assay represents a quantitative measurement of the major component(s) in the drug substance. For the drug product, similar validation characteristics also apply when assaying for the other active selected component(s). The same validation characteristics may also apply to assay associated with other analytical procedures as dissolution (Ermer and Miller, 2005).

1.3 Parameters of validation of analytical methods

1.3.1 Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degrades, matrix. The means of satisfying the criteria's of specificity differs for each types of analytical procedure. For identification, it would be proof of structure, where as in quality control, it is comparison to a reference substance. For purity test, to ensure that all analytical procedures allow an accurate statement of the content of the impurities of an analyte. For assay measurement to ensure that the signal measured comes only from the substance being analyzed. For example critical separations in chromatography should be investigated at an appropriate level. For critical separations, specificity demonstrated by the resolution of the two components which elute closest to each other. One practical approach to testing the specificity of analytical methods is to compare the response of sample containing impurities versus those not containing impurities. The basis of the test is the difference in results between the two types of sample. The assumption to this approach is that all the interference are known and available to the analyst for spiking studies. If the impurities or degradation product standards are unavailable, specificity may be demonstrated by comparing the test results of samples containing impurities or degradation products to second well-characterized procedure e.g. pharmacopoeia method or other validated analytical procedure (independent procedure). For the assay, the two results should be compared and for impurity tests profiles should be compared. Peak purity tests useful to show that the analyte chromatographic peak is not attributable to more than one component (British Pharmacopeia, 2014).

1.3.2 Linearity

Linearity of an analytical procedure is its ability to obtain test results which are directly proportional to the concentration of analyte in the sample. The function of the standard curve is to allow the prediction of a sample concentration interpolated from the standard and response signal. This predictive feature does not require linearity of the using response curve, but only that be reasonable description of the correlation between responses and concentration. The test method response curve is characterizes by comparing the goodness of fit of calculated concentration with actual concentration of standards. For linearity response, this value would be the correlation coefficient derived from a linear regression using least squares. In case of nonlinear curve, required curve fitting calculation with corresponding goodness-of-fit determination. Plotting the test results graphically as function of analyte concentration on appropriate graph paper may be an acceptable alternative to the regression line calculation (British Pharmacopeia, 2014).

According to ICH linearity can be established by minimum 5 concentrations. The regression line equation applied to results, should have an intercept not significantly from zero, if it does, it should be demonstrated that there is no effect on accuracy of analytical method (ICH, 2005).

1.3.3 Range

Range of analytical procedure is the interval between the upper and lower concentration of analyte in the sample for which it has been demonstrated that analytical procedure has a suability level of precision, accuracy and linearity. The specified range derived from linearity studies depends on the intended applications of the procedure, by conforming that the analytical procedure provides an

acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the rang of the analytical procedure. ICH recommended that minimum specified range for assay of drug substance or finish product from 80% to 120% of the test concentration and for content uniformity 70%to 130% of the test concentration based on the nature of the dosage form. In case of dissolution testing +/-20% over specified range. For impurity the reporting level of the impurity to 120% of the specification (Garcia et al., 2011).

1.3.4 Accuracy

It's the closeness of agreement between the values found from the analytical procedure to reference value. There are several methods for determination of analytical procedure, for assay of drug substance by comparison the results of the proposed analytical procedure with those of second well-characterized procedure. For assay of drug product by application of the analytical procedure to synthetic mixtures of the drug product component to which known quantities of the drug substance to be analyzed have been added, if it is impossible to obtain all the drug components, it may be acceptable either adding known amount of analyte to the drug product or to compare the results obtained from a second well characterized procedure (WHO, 2006).

ICH recommended that the accuracy can be done by using minimum of 9 determinations over minimum of 3 concentration levels covering the specified range. The accuracy reported as percent by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals (ICH, 2005).

1.3.5 Precision

It is the closeness of agreement between a series of the measurements obtained from the same homogeneous sample under the prescribed conditions. According to ICH precision classified into: repeatability, intermediate precision and reproducibility.

Validation of tests for assay and quantitative determination of impurities includes an investigation of precision. Precision investigation by using homogeneous samples, if it is not possible to obtain homogeneous sample, artificial prepared samples or sample solution should be used. Repeatability (intra assay procedure) expresses the precision under the same operating condition over short interval of time. It is assessed by using a minimum of nine determinations covering the specified range for the procedure (e.g., 3 concentrations/3replicates each). Intermediate precision defined as precision within-laboratories variation: different days, different analysts and different equipment. Reproducibility is the precision between laboratories. It is assessed by means of an inter-laboratory trial. The precision of analytical procedure expressed by the variance, standard deviation or coefficient of variation of a series of measurements (ICH, 2005).

1.3.6 Detection limit

Detection limit is the lowest content of the analyte in a sample which can be detected but not necessary quantitated as an expected value. There are many approaches to determination of the limit of detection possible, depending upon with the procedure is non instrumental or instrumental. Firstly, depending on visual evaluation and that for non-instrumental procedure the detection limit is determined by the analysis of the sample of known concentrations of the analyte and by established the minimum level at which the analyte can be reliable.

Secondly, based on signal-to-noise and that for the method that has baseline. Determination of the signal-to-noise ratio is performed by comparing the measured signal of the samples with known low concentrations of analyte with those of blank sample and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable as detection limit. Based on the standard deviation of the response and the slope:

$$DL = \frac{3.3\sigma}{S}$$

Where σ= the standard deviation of the response.

S= the slope of the calibration curve

1.3.7 Quantitation limit

The lowest amount of the analyte in the sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of the quantitative assays for low levels of compounds in sample matrices, and particularly for determination of impurities and/or degradation products. Quantitation limit for analytical method can be determined based on standard deviation of the response and slope expressed as (QL):

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

Where σ= the standard deviation of the response.

S= the slope of the calibration curve.

There are different methods for carry out the standard deviation;

Based on standard deviation of the blank by measuring the magnitude of the analytical background response is performed by analyzing an appropriate number of the blank samples and calculating the standard deviation of these responses. Based on the calibration curve using samples containing an analyte in the range of QL. The residual standard deviation of a regression lines may be used as the standard deviation (Ermer and Miller, 2005).

1.3.8 Robustness

Robustness of analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in the method parameters and provides an indication of its reliability during normal usage. The analytical conditions should be suitable for control or a precautionary statement should be included in the procedure. The goal of that is to ensure that the validity of the analytical procedure is maintained wherever used. For example in case of liquid chromatography the variation by influence of variation of pH in the mobile phase or changing in the composition, using different columns and variation in temperature or flow rate (WHO, 2006).

1.3.9 System suitability testing

System suitability testing is to see if the operating system is performing properly or not. As in case of HPLC, an acceptable approach is to prepare a solution containing the analyte and a suitable test compound. If the method being used is to control the level of impurities, the minimum resolution between the active component and the most difficult to resolve impurities should be given (ICH, 2005).

1.4 Spironolactone

Spironolactone is 7α-acetyle thio-3-oxo-17α-pregn-4-ene-21,17β-carbolactone. Its molecular formula is C_{24} $H_{32}O_4S$ having a molecular weight 416.58 gm/mole. It has following structure:

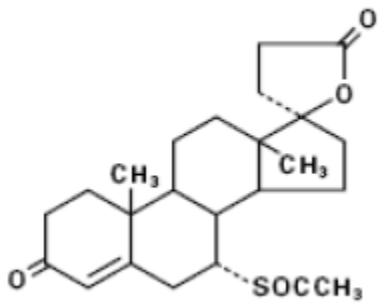

Figure (1.1): ***7α-acetyle thio-3-oxo-17α-pregn-4-ene-21,17β- carbolactone***

Spironolactone is light, cream-colored to light-tan crystalline powder, melting point 198-202, optical rotation -33.5 degree (chloroform) and it's practically insoluble in water, soluble in alcohol, and freely soluble in benzene and chloroform. Spironolactone (aldactone) is potassium -aspiring diuretic agent and has half-life of about 16 hour. Diuretics agents are drugs that increase renal excretion of water and solutes (mainly sodium salt). The main function of diuretics therapy are to decrease fluid volume of the body and to adjust the water and electrolyte balance. On its own, spironolactone is only a weak diuretic, but it can be combined with other diuretics (Laxman et al., 2010).

1.4.1 Preparation of spironolactone

Spironolactone is prepared by treating dehydroepiandrosterone (prepared from cholesterol or sitosterol) with acetylene to form the 17α-ethynyl-17β-hydroxy derivative, which is carbonated to the 17α-propiolic acid. Reduction of the

unsaturated acid in alkaline solution yields the saturated acid, which cyclizes to the lactone on acidification. Bromination to the 5, 6-dibromo compound, followed by oxidation of 3-hydroxyl group to the ketone and dehydrobromination to the 7α-hydroxyl derivative, produces spironolactone when esterified with thiolacetic acid.

1.4.2 Pharmacology of spironolactone

1.4.2.1 Pharmacodynamics

Spironolactone is antagonist of aldosterone acting through competitive binding of receptor at the aldosterone-dependent sodium- potassium exchange site in the distal convoluted renal tubule. It causes increased amounts of water and sodium to be excreted, while potassium is retained. It is used as diuretic and an antihypertensive agent and it used with other diuretics agents that acts more proximally in the renal tubule. Spironolactone provides effective therapy for edema and ascites through competing of spirononlactone with receptor sites. It is also effective in lowering the systolic and diastolic blood pressure in patient with primary hyperaldosteronism. Aldoctone inhibits the exchange of sodium for potassium distal renal tubule to help to prevent potassium loss. It has moderate anti-androgenic activity in humans by inhabitation of the interaction between dihydrotesterone and intracellular androgen receptor. It also inhabit several steps in ovarinsteroidogenesis resulting in lowered plasma levels of testosterone and other weak androgenic steroids. Through this activity spironolactone is effective in treatment of femalehirsutism (Sweetman, 2009).

1.4.2.2 Pharmacokinetics

The bioavailability of spironolactone in the human from early administered aldactone tablets exceed 90% when compared with an optimally –absorbed solution “spironolactone in polyethylene glycol 400”. Food may increase the

bioavailability of spironolactone. Spironolactone is rapidly and extensively metabolized. Approximately 25% to 30% of the dose admistered is converted to canrenone. Sulfur containing product are predominant metabolites and together with the spironolactone are thought to be primarily responsible for therapeutic effect of the drug. Canrenone attains plasma peak serum level at 2 to 4 hours followed by single oral administration. Canrenone plasma concentration declines in two distinct phases, being rapid in the first 12 hours and slower from 12 to 96 hours. The log-linear phase half-life of canrenone, following multiple doses of spironolactone, is between 13 and 24 hours. Both spironolactone and canrenoone are more than 90% bounded to plasma proteins.The metabolites of spironolactone are excreted primarily in urine, but also in bile (Sweetman, 2009).

1.4.3 Analysis of spironolactone

There are several international pharmacopoeias specify infrared and ultraviolet absorption spectrophotometry with comparison to standards and thin-layer chromatography as the methods for identifying spironolactone; ultraviolet absorption spectrophotometry and high-performance liquid chromatography (HPLC) with ultraviolet detection are used to assay its purity

The literature reviews regarding spironolactone suggest that various analytical methods were reported for its determination as drug in pharmaceutical formulation and in various biological fluids.

Tang et al. (2001) have developed nondestructive quantitative analytical method of spironolactone pharmaceutical powder by artificial neural network and near-infrared spectrometry. An artificial neural network model to predict unknown samples was based on the data from near- IR diffuse reflectance spectra of spironolactone pharmaceutical powders.

Mahrous et al. (1981) have developed colorimetric methods for the determination of spironolactone in tablets. Three different methods were developed for the colorimetric determination of spironolactone, either in its pure form or in tablets. The methods utilize either the alkaline picrate reagent, isonicotinic acid hydrazine solution or hydroxamic acid formation for the evaluation of spironolactone. The sensitivity of the proposed procedures was discussed and the results obtained were compared with the pharmacopeial methods.

Moussa and El-Kousy. (1985) have developed colorimetric method of some diuretic drugs: hydrochlorothiazide and spironolactone. Hydrochlorothiazide and spironolactone were determined in bulk and tablets by colorimetry. Hydrochlorothiazide reacted with $BuNH_2$ and $CoCl_2$ in anhydride giving a blue-violet color. The absorbance at 570 nm of 0.2-1.12 mg/ml hydrochlorothiazide obeyed Beer's law. Spironolactone determination was dependent on the reaction with isoniazid. The absorbance was measured at 450 nm. Beer's law was obeyed over the concentration range 0.004-0.020 mg/ml.

Shingbal and Rao. (1986) have developed colorimetric method for spironolactone and its dosage forms. Spironolactone was determined in tablets by a colorimetric method based on spironolactone dissolution in EtOH and mixing of an aliquot of this solution with p-nitrobenzene and sodium hydroxide solution and measured of the yellow solution at 400 nm. The color was stable for 6 hour at room temperature. Common tablet excipients did not interfere in the determination. The recovery was 98.8-99.76%.

Nevrekar (1984) has developed spectrophotometric estimation of spironolactone. Spironolactone was determined in tablets by dissolving in EtOH, diluted with MeOH, mixing with 2, 3, 5-triphenyltetrazolium chloride and Me_4NOH in MeOH,

heating the solution at 45 ºC for 55 min, and measuring the absorbance at 480 nm. Beer's law held for 0.01-0.07 mg/ml, and recoveries were 99.9-100.1%.

Shingbal and Prabhudesai (1984) have developed a spectrophotometric estimation method for spironolactone in pharmaceutical dosage form, based on reaction with isoniazid in $CHCl_3$ and MeOH in acid medium and measuring the absorbance of the resulting yellow solution at 375 nm. Beer's law was obeyed in the concentration range 0.005-0.040 mg/ml. the standard deviation was 0.613-0.652 and recovery 99%.

Parimoo et al. (1995) have developed a method for simultaneous determination of spironolactone with Hydroflumethiazide and spironolactone with Frusemide in combination formulation by UV absorption method. The λ max for spironolactone was found at 238 nm, for Hydroflumethiazide, at 273 nm, and for Frusemide at 276 nm. There were no interferences in their estimations.

Salem et al. (1991) have developed UV and UV derivative spectrophotometric determination of two-component mixtures. Two-component mixtures (spironolactone with hydrochlorthiazide, captopril with hydrochlothiazide, and spironolactone with furosemide) were assayed by UV (A), first derivative ($dA/d\lambda$) and second derivative ($d^2A/d\lambda^2$) spectrophotometric methods, applying "Zero-Crossing" technique of measurement. The methods were proved using laboratory prepared mixture. The utility of the methods in assay of dosage forms is also presented.

Dyade and Sharma (2001) have developed two methods for simultaneous spectrophotometric determination of spironolactone and hydroflumethiazide in tablet formulations. The methods were multi-wavelength spectroscopy and isoabsorptive point method. Spironolactone and hydroflumethiazide had absorption

maxima at 238 and 273 nm, respectively in MeOH. Both the drugs obeyed Beer's law in the concentration ranges employed for these methods.

Hong and Guang et al**. (**1999) have developed a method for determination of spironolactone by micellar enhanced spectrofluorimetry. In the presence of sodium dodecyl sulfate (SDS), the detection limit is $1.2 * 10^{-9}$mol/L, the linear range of $1.4*10^{-9} – 4.3*10^{-6}$mol/L is obtained. This method was successfully applied to the determination of spironolactone in tablets with satisfactory results.

Hardik and Sagar (2012) have developed a spectrophotometric method for simultaneous estimation of Furosemide and Spironolactone in combined tablet form. First order derivative spectroscopic method and absorbance ratio method were used. The amplitude at 350 nm in the first order derivative spectra were selected to determine Furosemide and Spironolactone, respectively and wavelength ranges 261.21 nm (iso-absorptive point) and 276 (λ max for Furosemide) were selected for absorbance ratio method. Beer's law obeyed in concentration ranges of 0.002-0.010 mg/ml and 0.005-0.025 mg/ml for furosemide and spironolactone for derivative method as well as absorbance ratio method. Recovery was found in the range 98.25%-102% for Furosemide and 98.8-100.9% for Spironolactone by first order derivative spectroscopic method and 99.4-102% for Furosemide and 98.25-100.55% for Spironolactone by absorbance ratio method.

Chaudhary et al. (2013) have developed simultaneous estimation of Metolazone and spironolactone Ratio Drevative spectrophotometric method based on resolving spectra overlapping of Metrolazone and Spironolactone by making use of the first-derivative of the ratios of their direct absorption on spectra. Methanol: water (1:4) was used as solvent for the method. Wavelength at 241.0 nm and 263.0 nm were selected in the ratio derivative spectra to determine Metolazone and Spirnolactone respectively. Beer's law was obeyed in the concentration ranges of 0.001-0.005

mg/ml and 0.005-0.025 mg/ml Metolazone and Spironolactone, respectively, in the method. Recovery was found in the range of 99.74-100.76% for Metolazone and spironolactone in the formulations.

Laxman et al. (2010) have developed RP-HPLC and spectrophotometric methods for simultaneous determination of spironolactone and Torsemide in pharmaceutical dosage form. Chromatographic analysis was carried out by isocratic technique on a reversed-phase C-18 Inertsil (250 mm * 4.60 mm) 5 micro column with mobile phase composed of methanol: water (80: 20). The UV spectrophotometric determinations (dualwavelength) were performed at 210 and 268 nm for spironolactone and 243 and 330.5 nm for torsemide. The recovery ranges were found from 98.12% and 101% for both drugs and RSD was < 2%.

El-Shahawi et al. (2013) have developed a new analytical method for spironolactone residues in industrial waste water and in drug formulations by cathodic stripping voltammetry. The method was successfully applied for the analysis of Spironolactone residues in industrial waste water. In pure form (98.2+/- 3.1%) and in drug formulations, e.g., Aldactone tablet (98.36 +/- 2.9%). The method was validated by comparison with HPLC and the official data methods.

Walash et al**. (**2013) have developed HPLC method for simultaneous determination of metolazone and spironolactone in raw materials, combined tablets and human urine. Good chromatographic separation was achieved within 5.0 min on 150 mm * 4.6 mm i.d., 5 micro m particle size Spherisorb-ODS 2 C-18 column. A mobile phase containing a mixure of methanol and 0.02 M phosphate buffer (70: 30) v/v at pH 3.0 was used. The analysis was performed at a flow rate of 1 ml/ min with UV detection at 235nm. The exipamide (XPM) was used as an internal standard (IS). The mean percentage recoveries of metolazone and spironolactone in spiked human urine were 99.33 +/- 2.37 % and 99.72 +/- 3.27 %, respectively. The

linearity of the method was found over the range 0.05 – 1.0 micro/ ml and 0.5-10.0 micro/ ml for metolazone and spironolactone, respectively.

1.4.4 The objectives of the proposed work

1- To develop simple UV method for determination of spironolactone in raw material and dosage form.
2- To develop simple HPLC method for determination of spironolactone in raw material and dosage form.
3- To validate the developed methods.
4- To compare HPLC and UV methods of analysis of spironolactone.

Chapter Two

2. Materials and methods

2.1 Chemicals and Reagents

- Spironolactone working standard (purity 99.4%) (Zheg and Xinhua pharmaceutical).
- Methanol, Acetonitrile HPLC GRADE reagents (Duksan).
- Dichloromethane pure, Sodium hydroxide pellets purified, Orthophosphoric acid, Potassium dihydrogen orthophosphate anhydrous A.R (CDH).
- Tetrahydrofuran, Chloroform, (ROMIL pure chemical).
- Hydrazinehydrogensulphate A.R (bening huagong chang).
- Hydroxyl amine hydrochloride A.R (Loba chemide).
- Ethanol absolute A.R (Chemical lab).

2.2 Instruments:

- UV-VIS recording spectrophotometer (UV-2201) Shimadzu, equibed with 1cm quartz cell.
- HPLC SHIMADZU CORP 03137 serial number LC202544:
  - Model of pump: LC-20Ab.
  - Model of detector: SPD-20A.
  - Model of oven: CTO – 20 AC.
  - Software: LC- Solution.
- PH/Temperature Bench Meter Mi 150 (Milwaukee).
- Analytical Balance (keranabs).

2.3 Determination of the wavelength of maximum absorption (λ_{max}) of spironolactone

2.3.1 Effect of pH on λ_{max} of spironolactone

Standard stock solution was prepared by weighed 25 mg of spironolactone and transferred to 100-ml volumetric flask by methanol, and put in ultrasonic bath for 30 sec for dissolution and completed the volumetric mark by methanol.

6.08020 g of potassium dihydrogen phosphate were weighed and dissolved in 200ml of purified water. To this solution, 200 ml of 0.2 M sodium hydroxide was added. The mixture was transferred to 1000-ml volumetric flask and completed to the mark by purified water to prepare pH 7.33 buffer solution. From this buffer solution a series of pH's were prepared (pH 2 to pH 12) by adjusting the resulting pH by 0.2 M sodium hydroxide or by orthophosphoric acid.

2ml of standard stock solution was transferred to 50-ml volumetric flask and completed to the mark by buffer solution (pH 2-12). The solution was filtered and scanned on UV visible spectrophotometer.

2.3.2 Effect of solvent on λ_{max} of spironolactone

25 mg of spironolactone working standard were weighed and transferred quantitatively by solvent (ethanol, chloroform, dichloromethane, acetonitrile and methanol) to 100-ml volumetric flask and completed to the mark with the solvent. 2 ml of solution was transferred to 50-ml volumetric flask and completed the volume to the mark by solvent and scanned on UV-Visible spectrophotometer.

2.3.3 Effect of derivatization on (λ_{max}) of spironolactone

2.3.3.1 Derivatization of spironolactone with hydroxylamine hydrochloride

0.1 mmole of spironolactone were weighed and dissolved by minimum amount of absolute ethanol in 50-ml volumetric flask. In test tube 0.1061 mmole of sodium acetate were weighed and dissolved by a few drops of purified water (solution 1).

In another test tube 0.11 mmole of hydroxylamine hydrochloride were weighed and dissolved with a few drops of water (solution 2). The solution 1 and solution 2 were added respectively to the volumetric flask with continuous shaking. The contents of the volumetric flask were put in water path (88 ºC-90 ºC) for 15 min with shaking. The solution was cooled in room temperature and completed to the mark by absolute ethanol.

2.3.3.2 Derivatization of spironolactone with hydrazine hydrogensulphate

0.0984 mmole of spironolactone were weighed and dissolved by minimum amount of absolute ethanol in 50-ml volumetric flask. In test tube 0.1 mmole of sodium acetate were weighed and dissolved by a few drops of purified water (solution 1). In another test tube 0.1062 mmole of hydrazinehydrogensulphate were weighed and dissolved with a few drops of water (solution 2). The solution 1 and solution 2 were added respectively to the volumetric flask with continuous shaking. The contents of the volumetric flask were put in water path (88 ºC-90 ºC) for 15 min with shaking. The solution was cooled in room temperature and completed to the mark by absolute ethanol.

2.4 Development and validation methods of spironolactone

2.4.1 Spectrophotometric analysis methods

2.4.1.1 Derivatization by Hydroxylamine

2.4.1.1.1. Method development

2.4.1.1.1.2 Selection of maximum absorption wavelength (λ_{max})

0.1 mmole of spironolactone were weighed and dissolved by minimum amount of absolute ethanol in 50-ml volumetric flask. In a test tube 0.1061 mmole of sodium acetate were weighed and dissolved by a few drops of purified water (solution 1).

In another test tube 0.11 mmole of hydroxylaminehydrochloride were weighed and dissolved with a few drops of water (solution 2). The solution 1 and solution 2 were added, respectively, to the volumetric flask with continuous shaking. The contents of the volumetric flask were put in water path (88 ºC-90 ºC) for 15 min with shaking. The solution was cooled in room temperature and completed to the mark by absolute ethanol. The maximum absorption wavelength was found to be 263.7 nm.

2.4.1.1.1.3 Preparation of standard solution

25.1 mg of spironolactone working standard was dissolved in 50-ml volumetric flask by absolute ethanol. 10 ml of sodium acetate solution (1mg/ml) and 4 ml of hydroxylamine hydrochloride solution (1mg/ml) were added, respectively, and shacked. The contents of the volumetric flask were put in water path (88-90 ^{0}C) for 15 min then cooled at room temperature and completed to the mark by absolute ethanol.

2.4.1.1.1.4 Preparation of the sample for assay

Equivalent to 25.0 mg of spironolactone were weighted from twenty crashed tablets powder. Then dissolved in 50-ml volumetric flask by absolute ethanol and treated in ultrasonic bath for 10 min. 10 ml of sodium acetate solution (1mg/ml) and 4 ml of hydroxyl amine hydrochloride solution (1mg/ml) were added, respectively. The contents of volumetric flask were put in water path (88 ºC-90 ^{0}C) for 15 min then cooled at room temperature and completed to the mark by absolute ethanol. 1.25-ml of filtrated solution were transferred to 25-ml volumetric flask and completed to the mark by ethanol absolute.

2.4.1.1.2 Method validation

The method was validated for linearity, accuracy, repeatability and intermediate precision, LOD and LOQ, in accordance with ICH guidelines.

2.4.1.2 Derivatization by Hydrazine

2.4.1.2.1 Method development

2.4.1.2.1.1 Selection of wavelength of maximum absorption

0.0984 mmole of spironolactone were weighed and dissolved by minimum amount of absolute ethanol in 50-ml volumetric flask. In a test tube, 0.1 mmole of sodium acetate were weighed and dissolved by a few drops of purified water (solution 1). In another test tube, 0.1062 mmole of hydrazine hydrogensulphate were weighed and dissolved with a few drops of water (solution 2). The solution 1 and solution 2 were added, respectively, to the volumetric flask with continuous shaking. The contents of the volumetric flask were put in water path (88 ºC-90 ºC) for 15 min with shaking. The solution was cooled in room temperature and completed to the mark by absolute ethanol. The maximum absorption wavelength was found to be 251.5 nm.

2.4.1.2.1.2 Preparation of standard solution

25.6 mg of spironolactone working standard was dissolved in 50-ml volumetric flask by absolute ethanol. 9 ml of sodium acetate solution (1mg/ml) and 8 ml of hydrazine hydrogen sulfate solution (1mg/ml) were added, respectively, and shacked. The contents of volumetric flask were putted in water path (88 ºC-90 ^{0}C) for 15 min then cooled at room temperature and completed to the mark by absolute ethanol.

2.4.1.2.1.3 Preparation of the sample for assay

Equivalent to 25.0 mg of spironolactone were weighed from twenty crashed tablets powder. Then dissolved in 50-ml volumetric flask by absolute ethanol and treated in ultrasonic bath for 10 min. 9 ml of sodium acetate solution (1mg/ml) and 8 ml of hydrazine hydrogen sulfate solution (1mg/ml) were added, respectively, and shacked. The contents of volumetric flask were put in water path (88 ^{o}C-90 ^{0}C) for 15 min then cooled at room temperature and completed to the mark by ethanol absolute. 1.25 ml of filtrated solution were transferred to 25-ml volumetric flask and completed to the mark by absolute ethanol.

2.4.1.2.2. Method validation

The methods was validated for linearity, accuracy, repeatability and intermediate precision, LOD and LOQ, in accordance with ICH guidelines.

2.4.2 High performance liquid chromatography (HPLC) method

2.4.2.1 Method development

2.4.2.1.1 Preparation of mobile phase

6.8 g of potassium dihydrogen phosphate were transferred to 1000 ml volumetric flask and dissolved by purified water and completed to the mark by purified water. PH was adjusted to pH 4 with orthophosphoric acid. Acetonitrile and buffer solution were mixed in the ratio of 1:1.

2.4.2.1.2 Selection of wavelength

20.6 mg of spironolactone working standard was dissolved in 100-ml volumetric flask by mobile phase. Sonication for 30 second and completed to the mark by mobile phase. Solution was diluted by pipetting 5ml into 100-ml volumetric flask and completed to the mark.

2.4.2.1.3 Preparation of standard stock solution

25.0 mg of spironolactone working standard (99.4% purity) were dissolved in 100-ml volumetric flask by mobile phase. Sonication for 30 second then completed to the mark by mobile phase.

2.4.2.1.4 Preparation of the sample for assay

Equivalent to 25 mg of spironlactone from 20 crushed tablet powder of spironlaction were weighted and dissolved in 100 ml volumetric flask by mobile phase. Sonication for 20 minutes then completed to the mark by mobile phase. 2.5 ml of solution were transferred to 25 ml volumetric flask, then completed to the mark by mobile phase.

2.4.2.1.5 Chromatographic conditions

Column: C18 (packing matter size 5um, dimension 4.6 *150mm, S/N 3LR97090).

Temperature: 40 ºC.

Flow rate: 1.5ml/minute.

Volume injection: 20µl.

Wave length: 240nm.

2.4.2.2 Method validation

The methods were validated for specificity, linearity, accuracy, repeatability and intermediate precision, LOD and LOQ, in accordance with ICH guidelines.

2.4.2.2.1 Specificity

The Specificity was performed by using, 100% standard solution and reported as average and RSD%.

2.4.2.2.2 Linearity

Aliquots of 2.00 ml, 2.25 ml, 2.50 ml, 2.75 ml and 3.00 ml standard solution of spironolactone from stock solution were transferred to series of 25 ml volumetric flask and made up to volume with mobile phase to obtain 20.0, 22.5, 25.0, 27.5 and 30.0 μg/ml (range from 80 to 120%).

2.4.2.2.3 Precision

The precision of the assay methods by repeatability and intermediate precision using, 100% standard solution and reported as average and RSD%

2.4.2.2.4 Recovery

Recovery studies were performed at three levels (80, 100 and 120 %) and the recovery of spironolactone calculated. Recoveries were carried out by preparating the above concentrations from stock standard solution and added to analyzed sample.

2.4.2.2.4 Limit of detection (LOD) and limit of quantification (LOQ)

They were calculated as 3.3 σ/S and 10σ/S, respectively. Where σ is standard deviation of the response and S is the slope of calibration curve.

From regaration equation:-

$$LOD = 3.3\sigma/S$$

$$LOQ = 10\ \sigma/S$$

σ = standard deviations of the response (y intercept)

S = mean of the slope of the calibration plot.

Chapter Three

3. Results and Discussion

3.1 Development and validation methods of spironolactone

In the present work spectrophotometric and high performance liquid chromatography (HPLC) analytical methods were developed and validated as assay methods for determination of spironolactone in raw materials and tablet dosage form.

3.1.1 Selection of wave length of maximum absorption

Table (3.1): The effect of pH on λ_{max}

pH	Absorbance	Wavelength of maximum absorption (nm)		
		Zero order	First order derivative	Second order derivative
2	0.295	245.5	-	-
3	0.330	244.2	-	-
4	0.345	244.2	238.1	239.2
5	0.338	245.3	-	-
6	0.312	244.2	-	-
7	0.333	245.5	238.1	241.5
8	0.318	244.2	-	-
9	0.312	245.5	242.7	242.7
10	0.288	247.9	-	-
11	0.310 1.369	247.9 207.0	-	-
12	0.281 0.215	247.9 293.4	-	-
Methanol+ SPI*	0.381	240.5	238.1	240.5
Methanol+SPI*+drop of NaOH	0.339 0.534	240.5 209.8	240.4	241.5
Methanol+SPI*+drop of HCl	0.410	240.4	238.1	239.2

*SPI for spironolactone

In developing of spectrophotometric and HPLC analytical methods different buffer solutions and solvents were used to select the suitable solvent for determination of spironolactone. The selection of solvent was based on solubility, shape of spectrum and cost of chemicals. Table (3.1) shows that the pH does not have large effect on maximum absorption wavelength in zero order, first and second derivative due to the neutral nature of structure of spironolactone. In scanned spectrum at pH 11, 12 and (methanol + SPI+ drop of 1M NaOH), two peaks were raised and that might be due to the absorption of buffer solution or degrading components from spironolactone.

In Table (3.2), where different solvents are used, shows that the best solvents were dichloromethane giving a maximum absorbance of 0.889 at wavelength 236.8 nm and acetonitrile, 0.818 at the same wavelength, ethanol solvent, however, has absorbance 0.778 at same wavelength.

Table (3.2): Effect of solvents on λ_{max}

Solvent	The wavelength of maximum absorption (nm)	Absorbance
Methanol	240.5	0.790
Dichloromethane	236.8	0.889
Ethanol	236.8	0.778
Acetone	214.7	0.059
Acetonitrile	236.8	0.818
Chloroform	244.2	0.713
Tetrahydrofuran	240.5	0.737

From previously mentioned information, the ethanol was chosen as a solvent for UV spectrophotometric methods when the shiff base was used that gives good yield in ethanol. Acetonitrile solvent and buffer solution of pH 4 in ratio 1:1 were chosen as mobile phase and diluent for HPLC method because they also give maximum absorption and good resolution peak in HPLC chromatogram.

In the spectrophotometric analysis two methods were developed and validated for assay analysis of spironolactone in tablet dosage form, which includes derivatization by hydrazine and derivatization by hydroxylamine. The wavelength of spironolactone in ethanol was found to be 236.5 nm and detection wavelength of spironolactone in hydroxylamine and hydrazine derivatization agents were found to be 263.7 nm and 251.5 nm, respectively. The shift in wavelength indicates that a complex, between spironolactone with hydroxylamine and spironolactone with hydrazine was formed due to new oxochrome (HO-N=C-R in the complex of spironolactone with hydroxylamine and H_2N-N=C-R in the complex of spironolactone with hydrazine).

Table (3.3): The effect of derivative on spironolactone on (λ_{max}):

		Absorbance	The wavelength of maximum absorption (nm)		
			Zero order	First order derivative	Second order derivative
Ethanol + SPI		0.778	236.8	-	-
Dervatizatin of SPI with	Hydrazine	3.241	251.5	249.6	245.0
	Hydroxyl Amine	3.172	263.6	236.9	246.9

The effect of derivatization of spironolactone with hydrazine and hydroxylamine is shown in Table (3.3).

3.1.2 Spectrophotometric analysis methods

3.1.2.1 Devatization by Hydrazine

3.1.2.1.1 Method development

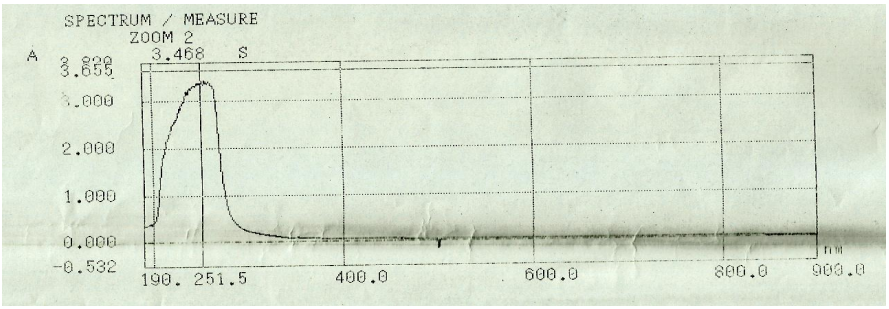

Figure (3.1): UV spectrum of derivatization by hydrazine

3.1.2.1.2 Method validation

All the validation parameters were done according to ICHQ2 (R1) guideline.

For linearity five concentrations of standard in range of 20 ppm to 30 ppm and their absorbance's 251.5 nm wavelength were recorded in Table (3.5).

Table (3.4): Calibration curve of spironolactone in method of Hydrazine:

Concentration ppm	Absorbance at 251. 5 nm	SD of the absorbance
20	0.045	0.001000
22.5	0.133	0.000577
25.0	0.217	0.000577
27.5	0.282	0.000577
30	0.356	0.000577
Average of standard deviation (SD)		0.000662

The R^2 value shown in Figure (3.2) indicate that the method was linear.

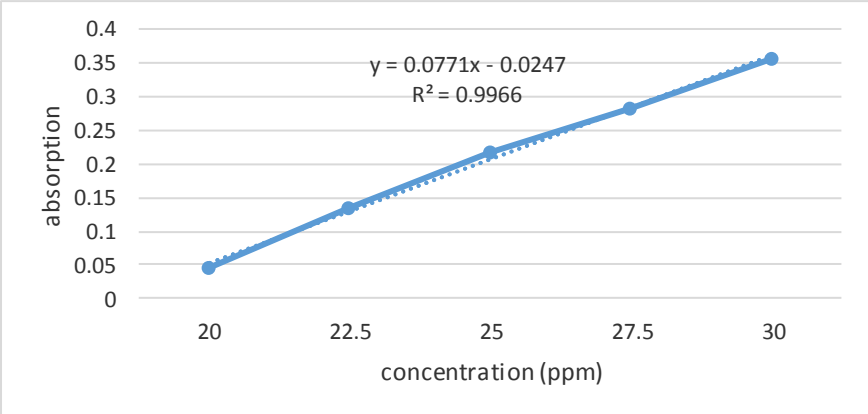

Figure (3.2): Calibration curve of spironolactone in derivatization by Hydrazine

Table (3.5): Recovery study of spironolactone in derivatization by Hydrazine

Level of the method %	Amount taken (µg/ml)	Amount added (µg/ml)	Total theoretical amount found (µg/ml)	Amount found (µg/ml)	Recovery %	Average of recovery %	Relative standard deviation %
80	25	20	45	46.08	102.41		
80	25	20	45	46.21	102.68	102.50	0.15362
80	25	20	45	46.08	102.41		
100	25	25	50	51.36	102.72		
100	25	25	50	51.31	102.61	102.65	0.06183
100	25	25	50	51.31	102.61		
120	25	30	55	55.08	100.14		
120	25	30	55	55.08	100.14	100.20	0.1052
120	25	30	55	55.18	100.32		

The recovery was done by using standard addition method and found in the range (100.2 – 102.5) % in concentration range 80% - 120% and that reflect that the inactive material does not interfere in analysis of spironolactone as mentioned in Table (3.5).

The intermediate precision was done by using 100% concentration solution The RSD % of intermediate precession of three analysts were found to be in the range (0.26-0.27) % (Table 3.6).

Table (3.6): intermediate precision of spironolactone in derivatization by Hydrazine**:**

Run	ABS of 1st analyst	ABS of 2st analyst	ABS of 3st analyst
1	0.219	0.226	0.211
2	0.22	0.227	0.211
3	0.219	0.226	0.210
Mean	0.2193	0.2253	0.2107
RSD %	0.26323%	0.25509%	0.27406%

The repeatability precision was done by using 100% concentration solution and RSD% were found to be 0.34% (Table 3.7).

Table (3.7): repeatability precision of the system of spironolactone in derivatization by Hydrazine:

Run	1	2	3	4	5	6	Mean	RSD%
Absorption	0.221	0.222	0.220	0.221	0.221	0.220	0.2208	0.3409

The RSD % values of intermediate precision and repeatability showed that the developed spectrophotometric method for assay of spironolactone by derivatization by hydrazine was precise according to ICH Q2 R1.

Limit of detection and limit of quantitation of the method were determined by the following equation:

$$LOD = 3.3\sigma/S$$

$$LOQ = 10\ \sigma/S$$

σ = standard deviations of the response

S = mean of the slope of the calibration plot.

$$LOD = 3.3 * 0.000551/0.0067 = 0.271321 \text{ ppm}$$

$$LOQ = 10 * 0.000551/0.0067 = 0.822185 \text{ppm}$$

The assay of spironolactone in tablet dosage form was found 97.15% by UV method of derivatization by Hydrazine (Table 3.8).

Table (3.8): assay of spironolactone in commercial formulation for dervatization by Hydrazine:

Drug	Amount taken μg/ml	Amount found μg/ml	% recovery	Average of recovery	% RSD
Yesalactone	25	24.21	96.84	97.15	0.28209
	25	24.33	97.31		
	25	24.33	97.31		

3.1.2.2 Derivatization by Hydroxylamine

3.1.2.2.1 Method development

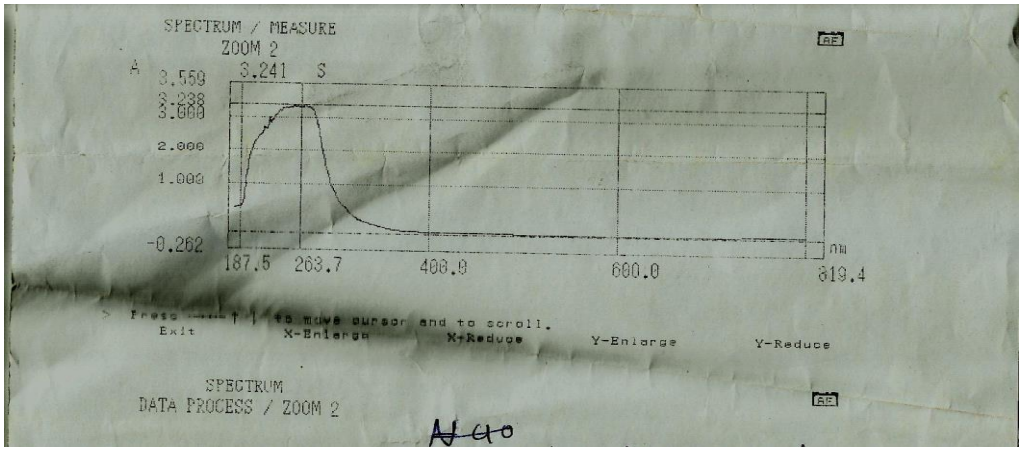

Figure (3.3): UV spectrum of spironolactone in derivatization by hydroxylamine

3.1.2.2.2 Method validation

Linearity of the method was determined by preparation five concentration of standard in range 20 ppm to 30 ppm and their absorbance's at wavelength 263.7 nm were recorded (Table 3.9).

Table (3.9): Calibration curve of spironolactone in dervatization by hydroxylamine:

Concentration ppm	Absorption at 263.7 nm	SD of the absorbance
20	0.125	0.000471
22.5	0.141	0.000000
25.0	0.159	0.000577
27.5	0.179	0.000577
30	0.190	0.000577
Average of Standard deviation (SD) of the absorbance		0.000551

The R^2 value of calibration curve graph showed that the developed spectrophotometric method for assay of spironolactone by derivatization by hydroxylamine was linear according to ICH Q2 R1 (Figure 3.4).

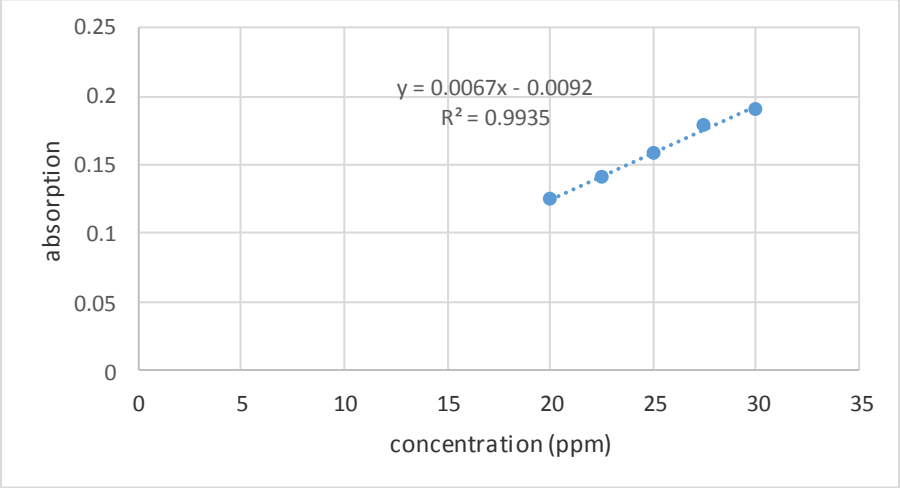

Figure (3.4): Calibration curve of spironolactone in derivatization by hydroxylamine

The repeatability precision was done by using 100% concentration solution and RSD% found to be 0.81% as mention in Table (3.10).

Table (3.10): Repeatability precision of the system of spironolactone in derivatization by Hydroxylamine:

Run	Absorption
1	0.155
2	0.155
3	0.157
4	0.158
5	0.155
6	0.156
Mean	0.156
RSD%	0.81084

The recovery was done by using standard addition method and found in the range (98.47-100.67) % and RSD (0.17-0.2) % (Table 3.11).

Table (3.11): Recovery study of spironolactone in dervatization by Hydroxylamine:

Level of the method %	Amount taken (µg/ml)	Amount added (µg/ml)	Total theoretical amount found (µg/ml)	Amount found (µg/ml)	Recovery %	Average of recovery %	RSD %
80	25	20	45	45.36	100.79		
	25	20	45	45.36	100.79	100.67	0.206
	25	20	45	45.19	100.43		
100	25	25	50	50.22	100.43		
	25	25	50	50.38	100.76	100.65	0.186
	25	25	50	50.38	100.76		
120	25	30	55	54.77	98.66	98.47	
	25	30	55	54.10	98.37		0.173
	25	30	55	54.10	98.37		

The intermediate precision was done by using 100% concentration solution and RSD% found to be in the range (0.374-0.385) % (Table 3.12).

Table (3.12): intermediate precision of spironolactone in dervatization by hydroxylamine:

Run	ABS of 1st analyst	ABS of 2st analyst	ABS of 3st analyst
1	0.155	0.154	0.150
2	0.154	0.153	0.150
3	0.154	0.153	0.149
Mean	0.1543	0.1533	0.1497
RSD %	0.37409%	0.37653%	0.38576%

The RSD % values of intermediate precision and repeatability showed that the developed spectrophotometric method for assay of spironolactone by derivatization by hydroxylamine was precise according to ICH Q2 R1.

Limit of detection and limit of quantitation of the method:

$$LOD = 3.3\sigma/S$$

$$LOQ = 10\ \sigma/S$$

σ = standard deviations of the response

S = mean of the slope of the calibration plot.

LOD = 3.3 *0.000551/0.0067 = 0.271321 ppm

LOQ = 10 *0.000551/0.0067 = 0.822185ppm

Table (3.13): assay of spironolactone in commercial formulation in derivatization by Hydroxylamine:

Drug	Amount taken μg/ml	Amount found μg/ml	% recovery	Average of recovery	% RSD
Yesalactone	25	24.55	98.22	98.00	0.3936
	25	24.55	98.22		
	25	24.39	97.55		

The assay of spironolactone in tablet dosage form was found 98.00 % by UV method of derivatization by hydroxylamine (Tablet 3.13).

The spectrophotometric analysis methods of spironolactone by derivatization by hydroxylamine and derivatization by hydrazine were applied to the analysis of spironolactone 25 mg tablet formulation (yesalactone) and the assay (average ±RSD) were found to be (98.00±0.39) % and (97.15±0.28) percentage, respectively, and the assay of spironolactone according to united states pharmacopeia (USP) was found to 98.6% and that reflect the high accuracy of UV spectrophotometric methods. According to the statistical analysis data of validation parameters of the two spectrophotometric methods, the derivatization by hydrazine method has a higher linearity, precision, accuracy, limit of detection and limit of quantitation than the derivatization by hydroxylamine method.

3.1.3 High performance liquid chromatography (HPLC)

In the high performance liquid chromatography (HPLC) method acetonitrile and buffer solution (pH 4) with (1:1) ratio were selected as the suitable solvent for the method after studying the solubility of spironolactone in different solvents and buffer solutions (pH 2-12). The detection wavelength was found to be 240 nm. Different ratios of buffer solution (pH 4) and acetonitrile were tried to get the suitable mobile phase. The experiment was conducted using acetonitrile and buffer solution (pH 4) with (1:1) ratio as the mobile phase and C18 as a column for HPLC method. The optimum conditions for selection of mobile phase and column were based on separation, shape, retention time and purity of the peak.

3.1.3.1 Method development

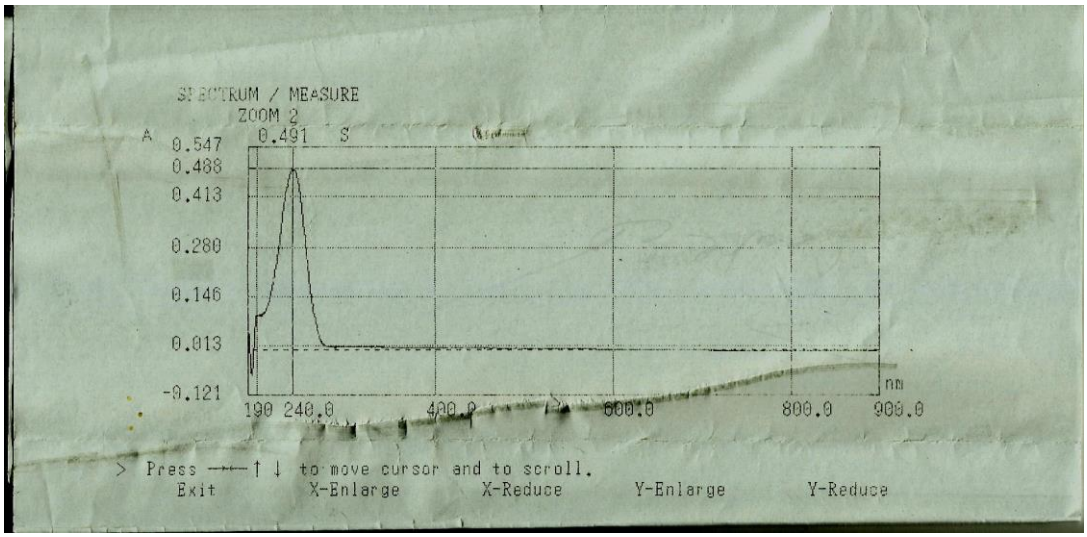

Figure (3.5): UV spectrum of spironolactone in HPLC method

3.1.3.2 Method validation

All the validation parameters were done according to ICH Q2 (R1) guideline:

Specificity of HPLC method were done by using 100% concentration solution and showed good resolution of the peak with retention time at 4.75 min (Figure 3.6).

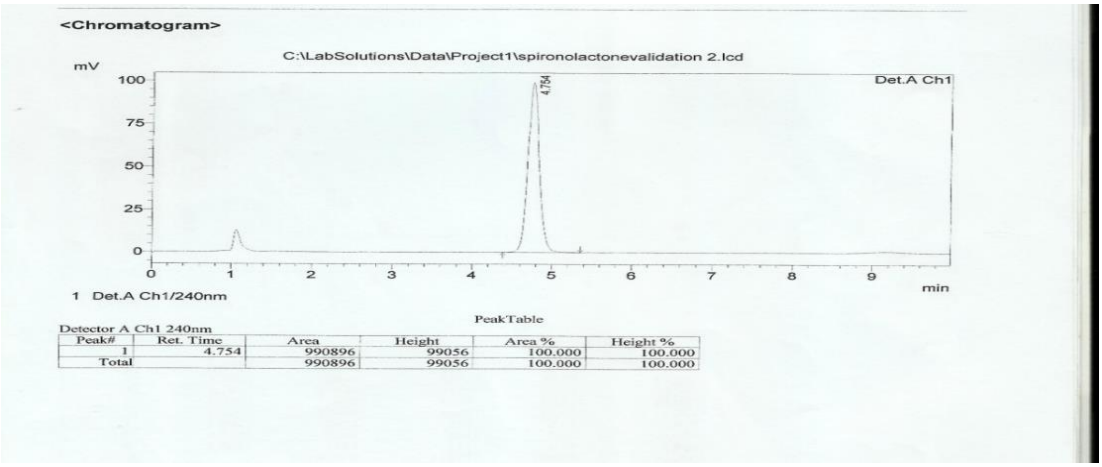

Figure (3.6): Specificity of HPLC method

Five concentrations of standard in range (20-30) ppm and the area under their absorption peaks at wavelength 240 nm were recorded in Table (3.14).

Table (3.14): Calibration curve of spironolactone in HPLC method:

Concentration ppm	Area under peak	SD of the area under peak
20	799643.7	5044.273
22.5	888063	12717.570
25.0	979287	10278.050
27.5	1076951	1549.059
30	1168108	1465.315
Average of SD of area under peak		6210.854

The R^2 value of HPLC method was bigger than 0.99 and that reflects linearity of the method according to ICH Q2R1 (Figure 3.7).

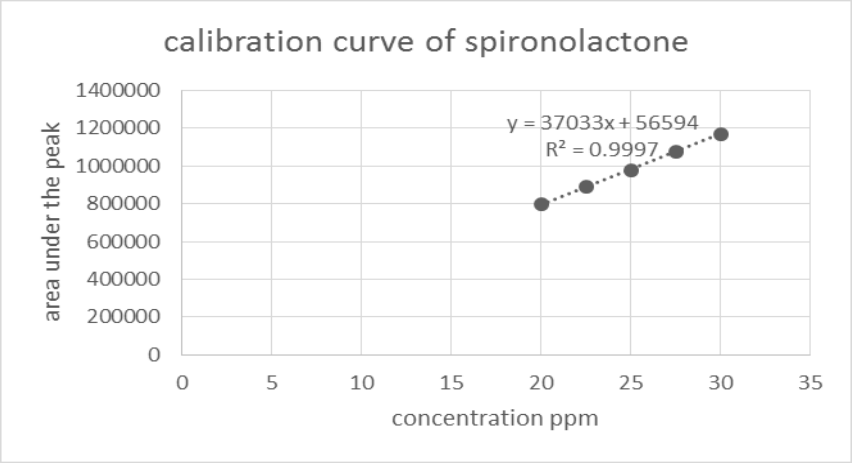

Figure (3.7): Calibration curve of spironolactone in HPLC method

All the relative standard deviation (RSD %) of the precision (intermediate precision and repeatability) values in HPLC method were found less than 2% which reflect the high precision of the method developed.

The repeatability precision was done by using 100% concentration solution and RSD% found to be 0.4355% (Table 3.15).

Table (3.15): Repeatability precision of the system in HPLC method:

Run	Area under the peak
1	991027
2	984376
3	984029
4	978274
5	985478
6	988032
Mean	985202.7
RSD%	0.4355

The intermediate precision was done by using 100% concentration solution and RSD% found to be in the range (0.25-0.59) % (Table 3.16).

Table (3.16): Intermediate precision of spironolactone in HPLC method

Run	area the under peak of 1st analyst	area the under peak of 2st analyst	Area the under peak of 3st analyst
1	1002754	1016599	1003482
2	993339	1005208	1001133
3	1000558	1007729	1006187
Mean	998883.7	1009845	1003601
RSD %	0.493126	0.59248	0.252002

The recovery was done by using standard addition method and found in the range (98.47-100.67) % and RSD (0.17-0.2) % (Table 3.17).

Table (3.17): Recovery study of spironolactone in HPLC method:

Level of method%	Area the under peak	Amount found %	Recovery %	Average of recovery	RSD of recovery %
80%	816649	82.24442	102.8055	101.99	0.722723
	805671	81.13883	101.4235		
	808246	81.39816	101.7477		
100%	995124	100.2186	100.2186	99.40	0.760866
	980161	98.71166	98.71166		
	985821	99.28167	99.28167		
120%	1214609	122.3228	101.9357	101.32	1.742522
	1223484	123.2166	102.6805		
	1183915	119.2316	99.3597		

Limit of detection and limit of quantitation of the method were calculated by the following equations:

$$LOD = 3.3\sigma/S$$

$$LOQ = 10\ \sigma/S$$

σ = standard deviations of the response

S = mean of the slope of the calibration plot.

LOD = 3.3 *(6210.854/37033) = 0.553447ppm

LOQ = 10 *(6210.854/37033) = 1.677113ppm

The assay of spironolactone in tablet dosage form was found to be 98.25 % by HPLC method is mentioned in Table (3.18).

Table (3.18): Assay of spironolactone in commercial formulation for HPLC method:

Drug	Amount taken μg/ml	Amount found μg/ml	% recovery	Average of recovery	% RSD
Yesalactone	25	24.40	97.60	98.25	0.59
	25	24.68	98.73		
	25	24.61	98.44		

At last according to the statistical analysis data of validation of HPLC method, the method was found to be linear in the concentration range of 20-30 ppm. In addition to that, it was found to be accurate, precise with acceptable value of LOD and LOQ.

In Table (3.19) is shown the summary of the validation parameter of UV spectrophotometric and HPLC methods.

Table (3.19): Summary of validation parameters of HPLC and spectrophotometric methods:

Parameter	Spectrophotometric Methods		HPLC method
	Dervatization by Hydroxylamine	Dervatization by Hydrazine	
Beer's law range	20 ppm-30 ppm	20ppm-30ppm	20ppm-30ppm
Wavelength	263.7 nm	251.0nm	240nm
Correlation coefficient	0.9935	0.9966	0.9997
Slope	0.0067	0.0771	37033
Intercept	0.0092	0.0247	56594
LOD ppm	0.271321	0.02833	0.553447
LOQ ppm	0.822185	0.085847	1.677113
Precision (RSD %)			
Repeatability	0.81084	0.3409	0.4355
Intermediate precision	0.3741 – 0.3858	0.2551 – 0.2741	0.2520 – 0.5925
Recovery %	98.47-100.67	100.20-102.65	99.40-101.99

3.2 Conclusion

The described analytical methods were validated according to ICH Q2 (R1) guideline and they meet the specific acceptance criteria. So these validated methods were found to be linear, accurate, precise, and sensitive and can be used for routine analysis for estimation of spironolactone in raw material and tablet dosage form.

3.3 Recommendations

1. Studying the stability of spectrophotometric and HPLC analysis methods (oxidation, light, acidity and alkalinity).

2. Studying the reproducibility of spectrophotometric and HPLC analysis methods.

3. Studying the robustness for spectrophotometric methods.

4. Appling the spectrophotometric methods and HPLC method for other commercial tablet dosage forms.

5. Appling the HPLC method for dissolution test.

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Appendix

Dervatization by Hydrazine

Method development

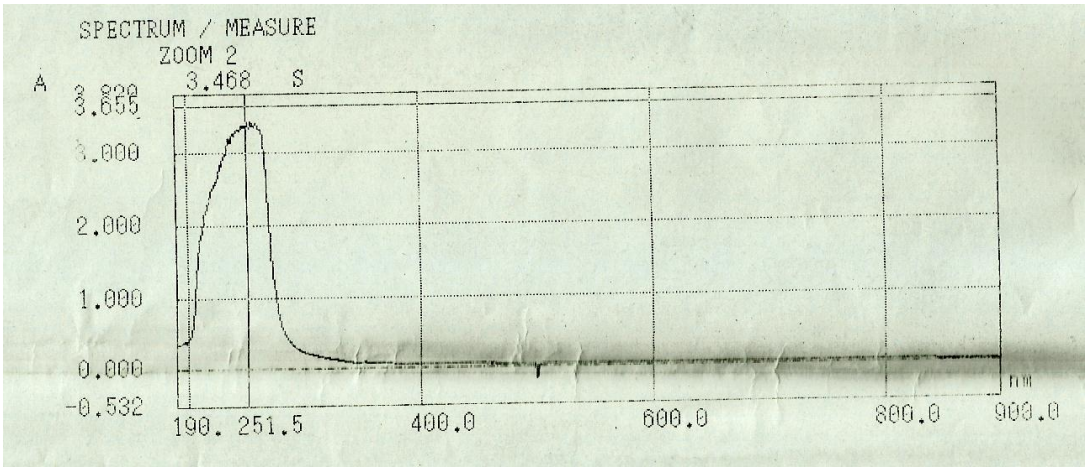


Figure (5.1) UV spectrum of spironolactone in dervatization by hydrazine

Dervatization by Hydroxylamine

Method development

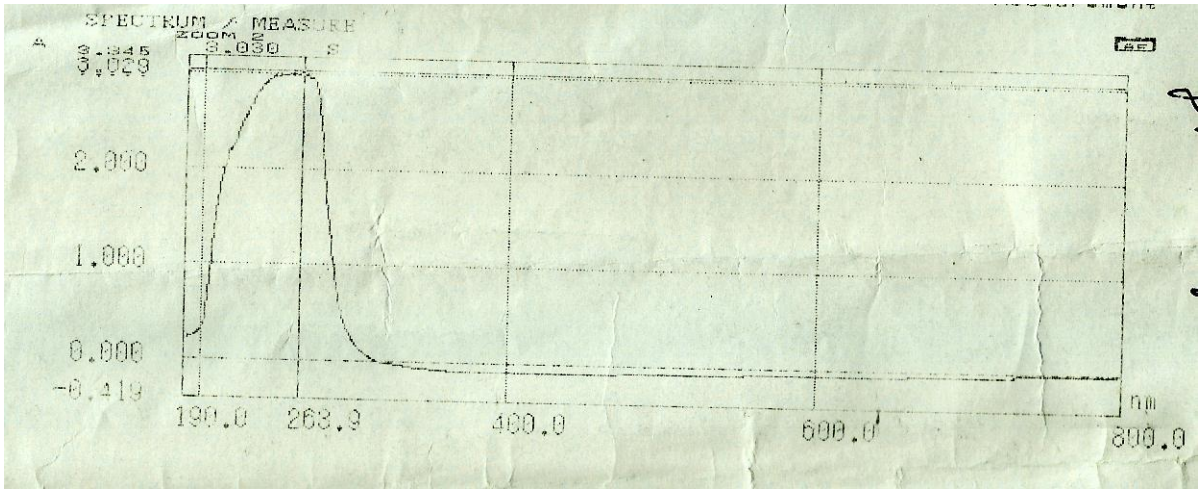


Figure (5.2): UV spectrum of spironolactone in derivatization of hydroxylamine

High performance liquid chromatography (HPLC) analysis method of spironolactone

Method development

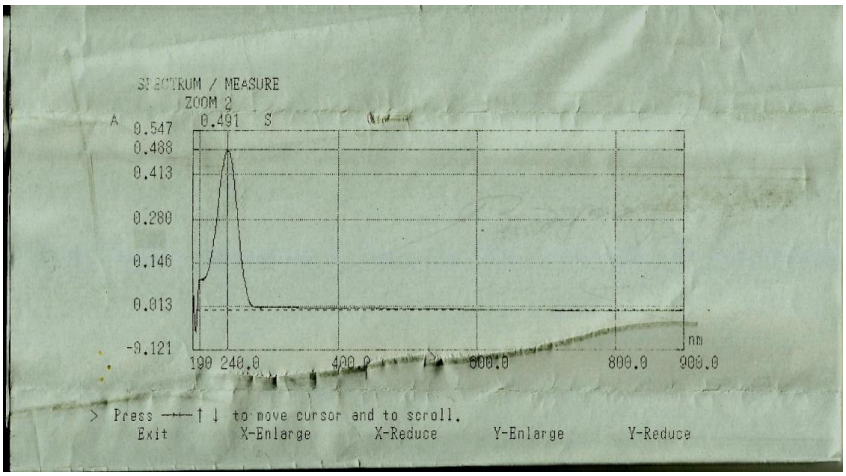
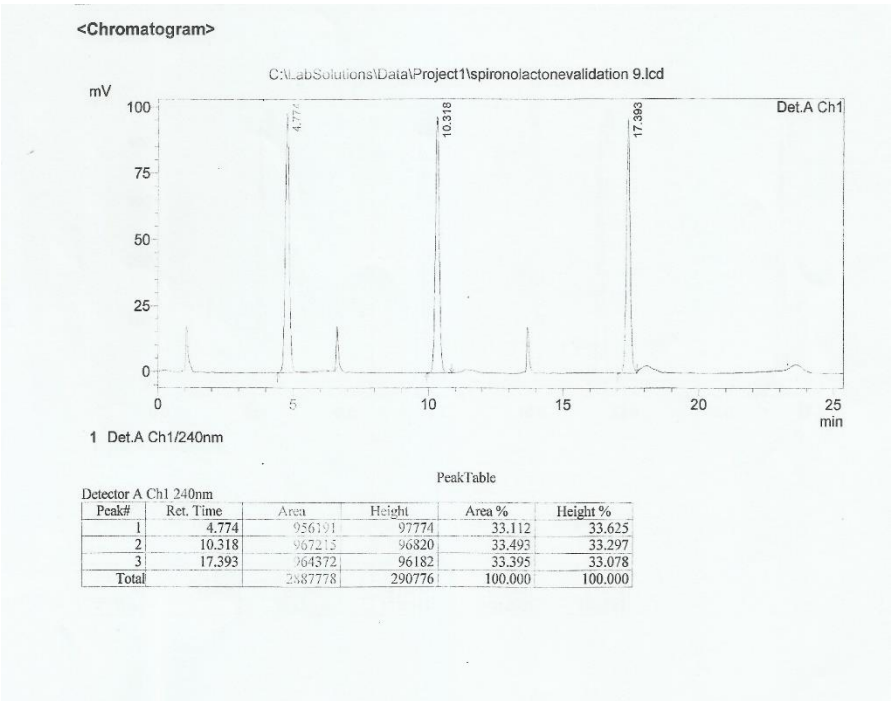


Figure (5.3): UV spectrum of spironolactone in HPLC method

Assay

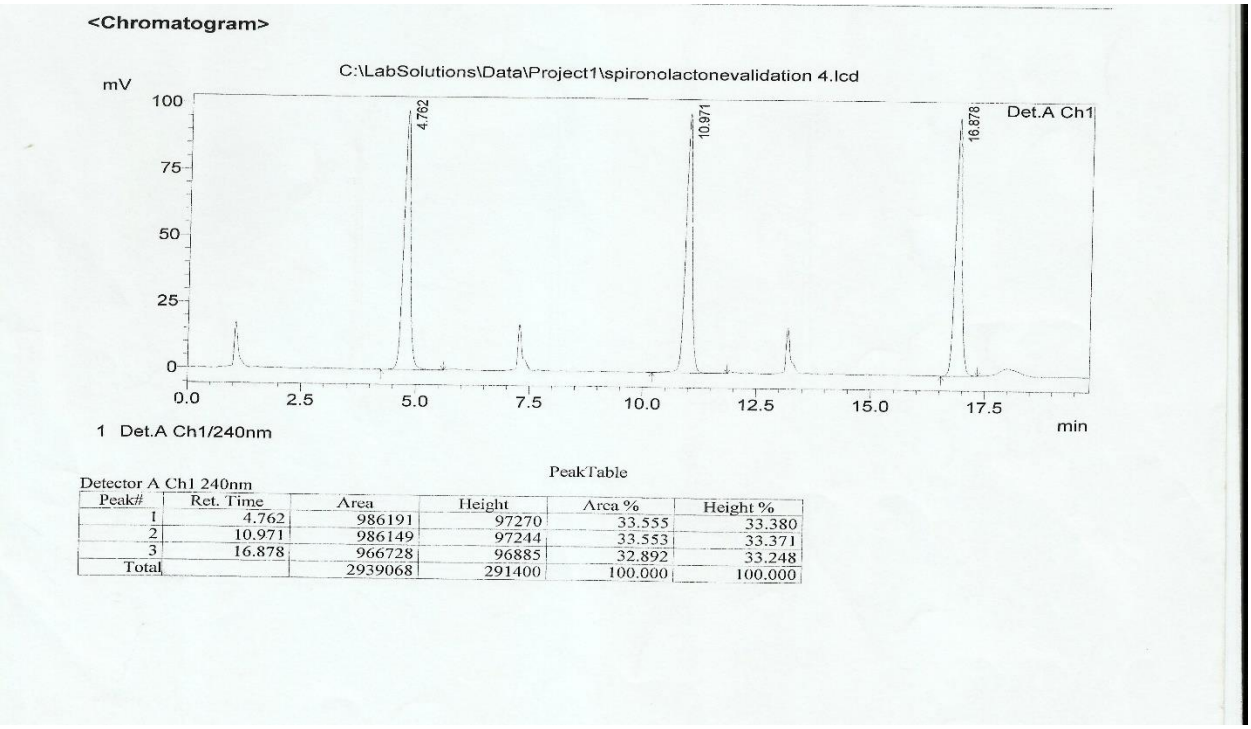


PeakTable

Detector A Ch1 240nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	4.774	956191	97774	33.112	33.625
2	10.318	967215	96820	33.493	33.297
3	17.393	964372	96182	33.395	33.078
Total		2887778	290776	100.000	100.000

Figure (5.4): Assay sample HPLC chromatogram



PeakTable

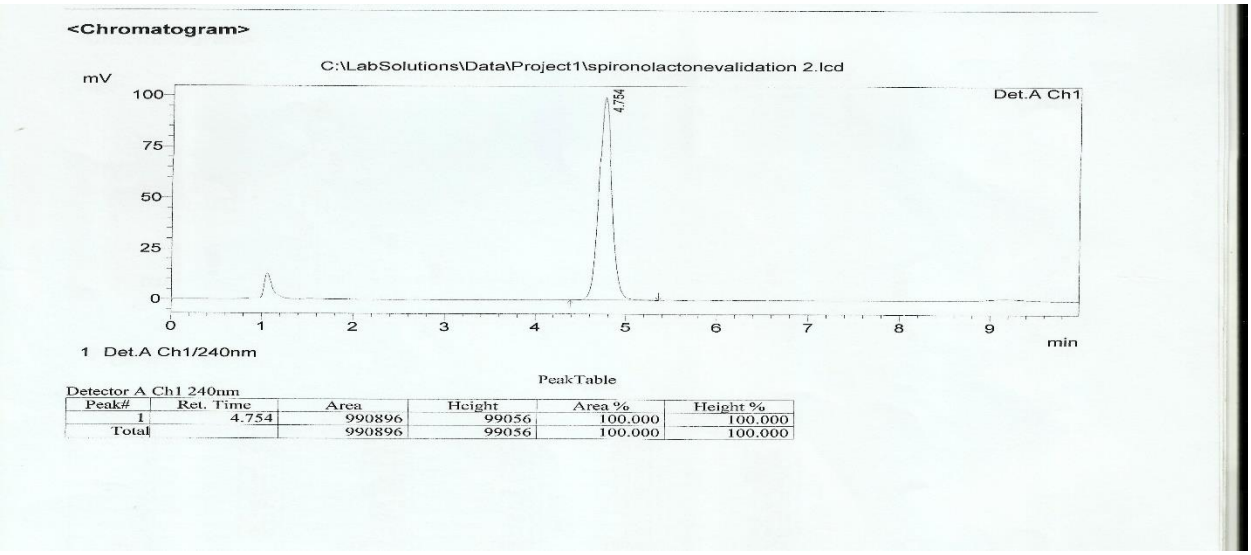
Detector A Ch1 240nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	4.762	986191	97270	33.555	33.380
2	10.971	986149	97244	33.553	33.371
3	16.878	966728	96885	32.892	33.248
Total		2939068	291400	100.000	100.000

Figure (5.5): Assay standard HPLC chromatogram

Method validation

Specificity



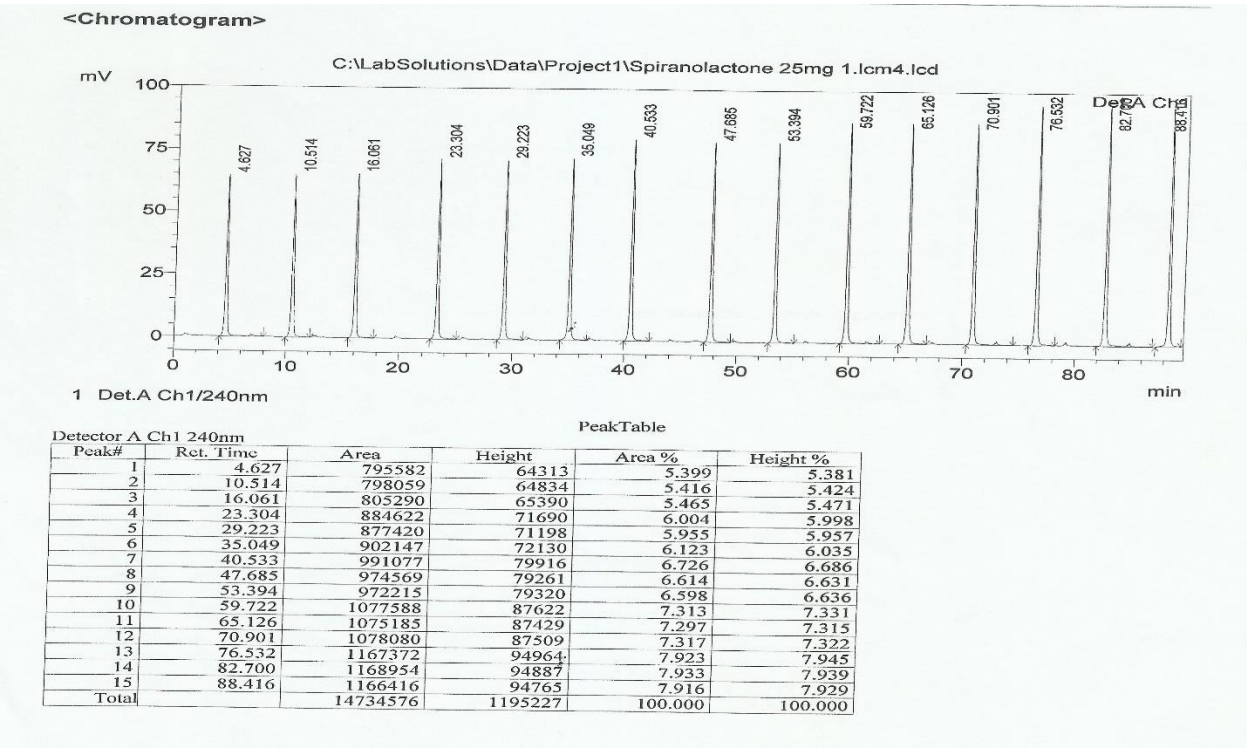
PeakTable

Detector A Ch1 240nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	4.754	990896	99056	100.000	100.000
Total		990896	99056	100.000	100.000

Figure (5.6): chromatogram of specificity of HPLC method

Linearity



PeakTable

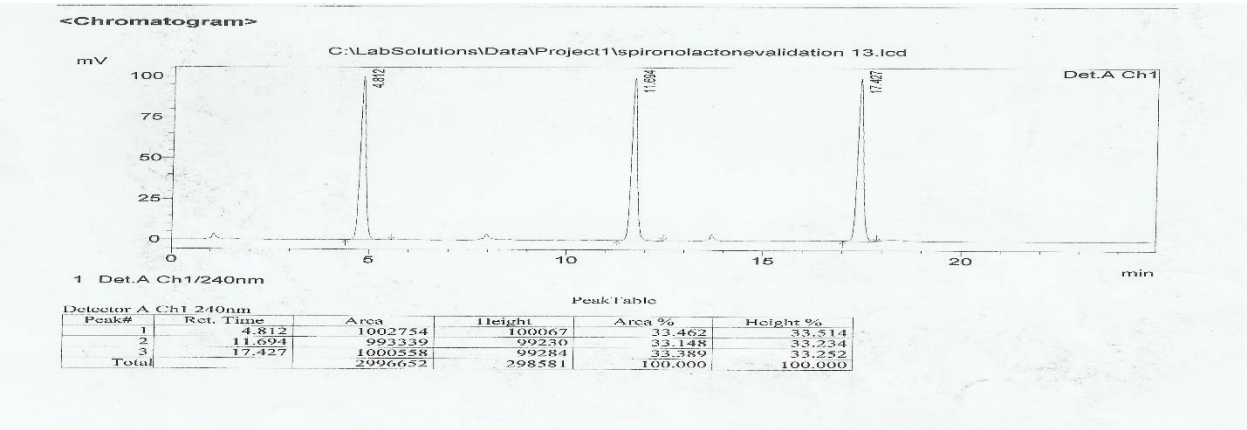
Detector A Ch1 240nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	4.627	795582	64313	5.399	5.381
2	10.514	798059	64834	5.416	5.424
3	16.061	805290	65390	5.465	5.471
4	23.304	884622	71690	6.004	5.998
5	29.223	877420	71198	5.955	5.957
6	35.049	902147	72130	6.123	6.035
7	40.533	991077	79916	6.726	6.686
8	47.685	974569	79261	6.614	6.631
9	53.394	972215	79320	6.598	6.636
10	59.722	1077588	87622	7.313	7.331
11	65.126	1075185	87429	7.297	7.315
12	70.901	1078080	87509	7.317	7.322
13	76.532	1167372	94964	7.923	7.945
14	82.700	1168954	94887	7.933	7.939
15	88.416	1166416	94765	7.916	7.929
Total		14734576	1195227	100.000	100.000

Figure (5.7): linearity chromatogram of HPLC method

Precision

Intermediate precision

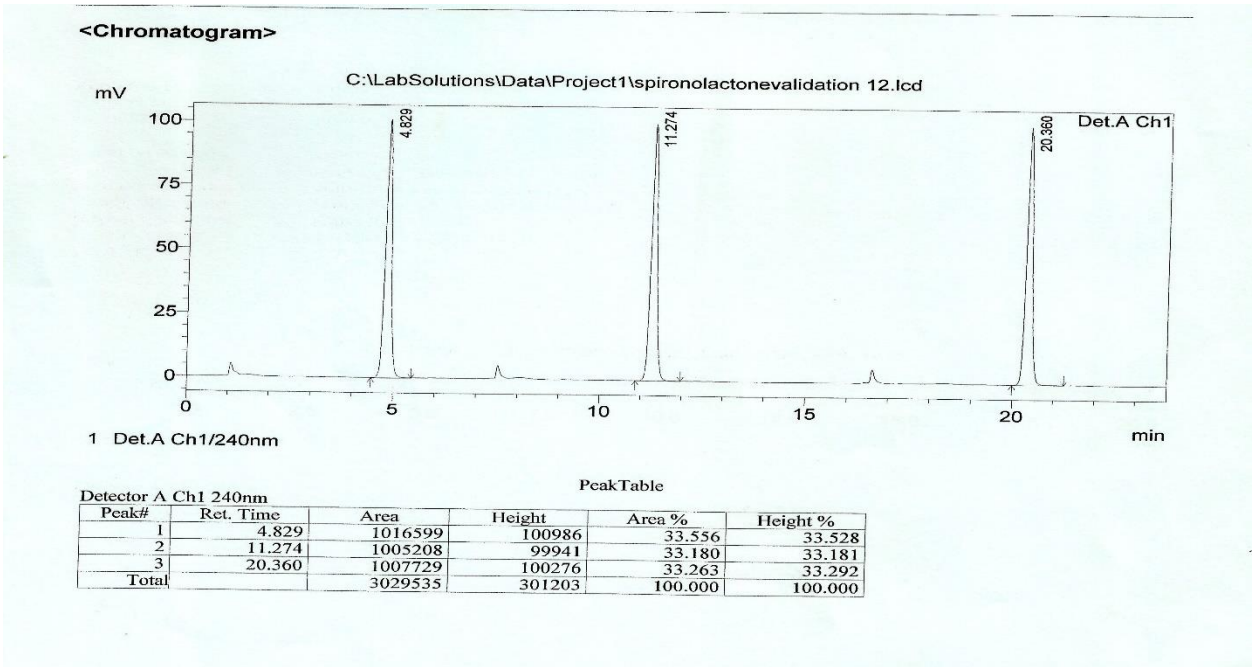


Peak Table

Detector A Ch1 240nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	4.812	1002754	100067	33.462	33.514
2	11.694	993339	99230	33.148	33.234
3	17.427	1000558	99284	33.389	33.252
Total		2996652	298581	100.000	100.000

Figure (5.8): intermediate precision (first analyst) chromatogram of HPLC method

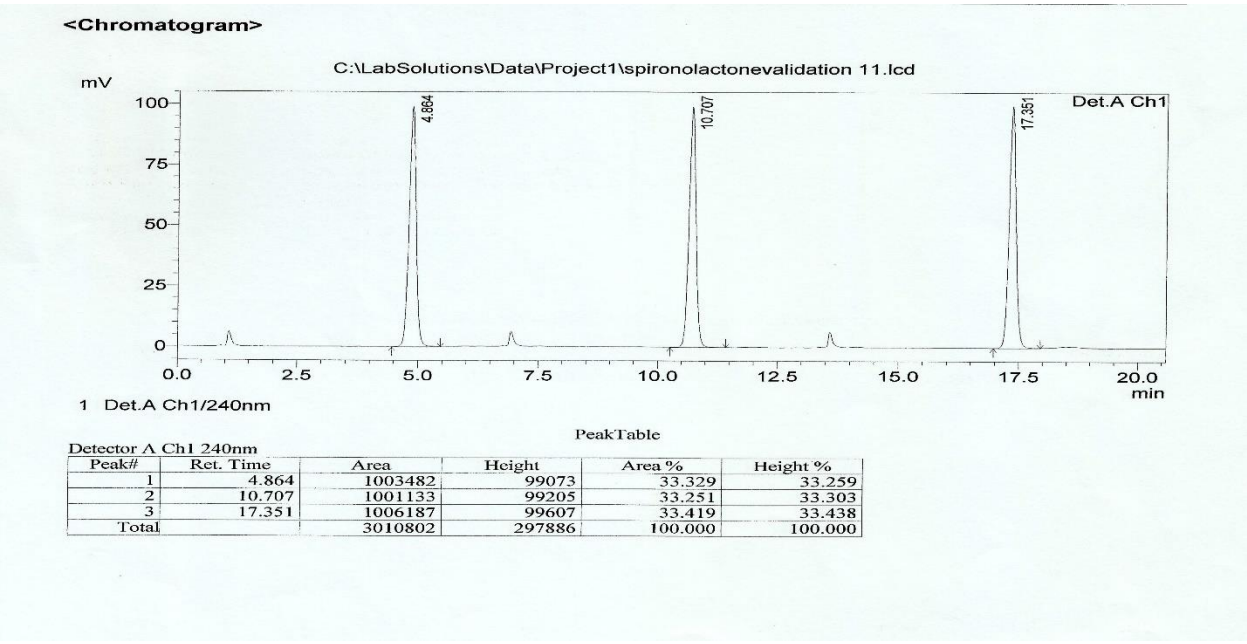


PeakTable

Detector A Ch1 240nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	4.829	1016599	100986	33.556	33.528
2	11.274	1005208	99941	33.180	33.181
3	20.360	1007729	100276	33.263	33.292
Total		3029535	301203	100.000	100.000

Figure (5.9): intermediate precision (second analyst) chromatogram of HPLC method



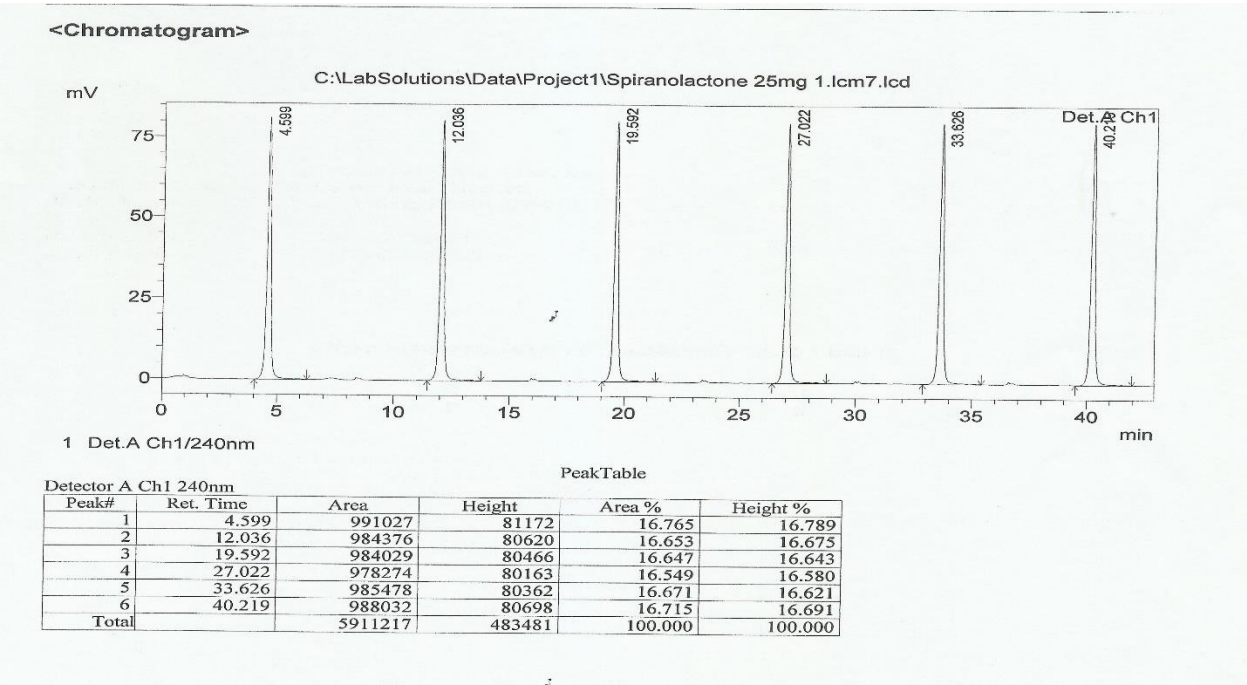
PeakTable

Detector A Ch1 240nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	4.864	1003482	99073	33.329	33.259
2	10.707	1001133	99205	33.251	33.303
3	17.351	1006187	99607	33.419	33.438
Total		3010802	297886	100.000	100.000

Figure (5.10): intermediate precision (third analyst) chromatogram of HPLC method

Repeatability



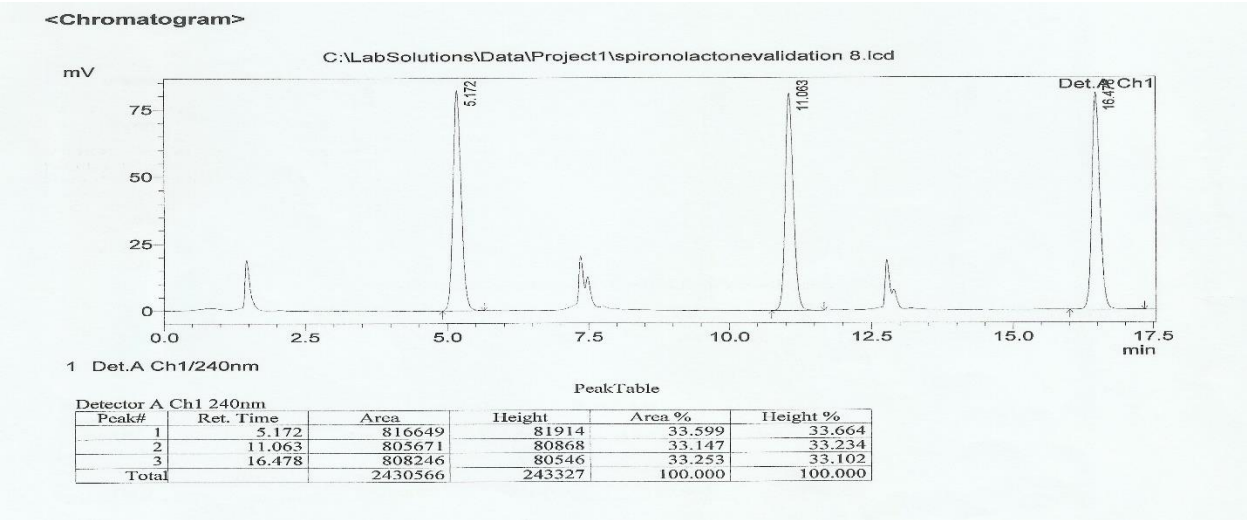
PeakTable

Detector A Ch1 240nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	4.599	991027	81172	16.765	16.789
2	12.036	984376	80620	16.653	16.675
3	19.592	984029	80466	16.647	16.643
4	27.022	978274	80163	16.549	16.580
5	33.626	985478	80362	16.671	16.621
6	40.219	988032	80698	16.715	16.691
Total		5911217	483481	100.000	100.000

Figure (5.11): repeatability chromatogram of HPLC method

Recovery

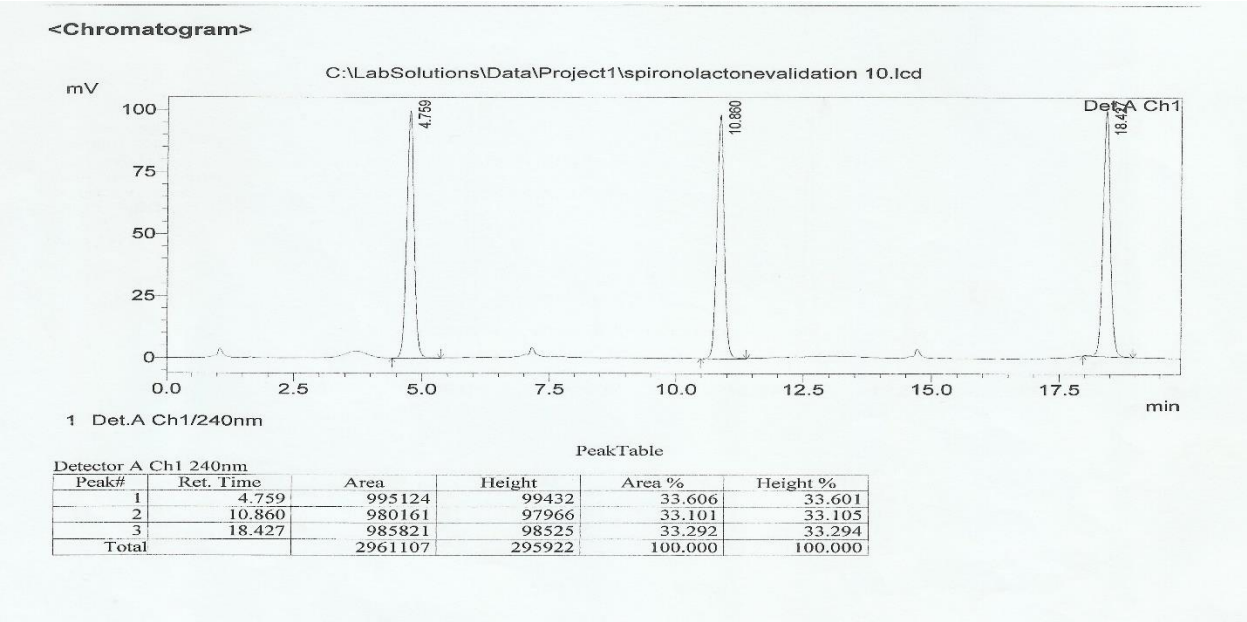


PeakTable

Detector A Ch1 240nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	5.172	816649	81914	33.599	33.664
2	11.063	805671	80868	33.147	33.234
3	16.478	808246	80546	33.253	33.102
Total		2430566	243327	100.000	100.000

Figure (5.12): Recovery chromatogram of 80% concentration solution

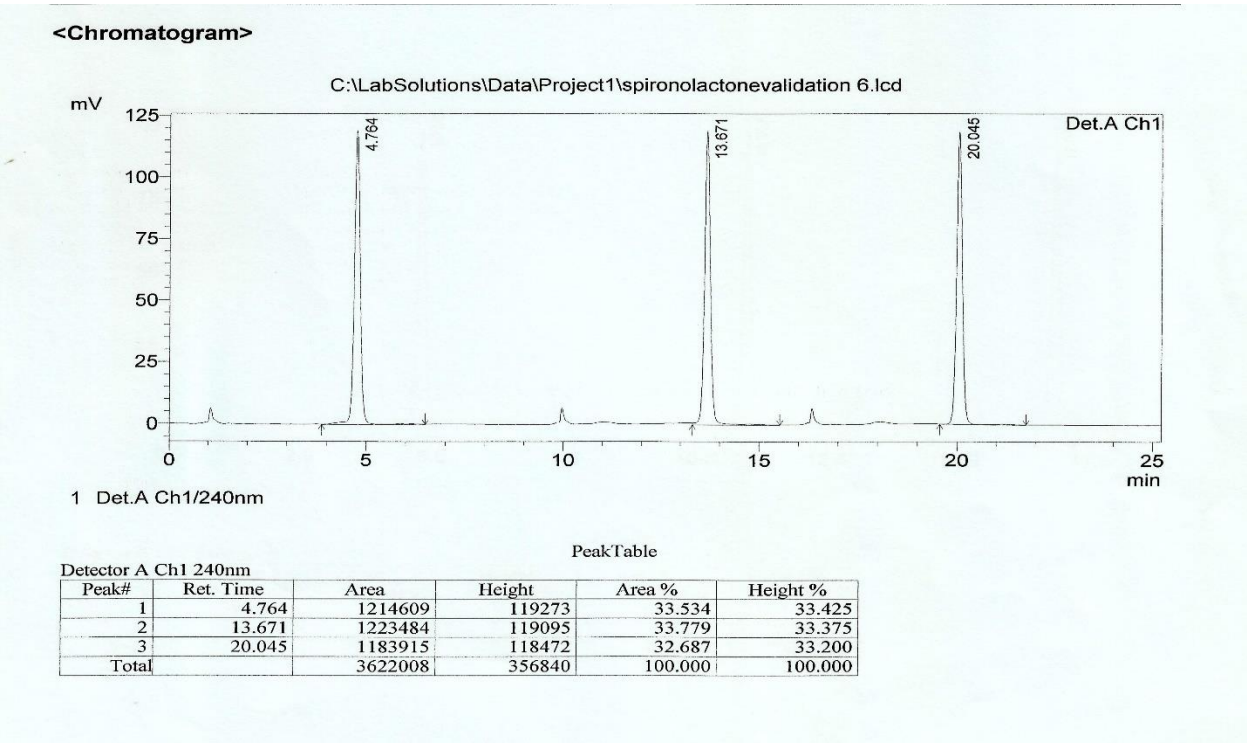


PeakTable

Detector A Ch1 240nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	4.759	995124	99432	33.606	33.601
2	10.860	980161	97966	33.101	33.105
3	18.427	985821	98525	33.292	33.294
Total		2961107	295922	100.000	100.000

Figure (5.13): Recovery chromatogram of 100% concentration solution



PeakTable

Detector A Ch1 240nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	4.764	1214609	119273	33.534	33.425
2	13.671	1223484	119095	33.779	33.375
3	20.045	1183915	118472	32.687	33.200
Total		3622008	356840	100.000	100.000

Figure (5.14): Recovery chromatogram of 120% concentration solution

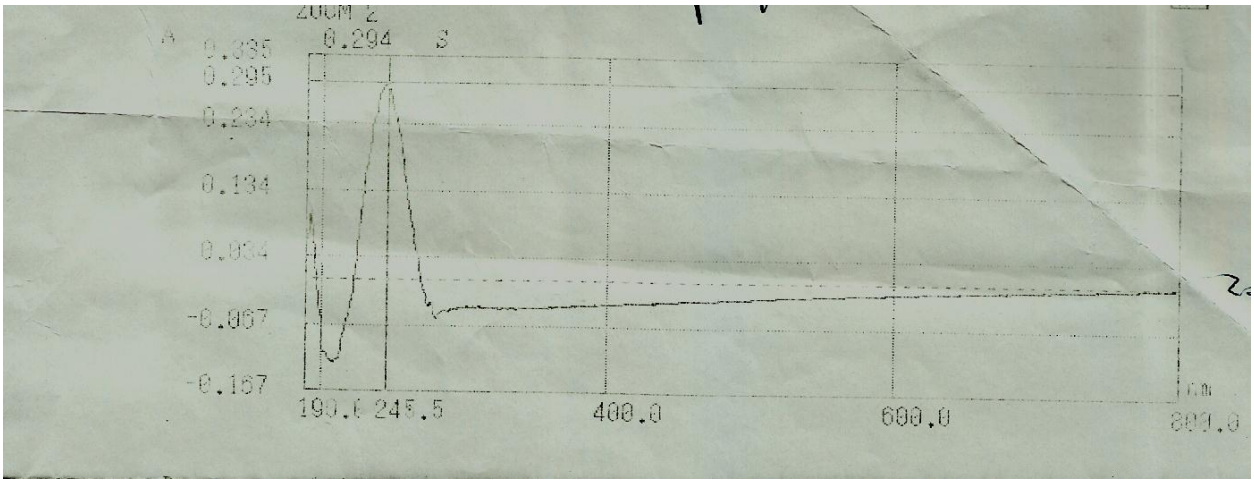


Figure (5.15): UV spectrum of pH 2

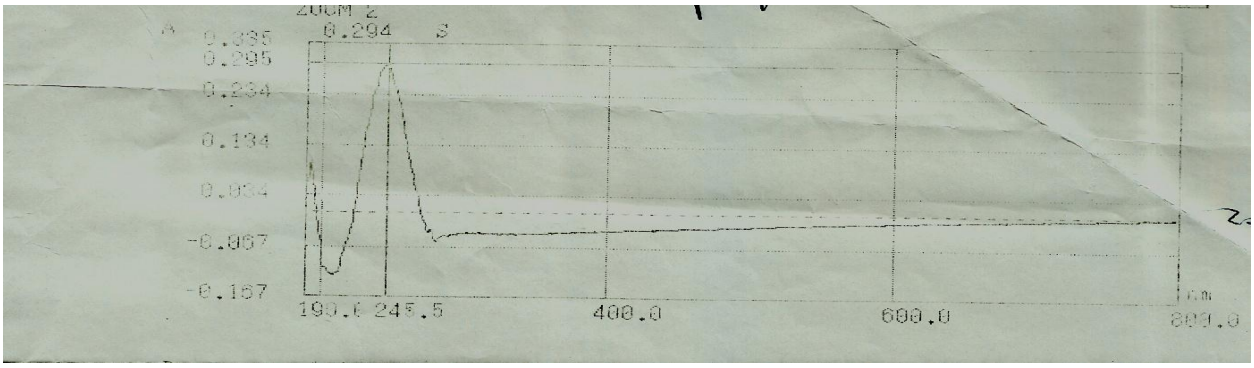


Figure (5.16): UV spectrum of pH 3

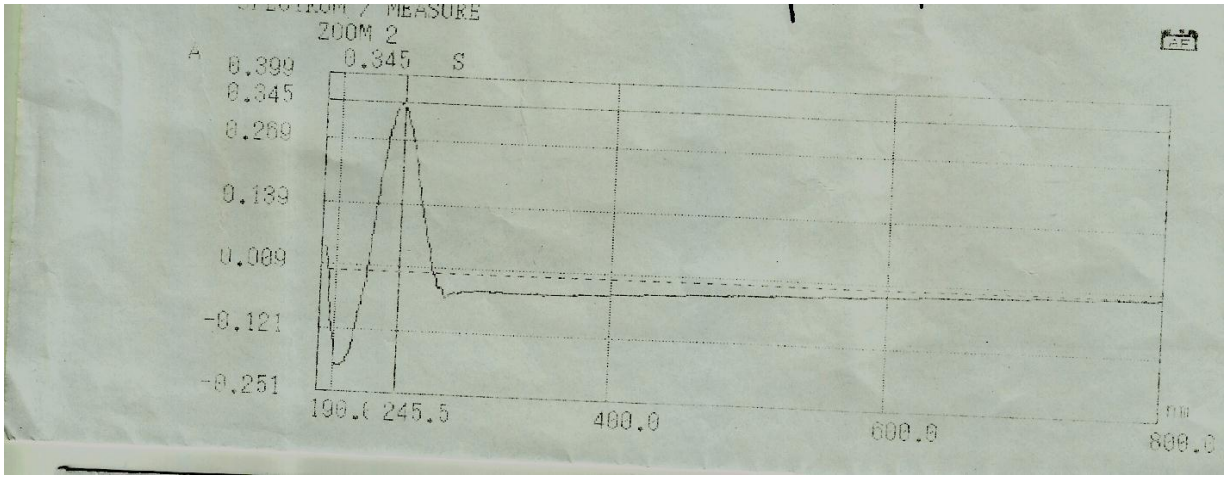


Figure (5.17): UV spectrum of pH 4

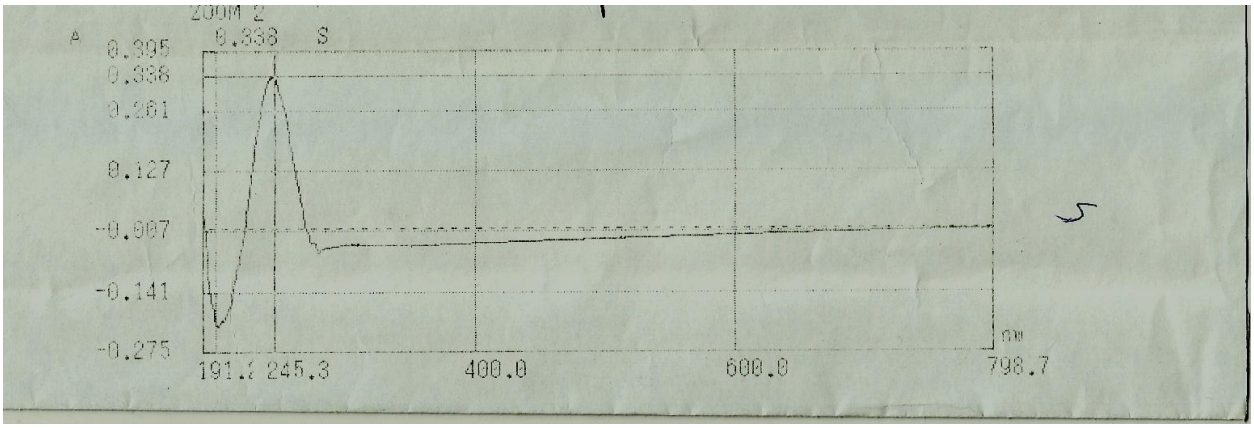


Figure (5.18): UV spectrum of pH 5

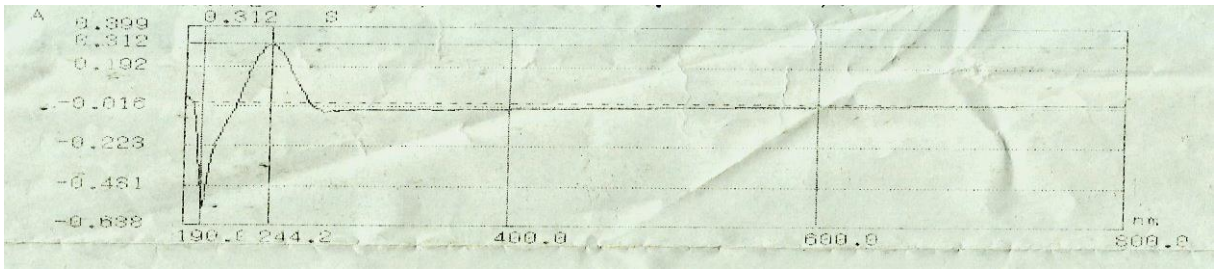


Figure (5.19): UV spectrum of pH 6

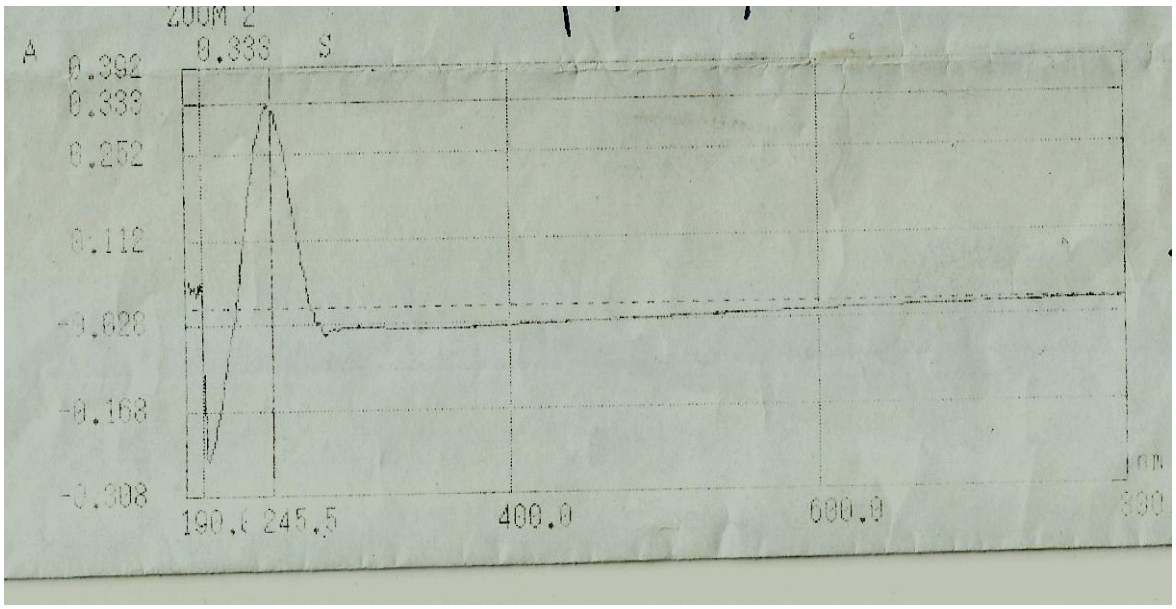


Figure (5.20): UV spectrum of pH 7

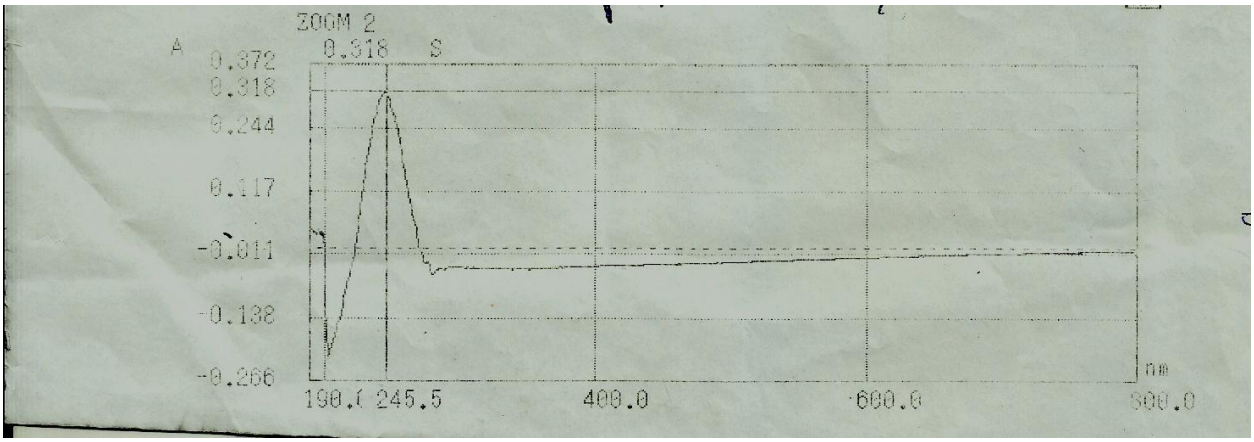


Figure (5.21): UV spectrum of pH 8

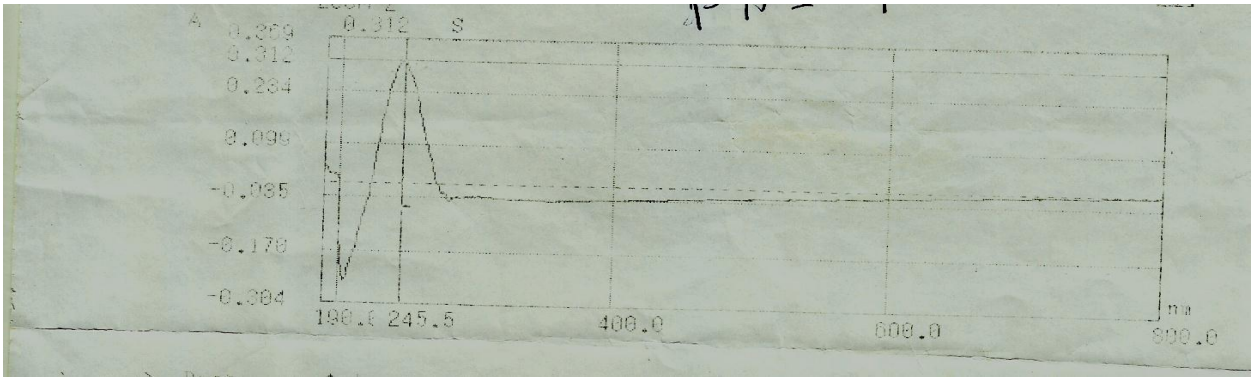


Figure (5.22): UV spectrum of pH 9

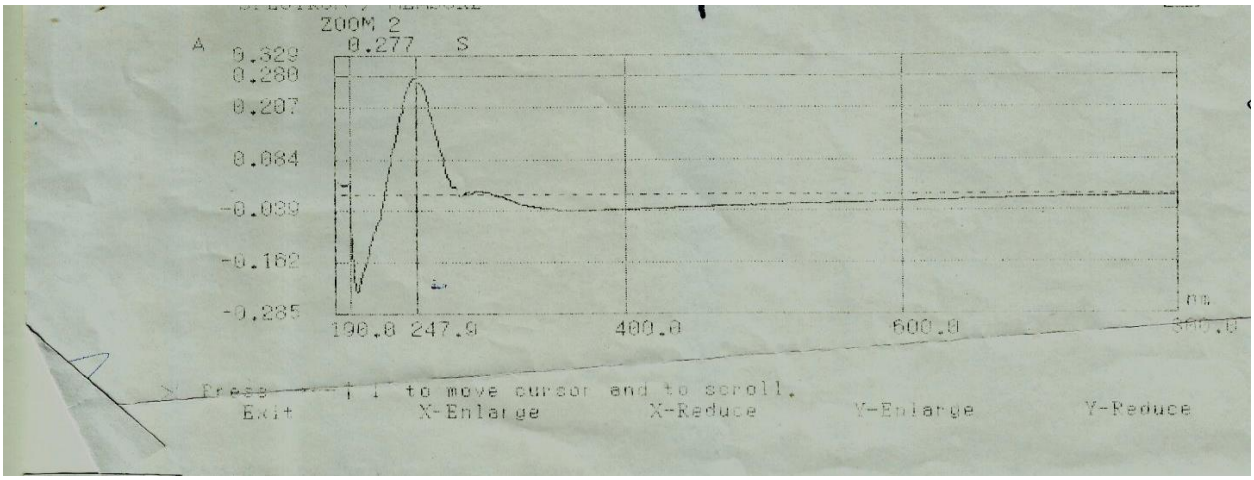
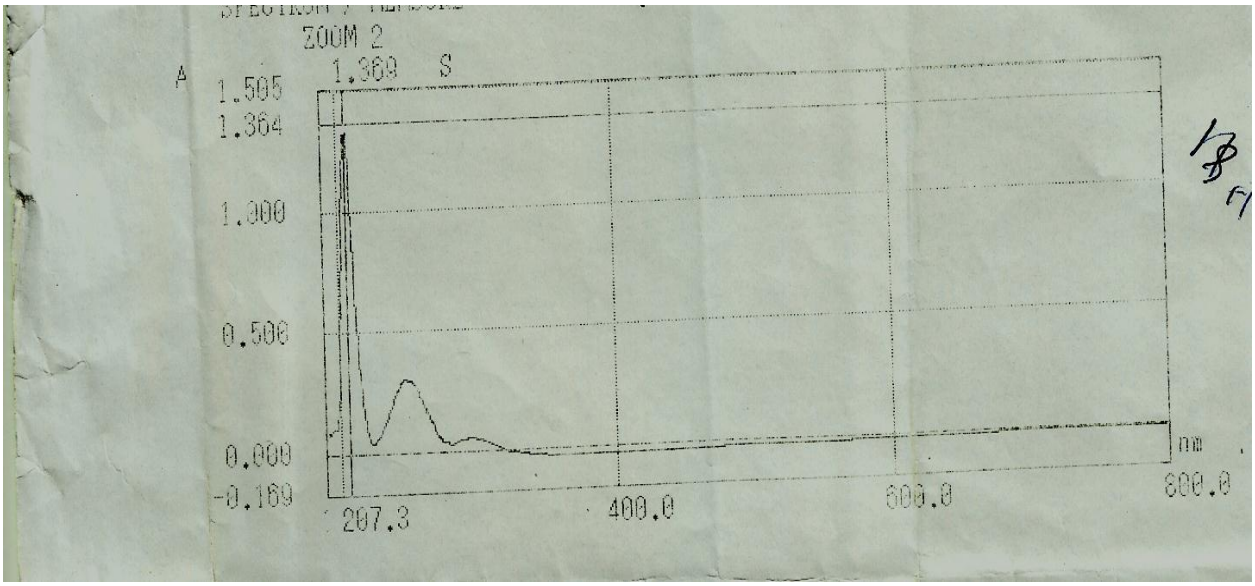


Figure (5.23): UV spectrum of pH 10



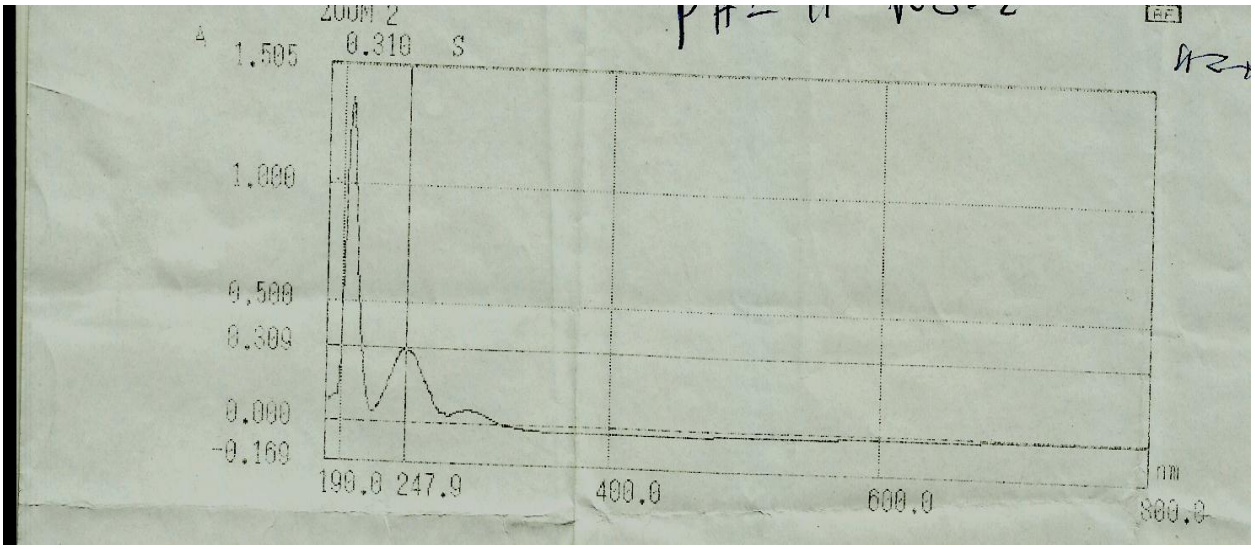
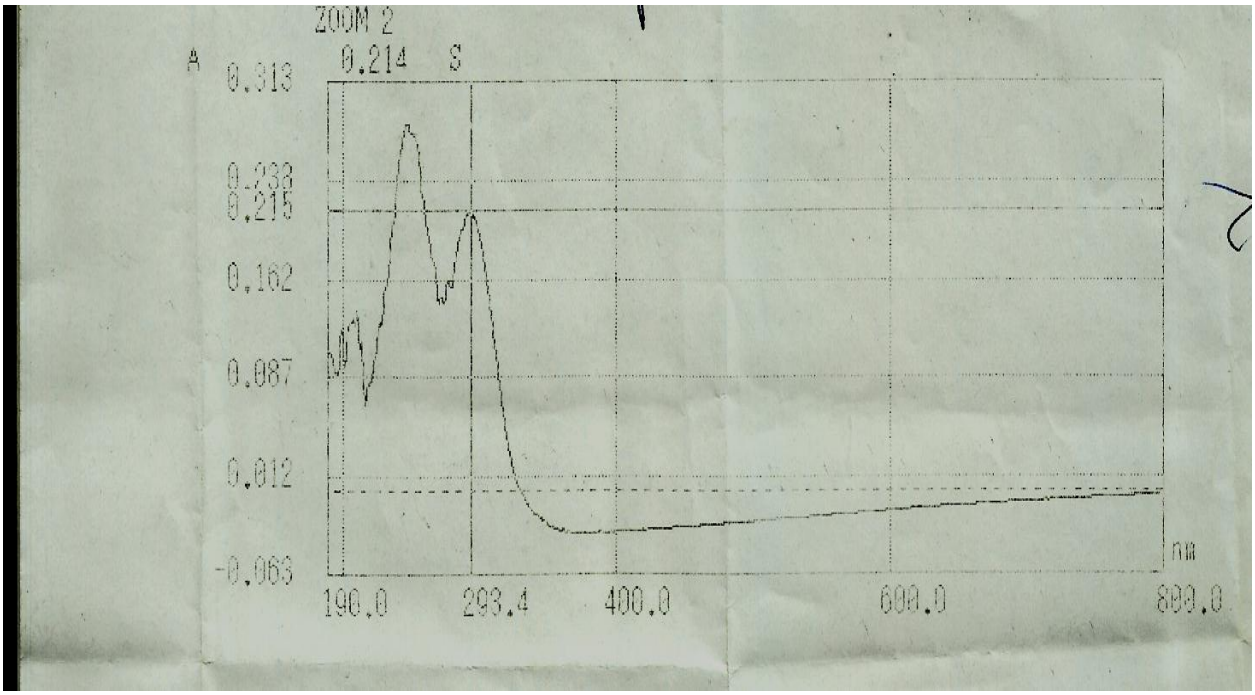


Figure (5.24): UV spectrum of pH 11



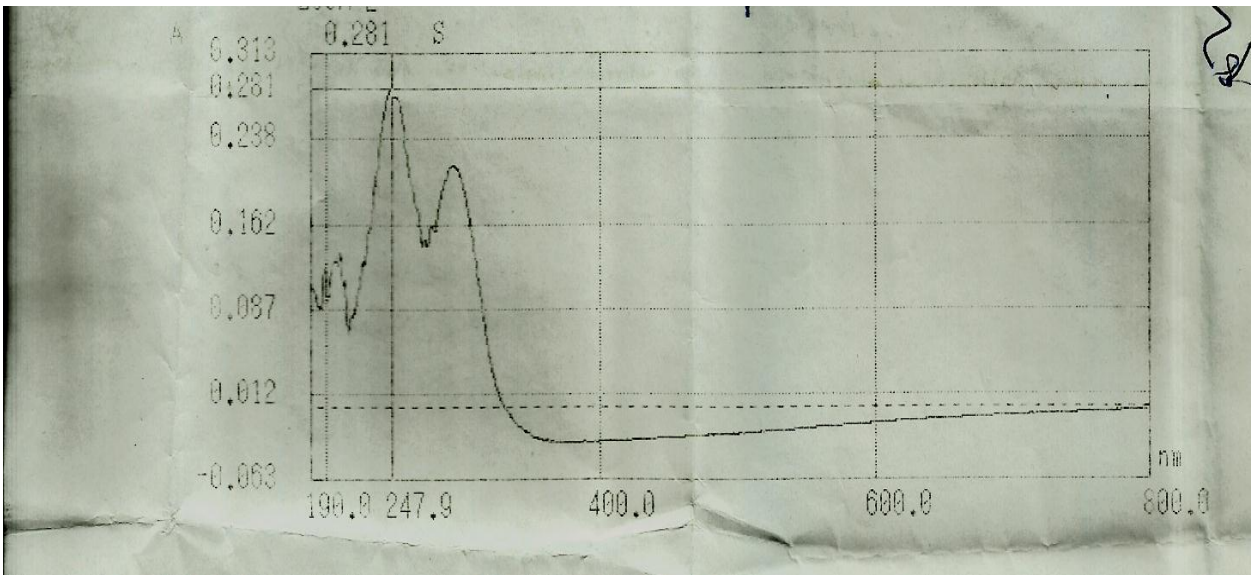


Figure (5.25): UV spectrum of pH 12

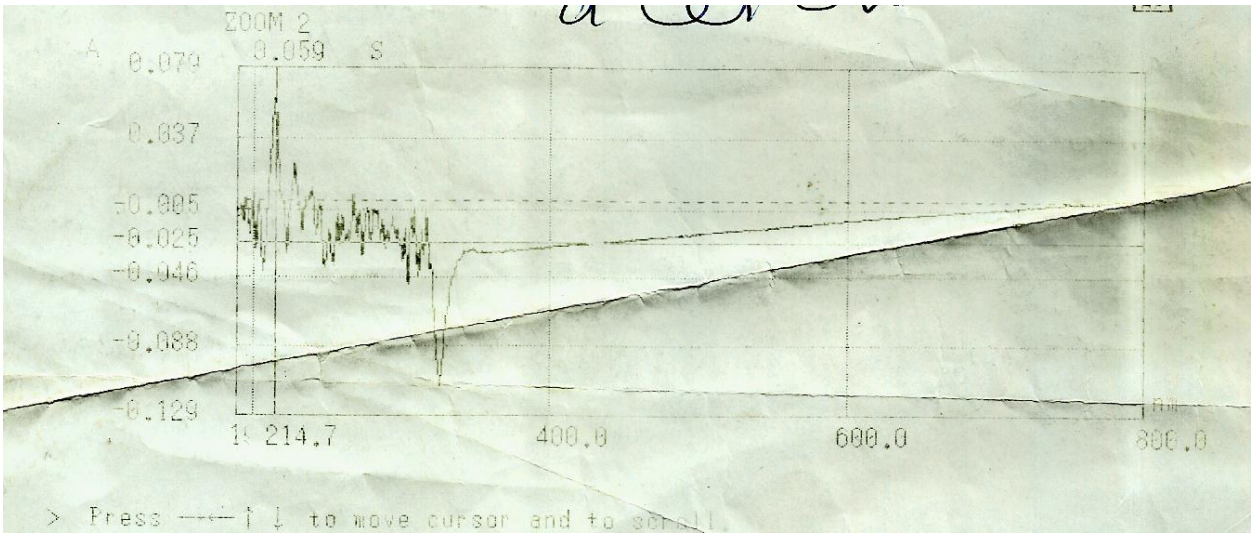


Figure (5.26): UV spectrum of acetone

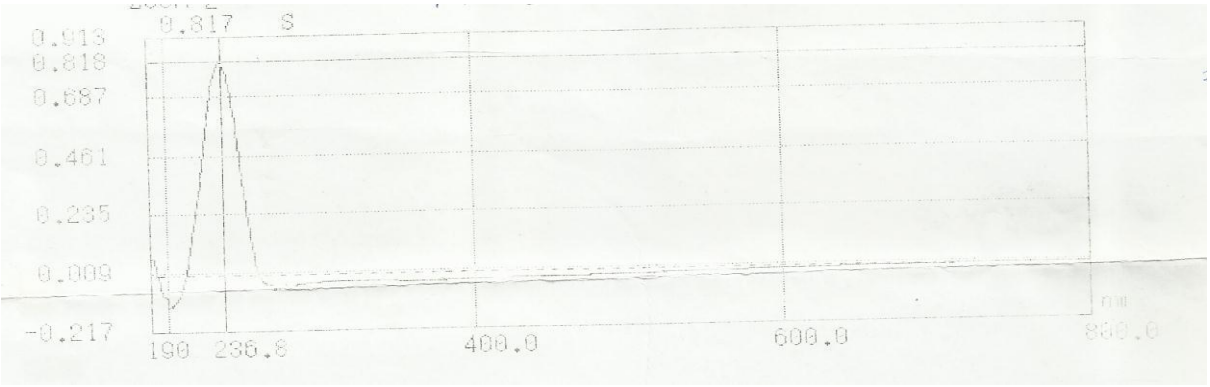


Figure (5.27): UV spectrum of acetonitrile

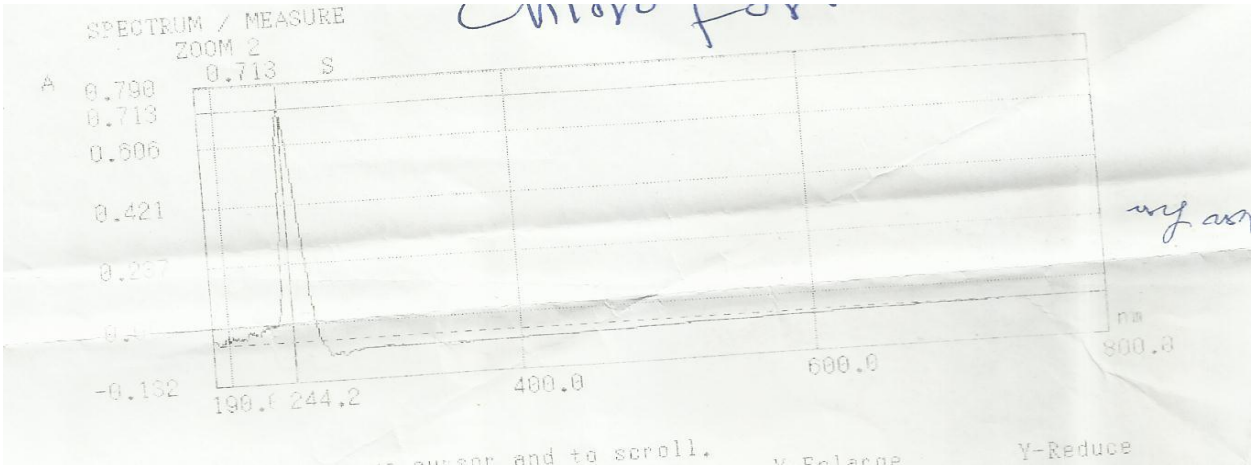


Figure (5.28): UV spectrum of chloroform

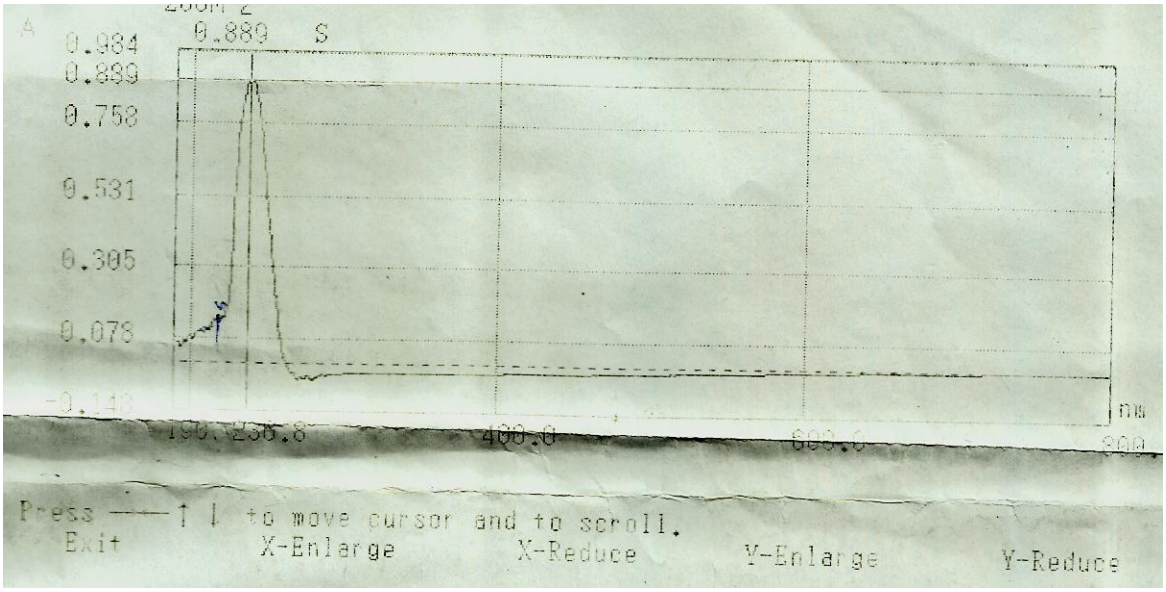


Figure (5.29): UV spectrum of dichloromethane

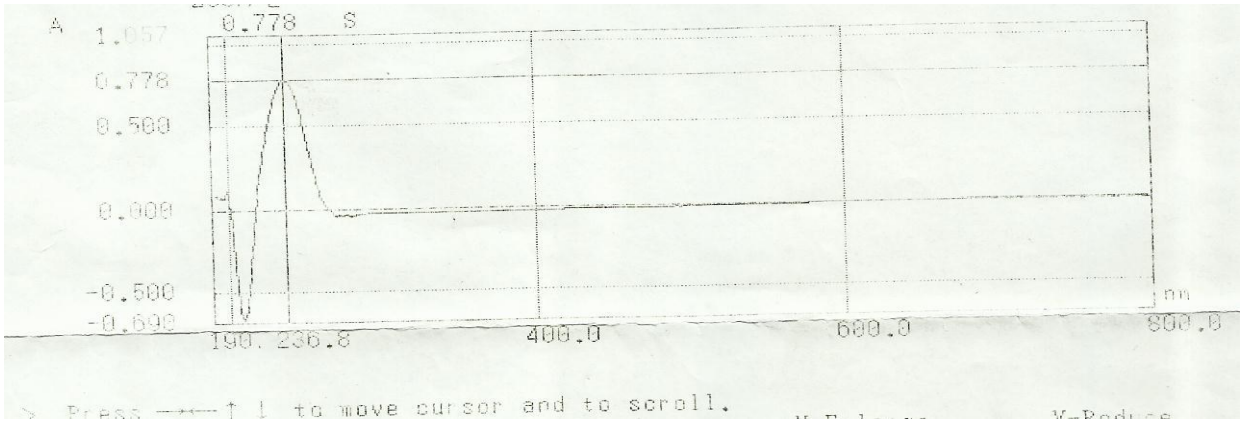


Figure (5.30): UV spectrum of ethanol

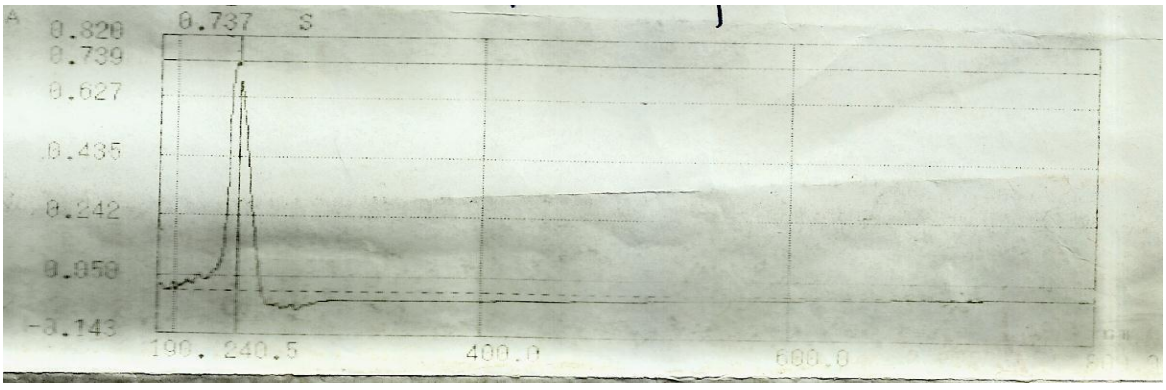


Figure (5.31): UV spectrum of tetrahydrofuran

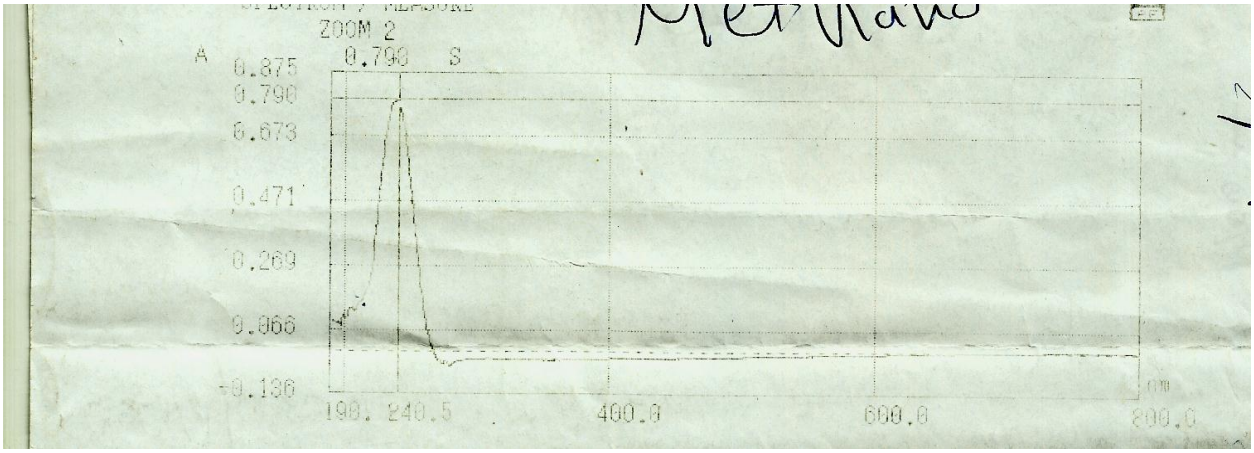


Figure (5.32): UV spectrum of methanol

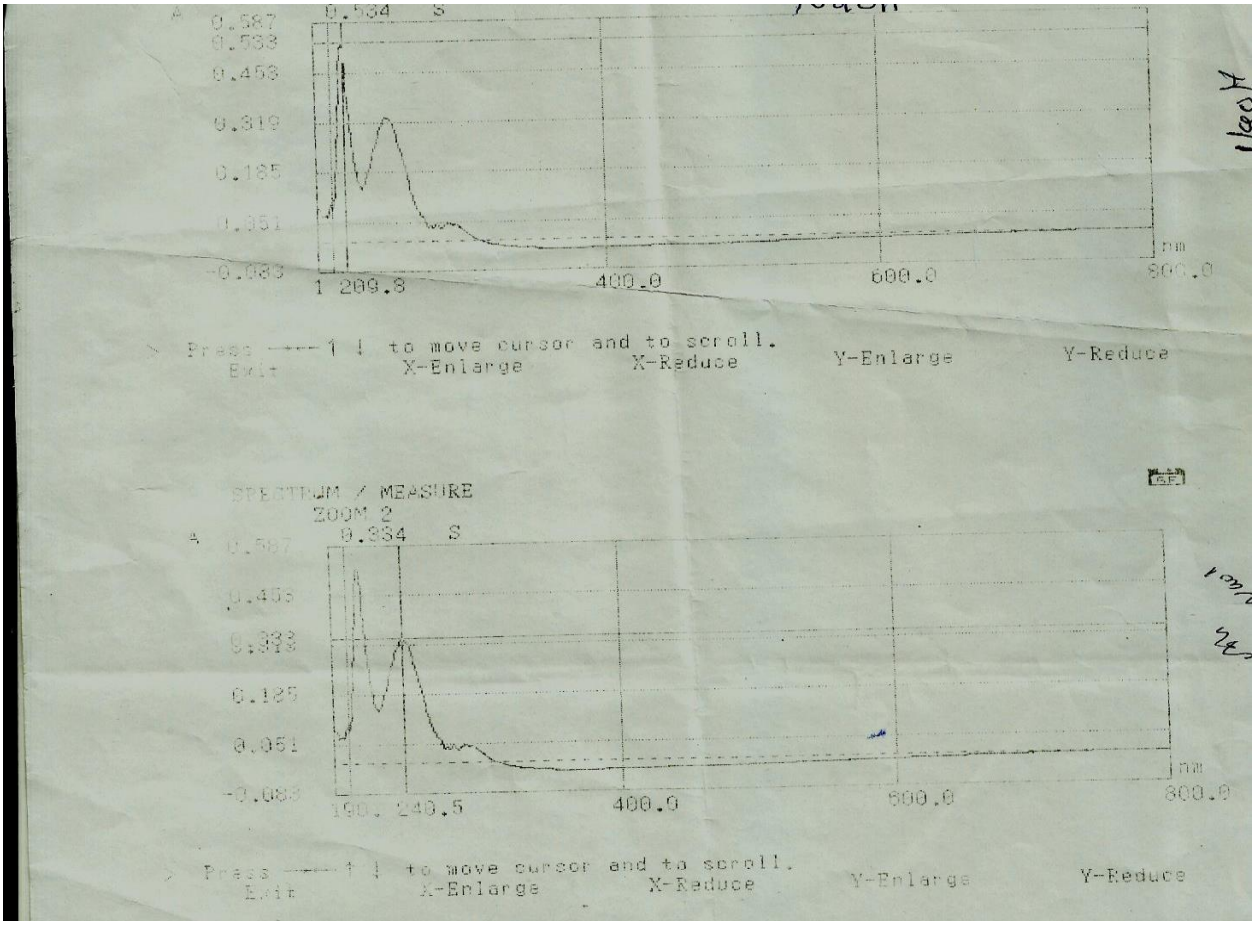


Figure (5.33): UV spectrum of methanol + drop of 0.1 M NaOH

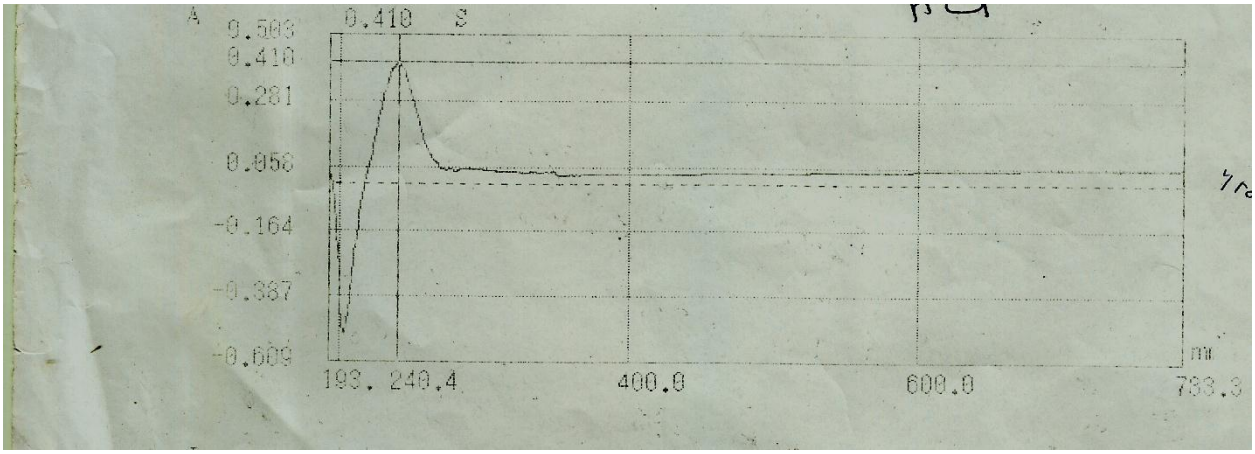


Figure (5.34): UV spectrum of methanol + drop of 0.1 M HCl