Technology Sudan University of Science and

College of graduate studies

Development and Validation of SOME Analytical Methods for Quantitative Determination of Allopurinol Drug

التطوير والتحقق لبعض الطرق التحليلية للتقدير الكمي لعقار الألوبيرينول

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الآية

قَالَ تَعَالَىٰ:

بسم الله الرحمن الرحيم

﴿ ...وَمَآ أُوتِيتُم مِّنَ ٱلْعِلْمِ إِلَّا قَلِيلًا ﴾

صدق الله العظيم

(سورة الإسراء الآية 85)

Dedication

This piece of work is dedicated to my father, who taught me that the best kind of knowledge to have is that which is learned for its own sake. It is also dedicated to my mother, who taught me that even the largest task can be accomplished if it is done one step at a time. It is also dedicated to my sisters, brothers and my friends.

Acknowledgment

First I would like to thank ALLAH for all things given to me. Iwould like to thank my supervisor Prof.Dr. Ahmed Elsadig Mohammed saeed and thank Dr. Elsadig Rudwan. Iwould also like to thank my colleagues in drug quality control laboratories, in national health laboratories, for their support, cooperation and help throughout this work.

Abstract

The aim of the present study was to develop and validate HPLC method and UV Spectrophotometeric method for the determination of allopurinol. In RP- HPLC a mixture of 70% buffer solution (mono basic ammonium phosphate 0.05M with 30% acetonitrile and methanol1:1) (v/v) was used as a modified mobile phase mixture. The column was C8 (250×4.6mm). The flow rate was set to 1ml/mint. The method was validated regarding linearity, range, limit of detection, limit of quantitation, precision, and accuracy. The obtained results showed that the validated method have good linearity, accuracy, precision and selectivity and reducing the retention time of allopurinol from (14.26) minutes to (3.0) minutes. Analytical method development results indicated that the assay (98.43%) and the limit of detection was (0.051µg/ml), limit of quantitation was (0.156µg/ml) and assay exhibited a linear over the range of (10-50µg/ml). The modified method of chromatography gave a linear relationship at a wavelength of 250 nm, linear correlation coefficient (0.9994). In the UV validated method using the buffer solution (pH 4.5). The method validated according to international conference on harmonization guideline, linearity range, and limit of detection, limit of quantitation, precision, and accuracy. The modified method of UV gave a linear relationship at a wavelength of 250 nm, linear correlation coefficient (0.9991).The validated method showed results the indicated limit of detection was (0.052µg/ml), limit of quantitation was (0.158µg/ml) and assay of UV validated method (100.15%) Respectively results Were compared with the official method were conducted analyzes in two different days to determine relative standard deviation which did not exceed 2%, the developed methods in present research successfully in the quantitation analysis of commercial preparation tablets of different

sources. The study summarized the results of the analysis are not different from those obtained by official method approved therefore developed method considered to be fit, the best because fast, inexpensive and more safe, which can be of use for routine analysis drug lab.

الخلاصة

الهدف من هذه الدراسة التطوير والتحقق من صحة طريقتي كروموتوغرافيا السائل ذات الأداء العالي ومطيافية الأشعة فوق البنفسجية لتقدير الألوبيرينول 100ملجرام .في كروموتوغرافيا الساثل ذات الأداء العالي تم تعديل الطور المتحرك بخلط 70٪ من المحلول المنظم (فوسفات الأمونيوم أحادية القاعدية بمولارية 0.05م واس هيدروجيني 4.5 مع 30٪ من الأسيتونترايل والميثانول بنسبة 1:1). تم تغير الكولوم من (C_{18}) الي (C_8) بطول (4.6مم × 250) ، حقنت كميات متساوية مكغ/مل . تم تعديل معدل التدفق إلى 1/مل ف الدقيقه. تم التحقق من صحة هذه الطريقة فيما يتعلق مع الخطية والمدى، وحد الكشف، وحد القياس الكمي ، والدقة .وأظهرت النتائج المتحصل عليها أن هذه الطريقة جيدة، ودقيقة ومضبوطة وانتقائية وخطية وكانت التراكيز قي المدى ما بين (10- 50مكغ/مل) وقد تم تقليل زمن الاستبقاء للألوبيرينول من 14.26 دقيقة الي 3.0 دقائق وكانت نتيجة فحص نقاؤة العينة (98.43٪)، وحد الكشف(0.05مكغ/مل) وحد القياس الكمي (0.158مكغ/مل). الطريقة المعدلة للكروماتوغرافيا أعطت علاقة خطية عند طول موجي 250 نانوميتر بمعامل ارتباط (0.9994).

في طريقة الأشعة فوق البنفسجية تم استخدام محلول منظم ذو أس هيدروجيني (4.5) وبالرجوع لموجهات المؤتمر العالي للتوافق للتحقق من صحة، الحد من الكشف، والحد من الكميات، والدقة، والمضبوطية وأعطت طريقة مطيافية الأشعة فوق البنفسجية علاقة خطية عند نفس الطول موجي (250نانوميتر) وبمعامل ارتباط (0.9991). وأشارت نتائج التحقق لحد الكشف (0.052مكغ / مل)، و حد الكشف القياسي (0.158مكغ / مل). وكانت نتيجة فحص نقاؤة العينة (100.15٪). تمت مقارنة النتائج مع نتائج أجريت بطرق التحليل الدستورية وتم اجراء التحاليل في يومين مختلفين وجهازين مختلفين لمعرفة درجة الأنحراف القياسي والتي لم تتجاوز 2%.

أستخدمت الطرق المستحدثة في البحث الحالي بنجاح تام في التحليل الكمي للمستحضر التجاري (أقراص) من مصادر مختلفة .

لخصت الدراسة الي أن نتائج التحليل لا تختلف عن تلك المتحصل عليها بالطرق الدستورية المعتمدة علية فأن الطرق المستحدثة تعتبر الأفضل لأنها سريعة وغير مكلفة وامنة مما يمكن من استخدامها في التحليل الروتيني لمعامل الأدوية.

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List of Abbreviations

B.NO: Batch Number

BP: British pharmacopeia

EP: European Pharmacopeia

EURACHEM: European Analytical Chemistry

EXP Date: Expire Date

FDA: Food and Drug Administration

GMP: Good Manufacturing Practice

HPLC: High Performance Liquid Chromatography

ICH: International Conference on Harmonization

ISO: International Standard Organization

Mf. Date: Manufacture Date

QA: Quality Assurance

QC: Quality Control

RSD: Relative Standard Deviation

SD: Standard Deviation

USP: United State Pharmacopeia

UV: Ultra Violet

WHO: World Health Organization

LOD: Limit of Detection

LOQ: Limit of Quantitation

CHAPTER ONE

1. INTRODUCTION

Method validation is very important parameters in pharmaceutical drug analysis in order to attain high reliable and confidence results. The accurate analytical results refer to the way of performing the method. Analytical method validation is required for herbal procedure, new process and reaction, active ingredients, impurity profiling and component of interest in different matrices. An analytical methodology consists of the techniques, method, procedure and protocol. This methodology required data for a given analytical problem, sensitivity, accuracy, range of analysis and precision to the analyst. Method validation is a vital parameter for assuring quality, achieving acceptance of products by the international agencies, mandatory requirement purposes for accreditation in accordance to ISO 17025 guidelines. The international official methodology for drug standards is used in conducting drug quality control such as USP, BP, …etc. The applicability of these official methods sometimes faces with various limitations in routine analysis such as economical factors or long time duration of the analysis. So any substitution for the official method to come over these limitations needs approaches to conduct validation of the method.

1.1. Quality Assurance

Quality assurance is a wide ranging concept covering all matters that individually or collectively influence the quality of a product. It is the totality of the arrangements made with the object of ensuring that pharmaceutical products are of the quality required for their intended use. Quality assurance therefore incorporates good manufacturing practice and other factors, such as a product design and development. Every government allocates a substantial proportion of its total health budget to medicines. In developing countries considerable administrative and

technical effort is directed to ensuring that patients receive effective medicines of good quality (World Health Organization, 2007).

1.2. Compendial Testing

To assure drug quality, various countries have published texts commonly called compendial or pharmacopoeia that list official test methods as well as specification for commonly used drug product. Three notable examples of such compendia are the United States pharmacopoeia USP, BP, EP, and JP. Compendia methods should be implemented as written except where scientifically justified changes are necessary. Although considerable efforts are ongoing to standardize pharmacopoeia, differences may exist between the USP and other pharmacopoeia. In these instances, testing should be done in accordance with the procedures described in the pharmacopoeia that governs the country or region for which the product is intended. The USP is used as important guide by a number of countries. It is also the most commonly used document by QC and QA departments. In the United States the QA function is critical to assure effective safe products are released to the market place. The QC analytical laboratory is the final step in a long line of process where many individuals from diverse departments take part to ensure the safety, efficacy and quality of drug products. Producing quality product requires not only a good testing laboratory but an organization that is empowered to identify problems and develop innovative solutions. Analytical testing is one of the more interesting ways for scientists to take part in the quality process by providing actual data on the identity, content, and purity of drug products.

1.3 Basic test for drug substances and drug product

1.3.1 Description of active ingredients and solid oral dosage form

A description test is a qualitative physical description of the drug product including the dosage form and any color, and any other identifying

marking. The description test is critical and if it is incorrect, that particular batch of product is immediately considered defective. Description testing is not included in the USP because the physical description of product is unique to the manufacturer. Generic products containing the same drug substances have their own identifying, marking different from those of the branded product.

1.3.2. Identification of active pharmaceutical ingredient

Identification testing is designed to confirm the identity or presence of the active ingredient by employing a variety of analytical techniques and methods for drug formulation. The drug substance may need to be extracted from the dosage form. Techniques such as IR spectroscopy may eliminate the need to isolate the active ingredient. Once the pure compound is obtained, a spectroscopy technique such as UV, IR, or melting point will be used to compare the sample identity to that of a standard that has been similarly prepared. The characteristics of the compound will help define which type of spectroscopy will be most useful. One of the most important goals of identification testing is that it must be specific enough to distinguish between compound with similar structures including starting materials and degradation products. In some cases, non specific methods are sometimes used in conjunction to obtain a positive identification. High-performance liquid chromatography (HPLC) is commonly used analytical technique with the retention time being indicative of the compound. However, HPLC retention time is not usually regarded as specific for identification testing, but can be used in conjunction with other tests such as thin-layer chromatography (TLC), IR spectroscopy, UV visible spectroscopy, or other physical test such as melting point. When retention time is used, the standard and sample must elute at similar times. Many drug substances are used in the form of the salt. The identification of these materials may also include a test for

the specific counter ion used in pharmaceutical is sodium, chloride, and pamoate ions.

1.3.3 Assays

The test which commonly performed in the QC laboratory is the assay. This test is used to determine the purity of an active substance present in the dosage form or the amount of an active ingredient present in dosage form. The information is used to support the manufactures claim on the label. Analytical techniques such as chromatography are typically used. Common methods for testing assay are UV spectroscopy, titration and HPLC. in order methods, automated UV systems or column chromatography may be employed, but recently have become obsolete. HPLC is the technique usually chosen because of its specificity. Some of the more interesting assay tests are those where more than one drug substance is present in the dosage form. As with identification testing, depending on the dosage form, different procedures may be needed. (Satinder Ahuja, 2001).

1.4 Validation of analytical methods

The objective of any analytical measurement is to obtain consistent, reliable and accurate data. Validated analytical methods play a major role in achieving this goal. The results from method validation can be used to judge the quality, reliability and consistency of analytical results which is an integral part of any good analytical practice. Validation of analytical methods is also required by most regulations and quality standards that impact laboratories. Analytical methods need to be validated, verified, or revalidated in the following instances:

- Before initial use in routine testing
- When transferred to another laboratory
- Whenever the conditions or method parameters for which the method has been validated change for example, an instrument with

(ICH, 1995) different characteristics or samples with a different matrix) and the change is outside the original scope of the method.

Method is validated in term of: specificity, selectivity, precision, repeatability, intermediate precision, reproducibility, accuracy, linearity, range, limit of detection, limit of quantitation, Robustness, ruggedness, ruggedness. (ICH, 1996)

1.5 Validation

Validation of analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for the intended analytical purpose. The definitions refer to "test result". The description of the analytical procedure should define what the test results for the procedure are. The test method should specify that one or number of individual measurements be made, and their average, or another appropriate function (such as the median or the standard deviation), be reported as the test result. It may also require standard correction to be applied, such as correction of gas volumes to standard temperature and pressure. Thus, a test result can be a result calculated from several observed values. In the simple case, the test result is the observed value itself. A test result also can be, but need not be, the final, reportable value that would be compared to the acceptance criteria of a specification. Validation of physical property methods may involve the assessment of chemometric models. However, the typical analytical characteristic used in method validation can be applied to the methods derived from the use of the chemo metric models. The effects of processing conditions and potential for segregation of materials should be considered when obtaining a representative sample to be used for validation of procedures. Typical analytical characteristics used in method validation. In the case of compendia procedures, revalidation may be necessary in the following

cases: a submission to the USP of a revised analytical procedure, or the use of an established general procedure with a new product or raw material. The ICH documents give guidance on the necessity for revalidation in the following circumstances: changes in the synthesis of the drug substance; changes in the analytical procedure. (kenkel, 2000)

1.5.1 Analytical Performance Characteristics

1.5.1.1. Accuracy and Recovery

The accuracy of an analytical method is the extent to which test results generated by the method and the true value agree. Accuracy can also be described as the closeness of agreement between the values that is adopted, ether as a conventional, true or accepted reference value found. The true value for accuracy assessment can be obtained in several ways. One alternative is to compare the results of the method with results from an established reference method. This approach assumes that the uncertainty of the reference method is known. Secondly, accuracy can be assessed by analyzing a sample with known concentration (e.g, a control sample or certified reference material) and comparing the measured value with the true value as supplied with the material. If certified reference materials or control samples are not available, a blank sample matrix of interest can be spiked with a known concentration by weight or volume. After extraction of the analyte from the matrix and injection into the analytical instrument, its recovery can be determined by comparing the response of the extract with the response of the reference material dissolved in a pure solvent. Because this accuracy assessment measures the effectiveness of sample preparation, care should be taken to mimic the actual sample preparation as closely as possible. If validated correctly, the recovery factor determined for different concentrations can be used to correct the final results. The concentration should cover the range and should include concentration close to the quantitation limit, one in the

middle of the range and one at the high end of the calibration curve. Another approach is to use the critical decision value as the concentration point that must be the point of greatest accuracy.

(kenkel, 2000)

1.5.1.2. Precision and Reproducibility

The precision of a method is the extent to which the individual test result of multiple injections of a series of standards agrees. The measured standard deviation can be subdivided into 3 categories: repeatability, intermediate precision and reproducibility. Repeatability is obtained when the analysis is carried out in a laboratory by an operator using a piece of equipment over a relatively short time span. At least six determination or three different matrices at two or three different concentration should be performed, and the RSD calculated. The ICH requires precision from at least six replications to be measured at 100 percent the test target concentration or from at least nine replications covering the complete specified range. The acceptance criteria for precision depend very much on the type of analysis. Pharmaceutical QC precision of greater than 1 percent RSD is easily achieved for compound analysis. Precision is largely dependent on the sample matrix, the concentration of the analyte, the performance of the equipment and the analysis technique. Reproducibility as defined by the (ICH) represents the precision obtained between different laboratories. The objective is to verify that the method will provide the same results in different laboratories. The reproducibility of an analytical method is determined by analyzing aliquots from homogeneous lots in different laboratories with different analysts, and by using operational and environmental conditions that may differ from, but are still within, the specified parameters of the method inters laboratory tests. Validation of reproducibility is important if the method is to be used in different laboratories. (ICH, 1996)

1.5.1.3. Linearity and Calibration Curve

The linearity of an analytical method is its ability to elicit test result that are directly proportional to the concentration of analytes in samples within a give range or proportional by means of well-defined mathematical transformation. Linearity may be demonstrated directly on the test substance (by dilution of a standard stock solution) and by using separate weighing of synthetic mixtures of the test product components, using the propose procedure. Linearity is determined by a series of 3 to 6 injection of 5 or more standards whose concentration span 80-120 percent of the expected concentration range. The response should be directly proportional to the concentration of the analytes or proportional by means of a well-defined mathematical calculation. A linear regression equation applied to the result should have an intercept not significantly different from zero. If a significant nonzero intercept is obtained, it should be demonstrated that this has no effect on the accuracy of the method. The ICH recommends, for accuracy reporting, the linearity curve's correlation coefficient, y-intercept, slope, of the regression line and residual sum of squares. A plot of the data should be included in the report. In addition, an analysis of the deviation of the actual data point from the regression line may also be helpful for evaluating linearity. Some analytical procedure, such as immunoassays, does not demonstrate linearity after any transformation. In this case, the analytical response should be described by an appropriate function of the concentration (amount) of an analyte in a sample.

1.5.1.4. Range

The range of an analytical method is the interval between the upper and lower levels (including these levels) that have been demonstrated to be determined with precision, accuracy and linearity using the method as written. For assay tests, the ICH requires the minimum specified range to

be 80 to 120 percent of the test concentration, and for the determination of an impurity the range to extend from the limit of quantitation, or from 50 percent of the specification of each impurity, whichever is greater, to 120 percent of the specification.

1.5.1.5. Limit of Detection

The limit of detection is the point at which a measured value is larger than the uncertainty associated with it. It is lowest concentration of analyte in a sample that can be detected but not necessarily quantified. The limit of detection is frequently confused with the sensitivity of the method. The sensitivity of an analytical method is the capability of the method to discriminate small differences in concentration or mass of the test analyte. In practical terms, sensitivity is the slope of the calibration curve that is obtained by plotting the response against the analyte concentration or mass. In chromatography, the detection limit is the injected amount that results in a peak with a height at least two or three times as high as the baseline noise level. Besides this signal noise method the (ICH) describes three more methods:

Visual inspection: The detection limit is determined by the analysis of samples with known concentration of analyte and by establishing the minimum level at which the analyte can be reliably detected. Standard deviation of the response based on the standard deviation of the blank: Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

Standard deviation of the response based on the slope of the calibration curve: A specific calibration curve is studied using samples containing an analyte in the range of the limit of detection. The residual standard deviation of a regression line, or the standard deviation of y-intercepts of regression lines, may be used as the standard deviation. (ICH, 1996)

1.5.1.6. Limit of Quantitation

The limit of qunantitation is the minimum injected amount that produces quantitative measurements in the target matrix with acceptable precision in chromatography, typically requiring peak heights 10 to 20 times higher than the baseline noise. If they require precision of the method at the limit of quantitation has been specified, the EURACHEM approach can be used. A number of samples with decreasing amounts of the analyte are injected six times. The calculate RSD percent of the precision is plotted against the analyte amount. The amount that corresponds to the previously defined required precision is equal to the limit of quantitation. It is important to use not only pure standard for this test but also spiked matrices that closely represent that unknown samples. For the limit of detection, the ICH recommends, in addition to the procedures as described above, the visual inspection and the standard deviation of the response and the slope of the calibration curve. (ICH, 1996)

$LOQ = 10\times \partial /S$, $LOD = 3.3\times \partial /S$

Description: σ is the standard deviation and S is the slope of the calibration curve. (Thompcon and Ellison, 2002)

1.5.1.7. Robustness

The robustness of analytical procedures is a measure of its capacity to remain unaffected by small but deliberate variation in procedural parameters listed in the procedure documentation and provides an indication of its suitability during normal usage. Robustness may be determined during development of the analytical procedure.

(U.S. Pharmacopeia), (International Pharmacopeia)

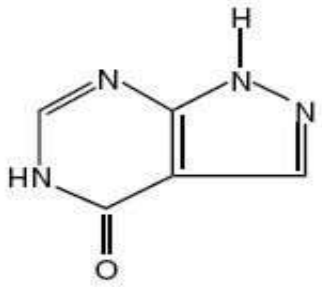
Table (1.1) Method validation acceptance criteria

Validation element	Recommendation	Acceptance Criteria
Linearity and range	Minimum 5 points Slope Y Intercept Correlation coefficient	 RSD≤2 Y close to zero $R^2 \geq 0.99$
Precision and repeatability	6 replicate analysis of sample at 100% test condition	RSD ≤2
Accuracy	Triplicate spiking of placebo at 80, 100,120% of label calim	98-102%
Intermediate precision	6 replicate analysis by different analyst on different day	RSD ≤2

1.6. Allopurinol

Common Brand: Zyloprim

Generic Name: Allopurinol



(C5H4N4O) 136.1

1H-pyrazolo [3,4-d]pyrimidin-4(2H)-one. (IUPAC)

1.6.1. Mechanism of action

Allopurinol is a purine analog; it is a structural isomer of hypoxanthine (a naturally occurring purine in the body) and is an inhibitor of the enzyme xanthine oxidase. (Pacher *et al*, 2006) Xanthine oxidase is responsible for the successive oxidation of hypoxanthine and xanthine, resulting in the production of uric acid, the product of human purine metabolism. In addition to blocking uric acid production, inhibition of

xanthine oxidase causes an increase in hypoxanthine and xanthine. While xanthine cannot be converted to purine ribotides, hypoxanthine can be salvaged to the purine ribotides adenosine and guanosine monophosphates. Increased levels of these ribotides may cause feedback inhibition of amidophosphoribosyl transferase, the first and rate-limiting enzyme of purine biosynthesis. Allopurinol, therefore, decreases uric acid formation and may also inhibit purine synthesis. (Cameron *et al*, 1993)

1.6.4. General Properties

A white or off white, almost odourless powder. The melting point is above 350. The molecular weight is 136.11. C: 44.12%, H: 2.96%, N: 41.16%, O: 111.75%. Very slightly soluble in water and alcohol practically insoluble in chloroform and in ether and dissolved in dilute solutions of acids and alkali hydroxides.

1.6.2. Pharmacology

A common misconception is that allopurinol is metabolized by its target, xanthine oxidase, but this action is principally carried out by aldehyde oxidase. (Reiter *et al*, 1990) The active metabolite of allopurinol is oxypurinol, which is also an inhibitor of xanthine oxidase. Allopurinol is almost completely metabolized to oxypurinol within two hours of oral administration, whereas oxypurinol is slowly excreted by the kidneys over 18–30 hours. For this reason, oxypurinol is believed responsible for the majority of allopurinol's effect. (Graham *et al*, 2007)

1.6.5. Side effects

Because allopurinol is not a uricosuric, it can be used in patients with poor kidney function. However, allopurinol has two important disadvantages. First, its dosing is complex. (Dalbeth *et al*, 2007)

Second, some patients are hypersensitive to the drug; therefore its use requires careful monitoring. Allopurinol has rare but potentially fatal adverse effects involving the skin. The most serious adverse effect is a hypersensitivity syndrome consisting of fever, skin rash, eosinophilia, hepatitis, worsened renal function, and, in some cases, allopurinol hypersensitivity syndrome. (Tsai and Yeh, 2010). Allopurinol is one of the drugs commonly known to cause Stevens Johnson syndrome and toxic epidermal necrolysis, two life-threatening dermatological conditions. (Roujeau and Kelly, 1995). More common is a less-serious rash that leads to discontinuing this drug. More rarely, allopurinol can also result in the depression of bone marrow elements, leading to cytopenias, as well as a plastic anemia. Moreover, allopurinol can also cause peripheral neuritis in some patients, although this is a rare side effect. Another side effect of allopurinol is interstitial nephritis. (Marc *et al*, *2003*). It is suspected to cause congenital malformations in a newborn infant whose mother was on allopurinol treatment through the pregnancy, and should be avoided whenever possible by women trying to conceive or during pregnancy. (Kozenko *et al*, 2011)

1.7. Quantification of allopurinol

Mattheus and coworkers a simple method for quantification of allopurinol and oxipurinol in human serum by HPLC with UV- detection (Mattheus *et al*, 2007)

Vani and coworkers described analytical method development and validation for the determination of ALP and alphalipoic using Reverse phase HPLC method bulk and tablet dosage form. (Vani *et al*, 2015)

Patel and coworkers development and validation of spectroscopic absorbavce correction method for simul taneous estimation of allopurinol and 1±lipoic acid in combination tablet (patel, *et al*, 2014)

Allopurinol quantified in USP (2015) by HPLC method. The column using 4-mm×30-cm contains packing (L1), the λ_{max} 254nm, flow rate is about 1.5 ml per minute, inject equal volumes about (15µ) and the mobile phase using 0.05M solution of monobasic ammonium phosphate.

BP quantified UV spectrophotomeric method at λ_{max} 250nm using hydrochloric acid in the reference cell and calculated the content of allopurinol taking 563 as the value of A (1%).

1.8. Aims and Objective

-The purpose of this study is to develop specific, sensitive, safe and selective and accurate methods for analysis of allopurinol.

-To develop, optimize and use direct spectrophotometer analytical method for the determination of allopurinol tablets by using acetate buffer (pH 4.5), and application on different samples marketed in Sudan.

-To validate HPLC and UV methods for determination allopurinol in tablet and compare the analytical results with official (USP and BP) methods.

CHAPTER TWO

2. MATERIALS, STANDARD, INSTRUMENTS AND METHODS

2.1 Materials

2.1.1 Chemical solvents and reagents

- Methanol HPLC grade, Scharlau, Spain
- Ethanol HPLC grade, Scharlau, Spain
- Acetonitrile HPLC grade, Scharlau Spain
- Hydrochloric acid (PRS), Barcelona, Spain
- Ortho-Phosphoric acid, 85%, Scharlau, Spain
- Sodium hydroxide pellets, reagent grade, ACS, ISO, Reag. Ph Eur, Scharlau, Spain
- Ammonia solution, Darmstadt, Germany
- Sodium acetate anhydrous, Scharlau Spain
- Mono basic ammonium phosphate, Scharlau Spain

2.2 Standard

Allopurinol working standard from Blue Nile Pharmaceutical Khartoum, Sudan N: BNP/ALP-120120/ MFG Date: 03/2012/ Exp Date: 01/2017/Loss on drying: 0.04%/Assay potency: 99.45%

2.3 INSTRUMETS

2.3.1. High Performance Liquid Chromatography

- Shimadzu liquid chromatography, with UV/Visible and diodray-detectors, isocratic and low pressure gradient pump and PC control (Japan). Two instruments with different Code (LO4, LO5)

2.3.2 Ultra violet - visible spectrophotometer

-Shimaduzu Model 1700-UV/visible spectroscopy double beam with PC control and capability of derivative mode (Japan).

- Perkinelmer UV/VIS Spectrometer lambada 25.

2.3.4 Infrared Spectroscopy

Infrared spectrometer, TF/IR -4100 Fourier transform serial number B187361016 Jasco Japan.

2.3.5 General equipments

Sensitive balance, Model: ADAM AAA 250LE _ENGLAN

Vacuum Pressure Perkin Elmer. USA

2.4 Samples

Table (2.1): Three samples of allopurinol were collected randomly from local pharmacies in Khartoum state.

NO	Dosage form	Tread. Name	Manufacture	B. No	Mf. Date	Exp. Date
1	Tablets	Cityuric 100mg	City pharm-Sudan	P20	11/2013	11/2015
2	Tablets	Alopron 100mg	Remedica - Cyprus	59046	03/14	3/2019
3	Tablets	Zyloric 100mg	Bad oldesloe, Germany	B8261 4D	APR 2014	APR 2019

2.5 Methods

2.5.1 Identification test for allopurinol

A standard IR runs a single spectrum. An FT-IR uses an interferometer and makes several scans and then uses Fourier Transforms to convert the interferogram into infrared spectrum. IR used monochromatic light where FTIR used polychromatic. In IR only narrow wave length are identified

using interferometer in place of grating. In IR single time scanning while in FT-IR number of scans more; scan 50 times in minute providing better resolution and compound using library database available it is more sensitive.

The FTIR spectra of reference standard, test sample of allopurinol were recorded with FT-IR spectrometer 4100 (Jasco, Japan) by KBr disk method, prepared by finely grinding 1part of allopurinol with about 250 part of dried potassium bromide. The mixture was compressed under 10 tons in vacuum pressure Perkin Elmer. FT-IR computerized spectrometer was used to obtain IR spectrum, the wave length range of 400 to 4000cm^{-1} scanning speed (2mm/sec), resolution 4cm^{-1} using detector (TGS) tri glycine sulfate.

2.5.2 Effect of solvent upon UV spectrum of allopurinol

Accurate weight 0.109g of allopurinol was dissolved in each dry 100 ml volumetric flask, with solvent (water, methanol, ethanol, acetonitrile, n.hexan, ethyl acetate, 0.1M HCL, 0.1 NaOH) 1ml of the above solution was diluted with 50% methanol to obtain 10µg/ml of allopurinol and scanned in range 200-400 nm.

2.5.3 Effect of acid and base upon UV spectrum of allopurinol

Accurate weight 0.101g of allopurinol was dissolved in 100 ml volumetric flask with hydrochloric acid 0.02M or 0.02M NaOH used as above to have a concentration of 10µg/ml both are scanned over the range of 200 to 400nm.

2.5.4 Effect of pH on the UV spectrum of allopurinol

Accurately weight of 0.105g of allopurinol transferred into 100 ml volumetric flask with methanol. 1ml was transferred in 100ml volumetric flask and the volume was complete to the mark with buffer and (methanol and acetonitrile 1:1). Aliquots was dissolved in different ratio, in pH

range from 1 to14, to obtain a concentration of 10µg/ml, the solution was scanned in the range over 200 to 400 nm.

2.6. Method Validation

2.6.1 Selection of solvent

Allopurinol was very slightly soluble in water, so after study the solubility profile of allopurinol in different solvents, methanol as solvent was selected as common solvent for developing spectral characteristics.

2.6.2 Preparation of standard solution

In UV method 0.025 g of allopurinol standard was weighed accurately by using sensitive balance, dissolved in 25ml volumetric flask with 5 ml sodium hydroxide 0.05M. The solution was sonicated for 10 minutes.10 ml of the acetate buffer was added, sonicated for 10mint. The solution was kept at room temperature, completed to the mark with water.

In HPLC method the same weight was taken dissolved in 25ml volumetric flask and 5ml sodium hydroxide 0.1M was added, shake by mechanical shaker for10 minutes.10ml of hydrochloric acid 0.1M. The solution was kept at room temperature and completed to the mark with water.

2.6.3 Linearity and range of the two methods

From stock solution was taken (1, 2, 3, 4, 5 ml) by volumetric pipette and transferred to 100 ml volumetric flask, sonicated and completed to the mark with acetate buffer (pH 4.5), to obtain concentration range of (10-50µg/ml) for determination of linearity of UV method.

In the HPLC method the same volumes transferred to 100 ml volumetric flask, sonicated and completed to the mark by mobile phase (mixed with70% monobasic ammonium phosphate pH 4.5, and (30%methanol and acetonitrile 1:1).

2.6.4 Accuracy and recovery of the two methods

2.6.4.1 Preparation of allopurinol standard stock solution

0.0781 g of allopurinol standard was weighted and transferred into the 25 ml volumetric flask, dissolved with 5ml NaOH 0.05M, Shaked by mechanical shaker for 10 minutes, 10ml from acetate buffer was added and shaked10 minutes. The solution was left to cool down at room temperature, completed to the mark with water and transferred 4ml in the 100ml volumetric flask, completed with acetate buffer (pH 4.5) for UV method.

In HPLC method the same weighed was taken dissolved in the 25 ml volumetric flask with 5ml from NaOH 0.05M, shaked by mechanical shaker 10 minutes. The solution was left to cool down at room temperature, completed to the mark with water and transferred 4ml in the 100ml volumetric flask, completed with (mix with70% monobasic ammonium phosphate pH 4.5 and 30%methanol and acetonitrile 1:1).

2.6.5 Precision of the two methodes

The precision of method was checked out through intraday precision and interday precision in two different instruments.

2.6.5.1 Intraday precision

Intraday precision was assessed on the same day by the same operator by preparing five different concentrations of allopurinol standard and after that each concentration level was repeatedly injected for six times.

Refer section (2.6.2) from the standard stock solution (1, 2, 3, 4, 5) was taken through volumetric pipette and transferred to 100ml volumetric flask then it was shaked thoroughly until complete dissolution of standard were established . Then it was repeatedly injected in instrument for six times for HPLC method. In UV validated method accurate weight of

0.025g of allopurinol standard was transferred into 25ml volumetric flask and the volume completed with water. Taken was 1ml of standard solution and completed with acetate buffer to obtain concentration of 10µg/ml of allopurinol standard and after that the concentration was repeatedly injected for six times.

2.6.5.2 Inter day precision (repeatability between days)

Was assessed on separate days by the same operator through preparing five concentration of allopurinol standard and six time repetition of the standard on instrument

Refer to section (2.6.2) from the stock solution (1, 2, 3, 4, 5) was taken through volumetric pipette and transferred to 100ml volumetric flask then it was shacked thoroughly until complete dissolution of standard were established . Then it was repeatedly injected in instrument for six times for HPLC method. In UV method accurate weight of 0.025g of allopurinol standard was transferred into 25 ml volumetric flask and the volume completed with water. Taken was 1ml of standard solution and completed with acetate buffer to obtain concentration of 10µg/ml of allopurinol standard and after that the concentration was repeatedly injected for five times.

2.6.6 Specificity and Robustness

Assay of tablet formulation.

A commercially available of 0.0125g of allopurinol tablet form different companies was dropped into 25 ml volumetric flask containing 5ml of sodium hydroxide 0.05M and 10 ml of hydrochloric acid 0.1M completed to the mark with water and transferred 4ml in the 100ml volumetric flask, completed with (70% mono basic ammonium phosphate and 30% methanol & acetonitrile 1:1) of HPLC method.

In UV method the same weight was taken dissolved in 25ml

ml volumetric flask, with 5ml NaOH 0.05M, Shaked by mechanical shaker for 10 minutes, 10ml from acetate buffer was added and shaked 10 minutes. The solution was left to cool down at room temperature, completed to the mark with water and transferred 4ml in the 100ml volumetric flask, completed with acetate buffer (pH 4.5) for UV method.

2.6.7 Sensitivity

The sensitivity depends upon experimental condition. The maximum sensitivity of which a method is capable is expressed in term of detection limit; sensitivity of allopurinol was calculated in the range of a concentration of 10-50µg/ml at 250nm.

2.7 Design and Validated of HPLC and UV Methods

2.7.1 The development Method

In this work HPLC method for determination of allopurinol in tablets was developed and validated. The developed of the method was done by selection of suitable solvents such as methanol and acetonitrile, suitable mobile phase (70% buffer PH 4.5and 30%methanol and acetonitrile11:1) and different columns C8, C18, and detection wavelength and analyte concentration. The detection wave length of 250nm was selected after scanning the standard solution range 200-400nm by using UV detector. The development of the UV method was done by selection suitable solvent (methanol) and suitable buffer such as acetate buffer pH 4.5.

2.7.1.1 Preparation of buffer solution

Accurate weighted of 11.503 g of mono basic ammonium phosphate was transferred and dissolved in 2 litters volumetric flask, sonicated for 30 minute. The solution left to cool at room temperature, completed to the mark with water. 70% of solution pH4.5 mixed with 30% mixture methanol and acetonitrile 1:1.

2.7.1.2 Chromatographic system

The liquid chromatography is equipped with a 250 nm and 250*4.6 column that contain (C_8) and the flow rate is about 1.0 ml per minute and the separately inject equal volumes 10 µl.

CHAPTER THREE

3. RESULTS AND DISCUSSION

3.1 Identification

3.1.1 Identification test for allopurinol

The IR spectrum of allopurinol showed characteristic peak at 1650-1800 cm^{-1} indicated the presence of (C=O) carbonyl group due to a mid in allopurinol structure, 1238.21cm^{-1} (C-N) stretching, 3082.04 cm^{-1} (N-H) stretching, 1550 -1610 cm^{-1} (N-H) bending,1587.31cm^{-1} due to band of (C=C) bending of aromatic. Form IR spectrum of allopurinol Figure (3.1)

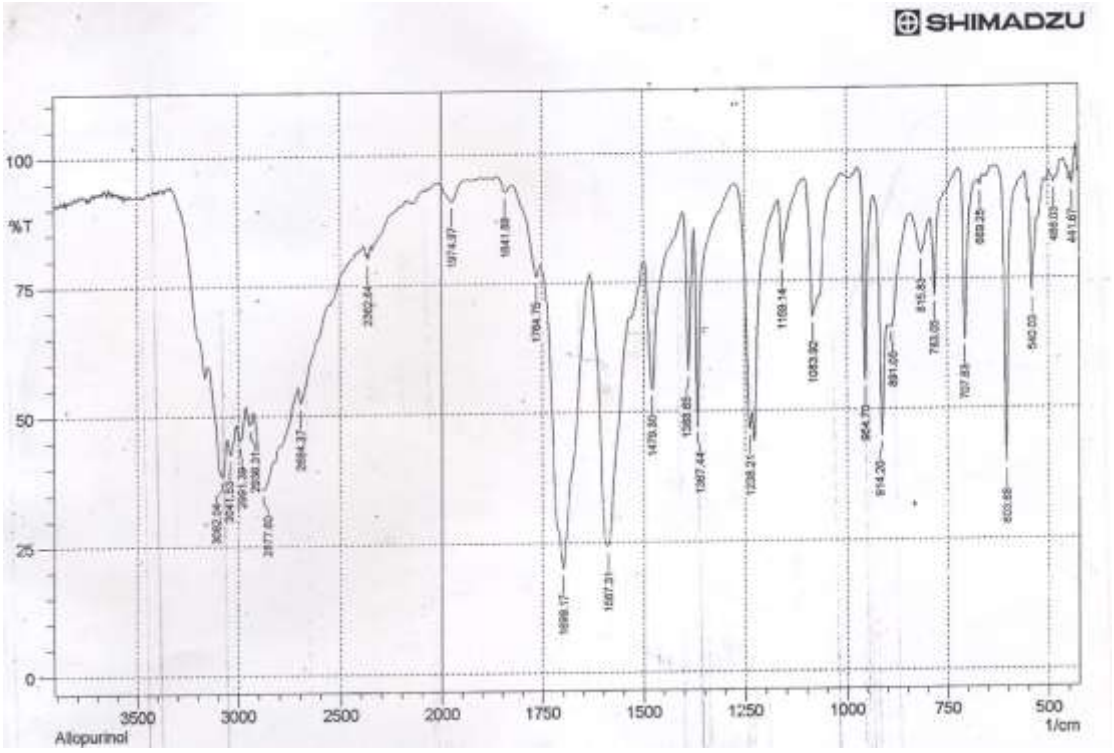


Figure (3. 1): IR spectrum of allopurinol

3.1.2 Effect of solvent upon UV spectrum of allopurinol

Water and many organic solvents such as methanol, ethanol, acetonitrile, n.hexan, ethyl acetate, etc. are used for this purpose. Theses solvents dissolved the sample well be optically transparent (not absorb incident radiation), and chemically inert and pure (Chandarasekhr et al, 2006).

Methanol selected as sutible solvent for developing characteristics of the drug. The selection assessing the solubility of drug in different solvents shown Figures (3.2) (3.3)

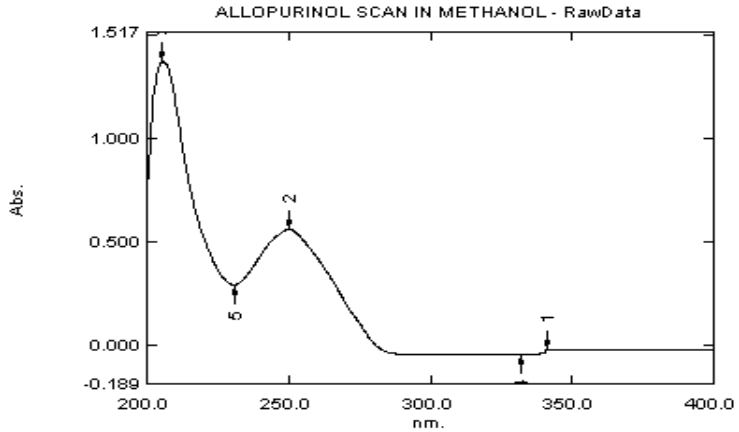


Figure (3.2): Methanol solvents effect on UV spectrum of allopurinol

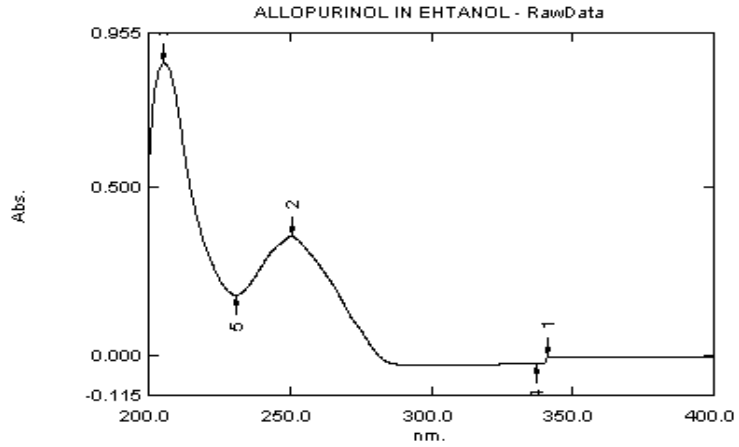


Figure (3.3): Ethanol solvents effect on UV spectrum of allopurinol

3.1.3 Effect of acid and base upon UV spectrum of allopurinol

Allopurinol maximum absorbance in acid media at wavelenghth 250 nm, is the absorbance is 0.485. Figure (3.4), the chromophores intact in extreme acid media and maximum absorbance in base media at wavelenghth 255nm and the absorbance is 0.470nm, as shown Figure (3.5).

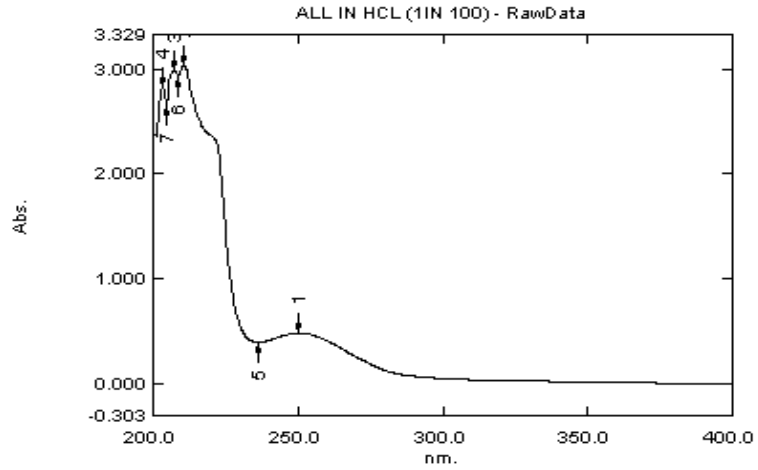


Figure (3.4) Effect acid media upon UV spectrum of allopurinol

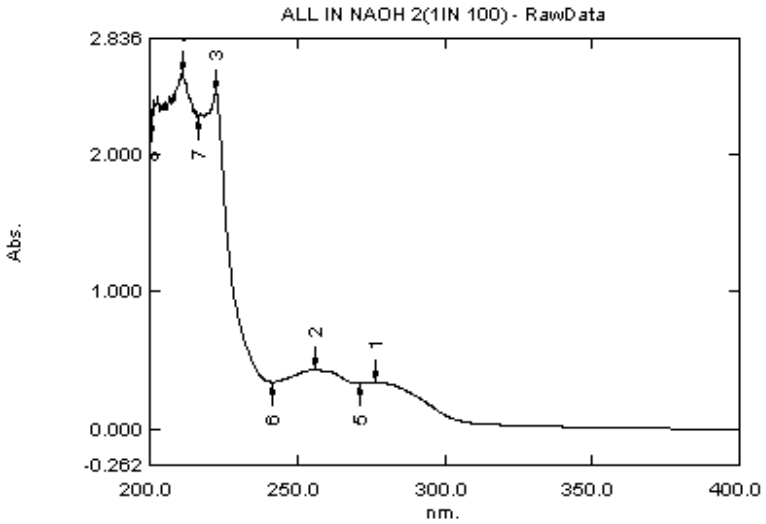


Figure (3.5) Effect base media upon UV spectrum of allopurinol

3.1.4 Effect of pH upon UV spectrum of allopurinol

The study of the effects of the pH on the sample a pperent in the cases of the high and low pH and scanned with UV spectrometer the result revealed that there is no peak apparent in pH lower than 2 and higher than pH 8 in wavelength of 250nm. But the perfect apparent peak was clear in the pH 4 in the wave length 250nm, shown Figures (3.6), (3.7) and (3.8).

Table (3.1) effect of pH upon UV spectrum of allopurinol

pH	Ratio buffer and methanol %	(λ)	ABS
1	30/70	221.00	2.012
2	40/60	221.00	1.994
3	50/50	249.80	0.436
4	30/70	250.40	0.356
5	60/40	254.80	0.307
6	40/60	266.00	0.099
7	50/50	249.8	0.469
8	50/50	221.0	1.998
9	80/20	260.4	0.032
10	20/80	252.4	0.309
11	30/70	264.2	0.365
12	60/40	221.8	1.899
13	70/30	215.4	2.910
14	80/20	222.0	1.848

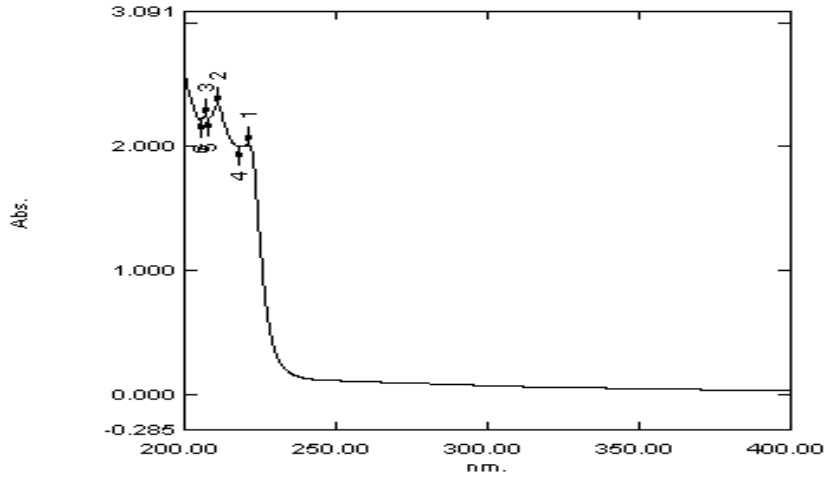


Figure (3.6): 30% buffer solution pH (1) and 70 % (methanol and acetonitrile1:1)

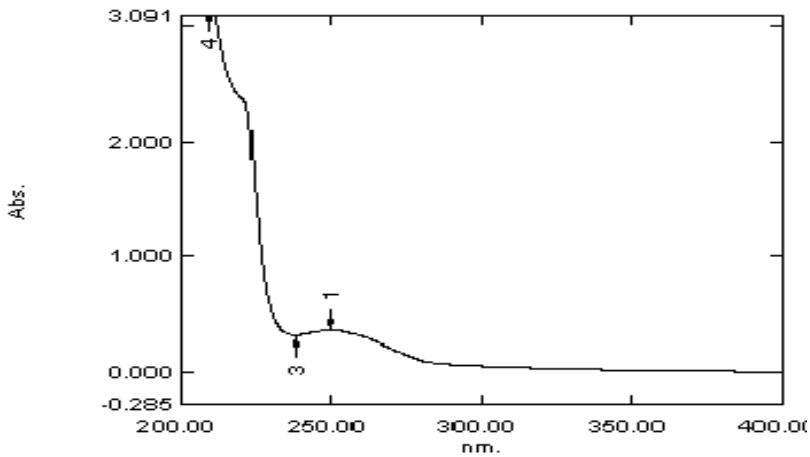


Figure (3.7): 20% buffer solution pH (4) and 80% (methanol and acetonitrile 1:1)

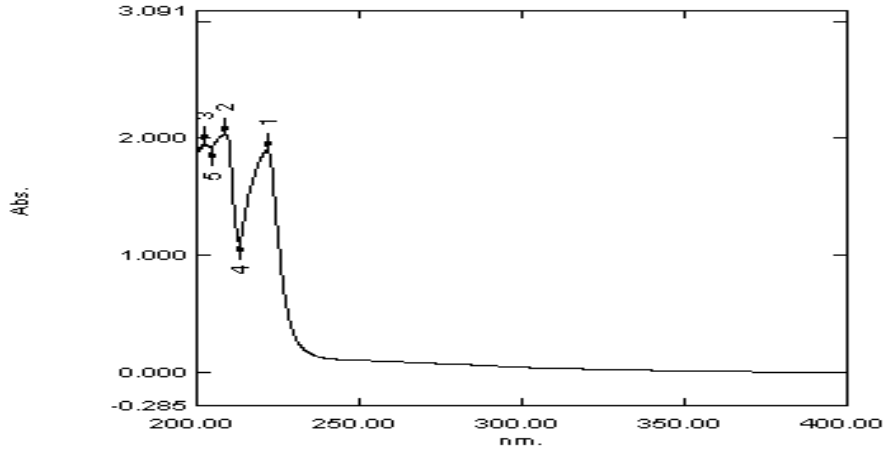


Figure (3.8): 60% buffer solution pH (12) and 40% (methanol and acetonitrile 1:1)

3.2 Methods Validation

3.2.1 Development and Validation of UV spectrophotometric

(UV-Vis or UV/Vis) refers to absorption spectroscopy or reflectance spectroscopy in the ultraviolet-visible spectral region. This means it uses light in the visible and adjacent (near- UV and near infrared) ranges. The absorption or reflectance in the visible rang directly affected the perceived color of the chemicals involved. In the region of the electromagnetic spectrum, molecules undergo electronic transition. This technique is complementary to fluorescence spectroscopy, in that fluorescence deals with transitions from the excited to ground state, while absorption measures transition from the ground state to the excited state. (Skoog et al, 2007)

The aim of this work to develop and to validated the UV spectrophotometeric method by using acetate buffer pH 4.5 instated of hydrochloric 0.1M showed Figures (3.2.1.1) (3.2.1.2) (3.2.1.3)

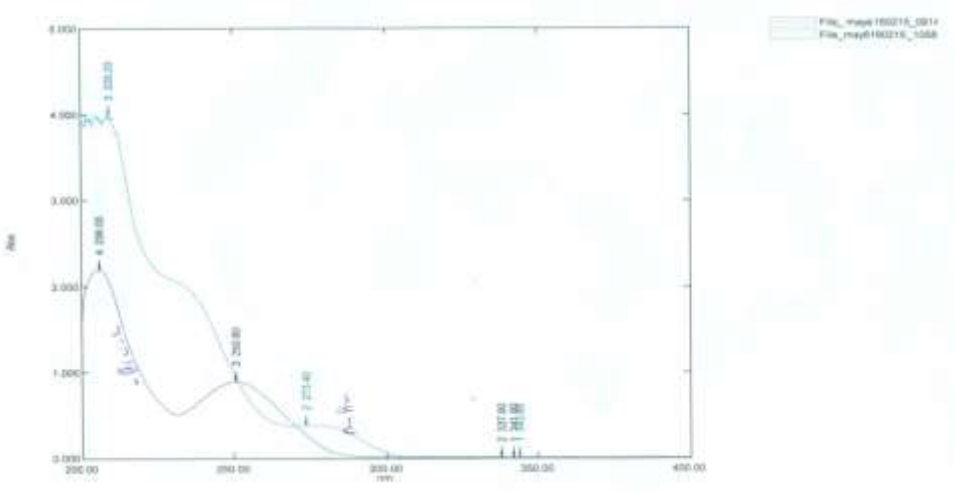


Figure (3.9): UV Spectrum of hydrochloric acid (0.1M) and acetate buffer (pH 4.5)

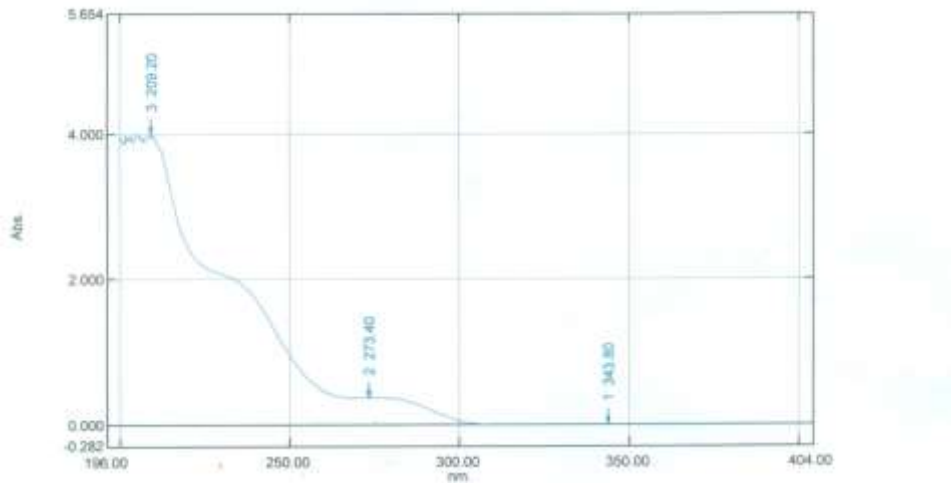


Figure (3.10): UV Spectrum of acetate buffer (pH 4.5)

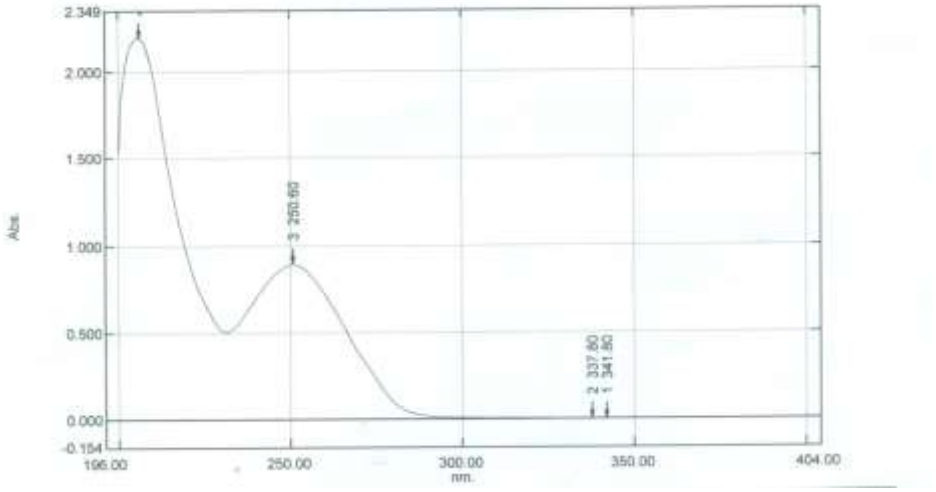


Figure (3.11): UV Spectrum of hydrochloric acid (0.1M)

3.2.1.1 Assay of tablet formulation

Validation of an analytical method is the process to establish that the performance characteristics of the developed method meet the requirements of the intended analytical application, the UV method was validated in the term of linearity, accuraty, precision, LOD, LOQ, and sensitivity. The method was capable to analyze the active ingredient in allopurinol without interference.

Table (3.2) Assay result of allopurinol tablets

Drug	Batch No	Label claim mg/tablet	Amount *found	Recovery%	RSD%
Cityuric	P20	100	100.15	100.15	0.175
Alopron	59046	100	102	102	0.98
Zyloric	B82614D	100	100.6	100,6	0.80

3.2.1.2 Recovery Studies (Accuracy)

Table (3.3) Recovery data of allopurinol

(Day one)

Standard and Sample	Abs	Actual Content	Theoretical	Recovery%
5 ml only std	0.391	100%	100%	100%
5 ml only sp	0.402	102.0%	100%	102%
5ml std 1ml sp	0.465	118.5%	120%	98.75%
5ml std 2ml sp	0.562	142.9%	140%	102%
5ml std 3ml sp	0.623	158.4%	160%	99%
5ml std 4ml sp	0.708	180.0%	180%	100%
5ml std 5ml sp	0.785	199.7%	200%	99.8%
				Mean= 100.2% RSD%=1.305

Table (3.4) Recovery data of allopurinol

(Day two)

Standard and Sample	**Abs**	**Actual Content**	**Theoretical**	**Recovery%**
5 ml only std	0.387	100%	100%	100%
5 ml only sp	0.395	101.5%	100%	101.5%
5ml std 1ml sp	0.465	119.4%	120%	99.1%
5ml std 2ml sp	0.540	137.3%	140%	99.2%
5ml std 3ml sp	0.620	157.6%	160%	98.5%
5ml std 4ml sp	0.690	179.0%	180%	99.4%
5ml std 5ml sp	0.790	202.7%	200%	101%
				Mean = 99.8% RSD% = 0.941

3.2.1.3 Precision

The precision (system method) of proposed method was evaluated the assay were performed by repeatability (intraday) and intermediate reported as RSD%. Tabulated in Table (3.5), (3.6)

Table (3.5) repeatability precision of system method

Run	Absorbance at 250nm
1	0.387
2	0.386
3	0.386
4	0.389
5	0.385
6	0.384
Mean	0.386
RSD%	0.445

Table (3.6) intermediate precision data of allopurinol

Run	Abs analyst day one	Abs analyst day two
1	0.391	0.389
2	0.389	0.387
3	0.390	0.390
4	0.390	0.386
5	0.388	0.387
Mean	0.3896	0.387
RSD%	0.292	0.299

3.2.1.4 Determination of linearity and range

The linearity for allopurinol was determined in term of correlation coefficient, which equal 0.998, which indicates the linearity of the method.

UV Spectrophotometer absorption of allopurinol standard solution gave linearity in the range (10-50 µg/ml)

Table (3.7) Standard curve of allopurinol

Conc.mg/ml	UV absorbance 250nm
0.01	0.576
0.02	1.119
0.03	1.683
0.04	2.209
0.05	2.689

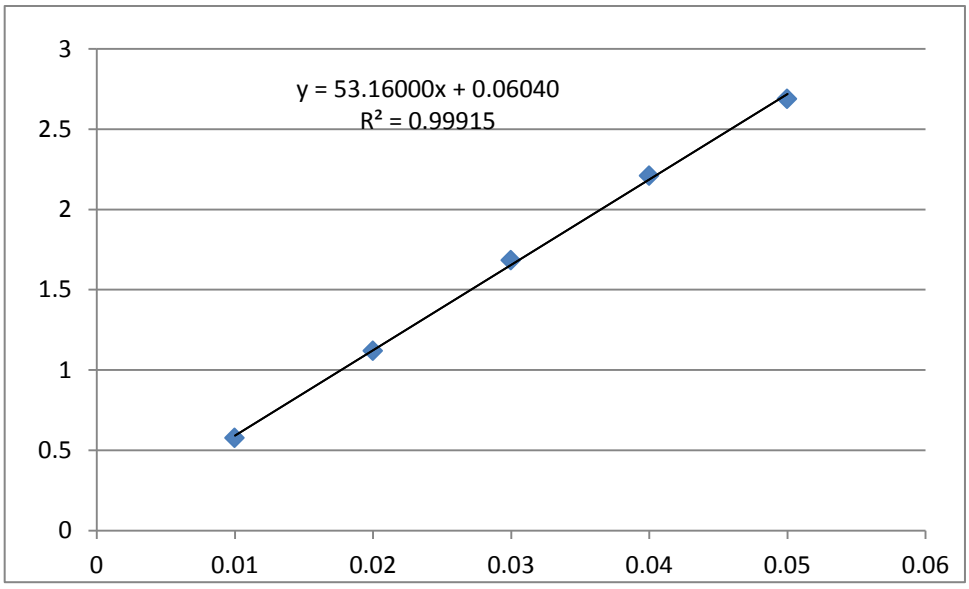


Figure (3.12) Calibration curve of allopurinol standard solution

3.2.1.5 Limit of detection and limit of Quantitation

The limit of detection LOD and limit of quantitation for allopurinol is determined from the recession graph of ALP, LOD = 0.051µg/ml and LOQ = 0.158µg/ml

3.2.2. Development and Validation of High Performance Liquid Chromatography Methods for determination of allopurinol

The traditional approach to HPLC optimization is to perform an experiment by trial and error or by change on control variable at time; such method can frequently require a very large number of experiments to identify the optimal condition. Recently computer assessed to HPLC separation has addressed the problem using factorial design strategies.

In this work with and optimize using buffer (pH 4.5) as mobile phase, and change was column from C_{18} to $C_{8.}$ Either in the method of HPLC reduces the retention time from 14.26 to 3.0 mints. As shown the Figures (3.13), (3.14)

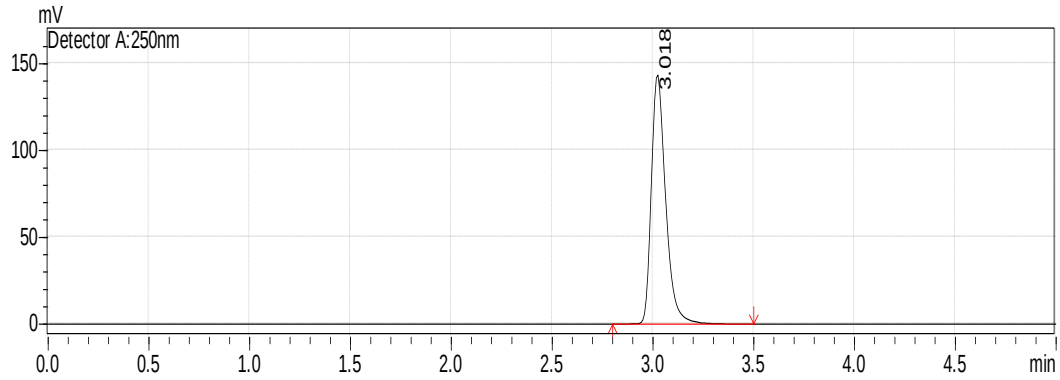


Figure (3.13) Typical HPLC chromatogram for allopurinol standard using developed method

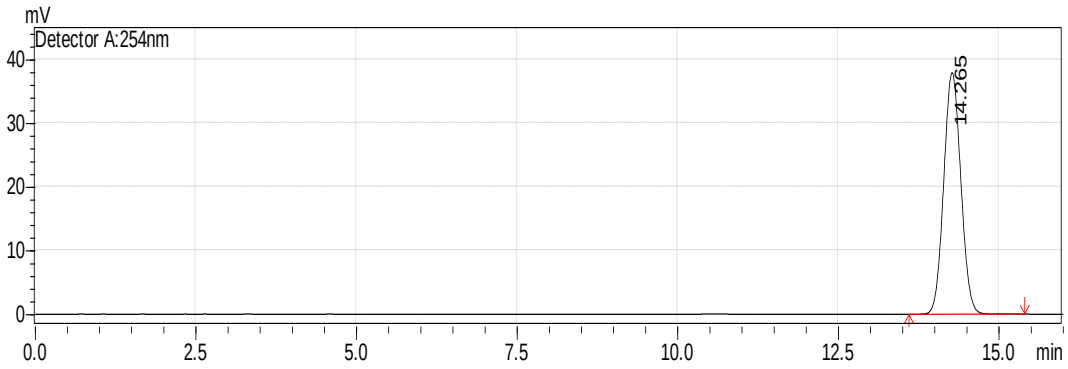


Figure (3.14) Typical HPLC chromatogram for allopurinol standard using official method USP

3.2.3.1 Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration of analyte in the sample. Linearity of detector response for allopurinol was established by analyzing serial dilutions of a stock solution of the working standard. Five concentrations such as 10,20,30,40 and 50µg/ml for allopurinol prepared and analyzed. The linearity graph was plotted. Showed Figure (3.8)

Table (3.8) calibration curve of allopurinol

Standard Concentration(mg/ml)	Average area
0.01	350824
0.02	713192
0.03	1063115
0.04	1409632
0.05	1733090

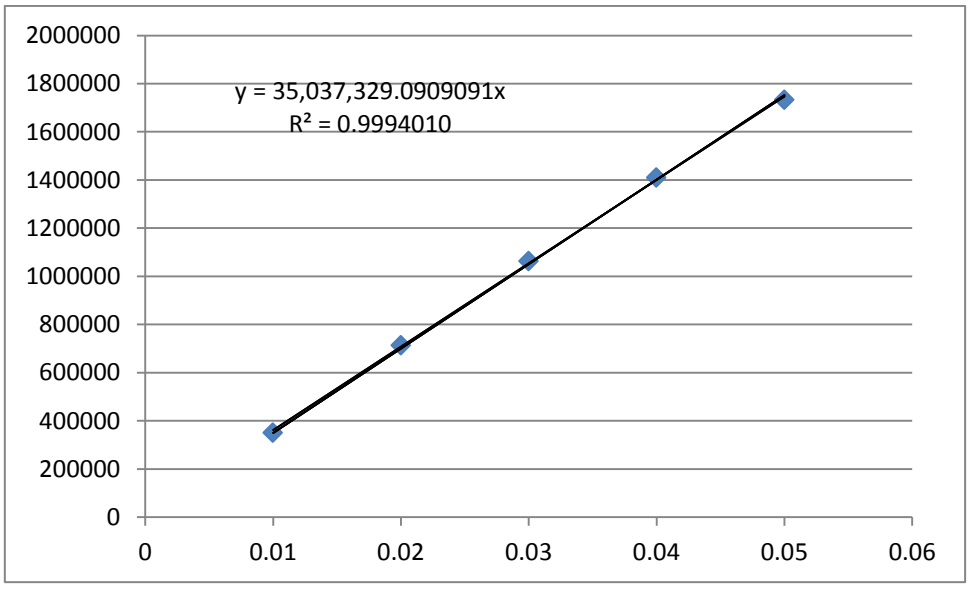


Figure (3.15) Linearity curve of allopurinol

3.2.3.2 Recovery Studies (Accuracy)

The accuracy of the method was assessed by determination of recovery for five concentrations covering the range of the method. The amount of allopurinol was recovered in the presence of placebo interference, was calculated. The mean recovery of allopurinol was (99.89%) and the RSD% was less than 2% which is satisfactory as shown in Table (3.9)

Table (3.9) Recovery of allopurinol (Instrument (1) LC-04)

Standard and Sample	**Area**	**Actual Content**	**Theoretical**	**Recovery%**
5 ml only std	249315	100%	100%	100 %
5ml only sp	251,262	100,18%	100%	100.18%
5mlstd 1mlsp	303020	121,72%	120%	101.45%
5mlstd 2mlsp	341181	138,5%	140%	98.9%
5mlstd 3mlsp	386223	158,6%	160%	98.70%
5mlstd 4ml sp	432775	179.6%	180%	99.70%
5mlstd 5ml sp	499644	200,70%	200%	100.35%
			Mean ± SD	99.89 ± 0.928
			RSD%	0.929

In the different instrument prepared five concentrations covering the range of the method. The amount of allopurinol was recovered in the presence of placebo interference, was calculated. The mean recovery of allopurinol was (99.66%) and the RSD% was less than 2%. which is satisfactory as shown in Table (3.10)

Table (3.10) Recovery of allopurinol (Instrument (2) LC-05)

Standard and sample	**Area**	**Actual Content**	**Theoretical**	**Recovery%**
5 ml only std	249315	100%	100%	100 %
5ml only sp	251,262	100.2%	100%	100.2%
5mlstd 1mlsp	303020	119.0 %	120%	99.17%
5mlstd 2mlsp	341181	137.23%	140%	98.02%
5mlstd 3mlsp	386223	158,7%	160%	99.10%
5mlstd 4ml sp	432775	181.0%	180%	100.50%
5mlstd 5ml sp	499644	201,28%	200%	100.64%
			Mean ± SD	99.66 ± 0.941
			RSD%	0.944

3.2.3.3 System suitability

The obtained results of system suitability showed that the HPLC systemis is capable of providing high recovery Table (3.11)

Table (3.11) System suitability data of allopurinol

No	Area of standard	Retention time	Tailing factor	Theoretical plate
1	1063014	3.050	1.323	6833.828
2	1063926	0.050	1.323	6824.699
3	1063525	3.050	1.323	6828.976
4	1062859	3.049	1.324	6841.952
5	1062575	3.049	1.326	6811.071
6	1062756	3.049	1.325	6839.484
Mean	1063115	3.049	1.324	6830.002
SD	514.16	1.22	0.001	11.26
RSD%	0.049	0.018	0.102	0.165

3.2.3.4 Precision

The Precision of analytical procedure expresses the closeness of agreement between a series of measurement obtained from multiple sampling of the same homogenous sample under prescribed condition. Repeatability of the method was checked by injecting replicate injections of 10µg/ml of the solution for 6 times on the same day. The mean and RSD% was calculated. From the data obtained, the developed RP-HPLC. Shown table (3.12), (3.13), (3.14)

Table (3.12) intraday precision of allopurinol concentration (10µg/ml)

No	Peak area at 250 nm	Retention time
1	350941	3.064
2	350580	3.061
3	350657	3.061
4	350797	3.062
5	350951	3.058
6	351016	3.057
Mean	350823	3.060
SD	176	0.025
RSD%	0.05	0.08

Table (3. 13) inter day precision parameter for day one

Day one for inter day precision	Retention time	Peak area
Average	3.060	350824
RSD%	0.080	0.050
Minimum	3.061	350580
Maximum	3.064	350951

Table (3. 14) inter day precision parameter for day two

Day two for inter day precision	Retention time	Peak area
Average	3.053	349088
RSD%	0.077	0.027
Minimum	3.051	349.066
Maximum	3.056	349102

3.2.3.5 Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters an indication of its reliability during normal usage. It was observed that the variations like used compared between the two devices and the results were convergent by calculating the mean, relative standard deviation less than 2% and both the RSD1+ RSD2 in the different instrument ≤ 3.0. Dose not has any significant effect on the method performance, which demonstrated that the developed RP-HPLC method is robust. Shown the table (3.15)

Table (3.15) Robustness for allopurinol

Area 5ml sp only ÷ average area 5 ml STD only × potency (99.41%)

Instrument (1) LC_04		**Instrument (2) LC_05**	
Average area 5 ml standard only 249315		Average area 5ml standard only246736	
Sp area1 251356	100.22%	Sp area1 248200	100%
Sp area2 251450	100.26%	Sp area2 248167	99.98%
Sp area3 251155	100.14%	Sp area3 248303	100.04%
Mean RSD%	100.2% 0.06%	Mean RSD%	100.0% 0.03%

3.2.3.6 Detection limit and quantitation limit

The limit of detection and limit of quantitation of allopurinol was determined by using the signal to noise ratio approach as defined in ICH guidelines. According to the determined signal to noise ratio the limit of detection and limit of quantitation for allopurinol was 0.051µg/ml and 0.156µg/ml, respectively.

3.3 Validated method of HPLC result of three samples of allopurinol

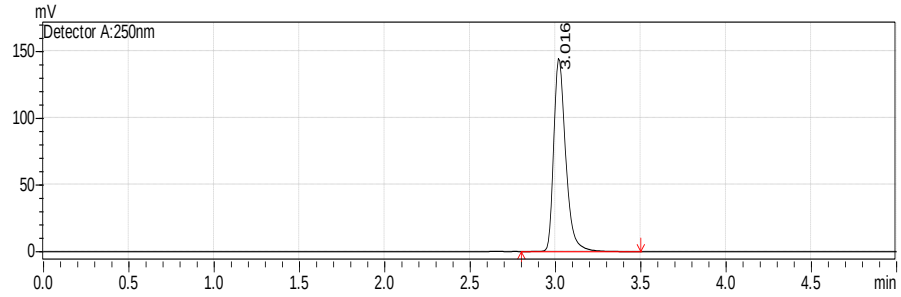


Figure (3.16) HPLC chromatogram for (Cityuric100 mg) tablets

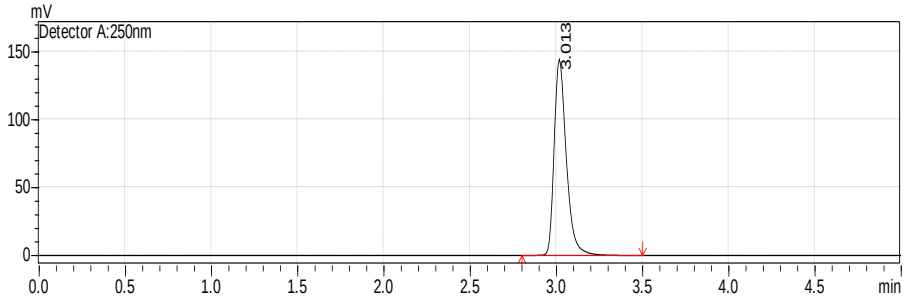


Figure (3.17) HPLC chromatogram for (Alopron100 mg) tablets

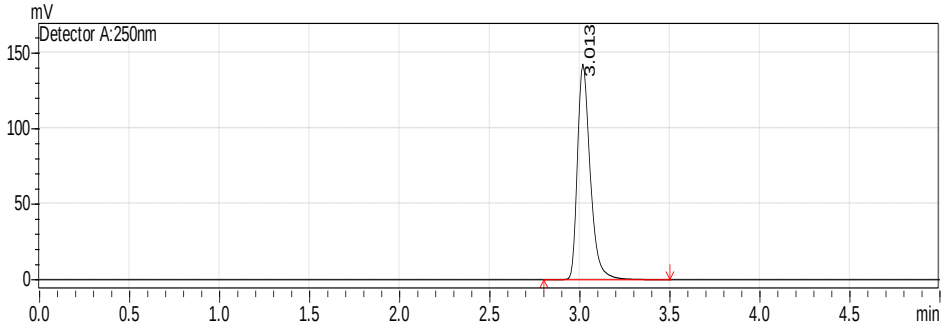


Figure (3.18): HPLC chromatogram for (Zyclric100 mg) tablets

3.3.1 Assay of samples

The assay comber between two methods validated applied (UV and HPLC) and then comber between official methods showed Table (3.16).

Table (3.16): The assay of allopurinol samples

NO	Samples	HPLC official method	HPLC developed method	UV official method	UV developed method
1	Cityluric100mg	100.31%	98.43%	99.80%	100.15%
2	Alopron100mg	100.2%	99.46%	100.2%	102.0%
3	Zyloric 100mg	99.30%	98.46%	101.1%	100.6%

3.4 Conclusion and Recommendation

The developed UV Spectrophotometer method was successfully a applied for the quantitative assay of commercial tablets. Validation of HPLC method was applied as indicating method recommended for routine work. The assay result of the developed methods was found to be similar to those obtained by official methods. Thus the proposed method for the estimation of allopurinol dosage form was found to be rapid, simple, accurate, precision and economical. High percentage of recovery shows that the method is free from the interference.

The verification of the validity of your method allopurinol assay .But the method of using the HPLC assay device was more accurate, and the accuracy of the linear method indexation by the UV, in the case of non availability of the device can be used as alteranative UVand get similar results.

The developed UV Spectrophotometeric method is suitable for routine analytical procedure for the analysis of allopurinol tablets, it is inexpensive and is time consuming and gave comparable results from HPLC can therefore be used in the event of unavailability of HPLC Developed HPLC in laboratory. method recommended for applying as routine analysis in quality control laboratories for the analysis of the allopurinol, and the shorter time than the official method and simple, safe, rapid, accurate and not expensive.

Follow up the development of new methods for the estimating the allopurinol in all pharmaceutical forms because it is one of the important pharmaceutical treatment of gout.

3.5 References

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APPENDIX

Appendix

Effected of pH upon UV spectrum of allopurinol

pH. 2

40% of buffer solution (mono basic ammonium phosphate), pH 2.0 and added 60% methanol with 1ml of the sample allopurinol

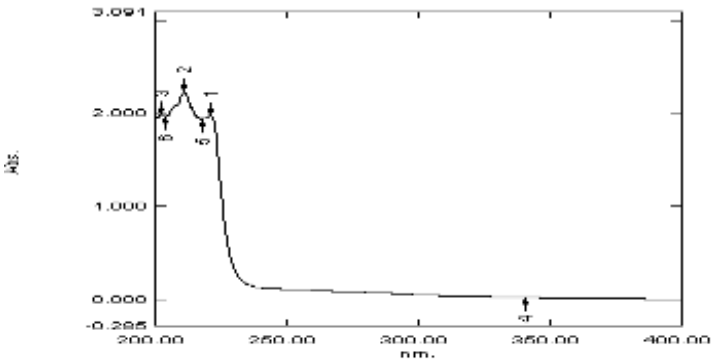


Figure (3.19): 40% buffer solution pH (2) and 60% (methanol & acetonitrile 1:1)

pH. 3

50% of buffer solution (mono basic ammonium phosphate), pH 3.0 and added 50% methanol with 1ml of the sample allopurinol

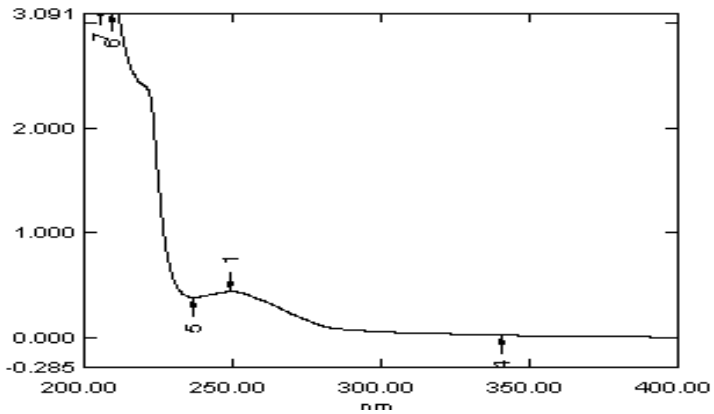


Figure (3.20): 50% buffer solution pH (3) and 50% (methanol and acetonitrile 1:1)

pH. 5

60% of buffer solution (mono basic ammonium phosphate), pH 5.0 and added 40% methanol with 1ml of the sample allopurinol

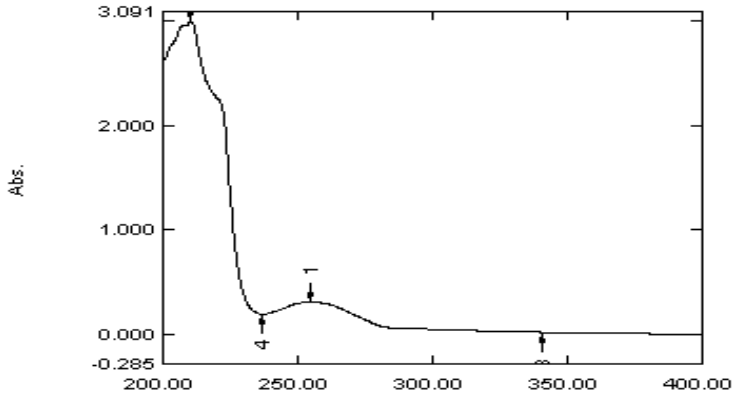


Figure (3.21): 60% buffer solution pH (5) and 40% (methanol and acetonitrile 1:1)

pH 6.0

40% of buffer solution (mono basic ammonium phosphate), pH 6.0 and added 60% methanol with 1ml of the sample allopurinol

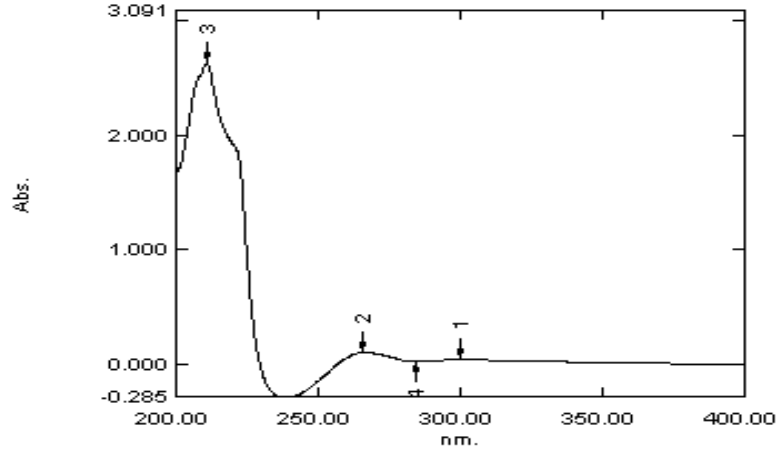


Figure (3.22): 60% buffer solution pH (5) and 40% (methanol and acetonitrile 1:1)

pH 7.0

50% of buffer solution (mono basic ammonium phosphate), pH 7.0 and added 50% methanol with 1ml of the sample allopurinol

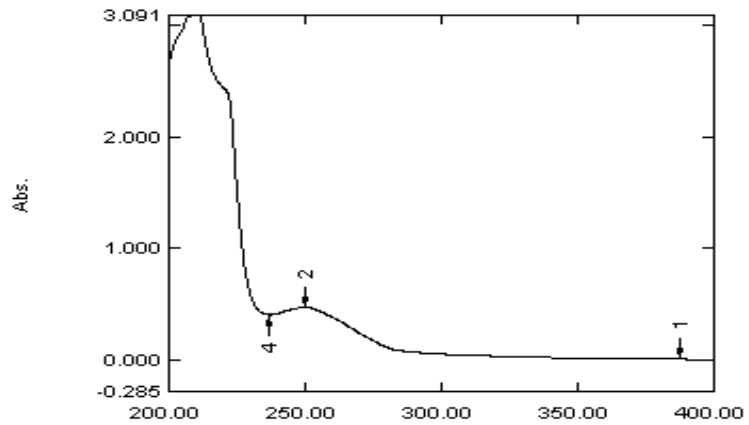


Figure (3.23): 60% buffer solution pH (5) and 40% (methanol & acetonitrile 1:1)

pH. 8

50% of buffer solution (mono basic ammonium phosphate), pH 7.0 and added 50% methanol with 1ml of the sample allopurinol

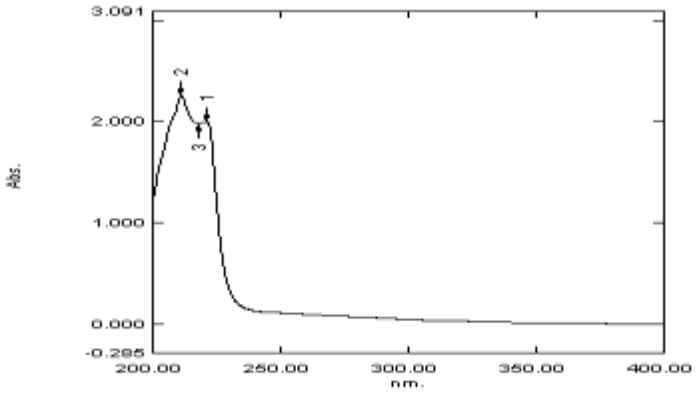


Figure (3.24): 50% buffer solution pH (8) and 50% (methanol and acetonitrile 1:1)

pH 9.0

80% of buffer solution (mono basic ammonium phosphate), pH 9.0 and added 20% methanol with 1ml of the sample allopurinol

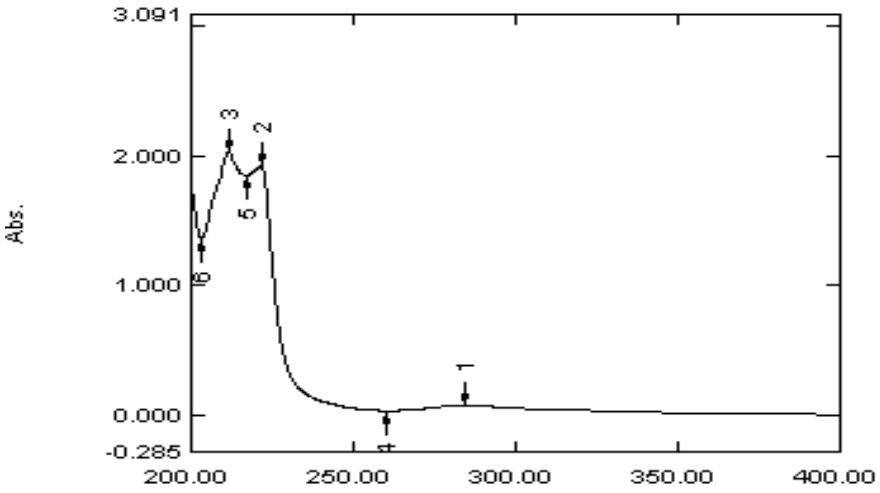


Figure (3.25): 80% buffer solution pH (9) and 20% (methanol and acetonitrile 1:1

pH 10.0

20% of buffer solution (mono basic ammonium phosphate), pH 10.0 and added 80% methanol with 1ml of the sample allopurinol

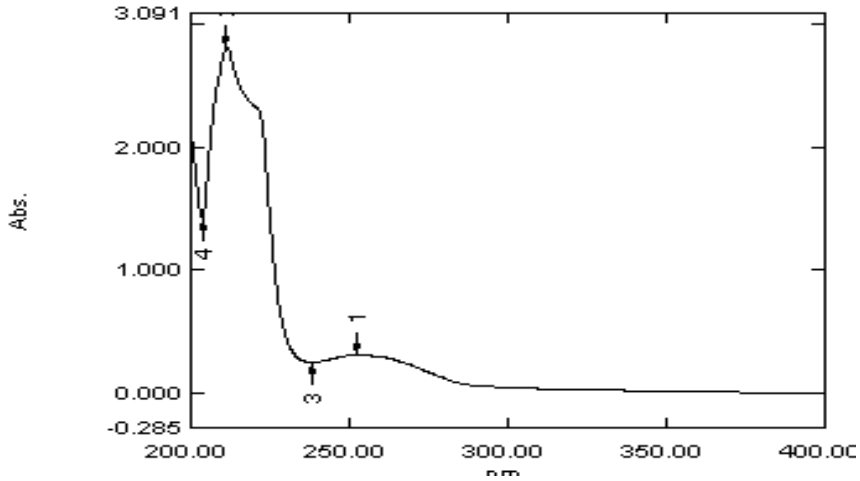


Figure (3.26): 20% buffer solution pH (10) and 80% (methanol and acetonitrile 1:1

pH 11.0

25% of buffer solution (mono basic ammonium phosphate), pH 11.0 and added 75% methanol with 1ml of the sample allopurinol

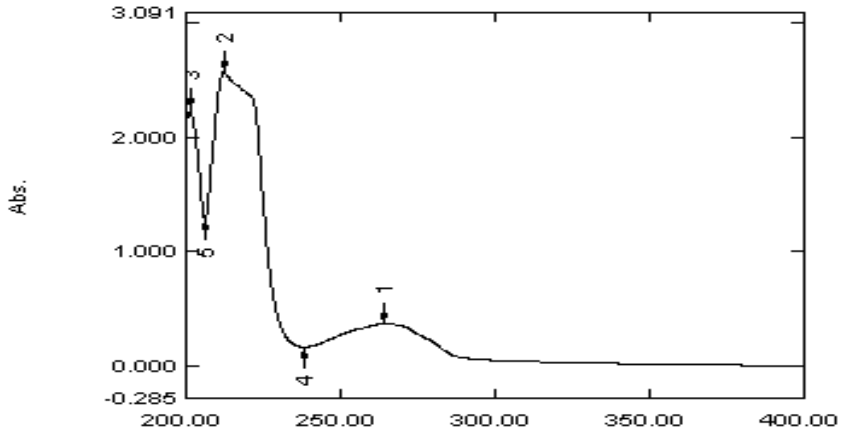


Figure (3.27): 25% buffer solution pH (11) and 75% (methanol and acetonitrile 1:1

pH 13.0

70%% of buffer solution (mono basic ammonium phosphate), pH (13.0) and added 30% methanol with 1ml of the sample allopurinol

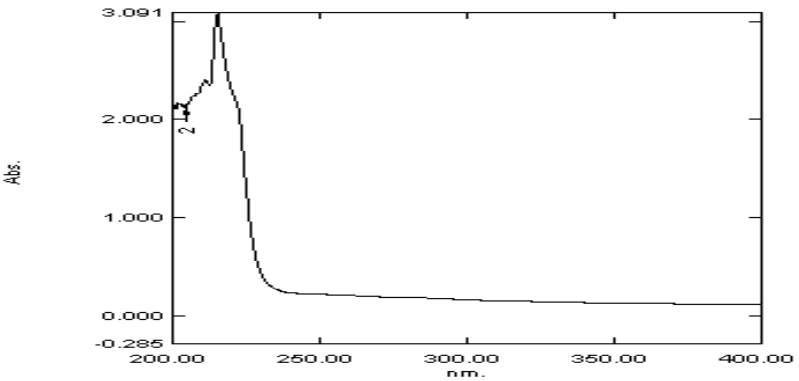


Figure (3.28): 70% buffer solution pH 13 and 30% (methanol and acetonitrile 1:1)

PH. 14

80% of buffer solution (mono basic ammonium phosphate), pH 14.0 and added 20% methanol with 1ml of the sample allopurinol

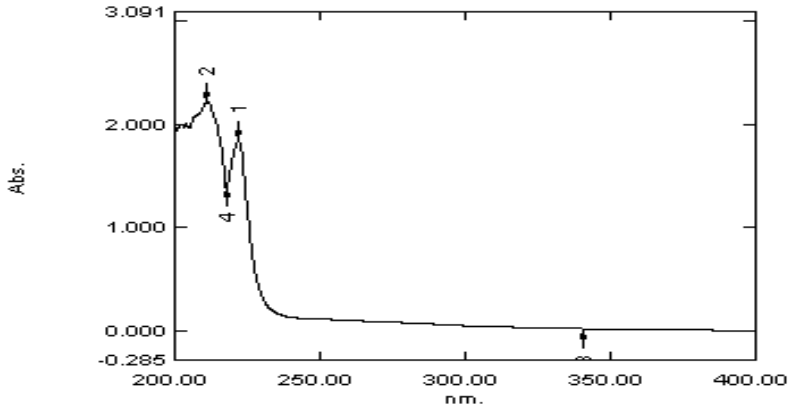


Figure (3.29): 80% buffer solution pH (14) and 20% (methanol and acetonitrile 1:1)

Validated method

Figure (3.30) Chromatogram of repeatedly injected standard (0.01 mg / ml)

Area 350657

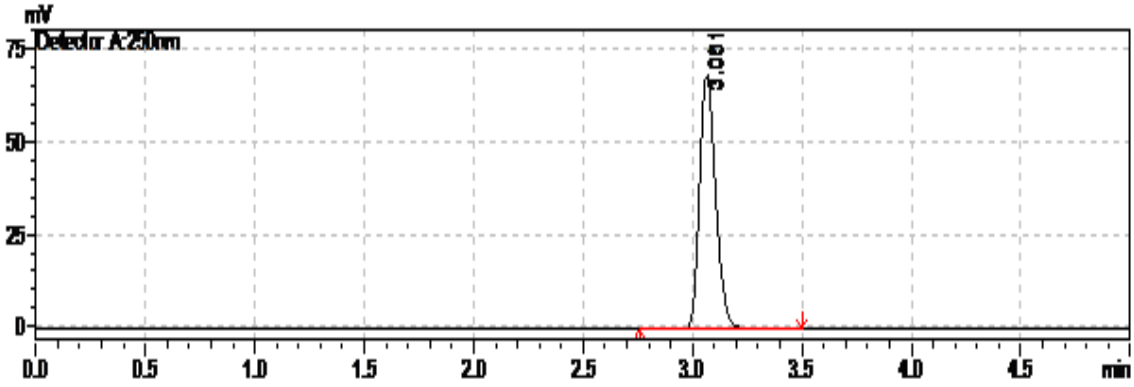


Figure (3.31) Chromatogram of repeatedly injected standard (0.02 mg / ml)

Area 713133

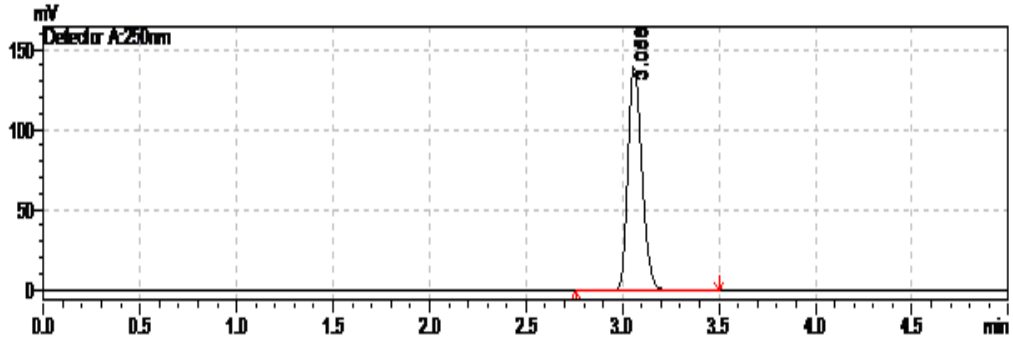


Figure (3.32) Chromatogram of repeatedly injected standard (0.03 mg / ml)

Area 1063525

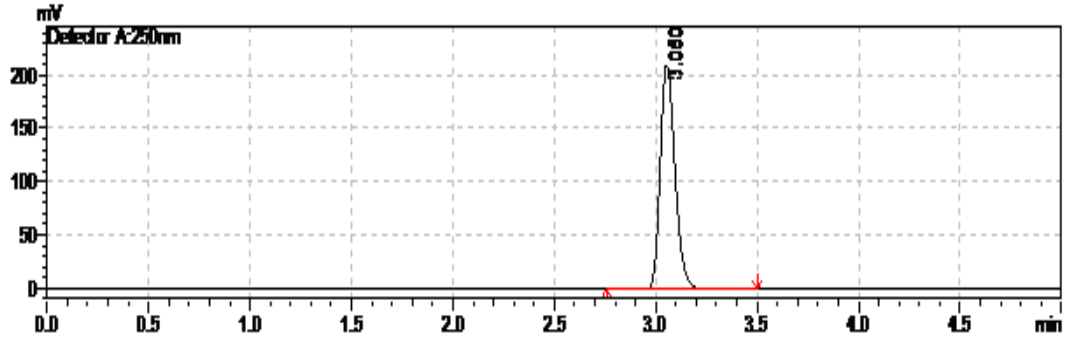


Figure (3.33) Chromatogram of repeatedly injected standard (0.04 mg / ml)

Area 1408715

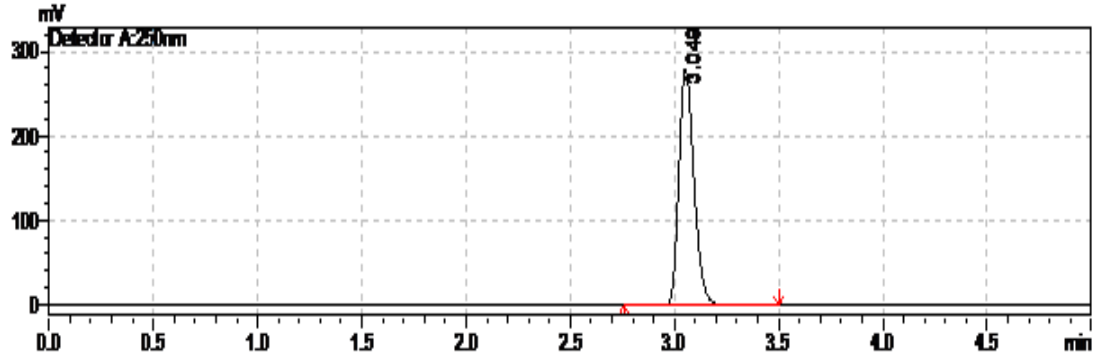


Figure (3.34) Chromatogram of repeatedly injected standard (0.05 mg / ml)

Area 1736061

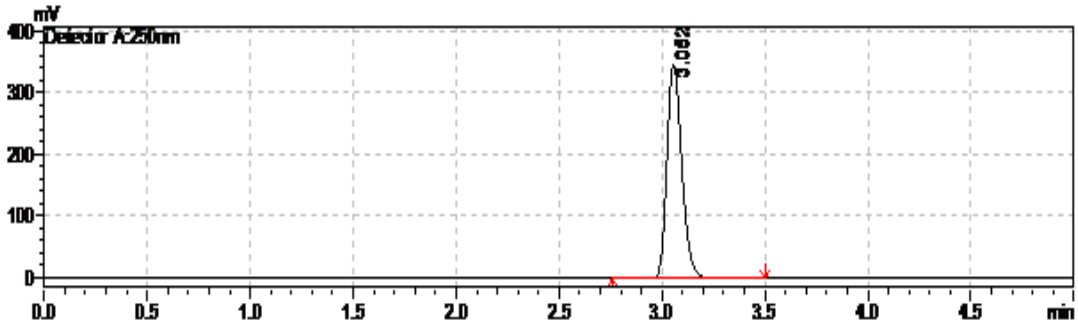


Table (3.17) linearity parameter for repeatedly injected standard (0.01mg/ ml)

Standard dilution 1	Retention time	Peak area
Average	3,060	350824
RSD%	0,080	0,050
Maximum	3,064	351016
Minimum	3,057	350580

Table (3.18) linearity parameter for repeatedly injected standard (0.02mg/ml) stock solution

Standard deviation	Retention time	Peak area
Average	3,053	713192
RSD%	0,077	0,027
Maximum	3,056	713536
Minimum	3,051	712965

Table (3.19) linearity parameter for repeatedly injected standard (0.03 mg/ml)

Standard deviation	Retention time	Peak area
Average	3,049	1063115
RSD%	0,018	0,049
Maximum	3,050	1063962
Minimum	3,049	1062575

Table (3.20) linearity parameter for repeatedly injected standard (0.04 mg/ml)

Standard deviation	Retention time	Peak area
Average	3,047	1409632
RSD%	0,049	0,112
Maximum	3,049	1412816
Minimum	3,045	1408715

Table (3.21) linearity parameter for repeatedly injected standard (0.05mg/ml)

Standard deviation	Retention time	Peak area
Average	3,048	1733090
RSD%	0,066	0,230
Maximum	3,052	1736016
Minimum	3,046	1725113

Instrument (1): LC-O4

Figure (3.35): Chromatogram of solution one for accuracy (5ml std and 1ml sp)

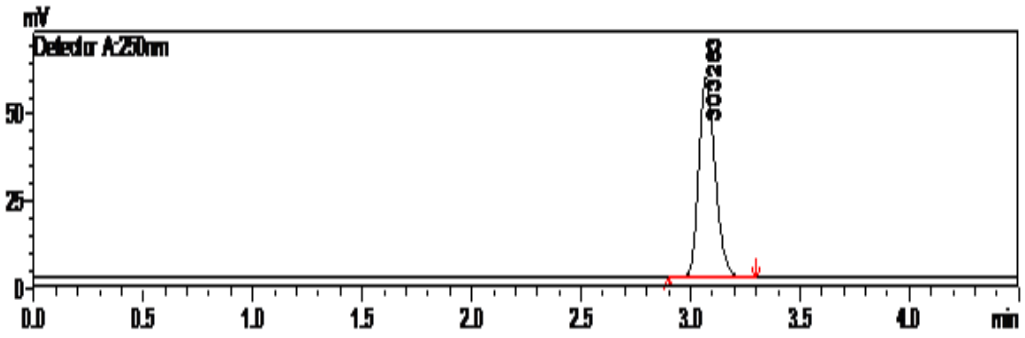


Figure (3.36): Chromatogram of spike solution two for accuracy (5ml std and 2ml sp)

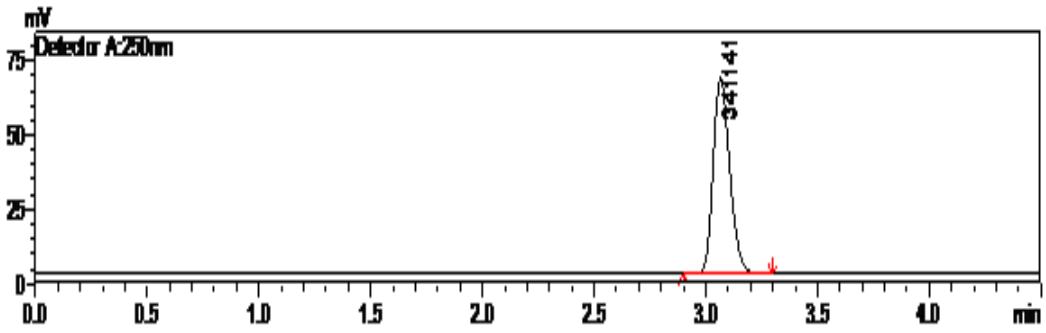


Figure (3.37): Chromatogram of spike solution three for accuracy (5ml std and 3ml sp)

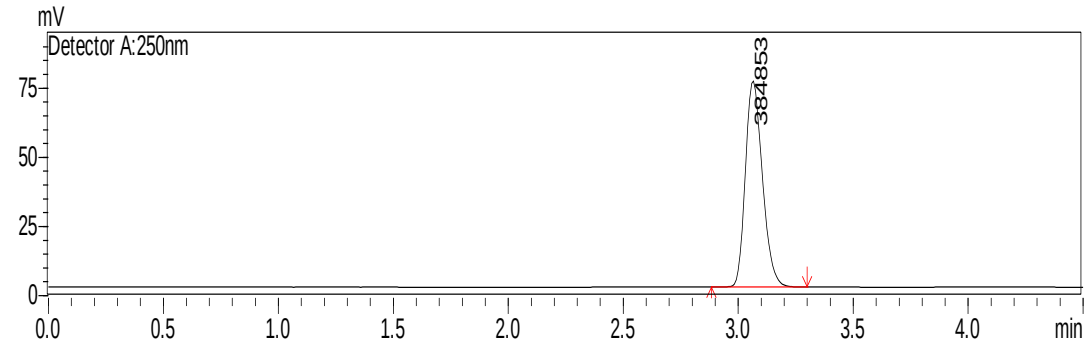


Figure (3.38): Chromatogram of spike solution four for accuracy (5ml std and 4ml sp)

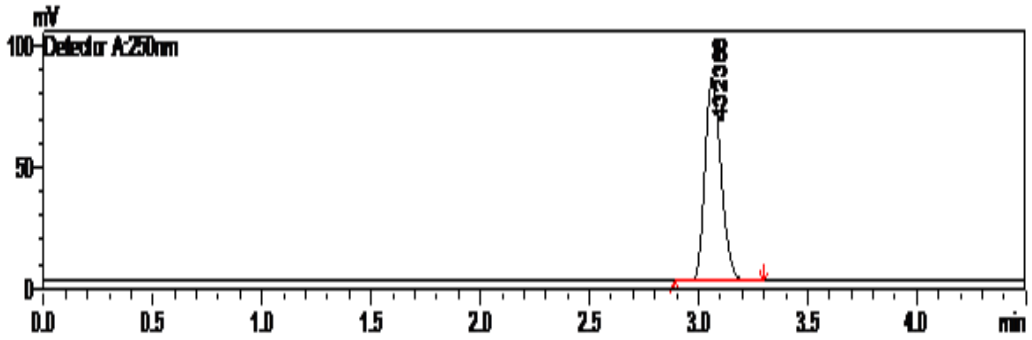


Figure (3.39): Chromatogram of spike solution five for accuracy (5ml std and 5ml sp)

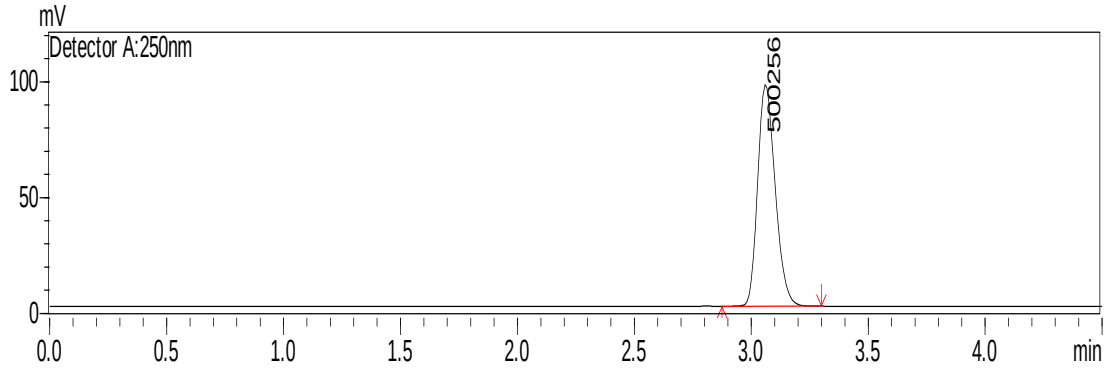
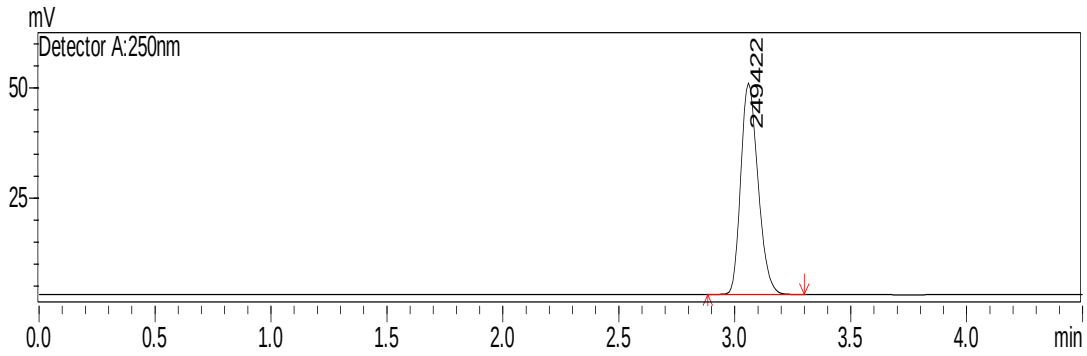


Figure (3.40): Chromatogram of standard solution for accuracy (5ml std only) Area 249422



Chromatogram of sample solution for accuracy

Instrument LC-05

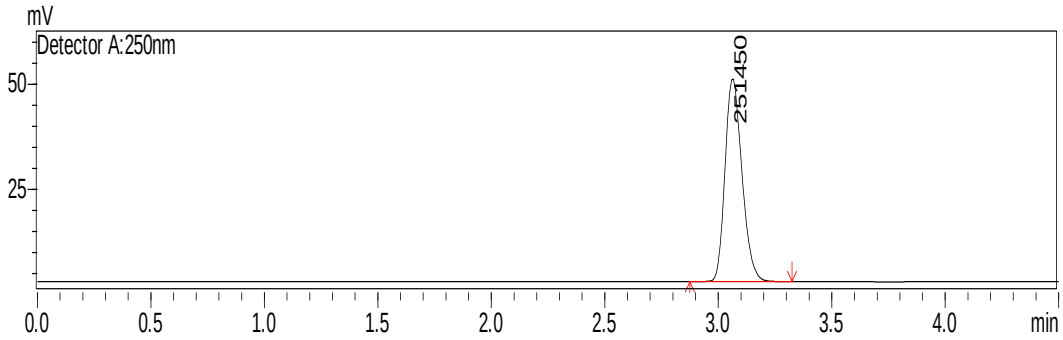


Figure (3.41): Chromatogram of spike solution one for accuracy (5ml std and 1ml sp) Area 293212

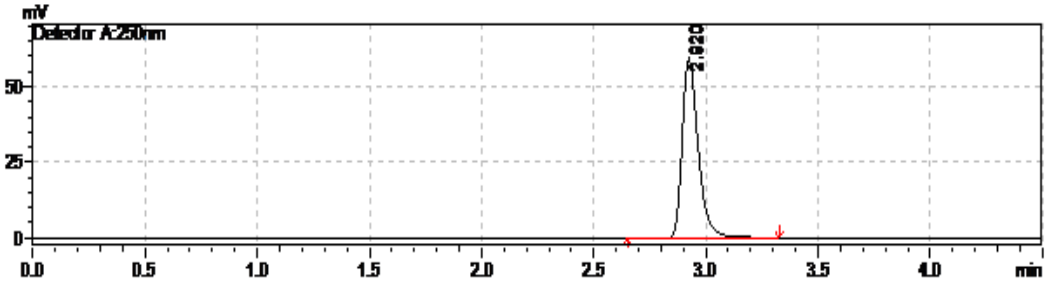


Figure (3.42): Chromatogram of spike solution Two for accuracy (5ml std and 2ml sp) Area 338521

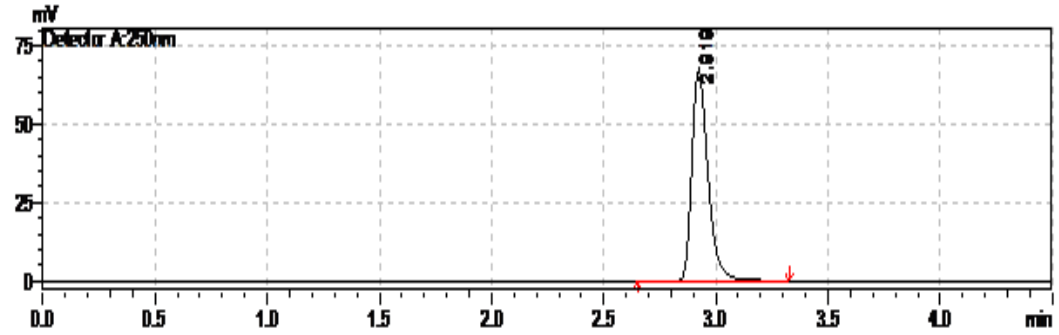


Figure (3.43): Chromatogram of spike solution Three for accuracy (5ml std and 3ml sp) Area 381347

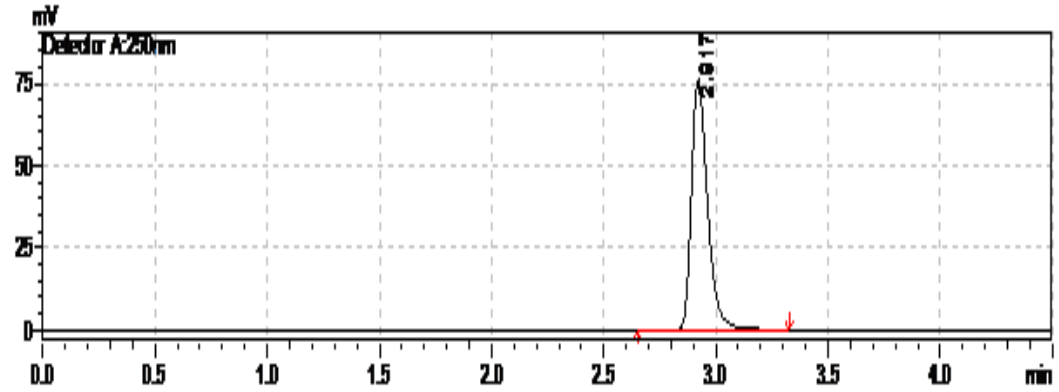


Figure (3.44): Chromatogram of spike solution four for accuracy (5ml std and 4ml sp) Area 426289

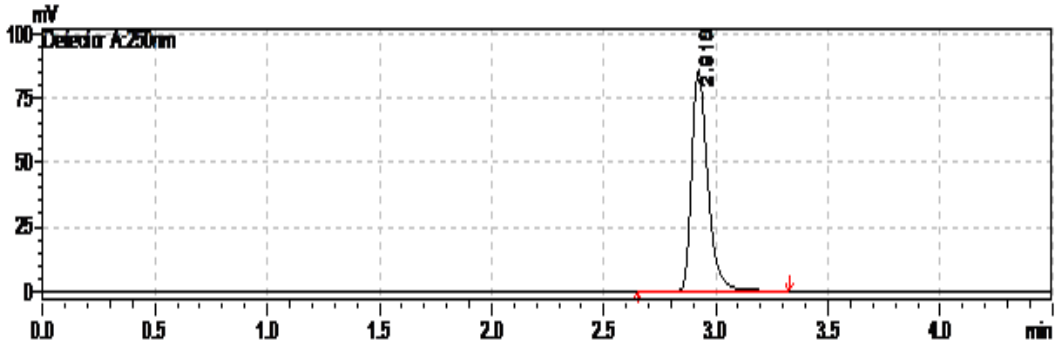


Figure (3.45): Chromatogram of spike solution five for accuracy (5ml std and 5ml sp) Area 495847

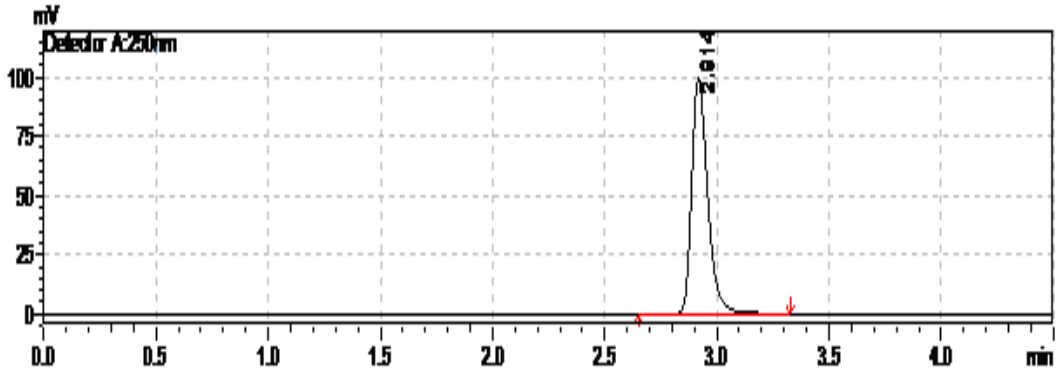


Figure (3.46): Chromatogram of standard solution for accuracy (5ml std

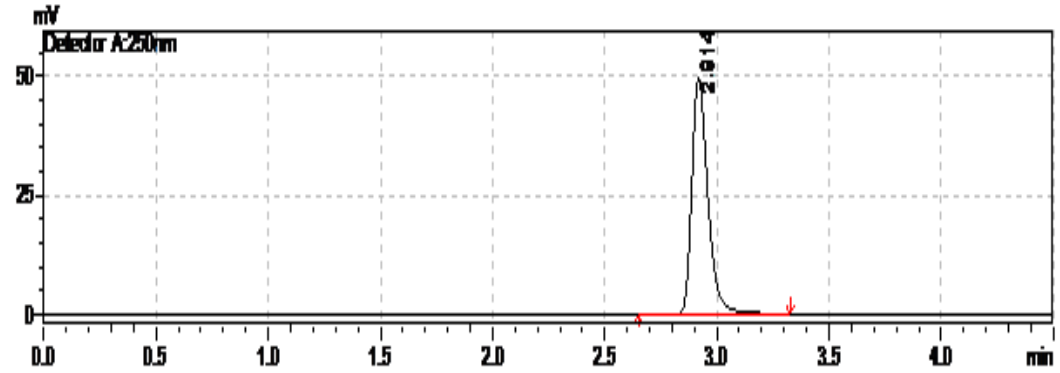


Figure (3.47): Chromatogram of sample solution for accuracy (5ml sp only) Area 248457