Sudan University of Science and Technology
College of Graduate Studies

DEVELOPMENT AND VALIDATION OF UV SPECTROPHOTOMETRIC METHOD FOR ESTIMATION OF METRONIDAZOLE TABLETS

تطوير وتحقق من تقدير الميترونيدازول بطريقة مطيافية الأشعة فوق البنفسجية

Dissertation Submitted For the Partial Fulfillment of the Requirements of M. Sc.in Chemistry

By

Sara Adam Abdelsamed Adam

(B.Sc.Honors in chemistry)

Supervisor

Dr.Kamal Mohamed Saeed

March, 2018

الإستهلال

بسم الله الرحمن الرحيم

قال تعالى :(تبارك الذي بيده الملك وهو علي كل شئ قدير(1) الذي خلق الموت والحياة ليبلوكم أيكم أحسن عملا وهو العزيز الغفور(2)

صدق الله العظيم

سورة الملك

DEDICATION

To:

My parents,

My brothers and my sisters,

My friends

Acknowledgement

All praise is raised to Allah the merciful and the helper who gave me the strength and determination to complete this work.

I would like to seize this opportunity to express my deep gratitude to Dr.Kamal Mohamed Saeed for his guidance advice and academic support.

I would also like to thank the quality control department of Marwa pharmaceuticals industries for their help and for giving me the chance to conduct this work.

I also feel very much obliged to the assistance, support or advice of many persons without which this work could not have been completed.

ABSTRACT

A simple,linear, precise and accurate UV-VIS method was developed and validated for rapid assay of metronidazole in pharmaceutical dosage forms.

The validation parameters (linearity, precision, accuracy, detection limit, quantitation limit) were validated according to the international conference of Harmonization specification.

The method shows linearity with regression equation:

Y= 0.057X and with a correlation coefficient R^2=0.999 Arelative standard deviation of precision0.09, 0.069 and 0.228 % was observed on analysis of six replicate samples of concentration 6, 8 & 10 ppm respectively a recovery percentage of 99.24 – 100.0 %, which indicates that the developed method was simple, rapid and precise.

Statistical data reveals the method is accurate and precise for the quantitation of Metronidazole.

The method was successfully applied for the routine analysis of Metronidazole in tablet dosage.

مستخلص البحث

تم تطويرطريقه تقدير الميترونيدازول بطريقة مطيافيه بسيطه ، انتقائيه ، خطيه ودقيقه في الاشكال الصيدلانيه.

المتغيرات المستخدمه للتأكد من صحة الطريقه المستخدمه في تقدير الميترونيدازول مثل: الخطية ، الدقه ، تم اعتمادها والتحقق من صحتها وفقا لتوصيات المؤتمر الدولي للتنسيق

في الخطية وجدت معادلة الانحدار $Y = 0.057\ X$

ومعامل الانحدار $R^2 = 0.999$

في الدقة وجد ان متوسط الانحراف المعياري 0.09 ,0.069 و0.228% من خلال علي تكرار التحليل لعدد 6 عينات بالتراكيز 6,8,10 ppmالتوالي ,

ووجد ان نسبة التحسن 99.24-100.0% والذي يدل علي ان الطريقة بسيطة ,سريعة ودقيقة.

البيانات الاحصائيه اثبتت دقة وانتقائية وحساسية الطريقه لتقدير الميترونيدازول.

هذه الطريقة تم تطبيقها بنجاح للتحليل الروتيني لتقدير الميترونيدازول في الاشكال الصيدلانية.

Table of Contents:

List of Tables

List of Figures

List of abbreviation

Abbreviation	Full form
ICH	International Conference on Harmonization
API	Active pharmaceutical ingredient
CFR	Code of Federal Regulations
cGMP	Current good manufacturing practices
AQC	analytical quality control
IND	Investigational New Drug
NDA	New Drug Application
MAA	Marketing Authorization Application
FDA	Food and drug administration
USP	United states pharmacopeia
IUPAC	International Union of Pure and Applied Chemistry
DL	Detection limit
QL	Quantitation limit
ppm	parts per million
ppb	parts per billion
VMP	Validation Master Plan
QC	Quality control

Chapter One

1-Introduction and literature Review

1.1-Definitions of Analytical Method Development and Validation

Analytical methods development , validation, and transfer are key elements of any pharmaceutical development program. This technical brief will focus on development and validation activities as applied to drug products. Often considered routine, too little attention is paid to them with regards for their potential to contribute to overall developmental time and cost efficiency.

These method-related activities are interrelated. They are iterative, particularly during early drug development phases. Parts of each process may occur concurrently or be refined at various phases of drug development. Changes encountered during drug development may require modifications to existing analytic methods. These modifications to the methods, in turn, may require additional validation or transfer activities, as shown below (Figure 1).

Figure 1

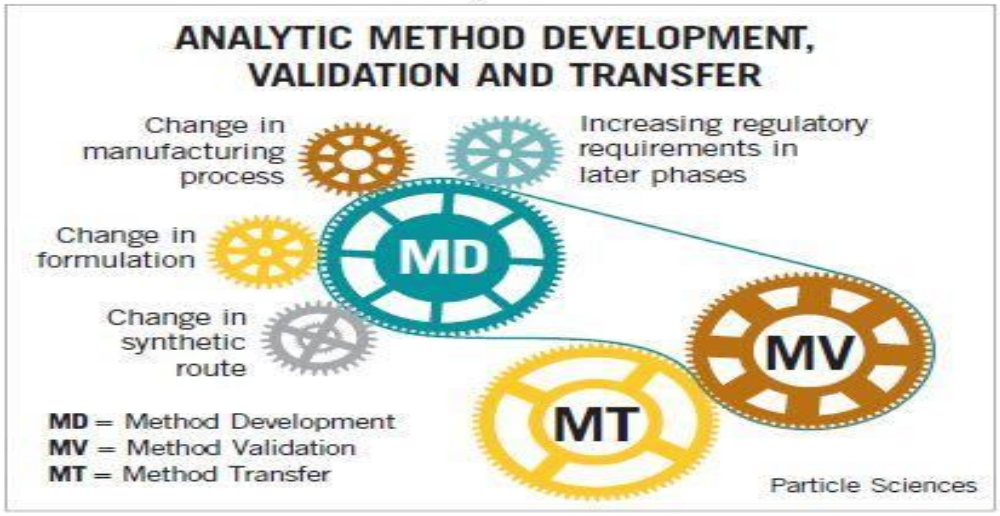

Figure 1.1: Analytical method development validation and transfer

Effective method development ensures that laboratory resources are optimized, while methods meet the objectives required at each stage of drug development. Method validation, required by regulatory

agencies at certain stages of the drug approval process, is defined as the "process of demonstrating that analytical procedures are suitable for their intended use"[1]. Method transfer is the formal process of assessing the suitability of methods in another laboratory. Each of these processes contributes to continual improvement of the methods and results in more efficient drug development.

Analytical methods are intended to establish the identity, purity, physical characteristics and potency of the drugs that we use. Methods are developed to support drug testing against specifications during manufacturing and quality release operations, as well as during long-term stability studies. Methods may also support safety and characterization studies or evaluations of drug performance. According to the International Conference on Harmonization (ICH), the most common types of analytic procedures are: (i) identification tests, (ii) quantitative tests of the active moiety in samples of API or drug product or other selected component(s) in the drug product, (iii) quantitative tests for impurities' content, (iv) limits tests for the control of impurities*(chemical and pharmaceutical research,2012)*.

Method development (Figure 2) is a continuous process that progresses in parallel with the evolution of the drug product. The notion of phase-appropriate method development is a critical one if time, cost, and efficiency are concerns. The goal and purpose of the method should reflect the phase of drug development. During early drug development, the methods may focus on API behavior. They should be suitable to support pre-clinical safety evaluations, pre-formulation studies, and prototype product stability studies. As drug development progresses, the analytical methods are refined and expanded, based on increased active pharmaceutical ingredient (API) and drug product knowledge. The methods should be robust and uncomplicated, while still meeting the appropriate regulatory guidelines.

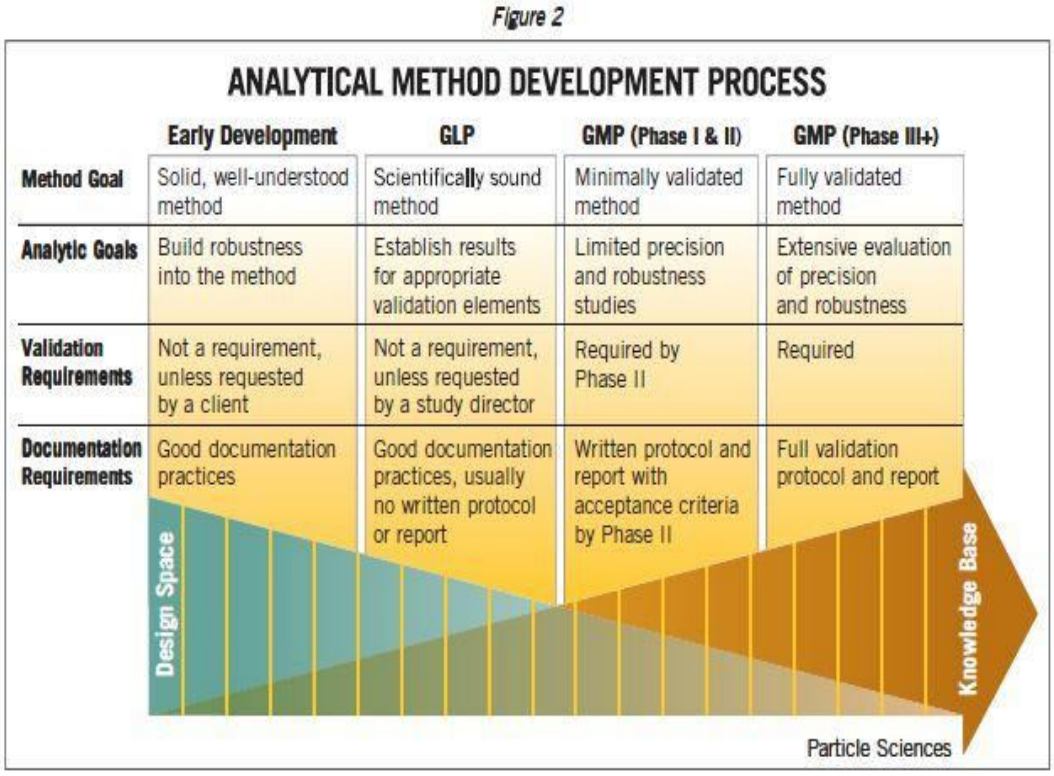

Figure 1.2: Analytical method development process

Scouting experiments are frequently performed during method development to establish the performance limits of the method, prior to formal validation experiments. These may include forced degradation studies, which are an integral part of development of a stability-indicating method. API is typically subjected to degradation by acid, base, peroxide, heat, and light. This allows for a determination of the capability of the method to separate and quantify degradation products, while providing insight into the main mechanisms of degradation. Once a stability-indicating method is in place, the formulated drug product can then be subjected to heat and light in order to evaluate potential degradation of the API in the presence of formulation excipients.

Additional experiments help to define the system suitability criteria that will be applied to future analytic sample sets. System suitability tests are a set of routine checks to assess the functionalities of the instrument, software, reagents, and analysts as a system[3]. Final method system suitability parameters may be determined from evaluations of method robustness performed under statistical design of experiments. The goal is to identify the critical parameters and to establish acceptance criteria for method system suitability.

Validation is defined as ‘finding or testing truth of something ‘. When analytical methods are used to generate results about the characteristics of drug related samples it is vital that the results are trustworthy: they may be used as the basis for decisions relating to administering the drug to patients, a validation study is performed on an analytical method to ensure that the results are always obtained .

1.1.4 Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the Pharmaceutical Manufacturing Handbook: Regulations and Quality. (ShayneC ,2002).

1.2-The important of validate analytical procedures

There are many reasons for the need to validate analytical procedures. Among them Are regulatory requirements, good science, and quality control requirements. The Code of Federal Regulations (CFR) 311.165c explicitly states that “the accuracy, Sensitivity, specificity, and reproducibility of test methods employed by the firm shall Be established and documented” of course, as scientists, we would want to apply Good science to demonstrate that the analytical method used had demonstrated Accuracy, sensitivity, specificity, and reproducibility. Finally management of the quality control unit would definitely want to ensure that the analytical methods that the department uses to release its products are properly validated for its intended Use so the product will be safe for human use. .(joelGH, et al, 2001)

1.3-Analytical method validation requirements for its intended use

All analytical methods that are intended to be used for analyzing any clinical samples will need to be validated. Validation of analytical methods is an essential but time – consuming activity for most analytical development laboratories. It is therefore important to understand the requirements of method validation in more detail and the options that are available to allow for optimal utilization of analytical resources in a development laboratory(ISO/IEC 17025,2005).

1.4-Current good manufacturing practices in twenty - first century

The overarching philosophy in current good manufacturing practices (cGMPs) of the twenty - first century and in robust modern quality systems is quality should be built into the product, and testing alone cannot be relied on to ensure product quality. From the analytical perspective, this will mean that

analytical methods used to test these products should have quality attributes built into them. To have quality attributes built into the analytical method will require that fundamental quality attributes be applied by the bench - level scientist. This is a paradigm shift that requires the bench - level scientist to have the scientific and technical understanding, product knowledge, process knowledge, and/or risk assessment abilities to appropriately execute the quality functions of analytical method validation. It will require (1) the appropriate training of the bench - level scientist to understand the principals involved with method validation and able to validate an analytical method and understand the principals involved with the method validation, (2) proper documentation and understanding and interpreting data, and (3) cross - functional understanding of the effect of their activities on the product and the customer (the patient). It is the responsibility of management to verify that skills gained from the training are implemented in day - to - day performance(International Conference on Harmonization,1996).

1.5-Strategy for the validation of methods

The validity of a specific method should be demonstrated in laboratory experiments using samples or standards that are similar to unknown samples analyzed routinely. The preparation and execution should follow a validation protocol, preferably written in a step-by-step instruction format. Possible steps for a complete method validation are listed in Table 1. This proposed procedure assumes that the instrument has been selected and the method has been developed. It meets criteria such as ease of use; ability to be automated and to be controlled by computer systems; costs per analysis; sample throughput; turnaround time; and environmental, health and safety requirements.

Develop a validation protocol, an operating procedure or a validation master plan for the validation

1. For a specific validation project define owners and responsibilities.
2. Develop a validation project plan
3. Define the application, purpose and scope of the method
4. Define the performance parameters and acceptance criteria
5. Define validation experiments
6. Verify relevant performance characteristics of equipment
7. Qualify materials, e.g. standards and reagents for purity, accurate amounts and sufficient stability
8. Perform pre-validation experiments

9. Adjust method parameters or/and acceptance criteria if necessary
10. Perform full internal (and external) validation experiments
11. Develop SOPs for executing the method in the routine
12. Define criteria for revalidation
13. Define type and frequency of system suitability tests and/or analytical quality control (AQC) checks for the routine
14. Document validation experiments and results in the validation report

1.6-Analytical method validation characteristics

Method will be developed and validated for use to analyze samples during the early development of an active pharmaceutical ingredient (API) or drug product. As drug development progresses from phase 1 to commercialization, the analytical method will follow a similar progression. The final method will be validated for its intended use for the market image drug product and transferred to the quality control laboratory for the launch of the drug product. However, if there are any changes in the manufacturing process that have the potential to change the analytical profile of the drug substance and drug product; this validated method may need to be revalidated to ensure that it is still suitable to analyze the API or drug product for its intended purpose.

Once an analytical method is developed for its intended use, it must be validated. The extent of validation evolves with the drug development phase. Usually, a limited validation is carried out to support an Investigational New Drug (IND) application and a more extensive validation for New Drug Application (NDA) and Marketing Authorization Application (MAA). Typical parameters recommended by FDA, USP, and ICH are as follow:

1.6.1-Linearity

ICH defines linearity of an analytical procedure as the ability (within a given range) to obtain test results of variable data (e.g., absorbance and area under the curve) which are directly proportional to the concentration (amount of analyte) in the sample. The data variables that can be used for quantitation of the analyte are the peak areas, peak heights, or the ratio of peak areas (heights) of analyte to the internal standard peak. Quantitation of the analyte depends on it obeying Beer's law for the spectroscopic method over a concentration range. Therefore, the working sample concentration and samples tested for accuracy should be in the linear range. There are two general approaches for determining the linearity of the method. The first

approach is to weigh different amounts of standard directly to prepare linearity solutions at different concentrations. However, it is not suitable to prepare solution at very low concentration, as the weighing error will be relatively high. Another approach is to prepare a stock solution of high concentration. Linearity is then demonstrated directly by dilution of the standard stock solution. This is more popular and the recommended approach. Linearity is best evaluated by visual inspection of a plot of the signals as a function of analyte concentration. Subsequently, the variable data are generally used to calculate a regression line by the least – squares method. At least five concentration levels should be used. Under normal circumstances, linearity is acceptable with a coefficient of determination (r 2) of ≥ 0.997. The slope, residual sum of squares, and y intercept should also be reported as required by ICH. The slope of the regression line will provide an idea of the sensitivity of the regression, and hence the method that is being validated. They intercept will provide an estimate of the variability of the method. For example, the ratios percent of they intercept with the variable data at nominal concentration are sometimes used to estimate the method variability. For the determination of potency assay of a drug substance or a drug product, the usual range of linearity should be ± 20% of the target or nominal concentration. For the determination of content uniformity, it should be ± 30% of the target or nominal concentration.(ICH Q2 2006).

1.6.2-Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple samples of the same homogeneous sample under prescribed conditions. Precision is usually investigated at three levels: repeatability, intermediate precision, and reproducibility. For simple formulation it is important that precision be determined using authentic homogeneous samples. A justification will be required if a homogeneous sample is not possible and artificially prepared samples or sample solutions are used.

Intermediate precision is defined as the variation within the same laboratory. The extent to which intermediate precision needs to be established depends on the circumstances under which the procedure is intended to be used. Typical parameters that are investigated include day - to - day variation, analyst variation, and equipment variation. Depending on the extent of the study, the use of experimental design is encouraged. Experimental design will minimize the number of experiments that need to be performed. It is important to note that ICH allows exemption from doing intermediate

precision when reproducibility is proven. It is expected that the intermediate precision should show variability that is in the same range or less than repeatability variation. ICH recommends the reporting of standard deviation, relative standard deviation (coefficient of variation), and confidence interval of the data

1.6.3- Accuracy

The International Convention on Harmonization (ICH) defines the accuracy of an analytical procedure as the closeness of agreement between the values that are accepted either as conventional true values or an accepted reference value and the value found. For drug substance, accuracy may be defined by the application of the analytical procedure to an analyte of known purity (e.g., a reference standard). For the drug product, accuracy will be determined by application of the analytical procedure to synthetic mixtures of the drug product components to which known amounts of analyte have been added within the range of the procedure. The ICH document also recommends assessing a minimum of nine determinations over a minimum of three concentration levels covering the specified range (e.g., three concentrations/three replicates).

Accuracy is usually reported as percent recovery by the assay (using the proposed analytical procedure) 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. The range for the accuracy limit should be within the linear range.

Typical accuracy of the recovery of the drug substance is expected to be about 99 – 101%. Typical accuracy of the recovery of the drug product is expected to be about 98 – 102%. Values of accuracy of recovery data beyond this range need to be investigated as appropriate.

1.6.4-Repeatability

Repeatability is a measure of the precision under the same operating conditions over a short interval of time, that is, under normal operating conditions of the analytical method with the same equipment. It is sometimes referred to as intra - assay precision. The ICH recommends that repeatability be assessed using a minimum of nine determinations covering the specified range for the procedure (e.g., three concentrations/three replicates as in the

accuracy experiment) or using a minimum of six determinations at 100% of the test concentration. Reporting of the standard deviation, relative standard deviation (coefficient of variation), and confidence interval is required. The assay values are independent analyses of samples that have been carried through the complete analytical procedure from sample preparation to final test result.

1.6.5- Reproducibility

Reproducibility measures the precision between laboratories. This parameter is considered in the standardization of an analytical procedure (e.g., inclusion of procedures in pharmacopeias and method transfer between different laboratories).To validate this characteristic; similar studies need to be performed at different laboratories using the same homogeneous sample lot and the same experimental design. In the case of method transfer between two laboratories, different approaches may be taken to achieve the successful transfer of the procedure. The most common approach is the direct - method transfer from the originating laboratory to the receiving laboratory. The originating laboratory is defined as the laboratory that has developed and validated the analytical method or a laboratory that has previously been certified to perform the procedure and will participate in the method transfer studies. The receiving laboratory is defined as the laboratory to which the analytical procedure will be transferred and that will participate in the method transfer studies. In the direct - method transfer, it is recommended that a protocol be initiated with details of the experiments to be performed and acceptance criteria (in terms of the difference between the means of the two laboratories) for passing the method transfer(G.C Hokanson. Table 2 provides examples of a set of method transfer data between two laboratories.

1.6.6-Selectivity and specificity

The ICH defines specificity as the ability to assess unequivocally an analyte in the presence of components that may be expected to be present. In many publications,are often used interchangeably. However, there are debates over the use of specificity over selectivity and some authorities, for example, the International Union of Pure and Applied Chemistry (IUPAC), have preferred the term selectivity , reserving specificity for those procedures that are completely selective. For pharmaceutical application, the above definition of ICH will be used. For identity test, compounds of closely related structures which are likely to be present should be discriminated from each other. This could be confirmed by obtaining positive results (by comparison with a known reference material) from samples containing the analyte, coupled with

negative results from samples which do not contain the analyte. Furthermore, the identification test may be applied to material structurally similar or closely related to the analyte to confirm that a positive response is not obtained. The choice of such potentially interfering materials should be based on sound scientific judgment with a consideration of the interferences that could occur. The specificity for an assay and impurity tests should be approached from two angles:

1. When Impurities Are Available The specificity of an assay method is determined by comparing test results from an analysis of sample containing the impurities, degradation products, or placebo ingredients with those obtained from an analysis of samples without the impurities, degradation products, or placebo ingredients. For a stability - indicating assay method, degradation peaks need to be resolved from the drug substance. However, these impurities do not need to be resolved from each other.

For the impurity test, the determination should be established by spiking drug substance or drug product with the appropriate levels of impurities and demonstrating the separation of these impurities individually and/or from other components in the sample matrix. Representative chromatograms should be used.

2. If Impurities are not available. Specificity may be demonstrated by comparing the test results of samples containing impurities or degradation products to a second well - characterized procedure or other validated analytical procedure (orthogonal method). This should include samples stored under relevant stress conditions (light, heat, humidity, acid/base hydrolysis and oxidation). For the assay method, the two results should be compared; for impurity tests, the impurity profiles should be compared. Peak homogeneity tests should be performed using PDA or mass spectrometry to show that the analyte chromatographic peak is not attributable to more than one component. Figure 2 illustrates the selectivity of a method to resolve known degradation peaks from the parent peak.

1.6.7- Range

The range of an analytical procedure is the interval between the upper and lower concentration of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity. The range is normally expressed in the same units as test results (e.g., percent, parts per million) obtained by the analytical procedure. For the assay of drug substance or finished drug product, it is

normally recommended to have a range of 80 – 120% of the nominal concentration.

For content uniformity, a normal range would cover 70 – 130% of the nominal concentration, unless a wider and more appropriate range (e.g., metered - dose inhalers) is justified.

For dissolution testing, a normal range is ± 20% over the specified range. If the acceptance criterion for a controlled - release product covers a region from 20% after Peak area 1 h, and up to 90% after 24 h, the validated range would be 0 – 110% of the label claim. In this case, the lowest appropriate quantifiable concentration of analyte will be used as the lowest limit as 0% is not appropriate. (Green,J.1996).

1.6.8-Detection limit (DL)

The detection limit (DL) is a characteristic for the limit test only. It is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated under the stated experimental conditions. The detection is usually expressed as the concentration of the analyte in the sample, for example, percentage, parts per million (ppm), or parts per billion (ppb). There are several approaches to establish the DL. Visual evaluation may be used for no instrumental (e.g., solution color) and instrumental methods. In this case, the DL is determined by the analysis of a series of samples with known concentrations and establishing the minimum level at which the analyte can be reliably detected. Presentation of relevant chromatograms or other relevant data is sufficient for justification of the DL. For instrumental procedures that exhibit background noise, it is common to compare measured signals from samples with known low concentrations of analyte with those of the blank samples. The minimum concentration at which the analyte can reliably be detected is established using an acceptable signal - to - noise ratio of 2: 1 or 3: 1. Presentation of relevant chromatograms is sufficient for justification of the DL. .(Wegscheider,H.1996)

Another approach estimates the DL from the standard deviation of the response and the slope of the calibration curve. The standard deviation can be determined either from the standard deviation of multiple blank samples or from the standard deviation of the y intercepts of the regression lines done in the range of the DL. This estimate will need to be subsequently validated by the independent analysis of a suitable number of samples near or at the DL:

$$DL = 3\sigma$$

Where: σ is the standard deviation of the response.

1.6.9-Quantization limit(QL)

The quantization Limit (QL) is a characteristic of quantitative assays for low levels of compounds in sample matrices, such as impurities in bulk drug substances and degradation products in finished pharmaceuticals. QL is defined as the concentration of related substance in the sample that will give a signal – to – noise ratio of 10 : 1. The QL of a method is affected by both the detector sensitivity and the accuracy of sample preparation at the low concentration of the impurities. In practice, QL should be lower than the corresponding ICH report limit. International conference of harmonization recommends three approaches to the estimation of QL. The first approach is to evaluate it by visual evaluation and may be used for no instrumental methods and instrumental methods. QL is determined by the analysis of samples with known concentrations of the analyte and by establishing the minimum level at which the analyte can be quantized with acceptable accuracy and precision. The second approach determines the signal - to - noise ratio by comparing measured signals from samples with known low concentrations of anlayte with those of blank samples. QL is the minimum concentration at which the analyte can be reliably quantified at the signal - to - noise ratio of 10 : 1. The third approach estimates QL by the equation

$$QL = 10\sigma / S$$

Where: σ is the standard deviation of the response and S is the slope of the calibration curve.

The slope S may be estimated from the calibration curve of the analyte. The value of σ may be estimated by (1) calculating the standard deviation of the responses obtained from the measurement of the analytical background response of an appropriate number of blank samples or (2) calculating the residual standard deviation of the regression line from the calibration curve using samples containing the analyte in the range of the QL. Whatever approach is applied, the QL should be subsequently validated by the analysis of a suitable number of samples prepared at the QL and determining the precision and accuracy at this level.(Rockville,Md.2007)

1.6.10-Robustness/ruggedness

The definition for applied is "The robustness/ruggedness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage

Robustness can be described as the ability to reproduce the (analytical) method in different laboratories or under different circumstances without the occurrence of unexpected differences in the obtained result(s), and a robustness test as an experimental set-up to evaluate the robustness of a method. The term ruggedness is frequently used as a synonym several definitions for robustness or ruggedness exist which is, however, all closely related. The one nowadays most widely applied in the pharmaceutical world is the one given by the International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH) and which was given above. a distinction between the terms ruggedness and robustness is made and ruggedness is defined there as the degree of reproducibility of the test results obtained under a variety of normal test conditions, such as different laboratories, different analysts, different instruments, different lots of reagents, different elapsed assay times, different assay temperatures, different days, etc. The latter definition will not be applied since detailed guidelines exist for the estimation of the reproducibility and the intermediate precision. The international conference of harmonization (ICH) guidelines also recommends that "one consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g. resolution tests) is established to ensure that the validity of the analytical procedure is maintainedwhenever used". The assessment of the robustness of a method is not required yet by the ICH guidelines, but it can be expected that in the near future it will become obligatory. Robustness testing is nowadays best known and most widely applied in the pharmaceutical world because of the strict regulations in that domain set by regulatory authorities which require extensively validated methods. Therefore most definitions and existing methodologies, e.g. those from the ICH, can be found in that field, as one can observe from the above. However, this has no implications for robustness testing of analytical methods in other domains and this guideline is therefore not restricted to pharmaceutical methods.

Robustness tests were originally introduced to avoid problems in inter laboratory studies and to identify the potentially responsible factors. This

means that a robustness test was performed at a late stage in the method validation since inter laboratory studies are performed in the final stage. Thus the robustness test was considered a part of method validation related to the precision (reproducibility) determination of the method However, performing a robustness test late in the validation procedure involves the risk that when a method is found not to be robust, it should be redeveloped and optimized. At this stage much effort and money have already been spent in the optimization and validation, and therefore one wants to avoid this. Therefore the performance of a robustness test has been shifting to earlier points of time in the life of the method. The Dutch Pharmacists Guidelines, the ICH Guidelines as well as some authors working in bio-analysis consider robustness a method validation topic performed during the development and optimization phase of a method, while others consider it as belonging to the development of the analytical procedure. (Senso,et al ,1997)

The robustness test can be viewed as a part of method validation that is performed at the end of method development or at the beginning of the validation procedure.
The exact position has relatively little influence on how it is performed.

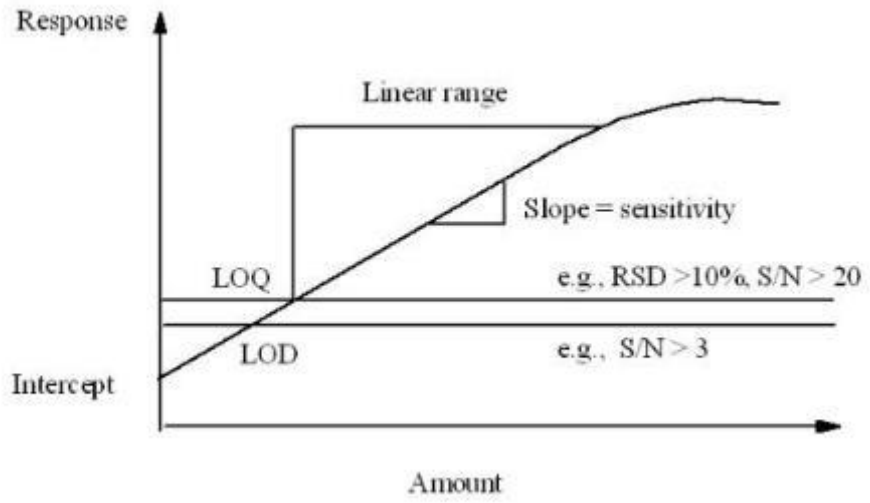

Figure1.3: Definitions for linearity, range, LOQ, LOD

1.7-Process of analytical method validation

The typical process that is followed in an analytical method validation is chronologically listed below:

1. Planning and deciding on the method validation experiments

2. Writing and approval of method validation protocol
3. Execution of the method validation protocol
4. Analysis of the method validation data
5. Reporting the analytical method validation
6. Finalizing the analytical method procedure

The method validation experiments should be well planned and laid out to ensure efficient use of time and resources during execution of the method validation. The best way to ensure a well - planned validation study is to write a method validation protocol that will be reviewed and signed by the appropriate person (e.g., laboratory management and quality assurance). The validation parameters that will be evaluated will depend on the type of method to be validated. Analytical methods that are commonly validated can be classified into three main categories: identification, testing for impurities, and assay.(U.S.FDA 2000).

Execution of the method validation protocol should be carefully planned to optimize the resources and time required to complete the full validation study. For example, in the validation of an assay method, linearity and accuracy may be validated at the same time as both experiments can use the same standard solutions. A normal validation protocol should contain the following contents at a minimum:

(a) Objective of the protocol
(b) Validation parameters that will be evaluated
(c) Acceptance criteria for all the validation parameters evaluated
(d) Details of the experiments to be performed
(e) Draft analytical procedure

The data from the method validation data should be analyzed as the data are obtained and processed to ensure a smooth flow of information. If an experimental error is detected, it should be resolved as soon as possible to reduce any impact it may have on later experiments. Analysis of the data includes visual examination of the numerical values of the data and chromatograms followed by statistical treatment of the data if required.

Upon completion of all the experiments, all the data will be compiled into a detailed validation report that will conclude the success or failure of the validation exercise. Depending on the company's strategy a summary of the validation data may also be generated. Successful execution of the validation will lead to a final analytical procedure that can be used by the laboratory to support future analytical work for the drug substance or drug product.(Krause,S.(2006).

1.8-Information required in analytical procedure:

The minimal information that should be included in a final analytical procedure is as follows:

(a) Rationale of the analytical procedure and description of the capability of the method. Revision of analytical procedure should include the advantages offered by the new revision.

(b) Proposed analytical procedure. This section should contain a complete description of the analytical procedure in sufficient detail to enable another analytical scientist to replicate it. The write - up should include all important operational parameters and specific instructions, for example, preparation of reagents, system suitability tests, precautions, and explicit formulas for calculation of the test results.

(c) List of permitted impurities and its levels in an impurity assay.

(d) Validation data. Either a detailed set or summary set of validation data is included

1.9-Type of analytical procedure:

Note: - characteristic not normally evaluated +characteristic normally evaluated.

(a) In cases where reproducibility has been performed, intermediate precision is not needed.

(b**)** Lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s).

(c) May be needed In some cases.

(e) Revision history.

(f) Signature of author, reviewer, management, and quality assurance.

1.10-Method verification:

The U.S. Food and Drug Administration (FDA) regulation 21 CFR 211.194(a)(2) specifically states that users of analytical methods in the U.S. Pharmacopeia/National Formulary (USP/NF) are not required to validate the accuracy and reliability of these methods but merely verify their suitability under actual conditions of use.

USP has issued guidance for verification in general chapter ⟨ 1226 ⟩. This proposal provides general information to laboratories on the verification of compendial procedures that are being performed for the first time to yield acceptable results utilizing the laboratories' personnel, equipment, and reagents.(Kaye CM,Sanky MJ.1980).

Verification consists of assessing selected analytical validation characteristics described earlier to generate appropriate, relevant data rather than repeating the validation process for commercial products. The guidance in this general chapter is applicable to applications such as titrations, chromatographic procedures (related compounds, assay, and limit tests), and spectroscopic tests. However, general tests (e.g., water, heavy metals, residue on ignition) do not typically require Verification.(Citac,S.and Eura,A 2002).

.

1.11-Method revalidation

There are various circumstances under which a method needs to be revalidated. Some of the common situations are described below:

1. During the optimization of the drug substance synthetic process, significant changes were introduced into the process. To ensure that the analytical method will still be able to analyze the potentially different profile of the API, revalidation may be necessary.

2. If a new impurity is found that makes the method deficient in its specificity, this method will need to be modified or redeveloped and revalidated to ensure that it will be able to perform its intended function.

3. A change in the excipient composition may change the product impurity profile. This change may make the method deficient in its specificity for the assay or impurity tests and may require redevelopment and revalidation.

4. Changes in equipment or suppliers of critical supplies of the API or final drug product will have the potential to change their degradation profile and may require the method to be redeveloped and revalidated.(ICH 1996).

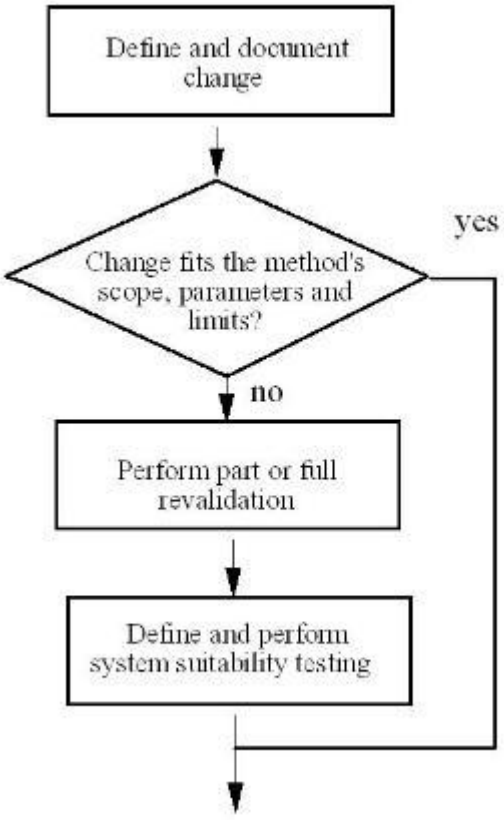

Figure1.4: Flow diagrams for revalidation

1.12-Steps in method validation

Successful acceptance of the validation parameters and performance criteria, by all parties involved, requires the cooperative efforts of several departments, including analytical development, QC, regulatory affairs and the individuals requiring the analytical data. The operating procedure or the Validation Master Plan (VMP) should clearly define the roles and responsibilities of each department involved in the validation of analytical methods.

The scope of the method and its validation criteria should be defined early in the process. These include the following specification:

- The analyte to be detected.
- The concentration levels.
- The sample matrices.
- The interfering substances expected,
- any specific legislative or regulatory requirements
- information be qualitative or quantitative
- the required detection and quantitation limits
- precision and accuracy is expected
- robust should the method be
- type of equipment should be used, Is the method for one specific instrument, or should it be used by all instruments of the same type
- the method be used in one specific laboratory or should it be applicable in all laboratories at one side or around the globe
- skills do the anticipated users of the method have

The method's performance characteristics should be based on the intended use of the method. It is not always necessary to validate all analytical parameters that are available for a specific technique. For example, if the method is to be used for qualitative trace level analysis, there is no need to test and validate the method's limit of quantitation, or the linearity, over the full dynamic range of the equipment. Initial parameters should be chosen according to the analyst's experience and best judgment. Final parameters should be agreed between the lab or analytical chemist performing the validation and the lab or individual applying the method and users of the data to be generated by the method. Table 2 gives examples of which parameters might be tested for a particular analysis task.

The scope of the method should also include the different types of equipment and the locations where the method will be run. For example, if the method is to be run on a specific instrument in a specific laboratory, there is no need to use instruments from other vendors or to include other laboratories in the validation experiments. In this way, the experiments can be limited to what is really necessary. (Peer,A.1998).

Table1.1: Validation parameters for specific task

	Major compounds Quantitative	Major compounds and traces quantitative	Traces qualitative	Traces Qualitative
limit of detection	No	No	Yes	No
limit of quantitation	No	yes	No	Yes
Linearity	Yes	yes	No	Yes
Range	Yes	yes	No	No
Precision	Yes	yes	No	Yes
Accuracy	Yes	yes	No	Yes
Specificity	Yes	yes	Yes	Yes

Basics of UV Spectrophotometry:

It is the branch of science dealing with the study of interaction between Electromagnetic radiation and matter. It is a most powerful tool available for the study of atomic and molecular structure/s and is used in the analysis of wide range of samples. Optical spectroscopy includes the region on electromagnetic spectrum between 100 Å and 400 μm. The regions of electromagnetic spectrum are shown in table 2.Ultraviolet-Visible spectrophotometry UV-Visible spectrophotometersis one of the most frequently employed techniques in pharmaceutical analysis. It involves measuring the amount of ultraviolet or visible radiation absorbed by a substance in solution. Instrument which measure the ratio, or function of ratio, of the intensity of two beams of light in the U.V-Visible region are called Ultraviolet-Visible spectrophotometers. In qualitative analysis, organic compounds can be identified by use of spectrophotometer, if any recorded data is available, and quantitative spectrophotometric analysis is used to ascertain the quantity of molecular species absorbing the radiation. Spectrophotometric technique is simple, rapid, moderately specific and applicable to small quantities of compounds. The fundamental law that governs quantitative spectrophotometric analysis is Beer -Lambert law(Rang HP,Dale M,(2003).

1.13.1- Beer's law: It states that the intensity of a beam of parallel monochromatic radiation decreases exponentially with the number of absorbing molecules. In other words, absorbance is proportional to the concentration.

1.13.2 Lambert's law: It states that the intensity of a beam of parallel monochromatic radiation decreases exponentially as it passes through a medium of homogeneous thickness. A combination of these two laws yields the Beer-Lambert law.

Table1.2:Regions of electromagnetic spectrum.

Region	Wavelength
Far (or vacuum)ultraviolet	10-200 nm
Near ultraviolet	200-400 nm
Visible	400-750 nm
Near infrared	0.75- 2.2 μm
Mid infrared	2.5-50 μm
Far infrared	50-1000 μm

1.13.3- Beer-Lambert law: When beam of light is passed through a transparent cell containing a solution of an absorbing substance, reduction of the intensity of light may occur. Mathematically,

Beer-Lambert law is expressed as: A=a b c

Where, A= absorbance or optical density

b= absorptivity or extinction coefficient

b= path length of radiation through sample (cm)

C= concentration of solute in solution.

Both b and a are constant so a is directly proportional to the concentration cWhen c is in gm/100 ml, then the constant is called A (1%, 1 cm)

$$A=A\ (1\%1\ cm)\ bc$$

Quantification of medicinal substance using spectrophotometer may carried out by preparing solution in transparent solvent and measuring it's absorbance at suitable wavelength. The wavelength normally selected is wavelength of maximum absorption (λmax), where small error in setting the wavelength scale has little effect on measured absorbance. Ideally, concentration should be adjusted to give an absorbance of approximately 0.9, around which the accuracy and precision of the measurements are optimal.

The assay of single component sample, which contains other absorbing substances, is then calculated from the measured absorbance by using one of three principal procedures. They are, use of standard absorptivity value, calibration graph and single or double point standardization. In standard absorptive value method, the use of standard A (1%, 1 cm) or E values are used in order to determine its absorptivity. It is advantageous in situations where it is difficult or expensive to obtain a sample of the reference substance. In calibration graph method, the absorbance's of a number of standard solutions of the reference substance at concentrations encompassing the sample concentrations are measured and a calibration graph is constructed. The concentration of the analyte in the sample solution is read from the graph as the concentration corresponding to the absorbance of the solution. The single point standardization procedure involves the measurement of the absorbance of a sample solution and of a standard solution of the reference substance. The concentration of the substances in the

sample is calculated from the proportional relationship that exists between absorbance and concentration.

$$C\ test1=(Atest\times Cstd)/Astd$$

Where C test and C std are the concentrations in the sample and Standard solutions respectively and A test and A std are the absorbance's of the sample and standard solutions respectively. For assay of substance/s in multi component samples by spectrophotometer; the following methods are being used routinely, which includes :-

- Simultaneous equation method
- Derivative spectrophotometric method
- Absorbance ratio method (Q-Absorbance method)
- Difference spectrophotometry
- Solvent extraction method

Dilution solvent should be as similar to mobile phase as possible.

1.14- Metronidazole:

Metronidazole is an antibiotic that fights bacteria and used to treat bacterial infections of the vagina, stomach, liver,skin and respiratory tract.it is the drug of choice for first episode of mild to moderate clostridium difficile colitis. Metronidazole is available by mouth, as acream, and intravenously.Metronidazole is began to be commercially used in 1960 in france. It is on the world health organization list of essential medicines, the most effective and safe medicines needed in ahealth system.it is available in most areas of the world.(Liu, and Yang , (2009).

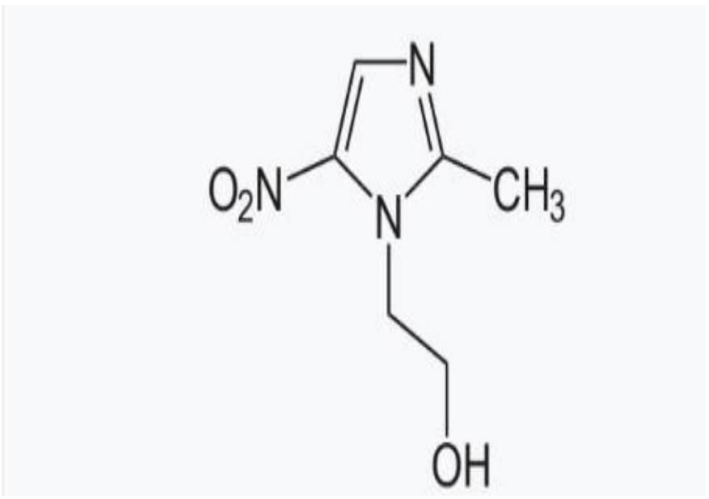

Figure1.6: Structure of metronidazole

1.14.2-Chemical data:

Table1. 3: Chemical data of metronidazole

Formula	$C_6H_9N_3O_3$
Molar mass	171.16 $g.Mol^{-1}$

1.14.3- Side effects and contraindications:

The common side effects include nausea, ametallic taste, loss of appetite, and headaches. Occasionly seizures or allergies to the medication may be occur. Some state that metronidazole should not be used in early pregnancy while other state doses for trichomoniasis are safe.it should not be used when breastfeeding.(Wegscheider H, 1996).

1.14.4- Mechanism of action:

Metronidazole is of the nitroimidazole class.it inhibits nucleic acid synthesis by disrupting the DNA of microbial cells. This function only occurs when metronidazole is partially reduced, and because this reduction usually happens only in anaerobic cells. It has relatively little effect upon human cells or aerobic bacteria (Rockville,M, (2007).

1.14.5-Doses:

General dosage information for the active ingredient contained in metronidazole:

Metronidazole: oral tablet, oral capsule, intravenous solution, hntravenous powder for injection, compounding powder, oral suspension.

1.14.6- Excipients:

Excipients are the additives used to convert pharmacologically active compounds into pharmaceutical dosage forms suitable for administration to patients.although excipients are the non- active ingredients,they are essential in the successful production of acceptable solid dosage forms such as tablets and powders.

1.14.7- Excipients used in metronidazole tablets:-

Excipients	functions
Maize starch	Binders to hold powders together to form granules for tableting
M.c.c	Disintigrants to facilitate the breakup of atablet in the gastrointestinal tract
Lactose	To act as abulking agent or filling material
Talc powder	To improve the flow of granules from the hopper to the die cavity to ensure uniform fill for each tablet
Magnesium stearate	Act as lubricant

1.15- Objective:

Based on the importance and advantages of UV-Vis method in pharmaceutical industry this work aimed to:

- To estimate and validate the UV-VIS method using the validation parameters: Pression , Accuracy ,Linearity , Range , Selectivity ,sensitivity , limit of detection ,limit of quantitation.

Chapter Two

2- Materials and Methods

2.1- Instrumentation and conditions:

2.1.1Absorption spectral measurements were carried out with a UV – Visible spectrophotometer (Shimadzu Model 1800) using UV Probe software version 2 was employed with spectral bandwidth of 1 nm and wavelength accuracy of 0.3 nm (with automatic wavelength correction with a pair of 5 cm matched quartz cells.

2.1.2 Digital heated ultrasonic path.

2.1.3 Sensitive balance Adam Equipment's.

2.2-Materials

*metronidazole were supplied by India and used as such.

*****Methanol used was from sharlabfine chemicals Ltd,India.

*Water used was generated by reverse osmosis.

*metronidazole Tablet used was supplied by marwa Pharmaceutical

2.3-Methods

2.3.1-Preliminary solubility studies of drugs

Solubility of the drug was determined at 28±1C^0. A small quantity of standard drugs were dissolved in different solvents like distilled water, methanol, acetone, By the solubility studies we determined that the drug was dissolved in methanol,water(4:1).

2.3.2Preparation of standard solutions

The metronidazole reference standard solution (1000 mg L^{-1}) was prepared by accurately weighing 50.0 mg of metronidazolereference in a 50.0 mL volumetric flask. The volume was completed with methanol. This flask was sonicated for 20 min. this solution was diluted in volumetric flask with methanol.1ml of the solution pipette and diluted to the mark with the solvent. The concentration of the solution was 20mg/L

2.3.3-Determination of maximum absorption $\lambda_{max.}$

From the stock solutions, a working standard was prepared. The absorption spectrum for MNZ the absorption spectrum was recorded using 10 mg L^{-1} solution and the maximum absorption was found to be 313 nm.

Chapter Three

3- Results and Discussion

3.1-Method validation parameters

3.1.1-Linearity

The linearity was determined by plotting concentration against the corresponding absorbance. The calibration curve was defined in the concentration interval in which the intensity of the spectrophotometer response was linearly proportional to the concentration of the analyzed substance:

General equation of regression line:

$$A = a.c + b$$

Where:*A* is the absorbance; *C*, concentration of sample; *a*, slope of the curve; and, *b*, y intercept of the curve.

The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method and the correlation coefficient (*r*) indicated the linearity of the method.

Table 3.1: Determination of linearity

Sample No.	**Concentration (ppm)**	**Absorbance**
1	4	0.221
2	6	0.335
3	8	0.455
4	10	0.562
5	12	0.670
SD = 0.136		
R^2 = 0.999		

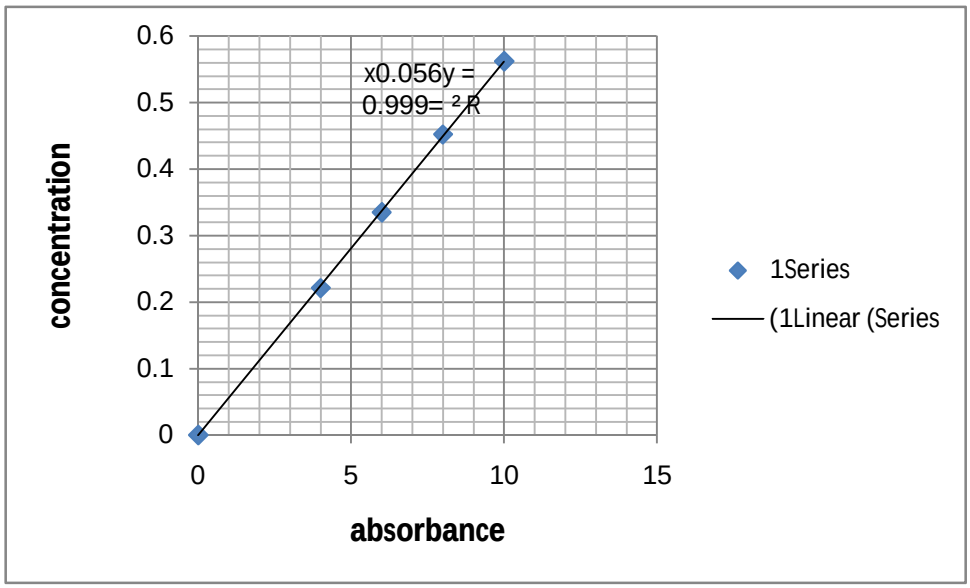

Figure3.1: Linear plot of metronidazole

Correlation Coefficient = 0.999

Slope = 0.056

Intercept =0

***Acceptance criteria**

Typically, a square of correlation coefficient (r2 > 0.99) demonstrate linearity. In addition y-intercept must not be significantly different from zero

3.1.2 Range:

Table 3.2: Determination of linearity range

Sample No.	Concentration (ppm)	Absorbance
1	1	0.076
2	2	0.119
3	4	0.231
4	6	0.345
5	8	0.460
6	10	0.566
7	12	0.689

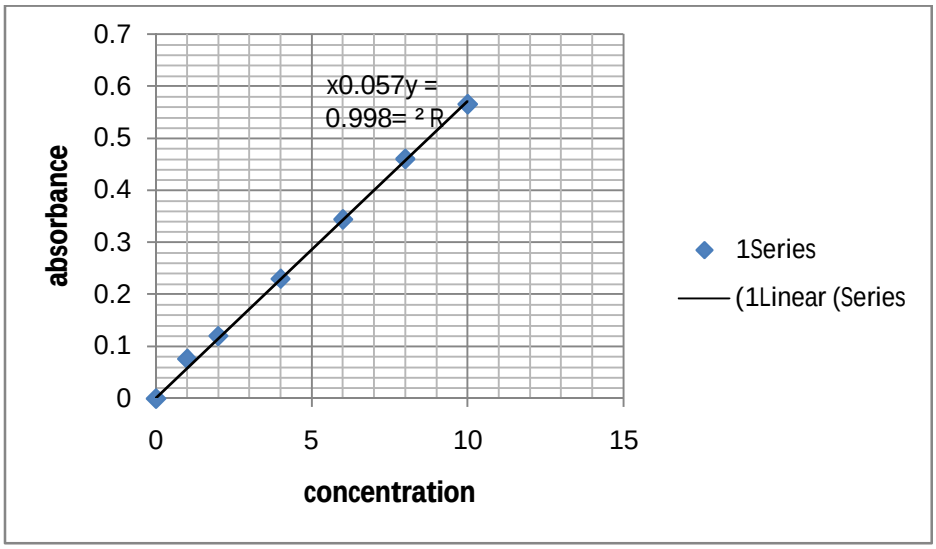

Figure3.2: Range of linearity

Correlation Coefficient	0.998
Slope	0.057
Intercept	**0**

***Acceptance criteria**

Typically, a square of correlation coefficient (r2 > 0.99) demonstrate linearity Ran. In addition y-intercept must not be significantly different from zero.

3.1.3 Precision

The intra-day precision was determined by analyzing the samples of metronidazole at concentrations of 5.0 µg mL^{-1}. Determinations were performed with 6 replicates on the same day. The precision is expressed as relative standard deviation (RSD) amongst responses. In order to be considered

precise

* **Acceptance criteria**

RSD of the method should be less than 2.0%.

3.1.3.1 Repeatability

Table 3.3: Determination of repeatability

Concentration	6 PPM	8 PPM	10 PPM
	0.344	0.460	0.565
	0.345	0.460	0.566
	0.344	0.459	0.566
	0.344	0.459	0.562
	0.344	0.460	0.564
	0.345	0.460	0.569
Mean	0.344	0.459	0.564
SD	0.00032	0.00032	0.00128
%RSD	0.09	0.069	0.22

*** Acceptance criteria**

RSD f the method should be less than 2.0%.

3.1.3.2 Intermediate precision

Table 3.4: Determination of intermediate precision

concentration	**Time**	
	09.30 a.m.	**12.30 p.m.**
standard 10 ppm 1	0.562	0.564
standard 10 ppm 2	0.562	0.565
standard 10 ppm 3	0.565	0.562
standard 10 ppm 4	0.564	0.562
standard 10 ppm 5	0.564	0.566
standard 10 ppm 6	0.569	0.569
Mean	0.564	0.564
SD	0.00112	0.00144
%RSD	0.198	0.255

3.1.4 Accuracy

The accuracy of the method was evaluated through the recovery test. Recovery tests were performed by adding known amounts of standard solutions to samples followed by analyses using the proposed method. Aliquots of standard and samples solutions were transferred to 10 mL volumetric flasks and final volumes were completed with methanol. The

percentage of recovery (R) was calculated as indicated by association of official analytical chemists International.

$$R = \left[\frac{(C_f - C_u)}{C_A}\right] \times 100$$

Where C_F represents the concentration of analyte measure in fortified test sample; C_U , the concentration of analyte measure in unfortified test sample; and, C_A , the concentration of analyte added to fortified test sample.

Table 3.5: Determination of accuracy

Concentration	absorbance	conc ppm	Recovery %	Mean	SD	RSD%
80%	0.231	4.01	99.5	99.7	0.204	0.205%
80%	0.23	4	100			
80%	0.231	4.01	99.6			
100%	0.279	4.85	97.0	99.2	1.56	1.57%
100%	0.289	5.02	100.5			
100%	0.288	5.00	100.17			
120%	0.344	5.98	99.7	100	1.08	1.08%
120%	0.35	6.08	101.4			
120%	0.341	5.93	98.8			

.

* **Acceptance criteria**

The measured recovery should be 98% to 102% of the amount added

3.1.5 Specificity & Selectivity

Specificity is the ability of the method to accurately measure a compound in the presence of other components such as impurities, degradation products and matrix components. The specificity of the proposed method was evaluated through the analysis of a placebo solution, which it was prepared with the excipients of the pharmaceutical formulation. Thus, the mixture of component inert was prepared in their usual concentration employed in tablets than the method was applied in order to check if any component of the formulation could generate a response or a read with absorption band similar to the drug. And the placebo didn't show any absorbance at the specified wave length.

3.1.6 Limit of detection:

The results of Linearity were used to find out the detection limit using the method of standard deviation of absorbance's and the slope ,in which the detection limit is expressed by the following relation according to International Conference on Harmonization guidelines.

LOD = 3.3 SD / a

Where *SD* represents the standard deviation

And *a* is the slope of calibration curve.

3.1.7-Limit of quantitation:

The results of linearity were used to find out the quantitation limit using the method of standard deviation of the absorbance's and the slope in which it expressed by the following relation

LOQ = 10 SD /a

Table 3.6: Determination of LOD and LOQ

LOD	LOQ
0.04007 µg/mL	0.12100µg/mL

3.1.8 Robustness:

Method robustness was performed by applying small changes in the wave length.

Table 3.7: Determination of robustness by using different wave length of light on 10 ppm solution of sample

	Wave length	absorbance	Assay	Mean	SD	RSD %
Sample 1	313	0.562	99.652174	99.4%	0.141	0.142%
	312	0.561	99.478261			
	314	0.560	99.304348			
Sample 2	313	0.561	99.304348	99.0%	0.41	0.41%
	312	0.561	99.304348			
	314	0.559	98.434783			
Sample 3	313	0.559	98.26087	98.4%	0.46	0.46%
	312	0.559	98.086957			
	314	0.560	98.956522			

3.2 CONCLUSION

In this study, the developed and validated UV-spectrophotometric alternative method for the determination of metronidazole in pharmaceutical formulations has the advantage of being fast, simple, cost-effective with high precision, and accuracy. These advantages encourage the application of this method in routine analysis of metronidazole

The proposed method for the determination metronidazole in solid dosage form was found to be precise, selective, rapid and economical. metronidazoleexhibited maximum absorption at 313 nm and obeyed Beer's law in the concentration range of 2-20 µg/ml. the proposed method for the determination of metronidazole showed linear regression

Y = 0.057x with a correlation coefficient (R^2) of 0.999(Figure 8). A relative standard deviation of

0.09, 0.069 and 0.228 % was observed on analysis of six replicate samples of concentration 6, 8 & 10 ppm respectively.

Our studies revealed a recovery percentage of 99.24 – 100.0 %, which indicates that the developed method was simple, rapid and precise. The proposed method can be used for the drug analysis in routine quality control & method proves to be more economical than the published standard methods.

3.3 Recommendations

1. Develop a validation master plan or an operating procedure for method validation.
2. Check all equipment and material for performance and quality. .
3. For non-routine methods: develop and use generic methods and customize them for specific non-routine tasks.
4. Analytic method development and validation are continuous and interconnected activities conducted throughout the drug development process. The practice of validation verifies that a given method measures a parameter as intended and establishes the performance limits of the measurement. Although apparently contradictory, validated methods produce results within known uncertainties. These results are crucial to continuing drug development, as they define the emerging sknowledge base supporting the product.
5. The time and effort that are put into developing scientifically-sound, robust, and transferrable analytic methods should be aligned with the drug development stage. The resources that are expended on method validation must be constantly balanced with regulatory requirements and the probability for product commercialization.

References

- Ashour, S.andKattan, N. (2010): Simultaneous determination of miconazole nitrate and metronidazole in different pharmaceutical dosage forms by gas chromatography and flame ionization detector (GC-FID),*International Journal of Biomedical Science*,**6**:13-18.
- Breaux,J. anJones,K.(2003) Pharmaceutical Technology, Analytical Technology and Testing.
- Citac, S. and Eura,A (2002): Working Group, International guide to quality in analytical chemistry.
- FDA Guidance for Industry –Analytical Procedures and Method Validation, Chemistry, Manufacturing, and Controls Documentation, Center for Drug Evaluation and Research (CDER) and Center for Biologics Evaluation and Research (CBER), August 2000.
- Green,A. (1996) practical guide to analytical method validation, *Analytical Chem. News & Features, pp. **305A–309***
- Hokanson,C.(1994):A life cycle approach to the validation of analytical methods during pharmaceutical product development
- Huber,Land S. George, (1993):Diode-array detection in high-performance liquid chromatography
- ICH Topic Q 2 (R1)
- International Conference on Harmonization (ICH)(1996):Technical Requirements for the Registration of Pharmaceuticals for Human Use, Validation of analytical procedures: definitions and terminology, Geneva
- ISO/IEC 17025,(2005): General requirements for the competence of testing and calibration laboratories
- Joel,G.H.(2012): Goodman and Gilman's the Pharmacological basis of therapeutics.
- Journal of Chemical and Pharmaceutical Research, 2012, 4(2):1090-1096
- Kaye CM, Sankey MJ: (1980) A rapid and specific semi-micro method involving high-pressure liquid chromatography for the assay of metronidazole in plasma, saliva, serum, urine and whole blood.
- Krause, S.(2006):A Guide for testing biopharmaceuticals, Part II: acceptance criteria and analytical method maintenance, Pharm. Tech.

- Liu ,H. Yang,R.(2009): Determination of common antibiotics and metronidazole in cosmetics by ultra performance liquid chromatography-tandem mass spectrometry. *Chinese Journal of Chromatography 2009;* ***27:50-53.***
- Peer, A .(1998) :Verified Methods Program, Manual on policies and procedures,Arlington,

- Renger,B, and Jehle,H.,(1995): Validation of analytical procedures in pharmaceutical analytical chemistry
- Rang HP, Dale M, (2003):*Pharmacology.6th Edn. Elsevier publication house, New Delhi; 2003, 666-671*
- Rockville,Md.(2007): The United States Pharmacopeial Convention.
- Remington, The Science and Practice of Pharmacy; Volume II; 21st Edition; Lippincott Williams & Wilkins; Philadelphia; 2010; p.1669.
- Seno,S. Ohtake and H. Kohno,(1997): Analytical validation in practice at a quality control laboratory in the Japanese pharmaceutical industry.
- U.S. FDA - Guidance for Industry (draft) Analytical Procedures and Methods Validation: Chemistry, Manufacturing, and Controls and Documentation, 2000
- U.S. FDA - Guidance for Industry, Bioanalytical Method
- U.S. EPA, Guidance for methods development and methods validation for the Resource Conservation and Recovery Act (RCRA) Program, Washington.
- Wegscheider,H.(1996):Validation of analytical methods, in: Accreditation and quality assurance in analytical chemistry.

- Winslow, P. and Meyer, R. (1997): Defining a master plan for the validation of analytical methods, J. Validation Technology