DEVELOPMENT AND VALIDATION OF SIMPLE AND SENSITIVE METHODS FOR THE DETERMINATION OF ACTIVE INGREDIENTS FROM PHARMACEUTICAL PREPARATION USING SPECTROPHOTOMETRIC AND HPLC TECHNIQUES

A thesis submitted to

THE MAHARAJA SAYAJIRAO UNIVERSITY OF BARODA
For the degree of

DOCTOR OF PHILOSOPHY

in

APPLIED CHEMISTRY

Guide
Dr. P. Padmaja Sudhakar

Submitted by
Shaligram Rane

APPLIED CHEMISTRY DEPARTMENT
FACULTY OF TECHNOLOGY AND ENGINEERING
THE MAHARAJA SAYAJIRAO UNIVERSITY OF BARODA
VADODARA- 390 001
GUJARAT (INDIA)

November, 2011

Tel: +91-265-2434188

APPLIED CHEMISTRY DEPARTMENT
Faculty of Technology & Engineering,
The Maharaja Sayajirao University of Baroda,
POST BOX No. 51, KALABHAVAN,
VADODARA-390 001 (India)

Date: -25th November, 2011

CERTIFICATE

This is to certify that the thesis entitled "**DEVELOPMENT AND VALIDATION OF SIMPLE AND SENSITIVE METHODS FOR THE DETERMINATION OF ACTIVE INGREDIENTS FROM PHARMACEUTICAL PREPARATION USING SPECTROPHOTOMETRIC AND HPLC TECHNIQUES**" submitted for Ph.D. Degree in Applied Chemistry by Mr. Shaligram Rane incorporates the original research work carried out by him under my supervision.

(Dr. Padmaja Sudhakar)
Research Guide

Head
Applied Chemistry Department

Dean,
Faculty of Technology and Engineering,
The M. S. University of Baroda,
Vadodara.

DECLARATION

I, hereby declare that the work presented in this thesis entitled "**DEVELOPMENT AND VALIDATION OF SIMPLE AND SENSITIVE METHODS FOR THE DETERMINATION OF ACTIVE INGREDIENTS FROM PHARMACEUTICAL PREPARATION USING SPECTROPHOTOMETRIC AND HPLC TECHNIQUES**" is carried out by me as an independent investigation. All the references of other workers are clearly indicated with the source of information under the references section.

Signature of the candidate

Shaligram Rane

Certified and forwarded through research guide

Dr. P. Padmaja Sudhakar
Research Guide,
Chemistry Department,
Faculty of Science,
The Maharaja Sayajirao University of Baroda,
Vadodara

Acknowledgements

First and foremost, I would like to express my deep and sincere gratitude and appreciation to my guide Dr. P. Padmaja Sudhakar, who introduced me to this topic and whose original and never ending flow of ideas and encouragement are in a large part responsible for the work described in this thesis. Inculcation of having a systematic approach in any scientific endeavor will help me in the long run. I shall always cherish the memories of my association with her.

I am sincerely thankful to Dean, Faculty Authority of Faculty of Technology & Engineering and to Prof. C.N. Murthy, Applied Chemistry Department, for providing me the laboratory facilities during their tenures and to Prof. P.T. Deota, Head Applied Chemistry Department.

I am very much thankful to Dr. D. P. Bharmabe, Applied Chemistry Department, without whose cooperation and moral support; it would not have been possible to carry out this work and also for providing all-round guidance including rules and regulations.

I am thankful to Prof. Uma Chudasama, Dr. Jayrang S. Dave, Dr. P.T. Deota, Dr. A.K. Prajapati and all other teaching and non-teaching staff members of the department for their kind help during my Ph.D. work.

I am also thankful to Prof. Dr. Dhake (Physics Dept.), Former Head, Physics Department for introduction and motivation at all levels during my Ph.D work.

I am sincerely thankful to Dr. Urmish Chudgar, Managing Director, Intas Biopharmaceuticals Ltd., Ahmedabad for allowing me to do my research work along with current job responsibilities.

I am grateful to Dr. Rustom Mody, and Dr. Kashmira Pagdiwalla for providing facilities at Intas Biopharmaceuticals Ltd. Dr. Rustom Mody has always provided the technical support and guidance during experimental design to completion of work for biopharmaceutical molecules.

I am also thankful to my earlier subordinates Dr. T.T. Rao, Mr. Diwan, Dr. Abhay Chheda, Mr. Tanaji Patil, Mr. Nanasaheb Gunjal, Mr. Vinay Balen, Mr. Vivek Madan for providing support of facility, knowledge sharing, providing sample etc. during my research work.

I am also thankful to my subordinates Mr. Ravindra Wagh, Mr. Deepak Gupta, Ms. Nilam Diwan, Mr. Hitesh Patel and Mr. Banger for support and sharing my existing job responsibilities.

I am also very thankful to my subordinates Mr. Alkesh Ajameri for support during analysis of samples,

I express my heartfelt thanks to our group Sushil Choudhari, Jignesh B. Patel, Meeta, Sansruti, Sreelatha Gaddam, Ajeetha V., Harneesh, Jitendra, Mayur Patel, Vinod Parimal and all my close colleagues of Applied Chemistry and Chemistry Department for their co-operation, encouragement and valuable tips throughout the entire research work. And my special thanks to Shilpi Kushawa for her help at the final stages of my thesis work.

I would like to express my deep sense of gratitude to my family and my wife for their patience and continued encouragement and I am highly grateful to my son Mohit for all her sacrifices and made possible to complete this work. I would like to say my heartfelt thanks to Mr. Barhate and family for moral support during my stay in Baroda. And I express my heartfelt thanks to my friend Sushil Choudhari for his co-operation, moral support, encouragement and valuable suggestions throughout the entire journey of my research work. Lastly I would like to acknowledge to my all friends and all my well wishers.

Shaligram Rane

Dedicated
to
my parents,
son and wife

By the grace of Lord Ganesha

CONTENTS

Chapter-1

INTRODUCTION

1.1. INTRODUCTION – ANALYTICAL CHEMISTRY

Analytical Chemistry plays a critical role in the development of a compound from its synthesis stage to its marketing stage as a part of a drug formulation and analysis [1].

Before the introduction of chromatographic methods into pharmaceutical analysis in the middle of the 20th century, almost exclusively classical methods such as titrimetry, gravimetry and later on UV spectrophotometry/colorimetry were available for this purpose. It was well known already in those years that, due to the poor specificity of these methods, the value of the percentage figures obtained in such a way for the active ingredient content were of limited value. Nevertheless, due to the lack of specific chromatographic methods these assay methods were considered to be among the most important characteristics of the quality of a bulk drug substance. The purity was checked by means of physical constants, mainly by the melting point and the width of the melting range, limit tests for signal (mainly inorganic) impurities, clarity and colour of the solution of the material, etc.

1.2. THE PRESENT STATE-OF-THE-ART: ASSAY METHODS IN THE CHROMATOGRAPHY ERA

1.2.1. Introduction

The invention and rapid spread of thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) in the 1960s and 1970s [2], respectively, created an entirely new situation in this field. The reasons for this are as follows: (1) both methods enable the detection, separation, identification and quantitative determination of organic impurities which were up to that time not measurable [3]; (2) the selective chromatographic methods were found to be suitable for the reliable determination of also the main component.

1.2.2. Non-specific methods

1.2.2.1. Titrimetric methods

Classical, non-specific methods are still used, especially in the European Pharmacopoeia [4]. Of these, the non-specificity of titrimetric methods is evident: in the majority of cases organic impurities contain the same functional group on which the titration of the drug material is based. Signs of some modernization are the spreading of non-aqueous titration methods expanding the field of application of titrimetric methods to (very) weak acids and bases as well as potentiometric (in the case of nitritometric titrations amperometric) end-point detection improving the precision of the methods. Advantages of these methods are saving time and labour, high precision and the fact that there is no need of using reference standards. However, due to their poor specificity the accuracy of titrimetric methods is also poor in the presence of related impurities.

1.2.2.2. Spectrophotometric/colorimetric methods

Another group of non-specific methods in pharmacopoeias are spectrophotometric methods based on natural UV–VIS absorption and to a lesser extent visual (VIS) spectrophotometric methods based on chemical reactions (colorimetric methods) [5]. The reason for their non-specificity is the same as in the case of titrimetric methods: most of the impurities of drugs contain the same or similar chromophoric groups as those of the drug material. The low time and labour consumption of the methods as well as good precision are advantages in this case also, especially if the method is based on natural absorption. There is no clear picture regarding the necessity of reference standards. In the majority of pharmacopoeial monographs of US Pharmacopoeia [6] the use of reference standards is prescribed, while in the European

Pharmacopoeia the calculation of the content is mainly (but not exclusively) based on specific absorbance values given in the monographs. Although the principles of the validation of the determination of specific absorbance have been set up [7], and this is the less time consuming approach, this can be the source of further analytical error, if not high-level spectrophotometers are used for the assay. It is worth mentioning that (although not too many) startlingly outdated colorimetric methods based on chemical reactions are still in use for the assay of bulk drug materials.

1.2.2.3. Other methods

Although some other non-specific methods (polarimetry, polarography, fluorimetry, etc.) do not play an important role in the assay of bulk drugs, it is to be noted that even the precision of these methods is by no means sufficient for this purpose.

1.2.3. Specific chromatographic methods

1.2.3.1. High-performance liquid chromatography

HPLC methods appeared for the first time for the assay of bulk drug materials in 1980 [8]. This has become the predominant method in USP XXVII [6] and—although to a lesser extent—it is one of the most widely used methods also in Ph. Eur. 4 [4]. The reason for this is that, in contrast to the above discussed non-specific methods the specificity of this method is excellent and at the same time sufficient precision is also attainable. Due to these advantageous features and the disadvantages of the methods discussed so far it can be stated HPLC is certainly one of the methods applicable for the assay of drug materials which can afford accurate results. However, it has to be mentioned that the high specificity, precision and accuracy are attainable only if lengthy system suitability tests are carried out prior to the HPLC assay. For this reason the price to be paid for the high specificity, precision and accuracy is also high:

the HPLC method is by about one order of magnitude more time consuming and labour extensive than the above discussed non-specific methods.

1.2.3.2. Gas chromatography (GC)

Due to the insufficient volatility and thermal stability of the majority of drug materials, gas chromatography can be used for their assay in a limited number of cases only. For the specificity, precision and accuracy as well as the time and labour consumption of this method the same considerations apply that are described for HPLC

1.2.3.3. Thin-layer chromatography–UV spectrophotometry

Before the introduction and widespread adoption of HPLC, the high specificity of TLC was often exploited for quantitative analytical purposes using spot elution followed by spectrophotometric measurement and is still prescribed in some cases in USP XXVII [6] inspite of being a labour-intensive and less precise method.

The low acquisition, operational and maintenance costs needed to successfully perform the TLC analytical technique are very important because it can provide product quality assessment capability in areas where laboratory facilities for pharmaceutical quality analysis are minimal or do not exist.

Kenyon et al. demonstrated that TLC can be used to provide a semi-quantitative yet versatile and robust testing of pharmaceuticals in a resource limited environment [9] The ease of deployment with low operational costs of the TLC based analytical techniques has been a key to the vast increase in its use to detect counterfeit/substandard medicines in markets particularly in resource constrained settings [10, 11].

Recent advancements in technology have contributed to a marked improvement of repeatability and reliability of TLC based testing. Automating the TLC sample

application step has markedly improved repeatability of the sample application process, and thereby the overall test procedure. In addition the detection technology has been developed to measure the intensity of a spot of interest on the plate by which comparisons to standards can be related to drug content. With the aid of software, the complex mathematics needed to calculate the drug content from the reflected light can be easily performed. These two key developments have made TLC-Densitometry a reliable method for pharmaceutical drug analysis. The separation media also have been improved by reducing the particle size and uniformity which has evolved into HPTLC. The HPTLC offers all of the advantages of TLC but with improved separation capacity by marked improvement in plate numbers which approach those afforded by the conventional HPLC columns.

The above developments have increased the acquisition costs for HPTLC plates but the new systems have brought improved versatility, throughput and robustness to the TLC technique while retaining the low running and maintenance costs.

1.2.3.4 Ultra Performance Liquid Chromagrography (UPLC)

Ultra Performance Liquid Chromatography (UPLC) could be considered to be a new direction of liquid chromatography. UPLC, as its first producer Waters proclaims, means "speed, resolution and sensitivity" [12]. As it is very well known from Van Deemter equations, the efficiency of chromatographic process is proportional to particle size decrease. According to his model describing band broadening, which describes relationship between height equivalent of theoretical plate (HETP) and linear velocity, one of the terms (path dependent term), is dependent on a diameter of particle packed into the analytical column. Smaller particle diameter can significantly reduce HETP which results in higher efficiency and the flatter profile of Van Deemter curve (Figure 1). Consequently, the mobile phase flow-rate increase does not have

negative influence to the efficiency as it could be observed at 10 or 5 μm particles [13-15]. The negative aspect of small particle packed columns used in HPLC is, however, high back-pressure generating.

In conventional HPLC the choice of particle size must be a compromise. The smaller the particle size, the higher the column back-pressure is occurring in the HPLC system. That could be a limitation of the use of such columns in HPLC systems. Small column diameters like 2.1 or 1.0 mm could also cause similar problems and disable their use under the conventional conditions. Throughout the history of HPLC there has been a trend to use smaller particles packing material. Due to the pressure limitation of conventional equipment, shorter columns packed with small particle diameter particles were used.

However, in order to use ultra high pressure chromatography routinely in the laboratory, some practical concerns, such as sample introduction, reproducibility and detection still needed an improvement. Ultra high pressure columns required extremely narrow sample plugs to minimize any sample volume contribution to peak broadening. To overcome these problems, Acquity UPLC system was developed because many of ultra high pressure systems used before needed in-house modification of commercial products by laboratory itself and also the own manufacturing of analytical columns [16-18] often capillary columns, as was stated above.

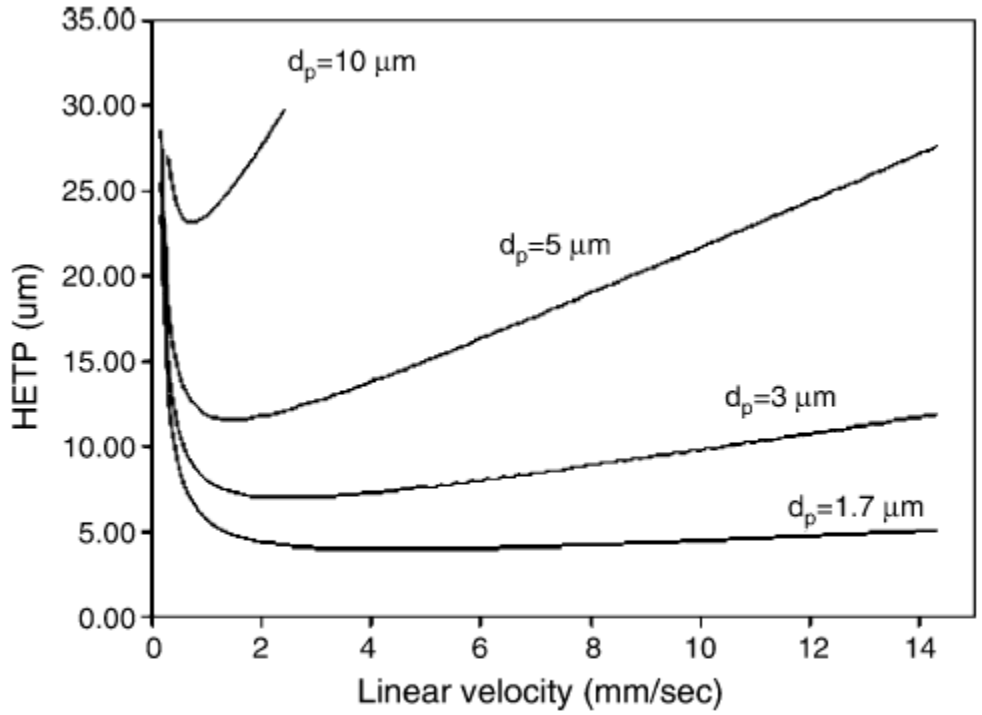


Fig. 1. Van Deemter curves for different particle sizes (10, 5, 3, 1.7 µm).

1.3 VALIDATION

The demonstration of the ability of an analytical method to quantify is of great importance to ensure quality, safety and efficacy of pharmaceuticals. Consequently, before an analytical method can be implemented for routine use, it must first be validated to demonstrate that it is suitable for its intended purpose.

1.4 LIQUID CHROMATOGRAPHIC METHOD

The actual meaning and utilization of the various phases of a validation are exemplified by a survey of published methods on LC analysis of drug substances and dosage forms.

- **Linearity-**

The response function for an analytical procedure is the existing relationship, within a specified range, between the response (signal, e.g. area under the curve, peak height, absorption) and the concentration (quantity) of the analyte in the sample. The

calibration curve should be described preferably by a simple increasing or decreasing) response function that gives reliable measurements, i.e. accurate results. The response function – or standard curve – is widely and frequently confounded with the linearity criterion.

The linearity criterion refers to the relationship between the quantity introduced and the quantity back-calculated from the calibration curve while the response function refers to the relationship between the instrumental response and the concentration.

For an analyst, the "test results" are, without ambiguity, the back-calculated measurements evaluated by the "regression line" that is in fact the calibration curve, established using appropriate statistics methodologies. Another aspect that is very important is the fit-for purpose principle [19]. The central idea is very logical: the purpose of an analytical procedure is to give accurate measurements in the future; so a standard curve must be evaluated on its ability to provide accurate measurements. A significant source of bias and imprecision in analytical measurements can be caused by the inadequate choice of the statistical model for the calibration curve. The statistical criteria such as $R2$, lack-of-fit or any other statistical test to demonstrated quality of fit of a model are only informative and barely relevant for the objective of the assay [19-23]. For that intend, several authors [24-26] have introduced the use of the accuracy profile based on the tolerance intervals (or prediction intervals) to decide if a calibration model will give quality results.

- **Accuracy**

In document ICH Q2R1 part 1 [27], accuracy is defined as: ". . . the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found."

Accuracy is usually established through spiked placebo studies (simulated samples) in which, placebo is fortified with drug at various concentrations above and below the target claim. Frequently 0, 80, 100 and 120% or 0, 75, 100 and 125% of claim are used. These samples are then passed through the processing scheme, assayed and the linearity of recovery is calculated with appropriate statistical analysis.

Under certain circumstances use of the spiked placebo method is impossible such as in academic settings or in government labs, which cannot obtain authentic placebo and its exact composition is unknown. Here the standard addition method (SA) should be used to verify accuracy by beginning with a sample and then adding known amounts of standard to it in order to derive a linearity expression. This method is also commonly practiced in impurity analysis for drug substance in which various levels of impurity are added to the lot of bulk drug showing lowest impurity levels. Linearity of recovery of degradation products likewise can be calculated following their addition to placebo for drug products.

Recovery studies can be performed using different columns or on different days are drug substance recovery studies are also performed which do not relate to method accuracy but only to reproducibility of standard preparation.

- **Precision**

The ICH Q2R1 Part 1 definition of precision is: "The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions."

Precision is expressed as standard deviation (s), variance (s2) or relative standard deviation (RSD) or coefficient of variation (CV). It measures the random error linked to the analytical procedure, i.e. the dispersion of the results around their average

value. The estimate of precision is independent of the true or specified value and the mean or trueness estimate. For ICH Q2R1 and ISO documents, three levels could be assessed:

(1) Repeatability which "expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision."

(2) Intermediate precision which "expresses within-laboratories variations different days, different analysts, different equipment, etc."

(3) Reproducibility which "expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology)."

The repeatability conditions involve the re-execution of the entire procedure to the selection and preparation of the test portion in the laboratory sample and not only the replicate instrumental determinations on a single prepared test sample. The latter is the instrumental precision which does not include the repetition of the whole analytical procedure.

The document of the FDA, also distinguish "within-run, intra batch precision or repeatability, which assesses precision during a single analytical run", and "between-run, inter-batch precision or repeatability, which measures precision with time, and may involve different analysts, equipment, reagents, and laboratories".

As can be seen in the regulatory documents what makes the difference between repeatability and intermediate precision is the concept of series or runs. These series or runs are composed at least of different days with eventually different operators and/or different equipments. A run or series is a period during which analyses are executed under repeatability conditions that remain constant. The rational to select the different factors which will compose the runs/series is to mimic conditions that will be encountered during the routine use of the analytical procedure.

It is evident that the analytical procedure will not be used only 1 day. So including the variability from one day to another of the analytical procedure is mandatory. Then during its routine use, will the analytical procedure be used by only one operator, and/or on only one equipment? Depending on the answers of these questions, different factors representing the procedure that will be used during the routinely performed analysis will be introduced in the validation protocol, leading to a representative estimation of the variability of the analytical procedure.

When the selection of the appropriate factors is made, an experimental design can be made in order to optimize the number of runs or series to account for the main effects of these factors with a cost effective analysis time. For example if the factor selected are days, operators and equipments, each of them at two levels, then a fractional factorial design allows to execute four runs or series in only 2 days. The design is shown in Table 1.1.

Table 1.1 Experimental design of four runs taking into account days, operators and equipments as sources of variability

Run 1	**Run 2**	**Run 3**	**Run 4**
Day 1	Day 1	Day 2	Day 2
Operator 1	Operator 2	Operator 1	Operator 2
Equipment 2	Equipment 1	Equipment 1	Equipment 2

Usually, precision is commonly expressed as the percent Relative Standard Deviation (RSD). The classical formula is:

$$\text{RSD}\ (\%) = 100 \times \frac{\sqrt{\sigma^2}}{\bar{x}}$$

When an RSD precision is expressed, the corresponding variance is used, e.g. repeatability or intermediate precision. The computed RSD is therefore the ratio of two random variables, giving a new parameter with high uncertainty. However, in the

case of validation of analytical procedure, because the true or reference value is known, then the denominator should be replaced by its corresponding true value μT. The RSD computed by this way depends only on the estimated precision (estimated variances), regardless of the estimated trueness.

The precision tests include tests for precision of the system which is measured by replicate analysis of a single standard solution, ordinarily run before initiation of sample analysis as part of a system suitability test. This precision measurement should be carried out on each day a particular analysis is performed giving rise to the expression of results for different days. Method precision is shown by replicate analysis of a pooled sample such as the thoroughly mixed contents from 20 capsules, 20 finely ground tablets or five ampoules. Each measured aliquot is carried through the entire sample preparation scheme and assayed. This measurement can be done on more than and also by using more than one column. Precision of recovery is based on multiple measurements made on placebos spiked at one concentration. Precision of linearity of recovery is the measure derived from the linearity of recovery study in which percents recovered at each concentration, possibly in replicate, are analysed to give the RSD. A third term in the context of precision is robustness or ruggedness.

The US Pharmacopeia [28] defines ruggedness as: "The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same sample 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.

In the chemical literature however, a ruggedness test was defined as [27, 29]: "An intra laboratory experimental study in which the influence of small changes in the operating or environmental conditions on measured or calculated responses is

evaluated. The changes introduced reflect the changes that can occur when a method is transferred between different laboratories, different experimentators, different devices, etc."

As part of a ruggedness test, the method precision is determined by assaying the same set of samples in different labs. Method ruggedness is also indicated by results from tests in which standard mixtures are chromatographed using mobile phase variations of l0-20% (organic/aqueous) and by use of one mobile phase with three to five columns of different age for analysis of a standard mixture. A final precision measure that is determined is a method precision in which different lots of bulk drug are assayed.

The steps of a ruggedness test

A ruggedness test requires an experimental design approach. It consists of the following steps:

- Selection and identification of the operational or environmental factors to be investigated;
- Selection of levels for the factors to be examined. In a ruggedness test 2 or 3 levels for each factor are normally considered. The ruggedness for the factors in the intervals between the factor levels is then investigated;
- Selection of the experimental design;
- Carrying out the experiments described in the design. This is the experimental part of the ruggedness test;
- Computation of the effect of the factors on the response(s) of the method, to derive which factors might have experimentally relevant effects;
- Statistical analysis of the results. In this part of the test statistically significant effects are identified;

- Drawing chemically relevant conclusions;
- When necessary giving advice for improvement of the performance of the method and definition of suitability criteria.

Selection of the factors

As a first step one selects a number of factors to examine. The selected factors should be chosen from the description of the analytical procedure or from environmental parameters which are not necessarily specified explicitly in the analytical method. The factors can be quantitative (continuous, numerical) or qualitative (discrete). The factors to be tested should represent those that are most likely to be changed when a method is transferred, for instance, between different laboratories, different devices, or over time, and that potentially could influence the response of the method. However it is not always obvious which factors will influence a response and which will not. This is one of the reasons why screening designs are used. They allow to screen a large number of factors in a relatively small number of experiments.

A list of different factors investigated in different publications is given [30-34]. The list is not exhaustive

Some salient features to be noted include:

- The selection of the factor "type of acid" in a ruggedness test could be accepted when only the pH is specified by the method rather than the acid used to bring the solution or the buffer up to the desired pH. Clearly, however, in such a case the method is poorly defined.
- A group of factors causing problems are HPLC columns. Some articles [31, 35, 36] propose to include the factor "batch of material" or "manufacturer of material" in a two level design and do this by comparing two columns. However, it is far from evident that these two selected columns are extreme levels for the whole

population of batches from one manufacturer or for the population of columns from different manufacturers. The problem could be tackled by examining more than two columns. One possibility is to consider the column factors in the same way as the factors "different laboratories, different analysts, different instruments".

Selection of the levels of the factors

In a second step the levels for the chosen factors are selected. For quantitative factors one considers a low and a high extreme level that is respectively smaller and larger than the nominal one. The nominal level is the level for the factor as it is given in the description of the procedure or the one that is most likely to occur in the case it is not specified in the analytical procedure. The levels for the factors are chosen in such a way that they represent the maximum difference in the values of the factors that could be expected to occur when a method is transferred from one laboratory to another without the occurrence of major errors [31].

A common error is to select levels that are too far apart from each other. In a ruggedness test one selects the extreme levels of the factors to be somewhat larger than the changes that would occur for this factor under normally changing conditions (different laboratories, etc.). In a number of published ruggedness tests one finds levels that are quite far from each other, much further than can occur by transferring a method between different laboratories. Since one does not know the effect of the factor in advance one will introduce a large possibility of finding a significant effect which is not relevant for the evaluation of the ruggedness. If in a method description the pH of the mobile phase is 5.0 then one normally should be able to work in an interval between 4.8 and 5.2. This then is the interval proposed to be examined in a ruggedness test and not for example 4.0 and 6.0. Examples of levels of factors that

seem too far from each other and that were tested in different ruggedness tests are given in Table 3.2.

Table 3.2 Some levels of factors that are tested with large intervals in a ruggedness test (HPLC methods)

Factor	**Levels as tested in the literature**
pH	Nominal ± 1 [31] Nominal ± 0.5 [32, 34, 36]
Flow rate	Nominal ± 0.3 ml/min [32] Nominal ± 0.5 ml/min [34, 36]
Wavelength (UV)	Nominal ± 8 to 12 nm [36]

Selection of the experimental design

To examine the ruggedness of the factors that were selected one could test these factors one variable at a time, i.e. change the level of one factor and keep all other factors at nominal level. The result of this experiment is then compared to the result of experiments with all factors at nominal level. The difference between the two types of experiments gives an idea of the effect of the factor in the interval between the two levels. The disadvantage of this method is that a large number of experiments is required when the number of factors is large.

For this reason one prefers to apply an experimental design. In the literature a number of different designs are described, such as saturated fractional factorial designs and Plackett-Burman designs, full and fractional factorial designs, central composite designs and Box-Behnken designs [37].

Decision rule

Most of the regulatory documents do not make any recommendation on acceptance limits to help the analyst to decide when an analytical procedure is acceptable. The only exception found concerns the FDA document on bio-analytical methods that

clearly indicates in the pre-study validation part: "The mean value should be within ±15% of the theoretical value, except at LLOQ, where it should not deviate by more than ±20%. The precision around the mean value should not exceed 15% of the CV, except for LLOQ, where it should not exceed 20% of the CV." Later, when referring to in-study validation, the same document indicates: "Acceptance criteria: At least 67% (4 out of 6) of quality control (QC) samples should be within 15% of their respective nominal value, 33% of the QC samples (not all replicates at the same concentration) may be outside 15% of nominal value. In certain situations, wider acceptance criteria may be justified."

Dosing range

For any quantitative method, it is necessary to determine the range of analyte concentrations or property values over which the method may be applied. ICH Q2R1 part 1 document defines the range of an analytical procedure as "the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity". The FDA Bio-analytical Method validation definition of the quantification range is "the range of concentration, including ULOQ and LLOQ that can be reliably and reproducibly quantified with accuracy and precision through the use of a concentration–response relationship", where LLOQ is the lower limit of quantitation and ULOQ is the upper limit of quantitation. Thus, the above mentioned definitions are quite similar because for both of them, the range is correlated with the linearity and the accuracy (trueness + precision). Moreover, both documents specify that the range is dependent on the specific application of the procedure. ICH Q2R1 part 2 states that the specified range is "established by confirming that the analytical procedure provides an acceptable

degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure". The range should be anticipated in the early stage of the method development and its selection is based on previous information about the sample, in a particular study. The chosen range determines the number of standards used in constructing a calibration curve.

ICH Q2R1 part 2 recommends the minimum specified ranges for different studies:

- for the assay of a drug substance or a finished (drug) product: normally from 80 to 120% of the test concentration;
- for content uniformity, covering a minimum of 70–130% of the test concentration, unless a wider more appropriate range, based on the nature of the dosage form (e.g. metered dose inhalers), is justified;
- for dissolution testing: ±20% over the specified range;
- for the determination of an impurity: from the reporting level of an impurity to 120% of the specification.

Therefore, the dosing range is the concentration or amount interval over which the total error of measurement – or accuracy – is acceptable. It is essential to demonstrate the accuracy of the results over the entire range. Consequently, and in order to fulfill these definitions, the proposition of ICH document to realize six measurements only at the 100% level of the test concentration to assess the precision of the analytical method should be used with precautions to be in accordance with the definition of the range. Accuracy, and therefore trueness and precision should be evaluated experimentally and acceptable over the whole range targeted for the application of the analytical procedure.

Limit of quantitation

ICH considers that the "quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products". ICH Q2R1 part 1 defines the quantitation limit of an individual analytical procedure as "the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy". Limit of quantitation (or quantitation limit) is often called LOQ. Both terms are used in regulatory documents, the meaning being exactly the same. ICH document defines only one limit of quantitation. But the quantification range of the analytical procedure has two limits: LLOQ and ULOQ.

ICH Q2R1 part 2 proposes exactly the same approaches to estimate the (lower) quantification limit as for the detection limit. A first approach is based on the well known signal-to-noise (s/n) ratio approach. A 10:1 s/n is considered by ICH document to be sufficient to discriminate the analyte from the background noise. The main problem appears when the measured signal is not the signal used to quantify the analyte. For example, in chromatography with spectral detection, the measured signal represents the absorption units, i.e. the signal height but for the quantitation the areas are generally used. Therefore, the quantitation limit is not expressing the lowest level of the analyte, but lowest quantified absorbance.

The other approaches proposed by ICH Q2R1 part 2 documents are based on the "Standard Deviation of the Response and the Slope" and it is similar to the approach used for detection limit computation. The computation ways for detection (DL) and quantitation limit (QL) are similar, the only difference being the multiplier of the standard deviation of the response:

$$DL = \frac{3.3\sigma}{S};\ \ QL = \frac{10\sigma}{S}$$

where σ is the standard deviation of the response and S = the slope of the calibration curve [19, 38-55].

System Suitability

System suitability tests include resolution factor, precision of standard analysis or precision of impurity analysis and can include such measures as tailing factor or standard linearity. Other parameters measured under system suitability can include capacity factor (k'), retention time (t), relative retention (a), number of theoretical plates (iV) or peak symmetry (s).

These terms have been adequately described in many reviews and in the USP [56].

Capacity factor (capacity ratio) k'

This value gives an indication of how long each component is retained on the column (ie how many times longer the component is retarded by the stationary phase than it spends in the mobile phase).k' is used in preference to retention time because it is less sensitive to fluctuations in chromatographic conditions (ie flow rate) and therefore ensures greater reproducibility from run to run. In practice the k value for the first peak of interest should be >l to assure that it is separated from the solvent.

$$k' = \frac{t_R - t_m}{t_m}$$

tm = unretained peak's retention time

tR = retention time of the peak of interest

Separation Factor (relative retention)

This describes the relative position of two adjacent peaks. Ideally, it is calculated using the capacity factor because the peaks' separation depends on the components' interaction with the stationary phase. Therefore considering peaks A and B Separation

factor calculation k for the later peak is always placed in the numerator to assure a value >l.

If the capacity factor is used then the separation factor should be consistent for a given column, mobile phase, composition and specified temperature, regardless of the instrument used. The separation factor gives no indication of the efficiency of the column.

Peak Resolution R

This is not only a measure of the separation between two peaks, but also the efficiency of the column. It is expressed as the ratio of the distance between the two peak maxima. (At) to the mean value of the peak width at base (Wb).

Specificity

Specificity studies include the subcategory selectivity. Selectivity implies that the method separates potential process impurities (I), degradation products (D) and structural analogues (A). Specificity, as a broader concept, also includes peak homogeneity. This means that a particular peak corresponds to a single chemical entity rather than several different molecules whether structural, geometrical or configurational isomers or unrelated compounds with overlapping retentions. This property can be indicated by diode-array detection in which spectra taken at various times while a peak is eluting are compared with standard spectra known to be due to a single entity. A second means of showing peak homogeneity is to collect the fraction as the peak elutes and run the sample in an alternate chromatographic system such as TLC or a different mode of LC. Alternatively a non-chromatographic stability indicating method such as capillary electrophoresis or certain electrochemical methods may verify that a collected peak and a standard substance are the same. When a method is shown to be specific for a particular compound, this implies that

the method is stability indicating. Further proof of this implication comes from stress studies in which drug product and/or drug substance are degraded chemically (acid, base, oxygen, air), thermally and photo-chemically. These forced degradations (FD) give rise to reaction products which can be separated from the parent compound and quantified. If this is done as part of a stability study, degradation kinetics (DK) can be established. A stressed placebo study can be included as well to show that no products resulting from possible excipient decomposition will interfere with measurement of components of interest [57].

1.5 CLEANING VALIDATION

In pharmaceutical industry the cleaning procedure is one of the most important tasks to avoid the cross contamination for subsequent batches manufactured in the same equipment. Analytical methods used to determine residuals or contaminants should be specific for the substance or the class of substances to be assayed (e.g., API residue, detergent residue) and be validated prior to cleaning validation [58-60]. Guidelines recommend thin layer chromatography (TLC), UV photometric, total organic carbon analysis (TOC), conductivity, gas chromatography (GC) and conventional high performance liquid chromatography (HPLC) methods for cleaning control or validation [61]. The use of other analytical methods, including capillary gas chromatography [62], over-pressured layer chromatography (OPLC) [63] or micellar electro-kinetic chromatography (MEKC) [64], have also been described. Ion mobility spectrometry (IMS) [65] and TOC [66] have the advantage of speed over the abovementioned methods but TOC is not specific and IMS is usually not available at pharmaceutical manufacturing facilities. Liquid chromatography–mass spectrometry (LC–MS) [67, 68] and ultra performance liquid chromatography–mass spectrometry (UPLC–MS) [69] techniques applied in pharmaceutical cleaning verification have the

advantage of improved sensitivity, selectivity and general applicability even for UV-inactive compounds. However, these techniques are more expensive than the other techniques mentioned above and not widespread yet in cleaning control analysis. Nowadays HPLC–UV is the most commonly applied technique for cleaning control and validation [70-75]. In liquid chromatography, the analysis time can be reduced by using small columns packed with sub-2μm particles. In addition, with sub-2μm particles, due to the higher efficiency and smaller retention volume, sensitivity is also improved, compared to conventional HPLC. However, extra column effects are more significant for scaled down separations, therefore it is essential to minimize extra column dispersion.

A dedicated low dispersion system for ultra-high pressure separation (UPLC) with the particle size of stationary phases reduced down to 1.7μm, small dwell and extra column volume is able to work up to 1000 bar (15,000 psi). In such a way the analysis time could be reduced down to 1–3 min, without the loss of resolution and sensitivity [76-77].

The cleaning procedures for the equipment must be validated according to good manufacture practice (GMP) rules and guidelines [78-79].

During the cleaning validation following factors should be taken into consideration: equipment construction material, sealing part and parts that offers greater risk of contamination. It is important to standardize cleaning procedures and cleaning material, verification of residues chemical products and post-cleaning microbial load. Other factors such as time that the equipment can be considered clean, sampling procedure and analysis of contaminating residues in the equipment should also be considered. The analysis method and selected sampling procedure should be validated

and presents adequate extraction-recovery to favor the analysis of possible contaminating residues [80].

The acceptable limit for residue in the equipments is not established in the current regulations. However, Food and Drug Administration (FDA) mention that the limit should be based on logical criteria, involving the risk associated to residues of a determining product [81]. The calculation of acceptable residual limit for active products in production equipments should be based on therapeutical doses, pharmacological activity and toxicological index. Several mathematical formulas were proposed that can be used to establish acceptable residual limit [80].

To summarise, GMP/GLP compliance is vital to the success of the pharmaceutical industries. In this regard sensitive and selective methods for checking purity of new drug candidates, monitoring changes during scale up or revision of synthetic procedures, evaluating new formulations, and running control/assurance of the final drug product. Cross contamination with active ingredients is also a matter of real concern. The Code of Federal Regulations (CFR) states that "Equipment and utensils shall be cleaned, maintained, and sanitized at appropriate intervals to prevent malfunctions or contamination that would alter the safety, identity, strength, quality, or purity of the drug product beyond the official, or other established requirements". Cleaning validation is required in the pharmaceutical field to avoid potential clinically significant synergistic interactions between pharmacologically active chemicals.

1.6 AIM AND OBJECTIVE

Thus the main objectives of our work are

Method development for cleaning validation using Loratadine as a model pharmaceutical

Method development and validation for protein based drugs in the presence of stabilizers used model drugs- erythropoietin, parathyroid Hormone. Sensitive and selective method development for pharmaceutical model drug- ketoconazole.

1.7 References

[1]. Vinod K. Gupta, Rajeev Jain, Keisham Radhapyari, Nimisha Jadon, Shilpi Agarwal, Voltammetric techniques for the assay of pharmaceuticals—A review, Analytical Biochemistry 408 (2011) 179–196

[2]. J. Cases (Ed.), Encyclopedia of Chromatography, Marcel-Dekker, New York, 2001.

[3]. S. G¨or¨og (Ed.), Identification and Determination of Impurities in Drugs, Elsevier, Amsterdam, 2000.

[4]. The European Pharmacopoeia, fourth ed., Council of Europe, Strasbourg, 2002.

[5]. S. G¨or¨og, Ultraviolet-Visible Spectrophotometry in Pharmaceutical Analysis, CRC Press, Boca Raton, 1995.

[6]. United States Pharmacopoeia, XXVII ed., The USP Convention Inc., Rockville, MD, 2004.

[7]. U. Rose, J. Fuchs, Pharmeuropa 10 (1998) 146–148.

[8]. United States Pharmacopoeia, XX ed., The USP Convention Inc., Rockville, MD, 1980.

[9]. T.A. Kenyon, A.S. Kenyon, T. Sibiya, Bull. World Health Organ. 72 (4) (1994) 615.

[10]. J.-M. Caudron, N. Ford, M. Henkens, C. Mace, K. Monroe, J. Pinel, Trop. Med. Int. Health 13 (8) (2008) 1062.

[11]. P.N. Newton, M.D. Green, F.M. Fernández, N.P. Day, N.J. White, Lancet Inf. Dis. 6 (2006) 602.

[12]. Ultra Performance LCTM by design, 2004. Waters Corporation, USA, 720000880EN LL&LW-UL.

[13]. J.J. Van Deemter, F.J. Zuiderweg, A. Klingengerg, J. Chem. Eng. Sci. 5 (1956) 272.

[14]. C.E. Meloan, Chemical separation principles, in: Techniques and Experiments, John Wiley & Sons, Canada, 1999.

[15]. Y. Kazachevich, H. McNair, Texbook on High Performance Liquid Chromatography, http://hplc.chem.shu.edu/NEW/HPLC Book/, 1996.

[16]. J.E. MacNair, K.C. Lewis, J.W. Jorgenson, Anal. Chem. 69 (1997) 983.

[17]. J.E. MacNair, K.D. Patel, J.W. Jogenson, Anal. Chem. 71 (1999) 700.

[18]. N. Wu, J.A. Lippert, M.L. Lee, J. Chromatogr. A 911 (2001) 1.

[19]. J.W. Lee,V. Devanarayan,Y.C. Barrett, R.Weiner, J. Allinson, S. Fountain, S. Keller, I. Weinryb, M. Green, L. Duan, J.A. Rogers, R. Millham, P.J. O'Brian, J. Sailstad, M. Khan, C. Ray, J.A.Wagner, Pharm. Res. 23 (2006) 312.

[20]. Analytical Methods Committee, Analyst 113 (1988) 1469.

[21]. S.V.C. de Souza, R.G. Junqueira, Anal. Chim. Acta 552 (2005) 25.

[22]. J. Ermer, H.-J. Ploss, J. Pharm. Biomed. Anal. 37 (2005) 859.

[23]. C. Hartmann, J.Smeyers-Verbeke, D.L. Massart, R.D.McDowall, J. Pharm. Biomed. Anal. 17 (1998) 193.

[24]. Ph. Hubert, J.-J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap, N. Cohen, P.-A. Compagnon,W. Dewe, M. Feinberg, M. Lallier,M. Laurentie, N. Mercier, G. Muzard, C. Nivet, L. Valat, J. Pharm. Biomed. Anal. 36 (2004) 579.

[25]. Ph. Hubert, J.-J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap, N. Cohen, P. A. Compagnon,W. Dewe, M. Feinberg, M. Lallier,M. Laurentie, N. Mercier,G.Muzard, C. Nivet, L.Valat, STP Pharma Pratiques 13 (2003) 101.

[26]. D. Hoffman, R. Kringle, J. Biopharm. Stat. 15 (2005) 283.

[27]. International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use, Topic Q2 (R1): Validation of Analytical Procedures: Text and Methodology, Geneva, 2005

[28]. The United States Pharmacopeia XXII, The National Formulary XVII, United States Pharmacopeial Convention, Rockville, 1990, p. 1712.

[29]. B.-A. Persson, J. Vessman, Trends Anal. Chem. 20 (2001) 526.

[30]. S.W. Sun, Experimental and statistical approach for validating a test procedure in a pharmaceutical formulation, Ph. D. thesis, Laboratoire de Chimie Analytique, Facult6 de Pharmacie, Montpellier, 1993.

[31]. J.A. Van Leeuwen, L.M.C. Buydens, B.G.M. Vandeginste, G. Kateman, P.J. Schoenmakers, M. Mulholland, RES, an expert system for the set-up and interpretation of a ruggedness test in HPLC method validation. Part 1: The ruggedness test in HPLC method validation, Chemometrics and Intelligent Laboratory systems, 10 (1991) 337-347.

[32]. Caporal-Gautier J., Nivet J.M., Algranti P., Guilloteau M., Histe M., Lallier M., N'Guyen-Huu J.J. and Russotto R., Guide de validation analytique, Rapport d'une commission SFSTP, STP Pharma Pratiques, 2 (1992) 205-239.

[33]. G. Wynia, P. Post, J. Broersen and F. Maris, Ruggedness testing of a gas chromatographic method for residual solvents in pharmaceutical substances, Chromatographia, 39(5/6) (1994) 355-362

[34]. M. Mulholland, J. Waterhouse, Development and evaluation of an automated procedure for the ruggedness testing of chromatographic conditions in highperformance liquid chromatography, J. Chromatogr., 395 (1987) 539-551.

[35]. M. Mulholland, J. Waterhouse, Investigation of the limitations of saturated fractional factorial experimental designs, with confounding effects for an HPLC ruggedness test, Chromatographia, 25 (1988) 769-774.

[36]. L. Abdel-Malek, Test de robustesse, Comett Euro training course in advanced HPLC and capillary electrophoresis, Montpellier, September 1993.

[37]. M. Mulholland, Ruggedness testing in analytical chemistry, TRAC, 7 (1988) 383-389.

[38]. ISO 5725, Application of the Statistics-Accuracy (Trueness and Precision) of the Results and Methods of Measurement—Parts 1 to 6, International Organization for Standardization (ISO), Geneva, 1994.

[39]. ISO 3534-1: Statistics—Vocabulary and Symbols. International Organization for Standardization (ISO), Geneva, 2006.

[40]. J. Vessman, J. Pharm. Biomed. Anal. 14 (1996) 867.

[41]. J. Vessman, Accred. Qual. Assur. 6 (2001) 522.

[42]. B.-A. Persson, J. Vessman, Trends Anal. Chem. 17 (1998) 117.

[43]. J.Vessman, R.I. Stefan, J.F.Van Staden, K. Danzer,W. Lindner, D.T. Burns, A. Fajgelj, H. M¨uller, Pure Appl. Chem. 73 (2001) 1381.

[44]. A.D. McNaught, A. Wilkinson, IUPAC Compendium of Chemical Terminology, second ed., Blackwell, Oxford, 1997.

[45]. H. Kaiser, Fresenius Z. Anal. Chem. 260 (1972) 252.

[46]. D.L. Massart, B.G. Vandeginste, S.N. Deming, Y. Michotte, L. Kaufman, Chemometrics, Elsevier, Amsterdam, 1988, p. 115.

[47]. A. Lorber, K. Faber, B.R. Kowalski, Anal. Chem. 69 (1997) 69.

[48]. K. Faber, A. Lorber, B.R. Lowaski, J. Chemometrics 11 (1997) 419.

[49]. K. Danzer, Fresenius J. Anal. Chem. 369 (1997) 397.

[50]. WELAC Guidance DocumentsWGD2, Eurachem/Western European Laboratory Accreditation Cooperation (WELAC) Chemistry, Teddington, UK, first ed., 1993.

[51]. ISO VIM. DGUIDE 99999.2, International Vocabulary of Basic and General terms in Metrology (VIM), third ed., ISO, Geneva, 2006 (under approval step).

[52]. The Fitness for Purpose of Analytical Methods, Eurachem, Teddington, 1998.

[53]. Analytical Methods Committee,AMCTechnical Brief 13, Royal Society of Chemistry, London, 2003; http://www.rsc.org/Membership/Networking/ Interest Groups/Analytical/AMC/TechnicalBriefs.asp.

[54]. F. Satterthwaite, Psychometrika 6 (1941) 309.

[55]. B. Boulanger, P. Chiap, W. Dewe, J. Crommen, Ph. Hubert, J. Pharm. Biomed. Anal. 32 (2003) 753.

[56]. The U.S. Pharmacopeia (21st edn), p. 1221. The U.S.P. Convention, Rockville, MD.

[57]. T.D. WILSON, Liquid chromatographic methods validation for pharmaceutical products Journal of Pharmaceutical & Biomedical Analysis Vol. 8, No. 5, pp. 389~400,1990

[58]. FDA, Guide to Inspections of Validation of Cleaning Processes, 1993.

[59]. Guide to Inspections of Validation of Cleaning Processes, Reference Material for FDA Investigators and Personnel, Food and Drug Administration, Washington, DC, July, 1993, pp. 1–6.

[60]. Cleaning Validation Guidelines—Good Manufacturing Practice, Health Products and Food Branch Inspectorate, Canada, 2004.

[61]. Active Pharmaceutical Ingredient Committee, Guidance on Aspects of Cleaning Validation in Active Pharmaceutical Ingredient Plants, 2000.

[62]. T. Mirza, R.C. George, J.R. Bodenmiller, S.A. Belanich, J. Pharm. Biomed. Anal. 16 (1998) 939–950.

[63]. Z. Katona, L. Vincze, Z. Vegh, A. Trompler, K. Ferenczi-Fodor, J. Pharm. Biomed. Anal. 22 (2000) 349–353.

[64]. M.B. Boca, E. Pretorius, C. Kgaje, Z. Apostolides, J. Pharm. Biomed. Anal. 46 (2008) 631–638.

[65]. R. Debono, S. Stefanou, M. Davis, G.Walis, Pharm. Technol. 26 (2002) 72–78.

[66]. K.M. Jenkins, A.J. Vanderwielen, J.A. Armstrong, L.M. Leonard, G.P.Murphy, N.A. Piros, J. Pharm. Sci. Technol. 50 (1996) 6–15.

[67]. E.L. Simmonds, W.J. Lough, M.R. Gray, J. Pharm. Biomed. Anal. 40 (2006) 631–638.

[68]. L. Liu, B.W. Pack, J. Pharm. Biomed. Anal. 43 (2007) 1206–1212.

[69]. K.J. Fountain, M. VanWingerden, D.M. Diehl, LC–GCNorth-Am. 25 (2007) 66–67.

[70]. J. Lambropoulos, G.A. Spanos, N.V. Lazaridis, J. Pharm. Biomed. Anal. 23 (2000) 421–428.

[71]. M.J. Nozal, J.L. Bernal, L. Toribio, M.T. Martin, F.J. Diez, J. Pharm. Biomed. Anal. 30 (2002) 285–291.

[72]. T. Mirza, M.J. Lunn, F.J. Keeley, R.C. George, J.R. Bodenmiller, J. Pharm. Biomed. Anal. 19 (1999) 747–756.

[73]. M.B. Boca, Z. Apostodiles, E. Pretorius, J. Pharm. Biomed. Anal. 37 (2005) 461–468.

[74]. T.T. Fazio, A.K. Singh, E.R.M. Keodor Hackmann, M.I.R.M. Santoro, J. Pharm. Biomed. Anal. 43 (2007) 1495–1498.

[75]. R. Klinkenberg, B. Streel, A. Ceccato, J. Pharm. Biomed. Anal. 32 (2003) 345–352.

[76]. M.E. Swartz, B. Murphy, Am. Lab. 37 (2005) 22–35.

[77]. M.E. Swartz, J. Liq. Chromatogr. 28 (2005) 1253–1263.

[78]. Guide to Inspections of Validation of Cleaning Processes, Reference Material for FDA Investigators and Personnel, US Food and Drug Administration, Washington, DC, July 1993, p. 1.

[79]. International Conference on Harmonisation, Harmonised Tripartite Guideline Q7A: Manufacturing Practice Guideline for Active Pharmaceutical Ingredients, US Food and Drug Administration, 2000.

[80]. G. Bismuth, S. Neumann, Cleaning Validation, first ed., Interpharm Press, Englewood, 1999.

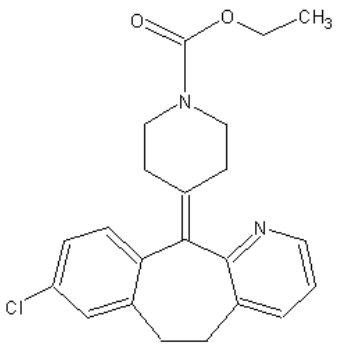
[81]. UNITED STATES Food and Drug Administration (FDA), Guide to Inspections Validation of Cleaning Processes, available at: http://www.fda.gov/ora/inspect ref/igs/valid.html (19/02/2003).

CHAPTER 2:
CLEANING VALIDATION METHOD DEVELOPMENT

2.1. INTRODUCTION

Loratadine is a tricyclic antihistamine, which has a selective and peripheral histamine H1-antagonistic action. Its anti-histaminic action is more effective than the other anti-histaminic drugs available commercially Loratadine is ethyl 4-(8-chloro-5,6-dihydro-11-H-benzo-[5,6]cyclohepta[1,2-b]pyridin-11-ylidene)-1-piperidine-carboxylate, molecular formula is $C_{22}H_{23}ClN_2O_2$ and molecular weight is 382.89.

Figure 2.1 Structure of Loratadine



Loratadine and pseudoephedrine sulfate are present together in dosage form prescribed to relieve symptoms of allergic rhinitis. Different analytical procedures have been reported for the determination of each one of them alone and for their simultaneous quantification in their mixtures.

Different methods were developed for determination of Loratadine which include polarographic methods [1, 2] and spectrophotometric methods [3-10]. Different chromatographic methods have been developed for the determination of Loratadine and its metabolite in human plasma. These include GC [11, 12], HPLC [13-16]. A densitometric method and HPLC method was developed for determination of Loratadine in pharmaceutical preparations [17].

Some methods have been developed for pharmacokinetic studies and they are applied to quantify Loratadine and its metabolite descarbo-ethoxy-Loratadine (LD) in plasma by HPLC [18].

During the analysis of different laboratory batches of Loratadine by a simple isocratic reversed-phase LC method, three unknown impurities were detected consistently in almost all the batches, whose area percentage ranged from 0.05 to 0.1%. A comprehensive study had been undertaken to isolate and characterize these impurities by spectroscopic techniques. The impurity profile study has to be carried out for any final product to identify and characterize all the unknown impurities that are present at a level of even below 0.05% [19]. The requirement of identifying and characterizing the impurities in the final product is extremely necessary in the wake of stringent purity requirements from the regulatory authorities leading to a HPLC method being developed by Krishna Reddy et al. [20].

A few validated LC methods for the quantitative determination of Loratadine and its related substances [21-24] are available.

After the manufacture of a pharmaceutical formulation has been completed it is a cGMP requirement that the equipment be cleaned prior to being used for the manufacture of a different product [25]. Cross contamination with active ingredients is a real concern. The Code of Federal Regulations (CFR) states that "Equipment and utensils shall be cleaned, maintained, and sanitized at appropriate intervals to prevent malfunctions or contamination that would alter the safety, identity, strength, quality, or purity of the drug product beyond the official, or other established requirements" [26]. Cleaning validation is required in the pharmaceutical field to avoid potential clinically significant synergistic interactions between pharmacologically active chemicals [27]. Since the issuance of the US Food and Drug Administration's "Guide to Inspection of Validation of Cleaning Process" in July 1993 [28], cleaning validations have received increasing attention. Various analytical methods have been used to validate the cleaning operations; which include HPLC–UV [29, 30], ion

mobility spectrometry (IMS) [31] total organic carbon (TOC) [32] and HPLC with evaporative light scattering detection (ELSD) [33].

To ensure that the sampling techniques chosen meet the established acceptance criteria, validation feasibility studies and method development must be performed. The two main sampling techniques available for cleaning validation are rinse and swab sampling. FDA prefers swab sampling to rinse sampling [34, 35].The residues are then determined by a suitable technique.

To the best of our knowledge no method has been reported for the determination of residual Loratadine during the control of cleaning procedures.

The main objective of this paper was thus to develop validated spectrophotometric and HPLC methods for determining residual levels of Loratadine. Validation has been done in terms of linearity, accuracy, precision, Limit of detection (LOD) and Limit of quantitation (LOQ).

2.2 LITERATURE REVIEW

Prior to the development of a specific UV spectrophotometric and HPLC methods for the quantification of trace amount of Loratadine in support to cleaning validation study, a literature review was conducted and the conditions reported in published articles were used for developing the swab technique as well as trace amount of loratadine. The initial conditions were used for the development of UV spectrophotometric and HPLC methods for use in quantification of Loratadine during cleaning validation study.

Following are the review of published articles from where we started the development work:

Table 2.1 Literature review for spectrophotometric methods

Sample preparation	Wave length	LOD/LOQ	Remarks	References
10 mg / 100 ml in ethanol	266 nm	3 - 22 mcg/mL	Ethanol is not suitable as rinsing agent during cleaning validation due to cost factor and inflammable than methanol	[36]
For derivative (Not for Loratadine)	--	--	Not considered due to derivative. We are interested in Loratadine	[37]
in Methanol	247 nm	1 mcg/mL to 20 mcg/mL	Best suitable and matching cleaning validation solvent as well as LOD and LOQ is good	New developed method

Table 2.2 Literature review for HPLC methods

Sample Preparation	Column	Mobile Phase	Method parameter	LOQ /LOQ	Ref.
Sensitivity range is 5.00–50.00 mcg/ml.	C18 µBondapak™ C18 125°A 10 µm 4.6×250 mm HPLC cartridge column Flow rate= 2ml/min	Mobile phase is acetonitrile: H_3PO_4 (35:65) using benzophenone as an internal standard.	Isocratic at 250 nm R_t of LR = 4.6	5-50 mcg/mL	[36]
	C18 column (Hichrom-RPB, 250 /4.6 mm i.d., 5 µm particle size, Hichrom Ltd., Flow rate= 1ml/min	mixture of 0.01 M KH_2PO_4 and acetonitrile in the ratio of 40:60 (v/v; pH 3.5)	Isocratic at 240 nm R_t of LR = 10		[38]
Sensitivity range is 5.00–100.00 mcg/ml.	Separation was performed on m-BondaPak C18 (300 /3.9 mm, 10 mm) column. Flow rate= 2ml/min	Mixture of H_2O: CH_3OH: H_3PO_4: $NH_4H_2PO_4$ (300:220:2:3 g) (v/v/v/w), 60 and 40% acetonitrile.	Isocratic at 247 nm R_t of LR = 3.5	5-50 mcg/mL	[37]

Sample Preparation	Column	Mobile Phase	Method parameter	LOQ /LOQ	Ref.
156 mg/100 ml methanol	Symmetry shield RP8 column has been developed and validated for Loratadine and related compounds measurement, the last ones under the 0.1% level.	Mixture of methanol-buffer A (65:35, v/v), being buffer A: H_3PO_4 10 mM (H_2O) brought up to pH 7.00 with tri-ethylamine.	Isocratic at 244 nm R_t of LR = 23	0.1 mcg but RSD was found 10%	[7]
0.4 mg/mL Dissolve Loratadine in diluent (400 mL of 0.05 N HCl and 80 mL of 0.6 M K_2PO_4 to a 1000-mL volumetric flask, dilute with a mixture of CH_3OH and acetonitrile (1:1) to volume, and mix.)	A 4.6-mm ×15-cm column that contains 5-µm packing L7 (C8). Flow rate 1 mL/min. Column temp. = 25 - 35	0.01 M K_2PO_4 0.6 M K_2PO_4 Mobile phase— Prepare a filtered and degassed mixture of 0.01 M K_2PO_4,CH_3OH, and acetonitrile (7:6:6). Adjust with 10% H_3PO_4 solution to an apparent pH of 7.2.	Gradient		[39]
0.5 - 20 mcg/mL	Phenomenex: Jupiter C18 (250 x 4.6 mm), 5 µ Flow rate = 1 ml/min	Buffer for mobile phase: To 2000 mL of aqueous solution of 0.28% $NaH_2PO_4.2H_2O$, 1 ml of triethylamine was added and pH adjusted to 3.5 with H_3PO_4. Mobile phase A consisted of 80 volumes of buffer and 20 volumes of acetonitrile whereas Mobile phase B consisted of 20 volumes of buffer and 80 volumes of acetonitrile.	Gradient timed gradient programme T(min) / mobile phase A(%): 0/72, 45/28, 50/28, and 60/72. At 247 nm 19 min. other peak of swab is at about 22 min.	0.5 - 20 mcg/mL . RSD was found 7% even though not claimed as our target is, RSD should be less than 2%	Developed method

2.3 QUANTIIFICATION OF LORATADINE AND METHOD VALLIDATION BY UV SPECTROPHOTOMETRIC METHOD

2.3.1 EXPERIMENTAL:

Materials, Reagents and Chemicals

HPLC grade methanol was purchased from Merck; Loratadine API obtained from Cadila Pharmaceuticals Ltd. was used for preparation of standard, samples and for swab study; Ultra pure water was obtained using Milli-Q® UF-Plus (Millipore) system.

Preparation of standard

Stock Standard Solution: Loratadine 1 mg/mL (1000 µg/mL) was prepared in methanol and used for further dilutions.

Preparation of sample

0.1 µg/mL; 0.2 µg/mL; 0.5 µg/mL; 1 µg/mL; 5 µg/mL; 10 µg/mL; and 20 µg/mL Loratadine solutions were prepared by diluting the stock standard solution in methanol accordingly. All dilutions were made using calibrated class A grade glassware.

Equipment

Ultraviolet spectrophotometric analyses were carried out on a Shimadzu UV 2400 (Shimadzu, Kyoto, Japan) spectrophotometer, in a 1cm quartz cuvette. The wavelength of 247nm was selected for the quantitation of Loratadine and the measurements were obtained against methanol as a blank.

2.3.2. RESULTS AND DISCUSSION

2.3.2.1 Method development

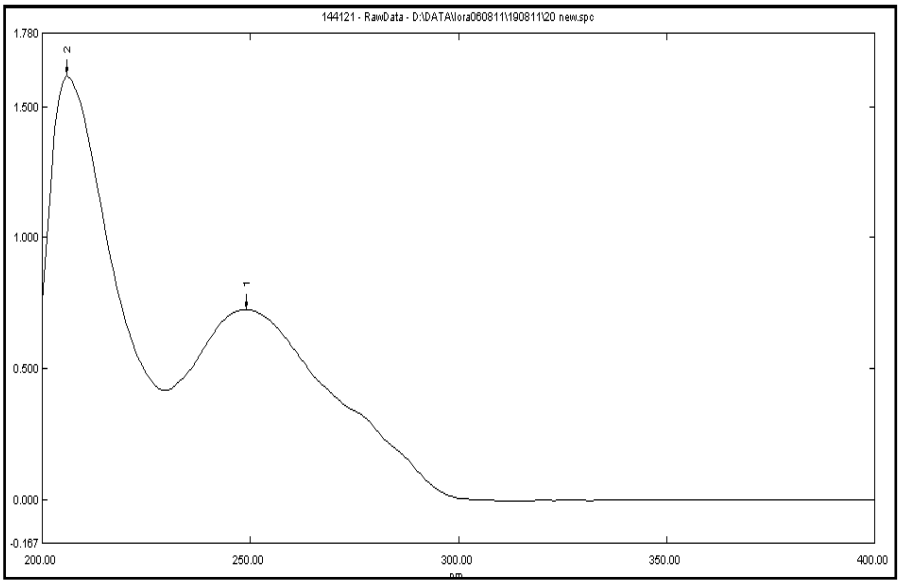
Initially solubility of Loratadine was checked in different solvents to simulate the cleaning agent during cleaning process of manufacturing equipment.

Loratadine is insoluble in water but soluble in alcohol, chloroform, and in acetone. Methanol was selected for preparation of standard and samples because it was ultimately to be used as cleaning solvent / rinsing solvent and also it is economic and safe as compare with other solvents.

A stock solution of Loratadine (1000 µg/mL) was prepared by dissolving 100 mg of Loratadine in 100 mL of methanol. Working standards of required concentrations were prepared by suitably diluting the stock solution in methanol.

Loratadine standard solution 10 µg/mL was prepared in methanol and scanned between 400nm and 200 nm, to finalize the wavelength for further experiments. It was observed that maximum absorbance occurred at 247 nm as shown in Figure 2.2.

Figure 2.2 UV spectrum of Loratadine



The applied wavelength and dilution pattern permitted good results for different concentrations of Loratadine. Based on the maximum absorbance, it was concluded

that in all future experiments the absorbance for Loratadine would be measured at 247 nm. Further experiments to validate the method for detection level, quantification level, linearity and range, accuracy and precision were then conducted.

2.3.2.2 Method validation

The sample preparation (in methanol) and maximum wavelength (247 nm) were validated according to the procedures described in ICH guidelines Q2 (R1) [40].

System suitability

General calibration norms were applied as system suitability parameters as mentioned in the pharmacopoeia:

Control of wavelength: The wavelength of the deuterium lamp emission lines was measured and the accuracy of the absorbance of Loratadine at the displayed wavelength was checked. Prior to this the wavelength scale using the absorption maxima of Holmium Perchlorate solution / Holmium Filter was verified.

Absorbance maxima should be observed at the following wavelengths

Table 2.3 Tolerance limit of absorbance at wavelength

Wavelength (nm)	Acceptance Criteria (nm)
241.15	240.15 – 242.15
287.15	286.15 – 288.15
361.50	360.50 – 362.50
536.30	533.30 – 539.30

Control of absorbance: This test was performed to check the absorbance control for individual and multi wavelength. The absorbance was checked using Potassium dichromate solution at its wavelength of maximum absorbance. A 0.06 % W/V Potassium dichromate solution was prepared in 100 mL 0.0005 M sulfuric acid. The absorbance values shall be measured at 235 nm, 257 nm, 313 nm and 350 nm respectively, it should be within limit as specified in below table

Table 2.4 Tolerance limit of absorbance at wavelength

Wavelength (nm)	Acceptance criteria	
	Maximum tolerance (As per IP)	Maximum tolerance (As per EP)
235	122.9 – 126.2	122.9-126.2
257	142.8 – 145.7	142.8-146.2
313	47.0-50.3	47.0-50.3
350	104.9-108.2	105.6-109.0
430	15.7 – 16.1	15.7 – 16.1

Limit of stray light: The absorbance of 1.2 % W/V KCl Solution at 198 nm (as per EP) and 200 nm (as per IP) should be greater than 2.0

Resolution: This test is performed to check the highest resolution between two maxima of absorbance at nearest wavelength. Resolution is checked by using 0.02 % V/V Toluene in Hexane solution. The ratio of absorbance at 269 nm to that at 266 nm should be at least 1.5

Limit of detection and limit of quantification

A diluted standard Loratadine solution of 0.1 µg/mL; 0.2 µg/mL; 0.5 µg/mL; 1 µg/mL; 5 µg/mL were prepared for verification of detection and quantification level of the developed method. Each diluted standard solution was measured in triplicate.

Table 2.5 Absorbance for diluted standard solution of Loratadine

Conc. of LR (µg/mL)	Absorbance values			Avg. value	% RSD
	Measurement-1	Measurement-2	Measurement-3		
0.1	0.001	0.007	0.008	0.005	70.99
0.2	0.002	0.005	0.008	0.005	60.00
0.5	0.014	0.013	0.015	0.014	7.14
1	0.028	0.031	0.029	0.029	5.21
2	0.084	0.089	0.086	0.086	2.91
5	0.183	0.185	0.187	0.185	1.08

Based on above table, detection level was found to be 0.5 µg/mL with %RSD about 7% while quantification level was found to be 1 µg/mL with %RSD about 5%.

To verify the quantification level six preparation of 1µg/mL were prepared and measured separately and %RSD was found to be 5%

Table 2.6 Quantification level Studies

Measurement no.	Absorbance
1	0.028
2	0.030
3	0.028
4	0.026
5	0.027
6	0.029
Avg. Value	0.028
%RSD	5.05

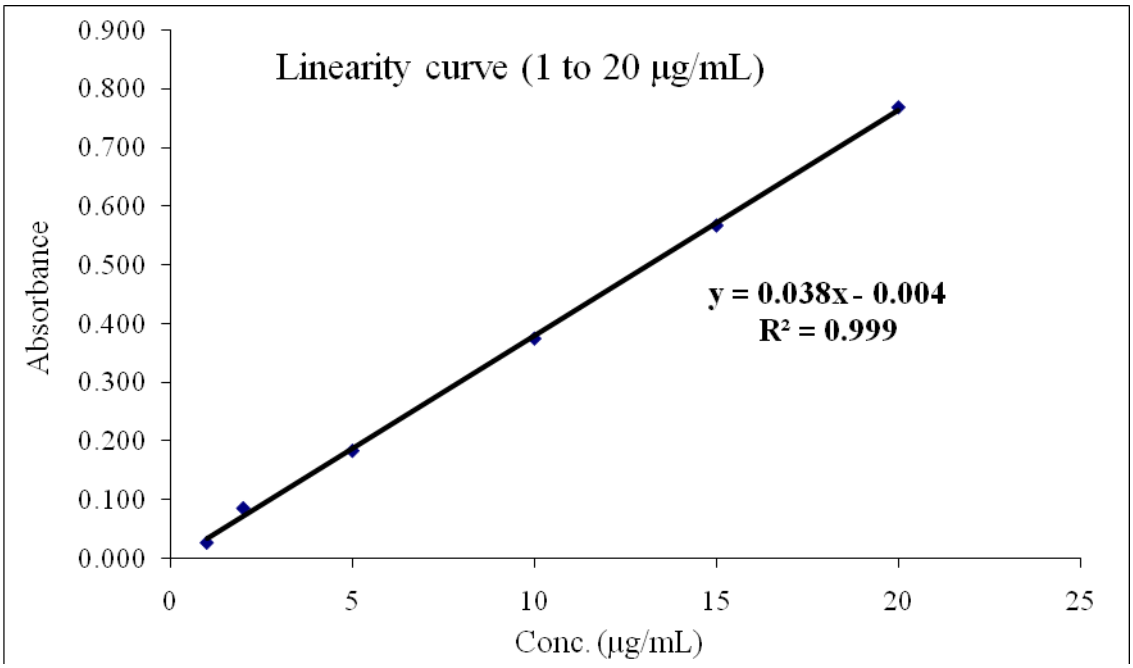
Linearity and range

Based on LOD and LOQ parameter, linearity concentration of Loratadine vs absorbance was plotted from LOQ level. Loratadine standard solution was used for preparation of different concentration ranging from 1 µg/mL, 2 µg/mL, 5 µg/mL, 10 µg/mL, 15 µg/mL and 20 µg/mL. Each diluted standard was measured in triplicate.

Table 2.7 Absorbance at different concentration of Loratadine for Linearity and Range Studies

Conc. of LR (µg/mL)	Absorbance Values				
	Measurement-1	Measurement-2	Measurement-3	Avg. value	%RSD
1	0.029	0.028	0.025	0.027	7.62
2	0.085	0.086	0.087	0.086	1.16
5	0.182	0.184	0.185	0.184	0.83
10	0.375	0.377	0.372	0.375	0.67
15	0.571	0.566	0.564	0.567	0.64
20	0.776	0.752	0.776	0.768	1.80

Figure 2.3 Linearity curve of Loratadine (Spectrophotometry)



Accuracy

Accuracy was studied by comparing the absorbance values of 5, 10, 15 and 20 µg/mL of spiked Loratadine solutions in methanol and the absorbance of 5, 10, 15 and 10 µg/mL of Loratadine standard solutions. . The percent recovery was found to be in the range of 95% to 102% and the percent RSD was found to be about 2% as seen from Tables 2.8 and 2.9.

Table 2.8 Absorbance for Standard diluted Loratadine solution:

Conc. of LR (µg/mL)	Measurement-1	Measurement-2	Measurement-3	Avg. value	% RSD	% Recovery
5	0.178	0.181	0.179	0.179	0.85	96.5
10	0.378	0.371	0.361	0.370	2.31	98.4
15	0.578	0.577	0.564	0.573	1.36	101.2
20	0.786	0.772	0.776	0.778	0.93	102.9

Table 2.9 Absorbance for spiked Loratadine solution in methanol

Conc. of LR (µg/mL)	Measure ment-1	Measure ment-2	Measure ment-3	Avg. value	%RSD	Back calc (µg/mL)	% Recovery
5	0.172	0.181	0.179	0.177	2.66	4.772	95.4
10	0.361	0.368	0.353	0.361	2.08	9.518	96.0
15	0.532	0.537	0.564	0.544	3.16	14.430	96.2
20	0.766	0.752	0.736	0.751	2.00	19.877	99.4

Precision

The repeatability was determined by performing five independent sample preparations of 10 µg/mL Loratadine standard. Single absorbance was measured. The percent RSD of absorbance was found about 1 %.

Table 2.10: Data for precision measurement

Measurement no.	Absorbance
1	0.379
2	0.372
3	0.377
4	0.368
5	0.372
Avg. Value	0.374
%RSD	1.18

2.4 QUANTIFICATION OF LORATADINE AND METHOD VALLIDATION BY HPLC

2.4.1 EXPERIMENTAL:

Materials, Reagents and Chemicals

HPLC grade methanol, acetonitrile were purchased from Merck; Loratadine API obtained from Cadila Pharmaceuticals Ltd. was used for preparation of standard, samples and for swab study; Analytical grade triethyl amine and ortho phosphoric

acid were purchased from Merck; Ultra pure water was obtained using Milli-Q® UF-Plus (Millipore) system.

Preparation of standard

Stock Standard Solution: Loratadine 1 mg/mL (1000 µg/mL) was prepared in methanol and used for further dilutions.

Preparation of sample

0.1 µg/mL; 0.2 µg/mL; 0.5 µg/mL; 1 µg/mL; 5 µg/mL; 10 µg/mL; and 20 µg/mL Loratadine solutions were prepared by diluting the stock standard solution in methanol accordingly.

All dilutions were made using calibrated class A grade glassware.

Mobile phase

Buffer for mobile phase: To 2000 mL of aqueous solution of 0.28% sodium dihydrogen phosphate dihydrate, 1 ml of triethylamine was added and pH adjusted to 3.5 with ortho phosphoric acid.

Mobile phase A consisted of 80 volumes of buffer and 20 volumes of acetonitrile while Mobile phase B consisted of 20 volumes of buffer and 80 volumes of acetonitrile.

Equipment

The HPLC analyses were carried out on an Agilent 1100 system, composed of a quaternary pump, auto sampler, UV detector and HP ChemStation software.

Chromatographic condition:

The chromatographic column used was Phenomenex: Jupiter C_{18} column (4.6mm ID X 250mm L), with 5µm sized particles.

Table 2.11 Chromatographic condition (HPLC)

Parameter	Standard Condition
Column	C18 (250 x 4.6 mm), 5 µ
Detector wavelength	247 nm
Flow Rate	1.0 ml/min
Injection Volume	30 µl

Table 2.12 Gradient Program for HPLC

Time	Solution A (%)	Solution B (%)
0	72	28
45	28	72
50	28	72
60	72	28

To get the optimum results, mobile phase with a flow rate of 1.0mL/min was used. The gradient program for mobile phase was optimized using a timed gradient programme.

2.4.2. RESULTS AND DISCUSSION

2.4.2.1 Method development

Initially the solubility of Loratadine was checked in different solvents to simulate the cleaning agent during cleaning process of manufacturing equipment.

Loratadine is insoluble in water but soluble in alcohol, chloroform, and in acetone. Methanol was selected for preparation of standard and samples because it was ultimately to be used as cleaning solvent / rinsing solvent and also it is economic and safe as compared with other solvents.

In the past many HPLC methods suffered from problems when analysing basic drugs, such as loratadine, since these compounds strongly interact with polar ends of HPLC column packing materials, causing severe peak asymmetry and low separation

efficiencies. High purity silica backbone and advances in bonding technology have alleviated the tailing problem of polar compounds in HPLC to a significant extent. Consequently, for the initial development a Phenomenex: Jupiter C18 column was used. This packing was selected because it has one of the lowest hydrophobicity and silanol activity as seen in commercial catalogues. The pH value of 3.5, in the mobile phase permitted a low ionization degree and therefore, a higher retention of the analytes. It provides more tools to obtain the separation. Moreover, it permits us to take advantage of the addition of a 'silanol blocker', such as triethylamine, to the mobile phase and this has proved to be necessary to obtain good peak symmetry in the present work. The critical point in developing the separation was to get a good resolution for peak from swab. Gradient elution was established because it is known to give better resolution

A stock solution of Loratadine (1000 µg/mL) was prepared by dissolving 100 mg of Loratadine in methanol. Working standards of required concentrations were prepared by suitably diluting the stock solution in methanol.

Loratadine standard solution 10 µg/mL was prepared in methanol and injected into HPLC system by applying the chromatographic condition.

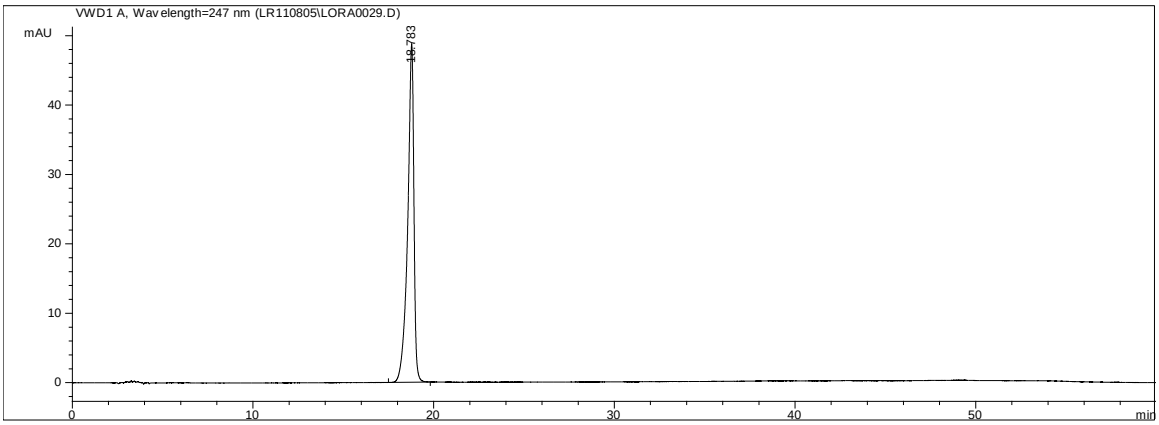
The applied chromatographic condition and dilution pattern permitted good results at different concentrations of Loratadine. No interference was observed. Based on the studied parameters, it was concluded that the developed method is optimum.

2.4.2.2 Method validation

System suitability

A diluted standard of Loratadine solution was injected to verify interference of mobile phase. The typical chromatogram of Loratadine is shown in Fig 2.4.

Figure 2.4 Typical chromatogram of Loratadine



Limit of detection and limit of quantification

A diluted standard Loratadine solution of 0.1 µg/mL; 0.2 µg/mL; 0.5 µg/mL; 1 µg/mL; 2 µg/mL and 5 µg/mL were prepared for verification of detection and quantification of method. Each diluted standard solution was measured in triplicate.

Table 2.13 Peak area of Loratadine at different concentrations

Conc. of LR (µg/mL)	Area of 1st replicate	Area of 2nd replicate	Area of 3rd Replicate	Avg. value	% RSD
0.1	6.1	5.6	5.3	5.7	7.13
0.2	12.2	12.0	12.1	12.1	0.83
0.5	32.4	32.5	32.1	32.3	0.64
1	56.1	56.2	56.1	56.1	0.10
2	141.4	143.1	143.6	142.7	0.81
5	280.2	286.3	285.7	284.1	1.18

From Table 2.13 it is evident that the detection level is 0.2 µg/mL while quantification level is 0.5 µg/mL with %RSD below 2%

Linearity and range

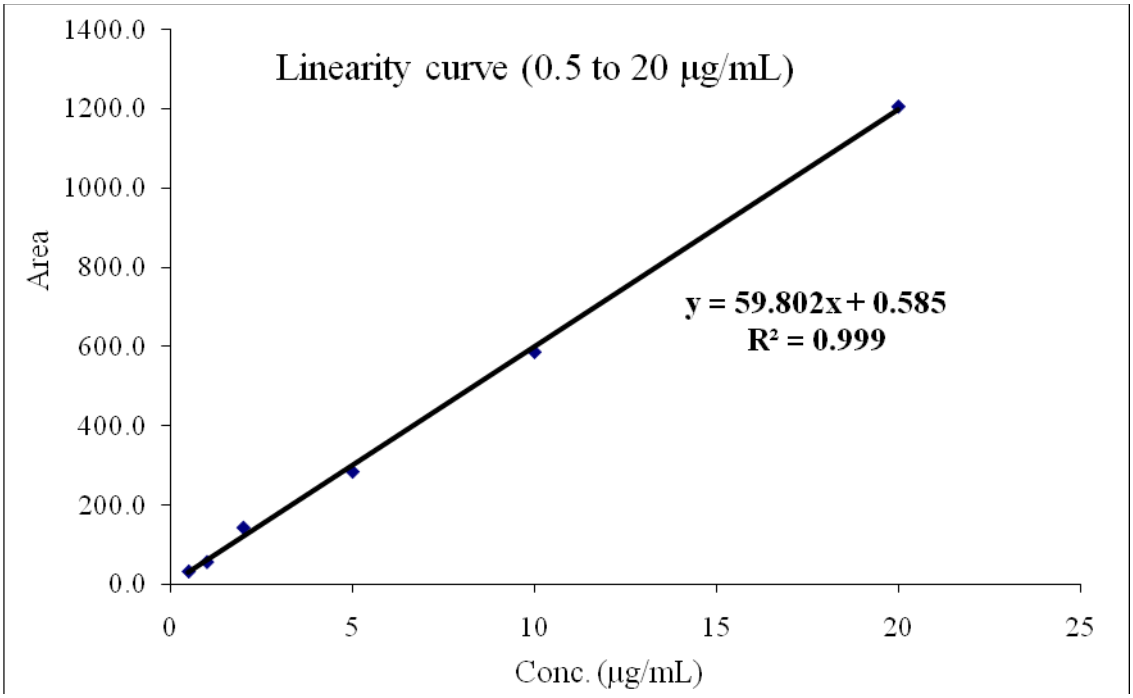
Generally linearity has to be established from the target value $\pm$20%. However since our objective was to develop a robust method for swab samples and to determine trace levels of Loratadine, LOQ level was chosen for linearity. Loratadine standard solution

was used for preparation of different concentrations ranging from 0.5 µg/mL, 1 µg/mL, 2 µg/mL, 5 µg/mL, 10 µg/mL, and 20 µg/mL. Each diluted standard was injected in triplicate and the observations are tabulated in Table 2.13 and Figure 2.14.

Table 2.14 Peak area of Loratadine at different concentrations for Linearity and range studies

Conc. of LR (µg/mL)	Area of 1st Replicate	Area of 2nd Replicate	Area of 3rd Replicate	Avg. value	%RSD
0.5	32.4	32.5	32.1	32.3	0.64
1	56.1	56.2	56.1	56.1	0.10
2	141.4	143.1	143.6	142.7	0.81
5	280.2	286.3	285.7	284.1	1.18
10	572.6	590.5	594.5	585.9	1.99
20	1173.6	1224.9	1215.9	1204.8	2.27

Figure 2.5 Linearity curve for Loratadine (HPLC)



Accuracy

Accuracy was studied by comparing the area of spiked solutions of 5, 10, and 20 µg/mL of Loratadine in methanol with the area of Loratadine standard in the range of 5, 10, and 10 µg/mL and the data are presented in Tables 2.16 and 2.17. The percent recovery was found to be in the range of 95% to 102%. The percent RSD was found to be about 2%.

Table 2.15 Peak area of standard Loratadine diluted solution:

Conc. of LR (µg/mL)	Area of 1st Replicate	Area of 2nd Replicate	Area of 3rd Replicate	Avg. value	%RSD	% Recovery
5	280	286	286	284	1.18	95.2
10	573	591	595	586	1.99	98.1
20	1174	1225	1216	1205	2.27	100.8

Table 2.16 Peak area of spiked Loratadine diluted solution in methanol

Conc. of LR (µg/mL)	Area of 1st Replicate	Area of 2nd Replicate	Area of 3rd Replicate	Avg. value	%RSD	Back calc (µg/mL)	% Recovery
5	284	291	282	286	1.65	4.8	95.7
10	578	591	595	588	1.46	9.8	98.4
20	1225	1169	1201	1198	2.34	20.0	100.2

Precision

The repeatability was determined by performing five independent sample preparations of 0.5 µg/mL Loratadine standard. Single injection was measured. The percent RSD of replicates was found to be ~1 % as seen from Table 2.18.

Table 2.17 Peak area of Loratadine standard at LOQ level

Measurement no.	Area of Replicate
1	27.5
2	27
3	27.2
4	27
5	27.4
6	27.1
Avg. Value	27.22
%RSD	0.84

2.5 CONCLUSION – COMPARATIVE STUDY OF UV SPECTROPHOTOMETRIC AND HPLC PERFORMANCE

Both UV spectroscopic and HPLC methods were demonstrated to be reliable for quantification of Loratadine. The UV spectroscopic method was found to be capable of giving faster analysis as compared with HPLC method. Since these methods are rapid and simple, they may be successfully applied to quality control analyses during cleaning validation activity as well as routine cleaning program to avoid any cross contamination or mix-ups.

Table 2.18 Results of regression equation / correlation coefficient and validation parameter

Statistical Parameter	**UV Results / Remarks**	**HPLC Results / Remarks**
Linearity and Range (µg/ml)	1 – 20	0.5 – 20
Regression Equation	y = 0.038x - 0.004	y = 59.802x + 0.585
Correlation coefficient	0.999	0.999
Wavelength	247 nm	247 nm
Analysis time (min)	30 min.	3 hours
Validation parameter		
Accuracy (Including spike recovery study)	Yes	Yes
Precision	Yes	Yes
LOD	0.5 µg/ml	0.2 µg/ml
LOQ	1 µg/ml	0.5 µg/ml

2.6 CLEANING VALIDATION

Now-a-days pharmaceutical products are manufactured in multi-use facility. FDA considered the potential for cross-contamination to be significant and to pose a serious health risk to the public. Cleaning validation program ensures absence of residues of reaction byproducts and degradants from the previous process/product. The most appropriate cleaning procedure has to be developed for the equipment to minimize the cross contamination and there is also necessity to develop and validate the sampling and chosen analytical methods for the compound(s) being cleaned for rinse sampling and swab sampling.

Develop a cleaning validation protocol for the product to be cleaned and the equipment being cleaned. Generate a cleaning validation report detailing the acceptability of the cleaning procedure for the equipment and the product.

How to sample surfaces for residues

After consideration of safety factor, daily dose and characteristic of products to be manufactured in the same train of equipment, acceptable residual limits have to be decided, for which appropriate methods need to be employed to determine the contamination levels actually present on the cleaned equipment. Such methods are usually categorised as direct or indirect, depending on whether the measurements are made directly or indirectly from the surface of interest. The FDA has outlined general methods of both types. Each has its advantages and disadvantages, but in general terms, direct surface sampling is generally more acceptable but on routine basis it is difficult to do this technique.

2.6.1 Rinse sampling

An indirect sampling method is rinse sampling. A small sample of the solution collected from the last rinse cycle of the cleaning process is analysed for the compound of interest and the residual limit is back-calculated according to the volume of solution and the contact area. There are generally two assumptions inherent in this method. The first is that the target residue is efficiently extracted into the rinsing solution. The second is that all parts of the contaminated surfaces are cleaned equally. The main reservations expressed by the FDA and other regulatory bodies about rinse sampling relate to these assumptions. Since the surface residues are not measured directly, the analyst cannot be sure that unacceptably high levels of residues have not been left in some areas of the equipment. For example, a very poor solvent will result in low contamination of the final rinse even if large amounts of residues have been left on the surface. There is an additional risk of system failure, where an (otherwise) entirely adequate procedure is incorrectly applied. This could mean that a dirty reactor is declared clean and used for some time before the malfunction is detected.

Even with these concerns, rinse sampling does have a number of advantages when implemented correctly and with adequate safety measures. One fairly significant advantage is the ease of collecting a part of the final rinse solution drained from the equipment. Another is that it allows evaluation of residues from all parts of the surface irrespective of the difficulty of reaching them with a swab. This makes rinse sampling ideal for clean-in-place (CIP) systems sealed systems or large-scale equipment that is difficult to disassemble.

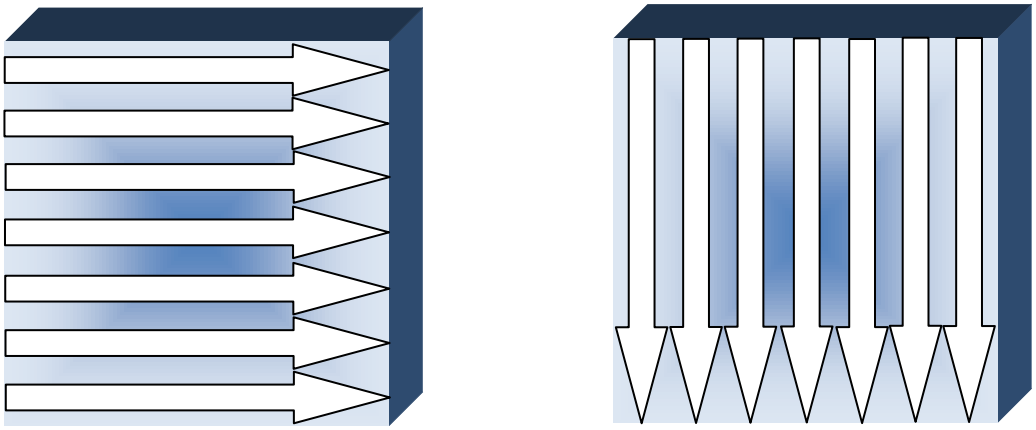
During cleaning validation program, rinse sample can be directly analysed by above methods and there is no need to develop specific preparation for rinse sample.

Depending upon the concentration and number of washing, final rinse sample can be analysed and based on results residue can be calculated.

2.6.2 Swab sampling / recovery study:

Direct surface sampling can be carried out in a number of ways, but the most common and widely accepted is swabbing. This involves wiping a predetermined area of the equipment with a swab that has been moistened with a solvent determined by the contaminating compound. Usually the surface is wiped with one side of the swab using a certain number of strokes, then the swap is flipped and the surface is wiped at 90° to the first series of stokes as shown in the following figures

Figure 2.6 Swab technique



Sideways **Downwards**

(one face of the swab) **(Other face of the swab)**

This process can be extended, for example by repeating the process at diagonal angles. Generally, the swabbing proceeds from less contaminated to more highly contaminated areas in order to prevent recontamination by the material already collected on the swab. The swab head is then immersed in a set amount of "recovery solvent".

Selection of swab is important part during cleaning validation program and to maximize the recovery, two types of swabs were studied–Himedia (having circle head) and Texwipe (having flat head) as shown in the following figures

Figure 2.7 Different head types of swabs

Swab – 1

Himedia Swab

Swab – 2

Texwipe Swab

The concentration of contaminant in the recovery solvent is then determined by analytical techniques and the amount of contamination on the swabbed surface is back calculated.

Amongst the advantages of the swabbing method is the fact that insoluble or slightly soluble residues on the surface are more readily removed by physical "rubbing" than is the case in (for example) rinsing. It also permits direct sampling from accessible, but hard-to-clean locations (although it can also be at a disadvantage for locations that are especially hard to clean or difficult to access). Generally, small sampling areas are used to determine residual limits, which are then extrapolated to estimate the level of contamination over the entire contact surface. This can lead to problems in large-scale reactors (especially in case of bulk drug – active pharmaceutical ingredient manufacturing facility) where the residues are not uniformly spread across all contact surfaces. Issues that need to be considered when using the swabbing technique

include the physical properties of the swabs, recovery levels and operator procedures. The swab material must not damage the surface or leave fibres behind; but most importantly it must not leach compounds that can interfere with the analytical procedures. One potentially significant interference that can lead to problems of the last type is the glue used to attach the swabbing head to the handle. To preclude this problem, thermal adhesion treatments are preferred over glues.

Recovery levels are determined by the solubility of the compound in the swabbing solvent, the wiping procedure and the physical nature of the surface. Ideally, they should be as close as possible to 100%, but greater than 70% is considered reasonable and as low as 50% is sometimes obtained. Lower values are generally considered unacceptable and then require improved procedures. To compensate for imperfect recovery, studies must be carried out to determine appropriate factors to correct the calculations for the actual residual limit. There are two major reasons for imperfect recovery. Firstly, not all of the contamination on a surface may be collected by the swabbing process; secondly, not all of the contamination on the swab may be passed to the recovery solution. The first of these can be influenced by the type of surface and its roughness, as well as the type of swab and the solvent. A second swab can be used to improve recovery, with the results from both swabs being combined. The second swab can employ a different solvent, with appropriate consideration of residues and toxicity. A common secondary solvent used after water is ethanol, since it dissolves many pharmaceutical compounds, is reasonably non toxic and evaporates readily leaving no residue.

2.6.2.1 Experimental

To simulate the manufacturing equipment, SS-316 plate (5.08 x 5.08 cm^2 area) was cut from the SS – 316 sheet and used for all recovery studies.

Recovery studies were performed on SS-316 plate (5.08 x 5.08 cm^2 area) by applying solutions of different concentrations (equivalent to 10 µg/mL, 15 µg/mL and 20 µg/mL) of Loratadine by using syringe and drying the plate in air. The plate was swabbed with a swab pre-moistened with methanol vertically and horizontally as shown in figure 2.6. The swab was then transferred to a beaker and sonicated with 3 x 10 mL of methanol. The methanol was then transferred into a 50 mL volumetric flask and made up to the mark with methanol. Recovered sample was analysed with both UV spectrophotometric and HPLC validated methods .

2.6.2.2 Recovery Studies

Recovery studies were done by applying different concentrations of Loratadine on a SS-316 plate (5.08 x 5.08 cm^2) with the help of a syringe and drying the plate in air. The plate was then swabbed with methanol vertically and horizontally using a swab sampler as shown in figure-2.6.

Two different brands (Himedia and Texwipe) of swabs were used to optimize the percentage recovery and are shown in figure-2.7.

The swab was then placed in a beaker and sonicated with methanol. The concentration of Loratadine in the methanol extract was determined by both the developed methods

Texwipe swab is more suitable than Himedia swab because surface area of Texwipe swab is more than Himedia circular swab, easy to handle and practically simple also Texwipe is made by special type of synthetic cotton whereas Himedia is made by cotton and leads more generation of particles.

One additional peak was observed during HPLC analysis and was identified to be due to swab in both the brands. Typical chromatograms are shown in figure 2.8 and 2.9.

Figure 2.8 Chromatogram of blank Texwipe swab

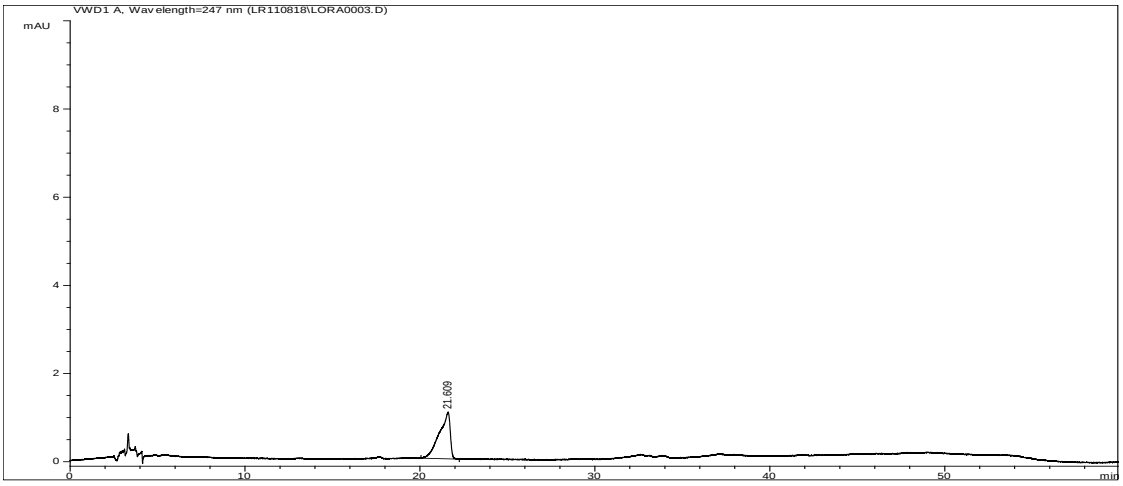
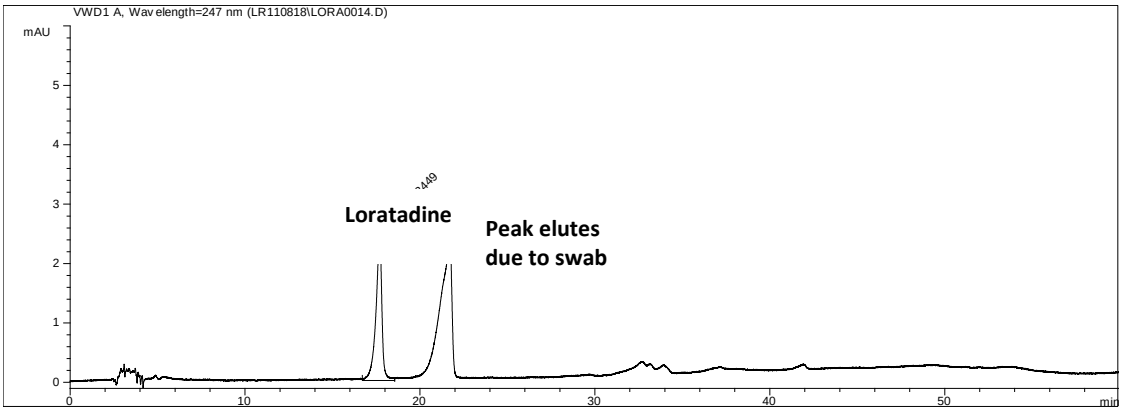


Figure 2.9 Chromatogram of Loratadine after swab from SS plate



There was no interference of this additional peak as it was observed at about 22 min. while Loratadine elutes at about 19 min. Additional peak was identified and it was due to swab used during execution. It was observed that recovery is better in Texwipe swab as compared with Himedia swab, thus suggesting that Texwipe swab is more suitable than Himedia swab. The recovery was found to be about 70%.

Table 2.19 Texwipe swab recovered sample by UV spectrophotometric method

Conc. of LR (µg/mL)	Absorbance values				% RSD	Back calc (µg/mL)	% Recovery
	Measurement -1	Measurement -2	Measurement -3	Avg. value			
6	0.167	0.159	0.182	0.169	6.90	4.6	76.0
12	0.375	0.359	0.358	0.364	2.62	9.7	80.7
24	0.704	0.701	0.705	0.703	0.30	18.6	77.6

The percent RSD was found to be less than 7% and percentage recovery was found to be more than 75%.

Table 2.20 Result of Texwipe swab recovered sample by HPLC method

Conc. of LR (µg/mL)	Area of 1st Replicate	Area of 2nd Replicate	Area of 3rd Replicate	Avg. value	% RSD	Back calc (µg/mL)	% Recovery
6	269.4	271	269.1	269.8	0.38	4.9	82.3
12	556.9	561.6	549.7	556.1	1.08	9.3	77.6
24	1120.3	1113.9	1081.1	1105.1	1.90	18.5	92.4

The percent RSD was found to be less than 2% and percentage recovery was found to be more than 75%.

2.7 CONCLUSION:

Swab recovery study was successfully developed and found satisfactory results. The UV spectrophotometric method was found to be capable of giving faster analysis with any other specific method. Since developed and validated method is rapid and simple, they may be successfully applied to quality control analyses of Loratadine during routine cleaning program to avoid any cross contamination or mix-ups.

2.8 REFERENCES:

[1] R. Johnson, J. Christensen, C.C. Lin, J. Chromatogr. B Biomed. Appl. 657 (1) (1994) 125-131.

[2] T. Radhakrishna, J. Satyanarayana, A. Satyanarayana, Indian Drugs 39 (6) (2002) 342-347.

[3] N.A. El-Ragehy, A.M. Badawey, S.Z El-Khateeb, Anal. Lett. 28 (13) (1995) 2363–2378.

[4] Z.H. Lin, Yaowu Fenxi Zazhi 16 (1) (1996) 53.

[5] S.J. Rajput, A.G. Vyas, Indian Drugs 35 (6) (1998) 352–355.

[6] F. Onur, C. Yucesoy, S. Dermis, M. Kartal, G. Kokdil, Talanta 51 (2) (2000) 269–279.

[7] F.J. Ruperez, H. Fernandez, C. Barbas, J. Pharm. Biomed. Anal. 29 (2002) 35-41.

[8] Guide to Inspections of Validation of Cleaning Processes, Reference Material for FDA Investigators and Personnel, Food and Drug Administration, Washington, DC, (1993), 1–6.

[9] PIC/S Guideline, Recommendations on Validation Master Plan, Installation and Operational Qualification, Non-sterile Process Validation, Cleaning Validation, (2004).

[10] Active Pharmaceutical Ingredients Committee (APIC) of CEFIC Guideline, Guidance on aspects of Cleaning Validation in active pharmaceutical ingredient plants, (2004).

[11] FDA-Guide to inspections validation of cleaning processes, (1993)

[12] J. Lambropoulos, G.A. Spanos, N.V. Lazaridis, J. Pharm. Biomed. Anal. 23 (2000) 421–428.

[13] M.J. Nozal, J.L. Bernal, L. Toribio, M.T. Martin, F.J. Diez, J. Pharm. Biomed. Anal. 30 (2002) 285–291.

[14] R. Debono, S. Stefanou, M. Davis, G. Walis, Pharmaceut. Technol. (2002) 72–78.

[15] J. Martens, J. Chromatogr. B. Biomed. Appl. 673 (2) (1995) 183–188.

[16] R. Ramanathan, A.D. Su, N. Alvarez, N. Blumenkrantz, S.K. Chowdhury, K. Alton, J. Patrick, Anal. Chem. 72 (6) (2000) 1352–1359.

[17] K.M. Jenkins, A.J. Vanderwielen, J.A. Armstrong, L.M. Leonard, G.P. Murphy, N.A. Piros, PDA J. Phar. Sci. Technol. 50 (1996) 6–15.

[18] J.A. Squella, J.C. Sturm, M.A. Diaz, H. Pessoa, L.J.N. Vergara, Talanta 43 (1996) 2029-2035.

[19] R. Johnson, J. Christensen, C.C. Lin, J. Chromatogr. B Biomed. Appl. 657 (1) (1994) 125-131.

[20] K.V.S.R. Krishna Reddy et.al. Journal of Pharmaceutical and Biomedical Analysis, (32) (2003) 29-39

[21] D. Zhong, H. Blume, Pharmazie, 49 (10) (1994) 736-739.

[22] R. Johnson, J. Christensen, C.C. Lin, J. Chromatogr. B Biomed. Appl. 657 (1) (1994) 125-131.

[23] T. Radhakrishna, J. Satyanarayana, A. Satyanarayana, Indian Drugs 39 (6) (2002) 342-347.

[24] F.J. Ruperez, H. Fernandez, C. Barbas, J. Pharm. Biomed. Anal. 29 (2002) 35-41.

[25] Guide to Inspections of Validation of Cleaning Processes, Reference Material for FDA Investigators and Personnel, Food and Drug Administration, Washington, DC, (1993), 1–6.

[26] PIC/S Guideline-Recommendations on Validation Master Plan, Installation and Operational Qualification, Non-sterile Process Validation, Cleaning Validation. (2004).

[27] Active Pharmaceutical Ingredients Committee (APIC) of CEFIC Guideline-Guidance on aspects of Cleaning Validation in active pharmaceutical ingredient plants. (2004)

[28] FDA – July 1993: Guide to inspections validation of cleaning processes – July 1993

[29] J. Lambropoulos, G.A. Spanos, N.V. Lazaridis, J. Pharm. Biomed. Anal. 23 (2000) 421–428.

[30] M.J. Nozal, J.L. Bernal, L. Toribio, M.T. Martin, F.J. Diez, J. Pharm. Biomed. Anal. 30 (2002) 285–291.

[31] R. Debono, S. Stefanou, M. Davis, G. Walis, Pharmaceut. Technol. (2002) 72–78.

[32] K.M. Jenkins, A.J. Vanderwielen, J.A. Armstrong, L.M. Leonard, G.P. Murphy, N.A. Piros, PDA J. Phar. Sci. Technol. 50 (1996) 6–15.

[33] D.S. Risley, K.F. Hostettler, J.A. Peterson, LC-GC 16 (1998) 562–568.

[34] FDA – Se 490.100: Process Validation Requirements for Drug Products and Active Pharmaceutical Ingredients Subject to Pre-Market Approval (CPG 713208)

[35] FDA – February, Guideline on General Principles of Process Validation. (1993).

[36] N.A. El Ragehy, A.M. Badawey, S.Z.-El Khateeb, Journal of Pharmaceutical and Biomedical Analysis 28 (2002) 1041–1053

[37] M.M. Mabrouk, H.M. El-Fatatry et al., Journal of Pharmaceutical and Biomedical Analysis 33 (2003) 597-604

[38] Impurity profile study of loratadine K.V.S.R. Krishna Reddy et.al Journal of Pharmaceutical and Biomedical Analysis 32 (2003) 29-39

[39] United States of Pharmacopoeia, volume 30, NF 25 page 2492

[40] ICH harmonized tripartite guideline, Validation of analytical procedures: text and methodology, Q2-R1 (2005)

CHAPTER 3

HPLC METHOD DEVELOPMENT AND VALIDATION OF PROTEIN BASED DRUGS

3.1 INTRODUCTION

The advent of recombinant DNA technology has led to a worldwide zeal to develop protein pharmaceuticals in the past three decades. These protein pharmaceuticals include functional regulators and supplements, enzyme activators and inhibitors, poly- and monoclonal antibodies, and various vaccines. In comparison with small chemical drugs, protein pharmaceuticals have high specificity and activity at relatively low concentrations. These features have made protein pharmaceuticals indispensable in combating human diseases.

Due to advances in analytical separation technology, recombinant proteins can now be purified to an unprecedented level (Bond et al., 1998). Highly purified protein pharmaceuticals significantly reduce the known and unknown potential side or even toxic effects. [1]

However, one of the most challenging tasks remaining in the development of protein pharmaceuticals is dealing with physical and chemical instabilities of proteins. Protein instability is one of the two major reasons why protein pharmaceuticals are administered traditionally through injection rather than taken orally like most small chemical drugs (Wang, 1996). Protein pharmaceuticals usually have to be stored under cold conditions or even freeze-dried to a solid form to achieve an acceptable shelf life. [2]

Pharmaceutical excipients may be added to a formulation to stabilize the protein, to aid in manufacture of the dosage form, for control or target delivery in the body, or provide tonicity to minimize pain upon injection. Examples include buffers, carbohydrates as bulking agents for lyophilization, polymers as viscosity agents for topical applications, and salts or sugars for adjusting solution osmolality into a

physiological range. Although it is often assumed that pharmaceutical excipients are essentially inert, some additives may have certain toxicological or biological activities, and therefore, may play a role in defining the overall safety profile of a drug. Although excipients are selected for their low toxicity, and are generally well tolerated, certain excipient classes such as antioxidants and preservatives may have some level of toxicity associated with them [3-5]. The overall safety profile of a drug or excipient is not determined independently from each other, since the combination of drug and excipient together defines the drug product tested in clinical trials [6]. Many pharmaceutical excipients are classified as "generally regarded as safe" or GRAS and typically have a long history of safe use as food additives [7].

One major hurdle for the formulation scientist to develop stable protein formulations is the limited number of excipients commonly used in parenteral formulations. The introduction of novel pharmaceutical excipients to stabilize proteins would be of great interest, however, the safety and efficacy of these new compounds would need to be evaluated as part of the drug approval process [6]. Novel pharmaceutical excipients have been designed to enhance protein stability, for example, low molecular weight multi-ions [8].

The various classes of pharmaceutical excipients commonly used to formulate and stabilize protein therapeutic drugs and vaccines are shown in Table 3.1,.(science protein excipients)

Table 3.1 Pharmaceutical excipients for use in protein formulations (categories, examples, comments

Category	Representative examples	General comments	Cautionary comments
Buffering agents	Citrate acetate, histidine, phosphate, Tris	Maintain solution pH Buffer-ion specific interactions with protein	pH may change with temperature Crystallizaiton during freezing.
Amino acids	Histidine, arginine, glycine, proline, lysine, methionline	Specific interactions with protein Antioxidant (His, Met) Buffering and tonicifying agents	Decomposition during storage
Osmolytes	Sucrose, trehalose, sorbitol, glycine, proline, glutamate, glyceron, ures	Natural compounds that stabilize proteins and macromolecules against environmental stress (temperature, dehydration	High concentration often required for stabilization Many additional osmolytes have been identified, but not currently approved for use as pharmaceutical excipients Destabilizing effects also reported
Sugars and cargohydrates	Sucrose, trehalose, sorbitol, mannitol, glucose, lactose	Protein stablilizer in liquid and lyophilized states Tonicifying agents	Reducing sugars react with proteins to form glycated proteins Nonreducing sugars can hydrolyze forming reducing sugars Impurities such as metals and 5-HMF
Protein and polymers	HSA, gelatin, PVP, PLGA, PEG	Lactose as a carrier for inhaled drugs Dextrose solutions during IV administration Competitive inhibitor of protein adsorption Lyophilization bulking agents	Trends toward use of recombinant sources of HSA and gelatins Drug delivery polymers may not be compatible with protein drugs.
Salts	Sodium chloride, Potassium chloride, Sodium sulfate	Drug delivery vehicles Tonicifying agents Stabilizing or destabilizing effects on proteins, especially with anions (Hofmeister salt series)	Concentration dependent effects Trace metals can cause oxidation May be corrosive to metal surfaces
Surfactants	Polysorbate 20 and 80	Competitive inhibitor of protein adsorption Competitive inhibitor of protein surface denaturation Liposomes as drug delivery vehicles	Lowers Tg' of solution (may affect lyophilization) Peroxide can cause oxidation May degrade during storage Complex behaviour during membrane filtration due to micelle formation
Chelators and anti-oxidants	EDTA, DTPA, amino acids (His, Met), ethanol	Bind metal ions Free radical scavengers	Certains antioxidant such as ascorbic acid and glutathione lead to protein stability Light exposure accelerates oxidation
Preservatives	Benzyl alcohol, m-cresol, phenol	Prevents microbial growth in multi-dose formulations	Inverse concentration dependant effects on protein destabilization vs antimicrobial effectiveness
Specific ligands	Metals, ligands, amino acids, polyanions	Binds protein and stabilizes native conformation against stress induced unfolding Binding may also affect protein's conformational flexibility	May involve use of novel excipients or an excipient with biological activity

The higher complexity of peptides and proteins compared to organic low molecular weight drug substances and the different ways to produce biotechnological products lead to special requirements concerning their quality assurance and analytical testing. Erythropoietin (EPO), the major physiological regulator of the red blood cell formation, is produced primarily by the kidneys and excreted in the urine. Production of EPO is stimulated under conditions of hypoxia and it exerts its biological effect by binding to specific receptors on erythroid progenitor cells in the bone marrow. Human EPO has an apparent molecular weight of 30,000 Da, consists of 165 amino acids and contains two disulfide linkages.

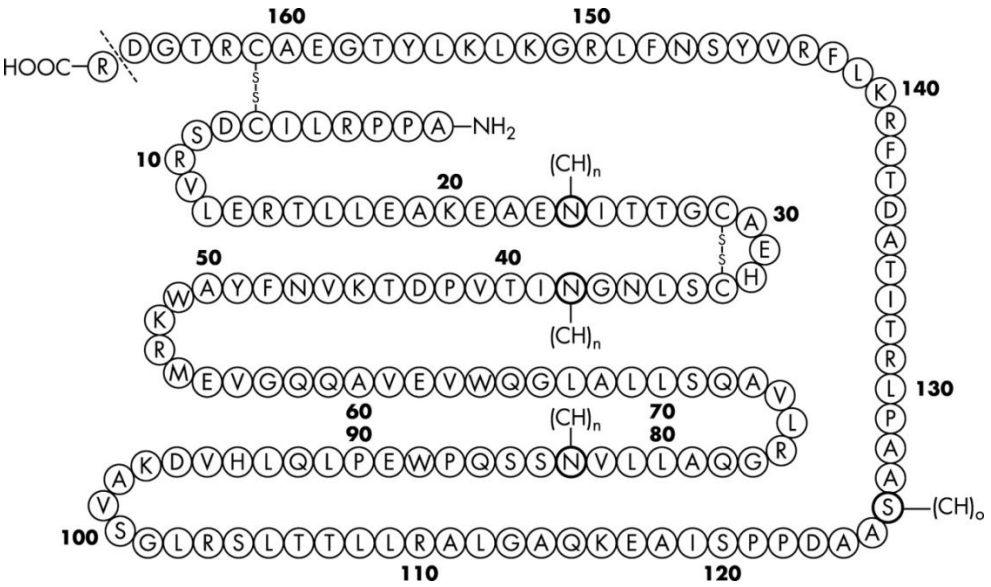
The first clinical trials were performed with human erythropoietin purified from urine. Today it is possible to produce human EPO by recombinant DNA technology (Lin et al.,1985) [9] (rHuEP0) in mammalian cell cultures receiving much better yields to supply the pharmaceutical market since its introduction in 1988. Recombinant human erythropoietin is therapeutically used for the treatment of anemia resulting from chronic kidney failure or from cancer therapy. Clinical trials with recombinant human erythropoietin showed its efficacy for reversing anemia related to advanced cancer or chemotherapy of cancer (Caro et al.,1989) [10]

Proteins or peptides can also be produced in quantities sufficient for pharmaceutical use by recombinant DNA technology in bacteria, yeasts, or in cell cultures (Nagata et al., 1980; Murray, 1980). [11, 12] The gene of interest is cloned into the appropriate host where the recombinant protein is then expressed. After synthesis of the recombinant protein by the ribosomes of the host cell, it is either directly secreted or has to be isolated after disruption of the host cell. Depending on the host cell also post-translational modifications of the recombinant protein occur. The recombinant protein is then purified to homogeneity in several steps. The pure substance is characterized and tested for the absence of impurities like host cell proteins or aggregates. Somatostatin was the first human hormone synthesized in cell cultures.

Nowadays it is possible to produce human erythropoietin (HuEPO) by recombinant DNA technology (Lin et al.,1985) [9] in mammalian cell cultures [e.g., Chinese hamster ovary (CHO) cells]. Much higher yields compared to the purification from urine are of great advantage due to the steadily increasing demand for the product since its introduction to the market in 1988. Hu EPO consists of a polypeptide sequence of 165 amino acids and a carbohydrate moiety, which contributes about

40% of the molecular weight and is attached at four glycosylation sites. Asn24, Asn38 and Asn83 are N-glycosylated whereas Ser126 was found to be O-glycosylated (Lai et al., 1986). [13] (science erythro)

Figure 3.1 Structure of EPO



The biological activity of EPO in vivo is affected by the glycosylation pattern (sialic acid content). Since production system and process conditions for rhEPO affect the glycosylation pattern the production process should be carefully validated and monitored to assure consistency of the biological activity throughout different production batches [14].

At present the content of rhEPO preparations is typically tested by complex in vivo potency assays which measure the relevant biological activity. For instance, the European Pharmacopoeia describes an assay for rhEPO bulk solutions in which the effect of rhEPO on mice kept under low oxygen conditions is monitored by measuring

incorporation of radio-labelled ferric chloride [15]. For assaying the content of rhEPO preparations in a routine setting these types of bioassays require a significant number of animals. A rapid and less resource demanding physico-chemical assay may not specifically mimic bioactivity but it would provide a wider forum for controlling the quality of these common pharmaceutical products. Moreover, from an analytical point of view content assays based on physicochemical technology will be more precise than bioassays. Developing a suitable physicochemical assay for rhEPO preparations is hampered by the low dose of the micro-heterogeneous glycoprotein in presence of relatively large amounts of excipients. Particular difficulties are encountered when human serum albumin (HSA) is present. The protein HSA is obtained from large pools of human plasma and cannot be considered chemically homogeneous. The physicochemical assays should have a high degree of selectivity and reproducibility for rhEPO assay. So far capillary electrophoresis (CE) methods have been developed to characterize the rhEPO glycoform pattern and a capillary zone electrophoresis method has been prescribed by the European Pharmacopoeia as an identification test for rhEPO in concentrated bulk solutions [15]. In addition to this method, another CE method has been developed that is capable of analysing rhEPO pharmaceutical preparations containing salts and HSA, and in the concentration range of 0.03–1.92 mg rhEPO/ml [16].

High-performance anion-exchange chromatography (HPAEC) separates proteins according to their negative electric charge and has been used for EPO assay with fluorimetric detection by D.M.A.M. Luykx et al. in pharmaceutical products. [17].

Srinivas R. Gunturi *et al*. have developed a method for the determination of rHu EPO aggregates in formulations by HPLC method with fluorescence detection [18].

Among the possible methods to eliminate HSA, immunoaffinity chromatography (IAC) is one of the most effective ones [19]. However, there is no method reported for the determination of EPO in the presence of HSA without any sample pretreatment**.**

HPLC in combination with UV-detection is a separation method that provides a powerful means for characterising the homogeneity of common biopharmaceuticals such as somatropin, insulin and interferons. Because of its high resolution, reversed-phase HPLC is often applied for quantification of the active pharmaceutical ingredient and for the analysis of closely related protein variants or degradation products (e.g., oxidised, deamidated) [20-22].

Although HPLC methodologies have been described previously, they have been developed either for analysis of purified r-Hu EPO monomeric protein [23-24] or for investigation of r-Hu EPO (monomer) metabolic pathways [25] in the absence of HSA.

3.2 CHALLENGES IN RP- HPLC / UPLC ANALYSIS OF PROTEINS AND PEPTIDES

RP-HPLC / UPLC analysis of biomolecules such as proteins and peptides can be a challenge as there are often problems associated with analytical systems such as excessive band broadening, peak tailing or misshaped bands, low recovery, ghost peaks and the appearance of one protein in two or more distinct bands [26]. Understanding the impact of process variables in RP-HPLC can help minimise or eliminate these undesirable effects.

The analysis of biochemical entities such as peptides, proteins, and oligo-nucleotides by RP-HPLC pose different challenges as compared to the analysis of small chemical

molecules since they have larger hydrodynamic radii and different functionalities in the molecules that may result in different interactions with RP-HPLC stationary and mobile phases. These factors must be considered in the development of an analytical method for proteins and peptides [26-27]

However, there is no method reported for the determination of EPO in the presence of HSA without any sample pretreatment

The objective of the study was to develop methods, using "RP-HPLC, and UPLC" techniques that enable quantification of EPO in medicinal formulations containing HSA.

Abbreviations used:

EPO – Erythropoietin

EPO-IRS – EPO Internal Reference Standard

DS (API) – Drug Substance (Active Pharmaceutical Ingredient)

EPO-DS – EPO Drug Substance

DP – Drug Product

EPO-DP – EPO Drug Product

RMP – Reference

rHu – Recombinant Human

HSA – Human Serum Albumin

EP – European Pharmacopoeia

3.3 QUANTIFICATION OF EPO AND METHOD VALIDATION

3.3.1 EXPERIMENTAL

Materials, reagents and chemicals

HPLC grade acetonitrile was purchased from Merck; tri-fluoro-acetic acid (TFA) was purchased from Sigma Aldrich. Ultra pure water was obtained using Milli-Q® UF-Plus (Millipore) system. Human Serum Albumin (HSA) with 20% globulin fraction was obtained from Baxter. EPO internal reference standard (EPO-IRS) having 0.8mg/mL concentration was procured from Intas Biopharmaceuticals Ahmedabad was used as standard and was qualified using EP reference standard. Formulated EPO (Drug Product) was used to prepare samples. Other chemicals, such as tri-sodium dihydrate, sodium chloride and citric acid used were of "highest purity" available.

Preparation of standard, mobile phase and dilution buffer

EPO-IRS was used for preparation of different working standards using dilution buffer or dilution buffer containing 2.5mg/mL of HSA.

Mobile phase 'A' consisted of 0.1% v/v TFA in Milli Q water and mobile phase 'B' consisted of 0.1% v/v TFA in acetonitrile.

Dilution buffer (Citrate buffer) containing 5.8 mg/mL tri-sodium dihydrate; 5.8 mg/mL sodium chloride and 0.06 mg/mL citric acid in "Milli Q water" was prepared and used so as to have a matrix similar to EPO formulation. Dilution buffer with HSA was prepared by diluting 2.5 mg/mL of HSA in dilution buffer. All dilutions were made using calibrated digital micro-pipettes.

Chromatographic condition

HPLC – An LC system equipped with an injection valve (quaternary), 215 UV detector and chemstation software was used for RP-HPLC method. A reverse -phase C_8 column (4.6 mm ID × 250 mm L, porosity 300º A, particle size 5 µm) with guard column (reverse-phase C_{18} column of 4.6 mm ID × 35 mm L, porosity 300º A, particle size 5 µm) was used for separation. To get the optimum results, mobile phase with a flow rate of 1.5 mL/min and column temperature at 45°C were used. The gradient programme for mobile phase was optimized using a timed gradient programme T (min)/%mobile phase A: 0/65, 4/65, 12/50, 14/50, 15/40, 16/65, 20/65.

UPLC – An LC system equipped with an injection valve (binary), a 210 UV detector and Empower software was used for RP-UPLC method. Reverse-phase C_{18} column (2.1 mm ID × 50 mm L, porosity 135ºA, particle size 1.7 µm) was used for separation. To get the optimum results, mobile phase flow rate was kept constant at 0.35 mL/min and column temperature at 60°C. The gradient programme for mobile phase was optimized using a timed gradient programme T (min)/%mobile phase A: 0/85, 0.12/85, 0.33/70, 0.62/64, 2.62/35, 3.19/0, 3.76/85 and 4.05/85.

3.3.2. RESULTS AND DISCUSSION

3.3.2.1. RP HPLC method

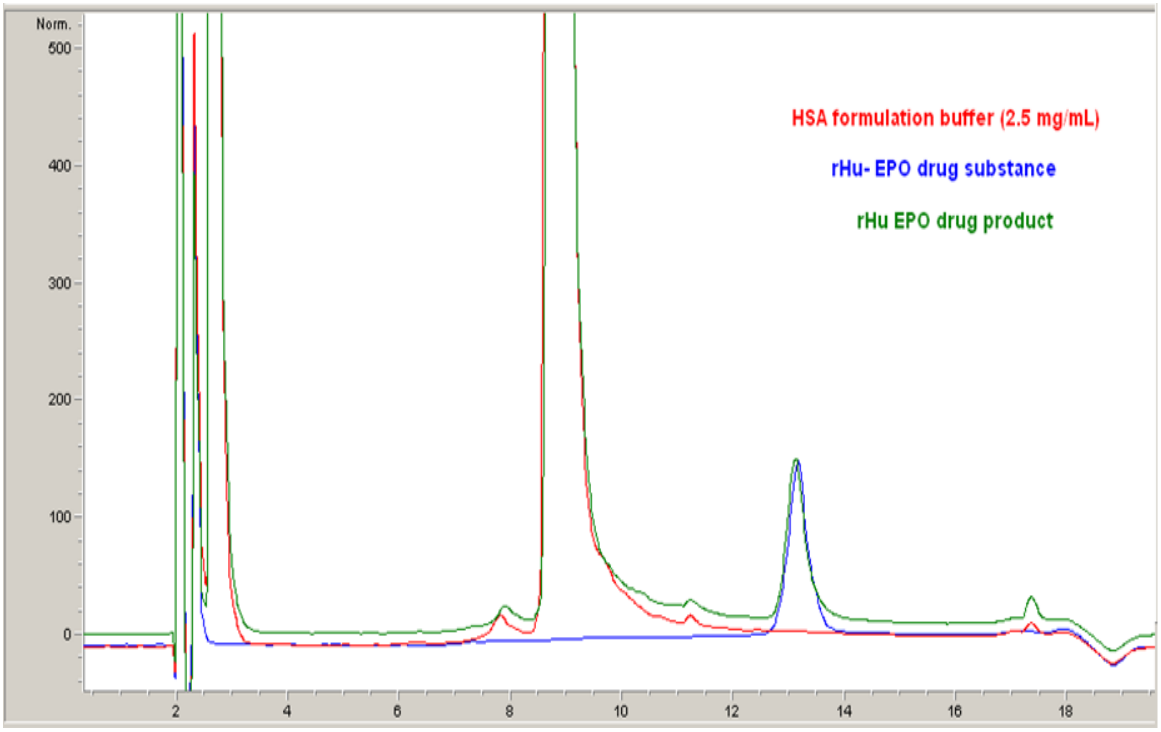
3.3.2.1.1 Method development

Initially, the gradient HPLC conditions were optimized for EPO in presence of HSA. Based on the different hydrophobic properties of both proteins in a non-polar stationary phase, an RP-HPLC in gradient mode was used. JADWIGA et. al. had reported a HPLC method with an analysis time of about 60 min., with retention times, of approximately 17 and 33 min for HSA and EPO, respectively The HPLC method

proposed by JADWIGA et al. [28] was taken into consideration for the experiments and efforts were made to minimize the analysis time which is a must for multi-product facility. The chromatographic separation was achieved by applying chromatographic conditions as described in above section 3.4.1.

The applied chromatographic conditions permitted a good separation of HSA and EPO at different concentrations of EPO each containing 2.5 mg/mL of HSA. No interference of HSA and other excipients was observed during the analysis as shown in Figure 3.2.

Figure 3.2 HPLC chromatograms of EPO-IRS, HSA in dilution buffer (2.5 mg/mL) and EPO-DP.



The capacity factor (k') of first peak (HSA) and second peak (EPO) were 3.24 and 5.24, respectively; while the resolution factor was 6.88. The asymmetry of the peak was found to be 1.29 and 5.29 for EPO and HSA, respectively; while the tailing factor

parameter was found to be 1.29 and 1.14 for EPO and HSA, respectively. For replicate injections of EPO-IRS the % RSD of the main peak area was found to be below 0.7%, and there was no variation in the retention time (less than 0.1 min).

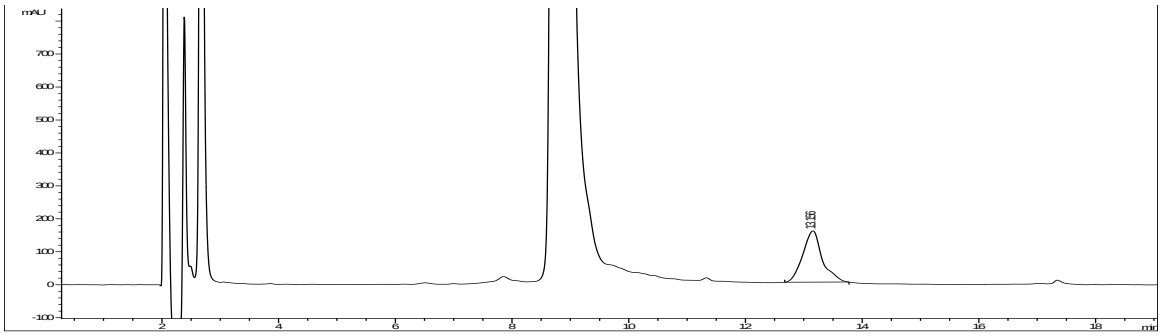
Based on the studied parameters, it was concluded that the EPO and HSA peaks were well resolved in the developed method and the tailing factor was within limits.

3.3.2.1.2 Method validation

- **System Suitability:**

The chromatographic separation, as explained above was carried out with HPLC to evaluate the chromatographic parameters. To check the suitability, a known amount of EPO-IRS was spiked to the dilution buffer and a chromatogram was run. Representative chromatogram is shown in Figure 3.3, which corresponds to the chromatographic separation of these substances. The % RSD for the main peak area of EPO-IRS(measured in triplicate) were found to be below 0.7%, while no variation in the retention time was observed (less than 0.1 minute). The peaks due to EPO and HSA were thus considered well resolved.

Figure 3.3: Chromatogram of EPO-DS spiked in dilution buffer containing HSA 2.5mg/mL



One sharp peak of EPO was eluted at 13.1 min along with the HSA peak that was eluted at 9.0min. The EPO peak was matched with the standard peak of EPO-IRS.

- **Specificity**

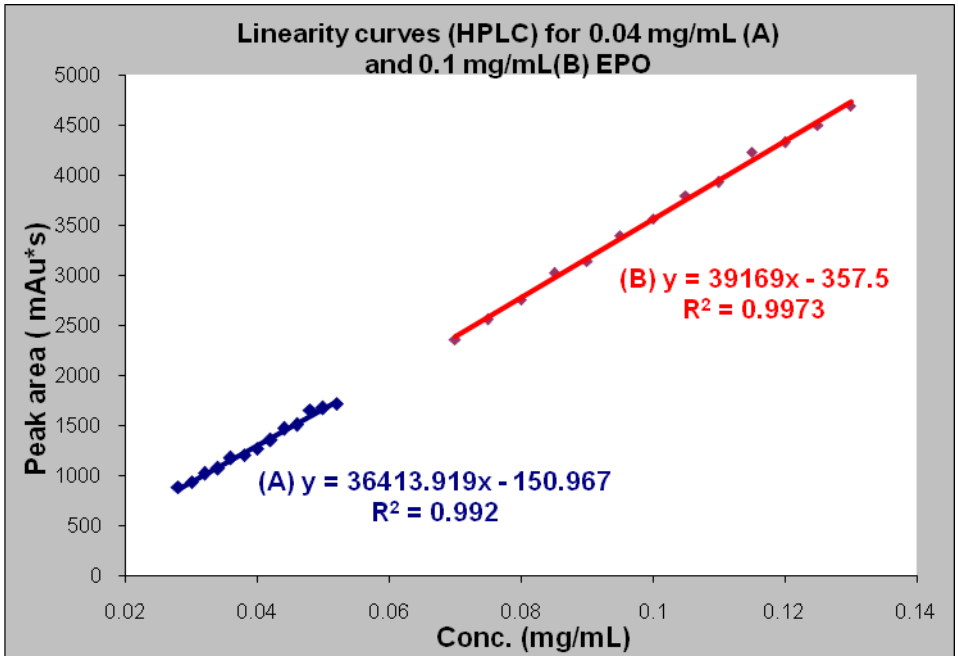
To evaluate possible interfering peaks, two different concentrations of EPO-IRS (0.04, and 0.1 mg/mL) in dilution buffer and HSA containing dilution buffer were injected into HPLC. No interference was observed as evidenced by the following observations:

  - No peak was observed in the integration window of the chromatogram for the sample of mobile phase (blank), HSA in dilution buffer, Dilution buffer.
  - Variation in Retention time of main peak between EPO-IRS and EPO-DP was less than 0.5 min.
  - Variation in terms of % recovery of EPO-IRS spiked in HSA dilution buffer was less than 5.0 % as compared to EPO-IRS spiked in mobile phase A.
  - There was 0.1 min variation in retention time of main peak of EPO-IRS in mobile phase as compared to the retention time of the main peak of EPO-IRS solution (0.1mg/mL).

- **Linearity and Range**

EPO-IRS was used for preparation of different concentrations of EPO-IRS ranging from 0.028 to 0.130 mg/mL, each containing 2.5 mg/mL HSA. Linearity curves were plotted for 0.04 and 0.1 mg/mL of EPO-IRS (Figure 3.4).

Figure 3.4 Linearity curves (HPLC) for (A) 0.04 mg/mL EPO-IRS and (B) and 0.1 mg/mL EPO-IRS



The correlation coefficient, slope, Y-intercept, regression equation of the calibration curve were determined and are shown in Table A. The percent RSD was found to be less than 2.0% while the percent recovery was found to be in the range of 97% to 103%.

- **Accuracy**

During accuracy study, we need to consider ±20% of the target concentration, therefore

accuracy was studied using two different sets of three different solutions, containing 0.032, 0.040 and 0.048 mg/mL and 0.08, 0.10 and 0.12 mg/mL of EPO-IRS. Each solution in its dilution buffer and in the mobile phase was spiked with HSA at a concentration of 2.5 mg/mL. From Table 3.2 it is evident that the percent recovery

was found to be more than 95% for 0.04 mg/mL of EPO while the percent recovery was found to be more than 99% for 0.1 mg/mL of EPO in presence of 2.5 mg/mL of HSA. The percent RSD was found to be less than 2.0%.

Table 3.2 % recovery of EPO-IRS spiked and control samples of 0.04 mg/mL

Type	Conc. of EPO-IRS in mg/mL	Injected amount of EPO-IRS (µg)	Avg. value of 3 inj. Main peak area of EPO-IRS (mAu*S)	%RSD	%Recovery
Spiked (0.040 mg/mL)	0.032	3.2	975	2.08	96.6
	0.040	4.0	1242	0.35	95.7
	0.048	4.8	1523	1.38	95.8
Spiked (0.10 mg/mL)	0.08	8.0	2896	0.76	103.8
	0.10	1.0	3642	0.86	102.1
	0.12	1.2	4441	0.28	102.1
Control (0.040 mg/mL)	0.032	3.2	1041	0.92	102.3
	0.040	4.0	1339	1.34	102.3
	0.048	4.8	1608	0.94	100.6
Control (0.10 mg/mL)	0.08	8.0	2753	0.24	99.3
	0.10	1.0	3566	0.31	100.2
	0.12	1.2	4326	0.15	99.7

- **Precision**

Precision was evaluated for intra-day (Repeatability) and inter-day (Intermediate precision) variation, and different makes of columns.

The repeatability was assessed with six independent sample preparations for each of the two different system suitability samples (0.04 mg/mL and 0.1 mg/mL of EPO-IRS) and single injection was injected from each preparation. The percent RSD of peak area, for each EPO IRS was found to be less than 0.9%.

Inter-day precision was determined by analysis of 0.04 mg/mL of EPO spiked with 2.5 mg/mL of HSA. The precision of the method was evaluated by performing five

different conditions (n = 30) (Table 3.4) and calculating the relative standard deviations (RSD). Three replicate injections of system suitability standards prepared independently were considered for the study. The percent RSD for the main peak area of EPO-IRS within each set and between different sets was found to be less than 2.0%. The percent recovery of each EPO -IRS was found to be between 95.0% - 105.0% and the maximum variation between sets was found to be 5.0%.

Table 3.3 % RSD of main peak area for the EPO-IRS samples

Sample details	Main peak area (mAu*s) / % recovery of EPO-IRS							%RSD
	Prep-1	Prep-2	Prep-3	Prep-4	Prep-5	Prep-6	Avg. value	
EPO-IRS (0.04 mg/mL)	1372	1345	1368	1362	1362	1336	1358	1.03
	104.6	102.7	104.3	103.9	103.9	102.1	103.6	0.9
EPO-IRS (0.1 mg/mL)	3683	3634	3635	3646	3595	3578	3629	1.03
	103.2	101.9	101.9	102.2	100.9	100.5	101.8	0.9

Table 3.4 Experimental matrix of intermediate precision

	Set 1	**Set 2**	**Set 3**	**Set 4**	**Set 5**
Equip. ID	System-1	System-1	System-1	System-1	System-2
Column	Column-1	Column-1	Column-1	Column-2	Column-1
Day	1	2	2	3	3

Table 3.5 % RSD of main peak area for different sets

Sample details	Set No.	Main peak area (mAu*s)							%RSD
		Prep-1	Prep-2	Prep-3	Prep-4	Prep-5	Prep-6	Avg. value	
EPO-IRS (0.04 mg/mL)	set-1	1372	1369	1378	1345	1368	1362	1366	0.83
	set-2	1323	1314	1299	1308	1299	1281	1304	1.12
	set-3	1342	1347	1346	1336	1328	1299	1333	1.36
	set-4	1359	1360	1381	1360	1354	1362	1363	0.69
	set-5	1363	1358	1357	1338	1351	1354	1353	0.65
EPO-IRS (0.1 mg/mL)	set-1	3683	3636	3659	3634	3635	3646	3649	0.53
	set-2	3578	3577	3535	3528	3499	3501	3536	0.99
	set-3	3572	3556	3540	3544	3528	3530	3545	0.47
	set-4	3632	3606	3639	3630	3625	3612	3624	0.35
	set-5	3676	3671	3671	3670	3658	3648	3666	0.29

Table 3.6 % recovery of EPO-IRS for different sets

Sample details	Set no.	% Recovery of EPO-IRS							% RSD
		Prep-1	Prep-2	Prep-3	Prep-4	Prep-5	Prep-6	Avg. value	
EPO-IRS (0.04mg/mL)	set-1	104.57	104.33	104.95	102.71	104.30	103.85	104.12	0.75
	set-2	101.22	100.60	99.58	100.19	99.51	98.33	99.90	1.01
	set-3	102.51	102.86	102.79	102.06	101.50	99.55	101.88	1.22
	set-4	103.65	103.75	103.76	103.76	103.34	103.87	103.69	0.18
	set-5	103.96	103.58	103.52	102.20	103.15	103.30	103.28	0.58
EPO-IRS (0.1mg/mL)	set-1	103.15	101.94	102.54	101.91	101.93	102.20	102.28	0.48
	set-2	100.47	100.46	99.38	99.19	98.45	98.50	99.41	0.90
	set-3	100.33	99.91	99.51	99.61	99.19	99.24	99.63	0.43
	set-4	101.86	101.19	102.03	101.79	101.67	101.35	101.65	0.32
	set-5	102.98	102.85	102.85	102.81	102.52	102.26	102.71	0.26

The % RSD for the main peak area between replicate injections for each EPO-IRS was less than 2.0 %. Maximum variation between the retention time of main peak for replicate injections of EPO-IRS was less than 0.2 min. The % recovery for each EPO-IRS was calculated using respective calibration curve and was found to be between 95.0% - 105.0%.

The above results and observations proved that the developed method is precise for the above mentioned EPO samples when analyzed with respect to, different days, different instruments and different brands columns (Table 3.5 and 3.6) and hence the parameter of precision stands validated.

- **Robustness**

Robustness was tested by varying age effect of mobile phase and test samples, column temperature and mobile phase composition.

Age effect of mobile phase and test samples held for seven days

Freshly prepared samples for system suitability (0.04 mg/mL and 0.1 mg/mL of EPO-IRS) and those prepared seven days ago were analyzed using both freshly prepared and seven day old mobile phase. There was not much variation in the results as seen from Table 3.7, with percent variation from initial day to 7 days being about 5% and percent RSD being less than 0.4%. There was no difference in retention time and percent recovery was found to be between 90% and 110%. It is thus recommended to use freshly prepared sample as well as mobile phase for analysis.

Column temperature effect

Experiments were conducted using system suitability samples with column temperature variation of $\pm$ 2ºC from the set temperature (60ºC) and the results are shown in Table 3.7. The percent RSD was found to be less than 0.7%, with no variation at lower temperature. However, 5% variation was observed at higher temperature and $\pm$ 0.1 minute difference in retention time. The percent recovery was found to be within acceptable limits (95 – 105%).

Mobile phase composition

Experiments were conducted using system suitability samples with mobile phase composition variation of ± 20% from the set percentage of TFA (0.1%). The percent variation between unaltered / initial condition and altered condition for EPO sample (done in triplicate) as seen from Table 3.7 was found to vary less than 2.0% and there was no variation in retention time.

Table 3.7 Comparison result for % recovery of EPO-IRS between unaltered / initial condition and altered condition for 0.1 mg/mL of EPO-IRS

Inj. No.	% Recovery of EPO-IRS peak						
	Initial sample	Stored sample & mobile phase	Stored sample & fresh mobile phase	58ºC Temp	62ºC Temp	0.08% TFA	0.12% TFA
Inj.1	100.33	89.14	88.74	100.10	99.05	100.33	99.30
Inj.2	99.91	89.45	87.52	100.00	98.94	101.72	98.89
Inj.-3	99.51	89.27	87.80	99.10	98.27	101.17	98.76
Av. value	99.92	89.29	88.02	99.73	98.75	101.07	98.98
Variation from initial day/unaltered condition (%)	-----	10.63	11.90	0.19	1.17	1.15	0.94

Interpretation:

% RSD for obtained main peak area between replicate injections for each EPO-IRS with altered and unaltered condition was less than 2.0. Maximum variation between the retention time of main peak for replicate injections of EPO-IRS for altered and unaltered condition was less than 0.2 min. Except for stability of sample and mobile phase, % recovery of each EPO-IRS for altered and unaltered condition was between 95.0 % - 105.0 %, and hence stability of sample and mobile phase can be considered

critical for the method under study. So for routine analysis freshly prepared sample as well as mobile phase is recommended.

3.3.2.2 UPLC method

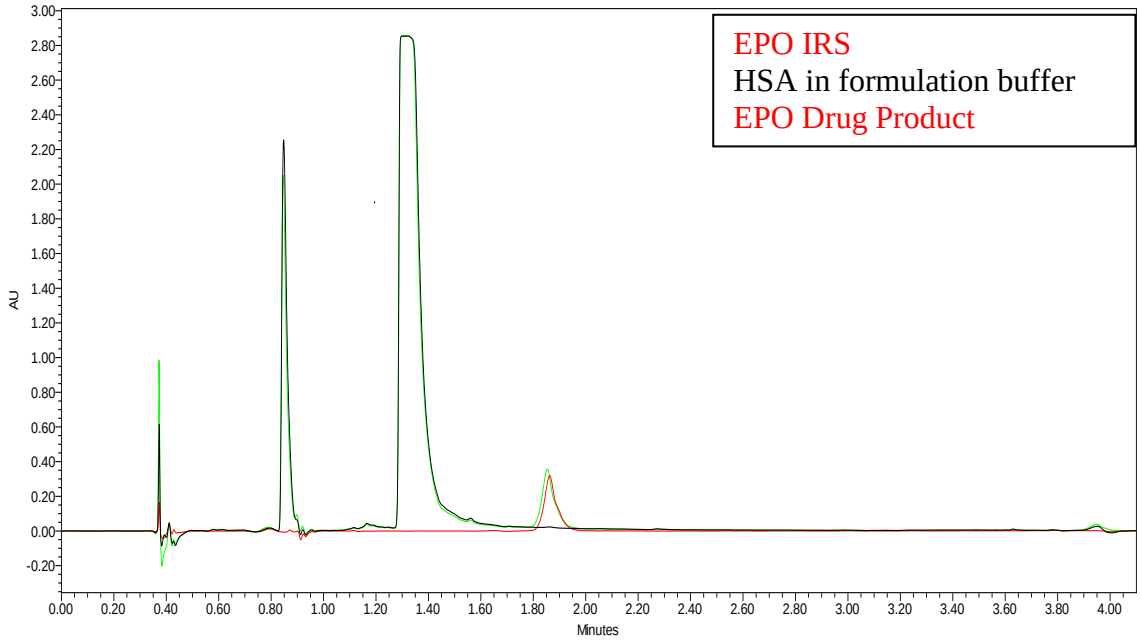
3.3.2.2.1 Method development

The basic chromatographic conditions like stationary phase, solvents and UV detector, employed in HPLC were taken into account while developing the new UPLC method. The stationary phase C_{18} was chosen in order to have similar polarity as that used in HPLC method. The injection volume was scaled down by about 10 fold to that used in HPLC. To get the optimum results, mobile phase flow rate was kept constant at 0.35 mL/min and column temperature was maintained at 60^{o}C.

The chromatographic separation was achieved as described in Section 3.4.1.

The applied chromatographic conditions permitted a good separation of HSA and EPO. Different concentrations of EPO-IRS in the range 2.5 to 150 µg with 2.5 mg/mL of HSA were studied and no interference of HSA and other excipients was observed during the analysis. Representative chromatograms are shown in Figure 3.5.

Figure 3.5 UPLC chromatogram of internal EPO-IRS, HSA in dilution buffer (2.5 mg/mL) & EPO-DP.



The capacity factor (k') was 2.45 and 3.9 for the first and second peak respectively, while the resolution factor was 5.35. The asymmetry of the peak was found to be 5.63 and 1.57 for HSA and EPO respectively. Tailing factor was found to be 3.68 and 1.33 for HSA and EPO, respectively. The percent RSD of the main peak area for replicate injections of EPO-IRS was found to be below 2.0% while no variation in the retention time was observed (less than 0.1 minutes).

3.3.2.2.2 Method Validation

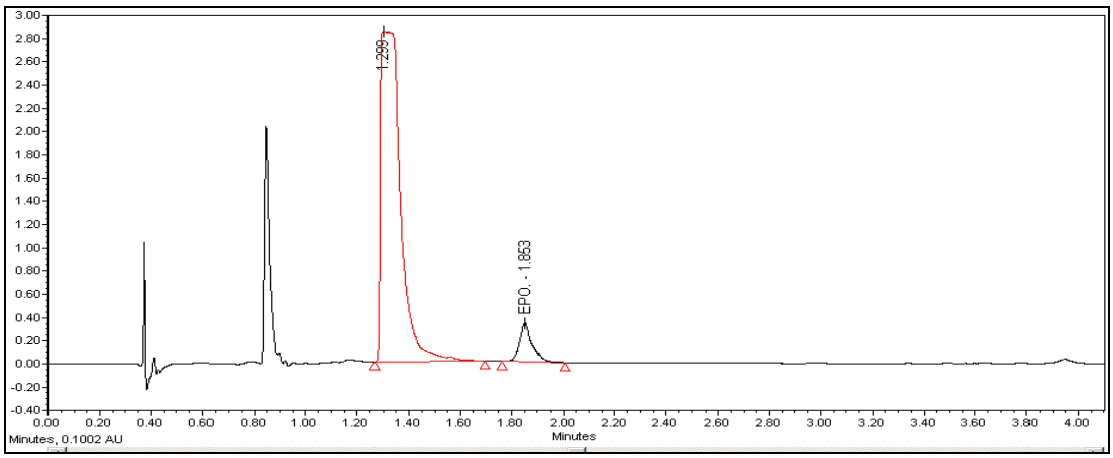
- **System Suitability:**

The chromatographic separation, as explained above was carried out with UPLC to evaluate the chromatographic parameters. A sample containing 0.1 mg/mL EPO-IRS in 2.5 mg/mL HSA in dilution buffer was run and the representative chromatogram is shown in Figure 3.6, which corresponds to the chromatographic separation of these

substances. The HSA peak was observed at 1.299 min and principal peak of EPO was obtained at 1.853 min, resolution between both being 5.35. Both peaks were well resolved in the developed method thus suggesting system suitability. The % RSD for the main peak area for replicate injections of EPO-IRS was found to be below 2.0% while no significant variation in the retention time was observed (less than 0.1 minutes).

It was concluded that the developed method is the optimum according to the studied parameters. The tailing factor to be controlled was within the limits established by these guidelines. Peak symmetry for the different mobile phase compositions and different flow rates was compared using the peak asymmetry factor *As*, measured at 10% of full peak height and the peak-tailing factor (PTF) measured at 5% of full peak height. Excellent columns give an As value of between 0.95 and 1.1 although values of < 1.5 are acceptable. PTF values of between 1.0 and 1.4 are also acceptable [29]

Figure 3.6 UPLC chromatogram of EPO-IRS, HSA and Dilution buffer



- **Specificity**

Separation selectivity is the ability of the method to elicit a response specific for the analyte in the presence of other components/substances that are present or are likely to be present with the analyte.

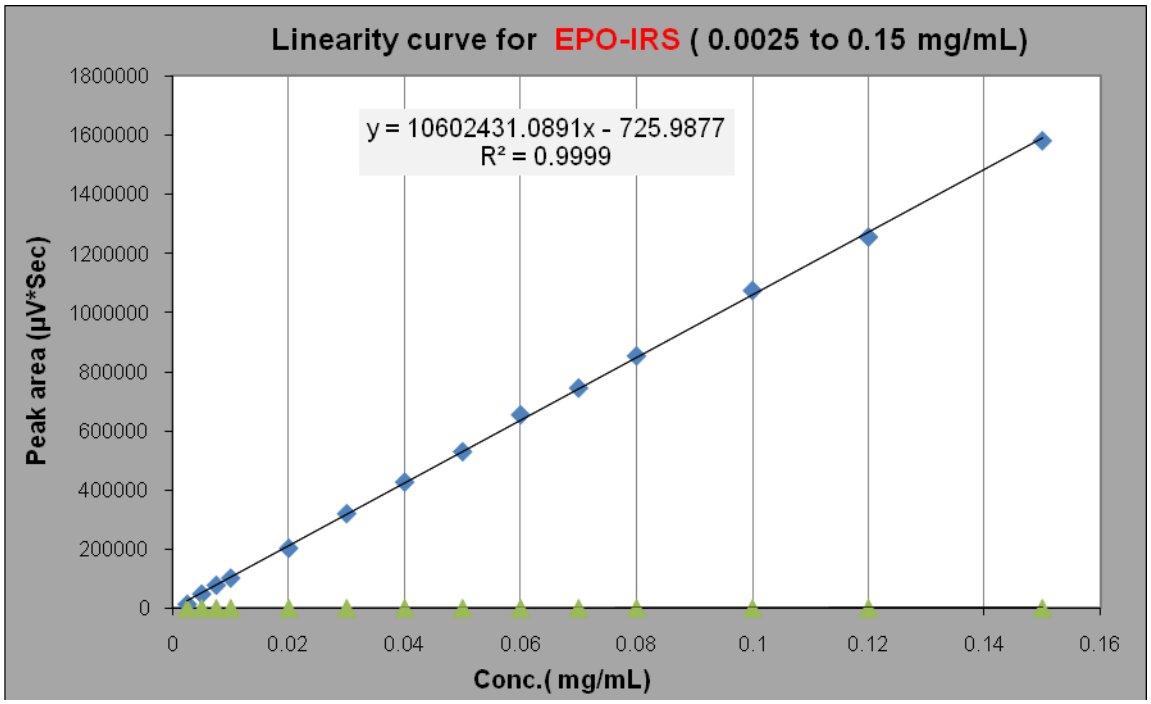
To address separation selectivity, 0.1 mg/mL of EPO-IRS in mobile phase (as positive control), HSA 2.5 mg/mL in dilution buffer, HSA 2.5 mg/mL in mobile phase, 0.1 mg/mL of EPO-IRS with 2.5mg/mL HSA in dilution buffer, mobile phase (Blank), Milli Q water and dilution buffer were injected into UPLC column.

HSA in dilution buffer and mobile phase was considered as the matrix components. Interference by the matrix components was evaluated by spiking known amount of EPO IRS in dilution buffer with HSA. No interference of matrix components was observed.

- **Linearity and Range**

EPO IRS was used for preparation of different working concentrations ranging from 0.0025 to 0.150 mg/mL, each containing 2.5 mg/mL of HSA. The peak area was plotted as shown in Figure 3.7.

Figure 3.7 Linearity curve (UPLC) for EPO-IRS



Calibration curves with concentration versus peak area were plotted with blank subtraction. The correlation coefficient, slopes and Y-intercepts and regression equation were determined and are shown in Table A. The correlation coefficient was found to be 0.999. The percent RSD was found to be less than 2.0% while the percent recovery was found to be in the range of 97% to 103%.

Table 3.8 Result of Linearity experiments:

Conc. of EPO-IRS in mg/mL	Avg. value of 3 inj. Main peak area of EPO-IRS (µV*Sec)	%RSD	%Recovery
0.0025	14542.67	3.94	57.60
0.0050	48853.33	0.16	93.52
0.0075	78670.33	0.81	99.85
0.0100	102880.67	1.19	97.72
0.0200	203637.00	0.26	96.38
0.0300	320597.00	0.06	101.02
0.0400	427237.67	0.19	100.91
0.0500	529824.33	0.09	100.08
0.0600	654388.33	0.25	102.98
0.0700	744953.00	0.14	100.47
0.0800	853514.33	0.50	100.71
0.1000	1074215.67	0.39	101.39
0.1200	1255101.33	0.26	98.71
0.1500	1580231.67	0.07	99.41

- **Accuracy**

Accuracy (% recovery) was studied with different working concentrations ranging from 0.0025 to 0.150 mg/mL of EPO-IRS. Each solution in its dilution buffer was spiked with HSA at a concentration of 2.5 mg/mL. The percent RSD was found to be less than 2.0%.

The percentage recovery in the concentration range 0.0075 to 0.15 mg/mL was greater than 95.0% while the recovery was found to be 93.52% and 57.60% for low EPO concentrations of 0.0050 and 0.0025 mg/L .

% Recovery was calculated by using the regression equation obtained from linearity curve of EPO.(y = mx+ c) where m =slope, c= intercept and y = average area of particular concentration of EPO-IRS

$$\text{Back calculated concentration (x)} = \frac{y}{m} - C$$

$$\%\ \text{Recovery} = \frac{\text{Back calculated concentration}}{\text{Concentration injected}} \times 100$$

Precision was evaluated by intra-day (Repeatability) and inter-day (Intermediate precision) variation. Repeatability (five replicates) was assessed independently for each of the three different concentrations (0.02 mg/mL, 0.04 mg/mL and 0.1 mg/mL). The percent RSD between areas of all five replicates as shown in Table 3.9 was less than 1.5% for all dilutions and percent recovery of all five replicates was more than 95%.

Table 3.9 % recovery of five replicates of EPO-IRS.

Sample mg/mL	Area of Principal Peak of EPO-IRS (diluted) sample solution (µV*Sec)					Avg Area µV*Sec	% RSD	% recovery
	Inj. 1	Inj. 2	Inj. 3	Inj. 4	Inj. 5			
0.02	203085	203689	204137	204133	197869	202583	1.32	96.11
0.04	426584	426986	428143	427039	424861	426723	0.28	101.19
0.1	1073542	1078738	1070367	1069226	1042460	1066867	1.32	101.18

Table 3.10 Back calculated concentration for five replicates of EPO-IRS:

Sample mg/mL	Back calculated Conc. of EPO-IRS (diluted) sample solution (mg/mL)					Average back calc. conc. Of EPO (mg/mL)	% RSD
	Inj. 1	Inj. 2	Inj. 3	Inj. 4	Inj. 5		
0.02	0.0193	0.0193	0.0194	0.0194	0.0188	0.0192	1.32
0.04	0.0405	0.0405	0.0406	0.0405	0.0403	0.0405	0.28
0.1	0.1018	0.1023	0.1015	0.1014	0.0989	0.1012	1.32

The % Recovery of all five replicates was more than 95% for all concentrations of EPO-IRS studied. The % RSD between areas of the five replicate injection for each concentration was not more than 2% while the % RSD for retention time of the five replicate injections of each concentration was not more than 2 %.

From the above results and observations it is established that the developed method has potential for the quantification of active substance in EPO-DP

- **Robustness**

To determine the robustness of the method, experimental condition (TFA concentration) was purposefully altered and the resolution between EPO-IRS and HSA was examined. The TFA concentration was changed between 0.08% and 0.12% from the standard composition of 0.1% which was originally used and the results are tabulated in Table 3.11

The percent recovery was found to be between 95% and 105%. The percent RSD for the area values obtained with altered and unaltered conditions of the parameter was found to be less than 2.5%, thus indicating that the developed method is robust and TFA concentration is not a critical parameter.

Table 3.11 Result of % RSD between the altered and unaltered condition for area, retention time and concentration.- Percentage TFA-0.08% and 0.12%, EPO-IRS-0.1mg/mL, Injection volume-5µL, Number of injections-2

Sample		Unaltered condition	Altered condition	Average	%RSD
TFA concentration : 0.12 % v/v					
EPO-IRS 0.1 mg/mL	Area	1074216	1104096	1089156	1.94
	RT	1.869	1.938	1.904	2.56
	Conc	0.1021	0.1047	0.1034	1.78
EPO-IRS 0.1 mg/mL	Area	1074216	1076856	1075536	0.17
	RT	1.869	1.841	1.855	1.07
	Conc	0.1021	0.1021	0.1021	0.00

3.3.3 COMPARATIVE STUDY OF HPLC AND UPLC PERFORMANCE

The performance parameters of both systems are shown in Table A. The runtime of UPLC was reduced by 4-fold to that of HPLC. The retention behaviors of HSA and EPO were similar in HPLC and UPLC columns. As expected, the UPLC method showed higher efficiency of analysis than HPLC method.

Both RP-HPLC and RP-UPLC methods were demonstrated to be validated for quantifying EPO in presence of another protein (HSA), which is often present in medicinal formulations using HSA as stabilizer. The HPLC and UPLC methods were validated showing satisfactory data for all the parameters tested. The reported UPLC method was found to be capable of giving faster analysis with good resolution, accuracy and precision than that achieved with conventional HPLC method. Both the chromatographic methods were found to be reliable. Since these methods are rapid and simple, they may be successfully applied to quality control analysis of EPO formulation containing HSA.

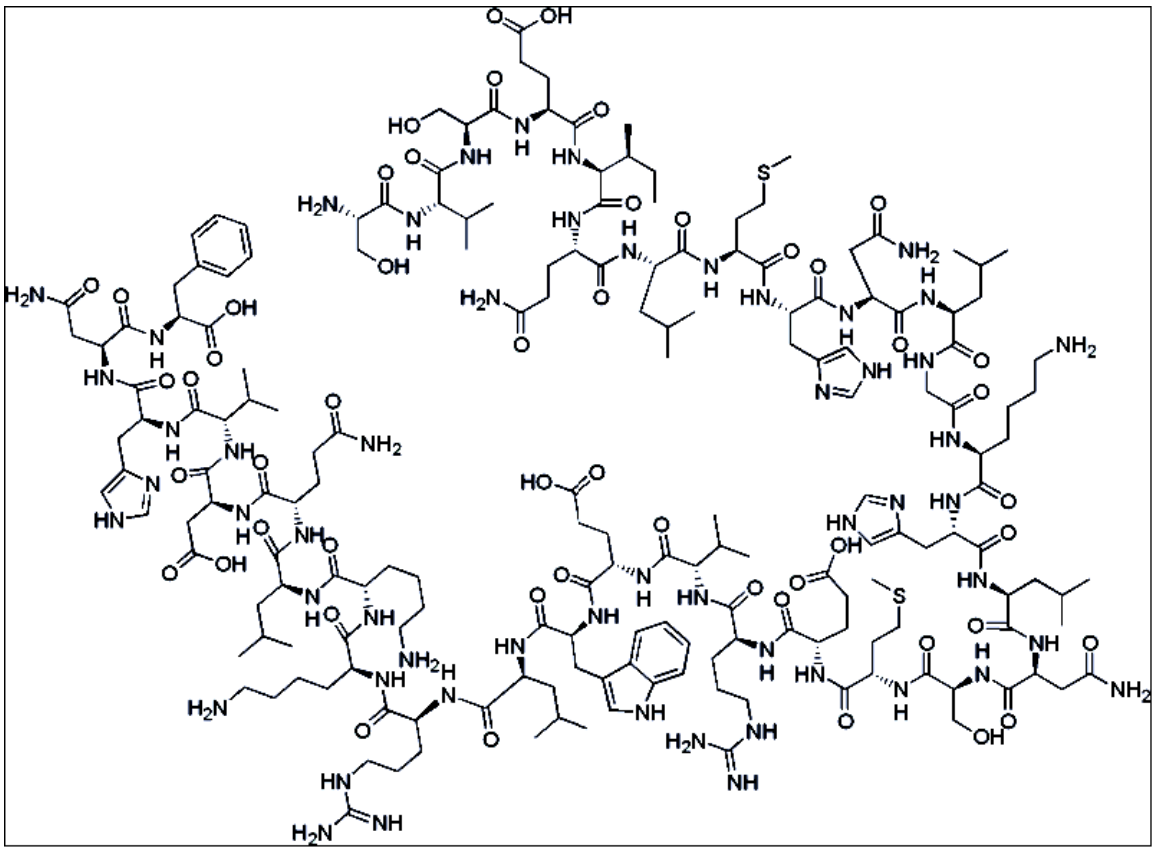
Table A: Results of regression equation/correlation coefficient and both methods comparative data

Statistical parameter	**HPLC for 0.04 mg/mL**	**HPLC for 0.1 mg/mL**	**UPLC**
Linearity & Range (Conc. in mg/mL)	0.028 to 0.052	0.07 to 0.13	0.0025 to 0.15
Regression Equation	y = 36413.9x – 150.967	y = 39168.8x – 357.502	y = 10602431.0x – 725.9877
Correlation coefficient	0.992	0.997	0.999
Total analysis time (min)	20	20	4
Retention time (min) For HSA For EPO	9 13		1.3 1.9
Sample size	100 µL		5 µL
Specificity	No interference		No interference
Accuracy	Recovery more than 95%		Recovery more than 97%
Precision	RSD less than 2%		RSD less than 1.5%
Robustness	Method is robust for all tested parameters except age effect of mobile phase and test samples		Method is robust for all tested parameters.

3.4 QUANTIFICATION OF PTH AND METHOD VALIDATION

Human parathyroid hormone (1–84) (hPTH) is a naturally occurring polypeptide composed of 84 amino acids [30], with overall basic properties (iso-electric point, p*I* >9). It has important biological activity as the major regulator of calcium ion homeostasis [31]. Efficient production methods have been reported through solution [32] and solid-phase [33] peptide synthesis as well as through recombinant DNA techniques [34].

Figure 8 Structure of PTH:



Teriparatide (recombinant DNA origin) injection [recombinant human PTH (1–34) termed as PTH] is a bone-forming agent for the treatment of osteoporosis. In the Fracture Prevention Trial (FPT), daily self-injections of teriparatide (20 and 40 µg) reduced the risk of new vertebral and non-vertebral fractures by 65% and 53%,

respectively, in postmenopausal women with advanced osteoporosis [35]. Once-daily injection of PTH induced pronounced increase in biochemical markers of bone turnover [36-41].

Immunoassay is a common technique for measurement of PTH in plasma. The measurement of PTH and its metabolites has been problematic due to the diversity of the circulating PTH metabolites, differences in the pharmacokinetic profiles of PTH and its metabolites and significant differences in specificity and sensitivity of PTH radioimmunoassay [**42-44**].

WHO International collaborative study of the proposed 1st international standard for recombinant human PTH (1-84) was done by RP-HPLC method in different laboratories [45]. Liquid chromatographic studies on separation of ten PTH amino acids were carried out using normal phase untreated silica gel plate, C-18 RP precoated plates and RP-HPLC by R. Bhushan et al. [46]. Separation, characterization and biological activity of PTH oxidized at methionine8 and methionine 18* was studied by A.L. Frelinger et al. [47]. PTH was oxidized with hydrogen peroxide and the biological activity of oxidation products was studied by Nabuchi et al. [48]. Methionine oxidation in PTH was also studied by Y. Nabuchi et al. by using RP-HPLC [49].

The PTH (1-34) formulation contains meta-cresol as antimicrobial preservative, which may interfere with OD_{280} UV detector of HPLC for either PTH or m-cresol as well as with colorimetric assays . An RP-HPLC/UPLC method which can specifically measure the protein component of Active Pharmaceutical Ingredient (API) with separation of meta-cresol from protein peaks; will be suitable for quantitation of the active substance in the presence of meta-cresol. The objective was to develop

methods, using RP-HPLC and UPLC techniques to enable quantification of PTH in medicinal formulations containing meta-cresol as well as a method to quantify meta-cresol.

PTH formulation (drug product) contains 250 µg/mL of PTH(API), 3mg/mL meta-cresol; 45.4mg/mL mannitol; 0.1mg/mL sodium acetate and 0.41mg/mL glacial acetic acid in water for injection

Abbreviations used:

PTH – Parathyroid Hormone

PTH IRS – PTH (1-34) Internal Reference Standard

DS (API) – Drug Substance (Active Pharmaceutical Ingredient)

PTH-DS – PTH Drug Substance

DP – Drug Product

PTH-DP – PTH Drug Product

RMP – Reference Medicinal Product (Innovator Product – Forteo). It is drug product

rHu – Recombinant Human

EP – European Pharmacopoeia

3.4.1 EXPERIMENTAL

Materials, reagents and chemicals

HPLC grade acetonitrile and methanol were purchased from Merck, tri-fluoro-acetic acid (TFA) was purchased from Sigma Aldrich. Ultra pure water was obtained using Milli-Q® UF-Plus (Millipore) system; meta-cresol was obtained from J.T. Baxter/ Hedinger. Reference Medicinal Product (herewith termed as RMP) having a concentration of 250 µg/mL and PTH-IRS obtained from Intas Biopharmaceuticals were used for preparation of standards in all experiments. All other chemicals such

as mannitol, sodium acetate and glacial acetic acid were of the highest purity available.

Preparation of mobile phase, dilution buffer and standard

Mobile phase 'A' consisted of 0.1% (v/v) TFA in Milli Q water and mobile phase 'B' consisted of 0.1% (v/v) TFA in acetonitrile. Dilution buffer containing 3 mg/mL meta-cresol; 45.4 mg/mL mannitol; 0.1 mg/mL sodium acetate and 0.41 mg/mL glacial acetic acid in "Milli Q water" was prepared and used so as to have a matrix similar to PTH formulation. Diluted PTH-IRS was prepared by using 400 µg/mL of PTH IRS using mobile phase A. Oxidized form of PTH was prepared by adding 4.0 µL of diluted 0.25 % H_2O_2 to 62.6 µL of PTH IRS (0.4 mg/mL) and mixing well. The solution was incubated for 40 minutes at room temperature and then quenched with 37.4 µL of 50 mg/mL methionine [50]. All dilutions were made using calibrated digital micro-pipettes.

Chromatographic condition

Agilent LC system (1100 and 1200 series) equipped with an injection valve (quaternary), 210 UV detector and Chemstation software was used for HPLC method. A reversed-phase C_{18} column (2.1mm ID × 100mm L, porosity 300°A, particle size 3µm) with guard column (reversed-phase C_{18} column of 2.1mm ID × 12.5mm L, porosity 300°A, particle size 5µm) was used for separation. To get the optimum results, mobile phase with a flow rate of 0.3mL/min was used and column temperature was maintained at 60°C. The gradient programme for mobile phase was optimized using a timed gradient programme T(min)/mobile phase A (%): 0/80, 6/80, 26.1/45, 28/0, 31/0, 31.5/80, and 40/80.

Waters LC system (ACQUITY) equipped with an injection valve (binary), 215UV detector and Empower software was used for RP-UPLC method. Reversed-phase C_8 column (2.1mm ID × 12.5mm L, porosity 300ºA, particle size 5µm) was used for separation. To get the optimum results, mobile phase flow rate was kept constant at 0.4mL/min, column temperature at 60^{o}C. The gradient programme for mobile phase was optimized using a timed gradient programme T(min)/mobile phase A (%): 0/80, 1.2/80, 4.8/0, 5/80, and 6/80.

3.4.2 RESULTS AND DISCUSSION

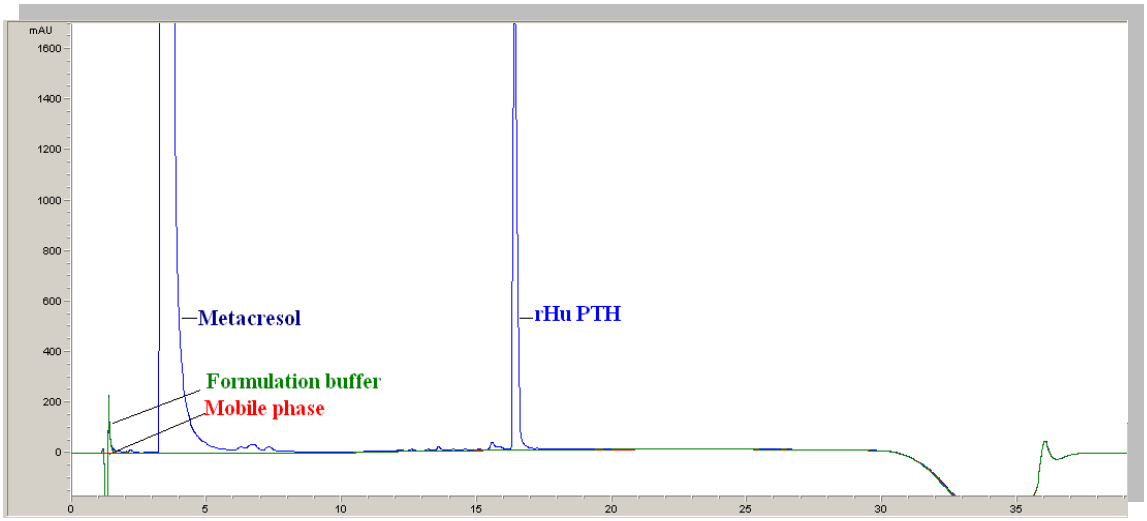
3.4.2.1. RP-HPLC Method

3.4.2.1.1. Method development

Initially, the gradient HPLC conditions were optimized for determination of PTH IRS in presence of meta-cresol. The chromatographic separation was achieved by applying chromatographic conditions described in Section 3.5.1

The applied chromatographic conditions permitted a good separation of meta-cresol and PTH at different concentrations of PTH. No interference of other excipients or oxidized impurities was observed as shown in Figure 3.9 and Figure 3.10.

Figure 3.9 Overlapped HPLC chromatograms of (A) mobile phase (as blank), (B) dilution buffer, (C) PTH-DP



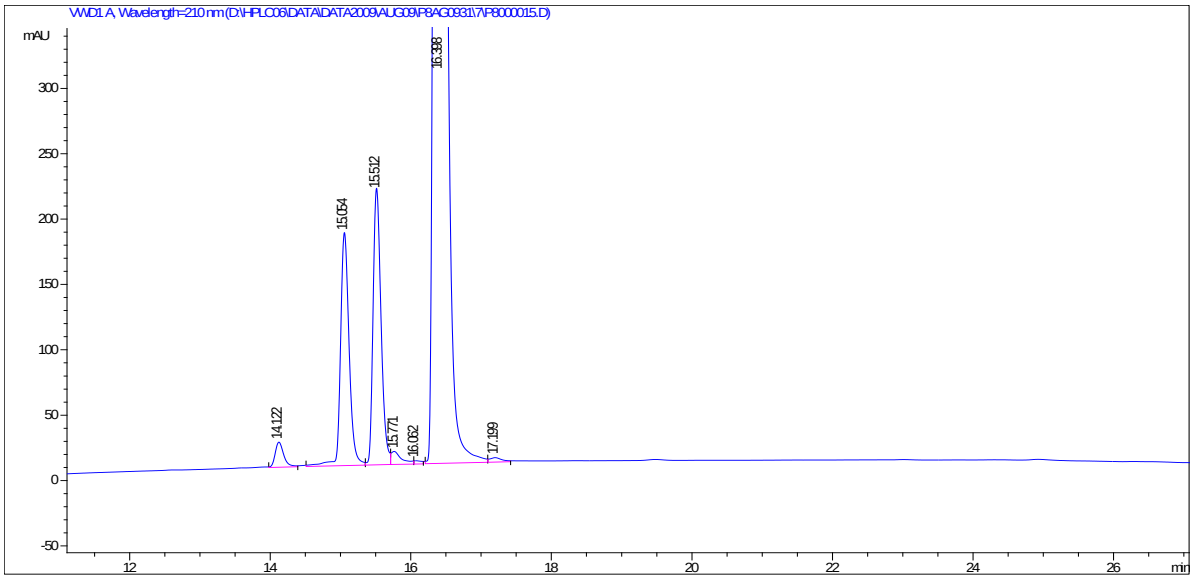
Mobile phase, dilution buffer, PTH-DP injected into HPLC separately

A) Mobile phase – 0.1% trifluoroacetic acid (TFA) in MilliQ water and 0.1% TFA in acetonitrile

B) Dilution buffer (without meta-cresol) – 45.4 mg/mL mannitol; 0.1 mg/mL sodium acetate and 0.41 mg/mL glacial acetic acid in Milli Q water

C) PTH–DP – 0.250 mg/mL of PTH-DS and 3 mg/mL meta-cresol

Figure 3.10 HPLC chromatogram of oxidized form of PTH



4.0 µL of diluted 0.25% H_2O_2, added to PTH-DS (0.4 mg/mL), mixed well, incubated for 40 minutes at room temperature, and then quenched with 37.4µL of 50 mg/mL methionine.

The capacity factor (k') of the first peak (meta-cresol) and second peak (PTH) were 1.60 and 11.5, respectively; while the resolution factor was 6.88. The asymmetry of the peaks for meta-cresol and PTH were found to be 0.26 and 1.06, respectively; while the tailing factor parameters for meta-cresol and PTH were found to be 3.62 and 1.36, respectively. Based on the studied parameters, it was concluded that the developed method is optimum. PTH, oxidized impurities and meta-cresol peaks were well resolved and the tailing factor was within limits.

3.4.2.1.2. Method validation

- **System suitability**

To verify the interference and resolution, PTH-DP, diluted PTH-DP and oxidized PTH-DS were injected into HPLC as mentioned below and the observations are tabulated in Tables 3.13 and 3.14:

Reference solution-1: Innovator product (commercial available in market) Forteo (RMP)(labeled concentration is 0.25 mg/mL) has been used as reference solution 1.

Reference solution-2: Reference solution -1 was diluted to get 0.005 mg/mL of PTH in mobile phase A.

Reference solution-3: PTH-IRS (0.4 mg/mL) was used as the stock solution for preparation of reference solution 3 (Oxidized solution). For preparing 0.25% H_2O_2, the commercially available 50% H_2O_2 was diluted 200 times. 5 µL of H_2O_2 was added to 995µL of MilliQ water and mixed well. 4µL of the diluted H_2O_2 solution was added to 62.6 µL of PTH-IRS (0.4 mg/mL) and mixed well. The solution was incubated for 40 minutes at room temperature and then quenched with 37.4µL of 50 mg/mL methionine. Thus the PTH-IRS gets diluted ~1.6 times and the final concentration of the sample is 0.25 mg/mL

Maximum variation of retention time between principal peak of reference solution 1 and 2 was found to be 0.1 minute (retention time is about 16.3 min.). The % recovery of the reference solution 2 when compared to 2% of the total area obtained with reference solution 1 was found to be 92.3%. The % RSD of the 2% solutions (reference solution 2) (for three replicate measurements) is 1.9%. The resolution between the principal peak and oxidized peak nearest to the principal peak is 3.9. The variation in retention time of principal peak of the standard reference solutions 1 and

2 is 0.1 minute. The % RSD for the total areas of the standard (Reference solution 1),for three measurements is 0.2%. The %RSD for the total areas of the of the standard (Reference solution 2) for two measurements is 1.9%. (Refer table 3.14)

- **Specificity**

To evaluate possible interfering peaks, PTH-IRS (250µg/mL) in mobile phase (as positive control); API; drug product (to verify the separation of interested protein from other components) and oxidized PTH-DS; (to confirm the separation of oxidized forms of protein from the interested protein) were injected into HPLC and no interference was observed as shown in Figure3.10.

Retention time of Forteo (RMP) was found to match exactly with the PTH-IRS and PTH-DP. Four very well resolved oxidized impurities were observed in the range of 11 to 20 minutes in the chromatogram of PTH-DS. There was no peak observed in the chromatogram of blank (mobile phase) and dilution buffer without meta cresol. There were 5 peaks observed in dilution buffer with meta cresol.

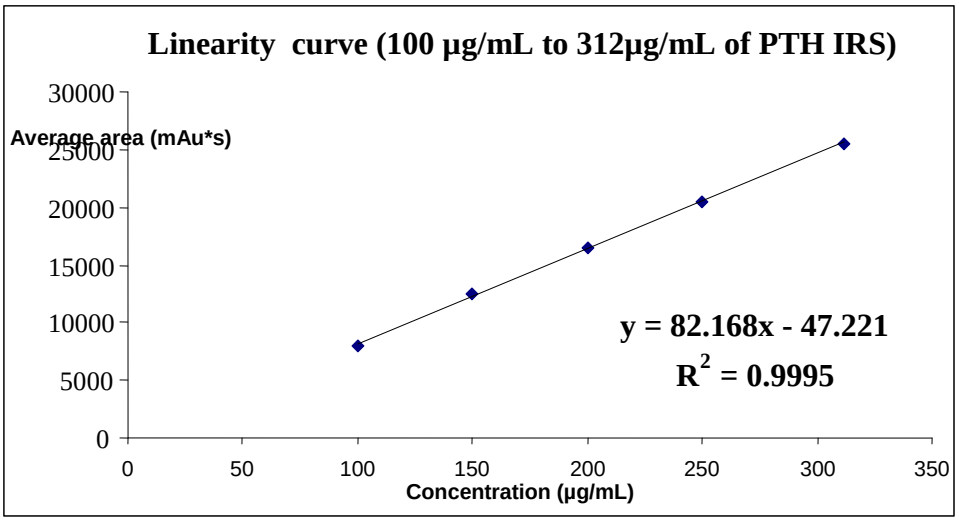
In the oxidised spike sample, peak of PTH was eluting after the main peak of Forteo (RMP), and the resolution obtained was more than 1.0. The %RSD between average purity percentage of 3µg, 4µg and 5 µg of 0.25 mg/mL PTH-IRS diluted in mobile phase and dilution buffer without meta cresol was found to be 0.12 and 0.07% respectively. The %RSD between average purity percentage of 3µg, 4µg and 5 µg of 0.25 mg/mL PTH-DP diluted in mobile phase and dilution buffer with meta-cresol was found to be 0.17 and 0.08% respectively. The %RSD between total areas of individual preparation of each amount of 3µg, 4µg and 5 µg of DS & DP diluted in mobile phase and dilution buffer was found to be not more than 2%. The % RSD between average purity percentage of 3µg, 4µg & 5 µg of PTH-DP diluted in mobile

phase and dilution buffer with metacresol was found to be 0.02%, 0.03% & 0.24% respectively.

- **Linearity and Range**

PTH IRS was used for preparation of different concentrations ranging from 100 to 312µg/mL. Linearity curve was plotted for peak area responses versus concentration of PTH and is shown in Figure3.11.

Figure 3.11 Linearity curve (HPLC) for PTH



PTH-IRS (400 µg/mL) was diluted with mobile phase A for preparation of different concentrations ranging from 100 to 312 µg/mL injected separately.

The correlation coefficient, slope, Y-intercept, regression equation of the calibration curve was determined and the results are shown in Table B. The percent RSD was found to be less than 2.0% while the percent recovery was found to be in the range of 97% to 103%.

Table 3.13 Area / % Recovery and % RSD

Injected Amount (µg)	Conc. mg/mL	Total Area 1 (Sol^n1)	Total Area 2 (Sol^n2)	Total Area 3 (Sol^n3)	Avg. Total area	SD	% RSD	Back calculated conc. (mg/mL)	% recovery.
0.04	0.002	84.5	87.5	72.4	81.4	7.9	9.8	0.04	104.6
0.05	0.0025	117	118	120	118.3	1.5	1.3	0.05	102.6
0.08	0.004	157	195	197	196	1.4	0.7	0.07	89.0
0.1	0.005	315	322	318	318	3.5	1.1	0.10	102.6
0.5	0.025	1936	1959	1964	1953	14.9	0.8	0.50	99.6
1	0.05	3799	4118	4065	3994	170.9	4.3	0.99	99.4
2	0.1	7931	7901	8074	7968	92.4	1.2	1.96	98.0
3	0.15	12318	12763	12389	12490	239.0	1.9	3.06	101.9
4	0.2	16374	16428	16638	16480	139.4	0.8	4.03	100.7
5	0.25	20458	20470	20447	20458	11.5	0.1	4.99	99.9
6.25	0.25	25570	25491	25501	25520	43.0	0.2	6.22	99.6

- **Accuracy**

Accuracy was studied by spiking PTH in the range of 200, 250 and 312 µg/mL in the mobile phase and calculating the percent recovery. The percent recovery as seen from Table 3.14 was found to be in the range of 95% to 105%. The percent RSD was found to be less than 2.0%.

Table 3.14 Area / % Recovery and % RSD

Conc. of PTH	Amount of protein injected (µg)	Area of main peak (mAu)			Average	% RSD	Back calculated amount (µg)	% Recovery from the graph
		Inj.1	Inj.2	Inj.3				
0.002 (0.8%)	0.04	84	76	76	79	5.75	0.05	116.5
0.0025 (1.0%)	0.05	92	93	91	92	0.66	0.05	99.3
0.004 (1.6%)	0.08	169	160	172	167	3.80	0.07	83.5
0.2 (80%)	4	15377	15384	15404	15388	0.09	4.14	103.4
0.25 (100%)	5	18845	18883	18880	18869	0.11	5.12	102.4
0.25 (125%)	6.25	22727	22783	22825	22778	0.22	6.23	99.7

- **Precision**

Precision was evaluated for intra-day (Repeatability) and inter-day (Intermediate precision) variation, and for different columns. Intra-day study was determined by using six independent preparations of the PTH-DS (250 µg/mL) and PTH-DP (250 µg/mL) as shown in Table 3.15. The percent RSD of main peak area was found to be less than 0.5%. Inter-day precision was determined by performing five different conditions along with five replicates for each condition which is equivalent to n = 25 (5 × 5) as shown in Table 3.17. The percent RSD of the main peak area was found to be less than 0.5% within each set and less than 2.0% between different sets. The percent recovery was found to be between 95.0% - 105.0 % and the maximum variation between sets was found to be less than 5.0%.

Table 3.15 Total area of PTH-DS and PTH-DP

Sample Details	Total area of PTH-IRS (DS) and PTH-DP (mAu*s)						Average	% RSD
	Sol^n.1	Sol^n.2	Sol^n.3	Sol^n.4	Sol^n.5	Sol^n.6		
0.25 mg/mL DS	18845	18845	18845	18845	18845	18845	18842	0.2
0.25 mg/mL DP	18364	18364	18364	18364	18364	18364	18368	0.2

Table 3.16 Percent purity of PTH–DS and PTH-DP.

Sample Details	Purity percentage of PTH-DS) and PTH-DP						Average Percent purity	% RSD
	Soln.1	Soln.2	Soln.3	Soln.4	Soln.5	Soln.6		
0.25 mg/mL DS	98.35	98.35	98.35	98.35	98.35	98.35	98.0	0.03
0.25 mg/mL DP	94.16	94.16	94.16	94.16	94.16	94.16	94.0	0.1

Table 3. 17 Experimental matrix of intermediate precision

	Set 1	**Set 2**	**Set 3**	**Set 4**	**Set 5**	**Set 6**
Equip. ID	System-1	System-1	System-1	System-2	System-1	System-2
Column	Column-1	Column-1	Column-1	Column-1	Column-1	Column-2
Day	Day-1	Day-1	Day-2	Day-2	Day-2	Day-2

- **Robustness**

The robustness is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its robustness during normal usage. Robustness was tested using three variables: flow rate, column temperature, and mobile phase composition.

Flow rate

Experiments were conducted using system suitability samples of concentrations 0.005 and 0.25 mg/mL prepared from Forteo (RMP), with flow rate variation of ±10% from the set flow rate (0.3 mL/min). The percent RSD was found to be less than 2%, with no variation and +0.1 minute difference in retention time but during lower flow rate, higher percentage recovery (~ 111%) was obtained as compared to higher flow rate, we found lower percentage of recovery (~ 93%). Based on recovery, it was concluded that flow rate is critical parameter.

Column temperature effect

Experiments were conducted using the same system suitability samples (as used in flow rate studies) with column temperature variation of +5ºC from the set temperature (60ºC). The percent RSD was found to be less than 2%, with no variation and +0.1 minute difference in retention time. The percent recovery was found to be within acceptable limits (95%-105%) and hence column temperature was not considered to be critical parameter.

Mobile phase composition

Experiments were conducted using system suitability samples(same as used in flow rate) with mobile phase composition variation of ± 20% from the set percentage of TFA (0.1%). Results for triplicate injections (% variation) between unaltered / initial condition and altered condition for PTH sample were found to vary less than 2.0% without variation in retention time and hence mobile phase composition was not considered to be critical parameter.

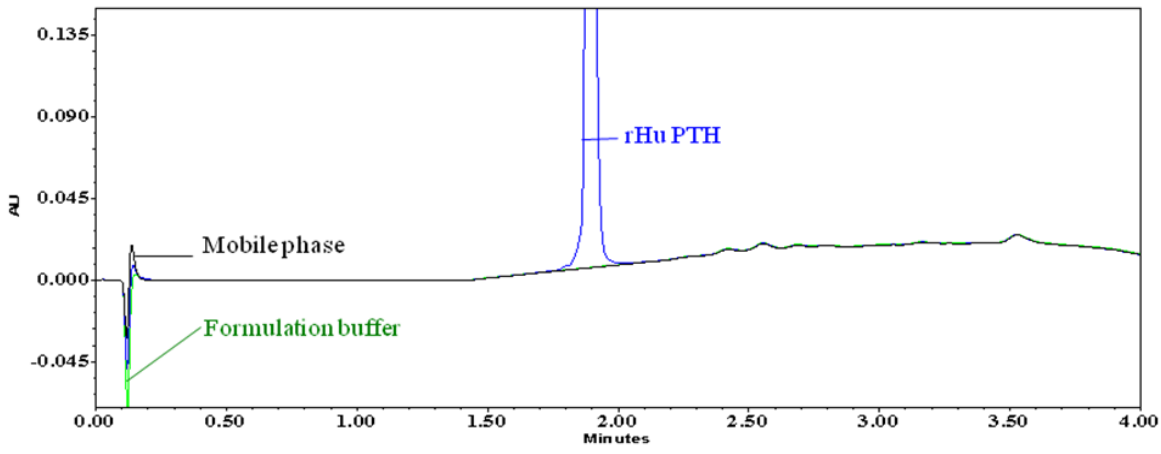
3.4.2.2. UPLC method

3.4.2.2.1. Method development

The basic chromatographic conditions like stationary phase, solvents and UV detector, employed in HPLC were taken into account while developing new UPLC method. The stationary phase C_8 was chosen in order to have similar polarity to that used in the method developed for HPLC. The injection volume was scaled down by about 5 fold as used in HPLC. To get the optimum results, mobile phase flow rate was kept constant at 0.4 mL/min and column temperature was maintained at 60^{o}C. The chromatographic separation was achieved as described in Section 3.5.1

The applied chromatographic conditions permitted a good separation of meta-cresol and PTH at different concentrations of PTH. No interference of other excipients or other oxidized impurities was observed during the analysis and are shown in Figure 3.12-3.14.

Figure 3.12 Overlapped UPLC chromatograms of (A) mobile phase, (B) dilution buffer and (C) PTH-DS (without meta-cresol)



Mobile phase, dilution buffer and PTH into HPLC separately

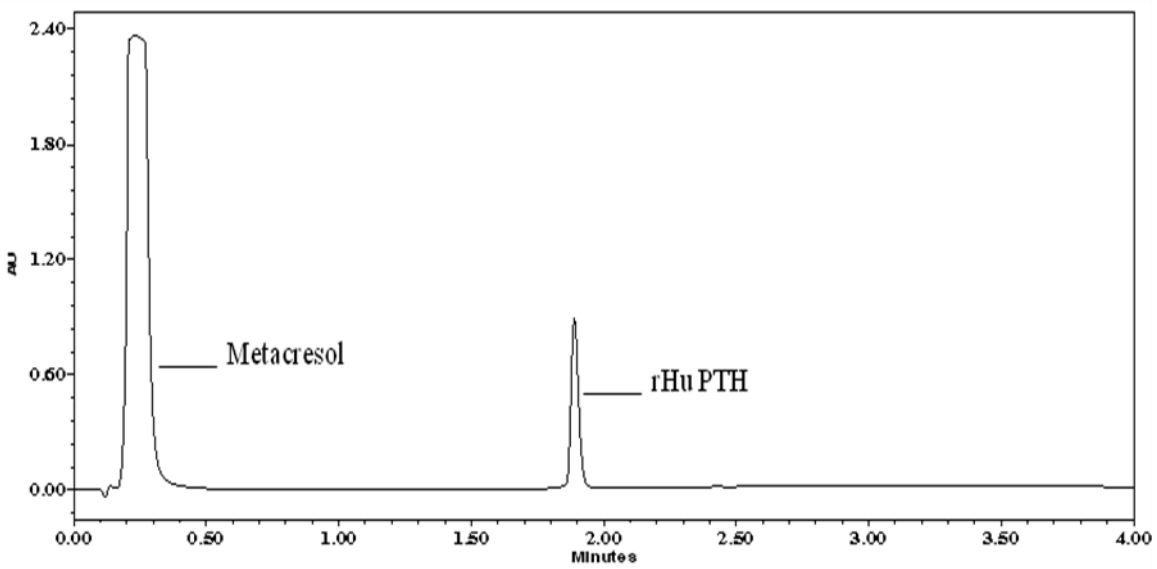
A) Mobile phase – containing 0.1% trifluoroacetic acid (TFA) in MilliQ water

and 0.1% TFA in acetonitrile

B) Dilution buffer (without meta-cresol) – containing 45.4 mg/mL mannitol; 0.1 mg/mL sodium acetate and 0.41 mg/mL glacial acetic acid in Milli Q water

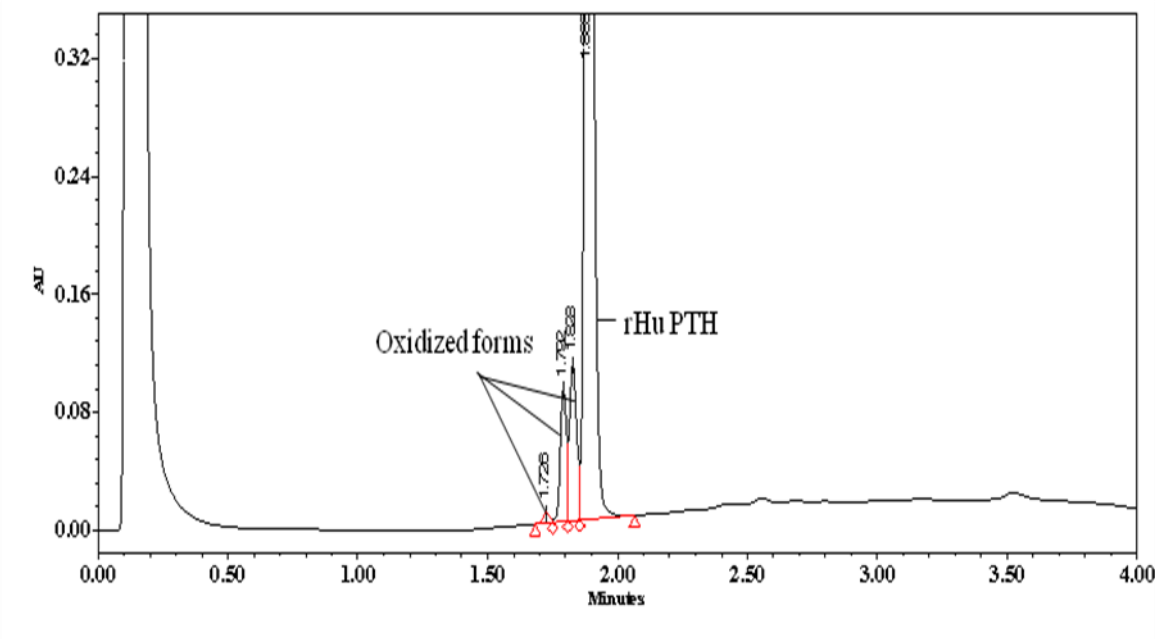
C) PTH–DS – containing 0.250 mg/mL of PTH in Milli Q water

Figure 3.13 UPLC chromatograms of (A) meta-cresol and (B) PTH-DP (with meta-cresol)



PTH–DP – containing 0.250 mg/mL of PTH and 3 mg/mL meta-cresol

Figure 3.14 UPLC chromatogram of oxidized form of PTH



4.0 µL of diluted 0.25% H_2O_2, added to PTH-IRS (0.4 mg/mL), mixed well, incubated for 40 minutes at room temperature, and then quenched with 37.4µL of 50 mg/mL methionine

The capacity factor (k') of the main peak (PTH) was 11.60; while tailing factor was found to be 1.24. It can be thus concluded that PTH, oxidized forms of PTH and meta-cresol peaks were well resolved in the developed method and the tailing factor was within limits.

3.4.2.2.2. Method Validation

- **System suitability**

Two types of system suitability were evaluated by analyzing PTH-IRS for the respective parameters through-out the validation study.

System suitability A – The RSD between the areas of the first three injections should not be more than 1 % while RSD between the areas of peak in five injections should not be more than 1 %.

System suitability B – The RSD between the areas of the first three injections should not be more than 1 %. The back calculated concentration based on average area of first three injections as well as last two injections of the standard sample should not show variation more than 5% of the pre-determined concentration (150µg /mL) as in the calibration curve.

System suitability A was evaluated with linearity and range and B was evaluated with other validation parameters.

- **Specificity**

To evaluate possible interfering peaks, diluted PTH-IRS (150µg/mL) in mobile phase (as positive control); API; PTH-DP (to verify the separation of interested protein from other components) and oxidized API; oxidized PTH-DP (to confirm the separation of oxidized forms of protein from the interested protein) were injected into UPLC and no interference was observed as seen from Figure 3.14. From Table 3.18 it is evident that the percent variation in peak areas of PTH-DS and oxidized PTH-DS was found to be less than 2%.

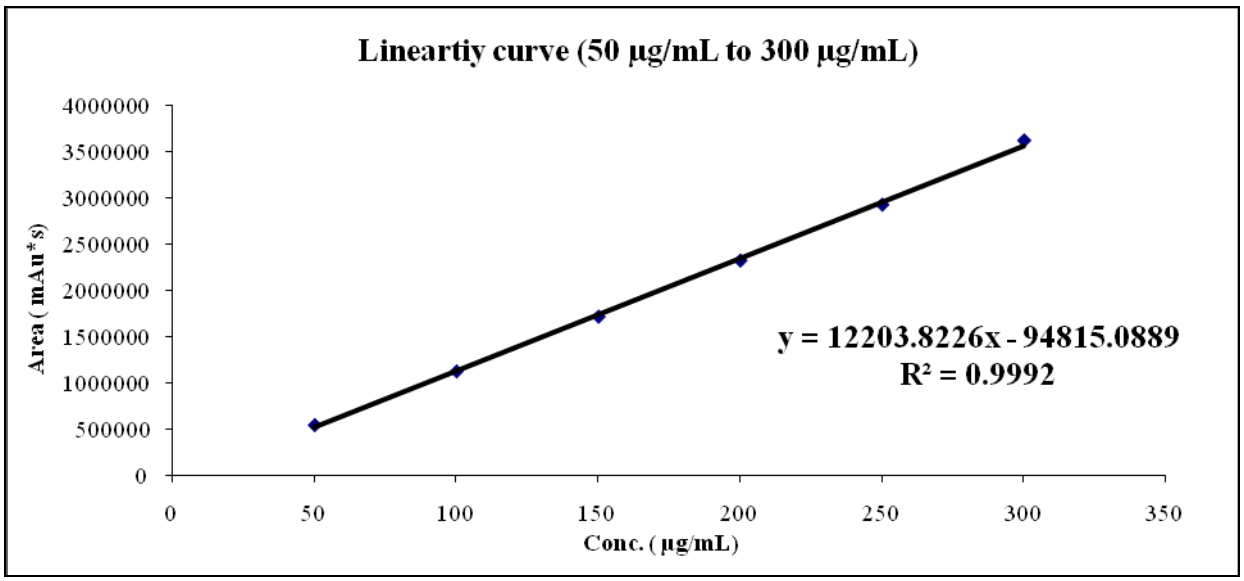
Table 3.18 – Area / % variation

Sample Name	Average total area of main peak (mAu*S)	Conc. of PTH (µg / mL)	% Variation
PTH-DS	1681222	145.5	1.5
Oxidized PTH-DS	1654234	143.3	
PTH-DP	1686672	146.0	1.6
Oxidized PTH-DP	1659430	143.7	

- **Linearity and Range**

PTH RS and samples were chromatographed using the set chromatographic conditions. Linearity curve was plotted using 50 to 300 µg/mL of PTH and is shown in Figure 3.15.

Figure 3.15: Linearity curve (UPLC) for PTH:



PTH-IRS (400 µg/mL) was diluted with mobile phase 'A' for preparation of different concentrations ranging from 50 to 300 µg/mL, injected separately.

The linearity of peak area responses versus concentration for PTH was studied and correlation coefficient, slopes and Y-intercepts and regression equation were

determined and the results are shown in Table B. The correlation coefficient was found to be 0.999. It is also observed from Table 3.19 that the percent RSD was found to be less than 2.0% while the percent recovery was found to be in the range of 98% to 105%.

Table 3.19 % RSD and % Recovery for the area of principal peak of PTH-IRS.

Conc. of PTH (µg/mL)	Area of main peak of PTH (mAu*s)			Average area (mAu*s)	% RSD	Back calculated conc. (µg/mL)	% recovery
	Prep:1	Prep:2	Prep:3				
50	--	548622	540801	544712	1.02	52.4	104.8
100	1115814	1135939	1123823	1125192	0.90	100.0	100.0
150	1719512	1699157	1726950	1715206	0.84	148.3	98.9
200	2317483	2321624	2322369	2320492	0.11	197.9	99.0
250	2924020	2947362	2899222	2923535	0.82	247.3	98.9
300	3611137	3671138	3565685	3615987	1.46	304.1	101.4

- **Accuracy**

Accuracy was studied using six different solutions, containing 50, 100, 150, 200, 250 and 300 µg/mL of PTH. Three replicates of each solution were spiked in the mobile phase. (n= 6 × 3 = 18) and the results are tabulated in Table 3.19. The percent recovery was found to be in the range of 95% to 105%. The percent RSD was found to be less than 2.0%. .

- **Precision**

Intra-day (Repeatability) and inter-day (Intermediate) precision were evaluated using different equipment and different lots of UPLC columns of same makes. Intra-day precision studies were performed by injecting six independent preparation of the PTH-IRS (150 µg/mL). The percent RSD of main peak area was found to be less than 0.5 % as seen from Table 3.20. Inter-day precision study was done under five different conditions along with six replicates for each condition which is equivalent

to n = 30 (5 × 6) as shown in Table 3.21 and the results are tabulated in Table 3.22. The percent RSD of the main peak area was found to be less than 0.5% within each set and less than 3.0% between different sets. The percent recovery was found to be between 95.0% - 105.0% and the maximum variation between sets was found to be less than 5.0%.

Table 3.20 Peak area (diluted PTH-IRS-0.15 mg/mL) and retention time for the principal peak of InRS.

Sample preparation	Area of main peak of PTH-IRS (mAu*s)	
	Set-1	Set-2
Prep-1	1679634	1.889
Prep-2	1671084	1.888
Prep-3	1663891	1.888
Prep-4	1675372	1.891
Prep-5	1667722	1.888
Prep-6	1668911	1.888
Avg. value	1671102	1.889
RSD	0.34	0.06

Table 3.21 Experimental matrix of intermediate precision

	Set 1	**Set 2**	**Set 3**	**Set 4**	**Set 5**
Equip. ID	System-1	System-1	System-2	System-2	System-1
Column	Column-1	Column-1	Column-1	Column-1	Column-2
Day	Day-1	Day-2	Day-3	Day-4	Day-4

Table 3.22 Intermediate precision study data

Sample preparation	Area of main peak of PTH-IRS (mAu*s)				
	Set-1	Set-2	Set-3	Set-4	Set-5
Prep-1	1679634	1677355	1392999	1635180	1689759
Prep-2	1671084	1667891	1398166	1643618	1677671
Prep-3	1663891	1676642	1400129	1650774	1688277
Prep-4	1675372	1674680	1395728	1658175	1684267
Prep-5	1667722	1682425	1396970	1641093	1675686
Prep-6	1668911	1677319	1391947	1644555	1692449
Avg. value	1671102	1676052	1395990	1645566	1684685
RSD (%)	0.34	0.28	0.22	0.49	0.40

- **Robustness**

The robustness is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its robustness during normal usage. Robustness was tested using three variables, age effect of mobile phase and test samples, column temperature and mobile phase composition.

Age effect of mobile phase and test samples held for seven days

Freshly prepared samples for system suitability (150 µg/mL of PTH) and those prepared seven days ago were analyzed using both freshly prepared and seven days, old mobile phase. There was not much variation in the results, with percent variation from initial day to 7 days being about 5% and percent RSD being less than 1.0%.

There was no difference in the retention time and percent recovery was found to be in between 95% and 105%, indicating that age of the mobile phase was not a critical parameter.

Column temperature effect

Experiments were conducted using system suitability samples with column temperature variation of + 5ºC from the set temperature (60ºC). The percent RSD was found to be less than 2%, with variation of + 0.1 minute in the retention time. The percent recovery was found to be within acceptable limits (95%–105%) suggesting that the variation in the results was within acceptable limits for all the parameters under study and indicating that column temperature was not a critical parameter.

Mobile phase composition

Experiments were conducted using system suitability samples with mobile phase composition variation of $\pm$ 20% from the set percentage of TFA (0.1%). Results for triplicate injections (% variation) between initial condition and altered condition for PTH sample were found to vary less than 2.0% and there was no variation observed in the retention time, suggesting that the mobile phase composition was not a critical parameter.

Table 3.23 % RSD for main peak area and back calculated concentration of PTH-IRS for all sets

Injection details	Main peak area of PTH-IRS (mAu*s)						
	Initial sample	Stored sample & mobile phase	Stored sample & fresh mobile phase	55ºC Temp	65ºC Temp	0.08% TFA	0.12% TFA
Inj.1	1667980	1689027	1681233	1736020	1683363	1697866	1670305
Inj.2	1668104	1682446	1679217	1733345	1677123	1689116	1669403
Inj.3	1672415	1680431	1682088	1733179	1670967	1680852	1674829
Inj.4	1661187	1681334	1678649	1732007	1664147	1677127	1682958
Inj.5	1636550	1679619	1678866	1735579	1780984	1675052	1685501
Avg. value for first 3 inj.	1669500	1683968	1680846	1734181	1677151	1689278	1671512
% RSD for first 3 inj.	0.15	0.27	0.09	0.09	0.37	0.50	0.17
Back calculated Conc. of 5 Injections in µg/mL	143.9	145.6	145.4	149.9	146.7	145.8	145.2
Variation from predetermined conc. (150 µg /mL)	4.1	2.9	3.1	0.1	2.2	2.8	3.2

3.4.3 COMPARATIVE STUDY OF HPLC AND UPLC PERFORMANCE

The performance parameters of both systems are shown in Table B. The runtime of UPLC was reduced by 7-fold to that of HPLC. The retention behaviors of meta-cresol, PTH, and oxidized impurities were similar in HPLC and UPLC columns. As expected, the UPLC method showed higher efficiency of analysis than HPLC method. Both RP-HPLC and RP-UPLC methods were demonstrated to be validated for

quantifying PTH respectively in presence of other excipients and oxidized impurities of PTH. The HPLC and UPLC methods were validated showing satisfactory data for all the parameters tested. The UPLC method was found to be capable of giving faster analysis with good resolution, accuracy and precision than that achieved with conventional HPLC method. Both the chromatographic methods described here were found to be reliable for quantifying PTH. Since these methods are rapid and simple, they may be successfully applied to quality control analyses of finished (formulated) product in presence of meta-cresol.

Table B Results of regression equation/correlation coefficient and both methods comparative data

Statistical parameter	RP-HPLC	UPLC
Linearity and range Concentration (µg/mL)	100 – 300	50 – 300
Regression Equation	y = 82.168x – 47.221	y = 12203.8226x – 94815.0889
Correlation coefficient (R^2)	0.999	0.999
Total Analysis time (min)	40	6
Retention time (min) For meta-cresol For PTH	 3.5 16.4	 0.3 1.9
Flow rate (mL/min)	0.3	0.4
Column	C18 (2.1 x 100 mm) 3µ, 300^{o}A	C8 (2.1 x 12.5 mm) 5µ, 300^{o}A
Column condition	C_{18}, 210 UV	C_8, 215 UV
Method	Gradient	Gradient
Sample size	20 µL	4 µL
Specificity	No interference	No interference
Accuracy	Recovery between 95% - 105%	Recovery between 95% - 105%
Precision	RSD <3.0%	RSD <1.0%
Robustness	Yes	Yes

3.5 QUANTIFICATION OF META-CRESOL AND METHOD VALIDATION

Introduction

Meta-cresol is widely used as bactericide in the biotechnological processing of pharmaceuticals; preservative in pharmaceutical formulations (injection solutions of insulin, somatropin, parathyroid hormone); pesticide for the treatment of the stems of fruit trees and plants. Exposure of humans is possible through the use of m-cresol as a preservative in pharmaceutical injection solutions.

Meta-cresol, para-cresol and m/p-cresol mixtures are absorbed across the respiratory and gastrointestinal tracts and through the skin, and are distributed throughout the body. The primary metabolic pathway for all cresol isomers is conjugation with glucuronic acid and inorganic sulfates. All isomers are mainly eliminated by renal excretion in form of above-mentioned conjugates. The oral LD50 of undiluted m-cresol in rats was 242 mg/kg bw. Clinical signs include hypoactivity, salivation, tremors, and convulsions. Neither mortality nor clinical signs of toxicity were seen following exposure to saturated vapour concentration of either m-cresol or p-cresol. Inhalation of aerosols may however cause death, and mean lethal concentrations in rats were reported to be 29 mg/m³ for p-cresol and 58 mg/m³ for m-cresol [51]. Reaction to meta-cresol in commercial preparations of insulin to humans was reported by Dennis et al.[52].

The analysis of cresol- like chemicals in use for a long period of time has evolved from a number of nonspecific colorimetric methods to more selective separation techniques using gas chromatography (GC) or high performance liquid chromatography (HPLC) [53-55].

The objective was hence to develop a rapid and simple RP-HPLC method with UV detection, useful for routine quality control of m-cresol in parathyroid hormone formulations (PTH).

To obtain the best chromatographic conditions, the mobile phase composition, column temperature and flow rate were optimised. The flow rate was varied from 0.8 mL min^{-1} to 1.2 mL min^{-1}. The column temperature was varied between 22°C to 30°C and the analysis at 30°C was preferred on the basis of improved peak symmetry and resolution. The % of mobile phase was varied ± 2% from the set parameters i.e. 60%±2% (58% to 62%). Isocratic chromatographic conditions were optimized for determination of meta-cresol in PTH pharmaceutical product. The applied chromatographic conditions permitted a good separation of meta-cresol and PTH at different concentrations of meta-cresol. No interference of other excipients was observed as shown in Figures 3.16-3.17.

Figure 3.16: Sample Chromatogram of principal peak of dilution buffer, meta-cresol standard and mobile phase:

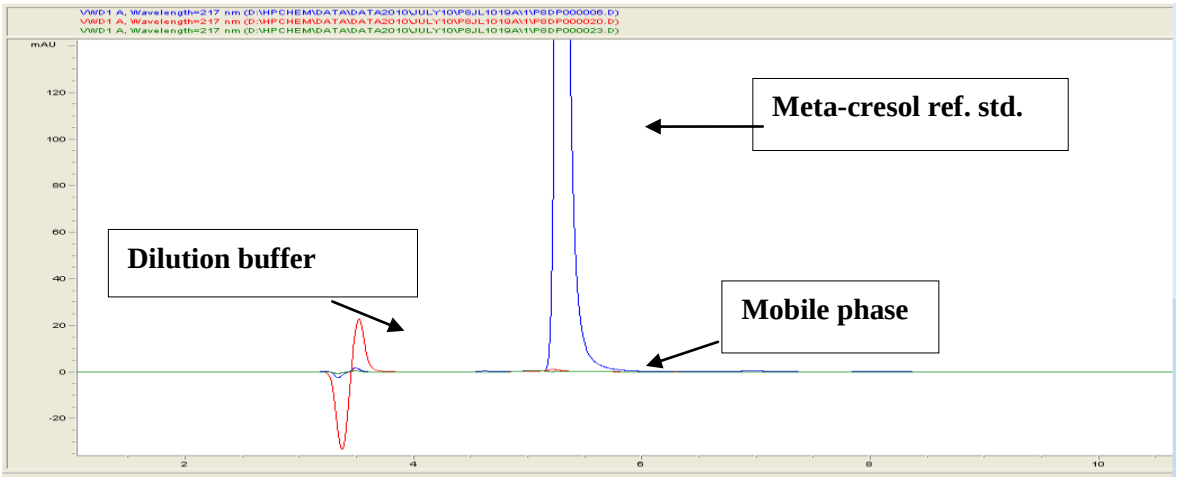
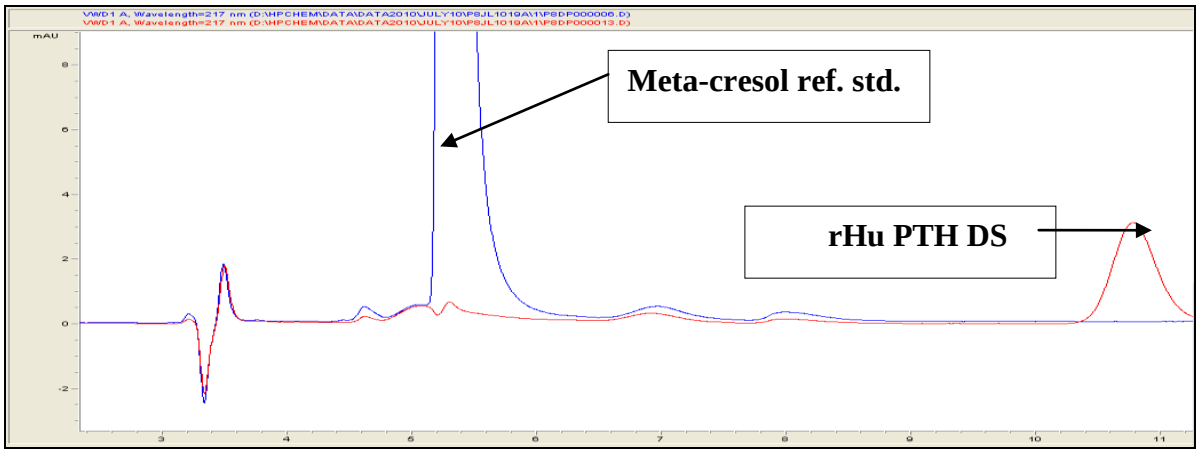


Figure 3.17: Sample Chromatogram (overlap) of principal peak of PTH API & meta-cresol standard:



3.5.1 EXPERIMENTAL

Material, Reagent & Chemicals:

HPLC grade acetonitrile and methanol were purchased from Merck; tri-fluoro-acetic acid was purchased from Sigma Aldrich. Ultra pure water was obtained using Milli-Q® UF-Plus (Millipore) system; meta-cresol was obtained from J.T. Baxter/ Hedinger and was used for preparation of different dilutions; PTH-DS having concentration of 400 µg/mL was used for diluted sample of PTH; PTH-DP was used as test sample. All chemicals i.e. mannitol, sodium acetate and glacial acetic were of the highest purity available.

Preparation of standard, mobile phase and dilution buffer:

Dilution buffer: Buffer containing 3mg/mL meta-cresol; 45.4mg/mL mannitol; 0.1mg/mL sodium acetate and 0.41mg/mL glacial acetic acid in Milli Q water was prepared. It is similar as excipients used for PTH formulation.

Diluted PTH standard: PTH (400 µg/mL) was used for preparation of different diluted samples.

Meta-cresol standard: 3 mg/mL was used for preparation of different dilutions.

Mobile phase consisted of 0.1% v/v TFA in 60% methanol.

All dilutions were made using calibrated digital micro-pipettes.

Chromatographic condition:

LC system equipped with an injection valve (quaternary), 217 UV detector and Chemstation software was used. A reversed-phase Jupiter C_{18} column (4.6mm ID × 250mm L, porosity 300ºA, particle size 5µm) with guard column (reversed-phase C_{18} column of 4.6mm ID × 12.5mm L, porosity 300ºA, particle size 5µm) was used for separation. To get the optimum results, mobile phase with a flow rate of 1.0mL/min was used and column temperature was maintained at 30^{o}C. The isocratic programme for mobile phase was optimized for 12 minutes.

3.5.2 RESULTS AND DISCUSSION

3.5.2.1 Method development:

The capacity factor (k') of the first peak (meta-cresol) and second peak (PTH) were 3.24 and 5.24, respectively; while the resolution factor was 6.88. The asymmetry of the peak for meta-cresol and PTH were found to be 1.29 and 5.29, respectively; while the tailing factor parameter for meta-cresol and PTH was found to be 1.29 and 1.14, respectively. For replicate injections of meta-cresol standard; the % RSD of the main peak area was found to be below 0.7%, and there was insignificant variation in the retention time (less than 0.1 min).

The PTH, and meta-cresol peaks were thus found to be well resolved and the tailing factor was within limits. As available PTH formulations in the market contain 100µg/mL of meta cresol the concentration range of m-cresol was selected from

75µg/mL to 120 µg/mL. Detection limit (LOD) and quantification limit (LOQ) were not done for the study.

- Usage of different column brands: –

Regarding the chromatographic procedure, different brands of reverse phase C_{18} columns were used (Jupiter column and Grace Vydac column) and compared in terms of percentage variation of principal peak area of meta-cresol standard. Experiments were conducted using system suitability samples. Percentage variation between principal peak of meta-cresol was not more than 5% in all samples when compare to the area of principal peak of meta-cresol from specificity samples. Retention time of principal peak of meta-cresol was found to be around 5.3 minutes and 3.9 minutes whereas principal peak of PTH was found to be around 10.9 minutes and 4.7 minutes on Jupiter and Grace Vydac column respectively. Principal peak in both samples was separated by base to base while overlapping their chromatograms. Integrable peak was found at the retention time of the principal peak of meta-cresol in the API dilution buffer without meta-cresol sample & mobile phase without affecting final results. Variation in retention time was observed in Jupiter & grace vydac column. Therefore it was decided to use Jupiter column for method validation.

3.6.2.2 Method validation

- **Specificity:**

Specificity of the method was validated in terms of interference of excipients including PTH. The excipients in the formulation include PTH, and others like mannitol, sodium acetate and glacial acetic acid). If peaks due to PTH, mannitol , sodium acetate, glacial acetic acid and other buffer excipients would be observed they could be considered as interfering peaks. To verify any interference, PTH, FB,

mobile phase and meta cresol standard were injected onto HPLC separately. Triplicate injections of three different concentrations of meta-cresol (75, 100 & 120 µg/mL) prepared in mobile phase and dilution buffer were tested for interference. No interference was observed as shown in Figure 3.16-3.17.

- **Linearity & Range:**

Meta-cresol standard (3mg/mL) was used for preparation of different concentrations ranging from 75 to 120µg/mL, by considering 100µg/mL as 100%. Five different concentrations were considered with three replicates of each concentration (n=15) Linearity curve was plotted for peak area responses versus concentration of meta-cresol as shown in Figure 3.18 and results are tabulated in Table C.

Figure 3.18: Linearity:

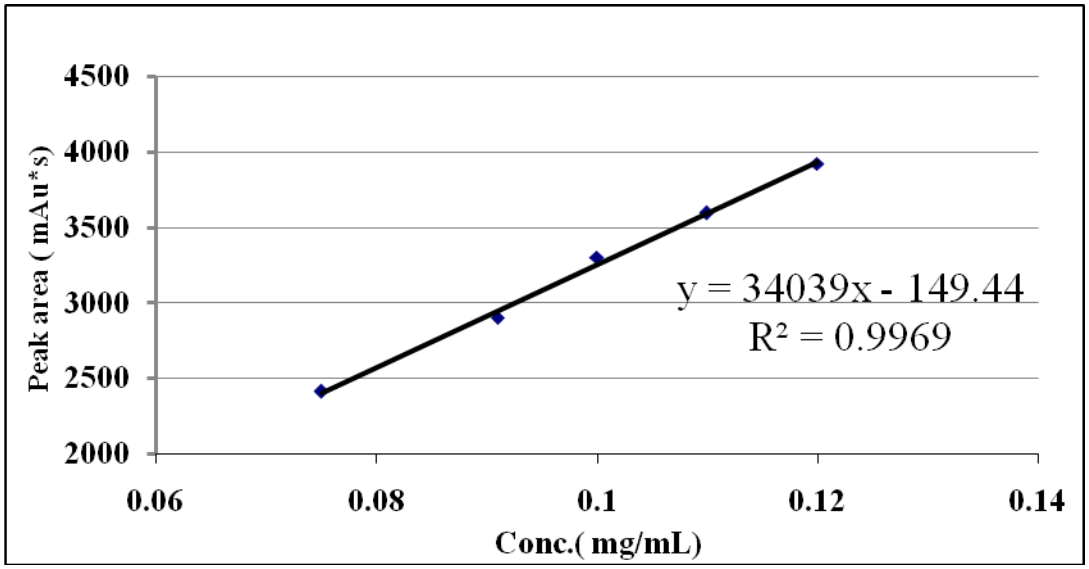


Table 3.24 Linearity and range:

Sample		Principal peak area			Average Total area	% RSD	Back calculated conc. (mg/mL)	% recovery
	Conc. mg/mL	Prep.1	Prep.2	Prep.3				
Standard-1	0.075	2460	2366	2410	2412	1.9	0.04	100.33
Standard-2	0.091	2910	2899	2899	2903	0.2	0.05	98.53
Standard-3	0.100	3230	3354	3315	3300	1.9	0.07	101.33
Standard-4	0.110	3534	3657	3596	2596	1.7	0.10	100.02
Standard-5	0.120	3841	3972	3963	2925	1.9	0.50	99.75

The correlation coefficient, slopes, Y-intercepts, regression equation of the calibration curve were determined and shown in figure 3.18 Table C. The percent RSD was found to be less than 2.0% while the percent recovery was found to be more than 98%

- **Accuracy:**

Accuracy was studied using two different sets of three different solutions, containing 90, 100 & 120 µg/mL of meta-cresol. Each solution was spiked in the mobile phase and injected onto HPLC (n=9); and the results are shown in Table 3.25. The percent recovery was found to be in between 98% and 102%. The percent RSD was found to be less than 1.0%.

Table 3.25 Accuracy studies-m-cresol:

Sample name	Conc. mg/mL	Area of principal peak area (mAu*s)				% RSD	Back calculated conc. (mg/mL)	% recovery
		Prep.1	Prep.2	Prep.3	Avg.			
Std.-1	0.091	3032	3028	3025	3028	0.1	0.093	102
Std.-2	0.100	3183	3182	3179	3181	0.1	0.098	98
Std.-3	0.120	4030	4031	4027	4029	0.1	0.121	101

- **Precision:**

Precision was evaluated based on Intra-day (Repeatability) and inter-day (Intermediate precision) variation and on different columns. The repeatability was assessed with six independent sample of 100µg/mL of meta-cresol. Single injection from each preparation was injected and results are shown in Table 3.26. The percent RSD of main peak area was found to be less than 2.0 %.

Table 3.26 Intraday precision m-cresol:

Inj. No.	Area of principal peak (mAu*s)	Avg. area of principal peak (mAu*S)	%RSD of principal peak	Rt of principal peak (min.)
1	3154	3262	1.8	5.3
2	3241			5.3
3	3302			5.3
4	3257			5.3
5	3308			5.3
6	3310			5.3

Using the experimental design and matrix shown in Table 3.27; intermediate precision was evaluated on different days with different equipments and with different columns. Three replicate injections of system suitability standards (100µg/mL of meta-cresol) prepared independently were considered for the study. Intra-day precision was determined for 100µg/mL of meta-cresol by performing five different conditions as mentioned in Table 27 (n = 15) and relative standard deviations (RSD) were calculated.

Table 3.27 Experimental design for intermediate precision:

	Set 1	**Set 2**	**Set 3**	**Set 4**	**Set 5**
Equipment used	System-1	System-2	System-2	System-1	System-1
Day	Day-1	Day-2	Day-2	Day-3	Day-3
Column	Column-1	Column-2	Column-1	Column-1	Column-1

The percent RSD for the main peak area of meta-cresol standard within each set and between different sets was found to be less than 2.0%. The percent recovery of meta-

cresol standard was found to be between 95% - 105% within each set and the maximum variation between sets was found to be 3.0%.

- **For different brand of column with same column chemistry:**

Table 3.28 % variation of principal peak area of m-cresol ref. std. compares with Jupiter column & Grace Vydac column:

Sample details	Area of principal peak of m-cresol (mAu*s)		% variation of principal peak area
	In Jupiter column	In Grace vydac column	
System Suitability (0.1mg/mL)	3207	3160	1.5
M-cresol ref. Std. (0.1 mg/mL)	3188	3154	1.1
Dilution buffer of rHu PTH Drug Product (0.1 mg/mL)	3154	3290	4.1
rHu PTH (1-34) Drug Product (0.1 mg/mL)	3249	3322	2.2

Table 3.29: Summary of results between different sets

Set No.	Average principal area (of four replicates – System suitability samples) (mAu*s)	% RSD	Retention time	Average principal area (of three replicates– 0.1mg/mL standard) (mAu*s)	%RSD
Set-1	3336	0.1	5.3	3306	0.9
Set-2	3345	0.2	5.3	3313	0.4
Set-3	3204	0.04	5.3	3207	0.3
Set-4	3206	0.2	5.3	3264	0.1
Set-5	3202	0.04	5.3	3289	0.4

Interpretation:

- **For Set-1 & Set-2:(Inter day, instrument)**

Variation between avg. area obtained for principal peak of set-1 and set-2 was 0.2%

Retention time (R.T) variation of principal peak between set-1 and set-2 was 0.0 min.

- **For Set-1 & Set-3: (Inter instrument)**

Variation between avg. area obtained for principal peak of set-1 & set-3 was 3.0 %

R.T variation of principal peak between set-1 & set-3 was 0.0 min.

- **For Set-1 & Set-4: (Inter day)**

Variation between average area obtained for principal peak of set-1 & set-4 was 1.3%

R.T variation of principal peak between set-1 & set-4 was 0.0 min.

- **For Set-1 & Set-5:(Inter column)**

Variation between average area obtained for principal peak of set-1 & set-5 is 0.5%

R.T variation of principal peak between set-1 & set-5 was 0.1 min.

- **Robustness:**

Robustness is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its robustness during normal usage. Robustness was tested using three variables:- Age effect of Mobile phase and Test Samples as well as Different Column brands.

Age effect of Mobile phase and Test Samples held for seven days: –

Freshly prepared samples (100µg/mL of meta-cresol) and those stored for seven days were analyzed using both freshly prepared and seven day old mobile phase. There was not much variation in the results, with percent variation from initial day to 7 days being about 5%. No variation in retention time was observed. Percentage variation of

principal peak area of freshly prepared meta-cresol standard was found to be higher than 5% when compared to principal peak area of aged meta-cresol. It was concluded that sample and mobile phase was not stable for a period of time and hence it is recommended to use freshly prepared sample as well as mobile phase before analysis.

Table C: Results of different test parameters:

Statistical Parameter	Details / Results
Linearity & Range (Conc. in µg/mL)	75 – 120
Regression Equation	y = 34039x – 149.44
Correlation coefficient	0.9969
Total Analysis time in minutes	12 minutes
Retention time in minutes For meta-cresol For PTH	 About 5.2 About 10.9
Flow rate	1ml/min
Column	C18 (4.6 x 250 mm) 5µ, 300oA, Guard column
Column condition	Flow rate = 1.0ml/min.
Mobile Phase:	0.1% TFA in 60% Methanol
Method	Isocratic
Validation parameter	
Specificity	No interference
Accuracy	Recovery more than 95%
Precision	RSD less than 2%
Robustness	Method is robust for all tested parameters.

3.5.3. Conclusion:

RP-HPLC method was demonstrated to be validated for quantifying meta-cresol in presence of other excipients. The HPLC method was validated showing satisfactory

data for all the parameters confirmed. The HPLC method was found to be capable of giving analysis with good resolution, accuracy and precision. The chromatographic method described here was found to be reliable for quantifying meta-cresol in PTH formulation. Since the method is simple and rapid, they may be successfully applied to quality control analysis of meta-cresol in PTH formulations.

3.6 REFERENCES

[1]. Bond, M., Jankowski, M., Patel, H., et al., 1998. Biochemicalcharacterization of recombinant factor IX. Semin. Hematol.35, 11–17

[2]. Wang, W., 1996. Oral protein drug delivery. J. Drug Target. 4,195–232. (science liquid protein)

[3]. G. Pifferi, P. Santoro, M. Pedrani, Quality and functionality of excipients, Farmaco 54 (1999) 1–14.

[4]. L.K. Golightly, S.S. Smolinske, M.L. Bennett, E.W. Sutherland III, B.H. Rumack, Pharmaceutical excipients. Adverse effects associated with inactive ingredients in drug products (Part I), Med. Toxicol. Adverse Drug Exp. 3 (1988) 128–165.

[5]. D. Jakel, M. Keck, Purity of excipients, in: M.L. Weiner, L.A. Kotkoskie (Eds.),Excipient Toxicity and Safety Drugs and the Pharmaceutical Sciences v.103, M. Dekker, New York, 2000, pp. 21–58.

[6]. M.L. Chen, Lipid excipients and delivery systems for pharmaceutical development: a regulatory perspective, Adv. Drug Deliv. Rev. 60 (2008) 768–777.

[7]. P.M. Gaynor, R. Bonnette, E. Garcia, L. Kahl, L. Valerio, FDA's approach to the GRAS provision: a history of processes, FDA Science Forum, 2006.

[8]. D.S. Maclean, Q. Qian, C.R. Middaugh, Stabilization of proteins by low molecular weight multi-ions, J. Pharm. Sci. 91 (2002) 2220–2229.

[9]. Lin, F.K., Suggs, S., Lin, C.H., Browne, J.K., Smalling, R., Ergie, J.C., Chen, K.K., Fox, G.M., Martin, F., Stavbinsky, Z., Badrawi, S.M., Lai, P.H. and

Goldwasser, E. (1985) Cloning and expression of the Human Erythropoietin gene Proc. Natl. Acad. Sci. USA 82, 7580-7584.

[10]. Caro, J., Schuster, S. and Ramirez, S. (1989) Regulating mechanisms involved in the expression of the erythropoietin gene. Contrib. Nephrol. 76, 7-13.

[11]. Nagata, S., Taira, H., Hall, A., Johnsrud, L., Streuli, M., Escodi, J., Boll, W., Cantell, K. and Weissmann, C. (1980) Synthesis in E. coli of polypeptide with human leucocyte interferon activity. Nature 284, 3 16-332.

[12]. Murray, K. (1980) Genetic engineering. Philos. Trans. R. Sot. London B 290, 369-386.

[13]. David Gilg, Bettina Riedl, Andreas Zier, Matthias F. Zimmermann Analytical methods for the characterization and quality control of pharmaceutical peptides and proteins, using erythropoietin as an example Pharmaceutics Acta Helvetiae 71 (1996) 383-394

[14]. Haselbeck A., Epoetins: differences and their relevance to immunogenicity, Curr. Med. Res. Opin. 19, (2003) 430-432.

[15]. L. Jin, S.L. Briggs, S. Chandrasekhar S, et al., Crystal structure of human parathyroid hormone 1-34 at 0.9-A resolution, J. Biol. Chem. 275 (35) (2000) 27238–27244.

[16]. G. N. Hendy, H. M. Kronengerg, et al., Nucleotide sequence of cloned cDNAs encoding human preproparathyroid hormone, Proc. Natl. Acad. Sci. USA 78 (12) (1981) 7365-7369.

[17]. Dion M.A.M. Luykx, Pieter J. Dingemanse, Soenita S. Goerdayal, Peter M.J.M. Jongen High-performance anion-exchange chromatography combined

with intrinsic fluorescence detection to determine erythropoietin in pharmaceutical products Journal of Chromatography A, 1078 (2005) 113–119

[18]. Srinivas R. Gunturi, Ibrahim Ghobrial, Basant Sharma, Development of a sensitive size exclusion HPLC method with fluorescence detection for the quantitation of recombinant human erythropoietin (r-Hu EPO) aggregates, Journal of Pharmaceutical and Biomedical Analysis 43 (2007) 213–221

[19]. Pilar Lara-Quintanar, Izaskun Lacunza, Jesus Sanz, Jose Carlos Diez-Masa, Mercedes de Frutos, Immuno-chromatographic removal of albumin in erythropoietin biopharmaceutical formulations for its analysis by capillary electrophoresis, Journal of Chromatography A, 1153 (2007) 227–234

[20]. J. Brange, L. Langkjaer, in: Y.J. Wang, R. Pearlman (Eds.), Stability and Characterization of Protein and Peptide Drugs, Plenum Press,New York, 1993, p. 315.

[21]. K.H. Buchheit, A. Daas, K.H. J¨onsson, Pharmeuropa BIO 1 (2002) 7.

[22]. R. Pearlman, T.A. Bewley, in: Y.J. Wang, R. Pearlman (Eds.), Stability and Characterization of Protein and Peptide Drugs, Plenum Press, New York, 1993, 1–58.

[23]. B.S. Kendrick, B.A. Kerwin, B.S. Chang, J.S. Philo, online size-exclusion high-performance liquid chromatography light scattering and differential refractometry methods to determine degree of polymer conjugation to proteins and protein-protein or protein-ligand association States, Anal. Biochem. 299 (2001) 136–146.

[24]. D. Gilg, B. Riedl, A. Zier, M.F. Zimmermann, Analytical Methods for the characterization and quality control of pharmaceutical peptides and proteins, Pharm. Acta. Helv. 71 (1996) 383–394.

[25]. R.D. Lange, R.B. Andrews, D.J. Trent, J.P. Reyniers, P.S. Draganac, W.R. Farkas, Preparation of purified erythropoietin by high performance liquid chromatography, Blood Cells. 10 (1984) 305–314.

[26]. K. D. Nugent, W. G. Burton, T. K. Slattery, B. F. Johnson, and L. R. Snyder, Separation of proteins by reversed-phase high-performance liquid chromatography : II. Optimizing sample pretreatment and mobile phase conditions, Journal of Chromatography A, vol. 443, pp. 381-397, 1988.

[27]. L. R. Snyder, J. J. Kirkland, and J. L. Glajch, Practical HPLC Method Development, 2nd Edition. John Wiley and Sons, Inc, New York. 1997.

[28]. JADWIGA DUDKIEWICZ WILCZY—SKA, IZA ROMAN and ELOBIETA ANUSZEWSKA THE SEPARATION OF EPO FROM OTHER PROTEINS IN MEDICAL PRODUCTS FORMULATED WITH DIFFERENT STABILIZERS, Acta Poloniae Pharmaceutica n Drug Research, Vol. 62 No. 3 pp. 177n182, 2005

[29]. L. R. Snyder, J. J. Kirkland, and J. L. Glajch, Practical HPLC Method Development, 2nd Edition. John Wiley and Sons, Inc, New York. 1997.

[30]. H.T. Keutmann, M.M. Sauer, G.N. Hendy, J.L.H. O'Riordan J.T. Potts, Biochemistry 17 (1978) 5723.

[31]. J.T. Potts Jr., H.M. Kronenberg, M. Rosenblatt, Adv. Protein Chem. 35 (1982) 323.

[32]. T. Kimura, M. Takai, K. Yoshizawa, S. Sakakibara, Bio-chem. Biophys. Res. Commun. 114 (1983) 493.

[33]. N.A. Goud, R.L. Mckee, M.K. Sardana, P.A. Dehaven, E.Huelar, M.M. Syed, R.A. Goud, S.W. Gibbons, J.E. Fisher, J.J. Levy, J.A. Rodkey, C. Bennett, H.G. Ramjit, L.H. Caporale, M.P. Caulfield, M. Rosenblatt, J. Bone Miner. Res.6 (1991) 781.

[34]. H. Gram, P. Ramage, K. Memmert, R. Gamse, H.P. Kocher, Bio/Technology 12 (1994) 1017.

[35]. R. M. Neer, C. D. Arnaud, et al Effect of parathyroid hormone (1–34) on fractures and bone mineral density in postmenopausal women with osteoporosis. The New England Journal of Medicine, 344 (2001) 1434–1441

[36]. J. Reeve, J. N. Bradbeer, M. Arlot, et al, hPTH 1–34 treatment of osteoporosis with added hormone replacement therapy: biochemical, kinetic and histological responses. Osteoporos International 1 (1991) 162–170

[37]. A. B. Hodsman, L. J. Fraher, T. Ostbye, et al, An evaluation of several biochemical markers for bone formation and resorption in a protocol utilizing cyclical parathyroid hormone and calcitonin therapy for osteoporosis. J Clin Invest 91 (1993) 1138–1148

[38]. A. B. Hodsman, L. J. Fraher, P. H. Watson, et al., A randomized controlled trial to compare the efficacy of cyclical parathyroid hormone versus cyclical parathyroid hormone and sequential calcitonin to improve bone mass in postmenopausal women with osteoporosis. J Clin Endocrinol Metab 82 (1997) 620–628

[39]. R. Lindsay, J. Nieves, C. Formica, et al. Randomised controlled study of effect of parathyroid hormone on vertebral-bone mass and fracture incidence among postmenopausal women on oestrogen with osteoporosis, Lancet 350 (1997) 550–555

[40]. N. E. Lane, S. Sanchez, G. W. Modin, et al., Parathyroid hormone treatment can reverse corticosteroid-induced osteoporosis. Results of a randomized controlled clinical trial, J Clin Invest 102 (1998) 1627– 1633

[41]. N. E. Lane, S. Sanchez, H. K. Genant, et al., Short-term increases in bone turnover markers predict parathyroid hormone-induced spinal bone mineral density gains in postmenopausal women with glucocorticoid- induced osteoporosis, Osteoporos Int 11 (2000) 434–442

[42]. E. K. Armitage, Parathyrin (Parathyroid Hormone): metabolism and methods for assay, Clin. Chem., 32 (1986) 418-424.

[43]. D. B. Endres, R. Villanueva, F.R. Singer, et al., Measurement of parathyroid hormone, Endocrinol Metab Clin North Am., 18 (1989) 611- 629.

[44]. G.V. Segre, J.T. Potts Jr., Differential diagnosis of hypercalcemia. Methods and clinical applications of parathyroid assays, Endocrinology, Vol. 2, W.B. Saunders company, Philadelphia, PA, 1989, 984-1001.

[45]. C. Burns, M. Moore, C. Sturgeon et al., WHO International Collaborative Study of the proposed 1st International Standard for Parathyroid Hormone 1-84, human, recombinant, (2009) WHO/BS/09.2115.

[46]. R. Bhushan, R. Agarwal, Liquid chromatographic separation of some PTH-amino acids, 12 (1998) 322-325.

[47]. A. L. Frelinger and James E. Zulls, Oxidized forms of parathyroid hormone with biological activity – Separation and characterization of hormone forms oxidized at methionine 8 and methionine 18*, The Journal of Biological Chemistry 259 (1984) 5507–5513.

[48]. Y. Nabuchi, E. Fujiwara et al., Oxidation of recombinant human parathyroid hormone: Effect of oxidized position on biological activity, Pharmaceutical Research 12 (1995) 2049–2052.

[49]. Y. Nabuchi, E. Fujiwara, H. Kuboniwa, et al., Kinetic study of methionine oxidation in human parathyroid hormone, Analytica Chimica Acta 365 (1998) 301- 307.

[50]. R.D. Lange, R.B. Andrews, D.J. Trent, J.P. Reyniers, P.S. Draganac, W.R. Farkas, Preparation of purified erythropoietin by high performance liquid chromatography, Blood Cells. 10 (1984) 305–314.

[51]. m/p Cresol category, UNEP Publication, internet link:, www.inchem.org/documents/sids/sids/m-p-cresols.pdf

[52]. Dennis Kim, James Baraniuk, Delayed-type hypersensitivity reaction to the meta-cresol component of insulin, Annals of Allergy, Asthma & Immunology, Volume 99, Issue 2, August 2007, Pages 194-195.

[53]. D. McNeil, Kirk-Othmer Encyclopedia of Chemical Technology", Vol. 6, 2nd Edition, John Wiley and Sons, New York; 1965 p. 434 – 444.

[54]. S. Husain, P. Kunzelmann, H. Schildknecht, Separation of isomeric alkyl phenols by high-performance liquid chromatographic and gas-liquid chromatographic techniques, J. Chromatogr. 1977; 13: 753 – 60.

[55]. "NIOSH Manual of Analytical Methods", Vol. 3, 2nd Edition, USDHEW, PHS, CDC, NIOSH, DHEW (NIOSH) Publication No. 77 - 157C, April 1977.

CHAPTER 4:

SIMPLE AND SENSITIVE SPECTROPHOTOMETRIC VALIDATED METHOD FOR ANTIFUNGAL BASED DRUGS

4.1 Introduction:

Ketoconazole, cis-1-acetyl-4-[4-[2-(2, 4-dichlorophenyl)-2-(1H-imidazole-1-ylmethyl)-1, 3-dioxolon-4-yl] methoxy piperazine (KC), (mol wt, 531.44), is a highly effective broad spectrum antifungal agent. Ketoconazole has been determined in pharmaceutical preparations and in biological fluids by spectroscopic [1-13], chromatographic [14-19] and electrochemical methods [20-24]. Ketoconazole is used in the treatment of a wide variety of superficial and systemic mycoses [15, 30]. Several techniques and procedures have been used for the determination of ketoconazole. Spectrophotometric methods based on acid-dye formation [31], charge-transfer complexation with 2,3-dichloro-5,6-dicyano-*p*-benzoquinone [DDQ; 12] or with iodine [3], complex formation with iron(III) [32] and with picric acid [1], densitometry [33], and first-derivative spectrometry [34] have been reported. Other techniques include spectrofluorimetry [13], infrared spectrometry [35], nuclear magnetic resonance spectrometry [36], polarography [14], adsorptive stripping voltammetry [37], capillary electrophoresis [38], and liquid chromatography [LC; 11, 39–41]. Nonaqueous titration methods for the determination of ketoconazole are described in the *British Pharmacopoeia* [42] and the *United States Pharmacopoeia* [43], whereas ketoconazole in tablets is determined by LC [42]. However, some of these methods need expensive equipment and/or are time consuming.

Application of uv-vis absorptiometry for quantitative determination of analytes has been widely used in the past and still is one popular method of analysis. The methods provide easy handling, simple analyte preparation procedures, and affordable cost instrumentation.

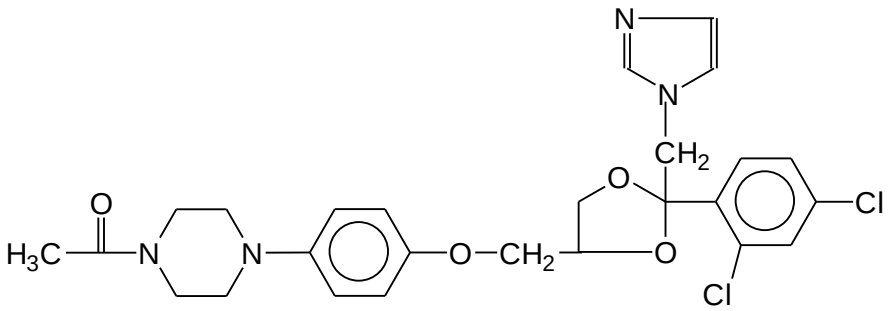
A large number of reagents are available and many new are synthesized that are able to provide methods of high sensitivity.

However, in the quantitative analysis of natural or complicated samples, selectivity is necessary, which can be obtained to some extent by the spectral selectivity that these methods offer, but not in a general sense.

Development of UV-Vis absorptiometry methods require reaction processes that involve either the consumption of a reagent or production of a product with high molar absorptivity absorbing at different wavelength region from that of reagent. Such high

molar absorptivity compounds may be (i) colored ion pairs, (ii) complexes containing ligands with conjugated π-electrons able to provide charge transfer transition, (iii) compounds that possess extensively delocalized π-electron system such as in oxidative coupling products with aromatic ring compounds, (iv) quinones, or quinonoid compounds, (v) specific molecular complexes, (vi) inorganic compounds like iodine and anions that form ion-pairs with bulky cationic dye molecules, or permanganate ions. Selectivity is usually obtained by masking reagents or by working at different conditions (pH range, temperatures, catalysts, inhibitors etc.) or even by using specific reagents.

Figure 4.1 Structure of Ketoconazole



Amplification reactions have been the subject of extensive research in analytical chemistry for more than a century. Amplification reactions provide a chemical means of enhancing the sensitivity of an analytical measurement.

Amplification reaction is defined as a reaction, which replaces the conventional reaction used in a particular determination so that a more favorable measurement can be made. The sequence can be repeated to provide a further favorable increase in measurement. The Liepert reaction for the determination of iodide is the most important amplification reaction, since many indirect and exchange reactions utilize same cycle of reaction

$$I^- \longrightarrow IO_3^- \longrightarrow 6\,I^- \quad \text{---------- (a)}$$

Where, each iodide gives rise to six iodine atoms

Iodine atom bonds with different number of oxygen atoms to give oxide anionic species, such as hypoiodous anion (IO^-), the iodic anion (IO_3^-), the metaperiodate anion (IO_4^-), the mesoperiodic anion (IO_5^{3-}) and the paraperiodic anion (IO_6^{2-}). The most stable ones are periodate and paraperiodic anions[1]. Paraperiodic acid (H_5IO_6) usually behaves as dibasic acid with dissociation constants $K_1 = 2.3\times10^{-2}$, $K_2 = 1.0\times10^{-6}$, and $K_3 = 6.3\times10^{-13}$. Iodine oxide ionic species are oxidation agents with a variety of electrochemical potentials ranging from ~1.7 to 0.26V depending on the pH and the specific iodine oxide species involved [2].

Periodate anion reacts with various chemical compounds or chemical species producing soft oxidation products. The oxidation reaction pathway depends on variables such as the type of reagents, the coexistence of other kinds of molecules in reaction mixtures, the activating conditions and so on [3]. The reaction rate of the soft oxidation process depends on the parameters of pH, temperature, solvent, activators, catalysts, enhancers/inhibitors, and molecular structure of reagents of the available functional groups, the configuration of the side groups in the skeleton of the molecule (i.e. in Malaprade reaction the $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ show considerable variation with anomeric configuration and type of pyranoside) [4]. These parameters can be

controlled to choose a desired type of reaction or kinetic pathway, thus leading to selective oxidation processes that are suitable in analytical work. The oxidation products or the excess of reagents can be detected or monitored by techniques of electrochemistry, spectrophotometry, luminometry, mass-spectrometry or through separation with chromatographic/detection techniques. In addition, some of the periodate oxidation reactions are rather slow, and are catalyzed by transition metal ions or various anions to provide catalytic–kinetic methods of analysis. In such cases the catalyst concentration in a sample can be determined with excellent sensitivity.

The above properties offered by periodate oxidation reactions are of utmost importance in developing micro-analysis methods for a great number of organic and inorganic species.

Periodate oxidation, apart from the quantitative determinations of analytes in aqueous- or solid-samples of geological or environmental or biological origin, can also be used, (a) for modifying solid polymer carbohydrate materials by generating surface-active functional groups for various applications in analytical methods such as the preparation of solid phases for column chromatography, or the formation of biosensors, or chemical grafting, or immobilization of reagents and enzymes on solid supports, or labeling reagents (b) for characterization of the kinetic properties of certain materials and (c) enabling selective dissolution process of a specific chemical component in multicomponent technical products for material analysis.

Furthermore, the Malaprade reaction has been used broadly in procedures of structure determination and characterization of polysaccharides and oligosaccharides (monosaccharide composition, anomeric structure analysis, sequence determination, inter monosaccharide linkages, linkage sites in the protein chains etc.) in saccharide natural biomaterials and plant materials.

It was as early as 1928 that Malaprade [5] observed that mannitol was decomposed by periodic acid and a few years later that Fleury and Lange [6] recognized the specific oxidative fission on 1,2-diols that stimulated many research groups to include periodate among the oxidizing agents in organic chemistry. Closely related fissions of C–C bond and other developments in this field of organic chemistry were reviewed [7]. Since then the mechanism of the reaction was elucidated by identification of the intermediate (product of esterification) and the products of structure break down, as well as the establishment of the kinetic rate constants of the steps involved and the equilibrium constant of the first step [8–12]. Testing the effect of structure parameters on the Malaprade reaction it was found that the rate of intermediate formation decreased with increasing substitution for steric reasons and that under certain conditions, the initial esterification was relatively slow, followed by fast cyclisation [13] and the rate of collapse of intermediate was increased with steric crowding; thus suggesting to be the dominant factor [14]. Many other reactions were, then, investigated and applied in different fields of chemistry and a number of reviews have been published [15- 20]. Recently A.G. Vlessidis et al have reviewed instrumental methods of analysis, pharmaceutical analysis, and chromatographic-separation methods that involve the periodate oxidation process [59].

Spectrophotometry methods of analysis

Various spectrophotometric methods of analysis involving periodate oxidation have been developed in UV–vis absorptiometry, kinetic-spectrophotometry, catalytic–kinetic-spectrophotometry, fluorimetry, phosphorimetry, and chemiluminescence-emission with static and FIA and/or SIA reactor systems.

The following reports (Table 4.1) are addressed to methods that involve periodate oxidation and are classified according to the redox reaction used for the quantitative absorptiometry method.

Table 4.1 Analytical parameters of spectrophotometric methods of analysis involving periodate oxidation.

Iodide Oxidation						
Analyte Reaction Parameters (Conditions)	**Method (Wavelength) [Mol. Abs I Mol^{-1} cm^{-1}] Reactor**	**Range (µg/mL) LOD (µg/mL)**	**RSD % At (x.x µg/mL)**	**Sample**	**Remarks**	**Ref.**
Periodate, Iodate KI $\xrightarrow{IO_4^-}$ I_3; KI $\xrightarrow{IO_3^-}$ I_3; (pH=3.5, Flow Rate 21ml/h Sample Vol.200-320 µL) IO^- (excess) $\xrightarrow{Mo(VI)}$ $[Imo_6O_{24}]^{5-}$	Sequential (352 nm) FIA	0.050-5.0 (P) 0.050-10 (I) $A=0.011+0.144C_p$ $A=0.002+0.117C_1$ **(3 σ)** **0.04 (P) 0.05 (I)**	(n=10) 0.8-0.65 (0.5-2.0 iodate) 0.92-0.81 (0.5-2.0 periodate)	River water Drinking water	**Recovery %:** 96-105 S^{2-}, SO_3^{2-}, NO_2^- interfere seriously	44
Sn(II), Sn(IV), S(total) Sn(II) $\xrightarrow{IO_4^-}$ Sn(IV) IO_4^- (excess) $\xrightarrow{Mo(VI)}$ $[Imo_6O_{24}]^{5-}$ KI $\xrightarrow{IO_3^-}$ I_3; (pH 2.2-3.5) I_3 $\xrightarrow{CHCl_3}$ I_3(extract) I_3 $\xrightarrow{SO^{2-}_3}$ 3I; I^- $\xrightarrow{IO_4^-}$ IO^-_3	Amplified Photometric of I_3^-(extract) (350 nm) Batch			Organotin compounds Artificial sea water	Amplification 6- fold or 144- fold	45
Au(I), Au(III), Pt(II), Pt(IV) Au(I) $\xrightarrow{IO_4^-}$ Au(III) Pt(II) $\xrightarrow{IO_4^-}$ Pt(IV) IO_4^- (excess) $\xrightarrow{Mo(VI)}$ $[Imo_6O_{24}]^{5-}$ KI $\xrightarrow{IO_3^-}$ I_3; (pH 0.4-4.3) I_3 $\xrightarrow{CHCl_3}$ I_3(extract) I_3 $\xrightarrow{SO^{2-}_3}$ 3I; I^- $\xrightarrow{IO_4^-}$ IO^-_3	Amplified (350nm) Batch			Binary mixtures	Amplification Au-8 – or 168-fold Pt: 6 or 126-fold $Au(III)+S_2O_3^{2-}$ ➔ Au(I) $Pt(IV)+S_2O_3^{2-}$ ➔ Pt(II)	46

Iodide Oxidation						
Analyte Reaction Parameters (Conditions)	**Method (Wavelength) [Mol. Abs I Mol^{-1} cm^{-1}] Reactor**	**Range (µg/mL) LOD (µg/mL)**	**RSD % At (x.x µg/mL)**	**Sample**	**Remarks**	**Ref.**
Arbutin $\xrightarrow{IO^-_4}$ Arbutin $_{oxi}$ + IO^-_3 (Borate buffer, pH 8.0, Room Temp 20-25 min) KI $\xrightarrow{IO^-_3(excess)}$ I_3; I_3 $\xrightarrow{CHCl_3}$ I_3(extract)	Indirect determination Arbutin photometric (351 nm) Batch	25-125 **25**	(n=3) 6.2 **(25)**	Authentic (arbutin)	**Recovery %:** 99-8 – 100.9 **Validation:** Pure authentic samples	47

4.1.1. Methods based on iodide oxidation

$IO_4^- + 11\ I^- + 8H^+ \rightarrow 4I_3^- + 4H_2O$

$IO_3^- + 8\ I^- + 6H^+ \rightarrow 3I_3^- + 3H_2O$

Periodate and iodate oxidation of iodide in FIA system at pH 3.5 is employed for the periodate and iodate determination followed by extraction in $CHCl_3$ and measurement of absorbance at 350 nm. Both analytes are determined simultaneously, but iodate alone in a mixture can be determined by masking periodate with molybdates [44].

Periodate oxidation of Sn(II) followed by masking the periodate excess with molybdates and further reaction of the iodate formed – during the periodate oxidation of Sn(II) – with potassium iodide was used to form I_3^- that is measured spectrophotometrically in the $CHCl_3$ extract at 350 nm for the indirect determination of Sn(II) with 6-fold amplification. The method can be extended to amplification by reduction of iodine to iodide, sequential oxidation of iodide to iodate and then following the previous procedure once again [45]. To determine Sn(II) and Sn(IV) together, the latter is reduced to Sn(II) with SO2 and the total is determined as Sn(II).

A similar sequential amplification procedure was followed for the indirect determination of gold (I) and platinum (II) in aqueous solution samples with 168-fold and 126-fold amplifications, respectively [46].

Periodate oxidation of arbutin followed by addition of iodide to reduce periodate excess at pH 8 and measurement of absorption of the chloroformic extract at 351 nm was used for the indirect quantitative determination of arbutin [47].

4.1.2. Manganese periodate oxidation reaction

$2Mn^{2+} + 5IO_4^- + 3H_2O \rightarrow 2MnO_4^- + 5IO_3^- + 6H^+$

The periodate oxidation of Mn(II) to permanganate in acid medium followed by spectrophotometric detection at 521 nm was employed for the determination of: (a) Mn(II) in copper-selenide [48], (b) average oxidation degree of manganese in manganites of trace rare earth elements [49], and (c) manganese in high carbon ferrochrome [50].

A mono-segmented FIA multi-commutation manifold coupled with photometric detection was reported for the determination of manganese in soybean digests [51]. The reaction rate is relatively slow, and the multi-commutation system improved the speed of analysis by eliminating the washing cycle.

The above system was implemented with micro-heater device to raise the oxidation temperature to 70°C in order to increase the rate of reaction for the determination of manganese in soybean digests [52]; this improved the speed of analysis to 30 s.

4.1.3. Ion-pair formation methods

Ion-pair formation with bulky organic cations is another trend used for spectrophotometric determinations. The absorption band shifts to higher wavelengths after ion-pair formation and provides easy spectrophotometry detection with absorbance band peak separated from that of the excess of reagent.

The following reports represent a small sample of ion-pair analytical methods. Ion-pair of tetramethyl ammonium cation with tri-iodide anion ($TMA+I_3^-$) formed by oxidation of TMAI by iodate or periodate at pH4 followed by photometry detection in $CHCl_3$ extract at 509, 358, and 258nm is used for the determination of periodate and iodate in artificial fresh water samples [53].

Ion-pair between periodate and amiloride followed by extraction in 4-methyl-2-pentanone at pH 4.0–5.5 and spectrophotometry detection at 354 nm was used for the

periodate determination in artificial fresh water. Iodate can also be determined by converting it to periodate by oxidation with persulphate [54].The extraction equilibria parameters of the above-mentioned ion-pair in cyclohexanone at pH 4 with spectrophotometry measurements at 354nm were obtained [55].

4.1.4. Charge transfer complex methods

A limited number of reports that use charge transfer complexes is available.

The complex between periodate or iodate and triphenyl tetrazolium chloride formed in acidic media followed by spectrophotometry detection at 295nm and 255 nm, respectively is used for the determination of periodate and iodate [56].

The Fe(III)[Fe(CN)6] complex formed by oxidation of Fe(II) with iodate or periodate or persulphate in the presence of potassium ferrocyanide to give the intense Prussian blue color is used for their determination in aqueous media [57].

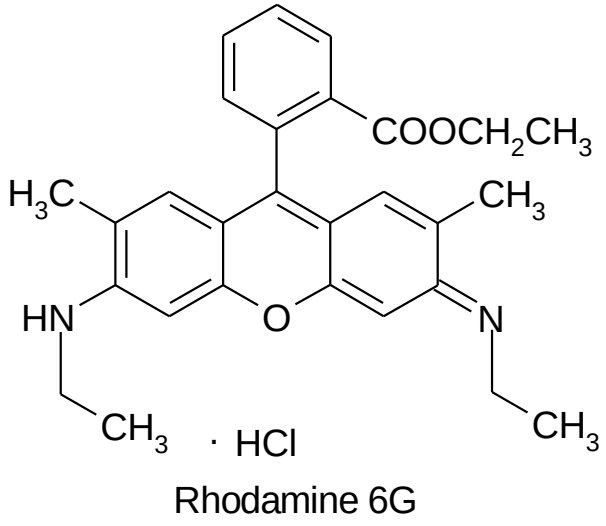
Molecular complex between sulphanilamide and metol, is used for the determination of the antibiotics dihydrostreptomycin, framycetin, and the acid hydrolysis product of chloramphenicol [58] followed by absorbance measurements at 520 nm. The procedure involves periodate oxidation of antibiotics followed by masking periodate excess by molybdates. In the presence of sulphanilamide and metol a molecular complex between oxidized metol and sulphanilamide is formed.

4.1.5. Malaprade reaction methods

All these reactions are two electron oxidations requiring one molecule of periodate in which the iodine atom is reduced from the +7 to the +5 valency state with the formation of iodate ion.

Recently a method was described for the determination of arsenic, after selective separation as AsI_3. In presence of chloride and acid, the reaction of arsenic (III) and the associated iodide with excess iodate caused the oxidation of the generated iodine and it's stabilization as ICl_2 species. The anionic iodine complex was extracted into benzene as ion pair with Rh6G (Rhodamine 6G) for spectrophotometric determination at 535 nm [25]. Structure of cationic dye – Rhodamine 6G is shown as below:

Figure 4.2 Structure of Rhodamine 6G



Rhodamine 6G

Similarly amplification reactions were used for the determination of mercury [26]. The method involved selective separation of mercury (II) as tetra-iodo mercury (II) and oxidation of the associated iodide to iodate using bromine water. The iodate then formed when reacted with iodide in the presence of chloride in acid medium facilitated the formation of anionic iodine complex for extraction as ion pair with rhodomine 6G in to benzene.

Since periodate is known to react with mercury to form mercury (II) para periodate and also has a favorable potential for oxidations of organic compound, the formation of

mercury (II) para periodate was put to advantage for the formation of anionic iodine complex for the determination of inorganic and organo-mercury (II) species present at trace levels[27].

It has been reported that electro-oxidation of ketoconazole (KC) takes place in aqueous as well as in non-aqueous media. Ketoconazole was initially oxidized with the loss of one electron to form KC (+1) cation radical. Also it has been reported that KC (+1) can be further oxidized with the loss of second electron to give some stable product [28].

It thus seemed worthwhile to examine the possibility of oxidation of KC with periodate so as to develop a sensitive spectrophotometric method for determination of KC.

4.2 Experimental

4.2.1 Instrumentation:

A UV 1601 spectrophotometer (Shimadzu, Tokyo) with a 10 mm matched silica cells was used for all spectral measurements. All pH values were measured on a Thermo Orion, pH meter (Model: 420 pH/mV) using a combined electrode.

4.2.2 Chemicals / Standard:

All chemicals i.e. Sodium periodate, Ammonium molybdate, Ammonia, Potassium iodide, Sulphuric acid, Toluene, Potassium iodate, Sodium chloride, Rhodamine 6G, Sodium sulphate, were of the highest purity available (AR grade) and used without further purification. Water (HPLC grade) was used to prepare all solutions. Ketoconazole USP grade material and tablet containing ketoconazole (Phytoral) were used for preparation of standard and assay solutions respectively.

Standard Ketoconazole solution:

A stock solution of standard KC was prepared by dissolving ~ 26.7mg of KC (USP grade) in HPLC grade water containing few drops of 0.5M sulphuric acid solution [28] and further diluted to 50 mL using HPLC grade water. Working standards were prepared by suitable dilution of an aliquot of the stock solution.

4.2.3 Procedure:

The method reported elsewhere was followed for oxidation of KC by periodate and formation of ICl_2^- [26]. To the sample solution containing not more than 0.0054 mg of KC in a 50 mL beaker, 1.5mL of 0.01M solution of sodium periodate was added, pH was adjusted up to 3 by addition of dil. ammonia solution. The solution was stirred well, followed by the addition of 1mL of 0.1 % of ammonium molybdenum solution and again the pH was adjusted to 3. The solution was transferred to a separatory funnel and 2mL of 0.1 M KI solution (freshly prepared) followed by 2mL of 0.5 M sulphuric acid solution was added. The solution was diluted to about 25 mL with water and was made to stand for 2 minutes. The solution was then shaken with 10 mL of toluene for few seconds. The organic layer was separated and washed twice with 10 mL of water. The aqueous layer and washings were discarded.

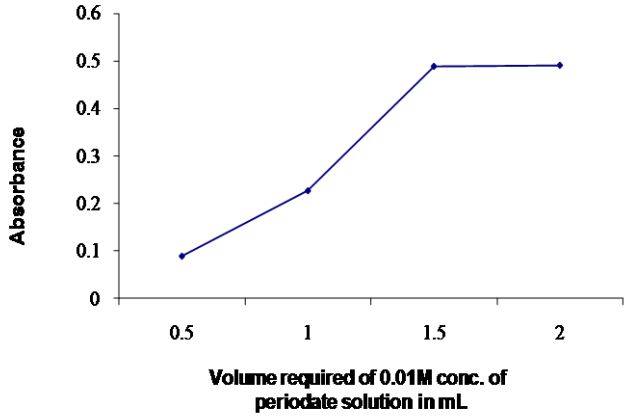
The toluene layer was shaken with 25 mL of solution containing 2mL each of 0.01% of potassium iodate solution and 2.5 M sulfuric acid solution, 4mL of 15% of sodium chloride solution and 2mL of 0.01% of Rh6G solution for 1minute. The toluene layer was separated into a dry test tube and about 1g of anhydrous sodium sulfate was added. The absorbance of the extract was measured at 535nm in 10mm cells against the reagent blank run through the entire procedure.

4.3 Results and Discussions:

4.3.1 Effects of periodate concentration:

To establish the optimum concentration of periodate required for complete oxidation of KC, reactions were carried out using 0.5 mL – 2mL of 0.01M sodium periodate. In each instance, 10 mL of 2.136 µg/mL KC was present in a total volume of 25 mL maintained at pH 3.0. A reagent blank was prepared for each concentrations of periodate. The results obtained for various volumes of periodate are shown in Figure3.

Figure 4.3: Effect of periodate concentration for oxidation of ketoconazole



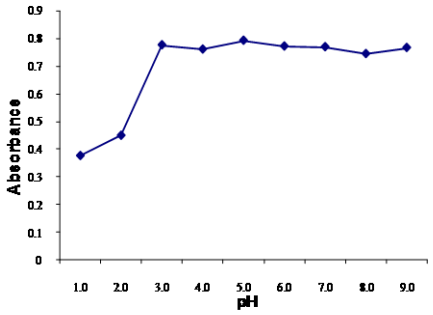
2.136 µg/mL KC- 10mL, periodate- x mL of 0.01M, pH-3, 0.1 % of ammonium molybdenum solution-1mL,0.1M KI-1mL, aqueous volume-25mL,toluene for extraction-10mL

From the graph it is evident that addition of 1.5 mL of 0.01M periodate solution is sufficient for the quantitative oxidation of KC. It was decided to use 1.5mL of 0.01M solution of periodate in all subsequent work.

4.3.2 Effect of pH:

The optimum pH for the oxidation of KC with periodate and for the liberation of iodine by reaction with iodide were evaluated. The variation of pH during oxidation of ketoconazole by periodate is shown in figure 4.

Figure 4.4: Effect of pH for oxidation of ketoconazole



2.136 µg/mL KC - 10mL, 0.01M periodate - 1.5 mL, pH - varied, 0.1 % of ammonium molybdenum solution - 1mL, 0.1M KI - 1mL,aqueous volume - 25mL, toluene for extraction-10mL

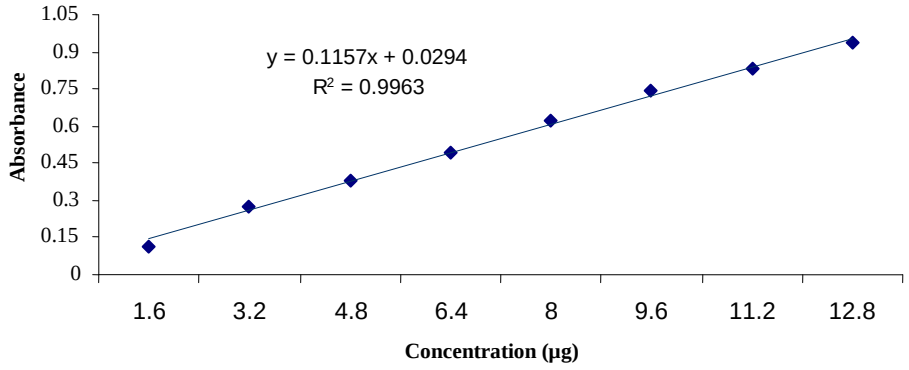
It is observed that the oxidation of KC by periodate was quantitative in the pH range 3.0 to 9.0. On the basis of these experiments it was decided to maintain the pH 3 for the oxidation of KC in all subsequent studies.

It was observed that the absorbance of the blank was low only when freshly prepared aqueous solution of periodate was used. This was possibly due to photo decomposition of periodate to iodate.

4.3.3 Calibration graph and molar absorptivity:

The adherence of oxidation of KC with periodate to Beer's law was next examined under the optimum conditions. Beer's law was obeyed over the concentration range of 0.2136 µg/mL to 1.7088 µg/mL with molar absorption coefficient of 5 x 10^5 L mol^{-1} cm^{-1} and a regression coefficient of 0.9963, indicating good linearity. The values of the regression equation are given in Table 4.3

Figure 4.5: Calibration graph



It compares favorably in sensitivity with the spectroscopic methods described in literature for the determination of ketoconazole as shown in Table 4.2.

Table 4.2: Comparative data of proposed method with literature survey of the spectrophotometric determination of ketoconazole

Reagents used	**λmax (nm)**	**Beer's law range in µg/mL or molar absorptivity**	**Experimental conditions involved**	**Reference**
Picric acid	410	1-58 µg/mL	Involves extraction into chloroform	[1]
Cu(II) and Co(II) complexes	720 and 612.5 respectively	35.36 ± 1.95 and 59.62 ± 1.87 respectively	Involves extraction into dichloromethane	[2]
Iodine	290	1-40 µg/mL	--	[3]
First-derivative ultraviolet	257	5.0 to 30.0 µg/mL	Zero crossing method	[4]
iron(III) chloride		1-15	--	[5]
iron (III) chloride and 1,10-phenanthroline	512	1.6-16	Redox complexation reaction	[6]
Tri-iodide ion and alizarin red	425	10^{-5}–10^{-2} M	Ion pairs	[29]
Amplification Method	535	0.2 – 1.7 µg/mL	Ion pair	Present Method

The detection limit of the method was found to be 0.127 µg/mL. The RSD were found to be 0.89%. The validation parameters are summarized in Table 4.3, which shows good repeatability and reproducibility of the proposed method. Precision were verified with six measurements on same day and on different days. RSD were found to be less than 2.0%.

Table 4.3: Spectral data for the amplification reaction of ketoconazole

Parameters	**Values**
λmax(nm)	535
Beer's law limits (µg/mL)	0.2-1.7
Molar absorptivity ($Lmol^{-1}cm^{-1}$)	5×10^5
Limit of detection(µg/mL)	0.127
Slope	0.1157
Limit of quantitation (µg/mL)	0.17
Intercept	0.0294
Correlation coefficient	0.9963
R.S.D of 6 determinations (%)	0.89

4.3.4 Reaction Sequence for the observed enhancement

In the method proposed the oxidation of KC with periodate would liberate 2 atoms of iodate. The reduction of iodate in acid medium with iodide ion would produce 12 atoms of iodine –

$$KC + IO_4^- \rightarrow KC^+ + IO_3^- \qquad (1)$$

$$KC^+ + IO_4^- \rightarrow KC^{+2} + IO_3^- \qquad (2)$$

$$IO_3^- + 5I^- + 6H^+ \rightarrow 3I_2 + 3H_2O \qquad (3)$$

As both un-reacted periodate and iodate formed due to reduction of periodate would react with iodide to liberate iodine, it was decided to mask the excess periodate using ammonium molybdate.

When 1.2 µg of ketoconazole was determined by the developed method it gave absorbance of 0.382. In accordance with equations 1 to 3, as 1.2 µg of ketoconazole would yield 3.45 µg of iodine and since the absorbance was found identical to that

obtained when 3.45 µg of iodine in toluene was directly subjected to determination, it was concluded that under the reaction conditions there was stoichiometric oxidation of ketoconazole in accordance with equations 1 to 3.

4.3.5 Application:

The method developed for the determination of KC was applied for establishing ketoconazole concentration levels in tablets. Samples to which known amount of KC were added were analyzed to ascertain whether the recovery was quantitative. Four tablets, each containing 200 mg of KC were crushed and powdered. A suitable amount of the powder (~ 5.78 mg) was weighed and dissolved in 50 mL of HPLC grade water containing a few drops of 0.5 M H_2SO_4 [28]. The excipients were separated by filtration and the filter paper was washed three times with water. The filtrate and washing solutions of the tablet were transferred quantitatively into a 50mL calibrated flask and diluted to the mark with HPLC grade water, and the developed method was followed. The recovery was calculated by comparing the concentration obtained from the spiked mixtures with that of the standard KC (USP grade material) added to the sample. The results of analysis of commercial dosage forms and the recovery study (standard addition method)) as shown in Table 4.4, indicates that the developed method is suitable for the assay of ketoconazole in commercial dosage forms.

Table 4.4: Results of determination of ketoconazole in its formulations

Sr. No.	Sample	Amount of drug in extract (µg/mL)	Amount of pure KC added (µg/mL)	Total found (µg/mL)	Recovery (%)
1.	Tablet 500mg	0.534	-	0.540	98.88
2.	Tablet 500mg	0.534	0.534	1.048	97.77
3.	Tablet 500mg	0.534	0.400	0.935	99.89

4.4 Conclusion

The method described provides a simple, fast and reliable means of determining ketoconazole in pharmaceutical preparations. The method developed has very high sensitivity (molar absorptivity= 5×10^5 $Lmol^{-1}cm^{-1}$). The method has been applied to establish the ketoconazole content in commercial tablet dosage forms.

4.5 References

[1]. S. Sadeghi, M. Shamsipur, A new extraction-spectrophotometric method for the determination of Ketoconazole from pharmaceutical preparations, Anal Lett., 31 (1998) 2691 – 2705.

[2]. A. Nairman, S. Yasser, Investigation of Ketoconazole Copper (II) and Cobalt(II) complexes and their spectrophotometric applications J. AOAC Int., 84 (2001) 563 – 568.

[3]. S. R. El-Shabour, K.M. Emara, P.Y. Khashaba, A. M. Mohamed, Charge transfer complexation for spectrophotometric assay of certain Imidazole antifungal drugs Anal. Lett, 31 (1998) 1367 – 1385.

[4]. E. R. M. Kedor-Hackmann, M. I. R. M. Santoro, A. K. Singh and A. C. Peraro, First-derivative ultraviolet spectrophotometric and high performance liquid chromatographic determination of ketoconazole in pharmaceutical emulsions Rev. Bras. Cienc. Farm.42 (2006) 91 – 98.

[5]. F. M. Abou-Attia, Y.M. Issab, F.M. Abdel-Gawad, S.M. Abdel-Hamid, Quantitative determination of some pharmaceutical piperazine derivatives through complexation with iron(III) chloride. Farmaco, 58 (2003) 573 – 579.

[6]. K. Farhadi, R. Maleki, Effect of surfactants on the AC voltammetry of 9,10-anthraquinone derivatives at glassy carbon electrode and its utilization for the determination of anthraquinones and cationic surfactants, Analytical Sciences,17 (2001) i867 – i869.

[7]. G. R. Rao, P. J. Rao,S. S. N. Murty, Spectrophotometric determination of ketoconazole Indian Drugs, 26 (1988) 1119 – 1120.

[8]. M.A. Abounassif, B.M. El-Shazly, D1-differential potentiometric and 1H-NMR spectroscopic determination of ketoconazole and its formulations. Anal. Lett., 22 (1989) 2233 – 2247.

[9]. S.S. Zarapkar, A.V. Rane and S.H. Rane, HPLC determination of ciprofloxacin HCl and tinadazole in pharmaceutical preparation, Indian Drugs, 36-5 **(1999)** 408 – 410.

[10]. O.H. Abdelmageed, P.Y. Kashaba, Spectrophotometric determination of clotrimazole in bulk drug and dosage forms Talanta, 40 (1993) 1289 – 1294.

[11]. E. L. M. Kedor-Hackmann, M.M. F. Nery, M. I. R. M. Santoro, Determination of ketoconazole in pharmaceuticals preparations by ultraviolet spectrophotometry and high performance liquid chromatography. Anal. Lett., 27 (1994) 363 – 376.

[12]. K. Kelani, L.I. Bebawy, Spectrophotometric determination of some n-donating drugs using DDQ, Anal. Lett., 30 (1997) 1843 – 1860.

[13]. A. A. A. El-Bayoumi, M.E. El-Shanawany, El-Sadek and A.A. El-Sattar, Synchronous spectrofluorimetric determination of famotidine, fluconazole and ketoconazole in bulk powder and in pharmaceutical dosage forms Spectroscop. Lett., 30 (1997) 25 – 46.

[14]. Z. Fijalek, J. Chodkowski, M. Warowna, Polarographic examination of drugs derived from imidazol. I. Clotrimazole and Ketoconazole Acta Polon.Pharm, 49 (1992) 1 – 5.

[15]. E. W. Gascoigue, G. J. Barton, M. Michaels, W. Mendlermans, J. Heykants, The kinetics of ketoconazole in animals and man Clin. Res. Rev., 1 (1981) 177 – 182.

[16]. K. Selinger, D. Matheou, H.M. Hill, High-performance liquid chromatographic method for the determination of miconazole in vaginal fluid J. Chromatogr., 434 (1988) 259 – 264.

[17]. M.A. Al-Meshal, Determination of ketoconazole in plasma and dosage forms by high-performance liquid chromatography and a microbiological method Anal. Lett., 22 (1989) 2249 – 2263.

[18]. A.M. Dipietra, V. Cavrini, V. Anderisano, R. Gatti, HPLC analysis of imidazole antimycotic drugs in pharmaceutical formulations J.Pharm. Biomed. Anal., 10 (1992) 873 – 879.

[19]. F. Alhaique, C. Anchisi, A.M. Fadda, A.M. Maccioni, V. Travagli, Miconazole, ketoconazole and liposomal preparations: a reserved-phase HPLC determination. Acta Technol. Legis Med., 4 (1993) 169 – 175.

[20]. R. T. Sane, R. V. Tendolkar, D. P. Gangal, K.D. Ladage, R.M. Kothurkar, An extractive colorimetric method for the determination of ketoconazole from pharmaceutical preparations, Indian J. Pharm. Sci., 50 (1988) 347 – 348.

[21]. M. Shamsipur, K.H. Farhadi, Electrochemical Behavior and Determination of Ketoconazole from Pharmaceutical Preparations Electroanalysis,12 (2000), 429 – 433.

[22]. M. Shamsipur, K.H. Fahadi, Adsorptive stripping voltammetric determination of ketoconazole in pharmaceutical preparations and urine using carbon paste electrodes Analyst, 125 (2000) 1639 – 1643.

[23]. M. Shamsipur, K.H. Fahadi, Electroxidation of Ketoconazole in acetonitrile and its determination in pharmaceutical preparations Chem. Anal., 46 (2001) 387 – 395.

[24]. M. Shamsipur, F. Jalali, Preparation of a ketoconazole ion-selective electrode and its application to phramaceutical analysis Anal Sci., 16 (2000) 549 – 552.

[25]. K. Palanivelu, N. Balasubramanian, T.V. Ramakrishna, A chemical enhancement method for the spectrophotometric determination of trace amounts of arsenic, Talanta, 39 (1992) 555 – 561.

[26]. P. Padmaja, N. Balasubramanian, T.V. Ramakrishna, Chemical enhancement method for the determination of mercury by spectrophotometry after iodide extraction. Talanta. 41 (1994) 255 -260.

[27]. P. Padmaja, N. Balasubramanian, T.V. Ramakrishna, Determination of submicrogram amounts of inorganic and organomercuric compounds by absorption spectrophotometry Intern. J. Environ. Anal. Chem. 63 (1995) 47 – 59.

[28]. M. Shamsipur, K. Farhadi, Electrochemical behavior and determination of ketoconazole from pharmaceutical preparations, Electroanalysis, 12 (2000) 429 – 433.

[29]. K. Farhadi, R. Maleki, Triiodide ion and alizarin red S as two new reagents for the determination of clotrimazole and ketoconazole, J Pharmaceut Biomed Anal., 30 (2002) 1023 – 1033.

[30]. (30) Odds, F.C., Milne, L.J.R., Gentles, J.C., & Ball, E.H. (1980) J. Antimicrob. Chemother. 6, 97–104

[31]. (31) Zarapkar, S.S., & Halkar, U.P. (1991) Indian Drugs 28, 265–269; Anal. Abstr. 53, 11G163

[32]. Abdel-Gawad, F.M. (1997) Farmaco 52, 119–122; Anal. Abstr. 59, 9G79

[33]. Roychowdhury, U., & Das, S.K. (1996) J. AOAC Int. 79, 656–658

[34]. El-Ragehy, N.A., & El-Bagary, R.I. (1993) Bull. Fac. Pharm. Cairo Univ. 31, 327–332

[35]. Atay, O., & Seluck, F. (1996) Anal. Lett. 29, 2163–2176

[36]. Abounassif, M.A., & El-Shazly, B.E. (1997) Anal. Lett. 22, 2233–2247

[37]. Cheng, Q., Peng, T.Z., Hu, X.B., & Yang, L.J. (1998) Fenxi Huaxue 26, 1315–1318

[38]. Zhang, C.X., Von-Heeren, F., & Tharmann, W. (1995) Anal. Chem. 67, 2070–2077

[39]. Al-Meshal, M.A. (1990) Anal. Lett. 22, 2249–2263

[40]. Riley, C.M., & James, M.O. (1986) J. Chromatogr. B 50 287–294

[41]. Yuen, K.H., & Peh, K.K. (1998) J. Chromatogr. B 715, 436–440

[42]. British Pharmacopoeia, Her Majesty's Stationary Office, London, UK

[43]. United States Pharmacopoeia, U.S. Pharmacopoeial Convention, Rockville, MD.

[44]. A.A. Ensafi, M.A. Chiamjangali, Spectrochim. Acta Part A 59 (2003) 2897–2903.

[45]. M.S. El-Shahawi, A.Z. Abu Zuhri, Bull. Chem. Soc. Jpn. 71 (3) (1998) 597–601.

[46]. M.S. El-Shahawi, A.B. Farag, Anal. Chim. Acta 307 (1) (1995) 139–144.

[47]. B.N. Barsoom, A.M.E. Abdelsamad, N.M. Adib, Spectrochim. Acta Part A 64 (2006) 844–852.

[48]. L.V. Ermakova, V.A. Podgornaya, Ind. Lab. 54 (9) (1989) 995–997.

[49]. A.G. Soldatov, S.V. Shiryaev, S.N. Barilo, J. Anal. Chem. 56 (12) (2001) 1077–1079.

[50]. S.-F. Qi, Y. Wang, J.-H. Deng, H. Zhao, Yezin Fenxi/Metall. Anal. 27 (12) (2007) 63–66.

[51]. M. Smiderie, B.F. Reis, F.R.P. Rocha, Anal. Chim. Acta 386 (1–2) (1999) 122–129.

[52]. M. Smiderie, M.C. Tomazzini, B.F. Reis, Quim. Nova 27 (1) (2004) 50–54.

[53]. M.S. El-Shahawi, F.A. Hasemi, Talanta 43 (1996) 2037–2043.

[54]. M.S. El Shahawi, Anal. Chim. Acta 356 (1997) 85–91.

[55]. S.M. Aldhaheri, Talanta 46 (1998) 1613–1618.

[56]. M. Kamburova, Talanta 39 (8) (1992) 997–1000.

[57]. S.A. Rahim,W.A. Bashir, Microchem. J. 29 (1) (1984) 87–91.

[58]. C.S.P. Sastry, T.E. Divakar, U. Viplava Prasad, Talanta 33 (2) (1986) 164–166.

[59]. A. G. Vlessidis, Nicholaos P. Evmiridis, Periodate oxidation and its contribution to instrumental methods of micro-analysis—A review, Analytica Chimica Acta 652 (2009) 85–127

Chapter 5

CONCLUSION AND FUTURE SCOPE OF WORK

In pharmaceutical industry, an important step consists in the removal of possible drug residues from the involved equipments and areas. The cleaning procedures must be validated and methods to determine trace amounts of drugs have, therefore, to be considered with special attention. On the basis of this study of cleaning validation using Loratadine as model drug, it appears that the use of UV spectrophotometry and reverse phase HPLC with UV detection for the quantification of API residues in cleaning validation samples in product formulation area is practical and can be used as part of a cleaning validation program in pharmaceutical manufacture of Loratadine.

Difficulties in assessing protein drug products by physico-chemical methods are often related to the presence of large amounts of excipients that interfere with the detection and separation of the active ingredient. The developed RP-HPLC and UPLC methods, were capable of quantifying recombinant erythropoietin in the presence of large amounts of HSA present in the formulations.

Similarly a rapid and sensitive reverse phase high performance liquid chromatography (RP–HPLC) and Ultra performance liquid chromatography (UPLC) method with UV detection for quantification of parathyroid hormone (PTH) in presence of meta-cresol as a stabilizer in a pharmaceutical formulation was also developed and validated.

A rapid and sensitive RP-HPLC method with UV detection for quantification of meta-cresol in pharmaceutical preparation of parathyroid hormone (PTH) has also been developed and validated.

The developed HPLC and UPLC methods for the assay of protein drugs in formulations were simple, highly sensitive, precise and accurate and have the potential of being useful for routine quality control. The time reducing and solvent

saving characteristics of UPLC method are very advantageous, compared to the most widely used conventional HPLC technique.

The important features and novelty of the proposed method for assay of ketoconazole include the use of amplification reactions for the first time for the assay of pharmaceutical formulations which led to very high sensitivity (molar absorptivity of 5 × 105 $Lmol^{-1}cm^{-1}$). Though the applicability of the method in ketoconazole formulations has been demonstrated, the application of the method can be extended for the determination of trace amounts of ketoconazole in plasma and water samples

List of Presentations

1. S. Rane, P. Padmaja, A new spectrophotometric method for the determination of ketoconazole based on amplification reactions, 19th Gujarat science congress on recent advances in science and technology at Vallabh Vidyanagar organized jointly by Sardar patel university and Gujarat science academy, Local chapter – Vallabh vidhyanagar – February, 2005 (Oral Presentation)
2. S. Rane, P. Padmaja, A spectrophotometric method for the determination of trace quantity of Loratadine during cleaning validation, Indian Chemical Society Vadodara Chapter, 4th All Gujarat Research Scholars Meet (AGRSM-2006) (Oral Presentation)
3. Shaligram Rane, Change Control and Process Validation

   IBC 2nd Annual BIOPROCESS INTERNATIONAL China conference, August 30- September 1, 2010 at the Westin Hotel Financial Center in Beijing, China. (Oral Presentation)

List of

Research Publications

1. Shaligram S. Rane, P. Padmaja, Spectrophotometric method for the determination of Ketoconazole based on amplification reactions, Journal of Pharmaceutical Analysis, .- In press
2. Shaligram S. Rane, Alkesh Ajameri, Rustom Mody, P. Padmaja, Development and validation of RP-HPLC and RP-UPLC methods for quantification of erythropoietin formulated with human serum albumin. Journal of Pharmaceutical Analysis, .- In press
3. Shaligram S. Rane, Alkesh Ajameri, Rustom Mody, P. Padmaja, Development and validation of RP-HPLC and RP-UPLC methods for quantification of parathyroid hormones (1-34) formulated with meta-cresol. for Journal of Pharmaceutical Analysis, .- Communicated and primary accepted
4. Shaligram S. Rane, Alkesh Ajameri, Rustom Mody, P. Padmaja, Validation of simple RP-HPLC method developed for the quantification of meta-cresol in parathyroid hormones formulation, Pharmaceutical Methods .-In press
5. Shaligram S. Rane, P. Padmaja, Development and Validation of Spectrophotometric & HPLC methods for detection and quantification of the Loratadine on swabs collected from pharmaceutical manufacturing equipment placebo in support of cleaning validation.- Communicated