CHAPTER 4

BIOAVAILABILITY ENHANCEMENT AND BIOANALYTICAL METHOD FOR TROVAFLOXACIN
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Bioavailability enhancement and bioanalytical method for trovafloxacin

BIOAVAILABILITY ENHANCEMENT: A NEW APPROACH

In pharmaceutical industry, the product development has evolved from herbal teas to stable pure formulations containing known amounts of chemical ingredients that have been defined as drugs. Now a day, it is understood that percent chemical strength is not the only criteria for chemical efficiency. A dosage form must contain not only the correct amount of the labeled drugs but also release the active ingredient upon administration to the patient. Thus clinical effectiveness and bioavailability were added to the criteria for effective drug product development. A drug therefore, should not only be safe and beneficial but its therapeutic claims must be based upon sound clinical evidence.

HISTORY

During the 1940s when Indian Drugs Act 1940 was passed the primary goal in the formation of drug products was to ensure that the dosage form contained the proper amount of active ingredient. But in 1945 Oser et al made suggestion that the official standards may not be adequate in assessing the performance of a drug product. The author reported that the physiological availability of the drug varied considerably among the tablets examined.

So it becomes necessary to put forward some steps in measurement of the release of drug from its dosage form.

In 1948, a laboratory procedure for determining the disintegration time of a tablet was adopted as an official test by the British Pharmacopoeia followed by the United States Pharmacopoeia and the National Formulary. In 1950 this test proved to be a valuable tool but provided no information concerning the rate of dissolution of drug from its dosage form.

Bedicottet al in 1958 showed some limitations of disintegration test and in 1964 Middletoletal showed the significance of dissolution test and their relationship between in vitro and in vivo release of drug product.

In another work Morrison and Compbell concluded that tablet disintegration test had limited usefulness in measuring bioavailability and must eventually be replaced by more critical tests.

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DEFINITIONS

Now it would be worthwhile to define the various terminologies that will be used in the description of the subject.

Bioavailability
Bioavailability is a measure of the rate and the extent of absorption of the active form or forms of a drug from its formulation as reflected by the concentration – time curve of the administered drug in systemic circulation.

Bioequivalence
Two formulations of a drug are said to be bioequivalent if the rate and the extent to which it reaches the systemic action after administration of their respective formulations and are statistically comparable. In general, two products may be said to be bioequivalent if 90% Westlake’s confidence interval for C_{max}, T_{max}, mean AUC_{(0-t)} are within ± 20% of that of the reference product. The margin may be reduced to ± 10% for drugs with a very low therapeutic index, such as antiarrhythmic, antiepileptic and anticoagulant drugs.

Pharmaceutical Equivalence
“Pharmaceutical equivalence” means the drug products that contain identical amounts of the identical active drug ingredient, i.e. the same salt or ester of the same drug, in identical dosage forms, but do not necessarily contain the same ingredient, and that meet the identical compendial or other applicable standard of identity, strength, quality and purity, including potency and where applicable, content uniformity, disintegration time and/or dissolution rates.

Pharmaceutical Alternative
Pharmaceutical alternative means drug products that contain identical therapeutic moiety, its precursor, but not necessarily in the same amount or dosage form or as the same salt or ester. Each such drug product individually meets either the identical or its own respective compendial or other applicable standard of identity, strength, quality and purity, including potency and where applicable, content uniformity, disintegration times and/or dissolution rates.

Therapeutic equivalent
A medicinal product is therapeutically equivalent with another product if it contains the same active substances or therapeutic moiety and, when administered to the same
individual, shows the same efficacy and toxicity as that product, whose efficacy and safety has been established.

\( C_{\text{max}} \):
This is the maximum drug concentration achieved in systemic circulation following drug administration.

\( T_{\text{max}} \):
It is the time required to achieve maximum drug concentration in systemic circulation.

**Area Under Curve (AUC):**
Area under the curve is the total area under the biological fluid (serum, blood, etc.) in the concentration-time curve as determined by the trapezoidal rule.

\( \text{AUC}_{(0-t)} \):
Area under the plasma concentration-time curve from drug administration to the last quantifiable concentration to be calculated using trapezoidal rule.

\[
\text{AUC}_{(0-t)} = \int_{t=0}^{C_t} \frac{(C_t + C_{t-1})(t - t-1)}{2}
\]

\( \text{AUC}_{(0-\infty)} \):
To avoid the dependence on the time of last sampling, the AUC is extrapolated to infinite time and is determined by the formula,

\[
\text{AUC}_{(0-\infty)} = \text{AUC}_{(0-t)} + \left( \frac{C_t}{K_{el}} \right)
\]

Where, \( C_t \) = last quantifiable concentration in elimination phase
\( K_{el} \) = Elimination rate constant

**Elimination Rate Constant:**
Terminal elimination rate constant is estimated as the absolute value of the slope of a simple linear regression of the natural logarithm of the plasma concentration during the terminal phase of the plasma-concentration profile.

**Pharmacodynamic Evaluation:**
It is the measurement of effect on a physiological process as a function of time, after administration of two different products to serve as a basis for bioequivalence assessment.
Analysis of Variance (ANOVA):
ANOVA is a statistical technique to identify sources of variances and estimate the
degree of variability. In most bioavailability studies, there are three readily identified
sources of variance namely formulation (treatment), subject and period; hence it is a 3-
way ANOVA.

Validation of Analytical Method:
Validation of an analytical method is the process by which it is established, by
laboratory studies, that the performance characteristics of the method or process meet
the requirements for the intended applications.

Drug Delivery System:
Drug delivery system is defined as therapeutic system which release drug at
predetermined rate for a fixed time either in systemic circulation or to a specified target
organ.

Elimination Half Life:
The half life \( t_{1/2} \) is the time taken by the plasma concentration or the amount drug in
the body to be reduced by 50 %.

\[
\frac{t_{1/2}}{K_{el}} = 0.693
\]

Pharmacokinetics:
Pharmacokinetics means the study of the fate of an active drug substance within the
organism and covers the absorption, distribution, biotransformation, and excretion of
the substance.

Minimum Effective Concentration (MEC):
This is the drug concentration in blood, plasma, or serum that has to be attained before
the desired biologic response can be achieved. If the drug concentration in the systemic
circulation does not reach the MEC, the drug product is considered clinically
ineffective. The minimum effective dose is the dose required to achieve the minimum
effective concentration\(^{10}\).

Therapeutic Level:
This is the drug concentration in blood, plasma, or serum that produces the optimum
intensity of the biologic response desired by the physician during drug therapy. The
therapeutic level always lies between the MEC and the minimum toxic level.
Minimum Toxic Level:
This is the drug concentration in blood, plasma, or serum above which toxic side effects are exhibited. The minimum toxic level can also be called the minimum safe level. The minimum toxic dose is the dose above which, toxic effects are experienced.

Onset:
This is the time required to achieve the MEC following the administration of the dosage form.

**Figure 4.1**

**BLOOD DRUG LEVEL VS TIME CURVE IN BIOAVAILABILITY STUDIES**

Pharmacological response and therapeutic effectiveness for a drug can be related directly to its observed blood levels. Bioavailabilities play a key role in explaining intra and inter subject variability in drug blood concentrations. Drug elimination processes, including metabolism and excretion, is also major contributing factors.
The bioavailability of a given drug entity can vary significantly depending upon the route of administration and the type of the dosage form. 

**Factors related to the drug and its dosage form:**
Size of the administered dose, multiplicity of the dose (dose regimen), and type of the dosage form and its route of administration.

**Factors related to the drug:**
Chemical structure, crystalline state, particle size, solubility, pKa, and dissociation constant.

**Factors related to the formulations:**
Effect of additives (binders, diluters disintegrating agents, lubricants etc.), influence of manufacturing (mixing, milling, granulation, compression etc.), and type of dosage form (solution, suspension, capsules, tablets etc.)

**Factors related to patients:**
Age, sex, disease state, and abnormal genetic changes

**Physiological factors related to the absorption:**
Gastrointestinal barrier, gastrointestinal pH, gastrointestinal motility and emptying, vascularity and blood flow, drug instability in the gastrointestinal tract, drug interaction and complexion, and mal-absorption.

**Variations related to the drug disposition processes:**
Pre systemic metabolism and binding partition in the body fat, metabolism and biotransformation, and excretion.

**BIOAVAILABILITY AND BIOLOGICAL RESPONSE**

There are two assumptions, which together form the foundation for the validity of bioavailability testing and the general approach to the pharmacokinetics. The first assumption is that drug concentration in blood is related directly to its concentration at the site of action in any part of the body. Drug plasma levels are in dynamic equilibrium with the levels in all tissues and body fluids. The equilibrium does not necessitate that drug levels in all body compartments be the same, but rather, are dependent on the drug concentration in the systemic circulation, and that the drug diffuses reversibly between the blood and the tissues.
The second assumption, in correlating drug blood levels with the biologic response, is that the intensity of the pharmacological activity of the drug is related directly to its concentration at the site of action.

MEASUREMENT OF BIOAVAILABILITY

The objective of bioavailability determinations is to demonstrate the biological availability of drug products in man. This encompasses the measurement of the rate at which a dosage form releases its active drug in vivo, the time it takes for the drug to appear in the systemic circulation, the percent of the dose released and the concentration in the blood.

BIOAVAILABILITY ENHANCEMENT

Conventional Approach

The drug manufacturers have tried to improve the bioavailability of drugs by physicochemical characteristics of the drug by the following practices

- micronisation of the active drug i.e. controlling the particle size
- deaggregation of the micronised particles by the use of protective colloids
- solublisation of the active drug by
  1. chemical derivatisation
  2. use of inclusion compounds such as with beta-cyclodextrine
  3. use of co-solvents and surfactant system
  4. complexation
  5. by solid phase manipulation
- Targeting and/or sustained release of the drug by
  1. film coating
  2. using polymorphic matrices for sustained leaching
- Pro-drug approach with a view to increase absorption (both the extent as well as the rate), prevent first pass effect and target the drug on to the tissue or organ which needs it most and passes requisite enzyme system to bio-reverse or release the active drug.
However, all these practices have their own problems and limitations.

**New Concept**

In the field of bioavailability a new approach was conceptualised while surveying the Ayurvedic literature\(^7\) for certain herbals of medicinal significance. Frequent and consistent repetition of certain herbal individually/as a group was noticed in large number of prescriptions recommended for a variety of diseases. One of the groups of herbals which is documented\(^8\) [very frequently as essential part of the prescriptions] is "TRIKATU" – the three acrids viz., Long pepper (Piper longum), Black pepper (Piper nigrum Linn.), and Ginger (Zingiber officinalis Rosc.) in equal proportions by weight.

Some modern Ayurvedic practitioners have tried to explain the scientific basis underlying the use of these herbals. According to Dutt and King\(^9\) (1900) these herbals are added to formulations often without reason and sometimes only for the sake of rhyme. Lakshmipathi\(^10\) (1940) reported the usefulness of these acrides in the maintenance of balance of *kapha, vatta and pitta*, the three humours of body according to Ayurveda. Bose\(^11\) (1928) was the only one who while describing the anti-asthmatic activity of vasaka leaves mentioned that addition of long pepper increases its efficacy.

Several experiments on modern drugs were performed to observe any enhancement in bioavailability, when these drugs were co-administered with the long pepper, black pepper and ginger.

The results of these experiments were the evidences describing use of these herbals or their active principles as bioavailability enhancer. It is also evident that these herbals were not included in the Ayurvedic formulations for the sake of rhyme but that they had definite role to play in increasing drug efficiency.

To check the enhancement in bioavailability of a modern drug, when co-administered with black pepper, the next chapter in this thesis is planned.
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Bioavailability enhancement and bioanalytical method for trovafloxacin

CHOICE OF DRUG

Among the various types of diseases, systemic infections due to microorganisms are prominent. There are many chemical compounds (synthetic drugs) which are effective against the microorganisms and called as an anti-bacterial drugs e.g. Acrosaxacin, Cinoxacin, Enoxacin and Ciprofloxacin etc. These drugs are also called as fluoroquinolones because of the fluorine in their chemical structure. These drugs are chemically related to nalidixic acid and are effective orally.

Fluoroquinolones are bactericidal and like nalidixic acid probably act by inhibiting bacterial DNA replication. The currently available fluoroquinolones are Acrosaxacin, Cinoxacin, Enoxacin, Ciprofloxacin, Norfloxacin, Ofloxacin and Pefloxacin. Since the bacteria can develop resistance to fluoroquinolones, new compounds are synthesized. The currently available new anti-bacterial drug is Trovafloxacin.

Trovafloxacin is a new synthetic fluoroquinolone anti-bacterial agent with a broad spectrum of activity against Gram-positive and Gram-negative bacteria and could be differentiated from Ciprofloxacin, Ofloxacin and other marketed fluoroquinolones by its greater potency against many clinically significant species of Gram-positive organisms, most notably against streptococci such as Streptococcus pneumoniae. Trovafloxacin administered orally was able to control systemic infections of Gram-negative organisms in mice; it was appreciably more potent than Temafloxacin, Ciprofloxacin and Ofloxacin in protecting mice against lethal infections with S. pneumoniae or S. pyogenes.

Since, Trovafloxacin is new synthetic fluoroquinolone having more potency than currently used fluoroquinolone, it was selected to check the enhancement in bioavailability when co-administrated with black pepper in present research work.
ROLE OF ASSAY METHOD IN BIOAVAILABILITY

Use of analytical methods to quantitate the levels of a drug and/or its metabolites are the important steps in bioavailability testing. The analytical method must be selective for the unchanged drug in the presence of its metabolites and other background interference in the sample matrix. It also must be sensitive enough to measure the expected low drug levels, especially in the last samples collected. The precision of the assay should be evaluated prior to the study and the coefficient of variation of the method must be considered in the design of the experimental protocol.

STATISTICAL ASPECTS OF BIOAVAILABILITY STUDIES

Statistical methods are used to estimate the certainty of statement and precision of measurement about the population after observing a random sample of its members. It is therefore absolutely essential that the following points be duly taken into consideration in order to improve the quality of the study.

- Statistical considerations must start at the stage of developing a protocol.
- The protocol must include reliability of measurement parameters, adequate controls, appropriate treatment of data and lead to clear conclusions.
- The method of statistical analysis should be appropriate for the type of the study undertaken depending on the objective of the study.

In bioavailability studies, it is normally assumed that each subject remains biologically constant throughout the study. Since this is rarely true, and since no two subjects are identical, it becomes critically important that the experimental data be analyzed by an appropriate statistical test. The intended statistical analysis should dictate the study design so that design constraints do not compromise study objectives. To decide whether one formulation becomes significantly different to another, statistical parameters are applied such as Analysis of variation, Confidence intervals etc.
ANALYSIS OF VARIANCE (ANOVA)

ANOVA is often used to test the hypothesis that the means of two populations are equal. The most important use of ANOVA is to identify sources of variation and estimate the size of that variability. In most bioavailability studies there are three readily identified sources of variation: formulation, subjects and periods. Interest is usually in only the formulations. The others are nuisance variances to be identified and removed. In a typical crossover model, there are two sequences of formulation; half the subjects get the test, then the reference, the other half get the reference, then test. These sequences may also be an effect in the statistical model. The often-used statistical model for measurement of variability in a pharmacokinetic model like AUC is

\[
AUC = M + S_j + s_{1(i)} + P_i + F_k + e_{1(i)jk}
\]

Where \( M \) is the overall mean, \( S_j \) is sequence effect, \( s_{1(i)} \) is the subject in sequence effect, \( P_i \) is period effect, \( F_k \) is formulation effect, and \( e_{1(i)jk} \) is the error term. By error term is meant all those other sources of variation, which are not identified. Subjects are said to be "in sequence" because each subject occurs with only one sequence, whereas each sequence occurs with every formulation and period. Often effects are classified as "fixed" or "random", with fixed effects those about which interferences are made and the variances of random effects are estimated. But for purposes of removing the variability of ANOVA this classification is not needed.

Since a mean has to be estimated, the degrees of freedom (df) is 1 less than the number in the classification. Thus the df for the total sum of squares (SS) is \( N-1 \); the df for formulation, period and sequences is 1. The SS represent the total variation about the mean of that effect. The SS of the total is number from which the variance of the data set is computed. The SS of all the effects add up to this total SS. Thus the total SS has the identifiable sources of variations plus the error SS. The mean square (MS) is SS divided by df and is the quantity used to compute the F-statistic. For formulation, period and subject (sequences) the error MS is used to compute F. The F-statistic for sequence is obtained by dividing the sequences MS by the subject (sequences) MS.
Although the interest does not lie in testing hypotheses, the F-statistic and its accompanying probability level give some idea of the relative strengths of the effects. The usefulness of ANOVA is that it is a way to identify sources of variation and evaluate their contribution to the total variability. Periods, for example, are mostly a random effect; they depend on availability of the clinic and other logistic problems. As such they will from time to time reach a level of significance. But if periods are large part of the total variation, one should probably look for something in the experimental set up that is not as well controlled as it should be. Likewise, subjects are usually assigned to sequences at random and the sequence effect will occasionally be significant. However, one problem with these simple two period, two formulation design is that interactions between the factors cannot be evaluated. The sequence effect is exactly the same as the formulation by period interaction. Thus, a large sequence effect may indicate that the difference between the formulations is not the same in both the periods. The treatment effect is a pointer to the variability between the formulations and would mean a large difference between the two formulations.

The most important use of ANOVA in bioavailability – bioequivalence studies is estimating the standard deviation of the error. Since all subjects have had both formulations in both periods, the standard deviation for the ANOVA may be used to compute confidence intervals on the mean differences.

**CONFIDENCE INTERVAL IN BIOAVAILABILITY-BIOEQUIVALENCE STUDIES**

The calculation of confidence interval plays a very important role in bioavailability /bioequivalence studies. According to the scientific community hypothesis tests are inappropriate in that products that are very close, but with small variance, may be deemed different, whereas products that are widely different, but with large variance may be considered as equivalent.

For non-transformed data the confidence interval is calculated using the formula:

$$\text{Limit Term} = \Delta \pm t \sqrt{\text{error term for MSS} \left(\frac{1}{N_1} + \frac{1}{N_2}\right)}$$

Where,
Δ = The difference between the standard and test formulation.

\( t \) = The ‘t’ value from the student t table for the given degrees of freedom.

\( N_1 \) and \( N_2 \) is the number of subjects taking the formulation in treatment one and treatment two respectively.

The confidence interval can be expressed as an approximate percentage relative bioavailability by dividing the lower and the upper limits by the average for the test formulation.

The lower and upper limits are calculated as:

\[
\text{Lower Limit} = \text{Mean value of Test} + \Delta - \sqrt{\text{error term for MSS} \left( \frac{1}{N_1} + \frac{1}{N_2} \right)}
\]

\[
\text{Upper Limit} = \text{Mean value of Test} + \Delta + \sqrt{\text{error term for MSS} \left( \frac{1}{N_1} + \frac{1}{N_2} \right)}
\]

The percentage confidence interval is calculated as follows:

\[
\text{Lower Interval} = \frac{\text{Lower limit}}{\text{Mean value for test}} \times 100
\]

\[
\text{Upper Interval} = \frac{\text{Upper limit}}{\text{Mean value for test}} \times 100
\]

The hypothesis that is now applied is that the

\( H_0: \frac{A}{B} < 0.8 \) and \( H_0: \frac{A}{B} > 1.25 \)
The conclusions are based on the confidence interval approach and are identical to two-sided 't' tests each performed at 5% confidence level. Now if either or both of the above hypothesis are not rejected, the products are not considered to be equivalent.

**Confidence Interval for Log Transformed Data**

The computation of the confidence interval is more direct when using the log-transformed data. The antilog of the average results gives directly the results. The results for the log transformed and the untransformed data are nearly similar. However, in cases where the confidence intervals are very close to the lower and/or upper limits, the two analyses may result in different conclusions.
BIOANALYTICAL METHOD

The present chapter describes the development and validation of bioanalytical method for the assay of Trovafloxacin from human plasma. The method was developed for application to assay the plasma samples collected during the bioavailability studies of trovafloxacin. The method developed is based on extraction of the drug from plasma by breaking the drug conjugate complex to release the drug which is then extracted in dichloromethane. The organic layer was evaporated to dryness. The residue was reconstituted and injected into the chromatographic system. The analysis was carried out on a Zorbax C$_{18}$ (250 x 4.6 mm, 5µ) column with a mobile phase comprising tri-ammonium citrate (0.05 M): Acetonitrile (ACN): Methanol (MeOH): tetrabutyl ammonium hydroxide (TBAH): tri-ethylamine (TEA) in the volume ratio of 70:20:10:1:0.2 (v/v) at pH 3.7. The detection was carried out at 271 nm, the wavelength of maximum absorption of trovafloxacin.

EXPERIMENTAL

Preparation of solutions

a) Preparation of 0.025 M KH$_2$PO$_4$ Buffer:
Accurately weighed 0.850 g of KH$_2$PO$_4$ and 0.887 g of Na$_2$HPO$_4$ were taken in a 250 cm$^3$ volumetric flask and dissolved in appropriate volume of water. It was then diluted to the mark with water. The pH of this buffer was adjusted to 3.0 with concentrated HCl.

b) Preparation of 0.05 M tri-Ammonia citrate solution:
Accurately weighed 12.161 g of tri-ammonia citrate was transferred to a 1000 cm$^3$ standard volumetric flask. It was dissolved in water and volume was made up to the mark with water and mixed well. This gave 0.05 M of tri-ammonia citrate solution.

c) Stock standard solution:
Accurately weighed 131.2 mg of mesylate salt of trovafloxacin (C$_{20}$H$_{15}$F$_3$N$_4$O$_7$HCl) was transferred into 100 cm$^3$ volumetric flask. The drug was dissolved in 0.025M KH$_2$PO$_4$ buffer and the volume was made with KH$_2$PO$_4$ buffer.
and mixed well. This gave a stock standard solution of the strength of 1000 µg/cm³ (solution A).

Profile of trovafloxacin is as follows:

**TROVAFOXACIN**

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>(1α, 5α, 6α)-7-(6-Amino-3-azobicycle[3.1.0]hex-3-yl)-1-(2,4-difluorophenyl)-6-fluro-1,4,dihydro-4-oxo-1.8-naptaylidine 3-carboxylic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Weight</td>
<td>416.36</td>
</tr>
<tr>
<td>Empirical Formula</td>
<td>C₂₀H₁₅F₃N₄O₃</td>
</tr>
<tr>
<td>Physical Characteristics</td>
<td>White Crystalline Powder</td>
</tr>
<tr>
<td>Solubility</td>
<td>Soluble in alcohol, sparingly soluble in water</td>
</tr>
<tr>
<td>Melting Point</td>
<td>246°C</td>
</tr>
<tr>
<td>Therapeutic category</td>
<td>Anti-bacterial</td>
</tr>
</tbody>
</table>

**Structural Formula**

![Structural Formula Image](image)

**Salt**

Trovafloxacin mesylate (C₂₀H₁₅F₃N₄O₃.HCl)
PLASMA drug concentrations of trovafloxacin were estimated by a specific high performance liquid chromatographic method employing a reverse phase mode of separation and a UV detector. The method was developed for application to the assay the plasma samples collected during the bioavailability study of trovafloxacin.
A method after development has to pass through the perils of method validation before being used.

**CHROMATOGRAPHIC PARAMETERS**

**Instrument**
A JASCO High Performance Liquid Chromatograph equipped with an PU-980 precision isocratic pump, a 7725i Rheodyne injection port fitted with a 100 μl loop and UV-970 variable wavelength detector. Data acquisition was performed by means of Borwin chromatography software (version 1.22).

**Column**
The separation of trovafloxacin was carried out on a Zorbax C_{18} column of dimensions 250 mm x 4.6 mm and guard column Zorbax C_{18} of dimensions 10 mm x 4.6 mm.

**Mobile Phase**
The mobile phase comprised tri-ammonium citrate (0.05 M): ACN: Methanol: tetrabutyl ammonium hydroxide (TBAH): tri-ethylamine (TEA) in the volume ratio 70:20:10:1.0.2. The pH of the mobile phase was adjusted to 3.7 using glacial acetic acid.

**Sample Preparation**
Plasma samples (1 cm³) were taken into a series of stoppered tubes and to them different concentrations of trovafloxacin were spiked. To these tubes 0.1 cm³ of 0.1 M NaOH was added. The mixtures were then vortexed for 1 minute. To these stoppered tubes, 10 cm³ of methylene dichloride was added, and these tubes were shaken for 10 minutes. These tubes were then centrifuged at 2000 rpm for 10 minutes. After centrifugation the aqueous layer was discarded, and 8 cm³ organic layer was separated. The organic layer was transferred to a centrifuge tube and evaporated to dryness in a low volume evaporator under the stream of N₂ at 40°C. A blank was
prepared in the same way without the drug solution. The residue was then reconstituted in 0.4 cm$^3$ mobile phase and 100 μl of this was injected into the chromatographic system.

**OPTIMIZED CONDITIONS FOR ANALYSIS**

<table>
<thead>
<tr>
<th>Pump</th>
<th>JASCO PU-980 isocratic HPLC pump</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td></td>
</tr>
<tr>
<td>Analytical</td>
<td>Zorbax ODS 5μ, (250 x 4.6 mm)</td>
</tr>
<tr>
<td>Guard</td>
<td>Zorbax ODS 5μ, (10 x 4.6 mm)</td>
</tr>
<tr>
<td>Detector</td>
<td>JASCO UV-970</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>Tri-ammonium citrate (0.05M): ACN: Methanol: tetrabutyl ammonium hydroxide (TBAH): tri-ethylamine (TEA) in the volume ratio 70:20:10:1:0.2 (v/v), pH adjusted to 3.7 using glacial acetic acid.</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1 cm$^3$/minute</td>
</tr>
<tr>
<td>Injection volume</td>
<td>100 μl</td>
</tr>
<tr>
<td>Detection wavelength</td>
<td>271 nm</td>
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</table>

**PREPARATION OF CALIBRATION CURVE FOR TROVAFLOXACIN FROM BLANK PLASMA**

For the preparation of calibration curve the stock solution of trovafloxacin 'solution A' was diluted with KH$_2$PO$_4$ buffer to yield a standard concentrations of 10 μg/cm$^3$, 50 μg/cm$^3$, 100 μg/cm$^3$, 200 μg/cm$^3$, 300 μg/cm$^3$, 400 μg/cm$^3$, 500 μg/cm$^3$. These standard solutions were used to prepare plasma standards ranging from 0.1 μg/cm$^3$ to 5μg/cm$^3$ by spiking 10 μl of the corresponding standard solutions into 1 cm$^3$ of blank plasma. The standards were prepared fresh on each day of validation from a freshly prepared stock solution.

The calibration plasma standards were analyzed by using the method described above. The AUC values obtained for the trovafloxacin peak were used for further calculations and statistical analysis. The linearity experiments were carried out seven times on seven different days. The plot of linear working range of trovafloxacin is
shown in figure 4.1. The results of calibration curve for trovafloxacin in plasma showing the linear working range are tabulated in Table 4.1, which gives the calibration data along with the standard deviation and coefficient of variation values for peak area values at each level. The peak area values were found to be linear in the range of 0.1 µg/cm³ to 5 µg/cm³. The data was further considered for statistical validation and regression analysis shown in Table 4.2.

The regression analysis of the calibration data was carried out to determine the relationship between the dependent variable (peak area value) and the independent variable (concentration).

Table 4.1

<table>
<thead>
<tr>
<th>Sr No.</th>
<th>Concentration (µg/cm³)</th>
<th>Mean Peak Area Value</th>
<th>S.D.</th>
<th>C. O. V. (%)</th>
<th>No. of Observations</th>
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<td>2332676</td>
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<td>158185</td>
<td>4.68</td>
<td>7</td>
</tr>
<tr>
<td>8</td>
<td>5.0</td>
<td>4212213</td>
<td>143116</td>
<td>3.40</td>
<td>7</td>
</tr>
</tbody>
</table>
Table 4.2

**BIO ANALYTICAL METHOD FOR TROVAFLOXACIN**

**REGRESSION ANALYSIS DATA**

<table>
<thead>
<tr>
<th>Concentration of trovafloxacin (µg/cm³)</th>
<th>Y Value Observed</th>
<th>Y Value Calculated</th>
<th>Residual Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>80388</td>
<td>83207</td>
<td>2819</td>
</tr>
<tr>
<td>0.5</td>
<td>400343</td>
<td>416033</td>
<td>15690</td>
</tr>
<tr>
<td>1</td>
<td>812253</td>
<td>832065</td>
<td>19812</td>
</tr>
<tr>
<td>2</td>
<td>1683404</td>
<td>1664130</td>
<td>-19274</td>
</tr>
<tr>
<td>3</td>
<td>2332676</td>
<td>2496195</td>
<td>163519</td>
</tr>
<tr>
<td>4</td>
<td>3383388</td>
<td>3328261</td>
<td>-55127</td>
</tr>
<tr>
<td>5</td>
<td>4212213</td>
<td>4160326</td>
<td>-51887</td>
</tr>
</tbody>
</table>

**REGRESSION OUTPUT**

<table>
<thead>
<tr>
<th>Constant</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard Error of Y Estimate</td>
<td>69166.52</td>
</tr>
<tr>
<td>R Squared</td>
<td>0.998</td>
</tr>
<tr>
<td>Number of Observations</td>
<td>8</td>
</tr>
<tr>
<td>Degree of Freedom</td>
<td>7</td>
</tr>
<tr>
<td>X Coefficient</td>
<td>832065.2</td>
</tr>
<tr>
<td>Standard Error of Coefficient</td>
<td>9304.45</td>
</tr>
</tbody>
</table>
The regression equation \( y = n x + C \) was found to be
\[
Y = 832065.2 x + 0
\]
Where
\( Y \) = dependent variable (absorbance)
\( M \) = slope of the regression line.
\( x \) = independent variable (concentration)
\( C \) = intercept on y axis

The regression equation indicates that one unit increase in the concentration of trovafloxacin will result in an increase in the peak area value by 832065.2.

The standard error of the \( Y \) estimate which quantifies the scatter of the points about the regression line was found to be 69166.52.

The correlation coefficient (r) of the standard calibration curve was found to be 0.998. This indicates that 99.80% (coefficient of determination \( r^2 \times 100 \)) of the variable in the response is explained by the variation in the drug concentration.

**ACCURACY AND PRECISION**

To ascertain the recovery of the experimental levels of drug concentration corresponding to lower middle and higher quantifiable concentrations of trovafloxacin were spiked in plasma and analyzed. The percentage recovery was then determined. The results demonstrate that the recovery of the drug was nearly 100%.

### Table 4.3

**BIO ANALYTICAL METHOD FOR TROVAFLOXACIN**

<table>
<thead>
<tr>
<th>Concentration in (µg/cm³)</th>
<th>% Recovery*</th>
<th>Standard Deviation</th>
<th>Coefficient of Variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>96.61</td>
<td>2538</td>
<td>3.16</td>
</tr>
<tr>
<td>2.0</td>
<td>101.16</td>
<td>58012</td>
<td>3.45</td>
</tr>
<tr>
<td>5.0</td>
<td>101.25</td>
<td>143116</td>
<td>3.40</td>
</tr>
</tbody>
</table>

*Each value is a mean of five determinations.*
The results obtained for repeat analysis carried out to ascertain the precision of the method are presented in Table 4.4.

<table>
<thead>
<tr>
<th>Day</th>
<th>0.5 µg/cm^3</th>
<th>2.0 µg/cm^3</th>
<th>4.0 µg/cm^3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.48</td>
<td>2.04</td>
<td>3.92</td>
</tr>
<tr>
<td>2</td>
<td>0.49</td>
<td>2.03</td>
<td>4.01</td>
</tr>
<tr>
<td>3</td>
<td>0.51</td>
<td>1.89</td>
<td>4.06</td>
</tr>
<tr>
<td>4</td>
<td>0.50</td>
<td>1.98</td>
<td>4.10</td>
</tr>
<tr>
<td>5</td>
<td>0.52</td>
<td>2.02</td>
<td>4.12</td>
</tr>
<tr>
<td>Mean</td>
<td>0.50</td>
<td>1.99</td>
<td>4.04</td>
</tr>
<tr>
<td>S. D.</td>
<td>0.02</td>
<td>0.06</td>
<td>0.08</td>
</tr>
<tr>
<td>% C.O.V.</td>
<td>3.16</td>
<td>3.08</td>
<td>1.98</td>
</tr>
<tr>
<td>% Recovery</td>
<td>100.00%</td>
<td>99.60%</td>
<td>101.05%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Day</th>
<th>0.5 µg/cm^3</th>
<th>2.0 µg/cm^3</th>
<th>4.0 µg/cm^3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.51</td>
<td>2.03</td>
<td>4.10</td>
</tr>
<tr>
<td>2</td>
<td>0.49</td>
<td>2.02</td>
<td>3.93</td>
</tr>
<tr>
<td>3</td>
<td>0.49</td>
<td>1.95</td>
<td>4.05</td>
</tr>
<tr>
<td>Mean</td>
<td>0.50</td>
<td>2.00</td>
<td>4.03</td>
</tr>
<tr>
<td>S. D.</td>
<td>0.01</td>
<td>0.04</td>
<td>0.09</td>
</tr>
<tr>
<td>% C.O.V.</td>
<td>2.33</td>
<td>2.18</td>
<td>2.17</td>
</tr>
<tr>
<td>% Recovery</td>
<td>99.333%</td>
<td>100.000%</td>
<td>100.667%</td>
</tr>
</tbody>
</table>
CHAPTER 4

Bioavailability enhancement and bioanalytical method for trovafloxacin

SENSITIVITY

To determine the lowest limit of quantification, the drug was spiked in plasma at lower level concentrations. The response of seven such observations was found and the coefficient of variation was determined. It was seen that at concentrations of 0.1 \( \mu g/cm^3 \), the coefficient of variation was less than 20%. Thus the lower limit of quantification was found to be 0.1 \( \mu g/cm^3 \) with coefficient of variation value less than 20%.

SPECIFICITY AND SELECTIVITY

The absence of peak in the blank at the retention time of trovafloxacin indicates the specificity of the method from plasma.

SAMPLE STABILITY

Plasma samples of trovafloxacin from volunteers were stored at \(-20^\circ C \pm 5^\circ C\). The samples to be analyzed were removed from the deep freezer and thawed at room temperature and processed. The samples were found to be stable till the completion of analysis.

QUALITY CONTROL SAMPLES

Pools of plasma samples spiked with the drug at three different concentrations were prepared and stored along with the volunteer plasma samples. These samples were analyzed with the volunteer plasma samples and the concentration of the drug was determined. The coefficient of variation for the levels was found to be less than 15%.
RESULTS AND DISCUSSION

The results of the above mentioned method validation can be summarized as follows:

**Linearity Range**
0.1 µg/cm³ to 5.0 µg/cm³

**Reproducibility**
The mean accuracy was in the range 99.60 % to 101.05 % for inter-day quality control samples and 99.33 % to 100.67 % for intra-day quality control samples. For quality control samples the inter-day precision was in the range 1.98 % to 3.16 % and the intra-day precision was 2.18 % to 2.33 %.

**Sensitivity**
The lowest limit of quantification was 0.1 µg/cm³.

**Specificity**
There were no interfering peaks observed at the retention time of the drug trovafloxacin.

**Recovery**
The mean recovery at the concentration of 0.5 µg/cm³, 2.0 µg/cm³ and 4.0 µg/cm³ was 100.00 % (Low), 99.60 % (Medium), 101.05 % (High) respectively.
Typical Chromatograms of the standard trovafloxacin blank plasma, spiked plasma sample and subject plasma samples are shown as follows.

**STANDARD RUN**

![Typical Chromatogram of the standard trovafloxacin blank plasma.](image)

**BLANK RUN**

![Typical Chromatogram of the spiked blank plasma.](image)

**SPIKED STANDARD RUN**

![Typical Chromatogram of the spiked standard run.](image)

2.0 hr Subject plasma

![Typical Chromatogram of the subject plasma sample.](image)
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Bioavailability enhancement and bioanalytical method for trovafloxacin


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