Bioanalytical Method Development and Validation of Griseofulvin Nanoparticles using RP-HPLC

Selvadurai Muralidharan[1*], Vijayan Venugopal[2], Jayaraja Kumar[2], Joshua Ho Chen Chiang[1], Julin Bee Chu Chin[1] and Siew Chiu Ghan[1]

[1]Unit of Pharmaceutical chemistry, Faculty of Pharmacy, AIMST University, Semeling, Bedong, Malaysia.

[2]Unit of Pharmaceutical Technology, Faculty of Pharmacy, AIMST University, Semeling, Bedong, Malaysia.

ABSTRACT

Objective: To prepare griseofulvin nanoparticles by emulsification solvent evaporation method and to evaluate its physical characters and bioavailability-bioequivalence properties. **Methods:** Bioanalytical method development of griseofulvin nanoparticle by using RP-HPLC method. The particle size and zeta potential was determined by scanning electron microscopy and Zetasizer, respectively and *in vitro* study was done by ultra centrifugation method. The release kinetics was also studied by fitting into few mathematical models. The bioanalytical study in rats was carried out. **Results:** The *in vitro* release studies showed that after the initial burst, all the drug-loaded batches provided can be substantiated by the fact that release profile of drug molecules, irrespective of their chemical nature was almost linear with time. For the polymer like PMMA, drug particles present in the surface of matrix is initially released into medium generating many pores and cracks which facilitate further drug release. Bioavailability-bioequivalence studies showed that the release of drug from nanoparticles was diffusion-controlled, and the mechanism of drug release was Fickian. The bio-distribution of these particles after intravenous injection in rats showed that of griseofulvin nanoparticles containing PMMA altered the bio-distribution pattern. **Conclusion:** Griseofulvin nanoparticles are proven to be an optimized drug delivery system with potential significance as an antifungal medicament. The preparation showed optimal bioavailability-bioequivalence characteristics in a rat model.

Key words: Bioanalytical method development, Bioavailability, Griseofulvin, HPLC, Nanoparticles.

INTRODUCTION

Nanoparticles are solid colloidal particles in the size range of 1-1000 nm. Nanoparticles are precisely designed to absorb or encapsulated a drug substances. The predominant methods to deliver drugs are oral and injection. The drug is expected to circulate whole body affecting the organ and cell that are dysfunctioning as well as those which are healthy.[1] It may cause serious side effects. The efficacy of drug is narrow by their potential to reach the site of therapeutic action, only a small amount of dose reaches the target site and majority of drug is distributed to the rest of the body with its physiochemical and biochemical properties, therefore developing a drug delivery system that optimizes the action of drug while reducing the side effects *in vivo* is a challenging task.

When drugs is loaded into nanoparticles through physical encapsulation, adsorption or chemical conjugation, the pharmacokinetics and therapeutic index of the drugs can be significantly improved in contrast to the free drug counterparts.[2] Many advantages of nanoparticle-based drug

***Address for correspondence:**
Dr. Selvadurai Muralidharan, Unit of Pharmaceutical chemistry, Faculty of Pharmacy, AIMST University, Semeling, Bedong, Malaysia.
E-mail: murali23pharm@hotmail.com
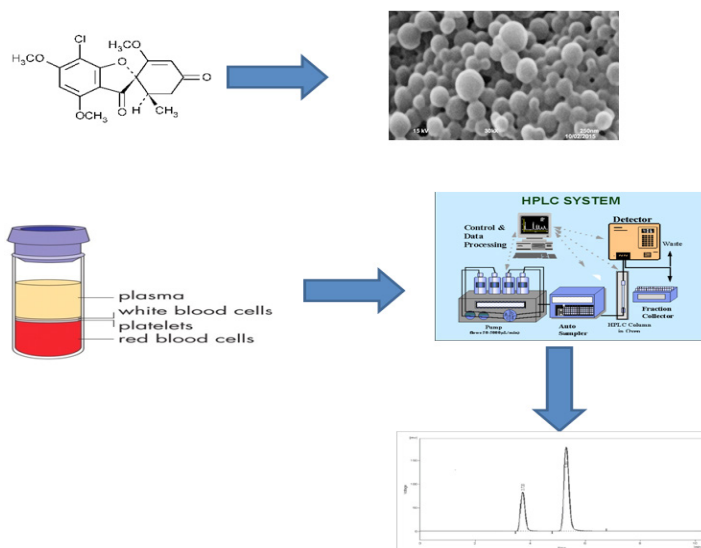
Graphical Abstract

delivery have been recognized, including improving serum solubility of the drugs, prolonging the systemic circulation lifetime, releasing drugs at a sustained and controlled manner, preferentially delivering drugs to the tissues and cells of interest, and concurrently delivering multiple therapeutic agents to the same cells for combination therapy.

Griseofulvin is an anti-dermatophytous drug. Currently, because of numerous oral complications, its application is restricted. griseofulvin insoluble in water and oral bioavailability of griseofulvin with poor solubility is limited. In attempt to increase bioavailability and efficacy, nanoparticle formulation have been more and more frequently employed over recent years.

The literature survey shown only limited nanoparticles methods of measuring griseofulvin have been reported.[3-9] To the best of our knowledge no reports were found for the validation of griseofulvin in drug free animal plasma. The objective of this study was to develop and validate an assay for the griseofulvin using RP-HPLC.

MATERIALS AND METHODS

Preformulation Studies

Preformulation testing is the first step in the rationale development of dosage forms of drug substance. It can be defined as an investigation of physical and chemical properties of drug substance alone and when combined with excipients. The overall objective of preformulation testing is to generate information useful to the formulator in developing stable, efficacious and safe dosage form.

Hence preformulation studies were carried out on the obtained samples of drug for identification and compatibility studies.

Identification of Drugs:

The obtained sample was examined by infrared absorption spectral analysis and was compared with the reference standard IR spectrum of griseofulvin.

Solubility studies

Solubility of pure griseofulvin
About 10 mg of pure griseofulvin was dissolved in 10 mL of solvent like water, 0.1 N HCL, methanol, ethanol, Dichloro methane and phosphate buffer (pH 7.4). The solubility was observed by sedimentation of particles (Gopinath, 2011).

Preparation of the standard calibration curves

Standard calibration curve of griseofulvin
Griseofulvin (10 mg) was dissolved in 100 mL DCM. From these stock solution aliquots of 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mL were withdrawn in 10 mL volumetric flask and diluted up to the volume with pH 7.4 phosphate buffer solutions to give concentrations of 5, 10, 15, 20, 25, and 30 mg/mL. Absorption of each solution was measured at 291 nm using Shimadzu HPLC and pH 7.4 phosphate buffers as a reference standard.

Compatibility Studies

The compatibility of drug and polymers under experimental condition is important prerequisite before formulation.

Incompatibility between drugs and excipients can alter stability and bioavailability of drugs, thereby, affecting its safety and efficacy. Study of drug–excipients compatibility is an important process in the development of a stable dosage form. Drug–excipients compatibility testing at an early stage helps in the selection of excipients that increases the probability of developing a stable dosage form (Prasanta, 2012).

FTIR Spectra

The FT-IR spectra were by using SHIMADZU FTIR-8400S spectrophotometer and the spectrum was recorded in the region of 4000-400 cm^{-1}. The samples (drug, polymer and mixture of drug and polymers) were mixed with 200-400 mg of potassium bromide (KBr). The samples were compressed as discs by applying pressure of 5 tons for 5 minutes in a hydraulic press. The prepared pellet was placed in the light path and the spectrum was recorded (Maltesen, 2009).

Method of preparation of griseofulvin PMMA nanoparticles

Griseofulvin nanoparticles were prepared by emulsification solvent evaporation technique. Required quantity of griseofulvin was dissolved in DCM and required quantity of PMMA was dissolved in 5 ml of ethanol. The drug solution was added in drops to the 1% PVA solution and emulsified under high pressure homogenizer at 20,000 rpm for 15 mins. To complete the precipitation process, 200 ml of water was added and mixed in a magnetic stirrer. Organic solvent was removed by using rotovapor. The thus formed nanoparticles suspension was freeze-dried under -20ºC to get free flowing powder of nanoparticles.

Evaluation of nanoparticles

Morphology of Nanoparticles
Morphology of Nanoparticles was observed by Scanning Electron Microscope (Masotti, 2007). A small amount of nanoparticle samples was spread on a metal stub. The stub was then coated with conductive gold by Hitachi 1010 ion sputter and was examined under Hitachi 3000N scanning electron microscope (JSM 5610 LV SEM, JEOL, Japan) chamber. The image was photographed at an acceleration voltage of 20 kV with a chamber pressure of 0.6 mmHg.

Nanoparticles size
Nanoparticles size was determined using a Zetasizer 300 HS (Malvern instruments UK). Samples were diluted with distilled water and measured at a temperature of 25ºC (Mansour Mansouri, 2011). The diameter was calculated from the autocorrelation function of intensity of light scattered from nanoparticles. The Particles measured are in triplicate.

Poly dispersibility index
The PDI was calculated for dispersion homogeneity ranging from 0 to 1. The value close to 0 indicated a homogeneous dispersion and greater than 0.3, high heterogeneity. The PDI was calculated by using the formula (Nidhin, 2008),

$$PDI=[D_{0.9} - D_{0.1}] \div D_{0.5}$$

Where $D_{0.9}$, $D_{0.1}$, $D_{0.5}$ is particle diameter determined at 90th, 10th, 50th percentile of undesired particles respectively.

Zeta potential
The charge of the Nanoparticles was determined by measuring the zeta potential by laser dropper anemometry using Zetasizer 3000HS. Nanoparticles were diluted with distilled water and the samples were placed in the electrophoretic cell where the potential of 150 mV was established.

In vitro release studies
In vitro release of nanoparticles was determined by using Franz diffusion cell. The cell has 20 ml receptor volume. The area of diffusion was 5 cm². The cell was placed in between the cell stirrer and the water bath where the temperature was maintained at 32 ± 0.5°C. Cellophane membrane (molecular weight cut-off: 6000-8000) previously soaked in receptor medium was clamped in between the donor and the receptor chamber of diffusion cell. A suitable aliquot of the formulation (100 mg of nanoparticles equivalent to 10 mg drug) was added to the donor chamber of the diffusion cell which was occluded with a paraffin film. The receptor medium (7.4 phosphate buffer) was stirred by magnetic bar. 1 ml sample was withdrawn from the receptor compartment at the following time intervals: 2, 4, 6, 8, 18, 20, 22 and 24 h and replaced by equal volume of the fresh receptor fluid (Anto Shering, 2011). The samples withdrawn were centrifuged (20,000 rpm, for 30 minutes, at cool temperature). The drug content of supernatant was estimated by using HPLC technique.

Optimization of chromatographic conditions for the estimation
Proper selection of the chromatographic method depends upon the nature of the sample (ionic or neutral molecule), its molecular weight and solubility. The drugs selected for the present study are polar in nature and hence either reverse phase or ion pair or ion exchange chromatography can be used. For the present study reverse phase HPLC

methods are considered to be more suitable because they are extremely specific, linear, precise, accurate, sensitive and rapid methods.

Preparation of standard and sample solutions

Preparation of standard and sample griseofulvin solutions

Standard stock solution of griseofulvin

10 mg of griseofulvin working standard was accurately weighed and transferred into a 10 ml volumetric flask and dissolved in methanol-water mixture (1:1) and made up to the volume with the same solvent to produce a 1mg/ml of griseofulvin. The stock solution was stored in refrigerator at -20 ± 2^0C until analysis.

The stock solution was diluted to suitable concentrations for spiking plasma to obtain calibration curve (CC) standards and quality control (QC) samples.

Calibration Curve Standards and Quality Control Samples

Working solutions for calibration and controls were prepared from the stock solution by an adequate dilution using water. Calibration standards for control plasma were prepared by spiking this stock solution to obtain the concentration levels of 100, 200, 400, 600, 800 and 1200 ng/ml in plasma. Quality control samples were prepared as bulk, at a concentration of 100 ng/ml (LQC), 400 ng/ml (MQC) and 1200 ng/ml (HQC).

These samples were stored below -50°C until use.

Plasma samples

Calibration standards, validation QC samples and plasma samples were prepared by adding 0.5 ml plasma to 2.0 ml centrifuge tube and 0.5 ml of precipitating agent (10% w/v tricholoroacetic acid) was added and then vortex for 2 min. The resulting solution was centrifuged at 4000 rpm for 7 min. The supernatant layer was separated and estimated by HPLC.

Validation of HPLC methods

Validation (10) is a process which involves confirmation or establishment by laboratory studies that a method/procedure/system/analyst can give the required accuracy, precision, sensitivity, ruggedness, etc. In the most basic form, validation of an analytical procedure demonstrates that the procedure developed is suitable for its intended purpose. Validation of the method was carried out after the development of the HPLC methods.

Validation parameters tested were Selectivity/ Specificity, Sensitivity, Linearity, Precision and Accuracy, Within-batch precision and accuracy, Intra-day precision and accuracy, Between batch / Inter-day precision and accuracy, Stabilities, Short Term Stock Dilution Stability, Long Term Stock Solution Stability, Freeze Thaw Stability, Bench Top (BT) Stability, Long-Term (LT) Stability, Autosampler Stability, Recovery, Ruggedness, Robustness

Selectivity/Specificity

A method is said to be specific when it produces a response only for a single analyte. Method selectivity is the ability of the method to produce a response for the analyte in the presence of other interferences. In order to prove that the method chosen was specific and selective the following two sets of samples were processed and injected into the HPLC using the extraction procedure.

- Blank samples from six different lots of biological matrix (plasma containing K2EDTA as anticoagulant).
- Samples from the same six lots of biological matrix mentioned in step 1 Spiked with the analyte at the lower limit of quantification (LLOQ) of the calibration curve and with the internal standard at the concentration level in the study.

To calculate % interference, the response obtained for each sample in step 1 was compared with the response obtained for each corresponding sample in step 2.

% Interference=(Peak area response of blank/Peak area response of LLOQ)X100

Sensitivity

It is expressed as limit of quantitation. It is the lowest amount of analyte in a sample matrix that can be determined. The lower limit of quantification for griseofulvin 100 ng/ml.

Linearity

Linearity and range of the methods were analyzed by preparing calibration curves using different concentrations of the standard solution containing the internal standard. The calibration curve was plotted using response factor and concentration of the standard solutions.

Linearity was established using four calibration curves over the range of (100 to 1200 ng/ml for griseofulvin) using the weighted least square regression analysis.

Precision and Accuracy

The precision and accuracy of the method was determined by analyzing two batches each consisting of one set of

calibration curve with six replicates of quality control samples at four concentration levels [Quality Control samples at Low (QCL), Middle (QCM) and High(QCH)].

Precision

Precision is expressed as the percentage coefficient of variation (%CV) which is calculated as per the following expression:

$$\% \ CV = (Standard \ Deviation \ / Mean) x100$$

Intra-run Precision

Intra-run precision was determined by calculating the percentage coefficient of variation (%CV) of the results obtained in the same run.

Intra-day Precision

Intra-day precision was determined by calculating the percentage coefficient of variation (% CV) of the results obtained in the same day.

Inter-day Precision

Inter-day precision was determined by calculating the percentage coefficient of variation (% CV) of the results obtained over at least two days.

Accuracy

Accuracy is reported as % nominal of the analyzed concentration which is calculated as:

$$\% \ Nominal = (Measured \ Concentration / Actual \ Concentration) x100$$

Intra-run Accuracy

Intra-run accuracy was determined by calculating the percentage nominal of the calculated concentration from the actual values for quality control samples at each concentration level analyzed in a single run and the mean of percentage nominal at each level was reported.

Intra-day Accuracy

Intra-day accuracy was determined by calculating the percentage nominal of the calculated concentration from the actual values for quality control samples at each concentration level analyzed in a single day and the mean of percentage nominal at each level was reported.

Inter-day Accuracy

Inter-day accuracy was determined by calculating the percentage nominal of the calculated concentration from the actual value for quality control samples at each concentration level analyzed over at least two days and the mean of percentage nominal at each level was reported.

Stock Solution Stability

Short Term Stock Dilution Stability

The stability of stock dilutions of analyte and the internal standard was evaluated at room temperature. Aqueous stock dilution of the analyte and the internal standard were prepared. One portion of the stock dilution was placed in the refrigerator between 2-8°C, while the other portion was placed at room temperature for 24 h. Stock dilution stored at room temperature (stability samples) was compared with refrigerated stock dilutions considered as 'comparison samples'. Six replicate injections of the above solutions were made.

Acceptance Criteria: % stability should be within 90 to 110% or the % change should be ± 10%.

$$\% \ Stability = (Mean \ response \ of \ stability \ samples / Mean \ response \ of \ comparison \ samples) \times 100$$

$$\% \ Change = 100 - (Mean \ response \ of \ stability \ samples / \ Mean \ response \ of \ comparison \ samples \times 100)$$

Long Term Stock Solution Stability

The stability of the stock solution when stored in the refrigerator for a given period of time was determined. Stock solutions of the analyte and internal standard were prepared and stored in the refrigerator between 2-8°C for 7 days (stability stock). The stock solution stabilities of the analyte and the internal standard were determined with a comparison stock solution, which was prepared freshly. Five replicate injections of the above solutions were made. The response of comparison samples were corrected by multiplying with correction factor to nullify the difference between the measured weights or the dilutions made.

Correlation factor(CF)=Concentration of comparison stock/Concentration of stability stock

$$\% \ Stability = (Mean \ response \ of \ stability \ samples / Mean \ response \ of \ comparison \ samples) \times CF \times 100$$

$$\% \ Change = 100 - (Mean \ response \ of \ stability \ samples / \ Mean \ response \ of \ comparison \ samples) \times CF \times 100$$

Freeze Thaw Stability

This test was done to ensure that the analyte was stable in the biological matrix even after multiple freeze-thaw cycles.

- Six quality control samples each at low and high concentrations stored below −50°C for at least 24 h were removed from the deep freezer and were

allowed to thaw unassisted at room temperature (fT4 samples). These samples were frozen again below –50°C for at least 12 h.

- Another set of six quality control samples at low and high concentration levels (fT3 samples) were removed from the deep freezer along with the fT4 samples and thawed unassisted. Both sets of samples were replaced back into the deep freezer.
- At least after 12 h of freezing, fT4, fT3 and another set of six samples each at low and high concentration levels (fT2 samples) were removed from the deep freezer and thawed unassisted. All the samples were replaced back into the deep freezer.
- At least after 12 h of freezing, fT4 samples were taken out from deep freezer, thawed unassisted to room temperature and analyzed with freshly prepared calibration curve (CC) solutions.

Bench Top (BT) Stability

Six quality control samples each at Quality Control sample at Low concentration (QCL) and Quality Control sample at High concentration (QCH) levels were stored at room temperature for 3 and 6 h. The above samples were analyzed along with freshly prepared calibration curve standards by using the method being validated.

Long-Term (LT) Stability

To assess the stability of the analyte in the biological matrix under the same conditions of storage as that of the study samples for the time period between the date of first sample collection and the date of last sample analysis, the following test was performed.

Six samples of each quality control samples at low and high concentrations were stored below -50°C in the deep freezer. The stability of the analyte was evaluated by comparing each of the back calculated concentrations of stability Quality Control sample (QCs) with the mean concentrations of the respective QCs analyzed in the first accepted precision and accuracy batch.

Auto sampler Stability

To evaluate the stability of the samples in the autosampler after processing for the anticipated run time, six sets of quality control samples each at low and high concentrations were placed in the auto sampler for 24 h and 48 h. The quality control samples were retained in the autosampler to prove auto sampler stability. After the lapse of the test time, the samples placed in the auto sampler were injected into the system along with freshly prepared calibration

curve standards. The stability of the analyte was evaluated by comparing the back calculated concentration of stability samples from the freshly prepared calibration curve with their respective nominal concentrations.

To determine the auto sampler stability of the internal standard, the mean peak area obtained for the internal standard of the stability samples was compared with that of the mean of internal standard area of accepted non-zero calibration curve solution (CC's) and the percent change was calculated using the following expression:

%Change=100–{(Mean of internal standard peak area in the stability samples/Mean of internal standard peak area of the accepted non–zero CCs) x 100)}

Recovery

Absolute recovery of a bio analytical method is the measured response obtained from a certain amount of analyte added to and extracted from the biological matrix, expressed as a percentage of the response obtained for the true concentration of the pure authentic standard which has not been subjected to the extraction procedure.

To determine recovery of this method, six replicates of aqueous quality control samples (un extracted) with concentrations close to spiked Quality Control sample at Low concentration (QCL), Quality Control sample at Middle concentration (QCM) and Quality Control sample at High concentration (QCH) concentration (extracted) were prepared. These un-extracted samples were injected along with precision and accuracy batch.

%Recovery of analyte at each level was calculated using the following expression:

{[Individual analyte peak area of extracted QCs x Concentration of analyte added (un-extracted sample)]/ [Mean analyte peak area of aqueous QCs x Concentration of analyte added (extracted sample)]}x100

The mean and standard deviation for the percent recovery obtained and there by the percent variation (%CV) was calculated at each concentration level. The overall percent recovery was calculated as the mean of recoveries obtained at the three quality control levels (QCL, QCM and QCH). The overall percent variation (% CV) was also calculated.

The percentage recovery for the internal standard was also calculated. The peak area response of the internal standard obtained for the extracted QCM sample (analyzed in the precision and accuracy batch) was compared with the mean
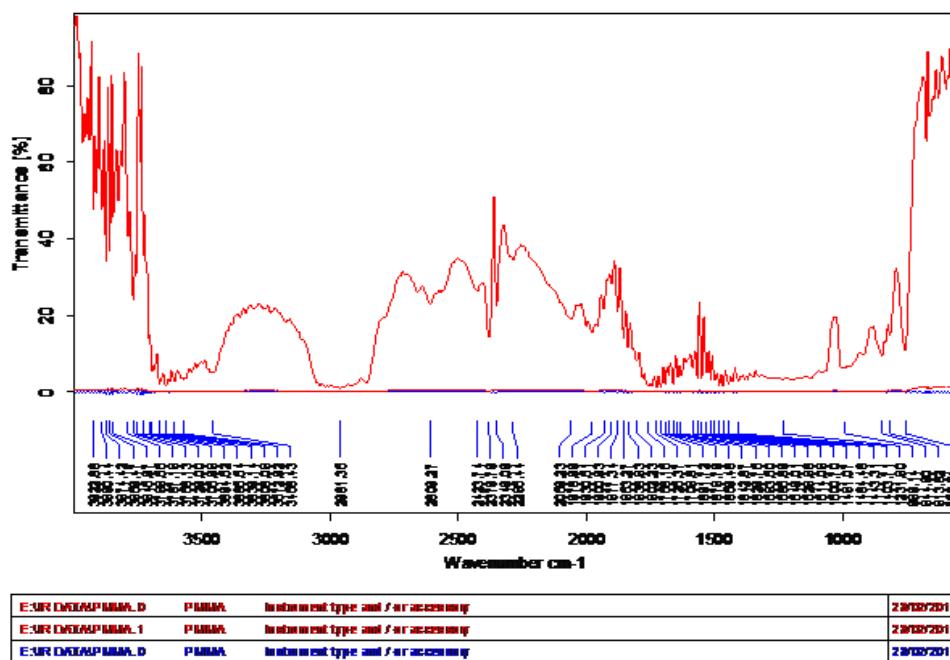
Figure 1: FTIR Spectrum of Griseofulvin

area response of the internal standard obtained for the un-extracted QCM samples.

Ruggedness

Ruggedness of the method was studied by changing the experimental conditions such as operators, instruments, source of reagents, solvents and column of similar type. Chromatographic parameters such as retention time, asymmetric factor, capacity factor and selectivity factor were evaluated.

Robustness

Robustness of the method was studied by injecting the standard solutions with slight variations in the optimized conditions namely, ± 1% in the ratio of acetonitrile in the mobile phase, ± 0.5 units in the pH of the buffer, ± 0.5 ml volume of the triethylamine in aqueous phase and ± 0.1 ml of the flow rate.

RESULTS AND DISCUSSION

Identification of Pure Drugs

Determination of IR spectrum of Griseofulvin

The FTIR studies showed that the significant peaks of griseofulvin where C-N stretching at 1444.02 cm-1,C=O cm^{-1} vibration at 1682.16 cm^{-1} ,C-O-C at 1085.35 cm^{-1},

N-H cm^{-1} at 3301.77 cm^{-1},C=C group vibration at 1630.68 cm-^{1} and O-H vibration at 2799.45 cm^{-1} (Figure 1). Based on that FTIR spectrum of griseofulvin functional groups peak was coincided with standard griseofulvin pure drug. Based on this result the drug was confirmed as in its pure form without by-products.

Solubility studies

Solubility of griseofulvin was performed in various solvents like water, 0.1 N HCL, methanol, ethanol, Dichloro methane and phosphate buffer (pH 7.4). From the above solvent griseofulvin was freely soluble in Dichloro methane and ethanol, whereas remaining solvents shows insoluble particle sediment in the bottom of test tube.

Preparation of griseofulvin polymeric nanoparticles

Polymeric nanoparticles of griseofulvin were prepared by emulsification solvent evaporation method with three different biodegradable polymer (Table 1). This method is comparatively easy to prepare than other methods. Suspension of polymer and drug in dichloromethane as organic phase. This organic solution was poured into an aqueous phase containing 1% PVA. The organic solvent used in these preparation rapidly partitioned into the external aqueous phase and the polymer precipitated around the drug particle. The subsequent evaporation of the entrapment solvent led to the formation of polymeric nanoparticles.

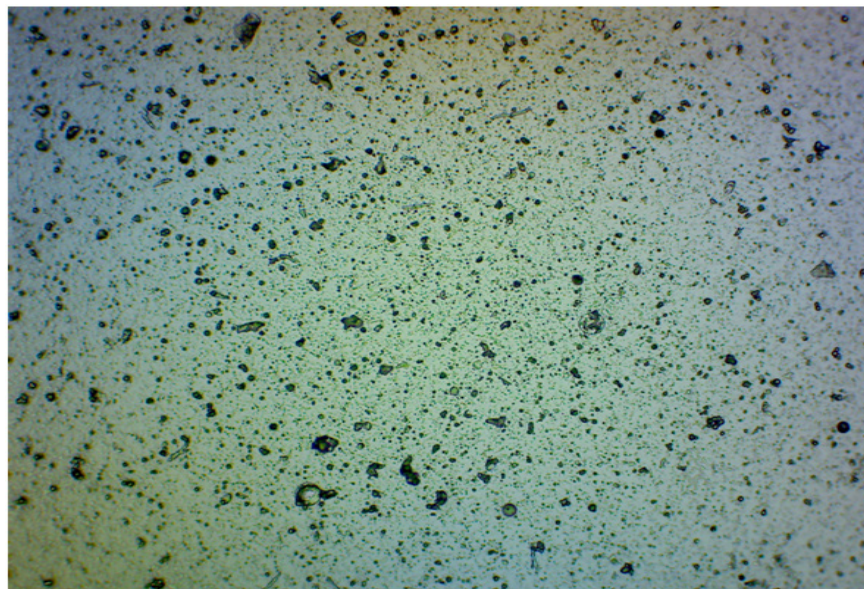| Table 1: Ingredients of formulation of Griseofulvin					
Ingredients	G1	G2	G3	G4	G5
Grisofulvin (mg)	100	100	100	100	100
PMMA (mg)	100	200	300	400	500
PVA (%)	1	1	1	1	1
Dichloro methane	q.s	q.s	q.s	q.s	q.s
Ethanol	q.s	q.s	q.s	q.s	q.s

Figure 2: Microscopic view of Griseofulvin nanoparticles

Morphological examination

The PMMA–griseofulvin nanoparticles had spherical shape with rough surface, as the polymer did not completely dissolved in organic solvent and secondly due to faster evaporation of solvent. In the case of G3, G4 formulations, it showed smooth surface of spherical shaped nanoparticles (Figure 2). The surface morphology of formulated nanoparticles depends on (1) a saturated solution of polymer produced irregular and rod shaped nanoparticles. 2) the diffusion rate of solvent is varying fast and solvent may diffuse into the aqueous phase before stabilization of nanoparticles and caused to aggregation of nanoparticles. In this formulation, the polymer was fully saturated and the diffusion rate of solvent was minimal, leading to formation of smooth, spherical and homogeneously distributed particle, which have a smooth surface and complete removal of solvent from the formulated nanoparticle with good quality.

Particle size and poly dispersity index

The nanoparticles size was very important factor for drug permeation through the skin. The explanation for this difference in the polymers concentration has been given in the literature and can be employed for the charged co- polymer nanoparticles as well. Particle size is often used to characterize the nanoparticles facilitation via skin and understanding of aggregation (Duane, 2000), In the case of large surface area, the attractive force between the particles and chance for possible aggregation in smaller sized particles. To overcome such aggregation, addition of a surfactant in the preparation was necessary. 1% PVA appeared to be the most suitable surfactant for reducing aggregation between nanoparticles, as it suspends quickly after formation (Duane, 2000). The formulations G1-G5 shows the particle size range between 108.6 ± 3.4 nm to 220.6 ± 1.2 nm. It is indicated that the particle size increases with increase in concentration of polymer (Table 2).

The particle size data showed that the nanoparticle produced submicron size and had low polydispersity, which indicate relative narrow size distribution. The PDI of all formulations was found to be in the range of 0.14-0.44, which concluded that prepared nanoparticle was monodispersed in nature (Table 2).

Table 2: Analytical Study of Griseofulvin nanoparticle

Formulation code	Particle size (nm)	PDI	Zeta potential (mV)	Entrapment efficiency (%)
G1	220.6±1.2	0.44	-30.52±3.20	81.4±1.8
G2	192.2±0.6	0.16	-22.04±1.82	84.1±1.0
G3	119.2±1.2	0.06	-29.17±0.46	97.7±1.4
G4	108.6±3.4	0.16	-27.61±3.42	90.7±1.4
G5	140.2±0.6	0.14	-30.64±1.84	83.6±0.6

Table 3: Percentage drug release

Formulation code	Percentage drug release					
	2 h	4h	8h	10h	12h	24h
G1	9.2±0.6	16.5±0.8	25.0±1.4	39.9±1.8	51.6±0.2	76.9±0.2
G2	9.8±1.2	15.4±1.4	24.8±0.4	37.6±0.2	49.4±1.8	77.1±1.4
G3	11.4±0.6	20.6±1.8	28.4±1.4	40.6±1.2	50.4±0.2	73.6±0.6
G4	10.6±1.2	17.4±0.4	29.8±1.4	41.0±1.2	54.1±0.8	70.2±1.2
G5	9.4±0.8	17.7±1.4	28.2±1.0	39.6±0.2	52.4±0.6	79.6±0.4

Entrapment efficiency

The entrapment efficiency is the functional characteristic of polymers, drug and surfactant etc. the entrapment efficiency was high in the case of G1-G5 formulations, due to high affinity of drug and the polymer in the same solvent. The low entrapment efficiency of remaining formulations was due to high affinity of drug and polymer in different solvents, i.e., drug in organic and polymer in aqueous phase. High entrapment efficiency has been shown by G1, G2, G3, G4 and G5 was in the range of 81.4 ± 1.8% to 97.7 ± 1.4%. (Table 2). The entrapment efficient depends on the polymer- drug concentration and the method used to prepare nanoparticles. The hydrophobic polymer of PMMA encapsulate large amount of hydrophobic drugs. High entrapment observed in PMMA, due to its poor aqueous solubility. The polymeric matrix decreases the drug leakage and drug release.

Zeta potential

Zeta potential of griseofulvin polymeric nanoparticles is presented in the Table 2. Zeta potential is an essential factor to evaluate the stability of nano dispersion. Zeta potential values mainly reflect the electrical repulsion between the particles (Muller, 2000). The average zeta potential value of griseofulvin polymeric nanoparticle was in the range of -22.04 ± 1.82 mV to -30.64 ± 1.84 mV. Even though a high zeta potential could provide an electric repulsion between the particles. Poloxamer 407 also provides the stearic stability for maintaining stability in polymeric nanoparticles. Surface charge of nanoparticles

influences their skin penetration. Kohli reported that only the negative charged particles were able to penetrate the SC to reach the inner epidermis. Whereas griseofulvin polymeric nanoparticles shows -22 to – 30 mV with small particle size, which abundantly influence the penetration of nanoparticles through stratum corneum.

In-vitro release

The *in vitro* release of griseofulvin from different biodegradable nanoparticles is shown in Table 3. The quantity of drug release in the all formulations (G1-G5) of polymeric nanoparticle was as very low and in the range of 9.2 ± 0.6 % to 11.4 ± 0.6 % in the initial period (2h). From this, it is obvious that the decreased percentage of drug release was due to the formulation of more compact wall around the drug by the biodegradable polymer and it significantly possess the sustained drug release for a prolonged period of time. At the end of 24 h limited percentage of drug was released in the range of 70.2 ± 1.2 to 79.6 ± 0.4 % (Table 3, Figure 3).

The drug release was attributed to the physical and chemical properties particularly on the P^{ka} and solubility profile of drug. For the polymer like PMMA that possess plastic and hydrophobic properties, drug particles present in the surface of matrix is initially release into medium generating many pores and cracks which facilitate further drug release. The fact can be substantiated by the fact that release profile of drug molecules, irrespective of their chemical nature was almost linear with time. *In vitro* medium mimics the pH and salt concentration in the body particularly for hydrophobic
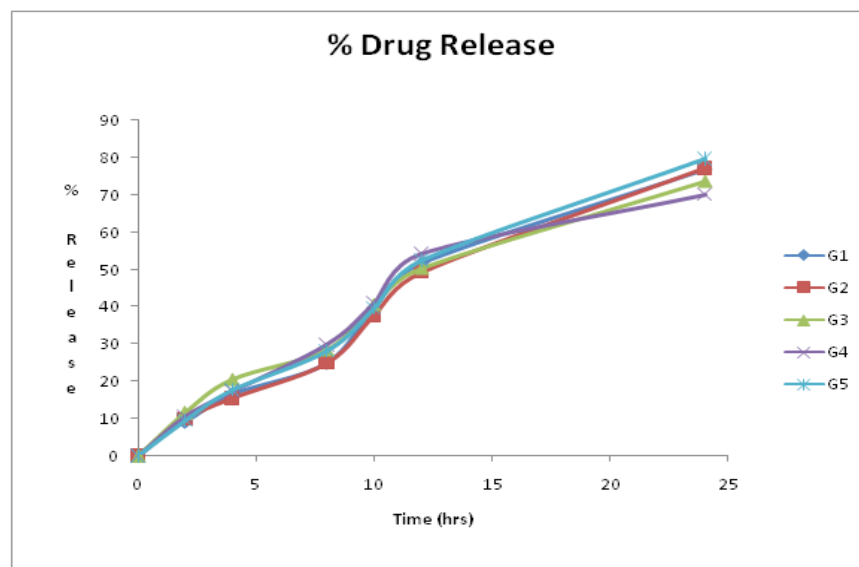
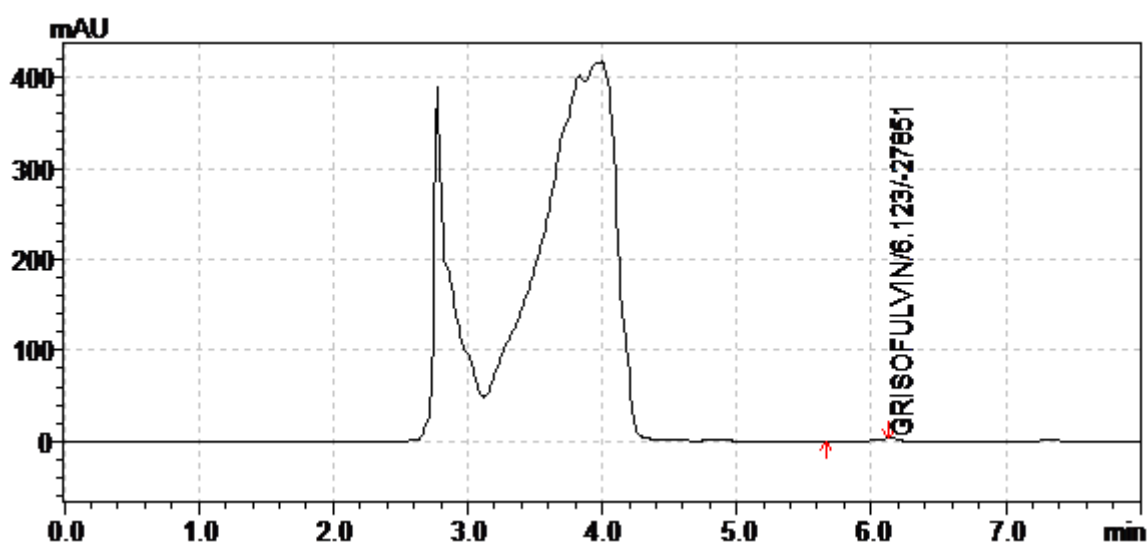**Figure 3: Release profiles of Griseofulvin**

Figure 4: Typical chromatogram of blank plasma

drugs, it is critical during dissolution testing that sink condition were maintain and pH and salt concentration of biological fluid were appromatially.

Validation of HPLC methods

Estimation of the drugs selected in plasma samples from the rabbits was carried out using optimized chromatographic conditions. The validation parameters such as accuracy, precision (repeatability and reproducibility), linearity and range, sensitivity (limit of detection and limit of quantitation), robustness/ruggedness, stability, selectivity/specificity and system suitability were evaluated.[10]

Griseofulvin

Specificity
HPLC-UV analysis of the blank rabbit plasma samples showed the separations of griseofulvin, no interference with either of these were observed. Hence the specificity of the method was established by comparison with rabbit plasma (control). Representative chromatograms of extracted blank plasma, blank plasma fortified with internal standard (IS) are shown in Figure 4 indicating no interference in the blank plasma and in drug-free rabbit plasma at the retention time of 5.5 for the drug griseofulvin (Figure 4).

Table 4: Concentrations-response linearity data for Griseofulvin

|	Nominal Concentration (µg/ml)					
	100	200	400	600	800	1200
1	98.5321	196.3216	398.6531	598.6947	798.5621	1198.3654
2	99.6348	198.3075	399.017	599.3015	799.6531	1197.9654
3	98.1027	198.2349	398.6254	598.9124	799.9205	1199.3025
4	99.5281	199.0347	397.0312	599.0321	799.3214	1199.6348
5	98.3042	198.3964	398.6597	598.4786	799.4341	1199.0321
Mean	98.82	198.05	398.39	598.88	799.37	1198.86
S.D (±)	0.71	1.02	0.78	0.31	0.51	0.68
C.V (%)	0.7206	0.5159	0.1959	0.0526	0.0638	0.0570
% Nominal	98.82	99.02	99.59	99.81	99.92	99.90
N	5	5	5	5	5	5
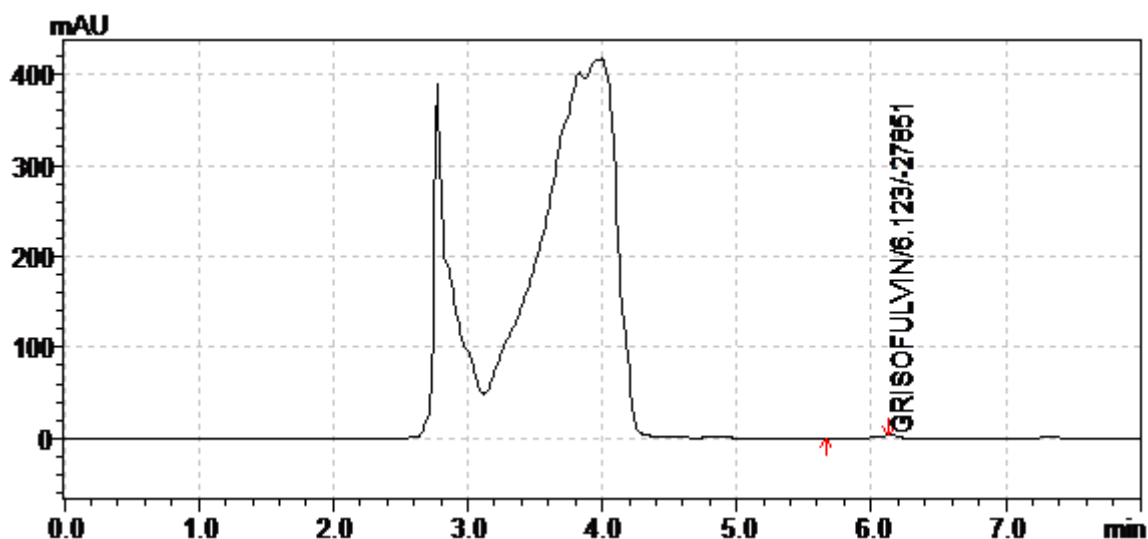
Figure 5: Typical chromatogram of standard

Sensitivity

It is expressed as limit of quantitation. It is the lowest amount of analyte in a sample matrix that can be determined. The Lower limit of quantification for griseofulvin is 100 ng/ml.

Linearity

A regression equation with a weighing factor of 1/concentration^2 was judged to produce the best fit for the concentration/detector response relationship for griseofulvin in rabbit plasma. The linearity range for griseofulvin was found to be 100, 200, 400, 600, 800 and 1200 ng/ml. The results are given in Table 4. The correlation coefficient (r^2) was greater than 0.99.

Precision and Accuracy

The precision of the assay was measured by the percent coefficient of variation over the concentration range of LOQ, low, middle and high quality control sample of

griseofulvin during the course of validation. The accuracy (Figure 5) of the assay was defined as the absolute value of the ratio of the calculated mean values of the LOQ, low, middle and high quality control samples to their respective nominal values, expressed as percent. The results are given in Table 5a–5c.

Stabilities

The stability studies of plasma samples spiked (Figure 6) with selected drugs were subjected to three freeze-thaw cycles, short term stability at room temperature for 3 h and long term stability at − 70°C over four weeks. In addition, stability of standard solutions was performed at room temperature for 6 h and freeze condition for four weeks. The mean concentrations of the stability samples were compared to the theoretical concentrations. The results indicate that selected drugs in plasma samples can be stored for a month without degradation in frozen state. The results of short term storage at room temperature

Table 5a: Within Batch Precision and Accuracy for Griseofulvin

	Nominal Concentration (µg/ml)		
	LQC	MQC	HQC
	100	400	1200
1	99.8624	399.8524	1199.3054
2	98.1027	398.6307	1198.3649
3	99.3061	398.6578	1197.0352
4	98.0325	399.5821	1199.6587
5	99.0022	398.342	1199.3075
Mean	98.86	399.01	1198.73
S.D (±)	0.78	0.66	1.06
C.V (%)	0.79	0.16	0.08
% Nominal	98.86	99.75	99.89

Table 5b: Between Batch/Inter day Precision and Accuracy for Griseofulvin

	Nominal Concentration (µg/ml)		
	LQC	MQC	HQC
	100	400	1200
1	98.6325	399.0324	1199.3054
2	99.4217	398.7563	1198.5287
3	98.6324	398.632	1198.1202
4	99.0368	399.0548	1199.6589
5	98.7134	398.6245	1197.6325
Mean	98.88	398.82	1198.64
S.D (±)	0.34	0.21	0.83
C.V (%)	0.34	0.05	0.06
% Nominal	98.88	99.70	99.88
N	5	5	5

Table 5c: Intra Day Precision and Accuracy for Griseofulvin

	Nominal Concentration (µg/ml)		
	LQC	MQC	HQC
	100	400	1200
1	99.6524	399.2304	1199.3065
2	98.7658	398.6347	1199.8657
3	99.3012	398.6358	1198.6356
4	98.1047	399.6256	1198.0323
5	99.0323	398.9425	1197.8638
Mean	98.97	399.01	1198.74
S.D (±)	0.58	0.42	0.84
C.V (%)	0.59	0.10	0.07
% Nominal	98.97	99.75	99.89
N	5	5	5
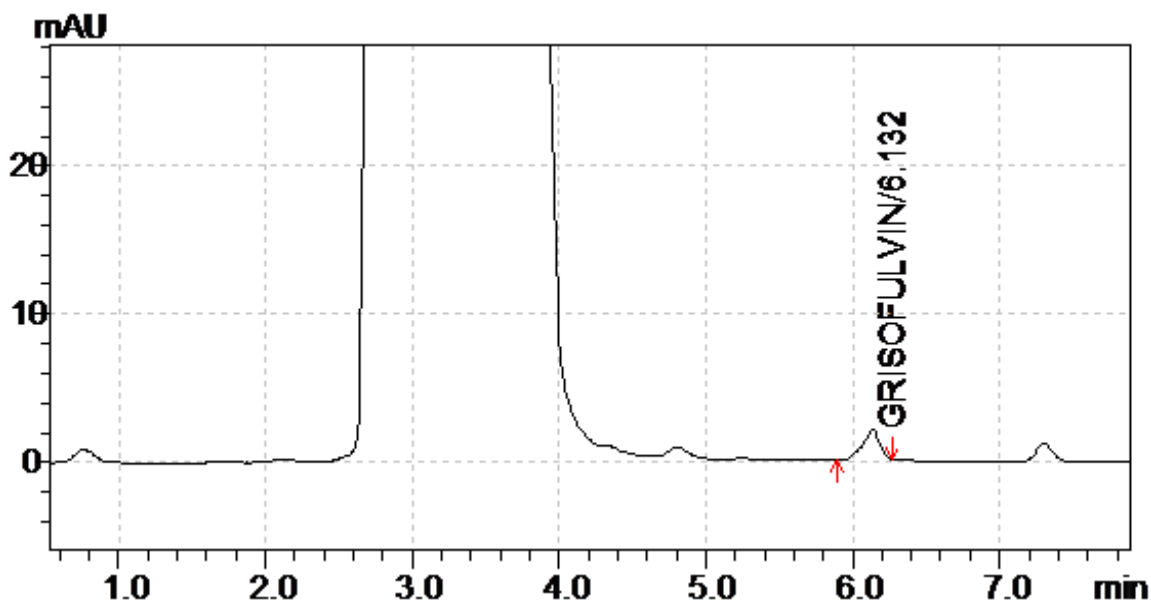
Figure 6: Typical chromatogram of spiking plasma

Table 6a: Stock Stability of Griseofulvin

S.No	Drug area 0 h	Drug area 3 h	Drug area 6 h
1	785532	798652	779865
2	798655	783124	789421
3	779324	798651	792634
4	789541	792302	782143
5	779326	789957	793257
Mean	786476	792537	787464
S.D	8076.00	6520.94	6127.30
C.V(%)	1.03	0.82	0.78

Table 6b: 20°C Stability of Griseofulvin in plasma

S.No	Nominal Concentration (µg/ml) LQC	HQC
1	99.3024	1199.6354
2	98.6527	1199.8537
3	99.6382	1199.6358
4	98.125	1198.5325
5	99.3651	1198.1204
Mean	99.01	1199.15
S.D	0.61	0.77
C.V(%)	0.62	0.064
% Nominal	99.01	99.92
N	5	5

Table 6c: Short Term Room Temperature Stability of Griseofulvin

S.No	Nominal Concentration (µg/ml) LQC	HQC
1	99.5321	1199.6325
2	98.9754	1198.4537
3	99.1027	1198.6322
4	98.6429	1198.4230
5	99.653	1197.6598
Mean	99.18	1198.56
S.D	0.41	0.70
C.V(%)	0.41	0.05
% Nominal	99.18	99.88
N	5	5

Table 6d: Auto sampler Stability of Griseofulvin in plasma

S.No	Nominal Concentration (µg/ml) LQC	HQC
1	98.6324	1199.6324
2	99.6528	1198.6789
3	98.9754	1198.4976
4	99.6327	1199.3207
5	98.6325	1198.3645
Mean	99.10	1198.89
S.D	0.51	0.55
C.V(%)	0.51	0.04
% Nominal	99.10	99.90
N	5	5

Table 6e: Freeze/thaw cycle stability of Griseofulvin

S.No	Nominal Concentration (µg/ml) LQC	HQC
1	99.3265	1199.6327
2	98.6574	1198.7654
3	99.0325	1199.6322
4	98.4628	1198.3647
5	99.3281	1198.6351
Mean	98.96	1199.00
S.D	0.39	0.58
C.V(%)	0.39	0.04
% Nominal	98.96	99.91
N	5	5

stability and freezethaw cycles indicate no degradation of selected drugs in plasma as well as in sample solution and hence plasma samples could be handled without special precautions. The results are given in Table 6a-6f.

Recovery

Analyte recovery from a sample matrix (extraction efficiency) is a comparison of the analytical response from an amount of analyte added to that determined from the

Table 6f: Long term stability for four weeks of Griseofulvin

S.No	Long Term Stability for four weeks	
	Nominal Concentration (µg/ml)	
	LQC	HQC
1	99.8652	1199.6327
2	98.6347	1198.4621
3	98.4301	1197.6358
4	99.3268	1199.7562
5	98.5227	1198.6327
Mean	98.95	1198.82
S.D	0.61	0.88
C.V(%)	0.62	0.07
% Nominal	98.95	99.90
N	5	5

sample matrix. The detailed results are presented in Table 7. The results indicate that the recovery of griseofulvin was consistent at all levels (Figure 7).

Ruggedness and robustness

The ruggedness and robustness of the methods were studied by changing the experimental conditions. No significant changes in the chromatographic parameters were observed when changing the experimental conditions (operators, instruments, source of reagents and column of similar type) and optimized conditions (pH, mobile phase ratio and flow rate).

This thesis deals with the studies carried out by the writer for the past one year on the Formulation and BA-BE Method Development and Validation of griseofulvin nanoparticle.

Table 7: Recovery study of Griseofulvin

	Nominal Concentration (µg/ml)					
	LQC Response		MQC Response		HQC Response	
	Extracted	Unextracted	Extracted	Unextracted	Extracted	Unextracted
1	98625	112387	99865	118972	99365	124792
2	99469	114528	98647	116534	98635	117985
3	97671	120354	99324	117856	99324	120457
4	98650	111986	99965	123967	98632	117985
5	99326	121756	99304	123654	99637	120652
Mean	98748.21	116202.20	99421.00	120196.60	99118.60	120374.20
S.D (±)	713.89	4561.17	528.02	3411.83	458.88	2784.67
C.V (%)	0.72	3.92	0.53	2.84	0.46	2.31
N	5		5		5	
% Recovery	84.9		82.7		82.3	
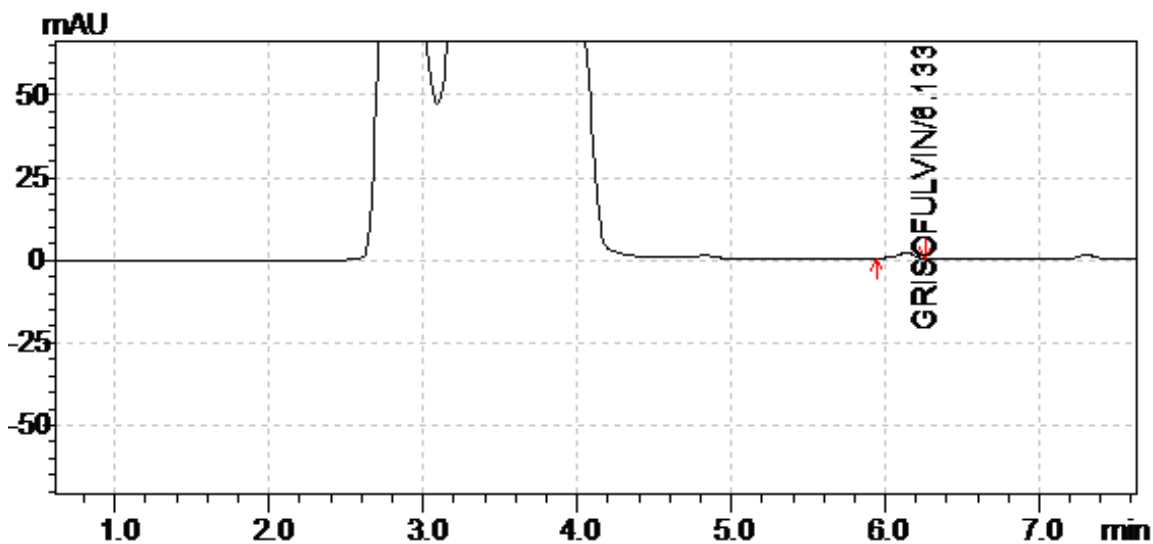
Figure 7: Typical chromatogram of Recovery studies

Thesis begins with a brief account of the pharmacokinetic model, *in vitro* dissolutions and estimation of drugs in biological medium. The methods used for the this model development, validation, the steps involved in bio analytical method development, *in vitro* dissolution methods and their importance have also been discussed. A review of literature on bio-analytical model development available for the drugs in biological fluids is presented.

how bio-analytical development necessitates development of new drugs, bio analytical method development and validation are discussed. The objectives of the present study, namely, to optimize the chromatographic conditions, to develop and validate the methods to estimate the selected drugs in the biological fluids by HPLC. It also deals with the experimental procedures adopted. It describes in detail the procedures adopted for the bioequivalence study design & data handling, optimization and validation of the chromatographic conditions for the estimation of the drugs in plasma and selected nanoparticle formulation, bio-analytical model development.

CONCLUSION

The present study deals with the investigation of bio-analytical method in the development of dosage forms and

Highlights of Paper

- The *in vitro* release studies showed that after the initial burst, all the drug-loaded batches provided can be substantiated by the fact that release profile of drug molecules, irrespective of their chemical nature was almost linear with time.
- The present study deals with the investigation of bio-analytical method in the development of dosage forms and how bio-analytical development necessitates development of new drugs, bio analytical method development and validation are discussed
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Author Profile

- **Dr. Selvadurai Muralidharan:** Has been completed M.Pharm and Ph.D in the department of Pharmaceutical Analysis in Dr. M.G.R. Medical university, India. Presently working as a senior lecturer in Asian Institute of medicine Science and Technology University (AIMST), Malaysia.

- **Dr. Vijayan Venugopal:** Has been completed M.Pharm and Ph.D in the department of Pharmaceutics in Acharya Nagarjuna University, India. Presently working as a senior lecturer in Asian Institute of medicine Science and Technology University (AIMST), Malaysia.

- **Dr. Jayaraja kumar:** Has been completed M.Pharm in the department of Pharmaceutics and Ph.D in Acharya Nagarjuna University, India. Presently working as a senior lecturer in Asian Institute of medicine Science and Technology University (AIMST), Malaysia.

REFERENCES

1. Nelson Ochekpe A, Patrick Olorunfemi O, Ndidi Ngwuluka C. Nanotechnology and Drug Delivery, Part 2: Nanostructures for Drug Delivery. Tropical Journal of Pharmaceutical Research 2009; 8(3): 275-87.

2. Waree Tiyaboonchai. Chitosan Nanoparticles: A Promising System for Drug Delivery. Naresuan University Journal 2003; 11(3): 51-66.

3. Li Zhiyi, Jiang Jingzhi, Liu Xuewu, Zhao Shunxuan, Xia Yuanjing, Wang Jian. Preparation of griseofulvin microparticles by supercritical fluid expansion depressurization process. Powder Technology 2008; 182(3): 459-65.

4. Pratap Jadon S, Virendra Gajbhiye, Rajesh Jadon S, Kavita Gajbhiye R, Narayanan Ganesh. Enhanced Oral Bioavailability of Griseofulvin via Niosomes. AAPS Pharm Sci Tech. 2009; 10(4): 1186–92.

5. Gopi Vudathala K, James Rogers A. Microencapsulation of Solid Dispersions: Release of Griseofulvin from Griseofulvin: Phospholipid Coprecipitates in Microspheres. Pharmaceutical Research 1992; 9(6): 759-63.

6. Siddharth Jambhrunkar, Zhi Qu, Amirali Popat, Surajit Karmakar, Chun Xu, Chengzhong Yu. Modulating *in vitro* release and solubility of griseofulvin using functionalized *Mesoporous silica* nanoparticles. Journal of Colloid and Interface Science 2014; 434(1): 218-25.

7. Ulla Brinkmann-Trettenes, Annette Bauer-Brandl. Solid phospholipid nano-particles: Investigations into formulation and dissolution properties of griseofulvin. International Journal of Pharmaceutics 2014; 467(1-2): 42-7.

8. Getu Kahsay, Aremu Olajire Adegoke, Ann Van Schepdael, Erwin Adams. Development and validation of a reversed phase liquid chromatographic method for analysis of griseofulvin and impurities. Journal of Pharmaceutical and Biomedical Analysis 2013; 80(1): 9-17.

9. Nidhi Aggarwal, Shishu Goindi. Preparation and evaluation of antifungal efficacy of griseofulvin loaded deformable membrane vesicles in optimized guinea pig model of *Microsporum canis*—Dermatophytosis. International Journal of Pharmaceutics 2012; 437(1-2): 277-87.

10. FDA Guidance for Industry. Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration, Centre for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM); 2000 May.