ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE FORMULATED CAPSULE DOSAGE FORM CONTAINING TACROLIMUS, MYCOPHENOLATE MOFETIL AND PREDNISOLONE

A

Thesis

Submitted to

For the award of

DOCTOR OF PHILOSOPHY (PhD)

in

Pharmaceutical Sciences

By

Pardeep Kumar

(41100147)

Supervised By
Dr. Amit Bhatia

Co-Supervised By
Dr. Surajpal Verma

LOVELY FACULTY OF APPLIED MEDICAL SCIENCES
LOVELY PROFESSIONAL UNIVERSITY
PUNJAB
2019

DECLARATION

I declare that the thesis entitled **"Analytical Method Development and validation for The Formulated Capsule Dosage form Containing Tacrolimus, Mycophenolate Mofetil and Prednisolone"** has been prepared by me under the guidance of **Dr. Amit Bhatia,** Assoc. Prof., Department of Pharmaceutical Sciences and Technology, Maharaja Ranjit Singh Punjab Technical University, Bathinda, Punjab, **Dr. Surajpal Verma,** Assoc. Prof., LSPS, Lovely Professional University, Phagwara, Punjab, India No part of this thesis has formed the basis for the award of any degree or fellowship previously.

Pardeep Kumar
Department of Pharmaceutical Sciences,
Lovely Professional University
Phagwara, Punjab

Date:

CERTIFICATE

I certify that **Pardeep Kumar** has prepared her thesis entitled **"Analytical Method Development and Validation for the Formulated Capsule Dosage Form Containing Tacrolimus, Mycophenolate Mofetil and Prednisolone"** for the award of Ph. D degree of Lovely Professional University**,** under my guidance. He has carried out the work at the Department of Management, Lovely Professional University.

Dr. Amit Bhatia
M. Pharm, PhD
Assoc. Prof., Department of Pharmaceutical Sciences and Technology,
Maharaja Ranjit Singh Punjab Technical University,
Bathinda, Punjab-151001
Date:

Dr. Surajpal Verma
M. Pharm, PhD
Assoc. Prof., Lovely School of Pharmaceutical Sciences,
Lovely Professional University, Phagwara, Punjab, India- 144411
Date:

ABSTRACT

In current scenario, treatment of any disease depends upon two major factors i.e. patient compliance and effective dosage regimen. The effective dose delivered by a dosage form to a patient depends on various parameters, which can be assessed by an effective and economic analytical method. In the case of solid organ transplantation, to inhibit the rejection, the Mycophenolate mofetil (MMF), Tacrolimus (TAC) and are used in the combination of corticosteroids like Prednisolone (PRED) are used. The combination of MMF, TAC with PRED provides long-term graft survival in better way than the other combination. In the present study a precise analytical method for estimating the combination of immunosuppressant drugs including mycophenolate mofetil (MMF), tacrolimus (TAC) and prednisolone (PRED) through RP-HPLC was developed. The mobile phase contained a mixture of acetonitrile and 0.35% triethylamine (pH 4.2) with orthophosphoric acid (70:30). As per ICH guidelines (Q2R1) the optimized RP-HPLC method was validated with respect to linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, precision, repeatability, robustness, ruggedness. The results of the recovery study were found to be within the acceptance criteria (96.93-103.99%), which indicated a good degree of sensitivity of the developed method in detection of analytes in a sample. The retention time (RT) of PRED, MMF and TAC was 2.243, 3.391, 6.698 respectively. This method is also used to analysed the concentration of drugs in blood plasma after administration of formulated granules.

Keywords: Dosage regimen, mycophenolate mofetil, prednisolone, RP-HPLC, tacrolimus, retention time.

Dedicated to

My Loving Parents

My Supporting Wife

&

My Adorable children

“Vasavi, Aarush & Manasvi”

ACKNOWLEDGEMENT

At this movement of my substantial enhancement, before I get into the thick of the things, I would like to add a few heartfelt words for the people who gave their unending support with their unfair humour and warm wishes. I am vastly indebted to many people who have helped and inspired me, in various ways, to start, to continue, and complete this work.

First and foremost, my gratitude goes to my mentor and supervisor, **Dr. Amit Bhatia**, Associate Professor, Department of Pharmaceutical Sciences and Technology, Maharaja Ranjit Singh Punjab Technical University, Dabwali Road, Bathinda, Punjab, India and co-supervisor, **Dr. Suraj Pal Verma**, Associate Professor, Lovely School of Pharmaceutical Sciences, Lovely Professional University, Phagwara, Punjab, India for supporting me at the conception of this work and strengthens my resolve in so many direct and indirect ways. Their thoughtful and valuable reviews and suggestions have helped enormously to improve the work. I cannot find words to describe the debt I owe to my supervisor and co-supervisor, for having created a stimulating atmosphere of academic excellence, the basic element of any long-lasting endeavor.

I also feel very much obliged to Dr. Yashwant L. Chaudhary, for his thoughtful and valuable reviews and suggestions have helped enormously to improve the work.

I also feel very much obliged to Sophisticated Analytical Instrumentation Facility, CIL and UCIM, Panjab University, Chandigarh for conduction of various characterization tests. I can't afford for not thanking Mr. Vishal Sharma, Assistant Professor, UIET, Panjab University, Chandigarh for helping me out of bounds for completing this research study.

I would like to make a very special mention of my supporting wife **Dr. Cherry Bhargava** who has been constantly a motivating and guiding force in my present research. This research could not have been possible without her thorough support. My loving parents deserve great acknowledgement in true sense who have always been a force to keep me riveted to my dedication towards the present research. Vasavi and Manasvi, my daughters and son Aarush are thanked and apologized too for snatching their time needed to conduct this research.

I want to thank **Dr. Monica Gulati**, Senior dean and faculty and staff of Lovely school of pharmaceutical sciences for giving me the opportunity to do research on the topic of "Analytical Method Development and validation for The Formulated Capsule Dosage Form Containing

Tacrolimus, Mycophenolate Mofetil and Prednisolone”. This has helped broaden my knowledge on the field.

I am deeply grateful to honourable chancellor **Mr. Ashok Mittal**, and Pro- chancellor **Ms. Rashmi Mittal**, Lovely Professional University for providing me required facilities to carry out this project.

I express my hole hearted thanks to **Dr. Amit Mittal**, Associate Professor and Head of Department, School of Pharmaceutical Sciences, Lovely Professional University, Phagwara, for giving me opportunity to work on this project.

I am thankful to management, seniors and colleagues especially Dr. Bimlesh, Mr. Gurvinder Singh, Mr. Rajesh, Ms. Charanjit of Lovely Professional University for constantly keep pushing me to move higher and higher. I am thankful to all the Teaching Staffs for providing me all the help and support during my work. I am grateful to all Non-teaching Staff of Library, Store and Pharmaceutical Laboratories for providing all facilities required for the successful completion of my thesis work.

Besides, I thank all my friends, well-wishers, respondents and academicians who helped and guided me throughout my journey from inception to completion.

A warm thanks to all.

Date: 30th April, 2019

Place: Ludhiana (Pardeep Kumar)

List of abbreviations

Abbreviation	Full Form
e.g.,	For example
ESRD	End Stage Kidney Disease
DWFG	Death With Functioning Graft
AZA	Azathioprine
ATG	Anti-Thymocyte Globulin
ALG	Anti-Lymphocyte Globulin
CyA	cyclosporine
MMF	Mycophenolate mofetil
TAC	Tacrolimus
IL-2	interleukin-2
IL-1	interleukin-1
MHC II	Major Histocompatibility Complex class II
PKC	Protein Kinase C
NFAT	Nuclear Factor of Activated T-cell
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
TNFα	Tumor Necrosis Factor alpha
DGF	Delayed Graft Function
CMV	Cytomegalovirus
IVIG	Intravenous immunoglobulin
APC	Antigen Presenting Cell
GRE	Glucocorticoids Response Elements
CNIs	Calcineurin Inhibitors
NFATc	Cytosolic Nuclear Factor of Activated T cells
TMA	Thrombotic Microangiopathy
FKBP	FK binding protein
mTOR	Mammalian Target of Rapamycin
6-MP	6-mercaptopurine
DNA	Deoxyribonucleic Acid
IMPDH	Inosine Monophosphate Dehydrogenase

HUS	Hemolytic-uremic syndrome
°C	Degree Celcious
RH	Relative Humidity
mg	Milli Gram
dL	Deci Liter
kg	Kilo Gram
vs	Versus
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
mL	Mili Liter
UPLC	Ultra Performance Liquid Chromatography
MS	Mass Spectrometery
mM	Mili Mole
v/v	Volume by vloume
w/v	Weight by vloume
ng	Nano Gram
nm	Nano Meter
µg	Micro Gram
UV	Ultra Violet
AUC	Area Under the Curve
ER	Extended Release
API	Active Pharmaceutical Ingredient
MPA	Mycophenolic acid
MPAG	Mycophenolic Acid Glucuronide
NSAIDs	Nonsteroidal anti-inflammatory drugs
LOD	Limit of detection
LOQ	Limit of quantification

TABLE OF CONTENT

CHAPTER 1 1

INTRODUCTION 1

1.1. Immunity 1

1.2. Immune System 2

1.3. Organ Transplantation 4

1.4. Immunosuppressant Drugs 7

1.5. Recommended Dosage 21

1.6. Special Conditions 24

1.7. Other Therapeutic Conditions 25

1.8. Side Effects 25

1.9. Drug Interactions 26

1.10. Capsule Dosage Form 26

1.11. Advantages of Capsule Dosage Form 31

1.12. Disadvantages of Capsule Dosage Form 32

1.13. Stability / Stable Formulation: A Preview 32

1.14. Routes of Degradation 33

1.15. General Guidelines for Collection of Stability Data 35

1.16. Capsule Formulation Excipients (In Case of Amorphous Powder API) 36

CHAPTER 2 38

REVIEW OF LITERATURE 38

2.1. Literature Review on Renal Transplant 38

2.2. Literature review on drug utilization in kidney transplant patients 40

2.3. Literature review on the comparison of immunosuppressant drugs and their effect 40

2.4. Literature review on simultaneous estimation of drugs by HPLC 49

CHAPTER 3 56

RATIONALE, AIM AND OBJECTIVES 56

3.1. The rationale of the study 56

3.2. AIM 56

3.3. Objectives of the study 56

CHAPTER 4 57
MATERIALS AND EQUIPMENT 57
4.1. Materials 57
4.2. Equipment 59
CHAPTER 5 60
DRUG PROFILE 60
5.1. Drug Profile: Mycophenolate mofetil 60
5.2. Drug Profile: Tacrolimus 68
5.3. Drug Profile: Prednisolone 76
CHAPTER 6 81
EXPERIMENTAL WORK 81
6.1. Preformulation Studies 81
6.2. Compatibility Studies of Drugs with Various Excipients 86
6.3. Formulation Development 87
6.4. Evaluation of the capsules 90
6.5. Stability of In-house product 91
6.6. Prototype Formula Development 91
6.7. Formula Optimization Studies 95
6.8. Dissolution Method Development 96
6.9. Dissolution Method Validation 97
6.10. Assay Method development and validation 98
6.11. Method Validation 100
CHAPTER 7 105
RESULT AND DISCUSSION 105
7.1 Initial Physicochemical Characterization 105
7.2 X-Ray – Diffraction Studies 108
7.3 Hygroscopicity Study 110
7.4 Flow Properties 113
7.5 Identification of the Drugs 114
7.6 Prototype Formulation Development 123
7.7 Compatibility studies of the MMF with various excipients 123

7.8 Chemical Compatibility Study ... 129
7.9 Preparation and formulation of capsules ... 134
7.10 Evaluation of powder and granules of all formulation (C1-C14) ... 136
7.11 Evaluation of Capsules (all formulation) ... 138
7.12 Prototype Formula Development ... 139
7.13 Formula Optimization Studies ... 142
7.14 Drug release profile of the optimized formulation ... 147
7.15 Accelerated Stability Study ... 153
7.16 Dissolution Method Development ... 155
7.17 Dissolution Method Validation ... 158
7.18 Assay method development ... 168
7.19 Validation of the Assay method ... 170
CHAPTER 8 ... 187
***IN-VIVO* PHARMACOKINETIC STUDIES** ... 187
8.1. Methods ... 187
8.2. Study Design ... 190
8.3. Preparation of Equivalent formulation for Rat Model ... 190
8.4. Pharmacokinetic studies of prepared formulation ... 190
8.5. Statistical Analysis ... 191
CHAPTER 9 ... 195
SUMMARY AND CONCLUSION ... 195
CHAPTER 10 ... 197
REFERENCES ... 197
APPENDICES ... 220

LIST OF TABLES

Table 1.1: Classification depends upon the type of graft and uses in autoimmune diseases 12
Table 1.2: Summary of Immunosuppressant Drugs 22
Table 1.3: Capsule size and body fill volumes 29
Table 1.4: General cases 35
Table 1.5: Current WHO definition of climatic zones 35
Table 4.1: List of materials 57
Table 4.2: List of equipment 59
Table 6.1: Hygroscopicity Classification 84
Table 6.2: Prototype formula for the capsule formulation 89
Table 6.3: Processing variables that were kept constant during the formulation of all the batches 90
Table 6.4: Commonly used binders and their concentrations of use 93
Table 6.5:Commonly used disintegrants and their concentrations of use 94
Table 7.1: Observation chart of moisture uptake by the MMF 110
Table 7.2: Observation chart of moisture uptake by the TAC 111
Table 7.3: Observation chart of moisture uptake by the PRED 112
Table 7.4: Flow Properties of the MMF 113
Table 7.5: Flow Properties of the TAC 114
Table 7.6: Flow Properties of the PRED 114
Table 7.7: FTIR Interpretation data of Mycophenolate 117
Table 7.8: FTIR Interpretation data of Tacrolimus 118
Table 7.9: FTIR Interpretation data of Prednisolone 119
Table 7.10: Compatibility study of the MMF with different excipients 124
Table 7.11: Compatibility study of the TAC with different excipients 126
Table 7.12: Compatibility study of the PRED with different excipients 128
Table 7.13: Composition of different trial of capsules 134
Table 7.14: Different properties of powder/ granules of all formulations 136
Table 7.15: Particle size of the optimized formulation by sieve 137
Table 7.16: Properties of capsules with different excipients and concentrations 138

Table 7.17: Properties of selected formulation 139
Table 7.18: Evaluation of capsules 139
Table 7.19: Physical characteristics of batches prepared with a different binder 140
Table 7.20: Proposed ingredients for the capsule formulation 142
Table 7.21: Observation of different capsule formulation processes 142
Table 7.22: Physical characteristics of batches prepared with different concentrations of HPMC K-100 143
Table 7.23: Physical characteristics of batches prepared with different concentrations of SSG 145
Table 7.24: Physical characteristics of batches prepared with different concentrations of Magnesium stearate and Aerosil 146
Table 7.25: Dissolution profile of C14 Capsules Formulation in 0.1N HCl at 25 RPM 148
Table 7.26: Dissolution profile of C14 Capsules Formulation in 0.1N HCl at 50 RPM 149
Table 7.27: Dissolution profile of C14 Capsules Formulation in pH 2.0 HCl Buffer at 50 RPM 150
Table 7.28: Dissolution profile of C14 Capsules Formulation in pH 2.0 HCl Buffer at 25 RPM 151
Table 7.29: Observation of stability analysis of the Optimized batch capsules (C14) 154
Table 7.30: Solubility of the PRED, MMF and TAC at different pH 155
Table 7.31: Calibration Data 159
Table 7.32: Precision results showing repeatability 162
Table 7.33: Intraday precision 163
Table 7.34: Interday precision 163
Table 7.35: Linearity table of Prednisolone, Mycophenolate and Tacrolimus 164
Table 7.36: Change in wavelength 166
Table 7.37: Change in flow rate 167
Table 7.38: Calibration data 168
Table 7.39: Results showing Specificity 170

Table 7.40: Linear regression data for the calibration curve in 0.1N HCl (n=6) 171
Table 7.41: Accuracy of HPLC method 174
Table 7.42: Change in wavelength 175
Table 7.43: Change in flow rate 175
Table 7.44: Data showing LOD and LOQ values 176
Table 7.45: Acid degradation 177
Table 7.46: Base degradation 178
Table 7.47: Oxidative degradation 179
Table 7.48: Thermal degradation of Prednisolone 179
Table 7.49: UV degradation of Prednisolone 180
Table 7.50: Stability testing of formulation C14 (n=3) 184
Table 7.51: RT/ RRT data 185
Table 8.1: In-vivo Study design 191
Table 8.2: Standard curve data of Prednisolone, Mycophenolate, and Tacrolimus in rat plasma 192
Table 8.3: The individual plasma drug concentrations with respect to time 193
Table 8.4: Pharmacokinetic parameters of the prepared capsule formulation 194

LIST OF FIGURES

Figure 1.1: Types of Immunity 1
Figure 1.2: Kidney Allograft Loss after First Year 7
Figure 5.1: Structure of Mycophenolic acid (A), Mycophenolate mofetil (B) 60
Figure 5.2: Structure of Tacrolimus 69
Figure 5.3: Structure of Prednisolone 76
Figure 6.1: Ideal flow for formula development of immediate release capsule dosage form 88
Figure 7.1: DSC thermogram of Mycophenolate drug 105
Figure 7.2: DSC thermogram of Tacrolimus drug 106
Figure 7.3: DSC thermogram of Prednisolone drug 106
Figure 7.4: X-Ray Diffraction pattern of the TAC 108
Figure 7.5: X-Ray Diffraction pattern of the PRED 108
Figure 7.6: X-Ray Diffraction pattern of the MMF 109
Figure 7.7: X-Ray Diffraction pattern of the physical mixture (1:1:1) 109
Figure 7.8: Moisture uptake by the MMF concerning time 110
Figure 7.9: Moisture uptake by the TAC concerning time 111
Figure 7.10: Moisture uptake by the PRED concerning time 112
Figure 7.11: The absorption maxima of MMF was observed at 254 nm 115
Figure 7.12: The absorption maxima of PRED was observed at 254 nm 116
Figure 7.13: The absorption maxima of TAC was observed at 254 nm 116
Figure 7.14: FTIR Spectra of the MMF 117
Figure 7.15: FTIR spectrum of Tacrolimus 118
Figure 7.16: FTIR spectrum of Prednisolone 119
Figure 7.17: Proton NMR of MMF 120
Figure 7.18: MMF structure showing data in ppm 120
Figure 7.19: Proton NMR of Prednisolone 121
Figure 7.20: Prednisolone structure showing data in ppm 121
Figure 7.21: Proton NMR of Tacrolimus 122
Figure 7.22: Tacrolimus structure showing data in ppm 122
Figure 7.23: FTIR spectrum of Talc 129

Figure 7.24: FTIR spectrum of Sodium Starch glycollate 130
Figure 7.25: FTIR spectrum of Magnesium stearate 130
Figure 7.26: FTIR spectrum of Aerosil 131
Figure 7.27: FTIR spectrum of lactose 131
Figure 7.28: FTIR spectrum of Starch 132
Figure 7.29: FTIR spectrum of physical mixture of excipient 132
Figure 7.30: FTIR spectrum of a physical mixture of excipient and Drugs 133
Figure 7.31: PSD of the lubricated blend 137
Figure 7.32: Dissolution profile of In-house capsules using a different binder 140
Figure 7.33: Dissolution profile of In-house capsules using a different disintegrating agent 141
Figure 7.34: Dissolution profile of In-house capsules using a different lubricating agent 141
Figure 7.35: Dissolution profile of formulations using different concentrations of HPMC K-100 144
Figure 7.36: Dissolution profile of formulations using different concentrations of SSG 145
Figure 7.37: Dissolution profile of formulations using different concentrations of Magnesium Stearate and combined with Aerosil 147
Figure 7.38: Dissolution profile of C14 Capsules Formulation in 0.1N HCl at 25 RPM 148
Figure 7.39: Dissolution profile of C14 Capsules Formulation in 0.1N HCl at 50 RPM 149
Figure 7.40: Dissolution profile of C14 Capsules Formulation in pH 2.0 HCl Buffer at 50 RPM 151
Figure 7.41: Dissolution profile of C14 Capsules Formulation in pH 2.0 HCl Buffer at 25 RPM 151
Figure 7.42: Comparison of the release rate and agitation of C14 in 0.1N HCl 152
Figure 7.43: Comparison of the release rate and agitation of C14 in pH 2.0 HCl Buffer 152

Figure 7.44: F2 values of the optimized C14 at different RPM and in different media 153
Figure 7.45: Solubility of the PRED, MMF and TAC in different media........ 155
Figure 7.46: Dissolution Profile of Reference Product in Different Media at 50 RPM, USP II 157
Figure 7.47: HPLC scan of placebo 157
Figure 7.48: Calibration curves of a) PRED, b) MMF, and c) TAC in 0.1N HCl 160
Figure 7.49: Data for overall recovery in 0.1 N HCl........ 161
Figure 7.50: Accuracy and Ruggedness data 162
Figure 7.51: Linearity curve of a) PRED, b) MMF and c) TAC by RP-HPLC in 0.1N HCl 165
Figure 7.52: Calibration curve for a) PRED, b) MMF and c) TAC 169
Figure 7.53: Linearity curve for a) PRED, b) MMF and c) TAC in HPLC method validation 172
Figure 7.54: Degradation data of PRED, MMF and TAC 180
Figure 7.55: HPLC scan of optimized formulation 181
Figure 7.56: HPLC scan of PRED (standard) 181
Figure 7.57: HPLC scan of TAC (standard)........ 182
Figure 7.58: HPLC scan of MMF (standard) 182
Figure 7.59: HPLC scan of placebo 183
Figure 7.60: Stability of C14 formulation at different time intervals........ 184
Figure 7.61: USP Theoretical plates in Robustness condition (>3000) 185
Figure 7.62: USP Tailing in Robustness condition (<2.0) 186
Figure 8.1: Standard curve of Prednisolone, Mycophenolate, and Tacrolimus by RP-HPLC 193
Figure 8.2: Time courses of plasma levels after oral administration of PRED, MMF, and TAC 194
Figure 8.3: HPLC chromatogram of drug concentration in plasma 194

CHAPTER 1

INTRODUCTION

1.1. Immunity

The term immunity is derived from Latin immunes, meaning free from burden. In biology, the burden is a disease that is caused by a variety of microorganism (e.g., viruses, bacteria, fungi, protozoa, etc.) and the physiological role of the immune system is to keep them at bay. Immunity defined as the state of resistance or insusceptibility exhibited by the host to toxic molecules, micro-organism and foreign cells.[1]

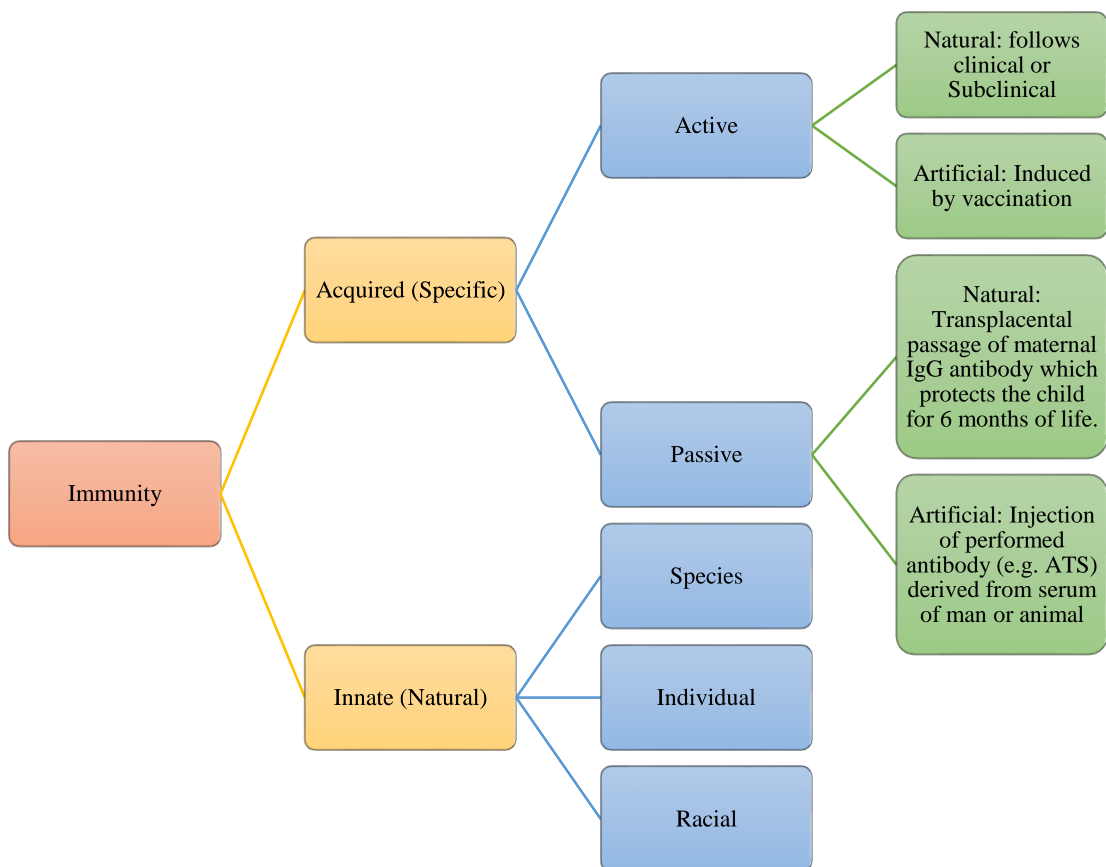


Figure 1.1: Types of Immunity

1.2. Immune System

There are two systems of immunity: innate immunity and acquired/ adaptive immunity which collaborate to protect the body. The innate immune system contains two types of mechanism, i.e., molecular and cellular mechanism. This system prevents or eliminates the virus before the infection. The second one is the adaptive immune system; this is enquired when there is any invading virus and adapts to recognize, remove and then remember the invading; which causes disease.

The inner immune system prevents or inhibits virus which causing infection in the body. The cell which is present in innate immunity, defend against the diseases.[2]

1.2.1. Cell and Organ of the Immune System

Many cells, organs, and tissues of the immune system protect from the virus. These cells, organs, and tissues are present in the whole body. By the function, these can be described into two categories:

Type 1 lymphoid organ: The first type of lymphocytes organ gives proper microenvironment for the generation or the action of lymphocytes cell.

Type 2 lymphoid organ: The second type lymphoid organ cell are retaining antigen generally from vascular or nearby spaces and is the place where effective interaction between mature lymphocytes and antigen takes place. Blood vessels and lymphatic system connect that organ, uniting them into a whole function.[2, 3]

1.2.2. The Normal Immunity Response of The Body

The primary role of the immune system is to protect the human body from infectious diseases. Concluding it, the immune system is entirely responsive to attacking pathogens while holding the capacity to recognize self-antigens to which it is tolerant. Security from infections and disease by the collective efforts from the innate and adaptive immune system.

1.2.3. Cells of Immunity System

Small lymphocytes are with a dense nucleus surround by a thin layer of cytoplasm containing a few mitochondria, ribosomes, and other organelles. These cells are resting to serve as a repository of genetically derived information about the recognition of antigens. The lymphocyte usually has one gene capable of expression committed to that antigen. When activated, the antigen recognizes it and divides to produce a clone. T-cell responds directly to the antigen to form clones. T-cells are stimulated by few antigens, viruses, acid-fast bacilli, fungi, different cell, and neoplastic cell. B-cell requires the cooperation of a T-cell before they can form in clone in response to antigen. A broader range of antigens stimulates B-cell. B-cell or T-cell antigen recognition involves a combination of the antigen with an antibody-like component on the cell membrane.[2]

1.2.3.1. Immune Responses

There are two major parts of immune responses: The humoral immune system and the cellular immune system.

1.2.3.1.1. Humoral Immunity

Antigens introduced into the tissue through a wound or by injection and carried to a regional lymph node. Orally ingested antigens go to gut-associated lymphoid tissue. Antigens in the bloodstream arrive at the spleen. In these tissues, the antigen stimulates proliferation specifically committed lymphocytes of the type. If the antigen has not previously been faced, lymphocytes in a mass of the proliferating cell synthesize a specific IgM against the antigens and release it into the plasma.[4, 5]

1.2.3.1.2. Cellular Immunity

It is responsible for the function such as organ transplantation rejection, the killing of tumor or virus-infected cells, and hypersensitivity reactions.[4]

1.3. Organ Transplantation

In the immunology, we generally use a transplantation term, which usually means that the transfer of body cells, tissue, or any organs from one site to another in the body. Lots of problems can be avoided by implanting the healthy organ, tissue or cells from one organism to another human body.[1]

Animal tissue and organs are similar; so, they can be grafted from another animal of the same species and can be rejected because of transplantation antigens; present in all animal and human cells.

Type of transplants:

Autograft: a tissue of one site engrafted to another location in the same individual.

Isograft: Graft placed on another individual of the same genetic constitution (e.g., monozygotic twins).

Allograft: Graft transfer within the same species but between two genetically different members.

Xenograft: Graft between members of different species.[1]

1.3.1. Allograft Reaction

Rejection or transplant of the allograft by one recipient to another is called allograft reaction. The most useful and successful organ transplant of the human body is kidney transplantation in man is that of the kidney. There are many organ transplants are tried, such as bone marrow, renal and heart transplantation, but with some success, the most successful and comfortable transplantation is kidney transplantation.[2]

1.3.1.1. Kidney Transplantation

In kidney transplantation, donor [living or died (deceased or cadaver donor)] kidney transplants to the ESRD (end-stage kidney disease) patients using the surgical procedure.

Based on the source of the donor organ, the kidney transplantation classified as a) living-donor transplantation and b) deceased-donor (cadaveric) transplantation. One of the risks of a renal transplant is that the body will reject (fight) the new kidney. To help keep the body from fighting new kidney, renal transplant patients require taking a lifelong immunosuppressant drug to prevent rejection.[6]

In kidney transplantation, quality of life and patient survival rate are better than patients who use dialysis. Subsequently, kidney transplantation is a treatment of choice for some individuals with ESRD.

1.3.2. History of Kidney Transplantation

In 1902, Emerich Ullmann performed the first successful experiment for kidney transplantation on the dog's neck, and it remains functional for five days. Then Alexis Carrel develops the technique of vascular sutures for which the Nobel Prize awarded him in Physiology or Medicine in 1912.

In humans, a first kidney transplant was attempted by Dr. Yuri Voronoy in 1933, in the Soviet Union. A kidney was removed from the deceased donor 6 hours before the operation and replanted into the thigh. This patient died two days later [7]. Ruth Tucker undergoes the first successful renal transplant at Little Company of Mary Hospital in Evergreen Park, Illinois. Because of the unavailability of immunosuppressive therapy, the transplanted kidney rejected within a year.

The first kidney transplantation in living patients was performed by Jean Hamburger in 1952, in France. Transplanted kidney survives only three weeks [8]. In 1954, Joseph Murray and team, at the Peter Bent Brigham Hospital, Boston, performed the first successful human kidney transplantation between the identical Herrick twins. After the positive transplantations between identical twins, the utilization of renal transplantation in people were expanding. Along with this, the improvement of immune science and the immunosuppressant medication has ventured into the focus of interest.

In India, during the 1950's the initial experimentation for kidney and liver transplantation was done in dogs by Dr. P K Sen and his team at King Edward VII

Memorial (KEM) Hospital, India. In India, first human kidney transplant performed at the KEM Hospital, India on May 1965, on a patient who had hypernephroma, using a cadaver donor. However, this patient was surviving only for 11 days after transplant.

After 17 years of identical twins transplant done by Murray et al., in 1954 Dr. Johny and Dr. Mohan Rao of CMC Hospital, Vellore, performed first successful live donor renal transplant in January 1971 [9].

In Gujarat, first live kidney transplantation was done at Muljibhai Patel Urological Hospital, Nadiad in 1980 [10].

1.3.3. Results of Kidney Transplantation

Kidney transplantation’s success measured by the survival rate of patient and survival of graft. The care of transplanted patients is divided into two: first early and second late post-transplant period. In the early transplant period, acute allograft rejection episodes are the most common. In this period, relatively a large amount of immunosuppressive medication must be administered while in the late post-transplant period, immunosuppressive medication load was lower as compared to the early transplant period. Patient’s death rate and return to dialysis is a crucial determinant point for identification of success rate of late renal allograft failure [11].

1.3.4. Causes of Graft Loss

The first three post-transplant months are known as an early post-transplant period. In the first few transplant days, medical issues are predominating while immunological and medical problems tend to dominate later on. In the early post-transplant period, causes of graft loss or graft dysfunction are either non-immunological or immunological. The non-immunological causes include acute tubular necrosis, vascular (obstruction or stenosis), urological or infections. While immunological causes include, antibody-mediated, T cell-mediated acute rejection, thrombotic microangiopathy, and nephrotoxicity, etc. [11].

The late post-transplant period refers to more than one year after transplantation. The significant causes of graft loss in late post-transplant periods are the chronic graft dysfunction or the death with functioning graft (DWFG).

The causes responsible for graft loss and rates of graft loss, estimated by the US Renal Data Systems report of 2003, are described in Figure 1.2.

Due to development of new immunosuppressive medications, the incidence rate of acute rejection and early graft failure has declined dramatically, and because of this, the one-year graft survival rate is increased up to 95%, in most of the transplant centers [11].

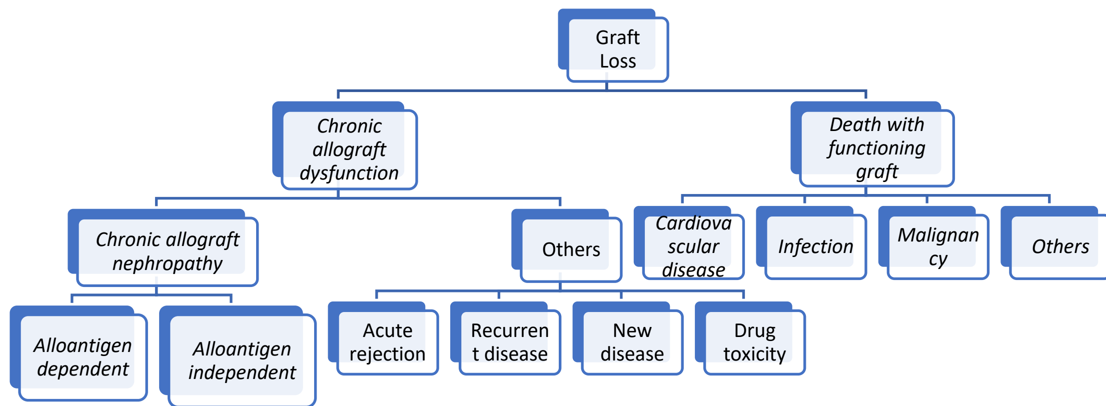


Figure 1.2: Kidney Allograft Loss after First Year

1.4. Immunosuppressant Drugs

Immunosuppressant drugs, which are also called anti-rejection drugs, are used to prevent the body from rejecting a transplanted organ. These drugs are used to dull the immune response in organs transplantation like kidney, renal, heart transplantation and autoimmune diseases.[12]

These drugs have met with a high level of clinical accomplishment in treating conditions, for example, acute immune rejection of organ transplants and severe autoimmune diseases. Nevertheless, such treatments require the long-lasting use and nonspecifically whole immune system would be suppressed. The long term use results

in more exposure to infectious disease and malignancy. The restricting usefulness of calcineurin inhibitors and glucocorticoids, specifically, due to nephrotoxic and diabetogenic, individually, in an assortment of clinical settings.

1.4.1. Development of Immunosuppressant Drugs

In 1948, the pharmacological actions of adrenal cortical hormones drew the intensions world widely as used first time in patients who have rheumatoid arthritis at the Mayo Clinic [13]. In 1951, the study in rabbits showed that the cortisone dependent treatment increases the survival rate of skin grafts done by Billingham and Morgan. Thus, setting the phase for the utilization of steroids to anticipate allograft dismissal [14].

Bone marrow infusion and lethal irradiation stimulate the resilience in adults (instead of new-born). It trialed by attempts at human immunosuppression for organ transplants were with early allograft bone marrow rescue and full body irradiation. These methods completed in Paris, Boston, and somewhere else in the late 1950s. This routine was too hard to even think about controlling the regimen, and graft-versus-host disease was inescapable. During the availability of the Azathioprine, the sublethal portion of radiation utilized, and it discovered that the sub-lethal irradiation alone in human patients was very immunosuppressive which was being used until 1962. Upon the same study, 12 patients were tested in Boston, and the only a single patient found who received the graft from non-identical twin and have long term survival. In Paris, similar success obtained with sibling grafts [13].

During the 1960 studies done by Schwartz and Dameshek with 6-mercaptopurine (6-MP) shows a delay of skin graft rejection in rats [15]. In 1960, Calne got new derivatives of 6-MP while visited Boston for research with Murray, Hitchings, Elion of Burroughs Wellcome. From these derivatives, Azathioprine (AZA) (initially known as BW57-322) shows promising results and less toxic than 6-MP in dog kidney transplants [16]. After this, in 1962 Azathioprine was first used clinically at the Peter Bent Brigham Hospital, Boston. Sooner, AZA brought into renal transplantation in a quickly expanding number of renal transplant units throughout the world. In 1963, Murray et al. and Starzl et al.

individually performed studies using AZA along with steroids showed better results in prevention of kidney graft [17, 18].

The anti-thymocyte globulin (ATG), anti-lymphocyte globulin (ALG) and polyclonal antibody preparations, were developed in the mid-1970s. These drugs were prescribed with AZA and prednisolone as the baseline regimen or used for induction or the treatment of steroid-resistant rejection. The success rate was increased up to 50% at one year, and the mortality rate was typically 10% to 20%.

AZA and steroids were the backbones of immunosuppression in renal transplantation for many years until the introduction of cyclosporine (CyA) in the early 1980s. Because of poor results in kidney transplantation before CyA, statistically significant improvement in graft survival rates observed after CyA. Graft survival rate was increased up to 80% at one year. Mortality rates decreased with the more effective immunosuppressant drug, less use of corticosteroids, and overall improvements in surgical and medical care.

After the introduction of CyA, the standard immunosuppressive regimen consisted of CyA and prednisone, often combined with AZA, now used as an adjunctive agent in so-called triple therapy. Although CyA is having a significant effect on both patient survival and graft survival rate, its impact on acute and chronic Nephrotoxicity was a significant detriment.

In 1985, OKT3 (now known as muromonab-CD3), the 1st monoclonal antibody having the capacity to treat first acute rejection events. Though, this drug had toxicity, so its use was limited to those in which patients were not responding to high dose steroids while in some programs it was also used as an induction agent.

The transplant community shares the success rate up to 90% and minimal mortality in many centers with a limited armamentarium of medications 'CyA, AZA, corticosteroids, and the antibody preparations' in the initial period of the 1990s.

Mycophenolate mofetil (MMF) introduced in 1995, after three randomized controlled trials (equal graft and patient survival at 12 months), this drug showed a decrease in

acute rejections significantly within the first six months [19-21]. Over the next decade, AZA gradually replaced MMF as an immunosuppressive agent.

In 1997, Neoral (microemulsion preparation of CyA), was available in the market. The microemulsion formulation of CyA shows more effectiveness than the more established formulations. These results encouraged the individual adapting and monitoring of CyA treatment, which converted into a less occurrence of acute rejection chances and improved graft survival, without expanding unfavorable impacts identified with CyA toxicity in renal transplantation [22-24]. After the development of microemulsion of CyA few studies were performed to compare MMF to AZA in a combination of microemulsion formulation and steroid as background immunosuppression in renal transplant. But, one study conducted by MYSS and its follow up studies shows that there is no significant difference in acute rejection rate or graft survival in two groups [25, 26].

Tacrolimus (TAC) introduced in 1997, after many clinical trials, it founds that TAC was progressively viable in patients with steroid-resistant rejection episodes and its capacity to produce equivalent patient and graft survival as CyA [27-30]. So, within a few years, CyA was replaced by TAC as an immunosuppressive agent.

After next year’s the introduction of new drugs like Sirolimus (1999), Mycophenolate Sodium (2004) and some new antibody preparations like Daclizumab (1999), ATG (1999) and Basiliximab (2000) the safety and efficacy of kidney transplant recipients increased to 95% [11]. Drugs which inhibit cellular/humoral or both immune response and have their principal use in organ transplantation and autoimmune.[12]

1.4.2. Immunosuppressive therapy for organ transplantation

The allogeneic transplantation survival rate requires a degree of immunosuppressant. Immunosuppressive treatment has a disadvantage due to its non-specificity. With this property, it gives generalized immunosuppression towards all antigens, allograft, and subject the patients towards increased risk of other infections. Also, many immunosuppressive measures aimed at slowing the proliferation of activated lymphocytes. This treatment affects many rapidly dividing nonimmune cells, leading to

severe or even life-threatening complication. Patients on extended – term immunosuppressive therapy are at increased risk of cancer, hypertension, and metabolic bone disease

Also, numerous immunosuppressive treatments went for slowing down the multiplication of activated lymphocytes. This treatment influences multiple rapidly dividing nonimmune cells, prompting to major complication which leads to death. Patients on long – term immunosuppressive treatment are having a significant risk of infectious diseases.[2]

1.4.3. The success of immunosuppressive drugs

The accomplishment of organs transplantation is altogether subject to the utilization of immunosuppressive medications that control the alloimmune or isoimmune reaction. The most frequent combination therapy utilized for kidney transplant patients includes three specialists, every one of which has a distinct method of activity:

- A drug that inhibits T cell activation;
- An antiproliferative;
- An anti-inflammatory agent. [31]

1.4.4. Classification of Immunosuppressive Drugs

The characteristic classes of immunosuppressants are:

Antiproliferative drugs: also known as cytotoxic drugs; e.g., Cyclophosphamide, Azathioprine, Mycophenolate mofetil (MMF), Chlorambucil and Methotrexate.

Calcineurin inhibitors (Specific T-cell inhibitors); e.g., Tacrolimus and Cyclosporine (Ciclosporin).

Glucocorticoids; e.g., Prednisolone and others

Antibodies; e.g., Rho (D) immunoglobulin, anti-thymocyte globulin (ATG), and Muromonab CD3 [32]

Table 1.1: Classification depends upon the type of graft and uses in autoimmune diseases

These drugs can be classified depending upon the graft	
Renal Transplant	Basiliximab, Daclizumab, Muromonab CD3, Tacrolimus, Sirolimus
Liver	Muromonab CD3, Tacrolimus
Heart	Muromonab CD3, Tacrolimus
Pancreas	Tacrolimus
Small bowl	Tacrolimus
Bone Marrow	Tacrolimus
Drugs used in autoimmune diseases	
Azatioprine	rheumatoid arthritis, ulcerative colitis
Cyclosporin	psoriasis, rheumatoid arthritis, multiple sclerosis, diabetes, and myasthenia gravis
Glatiramer acetate	relapsing-remitting multiple sclerosis
Sirolimus	psoriasis

1.4.4.1. Antiproliferative Drugs (Cytotoxic immunosuppressants)

Certain cytotoxic drugs used in cancer chemotherapy, they have also applied for immunosuppressant action, they act like the T and B lymphocytes cell and known as an antiproliferative drug. [32]

1.4.4.2. Calcineurin Inhibitors (Specific T-cell inhibitors)

It significantly and specifically restrains T lymphocyte proliferation, interleukin-2 (IL-2), and other cytokine generation and the reaction of inducer T cells to interleukin-1 (IL-l), with no impact on suppressor T-cells which arrested in the quiescent state of the cell cycle (G0/ G1). The CD4 molecule associated with a T-cell receptor on helper T-cells anchors the Major Histocompatibility Complex class II (MHC II) carrying the antigen peptide. So that it can activate the T-cell receptor Stimulation of T cell receptor, produces a cascade of Ca2+ dependent events and protein kinase C (PKC) activation. The Ca^{2+} ions after binding to calmodulin activate the calcineurin which dephosphorylates regulatory protein 'nuclear factor of activated T-cell' (NFAT), permitting its intranuclear migration and transcription of cytokine genes leading to the production of IL-2 along with other interleukins, GM-CSF, TNFα, interferon, etc. Cyclosporine enters target cells and binds to immunophilin class of protein (cyclophilin). This complex bind and inactivates the calcineurin response of helper T cell to antigenic stimulation fails. Cyclosporine also enhances expression of an inhibitor of IL-2 which attenuates IL-2 is excited T -cell proliferation and production of killer lymphocyte. Cyclosporine is most active when administered before antigen exposure, but can, also, suppress the responses of primed helper T cells; hence useful in autoimmune diseases as well. [32]

1.4.4.3. Glucocorticoids

The steroid is the first class of drug which used in the case of immunosuppressant, and it also used in the case of anti-inflammatory response, blocks several parts of the immune response. They are causing inhibition MHC expression and the reproduction of T lymphocyte cell on the body. This expression is generally shown by some interleukins and other like cytokine genes which are controlled by corticosteroids and production of the same attractive molecules are suppressed.

1.4.4.4. Immunosuppressant Antibodies

Antibodies are the second class of the drugs which used for the organ transplantation and another immunity disease. The first drug Muromonab CD3 used for this propose.

The action of this drug It is a murine monoclonal antibody against the CD3 glycoprotein located on helper T-cells. They causing for binding of muromonab CD3 with CD3 antigen prevent the binding of MHC II-antigen form complex with T-cell receptor. The response to this monoclonal antibody is less variable than to the many colons are anti-thymocyte globulin. They generally produce less allergic reactions. [32] Tacrolimus and Mycophenolate Mofetil (MMF) are immunosuppressive drugs. It uses to suppress the immune response consequent to organ transplantation and autoimmune diseases.

1.4.5. Few commonly used immunosuppressant drugs

1.4.5.1. Induction Antibody Preparations

1) Muromonab-CD3: It is a murine monoclonal antibody.

Use: It is used for acute rejection of renal allograft in case of (corticosteroid-resistant acute allograft rejection), and to deplete T cells from benefactor bone marrow before preceding transplantation.

Mechanism of action: OKT3 produce its immunosuppressant drug effect by binding the CD3 glycoprotein (T-cell receptor-associated protein), and starts initial activation and release of a cytokine, followed by inhibition of functions and T-cell reduction.

Adverse effects: It produces anaphylactic reactions, infections, high fever, cerebral edema, seizures, aseptic meningitis, encephalopathy, and headache. Because of more harmful effects of OKT3 and development of more tolerable rabbit anti-thymocyte globulin and antagonists of interleukin- 2 receptors, OKT3 rarely used. [30]

2) Anti-thymocyte Globulin (ATG)

Based on the derived source, ATG divided into two types; one derived from horses (ATGAM) and second is rabbits (thyroglobulin) derived.

Use: ATG is generally used in combination to prevent early allograft rejection. ATG is also used to treat corticosteroid-resistant acute rejection and severe rejection episodes.

For shorter hospital stay and delayed graft function (DGF), the intraoperative administration of ATG was used and have a lower incidence of rejection. [33]

Mechanism of action: They produce their immunosuppressant effect by binding to various cell surface indicators, and starts the disintegration of lymphocytes.

Adverse effect: Chills, skin rashes, fever, thrombocytopenia, leucopenia, and infections due to cytomegalovirus (CMV) or other viruses will produce after the usage of ATG.

Several studies conducted to compare thyroglobulin with ATGAM shows that thymoglobulin shows better graft survival and prevents the rejection more effectively, than ATGAM [34].

3) Interleukin-2 receptor antagonists

Interleukin-2 receptor antibodies are Daclizumab and Basiliximab. Basiliximab is consists of 75% human protein and 25% murine, and hence it is designated as ‘chimerized.’ While Daclizumab is consist of 90% human protein, and hence it is designated as ‘humanized.’

Use: IL-2-receptor antagonists in combination with cyclosporin and corticosteroids to prevent early chances of rejection after transplant and is used as a precaution in low-to-moderate risk. [35]

Mechanism of action: Daclizumab and Basiliximab act anti to CD25 antibodies. Both these drugs bind with the interleukin-2 receptor (α chain) on activated T cells and delays the proliferation of T cells. Inhibition of this receptor stops the capacity of any antigenic stimulus to activate the T-cell reaction system.

Adverse effects: Both daclizumab and basiliximab are well-tolerated drugs. Commonly observed adverse events of this class of drugs relate with GI tract.

4) Rituximab

It is monoclonal antibody focused against the CD20 antigen

Use: It used in the treatment of autoimmune diseases, lymphomas, and leukemia. It is also used in kidney transplant patients in combination with Intravenous immunoglobulin (IVIG) and plasmapheresis to treat antibody-mediated rejection. [36]

Mechanism of action: It produces its effect by binding to CD20 and downregulates the B cell receptor.

Adverse effect: It produces severe infusion reaction, bowel obstruction, cardiac arrest, infections, cytokine release syndrome, tumor lysis syndrome, perforation, and pulmonary toxicity.

1.4.5.2. Maintenance Immunosuppressive Drugs

1) Glucocorticoids

The glucocorticoids are used in transplantation and various autoimmune disorders as an immunosuppressive agent. They are one of the support systems for lessening rejection scenes.

Use: The glucocorticoids are used to decreases acute rejection of an allograft, in autoimmune conditions like temporal arthritis, asthma, refractory rheumatoid arthritis, and systemic lupus erythematosus.

Mechanism of action: It delivers an immunosuppressive impact by blocking T-cell and antigen presenting cell (APC) determined cytokine articulation. Glucocorticoids tie to a cytoplasmic receptor to frame a complex, which transfer it into the nucleus and ties to glucocorticoids response elements (GRE) in the supporter areas of cytokine genes. Glucocorticoids additionally repress the translocation of translation factors in the nucleus. In this manner, the production of a few cytokines (interleukin-1, 2, 3, 6, Tumor necrosis factor -α, gamma interferon) is repressed [37].

Adverse effect: It is diabetogenic and can cause weight gain, vascular necrosis hypercholesterolemia, cataracts, osteoporosis, and hypertension.

2) Calcineurin Inhibitors (CNIs)

I) Cyclosporine: A cyclic peptide obtained from *Tolypocladium inflatum*.

Use: Cyclosporine is used to prevent rejection of solid organ transplants along with some autoimmune diseases like rheumatoid arthritis and also used for xerophthalmia and recalcitrant psoriasis.

Mechanism of action: CyA especially dominates cell interceded immune responses. Though, humoral immunity influenced to a far lesser degree. After diffusing into the T cell, CyA ties to a cyclophilin (immunophilin) to form complex that binds to calcineurin and leads to dephosphorylation of NFATc (cytosolic Nuclear Factor of Activated T cells). Since the CyA-calcineurin complex can't play out this response, NFATc can't enter the nucleus to advance the reactions that required for the combination of various cytokines, including interleukin-2. The final product is a reduction in IL-2, which is the essential substance for T lymphocytes.

Adverse effects: CyA produces acute and chronic nephrotoxicity, neurotoxicity (tremor, dysesthesias, insomnia, headache), electrolyte disorders (hyperuricemia, hyperkalemia, hypomagnesemia), gingival hyperplasia, Hypertrichosis, thrombotic microangiopathy (TMA), new onset diabetes, hypertension, hirsutism, bone pain syndrome, and hyperlipidemia.

II) Tacrolimus: A macrolide obtained from *Streptomyces tsukubaensis*.

Use: TAC is also used to prevent rejection of solid organ transplants and in severe eczema that does not respond to established therapies. TAC is prescribed with corticosteroid and/or with an anti-metabolite.

Mechanism of action: The immunosuppressive activity is due to binding with immunophilin, FKBP-12 (FK binding protein).

Adverse effects: Adverse effect produced by TAC include nephrotoxicity, neurotoxicity (tremor, seizures, and hallucinations), insulin-dependent diabetes mellitus, hypertension, electrolyte disorders (hyperkalemia, hypomagnesemia, hyperuricemia), hyperlipidemia, and anaphylactic reactions.

TAC therapy associated with lesser episodes of rejection as compare to CyA and also with TAC therapy, lower doses of corticosteroids are required to produce similar immunosuppression, and hence TAC therapy reduces the probability of steroid-associated adverse effects. Thus in most of the transplant centers, CyA is replaced with TAC.

3) Mammalian Target (mTOR) Inhibitors

I) Sirolimus: A macrolide obtained from *Streptomyces hygroscopicus*.

Use: Sirolimus used in renal transplantation along with CyA and corticosteroids. Sirolimus is also used as Sirolimus-coated stents in cardiac patients to reducing proliferation of the endothelial cells and hence prevent restenosis.

Mechanism of action: Sirolimus binds to the cytoplasmic FK binding protein and mTOR to form a complex, which prompts in the reduction of activated T cells. Contrasting CyA and TAC, sirolimus does not owe its impact to bringing down interleukin-2 production in any case, instead, to restraining the cellular reactions to interleukin-2.

Adverse effects: Hyperlipidemia is the most common side effect of sirolimus. Other side effects include nephrotoxicity, headache, nausea and diarrhea, leucopenia, and thrombocytopenia. Clinical monitoring of plasma concentration of sirolimus must be monitored closely.

II) Everolimus: It is considered to be the derivative of sirolimus.

Use: Used in kidney transplantation and evidence of advanced renal cell carcinoma. Because of anti-neoplastic and antiviral benefits, so used against cancer and viral diseases (Cytomegalovirus and BK polyomavirus infections). [38]

Mechanism of action: Same as Sirolimus.

Adverse effects: It produces adverse effects which include hyperlipidemia, impaired or delayed wound healing, nephrotoxicity, angioedema and increased risk of kidney venous and arterial thrombosis.

4) Anti-metabolites

I) Azathioprine

Azathioprine is a prodrug of 6-mercaptopurine (6-MP) and then converted into thio inosinic acid.

Mechanism of Action: It inhibits the thioguanine nucleotides synthesis, and metalloproteinase enzyme leads to interferes with DNA synthesis.

Adverse events: The significant toxicity of Azathioprine is bone marrow suppression. Other adverse effects of AZA include pancreatitis, macrocytosis, and liver toxicity.

II) Mycophenolate acid

Mycophenolate acid has replaced AZA in case of prolonging graft survival as it shows better safety and efficacy. [39] Mycophenolate acid is available in two different formulations: mycophenolate sodium and mycophenolate mofetil.

Mechanism of action: Both forms quickly hydrolyzed in the gastrointestinal tract to mycophenolic acid. It is non-competitively, selectively inhibits the enzyme inosine monophosphate dehydrogenase (IMPDH), needed for the growth of T and B lymphocytes, which hinders the synthesis of guanosine phosphate. Therefore, similar to 6-MP, it decreases the proliferation of T and B cells.

Adverse event: It shows common adverse effects like others along with anemia, CMV infection, and leucopenia. [40]

Year of introduction, IS type, mechanism of action, site of action and adverse events of Immunosuppressant drug, are summarized in Table 1.2.

1.4.6. Immunosuppressive Drugs and Long-term Graft Outcome

Immunosuppressive medications are necessary for counteractive action of acute rejection. Though, their job in anticipation of late graft loss isn't clear. Exploratory examinations in animals uncovered that immunosuppressive medications could inhibit

continuing allograft failure in some instances, yet there is no proof in humans. Before the 1980s, a combination (corticosteroids and AZA along with anti-lymphocyte antibody) was in use in the initial few weeks of post-transplantation. Different clinical investigations show fibrosclerotic changes and signs of dysfunction in surviving grafts. There is a reliable connection of acute rejection scenes with later dysfunction lead to the speculation that these late changes may have immune pathogenesis. [41, 42]

1.4.7. Complication after Kidney Transplant

The main complications are diabetes mellitus, rejection, and infections after renal transplantation.

1.4.7.1. Diabetes Mellitus

Diabetes means the body experiences issues in maintaining typical glucose levels. Immunosuppressant drugs which are prescribed to prevent rejection episode such as Prednisone, TAC, CyA can cause diabetes, which called a new onset of diabetic mellitus (NODAT). If blood sugar level does not controled then it effects on patient's survival rate and also kidney function, so it is required to begin with insulin or antidiabetic medication to control glucose when side effects like expanded thirst, expanded recurrence of pee, obscured vision, and disarray continued and blood glucose level shows high.

1.4.7.2. Rejection

For patients who underwent transplantation, transplanted kidney is a foreign organ, so patients own immune system oppose this kidney and patient's bodies starts immunological response against this organ. So, to prevent the rejection episode transplant patients has to take the medication for the rest of his life. It is required to proper management of Immunosuppressant drug in transplant patients to prevent rejection.

Rejection episode is divided into three: Hyper, Acute, and Chronic rejections. In Hyper-acute rejection, the body immediately destroys the graft and is a very rare form of

rejection and is reported immediately after transplant. Acute rejection more often than occurs in an initial couple of months after transplantation, yet it can happen any time after the transplant. Acute rejection can be cured with the utilization of increased doses of immunosuppressant medicine to curb the body's desire to discard the organ. Chronic rejection is happening months, or even a very long time after the transplant; this type of rejection causes transplanted kidney to stop working gradually at a slow rate. Still, we have no any medication that prevents or cure this chronic rejection episode.

1.4.7.3. Infections

Immunosuppressive medications prescribe to transplant patients which help to prevent rejecting of transplanted kidney by patients own body. However, these drugs also reduce the body’s immune system which may lead to increase susceptibility to various infections after transplant surgery. Few common infections after transplantation are a bacterial infection, fungal infection, viral infection including CMV and herpes infection. Infection after transplantation may affect the patient and graft and also increase the hospitalization of transplant patients. So, prevention of infection requires extra care for long term graft survival and patient’s survival.

Other complications after transplantation are Delayed function, Drug toxicity, and Urologic problems. Drug prescribe to transplant patients also produce some difficulties like Cancer (skin, organ), Gastrointestinal problems, Weight gain, Joint problems, Cataracts, Hyperlipidemia, Acne, Tremors, Gum overgrowth, etc.

1.5. Recommended Dosage

Immunosuppressant drugs are under schedule-I (prescription is required). They generally come in solid and liquid dosage forms. The suggested dose relies upon the kind and type of immunosuppressant utilized. Dosages might be distinctive for different patients.[43]

Table 1.2: Summary of Immunosuppressant Drugs

Year of introduction	IS type	Drugs	Targets	Mode of action (inhibition)	Side effects
----	Corticosteroid	prednisolone, methylprednisolone	T-cells, Macrophages, Neutrophils, Endothelial cells, Fibroblasts	Cytokine release (IL-1 and IL-2) MHC-II expression Adhesion Expression of the adhesion molecules Collagen synthesis	Osteoporosis, Diabetes, Dyslipidemia, Hypertension, Cataract
----	Antimetabolites	Azathioprine	Purine analog myelocytes	DNA synthesis Promyelocytes proliferation	Neutropenia
1984	Calcineurin inhibitors (CNI)	Cyclosporine A	Calcineurin phosphatase enzyme	IL-2 synthesis, T-cell activation	Nephrotoxicity, Hemolytic-uremic syndrome (HUS), Neurotoxicity, hypertension, Hypertrichosis, Gum hypertrophy, tremor, TMA, Hyperlipidemia
1997		Neoral			
1997		Tacrolimus			
1995	Purine synthesis inhibitors	Mycophenolate mofetil (MMF)	IMPDH (inosine monophosphate dehydrogenase)	De novo purine synthesis B and T-cell proliferation	GI side-effects (abdominal pain, diarrhea), Leucopenia, Anemia
2004		Mycophenolate sodium (MPS)			
1999	mTOR-inhibitors	Sirolimus	T-cells, Endothelial cells	T-cell proliferation	Hyperlipidemia, Anemia, Thrombocytopenia
2005		Everolimus			

1999	Polyclonal antibodies	Anti-thymocyte globulin (ATG)	T and/or B cells (activated) Platelets	Blocking adhesion of lymphocytes and platelets to the endothelium	Cytokine release syndrome, Pancytopenia, Anaphylaxis, Allergy
1985	Monoclonal antibodies	OKT3	CD3, CD25	Block α chain of the IL-2 receptor and hence T cell replication	GI toxicity
2000		Basiliximab			
1999		Daclizumab			

1.6. Special Conditions

Individuals who have certain sicknesses or disorders, or who are taking some different medications may have issues if they take immunosuppressant drugs along with their previous drugs. Before consuming these medications, patients have to discuss with the doctor about any of the conditions:

1.6.1. Allergies

Any individual who has had irregular responses to immunosuppressants in the past should tell his or her doctor before taking the medications once again. The doctor should discuss any sensitivities towards food, colors, additives, or different substances.

1.6.2. Pregnancy

It is supposed that the Azathioprine associated with congenital disabilities. The British National Formulary, however, states: Transplant patients immunosuppressed with azathioprine should not discontinue it on becoming pregnant; there is no evidence that azathioprine is teratogenic. There is less experience of cyclosporin in pregnancy, but it does not appear to be any more harmful than azathioprine. The use of these drugs during pregnancy needs supervision in specialist units. Any risk to the offspring of azathioprine-treated men is small. However, patients who are on any immunosuppressive medication ought to counsel with their doctor before conceiving a child, and they ought to advise the physician at once when there is any sign of pregnancy. [43]

The producers of this drug suggest the use of acceptable contraception along with the utilization of this medication, and for about two months following the last dose. The use of tacrolimus within pregnancy should be avoided, based on animal studies. So, contraception should be used along with tacrolimus and for about a month and a half after the last dose. The use of corticosteroids amid pregnancy has not yet evaluated. There is some proof that the utilization of these medications during pregnancy may influence the child's development; nonetheless, this outcome isn't sure and may be diverse by the drug utilized. Patients taking any steroid medication ought to counsel with their doctor before beginning a family, and ought to tell the physician without a

moment's delay if they think they are pregnant. The majority of these medications have not evaluated yet in people amid pregnancy. Ladies who are pregnant or who may end up pregnant and who need to take immunosuppressants ought to counsel their doctors.[43]

1.6.3. Lactation

During the lactation period, it is not suggested that they feed their children's as immunosuppressant drugs generally pass into breast milk and may cause problems in nursing babies.

1.7. Other Therapeutic Conditions

Peoples who suffer from the following conditions and are on immunosuppressant medication may have some severe problems:

Peoples having intestinal problems may report less effectiveness of immunosuppressants if they take oral dosage as there were problems in the absorption of the drug. If a patient is suffering from the renal or hepatic disorder, these drugs may produce some severe side effects in the body as their bodies are slowly excreting the drug. IF the peoples recently suffered from shingles (herpes zoster), chickenpox may develop severe infections in the other parts of their body if they consume these drugs.

1.8. Side Effects

Elevated risks of infectious disease are a typical symptom of all immunosuppressant drugs. Taking such anti-infection agents as co-trimoxazole prevents some of these infections. Immunosuppressant drugs are associated with elevated risks of malignant growth because the immune system protects the body against certain types of disease. For instance, the long-term utilization of immunosuppressant drugs associated with the high risk to develop skin cancer because of the combination of the medications and exposure to sunlight. Additional symptoms of immunosuppressant drugs are minor and disappear as the body acclimates to the prescription. These symptoms are a loss of hunger, more hair growth, and trembling or shaking of the hands. There is no need for any medical treatment unless these symptoms proceed or cause issues.[43]

1.9. Drug Interactions

There are chances of drug-drug interactions if a patient is on multiple medications. Due to these interactions, the efficacy of one or both the drugs may change, or sometimes the chances of severe side effects may increase. The use of other medications sometimes suppresses the effect of immunosuppressant therapy. If a patient is on cyclosporine or tacrolimus, then he/ she has to take care of the possibility of interaction chances. Some other examples are:

- Azathioprine along with allopurinol shows more significant effects in humans.
- Cyclosporin effects also intensify if consumed along with estrogens, androgens, ketoconazole, cimetidine, erythromycin, etc.
- If sirolimus in combination with cyclosporin, is consumed; the blood level of sirolimus reaches a toxic level. Though these drugs are the part of the combination for the immunosuppression, the sirolimus should be taken at least 4 hours after the dose of cyclosporin.
- Tacrolimus is excreted mainly through urine. When used with other medications which alter the renal functions leads to an increased concentration in blood. So, it should be limited along with such drugs (cyclosporin, gentamicin, amikacin, amphotericin B, etc.) which have a direct effect on kidneys.
- The chances of cancer or other infections are more if consumed with other medications which have a direct effect on the body's immune system like prednisone, chlorambucil, cyclophosphamide, mercaptopurine, and muromonab-CD3. These drugs also used to prevent graft rejection. [43]

1.10. Capsule Dosage Form

The word 'capsule' in the English language is derived from the Latin word 'capsula,' which means a small box or container.[44] In the 19th century, the discovery of substances in powder form like the alkaloids suddenly opened new therapeutic possibilities. With the new substances, new dosage forms were created (like in 1834 the hard gelatin capsule invented by Mothes and in 1843 the tablet invented by Brockedown). Capsules are solid dosage forms containing drug and usually appropriate filler(s), enclosed in a hard or soft gelatin shell. The gelatin shell readily ruptures and

dissolves following oral administration, and in most cases, the drug is released from a capsule faster than from a tablet. [45]

Hard gelatin capsules consist of two pieces in the form of cylinders closed at one end: the shorter piece, called the 'cap,' fits over the open end of the longer piece, called the 'body. Both soft and hard gelatin capsules contain gelatin, water, colorants and optional materials such as process aids and preservatives; also, soft capsules contain various plasticizers. Capsules can also be produced from hydroxypropyl methylcellulose to produce a shell with low moisture content. [45, 46]

The chance to process powders on a large scale with prolonged stability compared to liquid or semi-solid dosage forms opened all possibilities of industrial production.

Nowadays, solid dosage forms are still prevalent because they have a high metering accuracy, the application of them is straightforward and comfortable, and their stability is excellent.

1.10.1. Properties of the hard gelatine capsule

Hard gelatin capsules are readily soluble in water at 37°C. When the temperature falls below this, their rate of solubility decreases. At below about 30°C they are insoluble and absorb water, swell and distort. So, solubility is an essential factor to take into account during disintegration and dissolution testing. Because of this most pharmacopeias have set a limit of 37 ° ± 1°C for the media for carrying out these tests. Capsules made from hydroxypropyl methylcellulose have a different solubility profile, being soluble at temperatures as low as 10°C. [45]

1.10.2. Capsule sizes

Hard gelatin capsules have a range of fixed sizes; the standard industrial sizes in use today for human medicines are from 0 to 4. For a powder, the most straightforward way in which to estimate the fill weight is to multiply the body volume by its tapped bulk density. For liquids, the calculations of total filled weight are multiplying the specific gravity of the fluid by the capsule body volume x 0.8. To accommodate individual needs, some standard sizes are produced, termed 'elongated sizes,' that typically have an

extra 10% of fill volume over the standard sizes. [45, 47] The capsule size and body fill volumes are mentioned in Table 1.3.

1.10.3. Capsule shell filling

Hard gelatin capsules may be filled with a large variety of materials of different physicochemical properties. Gelatin is a relatively inert material. The substances to be avoided are those which are known to react with it or those that interfere with the integrity of the shell. Materials for filling into hard gelatin capsules include dry solids, powders, pellets, granules, tablets, semisolids, thermo softening mixtures, thixotropic mixtures, pastes, and non-aqueous liquids. If the dose of the drug to be placed in a single capsule is inadequate to fill the volume of the capsule, a diluent is necessary to add the proper degree of bulk to the drug to produce the appropriate fill. When the amount of drug to be administered in a single capsule is large enough to fill a capsule, a diluent may not be required. In many instances, the amount of drug is filled in a single capsule as a dose of that particular medication. [44, 45]

However, when the amount of drug representing a usual dose is too large to be placed in a single capsule, two or more capsules may be required to provide the desired dose of the particular drug. [48]

1.10.4. Capsule-filling machines

There are some basic sets of operations which are carried out whether capsules are being prepared on the bench for extemporaneous dispensing or on high-speed automatic machines for industrial products. The major difference between the various methods available is how the dose of material is filled into the capsule body. [45, 49]

1.10.5. Filling of powder formulations

There are two basic methods for filling capsules. These are bench scale filling and industrial filling methods. [45]

Table 1.3: Capsule size and body fill volumes

Size	Avg. weight of empty capsule (mg)	Capacity (in mL)	Capacity (in mg)
000	163	1.37	1000
00	118	0.91	735
0	95	0.68	500
1	75	0.5	400
2	60	0.37	300
3	47	0.3	200
4	38	0.21	100

1.10.6. Bench-scale filling

There is a requirement for filling small quantities of capsules, from 50 to 10,000 in community pharmacy, in hospital pharmacy, or industry for proper prescriptions or trials. There are several simple pieces of equipment available for doing this. These normally consist of sets of plastic or metal plates which have predrilled holes to take from 30 to 100 capsules of a specific size. Empty capsules are placed into the holes, either manually or with a simple loading device. The bodies are locked in their plate using a screw, and the caps in their plate are removed. The powder is placed on to the surface of the body plate and is spread with a spatula so bodies can be filled. The uniformity of fill weight is very dependent upon good flow properties of the powder. The cap plate is repositioned over the body one, and the capsules are rejoined using manual pressure. [45, 50]

1.10.7. Industrial-scale filling

The machines for the industrial-scale filling of hard gelatin capsules come in great variety of shapes and sizes, varying from semi- to fully automatic and ranging in output from 5000 to 15, 000 per hour. The dosing systems may have two groups:

• Dependent dosing systems that use the capsule body directly to measure the powder. Uniformity of fill weight can only be attained if the capsule is filled properly. For filling the auger is normally used. [45, 47]

• Independent dosing systems where the powder is measured independently of the body in a special measuring device. Weight uniformity is not dependent on filling the body. With this system, the capsule can be partially filled. [45, 51]

1.10.8. Bioavailability of Powder-filled capsules

Provided the hard gelatin shell dissolves rapidly in the gastrointestinal fluids and the encapsulated mass disperses rapidly and efficiently, a relatively large effective surface area of the drug will be exposed to the gastrointestinal fluids, thereby facilitating dissolution. The overall rate of dissolution of drugs from capsules appears to be a complex function of the rates of different processes. It includes the dissolution rate of the gelatin shell, the rate of penetration of the gastrointestinal fluids into the encapsulated mass, the rate at which the mass de-aggregates (disperses) in the gastrointestinal fluids, and the rate of dissolution of the dispersed drug particles. The inclusion of excipients such as diluents, lubricants, and surfactants in a capsule formulation can have a significant effect on the rate of dissolution of drugs, particularly those that are poorly soluble and hydrophobic. [45, 50]

The diluent should not tend to adsorb or complex with the drug, as either can impair absorption from the gastrointestinal tract. Both the formulation and the type and conditions of the capsule-filling process can affect the packing density and liquid permeability of the capsule contents. In general, an increase in packing density of the encapsulated mass will probably result in a decrease in liquid permeability and dissolution rate, particularly if the drug is hydrophobic, or if a hydrophilic drug mixed with a hydrophobic lubricant such as magnesium stearate. If the encapsulated mass is

tightly packed, and the drug is hydrophobic, then a decrease in dissolution rate with a concomitant reduction in particle size would be expected, unless a surfactant had been included to facilitate liquid penetration. In summary, formulation factors [45, 50] that can influence the bioavailability of drugs from hard gelatin capsules include:

- The surface area and particle size of the drug (particularly the effective surface area exhibited by the drug in the gastrointestinal fluids);
- The use of the salt form of a drug in preference to the parent weak acid or base;
- The crystal form of the drug;
- The chemical stability of the drug (in the dosage form and gastrointestinal fluids);
- The nature and quantity of the diluent, lubricant and wetting agent;
- Drug-excipient interactions (e.g., adsorption, complexation);
- The type and conditions of the filling process used;
- The packing density of the capsule contents;
- The composition and properties of the capsule shell (including enteric capsules);
- Interactions between the capsule shell and its contents.

1.11. Advantages of Capsule Dosage Form

A capsule has some benefits compared to a tablet:

Developing a capsule formulation is in most cases not as complicated as for a tablet formulation. A powder mixture can be filled directly into a capsule shell without a granulation and a compression process. For this reason, a capsule formulation often is the first dosage form for early clinical studies in the industry, and the filling of capsules by hand is a common practice in pharmacies for a particular medication.[52]

For blinding purposes, an active ingredient can be simply encapsulated. Once the shell is soaked and dissolved in the stomach, the active component may in some cases be available in a loose, dispersed and, for this reason, in an early and well absorbable state if the permeability through a biomembrane. [52]

Different colors of the capsule shells allow the patients to distinguish their medications. A bad taste of a substance can be masked by a capsule shell (e.g., chloramphenicol,

tetracycline). When a small-sized capsule has to be taken orally, the swallowing may in some instances be more comfortable because after contact with the saliva it gets more slippery than a tablet. [53]

1.12. Disadvantages of Capsule Dosage Form

If a significant amount of a compound has to be given to a patient, the size of the capsule can quickly get too big compared to the same amount compressed to an oblong tablet.

Some highly efflorescent and hygroscopic materials should not be filled into capsule shells because efflorescent substances may cause the capsules to soften, whereas hygroscopic powders may dry the capsule shell to excessive brittleness.

A significant disadvantage of the capsule, however, is the fact that producing a capsule formulation is more expensive compared to a tablet formulation because the capsule shell has to be bought additionally. Furthermore, a tablet rotary press can produce up to one million tablets per hour whereas the maximum production speed of a dosing disk capsule filler reaches about 200’000 capsules per hour. Thus, there are some reasons for the economic and marketing point of view to prefer a capsule or tablet formulation.

1.13. Stability / Stable Formulation: A Preview

The term ‘stability’ concerning a drug dosage form, refers to the chemical and physical integrity of the dosage unit and, when appropriate, the ability of the dosage unit to maintain protection against microbiological contamination. The shelf life of the dosage form is the time lapse from initial preparation to the specified expiration date. The monograph specifications of identity, strength, quality, and purity apply throughout the shelf life of the product.

The stability parameters of a drug dosage form can be influenced by environmental conditions of storage (temperature, light, air, and humidity), as well as the package components. Significant factors affecting the stability are:

- Temperature
- Moisture content (Humidity)
- Presence of oxygen

- Light
- pH

One of the main contributors to the degradation of an active drug substance in a pharmaceutical formulation is the presence of moisture. Capsules, which are mostly dry dosage forms containing only minute amounts of water, commonly have a much longer shelf life than other formulations, such as oral and parenteral liquids.

Nonetheless, it cannot be taken for granted that all capsules will have a long shelf life. The choice of excipients, for example, is an important factor in this respect. Some excipients are hygroscopic, and even minute amounts of moisture can decrease the stability of the drug. This is especially important for effervescent capsules; the packaging material plays an essential role in the protection of this capsule form from moisture.

The importance of stability in the development of pharmaceutical dosage forms is well recognized in the pharmaceutical industry. Increasing filing of ANDA by generic drug manufacturer has resulted in the submission of stability data to FDA. For assure quality and safety the stability data are required. The application of certain physiochemical principles in the performance of stability study has proven to be considerable advantage in the development of stable dosage forms.

For a drug substance to be developed into a capsule dosage form, the objective may be achieved by investigating the stability of the drug under the following conditions.

- Solid state stability of drug alone
- Compatibility studies (stability in the presence of excipients)
- Solution phase stability (stability in gastrointestinal fluid and granulating solvents used during the manufacturing process)

1.14. Routes of Degradation

1.14.1. Hydrolysis

In this type of reaction, the active drug undergoes decomposition following reaction with the solvent present. Usually, the solvent is water, but sometimes the response

involves pharmaceutical co-solvents such as ethyl alcohol or polyethylene glycol. These solvents act as nucleophiles attacking the electropositive center in drug molecule. The examples are esters in Aspirin and Alkaloids, lactones in Pilocarpine and Spironolactone, and malonic ureas in Barbiturates.

1.14.2. Oxidation

Oxidation reactions are important pathways of drug decomposition. In pharmaceutical dosage forms, oxidation is usually facilitated through reaction with atmospheric oxygen under ambient conditions; a process commonly referred to as autoxidation. The mechanism of oxidation reactions is generally involved, involving multiple pathways for the initiation, propagation, branching, and termination. Acids and bases catalyze many oxidation reactions. Some functional groups subjected to autoxidation in drugs are phenols in steroids, thiols in chlorpromazine, and amines in morphine and clozapine.

1.14.3. Photolysis

Ordinary room light or sunlight may cause substantial degradation of drug molecules. The energy from light radiations is absorbed by a molecule to produce a photolytic reaction. If that energy is sufficient to achieve activation, degradation of the molecule is possible. A dramatic example of photolysis is the photodegradation of sodium nitroprusside in aqueous solution.

1.14.4. Dehydration

In dehydration, the elimination of water molecule from the active substances takes place. The driving force for this type of dehydration is the formation of a double bond that can then participate in electronic resonance with neighboring functional groups. Water removal does not create new bonds but often changes the crystalline structure of the drug. Dehydration reactions involving water of crystallization may potentially affect the absorption rate. Prostaglandin E2 and tetracycline degrade by dehydration.

1.15. General Guidelines for Collection of Stability Data

In general case, stability conditions are defined in three types. The conditions are given in the table that covered, storage condition and period of the study. The accelerated study was done at 40°C ± 2°C/75% RH ± 5% RH, to know the results of the study in a short duration of time. The results of accelerated stability study were then extrapolated to estimate the stability under ordinary conditions. A long-term study was mainly done at 25°C ± 2°C/60% RH ± 5% RH and the results were collected after 12 months. Sometimes intermediate stability studies at 30°C ± 2°C/65% RH ± 5% RH, was done and the data was collected after six months.

Table 1.4: General cases

Study	Storage condition	The minimum period covered by data at submission
Long term*	25°C ± 2°C/60% RH ± 5% RH or 30°C ± 2°C/65% RH ± 5% RH	12 months
Intermediate**	30°C ± 2°C/65% RH ± 5% RH	6 months
Accelerated	40°C ± 2°C/75% RH ± 5% RH	6 months
*It is up to the applicant to decide whether long-term stability studies are performed at 25 ± 2°C / 60% RH ± 5% RH or 30°C ± 2°C / 65% RH ± 5% RH. **If 30°C ± 2°C/65% RH ± 5% RH is the long-term condition, there is no intermediate condition		

Table 1.5: Current WHO definition of climatic zones

Climatic zone	**Definition**	**Storage condition**
I	Temperate climate	21°C/45% RH
II	Subtropical and Mediterranean climates	25°C/60% RH
III	Hot, dry climate	30°C/35% RH
IVA	Hot, humid climate	30°C/65% RH
IVB	Hot and very humid climate	30°C/75% RH

1.16. Capsule Formulation Excipients (In Case of Amorphous Powder API)

In a capsule formulation, a range of excipient materials is usually required along with the active ingredient to give the capsule the desired properties. For example, the reproducibility and dose homogeneity of the capsules are dependent on the properties of the powder mass. The capsule should also be sufficiently strong to withstand handling but should disintegrate after intake to facilitate drug release. The choice of excipients will affect all these properties.

1.16.1. Filler

Fillers are used to making capsules of sufficient size for easy handling by the patient and to facilitate production. Capsules containing a very potent active substance would be small without additional excipients. The good filler will have good compactability and flow properties, pleasant taste, will be non-hygroscopic and preferably chemically inert. It may also be advantageous to have a filler that fragments easily since this counteracts the adverse effects of lubricant additions to the formula.

1.16.2. Binder

A material with a high bonding ability can be used as a binder to increase the mechanical strength of the capsule. A binder is usually a ductile material prone to undergo plastic (irreversible) deformation. Typically, binders are polymeric materials, often with disordered solid-state structures. Of particular importance is the deformability of the peripheral parts (asperities and protrusions) of the binder particles. Thereby, this group of materials has the capacity of reducing inter-particulate distances within the capsule, improving bond formation. If the entire bulk of the binder particles undergo extensive plastic deformation during compression, the interparticular voids will, at least partly filled and the capsule porosity will decrease. Binders increase the contact area between the particles, which promotes the creation of interparticular bonds and subsequently increases the capsule strength. However, the effect of the binder depends on both its properties and those of the other compounds within the capsule. A binder is often added to the granulation liquid during wet granulation to improve the cohesiveness and compactability of the powder particles, which assists the formation of agglomerates or

granules. The soluble binder is commonly added, during a granulation process, is more effective than used in dry powder form during direct compression.

1.16.3. Glidant

Glidants are added to increase the flowability of the powder mass, reduce interparticular friction and improve powder flow in the hopper shoe of the capsule filling machine. A glidant will only work at a certain range of concentrations. Above a specific level, the glidant will in fact function to inhibit flowability. A glidant's effect is due to the counter-action of factors that cause poor flowability of powders. For instance, correcting surface irregularity, reducing interparticular friction and decreasing surface charge. The result is a decrease in the angle of repose which is an indication of an enhanced powder's flowability.

CHAPTER 2

REVIEW OF LITERATURE

2.1. Literature Review on Renal Transplant

In the immunology, we generally use a transplantation term, which generally means that the transfer of body cells, tissue, or any organs from one site to another in the body. Lots of problems are eliminated by implanting the healthy organ, tissue or cells from one body to another human body.[1]

2.1.1. Schulz, *et al,* (1998).

A studied case report in which simultaneously pancreas-kidney was transplanted in December 1995 and March 1997. Twenty-five patients were under the immunosuppressive therapy which was provided as follows 250 mg of Prednisolone, 5 mg of tacrolimus, 3 g of MMF ATG (6mg/kg). This regimen proved that MMF and Prednisolone had been provided in transplantation from a long time. [54]

2.1.2. Schulz, *et al,* (1999).

A studied case report in which simultaneously pancreas-kidney was transplanted in December 1996 and October 1997. Thirty-eight patients were under immunosuppressive therapy which was given as 250 mg of Prednisolone, 5 mg of tacrolimus, 4g of MMF, ATG (6mg/kg). This study confirmed that MMF and Prednisolone are active together. [55]

2.1.3. Agarwal, *et al,* (2000).

Studied a case report of a 10-year-old male child that had undergone renal transplantation. The patient was maintained on the triple regimen of azathioprine, cyclosporine, and prednisolone. Due to the complications developed cyclosporine was replaced with Mycophenolate Mofetil. The renal function improved with this triple therapy MMF (750 mg/day), prednisolone and azathioprine. After seven days, the patient was maintained on the mixture of prednisolone and MMF and azathioprine was withdrawn. Even after nine months, the patient continued the renal function quite well

with a serum creatinine of 0.9 mg/dl despite the low immunosuppression being administered. [56]

2.1.4. Kim, *et al,* (2000).

The study described the incorporation of mycophenolate mofetil in the conventional regimen therapy for the prevention of rejection of organ transplantation. A non-randomized prospective study was conducted to compare the efficacy of already available double drug regimen of cyclosporine and prednisolone. For renal transplant patients, the MMF was found as a useful third agent. It was connected with a decrease in the rejection rate of the allograft. [57]

2.1.5. Bagga, *et al,* (2003).

Potentially observed the long-term therapy of MMF as a steroid-sparing agent. Nineteen patients of the average age of 99.1 months were under the long-term therapy of steroids and cyclophosphamide previously. Then MMF was incorporated into the regimen with Prednisolone that was given at alternate days. It was concluded that MMF with tapering doses of Prednisolone was a promising intervention. [58]

2.1.6. Borrows, *et al,* (2005).

The authors studied a 7-day steroid-sparing regimen, tacrolimus, and MMF. In this, an attempt was made to eliminate steroids from the therapy. One hundred patients were examined under the proposed regimen, and they were given tacrolimus 0.15mg/ kg and 750 mg of MMF administered twice daily which was reduced to 2 g depending on the white count. The steroid protocol included 500 mg of methylprednisolone iv followed by Prednisolone 1mg/kg/day which was reduced to 0.5 mg/kg/day. After 7th-day steroids were eliminated from the protocol. The subject maintained graft for a while, but steroids were incorporated to treat the acute rejections. Oral Prednisolone was continued if the rejection was in the first week. So, it was concluded that a low dose of steroids was beneficial withthe regimen of MMF and tacrolimus. [59]

2.1.7. Kalble, *et al,* (2005).

The author presented guidelines that provided insight into the issues surrounding the

renal transplantation. A review on the immunosuppressive therapy with their origin and starting year of their clinical utilization with the mechanism of actions, advantages of one treatment over the other**.** [60]

2.1.8. Taylor, *et al,* (2005).

A review on the immunosuppressive therapy was presented that showed that effective immunosuppression is essential for the successful solid organ transplantation. Details of the individual agents that are involved in the immunosuppressive therapy have been given with a focus on their mechanism of action. [61]

2.2. Literature review on drug utilization in kidney transplant patients

With the development of more effective immunosuppressant drugs for kidney transplant patients, usage of immunosuppressant drugs is changed than the previous years in all over the world. Few studies were conducted to identify the changes in drug utilization which are described as bellow.

2.2.1. Meier-Kriesche, *et al,* (2003).

In this study, they found that usage of induction immunosuppressant has increased from 46% in 1995 to 72 % in 2004. Authors also found that anti-thymocyte globulin was the most commonly used induction immunosuppressant (37% of transplant recipients). According to the report, calcineurin inhibitors (CNIs) were the main maintenance immunosuppressant, being prescribed to 93% of patients. They observed trend was a move towards the TAC among CNI. In 2004, usage of TAC was increased to 72 % as compared to only 21% patient received CyA. Same way among antiproliferative agents, the use of MMF was increased (81%) compared to AZA. There was continues to improve the utilization of the combination of TAC/MMF; it was the most frequently used discharge regimen (60%).[62]

2.3. Literature review on the comparison of immunosuppressant drugs and their effect

With the development of the new medication, graft and patient survival rate after the transplant is increasing. Now a day one-year patient survival rate is increased to 95%.

Many trials were conducted to compare the drugs, the impact of drugs on patients and graft survival rate, kidney functions and a side effect of drugs. Some of them mention below:

2.3.1. Vincenti, *et al,* (2007).

The author had performed a 6-month, randomized, open-label, multicenter trial on 682 patients to compare CyA micro-emulsion with TAC. At 6-month, they observed the incidence of treated diabetes was significantly higher in the TAC group (48/286, 16.8%) as compared to the CyA group (25/281, 8.9%; P = 0.005) and incidence of graft loss, BPAR, or death occurred in 34 TAC patients (9.8%) and 43 CyA patients (12.8%, P = 0.211). In their study, in CyA arm 59% (20/34) rejection episodes were mild graded (Grade IA or IB), 38% (13/34) were moderately graded (Grade IIA) and 3% (1/34) were severe graded (Grade > 2B) as compared to 46% (11/24), 29% (7/24) and 25% (6/24) in the TAC group, respectively.

At 6 months, they did not find any significant difference between both TAC and CyA group for mean GFR (65.9 ± 23.1 vs. 63.6 ± 20.7, P = 0.285) but for mean creatinine they found a significant difference between both groups (133 ± 57 µmol/L vs. 139 ± 58 µmol/L, P = 0.005). The average blood pressure was almost alike between both treatment groups at six months while median LDL-cholesterol, triglycerides, and total cholesterol were higher in the CyA arm than in the TAC arm. They observed increased CMV infection in CyA arm as compared to the TAC arm (P = 0.003). [14]

2.3.2. Larson, *et al,* (2006).

The author had performed a study on recipients who suffered transplantation between April 2001 and January 2004 at Mayo Clinic, Rochester. In this study, a total of 165 patients was enrolled, and among them, 84 patients were randomized to TAC therapy and 81 patients to sirolimus therapy.

During the study, 38% (30 patients) of sirolimus group and 16% (13 patients) of the TAC group discontinued the assigned study medication. In Kaplan-Meier analysis, one-year patient survival rate (96% vs. 98%, P = 0.42) and graft survival rate (92% vs. 94%, P = 0.95) were non-significant between TAC group and sirolimus group. The similar

non-significant difference in the rejection rates was also observed between the groups (P = 0.51). For tubular atrophy, interstitial fibrosis, and glomerulopathy difference were also nonsignificant between both groups. They observed a higher incidence of chronic vascular changes in the TAC group (43% in TAC vs. 26% in Sirolimus, P = 0.03).

The incidence of polyomavirus infection was non-significant between both groups (P = 0.37) but systemic CMV infection was significantly higher in TAC group (TAC = 12% vs. Sirolimus = 3%, P = 0.02). The incidence of NODAT was non-significant between both group (TAC = 10% vs. Sirolimus = 7.5%, P = 0.78). Sirolimus was linked with an enhanced incidence of wound healing complications as compared to TAC. By these results, they conclude that sirolimus + MMF + prednisone regimen having a similar patient and graft survival as compared to a regimen of TAC + MMF + prednisone but having low acute rejection rates at one year after transplant. [63]

2.3.3. Webster, *et al,* (2005).

The author had comprehensively searched Embase (1980-October 2003), Medline (1966-October 2003), conference proceedings, and Cochrane Collaboration resources and included all randomized trials comparing TAC with CyA solution or CyA micro-emulsion as initial immunosuppressive therapy. By this way, they enrolled 4102 randomized participants from 123 reports of 30 trials.

The study reported that, at 6 months, TAC was associated with significant reduction of graft censored for death (44%; CI = 0.36 to 0.86, RR = 0.56, 95%) and similar result was also observed after three years (29%; CI = 0.52 to 0.96, RR = 0.71, 95%). They also observed TAC treated recipients had expressively lower rejection (confirmed by biopsy or diagnosed by clinically) beyond three months as compared to CyA. For steroid-resistant rejection, they also find TAC therapy was associated with a 55% reduction at six months as compared to CyA therapy (RR = 0.45, 0.33 to 0.60). At six months, mean creatinine was significantly lower in TAC-treated patients as compared to CyA treated patients.

For NODAT, they observed TAC was significantly associated with risk of NODAT at 6-month (RR = 2.56, 1.37 to 4.78), 1-year (RR = 1.86, 1.11 to 3.09), and 3-year (RR =

2.01 to 7.41) after transplant. TAC treatment was more prone to have vomiting, tremor, dyspepsia, headache, hypomagnesemia, and diarrhea than CyA treatment while CyA treatment was more prone to have hirsutism, constipation, and gingival hyperplasia.

By this study, they conclude TAC is superior to CyA in preventing acute rejection and have improved early graft survival, but TAC treatment has more diabetes, neurological, and GI side effects. [64]

2.3.4. Kramer, *et al,* (2005).

They performed a randomized, comparative six-month trial to compare TAC and CyA. Both drugs were given in combination with AZA and steroids. In this study, they enrolled 286 patients in the TAC arm and 271 in the CyA arm in aged 16 to 60 year as intent to treat population (ITT). Among these patients, 237 patients (82.9%) in the TAC treatment group and 222 patients (81.9%) in the CyA group were assessed at two-year follow-up.

Calculated on ITT populations, mortality was significantly lower in TAC group as compare to CyA (2.0% vs 3.3%; $P < 0.05$) while graft loss rate in both groups were non-significant after 2 years (9.3% vs 11.2%; $P = 0.12$). Biopsy-proven acute rejection was significantly lower in TAC as compare to CyA (19.6% vs 37.3%, $P < 0.0001$) at first 6 month, but was non-significant during 7–12 and 13–24 months follow-up (4.7% and 0.9% with CyA and 1.7% and 0.8% with TAC, respectively). At 24 months after transplant, composite endpoint consisting of biopsy-proven acute rejection, patient death, and graft loss was reported significantly less frequently in TAC patients than in CyA patients.

In their study, a serum creatinine concentration was significantly better in the TAC group as compared to the CyA group (136.9 vs. 161.6 mmol/L; $P < 0.01$). If considering stable maintenance regimen then more patients of TAC group were stable as compared to CyA group (82.5% vs. 66.2%, respectively) at two years while more patients in the TAC group were off steroids and received CNI monotherapy and fewer TAC patients remained on a triple immunosuppressive regimen.

They also compare cardiovascular risk profile and found that TAC having beneficial effect on lowering cholesterol (5.24 ± 1.04 vs 5.49 ± 1.04 mmol/L, $P < 0.01$) and triglycerides (1.59 ± 0.86 vs 1.75 ± 1.03 mmol/L, $P < 0.05$) at 2 year after transplant. They also measure New-onset diabetes mellitus at 2 year but difference between both drugs was statistically nonsignificant [TAC = 3.6% (08 Patients) vs CyA = 1.9% (04 patients)]. In this study, they did not find any significant difference in blood pressure in both treatment groups at two years. By this study, they conclude that TAC is highly efficient as a baseline immunosuppressant and produce a long-term beneficial effect on graft function and graft survival. [65]

2.3.5. Vincenti, *et al,* (2002).

The author had performed a five-year crossover study to measure the effect of TAC and CyA on graft survival. In Intent-to-treat analysis, they did not find significant difference between both treatment groups for graft survival (64.3% vs. 61.6%; $P = 0.558$) and patient survival (79.1% vs. 81.4%; $P = 0.472$) at 05 year. The rate of crossover and treatment failure was significantly lower in patients randomized to receive TAC-based therapy (9.3% vs. 27.5%; $P < 0.001$ and 43.8% vs. 56.3%; $P = 0.008$, respectively). It was found significant improvement in graft survival in TAC treatment arm. Due to rejection, the crossover was calculated as the graft failure (i.e., 63.8% vs. 53.8% and P was found as 0.014). They also compare both groups for the requirement for medications to control hyperlipidemia and hypertension and found that TAC therapy was also associated with a significantly reduced the usage of this concomitant medication. They found that there was a substantial rate of reversal of TAC associated insulin dependence. [66]

Based on results, they conclude TAC-based therapy was significantly associated with lowering the risk of graft failure, without an increase in the incidence of adverse events associated with a long-term immunosuppressant.

2.3.6. Knoll, *et al,* (1999).

The author had performed a meta-analysis of randomized trials to compare TAC vs. CyA and for that, they reviewing Medline database, Embase database, Cochrane

Library, Transplantation and Transplantation Proceedings journal, and in this study, they used eight articles out of 499 articles based on exclusion criteria. Thus, the final analysis was based on 1037 patients. They found that there was a non-significant effect of TAC on graft loss at one year as compared to CyA (OR 0.95; 95% CI 0.65 to 1.40). Similarly, the non-significant difference for mortality was also observed between both treatment arms at one year (OR 1.07; 95% CI 0.47 to 2.48). In their study, they found TAC treatment was associated with a significant reduction in episodes of acute rejection as compare to CyA therapy (OR 0.52; 95% CI 0.36 to 0.75) and also the use of anti-lymphocyte antibodies to treat rejection was significantly lower in patients receiving TAC (OR 0.37; 95% CI 0.25 to 0.56). In this study, they reported a higher proportion of patients treated with TAC had NODAT as compare to CyA group at one year after transplant (OR 5.03; 95% CI 2.04 to 12.36). They perform a sensitivity analysis between two groups and found treatment with TAC did not have a significant effect on graft loss (odds ratio 0.68; 95% CI 0.38 to 1.22) or patient mortality (OR 0.80; 95% CI 0.20 to 3.21). [67]

2.3.7. Mayer, *et al,* (1997).

The author had performed a multicenter, randomized trial to compare the 12-month efficacy and safety of TAC- and CyA-based immunosuppressive regimens in the prevention of renal allograft rejection. In this study, a total of 448 renal transplant recipients was enrolled. Among these patients, 303 patients were on TAC arm and 145 patients on CyA arm. All patients were also receiving AZA and corticosteroids. At the end of study, they observed, acute (25.9% vs. 45.7%; $P < 0.001$) and corticosteroid-resistant rejection (11.3% vs. 21.6%; $P = 0.001$) were considerably poorer in TAC therapy. Actuarial 1-year graft survival rate (82.5% vs. 86.2%; $P = 0.380$) and patient survival rate (93.0% vs. 96.5%; $P = 0.140$) was statistically non-significant between both treatment groups.

In this study, they observed renal impairment, infections, gastrointestinal complaints and neurological complications in both treatment group frequently but mostly reversible. In TAC treated group, they reported higher incidences of diarrhea, elevated serum creatinine, hyperglycemia, tremor, angina pectoris, and diabetes mellitus while

in CyA group, they more reported gingival hyperplasia, acne, hirsutism, and arrhythmia. By this study, they conclude that TAC therapy was associated with a significant reduction in the incidence of rejection episodes and may have critical long-term suggestions on graft survival. [68]

2.3.8. Sollinger (1995).

The U.S. Renal Transplant Mycophenolate Mofetil Study Group compared the two doses of MMF to AZA in patients receiving CyA, steroids, and ATG induction therapy. This double-blind, multicenter, randomized study was conducted in adult patients to monitor and explore the ability and safety of MMF for the prevention of acute rejection.

In this study, a total of 499 patients was randomized in three treatment group based on dose and treatment. One group received 2 g MMF (1 g MMF, twice a day), the second group received 3 g MMF (1.5 g MMF, twice a day) and the third group received AZA (1-2 mg/kg/day). All patients were received CyA, corticosteroids, and ATG.

They reported 47.6% of AZA-treated patients had biopsy-proven acute rejection episodes or treatment failure while 31.1% of 2 g MMF treated patients ($P = 0.0015$) and 31.3% of 3 g MMF treated patients ($P = 0.0021$) reported with biopsy-proven acute rejection episodes or treatment failure. They also compared the time of first biopsy-proven rejection episode or treatment failure and found that 2 g MMF treated patients and 3 g MMF treated patients developed significantly longer time after transplant as compared to AZA-treated patients ($P = 0.0036$, $P = 0.0006$, respectively).

They also reported, AZA-treated patients required more antirejection treatment as compared to patients treated with 2 g MMF and 3 g MMF (AZA = 44.5% vs2 g MMF = 24.8%, and 3 g MMF = 21.1%). The usage of antilymphocyte agents as a part of antirejection therapy was higher in the AZA-treated patients (20.1%), while 10.3% in 2 g MMF treated group and 5.4% in 3 g MMF treated patients. At six months after transplant, patient survival and graft survival were similar in all three treatment groups. [69]

2.3.9. European Mycophenolate Mofetil Cooperative did a study

The European Mycophenolate Mofetil Cooperative Study Group compared 2 g MMF treated and 3 g MMF treated patients with placebo-treated patients. All patients received CyA and prednisone along with this drug. In this study total 491 patients were enrolled, and among them, 166 patients were randomized in the placebo, while 165 patients in 2 g MMF, and 160 patients in 3 g MMF treatment group.

In this study, they observed 2 g MMF treated recipients having significantly lower biopsy-proven rejection as compared to placebo-treated recipients (17.0% in 2 g MMF, 13.8% in 3 g MMF and 46.4% in Placebo-treated, $P < 0.0001$). Antirejection therapy requirement for rejection episode was also higher in the placebo-controlled group (51.8%) as compared to 2 g MMF treated group (28.5%) and 3 g MMF treated group (24.4%).

This study reported 10.2% placebo-treated patients were died or lost their graft at 06 months after transplant while 6.7% of 2 g MMF and 8.8% of 3 g MMF treated patients were died or lost their graft at 06 months after transplantation. In totality, the frequency of contrary events was almost alike in all treatment groups, although opportunistic infections, leucopenia, and gastrointestinal problems, were more common in the MMF groups and more in 3 g MMF treated patients as compared to 2 g MMF treated patients. By this study, they conclude that at six months after transplant, MMF treatment was significantly associated with a reduction of biopsy-proven rejection rate or another treatment failure. MMF therapy was well tolerated, but 3 g dose was somewhat less well tolerated as compared to 2 g dose. [70]

2.3.10. Goldfarb-Rumyantzev, *et al,* (2006).

In this retrospective study, patients who underwent renal transplantation between 01 January 1995 and 31 December 2000 and registered in US Renal Data System were selected and by this way, data of 31,012 patients were collected. All patients were followed up till 31 December 2000. Among these patients, 17,108 patients were treated with PCM, 7225 with PTM and 6679 with PCA.

Survival Analysis:

In Cox model, PTM and PCA therapy was linked with enhanced risk of allograft failure using PCM as a reference (HR = 1.08; $P < 0.05$ and HR = 1.14; $P < 0.001$, respectively). For recipient's mortality, they did not find significant difference between PTM and PCM treated recipients (HR = 0.99; P = 0.9), but PCA treated recipients had higher rate of mortality as compared to PCM treated recipients (HR = 1.15; $P < 0.005$).

Living versus Deceased Donor:

In deceased-donor recipients, author did not find significant difference for graft outcome between PTM and PCM treated patients (HR = 1.04; P = 0.284), but PCA therapy was associated increased risk of graft failure by 18% (95% CI = 1.1 to 1.27; HR = 1.18; $P < 0.001$). In living-donor recipients, PTM therapy was associated with higher graft loss as compared to PCM therapy (95% CI 1.06 to 1.41, HR = 1.22; $P < 0.01$) but not PCA (HR = 1.05; P = 0.51)

Adult versus Pediatric Recipients:

In this study, recipients were divided in pediatric (Age < 18 years) and adult (Age > 18 years) group. In pediatric patients, graft or recipient outcomes were similar between all treatment groups. However, in adults, PTM therapy was associated with higher graft failure (HR = 1.08; $P < 0.05$) as compared to PCM therapy but not for recipient's survival. While PCA therapy was associated with increased risk for patient's death (HR = 1.14; $P < 0.01$) and graft failure (HR = 1.14; $P < 0.001$), as compared to PCM therapy.

Effect of Induction Therapy:

In this study, recipients were divided in with and without induction therapy treatment. In Cox model, considering PCM as a reference, PCA and PTM therapy was associated with higher graft failure (HR = 1.15; $P < 0.001$ and HR = 1.07; $P < 0.05$, respectively). However, for recipient survival, only PCA regimen was associated with significant risk (HR = 1.14; $P < 0.01$).

Serum Creatinine Levels:

They compared average creatinine values at six months, one year, three years, five years and seven years in the three study groups. Creatinine value in the PTM group seems to be consistently lower than in PCM or PCA groups, except for the 7-year follow-up.

Acute Rejection Episodes:

For acute rejection, they did not find a significant difference between all treatment groups. They conclude that the PCM regimen is associated with lower risk for graft failure as compared with PTM and lower risk for graft failure and recipient death compared with PCA. [71]

2.3.11. Kunz, et al, (1997).

Kunz R *et al.* perform a study to compare the effect of triple immunosuppressive maintenance therapy (CyA, AZA, and prednisolone) with dual therapy (CyA and prednisolone) in renal transplant patients for identifying the effect on graft failure, acute rejection episodes, and mortality. They performed this study by reviewing MEDLINE, reference lists, Science Citation Index, and expert files which were published between 1984 and 1995. They review a total of 449 originally identified studies. After excluding studies on the bases of exclusion criteria, data of five controlled trials were used for this study. Results of this study, did not find statistically significant difference for graft failure (odds ratios (OR) = 0.82; 95% confidence intervals (CI) = 0.61-1.16), graft survival (OR = 0.83; 95% CI = 0.57-1.21), or acute rejection (OR = 1.02; 95% CI = 0.76-1.36) between triple-drug therapy and double-drug therapy. In this study, they found that a patient's withdrawal rate is low in triple therapy, so it shows the more stable immunosuppressive effect on triple therapy. By this study, they conclude that there is no statistically significant difference in the long-term management of renal transplant recipients between the two treatment regimens. [72]

2.4. Literature review on simultaneous estimation of drugs by HPLC

2.4.1. Defrancesco, *et al,* (2007).

Developed an assay method for the pharmacokinetic studies and therapeutic drug

monitoring for the simultaneous estimation of multiple immunosuppressants by liquid chromatography-tandem mass spectroscopy. This study provided evidence that the simultaneous estimation of MMF and Prednisolone was done. [73]

2.4.2. Gopalakrishnan, *et al,* (2010).

Developed a reverse-phase high-performance liquid chromatography (*RP-HPLC*) method for prediction of Mycophenolate Sodium in pharmaceutical dosage forms. They used USP L7 octyl silane which is chemically bonded to porous silica C8 (4.6X250 mm) with mobile phase. The mobile phase consisted of acetonitrile and buffer having fraction ratio of 50:50 v/v. The measured flow rate was reported as 1.5 mL/min. The photodiode array detector was used for detection purpose at 254 nm. For Mycophenolate Sodium, they had reported the retention time at 4.872 min. A linear response is recorded using this technique with 288-468 µg/ml concentration range. The recovery study was performed, and %RSD was reported as 0.2423. This proposed technique was assessed statistically. It is suitable for general quality control analysis of Mycophenolate Sodium. This technique has reported various advantages such as:

- Reduced retention time
- The mobile phase is economical and easily available
- Peaks are of good resolution.
- Short run-time.
- Fast quantification

So, all the properties mentioned above and result, make this technique feasible and suitable for Mycophenolate Sodium quantification without any interventions, in pharmaceutical dosage forms[74]

2.4.3. Patel, *et al,* (2011).

For exploring tacrolimus in capsules, a reverse-phase high-performance liquid chromatography (*RP-HPLC*) method was developed, and further, it was validated. For analysis, a reverse phase C18 column was taken with ultraviolet detection at 213 nm, at ambient temperature. A 100% acetonitrile was used as mobile phase with a persistent

flow of 0.9 ml/min. Across the full concentration range, a linear response was recorded by the assay. The advantages of this technique were found as:

- Simple procedure
- Rapid performance
- Precise and Economic

So, the proposed method is better suited in quality control purpose and simultaneous quantification of Tacrolimus in bulk drug as well as a dosage form. [75]

2.4.4. Upadhyay, *et al,* (2012).

In whole blood of human and Wister rats, tacrolimus was estimated using UPLC-MS-MS technique. It is an ultra-high-performance liquid chromatography-mass spectrometry (UPLC–MS-MS) method. This technique was performed as a pre-clinical study in twenty-five rats and for bioequivalence twenty healthy Indian subjects were taken. For protein precipitation of the analyze, the 50ml blood was taken from human and rat, and sirolimus was prepared as an internal standard with $ZnSO_4$ from that blood. Then the solid phase extraction was carried out. [76]. It was performed under isocratic conditions on a Waters Acquity UPLC BEH C18 column (50 32.1 mm, 1.7 mm) with the help of 10 mM ammonium acetate (pH 6.0) and methanol (5:95, v/v) and detection were performed using MS-MS method. Under positive ionization mode, various reactions were monitored, and quantitation of the analytes was completed. The technique was validated at a dynamic concentration range of 0.200–200 ng/ml and it reported the chromatographic run time of 1.2 min. Across the three levels of control, more than 96% tacrolimus was extracted as recovery. Using variation coefficient, matrix effect was evaluated. [76]

2.4.5. Qingping, *et al*, (2012).

Studied and developed a technique based on the RP-HPLC method, for assessment of KF506 in capsules and its related substances. This method was proved as an essential tool for exploring the quality of KF506. It was reported that this proposed method of validation was proved as a stable and robust technique for assessing and predicting the stability study of pharmaceutical capsules as well as quality monitoring of manufactured capsules. [77]

2.4.6. Kunithala, *et al,* (2012).

Developed a UPLC based technique for the prediction of the dosage form of Mycophenolate using Potassium dihydrogen phosphate mobile phase: acetonitrile with fractional ratio 35:65 v/v. At wavelength 228 nm, the ultraviolet-visible detector was used. The column used was C18 (4.6 x 100mm, 3.5 mm, Make: XBridge). The reported results were:

- the flow rate was reported as 0.2 ml/min.
- The linearity range was found to be 10-50 µg/ml.

Based on peak area and absorbance effect, the quantification was recorded with ultraviolet detections at 216nm. [78]

2.4.7. Kumari, *et al,* (2012).

Developed a separation method of Mycophenolate mofetil with impurity-c using RP-HPLC technique. They had validated the technique for exploring Mycophenolate mofetil activity in Mycophenolate mofetil capsule formulation. This technique was conducted successfully using the following configuration:

- Hypersil BDS C18(250mm x 4.6mm x 5.0 µm) column
- Mobile phase: phosphate buffer (7.0 pH) and acetonitrile (65:35 v/v)
- The flow rate of 2mL/min
- Eluate was assessed using PDA Detector at 250 nm.
- Retention time was 6.520. [79]

As per ICH regulations and guidelines, the method was validated, and the response was recorded. Linearity, accuracy and precision, ruggedness and robustness were taken as validation parameters. The results showed that degradation products were separated well and it was marked as a reliable method for routine analysis of Mycophenolate mofetil Capsule formulation. [79]

2.4.8. Abhilash, *et al,* (2013).

Developed a reverse phase UPLC technique for dosage form prediction of Mycophenolate. The used configuration for the proposed technique was:

- Mobile phase: Potassium dihydrogen phosphate: acetonitrile, ratio 35:65 v/v
- Flow rate: 0.2 ml/min. on UPLC system
- Ultra Violet based visible detector; wavelength 228 nm.
- Column: C18 (4.6 x 100mm, 3.5 mm).

It was found that the linearity range was 10-50 µg/ml. Based on absorbance and peak, quantification was achieved with UltraViolet detections at 216 nm. [80]

2.4.9. Musuamba, *et al,* (2013).

Studied and developed a rejection prevention technique after solid organ transplantation. In this study, the immunosuppressive agents such as Mycophenolic acid (MPA) and tacrolimus were used with corticosteroids. They designed a method based on D-optimality to describe the PK of the two drugs. By biochemical test reports, patients' demographics and physiological properties, precision and accuracy were reported.

The Pharmacokinetic profiles of MPA and TAC were taken from 65 stable adult renal allograft recipients, after 15 days of transplant. The used configuration for the proposed technique was:

- POPED software was used for sample schedule, based on D-optimality criterion.
- Using nonlinearly mixed effect modeling technique, a PK model describing MPA and TAC was formulated.
- The optimal blood pre-dose time was estimated at 0 and 0.24, 0.64, 0.98, 1.37, 2.38, and 11 hours after oral intake of mycophenolate and TAC.
- 2-compartment model with first-order elimination to describe PK of MPA and TAC.
- Transit compartment model for MPA absorption assessment.
- First order absorption with lag time for TAC.

The result of this proposed technique reported that:

- Precise and accurate
- Hematocrit levels and CYP3A5 genetic polymorphism significantly influenced TAC clearance

- Pharmaceutical formulation and MRP2 genetic polymorphism found as co-variates on MPA absorption and elimination, respectively.
- Proper estimation of MPA and TAC PK parameters
- Very high unexplained variability. The influence of some relevant covariates could be shown. [81]

2.4.10. Yamaguchi, *et al,* (2013).

The author examined a limited sampling strategy (LSS) for estimation of MPA-$AUC_{0–12\,h}$. For oral administration, the Mycophenolate mofetil (MMF) is used which further prevents rejection in renal transplant recipients. In vivo, it is converted into active metabolite: mycophenolic acid (MPA). The limited sampling strategy was used because frequent blood samples were required. This technique was conducted on Japanese living-related renal transplant recipients. The critical factors of the proposed technology were:

- The area under the concentration-time curve ($AUC_{0–12\,h}$) of MPA for predicting acute rejection.
- Prediction of MPA-$AUC_{0–12\,h;}$ using only a trough level (C_0) and two points including C_0 in Japanese living-related renal transplant recipients with concomitant extended-release tacrolimus (ER-TAC).

The present study suggested that:

- Better estimation of MPA-$AUC_{0–12\,h}$ using two points including C_0 as compared with the only C_0 regardless of transplant progress.
- Highest estimation of MPA-$AUC_{0–12\,h}$ by adding to C_0 were C_4 at pre-transplantation (Tx) and one-month post-Tx, and C_6 at three months post-Tx.
- A substantial difference in the reciprocal of serum creatinine (1/Scr) and Alb between pre-Tx and post-Tx.

The proposed method was found to be practical and useful for monitoring of mycophenolic acid levels. [82]

2.4.11. Wene, *et al,* (2015).

Investigated the pharmacokinetics of mycophenolate mofetil (MMF) in Chinese adults after renal transplantation.

- An enzyme multiplied immunoassay technique
- Designed a limited sampling strategy for plasma levels of mycophenolic acid (MPA-AUC).
- Fifty-eight renal transplantation patients with an organ donated after cardiac death used a triple immunosuppressant approach of MMF, tacrolimus, and prednisone.
- On 7th day after transplantation, plasma samples were taken at initially 0 hours (pre-dose) and at 0.5, 1, 1.5, 2, 4, 6, 8, 10, and 12 hours post-dose (C_{0h}, $C_{0.5h}$, C_{1h}, $C_{1.5h}$, C_{2h}, C_{4h}, C_{6h}, C_{8h}, C_{10h}, and C_{12h}, respectively).
- Enzyme multiplied immunoassay technique was used to explore mycophenolic acid concentration
- Model equations and multiple regression analyses were conducted to find MPA-$AUC_{0–12h}$.

The findings of the proposed techniques were enlisted as:

- 3-point equation: MPA-AUC = 7.951 + 4.04C_{6h} + 1.893C_{2h} + 4.542C_{10h} (adjusted $r^2 = 0.863$)
- 4-point equation: MPA-AUC = 4.272 + 4.074C_{6h} + 1.896C_{2h} + 4.680C_{10h} + 0.859$C_{0.5h}$ (adjusted $r^2 = 0.918$).
- The % mean prediction error was -0.2%, % indicate an absolute error was 8.7%, and % root mean squared prediction error for the best-fit formula was found as 14.2% using C_{6h}, C_{2h}, C_{10h}, and $C_{0.5h}$.

The best equation for estimating MPA-$AUC_{0–12h}$ is 4.272 + 4.074C_{6h} + 1.896C_{2h} + 4.680C_{10h} + 0.859$C_{0.5h}$. [83]

CHAPTER 3

RATIONALE

3.1. The rationale of the study

In the case of solid organ transplantation, to inhibit the rejection, the combined drug therapy is used. This combined therapy also uses corticosteroids. From the literature it was found that the combination of MMF, TAC with PRED provides long-term graft survival in better way than the other combination.

Therefore, in the present study, an attempt will be made to provide the simultaneous estimation of these drugs in bulk or formulations along with a cost-effective and elegant formulation of immunosuppressants, which can be safely and effectively used by patients. This analytical method can also be used for the determination of drug concentrations in blood.

3.2. AIM

Analytical Method Development and Validation for the Formulated Capsule Dosage Form Containing Tacrolimus, Mycophenolate Mofetil and Prednisolone.

3.3. Objectives of the study

- To develop an immediate release capsule formulation of mycophenolate mofetil, tacrolimus, and prednisolone.
- Development and validation of the analytical method.
- Optimization of the formulation by formulation and process variables.
- *In vitro* evaluation and comparison of the developed formulation with the reference product.
- Stability testing of the developed formulation.

CHAPTER 4

MATERIALS AND EQUIPMENT

4.1. Materials

Table 4.1: List of materials

Material	**Manufacturer**
MMF Active Pharmaceutical Ingredient (API)	Biocon Ltd., India
TAC Active Pharmaceutical Ingredient (API)	Biocon Ltd., India
PRED Active Pharmaceutical Ingredient (API)	Jackson Pharmaceuticals, India
Povidone K-30	HiMedia Lab, India
MCC	LOBA Chemie, India
Starch Maize	LOBA Chemie, India
Hydroxy Propyl Cellulose	LOBA Chemie, India
Anhydrous lactose	DMV, USA
Sodium starch glycolate	LOBA Chemie, India
Magnesium stearate	LOBA Chemie, India
Anhydrous acetate	LOBA Chemie, India
Sodium Hydroxide	Thomas Baker, India
Potassium dihydrogen phosphate	HiMedia Lab, India
Distilled Water	In – house

Hydrochloric Acid	LOBA Chemie, India
Acetic acid	HiMedia Lab, India
Triethylamine (TEA)	Qualigens, India
Orthophosphoric acid (OPA)	Qualigens, India
Sodium acetate	LOBA Chemie, India
Acetonitrile (HPLC grade)	LOBA Chemie, India
Methanol (HPLC grade)	Merck, India
Hydrogen peroxide	LOBA Chemie, India
Aerosil 200	CHD Vadodara, India
Talc	LOBA Chemie, India

4.2. Equipment

Table 4.2: List of equipment

Equipment	Model/Company
HPLC Systems	SPDM20A, Shimadzu
Dissolution Apparatus	Lab India DS8000, Bhushan Eng. and Sci. Traders, India
UV-Visible Spectrophotometer	Shimadzu, UV-1800, India
Stability Chamber	REMI, Elektrotechnik, India
Digital Weighing Balance	Shimadzu, Japan
pH Meter	Thermo Electron Corporation, USA
Magnetic Stirrer	REMI 2MLH, Elektrotechnik, India
Hot Air Oven	Q5247, Navyug, India
Sieves (ASTM standard)	Jayant Test Sieves (Jayant Scientific, India)
Digital Ultra Sonicator	Labman Scientific Instruments, India
IR	FTIR-8400S, Shimadzu, India
IR	Bruker VERTEX 70v, India
Photostability chamber	Thermolab, India
NMR	Bruker AVANCE II (400MHz) spectrometer, India

CHAPTER 5

DRUG PROFILE

5.1. Drug Profile: Mycophenolate mofetil

5.1.1. Description

"Mycophenolate mofetil the clinically usable molecule of Mycophenolic acid (MPA). Its 2-morpholinoethyl ester and was developed by South African geneticist A. Allison to increase the oral and intravenous bioavailability of MPA[84].

5.1.2. Structure of Mycophenolate mofetil and Mycophenolic acid

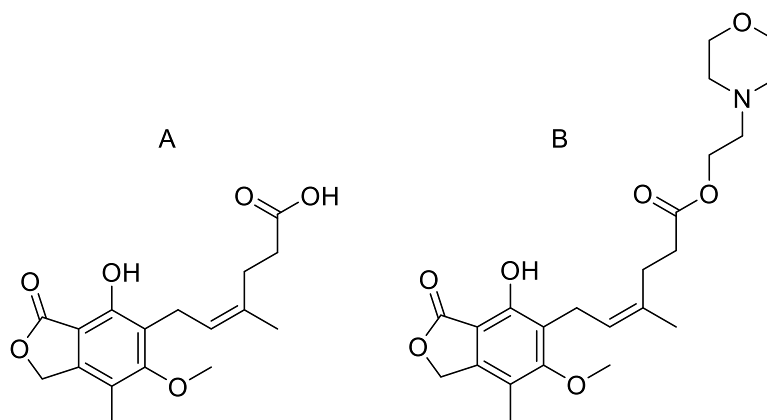


Figure 5.1: Structure of Mycophenolic acid (A), Mycophenolate mofetil (B)

5.1.3. IUPAC Name of MPA and MMF

MPA: (Z)-6-(4-hydroxy-6-methoxy-7-methyl-3-oxo-1,3-dihydroisobenzofuran-5-yl)-4-methylhex-4-enoic acid

MMF: 2-morpholinoethyl (Z)-6-(4-hydroxy-6-methoxy-7-methyl-3-oxo-1,3-dihydroisobenzofuran-5-yl)-4-methylhex-4-enoate

5.1.4. CAS number

128794-94-5

5.1.5. Molecular weight

Average: 433.4947

Monoisotopic: 433.210052351

5.1.6. Chemical Formula

$C_{23}H_{31}NO_7$

In the case of solid organ transplantation, to inhibit the rejection, the Mycophenolate or Mycophenolic acid (MPA), is used. MPA is produced by fungi of the genus *Penicillium.* It is *a* fermented by-product and was having antibacterial, antifungal, and immunosuppressive potentials. [85] MPA was first discovered in 1893 by Bartolomeo Gosio, an Italian scientist and was isolated in pure form. C.L. Alsberg and O.M. Black were successfully established the re-synthetic process in 1912. Later, it was found to have broad-spectrum properties like antiviral, antifungal, antibacterial, anticancer, and antipsoriasis[86].

On 3rd May 1995, the Food and Drug Administration (FDA), a federal agency of the United States has approved to use Mycophenolate mofetil as an immunosuppressant with corticosteroids in kidney transplantation[87]. Mycophenolate sodium, the sodium salt of MPA (Myfortic), developed by Novartis, now available as delayed-release tablets for oral administration. The chemical structure of Mycophenolic Acid active and parent compound is mentioned as in Figure 5.1.

5.1.7. Pharmacokinetics

Mycophenolate sodium, a sodium salt of MPA and Mycophenolate mofetil (Cellcept) are quickly absorbed and rapidly converted by tissues and in the blood into MPA the active form of the drug.

5.1.8. Absorption

Absolute bioavailability refers to the quantity of the drug obtainable to the body or system. In the case of mycophenolate mofetil, the average absolute bioavailability of

oral in comparison to intravenous resulted as 94%. Whereas, in the case of patients with a stable kidney transplant, the delayed release tablet had 72% absolute bioavailability.

5.1.9. Protein binding

At clinically relevant concentrations, the active metabolite MPA has proven as 98% plasma albumin binding rate, whereas mycophenolic acid glucuronide (MPAG), the phenolic glucuronide of MPA is having protein albumin binding rate as 82%.

More than 99 percent of the active drug reaches in the plasma after conversion from mycophenolate mofetil to MPA during intravenous administration within 2 minutes or one hour after oral administration [88-90]. For MPA quantitation serum or plasma is used extensively.

5.1.10. Metabolism

The mycophenolate mofetil experiences absolute metabolism to the active metabolite MPA, when follows oral as well as intravenous dosing. After oral dosing, the Metabolism to MPA occurs pre-systemically. MPA is metabolized primarily by glucuronyl enzyme to create the phenolic glucuronide of MPA (MPAG), that is pharmacologically inactive. The phenolic glucuronide of MPA is transformed into Mycophenolic Acid using enterohepatic recirculation. The other metabolites of the 2-hydroxyethyl-morpholino moiety, that are also retrieved in urine, which furthers oral administrates mycophenolate mofetil to healthy subjects are listed as:

N-(2-carboxymethyl)-morpholine

N-oxide of N-(2-hydroxyethyl)-morpholine

N-(2-hydroxyethyl)-morpholine

For biotransformation of MPA to 6-O-desmethyl-mycophenolic acid, the liable parts are: Cytochrome P450 isozymes, CYP3A4/5, CYP2C8 (to a lesser extent) 7-*O*-glucoside another inactive metabolite is also produced in liver but small quantities along with acyl glucuronide (AcMPAG) that may lead to the gastrointestinal side effects [91, 92].

The second plasma peak of MPA is observed during 4–12 hours after drug administration. This is because of enterohepatic recirculation of MPAG. The renal system generally excretes MPAG, but the concentration is quickly increased in case of severe renal impairment [93]. It has been observed that the free drug concentration (unbound) of the MPA is ranged from 1.25 to 2.5 % and the rest of the drug is bounded with the albumin in the circulation. The free drug concentration is increased in case of the level of albumin in the blood is abnormally low (hypoalbuminemia), or there is too much bilirubin in the blood (hyperbilirubinemia) and uremia [94, 95]. Majorly, it has been observed that the MPAs effect is related to the free drug concentration and not with the total blood concentration [89].

In some cases, it has been observed that the patient is over immunosuppressed even though the aggregate MPA concentration is well within the restorative range. This is only because if the patient is suffering from severe renal problems, which leads to an increase in free drug concentration [94, 96].

5.1.11. Route of elimination

In urine, a decidedly less amount (less than 1%) of the drug is excreted as MPA. The recovery of mycophenolate mofetil, during oral administration, is as per the following percentage:

93% of the dose in urine

6% of the dose in feces

Whereas, in urine, the excretion percentage of administrated dose as phenolic glucuronide of MPA, i.e., MPAG is 87%.

5.1.12. Half-life

The half-life of MPA is 18 hours and metabolized via phase II process to its inactive glucuronidated in the 7-*O*-glucuronide mycophenolic acid (MPAG) [97].

5.1.13. Mechanism of action

MPA non-competitively selectively inhibits the enzyme inosine monophosphate dehydrogenase (IMPDH), needed for the growth of T and B lymphocytes [98]. IMPDH is rate-limiting and is responsible for the production of guanosine nucleotides in the cell. DNA synthesis and cellular proliferation require guanosine nucleotides, which is synthesized in most cell types via IMPDH and another salvage pathway. However, MPA blocks the IMPDH pathways in lymphocytes selectively because there is no salvage pathway. Ultimately, inhibits lymphocyte proliferation[99, 100]. IMPDH exists in two isomeric forms (type-I and type-II), and type II isoform is selectively inhibiting by the MPA. This type -II isoform is generally found in activated lymphocytes only [101].

5.1.14. Toxicity

Oral (LD50): Acute: 352 mg/kg [Rat], 1000 mg/kg [Mouse], and >6000 mg/kg Rabbit.

The feasible indication of acute overdose contains the following symptoms:

a) hematological abnormalities

It may include leukopenia and neutropenia

b) gastrointestinal symptoms

It may include dyspepsia, abdominal pain, diarrhea, nausea, and vomiting.

5.1.15. Drug monitoring

The systemic concentration is monitored due to following reasons

- The direct relationship between dose and pharmacological response
- These drugs generally have a small therapeutic window
- Drugs concentration display a high degree of inconsistency in intra and inter patients
- Difficulty in distinguishing between pharmacological response and side effects
- These drugs are administered for the rest of the entire life. So, increased chances of poor or noncompliance

- There are significant changes for the drug-drug interactions

The probability of drug-drug interactions is not only with non-immunosuppressive, may occur among the different classes of immunosuppressants. For example, CsA inhibits the excretion of mycophenolic acid glucuronide (MPAG), a mycophenolic acid metabolite into bile via interacting with the multidrug resistance-associated protein 2 (Mrp2) transporter which results in decreased concentrations of MPA when used in combination [102, 103].

5.1.16. Adverse Effects

Almost all drugs have some of the adverse effects associated with them, and in case of immunosuppressants, the adverse effects increase the risk of infections. Ester prodrug, mycophenolate mofetil, and mycophenolate sodium, the sodium salt of MPA are the same. The most common side effects are diarrhea, loss of appetite, nausea, hand trembling, vomiting, increased hair growth, and abdominal pain [94]. These effects generally stop as the body adjusts to the dose of the drug. Increased risk of bone marrow suppression, cytomegalovirus, anemia, candida and herpes simplex infections are also there [96, 104].

5.1.17. Drug -Interactions

Drug interactions may make the drug less effective, may lead to unwanted side effects or sometimes may increase the action of a particular drug. So, drug interactions generally divided into three broad categories:

■ Drug-drug interactions: It is the most common type and occur when two or more drugs interact with each other, leads to an unexpected side effect.

■ Drug-food interactions: It generally results from drugs when mixed with foods or beverages.

■ Drug-condition interactions: Sometimes also known as 'Drug-disease interactions,' in this existing medical condition sometimes can influence the way a medication works and may lead to harmful effects.

The combination with CsA generally lowers the concentrations of MPA. This is because of limited enterohepatic recirculation of MPAG and MPA [105] [106]. The co-administration with the antibiotics (cefuroxime, tobramycin, and Mycostatin) also decrease MPA bioavailability via a similar mechanism [107]. When MPA used along with other immunosuppressants the may increase the bioavailability via inhibiting its glucuronide formation[108], however, further studies are required for the confirmation of this possible drug interactions. MPA when co-administered with a corticosteroid such as dexamethasone, results in the decrease in the blood concentration. This is because of extending the enhancing the activity of the metabolizing enzyme. On the other hand, if used along with NSAIDs, increase in MPA concentrations reported. This is due to inhibiting MPA glucuronidation process [109]. Antacids also lower the MPA concentrations by decreasing the rate of absorption in the GIT. Calcium and iron preparations also interfere with the absorption of MPA results in decreased concentrations [110]. Some of the drugs like salicylic acid and furosemide also increases the free drug concentrations via altering the binding with albumin [26].

5.1.18. Preanalytic Variables

Serum or plasma is used extensively for the measurement of MPA and MPAG because more than 99 percent drug is reaching the blood circulation within one hour after oral administration [93]. For the analysis, the plasma obtained from EDTA anticoagulated whole blood is preferred because the same sample can be used to determination of CsA, tacrolimus, and sirolimus whole blood concentrations [111].

The free drug and its glucuronide in whole blood are stable for 4 hours at room temperature [112], and in plasma, they are stable for four hours at 4°C and can be stored at –20°C for 11 months [111, 113]. Samples are stable enough that can be thaw and refreeze up to four times and there is no significant loss of drug concentrations [114, 115].

Monitoring of mycophenolate mofetil during systemic infusion, the blood sample should be freeze immediately, and plasma should be separated within first 30 minutes as the MMF is very unstable molecule and converts to MPA via temperature-dependent degradation process [116, 117]. MPA trough concentration is a good indicator for the

total drug concentration monitoring on a regular basis [118]. It is amazing that some of the studies related to the area under curve (AUC) measurement for total drug exposure and acute graft rejections are more predictive than trough concentrations [119-121]. MPA trough concentrations can change impressively liable on time after transplantation [121]. Nonetheless, the practical complexity outshined the superiority of AUC measurements, such as multiple sample collection at different intervals.

5.1.19. Methods of Analysis

Analysis of a drug is always playing an important part in various fields of pharmaceutical and medical sciences. So, a method is required which should be suitable and validated for the analysis of drugs in bulk, in drug delivery, in vitro, and in vivo.

After getting approval form, US-FDA as immunosuppressant therapeutic drug monitoring (TDM) is not so important. But ongoing studies suggest that the individualized dosing is beneficial because of wide variations in fixed dose treatment [122, 123]. A roundtable meeting which was held in New York in December 2004, prescribed therapeutic drug monitoring depending on the interpatient inconsistency and significant drug interactions during combined immunosuppressive therapy [124].

During the first CAP proficiency survey of 2008 in the US, only a few (less than 40 laboratories) measures the MPA out of them only 40 percent of the lab's measures MPA by HPLC and 30 percent labs uses HPLC-MS methods. For the measurement of total MPA and free MPA via automated enzyme receptor assay Roche develops the method using COBAS INTEGRA system [125, 126]. For plasma samples, several HPLC method combined with UV, MS, and fluorimetric system have been reported to measure MPA [113, 114, 127-134] The described HPLC methods generally differ in sample extraction, the column used, run- time, LOD and LOQ [127, 130-132, 135-137]. In some cases, the ultrafiltration technique is used for the separation of protein-bound MPA for the measurement of free MPA by HPLC method [92, 138, 139]. The free MPA is customarily harder to quantify and does not emit an impression of being superior to total MPA in figuring the actual responses in most transplant patients [140]. Some companies are working in the way so that direct measurement of the drug can possible from serum or plasma samples. For example, Siemens has developed ACMIA for direct

measurement of MPA and Microgenics developed CEDIA to measure MPA on various instruments [141, 142]. EMIT 2000 immunoassay was developed by SYVA and widely used. This assay can be performed on various instruments and uses antibodies which cross-reacts with acyl glucuronide and produces up to 30 percent MPA values than by HPLC method [143-147]. The values are even more in impaired renal function patients due to increased acyl glucoronide[144, 148]. The positive inclination on account of acyl glucuronide cross-reactivity may turn out to be gainful since this metabolite has in vitro anti- IMPDH activity[95, 122].

5.1.20. Analytical Considerations

For trough MPA plasma concentrations accepted therapeutic range is 1.0–3.5 mg/L[111, 149, 150]. The current analytical methods now have good precision, and this range can be easily measured. The concentration of free MPA is often 2% of the total MPA level and can be determined by HPLC-UV methods [151]. In these circumstances, the useful functionality of the free MPA assay should be cautiously validated. The other methods can be validated against HPLC as this is the reference technique for measuring. This is only because HPLC is exceptionally particular for drug and is free from co-administered medication interventions [116, 127-129]. To measure MPA concentrations, more and more immunoassays are available, and care should be taken as metabolite cross-reactivity, and assay bias parameters cannot be ignored while measuring MPA concentrations."

5.2. Drug Profile: Tacrolimus

5.2.1. Description

"Tacrolimus is one type of macrolide antibiotic, which is having a molecular weight as 822. The tacrolimus is also known as FK-506. The figure 5.2 depicts the structure of Tacrolimus. The fungus *Streptomyces tsukubaensis* is originally isolated from FK-506 or Tacrolimus [152]. In the year 1994, the Tacrolimus with brand PROGRAF was accepted by the USA to use in liver transplantation and year, 1997, it was approved for use in kidney transplantation.

In comparison to cyclosporin A (CsA), the potency of Tacrolimus is 100 times more. It has properties like reduction in acute and chronic rejection. It has long-term graft survival in better way[153]. As per data analytics of the year 2004 hospital patients, approximately ½ of heart transplant patients and more than 2/3rd of kidney and liver transplant patients have received Tacrolimus before the discharge from hospital[11].

5.2.2. Structure of Tacrolimus

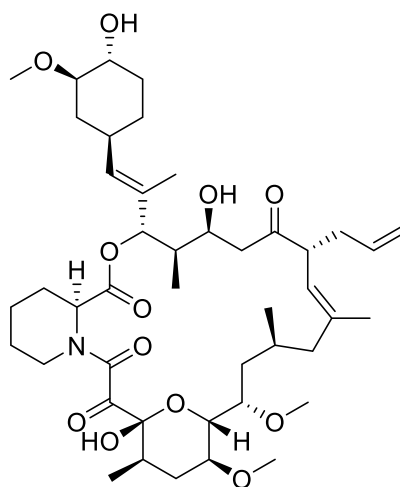


Figure 5.2: Structure of Tacrolimus

5.2.3. Properties of Tacrolimus

The various properties of Tacrolimus are enlisted in the following section, which includes IUPAC name, CAS number, molecular weight, and chemical formula, etc.

5.2.4. IUPAC Name

(1R,9S,12S,13R,14S,17R,21S,23S,24R,25S,27R)-1,14-dihydroxy-12-[(1E)-1-[(1R,3R,4R)-4-hydroxy-3-methoxycyclohexyl]prop-1-en-2-yl]-23,25-dimethoxy-13,19,21,27-tetramethyl-17-(prop-2-en-1-yl)-11,28-dioxa-4-azatricyclo[22.3.1.0^{4,9}]octacos-18-ene-2,3,10,16-tetrone

5.2.5. CAS number

104987-11-3

5.2.6. Molecular Weight

Average: 804.0182

Monoisotopic: 803.481976677

5.2.7. Chemical Formula

$C_{44}H_{69}NO_{12}$

5.2.8. Pharmacokinetics

For both, intravenous as well as oral administration the Tacrolimus is available. From the gut, the oral absorption of Tacrolimus is highly variable and inferior, as in the case of cyclosporin A, which is also having the same properties. The average of oral absorption of Tacrolimus is 25%[154]. Within 1.5 hours to 4 hours, the peak blood concentration occurs. In the plasma, Tacrolimus is primarily bound to albumin, α1-acid glycoprotein. However, within erythrocytes, most of the Tacrolimus is reported [153]. The CYP3A (cytochrome P450 isoenzymes) are used to metabolize Tacrolimus. The cytochrome P450 isoenzymes are mostly located in intestine and liver, which are used for metabolism. As the case of cyclosporin A, the bioavailability of Tacrolimus is also influenced by cytochrome P450 isoenzymes. In intestinal enterocytes, there is a multidrug efflux pump present which also influences the bioavailability of Tacrolimus.

The reactions which are responsible for the biotransformation of Tacrolimus are [155]:

- Demethylation
- Hydroxylation
- Oxidative

In vitro studies, minimum of nine metabolites have been identified.[156], and except 31-o-demethyl tacrolimus (M-II), reports negligible concentration with immunosuppressive activity. In vitro study, it has been reported that M-II and parent compound have almost the same immunosuppressive activity[157]. The metabolites show ten to twenty percent of whole blood Tacrolimus concentrations [158]. By biliary emission into feces, the Tacrolimus is reduced. The adjustments in dosage are required

for the patients who suffer from hepatic dysfunction. A negligible Tacrolimus that is reported in Urine and blood concentration is not changed during kidney dysfunction.

5.2.9. Adverse Effects

With cyclosporin A (CsA), the Tacrolimus reports various dose-based adverse effects, which are enlisted as[61].

- nephrotoxicity
- neurotoxicity
- hepatotoxicity
- hypertension
- glucose intolerance

In kidney transplantation, it has been reported that there is less problem of Nephrotoxicity with Tacrolimus, as compare to cyclosporin A (CsA).[159] But the Diabetogenesis is more visible with tacrolimus (about three times) than with cyclosporin A [160]. In comparison to cyclosporin A, the other adverse effects such as Hyperkalemia, hyperuricemia, hyperlipidemia, hirsutism, and gingival hypertrophy are also reported with Tacrolimus. [161] With the use of Tacrolimus, it has been analyzed that Alopecia is also reported. [61]

5.2.10. Drug Interactions

As cytochrome P450 system is mainly used for the metabolism of Tacrolimus, so most of the drugs which are reported for cyclosporin A are also applicable to Tacrolimus[154]. The Tacrolimus concentration in blood is also reduced by St John's wort.

5.2.11. Preanalytic Variables

For the purpose of Tacrolimus quantitation, the mostly used specimen is EDTA-anticoagulated whole blood is mostly used, for the same reason as for cyclosporin A. For stability of whole blood sample, it requires: Almost 1 week, if transported without coolant [162, 163], 1–2 weeks at room temperature [163, 164], 2 weeks at refrigerator temperatures [164], almost 1 year at −70°C [164].

For routine monitoring, the trough blood samples of Tacrolimus are widely used. It is believed that trough blood tacrolimus represents the total drug exposure[165]. The latest analysis with cyclosporin A challenges the notion mentioned above. It has been proposed the after dose; alternative draw times are within 1hour to 6 hours[166]. There is a difference in opinions by investigators about the correlation between total drug experience and trough Tacrolimus. Some investigators oppose the correlation whereas some investigators are in favor of this correlation [167, 168]. Overall, it has been reported that total drug exposure can be predicted using trough Tacrolimus. After dosage, Tacrolimus may not improve the conditions dramatically. Unless the scenario of predictive relationship between trough Tacrolimus and total drug exposure is fully resolved, the level of trough Tacrolimus can be used. The trough Tacrolimus is having the advantage of reproducibility and convenience.

5.2.12. Methods of Analysis

For organ transplant, the monitoring of Tacrolimus plays an integral and important role, because it is having a narrow therapeutic index and its blood to dose concentration is variable. The different ways to measure Tacrolimus are using:

- Enzyme-linked immunosorbent assay (ELISA)
- Semi-automated and automated immunoassay

High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS)

The whole blood pre-treatment is required by the Enzyme-linked immunosorbent assay and semi-automated immunoassays, which is to be done manually. But the pre-treatment step is not required by the dimension of Affinity chrome-mediated immunoassay (ACMIA), which allows the instructor to place the whole blood samples directly on the instrument.

Using modern High-Performance Liquid Chromatography-Mass Spectrometry systems, sample extractions can be semi-automated[169]. The Enzyme-linked immunosorbent assay takes approximately 4 hours for the complete process. It requires various manual processes and is used by very less clinical laboratories.

The College of American Pathologists (CAP's) proficiency testing (PT) Surveys reports show that 88% of US laboratories use Abbott microparticle enzyme immunoassay (MEIA) II on the IMx instrument. The earlier version (MEIA I) has a detection limit of 5μg/L, whereas the upgraded version (MEIA II) has improved detection limit of 2μg/L. So, laboratories use MEIA II as compare to MEIA I.

The Tacrolimus Siemen's Svya Enzyme multiplied immunoassay technique has the following applications:

- Dade Behring instrumentation
- COBAS Integra 400 [170]
- Beckman Synchron LX20 PRO [171]
- Bayer ADVIA 1650 [172]

The Siemen's Syva Enzyme multiplied immunoassay technique EMIT is currently available outside the United States of America. For applications for Beckman, Hitachi, and Olympus instruments, etc., the Microgenics released Cloned Enzyme Donor Immuno Assay (CEDIA) for tacrolimus.

In July 2006, the Dade-Behring laboratories have released an antibody conjugated magnetic immunoassay (ACMIA) to measure tacrolimus. The monoclonal antibody that is used in Svya EMIT, the same is used in ACMIA, to explore the value of Tacrolimus. The antibody conjugated magnetic immunoassay (ACMIA) uses:

- Dimension family of analyzers
- V-Twin
- Viva-E drug-testing analyzers.

The Abbott Core laboratories developed a chemiluminescent immunoassay i.e. ARCHITECT i1000SR. It is used for in vitro diagnostics [173]. The laboratories which do not use MEIA II, they use the High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) techniques. As the molecule does not have any chromophore, Tacrolimus cannot be explored and measured by High-Performance Liquid Chromatography using UV. For Consensus documents and parent drug, the High-

Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) techniques are mostly used [174]. It has been reported that Tacrolimus can be quantitated by using High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) techniques or HPLC-MS/MS, having detection limit <0.5ng/mL [169, 175]. The major benefit of High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) over immunoassays is the property to measure other immunosuppressant drugs in the same whole blood sample simultaneously, for example, sirolimus, everolimus and CsA[176].

5.2.13. Metabolite Cross-Reactivity

The tacrolimus metabolites possess significant cross-reactivities with all the immunoassays, such as M-II, M-III and M-V metabolites of tacrolimus cross-react with ELISA, MEIA II and EMIT. Where the M-II is 31-o-demethyl, M-III is 15-o-demethyl, and M-V is 15,13-di-o-demethyl [177]. The cross-reactivity of Cloned Enzyme Donor Immunoassay (CEDIA) is reported with M-I (13-o-demethyl), but CEDIA does not possess the cross-reactivity with M-II (31-o-demethyl) or M-III (15-o-demethyl). It has not been reported the cross-reactivity of M-V and CEDIA[178]. As both ACMIA and EMIT, hold the identical monoclonal antibody, S, it is expected that both ACMIA and EMIT possess metabolite cross-reactivity.

Due to metabolite cross-reactivity, the extent of positive bias is determined by the transplant group studied. In the patient with normal liver function, metabolite cross-reactivity is generally, not a big issue because the parent drug has relatively higher metabolite concentration [179]. However, the assay interference and erroneous high blood tacrolimus concentration arise after liver transplant or during diminish liver function, as metabolites incline towards accumulation during those conditions [180]. Overall, in kidney and liver transplant patients, in comparison to tacrolimus obtained by HPLC-MS technique, MEIA II possesses 15-20% higher tacrolimus, the EMIT reports 17% higher tacrolimus and CEDIA results in 19% higher tacrolimus [171, 177, 178, 181, 182]. In overall positive bias, the calibration error may also include.

5.2.14. Analytical Considerations

After kidney and liver allograft transplants, while measuring with HPLC-MS technique, the recommended therapeutic range is 5–20μg/L for whole blood tacrolimus[183]. With other immunosuppressive agents such as sirolimus, the tacrolimus reports the desired target concentration as <5μg/L. Keeping a view of this, the performance characteristics of tacrolimus assay at each laboratory is determined at concentrations <5μg/L and it is equally important to mention at this concentration about the lower detection limit and imprecision (%CV). It has been reported that functional sensitivity of CEDIA and MEIA II, (between-day CV < 20%) is approximately 2μg/L [177, 181, 184, 185], whereas the detection limit of EMIT is reported as 3μg/L [171]. During this study (MEIA II tacrolimus assay) it was found that the tacrolimus concentrations ranging from 0.8 to 1.7μg/L on MEIA II.

In case of patients, who are not receiving tacrolimus using MEIA, Homma et al. [186] also reported the false-positive results, when tacrolimus is determined and measured in a whole blood sample of such patients. When the hematocrit value is <25%, then the false elevated tacrolimus concentration is reported through MEIA II [187, 188]. The change in hematocrit values does not affect the EMIT for tacrolimus [188]. In under-immunosuppressed patients, because of low hematocrit values, the therapeutic tacrolimus blood concentration could be caused due to MEIA II hematocrit bias. After the transplant, when the hematocrit values possess the smallest concentrations, this would be more problematic. With patients with extensive variable hematocrit values, it would be more difficult. The reliability of MEIA II becomes a challenging issue for low whole blood concentrations. Using HPLC-MS/MS technique, MEIA II possess stronger between-day imprecision and a lesser correlation, at tacrolimus concentrations <9μg/L[189]. At lower drug concentration, recovery experiments also signify the notable over-estimation of tacrolimus using MEIA II [189]. At low tacrolimus concentration, the poor precision is noted, for a longitudinal immunosuppressive drug study. The major cause of imprecision is found to be time-dependent within-laboratory changes or variation in assay standardization [190]. Therefore, while selecting an assay to monitor whole blood tacrolimus concentrations, these performance variables are essential to consider."

5.3. Drug Profile: Prednisolone

5.3.1. Description

"With general characteristics of the corticosteroids, the Prednisolone is a glucocorticoid. Except for adrenal deficiency, Prednisolone is used for all situations, where routine systemic corticosteroid therapy is specified. In solid organ transplantation, for induction and maintenance of immunosuppressive regimens, Prednisolone and prednisone are essential constituents. The pharmacokinetics of prednisolone and prednisone are very complicated. Prednisolone is the active drug moiety while prednisone is both a pro-drug and inactive metabolite of prednisolone. In transplantation, when parameters are calculated about total drug concentration, prednisolone and prednisone reveal concentration-dependent non-linear pharmacokinetics[191].

5.3.2. Structure of Prednisolone

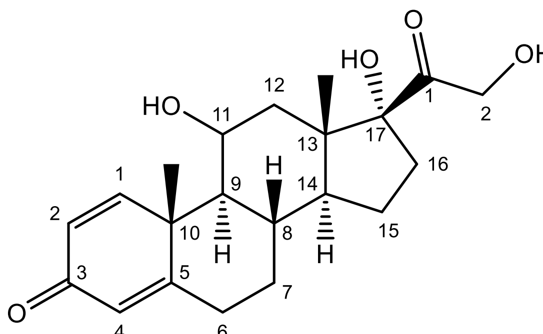


Figure 5.3: Structure of Prednisolone

The chemical characteristics of prednisolone are enlisted in the following section.

5.3.3. IUPAC Name

(8S,9S,10R,13S,14S,17R)-11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13-dimethyl-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-3-one

5.3.4. CAS Number

50-24-8

5.3.5. Molecular weight

Average: 360.444

Monoisotopic: 360.193674006

5.3.6. Chemical Formula

$C_{21}H_{28}O_5$

5.3.7. Pharmacokinetics

Prednisolone is promptly immersed by the gastrointestinal tract. In the normal case, the peak plasma concentration is reached within 1 hour to 2 hours, and for transplant recipients after administration, it takes normally 1 hour to 3 hours. Prednisolone acceptance from the gastrointestinal tract is generally substantial, although the majority reported data is from non-transplant recipients' studies [192, 193]. It has a high protein binding rate, i.e. approximately more than 90%. Using phase I and phase II biotransformation, the hepatic metabolism primarily expelled Prednisolone from the body. It is reported by animals' in vitro kidney perfusion studies that tissue may also be competent in metabolizing these corticosteroids [194]. Various resulting hydrophilic inactive metabolites are excreted by the kidneys, consequently [195]. Prednisolone and prednisone concentrations reduce in parallel and the terminal phase of elimination [196].

5.3.8. Pharmacodynamics

The Prednisolone is a synthetic glucocorticoid, and it is used as an anti-inflammatory or immunosuppressive agent. Using 11β-hydroxysteroid dehydrogenase (11β-HSD), Prednisolone and prednisone experience inter-conversion (reversible metabolism)[195, 197].

Various conditions such as systemic lupus erythematosus, seasonal or perennial allergic rhinitis, allergic corneal marginal ulcers, symptomatic sarcoidosis, bullous dermatitis herpetiformis, idiopathic thrombocytopenic purpura in adults, leukemias and lymphomas in adults, and ulcerative colitis, congenital adrenal hyperplasia, psoriatic arthritis are treated by Prednisolone. Adrenocortical steroids such as Glucocorticoids

can cause reflective and diverse metabolic effects. Additionally, the immune response of the body is also modified to diverse stimuli.

5.3.9. Mechanism of action

At inflammation site, the prednisolone can constrain leukocyte infiltration. It can interfere with inflammatory response and further overcome humoral immune responses. The anti-inflammatory actions of glucocorticoids involve phospholipase A2 inhibitory proteins and lipocortins. The biosynthesis of potent mediators of inflammation such as prostaglandins and leukotrienes are controlled by anti-inflammatory actions of glucocorticoids. By restraining the capillary dilatation and permeability of the vascular structures, the inflammatory reaction is decreased by Prednisolone. The collection of polymorphonuclear leukocytes and macrophages are restricted by these compounds. It further releases vasoactive kinins.

The latest research indicates that corticosteroids may constrain the release of arachidonic acid from phospholipids, that further reduces the formation of prostaglandins. Prednisolone is an agonist of the glucocorticoid receptor. In the process of binding, the corticoreceptor-ligand complex transports itself into the cell nucleus, where it binds to many glucocorticoid response elements (GRE) in the promoter region of the target genes. The basic transcription factors then interact with DNA bound receptor. It causes an increase or reduction in the expression of target genes, including suppression of IL2 (interleukin 2) expression.

5.3.10. Adverse Effects

Prednisolone and prednisone therapy have been involved in a wider range of toxicities. There are some principal somatic effects such as skin fragility, bodyweight gain, infections and fractures [198]. Some cardiovascular and metabolic effects are reported such as hypertension, hyperglycemia and dyslipidemia [195].

5.3.11. Drug Interactions

Prednisolone divulges metabolic (CYP3A) and transporter (P-gp) routes with ciclosporin, tacrolimus, and sirolimus. Prednisolone is the substrates and competitive

inhibitors of CYP3A4 [199]. In comparison with prednisone, the Prednisolone is the substrates of P-gp and transferred more effectively [200]. In vitro studies, the Ciclosporin, tacrolimus, and sirolimus have all been shown to be substrates and competitive inhibitors of CYP3A4 and P-gp. [201]. In vivo, ciclosporin has been related with enhanced intestinal CYP3A4 activity and reduced intestinal and hepatic P-gp activity. It is reported by in vivo studies of tacrolimus, that it does not change CYP3A4 or P-gp activity at clinically relevant doses [201]. It is also conveyed that Corticosteroids have an induction effect on the uridine diphosphate glucuronosyltransferase enzymes and multidrug resistance-associated protein two involved in mycophenolate mofetil disposition [201].

5.3.12. Analytical Considerations

The analytical aspects of the prednisolone/prednisone metabolism have reviewed by Frey Fl. in 1987 [202]. From that analysis, the following relevant conclusions can be derived for the presentation outline. Prednisone and prednisolone were introduced into clinical medicine about 64 years ago. At that time only limited information was required about the pharmacokinetics and metabolism of new xenobiotics. Thus, the sum of the metabolites identified so far in urine and feces accounts for only about 50% of the dose of glucocorticoid administered and their biological relevance is largely unknown [203-206]. Prednisolone is considered to be by far the most active steroid when it or its interconvertible 11keto metabolite, prednisone is given [207]. Therefore, pharmacokinetic studies should specifically assess prednisolone concentrations in biological fluids. Since healthy volunteers and patients given a single dose of an exogenous glucocorticoid [208, 209] or patients on long term therapy with a reasonable dose of prednisone release endogenous cortisone and cortisol [210], high performance liquid chromatography (HPLC) methods had to be developed to specifically distinguish prednisolone from its metabolites and from endogenous glucocorticoids [211-213]. Prednisolone possesses nonlinear binding to albumin and transcortin. The free fraction of nonlinear binding is ranging from less than 0.1 to 0.5 [211, 214-216]. The disease states and concomitant drug therapy affect the concentration of these drugs [217-219]. There is confirmation that the unbound species of prednisolone is responsible for its

biological effect [220-222]. Thus, pharmacokinetic investigations must focus on unbound rather than total concentrations of prednisolone."

CHAPTER 6

EXPERIMENTAL WORK

6.1. Preformulation Studies

Before the development of any dosage form of a new drug or combination of drugs, a contender drug should come across a phase called preformulation. Preformulation is the physicochemical depiction of the solid and solution properties of compounds. Preformulation testing incorporates all examinations approved on the new medicinal compound with a specific extreme goal to produce useful information for succeeding formulation of stable and bio pharmaceutically sensible dosage forms.

Preformulation studies should not be conducted on a checklist basis. Rather, they should form the basis of a controlled investigation into the physicochemical characteristics of the candidate drugs. The preformulation stage is a basic learning time about applicant drugs. The choice made on the data generated during this stage can profoundly affect the resulting improvement of the compounds. Accordingly, it is crucial that the preformulation ought to be executed carefully as possible to empower reasonable choices to be made. The amount and nature of the drugs can influence the information produced; so, can the equipment accessible and the expertise of the person directing the examinations. Preformulation studies ought not to be directed on an agenda premise. Or maybe, they should frame the premise of a controlled examination concerning the physicochemical qualities of the confident medications.

There are numerous methods which can be applied to characterize compounds. In the Gold Sheet (1985), the U.S. Food and Drug Administration (FDA) indicated that the principle physicochemical techniques that could be utilized for the identification of compounds should include.

- Melting point
- Infrared spectroscopy
- Particle size determination
- Hygroscopicity
- Flow property

- XRD
- Thermal analytical techniques (e.g., DSC, DTA, TGA, etc.)
- Phase solubility analysis
- Solution-pH profile determination

All the drugs (MMF, PRED and TAC) were evaluated based on these parameters, and the findings of these studies will help in formulating a stable oral capsule dosage form at various stages of formulation development.

6.1.1. Physical Appearance

The physical appearance was observed according to ICH.

6.1.2. Melting point

Differential Scanning Calorimetry was used to determined melting point.

6.1.3. Particle Size Analysis by Sieve Shaker

Note down the weight of each of the sieve or tare each nest sieve to the lowest to 0.1 g. Take the known quantity of the test sample on the topmost sieve (coarsest), and close the lid. Whisk the nest for 5 minutes. Then carefully remove each sieve from the nest so that loss of material would not take place. Take the weight of each sieve along with the material. Then calculate the remaining content on each of the sieve and last collecting pan. Reassemble the nest, and agitate again for 5 minutes with the content. Finally. Remove each sieve and weigh again for the calculation of content residing on each of the sieves. Repeat the experiment till you got the results according to endpoint criteria (loss should not be more than 5% of the initial weight).

Endpoint criteria Determination: The sieving analysis is only be completed when its weight does not change much (5% of previous weight or 0.1g) from the previous weight on that sieve. If it is less than 5%, then the endpoint can be increased to not more than 20% of the previous weight on that sieve. If weight change (more than 50%) is found on any of the sieves, the test should be repeated by adding additional sieves (coarser sieve(s) in between the two that carry larger weight).

6.1.4. X-Ray – Diffraction Studies

The X-ray diffraction (XRD) pattern of powder samples was recorded on a scanning powder X-ray diffractometer using an X' Pert PRO instrument (PANalytical, Netherlands), equipped with an X' Pert PRO Data Collector software. For the physical mixture analysis 1:1:1 ratio has been used.

6.1.5. Hygroscopicity Study

Numerous compounds and salts are sensitive to the presence of water vapors or moisture. At the point when compounds associate with moisture, they hold the water by bulk or surface adsorption, capillary condensation, chemical reaction and, in extraordinary cases a solution (deliquescence). Moisture is likewise a vital factor that can influence the stability of the competitor drugs and their formulations. Sorption of water particles onto a candidate drug (or excipient) can regularly incorporate hydrolysis. The impact that moisture has on stability relies upon how explicitly it is bound, i.e., it relies upon whether the moisture is in a free or bound state.

The active pharmaceutical ingredient and non-active ingredient can exist in either crystalline or amorphous form. Additionally, within crystalline forms, the compound can exist in anhydrous or hydrate forms.[223] The hygroscopic nature of the compounds generally varies upon the solid-state under analysis. That is, both amorphous and crystalline forms of a compound are liable for adsorption/ absorption of more moisture upon comparing their respective crystalline and hydrate forms.[224] Hence, it is very important to determine the hygroscopicity of the respective solid-state samples. It also ensures that the compound would not undergo any change during experiment.[225] If a compound is very hygroscopic, proper care should be taken to process it in such a way to minimize the effect of moisture.

Table 6.1: Hygroscopicity Classification

S. No.	Class	Type	Remarks
1	Class 1	Non hygroscopic	Essentially no moisture increases below 90% RH; less than 20% (w/w) increase in moisture content above 90% RH in 1 week
2	Class 2	Slightly hygroscopic	Essentially no moisture increases below 80% RH; less than 40% (w/w) increase in moisture content above 80% RH in 1 week
3	Class 3	Moderately hygroscopic	Moisture content does not increase >5% (w/w) below 60% RH; less than 50% (w/w) increase in moisture content above 80% RH in 1 week
4	Class 4	Very hygroscopic	Moisture content will increase as low as 40–50% RH; greater than 20% (w/w) increase in moisture content above 90% RH in 1 week

6.1.6. Flow Properties

6.1.6.1. Angle of repose

The Funnel method was selected to determine the Angle of repose of granules. The predetermined quantity of granules was taken in the funnel and kept in such a way that its tip touches the apex of the heap of the granules. The granules were flown freely onto the surface through the funnel. Finally, cone's diameter was measured, and the angle of repose was calculated using the following equation:

$$\tan \theta = \text{height/radius}$$

Where height and radius are of the cone.

6.1.6.2. Bulk Density

A known quantity of each of the drug was taken in a 100 ml graduated cylinder. Note down the height/ level of the sample without compacting. The following equation was used for the calculation of bulk density:

Bulk density = Mass of the sample taken/ Volume occupied by the sample

6.1.6.3. Tapped Density

A known quantity of sample was taken in a 100 ml graduated cylinder. The tapping of the cylinder was done mechanically using the tapped density apparatus. In this method, the cylinder was raised to a height of 14±2 mm and then allowing it to drop under its weight at a normal rate of 250 drops per minute. The sample was tapped in the cylinder up to 1, 2, 3, 5 mins. (250, 500, 750, 1250 times) Initially and then measured the final volumes. For the tapped density calculations, the following equation was used:

Tapped density = Mass of the sample/ Final volume after tapping

The interparticulate interactions have an impact on the bulking properties are also altered the powder flow. So, a comparison of these two (tapped and bulk densities) can be used as an index of the ability of the flow of powder.

6.1.6.4. Powder Compressibility

Greater the interparticulate interactions, greater is the differences between tapped and bulk densities results in poorer flow properties of the substance. Carr's Index and Hausner Ratio are measuring the relative importance of interparticulate interactions as well as porosity of sample. Percentage compressibility of the granules was determined by Carr's Index (Aulton, 1998). Both are calculated by the following equation

Carr's compressibility Index:

Carr's compressibility Index = $(T_d – P_d)/ Td \times 100$

Where, T_d is Tapped density and P_d is Poured/Bulk density

Hausner Ratio (HR):

$HR = T_d / P_d$

Where, T_d is Tapped density, and P_d is Poured/Bulk density

6.1.7. Identification of the PRED, MMF, and TAC

6.1.7.1. By UV Spectral Analysis

All the drugs were analyzed using Acetonitrile. Each drug standard (5 mg) was weighed accurately and transferred into a 10 ml volumetric flask. The solution was sonicated for 5 minutes, and then the volume was made up with a further quantity of acetonitrile. All the scans were taken using UV-VIS spectrophotometer UV-1800, Shimadzu, India.

6.1.7.2. Infrared Spectral Analysis

Infrared spectroscopy of the PRED, MMF, TAC was studied for identification purpose. Bruker: VERTEX 70v and FTIR-8400S, Fourier transform infrared Spectrophotometer, Shimadzu was used for this purpose.

Method: Approximately 2 g of each drug was triturated with 400 mg of finely powdered and dried potassium bromide individually. These quantities are usually sufficient to give a disc 10-15 mm diameter and a spectrum of suitable intensity. The mixture was carefully ground, spread uniformly in a suitable die and submitted to a pressure of about 800 MPa (8 $t \cdot cm^{-2}$). A background scan was performed using KBr disc without the drug samples and then the scan for KBr disc containing the PRED, MMF, and TAC individually in the range of 4000-400cm^{-1}.

6.1.7.3. NMR Analysis

All the APIs (PRED, MMF, TAC) were analyzed for ^{1}H-NMR using Bruker AVANCE-II 400 MHz spectrometer using $CDCl_3$ and DMSO as NMR solvents, TMS was used as internal standard and chemical shifts reported in parts per million (ppm).

6.2. Compatibility Studies of Drugs with Various Excipients

6.2.1. Physical Compatibility Study

A physical compatibility study was designed to determine the interaction of the drug with various excipients. The samples, i.e. drug alone, Excipients alone and homogeneous mixture of drug and each excipient were kept at accelerated conditions of 60°C in sealed glass vials, and 40°C/75% RH in open glass vials (punctured to enable

exposure to RH conditions for four weeks). These samples were then periodically examined against a control sample kept at 4°C.

Control (2-8°C)	**Sealed vials**
40°C, 75% RH (open)	Open vials
40°C, 75% RH (Close)	Sealed vials
60°C (open)	Open vials
60°C (Close)	Sealed vials

The ratio for the physical mixture of drug and the excipients was selected based on the probable concentration of the excipients in the capsule formulation.

6.2.2. Chemical Compatibility Study

6.2.2.1. FTIR of different excipients and compatibility study

FTIR spectrums of drug and mixture of drug formulation were obtained using an FTIR spectrophotometer. The samples were prepared by the potassium bromide disk method and measurements were attempted with the accumulation of 20 scans and a resolution of 4 cm^{-1} over the range of 400–4000cm^{-1}. After running the spectra, significant peaks relating to major functional groups were identified; spectra of the subsequent sample of the same compound were compared with the original.

6.3. Formulation Development

From the literature and compatibility studies of the excipients, the most favorable excipients were short-listed. All the excipients chosen are well known for their suitability and fitness of purpose. Each excipient is controlled by the pharmacopeial specification. The final list of excipients to be used with their probable functions is in Table 6.2.

6.3.1. Prototype Formula Development

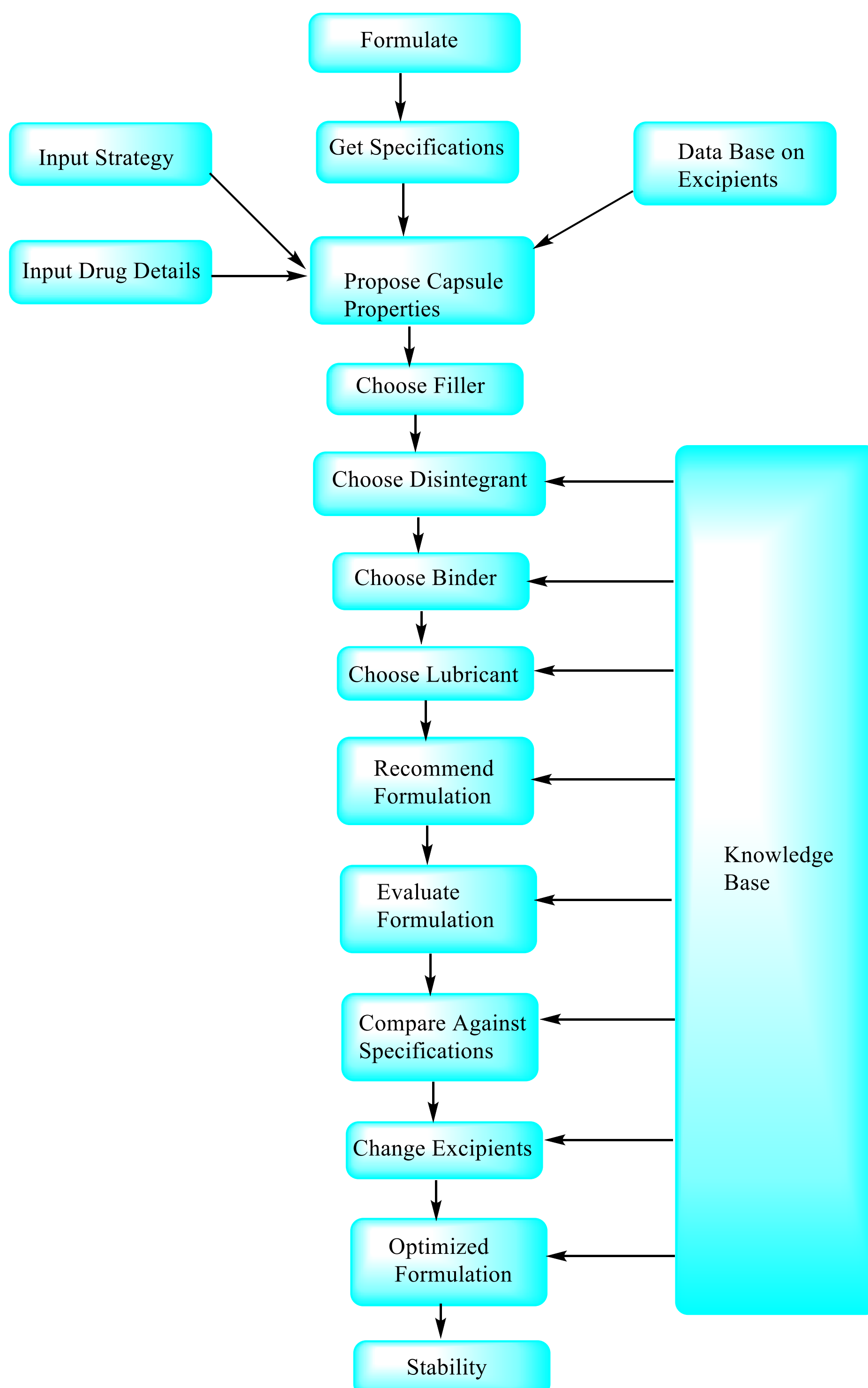


Figure 6.1: Ideal flow for formula development of immediate release capsule dosage form

6.3.2. Selection of Average Weight

As this formulation was not available in the market, so we decided to keep the average weight of our product that is approx. 447.5 mg.

6.3.3. Preparation of capsules

Capsules were prepared using direct, dry and wet granulation method. All 14 batches were prepared and analyzed for the chosen responses.

Table 6.2: Prototype formula for the capsule formulation

S. No	Inactive Ingredient	Weight/ capsule (mg)	Weight/ 20 capsules (mg)	Weight/ 100 capsules (mg)
1	Mycophenolate Mofetil	100	2000	10000
2	Prednisolone	5	100	500
3	Tacrolimus	0.5	10	50
4	SSG	70	1400	7000
5	Talc	5	100	500
6	HPMC K 100	10	200	1000
7	Purified Water	q.s.	q.s.	q.s.
8	Lactose	120	2400	12000
9	Maize starch	125	2500	12500
10	Aerosil	10	200	1000
11	Magnesium stearate	2	40	200
Total weight		447.5	8950	44750

6.3.4. Manufacturing procedure

1. First weigh the required quantities of PRED, MMF and TAC. Then pass these powder drugs through 40# sieve.
2. Mix this drug mixture with lactose powder uniformly in mortar and pestle.
3. Prepare HPMC K 100 solution as specified in prototype formula.
4. Add this solution dropwise in mortar to get cohesive mass.
5. Screen prepared cohesive mass through 12# granulating sieve and collect it on

granulating tray.

6. The prepared granules were subjected for drying at 60°C until LOD reached 1.5-2% w/w.
7. Pass 50 % dried granules through 16# sieve to get uniform particle size and continue drying for 30 min.
8. Magnesium stearate weighed and passed through # 60 mesh screen and was mixed with doubling cone blender for 5 min at 10 rpm.
9. The capsules were filled using these granules.

Table 6.3: Processing variables that were kept constant during the formulation of all the batches

S. No	Processing variables	Constant
1	Amount of DM water used for binder solution	75 ml
2	Mixing time of the ingredients	10 min
3	Binder solution adding time	3 min
5	The drying temperature of the granulation	60º C
6	Loss on drying of the blend	Not more than 2 w/w
7	Mixing time of granules and Extra granular material in a double cone blender	15 min
8	Mixing time of magnesium stearate to the blend in a double cone blender	5 min

The granules were filled into capsules to a unit fill weight of 447.5 mg.

6.4. Evaluation of the capsules

6.4.1. Weight Variation

Twenty capsules of each formulation were weighed individually and mean weight and percentage relative standard deviation was calculated.

Average weight of capsule contains	Percentage deviation
Less than 80 mg	10
More than 80 or less than 250 mg	7.5
250 mg or more	5

Six capsules of each formulation were also examined for their thickness using sliding Vernier caliper, and the mean thickness value was calculated.

6.4.2. Disintegration Time

Six capsules of each formulation were used to determine disintegration time. DI water was used as a disintegration medium and temperature was maintained at 37±2°C.

6.4.3. Dissolution profile

Dissolution was performed on capsules (n=6) using USP apparatus 2 (Paddle) 50 rpm in 900 ml of 0.1 N HCl (pH =1.2) maintained at 37± 0.5° C. 10 ml of the samples were withdrawn at the interval of 5, 10, 15, 20, 25, 30 and 35 min and equal volume of fresh dissolution medium maintained at same temperature was added to maintain the sink conditions, and filtered through 0.22 µ nylon filter, and analyzed by HPLC method.

6.5. Stability of In-house product

Accelerated stability studies according to the ICH guidelines

The capsules were packed in HDPE bottle and kept in stability chamber maintained at 40° C and 75 % Relative Humidity for 90 days. Samples were withdrawn at an interval of 0, 30, 60 and 90 days. The samples were analyzed for their drug content, disintegration time, hardness and dissolution in 0.1N HCl (pH =1.2) in USP II at 50 rpm. (As this is the official apparatus and media in USFDA).

6.6. Prototype Formula Development

6.6.1. Selection of Excipients

Historically, pharmaceutical excipients have been regarded as inert derivatives, but this is no longer the case. Each additive must have a clear justification for inclusion in the formulation and must perform a defined function in the presence of all the action and any other excipients included in the formulation.

Traditionally, pharmaceutical excipients have been viewed as inactive derivatives, yet this is not true anymore. Every added substance must have a clear reason for

incorporation in the formulation and must play out a defined work within the formulation along with all the active and some other excipients incorporated.

According to the International Pharmaceutical Excipient Council (IPEC), the pharmaceutical excipient is a substance other than the API or prodrug, which have been incorporated in the drug delivery system after evaluation for its safety and may include

- aid processing of the system during manufacture, or
- protect, support or enhance stability, bioavailability or patient acceptability, or
- assist in product identification, or
- enhance any other attribute of the overall safety and effectiveness of the drug product during storage or use

6.6.2. Suitable Filler Selection

There is a number of general principles for choosing a diluent depending upon the compaction properties of the API. If the material is greatly plastic, it is recommended to include a diluent that compacts by brittle fracture; also, a brittle drug substance ought to be joined with plastic filler. In these considerations, the drug solvency should be considered. A soluble drug is typically combined with an insoluble filler to improve the disintegration process.

Apart from the other properties, the fillers should have the following properties

- They should be inert and physically and chemically compatible with the active substance and the other excipients being used in the formulation
- They should be physiologically inert
- They should not have an unacceptable microbiological burden
- They should not have a deleterious effect on the bioavailability
- They should have regulatory acceptability in all countries where the product is to be marketed

Therefore, out of many diluents, one insoluble and one soluble diluent were evaluated. The fillers evaluated were

- Lactose Monohydrate
- Microcrystalline Cellulose.

6.6.3. Suitable Binder Selection

It is conceivable to granulate a powder essentially by including water or organic solvent, under one condition that the fluid can wet the powder surface, it will form liquid bridges. At the point when the granule dries, crystallization of any solids that had soluble in the fluid will form a solid bond between the particles. These bonds are generally weak and form the friable granules; frequently the granules won't be adequately robust to tolerate the drying procedure. So, it is necessary to incorporate the binders to the granulation process to expand the granule quality.

It is important that the granulating agent must form a film under a particle surface. Rowe (1989) has proposed that the choice should be based on their spreading coefficients, where the spreading coefficient is characterized as the contrast between the work of binder and the substrate and cohesion property of binder.

Table 6.4: Commonly used binders and their concentrations of use

S. No	**Granulating agent**	**Normal usage concentration (%)**
1	Starch	5-25
2	Pre-gelatinized starch	5-10
3	Acacia	1-5
4	Povidone (PVP) K 30	2-5
5	Hydroxypropyl methyl cellulose (HPMC)	2-8
6	Methyl Cellulose	1-5

The synthetic polymers have almost totally superseded the use of natural products in modern formulations.

Therefore, the following binders were considered for use in the prototype formulation.

1. Hypromellose
2. PVP K-30

Tests dissolution helps in the selection of binder.

6.6.4. Suitable Disintegrant Selection

Disintegrant plays a vital role in the formulation of an immediate release solid dosage form. To maximize the dissolution rate of a drug substance from a capsule, it is necessary to overcome the cohesive strength produced by the filling process and break the capsule into the primary particles as rapidly as possible. This is achieved by adding disintegrants, which will induce this process.

Table 6.5:Commonly used disintegrants and their concentrations of use

S. No.	Disintegrant	Normal usage Concentration (%)
1	Starch	5 – 10
2	Microcrystalline Cellulose	-
3	Insoluble Ion-exchange Resin	-
4	Sodium Starch Glycollate	2 – 8
5	Crosscaramllose	1 – 5

Since the immediate release capsules require fast disintegration, the following commonly used and commercially available disintegrants were evaluated:

1. Sodium Starch Glycollate
2. Starch

6.6.5. Lubricant Concentration Optimization

Magnesium Stearate, the lubricant of choice is commonly used between the concentrations of 0.2% to 2%; It may be combined with other (Aerosil) within the same range. Batches were prepared and evaluated in the concentration range of 0.5% to 2% individual and combination.

6.7. Formula Optimization Studies

The major objective of the product optimization stage is to ensure that the product selected for further development (the intended commercial product) is fully optimized and complies with the design specifications and critical quality parameters. The key output from this stage of development is

- A quantitative formula defining the grades and quantities of each excipient and the quantity of candidate drug;
- Defined pack;
- The defined drug, excipient and component specifications;
- Defined product specifications.

The approach to product optimization will depend on the nature of the product to be developed. The concentrations of various excipients selected

6.7.1. Effect of Process

Capsules may be prepared using the following processes

1. Direct powder filing
2. Dry Granulation filling
3. Wet Granulation filling

Factors that require consideration during the development of capsule formulation are the physical properties of the drug (bulk density and tapped density), granule properties, the bioavailability of the active ingredient and the stability of the formulation.

Based on the data appended in table 4.16 the MMF was found to have poor flow (C.I lies between 25-35). So, it cannot be directly filled to form a capsule.

For formulating a capsule dosage form, the drug should be processed to enhance flowability so that it can be filled uniformly. The following processes were evaluated.

1. Direct powder filling - The MMF was blended with the excipients of table 8.21 and evaluated for various parameters. The result is compiled in Table 8.22

2. Dry granulation filling - The dry granulation process was also evaluated as an alternative to direct powder filling to achieve the capsule filling process with a drug having the characteristic of low bulk density. The MMF was compacted with excipients of Table 8.21 and evaluated for various parameters. The result is compiled in Table 8.22

3. Wet granulation - The wet granulation process was also evaluated as an alternative to dry granulation process and direct filling, to achieve capsule filling process with the drug having the characteristic of low bulk density The MMF was granulated with excipients of table 8.21 and evaluated for various parameters. The result were compiled in table 8.22

6.8. Dissolution Method Development

6.8.1. Selection of a suitable apparatus

The preferred apparatus for a capsule dosage form is by paddle type (USP-II) as mentioned in USFDA guidance for dissolution of generic drugs.

6.8.2. Selection of a suitable Dissolution Media

0.1 N HCl (pH =1.2) was selected as the dissolution media as mentioned in USFDA guidance for dissolution of generic drugs.

6.8.3. Placebo Interference

Placebo of the capsule formulation was analyzed by developed HPLC method to see if it has any interference in the estimation of the PRED, MMF, and TAC.

A weighed amount of the placebo was taken and dissolved in 900 ml of 0.1N HCl (pH =1.2) and sonicated for 15 min to facilitate maximum solubility of the placebo in the dissolution media. 2 ml of this solution was pipetted out into a 50 ml volumetric flask, and the volume was made up to 50 ml with 0.1N HCl.

6.9. Dissolution Method Validation

Dissolution studies were carried out by using a rotating basket method where 0.1 N HCl was used as dissolution medium. All the formulations were placed in one media vessel, with a specified interval of time 5 ml of the sample was withdrawn from dissolution media and same amount was replaced with fresh media. The percent drug release was measured by using HPLC method.

6.9.1. Specificity and selectivity

All the three-drug solutions (according to the API weight) were prepared in 0.1 N HCl media along with and without common excipients (lactose, magnesium stearate, talc, HPMC) separately. All the solutions were injected to HPLC and checked for any interference by dissolution mediums or placebo. In a separate study, drug concentrations were prepared independently from pure drug stock solution in selected media and analyzed ($n = 6$). The standard deviations were determined in both cases.

6.9.2. Accuracy

The accuracy of the method was determined in terms of % recuperation of standard. Recuperation studies were carried out by extending the standard drug solution concentration at the level of lower, medium and higher for each drug in the pre-analyzed sample ($n = 9$). Accuracy was assessed as the standard deviation, percentage RSD at each level; overall standard deviation and overall % RSD and compiled % recovery was determined.

6.9.3. Precision

Repeatability was determined by using different levels of drug concentrations (same concentration levels taken in accuracy study), prepared from independent stock solutions and analyzed (n=6). The intra-day and inter-day variation for determination of all the three drugs were carried out with concentrations over three levels (low, medium and high) in the same day and three consecutive days where repeatability was determined with a lower concentration and injected six times and % RSD was calculated. The percent relative standard deviation (% RSD) of the predicted concentrations from the regression equation was taken as precision.

6.9.4. Linearity

To establish linearity of the proposed methods, ten separate series of solutions of mycophenolate mofetil, prednisolone, and tacrolimus 10-100 μg/mL concentrations were prepared. All the dilutions were filtered through 0.22 μ nylon filter and injected. For this, each concentration was used in triplicate. Least square regression analysis was done for the obtained data.

6.9.5. Robustness and Ruggedness

These terms refer to the capability of an analytical method to remain unchanged by deliberately changing the method parameters like a change in flow rate and change in wavelength. The concept of remaining unchanged by deliberately varying the method parameters has two possible elucidations such as- (a) no change of the identified measure of the analyte in a specific test disregarding the variation in the method parameter or (b) no change is observed in the critical performance characteristics disregarding the variation in the method parameter. For the calculation of robustness, the sample with the lowest concentration was analyzed by deliberately changing the flow rate about ±15%, i.e., 1 and 1.4 mL/min and changing the wavelength by ±5 nm, i.e., 245 and 255 nm. The robustness was studied by analyzing the sample containing lower concentration with deliberate variation in the method parameters. Robustness of the method was studied by a change in wavelength or change in flow rate. The change in the responses of drugs was noted in terms of %RSD. The ruggedness was studied by analyzing the same samples of three drugs by changing the analyst. The change in the responses of drugs was noted in terms of %RSD.

6.10. Assay Method development and validation

6.10.1. Reagents and solvents

Samples of Mycophenolate Mofetil and Tacrolimus were the gift samples from Biocon Ltd., (Bangalore, India). Prednisolone was the gift sample from Jackson Laboratories Private Limited, (Amritsar, Punjab, India). HPLC grade solvents, Acetonitrile, and other chemicals were purchased from Thermo Fisher Scientific (Vadodara, Gujarat, India). For the entire HPLC method, in-house produced double-distilled water was

used. Analytical grade Orthophosphoric acid, Triethylamine was obtained from Merck (Worli, India, India). Triethylamine buffer solution was prepared and filtered through a 0.22µ filter (Millipore, USA).

6.10.2. Instrumentation and chromatographic conditions

The HPLC (Shimadzu, Kyoto, Japan) instrument was equipped with two LC-10 ATVP pumps, SPD-10AVP UV-vis detector, injector with a 20 μL loop. The HPLC column used for analysis was Kinetex Polar, C18, 5 µm, 4.6 × 250 mm. Column. The results were acquired and processed using Shimadzu LC-solution version 6.42 software for data acquisition and processing. The mobile phase was a mixture of Acetonitrile and 0.35% Triethylamine pH 4.2 with Orthophosphoric acid (70:30). Injection volume was 20 µL which was injected into the column using a syringe and the linear gradient flow rate was set at 1.2 mL/min. The drugs were detected at 254 nm for Prednisolone and Mycophenolate and Tacrolimus.

6.10.3. Calibration curves

6.10.3.1. Preparation of standard stock solution

Fifteen mg MMF, 5 mg PRED and 0.5 mg TAC were accurately weighed and put into 10 mL volumetric flask containing 5 mL of diluents (Acetonitrile) and sonicated for 10 min then the volume was adjusted with diluents up to the mark.

6.10.3.2. Preparation of sample solution

Sample solution of MMF with different concentrations from 50-150 µg/mL was prepared from the above stock solution and diluted with Acetonitrile.

Sample solution of PRED with different concentrations from 0.5-50 µg/mL was prepared from the above stock solution and diluted with Acetonitrile.

Sample solution of TAC with different concentrations from 0.05-5 µg/mL was prepared from the above stock solution and diluted with Acetonitrile.

6.11. Method Validation

6.11.1. Specificity

Specificity of the HPLC method was demonstrated by the separation of the analytes from other potential components such as impurities, degradants or excipients. A volume of 20µL of individual ingredients solutions (Placebo, API, Excipients) was injected, and the chromatogram was recorded.

6.11.2. Precision

6.11.2.1. System precision

System precision of the system was determined by injecting six replicates of the standard solution (injection volume, 20 µL) and measurement carried out of peak areas of the main peak. Data were treated to calculate % RSD.

6.11.2.2. Method precision

Method precision of the method was determined by injecting six replicates (injection volume, 20 µL) of the sample solution from a single batch of capsules individually and measurement carried out of peak areas of the main peak. Data were treated to calculate % RSD.

6.11.2.3. Intermediate precision

Intermediate precision of the method was determined by injecting six replicates (injection volume, 20 µL) of the sample solution from a single batch of capsules individually as per the method by a different analyst on the different instrument using different column and on a different day. Measurements carried out of peak areas of the main peak. Data were treated to calculate % RSD.

6.11.3. Linearity

For linearity, mycophenolate mofetil, prednisolone, and tacrolimus concentrations were prepared (as discussed in preparation of sample solutions). All the dilutions were filtered through 0.22 µ nylon filter and injected. For this, each concentration was used in triplicate. To assess the linearity of the method data were plotted in the form of

linearity curve and slope, intercept, and a correlation coefficient of the curve has been calculated.

6.11.4. Accuracy

Accuracy studies were carried out by applying the method to placebo samples to which known amount of each drug (PRED, MMF, and TAC) corresponding to 80, 100, and 120 level have been added. At each level of the amount, samples prepared in triplicate and determination was performed. Data were treated to calculate % RSD at each level and overall.

6.11.5. Robustness and Ruggedness of the method

These terms refer to the capability of the analytical method to remain unchanged by deliberately changing the method parameters like by changing the flow rate, change in wavelength, etc. The concept of remaining unchanged by deliberately varying the method parameters has two possible elucidations: it can be taken as:

(a) no change of the identified measure of the analyte in a specific test disregarding the variation in the method parameter or

(b) no change is observed in the critical performance characteristics disregarding the variation in the method parameter.

For the calculation of robustness, the sample of lowest concentration was analyzed by deliberately changing the flow rate about ±15%, i.e., 1 and 1.4 mL/min and changing the wavelength by ±5 nm, i.e., 245 and 255 nm.

The robustness was studied by analyzing the sample of lower concentration with deliberate variation in the method parameters. The change in the responses of drugs was noted in terms of % RSD. Robustness of the method was studied by a change in wavelength or change in flow rate.

The ruggedness was studied by analyzing the same samples of three drugs by changing analyst. The change in the responses of drugs was noted in terms of % RSD.

6.11.6. Limit of detection and limit of quantitation

The LOD and LOQ of developed method were contemplated according to ICH rules. A few methodologies for deciding the LOD and LOQ are conceivable, contingent upon the strategy, i.e. a non-instrumental or instrumental. Among them here employed method was,

$$LOD = 3.3\sigma/S \text{ and}$$

$$LOQ = 10\sigma/S$$

Where, σ = the standard error of response, S = the slope of the calibration curve.

6.11.7. Stability of analytical solution

To determine the stability of the analytical solution, standard solution and sample solution to be analyzed initially and at different time intervals at 25ºC for around 24 hours and/or standard solution and a sample solution to be analyzed initially and at different time intervals at 5ºC for around 24 hours. For that six injections of standard solution were injected in a column for the determination of system suitability and one injection of each standard solution as well as sample solution were injected at different time intervals for around 24 hours.

6.11.8. Forced degradation studies

To perform the forced degradation studies first injected six injections of standard solution (for system suit). Then the sample solution and placebo solution were treated separately in each condition as followed:

(a) Two milliliters of 1N HCl was added, and the mixture was heated at 70°C for 30 minutes and neutralized by addition of 1N NaOH solution and 10 mL of diluent. (Acid-induced degradation)

(b) One milliliter of 1N NaOH was added and the mixture placed at room temperature for 5 minutes. (Base induced degradation)

(c) Two milliliters of 30% w/v H_2O_2 was added, and the mixture was heated at 70°C for 15 minutes. (Hydrogen peroxide H_2O_2 induced degradation)

(d) The samples were placed in a UV chamber at 6500 LUX for 15 days. (UV induced degradation)

(e) The samples were placed in an oven at 105°C for 15 days. (Thermal induced degradation) In all degradation studies, ten µL of the resultant solutions were injected in column and chromatograms were run as described in section 2.2. The peak area of each peak has been determined and peak purity determined in each case. Data were treated to calculate the degradation in each case.

6.11.9. Analysis and stability testing of formulations

To determine the content of individually available formulations of each drug the twenty tablets/capsules were weighed and their mean weight determined. Powder (tablet/capsule) equivalent to 2.0 mg of each drug was accurately weighed and transferred into a 10 mL volumetric flask, containing 5.0 mL of diluent (a mixture of water: acetonitrile, 20:80% v/v). To ensure complete extraction of the drug, it was sonicated for 30 min and diluted to 10 mL with diluent. The resulting solution was centrifuged at 2000 rpm for 10 min, and the supernatant was analyzed for drug content. Twenty microliters of the filtered solution were injected into the chromatographic conditions as mentioned. The analysis was repeated in three replicates, and the possibilities of excipient interference in the analysis were studied. For stability studies, prepared capsules (in closed high-density polyethylene containers) were stored at accelerated conditions (40°C/75% RH), and drug content was analyzed after 1, 2, and 3 months.

6.11.10. System suitability

System suitability in each parameter of validation has been determined, and the acceptance criteria for the system suit were as followed:

- Tailing factor of PRED, MMF, and TAC peak from the standard solution should not be more than 2.0.

- Theoretical plates of PRED, MMF, and TAC peak from the standard solution should not be less than 3000.
- % RSD of the area of PRED, MMF, and TAC peak from the five injections of the standard solution should not be more than 2.0.

CHAPTER 7

RESULT AND DISCUSSION

7.1 Initial Physicochemical Characterization

7.1.1 Physical Appearance

Immunosuppressant MMF is a white to off-white crystalline powder, TAC is white crystals, or crystalline powder and PRED is white or practically white crystalline powder.

7.1.2 Melting point

Differential Scanning Calorimetry was used to determined melting point.

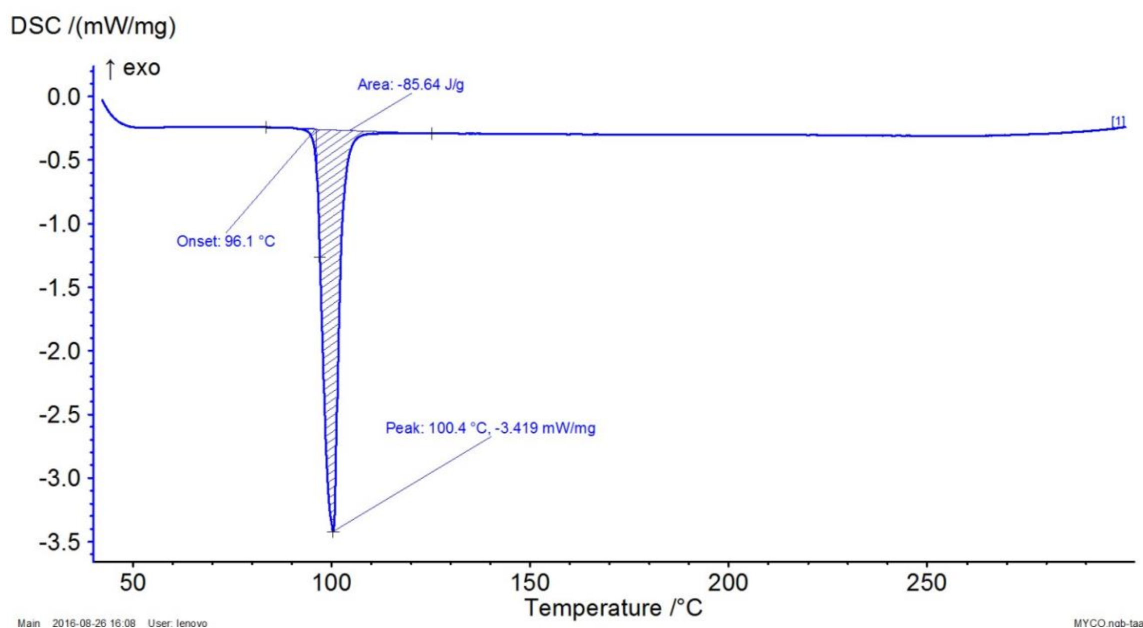


Figure 7.1: DSC thermogram of Mycophenolate drug

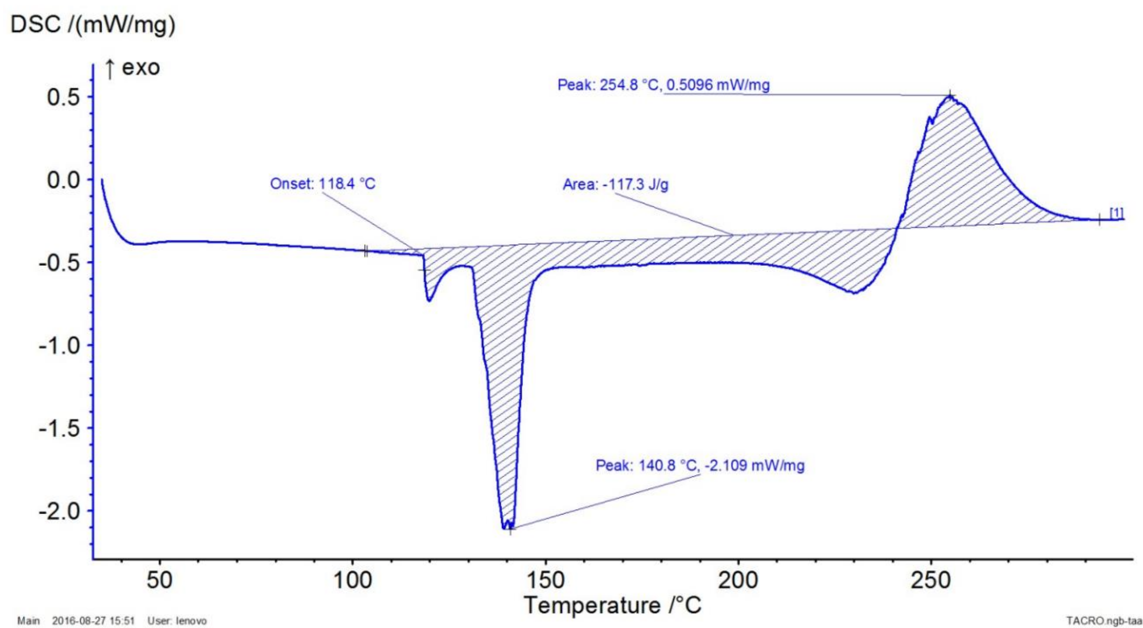


Figure 7.2: DSC thermogram of Tacrolimus drug

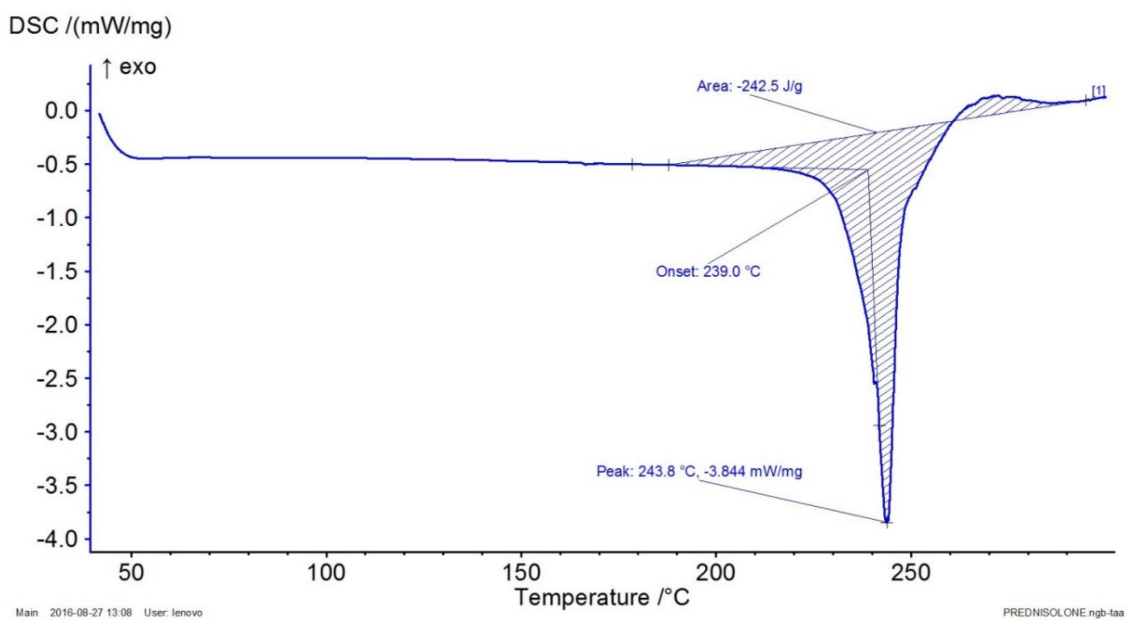


Figure 7.3: DSC thermogram of Prednisolone drug

Result

The melting endotherm of the MMF was obtained at 100.4 °C (Lit 99-100 °C). Therefore, it was concluded that exposure to high temperatures should be avoided and

the loss on drying percentage of the blends prepared should be taken at temperatures fairly below the melting point of the drug.

The DSC curves of the tacrolimus in the heating revealed the existence of two endothermic phase transitions. The first endothermic peak occurred in the range temperature of 90–110˚C and must be related to the evaporation of solvents, including water. The second endothermic peak, which corresponds to the melting point of tacrolimus, showed variations in the temperature of phase transition as a function of the heating rate for all samples. The comparison between the peaks of fusion in different heating rate showed a variation between 117.3±1.0°C and 140.0±1.0°C.

The melting endotherm of the PRED was obtained at onset 239° C (Lit. 237-240° C).

7.1.3 Particle size determination

In the case of capsules, particle size and shape of the drug substance influence the flow and the mixing efficiency of powders and granules. Particle size can also be a factor in stability; fine materials are relatively more open to attack from atmospheric oxygen, the humidity, and interacting excipients than are coarse materials due to high surface area.

Classical methods for measuring particle size are as follows:

- Microscopy
- Sieving or screening

7.1.3.1 Particle Size Analysis by Sieve Shaker

Result

Lump formation of the PRED, MMF and TAC occurred on the top sieve (# 60) indicating the adhesive nature of the all the drugs. Thus, this method could not be employed for particle size determination.

7.2 X-Ray – Diffraction Studies

The X-ray diffraction (XRD) pattern of powder samples was recorded on a scanning powder X-ray diffractometer using an X' Pert PRO instrument (PANalytical, Netherlands), equipped with an X' Pert PRO Data Collector software.

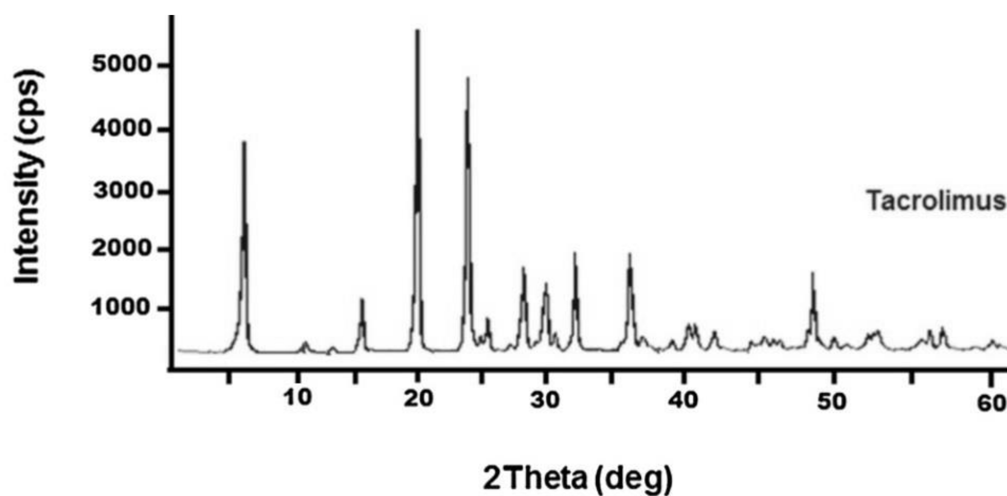


Figure 7.4: X-Ray Diffraction pattern of the TAC

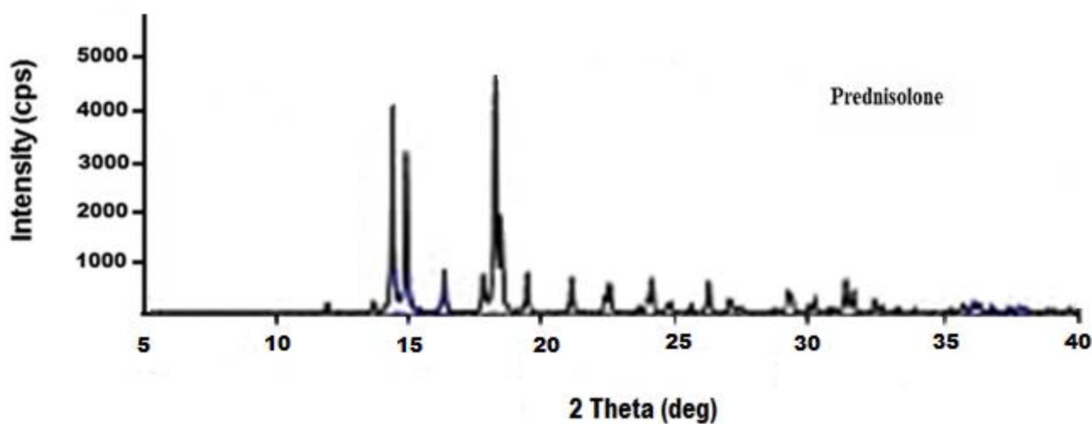


Figure 7.5: X-Ray Diffraction pattern of the PRED

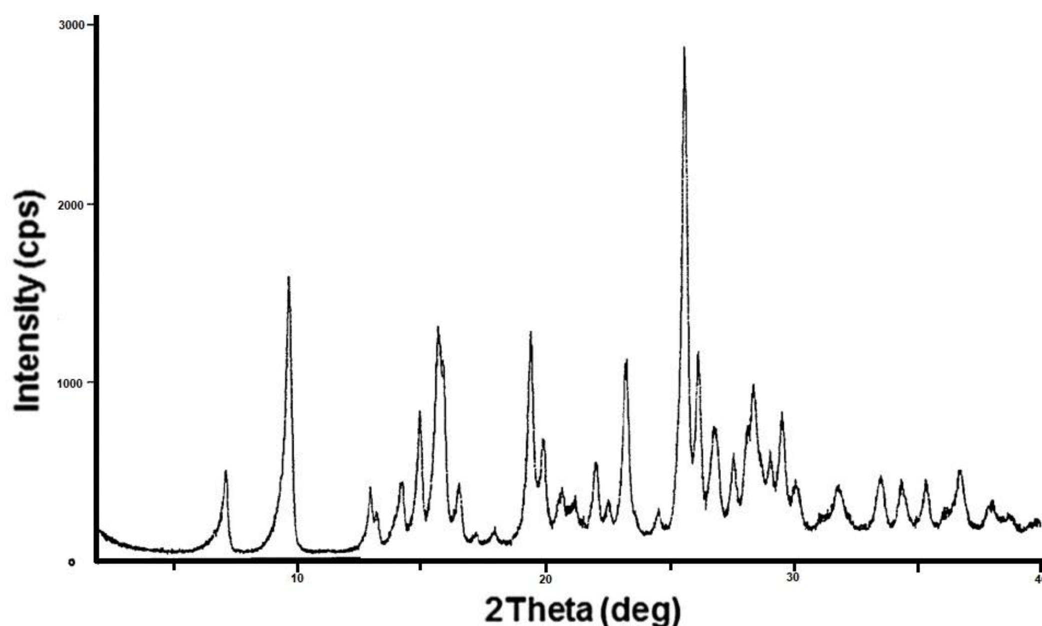


Figure 7.6: X-Ray Diffraction pattern of the MMF

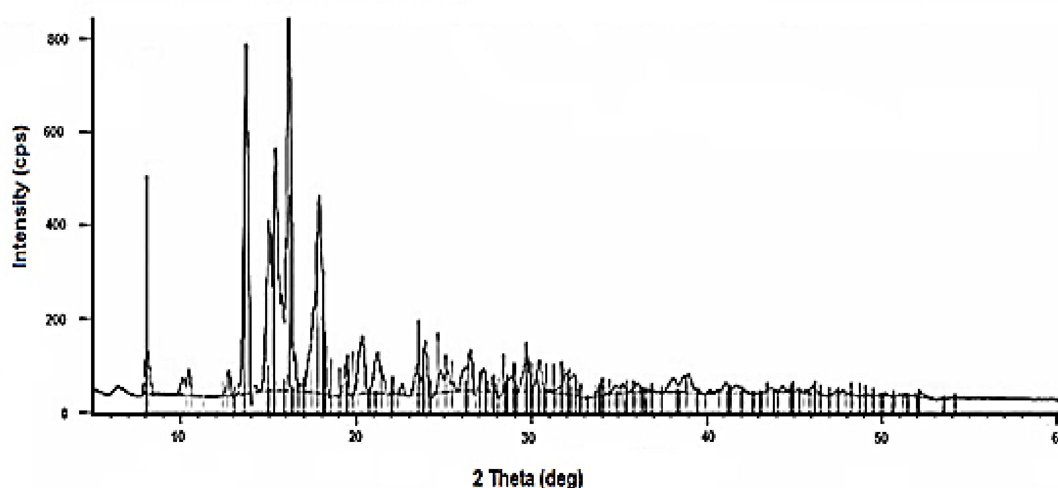


Figure 7.7: X-Ray Diffraction pattern of the physical mixture (1:1:1)

Result

The diffraction pattern of TAC shows characteristic peaks at 2θ values of 6.5, 11.2, 16, 20 and 24.5. PRED shows peaks at 10, 15.2, 16, 17.3 and 20.2. MMF shows at 6.6, 9.8, and 13.2, 14.1, 15, 16.3, 17.4. The 2θ values obtained were found matching to that present in the literature and confirms the crystalline nature of the TAC, PRED and MMF.

7.3 Hygroscopicity Study

Numerous compounds and salts are sensitive to the presence of water vapors or moisture. At the point when compounds associate with moisture, they hold the water by bulk or surface adsorption, capillary condensation, chemical reaction and, in extraordinary cases a solution (deliquescence).

If a compound is very hygroscopic, proper care should be taken to process it in such a way to minimize the effect of moisture.

Table 7.1: Observation chart of moisture uptake by the MMF

Humidity (%)	Petri dish No.	The weight of Petri dish (g)	The weight of MMF (g)	Total Wt. (g)	Wt. after one week (g)	% Moisture uptake
10	1	24.5	1	25.5	25.6	0.3
	2	28.2	1	29.2	29.3	0.3
30	3	30.2	1	31.2	31.3	0.3
	4	24.4	1	25.4	25.5	0.3
75	5	28.4	1	29.4	29.5	0.3
	6	26.6	1	27.6	27.7	0.3

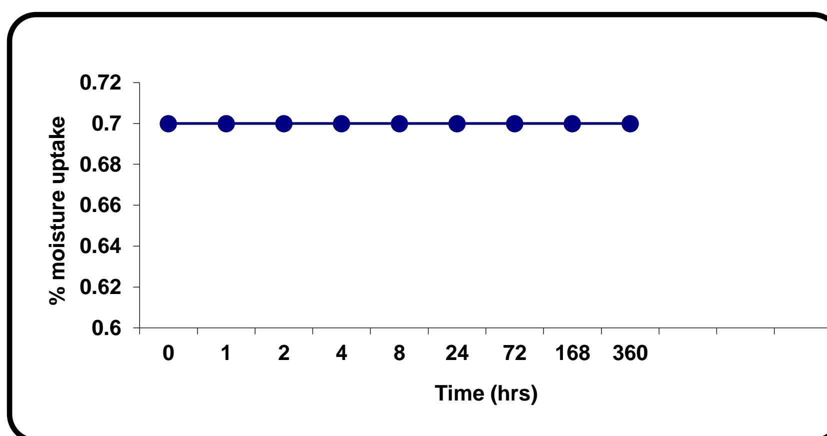


Figure 7.8: Moisture uptake by the MMF concerning time

Table 7.2: Observation chart of moisture uptake by the TAC

Humidity (%)	Petri dish No.	The weight of Petri dish (g)	The weight of TAC (g)	Total Wt. (g)	Wt. after one week (g)	% Moisture uptake
10	1	18.2	1	19.2	19.3	0.3
	2	19.0	1	20.0	20.1	0.3
30	3	17.3	1	18.3	18.4	0.3
	4	16.9	1	17.9	18	0.3
75	5	17.1	1	18.1	18.2	0.3
	6	16.9	1	17.9	18	0.3

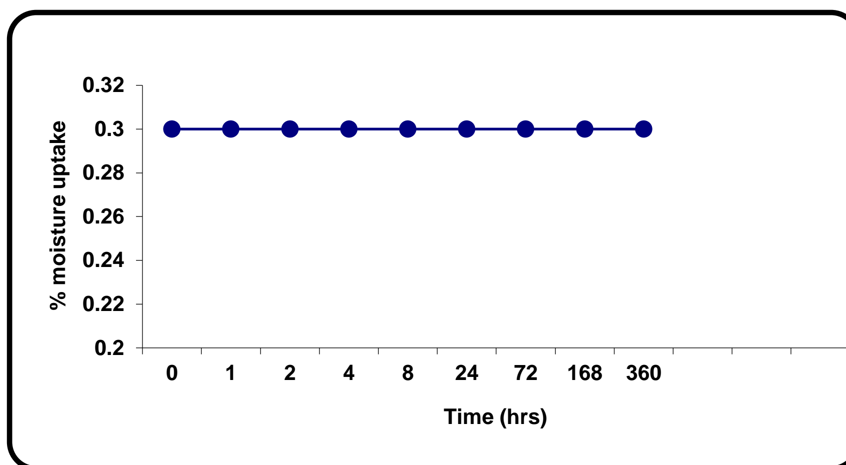


Figure 7.9: Moisture uptake by the TAC concerning time

Table 7.3: Observation chart of moisture uptake by the PRED

Humidity (%)	Petri dish No.	The weight of Petri dish (g)	The weight of PRED (g)	Total Wt. (g)	Wt. after one week (g)	% Moisture uptake
10	1	25.5	1	26.5	26.7	0.7
	2	26.2	1	27.2	27.4	0.7
30	3	27.2	1	28.2	28.4	0.7
	4	27.4	1	28.4	28.6	0.7
75	5	25.4	1	26.4	26.6	0.7
	6	26.6	1	27.6	27.8	0.7

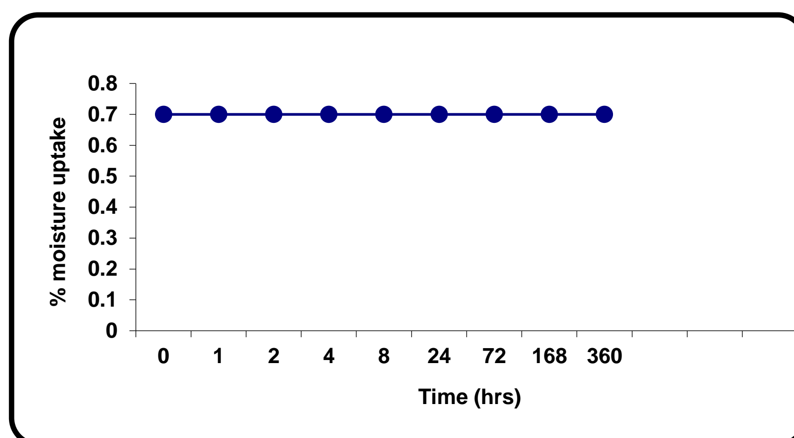


Figure 7.10: Moisture uptake by the PRED concerning time

Result

Based on moisture uptake data, it can be concluded that the MMF and TAC are not hygroscopic as the weight gain when exposed to 75%RH, was found to be negligible. But in the case of PRED, it is little hygroscopic. This will broaden the limits of relative humidity in the working area.

7.4 Flow Properties

For the successful manufacturing, the solid dosage form (i.e., Capsule) the good flow properties of powder/ granules is essential. At the preformulation stage, information produced on low properties can be of used wisely in the improvement of the formulation development. The information produced can give direction on the determination of the excipients to utilize, the plan of formulation type and method to use, for instance, granulation. It is imperative that once the propensity and size dispersion of the test compound has been determined, the flow properties are calculated if the expected formulation is solid dosage based.

7.4.1 The angle of Repose, Bulk Density, Tapped Density, Compressibility Index and Hausner's Ratio

Certain natural and derived properties are evaluated to assess the flowability of the blend which in turn affect the critical parameters like the uniformity of weight in the dosage form and weight variation in the final formulation. The natural properties include an angle of repose, bulk density, and tapped density while compressibility index and Hausner's ratio are derived from these properties.

Table 7.4: Flow Properties of the MMF

S. No.	Parameter	Result
1	Angle of Repose (θ)	37
2	Bulk Density (gm/ml)	0.278
3	Tapped Density (gm/ml)	0.419
4	Compressibility Index	33.67
5	Hausner's Ratio	1.51

Table 7.5: Flow Properties of the TAC

S. No.	Parameter	Result
1	Angle of Repose (θ)	33.2
2	Bulk Density (gm/ml)	0.298
3	Tapped Density (gm/ml)	0.421
4	Compressibility Index	31.21
5	Hausner’s Ratio	1.3

Table 7.6: Flow Properties of the PRED

S. No.	Parameter	Result
1	Angle of Repose (θ)	24.67
2	Bulk Density (gm/ml)	0.267
3	Tapped Density (gm/ml)	0.338
4	Compressibility Index	40.44
5	Hausner’s Ratio	1.37

Result

All the flow parameters like angle of repose, compressibility and Hausner’s ratio are suggestive of poor flow of the MMF, TAC, and PRED. Therefore, it was concluded that to formulate the drug in a capsule dosage form; the drug should be processed with excipients in such a way that increases flowability.

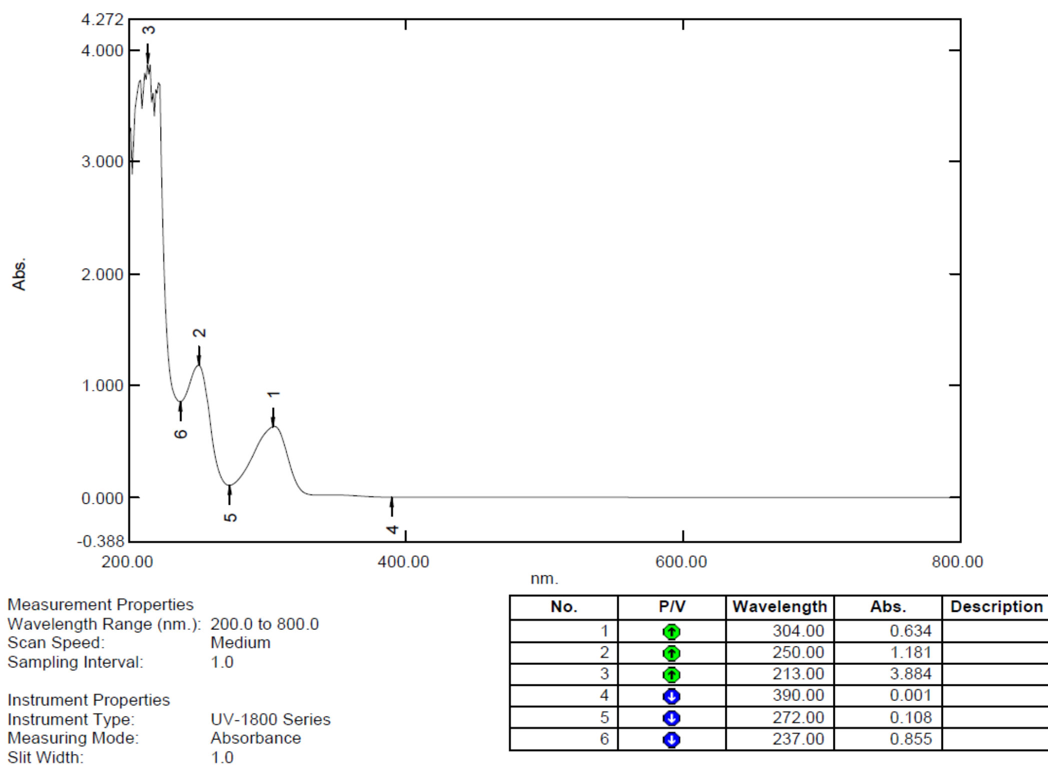
7.5 Identification of the Drugs

7.5.1 UV Analysis

7.5.1.1 UV analysis of Mycophenolate

Determination of Absorption Maxima (λ_{max}) of Mycophenolate in Acetonitrile: 5 mg of the standard was weighed accurately and transferred into a 10 ml volumetric flask. The

solution was sonicated for 5 minutes, and then the volume was made up with a further quantity of acetonitrile.



No.	P/V	Wavelength	Abs.	Description
1	↑	304.00	0.634	
2	↑	250.00	1.181	
3	↑	213.00	3.884	
4	↓	390.00	0.001	
5	↓	272.00	0.108	
6	↓	237.00	0.855	

Figure 7.11: The absorption maxima of MMF was observed at 254 nm

7.5.1.2 UV analysis of Prednisolone

Determination of Absorption Maxima (λ_{max}) of Prednisolone in Acetonitrile: 5 mg of the standard was weighed accurately and transferred into a 10 ml volumetric flask. The solution was sonicated for 5 minutes, and then the volume was made up with a further quantity of acetonitrile.

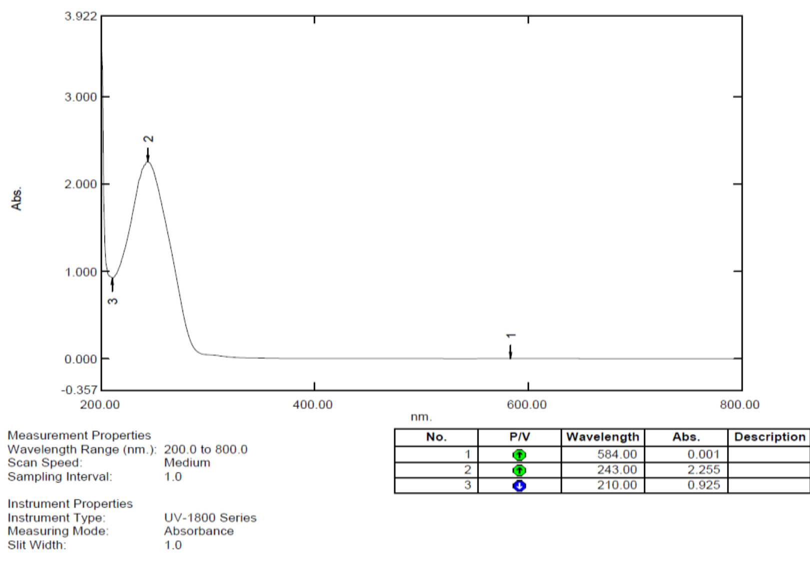


Figure 7.12: The absorption maxima of PRED was observed at 254 nm

7.5.1.3 UV analysis of Tacrolimus

5 mg of the standard was dissolved in acidified acetonitrile (using sulphuric acid) and sonicated for 10 minutes. Then the volume was made up with a further quantity of acetonitrile up to 10 ml.

No maxima peak was observed in the case of Tacrolimus. Even after heating the standard solution.

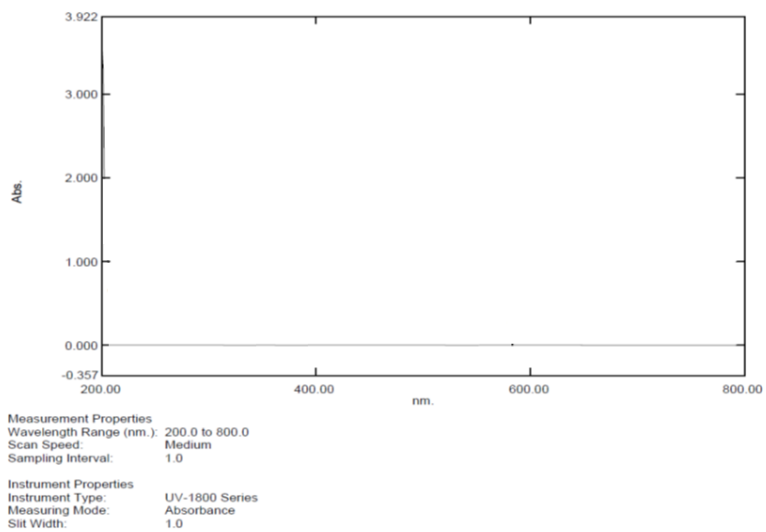


Figure 7.13: The absorption maxima of TAC was observed at 254 nm

7.5.2 Infrared Analysis

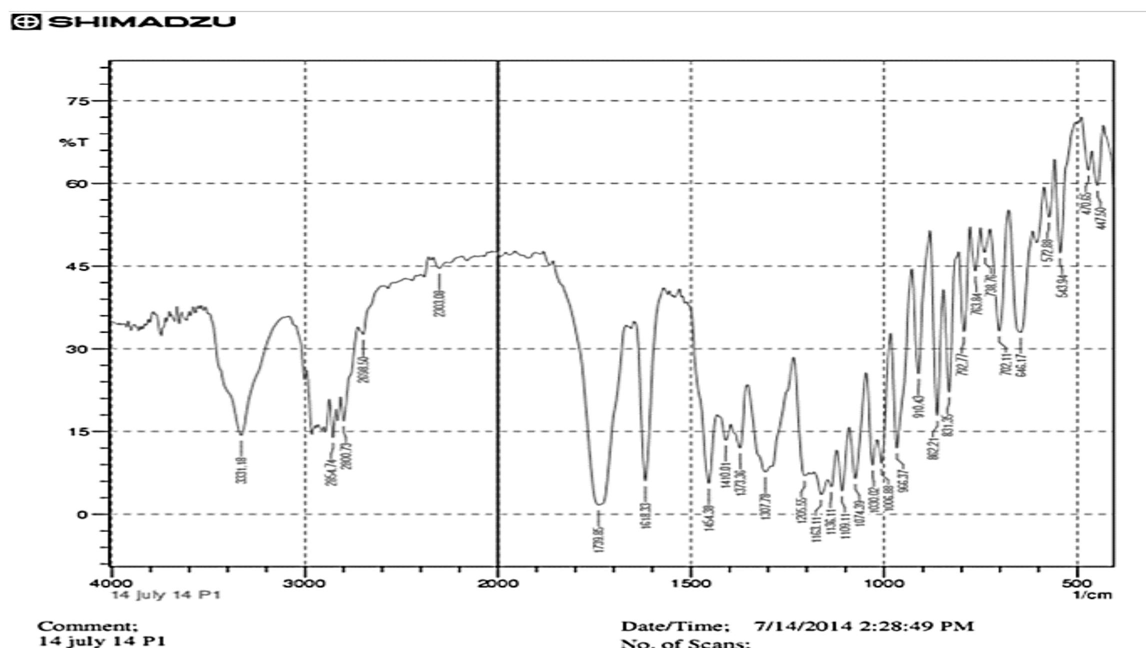


Figure 7.14: FTIR Spectra of the MMF

Table 7.7: FTIR Interpretation data of Mycophenolate

Characteristic peaks	**Reported (cm^{-1})**	**Observed (cm^{-1})**
O–H stretching	3329	3317.31
C-H stretching	2801-2960	2798.84-2891.94
C=O (ester) stretching	1740	1733.48
C=C group	1619-1456	1616.83-1451.30
O-C-C stretching	1076	1070.70
C-C–O (ester) stretching	1205	1158.38
C–O–C (ether) stretching	1076	1070.70

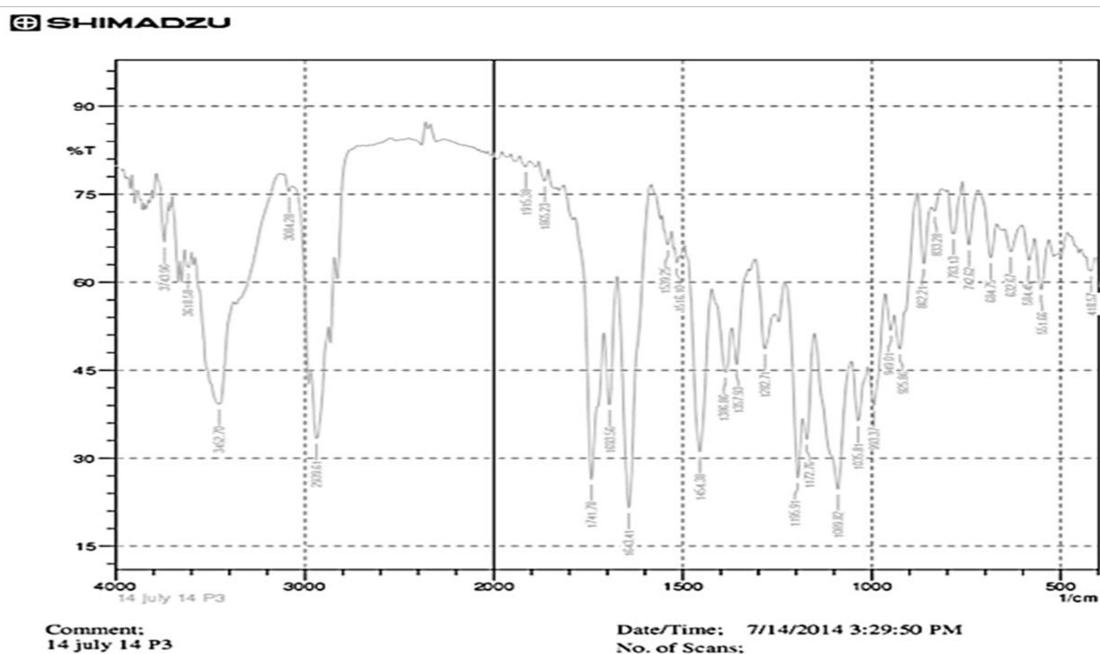


Figure 7.15: FTIR spectrum of Tacrolimus

Table 7.8: FTIR Interpretation data of Tacrolimus

Characteristic peaks	**Reported (cm^{-1})**	**Observed (cm^{-1})**
O–H stretching	3450	3,452.7
C=O (ester) stretching	1733	1,741.78
C=O (ketone) stretching	1690	1,693.56
C=O (keto-amide)	1638	1,643.41
C=C stretching	1638	1,634.76
C–O (ester) stretching	1184	1195.91
C–O–C (ether) stretching	1091	1,089.82

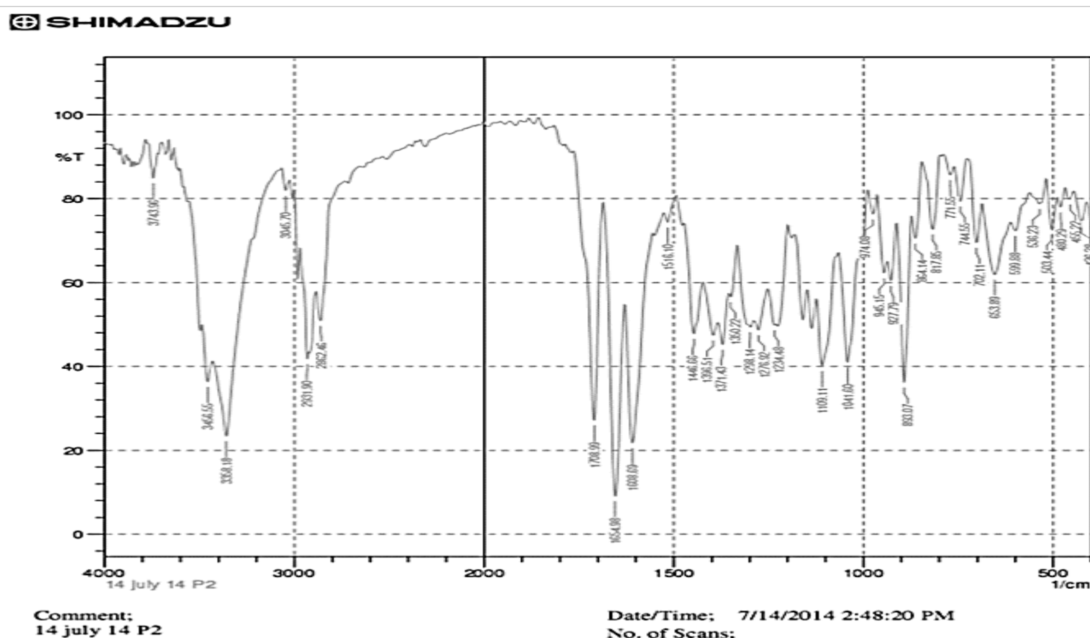


Figure 7.16: FTIR spectrum of Prednisolone

Table 7.9: FTIR Interpretation data of Prednisolone

Characteristic peaks	**Reported (cm^{-1})**	**Observed (cm^{-1})**
C=O stretching	1,654	1708.00, 1654.98
OH bending	1430	1446.66
C–O stretching	1,260	1276.92
C–C(O)	1111	1109.11

Result

Infrared spectroscopy of the MMF, TAC, and PRED was studied (as shown in figure 7.13-7.15) for identification purpose. Peaks were found according to functional groups present in the compounds as reported in the literature.

7.5.3 NMR analysis

^{1}H NMR of MMF

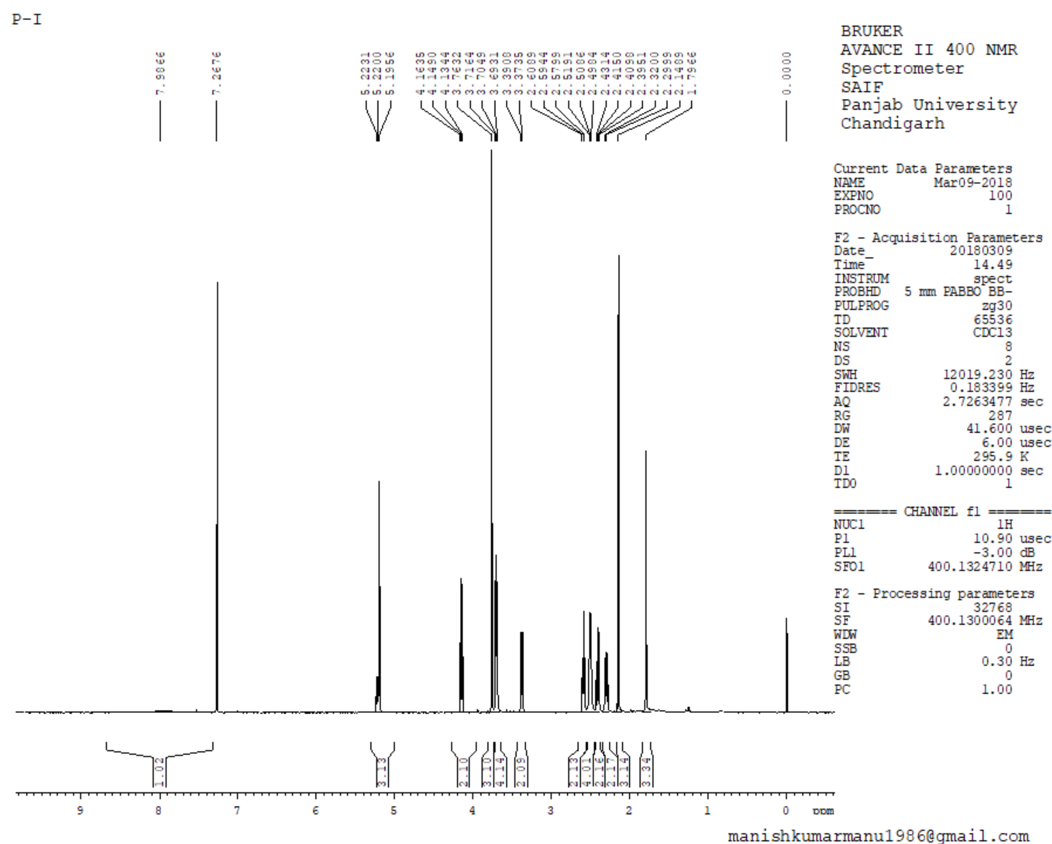


Figure 7.17: Proton NMR of MMF

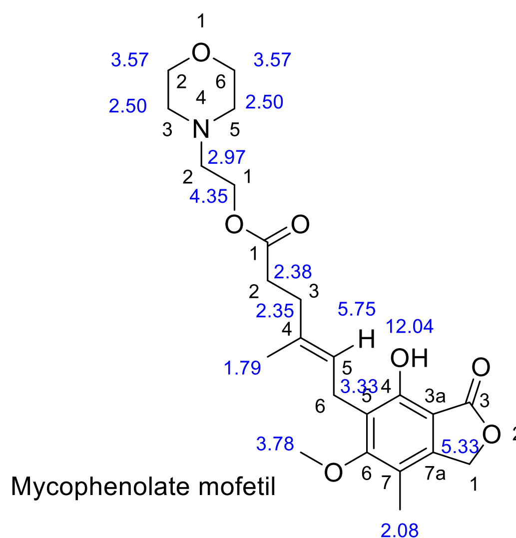


Figure 7.18: MMF structure showing data in ppm

^{1}H-NMR (400MHz) C*D*Cl$_3$: 1.79 (*s*, 3*H*, C*H*$_3$-C=), 2.14 (*s*, 3*H*, C*H*$_3$-Ar), 2.29–2.32 (*m*, 2*H*, C*H*$_2$-C[CH$_3$]=), 2.41–2.44 (*m*, 2*H*, C*H*$_2$CO), 2.60 (*m*, 4*H*, 2C*H*$_2$-N), 2.67 (*m*, 2*H*, C*H*$_2$-N), 3.39 (*d*, *J* = 6.8 Hz, 2*H*, C*H*$_2$-Ar), 3.77-3.77 (*m*, 4*H*,C*H*$_2$O), 3.77 (*s*, 3*H*, OC*H*$_3$), 4.21 (*t*, *J* = 5.0 Hz, 2*H*, C*H*$_2$O), 5.21 (*s*, 2*H*, C*H*$_2$O), 5.22–5.24 (*m*, 1*H*, C*H*=), 7.97 (*br*, *s*, 1*H*, ArO*H*).

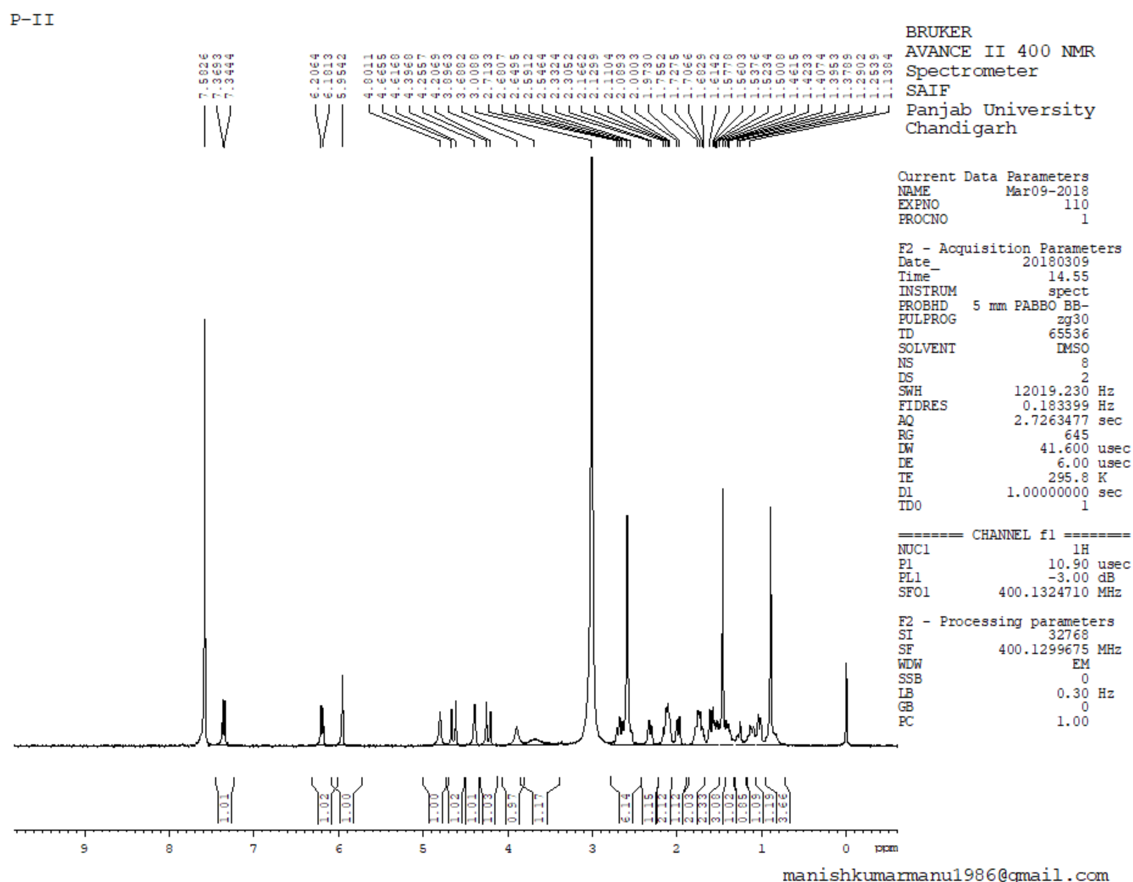


Figure 7.19: Proton NMR of Prednisolone

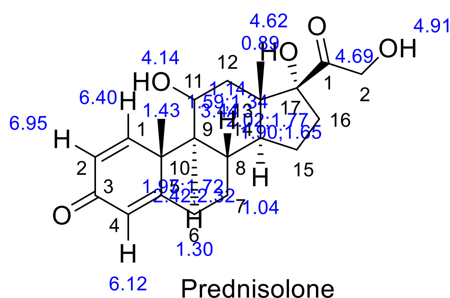


Figure 7.20: Prednisolone structure showing data in ppm

^{1}H NMR (400 MHz, DMSO) 1.06-1.09 (dd J = 11.20 and 2.80Hz, 1H), 1.13–1.24 (m, 5H), 1.45–1.50 (m, 4H), 1.62-1.70 (m, 2H), 1.91-2.09 (m, 3H), 2.19-2.29 (m, 2H), 2.36-2.39 (m, 1H), 2.47–2.54 (m, 1H), 2.54–2.63 (m, 1H), 4.48 (m, 1H), 5.95 (s, 1H), 6.20 (d, J =10.00 Hz, 1H)), 7.36 (d, J = 8.40 Hz, 1H).

The spectra are taken in DMSO; there might be an impurity in the DMSO.

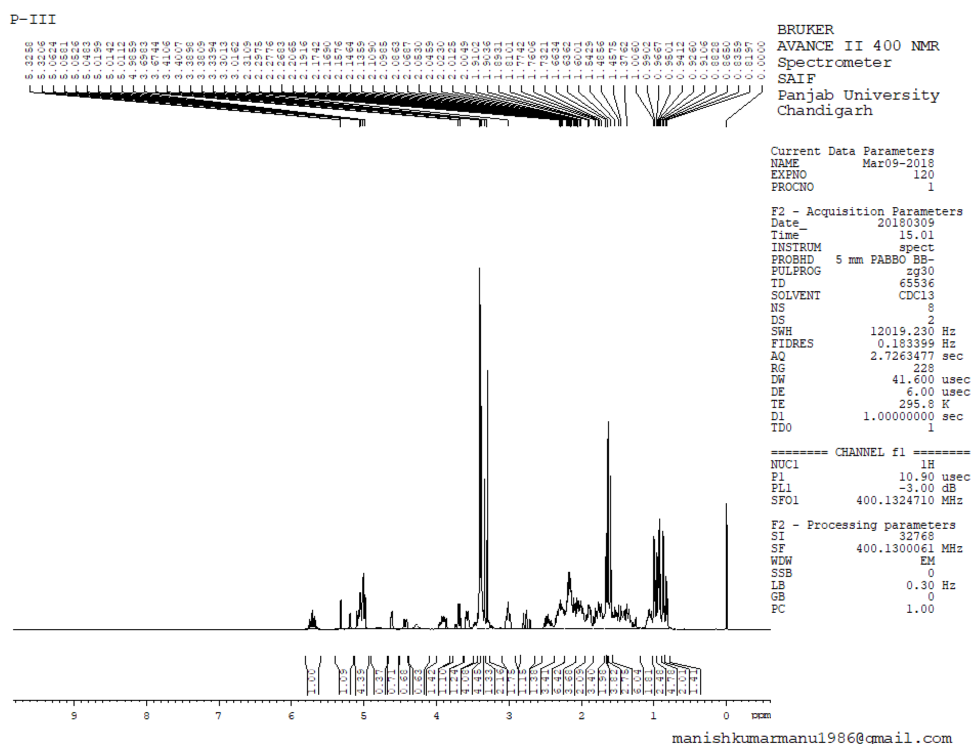


Figure 7.21: Proton NMR of Tacrolimus

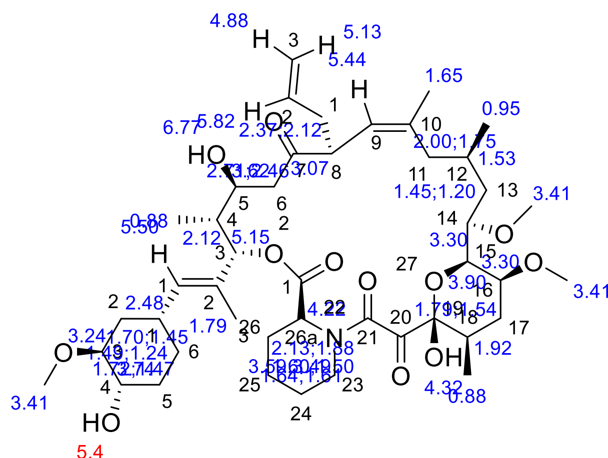


Figure 7.22: Tacrolimus structure showing data in ppm

^{1}H NMR ($CDCl_3$, 400 MHz) 5.76-5.67 (m, 1 H), 5.33 and 5.20 (d, *J* = 2.1, 1 H), 5.10 (br d, J=9.0,1H), 5.05 (br d, J=12.3,1H), 5.01(br d, J=10.1,1H), 4.88 and 4.26 (br s, 1 H), 4.63 (br d, *J* = 5.2, 1 H), 4.44 and 3.72 (m, 1 H), 3.97-3.90 (m, 1 H), 3.89 and 3.70 (m, 1 H), 3.61-3.58 (m, 1 H), 3.49-3.40 (m, 3 H), 3.419, 3.417, 3.399, 3.390, 3.347, and 3.309 (s, total of 9 H), 3.05-3.00 (m, 3 H), 2.81 and 2.74 (dd, *J* = 16.1, 2.8, 1 H), 2.52-2.44 (m, 1 H), 2.38-2.26 (m, 3 H), 2.23-2.14 (m, 3 H), 2.12-1.99 (m, 4 H), 1.94-1.88 (m, 2 H), 1.83-1.72 (m, 4 H), 1.65-1.30 (m, 10 H), 1.67 and 1.65 (br s, 3 H) 1.65 and 1.61 (br s, 3 H), 1.10-1.03 (m, 2 H), 1.01, 0.97, 0.94, 0.93, 0.88, 0.83 (d, *J* = 6.4, 6.6, 6.5, 7.2, 7.1, 6.5, total of 9 H).

Interpretation: Because it is a macrolide, all the signals broadened to a similar extent, we could not get all the values from the ^{1}H-NMR spectra.

Result

NMR spectroscopy of the MMF, TAC, and PRED was studied (as shown in figs 8.15-8.20) for identification purpose. Peaks were found according to the chemical structure of the compounds.

7.6 Prototype Formulation Development

A capsule formulation should possess the following properties to optimize the technical feasibility, stability, and bioavailability of the formulation

- Compatibility of the drug substance with excipients
- Flowability
- Compactability
- Lubricity
- Appearance
- Disintegration
- Dissolution

7.7 Compatibility studies of the MMF with various excipients

7.7.1 Physical Compatibility Study

Table 7.10: Compatibility study of the MMF with different excipients

Sr. No.	Parameter	Ratio	Initial	Ctrl sample (30 days)	40 °C/ 75 % RH – Open (30 days)	40 °C/ 75 % RH – Closed (30 days)	60°C Open (30 days)	60°C Closed (30 days)
1.	MMF: Lactose monohydrate	(1:10)	White powder	√	√	√	√	√
2.	MMF: PVP	(1:5)	White powder	√	L	√	√	√
3.	MMF: Pregelatinized Starch	(1:5)	White powder	√	√	√	√	√
4.	MMF: Croscarmellose sodium	(1:5)	White powder	√	√	√	√	√
5.	MMF: Sodium Starch Glycolate	(1:5)	White powder	√	√	√	√	√
6.	MMF: MCC PH 200	(1:10)	White powder	√	√	√	√	√
7.	MMF: Magnesium Stearate	(1:1)	White powder	√	√	√	√	√
8.	MMF	Ctrl	White powder	√	√	√	√	√
9..	Lactose monohydrate	Ctrl	White powder	√	L	√	√	√
10.	PVP	Ctrl	White powder	√	√	√	√	√
11.	MCC PH 200	Ctrl	White powder	√	√	√	√	√

12.	Pregelatinized Starch	Ctrl	White powder	√	√	√	√	√
13.	Croscarmellose sodium	Ctrl	White powder	√	√	√	√	√
14.	Sodium Starch Glycollate	Ctrl	White powder	√	√	√	√	√
15.	Magnesium Stearate	Ctrl	White powder	√	√	√	√	√

Ctrl= Control, L= LUMP FORMATION, √ = OK

Table 7.11: Compatibility study of the TAC with different excipients

Sr. No.	Parameter	Ratio	Initial	Ctrl sample (30 days)	40 °C/ 75 % RH – Open (30 days)	40 °C/ 75 % RH – Closed (30 days)	60°C Open (30 days)	60°C Closed (30 days)
1.	TAC: Lactose monohydrate	(1:10)	White powder	√	√	√	√	√
2.	TAC: PVP	(1:5)	White powder	√	L	√	√	√
3.	TAC: Pregelatinized Starch	(1:5)	White powder	√	√	√	√	√
4.	TAC: Croscarmellose sodium	(1:5)	White powder	√	√	√	√	√
5.	TAC: Sodium Starch Glycolate	(1:5)	White powder	√	√	√	√	√
6.	TAC: MCC PH 200	(1:10)	White powder	√	√	√	√	√
7.	TAC: Magnesium Stearate	(1:1)	White powder	√	√	√	√	√
8.	TAC	Ctrl	White powder	√	√	√	√	√
9..	Lactose monohydrate	Ctrl	White powder	√	L	√	√	√
10.	PVP	Ctrl	White powder	√	√	√	√	√
11.	MCC PH 200	Ctrl	White powder	√	√	√	√	√

12.	Pregelatinized Starch	Ctrl	White powder	√	√	√	√	√
13.	Croscarmellose sodium	Ctrl	White powder	√	√	√	√	√
14.	Sodium Starch Glycollate	Ctrl	White powder	√	√	√	√	√
15.	Magnesium Stearate	Ctrl	White powder	√	√	√	√	√

Ctrl= Control, L= LUMP FORMATION, √ = OK

Table 7.12: Compatibility study of the PRED with different excipients

Sr. No.	Parameter	Ratio	Initial	Ctrl sample (30 days)	40 °C/ 75 % RH – Open (30 days)	40 °C/ 75 % RH – Closed (30 days)	60°C Open (30 days)	60°C Closed (30 days)
1.	PRED: Lactose monohydrate	(1:10)	White powder	√	√	√	√	√
2.	PRED: PVP	(1:5)	White powder	√	L	√	√	√
3.	PRED: Pregelatinized Starch	(1:5)	White powder	√	√	√	√	√
4.	PRED: Croscarmellose sodium	(1:5)	White powder	√	√	√	√	√
5.	PRED: Sodium Starch Glycolate	(1:5)	White powder	√	√	√	√	√
6.	PRED: MCC PH 200	(1:10)	White powder	√	√	√	√	√
7.	PRED: Magnesium Stearate	(1:1)	White powder	√	√	√	√	√
8.	PRED	Ctrl	White powder	√	√	√	√	√
9..	Lactose monohydrate	Ctrl	White powder	√	L	√	√	√
10.	PVP	Ctrl	White powder	√	√	√	√	√
11.	MCC PH 200	Ctrl	White powder	√	√	√	√	√

12.	Pregelatinized Starch	Ctrl	White powder	√	√	√	√	√
13.	Croscarmellose sodium	Ctrl	White powder	√	√	√	√	√
14.	Sodium Starch Glycollate	Ctrl	White powder	√	√	√	√	√
15.	Magnesium Stearate	Ctrl	White powder	√	√	√	√	√

Ctrl= Control, L= LUMP FORMATION, √ = OK

7.8 Chemical Compatibility Study

7.8.1 FTIR of different excipients and compatibility study

FTIR spectrums of drug and mixture of drug formulation were obtained using an FTIR spectrophotometer. The samples were prepared by the potassium bromide disk method and measurements were attempted with the accumulation of 20 scans and a resolution of 4 cm^{-1} over the range of 400–4000cm^{-1}. After running the spectra, significant peaks relating to major functional groups were identified; spectra of the subsequent sample of the same compound were compared with the original.

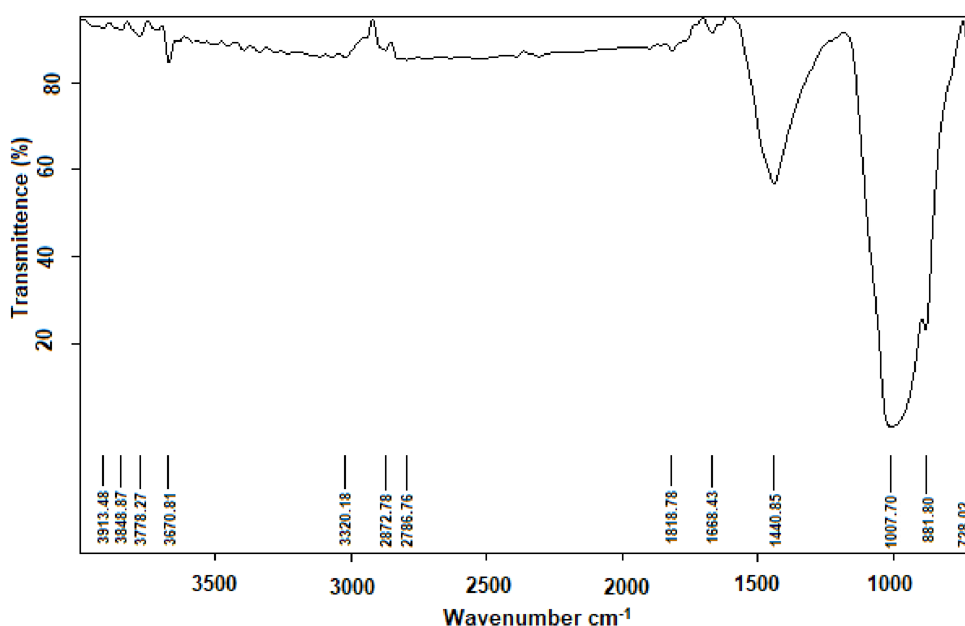


Figure 7.23: FTIR spectrum of Talc

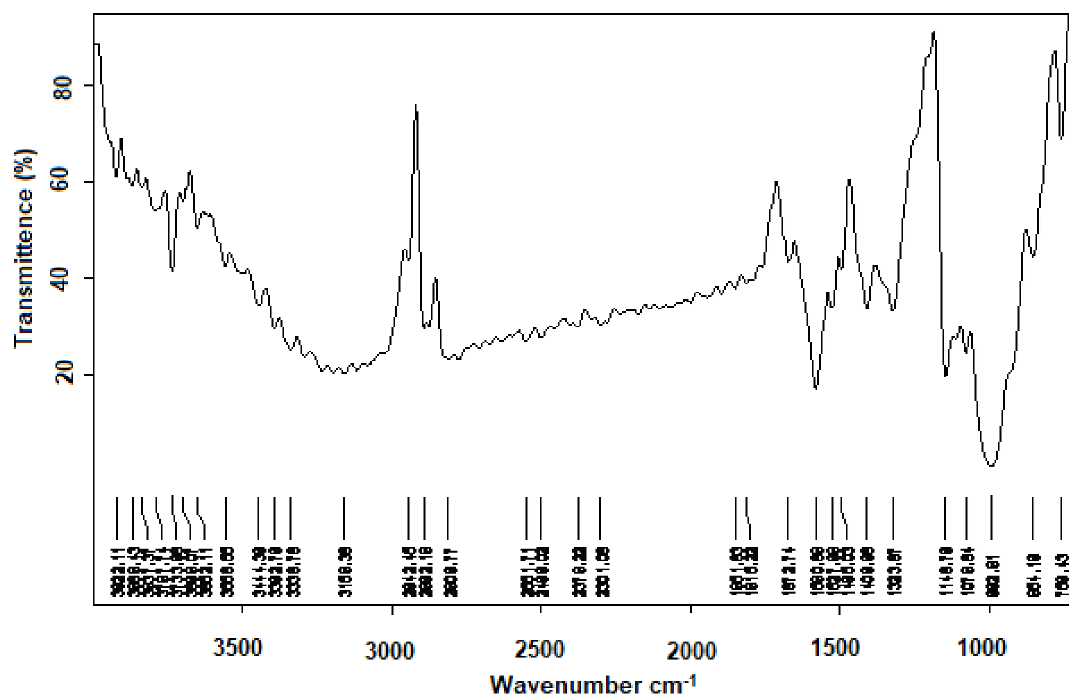


Figure 7.24: FTIR spectrum of Sodium Starch glycollate

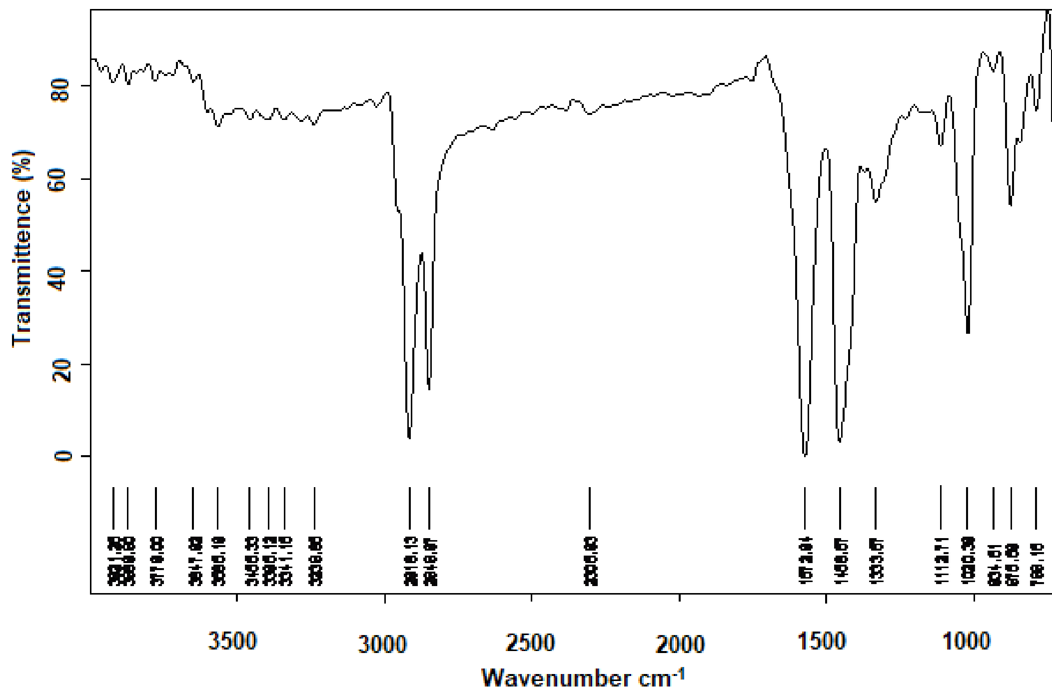


Figure 7.25: FTIR spectrum of Magnesium stearate

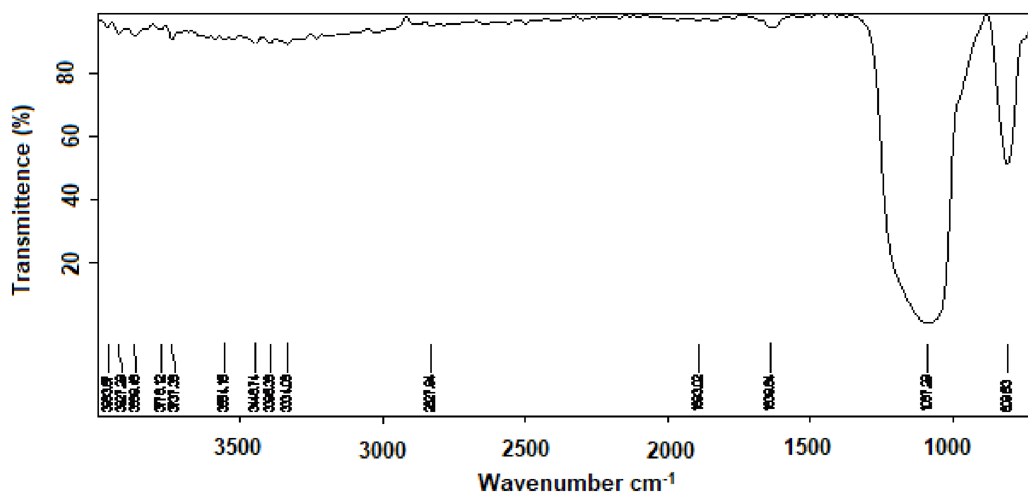


Figure 7.26: FTIR spectrum of Aerosil

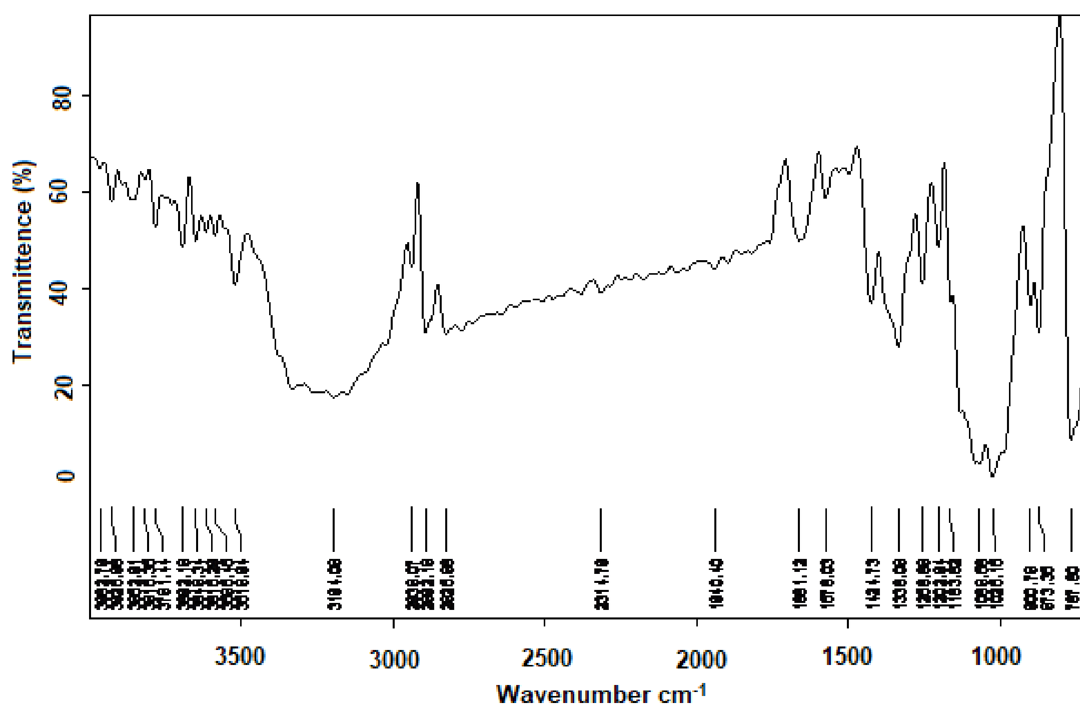


Figure 7.27: FTIR spectrum of lactose

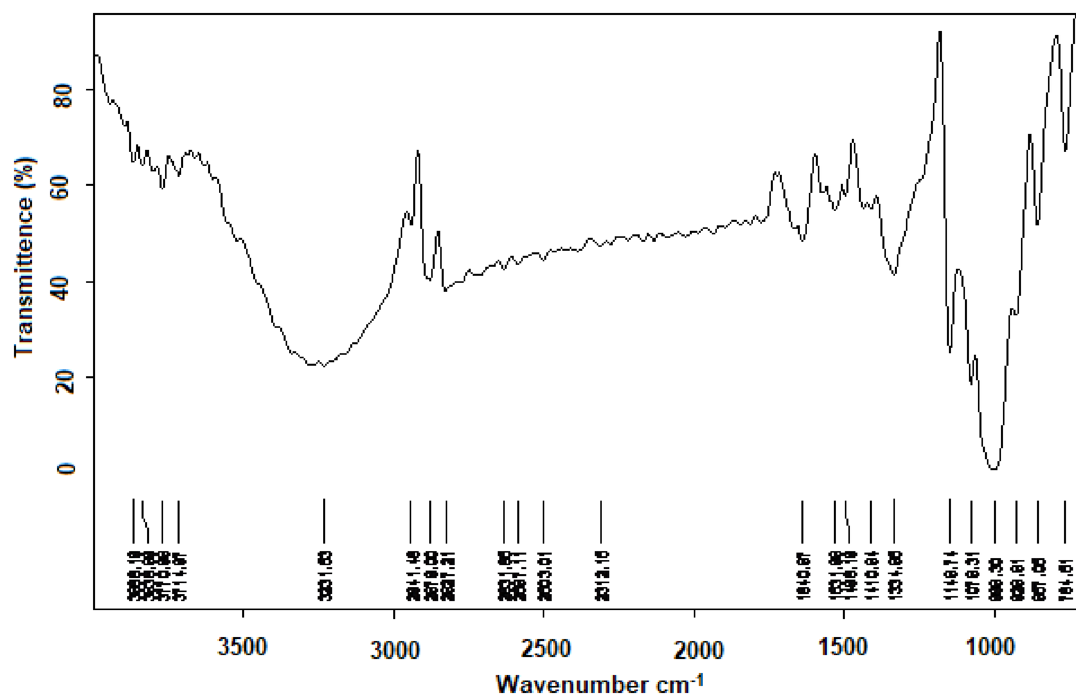


Figure 7.28: FTIR spectrum of Starch

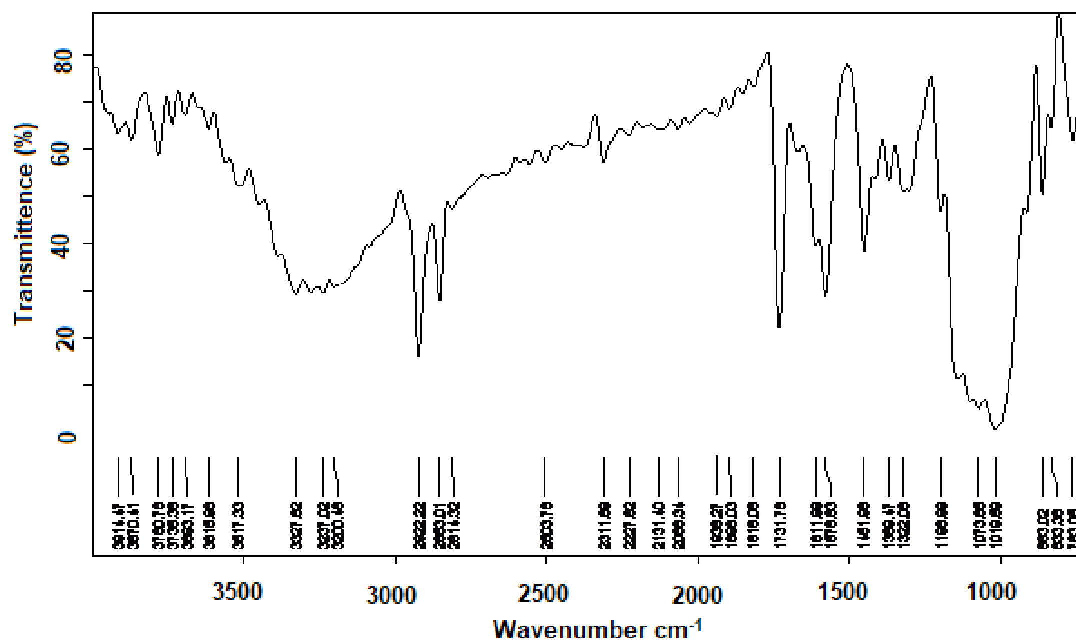


Figure 7.29: FTIR spectrum of physical mixture of excipient

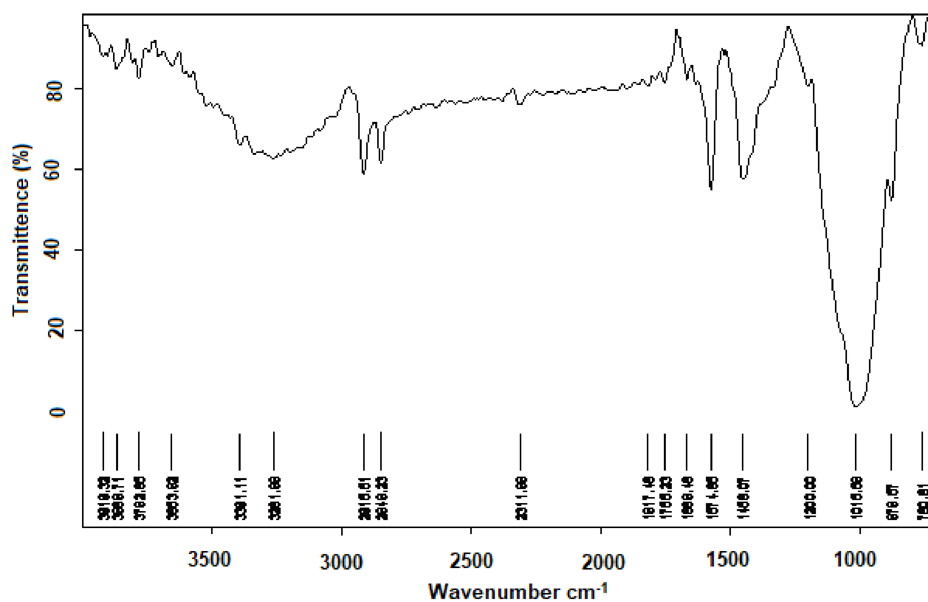


Figure 7.30: FTIR spectrum of a physical mixture of excipient and Drugs

From the above Figure 8.21, it is revealed that corresponding peaks of drugs are present in the above spectra along with excipients peaks. Hence no interaction was observed in this mixture.

Result

No physical change in the appearance of the drug-excipients mix was noticed as there is no change in the major peaks was obsearved. Therefore, these excipients will be used in a capsule formulation. However, DSC is not the ultimate tool for confirmation of compatibility, and it should be established by solid-state stability studies.

Characteristic peaks	Reported (cm^{-1})	Observed (cm^{-1})
O–H stretching	332	3321.11
C-H stretching	2801-2960	2915.51-2848.23
C=O stretching ester	1740	1766.23
C=O stretching ketone	1690	1668.48
O-H bending	1430	1468.07

7.9 Preparation and formulation of capsules

Table 7.13: Composition of different trial of capsules

MMF: 100mg, PRED: 5mg and TAC: 0.5mg

F-code	Aerosil 200 (mg)	Crosscaramllose (mg)	Mg Stearate (mg)	MCC (mg)	PVP K-30 (mg)	Lactose	SSG	Talc	HPMC K 100	SSC	Maize starch	method
C1	10	15	2	200	10	-	15	5	-	-		Direct
C2	50	50	5	150	50	-	70	10	-	-		Direct
C3	-	10	5	200	3	-	-	1.5	-	1.5		Direct
C4	-	20	2	-	50	126	100	5	10	-		Direct
C5	-	20	2	-	50	126	100	5	10	-		Wet
C6	-	20	2	-	-	126	100	5	10	-		Direct
C7	-	20	2	-	-	126	100	5	10	-		Wet
C8	-	20	2	-	-	126	100	5	10	-		Direct
C9	-	20	2	-	-	126	100	5	10	-		Wet

C10	-	20	2	-	2.5	126	100	5	10	-		Direct
C11	-	-	7	50	-	85	-	8	-	-	150	Direct
C12	-	20	2	-	-	126	70	5	10	-	-	Direct
C13	-	20	2	-	10	120	70	5	10	-	125	Direct
C14	10	-	2	-	-	120	70	5	10	-	125	Wet

7.10 Evaluation of powder and granules of all formulation (C1-C14)

The capsules were prepared according to the master formula. Granulation is the key process in the production of many dosage forms involving the controlled release of a drug from coated or matrix type particles.

A granule is a total of segment particles that are held together by the presence of bonds of finite strength. Physical properties of granules, for example, particular surface, shape, size, hardness, surface attributes and so on can essentially influence the rate of disintegration/ dissolution of drugs contained in heterogeneous dose forms. The granules of various formulations were assessed for the angle of repose, bulk density, compressibility index, and drug contents.

Table 7.14: Different properties of powder/ granules of all formulations

F-Code	Angle of Repose	Bulk Density (g/ml)	Tapped Density (g/ml)	Compressibility Index	Hausner's Ratio	Drug Content
C1	25.00 ± 0.03	0.434	0.576	24.65	1.33	99 ± 0.05
C2	27.52 ± 0.03	0.436	0.580	24.83	1.33	100.5 ± 0.03
C3	23.87 ± 0.09	0.452	0.591	23.52	1.31	98.54 ± 0.02
C4	28.47 ± 0.02	0.432	0.582	25.77	1.35	99.45 ± 0.05
C5	24.93 ± 0.03	0.434	0.576	24.65	1.33	102 ± 0.01
C6	25.29 ± 0.06	0.438	0.582	24.74	1.33	100 ± 0.05
C7	27.52 ± 0.03	0.432	0.582	25.77	1.35	99.61 ± 0.03
C8	23.87 ± 0.09	0.454	0.582	21.99	1.28	101.02 ± 0.03
C9	32.47 ± 0.02	0.423	0.621	31.88	1.47	96.21 ± 0.02
C10	24.93 ± 0.03	0.465	0.591	21.32	1.27	98.03 ± 0.03
C11	24.29 ± 0.06	0.438	0.582	24.74	1.33	101 ± 0.04
C12	26.52 ± 0.03	0.432	0.582	25.77	1.35	99 ± 0.02
C13	25.93 ± 0.03	0.434	0.576	24.65	1.33	98.10 ± 0.06
C14	25.87 ± 0.09	0.434	0.574	24.39	1.32	100± 0.05

All values are expressed as Mean ± SE, n=5

7.10.1 Particle Size Distribution by Sieve [for optimized formulation]

Table 7.15: Particle size of the optimized formulation by sieve

Sr. No.	Sieve No.	Wt. of Sieve		Sample Retained (g)	% Retained	Cumulative Retained (%)
		Initial	Final			
1	# 40	329.86	332.22	2.36	7.87	7.87
2	# 80	346.52	355.25	8.73	29.10	36.97
3	# 120	338.15	343.33	5.18	17.27	54.23
4	Pan	504.34	517.9	13.56	45.20	99.43

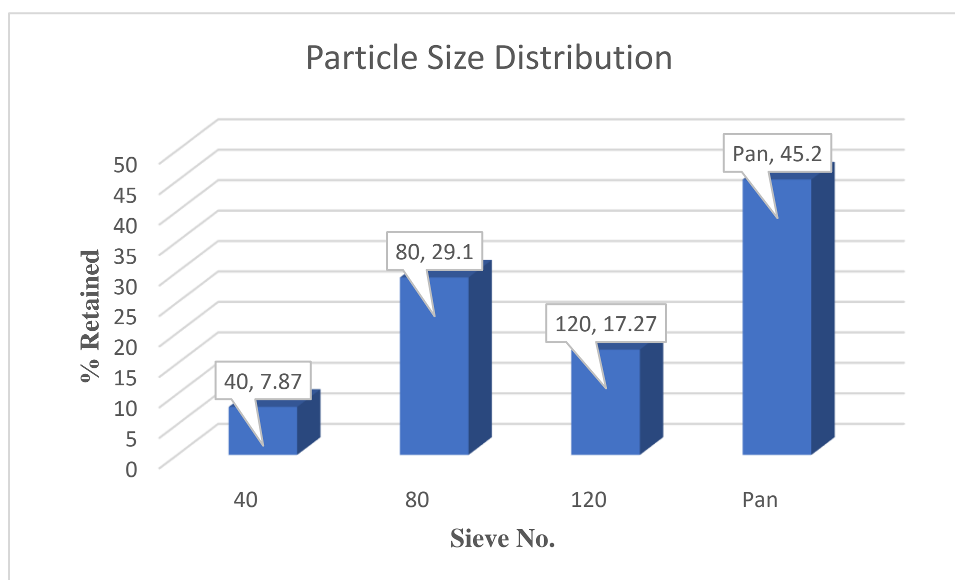


Figure 7.31: PSD of the lubricated blend

Result

For uniform filling of the dies during filling, about 50% fines should be present in the blend, and the other 50% is constituted by granules to enhance the flow. The blend has 45.20 % fines and 54.24 % of the granular part, which accounts for the uniform weight of the capsules and good flow properties of the blend.

7.11 Evaluation of Capsules (all formulation)

The capsules of different formulations were subjected to the various evaluation tests like uniformity of weight, drug content.

7.11.1 Uniformity of weight

The average percentage deviation of ten capsules of each formula is determined. As per IP limit, the percentage deviation for capsules of more than or equal to 300 mg is ±7.5%. The percentage deviation of all capsule formulations was found within the above limit, and hence all formulations pass the test for uniformity of weight as per official requirement (IP 1996).

7.11.2 Drug content of capsules

The drug content determinations were made in triplicate for all formulations and the results given in Table 8.17. Good uniformity in drug content was found among different batches of the capsules, and the percentage of drug content was ranged from 96.21±0.02 to 102.12±0.8.

The C14 further is selected for optimization as it shows good flow properties as compared to C1-C13 formulations.

Table 7.16: Properties of capsules with different excipients and concentrations

F-Code	Weight (mg)	Drug content* (%)	F-Code	Weight (mg)	Drug content* (%)
C1	362.5±0.03	99±0.05	C8	368.5±0.03	101.02±0.03
C2	490.5±0.02	100.5±0.03	C9	368.5±0.02	96.21±0.02
C3	326.5±0.03	98.54±0.02	C10	371±0.05	98.03±0.03
C4	418.5±0.02	99.45±0.05	C11	405.5±0.03	101±0.04
C5	418.5±0.02	102±0.01	C12	338.5±0.02	99±0.02
C6	368.5±0.03	100±0.05	C13	467.5±0.02	98.10±0.06
C7	368.5±0.03	99.61±0.03	C14	447.5±0.03	100±0.05

* All values are expressed as Mean ± SE, n=3

Table 7.17: Properties of selected formulation

F-Code	Weight (mg)	Drug content* (%)
C14(44)	446.50±0.03	101.02±0.03
C14(44)	446.75±0.02	96.21±0.23
C14(44)	447.3±0.05	98.03±0.05
C14(44)	447.5±0.02	99.00±0.03
C14(44)	447.75±0.02	101.00±0.03
C14(44)	447.10±0.03	99.00±0.05
C14(44)	446.50±0.05	98.10±0.03
C14(44)	447.50±0.02	100.00±0.03
C14(44)	448.10±0.03	100.50±0.03
C14(44)	447.50±0.02	100±0.05

* All values are expressed as Mean ± SE, n=3

Table 7.18: Evaluation of capsules

Parameter	**Weight uniformity**	**Drug content**
Mean	447.25	99.29
S.D.	0.53	1.53
% RSD	0.12	1.54
Min	446.53	96.44
Max	448.13	101.05

7.12 Prototype Formula Development

7.12.1 Suitable Filler Selection

The drug release of capsules prepared by using Lactose Monohydrate was much higher as compared to the reference product, whereas capsules prepared by Microcrystalline Cellulose showed comparable drug release to the reference product.

7.12.2 Suitable Binder Selection

The commonly used granulating agents are listed in the table 8.18.

Table 7.19: Physical characteristics of batches prepared with a different binder

Parameters	**Hypromellose**	**PVP K30**
B.No.	C14 (24)	C14 (25)
Dissolution condition		
Media/Volume	0.1 N HCl (pH 1.2), 900 ml	
Apparatus	USP # II	
RPM	50	
	% Drug release	
Time (Min)	C14 (24)	C14 (25)
0	0	0
5	93	67
10	97	88
15	99	87
30	100	93
45	100	93

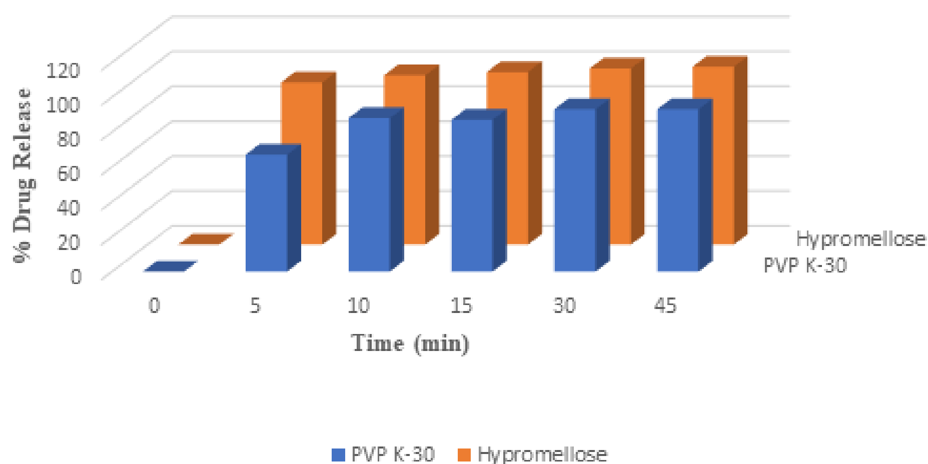


Figure 7.32: Dissolution profile of In-house capsules using a different binder

Result

The incorporation of PVP K30 slowed down the release rate. Hence, HPMC was selected as the binder.

7.12.3 Suitable Disintegrant Selection

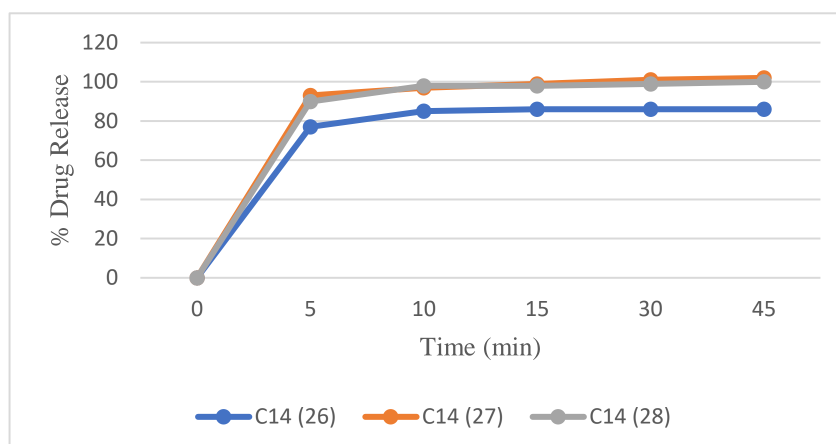


Figure 7.33: Dissolution profile of In-house capsules using a different disintegrating agent

Result

The drug release of capsules prepared with Starch and Sodium Starch Glycolate was much higher as compared to the reference product, whereas capsules prepared by Croscarmellose showed comparable drug release to the reference product.

7.12.4 Suitable lubricant selection

Three different batches were prepared using 1 % concentration of each of Magnesium Stearate, Aerosil, and Stearic Acid.

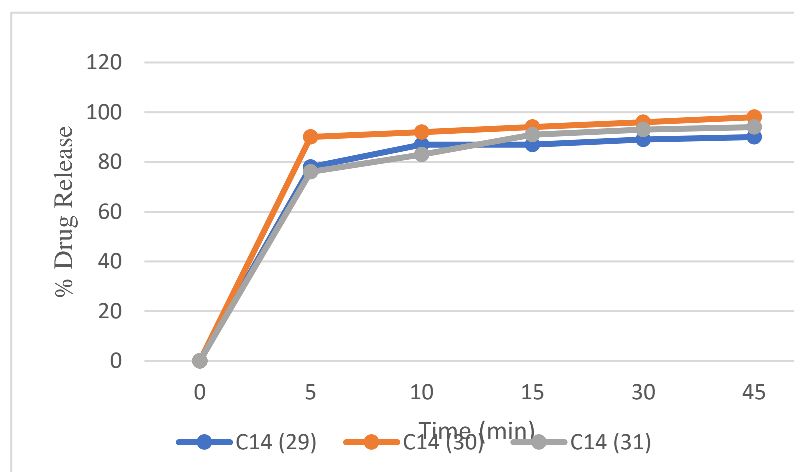


Figure 7.34: Dissolution profile of In-house capsules using a different lubricating agent

Magnesium Stearate and Aerosil was selected, being the most commonly used and efficient lubricant properties. Other lubricants evaluated were ruled out based on poor lubrication properties and higher concentrations required in comparison to both.

7.13 Formula Optimization Studies

7.13.1 Effect of Process

The following formula was chosen to perform the above processes.

Table 7.20: Proposed ingredients for the capsule formulation

Ingredients	Function
PRED, MMF, TAC	Active
SSG	Super Disintegrant
HPMC K 100	Binder
Lactose	Filler
Magnesium Stearate	Lubricant
Aerosil	Lubricant

Table 7.21: Observation of different capsule formulation processes

Process	Batch No.	Bulk Density g/cm^3	Tap Density g/cm^3	Carr's Compressibility Index (CI)	Hausner ratio	Flow
Direct Filling	C14 (32)	0.38	0.60	36.67	1.58	Extremely poor flow
Dry Granulation	C14 (33)	0.45	0.63	28.57	1.40	Poor flow
Wet Granulation	C14 (34)	0.43	0.58	24.65	1.32	Good flow

Result

Based on the above data the blend obtained with direct filling was found to have extremely poor flow (C.I > 35), hence it cannot be filled directly into capsules. The blend obtained from dry granulation was found to have poor flow (C.I. lies between 25-35), hence it cannot be filled into capsules, and the blend obtained from wet granulation was found to have good flow (C.I. is lying between 15-25).

7.13.2 Binder Concentration Optimization

HPMC is used as a capsule binder in the concentration range of 2-5%. For developing the formulation, the lower concentrations generally used were first evaluated.

1. 2% w/w
2. 3% w/w
3. 4% w/w

Table 7.22: Physical characteristics of batches prepared with different concentrations of HPMC K-100

Ingredients/ B.No.	**C14 (35)**	**C14 (37)**	**C14 (36)**
HPMC K-100	2 %	3 %	4 %
Dissolution condition			
Media/Volume	0.1 N HCl (pH 1.2), 900 ml		
Apparatus	USP # II		
RPM	50		
%-Drug release			
Time (min)/ B. No.	**C14 (35)**	**C14 (37)**	**C14 (36)**
0	0	0	0
5	84	93	89
10	92	97	100
15	88	99	102
30	93	101	100
45	91	102	100

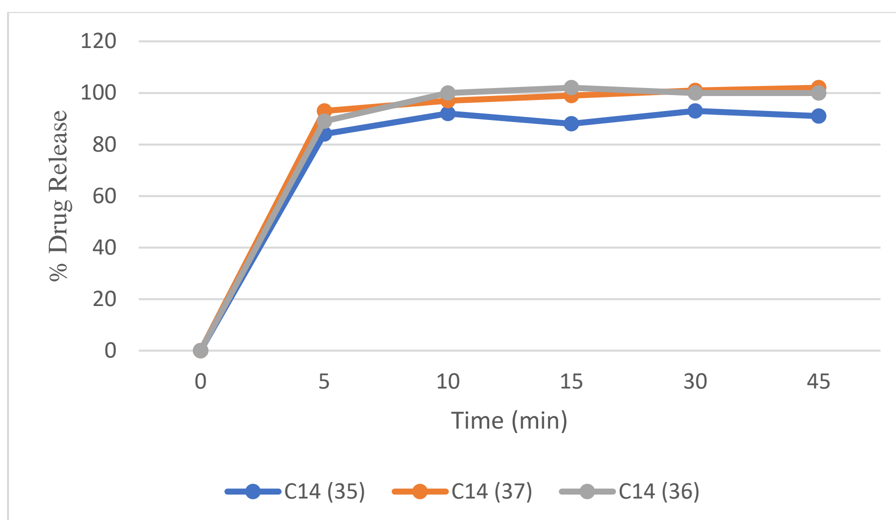


Figure 7.35: Dissolution profile of formulations using different concentrations of HPMC K-100

Result

The tests like dissolution were used to optimize the binder concentration. The percentage drug release profile of different formulations using 2%, 3% and 4% concentration of HPMC K-100 was found comparable to the reference product. It was concluded that the concentration of binder in the range of 2-4% has no considerable effect on the release profile. The batch with 3% HPMC was closer to the reference profile. Hence this concentration was chosen.

7.13.3 Disintegrant Concentration Optimization

SSG is generally used in the concentration range of 1 – 8 % in the formulation of a capsule dosage form. Therefore, batches were prepared and in concentrations ranging from 2% - 8%.

Table 7.23: Physical characteristics of batches prepared with different concentrations of SSG

Ingredients/ B. No.	**C14 (38)**	**C14 (39)**	**C14 (40)**
SSG	2 %	5 %	8 %
Dissolution condition			
Media/Volume	0.1 N HCl (pH 1.2), 900 ml		
Apparatus	USP # II		
RPM	50		
%-Drug release			
Time (min) B. No.	**C14 (38)**	**C14 (39)**	**C14 (40)**
0	0	0	0
5	77	93	90
10	85	97	98
15	86	99	98
30	86	101	99
45	86	102	100

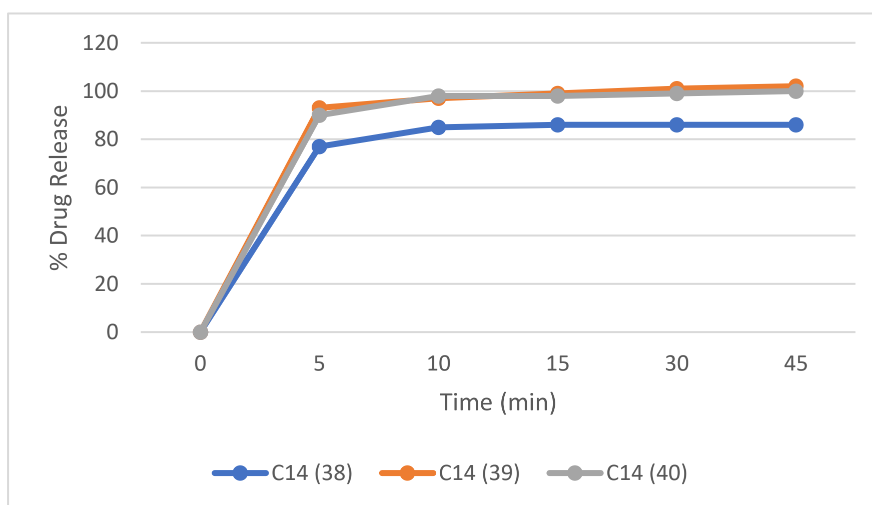


Figure 7.36: Dissolution profile of formulations using different concentrations of SSG

Result

The tests dissolution was used to optimize the disintegrants concentration. The release profile from the formulation with 2 % SSG concentration was considerably slower than the reference product, whereas the release profile obtained in the formulations with 5% and 8% SSG concentration showed comparable dissolution profile to the reference product.

7.13.4Lubricant Concentration Optimization

Table 7.24: Physical characteristics of batches prepared with different concentrations of Magnesium stearate and Aerosil

Ingredients/ B. No.	**C14 (41)**	**C14 (42)**	**C14 (43)**	**C14 (44)**
Magnesium Stearate	0.5 %	1.0 %	2.0 %	0.6%
Aerosil	-	-	-	2.0 %
Dissolution condition				
Media/Volume	0.1 N HCl (pH 1.2), 900 ml			
Apparatus	USP # II			
RPM	50			
%-Drug release				
Time (min) /B. No.	**C14 (41)**	**C14 (42)**	**C14 (43)**	**C14 (44)**
0	0	0	0	0
5	78	90	76	93
10	87	92	83	97
15	87	94	91	99
30	89	96	93	101
45	90	98	94	102

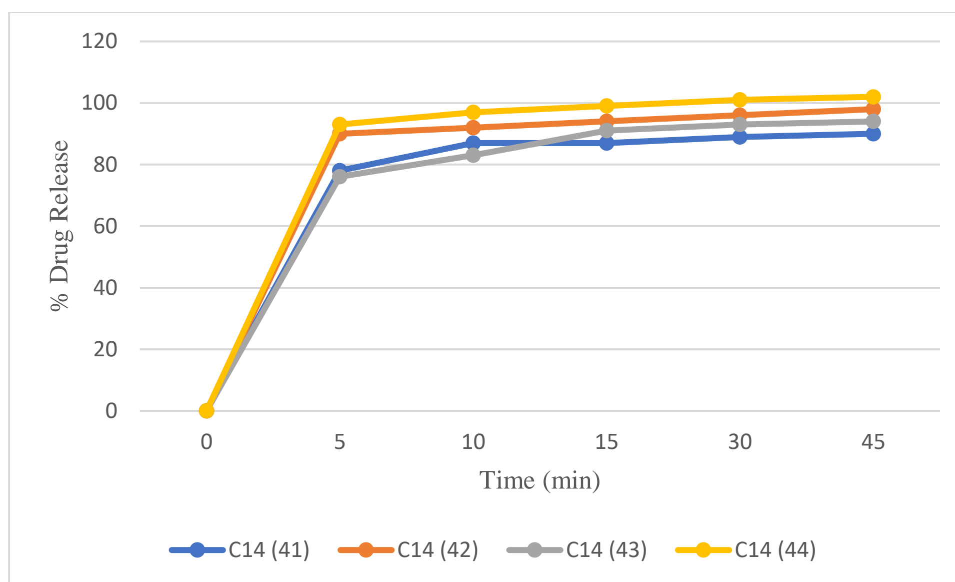


Figure 7.37: Dissolution profile of formulations using different concentrations of Magnesium Stearate and combined with Aerosil

Result

The batches prepared by using 0.5% and 2.0% concentration of lubricant demonstrated slow drug release as compared to the reference product, whereas the batch prepared with 0.6% Magnesium Stearate and 2.0 % Aerosil showed comparable release rate to the reference. Magnesium stearate being hydrophobic, form a coating over the granules and retard their dissolution. Therefore 2% concentration showed poor release whereas with 0.5% concentration sticking was observed on the upper and lower punches which resulted in the loss of drug and slowing of release rate. Therefore, the combination shows good results.

7.14 Drug release profile of the optimized formulation

Result

The pH of the gastric tract is variable. For a formulation to be bioequivalent to the reference product, it must dissolve and get absorbed at the same rate and from the same location as the GI tract. For this to happen effectively, the product should have a similar rate of dissolution in different pH media and at different paddle RPM of the in vitro dissolution tester. The F2 values ranging from 83 to 99 were obtained in all the media

tested. As the F2 values lie above 50 in all the Media tested it may be concluded that the optimized formulation is similar.

Table 7.25: Dissolution profile of C14 Capsules Formulation in 0.1N HCl at 25 RPM

Vessel No.	% Drug Dissolved in								
	5 min	10 min	15 min	20 min	25 min	30 min	35 min	40 min	45 min
1	46.0	68.0	80.0	89.0	92.0	95.0	96.0	97.0	98.0
2	46.0	69.0	81.0	90.0	91.0	94.0	95.0	96.0	97.0
3	46.0	70.0	82.0	89.0	92.0	97.0	97.0	98.0	99.0
4	45.0	69.0	81.0	90.0	93.0	97.0	97.0	98.0	99.0
5	45.0	69.0	82.0	90.0	92.0	94.0	96.0	97.0	98.0
6	47.0	66.0	82.0	89.0	92.0	93.0	95.0	96.0	97.0
Mean	**45.8**	**68.5**	**81.3**	**89.5**	**92.0**	**95.0**	**96.0**	**97.0**	**98.0**
SD	0.8	1.4	0.8	0.5	0.6	1.7	0.9	0.9	0.9
% RSD	1.6	2.0	1.0	0.6	0.7	1.8	0.9	0.9	0.9
% SEM	0.3	0.6	0.3	0.2	0.3	0.7	0.4	0.4	0.4

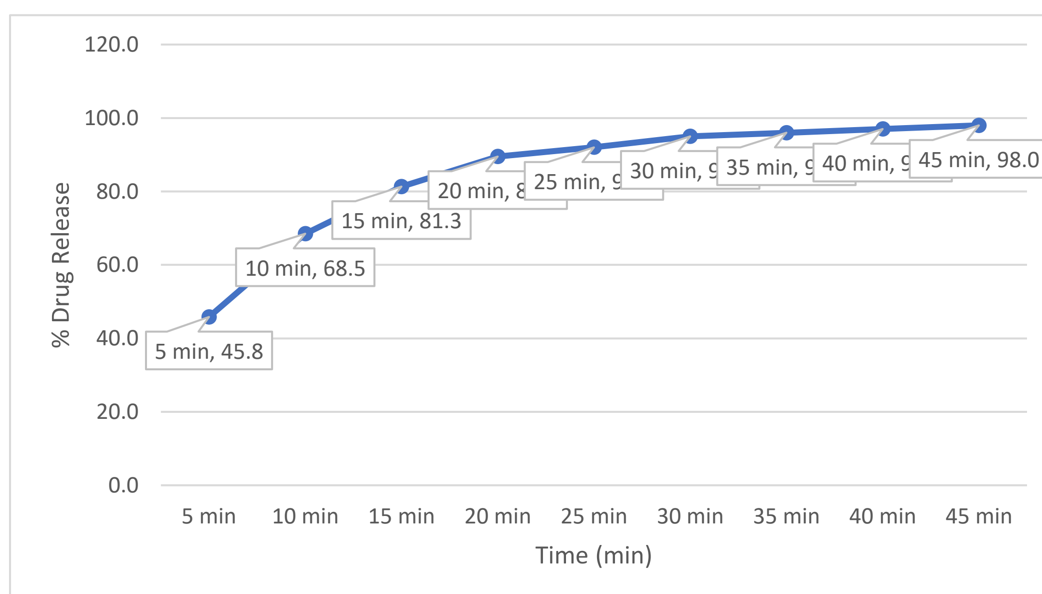


Figure 7.38: Dissolution profile of C14 Capsules Formulation in 0.1N HCl at 25 RPM

Table 7.26: Dissolution profile of C14 Capsules Formulation in 0.1N HCl at 50 RPM

Vessel No.	% Drug Dissolved in								
	5 min	**10 min**	**15 min**	**20 min**	**25 min**	**30 min**	**35 min**	**40 min**	**45 min**
1.0	45.0	68.0	80.0	92.0	95.0	96.0	99.0	100.0	100.0
2.0	46.0	69.0	81.0	91.0	94.0	95.0	98.0	99.0	100.0
3.0	46.0	66.0	82.0	92.0	94.0	97.0	100.0	100.0	100.0
4.0	45.0	66.0	81.0	93.0	94.0	97.0	100.0	100.0	100.0
5.0	45.0	68.0	82.0	92.0	94.0	96.0	99.0	100.0	100.0
6.0	44.0	66.0	82.0	92.0	93.0	95.0	98.0	99.0	100.0
Mean	**45.2**	**67.2**	**81.3**	**92.0**	**94.0**	**96.0**	**99.0**	**99.7**	**100.0**
SD	**0.8**	**1.3**	**0.8**	**0.6**	**0.6**	**0.9**	**0.9**	**0.5**	**0.0**
% RSD	**1.7**	**2.0**	**1.0**	**0.7**	**0.7**	**0.9**	**0.9**	**0.5**	**0.0**
% SEM	**0.3**	**0.5**	**0.3**	**0.3**	**0.3**	**0.4**	**0.4**	**0.2**	**0.0**

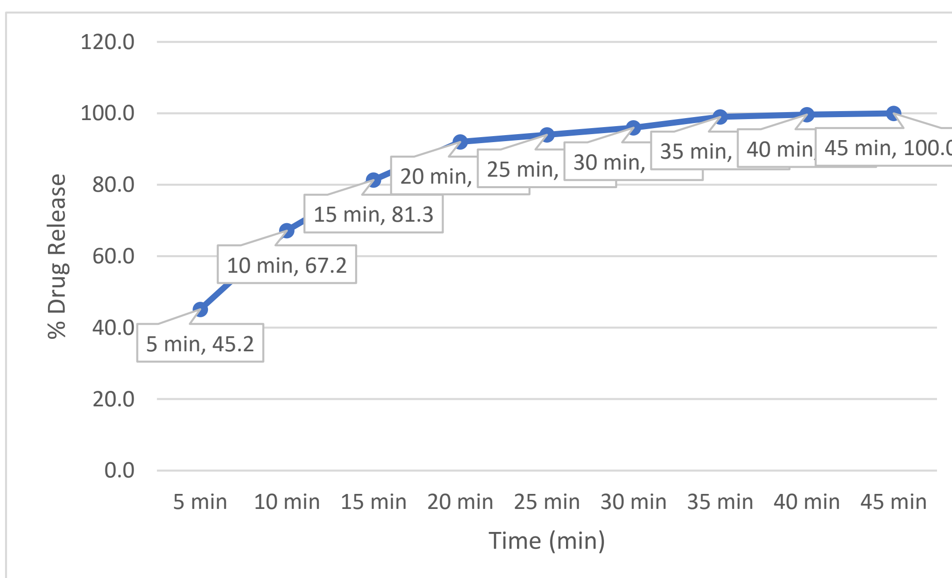


Figure 7.39: Dissolution profile of C14 Capsules Formulation in 0.1N HCl at 50 RPM

Table 7.27: Dissolution profile of C14 Capsules Formulation in pH 2.0 HCl Buffer at 50 RPM

Vessel No.	**% Drug Dissolved in**				
0	**5 min**	**10 min**	**15 min**	**30 min**	**45 min**
1	79.0	88.0	97.0	99.0	99.0
2	80.0	87.0	97.0	100.0	100.0
3	80.0	88.0	96.0	98.0	99.0
4	79.0	87.0	95.0	98.0	98.0
5	79.0	87.0	92.0	97.0	98.0
6	80.0	87.0	98.0	98.0	98.0
Mean	**79.5**	**87.3**	**95.8**	**98.3**	**98.7**
SD	0.5	0.5	2.1	1.0	0.8
% RSD	0.7	0.6	2.2	1.1	0.8
% SEM	0.2	0.2	0.9	0.4	0.3

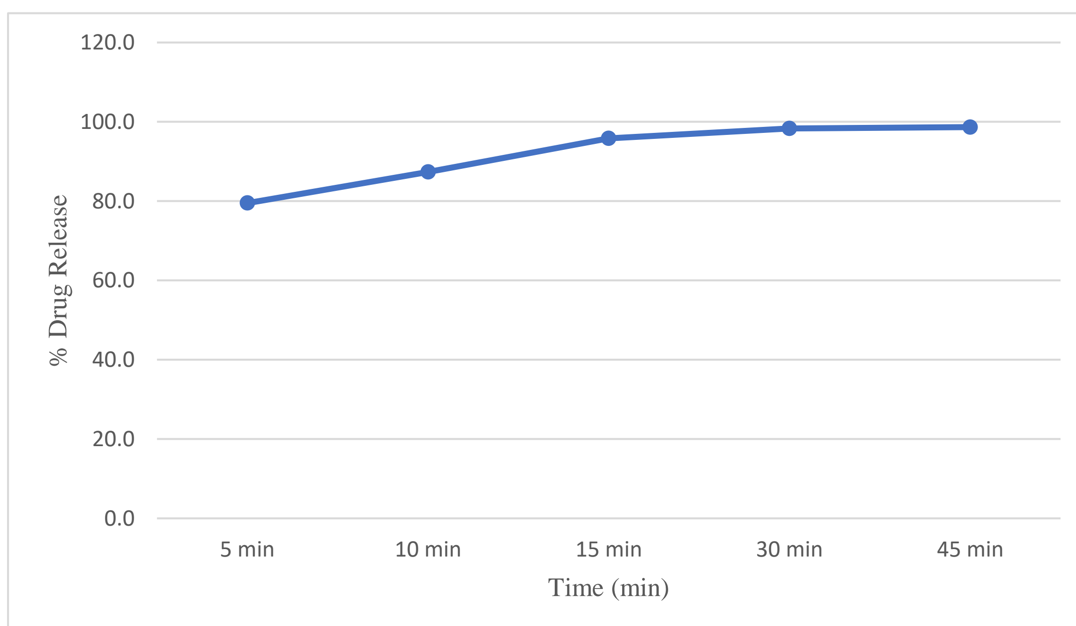


Figure 7.40: Dissolution profile of C14 Capsules Formulation in pH 2.0 HCl Buffer at 50 RPM

Table 7.28: Dissolution profile of C14 Capsules Formulation in pH 2.0 HCl Buffer at 25 RPM

Vessel No.	% Drug Dissolved in				
0	**5 min**	**10 min**	**15 min**	**30 min**	**45 min**
1	40.0	59.0	74.0	79.0	85.0
2	39.0	58.0	73.0	78.0	83.0
3	41.0	59.0	72.0	80.0	83.0
4	41.0	59.0	74.0	81.0	84.0
5	40.0	60.0	74.0	80.0	84.0
6	40.0	69.0	72.0	80.0	83.0
Mean	**40.2**	**60.7**	**73.2**	**79.7**	**83.7**
SD	0.8	4.1	1.0	1.0	0.8
% RSD	1.9	6.8	1.3	1.3	1.0
% SEM	0.3	1.7	0.4	0.4	0.3

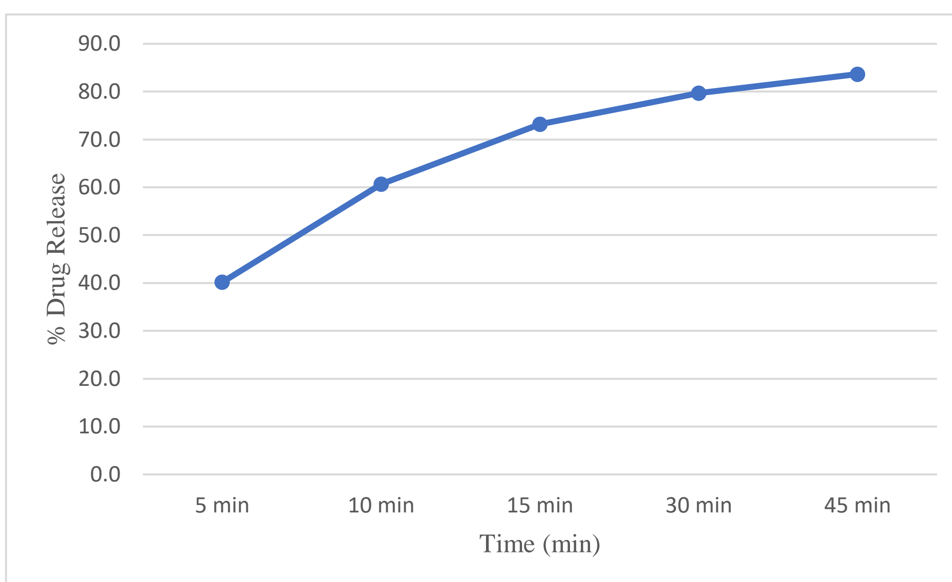


Figure 7.41: Dissolution profile of C14 Capsules Formulation in pH 2.0 HCl Buffer at 25 RPM

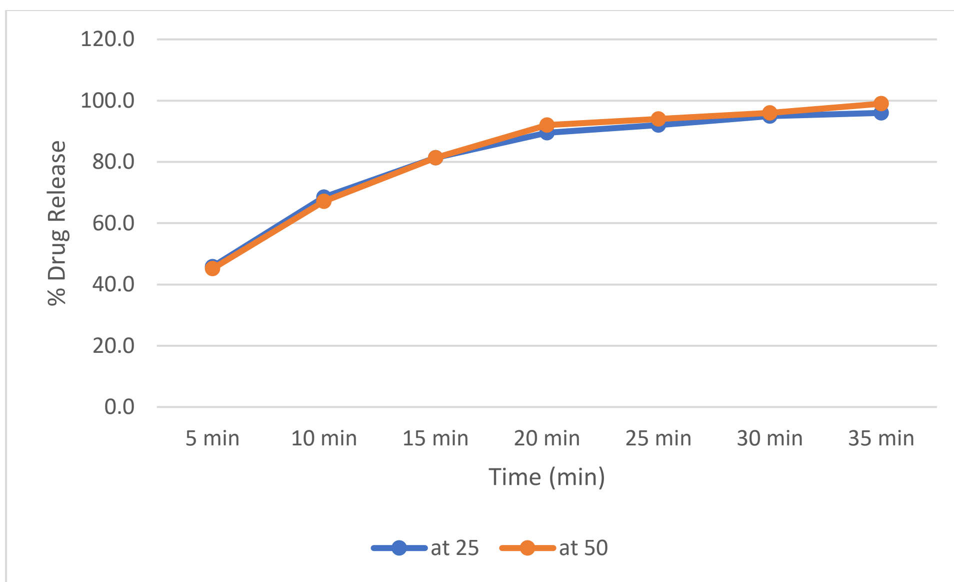


Figure 7.42: Comparison of the release rate and agitation of C14 in 0.1N HCl

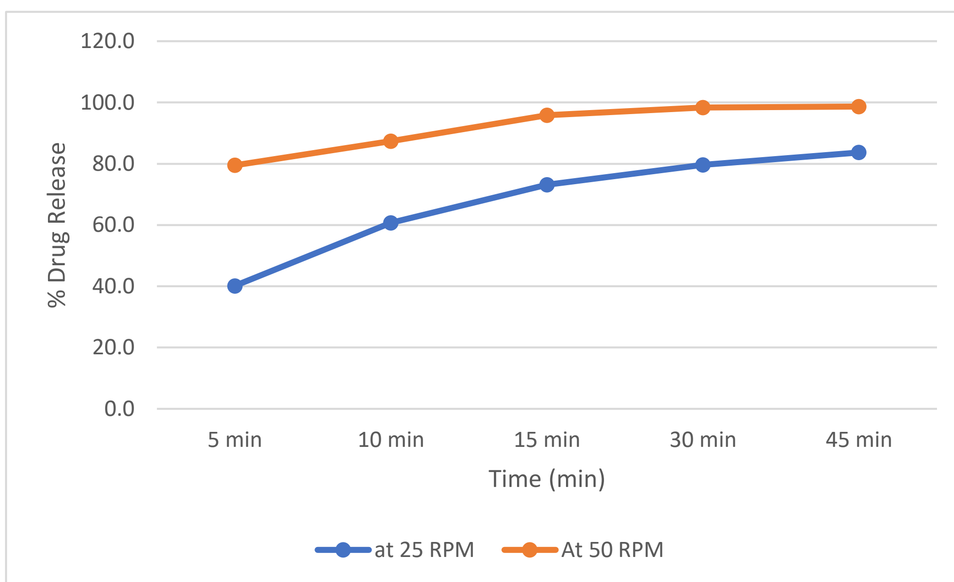


Figure 7.43: Comparison of the release rate and agitation of C14 in pH 2.0 HCl Buffer

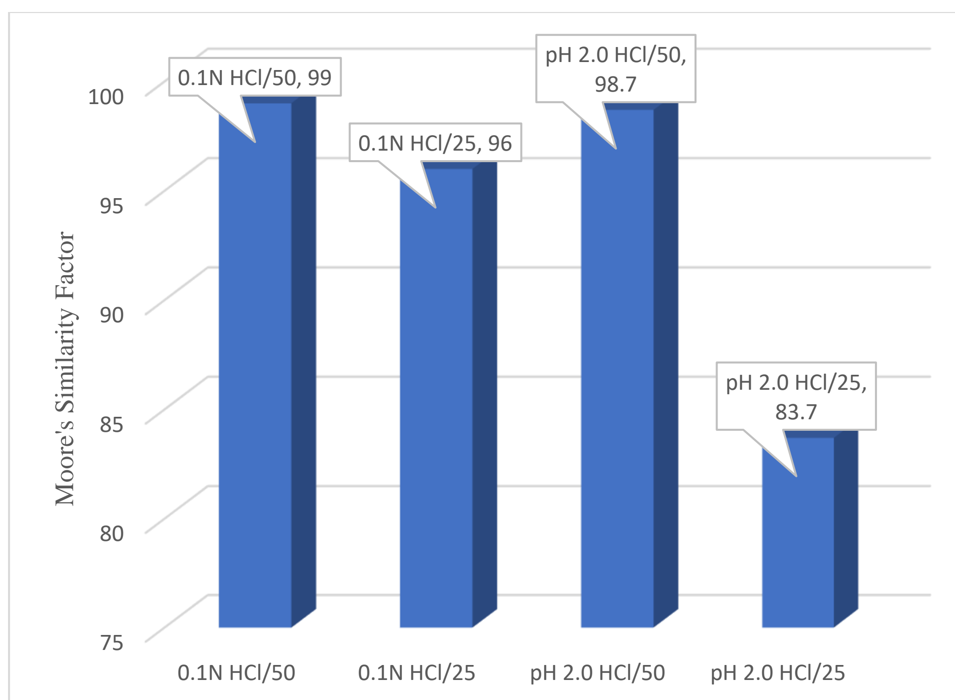


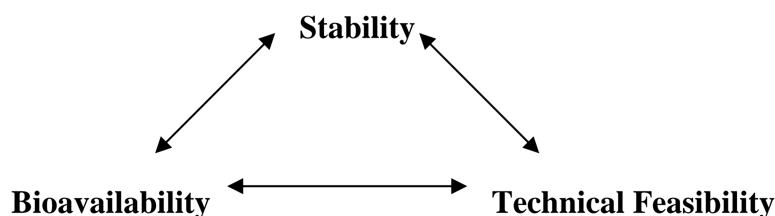
Figure 7.44: F2 values of the optimized C14 at different RPM and in different media

7.15 Accelerated Stability Study

The purpose of stability testing is to provide evidence of how the quality of a drug substance or formulated product varies with time under the influence of a variety of environmental factors such as temperature, light, and humidity. The ultimate goal of stability testing is the application of appropriate testing to allow the establishment of recommended storage conditions, retest periods and shelf lives.

It is necessary to establish the 'fitness for purpose' of the product throughout proposed shelf life, that is, to establish that all those attributes affecting product performance in use are not unacceptably changed during the period of storage up to the proposed expiry date. Testing must include factors affecting drug potency, the formation of degradation products and the microbiological and physical integrity of the product. It may also be required to measure other quality parameters considered to be important, such as organoleptic and aesthetic properties of the product.

When formulating any pharmaceutical dosage form, it is important to remember that there is an equilibrium between the bioavailability of the product, its chemical and physical stability and the technical feasibility of producing it.



Any change made to a formulation in an attempt to optimize one of these properties is likely to affect the other two parameters that must be considered. This is especially true for solid dosage forms.

The stability of a product can be evaluated if it's degradation impurities, it's assayed, it's dissolution and disintegration time does not increase considerably after 6M of accelerated stability testing at 40°C and 75% RH as per the ICH guidelines.

7.15.1 Accelerated Stability of optimized formulation C14

Table 7.29: Observation of stability analysis of the Optimized batch capsules (C14)

Time	Assay (% w/w) 40°C/75% RH			Assay (% w/w) 5 ± 3°C		
	PRED	MMF	TAC	PRED	MMF	TAC
Initial	101.7 ± 0.2	98.6 ± 1.4	98.6 ± 2.1			
1 M	101.2 ± 0.3	100.7 ± 0.2	98.6 ± 1.6	100.6 ± 0.4	100.7 ± 0.2	98.1 ± 1.9
2 M	101.3 ± 0.3	101.4 ± 0.4	97.2 ± 2.8	100.4 ± 0.2	101.4 ± 0.4	97.1 ± 1.1
3 M	100.7 ± 0.5	100.2 ± 0.2	96.1 ± 1.3	100.1 ± 0.4	100.2 ± 0.2	98.1 ± 1.5

n=3

Result

The assay of the PRED, MMF and TAC was varying from an initial value of 101.7 to 100.7, 98.6 to 100.2 and 98.6 to 96.1 % respectively, on three months/ 40°C/75% RH and at refrigerated (5 ± 3°C) conditions in HDPE bottles. Evaluation of stability data indicates that there is no significant change at the end of 3 Month at 40°C/75% RH in comparison to initial data. Hence the product was assumed to be stable, though the results of 6-month data will confirm its overall stability.

7.16 Dissolution Method Development

7.16.1 pH Solubility Profile Determination

Table 7.30: Solubility of the PRED, MMF and TAC at different pH

		Solubility (mg/ml)		
Sr. No.	**Media**	**MMF**	**TAC**	**PRED**
1	0.1N HCl (pH 1.2)	95 ± 0.17	0.00666 ± 0.057	4.51 ± 0.23
2	pH 2.0 HCl Buffer	92.3 ± 0.57	0.00143 ± 0.087	2.20 ± 0.13
3	Purified Water	6.7 ± 0.27	0.00933 ± 0.057	0.25 ± 0.03

All the drugs belong to class II. So, they have low solubility in water.

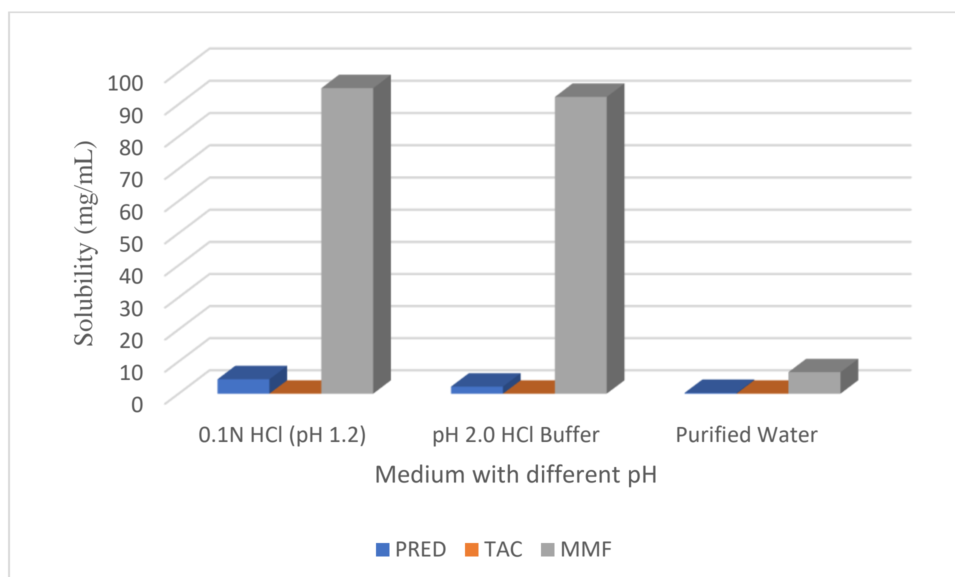


Figure 7.45: Solubility of the PRED, MMF and TAC in different media

Result

The solubility of the MMF decreases with the increase in the pH of the dissolution media. The MMF has high solubility as compared to PRED and TAC in 0.1N HCl, pH 2.0 HCl buffer, and sink conditions are maintained in these media. Whereas, in distilled water sink conditions were not observed, therefore; the PRED, MMF and TAC can be evaluated in any of the above media except distilled water. 0.1N HCl being easiest to prepare was selected to analyze the drug content and its formulations.

7.16.2 Selection of a suitable apparatus

The preferred apparatus for a capsule dosage form is paddle (USP Apparatus II). Hence it was decided to evaluate USP type II dissolution apparatus.

7.16.3 Selection of a suitable Dissolution Media

Based on these assumptions the references product was evaluated in the following media

- 0.1N HCl (pH 1.2)
- pH 2.0 HCl Buffer
- Distilled Water

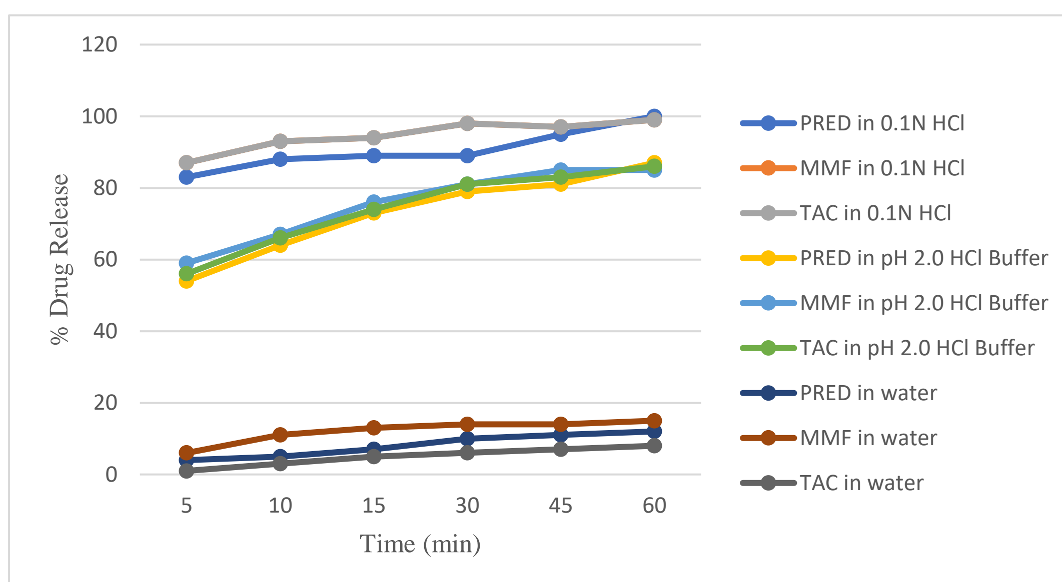


Figure 7.46: Dissolution Profile of Reference Product in Different Media at 50 RPM, USP II

Result

The evaluation of the product in these media reveals that 100% release is obtained in 0.1N HCl while Distilled water showed poor drug release.

7.16.4 Placebo Interference

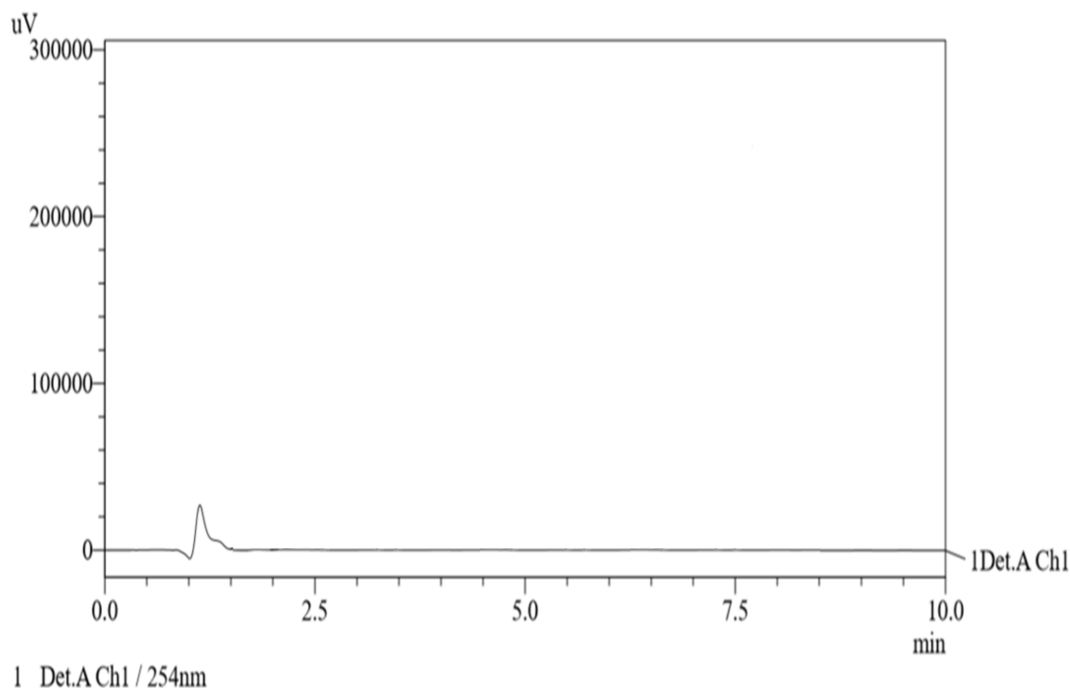


Figure 7.47: HPLC scan of placebo

The placebo solution does not show any signal in the range of HPLC method. Therefore, no placebo interference was encountered during the dissolution of the prototype formulation.

7.16.5 Selection of agitation rate

All compendial dissolution apparatus can be operated at different agitation intensities. The three most outstanding aspects to consider when deciding at which level the tests should be performed are

- Correlation to the *in vivo* data
- Variability of dissolution results and

- Regulatory guidelines and pharmacopoeial recommendations

The agitation rate of 25 and 50 rpm were evaluated using the selected dissolution medium and was found to give more than 80% drug release in 20 minutes at 50 rpm. At 25 rpm incomplete release was obtained. Thus, it was decided to employ 50 rpm. Since complete release was observed in the case of 50 rpm, 75 rpm was not evaluated.

7.17 Dissolution Method Validation

For media optimization, various aqueous media like 0.1N HCl, HCl buffers (pH 2.0), and water were investigated.

The HPLC (Shimadzu, Kyoto, Japan) instrument was equipped with two LC-10 ATVP pumps, SPD-10AVP UV-vis detector, injector with a 20 μL loop. The HPLC column used for analysis was Kinetex Polar, C18, 5 µm, 4.6 × 250 mm. Column. The results were acquired and processed using Shimadzu LC-solution version 6.42 software for data acquisition and processing. The mobile phase was a mixture of Acetonitrile and 0.35% Triethylamine pH 4.2 with Orthophosphoric acid (70:30). Injection volume was 20 µL which was injected into the column using a syringe and the linear gradient flow rate was set at 1.2 mL/min. The drugs were detected at 254 nm.

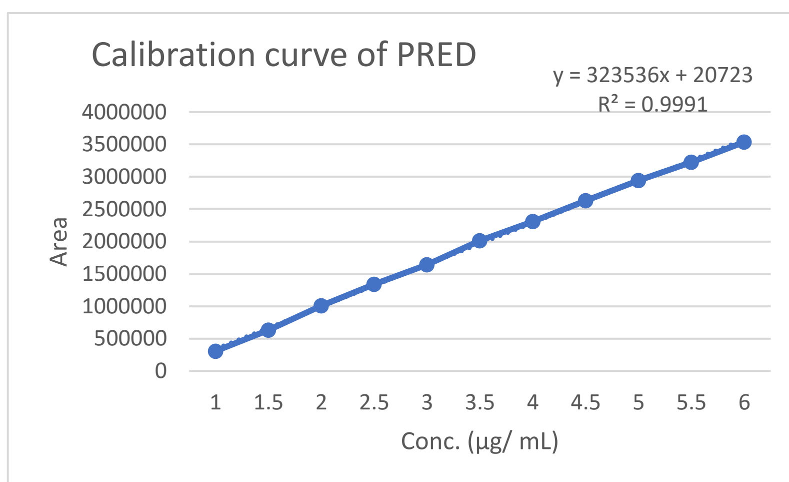
Addition of varying amounts of the methanol to various aqueous media did not improve the sensitivity of the methods, and the final decision of using 0.1N HCl as a media was based on the criteria like; sensitivity of the method, cost of solvents, ease of preparation and applicability of the method to dissolution samples.

7.17.1 Calibration curve

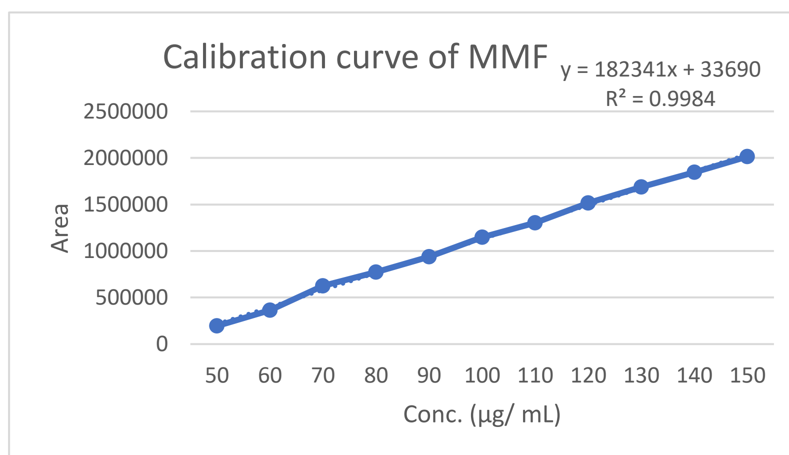
All the dilutions were filtered through 0.22 µ nylon filter and injected. For this each concentration was used in triplicate. Calibration curve was plotted and r^2 was determined.

Table 7.31: Calibration Data

Conc. (µg/ml)	Area PRED	Conc. (µg/ml)	Area MMF	Conc. (µg/ml)	Area TAC
1.0	307741	50	194369	0.05	112939
1.5	630236	60	363568	0.1	239767
2.0	1006100	70	624611	0.15	427003
2.5	1338654	80	773495	0.2	527224
3.0	1639917	90	937760	0.25	644617
3.5	2011201	100	1147992	0.3	792952
4.0	2311672	110	1301459	0.35	919790
4.5	2631714	120	1517078	0.4	1045456
5.0	2943512	130	1686370	0.45	1167147
5.5	3224389	140	1844563	0.5	1286739
6.0	3536187	150	2013855	0.55	1408430



a



b

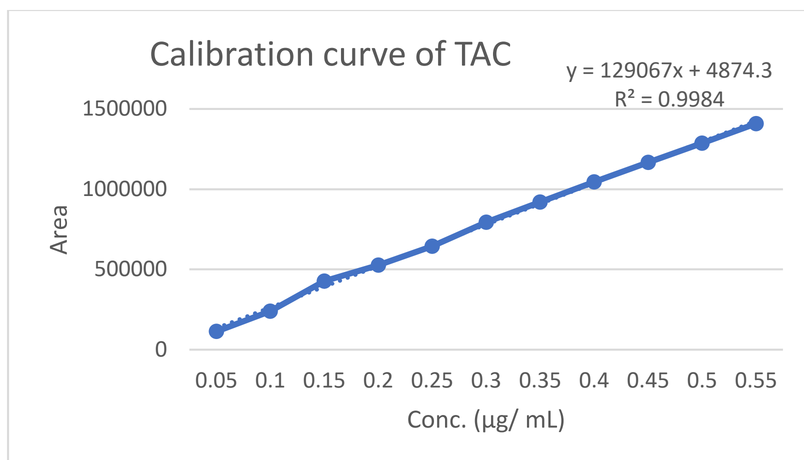


Figure 7.48: Calibration curves of a) PRED, b) MMF, and c) TAC in 0.1N HCl

7.17.2 Specificity and selectivity

Specificity of the HPLC method was demonstrated by the separation of the analytes from other potential components such as impurities, degradants or excipients. A volume of 20µL of individual ingredients and excipients solution were injected and the chromatogram was recorded**.**

The test results obtained were compared with the results of those obtained for standard drug. It was shown that those ingredients were not interfering with the developed method.

The calculated *t*-values were found to be less than that of the tabulated *t*-values, indicating that statistically there was no significant difference between the mean absorbance of solutions prepared from pure drug samples and the formulation samples. Therefore, proposed analytical methods are specific and selective for the drug.

7.17.3 Accuracy

Result

The excellent mean %recovery values, close to 100%, and their low standard deviation values (% RSD < 2) represent high accuracy of the analytical methods.

Figure 7.49: Data for overall recovery in 0.1 N HCl

Level of addition	Amount of Drug (µg/ml)	Area PRED	Amount Recovered	% Recovery	Statistical Analysis		Amount of Drug (µg/ml)	Area MMF	Amount Recovered	% Recovery	Statistical Analysis		Amount of Drug (µg/ml)	Area TAC	Amount Recovered	% Recovery	Statistical Analysis	
					Mean	%RSD					Mean	%RSD					Mean	%RSD
80%	4	1362329	4.15	103.75	103.91± 0.14	0.14	80	745687	79.88	99.85	99.83± 0.05	0.05	0.4	517700	0.392	98	98.88± 0.88	0.88
80%	4	1363285	4.16	104			80	744743	79.82	99.775			0.4	520734	0.399	99.75		
80%	4	1363105	4.16	104			80	744602	79.9	99.875			0.4	515996	0.395	98.75		
100%	5	1588900	4.85	97	97.26± 0.64	0.06	100	938708	99.86	99.86	99.85± 0.03	0.03	0.5	647394	0.496	99.2	99.20± 0.60	0.6
100%	5	1590971	4.84	96.8			100	939273	99.82	99.82			0.5	651493	0.499	99.8		
100%	5	1589885	4.9	98			100	938403	99.88	99.88			0.5	642681	0.493	98.6		
120%	6	1965186	6	100	100.05± 0.09	0.09	120	1091284	119.64	99.70	99.70± 0.04	0.04	0.6	820059	0.629	104.83	104± 0.76	0.73
120%	6	1966305	6	100			120	1089726	119.7	99.75			0.6	808380	0.62	103.33		
120%	6	1968448	6.01	100.17			120	1088178	119.6	99.67			0.6	812113	0.623	103.83		

Figure 7.50: Accuracy and Ruggedness data

Analyst 1					
Conc. (µg/ml)	**Area**	**Conc. (µg/ml)**	**Area**	**Conc. (µg/ml)**	**Area**
	PRED		**MMF**		**TAC**
5	159327	100	814082	0.5	64469
5	159108	100	814278	0.5	64637
5	159173	100	814789	0.5	64701
5	159047	100	814657	0.5	64286
5	159047	100	814816	0.5	64732
5	159091	100	814905	0.5	64460
Mean	159132	**Mean**	814588	**Mean**	64547.5
SD	106.22	**SD**	331.59	**SD**	171.94
%RSD	0.067	**%RSD**	0.419	**%RSD**	0.266
Analyst 2					
5	159379	100	737286	0.5	64419
5	159383	100	739687	0.5	64428
5	159341	100	738565	0.5	64346
5	159412	100	738156	0.5	64256
5	159216	100	739007	0.5	64409
5	159421	100	738441	0.5	64654
Mean	159359	**Mean**	738524	**Mean**	64418.7
SD	75.37	**SD**	807.77	**SD**	132.14
%RSD	0.0473	**%RSD**	0.109	**%RSD**	0.205

7.17.4 Precision

Data for System Precision, Method Precision, and Intermediate Precision

Table 7.32: Precision results showing repeatability

conc. (µg/ml)	**PRED**	**conc. (µg/ml)**	**MMF**	**conc. (µg/ml)**	**TAC**
5	162327	100	614082	0.5	65469
5	162741	100	612433	0.5	65284
5	162182	100	612691	0.5	65723
5	162112	100	613153	0.5	65554
5	162415	100	614420	0.5	65688
5	162455	100	613002	0.5	65579
Mean	162372	**Mean**	613297	**Mean**	65549.5
SD	223.886	**SD**	787.254	**SD**	159.465
%RSD	0.13788	**%RSD**	0.12836	**%RSD**	0.24327

Table 7.33: Intraday precision

conc. (µg/ml)	PRED	conc. (µg/ml)	MMF	conc. (µg/ml)	TAC
5	159327	100	814082	0.5	64469
5	159108	100	814278	0.5	64637
5	159173	100	811789	0.5	64701
5	159047	100	809657	0.5	64286
5	159047	100	807816	0.5	64732
5	159091	100	805905	0.5	64460
Mean	159132	**Mean**	810588	**Mean**	64547.5
SD	106.219	**SD**	3398.46	**SD**	171.938
%RSD	0.06675	**%RSD**	0.41926	**%RSD**	0.26637

Table 7.34: Interday precision

conc. (µg/ml)	PRED	conc. (µg/ml)	MMF	conc. (µg/ml)	TAC
5	159141	100	746736	0.5	64497
5	159079	100	740286	0.5	64419
5	159216	100	737007	0.5	64209
5	159121	100	736441	0.5	64354
5	158770	100	731683	0.5	64523
5	159047	100	747816	0.5	64332
Mean	159062	**Mean**	739995	**Mean**	64389
SD	154.412	**SD**	6282.39	**SD**	116.098
%RSD	0.09708	**%RSD**	0.84898	**%RSD**	0.18031

Result

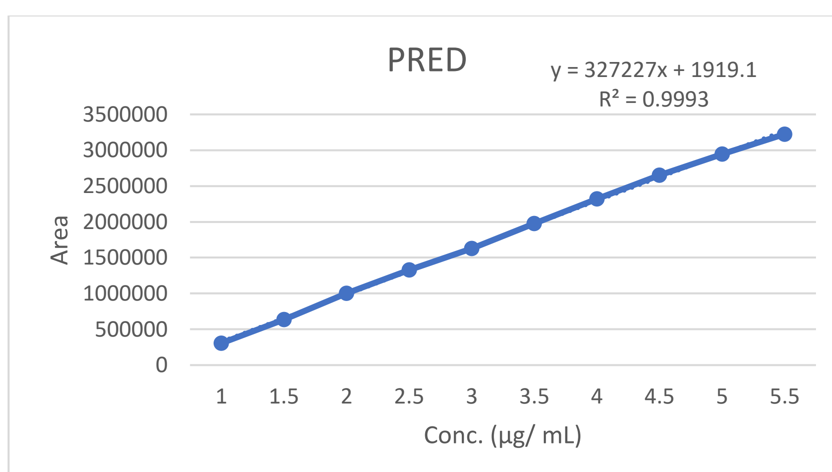
Precision was determined by studying the repeatability and intermediate precision. Repeatability (% RSD) of PRED, MMF and TAC ranged from 0.66 to 1.02; 0.41 to 0.87; and 0.26 to 1.03 respectively in 0.1N HCl at all three (Table 8.33 to 8.35). Repeatability results indicated the precision under the same operating conditions over a short interval of time and inter-assay precision. Intermediate precision expresses within-laboratory variations in different days and different instruments. In the intermediate precision study, %RSD values were not more than 2.0% in all the cases. RSD values

found for both the analytical methods were well within the acceptable range indicating that these methods have excellent repeatability and intermediate precision.

7.17.5 Linearity

Table 7.35: Linearity table of Prednisolone, Mycophenolate and Tacrolimus

PRED			MMF			TAC		
Conc. (µg/ml)	Area	SD	Conc. (µg/ml)	Area	SD	Conc. (µg/ml)	Area	SD
1.0	305579	4048.35	50	192027	2066.73	0.05	112687	407.65
1.5	634971	6180.71	60	370156	7401.62	0.1	239156	535.33
2.0	1003729	2957.65	70	612808	10224.46	0.15	421809	4883.27
2.5	1329168	21710.31	80	768420	9407.89	0.2	524716	5618.78
3.0	1626347	16692.96	90	942060	4499.84	0.25	654758	10412.89
3.5	1976215	30299.37	100	1134766	14594.69	0.3	795547	2262.62
4.0	2321734	8966.31	110	1290143	11643.82	0.35	921787	1761.69
4.5	2651374	17168.08	120	1497025	20125.89	0.4	1042911	2222.67
5.0	2943142	366.56	130	1668172	15772.36	0.45	1170387	4833.77
5.5	3224405	510.70	140	1844333	2933.77	0.5	1286536	3677.22
6.0	3505668	510.70	150	2020494	2933.77	0.55	1402685	3677.22



a

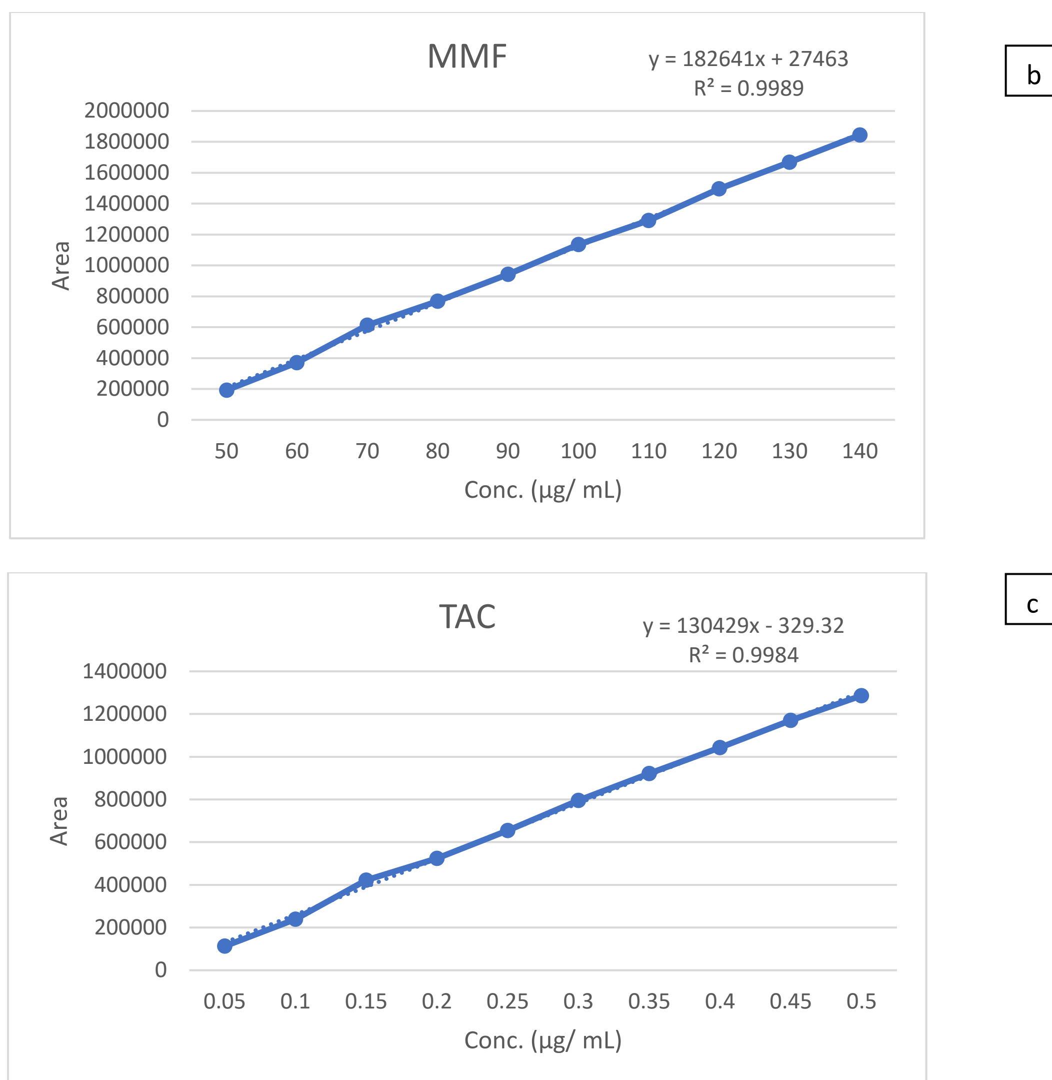


Figure 7.51: Linearity curve of a) PRED, b) MMF and c) TAC by RP-HPLC in 0.1N HCl

Result

The linearity range for PRED, MMF and TAC estimation was found to be 1.0-6.0; 50–150; 0.05=0.55 µg mL^{-1} respectively (r^2 = 0.99) in 0.1N HCl. Lower values of parameters like a standard error (SE) of slope and intercept indicated high precision of the proposed methods. Also, the mean slope and intercept values are within the 95% confidence interval. The goodness of fit of the regression equations was supported by high regression coefficient values and lower calculated *F*-values.

7.17.6Robustness

The Percentage RSD should not be more than 2. The % RSD obtained for change of flow rate, change in wavelength was found to be below 2, which was within the acceptance criteria. Hence the method was robust.

Table 7.36: Change in wavelength

Conc. (µg/ml)	**249 nm**			**254 nm**			**259 nm**		
	Area			**Area**			**Area**		
	PRED	**MMF**	**TAC**	**PRED**	**MMF**	**TAC**	**PRED**	**MMF**	**TAC**
PRED:5.0	177665	903237	93355	157397	712879	67928	137109	516520	42500
MMF:100	177502	903786	93542	157193	712900	67978	136963	516213	42413
TAC: 0.5	177493	903279	93561	157280	712792	68212	137026	516305	42663
Mean	177553.33	903434.00	93486.00	157290.00	712857.00	68039.33	137032.67	516346.00	42525.33
SD	96.81	305.56	113.85	102.37	57.26	151.61	73.23	157.55	126.91
%RSD	0.05	0.03	0.12	0.07	0.01	0.22	0.05	0.03	0.30

Table 7.37: Change in flow rate

Flow rate	**1.0 ml/min**			**1.2 ml/min**			**1.4 ml/min**		
Conc. (µg/ml)	**Area**			**Area**			**Area**		
	PRED	MMF	TAC	PRED	MMF	TAC	PRED	MMF	TAC
PRED:5.0	160298	796699	64607	159047	788571	64292	157796	778842	64376
MMF:100	160351	796500	64893	159156	788276	64504	157961	778652	64314
TAC:0.5	160453	796512	64732	159198	788249	64382	157943	778986	64533
MEAN	160367.33	796570.33	64744.00	159133.67	788365.33	64392.67	157900.00	778826.67	64407.67
SD	78.78	111.59	143.38	77.94	178.62	106.40	90.52	167.53	112.88
%RSD	0.05	0.01	0.22	0.05	0.02	0.17	0.06	0.02	0.18

7.18 Assay method development

7.18.1 Development of the optimal mobile phase

The assay procedure was optimized with a view to developing a stability indicating assay method to quantify the PRED, MMF and TAC from marketed formulation and manufactured capsules. To obtain good separation, various compositions of triethylamine buffer pH 4.2 and acetonitrile were tried, but the best results were obtained with the mobile phase consisting Acetonitrile and 0.35% Triethylamine pH 4.2 with Orthophosphoric acid (70:30). The injection volume used in method was 20 µL for each injection at a flow rate of 1.2 mL/min. The scanning of the standard solution represented wavelength maxima at 254 nm.

7.18.2 Calibration curves

The linear regression data for the calibration curves (n = 5) as shown in table 8.38 showed a good linear relationship over concentration range (PRED: 1-5.5, MMF: 50-150 and TAC: 0.05-0.5) µg mL^{-1} concerning the peak area. The linearity of the calibration graphs and adherence of the system to Beer's law was validated by the high value of correlation coefficient (R± S.D. = 0.999 ± 0.0002). No significant difference was observed in the slopes of standard curves (ANOVA, $P > 0.05$).

Table 7.38: Calibration data

Conc. (µg/ml)	Area	Conc. (µg/ml)	Area	Conc. (µg/ml)	Area
	PRED		**MMF**		**TAC**
1.0	308088	50	191255	0.05	112906
1.5	641963	60	378165	0.1	238768
2.0	1004673	70	607131	0.15	417311
2.5	1304329	80	757564	0.2	528644
3.0	1631418	90	946736	0.25	665423
3.5	1958914	100	1119108	0.3	796579
4.0	2328878	110	1278197	0.35	922450
4.5	2663406	120	1476827	0.4	1041923
5.0	2943134	130	1659699	0.45	1175943
5.5	3224924	140	1841291	0.5	1290107
6.0	3506714	150	2022883	0.55	1404271

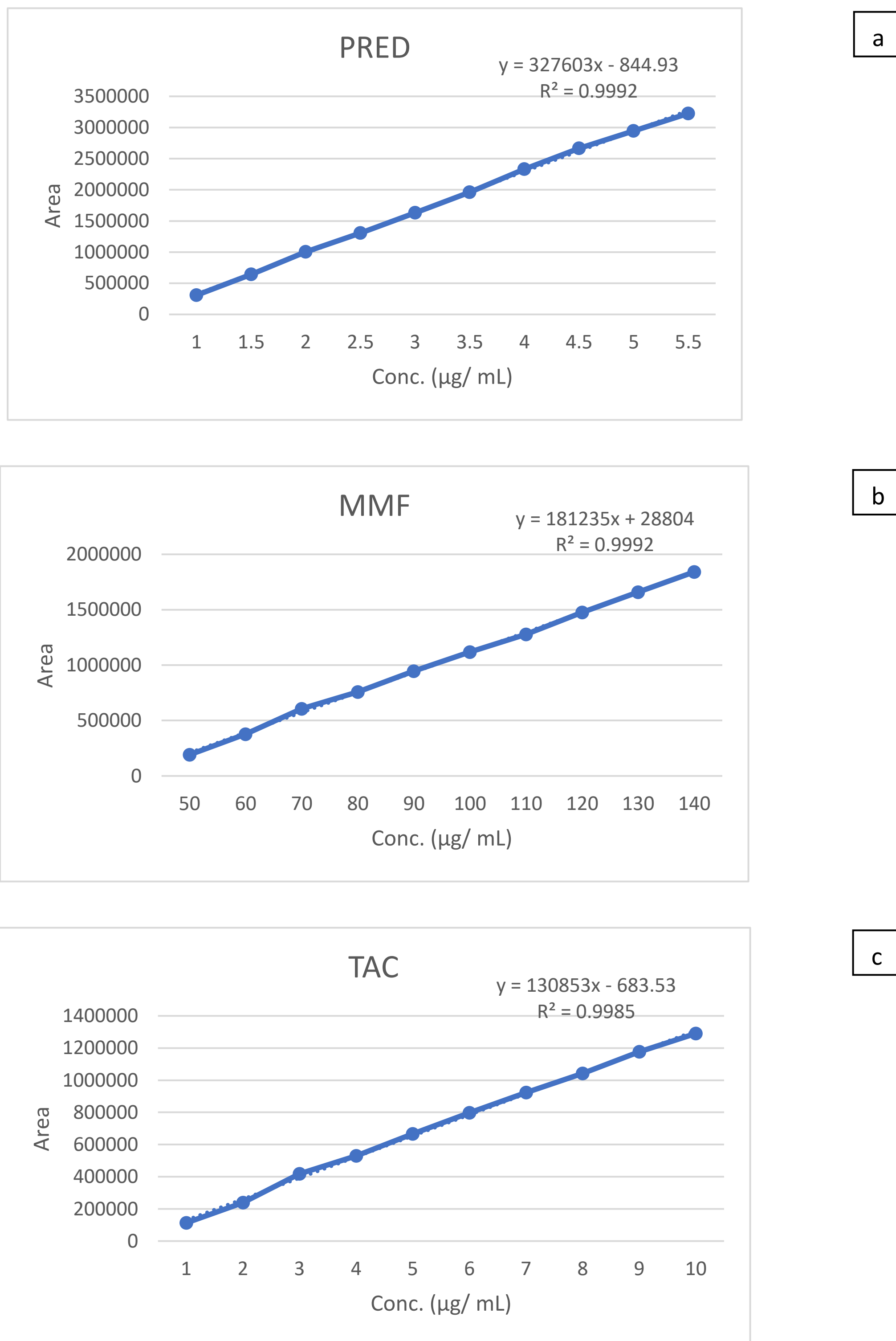


Figure 7.52: Calibration curve for a) PRED, b) MMF and c) TAC

7.19 Validation of the Assay method

7.19.1 Specificity

In the interference studies, diluents run as well as placebo run were not produced any peak or fluctuation at the run time of the main peak of mycophenolate mofetil. The % RSD of six injections of standard solution of PRED, MMF and TAC were found to be 0.068, 0.019 and 0.109; hence the system is suitable for the studies.

Table 7.39: Results showing Specificity

Conc. (µg/ml)	**Area**	**Conc. (µg/ml)**	**Area**	**Conc. (µg/ml)**	**Area**
	PRED		**MMF**		**TAC**
5	153647	100	726475	0.5	62202
5	153695	100	726409	0.5	62109
5	153497	100	726462	0.5	62111
5	153482	100	726385	0.5	62156
5	153436	100	726767	0.5	62287
5	153482	100	726434	0.5	62136
Mean	153540	Mean	726489	Mean	62166.8
SD	104.75	SD	140.32	SD	68.13
%RSD	0.068	%RSD	0.019	%RSD	0.109

7.19.2 Precision

7.19.2.1 System Precision

In the system precision; the USP tailing, USP plates and % RSD of the six injections of standard solution were found to be 1.40, 6246 and 0.75 respectively.

7.19.2.2 Method Precision

For the method precision; the USP tailing, USP plates and % RSD of the five injections of standard solution were found to be 1.32, 6564 and 0.56 respectively. The % RSD of the six sample solutions was found to be 0.67. The purity angle and purity threshold for the main peak were found to be 0.289 and 0.827 respectively.

7.19.2.3 Intermediate Precision

In the intermediate precision studies, the USP tailing, USP plates and % RSD of the five injections of standard solution were found to be 1.79, 3649 and 0.17 respectively. The % RSD of the six sample solutions was found to be 0.76. The purity angle and purity threshold for the main peak were found to be 0.324 and 0.637 respectively.

7.19.3 Linearity

For linearity, mycophenolate mofetil, prednisolone and tacrolimus 50-150; 1.0-6.0; 0.05-0.55 µg/mL concentrations were prepared. All the dilutions were filtered through 0.22 µ nylon filter and injected. For this each concentration was used in triplicate. Calibration curve was plotted and r^2 was determined.

Table 7.40: Linear regression data for the calibration curve in 0.1N HCl (n=6)

PRED			**MMF**			**TAC**		
Conc. (µg/ml)	**Area**	**SD**	**Conc. (µg/ml)**	**Area**	**SD**	**Conc. (µg/ml)**	**Area**	**SD**
1.0	305579	4048.35	50	192027	2066.73	0.05	112687	407.65
1.5	634971	6180.71	60	370156	7401.62	0.10	239156	535.33
2.0	1003729	2957.65	70	612808	10224.46	0.15	421809	4883.27
2.5	1329168	21710.31	80	768420	9407.89	0.20	524716	5618.78
3.0	1626347	16692.96	90	942060	4499.84	0.25	654758	10412.89
3.5	1976215	30299.37	100	1134766	14594.69	0.30	795547	2262.62
4.0	2321734	8966.31	110	1290143	11643.82	0.35	921787	1761.69
4.5	2651374	17168.08	120	1497025	20125.89	0.40	1042911	2222.67
5.0	2943142	366.56	130	1668172	15772.36	0.45	1170387	4833.77
5.5	3224405	510.70	140	1844333	2933.77	0.50	1286536	3677.22

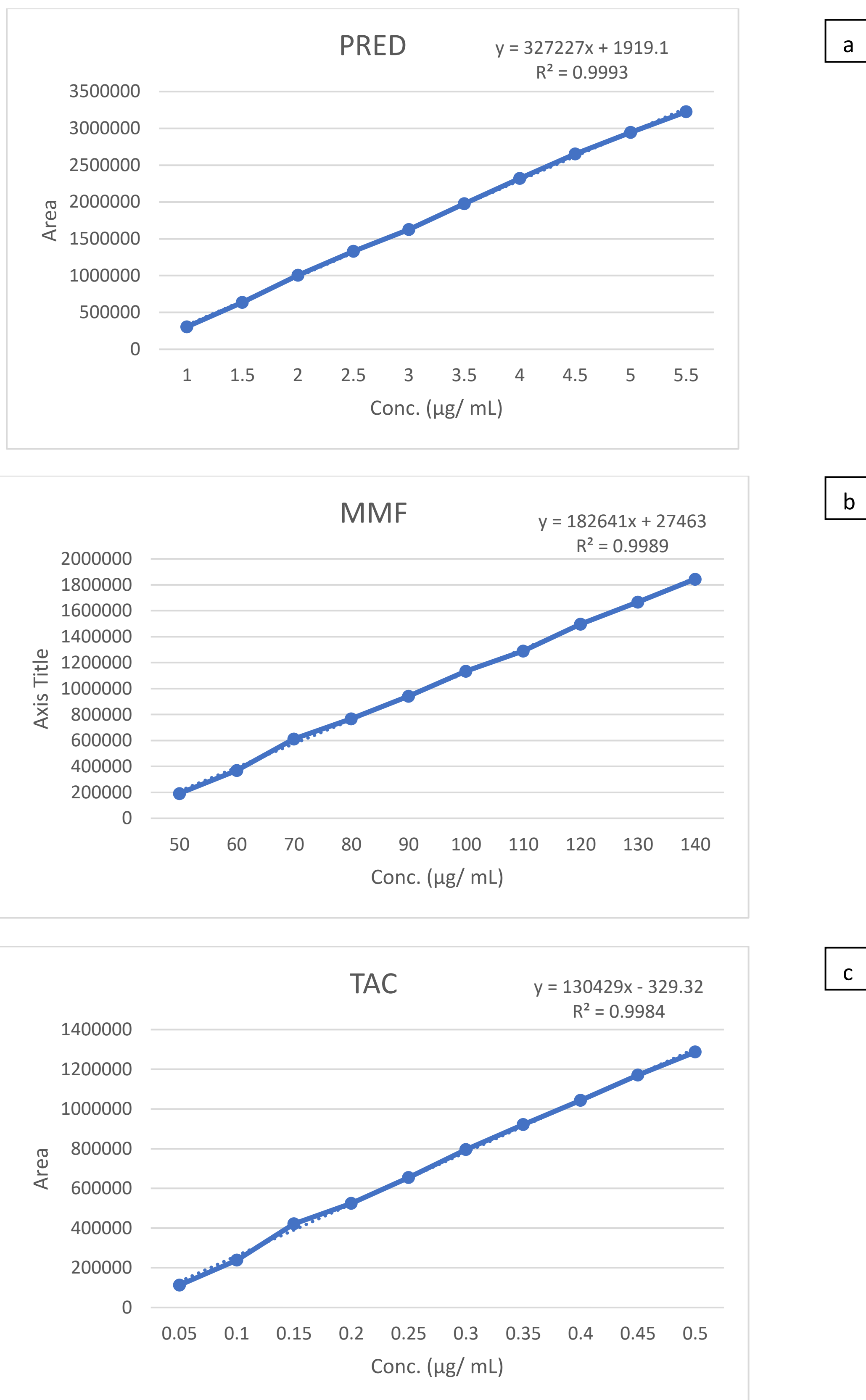


Figure 7.53: Linearity curve for a) PRED, b) MMF and c) TAC in HPLC method validation

7.19.4 Accuracy

The standard deviation and % RSD of three replicates of each level individually; for 80%, 100%, 120% for PRED, MMF and TAC were found to be (0.038, 0.036; 0.081, 0.083; and 0.461, 0.464), (0.065, 0.067; 0.046, 0.046; and 0.675, 0.680), and (0.086, 0.086; 0.148, 0. 153; and 0.761, 0.731) respectively.

7.19.5 Robustness of the method

The robustness was studied by analyzing the sample of lower concentration with deliberate variation in the method parameters. The change in the responses of drugs was noted in terms of %RSD. Robustness of the method was studied by change in wavelength or change in flow rate.

The ruggedness was studied by analyzing the same samples of three drugs by changing analyst. The change in the responses of drugs was noted in terms of % RSD.

The low values of % RSD after introducing small deliberate changes in the developed HPLC method indicated the robustness of the method.

Table 7.41: Accuracy of HPLC method

Level of addition	Amount of Drug added (µg/ml)	PRED				Amount of Drug added (µg/ml)	MMF				Amount of Drug added (µg/ml)	TAC			
		% Rec	Mean	SD	%RSD		% Rec	Mean	SD	%RSD		% Rec	Mean	SD	%RSD
80%	4	103.93				80	98.31				0.4	99.29			
80%	4	104.00	103.973	0.038	0.036	80	98.18	98.217	0.081	0.083	0.4	99.87	99.373	0.461	0.464
80%	4	103.99				80	98.16				0.4	98.96			
100%	5	96.99				100	99.78				0.5	99.32			
100%	5	97.12	97.053	0.065	0.067	100	99.84	99.790	0.046	0.046	0.5	99.95	99.290	0.675	0.680
100%	5	97.05				100	99.75				0.5	98.6			
120%	6	99.99				120	97.08				0.6	104.83			
120%	6	100.05	100.067	0.086	0.086	120	96.99	96.953	0.148	0.153	0.6	103.34	103.997	0.761	0.731
120%	6	100.16				120	96.79				0.6	103.82			

Table 7.42: Change in wavelength

Conc. (µg/ml)	249 nm			254 nm			259 nm		
	Area			Area			Area		
	PRED	**MMF**	**TAC**	**PRED**	**MMF**	**TAC**	**PRED**	**MMF**	**TAC**
PRED: 5	1776659	907237	933558	1573979	712879	679282	1371098	518520	425005
MMF: 50	1775023	904786	935429	1571931	712900	679780	1369639	517013	424131
TAC: 0.5	1774938	902279	935611	1572804	709292	682122	1370269	516305	426633
Mean	1775540	904767	934866	1572905	711690	680395	1370335	517279	425256
SD	970.01	2479.05	1136.41	839.12	1695.90	1238.21	731.76	1131.26	1269.79
%RSD	0.0546	0.274	0.122	0.0534	0.238	0.182	0.053	0.219	0.299

Table 7.43: Change in flow rate

Flow rate	1.0 ml/min			1.2 ml/min			1.4 ml/min		
Conc. (µg/ml)	Area			Area			Area		
	PRED	**MMF**	**TAC**	**PRED**	**MMF**	**TAC**	**PRED**	**MMF**	**TAC**
PRED: 5	1602980	796699	646079	1590471	788571	651924	1577962	779842	643768
MMF: 50	1603518	796200	648935	1591569	788276	645042	1579619	778152	641149
TAC: 0.5	1604539	793512	651320	1591989	785249	643826	1579439	776986	635332
Mean	1603679	795470	648778	1591343	787365	646931	1579007	778327	640083
SD	791.87	1714.22	2624.02	639.99	1501.31	3565.55	909.17	1435.99	4317.85
%RSD	0.049	0.216	0.405	0.040	0.191	0.551	0.058	0.185	0.675

7.19.6 Limit of detection and limit of quantitation

The LOD and LOQ were calculated on the basis of standard deviation of the response and the slope (s) of the calibration curve at approximate levels of LOD and LOQ. The obtained results were found to be within the limit.

Table 7.44: Data showing LOD and LOQ values

Sr. No.	Sample	LOD (µg/mL)	LOQ (µg/mL)
1.	Mycophenolate	11.4163	32.564540
2.	Prednisolone	0.442067	1.339597
3.	Tacrolimus	0.038667	0.117171

7.19.7 Stability of analytical solution

The stability of the solutions was monitored at 0 h, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h and 24 h. The results indicated that there is no significant (within ±2%) change in solutions. Hence, all the solutions were stable at room temperature for 24 hours.

7.19.8 Forced degradation studies

The % degradation in acid degradation, base degradation, peroxide degradation, photolytic degradation, and thermal degradation were determined and tabulated as follows:

7.19.8.1 Preparation of solution for acid degradation

Process: Acid decomposition study was performed by treating three drugs (1 ml) in 1 ml of 0.1M HCl for 2 hr at 80 ˚C. After 2 hr solution neutralized with 1 ml of same strength of base and finally made up to 10 ml volume with water, sonicated and filtered through 0.22µm membrane filter and injected in to HPLC system. The further treatment was not monitored as TAC was already degraded by more than 30%.

Table 7.45: Acid degradation

Conditions	Conc. (µg/ml)	Time period	Peak area (PRED)		% Degradation	Peak area (MMF)		% Degradation	Peak area (TAC)		% Degradation
			Before	After		Before	After		Before	After	
Acid degradation	50	15 min	1143328	1129325	1.22	704737	696231	1.21	495479	462319	6.69
		30 min		1116541	2.34		674321	4.32		441289	10.94
		45 min		1109325	2.97		647621	8.10		419832	15.27
		60 min		1100024	3.79		619872	12.04		397421	19.79
		90 min		1091141	4.56		604213	14.26		376541	24.00
		120 min		1070832	6.34		587431	16.65		332131	**32.97**

7.19.8.2 Preparation of solution for basic degradation

Process: Alkali decomposition study was performed by treating working solution of three drugs (1 ml) in 1 ml of 0.1M NaOH for 1 hr at 80 ºC. After 1 hr solution neutralized with 1 ml of same strength of acid and finally made up to 10 ml volume with water, sonicated and filtered through 0.22µm membrane filter and injected in to HPLC system.

Table 7.46: Base degradation

Conditions	Conc. (µg/ml)	Time period	Peak area (PRED)		% Degradation	Peak area (MMF)		% Degradation	Peak area (TAC)		% Degradation
			Before	**After**		**Before**	**After**		**Before**	**After**	
Basic degradation	50	15 min	1143328	1129751	1.19	704737	674287	4.32	495479	448312	9.52
		30 min		1109923	2.92		634521	9.96		412311	16.79
		45 min		1102001	3.61		599927	14.87		378218	23.67
		60 min		1096641	4.08		556234	21.07		323218	**34.77**

7.19.8.3 Preparation of solution for oxidative degradation

Process: Oxidative decomposition study was performed by treating the working solution of three drugs (1 ml) in 1 ml 30% H_2O_2 for 2.5 hr at 80 ºC. After 2.5 hr volume made up to 10 ml with water, sonicated and filtered through 0.22µm membrane filter and injected into HPLC system. The data was given in table 7.47.

7.19.8.4 Preparation of solution for thermal degradation

Process: Thermal decomposition study was performed by treating the working solution of three drugs (1 ml) for 15 days at 105 ºC. After 15 days volume made up to 10 ml volume with water, sonicated and filtered through 0.22µm membrane filter and injected into HPLC system. The data was given in table 7.48.

Table 7.47: Oxidative degradation

Conditions	Conc. (µg/ml)	Time period	Peak area (PRED)		% Degradation	Peak area (MMF)		% Degradation	Peak area (TAC)		% Degradation
			Before	After		Before	After		Before	After	
Oxidative degradation	50	15 min	1163223	1158116	0.44	714787	713923	0.12	499478	488312	2.24
		30 min		1152876	0.89		711321	0.48		470311	5.84
		45 min		1144231	1.63		708754	0.84		452218	9.46
		60 min		1132116	2.67		702137	1.77		434218	13.07
		90 min		1121356	3.59		697638	2.40		409480	18.02
		120 min		1107289	4.81		679213	4.98		378862	24.15
		150 min		1100116	5.43		651213	8.89		336424	**32.64**

Table 7.48: Thermal degradation of Prednisolone

Conditions	Conc. (µg/ml)	Time period	Peak area		% Degradation	Peak area		% Degradation	Peak area		% Degradation
			Before	After		Before	After		Before	After	
Thermal degradation	50	50 days	1143123	1035023	6.71	704737	663527	2.51	495479	334023	21.61
			1042030	1037651		697893	663606		484662	339775	
			1143328	1039036		696961	668593		472176	342972	
			1121215	1038497		678227	678588		389809	347423	
			1116216	1037130		678322	675817		390612	332993	
			1104599	1035632		673263	675547		371560	344354	

7.19.8.5 Preparation of solution for UV degradation

Process: UV degradation was performed by exposing the working solution of three drugs in (1 ml) to UV radiation at 254 nm for 15 days. After 15 days volume made up to 10 ml volume with water, sonicated and filtered through 0.22µm membrane filter and injected into HPLC system.

Table 7.49: UV degradation of Prednisolone

Conditions	Conc. (µg/ml)	Time period	Peak area		% Degradation	Peak area		% Degradation	Peak area		% Degradation
			Before	After		Before	After		Before	After	
UV degradation	50	15 days	1143123	1062243	4.46	704737	557778	16.62	495479	403838	8.83
			1042030	1058682		697893	561829		484662	350040	
			1143328	1061056		696961	584470		472176	454526	
			1121215	1062551		678227	568859		389809	334101	
			1116216	1064206		678322	571652		390612	417362	
			1104599	1064434		673263	598577		371560	414498	

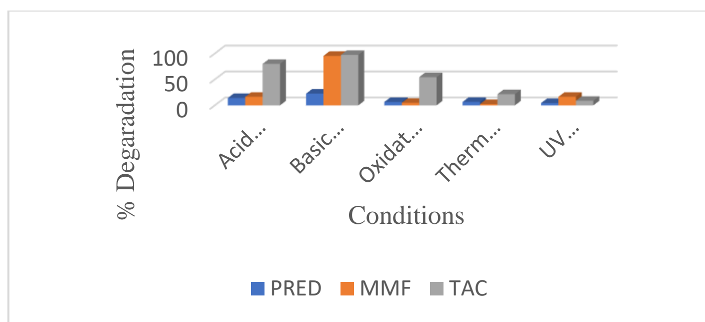


Figure 7.54: Degradation data of PRED, MMF and TAC

Significant degradation was observed in acidic, basic, UV and thermal condition. Major degradation was observed in basic condition. Tacrolimus highly degraded in acidic, basic, UV and thermal condition as compare to rest of drugs.

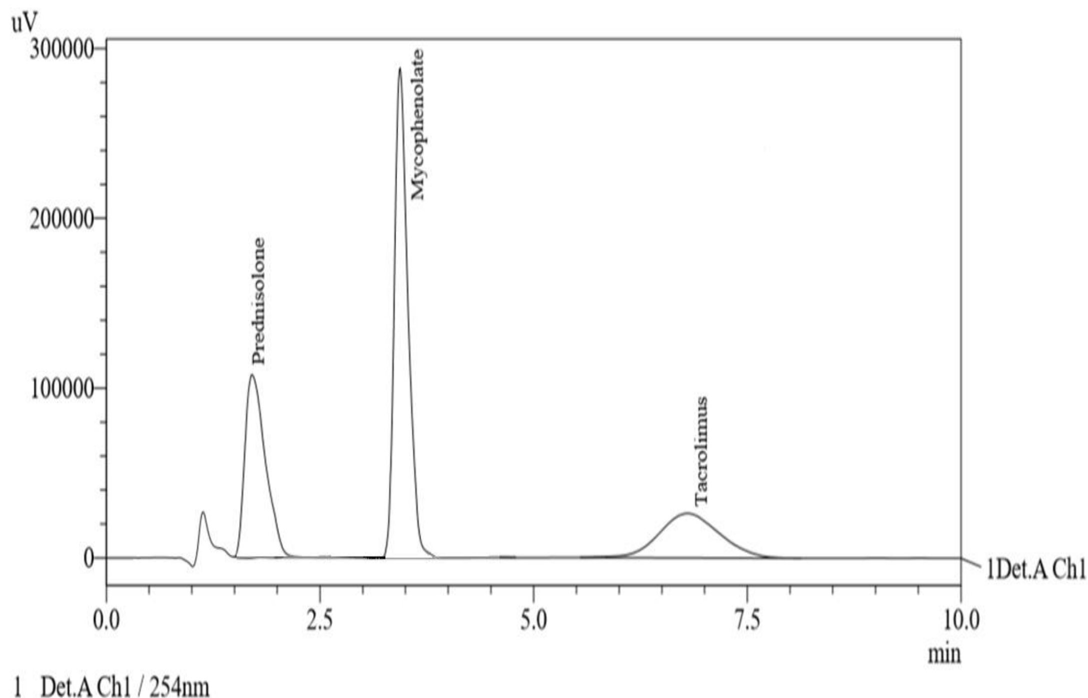


Figure 7.55: HPLC scan of optimized formulation

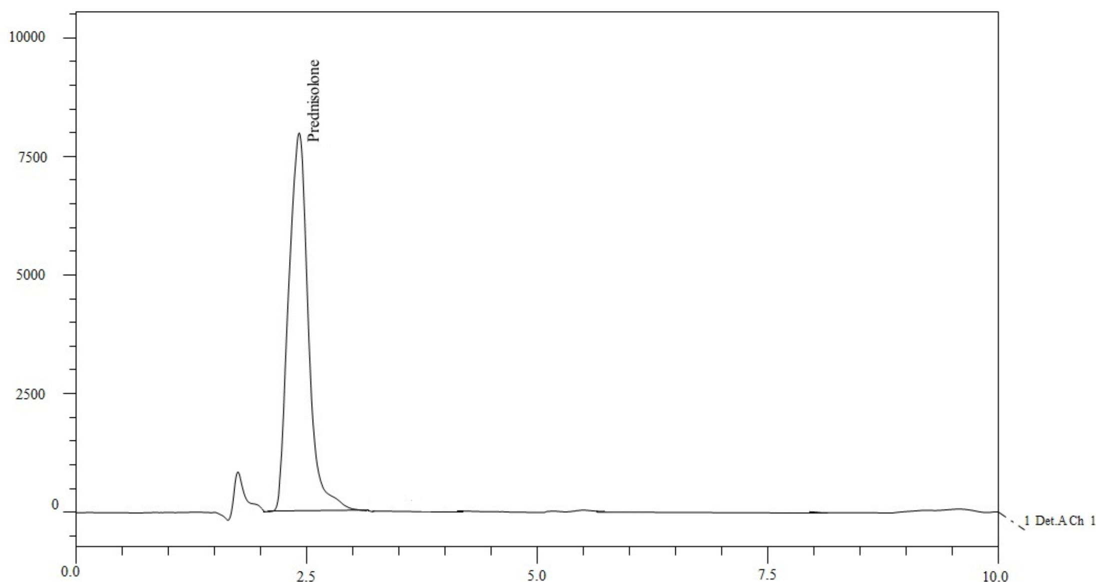


Figure 7.56: HPLC scan of PRED (standard)

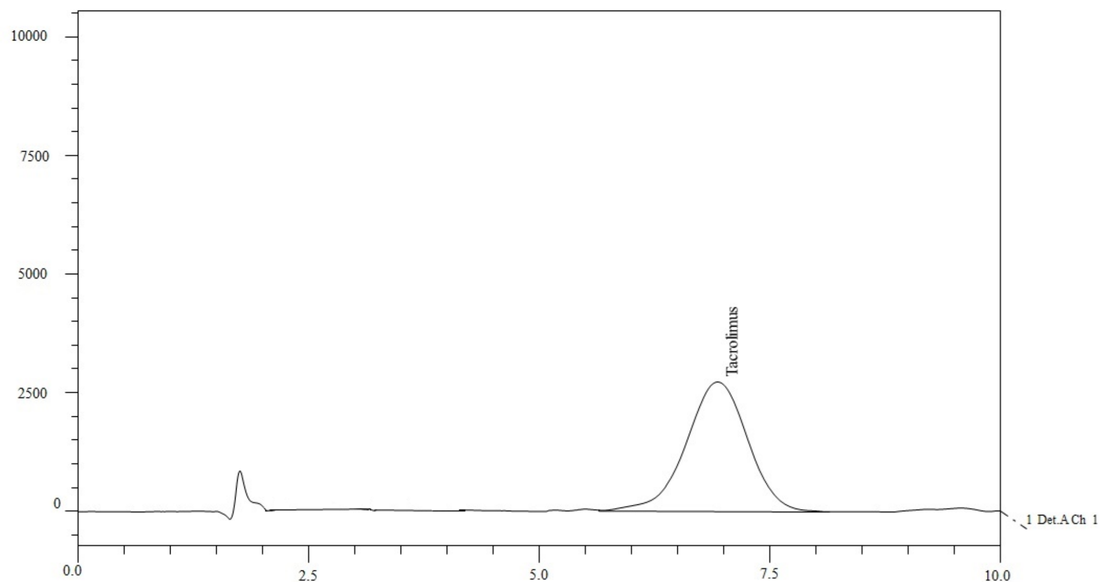


Figure 7.57: HPLC scan of TAC (standard)

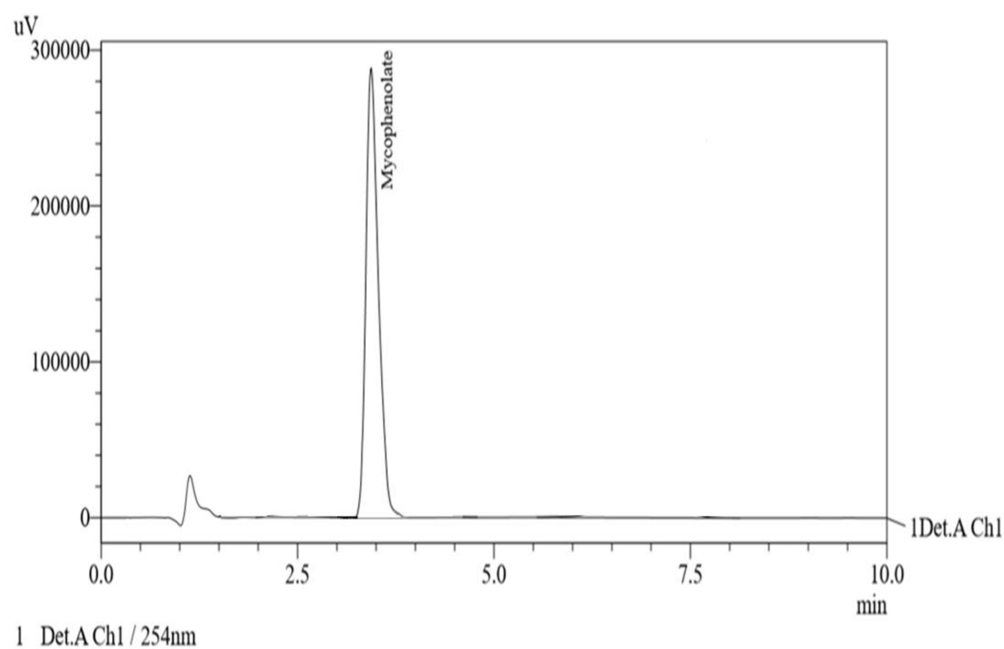


Figure 7.58: HPLC scan of MMF (standard)

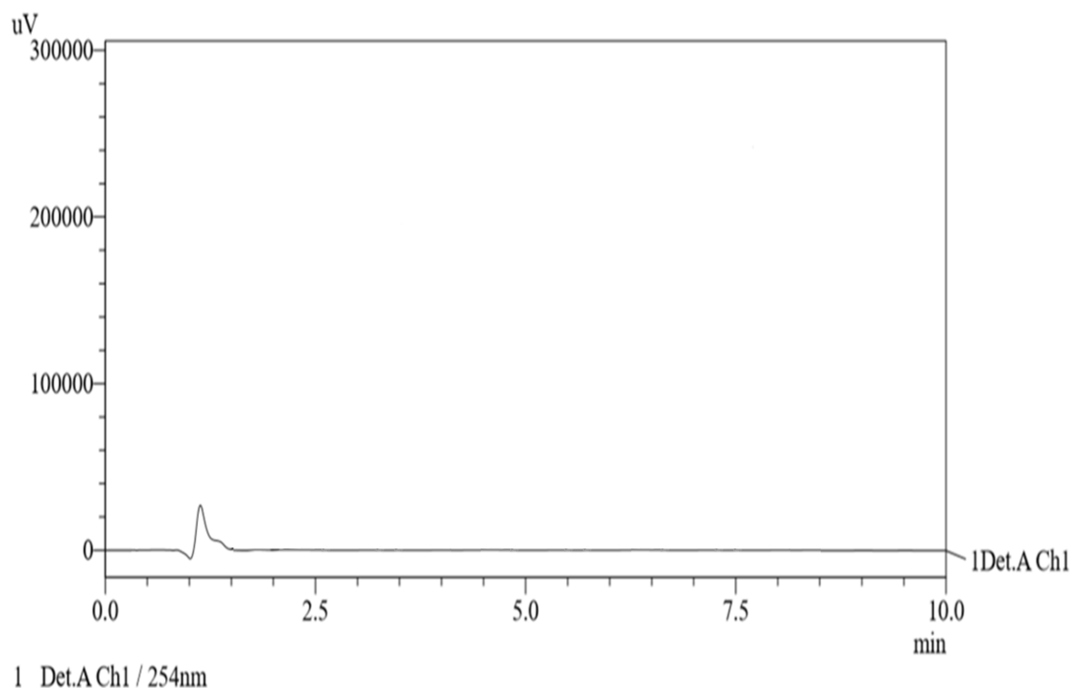


Figure 7.59: HPLC scan of placebo

7.19.9 Analysis and stability testing of formulations

A single peak was observed in the chromatogram of the PRED, MMF and TAC samples extracted from capsules. The peak purity was assessed. Good correlation (r=0.99) between the standard and the sample spectra of PRED, MMF and TAC. The absence of interference peaks of degradation products, impurities, and excipients indicate the specificity of the method. The drug contents of the PRED, MMF and TAC samples analyzed varies from 101.7 to 100.7, 100.6 to 100.2 and 98.6 to 98.1% respectively and summarized in Table. 8.61. In no case, the student's t-test showed a significant difference (P>0.05) between the drug content of the samples exposed to accelerated storage conditions as compared to initial drug content. So, no degradation occurred in the formulation during accelerated storage. The low S. D. value indicated the suitability of this method for routine analysis.

Table 7.50: Stability testing of formulation C14 (n=3)

Capsule Formulation	Drug content ± S.D., accelerated storage (40ºC/ 75% RH)			
	Initial (control)	1 month	3 months	6 months
PRED	101.7 ± 0.2	101.2^{b}±0.3	101.3^{b}±0.3	100.7^{b}±0.5
MMF	100.6 ± 1.4	100.7^{b}±0.2	100.4^{b}±0.4	100.2^{b}±0.2
TAC	98.6 ± 2.1	98.6^{b}±1.6	98.2^{b}±2.6	98.1^{b}±1.3

^{b} p> 0.05 v.s. intial (control), students t- test.

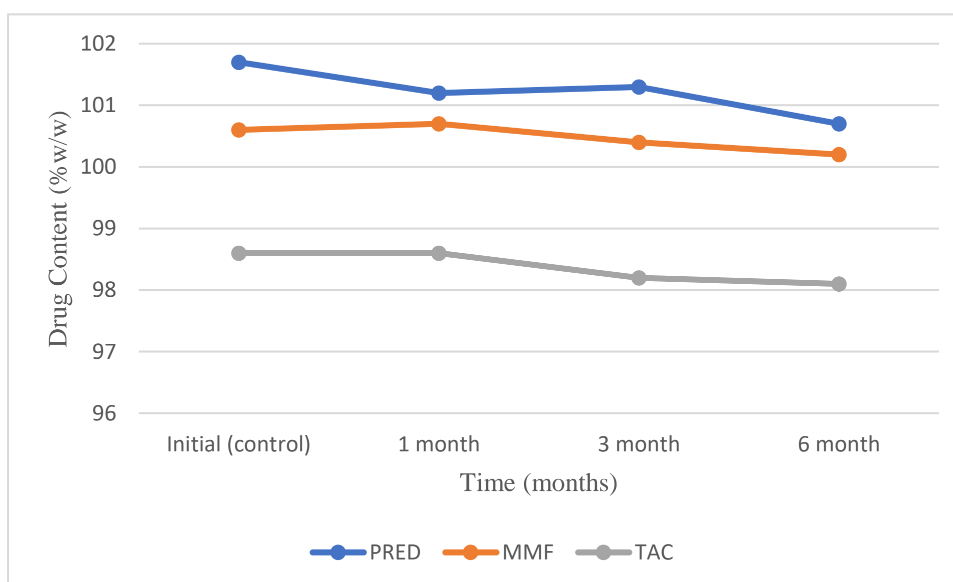


Figure 7.60: Stability of C14 formulation at different time intervals

7.19.10 System suitability

System suitability in each parameter of validation has been determined.

	Theoretical Plates			Tailing Factor		
	PRED	MMF	TAC	PRED	MMF	TAC
	5802.345	6207.287	3850.754	1.402	1.676	1.046
	5819.469	6254.456	3841.382	1.396	1.679	1.047
	5828.778	6258.51	3802.237	1.409	1.685	1.042
	6537.004	7027.706	3807.745	1.298	1.502	1.071
	6147.222	6553.147	3919.667	1.355	1.611	1.100
	6220.592	6635.247	3897.115	1.347	1.599	1.092
Mean	6059.235	6489.392	3853.150	1.368	1.625	1.066
SD	296.186	317.240	47.234	0.0427	0.071	0.025

7.19.10.1 Retention time and relative retention time

The retention time (RT) of PRED, MMF and TAC is 2.243, 3.391, 6.698 respectively.

Table 7.51: RT/ RRT data

Sr. No.	RT/RRT	Time
1.	MMF (RT)	3.391
2.	RRT1(PRED)	0.661
3.	RRT2(TAC)	1.975

The RRT data was calculated with respect to the MMF retention time.

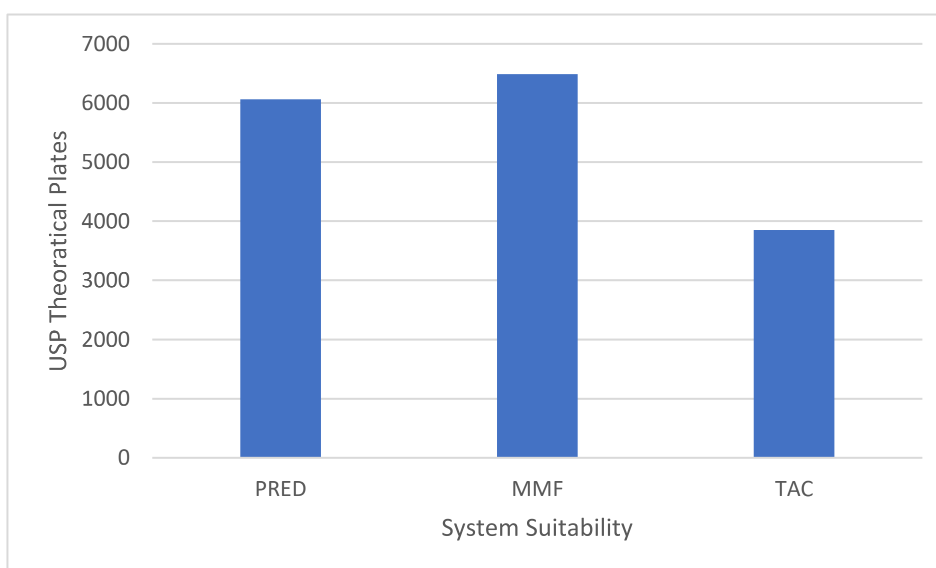


Figure 7.61: USP Theoretical plates in Robustness condition (>3000)

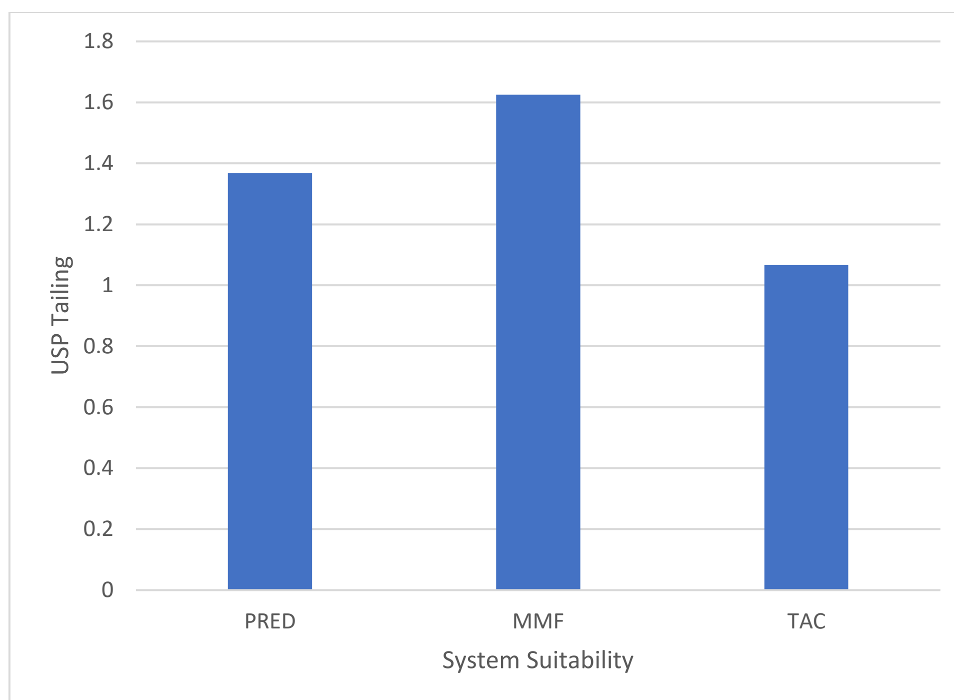


Figure 7.62: USP Tailing in Robustness condition (<2.0)

Result:

The system suitability data like theoretical plates, tailing factor and % RSD from the five injections of standard solutions were found to be (6059.235, 6489.392, 3853.150); (1.368, 1.625, 1.066); and (0.067, 0.046, and 0.680) respectively.

CHAPTER 8

IN-VIVO PHARMACOKINETIC STUDIES

To evaluate the in-vivo characteristics of optimized formulation, pharmacokinetic studies were performed.

8.1. Methods

8.1.1. Animals

Male Wistar rats were procured from animal house of NIPER, S.A.S. Nagar. The protocol of experimental work was approved by the Institutional Animal Ethics Committee (IAEC) of Lovely Professional University having IAEC approval no. LPU/IAEC/2018/Protocol No.: 31 and all animal experiments conducted were approved by the Committee for Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Government of India. Before experimentation, the animals were acclimatized in the Central Animal House Facility, Lovely Institute of Technology (Pharmacy), Lovely Professional University, Phagwara, Punjab (144411) (Reg. No.: 954/PO/Re/S/06/CPCSEA) for one week before the start of experimentation. Wistar rat weighing between 250-400 gm were selected for pharmacokinetic studies.

All the approved Wistar rats (37) were distributed in seven groups and received different treatments. The distribution and group details were mentioned in table 7.1.

8.1.2. Hydrolytic study

- The plasma was collected from male Wistar rats each weighing 350-400 g using a heparinized centrifuge tube and the plasma fraction was obtained by centrifugation at 4000 rpm for 10 min.

- Ten microliters of each of the Mycophenolate mofetil (MMF), Tacrolimus (TAC) and Prednisolone (PRED) stock methanolic solution was added to 5mL of plasma to start the reaction after preincubation for 5 min at 37ºC, and the concentration of MMF, TAC, and PRED in plasma was finally adjusted to 30 µM.

- After incubation under the air blow at 37ºC with shaking, 0.5mL of the incubation mixture was withdrawn at 2, 5, 10, and 20min, and immediately mixed with 1 mL of ice-cold methanol to stop the reaction at each sampling time.
- The average protein concentrations of plasma were determined by Lowry assay [226], which involved a two-step procedure. The first step was the Biuret reaction, and it involved the copper ion reduction (Cu^{2+} to Cu^{+}) in alkaline solutions by proteins. The second step was the Folin–Ciocalteu reagent reduction [227], that produces the specific blue color having absorbance maxima at 750 nm. This assay also shows the protein sequence variation, as color development was according to tyrosine, tryptophan, and to a lesser extent histidine, cysteine and cystine residues. [228, 229] The preparation of reagents and detailed procedure which has been used as described by Lowry et al. [226]

8.1.3. Pharmacokinetic study

8.1.3.1. Dosing Procedure

Granules were dosed intact to the rat by the following procedure. Rats were partially anesthetized using chloroform. Rats were restrained by grasping the scruff of the neck with one hand and the rear with the other hand. Rat's tail was wrapped around a small finger to secure the lower portion of the rat. The granules were placed in the center of the mouth using the holder follow the roof of the mouth to the opening of the esophagus. The rat's head was tilted back with the shaft of the sample holder. This straightens the esophagus and makes insertion of the makes the insertion of easier. After dosing, rats were dose with a few ml of water as this further facilitate movement of the granules into the stomach.

8.1.3.2. Blood Sampling Procedure

Collection Site: Tail Vein

The acceptable quantity and frequency of blood sampling were determined by the circulating blood volume and the red blood cell (RBC) turnover rate. Excessive blood collection may result in hypovolemic shock, physiological stress and even death of the

animal. Because it was necessary to take multiple samples, smaller blood volumes, i.e. 300 pi were drawn five times. Without fluid replacement, the maximum blood volume which can be safely removed for a one-time sample is 10% of the total blood volume or 5.5-7 ml/kg. For a 300 g rat, this was equivalent to 1.7-2.1 ml. For a 300 g rat if subcutaneous fluid replacement is done then collection volume can be increased equivalent to 2.5-3.2 ml.

Animal recovery: If sampling was done every two weeks, up to 10% of the total blood volume may be drawn or 5.5-7 ml/kg (4). For a 300 g rat, this is equivalent to about 1.7-2.1 ml every two weeks.

8.1.3.3. Sampling Procedure

• Tail vain sampling is recommended for collecting a large volume of a blood sample (up to 2ml /withdrawal)

• The animal was restrained properly.

• The tail was not rubbed from the base to the tip as it may result in leukocytosis. If the vein was not visible, the tail is dipped into warm water (40°C).

• Local anesthetic cream was applied on the surface of the tail 30 min before the experiment.

• A 23 G needle inserted into the blood vessel and blood is collected using a syringe with a needle. In case of difficulties, 0.5 to 1 cm of the surface of the skin is cut open, and blood is collected with a syringe with a needle.

• Having completed blood collection, silver nitrate ointment was applied to stop the bleeding.

• Each sample was immediately placed in an appendrop tube containing potassium EDTA equivalent to 2 mg/ml and refrigerated.

• Blood sample was frozen -20 ^{0}C until analyzed.

• Plasma Sample obtained by centrifugation of blood samples at 4000 RPM for 10 minutes.

8.2. Study Design

For this study, 36 rats were used and divided into six groups, O-E. After a single treatment blood sample were collected at 5, 15, 30, 60, and 180 min. after drug administration. All the blood samples will be immediately centrifuged to obtain the plasma fraction and will be stored at -20 ^{0}C until analysis. The area under the plasma concentration-time curves (AUC), C_{max} and T_{max} after oral administration will be calculated using the trapezoidal rule up to the last measured plasma concentration (3 h after dosing).

8.3. Preparation of Equivalent formulation for Rat Model

Rat LD_{50} of MMF, TAC and PRED is 352 mg/kg, 134-194 mg/kg and 10000 mg/kg respectively. Proportional Dose proportional formulation was prepared for ingestion into rat model. The whole process was similar to that of the original formulation.

Preparation of plasma samples: The plasma samples were subjected to liquid-liquid extraction before they were subjected to HPLC analysis. 0.5 mL plasma was taken to which 0.5 mL of chloroform was added. The mixture was vortexed for 2 min and then centrifuged for 10 min at 6000 rpm at 4 ℃. The supernatant was collected and evaporated to dryness and reconstituted with 0.5 mL of mobile phase. The developed HPLC method was used for drugs quantification in plasma samples. The in-vivo Study design is mentioned in table 7.1.

8.4. Pharmacokinetic studies of prepared formulation

The immediate release formulation of MMF, TAC, and PRED was evaluated for pharmacokinetic studies in rat for 3 hrs after oral administration.

8.5. Statistical Analysis

All data are reported as mean ± SD (standard deviation). Non-Compartmental analysis of plasma data was evaluated. The pharmacokinetic parameters of prepared formulation in the rat after oral administration of the drug was recorded.

Table 8.1: In-vivo Study design

Groups	**Treatment**	**Dose**	**Number of animals**
For Hydrolytic Study			
1	--	--	1
For Pharmacokinetic Study			
O	Control		6
A	MMF	25.830 $mgkg^{-1}$ (Oral)	6
B	TAC	0.052 $mgkg^{-1}$ (Oral)	6
C	PRED	1.030 $mgkg^{-1}$ (Oral)	6
D	MMF+ TAC+PRED	26.912 $mgkg^{-1}$ (Oral)	6
E	Placebo		6
		Total Animals	**37**

Results

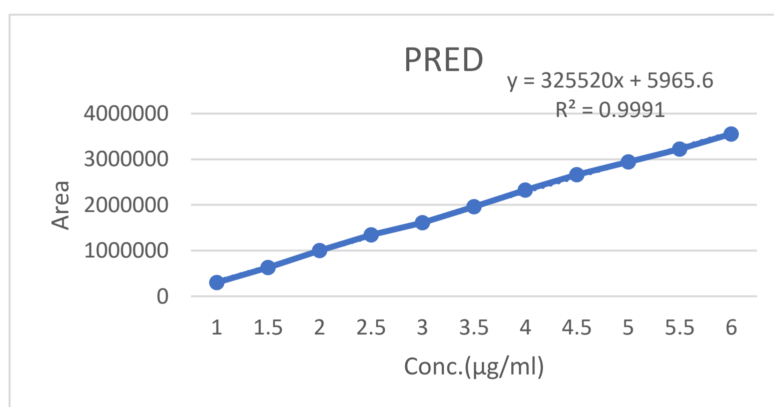
The present study revealed that the developed bioanalytical method was sensitive. The results of pharmacokinetic parameters are shown and discussed below.

Administration of prepared formulation was not able to affect the level of tissue protein significantly, even no alteration in the level of protein was observed in any group. The hydrolysis of drugs: 50.7% of MMF was hydrolyzed to MPA within 2min, and 97.3% was hydrolyzed within 10 min in rat plasma, TAC was poorly absorbed from the gut and generally hydrolyzed in acidic conditions. Whereas, no change in the initial concentration of prednisolone was found. The average protein concentration in plasma was 69.4 mg mL^{-1}.

For the standard curve, each drug was spiked into the plasma and recorded using developed RP-HPLC method and given in Table 8.2.

Table 8.2: Standard curve data of Prednisolone, Mycophenolate, and Tacrolimus in rat plasma

Conc. (µg/ml)	Area	Conc. (µg/ml)	Area	Conc. (µg/ml)	Area
	PRED		MMF		TAC
1.0	300909	50	190458	0.05	112217
1.5	632714	60	368734	0.1	258934
2.0	1000415	70	606681	0.15	411114
2.5	1344521	80	774200	0.2	518280
3.0	1607707	90	941683	0.25	654234
3.5	1958530	100	1137197	0.3	777109
4.0	2324651	110	1290774	0.35	923121
4.5	2659003	120	1497170	0.4	1041353
5.0	2942779	130	1658447	0.45	1168071
5.5	3223903	140	1847145	0.5	1282761
6.0	3554792	150	2060250	0.55	1398771



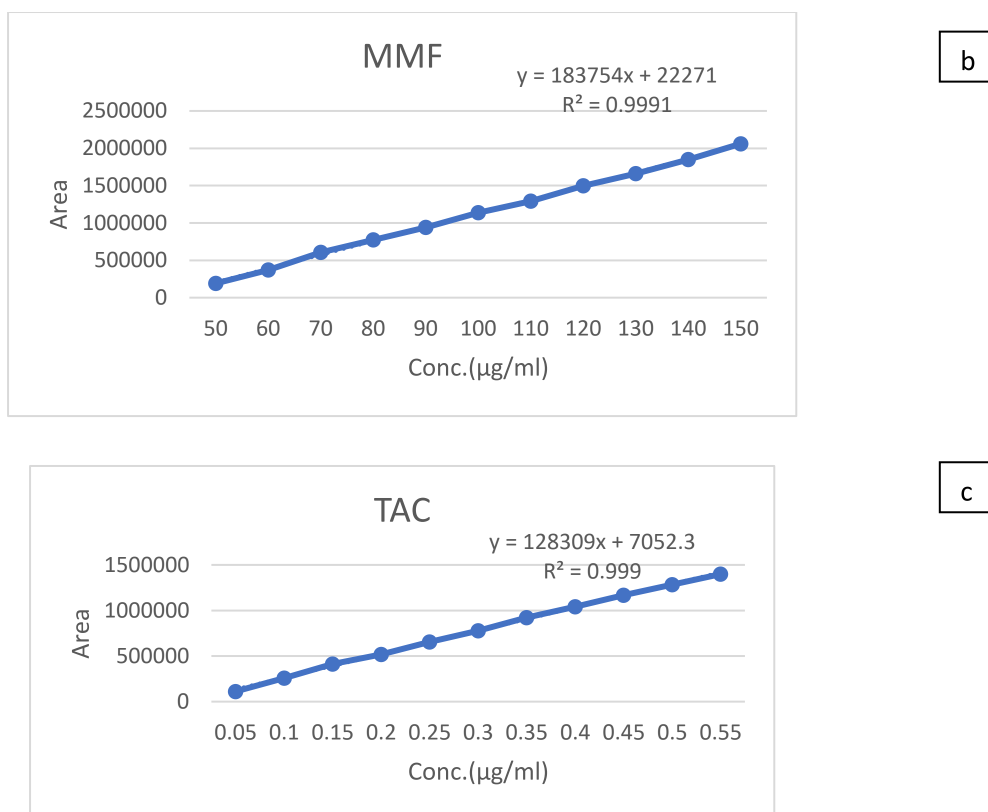


Figure 8.1: Standard curve of Prednisolone, Mycophenolate, and Tacrolimus by RP-HPLC

The individual concentration of each of the drug was calculated and data was recorded in Table 8.3.

Table 8.3: The individual plasma drug concentrations with respect to time

	PRED				MMF				TAC			
Time (min.)	Avg Area	Conc. *	Std Dev	% RSD	Avg Area	Conc. *	Std Dev	%RSD	Avg Area	Conc. *	Std Dev	%RSD
5	62866.7	2	45.09	0.07	63280	2	20	0.03	38130.7	3	95.3	0.25
15	128350	4	51.5	0.04	172657	8	383.8	0.22	155053	12	96.27	0.06
30	488254	15	57.27	0.01	756946	40	64.93	0.01	468035	36	57.33	0.01
60	292051	9	62.78	0.02	611152	32	154.04	0.03	233363	18	66.58	0.03
120	193758	6	54.63	0.03	428540	22	425.32	0.1	181433	14	201	0.11
180	128293	4	25.17	0.02	354908	18	75.99	0.02	76520.8	6	1.39	0

*Conc is in µg/mL

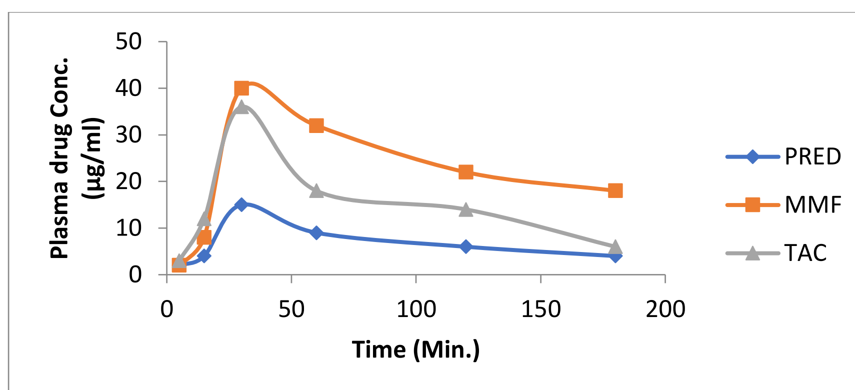


Figure 8.2: Time courses of plasma levels after oral administration of PRED, MMF, and TAC

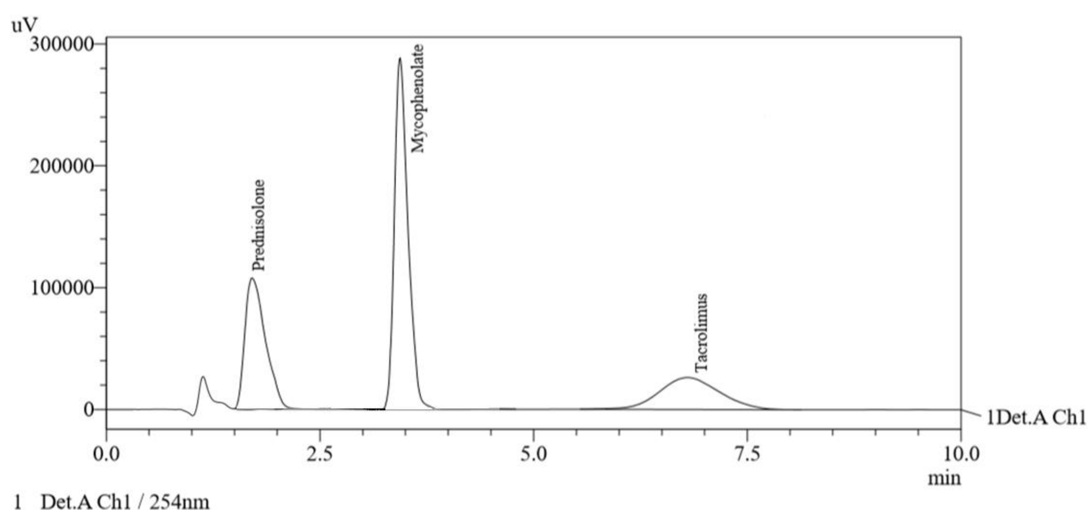


Figure 8.3: HPLC chromatogram of drug concentration in plasma

Table 8.4: Pharmacokinetic parameters of the prepared capsule formulation

Parameters	PRED	MMF	TAC
T_{max} (min)	30	30	30
C_{max} (µg/mL)	15	40	36
AUC_{0-3} (µg hr/mL)	1287.5	4315	2812.5
$AUC_{0-\infty}$ (µg hr/mL)	1323.86	4765	2852.5

It is clear that C_{max} of PRED, MMF, and TAC was 15, 40 and 36 µg/mL. The T_{max} of each drug has been achieved at 30 mins of formulation administration. The AUC_{0-3} for PRED, MMF, and TAC was 1287.5, 4315 and 2812.5 µg hr/mL and $AUC_{0-\infty}$ was 1323.86, 4765 and 2852.5 µg hr/mL respectively.

CHAPTER 9

SUMMARY AND CONCLUSION

Chemically MMF is 2-morpholinoethyl (Z)-6-(4-hydroxy-6-methoxy-7-methyl-3-oxo-1,3-dihydroisobenzofuran-5-yl)-4-methylhex-4-enoate. Pharmacologically, it belongs to Antiproliferative drugs and provides the immunosuppression.

Chemically TAC is (1R,9S,12S,13R,14S,17R,21S,23S,24R,25S,27R)-1,14-dihydroxy-12-[(1E)-1-[(1R,3R,4R)-4-hydroxy-3-methoxycyclohexyl]prop-1-en-2-yl]-23,25-dimethoxy-13,19,21,27-tetramethyl-17-(prop-2-en-1-yl)-11,28-dioxa-4-azatricyclo [22.3.1.0^{4,9}]octacos-18-ene-2,3,10,16-tetrone. Pharmacologically, it is a Calcineurin inhibitor and provides the immunosuppression.

Chemically PRED is (8S,9S,10R,13S,14S,17R)-11,17-dihydroxy-17-(2-hydroxyacetyl)- 10,13-dimethyl-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-3-one. Pharmacologically, it is a corticosteroid.

These drugs are used generally in the combination with other drugs either from the same category of other to overcome the solid organ transplant rejection. Clinically there are many combinations tried; in some cases, these experiments are successful to reduce the solid organ transplant rejection rate. It was found that the acute rejection is more than the later stage rejection as the patient restrict to respond towards initial therapy. So, a regular hunt is going on to find a new effective immunosuppressant. Out of these drugs it was found that the Tacrolimus (TAC), Mycophenolate mofetil (MMF) along with Prednisolone (PRED) provides effective treatment in case of sloid organ transplantation. None of the combination is available in the market.

An attempt has been made to provide the simultaneous estimation of these drugs in bulk or formulations along with a cost-effective and elegant formulation of immunosuppressants with the concern of patient compliance.

The formulation was prepared using wet granulation method using HPMC K-100 for rapid solubility.

The capsule formulation (containing granules) C14 was selected as it shows maximum drug release Prednisolone: 99.9931, Mycophenolate mofetil: 98.2221, Tacrolimus: 100.0001 after 30 minutes.

Further, in-vivo studies were carried out on optimized formulation (C14). The C_{max} of PRED, MMF, and TAC was 15, 40 and 36 µg/mL. The AUC_{0-3} for PRED, MMF, and TAC was 1287.5, 4315 and 2812.5 µg hr/mL and $AUC_{0-\infty}$ was 1323.86, 4765 and 2852.5 µg hr/mL respectively.

The future prospective of the present work can be combination of different polymers which can enhance the solubility of these drugs; may also enhances the bioavailability and efficacy of this combined formulation to a greater extent.

CHAPTER 10

REFERENCES

1. Chakraborty, P., *A textbook of Microbiology*. 2005: New Central Book Agency.
2. Owen, J.A., Punt, J., and Stranford, S.A., *Kuby immunology*. 2013: WH Freeman New York.
3. Rankin, L.C. and Artis, D., *Beyond Host Defense: Emerging Functions of the Immune System in Regulating Complex Tissue Physiology.* Cell, 2018. **173**(3): p. 554-567.
4. Ballington, D.A., Laughlin, M.M., and McKennon, S.A., *Pharmacology for Technicians*. 1999: EMCParadigm.
5. Moran, I., Avery, D.T., Payne, K., et al., *B cell–intrinsic requirement for STK4 in humoral immunity in mice and human subjects.* J Allergy Clin Immunol, 2019: p. 1-4.
6. Qaqish, S.S., Zuckerman, J.E., Danovitch, G.M., et al., *Acute Kidney Injury in a Patient Following Kidney Transplantation.* Am J Kidney Dis, 2019. **73**(1): p. A15-A19.
7. Danovitch, G.M., *Handbook of kidney transplantation*. 2009: Lippincott Williams & Wilkins.
8. Larsen, C.P., Elwood, E.T., Alexander, D.Z., et al., *Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways.* Nature, 1996. **381**(6581): p. 434-438.
9. Flechner, S.M., Modlin, C.S., Serrano, D.P., et al., *Determinants of chronic renal allograft rejection in cyclosporine-treated recipients.* Transplantation, 1996. **62**(9): p. 1235-1241.
10. Ravichandran, N., *Nadiad Kidney Hospital: Revamping Systems and Processes for Growth.* Vikalpa, 2008. **33**(4): p. 111-124.
11. Meier-Kriesche, H.U., Li, S., Gruessner, R., et al., *Immunosuppression: evolution in practice and trends, 1994–2004.* American Journal of Transplantation, 2006. **6**(5p2): p. 1111-1131.

12. Katzung, B.G. and Trevor, A.J., *Basic & clinical pharmacology*. 2015: McGraw-Hill New York, NY.
13. Matas, A.J., Smith, J.M., Skeans, M.A., et al., *OPTN/SRTR 2012 Annual Data Report: kidney.* Am J Transplant, 2014. **14 Suppl 1**(S1): p. 11-44.
14. Vincenti, F., Friman, S., Scheuermann, E., et al., *Results of an international, randomized trial comparing glucose metabolism disorders and outcome with cyclosporine versus tacrolimus.* Am J Transplant, 2007. **7**(6): p. 1506-1514.
15. Larson, T.S., Dean, P.G., Stegall, M.D., et al., *Complete avoidance of calcineurin inhibitors in renal transplantation: a randomized trial comparing sirolimus and tacrolimus.* Am J Transplant, 2006. **6**(3): p. 514-522.
16. Trompeter, R., Filler, G., Webb, N.J., et al., *Randomized trial of tacrolimus versus cyclosporin microemulsion in renal transplantation.* Pediatr Nephrol, 2002. **17**(3): p. 141-149.
17. Meier-Kriesche, H.U., Morris, J.A., Chu, A.H., et al., *Mycophenolate mofetil vs azathioprine in a large population of elderly renal transplant patients.* Nephrol Dial Transplant, 2004. **19**(11): p. 2864-2869.
18. Kumar, M.S.A., Saeed, M.I., Ranganna, K., et al., *Comparison of four different immunosuppression protocols without long-term steroid therapy in kidney recipients monitored by surveillance biopsy: five-year outcomes.* Transplant immunology, 2008. **20**(1-2): p. 32-42.
19. Johnson, C., Ahsan, N., Gonwa, T., et al., *Randomized Trial of Tacrolimus (prograf) In Combination With Azathioprine or Mychophenolate Mofetil Versus Cyclosporine (neoral) With Mycophenolate Mofetil After Cadaveric Kidney Transplantation.* Transplantation, 2000. **69**(5): p. 834-841.
20. Ciancio, G., Burke, G.W., Gaynor, J.J., et al., *A randomized long-term trial of tacrolimus/sirolimus versus tacrolimums/mycophenolate versus cyclosporine/sirolimus in renal transplantation: three-year analysis.* Transplantation, 2006. **81**(6): p. 845-852.
21. Lv, C., Chen, M., Xu, M., et al., *Influencing factors of new-onset diabetes after a renal transplant and their effects on complications and survival rate.* PloS one, 2014. **9**(6): p. e99406.

22. Cosio, F.G., Kudva, Y., van der Velde, M., et al., *New onset hyperglycemia and diabetes are associated with increased cardiovascular risk after kidney transplantation.* Kidney Int, 2005. **67**(6): p. 2415-2421.
23. Hagen, M., Hjelmesaeth, J., Jenssen, T., et al., *A 6-year prospective study on new onset diabetes mellitus, insulin release and insulin sensitivity in renal transplant recipients.* Nephrol Dial Transplant, 2003. **18**(10): p. 2154-2159.
24. David-Neto, E., Lemos, F.C., Fadel, L.M., et al., *The dynamics of glucose metabolism under calcineurin inhibitors in the first year after renal transplantation in nonobese patients.* Transplantation, 2007. **84**(1): p. 50-55.
25. Hur, K.Y., Kim, M.S., Kim, Y.S., et al., *Risk factors associated with the onset and progression of posttransplantation diabetes in renal allograft recipients.* Diabetes Care, 2007. **30**(3): p. 609-615.
26. Porrini, E., Moreno, J.M., Osuna, A., et al., *Prediabetes in patients receiving tacrolimus in the first year after kidney transplantation: a prospective and multicenter study.* Transplantation, 2008. **85**(8): p. 1133-1138.
27. Ram, R., Dakshina Murty, K., and Prasad, N., *Time table of infections after renal transplantation–South Indian experience.* Indian J Nephrol, 2005. **15**(Suppl 2): p. S14-S21.
28. Alangaden, G.J., Thyagarajan, R., Gruber, S.A., et al., *Infectious complications after kidney transplantation: current epidemiology and associated risk factors.* Clin Transplant, 2006. **20**(4): p. 401-409.
29. Parada, B., Figueiredo, A., Nunes, P., et al. *Pediatric renal transplantation: comparative study with renal transplantation in the adult population*. in *Transplantation proceedings*. 2005. Elsevier.
30. Abou-Jaoude, M.M., Khoury, M., Nawfal, N., et al., *Effect of recipient age on the outcome of kidney transplantation.* Transpl Immunol, 2009. **20**(3): p. 118-120.
31. Roitt, I.M., Brostoff, J., and Male, D., *Immunology*. 8 ed. 2012: Saunders.
32. Tripathi, K., *Essentials of medical pharmacology*. 2013: JP Medical Ltd.
33. Meier-Kriesche, H.U., Ojo, A.O., Cibrik, D.M., et al., *Relationship of recipient age and development of chronic allograft failure.* Transplantation, 2000. **70**(2): p. 306-310.

34. Mourad, G., Garrigue, V., Squifflet, J.P., et al., *Induction versus noninduction in renal transplant recipients with tacrolimus-based immunosuppression.* Transplantation, 2001. **72**(6): p. 1050-1055.

35. Ciancio, G., Burke, G.W., Suzart, K., et al., *Daclizumab induction, tacrolimus, mycophenolate mofetil and steroids as an immunosuppression regimen for primary kidney transplant recipients.* Transplantation, 2002. **73**(7): p. 1100-1106.

36. Lebranchu, Y., Bridoux, F., Buchler, M., et al., *Immunoprophylaxis with basiliximab compared with antithymocyte globulin in renal transplant patients receiving MMF-containing triple therapy.* Am J Transplant, 2002. **2**(1): p. 48-56.

37. Tesi, R.J., Elkhammas, E.A., Davies, E.A., et al., *Renal transplantation in older people.* Lancet, 1994. **343**(8895): p. 461-464.

38. Gjertson, D., *A multi-factor analysis of kidney graft outcomes at one and five years posttransplantation: 1996 UNOS Update.* Clinical transplants, 1996: p. 343-360.

39. Kendrick, E., *Cardiovascular disease and the renal transplant recipient.* Am J Kidney Dis, 2001. **38**(6 Suppl 6): p. S36-S43.

40. Roodnat, J.I., Zietse, R., Mulder, P.G., et al., *The vanishing importance of age in renal transplantation.* Transplantation, 1999. **67**(4): p. 576-580.

41. Ismail, N., Hakim, R.M., and Helderman, J.H., *Renal replacement therapies in the elderly: Part II. Renal transplantation.* Am J Kidney Dis, 1994. **23**(1): p. 1-15.

42. Kappes, U., Schanz, G., Gerhardt, U., et al., *Influence of age on the prognosis of renal transplant recipients.* Am J Nephrol, 2001. **21**(4): p. 259-263.

43. Rathee, P., Chaudhary, H., Rathee, S., et al., *Immunosuppressants: A Review.* The Pharma Innovation, 2013. **1**(12, Part A): p. 90-101.

44. Podczeck, F. and Jones, B.E., *Pharmaceutical capsules*. 2004, London, UK: Pharmaceutical Press.

45. Michael, E.A., *Pharmaceutics: The science of dosage form design*. 2002, London, UK: Churchill Livingstone. 205-210, 365-395.

46. Sharp, J., *Quality in the Manufacture of Medicines and other Healthcare Products*. 2000, London, UK: Pharmaceutical press.
47. Durgin, J.M. and Hanan, Z.I., *Pharmacy practice for technicians*. 4 ed. 2008, Clifton Park, NY, USA: Cengage Learning.
48. Allen, L. and Ansel, H.C., *Ansel's pharmaceutical dosage forms and drug delivery systems*. 2010: Lippincott Williams & Wilkins.
49. Stellman, J.M., *Encyclopaedia of occupational health and safety*. Vol. 1. 1998: International Labour Organization.
50. Gad, S.C., *Pharmaceutical manufacturing handbook: production and processes*. Vol. 5. 2008: John Wiley & Sons.
51. Bennett, B. and Cole, G., *Pharmaceutical production: an engineering guide*. 2003: IChemE.
52. Desai, D., Ranadive, S., Lozano, R., et al., *Dissolution instability of encapsulated marketed tablets.* International journal of pharmaceutics, 1996. **144**(2): p. 153-158.
53. Mallory, A. and Schaefer, J.W., *Clinitest ingestion.* Br Med J, 1977. **2**(6079): p. 105-107.
54. Schulz, T., Martin, D., Heimes, M., et al. *Tacrolimus/mycophenolate mofetil/steroid-based immunosuppression after pancreas-kidney transplantation with single shot antithymocyte globulin*. in *Transplantation proceedings*. 1998. Elsevier.
55. Schulz, T., Konzack, J., and Büsing, M. *Mycophenolate mofetil/prednisolone/single-shot ATG with tacrolimus or cyclosporine in pancreas/kidney transplantation: first results of an ongoing prospective randomized trial*. in *Transplantation proceedings*. 1999. Elsevier.
56. Agarwal, D., Gulati, S., Mehta, A., et al., *Mycophenolate mofetil as an effective alternative to cyclosporin in post-transplant haemolytic uraemic syndrome.* Nephrology Dialysis Transplantation, 2000. **15**(12): p. 2064-2065.
57. Kim, H., Park, S., Kim, H., et al. *Comparison of the safety and efficacy of mycophenolate mofetil, prednisolone and cyclosporine and conventional cyclosporine and prednisolone in kidney transplantation*. in *Transplantation proceedings*. 2000. Elsevier.

58. Bagga, A., Hari, P., Moudgil, A., et al., *Mycophenolate mofetil and prednisolone therapy in children with steroid-dependent nephrotic syndrome.* Am J Kidney Dis, 2003. **42**(6): p. 1114-1120.

59. Borrows, R., Loucaidou, M., Van Tromp, J., et al. *Steroid sparing in renal transplantation with tacrolimus and mycophenolate mofetil: three-year results*. in *Transplantation proceedings*. 2005. Elsevier.

60. Kalble, T., Lucan, M., Nicita, G., et al., *EAU guidelines on renal transplantation.* Eur Urol, 2005. **47**(2): p. 156-166.

61. Taylor, A.L., Watson, C.J., and Bradley, J.A., *Immunosuppressive agents in solid organ transplantation: Mechanisms of action and therapeutic efficacy.* Crit Rev Oncol Hematol, 2005. **56**(1): p. 23-46.

62. Meier-Kriesche, H.U., Steffen, B.J., Hochberg, A.M., et al., *Long-term use of mycophenolate mofetil is associated with a reduction in the incidence and risk of late rejection.* Am J Transplant, 2003. **3**(1): p. 68-73.

63. Larson, T.S., Dean, P.G., Stegall, M.D., et al., *Complete avoidance of calcineurin inhibitors in renal transplantation: a randomized trial comparing sirolimus and tacrolimus.* Am J Transplant, 2006. **6**(3): p. 514-22.

64. Webster, A.C., Woodroffe, R.C., Taylor, R.S., et al., *Tacrolimus versus ciclosporin as primary immunosuppression for kidney transplant recipients: meta-analysis and meta-regression of randomised trial data.* Bmj, 2005. **331**(7520): p. 1-11.

65. Krämer, B.K., Montagnino, G., del Castillo, D., et al., *Efficacy and safety of tacrolimus compared with cyclosporin A microemulsion in renal transplantation: 2 year follow-up results.* Nephrol Dial Transplant, 2005. **20**(5): p. 968-973.

66. Vincenti, F., Jensik, S.C., Filo, R.S., et al., *A long-term comparison of tacrolimus (FK506) and cyclosporine in kidney transplantation: evidence for improved allograft survival at five years1.* Transplantation, 2002. **73**(5): p. 775-782.

67. Knoll, G.A. and Bell, R.C., *Tacrolimus versus cyclosporin for immunosuppression in renal transplantation: meta-analysis of randomised trials.* Bmj, 1999. **318**(7191): p. 1104-1107.

68. Mayer, A.D., Dmitrewski, J., Squifflet, J.-P., et al., *Multicenter randomized trial comparing tacrolimus (fk506) and cyclosporine in the prevention of renal allograft rejection1: A report of the european tacrolimus multicenter renal study group.* Transplantation, 1997. **64**(3): p. 436-443.
69. Sollinger, H., *Mycophenolate mofetil for the prevention of acute rejection in primary cadaveric renal allograft recipients. US Renal Transplant Mycophenolate Mofetil Study Group.* Transplantation, 1995. **60**(3): p. 225-232.
70. Group, E.M.M.C.S., *Mycophenolate mofetil in renal transplantation: 3-year results from the placebo-controlled trial1.* Transplantation, 1999. **68**(3): p. 391-396.
71. Goldfarb-Rumyantzev, A.S., Smith, L., Shihab, F.S., et al., *Role of maintenance immunosuppressive regimen in kidney transplant outcome.* Clin J Am Soc Nephrol, 2006. **1**(3): p. 563-574.
72. Kunz, R. and Neumayer, H.H., *Maintenance therapy with triple versus double immunosuppressive regimen in renal transplantation: a meta-analysis.* Transplantation, 1997. **63**(3): p. 386-392.
73. Difrancesco, R., Frerichs, V., Donnelly, J., et al., *Simultaneous determination of cortisol, dexamethasone, methylprednisolone, prednisone, prednisolone, mycophenolic acid and mycophenolic acid glucuronide in human plasma utilizing liquid chromatography-tandem mass spectrometry.* J Chromatogr B Analyt Technol Biomed Life Sci, 2007. **859**(1): p. 42-51.
74. Gopalakrishnana, S., Vadivela, E., Krishnavenia, P., et al., *A novel reverse phase-HPLC method development and validation of Mycophenolate sodium-an immunosuppressant drug.* Research Journal of Pharmaceutical, Biological and Chemical Sciences, 2010. **1**(4): p. 200-207.
75. Patel, P., Panchal, S., Mehta, T., et al., *Reversed-phase high performance liquid chromatographic (RP-HPLC) method for determination of tacrolimus in bulk and pharmaceutical formulation.* Int. J. Pharm. Pharm. Sci, 2011. **3**(4): p. 220-222.
76. Upadhyay, V., Trivedi, V., Shah, G., et al., *Rapid and Sensitive UPLC–MS-MS Determination of Tacrolimus in Wistar Rats and Human Blood.* J Chromatogr Sci, 2012. **52**(1): p. 59-67.

77. Shi, Q., Li, J., and Ding, F., *Development and validation of method for the determination of related substances of Tacrolimus in Tacrolimus capsules and degradation studies.* Int J ChemTech Res, 2012. **4**: p. 1543-1552.
78. Kunithala, V.K., Chinthakindi, K.K., Vemula, S.K., et al., *A new rapid and simple analytical method development and validation of estimation the mycophenolate in dosage form by UPLC technique.* Asian J Pharm Clin Res, 2012. **5**: p. 233-237.
79. VijayaKumari, M., Anusha Devi, V., Nareshbabu, A., et al., *Anjaneyulu. New stability indicating RP-HPLC method for estimation of Mycophenolate mofetil capsule in pharmaceutical dosage form.* IRJPS, 2012. **5**(3): p. 149-154.
80. Abhilash, P.R., Kumar, K.V., and Kumar, V.S., *Development and validation of new RP-UPLC method for the quantitative analysis of mycophenolate in tablet dosage form.* Br J Pharm Res, 2013. **3**(3): p. 363-373.
81. Musuamba, F.T., Mourad, M., Haufroid, V., et al., *A simultaneous d-optimal designed study for population pharmacokinetic analyses of mycophenolic Acid and tacrolimus early after renal transplantation.* J Clin Pharmacol, 2012. **52**(12): p. 1833-1843.
82. Yamaguchi, K., Fukuoka, N., Kimura, S., et al., *Limited Sampling Strategy for the Estimation of Mycophenolic Acid Area under the Concentration–Time Curve Treated in Japanese Living-Related Renal Transplant Recipients with Concomitant Extended-Release Tacrolimus.* Biol Pharm Bull, 2013. **36**(6): p. 1036-1039.
83. Cai, W., Ye, C., Sun, X., et al., *Limited Sampling Strategy for Predicting Area Under the Concentration–Time Curve for Mycophenolic Acid in Chinese Adults Receiving Mycophenolate Mofetil and Tacrolimus Early After Renal Transplantation.* Ther Drug Monit, 2015. **37**(3): p. 304-310.
84. Lee, W.A., Gu, L., Miksztal, A.R., et al., *Bioavailability improvement of mycophenolic acid through amino ester derivatization.* Pharmaceutical research, 1990. **7**(2): p. 161-166.
85. Quinn, C.M., Bugeja, V.C., Gallagher, J.A., et al., *The effect of mycophenolic acid on the cell cycle ofCandida albicans.* Mycopathologia, 1990. **111**(3): p. 165-168.

86. Kitchin, J.E., Pomeranz, M.K., Pak, G., et al., *Rediscovering mycophenolic acid: a review of its mechanism, side effects, and potential uses.* J Am Acad Dermatol, 1997. **37**(3 Pt 1): p. 445-449.

87. Kim, M., Rostas, S., and Gabardi, S., *Mycophenolate fetal toxicity and risk evaluation and mitigation strategies.* Am J Transplant, 2013. **13**(6): p. 1383-1389.

88. Korecka, M., Nikolic, D., Van Breemen, R.B., et al., *The apparent inhibition of inosine monophosphate dehydrogenase by mycophenolic acid glucuronide is attributable to the presence of trace quantities of mycophenolic acid.* Clinical chemistry, 1999. **45**(7): p. 1047-1050.

89. Shipkova, M., Armstrong, V.W., Wieland, E., et al., *Identification of glucoside and carboxyl-linked glucuronide conjugates of mycophenolic acid in plasma of transplant recipients treated with mycophenolate mofetil.* Br J Pharmacol, 1999. **126**(5): p. 1075-1082.

90. Nowak, I. and Shaw, L.M., *Mycophenolic acid binding to human serum albumin: characterization and relation to pharmacodynamics.* Clin Chem, 1995. **41**(7): p. 1011-1017.

91. Kaplan, B., Meier-Kriesche, H.U., Friedman, G., et al., *The effect of renal insufficiency on mycophenolic acid protein binding.* J Clin Pharmacol, 1999. **39**(7): p. 715-720.

92. Shaw, L.M., Korecka, M., van Breeman, R., et al., *Analysis, pharmacokinetics and therapeutic drug monitoring of mycophenolic acid.* Clin Biochem, 1998. **31**(5): p. 323-328.

93. Holt, D.W., *Monitoring mycophenolic acid.* Ann Clin Biochem, 2002. **39**(Pt 3): p. 173-183.

94. Mourad, M., Malaise, J., Eddour, D.C., et al., *Pharmacokinetic basis for the efficient and safe use of low-dose mycophenolate mofetil in combination with tacrolimus in kidney transplantation.* Clinical chemistry, 2001. **47**(7): p. 1241-1248.

95. Schutz, E., Shipkova, M., Armstrong, V.W., et al., *Identification of a pharmacologically active metabolite of mycophenolic acid in plasma of*

transplant recipients treated with mycophenolate mofetil. Clin Chem, 1999. **45**(3): p. 419-422.

96. Sollinger, H.W., *Mycophenolates in transplantation.* Clin Transplant, 2004. **18**(5): p. 485-492.
97. Wieland, E., Shipkova, M., Schellhaas, U., et al., *Induction of cytokine release by the acyl glucuronide of mycophenolic acid: a link to side effects?* Clin Biochem, 2000. **33**(2): p. 107-113.
98. Franklin, T.J. and Cook, J.M., *The inhibition of nucleic acid synthesis by mycophenolic acid.* Biochem J, 1969. **113**(3): p. 515-524.
99. Wu, J.C., *Mycophenolate mofetil: molecular mechanisms of action.* Perspectives in drug discovery and design, 1994. **2**(1): p. 185-204.
100. Eugui, E.M. and Allison, A.C., *Immunosuppressive activity of mycophenolate mofetil.* Ann N Y Acad Sci, 1993. **685**(1): p. 309-329.
101. Allison, A.C. and Eugui, E.M., *Purine metabolism and immunosuppressive effects of mycophenolate mofetil (MMF).* Clin Transplant, 1996. **10**(1 Pt 2): p. 77-84.
102. Filler, G., Lepage, N., Delisle, B., et al., *Effect of cyclosporine on mycophenolic acid area under the concentration-time curve in pediatric kidney transplant recipients.* Ther Drug Monit, 2001. **23**(5): p. 514-519.
103. van Gelder, T., Klupp, J., Barten, M.J., et al., *Comparison of the effects of tacrolimus and cyclosporine on the pharmacokinetics of mycophenolic acid.* Ther Drug Monit, 2001. **23**(2): p. 119-128.
104. Taylor, A.L., Watson, C.J., and Bradley, J.A., *Immunosuppressive agents in solid organ transplantation: Mechanisms of action and therapeutic efficacy.* Critical reviews in oncology/hematology, 2005. **56**(1): p. 23-46.
105. Vidal, E., Cantarell, C., Capdevila, L., et al., *Mycophenolate mofetil pharmacokinetics in transplant patients receiving cyclosporine or tacrolimus in combination therapy.* Pharmacol Toxicol, 2000. **87**(4): p. 182-184.
106. Shipkova, M., Armstrong, V.W., Kuypers, D., et al., *Effect of cyclosporine withdrawal on mycophenolic acid pharmacokinetics in kidney transplant recipients with deteriorating renal function: preliminary report.* Ther Drug Monit, 2001. **23**(6): p. 717-721.

107. Schmidt, L.E., Rasmussen, A., Norrelykke, M.R., et al., *The effect of selective bowel decontamination on the pharmacokinetics of mycophenolate mofetil in liver transplant recipients.* Liver Transpl, 2001. **7**(8): p. 739-742.
108. Undre, N., Van Hooff, J., Christiaans, M., et al. *Pharmacokinetics of FK 506 and Mycophenolic Acid After the Administration of a FK 506–Based Regimen in Combination With Mycophenolate Mofetil in Kidney Transplantation*. in *Transplantation proceedings*. 1998. Elsevier.
109. Vietri, M., Pietrabissa, A., Mosca, F., et al., *Mycophenolic acid glucuronidation and its inhibition by non-steroidal anti-inflammatory drugs in human liver and kidney.* Eur J Clin Pharmacol, 2000. **56**(9-10): p. 659-664.
110. Kato, R., Ooi, K., Ikura-Mori, M., et al., *Impairment of mycophenolate mofetil absorption by calcium polycarbophil.* J Clin Pharmacol, 2002. **42**(11): p. 1275-1280.
111. Shaw, L.M., Holt, D.W., Oellerich, M., et al., *Current issues in therapeutic drug monitoring of mycophenolic acid: report of a roundtable discussion.* Ther Drug Monit, 2001. **23**(4): p. 305-315.
112. Irene, T., Frances, C., Hama, K., et al., *Manual and automated (robotic) high-performance liquid chromatography methods for the determination of mycophenolic acid and its glucuronide conjugate in human plasma.* Journal of Chromatography B: Biomedical Sciences and Applications, 1996. **675**(1): p. 119-129.
113. Streit, F., Shipkova, M., Armstrong, V.W., et al., *Validation of a rapid and sensitive liquid chromatography-tandem mass spectrometry method for free and total mycophenolic acid.* Clin Chem, 2004. **50**(1): p. 152-159.
114. Saunders, D.A., *Simple method for the quantitation of mycophenolic acid in human plasma.* J Chromatogr B Biomed Sci Appl, 1997. **704**(1-2): p. 379-382.
115. P Verma, S., J Gilani, S., Bala, I., et al., *Validated, stability indicating HPTLC method for the determination of mycophenolate mofetil in human plasma.* Current Analytical Chemistry, 2011. **7**(3): p. 216-219.
116. Tsina, I., Kaloostian, M., Lee, R., et al., *High-performance liquid chromatographic method for the determination of mycophenolate mofetil in human plasma.* J Chromatogr B Biomed Appl, 1996. **681**(2): p. 347-353.

117. Shipkova, M., Armstrong, V.W., Kiehl, M.G., et al., *Quantification of mycophenolic acid in plasma samples collected during and immediately after intravenous administration of mycophenolate mofetil.* Clin Chem, 2001. **47**(8): p. 1485-1488.
118. Mahalati, K. and Kahan, B.D., *Pharmacological surrogates of allograft outcome.* Ann Transplant, 2000. **5**(2): p. 14-23.
119. Filler, G. and Mai, I., *Limited sampling strategy for mycophenolic acid area under the curve.* Ther Drug Monit, 2000. **22**(2): p. 169-173.
120. Pawinski, T., Hale, M., Korecka, M., et al., *Limited sampling strategy for the estimation of mycophenolic acid area under the curve in adult renal transplant patients treated with concomitant tacrolimus.* Clin Chem, 2002. **48**(9): p. 1497-1504.
121. Le Guellec, C., Büchler, M., Giraudeau, B., et al., *Simultaneous estimation of cyclosporin and mycophenolic acid areas under the curve in stable renal transplant patients using a limited sampling strategy.* European journal of clinical pharmacology, 2002. **57**(11): p. 805-811.
122. van Gelder, T. and Shaw, L.M., *The rationale for and limitations of therapeutic drug monitoring for mycophenolate mofetil in transplantation.* Transplantation, 2005. **80**(2 Suppl): p. S244-S253.
123. Brunet, M., Cirera, I., Martorell, J., et al., *Sequential determination of pharmacokinetics and pharmacodynamics of mycophenolic acid in liver transplant patients treated with mycophenolate mofetil.* Transplantation, 2006. **81**(4): p. 541-546.
124. van Gelder, T., Le Meur, Y., Shaw, L.M., et al., *Therapeutic drug monitoring of mycophenolate mofetil in transplantation.* Ther Drug Monit, 2006. **28**(2): p. 145-154.
125. Domke, I., Engelmayer, J., Langmann, T., et al. *Measurement of total and free mycophenolic acid with new enzyme receptor methods on COBAS INTEGRA systems*. in *Clinical Chemistry*. 2005. Amer Assoc Clinical Chemistry 2101 L Street Nw, Suite 202, Washington, DC 20037-1526 USA.
126. Bunch, D. and Wang, S. *Therapeutic drug monitoring of plasma and serum Mycophenolic acid by Roche Integra 400 in comparison with a high*

performance liquid chromatography method. in *Clinical Chemistry*. 2008. Amer Assoc Clinical Chemistry 2101 L Street Nw, Suite 202, Washington, DC 20037-1526 USA.

127. Teshima, D., Kitagawa, N., Otsubo, K., et al., *Simple determination of mycophenolic acid in human serum by column-switching high-performance liquid chromatography.* J Chromatogr B Analyt Technol Biomed Life Sci, 2002. **780**(1): p. 21-26.
128. Sparidans, R.W., Hoetelmans, R.M., and Beijnen, J.H., *Liquid chromatographic assay for simultaneous determination of abacavir and mycophenolic acid in human plasma using dual spectrophotometric detection.* Journal of Chromatography B: Biomedical Sciences and Applications, 2001. **750**(1): p. 155-161.
129. Renner, U.D., Thiede, C., Bornhäuser, M., et al., *Determination of mycophenolic acid and mycophenolate mofetil by high-performance liquid chromatography using postcolumn derivatization.* Analytical chemistry, 2001. **73**(1): p. 41-46.
130. Na-Bangchang, K., Supasyndh, O., Supaporn, T., et al., *Simple and sensitive high-performance liquid chromatographic method for the determination of mycophenolic acid in plasma.* Journal of Chromatography B: Biomedical Sciences and Applications, 2000. **738**(1): p. 169-173.
131. Hosotsubo, H., Takahara, S., Kokado, Y., et al., *Rapid and simple determination of mycophenolic acid in human plasma by ion-pair RP-LC with fluorescence detection.* J Pharm Biomed Anal, 2001. **24**(4): p. 555-560.
132. Srivatsan, V., Dasgupta, A.K., Kale, P., et al., *Determination of mycophenolic acid in human plasma by high-performance liquid chromatography.* J Chromatogr A, 2004. **1031**(1-2): p. 259-264.
133. Plätzer, M., Jahn, K., Wohlrab, J., et al., *Quantification of mycophenolate mofetil in human skin extracts using high-performance liquid chromatography–electrospray mass spectrometry.* Journal of Chromatography B: Biomedical Sciences and Applications, 2001. **755**(1): p. 355-359.
134. ICH. *Q2 (R1): Validation of analytical procedures: text and methodology*. in *International Conference on Harmonization, Geneva*. 2005.

135. Kuhn, J., Prante, C., Kleesiek, K., et al., *Measurement of mycophenolic acid and its glucuronide using a novel rapid liquid chromatography–electrospray ionization tandem mass spectrometry assay.* Clinical biochemistry, 2009. **42**(1): p. 83-90.

136. Shen, B., Li, S., Zhang, Y., et al., *Determination of total, free and saliva mycophenolic acid with a LC-MS/MS method: application to pharmacokinetic study in healthy volunteers and renal transplant patients.* J Pharm Biomed Anal, 2009. **50**(3): p. 515-521.

137. Benech, H., Hascoet, S., Furlan, V., et al., *Development and validation of an LC/MS/MS assay for mycophenolic acid in human peripheral blood mononuclear cells.* J Chromatogr B Analyt Technol Biomed Life Sci, 2007. **853**(1-2): p. 168-174.

138. Jain, A., Venkataramanan, R., Hamad, I.S., et al., *Pharmacokinetics of mycophenolic acid after mycophenolate mofetil administration in liver transplant patients treated with tacrolimus.* J Clin Pharmacol, 2001. **41**(3): p. 268-276.

139. Zhong, Y., Jiao, Z., and Yu, Y., *Simultaneous determination of mycophenolic acid and valproic acid based on derivatization by high-performance liquid chromatography with fluorescence detection.* Biomed Chromatogr, 2006. **20**(4): p. 319-326.

140. Weber, L.T., Shipkova, M., Armstrong, V.W., et al., *The pharmacokinetic-pharmacodynamic relationship for total and free mycophenolic Acid in pediatric renal transplant recipients: a report of the german study group on mycophenolate mofetil therapy.* J Am Soc Nephrol, 2002. **13**(3): p. 759-768.

141. Goss, S., Driscoll, J., McClafferty, T., et al. *Performance of the mycophenolic acid method on the Dade Behring Dimension (R) clinical chemistry system*. in *Clinical Chemistry*. 2006. Amer Assoc Clinical Chemistry 2101 L Street Nw, Suite 202, Washington, DC 20037-1526 USA.

142. Lao, W., Nimmagadda, S., Ruzicka, R., et al. *Development of application protocols for CEDIA (R) Mycophenolic Acid assay on the Hitachi 911, Olympus AU640, and MGC 240 clinical chemistry analyzers*. in *Clinical Chemistry*. 2006.

Amer Assoc Clinical Chemistry 2101 L Street Nw, Suite 202, Washington, DC 20037-1526 USA.

143. Shipkova, M., Schütz, E., Armstrong, V., et al. *Overestimation of mycophenolic acid by EMIT correlates with MPA metabolite*. in *Transplantation proceedings*. 1999. Elsevier.

144. Weber, L.T., Shipkova, M., Armstrong, V.W., et al., *Comparison of the Emit immunoassay with HPLC for therapeutic drug monitoring of mycophenolic acid in pediatric renal-transplant recipients on mycophenolate mofetil therapy.* Clin Chem, 2002. **48**(3): p. 517-525.

145. Beal, J.L., Jones, C.E., Taylor, P.J., et al., *Evaluation of an immunoassay (EMIT) for mycophenolic acid in plasma from renal transplant recipients compared with a high-performance liquid chromatography assay.* Ther Drug Monit, 1998. **20**(6): p. 685-690.

146. Schütz, E., Shipkova, M., Armstrong, V., et al. *Therapeutic drug monitoring of mycophenolic acid: comparison of HPLC and immunoassay reveals new MPA metabolites*. in *Transplantation proceedings*. 1998. Elsevier.

147. Westley, I.S., Sallustio, B.C., and Morris, R.G., *Validation of a high-performance liquid chromatography method for the measurement of mycophenolic acid and its glucuronide metabolites in plasma.* Clin Biochem, 2005. **38**(9): p. 824-829.

148. Premaud, A., Rousseau, A., Le Meur, Y., et al., *Comparison of liquid chromatography-tandem mass spectrometry with a commercial enzyme-multiplied immunoassay for the determination of plasma MPA in renal transplant recipients and consequences for therapeutic drug monitoring.* Ther Drug Monit, 2004. **26**(6): p. 609-619.

149. Oellerich, M., Shipkova, M., Schutz, E., et al., *Pharmacokinetic and metabolic investigations of mycophenolic acid in pediatric patients after renal transplantation: implications for therapeutic drug monitoring. German Study Group on Mycophenolate Mofetil Therapy in Pediatric Renal Transplant Recipients.* Ther Drug Monit, 2000. **22**(1): p. 20-26.

150. Shaw, L.M., Korecka, M., Aradhye, S., et al., *Mycophenolic acid area under the curve values in African American and Caucasian renal transplant patients are comparable.* J Clin Pharmacol, 2000. **40**(6): p. 624-633.

151. Mandla, R., Line, P.D., Midtvedt, K., et al., *Automated determination of free mycophenolic acid and its glucuronide in plasma from renal allograft recipients.* Ther Drug Monit, 2003. **25**(3): p. 407-414.

152. Goto, T., Kino, T., Hatanaka, H., et al., *Discovery of FK-506, a novel immunosuppressant isolated from Streptomyces tsukubaensis.* Transplant Proc, 1987. **19**(5 Suppl 6): p. 4-8.

153. Zahir, H., Nand, R.A., Brown, K.F., et al., *Validation of methods to study the distribution and protein binding of tacrolimus in human blood.* J Pharmacol Toxicol Methods, 2001. **46**(1): p. 27-35.

154. Venkataramanan, R., Swaminathan, A., Prasad, T., et al., *Clinical pharmacokinetics of tacrolimus.* Clin Pharmacokinet, 1995. **29**(6): p. 404-430.

155. Iwasaki, K., Shiraga, T., Nagase, K., et al., *Isolation, identification, and biological activities of oxidative metabolites of FK506, a potent immunosuppressive macrolide lactone.* Drug Metab Dispos, 1993. **21**(6): p. 971-977.

156. Kelly, P. and Kahan, B.D., *Review: metabolism of immunosuppressant drugs.* Curr Drug Metab, 2002. **3**(3): p. 275-287.

157. Tamura, K., Fujimura, T., Iwasaki, K., et al., *Interaction of tacrolimus(FK506) and its metabolites with FKBP and calcineurin.* Biochem Biophys Res Commun, 1994. **202**(1): p. 437-443.

158. Beysens, A., Wijnen, R., Beuman, G., et al. *FK 506: monitoring in plasma or in whole blood?* in *Transplantation proceedings*. 1991.

159. Artz, M.A., Boots, J.M., Ligtenberg, G., et al., *Conversion from cyclosporine to tacrolimus improves quality-of-life indices, renal graft function and cardiovascular risk profile.* Am J Transplant, 2004. **4**(6): p. 937-945.

160. Mentzer Jr, R.M., Jahania, M.S., Lasley, R.D., et al., *Tacrolimus As A Rescue Immunosuppressant After Heart And Lung Transplantation1.* Transplantation, 1998. **65**(1): p. 109-113.

161. Laskow, D.A., Neylan, J.F., 3rd, Shapiro, R.S., et al., *The role of tacrolimus in adult kidney transplantation: a review.* Clin Transplant, 1998. **12**(6): p. 489-503.
162. Annesley, T.M., Hunter, B.C., Fidler, D.R., et al., *Stability of tacrolimus (FK 506) and cyclosporin G in whole blood.* Ther Drug Monit, 1995. **17**(4): p. 361-365.
163. Alak, A.M. and Lizak, P., *Stability of FK506 in blood samples.* Ther Drug Monit, 1996. **18**(2): p. 209-211.
164. Freeman, D.J., Stawecki, M., and Howson, B., *Stability of FK 506 in whole blood samples.* Ther Drug Monit, 1995. **17**(3): p. 266-267.
165. Holt, D.W., *Therapeutic drug monitoring of immunosuppressive drugs in kidney transplantation.* Curr Opin Nephrol Hypertens, 2002. **11**(6): p. 657-663.
166. Staatz, C.E. and Tett, S.E., *Clinical pharmacokinetics and pharmacodynamics of tacrolimus in solid organ transplantation.* Clin Pharmacokinet, 2004. **43**(10): p. 623-653.
167. Wong, K.M., Shek, C.C., Chau, K.F., et al., *Abbreviated tacrolimus area-under-the-curve monitoring for renal transplant recipients.* Am J Kidney Dis, 2000. **35**(4): p. 660-666.
168. McAlister, V.C., Mahalati, K., Peltekian, K.M., et al., *A clinical pharmacokinetic study of tacrolimus and sirolimus combination immunosuppression comparing simultaneous to separated administration.* Ther Drug Monit, 2002. **24**(3): p. 346-350.
169. Lensmeyer, G.L. and Poquette, M.A., *Therapeutic monitoring of tacrolimus concentrations in blood: semi-automated extraction and liquid chromatography-electrospray ionization mass spectrometry.* Ther Drug Monit, 2001. **23**(3): p. 239-249.
170. Akbas, S., Yavuz, A., Tuncer, M., et al. *Evaluation of the new EMIT tacrolimus assay in kidney and liver transplant recipients*. in *Transplantation proceedings*. 2004. Elsevier.
171. LeGatt, D.F., Shalapay, C.E., and Cheng, S.B., *The EMIT 2000 tacrolimus assay: an application protocol for the Beckman Synchron LX20 PRO analyzer.* Clin Biochem, 2004. **37**(11): p. 1022-1030.

172. Stephen, D., Rooke, P., Clark, S., et al., *Evaluation of the Dade Behring Syva® EMIT 2000 tacrolimus assay on the Bayer Advia 1650.* Annals of clinical biochemistry, 2003. **40**(6): p. 697-700.

173. Baugher, B., Drengler, S., Rahn, T., et al., *Evaluation of an Assay in Development for Tacrolimus on the Abbott ARCHITECT® Analyzer.* Clinical Chemistry, 2006. **52**(S6): p. A59.

174. Oellerich, M., Armstrong, V.W., Kahan, B., et al., *Lake Louise Consensus Conference on cyclosporin monitoring in organ transplantation: report of the consensus panel.* Ther Drug Monit, 1995. **17**(6): p. 642-654.

175. Keevil, B.G., McCann, S.J., Cooper, D.P., et al., *Evaluation of a rapid micro-scale assay for tacrolimus by liquid chromatography-tandem mass spectrometry.* Ann Clin Biochem, 2002. **39**(Pt 5): p. 487-492.

176. Deters, M., Kirchner, G., Resch, K., et al., *Simultaneous quantification of sirolimus, everolimus, tacrolimus and cyclosporine by liquid chromatography-mass spectrometry (LC-MS).* Clin Chem Lab Med, 2002. **40**(3): p. 285-292.

177. Iwasaki, K., Shiraga, T., Matsuda, H., et al., *Further metabolism of FK506 (tacrolimus). Identification and biological activities of the metabolites oxidized at multiple sites of FK506.* Drug metabolism and disposition, 1995. **23**(1): p. 28-34.

178. CEDIA®, *Tacrolimus Assay (package insert).* 2005: Fremont, CA.

179. Staatz, C.E., Taylor, P.J., and Tett, S.E., *Comparison of an ELISA and an LC/MS/MS method for measuring tacrolimus concentrations and making dosage decisions in transplant recipients.* Ther Drug Monit, 2002. **24**(5): p. 607-615.

180. Gonschior, A.K., Christians, U., Winkler, M., et al., *Tacrolimus (FK506) metabolite patterns in blood from liver and kidney transplant patients.* Clin Chem, 1996. **42**(9): p. 1426-1432.

181. Oellerich, M., Armstrong, V.W., Schutz, E., et al., *Therapeutic drug monitoring of cyclosporine and tacrolimus. Update on Lake Louise Consensus Conference on cyclosporin and tacrolimus.* Clin Biochem, 1998. **31**(5): p. 309-316.

182. Cogill, J.L., Taylor, P.J., Westley, I.S., et al., *Evaluation of the tacrolimus II microparticle enzyme immunoassay (MEIA II) in liver and renal transplant recipients.* Clin Chem, 1998. **44**(9): p. 1942-1946.

183. Busuttil, R.W., Klintmalm, G.B., Lake, J.R., et al., *General guidelines for the use of tacrolimus in adult liver transplant patients.* Transplantation, 1996. **61**(5): p. 845-847.
184. Schambeck, C.M., Bedel, A., and Keller, F., *Limit of quantification (functional sensitivity) of the new IMx Tacrolimus II microparticle enzyme immunoassay.* Clin Chem, 1998. **44**(10): p. 2217.
185. *Tacrolimus II (package insert).* 2003, Abbott Laboratories, Diagnostics Division: Abbott Park, IL.
186. Homma, M., Tomita, T., Yuzawa, K., et al., *False positive blood tacrolimus concentration in microparticle enzyme immunoassay.* Biol Pharm Bull, 2002. **25**(8): p. 1119-1120.
187. Kuzuya, T., Ogura, Y., Motegi, Y., et al., *Interference of hematocrit in the tacrolimus II microparticle enzyme immunoassay.* Ther Drug Monit, 2002. **24**(4): p. 507-511.
188. Akbas, S.H., Ozdem, S., Caglar, S., et al., *Effects of some hematological parameters on whole blood tacrolimus concentration measured by two immunoassay-based analytical methods.* Clinical biochemistry, 2005. **38**(6): p. 552-557.
189. Ghoshal, A.K. and Soldin, S.J., *IMx tacrolimus II assay: is it reliable at low blood concentrations? A comparison with tandem MS/MS.* Clinical biochemistry, 2002. **35**(5): p. 389-392.
190. Steele, B.W., Wang, E., Soldin, S.J., et al., *A longitudinal replicate study of immunosuppressive drugs: a College of American Pathologists study.* Arch Pathol Lab Med, 2003. **127**(3): p. 283-288.
191. Bergmann, T.K., Barraclough, K.A., Lee, K.J., et al., *Clinical pharmacokinetics and pharmacodynamics of prednisolone and prednisone in solid organ transplantation.* Clin Pharmacokinet, 2012. **51**(11): p. 711-741.
192. Pickup, M.E., *Clinical pharmacokinetics of prednisone and prednisolone.* Clin Pharmacokinet, 1979. **4**(2): p. 111-128.
193. Frey, B.M. and Frey, F.J., *Clinical pharmacokinetics of prednisone and prednisolone.* Clin Pharmacokinet, 1990. **19**(2): p. 126-146.

194. Rocci, M.L., Jr., Szefler, S.J., Acara, M., et al., *Prednisolone metabolism and excretion in the isolated perfused rat kidney.* Drug Metab Dispos, 1981. **9**(3): p. 177-182.

195. Czock, D., Keller, F., Rasche, F.M., et al., *Pharmacokinetics and pharmacodynamics of systemically administered glucocorticoids.* Clin Pharmacokinet, 2005. **44**(1): p. 61-98.

196. Garg, V. and Jusko, W.J., *Bioavailability and reversible metabolism of prednisone and prednisolone in man.* Biopharm Drug Dispos, 1994. **15**(2): p. 163-172.

197. Diederich, S., Eigendorff, E., Burkhardt, P., et al., *11β-Hydroxysteroid dehydrogenase types 1 and 2: an important pharmacokinetic determinant for the activity of synthetic mineralo-and glucocorticoids.* J Clin Endocrinol Metab, 2002. **87**(12): p. 5695-5701.

198. Coutinho, A.E. and Chapman, K.E., *The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights.* Mol Cell Endocrinol, 2011. **335**(1): p. 2-13.

199. Pichard, L., Fabre, I., Daujat, M., et al., *Effect of corticosteroids on the expression of cytochromes P450 and on cyclosporin A oxidase activity in primary cultures of human hepatocytes.* Mol Pharmacol, 1992. **41**(6): p. 1047-1055.

200. Yates, C.R., Chang, C., Kearbey, J.D., et al., *Structural determinants of P-glycoprotein-mediated transport of glucocorticoids.* Pharm Res, 2003. **20**(11): p. 1794-1803.

201. Lam, S., Partovi, N., Ting, L.S., et al., *Corticosteroid interactions with cyclosporine, tacrolimus, mycophenolate, and sirolimus: fact or fiction?* Ann Pharmacother, 2008. **42**(7): p. 1037-1047.

202. Frey, F.J., *Kinetics and dynamics of prednisolone.* Endocr Rev, 1987. **8**(4): p. 453-473.

203. Caspi, E. and Pechet, M.M., *The metabolism of 1-dehydro-steroids in man: isolation of 6 urinary products following the administration of 11beta, 17alpha, 21-trihydroxy-1, 4-pregnadiene-3, 20-dione.* Arch Biochem Biophys, 1957. **68**(1): p. 236-237.

204. Frey, F.J. and Frey, B.M., *Urinary 6 beta-hydroxyprednisolone excretion indicates enhanced prednisolone catabolism.* J Lab Clin Med, 1983. **101**(4): p. 593-604.

205. Slaunwhite Jr, W. and Sandberg, A.A., *Disposition of radioactive 17α-hydroxyprogesterone, 6α-methyl-17α-acetoxyprogesterone and 6α-methylprednisolone in human subjects.* J Clin Endocrinol Metab, 1961. **21**(7): p. 753-764.

206. Vermeulen, A., *The metabolism of 4-^{14}C prednisolone.* J Endocrinol, 1959. **18**(3): p. 278-291.

207. Tsang, S.Y., Garovoy, M.R., and Benet, L.Z., *Immunosuppressive activity of prednisone and prednisolone and their metabolic interconversion in the mixed lymphocyte reaction.* Int J Immunopharmacol, 1985. **7**(5): p. 731-737.

208. Oosterhuis, B., ten Berge, I.J., Schellekens, P.T., et al., *Prednisolone concentration-effect relations in humans and the influence of plasma hydrocortisone.* J Pharmacol Exp Ther, 1986. **239**(3): p. 919-926.

209. Stuck, A.E., Frey, B.M., and Frey, F.J., *Kinetics of prednisolone and endogenous cortisol suppression in the elderly.* Clin Pharmacol Ther, 1988. **43**(4): p. 354-362.

210. Frey, F.J., Amend, W.J., Jr., Lozada, F., et al., *Endogenous hydrocortisone, a possible factor contributing to the genesis of cushingoid habitus in patients on prednisone.* J Clin Endocrinol Metab, 1981. **53**(5): p. 1076-1080.

211. Frey, B.M. and Frey, F.J., *Simultaneous measurement of prednisone, prednisolone and 6β-hydroxyprednisolone in urine by high-performance liquid chromatography coupled with a radioactivity detector.* J Chromatogr B Biomed Sci Appl, 1982. **229**(2): p. 283-292.

212. Frey, F.J., Frey, B.M., and Benet, L.Z., *Liquid-chromatographic measurement of endogenous and exogenous glucocorticoids in plasma.* Clin Chem, 1979. **25**(11): p. 1944-1947.

213. Rose, J.Q. and Jusko, W.J., *Corticosteroid analysis in biological fluids by high-performance liquid chromatography.* J Chromatogr, 1979. **162**(3): p. 273-280.

214. Behm, H.L. and Wagner, J.G., *Errors in interpretation of data from equilibrium dialysis protein binding experiments.* Res Commun Chem Pathol Pharmacol, 1979. **26**(1): p. 145-160.

215. Boudinot, F.D. and Jusko, W.J., *Plasma protein binding interaction of prednisone and prednisolone.* J Steroid Biochem, 1984. **21**(3): p. 337-339.

216. Milsap, R.L., George, D.E., Szefler, S.J., et al., *Effect of inflammatory bowel disease on absorption and disposition of prednisolone.* Dig Dis Sci, 1983. **28**(2): p. 161-168.

217. Frey, B., Hugentobler, T., Bührer, M., et al., *Transcortinkonzentrationen im Plasma von Normalpersonen und Patienten mit Nieren-oder Lebererkrankungen.* Klin Wochenschr, 1984. **62**(19): p. 936-938.

218. Gustavson, L.E. and Benet, L.Z., *The macromolecular binding of prednisone in plasma of healthy volunteers including pregnant women and oral contraceptive users.* J Pharmacokinet Biopharm, 1985. **13**(6): p. 561-569.

219. Lewis, G.P., Jusko, W.J., Graves, L., et al., *Prednisone side-effects and serum-protein levels. A collaborative study.* Lancet, 1971. **2**(7728): p. 778-780.

220. Frey, B.M. and Frey, F.J., *The effect of altered prednisolone kinetics in patients with the nephrotic syndrome and in women taking oral contraceptive steroids on human mixed lymphocyte cultures.* J Clin Endocrinol Metab, 1985. **60**(2): p. 361-369.

221. Ogawa, K., Sueda, K., and Matsui, N., *The effect of cortisol, progesterone, and transcortin on phytohemagglutinin-stimulated human blood mononuclear cells and their interplay.* J Clin Endocrinol Metab, 1983. **56**(1): p. 121-126.

222. Slaunwhite, W.R., Lockie, G.N., Back, N., et al., *Inactivity in vivo of transcortin-bound cortisol.* Science, 1962. **135**(3508): p. 1062-1063.

223. Hilfiker, R., Blatter, F., and von Raumer, M., *Relevance of solid-state properties for pharmaceutical products*. Polymorphism: In the pharmaceutical industry. Vol. 1. 2006, Weinheim, Germany Wiley-Vch Verlag GmbH & Co. KGaA.

224. Petit, S. and Coquerel, G., *The amorphous state*. Polymorphism: In the pharmaceutical industry. Vol. 1. 2006, Weinheim, Germany WIiley-Vch Verlag GmbH & Co. KGaA.

225. Yoshinari, T., Forbes, R.T., York, P., et al., *Moisture induced polymorphic transition of mannitol and its morphological transformation.* Int J Pharm, 2002. **247**(1-2): p. 69-77.
226. Lowry, O.H., Rosebrough, N.J., Farr, A.L., et al., *Protein measurement with the Folin phenol reagent.* J Biol Chem, 1951. **193**(1): p. 265-275.
227. Peterson, G.L., *Review of the Folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall.* Anal Biochem, 1979. **100**(2): p. 201-220.
228. Wu, A.M., Wu, J., and Herp, A., *Polypeptide linkages and resulting structural features as powerful chromogenic factors in the Lowry phenol reaction. Studies on a glycoprotein containing no Lowry phenol-reactive amino acids and on its desialylated and deglycosylated products.* Biochem J, 1978. **175**(1): p. 47-51.
229. Peterson, G.L., *A simplification of the protein assay method of Lowry et al. which is more generally applicable.* Anal Biochem, 1977. **83**(2): p. 346-356.

Research publications

- **P. Kumar**, V. Mishra, S. Verma, A. Bhatia, "Development of RP- HPLC Method for Simultaneous Estimation of Mycophenolate Mofetil and Tacrolimus", Journal of Materials and Environmental Sciences. 2018: 9(4), 1357-1365.
- **P. Kumar**, V. Mishra, S. Verma, A. Bhatia, "Simultaneous estimation via RP-HPLC method for the immunosuppressant drug combination: Mycophenolate mofetil, Tacrolimus with prednisolone," Pertanika- Journal of Science and Technology. 2019: 27(1), 371-385.
- S. Verma, J. Kaur, **P. Kumar Sharma**, K. Kumar Namdev, I. Bala, "Development and Validation of a Novel Dual Wavelength UV-Spectrophotometric Method for the Simultaneous Estimation of Mycophenolate Mofetil and Prednisolone", Journal of Applied Spectroscopy. 2014: 81(3), 403-407.

Conference publications

- Presented a poster on "Development of Liquid Chromatographic Method for Simultaneous Determination of Mycophenolate Mofetil and Tacrolimus in Unit Dosage Form" in 8th National IPA Student Congress 2016 "Skill India: Emerging Horizons for Pharma Professionals" held at ISF college, Moga (2016).
- Presented a poster on "Simultaneous Estimation of Prednisolone and Mycophenolate mofetil" in National conference on "Advancement Opportunities and Future vision of Indian Scenario of Pharmaceutical Education & Research" held at Rayat Bahra institute of pharmacy, Hoshiarpur (2015).

CENTRAL ANIMAL HOUSE FACILITY (CAHF)

Lovely School of Pharmaceutical Sciences, Lovely Professional University

Ludhiana- Jalandhar G.T. Road, Phagwara (Punjab), 144402

Registration Number -954/ac/06/CPCSEA

CERTIFICATE

This is to certify that the project titled "*Determination of pharmacokinetic parameter by using bioanalytical method for the developed capsule formulation*" has been approved by the IAEC.

Name of Principal Investigator: Dr. Surajpal Verma

IAEC approval number: LPU/IAEC/2018/ 31

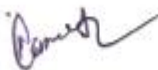
Date of Approval: 14.7.2018

Animals approved: 37 rats

Remarks if any: -

Dr. Monica Gulati

Biological Scientist,
Chairperson IAEC



Dr. Navneet Khurana

Scientist, COD Pharmacology

Mr. Bimlesh Kumar

Scientist In-Charge of Animal House,
Member Secretary IAEC