ANALYTICAL AND BIOANALYTICAL METHOD DEVELOPMENT OF MONOMETHYL FUMARATE AND ITS APPLICATION FOR PHARMACOKINETIC STUDY

To be submitted as Major report in partial fulfilment of the requirement

for the degree of

Masters in Technology In

Industrial Biotechnology

Submitted by

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(2K18/IBT/05)

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CERTIFICATE

This is to certify that the dissertation entitled **"Analytical and bioanalytical method development of monomethyl fumarate and its application for pharmacokinetic study"** (2K18/IBT/05) in the partial fulfillment of the requirements for the reward of the degree of Master of Technology, Delhi Technlogical University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work carried out by her under my guidance. The information and data enclosed in this thesis is original and has not been submitted elsewhere for honoring of any other degree.

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This is to certify that the thesis of Major Project II entitled **"Analytical and bioanalytical method development of monomethyl fumarate and its application for pharmacokinetic study"** in the partial fulfilment of the requirements for the reward of the degree of Mater of Technology, Delhi Technological University (Formerly Delhi college of Engineering, University of Delhi), is an authentic record of the my own work carried out under my guidance of the my project supervisor **Dr. Navneeta Bharadwaj**, Assistant Professor, Plant Biotechnology, Department of Biotechnology, DTU. The information and data enclosed in this thesis is original and has not been submitted elsewhere for honoring of any other degree.

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CERTIFICATE

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She has been hardworking and sincere in her entire period. I further certify that she bears a good moral character and I wish her a successful career.

For Sphaera Pharma Pvt Ltd

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ABSTRACT

Monomethyl fumarate is the pharmacologically active metabolite of immune modulator dimethyl fumarate. It is used for the treatment of relapsing forms of multiple sclerosis, relapsing-remitting disease, and active secondary progressive disease, in adults. The major objective of this thesis was to develop selective and sensitive method for HPLC and LCMS analysis of MMF, to study its pharmacokinetics in rat plasma. The chromatographic separation was achieved on a HYPERSIL GOLD, 50 ×4.6 mm,5u C18 by using a 70:30 (v/v) mixture of acetonitrile and 10mM ammonium formate as the mobile phase at a flow rate of 1 mL/min at wavelength of 265 nm.

Keywords: Monomethyl fumarate, LCMS, HPLC, Pharmacokinetics

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LIST OF ABBREVIATIONS

MMF	Monomethyl Fumarate
DMF	Dimethyl Fumarate
MS	Mass Spectrometer
HPLC	High pressure liquid chromatography
PK	Pharmacokinetics
ACN	Acetonitrile
DMSO	Dimethyl sulfoxide
NS	Normal saline
SPE	Solid phase extraction
LLE	Liquid phase extraction
PEG	Polyethylene glycol
FA	Formic acid
conc.	Concentration
Min.	Minute
µL	Micro litre
ml	Milli litre
mg	Mili gram
mM	Milli molar
%	Percentage
^{o}C	Degree centigrade

CHAPTER - 1

INTRODUCTION

Monomethyl fumarate (MMF) is the pharmacologically active metabolite of immune modulator dimethyl fumarate (DMF). Monomethyl fumarate is rapidly formed by hydrolysis of dimethyl fumarate (Dibbert et al., 2013). It is commercially available under the brand name Bafiertam for the treatment of relapsing forms of multiple sclerosis, relapsing-remitting disease and active secondary progressive disease in adults. Multiple sclerosis is a disease in which the protective covering of nerves is eaten away by the immune system, it is a neurodegenerative disease. Recently the U.S. Food and Drug Administration (FDA) approved Bafiertam bioequivalent to Biogen's dimethyl fumarate in April,2020. Dimethyl fumarate is the methyl ester of fumaric acid which works as hypoxic cell radiosensitizer. Fumaric acid and other esters of it can be used to cure multiple sclerosis (Moharregh et al., 2009). Dimethyl fumarate acts as anti-inflammatory and neuro protective agent as it activates Nrf2 antioxidant also its active metabolite monomethyl fumarate release transcription factor Nrf2 from cytoplasmic repression and proteasomal degradation by alkylation of Nrf2 repressor keap1 (kelch-like erythroid cell derived protein with CNC homology associated protein) (Davies et al., 2016).

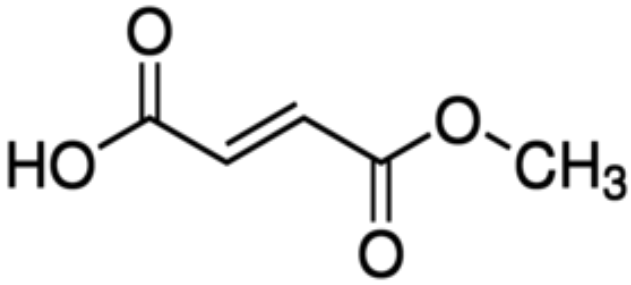

Fig. 1: Chemical structures of monomethyl fumarate

Till now only few analytical methods were reported for the determination of DMF along with its metabolite MMF (Junnotula et al., 2016). Till date, no LC–MS/MS method which completely describes the method development and validation procedures for the determination of MMF in human plasma. But for pharmacokinetic studies of DMF also

and for its bioequivalence a sensitive as well as selective analytical method to quantify MMF concentrations in plasma is required.

1.1 Solubility

Monomethyl fumarate is commercially available in crystalline form. A stock solution can be prepared by dissolving it in different solvents, which should be purged with an inert gas. Monomethyl fumarate is soluble in many organic solvents such as dimethyl sulfoxide, ethanol, polyethylene glycol and dimethyl formamide (caymanchem.com).

1.2 Bioanalytical Analysis

During the manufacturing, processing and storage organic impurities in drug can rise. Few methods have been reported for the analysis of Dimethyl fumarate and other fumaric acid esters separately (Trivedi et al., 2012) (Liu S, 1998). However, no combined validated stability-indicating reversed phase HPLC (RP- HPLC) method has been used for the separation and quantitative analysis. For the identification and quantification of a broad range of MW analytes, detection levels from ng/mL to pg/mL and structural information Mass Spectrometry is preferred (Jocelyn et al., 1997).

1.3 Pharmacology

Pharmacology is the study of how a drug affects a biological system and how the body responds to the drug. These effects can be therapeutic or toxic, depending on many factors (Kwon, 2001). Many factors influence the transport of pharmaceutical drug across cell membrane including its size, solubility, shape and degree of ionisation of drug. Some of the drugs might strongly binds to the plasma or tissues in the body. Consequently, only the free form of the drug is capable to pass through the membranes. At the steady state, the concentration of unbound drugs are the same on both sides of the membrane and pH difference across the membrane also play important role in drug transfer only if the compound is ionisable under physiological conditions (Karch, 2008)

1.4 Applications of MMF

Monomethyl fumarate and prodrugs of monomethyl fumarate are useful for treating neurodegenerative, inflammatory, and autoimmune diseases including multiple sclerosis, psoriasis, irritable bowel disorder, ulcerative colitis, arthritis, chronic obstructive

pulmonary disease, asthma, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (Raillard et al., 2014). It also prevents retina from light induced retinopathy (Dan Jiang, et al., 2019).

1.5 Objectives

- To determine the solubility of Monomethyl fumarate.
- HPLC method development for MMF
- LCMS/MS method development for MMF
- Pharmacokinetic study of Monomethyl fumarate on rat plasma

CHAPTER- 2

REVIEW OF LITERATURE

Monomethyl fumarate is a medication used for the treatment of relapsing forms of multiple sclerosis, to include clinically isolated syndrome, relapsing-remitting disease and active secondary progressive disease, in adults (wikipedia.org). Monomethyl fumarate is a compound that can cross the blood-brain barrier. It alters the NFE2L2 (Nuclear factor erythroid 2-related factor 2) transcription factor (Dodson et al., 2019). NFE2L2 is a basic leucine zipper protein which regulates the expression of antioxidant proteins that protect against oxidative damage triggered by injury and inflammation. Monomethyl Fumarate also protects the retina from light-induced retinopathy (Mark E. Pennesi). MMF can completely protect the retina from LIR in BALB/c mice (Dan Jiang et al., 2019).

2.1 Solubility of MMF

Monomethyl fumarate is available in the form of crystalline solid. Monomethyl fumarate is soluble in organic solvents. The solubility of monomethyl fumarate in ethanol is approximately 0.5 mg/ml and approximately 10 mg/ml in DMSo and DMF (Tocris, 2016). Further dilutions of the stock solution should be made prior to performing biological experiments. Ensure that the residual amount of organic solvent is insignificant, since organic solvents may have physiological effects at low concentrations. organic solvent-free aqueous solutions of monomethyl fumarate can be prepared by directly dissolving the solid in aqueous buffers. The solubility of monomethyl fumarate in PBS, pH 7.2, is approximately 1 mg/ml (Tang et al., 2008).

2.2 Pharmacokinetics of Fumarates

In general term pharmacokinetics means “what body does to the drug” (Kolthammer). Pharmacokinetics study is important because it gives useful indication for drug research and development. It also supports the studies of preclinical toxicology in animals (R. Urso et al., 2002). Pharmacokinetics is proposed to study the absorption, distribution, biotransformation (metabolism) and the elimination of drugs in humans and animals (Rescigno, 1966).

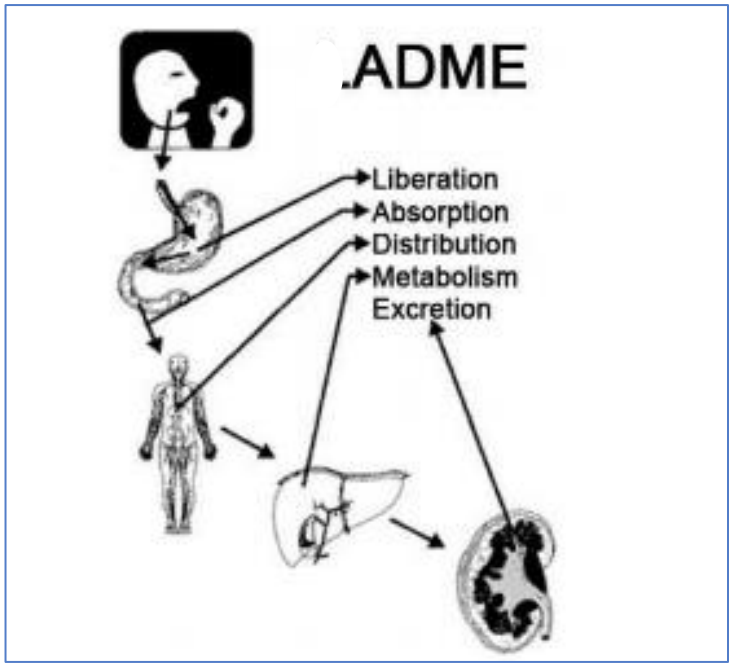

Fig 2: ADME Processes that take place after Drug Administration

1) **Absorption** = how the drug gets from its site of administration into the blood. Many factors affect the absorption phase for ex. molecule size, degree of lipid solubility, route of administration. Absorption is not applicable for drugs given by IV injection since they pass directly into the blood and therefore do not need to be absorbed.

2) **Distribution** = how the drug moves from the blood to other parts of the body, for ex. tissues and organs.

3) **Metabolism** = how the drug is broken down or transformed by the body into smaller molecules known as metabolites. Metabolites can be pharmacologically active, toxic or neither.

4) **Excretion** = how the drug is removed from the body (Rang et al., 1995)

From single concentration profile, we can observe some PK parameters to describe the drug exposure in the body and the rate and extent of absorption. Some of common measurements in considered in PK analysis are Cmax, Tmax, AUC (area under curve), t 1/2 and bioavailability (Twitchett et al., 2012).

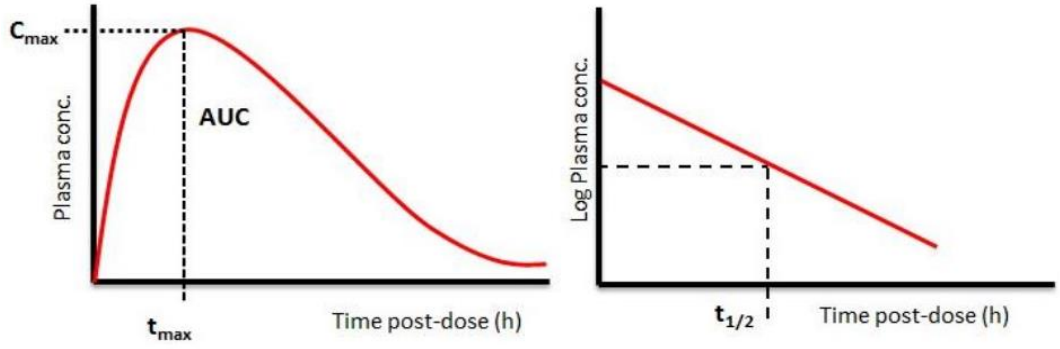

Fig 3: Common measurements used in PK analysis

- Bioavailability is the term used to indicates the proportion of the drug absorbed into the systemic circulation.
- Cmax = the maximum concentration recorded
- tmax = the time take to reach Cmax
- AUC = a measure of the exposure to the drug
- t1/2 (elimination half-life) = the time taken for the plasma concentration to fall by half its original value
- Dose Proportionality means that there is a constant ratio between the dose given and the PK profile.

2.3 Analytical Issues in PK

Biological samples are immensely complex because of the influence of many endogenous substance. Quantitation of administered drug in biological fluids can also be a challenge because of the low quantity of the target analytes (Sze mun, 2005). The ideal analytical method to monitor the concentration of a compound in plasma would enable isolation of the analytes from the matrix in a fast, inexpensive reproducible and simple way, while yielding high recoveries & avoiding degradation of the analytes (Alderley Park, 2004). After the collection of plasma from a body, the samples have to undergo some extraction and clean up processes before the instrumental analysis by LC-MS. This step is must to assure that the mass spectrometer is not contaminated & that it remains operational (Wang et al., 2004). Among the various clean-up processes, solid phase extraction (SPE) is a

common technique adopted for isolating analytes of interest from a wide variety of matrices including urine and blood (Lindegardh, N., et al., 2007). SPE is useful for removing matrix interference but it does require considerable method development and optimization.

2.4 Instrumental techniques

Most of the detection techniques for the analysis of organic chemicals are based on mass spectrometry (MS), which has become the preferred technique in bioanalysis & environmental analysis because of the inherent complexity of sample matrices. The LC-MS/MS instruments, mainly triple quadrupole & to a lesser extent ion trap, are today prevalent choices for reliable determination of rising polar organic compounds in environment (L. Kantiani, et al., 2012). The immensely high selectivity & sensitivity of MRM techniques allow trace constituents of complex mixtures to be determined. Among all possible ionization techniques, ESI (electrospray ionization) is by far most widely used as compared to atmospheric 36 pressure chemical ionization (APCI). Due to the high sensitivity and selectivity demonstrated by LC-MS/MS for the assays of chemicals in complex matrices - LCMS/MS was adopted.

2.4.1 High Performance Liquid Chromatography (HPLC)

HPLC is also known as high performance liquid chromatography. It is a type of chromatography which employs a liquid mobile phase and a very finely divided stationary phase. In order to obtain satisfactory flow rates, the liquid must be pressurized to several hundred pounds per square inch or more. This chromatography technique improved the performance if compared to classical column chromatography that's why known as high-performance chromatography. Most of the drugs in multicomponent dosage forms can be analysed by HPLC method because of its various advantages like specificity, accuracy, fast, precision, and ease of automation in this method (Bhardwaj, et al., 2015). HPLC method reduces tedious extraction and isolation procedures.

Some of the advantages of HPLC are:

- Speed (analysis can be accomplished in 20 min or less),
- Greater sensitivity (various detectors can be employed),
- Improved resolution (wide variety of stationary phases)

- Columns are reusable (expensive columns but can be used for different analysis),
- Ideal for substances of low volatility,
- Easy sample recovery, handling and maintenance,
- Instrumentation lends itself to automation and quantitation (less time and labour),
- Precise & reproducible, and
- Calculations done by integrator itself

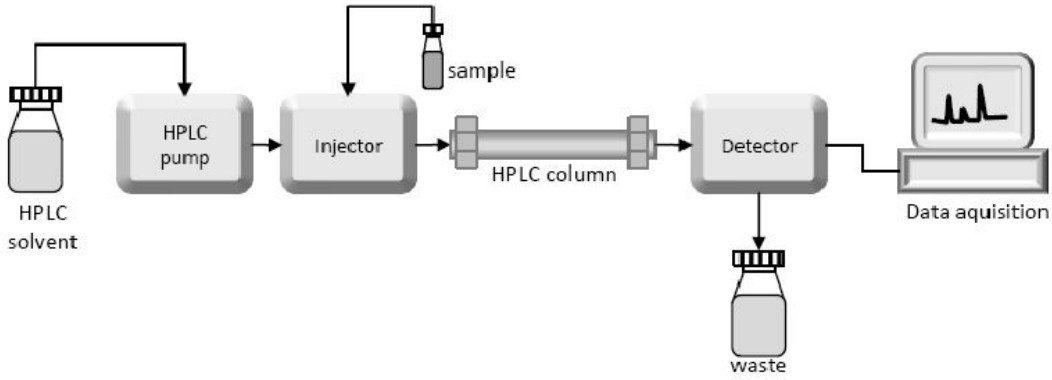

Fig 4. HPLC block diagram

HPLC instruments consist of a reservoir of the mobile phase, an injector, a separation column, a pump & a detector. Components are separated by injecting the samples into the column. The different compounds in the mixture move through the column and get separated because of the differences in their partition behaviour among the mobile phase & the stationary phase. The mobile phase must be degassed to remove the formation of air bubbles. The pump provides a steady high pressure without pulsation and can be programmed to vary the composition of the mobile phase during the course of separation. The detector relies on the change in refractive index, UV-VIS absorption, and fluorescence after excitation with a suitable wavelength in order to detect the separated compounds.

C18 Column

C18 has 18 carbons in the column packing that are bonded to the silica (Si). In general, C18 column retains more than C8 column, for instance, if a similar compound eluted on these two columns, it will elute later on the C18 column. The reversed-phase HPLC column is the most adaptable and commonly used type of column and it can be used for

a broad range of different types of analytes. Normal-phase HPLC columns have polar packing. C18 column is dense and because of denser packing of column surface area get increased which leads mobile phase to travel per unit of length of the column.

2.4.2 Mass Spectrometry

Mass spectrometry is fast becoming an indispensable field for analysing biomolecules. It generates multiple ions from the sample under investigation, it then separates them according to their specific mass-to-charge ratio (m/z), and then records the relative abundance of each ion type (Sagar Aryal, 2020). The mass spectrometer is composed of three components, an ion source, mass analyser, detector system, provides both qualitative and quantitative information about the composition of both organic and inorganic compounds in complex samples and computer system for acquiring the digitalised data.

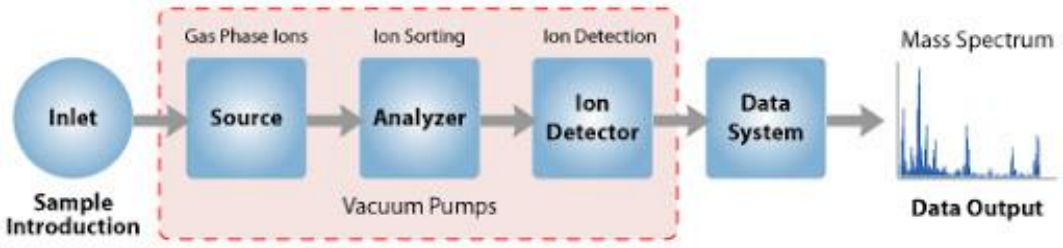

Fig 5: Components of a Mass Spectrometer

1) **Ion Source:** Produce gaseous ions from the substance being studied.
2) **Analyzer:** Resolves the ions into their characteristics mass components according to their mass-to-charge ratio.
3) **Detector System:** Detects the ions and recording the relative abundance of each of the resolved ionic species.

A mass spectrum is a representation of signal intensity versus m/z ratio versus intensity in a sample that has successfully been transferred into ions in gas phase. The advantages of MS are identification and quantification of a broad range of MW analytes, detection levels from ng/mL to pg/mL and structural information (R Waddell Smith, 2013).

However, the disadvantage of using MS is the inability to separate isobaric or isomeric compounds.

Precursor-ion scan

In this scanning, the second stage of mass spectrometry is fix to transmit single m/z ratio, namely that of the product (fragments) ion of interest, while the Ist stage is fix to scan through the mass range of interest, with the fragmentation of ions passing through MS1 being again carried out in MS2, the collision cell. A signal is seen at the detector only when ions are being transmittted by both MS1 and MS3.

Product-ion scan

The first stage of the mass spectrometry MSI is used to isolate ions of interest in the LC–MS, this is often the molecular species from the analytes. Fragmentation of ion is then affected; the way by which this is achieved is depend on types of instruments being used but it is often by collision with gas molecules in the collision cell. The second-stage of mass spectrometer is scanned to provide mass spectrum of the ions formed in collision cells.

Selected reaction monitoring

The fragmentation of a selected precursor ion to a selected product ion is monitored. It is carried out by setting each of the stages of mass spectrometry to transmit single ion, i.e. precursor ion by the MS1 and the product ion by the MS3.

Atmospheric pressure ionization (API) techniques

Once the target analytes have been appropriately chromatographically separated through the Liquid chromatography column, they move into the MS detector for the detection & measurements (Downard, 2004). The two most applicable interfaces for the analysis are electrospray ionization and atmospheric pressure chemical ionization (Botitsi, et al., 2011).

2.5 Sample Preparations

Trace analysis of organic contaminants is consistently challenging because of the complexity and variability of sample matrix. Because of matrix effect, it might exert a detrimental impact on important method parameters as limit of detection, limit of

quantification, linearity, accuracy & precision, pre-treatment of samples involves isolation of analytes (Junnotula, et al., 2016).

2.5.1 Liquid-Liquid Extraction (LLE)

Liquid-Liquid extraction is a typical technique to extract organic compounds from liquid state samples. The basic principle for LLE lies in the partition of target analytes into two immiscible liquid phase (L. Chimuka, et al., 2004). Due to its tedious procedure and large amount of organic solvent consumed, LLE is being replaced by other extraction methods.

2.5.2 Solid Phase Extraction

Compared to LLE, Solid phase extraction is a modern extraction method and has become most common sample preparation method in trace level analysis (Cheng, et al., 1997). SPE offers lower solvent consumption, shorter processing times, automation options, higher recoveries, and simpler procedures than LLE. The SPE method requires a measured volume of the liquid sample to be passed through a cartridge tube packed with a suitable solid adsorbent material. The chemicals in the sample are adsorbed onto the solid surface from which they are eluted by properly selected solvent. The sample is loaded at the top of the tube & drawn through the bed by a syringe or vacuum. The tube is washed with a nonpolar solvent for polar analytes, and with a polar solvent for non-polar analytes. Finally, the analytes are eluted out from column by a suitable solvent. The sample extracts may be concentrated further by evaporation of the solvent.

CHAPTER – 3

MATERIAL AND METHODS

3.1 Reagents and Materials

The reference sample of monomethyl fumarate were procured from Sphaera Pharma. For solubility testing different solvents were used such as Isopropyl alcohol, LCMS grade Methanol, LCMS grade ACN, DMSO, Normal saline (NS) and also Rankem water. For HPLC analysis ACN were purchased from J.T. Baker. Analytical grade ammonium acetate, ammonium formate and formic acid were purchased from Merck Ltd. The instrument used was HPLC Alliance Waters e2695 with Empower software, column - HYPERSIL GOLD,50 X 4.6mm and Sciex API 3200 LC-MS/MS coupled with Agilent 1200 – infinity II quaternary pump.

3.2 Solubility test

To test solubility of our reference sample i.e. MMF eight different solvents were taken including water. From reference sample 0.5 mg were weighed in eight different Eppendorf labelled IPA, NS, Water, MeOH, ACN, DMSO separately. To each Eppendorf add respective solvent drop-by-drop i.e 10 -10 µl and vortex for dissolving till it completely dissolves. Note the volume of solvent at which MMF is completely dissolved and also if its undissolved.

3.3 Mass Spectrometry

For MS stock solution of 1 mg/ml were prepared in methanol and injected to identify the polarity of monomethyl fumarate by positive and negative scanning.

3.4 High Performance Liquid Chromatography of MMF

For determination and quantification of MMF HPLC is performed. Also, to know its retention time.

3.4.1 Selection of solvent

Based on the sample's solubility, stability and suitability different mobile phase compositions were tried to achieve for good separation and resolution with sharp peaks.

After studying we were able to find that Methanol and water is a suitable solvent.

3.4.2 Selection of detection wavelength

The sensitivity of an HPLC method that use Photodiode array detector depends upon the proper selection of the wavelength. An ideal wavelength is one which gives good response for all the components to be detected. The UV spectrums of 1 mg/ml of standard MMF in selected solvents were recorded individually. The spectrums were superimposed to get overlay spectrums. From this overlain spectrum detection wavelength 265 nm was fixed because at this wavelength it shows good absorbance.

3.4.3 Optimized chromatographic conditions

Based on the studies, the following chromatographic conditions were selected.

Column: HYPERSIL GoLD, 50 × 4.6mm, 5u

Mobile phase A: 10 mm ammonium formate (30)

Mobile phase B: ACN (70)

Diluent: Water

Detection wavelength: 265 nm

Flow rate: 1 ml / min

Injection volume: 10 µl

Run time: 4 min

Retention Time: 0.674 min

To run the HPLC Purging and equilibration are two important steps. In purging flow rate is generally high than equilibration i.e. 6ml/min, whereas in equilibration it is 0.5 to 1ml/min.

3.5 Liquid chromatography–mass spectrometry (LC-MS/MS)

Quantitation of MMF was achieved with MS–MS detection in negative ion mode for the analytes using Sciex API 3200 LC-MS/MS system. The source parameters i.e. the nebulizer gas (gas 1), curtain gas, auxiliary gas (gas 2) and collision gas were optimized while tunning with 100ng solution of MMF. The compound parameters such as declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) were also determined by compound optimization. Detection of the ions was carried out in the multiple reaction monitoring mode (MRM) by monitoring

the transition pair of m/z 128.8 precursor ion to the m/z 85. Quadrupole 1 and Quadrupole 3 were set on unit (Ramanatham, et al., 2017). The analysis data obtained were processed by Analyst software™

3.5.1 Preparations for calibration curves standard and quality

The stock solutions of MMF were prepared at a concentration of 1 mg/mL in LCMS grade methanol. Final stocks were prepared for MMF for the preparation of calibration curve standards (CCs) and quality control (QC) samples in diluent (acetonitrile and LCMS grade water 70:30, v/v) to produce working standard. The prepared CC concentrations were 3.9, 7.8, 15.62, 31.25, 62.5, 125, 250, 500, 1000, 2000, 4000 and 8000 ng/ml. Aqueous linearity was prepared to check analytes concentration with reconstitution solution (RS). For aqueous CC 2µl analyte and 98 µl of RS were mixed and transferred to HPLC vials and checked for the aqueous linearity.

3.6 Extraction procedure

2µl of standard + add 23µl of plasma
↓
Add 100µl of 50Mm ammonium acetate with 0.5% formic acid
↓
Vibramax (vortex) for 1 min
↓
Conditioning: Add 1ml MeoH to starta X cartridge
↓
Equlibration: Add 1 ml water
↓
Add 1mL 50Mm ammonium acetate with 0.5% formic acid
↓
Load sample to starta X cartridge
↓
Washing-1: Add 1 ml 50Mm ammonium acetate with 0.5% formic acid
↓
Washing- 2: Add 1ml LCMS grade water
↓
Elution: Add 250 µl of Acetonitrile twice to elute compound
↓
Keep the sample LV until it dry
↓
Reconstitute with mobile phase (ACN: 0.1% formic acid) (80:20)
↓
Transfer to HPLC vial and inject in LC-MS/MS

CHAPTER - 4

RESULTS AND DISCUSSIONS

4.1 Solubility assay

Solubility of monomethyl fumarate were tested in different solvent. MMF was almost soluble in every selected solvent, result is in below table. From this study we can interpret that MMF is readily soluble in DMSo, IPA and Methanol and insoluble in normal saline.

Solvents	Solubility (w/v)	Solubility
IPA	30 mg/ml	Highly soluble
DMSo	50 mg/ml	Highly soluble
Normal Saline (NS)	1 mg/ml	Not soluble
Methanol	25 mg/ml	Highly soluble
ACN	10 mg/ml	Moderately soluble
Water	1 mg/ml	Moderately soluble

Table 1: Solubility data of MMF with different solvents

4.2 High Performance Liquid Chromatography of MMF

HPLC method was performed for the qualitative analysis and determination of retention time of MMF. Few HPLC columns such as Zorbax Hilic Agilent, Zorbax Eclipse, Zorbax SB-aq and Hypersil gold, ACN with changing buffers (5mM ammonium acetate, 10 mM ammonium acetate, 10mM ammonium formate, Methanol) organic modifiers were used, since the objective of the method is to quantify Monomethyl fumarate drug. Separation of these components their peak shape and interference from blank sample were monitored in all trials. out of all trials mobile phase ACN:10mM Ammonium formate (70:30) and

Hypersil Gold 50 × 4.6mm, 5u column gave sharp peak at wavelength 265 nm. A better symmetric peak of fumaric acid was obtained when the column temperature was 35-40˚C.

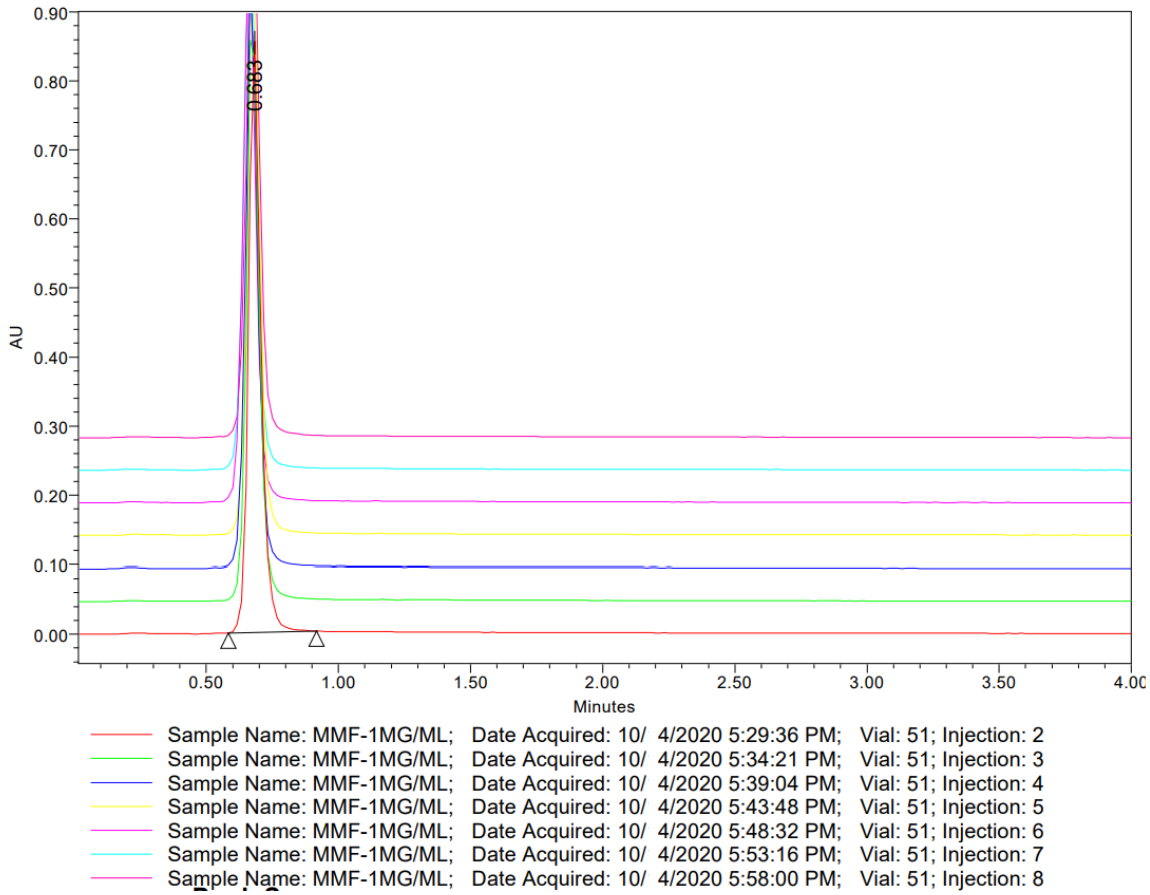

Fig: 6 – HPLC analysis of MMF

	Sample Name	Vial	Inj	Retention Time (min)	Area	% Area
1	MMF-1MG/ML	51	8	0.678	2837105	100.00
2	MMF-1MG/ML	51	2	0.683	2773156	100.00
3	MMF-1MG/ML	51	3	0.674	2861318	100.00
4	MMF-1MG/ML	51	4	0.670	2847248	100.00
5	MMF-1MG/ML	51	5	0.680	2852868	100.00
6	MMF-1MG/ML	51	6	0.664	2818238	100.00
7	MMF-1MG/ML	51	7	0.666	2782745	100.00
Mean				0.674	2824668.1	
Std. Dev.				0.007	34774.0	

	Sample Name	Vial	Inj	Retention Time (min)	Area	% Area
% RSD				1.04	1.2	

Table 2: Peak summary

4.3 Mass Spectrometry

A mass spectrometer is used to measure the mass of a molecule after it converts the molecule to a gas-phase ion. The MMF reference sample we took was pure compound and from current mass spectrum we can check the mass and ionization of MMF. We got good ionization and optimum molecular mass at negative scan side of spectra instead of positive.

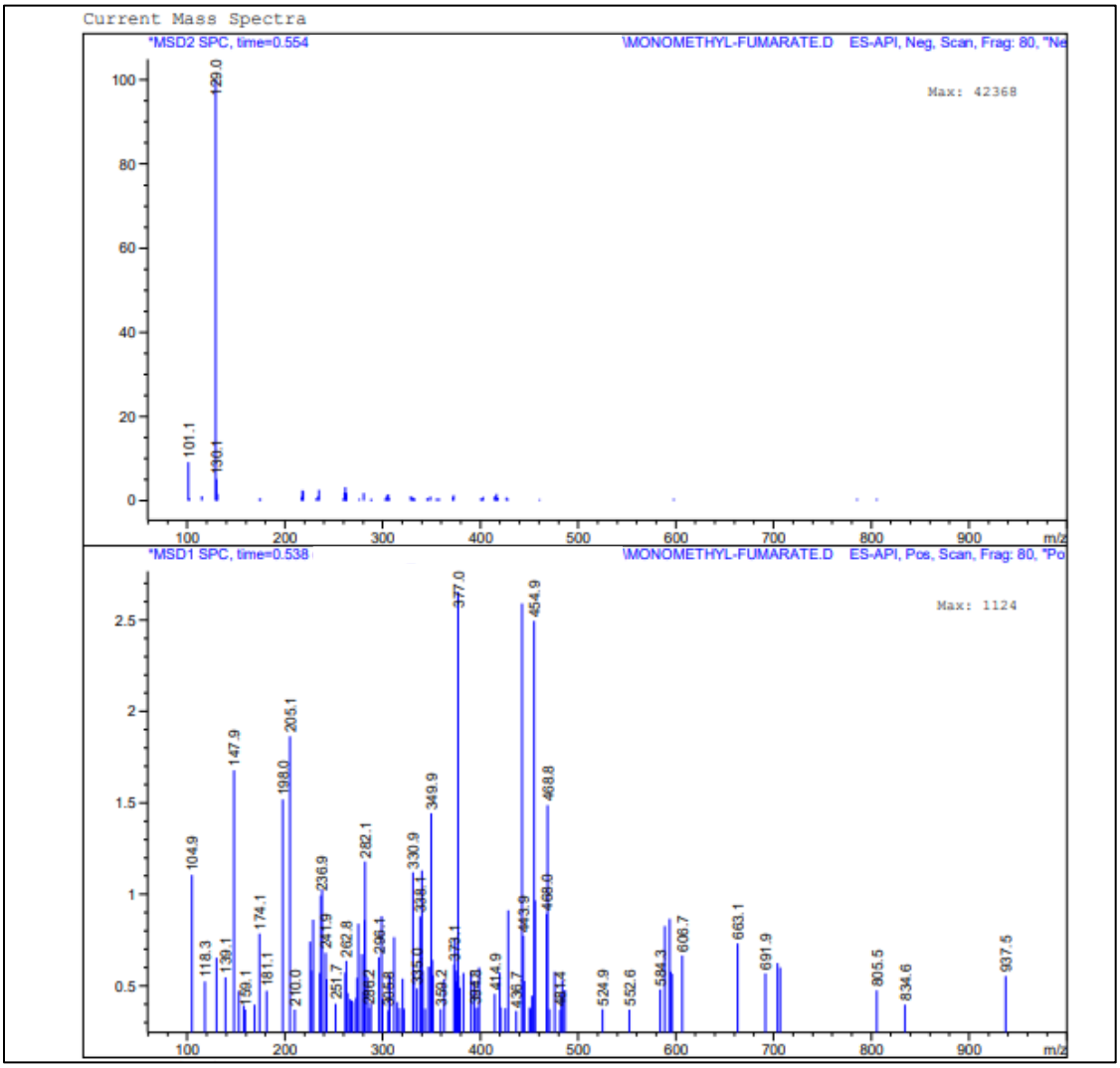

Fig 7: Mass spectra of MMF (Single quad mass)

4.4 LC/MS Analysis

MS parameters were optimized by infusing the standard analyte solution of 100 nanogram per mL into the mass spectrometer having electrospray as the source of ionization and operated in the multiple reaction monitoring (MRM) mode. Protonated form of analyte is

the parent ion in Quadrupole 1 spectrum and was used as the precursor ion to obtain Quadrupole 3 product ion spectra. The most sensitive mass transition was monitored in m/z range of 128.8 to 85 for monomethyl fumarate (shown in fig – 8 and 9).

The source parameters viz. the nebulizer gas, curtain gas, auxiliary gas & collision gas were set at 70, 10, 40 and 3 psi, respectively. The compound parameters such as the declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) were -30, -16, -8, -2 V. LC–MRM is a very powerful tool for pharmacokinetic studies because it provides sensitivity as well as selectivity requirements for bioanalytical methods. Thus, for the assay development, MRM technique was chosen.

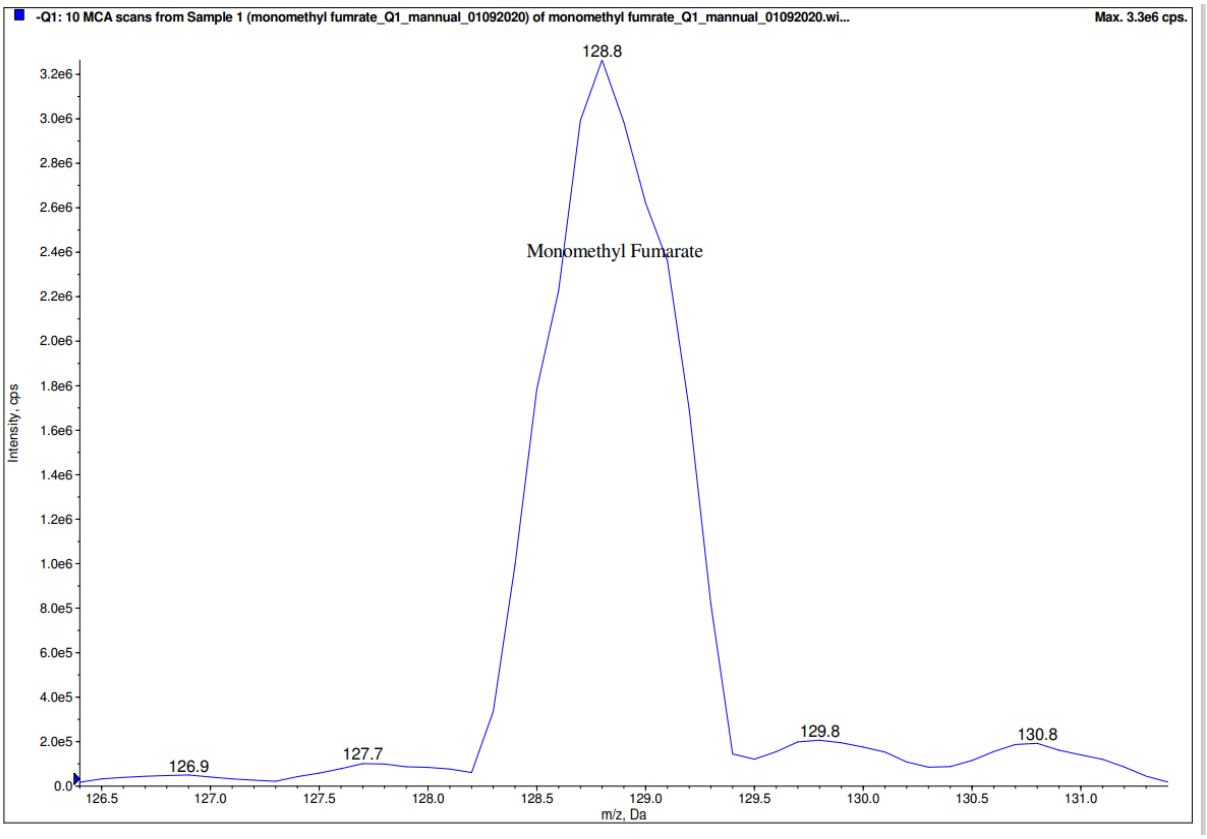

Fig: 8- Monomethyl fumarate (Q1) manual tunning parent ion

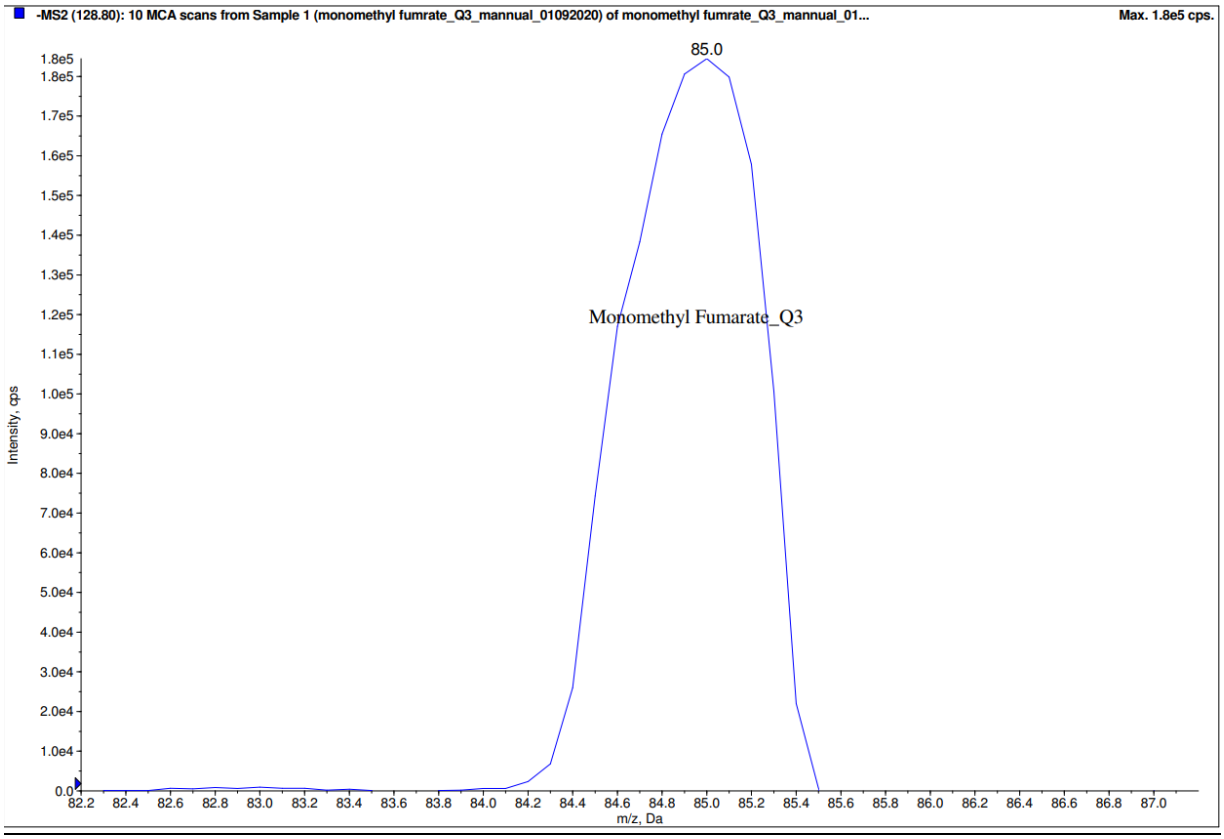

Fig: 9- Monomethyl fumarate (Q3) manual tunning daughter ion

4.5 System suitability

Before the start of the analysis system suitability was performed. For that a mixture of analyte at fixed concentrations constitutes the system suitability solution and it is injected 6 injections for system suitability.

4.6 Aqueous linearity

Standard serial dilution was prepared from 48.8 ng to 1,00,000 ng concentration. From this serial dilution aqueous linearity was prepared from 3.99 ng to 8000 ng (shown in table below) i.e. Cal 01 to Cal 12. All the calibration curve showed the linearity and R^2 value is 0.99.

	STD-12	STD-11	STD-10	STD-09	STD-08	STD-07	STD-06	STD-05	STD-04	STD-03	STD-02	STD-01
Stock Concentration (ng)	1000000.000	100000.000	50000.000	25000.000	12500.000	6250.000	3125.000	1562.500	781.250	390.625	195.313	97.656
Diluent (mL)	0.100	0.500	0.500	0.500	0.500	0.500	0.500	0.500	0.500	0.500	0.500	0.500
Made upto (mL)	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Final concentration (ng)	100000.000	50000.000	25000.000	12500.000	6250.000	3125.000	1562.500	781.250	390.625	195.313	97.656	48.828
Stock Name	STD-12	STD-11	STD-10	STD-09	STD-08	STD-07	STD-06	STD-05	STD-04	STD-03	STD-02	STD-01

Table 3: Standard serial dilution

	STD-12	STD-11	STD-10	STD-09	STD-08	STD-07	STD-06	STD-05	STD-04	STD-03	STD-02	STD-01
Stock Concentration	100000.000	50000.000	25000.000	12500.000	6250.000	3125.000	1562.500	781.250	390.625	195.313	97.656	48.828
Stock solution (mL)	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002
Plasma Volume (mL)	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
Final concentration (ng)	**8000.000**	**4000.000**	**2000.000**	**1000.000**	**500.000**	**250.000**	**125.000**	**62.500**	**31.250**	**15.625**	**7.813**	**3.906**
Stock Name	**Cal 12**	**Cal 11**	**Cal 10**	**Cal 09**	**Cal 08**	**Cal 07**	**Cal 06**	**Cal 05**	**Cal 04**	**Cal 03**	**Cal 02**	**Cal 01**

Table 4: Working standard

4.7 Recovery

The recoveries of the analytes were expected good and reproducible by solid phase extraction (SPE) method. The mean overall recoveries of monomethyl fumarate are expected 65 – 70 %. And stock solutions of MMF were stable for 30-35 days at 4◦C in refrigerator hence % stability of MMF is approx 99%.

	Sample Name	Sample Type	Analyte Peak Area (counts)	Analyte Concentration (ng/mL)	Calculated Concentration (ng/mL)	Accuracy (%)	Use Record
1	BLANK	Blank	0	0.000	N/A	N/A	
2	CAL 01	Standard	1630	62.400	< 0	N/A	☐
3	CAL 02	Standard	0	125.000	No Peak	N/A	☐
4	CAL 03	Standard	3160	250.000	241.658	96.66	☒
5	CAL 04	Standard	3695	500.000	550.973	110.19	☒
6	CAL 05	Standard	4404	1000.000	961.519	96.15	☒
7	CAL 06	Standard	6025	2000.000	1899.866	94.99	☒
8	CAL 07	Standard	9133	4000.000	3699.132	92.48	☒
9	CAL 08	Standard	17878	8000.000	8761.491	109.52	☒

Fig 10: Extraction CC with rat plasma

CHAPTER-5

CONCLUSION

The HPLC method developed for the estimation of monomethyl fumarate is very useful tool for monitoring the quality of MMF and its pharmaceutical forms. The method was found to be precise, and accurate. The method can be used for checking the quality of the manufactured capsules as well as for stability studies of the pharmaceutical capsules. Run time is only 4 min, which makes it attractive procedure for analysis of purity of MMF. For the quantification of monomethyl fumarate in plasma LC-MS/MS methods is specific and sensitive method. out of all extraction method SPE method gave consistent and reproducible recoveries for the analytes. Since MMF is very effective drug for the treatment of Multiple Sclerosis so for its pharmacokinetic studies and during study to increase its bioavailability derived HPLC and LS-MS methods are beneficial.

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