I.1 Drug profile and pharmacology (Moffat, 2005; Quetiapine a and b)

Generic name: Quetiapine fumarate

Brand name: Seroquel

Category: Atypical antipsychotic drug

Description: QF is indicated for the treatment of schizophrenia as well as for the treatment of acute manic episodes associated with bipolar I disorder.

Chemical name: Bis[2-(2-[4-(dibenzo[b,f][1,4]-thiazepin-11-yl)piperazin-1-yl]ethoxy) ethanol] fumarate

Appearance: A white to off white crystalline powder

Chemical formula: \( \text{C}_{42}\text{H}_{50}\text{N}_{6}\text{O}_{4}\text{S}_{2}\cdot\text{C}_{4}\text{H}_{4}\text{O}_{4} \)

Molecular weight: 883.1

Chemical structure:

![Chemical structure of Quetiapine fumarate](image)

Solubility: QF exhibits moderate pH dependent solubility (94.3 mg/mL to 2.37 mg/mL at pH values from 1 to 9). It has an aqueous solubility of 3.29 mg/ml at 25°C.

Log P: Log P value for QF vary with pH (0.45 in water, 1.37 at pH 5, 2.65 at pH 7 and 2.59 at pH 9).

pKa: Weak acid with pKa of 3.3 and 6.8

Melting point: 172 – 175 °C

Mechanism of action: QF, an atypical antipsychotic agent interacts with a broad range of neurotransmitter receptors. QF exhibit affinity for brain serotonin (5HT2)
and dopamine D1 and D2 receptors; this combination of antagonism at D2 receptors (improve positive symptoms) and serotonin receptor (improve negative symptoms) is likely to contribute antipsychotic activity with lower extrapyramidal side effects compared to typical antipsychotics.

Absorption: QF is well absorbed and extensively metabolised by the liver following oral administration. QF has an absolute bioavailability of 9%.

Distribution: QF is approximately 83% bound to plasma proteins.

Metabolism: QF is extensively metabolised by the liver. The major metabolic pathways are sulfoxidation, mediated by cytochrome P450 3A4 which leads to formation of pharmacologically inactive metabolites.

Elimination: Elimination of quetiapine is mainly via hepatic metabolism. QF is excreted as its inactive metabolite: 73% in urine and 20% in faeces. 1% of an administered dose is excreted unchanged. The elimination half-life of QF is 6 h.

Side effects: Dry mouth, dizziness, headache, hypotension, tardive dyskinesia, leukopenia, neutropenia, agranulocytosis and Cerebrovascular adverse reactions.

Dosage: Initial dose starts with 25 mg twice daily, may increase dosage by 25-50 mg 2-3 times a day on the second and third day, as tolerated. The target dose range is 300-400 mg daily after a week in to two divided dose with a maximum of 750 mg.
Section I/ Chapter 3
Intranasal delivery of quetiapine fumarate loaded microemulsion for brain targeting: Formulation and pharmacokinetic consideration

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Intranasal delivery of quetiapine fumarate loaded microemulsion for brain targeting: formulation and pharmacokinetic consideration

Graphical abstract of the work presented in chapter 3.

Abstract

Systemic drug delivery in schizophrenia is a major challenge due to presence of obstacles like, blood-brain barrier and P-glycoprotein. Quetiapine fumarate (QF), a substrate to P-glycoprotein under goes extensive first pass metabolism, thus leading to limited absorption and necessitating frequent oral dosing. The aim of this study was to develop QF based microemulsion (ME) with and without chitosan (CH) to investigate its potential use in improving the bioavailability and brain targeting efficiency. QF loaded ME and mucoadhesive ME showed globule size, pH and viscosity in the range of 29-47 nm, 5.5-6.5 and 17-40 cP respectively. CH-ME showed highest ex-vivo nasal diffusion (78.26 ± 3.29%) in 8 h with no sign of structural damage upon histopathological examination. Circular plume with an ovality ratio closer to 1.3 for CH-ME depicted ideal spray pattern. Significantly higher brain/blood ratio and 2.7 and 3.8 folds higher nasal bioavailability in brain with CH-ME in comparison to QF-ME and drug solution suggested preferential nose to brain transport (80.51 ± 6.46%) bypassing blood-brain barrier. Gamma scintigraphy data showed satisfactory correlation with in vivo pharmacokinetics of CH-ME thus revealing prolonged retention of QF at the site of action with CH-ME and superiority of CH as permeability enhancer.
3.1 Materials and methods

3.1.1 Materials

Quetiapine fumarate (QF) and risperidone were received as a gift sample from Torrent Pharmaceuticals Ltd. (Ahmedabad, India). Verapamil hydrochloride was received from Cadila Pharmaceuticals Ltd. (Ahmedabad, India) as a gift sample. Cremophor EL, Cremophor RH 40 and Polyethylene glycol 400 were received as gift samples from Signet chemicals corporation Pvt. Ltd. (Mumbai, India). Capmul MCM EP, AccononMC8-2 and Captex 200-P were provided as gift samples from Abitec Corporation Ltd. (Mumbai, India). Capryol 90, Labrafac lipophile WL1349, Lauroglycol 90, Plurol Oleique CC 497, Labrasol and Transcutol-P were gift samples received from Gattefosse Pvt. Ltd. (Mumbai, India). Tween 80, Sorbitol sesquioleate, Chitosan (low molecular weight, degree of deacetylation: 75–85%) and Methyl-β-cyclodextrin were purchased from Sigma- Aldrich (Bangalore, India). All other chemicals and reagents were of highly purified grade and were used without further purification.

3.1.2 Physical characterization and identification of QF

Prior to formulation and development, identification of drug and to understand its physical properties is very essential to develop a scientific basis for a drug molecule to form into a dosage form. Such initial examination helps to determine the solution as well as solid state stability of the product. Identification and characterization parameter for the procured QF sample including organoleptic properties, solubility, partition coefficient, loss on drying, melting point, infrared spectrum and X-ray diffraction studies is given below with the details.

3.1.2.1 Organoleptic properties

Nature, color, taste and odour were determined under organoleptic properties.

3.1.2.2 Physical characterization

**Saturation solubility:** The saturation solubility of QF was determined by means of mechanical shaker method. Excess amount of QF was added into 10 ml of distilled water to obtain a saturated solution, which was then placed in a mechanical shaker for 48 h at room temperature to attain the equilibrium. Resultant solution was filtered with 0.22 μm syringe filter and amount of soluble drug was identified by developed HPLC method.
Partition coefficient: Shake flask method was employed to find log P value of QF. For this in one conical flask, octanol and water were mixed together in the equal quantity and to this mixture excess of QF was added. After continuous shaking at 100 rpm for 48 h at room temperature, mixture was removed from the shaker and transferred into separating funnel and allowed to separate two solvents for 24 h. After equilibrium both the solvents were separated, filtered and analyzed for drug concentration.

Differential scanning calorimetry (DSC) analysis: DSC analysis of QF was performed using DSC Q20 (V24.9 build 121, TA instrument, USA). Sample (2 to 4 mg) was sealed into standard aluminum pans and analysis was performed at heating rate of 10 °C/min from 25 to 190 °C under a nitrogen atmosphere with a flow rate of 50 ml/min. An empty sealed aluminum pan was used as reference.

Fourier Transformed Infrared (FTIR) Spectroscopy: FTIR spectra for QF was recorded using FTIR spectrophotometer (Spectrum GX, Perkin Elmer, U.S.A.). The sample was prepared by potassium bromide disc method and scanned in the range of 4000 cm\(^{-1}\) to 400 cm\(^{-1}\). The obtained FTIR spectrum was compared with the Pharmacopoeial standard spectra.

X-ray Diffraction (XRD) Analysis: X-ray diffraction measurement of QF was performed using X-Ray diffractometer (X’PERT MPD, Philips, Holland) having X-ray generator with a copper anode, using Xe-filled counterate as a detector. Analysis was performed in continuous mode with step size of 0.017° over an angular range (2θ) of 3° to 50°. The sample was mounted onto a specific device before the measurement. Obtained diffractogram was analyzed using JCPDF database diffractometry software (Shah et al., 2016c).

3.1.3 Analytical method development and validation for QF
3.1.3.1 Development of UV visible (UV-Vis) spectrophotometric method for QF
UV-Vis spectrophotometric analysis was performed on a Shimadzu UV-1800 spectrophotometer (Tokyo, Japan). Various reagents and stock solutions were prepared to determine calibration curve of QF in different solvent and reagents. Each spectrum was performed in triplicate.
Stock solution of QF: Primary stock solution of QF was prepared by dissolving 10 mg of QF into 10 ml of methanol in a volumetric flask and then volume was made upto 100 ml with methanol to get final concentration of 100 µg/ml. This stock solution was scanned against methanol in the range of 200–400 nm using UV-Vis spectrophotometer to determine $\lambda_{\text{max}}$ values.

A). Calibration curve of QF in methanol: Initially standard solutions were prepared by pipetting required amount of stock solution into 10 ml of volumetric flask. Final volume was made up with the methanol upto 10 ml to obtain concentration of 2, 4, 6, 8, 10 and 12 µg/ml. The absorbances of standard solutions were measured using UV-Vis spectrophotometer against methanol as a blank at the wavelength where maximum absorbance was found for the stock solution of QF. Measurements were taken in a triplicate from which average absorbance values were calculated.

B). Calibration curve of QF in phosphate buffer saline (PBS) pH 6.4: Since in vitro diffusion studies were performed using PBS pH 6.4 as diffusion medium, calibration curve was made to estimate amount of QF in this media. PBS pH 6.4 was prepared by dissolving 1.79 gm of disodium hydrogen phosphate, 1.36 gm of potassium dihydrogen phosphate and 7.02 gm of sodium chloride in 1000 ml of distilled water. Standard solution of 2, 4, 6, 8, 10 and 12 µg/ml were prepared in PBS pH 6.4 from stock solution of 100 µg/ml of QF and absorbances were measured as described above against PBS pH 6.4 as blank.

3.1.3.2 HPLC method development and validation for QF

HPLC method was developed for the analysis of QF using HPLC LC-2010C HT (Shimadzu, Japan) during evaluating various parameters like drug content, ex vivo diffusion studies etc. for various preparations of QF.

A). Chromatographic conditions

Instrument: HPLC LC-2010C HT (Shimadzu, Japan)

Software: Labsolutions software

Column: Kinetex C18, Phenomenex (250 mm x 4.6 mm, I.D - 5 µm)

Mobile phase: Acetonitrile: Potassium dihydrogen orthophosphate (30:70 v/v), pH 6

Detector: UV detector
Wavelength: 250 nm
Flow rate: 1 ml/min
Run time: 12 min
Injection volume: 10 µl
Column temperature: 25 ± 1.0 °C

B). Preparation of mobile phase: To prepare 1M solution, 13.60 g of KH$_2$PO$_4$ was dissolved into 100 ml of volumetric flask containing milli-Q water and mixed gently. This solution was further sonicated for 10 min in an ultrasonic bath and further it can be used as a primary stock solution to prepare 10 mM of KH$_2$PO$_4$, pH 6 buffer solution (1 ml of 1M KH$_2$PO$_4$ solution in 100 ml of milli-Q water). This buffer solution was then filtered through 0.22 µm membrane filter using Millipore vacuum filter assembly. Filtered buffer was then transferred to schott bottle and degassed by sonication for 15 min. Acetonitrile (ACN) was also placed in separate schott bottle and sonicated.

C). Preparation of stock solution of risperidone (Internal standard): Bioanalysis samples having drug spiked in plasma are subjected to extraction by organic solvents. To determine the efficiency of extraction procedure, internal standard is added to each sample. The extraction efficiency calculated as % recovery should be more than 70%. 5 mg of risperidone was accurately weighed and transferred to 100 ml volumetric flask containing methanol.

D). Preparation of stock solution of QF: Accurately weighed amount (10 mg) of QF was dissolved into 100 ml of HPLC grade methanol and from this solution standard solutions of QF were prepared for calibration curve.

E). Calibration curve for QF: Stock solution of QF (100µg/ml) was diluted serially with mobile phase (30:70 v/v, ACN: KH$_2$PO$_4$) to obtain standard solutions in concentration range of 0.2 to 50 µg/ml (0.2, 0.5, 1, 2, 5, 10, 20 and 50). 10 µl of stock solution of internal standard was added to each calibration sample of QF. 10 µl of the calibration sample were injected into the HPLC system. Standard curve was constructed by plotting the concentration versus peak areas of QF. The correlation coefficients, slopes and Y-intercepts of the calibration curve were determined.
F). **Preparation of Quality Control (QC) samples:** The samples having concentrations of 0.4, 8 and 40 μg/ml were considered to represent low, mid and high QC samples, respectively. The QC samples were prepared by appropriate dilution from stock solutions and to these solutions 10 μl of internal standard solution was added and analysed by HPLC.

G). **Method validation:** Developed method was validated for system suitability, linearity, accuracy and precision.

**System suitability:** To evaluate the resolution and reproducibility, system suitability test was performed by taking six replicate analyses of the QF at a concentration of 10 μg/mL. Peak area, retention time, theoretical plates and tailing factor were measured under system suitability.

**Linearity:** The linearity was evaluated by linear regression analysis, from which correlation coefficient and calibration equation were calculated by the least square method.

**Accuracy and Precision:** Accuracy was calculated by comparing the observed concentration with the true concentration value of low, mid and high QC samples. Intra and inter-day precision was determined by repeated analysis of QC samples on same day and on three consecutive days. Precision was determined based on % RSD values.

### 3.1.4 Bioanalytical method development and validation for QF

The chromatographic conditions for bioanalytical method development and validation were same as those of analytical method development and validation as mentioned above in section 3.1.3.2. Two different extraction methods viz., protein precipitation and liquid-liquid extraction were carried out to determine maximum % recovery of the drug from rat plasma. To calculate % recovery, areas for rat plasma containing spiked QF (Extracted sample) and unextracted sample (QF in mobile phase without rat plasma) were determined. Further the ratio of area value between extracted and unextracted will give % recovery of QF from rat plasma.

A). **Preparation of Spiked plasma samples for calibration curve of QF:** Drug solution was spiked in blank rat plasma such that each 100 μl of plasma contains
desired concentration of drug in range of 0.1 – 50 μg/ml and vortexed for 1 min. 25 μl of internal Standard (Risperidone, 50 μg/ml) was added to spiked plasma sample and vortexed for 1 min. To this 25 μl of 0.05 M sodium carbonate and mixed well. Liquid-liquid extraction method was followed for extraction of drug from plasma samples. 2 ml of diethyl ether (extracting solvent) was to each cap tube and vortexed for 1 min. The mixture was then centrifuged at 8000 rpm for 10 mins at 4 °C. After centrifugation, supernatant organic layer was transferred to round bottom Ria tube and evaporated to dryness under a stream of nitrogen at room temperature. Dried extract was reconstituted with 100 μl of mobile phase mixture (30:70 v/v, ACN:KH₂PO₄) from which 20 μl of aliquot was injected and analyzed for QF content by HPLC method. 0.3, 8 and 30 μg/ml were considered to represent low, mid and high QC samples. These samples were also prepared in similar way as described above.

B). Method validation: Parameters like linearity, extracted recovery, accuracy and precision and stability study were performed under bioanalytical method validation.

Linearity: Linearity of response was evaluated using the least square linear regression analysis.

Recovery: Recovery of extraction procedure was calculated by analysing six extracted samples of low, mid and high quality control samples and then comparing it with unextracted samples. The recovery was also calculated by using following equation.

\[
\text{%Absolute recovery} = \left( \frac{\text{Area Extracted}}{\text{Area Unextracted}} \right) \times 100
\]

Accuracy and Precision: Accuracy and precision were determined by analysing the quality control samples at a minimum of 3 concentrations (low, mid and high), representing the entire range of calibration curve but not a part of calibration curve. Intra and inter-day accuracy and precision were calculated by injecting six replicates of Low, mid and high quality control samples in HPLC system for three consecutive days. Accuracy was estimated by comparing the observed concentration calculated from the calibration curve with the true concentration value of low, mid and high quality control samples. Precision was determined based on % RSD values.
Stability Studies: Bench Top stability: Six replicates of high and low quality control were analysed after 0 h and 6 h at room temperature and the deviation between two readings were calculated.

Dry extract stability: Six replicates of high and low quality control were analysed after 0 h and 24 h after storing at -80°C and the deviation between the two readings were calculated.

Long term stability: Six replicates of low and high QC were stored at -80°C for 30 days. The deviation in reading obtained at 1st and 30th day was analysed.

3.1.5 Formulation, optimization and characterization of QF loaded ME for intranasal administration

As discussed in the introduction (section 1.5.3) that QF has showed improved efficacy against positive and negative symptoms in schizophrenia and hence widely accepted as first line drug for treating psychotic symptoms. However, Oral administration with QF shows poor oral bioavailability of 9% due to extensive first pass metabolism leading to limited absorption and poor therapeutic effect. In addition to that as discussed earlier, QF being a Pgp substrate, it also aid into limited absorption when given orally. Thus the objective of this investigation is to develop intranasal ME of QF for brain targeting and to improve bioavailability with enhanced targeting efficiency. Different methodologies and formulation aspects in this section have been followed from the published research work by Shah et al., 2015b and 2016b.

3.1.5.1 Screening of oil, surfactant and cosurfactant by solubility study

To screen out the oil, surfactant and cosurfactant for final formulation, solubility of QF was determined in oils (Capmul MCM EP, Capryol 90, Labrafac lipophile WL 1349, Lauroglycol 90 and Plurol olique CC 497), surfactants (Acconon MC8-2, Cremophor EL, Cremophor RH 40, Labrasol and Tween 80) and cosurfactants (Captex 200-P, Polyethylene glycol 400, Sorbitan sesquioleate and Transcutol-P). Excess of drug was added into each 2ml of cap tube containing different oils, surfactants and cosurfactants, then mixed using a cyclo mixer (CM 101, REMI, Mumbai, India). The cap tubes were then kept for continuous stirring on Rotatest shaker (R100/TW, Luckham, England) at room temperature for 48 h. After
equilibrium, samples were removed from shaker and centrifuged (R2, REMI) at 10,000 rpm for 10 min. The amount of drug solubilized in each vehicle was determined by collecting supernatant from each cap tubes, and samples were diluted using methanol. Concentration of QF in each sample was determined by UV spectroscopy. Solubility study was carried out in triplicate. The oil, surfactant and cosurfactant were taken for further studies into which maximum amount of QF was soluble (Shah et al., 2015b).

3.1.5.2 Construction of pseudoternary phase diagrams
Pseudoternary phase diagrams were constructed to obtain the appropriate concentration for excipients to be incorporated and region into which maximum amount of ME formation takes place. From the result of solubility study, three ME systems were prepared by spontaneous emulsification method using Capmul MCM EP (Oil phase), Tween-80 (Surfactant), Transcutol-P (Cosurfactant) and distilled water (Aqueous phase). Smix was taken in different ratios (1:1, 2:1 and 3:1) to obtain three different ternary phase diagrams. For each phase diagram, oil and Smix were mixed at ambient temperature in nine different ratios starting from 9:1, 8:2, 7:3 up to 1:9 (% w/w). To these, distilled water was added dropwise under continuous stirring until formation of transparent o/w ME takes place. After equilibrium, samples were observed visually for being clear or turbid. Samples exhibiting a transparent and homogeneous state were assigned to a ME region in the phase diagram (Shah et al., 2016b). Pseudoternary phase diagrams were constructed using Triplot software Ver. 4.12 (David Graham and Nicholas Midgley, Loughborough, Leicestershire, UK).

3.1.5.3 Optimization of ME formulation
From three different Smix ratios, the one showing maximum region for ME formulation was considered as an optimized Smix ratio for ME formulation. Different formulation trials were carried out by maintaining oil (% w/w) constant, and % w/w for Smix and water were changed accordingly. All the trials were characterized for globule size, polydispersity index (PDI) and transparency in order to obtain optimized formulation. From the above trials, formulation that showed satisfactory results was subjected to centrifugation (Legend X1R, Thermo Scientific, Waltham, MA) test at different speeds like 5000, 10000 and 15000 rpm for 30 min and observed for phase separation, if any (Mandal and Mandal, 2010).
3.1.5.4 Solubility of QF in optimized ME
An excess of QF was added into the 2ml of cap tube containing optimized ME system and allowed to stir continuously on Rotatest shaker at 25 °C for 48 h. After equilibrium, ME system was centrifuged at 10000 rpm for 10 min. Quantity (mg/ml) of solubilized drug was determined in order to estimate the amount (mg/ml) of QF that can be incorporated into ME system.

3.1.5.5 Screening of mucoadhesive agent
Mucoadhesive agents help to retain the formulation at the site of administration and prevent MCC. As per literature, selected polymers such as chitosan and cyclodextrin were considered for mucoadhesion since they are reported to enhance the residence time at the absorption site thereby improving bioavailability (Khan et al., 2009; Bshara et al., 2014). For the study, dispersions of mucoadhesive polymers were prepared by soaking them in water at room temperature for overnight and were allowed to solubilize till become clear and transparent mixture.

3.1.5.6 Preparation of QF loaded ME and MME
The QF loaded ME and MME was prepared by titration method. Individual formulation was formulated as per final composition shown in Table 11. For this QF was allowed to solubilize in the mixture of Capmul MCM EP and Smix [(3(1:1):1) i.e., (Labrasol: Tween80): Transcutol-P] on magnetic stirrer (Digimag M2D, Eltek, Mumbai, India). To the above mixture distilled water was added and stirred until transparent and homogenous mixture of QF-ME is formed.

Chitosan (CH) and methyl-β-cyclodextrin (MeβCD) were used to prepare QF loaded MME viz., CH-ME and MeβCD-ME respectively. Initially, QF-ME was formulated using minimum quantity of water (50% w/w of the total water quantity was incorporated in the ME) and this was mixed with either CH (1% w/w) or MeβCD (6% w/w) dispersed in remaining 50% w/w of water to get 0.5% and 3%w/w loaded CH-ME and MeβCD-ME respectively.

3.1.5.7 Characterization of QF loaded ME and MME
Characterization of ME and MME were divided into qualitative and physicochemical parameters.
1). Qualitative test

**A). Dye solubility test:** It is also known as staining test. To identify the continuous phase water-soluble dye, methyl orange was sprinkled over ME and observed under microscope (Olympus BX 41, Center Valley, PA). A drop of oil-soluble dye, Sudan III, was sprinkled on a glass slide containing ME and was observed under a microscope (Kamila et al., 2009).

**B). Dilution test:** Dilution test is generally performed to check that the ME is only miscible with the liquid that forms its continuous phase. o/w ME gets easily miscible when it is diluted with water and vice versa for w/o ME upon dilution with oil.

**C). Centrifugation test:** To assess the physical stability of the ME, formulations were subjected to accelerated centrifugation cycle i.e. 5000, 10000 and 15000 rpm for 30 min to observe change in homogeneity if any.

2). Physicochemical parameters

Below mentioned physicochemical parameters were performed in triplicate.

**A). %Transmittance (%T):** %T of the prepared ME and MME were measured using UV-Vis spectrophotometer at 650 nm against distilled water as a blank.

**B). Particle size, PDI and zeta potential measurements:** Particle size, PDI and zeta potential measurements were performed using Zetasizer (Nano-ZS90, Malvern, Worcestershire, UK) by taking 1ml of ME and MME individually into clear polystyrene cuvettes for globule size and disposable folded capillary cell for zeta potential, respectively. The dynamic light scattering measurements were taken at scattering angle of 90° using helium-neon laser as a light source at the wavelength of 633 nm where diffusion of particle due to Brownian motion gets converted into particle size and then calculated by inbuilt software using the Stokes-Einstein equation. In case of zeta potential, due to application of an electric field, particles move with a velocity related to their zeta potential. This velocity is measured using a laser interferometric technique called phase analysis light scattering, which enables electrophoretic mobility of the particles and gets converted to the zeta potential by inbuilt software based on the Helmholtz-Smoluchowski equation (Malvern Zetasizer nano ZS90).
C. **pH:** With respect to intranasal formulations, pH is very crucial aspect to be determined since formulation comes in direct contact with nasal mucosa. pH of the formulations falling out of the nasal secretion pH (4.5-6.5) may cause nasal irritation. pH of the ME and MME was determined by taking 10 ml of formulation in a beaker. pH was measured at room temperature using a calibrated digital pH meter (EUTECH pH Tutor, Singapore).

D). **Viscosity:** Rheological behaviour of the ME and MME was determined using Brookfield viscometer (DV-II+, Brookfield Engineering Laboratories, Middleborough, MA). Viscosity determinations were performed at 30 rpm using spindle S18 at 25 ± 2 °C.

E). **Drug content:** Formulation equivalent to 2 mg of QF was diluted 100 times with methanol. Drug content in ME and MME was determined by means of HPLC method.

### 3.1.5.8 In vitro drug permeation study

An in vitro permeation study of QF formulations was determined using Franz diffusion cell (Hanson Research – Telemodul 40S, Chatsworth, CA) with a receptor volume capacity of 12.5 ml through cellulose acetate membrane (Dialysis membrane 70, HIMEDIA (Mumbai, India); molecular weight cut off 12000-14000 Daltons; pore size: 2.4 nm). PBS (pH 6.4) was used as a dialyzing medium. Cellulose membranes were soaked for overnight into PBS at room temperature and then sandwiched between the receptor and donor compartment. Comparative drug permeation study was performed by taking drug solution (DS; 10 mg/ml aqueous solution of QF), QF-ME, CH-ME and MeβCD-ME equivalent to 10 mg of QF in the donor compartment. The receptor compartment was filled with PBS, maintained at 37±1 °C by circulating water bath. PBS in the receptor compartment was stirred continuously with magnetic bead in a manner that membrane touches the receptor medium surface. Aliquots of 1ml were withdrawn at different time intervals (0.5, 1, 2, 4, 6, 8 and 12 h) and replaced with an equal volume of prewarmed PBS. Samples were filtered using syringe filter (Millex-GV, 0.22 µm, Millipore, Mumbai, India); and after suitable dilutions, samples were analyzed for drug content by HPLC. Study was performed in triplicate, and percentage drug diffused (mean values) was plotted versus time (h) (Shah et al., 2015b).
3.1.5.9 *Ex vivo* nasal permeation study

Nasal permeation study was carried out using Franz diffusion cell with a receptor volume capacity of 12.5 ml using goat nasal mucosa as a dialyzing membrane. The freshly excised goat nasal mucosa was rinsed thoroughly with PBS pH 6.4, from which superior nasal membrane was identified. Nasal mucosa having thickness of 0.3 mm (measured using vernier calliper, CD-6” CSX digital, Mitutoyo Corp., Kanagawa, Japan) was mounted on the diffusion cell with mucosal surface facing donor compartment and serosal surface facing receptor chamber. Previously published researches have revealed presence of Pgp and tight junctions on nasal mucosa which is a primary barrier to drug absorption from nasal cavity. Presence of such barriers play active role in effluxing Pgp substrates thereby limiting the brain uptake after intranasal administration (**Kandimalla and Donovan, 2005; Graff and Pollack, 2003; Graff and Pollack, 2005**). QF, a substrate to Pgp shows poor concentration at brain interface. **Sadeque et al., 2000 and Amin, 2013** reported inhibition of Pgp substrates using Pgp inhibitors like quinidine, verapamil and ketoconazole would result into increased drug delivery to the brain. Hence in the present work verapamil was taken to study the difference in the nasal diffusion between QF solution (DS) and same solution containing verapamil (DS+VH) as a Pgp inhibitor. Comparative nasal diffusion study was performed in triplicate by taking QF-ME, CH-ME, MeβCD-ME, DS and DS+VH equivalent to 10 mg of QF in the donor compartment. Receptor chamber was filled with PBS pH 6.4 and other conditions (temperature and setup of donor and receptor cell) were maintained similar as described above. To maintain minimum living condition of nasal tissue, during experiment the diffusion media was gassed with oxygen using laboratory aerator (Elit801, Dynamic Aqua-supply, Surrey, Canada). Samples were withdrawn at different time intervals up to 12 h, analyzed for drug content by HPLC and percentage drug permeated was calculated (**Pund et al., 2013**).

Flux (μg/cm²/h) and diffusion coefficients (cm²/h) values were calculated from the slope of plot obtained between amount of drug permeated/unit area (μg/cm²) of mucosal membrane versus time (h). The data obtained from nasal diffusion study was fitted to different kinetic models *viz.*, zero order (cumulative percentage of drug release versus time), first order (log cumulative of drug remaining versus time) and Higuchi model (cumulative percentage of drug release versus square root of time) (**Costa and Lobo, 2001**).
3.1.5.10 Ex vivo intestinal diffusion study

Pgp has been reported to be localized in the cells of tissues that have specific barrier functions, i.e. BBB, blood-nerve barrier, blood-placental barrier and intestinal mucosa, where it performs the defensive function. The endothelial cells of BBB and intestinal barrier are connected by tight junctions thus limiting the paracellular permeability (Flens et al., 1996; Schinkel and Jonker, 2003). Deli, 2009 reported that the tight junction components are expressed in brain, in the epithelium of nasal region and small intestine. Hence to confirm the Pgp inhibition and to overcome tight junction, intestinal diffusion study was undertaken to determine the connection with the results of nasal permeation study. Goat small intestine was taken and cleaned with PBS pH 6.4 to remove adhered tissues. Small intestine was cut into 6 cm long segments. One end of segment was tied with thread and from other end formulation equivalent to 16 mg of QF was filled and closed with thread. Each intestinal segment was dipped into beaker containing 20 ml of PBS pH 6.4 (37±1 °C) and comparative intestinal diffusion study was performed in triplicate by taking QF-ME, CH-ME, MeβCD-ME, DS and DS+VH. PBS was allowed to stir continuously on multiple magnetic stirrer in a manner such that the filled portion of the intestinal segment remains in contact with PBS. Sampling at different time intervals and analysis were performed similarly as described in nasal permeation study.

3.1.5.11 Measurement of ex vivo mucoadhesive strength

Mucoadhesive strength of QF-ME, CH-ME and MeβCD-ME were evaluated by Texture analyzer (TA.XT.Plus, Stable Micro Systems, Surrey, UK) using goat nasal mucosa. Cleaned piece of nasal mucosa was attached both on the base of texture analyzer and to the stainless steel probe using two side adhesive tape attached to the mobile arm of the texture analyzer. Individual formulation (0.1 ml) was placed on the nasal mucosa attached to a lower surface of the base. The mobile arm was lowered at a rate of 0.5 mm/s until (pre-test speed) contact with the formulation was made. After contact was made test speed was lowered to 0.1 mm/s and contact force of 100 g was applied for 100 s, consequently the probe was withdrawn from the membrane at a post test speed of 0.1 mm/s. After the adhesive bond was formed, the force (g) required to separate the bond was recorded as a mucoadhesive strength (Kharia and Singhai, 2015).
3.1.5.12 Trans epithelial electrical resistance (TEER) measurement

As discussed in the above section 3.4.10 that the endothelial cells of BBB and intestinal barrier are connected by tight junctions and the presence of Pgp is also reported on the intestinal and nasal mucosal apical surface thus limiting the transport of drug. As per report by Deli, 2009 explicating that the small intestine, nasal epithelium and brain have similar tight junction component structure. He reported that a Caco-2 cell morphologically resembles the tight junction physiology present at BBB, nasal and intestinal barrier. In one dissertation report on nasal drug delivery by Wadell, 2002 mentioned that monolayers of human intestinal Caco-2 cell line can be used to predict drug transport by the different pathways across the intestinal epithelium and this cell lines is often used to study the integrity of nasal epithelial tight junctions. Hence to investigate the effect of formulation on the integrity of tight junctions and on paracellular transport in Caco-2 cell monolayers, TEER measurements were performed. TEER can be explained by prevention of passage of small electrolytes by tight junction. Alteration in TEER value for the tightness of the cell layer was measured.

To study the effect of CH as penetration enhancer and effect of Verapamil as Pgp inhibitor against QF-ME and DS on integrity of tight junctions in Caco-2 cell monolayers, cells were cultured on tissue culture treated polycarbonate inserts in Transwell 12-well plates at pH 6.4. At initial stage i.e. called as post-confluence stage, tight junctions are expressed at the junctions between Caco-2 cells. When Caco-2 cells are grown to confluence after period of 14-16 days i.e. when resistance across the insert membrane ranged from 600 to 800 Ω cm² (control group), they can be used for TEER measurements. To each of the inserts 1 ml of the formulations (CH-ME, QF-ME, DS and VH+DS) was added individually and allowed to incubate for 30 min. After this TEER measurements were performed (Millicell electrical resistance system, Millipore Corp, MA, USA) upto 4 h at every 30 min interval to evaluate any change in the TEER value for the tightness of cell monolayer against blank or control. Experiment was performed in the triplicate (Yeh et al., 2011).

3.1.5.13 Morphological characterization

The morphology of CH-ME was carried out using transmission electron microscope (TEM; Philips, Tecnai 20, Holland) at an acceleration voltage of 200 kV and viewed at a magnification of 50,000×. The size of the CH-ME was measured using
AnalySIS® software (Soft Imaging Systems, Reutlingen, Germany). In order to perform TEM observations, CH-ME was diluted with water (1:10). Drop of diluted CH-ME was then directly deposited on the carbon coated copper grid, stained by 1% aqueous solution of phosphotungestic acid. CH-ME sample was allowed to adsorb on the holey film grid and observed after drying (Samia et al., 2012).

3.1.5.14 Contact angle measurement
An instrument called optical contact angle (15 Pro, Data Physics, Germany) with an inbuilt facility for imaging and having fixed syringe (Hamilton 500 ml) with an outer needle diameter of 0.52mm was used to determine the contact angle between a drop of ME and MME using suitable surface mimicking nasal mucosa (flat piece of goat nasal mucosa of 0.4mm thickness). Syringe was filled with ME and a drop of 5 µl of ME with a flow rate of 1 µl/s was allowed to fall on mucosal surface, and contact angle was captured and recorded immediately with inbuilt software SCA 20 (Filderstadt, Germany). Similarly, contact angle for CH-ME was also determined (Shah et al., 2015b).

3.1.5.15 Nasal cilio-toxicity study
Histopathology study was performed on goat nasal mucosa in order to determine the pathological changes on the nasal mucosa. Three pieces of nasal mucosa with even thickness (0.3 mm) were selected. First piece was treated with positive control (Isopropyl alcohol) for 1 h. Second and third piece of mucosa were treated with negative control (PBS pH 6.4) and CH-ME respectively for 1 h. After treatment for 1 h, all mucosa were rinsed with PBS and fixed into 10% v/v formalin solution for overnight. All pieces of mucosa were embedded in paraffin blocks and cut by a microtome into sections having thickness of 5 µm. Sections were stained with hematoxylin–eosin and observed under inverted microscope (Olympus-IX51, USA) to evaluate any damage to mucosa (Kumar et al., 2009).

3.1.5.16 Nasal spray pattern and plume geometry
To evaluate the spray characterization parameters for the developed formulation, 10 ml of CH-ME was filled into mechanical nasal spray pump capable of delivering 50 µl of formulation per actuation (Aptar Pharma Pvt. Ltd.). Spray pattern and plume geometry for CH-ME was measured using an instrument called EnVision Pharma QS system (Oxford Lasers Pvt. Ltd., UK). EnVision QC analysis software measures
various properties like cone angle, plume width, plume length and spray pattern (cross section of the plume). Instrument makes use of fully integrated actuator to eliminate the variability arising due to hand actuation. A laser light sheet slices through the spray plume and high speed movie of the spray cloud is captured by digital camera from which various images are combined using automated software to measure the cone angle and plume geometry. In case of spray pattern, images of spray plume are combined and ellipse is fitted to the resulting ensemble. The maximum and minimum chords across the ellipse are measured by software and ratio between them is calculated which is known as ovality ratio (Suman, 2009; Trows et al., 2014).

3.1.5.17 Pharmacokinetic study (Shah et al., 2016b)
All animal experiments were approved and performed in accordance with the guidelines of the Institutional Animal Ethics Committee (Registration No: 1661/PO/A/12/CPCSEA under CPCSEA, Delhi, India).

Animal handling drug administration: Sprague Dawley rats weighing between 250-300 g were selected for the biodistribution study. Animals were divided into five groups each consisting of 20 animals. First four groups (CH-ME, QF-ME, DS+VH and DS) were received intranasal (IN) formulations and fifth group was administered QF-ME intravenously (IV) equivalent to 2.3 mg/kg dose of QF respectively. For IN administration groups, formulations equivalent to 6 mg/ml of QF were prepared and 50 μl of formulation was administered into each nostril of anaesthetized rats. Rats were held from back in slanted position during IN administration. Similarly for IV administration group, 100 μl QF-ME (6 mg/ml of QF) was injected through rat tail vein. At each of the following time points: 15, 30, 60, 120 and 240 min (n = 4, each time point), blood samples were collected from retino-orbital vein into heparinized tubes. The rats were than sacrificed humanely by cervical dislocation method at said time intervals and brain tissue was collected and placed in a tube containing 1 ml of PBS pH 6.4.

Extraction of QF from blood and brain samples: After collecting blood samples from the rat, samples were centrifuged at 8000 rpm for 10 min at 4 °C to obtain plasma. Each brain tissue sample containing 1 ml of PBS pH 6.4 was homogenized (Kinematica AG, Polytron PT 1600 E, Switzerland) at 10000 rpm for 1 min. After
homogenization, brain homogenates were centrifuged as mentioned for blood samples to obtain supernatant. Plasma samples and brain supernatants were stored in freezer at −80.0 °C until HPLC analysis. For analysis, 25 μl of internal standard (Risperidone, 50 μg/ml) was spiked into 100 μl of plasma sample and mixed. To this, 25 μl of 0.05 M sodium carbonate and 2 ml of diethyl ether were added and vortexed for 1 min. Samples were centrifuged at 8000 rpm for 10 min at 4 °C and then freeze at −80.0 °C for 5 min. Supernatant organic layer was separated and was evaporated to dryness under a stream of nitrogen at room temperature. Dried extract was reconstituted with 100 μl of mobile phase mixture (30:70 v/v, ACN:KH₂PO₄) from which 20 μl of aliquot was injected and analyzed for QF content by HPLC method. Similar procedure was followed to analyze QF content in brain samples.

**Pharmacokinetic analysis:** Concentration versus time profiles of QF for brain and plasma after IN and IV administration were determined. Pharmacokinetic parameters (C<sub>max</sub>, T<sub>max</sub>, AUC<sub>0-240</sub> and T<sub>1/2</sub>) were calculated for each treatment group by pharmacokinetic software (PK Functions for Microsoft Excel, Pharsight Corporation, Mountain view, CA). Brain targeting efficiency, direct transport percentage and nasal bioavailability of QF following IN administration were calculated using following equations (Haque et al., 2014).

Drug targeting efficiency (DTE %) represents time average partitioning ratio.

\[
\%\text{DTE} = \left( \frac{\text{AUC}_{\text{brain}}}{\text{AUC}_{\text{blood}}} \right)_{\text{IN}} \times 100
\]

Direct transport percentage (DTP %) explains nose to brain direct transport.

\[
\%\text{DTP} = \frac{B_{\text{IN}} - B_{X}}{B_{\text{IN}}} \times 100
\]

Where, \(B_{X} = (B_{\text{IV}}/P_{\text{IV}}) \times P_{\text{IN}}\)

Here, \(B_{X}\) is the brain AUC fraction contributed by systemic circulation through BBB following IN administration. \(B_{\text{IV}}\) is the AUC<sub>0-240</sub> (brain) following IV administration. \(P_{\text{IV}}\) is the AUC<sub>0-240</sub> (blood) following IV administration. \(B_{\text{IN}}\) is the AUC<sub>0-240</sub> (brain) following IN administration. \(P_{\text{IN}}\) is the AUC<sub>0-240</sub> (blood) following IN administration.
Nasal bioavailability was calculated as ratio of AUC\textsubscript{IN}/AUC\textsubscript{IV}.

3.1.5.18 \textit{In vivo} qualitative biodistribution by gamma scintigraphy study

Gamma scintigraphy method provides a noninvasive platform to study qualitative biodistribution and helps to predict the \textit{in vivo} fate of the developed formulation. As described in section 3.1.5.17, the scintigraphy studies were performed after approval of protocol by Institutional Animal Ethics Committee.

\textbf{Radiolabeling:} Formulations (QF-ME, CH-ME, DS and DS+VH) were labeled with technetium-99m ($^{99m}$Tc) by direct labeling method. Stannous chloride was used in the study as powerful reducing agent as it reduces $^{99m}$Tc from the +7 oxidation state to the more reactive +5 oxidation state to promote binding (Patil \textit{et al.}, 2010). To a Ria vial containing individual formulation, 0.2 ml of 2\% w/w stannous chloride solution, 0.05M sodium bicarbonate solution for adjusting pH to 5.5 and 0.3 ml of pertechnetate (5 mCi) was added and allowed to incubate for 10 min at room temperature. Final volume was made upto 1.0 ml with saline solution and the resultant formulation had 100 \mu Ci/20 \mu l activity. Radiolabeling efficiency/purity was determined by ascending instant thin layer chromatography using silica gel coated fiber sheets. 10 \mu l of the radiolabeled formulation complex was spotted on the TLC strip at 1 cm above the bottom. The strips were allowed to develop in 100\% acetone and the solvent front was made to reach up to 5-6 cm top. Control TLC strip \textit{i.e.} spot of 10 \mu l pertechnetate alone was also developed. The strip was cut into two halves and radioactivity in each half was measured using shielded gamma scintillation Counter. The radiolabeling efficiency \textit{i.e.} \% radiolabeling was than calculated as a ratio of radioactivity counts present in the lower part of the strip against total counts present in the strip (Saha, 2010).

\textbf{Administration of radiolabeled complex to the animals:} Sprague Dawley rats weighing between 250-300 g were selected for the gamma scintigraphy study. Animals were divided into five groups as mentioned in the above pharmacokinetic section. 20\mu l of radio labeled drug formulation were administered to anaesthetised rats intranasally. For intravenous group, radio labeled QF-ME was administered to rat by IV route. After period of 15 min anaesthetised rats were placed on the imaging board to study the distribution of the radiolabeled drug. Animals were placed in a posterior–anterior position and emitted radiation was captured using Single
Photoemission Computerized Tomography gamma camera (Brivo NM 615 gamma camera, GE healthcare, UK). Following IN and IV administration of the radiolabeled formulation, the scintigraphy images were recorded after 15 and 120 min of dosing (Patil et al., 2010; Kumar et al., 2014).

### 3.1.5.19 Stability study
Optimized formulation was subjected to stability study to evaluate physical and chemical stability under different storage conditions namely refrigeration condition (4-8°C), room temperature and 40±2°C/75±5% RH (Humidity Chamber, EIE Instruments Ltd., Ahmedabad, India) upto six months. Samples were removed at 0, 1, 3 and 6 months of interval and checked for %T, globule size, zeta potential, pH, viscosity and drug content. After period of three and six months samples were subjected to accelerated centrifugation cycle at 15000 rpm for 30 min to observe for phase separation if any (Kesavan et al., 2013).

### 3.1.6 Statistical analysis
All data are reported as mean ± S.D and the differences between the groups were tested using student’s t test (paired t test, two tailed) at the level of $p<0.05$ considering significant and $p>0.05$ non-significant. In case of multiple comparison, more than two groups were compared using ANOVA (mean of each column is compared with mean of control column using dunnett test) and the differences were considered to be significant and highly significant at the level of $p<0.05$ and $p<0.01$ respectively with the help of GraphPad Pism Version 6.01 software.
3.2 Results and discussion

3.2.1 Physical characterization and identification of QF

3.2.1.1 Organoleptic properties

The organoleptic properties of the received sample of QF were found to be in accordance with the official monograph of QF. It was found to be crystalline, white to off white coloured solid powder, with characteristic odour and pungent taste.

3.2.1.2 Saturation solubility

The solubility of QF in distilled water (pH: 5-6) was found to be 15.26 ± 0.23 mg/ml which was in agreement with the reported value of 15.6 mg/ml (Moffat, 2005).

3.2.1.3 Partition coefficient

Partition coefficient value \(i.e.\) log P o/w of QF was found to be 1.89 ± 0.17 which matches with the reported value of 1.37 to 2.65 at pH between 5 to 7.

3.2.1.4 Differential scanning calorimetry analysis

DSC analysis of QF was performed at heating rate of 10 °C/min from 25 to 190 °C and thermogram was recorded as shown in Figure 13. DSC curve for QF showed sharp endothermic melting event at 175.10 °C, indicating crystalline nature. Obtained melting point value was in the range of the reported melting point value (172 – 175 °C) of QF (Moffat, 2005).

![Figure 13. DSC thermogram of QF.](image-url)
3.2.1.5 Fourier Transformed Infrared (FTIR) Spectroscopy

FTIR spectra of QF is shown below in Figure 14. The characteristic absorption bands for QF at 3440, 2927, 2855, 1600 and 768 cm\(^{-1}\) are assigned to stretching of OH, aromatic H, C-H, C-N, and C-S groups respectively. Obtained IR spectra was compared with reference spectra to confirm presence of absorption bands.

![Figure 14. FTIR spectrum of QF.](image1)

3.2.1.6 X-ray Diffraction Analysis

Diffractogram for QF (Figure 15) is showing sharp peak at 2\(\theta\) scattered angles of 16.13\(^{\circ}\), 20.01\(^{\circ}\), 21.03\(^{\circ}\), 22.15\(^{\circ}\) and 23.19\(^{\circ}\) thus confirming crystalline nature of drug.

![Figure 15. XRD pattern of QF.](image2)
**Outcome:** Overall, on the basis of above physical characterization and identification parameters it was concluded that the obtained drug sample was an authentic sample of QF.

### 3.2.2 Analytical method development for QF

#### 3.2.2.1 UV-Vis spectrophotometric method for QF

The $\lambda_{\text{max}}$ value of 250 nm was found when stock solution of QF (100 µg/ml) in methanol was scanned in the range of 200–400 nm in UV-Vis spectrophotometer. Hence $\lambda_{\text{max}}$ value of 250 nm was considered further for calibration curve and in HPLC method.

**A). Calibration curves of QF in methanol and PBS pH 6.4:**

The calibration curves for QF in methanol and PBS pH 6.4 are shown in Figure 16 (a) and 16 (b) respectively. The calibration curve was plotted in the range of 2-12 µg/ml which was found to be linear and the equations with correlation coefficient values ($> 0.99$) were summarized in the figures. % RSD was less than 2% in both curves.

![Calibration curve for QF in methanol](a)

\[
y = 0.0743x + 0.0114 \\
R^2 = 0.9992
\]

![Calibration curve for QF in PBS pH 6.4](b)

\[
y = 0.0797x - 0.0332 \\
R^2 = 0.9987
\]

Figure 16. Calibration curve for QF (a) in methanol and (b) in PBS pH 6.4.
3.2.2.2 HPLC method development and validation for QF

HPLC method for QF was developed and validated to quantify the amount of drug for the purpose of drug content, \textit{in vitro} and \textit{ex vivo} parameters of the developed formulation. Acetonitrile: 10 mM KH2PO4, pH 6 (30:70 v/v) was used as a mobile phase. UV detector was used at wavelength of 250 nm to analyze QF. Chromatogram of the mobile phase and QF is shown in below Figure 17. The retention time for QF was found to be within 9.4-9.6 min and the total run time was 12 min.

![HPLC chromatogram](image)

**Figure 17.** HPLC chromatogram (A) mobile phase devoid of QF and (B) QF.

\textbf{A). Calibration curve for QF:} Calibration curve for QF was found to be linear in the concentration range of 0.2-50 µg/ml. The linear regression data is shown in Figure 18 which depicts standard plot of mean area value versus concentration.
B). Method validation

System suitability: The acceptance criteria in the system suitability test were ± 2% for the % CV for the parameters studied. Table 10 explicate the % CV value for the peak area, retention time, theoretical plates and tailing factor under system suitability of QF.

<table>
<thead>
<tr>
<th>QF (10 μg/ml) (n=6)</th>
<th>Theoretical plate</th>
<th>Peak area</th>
<th>Retention time (min)</th>
<th>Tailing factor</th>
<th>Capacity factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean value</td>
<td>15682.26</td>
<td>1068721</td>
<td>9.58</td>
<td>1.26</td>
<td>1.32</td>
</tr>
<tr>
<td>%CV</td>
<td>1.27</td>
<td>1.63</td>
<td>0.41</td>
<td>0.24</td>
<td>0.52</td>
</tr>
</tbody>
</table>

Linearity: The calibration curve was constructed in the range of 0.2 to 50 μg/ml i.e. eight concentrations in triplicate including the LOQ. The mean peak area of the drug was plotted against the concentration. The regression equation was found to be $Y = 10793x - 2339.5$ with the correlation coefficient ($r^2$) of 0.9999. The results showed linear correlation between the peak area and concentration of QF.

Accuracy and precision: Accuracy and precision was calculated for intra-day and inter-day, as shown in Table 11. The intra-day precision for low, mid and high quality control samples was found to be 6.48%, 4.59% and 7.22% respectively.
Inter-day precision (% CV) of low, medium and high quality control samples were found to be 4.13%, 6.37% and 5.32% respectively. Accuracy of intra-day quality control samples ranged from 96.51% to 98.22% and for inter-day samples 98.12% to 99.02%.

Table 11. Intra and inter-day accuracy and precision of QF by HPLC.

<table>
<thead>
<tr>
<th>Nominal concentration (μg/ml)</th>
<th>Mean observed concentration (μg/ml)</th>
<th>Precision (% CV)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intra-day (n=6)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LQC 0.4</td>
<td>0.381</td>
<td>6.48</td>
<td>97.05</td>
</tr>
<tr>
<td>MQC 8</td>
<td>7.92</td>
<td>4.59</td>
<td>98.22</td>
</tr>
<tr>
<td>HQC 40</td>
<td>39.59</td>
<td>7.22</td>
<td>96.51</td>
</tr>
<tr>
<td><strong>Inter-day (n=18)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LQC 0.4</td>
<td>0.39</td>
<td>4.13</td>
<td>99.02</td>
</tr>
<tr>
<td>MQC 8</td>
<td>7.86</td>
<td>6.37</td>
<td>98.12</td>
</tr>
<tr>
<td>HQC 40</td>
<td>39.79</td>
<td>5.32</td>
<td>98.56</td>
</tr>
</tbody>
</table>

As per the guidelines of ICH Q2, coefficient of variation (%CV) should not exceed 15% at all concentration and for accuracy the mean observed concentration should lie within 15% of actual concentration. The obtained values lie within the limits of the guidelines, hence the method was found to be precise and accurate in the calibration range.

### 3.2.3 Bioanalytical method development and validation for QF

Bioanalytical method for QF was developed by keeping chromatographic conditions similar as per analytical method of QF. Among two different extraction methods, liquid-liquid extraction method showed maximum recovery of QF (89.23 ± 4.16%) from rat plasma when diethyl ether was used as an extracting solvent. Bioanalytical method was found to be specific, as QF and risperidone (internal standard) were well resolved with retention time of 9.63 for QF and 5.90 for risperidone. In below Figure 19 representative chromatogram of blank mobile phase (A), blank rat plasma (B) and QF spiked into rat plasma with risperidone (C) is presented. The method was thus found to be specific.
Figure 19. HPLC chromatogram (A) mobile phase (B) blank rat plasma and (C) QF and risperidone in rat plasma.
**A). Calibration curve of QF in rat plasma:** Calibration curve for QF in rat plasma was developed in the concentration range of 0.1 – 50 μg/ml as shown in the below Figure 20. Standard plot depicts the mean area value versus concentration with regression equation and correlation coefficient value of 0.9968.

![Figure 20. Calibration curve of QF in rat plasma.](image)

**B). Method validation**

*Linearity:* The least square linear regression analysis of peak area ratios of QF versus concentration was obtained with regression equation of $Y = 117480x + 20554$ and bioanalytical method was found to be linear in the concentration range of 0.1 – 50 μg/ml with $r^2$ value of 0.9999 thus showing linear correlation.

*Recovery:* Recovery of the extraction method indicates the amount of analytes that can be recovered from the plasma samples after extraction using organic solvents. Six extracted samples of low, middle and high quality control were analysed and their peak areas were compared with corresponding concentrations of unextracted samples. Table 12 shows the % recovery of QF from plasma samples.

<table>
<thead>
<tr>
<th>Concentrations μg/ml (n=6)</th>
<th>Mean peak areas of extracted samples</th>
<th>Mean peak areas of unextracted samples</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>LQC 0.3</td>
<td>45349.76</td>
<td>49247.22</td>
<td>91.27</td>
</tr>
<tr>
<td>MQC 8</td>
<td>897834.06</td>
<td>994785.21</td>
<td>88.02</td>
</tr>
<tr>
<td>HQC 30</td>
<td>3062426.76</td>
<td>3401354.89</td>
<td>90.24</td>
</tr>
</tbody>
</table>
The obtained results indicate that the liquid-liquid extraction method for extraction of QF from plasma samples using diethyl ether as extracting solvent gives good recovery of QF from plasma and hence can be used for extraction of drug after *in vivo* study.

**Accuracy and precision:** Table 13 shows the intra and inter-day precision and accuracy for low, mid and high quality control samples. The intra-day precision (% CV) of low, mid and high QC samples of QF were found to be 5.22%, 7.32% and 6.497%. The inter-day precision (%CV) were found to be 4.94%, 5.98% and 7.92% respectively. The intra-day accuracy value for QF was found to be between 96.58%-99.11% and inter-day accuracy was found to be between 96.61% to 99.02%.

**Table 13.** Intra and inter-day accuracy and precision of QF (Plasma sample).

<table>
<thead>
<tr>
<th>Nominal concentration (μg/ml)</th>
<th>Mean observed concentration (μg/ml)</th>
<th>Precision (% CV)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intra-day (n=6)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LQC 0.3</td>
<td>0.288</td>
<td>5.22</td>
<td>97.39</td>
</tr>
<tr>
<td>MQC 8</td>
<td>7.69</td>
<td>7.32</td>
<td>96.58</td>
</tr>
<tr>
<td>HQC 30</td>
<td>29.87</td>
<td>6.49</td>
<td>99.11</td>
</tr>
<tr>
<td><strong>Inter-day (n=18)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LQC 0.3</td>
<td>0.282</td>
<td>4.94</td>
<td>99.02</td>
</tr>
<tr>
<td>MQC 8</td>
<td>7.84</td>
<td>5.98</td>
<td>98.37</td>
</tr>
<tr>
<td>HQC 30</td>
<td>29.71</td>
<td>7.92</td>
<td>96.61</td>
</tr>
</tbody>
</table>

The intra-day precision (% RSD) of all QC samples ranged between 5% to 8% and inter-day precision ranged from 4% to 8%. As per the Bioanalytical method validation guidelines of **ICH Q2 R1**, coefficient of variation (indication of precision) around mean observed concentration should not exceed 15% and for accuracy the mean observed concentration should lie within 15% of the actual concentration. The obtained values are within the limits of specified guidelines. Hence, overall results indicate that the method was precise and accurate in the entire calibration range.
Stability study: Table 14 shows the result of bench top stability study, dry extract stability study and long term stability study. The obtained results indicate that QF is stable in plasma upon storage at -80°C for up to one month.

Bench top stability: The deviation observed after keeping the high and low QC samples for 6 h were 7.49 % and 4.38 % respectively.

Dry extract stability: The deviation in estimated concentration after storing plasma sample for 24 h at -80°C for high and low QC samples were 7.91% and 6.83 % respectively.

Long term stability: The deviation obtained after analysing the high and low QC samples stored at -80°C for 30 days were found to be 8.41 % and 3.73% respectively.

Table 14. Stability data for QF in rat plasma.

<table>
<thead>
<tr>
<th>QC samples</th>
<th>Mean concentration observed at 0 h (μg/ml)</th>
<th>Mean concentration observed at last h (μg/ml)</th>
<th>Deviation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bench top stability (n=6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HQC</td>
<td>29.78</td>
<td>29.44</td>
</tr>
<tr>
<td></td>
<td>LQC</td>
<td>0.292</td>
<td>0.285</td>
</tr>
<tr>
<td></td>
<td>Dry extract stability (n=6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HQC</td>
<td>29.82</td>
<td>29.65</td>
</tr>
<tr>
<td></td>
<td>LQC</td>
<td>0.287</td>
<td>0.263</td>
</tr>
<tr>
<td></td>
<td>Long term stability (n=6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HQC</td>
<td>29.61</td>
<td>29.25</td>
</tr>
<tr>
<td></td>
<td>LQC</td>
<td>0.249</td>
<td>0.252</td>
</tr>
</tbody>
</table>

Outcome: From the above results of UV and HPLC analysis of QF, it was concluded that the developed methods were highly stable, accurate and precise to quantify QF from biological samples, formulation and diffusion studies. Bioanalytical method showed superior stability of QF in rat plasma over a period of one month thus proving its accuracy.
3.2.4 Formulation, optimization and characterization of QF loaded ME and MME for intranasal administration

3.2.4.1 Solubility study

The components selected for the formulation of ME should be pharmaceutically acceptable, non-irritant and nonsensitizing to the skin and fall under the GRAS category. As per Figure 21, solubility of QF was found to be highest in Capmul MCM EP (25.41 ± 0.74 mg/ml) among all other oils. In case of o/w ME system, solubility of drug into oil phase is the most important criterion. Capmul MCM EP is also reported in enhancing the bioavailability of poorly absorbed drugs; hence Capmul MCM EP was selected as an oil phase (Lawrence and Rees, 2000). Among surfactants, Tween 80 (16.53 ± 1.42 mg/ml) and labrasol (14.31 ± 0.82 mg/ml) showed maximum solubility for QF and least was in remaining surfactants.

Figure 21. Solubility of QF in oils, surfactant and cosurfactants (n=3).

Tween 80 is reported to inhibit Pgp activity (He et al., 2010). Labrasol also showed satisfactory solubility for QF. In addition, it is reported to increase the permeability of tight junction and being widely used to enhance the oral, transdermal and intranasal absorption of poorly absorbed drug (Piao et al., 2010). The most important criteria in selecting surfactant is the hydrophilic lipophilic balance (HLB), the value of which should be more than 10 to form stable o/w ME system. In present work tween 80 and labrasol with HLB value of 14 and 15 were selected as
surfactants due to their nonionic nature. However use of nonionic surfactants in pharmaceutical formulations is gradually increasing due to their highly stable and least toxic nature. It is also reported that the combined use of surfactant may provide better HLB, superior solubilization and stable o/w ME formulation since the ability of surfactant to partition into oil-water interface have been increased (Cannon, 2011). Many times surfactants used in the formulation may not lower the interfacial tension sufficiently to form stable ME system. In such situations cosurfactant is added to decrease the blending stress of interface and to increase the fluidity of interfacial film around the ME droplets. Due to amphiphilic nature of cosurfactant, they accumulate substantially at the interfacial layer and intercalate between surfactant molecules thereby decreasing polar head group interactions (Kawakami et al., 2002; Sood et al., 2014). Transcutol-P with the HLB value of 4.2 showed highest solubility of 20.15 ± 0.87 mg/ml for QF among all other cosurfactants. Transcutol-P has an ability to form transparent and stable ME, hence it was selected as cosurfactant.

3.2.4.2 Pseudoternary phase diagrams
It is very important to select the ratio of oil to Smix in the formation of ME system. Pseudoternary phase diagrams help in selecting ME region and appropriate concentration ranges that can result in large existence area of ME. Figure 22(A) indicates ternary diagram consisting of Capmul MCM EP, three different ratios 1:1, 2:1 and 3:1 of Smix (Tween 80: Transcutol-P) and distilled water with shaded region denoting ME region. It was noted that at Smix ratio 3:1 the existence of ME area was higher when compared to remaining Smix ratios. This could be explained by the fact that as concentration of Tween 80 increases, it resulted in maximum solubilization of water into ME, thereby showing larger area. Based on visual observation it was found that nine different oil to Smix ratios of Smix system 3:1 were highly viscous since the amount of Tween 80 was higher. Systems being less viscous in nature allow good tolerance and are easy to handle compared to highly viscous formulations. Hence another ternary plot was constructed using combination of surfactants (Labrasol + Tween 80), since labrasol also showed satisfactory solubility for QF (14.31 ± 0.82 mg/ml). In the ternary plot (Figure 22A), highest ME formation region was observed with Smix 3:1 (Tween 80: Transcutol-P).
Figure 22. Pseudo ternary phase diagrams; (A) Capmul MCM EP, S\text{mix} (Tween 80: Transcutol-P) and water and (B) Capmul MCM EP, 3:1 S\text{mix} (Labrasol + Tween 80: Transcutol-P) and water.
Hence it was taken further and surfactant part (3 out of Smix ratio 3:1) was divided into three different ratios viz., 1:1, 2:1 and 3:1 of surfactant mixture (Labrasol + Tween 80) and ternary plot was made consisting of Capmul MCM EP, 3:1 Smix (Labrasol + Tween 80: Transcutol-P as 3(1:1):1, 3(2:1):1 and 3(3:1):1) and distilled water as shown in Figure 22B. Highest ME region was found when surfactant mixture was 1:1 (Labrasol: Tween 80). From Figure 22B it was observed that, as the labrasol concentration was increasing in surfactant mixture the ME region was decreasing. This can be explained as, upon increasing the surfactant concentration to a certain extent, it results in enhanced water penetration into the oil droplets causing interfacial disruption and ejection of oil droplets into the aqueous phase leading to decreased ME region (Pouton, 1997). Hence Smix ratio 3(1:1):1 (Labrasol + Tween 80: Transcutol-P) was selected as optimized Smix ratio for further preparations.

3.2.4.3 Optimization and preparation of ME and MME

From the result of ternary phase diagrams, Smix ratio 3(1:1):1 with Surfactant mixture 1:1 (Labrasol: Tween 80) showed highest ME formation region. In this Smix ratio 3(1:1):1, all nine different oil to Smix ratios (9:1, 8:2, 7:3 up to 1:9% w/w) were diluted with water by serial dilution method and were observed for homogeneity and phase separation over the period of 24 h. It was observed that oil to Smix ratio 2:8 showed good transparency and was found to be stable over the period of 24 h. Oil to Smix ratios from 9:1 to 4:6 were highly turbid when observed visually. This could be explained by the fact that in the presence of higher oil content, i.e. oil concentration > 30% result in turbid and crude emulsion (Hong et al., 2006).

In case of oil to Smix ratio 1:9, two phases were found to be separated because the amount of water incorporated to form stable ME system was higher which might have resulted into ejection of oil droplets into aqueous phase leading to formation of unstable ME after certain period of time (Pouton, 1997). Hence, oil to Smix ratio of 2:8 containing 6% w/w oil, 30% w/w Smix and 64% w/w distilled water was selected for further optimization by maintaining oil concentration constant and Smix concentration was increased. By keeping oil concentration at 6% w/w, ten different batches of ME as shown in Table 15 were prepared whereby Smix concentration was increased from 30% w/w to 48% w/w (2% w/w Smix was increased every time). Water concentration was brought down from 64% w/w to 46% w/w (2% w/w
Water was reduced every time. All the trials shown below were characterized for size, PDI and %T. Since the surfactants used were nonionic in nature, zeta potential was not evaluated for different trials.

Table 15. Optimization and characterization of ME (n=3).

<table>
<thead>
<tr>
<th>(%) w/w</th>
<th>Oil</th>
<th>Smix</th>
<th>Water</th>
<th>%T</th>
<th>Size (nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>6</td>
<td>30</td>
<td>64</td>
<td>98.04 ± 0.14</td>
<td>189.0 ± 1.32</td>
<td>0.654 ± 0.021</td>
</tr>
<tr>
<td>F2</td>
<td>6</td>
<td>32</td>
<td>62</td>
<td>98.35 ± 0.27</td>
<td>167.9 ± 0.59</td>
<td>0.469 ± 0.018</td>
</tr>
<tr>
<td>F3</td>
<td>6</td>
<td>34</td>
<td>60</td>
<td>98.43 ± 0.03</td>
<td>127.8 ± 1.45</td>
<td>0.305 ± 0.032</td>
</tr>
<tr>
<td>F4</td>
<td>6</td>
<td>36</td>
<td>58</td>
<td>98.69 ± 0.37</td>
<td>106.9 ± 0.89</td>
<td>0.330 ± 0.038</td>
</tr>
<tr>
<td>F5</td>
<td>6</td>
<td>38</td>
<td>56</td>
<td>99.18 ± 0.19</td>
<td>84.41 ± 0.65</td>
<td>0.304 ± 0.026</td>
</tr>
<tr>
<td>F6</td>
<td>6</td>
<td>40</td>
<td>54</td>
<td>99.49 ± 0.21</td>
<td>52.39 ± 0.37</td>
<td>0.286 ± 0.011</td>
</tr>
<tr>
<td>F7</td>
<td>6</td>
<td>42</td>
<td>52</td>
<td>99.94 ± 0.42</td>
<td>42.84 ± 1.16</td>
<td>0.264 ± 0.007</td>
</tr>
<tr>
<td>F8</td>
<td>6</td>
<td>44</td>
<td>50</td>
<td>100.12 ± 0.05</td>
<td>30.90 ± 1.06</td>
<td>0.175 ± 0.023</td>
</tr>
<tr>
<td>F9</td>
<td>6</td>
<td>46</td>
<td>48</td>
<td>100.03 ± 0.33</td>
<td>22.61 ± 1.74</td>
<td>0.223 ± 0.041</td>
</tr>
<tr>
<td>F10</td>
<td>6</td>
<td>48</td>
<td>46</td>
<td>99.87 ± 0.31</td>
<td>20.70 ± 0.89</td>
<td>0.249 ± 0.037</td>
</tr>
</tbody>
</table>

At lowest concentration of Smix (30% w/w) globule size was higher (189.0 ± 1.32 nm) and at higher concentration of Smix (48% w/w) globule size was lower (20.70 ± 0.89 nm) with %T more than 98% for all batches. Effect of Smix concentration on zeta potential was found to be negligible since the surfactant and cosurfactant used were nonionic in nature. Batch with oil: Smix: water ratio of 6:44:50% w/w was selected as optimized batch as it showed globule size of 30.90 ± 1.06 nm with PDI value of 0.175 ± 0.023. No phase separation was observed upon centrifugation at 5000, 10000 and 15000 rpm indicating superior stability. Batches which showed globule size lower than 30.90 ± 1.06 nm were having Smix concentration more than
44% w/w which may cause irritation to nasal mucosa due to presence of higher amount of surfactant. Hence, oil:Smix:water ratio of 6:44:50% w/w was selected as a final formulation for QF-ME into which 23.39±0.47mg/ml of QF was found to be soluble indicating 23 mg/ml of QF (10 mg/ml QF) can be incorporated into final ME system.

CH-ME and MeβCD-ME were prepared using 1% w/w CH and 6% w/w MeβCD in a way that final formulations will have 0.5% w/w CH-ME and 3% w/w MeβCD-ME respectively. Compositions for QF-ME, CH-ME and MeβCD-ME are as per formula shown in Table 16.

Table 16. Optimized composition of QF-ME, CH-ME and MeβCD-ME.

<table>
<thead>
<tr>
<th>Ingredients (% w/w)</th>
<th>QF-ME</th>
<th>CH-ME</th>
<th>MeβCD-ME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quetiapine Fumarate (mg/ml)</td>
<td>23</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>Capmul MCM EP</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Labrasol</td>
<td>44</td>
<td>44</td>
<td>44</td>
</tr>
<tr>
<td>Tween 80 (3(1:1):1) Transcutol-P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Chitosan</td>
<td>-</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>Tri methyl beta cyclodextrin</td>
<td>-</td>
<td>-</td>
<td>3</td>
</tr>
</tbody>
</table>

3.2.4.4 Characterization of QF loaded ME and MME

Qualitative and physicochemical parameters were performed for the developed formulation as shown below.

1). Qualitative test

A). Dye solubility test: With water-soluble dye, methyl orange, it was found to be distributed uniformly throughout the ME and showed continuous colored background of ME with dye confirming its o/w nature when observed under microscope. In case of oil-soluble dye, Sudan III, oil globules were found to be red.
colored and continuous phase was colorless when observed under microscope (Shah et al., 2015b).

**B). Dilution test:** From this test it was observed that when formulations were diluted with water, they could be easily diluted and remained clear and transparent. However upon dilution with oil, they became turbid hence confirming that the developed formulations were o/w in nature.

**C). Centrifugation test:** Neither phase separation nor change in the homogeneity was observed even after accelerated centrifugation at 15000 rpm for 30 min, thus confirming the superior physical stability of the formulations.

2). **Physicochemical parameters**

Characterization parameters like %T, size, PDI, zeta potential, pH, viscosity and drug content were performed for QF-ME, CH-ME and MeβCD-ME in triplicates.

A). **%Transmittance:** The %T for all three QF loaded ME systems was in the range of 95–97% indicating optical clarity of all systems.

B). **Size, PDI and zeta potential measurements:** The globule size of 29.75 ± 0.99, 35.31 ± 1.71 and 46.55 ± 1.9 nm with PDI of 0.221 ± 0.01, 0.249 ± 0.03 and 0.233 ± 0.02 for QF-ME, CH-ME and MeβCD-ME respectively, indicated all three systems showed narrow size distribution approaching a monodisperse system (Figure 23). Globule size is one of the major factor for intranasal delivery of drugs to the brain, since globules with smaller size (< 100 nm) will have larger surface area which in turn increase rate of drug absorption. At smaller size, higher amount of drug can be transported across the brain through olfactory region in nasal mucosa thereby preventing the availability of drug at non-targeting sites leading to enhanced therapeutic effect at reduced frequency of dosing and lower side effects (Bshara et al., 2014). Slight positive and/or close to neutral zeta value of 2.77 ± 0.51 mV for QF-ME could be attributed to nonionic nature of surfactant and cosurfactant. Zeta potential values of 20.29 ± 1.23 mV and 8.43 ± 0.7 mV for CH-ME and MeβCD-ME respectively, were probably due to the cationic nature of mucoadhesive agents. This phenomenon could be explained by the fact that, upon addition of cationic mucoadhesive agent, it would get adsorbed onto the surface of particle leading to positively charged surface with high zeta potential value and stability.
C). **pH**: pH for all QF loaded ME systems was within the range of 5.5 to 6.5 which is falling within the normal pH range (4.5 to 6.5) of human nasal mucosa thereby suggesting non-irritant nature of the formulations (Washington et al., 2000).

**D). Drug content**: Assay values within the range of 93–98% indicated that higher amount of QF was present in all ME systems.

**E). Viscosity**: Data obtained from viscosity study revealed that viscosity for the MME system was higher than the ME system. Viscosity plays an important role in case of intranasal formulation since it is related with residence time in the nasal mucosa. An optimum viscosity is required for the formulation to overcome MCC and have better penetration rate to enable hassle free administration. Viscosity values for QF-ME, CH-ME and MeβCD-ME was 17.5 ± 0.69, 38.5 ± 1.26 and 33.3 ± 0.93 cP respectively, which was lower than 50 cP and hence ensuring well tolerance upon intranasal administration. It is often seen that formulation with higher viscosity value results in enhanced residence time but face difficulties in administration and might show diminished absorption due to lower diffusion of drug from the formulation, while systems with lower viscosity can be administered easily but have to face faster MCC thereby lower contact time of the applied formulation with mucosa (Furubayashi et al., 2007).
3.2.4.5 *In vitro* drug permeation study

Figure 24 shows comparative *in vitro* diffusion profile of QF as percentage drug diffused versus time (h) for CH-ME, MeβCD-ME, QF-ME and DS. The QF diffused from DS was lowest (<35% upto 12 h) among all other formulations. Probable reason could be due to poor aqueous solubility of QF which could not diffuse across membrane into diffusion media hence resulted into lower %drug permeation. From Figure 24, it was revealed that the percentage drug diffused was highest in case of CH-ME when compared to MeβCD-ME, QF-ME and DS. All formulations showed maximum release for QF upto 6 to 7 h after which diffusion profile remained fairly constant upto 12 h. CH-ME showed almost 65% of diffusion upto 12 h which was 1.6 times higher than QF-ME. It was concluded that the extent of QF diffused from MME was greater than ME and DS. Perhaps, it would be not suitable to rely on findings from *in vitro* study using cellulose acetate membrane being an artificial membrane (Shah et al., 2015b).

![Figure 24](image)

**Figure 24.** Comparative *in vitro* diffusion profile of CH-ME, MeβCD-ME, QF-ME and DS in PBS, pH 6.4 (*n=3*).

3.2.4.6 *Ex vivo* nasal and intestinal diffusion study

Nasal diffusion study was performed using goat nasal mucosa as an alternative to human nasal mucosa for various QF loaded systems. Figure 25 depicts comparative nasal diffusion as percent drug diffused versus time (h). The amount of drug diffused
from CH-ME and DS + VH was highest when compared to other groups. As shown in Figure 25 and 26, more than 75% of drug was diffused from CH-ME and DS + VH after 6 h, whereas amount of drug diffused from DS was up to 40% only. It was observed that amount of QF diffused form various groups after 8 h, remains fairly constant up to 12 h. Based on *ex vivo* nasal data, flux and diffusion coefficient values of CH-ME was found to be $1.526 \pm 0.027 \mu g/cm^2/h$ and $(8.32 \pm 0.12) \times 10^{-4} \, cm^2/h$ respectively whereas for DS + VH it was $1.573 \pm 0.031 \mu g/cm^2/h$ and $(8.54 \pm 0.16) \times 10^{-4} \, cm^2/h$ respectively which was slightly higher than CH-ME. The order of diffusion coefficient was found to be DS + VH > CHME > MeβCD-ME > ME > DS, indicating enhanced diffusion with ME system compared to pure DS.

When compared to CH-ME and MeβCD-ME, CH-ME showed higher flux and diffusion coefficient indicating improved penetration enhancing and mucoadhesion performance with CH, which might have contributed for modulation of tight junction integrity and thus resulting in an enhanced paracellular transport (Illum, 1998; Yeh et al., 2011). This finding was in accordance with the outcome of Zerrouk et al., 2006, who described enhanced effect on opening of epithelial tight junction with CH in comparison to cyclodextrins, as a result of which they found higher permeation effect due to increased paracellular permeability by CH.
In the below Table 17, flux and diffusion coefficient values calculated based on data obtained from ex vivo nasal diffusion study for various QF loaded formulations are shown. DS + VH showed diffusion coefficient closer to CH-ME, whereas DS alone showed lowest diffusion coefficient in case of ex vivo nasal and intestinal diffusion study. This could be explained by the fact that QF a substrate to Pgp could not diffuse across nasal and intestinal epithelial membrane due to presence of Pgp efflux pump. In presence of VH, a Pgp inhibitor, the efflux pump activity is altered thereby preventing the drug from being pumped back to mucosal side resulting in higher diffusion for DS + VH in comparison to DS alone. This finding can be supported by the outcomes of Al-Mohizea, 2010 who studied the effect of VH on intestinal permeability of furosemide and confirmed the inhibitory effect on Pgp efflux pump (Al-Mohizea et al., 2014). Ex vivo nasal and intestinal diffusion data showed less than 45% of QF was diffused from DS at the end of 12 h which gave lowest diffusion coefficient. This could be attributed to various factors like presence of obstacles on the mucosal surface, poor lipophilicity, pH dependent solubility and Pgp substrate nature of QF. From the ex vivo nasal diffusion data, regression coefficients of all QF loaded carriers were compared and found that the release pattern of QF from the CH-ME across the nasal mucosa followed Higuchi order ($r^2 > 0.9923$) rather than zero order and first order release.
### Table 17. Flux and diffusion coefficients of QF loaded ME and MME.

<table>
<thead>
<tr>
<th>S.No</th>
<th>QF loaded formulations</th>
<th>Flux, $J_{ss}$ (mcg/cm²/h)</th>
<th>Diffusion coefficient $K_p \times 10^{-4}$ (cm²/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CH-ME</td>
<td>1.526 ± 0.027</td>
<td>8.32 ± 0.12</td>
</tr>
<tr>
<td>2</td>
<td>ME β CD-ME</td>
<td>0.927 ± 0.015</td>
<td>5.03 ± 0.08</td>
</tr>
<tr>
<td>3</td>
<td>QF-ME</td>
<td>0.662 ± 0.015</td>
<td>3.59 ± 0.08</td>
</tr>
<tr>
<td>4</td>
<td>DS</td>
<td>0.618 ± 0.012</td>
<td>3.34 ± 0.06</td>
</tr>
<tr>
<td>5</td>
<td>DS + VH</td>
<td>1.573 ± 0.031</td>
<td>8.54 ± 0.16</td>
</tr>
</tbody>
</table>

#### 3.2.4.7 Measurement of *ex vivo* mucoadhesive strength

Force required to detach the formulation from the mucosal surface is determined as a mucoadhesive potential of the formulation. Figure 27 depicts the comparative representation of mucoadhesive force or maximum force for QF-ME (10.4 g), CH-ME (33.5 g) and MeβCD-ME (24.1 g) respectively. Higher values of force for CH-ME and MeβCD-ME compared to QF-ME could be attributed to presence of mucoadhesion property of CH having ionisable group (NH₃⁺) and MeβCD forming hydrogen bonds with negatively charged groups of sialic acid on the mucosal surface respectively thereby allowing electrostatic interaction (Bshara et al., 2014). Overall, the highest value of force was found with CH-ME proving longer residence time on nasal mucosa compared to other formulations.
3.2.4.8. Trans epithelial electrical resistance (TEER) measurement

CH is reported to adhere to epithelial surfaces and impart transient opening of tight junctions between adjacent cells (Smith et al., 2004). Caco-2 cell line was used to evaluate the effect of CH on the integrity of tight junction and effect of verapamil as Pgp inhibitor on diffusion of QF. Caco-2 cells were cultured on tissue culture treated polycarbonate inserts in Transwell 12-well plates till confluence monolayer was obtained, which resemble intestinal cells morphologically with formation of strong and dense ring network like structure of claudins between adjacent cells (tight junctions) (Yeh et al., 2011). Presence of tight junction between the adjacent epithelial cells prevents the passage of molecules/drugs/electrolytes across the epithelium leading to generation of TEER. TEER falls when tight junction open and molecules diffuse paracellularly. TEER measurement was performed for all Transwell plates after 15 days and resistance was found to be between 600 – 700 Ω cm$^2$ indicating formation of tight junction between cells and this value was considered as control in the study.
As shown in Figure 28, CH-ME treated inserts showed highest reduction in the TEER value i.e. TEER value reduced from 720.53 ± 8.26 Ω cm² (at 0 min) to 321.18 ± 26.86 Ω cm² (at 30 min). DS + VH also showed decrease in TEER value but in comparison to CH-ME, the reduction was lesser and higher when compared to QF-ME and DS. DS showed TEER value in between 500 to 600 Ω cm² (almost similar to control) from 0 min till 240 min thus indicating it did not have any effect on integrity of tight junction and showed very poor diffusion. TEER value was decreased upto initial 30 min after which gradual recovery was observed with CH-ME, ME and DS + VH treated groups. This suggests absence of disruptive or harmful effect on tight junctions. Results from Figure 28 suggested that the CH induce opening of tight junction between Caco-2 cells transiently and reversibly by interacting with specific membrane proteins on the epithelial junctional complex.

![Figure 28. TEER measurements of CH-ME, QF-ME, DS + VH and DS on Caco-2 cell monolayers (n=3).](image)

3.2.4.9 Morphological characterization

Morphological characterization of CH-ME using TEM analysis revealed that the globules were spherical (Figure 29) and were within size range of 50–100 nm. This data was in agreement with the size distribution performed by photon correlation
spectroscopy which confirmed that the globules were having uniform size distribution with PDI value below 0.3 indicating absence of aggregation.

![Figure 29. TEM analysis of CH-ME.](image)

### 3.2.4.10 Contact angle measurement

Contact angle measurement helps to identify the degree of wetting between formulation and surface of interest. Figure 30, A and B shows contact angle for QF-ME and CH-ME respectively.

![Figure 30. Contact angle images: (A) QF-ME and (B) CH-ME.](image)

Contact angle was found to be nearly 29° for QF-ME and around 44° for CH-ME, indicating good wettability and prolonged residence time on the mucosal surface. Higher contact angle value for CH-ME when compared to QF-ME could be due to the enhanced viscosity of CH-ME which might result into prolonged contact time when compared to QF-ME (Yuan and Lee, 2013; Carvalho et al., 2010).
3.2.4.11 Nasal cilio-toxicity study

Nasal toxicity study is useful to study the toxicity effects of formulation on the integrity of nasal mucosa. Figure 31 represents three sections of nasal mucosa viz., positive control treated (A), negative control treated (B) and mucosa treated with CH-ME (C). The mucosa treated with isopropyl alcohol showed extensive damage to nasal mucosa with alteration on the surface of epithelium and internal tissue damage. Negative control (PBS pH 6.4) treated mucosa was found to be intact with preserved structure. After treating mucosa with CH-ME, neither cell necrosis nor structural damage was observed.

Figure 31. Nasal toxicity showing optical microscopic images of goat nasal mucosa
(A) Positive control treated, (B) Negative control treated and (C) CH-ME.
These observations were in accordance with the pH value of CH-ME (5.61 ± 0.16) which was within the pH range of human nasal mucosa (4.5–6.5) indicating its safety for nasal administration (Washington et al., 2000).

### 3.2.4.12 Nasal spray pattern and plume geometry

It is never the formulation alone which plays major role in nasal delivery system, the delivery device is equally important especially for drug delivery to olfactory region of nose (Trows et al., 2014). Figure 32 depicts plume geometry and Figure 33A and B depicts spray pattern images of CH-ME, whereby spray pattern measurements were performed at two distances from the actuator tip viz., 3 cm and 6 cm respectively. Plume geometry testing provides side view of the emitted spray parallel to the axis of the plume which includes cone angle, plume width and length measurements of the spray event. The cone angle occurs near or at the actuator tip and it is based on the conical region of the plume extending from a vertex (FDA, 2003). Cone angle was found to be 40.85° as seen in Figure 32. Trows et al., 2014 investigated the influence of viscosity on plume geometry and reported decrease in cone angle with increase in viscosity. They observed wide angled plume with fine droplets having higher cone angle (82.1°) at 0% polymer concentration (only water), whereas at 5% concentration viscosity was higher, cone angle value was lowest (13.2°) with jet-like plume and larger droplets. Very low value of cone angle may lead to development of poor spray which was not observed in our case.

![Figure 32. Plume geometry showing side view of the emitted CH-ME spray.](image-url)
3.2.4.13 In vivo pharmacokinetic study

Mean plasma and brain concentrations versus time profile of QF after IN and IV delivery were evaluated as shown in Figure 34 and 35 respectively, from which various pharmacokinetic parameters were calculated as represented in Table 18.
Following IV administration of QF-ME, QF concentration was found to be significantly higher at all the time points in plasma compared to other QF loaded systems administered intranasally where it was found lower (Figure 34).

**Figure 34.** Mean QF concentration-time profile after intranasal and intravenous administration in rat plasma (Data represents mean ± SD, n=4).

Figure 35. Mean QF concentration-time profile after intranasal and intravenous administration in rat brain (Data represents mean ± SD, n=4).
Concentration for QF in the brain following IN administration of CH-ME was found to be significantly higher at all the time points which was followed by VH+DS while remaining systems showed comparatively lower concentration (Figure 35). This revealed that distribution of drug in systemic circulation was lower with formulation administered intranasally than those administered by IV route indicating preferential nose to brain transport. Figure 36 depicted that the brain/blood ratio for CH-ME (IN) remained significantly higher at all the time points with respect to QF-ME (IN), DS (IN) and QF-ME (IV) indicating higher and prolonged retention of drug at site of action. The retention was around 3 to 5 times higher with CH-ME (IN) compared to QF-ME (IV) thus proving the superiority of CH as permeability enhancer for nose to brain delivery system (Florence et al., 2011). The brain/blood ratio of QF at 15min was in the order of CH-ME (IN) N QF-ME (IN) N DS (IN) N DS + VH (IN) N QFME (IV) i.e. $1.02 \pm 0.09$ N $0.59 \pm 0.08$ N $0.54 \pm 0.08$ N $0.44 \pm 0.05$ N $0.11\pm0.02$ respectively, indicating direct nose to brain transport of QF bypassing BBB (Mittal et al., 2016; Haque et al., 2014).

![Figure 36. Brain/blood concentration ratio of intranasally administered QF-ME, CH-ME, DS + VH and DS and intravenously administered QF-ME at different time intervals (Data represents mean ± SD, n=4).](image)

Table 18 explicates various pharmacokinetic parameters of QF following IN and IV administration along with brain targeting efficiency and direct transport percentage.
Table 18. Pharmacokinetic, brain targeting efficiency and direct transport percentage of QF following IN and IV administration \((n = 4)\).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Tissue</th>
<th>Formulation and route of administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>QF-ME (IN)</td>
</tr>
<tr>
<td>C\textsubscript{max} (ng/ml)/(ng/gm)</td>
<td>Plasma</td>
<td>178.32 ± 14.06</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>106.67 ± 35.53</td>
</tr>
<tr>
<td>T\textsubscript{max} (min)</td>
<td>Plasma</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>15</td>
</tr>
<tr>
<td>AUC\textsubscript{0-240} (ng min ml\textsuperscript{-1})/(ng min gm\textsuperscript{-1})</td>
<td>Plasma</td>
<td>13902.74 ± 2370.79</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>9785.12 ± 3161.44</td>
</tr>
<tr>
<td>T\textsubscript{1/2} (h)</td>
<td>Plasma</td>
<td>1.21 ± 0.30</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>1.16 ± 0.32</td>
</tr>
<tr>
<td>DTE (%)</td>
<td>Brain</td>
<td>371.20 ± 12.02</td>
</tr>
<tr>
<td>DTP (%)</td>
<td>Brain</td>
<td>68.66 ± 6.84</td>
</tr>
<tr>
<td>Nasal bioavailability (%)</td>
<td>Plasma</td>
<td>32.66 ± 5.24</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>118.65 ± 14.26</td>
</tr>
</tbody>
</table>
Lower $T_{\text{max}}$ value of 15 min ($C_{\text{max}} - 243.19 \pm 26.97 \text{ ng/gm}$) in the brain compared to $T_{\text{max}}$ of 30 min ($C_{\text{max}} - 348.90 \pm 24.62 \text{ ng/ml}$) in plasma with CH-ME (IN) may be attributed to direct nose to brain transport following IN administration. Lowest $C_{\text{max}}$ value of 65.44 ± 24.24 ng/gm in the brain with DS (IN) could be attributed to MCC which cleared the instilled formulation rapidly and hence $C_{\text{max}}$ (185.67±28.56 ng/ml) in plasma was also found to be lesser revealing addition of mucoadhesive agent plays a key role in improving brain uptake (Vyas et al., 2006). Nearly 3 folds higher $\text{AUC}_{0-240}$ (20024.80 ± 2374.98 ng min gm$^{-1}$) and 2.7 folds higher $C_{\text{max}}$ (178.86 ± 22.13 ng/gm) in brain with DS + VH (IN) compared to $\text{AUC}_{0-240}$ (6557.42 ± 726.22 ng min ml$^{-1}$) and $C_{\text{max}}$ of 65.44 ± 24.24 ng/gm in brain with DS (IN) proved inhibitory effect of VH on efflux transporters. This finding is in accordance with the outcomes of Amin, 2013 who stated that use of Pgp inhibitors may have great impact on altering the pharmacokinetics of Pgp substrate molecules. The $\text{AUC}_{0-240}$ and $C_{\text{max}}$ values in the brain after IN administration of QF loaded carriers were in the order of CH-ME > DH + VH > QF-ME > DS, depicting significantly higher $\text{AUC}_{0-240}$ (26493.21 ± 2762.87 ng min gm$^{-1}$) with CH-ME compared to other carriers. Nearly 2.7 folds higher $\text{AUC}_{0-240}$ in the brain with CH-ME (IN) compared to QF-ME (IN) could be attributed to presence of CH which reduced MCC thereby providing longer residence time. This statement was in agreement with the data obtained from contact angle study (Figure 30B) where CH-ME showed 1.5 folds higher contact angle with mucosa compared to QF-ME which would have resulted into enhanced residence time at the site of action.

The DTE (%) values among all nasally administered formulations were found to be highest with CH-ME (453.69 ± 10.17) which showed higher value (> 1) for drug targeting index, confirming direct nose to brain pathway. Nearly 1.7 folds higher DTE was observed with CH-ME (IN) compared to DS (IN) indicating higher targeting efficiency by virtue of bioadhesion and lipophilic nature of delivery system (Florence et al., 2011). Probable reason could also be presence of synergistic effects taking place i.e. firstly, penetration enhancement effect of surfactants and CH on opening of tight junction and Pgp and secondly, globule size of CH-ME was lower than that of axons in the filia olfactoria leading to intracellular axonal transport into olfactory neurons by endocytic mechanism as reported by Bshara et al., 2014. Nasal bioavailability of QF in the brain following CH-ME (IN) (316.67 ± 19.38%) and DS + VH (IN) (242.61 ± 22.73%) were found to be 3.8 and 2.9 folds higher respectively.
compared to DS (IN) (83.15 ± 9.82%). This outcome was in agreement with the finding of Patel et al., 2015 who also found 3.4 folds higher bioavailability with IN mucoadhesive ME compared to IN drug solution. As shown in Table 18, nasal bioavailability of QF in plasma was too low compared to brain following CH-ME (IN), QF-ME (IN) and DS+VH (IN) which showed larger difference among brain and plasma bioavailability. This can be explained by the fact that, part of nasally administered drug makes use of olfactory and trigeminal nerve for drug transport from nasal epithelium to brain, bypassing BBB, which may not occur in case of systemic circulation (Dhuria et al., 2010). Nearly 1.5 folds higher DTP value of CH-ME (80.51 ± 6.46%) compared to DS (54.08 ± 5.77%) suggested that the transport of QF from CH-ME to rat brain occurs probably via olfactory pathway bypassing BBB along with attribution of factors like increased permeation across nasal mucosa, reduced MCC and modulation of tight junction and Pgp efflux transporters. Above findings are consolidating the outcomes of previous reported results stating IN mucoadhesive formulation increases nose to brain uptake of drugs (Kumar et al., 2008; Haque et al., 2014; Patel et al., 2016).

3.2.4.14 In vivo qualitative biodistribution by gamma scintigraphy study
In order to visualize qualitative biodistribution following IN and IV administration of radiolabelled formulations, gamma scintigraphy study was performed. CH-ME, QF-ME, VH+DS and DS were effectively radiolabeled by direct labeling method using reduced $^{99m}$Tc. Radiolabeling efficiency/purity was within 97 to 98.5% for all the formulations when evaluated for reduced/hydrolyzed (R/H) $^{99m}$Tc and free $^{99m}$Tc. The gamma scintigraphic images of rats at 15 and 120 min post administration is depicted in Figure 37. From figure it was observed that, IN $^{99m}$Tc labeled CH-ME showed highest brain uptake (estimated count of 3248 at the region of interest i.e. brain) as compared to IN $^{99m}$Tc labeled QF-ME (count of 1250) at 15 min post administration. In other words 2.6 times higher radioactivity was present in the brain with IN $^{99m}$Tc labeled CH-ME in comparison to IN $^{99m}$Tc labeled QF-ME. This data was in agreement with the quantitative nasal bioavailability data between CH-ME (316.67 ± 19.38) and QF-ME (118.65 ± 14.26), which showed 2.7 folds higher nasal bioavailability with CH-ME.
Figure 37. Scintigraphy images at 15 and 120 min post administration of IN (A) CH-ME, (B) VH+DS, (C) QF-ME, (D) DS and IV (E) QF-ME.
Similarly when compared to IN $^{99m}$Tc labeled DS, radiolabeled IN VH+DS showed 4 times higher radioactivity in the brain and the outcome was in accordance with the pharmacokinetic findings where by VH+DS showed 3 folds higher nasal bioavailability and AUC in the brain when compared to DS. It was also observed that at 120 min post administration, the radioactivity in the brain was not visible and counts were decreased almost upto 4-6 folds lower with all the formulations given intranasally indicating that the formulations get cleared as time increases which can be observed in Figure 35 (Mean brain concentration versus time). Since QF is substrate to Pgp, very poor radioactivity in the brain was observed with $^{99m}$Tc labeled DS and with verapamil as Pgp inhibitor, the radioactivity for QF was higher. IV administration of radiolabeled QF-ME showed negligible counts in the brain i.e. 149 and 107 at 15 and 120 min respectively. As per literature other than olfactory and trigeminal pathway, one more pathway for drug uptake to brain via nasal route is reported i.e. systemic pathway by which some of the drug is absorbed into the systemic circulation and subsequently reaches the brain by crossing BBB (Vyas et al., 2006). This could be the reason behind presence of radioactivity in the stomach as seen in Figure 37. Overall, qualitative data obtained from gamma scintigraphy study was in agreement with the quantitative data of in vivo pharmacokinetics in rats and thus confirming the potential of CH as permeability enhancer and VH in inhibiting the efflux transporters thereby improving the bioavailability of QF.

3.2.4.15 Stability study

Stability study data indicated that the CH-ME remained stable over a period of six months at three different temperature conditions. Upon visual observation and on accelerated centrifugation at 15000 rpm for 30 min, CH-ME exhibited no signs of instability such as phase separation, flocculation, creaming and precipitation of drug. As shown in Table 19, values for pH were within the pH range (4.5–6.5) of nasal fluid explicating well tolerance upon nasal administration. Even after storage for three and six months, there was only negligible change in %T, globule size, zeta potential, viscosity and drug content, in comparison to the initial values which indicated that the CH-ME was stable over the period of six months.
Table 19. Stability study of CH-ME upto six months (n=3).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Time (month)</th>
<th>Temperature (°C)/ RH(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4-8 °C</td>
</tr>
<tr>
<td>% T</td>
<td>One</td>
<td>99.38 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>Three</td>
<td>98.12 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>Six</td>
<td>99.48 ± 0.14</td>
</tr>
<tr>
<td>Globule size (nm)</td>
<td>One</td>
<td>36.1 ± 1.31</td>
</tr>
<tr>
<td></td>
<td>Three</td>
<td>33.2 ± 0.97</td>
</tr>
<tr>
<td></td>
<td>Six</td>
<td>35.8 ± 1.26</td>
</tr>
<tr>
<td>PDI</td>
<td>One</td>
<td>0.245 ± 0.029</td>
</tr>
<tr>
<td></td>
<td>Three</td>
<td>0.262 ± 0.016</td>
</tr>
<tr>
<td></td>
<td>Six</td>
<td>0.243 ± 0.006</td>
</tr>
<tr>
<td>Zeta potential (mV)</td>
<td>One</td>
<td>22.09 ± 0.72</td>
</tr>
<tr>
<td></td>
<td>Three</td>
<td>19.45 ± 0.97</td>
</tr>
<tr>
<td></td>
<td>Six</td>
<td>20.41 ± 1.77</td>
</tr>
<tr>
<td>pH</td>
<td>One</td>
<td>6.21 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Three</td>
<td>5.97 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>Six</td>
<td>6.02 ± 0.22</td>
</tr>
<tr>
<td>Viscosity (cP)</td>
<td>One</td>
<td>37.22 ± 0.98</td>
</tr>
<tr>
<td></td>
<td>Three</td>
<td>36.82 ± 0.84</td>
</tr>
<tr>
<td></td>
<td>Six</td>
<td>39.07 ± 1.04</td>
</tr>
<tr>
<td>Drug content</td>
<td>One</td>
<td>98.39 ± 1.19</td>
</tr>
<tr>
<td></td>
<td>Three</td>
<td>98.42 ± 0.72</td>
</tr>
<tr>
<td></td>
<td>Six</td>
<td>98.87 ± 1.12</td>
</tr>
</tbody>
</table>
3.3 Conclusion

In the present work QF based ME with and without CH was developed to investigate its potential use in improving the bioavailability and brain targeting efficiency following noninvasive intranasal administration. CH-ME with spherical globules having mean size of 35.31 ± 1.71 nm, pH value of 5.61 ± 0.16 showed highest ex vivo nasal diffusion (78.26 ± 3.29%) with no sign of structural damage upon histopathological examination. Caco-2 cell line study demonstrated that CH-ME induced reversible opening of tight junctions and hence showed higher diffusion for QF via paracellular route. Circular plume with an ovality ratio closer to 1.3 for CH-ME depicted ideal spray pattern. Significantly higher brain/blood ratio of CH-ME in comparison to QF-ME and drug solution following intranasal administration revealed prolonged retention of QF at site of action suggesting superiority of CH as permeability enhancer for preferential nose to brain transport bypassing BBB. Gamma scintigraphy study showed 5.9 folds higher radioactivity in the brain with radiolabeled CH-ME compared radiolabeled drug solution, hence proving larger extent of drug transport across the brain with intranasal administration of CH-ME. Enhanced brain uptake of QF following noninvasive intranasal delivery of CH-ME confirmed higher extent of transport with improved bioavailability which may help in decreasing the frequency of dosing and probably maximize the therapeutic index. These results suggested the suitability of intranasal route for preferential nose to brain delivery of drugs utilizing olfactory and trigeminal pathway bypassing BBB. Quantitative and qualitative biodistribution data showed satisfactory correlation between AUC, nasal bioavailability and DTP for intranasally administered CH-ME. Overall on the basis of above findings, it can be concluded that CH-ME could be a promising approach for the brain delivery of QF via intranasal route. However, the findings still require preclinical and clinical studies to establish risk/benefit ratio, since the outcomes may differ in humans due to difference in the nasal physiology of human and animal.
3.4 Extrapolation of findings of CH-ME

From the above mentioned results of QF based ME and MME, especially the physicochemical, diffusion and pharmacokinetic parameters, it was found that CH-ME showed maximum permeation across goat nasal mucosa and higher intestinal permeability compared to QF-ME and DS. Highest diffusion of QF through alteration of tight junctions in Caco-2 cell monolayers was found with CH-ME. *In vivo* pharmacokinetics also showed maximum DTE, DTP and nasal bioavailability for QF with CH-ME over other groups taken into study. Hence from the above studies it was confirmed that chitosan was playing major role in enhancing the drug permeation across BBB by overcoming the biological obstacles, as reported previously by Kumar et al., 2008; Florence et al., 2011; Bshara et al., 2014.

Promising results with chitosan based ME *i.e.* CH-ME, prompted to investigate whether the effect exerted by chitosan was irrespective of the type of formulation developed. To investigate this, chitosan based polymeric nanoparticles of QF were formulated and evaluated for various physicochemical, *ex vivo* and *in vivo* parameters in order to compare with CH-ME.

In the next chapter (Chapter 4) formulation and evaluation of QF loaded chitosan nanoparticles (QF-NP) with final optimized method for the formulation and short description on the characterization parameters will be discussed. Comparative parameters between two different carrier systems will also be discussed with their outcome.

Preliminary screening of the formulation parameters with detailed methodology, results and discussion is described in research paper entitled “Application of Box-Behnken design for optimization and development of quetiapine fumarate loaded chitosan nanoparticles for brain delivery via intranasal route” by Shah et al., 2016c, attached in Annexure 1.