

# Enhancement of Oral Bioavailability of Rosuvastatin Using Combinational Approach of Thermal Fraction of Clarified Butter

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## Abstract

**Background:** Thermal fractionation is an effective technique similar to chromatographic separation. Clarified butter (CB) is a mixture of fatty acid which shown different physiochemical properties if fractionated by various means. The present investigation was a study of the suitability of 30°C and 50°C thermal fractions of CB in various ratios for improvement of absorption of poorly aqueous soluble drug rosuvastatin (RSC). This work also tries to address the difficulty in *in vitro* dissolution data correlation with *in vivo* absorption characteristics in a practical situation. **Aim:** The author aims to get the best combination of 30°C and 50°C fractions which give desirable drug release with effective permeation. **Materials and Methods:** The 3<sup>2</sup> factorial design approach was used to formulate rosuvastatin CB complex using thermal fractions in various ratios. Drug CB complex was evaluated in various parameters such as contact angle, partition coefficient, saturation solubility, dissolution, and permeation characteristics. The change in physical and chemical properties of drug complex prepared with various ratios of the thermal fraction was also studied by X-ray diffraction and Liquid Chromatography-Mass Spectroscopy. **Results and Discussion:** The weight ratio of thermal fraction of CB (TFCB) fractionated at 30°C and TFCB fractionated at 50°C in 1:1.5 w/w in formulation RAE-B2 was found suitable to improve the absorption characteristics of rosuvastatin. The *ex vivo* permeation studies showed 90.68% permeation of rosuvastatin from RAE-B2 formulation which was found to higher than other formulation as well as pure rosuvastatin. **Conclusion:** The result suggested that the drug complex prepared using 30°C and 50°C fractions at 1:1.5 shown optimum drug release with desired permeation.

**Key words:** Rosuvastatin, clarified butter, ghee, factorial design, contact angle, thermal fractionation

**Abbreviation list:** BSC: Biopharmaceutical classification system, CB: Clarified butter, CE: Collision energy, FA: Thermal fraction of clarified butter fractionated at 30°C, FE: Thermal fraction of clarified butter fractionated at 50°C, LCMS: Liquid Chromatography-Mass Spectroscopy, LDL: Low-density lipoproteins, RSC: Rosuvastatin, TFCB: Thermal Fraction of clarified butter, XRD: X-ray diffraction

## INTRODUCTION

Clarified butter (CB) is a dairy product prepared from cow and buffalo milk due to its varieties of application; it is a part of Indian culture, tradition, and rituals. CB is receiving space in an "Atharvaveda" handbook, and "Ayurveda" traditional medicinal book of India. Its popularity has attracted to the scientist to explore various properties rather than justify its traditional application. A typical household applied CB on the skin for a soothing effect in case of a burn, allergy, and insect bite. According

to Ayurveda, it has versatile medicinal applications and adjuvant/vehicle properties. Many research works confirmed

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its cutaneous penetrability with numbers of drugs such as Indomethacin, and Diclofenac sodium.<sup>[1]</sup>

In a typical practice, the author notices that CB is solidified in the winter session and liquefied in summer in the Indian climate. A highly attractive point is a more significant gap in CB melting and solidifying time. It is not a spontaneous process. Some fraction of CB is solidifying at a time, and rest in a liquid state. Finally, depends it on climate conditions, the whole CB is solidifying with a clear differentiated color band. This process is like chromatographic separations, which produce a color band.<sup>[2]</sup>

Rosuvastatin is used for treating hypercholesterolemia, mixed hyperlipidemia, mixed dyslipidemia, and hypertriglyceridemia through a competitive inhibitory effect on HMG-CoA reductase. Rosuvastatin inhibiting the synthesis of the very-low-density lipoprotein in the liver and increases the number of hepatic low dense lipid (LDL) receptors on the cell surface. Also to enhance the catabolism of LDL by more uptake of LDL, thus the total number of very-low-density lipoprotein and low-density lipoprotein levels reduced. Apart from the lipid-lowering effect of rosuvastatin, it has a potential anti-inflammatory characteristic.<sup>[3,4]</sup> Rosuvastatin is a statin drug categorized in the BCS Class II drug that shows low bioavailability of about 20% due to extensive excretion in the liver and lower permeability in the biological membrane. Rosuvastatin aqueous solubility is limited to only 7.8 mg/mL at 37°C with pKa of 4.6. Randomized 3<sup>2</sup> full factorial designs were used to optimize the combination of the thermal fraction of CB (TFCB) for oral bioavailability enhancement for rosuvastatin. The factorial design is a scientific approach consisting of two or more factors, each factor consisting of different possible levels or values. The factorial design was used to optimize the effect of independent variables and evaluate effects over the dependent variable.<sup>[5]</sup> Very few research articles have been published to explore the application of thermal fractionated individual components of CB. Most of the researches have been going in the direction of the use of whole CB. Surprisingly could not notice that CB is a combination of both saturated and unsaturated fatty acids with a minor component such as sterol, ester, vitamin, carotenoids, and the trace of minerals such as iron, calcium, and phosphorus.<sup>[6]</sup> The present research was tied to the full fill this gap. The present investigation aims to optimize the suitable combination ratio of TFCB in a range of 1:1–1:2 (TFCB 30°C; TFCB 50°C) with rosuvastatin to achieve desire drug dissolution profiles and enhance the permeation of rosuvastatin. The present article addressed the difficulty of *in vitro ex vivo* data correlation.

## MATERIALS AND METHODS

### Materials

CB was collected from local dairy farm Durg, Chhattisgarh, India. Rosuvastatin was purchased from Sigma-Aldrich

Private Limited Mumbai, India. Chloroform (PubChem compound ID number: 6212), methanol (PubChem compound ID number: 887), and ethanol (PubChem compound ID number: 702) were obtained from LobaChemie (Colaba, Mumbai). Potassium bromide, sodium hydroxide, and potassium dihydrogen orthophosphate were obtained from Sigma-Aldrich (India). All the other chemicals of analytical grade were used.

### Methods

#### Preparation of drug TFCB complex

Separation of TFCB at different temperatures was carried out, as described in our previous research article.<sup>[7]</sup> In the previous studies, effect of individual TFCB fraction on the dissolution of rosuvastatin was performed. The dissolution profiles play a key role in the permeation across the biological barrier. The dissolutions of rosuvastatin at gastric pH were retarded due to poor aqueous solubility. The previous study revealed that the rosuvastatin complex contained only thermal fraction TFCB 30°C (FA) showed a higher *in vitro* dissolution profile in simulated intestinal fluid pH 6.8, while the least dissolution was found with thermal fraction TFCB 50°C (FE). This was probably due to the higher lipophilic characteristic of FE which would restrict the initial solubility for dissolution. Therefore, to improve the oral bioavailability further plan of work, it is designed to make physical complex between rosuvastatin and these two thermal fractions FA and FE in different weight ratio combinations.

The evaporation crystallization method was used to prepare the physical complex of rosuvastatin using fractions of FA and FE at different weight ratios (w/w) to obtain maximum bioavailability. A constant gram equivalent weight ratio (1:1) was taken between rosuvastatin and combined thermal fractions FA and FE. The different weight ratio of fractions were ground separately and dissolved in chloroform. The solvent was evaporated at 35°C in a rotary evaporator (Biological Museum, India). The resultant was transferred into a desiccator for 24 h to get solvent-free powder using a silica gel packet as a moisture absorber. The solvent-free drug complexes were collected and screening the various composition ratios (1:1, 1:1.2, 1:1.4, 1:1.25, 1:1.5, 1:1.75, 1:1.43, 1:1.71, and 1:2 w/w) designated as RAE-A1, RAE-A2, RAE-A3, RAE-B1, RAE-B2, RAE-B3, RAE-C1, RAE-C2, and RAE-C3, respectively for performance parameters.

#### Optimization of a combination of two thermal fractions

Rosuvastatin-loaded drug complex was prepared by combine both the fractions FA and FE adopting the evaporation crystallization method. A randomized, 3<sup>2</sup> full factorial design was used to prepare the optimized drug complex.<sup>[8]</sup> For the study, the effect of independent variables on dependent

variables, a non-linear quadratic polynomial model was generated for explicit evaluation using Design-Expert software (Stat-Ease, Inc., Minneapolis, Minnesota). The drug complex formed at three levels (+1, 0, -1) of FA (X1) and FE(X2), and quadratic polynomial models were generated for contact angle, percentage drug permeation as dependent variables. In all the drug complexes, the drug concentration was kept constant; the only ratio of and FE fraction varies. The effect of an independent variable over-dependent response contact angle and percentage drug permeation is presented in Table 1.

## Characterization of the drug complex

### Chemical composition study of TFCB and drug complexes by LCMS

Quantization examination was carried out by Liquid Chromatography-Mass Spectroscopy (LC-MS)/MS detection in positive ion modes for an analyte. Chromatography separation was performed on a Waters Alliance® 2795 HPLC system equipped with LC Column X bridge waters C18 250\*4.6 5 µm. The system was coupled to a Waters Micromass Q-ToF micro™ (Manchester, UK) equipped with ionization source electrospray positive (ES+) mode. The source temperature was set at 110°C with a cone gas flow of 30 l/h, a desolvation gas temperature of 300°C, and a desolvation gas flow of 500 l/h was employed. The capillary voltage was set at 3000 V for positive ion mode and the cone voltage to 30 V. The collision energy (CE) of 4 eV was used with a scan time of 0.4 s and with an inter-channel delay of 0.1 s. The injected sample volume was 20 µL. The mass spectra were illustrating in centroid mode. An aliquot of the sample (20 µL for positive ion mode) was injected onto a 520 mm × 4.6 mm Symmetry® C18 5 µm column (Waters Corporation). The mobile phase was used acetonitrile-water (85:15, v/v) over 10 min at a flow rate of 0.8 mL/min, and the temperature was set at 45 ± 1°C. The mass spectrometric data were collected in full scan mode from m/z 60 to 1500. Nitrogen (N<sub>2</sub>) and argon gases were used with supply pressure of 6–7 bar (90–100 psi) and 5–6 bar, respectively.<sup>[9]</sup>

### State of thermal fraction of CB: Crystallinity behavior study

The internal structures of thermal fraction and drug complex were studied using X-ray diffraction (XRD) analysis. The XRD patterns of samples were measured using a PANalytical Empyrean diffractometer (Malvern Instruments Ltd, Malvern, United Kingdom) outfitted with a Copper K $\alpha$  radiation source ( $\lambda = 1.5418 \text{ \AA}$ ) operated with an electrical supply at 40 kV and 30 mA. Diffraction patterns were collected over a  $2\theta$  range 5°–60° with step size 0.02° scanning speed of 4°/min and a step time of 17 s. The characteristic XRD spectra of the pure drug and drug TFCB complexes were analyzed and obtained at ambient conditions.<sup>[10]</sup>

### Wettability study of TFCB and drug TFCB complex by contact angle measurement

The contact angle measurement between the solid surface of the samples and vehicle was determined using a Phoenix 300 Standard (Surface Electro-Optics, Korea) goniometer using the sessile drop and touch to the surface parallel drop technique. The instrument was equipped with an automatic drop-controlled syringe system and fit out the camera to measure the speed of 70 (USB)/84 (1394) frames/s with direction adjustment. The camera-equipped with static contact angle measurement range is 0–180°; surface energy calculator, surface tension calculator, and high resolution (12 × zoom) lens (Surface Electro-Optics, Korea) with single and sequence image capture modes. The software was used for capturing images with the D-base function to access records and stored in dBase-format as well as export captured data to excel format. 50 mg of samples (drug TFCB complexes and pure rosuvastatin) accurately weighed and press using Manual Hydraulic Press (Suravi Instrumentation Pvt. Ltd. India) at pressure 1000 psi for 1 min to make a disk. The contact angles were measured for 60 s instantly after 2 µL vehicle dropped on the compressed sample's surface. All measurements were performed under ambient conditions of 28 ± 2°C and relative humidity of about 40 ± 5%. The data are an average of three values.<sup>[11]</sup>

**Table 1:** Physicochemical properties of clarified butter and thermal fraction

Physicochemical parameter	Clarified butter (CB) at room temperature	TFCB 30°C (FA)	TFCB 50°C (FE)
Color	Creamy yellow	Creamy yellow	Transparent yellow
Specific gravity (g/cc)*	0.889±0.14	0.894±0.11	0.980±0.18
Acid value* (mg/g)	0.367±0.07	0.266±0.08	0.297±0.04
Ester value* (mg KOH/g)	234.21±0.99	233.03±0.91	232.37±0.54
Hydroxyl value* (mg KOH/g)	16.266±0.15	16.21±0.06	16.15±0.05
Iodine value* (g/100g sample)	29.336±0.49	31.003±0.65	8.123±0.51
Peroxide value* (mEq/kg)	4.55± 0.36	4.82±0.11	7.49±0.59
Saponification value* (mg KOH/g)	215.23±0.11	215.33±0.16	218.16±0.30
Melting range (°C)	37.1 – 37.6	30.1 – 31.2	38.4 – 39.3
Solidification range (°C)	22.1 – 23.3	19.2 – 19.8	18.2 – 17.3

\*Mean SD of three replicates

### Saturation solubility studies

A saturation solubility study was performed to determine the enhancement of the drug solubility by physical complexation with TFCB. Excess amounts of pure drug rosuvastatin were added to 10 mL of 0.2 M phosphate buffer of pH 6.8 in a 50 mL volumetric flask. The samples were stirred at 50 rpm using a magnetic stirrer (Multistirrer-6, VELP Scientifica, Italy) equipped with a thermostat hot plate for 24 h at constant temperature  $37 \pm 2^\circ\text{C}$ . After, the required dilutions of samples were filtered using 0.45 mm filters and analyzed spectrophotometrically at 250 nm. The same procedure was repeated for all other drug TFCB complex.

### Partition coefficient between n-octanol: phosphate buffer

All the drug TFCB complexes and pure rosuvastatin were dissolved in n-octanol and 0.2 M phosphate buffer, pH 6.8 in equal quantity. The solution was forcibly shaken for 1 h with 0.2 M phosphate buffer, pH 6.8, at room temperature. The concentration of rosuvastatin was measured in both phases, using UV-spectrophotometer at 250 nm. The partition coefficient was calculated according to equation 1.<sup>[12]</sup>

$$\text{Partition coefficient} = \frac{\text{Rosuvastatin concentration in octanol}}{\text{Rosuvastatin concentration in phosphate buffer}} \quad (1)$$

### In vitro dissolution studies of all drug TFCB complexes

*In vitro* drug release studies were performed for all drug TFCB complexes. The test was performed using eight stations dissolution test apparatus USP-1 (Electro lab India). The simulated intestinal fluid pH 6.8 phosphate buffer was used as the dissolution medium. A 5 mL sample was withdrawn for up to 60 min and replaced with a fresh phosphate buffer, pH 6.8. In the first 30 min of the experiment, samples were collected periodically at 5 min intervals and 15 min intervals from 30 to 60 min. Collected samples were filtered through a 0.45 mm filter paper. The temperature of the media was maintained at  $37 \pm 0.5^\circ\text{C}$  and the stirring rate was set at 50 rpm. Drug analysis was performed using Ultraviolet (UV)-spectrophotometer (UV-1800, Shimadzu, Japan) equipped with UV probe software. Absorbance maxima of rosuvastatin was measured at 250 nm.<sup>[13,14]</sup>

### Ex vivo everted sac modification method

An intestinal permeability study was performed using an gut sac. The excised goat intestine was purchased immediately after slaughtering the goat from the local slaughterhouse and kept in normal saline which cooled with ice. The middle small intestine was removed by cutting each end and placed into pH 6.8 artificial intestinal fluid with continuously

provide oxygen using an oxygen pump. Residual blood clots were washed out with artificial intestinal fluid. Pieces of intestinal segments of 20 cm were everted through a glass rod and tie to one end of the intestine with cotton thread and the other end was attached to a cannula then place in ice-cold normal saline solution. To determine, drug permeation through a mucosal membrane, the segment of the intestine was everted as the serosal side present inside and the mucosal side comes out. 50 mL of 0.2 M phosphate buffer pH 6.8 was placed inside the intestine and immersed in 900 mL of buffer solution containing drug sample in 1000 mL beaker placed over magnetic stirrer, 2 L capacity equipped with a hot plate (FEC, Delhi, India). The temperature was maintained at  $37 \pm 0.5^\circ\text{C}$  with continuously stirring with magnetic bead to provide peristaltic movements. 1 mL of aliquots of the sample was withdrawn at predetermined time intervals from the sac and simultaneously, the same volume was replaced with a fresh medium to maintain the sink condition. The experiment was carried out for up to 6 h. The concentration of the drug from the physical complex into the sac was measured. The absorbance maxima of rosuvastatin in the simulated intestinal fluid was analyzed by UV-Spectrophotometer at 250 nm. All experiments were performed in triplicate.<sup>[15]</sup>

### Calculation of the apparent permeability coefficients

The apparent permeability coefficient ( $P_{app}$ ) was calculated according to equation 2.

$$P_{app} = \frac{dQ}{Dt} \times \frac{1}{AC} \quad (2)$$

where  $dQ/Dt$  (mg/s) is the amount of drug diffusion per unit time across the biological membrane,  $P_{app}$  (cm/s) is the apparent permeability coefficient,  $A$  ( $\text{cm}^2$ ) is the mucosal surface area available for permeation, and  $C$  (mg/mL) is the initial concentration of the drug available on the outside of everted gut sacs. The standard deviation and mean value of the apparent permeability coefficient were determined and expressed  $10^{-6}$  cm/s.

### Calculation of the apparent permeability

The following equation was used to calculate the apparent permeability (3):

$$\text{Apparent permeability (mg/cm}^2\text{)} = \frac{\text{Conc.} \times \text{Volume}}{\text{Mucosal surface area}} \quad (3)$$

The mucosal surface area was calculated using the following equation:

$$\text{Mucosal surface area (cm}^2\text{)} = \text{Intestine circle circumference} (\pi \text{ diameter}) \times \text{intestine length (cm)} \quad (4)$$

### Calculation of the percentage of drug recovery and drug retained

The following equation was used to calculate the percentage of drug recovery, according to equation (5):<sup>[16]</sup>

$$\% \text{ Drug recovery} = \frac{C_{r,\text{end}} \times V_r + C_{d,\text{end}} \times V_d}{C_o \times V_r} \times 100 \quad (5)$$

where  $C_{r,\text{end}}$  and  $C_{d,\text{end}}$  (mg/mL) are measured drug concentrations inside and outside the sacs respectively at the end of the experiment;  $C_o$  (mg/mL) is the initial concentration of the drug outside the everted gut sacs;  $V_d$  and  $V_r$  (mL) are the volumes of the serosal and mucosal media, respectively.

The percentage of drug retained on the tissues of intestinal membrane was calculated by using following equation 6:

$$\% \text{ Drug retained} = 100 - \% \text{ Drug recovery} \quad (6)$$

### Statistical analysis

In the physicochemical evaluation, the sample size was  $n = 3$ . Significance was accepted at  $P \leq 0.05$ . All the data were expressed as a mean  $\pm$  SD. In physical characterization, the sample size was  $n = 3$ . The *t*-test was performed to determine the significant difference between the mean of the two groups. All the statistical analyses were carried out in GraphPad Prism 6 Demo software.

## RESULTS

### Preparation of drug TFCB complex

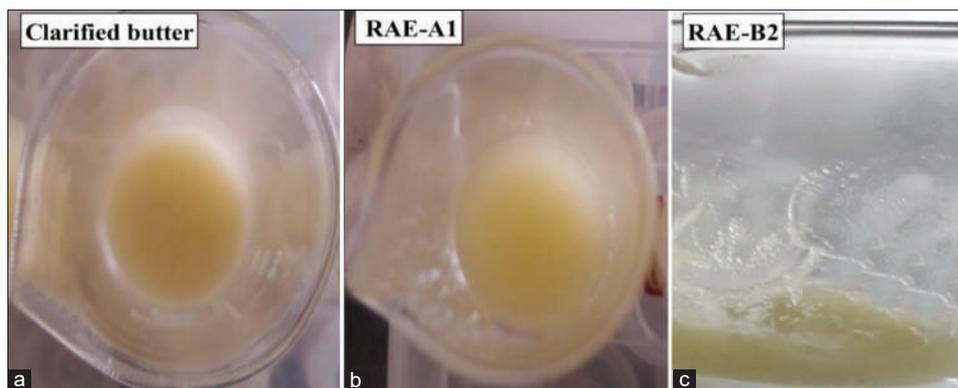
In the present investigation, TFCB separated at 30°C, and 50°C were taken to prepare the drug TFCB complex. The selection was done based on their fatty acid composition and physicochemical properties reported in Table 1. The prepared formulations were yellow in color with a slight intensity difference. Photograph of the formulation presented in Figure 1.

### Optimization of the ratio of TFCB for drug TFCB complex

The present study aims to investigate the effect of various ratios of FA and FE at a constant drug content level over the release and permeation profile of rosuvastatin. A randomized 3<sup>2</sup> full factorial design approach has been adopted to study the effect of the ratio of TFCB over two selected-response contact angles and drug permeation. These two parameters are dependent variables over the ratio of TFCB in the drug TFCB complex. The information of variables with limit and their effect on response is represented in Table 2.

The contact angle is an indicator of the wettability of solid onto the liquid surface and indicates about initial solubility of a drug in dissolution media. A maximum value of contact angle, that 71.89° obtained for the formulation REA-C3 prepared with (1:2 w/w) ratio of FA and FE while the least contact angle 33.38° obtained for the formulation (REA-A1) prepared with an equal proportion (1:1w/w) FA and FE. The equation  $Y = 65.60143 + 20.61929 X_1 - 3.59786 X_2 + 0.115446 X_1 X_2 - 0.578333 X_1^2 - 0.02406 X_2^2$  described a relationship between the ratios of the fraction with the contact angle ( $R^2$  value of 0.9493). A fit model summary generated by the factorial design approach suggested the quadratic model was significant with *P*-value and *F* value 0.0370 and 11.24, respectively, as represented in Table 3. There is only a 3.70% chance that an *F* value of this large cloud occurs due to noise. The effect of the ratio of FA and FE over contact angle is depicted in Figure 2.

The drug permeation is an indicator of the mass transfer of the drug toward the blood, which depends on the drug's solubility and partition coefficient. A maximum value of percentage drug permeated was obtained in 1 h for the RAE-B2 (90.68%) prepared with a 1:1.5 ratio of FA and FE while the least percentage drug permeation (52.46%) obtained for the RAE-A1 prepared with an equal proportion (1:1) ratio of FA and FE. A relationship between the fraction with percentage drug permeation was described by the equation  $52 X_1 X_2 + 0.1516 67 X_1^2 + 0.0067 71 X_2^2$  with an  $R^2$



**Figure 1:** Photograph of Clarified butter and drug-TFCB complex taken with a normal camera. The quantity of the drug was equal in both RAE-A1 and RAE-B2. (a) Clarified butter (CB). (b) RAE-A1 prepared by 1:1 w/w weight ratio of TFCB 30°C and TFCB 50°C. (c) RAE-B2 prepared by 1:1.5 w/w weight ratio of TFCB 30°C and TFCB 50°C

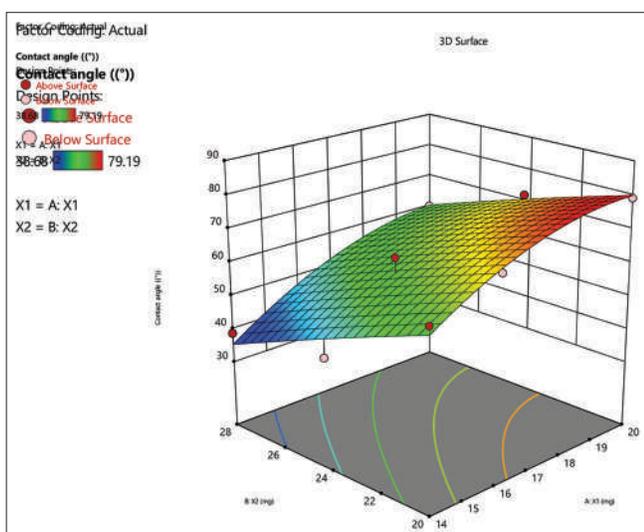
**Table 2:** Limits of the independent variables and their responses dependent variables

Independent variable	Notations	Limits		
		-1	0	1
RSC to FA ratio (w/w)	X1	14	16	20
RSC to FE ratio (w/w)	X2	20	24	28
Variable levels in coded form		Contact angle (°)		
Batch code	X1	X2	Percentage drug permeation	
RAE-A1	20	20	33.38	52.46
RAE-A2	20	24	38.68	59.18
RAE-A3	20	28	42.63	71.34
RAE-B1	16	20	45.43	79.04
RAE-B2	16	24	60.12	90.68
RAE-B3	16	28	62.61	86.46
RAE-C1	14	20	64.81	84.35
RAE-C2	14	24	70.12	81.47
RAE-C3	14	28	71.89	75.55

The ratio between independent variables and their response to contact angle and drug permeation.

**Table 3:** ANOVA Statistics and coefficients of contact angle obtained for the three-Level factorial design

Source	Sum of squares	df	Mean square	F-value	P-value	Significant
Model	1528.01	5	305.60	11.24	0.0370	
A-X1	749.95	1	749.95	27.59	0.0134	
B-X2	734.30	1	734.30	27.01	0.0138	
AB	7.96	1	7.96	0.2929	0.6260	
A <sup>2</sup>	41.28	1	41.28	1.52	0.3056	
B <sup>2</sup>	0.2965	1	0.2965	0.0109	0.9234	
Residual	81.55	3	27.18			
Core total	1609.56	8				



**Figure 2:** Effect of ratio of TFCB 30°C and TFCB 50°C, over contact angle. The contact angle increases with increasing the concentration of TFCB 50°C

value of 0.9928. A fit model summary generated by the factorial design approach suggested that the quadratic model

was significant with *P*-value and *F* value 0.0021 and 82.67, respectively. The data are reported in Table 4. There is only a 0.21% chance that an *F* value of this large cloud occurs due to noise. A relationship between percentage drug permeation and concentration of FA and FE is shown in Figure 3.

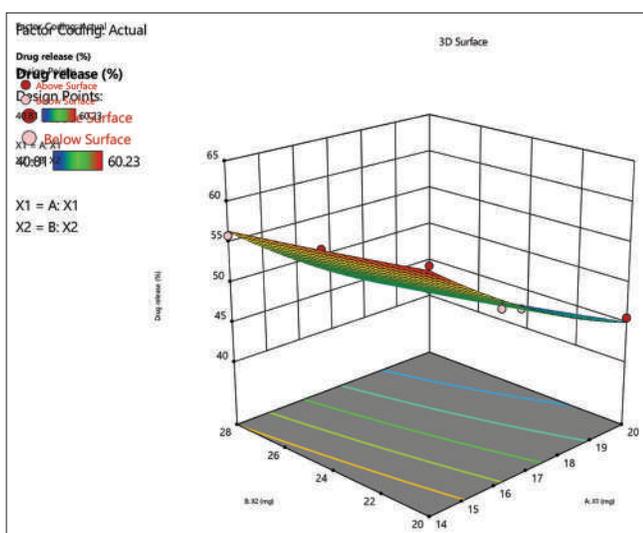
### Characterization of the drug complex

#### Chemical composition study of TFCB and complex by LCMS

The major obstacle and rate-limiting step to improve oral bioavailability is the solubility of the drug in the biological fluid. The interesting point is to achieve the desired bioavailability needs a balance of lipophilicity and hydrophilicity in a single formulation. This balance was well affected by the chemical composition of the excipient and the drug is taken for making a formulation. The identification purpose of LC-MS was performed for both drug and complexes. The LC-MS chromatogram of rosuvastatin presents in Figure 4. The chromatogram shows five abundant peaks with better separation, during optimization of the mass

**Table 4:** Statistics and Coefficients for the percentage drug permeation models obtained for the three-level factorial design

Source	Sum of squares	df	Mean square	F-value	P-value	significant
Model	368.38	5	73.68	82.67	0.0021	
A-X1	344.28	1	344.28	386.29	0.0003	
B-X2	20.63	1	20.63	23.14	0.0171	
AB	0.2800	1	0.2800	0.3142	0.6143	
A <sup>2</sup>	2.84	1	2.84	3.19	0.1723	
B <sup>2</sup>	0.0235	1	0.0235	0.0263	0.8814	
Residual	2.67	3	0.8912			
Core total	371.05	8				



**Figure 3:** The relationship between the concentration of TFCB 30°C and TFCB 50°C on drug permeation. The percentage of drug permeation increased with increasing the concentration of TFCB 50°C up to a certain limit than further decreased

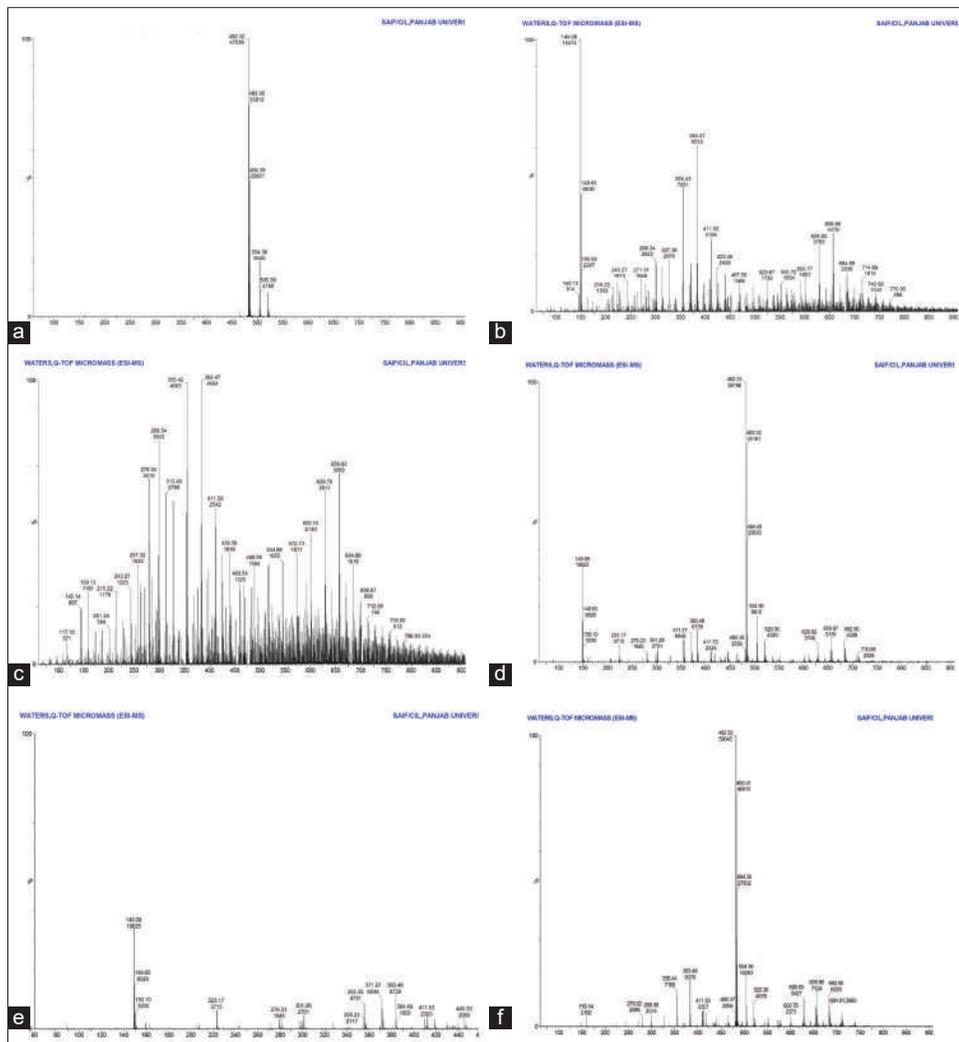
spectrometric data. Strong signals were observed in the form of  $[M+H]^+$  molecular ions and its mass/charge ( $m/z$ ) ratios of 482.32 at the CE values (4.7 eV). The ions resulted from collision-induced dissociation. At the higher CE (5.09 eV), the fragment ion at  $m/z$  299.36 was formed. Therefore, the ion transitions  $m/z$  482.1  $\rightarrow$  258.1 were selected for multiple reaction monitoring (MRM) of the rosuvastatin. In the chromatogram of rosuvastatin base peak was obtained at 482.32  $m/z$  ratios with the highest percentage relative abundance and intensity of 47039 at CE value 4.7 eV. The fragmented ion was also obtained at 482.32, 483.30, 484.39, 504.35, and 505.39 ( $m/z$ ) ratio as shown in Figure 4a.

In the chromatogram of FA, a most abundant peak was obtained at 149.08  $m/z$  ratios with an intensity of 15474. Several fragmented ions were also recognized at various  $m/z$  ratios. Some were at 149.65 and 150.09. Based on the base peak at 149.08  $m/z$  ratio, oleic acid was identified as a major constituent of the FA fraction as shown in Figure 4b. In the chromatogram of FE, a most abundant peak was obtained at 279.34  $m/z$  ratios with relative intensity 3010. A fragmented

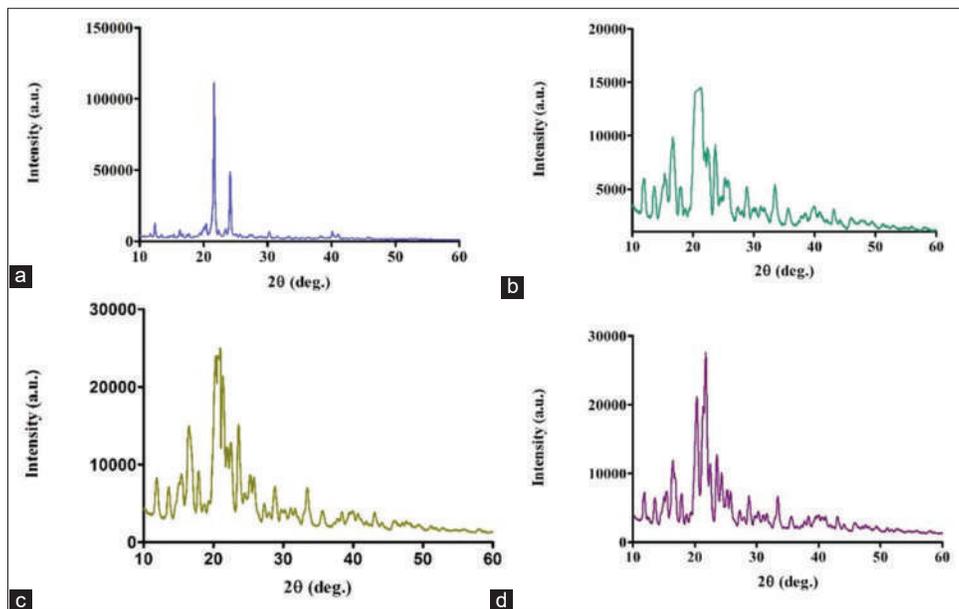
ion at  $m/z$  ratio 299.34 was also identified as shown in Figure 4c. Based on the literature a linoleic acid was produced a base peak at arrange at 250 to 300  $m/z$  ratio.<sup>[17]</sup> Hence, linoleic acid was identified as a major constituent of the FE fraction. The prepared drug TFCB complexes, RAE-A1, RAE-B2, and RAE-C3 were also studied in LCMS. A chromatogram is presented in Figure 4. The characteristic peak for rosuvastatin was found in all three chromatograms and the percentage relative abundance was the same with a slightly acceptable variation in relative intensity. A characteristic oleic acid and linoleic acid peaks were also found in all chromatograms of complexes. The  $m/z$  ratio of the oleic acid in the complex RAE-A1, RAE-B2, and RAE-C3 was found at 149.09, 149.09, and 159.14 with the relative intensity of 19625, 19625, and 2192, respectively. The presence of linoleic acid was also confirmed by the LCMS chromatogram of complexes. The  $m/z$  ratio for linoleic acid was found 279.23, 279.23, and 279.22 with correspondent intensity values of 1945, 1945, and 2088 for RAE-A1, RAE-B2, and RAE-C3, respectively.

### Crystallinity behavior study of TFCB and complex by XRD

The crystalline nature of rosuvastatin limits its solubility in gastric fluid and blood. The effect of formation of the physical complex using TFCB over crystallinity of rosuvastatin was studied using Powder XRD (PXRD). The diffractogram of pure rosuvastatin and formulation prepared in a different weight ratio of FA and FE is presented in Figure 5. The X-ray diffractogram of rosuvastatin showed sharp diffraction peaks at  $2\theta$  values of 12.34, 21.61, 24.16, and 30.20 with a weak diffraction peak at 11.90° and 13.68° as shown in Figure 5a. The intensity of the tallest peak for rosuvastatin was 111356 a.u. Drug TFCB complexes RAE-A1, RAE-B2, and RAE-C3 were taken in XRD studies, to confirm the pattern of change of state of material concerning composition ratio (1:1, 1:1.5, and 1:2) of FA and FE. Drug TFCB complex RAE-A1 showed a sharp peak at  $2\theta$  values of 16.65, 20.38, 21.38, and 23.64. The intensity of the tallest peak was 16651 a.u. as depicted in Figure 5b. The XRD patterns of drug TFCB complexes RAE-B2 are shown in Figure 5c. A sharp peak and strong diffraction signal were obtained at 16.49, 20.96,



**Figure 4:** The LCMS spectra of: [A] Rosuvastatin, [B] Thermal Fraction FA, [C] Thermal fraction FE, [D] RAE-A1, [E] RAE-B2, [F] RAE-C3.



**Figure 5:** Powder X-ray diffractogram of various samples: (a) Rosuvastatin, (b) RAE-A1, (c) RAE-B2, (d) RAE-C3

and 21.04 with an intensity of the tallest peak 24980 a.u. The reflection intensities of the X-ray diffractogram pattern of drug TFCB complex RAE-C3 was found centered at 16.47°, 20.33°, 21.77°, and 23.56° (2 $\theta$ ) with an intensity of tallest peak 27589 a.u. as depicted in Figure 5d.

The result of the study confirming about decrement of the relative crystallinity of rosuvastatin by the formation of the drug TFCB complex with CB fractions in a specific weight ratio. The major diffraction peaks obtained for rosuvastatin were shifted to the lower side by the formation of a physical complex. It was interesting that this shifting was more in RAE-A1. The disappearance of the minor diffraction peak obtained at 11.90° and 13.68° for the rosuvastatin in the diffractogram of RAE-A1 prepared by 1:1 ratio of TFCB indicate the conversion of the crystalline to the amorphous-like state of the rosuvastatin. The order of change of state of rosuvastatin from crystalline to amorphous was RAE-A1>RAE-B2>RAE-C3.

### Saturation solubility study of TFCB & complex

The intestine is a major absorption site for the rosuvastatin therefore saturated solubility of drug and drug TFCB complex measured in 0.2 M phosphate buffer pH 6.8. The rosuvastatin possesses very limited solubility in tested media (11.23  $\pm$  0.97  $\mu$ g/mL). The highest saturation solubility was showed by drug TFCB complex RAE-A1 (22.31  $\pm$  0.94  $\mu$ g/mL) and lowest by RAE-C3 (15.39  $\pm$  1.40  $\mu$ g/mL). The range of percentage solubility enhancement by the formation of the physical complex was 37–98%. The higher aqueous solubility was found in the 1:1 ratio and lowest in the 1:2 ratio of fraction, respectively. The figure was significantly higher than the aqueous solubility of rosuvastatin alone in the same media. The saturation solubility of the rosuvastatin and drug TFCB complex is summarized in Table 5.

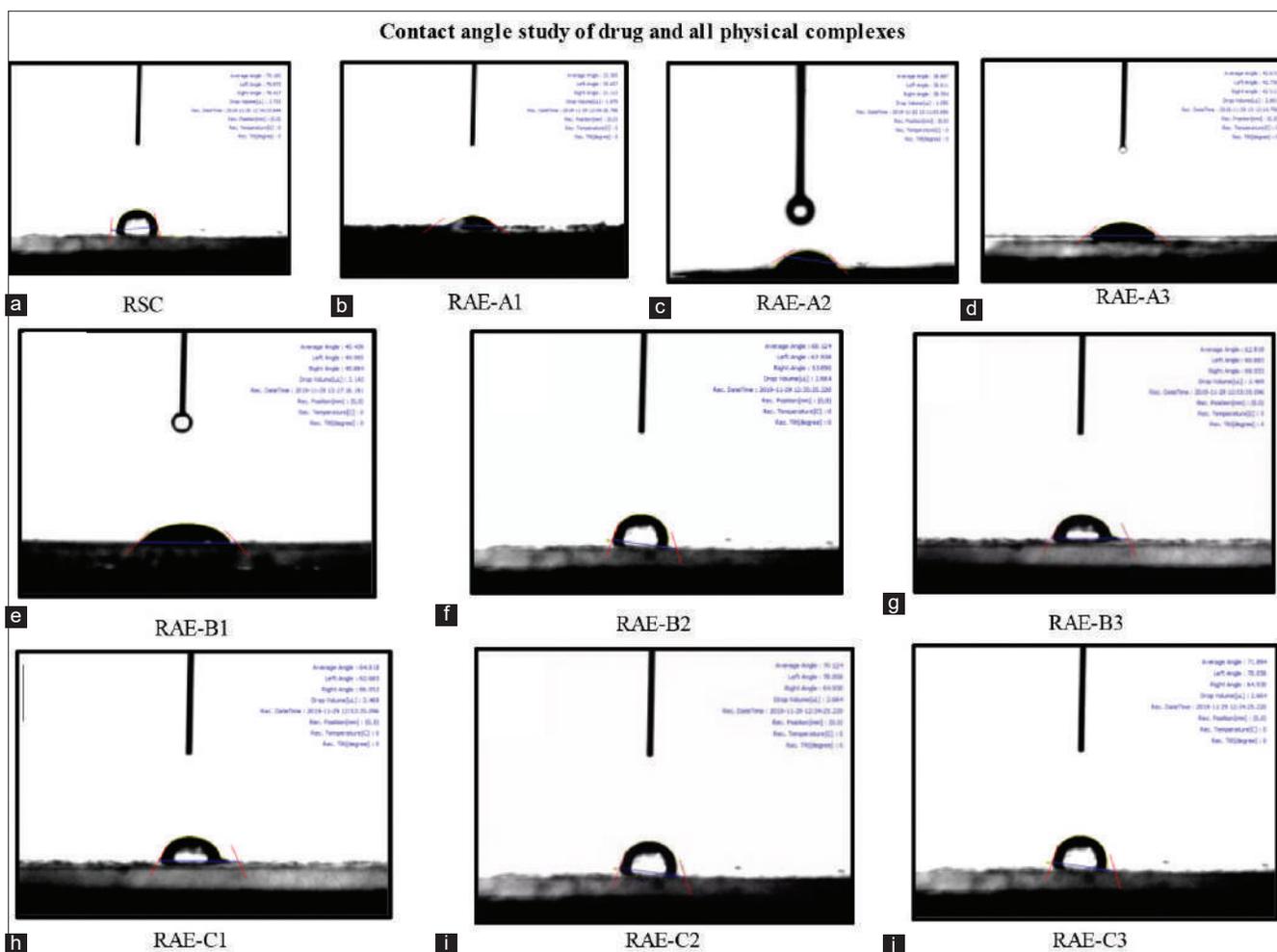
### Wettability study of TFCB and complex by contact angle measurement

The contact angle equals 90° indicates half wettability of solid in dissolution medium. In this investigation, the contact angle of rosuvastatin was found at 79.19° with higher surface energy (13.68 mN/m) indicating moderate wetting in media. The total surface free energy was always closely correlated with the contact angle; the lower spreading coefficient (–59.15 mN) and lower the work of adhesion (86.44 mN/m) confirming the lipophilic nature of the rosuvastatin. Preparation of drug TFCB complexes with CB fraction significantly decreases the contact angle and increases the correspondent surface free energy. The images related to contact angle are shown in Figure 6. Among the prepared complexes highest contact angle 71.81° was recorded for RAE-C3 and the lowest contact angle of 33.38° for RAE-A1. The highest was also lower than the contact angle of the rosuvastatin (79.19°). The data confirming the change of nature of rosuvastatin from lipophilic to hydrophilic. The summary of contact angle with correspondent surface free energy, spreading coefficient, and work of adhesion was presented in Table 5. The order of contact angle was found as RAE-A1< RAE-A2< RAE-A3< RAE-B1< RAE-B2< RAE-B3< RAE-C1< RAE-C2< RAE-C3. The contact angle of the RAE-B2 prepared with a 1:1.5 weight ratio of FA and FE confirming the intermediate contact angle in between REA-A1 and REA-C3 prepared with a 1:1 and 1:2 weight ratio of FA and FE, respectively. From the study of Table 5, conclusion was drafted that the equal quantity of the CB fraction provides a significant alteration of the contact angle and solubility characteristics. Approximate about 98% aqueous solubility of rosuvastatin was increasing after preparation of complex at 1:1 weight ratio of FA and FE.

**Table 5:** Contact angle, surface free energy, spreading coefficient, and work of adhesion of solid surface, saturated solubility, percentage solubility increases, and partition coefficient of tested complex

Physical complexes	Contact angle (°)	Surface free energy (mN/m)	Spreading coefficient (mN/m)	Work of adhesion (mN/m)	Saturation solubility ( $\mu$ g/mL)	Percentage solubility increase	Partition coefficient
RSC	79.19	13.648	–59.153	86.448	11.23 $\pm$ 0.97	--	0.33 $\pm$ 0.03
RAE-A1	33.38	60.787	–12.013	133.587	22.31 $\pm$ 0.94	98.66	0.71 $\pm$ 0.01
RAE-A2	38.68	56.826	–15.975	129.626	20.63 $\pm$ 1.34	83.70	0.77 $\pm$ 0.03
RAE-A3	42.63	53.561	–19.239	126.361	20.42 $\pm$ 1.06	81.83	0.84 $\pm$ 0.02
RAE-B1	45.43	53.019	–19.781	125.819	19.55 $\pm$ 1.28	74.09	0.87 $\pm$ 0.03
RAE-B2	60.12	51.081	–21.719	123.881	18.23 $\pm$ 1.05	62.33	0.92 $\pm$ 0.02
RAE-B3	62.61	32.769	–41.824	103.776	18.05 $\pm$ 1.55	60.73	1.23 $\pm$ 0.04
RAE-C1	64.86	30.976	–43.764	101.534	16.71 $\pm$ 1.84	48.80	1.37 $\pm$ 0.01
RAE-C2	70.12	22.625	–50.175	95.425	16.24 $\pm$ 0.75	44.61	1.58 $\pm$ 0.02
RAE-C3	71.89	14.995	–57.805	87.795	15.39 $\pm$ 1.40	37.04	1.63 $\pm$ 0.03

\*Data are presented as mean $\pm$ SD; (n=3)



**Figure 6:** Contact angle measurement sample at various ratio of FA and FE: (a) RSC, (b) RAE-A1 [1:1.1], (c) RAE-A2 [1:1.2], (d) RAE-A3 [1:1.4], (e) RAE-B1 [1:1.25], (f) RAE-B2 [1:1.5], (g) RAE-B3 [1:1.75], (h) RAE-C1 [1:1.43], (i) RAE-C2 [1:1.71], (j) RAE-C3 [1:2]

### Partition coefficient between n-octanol: phosphate buffer

The partition coefficient of rosuvastatin between n-octanol and 0.2 M phosphate buffer pH 6.8 was found  $0.33 \pm 0.03$  which was very low as compared to all drug TFCB complexes tested. The value of the partition coefficient is summarized in Table 5. The complex prepared with the highest amount of FE with the least amount of FA produced the highest partition coefficient value ( $1.63 \pm 0.03$ ). The intermittent partition coefficient was produced by RAE-B2 ( $0.92 \pm 0.02$ ). All the prepared complexes in different drug TFCB weight ratios shown a better partition coefficient than rosuvastatin in tested media.

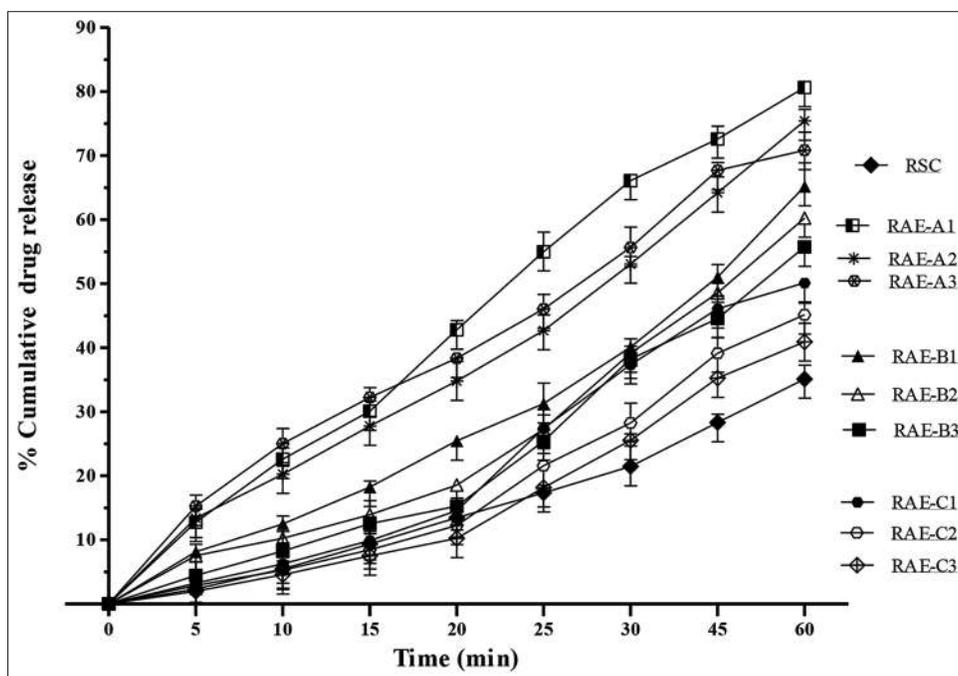
### *In vitro* dissolution study of drug TFCB complex

The result of *in vitro* drug release studies of rosuvastatin and prepared complex is depicted in Figure 7. Rosuvastatin

alone confirming only 35.12% drug dissolution at the same tested media. The percentage dissolution of rosuvastatin was significantly increased by the formation of the drug TFCB complex in a range of 40.91–80.62%. The lowest and highest percentage dissolutions were recorded for formulation RAE-C3 (40.91%) and RAE-A1 (80.62%), respectively. The result of the study was found probably due to better solubility of the drug by the formation of the drug complex in intestinal fluid. The release data are presented in Table 6.

### *Ex vivo* everted sac permeation study of drug TFCB complex

The everted sac model is a well-accepted model for testing of *ex vivo* permeation profile due to the similarity of the human intestinal tract. The permeation data are presented in Table 6. Initially, permeation was increased by increasing the quantity of fraction FE, followed by decrement as depicted in Figure 8. The highest permeation was obtained in drug TFCB complex RAE-B2. The apparent permeability coefficient of RAE-B2



**Figure 7:** Percentage cumulative drug release from pure drug and prepared physical complexes. The highest percentage of drug release was obtained for complex RAE A1 and least for RAE C3. Pure rosuvastatin produced only 35.12% drug release in a 60-min study

**Table 6:** Apparent permeability coefficients ( $P_{app}$  cm/s), percentage drug release, and drug retention of rosuvastatin in all physical complexes across everted gut sac model

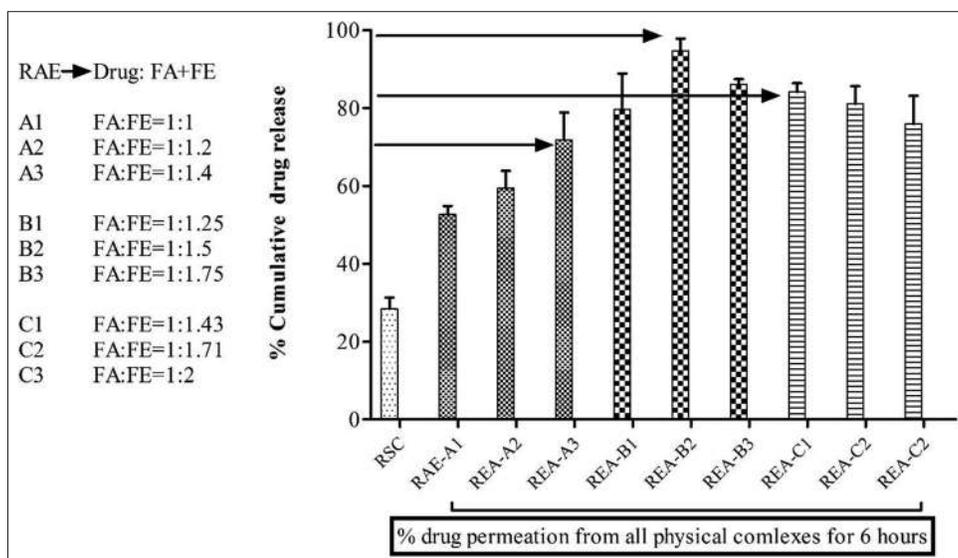
Physical complexes	Percentage drug release	Everted gut sac model			
		Percentage drug permeation	Percentage drug retention	Apparent permeability coefficient ( $10^{-6}$ cm/s)	Apparent permeability ( $\text{mg}/\text{cm}^2$ )
RSC	35.12 $\pm$ 1.62	28.32 $\pm$ 2.12	3.65 $\pm$ 1.78	0.75 $\pm$ 0.76	0.944 $\pm$ 0.09
RAE-A1	80.62 $\pm$ 2.63	52.46 $\pm$ 1.86	5.29 $\pm$ 1.78	1.67 $\pm$ 1.26	1.392 $\pm$ 0.80
RAE-A2	75.39 $\pm$ 3.51	59.18 $\pm$ 1.89	4.12 $\pm$ 1.78	1.89 $\pm$ 1.13	1.578 $\pm$ 0.42
RAE-A3	70.81 $\pm$ 2.84	71.34 $\pm$ 0.99	2.36 $\pm$ 1.65	2.28 $\pm$ 1.56	1.903 $\pm$ 0.58
RAE-B1	65.64 $\pm$ 0.76	79.04 $\pm$ 1.60	1.89 $\pm$ 1.70	2.52 $\pm$ 1.18	2.108 $\pm$ 0.74
RAE-B2	60.23 $\pm$ 1.03	90.68 $\pm$ 1.46	1.31 $\pm$ 1.26	3.02 $\pm$ 1.26	2.525 $\pm$ 0.80
RAE-B3	55.72 $\pm$ 3.16	86.46 $\pm$ 1.26	1.76 $\pm$ 1.69	2.76 $\pm$ 0.74	2.306 $\pm$ 0.74
RAE-C1	50.12 $\pm$ 1.82	84.35 $\pm$ 0.93	6.84 $\pm$ 1.78	2.69 $\pm$ 1.05	2.249 $\pm$ 0.58
RAE-C2	45.13 $\pm$ 1.14	81.47 $\pm$ 1.15	7.32 $\pm$ 1.40	2.60 $\pm$ 0.85	2.173 $\pm$ 0.76
RAE-C3	40.91 $\pm$ 1.94	75.55 $\pm$ 2.02	9.98 $\pm$ 1.36	2.44 $\pm$ 0.95	2.015 $\pm$ 0.95

\*Data are presented as mean $\pm$  SD; (n= 3)

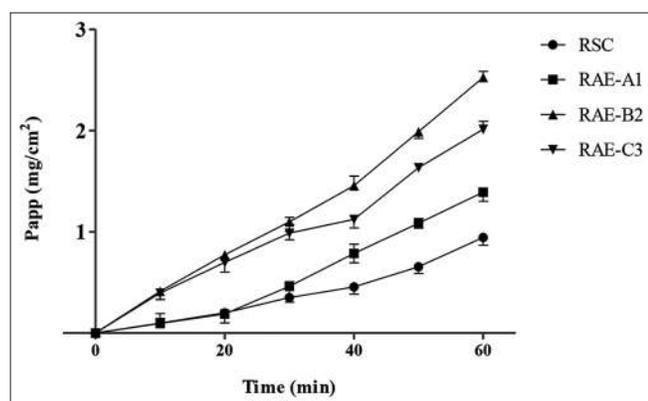
was 3.02 ( $10^{-6}$  cm/s) with apparent permeability of 2.52 mg/cm<sup>2</sup> as depicted in Figure 9. Which was significantly higher than the value obtained for rosuvastatin alone in the same tested media. A drug TFCB complex (RAE-C3) prepared with a 1:2 weight ratio of FA and FE confirming better permeation than a drug TFCB complex RAE-A1 prepared with a 1:1 weight ratio of FA and FE. This could happen probably due to the variation in aqueous solubility and partition coefficient of the formulation.

## DISCUSSION

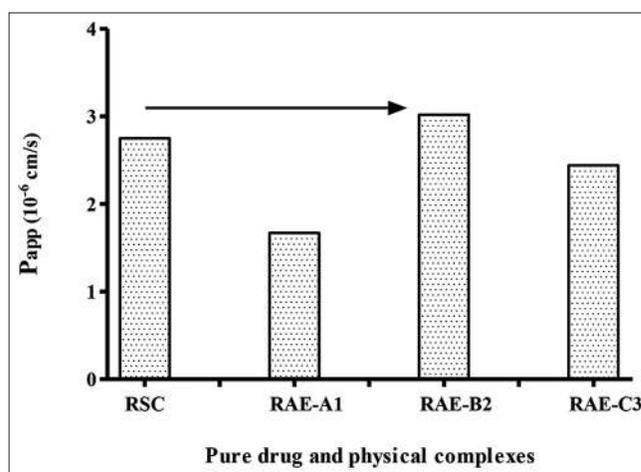
Many opportunities remain to discover the novel application of CB with a small modification in its composition. Separation of their thermal fraction might used in the present investigation to discover its application on the enhancement of rosuvastatin oral bioavailability. A novel concept, to use a combination of thermal fraction and report its effect on dissolution and permeation in a comparative mean presented in the article.



**Figure 8:** Percentage drug permeation from pure drug and physical complexes across everted gut sac and TFCB complexes



**Figure 9:** Apparent permeability  $P_{app}$  ( $\text{mg}/\text{cm}^2$ ) of pure drug and prepared complexes



**Figure 10:** Apparent permeability coefficients  $P_{app}$  ( $\text{cm/s}$ ) of pure drug and prepared complexes

A thermal fractionation has successfully separated the CBs fatty acid according to their fat profile confirmed by the LCMS chromatogram. Oleic acid and linoleic acid were found out as major constituents of FA and FE fraction, respectively. The carbon chain length of both the fatty acid is 18 but differ in their degree of saturation. Oleic acid having a single unsaturated bond where linoleic acid, possesses two double bonds. This difference might affect the dissolution and permeation characteristics of rosuvastatin after developing a physical complex with these two fractions in various ratios. With the help of these data, we expect the variability in the apparent permeability coefficient of these selected formulations. The order of apparent permeability was found as RAE-B2 > RAE-C3 > RAE-A1 as shown in Table 6 and depicted in Figure 10. A saturated fatty acid might enhance the solubility and unsaturated fatty acid provided lipophilicity.<sup>[18,19]</sup> This two feature of fatty acid of CB is very helpful for low aqueous soluble and low bioavailable drug-like rosuvastatin. In the present investigation, monounsaturated (oleic acid) and polyunsaturated (linoleic acid) were tried in a combination to get both the phenomenon hydrophilicity and hydrophobicity in a single formulation.

PXRD is one of the most important instrument used in the study of the crystal lattice structure that produces a specific diffraction pattern. Polymorphisms of certain drugs have a considerable impact on solubility, dissolution, and bioavailability.<sup>[20]</sup> Physical states of formulation play a role in their performance. XRD technique is a powerful tool for the study of the nature of the material. The report suggested that an amorphous form of solid materials are shown higher bioavailability than crystalline material due to the solubility characteristic differences,<sup>[21]</sup> Rosuvastatin is crystalline in nature and needs to convert into an amorphous form for better solubility.<sup>[22]</sup> XRD result shown in Figure 5 reveals that a transformation of the state of materials by the formation of drug-TFCB complex has occurred. Rosuvastatin characteristic sharp peak was found at lower  $2\theta$  with low intensity in the prepared complexes. The boarding of the peak was also given a clear indication of a change of state of the material.

Order of change of state from crystallinity to amorphous was found RAE-A1>RAE-B2>RAE-C3. This data are entirely agreed upon with a contact angle of these formulations. The formulation of RAE-A1 was higher solubility therefore shown a lower contact angle. The partition coefficient  $P_{o/w}$  of formulation RAE-B2 was near about 1 that indicates the balance of hydrophilic-lipophilic characteristics in a single. Formulation that is the probable explanation for better permeation characteristics by this formulation. The report published by Stott *et al.* (2001) in a study of binary mixtures of propranolol with fatty acid is agreed with the finding of the present study.<sup>[23]</sup>

Crystallinity decreases with the addition of long-chain saturated fatty acid. Suggesting that fatty acid interrupted the association of the drug with CB fraction and comparatively a less-organized structure was formed. The recrystallization of drug molecules affects the steric hindrance of the saturated fatty acid that might be increasing with the fatty chain length, which might be responsible for the lower crystallinity.<sup>[24]</sup> The present investigation revealed that the physical complexation of rosuvastatin with CB fractions could help increase solubility and permeation in a biological system.

The contact angle plays an important role in wettability in biological fluid and, ultimately, affect the quantity of drug solution availability at the site of drug absorption. A final target for biopharmaceutical pharmacists is to improve ultimate bioavailability through absorption enhancement. The obtained data suggested that the weight ratio of FA and FE well affected the contact angle and permeation characteristics. The contact angle is directly correlated with the solubility in tested media. The lower contact angle indicate better solubility obtained with a 1:1 ratio of CB fraction. The contact angle with increasing the proportion of TFCB 50°C fraction in complex and vice versa. To obtain the highest permeability across the biological membrane also need acceptable dissolution characteristic with lipophilicity to partition between GIT-blood barriers. The best ratio of tested two thermal fractions was studied for preparing the drug TFCB complex for oral administration to get the best hydrophilic-lipophilic balance characteristic property in a single formulation. The desired contact angle for solubility in biological fluid should be less than 45° and more than 50% drug release characteristics.<sup>[25]</sup> Among the tested formulation, RAE-B2 fulfills the above criteria with a small deviation. The contact angle and the release obtained with RAE-B2 were 60.12 and 60.23%, respectively. The addition of a higher amount of FE and the lower the amount of FA increases the contact angle, which could be ascribed to the hydrophobic groups of FE. In oral absorption, the need to balance both properties because of the aqueous nature of gastric fluid and the lipoidal nature of the GIT, which acts as a biological barrier to drug absorption.<sup>[26]</sup> 3<sup>2</sup> full factorial design approach giving liability to check multiple responses on multiple factors.<sup>[27]</sup> The factorial design approach revealed that change in contact angle by changing composition in drug TFCB complex directly affected the permeation across

biological barriers.<sup>[28]</sup> Also reported the applicability of the contact angle measurement as an alternative approach for understanding dissolution rate enhancement for an oral tablet. A relationship of these two dependent responses with lipid composition in the drug TFCB complex was shown quadratic fit model within acceptable R<sup>2</sup> value confirming the effect.

An extrapolation of *in vitro* data into *ex vivo* studies is always challengeable to the researcher because both the study having different rate parameters. *In vitro* dissolution is purely based on wetting and solubility characteristics of the targeted drug, but *ex vivo* permeation study is governed not only by the solubility characteristic but also lipoidal membrane which acts as a rate-limiting barrier. The present investigation also found a gap between *in vitro* dissolution data and *ex vivo* permeation profile. *In vitro* data were not extrapolated in *ex vivo* studies. The RAE-A1 produced the highest percentage of drug release but unsuccessful to produce maximum drug permeation. An *ex vivo* permeation data were abysmal with relation to FA and FE ratio. The permeation was initially increased by increasing the proportion of FE in a drug complex upto 1:1.5 (FA:FE) then further decrement recorded. A probable explanation for this uncertainly was based on the hydrophilic-lipophilic balance of formulation. In an RAE-A1, both oleic and linoleic fatty acid are present in equal proportion, therefore, a more hydrophilic nature restrict the permeation from the lipoidal membrane. Similarly, in the RAE-C3 quantity of linoleic acid was twice that of oleic acid which produces more lipophilicity; therefore, the formulation did not produce desired aqueous solubility in the biological fluid that was the initial barrier for dissolution. In a combination of 1:1.5 (FA: FE) provide better permeability than other tested ratio 1:1 and 1:2, probably due to the presence oleic acid and linoleic acid at ratio 1:1.5. More prominent linoleic acid having *cis* double bond unsaturation helpful in kinks isomer formation. These kinks are free volume generated on the hydrocarbon phase of the lipid composition of the biological membrane having an ability to alter the cellular matrix.<sup>[18,29]</sup> This structural defect by a specific combination of fatty acid permits the drug molecule across the membrane by alteration in fluidity and finally improves the bioavailability.

## CONCLUSION

Thermal fractionation is a unique separation technique for fatty acids. LC-MS spectrograms reveal that fraction having a different chemical composition; therefore, its physicochemical property was also varied and shows the different responses in the tested biological medium. By changing the ratio of FA and FE fraction makes a balance between hydrophilicity and hydrophobicity that is the most important factor to get maximum oral bioavailability. The FA fraction probably able to produce a better dissolution profile due to more saturated fatty acid content but the FE fraction

confirming practically higher permeability through the biological membrane due to the presence of unsaturated fatty acid. So need a balancing between hydrophobic property and hydrophilic property in a single formulation. A drug physical complex prepared with FA and FE in ratio 1:1.5 w/w full fills the need. The finding of the study may attract the researcher to find out novel applicability of CB rather than conventional use in holistic medicine. Thermal fractions may also provide a new class of pharmaceutical excipient as an alternative to other conventional lipid and lipid substitutes.

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## DECLARATION

### Ethics approval and consent to participate

Not Applicable.

### Consent for publication

Not applicable.

### Availability of data and materials

All data and materials are available on request.

### Competing interests

The authors declare that they have no competing interests.

### Funding

The authors report no financial conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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