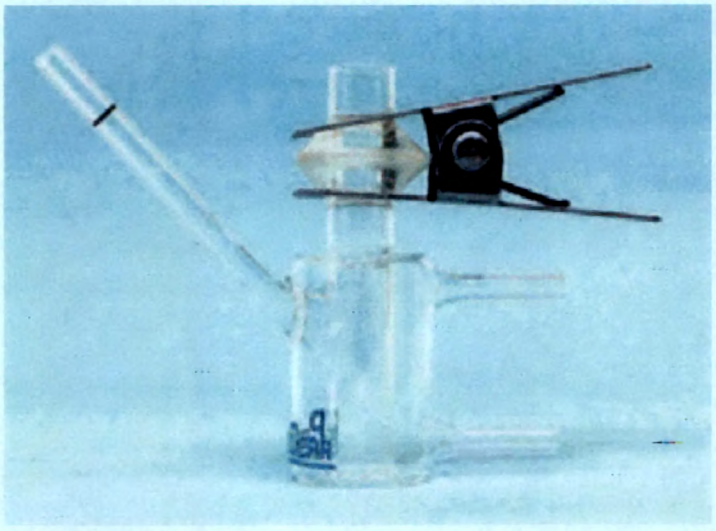
Chapter 5



Invitro Diffusion

5.1 INTRODUCTION

In vitro diffusion of formulations is a valuable tool to predict the behaviour of a particular formulation with respect to drug transport across the membrane. According to Gemmell and Morrison 1957, *in vitro* models may have limitations in terms of prediction of drug transport across the mucosal membrane nevertheless: under the testing conditions *in vitro* studies can be helpful to access the relative drug transport behaviour across the mucosa. Various parameters pertaining to formulations such as flux, partition coefficient and diffusion coefficient can be derived using *in vitro* evaluation techniques. The *in vitro* diffusion studies can also be used as a screening tool to screen the best formulation out of many. One of the disadvantages of *in vitro* evaluation techniques is that the method does not mimic the behavior of living organs/ tissues, for example, degradation of drug compound in the presence of enzymes, capricious blood supply or metabolism etc. As like other topical formulations, *in vitro* diffusion studies have to be carried out for nasal delivery systems. This will provide relatively quantitative data for comparison and evaluation of these formulations (Gavini et al 2005). In this study, all the test formulations were accessed for *in vitro* diffusion across the sheep nasal mucosa in triplicate and the parameters were calculated.

A. Percent drug diffused

The percent drug diffused across the sheep nasal mucosa at predetermined sampling time interval using formula mentioned below.

$$\% \text{ Drug diffused} = \frac{\text{Amount of drug in receptor compartment at t time}}{\text{Amount of drug loaded in the donor compartment}} \times 100$$

B. Kinetics of release

In order to investigate the mechanism of drug release from the formulation, the release rates were integrated into each of the following equation and the regression coefficient was calculated.

i. Zero order equation

$Q = K_0 t$

Where Q - Amount of drug released at time t

t - Time in hours

K_0 - Zero order release rate constant

First order equation

$Q = Q_0 \, e^{-K_1 t}$

Where Q - Amount of drug released at time t

t - Time in hours

K_1 - First order release rate constant

ii. Higuchi's equation

$Q = K_H \times \sqrt{t}$

Where Q - Amount of drug released at time t

t - Time in hours

K_H - Zero order release rate constant

The order of release was determined by performing the regression over the mean values of percent drug diffused vs. time (for zero order), log percent drug diffused vs. time (for first order) and percent drug diffused vs. square root of time (for higuchi order)

C. Flux: (Martin 1991)

The amount M of material flowing through a unit cross section S of a barrier in unit time t, is known as the flux, J.

$J = dM / S.dt$

D. Diffusion coefficient:

The diffusion coefficient of the drug was calculated using the following equation (Adrian Williams 2003)

$J = D \times C_0 / h$

Where J - Flux

C_0 - Drug concentration in donor compartment

h - Thickness of the membrane

5.2 MATERIALS AND METHODS:

Franz diffusion cell

The propose *in vitro* studies were carried out using franz diffusion cell. This cell consists of a hollow glass tube in the center having diameter of 10mm. The cell has two compartments viz. i) donor compartment and ii) receptor compartment. The donor compartment is used for holding the test formulation while the receptor compartment holds the respective diffusion media. The hydrodynamic characteristics

of the franz diffusion cell was established using benzoic acid disc method (Chein and Valia 1984)

Preparation of membrane:

The freshly excised sheep nasal mucosa, except septum part was collected from the slaughter house in PBS pH 6.4. The membrane was kept in PBS pH 6.4 for 15 minutes to equilibrate. The superior nasal conche was identified and separated from the nasal membrane and made free from adhered tissues. Selective samples of tissues of 0.2mm thickness were taken for the studies. The excised nasal membrane was then mounted on franz diffusion cell. The tissue was stabilized using phosphate buffer pH 6.4 in both the compartments and allowed to stir/ stabilize for 15 minutes with continuous stirring on a magnetic stirrer. After 15 minutes, solution from both the compartments was removed and the diffusion media was filled in the acceptor compartment. The mounting of the nasal mucosa was done using pharmaceutical grade grease at the brim of the donor compartment to avoid the leakage of the test sample and supported with rubber bands crossover the cell. The temperature of the receiver chamber containing diffusion media was controlled at 37° ± 1° c under continuous stirring with teflon coated magnetic bar at constant rate, in such a way that the nasal membrane surface just flushes the diffusion media.

Reagents:

Medium 1: 6.8 gm of pottasium dihydrogen phosphate was dissolved in 500ml of distilled water. 200ml of methanol and 4ml of Tween 80 was added and the volume was made up with distilled water and the pH was adjusted to 5 with ortho phosphoric acid.

Medium 2: 6.8 gm of pottasium dihydrogen phosphate was dissolved in 500ml of distilled water, 2ml of Tween 80 was added and the volume was made up with distilled water and the pH was adjusted to 5 with ortho phosphoric acid.

5.3 INVITRO DIFFUSION STUDY OF FORMULATIONS:

5.31. CLOBAZAM FORMULATIONS:

The *in vitro* drug diffusion study was performed using Franz diffusion cell with a diameter of 10 mm and mucosa thickness (height) 0.2 mm (Willimann H et al 1992). The saturation solubility of clobazam in various phosphate buffer pH 4, pH 5 and pH

6 were assessed and 20% v/v methanolic phosphate buffer pH 5 containing 0.4% v/v Tween 80 was used as diffusion media. (Chen et al 2005 and Patel et al 2006)
0.5 ml of CZS, CZME1, CME2, CMME11, CMME12, CMME21 and CMME22 was placed in the donor compartment along with 0.5 ml of diffusion media. Recipient compartment containing 12 ml of medium was stirred with teflon coated magnetic stirrer. Samples from the receptor compartment were withdrawn at predetermined time intervals and analyzed using HPLC method discussed in section 3.4.1.2. Each sample removed was replaced by an equal volume diffusion media. Each study was carried for a period of 6hrs, during which the drug in receiver chamber (μg/ml) across the sheep nasal membrane calculated at each sampling point. The formulations were studied in triplicate for diffusion studies and the mean cumulative values for % drug release were shown in Table 5.1. The release kinetics of diffusion was studied by calculating the regression coefficient for zero order, Higuchi's equation and first order equations and shown graphically (Graph 5.1, 5.2 & 5.3) and recorded in Table 5.2. The diffusion coefficients and flux for clobazam were calculated and were tabulated in Table 5.2.

5.3.2. CLOPIDOGREL BISULPHATE FORMULATIONS:

Phosphate buffer pH 5 containing 0.2% v/v Tween 80 (Tiwari et al 2007) was used as diffusion media for invitro study of clopidogrel bisulphate formulations. The diffusion study of CSS, CSME2, CSME3, CSMME21, CSMME22, CSMME31 and CSMME32 was carried out and samples were withdrawn at predetermined time intervals and analyzed using HPLC method discussed in section 3.4.2.2. The formulations were studied in triplicate for diffusion studies and the mean cumulative values for % drug release were shown in Table 5.3. The release kinetics of diffusion was studied by calculating the regression coefficient for zero order, Higuchi's equation and first order equations and shown graphically (Graph 5.4, 5.5 & 5.6) and recorded in Table 5.4. The diffusion coefficients and flux for clopidogrel bisulphate were calculated and were tabulated in Table 5.4.

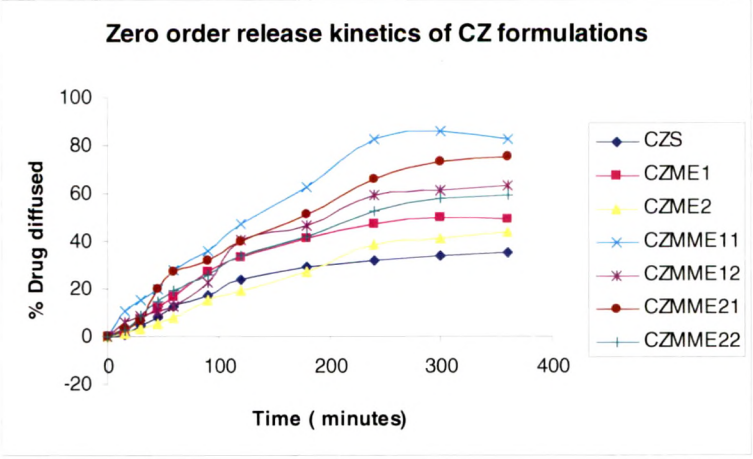
5.4 RESULTS Table 5.1 *In vitro* diffusion study of Clobazam formulations

Time (min)	Root time (min)	% Drug Diffused (%w/w)						
		CZS	CZME1	CZME2	CZMME11	CZMME12	CZMME21	CZMM22
15	3.87	0.36 ± 0.06	2.06 ± 0.18	1.15 ± 0.03	10.99 ± 1.54	5.84 ± 0.94	3.34 ± 0.36	2.90 ± 0.31
30	5.48	4.75 ± 0.41	7.39 ± 0.67	3.50± 0.33	15.63 ± 1.03	8.61 ± 0.70	6.41 ± 0.40	8.59 ± 1.19
45	6.71	8.06 ± 0.07	12.18 ± 0.42	5.65 ± 1.34	19.74 ± 1.86	10.63 ± 0.67	19.83 ± 0.79	14.37 ± 2.61
60	7.75	12.38 ± 1.1	16.68 ± 1.38	7.90 ± 2.51	28.92 ± 2.2	12.84 ± 1.47	27.18 ± 1.96	19.11 ± 3.21
90	9.49	17.37 ± 1.4	27.04 ± 1.62	15.01 ± 1.96	35.89 ± 1.6	22.51 ± 2.73	31.68 ± 2.71	25.97 ± 1.80
120	10.95	23.77 ± 2.5	33.28 ± 1.75	19.15 ± 2.27	47.63 ± 1.79	40.57 ± 2.81	40.31 ± 1.70	33.79 ±1.96
180	13.42	29.58 ± 2.64	41.28 ± 2.76	27.13 ± 1.92	62.55 ± 1.46	46.87 ± 2.14	51.49 ± 1.88	41.82± 2.55
240	15.49	32.28 ± 1.88	47.36 ± 3.52	38.99 ± 2.89	83.38 ± 1.99	59.22 ± 2.44	65.85 ± 2.72	52.33 ±1.85
300	17.32	34.17 ± 1.61	49.86 ± 2.06	41.03 ± 2.54	86.18 ± 2.50	61.39 ± 2.37	73.58 ± 3.17	58.31± 1.93
360	18.97	35.43 ± 1.91	49.56 ± 2.99	44.14 ± 1.18	82.36 ± 3.19	63.62 ± 1.91	75.01 ± 2.87	59.31 ±1.44

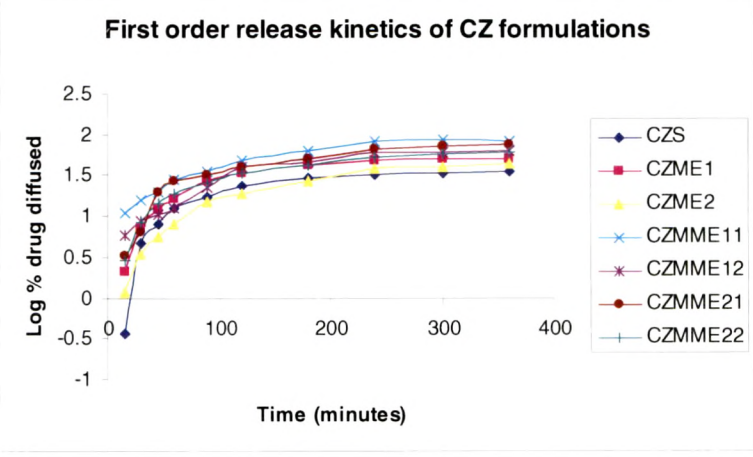
Table 5.4 Diffusion coefficient and regression coefficients of clobazam formulations

S.No	Formulation	Flux (µg/min)	Diffusion coefficient (mm^2/min)	Zero order	First order	Higuchi order
1.	CZS	0.0328	4.37E-05	0.8728	0.4978	0.9608
2.	CZME1	0.0439	5.85E-05	0.8733	0.6206	0.9599
3.	CZME2	0.0401	5.35E-05	0.9787	0.8717	0.985
4.	CZMME11	0.0775	1.03E-04	0.9177	0.8076	0.9584
5.	CZMME12	0.0569	7.58E-05	0.9668	0.7514	0.983
6.	CZMME21	0.0643	8.58E-05	0.9363	0.6616	0.9857
7	CZMME22	0.0506	6.75E-05	0.9404	0.6888	0.9913

Graph 5.1 Zero order release kinetics of CZ formulations



Graph 5.2 First order release kinetics of CZ formulations



Graph 5.3 Higuchi order release kinetics of CZ formulations

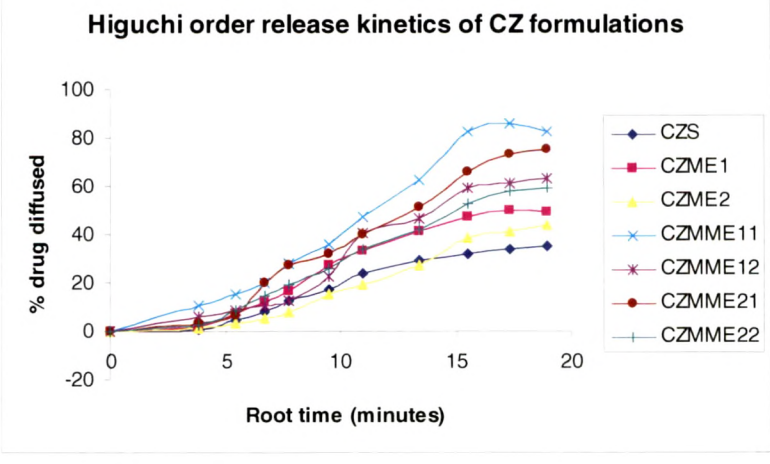


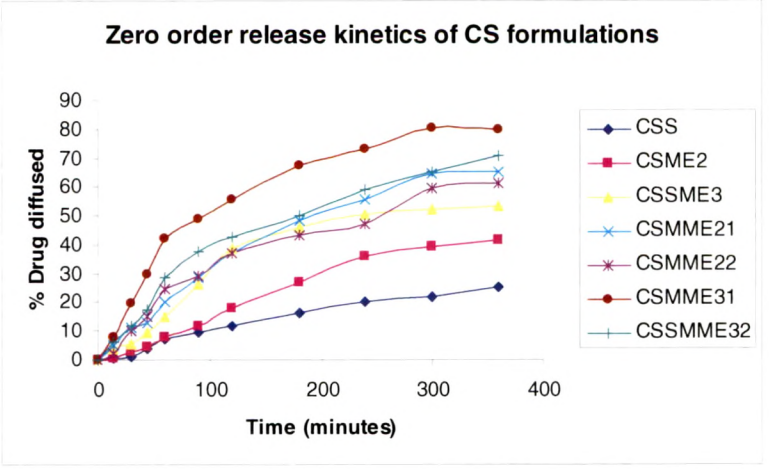
Table 5.3 *In vitro* diffusion study of Clopidogrel bisulphate formulations

Time (min)	Root time (min)	% Drug Diffused (%w/w)						
		CSS	CSME2	CSME3	CSMME21	CSMME22	CSMME31	CSMM32
15	3.87	0.67 ± 0.06	0.50 ± 0.03	3.65 ± 0.66	5.34 ± 0.53	2.06 ± 0.47	7.99 ± 0.44	5.94 ± 0.71
30	5.48	1.382 ± 0.22	2.53 ± 0.42	5.59 ± 0.85	11.4 1± 1.24	10.39 ± 0.64	19.63 ± 1.16	11.61 ± 1.14
45	6.71	4.56 ± 0.65	4.63 ± 0.84	9.38 ± 1.32	12.83 ± 1.31	15.18 ± 1.03	29.86 ± 0.89	17.63 ± 1.26
60	7.75	7.83 ± 1.02	8.10 ± 1.26	15.11 ± 1.24	20.18 ± 0.85	24.68 ± 0.24	42.19 ± 1.21	28.84 ± 1.81
90	9.49	9.80 ± 1.31	11.96 ± 2.2	26.39 ± 1.52	28.68 ± 1.11	29.03 ± 0.79	48.89 ± 1.34	37.51 ± 1.53
120	10.95	11.78 ± 2.14	18.16 ± 0.95	38.78 ± 2.13	37.30 ± 2.17	37.28 ± 1.07	55.64 ± 2.31	42.56 ± 1.42
180	13.42	16.58 ±2.31	27.19 ± 1.34	45.85 ± 1.86	48.49 ± 1.74	43.28 ± 2.54	67.55 ± 1.64	49.87 ± 0.96
240	15.49	20.28 ± 1.65	35.99 ± 1.16	50.53 ± 1.93	55.84 ± 0.85	47.36 ± 2.11	73.38 ± 1.85	59.22 ± 1.11
300	17.32	22.18 ± 1.84	39.35 ± 1.54	52.31 ± 0.95	64.58 ± 1.38	59.86 ± 1.76	80.18 ± 2.14	65.39 ± 1.43
360	18.97	25.43 ± 1.21	41.49 ± 0.96	53.34 ± 2.6	65.01 ± 1.53	61.56 ± 1.51	80.12 ± 1.75	70.62 ± 1.52

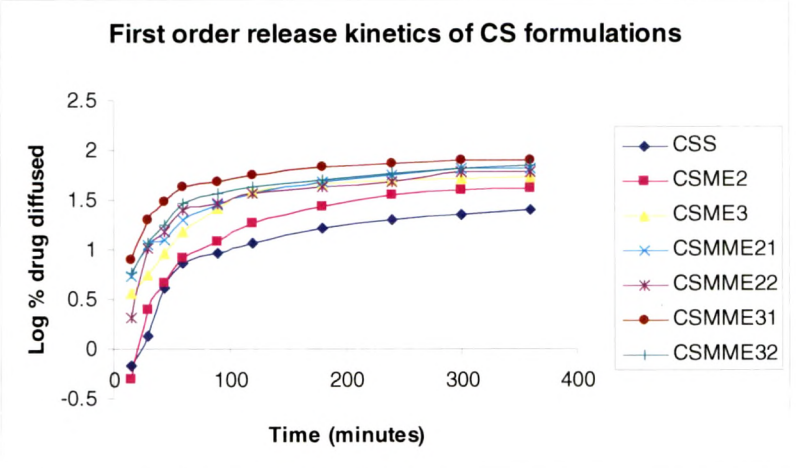
Table 5.4 Diffusion coefficient and regression coefficients of clopidogrel bisulphate formulations

S.No	Formulation	Flux (µg/min)	Diffusion coefficient (mm^2/min)	Zero order	First order	Higuchi order
1.	CSS	0.0217	2.17E-05	0.9604	0.6768	0.9937
2.	CSME2	0.0387	3.87E-05	0.966	0.6914	0.9844
3.	CSME3	0.0485	4.85E-05	0.8556	0.6984	0.9386
4.	CSMME21	0.0551	5.51E-05	0.9415	0.7675	0.9875
5.	CSMME22	0.0496	4.96E-05	0.9188	0.589	0.9797
6.	CSMME31	0.0669	6.69E-05	0.8748	0.6361	0.9625
7	CSMME32	0.0554	5.54E-05	0.915	0.6954	0.9751

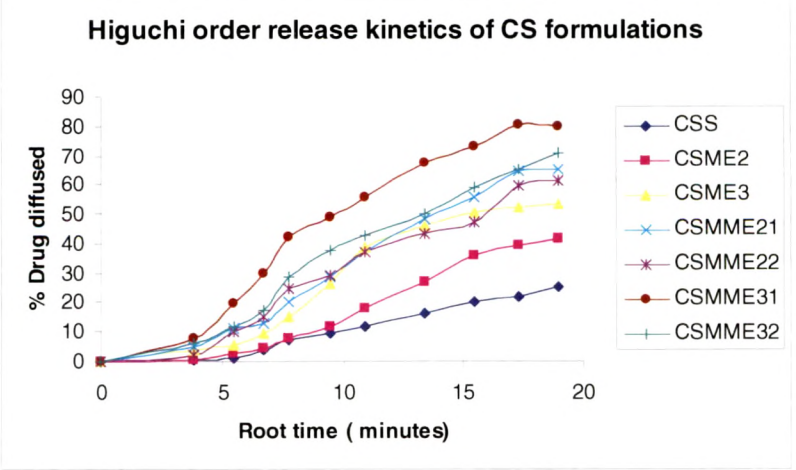
Graph 5.4 Zero order release kinetics of CS formulations



Graph 5.5 First order release kinetics of CS formulations



Graph 5.6 Higuchi order release kinetics of CS formulations



5.5 DISCUSSION

CLOBAZAM

The prepared formulations of clobazam were subjected to *in vitro* diffusion studies through sheep nasal mucosa for 6 hours. The % cumulative drug diffused across nasal mucosa from the formulations were calculated and recorded in Table 5.1. The kinetic pattern of the diffusion was studied by fitting % drug diffused in given time in different order kinetics like zero order, first order and higuchi order. The release kinetics of clobazam from different formulations was shown in Graph 5.1- 5.3. Regression coefficients of all formulations in different orders were compared and found that the release pattern of clobazam from the formulation across the nasal mucosa followed higuchi order rather than zero order and first order. This was concluded by higher regression coefficient value in curve fitting. Diffusion coefficient and flux for all the formulations were calculated and recorded in Table 5.2. Among the CZ microemulsions, CZME1 showed higher % drug diffused which was reflected in higher flux and diffusion coefficient value than CZME2. Both carbopol containing (CZME11 & CZMME21) and chitosan containing (CZMME12 & CZMME22) microemuslions were subjected to invitro diffusion studies and it was observed that the carbopol containing microemulsions showed higher % drug release than the chitosan containing microemulsions. This may be explained by bioadhesion and absorption enhancement property of carbopol across the mucosal membrane by opening tight epithelial junctions of the mucosal membranes like nasal membrane (Morimoto K et al 1985) and intestinal membrane (Gerrit Borchard et al 1996). This renders carbopol as a key ingredient in drug delivery systems across the mucosal membranes. Luben et al 1994 reviewed the applications of the bioadhesive polymers in delivery systems. The CZMME11 was found to have highest flux (0.0775 µg/ min) and diffusion coefficient (1.03E-04 mm^2/ min). The clobazam formulations which were selected for further studies and their characteristic parameters were shown in Table5.5

Table 5.5 Clobazam formulations

S.No	Parameters	CZS*	CZSME(P)	CZME	CZMME
	Composition				
1.	Capmul MCM (O)/ content (%v/v)	--	5.0	5.0	5.0
2.	Acconan CC6(S) / content (%v/v)	--	22.5	22.5	22.5
3.	Tween 20 (Cos) / content (%v/v)	--	7.5	7.5	7.5
4.	Surfactant/ cosurfactant ratio	--	3:1	3:1	3:1
5.	Distilled water (AP) / content (%v/v)	--	65	65	65
6.	Drug / conc.(mg/ml/)	3.0	3.0	3.0	3.0
7.	Carbopol P940(MA) /content (%w/v)	--	--	--	0.5
	Characterization				
8.	% Assay	98.97 ± 0.4	--	99.35 ± 0.5	99.19 ± 0.3
9.	Zeta potential(mV)	--	-9.69 ± 3.34	-8.45 ± 5.05	-15.2 ± 3.46
10.	Globule size(nm)	--	12.25 ± 4.9	16.47 ± 5.4	19.79 ± 6.2
11.	Poly dispersity index	--	0.122	0.168	0.181
12.	% Transmittance at 630 nm	--	99.6 ± 0.3	99.2 ± 0.4	--
13.	pH	5.531 ± 0.02	5.253 ± 0.15	5.68 ± 0.23	5.43 ± 0.2
14.	Viscosity at 33°C(cP)	--	7.52 ± 0.61	7.73 ± 0.43	25.8 ± 0.71
15.	Refractive index at 22°C	--	1.380	1.378	--
16.	Diffusion coefficient (mm^2/min)	4.37E-05	--	5.85E-05	1.03E-04
17.	Flux (µg/min)	0.0328	--	0.0439	0.0775

CZS* is mixture of propylene glycol, PEG 200, ethanol & Tween 20 (60%, 20%, 12%, 8% v/v).

CLOPIDOGREL BISULPHATE

The prepared formulations of clopidogrel bisulphate were subjected to *in vitro* diffusion studies through sheep nasal mucosa for 6 hours. The % cumulative drug diffused across nasal mucosa from the formulations were calculated and recorded in Table 5.3. The kinetic pattern of the diffusion was studied by fitting % drug diffused in given time in different order kinetics like zero order, first order and higuchi order. The release kinetics of clopidogrel bisulphate from different formulations was shown in Graph 5.4 - 5.6. Regression coefficients of all formulations in different orders were compared and found that the release pattern of clopidogrel bisulphate from the formulation across the nasal mucosa followed higuchi order rather than zero order and first order. This was concluded by higher regression coefficient value in curve fitting. Diffusion coefficient and flux for all the formulations were calculated and recorded in Table 5.4. Among the CS microemulsions, CSME3 showed higher % drug diffused which was reflected in higher flux and diffusion coefficient value than CSME2. Both carbopol containing (CSMME21 & CSMME31) and chitosan containing (CSMME22 & CSMME32) microemuslions were subjected to invitro diffusion studies and it was observed that carbopol containing microemulsions showed higher % drug release than chitosan containing microemulsions. This may be explained by bioadhesion and absorption enhancement property of carbopol across the mucosal membrane by opening the tight epithelial junctions of the mucosal membranes like nasal membrane (Morimoto K et al 1985) and intestinal membrane (Borchard et al 1996). The CSMME31 was found to have highest flux (0.0669 µg/ min) and diffusion coefficient (6.69E-05 mm^2/ min). The clopidogrel bisulphate formulations which were selected for further studies and their characteristic parameters were shown in Table 5.6.

The promising and stable clobazam, clopidogrel bisulphate formulations listed in Table 5.5 and Table 5.6 and the nasal gel of insulin like growth factor-1 were taken up for further studies.

Table 5.6 Clopidogrel bisulphate formulations

S.No	Parameters	CSS*	CSSME(P)	CSME	CSMME
	Composition				
1.	CapmulGMO (O) / oil content (%v/v)	--	2.5	2.5	2.5
2.	Tween 20 (S) / content (%v/v)	--	30	30	30
3.	PEG200 (Cos) / content (%v/v)	--	10	10	10
4.	Surfactant/ cosurfactant ratio	--	3:1	3:1	3:1
5.	AcetateBuffer(pH5)(AP)/content (%v/v)	--	57.5	57.5	57.5
6.	Drug/ conc.(mg/ml/)	4.0	--	4.0	4.0
7.	Carbopol P940 (MA)/content (%w/v)	--	--	--	0.5
	Characterization				
8.	% Assay	99.35 ±0.4	--	99.12 ± 0.3	99.31±0.5
9.	Zeta potential(mV)	--	- 4.19 ± 6.4	-7.18 ± 5.05	-22.5 ± 4.6
10.	Globule size(nm)	--	12.59 ± 3.5	13.73 ± 5.4	13.49 ± 4.3
11.	Poly dispersity index	--	0.111	0.134	0.125
12.	% Transmittance at 630 nm	--	98.9 ± 1.2	99.91 ± 0.4	--
13.	pH	4.731 ± 0.11	5.017 ± 0.22	5.513 ± 0.14	5.02 ± 0.17
14.	Viscosity at 33°C(cP)	--	7.11 ± 0.45	7.37±0.41	26.7± 0.71
15.	Refractive index at 22°C	--	1.398	1.399	--
16.	Diffusion coefficient (mm^2/min)	2.17E-05	--	4.85E-05	6.69E-05
17.	Flux (µg/min)	0.0217	--	0.0485	0.0669

CSS* is mixture of propylene glycol and distilled water (3:1)

5.6. REFERENCES

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