Chapter 7

In-vivo study

7.1. Introduction:

The in vivo fate of the microemulsion and SLN particles will depend mainly on the administration route, interaction of the microemulsion and SLN with the biological surroundings (adsorption of biological material on the particle surface and desorption of the formulation components in to the biological surroundings, enzymatic processes like lipid degradation by lipases and esterases).' Microemulsions are composed of high and low HLB surfactant along with oil and water phase which are physiologically compatible. SLN are also composed of physiologically compatible lipids (Mehnert,W.,Mader et al., 2001). Therefore, pathways for transportation and metabolism are present in the body. Lipases present in various organs and tissues split the ester linkage and form partial glycerides or glycerol and free fatty acids. The degradation of SLN depends largely upon the chain length of the triglyceride used as a lipid material. Longer the triglyceride chain, slower the degradation rate. The effect of surfactants can accelerate the degradation rate (cholate salts) or hinder it due to steric stabilization (e.g poloxamer 188 and 407). Although *in- vitro* studies give an idea about the release mechanism of drug from the SLN matrices, the results can not be extrapolated to get an idea about the *in-vivo* behavior. Thus, pharmacokinetic behavior of acyclovir and efavirenz in the plasma was studied by administering the SLN dispersions orally to rats. The SLN dispersion for *in-vivo* studies was selected on the basis of *in-vitro* release pattern. Since glyceryl distearate SLN (for Acyclovir, Batch No-Acy-SLN-opt)) and glyceryl tristearate (for efavirenz, Batch no-Efa-SLN-opt) dispersions are the optimum formulation and gave the maximum permeation coefficient and higher flux value compare to others, they were subjected to *in-vivo* testing.

7.2. Calculation of dose

The dose required to be administered for the study was calculated using the formula

$$\text{HED (mg/Kg)} = \text{Animal dose} \times \left\{ \frac{\text{Animal weight}}{\text{Human weight}} \right\}^{0.3}$$

HED	= Human Equivalent Dose
Animal weight	= 0.25 Kg (average)
Human weight	= 60.0 Kg (average)

So, Animal dose is approximately 19mg for both acyclovir and efavirenz for a rat of 250 gm weight.

7.3. In-vivo absorption study:

Absorption studies were performed in male albino rats weighing 280-350g. All experiments and protocols described in present studies were approved by 'The Institutional Animal Ethics Committee' (IACC) of M S University of Baroda and are in accordance with committee for purpose of 'Control and Supervision of Experiments on Animals' (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. The departmental ethical committee approved the animal experiments. The animals were divided into ten groups each group consist of 4 rats. The rats were fasted overnight prior to the experiment but had free access to water. Acyclovir microemulsion (Batch no- ME04 and ME11) and SLN (Acy-SLN-opt) dispersion was administered orally by oral snode in equivalent dose of 19mg/kg of acyclovir to three different group of rats. In similar fashion, efavirenz microemulsion (Batch no: ME13and ME23) and SLN dispersion (Batch no- Ef-SLN-opt) given orally in equivalent dose of 19mg/Kg to another three group of rats. Tablet (OCUVIR tablets) and capsule (EPIVIR capsules) suspension was administered (same dose) separately in the same manner to the seventh and eighth group of rat respectively. Intravenous administration of the acyclovir injection (same dose) was also given to ninth group of rat. Efavirenz solution (5mg/ml) was made in DMSO, ethanol and PEG 400 in water (0.2:0.2:0.2:99.4) and filtered through 0.2µm filter paper and given intravenously (same dose) to tenth group of rats. The blood samples were collected from retro-orbital vein using heparinised needle at 0, 5, 10, 30, 45, 60, 120, 240 minute after intravenous administration and 0, 0.5, 1, 2, 3, 4, 6, 12, 24 hrs after oral administration. The blood samples were collected into heparinised micro centrifuge tube. Then the samples were subjected to centrifugation on laboratory

centrifuge (Sigma, 3K30) at 10,000 rpm for 10 min at 0^0C temperature and supernatant plasma collected into another micro centrifuge tube and kept at -20^0C until analysis.

7.4. HPLC analysis of plasma sample:

7.4.1. Acyclovir:

The concentration of acyclovir was found out by HPLC analysis. The HPLC system consisted of Hewlett-Packard (Aglient) 1100 series components including a quaternary pump, auto sampler and variable wavelength UV detector (Palo Alto, CA, USA). Chromatographic separations were achieved using an Innertsil ODS-3V column (250 x 4.6mm, 5µm) (GL science, Japan). The mobile phase used for the plasma sample was 20mM Ammonium acetate with 5mM Octane sulphonic acid sodium salt in water (pH adjusted to 3 by acetic acid) - methanol (98:2, v/v). Filtration of the buffer was done using 0.2µ nylon 6.6 membrane filters and degassing by sonication.

200µl of the thawed plasma samples mixed with 100µl of water and mix for 1minute using vibromixer (SPINIX, India). To this add 20µl of 35% perchloric acid and mix for 1minute for protein precipitation. Then this mixture was centrifuged at 10,000rpm for 10minute using a Biofuge Pico Micro centrifuge (Heraeus Instruments, Hanau, Germany). After centrifugation, 50µl of supernatant solution was injected into HPLC. The linearity of the method was found suitable in the range of 0.05- 10mcg/ml. (R^2=1)

7.4.2. Efavirenz:

The concentration of efavirenz was found out by HPLC analysis. The HPLC system consisted of Dionex (Dionex Corporation Inc, Germany) components including a quaternary pump and variable wavelength UV detector. Chromatographic separations were achieved using a Hypersil ODS column (250 x 4.6mm, 10µm) (Thermo Electron Corporation). The mobile phase used for the plasma sample was 58% acetonitrile in 0.1% ammonium bicarbonate in water (pH adjusted to 7.4 by 0.1N HCl or 0.1N NaOH).. Filtration of the buffer was done using 0.2µ nylon 6.6 membrane filters and degassing by sonication.

200µl of the thawed plasma samples mixed with 100µl of mobile phase and mix for one minute using vibromixer (SPINIX, India). Then the mixture was basified by the addition

of 20µm of 0.1N NaOH and mixed for one minute. Then the mixture was extracted with 5ml oh methylene chloride. The resultant mixture was centrifuged at 10,000rpm for 10minute at 0^0C temperature. The organic layer was separated and the solvent was evaporated under a stream of nitrogen. The residues were reconstituted in a 4:1 mixture of acetonitrile and 0.1% NH_4HCO_3, pH 7.4. 20µl of the reconstituted solution was injected into HPLC. The linearity of the method was found suitable in the range of 0.05-3µg/ml. (R^2=0.9995)

7.5. Pharmacokinetic data analysis

Plasma concentrations of acyclovir and efavirenz were obtained from rats at each time point was determined to provide mean concentration and standard deviation (SD). Plasma pharmacokinetic parameters were obtained from the pooled concentration- time data of each experiment with statistical moment algorithm using reported statistical equation (Geibaldi et al, 1984).

The concentration at zero time (Co) was calculated using intercept of the graph using log transformed data versus time graph and using the following equation.

Co= Antilog (intercept).

The elimination rate constant was the slope of the graph (log transformed data vs time) multiplied by 2.303

Kel= 2.303 x slope.

The elimination half life ($T_{1/2}$) was calculated using the following formula:

$T_{1/2}$= 0.693/ Kel

The area under the curve (AUC_{0-24}) from 0 to 24 h was calculated using the linear trapezoidal method. The areas of each trapezoid are calculated and sum of all trapezoids gave AUC of total curve upto last determined time concentration. The AUC_{0-8} was calculated by dividing the concentration of 24-h point (C_{24}) by the elimination rate constant (k) as follows:

$AUC_{0-8} = AUC_{0-24} + C_{24}/k$

After oral administration, the elimination rate constant was estimated by linear regression analysis of the log transformed plasma level of the terminal deposition phase. The area under the curve was estimated by the trapezoid rule. From linear regression analysis slope

and intercept were obtained. The concentration at zero time, elimination rate constant and AUC were determined in the similar manner as for intravenous administration.
The absolute bioavailability in 24 h and at infinity at the same dose was calculated as:

Absolute bioavailability = $(AUC_{0-8})_{\text{oral ME or SLN}} / (AUC_{0-8})_{\text{i.v}} \times 100$

The relative bioavailability in 24 h and at infinity at the same dose was calculated as:

Relative bioavailability = $(AUC_{0-8})_{\text{oral, ME or SLN}} / (AUC_{0-8})_{\text{oral, Tab or Cap}}$

7.6. Statistical analysis

The data from different formulations were compared for statistical significance by one-way analysis of variance (ANOVA). Differences were considered to be statistically significant when $P < 0.01$.

7.7. Results:

7.7.1. Acyclovir:

In order to investigate the oral absorption of acyclovir from the microemulsion and SLN, acyclovir microemulsion (ME04 and ME11) and SLN (Batch no: Acy-SLN-opt) was orally administered to fasted rats, and the concentrations of acyclovir in the plasma were determined up to 48 h after oral administration. The acyclovir tablet (OCUVIR) was also administered as control sample. The plasma concentrations versus time profiles are shown in Table 7.1 and Figure7.1 and 7.2. The pharmacokinetic parameters are shown in Table 7.2. It was observed that Cmax of tablet formulation was 0.813mcg/ml achieved after 30miniute where as 1.64 mcg/ml achieved for microemulsion formulation (ME04) after 2hours. In case SLN formulation release rate is slower as compared to the tablet or microemulsion formulation. Cmax of the SLN was achieved 1.25mcg/ml after 4 hours. This may be due to the slow diffusion of acyclovir from the dispersed oil globules to the continuous medium. But the release of drug is more and sustained from microemulsion formulation than from tablets.

Table7.1: Concentration of acyclovir in rat plasma at different time.

Time Point	Concentration (µg/ml)				
	IV injection	Tablet	ME04	ME11	Acy-SLN-opt
10min	33.73 ± 3.45	-	-	-	-
20min	31.43 ± 2.42	-	-	-	-
30 min	27.29 ± 1.98	0.813 ± 0.32	0.62 ± .22	0.92 ±0.24	0.21 ± 0.11
45 min	24.01 ± 2.06	-	-	-	-
1Hr	19.61 ± 3.65	0.494 ± 0.21	0.76 ±0.24	1.76 ± 0.38	0.53 ± 0.24
1 Hr 30 min	10.66 ± 4.35	-	1.09 ±0.42	-	-
2 Hr	8.249 ± 2.45	0.303 ± 0.14	1.64 ±0.38	1.60 ± 0.26	0.86 ± 0.28
3 Hr	3.998 ± 1.22	0.025 ± 0.006	1.44 ±0.26	1.55 ± 0.34	1.02 ± 0.39
4 Hr	1.703 ± 0.42	0.012 ± 0.008	1.11 ±0.34	1.56 ± 0.42	1.25 ± 0.27
6 Hr	-	0	0.89 ±0.32	1.46 ± 0.28	1.06 ± 0.14
12 Hr	-	-	0.68 ±0.28	0.50 ± 0.14	0.89 ± 0.32

Cont…

Time Point	Concentration (µg/ml)				
	IV injection	Tablet	ME04	ME11	Acy-SLN-opt
24 Hr	-	-	0.12 ±0.08	0.37 ± 0.22	0.59 ± 0.23
48Hr	-	-	-	0.108 ± 0.09	0.15 ± 0.11

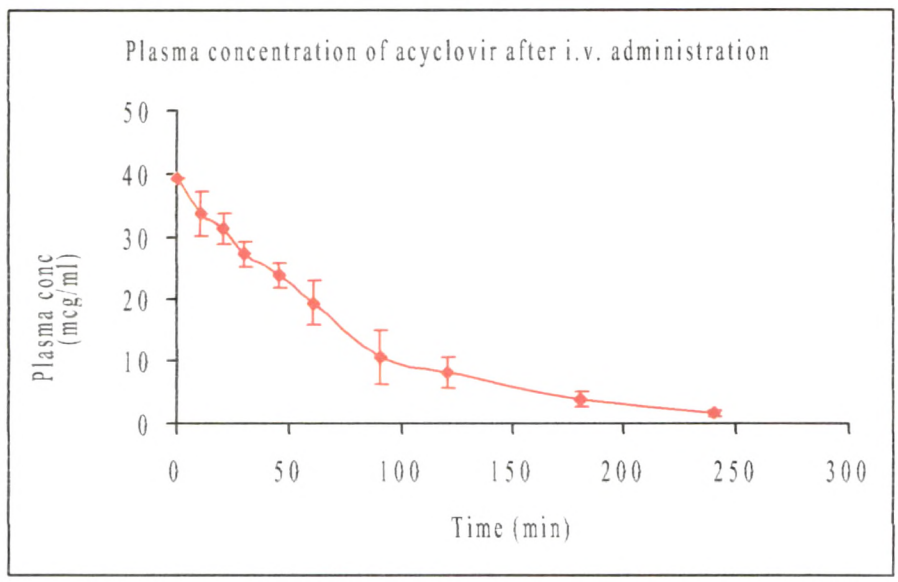

Figure7.1: Plasma concentration of acyclovir after i. v. administration

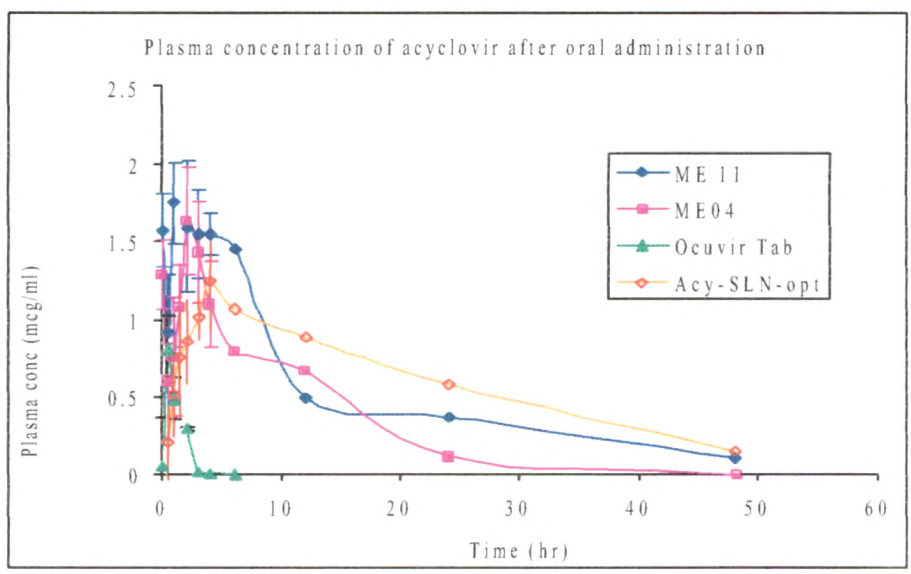

Figure7.2: Plasma concentration of acyclovir after oral administration

Table7.2: Summary of pharmacokinetic data of acyclovir in rats following i.v. and oral administration of 19mg/kg of acyclovir.

Formulation / Parameter	i. v.,	Ocuvir Tablet	ME04	ME11	Acy-SLN-opt
C_0 (µg/ml)	39.436	-	-	-	-
Kel (1/hr)	0.01289	0.00207	0.001352	0.00092	0.0004606
Tmax (hr)	-	0.50	2.00	1.00	4.0
Cmax(µg/ml)	-	0.813	1.64	1.76	1.25
$T_{1/2}$ (hr)	0.8955	5.57	8.35	12.53	25.06
AUC_{0-t} (µg. hr/ml)	2519.62	54.465	980.90	1529.88	1730.56
AUC_{0-8} (µg. hr/ml)	2651.67	60.254	1067.783	1647.12	2047.54
Absolute bioavailability	-	2.27	40.26	62.12	77.21
Relative bioavailability	-	-	17.72	27.34	33.98

7.7.2. Efavirenz:

In order to investigate the oral absorption of efavirenz from the microemulsion and SLN, efavirenz microemulsion (ME13 and ME23) and SLN (Efa-SLN-opt) was orally administered to fasted rats, and the concentrations of acyclovir in the plasma were determined up to 24 h after oral administration. The efavirenz capsules (EPIVIR) was also administered as a control sample. The plasma concentrations versus time profiles are shown in Table7.3 and Figure7.3 and 7.4. The pharmacokinetic parameters are shown in Table7.4. It was observed that Cmax of capsule formulation was 3.212mcg/ml achieved after 60miniute where as 2.64 mcg/ml achieved for microemulsion formulation (ME13) after 2hours. In case SLN formulation release rate is slower as compared to the capsule or microemulsion formulation. Cmax of the SLN achieved was 1.25mcg/ml after 4 hours. This may be due to the slow diffusion of acyclovir from the dispersed oil globules to the continuous medium. But the release of drug is more and sustained from microemulsion formulation than from tablets.

Table7.3: Concentration of efavirenz in rat plasma at different time.

Time Point	**Concentration (µg/ml)**				
	i.v. injection	ME13	ME23	Epivir Cap	Efa-SLN-opt
10min	16.848 ± 4.265	-	-	-	-
20min	13.232 ± 3.642	-	-	-	-
30 min	10.618 ± 2.854	0.725 ± 0.465	0.612 ± 0.286	0.622 ± 0.324	0.212 ± 0.054
45 min	9.506 ± 3.652	-	-	-	-
1Hr	8.932 ± 1.984	1.428 ± 0.528	0.764 ± 0.344	3.212 ± 0.846	0.525 ± 0.116
1 Hr 30 min	6.412 ± 2.126	-	-	-	-
2 Hr	6.256 ± 1.954	2.640 ± 0.482	1.640 ± 0.384	1.425 ± 0.284	0.864 ± 0.186
3 Hr	2.457 ± 0.659	1.556 ± 0.329	2.248 ± 0.486	0.654 ± 0.342	1.024 ± 0.245
4 Hr	1.645 ± 0.856	1.126 ± 0.242	1.785 ± 0.462	0.352 ± 0.164	1.254 ± 0.289
6 Hr	0.446 ± 0.254	0.742 ± 0.326	1.108 ± 0.314	0.098 ± 0.028	0.889 ± 0.462
12 Hr	-	0.545 ± 0.224	0.680 ± 0.244	-	0.478 ± 0.274
24 Hr	-	0.179 ± 0.068	0.212 ± 0.092	-	0.312 ± 0.164
48 Hr	-	-	-	-	0.117 ± 0.086

Table7.4: Summary of pharmacokinetic data of efavirenz in rats following i.v. and oral administration of 19mg/kg of efavirenz.

Formulation / Parameter	i. v.,	ME13	ME23	Epivir Cap	Acy-SLN-opt
C_0 (µg/ml)	16.281	-	-	-	-
Kel (1/hr)	0.0099	0.00138	0.00115	0.01515	0.00069
Tmax (hr)	-	2	3	1	4
Cmax(µg/ml)	-	2.640	2.248	3.212	1.254
$T_{1/2}$ (hr)	1.15	8.35	10.03	1.003	16.71
AUC_{0-t} (µg. hr/ml)	1342.94	964.515	1143.51	288.63	1142.025
AUC_{0-8} (µg. hr/ml)	1387.98	1094.05	1161.92	373.73	1304.13
Absolute bioavailability	-	78.82	83.71	26.92	93.95
Relative bioavailability	-	2.93	3.11	-	3.49

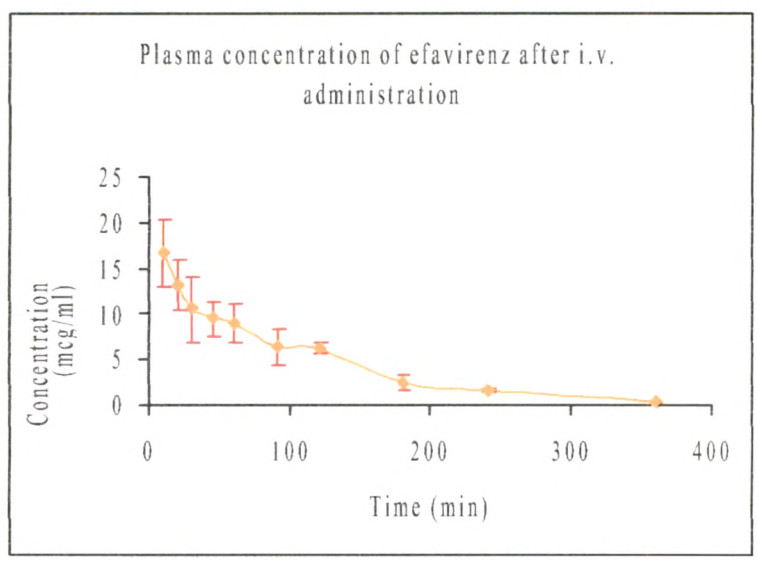

Figure7.3: Plasma concentration of efavirenz after i.v. administration.

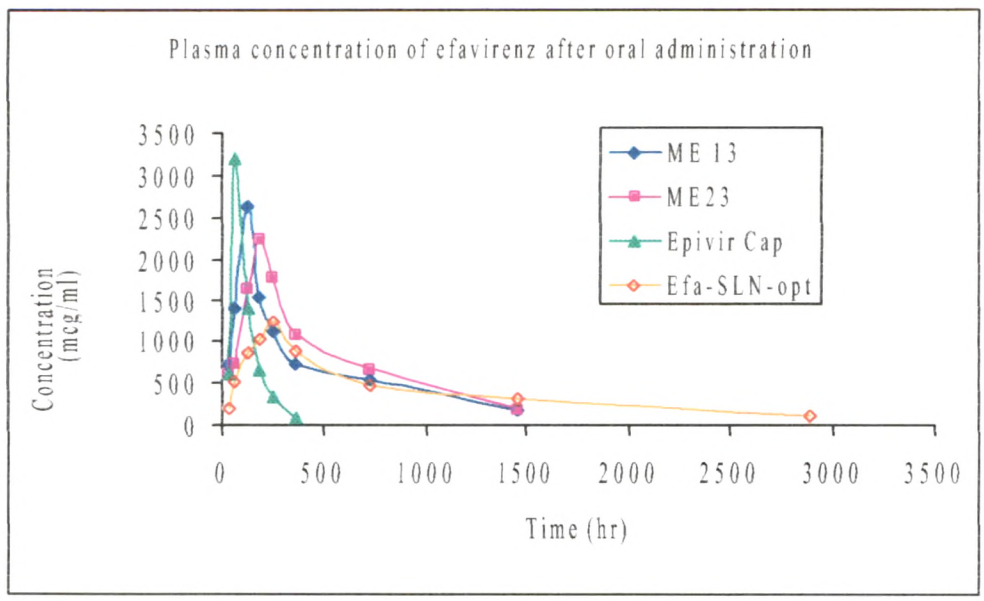

Figure7.4: Plasma concentration of efavirenz after oral administration

Statistically significant difference ($P<0.01$) was found from ANOVA analysis between capsule to other oral formulations.

7.8. Discussion:

Microemulsion and SLN formulations are considered to be a promising formulation technique, because the enhanced absorption of poorly-soluble drug becomes more and more important to overcome problem recently. The reality of this type of formulation was proved by the launch of the immunosuppressant (cyclosporine) microemulsion formulation (Sandimmune, Neoral®). However, much remains unknown about microemulsion formulations. For example, we hardly know what kind of microemulsion formulation is effective. As it was reported that different oral dosage form does not alters the bioavailability of acyclovir (Product literature, Zovirax), the comparison of microemulsion or SLN formulation was made against the commercial tablet (for acyclovir) preparation, not against suspension of acyclovir. For efavirenz only capsule preparation are available in the market. Our in vivo study showed that microemulsion and

SLN formulations exhibited significantly higher absorption compare to the commercially available tablet (for acyclovir) or capsule (for efavirenz),

The enhanced absorption may be explained in terms of: (1) the huge specific surface area of the microemulsion droplets (mean droplet size ~ 40nm), acting advantageously in contact with the gastrointestinal tract, (2) improved permeation of the acyclovir due to presence of surfactant which reduces the interfacial tension nearly to zero and (3) stability of the microemulsion in the gastrointestinal tract.

The elimination rate constant of acyclovir was found 0.01289, 0.00207, 0.001352, 0.00092 and 0.0004606 for i.v, commercial tablet preparation (ocuvir), ME04, ME11 and Acy-SLN-opt respectively. Similarly, elimination rate constant of efavirenz was found as 0.0099, 0.00138, 0.00115, 0.01515 and 0.00069 for intravenous, ME13, ME23, epivir cap and Efa-SLN-opt formulations respectively. The less value for SLN indicates the lesser degradation of acyclovir in the GI region. The Tmax was achieved much faster in case of tablet as compared to the microemulsion or SLN formulations. This may be due to the less solubility of the drug in the aqueous environment as compared to the lipid environment in the microemulsion or SLN formulations. It was also observed that $T_{1/2}$ of SLN or microemulsion formulations was much greater as compared with commercial tablet preparation. The $T_{1/2}$ obtained was 8.35hr, 12.53 hr, 25.06hr and 5.57hr for ME04, ME11, SLN and tablet preparations respectively. This may be due to the protection of the drug from chemical as well as enzymatic degradation in the oil droplets. Statistically significant difference ($P<0.01$) was found from ANOVA analysis between tablet (for acyclovir) or capsules (for efavirenz) to other oral formulations.

The most striking effect was obtained for the tween 80 formulation (ME11). The most important reason seems to be the highly-viscous characteristics of this ME, which may lead to prolonged retention of the formulation in the intestine and the sustained release of drugs. The absorption by labrasol microemulsion was increased (17.4 times) as compared to the commercial tablets. From SLN formulation drug get diffused slowly but in sustained manner. Although Cmax of this is not so high as compared to the microemulsion formulation, but total AUC is much higher than the microemulsion formulation. For Acyclovir it was found as 2047.54(μg. hr/ml) as compared to 1067.78

(μg. hr/ml) (for ME04) and 1647.12(μg. hr/ml) (for ME11). Similarly, for AUC (μg. hr/ml) of efavirenz was found as 1304.13 (from SLN) as compared to 1094.05 and 1161.90 for ME13 and ME23 respectively.

In case of both drugs, $T_{1/2}$ was increases in case of microemulsion or SLN as compared to conventional tablet or capsule preparation. This may be due the delayed absorption of acyclovir or efavirenz at jejunum or ileum region also through lymphatic transport. The similar findings also observed by Shicheng Yang et al (Shicheng Yang et al 2004) for Paclitaxel.

The absolute bioavailability of acyclovir tablet (OCUVIR) was found 2.27% whereas ME04 which was composed of labrasol, plurol olique and labrafac showed a marked increase of bioavailability and that was 40.26%. Surprisingly, Tween 80 based microemulsion (ME11) showed greater bioavailability (62.12%) than labrasol based microemulsion. But the maximum bioavailability was shown in case of SLN (77.21%) although T_{max} of this formulation was quite high (4hr) and C_{max} was quite low (1.2μg/ml). The release of drug was slow and in sustained manner for a longer period of time.

In case of efavirenz also, the bioavailability was quite improved in case of microemulsion as well for SLN. The absolute bioavailability of commercial capsule (EPIVIR) was found 26.92%. Whereas labrasol based microemulsion (ME13) and cremophor based microemulsion (ME23) showed 78.82% and 83.71% bioavailability respectively. But the greater bioavailability was observed from SLN, similar to acyclovir SLN, as compared to commercial capsule or microemulsion formulation. The absolute bioavailability was 93.95% in case of SLN preparation (Efa-SLN-opt).

To design an effective ME formulation, we must know why ME can promote the absorption. One reason is the increase in the interfacial area, which facilitates drug release from the droplets. Being in the equilibrium state may be another reason, because it can offer "soft" carriers. It should also be kept in mind that most surfactants act as absorption enhancers (S. Muranishi et al., 1990) (V.H.L. Lee et al., 1991). Another effect of microemulsion formulations reported is the reduction in the variability. This effect seems to be closely related to the rapid and enhanced absorption. The reduction in the variability also seems to have resulted from the lesser effect by bile salts or foods. Our

investigation showed that the absorption from all microemulsion or SLN formulations received a lesser effect by the fed state than those of the suspension of tablet or capsule. This may be because the microemulsion droplets formed in the small intestine resemble bile salt micelles, which aid the absorption of poorly-soluble drugs. Secretion of the bile salts is affected by the fed state and its variability between individuals is very large. However, if drugs are administered in the form of the microemulsion or SLN formulation, the absorption is hardly affected by the amount of the bile salts. (Kohsaku Kawakami et al., 2002).

The other possible reason to improve the bioavailability is due to the small particle size and positive charge. The effect of the emulsion droplet size on the affinity between the droplets and the intestinal mucosa was investigated by Gershanik et al., who found that the optimal droplet size was in the range of 100–500 nm (T. Gershanik et al., 1998). However, because the lesser effect of the small droplets was explained by the immediate neutralization of positive charges on the droplets by mucin. Our developed microemulsion formulation showing the positive in all cases and SLN dispersion in 0.1N HCl also shows a positive charge.

The other possible reason for increasing the bioavailability, bile salts can decrease duodenal and jejunal brush-border membrane vesicle integrity, increase membrane fluidity and passive proton permeability (D. L. Zhao et al 1990) , which might increase the absorption of acyclovir or efavirenz in the gut. These deductions can be supported by reported evidences showing a synergistic anti-tumor effect of mitomycin C and bile salts against L1210 cells owing to a probable increase in membrane fluidity by bile salts that resulted in an enhanced uptake of mitomycin C by the cells (T. Tamura et al., 1995 Other possible reasons for the enhanced uptake of acyclovir or efavirenz from the GI tract might be the solubilization of the drug in the microemulsion or lipid carrier in SLN (D. S. Chervinsky et al., 1993), (K. Matsuoka et al., 2002) (K. Kawakami et al., 2002) (J. M. Dintaman et al., 1999) and protection of the drug from chemical as well as enzymatic degradation in the oil droplets. The medium chain length fatty acid lipid components of the microemulsion system are also putative absorption enhancing agents and may therefore; increase the permeability of the intestinal wall to the drugs (Lundin et al., 1997; Yamamoto et al., 1997; Lindmark et al., 1998).

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