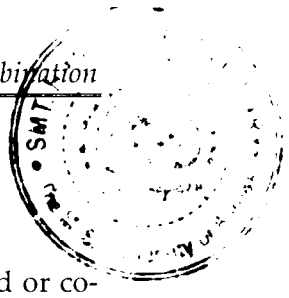
Chapter 9
Development of Combination



9.1 Introduction

Fixed-dosed combination products, with two or more drugs combined or co-formulated in a single dosage form, are becoming popular because of simplified treatment regimens, improved clinical effectiveness, enhanced patient adherence and reduced administrative costs

Available for decades, combination therapy is commonly used in treatment of almost every area of diseases, especially hypertension, HIV/AIDS, tuberculosis, diabetes and pain management, etc. However, given the added complexity of multi-drug regimens, combination therapy is usually hard to follow. Fixed dose combination (FDC) products, i.e. finished products with two or more drugs combined or co-formulated in a single dosage form, have their clinical, commercial and compliance advantages. Clinically, combination products can treat a single disease or target more than one therapeutic area. Ideally, combination products can provide a synergistic effect of individual drugs with reduced side effects. Commercially, combination products lower the cost of care by simplified packaging, fewer dispensing fees and less co-pay. From a compliance point of view, combination products provide a single pill, reducing the number of pills taken on a daily basis and therefore enhancing patient compliance. On the down side, combination products may reduce the range of treatment options available to physicians and patients. Hopefully, in most cases, the reduced dose regimen could be compensated by the designed synergistic effect and reduced side effects in the combination products.

The development of FDCs is becoming increasingly high either to improve compliance or to benefit from the added effects of the two or more active drugs given together. They are being used in the treatment of a wide range of

conditions and are particularly useful in the management of chronic conditions. FDCs should always be based on convincing therapeutic justification. Each fixed dose combination should be carefully justified and clinically relevant (e.g. in cases when each component of the FDC has several possible dosages, dosages that have shown benefit on clinical outcomes may be preferable).

Tacrolimus and corticosteroids have been investigated for utilization as combination because of their non overlapping mechanisms of action. The combination has been shown to be more effective than monotherapy. Further, reports have shown of them being chemically and physically compatible (Hebert et al, 2006, Levitt 2003)

9.2 Methods

9.2.1 Physical and Chemical Compatibility Testing

The two drugs HP and Tac are mixed in a fixed ration of 1:3 and filled in class I vials and sealed. The sealed vials are evaluated for physical appearance, by DSC and by HPLC on day 0. They are kept under stress conditions at 50°C for 1 month and evaluated again for physical appearance, by DSC and by HPLC.

The DSC of samples was carried out by scanning the samples using differential scanning colorimeter (Mettler 2.0). Thermograms were analyzed using Mettler Toledo star SW 7.01. An empty aluminium pan was used as the reference for all measurements. During each scan, 2 to 3 mg of sample was heated, in a hermetically sealed aluminium pan, at a heating rate of 10° C/min, from 0° C to 220° C.

Any chemical interaction, degradation and presence of additional impurities was evaluated by HPLC. The drug mixture was dissolved in methanol and diluted suitably. The diluted samples were analyzed according to the conditions specified in table 9.1. The chromatograms were analyzed for changes in peak areas, retention time and presence of additional peaks. The data is recorded in table 9.3.

9.2.2 Preparation and Characterization of Combination Microemulsion Cream

The drug loaded microemulsions for HP and Tac were prepared according to the optimized formula described in section 4.4. Cetomacrogol cream was prepared as described in section 4.3.4. The drug loaded microemulsion were incorporated in cetomacrgol cream base as described in section 4.3.4 to give a final concentration of 0.035% HP and 0.1% Tac in the cream. The cream was characterized for physical appearance, pH, viscosity and assay for both the drugs as described in section 4.3.5.

9.2.3 Drug Diffusion across Artificial Membrane

The in vitro diffusion study was carried out for the developed fixed dose combination cream in a manner as described in section 5.2.1.

9.2.4 Ex-vivo Drug diffusion and skin retention study

Ex-vivo drug diffusion and skin retention study was carried out for the developed fixed dose combination cream in rodent and human cadaver skin in a manner as described in section 5.2.2

9.2.5 Pharmacodynamic Study in Murine Model

BALB/c mice were sensitized and elicited with hapten TNCB as described in section 7.2.2 and treatment groups included sequential treatment with HP

cream 0.05% (marketed preparation) and Tacrolimus ointment 0.1% (marketed preparation and treatment with HP 0.035% + Tac 0.1% ME based Combination cream b.i.d. (See section 7.2.3).

Dermatitis Score

The skin symptoms were evaluated on day 0, 7, 14, 21, 28 and 35 after TNCB challenge and drug treatment as described in section 7.2.4.

Ear Swelling Studies

Ear thickness was measured with a dial thickness gauge on days 0, 7, 14, 21, 28 and 35 at 3 and 24h post application as described in section 7.2.5.

Measurement of serum IgE

Blood was collected by cardiac puncture under ether anesthesia at 24 h after each challenge on days 0, 7, 14, 21, 28 and 35 and serum IgE levels quantified as described in section 7.2.6.

Histopathology

Excised skin specimens from hapten elicited/ drug treated ear of mice were fixed, sections were prepared and stained with hematoxylin and eosin as described in section 7.2.7.

Cytokine gene expression by RT-PCR analysis

The expression of Th1 (IL-2, IFN- g) and Th2 (IL-4 and Il-10) cytokine mRNAs were semi quantitatively evaluated by means of reverse transcriptase polymerase chain reaction (RT-PCR) using β- actin as an internal reference as described in section 7.2.8.

9.3 Results

Analytical Method

Table 9.1: Experimental conditions for simultaneous evaluation of HP and Tac

	Halobetasol Propionate	**Tacrolimus**
Column	Phenomenex C18 (250 × 4.9mm, 5μ)	Phenomenex C18 (250 × 4.9mm, 5μ)
Mobile phase	Acetonitrile: Water (55:45)	Acetonitrile: Water (55:45)
Flow rate	1 ml/min	1 ml/min
UV detection	at 239 nm	at 210 nm
Injection Volume	20 μL	20 μL
Retention time	~17.3 min	~ 13.2 min

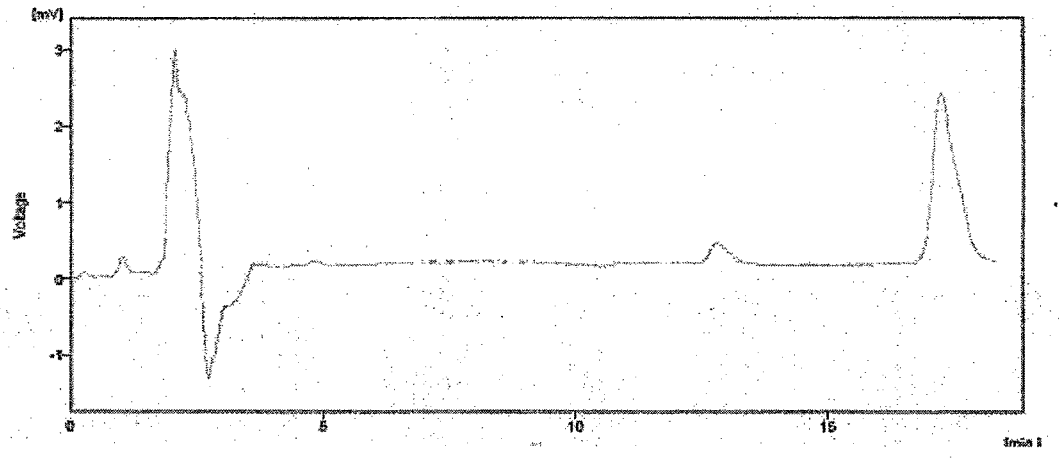


Fig. 9.1: Chromatogram of HP at 239 nm

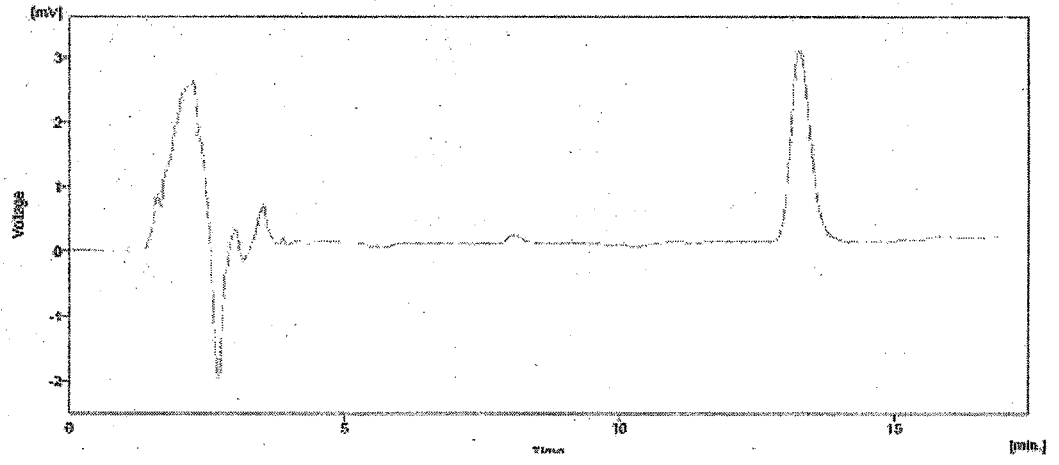


Fig. 9.2: Chromatogram of Tac at 210 nm

Compatibility Study

By HPLC

Table 9.2: Compatibility between HP and Tac in accelerated studies as evaluated by physical appearance and HPLC

Sample details	Physical Appearnce	UV wavelength	RT (min)	Area (mV)	% Recovery	Theoretical Plates	Any other peak
HP + Tac Mix (0 day)	White smooth powder	239 (HP)	17.3	357.75	95.1	6253	Small additional Peak at 13 min RT ~ tac
HP + Tac Mix (1M)	White smooth powder	239 (HP)	17.3	361.2	95.8	6158	Small additional Peak at 13 min RT ~ tac
HP + Tac Mix (0 day)	White smooth powder	210 (Tac)	13.2	323	102.7	6200	-
HP + Tac Mix (1 M)	White smooth powder	210 (Tac)	13.3	315.5	98.9	7673	-

DSC thermograms

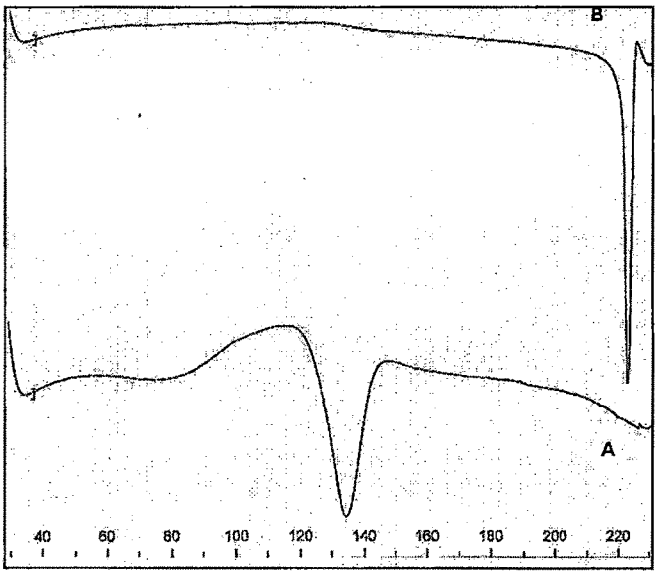


Fig. 9.3: DSC thermograms a) Tacrolimus b) Halobetasol propionate

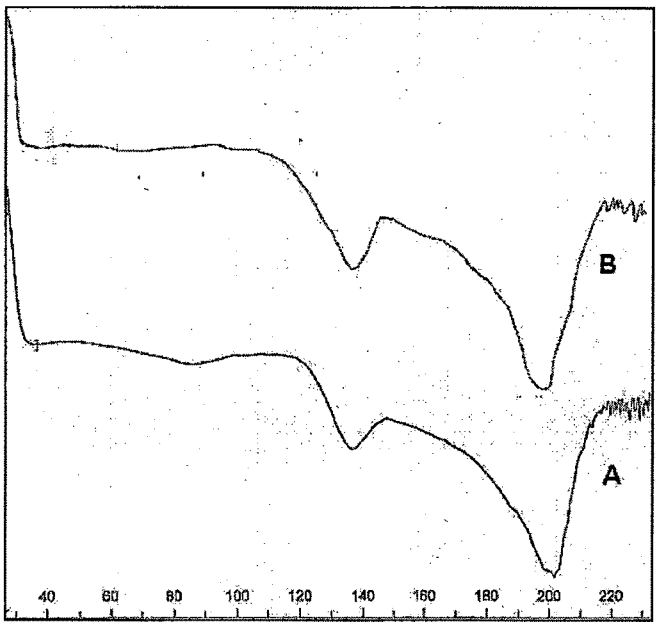


Fig. 9.4: DSC thermograms

a) Tac + HP mix at time 0. b) Tac + HP mix at time :1 month

Table 9.3: Characterization of HP (0.035%) + Tac (0.1%) ME based combination cream

Test	HP (0.035%) + Tac (0.1%) ME based combination cream
Appearance	White smooth textured
pH at 25°C	5.35
Assay (%) for HP	95.1 ± 1.35
Assay (%) For Tac	102.7 ± 2.68
Viscosity at 25°C (KcP)	36.97 ± 1.93

Table 9.4: *In vitro* diffusion study of HP, Tac and Combination formulations

Time (h)	% Drug Diffused (w/v) ± SD*			
	HPMEC (0.035%)	Tac MEC 0.1%	Combination ME Cream (HP 0.035%)	Combination ME Cream (Tac 0.1%)
0.00	0.00	0.00	0.00	0.00
0.25	5.00 ± 1.0	3.67 ± 1.53	3.00 ± 1.00	3.33 ± 1.15
0.50	10.33 ± 4.93	8.00 ± 2.00	8.67 ± 3.52	9.00 ± 1.0
1.00	14.00 ± 2.65	15.00 ± 2.00	12.33 ± 2.51	14.0 ± 3.6
2.00	24.67 ± 5.13	24.66 ± 4.04	22.67 ± 2.08	27.33 ± 3.2
4.00	38.67 ± 3.51	40.66 ± 5.13	50.33 ± 5.50	43.33 ± 10.01
6.00	68.66 ± 8.08	68.66 ± 12.85	78.34 ± 3.05	71.34 ± 11.93
8.00	82.00 ± 6.24	82.00 ± 6.24	86.67 ± 3.21	84.34 ± 5.03
24.00	88.34 ± 1.15	86.66 ± 2.51	88.33 ± 0.06	88.4 ± 3.05

*n=3

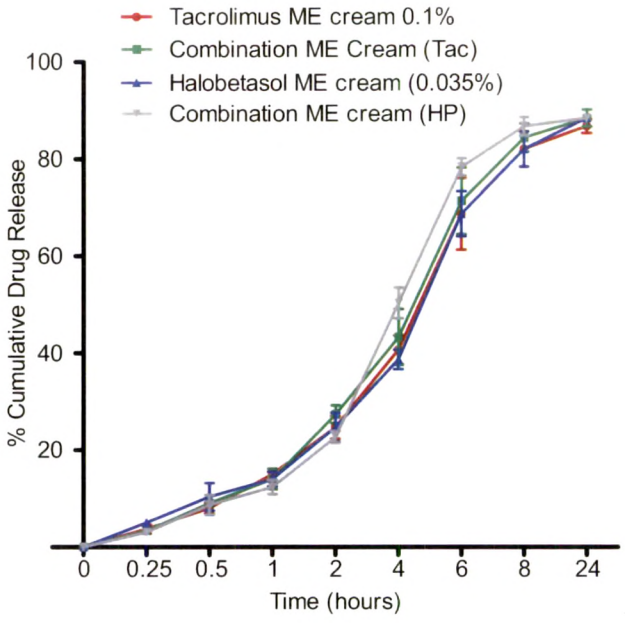


Fig.9.5: Comparative cumulative drug release through semi permeable cellulose acetate membrane. Values are mean ± SD for n=3 experiments. Release profiles were statistically compared using Two Way ANOVA, Bonferroni post test with $p<0.05$ considered significant.

Table 9.5: Skin retention data of HP, Tac and Combination formulation through rodent skin and human cadaver skin

Formulation	**Rodent skin**	**Human cadaver skin**
	Quantity (mcg) retained ± SD*	
HPMEC (0.035%)	20.33 ± 4.04	13.33 ± 4.16
Tac MEC 0.1%	13.30 ± 3.51	8.90 ± 2.74
Combination ME Cream (HP 0.035%)	22.33 ± 2.91	18.00 ± 1.53
Combination ME Cream (Tac 0.1%)	14.67 ± 2.03	10.33 ± 1.20

*n=3

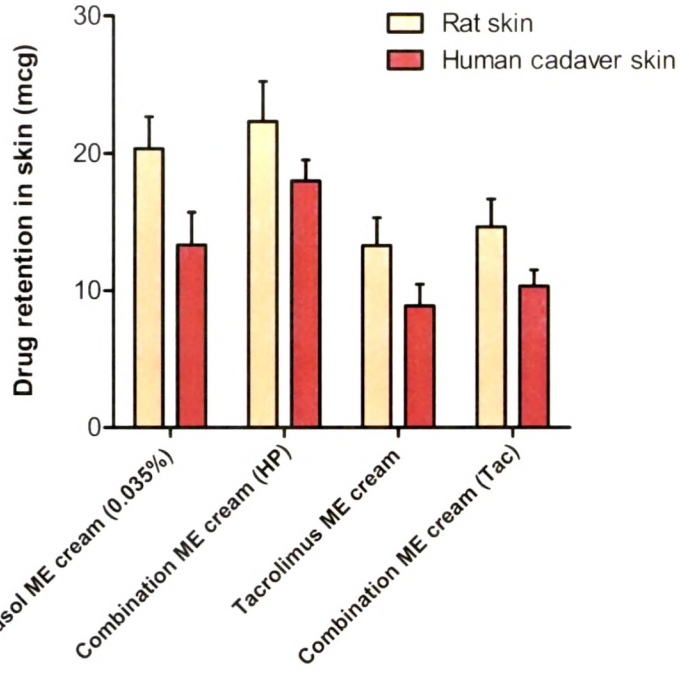


Fig. 9.6: Comparative drug retention in rat skin and human cadaver skin following 24 h application. The columns and the error bars represent means ± SEM for n=3. Results were statistically compared using Two Way ANOVA, bonferroni's post test $p<0.05$ considered as significant.

Table 9.6: Comparative dermatitis score in mice during the course of study after elicitation with inducing agent (TNCB) and treatment with different formulations

Day	Elicitation with TNCB	HPMEC (0.035%)	Tac MEC 0.1%	Combination ME cream (HP 0.035% + Tac 0.1%)	Sequential treatment HP 0.05% and Tac 0.1%
0	0.00				
7	0.33 ± 0.33				
10	2.33 ± 0.66				
14	3.00 ± 0.57				
21	8.00 ± 0.57				
28	8.33 ± 0.88	4.00 ± 0.57	4.00 ± 0.57	3.33 ± 0.88	3.67 ± 0.33
35	7.66 ± 0.88	2.00 ± 0.57	2.33 ± 0.33	2.33 ± 0.88	2.00 ± 0.57

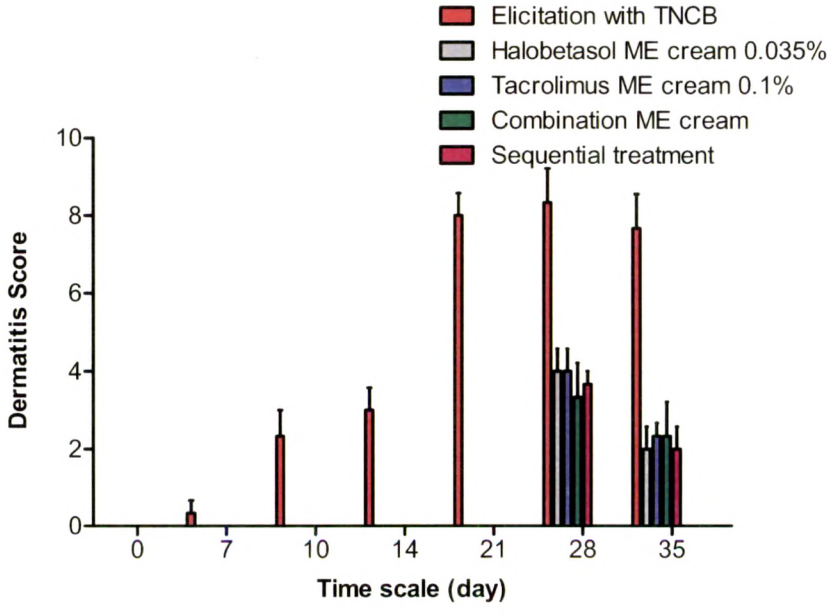


Fig. 9.7: Comparative dermatitis score in mice during the course of study after elicitation with inducing agent (TNCB) and treatment with different formulations. The columns and the error bars represent means ± SEM for n=3 animals. * ($p<0.05$) significant difference in comparison elicitation with TNCB.

Table 9.7: Comparative serum total IgE levels during the course of study after sensitization and elicitation with hapten (TNCB) and treatment with different formulations

Day	Elicitation with TNCB	HPMEC (0.035%)	Tac MEC 0.1%	Combination ME cream (HP 0.035% + Tac 0.1%)	Sequential treatment HP 0.05% and Tac 0.1%
0	243 ± 59				
7	1048 ± 200				
10	3658 ± 947				
14	5961 ± 1109				
21	5283 ± 1187				
28	4692 ± 1335	4886 ± 414	4553 ± 504	5516 ± 298	4719 ± 351
35	4765 ± 891	4925 ± 985	4592 ± 874	4587 ± 299	4632 ± 367

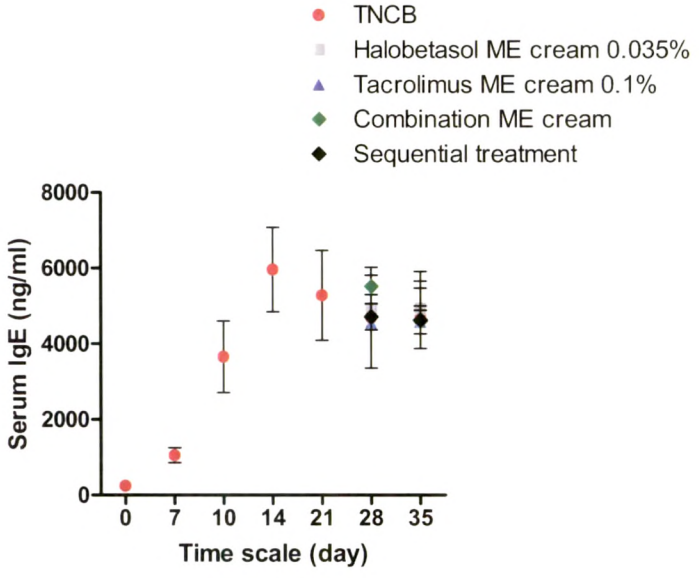


Fig 9.8: Comparative serum total IgE levels during the course of study after sensitization and elicitation with hapten (TNCB) and treatment with different formulations. Values are mean ± SD for n=3 animals. Results were statistically compared using Two Way ANOVA, Bonferroni post test with $p<0.05$.

Table 9.8: Ear swelling response in mice recorded 3 h post application of formulations

Day	Elicitation with TNCB	HPMEC (0.035%)	Tac MEC 0.1%	Combination ME cream (HP 0.035% + Tac 0.1%)	Sequential treatment HP 0.05% and Tac 0.1%
0	3.00 ± 0.57				
7	8.67 ± 1.20				
10	11.33 ± 1.76				
14	15.33 ± 0.88				
21	17.67 ± 1.46	8.00 ± 2.51	9.67 ± 1.67	7.33 ± 2.02	8.67 ± 2.33
28	17.67 ± 1.76	5.66 ± 1.20	7.67 ± 0.88	4.00 ± 0.57	4.33 ± 0.88
35	17.66 ± 3.48	3.67 ± 0.66	4.00 ± 0.57	3.00 ± 0.57	3.67 ± 0.33

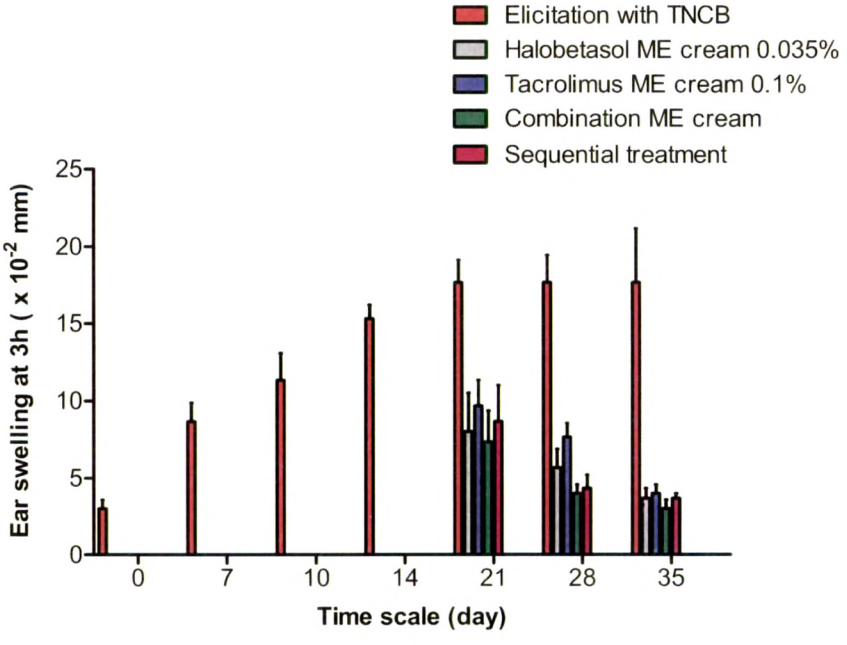


Fig. 9.9: Ear swelling response in mice recorded 3 h post application of formulations. (The difference in ear thickness is plotted and the columns and error bars represent means ± SEM for n=3 animals). * ($p<0.05$) significant difference in comparison elicitation with TNCB.

Table 9.9: Ear swelling response in mice recorded 24 h post application of formulations

Day	Elicitation with TNCB	HPMEC (0.035%)	Tac MEC 0.1%	Combination ME cream (HP 0.035% + Tac 0.1%)	Sequential treatment HP 0.05% and Tac 0.1%
0	2.33 ± 0.88				
7	7.33 ± 2.40				
10	9.00 ± 1.15				
14	12.67 ± 1.85				
21	11.00 ± 2.08	3.33 ± 0.33	4.66 ± 0.88	4.00 ± 0.57	4.33 ± 0.33
28	12.00 ± 1.52	2.33 ± 0.33	5.33 ± 0.88	2.00 ± 0.57	1.67 ± 0.33
35	11.00 ± 2.51	1.33 ± 0.33	1.99 ± 0.33	1.33 ± 0.33	1.33 ± 0.33

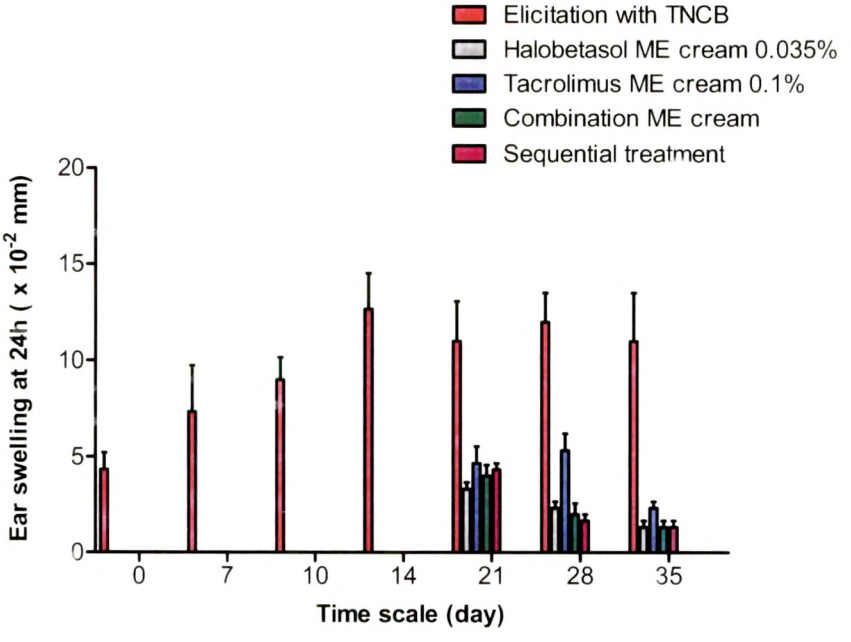
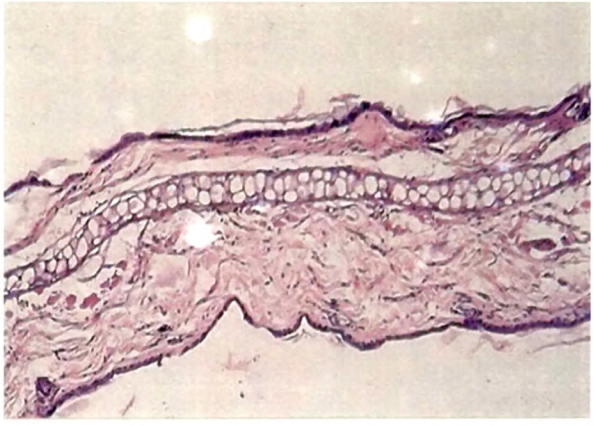
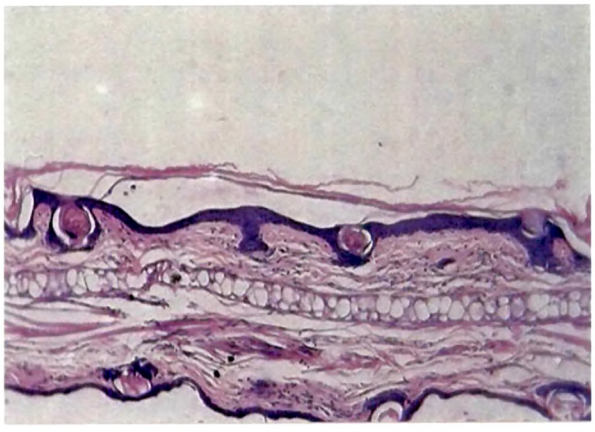


Fig. 9.10: Ear swelling response in mice recorded 24 h post application of formulations. (The difference in ear thickness is plotted and the columns and error bars represent means ± SEM for n=3 animals). * ($p<0.05$) significant difference in comparison elicitation with TNCB.

Fig 9.11:



(a)



(b)

Fig. 9.11(a-b): Histopathology of ear skin lesions caused by repeated application of TNCB and after treatment with formulations in BALB/C mice. The skin tissues were stained with hematoxylin and eosin and magnification is 10X.

a) After Combination ME cream treatment on day 35

b) After sequential treatment on day 35

Table 9.10: Cytokine gene expression levels by RT –PCR at the end of study on day 35. The expression level is expressed as a ratio to internal reference beta-actin expression

Gene	Basal	Elicitation with TNCB	HPMEC (0.035%)	TAC MEC 0.1%	Combination ME cream (HP 0.035% + Tac 0.1%)	Sequential treatment HP 0.05% and Tac 0.1%
IL2	0.063 ± 0.03	0.33 ± 0.02	0.027 ± 0.027	0.143 ± 0.037	0.020 ± 0.015	0.018 ± 0.02
IL4	0.000	0.678 ± 0.21	0.000	0.293 ± 0.120	0.000	0.000
IL10	0.113 ± 0.03	0.264 ± 0.02	0.293 ± 0.094	0.167 ± 0.045	0.345 ± 0.167	0.402 ± 0.278
IFN g	0.000	0.065 ± 0.03	0.000	0.000	0.000	0.000

Table 9.11: Stability Study of Combination ME Cream

System	Period (month)	Room temperature					2-8° C				
		Appearance	pH	Assay (%)		Viscosity (KcP)	Appearance	pH	Assay (%)		Viscosity (KcP)
				HP	Tac				HP	Tac	
Combination ME Cream (HP 0.035% + TAC 0.1%)	0	White smooth texture	5.35	95.1 ± 1.35	102.7 ± 2.68	36.97 ± 1.93	White smooth texture	5.35	95.1 ± 1.35	102.7 ± 2.68	36.97 ± 1.93
	1	Same	5.3	95.5 ± 2.4	100.3 ± 4.1	35.46 ± 1.25	Same	5.4	96.1 ± 3.5	101.3 ± 4.0	37.65 ± 3.8
	2	Same	5.3	94.35 ± 1.6	96.8 ± 2.3	34.89 ± 1.4	Same	5.2	94.3 ± 2.2	95.8 ± 3.3	34.68 ± 2.9
	3	Same	5.4	93.56 ± 2.7	95.4 ± 3.6	35.69 ± 2.6	Same	5.3	94.1 ± 3.9	97.5 ± 2.1	35.8 ± 1.5

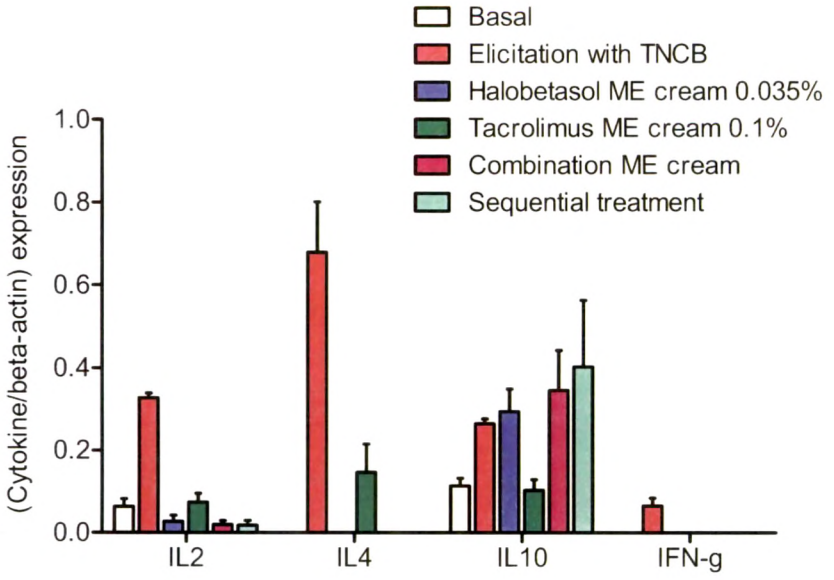


Fig. 9.12: Cytokine gene expression levels by RT –PCR at the end of study on day 35. The expression level is expressed as a ratio to internal reference beta-actin expression and the columns and error bars represent mean ± SEM for n=3 animals

9.4 Discussion

An attempt was made to investigate a combination cream of HP and Tac. Although, several researchers have advocated the use of combination in treatment of dermatitis, such a combination is not available commercially (Nakahara et al, 2004, Torok et al, 2003, Tanojo et al, 2003). In fact, no chemical incompatibility between corticosteroids and tacrolimus has been reported (Pappas et al, 2009). Clinically a combination in single formulation is not used but often sequential treatment with these two drugs is employed where tacrolimus is applied at night and corticosteroid in the day time.

An accelerated drug – drug compatibility study was carried out to explore any potential chemical or physical incompatibility between the two drugs. The drugs were mixed in 1:3 ratio and kept at 50°C for 1 month and evaluated for physical appearance, by DSC and by HPLC. It was found that mixture does not exhibit any change in color or other physical attributes. The DSC thermograms (Fig. 9.3 and 9.4) show the peaks of pure drugs and combination initially and after 1 month of stress study. The tacrolimus peak does not shift much when in combination however, the peak of HP shifts around 10 °C. This can be justified since, in presence of a melted component (in this case Tac), the endothermic peak of melting of the other component may shift to a lower temperature. Further, the shift of HP peak towards a lower temperature remains same after 1 month stress study. Hence, this peak shifting is because of combination of two products and does not indicate any degradative change. Chemical compatibility was further confirmed by HPLC where no extra peaks or significant change in area of peaks were observed. Hence it can be concluded from the study that HP and Tac are physically and chemically compatible with each other.

A fixed dose combination ME based cream of HP and Tac was developed wherein the concentration of HP is 0.035% and Tac is 0.1%. The lower dose of HP was found to be effective in earlier studies. Hence, 0.035% of HP was investigated for combination.

The analytical method was developed based on previous studies using acetonitrile and water system. Tac and HP both were found to give a peak in the same solvent system and was found to be linear also. But, the wavelength for detection was not the same. Tac absorbed at 210 and HP at 239 nm. Hence, whenever a combination cream was evaluated, two injections were carried out and detections were made at 210 and 239 nm for Tac and HP respectively.

The MEs for both the drugs were prepared according to optimized formulae and procedure given in chapter 4. The drug loaded microemulsions were incorporated into cetomacrogol cream base by replacing an equivalent quantity of aqueous phase to give a final concentration of 0.035% of HP and 0.1% of Tac.

The fixed dose combination ME based cream was characterized and details are recorded in table 9.3. A stability study on the developed combination cream was also done. It was found that the combination was stable for at least 3 months with no loss in potency as indicated by the assay.

An *in-vitro* drug release study through semi-permeable cellulose acetate membrane was carried out and the release of both the drugs was compared to their individual ME based creams. The drug release follows a very similar pattern and shows no significant change for any of the drugs (Table 9.4).

Ex-vivo drug diffusion and skin retention studies were also done through rat skin and human cadaver skin. It was found that the both the drugs exhibited slightly higher drug deposition in skin in comparison to individual cream (Table 9.5). Although the increase was not statistically significant, but such an increase in drug permeation when corticosteroid and tacrolimus are used in combination has been reported (Tanojo et al, 2003).

Pharmacological evaluation of the combination was carried out in earlier used hapten induced mice model of dermatitis. It was found that there is no statistical significant difference in the dermatitis score (Table 9.6 and fig. 9.7) between the treatment modalities. The individual ME creams, combination ME cream and sequential treatment showed almost similar therapeutic response and the test failed to reveal any distinguishing difference.

Serum IgE levels are reported to be elevated with the use of both drugs, tacrolimus as well as corticosteroid. The combination treatment showed an elevated serum IgE levels in comparison to other treatment modalities (fig.9.8).

When ear swelling response was characterized at 3 h post application it was found that a more pronounced decrease in ear swelling was observed on the first day of therapy with combination cream than individual ME cream (Table 9.8). The possible reason could be the non-overlapping mechanisms of actions of the two drugs which helped in better control of ear swelling. The difference between treatment modalities becomes almost nil with 2 weeks of treatment.

Ear swelling was also measured at 24h post application to judge late phase reaction. It was observed that the treatment modalities with corticosteroid had a better control of ear swelling in the late phase (Table 9.9). It was

observed that tacrolimus alone could control the ear swelling effectively only after 2 weeks of therapy. However, there is no significant difference between combination ME cream and sequential treatment.

Histopathology reflects that treatment with a combination may limit the atrophic effects of corticosteroid which are more prominent with sequential therapy.

Cytokine gene expression was evaluated by RT-PCR technique. It was observed that the corticosteroid containing formulations were more effective in suppressing inflammatory cytokine like IL-4. The anti-inflammatory cytokine IL-10 was elevated with combination ME cream and after sequential treatment also. This may have been because of non-overlapping mechanism of regulation of gene expression by corticosteroids and tacrolimus. IL-2 expression is also suppressed with combination and sequential treatment and levels go below the basal levels after 2 weeks of treatment. IFN-g shows an elevation after elicitation with hapten but remains undetectable in all other treatment groups (Table 9.10).

The developed combination microemulsion based cream did not demonstrate any significant benefit over sequential treatment. But, a combination may prove to be better choice of treatment for immediate control of severe cases of AD. The advantage would be reflected in better control of side effects of the individual drug. The burning sensations and pruritus associated with tacrolimus would be effectively controlled by corticosteroid and tacrolimus does not show atrophic effects like corticosteroids. The developed formulations are likely to alleviate drawbacks of current management of disease and provide a patient friendly aqueous based dosage form of drugs.

9.5 References

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