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Nanoemulsion Formulation of Acyclovir: Assessing Dermal Safety Through Irritation and Sensitization Tests

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Abstract

The field of skin sensitization and dermal toxicity is evolving to accurately predict dermal responses in humans for topically applied pharmaceutical formulations. Nanoemulsions are nano globular pharmaceutical systems having globules size in nanometre and hence they have high potential to increase the permeation of drugs through the skin. The objective of this study is to understand how a nanoparticulate matrix of a pharmaceutical formulation effects skin sensitization and dermal toxicity when compared to conventional macroemulsion formulation. Interpretation of dermal toxicity and skin sensitization studies on a nanoemulsion of Acyclovir was performed and compared with macroemulsions and conventional marketed formulation. Nanoemulsion was developed through cold emulsification process using inline homogenization technique. In this study it was found that nanoemulsion did not induce any sensitization reactions while comparing the same with existing marketed formulations and graded as weak in sensitization score and rate. Acute dermal toxicity test in Guinea pigs did not show any overt signs of toxicity following a 15-day period time. Basis this study, the finding suggests that the nanoemulsion gel does not cause any skin irritation, skin sensitization or dermal toxic effects following dermal applications.

Keywords: Nanoemulsion, dermal irritation, acute dermal toxicity, skin sensitization

Introduction

This has been established through lot of studies that nanoemulsions can increase the diffusion profile of a topical dosage form and hence increase the availability of drug at site of application. Nanoemulsions comprise of nanoparticulate systems or lipid systems which includes

emulsions, microemulsion, and liposomes. However, all of these processes have shown significant increase in diffusion and drug targeting but at the same time these products have shown cases of toxicity or irritation at the site of application (Souto EB et al., 2022). This has also been established that surface charge and mean particle size increases the adhesion of nanoemulsion to the cell membrane and their internalization to release the drug inside cells. These interactions interfere with cell metabolism and lead to irritation and toxicity (Silva A.M etal., 2019).

Surfactant system in nanoemulsion and microemulsion also plays a major contribution in imparting toxicity and irritation potential, surfactants must be chosen wisely amongst anionic or cationic emulsifiers. The physicochemical properties of surfactants are a crucial factor in eliciting skin irritation. Anionic surfactants are broadly accepted as potent irritants to human and animal skin. Cationic surfactants are more cytotoxic than anionic (Effendy I et al., 1995). Few studies also suggested that skin irritation is not simply related to the total concentration of surfactants in emulsions and nanoemulsion, but rather to the combination of surfactants present in the emulsion (Dillarstone A et al., 1993). Authors have also demonstrated that a combination of sodium lauryl glutamate (SLG), a mild surfactant, with Sodium lauryl sulphate (SLS) induced less skin irritation than SLS, alone as assessed by visual scoring and an evaporimeter (Lee H L et al., 1994).

In the current study, nanoemulsion of Acyclovir was prepared through cold emulsification process using non ionic emulsifiers, the prepared nanoemulsion has shown particle size, zeta potential and Polydispersibility index as per criteria of nanoemulsion, further the formation of nanoemulsion has been established through transmission electron microscopy (TEM). (Bhatt et al 2024) Nanoemulsion has also shown increase in diffusion profile significantly when compared with innovator product Zovirax. To establish the non-invasive characteristics of nanoemulsion skin sensitization and acute dermal toxicity studies were conducted and compared with conventional emulsion product available in market (Zovirax). Acute dermal toxicity and skin sensitization studies were conducted on Wistar rats and guinea pigs respectively.

Acute dermal toxicity data provides insight of the adverse effects occurring within a short time of dermal application of a single dose of a substance or multiple doses given within a 24–hour period. Acute dermal toxicity studies help in establishing the safety and toxicity of the test agent Organization for Economic Cooperation and Development (OECD) guidelines. (OECD. (2017))

Skin sensitization testing identifies the potential for a substance to cause allergic contact dermatitis. These studies are also used to determine levels of sensitizers that will not produce allergic sensitization. (Strickland et al., 2019) , (Han., 2012)

Materials and Methods

The nanoemulsion of Acyclovir was developed at R&D centre of Piramal Pharma Ltd, Mumbai. The animal studies were performed at the department of Institute for Industrial Research and Toxicology (registration no. 1303/PO/Rc/09/CPCSEA) at Ghaziabad, UP. The institutional animal ethics Committee (IAEC) of Delhi approved the trial protocols having Approval No: IIRT/IAEC/30/2024/b/030 for skin sensitization and IIRT/IAEC/30/2024/b/031 for acute dermal toxicity of nanoemulsion PB020 and Innovator product Zovirax.

18 Healthy, adult Albino rats (Wistar) (weighing 200±20g, 8-12 weeks of age, Female-Nulliparous and non-pregnant) and 66, healthy adult guinea pigs (Cavia porcellus) (weighing 300-400 g, 13-15 weeks of age, male were obtained from Animal House, Institute for Industrial Research and Toxicology. IAEC SOPs and CPCSEA regulations were followed for all animals.

Chemicals

Active ingredient acyclovir was provided as gift sample by Strides pharma, Bengaluru, India. Other excipients, Sepineo P 600 and Tween 20 (Seppic Inc, USA) .Glycerin (Adani Wilmar, Mumbai), light liquid paraffin (APAR industries, Mumbai), propylene glycol (Shandong Shida Chemical group Co. Ltd, China), butyl hydroxy toluene (Camlin fine sciences, Mumbai) hydrogenated castor oil (Nature Tech ingredients Mumbai) Benzyl alcohol (DCM Shriram industries Ltd, Mumbai) were used from approved and reliable vendors of Piramal Pharma Ltd.

Development and Characterization of nanoemulsion

Nanoemulsions of acyclovir was formulated by cold emulsification process to control the impurity profiling of the finished product and enhance the absorption rate. The optimized acyclovir nanoemulsion formulations were characterized and validated for globule size, zeta potential, morphology, and in vitro absorption profile and was compared to the innovator product. Selection of surfactant and cosurfactant was done and process was optimized to get a uniform nanoemulsion. Comparison of nanoemulsion was done with innovator product for all the physicochemical parameters and it was concluded that innovator product Zovirax was a conventional emulsion and optimized batch PB020 was a nanoemulsion.

Table 1- Comparison of physicochemical properties of optimized nanoemulsion and innovator product

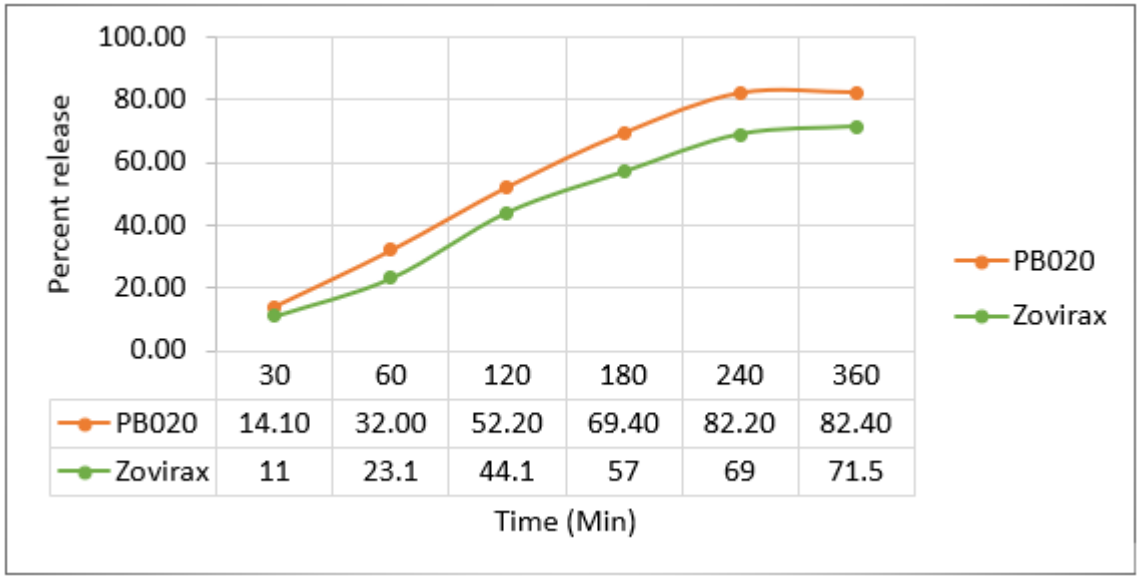
Each value is a mean of three determination ± SD (n= 3)

The physicochemical analysis revealed that batch PB020 is a very stable nanoemulsion basis

Formulation	*Assay (%)*	*Impurity of Acyclovir - Guanine (%)*	*Zeta Potential (mV)*	*Droplet size (nm)*	*Polydispersibility index (PDI)*	*pH*
Zovirax	97.20 ± 0.30	0.14± 0.02	-5.36 ± 32.50	8630.00 ± 2000.00	0.557 ± 0.082	4.60± 0.40
PB-020	99.50 ± 0.40	0.12 ± 0.01^{ns}	-32.20 ± 5.93	66.20 ± 10.00	0.251 ± 0.007	4.73 ± 0.33

Zeta potential and Polydispersibility index values and Zovirax is a conventional emulsion with droplet size in micrometers and has high Polydispersibility index. In vitro diffusion studies were performed using Franz diffusion cells using synthetic membranes Axiva (Polyvinylidene difluoride 0.45µm). 0.1N sodium hydroxide and Iso propyl alcohol (60:40) was used as receptor media, temperature was kept at 32±0.5°C throughout experiment and stirring rate

was maintained at 600 rpm throughout the experiment. Nanoemulsion has shown a better diffusion profile when compared with innovator product Zovirax, the diffusion profile has established that reduction in globule size has increased the rate of drug penetration of nanoemulsion



	30	60	120	180	240	360
PB020	14.10	32.00	52.20	69.40	82.20	82.40
Zovirax	11	23.1	44.1	57	69	71.5

Fig 1: In vitro diffusion profile of Innovator product Zovirax vs Optimized nanoemulsion PB020

EXPERIMENTAL DESIGN TO ESTABLISH NON-IRRITANCY PROFILE OF NANOEMULSION

Acute dermal irritation study:

The acute dermal irritation/corrosion study was carried out in accordance with the OECD Guideline 402 method. The toxicity of test compound PB020 which was an optimized nanoemulsion and conventional cream formulation (Zovirax -GSK) following dermal administration was assessed. Nine female rats were used for limit test for each of two products. The animals were housed in cages for five days to adapt to the laboratory environment. Thereafter, the animals were fasted for three to four hours without food or water. After the fasting period, the animals were weighed and assigned the groups as per the product description (table 1). Before initiating main study, range finding study was initiated, One rat from both the group (2 products) were used for range finding study where the product at a dose of 200 mg/Kg, 1000mg/Kg and 2000mg/Kg body weight was applied on the dorsum (shaved skin), covering not less than 10% of total body area. No evidence of toxicity was seen in range finding study, basis the results of range finding study main study was initiated. Two rats from each product group was selected for main study and the product at a dose of 200 mg/Kg, 1000mg/Kg and 2000mg/Kg body weight was applied on the dorsum (shaved skin), covering not less than 10% of total body area. In all the groups (Table 2). During the 24 h exposure period, animals were caged individually in order to avoid oral ingestion of the test substance by other animals. The test substance was held in contact with the skin throughout the 24 h exposure period by a porous gauze dressing. The test site was further covered to retain the gauze in place and ensure that the animals did not ingest the test substance (Fig 1).

Table 1.0: Animal identification for respective products

Product	Total No. of Rats	Animal ID for all the 9 rats for respective products
Zovirax	9	2024522-01, 2024522-02, 2024522-03, 2024522-04, 2024522-05, 2024522-06, 2024522-07, 2024522-08, 2024522-09
PB 020	9	2024523-01, 2024523-02. 2024523-03, 2024523-04, 2024523-05, 2024523-06, 2024523-07, 2024523-08, 2024523-09

Table 2.0 : Animal identification for dose range finding and main study

Animal ID No. of respective product		No. of Animals	Dose level (mg/kg body weight)	Study
2024522-01 2024523-01		1	200	Dose Range Finding
2024522-02 2024523-02	2024522-03 2024523-03	2	200	Main Study
2024522-04 2024523-04		1	1000	Dose Range Finding
2024522-05 2024523-05	2024522-06 2024523-06	2	1000	Main Study
2024522-07 2024523-07		1	2000	Dose Range Finding
2024522-08 2024523-08	2024522-09 2024523-09	2	2000	Main Study

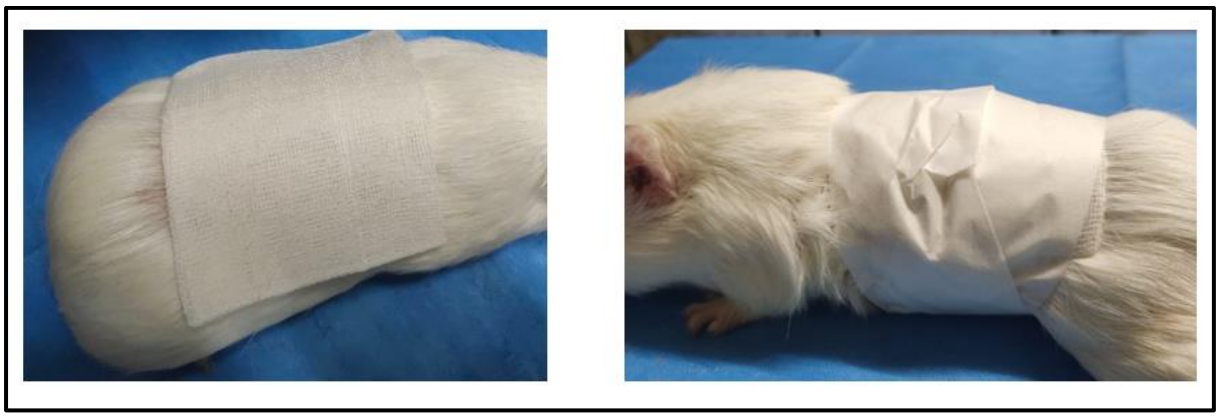


Fig 1: Gauze dressing of Wistar rats after application of test substance

Skin Sensitization

The skin sensitization test was carried out in accordance with the OECD Guideline 406. The test compound PB020 and marketed formulation, Zovirax -GSK, following dermal administration was assessed. Total 21 animals were used for skin sensitization study for all the four products. One day before the first induction, the guinea pigs were assigned to 1 of the 3 groups as mentioned in Table 3. Since control group was distilled water exposure (without any drug) so control group was run only once and compared with the data of two products of Treatment group. Buehler test method was adopted to evaluate the skin sensitization potential

(Buehler, 1965). The left flank of each guinea pig was shaved using electric clippers and an electric shaver. Only those animals without injury or irritation of the skin were used in the test. The pilot study was conducted To determine the high dose that causes mild skin irritation as well as the highest non-irritant dose, pilot study was carried out on 6 guinea pigs (3 guinea pigs for each sample), the test item was applied on the shaven flank of guinea pigs at the doses of 0.1ml, 0.2ml, 0.5ml in occlusive test patch system. The test item was held in contact with the skin for 6 hr by an occlusive patch and bandage dressing. dressings were removed and the animals were observed for dermal reactions. On the basis of pilot study observation, the high dose 0.5ml was selected for induction exposure and 0.2 ml was selected for challenge exposure. After satisfactory pilot study. Induction exposure study was initiated where one flank of each of 60 acclimated animals (for both products and both groups) as mentioned in table 3 was closely clipped of hair, without any abrasion, 24 hr before the induction exposure. A cotton pad about 4-6 cm2 in size was loaded with the test item at a dose of 0.5 ml and applied to the shaven area of animals of treatment group. To ensure dressing patch in place, animals were observed for 6hours. Control group containing distilled water was also exposed similarly. (OECD 2022).

Images were captured during studies using Ultra high-speed camera v1840 at 10X resolution.

Reliability Check

The sensitivity and reliability of the above method were validated. The experiment was carried out as detailed above using 5 male guinea pigs. Mercaptobenzothiazole was used as the sensitizer. A concurrent control group (5 male) were also maintained. It was concluded that the method employed was sensitive and reliable.

Table 3 : Allocation of Animals for different products

Product	Pilot study	Treatment Group	Control
Zovirax	3	20	20
PB020	3	20	

RESULTs

Nanoemulsion

The physicochemical analysis of PB020 and Zovirax (GSK) has revealed that developed nanoemulsion, PB020 is a nanoemulsion because of its Nano globule size, higher zeta potential and lower Polydispersibility index. Invitro diffusion studies also revealed that Nanoemulsion has better diffusion profile than conventional emulsion of Zovirax.

Acute dermal toxicity:

The animals were observed twice daily for 14 days for signs of irritation, general behaviour, and possible mortality. The animals were coded as in Table 1 and experiment was designed as mentioned in Table 2. All the animals were daily observed for clinical signs toxicity and mortality for a period of 14 days. The body weight was recorded on day 0 (pre-treatment), 7^{th} and 14^{th} (post treatment) Table 4 and table 5. On the 15th day, the rats were sacrificed and the organs were carefully taken out and weighed. Histopathological examination of animals was performed at the termination of the study on day 15. (Hajare 2022).

Necropsy was carried out on all the animals at the end of the study to observe any gross pathological changes. All the external organs including Skin and and Internal organs like

abdominal, adrenal and thoracic cavity, Genital organs, excretory system, thyroid and Cranial cavity were found intact without any pathological changes.

Table 4: Body weight change during acute dermal toxicity study to Zovirax cream

Animal ID	Dose level mg/kg body weight	Day 0	Day 7	% Gain/Loss	Day 14	% Gain/Loss
2024522-01	200	200	205	2.50	208	3.85
2024522-02	200	180	190	5.56	194	7.22
2024522-03	200	190	196	3.16	202	5.94
2024522-04	1000	202	209	3.47	212	4.72
2024522-05	1000	203	208	2.46	212	4.25
2024522-06	1000	204	209	2.45	214	4.67
2024522-07	2000	198	208	5.05	209	5.26
2024522-08	2000	188	199	5.85	202	6.93
2024522-09	2000	182	187	2.75	192	5.21

Table 5: Body weight change during acute dermal toxicity study of PB020

Animal ID	Dose level mg/kg body weight	Day 0	Day 7	% Gain/Loss	Day 14	% Gain/Loss
2024523-01	200	190	192	1.05	198	4.04
2024523-02	200	185	188	1.62	190	2.63
2024523-03	200	201	203	1.00	204	1.47
2024523-04	1000	198	205	3.54	208	4.81
2024523-05	1000	195	204	4.62	207	5.80
2024523-06	1000	202	208	2.97	210	3.81
2024523-07	2000	205	206	0.49	211	2.84
2024523-08	2000	198	202	2.02	204	2.94
2024523-09	2000	200	208	4.00	210	4.76

Skin sensitization:

None of the animals of treatment group of Zovirax, nanoemulsion PB020 and control group presented any skin reaction at 30 and 54 hrs after application of the challenge patch. Since none of the animals of treatment and control groups presented erythematous responses, a grade of '0' was given as per Magnusson and Klingman grading scale, to all the animals at both the time points of observation after the challenge patch application. Magnusson etal., 1970. Table 6. Observation of challenge study of skin sensitization has been shown in Fig 2 and Fig 3.

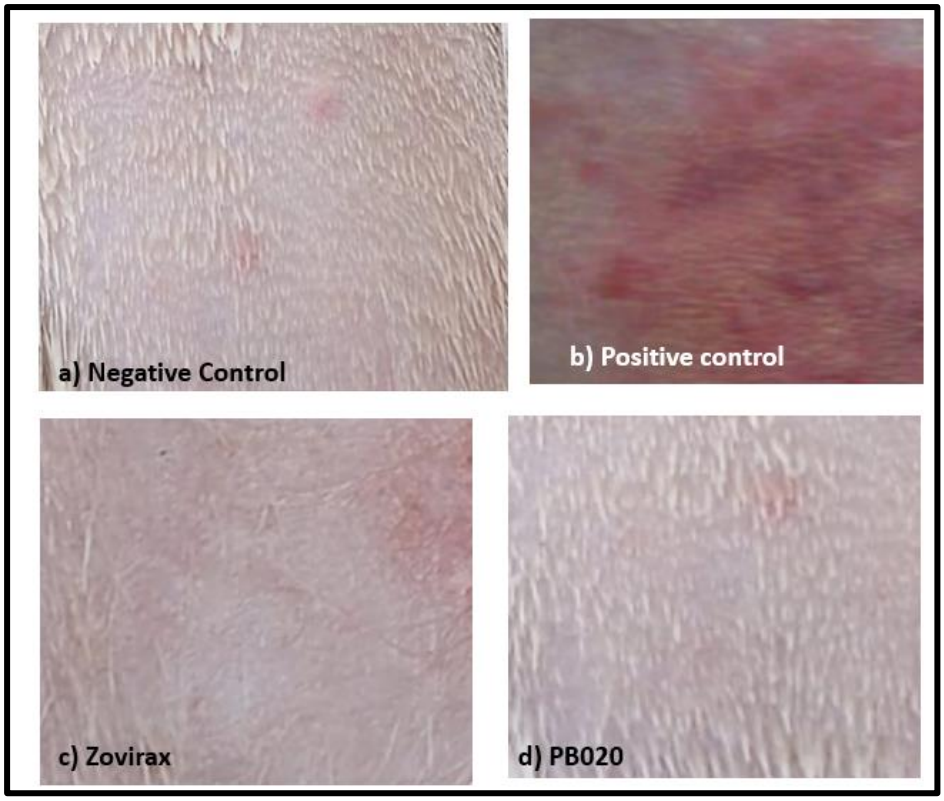


Fig 2: Skin sensitization challenge test after 30 h of treatment with a) water (negative control, b) Mercaptobenzothiazole (positive control, c) Zovirax, d) PB020: (10X Magnification)

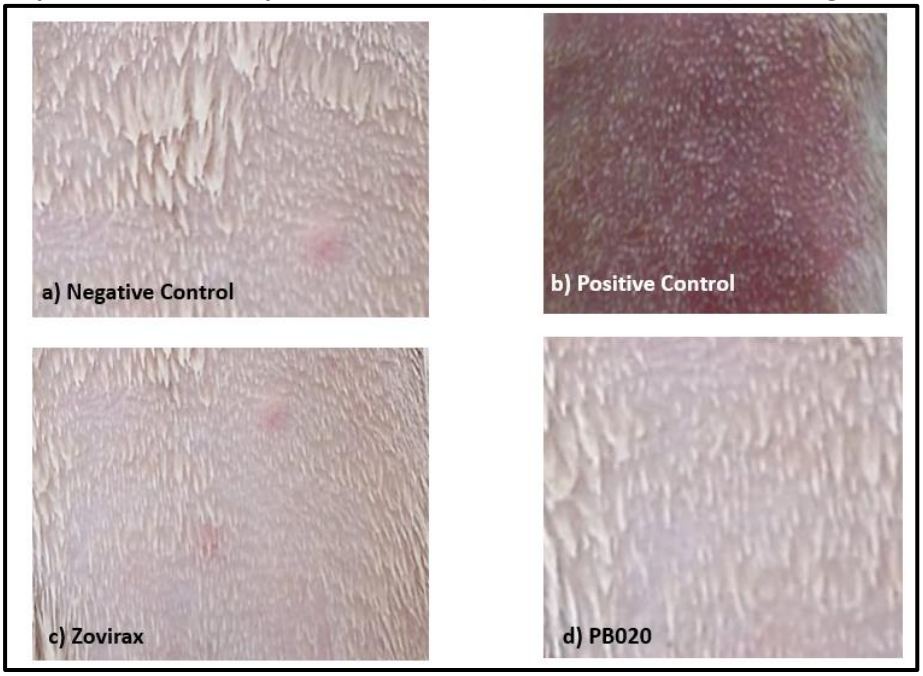


Fig 3: Skin sensitization challenge test after 54 h of treatment with a) water (negative control, b) Mercaptobenzothiazole (positive control, c) Zovirax, d) PB020: (10X Magnification)

Table 6 : Magnusson and Kligman Grading Scale for the Evaluation of Challenge Patch Test Reactions

S.No	Observation	Grade
1	No Visible Change	0
2	Discrete or patchy erythema	1
3	Moderate and confluent erythema	2
4	Intense erythema and swelling	3

Based on the results obtained from study, it was concluded that the nanoemulsion PB020 and Zovirax are non-hazardous and non-toxic to Wistar albino rats at the tested dose level .And both the formulation nanoemulsion PB020 and innovator product Zovirax did not show any erythematous responses in Guinea pigs and hence considered as Non skin sensitizer.

Discussion

Nanoemulsions, because of its smaller globule sizes, surfactant nature and high penetration efficiency. In this study, we have studied that nanoemulsion formulation are non invasive if right choice of surfactant and actives is kept in mind before finalizing the final formulation of nanoemulsion, Optimized formulation of nanoemulsion of acyclovir having particle size in the range of 60-100nm were evaluated in the current study and found not irritating to the skin as per acute dermal toxicity and skin sensitization test recommended as per OECD guidelines . The nanoemulsion was compared with the innovator product which was a conventional macroemulsion. None of the treatment group exhibited acute toxicity and skin sensitization was shown by positive control group only. This study has demonstrated that nanoemulsion formulation if developed considering the appropriate surfactant, solubilizers and penetration enhancer may lead to development of a non-irritating formulation. This study opens up the avenues for development of nanoemulsion for increasing the efficacy and stability of dosage form.

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