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Research Article

Theme: Recent Advances in Dissolution and In Vitro Release of Dosage Forms
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Comparison of *In Vitro* Release Rates of Acyclovir from Cream Formulations Using Vertical Diffusion Cells

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Abstract. Acyclovir, indicated in the treatment of *herpes labialis* ("*cold sores*"), is formulated as semisolid topical dosage forms and marketed in numerous countries. Since the formulations of the various acyclovir products may differ from country to country, this study was undertaken to compare the *in vitro* release of acyclovir from various generic cream products available on the South African and Indian markets using the respective brand/innovator product as the reference product. The *in vitro* studies were carried out using vertical diffusion cells with a diffusional surface area of 1.767 cm^2 and various commercially available membranes. Normal saline was used as receptor fluid and the temperature maintained at 32± 0.5°C. The *in vitro* release comparisons were based on the recommendations described in the US Food and Drug Administration Draft Guidance for acyclovir ointment and the SUPAC-SS Guidance for non-sterile semisolid dosage forms. The release rates (slope) of the test (*T*) and the relevant reference product (*R*) were monitored and compared. The comparative release of acyclovir from the various generic formulations compared with the reference product was found to be within the limits of 75–133.33% with a 90% confidence interval. These experiments indicate that the generic acyclovir cream formulations exhibited release rates that were comparable to the innovator product and could be considered to be bioequivalent.

KEY WORDS: acyclovir cream; FDA Guidance; *in vitro* release; membranes; vertical diffusion cells.

INTRODUCTION

Apart from the vasoconstrictor assay for the assessment of bioequivalence of topical corticosteroid products (1), the only means whereby a generic company can demonstrate bioequivalence of a topical dosage form intended for local and//or regional activity is through comparative clinical trials with a clinical endpoint using a randomized, double blind, parallel, placebo-controlled study design comparing the generic product *versus* the reference listed drug (RLD) in the USA. This has resulted in a dearth of generic topical products reaching the market since conducting clinical end-point trials is lengthy and expensive. Much effort has, however, been directed towards the development and validation of alternative approaches to demonstrate bioequivalence (2).

In vitro release testing of active ingredients from topical dosage forms can be conducted to characterize performance characteristics of a finished topical dosage form as a quality control procedure and also for justification for scale-up and post-approval changes (3). However, *in vitro* studies have generally not found acceptance by most regulatory agencies to establish bioequivalence until recently when the FDA published a draft guidance on Acyclovir (4) which makes provision for an *in vitro* option to establish bioavailability or bioequivalence of, specifically, acyclovir topical ointments, only.

For over three decades, vertical diffusion cells have been regarded as the single most powerful *in vitro* model for monitoring the release of active ingredient from semisolid and transdermal dosage forms and for predicting bioavailability and bioequivalence (5). These cells have been used with various synthetic membranes such as cellulose acetate/nitrate mixed ester, polysulfone, or polytetrafluoroethylene to separate the donor and receiver side for performing *in vitro* drug release testing. Although dermatomed human skin has also been used (5), human skin has largely been used to monitor drug diffusion from transdermal preparations (6). Whereas artificial membranes do not model the lipid perturbation effects undergone by biological samples, inferences regarding partitioning and diffusion phenomena can be made. Previously reported human skin penetration studies involving ayclovir creams (5) indicated that some generic creams might be bioinequivalent to the innovator, and those authors also mentioned that the use of human skin is prone to inconsistent diffusion and that the study protocol needs standardization of skin membranes. Hence, synthetic membranes may be preferred to skin tissue as they are more easily resourced, less expensive, and structurally simpler. This

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means large-scale studies can be more readily undertaken while mechanisms can be deconvoluted more readily (7). Furthermore, synthetic membranes exhibit superior permeation data reproducibility as *in vivo* variables such as skin age, race, sex, and anatomical site are eliminated (8). Nevertheless, the results of artificial membrane studies still tend to yield useful data (9,10).

Penetration of a drug molecule through skin layers is a complex process, typically rate-limited by the *stratum corneum* (SC). The SC layer of the skin is composed of terminally differentiated corneocytes embedded in a complex lipid matrix comprising primarily ceramides, cholesterol, and free fatty acids (11). Hence, the delivery of drug by passive diffusion and the pharmacological effects elicited are dose related—the more permeation of the drug through the skin, the greater the therapeutic effect. Trottet et al (5) undertook a study of 139 acyclovir cream formulations and concluded that a 40% propylene glycol concentration in the cream formulation enhanced the availability of acyclovir by 10-fold. Hence, formulating a topical dosage form that enhances skin permeation is predicted to result in improved therapeutic benefit on application.

The temperature during *in vitro* release studies is usually set at 32°C to reflect normal skin temperature, and the most discriminating test conditions are recommended for such *in vitro* studies. The amount of drug released from the sample at different time intervals is determined, and the slope of the straight line obtained by plotting cumulative amount of drug release across 1 cm^2 membrane *versus* the square root of time provides an indication of the release rate and/or associated release kinetics.

Since most regulatory authorities require clinical endpoint studies to confirm the safety and efficacy of generic topical products except for topical corticosteroid products where the vasoconstriction assay can be used (1,12), an acceptable *in vitro* method would be of great benefit. The objective of this study was therefore to evaluate the *in vitro* release rates of acyclovir from generic creams approved in South African and Indian markets and to compare them with the innovator brand products available in the respective markets using vertical diffusion cells in order to establish whether those approved generic products could be shown to be equivalent based on *in vitro* data. Recently, a regulatory guidance permitting the use of *in vitro* data to consider a biowaiver for topical acyclovir ointments was issued by the US FDA (4). Hence, based on the data generated for acyclovir creams in this study, these data should provide useful and compelling information to establish an additional guidance for biowaivers for acyclovir cream products using an *in vitro* method. Furthermore, this study involved the investigation of different types of membranes in order to facilitate the choice of an appropriate membrane for the assessment of acyclovir creams in the quest to establish suitable conditions for the application of *in vitro* release rates as an indicator of bioequivalence.

Acyclovir is an acyclic nucleoside analog which has a high activity and selectivity for herpes viruses, particularly *herpes simplex* virus types 1 and 2 and varicella zoster virus *or herpes labialis* ("*cold sores*") (13). Commercially available creams contain 5% acyclovir, and the innovator and generic creams marketed in the South African and Indian markets were selected for assessment.

MATERIALS AND METHODS

Vertical Diffusion Cell and Assembly

In vitro permeability studies were performed using six vertical cells (1.767 cm^2 diffusional surface area) and a PermeGear diffusion system (PermeGear, Inc. Hellertown, PA, USA). The diffusion cells and apparatus were assembled with donor and receptor chambers separated by a selected synthetic membrane. The receptor chamber was filled with 12.0 ml of normal saline to maintain the physiological condition of human skin. The temperature of the receptor fluid was maintained at 32°C by a water jacket connected to an external water bath. The receptor solution was continuously stirred using a 10 × 2.5 mm magnetic stirrer bar.

HPLC Conditions

Since the studies were carried out in different laboratories, two HPLC systems were used. One system comprising a Waters Alliance Model 2690 separation module equipped with a 2996 photo diode array detector (PDA), Pro2 Empower data acquisition system (Waters, Milford, USA), and the other a UFLC Shimadzu Model LC 20AD Prominence liquid chromatography system equipped with SPD-M20A diode array detector and an LC Solution data acquisition system (Shimadzu Corporation, Kyoto, Japan). The chromatographic separation was achieved using a Luna C8 (2) (5 μ, 150 mm×4.6 mm i. d.) column (Phenomenex, USA). The concentrations of acyclovir were

Table I. Acyclovir Creams

Country	Company	Product (as per label)	Batch	Expiry date	Excipients
South Africa	GlaxoSmithKline South Africa (Pty) Ltd	Zovirax cream (0.05 g acyclovir per gram)	C555747	11/2014	NA
	Cipla Life Sciences (Pty) Ltd.	Acitop (Acyclovir USP 5%*w*/*w*)	GM44	02/2014	Chlorocresol—0.12%
	Adcock-Ingram (Pty) Ltd	Adco-Acyclovir topical cream (each 1 g contains 0.05 g acyclovir)	A42	10/2013	Benzyl alcohol—0.75%
	Ranbaxy (South Africa) (Pty) Ltd.	Lovire cream (each 1 g contains acyclovir 50 mg)	1003	6/2012	Methyl paraben—0.15% Propyl paraben—0.08%
India	Cipla Ltd	Acivir cream (Aciclovir cream BP 5%*w*/*w* contains Acyclovir IP 5%*w*/*w*)	U428	02/2014	NA

NA not available

Table II. Vertical Diffusion Cell Conditions for *In vitro* Release of Acyclovir Cream Formulations

Parameter	Conditions
Average diffusional surface area	1.767±0.1 cm^2
Average receptor volume	12.0±0.1 ml
Temperature	32±0.5°C
Membranes	Nylon; Tuffryn; Durapore; Nitrocellulose; STRAT-M; Fluoropore
Receptor medium	Normal Saline
Dose	~300 mg
Sampling time	0.5, 1, 2, 3, 4, 5, and 6 h
Sample volume	100 µl
Sample analysis	HPLC with PDA detection (254 nm)

determined by HPLC using a mobile phase of methanol: 0.1% formic acid in water (5:95), pumped at a flow rate of 1.0 ml/min and the eluate monitored at a wavelength of 254 nm. The injection volume was 10 µL, and the chromatography was carried out on a Luna C8 (2) (5 µ, 150 mm×4.6 mm i. d.) column. Samples were injected at ambient temperature during analysis.

Chemicals and Formulations

HPLC grade methanol (UV cut-off 215 nm) and acetonitrile (*UV cut-off* 190 nm) were purchased from Romil Ltd (Cambridge, UK) and Rankem Ltd (Mumbai, India). HPLC grade water was generated in a Milli-Q System (Millipore, Milford, CT, USA). Normal saline (0.9%*w/v* NaCl) purchased from local pharmacy was used as receptor fluid. Zovirax cream was purchased from GlaxoSmithKline South Africa (Pty) Ltd, South Africa; Adco-Acyclovir topical cream from Adcock-Ingram (Pty) Ltd, South Africa; Acitop from Cipla Life Sciences (Pty) Ltd, South Africa; Lovire Cream from Ranbaxy (South Africa) (Pty) Ltd, South Afica; Acivir cream was from Cipla Ltd, India; and Acyclovir Reference Standard (RS) 99% was purchased from Sigma-Aldrich Co., (St. Louis, USA). Formic acid 99.9% was obtained from Associated Chemical Enterprises (Johannesburg, South Africa) and Thermo Electron LLS India Pvt. Ltd (Mumbai, India).

Acyclovir release testing was carried out using various synthetic membranes, Magna Nylon 0.22 µm, 25 mm (Cat. No. R02SP02500, Batch No. 293240, GE Water & Process Technologies, USA); Tuffryn membrane filters HT-450, 0.45 µm, 47 mm (Cat. No. 66223, Batch No. T12821) purchased from Pall Life Sciences (Ann Arbor, Michigan); Durapore 0.45 µm, 47 mm (Cat. No. HVLP04700, Lot No. R1DA62310K, Millipore, Ireland); Nitrocellulose 0.025 µm, 47 mm (Cat. No. VSWP04700, Lot No. R1MA22632, Millipore, Ireland); Fluoropore 0.2 µm, 25 mm (Cat. No. FGLP02500, Lot No. R1NA25271, Millipore, Ireland); and Strat-M 47 mm (Ref No. SKBM047TP, TD1EA0014, Millipore, USA) membrane filters.

Syringes (2 ml) with sampling needles were purchased from local pharmacies. Analytical balances, type AG 135, and Micro Balance MX5 (Mettler Toledo, Switzerland) and Shimadzu AUW220D (Shimadzu Corporation, Kyoto, Japan) were used for weighing standards and samples. An electronic pipette (model 71050XET supplied by Biohit PLC, Helsenki,

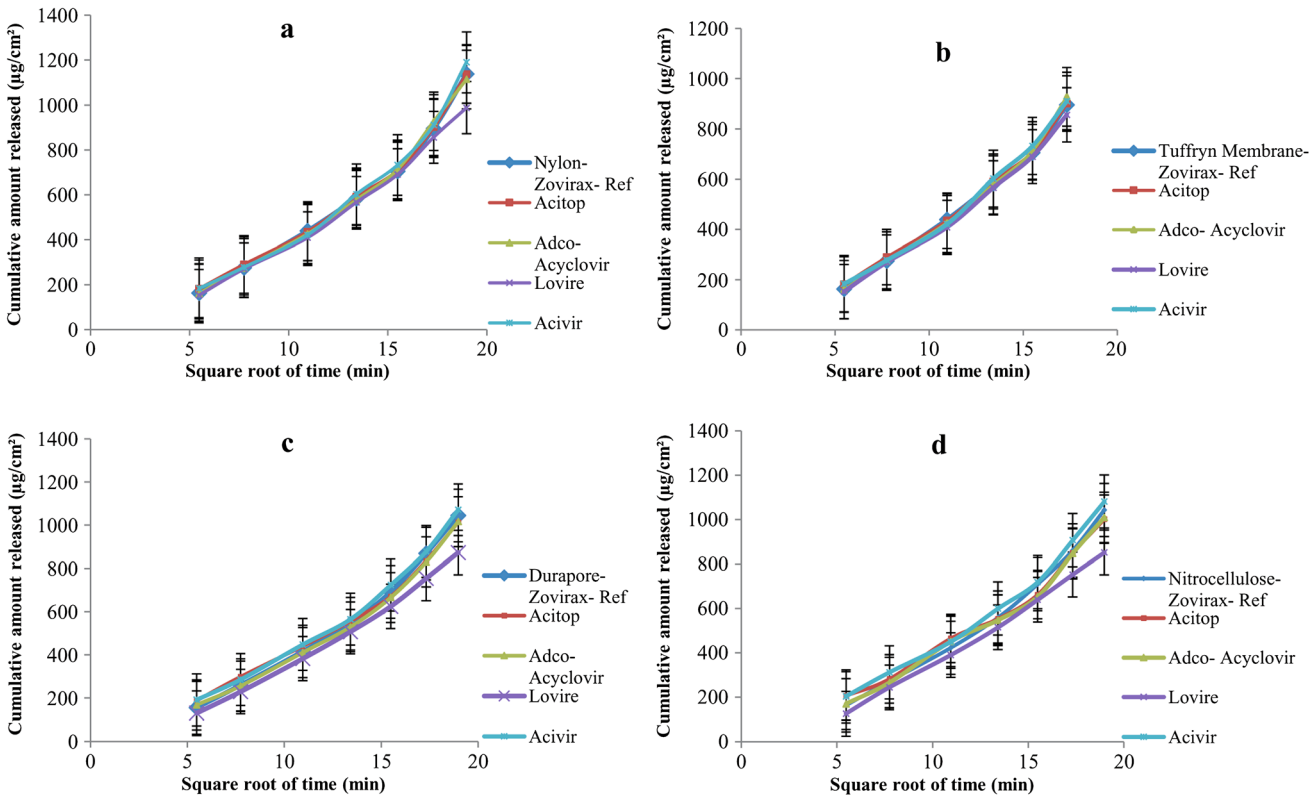

Fig. 1. a–d Cumulative amount of acyclovir released from various cream formulations using various membranes **a** Nylon. **b** Tuffryn. **c** Durapore. **d** Nitrocellulose

Table III. Comparison (Slopes) of Various Synthetic Membranes after 6 h (n=12) from Acyclovir Generic Cream Formulations Compared with Zovirax Cream (Innovator)

Generic *vs* innovator	Nylon	Tuffryn	Durapore HVLP	Nitrocellulose VSWP	Strat-M	Fluoropore FGLP
Zovirax	62.29 (±1.9)	62.21 (±1.64)	61.92 (±2.16)	61.81 (±2.61)	0.32 (±0.01)	Below LOQ
Acitop	62. 12 (±2.38)	61.29 (±1.65)	61.17 (±3.88)	61.02 (±3.62)	0.32 (±0.01)	Below LOQ
Zovirax	62.56 (±2.03)	62.17 (±1.46)	62.00 (±1.16)	61.59 (±1.76)	0.32 (±0.01)	Below LOQ
Adco-Acyclovir	62.18 (±1.96)	62.09 (±1.50)	61.37 (±1.44)	61.14 (±1.94)	0.31 (±0.02)	Below LOQ
Zovirax	62.98 (±0.97)	62.19 (±0.96)	62.15 (±1.21)	61.97 (±1.31)	0.33 (±0.01)	Below LOQ
Lovire	56.77 (±1.49)	57.36 (±2.39)	56.19 (±1.86)	55.89 (±2.46)	0.28 (±0.01)	Below LOQ
Zovirax	62.11 (±1.73)	61.99 (±2.45)	61.46 (±2.58)	61.25 (±2.42)	0.33 (±0.01)	Below LOQ
Acivir	67.78 (±4.07)	63.96 (±1.88)	62.86 (±0.56)	62.24 (±2.76)	0.33 (±0.02)	Below LOQ

Values in parentheses indicate mean±SD, *LOQ* limit of quantitation

Finland, and Eppendorf Xplorer supplied by *Eppendorf AG*, *Hamburg*, *Germany*) was used to transfer standard and sample solutions for dilutions.

In Vitro Studies

Table I shows the acyclovir creams tested using the vertical diffusion cells and system. The studies were conducted in accordance with the FDA SUPAC-SS guidance (14). The static diffusion cells maintained at 32°C±0.5 were assembled, and various selected synthetic membranes were investigated. The relevant membranes were pre-treated by immersing in the receptor fluid (0.9% normal saline) for a period of 30 min and blot dried prior to use. An accurately weighed amount (approximately 300 mg) of each acyclovir cream formulation was applied using a calibrated pipette, and the cells were covered with *Parafilm* M sealing film to prevent evaporation of vehicle and ensure integrity of the formulations throughout the respective study periods. Aliquots of 100 μl were sampled at intervals of 0.5, 1, 2, 3, 4, 5, and 6 h and replaced with fresh receptor fluid to maintain sink conditions. Each formulation was tested in triplicate (n=3), and the diffusion cell conditions are summarized in Table II.

RESULTS AND DISCUSSION

The various acyclovir creams were tested using the previously mentioned synthetic membranes using a solution of normal saline as the receptor fluid, and the cumulative amounts of acyclovir released over a period of 6 h were plotted against the square root of time. Comparative release rates of acyclovir from various cream formulations using the various synthetic membranes are depicted in Fig. 1a–d.

The acyclovir release rates from the creams were analyzed using the "Wilcoxon Rank Sum/Mann–Whitney statistical test" as described in the FDA's SUPAC guidance (14). The class interval ranges for each formulation were calculated using linear regression analyses, and the respective release rates were determined from the relevant slopes of the regression lines as shown in Table III.

The release of acyclovir for each generic cream was compared with the release from the reference product, Zovirax (n=6) using each of the six synthetic membranes, Nylon, Tuffryn, Durapore HVLP, Nitrocellulose VSWP, Strat-M, and Fluoropore. A 90% confidence interval for the ratio of the median *in vitro* release rate (slope) for test (T) over the median *in vitro* release rate for reference (R), expressed in percentage terms, was computed. For all the creams tested, the 90% confidence intervals were within the specified limits from 75 to 133.33% (14) except when Strat-M or Fluoropore membranes were used. The results are depicted in Table IV. Using the latter, two membranes resulted in very low concentrations of acyclovir diffusing through those particular membranes, and in most cases, the concentrations were below the limits of detection. Since the first stage confidence intervals complied with the SUPAC guidance, no further *in vitro* testing was necessary.

Plots of the cumulative release of acyclovir *versus* the square root of time for each cream formulation through nylon (0.22 μm), Tuffryn (0.45 μm), Durapore HVLP (0.45 μm), Nitrocellulose VSWP (0.025 μm), Strat-M, and Fluoropore FGLP (0.2 μm) membranes were linear with resultant R^2> 0.95 for all the plots. Acyclovir release from the generic formulations was best described by the Higuchi model,

Table IV. Ninety Percent Confidence Interval Values (8th and 29th Terms) of Acyclovir Released from Various Synthetic Membranes after 6 h from Acyclovir Generics (Cream Formulations) Compared with Zovirax Cream (Innovator)

Membrane type	Acitop (Cipla, South Africa)		Adco-Acyclovir (Adcock-Ingram, South Africa)		Lovire (Ranbaxy, South Africa)		Acivir (Cipla, India)	
Nylon	99.43	103.05	96.89	102.81	88.45	94.13	99.85	103.67
Tuffryn	94.55	99.45	95.15	100.12	87.62	90.03	96.65	99.66
Durapore HVLP	88.44	97.83	95.73	100.32	86.01	89.20	97.17	100.14
Nitrocellulose VSWP	87.02	95.33	98.91	102.34	85.49	88.85	98.07	104.43
Strat-M	Below LOQ							
Fluoropore FGLP	Below LOQ							

LOQ limit of quantitation

Table V. Cumulative Amount of Acyclovir Released (μg/cm^2) from Various Synthetic Membranes after 6 h (n=12) from Acyclovir Generic Cream Formulations Compared with Zovirax® Cream (Innovator)

Generic *vs* innovator	Nylon	Tuffryn	Durapore HVLP	Nitrocellulose VSWP	Strat-M	Fluoropore FGLP
Zovirax	1,034.09 (±19.10)	1,030.13 (±9.78)	1,023.22 (±23.18)	1,022.16 (±29.44)	4.85 (±0.06)	Below LOQ
Acitop	1,025.46 (±32.47)	1,015.76 (±31.21)	1,013.89 (±22.59)	1,002.32 (±29.97)	4.85 (±0.19)	Below LOQ
Zovirax	1,034.64 (±15.73)	1,028.23 (±15.93)	1,024.08 (±17.97)	1,018.68 (±18.21)	4.84 (±0.08)	Below LOQ
Adco-Acyclovir	1,028.81 (±36.13)	1,024.44 (±26.12)	1,016.65 (±19.12)	1,009.81 (±15.68)	4.84 (±0.14)	Below LOQ
Zovirax	1,036.39 (±13.23)	1,031.47 (±13.27)	1,024.05 (±24.28)	1,021.33 (±28.31)	4.87 (±0.06)	Below LOQ
Lovire	897.78 (±28.80)	847.84 (±18.62)	833.01 (±21.69)	826.60 (±9.03)	4.27 (±0.11)	Below LOQ
Zovirax	1,033.91 (±14.82)	1,021.54 (±34.23)	1,017.09 (±38.27)	1,014.67 (±19.74)	4.86 (±0.13)	Below LOQ
Acivir	1,235.45 (±25.93)	1,188.33 (±43.51)	1,102.11 (±18.07)	1,081.59 (±25.64)	4.90 (±0.17)	Below LOQ

Values in parentheses indicate mean±SD, *LOQ* limit of quantitation

where the rate controlling step for drug release is diffusion through the topical cream base. As shown in Table V, the release profiles obtained from Zovirax, Acitop, Adco-Acyclovir, Lovire, and Acivir creams showed similar patterns through Nylon, Tuffryn, Durapore, and Nitrocellulose membranes, whereas Strat-M membranes produced a low (4.84 μg/cm^2) acyclovir release, and Fluoropore membrane showed the lowest (below LOQ) acyclovir release from the cream formulations. The formulations evaluated according to the US FDA SUPAC guidance (14) show that, based on their *in vitro* release, the creams meet the bioequivalence requirements. This study compared the *in vitro* release of acyclovir from several topical dosage forms containing 5% of active ingredient where each of the products had been approved for marketing in the respective countries. Although bioequivalence is usually confirmed by clinical end-point studies in the case of topical dosage forms where the test and reference products are pharmaceutical equivalents and thus therapeutic equivalents, the data from the *in vitro* release studies on the acyclovir creams confirm that these products can also be confirmed to be bioequivalent in line with the recent FDA Draft Guidance for acyclovir (4). However, it should be noted that, in terms of the FDA guidance for acyclovir ointments, all of the following criteria must be met:

i. The test and Reference Listed Drug (RLD) formulations must be qualitatively and quantitatively the same (Q1/Q2).
ii. Acceptable comparative physicochemical characterization of the test and RLD formulations must be shown.
iii. Acceptable comparative *in vitro* drug release rate tests of acyclovir from the test and RLD formulations.

This later condition has clearly been met in these studies.

CONCLUSION

All the creams were evaluated according to recommendations in two US FDA guidance (4,14), and the results indicated that they showed acceptable comparative *in vitro* acyclovir release rates using four of the six synthetic membranes and thus could provide useful information for the development of a regulatory guidance for a biowaiver for acyclovir creams based on the precedent of the recently published Draft Guidance for Acyclovir Topical Ointment (4). However, Strat-M and Fluoropore membranes are not recommended in view of extremely low diffusion rates of acyclovir through those membranes.

The vertical diffusion cell apparatus was used to assess the release of acyclovir from the topical formulations of the five different acyclovir cream formulations using various synthetic membranes. Although there are various types of synthetic membranes which are commercially available, each type of membrane, because of its different physicochemical properties, may have a different effect on diffusion rates. Hence, the choice of the most appropriate membrane for a particular topical drug product is essential in order to characterize the release of that particular compound and permit valid comparisons to be made between products and an appropriate reference standard. Strat-M and Fluoropore membranes are hydrophobic in nature, whereas Nylon, Tuffryn, Durapore, and Nitrocellulose membranes are less hydrophobic. The latter membrane types are thus preferable for *in vitro* testing of relatively polar drugs such as acyclovir. The Nylon, Tuffryn, Durapore, and Nitro cellulose membranes showed similar release profiles of acyclovir from cream formulations, whereas the hydrophobic membranes Strat-M and Fluoropore resulted in significantly different diffusion rates (very low release profiles of acyclovir), and consequently, the latter two types of membranes are deemed unsuitable for the assessment of the release of acyclovir using normal saline as receptor fluid.

All tested acyclovir 5% generic creams, Acitop, Adco-Acyclovir, Lovire, and Acivir, were found to be *in vitro* equivalent to the innovator, Zovirax cream. Inspection of data indicates that good precision and reproducibility were obtained where the %RSD values were less than 5%.

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