

Comparative *ex vivo* and *in vitro* Permeation Kinetics of Tocopherol in Liquid Formulations

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Abstract

Introduction: There is a growing interest to save animals and cost in transdermal permeation studies, especially during the early screening phase of formulation development. The objective of this study was to establish a relationship between permeability of tocopherol (T) through synthetic and animal membranes and to predict *ex vivo* data from synthetic membrane results. **Materials and Methods:** The permeability of T from different liquid formulations (T1-T13) was studied using Spectra/Por® and neonatal rat skin. Isopropyl myristate (ISPM), polyethylene glycol 400 (PEG)-ethanol (1:1 v/v), ethanol, and propylene glycol (PG) were tested individually as carriers, as well as mixtures with dimethyl sulfoxide (DMSO) and tocopheryl polyethylene glycol succinate (TPGS) as solubility and permeation enhancers. The permeation study was conducted in Franz diffusion cells over 24 h. The permeability coefficient (Ps) was calculated from the observed flux value (J). Good correlation was observed for the *in vitro* and *ex vivo* studies regarding J and Ps values. **Results:** The calculated Ps and J from the *ex-vivo* study were in-line with the corresponding values calculated from Spectra Por® ($r = 0.84$ and 0.82 , for Ps and J, respectively) and followed the same order. The effect of vehicles tested on the permeation of T followed the same rank: PG > ethanol > PEG-ethanol > ISPM, for both the animal and synthetic membrane. The predicted Ps and J values for the neonatal rat skin, based on the correlation with Spectra Por®, followed also the same rank order, yet not the same observed values. **Conclusion:** Spectra Por® could serve as a good surrogate for the animal skin models for drug permeation studies during the early formulation and screening phase.

Key words: Correlation, neonatal rat skin, permeation, synthetic membrane, tocopherol, Vitamin E

INTRODUCTION

Alpha-tocopherol (T) plays a very important role in protecting skin and other organs since it is the most active lipophilic antioxidant in biological membranes^[1] and together with ascorbic acid constitutes the “antioxidant network” that protect skin against oxidative damage.^[2] Therefore, T and tocopherol acetate (TA) are widely used ingredients in the over-the-counter products for delaying/treatment of skin aging, as well as locally acting medicated formulations. Several studies from this laboratory were focused on the evaluation of T/TA in marketed and developed preparations.^[3,4] Results of activation of the prodrug TA into the active form T following topical administration are controversial, depending on the formulation factors and skin model investigated.^[5] Consequently, the use of unesterified T, similar to that found in natural sources, has provided the most consistent data concerning its topical efficacy.^[6] The results of previous studies from this laboratory and

others^[4,7] pointed out the poor permeation of T/TA through different model membranes as well as *in vivo*, owing to its highly lipophilic nature. Gabbanini *et al.* observed that T exhibited the least permeation coefficient (almost zero), in comparison with other water-soluble and fat-soluble vitamins following *in vitro* release through reconstructed human epidermis and synthetic membrane.^[7] The permeation of T/TA was investigated in our previous studies employing neonatal mouse skin model (*ex vivo*) and *in vivo*. Although the human skin remains the membrane of choice for *in vitro* permeation studies, however following the growing interest to save human and animals from invasive and unjustified

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in vivo/ex vivo studies, several synthetic membranes have been suggested as alternatives, based on their reproducibility, ease of use, and handling. This trend would be feasible and acceptable at least during the formulation development phase to optimize the final formulation, where a large number of screening studies deem necessary. Accordingly, synthetic membranes consisting of diverse nature have been used such as silicone, cellulose, and polysulfone membranes of a diverse range of pore size and thickness. However, previous results concluded that drug fluxes did not show a strong correlation with membrane parameters.^[8]

In a previous study of transmembrane diffusion and permeation of ibuprofen across several synthetic membranes, the authors concluded that the porous membranes were categorized into high-flux (8–18 mg/cm²/h) and low-flux (0.1–3 mg/cm²/h) membranes. Furthermore, the authors pointed out that the drug fluxes observed did not show strong correlations with membrane parameters, and thus, investigators should be careful in choosing membrane for formulation quality assessment.^[8] Furthermore, Miki *et al.* studied the permeation of 9 drugs through excised human skin, in comparison with synthetic copolymer membranes, and concluded that the synthetic membranes could be useful for prediction of human skin permeability.^[9] Christensen *et al.* investigated the diffusion of hydrocortisone from several commercial formulations through synthetic membrane, mouse skin, and TMEpiDermTM cultured skin, and the results indicated that hydrocortisone penetration through synthetic membrane was 10 times greater than through mouse skin and EpiDermTM. The authors concluded that the shape, pattern, and rank order of hydrocortisone diffusion from each commercial product were similar through each membrane.^[10]

Luo *et al.* in studying the penetration of ibuprofen from different formulations in human skin and other synthetic membranes concluded that the formulation which delivered the highest amount of the drug in human skin was also significantly more efficient than other tested formulations in delivering the drug through synthetic membranes.^[11] This finding further highlights the usefulness of membranes other than human skin model as a means for preliminary screening and discrimination between developed formulations.

The objectives of the present study were to study the T permeation from liquid formulations using Spectra Por[®] as a model synthetic membrane, in comparison with *ex vivo* data performed on neonatal rat skin as animal membrane to explore any *in vitro–ex vivo* correlation. Spectra/Por[®] membrane was chosen based on good chemical resistance to the following groups: Halogenated hydrocarbons, alcohols, ketones, esters, oxides, and solvents containing nitrogen. The ultimate unique goal is to establish a useful correlation between the *ex vivo* and *in vitro* data which will save animals and cost during the formulation development phase. Furthermore, the effect of liquid carriers and/or permeation enhancers will be investigated.

MATERIALS AND METHODS

Chemicals and reagents

Methanol, propylene glycol (PG), and dimethyl sulfoxide (DMSO) (Merck KGaA, Germany); polyethylene glycol 400 (PEG) (Loba Chemie, India); alpha-tocopherol (Sigma Aldrich Chemie GmbH, Germany); isopropyl myristate (ISPM) (Fluka Chemie, Switzerland); dimethylformamide (DMSO) (Riedel-de Haen Laborchemikalien, GmbH & Co. KG, Germany); D- α -tocopheryl polyethylene glycol 1000 - succinate (TPGS) (Courtesy of ISOICHEM S.A., France); Spectra/Por[®] 3 Dialysis Membranes, MWCO 3500 (Spectrum Laboratories, USA); purified water from Milli-Q synthesis, resistivity 18.2 M Ω cm at 25°C (Millipore SAS, France); and methanol, acetonitrile, hexane, and ethanol used in the study were of high-performance liquid chromatography (HPLC) grade (Merck, Darmstadt, Germany).

Analysis of alpha-tocopherol

The method employed in this study was the same used in our previously developed HPLC technique for drug analysis.^[3] Briefly, the method involved Waters 2690 HPLC (Waters 2690 Separations Module Milford, MA, USA) with variable wavelength PDA detector and a disposable guard column C-18 and RP Waters Symmetry C-18 column (4.6 × 150 mm, 5 μ m particle size). The mobile phase system was methanol-water (97:3 %v/v) at a flow rate of 1.5 mL/min, injected samples were 50 μ L each, and the eluents were monitored at 290 nm.

Effect of solvent carrier on tocopherol permeation

Liquid formulations of T in different solvents were prepared to contain 5% w/w in each solvent. The tested solvents were ISPM, PEG-ethanol (1:1 v/v), ethanol, and PG, using undiluted T as control.

Preparation of tocopherol formulations

The formulations (T1-T13) were made by simple mixing of T with other ingredients to get a final concentration of 4.75–9.5% w/v [Table 1]. To exclude the effect of TPGS on increasing the amount of T permeated following hydrolysis of TPGS, one formulation was prepared to be free from T as a control (T3). All experiments were carried out in containers protected from light by aluminum foils under dimmed conditions. Chemical stability was checked after 3 months at room temperature, and no appreciable loss in activity under these conditions was observed.

Permeation study

This study adopted exactly the previously mentioned conditions employed for investigation of the permeation of

Table 1: Composition of the tested tocopherol formulations

Formulations	PG (mL)	Ethanol (mL)	DMSO (mL)	TPGS (g)	Tocopherol (T) (mL)
T1	1.5	2.5	5.0	0.5	1.0
T2	1.5*	2.5	5.0	0.5	1.0
T3	1.5	2.5	5.0	0.5	0.0
T4	1.5	2.5	5.0	1.0	1.0
T5	1.5	2.5	5.0	1.5	1.0
T6	1.5	2.5	5.0	0.25	1.0
T7	1.5	2.5	5.0	0.5	0.5
T8	1.5	2.5	4.5	0.5	1.0
T9	1.5	2.0	4.5	0.5	1.0
T10	0.0	3.0	5.0	1.5	0.5
T11	0.0	3.0	4.5	1.5	1.0
T12	0.0	4.5	4.5	0.5	0.5
T13	0.0	3.5	4.5	1.5 mL	0.5

*In formulation T2, PEG 400 was used instead of PG

T from different formulations through neonatal rat skin.^[4] In summary, the permeation study was conducted in vertical Franz diffusion cells (PermeGear Inc., USA). The synthetic/animal membrane was mounted between the compartments of diffusion cells, and the effective diffusion area was 0.64 cm². The donor compartment contained 1 mL of the tested formulations, while 5 mL of 50% ethanol in water (v/v) served as the receptor medium. The diffusion cell was maintained at 32°C. At predetermined time intervals, samples were collected (0.2 mL) from the receptor compartment, diluted with methanol, and assayed for T content by the HPLC method described earlier.^[3] The withdrawn samples were replaced with an equal volume of the receptor fluid maintained at 32°C at each sampling time. The synthetic membrane was washed and soaked in distilled water overnight before using in the permeation study.

Regarding the animal model, the neonate rat skin epidermis was used as a model. The rats used were Sprague–Dawley, 2–3 days old and weighing 5–10 g. The animals were obtained from the Health Sciences Center (HSC) and Animal Resource Center and were kept at 20 ± 2°C with free access to food and water. The experiment protocols were approved by the local HSC and Animal Resource Center and were in compliance with international guidelines for the care and use of experimental animals.

The animals were euthanized using carbon dioxide asphyxiation before experiments, and the epidermis was removed by a heat separation technique as described in our previous study.^[4] The entire skin of neonate rats was soaked in water at 60°C for 60 s, followed by careful removal of the epidermis. The epidermis was washed with water and examined for any physical damage, and the intact epidermis was used for the *ex vivo* permeation studies. The epidermis was mounted in the diffusion cells, with the epidermal side

facing the donor compartment and the subcutaneous side facing the receptor compartment.

Permeation kinetics of tocopherol across the tested membranes

The transmembrane diffusion profile was determined by the analysis of the cumulative amount of T in the receptor compartment after predetermined time intervals, namely, 2, 4, 6, and 24 h.

The effective diffusion area of the diffusion cell was 0.64 cm², and the volume of donor compartment was 1 mL and that of the receiver chamber was 5 mL. The diffusion cell was maintained at 32°C. At the predetermined time intervals, permeate samples were collected (0.3 mL) from the receptor compartment, diluted with methanol, and assayed for Vitamin E content by the HPLC method described above. The samples were replaced with an equal volume of the receptor fluid maintained at 32°C. Several fluids were initially tested in the receptor compartment, including buffer pH 7.2 and hydroalcoholic solutions with variable proportions of ethanol. A mixture of ethanol in water (60% v/v) was found optimum and used for the subsequent permeation experiments.

Assuming a steady state condition, the diffusion process follows Fick's first law:

$$dQ/dt = P_s(CD - CR) \quad (1)$$

Where P_s (cm/h) is the skin permeability coefficient, and CD and CR (g cm⁻³) are vitamin concentrations in the donor and receptor chambers, respectively, while Q is the cumulative amount diffused through the membrane per unit area (µg cm⁻²).

Integration of Equation (1) gives:

$$Q = P_s(CD - CR) t \quad (2)$$

In addition, the linear relationships between Q and time, assuming infinite dose conditions ($CD \gg CR$), were assessed. Furthermore, the flux J ($\mu\text{g cm}^{-2} \text{h}^{-1}$) which was calculated from the slope of Q versus time plots during the steady state of the permeation study was further utilized to determine the skin permeability coefficient as shown in Equation (3):

$$P_s = J/CD \quad (3)$$

Comparison of the permeability data generated from the synthetic membrane versus rat skin

Evaluation and screening of cosmetic and medicated topical formulations usually need several experimental trials involving animals. Therefore, it is more advantageous to save the animals from being used as a model for permeation at this early stage of development and use synthetic membrane instead. Trials were performed in the present study to establish a correlation between a synthetic membrane made from pure regenerated natural cellulose (Spectra/Por®) and neonatal rat epidermis as a model for the permeation study. Therefore, the reported permeation parameters determined in the current investigation using Spectra/Por® membrane were compared with the corresponding values generated from permeation of identical tocopherol-containing formulations through the epidermal rat skin.

Prediction of *ex vivo* permeation parameters from *in vitro* data

One of the objectives of this study was to establish a correlation between the *in vitro* and *ex vivo* data to serve as a tool for prediction of the *ex vivo* permeation parameters (J and P_s) from the observed *in vitro* results. Therefore, the possible correlation between the data generated from the tested solvent systems in both permeation models with regard to each of J and P_s will be explored. By this way, we may be able to predict the vitamin permeation parameters J and P_s *ex vivo*, based on the corresponding values actually estimated for the four solvent carrier systems: ISPM, ethanol, PEG-ethanol, and PG.

RESULTS AND DISCUSSION

Effect of solvent system on permeation of tocopherol (T)

Ethanol and PG were included in the studied formulations, based on their superior effect on cumulative T permeation through the animal model and Spectra/Por® [Figures 1 and 2], compared with the other tested solvents (ISPM and ethanol-PEG 400). Furthermore, permeation kinetics of T was calculated in both model membranes, filling the

donor compartment with 1 ml of undiluted T as a control [Figure 3]. It could be concluded from Figures 1 and 2 that the permeation of T through the tested synthetic membrane was generally higher than the corresponding profile depicted with the neonatal epidermis. The same was also true for the control T (without any solvent) [Figure 3]. This finding points out that Spectra/Por® is a discriminative membrane and could differentiate between the different solvent systems. Moreover, the ranking of the tested solvent systems was the same for both the animal and synthetic membrane according to the decreasing order: PG > ethanol > PEG-ethanol > ISPM. This finding is an additional advantage for using Spectra/Por as a surrogate for evaluation of tocopherol, as a model hydrophobic molecule, permeation in the early stages of formulation instead of the *ex vivo* and may be also the *in vivo* experiments.

These results are in agreement with a recently published data,

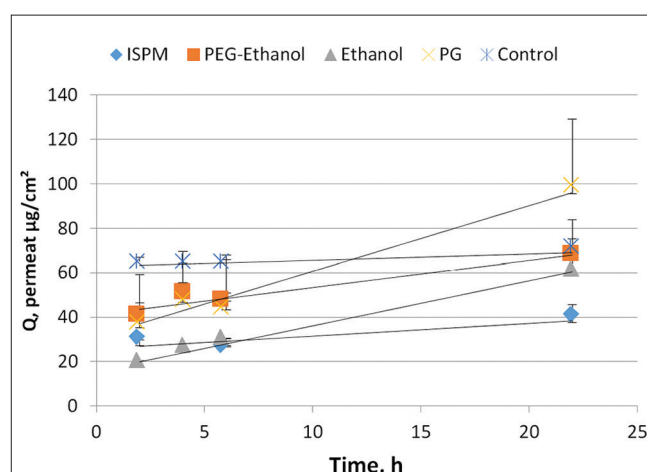


Figure 1: Cumulative amount of tocopherol in different vehicles (5% w/v) permeated *in vitro* through Spectra/Por®

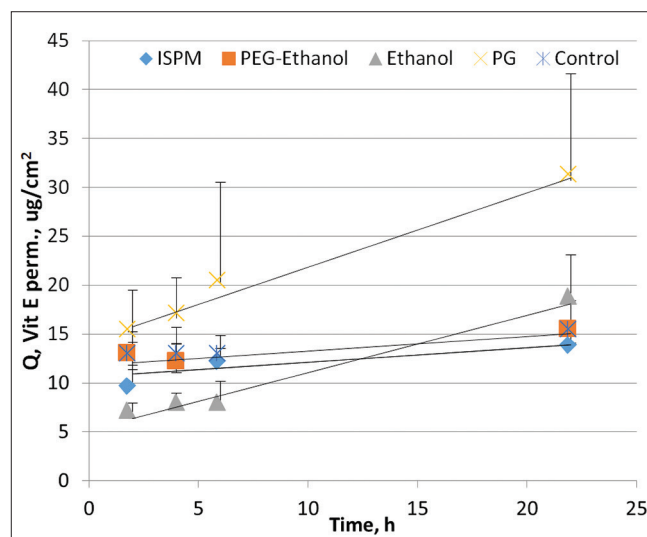


Figure 2: Cumulative amount of tocopherol in different vehicles (5% w/v) permeated *ex vivo* through the neonatal rat epidermis

where the authors pointed out the more permeable nature of synthetic membranes and porcine skin models to ibuprofen, compared with human skin.^[11]

Comparison of the permeability data generated from the synthetic membrane versus rat skin

Evaluation and screening of cosmetic and medicated topical formulations usually need several experimental trials involving animals. Therefore, it is more advantageous to save the animals from being used as a model for permeation studies at early stages of product development by using synthetic membranes, as pointed out also by several researchers, e.g.^[11] Trials were performed in the present study to establish a correlation between a synthetic membrane made from pure, regenerated, natural cellulose (Spectra/Por® 3) and neonatal rat epidermis as a model for permeation study. Therefore, the reported permeation parameters determined in the current investigation using Spectra/Por® membrane were compared with the corresponding values generated from permeation of identical tocopherol-containing formulations through the rat skin epidermis.

The composition of the tested T formulations, designated as T1-T13, is summarized in Table 1. The common ingredients are PG, ethanol, DMSO, TPGS, and T. T2 contains PEG 400 instead of PG, and T3 was free from T to ensure that no T is liberated from TPGS and reached to the receptor compartment. The T content was consistent, and formulations were stable throughout the study period. The cumulative amount of diffused T through each type of the tested membranes per unit area ($\mu\text{g}/\text{cm}^2$) was plotted versus time [Figures 4 and 5]. Subsequently, the permeation coefficient (Ps) and flux (J) values generated from the *in vitro* and *ex vivo* studies are summarized in Tables 2 and 3. From these results, it is obvious that the permeation of T from the different formulations through the synthetic membrane is generally following a similar pattern as the animal model. Moreover, Spectra/Por exhibited excellent power to differentiate between the tested formulations regarding the permeation of T, similar to the general pattern of the animal model. This also demonstrates the potential of the suggested synthetic membrane as a useful model to study the permeation of T, at least for the initial formulation phase.

It is worth mentioning here that the generally observed small cumulative amounts of T in this study are due to the fact that tocopherol is characterized by large molecular weight and extremely hydrophobic nature ($\log P = 9.3\text{--}13.4$);^[12] both will eventually hinder dramatically the permeation process. This is in agreement with previous studies, where the Ps of T was almost zero, in comparison with other water-soluble and water-insoluble vitamins employing reconstructed human epidermis and synthetic membranes.^[7]

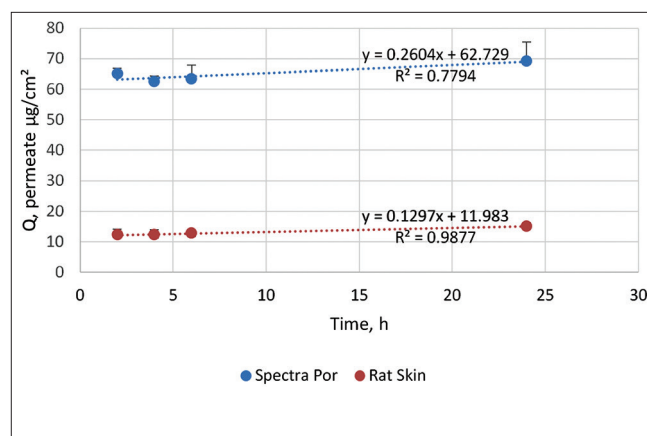


Figure 3: Cumulative amount of tocopherol permeated *ex vivo* through the neonatal rat epidermis and *in vitro* through Spectra/Por®, from the control tocopherol (1 mL = 0.95 g without any solvent)

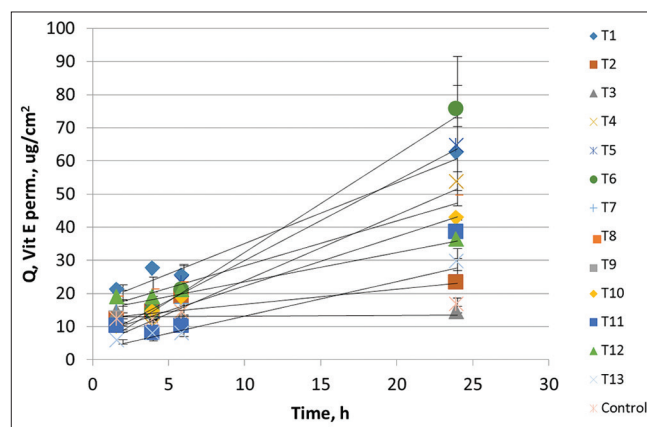


Figure 4: Cumulative amount of tocopherol permeated through the neonatal epidermis versus time

Correlation between actual and predicted permeation parameters

The flux values (J) and the permeation coefficients (Ps) calculated from the *in vitro* and *ex vivo* studies were well correlated. Linear relationships were observed for both parameters with r values equal to 0.84 and 0.82 for J and Ps, respectively [Figures 6 and 7]. The established straight lines were tested to predict the *ex vivo* parameters J and Ps from the determined *in vitro* data generated from the tested four solvent systems, namely, PG, ethanol, PEG-ethanol, and ISPM. The predicted and the actual values of J and Ps are summarized in Table 4, which indicate that although both predicted and actual values are not identical, they have the general trend. This observation is reflected in the same rank of each of the predicted and actual J and Ps according to the following order:

PG > ethanol > PEG-ethanol > ISPM. This finding also highlights the usefulness of the Spectra® Por as a successful alternative for mammalian skin models, capable of discriminating

Table 2: Permeation coefficients and flux values of the monophasic-TPGs formulations (T1-T13) - Spectra/Por® membrane

Formulation	J, Slope (µg/cm ² /h)	Cd, µg/ml	Ps (cm/h), J/Cd
T1	0.8884	90470	9.82E-06
T2	0.9384	90470	1.04E-05
T3*	-	-	-
T4	1.4532	86360	1.68E-05
T5	1.5721	82600	1.90E-05
T6	1.3616	92680	1.47E-05
T7	1.4984	47500	3.15E-05
T8	1.0116	95000	1.06E-05
T9	1.1886	95000	1.25E-05
T10	0.6816	47500	1.43E-05
T11	0.8213	95000	8.65E-06
T12	0.4982	47500	1.05E-05
T13	0.7273	47500	1.53E-05

*Tocopherol=0.0%

Table 3: Permeation coefficients and flux values of the monophasic-TPGs formulations (T1-T13)- neonatal rat skin

Formulation	J, Slope (µg/cm ² /h)	Cd, µg/ml	Ps (cm/h), J/Cd
T1	1.8297	90470	2.02E-05
T2	0.4374	90470	4.83E-06
T3*	-	-	-
T4	1.9749	86360	2.29E-05
T5	2.4148	82600	2.92E-05
T6	2.9461	92680	3.18E-05
T7	1.0026	47500	2.11E-05
T8	1.2837	95000	1.35E-05
T9	1.022	95000	1.08E-05
T10	1.6316	47500	3.43495E-05
T11	1.4571	95000	1.53379E-05
T12	0.8835	47500	0.0000186
T13	1.0948	47500	2.30484E-05

*Tocopherol=0.0%

between different formulations to guide the choice of optimum formulations for the *ex vivo* and *in vivo* studies.

CONCLUSION

Good correlation was observed between the *in vitro* and *ex vivo* J and P_s values resulting from the studied formulations. The calculated P_s and J for T from the T formulations after the *ex vivo* study were in-line with the corresponding values calculated from Spectra/Por® (r=0.84 and 0.82, for P_s

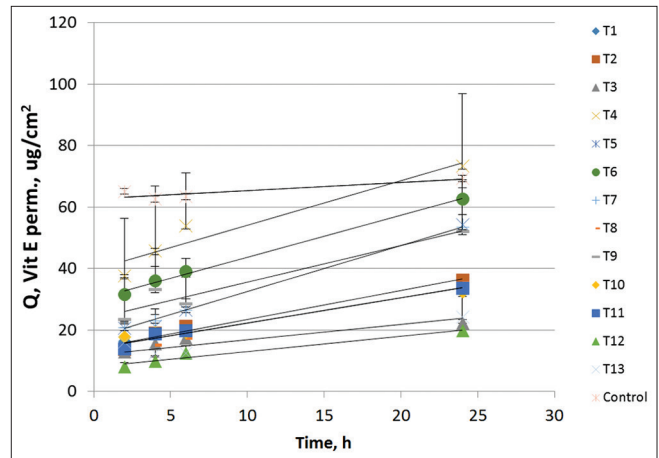


Figure 5: Cumulative amount of tocopherol permeated through Spectra/Por® versus time

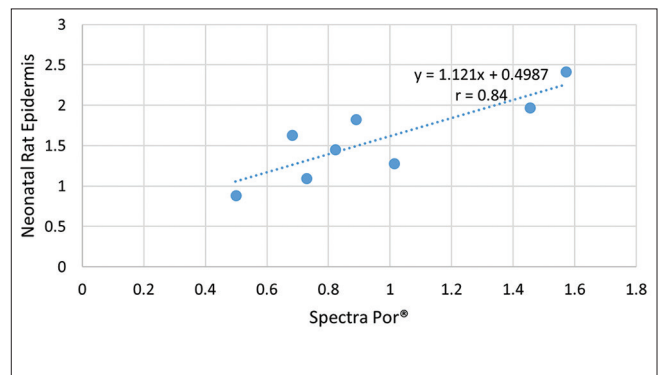


Figure 6: Correlation between J value calculated from neonatal rat skin and Spectra Por®, r = 0.84

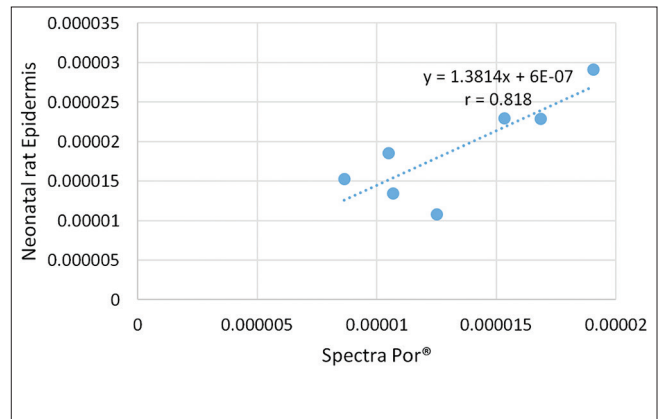


Figure 7: Correlation between P_s value calculated from neonatal rat skin and Spectra/Por®, r = 0.818

and J, respectively). The effect of vehicles tested on the permeation of T followed the same rank: PG> ethanol> PEG-ethanol> ISPM for both the animal and synthetic membrane. Furthermore, the predicted P_s and J values for the neonatal rat skin model, based on the correlation established between the two model membranes, followed also the same rank order, yet not the same observed values. The permeation efficiency of T from different vehicles and formulations through Spectra/

Table 4: Calculated and observed J and Ps values from the *in vitro* and *ex vivo* experiments

Vehicle	Permeation coefficient, Ps (cm/h)		Flux, ($\mu\text{g}/\text{cm}^2/\text{h}$)	
	Calculated Ps	Observed Ps	Calculated J	Observed J
ISPM	1.6177E-05	1.78E-06	1.13002	0.0888
PEG-ethanol	3.4339E-05	3.01E-06	1.866965	0.1507
Ethanol	5.6679E-05	1.0768E-5	2.773406	0.5384
PG	8.2318E-05	1.3722E-5	3.813694	0.6861

Por® was parallel to that estimated from *ex vivo* neonatal rat epidermis. The synthetic membrane demonstrated good discrimination between the tested formulations and therefore could be a successful surrogate to the animal models, at least in the initial formulation development and screening phase.

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