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Transdermal penetration of zalcitabine, lamivudine and synthesised *N*-acyl lamivudine esters

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Abstract

The objective of this study was to determine the *in vitro* transdermal permeation through human epidermis of zalcitabine, lamivudine and the synthesised *N*-acyl lamivudine esters, with and without the use of Pheroid™ as delivery system and to establish a correlation, if any, with selected physicochemical properties. Six *N*-acyl lamivudine esters were prepared by acylation of lamivudine with six different acid chlorides. The experimental aqueous solubility, log *D* and *in vitro* transdermal flux values were determined for these compounds. There was an inverse correlation between the aqueous solubility and the log *D* values. The median flux of zalcitabine (0.442 μmol/cm^2 h) in PBS was lower than that of lamivudine (4.289 μmol/cm^2 h), but in Pheroid™, lamivudine (0.011 μmol/cm^2 h) had a slightly lower median flux than zalcitabine (0.015 μmol/cm^2 h). Entrapment of compounds in Pheroid was confirmed by confocal laser scanning microscopy.
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Keywords: Zalcitabine; Lamivudine; *N*-acyl lamivudine esters; Skin penetration; Transdermal delivery

1. Introduction

As at the end of 2005, approximately 40.3 million people were living with HIV/AIDS (UNAIDS, 2006) which is generally treated with compounds like zalcitabine (**1**) and lamivudine (**2**). (**2**) is also used for treatment of hepatitis B (De Clercq, 2004). The most common adverse effects occurring with (**1**) and (**2**) are abdominal pain, insomnia, nausea, vomiting, diarrhoea (Gregg, 1999; Sweetman, 2002) and fatigue, as well as peripheral neuropathy (Merigan et al., 1989), cardiomyopathy, pancreatitis and mouth ulcers with (**1**) (Brinkman and Ter Hofstede, 1999; Flexner, 2006).

The skin, although an ideal site for drug administration, is also a major barrier to this process. Effective drug therapies must therefore overcome the challenge of finding a technology to administer, measure and deliver the required quantity of drug into or through the skin.

Research has found that a drug should have optimal permeation if it has reasonable solubility in both water and oils and has an aqueous solubility of more than 1 mg/ml and a log *P* in the range of 1–2 (Hadgraft, 1996; Roberts and Sloan, 2000). According to Guy and Hadgraft (1989) there are potential problems in achieving steady plasma concentrations if compounds have log *P* values higher than 2. A preferentially oil soluble drug may have difficulty leaving the stratum corneum and in contrast, an extremely polar drug will have trouble partitioning into the stratum corneum from its vehicle. Membranes are more permeable by the unionized forms, because of their greater lipid solubility (Abdou, 1989; Smith, 1990). In work done in our laboratories it was indicated that the compounds with the highest aqueous solubility presented with the greatest transdermal fluxes (Goosen et al., 2002; Monene et al., 2005; Gerber et al., 2006).

The adverse effects of (**1**) and (**2**) encouraged this study into the development of a transdermal delivery system with the aim of avoiding the poor palatability (of the liquid (**2**) formulation) (Schiffman et al., 1999) and hepatic first pass metabolism, improving patient compliance and bioavailability as well as decreasing the administered dose. There are numerous systems, like reservoir devices, matrix diffusion-controlled devices, multiple polymer devices, and multilayer matrix systems, available that effectively deliver drugs across the skin (Hadgraft and Lane, 2006) and some attention has also been given to the use of

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Pheroid™ to improve transdermal permeation. The development of compounds with enhanced physicochemical properties for greater transdermal delivery has been of interest.

Pheroid™ is a patented system consisting of an exceptional submicron emulsion formulation. Currently Pheroid™ comprise mostly of essential and plant fatty acids, i.e., ethyl esters of the essential fatty acids, oleic, linolenic and linoleic acids, which are emulsified in water and saturated with nitrous oxide. The essential fatty acids in the Pheroid™ system replenish the lack of lipids in the skin by means of merging with phospholipids and maintaining the integrity of the epidermal permeability barrier (Junginger et al., 1991; Touitou et al., 1994). Oleic acid increases transdermal permeation due to its kinked structure, by briefly disrupting the packed formation of the intercellular lipids (Touitou et al., 1994). Pharmacologically active compounds and other beneficial molecules can be entrapped, transported and delivered by Pheroid™ (Saunders et al., 1999), which in turn may increase therapeutic action (Grobler, 2004).

During this study, the aim was to determine the transdermal permeation of (**1**), (**2**) and the synthesised *N*-acyl lamivudine esters, with and without the use of Pheroid™ as delivery system and to establish a correlation, if any, with selected physicochemical properties.

2. Materials and methods

2.1. Materials

Aspen Pharmacare (Port Elizabeth, South Africa) and Roche Diagnostics GmbH (Mannheim, Germany) generously sponsored (**2**) and (**1**), respectively. The acid chlorides were purchased from Fluka and Sigma–Aldrich South Africa Ltd. HPLC grade acetonitrile was obtained from LabChem South Africa Ltd. All the other reagents and chemicals were of analytical grade.

2.2. General procedures

The ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini 300 spectrometer in $CDCl_3$, at a frequency of 300.075 and 75.462 MHz, respectively with tetramethylsilane as internal standard. The MS spectra were recorded on an analytical VG 7070E mass spectrometer fast atom bombardment (FAB) at 70 eV as ionisation technique. Melting points were determined by differential scanning calorimetry (DSC) with a Mettler Toledo DSC822e700 instrument.

2.3. High-pressure liquid chromatography (HPLC)

The HPLC system consisted of a HP (Hewlett Packard) Agilent 1100 series auto sampler, HP Agilent 1100 series variable wave detector (VWD) and HP Agilent 1100 series pump. A Phenomenex (Luna C-18, 150 mm × 4.60 mm, 5 μm) column was used together with a Securityguard pre-column (C-18, 4 mm × 3 mm) insert (Phenomenex) in order to prolong column life and the Agilent Chemstation for LC Systems software package was used for data analysis. The best UV maxima were determined with detection at 270 nm for (**1**) and (**2**) and 250 nm for the *N*-acyl lamivudine esters. The flow rate was 1.0 ml/min while the mobile phase compositions and retention times for each of the compounds are presented in Table 1. Orthophosphoric acid (OPA) and 0.2% triethylamine were used for (**1**) and (**2**) to adjust the pH of the mobile phase to 7.0. A different volume was injected for each of the compounds in order to compensate for the difference in concentration for each of the various derivatives and a correction calculation made. Recycling of the mobile phase did not adversely affect the HPLC analysis. Calibration curves were constructed ranging from concentrations of 1.97–475.0 μg/ml.

Table 1
HPLC conditions

Compound	Mobile phase H_2O:acetonitrile	Retention time (min)
Zalcitabine (**1**)	95:5	4.36
Lamivudine (**2**)	90:10	2.91
N-Acetyllamivudine-5′-acetate (**3**)	70:30	2.49
N-Propionyllamivudine-5′-propionate (**4**)	60:40	3.90
N-Butyryllamivudine-5′-butyrate (**5**)	40:60	3.43
N-Hexanoyllamivudine-5′-hexanoate (**6**)	30:70	5.32
N-Octanoyllamivudine-5′-octanoate (**7**)	10:90	5.10
N-Decanoyllamivudine-5′-decanoate (**8**)	10:90	12.53

2.4. HPLC conditions

2.4.1. Integrity

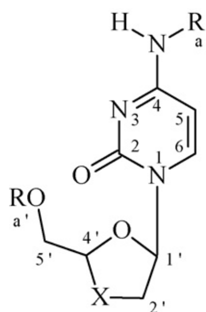
A Model 6401 LCR Databridge was used to determine the integrity of the skin before and after the transdermal procedure. Resistance readings (kΩ) were taken by immersing two stainless-steel test probe lead tips into the PBS filled donor and receptor compartments. The range of electrical resistance obtained in this study was between 2.2 and 31.0 kΩ.

2.5. Confocal laser scanning microscopy (CLSM)

The compounds containing Pheroid™ were viewed with the use of CLSM. The CLSM used was a PCM 2000 with Nikon inverted microscope and digital camera DXM 1200 equipped with a Helium/Neon Spectra-physics laser (red) with an excitation of 505 nm and emission of 568 nm and a Argon Spectra-physics laser (green) with an excitation of 488 nm and emission of 515 nm. An ApoPlanar oil immersion objective with a magnification of 60× and numerical aperture of 1.4 was used to determine the size of the Pheroid™. The Pheroid™ were stained with Nile Red, placed on a glass slide and covered with a glass cover-slip. The glass slide and cover-slip were sealed together using adhesive to prevent the Pheroid™ from drying out. The slides were inverted and images were capture through the cover-slip side of the prepared samples.

2.5.1. Acylation

To a well-stirred mixture of (**2**) (1.0 mol) in dry pyridine was added an acid chloride (5.0 mol) to form a clear solution. Stir-



(**1**): R = H, X = CH_2
(**2**): R = H, X = S
(**3**): R = $COCH_3$, X = S
(**4**): R = $COCH_2CH_3$, X = S
(**5**): R = $CO(CH_2)_2CH_3$, X = S
(**6**): R = $CO(CH_2)_4CH_3$, X = S
(**7**): R = $CO(CH_2)_6CH_3$, X = S
(**8**): R = $CO(CH_2)_8CH_3$, X = S

Fig. 1. Compounds (**1**–**8**).

ring was continued for 2 h where after distilled water was added to stop the reaction followed by an excess of dichloromethane. The organic phase was collected and washed with water at least three times to get rid of the pyridine. The organic phase was then dried over anhydrous $MgSO_4$, the dichloromethane removed under vacuum and the resulting product was collected. The prepared compounds were purified using column chromatography on silica gel (with EtOAc:DCM (9:1) for (**3**–**5**) and (**8**); EtOAc for (**6**) and (**7**); MeOH:DCM (8:2); for (**4**); EtOH:DCM (1:9) for (**5**) and diethyl ether for (**7**)) followed by recrystallisation from EtOAc or MeOH (DCM = dichloromethane) (Fig. 1).

*2.5.1.1. N-acetyllamivudine-5′-acetate(**3**).* A yield of 2.1 g (28.7%) off-white crystalline compound was obtained. m.p. 168.06 °C, $C_{12}H_{15}N_3O_5S$, ^{1}H NMR δ (ppm) 2.09 (s, 3H, H-b), 2.26 (s, 3H, H-b′), 3.16 (dd, 1H, J = 12.5, 3.3 Hz, H-5′b), 3.60 (dd, 1H, J = 12.5, 5.4 Hz, H-5′a), 4.40 (dd, 1H, J = 12.4, 3.1 Hz, H-2′b), 4.58 (dd, 1H, J = 12.5, 5.1 Hz, H-2′a), 5.37 (dd, 1H, J = 5.1, 3.1 Hz, H-1′), 6.29 (dd, 1H, J = 5.3, 3.3 Hz, H-4′), 7.43 (d, 1H, J = 7.6 Hz, H-5), 8.10 (d, 1H, J = 7.6 Hz, H-6), 10.07 (s, 1H, OH). ^{13}C NMR δ (ppm) 20.63 (C-b), 24.81 (C-b′), 38.78 (C-4), 63.57 (C-2), 84.69 (C-5′), 87.89 (C-4′), 96.47 (C-1′), 144.23 (C-5/C-6), 154.79 (C-2′), 163.13 (C-6/C-5), 170.14 (C-a), 171.08 (C-a′). FAB 314 (($M + H^+$) 35.5%), 154 (100.0%), 136 (56.0%), 120 (9.5%), 107 (22.5%). ν_{max} (KBr, cm^{-1}) 1059.81, 1134.49, 1184.38, 1656.81, 1715.40, 1741.19, 3256.47.

*2.5.1.2. N-propionyllamivudine-5′-propionate(**4**).* A yield of 4.7 g (61.9%) orange glass compound was obtained. m.p. 217.45 °C, $C_{14}H_{19}N_3O_5S$, ^{1}H NMR δ (ppm) 1.16 (t, 6H, J = 7.6 Hz, H-c, H-c′), 2.38 (m, 2H, H-b), 2.51 (m, 2H, H-b′), 3.17 (dd, 1H, J = 12.5, 3.2 Hz, H-5′b), 3.61 (dd, 1H, J = 12.6, 5.4 Hz, H-5′a), 4.42 (dd, 1H, J = 12.5, 3.1 Hz, H-2′b), 4.59 (dd, 1H, J = 12.5, 4.7 Hz, H-2′a), 5.35 (dd, 1H, J = 4.8, 3.1 Hz, H-1′), 6.31 (dd, 1H, J = 5.4, 3.3 Hz, H-4′), 7.43 (d, 1H, J = 7.5 Hz, H-5), 8.12 (d, 1H, J = 7.6 Hz, H-6), 9.48 (br s, 1H, OH). ^{13}C NMR δ (ppm) 8.68 (C-c), 8.95 (C-c′), 27.30 (C-b), 30.67 (C-b′), 38.92 (C-4), 63.34 (C-2), 84.93 (C-5′), 87.86 (C-4′), 96.32 (C-1′), 144.24 (C-5/C-6), 154.94 (C-2′), 162.84 (C-6/C-5), 173.66 (C-a), 174.38 (C-a’). FAB 342 (($M + H^+$) 47.5%), 168 (100.0%), 154 (15.5%), 136 (22.0%), 112 (66.0%). ν_{max} (KBr, cm^{-1}) 1053.16, 1163.35, 1569.82, 1743.77, 3560.03.

*2.5.1.3. N-butyryllamivudine-5′-butyrate(**5**).* A yield of 1.3 g (16.3%) white crystalline compound was obtained. m.p. 106.36 °C, $C_{16}H_{23}N_3O_5S$, ^{1}H NMR δ (ppm) 0.91 (t, 6H, J = 7.4 Hz, H-d, H-d′), 1.66 (m, 4H, H-c, H-c′), 2.31 (t, 2H, J = 7.3 Hz, H-b), 2.47 (t, 2H, J = 7.2 Hz, H-b′), 3.15 (dd, 1H, J = 12.5, 3.2 Hz, H-5′b), 3.58 (dd, 1H, J = 12.5, 5.4 Hz, H-5′a), 4.38 (dd, 1H, J = 12.5, 3.2 Hz, H-2′b), 4.62 (dd, 1H, J = 12.5, 4.8 Hz, H-2′a), 5.35 (dd, 1H, J = 4.9, 3.1 Hz, H-1′), 6.29 (dd, 1H, J = 5.4, 3.3 Hz, H-4′), 7.45 (d, 1H, J = 7.6 Hz, H-5), 8.10 (d, 1H, J = 7.6 Hz, H-6), 9.34 (s, 1H, OH). ^{13}C NMR δ (ppm) 13.51 (C-d), 13.57 (C-d′), 18.24 (C-c), 18.27 (C-c′), 35.83 (C-b), 38.89 (C-b′), 39.35 (C-4), 63.31 (C-2), 84.86 (C-5′), 87.89 (C-4′), 96.27 (C-1′), 144.25 (C-5/C-6), 154.82 (C-2′), 162.82 (C-6/C-5), 172.84 (C-a), 173.54 (C-a′). FAB 371 (($M + H^+$) 38.0%), 189 (12.0%), 182 (100.0%), 138 (9.0%), 112 (40.5%). ν_{max} (KBr, cm^{-1}) 1065.04, 1134.14, 1159.52, 1655.52, 1719.59, 1742.69, 3246.02.

*2.5.1.4. N-hexanoyllamivudine-5′-hexanoate(**6**).* A yield of 0.9 g (24.4%) white crystalline compound was obtained. m.p. 133.84 °C, $C_{20}H_{31}N_3O_5S$, ^{1}H NMR δ (ppm) 0.86 (t, 6H, J = 6.86 Hz, H-f, H-f′), 1.30 (m, 8H, H-e, H-e′, H-d, H-d′), 1.64 (m, 4H, H-c, H-c′), 2.35 (t, 2H, J = 7.5 Hz, H-b), 2.46 (t, 2H, J = 7.5 Hz, H-b′), 3.16 (dd, 1H, J = 12.5, 3.3 Hz, H-5′b), 3.60 (dd, 1H, J = 12.5, 5.4 Hz, H-5′a), 4.39 (dd, 1H, J = 12.4, 3.2 Hz, H-2′b), 4.63 (dd, 1H, J = 12.5, 4.9 Hz, H-2′a), 5.36 (dd, 1H, J = 4.9, 3.1 Hz, H-1′), 6.30 (dd, 1H, J = 5.3, 3.2 Hz, H-4′), 7.44 (d, 1H, J = 7.5 Hz, H-5), 8.10 (d, 1H, J = 7.6 Hz, H-6), 9.14 (s, 1H, OH). ^{13}C NMR δ (ppm) 13.80 (C-f, C-f′), 22.23 (C-e), 22.29 (C-e′), 24.48 (C-d, C-d′), 31.14 (C-c), 31.18 (C-c′), 33.96 (C-b), 37.59 (C-b′), 38.88 (C-4), 63.37 (C-2), 84.84 (C-5′), 87.94 (C-4′), 96.21 (C-1′), 144.25 (C-5/C-6), 154.82 (C-2′), 162.74 (C-6/C-5), 173.04 (C-a), 173.53 (C-a′). FAB 426 (($M + H^+$) 40.5%), 210 (100.0%), 154 (17.0%), 136 (22.5%), 112 (70.0%). ν_{max} (KBr, cm^{-1}) 1110.53, 1152.38, 1183.82, 1663.06, 1703.02, 1732.37, 3337.89.

*2.5.1.5. N-octanoyllamivudine-5′-octanoate(**7**).* A yield of 2.1 g (49.9%) light yellow crystalline compound was obtained. m.p. 93.89 °C, $C_{24}H_{39}N_3O_5S$, ^{1}H NMR δ (ppm) 0.82 (t, 6H, J = 6.3 Hz, H-h, H-h′), 1.27 (m, 16H, H-g, H-g′, H-f, H-f′, H-e, H-e′, H-d, H-d′), 1.65 (m, 4H, H-c, H-c′), 2.36 (dt, 4H, J = 25.2, 7.5 Hz, H-b, H-b′), 3.16 (dd, 1H, J = 12.6, 3.2 Hz, H-5′b), 3.62 (dd, 1H, J = 12.5, 5.4 Hz, H-5′a), 4.38 (dd, 1H, J = 12.4, 3.1 Hz, H-2′b), 4.64 (dd, 1H, J = 12.5, 4.9 Hz, H-2′a), 5.37 (dd, 1H, J = 4.9, 3.1 Hz, H-1′), 6.31 (dd, 1H, J = 5.3, 3.2 Hz, H-4′), 7.45 (d, 1H, J = 7.6 Hz, H-5), 8.05 (d, 1H, J = 7.6 Hz, H-6), 9.42 (br s, 1H, OH). ^{13}C NMR δ (ppm) 13.98 (C-h, C-h′), 22.51 (C-g, C-g′), 22.53 (C-f, C-f’), 24.82 (C-e), 28.84 (C-e′), 28.90 (C-d), 28.97 (C-d′), 29.01 (C-c), 31.58 (C-c′), 34.02 (C-b), 37.62 (C-b′), 38.92 (C-4), 63.34 (C-2), 84.93 (C-5′), 87.95 (C-4′), 96.13 (C-1′), 144.43 (C-5/C-6), 154.63 (C-2′), 162.78 (C-6/C-5), 173.04 (C-a), 173.62 (C-a′). FAB 483 (($M + H^+$) 32.0%), 239 (100.0%), 138 (19.5%), 127 (70.0%), 112 (88.0%). ν_{max} (KBr, cm^{-1}) 1053.34, 1140.31, 1185.29, 1665.79, 1713.87, 1737.95, 3305.49.

*2.5.1.6. N-decanoyllamivudine-5′-decanoate(**8**).* A yield of 1.9 g (39.0%) off-white crystalline compound was obtained. m.p. 88.61 °C, $C_{28}H_{47}N_3O_5S$, ^{1}H NMR δ (ppm) 0.84 (t, 6H, J = 6.73 Hz, H-j, H-j′), 1.25 (m, 24H, H-i, H-i′, H-h, H-h′, H-g, H-g′, H-f, H-f′, H-e, H-e′, H-d, H-d′), 1.65 (m, 4H, H-c, H-c′), 2.39 (dt, 4H, J = 26.2, 7.5 Hz, H-b, H-b′), 3.17 (dd, 1H, J = 12.5, 3.3 Hz, H-5′b), 3.60 (dd, 1H, J = 12.5, 5.4 Hz, H-5′a), 4.40 (dd, 1H, J = 12.4, 3.2 Hz, H-2′b), 4.63 (dd, 1H, J = 12.5, 4.9 Hz, H-2′a), 5.36 (dd, 1H, J = 4.9, 3.2 Hz, H-1′), 6.30 (dd, 1H, J = 5.3, 3.2 Hz, H-4′), 7.45 (d, 1H, J = 7.6 Hz, H-5), 8.10 (d, 1H, J = 7.6 Hz, H-6), 9.06 (s, 1H, OH). ^{13}C NMR δ (ppm) 14.03 (C-j, C-j′), 22.60 (C-i, C-i′), 24.82 (C-h, C-h′), 29.03 (C-g, C-g′), 29.06 (C-f, C-f′), 29.17 (C-e, C-e′), 29.19 (C-d), 29.26 (C-d′), 29.35 (C-c), 31.80 (C-c′), 34.02 (C-b), 37.66 (C-b′), 38.91 (C-4), 63.38 (C-2), 84.88 (C-5′), 87.96 (C-4′), 96.20 (C-1′), 144.27 (C-5/C-6), 154.83 (C-2′), 162.71 (C-6/C-5), 173.05 (C-a), 173.46 (C-a′). FAB 538 (($M + H^+$) 44.0%), 266 (100.0%), 155 (59.0%), 138 (20.0%), 112 (78.0%). ν_{max} (KBr, cm^{-1}) 1042.86, 1136.83, 1191.03, 1667.39, 1709.61, 1726.46, 3312.65.

2.6. Physicochemical properties

2.6.1. Solubility determination

The aqueous solubility of compounds (**1**–**8**) was obtained by preparing saturated solutions in a phosphate buffer solution (PBS) at pH 5 and 7. The slurries were stirred with magnetic bars in a water bath at 32 °C for 24 h. An excess of solute was present at all times to provide saturated solutions. The solutions were filtered after 24 h, diluted (**1**–**3**) and analysed directly by HPLC to determine the concentration of solute dissolved in the solvent. The experiment was done in triplicate.

2.6.2. Experimental log *D*

Equal volumes of *n*-octanol and PBS (pH 5 and 7) were saturated with one another under vigorous stirring for at least 24 h and then separated. An excess of compounds (**1**–**8**) was dissolved in 0.5 ml pre-saturated *n*-octanol and 0.5 ml pre-saturated PBS buffer, stoppered and agitated for 75 min. thereafter at 25 °C (**1**) and (**2**) were centrifuged at 4000 rpm for 20 min, (**3**–**5**) were centrifuged at 12000 rpm for 20 min and (**6**–**8**) were centrifuged at 12000 rpm for 40 min. An excess solute was present at all times. The *n*-octanol and aqueous phases were separately analysed by HPLC. The aqueous and the *n*-octanol phases were diluted with PBS and MeOH, respectively, prior to being analysed by HPLC. The log *D* values (log (octanol:PBS at both pH 5 and 7 partition coefficient)) were calculated as logarithmic ratios of the concentrations in the *n*-octanol phase to the concentrations in the PBS. The experiment was done in triplicate.

2.7. Permeation experiments

2.7.1. Preparation of donor solutions

Donor solutions of compounds (**1**–**8**) were obtained by the equilibration of excess amounts of solvent in PBS at pH 5 and Pheroid™ (formulated by the Department of Pharmaceutics, School of Pharmacy, North-West University, Potchefstroom, South Africa) in a water bath. The slurries were prepared in stoppered flasks; stirring in a water bath at 32 °C over a period of 24 h, in order for solvent saturation to occur. An excess amount of solute was present at all times.

2.7.2. Skin preparation

Female human abdominal skin, obtained after cosmetic procedures, was used for the permeation studies (human ethics approval reference number 04D08). A scalpel was used to separate the skin from the fat layer; subsequently the epidermis was removed by means of immersion in 60 °C HPLC water for 60 s (Kligman and Christophers, 1963).

The epidermis was gently teased away from the skin with forceps. Special care was taken that the integrity of the epidermis was not ruptured, as this would compromise the validity of the results. The epidermis was placed in a bath filled with HPLC water and carefully set on Whatman® filter paper, left to air dry and was wrapped in foil. The foil containing the epidermis was stored in a freezer at −20 °C and was used within 3 months after being prepared. Prior to use, the epidermis was thawed and examined (visually and by measuring the electrical resistance) for any defects, before it was mounted on the Franz diffusion cells.

2.7.3. Method for skin permeation

Vertical Franz diffusion cells with 2.0 ml receptor compartments and 1.0751 cm^2 effective diffusion area was used for the permeation studies. The epidermal skin layer was carefully mounted on the lower half of the Franz cell with the stratum corneum facing upwards. Leakage of the diffusion cells were prevented by applying vacuum grease. A clamp was used to fasten the upper and lower parts of the Franz cell together, with the epidermis separating the donor and receptor compartments. The receptor compartments were filled with isotonic PBS (pH 7.4). Special care was taken that no air bubbles came between the buffer solution and the epidermis. The donor compartments filled with 1.0 ml PBS (pH 5) were equilibrated at 37 °C for 1 h in the water bath and the integrity (electrical resistance) of the epidermis measured, prior to the addition of the saturated solutions. Only the receptor compartments were submerged in the water and were equipped with stirring magnets. After a period of 1 h, 1.0 ml of freshly prepared saturated solution (in PBS (pH 5) or Pheroid™) was added to each donor compartment, which was immediately covered with Parafilm® to prevent the evaporation of any constituents from the saturated solution for the duration of the experiment. An excess amount of solute was present in the donor compartments at all times during the experimental procedure.

The entire receptor volumes were withdrawn and replaced with 37 °C fresh buffer solution (pH 7.4) after 2, 4, 6, 8, 10, 12 and 24 h. The entire receptor volumes were withdrawn to mimic sink conditions as they occur in the human body. The experiments were conducted over 24-h periods.

The withdrawn samples were assayed immediately by HPLC to determine the drug concentration of compounds (**1**–**8**) that had permeated the epidermis. No extra peaks were observed in the chromatograms of the receptor phase of compounds (**1**–**8**) in

PBS. Except for the cells that leaked, all data obtained were used (7–10 cells/compound). At least six data points on the steady-state part of the curve were used. Since the data did not follow a normal distribution, but a skewed distribution the median flux was statistically obtained.

2.8. Statistical methods

When studying the correlation between median flux and the physicochemical properties the Spearman rank correlation was used, since this is a more robust estimate of correlation. The Pearson correlation coefficient was not used due to large variations (big spread) in some of the variables. The Spearman correlation is inversely related (strongly negative) if it is close to −1 and has a direct relationship (strongly positive) if it is close to 1. A 5% level of significance was used to test the hypothesis of zero correlation. Thus, if the *p*-value was less than 0.05 the Spearman correlation can be seen as statistically significant, hence either a significant positive or negative value was found. The correlations between flux and the physicochemical properties for both PBS and Pheroid™, together with the *p*-values are reported in Table 3.

3. Results and discussion

3.1. Structures of the products

3.1.1. N-propionyllamivudine-5′-propionate(4)

In the IR spectrum the ether (C–O) stretching vibration was at 1053.16 cm^{-1} and the stretching vibrations of two carbonyl groups (C=O) were at 1659.82 (mono amide) and 1743.77 cm^{-1} (acetate). These data verify the structure of (**4**).

The ^{13}C NMR data of (**4**) were similar to that of (**2**), except that the carbonyl carbon atoms moved slightly: C-4 and C-2 have shifted to a slightly lower magnetic field at δ 38.92 and 63.34, respectively in the ^{13}C NMR spectrum. The carbonyl carbon atoms were indicated by the signals at δ 173.66 and 174.38 representing the carbon atoms C-a/C-a′ and C-a′/C-a, respectively. In the IR spectrum the stretching vibrations of two carbonyl groups (C=O) were at 1659.82 (mono amide) and 1743.77 cm^{-1} (acetate). The structural properties of the remaining *N*-acyl lamivudine esters were consistent with that of (**4**) and the structures of all the *N*-acyl lamivudine esters were determined in the same fashion.

3.2. Physicochemical properties

The aqueous solubility of compounds (**1**–**5**) was higher at pH 5 than at pH 7. Hence, pH 5 was selected for diffusion studies since unionized compounds are more lipophilic and penetrate the stratum corneum with less difficulty than the ionized species (Abdou, 1989; Smith, 1990; Jack et al., 1991). The aqueous solubility of (**2**) at both pH 5 and 7 was higher than that of (**1**), but was distinctly higher than that of the synthesised compounds (**3**–**6**). (**4**) has the highest aqueous solubility value of all the *N*-acyl lamivudine esters. The aqueous solubility on a mass (mg/ml) as well as on a molar basis (mM) of (**1**) in both pH

Table 2
Aqueous solubility, partition coefficient and median flux of compounds (**1**–**8**)

Compound	Aqueous solubility at pH 5 (mg/ml)	Aqueous solubility at pH 7 (mg/ml)	Aqueous solubility at pH 5 (mM)	Aqueous solubility at pH 7 (mM)	Log *D* at pH 5	Log *D* at pH 7	Median flux in PBS (μmol/(cm^2 h))	Median flux in Pheroid™ (μmol/(cm^2 h))
(**1**)	114.36 ± 9.36	91.57 ± 3.79	541.42	433.53	−1.50	−1.78	0.442	0.015
(**2**)	144.78 ± 29.61	100.16 ± 4.74	631.53	436.90	−1.19	−1.15	4.289	0.011
(**3**)	3.98 ± 0.05	3.36 ± 0.05	12.70	10.72	0.12	0.25	0.046	1.3×10^{-3}
(**4**)	8.34 ± 0.12	6.16 ± 0.50	24.43	18.04	1.70	1.88	0.013	9.3×10^{-3}
(**5**)	0.28 ± 0.00	0.20 ± 0.01	0.76	0.54	2.51	2.55	1.8×10^{-3}	7.0×10^{-4}
(**6**)	$1.0 \times 10^{-4} \pm 0.00$	$1.0 \times 10^{-4} \pm 0.00$	2.0×10^{-4}	2.0×10^{-4}	4.55	4.88	2.0×10^{-4}	2.0×10^{-4}
(**7**)	Insoluble[a]	Insoluble[a]	Insoluble[a]	Insoluble[a]	–	–	–	–
(**8**)	Insoluble[a]	Insoluble[a]	Insoluble[a]	Insoluble[a]	–	–	–	–

– Data not available.
[a] Solubility less than the detection limit of HPLC.

5 and 7 was lower than that of (**2**). The octanol–PBS partition coefficient (log *D*) of compounds (**2–6**) was lower at pH 5 than at pH 7. Of all compounds (**1**) had the lowest log *D* at both pH 5 and 7. The log *D* of (**2**) at both pH 5 and 7 was lower than that of compounds (**3–6**). Table 2 shows that in the straight chain series (**2**), (**3**), (**5**) and (**6**) the aqueous solubility on a molar basis decreases and the log *D* increases with an increase in alkyl chain length, which is in accordance with the findings of Flynn and Yalkowsky (1972), Abdou (1989) and Gerber et al. (2006). (**7**) and (**8**) were highly lipophilic and insoluble in an aqueous medium and therefore their aqueous solubility and log *D* values could not be determined experimentally by HPLC or otherwise.

3.3. Transdermal properties

The median flux of (**2**) in PBS was 10 times higher than that of (**1**), but in Pheroid™, (**1**) had a slightly higher median flux than (**2**). In both PBS and Pheroid™, the median flux of (**2**) was higher than that of the compounds (**3–6**). Of all the compounds (**3–6**), (**3**) in PBS and (**4**) in Pheroid™ presented the highest flux. In PBS, the flux values of the compounds (**3–6**) decreased as the alkyl chain length increased. Hence, it seems that as the compound became more lipophilic and its aqueous solubility decreased, it had trouble leaving the stratum corneum and permeating into the aqueous receptor phase. In Pheroid™, the same phenomenon as in PBS occurred with the *N*-acyl lamivudine esters, except for (**4**) that had an increase in flux (with almost the same flux value as **2**).

When comparing flux in PBS with that in Pheroid™ it is observed that all the compounds have lower flux in Pheroid™ except (**5**). Hence, Pheroid™ does not improve transdermal flux of this series of compounds.

In this study a direct correlation between the aqueous solubility and transdermal flux was found. It is furthermore interesting to note that the three compounds, i.e. (**1**), (**2**) and (**4**) which had the highest median flux values in Pheroid™, also had the highest aqueous solubility values. Hence, it seems that when using Pheroid™ as delivery system to increase transdermal permeation, a more hydrophilic drug should be used.

The predicted flux, attained from using the Potts and Guy equation (Hadgraft et al., 2000) did not correlate well with the experimental flux values obtained in this study. Thus, the only reliable way of determining these data is by experimental means (Table 3).

A strong statistically significant correlation was observed between flux in both PBS and in Pheroid™ and each of molecular weight, aqueous solubility (at pH 5 and 7), and log *D* (at pH 5 and 7); as was determined with a 5% level of confidence using the Spearman correlation. Although there were no correlations found between median flux in both Pheroid™ and PBS and each of melting point and integrity of the skin (before and after), there was a better correlation between median flux and each of melting point and integrity of the skin (before and after) in Pheroid™ than in PBS.

Yellow spots were observed (Fig. 2) in the confocal laser scanning microscopy (CLSM) micrographs which confirmed that the compounds were entrapped in Pheroid™. As seen in Fig. 3, the more hydrophilic compounds had a decrease in microsponge size as they became more lipophilic from compounds (**1–5**) and thereafter an increase was noticed from (**5–8**). Hence, it seems that hydrophilic drugs permeate easier when entrapped in Pheroid™ than lipophilic compounds.

The aqueous solubility, log *D* and flux values of (**7**) and (**8**) could not be determined, because these two compounds are highly lipophilic and do not dissolve in PBS in either pH 5 or 7. With CLSM it is observed that (**7**) and (**8**) were entrapped in Pheroid™ and therefore if the receptor phase was milky, due to damaged skin, and injected on the HPLC the chromatograms showed small peaks in the first 6 h. Hence, during normal diffusion studies in Pheroid™ no peaks were visible for (**7**) and (**8**) and we can speculate that these compounds had trouble leaving the epidermis.

Previous investigations on (**1**) done by Kim and Chien (1996b) found higher flux values than the flux values obtained in this study (0.442 μmol/(cm^2 h)), due to the use of penetration enhancers such as ethanol and oleic acid, i.e. 0.61 (mg cm^2)/h (2.89 μmol/ (cm^2 h)) through human cadaver skin at 37 °C and 1.88 (mg cm^2)/h (8.90 μmol/(cm^2 h)) through hairless rat skin at 37 °C. Kim and Chien (1996a) had an remarkably lower flux value for (**1**) than their study in Kim and Chien (1996b) and this study, due to the use of water with a pH of almost 7 where (**1**) is more unionized, i.e. 3.19 (μg cm^2)/h (0.02 μmol/(cm^2 h)) through hairless rat skin at 37 °C.

Kim and Chien (1995) stated that the target rate for maintaining therapeutic blood level for (**1**) is 0.14 (mg cm^2)/h

Table 3
Spearman correlations between median flux and the physicochemical properties of compounds (**1**–**6**) in PBS and Pheroid™

Item	Physicochemical properties	Spearman correlation	*p* correlation	Spearman correlation	*p* correlation
1	Molecular weight (g/mol)	−0.9429	0.0048	−0.9429	0.0048
2	Aqueous solubility (pH 5) (mg/ml)	0.9429	0.0048	0.9429	0.0048
3	Aqueous solubility (pH 7) (mg/ml)	0.9429	0.0048	0.9429	0.0048
4	Log *D* (pH 5)	−0.9429	0.0048	−0.9429	0.0048
5	Log *D* (pH 7)	−0.9429	0.0048	−0.9429	0.0048
6	Melting point (°C)	0.4286	0.3965	0.6571	0.1562
7	Integrity before (kΩ)	0.2000	0.7040	−0.4286	0.3965
8	Integrity after (kΩ)	0.0857	0.8717	−0.2571	0.6228

Note: Although it seems highly improbable to obtain the very similar values for the Spearman correlation of such diverse properties as items **1**–**5** in this table, it was indeed found and confirmed in this study. This may be attributed to the data following the same pattern and the ranking process of the Spearman procedure.

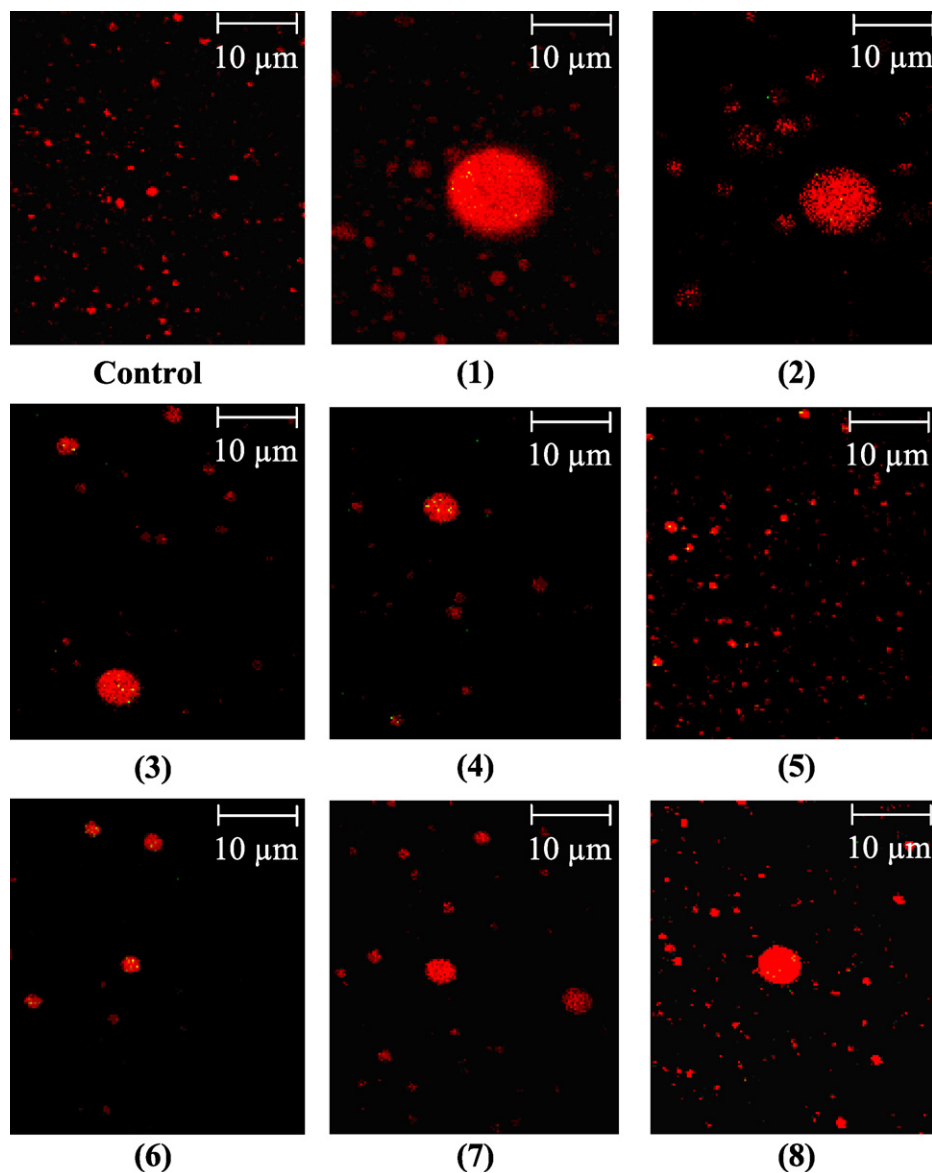


Fig. 2. The CLSM micrographs of the microsponge Pheroid^{TM} (control) and compounds (**1**–**8**) in entrapped in Pheroid^{TM} microsponges.

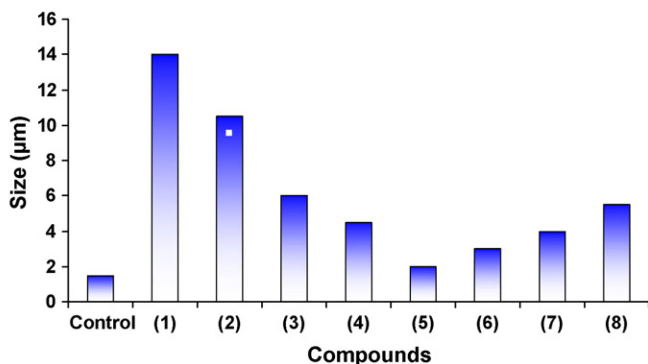


Fig. 3. A representation of the difference in microsponge size between the control and compounds (**1**–**8**) in microsponge Pheroid^{TM} formulation.

(0.663 μmol/(cm^2 h)), which median flux of (**1**) (0.442 μmol/(cm^2 h)) in this study does not achieve.

4. Conclusion

The *N*-acyl lamivudine esters were successfully synthesised and the structures were verified by ^{1}H and ^{13}C NMR and MS spectroscopy. The higher flux values in both PBS and Pheroid^{TM} of (**1**), (**2**) and (**4**) can be due to the aqueous solubilities being higher than 1 mg/ml. The aqueous solubility values of compounds (**5**) and (**6**) were far less than 1 mg/ml and they had log *D* values between higher than 2, which lead to lower flux values, due to the fact that the lipophilic compounds had difficulty leaving the stratum corneum. The more lipophilic *N*-acyl lamivudine esters may exhibit lower flux values than (**2**), since they are less water soluble. Pheroid^{TM} does not improve transdermal flux of

this series of compounds, although entrapment has taken place in Pheroid™ as seen in the micrographs taken in Fig. 2. The flux of (**2**) is also higher than that of any of the *N*-acyl lamivudine esters prepared in this study. Their transdermal application thus has no practical advantage.

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