Investigation of Permeation of Acyclovir through Skin Using Alaptide

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Received: 31 January 2017; accepted: 04 February 2017

The investigation deals with the affection of the permeation of acyclovir through full-thickness pig ear skin using a Franz diffusion cell from the donor vehicles of phosphate buffer (pH 7.4) and propylene glycol–water (1:1) using synthesised (*S*)-8-methyl-6,9-diazaspiro[4.5]decane-7,10-dione, alaptide as a transdermal permeation enhancer. Alaptide was applied in ratio 1:10 (*w*/*w*) relative to the amount of acyclovir. At the first hour after application, the permeated amount of acyclovir from propylene glycol–water system, simulating semisolid dosage forms, was ca. four-fold higher than from the formulation without alaptide. Despite that the enhancement ratio of alaptide in a steady state was 1.7, the pseudo-enhancement ratio of alaptide in the time range of first to third hour was 2.3. Both enhancement ratios indicate that alaptide modifies skin structure, while the short-term application of the alaptide formulation seems to be more advantageous.

Keywords: Acyclovir, Alaptide, Permeation, Skin, Franz diffusion cell, HPLC determination

Introduction

Transdermal therapeutic systems are drug delivery systems designed for systemic administration. Due to their pharmacokinetic advantages, they are a good alternative to traditional formulations with systemic administration. To reach the blood capillaries and achieve systemic therapeutic effect, a drug must be able to cross the skin barrier, especially the outermost layer, *stratum corneum* (SC), responsible for barrier function and formed by corneocytes and an intercellular lipid matrix. Unfortunately, transdermal drug delivery often faces the problem of insufficient or no permeation of drug substances through the skin. The use of chemical permeation enhancers (CPEs) is one of the approaches for facilitating drug delivery through the skin — modification of SC [1]. Small polar molecules containing a characteristic fragment of heteroatoms X−CO−N=, where X is $-CH_2-$, $-NH_2$, or −NH−, may break intermolecular *H*-bonds that hold ceramides together in the SC [2].

Based on the hypotheses of the mechanism of action of CPEs, 8-substituted 6,9-diazaspiro[4.5]decan-7,10-diones were prepared and evaluated as potential CPEs. One of them, (*S*)-8-methyl-6,9-diazaspiro[4.5]decan-7,10-dione, known by the INN of "alaptide" (Figure 1) is an original Czech compound prepared in the 1980s [3]. Alaptide was designed as an analogue of melanocyte-stimulating hormone release-inhibiting factor [4] and is able to influence the creation and function of keratinocytes. It was found that alaptide has significant skin curative activity [4] without any observed toxicity [4]. In addition, it demonstrated the excellent enhancement activity in a number of in vitro tests [5–9].

Acyclovir (Figure 1) is an antiviral drug from the class of antimetabolites that was discovered in 1977. It is used for the treatment of herpes simplex virus infections, chickenpox, and shingles. Other uses include prevention of cytomegalovirus infections following transplant and infections due to Epstein–Barr virus.

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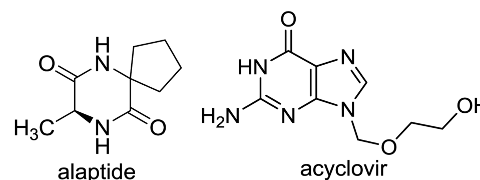

Figure 1. Structures of (*S*)-alaptide and acyclovir

Acyclovir is suitable for topical and systemic therapy [10, 11]. Since it is poorly water-soluble and has poor oral bioavailability [11], the effect of alaptide as a potential CPE on the permeation of acyclovir substance through the skin has been investigated.

Experimental

In Vitro Transdermal Permeation Experiments. Skin samples were obtained from porcine ear. Full-thickness skin was cut in fragments and stored at −20 °C until utilized. Skin samples were slowly thawed (at 4 °C overnight and then at ambient temperature) before each experiment [12, 13]. The penetration enhancing effect of alaptide was evaluated in vitro, using a vertical Franz diffusion cell (SES — Analytical Systems, Bechenheim, Germany) with a donor surface area of 0.6359 cm^2 and a receptor volume of 5.2 mL (see Figure 2). The skin was mounted between the donor and receptor compartments of the Franz diffusion cell with the epidermal side up. The receptor compartment was filled with phosphate buffered saline (pH 7.4) and maintained at 34.0 ± 0.5 °C [12, 14], using a circulating water bath. The receptor compartment content was continuously stirred using a magnetic stirring bar. The skin was kept in contact with the receptor phase for 0.5 h prior to the experiment. Acyclovir was purchased from Sigma-Aldrich (St. Louis, MO, USA); all other reagents and solvents were purchased from Merck (Darmstadt, Germany). Donor samples were prepared by dissolving acyclovir (10 mg) and alaptide (1 mg) either in phosphate buffered saline (pH 7.4) or in propylene glycol (0.5 mL), to which water (0.5 mL) was added

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DOI: 10.1556/1326.2017.00242
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First published online: 21 March 2017

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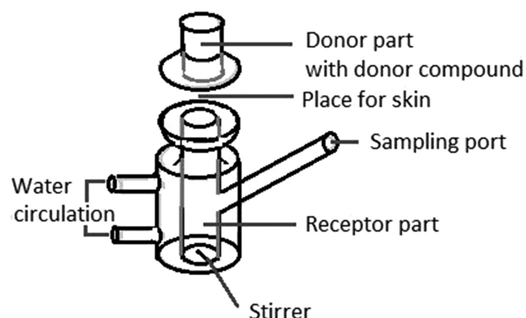

Figure 2. Scheme of vertical Franz diffusion cell

consequently to create a mixture of propylene glycol–water 1:1. These mixtures were shaken vigorously and then sonicated for 10 min at 40 °C; then, these stable systems (dissolved acyclovir in enhancer emulsion) were applied to the skin surface, and the donor compartment of the cell was covered by Parafilm®. Control samples were prepared in the same manner without alaptide. Samples (0.5 mL) of the receptor phase were withdrawn at predetermined time intervals (30, 60, 90, 120, 180, 240, 360, 480, 720, and 1440 min), and the cell was refilled with an equivalent amount of fresh buffer solution. A minimum of five determinations were performed using skin fragments from at least two animals for each compound, and the data were expressed as means ± SD. The samples were immediately analysed by high-performance liquid chromatography (HPLC).

Apparatus and Analytical Conditions

The analysis of the samples was performed using an Agilent 1200 series HPLC system, equipped with a diode array detection system, a quaternary model pump, and an automatic injector (Agilent Technologies, Santa Clara, CA, USA). Data acquisition was performed using ChemStation chromatography software. A Gemini C6-Phenyl 110A 5 μm, 250 × 4.6 mm (Phenomenex, Torrance, CA, USA) chromatographic column was used. The total flow of the column was 1.0 mL min^{-1}; injection was 10 μL; column temperature was 40 °C; and sample temperature was 20 °C. The detection wavelength of 254 nm was chosen, the time of analysis was 6 min. A mixture of acetonitrile (HPLC grade, 5.0%) and H_2O (HPLC — Milli-Q Grade, 95.0%) was used as a mobile phase. The retention time (t_R) of acyclovir was 4.91 ± 0.05 min; the limit of detection (LOD) was 0.0031 μg mL^{-1}; and the limit of quantification (LOQ) was 0.0104 μg mL^{-1}. A calibration curve is illustrated in Figure 3, the representative chromatogram of acyclovir standard is shown in Figure 4A, and the chromatogram of a permeated sample of acyclovir is illustrated in Figure 4B.

As a result of the sampling, the receptor compartment concentration of alaptide was corrected for sample removal and replenishment using equation: $C'_n = C_n\ (V_t/V_t - V_s)\ (C'_{n-1}/C_{n-1})$, where C'_n is corrected drug concentration in the nth sample; C_n, measured drug concentration in the nth sample; C'_{n-1}, corrected drug concentration in the $(n-1)^{th}$ sample; C_{n-1}, measured drug concentration in the $(n-1)^{th}$ sample; V_t, total volume of receptor solution; V_s, volume of the sample; and $C'_1 = C_1$ [15]. The corrected data were expressed as the cumulative drug permeation (Q_t) per unit of skin surface area using equation: $Q_t = C'_n/A$, where $A = 0.6359$ cm^2 in the experiment. From the slope of the linear portion of the curve [16] representing the dependence of the cumulative amount of the drug (Q_t [μg]) per unit area on time (t [h]), steady-state permeation flux (J [μg h^{-1} cm^{-2}]) was determined. Similarly, the lag time (t_{lag} [h]) was determined by extrapolating the linear portion of the cumulative amount of permeation per unit area (Q_t) versus time (t [h]) curve to the abscissa [17]. The permeability coefficient (K_p [cm/h]) can be calculated according to $K_p = J/C_d$, where C_d is drug concentration in the donor compartment. It is assumed that, under sink conditions, drug concentration in the receptor compartment is negligible compared to that in the donor compartment [18, 19]. The enhancement effect was expressed as an enhancement ratio (ER) that was calculated by the formula $ER = J_{ss\text{-}x}/J_{ss\text{-}k}$, where $J_{ss\text{-}x}$ is steady-state permeation flux with CPE and $J_{ss\text{-}k}$ is steady-state permeation flux without CPE [20]. All the calculated data are listed in Tables 1 and 2 and illustrated in Figure 5.

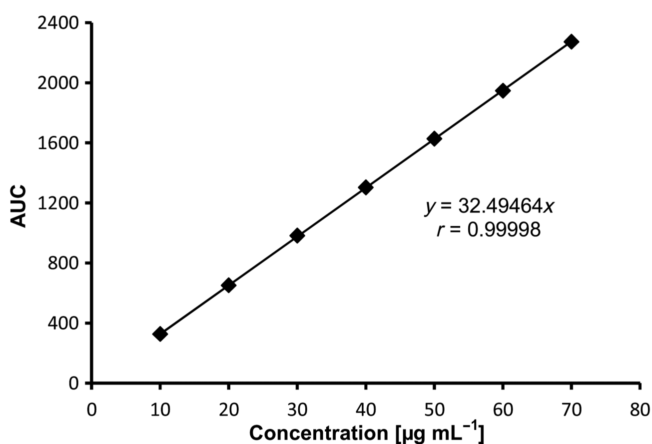

Figure 3. Calibration curve of acyclovir

Results and Discussion

Alaptide originally developed by Kasafirek was prepared according to Scheme 1 from protected L-alanine and ethyl ester of cycloleucine [21]. Due to the lack of active

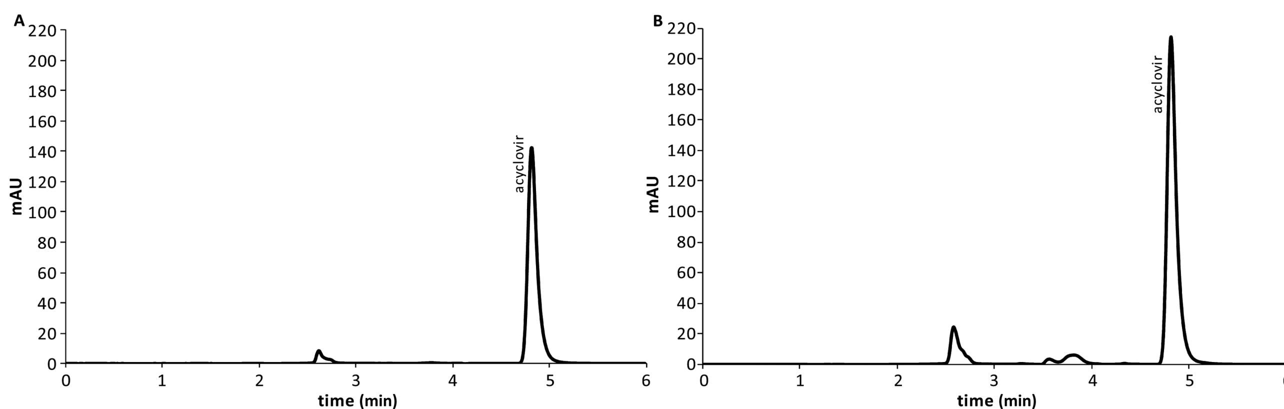

Figure 4. Chromatograms of acyclovir: standard at concentration 30 μg mL^{-1} (A); sample of permeated acyclovir from propylene glycol–water 1:1 at 12th hour (B)

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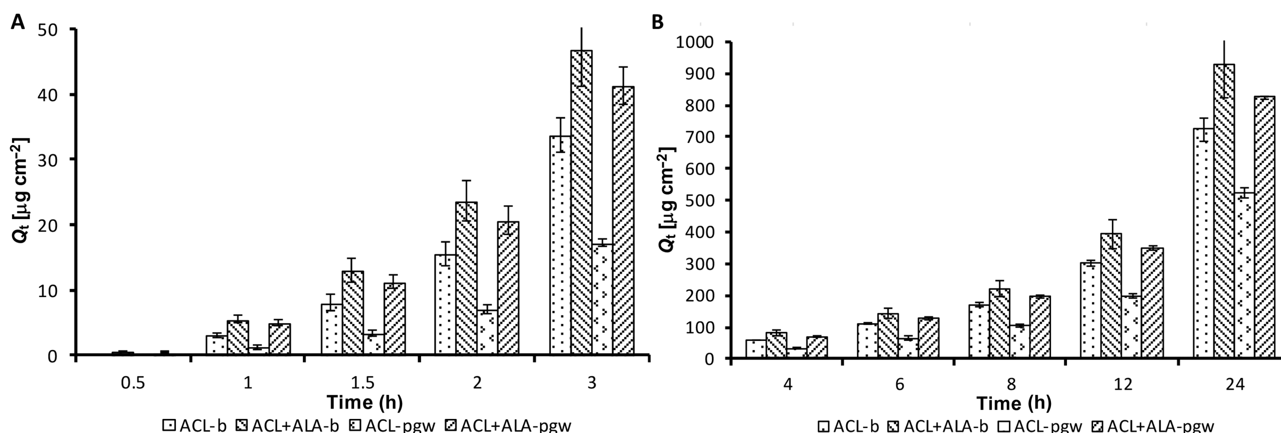
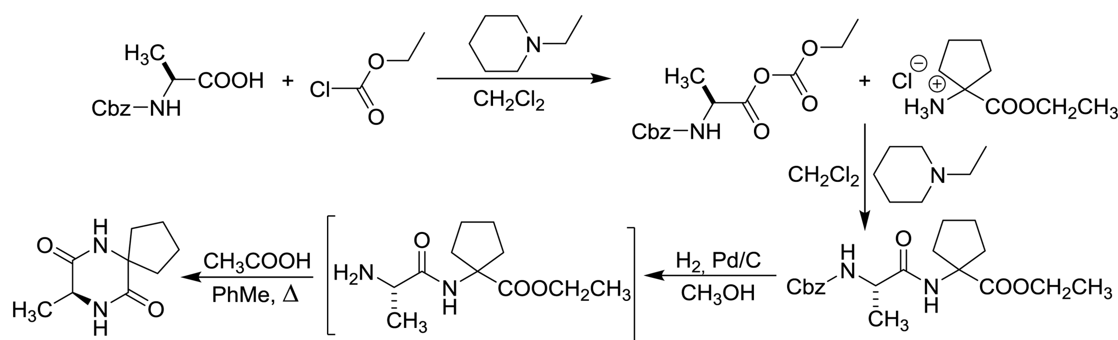


Figure 5. In vitro profile of cumulative permeated amounts Q_t per unit area (μg cm^{-2}) of acyclovir (ACL) alone and after addition of alaptide (ALA) in ratio 10:1 (ACL + ALA) from buffer pH 7.4 (b) and propylene glycol–water 1:1 (pgw) system through skin in time. Q_t values are expressed as mean ± SD (n = 5 experiments)



Scheme 1. Synthesis of alaptide

chromophore, the process of synthesis was extensively optimized using mass spectrometry.

In vitro skin permeation experiments were performed using static Franz diffusion cells [14] within 24 h. Full-thickness pig ear skin was selected for in vitro evaluation of permeation. This tissue is a suitable in vitro model of the human skin [22, 23], because porcine skin has shown to be histologically and biochemically similar to the human skin [24]. First, the permeation of acyclovir through the skin without and with alaptide was tested from the donor vehicle of phosphate buffer (pH 7.4) as a vehicle that maintains no permeation. The values obtained from the permeation experiments were expressed as the cumulative permeated amount of the drug (Q_t [μg]) per unit of skin surface area (see Table 1). The dependences of the cumulative permeated amount of the drug per unit of skin surface area in time are illustrated in Figure 5 that is divided into parts A and B for better lucidity.

Table 1. Cumulative permeated amounts Q_t of acyclovir per unit area (μg/cm^2) from buffer (pH 7.4) and propylene glycol–water 1:1 (pgw) without and with alaptide as CPE achieved in in vitro transdermal permeation experiments using Franz diffusion cell. Q_t values are expressed as mean ± SD (n = 5 experiments)

Time (h)	Cumulative permeated amounts Q_t per unit area (μg/cm^2)			
	Acyclovir		Acyclovir + alaptide	
	Buffer	pgw	Buffer	pgw
0.5	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 0.1	0.6 ± 0.1
1.0	3.1 ± 0.4	1.3 ± 0.4	5.6 ± 0.6	5.0 ± 0.4
1.5	8.1 ± 1.3	3.4 ± 0.4	13.0 ± 1.8	11.2 ± 1.0
2.0	15.6 ± 1.9	7.1 ± 0.7	23.6 ± 3.0	20.6 ± 2.2
3.0	33.7 ± 2.7	17.2 ± 0.6	46.8 ± 5.6	41.3 ± 2.9
4.0	59.9 ± 1.2	33.3 ± 3.0	80.4 ± 9.3	71.1 ± 2.5
6.0	111.3 ± 4.1	65.0 ± 5.8	142.9 ± 16.8	126.4 ± 5.0
8.0	170.8 ± 5.6	105.5 ± 5.8	221.4 ± 25.0	197.0 ± 5.0
12.0	300.7 ± 10.6	199.0 ± 5.5	392.4 ± 44.3	348.5 ± 6.1
24.0	721.9 ± 36.8	523.7 ± 17.2	925.7 ± 104.1	823.3 ± 6.4

As it is evident that the addition of alaptide caused acyclovir permeation enhancement, subsequently, permeation experiments from propylene glycol–water (1:1) system as a vehicle simulating semisolid formulations (gels, hydrocreams) were performed. It is important to note that previous studies have indicated that propylene glycol itself (or a propylene glycol–water system) does not interfere with membranes [25, 26]. The results, cumulative permeated amounts of acyclovir without and with alaptide, are summarized in Table 1 and illustrated in Figure 5. Other permeation parameters of acyclovir without and with alaptide from propylene glycol–water (1:1), steady-state permeation fluxes (J [μg h^{-1} cm^{-2}]), lag times (t_{lag} [h]), permeability coefficients (K_p [cm/h]), and enhancement ratios (ER) are mentioned in Table 2 (for time period 1–3 h, pseudo steady state) and Table 3 (for time period 6–12 h, real steady state).

The permeated amount of acyclovir with alaptide from propylene glycol–water increased rapidly already at the 30th minute similarly as from buffer. The permeated amount of acyclovir with alaptide at the 60th minute reached approx. four-fold higher values than from formulation without alaptide (see Table 1). Similarly, a sharp enhancement of transdermal permeation was found, e.g., for permeation of NSAIDs or antipyretics, such as ibuprofen, nimesulide, acetylsalicylic acid,

Table 2. Pseudo-permeation parameters of acyclovir in the range first to third hour without and with alaptide from propylene glycol–water (1:1): steady-state permeation fluxes (J''), corresponding lag times (t_{lag}'), permeability coefficients (K_p'), and enhancement ratio (ER′). All values are expressed as mean ± SD (n = 5 experiments)

Sample	$J'_{1–3h}$ (μg cm^{-2} h^{-1})	$t_{lag}'_{1–3h}$ (h)	$K_p'_{1–3h} \times 10^{-3}$ (cm h^{-1})	$ER'_{1–3h}$
Acyclovir	8.2 ± 0.3	1.0 ± 0.02	0.8 ± 0.03	1
Acyclovir + alaptide	18.5 ± 1.4	0.8 ± 0.03	1.9 ± 0.1	2.3 ± 0.2

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Table 3. Permeation parameters of acyclovir in the range sixth to 12th hour without and with alaptide from propylene glycol–water (1:1): steady-state permeation fluxes (J), corresponding lag times (t_{lag}), permeability coefficients (K_p), and enhancement ratio (ER). All values are expressed as mean ± SD (n = 5 experiments)

Sample	J (μg cm^{-2} h^{-1})	t_{lag} (h)	$K_p \times 10^{-3}$ (cm h^{-1})	ER
Acyclovir	22.5 ± 0.7	3.2 ± 0.2	2.3 ± 0.07	1
Acyclovir + alaptide	37.2 ± 0.5	2.6 ± 0.08	3.7 ± 0.05	1.7 ± 0.02

or paracetamol [5–9]. It can be stated that acyclovir without alaptide permeated moderately in comparison with acyclovir with added alaptide; in the whole investigated time range, in every time, the corresponding Q_t values related to the system without and with alaptide were statistically different from each other (see Figures 5A and 5B). It should be noted that the Q_t values obtained from propylene glycol–water have narrower variance than permeated amounts acquired from buffer. On the other hand, Q_t values related to the buffer and propylene glycol–water system with alaptide are not statistically different.

The effectivity of alaptide as a potential CPE can also be confirmed by parameters calculated from propylene glycol–water system that are mentioned in Tables 2 and 3. The standard steady-state parameters calculated for the time period 6–12 h are shown in Table 3. Parameters calculated for the time period 1–3 h mentioned in Table 2 are more significant, because they reflect significantly increased observed permeation flux and permeability coefficient (approx. 2.3) immediately after application, which is more favorable than after long-term application due to the stability of semisolid drug dosage forms and compliance of patients. These facts indicate that alaptide is able to enhance permeation of acyclovir through the skin.

Conclusions

(*S*)-8-Methyl-6,9-diazaspiro[4.5]decane-7,10-dione, alaptide, was prepared by optimized synthesis in 76% overall yield. The ability of alaptide to enhance the penetration of acyclovir was investigated. Based on the presented results, it can be assumed that the contribution of alaptide to the enhanced permeation of acyclovir through the skin is significant, especially immediately after application but also for long-term application. The structure of alaptide can be classified as a hybrid between the derivatives of urea and 2-pyrrolidone; therefore, the supposed mechanism of enhancement action can be as follows. As an urea-like derivative, it can demonstrate moisturizing effect on the SC [26–28], and as a 2-pyrrolidone-like derivative, it can exhibit interactions preferentially in the keratin region [27, 29]. However, the exact mechanism of action of alaptide and effects of mutual interactions of acyclovir, alaptide, and the skin should be investigated in detail.

Acknowledgment. This study was supported by IGA VFU Brno 302/2015/FaF, the Technology Agency of the Czech Republic TA04010065, and the institutional support RVO:68081715 of the Institute of Analytical Chemistry of the CAS, v.v.i.

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