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HPLC method for determination of *in vitro* delivery through and into porcine skin of adefovir (PMEA)

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Abstract

A simple HPLC/UV method for the determination of the transdermal permeation and dermal penetration of a broad-spectrum antiviral drug adefovir (PMEA) was developed. The separation was achieved on a C18 column with the mobile phase composed of 10 mM KH_2PO_4 and 2 mM Bu_4NHSO_4 at pH 6.0 and 7% acetonitrile. The method was validated with respect to selectivity, linearity (0.1–50 μg/ml), precision, accuracy, and stability. Transdermal permeation of 2% PMEA was studied *in vitro* using the Franz diffusion cell and porcine skin. The flux values were 1.8, 3.0, and 0.6 μg/cm^2/h from aqueous donor samples at pH 3.4 and 7.4, and isopropyl myristate, respectively. The respective skin concentrations at 48 h were 294, 263, and 971 μg/g from these vehicles. These results will serve as a lead for further studies on transdermal and topical delivery of antivirals from the group of acyclic nucleoside phosphonates.

Keywords: Adefovir; Acyclic nucleoside phosphonate; Antiviral; Transdermal transport; Dermal absorption; HPLC/UV

1. Introduction

Adefovir (PMEA, 9-(2-phosphonomethoxyethyl)adenine, Fig. 1) is an acyclic nucleoside phosphonate analog with a broad-spectrum activity against herpes-, retro-, and hepadnaviruses. In 2002, its bis(pivaloyloxymethyl) ester prodrug PMEA dipivoxil was approved for treatment of hepatitis B in adult HBeAg-positive and negative patients, and also in adult patients with clinically proven lamivudin-resistant HBV mutants. The main concern of this drug is nephrotoxicity, which limits the daily dose to 10 mg. For recent reviews on PMEA, see Refs. [1–3].

At present, novel antiviral treatments, together with strategies to enhance the response to current therapies, are being explored. Transdermal drug delivery offers numerous advantages over conventional routes of administration. They include therapeutic benefits such as sustained delivery of drugs to provide a steady plasma profile and hence reduced side effects. Avoidance of the first-pass metabolism and circumvention of the gastrointestinal tract results in higher bioavailability, and lower drug and food interactions. Transdermal application is convenient, and the patch can be simply removed in the case of an adverse reaction. Together with reduced dosing schedule, these advantages offer the potential for improved patient compliance. For recent reviews on transdermal drug delivery, see Refs. [4–6].

In the case of PMEA, transdermal delivery would provide a steady plasma profile without peaks and troughs, which might allow for higher dosing without manifestation of kidney toxicity. Gastrointestinal disturbance, which is another side effect, can be avoided by percutaneous application. Considering the chronic nature of PMEA administration and the requirement for a substantial commitment from the patients, less frequent application would be advantageous as well.

Moreover, PMEA was reported to be active against several herpes viruses including HSV-1 and HSV-2 in cell culture [7,8], and in mice in vivo [7]. Thus, topical application of PMEA might be one of the therapeutic approaches. However, no data on PMEA permeation through the skin or into the skin are currently available.

Several chromatographic methods have been used for the determination of PMEA in biological samples including UV

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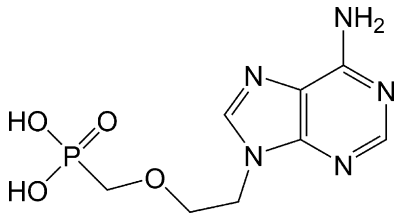

Fig. 1. Chemical structure of adefovir (PMEA).

detection [9–11], fluorescence detection of derivatized PMEA [12–14], radiochromatography [15], and highly sensitive liquid chromatography-tandem mass spectrometry methods [16,17]. None of them, however, focused on the skin as the biological matrix.

The purpose of this study was to develop and validate a chromatographic method for the routine determination of PMEA, suitable conditions for the *in vitro* transdermal permeation experiment, and a method for the extraction of PMEA from the skin. Subsequently, we aimed at obtaining basic information about the transdermal permeation and skin uptake of PMEA from three different donor vehicles.

2. Experimental

2.1. Chemicals

PMEA was kindly provided by Prof. A. Holý (Academy of Sciences, Prague, Czech Republic). Stock standard solution was prepared in the acceptor phase, i.e. phosphate buffered saline at pH 7.4 (PBS, composed of approximately 13 mM NaH_2PO_4, 53 mM Na_2HPO_4 and 75 mM NaCl) with 0.03% NaN_3 (0.5 mM), in a concentration of 100 μg/ml and it was stored at −20 °C for up to 1 month. The final concentrations of the calibration and quality control (QC) samples were prepared by diluting the stock solution in the blank acceptor phase that was in contact with the skin for 48 h under the same conditions as in the permeation study.

HPLC-grade acetonitrile and methanol, Bu_4NHSO_4, and NaN_3 were purchased from Sigma–Aldrich (Schnelldorf, Germany). KH_2PO_4, NaH_2PO_4, Na_2HPO_4, NaCl and were purchased from LachNer (Neratovice, Czech Republic). Isopropyl myristate (IPM) was purchased from Kulich (Hradec Králové, Czech Republic). Ultrapure water was obtained using Milli-Q Water Filtration System (Millipore, Bedford, MA).

2.2. HPLC conditions

The samples were analyzed with a system consisting of a Shimadzu LC-20AD high-pressure pump, Shimadzu SIL-20AC autosampler (Kyoto, Japan), LCD 2083 UV detector (Ecom, Prague, Czech Republic), and CSW v. 1.7 for Windows integrating software (Data Apex, Prague, Czech Republic). A LiChroCART 250-4 column with Purospher STAR, RP 18e, 5 μm (Merck, Darmstadt, Germany) with a guard column LiChroCART 4-4 with the same sorbent was used for separation of PMEA. The column temperature was maintained at 40 °C. The optimized mobile phase consisted of 10 mM KH_2PO_4 and 2 mM Bu_4NHSO_4 adjusted with NaOH at pH 6.0 with 7% acetonitrile at a flow rate of 1.5 ml/min. The detector wavelength was set at 260 nm and the volume of injection was 20 μl.

2.3. Method validation

The method was validated according to the FDA guidelines for validation of bioanalytical methods [18]. The selectivity was investigated by analyzing six blank samples of the skin extracts and acceptor phase compared to those spiked with PMEA at the lower limit of quantification (LLOQ). LLOQ was defined as the lowest concentration of the analyte with the response at least 5 times the response compared to blank sample, and a precision of <20% and accuracy of 80–120%.

Standard calibration curve was generated by spiking the skin extracts in acceptor phase with PMEA to produce eight concentration levels ranging between 0.1 and 50 μg/ml. The calibration curve was constructed by plotting the peak areas against concentration and analyzed by linear regression analysis.

Accuracy and precision were determined by analyzing five separate measurements of QC samples (0.1 = LLOQ, 0.5, 5 and 50 μg/ml of PMEA) on three different days.

The stability of PMEA was assessed by analyzing QC samples at three concentration levels (0.5, 5 and 50 μg/ml of PMEA, five samples each) exposed to different storage conditions including stability at −20 °C for 30 days, 4 °C for 30 days, and at 37 °C for 6 days.

2.4. Donor solutions

Donor samples were prepared by dispersing 20 mg of PMEA in 1 ml of water, PBS (adjusted at pH 7.4 with NaOH), and IPM, respectively. The samples were stirred for 5 min at 50 °C, allowed to equilibrate at 37 °C for 48 h, and redispersed before the application to the skin if needed.

For the determination of the solubility of PMEA in the vehicles (C_{veh}), the samples were prepared in triplicate, and allowed to equilibrate. After 48 h, they were centrifuged at 10,000 × *g* for 5 min, the supernatant was diluted with PBS, if needed, and analyzed for PMEA content. The pH of the samples was measured using a microelectrode HC153 (Fisher Scientific, Pardubice, Czech Republic).

2.5. Skin preparation

Porcine ears were purchased from a local slaughterhouse. Full-thickness dorsal skin was excised by blunt dissection and hairs were removed using a clipper. The skin was than immersed in 0.05% sodium azide solution in saline for 5 min for preservation. The skin fragments were stored vacuum-sealed at −20 °C for maximum of 2 months.

2.6. Permeation experiments

The skin permeability of PMEA was evaluated *in vitro* using the Franz diffusion cells [19]. The skin fragments were slowly thawed immediately before use, cut into pieces ca 2 cm × 2 cm,

mounted into the cells dermal side down and sealed with silicone grease. The diffusion area was 1 cm^2. The acceptor compartment of the cell was filled with PBS at pH 7.4 with 0.03% sodium azide as a preservative and allowed to equilibrate in a 32 °C water bath for 30 min. The precise volume of the acceptor compartment (approximately 18 ml) was measured for each cell and was included into the calculations. The donor sample of 150 μl volume was applied on the skin surface, and the donor compartment of the cell was occluded with a cover glass. The acceptor phase was stirred at 32 °C throughout the experiment. Samples of the acceptor phase of 600 μl volume were withdrawn at predetermined intervals over 48 h, each time being replaced with fresh acceptor phase, and analyzed by HPLC.

2.7. Determination of PMEA in the skin

At the end of the permeation experiment (48 h), the diffusion cells were dismounted and the skin surface washed three times with 0.5 ml PBS to remove the residual donor samples. 1 cm^2 of the skin exposed to the donor compartment was punched out, blotted dry, precisely weighed, placed into a vial and extracted with 5.0 ml PBS at 32 °C for 48 h. The extract was filtered and analyzed for PMEA content, C_{skin} (μg/g).

The efficiency of PMEA extraction from the skin was determined by adding 20 μl PMEA solution (10, 50, and 200 μg) to the surface of a skin fragment of 1 cm^2. The fragment was maintained unoccluded at 32 °C for 48 h to allow for penetration of PMEA into deeper skin layers, and then it was extracted as described above. The experiment was performed in triplicate and compared to the pertinent control samples of PMEA treated likewise, i.e. without the skin.

2.8. Data treatment

The cumulative amount of PMEA having penetrated the skin, corrected for the acceptor sample replacement, was plotted against time. The steady state flux (J, μg/cm^2/h) was calculated from the linear region of the plot and $lagT$ by extrapolation of the linear part to x-axis. The skin permeability coefficient (P, cm/h) was calculated by dividing J by C_{veh}. The data are presented as means ± SD (n = 4–6) obtained using the skin fragments from at least two animals. Statistical significance was determined using One Way Analysis of Variance with Student–Newman–Keuls post test.

3. Results

3.1. Chromatography

Fig. 2 shows typical chromatograms of a blank acceptor phase sample, a spiked one at LLOQ and a corresponding sample from the permeation study. The acceptor phase samples were analyzed directly without any treatment. Endogenous skin components and sodium azide, a preservative, were well separated from PMEA, which typically eluted at 6.4 min, using a simple isocratic run.

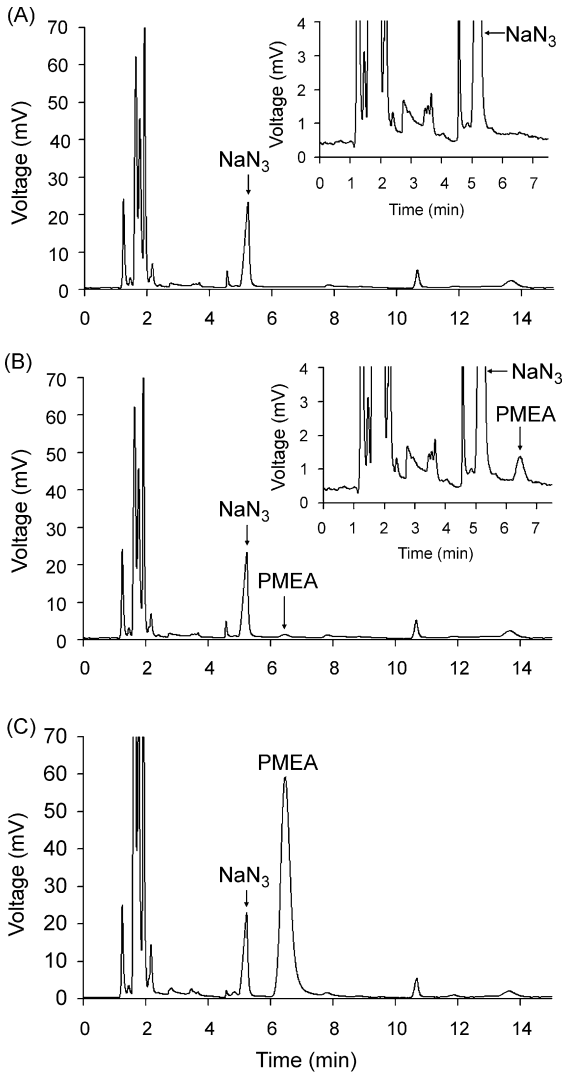

Fig. 2. Typical chromatograms of the acceptor phase from the Franz diffusion cells (A) blank, (B) spiked with PMEA at LLOQ = 100 ng/ml, both expanded in the inserts, and (C) after topical application of PMEA. PMEA eluted at 6.4 min, sodium azide at 5.3 min, the other peaks correspond to the endogenous skin components (2.5 mV = 1 mAU).

3.2. Linearity and lower limit of quantification (LLOQ)

The calibration curve (AUC = 102.99 ± 0.12c + 0.70 ± 2.30) was linear ($p < 0.0001$, $R^2 = 0.99996$) in the range of concentrations of 0.1–50 μg/ml. For each point of the calibration curve, the concentrations back-calculated from the equation of the regression analysis were within acceptable limits for precision (<6.8%) and accuracy (98–102%, except for LLOQ, see below).

LLOQ of this method was 100 ng/ml. The analyte response at this concentration level was >5 times the baseline noise (Fig. 2B). The precision and accuracy at this concentration were acceptable, with <6.8% for the RSD and <116% recovery (Table 1).

3.3. Precision and accuracy

The results for the within-day and between-day precision and accuracy of the QC samples are listed in Table 1. Mean recov-

Table 1
Within-day and between-day precision and accuracy of PMEA in the acceptor phase

C_{added} (μg/ml)	Within-day			Between-day		
	C_{found} (μg/ml)	RSD	Recovery (%)	C_{found} (μg/ml)	RSD	Recovery (%)
50	50.71 ± 0.03	0.06	101.42	50.91 ± 0.28	0.56	101.82
5	4.95 ± 0.03	0.57	99.07	4.96 ± 0.04	0.74	99.25
0.5	0.494 ± 0.007	1.45	98.80	0.503 ± 0.014	2.86	100.63
0.1 (LLOQ)	0.114 ± 0.008	6.81	113.59	0.116 ± 0.007	6.41	116.35

$n = 5$ (each day).

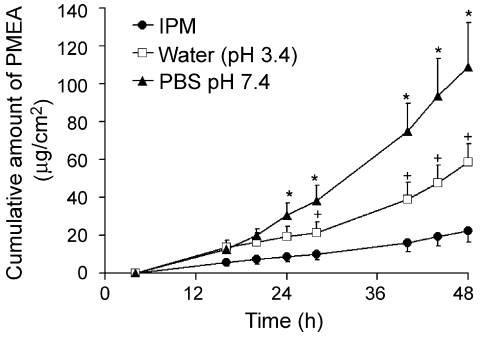

Fig. 3. Permeation profiles of PMEA through porcine skin *in vitro*. Data presented as mean ± S.E.M. Significant difference at $p < 0.05$: *IPM vs. PBS; +water vs. PBS.

eries for the within-day assay ranged from 98.80 to 101.42% and RSD ranged from 0.06 to 1.45%. Mean recoveries for the between-day assay were from 99.25 to 101.82% and RSD from 0.56 to 2.86%. These values are well within the acceptance criteria recommended by the FDA guideline [18].

3.4. Stability

No significant degradation of PMEA under the studied concentrations was observed since the concentrations of the QC samples deviated by no more than 3.8, 6.2 and 7.8% relative to the nominal concentrations after 30 days at −20 °C, 30 days at 4 °C, and 6 days at 37 °C, respectively.

3.5. Transdermal permeation of PMEA

Permeation profiles of PMEA, i.e. cumulative amounts of PMEA permeated through full-thickness porcine skin plotted against time, are shown in Fig. 3. The *J*, *P* and *lagT* values for permeation of 2% PMEA from the three donor vehicles are summarized in Table 2 together with C_{veh} and pH of the sample.

3.6. Dermal penetration of PMEA

The efficiency of extraction of PMEA from the skin was 96 ± 6, 98 ± 4, and 97 ± 2% at 10, 50, and 200 μg/cm², respectively. The highest C_{skin} of PMEA was obtained after topical application of the IPM suspension, followed by both aqueous donor samples (Table 2).

4. Discussion

The acyclic nucleotide phosphonates are an interesting group of compounds displaying various biological activities, including antiviral, cytostatic, antiparasitic and immunomodulatory. Due to a polar character of the phosphonate group, their resorption from gastrointestinal tract is restricted and the search for new prodrug types with an enhanced oral bioavailability is one of the current perspectives in this field. Another possibility to improve the pharmacokinetic parameters is to deliver these drugs through the skin.

In this study, we aimed at developing an HPLC method for rapid and precise determination of transdermal permeation and dermal penetration of PMEA. PBS at pH 7.4 was selected as the acceptor phase for permeation studies as a simple mimic of the *in vivo* conditions. Due to high solubility of PMEA in PBS, this acceptor phase was suitable to maintain sink conditions throughout the experiment. The disadvantage of this phase was, however, its ability to extract many potentially interfering substances from the skin due to its relatively large volume, temperature, pH, and long contact with the skin.

The majority of the previously published methods was designed to separate PMEA from its prodrugs or metabolites and applied gradient elution. For the permeation experiments, however, we preferred a simple isocratic elution as the search for conditions that influence adefovir behavior would involve a large number of samples. To achieve the best selectivity, efficiency and peak shape, various concentrations of an organic solvent, buffer strength and pH, and concentration of the ion-

Table 2
Permeation characteristics of 2% PMEA through full-thickness porcine skin *in vitro* and its concentration in the skin.

Donor vehicle	pH	C_{veh} (μg/ml)	J (μg/cm²/h)	P (cm/h)	$lagT$ (h)	C_{skin} (μg/g)
Water	3.4	2486 ± 52	1.8 ± 0.5	7.2×10^{-4}	18 ± 6	294 ± 75
PBS	7.4	18920 ± 279	3.0 ± 1.2	1.6×10^{-4}	14 ± 1	263 ± 159
IPM	–	0.11 ± 0.01	0.6 ± 0.3	5.45	14 ± 1	971 ± 162

Data are presented as means ± SD, $n = 4$–6.

pairing agent were investigated. The substances extracted from the skin behaved similarly to PMEA and some of them partially coeluted. Decreasing the concentration of the buffer and the ion-pairing reagent increased the retention of PMEA and improved separation from the majority of the extracted substances. The 10 mM buffer with 2 mM Bu_4NHSO_4 offered acceptable retention times. The final selection of a 250 mm column and an adjustment of pH to 6.0 resulted in an acceptable resolution of PMEA and the minor skin components (Fig. 2) and gave the best results regarding the interindividual variability of the skin samples. This method was validated and applied to the permeation experiment. The conditions used in the stability study reflected the situations encountered during the real sample handling, e.g. the samples had to be stable for at least 6 days at 37 °C as they were maintained for 2 days at this temperature to equilibrate, then the permeation experiment was performed at 32 °C for 2 days, and the skin was subsequently extracted at 32 °C for another 2 days to determine the amount retained in the skin.

The permeation rate of PMEA through the skin was surprisingly high for a phosphonate moiety-containing drug. It seems that the aqueous acceptor phase at pH 7.4 acted as an effective sink for the compound, allowing the majority of PMEA that reached the dermis to then dissolve in the acceptor, removing it from the skin. Currently, there are no data on PMEA permeation through the skin for comparison. The only member of the group of acyclic nucleoside phosphonates that has been studied with regard to the transdermal and dermal delivery is cidofovir. The mean J values of cidofovir permeation through porcine skin *in vitro* were 3.6 and 5.3 μg/cm^2/h from microparticles and cidofovir solution, respectively [20,21]. This roughly corresponds with our results with PMEA.

Of the three test donor vehicles, highest permeation rate was achieved from PBS at pH 7.4 (J = 3.0 μg/cm^2/h), which is almost twice higher than that from a non-buffered aqueous suspension (J = 1.8 μg/cm^2/h). The pH value of the unbuffered aqueous donor sample was 3.4. Thus, majority of PMEA was in the form of a zwitterion with a cation at N1 of adenine cycle and an anion at the phosphonate oxygen. In PBS at pH 7.4, phosphonate dianion was the prevailing species, and hence the higher permeation rate at pH 7.4 might be considered unexpected. However, the C_{veh} values were not the same—the solubility of PMEA in water at pH 3.4 was considerably lower than that at pH 7.4 (see Table 2). Consequently, the P value, which is independent of the donor concentration, was approximately 4.5 times lower at pH 7.4. This might be explained either by higher diffusivity of the zwitterion than that of a dianion or by lower PMEA thermodynamic activity in the donor sample at pH 7.4. The solubility of the dianion of this drug, however, is high; thus, a compromise between maximal thermodynamic activity and unwanted effects or irritation during potential clinical application must be accepted. PMEA behavior at different pH and concentration will be studied further. Unlike the transdermal permeation, the skin uptake of PMEA from both aqueous samples was comparable—approximately 300 μg/g of the tissue.

In a model lipophilic vehicle, IPM, 0.11 μg/ml of PMEA was dissolved, which is four orders of magnitude less than in the aqueous vehicles. Nevertheless, the apparent permeation rate from this vehicle was 0.6 μg/cm^2/h, giving the P value of 5.45 cm/h. Since the *lagT* values from these vehicles did not differ significantly, this high P value cannot be caused by an increase of PMEA diffusion coefficient. Thus, the main reason might be much greater partitioning ability of PMEA from this lipophilic vehicle into the stratum corneum than from the aqueous ones, where PMEA is much more soluble. IPM can also penetrate into the stratum corneum and change its solvent properties. Another possible explanation of such high P value is that IPM may penetrate faster than adefovir into the stratum corneum and produce supersaturated donor sample.

Despite its lower permeation through the skin, the skin uptake of PMEA from IPM was approximately three times greater than that from both hydrophilic vehicles, almost 1 mg/g of the tissue. These findings imply that the levels reaching the target site are far higher than the EC_{50} or IC_{50} values of PMEA for herpes viruses, which are typically in the range of tens of micrograms per ml [7,22–24]. IPM appears to be an ideal vehicle for topical application as the drug remained concentrated within the skin and the systemic absorption was low.

Although the permeation rates achieved in this preliminary study are not sufficient to reach effective plasmatic concentrations, they might be further improved, e.g. by the addition of permeation enhancers [4,25,26]. Moreover, the pH dependence of the PMEA permeability and the effect of various vehicles merit further investigation as well.

5. Conclusion

A simple and precise method for determination of transdermal and dermal absorption of PMEA was developed and validated. The method was successfully applied to determine the flux of PMEA through full-thickness porcine skin *in vitro* and its concentration in the skin. These results will serve as a lead for further studies with acyclic nucleoside phosphonates. Generally, this simple isocratic method may be used for determination of PMEA in other biological matrices depending on whether the concentration is above the quantification limit of this method, what method of sample preparation is used, and whether prodrugs/metabolites/other drugs will be simultaneously determined.

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