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Determination of cidofovir in both skin layers and percutaneous penetration samples by HPLC

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Abstract

The aim of this study was to develop a direct, simple and rapid high performance liquid chromatographic method for the determination of cidofovir in both skin layers and percutaneous penetration experiments. Samples were chromatographed on a reversed phase encapped column 250 × 4 mm C_8 LiChrospher Select B. The phase mobile consisted on 3% of acetonitrile and 97% of 1.5 mM of tetrabutylammonium dihydrogen phosphate (TADP) and 3.5 mM of disodium hydrogenphosphate adjusted to pH 6. Detection was at 274 nm and the run time was 14 min. The limit of detection was 0.06 μg/ml. The detector response was found to be linear in the concentration range 0.1–10 μg/ml. This assay is a selective, sensitive and reproducible method for the quantification of cidofovir in skin layers and in the receptor compartment of Franz-type diffusion cells after percutaneous studies. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: HPLC; Cidofovir; Percutaneous permeation; Skin recovery

1. Introduction

Cidofovir (1-[(*S*)-3-hydroxy-2-(phosphonomethoxy)propyl] cytosine) (HPMPC) is an acyclic nucleotide analogue with potent in vitro and in vivo activity against a broad spectrum of herpes viruses [1]. Unlike nucleoside analogues, cidofovir contains a phosphonate group and its conversion to the active triphosphate analogue in cells, is done by host enzymes [2] and is, therefore, independent of viral infection. By bypassing the need of virus encoded activation, cidofovir remains effective against thymidyne kinase-deficient or altered strains of HSV [3]. Additionally, in vitro studies have shown that cidofovir and its metabolites have long intracellular half-live [4].

Its ability to protect uninfected cells has a great potential in the treatment of recurrent herpes simplex virus infections, HSV-1 and HSV-2, where viral outbreaks lead to secondary cell infection [5]. In this way, topical administration of cidofovir has been shown to be effective in the treatment of cutaneous infections of herpes simplex viruses HSV-1 and HSV-2 in animal models

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[6–8]. Besides, acyclovir-resistant HSV-1 in human provided responsive to treatment with topical cidofovir [9]. Consequently, several authors have studied the way to deliver greater amounts of this drug to the target site of virus infections, the basal epidermis, in order to increase the efficacy of the topical therapy. Therefore, the quantification of cidofovir within the skin and after the in vitro percutaneous penetration studies are essential for topical and transdermal research [10].

The search of suitable techniques for selective and sensitive determination of cidofovir in biological samples is of great interest, since the complexity of these samples, including large numbers of endogeneous compounds, makes selective detection quite difficult. In previous works, different methods have been described to quantify cidofovir in biological samples, although in general, high-performed liquid cromatography (HPLC) has been the most employed one [11–14]. However, the polar character of the cytosine group makes selective detection at low concentration levels exceedingly difficult. Therefore, most of these HPLC methods used accurate and selective detection systems such as radiochemical detection [11,12,15], although their use required a considerable investment of time. Other method proposed [13] involved a precolumn derivatisation with phenacyl bromide and fluorencence detection. This method showed a great sensitivity and highly selectivity, although the derivatisation efficiency is low (about 45% of cidofovir was converted to the fluorescence derivative) and highly variable (41–45%). In addition, the derivatizacion procedure described in this method is unable to improve the chromatographic properties of cidofovir when skin samples were analysed.

The aim of this paper was to develop an analytical HPLC method with UV detection for the simple, sensitive, specific and rapid determination of cidofovir in both skin layers and in vitro percutaneous penetration samples.

2. Materials and methods

2.1. Materials

Cidofovir (Fig. 1) was kindly supplied by Gilead Sciences (Foster City, CA, USA). Thetrabutylammonium dihydrogenphosphate was obtained from Fluka Chemika (Switzerland). Acetonitrile, disodium hydrogenphosphate and other solvents used were of HPLC analytical grade and were supplied by Merck (Darmstadt, Germany).

2.2. Calibration standards

A stock solution of cidofovir with a concentration of 10 μg/ml was prepared by dissolving 5 mg de cidofovir in water. Ten standard solutions were made by further dilution of the stock solution with appropriate volumes of water. The concentration range of cidofovir for the standard curve samples was between 0.01 and 10 μg/ml. All solutions of the drug were kept at 4 °C.

2.3. Instrumentation and chromatographic conditions

The apparatus used for the HPLC analysis was a Hewlett–Packard (Waldbronn, Germany) system equipped with a HP 1050 quaternary pump, a HP 1050 autosampler and a HP 1050 diode-array detector set at 274 nm. Data acquisition and treatment were performed with a Hewlett–Packard computer using CHEMSTATION G2170 AA for chromatografic analysis and SPSS for statistic ones.

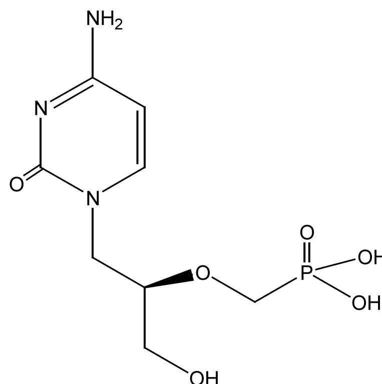

Fig. 1. Chemical structure of cidofovir.

The samples were cromatographed on a reversed-phase 250 × 4 mm C_8 LiChrospher Select B (5 μm) provided by Merck. A 4 × 4 mm precolumn of the same material was also used. The mobile phase consisted of 3% of acetonitrile and 97% of 1.5 mM of tetrabutylammonium dihydrogen phosphate (TADP) and 3.5 mM of disodium hydrogenphosphate at flow rate of 1 ml/min. The mobile phase was adjusted to pH 6.0 with concentrated phosphoric acid. The column was termostated at 40 °C. Under these experimental conditions, the run time was 14 min and the injection volume was 100 μl.

For ruggedness studies, different reversed phase columns, such as Hypersil ODS (3 μm particle size; 10 × 0.46 cm; Teknokroma, Spain) and Sperisorb C_{18} (10 × 0.46 cm; Teknokroma, Spain) were used. A 1 × 0.46 cm precolumn of the same materials was used, respectively, in each case. Similarly, the influence of mobile phase (percentage of acetonitrile ranged from 1 to 5%) and column temperature (30 °C) on the analytical procedure were also evaluated.

2.4. Instrument calibration

Calibration curves were prepared using concentrations of 0.1, 0.2, 0.4, 0.6, 0.8, 1, 2, 4, 6, 8, 10 μg/ml of cidofovir. They were determined by least-squares linear regression analysis (weighting, $1/x^2$). Peak areas of cidofovir versus the corresponding drug concentration in water were plotted.

The linearity of the method was confirmed by statistical comparison among the sloped obtained, the intercepts of calibration curves with zero and the correlation coefficients with 1. Moreover, a Student t-test was used to compare the back-calculated concentrations with each calibration curve versus the nominal ones.

2.5. Specificity

The specificity of the assay was verified against endogenous compounds of the skin. Several blank porcine skin samples from different animals were tested for the absence of interfering compounds. The retention times of endogenous compounds were compared with that of cidofovir.

2.6. Accuracy

Accuracy of the assay method was defined as the percentage of the systematic error, which is calculated as standardised agreement between the measured value and the true value. Accuracy values were studied at 0.1, 1 and 10 μg/ml.

2.7. Precision

In this work, precision of the method was tested as both within-day and between-day reproducibility of the assay. Precision of a method was expressed as the relative standard deviation (R.S.D.) of replicate measurements. To be acceptable, the measures should be lower than 10% at all concentrations [16].

This study was developed with three concentrations of cidofovir in water (0.1, 1, 10 μg/ml). Several aliquots of each samples were tested the same day to determine the within-day reproducibility. Aliquots of the same sample were tested once a day, during 5 days, to determine between-day reproducibility.

2.8. Determination of the limits of quantification and detection

The limit of quantification (LOQ) was defined as the lowest drug concentration, which can be determined with an accuracy and precision $<$ 20%. The limit of detection (LOD) was defined as the sample concentration resulting in a peak area of three times the noise level. In this work, the LOD of the assay method was determined by the analysis of the peak baseline noise in ten blank samples.

2.9. Application of the method

2.9.1. Quantification of cidofovir in the receptor side of the diffusion cells

This method was used to determinate the in

vitro percutaneous penetration of cidofovir through porcine skin using the Frank-type diffusion cells (FDC-400, Grown Glass Company, Somerville, NY). Porcine ears were obtained from the local slaughterhouse and after cleaning them under cold running water, the outer region of the ear was cut. The whole skin was dermatomed to 1.2 mm (Aesculap, Tuttlingen, Germany). The skin samples were clamped between the two chambers of Frank-type diffusion cells, with the stratum corneum facing the donor compartment and the dermis facing the receptor one. The Cidofovir solution was placed in the donor compartment and 0.4 ml samples were taken for the receptor compartment at different times (2, 8, 16, 24 h) and replaced by the same volume. Samples were filtered and analysed immediately.

2.9.2. Quantification of cidofovir in skin layers

The quantitative determination of cidofovir at different depths from skin surface was performed on horizontal slices (40 μm) of the skin sample. After the permeation experiments, the skin was removed, cleaned three times with distilled water, included in O.C.T. (Tissue-Tek®, Sakura, Zoeterwoude, The Netherlands) and frozen in liquid N_2. Ten 40 μm slices were cut at −25 °C parallel to the skin surface (2800 Frigocut E, Reichert-Jung, Germany) and stored at −20 °C until analysis.

Cidofovir was extracted from each skin slice with 300 μl of distilled water, at 40 °C for 15 min; during this time the tubes were vortexed twice for 10 s. After cooling, the mixture was centrifugated at 8000 rpm for 10 min. The supernatant was filtered through a 0.45 μm nylon filter (Lida, USA) and analysed by the HPLC method described in this work.

For the validation of cidofovir extraction from the skin, different known amount of cidofovir in water were directly added to a series of 40 μm slices of blank skin (which had not previously been in contact with cidofovir) and extracted as previously described. The extraction recovery was determined by computing the ratio of the amount of cidofovir extracted from spiked skin to the amount of cidofovir added. The skin slices were from the different animals and from different depths.

3. Results and discussion

In this paper, it has been described and validated a simple, sensitive and specific HPLC method with UV detection for the quantitative determination of cidofovir in skin layers at different depths and after in vitro percutaneous penetration assays. Methods employed for the quantification of drugs from this kind of samples have to be specific, since these samples are usually contaminated with skin endogenous compounds, as a large numbers of UV-absorbing nucleotides and nucleosides. Moreover, these methods also have to show enough sensitivity, due to the little volume of sample obtained in these experiments.

The HPLC method described in this paper was based on that developed by Oliyai et al. [14], although same modifications were made to obtain better sensibility for the very diluted samples in the permeation studies and avoid interference's with the skin compounds. Traditionally, the first attempt to avoid this problem involved modifications in the sample extraction procedure of drug from biological samples. However, even with extensive sample clean-up, the sensitivity and selectivity of the ultraviolet detection may not be sufficient for the detection of cytosine containing compounds in biological mediums. Nevertheless, it is interesting to note that tailing factors more than unity were observed under these chromatographic conditions. This problem is probably due to an ionic interaction between certain polar functional groups, such as cytosine group of cidofovir, and free silanols of column packing. Tailing factor is a measure of peak symmetry of chromatographic peaks and, therefore, of the column efficiency. As peak asymmetry increases the integration of trace components becomes less reliable. Therefore, modifications in the type of stationary phase and the chromatographic conditions can aid to solve our problem.

In order to increase the selectivity of the method we have replaced the C_{18} reversed phase column with a slightly more polar encapped stationary phase; the Lichrospher C_8 60 RP-select B column. This column is a pH stable column packed with a monofunctional phase bonded to extremely pure spherical silica particles, that ex-

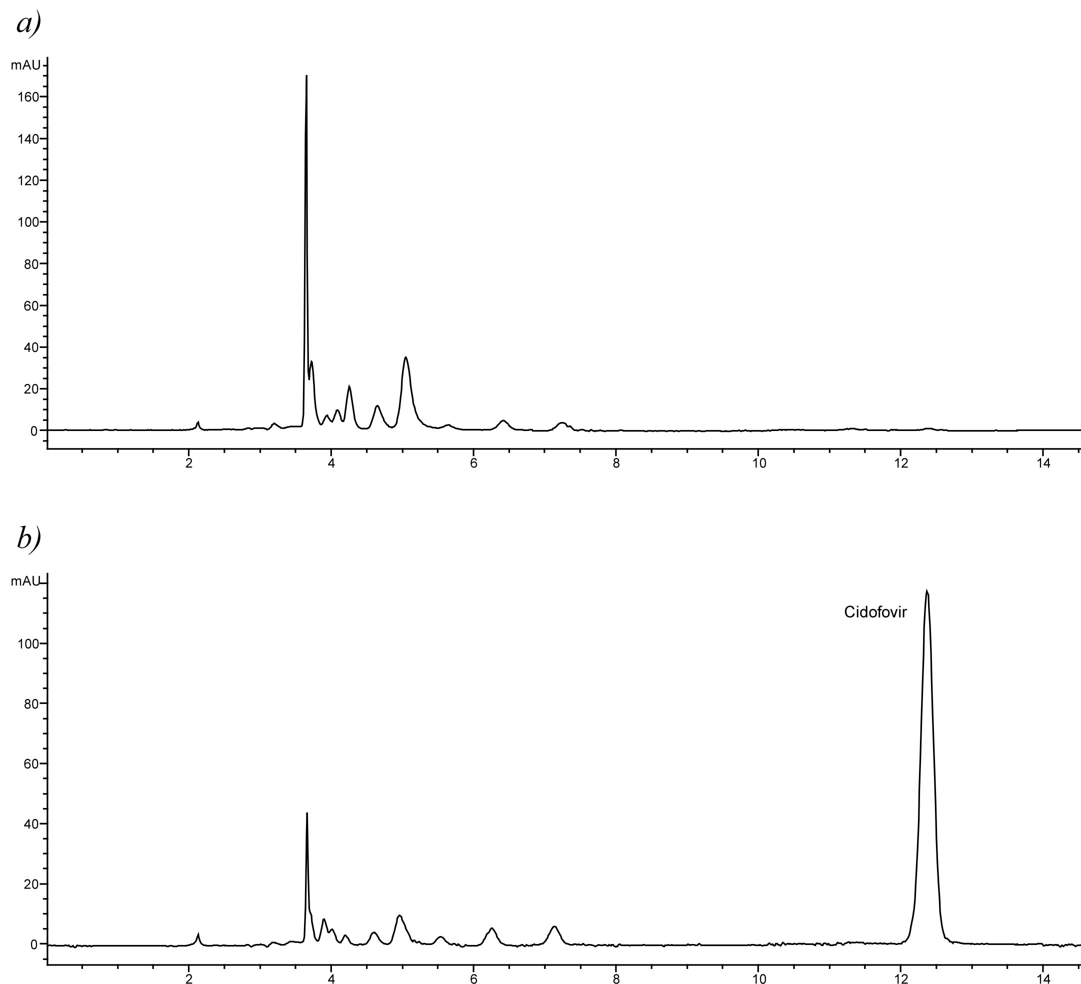

Fig. 2. Chromatograms obtained from the analysis of samples from skin layers, (a) blank sample; (b) sample obtained after the topical application of a cidofovir solution.

hibits a good peak shape. We have also included an organic solvent, acetonitrile, in the mobile phase composition. Under these conditions the selectivity of the chromatographic parameters also allow decreasing the flow rate and the concentration of the ion-pair reagent. Therefore, an increase in the column half-life can be expected.

The retention of cidofovir in the stationary phase was 10.32 ± 0.08 (k'value) and acceptable asymmetry coefficient was obtained (the asymmetry coefficient was 0.90 ± 0.03). Under the chromatographic conditions used, cidofovir has a retention time of 12.2 ± 0.4 min. Representative chromatograms for samples obtained in permeation studies and from skin slices, after applying topically the cidofovir solution, are shown in Figs. 2 and 3. There was a clear resolution of cidofovir ($R_s = 2.73 \pm 0.09$) without endogenous sources of interference.

The assay performance of the present method was assessed by all following criteria: linearity, accuracy, precision, LOD, LOQ, stability and applicability in percutaneous permeation studies. The assays exhibited linearity between the response (y) and the corresponding concentration of piroxicam (x) on three different days over the

0.1–10 μg/ml range (typical equation: $y = 158.76x + 2.26$). Linear regression analysis showed correlation coefficients greater than 0.999 in all curves ($n = 3$). For each point of calibration standards, the concentrations were back calculated from the equation of the regression curves, and R.S.D. were computed. The obtained values were below 10% for all concentrations. For each calibration curve, the slope was statistically different from 0, and the intercept was not statistically different from 0. Moreover, a linear regression of the back-calculated concentrations versus the nominal ones provided a unit slope and intercept equal to 0 (Student t-test).

Accuracy values studies at low, medium and high cidofovir concentrations (Table 1) were always within acceptable limits ($< 15\%$) [16]. The results for within-day and between-day precision

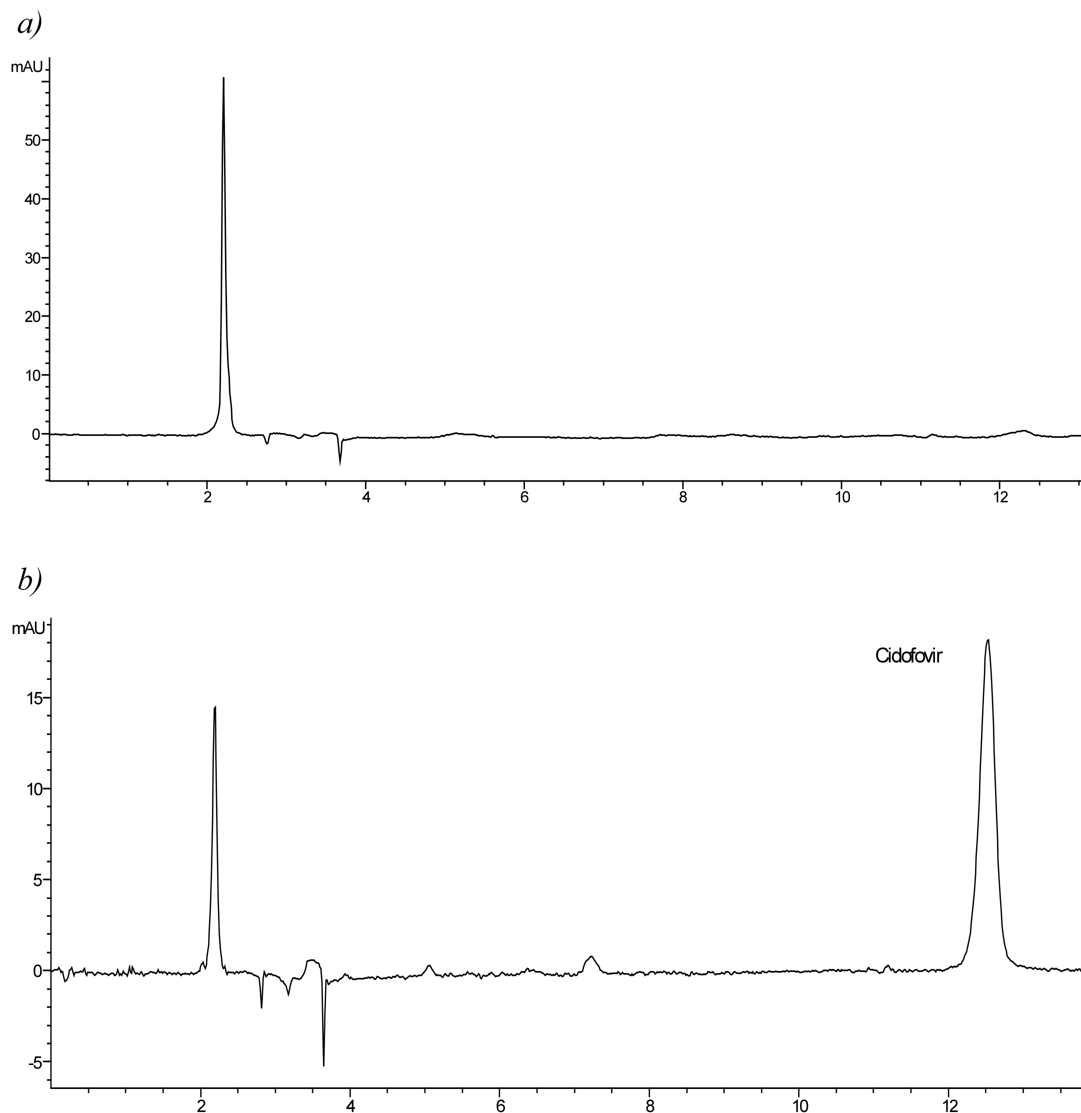

Fig. 3. Chromatograms obtained from the analysis of (a) blank sample and (b) sample after 16 h in a cidofovir transdermal permeation study.

Table 1
Accuracy of the method for determining cidofovir concentration (expressed as relative error in %)

Concentration added (μg/ml)	Concentration found (Mean ± S.D.)(μg/ml)	Relative error (%)
10	10.091 ± 0.037	0.91
1	1.049 ± 0.006	4.9
0.1	0.091 ± 0.002	9

$n = 5$.

are presented in Table 2 and the R.S.D. values were always below 5%. These data clearly indicate that the assay method is reproducible within the same day and between days [16].

The LOD of cidofovir was 0.06 μg/ml, although this value is higher than that reported by Eisenberg et al. (1996) in plasma, the method proposed in this work does not involve any previous sample preparation and is specific for cidofovir detection from skin samples. Besides, the estimated LOQ for this method was found to be 0.08 μg/ml, a value smaller than that reported by Lalezari et al. [17] in plasma (0.22 μg/ml) and Cundy et al. [18] in urine (0.5 μg/ml).

Stability studies carried out in water indicated that samples were stable for at least several weeks when stored at 4 °C.

For the ruggedness and robustness study, different analytical columns (Hypersil ODS and Spherisorb C_{18}) and guard columns were successfully used with no significant variations in the chromatography results. If the temperature of the column was decreased to 30 °C, the elution times were modified. Slight modifications in the percentage of acetonitrile (1–5%) in the mobile phase also altered the cidofovir retention times. Variance in the flow rate resulted in changes in the retention times. Nevertheless, no single parameter, extended to the specified limits, resulted in a dramatic adverse effect on the system suitability.

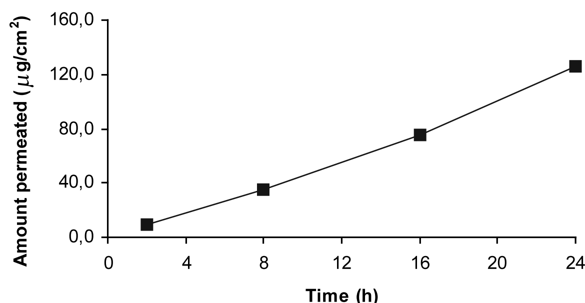

Fig. 4. Percutaneous permeation profile of cidofovir across porcine skin (24 h).

The applicability of this method has been demonstrated for the cidofovir quantification in porcine skin layers and in the receptor compartment of the diffusion cells after in vitro percutaneous penetration studies. Fig. 4 represented the amount of cidofovir found in the receptor side of the diffusion cells during percutaneous permeation studies, after a topical application of cidofovir solution. Previously to the cidofovir quantification in the porcine skin layers, the drug was extracted from skin slices with the method described previously. Satisfactory recoveries were obtained for all samples tested, always above 94%. Fig. 5 showed the cidofovir concentration determined in porcine skin slices, at different depths, after 24 h of topical application of a cidofovir solution.

Table 2
Between-day and within-day variability of the HPLC method for determining cidofovir concentrations

Concentration added (μg/ml)	Between-day variability ($n = 5$)		Within-day variability ($n = 5$)	
	Concentration found (Mean ± S.D.) (μg/ml)	R.S.D. (%)	Concentration found (Mean ± S.D.) (μg/ml)	R.S.D. (%)
0.1	0.091 ± 0.003	0.20	0.091 ± 0.002	1.65
1	1.056 ± 0.015	1.42	1.049 ± 0.006	0.54
10	9.983 ± 0.020	3.29	10.910 ± 0.037	1.65

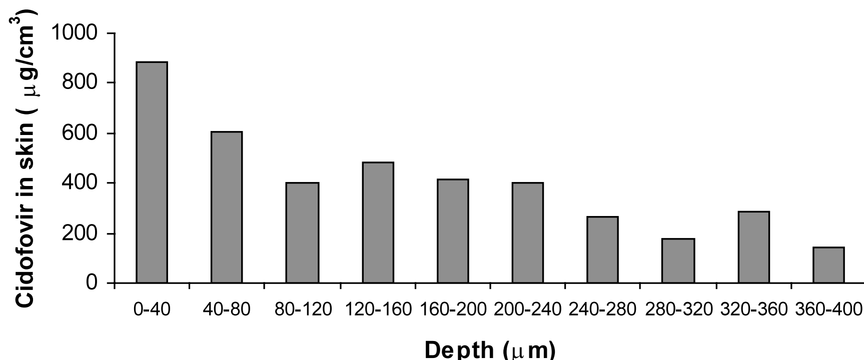

Fig. 5. Distribution of cidofovir in porcine skin layers after topical application of a cidofovir solution during 24 h.

4. Conclusions

A simple chromatographic method has been developed for the rapid and precise determination of cidofovir in both skin layers and percutaneous permeation samples. The simplicity of the technique (without any extraction procedure) and the high sensitivity makes this technique particularly attractive for this purpose.

This method was sensitive, accurate and has a good level of precision. The results described in this paper showed that this assay is suitable for the determination of cidofovir in the different strata of porcine skin and in the receptor side of the diffusion cell after skin permeation experiments.

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