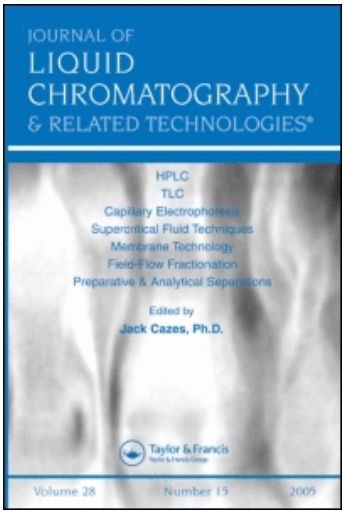

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HPLC DETERMINATION OF ACYCLOVIR IN SKIN LAYERS AND PERCUTANEOUS PENETRATION SAMPLES

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ABSTRACT

The aim of this study was to develop a direct, simple, and rapid high performance liquid chromatographic (HPLC) method for the determination of acyclovir (ACV) after in vitro percutaneous permeation studies. Samples were chromatographed on a reversed phase encapped column 250×4 mm C_8 LiChrospher Select B. The phase mobile was a mixture of acetonitrile–ammonium acetate 0.05 M (1 : 99, v/v) pH 6.5. Detection was at 252 nm and the run time was 12 min. The limit of detection was 0.006 μg/mL. The detector response was found to be linear in

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the concentration range 0.05 to 10 μg/mL. This assay is a selective, sensitive, and reproducible method for the quantification of ACV in skin layers and in the receptor compartment of Frank-type diffusion cells after percutaneous permeation studies.

Key Words: HPLC; Acyclovir; Percutaneous permeation; Skin recovery

INTRODUCTION

Acyclovir (ACV), 9-[(2-hydroxyethoxy)methyl]-guanine, a synthetic analogue of 2′-deoxiguanosine (Fig. 1), is one of the most effective and selective agents against viruses of the herpes group. Acyclovir is active against herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), varicella zoster virus, and to a lessen extent, against Esptein-Barr virus and cytomegalovirus.[1] The mechanism of action of this drug has been extensively studied, and its antiviral activity has been shown to result from the inhibition of herpes virus DNA replication.[2]

This drug is clinically used against the cutaneous infections due to HSV-1, although it has been suggested that ACV topical therapy has low efficacy, due to the lack of penetration of a sufficient amount of the drug to the basal epidermis.[3] Consequently, several authors have studied the way to deliver greater amounts of this drug to the target site of HSV infections, the basal epidermis. In this way, the quantification of drugs within the skin and after the in vitro percutaneous penetration studies is essential for topical and transdermal research.[4]

The search of suitable techniques for selective and sensitive determination of ACV in biological samples is of great interest, since the complexity of these samples, including large numbers of endogeneous compounds, make selective detection quite difficult. In previous works, different methods have been employed to quantify ACV in biological samples. The amount of the drug in these samples was determined using UV spectrophotometry,[5] thin layer

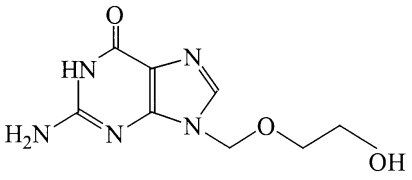

Figure 1. Chemical structure of ACV.

chromatography (TLC),[6] capillary electrophoresis,[7] gas chromatography,[8] electrospray ionization mass spectrometry (ESI-MS),[9] radioimmunoassay (RIA),[10] and high performance liquid chromatography (HPLC).[11] In general, HPLC methods have been the most employed for the analysis of ACV in biological samples, although the complexity and heterogeneity of skin tissue requires the development of specific and reliable methods.

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

EXPERIMENTAL

Chemicals, Reagents, and Solutions

Acyclovir was a gift from Glaxo-Wellcome (Madrid, Spain). Ammonium acetate, acetonitrile, and all solvents and reagents used were of HPLC analytical grade and were supplied by Merck (Darmstadt, Germany).

Calibration Standards

A stock solution of ACV with a concentration of 10 μg/mL was prepared by dissolving 5 mg of ACV in water. Eight standard solutions were made by further dilution of the stock solution with appropriate volumes of water. The concentration range of ACV for the standard curve samples was between 0.05 and 10 μg/mL.

All solutions of the drug were kept at 4°C.

Instrumentation and Chromatographic Conditions

The instrument used was a Hewlett-Packard (HP) system (Waldbronn, Germany) equipped with a HP 1100 quaternary pump; a HP 1100 autosampler and a HP 1100 diode-array detector set at 252 nm (λ_{max}). Data acquisition and treatment were performed with a Hewlett-Packard computer using Chemstation 3D (Hewlett-Packard, Waldbronn, Germany) for chromatographic analysis and SPSS for statistic ones.

The samples were chromatographed 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 a mixture of acetonitrile–ammonium acetate 0.05 M (1 : 99, v/v) at a flow rate of 1 mL/min.

The column was thermostated at 20°C. Under these experimental conditions, the run time was 12 min and the injection volume was 100 μL.

For ruggedness and robustness studies, different reversed phase columns, such as Hypersil ODS (3 μm particle size; 10 × 0.46 cm; Teknokroma, Spain) and Spherisorb C_{18} (10 × 0.46 cm; Tecnokroma, 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 (35°C) on the analytical procedure were also evaluated.

Application of the Method

Quantification of Acyclovir in the Receptor Side of the Diffusion Cells

This method was used to determinate the in vitro percutaneous penetration of ACV 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 ACV suspension was placed in the donor compartment and 0.4 mL samples were taken from the receptor compartment at different times (4, 6, 16, 24, 40, 72, and 88 h) and replaced by the same volume of buffer. Samples were filtered and analysed immediately.

Quantification of Acyclovir in Skin Layers

The quantitative determination of ACV at different depths from skin surface was performed on horizontal slices (30 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. Twenty 30 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.

Acyclovir was extracted from each skin slice with 200 μL of distilled water at 60°C ± 5°C for 15 min; during this time the tubes were vortexed twice for 10 s. After cooling, 100 μL of 1 N $HClO_4$ were added to precipitate residual proteins and the mixture was centrifugated at 8000 rpm for 10 min. The supernatant was filtered through a 0.45 μm nylon filter (Lida, USA) and analyzed by the HPLC method described in this work.

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For the validation of ACV extraction from the skin, different known amounts of ACV in water were directly added to a series of 30 μm slices of blank skin (which had not previously been in contact with ACV) and extracted as previously described. The extraction recovery was determined by computing the ratio of the amount of ACV extracted from spiked skin to the amount of ACV added. The skin slices were from the different animals and from different depths.

RESULTS AND DISCUSSION

Validation of the Method

Chromatography

The quantitative determination of ACV in skin layers, at different depths and in the receptor side of diffusion cells, required specific methods, since these kinds of samples were usually contaminated with skin endogenous compounds. With the method described in this work, the analytical peaks of ACV were well resolved (Figs. 2 and 3). The retention of ACV in the stationary phase was 8.17 ± 0.05 (k' value) and acceptable asymmetry coefficient was obtained (the asymmetry coefficient was 0.75 ± 0.02). Under the chromatographic conditions used, ACV has a retention time of 10.2 ± 0.4 min.

Selectivity of the Assay

The selectivity of the assay was determined by the individual analysis of blank samples from skin layers and from the receptor side of the diffusion cells. Under these chromatographic conditions, no endogenous sources of interference were observed in the medium and the resolution ($R_s = 6.30 \pm 0.12$) of ACV was satisfactory.

Sensitivity of the Assay

Detection and quantification limits (LOD and LOQ) were determined by the analysis of the peak baseline noise in 10 blank samples. The LOD and LOQ were 0.006 μg/mL and 0.009 μg/mL, respectively.

Linearity of the Assay

Linearity was determined by plotting a standard curve from the ACV peak areas vs. the corresponding drug concentrations in water.

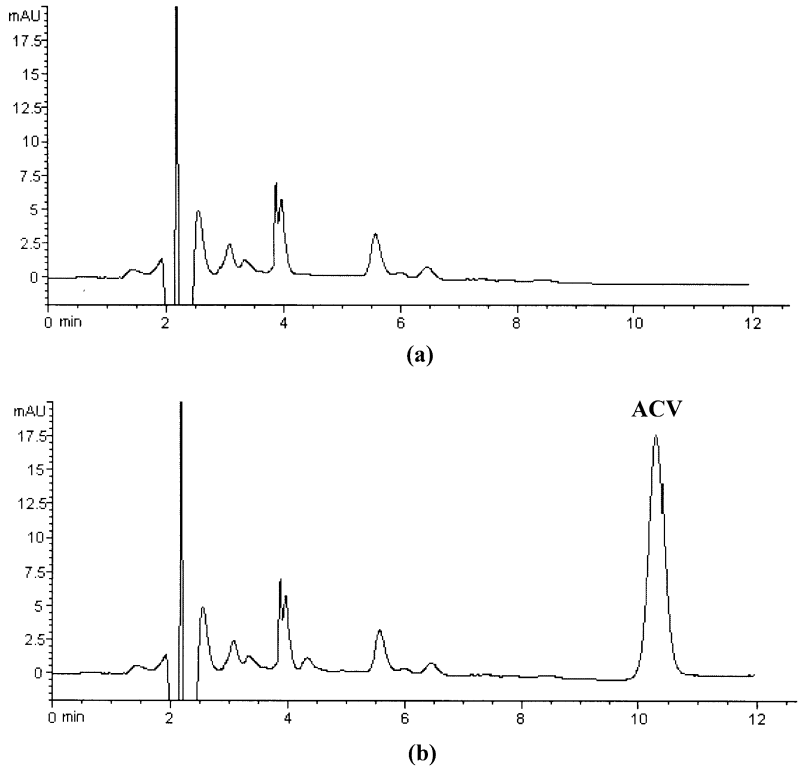

Figure 2. Chromatograms obtained from the analysis of (a) a blank sample and (b) a 6 h sample from the receptor side of the diffusion cells after ACV permeation studies.

Standard curves were found to be linear on three different days over the range 0.05–10 μg/mL (typical equation: $y = 310.86x + 0.948$). Linear regression analysis showed correlation coefficients greater than 0.999 in all curves ($n = 3$). For each calibration curve, the slope was statistically different from zero, and the intercept was not statistically different from zero. Moreover, a linear regression of the back-calculated concentrations vs. the nominal ones provided a unit slope and intercept equal to zero (Student *t*-test).

Accuracy of the Assay

Accuracy of the assay method was defined as the percentage of the systematic error, which is calculated as standardized agreement between the

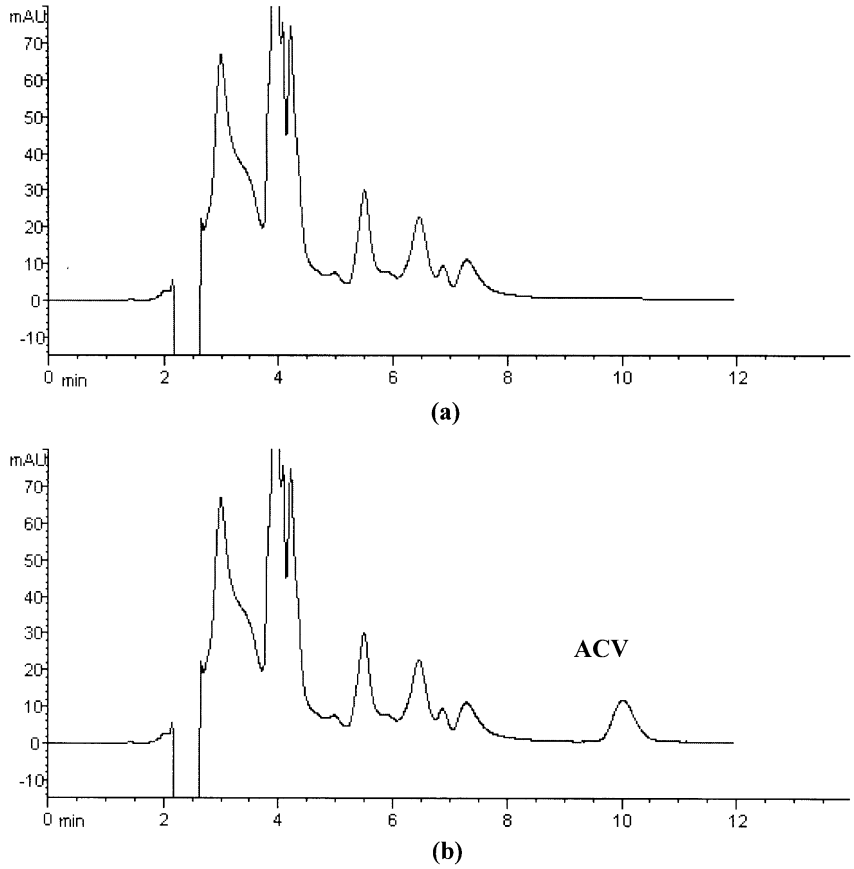

Figure 3. Representative chromatograms obtained by extraction from (a) blank skin slice and (b) slice of skin after an ACV permeation study.

measured value and the true value. Accuracy values studies at low, medium, and high ACV concentrations were always <5% (Table 1). These values were within acceptable limits (<15%).[12]

Precision of the Method

Precision of a method was expressed as the R.S.D. (relative standard deviation) of replicate measurements. In this work, precision of the method was tested on both within-day and between-day reproducibility in the assay. Within-day

Table 1. Accuracy of the Method for Determining ACV Concentrations (Expressed as Relative Error in %) ($n = 5$)

Concentration Added ($\mu g\,mL^{-1}$)	Concentration Found (Mean ± S.D.) ($\mu g\,mL^{-1}$)	Relative Error (%)
0.05	0.048 ± 0.002	−4.003
0.5	0.487 ± 0.009	−2.560
5	4.911 ± 0.013	−1.781

variability of the assay method was determined by repeated analysis of three concentrations of ACV in water (0.05, 0.5, and 5 μg/mL) on the same day. Similarly, between-day variability was determined by repeated analysis of the control samples on three different days.

The results for within-day and between-day precision 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.[12]

Robustness and Ruggedness

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 increased to 35°C, the elution times were modified. Slight modifications in the percentage of acetonitrile (1–5%) in the mobile phase also

Table 2. Between-Day and Within-Day Variability of the HPLC Method for Determining ACV Concentrations

	Between-Day Variability ($n = 4$)		Within-Day Variability ($n = 4$)	
Concentration Added (μg/mL)	Concentration Found (Mean ± S.D.) (μg/mL)	R.S.D. (%)	Concentration Found (Mean ± S.D.) (μg/mL)	R.S.D. (%)
0.05	0.048 ± 0.002	4.167	0.045 ± 0.001	2.222
0.5	0.499 ± 0.017	3.367	0.501 ± 0.024	4.904
5	4.916 ± 0.004	0.084	5.057 ± 0.161	3.186

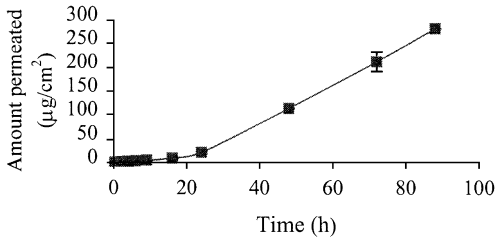

Figure 4. Amount of ACV found in the receptor compartment during 88 h of percutaneous permeation experiment. Each point represents the mean ± S.D. of three experiments.

altered the ACV 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.

Stability

Stability studies, carried out in water, indicated that samples were stable for at least several weeks when stored at 4°C. Anyway, no changes in the drug concentration were detected when the samples were stored at room temperature for two weeks.

Application of the Method

The application of this method has been demonstrated for the ACV quantification in porcine skin layers and in the receptor compartment of the diffusion cells, after in vitro percutaneous studies.

The amount of ACV found in the receptor compartment during a percutaneous permeation experiment after the topical application of an ACV suspension, is represented in Fig. 4. The experiment was carried out during 88 h and the ACV permeated increased considerably between 24 and 88 h.

Table 3. Recovery of ACV from Skin Slices

Acyclovir Added (μg/mL)	Acyclovir Extrated (μg/mL)	*n*	Recovery (%)	R.S.D. (%)
5.18 ± 0.03	4.94 ± 0.12	7	95.36	2.43
0.92 ± 0.01	0.91 ± 0.02	8	99.87	2.19
0.77 ± 0.01	0.76 ± 0.01	6	98.85	1.31

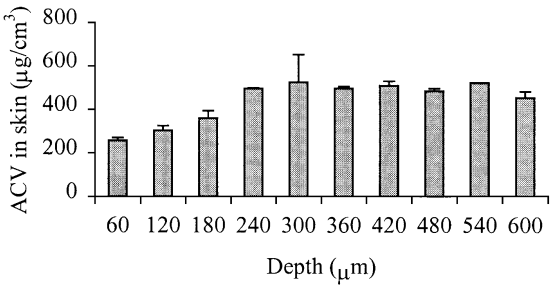

Figure 5. Distribution of ACV in porcine skin layers after topical application of an ACV suspension during 88 h. Each point represents the mean ± S.D. of three experiments.

Previously to the ACV quantification in the skin layers, the drug was extracted from skin slices (with the method described above), and satisfactory recoveries were obtained from all samples tested. The results, given in Table 3, were always above 95%.

Figure 5 shows ACV concentration determined in porcine skin slices at different depths, after 88 h of a percutaneous penetration experiment, using an ACV suspension. The accumulation of ACV in the skin increased up to 300 μm, and from this depth to 600 μm the profile remained practically constant.

CONCLUSIONS

A simple chromatographic method has been developed for the rapid and precise determination of ACV in both skin layers and percutaneous permeation samples. The simplicity of the technique, the short analysis time, and the high sensitivity makes this technique particularly attractive for this propose.

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

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