# Comparative Analysis of Acyclovir Esters Stability in Solutions: The Influence of the Substituent Structure, Kinetics, and Steric Effects

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ABSTRACT: Reversed-phase high-performance liquid chromatography has been applied to the determination of acyclovir (ACV) esters such as acetate, *iso*butyrate, pivalate, ethoxycarbonate, and nicotinate. All analyses were carried out at laboratory temperature using a column LiChrospher RP-18 (250 × 4 mm, 5 μm) and a proper mobile phase consisting of acetonitrile and phosphate buffer (pH 6 or 6.7) or acetonitrile and potassium dihydrogen phosphate, and acetic acid. The methods were validated by the determination of the following parameters: selectivity, precision, accuracy, and linearity. Kinetic studies on the hydrolysis were investigated in solutions at 310 K over the pH range 0.42–1.38. The pH-profiles indicated specific acid-catalyzed and spontaneous water-catalyzed degradation. The stability of the studied ACV esters were determined not only by steric factors. In the case of ethoxycarbonyl ester of ACV, the hydrolysis was a two-step reaction. © 2015 Wiley Periodicals, Inc. Int J Chem Kinet 47: 724–733, 2015

## INTRODUCTION

Owing to the prevalence of herpes viruses in the human population, and the fact that the infection is often asymptomatic, dedicated research and development work on effective drugs have been in progress for a long time. In the 1960s, trifluridine and vidarabine were applied to therapeutic use, i.e. drugs that are DNA polymerase inhibitors [1]. However, both of them are characterized by low selectivity and high toxicity. A new generation of an antiviral drug initiated by acyclovir (ACV), a guanosine analogue with marked selectivity in regard to the virus and the safe use, was a breakthrough. At a further stage, ganciclovir and pencyclovir as well as ester modifications of ACV (valacyclovir) and pencyclovir (famcyclovir) were developed and

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introduced into therapeutics [1]. The relatively low toxicity of ACV results from the mechanism of the action that requires its phosphorylation to a monophosphate. In this reaction, viral thymidine kinase is involved. ACV is not a substrate for the host's cellular-type kinase [2,3]. For the improvement of pharmacokinetic properties of drugs containing ACV, different preparations with modified release to allow a reduction in the frequency of drug administration, are being developed [4,5]. There is also research work being carried out to apply mucoadhesive microspheres composed of ethyl cellulose and Carbapol resulting in a significant prolongation of drug residence in the upper and middle sections of the digestive system in comparison with conventional microspheres [6]. In the case of ophthalmic drugs, the potential application of microspheres prepared by the spray drying method and nanocapsules made by the ionotropic gelation method (a combination of chitosan and tripolyphosphate) is being considered [7].

Another way to improve the effectiveness of the therapy with ACV is the chemical modification of the molecule toward the formation of an ester within the pseudosugar group. The only ACV ester approved for trading is valacyclovir (combined with L-valine), and it is rapidly converted into ACV. Its bioavailability is about 54%, and it is several times higher than for ACV [8]. This is a drug of high stability in an acidic environment, but it is metabolized intensively in the intestines and liver [8–10]. ACV esters have also been synthesized with other amino acids, such as glycine, alanine, glutamic acid, serine, and *iso*leucine [8,11]. Glycine and alanine esters proved to be too unstable at physiological pH [8,11]. In the intestinal lumen and in homogenizates of Caco-2 and liver cells, the ester of $\gamma$-glutaminic acid has been the most stable, whereas the stability of L-serine ester has turned out to be similar to valacyclovir [11]. Furthermore, L-serine and L-*iso*leucine esters have been recognized as promising ACV precursors because they have revealed the best absorption from the intestinal lumen [11]. For dipeptide derivatives, it has been demonstrated that direct binding with valine significantly stabilizes the ester binding that is influenced by steric properties of this amino acid [12]. The bivaline derivative is also characterized by higher solubility in water in comparison with ACV, and better bioavailability as well [12–14]. The low stability of amino acid esters in water solutions does not allow a drug being developed to be administered as an injection. Therefore, ACV esters with the aminomethylbenzoyl group have been analyzed to prove that the incorporation of an aromatic ring between the amino group and the carboxyl group stabilizes the molecule [15], whereas the insertion of a thiazole ring does not have such an effect [16,17]. For the purpose of use after oral and nasal mucosa administration, the stability of aliphatic (ACV) esters (*n*-butyrate, pivalate, valerate, and hexanoate) has been investigated. These esters are characterized by higher lipophilicity and reduced solubility in water in comparison with ACV [18]. Their stability has been investigated in plasma. It has been proven that the pivaloyl ester is more resistant to the impact of plasma esterase, and only the hexane ester has shown satisfactory properties relating to penetration through the nasal mucosa [18].

However, in the literature, there are no comprehensive studies on the stability of ACV esters in water solutions and the evaluation of the impact of hydrogen ions on the observed hydrolysis reaction. The main aim of this study is to determine kinetic parameters of the hydrolysis of ACV esters (acetate, *iso*butyrate, pivalate, etoxycarbonate, and nicotinate; Fig. 1) under conditions of constant ionic strength (0.50 mol $L^{-1}$) and at a temperature of 310 K (37°C). Additionally, to determine catalytic rate constants by a kinetic equation describing the observed reaction and analysis of the correlation between calculated parameters with the steric factors.

## EXPERIMENTAL

ACV was received from JELFA S.A. (Jelenia Góra, Poland). Esters of ACV (Fig. 1): acetyl (Ac-ACV), *iso*-butyryl (*i*But-ACV), pivaloyl (Piv-ACV), ethoxycarbonyl (Etc-ACV), and nicotinoyl (Nic-ACV) were synthesized in a laboratory, for the purpose of the present study [19]. Acetonitrile for HPLC (isocratic basis) and methyl 4-hydroxybenzoate (99%) were obtained from POCH S.A. (Gliwice, Poland) but sulfathiazole (98.0%) and sulfamerazine (99.0%) were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals of the highest purity were commercially available.

### Apparatus and Chromatographic Conditions

The high-performance liquid chromatography (HPLC) with a system consisting of a Rheodyne 7120, 20 μL fixed-loop injector, an LC 3-UV detector (Pye Unicam, England), an L-6000A pump (Merck-Hitachi, Germany), and an A/C transmitter with Chromed software (Medson, Poland), was used for the determination of tested substances and their decomposition product – ACV. As a stationary phase, a LiChrospher RP-18 column (250 × 4 mm, 5 μm; Merck, Germany) was

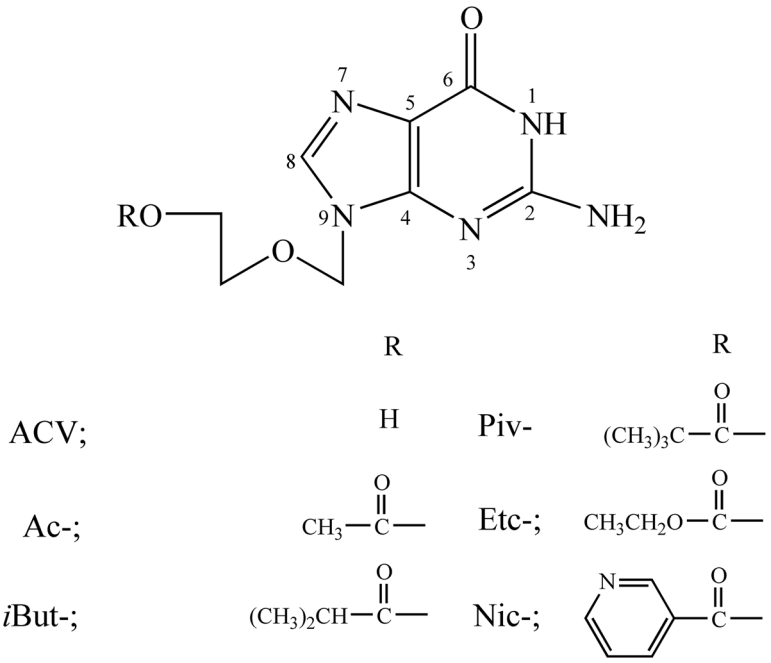



**Figure 1** Chemical structure of ACV esters.

**Table I** The Composition of the Mobile Phase and Solutions of Internal Standards for the Determination of Various Esters

| | | | Retention Time (min) | | |
|---|---|---|---|---|---|
| Compound | Mobile Phase Composition | Internal Standard Solution (μg $mL^{-1}$) | ACV | Compound | Internal Standard |
| Ac-ACV | ACN and 20 mmol $L^{-1}$ phosphate buffer pH 6 (25:75) | Sulfathiazole (120)* | 1.99 | 2.48 | 3.70 |
| *i*But-ACV | ACN and phosphate buffer 20 mmol $L^{-1}$ pH 6.7 (30:70) | Methyl 4-hydroxybenzoate (90)* | 1.95 | 3.28 | 7.15 |
| Piv-ACV | | | | 4.05 | |
| Etc-ACV | $KH_2PO_4$ 0.5 mol $L^{-1}$ – ACN – $CH_3COOH$ (99,9%) – $H_2O$ | Sulfamerazine (240)* | 2.09 | 4.55 | 6.20 |
| Nic-ACV | (40:200:5:755) | | | 3.50 | |

*In a mixture of ACN and phosphate buffer 0.2 mol $L^{-1}$ pH 6 (1:1).

used. The flow rate of the mobile phases (Table I) was 1.0 mL $min^{-1}$. The UV detection was carried out at 254 nm.

The pH values of the buffer solutions were measured on a HI 110 pH-Meter (HANNA Instruments, Romania).

## Validation of the HPLC Method

All analyses was carried out at laboratory temperature using a reversed phase technique (RP-HPLC) on the analytical column RP-18. The phosphate buffer pH 6 used as a component of the mobile phase was a mixture of $K_2HPO_4$ (17 mmol $L^{-1}$) and $KH_2PO_4$ (3 mmol $L^{-1}$), and the phosphate buffer pH 6.7 was the mixture of $KH_2PO_4$ (11 mmol $L^{-1}$) and $Na_2HPO_4$ (9 mmol $L^{-1}$), respectively.

After hydrolysis in acidic medium, the solutions of tested substances at a concentration of ca. 180 μg $mL^{-1}$ in mixtures with internal standards and the phosphate buffer pH 6 (0.2 mol $L^{-1}$) (1:1:1) were chromatographed using a column LiChrospher RP-18 (250 × 4 mm, 5 μm) and a proper mobile phase (Table I). Simultaneously, the retention times were

**Table II** The Quantitative Parameters and Statistical Data for Determination of ACV Esters by the Proposed HPLC Method: Calibration Curves

| Compound | Range ($\mu g\ mL^{-1}$) | $n$ | Slope ($b$) | Correlation Coefficient ($r$) | LOD ($\mu g\ mL^{-1}$) | LOQ ($\mu g\ mL^{-1}$) |
|---|---|---|---|---|---|---|
| Ac-ACV | 5.0–120.0 | 11 | $0.0130 \pm 0.0005$ | 0.9985 | 4.19 | 12.70 |
| *i*But-ACV | 5.0–150.0 | 10 | $0.0100 \pm 0.0003$ | 0.9994 | 3.20 | 9.70 |
| Piv-ACV | 10.0–150.0 | 9 | $0.0097 \pm 0.0004$ | 0.9984 | 5.75 | 17.42 |
| Etc-ACV | 10.0–200.0 | 11 | $0.0040 \pm 0.0001$ | 0.9989 | 5.57 | 16.87 |
| Nic-ACV | 10.0–150.0 | 9 | $0.0068 \pm 0.0002$ | 0.9994 | 3.62 | 10.70 |

determined for ACV (50 $\mu g\ mL^{-1}$ in the mobile phase) and nicotinic acid (50 $\mu g\ mL^{-1}$ in the mobile phase) as the ester degradation products.

The linearity was assessed in the range from ca. 5 to ca. 200 $\mu g\ mL^{-1}$ of the esters (Table II) in the appropriate mobile phase (Table I). For each sample, the test was performed three times. A statistical evaluation of the above relationships was described by the equation $y = bx$ (the intercepts where statistically insignificant). The limits of detection (LOD) and quantitation (LOQ) were determined by using the following formula:

$$\mathrm{LOD(LOQ)} = \frac{\kappa \cdot \mathrm{SD}_a}{b} \tag{1}$$

where $\kappa$ is 3.3 for LOD and 10 for LOQ, $SD_a$ is the standard deviation of the intercept, and $b$ is the slope.

The precision, accuracy, and reproducibility of the method was assessed by determining the content of tested substance in solution in the mobile phase, at two different concentrations ($\mu g\ mL^{-1}$; Ac-ACV: 25.0, 90.0; *i*But-ACV and Piv-ACV: 25.0, 125.0; Etc-ACV: 25.0, 175.0; Nic-ACV: 40.0, 125.0). The study was performed in two days (interday, intraday), and the test was repeated six times for each series of solutions.

## Kinetic Procedures

Hydrochloric acid solutions (0.05–0.50 mol $L^{-1}$) were prepared while maintaining a constant ionic strength ($\mu$ 0.50 mol $L^{-1}$) by adding varying amounts of the sodium chloride solution (2 mol $L^{-1}$). The solutions (9.5 mL) were equilibrated at the temperature of the study (310 K) prior to initiation of the reaction. The reaction was initiated by adding 0.5 mL of the ester solution in the same hydrochloric acid (ca. 3.6 mg $mL^{-1}$). At specified time points, a 0.2-mL of the reaction mixture was transferred into 5 mL tubes and neutralized by adding 0.2 mL of NaOH solution with a concentration equal to the concentration of the acid. Then, the mixture was cooled in ice water to stop the reaction. To each mixture, 0.2 mL of the internal standard solution was added and the contents of the tested compounds were determined by the HPLC method. The final concentration of tested substances in the mixture applied to the column was ca. 60 $\mu g\ mL^{-1}$.

The pH values of the hydrochloric acid at the temperature of the study were calculated from the activity coefficient data [20].

## Statistical Calculation

Statistical analyses of the results were performed using a spreadsheet of MS Excel program. The errors of the values were estimated and presented as a confidence interval for a level of significance $\alpha = 0.05$, standard deviation (SD), and coefficient of variation (RSD,%). Repeatability of results for series of assays was analyzed by the F-Snedecor test for comparing of variance and the *t*-student test for comparing of mean values. The correlation analyses were performed using the test of significance of the correlation coefficient ($r$). The assessment of significance of the regression coefficients was included in the regression analysis with the significance level $\alpha = 0.05$.

# RESULTS AND DISCUSSION

## HPLC Determination: Validation of the Method

Separately prepared conditions (Table I) were used to develop the RP-HPLC method for the determination of all five esters. According to the selectivity, the peaks position of the compounds: the signals of substrates, expected degradation products, as well as the internal standards were separately observed (Table I). In the case of Ac-, *i*But-, Piv-, and Etc-ACV, the ACV molecule and an aliphatic acid, which does not have UV absorption, were formed by hydrolysis. The retention time of nicotinic acid (a hydrolysis product of ester Nic-ACV) was compatible with the elution time of ACV (Table I). For the linearity study of the determination of esters, solutions of each compounds in

**Table III** The Precision and Accuracy of the Proposed HPLC Methods for the Determination of ACV Esters in Solution ($n = 6$)

| Compound | Declared Content ($\mu g\ mL^{-1}$) | Amount Found (mg) (Recovery, %) | | Repeatability $RSD_r$% |
|---|---|---|---|---|
| | | Interday | Intraday | |
| Ac-ACV | 25.0 | 23.2 ± 0.2 (92.8 ± 0.8) | 24.0 ± 0.3 (96.1 ± 1.1) | 0.37 |
| | 90.0 | 88.6 ± 1.1 (98.5 ± 1.3) | 85.8 ± 0.4 (95.3 ± 0.4) | 0.24 |
| *i*But-ACV | 25.0 | 23.8 ± 0.4 (95.4 ± 1.7) | 25.2 ± 0.3 (101.0 ± 1.1) | 0.37 |
| | 125.0 | 131.6 ± 1.6 (105.3 ± 1.3) | 129.4 ± 1.1 (103.5 ± 0.9) | 0.28 |
| Piv-ACV | 25.0 | 23.3 ± 0.4 (93.1 ± 1.5) | 23.0 ± 0.3 (92.2 ± 1.0) | 0.36 |
| | 125.0 | 117.3 ± 0.7 (93.8 ± 0.5) | 117.6 ± 1.2 (94.0 ± 0.9) | 0.31 |
| Etc-ACV | 25.0 | 24.2 ± 0.3 (96.6 ± 1.2) | 23.8 ± 0.4 (95.3 ± 1.5) | 0.35 |
| | 175.0 | 176.2 ± 0.6 (100.7 ± 0.4) | 177.6 ± 1.0 (101.5 ± 0.6) | 0.18 |
| Nic-ACV | 40.0 | 39.7 ± 0.7 (99.2 ± 1.8) | 42.0 ± 0.5 (104.9 ± 1.1) | 0.37 |
| | 125.0 | 124.6 ± 0.6 (99.7 ± 0.5) | 137.3 ± 1.4 (109.8 ± 1.1) | 0.31 |

appropriate mobile phases in the range of 5 to about 200 $\mu g\ mL^{-1}$ were prepared (Table II). The relationships of the observed signals analyzed as a function of the concentration of esters had the linear character described by the equation $y = bx$. The values of correlation coefficients ≥0.9984 indicated a strong correlation of the examined relationships. Calculated parameters of the equations were used to determine the limits of detection (LOD: 3.2–5.8 $\mu g\ mL^{-1}$) and quantification (LOQ: 9.7–16.9 $\mu g\ mL^{-1}$) of the tested compounds (Table II). Comparison of the regression coefficients (the slope) can sort the HPLC method in terms of sensitivity. Thus, sensitivity for the determination of the tested compounds was decreased in the order: Ac-ACV > *i*But-ACV > Piv-ACV > Nic-ACV > Etc-ACV (Table II). To assess the precision, accuracy, and repeatability of the methods, the esters were studied in two solutions, in significantly different concentrations. The tests were repeated the next day for parameter estimation and validation on an interday and an intraday. The values of the coefficients of variation less than 1.9% indicated an adequate precision for the research methods. The coefficients of variation for repeatability did not exceed 0.4% (Table III). The accuracy of the developed methods was evaluated on the basis of recovery in the range from 93% to 96% for Ac-ACV and from 99% to 110% for Nic-ACV (Table III). The results of statistical analyses have confirmed that the developed chromatographic conditions provide precise, accurate, and reproducible analyses of the esters studied in solutions.

## Observed and Catalytic Rate Constants

Kinetic studies of the decomposition of esters was carried out in hydrochloric acid (0.05–0.50 mol $L^{-1}$) pH (0.42–1.38) at a temperature of 310 K, which was accepted as suitable for testing at a temperature approaching body temperature (Table IV). All solutions were applied to a constant value of the ionic strength ($\mu$ = 0.50 mol $L^{-1}$) by the addition of an appropriate volume of sodium chloride solution (2.0 mol $L^{-1}$). The p$K_a$ of acyclovir (p$K_{a1}$ = 2.27, p$K_{a2}$ = 9.25) [21] concluded that esters of ACV exist in the protonated ($BH^+$) and neutral (B) forms in this pH range, and these forms were hydrolyzed. Therefore, in the acid medium the neutral forms are between 1.4% in pH solutions 0.42 and 11.4% in pH solutions 1.38. For the formation of the ionized forms ($BH^+$), the amine groups at the C2 position of ACV esters have been protonated. In the acidic medium, the observed hydrolysis reactions were the pseudo–first-order reactions (Fig. 2a) described by the following equation:

$$\ln P_t = \ln P_0 - k_{obs} \cdot t \tag{2}$$

where $P_t$ and $P_0$ are the ratio of the peak areas of the substrate to the internal standard peak determined for the respective time $t$ and $t_0$, $k_{obs}$ is the observed rate constant, and $t$ is the time of the reaction (Table IV).

The values of the observed rate constant ($k_{obs}$) corresponding to $k_{pH}$ values (acid–base catalysis) were used to determine the catalytic rate constants, describing the catalytic effect of hydrogen ions ($k_{H+}$) and the spontaneous hydrolysis by water ($k_{H_2O}$) on the hydrolysis of esters (Table IV). The overall reaction of esters hydrolysis in pH range 0.42–1.38 can be defined by the following equation:

$$k_{pH} = k_{H+} \cdot a_{H+} \cdot f_1 + k_{H_2O} \cdot f_1 \tag{3}$$

**Table IV** The Kinetic Parameters ($k_{obs}$) of Hydrolysis of ACV Esters in the Hydrochloric Acid Medium (310 K, $\mu = 0.50$ mol L$^{-1}$)

| Compound | $c_{HCl}$ (mol L$^{-1}$) | pH | $k_{obs} \pm \Delta k_{obs}$ (s$^{-1}$) | $-r$ | $n$ | Parameters of $k_{pH}$ Equation (3) |
|---|---|---|---|---|---|---|
| Ac-ACV | 0.05 | 1.38 | $(1.30 \pm 0.12) \times 10^{-5}$ | 0.9908 | 13 | $k_{H+} = (3.38 \pm 0.51) \times 10^{-4}$ |
| | 0.10 | 1.10 | $(2.40 \pm 0.15) \times 10^{-5}$ | 0.9928 | 18 | $SD = 1.96 \times 10^{-5}$ |
| | 0.20 | 0.81 | $(5.06 \pm 0.38) \times 10^{-5}$ | 0.9924 | 15 | $k_{H_2O}$ = ns |
| | 0.30 | 0.65 | $(6.32 \pm 0.66) \times 10^{-5}$ | 0.9904 | 11 | $r = 0.9920$ |
| | 0.40 | 0.52 | $(1.07 \pm 0.05) \times 10^{-4}$ | 0.9968 | 16 | $n = 6$ |
| | 0.50 | 0.42 | $(1.28 \pm 0.05) \times 10^{-4}$ | 0.9977 | 15 | |
| *i*But-ACV | 0.05 | 1.38 | $(6.02 \pm 0.22) \times 10^{-6}$ | 0.9979 | 17 | $k_{H+} = (1.72 \pm 0.14) \times 10^{-4}$ |
| | 0.10 | 1.10 | $(1.37 \pm 0.06) \times 10^{-5}$ | 0.9966 | 18 | $SD = 5.56 \times 10^{-6}$ |
| | 0.20 | 0.81 | $(2.53 \pm 0.07) \times 10^{-5}$ | 0.9989 | 13 | $k_{H_2O}$ = ns |
| | 0.30 | 0.65 | $(4.04 \pm 0.28) \times 10^{-5}$ | 0.9964 | 10 | $r = 0.9973$ |
| | 0.40 | 0.52 | $(4.91 \pm 0.28) \times 10^{-5}$ | 0.9976 | 10 | $n = 6$ |
| | 0.50 | 0.42 | $(6.45 \pm 0.55) \times 10^{-5}$ | 0.9937 | 11 | |
| Piv-ACV | 0.05 | 1.38 | $(1.07 \pm 0.29) \times 10^{-6}$ | 0.9728 | 7 | $k_{H+} = (2.30 \pm 0.18) \times 10^{-5}$ |
| | 0.10 | 1.10 | $(1.92 \pm 0.22) \times 10^{-6}$ | 0.9919 | 9 | $SD = 6.84 \times 10^{-7}$ |
| | 0.20 | 0.81 | $(3.33 \pm 0.26) \times 10^{-6}$ | 0.9945 | 11 | $k_{H_2O}$ = ns |
| | 0.30 | 0.65 | $(5.39 \pm 0.34) \times 10^{-6}$ | 0.9975 | 9 | $r = 0.9976$ |
| | 0.40 | 0.52 | $(6.60 \pm 0.54) \times 10^{-6}$ | 0.9950 | 10 | $n = 6$ |
| | 0.50 | 0.42 | $(8.57 \pm 0.31) \times 10^{-6}$ | 0.9990 | 10 | |
| Etc-ACV | 0.05 | 1.38 | $(2.48 \pm 0.33) \times 10^{-7}$ | 0.9614 | 22 | $k_{H+} = (2.55 \pm 0.25) \times 10^{-6}$ |
| | 0.10 | 1.10 | $(4.06 \pm 0.44) \times 10^{-7}$ | 0.9779 | 19 | $SD = 8.98 \times 10^{-8}$ |
| | 0.20 | 0.81 | $(5.47 \pm 0.64) \times 10^{-7}$ | 0.9703 | 22 | $k_{H_2O} = (1.90 \pm 0.57) \times 10^{-7}$ |
| | 0.30 | 0.65 | $(7.26 \pm 0.62) \times 10^{-7}$ | 0.9836 | 22 | $SD = 2.07 \times 10^{-8}$ |
| | 0.40 | 0.52 | $(9.53 \pm 0.93) \times 10^{-7}$ | 0.9833 | 18 | $r = 0.9975$ |
| | 0.50 | 0.42 | $(1.15 \pm 0.10) \times 10^{-6}$ | 0.9877 | 16 | $n = 6$ |
| Nic-ACV | 0.05 | 1.38 | $(2.67 \pm 0.30) \times 10^{-7}$ | 0.9888 | 11 | $k_{H+} = (2.76 \pm 0.73) \times 10^{-6}$ |
| | 0.10 | 1.10 | $(4.15 \pm 0.57) \times 10^{-7}$ | 0.9839 | 11 | $SD = 2.28 \times 10^{-7}$ |
| | 0.20 | 0.81 | $(6.43 \pm 0.71) \times 10^{-7}$ | 0.9894 | 11 | $k_{H_2O} = (2.19 \pm 1.35) \times 10^{-7}$ |
| | 0.30 | 0.65 | $(8.72 \pm 0.54) \times 10^{-7}$ | 0.9961 | 12 | $SD = 4.25 \times 10^{-8}$ |
| | 0.40 | 0.52 | $(9.86 \pm 0.71) \times 10^{-7}$ | 0.9902 | 19 | $r = 0.9899$ |
| | | | | | | $n = 5$ |

ns, not significant; $k_{H+}$ (mol$^{-1}$ L s$^{-1}$); $k_{H_2O}$ (s$^{-1}$).

or, by its kinetic equivalent:

$$k_{pH} = k_{H+} \cdot a_{H+} \cdot f_1 + k'_{H+} \cdot a_{H+} \cdot f_2 \qquad (4)$$

where $a_{H+}$ refers to the hydrogen ion activity, $f_1$ and $f_2$ are fractions of the ionic forms $BH^+$ and B, respectively:

$$f_1 = \frac{(a_{H+})^2}{(a_{H+})^2 + K_{a1} \cdot a_{H+} + K_{a1} \cdot K_{a2}} \qquad (5)$$

$$f_2 = \frac{K_{a1} \cdot a_{H+}}{(a_{H+})^2 + K_{a1} \cdot a_{H+} + K_{a1} \cdot K_{a2}} \qquad (6)$$

The p$K_a$ values of esters calculated by theoretical methods showed that the substituents have no effect on the p$K_{a1}$ values (MarvinSketch structure-based calculations). The analysis of compliance $k_{pH}$ values calculated (Eq. (3)) and designated experimentally confirmed the usefulness of taking account of the value of p$K_{a1} = 2.27$ in Eq. (3) (Fig. 2b) for the analysis of the relationship log $k_{pH}$ – pH. The analysis of the relationship between $k_{pH}/f_1$ values and the hydrogen ion activity ($a_{H+}$) as well as $k_{pH}/a_{H+}$ and the fraction of the neutral forms ($f_2$) of Ac-, *i*But-, and Piv-ACV esters showed that catalytic rate constants $k_{H_2O}$ are not statistically significant ($k_{H_2O} = 0$, Table IV) as well as $k_{H+}$ and $k'_{H+}$ are statistically equal ($k_{H+} = k'_{H+}$). Thus, for these esters Eq. (3) and (4) can take the form of

$$k_{pH} = k_{H+} \cdot a_{H+}(f_1 + f_2) = k_{H+} \cdot a_{H+} \qquad (7)$$

In the case of Etc- and Nic-ACV esters, the values of $k_{H+}$ and $k'_{H+}$ are not statistically equal ($k_{H+} \neq k'_{H+}$). The values of the catalytic rate constants describing

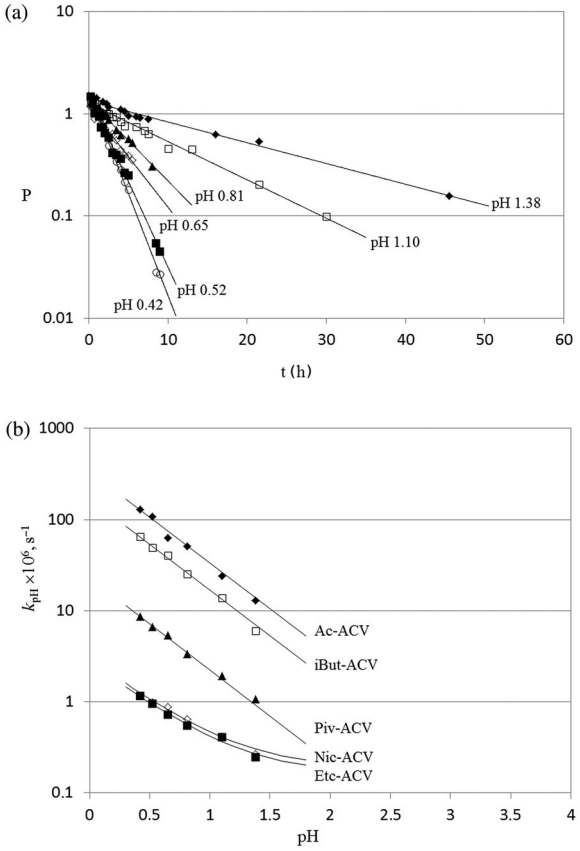



**Figure 2** Apparent pseudo–first-order plots for degradation of acyclovir acetate in hydrochloric acid (pH 0.42–1.38; μ 0.50 mol $L^{-1}$; 310 K) (a) and the log *k*-pH profiles for the hydrolysis of ACV esters in acid medium pH 0.42–1.38 at 310 K (the circles represent the experimentally determined values; the lines represent theoretical curves calculated from the $k_{pH}$ equation (3)) (b).

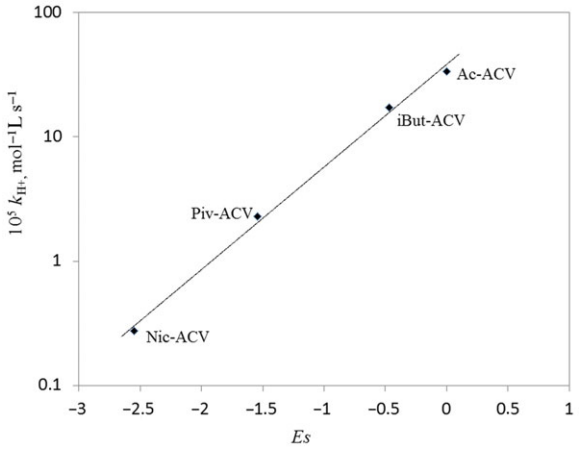



**Figure 3** Correlation between the steric factors (*Es*) and the catalytic rate constants $k_{H+}$ for acid-catalyzed hydrolysis of ACV esters: acetate, *iso*butyrate, pivalate, and nicotinate.

the catalytic effect of hydrogen ions on the hydrolysis of tested esters ($k_{H+}$) were calculated from Eq. (3) with a good correlation of the results (Table IV) since Eq. (4) involving the form of the neutral molecule (below 11.4%) was the cause of the high error in calculation of these values.

The log $k_{pH}$ – pH profiles (Fig. 2b) for the hydrolysis of ACV esters were constructed from the logarithm of rate constants $k_{pH}$ and the pH values at 310 K. The compatibility of the experiment with calculated values $k_{pH}$ (Eq. (3)) confirms the correctness of the assumptions made. Therefore, at 310 K in hydrochloric acid medium (pH 0.42–1.38) the hydrolysis of the esters is composed of

- hydrolysis of the protonated and neutral forms by hydrogen ions (all esters) and/or
- spontaneous hydrolysis of the protonated form by water (Etc-ACV and Nic-ACV).

The general scheme of the observed reaction can be summarized as follows:

$$BH^+ + H_3^+O \rightarrow \text{Products},\ k_{H+}$$

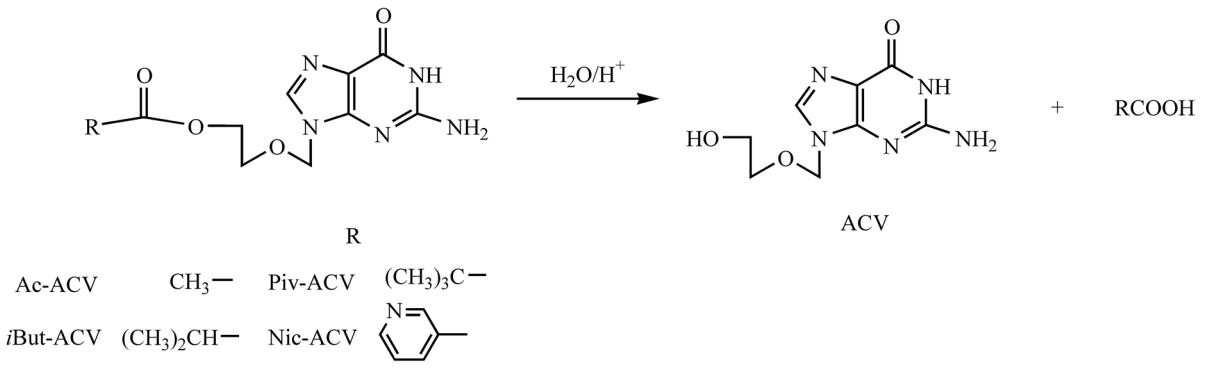
$$B + H_3^+O \rightarrow \text{Products},\ k'_{H+}$$

$$BH^+ + H_2O \rightarrow \text{Products},\ k_{H_2O}$$

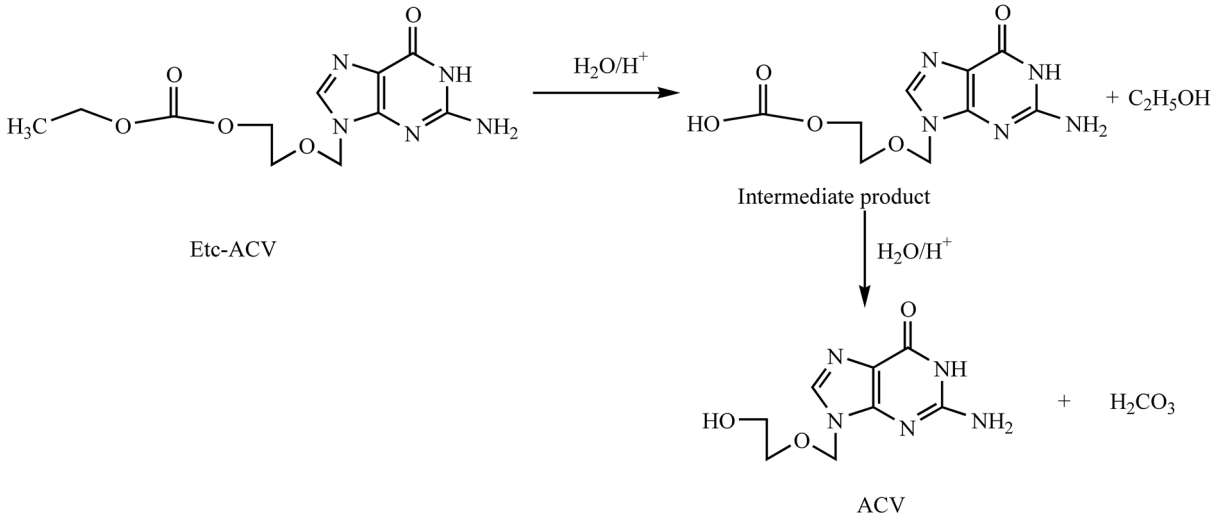
## The Influence of Steric Effects on the Stability and Mechanism of the Reaction

It is known that structural modifications influence the stability, physicochemical properties, and biological activity through polar, steric, or resonance effects. According to the Taft's analysis, the relative rates of acid-catalyzed hydrolysis or esterification are determined only by steric factors, whereas for base-catalyzed reaction both polar and steric factors (*Es*) are involved [22]. The steric effect can also be described by the *v* parameter, which is independent of the kinetic data but dependent on the van der Waals radius of the substituent group [23]. Figure 3 shows a good semilogarithmic correlation ($r = 0.9984$) between steric factors, which differentiate tested esters of ACV and the catalytic rate constants $k_{H+}$ describing the catalytic effect of hydrogen ions on the hydrolysis of these esters. The steric factors standardized for the $CH_3$ group – *Es(CH₃)* were taken from the literature: methyl 0.00 (for Ac-ACV); *iso*-propyl –0.47 (for *i*But-ACV);

a) **Mechanism A**




b) **Mechanism B**




**Scheme 1** Mechanism of acid-catalyzed hydrolysis of ACV esters.

*tert*-butyl –1.54 (for Piv-ACV); phenyl –2.55 (for Nic-ACV; 2-pyridinyl and phenyl substituents was considered as isosteric) [24]. This relationship confirms the influence of the steric effect on the stability of these four esters. If the polar effects of substituents are irrelevant, Taft's equation takes the following form:

$$\log\frac{k_s}{k_{CH_3}} = \delta Es \tag{8}$$

where $\log(k_s/k_{CH_3})$ is the ratio of the rate of the substituted reaction compared to the reference reaction and $\delta$ is the sensitivity factor for the reaction to steric effects. The sensitivity factor in the acid hydrolysis is 1 [25]. Therefore, the steric factors for a number of tested compounds can be calculated by taking into account the value of $k_{H+}$. And so, the calculated values of *Es(CH3)* were –0.29 (*iso*-propyl), –1.17 (*tert*-butyl), –2.12 (ethoxy), and –2.09 (3-pyridinyl), respectively. The negative values indicate that these substituents decrease the rate of hydrolysis of the examined esters (show a stabilizing effect) assuming only the influence of steric effects. It should be noted that if the steric and also inductive mesomeric effects are present in the hydrolysis, the calculated values of *Es* also take into account the influence of the electronic coupling between the substituent and the reaction center.

Computational studies suggest that the ester hydrolysis mechanisms of aliphatic and aromatic acids for the destruction of the ester bond could be different depending on the accepted model of the reaction [26–28]. The mechanism proposed for hydrolysis of methyl

acetate [26] in acidic conditions assumes the involvement of two water molecules and the formation of a tetrahedral intermediate. The protonation of carbonyl oxygen facilitates the addition of one water molecule at the carbonyl carbon to form a tetrahedral intermediate, which is stabilized by an additional water molecule via a hydrogen bond [26]. In this case, the hydrolysis proceeds in two steps, i.e. the formation of the tetrahedral intermediate and its decomposition. A similar two-step mechanism was proposed for acidic hydrolysis of ethyl acetate and ethyl benzoate studied by a model cluster consisting of an ester, a $H_3O^+$ cation and 15 water molecules [27]. However, in case of three explicit water molecules included in the modeled reaction, the hydrolysis proceeds in one step and the tetrahedral intermediate does not form in the acid-catalyzed hydrolysis of ethyl benzoate [28]. Calculated values of activation energy ($\Delta E^{\ddagger}$) of the hydrolysis reaction of methyl acetate, ethyl acetate, and ethyl benzoate in the proposed models of reaction were 18.3 [26], 19.18 [27], and 25.10 kcal mol$^{-1}$ [27], respectively. It is common knowledge that the rates of chemical reactions are dependent on their activation energies. The values of the catalytic rate constants of the hydrolysis of ACV esters in the present experiments are approximately two (*i*But-), 10 (Piv-), and 100 times (Etc-, Nic-) lower than the value determined for acetate ACV (Table IV), which indicates differences in the activation energies of their acid hydrolysis. Therefore, in accordance with [26,27], the stability of these esters is due to differences in activation energy of the reaction. This may provide an additional explanation for such a high stability of nicotinoyl and ethoxycarbonyl esters.

In case of the ethoxycarbonyl ester (carbonate ester), its stability should be explained by the difference in the mechanism of the reaction in comparison with the other esters (Scheme 1). That fact may result from the two-step reaction in which an unstable intermediate product has been formed. The intermediate peak was not observed as an additional peak in the chromatogram of HPLC, and therefore the kinetic parameters of the reaction of its formation and hydrolysis were not determined. This is a two-step reaction, and its rate is determined by the first stage of the hydrolysis, which is slower than the second stage. A rapid preequilibrium protonation step is followed by a slow bimolecular reaction, the attack of a water molecule and carbonyl-oxygen fission [29–31]. Subsequently, the reaction is followed by a fast unimolecular acyl-oxygen fission of the alkyl hydrogen carbonate [30,32]. The stability studies of the phenolic carbonate esters demonstrated that the carbonate esters appear to be less reactive than the corresponding carboxylic acid ester derivatives [33]. Thus, the mechanism of the hydrolysis reaction of the Etc-ACV reaction (Scheme 1b) is compliant with the mechanism proposed by Østergaard and Larsen [33].

## CONCLUSIONS

The validated RP-HPLC methods have been developed for stability studies of ACV esters (Ac-, *i*But-, Piv-, Etc-, Nic-) in acidic medium. The methods are precise, accurate, linear and simple for this purpose. The developed chromatographic parameters allow the determination of ACV esters in the mixture with degradation products. In the acidic medium (pH 0.42–1.38) at temperature 310 K, the hydrolysis of the protonated form by hydrogen ions and spontaneous hydrolysis of the protonated form by water were observed. The stability of the ACV esters is determined not only by steric properties of their chemical structure. The hydrolysis of the ethoxycarbonyl ester of ACV (Etc-ACV) is a two-step reaction.

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