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Syntheses, chemical and enzymatic stability of new poly(ethylene glycol)–acyclovir prodrugs

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Abstract

Two known antiherpetic agents, acyclovir and valaciclovir, were coupled with activated poly(ethylene glycol). In vitro drug release studies demonstrated the conjugates to be stable in buffer solutions at pH 7.4 and 5.5, while only PEG–valacyclovir$_2$ was stable in a buffer solution at pH 1.2. The ability of the macromolecular conjugate to release the free drug was also evaluated in plasma, in which the most stable prodrug also proved to be PEG–valacyclovir$_2$. The derivatives are degraded in the presence of proteolytic enzyme. The rate of hydrolysis was monitored by HPLC-analysis. © 2002 Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

Keywords: Macromolecular prodrugs; Acyclovir; Valacyclovir; PEG; Poly(ethylene glycol)

1. Introduction

Biological membranes are lipophilic and thus only relatively lipophilic molecules can penetrate such membranes. To obtain a successful formulation, the drug must both be water-soluble (i.e. hydrophilic) and lipid-soluble (i.e. hydrophobic). Since acyclovir is poorly soluble in water, we tried to improve its water solubility and chemical stability by linking it, via covalent bonding, to a water-soluble polymeric carrier, which we also felt would be a way to increase the duration of activity through slow release.

We chose poly(ethylene glycol), PEG, as the carrier polymer, because it is known to be non-toxic, non-antigenic, non-teratogenic, non-immunogenic and biocompatible. Available in a variety of molecular weights, PEG is a linear, uncharged, amphiphilic polymer that is soluble in water and in most organic solvents [1] and has solubilizing properties. It is rapidly eliminated from the body, and has been approved for a wide range of biomedical applications.

PEG may transfer its properties to another molecule when it is covalently bound to that molecule, which could result in toxic molecules becoming soluble and non-toxic, or hydrophobic molecules becoming soluble (when coupled to PEG). Coupling of a biological molecule to PEG usually contributes to its biological activity.

PEG has two hydroxylic end groups that do not allow direct attachment of acyclovir to the polymer. Therefore, we carried out derivatization of PEG to introduce a functional group suitable for conjugation to acyclovir ''via ester derivatives''.

Through PEG complexation the aqueous solubility of some lipophilic, water-insoluble drugs can be increased without changing their molecular structure; that is without affecting their intrinsic ability to permeate lipophilic biological membranes.

Acyclovir, a synthetic purine nucleoside analogue derived from guanine, is used mainly for the treatment of viral infections. The intravenous infusion of acyclovir as the sodium salt produces plasma acyclovir concentrations that demonstrate a two-phase pattern. Acyclovir is excreted by the kidney through filtration by the glomerules and tubular secretion. The terminal or beta-phase half-life is reported to be about 2–3 h in adults without renal impairment. In chronic renal failure, this value is increased and may reach 19.5 h in anuric patients. Approx. 15–30% of an oral dose of

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acyclovir is considered to be adsorbed from the gastrointestinal tract. The orally active prodrug valacyclovir has been developed in order to overcome this poor absorption.

The effectiveness of topical acyclovir in treating herpetic keratitis has been demonstrated. Acyclovir has also been used systemically to treat HSV-1 immunocutaneous infections in patients with depressed immune systems (e.g. who have undergone kidney, heart and bone marrow transplants), primary genital herpes and acute forms of herpes zoster [2].

In the topical treatment of herpetic keratitis, acyclovir must be applied as a 3% eye ointment. In the systemic treatment of HVS and VZV infections, it must be administered intravenously as a bolus infusion of 5 mg/kg every 8 h [2].

Because of its limited solubility in water (about 0.2% at 25 °C) [3,4], acyclovir cannot be administered as eye drops or intramuscolar injections (which are undoubtedly more practical in therapeutic use). For this reason, a number of acyclovir esters have been prepared in attempt to increase its solubility in water [5]. Studies on the rate of acyclovir release from these prodrugs have revealed that acyclovir is rapidly released at pH 7.4, whether esterase-type enzymes are present or not. As a result, it is important to use alternative approaches to increase the bioavailability of the drug.

We prepared two esters of acyclovir using PEG. Due to the two-phase behavior of PEG, the compounds we synthesized showed increased solubility in water as well as marked lipophylia, which resulted in an increased affinity for biological membranes.

In this paper we report:

1. The derivatization of PEG to obtain the chloro-PEG, an intermediate derivate for the synthesis of carboxyl-PEG;
2. The synthesis of the drug-polymer conjugates by linking acyclovir to carboxyl–PEG, using an ester bond, or by linking valacyclovir to chloro-PEG using a covalent C–N bond;
3. The results of in vitro studies on the release of the drug from the macromolecular prodrug in various media.

2. Experimental

2.1. Materials

Bromotripyrrolidinophosphoniumhexafluorophosphate (PyBrop®), triethylamine (TEA), 4-dimethylaminopyridine (DMAP), thionyl chloride, methanol HPLC grade, β-alanine, poly(ethyleneglycol) with two hydroxylic end groups of MW = 1500 were purchased from Fluka.

Human plasma, α-chymotrypsine and acyclovir {9-[(2-hydroxyethoxy)methyl]guanine} were obtained from Sigma Chemicals Co. (St. Louis, MO, USA).

Valacyclovir (Talavir®) is a product of Sigma Tau Industrie Farmaceutiche (Pomezia, Italia).

All the other chemicals and solvents used in this work were of the highest quality commercially available. Organic solvents were dried over molecular sieves (3 Å).

2.2. Analytical methods

Infrared spectra were recorded using a Perkin–Elmer 1720 IR Fourier Transform Spectrophotometer.

HPLC analyses were carried out with a Perkin–Elmer Series 4-high-performance liquid chromatography, equipped with a Rheodyne Model 7125 injector with a 20 μl loop and connected to a Perkin–Elmer Model LC 75 variable-wavelength UV detector. A LiChrosorb RP18 (Perkin–Elmer) 250 × 4.6 mm ID column packed with 10 μm particle size was used.

Potentiometric titrations were carried out using a TTT80 pH stat equipped with an ABU80 autoburetta, a titrigraph module REA 160 and a REA 070 pH stat unit, Radiometer.

Elemental analyses were carried out on a Carlo Erba model 1016 analyzer.

2.3. Preparation of PEG–Cl_2

10 mmol of PEG (20 mmol OH groups) were dissolved in toluene and dried by azeotropic distillation. Pyridine (20 mmol) was added, and thionyl chloride (60 mmol) freshly distilled from quinoline was added drop-by-drop for 30 min under reflux. The mixture was heated for 4 h, cooled to room temperature, filtered from pyridine hydrochloride, and toluene was evaporated in vacuo. The residue was dissolved in methylene chloride, dried over anhydrous K_2CO_3 and filtered. The filtrate was treated with alumina (50 g, activated by heating at 120 °C for 2 h), precipitated by cold diethyl ether and crystallized from ethanol (yield 94%).

Anal. Calc. for $C_{68}H_{135}Cl_2$: C, 52.62; H, 8.77; Cl, 4.62. Found: C, 52.59; H, 8.61; Cl, 4.97%.

IR (nujol) spectrum showed bands at 1100 cm^{-1} (stretching of $–CH_2–O–CH_2–$), 663 cm^{-1} (stretching of –C–Cl), no absorption for –OH at 3300–3500 cm^{-1}.

2.4. Covalent binding of β-alanine to PEG–Cl_2

To a solution of PEG–Cl_2 (6 mmol Cl groups) and pyridine (6 mmol) in DMF (20 ml), β-alanine (30 mmol) was added in small portions for 3 h under stirring, and the mixture was refluxed for 6 h. The solvent was then removed under reduced pressure, and the residue was taken up in methylene chloride, cooled,

filtered and precipitated using diethyl ether. The PEG-(β-ala-COOH)$_2$ was recrystallized from ethanol (yield 98%).

Purified PEG-(β-ala-COOH)$_2$ yielded 100% of free carboxyl groups, considering a polymer of MW 1500D.

Anal. Calc. for $C_{74}H_{147}N_2$: C, 53.57; H, 8.93; N, 1.70. Found: C, 53.08; H, 8.56; N, 1.58%.

IR (nujol) spectrum showed bands at 3290 cm^{-1} (–NH stretching of –NH_2), 3080 cm^{-1} (–OH stretching of –COOH), 1665 cm^{-1} (–C=O stretching of COOH), 1100 cm^{-1} (–CH_2–O–CH_2– stretching).

2.5. Synthesis of PEG–acyclovir$_2$

A solution of PyBroP® (15 mmol), DMAP (6 mmol) and TEA (6 mmol) in 10 ml of DMF was added drop-by-drop at 0 °C under a nitrogen atmosphere to a solution of PEG-(β-ala-COOH)$_2$ (6 mmol COOH groups) dissolved in DMF (20 ml).

Acyclovir (15 mmol) was added in small portions to this mixture, which was maintained at 0 °C for 10 min. The coupling reaction was carried out for 3 days at room temperature. The solvent was then removed under reduced pressure, and the residue was dissolved in methylene chloride and washed with brine. The organic layer was separated, dried over anhydrous K_2CO_3, concentrated and precipitated by the addition of diethyl ether.

The product was recrystallized twice from CH_2Cl_2/diethyl ether.

A 98% yield of the pure polymeric conjugate was obtained. The absence of the free drug and the content of acyclovir in the adduct (after hydrolysis in alkaline media) were evaluated by HPLC analysis.

Anal. Calc. for $C_{88}H_{161}N_{12}$: C, 51.01; H, 7.82; N, 8.17. Found: C, 50.85; H, 7.86; N, 8.03%.

IR (nujol) spectrum showed bands at 3290 cm^{-1} (–NH stretching), 1774 cm^{-1} (–C=O stretching of ester group), 1100 cm^{-1} (–CH_2–O–CH_2– stretching).

^{1}H NMR (DMSO): $\delta = 2.41$ ppm (br s; 4H, CH_2COO), 2.65 (br s; 8H, CH_2NH), 3.22 (s; CH_2O), 3.60 (m; O(CH_2CH_2O)$_n$], 4.18 (t; 4H, CH_2OCO), 5.25 (s; 4H, N–CH_2–O), 6.20 (br s; 4H, NH_2), 7.80 (s; 2H, H-8).

^{13}C NMR (DMSO): $\delta = 36.0$ ppm (*C*H_2COO), 44.1 (*C*H_2NH), 60.6 (N–*C*H_2–O), 61.5 (*C*H_2OCO), 71.0 (*C*H_2O), 72.1 [O(*C*H_2*C*H_2O)$_n$], 138.6 (C-5), 152.1 (C-8), 154.4 (C-4), 157.6 (C-2), 179.6 (C-6).

2.6. Synthesis of PEG–valacyclovir$_2$

Valacyclovir (15 mmol) was added in small portions to a solution of PEG–Cl_2 (6 mmol Cl groups) and pyridine (6 mmol) in DMF (20 ml) for 3 h, and the mixture was stirred for 5 h on an oil bath under reflux. The mixture was cooled and filtered, and the filtrate was evaporated to dryness. The residue was taken up in CH_2Cl_2, and the polymeric conjugate was precipitated using diethyl ether. The product was recrystallized twice from CH_2Cl_2/diethyl ether. A 98% yield of the pure polymeric conjugate was obtained. The absence of the free drug was evaluated by HPLC analysis. The content of acyclovir in the adduct was calculated by HPLC analysis, on the basis of the released acyclovir in alkaline media, after 30 min at 60 °C.

Anal. Calc. for $C_{92}H_{169}N_{12}$: C, 50.45; H, 7.76; N, 7.72. Found: C, 50.00; H, 7.36; N, 7.49%.

IR (nujol) spectrum showed bands at 3290 cm^{-1} (–NH stretching), 1774 cm^{-1} (–C=O stretching of ester), 1100 cm^{-1} (–CH_2–O–CH_2– stretching).

^{1}H NMR ($CDCl_3$): $\delta = 0.95$ ppm [d; 12H, (CH_3)$_2$CH], 1.45 [m; 2H, (CH$_3$)$_2$C*H*], 2.64 (t; 4H, CH_2NH), 3.17 (m; 4H, CH_2O), 3.60 [m; O(CH_2CH_2O)$_n$CH_2], 3.90 (m; 2H, C*H*COO), 4.20 (t; 4H, CH_2–OCO), 5.25 (s; 4H, N–CH_2–O), 6.10 (br s; 4H, NH_2), 8.24 (s; 2H, H-8).

^{13}C NMR ($CDCl_3$): $\delta = 24.7$ ppm [(*C*H$_3$)$_2$CH], 25.6 [(CH$_3$)$_2$*C*H], 44.1 (*C*H$_2$NH), 60.6 (N–*C*H$_2$–O), 61.7 (*C*HCOO), 68.1 (*C*H$_2$OCO), 71.0 (*C*H$_2$O), 72.3 [O(*C*H$_2$*C*H$_2$O)$_n$], 138.5 (C-5), 152.1 (C-8), 154.4 (C-4), 157.5 (C-2), 179.5 (C-6).

2.7. HPLC analysis on acyclovir

A single chromatographic method was used to detect acyclovir released from different PEG-adducts during hydrolysis studies in aqueous buffer solutions at different pH values, both in the presence of enzyme and in human plasma.

The released acyclovir was determined as described [4], with modification. The pH was adjusted to 6.15 to increase the affinity of the acyclovir for the mobile phase, thus enhancing resolution.

Benzoic acid was used as internal standard.

20 μl of each sample prepared as described above were injected into a reverse phase HPLC C18 column. A mixture of CH_3COONa 0.4 M and methanol (92:8 v/v) pH 6.15 was employed as the mobile phase, and the detection wavelength was 255 nm. The analyses of acyclovir in buffer and plasma were validated. The limit of quantification was approximately 3 μg of acyclovir per ml of buffer or plasma. The coefficient of variation calculated for the six samples analyzed did not exceed 5%. The precision of the assay method was calculated by determining the relative standard deviations of peak height ratios obtained from six replicate assays within a concentration interval of 5–100 μg/ml. The relative standard deviation ranged from 1 to 3.5% for intraday analysis and from 2 to 4% for interday analysis. The absolute recoveries of acyclovir and the internal standard in buffer and plasma were determined by comparing the slopes of the standard curves of the processed

buffer and plasma to those of the standard curves prepared in methanol. The recoveries of acyclovir and the internal standard in buffer was 91 ± 5 and 92 ± 6%, respectively, whereas in plasma the recoveries were slightly lower (82 ± 4 and 84 ± 6%, respectively).

2.8. Hydrolysis studies

The hydrolysis of PEG-adducts was studied in pH 1.2, 0.2 M HCl/NaCl/glycine, in pH 5.5, 0.2 M phosphate/citrate, and pH 7.4, 0.1 M phosphate buffer.

Equal known aliquots of adducts (5 mg/ml = 1 mg of acyclovir), each containing an appropriate quantity of benzoic acid as the internal standard, were dissolved in equal volumes of preheated buffer solutions at diverse pH values, maintained at 37 ± 0.1 °C and sampled at suitable intervals.

Each sample was directly analyzed by HPLC, and the amounts of acyclovir released were monitored. Each experiment was repeated three times.

2.9. Enzymatic hydrolysis studies

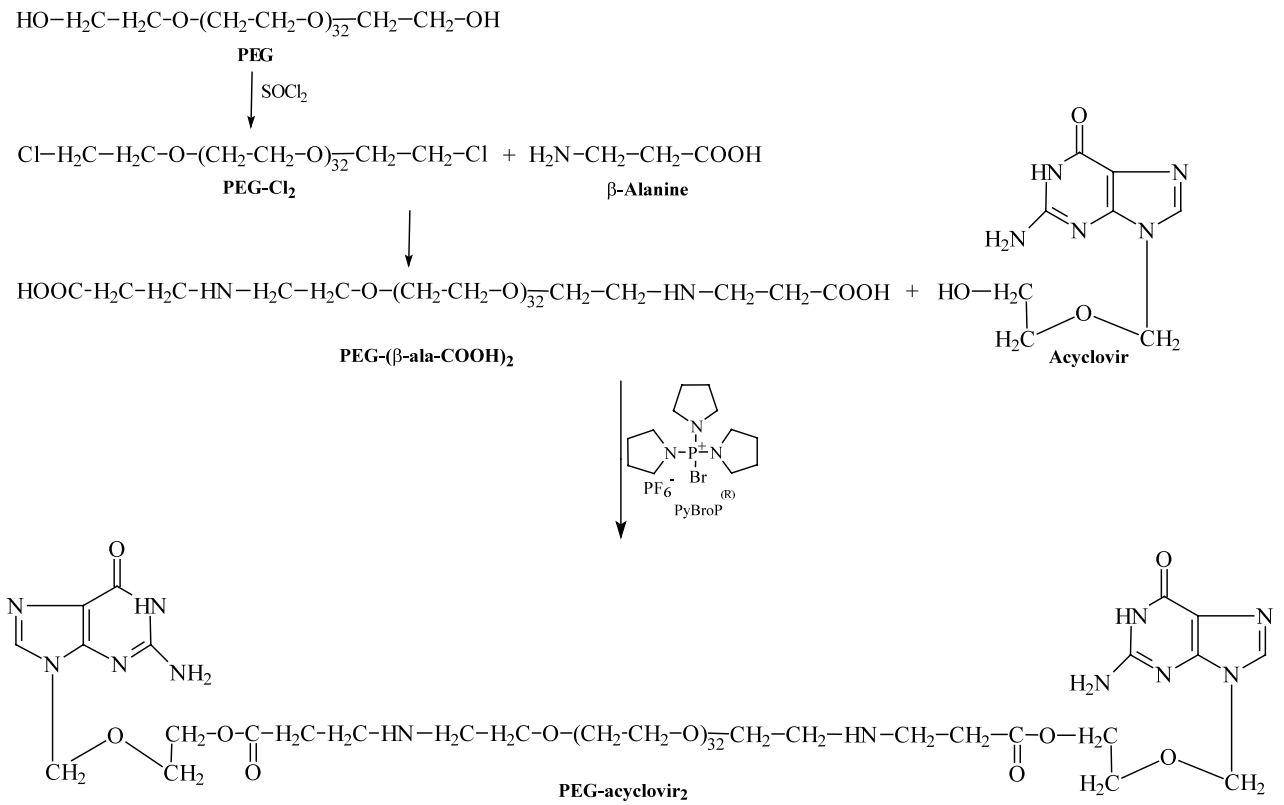
The hydrolytic stability to α-chymotrypsin of drug-PEG linkage of the polymeric conjugate was assessed in 0.08 M Tris buffer, 0.1 M $CaCl_2$ at pH 8 with 5 mg/ml of PEG-adduct, which contained an appropriate quantity of benzoic acid as internal standard.

200 μl of a α-chymotrypsin 10^{-5} M solution in 0.001 M HCl were added to 2 ml samples of PEG-adduct solution.

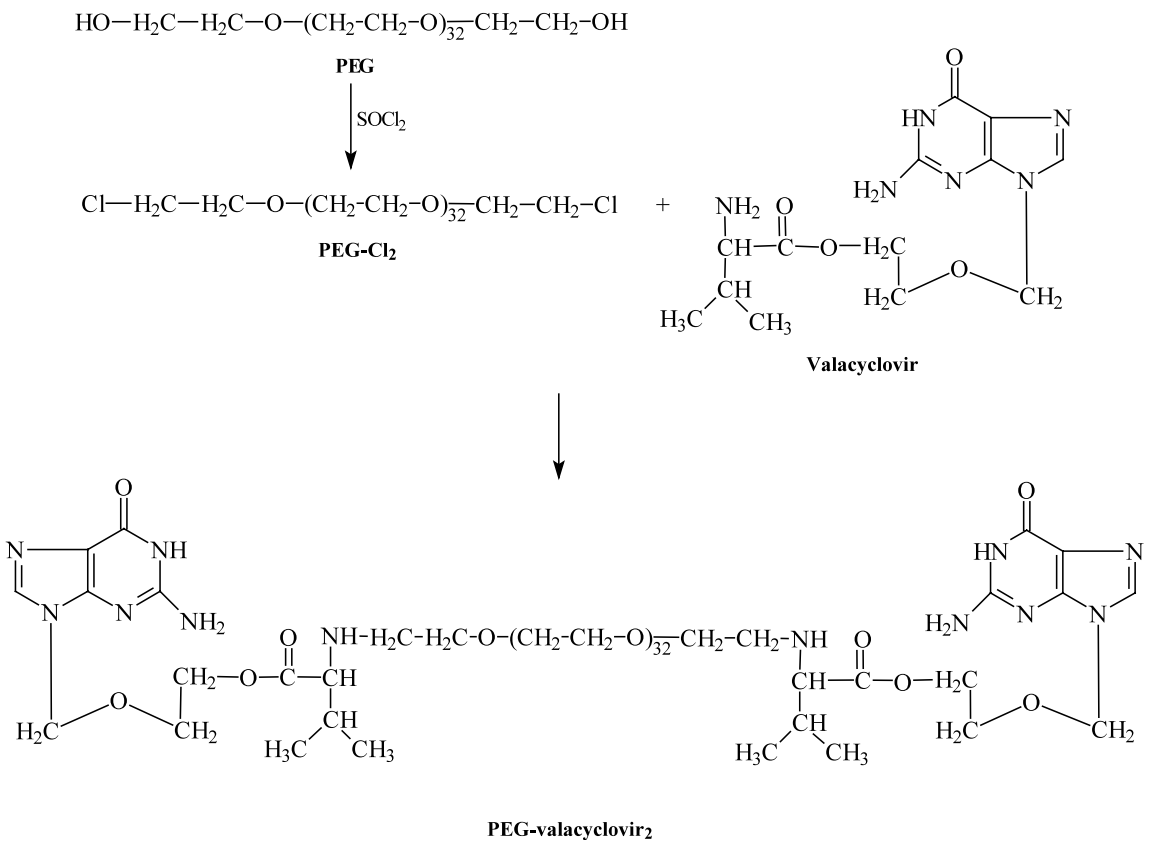
The solutions were incubated at 37 ± 0.1 °C and sampled by HPLC analysis at suitable intervals. To obtain control analogues, experiments were performed by adding the acidic solutions without enzyme to the conjugate solutions.

2.10. Plasmatic hydrolysis studies

Hydrolysis reactions of the polymeric prodrugs were studied in human plasma at 37 ± 0.1 °C. The reactions were started by adding 0.5 ml of aqueous solution of PEG-adducts (at the concentration of 10 mg/ml) to the (1 ml final) sample of plasma, which contained an appropriate quantity of benzoic acid as internal standard. The samples were held in a water bath at 37 ± 0.1 °C under continuous stirring. At suitable intervals, 0.1 ml of a 4% $ZnSO_4 \cdot 7H_2O$ solution in water was added to the (0.1 ml) sample followed by vortexing for 1 min to deproteinize the plasma. After centrifuging at 12 000 rpm for 4 min, the supernatant was filtered on a 0.45 μ CA membrane and analyzed by HPLC.



Scheme 1. Schematic synthetic procedure of PEG–acyclovir$_2$ conjugation.



Scheme 2. Schematic synthetic procedure of PEG–valacyclovir$_2$ conjugation.

3. Results

3.1. Synthesis of PEG-adducts

Chloro-PEG is typically used as an intermediate derivate for further transformations of functional groups.

Terminal hydroxyl groups are readily converted into chloride derivatives by treatment with thyonil chloride in toluene in presence of pyridine [6]. The product upon recrystallization is a white solid.

The carboxylic derivate of PEG was prepared by a covalent –C–N– bond between the chlorinate polymer and β-alanine. In both cases, purified PEG–Cl_2 and PEG–(β-ala-COOH)$_2$ yielded 100% of free chloride and carboxylic groups.

To introduce a cleavable bond at the conjugation site, an ester linkage between the hydroxyl group of acyclovir and the carboxylic group present in PEG–(β-ala-COOH)$_2$ was prepared (Scheme 1). In the activation of the carboxylic acid groups present in the PEG–(β-ala-COOH)$_2$, the use of the PyBrop® as coupling agent in presence of TEA, maximizes yield (98%) [7].

The coupling reaction was followed by a C_{18} reversed-phase column HPLC with UV detector ($\lambda = 255$ nm) and MeOH/H_2O [75:25] as the mobile phase with a flow rate of 1 ml/min (retention time PEG–adduct = 4.7 min).

Valaciclovir, Talavir® Sigma Tau Industrie Farmaceutiche, is a derivate of acyclovir. Because it is much more water soluble and much more bioavailable than the parent drug, it qualifies for oral administration in suitable quantities.

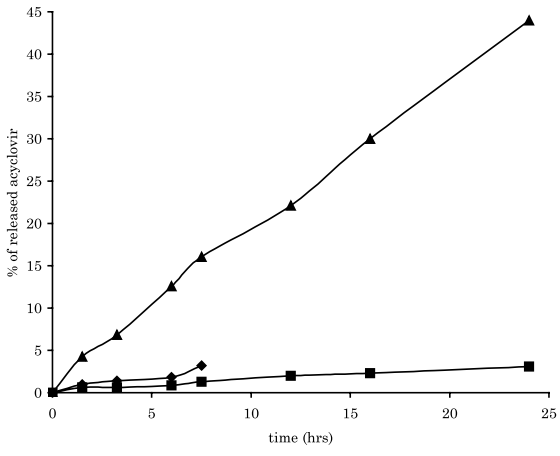


Fig. 1. Release of acyclovir in buffer solutions. ◆, pH 1.2, 0.2 M (HCl, NaCl, and glycine); ■, pH 5.5, 0.2 M phosphate/citrate, and ▲, pH 7.4, 0.1 M phosphate, at 37 °C from PEG–valacyclovir$_2$.

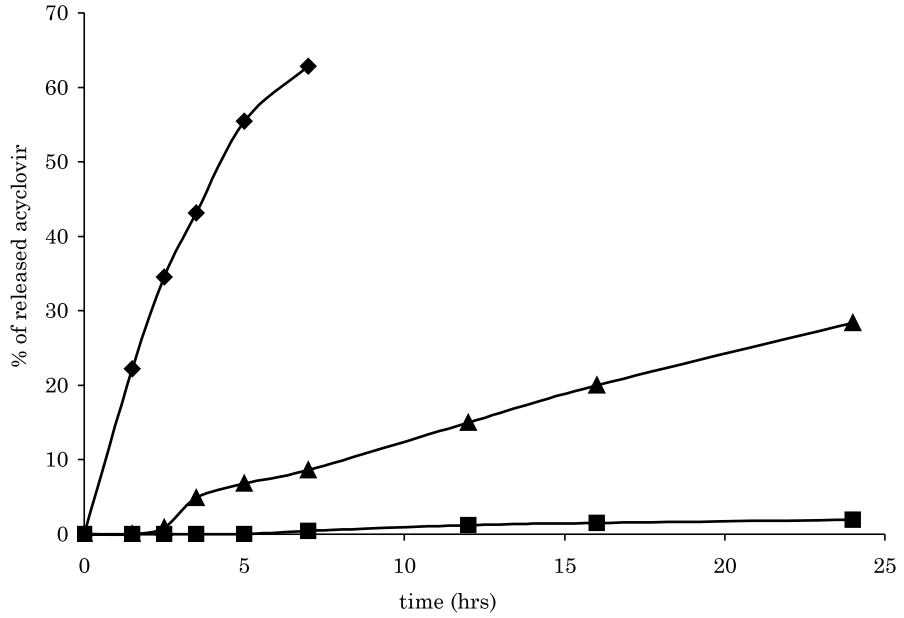


Fig. 2. Release of acyclovir in buffer solutions. ◆, pH 1.2, 0.2 M (HCl, NaCl, and glycine); ■, pH 5.5, 0.2 M phosphate/citrate; and ▲, pH 7.4, 0.1 M phosphate, at 37 °C from PEG–acyclovir$_2$.

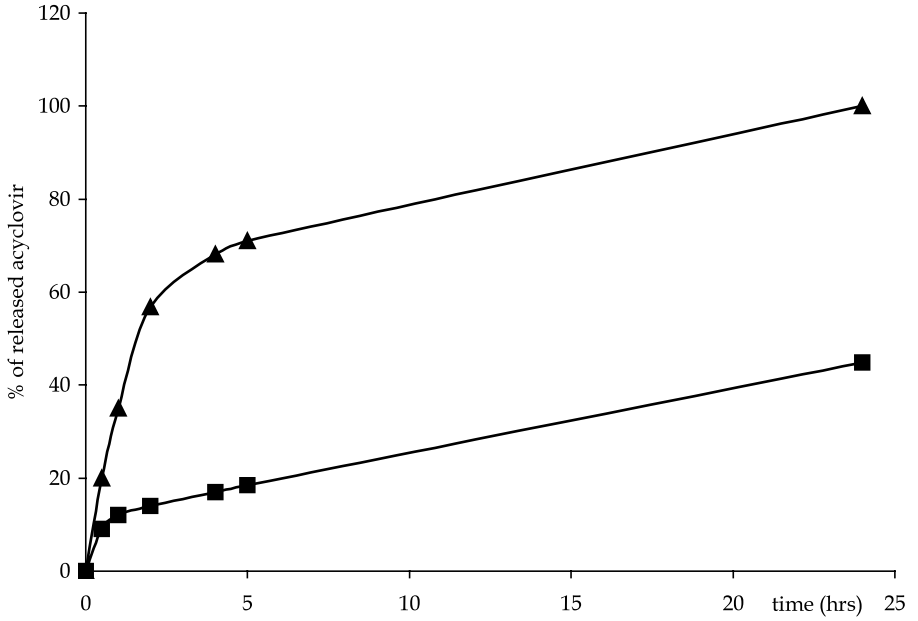


Fig. 3. Release of acyclovir in human plasma at 37 °C from PEG–acyclovir$_2$ (▲) and PEG–valacyclovir$_2$ (■).

The attachment of valacyclovir (valinic ester of acyclovir) to the PEG–Cl_2 was performed directly by the free aminic group of the valinic residue (Scheme 2).

The conjugation reaction was performed in DMF in presence of pyridine, with a yield of 88%.

The coupling reaction was followed by a C_{18} reversed-phase column HPLC with UV detector ($\lambda = 255$ nm) and MeOH/H_2O [75:25] as the mobile phase with a flow rate of 1 ml/min (retention time PEG–adduct = 4.7 min).

3.2. In vitro release studies

The hydrolysis rates of the two conjugates were studied in vitro in buffer solutions at pH 1.2 (simulated gastric juice), at pH 5.5 (endosomial compartments), at pH 7.4 (extracellular fluids), and at pH 8 in the presence and in the absence of α-chymotrypsin.

The release rates of acyclovir from the two adducts were also studied in plasma.

Fig. 1 depicts the hydrolysis rates of PEG–valacyclovir$_2$ at the three pH levels under consideration, while Fig. 2 depicts the same hydrolytic behavior for PEG–acyclovir$_2$. As can be seen, all products are stable at all pH levels except for PEG–acyclovir$_2$ at pH 1.2, which is 60% hydrolyzed after 6 h, while PEG–valacyclovir$_2$ in the same time period is only 2% hydrolyzed. The same diagrams show that 44% of acyclovir from PEG–valacyclovir$_2$ and 28% of acyclovir from PEG–acyclovir$_2$ are released after 24 h at pH 7.4.

The behavior of release rate (i.e. the fact that both the adducts are stable) at pH 5.5 is shown in Figs. 1 and 2.

The ability of macromolecular prodrug to release the free drug was also evaluated in plasma. As can be seen in Fig. 3, approximately 40% of PEG–valacyclovir$_2$ is hydrolyzed within 24 h, while around 100% of PEG–acyclovir$_2$ is hydrolyzed in the same period of time.

α-Chymotrypsin, a proteolytic pancreatic enzyme, is widely known to catalyze the hydrolysis of ester bonds [8]. Thus, the release of acyclovir in the presence of α-chymotrypsin at pH 8 was studied to determine the release rate and thus to evaluate the possible oral use of our products. The hydrolysis rates of PEG–valacyclovir$_2$ with or without α-chymotrypsin are shown on Fig. 4, while such rates for PEG–acyclovir$_2$ are shown on Fig. 5.

As can be seen in both cases, more of the product is released in the presence of α-chymotrypsin than in its absence.

The potential use of PEG–valacyclovir$_2$ in oral therapy is possible, while the use of PEG–acyclovir$_2$ is not

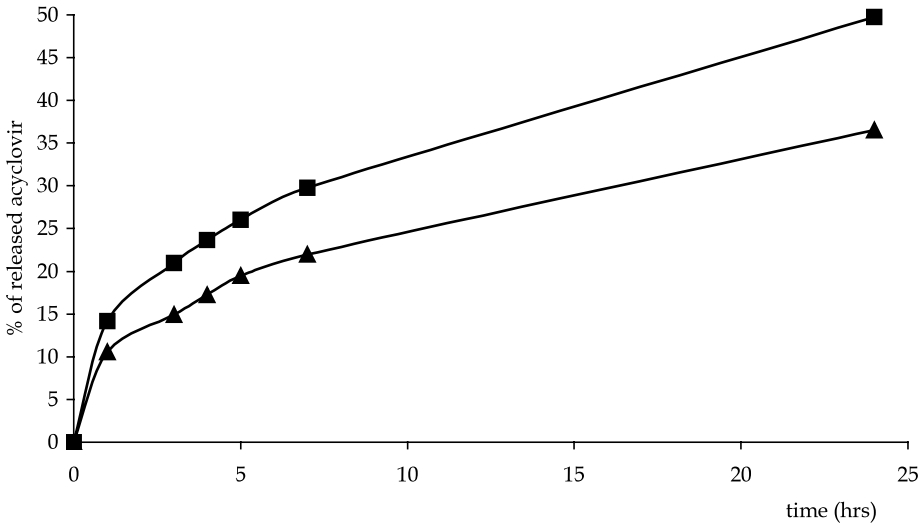


Fig. 4. Release of acyclovir from PEG–valacyclovir$_2$ in 0.08 M Tris buffer, 0.1 M $CaCl_2$ at pH 8 in absence (▲) or in presence (■) of α-chymotrypsin.

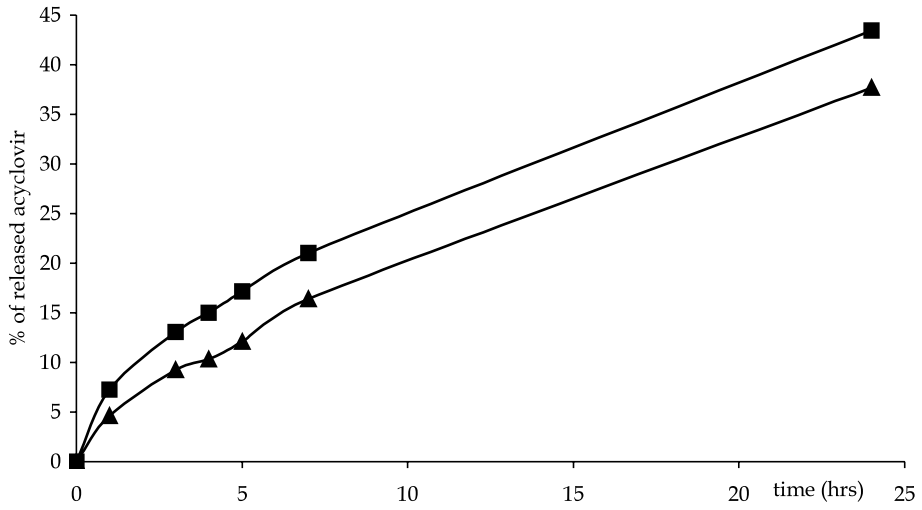


Fig. 5. Release of acyclovir from PEG–acyclovir$_2$ in 0.08 M Tris buffer, 0.1 M $CaCl_2$ at pH 8 in absence (▲) or in presence (■) of α-chymotrypsin.

possible because it is too unstable at pH 1.2, as was previously noted.

4. Conclusion

Of the two products we synthesized, PEG–valacyclovir$_2$ is more suitable for therapeutic use since it is more stable in various buffer and releases more of the free, active drug over time (40% in 24 h). As a result, the possibility of properly retaining this adduct in the bloodstream could represent an interesting way to improve the pharmacokinetics of acyclovir, particularly by increasing its half-life.

Thus, PEG–valacyclovir$_2$ could be used orally, by intramuscolar injection, or topically. PEG–acyclovir$_2$, on the other hand, may be suitable for administration (in all ways except orally) in those cases in which a rapid therapeutic effect is desired.

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