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Synthesis, enzymatic stability and oral bioavailability of poly (Ethylene glycol) acyclovir prodrug

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Abstract
Acyclovir a drug for the treatment of vericella-zoster infections (Shingles) orally in immune competent patients, intravenous in immune compromised patients and coupled to PEG through succinic anhydride spacer. The average molecular weight used for PEG_{1500} through. The procedure of chemical modification for PEG was conducted by two steps protocol (I) preparation of PEG_{1500} diacid (II) preparation PEG_{1500} Acyclovir. The in vitro drug release studies were performed in buffer solution with P^H values equal to 1.2, 5.5, 6.8 and 7.4. Enzymatic stability of synthesis polymeric-prodrug was assessed in both pepsin and α-chymotrypsin. It was found that prodrug was fairly stable in stimulated gastric juice (in the presence of pepsin), whereas it is susceptible to hydrolytic action of α- chymotrypsin releasing free acyclovir. It was found that drug release from the prodrug was nearly sustained over a period of 12 hrs and bioavailability was increased. After plasma hydrolysis studies were carried out 91% of acyclovir was released from polymeric backbone. The synthesized polymeric prodrug had capacity to prolong the release of free and active drug acyclovir also retained active drug in blood system for a longer time, improved pharmacokinetics of acyclovir, short half life, solubility and oral bioavailability.

Keywords: Synthesis, enzymatic stability, oral bioavailability, acyclovir prodrug

Introduction
Acyclovir, (2-Amino-1, 9 dihydro-9-[(2 hydroxy ethoxy) methyl]-6H-purine-6-one), is a synthetic purine nucleoside analogue and a highly selective antiviral drug. Acyclovir has short half-life of 2 to 3 hours it has to be administered 4-5 times a day resulting in patient non-compliance and also fluction in the blood drug levels that produce unwanted side effects. The drug is absorbed slowly and completely from the gastrointestinal tract with an oral bioavailability 15-30%. 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, on-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. PEG has the lowest level of protein or cellular absorption. These properties have been exploited in numerous ways include grafting PEG surfaces to prevent deposition of proteinaceous material. This can be further extrapolated to prevention of bacterial surface growth as well as conjugation to protein which prevents recognition by the immune system. A host of other applications of PEG have been reported [2] making PEG and its derivatives one of the most utilized polymers in the vast array of macromolecular organic compounds available It was proposed to couple acyclovir to PEG through a succinic anhydride spacer. The succinic spacer facilitates the attachment of the acyclovir to PEG and also will separate the drug from the polymeric back bone facilitating its release from the conjugate. 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. 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 intramuscular 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]. Its low oral and intravenous bioavailabilities [6]. This led to the development of ACV analogues [7, 8] as well as to a number of potential ACV prodrugs, most of them based on amino acid and dipeptide esters of ACV [5, 6, 9-12].

Materials and Methods

Materials

Acyclovir (9-[(2-hydroxyethoxy) methyl] guanine) was obtained from Matirx Laboratories limited, secundrabad as a gift sample, Dicyclohexyl cabodiimide (DCC) were obtained from Fluka. 4-Dimethyl aminopyridine, triethylamine (TEA) and poly (ethylene glycol) of MW=1500 were purchased from SD Fine Chemicals and used as such. Solvents like Dichloromethane, Diethylether, Acetonitrile, 1, 4 Dioxane were obtained from Ranbaxy fine chemicals.

Methods

Analytical methods

Infrared spectra were obtained from shimadzu IR fourier transform spectrophotometer. High-performance liquid chromatographic (HPLC) analyses were carried with Agilant series 1100, equipped with a Rheodyne Model injector and connected to a variable detector. The C_{18} Phenomenex (250×4.6 mm) column packed with 5µm particle size was used.

Preparation of PEG-diacid

PEG_{1500} (6g, 8mmol of OH groups), succinic anhydride (10mmol), DMAP (8mmol) and TEA (4mmol) were dissolved in dioxane (30ml) and left overnight at room temperature. The dioxane was evaporated in vacuo, the residue taken up in CCl_4, filtered and precipitated by ether. The mixture was dissolved in 50ml of cold dichloromethane, the unreacted succinic anhydride is insoluble in cold methylene chloride, it is removed by filteration. The methylene chloride in the mixture was removed using rotator evaporator. The residue was dried at 60ºC under reduced pressure. Yield 90%
TLC: $MeOH/CH_2Cl_2$, 1:5 v/v.

IR (KBr) cm^{-1}: 1732 (C=O, ester), 1647 (C=N), 1560 (COO), 1107 (CH_2-O-CH_2).

^{1}H NMR (DMSO) δ ppm: 3.48 – 3.66 (O-CH_2-CH_2)$_n$, 2.98 [s, 6H, (N-CH_3)$_2$], 2.51 (s, 8H, CH_2-COO-), 6.63 – 8.11 (m, 4H, pyridine H), 4.12 (t, 4H, CH_2-O-CO).

Preparation of PEG-acyclovir

PEG-diacid$_{1500}$ (4.46mmol, 7.6g) was dissolved in 200ml of anhydrous methylene chloride at room temperature. The solution was chilled to 0ºC DCC (7.6mmol, 3.66g), DMAP (1.7mmol, 0.79g) and acyclovir (1.77mmol, 4g) were added in that order and stirred for 2h at 0ºC. The reaction mixture was allowed to warm to room temperature and left for 16h. The solution was concentrated to about 100ml and filtered through celite. The filterate was washed with 0.1N Hydrochloric acid, dried over anhydrous magnesium sulphate and evaporated under reduced pressure to yield product as a white solid which was recrystallised from DMF/ether. The solid was filtered and washed with 2-propanol.

IR (KBr) cm^{-1}: 3367 (NH-stretching), 3404 & 3385 (NH_2), 2875 (CH stretching), 1734 (C=O, ester), 1645 (C=N), 1566 (-COO^-), 1112 (CH_2OCH_2)

^{1}H NMR (DMSO) δ ppm: 2.51 (s, 8H, CH_2-COO-), 3.37 (s, CH_2O), 3.51 [m, O (CH_2-CH_2-O)$_n$], 4.12 (t, 4H, CH_2-O-CO), 5.57 (s, 4H, N-CH_2-O), 5.58 (s, 4H, NH_2), 8.23 & 8.25 (s, 2H, H-8).

In vitro drug release studies

The drug release experiments via hydrolysis were carried out pH 1.2 (acidic buffer), pH 5.5 (phthalate buffer) and 7.4 (phosphate buffer). PEG-acy_2 was first put into dialtic bag and sealed, then was immersed into a buffer solution of 40ml. After a suitable interval time, 5.0ml of the solution released was withdrawn and another 5.0ml of fresh buffer solution was added for maintaining the system to a suitable volume. The amount of drug released was analysed by HPLC.

Enzymatic hydrolysis studies

The hydrolytic stability of PEG-acy to α-chymotrypsin was assessed in 0.08M Tris buffer, 0.1M $CaCl_2$ at pH 8.0 buffer solution. Two hundred microliters of α-chymotrypsin 10mg of acyclovir, and then was put into dialtic bag and sealed, incubated in a buffer solution of 40ml at 37±0.1ºC. After a suitable interval time 5.0ml of the solution released was withdrawn and 5.0ml of fresh buffer solution was added for maintaining the system with a stable volume. The amount of drug released was analysed by HPLC.

Plasma hydrolysis studies

The hydrolysis of the PEG-acy_2 polymeric pro-drug was studied in human plasma at 37±0.1ºC. The reactions were initiated by adding 100 µl of aqueous solution of PEG-acy_2 (at the concentration of 25mg/100ml) to the samples (1ml) of preheated plasma. The samples were kept in water bath at 37±0.1ºC, under continuous stirring. At suitable intervals, 4ml of acetonitrile was added in order to deproteinize the plasma. After immediate mixing and centrifugation FOR 10 min at 6000 rpm at 4ºC, 20 µl of the clear supernated liquid was analysed by HPLC to evaluate the amount of drug released. All the experiment was repeated in triplicate.

Oral bioavailability studies

Oral bioavailability of the synthesized polymeric drug conjugate was evaluated in two groups of three rabbits in a group. The animals were kept without food 24 h before the experiments. During the experiment, all rabbits were kept in restrain boxes in normal upright posture. PEG-acyclovir, equivalent to 10 mg/ml of acyclovir, was prepared by dissolving 250 mg in 25ml of polymeric prodrug conjugate in carboxmethyl cellulose suspension. An equivalent dose of Zovirax suspension was used as control. The blood samples were collected from the rabbits in the marginal ear vein at regular time interval.

A suspension of carboxymethyl cellulose (containing an amount of conjugate giving a dose of 25 mg/kg body weight of acyclovir) was administered orally to the first group of animals by gastric sonde. The second group of animal was treated in the same way with suitable volume of suspension of zovirax such as to have the same dose of drug, i.e., 25 mg/kg. At 0, 0.5, 1, 2, 4, 6, 8 and 12 h post-dosing, 3 ml blood samples were collected into tube containing a anticoagulant of 0.2 ml (3.8% w/v Trisodium citrate) and centrifuged at 6000 rpm for 10 min. The plasma was collected after centrifugation and the drug content after various time intervals was calculated using HPLC technique.

HPLC analysis

Reverse phase HPLC C18 column was used. A mixture of phosphate buffer pH 4.5 and acetonitrile (90:10 v/v %) was used as a mobile phase, and the detection wavelength was 254 nm. Paracetamol was used as internal standard, with a flow rate of 0.7ml/min. For analysing the plasma samples solid-phase extraction cartridges were used to avoid the interference.

Results and Discussion

Synthesis of PEG-diacid

PEG diacid was obtained in good yield (90%). The structure of PEG-diacid was confirmed by IR and ^{1}H NMR spectrum.

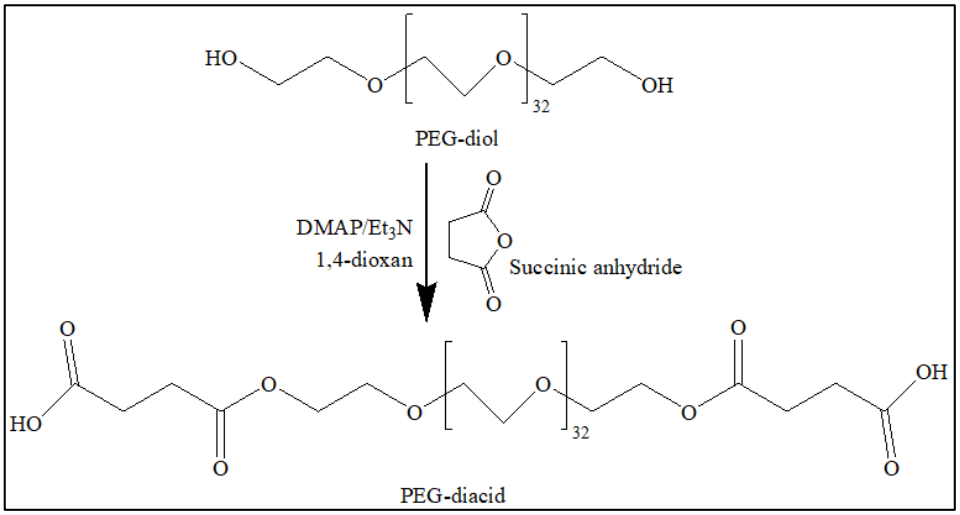

Fig 1: Schematic diagram for Synthesis of peg-diacid

The signal at 1732 cm^{-1} in the FT -IR spectrum corresponds to the carbonyl group of carboxylic acid, thus confirmed the formation of PEG diacid. It should be noted that the signals corresponding to the carbonyl group of succinic anhydride are approximately 1865 cm^{-1} due to an asymmetric C=O stretching mode and 1782 cm^{-1} due to a symmetric C=O stretching mode and both did not appear. The hydroxyl group in carboxylic acid terminals significantly has less intensity than that of PEG diol.

^{1}H NMR (DMSO) δ ppm: 3.48 – 3.66 (O-CH_2-CH_2) $_n$, 2.98 [s, 6H, (N-CH_3)$_2$], 2.51 (s, 8H, CH_2-COO-), 6.63 – 8.11 (m, 4H, pyridine H), 4.12 (t, 4H, CH_2-O-CO).

The singlet between 3.48- 3.66 ppm corresponds to the proton of (O-CH_2-CH_2)$_n$ of PEG-diacid. The singlet at 2.98 ppm corresponds to six protons of the (N-CH_3)$_2$ DMAP, which was used as a catalyst. The singlet at 2.51 ppm corresponds to eight protons of (CH_2-COO) $_4$. The multiplet between 6.63-8.11 ppm, corresponds to pyridine nucleus, present in DMAP. The triplet at 4.12 ppm, corresponds to four protons of (CH_2-O-CO) $_2$.

Synthesis of peg-acyclovir

To synthesis a peg-acyclovir conjugate peg_{1500} was used. Where succinic acid is used as spacer through an ester linkage between the PEG and acyclovir and separates the drug molecule from the polymeric backbone and facilitating the drug release. It was already proved that ester with PEG as an electron withdrawing substituent (alkoxy) in the α-position proved to be especially effective linking groups in the design of pro drugs which aids in the enzymatic hydrolysis of the ester carbonyl bond, so the release the parent drug in its active from without changing in the functional group.

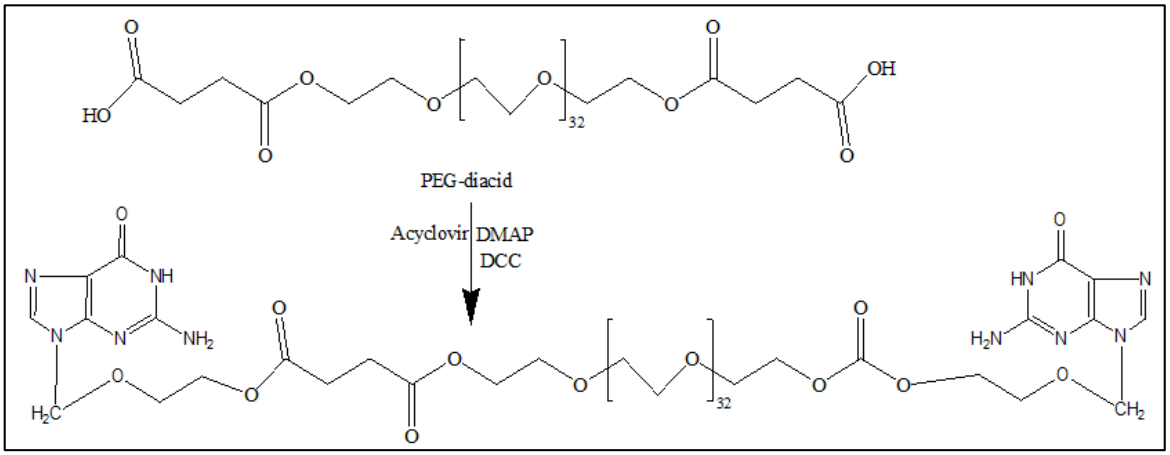

Fig 2: Schematic diagram for Synthesis of peg-acyclovir

The singlet at 2.51 ppm corresponds to eight protons of (CH2-COO)2. The singlet at 3.37 ppm corresponds to the proton of (CH2-O) of PEG-acyclovir. The multiplet at 3.51 ppm corresponds to (CH2-CH2-O)n, the triplet at 4.12 ppm corresponds to four protons of CH2-O-CO. The singlet at 5.57 ppm is due to presence of four protons of acyclovir (N-CH2-

O). The singlet at 5.58 ppm, assigned for four protons, due to the presence of –NH2 group in acyclovir. The peak present at 8.23 & 8.25 is due to the presence of –NH (H-8) in acyclovir.

In vitro drug release studies

The *In vitro* drug release studies were carried to obtain the potential use of PEG-acy as a drug delivery system for oral administration. *In vitro* hydrolysis studies were carried in the buffer solutions at physiological pH, stimulated gastric juice, pH 1.2, endosomal compartments, pH 5.5, and extracellular fluids, pH 7.4.

Samples which are withdrawn at regular intervals are analysed by HPLC to find the percentage of drug release from the polymeric drug conjugate.

Table 1: *In vitro* drug release studies in pH 1.2, pH 5.5and pH 7.4

Time in hours	pH 1.2	pH 5.5	pH 7.4
	% released	% released	%released
0		0	0
0.5	1.35	0.86	6.59
1	2.22	1.40	10.76
2	3.87	1.96	16.82
3	5.00	2.39	22.37
4	5.60	5.21	30.46
6	5.90	6.96	34.55
8	6.38	10.00	36.72
12	7.19	11.2	42.34

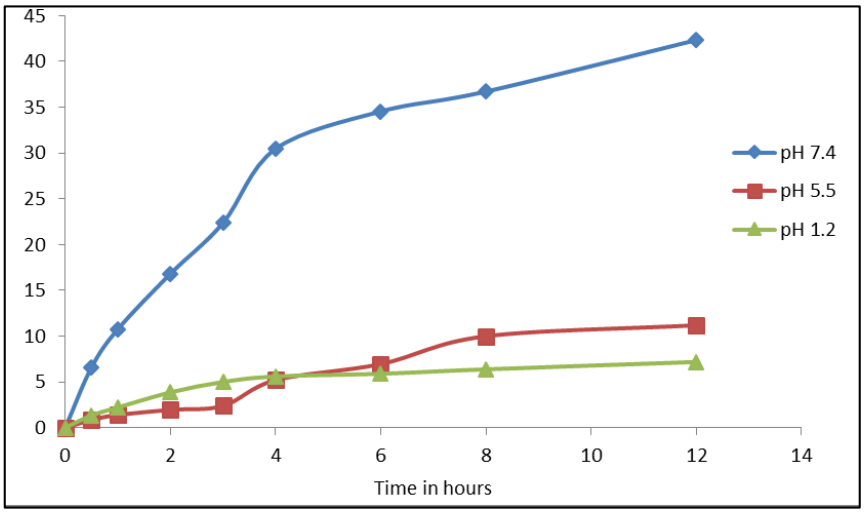

Fig 3: In vitro drug release studies in pH 1.2, pH 5.5 and pH 7.4

Table 2: In vitro drug release studies for Prodrug and marketed suspension

Time in hours	Prodrug Conc in µ gm/ml	Marketed suspension Conc in µ gm/ml
0	0	0
0.5	0.11	0.18
1	0.19	0.26
2	0.22	0.29
3	0.31	0.30
4	0.32	0.24
6	0.33	0.21
8	0.30	0.18
12	0.29	0.13

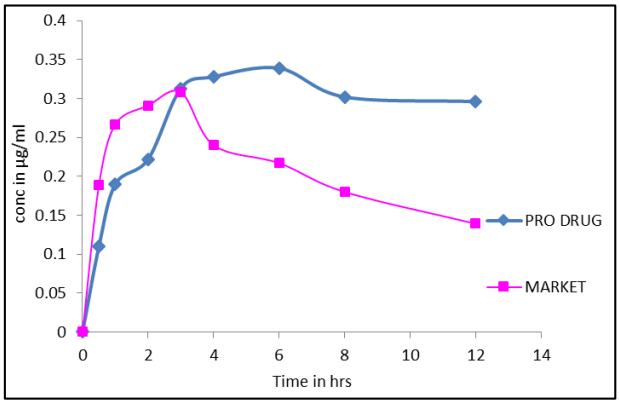

Table 3: In vitro drug release studies for Prodrug and marketed suspension pharmacokinetic data

Type of drug		$C_{max}{}^{c}$mgm/ml	$T_{max}{}^{c}$	$AUC_{0-t}{}^{c}$	$t_{1/2}{}^{c}$	Kel^{c}	$AUC_{0-\alpha}{}^{c}$
Acyclovir	Pro-drug	0.33 ± 0.12	6	3.40 ± 0.32	34.49 0.86	0.02±.014	20.27 ± 1.24
	Marketed drug	0.30 ± 0.15	3	2.50 ± 0.27	8.47 ± 0.72	1.76± 0.23	2.68 ± 0.87

The amount of drug released from the polymeric drug conjugate at pH 1.2 was only about 7.1% over 12h. In buffer 5.5 only about 11.2% of acyclovir is released. But at the pH of 7.4 about 42% of drug has been released over a period of 12h which shows that the acyclovir was released to a greater extent in the alkaline pH due to gelling effect of PEG with facilitating the release of acyclovir in a prolonged manner.

Oral bioavailability studies

The oral bioavailability of synthesized polymeric prodrug was tested in comparison with commercial suspension of drug (zovirax). The results of these experiments are reported in Fig As can be seen, whereas the suspension of free acyclovir gives a plasma concentration of drug that increases up to 3h and started to decrease considerably but for the synthesized polymeric prodrug the concentration of acyclovir increases gradually and reaches the maximum plasma concentration at 6h and which remains practically constant over a period of 12h. consequently the area under curve (AUC) value ($\mu g.ml^{-1}.\ h^{-1}$) calculated for the macromolecular prodrug is higher than that calculated for the suspension of drug with an increase of bioavailability.

Enzymatic hydrolysis studies

Enzymatic hydrolysis studies

The amount of drug released from the polymeric drug conjugate in stimulated gastric juice in the presence of pepsin was found to be 15% in 3h.

The α-chymotrypsin, a proteolytic pancreatic enzyme, to catalyze the hydrolysis of ester bond is well known and the possibility that PEG-acy can be good substrate for this enzyme (In which drug molecule are linked to polymeric backbone by ester linkages, via succinic spacer). It was found approximately 48% of the drug was released from the polymeric backbone. This clearly shows that the active drug released in a controlled manner from the polymeric backbone.

Time in hours	α-Chymotrypsin (%released)	Pepsin (%released)
0	0	0
0.5	8.06	6.13
1	12.37	9.82
2	18.53	11.19
3	29.34	14.38
4	36.91	-
6	42.33	-
8	46.66	-
12	48.31	-

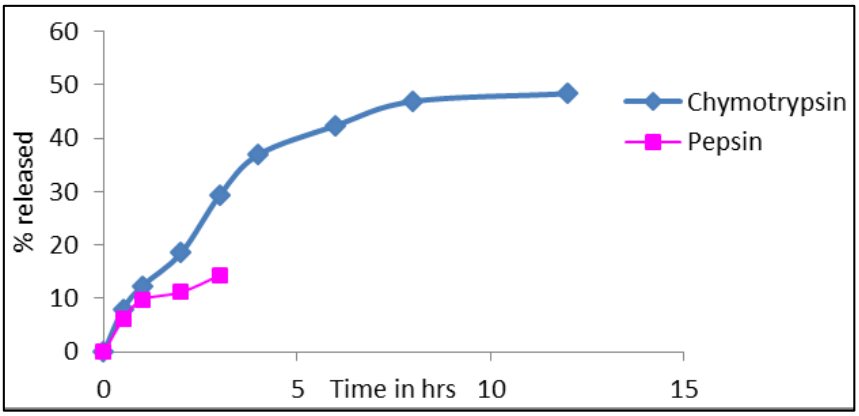

Fig 4: Enzymatic hydrolysis studies

Plasma hydrolysis studies

The macromolecular prodrug also has the ability to release the free drug was evaluated in human plasma which is shown in fig. Only about 12% of drug was released at the first hour, after 12h about 44% of drug has been released where at the end of 24h about 91% of drug has been released. This shown that polymeric prodrug can also be given iv route.

Table 4: Plasma hydrolysis studies

Time in Hours	Drug area	Internal standard area	Sample response factor	Standard response factor	% released
0	0	547.91	0	0.75	0
0.5	34.68	550.57	0.06	0.75	8.40
1	52.25	561.94	0.09	0.75	12.39
2	66.63	542.68	0.12	0.75	16.37
3	77.08	557.64	0.13	0.75	18.43
4	89.26	545.54	0.16	0.75	21.81
6	118.47	570.5	0.20	0.75	27.69
8	151.96	549.91	0.27	0.75	36.84
10	170.69	551.01	0.30	0.75	41.30
12	187.76	567.48	0.33	0.75	44.11
24	406.45	591.09	0.68	0.75	91.68

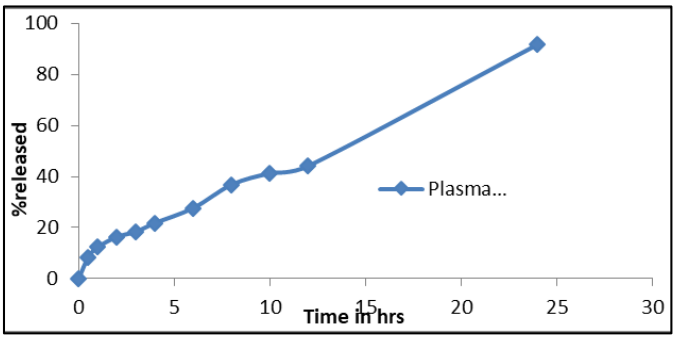

Fig 5: Plasma hydrolysis studies

Discussion

Polymeric drug delivery systems: in which a small molecular drug is covalently bounded to the back bone of polymers that is of hydrolysable in the body fluid, are currently recognized as an effective way to prolong the drugs pharmacological activity. The mechanism of this technology can make small molecular drugs gradually free from polymer matrixes if they are subjected to hydrolysis of body fluids.

Traditionally prodrugs are generally designed to be cleaved efficiently and rapidly ($t_{1/2}$<20min) by accelerated rate of conversition of the inert form to the biologically active parent moiety. Thus, the pharmacokinetic of the parent drug is only minimally affected by prodrug modification. In addition to this approach, an alternative solution to the problem of prodrug efficacy would be to extend the circulating life of the prodrug in plasma relative to its rate of hydrolysis, equivalent or greater potency should result with a gradual controlled release of the drug as long as therapeutic levels can be reached and maintained without causing toxicity. This method was well established for the delivery of anticancer drugs, showed significantly higher accumulation in tumors than within in normal tissue, irrespectively of tumor cells. The underlying mechanism appears to be combination of increased tumor vascular permeability and insufficient lymphatic drainage resulting in what is the termed “enhanced permeation and retention effect” (EPR). Acyclovir 9-[(2-hydroxyethoxy)methyl]guanine used in the treatment of varicella-zoster infections, herpes simplex infection and Prophylactic in patients who are to be treated with immunosuppressant drugs or radio theraphy and who are at risk of herpes virus infection owing to reactivation of latent virus.

Conclusion

An attempt was therefore proposed in the present investigation to improve the water solubility, chemical stability and the bioavailability of acyclovir by synthesizing a polymer pro-drug by linking the drug to a water soluble polymeric carrier namely poly (ethylene glycol) through succinic anhydride spacer. The synthesised polymeric-prodrug has the capacity to prolong the release of free and active drug acyclovir also has the possibility of retaining the active drug in the blood system for a longer time and improving the pharmacokinetics of acyclovir, like its short half-life, solubility and oral bioavailability.

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