Synthesis and evaluation of some polymeric drug delivery systems

Thesis submitted to
The Tamilnadu Dr. M.G.R. Medical University, Chennai,
for the award of the Degree of

Doctor of Philosophy

Submitted by

J.K. Ravichandran

July 2010

J. S. S. COLLEGE OF PHARMACY
Ootacamund–643 001

Dr. Shrishailappa Badami
Director
Sree Siddaganga College of Pharmacy
B.H Road, Tumkur -572102
Karnataka, India.

CERTIFICATE

This is to certify that the thesis entitled, " *Synthesis and evaluation of some polymeric drug delivery systems* " is a record of research work done by Mr. J.K.Ravichandran at J.S.S College of Pharmacy, Ootacamund, under my supervision during the years 2005-2010 and this thesis has not previously formed the basis for the award of any Degree, Diploma, Associateship, Fellowship or other similar title. I also certify that the thesis represents independent work done by the candidate and has not formed in part or fully the basis for the award of any other previous research degree.

Place: Ootacamund Supervisor

Date:

Prof. K. Elango, Ph. D.,
Principal
J.S.S. College of Pharmacy
Ootacamund-643001.

CERTIFICATE

This is to certify that this thesis entitled, " *Synthesis and evaluation of some polymeric drug delivery systems"* is a record of research work done by Mr. J.K.Ravichandran at J.S.S. College of Pharmacy, Ootacumund, under the supervision of Dr. Shrishailappa Badami, during the years 2005-2010.

Place: Ootacamund Principal

Date :

Prof. M.J. Nanjan, Ph. D.,
Director, Research and Postgraduate studies
J.S.S. College of Pharmacy
Ootacamund-643001.

CERTIFICATE

This is to certify that this thesis entitled, " *Synthesis and evaluation of some polymeric drug delivery systems"* is a record of research work done by Mr. J.K.Ravichandran at J.S.S. College of Pharmacy, Ootacumund, under the supervision of Dr. Shrishailappa Badami, during the years 2005-2010.

Place: Ootacamund M.J.Nanjan

Date :

DECLARATION

I hereby declare that the thesis entitled, " *Synthesis and evaluation of some polymeric drug delivery systems* " submitted by me for the degree of Doctor of Philosophy of The Tamilnadu Dr. M.G.R. Medical University, Chennai, is the result of my original and independent work done at J.S.S College of Pharmacy, Ootacamund, during the years 2005-2010 under the supervision of Dr. Shrishailappa Badami and has not formed the basis for the award of any Degree, Diploma, Associateship, Fellowship or other similar title previously.

Date: (J.K. Ravichandran)

ACKNOWLEDGEMENTS

*I express my deep sense of gratitude and respect to **Dr. Shrishailappa Badami,** Director. Sree Siddaganga College of Pharmacy, Tumkur, Karnataka, India for his valuable guidance, helpful suggestions, constant motivation and the moral support extended during the course of this study.*

*My sincere thanks are due to **Dr. K.Elango,** Principal, J.S.S. College of Pharmacy, for his support and the facilities extended to me to carry out this project.*

*I am deeply indebted to **Dr. M.J.Nanjan, Director,** Research and Post Graduate studies, J.S.S. College of Pharmacy, for his constructive suggestions, encouragement and moral support.*

*I am also thankful to **Dr.M.J.N. Chandrasekar**, Professor, Dept of Pharmaceutical Chemistry and **Mr. Puttarajappa S**, Administrative Officer and their team from J.S.S. College of Pharmacy for providing their moral support during the course of this work.*

It is my pleasure to thank my parents, wife Sumathi and my children Shyam and Gokul for their patient endurance during the course of this work.

*I am grateful to the management of M/s. **Hindustan Photo Films**, Ootacamund for their permission and support to carry out this work.*

*My sincere pranams and respectful regards to His Holiness **Jagadguru Sri Sri Shivarathri Deshikendra Mahaswamigalavaru** of Sri Suttur Mutt, with whose blessings, this work was completed.*

J.K.Ravichandran

Contents

1. Introduction

Improving the therapeutic index of drugs is a major impetus for innovation in many therapeutic areas such as cancer, inflammatory, and infective diseases. The search for new drug-delivery concepts and new modes of action are the major driving force in polymer therapeutics [1–3].

Today, the vast majority of clinically used drugs are low molecular weight compounds (typically under 500 $gmol^{-1}$) that exhibit a short half-life in the blood stream and a high overall clearance rate. These small-molecule drugs typically interact through a multiple but monovalent binding with a given receptor. Furthermore, they diffuse rapidly into healthy tissues and are distributed evenly within the body. As a consequence, relatively small amounts of the drug reach the target site, and therapy is associated with side effects. These disadvantages are especially pronounced with drugs that exhibit a narrow therapeutic index such as anticancer, antirheumatic, and immunosuppressive agents. Frequent sideeffects associated with these drugs are nephrotoxicity, bonemarrow toxicity, neurotoxicity, cardiotoxicity, mucositis, and gastrointestinal toxicity, which are dose-limiting and thus prevent effective treatment.

A number of macromolecular delivery systems are under investigation to circumvent these limitations and improve the potential of the respective drug. Generally, these can be classified as nanoparticulate drug-delivery systems or as drug–polymer conjugates. Particulate delivery systems in which the drugs are physically incorporated into nanoparticles include emulsions, liposomes, and noncovalent polymeric carrier systems. In drug–polymer conjugates, however, a drug is covalently linked to polymers such as proteins, polysaccharides, or synthetic polymers. The coupling of drugs to macromolecular carriers received an important impetus from 1975 onwards with the development of monoclonal antibodies by Milstein and Kohler [4] and from Ringsdorf's notion of a general drug-delivery system based on synthetic research work has focused on realizing drug conjugates with antibodies to selectively target cell-specific antigens or

receptors. This propagated the therapeutic concept of drug targeting that was founded on Paul Ehrlich's vision of "the magic bullet" which he proclaimed at the beginning of the last century. However, it took many years for the dawning era of "polymer therapeutics" to "kick-off".

In Ringsdorf's original model (Figure 1) [5] a number of drug molecules are bound to a macromolecule through a spacer molecule, which can incorporate a predetermined breaking point to ensure release of the drug at the site of interest. The polymer conjugate can additionally contain moieties, for example, antibodies or sugar moieties, which target disease related antigens or receptors. In addition, solubilizing groups can be attached to the polymer backbone to modify the bioavailability of the drug–polymer conjugate.

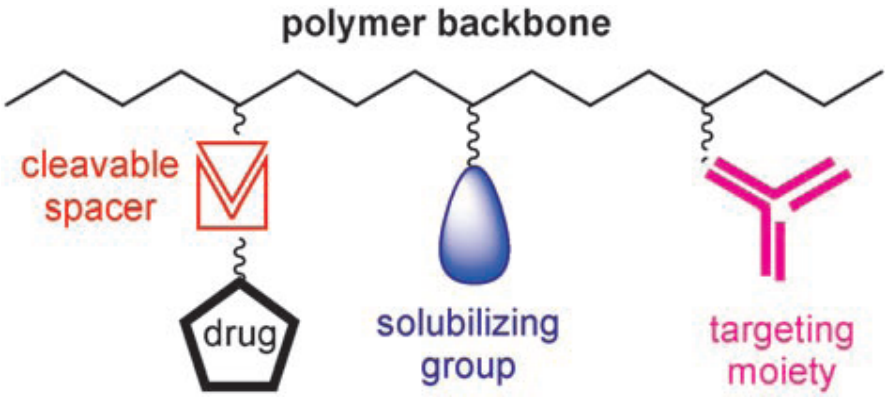


Figure 1. Ringsdorf's model for drug-delivery systems based on synthetic polymers.

Macromolecules chosen for the preparation of drug–polymer conjugates should ideally be water-soluble, nontoxic, and nonimmunogenic, as well as degraded and/or eliminated from the organism [6]. Finally, the macromolecular carrier should exhibit suitable functional groups for attaching the respective drug or spacer. Initially, N-(2-hydroxypropyl) methacrylamide (HPMA) copolymers were intensively studied as linear polymers for therapeutic applications according to the Ringsdorf model [7–9]. However, a spectrum of other synthetic polymers with

structural and architectural variations, including A) monofunctional linear, B) polyfunctional linear,C) starlike, and D) dendritic architectures are being investigated today (Figure 2).

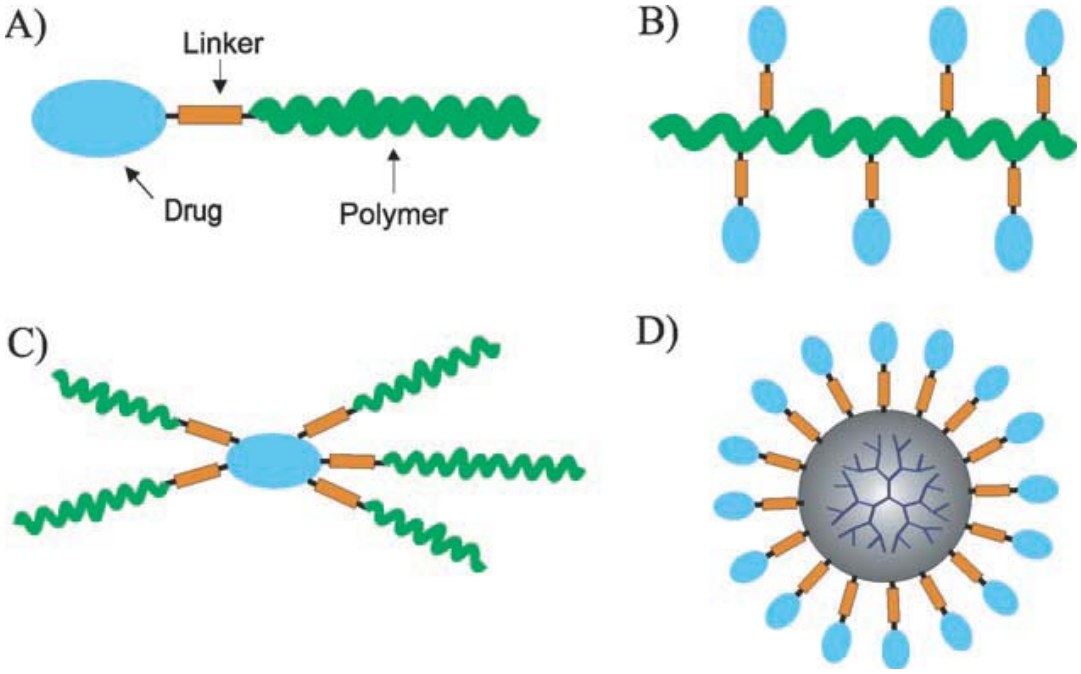


Figure 2. Selected structural and architectural types of drug–polymer conjugates

Conjugates of drugs and polymers as well as other polymeric carrier systems have collectively been termed polymer therapeutics, which primarily encompass polymer–protein conjugates, drug–polymer conjugates, and more recently supramolecular drug-delivery systems as well as other defined nanosized systems [10–12]. Anchoring of enzymes or biological response modifiers to polyethylene glycol components (PEGylation) has led to numerous polymer–protein conjugates with improved stability and pharmacokinetic properties. Several polymer–protein conjugates have received market approval (Table 1). The coupling of low molecular-weight anticancer drugs to polymers through a cleavable linker has been an effective method for improving the therapeutic index of clinically established agents, and the first candidates of anticancer drug–polymer conjugates are being evaluated in clinical trials.

Table 1: Polymer–protein conjugates with market approval

Trade name	Protein	Polymer	Indication	Marketed
adagen	adenosine deaminase	5 kDa PEG	severe combined immunodeficiency disease	Enzon
oncaspar	asparaginase	5 kDa PEG	acute lymphatic leukemia	Enzon
pegvisomant	GH antagonist	5 kDa PEG	excessive growth (acromegaly)	Pfizer
PEG-intron	interferon a2b	12 kDa PEG	hepatitis C	Schering-Plough
pegasys	interferon a2a	40 kDa PEG	hepatitis C	Roche
neulasta	granulocyte colony stimulating factor	20 kDa PEG	neutropenia	Amgen
SMANCS/ lipiodol	neocarzinostatin	copolymer of styrene maleic acid	hepatocellular cancer	Yamanouchi Pharmaceutical

The advance of well-defined polyvalent and dendritic polymers [13] has paved the way for designing tailor-made systems with self-assembling properties which are also classified as polymer therapeutics. These include: a) polyanionic polymers for the inhibition of virus attachment and as heparin analogues; b) polycationic complexes with DNA or RNA (polyplexes); and c) polymer micelles with covalently bound drugs as well as dendritic core–shell architectures for the encapsulation of drugs.

1.1 Macromolecules as Drug-Delivery Systems: Biological Rationale

Passive Drug and Specific Tissue Targeting: The EPR Effect

It has long been known that biopolymers play an essential role as free and membrane-bound “therapeutics”. Therefore, it is surprising that synthetic polymers were originally only discussed as plasma expanders, for example, pervirlon or poly (vinyl pyrrolidone) during the Second World War [14]. Passive accumulation of macromolecules and other nanoparticles in solid tumors is a phenomenon which was probably overlooked for several years as a potential biological target for tumor-selective drug delivery.

The rationale for using macromolecules as efficient carriers for the delivery of antitumor agents, even if they are not targeted towards an antigen or receptor on the surface of the tumor cell, is based on the pioneering work of Maeda and co-workers[15, 16] as well as Jain et al[17, 18]. The results of these studies gave detailed insight into the pathophysiology of tumor tissue that is characteristic of angiogenesis, hyper vasculature, a defective vascular architecture and an impaired lymphatic drainage.

Differences in the biochemical and physiological characteristics of healthy and malignant tissue are responsible for the passive accumulation of macromolecules in tumors. This feature has been termed “enhanced permeability and retention” (EPR effect) [19]. In general, low-molecular-weight compounds diffuse into normal and tumor tissue through the endothelia cell layer of blood capillaries. Macromolecules, however, cannot pass through the capillary walls of normal tissue. The entry of macromolecules into tumor tissue takes place in the capillaries where blood flow is diminished and nutrients transfer into the tissue. In contrast to the blood capillaries in most normal tissues, the endothelial layer of the capillaries in the tumor tissue is fenestrated and leaky so that macromolecules and other nanoparticles reach the malignant tissue. Tumor tissue generally has a defective lymphatic drainage system with the result that macromolecules are retained and can subsequently accumulate in solid tumors.

The size of the macromolecule is a crucial factor with respect to uptake by the tumor. The EPR effect is observed for macromolecules with molecular weights greater than 20 kDa. Therfore, there is a correlation between the half-life in plasma, the renal clearance, and the accumulation in the tumor of the respective macromolecule. In recent years, most of the research groups involved in the development of drug–polymer conjugates selected macromolecular carriers with molecular weights in the range of 20 to 200 kDa. It is generally assumed that in a healthy organism the renal threshold is in the range of 30–50 kDa to avoid leakage of body proteins into the bladder [20].

A number of preclinical studies have demonstrated that the physiochemical nature of the biopolymer or synthetic polymer has a strong influence on its pharmacokinetic profile and degree of accumulation in the tumor [21, 22]. The biodistribution and uptake by the tumor of the polymer in question is essentially dictated by its molecular weight, charge, conformation, hydrophobicity, and immunogenicity. Preclinical studies have shown that the size of the tumor influences the uptake rate of the polymer in solid tumors. Smaller tumor nodules accumulate larger amounts of the polymer than larger nodules [23]. This observation points to the possibility that polymeric imaging agents could help to detect small tumor nodules.

The influence of the different factors on the EPR mediated uptake of the polymer in solid tumors is not yet completely understood. As a general rule, a polymer with a molecular weight above the renal threshold (ca. 30 kDa) as well as a neutral charge ensures a long half-life in plasma. This prolonged plasma residence time is an important prerequisite for a significant accumulation of the circulating polymer in the tumor. A similar uptake mechanism is also apparent in other leaky tissues, such as inflamed or infected tissue, and can result in an enhanced uptake of macromolecules at the respective sites. In contrast to this simple passive targeting by size, cellspecific targeting using antibodies, oligosaccharides, and peptides has also been addressed by many research groups [24].

Cellular uptake of polymers, site-specific drug release, and implications for drug design

In general, macromolecules are taken up by the cell through receptor-mediated endocytosis, adsorptive endocytosis, or fluid-phase endocytosis (Figure3)[25]. During endocytosis a significant drop in the pH value takes place from the physiological value (7.2–7.4) in the extracellular space to pH 6.5–5.0 in the endosomes and to around pH 4.0 in primary and secondary lysosomes. A great number of lysosomal enzymes become active in the acidic environment of these

vesicles, for example, phosphatases, nucleases, proteases, esterases, and lipases.

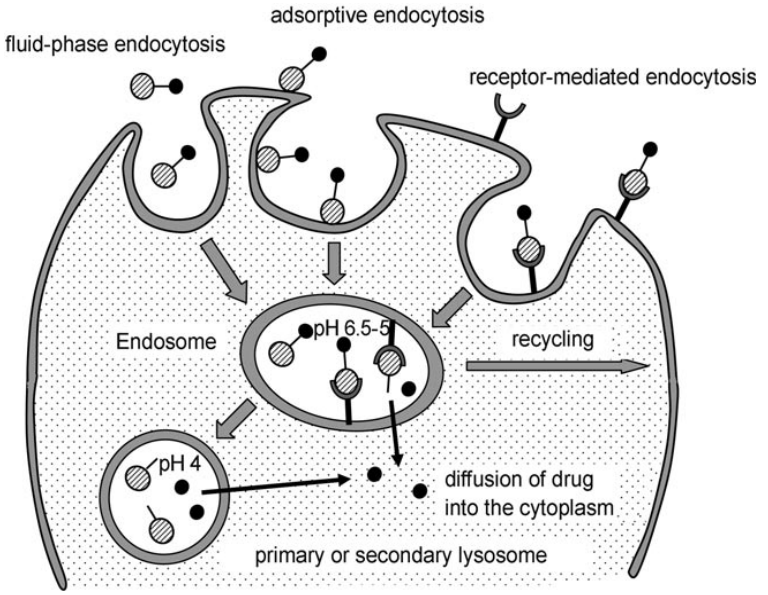


Figure 3. Endocytotic pathway for the cellular uptake of macromolecules

Drug–polymer conjugates or complexes should be sufficiently stable in the blood stream prior to the drug being liberated at the site of action. In principle, the polymer-bound drug can be released in the body by unspecific hydrolysis by enzymes, by reduction, or in a pH-dependent manner. In an ideal case, cleavage of the drug–polymer conjugate at the tumor site is triggered by a biochemical or physiological property unique for the individual tumor. Although such truly tumor-specific features are rarely encountered, the overexpression of certain enzymes, an acidic and hypoxic environment in solid tumors, as well as the endocytotic pathway of macromolecules offer several options for designing drug–polymer conjugates that are preferentially cleaved within the tumor.

The design of drug–polymer conjugates initially focused on incorporating enzymatically cleavable bonds that allow the prodrug to be cleaved intracellularly after cellular uptake. More recently, cleavage mechanisms involving triggering events that lead to a release cascade have been presented [26, 27]. The

advantage of this approach is a high local drug concentration with a potential increase in efficacy [28].

Both the low pH values in endosomes and lysosomes as well as the presence of lysosomal enzymes are therefore intracellular properties which have been exploited for releasing the polymer-bound drug specifically in tumor cells. Furthermore, the microenvironment of tumors has been reported to be slightly acidic in animal models and human patients: Non-invasive techniques have demonstrated that the pH value in tumor tissue is often 0.5–1.0 units lower than in normal tissue [29]. This difference could contribute to the extracellular release of drugs bound to polymers through acid-sensitive linkers, especially if the drug is trapped by the tumor for longer periods of time. Finally, drug–polymer conjugates can also be designed to slowly release the polymer-bound drug through hydrolysis under physiological conditions, as exemplified by conjugates of drugs and polyethylene glycol [30].

Polymer Conjugates for Protein Stabilization

Coupling polymers to therapeutically relevant proteins imparts several potential advantages: Conjugation can reduce the immunogenicity of the native protein, increase its stability, and prolong its biological half-life, thus resulting in less-frequent administration to the patient. Poly (ethylene glycol) (PEG) has mainly been the polymer of choice for preparing polymer–protein conjugates. In this “PEGylation” technology, linear or branched PEG derivatives are coupled to the surface of the protein [21, 31]. The companies Shearwater Polymers and Enzon initiated and refined this technology, which has resulted in the development of clinically as well as commercially successful products such as PEGylated asparaginase, PEGylated adenosine deaminase, PEGylated interferons, and PEGylated granulocyte colony stimulating factor [31–34].

Multivalent Interactions

In recent years, the development of multivalent drugs which are bridged by polymeric spacers has advanced dramatically [35, 36].The great potential of these systems is the high entropic gain in the formation of the multivalent complex. For example, the binding constants of bivalent interactions can be a factor of 1000 higher than monovalent binding, and for tri- and pentavalent interactions values up to 10^8 have been reported. This possibility allows for completely new ways to develop drugs; however, only a few efforts have been made so far to develop the first candidates for clinical trials.

A challenging approach to the application of multivalent interactions is the mimicry of functional biomacromolecules with therapeutic relevance. Several attempts have been made to mimic specific proteins (e.g., histones) or polysaccharides (e.g., heparin). In these cases, mimicry is mostly based on the surface charge of the polymer molecules. Applications range from DNA-transfection agents (polycationic systems) to anticoagulating, anti-inflammatory, and anti-HIV drugs (polyanionic systems).

1.2. Approaches and Applications

Polymer Conjugates of Therapeutically Relevant Proteins

Therapeutically relevant proteins such as antibodies, cytokines, growth factors, and enzymes are playing an increasing role in the treatment of viral, malignant, and autoimmune diseases. The development and successful application of therapeutic proteins, however, is often impeded by several difficulties, for example, insufficient stability and shelf-life, costly production, immunogenic and allergic potential, as well as poor bioavailability and sensitivity towards proteases.

An elegant method to overcome most of these difficulties is the attachment of polyethylene glycol chains onto the surface of the protein. PEGylation of the native protein increases its molecular weight and as a result prolongs the half-life in vivo, which in turn allows less frequent administration of the therapeutic

protein. In addition, the PEG chains mask the protein, which renders it more resistant to proteases and less immunogenic.A consequence of the PEGylation of proteins is generally a loss of the protein's biological activity. This loss, however, is outweighed by a substantial increase in the biological half-life of the PEGylated protein [20].

In the past few years two PEGylation processes have emerged: In the first method one or more linear PEG chains with a molecular weight between 5 and 12 kDa are bound to the surface of the protein (first generation PEGylated proteins). In the second method a single branched or a multibranched PEG chain is attached to a specific amino acid on the protein's surface (second-generation PEGylated proteins). In most cases activated PEG-carboxylic acids, for example, activated with N-hydroxysuccinimide, are bound to the ε-amino groups of lysine residues or the N-terminal amino group, but other chemical modifications with aldehyde, tresylate, or maleimide derivatives of PEG are also used.

The major drawback of first-generation PEGylated proteins was the heterogeneous nature of the pharmaceutical product, since in most cases multiple linear PEGs were attached to the protein. Despite this, several first-generation candidates received regulatory approval. The most prominent examples are adagen (PEGylated adenosine deaminase) for the treatment of severe combined immunodeficiency disease, oncaspar (PEGylated asparaginase) for the treatment of acute leukemia, and PEG-intron (PEGylated interferon α2b) for treating hepatitis C (Table 1).

Second-generation PEGylated proteins, in which a branched or linear PEG chain is attached to a site-specific amino acid on the protein, have the advantage in that they represent defined products with minimal alteration of the three-dimensional conformation of the protein. In 2002, granulocyte colony stimulating factor (G-CSF) PEGylated with a 20-kDa linear PEG chain (neulasta) was the first second-generation PEGylated system to receive market approval (Table 1).

Neulasta stimulates the production of white blood cells following bone-marrow depletion in the course of cancer chemotherapy. This treatment is more convenient than with the native protein, human recombinant G-CSF (neupogen); only one injection of neulasta is required every three weeks compared to daily injections of neupogen over two weeks [37]. Interferon α2a PEGylated with a 40-kDa branched PEG chain (pegasys) is a second-generation PEGylated system that has received market approval, and is a competitor of the firstgeneration conjugate PEG-intron (Table 1). Both PEG-intron and PEGasys have shown significantly better efficacy in the treatment of hepatitis C than the native interferon when combined with the antiviral agent ribavarin [32, 38]. Other examples of PEGylated proteins on the market or in advanced clinical trials are pegvisomant, a PEGylated form of the human growth hormone [39] and a PEGylated receptor and antibody fragment directed against tumor necrosis factors, a major mediator of inflammation (PEG-TNF-RI and PEG-anti-TNF Fab, respectively)[40,41]. Besides PEGylated proteins, one polymer–protein conjugate consisting of the anticancer protein neocarcinostatin and a synthetic copolymer of styrene and a maleic acid anhydride drug (Table 1) has been approved for the treatment of hepatocellular cancer in Japan [21].

Drug–Polymer Conjugates with Cleavable Linkers

The development of drug–polymer conjugates is a promising strategy to improve the therapeutic index of toxic drugs, especially in the field of cancer chemotherapy. Several drug–polymer conjugates are being investigated in phase I–III studies at present.

Although great efforts are being made to develop novel polymeric carriers, synthetic polymers that have been used in clinically evaluated drug conjugates have been mainly restricted to HPMA, PEG, and poly (glutamic acid) (PG). In addition, albumin, a biopolymer carrier, is being evaluated as a drug-delivery system in anticancer therapy. The cytostatic agents that have been primarily selected for preparing drug–polymer conjugates are doxorubicin, camptothecin, taxol, methotrexate, and platinum complexes.

Several drug–polymer conjugates with HPMA copolymers have been studied clinically. A doxorubicin–(HPMA copolymer) conjugate PK1 was the first drug–polymer conjugate to enter clinical trials [42]. PK1 has a molecular weight of approximately 28 kDa and contains doxorubicin (about 8.5 wt %) linked through its amino sugar to the HPMA copolymer by a tetrapeptide spacer, Gly-Phe-Leu-Gly. This peptide sequence is cleaved by lysosomal enzymes of tumor cells. Preclinical studies showed that the level of lysosomal enzyme expression in solid tumors, as well as their vascular permeability for macromolecules, correlated with the activity of this conjugate *in vivo* [43].

A phase I study revealed that the maximum tolerated dose (MTD) was 320 mgm^{-2} doxorubicin equivalents, which is a fivefold increase relative to the standard dose for doxorubicin [42]. The dose-limiting factors observed in this study were bone-marrow toxicity and mucositis. Other side effects, for example, nausea and diarrhea, were moderate. A noteworthy finding of this study was that no acute cardiotoxicity was observed even at these high doses. Two partial remissions and two minor responses were seen in four patients with lung, breast, and colorectal cancer. The recommended dose for phase II studies was 280 mgm^{-2} every three weeks. Phase II trials in breast, non-small-cell lung and colon cancer were initiated at the end of 1999; an interim report indicated positive responses in a few cases [44].

PK2 is a related compound to PK1, but incorporates an additional targeting ligand, namely, a galactosamine group that was designed to be taken up by the asialoglycoprotein receptor of liver tumor cells. In a phase I study, 31 patients with primary or metastatic liver cancer were evaluated [45]. The MTD of PK2 was 160 mgm^{-2} doxorubicin equivalents which is approximately half the MTD value of PK1, although the molecular weight and the loading ratio are very similar in both conjugates. Dose-limiting toxicity was associated with severe fatigue, neutropenia, and mucositis; a dose of 120 mgm^{-2} doxorubicin equivalents was recommended for phase II studies. Two partial remissions and one minor response were achieved in this study.

Two other HPMA conjugates with either taxol or camptothecin, respectively, entered phase I trials (Table 2).PNU-166945 is a water-soluble conjugate in which taxol at its 2-OH position is bound through a Gly-Phe-Leu-Gly linker to the polymer backbone. The camptothecin–(HPMA copolymer) conjugate consists of camptothecin linked at its 20-OH group to the HPMA copolymer through a Gly-6-aminohexanoyl-Gly spacer. Although preclinical results in tumorbearing mice have been promising, both conjugates have had limited success in early clinical trails because of their toxicity profile [46, 47].

Two drug–HPMA conjugates that have only recently entered phase I studies are AP5280 and AP5286, in which a diamine- or a diaminocyclohexaneplatinum (ii) moiety is bound to a dicarboxylate ligand that is coupled to the polymer through the tetrapeptide spacer Gly-Phe-Leu-Gly. This cathepsin B sensitive linker is also present in PK1, PK2, and PNU-166945[48, 49]. Preclinical assessment showed a high antitumor efficacy and a significantly increased MTD value for AP5280 compared to the clinical standards (cis- and carboplatin). In a phase I study the dose-limiting toxicity for AP5280 was vomiting (grade 3) at 4500 $mgPtm^{-2}$ (platinum equivalents); the dose recommendation for a phase II study was 3300 mg (Pt) m^{-2}. Five patients had a stabilization of their disease [50].

Another approach to doxorubicin–polylactide conjugates was recently reported by Sengupta et al [51]. These conjugates have been embedded into a biodegradable polylactide nanoparticle (ca.150 nm) to achieve better tumor selectivity through the EPR effect.

Prothecan, a camptothecin conjugate, is the first drug conjugate with polyethylene glycol that has been clinically assessed. Conjugating the 20-OH position of camptothecin with PEG through a glycine spacer[52–54] proved to have several advantages: a) the EPR effect results in a drug-targeting effect, b) esterifying the 20-hydroxy group of CPT stabilizes the drug in its active lactone form which otherwise tends to hydrolyze under physiological conditions and lead to the inactive hydroxycarboxylic acid form, c) incorporation of a glycine spacer

ensured a controlled release of the drug; and d) use of hydrophilic PEG leads to a highly water-soluble formulation of camptothecin.

Table 2- Drug–polymer conjugates in clinical trials

Compound	Spacer	Molecular weight (kDa)	Status of development
PK1, doxorubicin–(HPMA copolymer)	Gly-Phe-Leu-Gly	30	phase II
PK2, galactosaminated doxorubicin-(HPMA-copolymer)	Gly-Phe-Leu-Gly	30	Phase I discontinued
PNU-166945, taxol-((HPMA copolymer)	Ester	40	Phase I completed
MAG-CPT, camptothecin-(HPMA copolymer)	Gly-6-aminohexanoyl-Gly	30	Phase I completed
AP5280, diammineplatinum(II)-(HPMA copolymer)	Gly-Phe-Leu-Gly	25	Phase I completed
AP5286, diamminocyclohexaneplatinum (II)-(HPMA copolymer)	Gly-Phe-Leu-Gly	25	Phase I
Prothecan, camptothecin-PEG conjugate	Alanine ester	40	Phase II
CT-2103, taxol-polyglutamate conjugate	Ester	40	Phase II / III
CT-2106, camptothecin-polyglutamate conjugate	Gly-ester	50	Phase I
MTX-HSA, methotrexate-albumin conjugate	--	67	Phase II
DOXO-EMCH, 6-maleinimodcaproyl hydrazone derivative of doxorubicin	Acid-sensitive hydrazone	67 (albumin-bound prodrug)	Phase I completed

Preclinical results with prothecan showed it had better efficacy in animal models of human cancers than free camptothecin. Prothecan is currently being assessed in phase II studies for the treatment of gastric and gastroesophageal tumors after

a phase I study showed moderate nonhematologic toxicities at its MTD of 200 mgm^{-2} camptothecin equivalents [55].

PG-TXL (CT-2103), a poly(l-glutamic acid) conjugate of taxol is probably the most successful drug–polymer conjugate to date and is currently undergoing phase III trials in combination with standard chemotherapyagainst ovarian cancer and non-small-cell lung cancer[56]. PGTXL has a higher loading ratio (ca. 37 wt% taxol) than other drug–polymer conjugates, and the taxol is linked through its 2'-OH group to the poly (glutamic) acid backbone. Phase I and II studies of various cancers showed promising response rates, even for patients who were resistant to taxane therapy [57, 58]. The recommended dose of PG-TXL ranged from 175 to 235 mgm^{-2} (taxol equivalents) which is approximately twice as high as for free taxol. The dose-limiting toxicities of the conjugate are neurotoxicity and neutropenia. A noteworthy feature of PG-TXL is the biodegradability of the polyglutamic acid backbone and the liberation of taxol and taxol glutamic acid derivatives *in vitro* and *in vivo*, which, in part, appear to be mediated by cathepsin B [59]. A phase I study with an analogously constructed PEG conjugate with camptothecin has recently been completed successfully [60].

Besides synthetic polymers, albumin is also being investigated as a drug carrier in clinical trials. A methotrexate–albumin conjugate (MTX–HSA) was synthesized by directly coupling methotrexate to human serum albumin (HSA). This conjugate showed significant accumulation in rat tumors and high antitumor activity in selected nude mice models [61, 62]. Stomatitis proved to be dose-limiting above 50 mgm^{-2} MTX– HSA (MTX equivalents) in a phase I study [63]. Two patients with renal cell carcinoma and one patient with mesothelioma responded to MTX–HSA therapy (one partial remission, two minor responses). Renal cell cancer is a malignancy with low response rates to conventional chemotherapy. Phase II studies are ongoing.

New approaches have concentrated on forming a drug–albumin conjugate in vivo by binding prodrugs selectively to circulating albumin after intravenous administration [64–67]. This prodrug concept is based on two features: a) rapid and selective binding of a maleimide prodrug to the cysteine 34 position of endogenous albumin after intravenous administration, and b) release of the albumin-bound drug at the target site as a result of the incorporation of an acid-sensitive or an enzymatically cleavable bond between the drug and the carrier.

A first clinical candidate is the (6-maleimidocaproyl) hydrazone derivative of doxorubicin (DOXO-EMCH) which incorporates an acid-sensitive carboxylic hydrazone bond as a predetermined breaking point. DOXO-EMCH entered a phase I study in 2003 after demonstrating superior efficacy and an improved toxicity profile relative to free doxorubicin, the clinical standard [65]. Mice treated with doxorubicin at its MTD value (4x6 $mgkg^{-1}$) showed distinct kidney tumors (body weight loss of -10%), while the group treated with DOXO-EMCH at 4x12 $mgkg^{-1}$ doxorubicin equivalents showed no body weight loss and complete remission was achieved in nearly all the animals.

In a phase I study with DOXO-EMCH, 37 patients with advanced cancer were treated with an intravenous infusion of DOXO-EMCH once every 3 weeks at a dose of 20– 340 mgm^{-2} doxorubicin equivalents. Treatment with DOXO-EMCH was well tolerated up to 200 mgm^{-2}, without manifestation of drug-related side effects. Myleosuppression (grade 1–2), mucositis (grade 1–2), alopecia (grade 1–2), nausea and vomiting (grade 1), mouth dryness (grade 1), and fatigue (grade 1) have been noted at dose levels of 260, and myleosuppression (grade 2–3) as well as mucositis (grade 2–3) were dose-limiting at 340 mgm^{-2}. Of 29/37 evaluable patients, 13 had progressive disease, 13 had disease stabilization, a breast cancer and a liposarcoma patient had partial remission, and a patient with small-cell lung cancer had a complete remission. The recommended dose for phase II studies is 260 mgm^{-2}.

Although the clinical data for drug–polymer conjugates is limited to a few hundred patients, some general trends are apparent. The increase in the maximum tolerated dose (MTD) of the drug–polymer conjugates compared to the parent drug noted in preclinical studies is also manifested in clinical trials. Furthermore, no particular toxicity can be attributed to the polymer, and dose-limiting toxicities are comparable to the free drug. The significance of the molecular weight and of the cleavable linker of the drug–polymer conjugate remains unclear. Although the majority of nonbiodegradable polymers have molecular weights close to the renal threshold (30–50 kDa,), which allows enhanced permeation and retention in solid tumors, as well as a certain degree of renal clearance, a few recent examples of conjugates with albumin, polyglutamic acid, and PEG have molecular weights of 40–80 kDa. Whether the differences in the pharmacokinetic profile as a result of the different molecular weights influence the toxicity and tumor response needs to be evaluated in a larger population of patients.

The effectiveness of the predetermined breaking point incorporated in the drug–polymer conjugate also remains a matter of debate. The majority of drug–HPMA conjugates have made use of the tetrapeptide Gly-Phe-Leu-Gly, which is cleaved by lysosomal enzymes such as cathepsin B. However, preclinical data indicate that antitumor efficacy of such designed conjugates correlates with the expression of cathepsin B in the tumor,[43] a fact that has not been adequately addressed in clinical trials. Detailed knowledge of the expression of tumor-related proteases in individual tumor entities would certainly be helpful for the future development of cleavable drug–polymer conjugates. Whether drug–polymer conjugates that are cleaved by unspecific hydrolysis or at acidic pH values are more universally applicable needs to be addressed in clinical studies. Preliminary preclinical studies with doxorubicin–HPMA conjugates have indicated that an acid-sensitive linker is more effective than a cathepsin B sensitive one [68].

Incorporation of spacers in prodrug conjugates

Various spacers have been incorporated along with the polymers and copolymers to decrease the crowding effect and steric hindrance [69]. Steric hindrance describes how molecular groups interfere with other groups in the structure or other molecules during chemical conjugation. This effect is due to the interaction of molecules as dictated by their shape and/or spatial relationships. For example, molecular atoms that have affinity for one another may not be at an appropriate distance to attract each other due to their shape or they may have other atoms blocking them. The macroscale architecture of polymers causes steric hindrance for covalent conjugation with drugs in general and large peptides molecules in particular. Steric hindrance drives chemical transformations and may affect the chemical conjugation with bulkier and unstable molecules. A conjugation reaction involving polymers, peptides and unstable molecules, therefore, requires methodologies to reduce this effect. The most preferred method to decrease steric hindrance has been to alter the synthetic approach either by incorporating a spacer arm or by increasing the reactivity of the polymer or biomolecules [70].

The incorporation of a spacer arm can enhance ligand–protein binding and has application in prodrug conjugates and in biotechnology [70]. Ideal linkers possess the following characteristics: (1) stability in the physiological pH if the drug is to be delivered to the tumor vasculature and (2) they release the bioactive agent at an appropriate site of action. For example, amino acid spacers such as glycine, alanine, and small peptides are preferred due to their chemical versatility for covalent conjugation and biodegradability. Heterobifunctional coupling agents containing succinimidyl have also been used extensively as spacers. Therapeutic potential of a carboxypeptidase monoclonal antibody conjugate were reported using N-succinimidyl anhydrides [71–75]. The higher conjugation ratio of an antibody with a drug can result in a decrease in the ability of the antibody to bind to its specific receptor. This could be overcome by introducing a polymer spacer between the targeting antibody and the drug. The use of an intermediate polymer

with drug molecules carried in its side chains increases the potential number of drug molecules able to attach to that antibody by modification of only a minimum amount of existing amino acid residues [29]. In most of the bioconjugates, the NHS ester anhydride is reacted with primary $–NH_2$ of the peptide at slightly higher pH (7.5) to form an amide bond which links the maleimide group to the protein and releases NHS. N-hydroxysuccinimide released from the protein can be easily removed either by dialysis or by gel filtration using Sephadex columns such as G10 or G25. Thereafter, the maleimide group can be further reacted with the thiol containing moieties or proteins to form a thioether bond in the presence of a slightly acidic or neutral pH [75].

The higher conjugation ratio of an antibody with a drug can result in a decrease in the ability of the antibody to bind to its specific receptor. This could be overcome by introducing a polymer spacer between the targeting antibody and the drug. The use of an intermediate polymer with drug molecules carried in its side chains increases the potential number of drug molecules that are able to attach to the antibody by modification of only a minimum amount of existing amino acid residues [75].

The reactivity of functional polymers to couple with other biomolecules, which may be low, could be enhanced by first conjugating the polymer with reactive bis functional molecules. The resulting polymer–spacer conjugate moiety often enhances the reactivity and decreases steric hindrance for further coupling with drugs or biomolecules.

1.3. A Combined Approach: The PDEPT Concept

Polymer-directed enzyme–produg therapy (PDEPT) is a novel two-step antitumor approach that combines a polymeric prodrug and a polymer–enzyme conjugate to generate a cytotoxic drug at the tumor site [76]. PDEPT involves initial administration of the polymeric drug to promote tumor targeting before the activating polymer–enzyme conjugate is administrated. PDEPT has certain advantages compared to antibody-directed enzyme–produg therapy (ADEPT):

the relatively short residence time of the polymeric prodrug in the plasma allows subsequent administration of the polymer–enzyme conjugate without fear of activation of the prodrug in the blood stream, and also the polymer–enzyme conjugates could have reduced immunogenicity.

Two PDEPT approaches have been investigated with doxorubicin: In the first case, the polymeric prodrug PK1 (FCE 28068), which is currently under phase II clinical evaluation, was selected as a model prodrug in combination with an (HPMA copolymer)–(cathepsin B) conjugate. In the polymer-bound form, the (HPMA copolymer)–(cathepsin B) conjugate retained approximately 20–25% of the cathepsin B activity in vitro. After intravenous administration of the conjugate to tumorbearing B16F10 mice there was a 4.2-fold increase in its accumulation in tumors relative to the free enzyme. When PK1 and the PDEPT combination were used to treat established B16F10 melanoma tumors, the antitumor activity (%T/C, the survival time of treated versus control animals) for the PDEPT combination was 168% compared to 152% for PK1 alone, and 144% for free doxorubicin [77].

Another more successful PDEPT combination consisting of (HPMA-copolymer)–(methacryloyl-gly-gly-cephalosporin)– doxorubicin (HPMA-co-MA-GG-C-Dox) as the macromolecular prodrug and an HPMA copolymer conjugate containing the nonmammalian enzyme b-lactamase (HPMAco-MA-GG-b-l) as the activating component has been reported[85]. HPMA-co-MA-GG-C-Dox had a molecular weight of about 31600 Da and a doxorubicin–cephalosporin content of 5.85 wt%. Whereas free b-lactamase has a molecular weight of 45 kDa, the HPMA-co-MA-GG-b-L conjugate had a molecular weight in the range of 75–150 kDa. The HPMA-co-MA-GG-b-L conjugate retained 70% and 80% of its activity against the cephalosporin C and HPMAco-MA-GG-C-Dox substrates, respectively. Intraveneous administration of HPMA-co-MA-GG-C-Dox to mice bearing subcutaneously implanted B16F10 melanoma, followed after five hours by HPMA-co-MA-GG-b-L induced the release of free doxorubicin in the tumor. Whereas the PDEPT combination caused a significant decrease in the size of the

tumor (T/C=132%), neither free doxorubicin nor HPMA-co-MAGG- C-Dox alone displayed activity. Furthermore the PDEPT combination showed no toxicity at the doses used [78].

Multivalent Therapeutics

A fundamentally different approach to polymer therapeutics is based on the multiple interactions of ligands conjugated with a polymer which interact simultaneously with multiple receptor sites in protein complexes or multiple receptors on the cell surface. This concept is a close mimicry of biological interactions such as cellular recognition and signal transduction where multivalent processes play an important role. Although many interesting approaches have been reported, only a few clinical developments have so far been pursued.

Multivalent Drug Concepts (Toxins and Bacteria)

A number of multivalent inhibitors have been designed that are based on low-molecular-weight drugs and target dimeric or multimeric proteins that contain multiple identical receptor sites [35, 36]. For example, a pentavalent starlike carbohydrate ligand has been reported that fitted precisely into the binding pocket of the five subunits of the Shiga-like bacteria toxin, a close analogue of the cholera toxin [79]. An increase in the binding affinity by a factor of 10^7 was observed for this pentavalent interaction relative to the monovalent ligand. This example clearly demonstrates that dendritic and starlike molecules are perfect scaffolds for presenting ligands for multivalent interactions.

Another example is the binding of vancomycin derivatives or oligomers to the D-Ala-D-Ala motive of the bacteria cell wall. Whitesides and co-workers have reported on divalent and trivalent vancomycin derivatives which showed extremely high binding affinities. The trivalent model complex of vancomycin-D-Ala-D-Ala, with a binding constant of $4x10^{-17}$M, has a higher affinity than the avidin–biotin complex [80–82]. This concept of multivalent interactions with

vancomycin has been taken up in the pharmaceutical industry for *in vivo* and clinical studies 83].

Multivalent Interactions at Surfaces—Inhibition of Virus Attachment

The inhibition of virus attachment to cell surfaces is a fundamental problem for the prevention of viral infections, such as influenza and HIV. Traditional monovalent drugs cannot prevent the multiple adhesion of the virus to the cell surface. Therefore, the development of multivalent ligands that bind to membrane proteins of viruses is an important goal.

Several polymer architectures, including linear, starlike, and dendritic structures, have been considered as scaffolds for multivalent drugs [35, 36, 84-86]. Besides linear glycopolymers, various dendrimer structures have been investigated as multivalent ligands for sugar-binding proteins (for example, lectins), with multiple carbohydrate moieties attached at the exterior to form a so-called “sugar-coating”. For example, l-lysine dendrimers with 2 to 16 sialic acid units show enhanced binding affinities in the Limax flavus lectin precipitation assay and the hemaglutination assay of erythrocytes [87]. In these systems, four to six sialic acid residues appeared to be an optimal number of functional groups for antiviral activity against the influenza A virus. An approximately 200-fold increase in the binding affinity to the trivalent hemaglutinin as compared to the monovalent ligand was observed. The small size of dendrimers (3–5 nm) relative to the spacing of receptor sites on the virus surface is a major limitation of this approach; In comparison a high-molecular weight (106 Da) linear acrylamide polymer has shown in vitro an up to 108-fold increase in binding affinity, and hence is much more effective in blocking the attack of the influenza virus at the cell surface [88, 89]. However, the molecular weight of the polymer is too high to be cleared from the body by the kidneys, and rapid biodegradation is unlikely. In addition to its extremely high binding constant, the polymer can also sterically shield the virus particle when applied in combination with other monovalent ligands [90].

1.4. Polyanionic Polymers: Heparin Analogues

Heparin, a glycosaminoglycan, has been the drug of choice in the prevention and treatment of thromboembolic disorders for nearly 70 years. There is great interest in finding alternatives to both unfractionated heparin (UFH) and low-molecular-weight heparins (LMWH) because heparin has several disadvantages: First, it has to be isolated from mammalian organs, which implies a potential risk of contamination with pathogens such as viruses or prions, second, the increased use of heparin, especially of LMWH, means there is a growing shortage of the raw material, and third, heparin is a polydisperse mixture of molecules with different chain lengths and chemical structures. Numerous parameters, such as the animal species used for providing heparin, the method of isolation, and the purification step of the product, influence its respective composition and results in wide chemical and subsequent pharmacological variations between different heparin preparations.

In addition to their antithrombic activity, the characteristic feature of heparin and other natural sulfated polysaccharides are complement inhibition,[103] anti-inflammatory, antiangiogenic, antimetastatic, antiatherosclerotic, antiproliferative, antiadhesive, and antiviral effects. These additional modes of action can contribute to the overall therapeutic benefit of heparin in some cases. Consequently, heparin analogues with a similar or even improved pharmacological profile, but lacking the disadvantages of this animal product, are of interest. Besides partially synthetic sulfated linear polysaccharides, [91] fully synthetic sulfated linear polymers,[92] which are produced without a starting carbohydrate, may represent promising heparin mimetics[93].

Recently, a new type of polysulfated heparin analogue based on branched polysaccharides was described that possesses a much higher anticoagulant activity than its linear counterparts[94]. However, the accessibility of branched polysaccharides is problematic because of limited natural sources. Thus, a simple and efficient approach to highly branched polysulfated heparin analogues based on dendritic polyglycerols has been developed [95]. These polyglycerol

sulfates prolong the time of activated partial thromboplastin as well as thrombin and inhibit both the classical and alternative complement activation more effectively than heparin itself. In contrast to sulfated polysaccharides, their activities are not directly dependent on the molecular weight, which might be a result of the globular 3D structure of the dendritic polyglycerol sulfates. Since coagulation, complement activation, and inflammation are often present in the pathophysiology of numerous diseases, polyglycerol sulfates with both anticoagulant and anticomplementary activities represent promising candidates for the development of future drugs.

Recently, immunomodulatory and antiangiogenic properties of glucoseamine-modified polyamidoamine (PAMAM) dendrimers have been described. The use of dendrimeric glucosamine and dendrimeric glucosamine 6-sulfate together in a validated and clinically relevant rabbit model of scar tissue formation after glaucoma filtration surgery resulted in the long-term success of the surgery increasing from 30% to 80% [96].

Polycationic Polymers as DNA/RNA Transfection Agents

The search for nonviral alternatives remains a challenge because of problems associated with viral gene transfection, such as immune response and limited selectivity [97]. In the past decade several approaches were pursued in which cationic amphiphiles, polymers, or block copolymers and other pH-responsive polymers were used [98]. The colloidal surface and chemical properties of DNAand RNAcomplexes with polycations are responsible for controlling the extent and rate of delivery of genes to cells. However, additional hurdles on the cellular level have to be overcome on the surface of the polyplexes, such as size, charge, hydrophobicity, and buffering capacity, play a major role in the efficient transport and biological activity of the gene-based drugs [99].

The “proton- sponge hypothesis” postulates enhanced transgene delivery by cationic polymer–DNA complexes (polyplexes) containing proton-buffering polyamines through enhanced endosomal accumulation of chloride, which leads to osmotic swelling and lysis of the endosome [100]. For therapeutic applications,

however, an early endosomal escape mechanism, rather than lysosomal fusion, would be preferable to avoid the release of lysosomal enzymes into the cytosol[101].

The most frequently used cationic polymers for in vitro gene delivery are poly (ethylene imine) (PEI), poly (l-lysine), and chitosans. Another approach is the use of perfect polyamine-dendrimers [98, 102] to mimic the globular shape of the natural protein complex. However, the synthetic workload to obtain dendritic structures in the size-range of the natural histone complex (ca. 8 nm) [103] is tremendous. Also, the observation that a partially destroyed (hydrolyzed) dendritic backbone showed even higher transfection efficiencies [102] underlines the significance of readily available alternatives.

1.5. Supramolecular Drug–Polymer Complexes

One of the major problems in drug development is the poor solubility of many existing and new drugs. Very often the therapeutic effectiveness of these drugs is diminished by their inability to gain access to the site of action at an appropriate dose. Therefore, these drugs are either, not clinically used, delivered in large volumes of aqueous or ethanolic solutions, delivered in conjunction with surfactants, or chemically derivatized to soluble prodrugs. Unfortunately, all of these modifications can result in reduced efficacy or adverse effects. Many approaches for delivering hydrophobic compounds using polymeric carriers, such as block copolymers and dendritic polymers, have been explored [2, 11].

Block Copolymer Micelles

Polymeric micelles are generally more stable than micelles of small surfactant molecules and can retain the loaded drug for a longer period of time [104, 105]. The blockcopolymer micelles form spontaneously by self-assembly in water when the concentration of the amphiphilic block copolymer is above the critical micellar concentration (CMC) [106]. The driving force can be the hydrophobic interactions of the inner block, for example, a nonpolar poly(caprolactone) block (PCL), or ionic interactions, for example, a poly(aspartate) block (PAsp),

complexed to a negatively charged polymer such as DNA that forms a polyion micelle [107]. The outer block often consists of a polar poly(ethylene oxide) (PEO) block which forms the shell of the nanocarrier and protects its core. It has been demonstrated that PEO prevents the adsorption of proteins [108, 109] and hence forms a biocompatible polymeric nanocarrier shell. The size of these block-copolymer micelles is determined by thermodynamic parameters, but partial control over the size is possible by variation of the block length of the polymer [110]. Typically, these block-copolymer micelles are 20–50 nm in diameter with a relatively narrow distribution and are therefore similar in size to viruses, lipoproteins, and other naturally occurring transport systems [104].

A major obstacle for these nanocarrier systems is their nonspecific uptake by the reticuloendothelial systems (RES). The size and the surface properties of the nanocarriers based on block copolymers require careful design to achieve long circulation times in the blood and site-specific drug delivery [111]. The polarity and functionality of each block allow control over the spontaneously formed core–shell architecture. While terminal functionalities on the outer block (the shell) control biocompatibility and may incorporate potential targeting properties, the inner block of such nanocarriers can be used to complex or covalently couple active drug molecules. This core–shell concept is frequently used to dissolve nonpolar drugs. Examples of block copolymers that have poor solubility in water are the pluronics PEO-b-PPO or PEO-b-PPO-b-PEO.

Supramolecular constructs have also been generated by using block copolymers as shells for dendritic porphyrins [112]. These “blown up” micelles (ca. 100 nm) may have a much higher targeting specificity for tumor tissue as a result of an enhanced EPR effect. Kataoka and co-workers have recently reported a pHsensitive supramolecular nanocarrier for doxorubicin based on biocompatible block-copolymer micelles [113]. In contrast to drug–polymer conjugates, in which antitumor agents are covalently attached to a single macromolecule chain, doxorubicin was coupled through an acid-labile hydrazone linker to a PEO-b-PAsp copolymer. After spontaneous self-assembly of the drug-loaded

supramolecular nanocarrier, kinetic analysis clearly demonstrated the effective cleavage of the hydrazone bonds at pH-5, with concomitant release of doxorubicin. Release of doxorubicin was negligible under physiological conditions in cell culture medium (pH-7). The doxorubicin nanocarrier demonstrated in vitro cytotoxicity against a human small-cell lung cancer cell line (SBC-3) in a time-dependent manner, thus suggesting cellular uptake by endocytosis. The first candidates of antitumor drugs based on polymer micelles have entered clinical trials in Japan [114].

1.6. Dextran

Dextrans are glucose polymers which have been used clinically for more than five decades for plasma volume expansion, peripheral flow promotion, and as antithrombolytic agents [115]. Recently, however, dextrans have been investigated as potential macro molecular barriers for delivery of drugs and proteins, primarily to increase the longevity of therapeutic agents in the circulation. This is achieved mainly through relatively longer blood half-lives of high *Mw* dextran conjugates of therapeutic agents, compared with the intact drug or protein. Another application of dextrans as drug carriers has been for targeting drugs to specific sites of action via passive or active targeting. Finally, dextrans have been used to increase the in vitro stability and decrease the in vivo immunogenicity of proteins or enzymes. Whereas a majority of dextran-protein conjugates retain the pharmacologic activity of the protein to some degree, most of the dextran conjugates of small molecule drugs act as pro drugs, releasing the active drug *in vivo*.

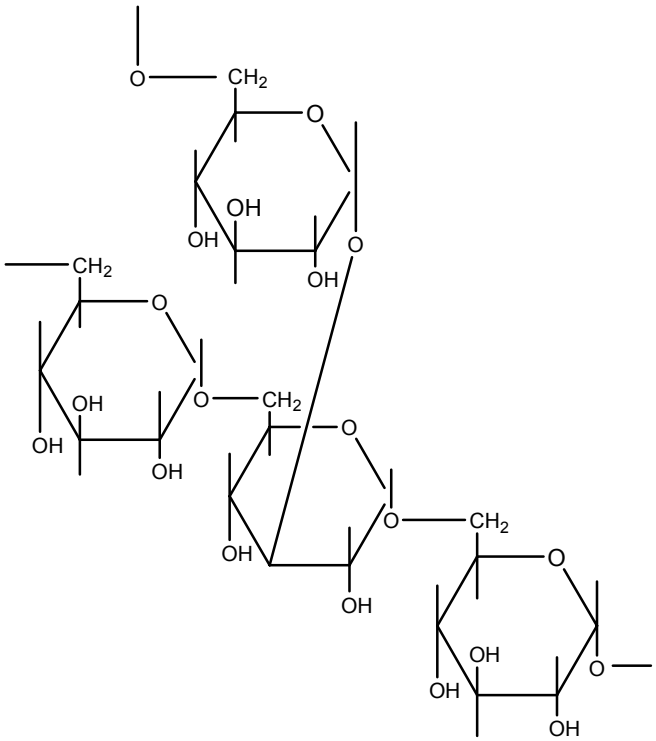
Physicochemical properties

Dextrans are produced by bacteria from sucrose or by chemical synthesis. Structurally, dextrans consist predominantly of linear α-l, 6-glucosidic linkage with some degree of branching via 1, 3 linkage [116]. The degree of branching is dependent on the source of dextrans and may vary from 0.5 to 60% [117]. Additionally, the source of dextrans determines the *Mw* distribution of the

polymers. Both the degree of branching and *Mw* distribution of dextrans affect the physicochemical properties of the polymer. For example, it has been reported that the degree of water solubility of dextrans decreases by an increase in the degree of branching of the macromolecules. In fact, dextrans with >43% branching (1,3-α linkage) have been considered water insoluble. However, most of the commercially available dextrans have a low degree of branching (0.5%), resulting in a high degree of water solubility.

Another physicochemical property of dextrans that significantly affects their in vivo behavior is the degree of polydispersity of these polymers. Although native dextrans have a large degree of polydispersity, dextrans of relatively narrow *Mw* distribution (polydispersity or weight average: number average *Mw* of: 5 2) may be obtained by partial depolymerization and fractionation of these polymers. Dextrans with average *Mw* of 4-200 kDa and polydispersity of 1.5 are now available commercially for research purposes. As for clinical dextrans, 10 or 6% solutions containing low (40 kDa) or high (70 kDa), respectively, are available in the US for blood flow enhancement and/ or plasma volume expansion. The range of molecular weights for the clinical dextran 40 kDa is between 10 and 90 kDa and that for the dextran 70 kDa is between 20 and 200 kDa. The effects of polydispersity of dextrans on the plasma and tissue distribution and elimination of these polymers are discussed in the following chapters.

In addition to high water solubility, dextran polymers are stable under mild acidic and basic conditions [118] and contain large number of hydroxyl groups for conjugation. These suitable physicochemical characteristics along with the low cost and a history of clinical use make dextrans attractive for use as drug or protein carriers.



Structure of Dextran

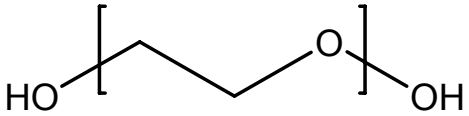
A few studies have reported the potential of dextran in colon-specific delivery of drugs via the oral route [119]. On the other hand by systemic administration, the pharmacokinetics of the dextran conjugates along with therapeutic agents are significantly affected by the kinetics of the dextran. Animal and human studies have shown that both the distribution and elimination of dextrans are dependent on molecular weight and the net charge. Dextran has been extensively evaluated as a polymeric vehicle for delivery of anticancer drugs to the tumor tissue through a passive accumulation of the dextran–anticancer conjugate [120]. Conjugates of dextrans with corticosteroids have been evaluated previously for the local delivery of steroids in colon as anti-inflammatory agents [121].

Potent anticancer agents, such as camptothecin have been conjugated to dextran to form prodrugs. Carrier and dose effects on the pharmacokinetics of T-0128, a camptothecin analogue-carboxymethyl dextran conjugate, were reported in control and tumor bearing rats [122]. Conjugation of drugs with

dextrans has exhibited prolonged effect, reduced toxicity, and immunogenicity. Most of the studies have been carried out in animals, however, with only a few experiments being performed on humans [123]. The multiple –OH groups on the polymeric dextran backbone provides possible functional sites for drug conjugation. Dextran of Mw 70,000 Da was conjugated to doxorubicin via an acidlabile linkage for intratumoral delivery [124]. Dextran is considered as a polymeric carrier because of its biocompatibility and biodegradability [125].

1.7. PEG Chemistry

PEG is a simple, water-soluble, non-toxic polymer that is nonimmunogenic and has been approved in certain products for human administration by mouth, injection and topical application. When attached to a drug, it can extend the length of action in the body from minutes to hours to days, depending on its molecular weight. Early attempts to attach low molecular-weight PEGs were limited because of unstable drug linkages and non-specific attachment of the PEG to the drug, resulting in impaired drug activity and problems of contamination leading to cross-linking and aggregation. Improvements in technology ‘however, has led to improved drug-to-PEG link stability and site-specific targeting, allowing prolonged performance of the drug in its PEG-drug conjugates form.



Poly(ethylene glycol)

A controlled release system that has been employed by several researchers and commercialized by Schering-Plough and Roche is that of using an injectable polymer vehicle. This protects the protein from rapid hydrolysis or degradation within the body, thus prolonging its action. Davies and Abuchowski carried out pioneering research on these systems wherein poly (ethylene glycol) (PEG)

chains were attached to drug chemically thus increasing their size and hence improving their delivery [126].

Reasons for PEGylation (the covalent attachment of PEG) of peptides and proteins are numerous and include shielding of antigenic and immunogenic epitopes, shielding receptor-mediated uptake by reticuloendothelial system (RES), and preventing recognition and degradation by proteolytic enzymes. PEG conjugation also increases apparent size of the polypeptide, thus reducing the renal filtration and altering biodistribution. An important aspect of PEGylation is the incorporation of various PEG functional groups that are used to attach the PEG to the peptide or protein [127]. It has been shown that PEGylation improves macromolecule solubility and stability by shielding it from carbohydrate and peptide receptor clearance mechanisms and immune system cells.

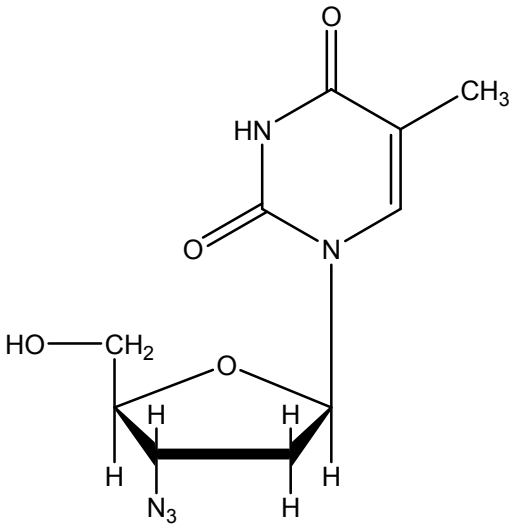
First generation chemistries are generally plagued by PEG impurities, restriction to low molecular weights, unstable linkages, and lack of selectivity in modification. The initial work of Davies and coworkers used cyanuric chloride to prepare activated PEG for attachment to proteins [128, 129]. Most first generation PEG chemistries are those that produce conjugates through acylation. Two widely used first generation activated mPEGs are succinimidyl carbonate and benzotriazole carbonate.

Second generation PEGylation chemistry were designed to avoid the problems of diol contamination, restriction to low molecular weight mPEG, unstable linkages, side reactions and lack of selectivity in substitution for example, mPEG-propionaldehyde[130]. There has been an increasing need for heterobifunctional PEGs, which are PEGs bearing dissimilar terminal groups. Such heterobifunctional PEGs bearing appropriate functional groups may be used to link two entities where a hydrophilic, flexible, and biocompatible spacer is needed. Preferred end groups for heterobifunctional PEGs are NHS esters, maleimide, vinyl sulphone, pyridyl disulfide, amine and carboxylic acids.

The development of polymer therapeutics has emerged as an exciting field of research for improving the therapeutic potential of low-molecular-weight drugs and proteins. PEGylation of therapeutically relevant proteins is an established technology, and it is likely that new PEGylated proteins will attain market approval in the next few years, considering that several hundreds of protein-based therapeutics are under preclinical or clinical development.

1.8. Zidovudine

Zidovudine (3'-azido-3'-dideoxythymidine, AZT, Retrovir) is a chemotherapeutic agent that is particularly effective against the **HIV** virus with an inhibiting mechanism of viral reverse transcriptase [131].



Zidovudine

Although AZT is clinically active in patients with AIDS or AIDS-related diseases and is still one of the few drugs clinically approved against HIV infection, both in monotherapy and more recently in combination with other antiviral drugs [132], doserelated toxic effects, especially on bone marrow, have been significant, necessitating dose reduction or discontinuance of treatment [133]. High plasma concentrations of the drug are responsible for serious side effects such as anaemia and leucopenia, while low doses prevent effective intracellular concentration, especially in the brain, and considerably limit drug effectiveness. Also, AZT has a very short plasma half-life (approximately 1h), which

necessitates frequent administrations to maintain therapeutic drug doses. The synthesis of a drugpolymer conjugate with AZT can achieve a good approach to control drug concentration in plasma, to limit its indiscriminate diffusion ability across cell membranes and to improve the retention of the drug in the body.

Absorption and Bioavailability

Zidovudine is well absorbed following oral administration. Bioavailability is between 60 and 70%. Peak plasma concentrations occur within 1 hour after dosing. In healthy volunteers, at a therapeutic dose of 300 mg twice daily, mean steady-state C_{max} of zidovudine in plasma were 2µg/ml. The mean area under the curve (AUC) over a dosing interval of 12 hours is 2.4 µg/ml [134].

Distribution

The estimated volume of distribution is 1.6 L/kg. Protein binding is 34-38%.

Metabolism / Elimination

The observed half-life is 1 hour. The mean systemic clearance of zidovudine is approximately 1.6 l/h/kg. 90% of zidovudine and its major metabolite, 5'-glucuronylzidovudine, is excreted in urine [135].

1.9. Literature review on the sustained release formulations of zidovudine

Giammona and coworkers [136] synthesized macromolecular prodrug of the known antiretroviral agent zidovudine and α, β-poly (*N*-2-hydroxyethyl)-DL -aspartamide (PHEA). A succinic spacer was present between the polymer and the drug. *In vitro* drug release studies at pH 1.1, 5.5 and 7.4 indicated that limited amounts of intact drug were released from the conjugate. At pH 1.1 and 7.4 succinylzidovudine was released, and this was hydrolysed to give free zidovudine. In the presence of α-chymotrypsin, zidovudine was released preferentially in comparison with the succinyl derivative. The amounts of released zidovudine and succinylzidovudine were greater in plasma than in aqueous buffer solutions. These results showed that after i.v. administration this drug-polymer conjugate can release zidovudine into the blood circulation for prolonged periods

Wannachaiyasit S and coworkers [137] synthesized dextrin-zidovudine (AZT) conjugate designed as a sustained release prodrug of AZT for parenteral administration. AZT was first reacted with succinic anhydride to form a succinoylated AZT which was subsequently coupled with dextrin to yield the dextrin-AZT conjugate. The release in vitro of free AZT and succinoylated AZT was investigated in buffer solutions at pH 5.5 and 7.4 and in human plasma. AZT and succinoylated AZT release from the conjugate was 1.4% (pH 5.5), 41.7% (pH 7.4) and 78.4% in human plasma after 24 h. Release was complete in human plasma after 48 h. A pharmacokinetic study in rats following intravenous administration of the conjugate showed prolonged plasma levels of AZT compared to free AZT. The use of the conjugate extended the plasma half-life of AZT from 1.3 to 19.3 h and the mean residence time from 0.4 to 23.6 h. Furthermore, the conjugate provided a significant greater area under the plasma concentration-time curve and reduced the systemic clearance of AZT. This study suggested the potential of this novel dextrin-AZT conjugate as a new intravenous preparation of AZT.

Gennara Cavallaro and coworkers [138] synthesized polymeric conjugates for specific liver targeting by conjugating sugar moieties and ganciclovir and acyclovir to α, β-poly[*N*-2-(hydroxyethyl)-dl-aspartamide] (PHEA). PHEA-galactopyranosylphenylthiocarbamide-mono-*O*-succinylganciclovir and PHEA-mannopyranosylphenylthiocarbamide-*O*-succinylacyclovir were synthesized according to a multi-step procedure which allowed for obtaining high product yield and process standardization. *In vitro* studies demonstrated that both acyclovir and ganciclovir are released from the polymeric adducts at a release rate, which depended on the incubation medium. Though a detailed study evidenced that the two bioconjugates undergo different hydrolysis pathways, in both cases high drug release rate was found in plasma, while the glycosidic moiety was not released. Pharmacokinetic studies carried out by intravenous administration of the bioconjugates to Balb/c mice demonstrated that the conjugation of glycosidic moieties promotes the disappearance of the polymer from the bloodstream. The two derivatives displayed a different pharmacokinetic profile. In particular, the mannosyl conjugation promoted the rapid disposition of the macromolecule in the kidneys and in the liver, while prevented the accumulation in the spleen. On the contrary, the galactosyl derivative was found to dispose in the liver at the same extent of the naked polymer.

Anthony R. Vortherms and coworkers [139] synthesized conjugates of three components namely folic acid, poly (ethyleneglycol) and 3 '-azido-3'-deoxythymidine (AZT). Folate-PEG units were coupled to AZT to facilitate delivery of the nucleoside into the cell. A convenient separation of the polydisperse PEGylated-folic acid regioisomers produced upon conjugation is described. This is to select for the active γ-regioisomer over the inactive α-regioisomer. In vitro cytotoxicity assays were conducted against an ovarian cell line (A2780/AD) that overexpresses the folate receptor (FR) and compared to a FR free control cell line. Compared to AZT a ≈ 20-fold greater potency against the resistant ovarian line was observed for the conjugates.

Yong Ju and coworkers [140] synthesized a series of bile acid–polyamine amides conjugated with 3′-azido-3′-deoxythymidine (AZT) as potential antitumor prodrugs in the form of phosphoramidates and evaluated their antitumor activities against two human cancer cells in vitro, cervix cancer HeLa cells and renal cancer 7860 cells. They found out that the synthesized conjugates improved antitumor activity. This probably may derived from the enhanced delivery efficiency of AZT due to bile acid–polyamine conjugates

Rong Zeng and coworkers [141] developed a novel approach to synthesize chitosan–*O*-isopropyl-5′-*O*-d4T monophosphate conjugate. Chitosan-d4T monophosphate prodrug with a phosphoramidate linkage was efficiently synthesized through Atherton-Todd reaction. In vitro drug release studies in pH 1.1 and 7.4 indicated that chitosan–*O*-isopropyl-5′-*O*-d4T monophosphate conjugate prefers to release the d4T 5′-(*O*-isopropyl) monophosphate than free d4T for a prolonged period. The results suggested that chitosan–*O*-isopropyl-5′-*O*-d4T monophosphate conjugate may be used as a sustained polymeric prodrug for improving therapy efficacy and reducing side effects in antiretroviral treatment.

Yiguang Jin and coworkers [142] synthesized an amphiphilic prodrug of anti-HIV nucleoside analogue, cholesteryl-phosphonyl zidovudine (CPNZ). An aqueous suspension containing CPNZ self-assemblies was obtained through injecting the ethanol solution of CPNZ and cholesteryl succinyl poly(ethylene glycol) 1500 (20:1, mol/mol) into water under agitation. The self-assemblies were nanoscale with ~100 nm in size, and remained stable for a long time. Degradation of CPNZ self-assemblies was investigated in various environments including buffered solutions, plasma and rabbit tissue homogenates. CPNZ was degraded very slowly in neutral solutions but rapidly in various plasma with the half-lives ($t_{1/2}$) of less than 20 h. Tissue homogenates degraded CPNZ with varied rates depending on enzyme activity. CPNZ self-assemblies showed potent anti-HIV activity on MT4 cell model. CPNZ was rapidly eliminated from circulation and distributed into the mononuclear phagocyte system (MPS) including liver,

spleen and lung after bolus intravenous administration of CPNZ self-assemblies followed slowly elimination. The MPS-targeted effect and high anti-HIV activity of CPNZ self-assemblies make them a promising self-assembled drug delivery system (SADDS).

Bonina and coworkers [143] synthesized glycosyl derivatives of 3′-azido-3′-deoxythymidine (AZT) in order to improve AZT retention in the blood to guarantee its sustained release and overcoming the necessity of multiple drug administrations. The esters synthesized link AZT, by a succinyl linker, to the C-3 position of glucose and to C-6 of galactose. Furthermore, the chemical and enzymatic stabilities of prodrugs were evaluated in order to determine both their stability in aqueous medium and their feasibility to undergo enzymatic cleavage by esterase to regenerate the original drug. The pharmacokinetic profiles of prodrugs, obtained after systemic administration, showed an interesting controlled release, in particular for prodrug attached with galactose, compared to the pharmacokinetic profile of AZT.

Rong Zeng and coworkers [144] developed a novel approach to improve the antiviral efficacy of nucleoside reverse transcriptase inhibitors (NRTIs) and reduce their side effects by constructing a nanosized NRTI monophosphate-polymer conjugate using d4T as a model NRTI. Firstly, a novel chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate with a phosphoramidate linkage was efficiently synthesized through Atherton–Todd reaction under mild conditions. The anti-HIV activity and cytotoxicity of the polymeric conjugate were evaluated in MT4 cell line. Then the conjugate nanoparticles were prepared by the process of ionotropic gelation between TPP and chitosan-d4T conjugate to improve their delivery to viral reservoirs, and their physicochemical properties were characterized by transmission electron microscopy (TEM), dynamic light scattering (DLS) techniques and X-ray diffraction (XRD). *In vitro* drug release studies in pH 1.1 and pH 7.4 suggested that both chitosan-d4T conjugate and its nanoparticles prefer to release d4T 5′-(*O*-isopropyl) monophosphate than free d4T for prolonged periods, which resulted in the enhancement of anti-HIV

selectivity of the polymeric conjugate relative to free d4T due to bypassing the metabolic bottleneck of monophosphorylation. Additionally, the crosslinked conjugate nanoparticles prevent the coupled drug from leaking out of the nanoparticles before entering the target viral reservoirs and provide a mild sustained release of d4T 5′-(*O*-isopropyl) monophosphate without the burst release. The results suggested that this kind of chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate nano-prodrugs may be used as a targeting and sustained polymeric prodrugs for improving therapy efficacy and reducing side effects in antiretroviral treatment.

Sriram and coworkers [145] synthesized a series of prodrugs of zidovudine in an effort to enhance spectrum of chemotherapeutic properties for the effective treatment of HIV/AIDS. The 5'-OH function of zidovudine was esterified with ciprofloxacin, norfloxacin, isoniazide, pyrazinamide acetic acid. The anti-HIV-1 activity of the esters was determined in CEM cell-line and zidovudine ester bearing pyrazinamide acetic acid was found to be the most potent compound with EC50 of<0.0636 microM, CC50 of>1000 microM and selectivity index (SI) of>15,723. Zidovudine prodrug bearing ciprofloxacin and norfloxacin moiety showed 100% inhibition against Mycobacterium tuberculosis H37Rv at 6.25microg/ml. The prodrugs were also found to exhibit antibacterial activity against 24 pathogenic bacteria. *In vitro* hydrolysis of the various esters in human plasma indicated that these agents were relatively stable toward plasma esterase with t1/2 ranging from 20 to 240 min.

C K Chu and coworkers [146] in an effort to increase the brain delivery of anti-HIV nucleosides, studied *in vitro* and *in vivo* pharmacokinetics of dihydropyridine derivatives of 3'-azido-2',3'-dideoxyuridine (AzddU, AZDU, or CS-87) and 3'-azido-3'-deoxythymidine (AZT, Zidovudine). *In vitro* studies of the prodrugs (AzddU-DHP and AZT-DHP) in human serum, mouse serum, and mouse brain homogenate indicated that the rates of serum conversion from prodrugs to parent drugs are species dependent: mouse brain homogenate greater than mouse serum greater than human serum. *In vivo* studies of AzddU-DHP and AZT-DHP

showed that the prodrugs have areas under the serum concentration-time curves (AUC) similar to those of the parent drugs. Thus, the relative brain exposure (re) for AzddU (5.47) and AZT (9.32) indicate a significant increase in exposure to the anti-HIV nucleosides following prodrug administrations. The results of extended half-lives of the synthesized prodrugs in human serum along with the higher relative brain exposure (re) values *in vivo* warrant studies in larger animals to determine the potential usefulness of the prodrugs in humans.

McGuigan and coworkers [147] synthesized some novel aryl phosphate derivatives of the anti-HIV nucleoside analogue AZT by phosphorochloridate chemistry. These materials were designed to act as membrane-soluble pro-drugs of the bio-active free nucleotides. *In vitro* evaluation revealed the compounds to have a pronounced, selective antiviral activity. The magnitude of the biological effect varied considerably with the nature of the phosphate-blocking group. Moreover, one of the compounds, a phosphoramidate, was particularly active in a cell line restrictive to the activity of AZT, due to poor phosphorylation therein. These data support the suggestion that the phosphate derivatives exert their biological effects via intracellular release of the nucleotide forms.

Piantadosi and coworkers [148] chemically linked amidoalkyl, oxyalkyl, and thioalkyl ether lipids to anti-HIV-1 nucleosides zidovudine and didonosine (AZT and DDI) through phosphate and phosphonate linkages. These conjugates showed promising *in vitro* anti-HIV-1 activity and had a 5-10-fold reduction in cell cytotoxicity compared to AZT alone. The most active compound, an amidoalkyl ether lipid-AZT conjugates was found to have a differential selectivity of 1793 in a syncytial plaque assay. In comparison, AZT alone has a value of 1281.

S Chang and coworkers [149] prepared a series of phosphoramidate monoesters of 3'-azido-3'-deoxythymidine (AZT) bearing aliphatic amino acid methyl esters and methyl amides and evaluated for anti-HIV-1 activity in peripheral blood mononuclear cells (PBMCs). These compounds, which showed no cytotoxicity at concentrations of 100 microM, were effective at inhibiting HIV-1

replication at concentrations of 0.08-30 microM. The D-phenylalanine and D-tryptophan derivatives exhibited equivalent or enhanced antiviral activity compared to their L-counterparts. In addition, except for the D-phenylalanine derivatives, the methyl amides had greater antiviral activity than the corresponding methyl esters. The results suggest that the biological activity and intracellular metabolism of nucleoside phosphoramidate monoesters are distinct from that of phosphoramidate diesters.

McGuigan and coworkers [150] synthesized phosphate triester derivatives of zidoveudine (AZT) as membrane-soluble pro-drugs of the bio-active nucleotides and evaluated them against HIV-1 *in vitro*. The phosphorus centre carried a trichloro- or trifluoroethyl group and a carboxyl-protected, amino-linked amino acid. The triester derivatives displayed potent anti-HIV activity and low host toxicity, but surprisingly this activity did not increase on the introduction of the haloalkyl moiety.

Mati Fridkin and coworkers [151] conjugated zidovudine to tuftsin the IgG-derived immunomodulating peptide, (Thr-Lys-Pro-Arg). Tuftsin is recognized by specific receptors on phagocytic cells, notably macrophages, and is capable of targeting proteins and peptides to these sites. The AZT-tuftsin chimera possesses the characteristic capacities of its two components. Thus, like AZT, it inhibited reverse transcriptase activity and HIV-antigen expression, and similarly to tuftsin, it stimulated IL-1 release from mouse macrophages and augmented the immunogenic function of the cells. Importantly, the conjugate was not cytotoxic to T-cells. The results suggest that the AZT-tuftsin conjugate might have potential use in AIDS therapy.

Patrick Vlieghe and coworkers [152] synthesized kappa-carrageenan-3'-azido-3'-deoxythymidine (AZT) conjugates. A succinate diester spacer was used to covalently couple AZT onto kappa-carrageenan, resulting in a tripartite prodrug. This polymeric carrier, through its own intrinsic anti-HIV activity, was expected to act not only as a drug delivery agent but also as an anti-HIV agent. Synergism

between the two drugs (kappa-carrageenan and AZT) was demonstrated when MT-4 cells were preincubated with the kappa-carrageenan-AZT conjugate prior to HIV-1-infection. A threshold of AZT loaded onto the kappa-carrageenan was required to achieve this synergistic effect. Such kappa-carrageenan-AZT conjugates could be of great therapeutic interest because these conjugates, which contain a low AZT concentration, present improved anti-HIV activities relative to free AZT.

2. Scope and objective

Conventional pharmacological therapy consisting of a number of non- specific administrations of a drug introduced at regular intervals may have contraindications such as variable drug concentrations in the blood and excessive drug presence also in healthy tissues, with toxic and side effects. Frequent high doses are often needed because of the drug's unfavorable physical and chemical properties, such as excessive or insufficient water-solubility, which restricts drug bioavailability, and also because of low drug specificity towards the affected organs.

Many antiviral drugs have remarkable therapeutic activity accompanied by serious undesirable side effects. In some very grave diseases such as AIDS, the side effects are a considerable problem, since the quality of life of patients with complications such as severe weight loss, herpes zoster and thrush is already strongly endangered [153]. The current problems in controlling severe viral infections as well as the lack of effective and safe therapeutic measures for such diseases have caused interest in systems such as macromolecular pro-drugs potentially able to solve heavier drawbacks of conventional antiviral therapy.

In the recent years preparation of polymer-drug conjugates in which drug molecules are linked to polymeric matrix through a covalent bond of limited stability in the physiological environment is receiving increased attention by several workers. This is believed to be one of the most promising ways to modify the pharmacokinetics of the drugs

Zidovudine (3'-azido-3'-dideoxythymidine, AZT, Retrovir) is a chemotherapeutic agent that is particularly effective against the HIV virus with an inhibiting mechanism of viral reverse transcriptase. Although AZT is clinically active in patients with AIDS or AIDS-related diseases and is still one of the few drugs clinically approved against HIV infection, both in monotherapy and more recently in combination with other antiviral drugs , dose related toxic effects, especially on bone marrow, have been significant, necessitating dose reduction or

discontinuance of treatment [154]. High plasma concentrations of the drug are responsible for serious side effects such as anemia and leucopenia, while low doses prevent effective intracellular concentration, especially in the brain, and considerably limit drug effectiveness. Also, AZT has a very short plasma half-life (approximately 1 h), which necessitates frequent administrations to maintain therapeutic drug doses. The synthesis of a drug polymer conjugate with AZT can achieve a good approach to control drug concentration in plasma, to limit its indiscriminate diffusion ability across cell membranes and to improve the retention of the drug in the body.

Poly (ethylene glycol), PEG, was chosen as the carrier polymer, because it is known to be non-toxic, non-teratogenic, non-immunogenic and biocompatible. PEG is available in a variety of molecular weights. It is a linear, uncharged and amphiphilic polymer. It is soluble in water and in most organic solvents 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). It has been also employed to improve biocompatibility, promote peptide immobilization, prolong protein drug circulation time, increase bioactivity and reduce immunogenicity (155].

Dextrans are glucose polymers which have been used clinically for more than five decades for plasma volume expansion, peripheral flow promotion, and as antithrombolytic agents [156]. Recently, dextrans have been investigated as potential macromolecular carriers for delivery of drugs and proteins, primarily to increase the longevity of therapeutic agents in the circulation. This is achieved mainly through relatively longer blood half-lives of high MW dextran conjugates of therapeutic agents, compared with the intact drug or protein. Another application of dextrans as drug carriers is for targeting drugs to specific sites of action via passive or active targeting. Dextrans have also been used to increase the *in vitro*

stability and decrease the *in vivo* immunogenicity of proteins or enzymes. Whereas a majority of dextran–protein conjugates retain the pharmacologic activity of the protein to some degree, most of the dextran conjugates of small molecule drugs act as prodrugs, releasing the active drug *in vivo*.

In the present investigation it was proposed, therefore, to couple Zidovudine to PEG_{1500} and Dextran-40 through succinic spacer and evaluate their *in vitro* hydrolysis studies in order to evaluate their ability to release the drug molecule at different pH values mimicking some biological compartments. The possibility of drug release from the macromolecular pro-drugs in human plasma and their oral bioavailability also were also proposed to be carried out.

Proposed plan of work

Stage I: Synthesis and characterization of pro-drugs

- Synthesis of 5'-O-succinylzidovudine
- Synthesis of PEG-O-succinylzidovudine and Dextran-O-succinylzidovudine

Stage II*: In vitro* hydrolysis studies in buffer solutions of

- pH 1.1 (simulated gastric juice)
- pH 5.5 (endosomal compartments)
- pH 7.4 (extraceullar fluid)
- Enzymatic hydrolysis at pH 8 in the presence and absence of α-chymotripsin
- Plasmatic hydrolysis studies

Stage III: Bioavailability studies

3. Experimental

3.1. Materials

Succinic anhydride and N, N'-dicyclohexylcarbodiimide (DCC) were purchased from Fluka; N, N'- dimethylformamide (DMF) (SD Fine Chemicals Limited, India) was dried using P_2O_5 and distilled under reduced pressure before use. Anhydrous tetrahydrofuran (THF) was purchased from M/s. Ranbaxy Chemicals, India and α-chymotrypsin (from bovine pancreas) and Zidovudine (AZT) were purchased from Sigma. All other chemicals were reagent grade and used as such.

Infrared spectra were obtained using a Perkin-Elmer 1720 IR Fourier Transform Spectrophotometer in potassium bromide disks. Ultraviolet (UV) spectra were recorded by using a Perkin-Elmer 330 Instrument equipped with a 3600 Station. Compounds were quantitatively dried before analysis on P_2O_5 under reduced pressure (10 mmHg) at room temperature for 48 h.

The H^1NMR spectra were obtained with a Varion 400 instrument operating at 250.13 MHz. Centrifugations were performed using a Centra MP4R IEC centrifuge. HPLC analyses were carried out on a system consisting of a Varian 9012 Liquid Chromatography equipped with a Rheodyne Injector 7125 (fitted with a 10 ml loop), a Kontron HPLC.

3.2. Synthesis of the macromolecular pro-drugs

Synthesis of 5'-O-succinylzidovudine

A solution of 1.0 g (3.75 mmol) of zidovudine , 0.6g (5.97mmol) of succinic anhydride and 0.63 ml of triethylamine (4.53 mmol), in 60 ml of dry THF was heated at reflux in an oil bath for 11h. The progress of the reaction was followed by TLC (CH_3OH:$CH_3COOC_2H_5$, 6:4, v/v) and after 6h, 0.6g of succinic anhydride and 0.3ml of triethylamine were added all at once. Then the solvent was evaporated in vacuo and the residue was taken up in 40ml of distilled water and acidified to pH 2 by 1M HCl. The acid aqueous solution was extracted with

CH_2Cl_2 (about 50 ml) several times. The progress of the extraction was verified by TLC as previously described. The organic fractions were collected, dried over anhydrous Na_2SO_4 at room temperature for 2 days, filtered and evaporated the white residue obtained with a 98% yield (w/w) was characterized by IR, Mass and H^1 NMR spectroscopy.

Synthesis of PEG-O-succinylzidovudine and

A solution of DCC (0.266g, 1.25mmol) in 1.8 ml of dry DMF was added drop wise at 0ºC to a solution of 5'-O-succinylzidovudine (0.361g, 0.984mmol) dissolved in dry DMF (3.5 ml). To the mixture kept at 0ºC for 30 min, a solution of PEG_{1500} (0.75g, 0.5mmol) in dry DMF (3.6ml) was added drop wise. The reaction mixture was maintained for 8 min at 0ºC, for 1 h at 9ºC and then set aside at room temperature for 3 days with constant stirring. After filtration, DMF was evaporated at reduced pressure. The white residue obtained with an 82% yield (w/w) was characterized by IR, and H^1-NMR spectroscopy.

Synthesis of DEX-O-succinylzidovudine

A solution of DCC (0.266g, 1.25mmol) in 1.8 ml of dry DMF was added drop wise at 0ºC to a solution of 5'-O-succinylzidovudine (0.361g, 0.984mmol) dissolved in dry DMF (3.5 ml). To the mixture kept at 0ºC for 30 min, a solution of Dextran 40 (4g, 0.1mmol) in dry DMF (3.6ml) was added drop wise. The reaction mixture was maintained for 8 min at 0ºC, for 1 h at 9ºC and then set aside at room temperature for 3 days with constant stirring. After filtration, DMF was evaporated at reduced pressure. The white residue obtained with an 82% yield (w/w) was characterized by IR, and H^1-NMR spectroscopy.

3.3. Estimation of Drug content

A stock solution of zidovudine was prepared by dissolving 100 mg of the drug in 100 ml of 0.1N NaOH. From the stock solution 10, 20, 30, 40 and 50 µg/ml dilutions were prepared using phosphate buffer of pH 7.4. The λ_{max} of the drug was determined by scanning the diluted solution between 200-400nm against a

blank reagent in a double beam spectrophotometer (model UV 160 A). The λ_{max} was found to be 266nm. A standard curve between concentration and absorbance was then plotted and its intercept (B) and slope (k) were calculated. Similar procedures were also adopted for the development of calibration curves for zidovudine in buffer solutions of pH 1.1, 5.5 and 7.4 in 0.1 N NaOH.

The amounts of zidovudine in the synthesized polymeric pro-drugs were determined by UV spectrophotometry. The pro-drug (100 mg) was taken in 100 ml 0.1N NaOH. The resulting solution was sonicated for 10 minutes and filtered. The filtrate (5 ml) was diluted to 50ml using 0.1N NaOH and kept for 24h at room temperature for the complete release of the drug from the polymer backbone. The absorbance was measured at 266 nm using 0.1N NaOH solution as a blank.

3. 4. *In vitro* hydrolysis studies

The synthesized PEG-drug and Dextran-drug conjugates on oral administration should undergo drug release in the biological media followed by absorption of the drug into the systemic circulation before eliciting their action. The rate and the extent of the drug release will decide the intensity and duration of the drug action in the system. *In vitro* drug release testing should provide the means to evaluate bioavailability and the information necessary for the development of more efficacious and therapeutically optimal dosage forms.

In vitro hydrolysis studies of polymer drug conjugates in buffer solutions at pH 1.1, 5.5 and 7.4

The hydrolysis of PEG-O-succinylzidovudine and DEX-O-succinylzidovudine adducts were studied in buffer solutions at pH 1.1 (HCl, NaCl and glycine), pH 5.5 (citric acid, Na_2HPO_4) and pH 7.4 (Na_2HPO_4, KH_2PO_4, NaCl). The pro-drugs (100 mg) containing a known quantity of the drugs was taken in 900ml of preheated dissolution media maintained at 37º0±0.1ºC. It was stirred at 100rpm over a period of 12h. Samples of 5ml were withdrawn at time intervals of 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10 and12h. After each sample was withdrawn, an equal quantity of fresh dissolution media was replaced. The absorbance of the samples

withdrawn after suitable dilution, were measured against the blank at λ_{max} of the drug. The amount of the drug released at different time intervals and percentage release was calculated. Each experiment was repeated in triplicate

In vitro hydrolysis of 5'-*O-succinylzidovudine in buffer solutions of pH* 1.1, 5.5 *and* 7.4

The hydrolysis of 5'-O-succinylzidovudine derivative was also carried out at pH 1.1, 5.5 and 7.4. Known quantities of 5'-O-succinylzidovudine were dissolved in equal volumes of preheated buffer solutions at each different pH values. The amount taken was equivalent to the amount of 5'-O-succinyl derivative present in polymeric pro-drugs solutions. The samples were maintained at 37º0±1ºC. Samples of 5ml were withdrawn at time intervals of 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10 and12h. The samples were analyzed and the amount of zidovudine released was estimated. Each experiment was repeated in triplicate.

3.5. Enzymatic hydrolysis studies

The capacity of α-chymotrypsin, a proteolytic pancreatic enzyme, to catalyse the hydrolysis of ester bonds is well known and the possibility that the synthesized pro-drugs (in which zidovudine is linked to the polymeric backbone by ester linkage through succinic spacer) can be a good substrate for this enzyme was therefore evaluated at pH 8.

The hydrolytic stability of drug-polymer linkage of the polymeric conjugates to α-chymotrypsin was assessed in at pH 8 (0.08 M Tris buffer, 0.1 M $CaCl_2$). To 2ml of PEG-O-succinylzidovudine solution, 200µl of α-chymorypsin 10M solution in 0.001 M HCl was added. The solutions were incubated at 37º0±1ºC and the amount of zidovudine released at different time intervals were estimated.

The same procedure was adopted to access the enzymatic hydrolysis of DEX-O-succinylzidovudine.

The hydrolytic stability of 5'-O-succinylzidovudine to α-chymotrypsin was also assessed under the same experimental condition that of the polymer-drug conjugates.

3.6. Plasmatic hydrolysis studies

The hydrolysis of the PEG-O-succinylzidovudine was studied in human plasma at 37º 0±1º C. The reactions were initiated by adding 100ml of aqueous solution of PEG-O-succinylzidovudine (at the concentration of 2g/100ml) to samples (1ml) of preheated plasma. The samples were kept in a water bath at 37º 0±1ºC, under continuous stirring. At suitable intervals, 4 ml of methanol was added in order to deproteinize the plasma. After immediate mixing and centrifugation for 10 min at 10000rpm at 4ºC, 10ml of the clear supernatant was analysed by HPLC to determine the amount of zidovidine released. Every experiment was repeated in triplicate.

The same procedure was adopted to access the plasmatic hydrolysis of DEX-O-succinylzidovudine.

Plasmatic hydrolysis of 5'-O-succinylzidovudine was performed in the same conditions as above. A 5'-O-succinylzidovudine stock solution was prepared in such a way as to have an equal zidovudine succinyl derivative concentration that of the polymeric conjugate solution used for the plasmatic hydrolysis studies. 100ml of this solution was added to samples (1ml) of preheated plasma and samples kept in a water bath at 37º 0±1ºC under continuous stirring. At suitable intervals, 4ml of methanol was added and after mixing and centrifugation for 10min at 10000rpm at 4ºC, 10ml of the clear supernatant was analysed by HPLC to determine the amount of zidovidine released from 5'-O-succinylzidovudine. Every experiment was repeated in triplicate.

3.7. Release kinatics

Whenever a new solid dosage form is developed or produced, it is necessary to ensure that drug dissolution occurs in an appropriate manner. The quantitative analysis of the values obtained in dissolution / release tests is easier when

mathematical formulas that express the dissolution results as a function of some of the dosage forms characteristics are used. Drug dissolution from solid dosage forms has been described by kinetic models in which the dissolved amount of drug (Q) is a function of the test time, t or $Q = f(t)$.

Zero order kinetics

It is used to express drug dissolution from pharmaceutical dosage forms that do not disaggregate and release the drug slowly. The amount of the drug released is independent of the initial concentration of the drug. This relation can be used to describe the drug dissolution of several types of modified release pharmaceutical dosage forms, as in the case of some transdermal systems, as well as matrix tablets with low soluble drugs, coated forms, osmotic systems, etc. The pharmaceutical dosage forms following this profile release the same amount of drug by unit of time and it is the ideal method of drug release in order to achieve a pharmacological prolonged action. The following relation can, in a simple way, express this model;

$$Q_t = Q_o + K_o\, t$$

where Q_t is the amount of drug dissolved in time t, Q_o is the initial amount of drug in the solution (most times, $Q_o = 0$) and K_o is the zero order release constant.

First order kinetics

The pharmaceutical dosage forms following this dissolution profile, such as those containing water-soluble drugs in porous matrices, release the drug in a way that is proportional to the amount of drug remaining in its interior. This model can be expressed by following equation:

$$\ln Q_t = \ln Q_o + K_1 t$$

where, Q_t is the amount of drug released in time t, Q_o is the initial amount of drug in the solution and K_1 is the first order release constant.

3.8. Stability studies

One of the major criteria for rational drug design, evaluation of dosage forms for drugs and their acceptance or rejection is the stability of the active component. Drug stability in pharmaceutical formulations may be detected in some instances by a change in physical appearance, color, odour, taste or texture of the formulation. Chemical changes that may occur which are not self evident may also affect drug stability. Scientific data pertaining to the stability of the formulation leads to the prediction of the expected shelf-life of the product.

Accelerated stability studies for the synthesized PEG and Dextran conjugates were carried out for three months. The pro-drugs containing equivalents of 100 mg of the parent drug (3 batches each) were filled manually in hard gelatin capsules (size 1) and then packed in transparent polyvinyl chloride blisters of 0.25 mm thickness. Three different temperature and humidity conditions, prescribed by the International Conference on Harmonization (ICH) for zone IV, were employed namely,

- 25^{o}C with 60% relative humidity (RH).
- 40^{o}C with 75% relative humidity (RH).
- Room temperature.

Samples were withdrawn at the end of 30, 60 and 90 days and evaluated for their physical parameters and *in vitro* drug release. The drug content in each batch was evaluated

3.9. Bioavailability studies

Bioavailability is defined as the rate and the extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the systemic circulation. Bioavailability studies provide pharmacokinetic information related to absorption, distribution, elimination and dose proportionality of active moieties.

Permission was obtained from the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) / Institutional Animal Ethics Committee (IAEC) to carry out the *in vivo* experiments, (*JSSCP / IAEC / PhD / PH. CHEM / 01 /2006 – 07*).

Ten healthy overnight fasted rabbits (approximately 2kg) were obtained from Central Animal House, J.S.S. College of Pharmacy, Ootacamund, India. Nine rabbits were divided into three groups, each group consisting of three rabbits. The pro-drugs PEG-O-succinylzidovudine and DEX-O-succinylzidovudine and 5'-O-succinylzidovudine were administered to the three groups of animals Standard zidovudine was administered to the 10^{th} animal.

AZT (30 mg/kg body weight) and polymeric prodrug (48 mg/kg equivalent to 10 g/kg of AZT) were suspended in 1 % carboxy methyl cellulose and the homogenous microsuspensions were administered orally using a conventional gastric delivery tube. Each drug solution was prepared immediately prior to administration. Blood sample (1.0 ml) was withdrawn immediately prior to drug administration from the marginal ear vein.

Further (1.0 ml) blood samples were withdrawn at intervals of 0.5, 1, 2, 3, 4, 6, 8, 10, 12, and 24 h period using a sterilized syringe. The blood samples collected in the centrifuge tube containing the anticoagulant O.2ml (3.8% w/v trisodium citrate) were centrifuged at 6000 rpm for 10 min. The plasma were separated and stored in a freezer pending assay. The separated plasma was processed for analysis by HPLC.

The plasma sample (0.25ml) was pipetted into a microcentrifuge tube and was spiked with frusemide internal standard (0.4ml, 50 µg/ml) and shaken for a few minutes. The mixture was then deprotenised by adding zinc sulphate (0.4ml, 10 % w/v). After vortexing for 60 sec, the sample was centrifuged for 10min at 6000 rpm. The supernatant liquid was separated and again centrifuged and 20 µl of the solution was injected onto the HPLC.

3.10. Validation of HPLC method

Validation is a process which involves confirmation or establishment by laboratory studies that a method / procedure / system / analyst can give the required accuracy, precision, sensitivity, ruggedness, etc. In the most basic form, validation of an analytical procedure demonstrates that the procedure developed is suitable for its intended purpose. Validation of the method was carried out after the development of the HPLC method. This section describes the procedure followed for the validation of the methods developed.

Validation parameters tested

- Accuracy
- Precision
- Selectivity / Specificity
- Linearity and range
- Sensitivity
- Robustness / Ruggedness and
- System suitability

Accuracy of the method was determined by relative and absolute recovery experiments.

The relative recovery of the drug was calculated by comparing the concentration obtained from the drug supplemented plasma to the actually added concentration. To 1.0 ml of drug supplemented plasma, 1.0 ml of standard zidovudine solution and 1.0 ml of internal standard solution were added. The preparation of solution was carried out adopting the procedure used for the preparation of the sample solution. The resulting solution was analysed and the response factor was calculated.

The absolute recovery of zidovudine was determined by comparing the response

factor of the drug obtained from the plasma with response factor obtained by the direct injection of zidovudine in mobile phase. The recovery of a drug in an assay is the response factor obtained from an amount of the drug added to and extracted from the biological matrix, compared to the response factor obtained for the true concentration of the pure authentic standard.

The *precision* is a measure of the degree of reproducibility of an analytical method and it describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix.

The intraday precision was evaluated by analysis of plasma samples containing drugs at three different concentrations containing the internal standard using nine replicate determined for three occasions were injected and chromatogram was recorded. The interday precision was similarly evaluated over a two week period and the response factor and wase calculated. From the mean concentration, standard deviation was calculated.

Selectivity is the ability *of* an analytical method to measure accurately and specifically the analyte *of* interest in the presence *of* components that may be expected to be present in the sample matrix. The selectivity was established to differentiate and quantify the analyte in the presence *of* other components in the sample.

Chromatograms of blank plasma samples obtained from different rabbits were compared with the chromatogram obtained from standard solutions. Each chromatogram was tested *for* interferences due to endogenous plasma components on the retention times *of* the selected drugs and internal standards.

Linearity is the ability of an analytical method to elicit test results that are directly proportional to the concentration of the analyte in samples within a given range and the Range is the interval between the upper and lower levels of the analyte that have been demonstrated to be determined with precision, accuracy and

linearity. Linearity and range of the method were analysed by preparing calibration curves using different concentrations of standard solutions containing the internal standard in plasma. The blank sample were prepared by spiking varying concentrations ranging from 12.5 ng/ml-280 ng/ml and it was extracted and analysed by the same procedure which was adopted for the preparation of the plasma sample. The peak area and response factor were calculated. The calibration curve was plotted using response factor and concentration of the standard solutions. Standard curve fitting is determined by applying the simplest model that adequately describes the concentration-response relationship using appropriate weighting and statistical tests for goodness of fit.

Limit of detection (LOD) is the lowest concentration *of* analyte in a sample that can be detected, but *not* necessarily quantitative, under the stated experimental conditions and determined by measuring the signal-to-noise ratio at 3: 1. The signal-to*noise* ratios were performed by comparing measured signals *of* known low concentrations *of* drugs with those *of* blank plasma samples.

Limit of quantification (LOQ) is the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions and determined by measuring the signal-to-noise ratios at 10:1. The signal-to-noise ratios were performed by comparing measured signals of known low concentrations of drugs with those of blank plasma samples.

System suitability of the methods was performed by calculating the chromatographic parameters namely, column efficiency, resolution, peak asymmetry factor and capacity factor on the repetitive injection of standard solutions.

Ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions. The ruggedness of the method was studied by changing the

experimental conditions. Chromatographic parameters such as retention time, asymmetric factor, capacity factor and selectivity factor were evaluated.

Robustness is defined as a measure of its capacity to remain unaffected by small but deliberate variations in method parameters. The robustness of the methods were studied by injecting standard solutions with slight variations in the optimized conditions were made and the standard solution was injected.

The separation factor, retention times and peak asymmetry were then calculated.

Estimation of Zidovudine

A Waters Breeze system was used for the analysis with the following chromatographic conditions.

Stationary phase	: Princeton SPHER® C_{18} (250 cm x 4.6 mm i.d, 5µ)
Mobile phase	: 0.1 % of Orthophosporic acid: Methanol
Solvent ratio	: 50: 50% v/v
pH	: 4.2
Detector	: Waters 2487 (Dual wavelength absorbance detector)
Detection	: 265 urn
Flow rate	: 1.0 mllmin
Sample size	: 50 µl
Needle wash	: Water: ACN HPLC grade
Column temperature	: Room temperature
Internal standard	: Frusemide

With the optimised condition, standard and sample solutions were injected and the chromatograms were recorded. The optimised condition used for estimation

provided a well defined separation between the drug, internal standard and endogenous components. The blank plasma samples showed no interference at retention time of the drugs and their internal standards. The retention time of zidovudine was found to be 5.34 minutes. The response factor (peak area ratio of drug peak area and the internal standard peak area) of the standard solution and the sample were calculated and the concentration *of* the zidovudine present in the plasma samples was calculated.

Pharmacokinetic Parameters

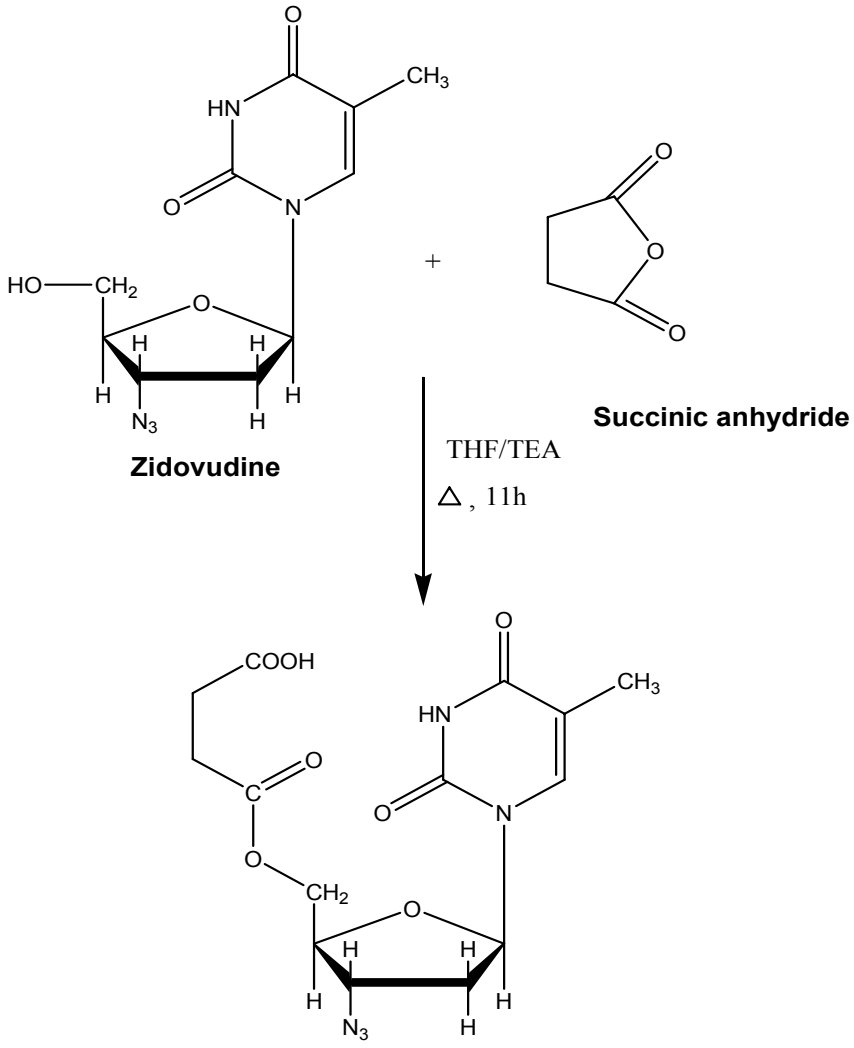
Pharmacokinetic parameters, namely, the peak height concentration, (C_{max}) time of the peak concentration (t_{max}), elimination half-life ($t_{½}$) and elimination rate constant (k_{eli}) were determined. The areas under the plasma concentration time curves (AUC_{0-12}, $AUC_{0-\infty}$) were also calculated.

4. Results and Discussion

4.1. Synthesis and characterization of the pro-drugs

Synthesis of 5'-O-Succinylzidovidine

The 5-O-succinylzidovudine was obtained by the reaction of AZT with succinic anhydride **(Scheme 1)**. The purity of 5'-O-Succinylzidovidine was verified by HPLC analysis. The analytical and spectral data were in agreement with the attributed structure.



5'-O-Succinylzidovudine

Scheme 1

IR spectrum (Figure 4) showed bands at : 3100 cm $^{-l}$ (the -OH stretching of COOH), 2108 cm $^{-1}$) (-N_3), 1733 cm^{-l} (the C=O stretching of ester group), 171 0 cm $^{-1}$ (the C =O stretching of -CO- NH-CO-), 1665 cm $^{-1}$(the C=O stretching of -COOH).

H^1-NMR ($CDCl_3$) (Figure 5) : δ 1.88 [s, 3 H, **CH_3** -C_5], 2.4 [m, 2 H-2'], 2.67 [m, 4H, CO-**CH_2 - CH_2** -CO-], 4.10 [m, 1 H-3'], 4.3 [m, 2H-5' and 1 H -CO-**NH**-CO-, which disappears by adding D_2O], 4.78 [m, I H-4'], 5.93 [t, 1 H-l '], 7.35 [s, 1 H-C_6], 10.33 [s, -COOH which disappears by adding D_2 O].

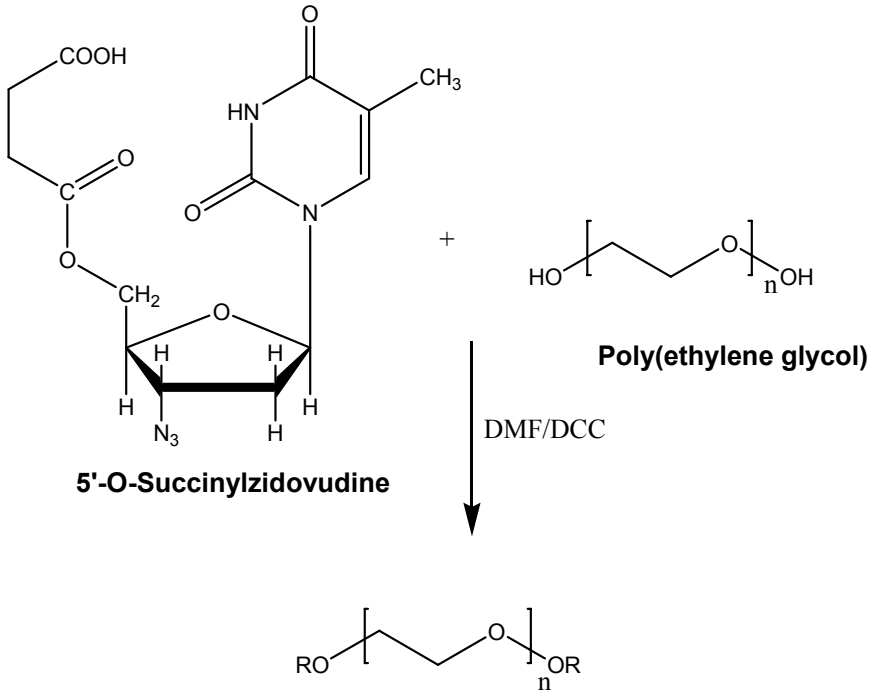
The structure of 5'-O-Succinylzidovidine was also confirmed by Mass spectra (Figure 6) where the N+1 peak was found at 368 m/z.

Synthesis of PEG-O-Succinylzidovudine

The 5-O-succinylzidovudine derivative was coupled to PEG by using *N,N'*-Dicyclohexylcarbodiimide (DCC) **(Scheme 2)**. The absence of both zidovudine and succinylzidovudine in the adduct, purified by dialysis, was confirmed by HPLC analysis. Analytical and spectral data were in agreement with the attributed structure.

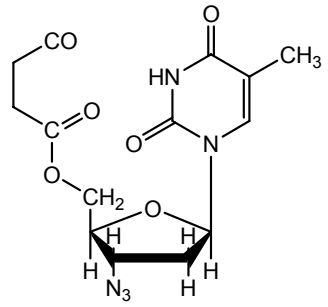
IR spectrum (Figure 7) showed bands at: 2900 cm $^{-l}$ (multiple CH_2), 2098 cm $^{-1}$) (-N_3), 1732 cm^{-l} (the C=O stretching of ester group), 1665 cm $^{-1}$(the C=O stretching of -COOH) and 1116cm^{-1} (CH_2 - O- CH_2)

H^1-NMR (DMSO D_6) (Figure 8) : δ 1.80 [s, 3 H, **CH_3** -C_5], 2.5 [m, 2 H-2'], 2.59 [m, 4H, CO-**CH_2 - CH_2** -CO-], 3.5[m, $(CH_2$ -CH_2 -O$)_n$ 4.24 [m, 1 H-3'], 4.3 [m, 2H-5'], 4.58 [m, I H-4'], 6.1 [t, 1 H-l'], 7.42 [s, 1 H-C_6].



PEG-5'-O-Succinylzidovudine

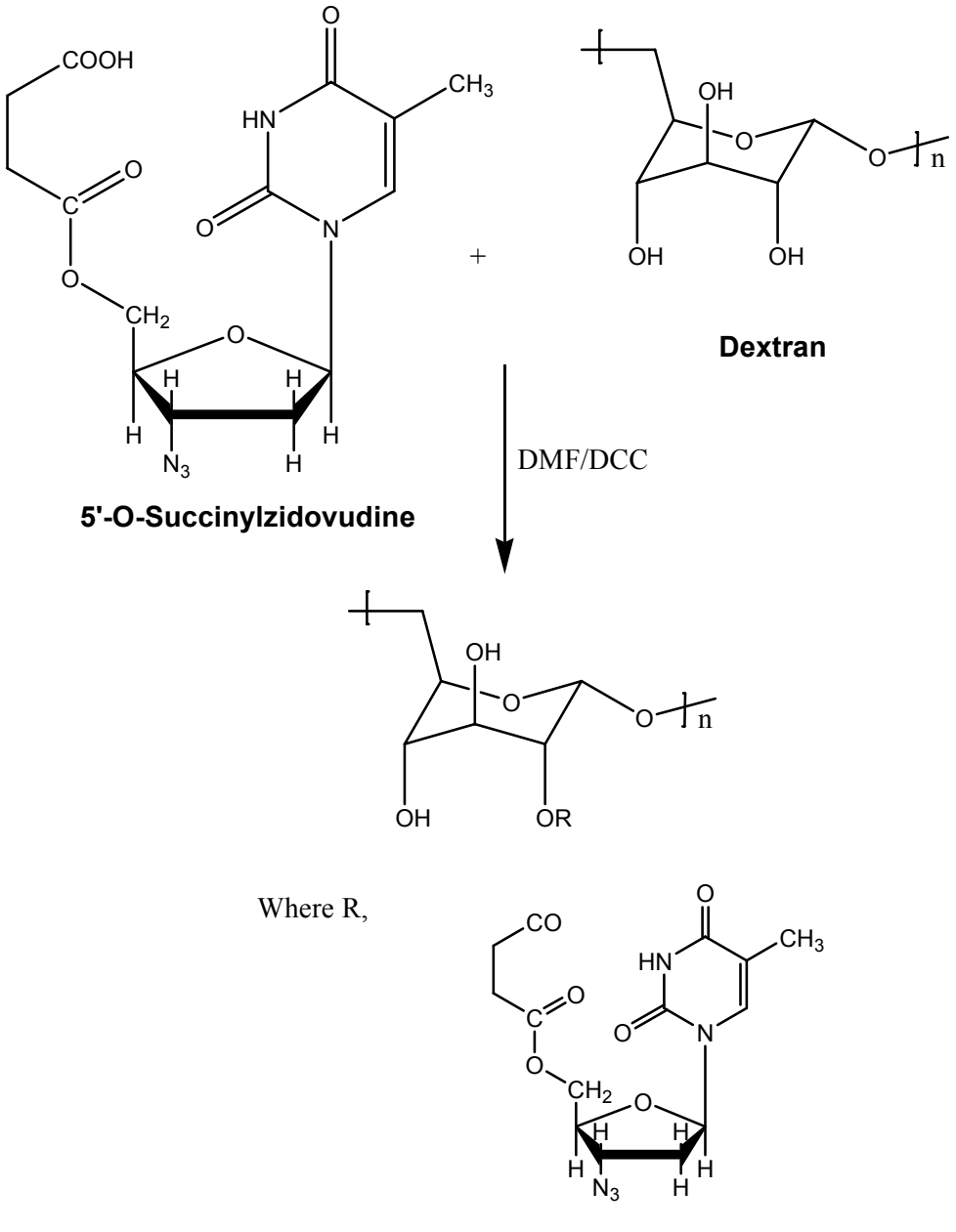
Where R,



Scheme 2

Synthesis of DEX-O-Succinylzidovudine

The 5'-O-succinylzidovudine derivative was coupled to Dextran-40 by using DCC **(Scheme 3)**. The absence of both zidovudine and succinylzidovudine in the adduct, purified by dialysis, was confirmed by HPLC analysis. Analytical and spectral data were in agreement with the attributed structure.



Scheme 3

IR spectrum (Figure 9) showed bands at: 3418 cm $^{-l}$ (the -OH stretching), 2098 cm $^{-1}$) (-N_3), 1720 cm^{-l} (the C=O stretching of ester group),

H^1-NMR (DMSO D_6) (Figure 10): δ 1.79 [s, 3 H, **CH_3** -C_5], 2.4[m, 2 H-2'],2.54 [m, CH_2 of dextran] 2.59 [m, 4H, CO-**CH_2 - CH_2** -CO-], 3.3 [m CH of dextran]3.96 [m, 1 H-3'], 4.3 [m, 2H-5'], 4.58 [m, I H-4'], 6.11 [t, 1 H-I'], 7.46 [s, 1 H-C_6].

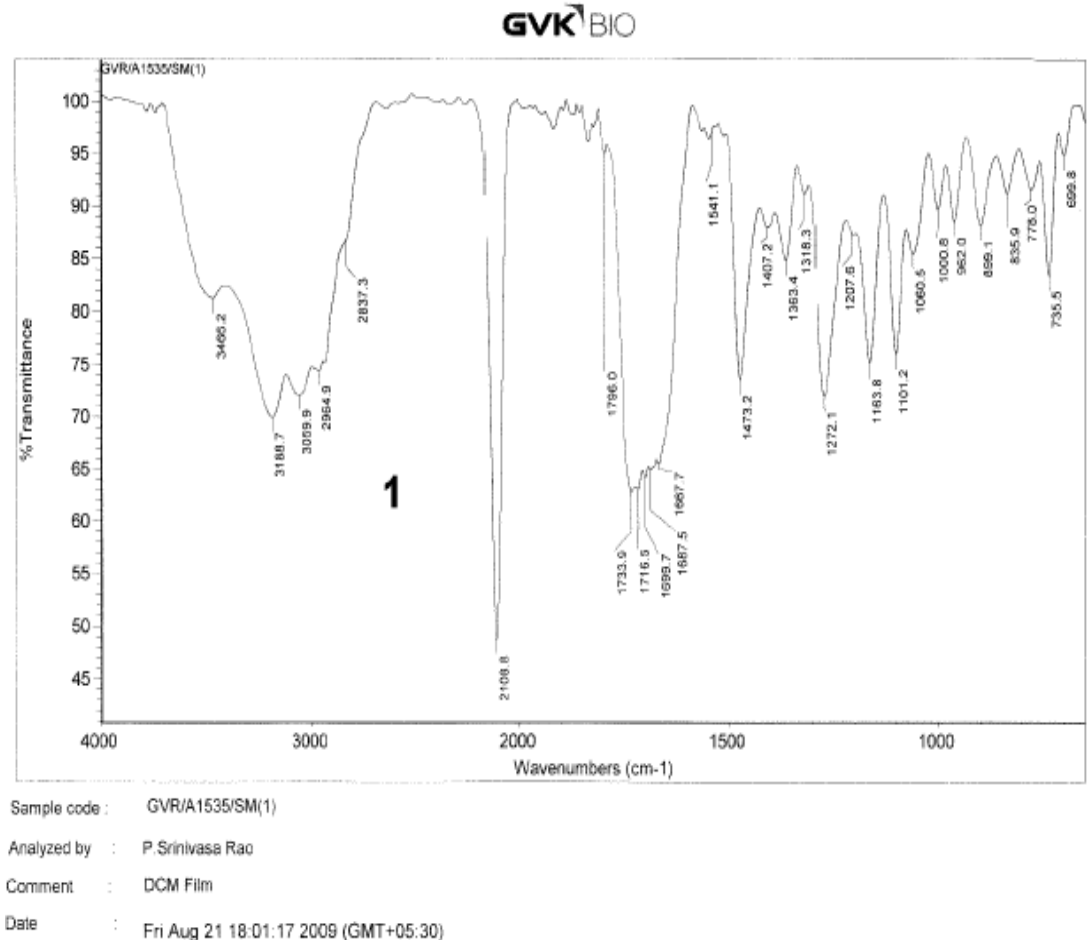
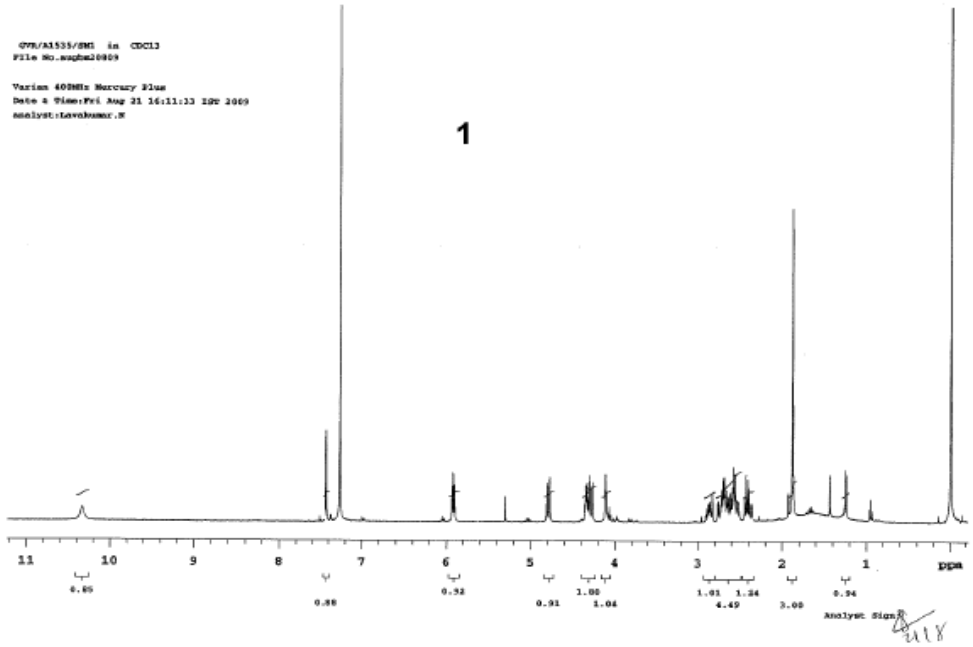


Figure 4 - IR spectra of 5' O-Succinylzidovudine



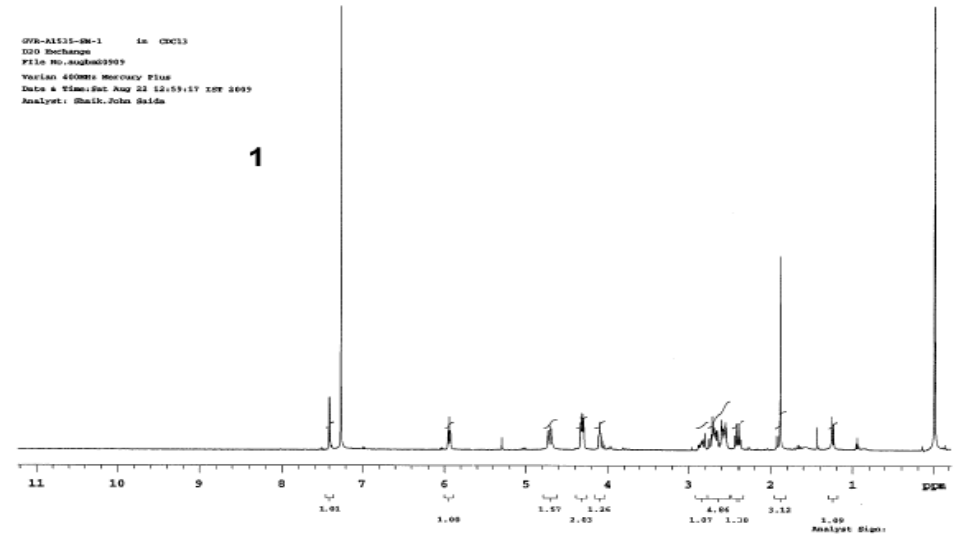


Figure 5- H^1 NMR spectra of 5' O-Succinylzidovudine

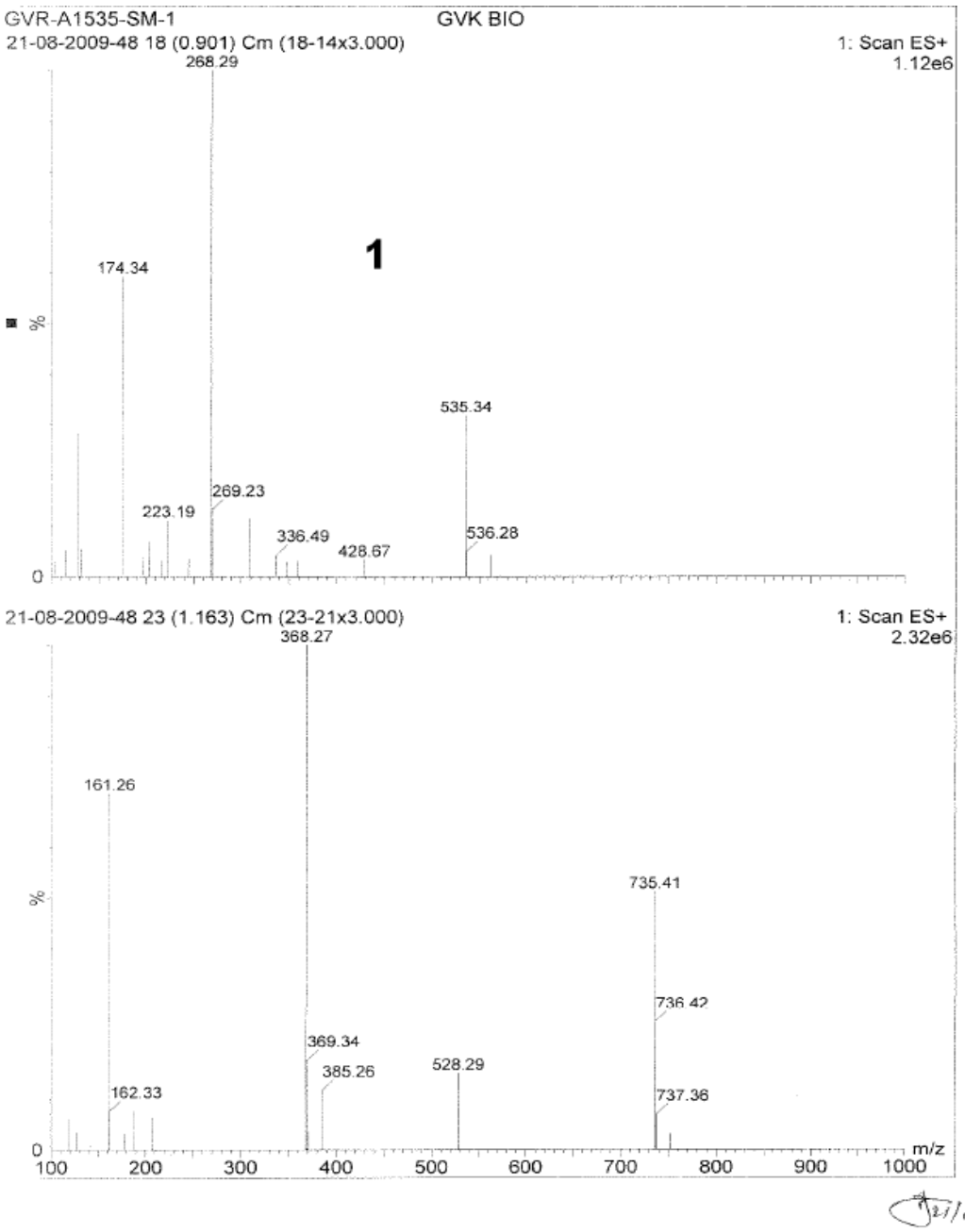


Figure 6 – Mass spectra of 5' O-Succinylzidovudine

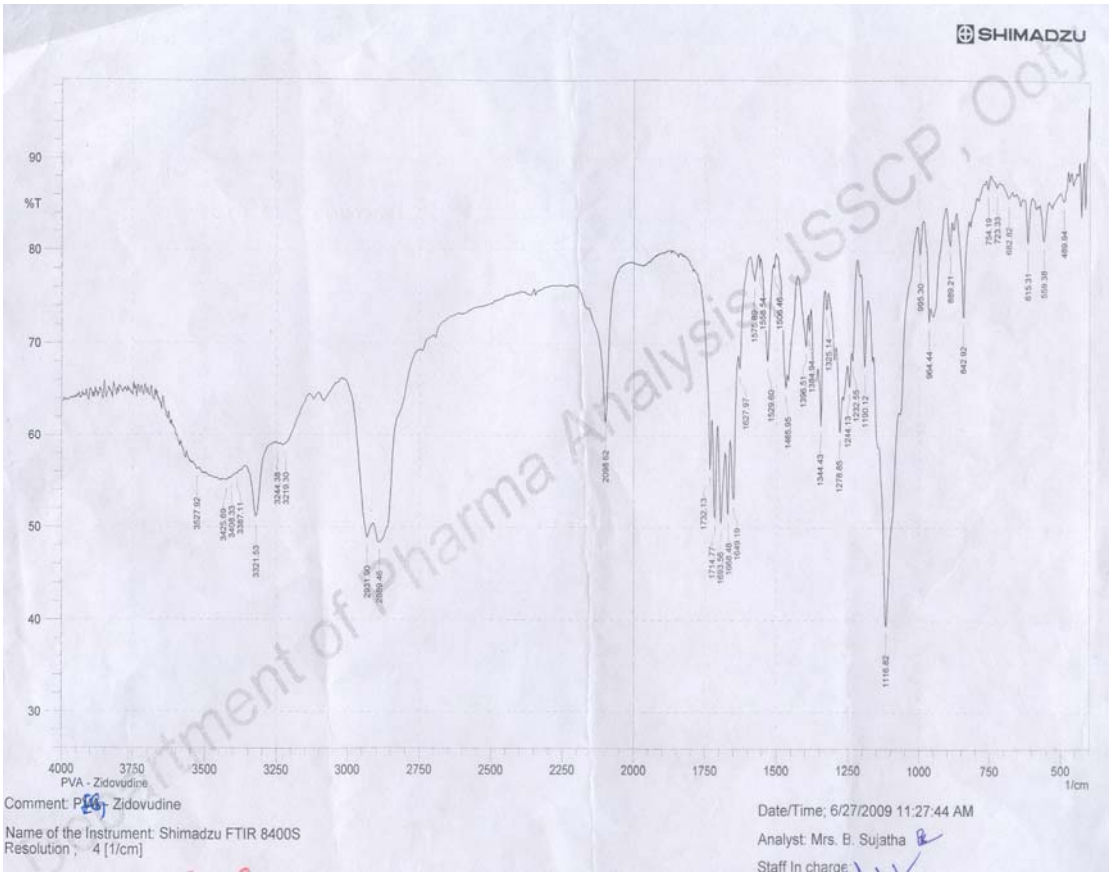


Figure 7 - IR spectra of PEG- O-Succinylzidovudine

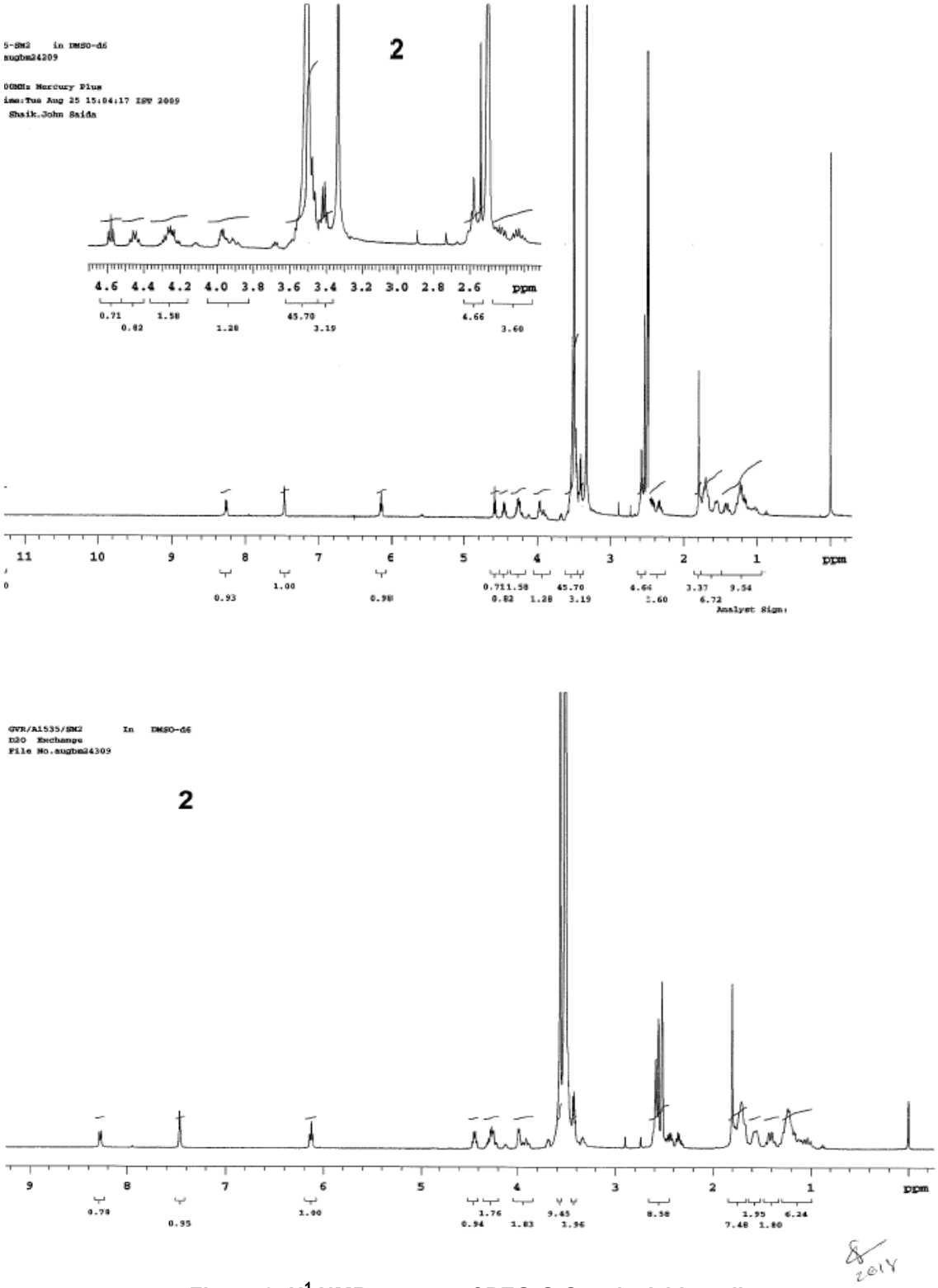


Figure 8- H[1] NMR spectra of PEG-O-Succinylzidovudine

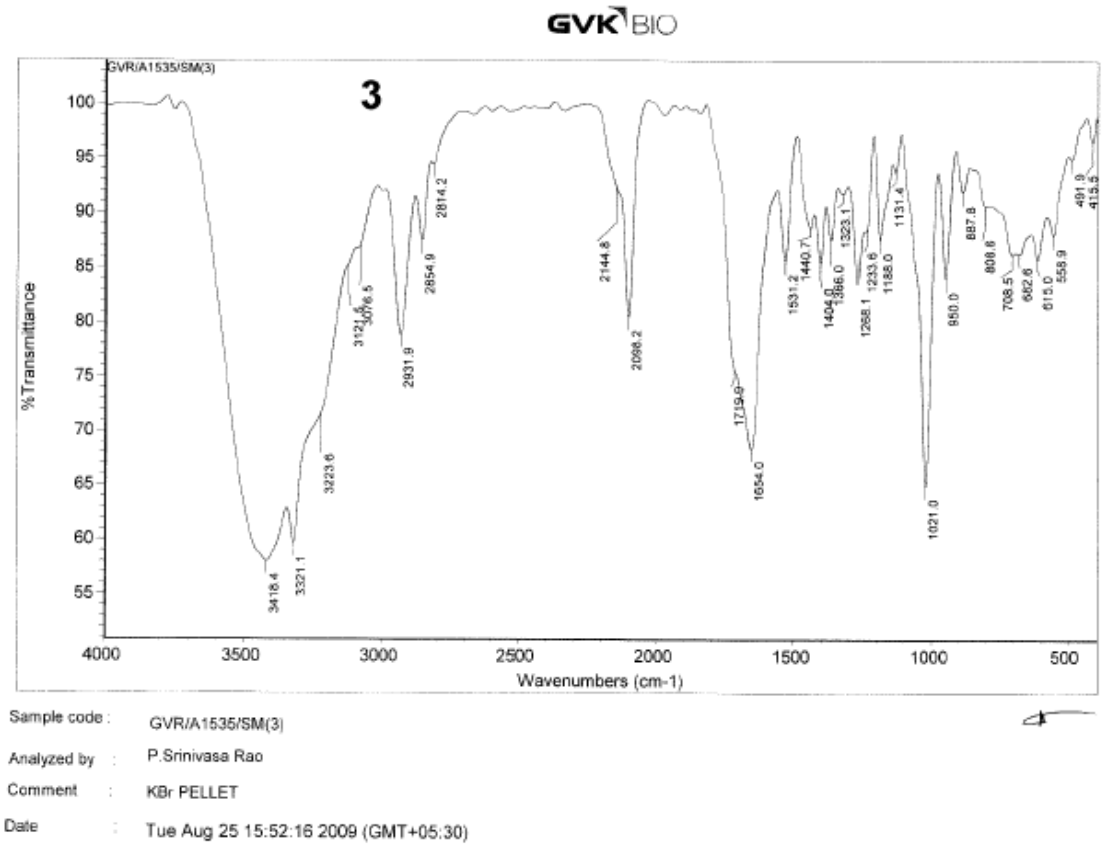


Figure 9 - IR spectra of DEX- O-Succinylzidovudine

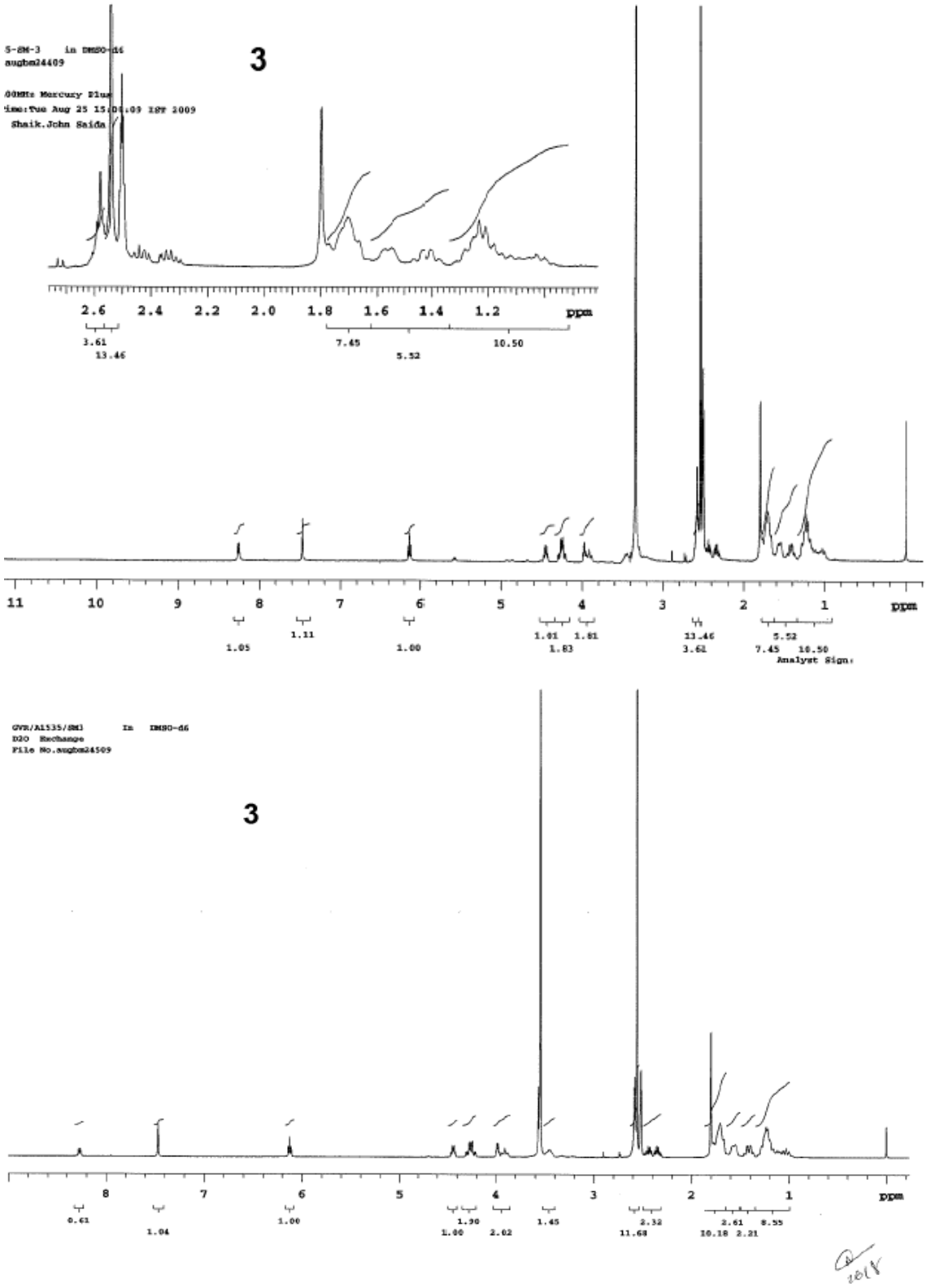


Figure 10- H[1] NMR spectra of DEX-O-Succinylzidovudine

4.2. *In vitro* drug release studies

In order to obtain some preliminary information about the potential use of the PEG-O-succinylzidovudine and DEX-O-succinylzidovudine as drug delivery systems for both oral and systemic administration, *in vitro* hydrolysis studies were performed by subjecting the conjugates to hydrolysis in buffer solutions at pH 1.1 (simulated gastric juice), at pH 5.5 (endosomal compartments) and at pH 7.4 (extracellular fluids) and at pH 8 in the presence and in the absence of α-chymotrypsin.

It was found that at pH 1.1 (Figure 11 and Table 3) both zidovudine and 5'-O-succinylzidovudine were released from the macromolecular prodrugs. The amount of zidovudine and O-succinylzidovudine released after 12 h was 6.26% and about 3.5% respectively from PEG-O-succinylzidovudine. The starting hydrolysis rates of zidovudine and succinylzidovudine from the polymeric conjugate were calculated to be 0.90 %/h and 0.45%/h, respectively. Similarly the amount of zidovudine and O-succinylzidovudine released from DEX-O-succinylzidovudine after 12 h was 1.43% of and about 0.25% with a starting release rate of 0.22%h and 0.05%h respectively.

At pH 5.5 only zidovudine was released from the polymeric prodrugs under the same experimental conditions. After 12 hours about 2.25% of zidovudine was released from PEG-O-succinylzidovudine with a starting release rate of 0.3%/h and only traces of zidovidine was released from DEX-O-succinylzidovudine (Figure 12 and Table 4).

The results of the zidovudine release from PEG-O-succinylzidovudine and DEX-O-succinylzidovudine in buffer solution of pH 7.4 indicated that, both zidovudine and 5'-O- succinylzidovudine were released (Figure 13 and table 5). After 12h about 14.65% of zidovudine with a starting release rate of 3.05%/h and 6.25% of 5'-O-succinylzidovudine with a starting release rate of 0.95%/h was released respectively. It was found that during the same period, about 5.22% of zidovidine and 3.25% of 5'-O- succinylzidovudine were released from

DEX-O-succinylzidovudine with a starting release rate of 0.86%/h 0.40%/h respectively.

The *in vitro* hydrolysis of 5'-O- succinylzidovudine was also carried at pH 1.1, 5.5 and 7.4 under the same experimental conditions used for the polymeric conjugate. It was found that the percentage of zidovudine release at pH 1.1, 5.5 and 7.4 were 4.25%, 6.80% and 3.25% respectively over a period of 12h (Figure 14 and Table 6).

It was found that, at all pH values considered, free zidovudine was always released from 5'-O-succinylzidovudine derivative. Even if 5'-O-succinylzidovudine was released from the macromolecular pro-drugs, it can in turn be hydrolysed to give free and active zidivudine. It was also found that the release rate of zidovudine from 5'-O-succinylzidovudine derivative decreased as the pH value increased. A different trend was noticed in the case of release of zidovudine from the polymer drug conjugates. The release of drug was minimum at pH 5.5 buffer solution. This drug release can be attributed to influence of pH value on the hydrolysis rate of low molecular weight ester compounds [157].

4.3. Enzymatic hydrolysis studies

At pH8 approximately 45.25% of zidovidine with a starting release rate of 10.56%h was released from PEG-O-succinylzidovudine within 12h in the presence of α-chymotrypsin (Figure 15 and Table 7), against less than 20% at the same pH value without the enzyme (Figure 16 Table 8). The amount of 5'-O-succinylzidovudine released in the presence of the enzyme was the same as that released in the absence of the enzyme.

The amount of zidovidine released from DEX-O succinylzidovudine was 27.35% within 12h in the presence of α-chymotrypsin (Figure 15 andTable 7) as against 11.20% in the absence of the enzyme (Figure and 16 Table 8). The amount of 5'-O-succinylzidovudine released in the presence of the enzyme was the same as that released in the absence of the enzyme. From the hydrolysis profiles a

starting rate of 4.86%/h was obtained for the release of AZT in the presence of α-chymotrypsin, against a value of 1.18%/h under the same conditions without the enzyme

These results confirm the capacity of macromolecular conjugates to release free drug by hydrolytic activity of α-chymotrypsin. The ester bond between zidovudine and the succinic spacer would seem more susceptible to this enzymatic hydrolysis than the ester bond between 5'-O-succinylzidovudine and the polymeric carrier.

The results of the hydrolysis of 5'-O-succinylzidovudine in the presence and in the absence of α-chymotrypsin (Figure 17 and Table 9). showed that about 16% of zidovudine with a starting release rate of 3.46%/h was released from the succinylderivative within 12h in the presence of α- chymotrypsin, whereas about 11% was detected under the same conditions in the absence of the enzyme with release rate of 2.12%/h.

These results suggest that α-chymotrypsin exerted its hydrolytic activity also towards the low molecular weight derivative of zidovudine. Besides the comparison of results obtained by subjecting to enzymatic hydrolysis the polymeric conjugate of zidovudine and low molecular weight succinyl derivative (under the same experimental conditions), it would seem that the presence of the macromolecular chain could offer a better interaction substrate-enzyme and thus improve the enzyme's catalytic activity.

Table3 -*In vitro* release of zidovudine and 5'-O-succinylzidovudine from the polymeric pro-drugs at pH 1.1

Time	PEG-O-succinylzidovudine		DEX-O-succinylzidovudine	
	zidovudine	5'-O-succinylzidovudine	zidovudine	5'-O-succinylzidovudine
0.5	0.40	0.20	0.09	0.02
1	0.90	0.45	0.22	0.05
2	1.20	0.70	0.29	0.07
3	1.80	1.20	0.41	0.10
4	2.22	1.40	0.49	0.12
6	2.90	1.90	0.59	0.14
8	3.87	2.50	0.92	0.17
10	4.90	3.10	1.18	0.19
12	6.26	3.50	1.43	0.25

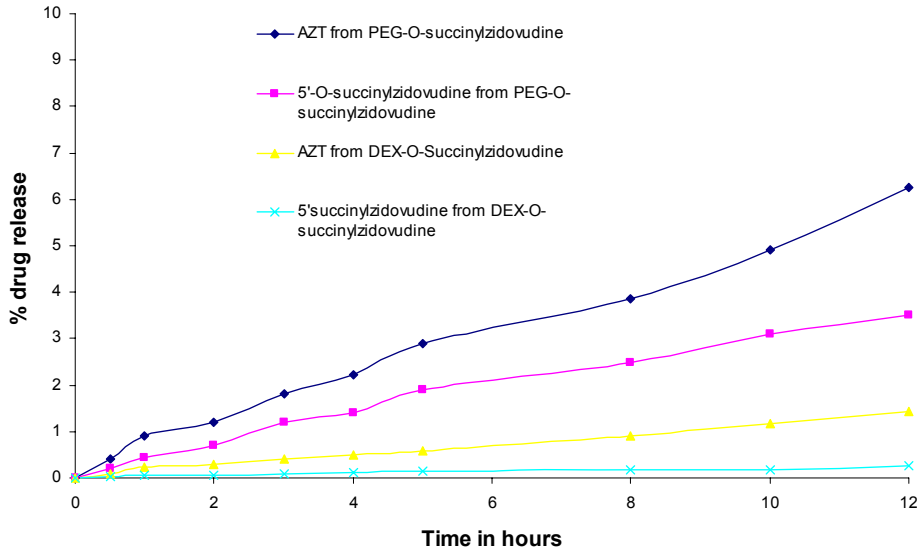


Figure 11 - *In vitro* release of zidovudine and 5'-O-succinylzidovudine from the polymeric pro-drugs at pH 1.1

Table 4 -*In vitro* release of zidovudine from PEG-O-succinylzidovudine in pH 5.5

Time	PEG-O-succinylzidovudine		DEX-O-succinylzidovudine	
	zidovudine	5'-O-succinylzidovudine	zidovudine	5'-O-succinylzidovudine
0.5	0.19	---	---	---
1	0.3	---	---	---
2	0.42	---	---	---
3	0.55	---	---	---
4	0.69	---	---	---
6	1.15	---	---	---
8	1.48	---	---	---
10	1.85	---		---
12	2.25	---	---	---

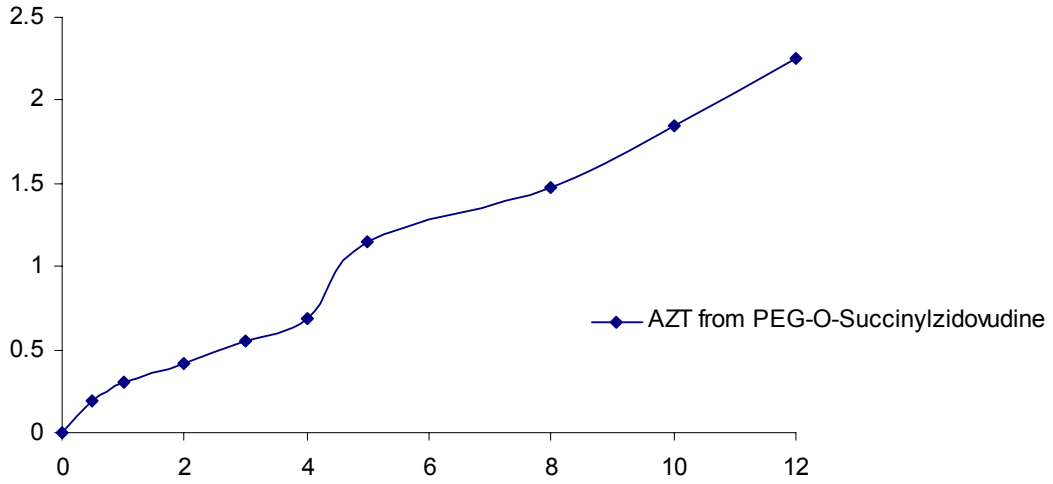


Figure 12 -*In vitro* release of zidovudine from PEG-O-succinylzidovudine in pH 5.5

Table5 - *In vitro* release of zidovudine and 5'-O-succinylzidovudine from the polymeric pro-drugs at pH 7.4

Time	PEG-O-succinylzidovudine		DEX-O-succinylzidovudine	
	zidovudine	5'-O-succinylzidovudine	zidovudine	5'-O-succinylzidovudine
0.5	2.56	0.5	0.42	0.23
1	3.05	0.95	0.86	0.4
2	3.8	1.25	1.14	0.54
3	4.75	1.51	1.35	0.65
4	5.85	1.86	1.78	0.84
6	7.13	2.37	2.20	1.13
8	10.58	4.12	3.82	1.86
10	12.84	5.16	4.56	2.44
12	14.45	6.25	5.22	3.25

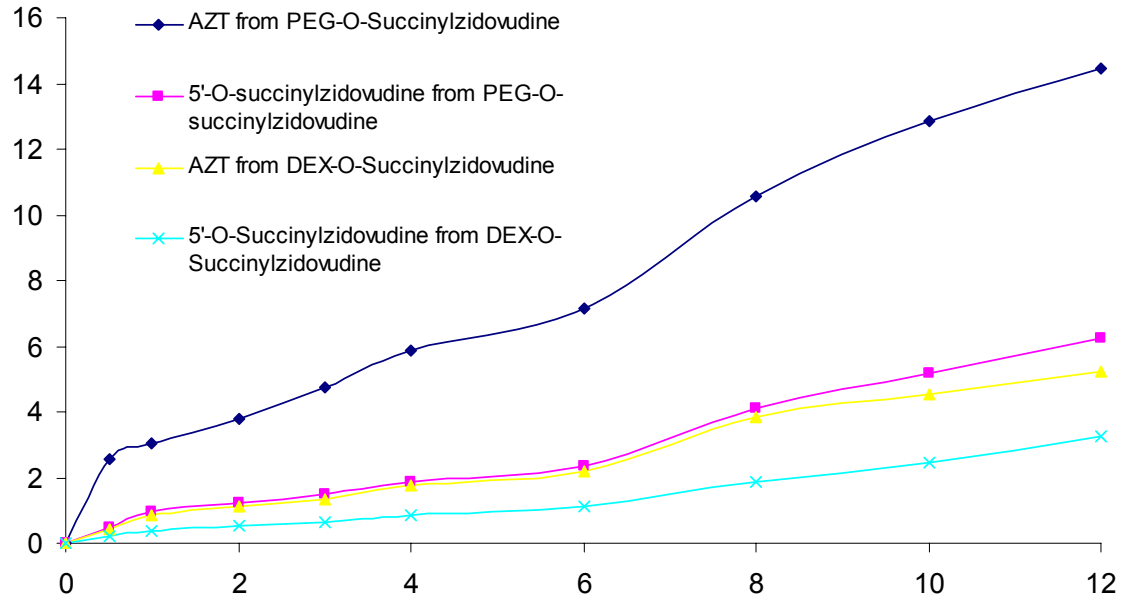


Figure 13 - *In vitro* release of zidovudine and 5'-O-succinylzidovudine from PEG-O-succinylzidovudine and DEX-O-succinylzidovudine in pH 7.4

Table 6 -*In vitro* release of zidovudine from 5'-O-succinylzidovudine at pH 1.1, 5.5 7.4.

Time	At pH 1.1	At pH 5.4	At pH 7.4
0.5	0.62	0.54	0.56
1	1.3	0.84	0.6
2	1.48	0.96	0.66
3	1.66	1.08	0.77
4	1.86	1.25	0.81
6	2.4	1.5	0.96
8	3.14	1.85	1.02
10	3.56	2.05	1.22
12	3.88	2.22	1.25

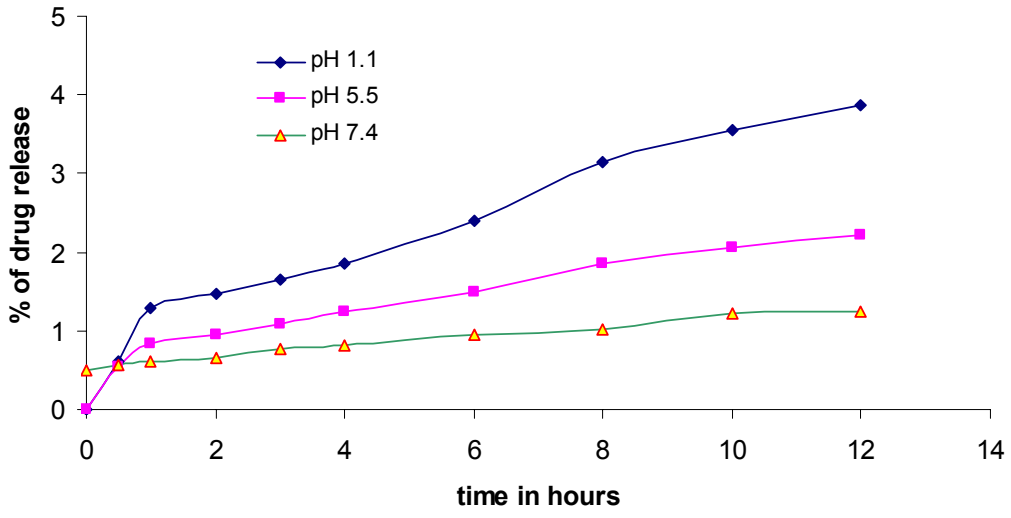


Figure 14 -*In vitro* release of zidovudine from 5'-O-succinylzidovudine at pH 1.1, 5.5 7.4.

Table7- *In vitro* release of zidovudine and 5'-O-succinylzidovudine from the pro-drugs at pH 8.0 in the presence of α- chymotripsin

Time	PEG-O-succinylzidovudine		DEX-O-succinylzidovudine	
	zidovudine	5'-O-succinylzidovudine	zidovudine	5'-O-succinylzidovudine
0.5	4.22	0.04	1.82	0.15
1	10.56	1.06	4.86	0.51
2	12.28	1.58	5.88	0.72
3	16.43	2.36	7.96	1.16
4	19.82	3.12	9.55	1.51
6	24.78	3.76	12.1	1.82
8	32.75	4.64	12.9	2.4
10	39.49	5.48	20.46	2.65
12	45.25	6.25	27.35	3.08

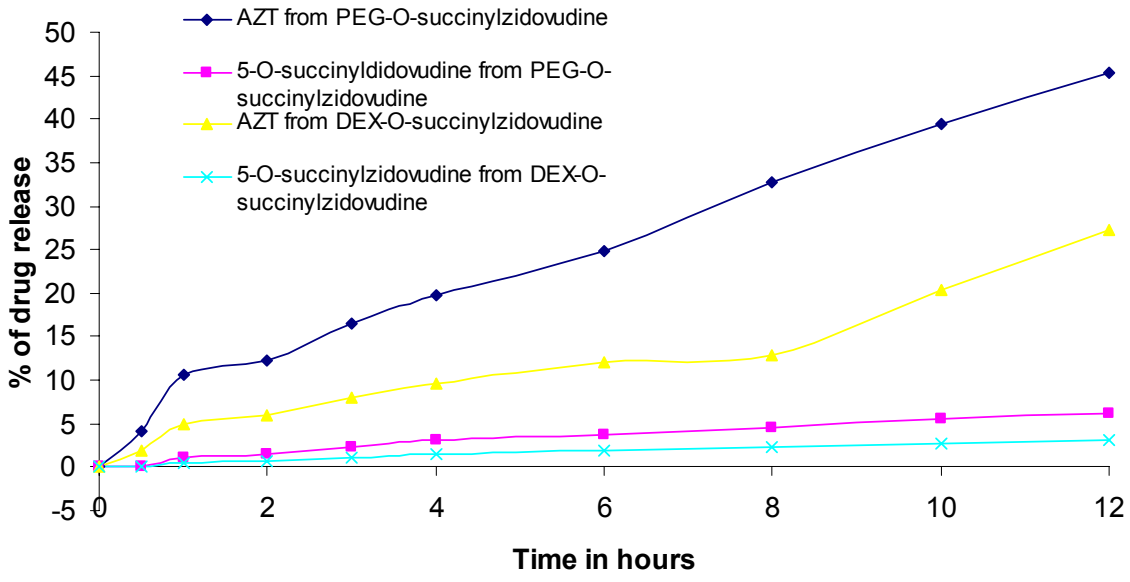


Figure 15 -*In vitro* release of zidovudine and 5'-O-succinylzidovudine from pro-drugs at pH 8.0 in the presence of α- chymotripsin

Table 8 -*In vitro* release of zidovudine and 5'-O-succinylzidovudine from the pro-drugs at pH 8.0 in the absence of enzyme

Time	PEG-O-succinylzidovudine		DEX-O-succinylzidovudine	
	zidovudine	5'-O-succinylzidovudine	zidovudine	5'-O-succinylzidovudine
0.5	0.22	0.05	0.14	0.02
1	1.98	1.05	1.18	0.52
2	3.85	1.62	1.65	0.71
3	6.45	2.40	3.82	1.12
4	9.52	3.16	5.68	1.49
6	10.28	3.72	7.96	1.82
8	13.68	4.66	9.2	2.35
10	15.48	5.5	10.10	2.68
12	19.45	6.30	11.20	3.10

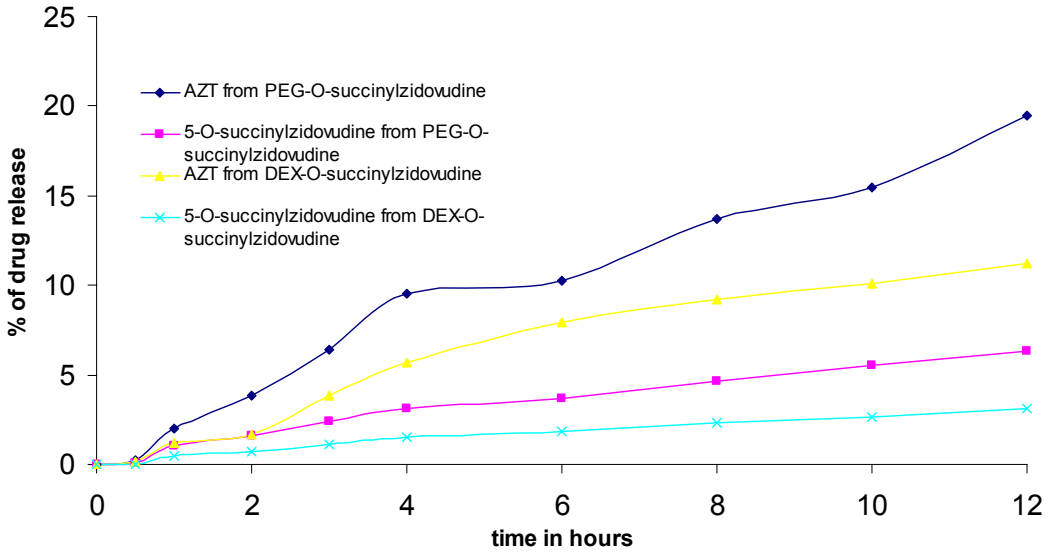


Figure 16 -*In vitro* release of zidovudine and 5'-O-succinylzidovudine from the pro-drugs at pH 8.0

Table 9- *In vitro* release of zidovudine from 5'-O-succinylzidovudine in pH 8.0 in the presence and absence of α- chymotripsin

Time	Zidovudine (in presence of enzyme)	Zidovudine (in absence of enzyme)
0.5	1.89	1.26
1	3.46	2.12
2	4.86	3.14
3	5.98	3.96
4	7.24	4.86
6	9.62	6.52
8	11.46	7.78
10	13.48	9.34
12	15.94	10.82

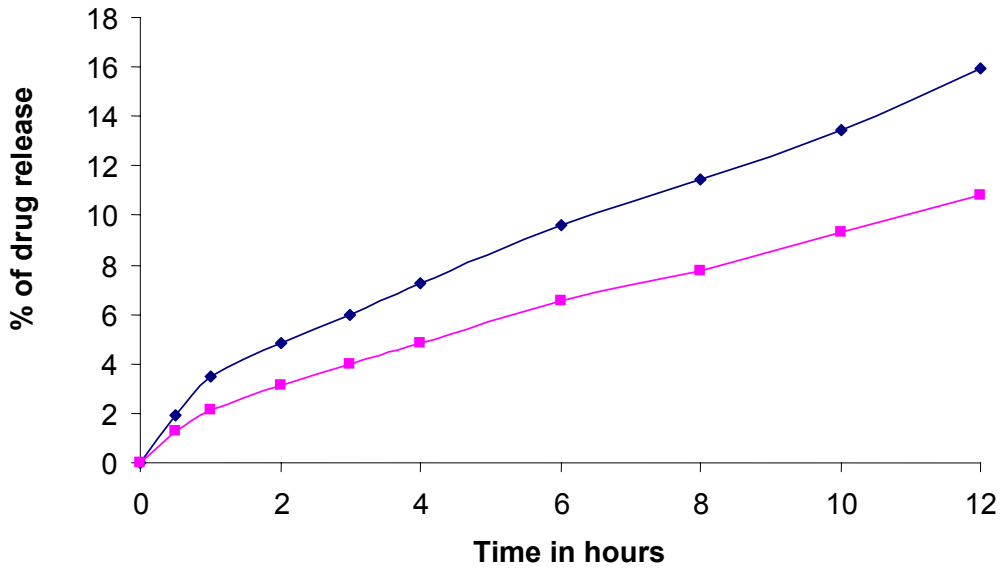


Figure 17- *In vitro* release of zidovudine (-) from 5'-O-succinylzidovudine (■) in pH 8.0 in the presence and absence of α- chymotripsin

4.4. Plasmatic hydrolysis studies

The ability of the synthesized macromolecular prodrugs to release free drug was also evaluated in plasma. It was found that nearly 60% of linked zidovidine was released from PEG-O-succinylzidovudine within 12h (Figure 18 and Table 10) and within the same time about 14% of succinylderivative was also released. Nearly 25.22% of zidovidine was released from DEX-O-succinylzidovudine. It was found that there was a burst release of 8.8% of zidovidine from PEG-O-succinylzidovudine and 4.27% from DEX-O-succinylzidovudine, which were greater than the amount zidivudine released considering all the *in vitro* hydrolysis experiments. This indicates the possibility of this macromolecular conjugates constituting a good substrate for the plasma enzymatic complex and its capacity to deliver free and active zidovudine in a prolonged manner.

The results of plasmatic hydrolysis of 5'-O-succinylzidovudine showed that about 30% of the drug derivative was hydrolysed to give free zidovudine within 12h with a burst release rate of 3.96%, indicating that the amount of 5'-O-succinylzidovudine released from the polymeric pro-drug can in turn be hydrolysed to give free zidovudine (Figure 19).

Table10- Release of zidovudine and 5'-O-succinylzidovudine from the pro-drugs in human plasma.

Time	PEG-O-succinylzidovudine		DEX-O-succinylzidovudine	
	zidovudine	5'-O-succinylzidovudine	zidovudine	5'-O-succinylzidovudine
0.5	3.89	0.48	1.89	0.12
1	8.80	0.78	4.27	0.20
2	10.20	1.54	6.95	0.41
3	10.65	2.76	8.42	0.63
4	11.20	3.92	9.36	0.98
6	21.65	5.28	15.48	1.24
8	25.34	8.76	17.60	2.36
10	40.15	11.4	21.35	3.52
12	59.30	13.64	25.22	4.89

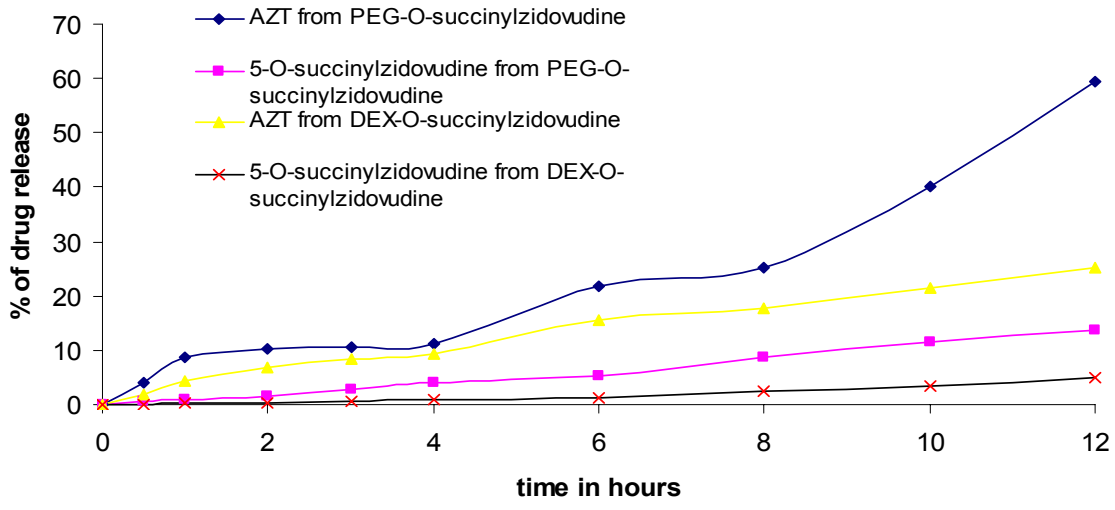


Figure 18 -Release of zidovudine and 5'-O-succinylzidovudine from the pro-drugs in human plasma .

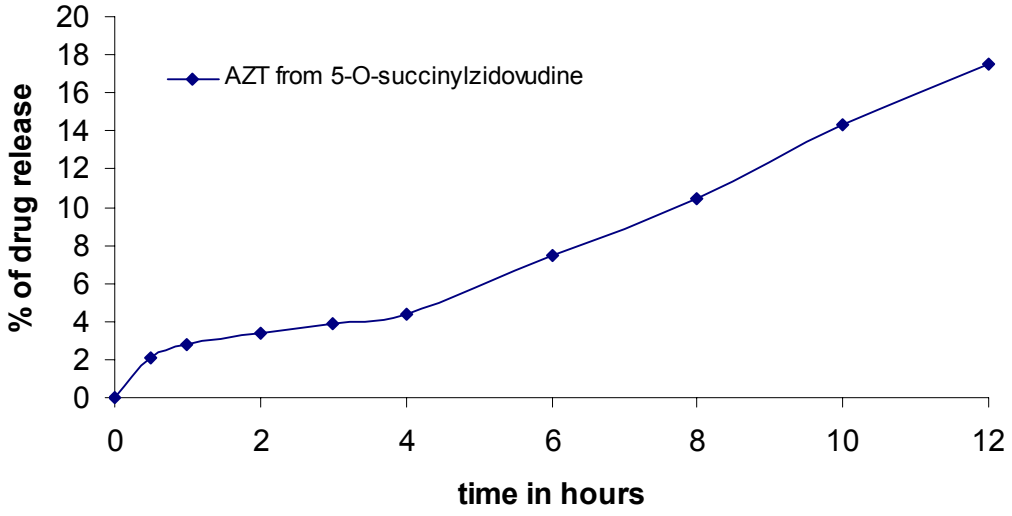


Figure 19 - Release of zidovudine from 5'-O-succinylzidovudine in human plasma

4.5. Release kinatices

The release of Zidovudine and 5'-O-succinylzidovudine from PEG-O-succinylzidovudine and DEX-O-succinylzidovudine was found to be pH dependent. With an increase in pH there was an increase in release of Zidovudine and 5'-O-succinylzidovudine except for the release of 5'-O-succinylzidovudine from DEX-O-succinylzidovudine at pH 7.4 and 8 which was almost comparable. Significant increase in release of zidovudine from PEG-O-succinylzidovudine and DEX-O-succinylzidovudine was found when *in vitro* release was carried out in the presence of α-chymotripsin at pH 8 however there was no effect on the release of 5'-O-succinylzidovudine. Release of Zidovudine and 5'-O-succinylzidovudine from PEG-O-succinylzidovudine follows first order kinetics at different pH. Howerver the release of zidovudine and 5'-O-succinylzidovudine from DEX-O-succinylzidovudine followes zero order kinatics (Tables 11-15 and Figures 20-37).

Time	PEG-O-succinylzidovudine						DEX-O-succinylzidovudine					
	AZT	% drug remaining	Log % drug remaining	5'-O-succinyl-AZT	% drug remaining	Log % drug remaining	AZT	% drug remaining	Log % drug remaining	5'-O-succinyl AZT	% drug remaining	Log % drug remaining
0.5	0.40	99.6	1.998	0.20	99.8	1.999	0.09	99.91	1.999	0.02	99.98	1.999
1	0.90	99.1	1.996	0.45	99.55	1.998	0.22	99.78	1.999	0.05	99.95	1.999
2	1.20	98.8	1.994	0.70	99.3	1.996	0.29	99.71	1.998	0.07	99.93	1.999
3	1.80	98.2	1.992	1.20	98.8	1.994	0.41	99.59	1.998	0.10	99.9	1.999
4	2.22	97.78	1.990	1.40	98.6	1.993	0.49	99.51	1.997	0.12	99.88	1.999
6	2.90	97.1	1.987	1.90	98.1	1.991	0.59	99.41	1.997	0.14	99.86	1.999
8	3.87	96.13	1.982	2.50	97.5	1.989	0.92	99.08	1.995	0.17	99.83	1.999
10	4.90	95.1	1.978	3.10	96.9	1.986	1.18	98.82	1.994	0.19	99.81	1.999
12	6.26	93.74	1.971	3.50	96.5	1.984	1.43	98.57	1.993	0.25	99.75	1.998

	R^2 Value			R^2 Value	
	PEG-O-succinylzidovudine			DEX-O-succinylzidovudine	
	zidovudine	5'-O-succinylzidovudine		zidovudine	5'-O-succinylzidovudine
Zero order	**0.9923**	**0.9989**	**Zero order**	**0.9858**	**0.9679**
First order	**0.9956**	**0.9869**	**First order**	**0.9761**	**0.392**

Table11- *In vitro* release of zidovudine and 5'-O-succinylzidovudine from the pro-drugs at pH 1.1

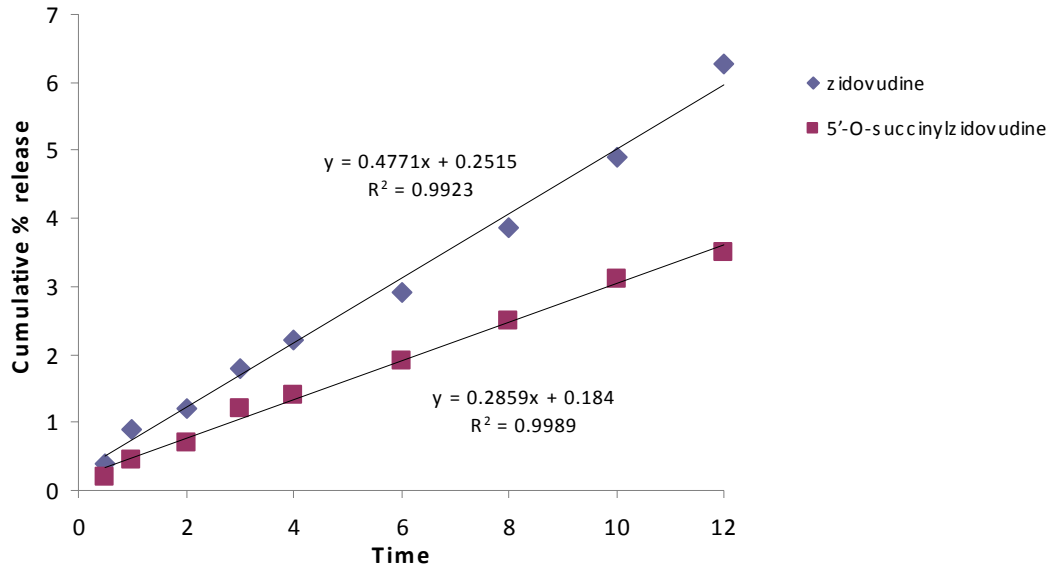


Figure 20- Zero order release of Zidovudine and 5'-O-Succinylzidovudine from PEG-O-Succinylzidovudine at pH 1.1

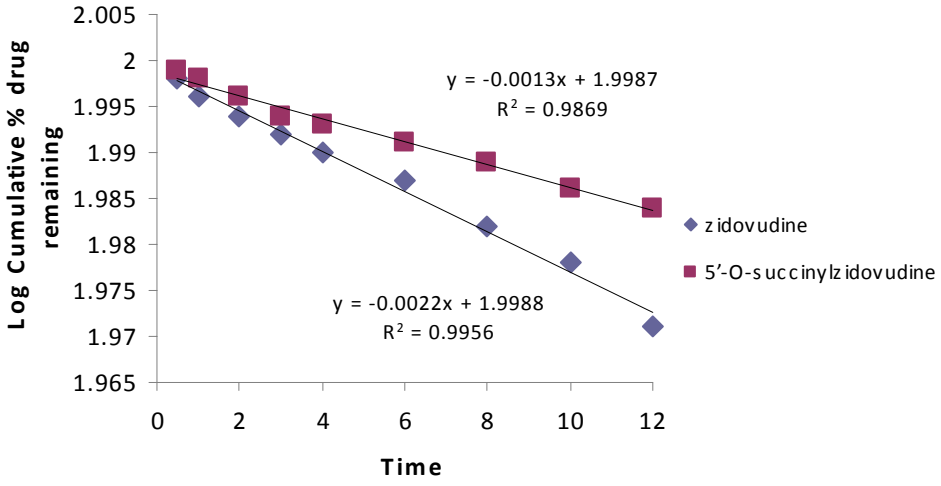


Figure 21- First order release of Zidovudine and 5'-O-Succinylzidovudine from PEG-O-Succinylzidovudine at pH 1.1

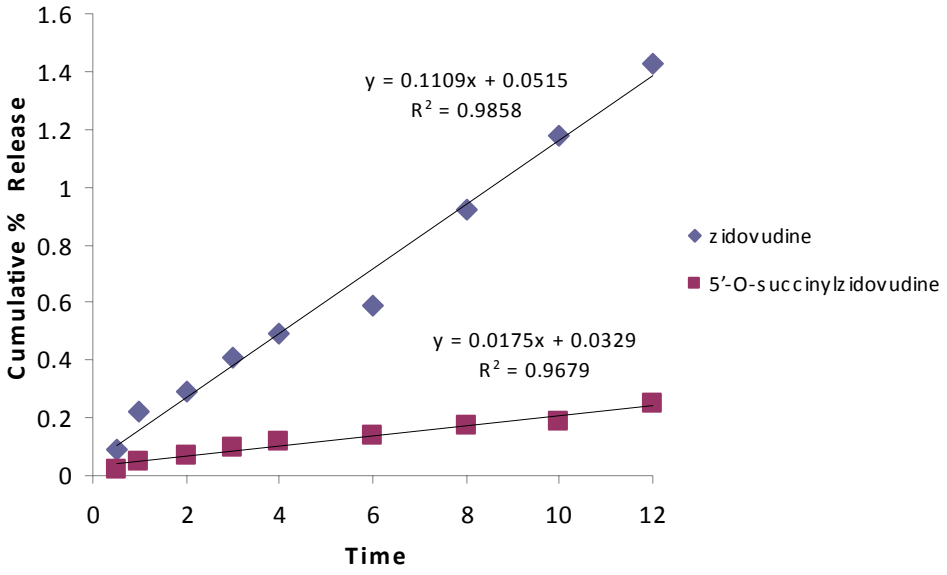


Figure 22- Zero order release of Zidovudine and 5'-O-Succinylzidovudine from DEX-O-Succinylzidovudine at pH 1.1

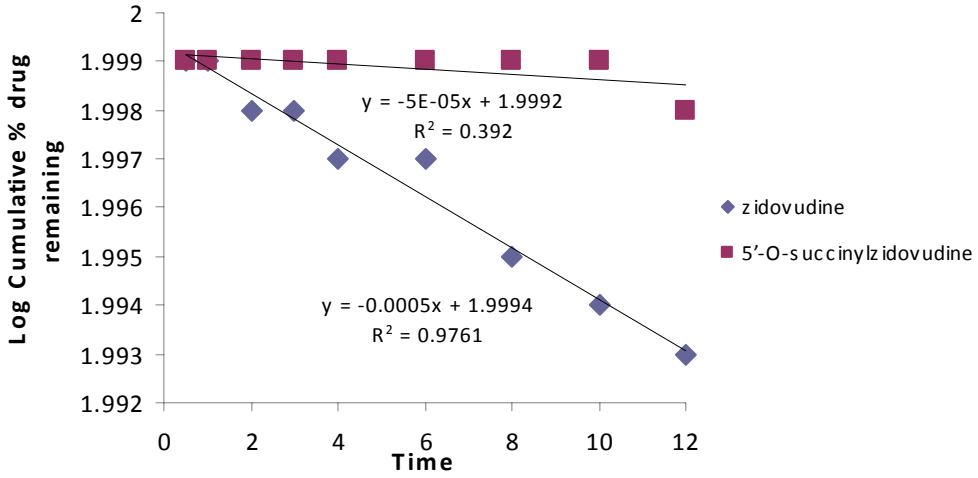


Figure**23 - First** order release of Zidovudine and 5'-O-Succinylzidovudine from DEX-O-Succinylzidovudine at pH 1.1

Time	PEG-O-succinylzidovudine						DEX-O-succinylzidovudine					
	AZT	% drug remaining	Log % drug remaining	5'-O-succinyl-AZT	% drug remaining	Log % drug remaining	AZT	% drug remaining	Log % drug remaining	5'-O-succinyl-AZT	% drug remaining	Log % drug remaining
0.5	2.56	97.44	1.988	0.5	99.5	1.997	0.42	99.58	1.998	0.23	99.77	1.998
1	3.05	96.95	1.986	0.95	99.05	1.995	0.86	99.14	1.996	0.4	99.6	1.998
2	3.8	96.2	1.983	1.25	98.75	1.994	1.14	98.86	1.995	0.54	99.46	1.997
3	4.75	95.25	1.978	1.51	98.49	1.993	1.35	98.65	1.994	0.65	99.35	1.997
4	5.85	94.15	1.973	1.86	98.14	1.991	1.78	98.22	1.992	0.84	99.16	1.996
6	7.13	92.87	1.967	2.37	97.63	1.989	2.20	97.80	1.990	1.13	98.87	1.995
8	10.58	89.42	1.951	4.12	95.88	1.981	3.82	96.18	1.983	1.86	98.14	1.991
10	12.84	87.16	1.940	5.16	94.84	1.976	4.56	95.44	1.979	2.44	97.56	1.989
12	14.45	85.55	1.932	6.25	93.75	1.971	5.22	94.78	1.976	3.25	96.75	1.985

	R^2 Value			R^2 Value	
	PEG-O-succinylzidovudine			DEX-O-succinylzidovudine	
	zidovudine	5'-O-succinylzidovudine		zidovudine	5'-O-succinylzidovudine
Zero order	0.9902	0.9765	Zero order	0.9817	0.9669
First order	0.9906	0.9771	First order	0.9813	0.9486

Table 12- *In vitro* release of zidovudine and 5'-O-succinylzidovudine from the pro-drugs at pH7.4

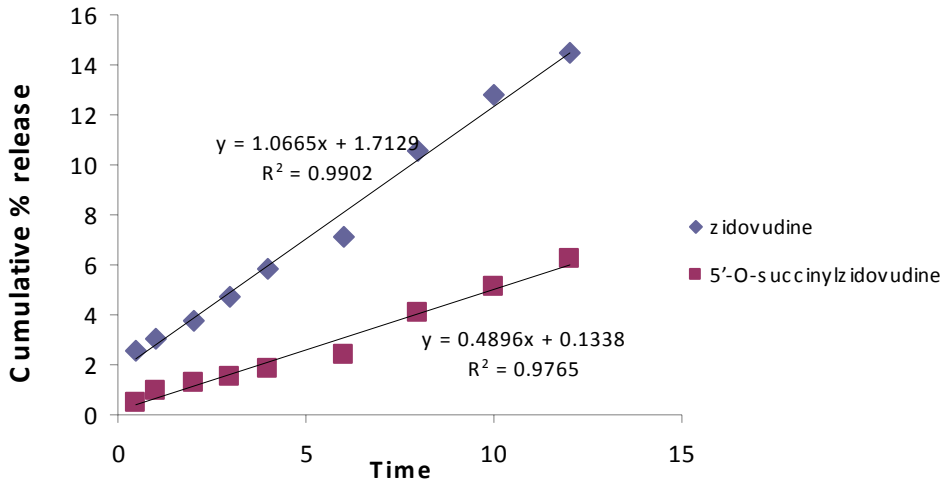


Figure 24- Zero order release of Zidovudine and 5'-O-Succinylzidovudine from PEG-O-Succinylzidovudine at pH 7.4

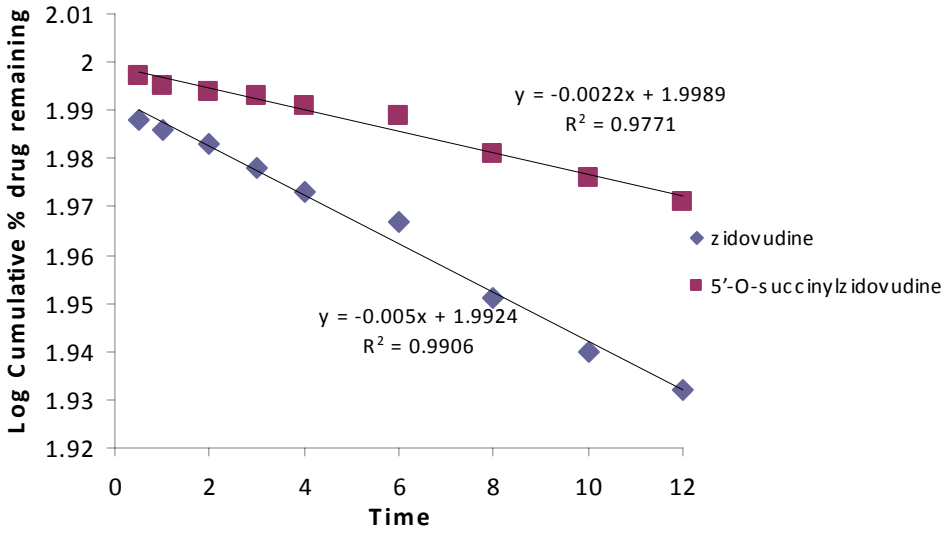


Figure 25- First order release of Zidovudine and 5'-O-Succinylzidovudine from PEG-O-Succinylzidovudine at pH 7.4

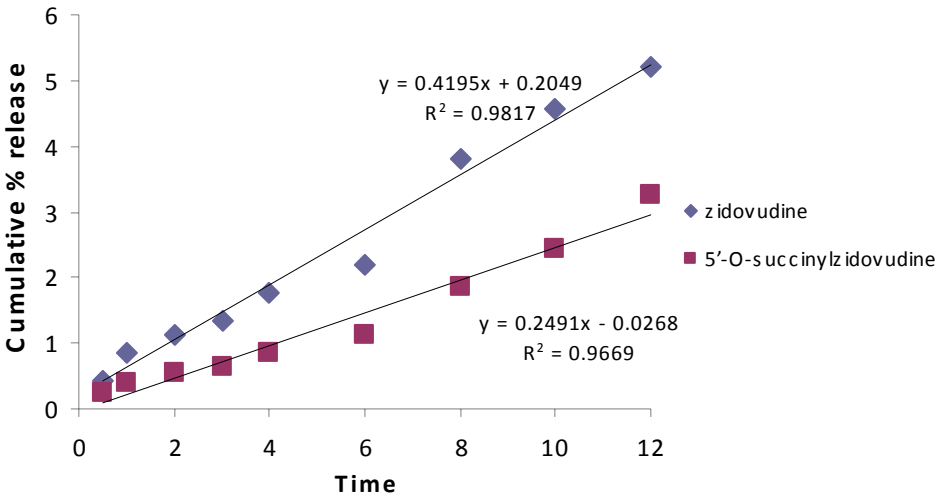


Figure 26- Zero order release of Zidovudine and 5'-O-Succinylzidovudine from DEX -O-Succinylzidovudine at pH 7.4

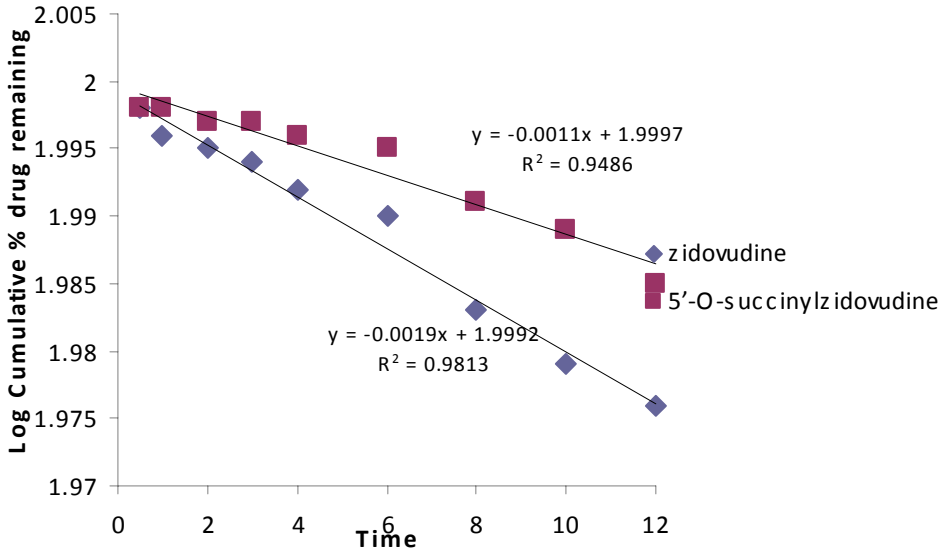


Figure 27- First order release of Zidovudine and 5'-O-Succinylzidovudine from DEX-O-Succinylzidovudine at pH 7.4

Time	PEG-O-succinylzidovudine						DEX-O-succinylzidovudine					
	AZT	% drug remaining	Log % drug remaining	5'-O-succinyl-AZT	% drug remaining	Log % drug remaining	AZT	% drug remaining	Log % drug remaining	5'-O-succinyl-AZT	% drug remaining	Log % drug remaining
0.5	4.22	95.78	1.981	0.04	99.96	1.999	1.82	98.18	1.992	0.15	99.85	1.999
1	10.56	89.44	1.951	1.06	98.94	1.995	4.86	95.14	1.978	0.51	99.49	1.997
2	12.28	87.72	1.943	1.58	98.42	1.993	5.88	94.12	1.973	0.72	99.28	1.996
3	16.43	83.57	1.922	2.36	97.64	1.989	7.96	92.04	1.963	1.16	98.84	1.994
4	19.82	80.18	1.904	3.12	96.88	1.986	9.55	90.45	1.956	1.51	98.49	1.993
6	24.78	75.22	1.876	3.76	96.24	1.983	12.1	87.9	1.943	1.82	98.18	1.992
8	32.75	67.25	1.827	4.64	95.36	1.979	12.9	87.1	1.940	2.4	97.6	1.989
10	39.49	60.51	1.781	5.48	94.52	1.975	20.46	79.54	1.900	2.65	97.35	1.988
12	45.25	54.75	1.738	6.25	93.75	1.971	27.35	72.65	1.861	3.08	96.92	1.986

	R^2 Value			R^2 Value	
	PEG-O-succinylzidovudine			DEX-O-succinylzidovudine	
	zidovudine	5'-O-succinylzidovudine		zidovudine	5'-O-succinylzidovudine
Zero order	0.9906	0.967	Zero order	0.9463	0.975
First order	0.9923	0.9764	First order	0.9315	0.9692

Table 13 - ***In vitro*** **release of zidovudine and 5'-O-succinylzidovudine from the pro-drugs at pH 8 (in the presence of α- chymotripsin)**

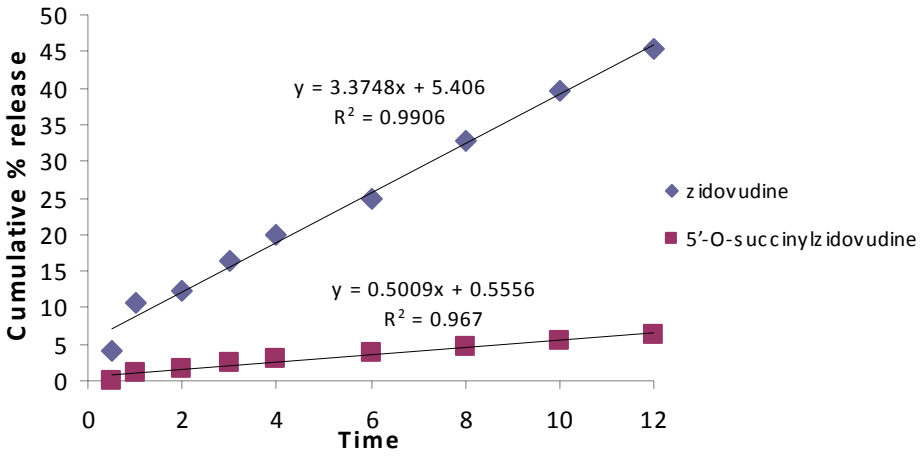


Figure 28 - Zero order release of Zidovudine and 5'-O-Succinylzidovudine from PEG-O-Succinylzidovudine at pH 8 (in presence α-chymotripsin)

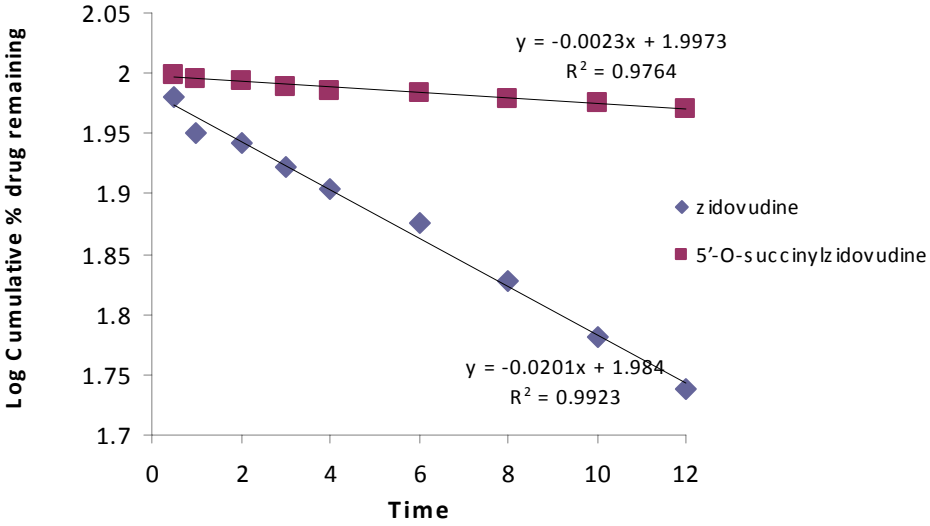


Figure 29 - First order release of Zidovudine and 5'-O-Succinylzidovudine from PEG-O-Succinylzidovudine at pH 8 (in presence α-chymotripsin)

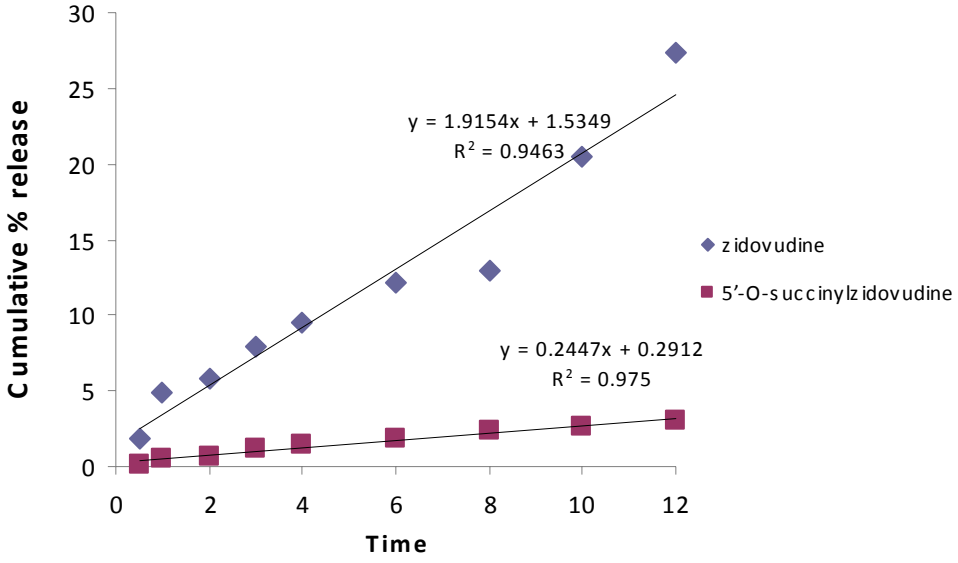


Figure 30 - Zero order release of Zidovudine and 5'-O-Succinylzidovudine from DEX-O-Succinylzidovudine at pH 8 (in presence α-chymotripsin)

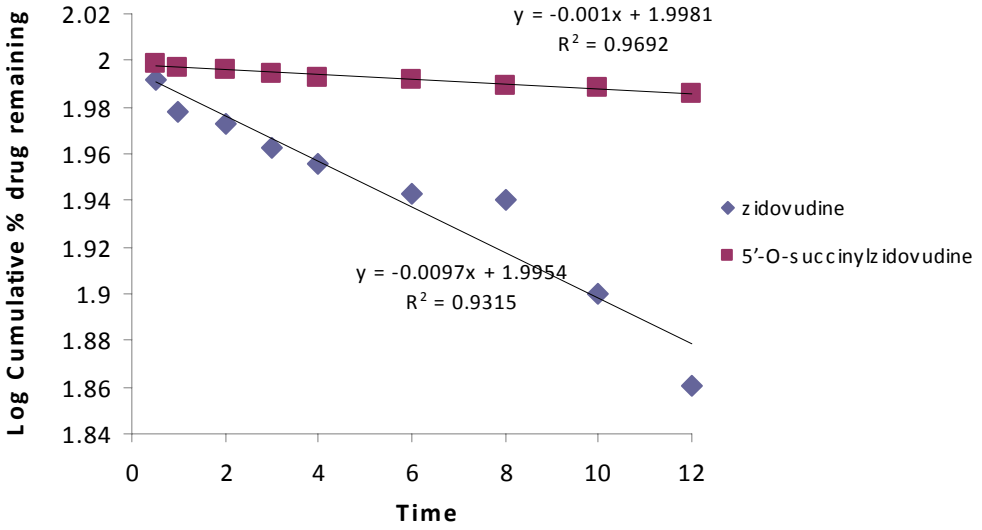


Figure 31 - Zero order release of Zidovudine and 5'-O-Succinylzidovudine from DEX-O-Succinylzidovudine at pH 8 (in presence α-chymotripsin)

Time	PEG-O-succinylzidovudine						DEX-O-succinylzidovudine					
	AZT	% drug remaining	Log % drug remaining	5'-O-succinyl-AZT	% drug remaining	Log % drug remaining	AZT	% drug remaining	Log % drug remaining	5'-O-succinyl-AZT	% drug remaining	Log % drug remaining
0.5	0.22	99.78	1.999	0.05	99.95	1.999	0.14	99.86	1.999	0.02	99.98	1.999
1	1.98	98.02	1.991	1.05	98.95	1.995	1.18	98.82	1.994	0.52	99.48	1.997
2	3.85	96.15	1.982	1.62	98.38	1.992	1.65	98.35	1.992	0.71	99.29	1.996
3	6.45	93.55	1.971	2.40	97.60	1.989	3.82	96.18	1.983	1.12	98.88	1.995
4	9.52	90.48	1.956	3.16	96.84	1.986	5.68	94.32	1.974	1.49	98.51	1.993
6	10.28	89.72	1.952	3.72	96.28	1.983	7.96	92.04	1.963	1.82	98.18	1.992
8	13.68	86.32	1.936	4.66	95.34	1.979	9.2	90.8	1.958	2.35	97.65	1.989
10	15.48	84.52	1.926	5.5	94.5	1.975	10.10	89.90	1.953	2.68	97.32	1.988
12	19.45	80.55	1.906	6.30	93.70	1.971	11.20	88.80	1.948	3.10	96.90	1.986

	R^2 Value			R^2 Value	
	PEG-O-succinylzidovudine			DEX-O-succinylzidovudine	
	zidovudine	5'-O-succinylzidovudine		zidovudine	5'-O-succinylzidovudine
Zero order	0.9702	0.9668	Zero order	0.9509	0.9762
First order	0.9764	0.9754	First order	0.9503	0.9745

Table 14- *Invitro* release of zidovudine and 5'-O-succinylzidovudine from the pro-drugs at pH 8.0 (in the absence of α- chymotripsin)

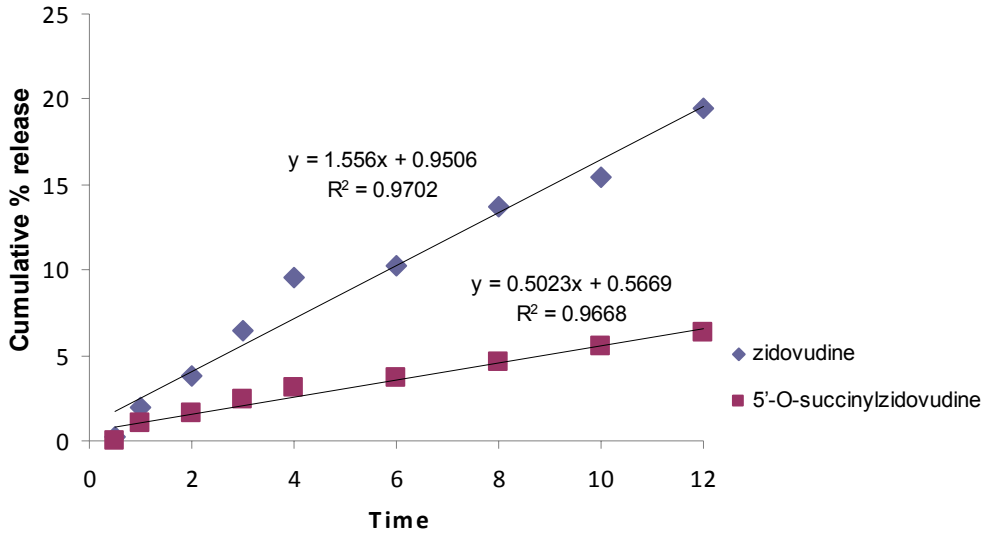


Figure 32 - Zero order release of Zidovudine and 5'-O-Succinylzidovudine from PEG-O-Succinylzidovudine at pH 8 (in absence α-chymotripsin)

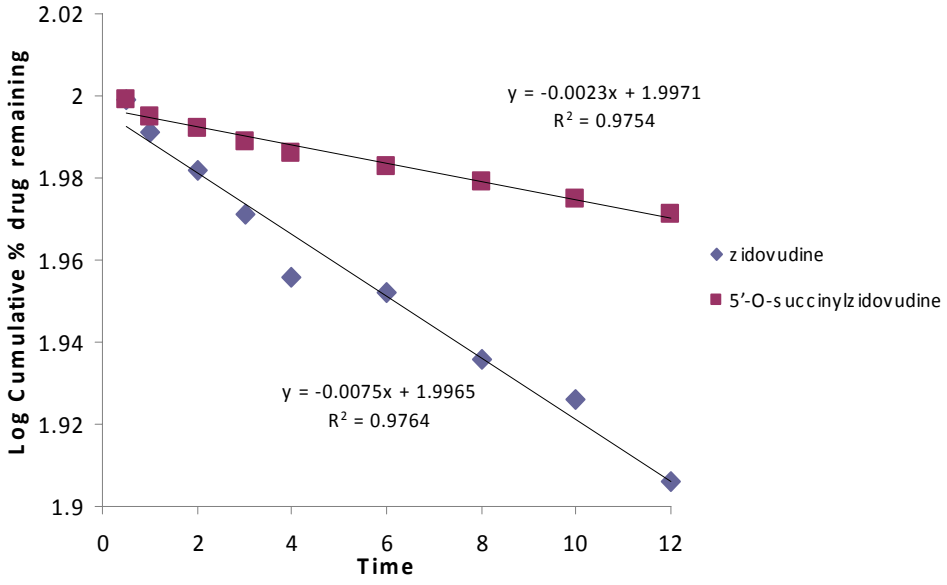


Figure 33 - First order release of Zidovudine and 5'-O-Succinylzidovudine from PEG-O-Succinylzidovudine at pH 8 (in absence α-chymotripsin)

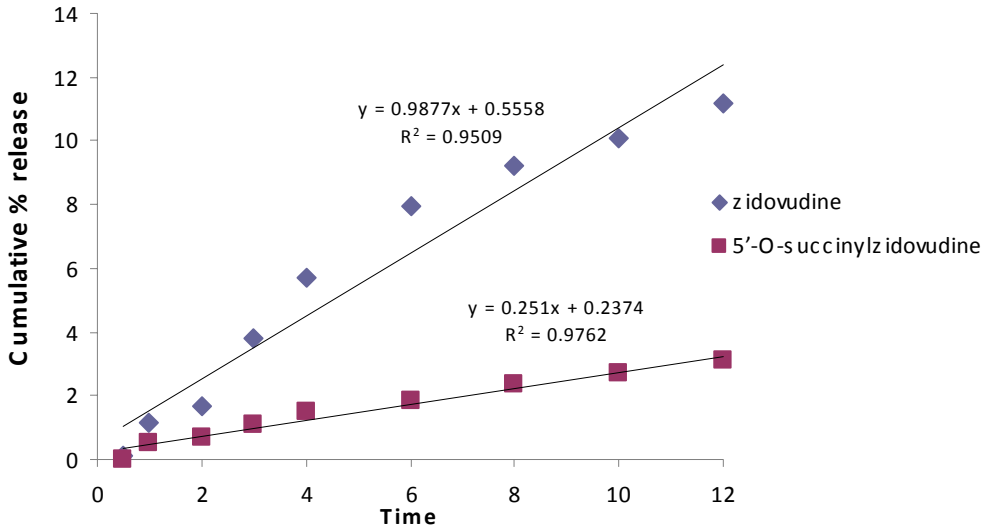


Figure 34 - Zero order release of Zidovudine and 5'-O-Succinylzidovudine from DEX-O-Succinylzidovudine at pH 8 (in absence α-chymotripsin)

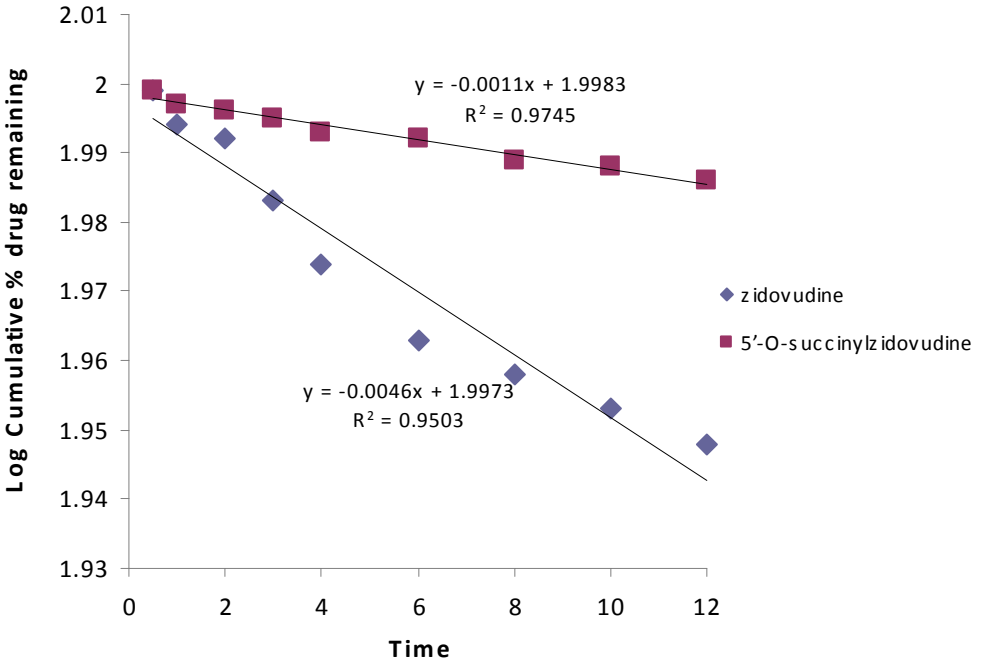


Figure 35 - First order release of Zidovudine and 5'-O-Succinylzidovudine from DEX-O-Succinylzidovudine at pH 8 (in absence α-chymotripsin)

Time	AZT	% drug remaining	Log % drug remaining	% drug remaining	% drug remaining	Log % drug remaining
	In the presence of α-chymotripsin			In the absence of α-chymotripsin		
0.5	1.89	98.11	1.991	1.26	98.74	1.994
1	3.46	96.54	1.984	2.12	97.88	1.990
2	4.86	95.14	1.978	3.14	96.86	1.986
3	5.98	94.02	1.973	3.96	96.04	1.982
4	7.24	92.76	1.967	4.86	95.14	1.978
6	9.62	90.38	1.956	6.52	93.48	1.970
8	11.46	88.54	1.947	7.78	92.22	1.964
10	13.48	86.52	1.937	9.34	90.66	1.957
12	15.94	84.06	1.924	10.82	89.18	1.950

	R^2 Value	
	Zidovudine (presence of α- chymotripsin)	Zidovudine (absence of α-chymotripsin)
Zero order	0.9916	0.9937
First order	0.9947	0.9953

Table 15- ***In vitro*** **release of zidovudine from 5'-O-succinylzidovudine at pH 8.0 in the presence and absence of α- chymotripsin**

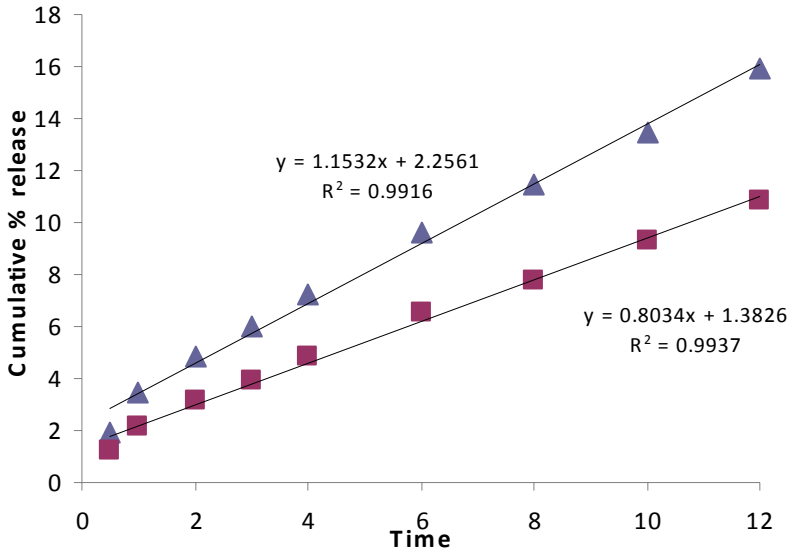


Figure 36 - Zero order release of Zidovudine from 5'-O-Succinylzidovudine at pH 8 in presence (--▲--) and absence (--■--) α-chymotripsin)

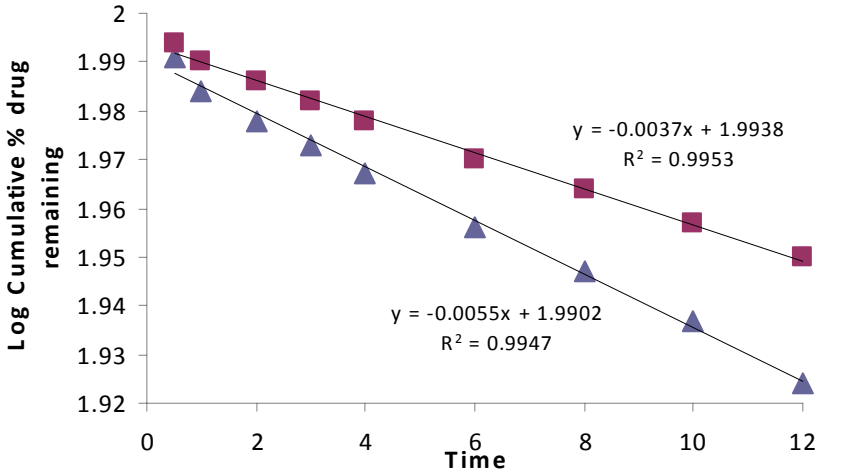


Figure 37 - First order release of Zidovudine from 5'-O-Succinylzidovudine at pH 8 in presence (--▲--) and absence (--■--) α-chymotripsin)

4.6 Stability studies

Studies were, therefore, carried out to assess the stability of the polymeric pro-drugs synthesized, at three different temperature and humidity conditions, prescribed by the ICH over a period of three months. Samples were withdrawn at different time intervals and analysed for their drug content and their *in vitro* drug release behavior obtained. Drug content and their *in vitro* drug release behavior are presented in Table 16. The results indicate no significant change occurs in the drug content in any of the pro-drugs during the storage period. Similarly no significant difference in their *in vitro* drug release behavior was observed. This indicates no physical and chemical changes occurr in the polymeric pro-drugs during the storage period and the pro-drugs synthesized are stable

Table 16- Drug content during stability study

Duration (In days)	PEG-O-succinylzidovudine	DEX-O-succinylzidovudine	5-O-succinylzidovudine
At 25°C / 60% RH			
0	22.45	8.43	72.25
30	23.10	8.12	71.62
60	22.80	8.35	72.48
90	22.62	8.40	71.86
At 40°C / 75% RH			
0	22.40	8.42	72.20
30	22.65	8.26	72.08
60	23.02	8.18	72.36
90	22.84	8.28	71.68
At room temperature			
0	22.45	8.42	72.24
30	22.86	8.38	72.38
60	23.05	8.30	71.80
90	22.75	8.18	71.95

4.7 Bioavailability Studies

Validation of optimized method

The accuracy of the optimised methods was determined by relative and absolute recovery experiments.
The optimized methods for the estimation of the drugs were found to be precise. This was evident from the coefficient of variation values, which were less than 10 % at all concentrations.

It was observed that the optimised methods were linear within a specific concentration range for individual drugs. The calibration curves were plotted between response factor and concentration of the standard solutions. The linearity range for zidovudine was found to be, 12.5, *15, 50,* 75, 125, 175,225,280 ng/ml respectively.

The Limit of Detection (LaD) value was found to be 5ng/ml for zidovudine and their Limit of Quantification (LOQ) value was 10ng/ml. This observation showed that the developed methods have adequate sensitivity. These values, however, may be affected by the separation conditions (e.g., column, reagents, and instrumentation and data systems), instrumental changes (e.g., pumping systems and detectors) and use of non HPLC grade solvents and may result in changes in signal to noise ratios.

The ruggedness and robustness of the methods were studied by changing the experimental conditions. No significant changes in the chromatographic arameters were observed when changing the experimental conditions (operators, instruments, source of reagents and column of similar type) and optimised conditions (pH, mobile phase ratio and flow rate).

System suitability parameters such as column efficiency (theoretical plates), resolution factor and peak asymmetry factor of the optimised methods were found satisfactory.

Bioavailability Studies

The bioavailability studies carried out for polymer-drug conjugates are given in Figures 38 and Tables 18-21. Typical chromatograms are given in Figures 39. A comparative analysis of pharmacokinetic parameters like the maximum blood level concentration (C_{max}), time for maximum blood level concentration (t_{max}), the areas under curve (AUC_{o-t}) and ($AUC_{0-\infty}$), elimination half-life ($t_{1/2}$) and elimination rate constent (keli) are given in Table 17.

An analysis of the data reveals that the maximum blood level drug concentration (C_{max}) obtained in the case of zidovudine was 985.76ng/ml within 2h, whereas the two synthesized polymeric pro-drugs and 5'-O-succinylzodovudine showed a lesser C_{max} with an increased $t_{1/2}$ when compared to the pure drug. However the maximum blood level drug concentration obtained from 5'-O-succinylzodovudine was 698.74.54ng/ml with a t_{max} of 2h. This was much higher when compared to the two polymeric pro-drugs synthesized.

If was found that for PEG-O-succinylzodovudine the C_{max} was 415.69 with a t_{max} of 4h whereas for DEX-O-succinylzodovudine it was nearly 610.55 ng/ml after a period of 6h. The delay in the t_{max} is thus due to the slower rate of drug release from the pro-drugs and consequent absorption. It was found that the absorption of the drug was comparatively more in the case of DEX-O-succinylzidovudine than that from PEG-O-succinylzidovudine. This may be due to the biodegradation of dextran itself in the system by the enzyme dexranase [158].

However it was found that the absorption of the drug was comparatively more in the case of DEX-O-succinylzidovudine than that from PEG-O-succinylzidovudine. This may be due to the biodegradation of dextran itself in the system by the enzyme dexranase [35].

Detectable concentration of the drug was observed till 12 h for all animals in the case of pro-drugs, whereas it was only 3h for the free drug. The area under the plasma concentration-time curve values showed that there is significant increase in

drug absorption for the pro-drugs when compared to test product, which confirms the complete release of the drug from the pro-drugs and its consequent absorption in the systemic circulation.

An analysis of pharmacokinetic data shows that there is a distinct difference in plasma concentration response between the free drug and the pro-drugs. There is a slower rate of drug absorption for the polymeric pro-drugs synthesised when compared to the free drug as evident from the delayed t_{max} values for the pro-drugs synthesised. Further, the extent of absorption of the pro-drugs is complete and is comparable with the free drugs. This reveals the potential of the polymeric pro-drug synthesised in the present study in sustaining the drug release in *in vivo* conditions also.

These polymeric pro-drugs are thus capable of avoiding or minimising the systemic side effects associated with free drugs. This is also expected to improve the patient compliance as it has the potential to reduce the frequency of drug administration.

Pro-drug	**Zidovudine**	**PEG-O-succinylzidovudine**	**DEX-O-succinylzidovudine**	**5'-O-succinylzidovudine**
C_{max}	985.75	415.69	610.55	687.45
t_{max}	2	4	6	2
$t_{1/2}$	1.26	2.06	2.85	1.25
keli	0.271	0.245	0.259	0.286
Auc_{0-t}	5180. 904	5079.55	5585.53	5059.987
$Auc_{0-\infty}$	5526.07	5626.36	5968.26	5468.98

Table 17 - Comparative pharmacokinetic parameters of the polymeric pro-drugs

Table 18- Plasma drug concentration of zidovudine

Time (hours)	Plasma concentration						Mean plasma Conc (ng/ml)	SD
	A1	A2	A3	A4	A5	A6		
0.5	320.42	346.55	286.78	380.76	406.78	348.03	348.22	42.52
1	606.48	652.43	666.7	608.42	694.56	720.73	658.22	45.81
2	912.43	978.23	994.76	1061.9	924.78	1042.4	985.75	60.40
3	598.5	630.7	662.7	584.63	608.4	638.37	620.55	28.71
4	296.43	348.26	368.43	343.46	381.7	413.62	358.65	39.65
6	176.45	149.36	120.86	158.43	136.45	210.59	158.69	31.69
8	48.56	42.84	47.75	60.75	54.66	47.05	50.26	6.39
10	16.4	18.85	20.4	15.78	16.76	21.91	18.35	2.45
12	4.26	4.84	4.68	4.22	4.34	5.02	4.56	0.33

Table 19 - Plasma drug concentration of zidovudine from PEG-O-succinylzidovudine

Time (hours)	Plasma concentration						Mean plasma Conc (ng/ml)	SD
	A1	A2	A3	A4	A5	A6		
0.5	65.45	68.3	74.65	69.32	72.75	72.83	70.55	3.45
1	122.64	128.48	108.58	124.76	122.38	116.52	120.56	7.04
2	232.4	252.7	224.56	248.42	244.7	240.94	240.62	10.48
3	356.46	378.3	388.52	412.4	362.76	412.22	385.11	23.91
4	388.68	405.7	453.65	382.42	448.38	415.31	415.69	29.82
6	328.56	336.57	298.56	288.5	347.76	350.8	325.14	26.02
8	184.7	192.28	164.72	168.58	200.48	169.96	180.12	14.54
10	102.56	110.78	98.88	86.78	126.32	107.98	105.55	13.19
12	45.4	48.48	39.56	50.64	48.56	44.56	46.20	3.95

Table 20- Plasma drug concentration of zidovudine from DEX-O-succinylzidovudine

Time (hours)	Plasma concentration						Mean plasma Conc (ng/ml)	SD
	A1	A2	A3	A4	A5	A6		
0.5	128.52	124.76	132.7	98.56	118.76	119.46	120.46	11. 97
1	270.43	278.56	231.65	254.76	238.56	317.48	265.24	31..25
2	352.76	445.98	452.2	358.76	368.48	364.7	390.48	45.75
3	465.23	478.43	523.45	448.22	512.56	435.47	480.56	28.50
4	558.5	580.56	612.4	474.63	568.4	568.91	560.45	25.71
6	584.56	610.43	628.36	648.56	568.3	623.09	610.55	29.60
8	326.8	348.4	296.46	288.52	306.38	354.76	320.22	27.54
10	128.76	129.88	132.46	136.78	134.56	118.96	130.23	6.26
12	40.78	42.34	36.5	38.88	42.52	34.96	39.33	3.12

Table 21- Plasma drug concentration of zidovudine from 5'-O-succinylzidovudine

Time (hours)	Plasma concentration						Mean plasma Conc (ng/ml)	SD
	A1	A2	A3	A4	A5	A6		
0.5	245.65	300.48	285.38	265.78	256.42	269.77	270.58	19.79
1	487.58	468.23	478.54	412.75	508.36	402.02	459.58	42.67
2	620.34	694.38	724.2	748.58	638.46	698.74	687.45	49.33
3	448.65	408.48	395.76	480.36	442.85	451.78	437.98	30.91
4	234.6	254.62	228.56	248.42	215.38	233.64	235.87	14.06
6	94.58	106.76	94.35	89.76	104.7	101.15	98.55	6.67
8	26.7	28.32	35.74	32.65	25.78	36.09	30.88	4.56
10	11.78	12.84	18.34	16.78	13.89	17.21	15.14	2.66
12	2.45	2.24	2.52	2.84	2.62	2.63	2.55	0.20

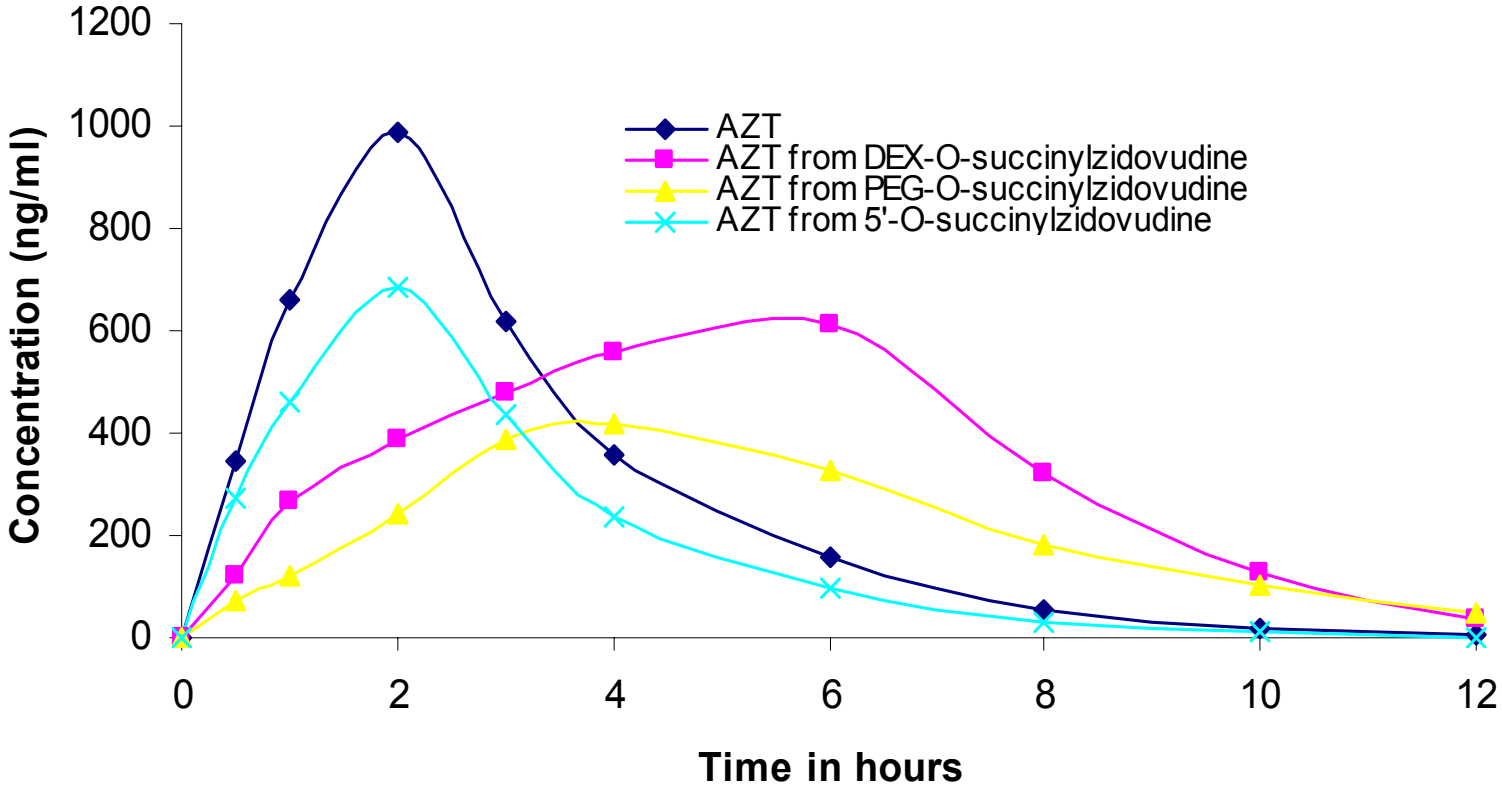
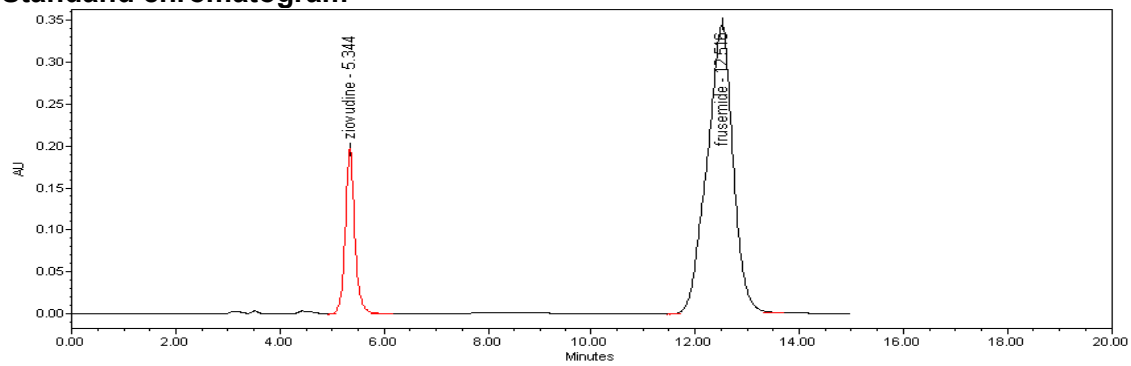
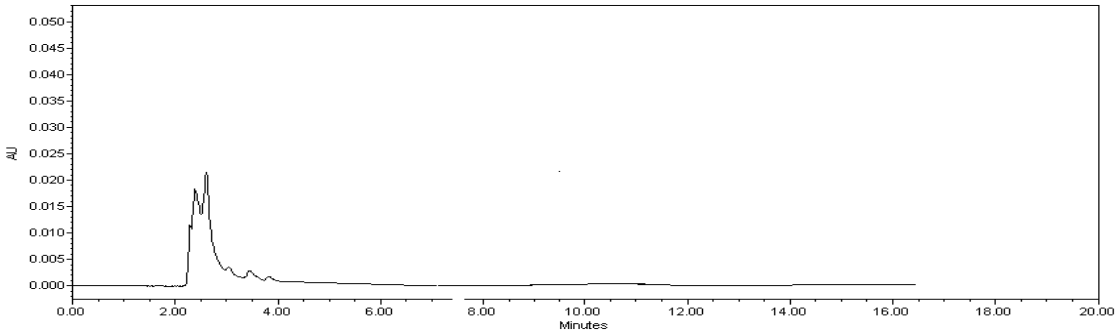


Figure 38 -Plasma concentration- time curve of zidovudine, PEG-O-succinylzidovudine, DEX-O-succinylzidovudine and 5'-O succinylzidovudine

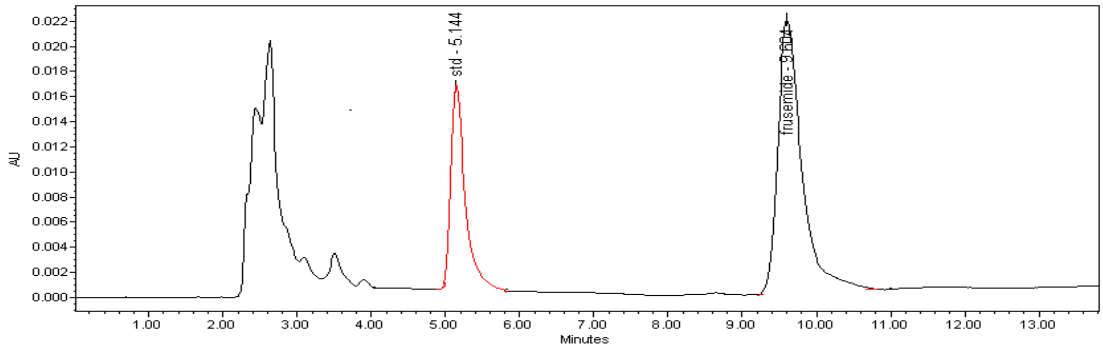
Standand chromatogram



Blank plasma



Control chromatogram



Sample chromatogram of the pro-drugs

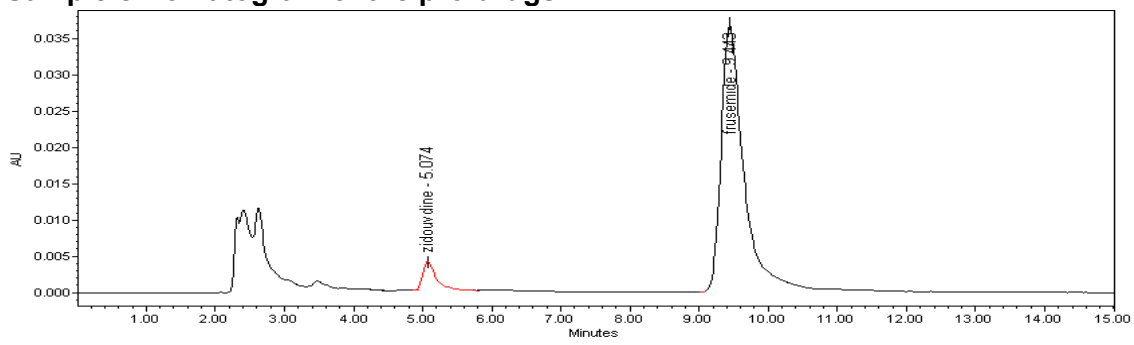


Figure 39- TypicalChromotograms

5. Summary and Conclusions

This thesis deals with the investigations carried out by the writer on the synthesis, characterization and evaluation of some polymeric pro-drugs.

The first chapter of the thesis deals with a brief introduction to controlled drug delivery systems, passive drug targeting and specific tissue targeting , cellular uptake of polymers, site specific drug release, polymer conjugates, incorporation of spacers in pro-drug conjugates, PEG chemistry and use of Dextran in drug delivery. The use of antiviral drug namely, zidivudine, problems associated with its use and a literature survey on the investigations that have been carried out on polymeric drug conjugates of zidovudine are briefly given.

The second chapter of the thesis deals with the scope and objective of the present investigations in detail. It explains, in particular, how when zidovudine is linked covalently to biocompatible polymers through succnic spacer should lead to a better drug delivery system capable of releasing of the drug in a sustained manner.

The third chapter of the thesis deals with the experimental procedures that are adopted in the preparation of the polymeric pro-drugs, estimation of the drug content, *in vitro* drug release studies, enzymatic hydrolysis, stability studies and bioavailability studies.

The fourth chapter of the thesis deals with the results obtained in the present study along with a detailed discussion of the results supported by chemical equations, tables, figures, etc.

The following are some of the important findings in the present study;

- Two macromolecular pro-drugs of the known antiviral drug zidovudine were synthesized by conjugating it with poly (ethylene glycol)$_{1500}$, and detrain through succinic spacer. The pro-drugs were thoroughly characterized in terms of IR, and H^1NMR.

- In order to obtain some preliminary information about the potential use of the PEG-O-succinylzidovudine and DEX-O-succinylzidovudine as drug delivery systems for both oral and systemic administration, *in vitro* hydrolysis studies were performed by subjecting the conjugates to hydrolysis in buffer solutions at pH 1.1 (simulated gastric juice), at pH 5.5 (endosomal compartments) and at pH 7.4 (extracellular fluids) and at pH 8 in the presence and in the absence of α-chymotrypsin. It was found that, at all pH values considered, free zidovudine was always released from 5'-O-succinylzidovudine derivative. Even if 5'-O-succinylzidovudine was released from the macromolecular pro-drugs, it was in turn be hydrolysed to give free and active zidivudine. The amount of zidovudine released from PEG-O-succinylzidovudine was more than that from DEX-O-succinylzidovudine. It was also found that the release rate of zidovudine from 5'-O-succinylzidovudine derivative decreased as the pH value increased. A different trend was noticed in the case of release of zidovudine from the polymer drug conjugates. The release of drug was minimum at pH 5.5 buffer solution. This drug release can be attributed to influence of pH value on the hydrolysis rate of low molecular weight ester compounds.

- It was found that nearly 45% and 28% and 16% of zidovudine was released from PEG-O-succinylzidovudine and DEX-O-succinylzidovudine and 5'-O-succinylzidovudine respectively. These results confirm the capacity of macromolecular conjugates to release free drug by hydrolytic activity of α-chymotrypsin. The ester bond between zidovudine and the succinic spacer would seem more susceptible to this enzymatic hydrolysis than the ester bond between 5'-O-succinylzidovudine and the polymeric carrier

- Stability studies carried out for the pro-drugs for a period of three months as per ICH guidelines indicate that no major physical and chemical changes occur in the polymeric pro-drugs during storage period indicating the stability of the pro-drugs.

- The ability of the synthesized macromolecular pro-drugs to release free drug was also evaluated in human plasma. It was found that nearly 60% of linked zidovidine was released from PEG-O-succinylzidovudine within 12h, were as nearly 25 of zidovidine was released from DEX-O-succinylzidovudine. The plasmatic hydrolysis of 5'-O-succinylzidovudine showed that about 30% of the drug derivative was hydrolysed to give free zidovudine within 12h indicating that the amount of 5'-O-succinylzidovudine released from the polymeric pro-drug can in turn be hydrolysed to give free zidovudine.

- The release of Zidovudine and 5'-O-succinylzidovudine from the macromolecular pro-drugs was found to be pH dependent. It was found that the release of Zidovudine and 5'-O-succinylzidovudine from PEG-O-succinylzidovudine follows first order kinetics at different pH. Howerver, the release of zidovudine and 5'-O-succinylzidovudine from DEX-O-succinylzidovudine followes zero order kinatics.

- An analysis of pharmacokinetic data shows that there is a distinct difference in plasma concentration response between the free drug and the pro-drugs. There is a slower rate of drug absorption for the polymeric pro-drugs when compared to the free drug as evident from the delayed t_{max} values for the pro-drugs. Further, the extent of absorption of the pro-drugs is complete and is comparable with the free drug. This reveals the potential of the polymeric pro-drugs in the present study in sustaining the drug release in *in vivo* conditions also. However it was found that the absorption of the drug was comparatively more in the case of DEX-O-succinylzidovudine than that from PEG-O-succinylzidovudine. This may be due to the biodegradation of dextran itself in the system by the enzyme dextranase. This reveals the potential of these pro-drugs in sustaining the drug release in *in vivo* conditions thus leading to drug release systems capable of avoiding or minimizing the side effects. The macromolecular pro-drugs are thus capable of reducing the frequency of drug administration and improve patient compliance.

6. References

1. L. Gros, H. Ringsdorf, H. Schupp, Polymeric antitumour agents on a molecular and on a cellular level? *Angew. Chem*. **1981,** 93, 311 –331.
2. R. Duncan, The Dawning Era of Polymer Therapeutics, *Nat. Rev. Drug Discovery,* **2003,** 2, 347–360.
3. R. Duncan, Targeting and intracellular delivery of drugs in Encyclopedia of Molecular Cell Biology and Molecular Medicine, Vol.14 (Ed.: R. A. Meyers), Wiley-VCH, Weinheim, **2005,** 163 –204.
4. G. Kohler, C. Milstein, Continuous cultures of fused cells secreting antibody of predefined specificity, *Nature* **1975**, 256, 495 –497.
5. H. Ringsdorf, Structure and properties of pharmacologically active polymers, *J. Polym. Sci. Polym. Symp.* **1975,** 51, 135–153.
6. A. Godwin, K. Bolina, M. Clochard, E. Dinand, S. Rankin, S. Simic, S. Brocchini, New strategies for polymer development in Pharmaceutical science- a short review, *J. Pharm. Pharmacol*, 2**001,** 53, 1175–1184.
7. B. Rihova, K. Kubackova, Clinical implications of N-(2-hydroxypropyl) methacrylamide copolymers, *Curr. Pharm. Biotechnol*, **2003,** 4, 311–322.
8. J. Kopecek, P. Kopeckova, T. Minko, Z. R. Lu, HPMA copolymer-anticancer drug conjugates, Design, activity, and mechanism of action, *Eur. J. Pharm. Biopharm*, **2000,** 50, 61–81.
9. R. Duncan, N-(2-Hydroxypropyl) methacrylamide copolymer conjugates. In Polymeric Drug Delivery Systems, (Ed.: G. S. Kwon), *Marcel Dekker, New York*, **2005**, 1–92.
10. M. Yokoyama, Drug targeting with nano-sized carrier systems, *Journal of Artificial Organs* , **2005**, 8, 77-84.
11. R. Haag, Dendritic Polyglycerol Sulfates as New Heparin Analogues and Potent Inhibitors of the Complement System, *Angew. Chem,* **2004**, 116, 280–284.
12. J. K. Vasir, M. K. Reddy, V. D. Labhasetwar, Nanosystems in drug targeting: Opportunities and challenges *Curr. Nanoscince*, **2005,** 1, 47–64.

13. R. Duncan, L. Izzo, Dendrimer biocompatibility and toxicity, *Adv. Drug Delivery Rev,* **2005,** 57, 2215–2237.
14. A. Gresser, The use of PVP as a polymeric carrier to improve the plasma half-life of drugs, *Dtsch. Med. Wochenschr*, **1963**, 88, 2217.
15. Y. Matsumura, H.Maeda**,** New Concept for Macromolecular Therapeutics in Cancer Chemotherapy, Mechanism of Tumoritropic Accumulation of Proteins and the Antitumor Agent Smancs, *Cancer Res*, **1986**, 46, 6387–6392.
16. H. Maeda, J. Wu, T. Sawa, Y. Matsumura, K. Hori, Tumor vascular permeability and the EPR effect in macromolecular therapeutics, a review, *J. Controlled Release,* **2000,** 65, 271–284.
17. R. K. Jain, Transport of Molecules in the Tumor Interstitium, *A Review*, *Cancer Res.*, **1987,** 47, 3039–3051.
18. R. K. Jain, Transport of molecules across tumor vasculature, *Cancer Metastasis Rev,* **1987**, 6, 559–593.
19. H. Maeda, Y. Matsumura, Tumoritropic and lymphotropic. Principles of macromolecular drugs, *Crit. Rev. Ther. Drug Carrier Syst,* **1989**, 6, 193-210.
20. P. Caliceti, F. M. Veronese, Pharmacokinetic and biodistribution properties of poly (ethylene glycol)-protein conjugate, *Drug Deliv.Rev,* **2003**, 55, 1261–1277.
21. K. Greish, J. Fang, T. Inutsuka, A. Nagamitsu, H. Maeda, Macromolecular Therapeutics: Advantages and Prospects with Special Emphasis on Solid Tumour Targeting*, Clin. Pharmacokinet*, **2003**, 42, 1089 –1105.
22. H. Maeda, T. Sawa, T. Konno, Mechanism of tumor-targeted delivery of macromolecular drugs, including the EPR effect in solid tumor and clinical overview of the prototype polymeric drug SMANCS, J*. Controlled Release* , **2001**, 74, 47 –61.
23. R. Satchi-Fainaro, Targeting tumour vasculature: reality or a dream? *J. Drug Targeting,* **2002**, 10, 529 –533.

24. R. Langer, Drug delivery and targeting. *Nature,* **1998**, 392, 5 –10.
25. S. Mukherjee, R. N.Ghosh, F. R. Maxfield, Endocytosis, *Physiol. Rev*. **1997,** 77, 759–803.
26. R. J. Amir, N. Pessah, M. Shamis, D. Shabat, Self-immolative dendrimers, Angew*. Chem*, **2003**, 115, 4632–4637.
27. K.Haba, M.Popkov, M.Shamis, R.A.Lerner, C.F.Barbas III, D.Shabat, *Single-Triggered Trimeric Pro-drugs*, Angew.Chem, **2005,** 117, 726–730.
28. M.Shamis, H.N.Lode, D.Shabat, Self-immolative dendrimer biodegradability by multi-enzymatic triggering, *Chem. Commun*, 2004**,** 21, 1614 – 1615.
29. I. F. Tannock, D. Rotin, Acid pH in Tumors and Its Potential for Therapeutic Exploitation , *Cancer Res*, **1989,** 49, 4373–4384.
30. R. B. Greenwald, C. D. Conover, Y. H. Choe, Poly (ethylene glycol) conjugated drugs and pro-drugs, a comprehensive review, *Crit. Rev. Ther. Drug Carrier Syst.* **2000**, 17,101 –161.
31. J. M. Harris, R. B. Chess, Effect of PEGylation on pharmaceuticals, *Nature Rev. Drug Discovery* , **2003**, 2, 214–221
32. S. C. Pedder, Pegylation of interferon alfa: structural and pharmacokinetic properties, *Semin. Liver Dis*, **2003**, 23, 19 –22.
33. T. K. Choueiri, T. E. Hutson, R. M. Bukowski, Evolving role of pegylated interferons in metastatic renal cell carcinoma, *Expert Rev. Anticancer Ther,* **2003**, 3, 823 –829.
34. G. Molineux, Pegfilgrastim: using pegylation technology to improve neutropenia support in cancer patients, Anticancer *Drug*s, **2003,** 14, 259 –264.
35. M. Mammen, S. K. Choi, G. M. Whitesides, Polyvalent Interactions in Biological Systems, Implications for Design and Use of Multivalent Ligands and Inhibitors*, Angew.Chem.Int.Ed*, **1998,** 37, 2754–2794.
36. S. K. Choi, Synthetic Multivalent Molecules, Wiley-Interscience, Hoboken, USA, **2004** .

37. J. Crawford, Once-per-cycle pegfilgrastim (Neulasta) for the management of chemotherapy-induced neutropenia, Semin. Oncol, **2003**, 30, 24 –30.
38. W. Vogel, Peginterferon-alpha 2a (40 kDa)/ribavirin combination for the treatment of chronic hepatitis C infection, Expert Rev, *Anti-Infect. Ther,* **2003**, 1, 423 –431.
39. V. Goffin, P. Touraine, Pegvisomant (Pfizer/Sensus), *Curr. Opin. Invest. Drugs*, **2004**, 5, 463 –468.
40. R.Fernandez-Botran, Soluble cytokine receptors: novel immunotherapeutic agents, Expert *Opin. Invest. Drugs*, **2000**, 9, 497 –514.
41. E. H. Choy, B. Hazleman, M. Smith, K. Moss, L. Lisi, D. G. cott, J. Patel, M. Sopwith,D. A. Isenberg, Efficacy of a novel PEGylated humanized anti-TNF fragment (CDP870) in patients with rheumatoid arthritis: a phase II double-blinded, randomized, dose-escalating trial, *Rheumatology* , **2002**,41, 1133 –1137.
42. P. A. Vasey, S. B. Kaye, R. Morrison, C. Twelves, P. Wilson, R.Duncan, A. H. Thomson, L. S. Murray, T. E. Hilditch, T.Murray, S. Burtles, D. Fraier, E. Frigerio, J. Cassidy, Clin. Phase I clinical and pharmacokinetic study of PK1 [*N*-(2-hydroxypropyl) methacrylamide copolymer doxorubicin]: First member of a new class of chemotherapeutic agents - Drug-polymer conjugates*, Cancer Res*, **1999**, 5, 83 –94.
43. P. M. Loadman, M. C. Bibby, J. A. Double, W. M. Al-Shakhaa, R. Duncan, Clin. Pharmacokinetics of PK1 and Doxorubicin in Experimental Colon Tumor Models with Differing Responses to PK1, *Cancer Res*, **1999**, 5, 3682 –3688.
44. V. Bilim, Technology evaluation: PK1, Pfizer/Cancer Research UK, *Curr.* Opin. Mol. Ther, **2003**, 5, 326 –330.
45. L.W. Seymour, D. R. Ferry, D. Anderson, S. Hesslewood, P. J. Julyan, R. Poyner, J. Doran, A. M. Young, S. Burtles, D. J. Kerr, Hepatic drug targeting: phase I evaluation of polymer-bound doxorubicin, *J. Clin. Oncol,* **2002,** 20, 1668 –1676.

46. M. Meerum Terwogt,W.W. ten Bokkel Huinink, J. H. Schellens, M. Schot, I. A. Mandjes, M. G. Zurlo, M. Rocchetti, H. Rosing, F. J. Koopman, J. H. Beijnen, Phase I clinical and pharmacokinetic study of PNU166945, a novel water-soluble polymer-conjugated prodrug of paclitaxel, *Anticancer Drugs* , **2001**,12, 315 –323.
47. N. E. Schoemaker, C. van Kesteren, H. Rosing, S. Jansen, M.Swart, J. Lieverst,D. Fraier,M. Breda, C. Pellizzoni, R. Spinelli, M. Grazia Porro, J. H. Beijnen, J. H. Schellens, W. W. ten Bokkel Huinink, A phase I and pharmacokinetic study of MAG-CPT, a water-soluble polymer conjugate of camptothecin, *Br. J. Cancer,* **2002**, 87, 608 –614.
48. E. Gianasi, M.Wasil, E. G. Evagorou, A. Keddle, G.Wilson, R.Duncan, HPMA copolymer platinates as novel antitumour agents: *in vitro* properties, pharmacokinetics and antitumour activity *in vivo, Eur. J. Cancer* , **1999**, 35, 994 –1002.
49. E. Gianasi, R. G. Buckley, J. Latigo, M. Wasil, R. Duncan, HPMA copolymers platinates containing dicarboxylato ligands. Preparation, characterisation and *in vitro* and *in vivo* evaluation, *J. Drug Targeting, 2002*, 10, 549 –556.
50. J. M. Rademaker-Lakhai, D. van den Bongard, D. Pluim, J. H. Beijnen, J. H. Schellens, A Phase I and Pharmacological Study with Imidazolium-**trans-**DMSO-imidazole-tetrachlororuthenate, a Novel Ruthenium Anticancer Agent, *Clin. Cancer Res*, **2004**, 10, 3717 –3727.
51. S. Sengupta, D. Eavarone, I. Capila, G. Zhao, N. Watson, T. Kiziltepe, R. Sasisekharan, Exploiting nanotechnology to target cancer, *Nature,* **2005**, 436, 568 –572.
52. R. B. Greenwald, A. Pendri, C. D. Conover, C. Lee, Y. H. Choe, C. Gilbert, A. Martinez, Y. Xia, D. Wu, M. Hsue, Camptothecin delivery systems: enhanced efficacy and tumor accumulation of camptothecin following its conjugation to polyethylene glycol via a glycine linker, *Bioorg. Med. Chem*, **1998**, 6, 551 –562.

53. D. Fraier, E. Frigerio, G. Brianceschi, M. Casati, A. Benecchi, C. James, Determination of MAG-camptothecin, a new polymer-bound camptothecin derivative, and free camptothecin in dog plasma by HPLC with fluorimetric detection, *J. Pharm. Biomed. Anal*, **2000**, 19, 505 –514.
54. J. W. Singer, R. Bhatt, J. Tulinsky, K. R. Buhler, E. Heasley, P. Klein, P. de Vries, Water-soluble poly-(L-glutamic acid)-Gly-camptothecin conjugates enhance camptothecin stability and efficacy *in vivo*, *J. Controlled Release,* **2001**, 74, 243 –247.
55. E. K. Rowinsky, J. Rizzo, L. Ochoa, C. H. Takimoto, B. Forouzesh, G. Schwartz, L. A. Hammond, A. Patnaik, J. Kwiatek, A. Goetz, L. Denis, J. McGuire, A.W. Tolcher, A Phase I and Pharmacokinetic Study of Pegylated Camptothecin as a 1-Hour Infusion Every 3 Weeks in Patients With Advanced Solid Malignancies, *J. Clin. Oncol*, **2003**, 21, 148 –157.
56. For further information, see: http.//www.cticseattle.com.
57. P. Sabbatini, C. Aghajanian, D. Dizon, S. Anderson, J. Dupont, J. V. Brown, W. A. Peters, A. Jacobs, A. Mehdi, S. Rivkin, A. J. Eisenfeld, D. Spriggs, Phase II study of CT-2103 in patients with recurrent epithelial ovarian, fallopian tube, or primary peritoneal carcinoma, *J. Clin. Oncol.* **2004**, 22, 4523 –4531.
58. J. W. Singer, B. Baker, P. De Vries, A. Kumar, S. Shaffer, E. Vawter, M. Bolton, P. Garzone, Poly-(L)-glutamic acid-paclitaxel (CT-2103) [XYOTAX], a biodegradable polymeric drug conjugate: characterization, preclinical pharmacology, and preliminary clinical data, *Adv. Exp. Med. Biol*, **2003**, 519, 81 –99
59. E. Auzenne, N. J. Donato, C. Li, E. Leroux, R. E. Price, D. Farquhar, J. Klostergaard, Superior Therapeutic Profile of Poly-L-Glutamic Acid-Paclitaxel Copolymer Compared with Taxol in Xenogeneic Compartmental Models of Human Ovarian Carcinoma, *Clin. Cancer Res*, **2002**, 8, 573 –581.
60. S. Sayid, J. Dupont, M. McNamara, J. H. Doroshow, P. S. D. Spriggs, E. Eastham, S. Stromatt, C. H. Takimoto, *Clin. Cancer Res*, **2003**, 9, 16.

61. A. Wunder, G. Stehle, H. Sinn, H. H. Schrenk, D. Hoff-Biederbeck, F. Bader, E. A. Friedrich, P. Peschke, W. Maier-Borst, D. L. Heene, Enhanced albumin uptake by rat tumors, *Int. J. Oncol*, **1997**, 11, 497 –507.
62. A. M. Burger, G. Hartung, G. Stehle, H. Sinn, H. H. Fiebig, Pre-clinical evaluation of a methotrexatealbumin conjugate (MTX-HSA) in human tumor xenografts *in vivo*, *Int. J. Cancer,* **2001**, 92, 718 –724.
63. G. Hartung, G. Stehle, H. Sinn, A. Wunder, H. H. Schrenk, H. S. L. KrPnzle, H. H. Fiebig,W. Maier-Borst, D. L. Heene,W. Queißer, Phase I Trial of Methotrexate-Albumin in a Weekly Intravenous Bolus Regimen in Cancer Patients, *Clin. Cancer Res*, **1999**, 5, 753 –759.
64. F. Kratz, R. Mueller-Driver, I. Hofmann, J. Drevs, C. Unger, A Novel Macromolecular Prodrug Concept Exploiting Endogenous Serum Albumin as a Drug Carrier for Cancer Chemotherapy, *J. Med. Chem*, **2000**, 43, 1253 –1256.
65. F. Kratz, A. Warnecke, K. Scheuermann, C. Stockmar, J. Schwab, P. Lazar, P. DrOckes, N. Esser, J. Drevs, D. Rognan, C. Bissantz, C. Hinderling, G. Folkers, I. Fichtner, C. Unger, Probing the Cysteine-34 Position of Endogenous Serum Albumin with Thiol-Binding Doxorubicin Derivatives. Improved Efficacy of an Acid-Sensitive Doxorubicin Derivative with Specific Albumin-Binding Properties Compared to That of the Parent Compound, *J. Med. Chem*, **2002**, 45, 5523 –5533.
66. A. M. Mansour, J. Drevs, N. Esser, F. M. Hamada, O. A. Badary, C. Unger, I. Fichtner, F. Kratz, A New Approach for the Treatment of Malignant Melanoma: Enhanced Antitumor Efficacy of an Albumin-binding Doxorubicin Prodrug That Is Cleaved by Matrix Metalloproteinase, *Cancer Res*, **2003**, 63, 4062 –4066.
67. A.Warnecke, F. Kratz, Maleimide-oligo (ethylene glycol) derivatives, synthesis and antitumor efficacy, *Bioconjugate Chem*, **2003**, 14, 377 –387.
68. B. Rihova, T. Etrych, M. Pechar, M. Jelinkova, M. Stastny, O. Hovorka, M. Kovar, K. Ulbrich, Synergistic action of doxorubicin bound to the

polymeric carrier based on N-(2-hydroxypropyl)methacrylamide copolymers through an amide or hydrazone bond*, J. Controlled Release,* **2001**, 74, 225 –232.

69. A.A. Vaidya, B. S. Lele, M.G. Kulkarni, R.A. Mashelkar, Enhancing ligand-protein binding in affinity thermoprescipitation, elucidation of spacer effects, *Biotechnol Bioeng* , **1999,** 64, 418-25.
70. J.J. Khandare, M.G. Kulkarni, Polymerizable macromers and preparation thereof, US patent (Council of Scientific and Industrial Research, New Delhi, **2004,** IN 6.822, 064.).
71. A. Esswein, E. Hanseler, Y. Montejano, K. S. Vitols, F. M Huennekens. Construction and therapeutic potential of a carboxypeptidase A. monoclonal antibody conjugate, *Adv Enzyme Regul,* **1991**, 31, 3-12.
72. R. G. Melton, J. M.B. Boyle, G.T. Rogers, P. Burke, K. D. Bagshawe, R. F. Sherwood. Optimization of small-scale coupling of A5B7 monoclonal antibody to carboxypeptidase G, *J Immunol methods*, **1993,** 158, 49-56.
73. F. Searle, C. Bier, R. G. Buckley, S. Newman, R. B. Pedley, K. D. Bagshawe, The potential of carboxypeptidase G,-antibody conjugates as anti-tumor agents. I, Preparation of antihuman chorionic gonadotrophin-carboxypeptidase G2 and cytotoxicity of the conjugate against JAR choriocar-cinema cells in vitro, *Br J. Cancer*, **1986,** 53, 337-84.
74. K. Ulbrich, V. Subr, Polymeric anticancer drugs with pH-controlled activation, *Adv Drug Deliv Rev,* **2004,** 56, 1023-50.
75. R. G. Melton, Preparation and purification of antibody-enzyme conjugates for therapeutic applications, *Adv. Drug. Deliv. Rev,* **1996,** 22, 289-301.
76. R. Duncan, S. Gac-Breton, R. Keane, R. Musila, Y. N. Sat, R. Satchi, F. Searle, Polymer-drug conjugates, PDEPT and PELT, basic principles for design and transfer from the laboratory to clinic, *J. Controlled Release* , **2001**, 74,135–146.
77. R. Satchi, T. A. Connors, R. Duncan, PDEPT: polymer-directed enzyme prodrug therapy, *Br. J. Cancer*, **2001**, 85, 1070 –1076.

78. R. Satchi-Fainaro, H. Hailu, J. W. Davies, C. Summerford, and R. Duncan, PDEPT: Polymer-Directed Enzyme Prodrug Therapy. 2. HPMA Copolymer-β-lactamase and HPMA Copolymer-C-Dox as a Model Combination, *Bioconjugate Chem*, **2003,** 14, 797 –804,
79. P. I. Kitov, J. M. Sadowska, G. Mulvey, G. D. Armstrong, H. Ling, N. S. Pannu, R. J. Read, D. Bundle, "Shiga-like toxins are neutralized by tailored multivalent carbohydrate ligands", *Nature,* **2000**, 403, 669 –672.
80. J. H. Rao, L. Yan, J. Lahiri, G. M.Whitesides, R. M.Weis, H. S. Warren, Binding of a dimeric derivative of vancomycin to l-Lys-d-Ala- d-lactate in solution and at a surface, *Chem. Biol*, **1999**, 6, 353 –359.
81. J. H. Rao, J. Lahiri, L. Isaacs, R. M. Weis, G. M. Whitesides, A Trivalent System from VancomycinoD-Ala-D-Ala with Higher Affinity Than AvidinoBiotin, *Science,* **1998**, 280, 708 –711.
82. J. H. Rao, J. Lahiri, R. M.Weis, G. M.Whitesides, Design, syntheses, and characterization of a high-affinity trivalent system derived from vancomycin and L-Lys-D-Ala-D-Ala, *J. Am. Chem. Soc*, **2000**, 122, 2698 –2710.
83. A. King, I. Philips, K. Kaniga, Comparative *in vitro* activity of telavancin (TD-6424), a rapidly bactericidal, concentration-dependent anti-infective with multiple mechanisms of action against Gram-positive bacteria, *J. Antimicrob. Chemother*, **2004**, 53, 797 –803.
84. R. Roy, Syntheses and some applications of chemically defined ultivalent glycoconjugates, *Curr. Opin. Struct. Biol*, **1996**, 6, 692 –702.
85. N. Rockendorf, T. Lindhorst, Glycodendrimers, *Top. Curr. Chem*, **2001**, 217, 201–238.
86. R. Roy, A decade of glycodendrimer chemistry**,** Trends in Glycoscience and Glycotechnology, *Glcycotechnol*, **2003**, 15, 291 –310.
87. D. Zanini, R. Roy, Synthesis of new α-thiosialodendrimers and their binding properties to the sialic acid ppecific lectin from *Limax flavus, J. Am. Chem. Soc*, **1997**, 119, 2088 –2095.

88. M. Mammen, G. Dahmann, G. M. Whitesides, Effective inhibitors of hemagglutination by influenza Virus synthesized from polymers having active ester groups. Insight into mechanism of inhibition, *J. Med. Chem*, **1995**, 38, 4179 –4190.
89. S. K. Choi, M. Mammen, G. M. Whitesides, Generation and in situ evaluation of libraries of poly (acrylic acid) presenting sialosides as side chains as polyvalent inhibitors of influenza-mediated hemagglutination, *J. Am. Chem. Soc*, **1997**, 119, 4103 –4111.
90. S. K. Choi, M. Mammen,G. M. Whitesides, Monomeric inhibitors of influenza neuraminidase enhance the hemagglutination inhibition activities of polyacrylamides, Presenting multiple C-sialoside sroups, *Chem. Biol.* **1996**, 3, 97 –104.
91. E. DeClercq in Carbohydrates and Carbohydrate Polymers (Ed.: M. Yalpani), ATL, Shrewsbury, **1993**, pp. 87 –100.
92. L. C. Hsu, Heparin-coated cardiopulmonary bypass circuits: current status, *Perfusion,* **2001**, 16, 417 –428.
93. G. Franz,D. Pauper, S. Alban, Bioactive carbohydrate polymers. Pharmacological activities of sulfated carbohydrate polymer, *Proc. Phytochem. Soc. Eur*, **2000**, 44, 47 –58.
94. S. Alban, G. Franz, Partial synthetic glucan sulfates as potential new antithrombotics: A review, *Biomacromolecules*, **2001**, 2, 354 –361.
95. H. Turk, R. Haag, S. Alban, Dendritic polyglycerol sulfates as new heparin analogues and potent inhibitors of the complement system, *Bioconjugate Chem,* **2004**, 15, 162 –167.
96. S. Shaunak, S. Thomas, E. Gianasi, A. Godwin, E. Jones, I. Teo, K. Mireskandari, P. Luthert, R. Duncan, S. Patterson, P. Khaw, S. Brocchini, Polyvalent dendrimer glucosamine conjugates prevent scar tissue formation, *Nat. Biotechnol*, **2004**, 22, 977 –984.
97. J. A. Wolff, The “grand” problem of synthetic delivery, *Nat. Biotechnol*. **2002**, 20, 768 –769.

98. J. Haensler, F. C. Szoka, Polyamidoamine cascade polymers mediate efficient transfection of cells in culture, *Bioconjugate Chem*. **1993**, 4, 372 –379.
99. D. Fischer, *Biomater. Delivery targeting proteins nucleic acids,* **2005**, 295 –321.
100. N. D. Sonawane, J. F. C. Szoka, A. S. Verkman, Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes, *J. Biol. Chem*, **2003**, 278, 44 826 –44 831.
101. J. P. Behr, , The proton sponge: a trick to enter cells the viruses did not think of, *Chimia,* **1997**, 51, 27 –30.
102. M. X. Tang, C. T. Redemann, F. C. Szoka, *In Vitro* gene delivery by degraded polyamidoamine dendrimers, *Bioconjugate Chem*, **1996**, 7, 703 –714.
103. K. Luger, A.W. Made, R. K. Richmond, D. F. Sargent, T. J. Richmond, Crystal structure of the nucleosome core particle at 2.8A resolution, *Nature*, **1997**, 389, 251 –260.
104. K. Kataoka, A. Harada, Y. Nagaski, Block copolymer micelles for drug delivery: design, characterization and biological significance, *Adv. Drug Delivery Rev*, **2001**, 47, 113 –131.
105. G. Hoerpel, PhD Thesis, Mainz, **1983**.
106. G. S. Kwon, M. Naito, K. Kataoka, M. Yokoyama, Y. Sakurai, T. Okano, Colloids Surf. B, *Biointerfaces*, **1994**, 2, 429 –434.
107. Y. Kakizawa, K. Kataoka, Block copolymer micelles for delivery of gene and related compounds*, Adv. Drug Delivery Rev*, **2002**, 54, 203 –222.
108. L. Deng, M. Mrksich, G. M. Whitesides, Using Self-Assembled Monolayers to Understand the Interactions of Man-Made Surfaces with Proteins and Cells, *J. Am. Chem. Soc*, **1996**, 118, 5136 –5137.
109. I. Szleifer, Polymers and proteins: interactions at interfaces, Curr*. Opin. Solid State Mater. Sci*, **1997**, 2, 337 –344.

110. M.C. Jones, M. Ranger, J.C. Leroux, pH-Sensitive Unimolecular Polymeric Micelles: Synthesis of a Novel Drug Carrier, *Bioconjugate Chem*, **2003**, 14, 774 –781.
111. K. Kataoka, G. S. Kwon, M. Yokoyama, T. Okano, Y. Sakurai, Block copolymer micelles as vehicles for drug delivery, *J. Controlled Release* , **1993**, , 24, 119 –132.
112. W. D. Jang, N. Nishiyama, G.-D. Zhang, A. Harada, D.L. Jiang, S. Kawauchi, Y. Morimoto,M. Kikuchi, H. Koyama, T. Aida, K. Kataoka, Supramolecular Nanocarrier of Anionic Dendrimer Porphyrins with Cationic Block Copolymers Modified with Polyethylene Glycol to Enhance Intracellular Photodynamic Efficacy, *Angew. Chem*, **2005**, 117, 423 –427.
113. Y. Bae, S. Fukushima, A. Harada, K. Kataoka, Design of Environment-Sensitive Supramolecular Assemblies for Intracellular Drug Delivery: Polymeric Micelles that are Responsive to Intracellular pH Change, *Angew. Chem*, **2003**, 115, 4788 –4791.
114. T. Nakanishi, S. Fukushima, K. Okamoto, M. Suzuki, Y.Matsumura, M. Yokoyama, T. Okano, Y. Sakurai, K. Kataoka, Development of the polymer micelle carrier system for doxorubicin, *J. Controlled Release* , **2001**, 74, 295 –302.
115. L. Thoren, The dextrans - clinical data*, Devel. Biol. Stand*. **1981**, 48, 157-167.
116. C. Larsen, Dextran prodrugs - structure and stability in relation to therapeutic activity, *Adv. Drug Deliv. Rev*, **1989**, 3, 103-154.
117. GJ. Walker, Dextrans, in: D.I. Manners (Ed.), Biochemistry of Carbohydrates II, Vol. 16, university Park Press, Baltimore, **1978**, 75-125.
118. E. Schacht, Polysaccharide macromolecules as drug carriers, in: L. Illum, S.S. Davis (Eds.), *Polymers in Controlled Drug Delivery*, Wright, Bristol, **1987**,. 131-151.
119. M. Sund, L.L. Xu, A. Rahman, B. F. Qian, M. L. Hammarstrom, A. Danielsson. Reduced susceptibility to dextran sulphate sodium-

induced colitis in the interleukin-2 heterozygous (IL-2) mouse, *Immunology, **2005***; 14:54–64.

120. Y. Takakura, M. Hashida. Macromolecular carrier systems for targeted drug delivery: pharmacokinetic considerations on biodistribution *Pharm Res*, **1996,** 13, 820–31.
121. R, Mehvar. Dextrans for targeted and sustained delivery of therapeutic and imaging agents, *J Control Release,* **2000**, 69, 1–25.
122. A..D. McLeod ,D.R. Friend, T.N. Tozer, Synthesis and chemical stability of glucocorticoid-dextran esters: potential prodrugs for colon-specific delivery*, Int J Pharm* , **1993**, 92, 105–14.
123. M. Harada, J. Murata, Y. Sakamura, H. Sakakibara, S. Okuno, T. Suzuki, Carrier and dose effects on the pharmacokinetics of T-0128, a camptothecin analogue-carboxymethyl dextran conjugate, in non-tumor- and tumor-bearing rats., *J Control Release,* **2001**, 71, 71–86.
124. K. Munechika, Y. Sogame, N. Kishi, Y. Kawabata, Y. Ueda, K.Yamanouchi, Tissue distribution of macromolecular conjugate, adriamycin linked to oxidized dextran, in rat and mouse bearing tumor-cells, *Biol Pharm Bull,* **1994**;17: 1193–8.
125. R. Vercauteren, E. Schacht, R. Duncan, Effect of the chemical modification of dextran on the degradation by rat-liver lysosomal-enzymes *J Bioact Compat Polym,* **1992**, 7, 346–57.
126. M. L. Nucci, R. Shorr, A. Abuchowski, The Therapeutic Value of Poly (Ethylene Glycol)-Modified Proteins, *Adv. Drug Delivery Rev,* **1991**, 6, 133–151.
127. M. J. Roberts, M. D. Bentley, J. M. Harris. Chemistry for peptide and protein PEGylation, *Adv. Drug Delivery Rev,* 2002**,** 54, 459-76.
128. A. Abuchowski, J. R. McCoy, N. C. Palczek, T. Van Es, F. F. Davis, Effect of covalent attachment of polyethylene glycol on immunogenecity and circulating life of bovine liver Catalase, *J. Biol. Chem*. **1977,** 252, 3582-3586.

129. A. Abuchowski, T. Van Es, N. C. Palczek, F. F. Davis, Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol, *J. Biol. Chem*. **1977,** 252, 3578-3581.
130. J. M. Harris, R. M. Herati, Prepration and use of polyethylene glycol propionaldehyde. *US Pataent*, **1993,** 252, 714.
131. H. Mirchandoni, yw. Chien, Drug delivery approaches for anti-HI V drugs, *Int. J. Pharm*, **1993**, 95, 1-21.
132. S.M. Hammer, Advances in antirctroviral therapy and viral load monitoring, *AIDS,* **1996***,* 10, SI-SII.
133. S.K. Aggarwal, S.R. Gogu, S.R.S. Rangon, K. Agrawal, Synthesis and biological evaluation of prodrugs of zidovudine (and notes therein), *J. Med. Chern*, **1990,** 33, 1505-1510.
134. M. R Blum , S. H. Liao , S.S. Good , P. , Pharmacokinetics and bioavailability of zidovudine in humans, *Am J Med*, **1988**, 85(2A), 189-94.
135. E.P. Acosta, L.M. Page, C. V. Fletcher, Clinical pharmacokinetics of zidovudine. An update, *Clin Pharmacokinet*, 1996**,** 4,:251-62.
136. G. Giammona , G. Cavallaro, G. Fontana, G. Pitarresi and B. Carlisi Coupling of the antiviral agent zidovudine to polyaspartamide and *in vitro* drug release studies, J. Controlled Release, **1998,** 54,321-331.
137. S. Wannachaiyasit, P. Chanvorachote, U. Nimmannit, A novel anti-HIV dextrin-zidovudine conjugate improving the pharmacokinetics of zidovudine in rats. AAPS PharmSciTech, **2008**. 9, 840-850.
138. G. Cavallaro, Laura Maniscalco, P. Caliceti S. Stefano. A. Semenzato, G. Giammona, Glycosilated Macromolecular Conjugates of Antiviral Drugs with a Polyaspartamide, *J. Drug Targeting*, **2004**,12,593 – 605.
139. R. Anthony, Robert P, Vortherms**,** D. D. Gao, O. Debrah J. Patrick, Synthesis, Characterization, and *In Vitro* Assay of Folic Acid Conjugates of 3'-Azido-3'-Deoxythymidine (AZT): Toward Targeted AZT Based Anticancer Therapeutics , *Nucleosides, Nucleotides and Nucleic Acids*, **2008**, 27, 173 – 185.

140. W. Dimao, J. Sanhao, W. Yan, J. Yong and Z. Yufen , Design, synthesis, and antitumor activity of bile acid–polyamine–nucleoside conjugates , *Bioorganic & Medicinal Chemistry Letters* , **2007**, 17, 2983-2986.

141. Lin Yang Rong Zeng, Chao Li, Gang Li, Renzhong Qiao, Liming Hu and Zelin Li, Novel synthesis and in vitro drug release of polymeric prodrug: Chitosan–*O*-isopropyl-5′-*O*-d4T monophosphate conjugate, *Bioorganic & Medicinal Chemistry Letters* , **2009**, 9, 2566-2569.

142. Yiguang Jin, Lei Xing, Ying Tian, Miao Li, Chunsheng Gao, Lina Du, Junxing Dong, and Hongxuan Chen, Self-assembled drug delivery systems. Part 4. *In vitro*/*in vivo* studies of the self-assemblies of cholesteryl-phosphonyl zidovudine*, Int. J.Pharm,* **2009**, , 20, 40-48

143. F. Bonina , C. Puglia ,M.G. Rimoli , L. Avallone, E. Abignente, G. Boatto , M. Nieddu, R. Meli ,M. Amorena, P. de Caprariis. Synthesis and *in vitro* chemical and enzymatic stability of glycosyl 3'-azido-3'-deoxythymidine derivatives as potential anti-HIV agents, *Eur J Pharm Sci*. **2002** ,16, 167-74.

144. Lin Yang, Liqiang Chen, Rong Zeng, Chao Li, Renzhong Qiao, Liming Hu and Zelin Li, Synthesis, nanosizing and in vitro drug release of a novel anti-HIV polymeric prodrug: Chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate, Bioorganic & Medicinal Chemistry, **2010,** 18, 17-123.

145. D. Sriram, N. Srichakravarthy, T. R. Bal, P. Yogeeswari, Synthesis of zidovudine prodrugs with broad-spectrum chemotherapeutic properties for the effective treatment of HIV/AIDS, *Biomed Pharmacother,* **2005**, 59 452-5.

146. C. K. Chu, V. S. Bhadti, K. J. Doshi, J. T. Etse, J. M. Gallo, F. D. Boudinot, R. F. Schinazi, Brain targeting of anti-HIV nucleosides: synthesis and in vitro and in vivo studies of dihydropyridine derivatives of 3'-azido-2',3'-dideoxyuridine and 3'-azido-3'-deoxythymidine, *J Med Chem.* **1990,** 33, 2188-92.

147. C. McGuigan, R. N. Pathirana, N. Mahmood, K. G. Devine, A. J. Hay, Aryl phosphate derivatives of AZT retain activity against HIV1 in cell lines which are resistant to the action of AZT, *Antiviral Res*, **1992**, 17,311-21.
148. C. Piantadosi, C. J. Marasco Jr, S. L. Morris-Natschke, K. L. Meyer, F. Gumus, J. R. Surles, K. S. Ishaq, L. S. Kucera, N. Iyer, C. A. Wallen ,Synthesis and evaluation of novel ether lipid nucleoside conjugates for anti-HIV-1 activity, *J Med Chem*, **1991,** 34,1408-14.
149. S Chang , G. W. Griesgraber, P. J. Southern, C. R. Wagner, Amino acid phosphoramidate monoesters of 3'-azido-3'-deoxythymidine: relationship between antiviral potency and intracellular metabolism, *J Med Chem*. **2001**,18 223-31.
150. C. McGuigan, K. G. Devine, T. J. O'Connor, D. Kinchington ,Synthesis and anti-HIV activity of some haloalkyl phosphoramidate derivatives of 3'-azido-3'-deoxythymidine (AZT): potent activity of the trichloroethyl methoxyalaninyl compound, *Antiviral Res*, **1991,** 15, 255-63.
151. Mati Fridkin, Haim Tsubery, Esther Tzehoval, Ami Vonsover, Laura Biondi, Fernando Filira, Raniero Rocchi, Tuftsin-AZT conjugate: potential macrophage targeting for AIDS therapy. *J Pept Sc*i, **2005,** 11, 37-44.
152. Patrick Vlieghe, Thierry Clerc, Christophe Pannecouque, Myriam Witvrouw, Erik De Clercq, Jean-Pierre Salles, Jean-Louis Kraus, Synthesis of new covalently bound kappa-carrageenan-AZT conjugates with improved anti-HIV activities, *J Med Chem*, **2002,** 14, 1275-83.
153. H. Mirchandoni, Y.W. Chien, Drug delivery approaches for anti-HIV drugs, *Int. J. Pharm*, **1993**, 951–21.
154. S.K. Aggarwal, S.R. Gogu, S.R.S. Rangon, K. Agrawal, Synthesis and biological evaluation of prodrugs of zidovudine (and notes therein), *J. Med. Chem*, **1990**, 33, 1505–1510/
155. J.M. Harris, Introduction to biotechnical and biomedical applications of poly (ethylene glycol), in: J.M. Harris (Ed.), Poly (ethylene glycol) Chemistry, Plenum Press, New York, **1992**, pp. 1–14.

156. L. Thoren, The dextrans-clinical data, *Devel. Biol. Stand*, **1981**, 48, 157–167.
157. A. Martin, Kinetics, in: Physical Pharmacy, 4th Ed., Physical principles in the pharmaceutical sciences, Lea and Febiger, Philadelphia, **1993,** 284-323.
158. Guoming Sun, Chih-Chang Chu, Synthesis, characterization of biodegradable dextran-allyl isocyanate-etylamine/polyethylene glycol-diacrylate hydrogels and their in vitro release of albumin, *Carbohydrate polymers*, **2006**, 65, 273-287.