Chapter 2
Literature Review

2.1 LEUKAEMIA

Leukaemia is a cancer of the blood or bone marrow and is characterized by an abnormal proliferation of blood cells, usually white blood cells (leukocytes). It is derived from the Greek word *leukos* meaning white and *aima* meaning blood. It is part of the broad group of diseases called hematological neoplasms. In leukaemia, normal control mechanisms in the blood break down and the bone marrow starts to produce large numbers of abnormal white blood cells, disrupting production of normal blood cells and affecting the vital functions that these blood cells carry out.

2.1.1 Causes of Leukaemia

Leukemia, like other cancers, results from somatic mutations in the DNA which activate oncogenes or deactivate tumor suppressor genes, and disrupt the regulation of cell death, differentiation or division. These mutations may occur spontaneously or as a result of exposure to radiation or carcinogenic substances and are likely to be influenced by genetic factors. Cohort and case-control studies have linked exposure to petrochemicals, such as benzene, and hair dyes to the development of some forms of leukemia.

Viruses have also been linked to some forms of leukemia. For example, certain cases of ALL are associated with viral infections by either the human immunodeficiency virus or human T-lymphotropic virus (HTLV-1 and -2, causing adult T-cell leukemia/lymphoma). However, a CNN Health report says children may be offered limited protection against leukemia by exposure to certain germs. Fanconi anemia is also a risk factor for developing acute myelogenous leukemia.

Until the cause or causes of leukemia are found, there is no way to prevent the disease. Even when the causes become known, they may not be readily controllable, such as naturally occurring background radiation, and therefore not especially helpful for prevention purposes.

2.1.2 Symptoms

Damage to the bone marrow, by way of displacing the normal bone marrow cells with higher numbers of immature white blood cells, results in a lack of blood platelets, which

are important in the blood clotting process. This means people with leukemia may become bruised, bleed excessively, or develop pinprick bleeds.

White blood cells, which are involved in fighting pathogens, may be suppressed or dysfunctional. This could cause the patient's immune system to be unable to fight off a simple infection or to start attacking other body cells.

Finally, the red blood cell deficiency leads to anemia, which may cause dyspnea. All symptoms can be attributed to other diseases; for diagnosis, blood tests and a bone marrow examination are required.

Some other related symptoms:

Fever, chills, night sweats and other flu-like symptoms, weakness and fatigue , swollen or bleeding gums, neurological symptoms (headaches), enlarged liver and spleen, frequent infection, bone pain, joint pain, dizziness, nausea, swollen tonsils, diarrhea, paleness, malaise and weight loss.

2.1.3 Types of leukaemia

Leukemia is a broad term covering a spectrum of diseases. It is also categorised as either acute or chronic, reflecting the speed of progression.

Leukemia is clinically and pathologically split into its acute and chronic forms.

Acute leukemia is characterized by the rapid increase of immature blood cells. This crowding makes the bone marrow unable to produce healthy blood cells. Acute forms of leukemia can occur in children and young adults. (In fact, it is a more common cause of death for children in the US than any other type of malignant disease). Immediate treatment is required in acute leukemias due to the rapid progression and accumulation of the malignant cells, which then spill over into the bloodstream and spread to other organs of the body. Central nervous system (CNS) involvement is uncommon, although the disease can occasionally cause cranial nerve palsies.

Chronic leukemia is distinguished by the excessive build up of relatively mature, but still abnormal, blood cells. Typically taking months to years to progress, the cells are

produced at a much higher rate than normal cells, resulting in many abnormal white blood cells in the blood. Chronic leukemia mostly occurs in older people, but can theoretically occur in any age group (Harrison). Whereas acute leukemia must be treated immediately, chronic forms are sometimes monitored for some time before treatment to ensure maximum effectiveness of therapy.

Furthermore, the diseases are classified into lymphoblastic or lymphocytic leukaemia, which indicate that the cancerous change took place in a type of marrow cell that normally goes on to form lymphocytes, and myeloid or myelogenous leukaemia, which indicate that the cancerous change took place in a type of marrow cell that normally goes on to form red cells, some types of white cells, and platelets.

Combining these two classifications provides a total of four main categories: acute lymphocytic leukaemia, chronic lymphocytic leukaemia, acute myelogenous leukaemia and chronic myelogenous leukaemia.

2.1.4 Treatment options for leukemia by type

Acute lymphocytic leukaemia (ALL) is the most common type of leukemia in young children. This disease also affects adults, especially those age 65 and older. The survival rates vary by age: 85% in children and 50% in adults.

Management of ALL focuses on control of bone marrow and systemic (whole-body) disease. Additionally, treatment must prevent leukemic cells from spreading to other sites, particularly the central nervous system (CNS). In general, ALL treatment is divided into several phases:

- **Induction chemotherapy** to bring about bone marrow remission. For adults, standard induction plans include prednisone, vincristine, and an anthracycline drug; other drug plans may include L-asparaginase or cyclophosphamide. For children with low-risk ALL, standard therapy usually consists of three drugs (prednisone, L-asparaginase, and vincristine) for the first month of treatment.
- **Consolidation therapy** to eliminate any remaining leukemia cells. This typically requires one to three months in adults and four to eight months in children.

Patients with low- to average-risk ALL receive therapy with antimetabolite drugs such as methotrexate and 6-mercaptopurine (6-MP). High-risk patients receive higher drug doses of these drugs, plus additional drugs.

- **Preventive therapy (CNS prophylaxis)** to stop the cancer from spreading to the brain and nervous system. Standard prophylaxis may include radiation of the head and/or drugs delivered directly into the spine.
- **Maintenance Chemotherapy** with chemotherapeutic drugs to prevent disease recurrence once remission has been achieved. Maintenance therapy usually involves lower drug doses, and may continue for two years.
- **Bone Marrow Transplantation**, allogeneic bone marrow transplantation may be appropriate for high-risk or relapsed patients.

Chronic lymphocytic leukaemia (CLL) most often affects adults over the age of 55. It sometimes occurs in younger adults, but it almost never affects children. Two-thirds of affected people are men. The five-year survival rate is 75% (Colvin, 2003). It is incurable, but there are many effective treatments.

The primary chemotherapeutic plan is combination chemotherapy with chlorambucil or cyclophosphamide, plus a corticosteroid such as prednisone or prednisolone. The use of a corticosteroid has the additional benefit of suppressing some related autoimmune diseases, such as immunohemolytic anemia or immune-mediated thrombocytopenia. In resistant cases, single-agent treatments with nucleoside drugs such as fludarabine, pentostatin, or cladribine may be successful. Younger patients may consider allogeneic or autologous bone marrow transplantation.

Acute myelogenous leukaemia (AML) occurs more commonly in adults than in children, and more commonly in men than women. The five-year survival rate is 40% (Colvin, 2003).

The strategy in management is to control bone marrow and systemic (whole-body) disease while offering specific treatment for the central nervous system (CNS), if

involved. In general, most oncologists rely on combinations of drugs for the initial, induction phase of chemotherapy. Such combination chemotherapy usually offers the benefits of early remission (lessening of the disease) and a lower risk of disease resistance. Consolidation or "maintenance" treatments may be given to prevent disease recurrence once remission has been achieved. Consolidation treatment often entails a repetition of induction chemotherapy or the intensification chemotherapy with added drugs. By contrast, maintenance treatment involves drug doses that are lower than those administered during the induction phase.

Chronic myelogenous leukaemia (CML) occurs mainly in adults. A very small number of children also develop this disease. The five-year survival rate is 90%.

Chemotherapy with drugs such as hydroxyurea (Hydrea), busulfan (Myleran) or imatinib mesylate (Gleevec). In general, CML treatment options are divided into two groups: those that do not increase survival and those that do. Chemotherapeutic drugs such as hydroxyurea (Hydrea) and busulfan (Myleran) can normalize the blood count for a period of time, but they do not increase survival.

One treatment that improves CML survival is allogeneic bone marrow transplantation, the use of high dose chemotherapy and radiation followed by infusion of a donor bone marrow.

Hairy cell leukaemia (HCL) is sometimes considered a subset of CLL, but does not fit neatly into this pattern. About 80% of affected people are adult men. There are no reported cases in young children. HCL is incurable, but easily treatable. Survival is 96% to 100% at ten years (Else et al., 2005). Patients who need treatment usually receive either one week of cladribine, given daily by intravenous infusion or a simple injection under the skin, or six months of pentostatin, given every four weeks by intravenous infusion. (Else et al., 2005).

Table 2.1 lists some of the most commonly used drugs in leukaemia with there formulations available and dosage regimen.

Table 2.1. Drugs used in leukaemia: their formulations and dosage regimen

Drug	Description	Formulations available	Type of Leukaemia primarily used	Dosage regimen in Leukaemia	Ref.
6-Mercaptopurine	A purine antagonist having specificity for the S phase of the cell-cycle. Oral absorption is incomplete and highly variable (5-35%); largely due to first pass metabolism in the liver.	50 mg tablet	AML, ALL, CML.	Induction: 2.5-5 mg/kg/day PO once daily. maintenance: 1.5-2.5 mg/kg/day PO once daily	McEvoy, 2006; Chabner et al, 2006
Chlorambucil	Alkylating agent of the nitrogen mustard and is not specific for cell cycle phase. Oral absorption is 70-80%	2mg tablet	CLM	Induction: Daily: 0.1-0.2 mg/kg/day Maintenance: Daily: 0.03-0.1 mg/kg/day	bccancer.bc.ca
Cyclophosphamide	Alkylating agent of the nitrogen mustard type is not cell cycle specific.	25 mg and 50 mg tablets. 200 mg, 500 mg, 1000 mg and 2000 mg vials.	AML, CLL, CML	Oral-100 mg/m^2 (range 75-100 mg/m^2) once daily for 14 consecutive days IV for 3 weeks- 600 mg/m^2 (range 500-1000 mg/m^2) for one dose on day 1	Bristol-Myers Squibb, 2004 Crom et al., 1987

Cytarabine	A synthetic pyrimidine nucleoside, is cell cycle phase-specific for the S-phase, ineffective when administered orally.	100 mg, 500mg, 1 g, and 2 g cytarabine vials.	AML, ALL, CML	IV- 200 mg/m^2 IV over 24 hours for 5 consecutive days High dose therapy- 3,000 mg/m^2 IV every 12 hours for 2-6 consecutive days SC- 20 mg/m^2 SC once daily for 10 consecutive days IT-50 mg IT for one dose once weekly	Mayne Pharma, 2003
Etoposide	A semisynthetic derivative of the podophyllotoxins, inhibits DNA topoisomerase II, is cell cycle dependent and phase specific, affecting mainly the S and G_2 phases	Soft gelatin 50 mg capsules; etoposide injection as multi-dose vials of 5, 25 and 50 ml at a concentration of 20 mg/mL	AML, ALL	50 mg – 100 mg PO once daily for 3-10 days (total dose per cycle 150 mg-1000 mg/m^2) 100 mg/m^2 IV once daily for 2 consecutive days starting on day 1 High dose therapy- 1800-2400 mg/m^2 IV continuous infusion over 24-34 h (total dose per cycle 1800-2400 mg/m^2	rxlist.com
Hydroxyurea (Hydrea)	Acts primarily as inhibitor of ribonucleotide reductase and is specific for S-phase cycle. Readily absorbed from GIT.	500 mg capsules	CML	daily: 20-30 mg/kg po	rxlist.com

Idarubicin	Anthracycline analogue of daunorubicin, inhibition of DNA topoisomerase II. Rapid but erratic absorption, about 30% bioavailability	5 mL (5 mg), 10 mL (10 mg) and 20 mL (20 mg) single use vials.	AML, ALL	Induction therapy-PFS Injection 12 mg/m ^{2} daily for 3 days by slow (10 to 15 min) intravenous injection	rxlist.com
Mitoxantrone	A synthetic antineoplastic anthracenedione for intravenous use. Is not specific for cell cycle.	Mitoxantrone for injection concentrate (2 mg/ml) 5 and 10 ml vials.	AML	2 mg/m^2 daily on Days 1-3 given as an intravenous infusion	rxlist.com; bccancer.bc.ca
Thioguanine	Is a purine antagonist, specific for the S phase of the cell cycle. Oral absorption is incomplete and variable (14-46%).	40 mg tablet	AML	2-3 mg/kg/day or 75-200 mg/m^2/day PO once daily in 1-2 divided doses for 5-7 days or until remission is attained.	rxlist.com; bccancer.bc.ca
Vincristine	Antimicrotubule agent, blocks mitosis by arresting cells in the metaphase. Cell cycle phase-specific for M phase and S phase. Has erratic oral bioavailability	A sterile, preservative-free, single use vial in 1mg/mL solution. 1, 2, and 5 mL vials	AML	0.8-1.4 mg/m2 IV for one dose daily on day 1 (total dose per cycle 0.8-1.4 mg/m2)	Novopharm., 1999

2.1.5 Drawbacks associated with conventional therapy

The conventional therapy used presently with these drugs to treat leukaemia suffers from serious drawbacks like- they give pains to the patients even with as little as effective concentration and cause severe side effects. These side effects are mainly caused by the lack of specificity of these anticancer drugs, that is, the anticancer drugs not only kill cancer cells but also inhibit normal cell growth and eventually lead to necrosis of normal cells. Moreover the conventional drugs kill the leukemia cells in the blood but are not effective in penetrating into the spinal chord or brain. Leukemia cells flourish into these central nervous systems hideouts, eventually causing fatal complications.

Drugs listed in Table 2.1 are used in leukaemia therapy. Mercaptopurine has incomplete and highly variable (5-35%) oral bioavailability; largely due to first pass metabolism in the liver (Lafolie et al., 1989). Idarubicin has erratic absorption and its bioavailability is about 30% (Hollingshead and Faulds, 1991). Hence taking these drugs by the oral route reduces the actual amount reaching the site of action. However, administering bolus doses of these drugs systematically has side effects (Feng and Chien, 2003). Doxorubicin has a number of undesirable side effects such as cardiotoxicity and myelosuppression which leads to a very narrow therapeutic index. Other crucial problems associated with the conventional drugs for leukaemia is drug resistance at the cellular level. Also, anticancer drugs generally feature large volumes of distribution, as cancer fighting drugs are toxic to both tumor and normal cells; the efficacy of chemotherapy is often limited by important side- effects.

Conventional drug delivery systems cannot release drugs in a sustained manner, the amount of released drug decreases with time, which plays a detrimental role in maintaining a constant level of drug in blood.

Controlling the release of the drug would decrease unwanted side effects that might occur due to the natural circadian fluctuations of chemical levels throughout the body (Hermida et al., 2001). The overall benefit of the improvements in disease treatment by using a modified release system would be an increase in patient compliance and quality of life.

2.2 NANOPARTICULATE DRUG DELIVERY SYSTEMS FOR CANCER DRUGS

One of the best ways to increase the efficacy and reduce the toxicity of cancer drugs is to direct the drug to its target and maintain its concentration at the site for a sufficient time for therapeutic action to take effect. The therapeutic index of most of the anti cancer drugs currently being used would be improved if they were more efficiently delivered to their biological targets through appropriate application of nanotechnologies (Kawasaki et al., 2005).

The nanometer size-ranges of these delivery systems offer certain distinct advantages for drug delivery. Due to their sub-cellular and sub-micron size, nanoparticles can penetrate deep into tissues through fine capillaries, cross the epithelial lining and are generally taken efficiently by the cells.

Nanoparticles may consist of either a polymeric matrix (nanospheres) or of a reservoir system in which an oily or aqueous core is surrounded by a thin polymeric wall (nanocapsules). Suitable polymers for nanoparticles include poly(alkyl cyanoacrylates), poly(methylidene malonate) and polyesters such as poly(lactic acid), poly(glycolic acid), poly(e-caprolactone) and their copolymers (Barratt, 2003).

Nanoparticles incorporating anticancer agents can overcome resistances to drug action, increasing the selectivity of drugs towards cancer cells and reducing their toxicity towards normal cells.

The accumulation mechanism of intravenously injected nanoparticles in cancer tissues relies on a passive diffusion or convection across the hyperpermeable tumor vasculature. Additional retention of the colloidal particles in the tumor inter- stitium is due to the compromised clearance via lymphatics. This so-called ''enhanced permeability and retention effect'' results in an important intratumoral drug accumulation that is even higher than that observed in plasma and other tissues (Maeda, 2001). Controlled release of the drug content inside the tumoral interstitium may be achieved by controlling the nanoparticulate structure, the polymer used and the way by which the drug is associated with the carrier. Certain types of nanoparticles are able to overcome multidrug resistance

mediated by the Pglycoprotein, such as poly(alkyl cyanoacrylate) nanoparticles (Authier et al., 2003). Nanoparticles loaded with anticancer agents can successfully increase drug concentration in cancer tissues and also act at cellular levels, enhancing antitumor efficacy. They can be endocytosed/ phagocytosed by cells, with resulting cell internalization of the encapsulated drug.

Nanoparticles of biodegradable polymers can provide controlled and targeted delivery of the drug with better efficacy and fewer side-effects. Lipophilic drugs, which have some solubility either in the polymer matrix or in the oily core of nanocapsules, are more readily incorporated than hydrophilic compounds, although the latter may be adsorbed onto the particle surface. Nanospheres can be formed from natural macromolecules such as proteins and polysaccharides, from non polar lipids, and from inorganic materials such as metal oxides and silica (Barratt, 2003).

Yoo et al. (2000) conjugated doxorubicin and prepared its Nanoparticles having 100 nm diameter. It was found that mice injected intravenously with both dextran-doxorubicin conjugates and the conjugates encapsulated in nanoparticles showed a decrease in the tumor volume. Biodegradable nanoparticle formulations of Paclitexel have been studied by Wang et al. (1996) and have shown comparable activity to traditional formulations and much faster administration.

Applications of nanoparticulate drug delivery systems for cancer drugs

- Nanoparticles can be made long circulating in blood (Muller et al., 2004, Wissing et al., 2004).
- Drug-loaded nanoparticles are able to penetrate the blood brain barrier, and have been shown to greatly increase therapeutic concentrations of anticancer drugs in brain tumors (Koziara et al., 2004).
- Nanoparticles improve the therapeutic index of the anti cancer drugs.
- Drug-loaded nanoparticles allow entering the cancer cell and act as an intracellular anti-cancer drug reservoir (Panyam, 2004).

2.2.1 Drawbacks associated with conventional Nanoparticles

The introduction of synthetic material into the body always affects different body systems, including the defence system. Synthetic polymers are usually thymus independent antigens with only a limited ability to elicit antibody formation or to induce a cellular immune response against them. Drug associated with the colloidal carriers modifies its biodistribution profile, as it is mainly delivered to the mononuclear phagocytes system (liver, spleen, lungs and bone marrow). Once in the bloodstream, surface non-modified nanoparticles, conventional nanoparticles, are rapidly opsonized and massively cleared by the fixed macrophages of the mononuclear phagocytes system organs.

The conventional nanoparticles are naturally concentrated within macrophages they can be used to deliver drugs to these cells. However, if the target site is not the macrophages, then their distribution has to be changed. The opsonization or removal of nanoparticulate drug carriers from the body by the mononuclear phagocytic system (MPS), also known as the reticuloendothelial system (RES), is a major obstacle to the conventional nanoparticles. The macrophages of the MPS have the ability to remove unprotected nanoparticles from the bloodstream within seconds of intravenous administration, rendering them ineffective as site-specific drug delivery devices (Gref et al., 1994). Nanoparticles having particle size < 100 nm and/or a hydrophilic surface are needed to reduce opsonization reactions and subsequent clearance by macrophages (Storm et al., 1995).

2.2.2 Sterically Stabilized Nanoparticles

Since the usefulness of conventional nanoparticles is limited by their massive capture by the macrophages of the mononuclear phagocytes system after intravenous administration, systems with modified surface properties to reduce the disposition of plasma proteins and the recognition by phagocytes have been developed (Storm et al., 1995). These are known as sterically stabilized carriers or "stealth carriers" and may remain in the blood compartment for a considerable time. The hydrophilic polymers poly(ethylene glycol), poloxamines, poloxamers, polysaccharides have been used to efficiently coat conventional nanoparticles' surface (Illum et al., 1997). The coating provides a dynamic

cloud of hydrophilic and neutral chains at the particle surface that keep away plasma proteins (Jeon et al., 1991). Poly (ethylene glycol) (PEG) has been introduced at the surface either by adsorption of surfactants or by using block or branched copolymers, usually with poly(lactide) (Gref et al., 1995). These Stealth^{TM} nanoparticles are characterized by a prolonged half-life in the blood compartment and by extravasation into sites where the endothelium is more permeable, such as solid tumors, regions of inflammation and infection. Consequently, such long-circulating Nanoparticles are supposed to be able to target directly most tumors located outside the mononuclear phagocytes system. The small size and the hydrophilicity of the carrier device, as well as a sustained release of the drug could improve the efficacy of anticancer chemotherapy. Coating conventional nanoparticles with surfactants allow them to act as long-circulating carrier or they can cross blood brain barrier (Moghimi et al.,2001). The surface characteristics of nanospheres prepared from poly(lactide-co-glycolide) copolymers have been optimized to reduce their interactions with plasma proteins and to increase their circulating half-life (Dunn et al., 1997). Biodegradable nanospheres prepared from PLGA coated with poly(lactide)- PEG diblock copolymers showed a significant increase in blood circulation time and reduced liver uptake in a rat model compared with non-coated PLGA nanospheres (Stolnik, 1994).

2.3 PLGA BASED NANOPARTICULATE DRUG DELIVERY SYSTEMS

There is significant interest in recent years in developing biodegradable nanoparticles as a drug delivery system (Sahoo and Labhasetwar, 2003). Biodegradable polymers can be either natural or synthetic. In general, synthetic polymers offer greater advantages than natural ones in that they can be tailored to give a wider range of properties (Lewis et al., 1990). The general criteria for selecting a polymer for use as a degradable biomaterial are to match the mechanical properties and the degradation rate to the needs of the application.

Although a number of different polymers have been investigated for formulating biodegradable nanoparticles, poly(D,L-lactide-co-glycolide) (PLGA) and poly lactic acid (PLA) are the most extensively studied polymers for controlled drug delivery applications (Bala et al., 2004). The lactide/glycolide polymers chains are cleaved by hydrolysis into natural metabolites (lactic and glycolic acids), which are eliminated from the body by the citric acid cycle (Shive and Anderson, 1997). PLGA is the most extensively studied and preferred polymer for drug delivery through NPs because of its ease of preparation, commercial availability, versatility, biocompatibility, and hydrolytic degradation into harmless products. PLGA polymers are approved by the U.S. Food and Drug Administration for human use (Edlund and Albertsson, 2002).

Applications of PLGA based Nanoparticulate drug delivery systems

1. Increase in cellular uptake

Cystatin-loaded poly(lactic-co-glycolic acid) (PLGA) nanoparticles were cytotoxic towards mammary MCF-10A neoT cells, but free cystatin at the same concentrations was not. Poly (lactide-co-glycolide) nanoparticles were rapidly internalized into MCF-10A neoT cells, whereas the uptake of free cystatin was very slow (Cegnar et al., 2004).

2. Delivery to lymph node

Poly(lactide-co-glycolide)(PLGA) nanoparticles have been investigated for lymph node drug delivery agents. By modifying the surface of the PLGA nanoparticles with block co-polymers, PLGA nanoparticles deliverd up to 17% of a subcutaneously injected dose to regional lymph nodes have been developed (Hawley et al., 1997).

3. Oral delivery

PLGA have been explored for the preparation of nanoparticulate formulations of insulin for oral delivery (Carino et al., 2000). Spray-dried PLGA nanoparticles have been investigated for the oral delivery of Amifostine (Pamujula et al., 2004). Heparin-loaded PLGA nanoparticles prepared by the emulsification evaporation process showed promise as new oral heparin delivery systems (Jiao et al., 2001).

4. Parenteral delivery

A sustained release formulation of Nimodipine using PLGA 50:50 and 85:15 was prepared for intravenous use (Mehta et al., 2007). Docetaxel loaded carboxylated PLGA–b–PEG NPs were prepared for systemic administration and were tested for tumor targeting efficiency and biodistribution in a xenograft mouse model of prostate cancer (Cheng et al., 2007).

5. Ophthalmic delivery

It has been observed that large particles may irrititate the eye. Consequently, smaller particles are preferred for ophtalmic delivery systems (Zimmer and Kreuter 1995). Yoncheva et al. (2003) investigated the possibility for incorporation of pilocarpine hydrochloride in PLGA nanoparticles for ocular delivery.

5. Treatment of tuberculosis

PLGA nanoparticles have been successfully prepared and used to encapsulate isoniazid, rifampicin and pyrazinamide, which are the three frontline drugs employed in the treatment of tuberculosis (Sharma et al., 2004)

6. Sustained release

Recombinant human granulocyte colony-stimulating factor (rhG-CSF) loaded in biodegradable PLGA nanoparticles exhibited a sustained release profile over an extended period of 15 days (Choi and Park, 2006). Therapeutic proteins were sustained released using various biodegradable polymeric formulations (Langer, 1990; Cohen et al., 1991). Cyclosporin A-loaded PLGA NP prepared by high-pressure homogenizer showed a zero-order release kinetics upto 3 weeks (Lee et al., 2002). Praziquantel-loaded PLGA nanoparticles released 25% of the entrapped drug in 24h (Mainardes and Evangelista, 2005). Bovine serum albumin (BSA) loaded PLGA NP were prepared by double emulsion technique and the release of BSA was sustained for a period of 4 weeks (Lamprecht et al., 2000). Double emulsion method based on high-pressure homogenization was used to prepare DNA-containing PLGA nanoparticles and the in-vitro release of DNA was sustained upto 250h (Rizkalla et al., 2006). The release of the anti-inflammatory agent dexamethasone from PLGA nanoparticles embedded in alginate hydrogel matrices was sustained over 2 weeks (Kim and Martin, 2006).

7. Long circulating carriers

Dunn et al. (1997) prepared biodegradable poly(lactide-co-glycolide) (PLGA) nanospheres in the size range 80–150 nm and then surface modified them with poly(ethylene glycol) (PEG) either by adsorption of Poloxamer 407 or Poloxamine 904. These surface modified NP were effective in prolonging the circulation of the PLGA nanospheres upto 180h in rabbits. The blood circulation half-life of Rose Bengal was significantly extended by its delivery in either Poloxamer 407 or Poloxamine 908 coated PLGA nanoparticles. In contrast, free Rose Bengal was cleared from the circulation within minutes of injection, with only 8% remaining in the blood after 5 min (Redhead et al., 2001). The effect of nanoparticle dose on the biodistribution and pharmacokinetics of conventional PLGA and stealth PLGA–mPEG nanoparticles was investigated and it was found that the PLGA nanoparticles were rapidly removed from blood, exhibiting biological half-lives in the range of 13–35 s, whereas the PLGA–mPEG nanoparticles exhibited prolonged residence in blood with biological half-lives in the order of 7 h (Panagi et al., 2001).

8. Cancer therapy

Cancer therapy is one of the most important applications of biodegradable nanoparticles made of PLGA. Paclitaxel has been encapsulated into PLGA nanoparticles (Mo and Lim, 2005). 9-nitrocamptothecin, a novel anticancer, lipophilic drug was loaded in PLGA nanoparticles for intravenous administration and its therapeutic index was improved (Derakhshandeh et al., 2006). Doxorubicin, a widely used anticancer drug, has been encapsulated into PLGA nanoparticles and shown to sustain the release of the drug for 30 days (Yoo et al., 2000). PLGA NPs for paclitaxel formulation were prepared with an improved therapeutic index and adverse effects associated with of current clinical formulation of TAXOL due to adjuvant Cremophor EL were reduced (Win and Feng, 2006).

2.3.1 Methods for Preparation of PLGA Nanoparticles

Commonly used methodologies for preparation of PLGA based NP include solvent evaporation (Scholes et al., 1993), the spontaneous emulsification/ solvent diffusion (Niwa, 1993), nanoprecipitation or solvent displacement (Bilati et al., 2005) and emulsion polymerization techniques. The details of these methods is shown in Fig. 2.1 and Table 2.2. The method of choice depends on the polymer and the drug type, as well as the required particle size distribution and polydispersity indices.

Solvent evaporation method

One of the most popular techniques for the preparation of PLGA nanoparticles is emulsification/solvent evaporation method. In the solvent evaporation method, the polymer is dissolved together with the drug in an organic solvent and the mixture is then emulsified to form either an oil-in-water nanoemulsion (for encapsulation of hydrophobic drugs) or water in-oil nanoemulsion (for encapsulation of hydrophilic drugs) using suitable surfactants. Nanoparticles are then obtained following evaporation of the solvent and can be concentrated by filtration, centrifugation or lyophilization. Vandervoort et al. (2004) added a homogenization step to the emulsification methods and influence of the homogenisation procedure on the size, the zeta potential value, drug encapsulation and drug release of the particles prepared was studied.

Spontaneous emulsification/solvent diffusion method

The spontaneous emulsification/solvent diffusion method is a modified version of the solvent evaporation technique, which utilizes a water-soluble solvent (e.g. methanol or acetone) along with a water-insoluble one such as chloroform. As a result of the spontaneous diffusion of the water-soluble solvent into the water-insoluble phase, an interfacial turbulence is created leading to the formation of nanoparticles.

Nanospheres containing indomethacin or 5-fluorouracil (as model water-insoluble and water-soluble drugs) were prepared using a spontaneous emulsification solvent diffusion method with a high speed homogenizer. The resulting particles were found to be 400-600 nm in diameter (Niwa et al., 1993)

Nanoprecipitation method

Nanoprecipitation, is a versatile and simple method. This is based on spontaneous formation of nanoparticles during phase separation, which is induced by slow addition of the diffusing phase (polymer-drug solution) to the dispersing phase (a non-solvent of the polymers, which is miscible with the solvent that solubilizes the polymer). The dispersing phase may contain surfactants. Depending on the solvent choice and solvent/non-solvent volume ratio, this method is suitable for encapsulation of both water-soluble and hydrophobic drugs, as well as protein-based pharmaceuticals (Bilati et al., 2005).

Paclitaxel could be incorporated at very high loading efficiencies, nearing 100%, using the nanoprecipitation method using acetone and PLGA having particles of 117–160 nm diameters (Fonseca et al., 2002).

Salting –out

This technique involves the addition of polymer and drug solution in a slightly water-miscible solvent such as acetone to an aqueous solution containing the salting out agent and a colloidal stabilizer under vigorous mechanical stirring. When this o/w emulsion is diluted with a sufficient volume of water, it induces the formation of nanoparticles by enhancing the diffusion of acetone into the aqueous phase (Allemann et al., 1992; Konan et al, 2002). The major advantage of salting out method is the incorporation of high amounts of polymer and drug, and easy scale-up of the process. The limitations of the

method are that it is suitable for the encapsulation of only lipophilic drugs and intensive purification of the nanospheres is required.

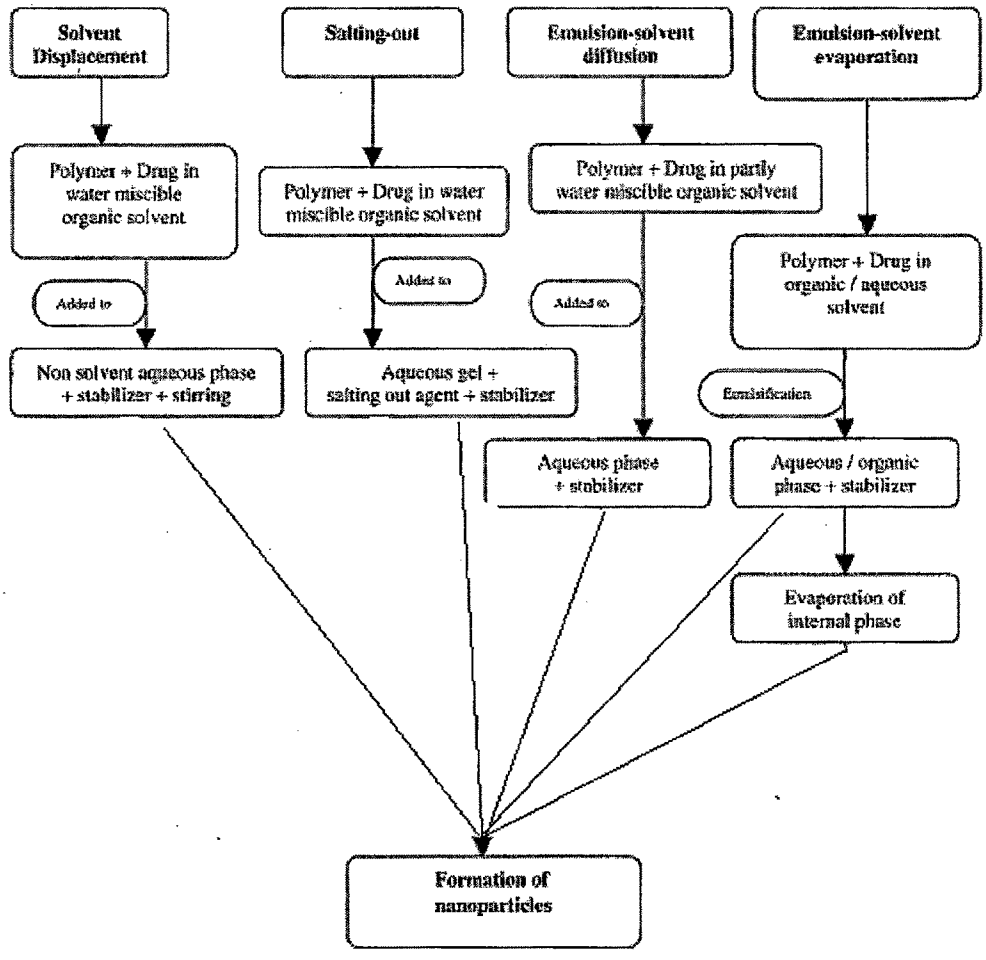


Fig. 2.1: Methods for preparation of PLGA Nanoparticles

Table 2.2

Methods for preparation of Nanoparticles

Method	Drug	Solvent	Stabilizer	Size (nm)	Reference
Solvent diffusion	Doxorubicin	Acetone	Pluronic F-127	200	Yoo et al., 2000
	-	Acetone/ DCM	PVA	200–300	Murakami et al., 1999
	Estrogen	Propylene carbonate	PVA or DMAB	100	Kwon et al., 2001
Solvent displacement	Tyrphostins	Acetone/ MC	Pluronic F68	123	Fishbein et al.2000
	-	Acetone/ ethyl acetate	Poloxamer 188	110	Jung et al., 2000
Nanoprecipitation	Isradipine	Acetone	Pluronic F68	110–208	Verger et al., 1998
	Procaine HCl	Acetonitrile	–	157	Govender et al., 1999
Solvent evaporation	Progesterone	Dichloromethane	–	193–335	Matsumoto et al., 1999
	Haloperidol	Dichloromethane	PVA	800	Cheng et al., 1998
	Paclitaxel	Methylene chloride	PVA	150-625	Suh et al., 1998
Multiple emulsion	Tetanus toxoid	Ethyl acetate	–	100	Tobio et al., 2000
	U-86983 (Anti-proliferative agent)	Dichloromethane	PVA	70–160	Song et al., 1998
	Bovine serum albumin	Acetone	PVA	100	Song et al., 1997
Interfacial deposition	Rose Bengal	Acetone	–	135	Redhead et al., 2000
Phase inversion	Insulin	Methylene chloride	–	500	Carino et al., 2000

2.3.2 Characterization of Nanoparticles

Characterization of the nanoparticle is important before putting them to pharmaceutical application. After preparation, nanoparticles are characterized at two levels. The physicochemical characterization consists of the evaluation of the particle size, size distribution, and surface properties (composition, charge, hydrophobicity) of the nanoparticles. The biopharmaceutical characterization includes measurements of drug encapsulation, in vitro drug release rates, and in vivo studies revealing biodistribution, bioavailability, and efficacy of the drug.

There are many sensitive techniques for characterizing nanoparticles, depending upon the parameter being looked at; laser light scattering (LLS) or photon correlation spectroscopy (PCS) for particle size and size distribution (Vandervoort et al., 2004); scanning electron microscopy (SEM), transmission electron microscopy (TEM) (Jeon et al., 2000) and atomic force microscopy (AFM) (Lamprecht et al., 2000) for morphological properties; X-ray photoelectron spectroscopy (XPS), Fourier transform infrared spectroscopy (FTIR),a and nuclear magnetic resonance spectroscopy (NMR) for surface chemistry; and differential scanning calorimetry (DSC) for thermal properties (Gref et al., 1995). Parameters such as density, molecular weight, and crystallinity affect release and degradation properties, where as surface charge, hydrophilicity, and hydrophobicity significantly influence interaction with the biological environment.

Particle size and Morphology: Nanoparticle size is critical not only in determining its release and degradation behaviour (Dunne et al., 2000) but also in determining the efficacy of the therapeutic agent by affecting tissue penetration or even intracellular uptake. Particle size can be determined by Photon correlation spectroscopy (Vandervoort and Ludwig, 2002), Scanning electron microscopy (Mi et al., 2003), Transmission electron microscopy (Stolnik et al., 1994), Atomic force microscopy (Lee et al., 2002,) and Fluorescent NP can be visualized by Confocal Laser Scanning Microscope (De and Robinson, 2004).

Surface chemistry analysis: X-ray photon spectroscopy (Jeon et al., 2000), Fourier transform infrared spectroscopy (Mainardes and Evangelista, 2005), Nuclear magnetic

spectroscopy (Gref et al., 1994) are the techniques employed to analyze the surface chemistry.

Crystallinity: The physical state of both the drug and the polymer are determined because this will have an influence on the in vitro and in vivo release characteristics of the drug. The crystalline behaviour of polymeric nanoparticles is studied using X-ray diffraction and thermo-analytical methods such as differential scanning calorimetry (Ubrich n et al., 2004) (DSC) and differential thermal analysis (DTA) (Oh et al., 1999). DSC and X-ray diffraction techniques are often combined to get useful information on the structural characteristics of both drugs and polymers.

Surface charge: Zeta potential is measure of the surface charge of the nanoparticles. The zeta potential value can influence particle stability and mucoadhesion as well as intracellular trafficking of nanoparticles as a function of pH. High zeta potential values, either positive or negative, should be achieved in order ensure stability and avoid aggregation of the particles. The extent of surface hydrophilicity can then be predicted from the values of zeta potential (Soppimath et al., 2001). Surface charge is generally determined by well-known electrophoresis method with the help of zetasizer (Panagi et al., 2001)

Hydrophobicity: Hydrophobicity determines the distribution of nanoparticles in the body after administration. Hydrophilic particles tend to remain in the blood for a longer time. Hydrophilicity is determined by water contact angle measurements or hydrophobic interaction chromatography (Kreuter, 1994)

2.3.3 Drug Loading and Encapsulation efficiency

Drug loading is crucial as it affects the amount of drug released. Nanoparticles are much smaller in size compared to microparticles and hence comparatively less amount of total drug can be loaded. Table 2.3 shows different drug loading, loading efficiency and their particle size. Yoo et al., (2000) chemically conjugated Doxorubicin to a terminal end group of poly(D,L-lactic-co-glycolic acid) (PLGA) by an ester linkage and the doxorubicin–PLGA conjugate was formulated into nanoparticles. There was a difference in loading efficiency and loading amount in the formulated nanoparticles with and without conjugation. The nanoparticles containing the conjugates showed greater loading

amount (1.9% w/w) and loading efficiency over 95% compared to those encapsulated with free doxorubicin, which exhibited only 0.66% w/w loading amount and loading efficiency of only 33.3 %. This was supported with the fact that doxorubicin–PLGA conjugate had a limited water solubility which suppress the tendency of the conjugate to escape out of nanoparticles into aqueous medium during the formulation process. On the other hand, the nanoparticles formulated with free doxorubicin exhibited lower loading efficiency and amount due to their greater aqueous solubility (Yoo et al., 2000).

Table 2.3
Drug loading, loading Efficiency and NP size by using PLGA 50:50

Sr. No.	Drug	Drug Loading (%)	Encapsulation Efficiency (%)	Size (nm)	Ref
1	Doxorubicin	0.66	33.3%	-	Yoo et al., 2000
2	Dox-Conjugated to PLGA	1.9	95%	230	Yoo et al., 2000
3	Triclosan	0.84	70.04	219	Segundo et al., 2005
4	Triclosan	3.93	85.12	246	Segundo et al., 2005
5	Triclosan	5.62	63.88	272	Segundo et al., 2005
6	Triclosan	11.46	71.40	308	Segundo et al., 2005
7	Triclosan	16.18	72.23	354	Segundo et al., 2005
8	Human Granulocyte Colony-Stimulating Factor	5.0	37.8	257	Choi et al., 2006
9	Cyclosporin A-	8.58	94.4	312	Lee et al., 2002
10	Enalaprilat	13.2	46.4	204	Ahlin et al. 2002

2.3.4 Drug release studies from PLGA Nanoparticles

The release profile of drugs from nanoparticles depends on the physicochemical nature of the drug molecules as well as the matrix (Niwa, 1993). Factors include mode of drug attachment and/or encapsulation (e.g. surface adsorption, dispersion homogeneity of drug molecules in the polymer matrix, covalent conjugation), the physical state of the drug within the matrix (such as crystal form), and parameters controlling matrix hydration and/or degradation. Generally, rapid release occurs by desorption, where the drug is weakly bound to the nanosphere surface. If the drug is uniformly distributed in the polymer matrix, the release occurs either by diffusion (if the encapsulated drug is in crystalline form, the drug is first dissolved locally and then diffuses out) or erosion of the matrix, or a combination of both mechanisms. Erosion can be further subdivided into either homogeneous (with uniform degradation rates throughout the matrix) or heterogeneous (where degradation is confined at the surface) processes (Panyam et al., 2004). Parameters such as polymer molecular weight distribution, crystallinity, hydrophobicity/hydrophilicity, melting and glass transition temperature, polymer blends and prior polymer treatment (e.g. oxygen-plasma treatment) all control the extent of matrix hydration and degradation. For instance, in the case of aliphatic polyesters, their degradation time is shorter for low molecular weight polymers, more hydrophilic polymers, more amorphous polymers and copolymers with high glycolide content. Larger particles have a smaller initial burst release and longer sustained release than smaller particles. In addition, the greater the drug loading the greater the burst and the faster the release rate.

The release characteristics of PLGA Nanoparticles are one of the most important features of the drug /polymer formulations because of the proposed application in sustained drug delivery. Table 2.4 lists the drugs formulated into Nanoparticles having sustained release from 4 hours to 96 days by use of PLGA.

Table 2.4
PLGA Nanoparticles for sustaining the release of the drug

Sr. No.	Type of PLGA	Drug	Drug release Duration	Reference
1.	PLGA, Resomer® RG 502, MW 8000	Triclosan	4 hours	Segundo et al., 2005
2.	PLGA, 50/50 (Resomer RG® 503 H MW 48 kDa	Chicken cystatin,	5 hours	Cegnar et al., 2004
3.	PLGA 50:50	Enalaprilat	24 hours	Ahlin et al. 2002
4.	PLGA, Resomer RG 503, 52 : 48, MW 40000	Pilocarpine HCl	24 hours	Vandervoort et al., 2004
5.	PLGA 85:15, 75:25 and 50:50	Norfloxacin	25 hours	Jeon et al., 2000
6.	Resomer RG 503	ciprofloxacin HCl	25 hours	Dillen et al., 2004
7.	PLGA 50/50 MW of 8300	G-CSF	7 days	Choi and Park, 2006
8.	PLGA 50:50	Cyclosporin A	21 days	Lee et al., 2002
9.	PLGA 50:50 MW 40 000	Bovine serum albumin	28 days	Lamprecht et al., 2000
10.	PLGA 85:15	Doxorubicin	35 days	Yoo et al., 2000
11.	PLGA) 50:50	dexamethasone phosphate BSA	70 days.	Song et al., 1997
12.	(PLGA) 50/ 50, Resomer	interferon-alpha (IFN-alpha)	96 days	Sanchez et al., 2003

For example, PLA nanoparticles containing 16.7% savoxepine released 90% of their drug load in 24 h, as opposed to particles containing 7.1% savoxepine, which released their content over 3 weeks (Leroux et al., 1996). The initial burst release was thought to be caused by poorly entrapped drug, or drug adsorbed onto the outside of the particles. When using polymers, which interact with a drug, like PLGA with a free COOH group and proteins, the burst release is lower and in some cases absent, and drug release is prolonged (Blanco et al, 1997; Nicoli et al., 2001).

The drug release from PLGA NP is a function of the lactide to glycolide ratio (Gasper et al, 1998). As the lactide content increases stronger hydrophobic interactions between the polymer and drug happen this retards the release of the drug. It has been reported that the water uptake capacity in PLGA increases as the glycolide ratio increases in the polymer. Rate of hydration of the polymeric materials is an important consideration for the drug release. The higher the glycolide contents in the copolymer, the faster the release of the entrapped drug.

Size of the particles also effect release of the drug. In general faster release from smaller particles seems to be due to the larger surface area and shorter length of diffusion path.

Segundo et al. prepared triclosan-loaded nanoparticles (TCS) by the emulsification–diffusion Process using poly(d,l-lactide-co-glycolide) (PLGA) and poly(d,l-lactide) (PLA). 75% of the TCS was released from PLGA-NPs at 30 min. It was observed that NPs with a greater content of TCS released faster. It has been reported that an increase in the amount of drug in the nanospheres not only increases the porosity of the system as the drug dissolves, but also, as in TCS-NPs, reduces the relative amount of polymeric material acting as a diffusional barrier. The rapid initial release of TCS was also attributed to the large surface to volume ratio of the NP geometry. Due to their size, a burst effect was observed for drug molecules entrapped into all of the NPs prepared. PLA-NPs released the drug at a slightly slower rate than PLGA-NPs with similar proportions of TCS (Segundo et al., 2005).

Choi et al. (2006) prepared PLGA nanoparticles loaded with recombinant human granulocyte colony-stimulating factor (rhG-CSF) by a spontaneous emulsion/solvent

diffusion method and the Nanoparticles showed initial burst releases of 27.6% at 12 h and 43.6% at day 1, followed by a sustained release period up to over 90% for a 1 week.

Lee et al. (2002) prepared PLGA NP of Cyclosporin A and studied its release for 21-days, during which only less than 50% of the encapsulated drug was released from the particles. No significant burst effect was observed. The prolonged CyA release from PLA and PLGA particles was attributed to the absence of surface drug crystals and the formation of a homogeneous matrix with the drug randomly distributed throughout the polymer particle at low drug loading in this system.

Lamprecht et al. (2000) used Bovine serum albumin (BSA) as a model protein drug having high solubility in water and used the double emulsion technique to load it in to PLGA NPs. NPs were investigated in order to characterize their properties as a controlled protein delivery system. The in vitro protein release profiles obtained for each formulation showed a three phase composition, a first initial burst release due to the drug desorption from the particle surface; a plateau for an certain period, resulting from the only diffusion of the drug dispersed into the polymer matrix; and a constant sustained release of the drug over four weeks resulting from the diffusion of the protein through the polymer wall as well as its erosion.

Jeon et al., (2000) prepared surfactant free PLGA nanoparticles by dialysis method without surfactant using various solvent and studied possibility of nanoparticles as a drug carriers using norfloxacin (NFX) as a hydrophobic drug. The authors concluded that the hydrophobic drug loaded into nanoparticles would release slower at higher drug contents which is differenced with hydrophilic water soluble drugs as NFX release was slower rate kinetics from the nanoparticles with higher drug contents. At low drug content, NFX was said to be present as a molecular dispersion inside the Nanoparticles (Gref et al., 1994).

Saxena et al. (2004) prepared indocyanine green (ICG)-loaded biodegradable nanoparticles by PLGA by a modified spontaneous emulsification solvent diffusion method. The release profile of ICG from ICG-loaded PLGA Nanoparticles appeared to have two components. First an initial exponential phase releasing 78% of ICG (within 8 h) followed by a slow phase releasing up to 80% of ICG (within next 16 h). The initial exponential release phase of ICG was said to be due to ICG which was adsorbed or close

to the surface of the nanoparticles and due to diffusion of the dissolved ICG within the PLGA core of the nanoparticle into the release medium. The large surface to volume ratio of the nanoparticle geometry and the water-soluble nature of ICG was also said to be the contributing factors for the initial release pattern of ICG from ICG-loaded PLGA Nanoparticles. The exponential type of release pattern for PLGA nanoparticles have been reported by several authors (Govender et al., 1999; Jeon et al., 2000).

2.3.5 Mechanism of drug release from PLGA NP

Higuchi's equation (Higuchi, 1961) was originally developed to explain the drug release from an ointment base. Nanoparticles can be considered as a homogeneous polymer matrix-type delivery system in which the drug molecularly dispersed and hence many authors have applied Higuchi's equation. Higuchi's equation can be extensive for matrix systems, considering that the depletion zone moves to the center of the NPs as the drug is released. The equation:

$$Q = (2ADCst)1/2 \qquad (2.1)$$

indicates that the cumulative amount of drug released (Mt) per unit surface (S) is proportional to the square roots of A, the total amount of drug in a unit volume of the matrix; D, the diffusion coefficient of the drug in the matrix; Cs, the solubility of drug in the polymeric matrix; and t, the time. The factor $(2ADCs)1/2S$ is the drug release rate constant (KH). When the Higuchi's equation was applied (Mt versus $t1/2$) by Segundo et al to Triclosan loaded NPs a straight line having $R2 > 0.99$ was obtained, suggesting that the release rate can be satisfactorily described by this model (Segundo et al, 2005).

Mode of drug release from the matrices of PLGA NP can be also fitted to the power law equation (Peppas, 1985) -

$$M_t / M_0 = K \cdot t^n \qquad (2.2)$$

where M_t / M_0 is the fraction of drug released up to time t, K is a constant incorporating the structural and geometric characteristics of the release device, and n is the release exponent indicative of the mechanism of release. The equation, proposed by Peppas in

1985, has proven useful for the study of the release mechanism. The exponent n describes the release kinetics; with n=0.5 for square root of time kinetics, n=1.0 for zero-order kinetics, and zero-order kinetics prevailing if n>0.66.

For instance in the work done by Lee et al the prepared PLGA NP of Cyclosporin A showed that the calculated values of n were greater than 0.66, indicating that the release of CyA from PLA/PLGA particles approximated zero-order release characteristics. The release of CyA was slow from these particles with low drug loading (Lee et al., 2002). The authors proposed two possible mechanisms involved in the release of CyA from the particles: the dissolution diffusion of drug from the matrices as well as the matrix erosion resulting from degradation of the polymers.

According to the model developed by Baker and Lonsdale, for a drug incorporated in a spherical matrix, a straight line is expected for the equation-

$$3/2[1-(1-Q)^{2/3}]Q \qquad (2.3)$$

where Q is the release percentage versus time plot if drug release from the spherical matrix is based on a diffusion mechanism (Baker, 1974).

2.4 FACTORIAL DESIGN

Conventional method of optimization involves varying only one parameter at a time and keeping the others constant and the conventional optimization method does not allow to study the effect of interaction of various parameters governing the process. Factorial design is useful models for studying the effect of several factors influencing the responses by varying them simultaneously and carrying out a limited number of experiments (Box et al., 1978).

Design of Experiment

The application of factorial design in the pharmaceutical formulations has played a key role in understanding relationship between the independent variables and the responses to them. The independent variables are controllable whereas responses are dependent (Montegomery, 2004).

For example in a 3^2 factorial design, the effect of two independent variables; X1 and X2 on the response variables can be studied. Each factor can be tested at three levels designated as -1, 0 and +1. The values of the factors will be transformed to allow easy calculation of co-efficient in polynomial equation (Hocking, 1976). To identify the effect of significant variables, the reduced model can be also generated. Interactive multiple regression analysis and F- statistics is utilized in order to evaluate the response. The regression equation for the two response will be calculated using the following equations-

$$\text{Response: } Y1 = b_0+b_1X1+ b_2X2+ b_3X1^2+ b_4X2^2+ b_5X1X2 \quad (2.4)$$

$$\text{Response: } Y2 = b_0+b_1X1+ b_2X2+ b_3X1^2+ b_4X2^2+ b_5X1X2 \quad (2.5)$$

Where Y1 and Y2 are the two responses, the responses in the above equation Y1 and Y2 are the quantitative effect of the formulation components or independent variables X1 and X2, b is the coefficient of the term X. Multiple regression is applied using Microsoft excel in order to deduce the factors having significant effect on the formulation properties. To identify the significant variables, the variables having p value > 0.05 in the

full model has to be discarded and then the reduced model can be generated for both the independent variables and each type of formulation.

Calculation of effects and interactions.

To calculate the effect *Ex* of a factor *x* all measurements where the factor is at its lower (−) level were subtracted from all those where the factor was at its upper (+) level and subsequently divided divided through half of the number of measurements used in the calculation. This results in the following formula (Box et al., 1978):

$$Ex=\sum x(+)-\sum x(-)/\ 4/2 \qquad (2.6)$$

To estimate an interaction between two factors one has to calculate the effect of the first factor at the lowest level of the second factor and subtract it from the effect of the first factor at the highest level of the second factor. An interaction between two factors is symbolized as factor 1×factor 2. To estimate the significance of the effects a *t*-test is performed. The test statistic *t* equals:

$$t= Ex/\,(\mathrm{SE})\mathrm{e} \qquad (2.7)$$

Ex is the effect of a factor and (SE)e is the standard error on the effect. The replicates of the centerpoint were used to estimate (SE)e. In this case, (SE)e equals *s*, the standard deviation on the results measured at the centerpoint. The calculated test statistic *t* is compared to a tabulated *t*-value at a significance level of 95% (_=0.05). If the calculated *t*-value is higher than the tabulated *t*-value the effect is considered to be significant.

Vandervoort and Ludwig prepared PLGA NP using different biodegradable stabilizers by 2^2 full factorial design. The different polymers were evaluated as stabilizers in the production of PLGA nanoparticles using a two level full factorial design with centerpoint. The two factors investigated were the concentration of PVA and the concentration of the polymer tested in the outer water phase. For the concentration of PVA, the upper (+), centerpoint (0), and lower (−) level values were 1% w/v, 0.5% w/v and 0% w/v, respectively. Interactions between the components PVA and a polymer was seen when the effect of the polymer was not the same at the two levels of PVA (Vandervoort and Ludwig, 2002).

2.5 CELL LINE STUDIES

2.5.1 Evaluation of Cellular Uptake by Flow Cytometry and Confocal Microscopy

Flow cytometry is used to quantify the amount of sample within the cells by using fluorescence (Durand 1981). In this method the cells are first incubated with test sample in culture medium and then their cellular uptake is evaluated (Herzenberg, 2002). The flow cytometer can indicate relative cell size and density or complexity by measuring forward- and side-scattered laser light, respectively. In addition, the flow cytometer can measure relative fluorescence from fluorescent probes which bind to specific cell-associated molecules. Flow cytometry measures the percentage of cells in a population with each (or multiple) fluorescent probe(s) attached. The cell sorter is capable of sorting specific cell populations from a mixture of cells based on fluorescence profiles (Bonner et al., 1972). For flow cytometry analysis, cells are incubated with test samples in a medium such as RPMI1640 supplemented with 10% FBS. After 4 h of incubation, the cells are washed with PBS and then harvested for further analysis. In order to quantify the amount of test sample within the cell, the cells are analyzed by a flow cytometry, with a forward scattering (FSC) range between 200 and 600 in a linear scale. .

Confocal laser scanning microscopy (CLSM) is a relatively new light microscopical imaging technique which has found wide applications in the biological sciences (Pawley,1990; Boyde, 1994). The primary value of the CLSM to the biologist is its ability to produce optical sections through a 3-dimensional (3-D) specimen - e.g., an entire cell or a piece of tissue - that, to a good approximation, contain information from only one focal plane. Therefore, by moving the focal plane of the instrument step by step through the depth of the specimen, a series of optical sections can be recorded (Lichtman, 1994). This property of the CLSM is fundamental for solving 3-D biological problems where information from regions distant from the plane of focus can obscure the image (thick objects). With biological specimens, either the epi-fluorescence or the epi-reflection mode is generally employed. As a valuable by-product, the computer-controlled CLSM produces digital images which are amenable to image analysis and processing, and can also be used to compute surface- or volume-rendered 3-D reconstructions of the specimen. Confocal microscopy is used to visualize cellular uptake

of drug and test sample by cancer cells at a specific excitation wavelength and an emitting wavelength (Yoo et al, 2004). In order to investigate selective cellular uptake of the test samples, the cell lines are incubated with the culture medium. The cells are examined by a confocal microscopy with an appropriate excitation wavelength and an emitting wavelength.

2.5.2 Cytotoxicity Assay

Cytotoxicity is determined by the use of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay (Cole, 1986; Freshney, 1994). MTT is a substance capable of being reduced by dehydrogenase enzymes present in active mitochondria of living cells (Mosmann, 1983). Cells are harvested on a flat bottom 96 well plate at a specific cell density. After incubating the cells in a logarithmic phase with different concentrations of Test sample for specified time, 20 µl of MTT dye is added to each well. After 4 h of incubation at 37 °C, the crystals are dissolved by the addition of 10% SDS. The percentage of cell viability is determined at 570 nm relative to non-treated cells using an automated microplate reader. Cell survival is estimated as a percentage of the corresponding control. Drug cytotoxicity is assayed by IC_{50}, corresponding to a 50% inhibition of cells.

The nanoparticles of Doxorubicin prepared by Yoo et al. (2002) exhibited slightly lower IC50 value against the HepG2 cell line compared to that of free doxorubicin. Mo et al. (2005) prepared PLGA nanoparticles loaded with paclitaxel and isopropyl myristate (IPM) as release modifier were prepared by a solvent evaporation method. Wheat germ agglutinin (WGA) was conjugated to the nanoparticle surface to give novel WIT-NP. In vitro cytotoxicity of WIT-NP against malignant (A549 and H1299) and normal (CCL-186) pulmonary cell lines was evaluated alongside control formulations. IC50 doses were determined by the MTT assay. The WIT-NP showed superior in vitro anti-proliferation activity against the A549 and H1299 cell lines than the control and clinical formulations. The enhanced cytotoxicity of the WIT-NP was attributed to a more efficient cellular internalization of the drug via WGA-receptor-mediated endocytosis and IPM-facilitated release of paclitaxel from the Nanoparticles in the cells. Cytotoxicity of the WIT-NP was due to paclitaxel-induced apoptosis and cell arrest in the G2/M phase.

2.6 RADIOLABELING STUDIES

2.6.1 Technetium-99m (^{99m}Tc)

Technetium was the first artificially produced element. It was isolated by Carlo Perrier and Emilio Segrè in 1937. Technetium was created by bombarding molybdenum atoms with deuterons that had been accelerated by a device called a cyclotron. Today, technetium is produced by bombarding molybdenum-98 with neutrons. Molybdenum-98 becomes molybdenum-99 when it captures a neutron. Molybdenum- 99, with a half-life of 65.94 hours, decays into technetium -99 through beta decay. While technetium has never been found to occur naturally on earth, its spectral lines have been observed in S-, M- and N-type stars.

Uses

The radioisotope most widely used in medicine is technetium-99m, employed in over half of all nuclear medicine procedures. It is an isotope of the artificially produced element technetium and it has almost ideal characteristics for a nuclear medicine scan (Cao and Suresh, 1998).

These characteristics are:

- Technetium-99m decays by a process called isomeric transition, a process in which 99mTc decays to 99Tc via the release of gamma rays and low energy electrons. Since there is no high energy beta emission the radiation dose to the patient is low.
- The low energy gamma rays emitted easily escape the human body and are accurately detected by a gamma camera. Once again the radiation dose to the patient is minimized.
- It has a half-life of six hours which is long enough to examine metabolic processes yet short enough to minimize the radiation dose to the patient.

- The chemistry of technetium is so versatile it can form tracers by being incorporated into a range of biologically-active substances to ensure that it concentrates in the tissue or organ of interest.

Technetium-99m is used to image the skeleton and heart muscle in particular, but also for brain, thyroid, lungs, liver, spleen, kidney, gall bladder, bone marrow, salivary and lachrymal glands, heart blood pool, infection and numerous specialized medical studies (Philips et al., 1992).

Technetium can concentrate in several organs depending on its chemical form, so there is no primary organ of concern. This is one reason why the short-lived isotope technetium-99m has such wide usage in nuclear medicine as a diagnostic tool. The low energy of the beta particle, the lack of significant gamma or X-rays, and the rapid excretion of technetium-99m from the body limit the potential for health effects.

Half-Life of technetium is 6.02 hours (Martin, 1976).

Technetium-99m is used in 20 million diagnostic nuclear medical procedures every year. Approximately 85 percent of diagnostic imaging procedures in nuclear medicine use this isotope. Depending on the type of nuclear medicine procedure, the Tc-99m is tagged (or bound to) a pharmaceutical that transports the Tc-99m to its required location. For example, when Tc-99m was chemically bound to Exametazime, the drug was able to be cross the blood brain barrier and flow through the vessels in the brain to see cerebral blood flow (it is also used for labeling white blood cells to visualize sites of infection (Puncher, 1994). Tc-99m Sestamibi was used for myocardial perfusion imaging (which shows how well the blood flows through the heart) (Wilson, 2004). Measurements of renal function and imaging was undertaken by tagged to Mercapto Acetyl Tri Glycine, known as a MAG3 scan.

Technetium-99m is made from the synthetic substance molybdenum-99 which is a by-product of nuclear fission. It is because of its parent nuclide, that technetium-99m is so suitable to modern medicine. Molybdenum-99 has a half-life of approximately 66 hours, and decays to Tc-99m, a negative beta, and an antineutrino (equation shown below). This

is a useful life since, once this product (molybdenum-99) is created, it can be transported to any hospital in the world and would still be producing technetium-99m for the next week. The betas produced are easily absorbed, and Mo-99 generators are only minor radiation hazards, mostly due to secondary X-rays produced by the betas (also known as bremsstrahlung).

$$^{99}\text{Mo (Negative Beta Decay)} \rightarrow {}^{99m}\text{Tc} + \beta^- + \nu^-$$

Where β^- = a negative beta particle (electron), and ν^- = an antineutrino.

^{99m}Tc will then undergo an isomeric transition to yield ^{99}Tc and a monoenergetic gamma emission.

$$^{99m}\text{Tc} \rightarrow {}^{99}\text{Tc} + \gamma$$

When a hospital receives molybdenum-99 generator, the technetium-99m from within can be easily chemically extracted. That same molybdenum-99 generator (holding only a few micrograms) can potentially diagnose ten thousand patients because it will be producing technetium-99m, strongly for over a week. The radioisotope is perfect for medicinal purposes. The short half life of the isotope allows for scanning procedures which collect data rapidly. The isotope is also of a very low energy level for a gamma emitter. Its ~140 keV of energy make its use very safe and substantially reduce the chance of ionization.

^{99m}Tc in Nuclear medicine

^{99m}Tc ("m" indicates that this is a metastable nuclear isomer) is used in radioactive isotope medical tests, for example as a radioactive tracer that medical equipment can detect in the human body (Cifford A et al., 1986). It is well suited to the role because it emits readily detectable 140 keV gamma rays, and its half-life is 6.01 hours (meaning that about seven eighths of it decays to ^{99}Tc in 24 hours) (Emsley, 2001). Klaus Schwochau's book *Technetium* lists 31 radiopharmaceuticals based on ^{99m}Tc for imaging and functional studies of the brain, myocardium, thyroid, lungs, liver, gallbladder, kidneys, skeleton, blood, and tumors.

Immunoscintigraphy incorporates ^{99m}Tc into a monoclonal antibody, an immune system protein capable of binding to cancer cells. A few hours after injection, medical equipment is used to detect the gamma rays emitted by the ^{99m}Tc; higher concentrations indicate where the tumor is. This technique is particularly useful for detecting hard-to-find cancers, such as those affecting the intestine. These modified antibodies are sold by the German company Hoechst (now part of Sanofi-Aventis) under the name "Scintium".

When ^{99m}Tc is combined with a tin compound it binds to red blood cells and can therefore be used to map circulatory system disorders. It is commonly used to detect gastrointestinal bleeding sites. A pyrophosphate ion with ^{99m}Tc adheres to calcium deposits in damaged heart muscle, making it useful to gauge damage after a heart attack. The sulfur colloid of ^{99m}Tc is scavenged by the spleen, making it possible to image the structure of the spleen (Cifford A et al., 1986).

Radiation exposure due to diagnostic treatment involving Tc-99m can be kept low. Because ^{99m}Tc has a short half-life and high energy gamma (allowing small amounts to be easily detected), its quick decay into the far-less radioactive ^{99}Tc results in relatively less total radiation dose to the patient, per unit of initial activity after administration. In the form administered in these medical tests (usually pertechnetate) both isotopes are quickly eliminated from the body, generally within a few days.

Technetium for nuclear medicine purposes is usually extracted from technetium-99m generators. ^{95m}Tc, with a half-life of 61 days, is used as a radioactive tracer to study the movement of technetium in the environment and in plant and animal systems.

2.6.2 Radiolabeling studies of Drugs and Drug loaded Nanoparticles

Radiolabeling is incorporation of a radioactive element into a compound in order to investigate its metabolism, fate and utilization. Radiolabeling studies on drugs and drug delivery systems have recently gained importance for studying their biodistribution and their fate in the body. Different nuclides used are ^{3}H, ^{14}C, ^{32}P, ^{99}MO, ^{131}I, ^{99}Tc, ^{67}Ga. In our studies we have used Technetium-99m (^{99m}Tc) to label free drugs and nanoparticleds to check their in vivo performance in mice and rats. Biodistribution of the test samples was studied in mice. Pharmacokinetic studies and blood Clearance was studied in rats.

Drug and nanoparticles are radiolabeled with Technetium-99m (^{99m}Tc)) by reducing pertechnetate (TcO4-) (2 mCi) with 100mg stannous chloride. To it Drug and/or nanoparticles are added and incubated at room temperature for 10 min and checked for labeling efficiency and stability before injecting in the animals (Theobald, 1990). The labeling efficiency of the ^{99m}Tc labeled drug and NP is determined by instant thin layer chromatography (ITLC) using ITLC–SG mini strips as described by Banerjee et al. (2005). Silica gel coated fibre sheets are used for ascending thin layer chromatography. The stability of labeled complexes in serum supports their stability in biological environment upon administration into the body. The stability of ^{99m}Tc labeled complexes of Drugs and nanoparticles is determined in vitro in human serum by ascending ITLC technique. The labeled complex is incubated with freshly collected human serum at 37°C. The stability is performed by determining the changes in labeling efficiency by subjecting the samples to ITLC at regular intervals up to 24 h and analyzing the chromatograms in gamma ray spectrometer (Reddy et al., 2004).

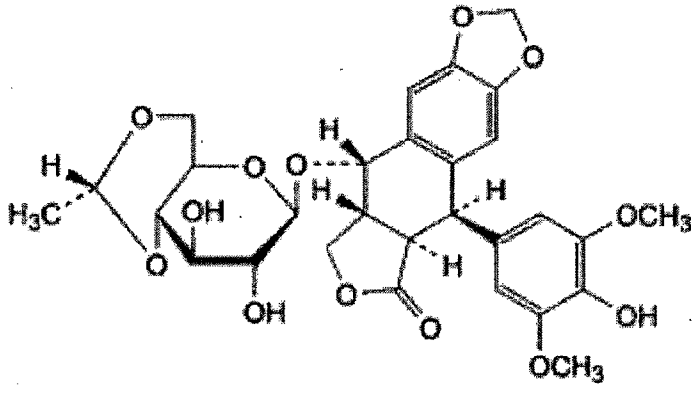
2.7 DRUG PROFILE

2.7.1 Etoposide

Description

Etoposide is a semisynthetic podophyllotoxin derived from the root of Podophyllum peltatum (the May apple or mandrake). It inhibits DNA topoisomerase II, thereby inhibiting DNA synthesis.

Structure



Chemical IUPAC name

4'-demethyl-epipodophyllotoxin 9-[4,6-O-(R)-ethylidene-beta-D-glucopyranoside], 4' -(dihydrogen phosphate)

Formula
$C_{29}H_{32}O_{13}$

Mol. mass
588.557 g/mol

Appearance
White or almost white crystalline powder.

Solubility

Practically insoluble in water, sparingly soluble in methanol, slightly soluble in alcohol and in methylene chloride.

Mechanism of Action

Etoposide is cell cycle dependent and phase specific, affecting mainly the S and G_2 phases (Haskell, 1990). It is known to cause single-strand breaks in DNA. Etoposide also causes DNA damage through inhibition of topoisomerase II and activation of oxidation-reduction reactions to produce derivatives that bind directly to DNA. Topoisomerase II carries out breakage and reunion reactions of DNA which are necessary for normal cellular function. Etoposide is cell cycle phase specific with predominant activity occurring in late S phase and G_2 (Chabner, 1989).

Pharmacokinetics

Etoposide has bioavailability of about 25-74% with large inter- and intrapatient variability. Bioavailability of low oral doses of 100 mg may be better than higher oral doses. Its volume of distribution is -17 L/m^2, 0.36 L/kg, 28-32% of body weight. Plasma protein binding is about 94%. Etoposide has a half life of 6 hours by oral route and 6-12 hours by intra venous route (Clark et al., 1987; Knoben et al., 1988).

Administration

It is given intravenously or orally in capsule form. If the drug is given by intravenous route it must be done slowly over a 30 to 60 minute period because it can lower blood pressure as it is being administered. Blood pressure is checked often during infusing with the speed of administration adjusted accordingly. Its dose is 75 mg/m2/day on days 1-7 (Bishop 1990).

Uses

Primarly used for Acute lymphocytic leukemia, Acute myelogenous leukemia, Lung cancer, Non-Hodgkin's lymphoma (Hainsworth et al., 1995).

Side Effects

Hypotension, alopecia, nausea and vomiting are most common side effects. Etoposide has an appreciable risk of causing hypersensitivity reactions. Anaphylactoid reactions occur in 0.7-2% of patients. Fewer hypersensitivity reactions may occur if the infusion rate is slow (ie, over at least 30 minutes). If a reaction occurs, the drug should be discontinued

and vasopressors, corticosteroids, antihistamines or plasma volume expanders administered. Patients can be rechallenged. Transient hypotension during infusion occurs in 1-2% of patients and is usually associated with rapid infusion and/or high doses. Rarely, etoposide may cause an increase in blood pressure. Stomatitis is likely to occur in patients treated with radiation to the head and neck region and has been the dose-limiting toxicity on high-dose etoposide protocols. Adverse gastrointestinal effects occur more frequently following oral administration. Phlebitis has occurred following the administration of undiluted etoposide (Winick et al., 1993; Murphy 1993; Pui et al., 1989).

Estimation of Etoposide

Different high performance liquid chromatographic (HPLC) methods for analysis of etoposide have been described especially for analyzing serum levels of etoposide. In general, these methods use a reverse-phase column with a methanol or acetonitrile-containing mobile phase with isocratic elution. Some of these methods with details of the solvent system is described in Table 2.5

Murthy and Reddy described assay of etoposide by UV-visible spectrophotometer,. The solvent system used was methanol: chloroform (50:50) and the wavelength selected for analysis was 286 nm (Murthy and Reddy, 2005).

Table 2.5
HPLC methods for estimation of Etoposide

Sr. No.	Detector	Mobile phase	Column	Flow rate	detection wavelength	detection limit	Ref
1	SPD-10AV ultraviolet detector	70% H2O:acetic acid (100:1) and 30% acetonitrile	5µm phenyl column	1.4 ml/min	239 nm	10 ng	Schaefer et al., 2001
2	Waters 996 Photodiode array detector	35% acetonitrile, 64% water, and 1% acetic acid,	C18 phenyl column	1.5 ml/min.	230 nm.	0.25 µg/ml	Lacayo et al., 2002
3	SPD-6AV ultraviolet detector	methanol: water: acetonitrile (55:42:3 v/v/v/	ODS M, 5µm (150X.6 mm ID)	1.5 ml/min.	229 nm	50 ng/ml	Kato et al., 2003
4	SPD-6AV ultraviolet detector	methanol/water/glacial acetic acid 49/50/1 v/v/v	10 µm Bondapak 3.9X300 mm reversed-phase phenyl column	1ml/min	235 nm	2µM	Schroeder et al., 2004
5	L-4000 UV detector	65% (v/v) methanol in distilled water.	µBondpak C18 reversed-phase column	0.7 ml/min.	254 nm.	0.1 µg/ml.	Gregianin et al., 2002
6	SPD-6AV Shimadzu UV–VIS Detector	Methanol: water (45:55 v/v	Bondapac C18 column	2.8 ml/min	220 nm	20 ng/ml	Shirazi, et al., 2001

Review of Etoposide in Novel Drug delivery systems

Etoposide is an antineoplastic agent which acts by forming a ternary complex with topoisomerase II and DNA, causing DNA breaks and cell death. Sengupta et al. demonstrated that encapsulation in liposomes increases the antitumour efficacy and reduces the adverse effects associated with etoposide. They evaluated the cationic liposomes of etoposide and studied the effect of cholesterol incorporation on the stability of the liposomes. Etoposide-encapsulated unilammellar liposomes were synthesized by thin film hydration followed by extrusion. Half-life (β) was 58.62 and 186 min in the case of free and liposomal etoposide, respectively. In the stability studies, incorporation of cholesterol progressively stabilized the formulation in serum. The use of sucrose at increasing concentrations as a cryoprotectant also increased the shelf stability of the formulation at 0°C (Sengupta et al., 2000).

Etoposide loaded tripalmitin (ETPL) nanoparticles were prepared by melt-emulsification and were studied for enhancing the tumor uptake of etoposide, in Dalton's lymphoma tumor bearing mice. The size of ETPL nanoparticles was 387 nm and possessed negative charge. Etoposide and ETPL nanoparticles were radiolabeled with ^{99m}Tc with high labeling efficiency (Reddy et al., 2005). The study signifies the advantage of incorporating etoposide into tripalmitin nanoparticles in controlling its biodistribution and enhancing the tumor uptake by several folds.

Reddy and Murthy (2005) studied etoposide-loaded nanoparticles prepared with 3 different glyceride lipids. All 3 glyceride nanoparticle formulations exhibited sustained release characteristics, and the release pattern followed the Higuchi equation. The spray-dried lipid nanoparticles stored in black polypropylene containers exhibited excellent long-term stability at 25°C and room light conditions. Such stable lipid nanoparticles with in vitro steric stability were suggested to be beneficial for intravenous administration as long circulating carriers for controlled and targeted drug delivery.

Lamprecht et al reported use of lipid nanocapsules (LNC) of Etoposide which were hypothesized to reverse multidrug resistance additionally by their P-glycoprotein (P-gp) inhibiting surfactant. These LNC loaded with Etoposide had mean diameter of 25 to

100 nm and were tested for the drug release and their efficiency to reduce cell growth in cell culture for C6, F98, and 9L glioma cell lines. A sustained release of etoposide was observed over a period of 1 week. The mechanism of action of etoposide LNC was proposed to be a cell uptake followed by a sustained drug release from the LNC in combination with an intracellular P-gp inhibition ensuring a higher anticancer drug concentration inside the cancer cells (Lamprecht et al., 2006).

Reddy et al have made polysorbate micelles and evaluated the possibility of enhancing Etoposide uptake in Dalton's lymphoma mouse (Reddy et al., 2006). Etoposide (ET) and etoposide loaded polysorbate 20 micelles (EPM) were radiolabeled with 99mTc by the reduction method using stannous chloride. Subcutaneous injection of EPM resulted in significantly higher tumor uptake (100 folds compared to ET 6 h post injection) ($p < 0.001$) and prolonged tumor retention. Tumor uptake was also confirmed by gamma imaging studies. EPM exhibited relatively high brain concentrations (7 fold 24 h post injection) compared to ET, suggesting the potential use of EPM in the treatment of brain malignancies.

Block ionomer complexes (BIC) composed of graft-comb copolymers of Pluronic and poly(acrylic acid) (Pluronic–PAA) and a model cationic surfactant, exadecyltrimethylammonium bromide (HTAB), were synthesized by mixing the polymer and surfactant in aqueous media.for etoposide. In such BIC loading capacities of about 6 to 15% by weight of the dispersed complex. Overall, these BIC wield a promise as responsive nanocarriers for pharmaceuticals (Oh et al., 2006).

Etoposide has been made into lipid nanosphers (LN) and studied for folate targeting (Patlolla et al., 2008). The study was focused on the targeting ability of lipid nanospheres using Folate-PEG-DSPE. etoposide-encapsulated lipid nanospheres were prepared with the help of soybean oil, egg phosphatidylcholine, and PEG-DSPE with and without Folate-PEG-DSPE. The anticancer activity of these formulations was assessed in KB cell line and it was found that Fol-LN was selectively taken up by the KB cells and the addition of 1 mM folic acid completely blocked this uptake. The IC50 values of etoposide solution, Fol-LNE, and Fol-LNE-comp (competition with 1 mM folic acid) were 33, 5, and 19 μM, respectively.

2.7.2 CYTARABINE

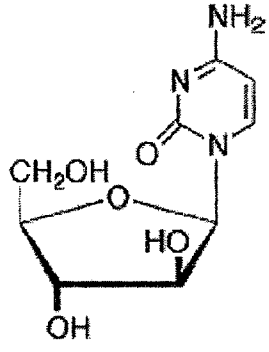
Description

Cytarabine is white to off-white, crystalline powder synthetic nucleoside which differs from the normal nucleosides cytidine and deoxycytidine in that the sugar moiety is arabinose rather than ribose on deoxyribose.

Synonyms

1-B-arabinofuranosylcytosine, arabinosylcytosine, ara-C, cytosine arabinoside

Chemical Structure



Chemical IUPAC Name

4-amino-1-[(2R,3S,4R,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]pyrimidin-2-one

Molecular formula

$C_9H_{13}N_3O_5$

Molecular weight

243.22

Solubility

It is freely soluble in water and slightly soluble in alcohol and in chloroform.

Melting Point

212-213°C

LogP/Hydrophobicity

-2.712

Mechanism of Action

Cytarabine, a synthetic pyrimidine nucleoside, is converted intracellularly, primarily by deoxycytidine kinase, to active cytarabine triphosphate (McEvoy, 2006). Activity occurs primarily as the result of inhibition of DNA polymerase via competition with deoxycytidine triphosphate, resulting in the inhibition of DNA synthesis. Incorporation of cytarabine into DNA and RNA may contribute to cytotoxic effects. Cytarabine also has antiviral and immunosuppressive properties. Cytarabine is cell cycle phase-specific for the S-phase; cytarabine may also block progression from the G1-phase to the S-phase. Both concentration and duration of exposure are critical for cytotoxicity (Hamada et al., 2002).

Cytarabine is cytotoxic to a wide variety of proliferating mammalian cells in culture. It exhibits cell phase specificity, primarily killing cells undergoing DNA synthesis (S-phase) and under certain conditions blocking the progression of cells from the G1 phase to the S-phase. Although the mechanism of action is not completely understood, it appears that cytarabine acts through the inhibition of DNA polymerase. A limited, but significant, incorporation of cytarabine into both DNA and RNA has also been reported. Extensive chromosomal damage, including chromatoid breaks, has been produced by cytarabine, and malignant transformation of rodent cells in culture has been reported. Deoxycytidine prevents or delays (but does not reverse) the cytotoxic activity

Pharmacokinetics:

Oral absorption of cytarabine is less than 20% and it is ineffective when administered orally. Plasma protein binding is 13%. Metabolism is rapid and extensive; primarily hepatic; it is also metabolized in the kidneys, GI mucosa, granulocytes, and other tissues by cytidine deaminase. Its half life is 1-3 hours. Following rapid intravenous injection of cytarabine labeled with tritium, the disappearance from plasma is biphasic. There is an

initial distributive phase with a half-life of about 10 minutes, followed by a second elimination phase with a half-life of about 1 to 3 hours. After the distributive phase, more than 80% of plasma radioactivity can be accounted for by the inactive metabolite 1-β-D-Arabinofuranosyluracil (ara-U). Within 24 hours, about 80% of the administered radioactivity can be recovered in the urine, approximately 90% of which is excreted as ara-U. Relatively constant plasma levels can be achieved by continuous intravenous infusion.

After subcutaneous or intramuscular administration of cytarabine labeled with tritium, peak plasma levels of radioactivity are achieved about 20 to 60 minutes after injection and are considerably lower than those after intravenous administration.

Cerebrospinal fluid levels of cytarabine are low in comparison to plasma levels after single intravenous injection. However, in one patient in whom cerebrospinal levels were examined after 2 hours of constant intravenous infusion, levels approached 40% of the steady-state plasma level. With intrathecal administration, levels of cytarabine in the cerebrospinal fluid declined with a first-order half-life of about 2 hours.

Uses:

Primary uses: acute lymphocytic Leukemia, acute myeloid Leukemia, chronic myelogenous Leukemia, meningeal Leukemia

Other uses: Lymphoma, Hodgkin's Myelodysplastic syndrome

Formulations Available

Cytarabine for Injection USP is available in vials containing 100 mg, 500mg, 1 g, and 2 g cytarabine. When necessary, the pH of Cytarabine for Injection USP was adjusted with hydrochloric acid and/or sodium hydroxide. Reconstitute with Bacteriostatic Water for Injection USP with benzyl alchohol for intravenous and subcutaneous use

Dosage and Administaration

Cytarabine is not active orally. The schedule and method of administration varies with the program of therapy to be used. Cytarabine may be given by intravenous infusion or

injection, subcutaneously, or intrathecally. Patients can tolerate higher total doses when they receive the drug by rapid intravenous injection as compared with slow infusion. This phenomenon is related to the drug's rapid inactivation and brief exposure of susceptible normal and neoplastic cells to significant levels after rapid injection. Normal and neoplastic cells seem to respond in somewhat parallel fashion to these different modes of administration and no clear-cut clinical advantage has been demonstrated for either.

- In the induction therapy of acute non-lymphocytic leukemia, the usual cytarabine dose in combination with other anticancer drugs is 100 mg/m^2/day by continuous IV infusion (days 1 to 7) or 100 mg/m^2 IV every 12 hours (days 1 to 7).

Side Effects

Cytarabine given intrathecally may cause systemic toxicity and careful monitoring of the hemopoietic system is indicated. Modification of other anti leukemia therapy may be necessary. Major toxicity is rare. The most frequently reported reactions after intrathecal administration were nausea, vomiting, and fever; these reactions are mild and self-limiting. Paraplegia has been reported (Saiki et al., 1972). Isolated neurotoxicity has been reported (Marmont et al., 1973). Blindness occurred in two patients in remission whose treatment had consisted of combination systemic chemotherapy, prophylactic central nervous system radiation and intrathecal cytarabine (Margileth et al., 1977).

When cytarabine is administered both intrathecally and intravenously within a few days, there is an increased risk of spinal cord toxicity. However, in serious life-threatening disease, concurrent use of intravenous and intrathecal cytarabine is left to the discretion of the treating physician (Smith et al., 1997).

Analytical methods for Cytarabine

Different methods have been developed of estimation of Cytarabine and its degradation products by HPLC (Larry et al., 1986). Boos (1991) developed a sensitive isocratic ion-pair HPLC method using a reversed-phase C_{18} column and phosphate buffer at pH 6 for the determination of the cytotoxic intracellular anabolite 1β-D-arabinofuranosylcytosine-triphosphate (Ara-CTP) of the antineoplastic drug cytarabine in leukaemic cells *in vivo.* The recovery from cells was more than 90%, the limit of detection was 25 ng ml^{-1}. The method enables intracellular drug monitoring of Ara-CTP with standard HPLC equipment.

Ramsauer et al. (1995) developed an ion-pair HPLC method for the determination of 1-β-D-arabinofuranosylcytosine-5'-stearyl phosphate (cytarabine-ocfosfate I) using a phenyl-bonded column under reversed-phase conditions with a mobile phase of acetonitrile-buffered water (pH 6.8) (50:50) for isocratic elution. A reproducible sample clean-up was achieved by solid-phase extraction to reach the low limit of detection of 2 ng/ml. Some of the recent methods developed for estimation of cytarabine have been shown in Table 2.6

Subramanian and Murthy (2004) developed U V spectrophotometric method for estimation of cytarabine using UV-1601 UV-VIS spectrophotometer. Two solvents, distilled water and methanol were used as solvents. The absorptivity at 274 nm was found suitable and selected for further investigations.

Fahmy et al. (2004) developed HPLC methods for the simultaneous determination of cytarabine (CYT) and etoposide (ETO), combination, as co-administered drugs. A 250 mm×4.6 mm C-18 column was used, mobile phase consists of a mixture of 0.02 M sodium dihydrogenphosphate aqueous solution adjusted to pH 6.0 (with 0.2 M orthophosphoric acid or sodium hydroxide) and acetonitrile in a ratio of (7:3) at a flow rate 1 ml/min, with UV detection at 254 nm and ambient temperature.

Table 2.6

HPLC methods for estimation of Cytarabine

Mobile phase	Flow rate	Column	Detector wavelength	Reference
0.005 M monobasic sodium phosphate in distilled water containing 5% (v/v) methanol	1.2 ml/ min	5-mm Spherisorb ODS2	272 nm	Ruckmani et al., 2000
Phosphate buffer and methanol (95:5)	1.0 ml/min	L1 (Octadecyl silane chemically bonded to porous silica	272 nm	Subramanian and Murthy, 2004
0.005 M monobasic sodium phosphate in distilled water containing 5% v/v methanol	1.2 ml/ min	Spherisorb ODS, C18, 5 mm	272 nm	Gomez et al., 2004
5% methanol and 95% 10 mmol L^{-1} phosphate buffer adjusted to pH 5.5	1.0 ml/ min	Diamonsil C_{18} column	270 nm	Zhang et al., 2006

Literature Review on Novel Drug Delivery Systems of Cytarabine

Teijón et al studied the release of cytarabine (ara-c) from poly(2-hydroxyethyl methacrylate) hydrogels cross-linked with different amounts of ethyleneglycol dimethacrylate (EGDMA). The drug was trapped in polymer discs by including it in the feed mixture of polymerization. The release was in accordance with Fickian behaviour. Total release of ara-C was reached after between 3 and 7 days depending on the percentage of EGDMA in the gels. A constant release rate of ara-C from the hydrogels was obtained, the time depending on the degree of cross-linking of the gels: 22 h for gels with 0.5% EGDMA, 32 h for gels with 5% EGDMA and 42 h for gels with 7% EGDMA; the amount of ara-C released being 50%, 80% and 85%, respectively, of the drug load of the gel discs. An increase of the release rate with the disc load was observed for each sort of hydrogel (Teijón et al, 1997).

Blanco et al. (1998 A) prepared slow releasing cytarabine (ara-C) from poly(2-hydroxyethyl methacrylate) and poly(2-hydroxyethyl methacrylate-co-N-vinyl-2-pyrrolidone) hydrogels cross-linked with different amounts of ethyleneglycol dimethacrylate (EDGMA). The developed hydrogel discs were subcutaneously implanted in the back of male Wistar rats. The release time of ara-C was between 3 days from pHEMA 0.5% and 16 days from H80/VP20/E15 gels.

Blanco et al. (1998 B) studied the release of Cytarabine (ara-C) from copolymeric poly(acrylamide-co-monomethyl itaconate) (A/MMI) hydrogels. Three different compositions of copolymers were studied, 90A/10MMI, 75A/25MMI and 60A/40MMI. The drug was trapped in gels by placing it in the polymerization feed mixture and discs loaded with 5-50 mg of ara-C were obtained. Drug release kinetics was examined as a function of temperature and drug load. The diffusion kinetics followed Fick's second law. The diffusion coefficients for the release of ara-C were between 5.7 × 10−11 and 9.6 × 10−11 m2 s−1. The gel was subcutaneously implanted in rats and the drug plasma concentration was determined, no drug was detected after 31 h.

Ruckmani et al. (2000) prepared niosome vesicles of cytarabine hydrochloride by lipid hydration method having sizes of the vesicles in between of 600 to 1000 nm. Niosomes exhibited a prolonged release profile over a period of 16 hr and the vesicular suspension was stable over a period of 4 weeks.

Gomez et al. (2004) prepared Cytarabine comatrices of albumin microspheres in a poly (lactide–co-glycolide) film. Cytarabine (ara-C) was included in albumin microspheres and these microspheres were immersed in a poly(lactide-co-glycolide) (PLGA) film to constitute a comatrix system to develop a prolonged form of release. The in vitro release study showed a slower kinetic release of the drug from PLGA comatrices, releaseing 80% of the included cytarabine on day 7. In vivo studies in rats the drug was detected in plasma up to 10 days.

Subramanian et al. (2004) prepared Cytarabine liposomes by thin film hydration method by using 33factorial design and studied the combined influence of three independent variables Drug (Cytarabine) / Lipid [Phosphatidyl choline (PC) and Cholesterol (Chol)] in the preparation of liposomes.

Craparo et al. (2004) prepared polymeric nanoparticles of cytarabine by Photo-Crosslinking of an acryloylated polyaspartamide in w/o microemulsion. Nanoparticles had mean diameter of 88 ± 13 nm, were spherical and had a negative surface charge. Cyt-loaded PHG nanoparticles were able to release it in a simulated physiological fluid (phosphate buffer at pH 7.4) and in blood plasma.

2.8 PROFILE OF POLYMERS

2.8.1 Poly(D,L-lactide-co-glycolide) PLGA

Nonproprietary Names:

Poly(lactide-co-glycolide)

Functional Category: (Lewis, 1990)

Bioabsorbable, Biocompatible, Biodegradable material

Trade Names:

Purasorb PLG (PURAC, USA.)

Medisorb 5050 DL (Alkermes Inc.)

Resomer RG 502 (Boehringer Ingelheim, Germany)

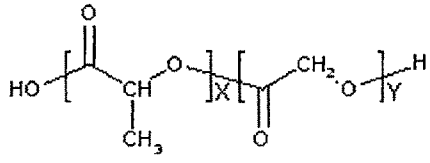
Lactel 5050 DL PLGA (Birmingham Polymers Inc.)

Chemical Name and CAS Registry Number:

CAS Number: 26780-50-7

Chemical Name: Poly(D,L-lactide-co-glycolide)

Structural Formula:



x - Number of units of Lactic Acid
y - Number of units of Glycolic Acid

Method of Manufacture: (Bala et al., 2002)

Generally, aliphatic polyesters like PLGA can be synthesized via polycondensation of hydrocarboxylic acids and catalytic ring-opening polymerization of lactones. Ring opening polymerization is preferred because polyesters with high molecular weights can be produced. Moreover, the dehydration of hydrocarboxylic acids to form lactones does

not have to be carried to a high degree of completion. Lactones can easily be purified owing to the differences of their physical and chemical properties from those of the corresponding hydrocarboxylic acid.

Description:

PLGA polymers are a group of synthesized copolymers of Lactic and Glycolic acid. They are nontoxic, and can be easily fabricated into variety of novel devices such as rods, screws, nails and cylinders. The polymers are available commercially in varying molecular weight and molecular ratio of Lactic and Glycolic acid. Co-monomer ratio of lactic acid and glycolic acid for PLGA range from 85:15 to 50:50.

Typical Properties: (Bala et al., 2002)

Description : White or nearly white amorphous powder

Odor: Odorless

Inherent Viscosity: 0.45dl/g 0.1% in chloroform, 25 ^{o}C.

Glass Transition Temp: 50 ^{0}C

Solubility: Methylene Chloride, Ethyl Acetate, Tetrahydrofuran, Acetone,

Chloroform

Polymer Composition: 51:49 molar ratio of

D,L lactide : glycolide

Acid Number: 9mg KOH/gm

Polymer composition and crystallinity play important roles in the solubility of these aliphatic polyesters. The crystalline homopolymers of glycolic acid are soluble only in strong solvents like hexafluoroisopropanol. The crystalline homopolymers of lactic acid also do not have good solubility in most organic solvents. However, amorphous polymers of D,L-lactic acid and co-polymers of lactic acid and glycolic acid are soluble in many organic solvents.

Stability and Storage Conditions:

Aliphatic polyesters like PLGA easily susceptible to hydrolysis in the presence of moisture. Hence they should be properly stored preferably refrigerated at around 4 ^{o}c or below. It is necessary to allow the polymers to reach room temperature before opening the container. After the original package has been opened, it is recommended to re-purge the package with high-purity nitrogen prior to resealing.

Safety:

PLGA and homopolymers of D,L-lactic acid and glycolic acid are used in parenteral pharmaceutical formulations and are regarded as biodegradable, biocompatible and bioabsorbable materials. Their biodegradation products are nontoxic, non-carcinogenic and non-teratogenic. In general, these polymers exhibit very little hazard.

Handling Precautions:

Observe normal precautions appropriate to the circumstances and quantity of material handled. Contact with eyes, skin and clothing, and breathing the dust of the polymers should be avoided. PLGA produces acid materials such as hydroxyacetic acid and/or lactic acid in the presence of moisture; thus contact with materials that will react with acids, especially in moist conditions, should be avoided.

Regulatory Status:

GRAS listed.

Applications in Pharmaceutical Formulation:

In aqueous environment, PLGA undergoes hydrolytic degradation, through cleavage of the ester linkages, into nontoxic hydrocarboxylic acids. Eventually, they are degraded to carbon dioxide and water via the citric acid cycle. Owing to their reputation as safe materials and their biodegradability, they are primarily used as biocompatible and biodegradable polymers for formulation of many types of implantable and injectable drug delivery systems for both human and veterinary use (Shive and Anderson, 1997). Examples of implantable delivery system include rods, cylinders, tubing, films, fibres, pellets, and beads. Examples of injectable drug delivery system include microcapsules,

microspheres, nanoparticles, and liquid injectable controlled release systems. The rate of biodegradation and drug release characteristics from these polymers can be controlled by changing the physic-chemical properties of the polymers, such as crystallinity, hydrophobicity, co-polymer ratio, and polymer molecular weight (Bala et al., 2004)

Biodegradation and toxicity of PLGA:

The PLGA copolymers degrade in the body by hydrolytic cleavage of ester linkage to lactic and glycolic acid. These monomers are easily metabolized in the body via Kreb's cycle and eliminated as carbon dioxide and water (Kwon et al., 2001; Jiao et al., 2002). The degradation process of polymers both in vivo and in vitro is affected by several factors, including preparation method; the presence of low molecular weight compounds, size, shape and morphology; intrinsic properties of the polymer (molecular weight, chemical structure hydrophobicity, crystallinity, and glass transition temperature of the polymer); physicochemical parameters (pH, temperature and ionic strength of the environment); site of implantation and mechanism of hydrolysis.

Bulk erosion is the main degradation pathway for PLGA copolymer. A three-phase mechanism for PLGA biodegradation has been proposed (Jain, 2000). Initially, a significant decrease in molecular weight of polymer is observed, with no appreciable weight loss and no soluble monomer products formed after random chain scission. This phase is followed by a decrease in molecular weight with rapid loss of mass and formation of soluble mono and oligomeric products. Finally, soluble monomer products are formed from soluble oligomer fragments, resulting in completer polymer degradation (Jain, 2000).

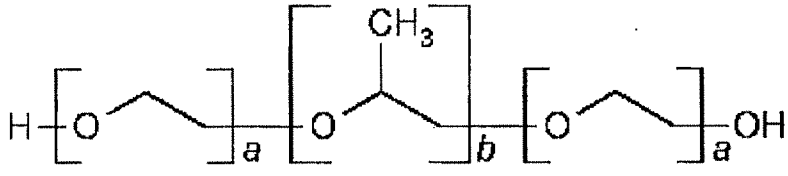
PLGA copolymer biodegradation products are formed at a very slow rate and hence do not affect normal cell function. These polymers have been tested for toxicity and safety in extensive animal studies and are currently being used in humans for resorbable sutures, bone implants and screws, and contraceptive implants (Katz, 2001; Hanafusa et al., 1995).

2.8.2 Pluronic F 68

Synonyms
Polyoxypropylene-polyoxyethylene Block Copolymer, Poloxamer 188

Description
Pluronic F68 is a difunctional block copolymer surfactant terminating in primary hydroxyl groups. A nonionic surfactant that is 100% active and relatively nontoxic.

Chemical Structure of Pluronic Block Copolymer



(a is poly(oxypropylene) and b is poly(oxyethylene, for F- 68 a= 80, b=27)

Molecular weight
8,400 g/mol

Colour
White to cream

pH value
6 – 7

Freezing point
Approx. 52 °C (1,013 hPa)

Bulk density
1,050 kg/m3

Viscosity, dynamic
19 mPa.s (77 °C)

Solubility in water
Soluble

Specific gravity, 77°C/25°C
1.06

Melt Point
52°C

HLB
>24

Structure and Synthesis

Pluronic block copolymers (also known as poloxamers) consist of ethylene oxide (EO) and propylene oxide (PO) blocks arranged in basic A-B-A structure; EOx-POy-EOx. This arrangement results in an amphiphilic copolymer, in which the number of ydsrophilic EO (x) and hydrophobic PO (y) units can be altered.

Pluronic block coplymers are synthesized by sequential addition of PO and EO monmers in the presence of an alkaline catalyst, such as sodium or potassium hydroxide(12). The reaction is initiated by polymerization of the PO block followed by the growth of EO chains at both the ends of the PO block. Anionic polymerization usually produces polymers with a relatively low polydispersity index (Mn/Mw). Further chromatographic fractionation was employed in procedures for the manufacture of highly purified block copolymer (Emanuele et al., 1996).

Pharmacokinetics

Pharmacokinetics and Biodistribution of 3H labelled P 85 was studied after a single I.V bolous administration in mice and it was found that it remained in the circulation for prolonged period. At sampling time of 19 h 17% of the dose of the block copolymer was found in the blood (Kabanov et al. 2002). In a separate study Kabanov and Alakhov compared Biodistribution of different block copolymers. Area under the curves (AUC) for blood, liver and spleen and tissue distribution coefficient (P organ/blood) were studied. As seen in the Table 2.7, the tissue distribution increased in the following order; F68<F108<P85<L61. this shows that concentration of block copolymer remain in the plasma for several hours after administration (Kabanov and Alakhov, 2000)

Table 2.7

Pharmacokinetics and tissue distribution of Pluronic block copolymer in mice

Pluronic block copolymer	Blood		Liver		Spleen	
	AUC	P organ/blood	AUC	P organ/blood	AUC	P organ/blood
F 68	0.111	-	0.225	2.03	0.066	0.59
P 85	0.026	-	0.079	3.03	0.032	1.23
L61	0.044	-	0.215	4.93	0.118	2.68

Pharmacological/ therapeutic effects of Pluronic F-68

Pluronics are non-toxic nonionic surface-active triblock copolymers. Poloxamer's structure, with a hydrophobic region bracketed by hydrophilic regions, has similarities to the phospholipid bilayer that comprises cellular membranes. Research indicates poloxamers associate themselves with a damaged membrane, coating exposed hydrophobic regions of the membrane and plugging water-enlarged pores (Maskarinec et al. 2002).

Pluronic F-68 has been shown to limit the damage from electroporation to membranes (Lee et al., 1992) and to improve tissue viability (Basakaran et al., 2001). Poloxamer-188 has also been reported to limit damage and improve recovery from mechanical, chemical, thermal, and radiation damage as well as ischemia reperfusion injury in a variety of tissue types that include neurons, muscles, and heart (Mezrow et al. 1992; Merchant 1998; Curry, 2004).

2.9 PROFILE OF THE CELL LINES

2.9.1 DU 145 Cells

(Human, Prostate, Carcinoma)

Morphology

Epithelial

Growth properties

Adherent

Organism

Homo sapiens (human)

Source

Organ: Prostate ; *Disease*: Carcinoma; *Derived from metastatic site*: brain

Cytogenetic analysis:

This is a hypotriploid human cell line. Both 61 and 62 chromosome numbers had the highest rate of occurrence in 30 metaphase counts.The rate of higher ploidies was 3%. The t(11q12q), del(11)(q23), 16q+, del(9)(p11), del(1)(p32) and 6 other marker chromosomes were found in most cells. The N13 was usually absent. The Y chromosome is abnormal through translocation to an unidentified chromosomal segment. The X chromosome was present in single copy (Stone et al., 1978; Mickey et al., 1977).

Isoenzymes:

AK-1, 1
ES-D, 1
G6PD, B
GLO-I, 2
Me-2, 1-2
PGM1, 1
PGM3, 2

Propagation:

The base medium for this cell line is ATCC-formulated Eagle's Minimum Essential Medium. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%.

Temperature: 37°C

Atmosphere: air, 95%; carbon dioxide (CO2), 5%

Subculturing:

Protocol:

1. Remove and discard culture medium.
2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin - 0.53 mM EDTA solution to remove all traces of serum which contains trypsin inhibitor.
3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes). Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37C to facilitate dispersal.
4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by pipetting.
5. Add appropriate aliquots of the cell suspension to new culture vessels.
6. Incubate cultures at 37C

Subcultivation ratio: A subcultivation ratio of 1:4 to 1:6 is recommended
Interval: Maintain cultures at a cell concentration between 6 X 10(3) and 6 X 10(4) cell/cm2.
Medium renewal: 2 to 3 times per week

Preservation:

Freeze medium: Complete growth medium, 95%; DMSO, 5%
Storage temperature: liquid nitrogen vapor phase

Doubling Time: 30-40 hours

2.9.2 L1210 CELLS

(Mouse, DBA/2, Ascitic Fluid, Leukemia, Lymphocytic)

Morphology

Lymphoblast

Growth properties

Suspension

Organism

Mus musculus (mouse)

Source:

Species: mouse, DBA/2 female;

Disease: lymphocytic leukemia

Strain: DBA subline 212

Tissue: Ascitic fluid;

Reverse transcriptase

positive

Cytogenetic analysis:

modal number = 40; range = 32 to 80. Mouse karyotype with a diploid stemline number of 40 chromosomes (Law et al., 1949).

Propagation:

ATCC complete growth medium: The base medium for this cell line is ATCC-formulated Dulbecco's Modified Eagle's Medium. To make the complete growth medium, add the following components to the base medium: horse serum to a final concentration of 10%.

Temperature:37°C

Atmosphere: air, 95%; carbon dioxide (CO2), 5%

Subculturing:

Protocol:

Cultures can be maintained by addition or replacement of fresh medium. Start cultures at 5 X 10 exp4 viable cells/ml.

McCoy's 5A + 10% FBS + 2mM L-Glutamine; maintain cultures between 1-5 x 10^5 cells/ml;

Splitting

1:3-1:6

Medium Renewal: Add fresh medium (20% to 30% by volume) every 2 to 4 days

Preservation:

Freeze medium: Complete growth medium, 95%; DMSO, 5%
Storage temperature: liquid nitrogen vapor phase

Doubling Time

8 to 10 hrs

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