The Incorporation of EO9 into Albumin Microspheres and their Evaluation as a Potential Anticancer Therapy

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Abstract

EO9 is one of a series of fully synthetic indoloquinones developed for their ability to act as potential bioreductive anticancer agents. Pre-clinical studies suggested that EO9 was an active drug which demonstrated preferential activity against solid tumour cell lines. However, disappointing results were seen in subsequent Phase II trials which failed to detect any significant antitumour activity. This was thought to be due, in part, to dose limiting renal toxicity combined with EO9's short half-life and instability. For these reasons EO9 was felt to be a suitable drug to consider for encapsulation into albumin microspheres for use in locoregional therapy. This would have the benefit of delivering a high concentration of the drug to the tumour site whilst at the same time minimising systemic exposure and therefore toxicity. In addition, the possible induction of hypoxia by the microspheres might enhance the drugs bioreductive activity.

A new technique was developed allowing the successful encapsulation of EO9 into human albumin microspheres. This was an emulsification process which took place at room temperature using 6.25% gluteraldehyde as the chemical cross-linking agent. The EO9-loaded microspheres produced by this method had a median size of 19.9μm with 70% of the microspheres being greater than 12μm in diameter. This would enable them to lodge in the first capillary bed they encounter and makes them suitable for use in locoregional therapy. The drug payload of the microspheres, determined by HPLC analysis, was 1.24 ± 0.2mg/100mg of microspheres, which represented the actual amount of chemically intact drug released from the microspheres. This appeared to be rapidly released following resuspension in comparison to other microsphere systems, but would be acceptable for a drug such as EO9, whose mode of action is related to peak drug concentration rather than prolonged drug exposure.

The antitumour activity of the EO9-loaded microspheres was compared to free drug when given by direct intratumoural injection in four tumour models. These were the subcutaneously grown human colonic xenografts; HT29 and BE and the murine adenocarcinomas of colon; MAC 16 and MAC 26. In the HT29 tumour no significant difference in antitumour activity was detected between the free drug (250μg) and the equivalent dose of drug-loaded microspheres. In the MAC 16 tumour twice the dose of EO9-loaded microspheres (500μg) was required to produce equivalent antitumour activity to the free drug. In the MAC 26 and BE tumours, antitumour activity was detected with the free drug, but no antitumour activity was seen with the EO9-loaded microspheres.

In an attempt to explain the differences which were seen in the antitumour studies between the free drug and the microspheres, pharmacokinetic analyses were performed in each of the tumour types. No significant differences were seen in the tumour pharmacokinetic profiles. Similarly, HPLC analysis failed to detect any obvious metabolites which might be indicators of drug activity. However, analysis was limited due to the lack of available data on EO9 metabolites in terms of standard curves and extraction efficiencies.

Direct intratumoural injection of the EO9-loaded microspheres is not the best way of assessing the potential of a drug delivery system. Therefore, a more clinically relevant model to assess the microspheres was developed. Preliminary *in vitro* and *in vivo* studies have established the sensitivity of the HSN rat sarcoma cell line to EO9. The liver of the Lister Hooded rat has subsequently been implanted with HSN cells and treated by the administration of free drug or microspheres via the hepatic artery. Further work is required to improve on both the consistency of tumour growth within the liver, as well as the technique of hepatic arterial injection, before definite conclusions can be made about whether encapsulating EO9 into microspheres improves drug delivery to the tumour following intra-arterial administration.

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I would like to thank everyone in the Imperial Cancer Research Fund laboratory, Edinburgh for their friendship over the three years I spent there. In particular, I would like to thank my supervisor, Dr Duncan Jodrell, for his support, encouragement and advice both during the research and subsequent writing of this thesis. Thanks also goes to Dr Jeff Cummings for his help, together with the other members of the Pharmacology Team. In particular, Alex MacLellan for help with the HPLC analysis for the pharmacokinetic studies, Janet Macpherson who introduced me to tissue culture and Rhona Aird who assisted with the tissue culture for the *in vivo* studies. Eric Miller helped with the histology and Samantha Carmichael helped with the simulations outlined in Chapter 4.

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Abbreviations

AUC	Area under the curve
CO_2	Carbon dioxide
DHFR	Dihydrofolate reductase
2, 7-DM	2, 7-diaminomitosene
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DTD	DT-Diaphorase
ERCP	Endoscopic retrograde cholangiopancreatography
FAA	Flavone Acetic Acid
FCS	Foetal Calf Serum
5FU	5-Fluorouracil
FUDR	Fluorodeoxyuridine
GI_{50}	50% Growth Inhibition
GST	Glutathione-S-transferase
HCR	Hypoxic Cytotoxicity Ratio
H+E	Haematoxylin + Eosin
HPLC	High Performance Liquid Chromatography
HSA	Human serum albumin
IC_{50}	Concentration causing 50% growth inhibition compared to control
ICRF	Imperial Cancer Research Fund
i.a.	intra-arterial
i.t.	intratumoural injection
LD_{10}	Lethal dose in 10% of the treated population
LD_{50}	Lethal dose in 50% of the treated population
LHRH	Leutenisizing hormone releasing hormone

LRP	Lung Cancer Resistance associated Protein
MAC	Mouse Adenocarcinoma of Colon
MDR	Multidrug Resistance Phenotype
MMC	Mitomycin C
MRP	Multidrug Resistance associated Protein
PBS	Phosphate Buffered Saline
SD	Standard deviation
SEM	Standard error of the mean
SDS	Sodium dodecyl sulphate
$T_{1/2}$	Half-life
T/C	Percentage change in tumour volume Treated group/Percentage change in tumour volume Control group
TNF	Tumour Necrosis Factor
UV	Ultraviolet
Vis	Visible
W/W	Amount of drug/100mg microspheres

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Introduction

1 Chapter 1

1.1 CANCER: AN INTRODUCTION

The term "cancer" is the collective name for a group of over 200 different diseases, each with its own characteristics and natural history. The group has in common growth regulatory defects which allow uncontrolled proliferation of a single clone of cells. This growth, resulting in the formation of a tumour mass, can produce local invasion as well as dissemination around the body.

Cancer is a major cause of morbidity and mortality in the western world, and is the second commonest cause of death after cardiovascular disease. In the UK it accounts for around 25% of all deaths. Current incidence rates mean that 1 in 3 people will develop cancer at some stage in their lives, the incidence increasing with age and of those, 50 - 60 % will probably die from their disease. Figure 1.1 details the ten most frequently diagnosed cancers for both males and females in Scotland (Scottish Cancer Incidence and Mortality Statistics 1998).

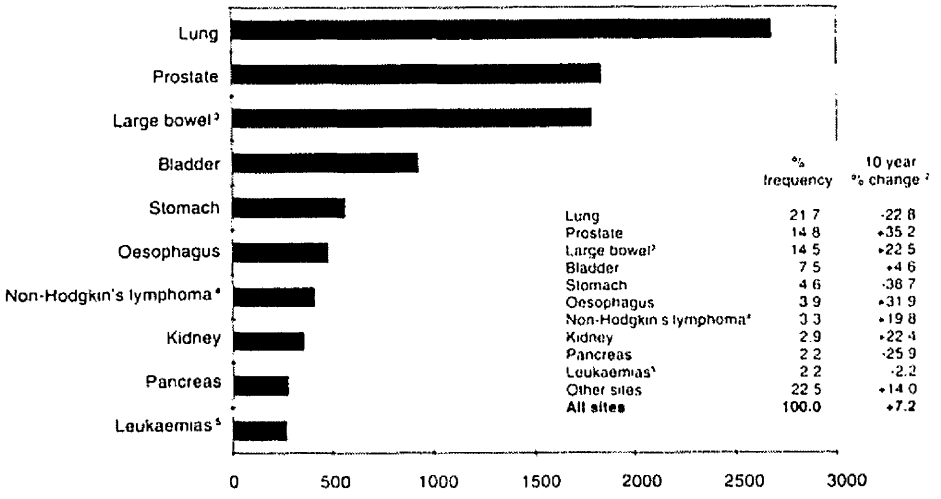
1.2 CANCER MANAGEMENT

The management of cancer requires a multi-disciplinary approach, and is dependent on both the stage and type of the disease. It can be divided into three main categories:

Curative

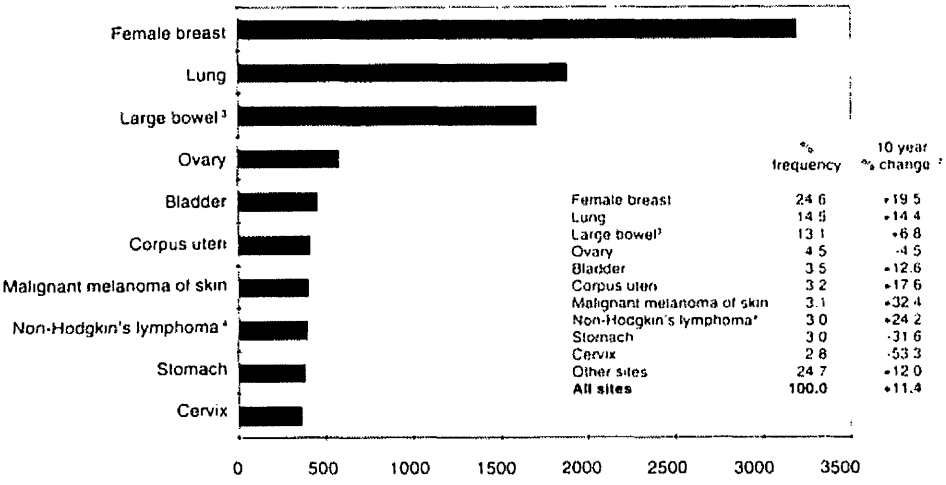
In the case of solid tumours such as colon or breast cancer this is usually when the cancer is still localised. Treatment is normally by surgery and/or radiotherapy. There are also some types of cancer; e.g. leukaemias, lymphomas, teratomas and some childhood tumours which can be cured with chemotherapy alone.

Males



	% frequency	10 year % change[2]
Lung	21 7	-22 8
Prostate	14 8	+35 2
Large bowel[3]	14 5	+22 5
Bladder	7 5	+4 6
Stomach	4 6	-38 7
Oesophagus	3 9	+31 9
Non-Hodgkin s lymphoma[4]	3 3	+19 8
Kidney	2 9	+22 4
Pancreas	2 2	-25 9
Leukaemias[5]	2 2	-2.2
Other sites	22 5	+14 0
All sites	100.0	+7.2

Females



	% frequency	10 year % change[2]
Female breast	24 6	+19 5
Lung	14 5	+14 4
Large bowel[3]	13 1	+6 8
Ovary	4 5	-4 5
Bladder	3 5	+12 6
Corpus uteri	3 2	+17 6
Malignant melanoma of skin	3 1	+32 4
Non-Hodgkin's lymphoma[4]	3 0	+24 2
Stomach	3 0	-31 6
Cervix	2 8	-53 3
Other sites	24 7	+12 0
All sites	100.0	+11.4

Figure 1.1 **The ten most frequently diagnosed cancers (male and female) registered in Scotland in 1996. Non-melanoma skin cancer is excluded (Scottish Cancer Incidence and Mortality Statistics, Scottish Health Statistics, 1998).**

Adjuvant

Systemic adjuvant therapy follows localised treatment and is given when dissemination is undetectable, but assumed to be present based on the histology of the tumour. When used in the correct setting, adjuvant treatment has been shown to improve survival. Examples include breast cancer (Bonadonna et al 1995, Early Breast Trialist's collaborative group 1992), colon cancer (Moertel et al 1990), and osteosarcoma (Link et al 1986).

Palliative

Palliative treatment is administered to relieve symptoms caused by the tumour. Local symptoms can be treated with localised therapy such as surgery or radiotherapy, but increasingly, systemic therapy is being used in this setting, to both improve patient well-being and prolong survival.

1.2.1 Systemic Therapy

Systemic therapy, which can be used at all stages in the management of cancer can be divided into 3 main categories:

1.2.1.1 Hormonal Therapy

Tumours which develop in organs which are under hormonal control, such as breast and prostate cancer, sometimes retain their hormone sensitivity. The aim of treatment is to prevent tumour growth either by decreasing endogenous hormone production or by blocking the hormone effect at its receptor. In the case of breast cancer, the former can be by ovarian ablation, the use of luteinising hormone releasing hormone (LHRH) analogues and aromatase inhibitors such as anastrazole, which switch off oestrogen production. The latter is achieved by the use of anti-oestrogens such as tamoxifen which block the receptor site. Similarly, in prostate cancer, androgen ablation can be achieved by orchidectomy or by the

use of an LHRH agonist which causes a reduction in androgen production. The use of an anti-androgen such as flutamide can prevent androgen binding to its receptor. (For detailed review the reader is referred to: Swain 1996, Erlichman et al 1997).

1.2.1.2 Biological/Cytokine Therapy

Cytokines are naturally occurring glycoproteins which, unlike endocrine hormones, can be secreted by various cell types throughout the body. Each cytokine may have several functions, but, because of the complex interactions which can occur between the various cytokines, these have often been difficult to define. Cytokines have two main roles in the treatment of cancer:

i) **Supporting treatment**

Growth factors such as G-CSF and GM-CSF can be used to reduce the severity of chemotherapy-induced neutropenia and to accelerate bone marrow recovery following transplantation. (For review, the reader is referred to: Petros et al 1996, Griffin 1997).

ii) **Antitumour activity**

Many cytokines are used as antitumour agents. Interferons are used in the treatment of various types of leukaemias, multiple myeloma, renal cell carcinoma and Kaposi's sarcoma. Interleukin-2 is used in the treatment of renal cell carcinoma and Tumour Necrosis Factor (TNF) is used in the isolated limb perfusion of patients with advanced melanoma in combination with cytotoxic drugs. (For review, the reader is referred to: Witt et al 1996, Bukowski et al 1996, Rosenberg 1997).

1.2.1.3 *Chemotherapy*

The history of cancer chemotherapy is relatively short. The first clinical studies took place in 1943 with the alkylating agent nitrogen mustard (DeVita 1978). Since then, intensive research has led to the discovery of a wide variety of chemotherapeutic agents. They can be classified in a number of ways depending on their mode of action or their effect on the cell cycle. They include the Alkylating agents, Heavy metals, Antimetabolites, Antitumour antibiotics, Topoisomerase inhibitors and Tubulin binding drugs (Figure 1.2, Table 1.1). A more detailed review of chemotherapy is not appropriate for this thesis, but further information can be found in Chabner et al 1996 and DeVita et al 1997.

1.3 DRUG RESISTANCE IN SOLID TUMOURS

In Scotland, as in the rest of the western world, solid tumours are the commonest type of malignant disease, with lung (21.7%), prostate (14.8%) and colorectal (14.5%) cancer being the most common in men and breast (24.6%), lung (14.5%) and colorectal (13.1%) cancer being the most common in women (Figure 1.1: Scottish Cancer Incidence and Mortality Statistics 1998). Despite the range of cytotoxic drugs now available which have made a considerable impact on leukaemias, childhood tumours and the rarer forms of adult cancer such as teratoma and lymphoma, solid tumours remain relatively chemoresistant. A variety of mechanisms have been identified in solid tumour resistance, though many remain to be elucidated.

One well characterised example of drug resistance is the P170 glycoprotein (or multidrug resistance phenotype (MDR)), a 170 kD transmembrane glycoprotein (Juliano et al 1976, Endicott et al 1989), which acts as a pump capable of ejecting cytotoxic agents from the cell.

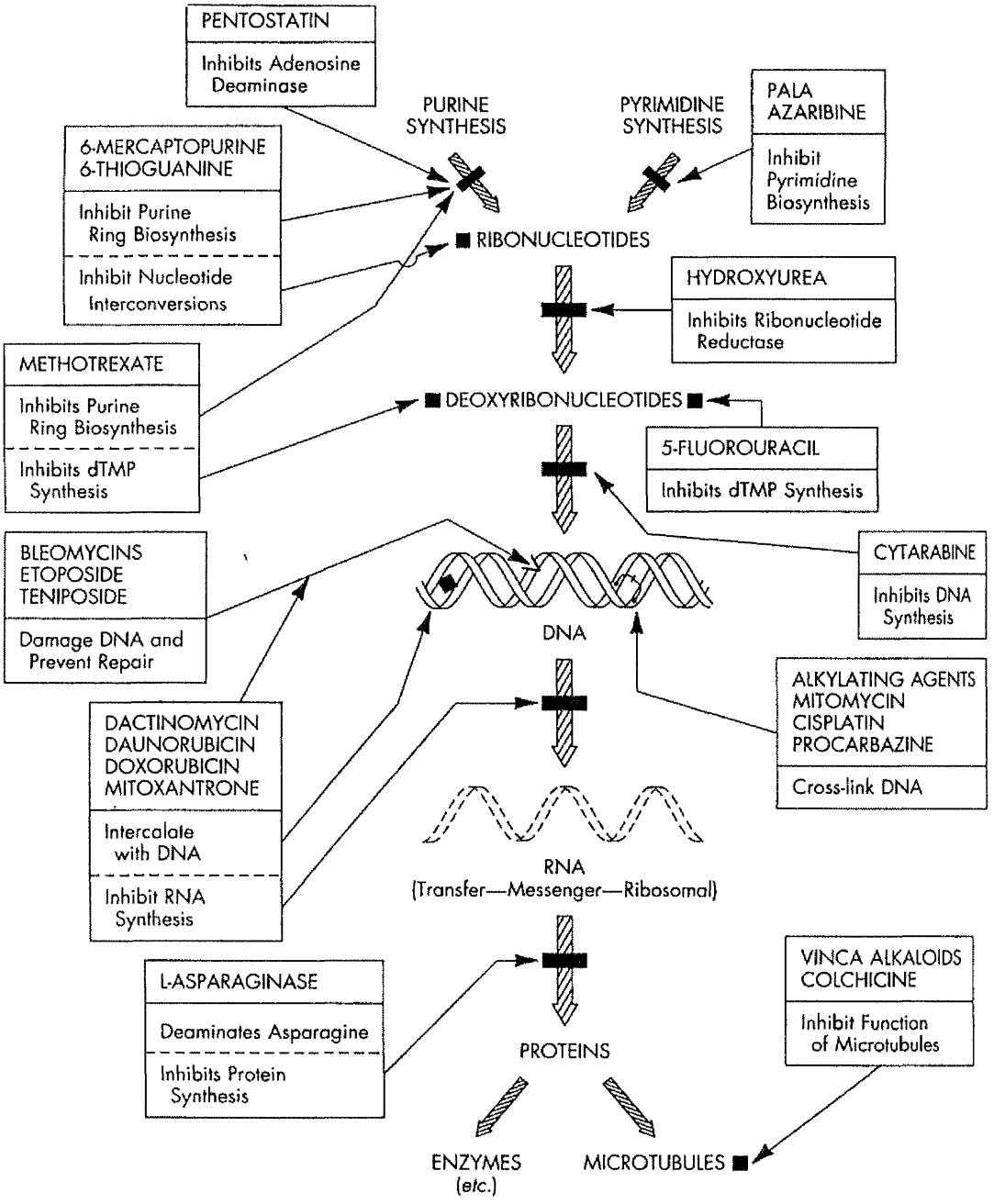


Figure 1.2 **Summary of the main classes of anticancer agents and their mechanisms of action.**

Class	*Example*	
Alkylating Agents	Nitrogen Mustards	Melphalan Chlorambucil
	Oxazophosphorine	Cyclophosphamide Ifosfamide
	Alkylaziridines	Busulphan
	Bioreductives	Mitomycin C
	Nitrosureas	Carmustine Lomustine
Heavy Metals	Cisplatin Carboplatin	
Antimetabolites	Antifolates	Methotrexate
	Antipyrimidines	5-Fluorouracil Cytosine arabinoside
	Antipurines	6 mercaptopurine Fludarabine
Antibiotics	Anthracyclines	Doxorubicin Daunorubicin
	Anthracenediones	Mitoxantrone
	Bleomycin	
Topoisomerase Inhibitors	Topo I inhibitor	Camptothecin Topotecan
	Topo II inhibitor	Etoposide Tenoposide
Tubulin Binding Drugs	Vinca alkaloids Taxanes	

Table 1.1 **A summary of the main classes of anticancer agents.**

The drugs affected by this pump are the natural product anticancer agents such as anthracyclines, vinca alkaloids, taxanes and podophyllotoxins, with exposure to one of the drugs resulting in cross-resistance to a number of the other agents (Moscow et al 1988). Various methods have been used to try and circumvent MDR, but they have met with limited clinical success e.g. verapamil and cyclosporin (For reviews see: Ford et al 1990, Raderer et al 1993). Other cellular proteins thought to be involved in resistance to natural product anticancer agents are the Multidrug Resistance associated Protein (MRP), associated with resistance to doxorubicin, etoposide and vincristine (Grant et al 1994) and the Lung cancer Resistance associated Protein (LRP) (Scheper et al 1993).

Tumour resistance to antimetabolites may occur as a result of an alteration in the drugs' intracellular target e.g. methotrexate, where resistant cells can show increased quantities of the target enzyme dihydrofolate reductase (DHFR) due to amplified gene expression (Schmike 1984) or contain variant forms of the DHFR enzyme, both of which affect enzyme inhibition by the methotrexate (Goldie et al 1980). Other mechanisms of resistance include impaired polyglutamation of methotrexate within the cell (Pizzorno et al 1989) leading to impaired intracellular retention of methotrexate and decreased uptake of methotrexate into the cell by reduced folate carriers (Goldman et al 1968). 5-Fluorouracil resistance can also be induced by the upregulation of its target enzyme, thymidylate synthase (Zang et al 1992).

Tumour resistance to alkylating agents can be associated with changes in the cellular systems which allow drug detoxification to occur. Glutathione, an important detoxifying non-protein thiol, can be found in increased concentration, associated with increased activity of the enzyme Glutathione-S-transferase (GST) in cells which are resistant to alkylating agents (Bellamy et al 1991, Tew 1994).

1.3.1 The Role of DNA Damage Recognition and Repair Mechanisms in Drug Resistance

Normal regenerating cell populations such as the bone marrow or the lining of the GI tract have the ability to monitor and repair damaged DNA or destroy cells with irreparable DNA rather than allow damaged cells to enter the cell cycle and potentially replicate this damage. P53 is one of the proteins found in the cell which, in response to DNA damage, causes either cell cycle arrest until the damage has been repaired or induces destruction of the damaged cell by apoptosis (Levine et al 1991, Keurbitz et al 1992). Mutations in the P53 gene are often found in tumour cells which may prevent normal cell regulation (Kemp et al 1994, Holstein et al 1991), resulting in cells with abnormal DNA, often as a result of treatment with radiation or drugs, being allowed to continue through the cell cycle and undergo cell division. Thus, as well as producing cells which do not readily undergo apoptosis, cells with altered phenotypes are produced. This may result in new drug resistance patterns developing, potentially leading to higher levels of tumour resistance and to more advanced patterns of tumour growth and spread.

1.3.2 Physical Barriers as a Mechanism of Drug Resistance

This important mechanism of drug resistance relates to the physical nature of the solid tumour and its associated blood supply (Curti 1993).

1.3.2.1 Tumour interstitium

The interstitial space within tumours is quite different to that found in normal tissues and varies considerably between tumour types and tumour sites (Gullino et al 1962, Gullino et al 1963). The interstitial space usually accounts for 14 - 34 % of normal tissue volume, but it is increased to 36 - 53 % in tumours (Gullino et al 1965, O'Connor et al 1984). This, together with the absence of lymphatic drainage, produces an increase in interstitial pressure which,

if it exceeds intravascular pressure, causes closure of blood vessels resulting in impeded transport of drugs and other molecules within the tumour (Roh et al 1991, Jain 1987). Marked variations in the protein, type IV collagen and glycosaminoglycan levels in the tumour interstitium are seen compared with the normal tissue (Gullino et al 1962, Gullino 1964, Choi et al 1971, Jain et al 1987). Variations in oxygen concentration and pH have also been documented (Thistlewaite et al 1985, Vaupel et al 1991).

1.3.2.2 Tumour vessels

If a tumour is to increase in size beyond $1mm^3$, it needs to develop its own blood supply (Chaplin 1992A). This is initiated by a variety of angiogenic factors released by the tumour or the hosts' inflammatory cells (Folkman 1993). The structure of tumour blood vessels differs from that of normal vasculature. The endothelial layer is often patchy with a variable basement membrane (Kaiser 1989) and the vessels lack both a nerve supply and smooth muscle layer (Dvorak et al 1988) which may make them less responsive to vasoactive drugs. The vessels are highly tortuous with abnormal branching patterns and arterio-venous anastomoses are often found which contribute to the slowing of blood flow within the tumour. Blood vessels are unevenly distributed throughout the tumour mass and because no adequate collateral circulation is present (Chaplin et al 1992A), some areas within the tumour receive a less than adequate blood supply (Jain et al 1994). Thus, the rapid growth of the tumour, together with the problems created by the inadequate vascular network and the interstitial tissue, can result in large areas of the tumour receiving an inadequate supply of essential nutrients, particularly oxygen, which has important implications for both tumour growth and treatment.

1.3.3 Tumour Oxygenation

The cell population of the tumour can be divided into three main groups with respect to their oxygen status (Thomlinson et al 1955, Workman 1992A):

1. **Well-oxygenated cells.** These cells are usually close to the vascular network so they receive an adequate amount of both oxygen and nutrients.
2. **Necrotic or anoxic cells.** In areas where the oxygen and nutrient levels are not sufficient to maintain viability, the cells die, resulting in the areas of necrosis commonly found within solid tumours.
3. **Hypoxic cells.** These are a population of cells who exist at an oxygen level which is just sufficient to maintain viability, but which is suboptimal for proper cell growth and metabolism. The presence of this reduced oxygen tension has been confirmed in a variety of human tumours using mini-electrodes. These studies also found that the outcome of treatment correlated with the degree of hypoxia which was detected within the tumour (Gatenby et al 1988, Vaupel et al 1991).

Hypoxic populations can arise as a result of two different mechanisms:

1. **Chronic or diffusion limited hypoxia.** This type of hypoxia can last for several days and arises because the diffusion distance for oxygen is limited by metabolic consumption to approximately 150 - 200 μm from the blood vessel. This results in zones of chronically hypoxic cells being found at the interface between the oxic and the anoxic cell populations (Thomlinson et al 1955, Hall 1994).
2. **Acute or perfusion limited hypoxia.** This is a transient, reversible form of hypoxia and may be the result of intermittent vascular occlusion caused by, e.g. a rise in the interstitial pressure of the tumour (Brown 1979, Chaplin et al 1986, Horsman et al 1992).

1.3.4 The Consequences of Hypoxia

Areas of hypoxic cells commonly occur within solid tumours. The reduced oxygen tension and the lack of nutrients means the cells will proliferate at a slower rate in relation to the rest of the tumour and lactic acid, produced as a result of anaerobic respiration, will result in a more acidic environment (Tannock et al 1989). Hypoxic cells have been found to be significantly more resistant to radiation than their normoxic counterparts because the lack of oxygen prevents the formation of the toxic free radicals which result in radiation induced DNA damage (Hewitt et al 1959, Hall 1994). Hypoxic cells may leave or move more slowly through the cell cycle which will affect their response to most conventional cytotoxic agents (Born et al 1976, Koch et al 1973, Tannock et al 1981). The distance the cells are from the tumour vasculature may also prevent them from receiving adequate concentrations of drug (Vaupel 1977). In addition, hypoxia has been shown to enhance the frequency of DHFR gene amplification which will increase the resistance of the cell to methotrexate (Rice et al 1986).

1.3.5 Overcoming the Effect of Hypoxia in the Treatment of Solid Tumours

Fractionation of radiation, which is the administration of small regular doses of radiation (usually on a daily basis), may result in improved tumour oxygenation due to a reduction in tumour mass as the treatment progresses. This should result in increased tumour sensitivity to the remaining radiation treatment (Souhami et al 1998, Coleman 1996).

Apart from fractionation, two other methods have been identified which attempt to overcome the effect of hypoxia in both the radiotherapeutic and chemotherapeutic treatment of solid tumours.

1.3.5.1 Increasing oxygen delivery to the tumour

The use of hyperbaric oxygen (Henk 1981), blood transfusions (Dische 1991) and oxygen carrying chemicals such as the fluorocarbons (Lustig et al 1989) have all been utilised to try to improve oxygen delivery to the tumour. While trials have shown some improvement in tumour response, it is unlikely that these methods alone will eliminate the hypoxic cell population. This is because the tumour adapts to the new oxygen level present within the tumour with the result that a chronically hypoxic region persists, but is located further away from the tumour blood supply (Hirst 1993).

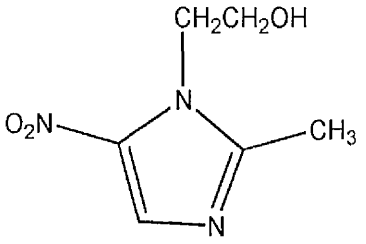
1.3.5.2 Using oxygen alternatives

1.3.5.2.1 Radiosensitisers

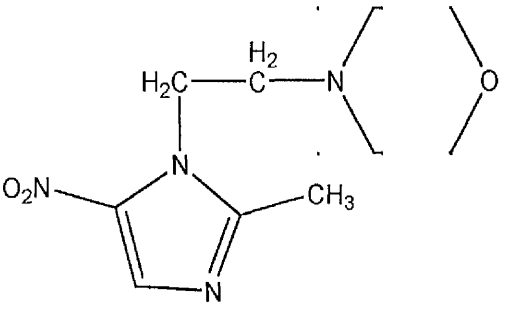
Radiosensitisers are unique agents which act as oxygen substitutes for hypoxic cells. The largest group of these are the electron affinic radiosensitisers whose efficiency is related to their electron affinity. They diffuse out of the tumour blood supply and are absorbed by hypoxic cells where they increase the production of free radicals in a manner similar to that of oxygen. They have no effect on well oxygenated cells.

The 5-nitroimidazole, metronidazole (Figure 1.3), was the first of these compounds to be tested. The median survival of the patients with glioblastoma treated with metronidazole in addition to radiotherapy was greater than those treated with radiotherapy alone (7 months v 3 months). However, because the study did not use a standard radiotherapy protocol, the maximum effect of the metronidazole could not be properly assessed (Urtasun et al 1976).

The 2-nitroimidazoles (Figure 1.4) are more electron affinic than metronidazole and therefore more efficient hypoxic cell sensitisers. Initial studies with oral misonidazole were limited by significant toxicities (nausea, vomiting and neuropathy) and while results



Metronidazole



Nimorazole

Figure 1.3 Structures of the 5-nitroimidazole radiosensitisers: Metronidazole and Nimorazole.

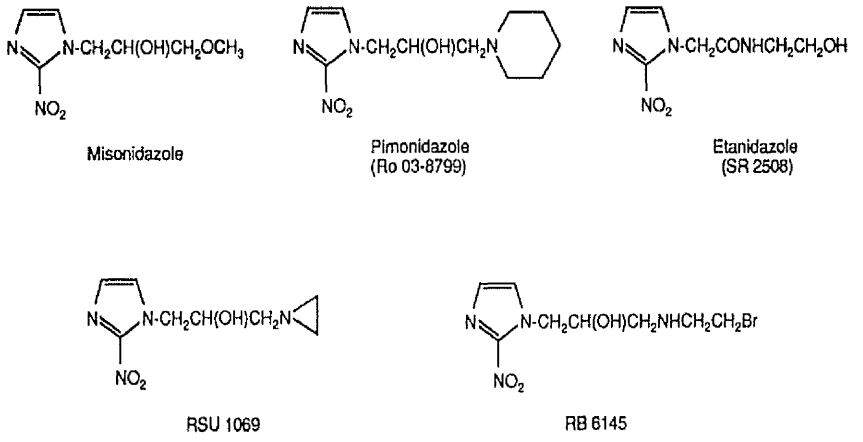


Figure 1.4 Structures of the 2-nitroimidazole radiosensitisers and bioreductive cytotoxins.

suggested that there was some benefit in the treatment of head and neck cancer, they were not reproducible (For review, see Dische 1985). Analogues of misonidazole have been developed, of which the lead compound is Etanidazole (SR 2508). Successful pharmacokinetic monitoring was carried out to minimise the dose dependent neurotoxicity (Coleman et al 1992) but, randomised phase III trials in head and neck cancer have so far failed to show any benefit in the group treated with Etanidazole in addition to standard radiotherapy (Lee et al 1995). Similarly, the addition of Pimonidazole (Ro 03-8799), a lipophilic 2-nitroimidazole, to radiotherapy has failed to show any benefit in a randomised phase III study in cervical carcinoma (Dische 1992). This lack of activity is thought to be the result of a drug-induced reduction in blood flow to the tumour, an effect which had been identified in preclinical studies (Chaplin et al 1992B).

A newer 5-nitroimidazole, Nimorazole (Figure 1.3), while a less potent sensitiser than the 2-nitroimidazoles, has produced a statistically significant improvement in survival in a phase III study in head and neck cancer carried out by The Danish Head and Neck Cancer Group (Overgaard et al 1991). This is thought to be related to the fact that it causes significantly less cumulative neurotoxicity than Etanidazole and Pimonidazole which has allowed the drug to be given throughout the whole of the radiotherapy treatment. Further studies are in progress with this agent.

1.3.5.2.2 Hypoxic cytotoxic agents (Bioreductive Drugs)

An alternative approach is to attempt to exploit the hypoxic environment found within tumours by using drugs which become chemically active under hypoxic conditions. The hypoxic environment promotes reductive reactions and drugs activated in this way have been termed bioreductives. They undergo metabolic activation by reduction via a number of enzyme pathways to produce cytotoxic species which are capable of covalently binding to DNA.

The enzymology of bioreductive activation, exemplified in Figure 1.5 by the bioreductive indoloquinone EO9 which will be discussed in detail later (section 1.7), is complex and occurs via 1 or 2 electron reduction steps. The 1 electron reductases include enzymes such as cytochrome P450 reductase, xanthine oxidase and cytochrome *b*5 reductase. This step is reversible in the presence of oxygen which means that under aerobic conditions the reductive metabolite formed will be back oxidised to the original product in a process known as redox cycling (Powis 1987). Although redox cycling results in the formation of toxic superoxide and hydroxyl radicals, these are less damaging to cells than the highly toxic drug metabolites that predominate in the hypoxic cells because the superoxide and hydroxyl radicals are rapidly inactivated by the cells normal protective enzymes (catalase and superoxide dismutase). The 2 electron reductases which include DT-Diaphorase and xanthine dehydrogenase are not usually oxygen sensitive. This means that the reactive intermediates formed by this process are formed independently of the oxygen status of the cell and therefore occur in both oxic and hypoxic tumour cells.

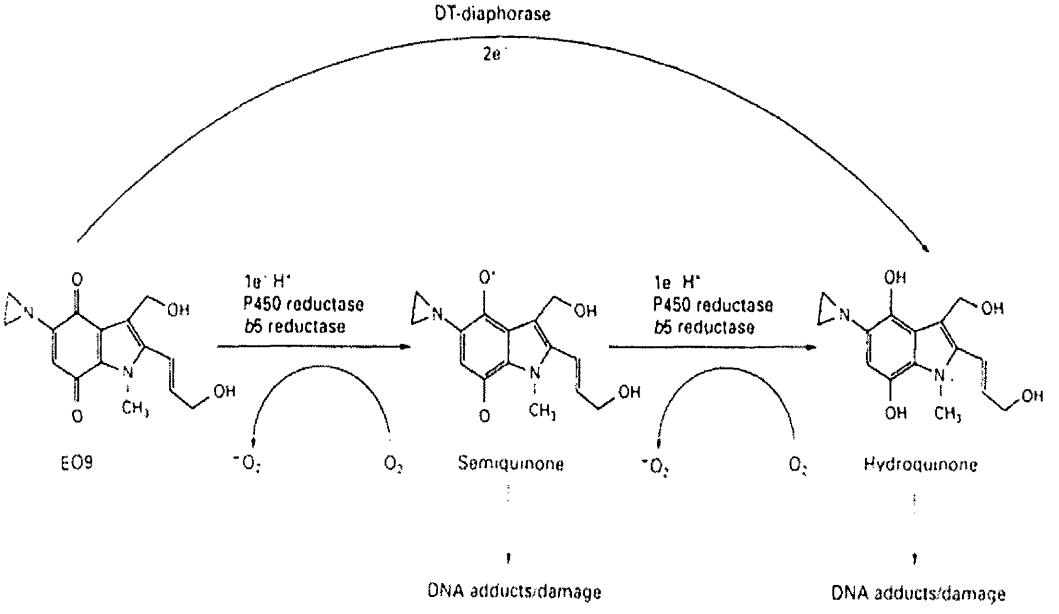


Figure 1.5 Possible routes for bioreductive activation of the indoloquinone. EO9 by 1 and 2 electron reduction.

1.4 CLASSIFICATION OF BIOREDUCTIVE DRUGS

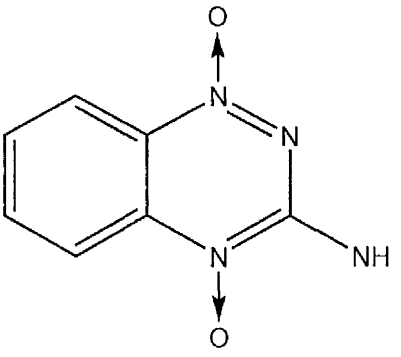
1.4.1 Nitroimidazoles

During the development of the nitroimidazoles as radiosensitisers, it became evident that some of the compounds being evaluated had a dual method of activation. As well as being radiosensitisers, the presence of an aziridine moiety resulted in bioreductive characteristics which also allowed them to act as cytotoxic alkylating agents.

The first compound RSU 1069 (Figure 1.4), a 2-nitroimidazole with an aziridine ring (Stratford et al 1986), entered clinical trials but there were problems with gastrointestinal toxicity which significantly limited the dose of drug which could be tolerated (Horwich et al 1986). A pro-drug of RSU 1069, RB 6145 (Figure 1.4) was developed which demonstrated antitumour activity in preclinical studies (Jenkins et al 1990) and appeared less toxic (Sebolt-Leopold et al 1992). The active metabolites of RU1069 are the nitroradical (1 electron reduced), the nitroso (2 electron reduced) and the hydroxylamine (4 electron reduced) anions which usually result in the formation of DNA monoadducts and occasionally DNA cross-linkage, although the precise nature of the covalent reaction products remain to be identified. In aerobic conditions the predominant reaction is, as expected, redox cycling using the 1 electron reductase enzymes (Workman, 1992B).

1.4.2 Benzotriazine-N-oxides

The lead compound of this potent group of specific hypoxic cell cytotoxins is SR4233 (3-amino-1,2,4-benzotriazine 1,4-dioxide, WIN 59075, tirapazamine) (Zeman et al 1986) (Figure 1.6). SR4233 undergoes a complex pathway of metabolic activation with 1 electron reduction producing an oxidising nitroxidal radical which then abstracts hydrogen from bases in the



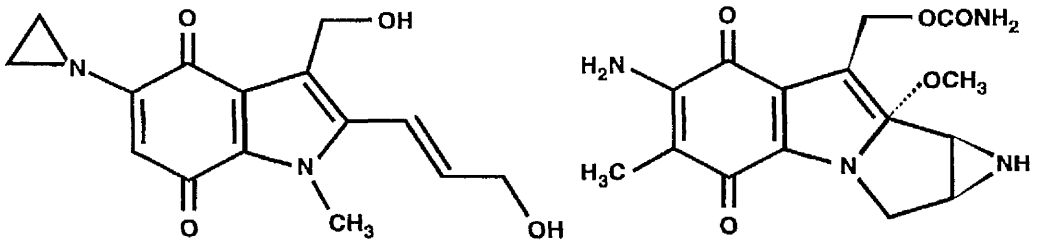
SR 4233

Figure 1.6 The structure of the lead Benzotriazine-N-oxide: SR4233 (3-amino,-1,2,4-benzotriazine1,4-dioxide; WiN 59075;tirapazamine).

DNA, resulting in both single and double strand breaks without the formation of DNA adducts (Laderoute et al 1988). Again this effect is lost in the presence of oxygen, as the nitroxidal radical is back-oxidised to the parent molecule via redox cycling. A Phase II study using SR4233 in combination with cisplatin has suggested an improved response rate and survival benefit in non small lung cancer when compared to historical data with single agent cisplatin (Treat et al 1998). This has been confirmed in the Phase III CATAPULT -1 trial where both the response rate (27.5% v 13.7%, $p<0.001$) and the median survival (34.6 weeks v 27.7 weeks, $p<0.0078$) were significantly better in the combined treatment arm (von Pawel et al 1998).

1.4.3 The Quinones

The quinone bioreductives (Figure 1.7) include Mitomycin C (MMC) often regarded as the prototype bioreductive alkylating agent, together with more recently developed drugs such as the indoloquinone, EO9, which will be discussed in more detail later (Section 1.7). Bioreduction of the quinones can occur by means of both 1 and 2 electron processes. 1 electron reduction results in the formation of the semiquinone free radical, which as previously indicated, in the presence of oxygen can undergo futile cycling back to the parent compound. 2 electron reduction results in the formation of a hydroquinone. In the case of MMC, reduction results in the activation at the C-1 position of the mitosene ring system which then alkylates at the N-2 position of guanine in the DNA. Subsequent loss of the carbamate group of the MMC results in the formation of a second centre at C-10 which then alkylates a second neighbouring guanine N-2. This can cause both inter- and intra-strand crosslinks, resulting in a bis-adduct which fits into the minor groove of the DNA molecule (Tomasz et al 1987). Similar reduction is thought to occur with EO9, but with the generation of 3 potential reactive centres due to the reduction of the aziridine ring and the hydroxyl groups.



EO 9 mitomycin C

Figure 1.7 The structures of the quinone bioreductives mitomycin C (MMC) and the indoloquinone, EO9.

1.5 DETERMINATION OF BIOREDUCTIVE ACTIVITY

The relative ability of cytotoxic drugs to kill hypoxic cells has been traditionally expressed as the hypoxic cytotoxicity ratio or HCR (Stratford et al 1989):

$$HCR = \frac{\text{Drug Concentration : 50\% Growth Inhibition (oxic conditions)}}{\text{Drug Concentration : 50\% Growth Inhibition (hypoxic conditions)}}$$

In the V79 cell line, MMC has an HCR of 2, which is weak compared to RSU 1069 and SR 4233 which in this cell line have HCRs of 66 and 50 respectively (Stratford et al 1989). However, when different cell lines are used, different results are seen, e.g. MMC has an HCR of 5 in the EMT6 mammary cell line (Kennedy 1987). The variation of the HCR across cell lines *in vitro* demonstrates the importance of testing drugs against more than one cell line when looking for activity. The difference in effect between cell lines is likely to be related to the heterogeneity of enzyme expression within the different cell types (Workman 1992B). This variation means that when assessing a tumour for a potential response to bioreductive therapy, the presence or absence of hypoxia which although obviously important, should not be the only deciding factor. The amount and type of bioreductive enzymes present within the tumour, together with the presence of enzymes which allow for DNA repair and which protect the cell from oxidative stress effects also need to be assessed (Workman 1992B).

Studies looking at bioreductive enzyme activity levels in tumours have shown that these are often elevated in comparison to normal surrounding tissues (Schlager et al 1990, Fitzsimmons et al 1996). This finding has lead to the concept of enzyme directed drug development which was first proposed by Workman and Walton. It suggests that if the enzyme profile of a tumour were known, it may be possible to modify the structure of a bioreductive drug to suit the catalytic preference of the reductase expressed in high levels in

the tumour and/or to avoid activation by any enzymes expressed in normal tissue (Workman et al 1990A). Enzyme profiling could also be used to individualise treatment by selecting patients for a particular drug based on their individual tumour enzyme profile (Workman 1994).

1.6 POTENTIAL USES OF BIOREDUCTIVE DRUGS

As single antitumour agents, bioreductive drugs are unlikely to have a significant impact on the tumour because they will only kill the small, slowly growing, hypoxic cell fraction present within the tumour. Therefore, to maximise their potential benefit to patients the following strategies might be employed:

1. Bioreductive drugs could be given in combination with more conventional therapy capable of killing the larger oxic part of the tumour, i.e. radiation or standard cytotoxic agents.
2. A bioreductive drug could be used which is capable of killing both oxic and hypoxic cells. One example of this is the cobalt (III)-nitrogen mustard complex, SN24771 (Wilson et al 1994).
3. The hypoxic region within the tumour could be enhanced. This can be achieved with a wide variety of agents which are capable of modulating tumour blood flow e.g. vasoactive drugs such as hydralazine, which by causing vasodilation of normal but not tumour related blood vessels results in tumour vascular shutdown (Chaplin 1989, Bremner et al 1990); biological response modifiers such as tumour necrosis factor (TNF) (Edwards et al 1991) and Flavone Acetic Acid (FAA) (Stratford et al 1990). The use of photodynamic therapy, which causes hypoxia as a result of damage to the vascular endothelium, has also been investigated (Adams et al 1994). A further option might be the use of drug delivery systems which may interfere with blood flow to the tumour (see later).

1.7 EO9

EO9 (3-hydroxy-5-aziridinyl-1-methyl-2-(1H-indole-4,7-dione)-prop-β-en-α-01; NSC 382459; E85/053) is the lead compound in a series of fully synthetic indoloquinones developed for their ability to act as potential bioreductive agents (Oostveen et al 1987). EO9 has a molecular weight of 288.3 and while similar to mitomycin C (MMC) in structure (Figure 1.7), it has shown a different antitumour profile in preclinical models. In *in vitro* studies EO9 demonstrated preferential cytotoxicity against solid tumours, particularly colon, central nervous system, melanoma, non-small cell lung and renal cell lines, with less than 10^{-8}mol/l of EO9 being required for 50% growth inhibition (GI_{50}) in the majority of sensitive cell lines. In contrast to the panel of solid tumour cell lines in which 49/56 demonstrated sensitivity to EO9, less activity was seen against leukaemia cell lines with the murine P388 leukaemia line being amongst the least sensitive cell line tested (GI_{50} = 4.8 x 10^{-7}mol/l) (Hendriks et al 1993). In the solid tumour cell panel, EO9 appeared to be a more potent drug than MMC with a mean GI_{50} of 1.7 x 10^{-8}mol/l compared to MMC which demonstrated a mean GI_{50} of 7.1 x 10^{-7}mol/l. EO9 also demonstrated activity in MMC resistant cell lines such as the MCF-7 ADR cell line (Hendriks et al 1993).

The effect of EO9 may be pH-dependent with a reduction in pH from 7.4 to 5.8 increasing cell kill from 40% to 95% in DLD-1 cells (Phillips et al 1992). In some cell lines hypoxic conditions also appear to increase EO9 activity. The concentrations of EO9 required to reduce cell survival to 10% of the control population in the EMT6 mouse mammary cell line were 3 and 10ng/ml for hypoxic and oxic cells respectively (Hendriks et al 1993).

Antitumour activity has been demonstrated *in vivo* following a single intraperitoneal injection of EO9 in 2 out of 4 drug refractory murine adenocarcinomas. The MAC 16 tumour (T/C = 50% at 2mg/kg) and the MAC 26 tumour (T/C = 66% at 3mg/kg) were both sensitive to EO9, whereas the MAC 13 and MAC 15A tumours were resistant to EO9. The

P388 murine leukaemia cell line was resistant to EO9 *in vivo* as it had been *in vitro* (Hendriks et al 1993).

In human tumour xenograft models, EO9 induced tumour regression in the gastric cancer GXF97 and the ovarian cancer MRI-H-207 xenografts following intravenous administration of the maximum tolerated dose of EO9 on 2 separate occasions 1 week apart. Growth delay was observed in the breast cancer xenograft MAXF449 and marginal antitumour activity was seen in the non-small cell lung cancer xenograft LXFL529. No activity was seen in the renal tumour xenograft RXL423 (Hendriks et al 1993).

In pre-clinical toxicology studies in Swiss CD1 mice, the LD_{50} (the dose that is lethal in approximately 50% of the mice) was 12.99mg/kg (95% confidence intervals: 11.6-15.4mg/kg) and 13.95mg/kg (12.3-17.0 mg/kg) in male and female animals. The corresponding LD_{10} (the dose that is lethal in approximately 10% of the mice) values were 9.23mg/kg (7.4-10.5mg/kg) and 9.91mg/kg (7.9-10.5mg/kg). No specific drug related macroscopic changes were observed at autopsy. The only toxicities seen following a single dose of 9mg/kg were transient anaemia and inflammatory changes both visibly and microscopically at the injection site. Following multiple intra-peritoneal treatments with 1.5mg/kg EO9 (daily for 5 days every week for 4 weeks), there was a transient reduction in body weight from day 9 until day 28. Minor changes in weight were also observed in the liver, testes, spleen and kidneys. Transient anaemia was again detected which was associated with an increase in neutrophil and a decrease in lymphocyte counts. The only abnormalities seen on microscopic examination were reversible changes in the gastrointestinal tract which showed congestion of the blood vessels and haemorrhagic spots in the jejunum and lymphoid hyperplasia in Peyer's patches. No bone marrow abnormalities were detected which contrasted with MMC where myelosuppression was the dose limiting toxicity (Hendriks et al 1993).

In Wistar rats, following both a single dose of 0.45mg/kg (one tenth of the mouse equivalent LD_{10}) intravenously and 4 weekly doses of 0.45mg/kg, no abnormalities were detected (Hendriks et al 1993).

EO9 was found to undergo rapid clearance in rodents following intravenous administration. At the maximum tolerated dose of 12mg/kg in male C3H/He mice, the initial plasma concentration (Co) was 1.8 ± 0.4μg/ml and the half life was 1.9 minutes with a large volume of distribution (Vd) of 7.5ml/g. In comparison, MMC at the same dose has a half life of 16 minutes with a peak plasma concentration 4 times greater than EO9. Using HLPC analysis neither EO9 nor its metabolites could be identified in the tumour or tissues, but around 20% of the intravenous dose was detected as metabolites in the urine, suggesting that rapid and extensive metabolism of the drug had occurred (Workman et al 1992C). Similarly, in Sprague-Dawley rats receiving 3mg/kg of EO9, the initial plasma concentration was 1.5 ± 0.1μg/ml with a half life of 3.0 ± 0.2 minutes and a volume of distribution of 2.2 ± 0.2ml/g (Workman et al 1992C).

1.7.1 Enzymology of EO9 Bioreductive Activation

As indicated previously (1.4.3), EO9 undergoes bioreductive activation in order to generate the active cytotoxic species. 1 or 2 electron reduction of the parent molecule results in the formation of either the semiquinone or the hydroquinone (Smitskamp-Wilms et al 1996) (Figure 1.5). However, in contrast to MMC, the role of the 2 electron reductase DT-Diaphorase in the bioactivation process appears to be much clearer. It is this relationship, to be discussed below, which prompted the enzyme directed drug development approach proposed by Workman and Walton (Workman et al 1990A).

1.7.1.1 DT-Diaphorase

DT-Diaphorase (NAD(P)H:(quinone acceptor) oxidoreductase (EC1.699.2)) is a flavoprotein which is ubiquitous among eukaryocytes and expressed in varying amounts in most tissues (Belinsky et al 1993). High levels of the enzyme normally occur in the stomach, bladder, colon and kidney, with low levels being documented in haematological tissues. DT-Diaphorase is thought to have two main physiological functions: causing reduction of the Vitamin K3 precursor and acting as a phase II drug metabolising enzyme (Ernster 1987). The latter function characterises it as a protective enzyme, playing an important role in the detoxification of chemically reactive metabolites such as the polycyclic hydrocarbons found in food, exhaust fumes and cigarettes. It also reduces the cells' exposure to oxidative stress by by-passing the 1 electron reductase pathways which produce the toxic free radicals and oxygen metabolites capable of causing further damage to the cell. There are several forms of DT-Diaphorase which are encoded by 4 gene loci, DIA 1-4 (Edwards et al 1980, Jaiswal et al 1990). Of these, Diaphorase 4 or NQO1 accounts for the majority of the expressed enzyme and is thought to be the most important in bioreductive activation (Riley et al 1992). It is found on chromosome 16 (Jaiswal et al 1988) but is absent in 4% of the European population (Edwards et al 1980). The protein occurs as a dimer *in vivo* (Ernster 1967), with more than 90% of the protein being found in the cytosol fraction of the cell and the remainder being located in the Golgi apparatus and the mitochondrial and microsomal membranes (Riley et al 1992). DT-Diaphorase accepts NADH or NADPH as a cofactor (Ernster 1967).

Elevated levels of DT-Diaphorase are found in rat hepatic preneoplastic lesions induced by chemical carcinogens (Schor 1978). It is also induced by heavy metals, quinones, and isocyanates as well as by smoking and alcohol (Schlager et al 1990). This suggests it may represent an early stress response (Riley et al 1992) or it may have a role in the early defence mechanisms of the cell against carcinogenesis (Beyer et al 1988). Increased concentrations of DT-Diaphorase are often found in tumour tissues (Smitskamp-Wilms et al 1995, Riley et al

1992) e.g. in lung cancers, DT-Diaphorase concentrations are up to 123 times higher than in normal tissue. Colon and breast tumours have also been noted to have up to 3 times the normal concentration of DT-Diaphorase (Schlager et al 1990).

1.7.1.2 DT-Diaphorase and EO9 sensitivity

A good correlation has been noted between the level of DT-Diaphorase expression and EO9 sensitivity in cell lines, with cells which have the highest levels of DT-Diaphorase being the most sensitive under oxic conditions (Collard et al 1995, Fitzsimmonds et al 1996, Phillips et al 1992, Plumb 1994A, 1994B, Robertson et al 1994, Smitskamp-Wilms 1994). This is in contrast to MMC and the quinone, porifromycin, where no correlation has been shown between levels of DT-Diaphorase and cell sensitivity (Robertson et al 1992). EO9 sensitivity can be abolished by the use of dicoumarol, but only in cells expressing high levels of DT-Diaphorase (Robertson et al 1994, Bando et al 1995, Smitskamp-Wilms 1994, Plumb et al 1994B).

The potential importance of DT-Diaphorase in EO9 activity has been demonstrated when human DT-Diaphorase genes were transfected into CHO (Chinese Hamster Ovary) cells. The subsequent sensitivity of the transfected cells to EO9 was found to correlate with their level of DT-Diaphorase expression (Gustafson et al 1996).

The human adenocarcinoma cell lines HT29 and BE have been extensively studied because of the difference in their DT-Diaphorase levels. The BE cell line has a single C to T mutation resulting in a proline instead of a serine in the DT-Diaphorase molecule. This results in cells which produce normal levels of DT-Diaphorase RNA and protein but the protein itself is inactive (Traver 1992). The HT29 cell line is rich in DT-Diaphorase and *in vitro* is 16.4-fold more sensitive to EO9 than the BE cell line under aerobic conditions (Walton et al 1992A).

The results appear to be similar *in vivo*. In the murine transplantable adenocarcinomas of colon, MAC 16 and MAC 26, it has been shown that MAC 16, which has 15-fold higher levels of DT-Diaphorase than MAC 26, is more sensitive in aerobic conditions to EO9 than MAC 26 (Workman et al 1990B, Walton et al 1992A).

The amount of DT-Diaphorase in xenografts has been shown to be lower than the DT-Diaphorase levels in the corresponding cell lines. This difference may be due to the presence of interstitial tissue in the tumour or microenvironmental factors *in vitro*, but suggests that it may be difficult to predict the activity of EO9 *in vivo* based only on *in vitro* data (Collard et al 1995).

In our laboratory the chemosensitivity of a panel of 4 solid tumours (the human colon xenografts HT29 and BE and the murine colon adenocarcinomas MAC 16 and 26) to EO9 did not correlate with the levels of DT-Diaphorase or the 1 electron reductases (cytochrome-P450 reductase and cytochrome-*b*5 reductase) when measured in the tumours using cytochrome C reduction and menindione as the intermediate electron acceptors. This is unlikely to be related to the known difference in reductive capacity between human and murine DT-Diaphorase (Lewis et al 1994) because this difference is small and would not manifest itself at the concentration of EO9 (100μg/ml) used in the study. When EO9 was incubated *in vitro* with tumour homogenates, no correlation was seen between antitumour activity and metabolite formation. A good correlation was seen between antitumour activity and the rate of disappearance of the parent drug (r^2=0.86), which suggested that the capacity of a tumour to metabolise EO9 rather than bioreductive enzyme expression was the most important determinant of antitumour activity (Cummings et al 1998).

The role of DT-Diaphorase in hypoxic conditions is less clear. Using the HT29 and BE cell lines, Plumb et al have shown a 1000 fold enhancement in EO9 cytotoxicity under hypoxic

conditions for the BE cell line, whereas HT29 demonstrated only a 2 to 5 fold increase in sensitivity under the same conditions (Plumb et al 1994C). This has been confirmed in other cell lines where a clear inverse relationship was demonstrated between cellular DT-Diaphorase activity and hypoxic sensitivity to EO9 (r^2=0.93) (Plumb et al 1994B). Others have also shown enhancement of activity under hypoxic conditions in cells which are low in DT-Diaphorase (Bando et al 1995, Robertson et al 1994). In DT-Diaphorase rich cells, the addition of dicoumarol, which inhibits DT-Diaphorase activity, inhibited the normal cell sensitivity to EO9 in oxic conditions, but increased sensitivity in the same cells under hypoxic conditions. In contrast the presence of dicoumarol had no effect on EO9 sensitivity in cells which were low in DT-Diaphorase in either oxic or hypoxic conditions (Plumb et al 1994B). This suggests a role for the 1 electron reductase enzymes, which are not inhibited by dicoumarol, in EO9 activation (Bando et al 1995, Plumb et al 1994B, 1994C).

The relative roles of the 1 and 2 electron reductases in the bioactivation of EO9 may be dependent on the cellular environment. This includes factors such as pH, oxygen tension, the cells proliferative state and the mechanisms present for cellular repair. In aerobic conditions, the effect of one electron reduction which is capable of generating the active species of EO9 is minimised due to rapid redox cycling. Two electron reduction, on the other hand, is oxygen-independent and therefore increases in relation to the level of enzyme present within the cell. Under hypoxic conditions redox recycling is prevented, thus one electron reduction allows the formation of active metabolites which are not subsequently reoxidised. This could account for the marked increase in activity seen in cells low in DT-Diaphorase when they are subject to hypoxic conditions.

Plumb et al postulate that it may be the semiquinone rather than the hydroquinone which is the active species of EO9. The increased activity in cells which are rich in DT-Diaphorase which is seen in oxic conditions may be the result of a transient increase in the concentration

of the semiquinone within the cell due to back oxygenation of the hydroquinone particle. This cannot occur under hypoxic conditions, hence the lack of enhancement seen in these cells in the presence of hypoxia. However, cells which are low in DT-Diaphorase demonstrate massive enhancement of activity under hypoxic conditions because the lack of redox cycling allows an increase in the semiquinone radical to occur within the cell (Figure 1.5) (Plumb et al 1994B). This theory is however, based on *in vitro* results and does not explain the, albeit slight, increase in activity noted in the DT-Diaphorase rich cells which still occurs in hypoxia. However, it would agree with studies which investigated the chemical properties and reactivities of the semiquinone and the hydroquinone radicals with oxygen using pulse radiolysis techniques. These results suggested that the semiquinone and the hydroquinone radicals are in equilibrium and that in the presence of oxygen the hydroquinone auto-oxidises, possibly via semiquinone mediated reactions (Butler et al 1996).

1.7.1.3 EO9 activity induced by DT-Diaphorase

EO9 activation by DT-Diaphorase appears to produce a reactive species capable of interaction with DNA. It can cause single strand breaks in DNA plasmids with maximum activity at 10-50μM of EO9. This is unaffected by superoxide dismutase, which indicates that the effect is mediated by an alkylating agent rather than a free oxygen radical (Walton et al 1991). Inter-strand cross-linking has also been demonstrated which appears to be pH-dependant, with cross-linking increasing as the pH rises from 5.5 to 7.0 (Bailey et al 1994, Maliepaard et al 1995). However, previous studies with EO9 *in vitro* have shown that in DLD-1 and MCF-7 cell lines an increase in pH actually causes a reduction in cytotoxicity, suggesting that inter-strand crosslinkage is not the main mechanism of action (Phillips et al 1992).

1.7.2 Clinical Evaluation of EO9

1.7.2.1 Phase I studies

Phase I studies have been carried out using two different schedules of EO9 under the auspices of the EORTC (Schellens et al 1994, Verweij et al 1994). In the first study, EO9 was given as a 5-minute infusion every 3 weeks to 32 patients with solid tumours (Schellens et al 1994). The maximum tolerated dose was 27mg/m^2. The dose limiting toxicity was proteinuria associated with salt and water retention which was reversible by day 15 in all but 2 of the patients who went on to develop acute renal failure. In 2 of the 4 patients with grade IV proteinuria, a renal biopsy was performed which showed changes suggestive of a minimal change glomerulopathy as well as degenerative changes in the tubules. This was not predicted in the preclinical data, but was thought to be related to the high levels of DT-Diaphorase found in the renal tissue of humans compared to other species (Riley et al 1992, Schlager et al 1990). No bone marrow toxicity was seen in any of the patients which confirmed the preclinical data. There was no cumulative toxicity and three patients had a partial response to EO9.

Pharmacokinetic analysis showed that there was wide inter-patient variability with the clearance varying from 3.2-24 l/min and the terminal half life varying from 0.8-29 minutes. The pharmacokinetics were related to the pharmacodynamic data with the area under the curve (AUC) of EO9 being the parameter which correlated best with toxicity (proteinuria).

The dose recommended for Phase II studies was 22mg/m^2, but, because of the unexpected renal toxicity, and the wide variability in patient pharmacokinetics, it was suggested that individual dose adjustments should be made based on the plasma concentration in order to combine maximum exposure with minimum toxicity (Schellens et al 1994). However, the multi-centre nature of the Phase II trial meant that it was not possible to allow individualisation of patient dosing.

In a second phase I study a weekly schedule was employed (Verweij et al 1994). The dose limiting toxicity was again proteinuria at the maximum weekly drug dose of 15mg/m^2 and 12mg/m^2 was recommended for the subsequent phase II trials. The weekly schedule reflected the highest dose intensity per course achievable, and it was therefore selected for the main Phase II study.

1.7.2.2 Phase II studies

Phase II studies were carried out employing the weekly schedule of 12mg/m^2 EO9 in patients with breast, colorectal, gastric and pancreatic cancer (Dirix 1996). Ninety-two patients entered the trials with the drug being generally well tolerated. Nausea and vomiting occurred in 26% and 13% of courses respectively. These were predominantly at grade I/II except for two documented grade III episodes of nausea and three documented episodes of grade III vomiting. Reversible proteinuria, the major toxicity, was documented in 45% of courses although there was only one documented case of grade III toxicity. However, no antitumour responses were seen in any of the patients, which suggested that this was not an active schedule in these tumour types.

A further study compared weekly (12mg/m^2) EO9 with a 3-weekly (22mg/m^2) schedule in 38 previously untreated patients with non small cell lung cancer (Pavlidis et al 1996). The major toxicity, proteinuria, was more severe in the 3 weekly arm (62.5 v 34.5% of courses). This arm also had increased asthenia (35 v 21%), nausea (27.5 v 15%) and vomiting (17.5 v 5%). Interestingly, although no responses were observed in either group, the 3 weekly chemotherapy arm had a higher proportion of patients with stable disease (53 v 26%).

1.7.3 Potential Reasons for Lack of EO9 Activity

EO9 has been shown to be an active drug both *in vitro* and *in vivo*. The lack of response in the clinical trials is disappointing. Several potential reasons for this lack of activity can be postulated:

1. The dose limiting toxicity of proteinuria found in both the weekly and the 3 weekly schedules is probably due to the high levels of DT-Diaphorase found in renal tissue and significantly limits the amount of drug which can be successfully administered to the patient.
2. EO9 is generally known to be unstable with a short half life *in vivo* (Workman et al 1992C). It is hydrolysed to the breakdown product EO5A which is known to be a poor substrate for DT-Diaphorase and is much less cytotoxic than EO9 (Bailey et al 1992).
3. There are problems in drug delivery to solid tumours as discussed previously (1.3.2). In particular, the often inadequate tumour vasculature, which, although providing a potentially enhancing hypoxic environment, may actually reduce the amount of drug reaching the tumour cells. The increase in interstitial tissue which can also be found in solid tumours may delay the arrival of the drug at the tumour cells. These factors will assume greater importance if, as is the case with EO9, the drug has a very short half-life. It is interesting that the only responses seen in clinical trials were in the Phase I, 3 weekly schedule which used higher doses of EO9. There were also more patients with stable disease in the 3 weekly arm of the phase II study in non-small cell lung cancer compared to the weekly arm (Pavlidis et al 1996).
4. If EO9 is eliminating only the hypoxic fraction of the tumour, then the remaining oxic fraction will continue to grow. This means that the usual methods of determining tumour response, which depend on change in tumour size, may not be the most appropriate as they are unlikely to detect any significant activity.

1.7.4 Potential Developments Using EO9

EO9 could be considered for use in locoregional therapy. This would have the benefit of providing a high concentration of EO9 where it was needed, at the tumour site, whilst at the same time reducing systemic exposure and therefore toxicity. In addition, depending on the method of drug delivery, enhancement of hypoxia may occur which could increase EO9's activity still further as has been shown using hydralazine in *in vivo* models (Bibby et al 1992). This hypothesis is the major theme of the work presented in this thesis.

EO9 may also have a role in combination with radiotherapy. Adams et al have shown that when EO9 was used as a post irradiation sensitiser, it resulted in enhancement of cytotoxic activity when administered following a 10 Gy dose of radiation (Adams et al 1992). However, this combination of methods, whilst potentially affecting both the oxic and hypoxic fractions of the tumour, would not eliminate all of the problems mentioned above.

1.8 LOCOREGIONAL THERAPY

Conventional systemic chemotherapy has a relatively low therapeutic index, which means that increasing the dose of drug administered to try to improve the antitumour effect will often result in a marked increase in systemic toxicity. Locoregional chemotherapy is an alternative method of drug administration which maximises the amount of drug which can be delivered to the target organ, thereby improving it's effectiveness, whilst at the same time, minimising systemic exposure and therefore toxicity. However, not all tumours are suitable for locoregional therapy as its utility depends on the site of the tumour to be targeted. One area where locoregional therapy has been extensively studied is in the treatment of liver tumours, either primary hepatomas or metastases from e.g. colorectal tumours.

1.8.1 Colorectal Cancer

Colorectal cancer is the third most common cancer in the western world. In Scotland alone, over 1700 people died from this disease in 1996 (Scottish Cancer Incidence and Mortality Statistics 1998). Liver metastases are found in up to 25% of people at presentation (Bengmark et al 1969). In 50% of patients with metastatic disease the liver may be the only site of involvement (Kemeny et al 1980). Survival in patients with hepatic involvement is poor with a mean survival of 3 - 9 months (Wood 1984). Surgical resection is one option for the treatment of liver metastases, but is unfortunately only suitable for a small percentage of patients (Bradpiece et al 1987, Ridge et al 1985). The mainstay of treatment for metastatic colorectal cancer is systemic therapy. The fluoropyrimidine, 5-Fluorouracil (5FU) is the most commonly used drug, but newer drugs such as irinotecan and oxaliplatin have become available which have demonstrated activity in metastatic colorectal cancer. For a detailed review of systemic therapy, the reader is referred to reviews by Coperchini (Coperchini et al 1995), Grem (Grem 1995), Meropol (Meropol et al 1995) and Steele (Steele 1995).

1.8.2 Locoregional Therapy and Colorectal Liver Metastases

There are several reasons why colorectal liver metastases may be suitable for treatment using locoregional therapy:

1. The liver is the only site of metastatic spread in 50% of patients (Kemeny et al 1980).
2. One theory for the spread of colorectal cancer is that it may occur in a "stepwise" manner (Weiss et al 1986, 1989). This theory suggests that tumour cells spread from the bowel via the portal vein and become trapped within the liver where metastatic deposits form. These metastatic deposits are then responsible for spread to the rest of the body; i.e. metastases are derived from metastases. Treating the liver with high dose therapy may therefore prevent or slow down the development of extra hepatic deposits.

3. The liver has a dual blood supply: the hepatic portal vein and the hepatic artery. The normal parenchymal tissue is supplied mainly by the hepatic portal vein, whereas metastases over 2 mm in diameter derive their own blood supply from the hepatic artery (Breedis et al 1954). This means that utilising the hepatic artery will incorporate a degree of selectivity into the treatment by increasing chemotherapy exposure to neoplastic cells relative to normal hepatocytes (Sigurdson et al 1987). Furthermore, tumour blood vessels tend to lack the smooth muscle layer normally found in precapillary arterioles (Dvorak et al 1988) and have a more fenestrated capillary lining which is deficient in basement membrane (Kaiser 1989). This difference in the tumour vasculature may affect drug diffusion and alter the responsiveness to vasoactive substances which provides a further area for exploitation when planning treatment (Goldberg et al 1991A and B).
4. Locoregional therapy to the liver can produce high hepatic drug concentrations which may be important for drugs such as 5-FU and fluorodeoxyuridine (FUDR) which have steep dose-response curves (Chen et al 1980). Locoregional administration also allows drugs with high hepatic extraction rates to be given in high doses to the liver whilst minimising systemic exposure (Collins 1984).

1.8.3 Approaches to Locoregional Therapy

1.8.3.1 Hepatic artery infusion

This involves the placement of an indwelling catheter into the hepatic artery. The first treatments used percutaneous catheters and external pumps which were poorly tolerated due to problems with arterial thrombosis, infection, bleeding and catheter dislodgement (Tandon et al 1973). The development of implantable pumps and subcutaneous ports have resolved many of these problems and have made this type of therapy a more feasible option (Fordy et al 1995). The main drugs used in hepatic arterial infusions have been FUDR,

which has a 95% first pass extraction rate in the liver and 5FU, which has a lower first pass extraction rate of 19 -55% (Ensminger et al 1978).

Several non-randomised Phase II trials have been carried out using hepatic arterial infusions of FUDR and 5FU in patients with metastatic colorectal carcinoma. In a collected series of nine of these studies involving 375 patients receiving FUDR, the mean response rate was 45% and the median survival was 17 months (Benson et al 1995).

There have been seven randomised Phase III trials comparing hepatic artery infusion with systemic therapy, and these are summarised in Table 1.2. The response rates were higher in the hepatic arterial arm, although all of the studies failed to detect a survival advantage. One study, which showed a higher response rate in the intrahepatic treatment arm compared with the intravenous arm (50% v 20%), documented an increase in extrahepatic progression in those treated with locoregional therapy compared to those treated with systemic therapy (56% v 37%), though this did not appear to affect survival (Kemeny et al 1987).

There were several reasons why it was not possible to determine a survival advantage:

1. Most of the studies contained only small number of patients which meant that they did not have the statistical power to detect a survival advantage.
2. In some of the studies (3/7) a crossover design was used. While this had the advantage of demonstrating that a response could be obtained using hepatic artery infusion in those who had progressed in the systemic arm of the trial, it prevented a meaningful comparison of survival between the 2 treatment arms.
3. Those patients in the systemic arms were often undertreated e.g. a lower dose of drug was used than we now know to be effective or they were treated with single agent 5FU rather than a combination of 5FU and Folinic Acid.

Group	Patient Numbers	HAI		IV		Cross-over
		Drug	% response	Drug	% response	
NCI (Chang et al, 1987)	64	FUDR	62	FUDR	17	No
MSKCC (Kemeny et al, 1987)	162	FUDR	50	FUDR	20	Yes
NCOG (Hohn et al, 1989)	143	FUDR	42	FUDR	10	Yes
Mayo Clinic (Martin et al, 1990)	74	FUDR	48	5-FU	21	No
French (Rougier et al, 1992)	163	FUDR	43	5-FU	9	No
City of Hope (Wagman et al, 1990)	91	FUDR	55	5-FU	20	Yes
English (Allen-Mersh et al, 1994)	100	FUDR	40	------	-----	No

Table 1.2 **Summary of Phase III randomised trials of hepatic artery infusion (HAI) versus systemic (IV) fluoropyrimidines for colorectal liver metastases.**

A meta-analysis of the literature has been performed to attempt to determine whether regional infusional chemotherapy using 5FU or FUDR conferred any survival advantage over systemic therapy. Only prospective randomised trials which used these drugs were considered and the trials had to have reported on survival from time of randomisation until death. Only 6 out of 149 studies which were reviewed met these criteria and were included in the analysis. The information on 1 and 2 year survival was either taken directly from the study or calculated using Kaplan - Meier survival curves. Survival was expressed as a percentage at 1 and 2 years post randomisation. The survival difference was 10% at 1 year ($p = 0.041$) and 6.4% at 2 years ($p = 0.124$) in favour of the regional chemotherapy group. In the studies which did not allow crossover the survival advantage increased to 18% at 1 year ($p=0.034$) and 7% at 2 years ($p=0.276$). These data suggest a statistically significant survival advantage for locoregional therapy when compared to systemic therapy (Harmantas et al 1996).

1.8.3.1.1 Hepatic artery infusion toxicity

The side effects of hepatic artery therapy are different to those seen with systemic therapy. Myelosupression and mucositis, the common side effects of systemic therapy, do not occur. The main toxicities are hepatobiliary toxicity and gastroduodenal ulceration (Kemeny et al 1984, Hohn et al 1986).

Biliary toxicity occurs because the bile ducts derive their blood supply from the hepatic artery (Northover et al 1979) and therefore receive a high dose of chemotherapy. Liver function tests need to be closely monitored and chemotherapy discontinued if they become abnormal. Endoscopic retrograde cholangiopancreatography (ERCP) has demonstrated the presence of lesions similar to those found in sclerosing cholangitis in 5-29% of patients who

became jaundiced during hepatic arterial chemotherapy (Kemeny et al 1984, Hohn et al 1985A, Shea et al 1986).

Gastroduodenal ulceration occurs because aberrant branches to these organs can arise from the hepatic artery. These should be carefully dissected out and ligated when the catheter is initially inserted into the artery (Hohn et al 1985B).

1.8.3.1.2 Reduction of hepatic artery infusion toxicity

Various methods have been employed to minimise the toxicity associated with hepatic arterial infusions. The hepatic arterial administration of dexamethasone may help reduce the portal triad inflammation which can result in bile duct ischaemia. In a study exploring the effect of dexamethasone during FUDR administration there was a trend towards a reduction in bilirubin elevation in the dexamethasone-treated group. The dexamethasone-treated group also demonstrated a significant increase in response rate (71% v 40% ($p=0.03$)) as well as a survival advantage (23 months v 15 months) when compared with the group treated with FUDR alone (Kemeny et al 1992). Circadian modification may also influence toxicity. In a non-randomised study, patients receiving the circadian modification tolerated almost twice the daily dose of FUDR with a decrease in hepatic toxicity when compared with those receiving standard infusions of FUDR (Hrushesby et al 1990). Alternation of the hepatotoxic FUDR with the less toxic 5FU may also result in a reduction in toxicity (Stagg et al 1991).

1.8.3.1.3 New approaches to infusional therapy

Extrahepatic disease develops in 40 -70% of patients undergoing hepatic arterial infusion, often when the patient is still responding in the liver. The use of combination systemic and hepatic therapy is undergoing further investigation as this may represent a more logical

approach to disease management (Kemeny et al 1993). Preliminary studies adding Folinic Acid to FUDR have shown an increase in response rates and survival when compared with FUDR alone. Further studies are underway to evaluate this further and to attempt to reduce the increase in associated biliary toxicity (Kemeny et al 1994).

1.8.3.2 Chemoembolisation

Chemoembolisation combines a chemotherapeutic agent with an embolic agent. The chemotherapeutic agent can either be co-administered with the embolic material or it can be incorporated into the embolic material prior to the embolisation procedure in what is termed a drug delivery system.

1.8.3.2.1 Chemotherapy and embolisation

Embolisation alone has been used as a therapeutic procedure for many years. It has very little effect on patient survival but it can be useful in providing symptomatic relief (Allison 1989). The benefit which is obtained is usually only temporary because of the rapid development of a collateral circulation which is not usually amenable to further embolic therapy. The addition of a chemotherapeutic agent, given by the same route during the embolisation procedure should, in theory, enhance the therapeutic effect of the embolic particles. This is because the reduction in blood flow caused by the embolic particles, in addition to producing ischaemia, should result in the prolonged exposure of the tumour to a higher concentration of the administered drug. This increase in exposure should increase drug extraction by the tumour and hence increase the antitumour effect.

Various types of embolic material have been used. These include both viscous material such as lipiodol (Bretagne et al 1988, Inoue et al 1993, Kobayashi et al 1987) and particulate material such as Ivalon (Chuang et al 1982), polyvinyl alcohol particles (Martinelli et al 1994)

and collagen (Daniels et al 1992). Biodegradable microspheres have also been utilised (Carr et al 1997, Civarelli et al 1994, Ensminger et al 1985, Lorenz et al 1989).

Most of the experience in chemoembolisation via the hepatic artery has been in the treatment of unresectable hepatocellular carcinoma where Phase II trials have demonstrated higher response rates than conventional systemic or intra-arterial therapy (response rates of 25 -50% and median survival of 5 -36 months) (Benson et al 1995). Experience is more limited in the treatment of colorectal metastases. The results of 4 of the studies are outlined in Table 1:3. The significance of the response rates and median survival is difficult to assess with such small numbers and further randomised studies with larger patient numbers are required.

1.8.3.2.2 Biodegradable microspheres

There are two types of biodegradable microsphere currently in use:

1.8.3.2.2.1 Starch microspheres

Starch microspheres are approximately 40μm in diameter and have a half-life at 37^{0}C *in vitro* in human plasma of 25 minutes (Hakansson et al 1997). If sufficient amounts are administered via the hepatic artery (mean dose of 835 mg) they are capable of causing transient vessel occlusion (Civarelli et al 1994). They are degraded by alpha-amylase which is present in normal human serum (Aronsen et al 1979, Hakansson et al 1997).

Co-administration of starch microspheres with cytotoxic drugs does appear to alter the pharmacokinetic profile of the administered drug. When compared to intra-arterial MMC alone, the peak systemic plasma concentration of MMC and the area under the curve were reduced in patients who received intra-arterial MMC in combination with starch microspheres (Ensminger et al 1985, Gyves et al 1983).

Group	*Patient Number*	*Cytotoxic Agent*	*Chemoembolic Agent*	*Response Rate*
Daniels et al, 1992	55	Cisplatin Dox MMC	Collagen	34%
Lang et al, 1993	46	Dox	Ethiodised Oil	CR 17% PD 13%
Martinelli et al, 1994	24	5FU+IFN versus no chemo	Polyvinyl Alcohol	25% (both groups)
Lyster et al, 1993	25	Cisplatin Dox MMC	Collagen	85%

Table 1.3 **Summary of four studies looking at the effect of chemoembolisation on colorectal liver metastases.**

Key:

Dox = Doxorubicin

MMC = Mitomycin C

5FU = 5 Fluorouracil

IFN = Interferon

CR = Complete Response

PD = Progressive Disease

This was also seen when BCNU was administered in combination with starch microspheres via the hepatic artery (Dakhil et al 1982). The transient occlusion caused by the microspheres may also cause a redistribution of blood flow towards the more resistant hypovascular lesions found within the liver, which should result in improved delivery of cytotoxic agents to these areas (Civalleri et al 1994).

A three-arm, prospective, randomised study of 61 patients compared the effect of chemoembolisation using starch microspheres and 5FU with simple hepatic embolisation using lypholised dura mater or gelfoam with symptomatic management only. There was no statistical difference in survival between each of the three groups. However, for the patients within each group who had less than 50% replacement of their liver by tumour (12-13 patients per group), there was a survival advantage for the chemoembolisation group (median of 23.6 months) compared with the other 2 groups (10.2 months and 10 months respectively) though this did not reach statistical significance (Hunt et al 1990). Study numbers were small and the absence of a chemotherapy only arm for comparison, makes any conclusion of limited value.

A non-randomised study which demonstrated significant responses in 4 out of 11 patients treated with MMC and degradable starch microspheres who had progressive disease following intra-arterial FUDR chemotherapy, was also too small for any conclusion to be reached with regard to the efficacy of MMC combined with starch microspheres (Lorenz et al 1989).

There is a need for a larger prospective randomised trial to be carried out in this area in order to determine whether combining chemotherapy with starch microspheres improves antitumour response and survival when compared with standard chemotherapy regimens in the treatment of colorectal liver metastases.

1.8.3.2.2.2 Albumin microspheres

Biodegradable albumin microspheres are similar in size to starch microspheres (20 - 40μm in diameter). In a dose finding study the maximum dose of microspheres which was safely administered via the hepatic artery was 350mg, although 4 out of 5 patients treated at this dose developed severe right upper quadrant pain and nausea. Therefore, 300mg of microspheres was recommended for further studies (Goldberg et al 1988). The median biological half-life of the albumin particles within the liver is 2.4 days (range 1.5 - 11.7 days). In some patients it is prolonged in tumour tissue compared with normal liver (Goldberg et al 1991C). The use of radiolabelled albumin microspheres has also shown that there is no significant arteriovenous shunting of microspheres to the lungs following hepatic arterial administration, despite the demonstration of arteriovenous malformations within the tumour tissue (Goldberg et al 1987).

Co-administration of intra-arterial Angiotensin II (Hemingway et al 1991A, 1991B) has been shown to further improve delivery of the microspheres to tumour deposits (Goldberg et al 1991A, 1991B). This is due to the lack of smooth muscle in the tumour blood vessels which reduces their responsiveness to such vasoactive agents. The administration of Angiotensin II therefore results in vasoconstriction of the normal vasculature, resulting in increased blood flow to the tumour areas, which results in an increased delivery of microspheres ± drug to these areas. Initial studies appear to suggest that the regional advantage of 5FU given over 24 hours can be significantly improved if it is co-administered with Angiotensin II and albumin microspheres. This is thought to be due to increased extraction of the drug by the tumour (Kerr et al 1991, Goldberg et al 1988, 1990).

1.8.3.2.3 Chemoembolisation toxicity

The side effects of embolic therapy differ from that of infusional therapy. The main toxicities are pain (60-100%), fever (50-90%), nausea (40%) vomiting and fatigue. Less commonly, cholecystitis (0-5%), gastritis (5-20%), abscess formation (5-10%) and renal failure (0-5%) can also occur. Most side effects resolve within 24-48 hours, although fatigue may persist for up to 3 weeks (Chuang et al 1982, Allison et al 1990).

1.8.3.2.4 Chemoembolisation using drug delivery systems

Drug delivery systems can be defined as any system which possesses the ability to target active molecules to specific sites in the body (Cummings et al 1993A). They allow drugs to be re-evaluated, many of which may have been unsuccessful, despite promising preclinical data, when given by the normal systemic route. The drug delivery system itself must be non toxic, and should not stimulate an immune response because this would prevent its repeated administration. It should, if possible, be a relatively non-specific system which would potentially allow the incorporation of different agents for the treatment of a variety of diseases. The drug delivery system must also be capable of delivering a therapeutically effective dose of drug to the target site without leakage or metabolism of the incorporated drug (Widder et al 1979). Furthermore, the formulation and production of the delivery system must be practical.

The ideal drug delivery system has yet to be designed, but there are numerous systems available which attempt to incorporate some of the above properties into their design. For the purposes of this thesis they can be divided into 2 main groups:

1.8.3.2.4.1 Vesicular systems

These are drug delivery systems of a more fluid consistency which are given systemically. They are manufactured from various products which include liposomes, non-ionic surfactant vesicles, polymeric based drug carriers and antibody based therapies but, because they do not normally undergo chemoembolisation, they will not be considered further in this thesis. (For detailed reviews the reader is referred to Cassidy et al 1993, Cummings et al 1993A).

1.8.3.2.4.2 Microparticulate systems

These are more rigid drug delivery systems which, following embolisation, release the cytotoxic agents which have been incorporated into their structure. They are only suitable for use in locoregional therapy. The two main types are microcapsules and microspheres.

i) *Microcapsules*

Microcapsules are vesicular-type structures in which a membrane encloses the incorporated drug. Ethyl cellulose is the most widely used material for the membrane structure (Kato et al 1980). They are large particles, over 100μm in diameter and they exert their effect by a combination of vascular embolism and prolonged drug release. Their main advantage is that, because of their structure, their level of drug entrapment is very high (80% W/W of drug). However, this drug load is released fairly rapidly compared to other chemoembolic agents and is largely complete within 1-2 hours of microcapsule administration (Goldberg et al 1991D).

The microcapsules which have been most commonly utilised are those containing MMC, as originally formulated by Kato, using the process of coacervation. This involves dispersing 2g of MMC into a solution containing 0.5g ethylcellulose, 0.5g polyethylene and 50mls cyclohexane at 80^{0}C. The solution is stirred and allowed to cool to room temperature, then

the microcapsules are washed and allowed to air dry before being sterilised. The microcapsules formed by this process have an average diameter of 224μm, and an irregular morphology. Approximately 84% of the MMC incorporated into the microcapsules is biologically active (Kato et al 1980).

Several clinical studies have been carried out using MMC microcapsules with some promising results. In a study of 56 patients with a variety of tumour types, an overall response rate of 77% was obtained in terms of both tumour reduction and symptom relief following locoregional administration (Kato et al 1981). In a study of 30 patients with unresectable hepatocellular carcinomas who were treated with MMC microcapsules at a dose of 0.5mg MMC/kg, 43% of patients had either a complete or a partial response to treatment. Toxicity was minimal and the median survival was 7 months with a 36% 1 year survival (Audisio et al 1990). In another non-randomised study of 32 patients with hepatocellular carcinoma treated with MMC microcapsules alone, a 35% response rate was documented (Sugita et al 1986).

Kato has collected data on 1013 patients who have been treated with microcapsules incorporating various drugs including MMC and cisplatin. A greater than 50% reduction in tumour size in 28% of 427 evaluable tumours was obtained with a median of 1 treatment, with MMC producing the best response rate. Systemic toxicity was rare with the most serious complications, as expected, being due to remote embolism of the particles. Two thirds of those being treated with microcapsules for intractable pain or haemorrhage were noted to have a significant improvement in their symptoms (Kato et al 1996).

Pharmacokinetic analysis comparing 20mg of free intra-arterial MMC with the equivalent microcapsule dose was carried out in 6 patients with colorectal liver metastases. The results showed that the peak plasma concentration was significantly lower when the MMC was

given in microcapsules (m/c) compared with free MMC (free): (80±75ng/ml (m/c) v 812±423ng/ml (free); $p<0.05$), whereas the plasma clearance (140±31 l/hour (m/c) v 46±8 l/hour (free); $p<0.05$) and volume of distribution (246±23 l (m/c) v 33±4 l (free); $p<0.05$) were significantly higher. The plasma MMC half life was also prolonged (0.39 ± 0.03hours (m/c) v 0.11 ± 0.02 hours (free); $p<0.05$) in the patients treated with microcapsules. The pharmacokinetic results and the lack of systemic toxicity suggested dose escalation of the MMC microcapsules was possible (Goldberg et al 1991D, Eley et al 1992).

When a Phase I study was attempted in 15 patients, escalating the dose of MMC to 30 and 40 mg in microcapsules did not increase systemic toxicity, but one third of the patients treated did experience significant local toxicity (3 developed pancreatitis and 2 developed gastroduodenal ulceration). This was probably due to local perfusion of these organs by the microcapsules (Anderson et al 1991A).

Both of these studies reported considerable problems in administration of the microcapsules. Their tendency to clump together due to their hydrophobic surface properties, combined with their large size has made this a very difficult procedure. The local toxicity which was documented may have occurred because of backflow as a result of the pressure which was required for successful particle injection and this has limited further studies (Anderson et al 1991A, Goldberg et al 1991D).

ii) *Microspheres*

Microspheres are solid porous structures which can be prepared by a variety of techniques including chemical stabilisation (Wilmott et al 1985), heat stabilisation (Fugimoto et al 1985A), coacervation (Golumbek et al 1993) and multiple emulsion solvent evaporation (Okada et al 1994). The process which is utilised depends not only on the matrix material (albumin (Fugimoto et al 1985A, 1985B, Allan et al 1993), gelatin (Narayani et al 1994, 1996),

transferrin (Chen et al 1988) and chitosan (Nishioka et al 1992)) but also on the drug which is to be encapsulated.

The method of microsphere preparation requires careful assessment in order to ensure that the encapsulated drug is not degraded or extensively cross-linked by the encapsulation process (Cummings et al 1985). When MMC is incorporated into albumin microspheres using heat stabilisation rather than chemical cross-linkage with gluteraldehyde, there is significant degradation of the MMC within the microspheres which is temperature dependant, ranging from 37% of the MMC undergoing degradation at 120^{0}C to 82% at 170^{0}C (Mehta et al 1988). The use of strong chemical cross-linking agents such as biacetyl can also result in complete degradation of the MMC within the microsphere (Mehta et al 1988).

Microspheres normally measure 10-50μm in diameter, their size being dependent on the method of preparation used. Selective intra-arterial administration is therefore the only practical route of administration as systemic therapy would result in entrapment within the lung. Targeting to an individual organ or tissue is referred to as "first order" targeting. *In vivo* studies with doxorubicin labelled microspheres have shown a high degree of "first order" targeting with 97% of the administered particles trapped in the kidney following intrarenal arterial injection and 93% of the particles trapped within the liver following hepatic arterial administration (McArdle et al 1988).

The use of vasoactive agents such as Angiotensin II can improve the degree of selectivity still further by targeting microspheres to the actual tumour deposits within the organ. This is referred to as "second order" targeting. In a study of 9 patients with liver metastases, the distribution of radiolabelled microspheres was studied both before and after the administration of Angiotensin II. The results showed that prior to the administration of Angiotensin II, the number of particles present in the normal liver was greater than in the

tumour deposits, but this was reversed following the administration of Angiotensin II with 2.8 times the number of particles being found in the tumour compared to the normal tissue (Goldberg et al 1991A, 1991B). The use of substances such as Angiotensin II to try to influence the pattern of microsphere distribution is referred to as "active" targeting.

1.8.3.2.5 The pharmacokinetic profile of microsphere encapsulated drug

The aim of locoregional therapy is to produce a high concentration of drug at the target site whilst minimising systemic exposure. This means that microspheres must not only be able to target well, but must also be associated with a suitable pharmacokinetic profile. The therapeutic advantage of locoregional administration can be defined pharmacokinetically as a redistribution ratio (Rd) in terms of the degree of reduced systemic exposure versus the increased target organ uptake (Collins et al 1982) and can be expressed as follows:

$$\text{Redistribution Ratio (Rd)} = \frac{\text{(AUC target tissue)/(AUC plasma) microspheres}}{\text{(AUC target tissue)/(AUC plasma) free drug}}$$

Intra-arterial doxorubicin has been shown to have a therapeutic advantage of 1.5-2 when compared to intravenous doxorubicin administration (Eksborg et al 1985). In contrast, when doxorubicin-loaded microspheres were compared with free intra-arterial drug, the ratio was 109 in favour of the microsphere group (Cassidy et al 1993). MMC-loaded microspheres administered by direct intratumoural injection demonstrated a redistribution ratio of 2.4 when compared with free drug given by the same route. (Cummings et al 1994).

A pharmacokinetic study, which compared doxorubicin-loaded microspheres (20-40μm diameter and 1% w/w drug loading) with free doxorubicin administered via the renal artery, showed a reduced peak plasma concentration with the microspheres compared to the free drug (16 ng/ml v 135ng/ml) as well as a reduced plasma AUC (20μg/ml v 1400μg/ml).

However, 1 hour after administration the renal concentration was greater in the microsphere-treated group compared to the free drug group (6.8μg/g v 4.5μg/g), which suggested continued drug release had occurred within the microsphere group (Kerr et al 1988).

Sprague-Dawley rats bearing Walker 256 tumour in the liver were treated via the hepatic artery with either doxorubicin-loaded albumin microspheres or the free drug equivalent 3 days after tumour implantation. The animals were culled at 7 days post-implantation and the tumours weighed and analysed. The mean tumour weight was found to be significantly lower in the doxorubicin-loaded microsphere group (0.45 ± 0.08g) compared to the free doxorubicin group (0.74 ± 0.12g) and the control group (1.01 ± 0.27g) ($p<0.05$). Fluorescent microscopy demonstrated considerable amounts of doxorubicin remaining in the liver of the microsphere group but not in the free drug group. This suggested that the slow release of doxorubicin from the microspheres may have been responsible for the improved antitumour effect which was seen (Goldberg et al 1992).

Limited pharmacokinetic studies have also been carried out using Mitomycin C- (MMC) loaded microspheres. The microspheres were similar in size to the doxorubicin microspheres (45± 8μm) with 5% MMC loading. Albino rabbits with VX-2 tumours growing on their hind leg were treated with MMC microspheres or the free drug equivalent, delivered via the femoral artery. The half-life of the MMC-loaded microspheres was longer than that of the free drug (20 minutes v 7-10 minutes). The drug was still detectable 6 hours after microsphere administration, while levels of free MMC were below the limit of detection by 2 hours. In the corresponding survival studies, 50% of the animals treated in the microsphere group had complete tumour regression, whilst in the free drug and control groups all the animals had progressive disease and died within 9 weeks of treatment (Fugimoto et al 1985A).

When MMC-loaded microspheres were compared with the equivalent dose of free MMC administered via the hepatic artery in Wistar rats, the concentration of MMC in the hepatic vein was constant for the first 2 hours in the microsphere-treated group, but was undetectable in the free drug group by 2 hours, which again suggested that prolonged drug release was occurring in the microsphere treated group (Fugimoto et al 1985A, 1985B). Histology of the treated livers confirmed that the microspheres had become trapped in the hepatic arterioles and these were still detectable 2 weeks after administration. Necrosis in the tissue adjacent to the trapped microspheres was also demonstrated in the MMC microsphere group, but was not seen in the blank microsphere or free drug groups (Fugimoto et al 1985A, 1985B).

Similarly, when MMC-loaded albumin microspheres were injected into the hepatic artery of Donryu rats bearing the AH-272 tumour and compared with free MMC and blank microspheres, histological analysis confirmed that necrosis was present only in the group treated with the MMC microspheres. The rats treated with the MMC-loaded microspheres also survived longer (21.9 days) compared with the group treated with free MMC (13.2 days) or the blank microsphere control group (9.6 days), though the numbers were small in each group (Morimoto et al 1989).

The concentration of methotrexate in the livers of dogs following intra-arterial injection of methotrexate-loaded albumin microspheres was also found to be greater than that seen following free drug administration. Methotrexate was still detectable 20 days following administration in the microsphere-treated group (0.2nmol/g), but not the free drug group which had undetectable levels of methotrexate by this time point. Histology again confirmed trapping of the microspheres within the hepatic arterioles (Feng et al 1993).

1.8.3.2.6 The effect of microsphere encapsulation on drug disposition

Microspheres are capable of altering a drug's disposition once it reaches the tumour. These observations were first made in the WAB/NOT rat bearing the syngeneic undifferentiated mammary carcinoma Sp-107, following intratumoural injection of doxorubicin-loaded microspheres. (Intratumoural injections are useful because they reproduce in a small animal the effect of chemoembolised particles and permit drug disposition variables to be studied without interference from targeting variables.) This study suggested that the antitumour effect of the doxorubicin-loaded albumin microspheres was 5 times greater than the equivalent amount of free drug (Willmott et al 1987A). When HPLC analysis of doxorubicin levels in both groups was carried out, the concentration of doxorubicin in the microsphere-treated group remained constant over a 72-hour period in contrast to the free drug group which showed a 5-fold decrease in doxorubicin concentration over the same time period. In addition, the microspheres appeared to stimulate bioreductive metabolism within the tumour, as determined by the presence of the 7-deoxglycone metabolites of doxorubicin (on HPLC analysis). These metabolites became detectable 16 - 24 hours after injection, increased by up to 155-fold at 48 hours and were still detectable 1 week later (Willmott et al 1987A, Cummings et al 1992A). This reaction was minimal in the free drug group and only happened in the microsphere group if the drug was actually incorporated into the microspheres prior to administration. Free drug co-administered with blank microspheres did not stimulate bioreductive metabolism (Cummings et al 1992A). Enzymatic studies have suggested that stimulation of the bioreductive metabolism of doxorubicin is thought to be due to the induction of hypoxia produced by the microspheres and is mediated through the activity of NADPH cytochrome P450 reductase (Cummings et al 1992B).

Further studies were carried out to determine whether the increased activity seen with the doxorubicin-loaded microspheres was due to either the sustained levels of parent drug within the tumour or the stimulation of bioreductive metabolism. The studies showed that it

was the proportion of the drug which was covalently complexed to the albumin matrix of the microsphere (via a molecule of gluteraldehyde) and which was slowly released as the microsphere was broken down, which was responsible for the enhanced antitumour activity (Cummings et al 1991). The bioreductive metabolism of the doxorubicin was shown to actually result in drug inactivation (Cummings et al 1992A, 1993B). Thus, although the doxorubicin-loaded microspheres have demonstrated greater antitumour activity than the equivalent amount of free drug, they are unable to take advantage of the hypoxic environment which is generated by the microspheres. Bioreductive drugs however might benefit from such an environment because it may enhance their activation.

MMC is regarded as the prototype bioreductive drug. Studies carried out by other groups, using encapsulated MMC either in microspheres or microcapsules, have demonstrated a degree of activity which is greater than for the free drug itself (Kato et al 1980, Fugimoto et al 1985A, 1995B, Morimoto et al 1989). The method of preparation for these microspheres however has not been optimal, as the heat stabilisation process often used in their manufacture may result in a significant degree of drug inactivation prior to use (Mehta et al 1988). Therefore a new method for the preparation of MMC microspheres was developed, which was based on a modification of the method used to prepare doxorubicin-loaded microspheres. This was carried out at room temperature using gluteraldehyde as the cross-linking agent. The resultant microspheres were found to have a mean diameter of 16.9μm with drug loading of 1.2% (w/w). Drug release was sustained over 20 hours with a burst effect (60%) apparent over the first 4 hours of drug release. HPLC analysis confirmed the presence of intact drug with no significant degradation occurring as a result of the microsphere preparation process (Allan et al 1993).

The pharmacokinetic parameters of the MMC-loaded albumin microspheres were determined in NMRI mice bearing the MAC 16 mouse adenocarcinoma of colon and

compared with free drug given by direct intratumoural injection. Both methods of drug administration achieved a similar level of total drug exposure as determined by the tumour AUC (46.9μg/ml/hr free MMC v 30.0μg/ml/hr MMC microspheres). The pharmacokinetic profiles were otherwise different. The peak level of MMC in the tumour was lower in the microsphere-treated group (20.5μg) compared with the free drug group (98.4μg). Peak plasma levels were also lower in the microsphere-treated group (2.34μg/ml) compared with the free drug group (6.03μg/ml) (Cummings et al 1994).

However, antitumour studies showed that the MMC-loaded microspheres had reduced antitumour activity compared with the free drug (The T/C value (the percentage change in tumour volume compared to day 0 of the treated group divided by the percentage change in tumour volume compared to day 0 for the control group) for free MMC was 39% compared to a T/C value of 8% for the MMC-loaded microspheres). The metabolite 2,7-Diaminomitosene (2,7-DM) was used as an indicator of metabolic activation in tumour tissue in these studies because this metabolite can only be formed as a result of quinone reduction. Levels of 2,7-DM have been demonstrated to correlate with cytotoxicity in human colon cell lines (Siegel et al 1990) and it has been shown to be a necessary prerequisite for the N-7 alkylation of deoxyguanosines located in the major groove of DNA (Prakash et al 1993). There was a 3-fold inhibition of 2,7-DM production in the microsphere-treated group compared to the free drug group. This was associated with the production of a different spectra of metabolites in the microsphere-treated group, which included elevated levels of the *cis*- and *trans*-hydro forms of 2,7-DM (Cummings et al 1994). The 1 electron reductases such as cytochrome P450 reductase, xanthine oxidase, and cytochrome *b*5 reductase result in the formation of metabolites such as the *cis*- and *trans*-hydro forms of 2,7-DM, while the 2 electron reductases such as DT-Diaphorase result in the formation of 2,7-DM. The altered pattern of metabolites seen following administration of the MMC-loaded microspheres

suggested an alteration in enzyme activity had occurred which has a negative effect on antitumour activity (Cummings et al 1994).

Although MMC is regarded as the archetypal anaerobic bioreductive agent, its hypoxic cytotoxicity ratio as indicated previously is low (HCR= 2) (Adams et al 1992). Drugs with increased selectivity towards hypoxic cells such as EO9 are more likely to benefit from the type of hypoxic environment which is generated by the microspheres. EO9 is also an attractive drug to consider for locoregional therapy, because as indicated previously, it is likely that, whilst producing a high concentration of drug at the tumour site, systemic exposure and therefore toxicity would be minimised using this approach.

1.9 OUTLINE OF THESIS

The aims of the studies presented in this thesis were therefore to:

1. Encapsulate EO9 into albumin microspheres.
2. Characterise the EO9-loaded microspheres.
3. Determine the antitumour activity and the pharmacokinetics of the EO9-loaded microspheres and to compare this with free EO9 given by direct intratumoural injection in a variety of subcutaneous colon tumours.
4. Develop a clinically relevant method of testing the EO9-loaded microspheres.

CHAPTER 2

The encapsulation of EO9 into human albumin microspheres

2 Chapter 2

2.1 INTRODUCTION

Microspheres are solid porous particles which can be manufactured from a wide variety of matrix materials. These have included proteins such as albumin (Fugimoto et al 1985A, 1985B, Morimoto et al 1989, Allan et al 1993, Cremers et al 1994A, 1994B, 1994C, Feng et al 1993), gelatin (Narayani et al 1994, 1996, Nastruzzi et al 1994), transferrin (Chen et al 1988) and caesin (Jayakrishnan et al 1994); polysaccharides such as starch (Ensminger et al 1985, Pfeifle et al 1985, Civalleri et al 1994, Teder et al 1995, Johansson 1996, Carr et al 1997, Hakansson et al 1997), chitosan (Nishioka et al 1992) and alginate and synthetic polymers such as glycolide and lactide (Ichihara et al 1989, Dordunoo et al 1995, Hagiwara et al 1996, 1997, Kumagai et al 1996). The primary function of the matrix is to protect the drug and to optimise its pharmacokinetic/pharmacodynamic profile. The material selected is important because it will determine the overall size and structure of the microsphere, its surface properties of charge and shape and its biodegradability, toxicity and antigenicity. It will also influence the incorporated drug's entrapment efficiency, release rate and overall stability (Chen et al 1994).

Albumin was first used as a matrix in the manufacture of microspheres in 1972 and remains the most widely used protein because of its many beneficial properties. These include high stability, biocompatability (Ratcliffe et al 1984) and biodegradability (Lee et al 1981), together with the ability to trap, in a relatively non specific manner, a wide variety of drugs (Kramer et al 1974). In common with the other proteins, albumin possesses functional groups such as COOH and NH_2, which form inter-molecular (albumin-albumin) covalent bonds during microsphere formulation. Albumin taken from different animal species produces microspheres with slightly differing physico-chemical properties (Egbaria et al 1992A,

1992B). The concentration of albumin used in microsphere preparation is important because it influences the final size of the microspheres and affects drug loading characteristics (Karunaker et al 1994).

Human albumin was selected for our microspheres because it is the least immunogenic in humans (Rhodes et al 1969). Like other types of albumin, it is also readily available, has excellent storage properties and can be used in several different types of microsphere manufacture.

Of the variety of methods available for microsphere preparation (For review see Yapel 1985, Chen et al 1994), emulsification is the one which is most commonly utilised for the preparation of albumin microspheres. It involves the use of a disperse phase (the drug suspended in an aqueous polymer solution) emulsified in a water immiscible continuous phase (usually an oil) which is then stabilised using a surfactant, followed by cross-linkage of the newly formed particles. It is suitable for both water soluble and insoluble drugs providing that the drug can be dispersed in the polymer solution.

Cross-linkage of the microsphere particles can be achieved in a variety of ways using heat or chemicals to form the cross-links. It has been shown that increasing the level of cross-linking alters the rate of particle biodegradation and the release of core material from the matrix (Arshady 1990). Heat stabilisation is the most commonly used method. This involves heating the albumin molecules to more than 100^{0}C which allows self cross-links to form between the reactive groups on the polypeptide side chains of the molecules. The level of cross-linkage increases with an increase in the reacting temperature (Arshady 1990). The main problem with this technique is that it can cause significant drug inactivation. This has been clearly demonstrated in the work by Mehta (Mehta et al 1988) who showed that heat stabilisation used in the manufacture of MMC microspheres actually resulted in degradation

of the MMC ranging from 37% at 120°C to 82% at 170°C. The use of strong chemicals to produce chemical cross-linkage such as biacetyl however can also result in complete degradation of the MMC within the particles (Mehta et al 1988).

Glutaraldehyde is the most commonly used chemical cross-linking agent and is the one utilised in our method. There is a little uncertainty as to the exact method by which the cross-linking occurs, but it would appear to involve the lysine residues as these are the only molecules in the albumin structure which are actually modified in the presence of gluteraldehyde (Peters et al 1977). Again, the degree of cross-linking, which will affect the properties of the microsphere, is directly proportional to the concentration of the gluteraldehyde present in the solution (Reddy et al 1990, Rubino et al 1993). This will be studied further during the development of the method for EO9-loaded microspheres.

Human albumin microspheres, prepared using the emulsification method with gluteraldehyde as the chemical cross-linking agent, have been extensively studied in our laboratory where the cytotoxic agents Adriamycin and MMC have been successfully encapsulated and characterised (Wilmott et al 1988, Cummings et al 1985, 1994, Allan et al 1993). As discussed previously (Chapter 1.5.4), EO9 was considered a rational choice to attempt to encapsulate into albumin microspheres. This was based not only on the problems associated with systemic administration of EO9 (instability and dose limiting systemic toxicity), but also because EO9 as a known bioreductive drug might demonstrate enhanced activity in an environment reduced in oxygen due to the chemo-embolic effect of the microspheres themselves.

2.1.1 Initial Experiment to Formulate EO9 Microspheres

The procedure described by Allan et al 1993, for the formulation of MMC-loaded microspheres was used initially to attempt to encapsulate EO9 into microspheres. This method of EO9 microsphere preparation is outlined in Figure 2.1, the principle of which, as discussed above, is the cross-linkage of albumin by gluteraldehyde during emulsification at room temperature. However, when this method was employed for EO9, two main problems were identified:

1. When the microspheres were incubated with trypsin at 37°C, microsphere digestion did not occur, making subsequent analysis of the drug content of the microspheres difficult. It also raised concerns about how biodegradable the particles would be *in vivo*, as trypsin digestion of microspheres *in vitro* is thought to reflect *in vivo* biodegradability. It was felt that this might be related to the concentration of gluteraldehyde used in the cross-linking step because, the higher the concentration of gluteraldehyde used, the greater the degree of cross-links which can occur (Reddy et al 1990, Rubino et al 1993). Therefore, microspheres were prepared using a range of concentrations of gluteraldehyde and the effect of this examined both in terms of microsphere yield and particle biodegradability (using trypsin digestion).

2. The isopropanol wash step following microsphere preparation was associated with pink discolouration of the supernatant. EO9 is pink, suggesting that there was drug loss from the microspheres and therefore alternatives to the use of isopropanol as one of the washing steps in the preparation of the microspheres were assessed.

This chapter will describe the studies performed to investigate and correct these problems with the method being described which lead to the successful incorporation of EO9 into albumin microspheres.

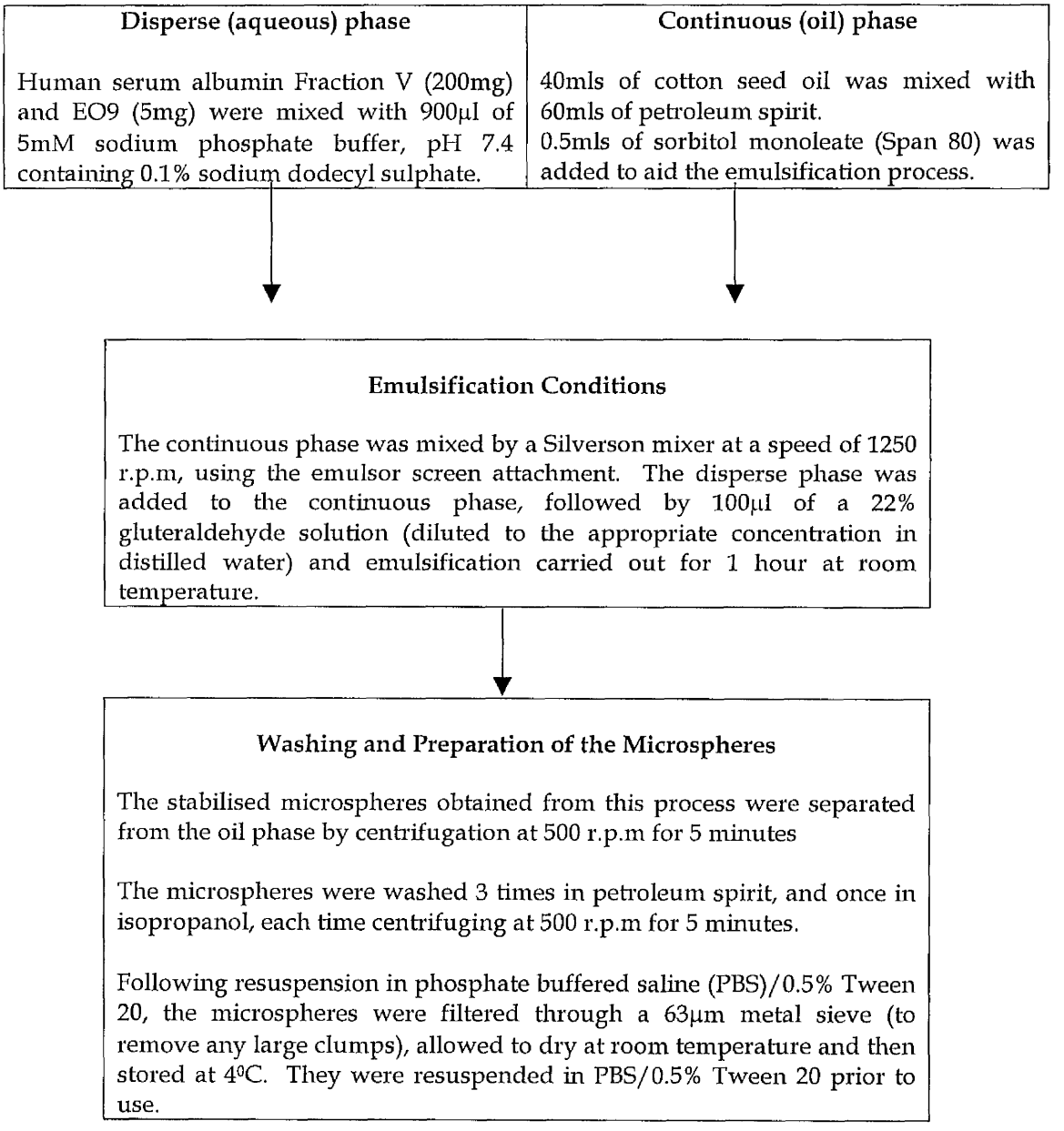


Figure 2.1 Flow chart outlining the method of Allan et al 1993, for the initial encapsulation of EO9 into albumin microspheres.

2.2 MATERIALS

All chemicals used were of the highest grade commercially available. These are listed, together with their suppliers in Appendix 1. Water was obtained from a Milli-U10 water purification system. All suppliers of the apparatus used throughout are also listed in Appendix 1. High Performance Liquid Chromatography (HPLC) analyses were performed using a Hewlett-Packard Model 1090 equipped with the following features: a PV5 ternary low pressure mixing solvent delivery system, a variable volume (10-250µl) automatic injector and autosampler, a heated column compartment and multidiode rapid scanning UV/Vis spectrophotometric detector. System control and data evaluation were performed using Hewlett-Packard Series 9000 300 "Chemstation" software.

2.3 METHODS

2.3.1 The Analysis of EO9 by High Performance Liquid Chromatography

The stationary phase was a Li-Chrosorb RP-18 (7µm particle size) HPLC cartridge column (column dimensions 25cm x 4mm) and a LiChrospher 100 RP-18 (5µm particle size) precolumn (column dimensions 4mm x 4mm) enclosed in a LiChrocart Manu-fix cartridge holder.

The mobile phase consisted of 10mM sodium phosphate buffer, pH 7.5, mixed with methanol in a ratio of 76:24. Elution was isocratic at a flow rate of 1ml per minute with the oven temperature maintained at 40^{0}C. The mobile phase was filtered before use through a 0.45µm filter for the methanol and a 0.6µm filter for the sodium phosphate buffer and sparged continuously with helium throughout chromatographic analysis.

Detection using a diode assay detector was at 280nm, close to the absorption maxima of both EO9 and the hydrolysis product EO5A (See Figure 2.2).

2.3.2 Effect of Process Variables on EO9-loaded Albumin Microspheres

2.3.2.1 Gluteraldehyde concentration

The original method produced microspheres which failed to undergo digestion in the presence of trypsin, making potential assessment of drug content in the microspheres difficult and raising concerns about the biodegradability of the particles *in vivo* (Chapter 2.1.1). This was thought to be related to the concentration of gluteraldehyde used in microsphere preparation, so microspheres were prepared as outlined above, but using different concentrations of glutaraldehyde in the cross-linking step. 100µl of 22%, 11%, 6.25%, 5.5%, and 2.5% gluteraldehyde solutions, prepared as previously described (Figure 2.1), were each compared for their effect on microsphere characteristics, which was determined in two ways:

a) The "yield" of microspheres: an approximation of the amount of filterable microspheres produced, which was assessed by visual inspection as being high, intermediate or low.

b) Microsphere digestion assessed by visual inspection and microscopy during a timed digestion with trypsin using the following method: 100µl of the final microsphere suspension was added to 2ml of 0.4%(w/v) trypsin in PBS solution containing penicillin and streptomycin (500 units/ml) to prevent possible drug degeneration occurring as a result of bacterial overgrowth and incubated at 37^{0}C for 4 and 16 hours. Digestion was graded as either complete or incomplete.

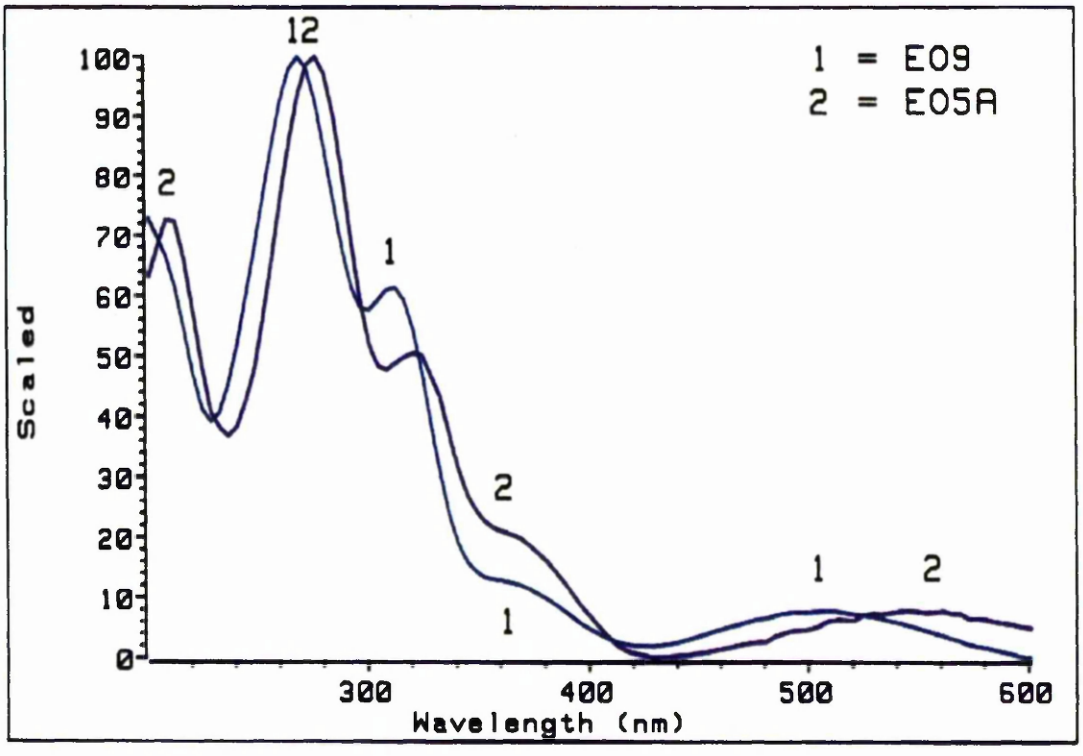


Figure 2.2 **Comparison of the UV/Visible absorption spectra of EO9 and the hydrolysis product EO5A.**

2.3.2.2 EO9 loss during the isopropanol wash

It had been noted that the supernatant following the isopropanol wash was pink suggesting premature release of EO9 from the microspheres. No loss of EO9 from the microspheres was detected in the preceding petroleum ether washes. In order to evaluate this, HPLC analysis (outlined in Chapter 2.3.1) was performed using a 20µl aliquot of the total isopropanol supernatant and standard curves used to determine the amount of EO9 present.

2.3.2.3 Microsphere resuspension

Because of the apparent loss of EO9 from the microspheres into the isopropanol wash, experiments were performed where this step was omitted. However, removing the isopropanol wash resulted in increased difficulty in resuspending the microspheres prior to their use. Alternative methods of particle resuspension were therefore investigated:

2.3.2.3.1 The effect of freeze drying on microsphere resuspension

The microspheres (both EO9-loaded and blank) were manufactured as in Figure 2.1. After 3 washes with petroleum ether the microspheres were allowed to dry at room temperature. They were then reconstituted in 1ml of ice cold PBS, snap frozen using solid carbon dioxide and methanol and freeze dried for 12 hours. The vacuum in these conditions results in the evaporation of water and solvent from the sample which reduces the requirement for multiple washes. After reconstitution in PBS/0.5%Tween 20, followed by filtering through a 63µm sieve, the supernatant from the EO9 and blank microspheres was obtained by vacuum filtration.

a) A 100µl aliquot of the supernatent from the reconstituted EO9 microspheres was taken and subjected to HPLC analysis in order to ensure that the EO9 which had been

incorporated and subsequently released from the microspheres had not been adversely affected by the freeze drying process.

b) 100µl of the blank microsphere suspension was taken following reconstitution and subjected to digestion for 16 hours at 37°C using trypsin in order to ensure that the freeze-drying process had not altered the ability of the microspheres to undergo digestion, which may suggest altered particle biodegradability.

2.3.2.3.2 Reconstitution of the microspheres following freeze drying

a) Microsphere resuspension

Resuspension in PBS/0.5% Tween 20 was assessed as being sub-optimal following freeze drying. Therefore, other agents were assessed and compared with PBS/0.5% Tween 20. The microspheres were resuspended in 4mls of either i) phosphate buffered saline (PBS), ii) 10mM sodium phosphate buffer (pH 7.5), iii) PBS with 0.5% Tween 20 or iv) PBS with 0.5% Tween 80. They were subsequently vortexed and filtered through a 63µm sieve. The amount of residue on the sieve was assessed and the microsphere sample was then analysed directly using light microscopy to inspect the degree of clumping and aggregation.

b) The effect of ultrasonication on microsphere resuspension

To improve microsphere resuspension further, the effect of ultrasonication was studied. The suspension of microspheres was divided into 2 aliquots (A and B). Aliquot A was sonicated for 3 minutes in an ultrasonic water bath and then filtered through a 63µm sieve. The amount of residue on the sieve was assessed and the microsphere suspension then inspected directly using light microscopy for the degree of clumping and aggregation. The sonicated samples were compared directly with microspheres which had not undergone the sonication process (Aliquot B).

2.4 RESULTS

2.4.1 High Performance Liquid Chromatographic analysis of Indoloquinone EO9

Using the HPLC analytical technique outlined in Chapter 2.3.1, EO9 has a retention time of 23-25 minutes with the hydrolysis product EO5A eluting at the earlier time of 16-18 minutes (Figure 2.3). Each has a distinct UV/Vis absorption spectra. EO9 shows UV absorption maxima at 268nm with a shoulder at 313nm and a visible absorption maximum at 505nm. In contrast, the spectra of EO5A exhibits bathochromic shift (a shift in the UV/Vis absorption spectra towards longer wave lengths) in UV absorption to maxima at 280 and 321nm and a bathochromic shift in the visible absorption maximum to 550nm (Figure 2.2).

2.4.2 Effect of process variables on EO9-loaded albumin microspheres

2.4.2.1 Gluteraldehyde concentration

a) Microsphere yield

At 22% and 11% glutaraldehyde by volume, the highest yield of microspheres was seen (Table 2.1). At the lowest concentration of 2.5%, the amount of microspheres produced fell dramatically. This was accompanied by the presence of a large amount of non-filterable material on the mechanical sieve, probably occurring as a result of inadequate cross-linkage of the albumin by the glutaraldehyde, which results in partially formed microspheres and aggregated albumin particles.

b) Microsphere digestion

The lower concentrations of glutaraldehyde (2.5%, 5.5% & 6.25% by volume) produced microspheres which underwent complete digestion with trypsin within 4 hours at 37^{0}C (Table 2.1). Digestion did not occur in the microsphere batches which used 11% and 22%

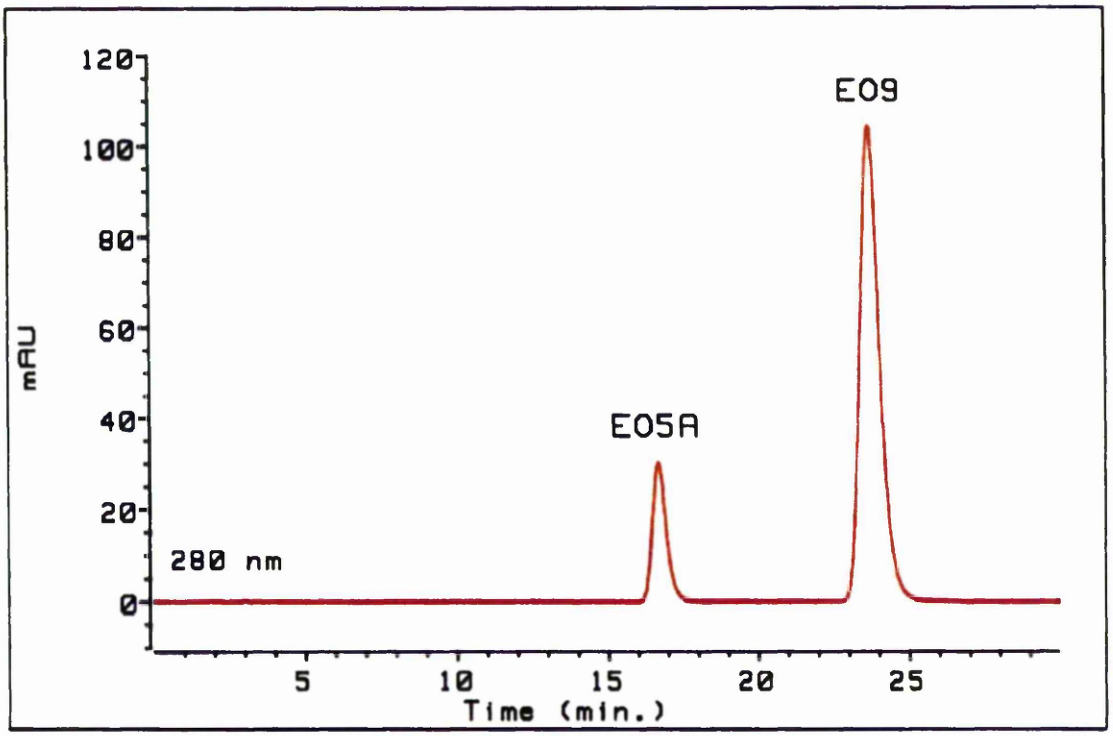


Figure 2.3 **Standard chromatographs of EO9 and the hydrolysis product EO5A.**

Gluteraldehyde Percentage (100μl volume)	*Microsphere Yield*	*Microsphere digestion after 4 hours incubation with trypsin at 37°C*
22	+++++	No
11	+++++	No
6.25	+++	Yes
5.5	+++	Yes
2.5	+	Yes

Key:

+++++	*high yield*
+++	*intermediate yield*
+	*low yield*

Table 2.1 The effect of gluteraldehyde concentration on a) Microsphere yield and b) Microsphere digestion with trypsin at 37^{0}C.

gluteraldehyde in their manufacture even after incubation with trypsin for 16 hours.

The concentration of 6.25% by volume of glutaraldehyde was chosen for the final microsphere method because it produced a high yield of microspheres which were amenable to digestion with trypsin.

2.4.2.2 Assessment of EO9 loss during the isopropanol wash

The HPLC trace obtained from the isopropanol wash showed three apparent peaks with retention times of 18.6 minutes, 20.6 minutes and 24.4 minutes respectively (Figure 2.4). The later peak had the same retention time and spectra as the EO9 standard. Although the other 2 peaks had different retention times, they had identical spectra to the EO9 standard (Figure 2.5). It was assumed that this was due to peak splitting of the parent compound as a result of the reduced solubility of EO9 in the isopropanol. On the basis of this assumption, the total peak area was used to calculate the amount of EO9 present in the isopropanol wash. Using a standard EO9 curve, this was calculated to be 37% of the total EO9 dose. This confirmed that significant loss of EO9 was occurring and the isopropanol wash was therefore omitted from subsequent method development.

2.4.2.3 Microsphere resuspension

2.4.2.3.1 The effect of freeze drying on microsphere resuspension

After 12 hours of freeze drying the microspheres, a fine powder was obtained. These microspheres resuspended more readily and passed through the sieve more easily than those which had not undergone the freeze drying process.

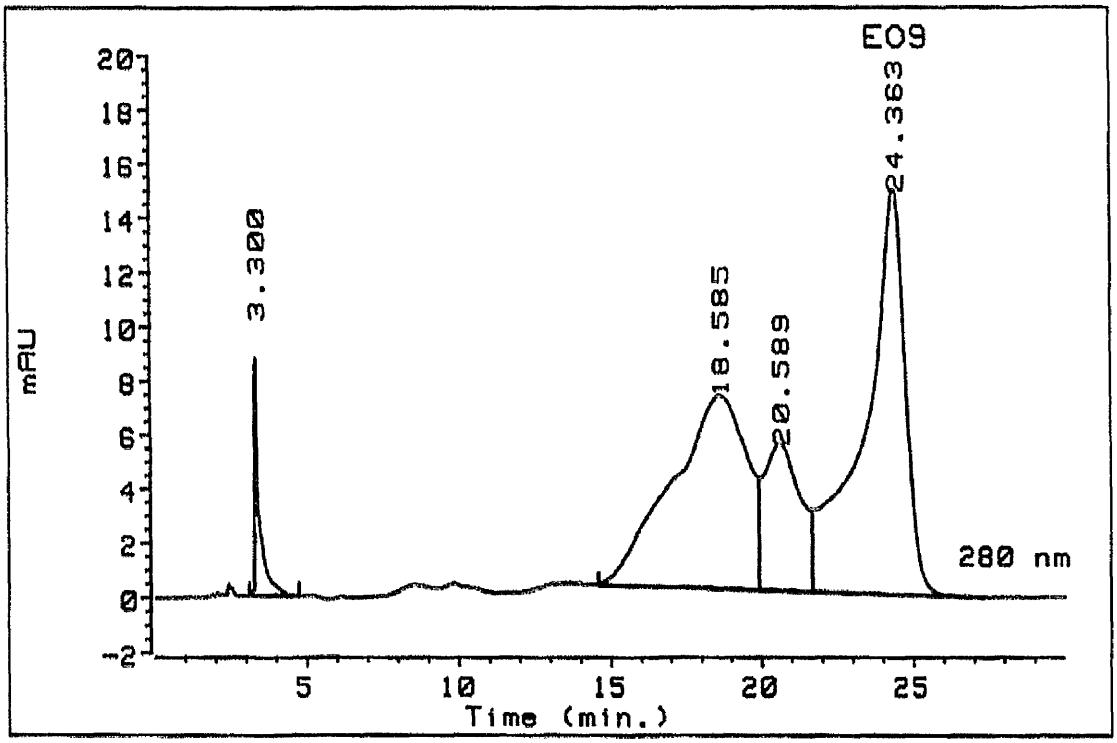


Figure 2.4 Chromatograph of the isopropanol wash produced during the preparation of the EO9-loaded microspheres.

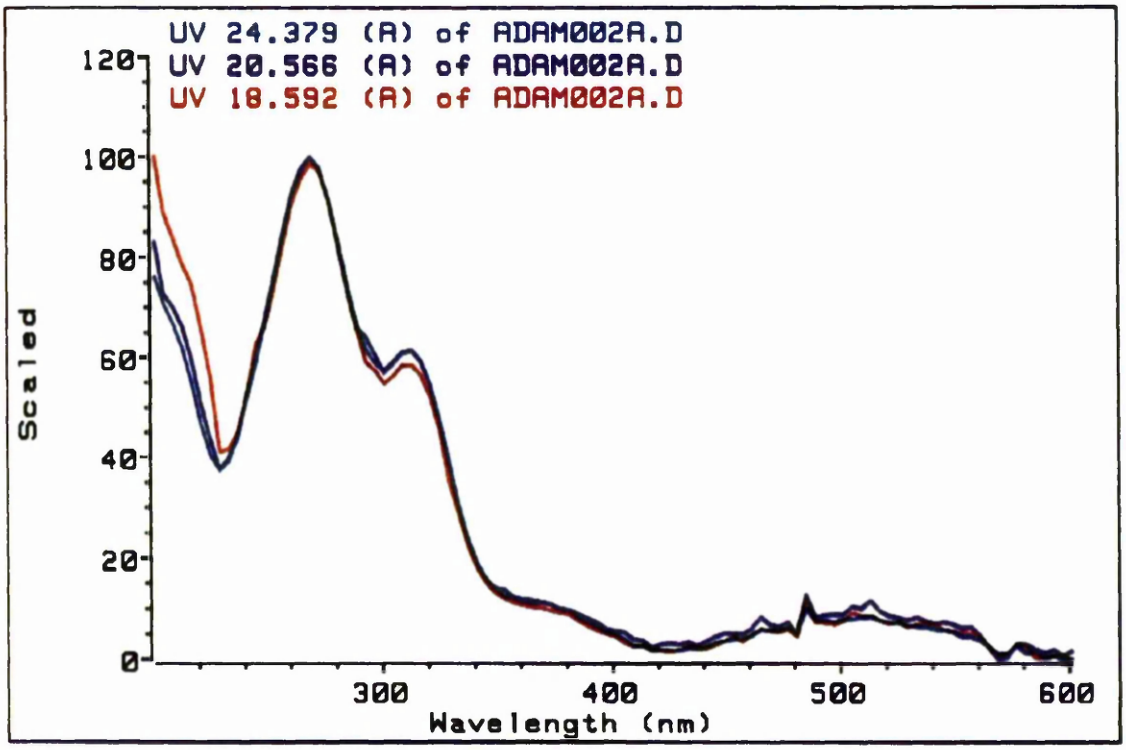


Figure 2.5 **Comparison of the UV/Visible absorption spectra of the 3 peaks found in the isopropanol wash produced during preparation of the EO9-loaded microspheres.**

a) HPLC analysis of the freeze dried EO9-loaded microsphere supernatant

HPLC analysis confirmed that the retention times (Figure 2.6) and the spectra (Figure 2.7) of the EO9 obtained from the freeze dried microspheres was identical to that of the freshly prepared EO9 sample. A small amount of the hydrolysis product, EO5A, was also identified but there was no evidence of any EO9 metabolites. Assessment of the purity of the EO9 peak obtained from the freeze dried microspheres using the Hewlett Packard "Chemstation" software confirmed a maximum possible purity factor of 1000. This was calculated by overlying the spectra taken from different points on the chromatographic peak of the sample and comparing it with the original parent compound (Figure 2.8).

b) Biodegradability of the freeze dried blank microspheres

Complete digestion of the freeze dried, blank microspheres occurred in the presence of trypsin at 37^{0}C. HPLC analysis of the blank digest mixture was identical to that obtained using freshly made blank microspheres (Figure 2.9), which suggested that freeze drying had not affected microsphere cross-links or structure.

Freeze drying also proved to be a suitable method for preparing the microspheres for long-term storage. The microspheres were stored in this form at 4^{0}C for up to 6 weeks. HPLC analysis of the supernatant of the resuspended EO9-loaded microspheres confirmed that the drug payload remained chemically intact throughout this period.

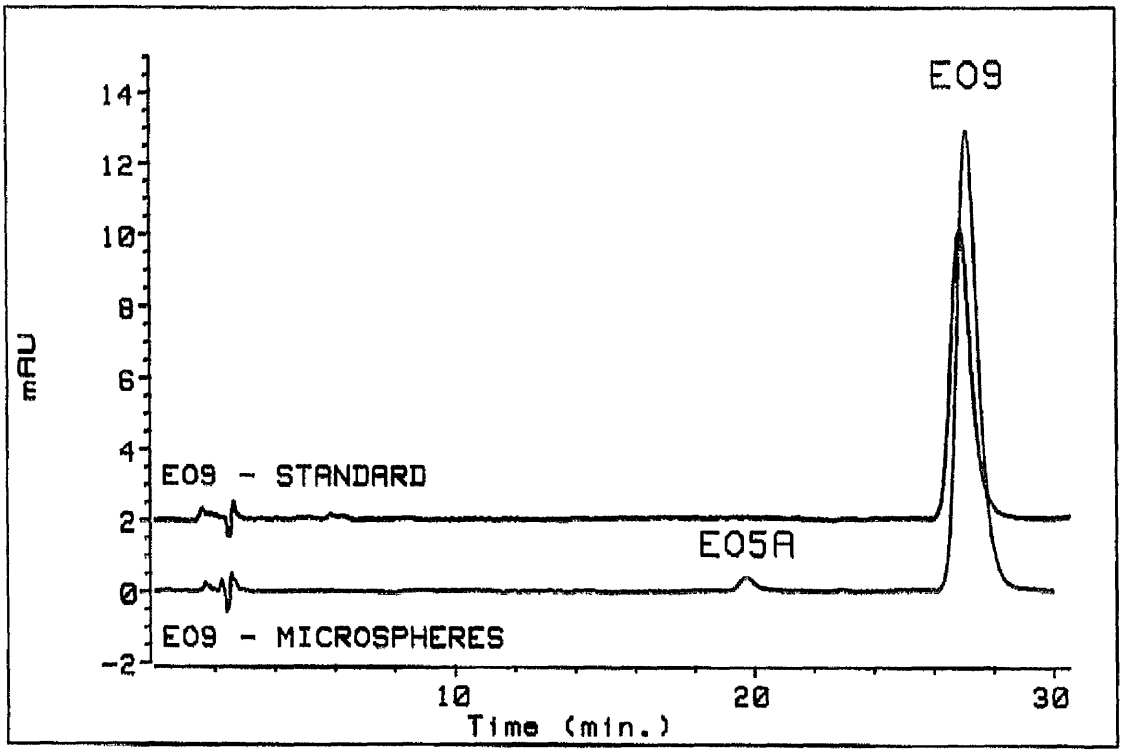


Figure 2.6 **Comparison of the chromatograph of the EO9 standard with the EO9 obtained from the freeze dried microspheres. In addition to EO9, a small amount of the hydrolysis product, EO5A, is identified in the microsphere sample.**

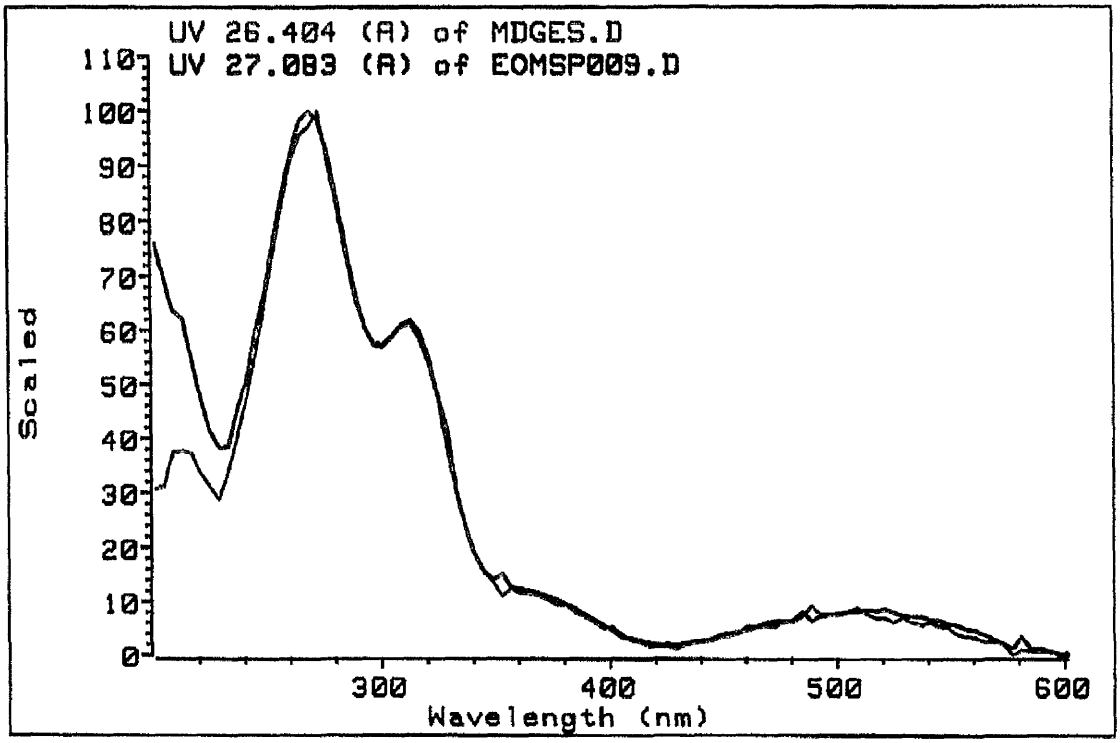


Figure 2.7 Comparison of the UV/Visible spectra of the EO9 standard (MDGES.D) with the EO9 obtained from the resuspended freeze dried microspheres (EOMSP009.D).

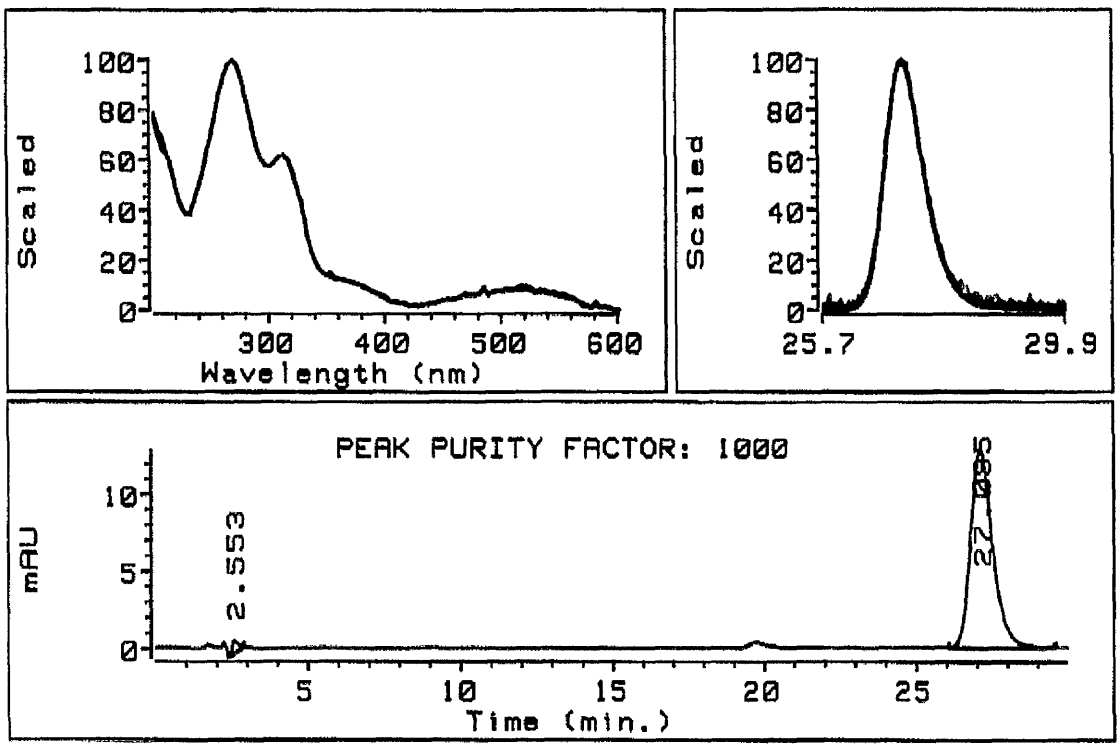


Figure 2.8 The peak purity assessment of the EO9 peak produced from the resuspended freeze dried microspheres. The peak is at the maximum purity level detectable by the HPLC "Chemstation" software.

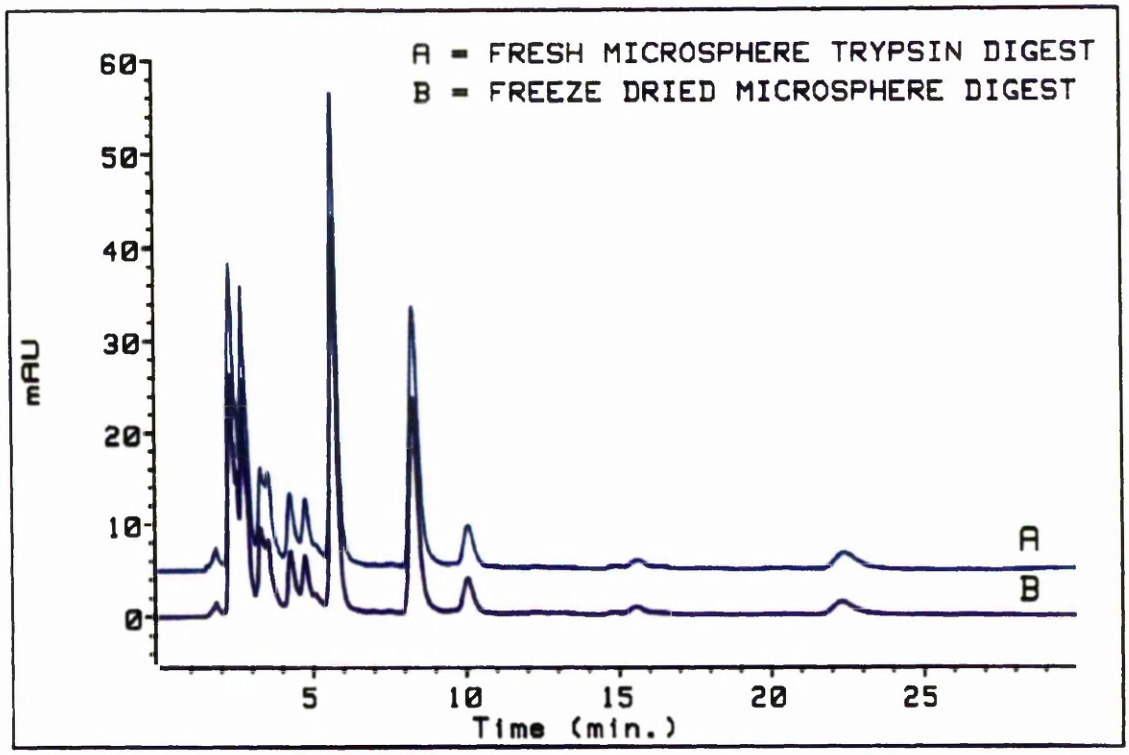


Figure 2.9 **Chromatographs of albumin microspheres digested overnight with trypsin. Identical chromatographs are obtained for freshly prepared (A), and freeze dried microspheres (B).**

2.4.2.3.2 Reconstitution of the microspheres following freeze drying

a) *Microsphere resuspension*

Following freeze drying the microspheres resuspended poorly in PBS and phosphate buffer. There was increased residue on the sieve and a greater degree of aggregation seen on direct microscopy. The two solutions which incorporated Tween products (PBS/0.5% Tween 20, PBS/0.5% Tween 80) produced improved microsphere resuspension with less residue on the sieve and less aggregation on direct microscopy, presumably because the Tween products help to emulsify any residual oil left on the microsphere surface. This experiment however failed to detect any difference between the two Tween products (Table 2.2 and Figure 2.10).

b) *The effect of ultrasonication on microsphere resuspension*

The effect of ultrasonication on microsphere particles resuspended in PBS/0.5%Tween 20 and PBS/0.5%Tween 80 showed that the microspheres that had been sonicated for 3 minutes resuspended more easily, gave less residue on the filter and were less aggregated on direct microscopy than the unsonicated microspheres (Table 2.2, Figure 2.10C and D, Figure 2.11).

The PBS/0.5%Tween 80 solution was found to be better than PBS/0.5% Tween 20 in resuspending the microspheres when sonication was used as part of the resuspension process.

2.4.3 Final Method of EO9 Albumin Microsphere Preparation

The final method of EO9-loaded albumin microsphere production, based on the above results, is outlined in Table 2.3. The characteristic appearances of blank and EO9-loaded microspheres, which were prepared using this method, are identical under light microscopy and are shown in Figure 2.11B.

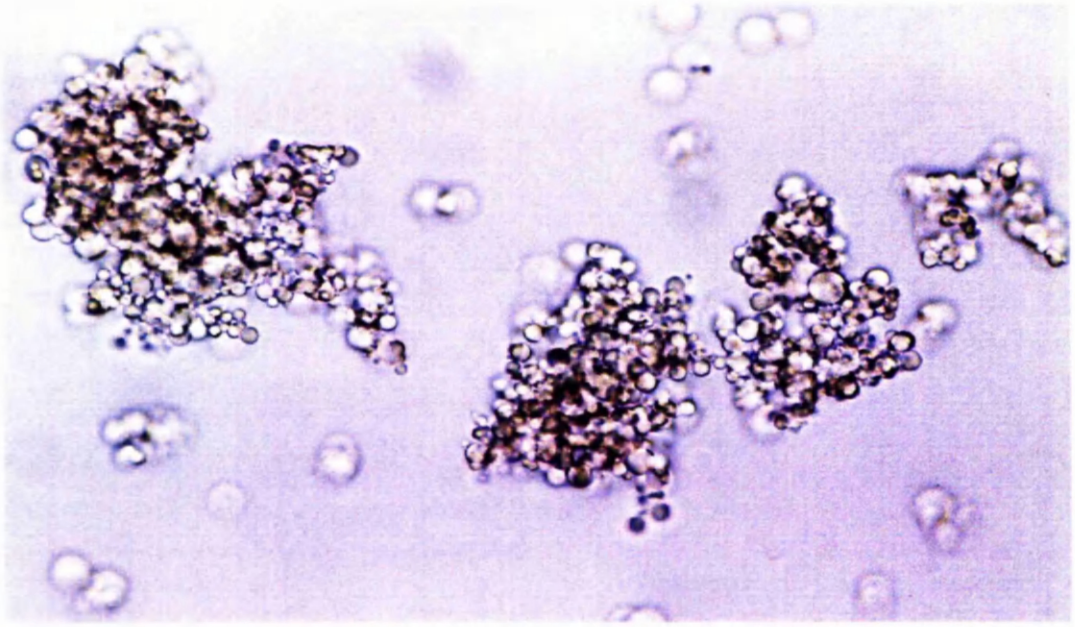
Resuspending Agent	*Residue on the sieve*	*Degree of aggregation On direct microscopy*	*Degree of aggregation post sonication*
Phosphate Buffered Saline (PBS)	+++	+++++	nd
Sodium Phosphate Buffer (10mM, pH 7.5)	+++++	+++++	nd
Phosphate Buffered Saline/0.5% Tween 20	+	+++	++
Phosphate Buffered Saline/0.5% Tween 80	+	+++	+

Key:

+++++	large residue/aggregation
+++	moderate residue/aggregation
+	minimum residue/aggregation
nd	not determined

Table 2.2 The effect of various resuspending agents and ultrasonication on microsphere resuspension.

(A)



(B)

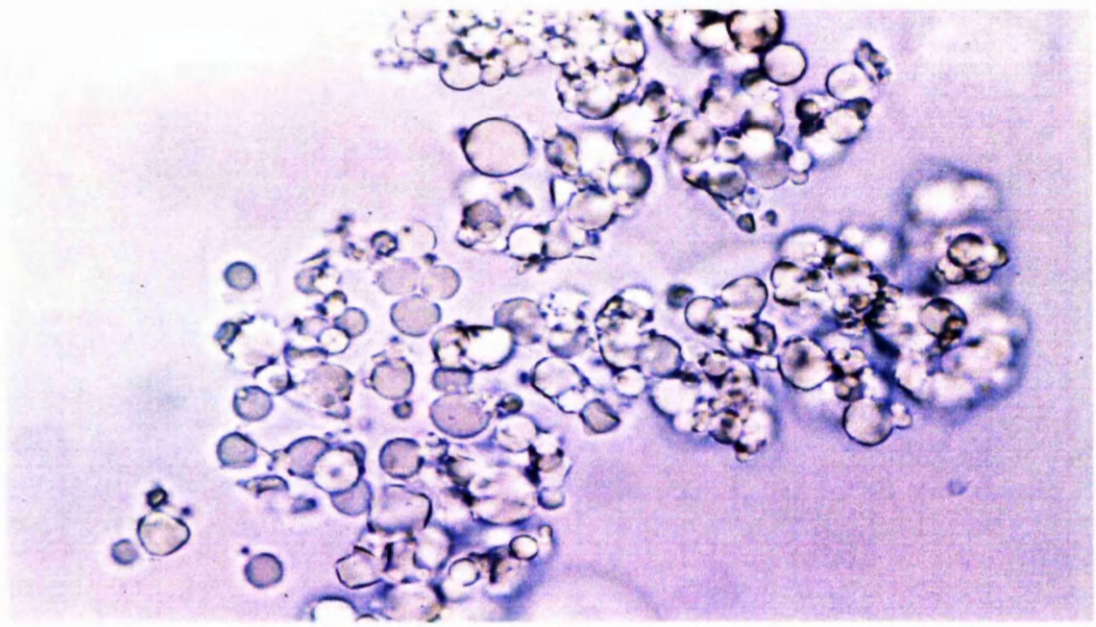
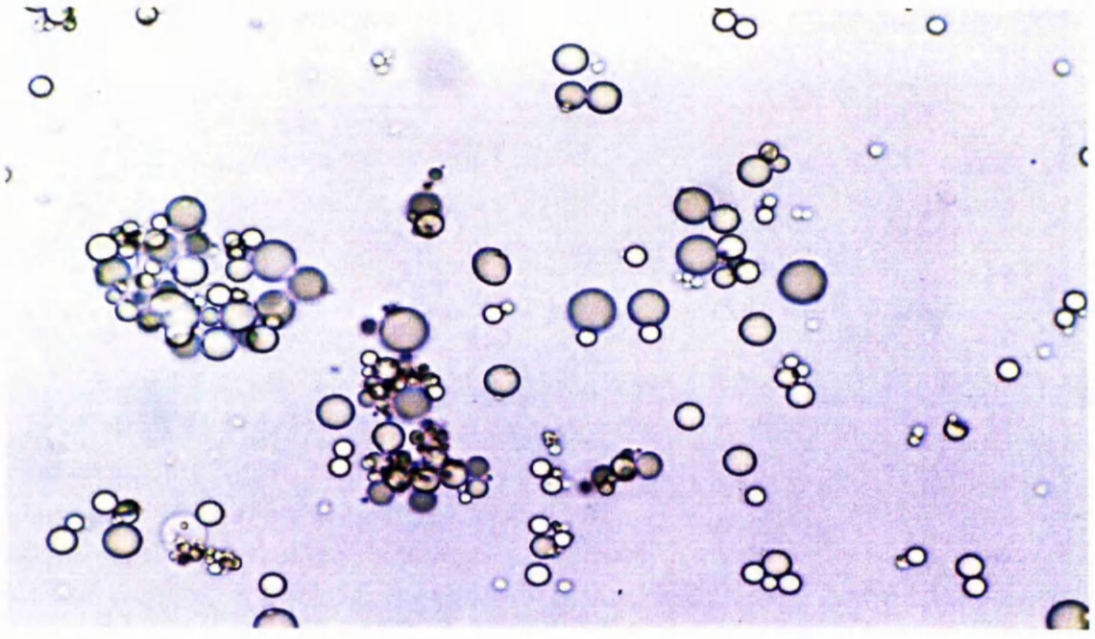


Figure 2.10 **Direct comparison using light microscopy of freeze dried microspheres resuspended in A) Phosphate Buffered Saline, and B) 10mm Sodium Phosphate buffer (pH 7.4).**

(C)



(D)

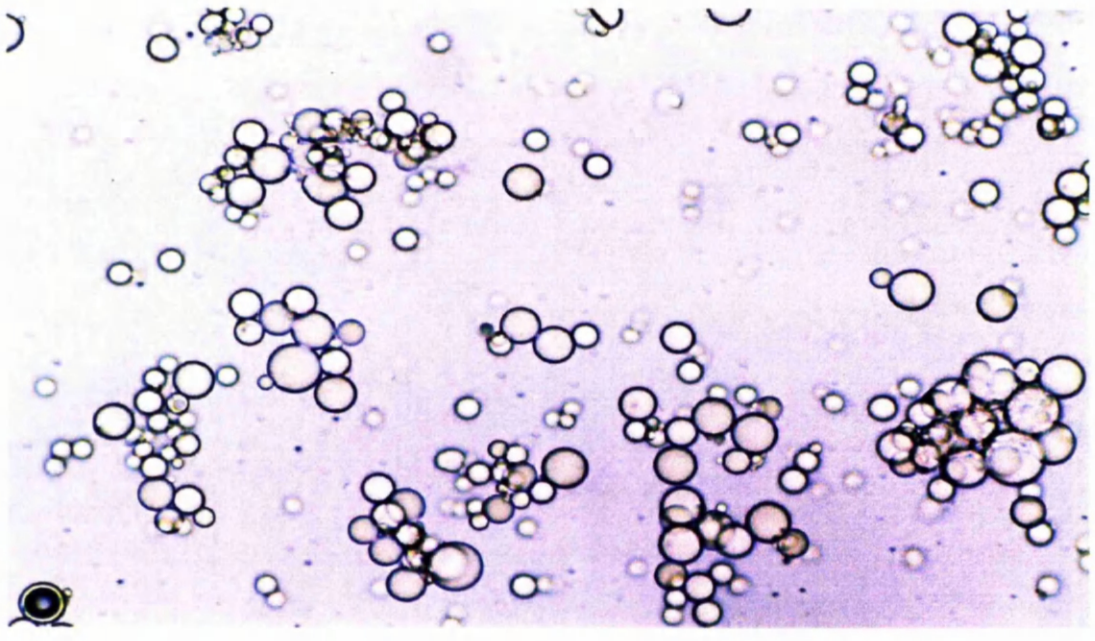
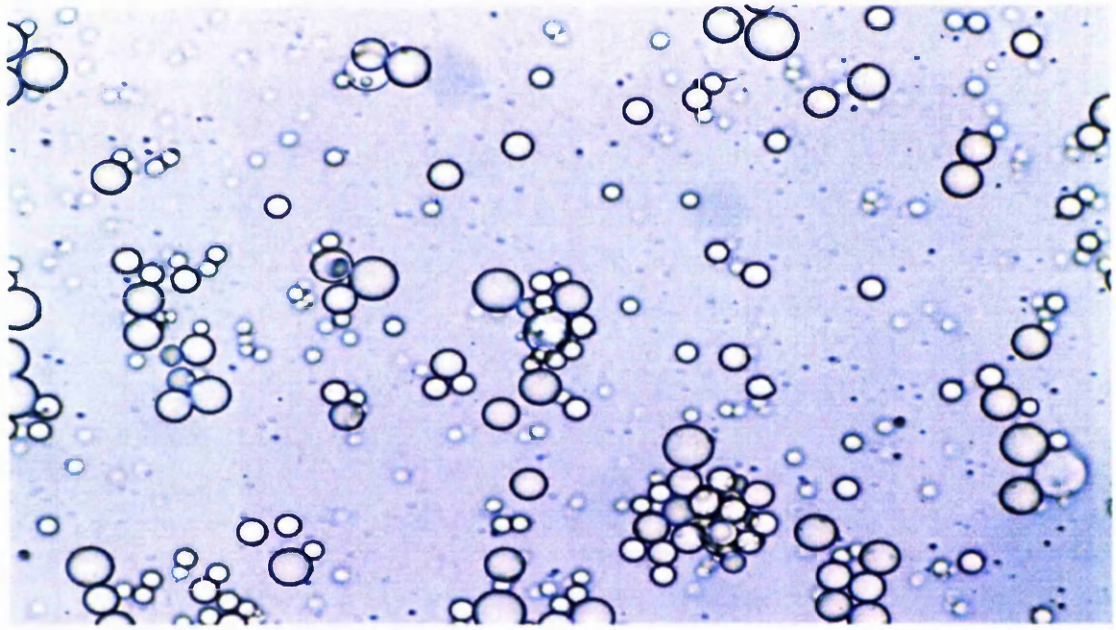


Figure 2.10 **Direct comparison using light microscopy of freeze dried microspheres resuspended in C) PBS/0.5% Tween 20, and D) PBS/0.5% Tween 80.**

(A)



(B)

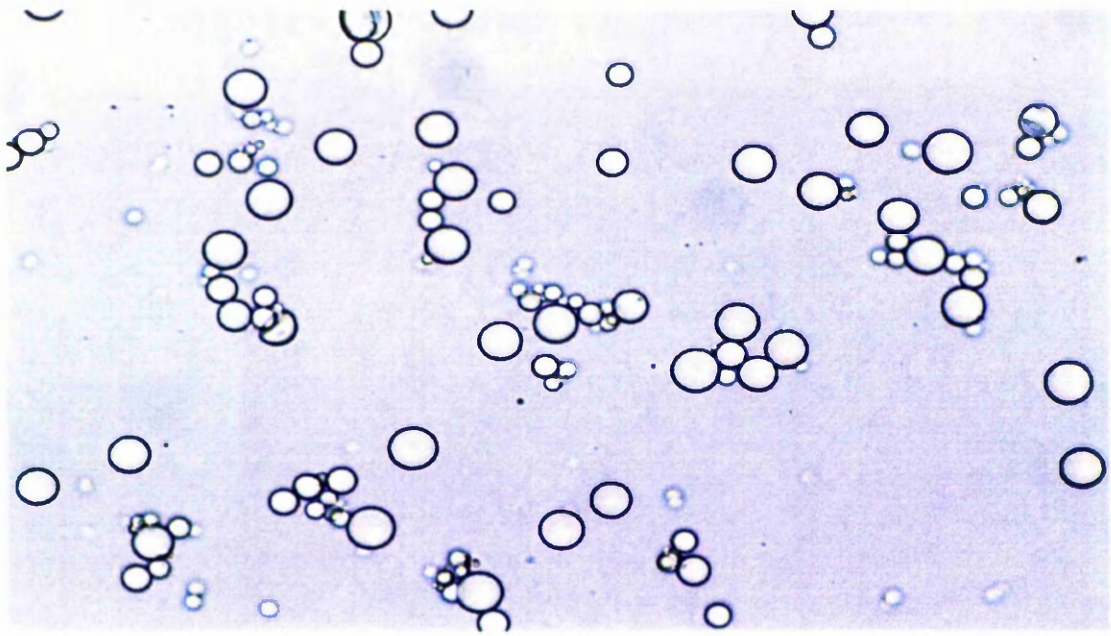


Figure 2.11 **Direct comparison using light microscopy of freeze dried microspheres resuspended in A) PBS/0.5% Tween 20, and B) PBS/0.5% Tween 80 after 3 minutes of ultrasonication.**

Step	*Method*
1	200mg of human serum albumin (fraction V) and 5mg of EO9 were added to 900μl of 5mM sodium phosphate buffer, pH 7.4 containing 0.1% sodium dodecyl sulphate as the disperse phase
2	The continuous phase consisted of 40ml of cotton seed oil mixed with 0.5mls of Span 80 (sorbitol monoleate) and 60mls of petroleum ether
3	The disperse phase was added to the continuous phase and emulsification carried out at 1600, 2000 or 2500r.p.m for 1 hour at room temperature. Stabilisation of cross-links was achieved by the addition of 100ml of a 6.25% gluteraldehyde solution
4	The resulting microspheres were washed three times in petroleum ether, centrifuging each time at 500r.p.m for 5 minutes
5	The microspheres were vacuum filtered and allowed to air dry. They were then resuspended in 1ml of ice cold PBS and freeze dried for 12 hours before being stored at 4°C
6	Prior to use, the microspheres were resuspended in PBS/0.5% Tween 80, sonicated in a waterbath for 3 minutes and then filtered through a 63μm metal sieve

Table 2.3 Final method for the production of EO9-loaded albumin microspheres.

2.5 DISCUSSION

The aim of this chapter was to develop a method for the successful encapsulation of EO9 into human albumin microspheres. The method previously developed in our laboratory for the encapsulation of MMC was used initially, not only because it has been well characterised, but also because it avoids the use of heat or strong chemicals for cross-linkage (Allan et al 1993). This is likely to reduce the risk of drug inactivation as a result of the encapsulation process which is important when chemically unstable drugs such as EO9 are being considered for encapsulation.

When this method (Allan et al 1993) was adopted with substitution of MMC by EO9, it was found to be sub-optimal and modifications were required in order to successfully encapsulate the EO9. In retrospect, this was perhaps not an unexpected outcome because EO9, although a bioreductive drug and pharmacologically similar to MMC, has different chemical properties. Furthermore, the method of MMC encapsulation was itself derived from the original method used to encapsulate the anthracyline, Adriamycin, which suggested that some modification would also be required for EO9 (Wilmott et al 1988).

Trypsin is a very proteolytic enzyme which digests albumin into well characterised fragments. Incubating the albumin microspheres with trypsin *in vitro* will therefore provide the best possible environment for microsphere digestion to occur. Failure of trypsin to digest the microsphere particles under these conditions suggests that the particles will be poorly biodegradable *in vivo*. Trypsin digestion is also useful as a means of preparing microspheres in a form which is amenable to analysis by HPLC. The use of HPLC in this situation will not only allow the chemical integrity of the drug to be assessed, but it will potentially also allow the total amount of drug which has been incorporated into the

microspheres to be determined. Incomplete digestion of the microsphere system is likely to result in inaccurate analysis of total drug content.

The inability of trypsin to digest the original microsphere particles was thought to be due to the high concentration of gluteraldehyde (12.5%) used in the cross-linking step. This was confirmed in subsequent experiments which assessed the effect of varying concentrations of gluteraldehyde on microsphere production and digestion. The highest concentration of gluteraldehyde did produce the highest microsphere yield, but failed to allow digestion of the microspheres by trypsin. Reducing the gluteraldehyde concentration resulted in fewer microspheres being produced, but allowed digestion to occur, thus making HPLC analysis possible (Table 2.1). The final concentration of gluteraldehyde which was chosen (6.25%) for future experiments was therefore a comprise between these 2 factors. It produced the highest yield of microspheres which underwent digestion in the presence of trypsin, thus allowing potential information on total drug content to be determined.

Isopropanol is important as one of the washing steps during microsphere preparation because it is miscible with both water and solvents and therefore provides a useful intermediate step between the solvent and water-based washes. The problem with this step was that resuspending the microspheres in the isopropanol turned the isopropanol "pink" which suggested that there was rapid release of the EO9 from the microspheres into the isopropanol. This had not been a significant problem with the MMC-loaded microspheres, presumably because MMC is more water soluble than EO9. HPLC analysis of the isopropanol wash confirmed this observation (Figure 2.4).

The isopropanol wash was removed from the method, which benefited the microspheres in terms of reducing the amount of drug lost during preparation (37% total EO9), but resulted in significant problems in microsphere resuspension prior to mechanical filtering. It proved

to be impossible to filter the microspheres when they had been resuspended in either petroleum ether or PBS. This aggregation of particles may, in part, be caused by the presence of residual aromatic hydrocarbon chains from the petroleum ether on the surface of the microspheres. Alternative methods of resuspending the microspheres were investigated.

Freeze drying was one of the first methods to be studied because freezing the particles in the presence of a vacuum may allow evaporation of the residual aromatic hydrocarbon chains present on the microsphere structure. It was easy to carry out and did not appear to have any adverse effect on either the EO9 (Figures 2.6, 2.7 and 2.8) or the microspheres (Figure 2.9). In addition to improving particle resuspension, it also proved to be a very useful means of preparing the microspheres for storage. It was possible to store microspheres prepared in this way for at least 6 weeks at 4°C without any loss of EO9 activity.

Despite the improvement produced as a result of the freeze drying process, the microspheres still had a greater tendency to aggregate than when the isopropanol wash was included in microsphere preparation. Various resuspending agents were compared and the effect of ultrasonication compared on the most efficient agents. The most effective reconstitution was produced by the combination of resuspension in detergent in the form of PBS/0.5% Tween 80 (presumably because the Tween component helps to emulsify any residual hydrocarbon chains from the pet ether which may still be present on the surface of the microspheres) and 3 minutes of vigorous mechanical resuspension using ultrasonication (Table 2.2 and Figures 2.10 & 2.11).

As a result of these experiments (summarised in Table 2.4), a new method for the successful preparation of the EO9-loaded microspheres has been developed and is outlined in Table 2.3. The characteristics of these new microspheres, which will influence both drug activity and particle disposition, are described in the next chapter.

Problem	*Solution*
Failure of microsphere Digestion using trypsin	Reduce Gluteraldehyde Concentration to 6.25%
Loss of EO9 into Isopropanol wash	Miss out Isopropanol wash
Aggregation of particles following Washing steps	Freeze Drying of particles
Aggregation of particles prior To utilisation	Resuspend in PBS/0.5% Tween 80 combined with ultrasonication

Table 2.4 Summary of steps involved in methods development of EO9 microspheres.

CHAPTER 3

The characterisation of EO9-loaded albumin microspheres

3 Chapter 3

3.1 INTRODUCTION

In Chapter 2 the development of a method for the successful encapsulation of the indoloquinone EO9 into human albumin microspheres was described. The aim of the experiments which are discussed in this chapter is to study some of the characteristics of the EO9-loaded microspheres.

Microsphere characteristics are important because they not only influence the disposition of the particles within the body, but they can also significantly affect the activity of the drug which has been incorporated (Chen et al 1994, Willmott et al 1987B). The microsphere properties which we considered to be important and which will be studied in this chapter can be summarised as follows:

1. Microsphere size.
2. Drug incorporation (drug payload).
3. Drug release rate.
4. Microsphere biodegradability.
5. The percentage of encapsulated drug released from the microspheres.
6. The retention of chemical integrity of the drug during and after the encapsulation process.

There are many inter-dependent factors which exert a significant influence on microsphere characteristics. These include the nature of the matrix material, the method of microsphere preparation, the physico-chemical nature of the drug and the ratio of drug to matrix material. It is not the place of this introduction to review each of these features in detail, as some of these factors are pre-determined, but they are summarised in relation to each of the above characteristics together with appropriate references in Table 3.1.

Microsphere Characteristic	*Influencing Factors*	*References*
Particle Size	Drug loading	Tomlinson et al 1985, Zeng et al 1994A
	Method preparation	Morimoto et al 1985, Natsume et al 1990, Reddy et al 1990, Anderson et al 1991B, Zeng et al 1994B, Oner et al 1993
	Viscosity of drug and solvent phase	Morimoto et al 1985, Reddy et al 1990, Karunakar et al 1994, Torrado et al 1989
	Temperature, pH & concentration of Albumin Solution	Karunakar et al 1994, Reddy et al 1990, Zeng et al 1994A
Drug Loading	Method of preparation	Gupta et al 1989, Gui 1994
	Particle size	Karunakar et al 1994
	Ratio of matrix to drug	Tomlinson et al 1985, Karunakar et al 1994
	Physico-chemical nature of drug	Fillipovic et al 1993
	Matrix material	Chen et al 1992, Ike et al 1991
Release Rate	Matrix material	Ike et al 1991, Cummings et al 1993A, Wang et al 1996
	Particle size	Yapel 1985, Karunakar et al 1994
	Drug payload	Bodmeier et al 1987
	Matrix cross-linkage/stabilisation	Reddy et al 1990, Cummings et al 1993A, Dilova et al 1993, Chuo et al 1996, Morimoto et al 1985, Yapel et al 1985, Kim et al 1986
	Physico-chemical nature of the drug	Karunakar et al 1993, Fillipovic et al 1993
	Ratio of drug to matrix	Kim et al 1986

Table 3.1 **A summary of the factors which can influence microsphere characteristics.**

3.1.1 Microsphere Size

The size of the microsphere particles is important because following administration their final location will, to a large extent, be determined by their size. This is shown in Table 3.2 which relates microsphere size to final destination after intravenous administration (adapted from Tomlinson et al 1983). Thus, in order to be suitable for locoregional administration via the hepatic artery, the particles need to be larger than 12μm so that they will remain trapped within the liver in the first capillary bed they encounter and not pass into the systemic circulation. Size will also influence the particles' biodegradability and the release profile of the encapsulated drug. Passive targeting, which relies on inherent microsphere properties, is also heavily dependent on this particular characteristic.

One of the most important factors in determining the size and the size distribution of the microspheres (in addition to the matrix material which is utilised and the nature of the drug being encapsulated) is the method by which the microspheres are actually prepared (Table 3.1). The emulsification process used in our method produces droplets, the size of which, will determine the size and range of the final particles which are produced. The size of the droplets is likely to be significantly influenced by the stirring speed of the mixer used in the emulsification method.

3.1.2 Drug Incorporation

The amount of drug which can be encapsulated into microspheres is usually expressed as the amount of drug per 100mg of microspheres (w/w). The aim in microsphere preparation is to try to encapsulate as high a drug content as possible in an attempt to maximise drug delivery to the tumour site. This is particularly important because there would appear to be physical limitations to the amount of microspheres which can be administered.

Microsphere Size (μm)	*Destination*
0.05	Spleen and bone marrow
0.1 - 2.0	Cleared from the blood stream by the reticuloendothelial system
2.0 - 12	Trapped in the liver, lung and spleen
>12	Lodge in the first capillary bed encountered (chemoembolisation)

Table 3.2 The relationship between microsphere size and final destination following intravenous administration.

(Adapted from Tomlinson 1983)

A dose of 350mg of blank albumin microspheres given via the hepatic artery was found to cause significant right upper quadrant pain in 85% of the patients treated, making 300mg the recommended maximum dose (Goldberg et al 1988). This clearly limits how much of the encapsulated drug the tumour site can receive and therefore, the higher the drug load, the more favourable the microsphere system is likely to be.

As expected, there are a number of factors which can influence drug loading, many of which are inter-related with other microsphere characteristics. Therefore, altering the drug loading capability of the microsphere system is likely to result in an alteration in other characteristics such as the drug's release pattern and the stability, size and structure of the microsphere particles (Table 3.1).

3.1.3 Drug Release Rate

The rate at which the drug is released from the microsphere system affects the concentration over time profile or exposure of the tumour cells to drug, which in turn influences the drug's overall effect. *In vitro* release of incorporated drug from microspheres is characteristically described as biphasic, with a fast initial release of drug known as the burst effect, followed by a slower second phase. It has been suggested by several groups that the initial burst effect of the drug may be the result of drug held loosely on the surface of the molecule being released and that the sustained release comes from particles which are trapped deeper within the microsphere (Gupta et al 1986, Reddy et al 1990). In the case of doxorubicin, a tri-phasic model has been devised to represent its release profile from albumin microspheres. Firstly, native drug which is superficially located and loosely attached accounts for the initial burst effect which is seen *in vitro*. Secondly, native drug which is strongly, but not covalently bound to the microsphere matrix using forces such as ionic bonds, is slowly released by dissociation and is responsible for the sustained release seen *in vitro*. Thirdly,

there is drug which is covalently bound to the albumin molecule via a molecule of gluteraldehyde. This is only released when the molecule undergoes biodegradation *in vivo*. Increasing the cross-links will therefore result in more drug being physically associated with the matrix material, thus resulting in a reduction in the burst effect and an increase in the drug available for release in the slower second phase (Chen et al 1994).

3.1.4 Microsphere Biodegradability

The biodegradability of the microsphere system influences the *in vivo* stability of the particles in terms of drug release rate as well as affecting the clearance of the microspheres from the body. For microspheres which are made of protein, the rate of biodegradability is a function both of the type of protein used for the matrix material and the incorporated drug, as well as the ratio of protein to drug and the amount of cross-linking agent used. Increasing the cross-linkage will increase the length of time the particles remain intact *in vivo*, which will also influence the release rate of the encapsulated drug (Davis et al 1987).

3.2 MATERIALS

All reagents, equipment and suppliers used are listed in Appendix 1.

3.3 METHODS

3.3.1 Microsphere size measurement

The mean diameter of the microsphere preparations was determined using a Malvern Particle Sizer; Series 2600C with a 63mm focal length and a 14.3 mm beam length. The instrument was calibrated with latex particles of known diameter prior to use. The freeze dried microsphere preparation was prepared and reconstituted using the final method

outlined in Table 2.3. A small amount of filtered suspension was then added to 10ml of degassed PBS and placed in the cell of the particle sizer for analysis. Each sample was analysed 3 times. The sample data recorded included the particle size distribution and the 50% size average. These recordings were determined for both EO9-loaded and blank microspheres manufactured at 3 different Silverson mixer speeds (1600, 2000, 2500r.p.m). There were 4-6 samples analysed within each group and the average result for each group was determined. Statistical analysis was carried out using the unpaired students t-test.

3.3.2 The Assessment of Drug Content in EO9-loaded Albumin Microspheres

3.3.2.1 Trypsin digestion of albumin microspheres

In order to determine the total amount of drug incorporated into the microsphere system, a known weight of microspheres were digested with trypsin as outlined in Chapter 2.3.2.1.b. 100µl of the digest mixture which was obtained from each sample was then subjected to HPLC analysis using the method previously described in Chapter 2.3.1. The EO9 microsphere digest was compared with the blank microsphere digest.

3.3.2.2 Timed trypsin digestion of EO9-loaded albumin microspheres

Preliminary HPLC results (section 3.4.2.1) failed to identify any EO9 in the digest mixture, though the hydrolysis product EO5A was identified. The breakdown and hydrolysis of EO9 could either be the result of the microsphere preparation procedure or the digestion process. In order to determine during which of the two procedures EO9 breakdown and hydrolysis was occurring, a timed trypsin digest was performed. 100µl samples of a known weight of resuspended EO9 microspheres were taken at 0, 30, 100, 150 and 240 minutes during the digestion procedure (section 2.3.2.1b), as well as at 24 hours, filtered if necessary (required during the early part of the procedure due to incomplete digestion) and then subjected to

HPLC analysis as described in 2.3.1. The amount of EO9 present at each time point was determined using previously constructed standard curves. Blank microspheres were compared with EO9-loaded microspheres.

3.3.2.3 Factors affecting trypsin digestion of EO9-loaded albumin microspheres

The previous experiment (for results, see section 3.4.2.2) confirmed that the encapsulated EO9 underwent breakdown and hydrolysis as a result of the trypsin digestion procedure rather than as a result of the encapsulation process. In order to determine which of the factors in the trypsin digest procedure were responsible for the hydrolysis and loss of EO9 the following set of experiments were performed.

3.3.2.3.1 The effect of 37^{0}C temperature on EO9 stability

1mg of EO9 was dissolved in 10ml of phosphate buffered saline containing penicillin and streptomycin (500units/ml) to prevent possible drug degeneration occurring as a result of bacterial overgrowth and placed in an incubator at 37^{0}C. 100µl samples were taken at 0, 2, 4, 6 and 18 hours, diluted 1 in 10 and 100µl of the diluent subjected to HPLC analysis as outlined in 2.3.1. This was compared with an identical sample placed at 4^{0}C over the same time period.

3.3.2.3.2 The effect of gluteraldehyde on EO9 stability

5mg of EO9 was dissolved in 900µl 5mM sodium phosphate buffer containing 0.1% sodium dodecyl sulphate and incubated at 37^{0}C with 100µl of a 6.25% gluteraldehyde solution. 100µl samples were taken at 0, 2, 4, 6 and 18 hours, diluted 1 in 100 in the above buffer and 100µl of the diluent subjected to HPLC analysis as outlined in 2.3.1. This was compared with EO9 incubated under the same conditions without gluteraldehyde.

3.3.2.3.3 The effect of trypsin on EO9 stability

1mg of EO9 was added to 2mls of phosphate buffered saline with penicillin/streptomycin (500units/ml) containing 0.4% trypsin and the sample incubated at 37°C. Samples were taken and processed as outlined above (3.3.2.3.2) and compared with EO9 incubated under the same conditions without trypsin.

3.3.3 In vitro Release of EO9 from Microspheres and Assessment of Drug Loading

In order to generate a release profile of the incorporated EO9 from intact microspheres and to examine its chemical integrity, a continuous "flow-through" system was employed (Cummings and Wilmott 1985). A known weight of freeze dried microspheres were resuspended in 4 mls of PBS/0.5% Tween 80 and immobilised on glass wool (2.0g) in a glass column (20cm x 3cm internal diameter). The column was eluted with phosphate buffered saline, pH 7.4 at a flow rate of 7.0mls per hour using a LKB Broma 2120 Varioperspex Peristaltic pump. Fractions were collected at 30 minute intervals for 6 hours using a LKB 7000 Ultrorac Fraction Collector. HPLC analysis was carried out on a 100µl aliquot from each fraction to detect and quantify EO9 and EO9 metabolites.

The amount of EO9 in each sample was determined using previously constructed standard curves to generate a drug release profile. Blank and drug-loaded microspheres prepared at mixer speeds of 1600, 2000 and 2500r.p.m were analysed and compared with free EO9. Six samples of blank and EO9-loaded microspheres were analysed for each mixer speed and the mean result and standard deviation calculated for each group. Statistical analysis was carried out using the unpaired students t-test.

The entrapment efficiency, calculated as the amount of drug eluted through the column (in milligrams) in comparison to the original drug weight used and expressed as a percentage, was determined for each sample. The mean and standard deviation were again determined for each group.

The mean drug loading for each group was also calculated, being defined as the amount of drug present (mg) per 100mg of freeze dried microspheres.

3.4 RESULTS

3.4.1 Microsphere Size Measurement

As discussed previously, the desired size for microspheres which could be potentially useful for hepatic arterial embolisation is greater than 12μm, so that they will remain trapped within the liver and not pass into the systemic circulation (section 3.1.1, Table 3.2). The median size of the EO9-loaded microspheres manufactured at the lower mixer speed of 1600r.p.m. was 19.9 ± 5.5μm (mean estimate of 50% particle size ± SD). This was significantly larger than the median size of the microspheres produced at the higher speeds of 2000r.p.m. (11.5 ± 0.8μm) and 2500r.p.m. (11 ± 1.6μm) ($p < 0.05$) (Figure 3.1, Table 3.3.). There was no significant difference in size between the particles produced at 2000r.p.m and 2500r.p.m. Both of these speeds (2000 and 2500r.p.m) produced microspheres of which more than 50% were smaller than required and therefore will not be considered further.

The addition of EO9 to the microspheres did not appear to affect microsphere size. No significant difference was detected between the blank and the EO9-loaded microspheres at each of the three mixer speeds.

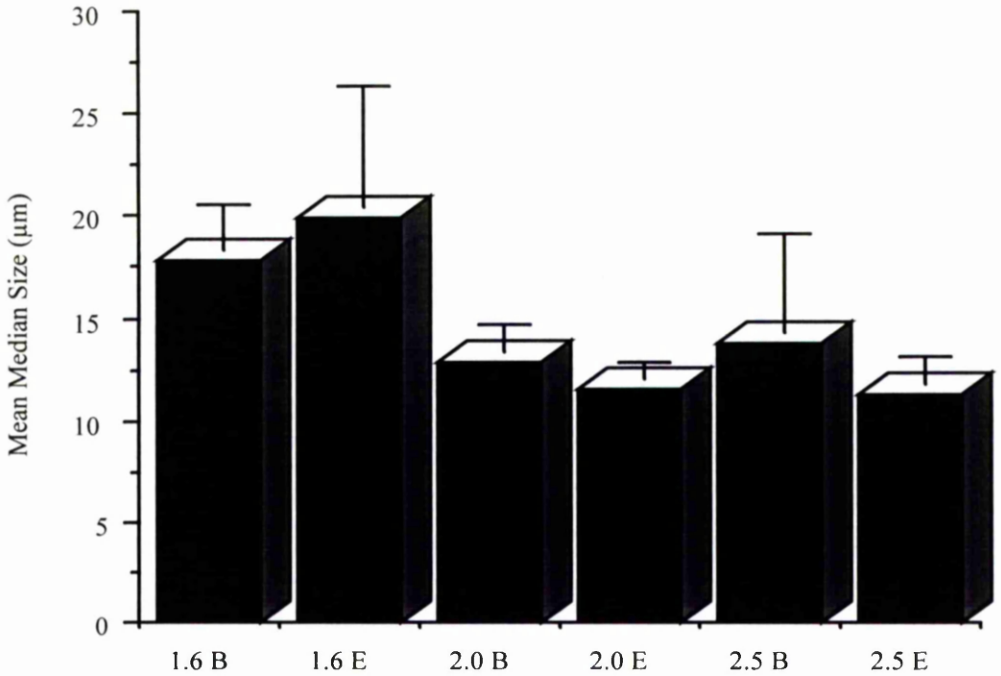


Figure 3.1 **The effect of mixer speed (1600, 2000 and 2500r.p.m) on microsphere size. Comparison of the mean median size (±SD) of blank (B) and EO9-loaded (E) microspheres. The EO9-loaded microspheres produced at 1600r.p.m were significantly larger ($p<0.05$) than the EO9-loaded microspheres produced at 2000 and 2500r.p.m.**

Mixer Speed	Microsphere Type	Mean Median Size ± Standard Deviation (μm)	Mean Population Range (10-90%) ± Standard Deviation (μm)
1600 RPM (n=4)	Blank Microspheres	17.8± 2.2	5.3± 1.8 - 38.2± 7.4
1600 RPM (n=6)	EO9-loaded Microspheres	19.9± 5.9	5.9± 2.1 - 41.9± 12.2
2000 RPM (n=4)	Blank Microspheres	12.8± 1.3	5.0± 2.1 - 25.8± 9.2
2000 RPM (n=4)	EO9-loaded Microspheres	11.5± 0.8	3.8± 0.6 - 20.4± 3.3
2500 RPM (n=4)	Blank Microspheres	14.1± 4.7	4.2± 1.5 - 31.2± 11.5
2500 RPM (n=4)	EO9-loaded Microspheres	10.7± 1.6	3.0± 0.3 - 25.5± 10.1

Table 3.3 **The effect of mixer speed on the size and population range of EO9-loaded and blank albumin microspheres. (The results shown are the mean and standard deviation for each group where n = 4 or 6).**

All three mixer speeds produced a wide range of particle sizes with a biphasic distribution (Figures 3.2 and 3.3). The peak at the smaller particle size appeared similar for all three speeds and is probably accounted for by partial cross-linkage of albumin particles as well as microsphere degradation products.

At 1600r.p.m approximately 70% of microspheres formed were larger than 12μm. Slower mixer speeds might be anticipated to give a greater yield of larger microspheres, but when this was attempted increased aggregation of the microspheres occurred which made them impractical for further use.

3.4.2 The Assessment of Drug Content in EO9-loaded Albumin Microspheres

3.4.2.1 Trypsin digestion of albumin microspheres

Comparison of the chromatograms comparing 16-hour trypsin digests of EO9-loaded microspheres with blank microspheres (Figure 3.4) showed that the digests for each were virtually superimposable, except for a peak on the EO9 microsphere digest at 20.52 minutes. The retention time and spectra of this peak was not that of EO9, but the hydrolysis product EO5A. Hence, there was no evidence of native EO9 in the digest mixture following overnight incubation with trypsin. Repeated experiments confirmed this result which suggested that EO9 was undergoing hydrolysis and breakdown either as a result of the encapsulation process or the digestion procedure. Subsequent experiments were performed in order to clarify the reason for the EO9 breakdown and hydrolysis.

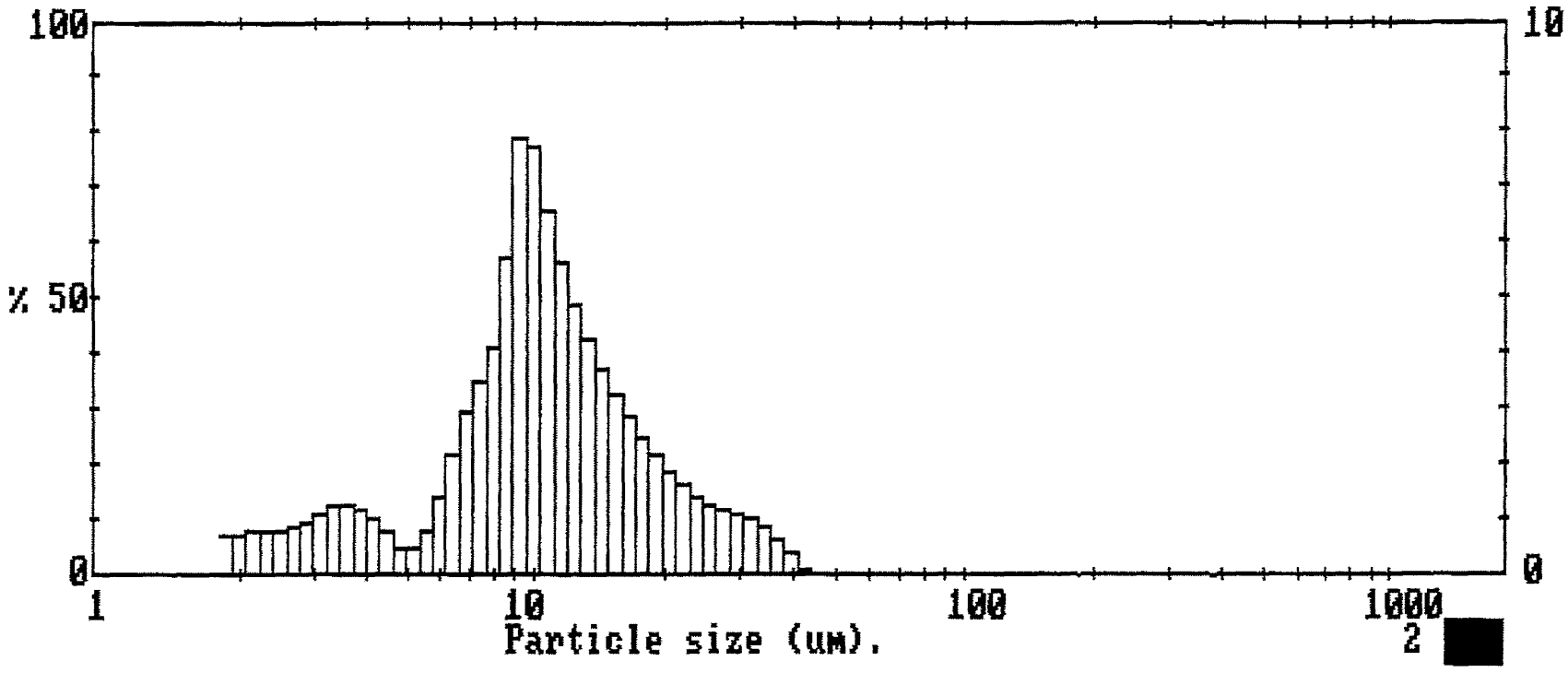


Figure 3.2 The size distribution of blank albumin microspheres manufactured at 2500r.p.m.

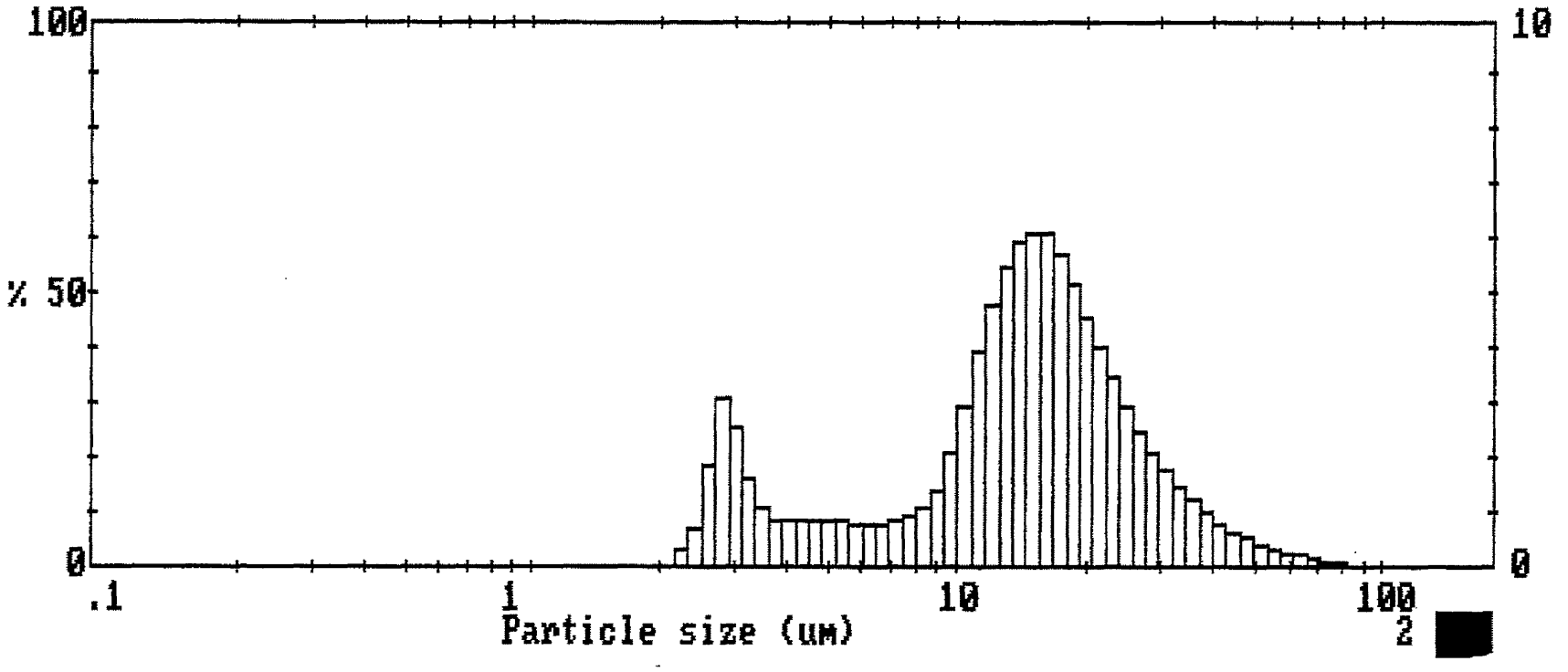


Figure 3.3 The size distribution of blank albumin microspheres manufactured at 1600r.p.m

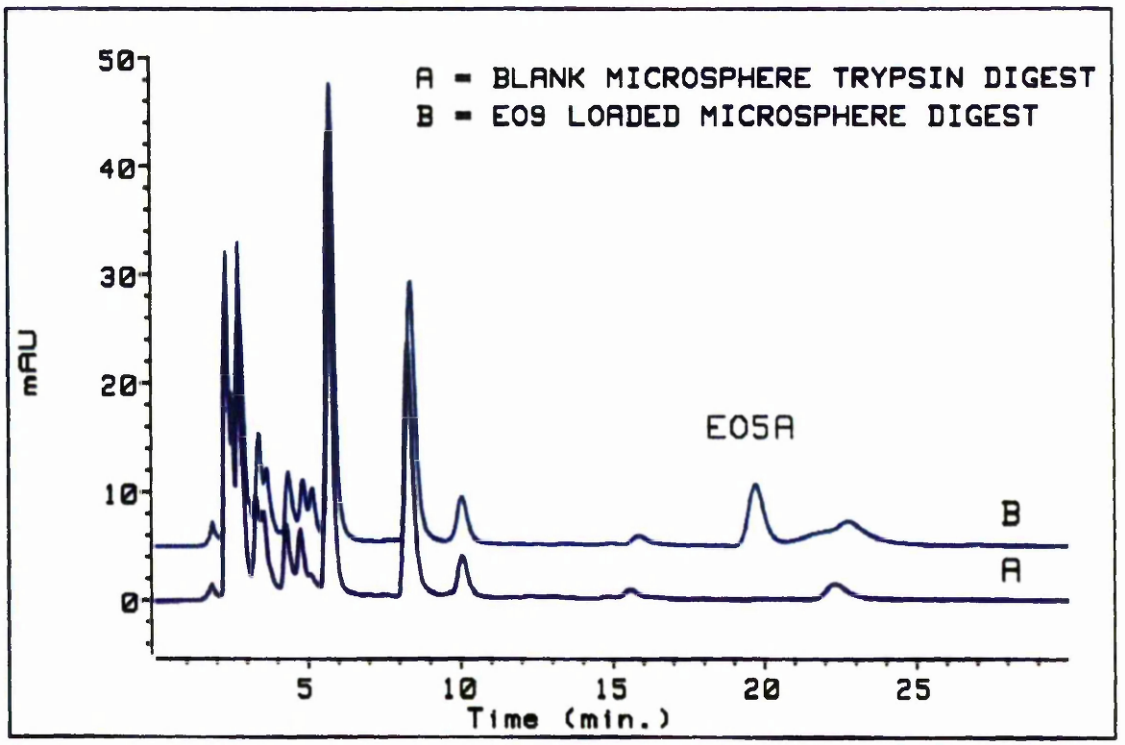


Figure 3.4 **Comparison of blank microsphere (A) and EO9-loaded microsphere (B) digests using HPLC analysis.**

3.4.2.2 Timed trypsin digestion of EO9-loaded albumin microspheres

In order to determine whether intact EO9 was present within the microspheres as digestion began, timed trypsin digests of EO9-loaded microspheres were carried out. The results showed that the amount of EO9 present in the filtrate increased initially, being maximal 30 minutes into the digestion process, but thereafter drug levels began to fall and were absent by 24 hours (Figure 3.5). The fall in the amount of EO9 was associated with the appearance and then increase in the hydrolysis product EO5A (Figure 3.5).

The amount of EO9 present within the digest at each time point was maximal at 30 minutes (Table 3.4). This could be considered a conservative estimate of the total amount of EO9 present in one batch of microspheres (approximately 1.25mg/100mg microspheres) because digestion of microspheres was incomplete at 30 minutes and the formation of the EO5A metabolite was already identified.

This experiment also confirms that free EO9 present in the digest mixture was hydrolysed to EO5A over time.

3.4.2.3 Factors affecting trypsin digestion of EO9-loaded albumin microspheres

The following experiments were carried out in an attempt to determine what factor in the digestion process was likely to be responsible for the hydrolysis and breakdown of the EO9 which had been observed in the previous experiments.

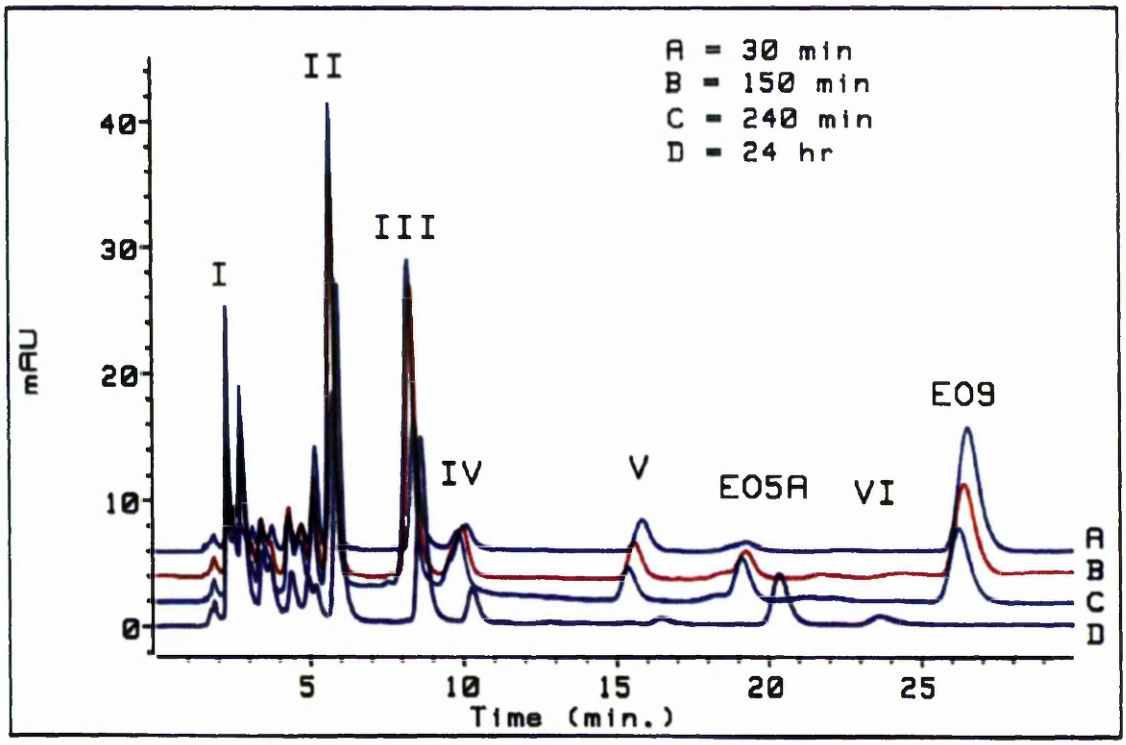


Figure 3.5 **HPLC analysis of timed trypsin digestion of EO9-loaded albumin microspheres which shows the loss of EO9 and the appearance of E05A as digestion proceeds.**

Time following commencement of microsphere digestion using trypsin (minutes)	*Total EO9 (mg/100mg microspheres) determined from the digest mixture using HPLC analysis*
0	0.60mg
30	1.25mg
100	1.12mg
150	0.74mg
240	0.66mg
24 hours	0.0mg

Table 3.4 **Assessment of the total amount of EO9 present (mg) in the EO9-loaded microspheres as determined from HPLC analysis of the trypsin/microsphere digest mixture. (This is a representative sample from duplicate experiments).**

3.4.2.3.1 The effect of temperature (37°C) on EO9 stability

The chromatograms shown in Figure 3.6 confirmed that incubation in digestion solution (minus trypsin) at 37°C resulted in the formation of a significant amount of the hydrolysis product EO5A in comparison to incubation at 4°C, whose chromatogram was similar to that obtained at 0 hours at 37°C. After 18 hours incubation the amount of EO9 had fallen to less than 50% of its original value ($t_{1/2}$ = ~14 hours), with a simultaneous increase in the amount of EO5A present. No other significant degradation products were detected.

3.4.2.3.2 The effect of gluteraldehyde on EO9 stability

When incubated at 37°C in phosphate buffer and 0.1% sodium dodecyl sulphate with added gluteraldehyde, EO9 did undergo hydrolysis to EO5A, but the rate of hydrolysis was not increased compared to incubation at 37°C in phosphate buffer and 0.1% sodium dodecyl sulphate alone (Figure 3.7). Gluteraldehyde therefore did not significantly increase EO9 hydrolysis. No other degradation products were identified and there was no evidence of EO9 complexation in the presence of the gluteraldehyde.

3.4.2.3.3 The effect of trypsin on EO9 stability

The results showed that the amount of EO9 fell significantly during an 18 hour incubation with trypsin at 37°C and that this was again associated with the formation of the hydrolysis product EO5A (Figure 3.8). In comparison to incubation at 37°C alone (outlined in 3.4.2.3.1), in the presence of trypsin, breakdown and hydrolysis appeared to occur more rapidly ($t_{1/2}$ = ~10 hours) with EO5A being present at detectable levels as early as 2 hours compared to 6 hours in the previous experiment.

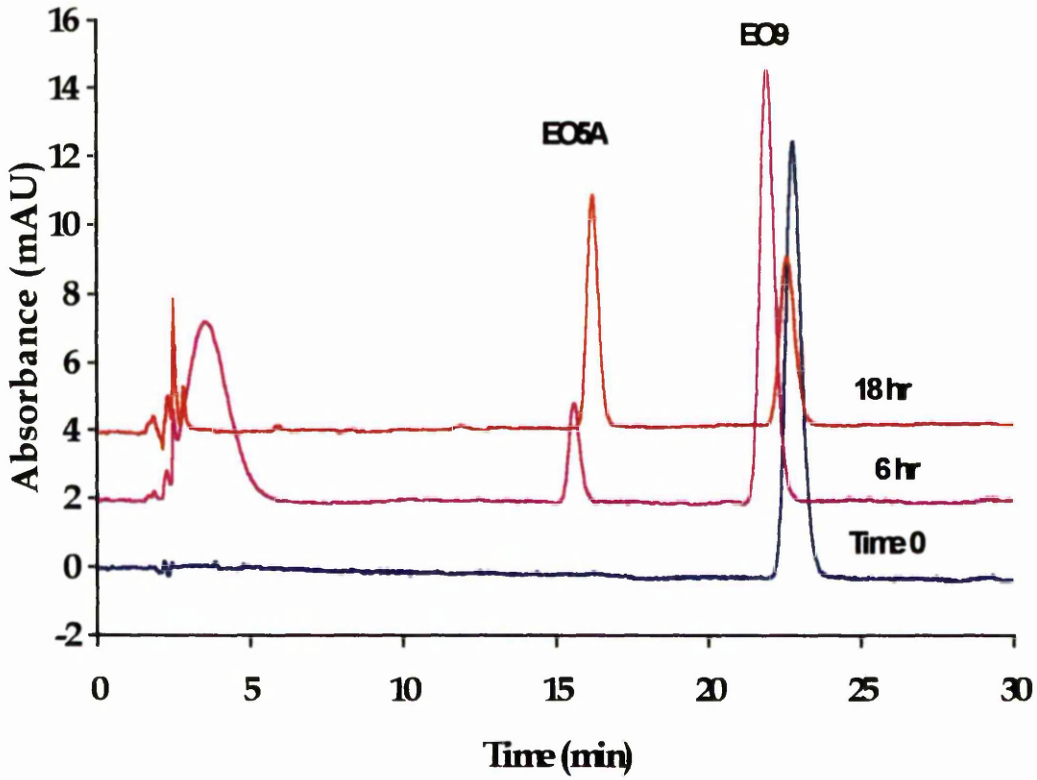


Figure 3.6 **The effect of incubation at 37°C on EO9 stability as assessed by HPLC analysis at 0, 6 and 18 hours.**

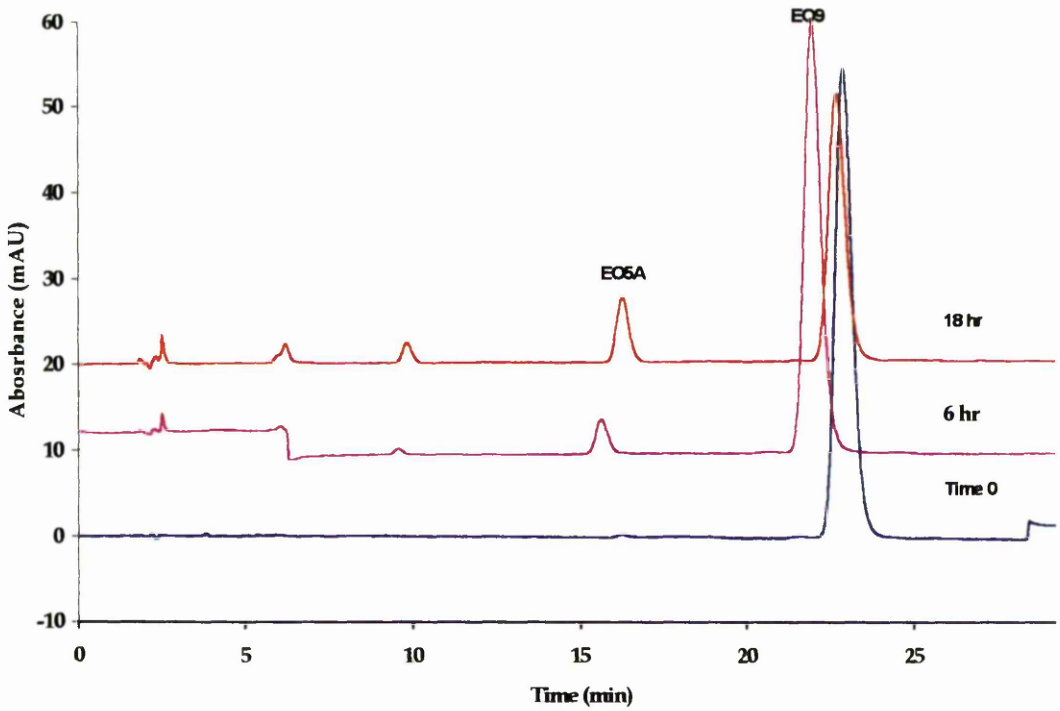


Figure 3.7 The effect of gluteraldehyde on EO9 stability at 37^{0}C as assessed by HPLC analysis at 0, 6 and 18 hours.

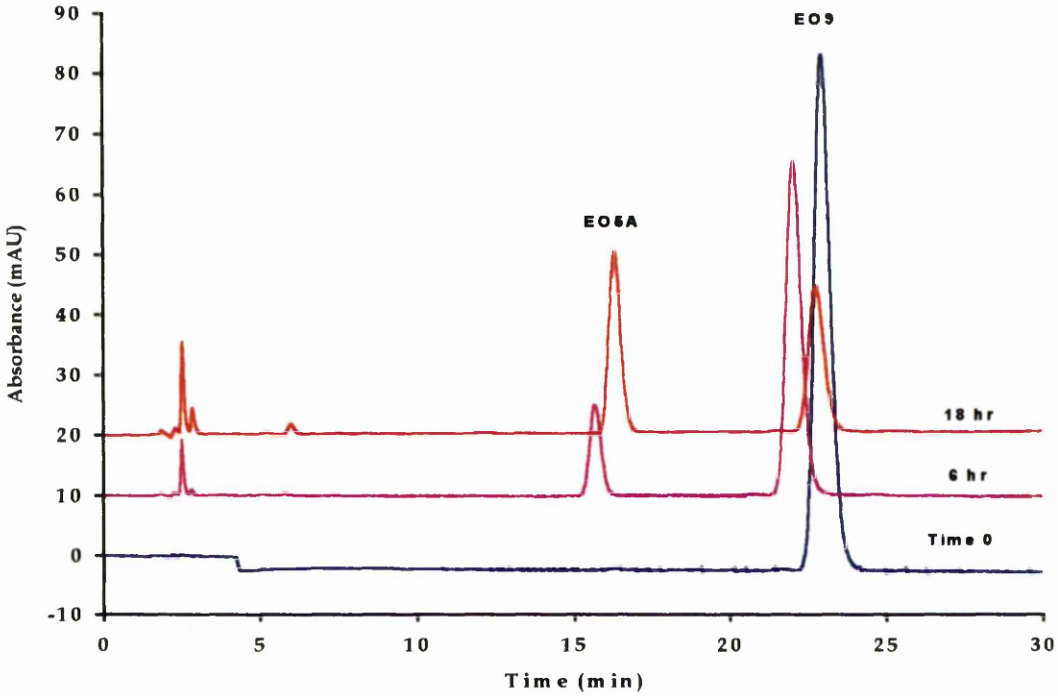


Figure 3.8 **The effect of trypsin on EO9 stability at 37^{0}C as determined by HPLC analysis. Comparison at 0, 6 and 18 hours.**

These experiments demonstrated that trypsin digestion of the EO9-loaded microspheres was unlikely to be useful in accurately determining the drug content of the microspheres because the prolonged exposure at 37°C, in the presence of trypsin, resulted in significant breakdown and hydrolysis of EO9. Therefore, an alternative method of quantification of drug loading was required.

3.4.3 *In vitro* Release of EO9 from Microspheres and Assessment of Drug Loading

The rate of drug release may vary between microspheres of different size and therefore, release profiles for the microspheres produced at 3 different mixer speeds were determined, together with the profile for free EO9 (Figure 3.9). These data showed that there must be rapid release of EO9 from the microspheres on washing, regardless of size, as the speed of transit of EO9 through the column from the microsphere samples was not statistically significantly different from the free drug samples ($p<0.05$). EO9 appeared to have been fully eluted from the column by 6 hours. HPLC analysis of the samples confirmed that the EO9 was chemically intact following it's release from the microspheres (Figure 3.10). EO5A can be identified in low concentration in the samples, but there was no other evidence of significant drug degradation.

Entrapment efficiency (EO9 eluted from column/original weight of EO9 used in microsphere preparation) was similar for each of the mixer speeds; 36 ± 7% for 1600r.p.m, 35 ± 2% for 2000r.p.m, and 34 ± 3% for 2500r.p.m (Table 3.5). Drug loading (EO9 eluted from the column/weight of microspheres applied to column) was also similar for the microspheres manufactured at 1600r.p.m and 2500r.p.m at 1.24 ± 0.2mg/100mg and 1.4 ± 0.12 mg/100mg freeze dried microspheres respectively (Table 3.5).

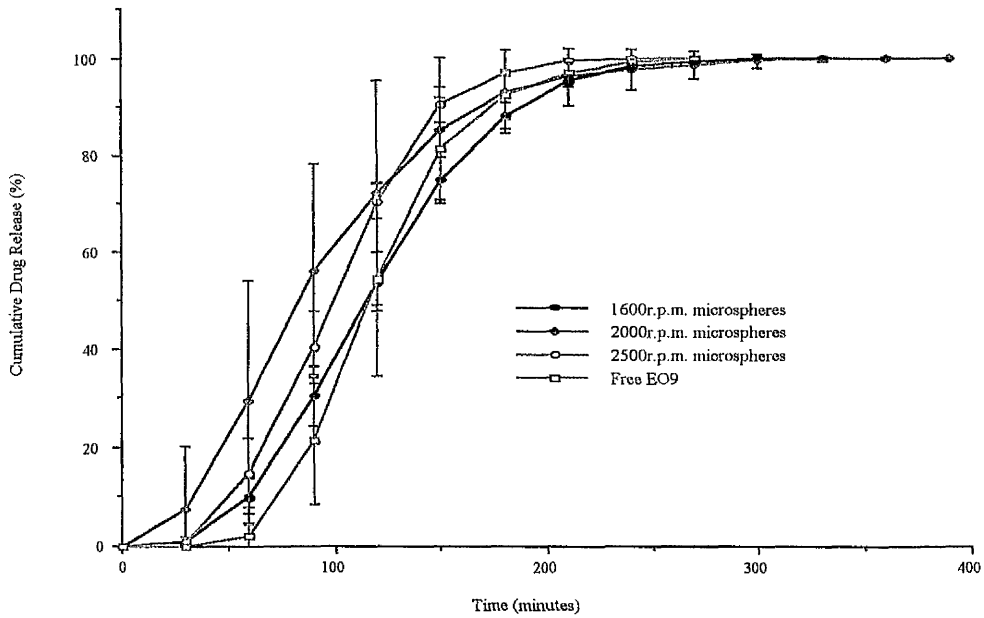


Figure 3.9 **The cumulative release (mean ± SD, n = 6) of EO9 from drug-loaded microspheres manufactured at 1600, 2000 and 2500 r.p.m. Comparison with free EO9.**

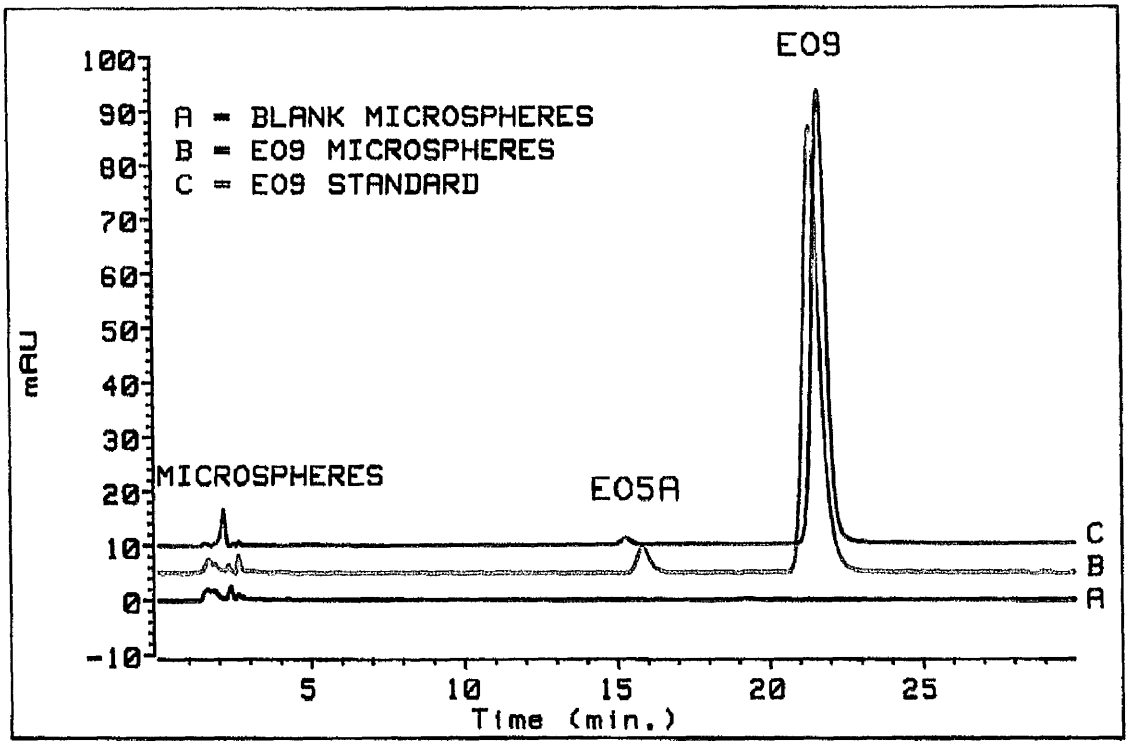


Figure 3.10 HPLC analysis of EO9 released from the drug-loaded microspheres (B). Comparison with blank microspheres (A) and free EO9 (C).

Silverson Mixer Speed (R.P.M)	1600	2000	2500
Entrapment Efficiency (%)	36 ± 7	35 ± 2	34 ± 3
Drug Loading (mg/100mg microspheres)	1.24 ± 0.2	Nd	1.4 ± 0.12

Table 3.5 **The entrapment efficiency (%) and drug loading (mg/100mg microspheres) of EO9-loaded albumin microspheres manufactured at 3 different mixer speeds (mean ± SD, n=6).**

3.5 DISCUSSION

The properties which are considered to be important in any drug delivery system and which require detailed consideration with regard to the EO9-loaded microspheres have already been outlined (see section 3.1) and include the following:

1. Microsphere size.
2. Drug incorporation (drug payload).
3. Drug release rate.
4. Microsphere biodegradability.
5. The percentage of encapsulated drug released from the microspheres.
6. The retention of chemical integrity of the drug during and after encapsulation process.

3.5.1 Microsphere Size

The size and the size distribution of the microspheres is significantly influenced by the method of microsphere production. Thus, the stirring speed of the mixer used in the emulsification process is likely to be important in determining the final size of the particles. This was confirmed in our experiments when microsphere sizes at three different mixer speeds (1600, 2000 and 2500r.p.m) were compared. The results showed that increasing the mixer speed did result in the production of statistically smaller microspheres (Table 3.3, Figures 3.1, 3.2 & 3.3). This agrees with others who have also shown an inverse relationship between mixer speed and microsphere size (Morimoto et al 1985, Reddy et al 1990, Anderson et al 1991B, Zeng et al 1994B).

No statistical difference was detected between the blank and EO9-loaded microspheres for each of the mixer speeds which suggested that the incorporation of this amount of EO9 did not significantly affect particle size (Table 3.3, Figure 3.1). This corresponds with previous

work carried out in our laboratory, looking at MMC-loaded microspheres, which detected no significant size difference between MMC-loaded and blank microspheres (Allan et al 1993). It does however, contradict the work of Tomlinson and Zeng (Tomlinson et al 1985, Zeng et al 1994A) who found that drug incorporation did influence microsphere size. Perhaps this effect is related to the nature or the amount of the drug which is being incorporated rather than a general effect.

The amount of drug incorporated into the microspheres may influence the final particle size, especially if the amount approaches the limit of drug solubility in the disperse (aqueous) phase. In general, increasing drug loading tends to increase the overall size of the particles (Tomlinson et al 1985, Zeng et al 1994A). This was not investigated in our experiments but, if the amount of EO9 was increased in an attempt to improve drug loading, the particles would need to be resized.

As indicated earlier (3.1.1), in order to be suitable for locoregional administration via the hepatic artery, the particles need to be greater than 12μm in diameter so that they will become trapped within the liver in the first capillary bed they encounter. A study in a rabbit model, using 99mTc labelled doxorubicin-loaded albumin microspheres which were 15-40μm in diameter, confirmed that 97% of the microspheres were trapped in the kidney after renal artery injection and that 93% were retained in the liver after hepatic artery injection (Kerr et al 1988, McArdle et al 1988). A further study in Hooded Lister rats which looked at the administration via the gastroduodenal artery of radiolabelled albumin microspheres with mean sizes of 12.5, 25 & 40μm respectively, confirmed that the best tumour to liver ratio was produced by the larger microspheres, athough increasing size resulted in increased difficulty in administration (Anderson et al 1991A). The EO9-loaded microspheres produced at 1600r.p.m have a mean median size of 19.9μm and an 80% population range of 5.9 - 38.2μm with approximately 70% of particles being greater than 12μm in diameter, which

makes them the most suitable of all the EO9-loaded microspheres produced using the present technique, for locoregional administration (Table 3.3).

3.5.2 Drug Incorporation

The amount of drug which can be encapsulated into microspheres (drug payload) is important because of the physical limitations with regard to the maximum amount of microspheres which can be administered (Section 3.1.2, Goldberg et al 1988).

There were problems in attempting to determine the level of drug loading in our microspheres. The original plan had been to utilise a method which had been used previously in our laboratory to determine the drug content of MMC-loaded microspheres (Allan et al 1993). This involved digesting the drug-loaded particles at 37^{0}C with trypsin and then subjecting the digest mixture to HPLC analysis in order to determine the drug content. Unfortunately, when this method was applied to the EO9-loaded microspheres, HPLC analysis failed to detect any EO9 in the digest mixture (Figure 3.4).

Subsequent studies carried out to establish the fate of the encapsulated EO9 showed that although EO9 was initially present at high levels, it underwent hydrolysis and breakdown fairly readily (Figure 3.5 & Table 3.4). This was shown to be due to a combination of temperature (37^{0}C) and the trypsin itself (Figures 3.6 and 3.8).

The lack of stability of EO9 at 37^{0}C meant that this digestion method could not be used to determine the drug payload of the microspheres. The timed digest however, did produce an estimate for the amount of EO9 present (1.25mg/100mg of microspheres). This is likely to be inaccurate both because of incomplete digestion of the particles at this early stage (30 minutes) and the development of the hydrolysis product EO5A which, in the absence of an available standard, is not quantifiable.

An alternative approach to determining the drug payload was to calculate the amount of drug released in each sample obtained from the "flow through" system (see section 3.3.3). This method has the advantage of being carried out at room temperature which will minimise the degradation associated with higher temperatures. It will only give an indication of the amount of free drug present in the system, so it may still therefore be, in real terms, an underestimate because it will fail to detect any EO9 complexed (if such complexes exist) to the albumin matrix which may become available *in vivo* when the microspheres are digested. HPLC analysis of these samples confirmed that the released drug was chemically intact with very little in the way of degradation being observed (Figure 3.10). Therefore the "flow through" system, despite its problems, was felt to be more accurate than the previous method and was used to calculate the drug payload of the various EO9 microspheres which were produced.

The drug payload did not seem to be significantly affected by the mixer speed with loading of 1.24 ± 0.2mg/100mg and 1.4 ± 0.2 mg/100 mg microspheres at speeds of 1600 and 2500r.p.m. respectively (Table 3.5). These results were noted to be similar to those obtained using the previous digestion method (1.25mg/100mg microspheres).

EO9 has not been encapsulated by any other group, therefore direct comparison of the drug loading obtained using our method with others is not possible. As mentioned previously, our result does not take into account any drug which may be complexed to the albumin matrix, which may be important as this has been shown to occur when doxorubicin is encapsulated into albumin microspheres (Cummings et al 1991). The values obtained however are comparable to results obtained by other groups using a similar method. Allan obtained drug loading of 1.8 ± 0.11% (w/w) with an average entrapment efficiency of 20% for MMC-loaded albumin microspheres (Allan et al 1993) and Chen obtained drug loading of 0.9 ± 0.28% (w/w) for doxorubicin-loaded albumin microspheres (Chen et al 1988).

MMC-loaded albumin microspheres prepared using a heat denaturation method produced microspheres of 45 ± 8μm with an increased drug loading of approximately 5% (Fugimoto et al 1985A & 1985B). Other MMC albumin microspheres formed by various heat denaturation methods also lie within the slightly higher range of 1.7 - 4.6% (Natsume et al 1990). Although the drug loading of these MMC microspheres is higher, *in vitro* studies carried out to determine drug release using dialysis tubing and sonication showed that only 10% of the incorporated drug was actually released in the first 24 hours and only 20% of the total incorporated drug was released in the first 3 days (Fugimoto et al 1985A). The *in vivo* picture obviously may be slightly different with drug being released as the microspheres are biodegraded although studies using these microspheres have shown that they remain intact within the liver parenchyma up to 2 weeks following intrahepatic administration (Fugimoto et al 1985A & 1985B). This would imply that the actual amount of drug released is small and occurs over a prolonged time period. It should also be remembered that this method of microsphere preparation results in significant levels of drug inactivation (Mehta et al 1988).

Thus, although the level of drug loading calculated for the EO9 microspheres is low when compared to other drug-loaded microspheres, it does represent the amount of chemically intact free drug which is actually released from the microspheres.

As mentioned previously, the payload is important because it will determine the maximum amount of drug which can be made available to the tumour due to the physical limits on the amount of microspheres which can be administered (300mg is the maximum recommended dose (Goldberg et al 1988)). Thus, if drug loading is in the region of 1-1.5%, it means the maximum amount of drug which can be given is 3-4.5mg. This is exemplified by doxorubicin-loaded albumin microspheres which have a payload of about 1%, resulting in the maximum administration of 3mg of doxorubicin, a dose which is 20-40 times less than the normal systemic dose (Kerr et al 1991). The drug payload may be less important

however if the drug disposition, as a result of the encapsulation process, is altered in favour of the microsphere system. Studies have shown that encapsulated doxorubicin undergoes altered metabolism due to the stimulation of quinone reduction which is probably a result of the hypoxia induced by the microsphere system (Willmott et al 1987A, Cummings et al 1992A & 1992B). Unfortunately this has a negative effect on doxorubicin activity but, for bioreductive drugs such as EO9, this effect may be beneficial and may result in increased activity despite lower levels of the drug being present.

In our method, because of time constraints, we only studied the drug loading obtained when one weight of EO9 (5mg) was used in the preparation of the microspheres. It would be useful to know, particularly if the microspheres have antitumour activity, whether the drug payload could be increased if the amount of EO9 used in the initial stages was increased. This would mean more EO9 could be administered for the same weight of microspheres. Re-characteristion of the microspheres in terms of particle size and drug release rate as well as the level of drug loading would be required.

3.5.3 Drug Release Rate

The release of EO9 from the loaded microspheres was also assessed using the "flow through" system outlined in section 3.3.3. It has two main advantages over the normal dialysis method used by others: it offers haemodynamic features which are closer to the physiological situation in the body and it generates a differential release profile unlike conventional methods, thus revealing the intrinsic release characteristics of the sustained release system.

Results from this study are similar for the microspheres manufactured at the three different speeds. There is rapid drug release from the microspheres (Figure 3.9) with no significant

difference between the microsphere samples and the free EO9 samples. The release is therefore presumably predominantly a "burst" effect, which suggests that most of the drug is present at or near the surface of the microsphere and that there is little in the way of any binding to the matrix material.

By comparison with other microsphere systems, the EO9 appears to be released more quickly from our microspheres. A similar system used by Allan for MMC-loaded microspheres showed that 60% of the encapsulated drug was released in the first 3 hours as an initial "burst" effect, followed by sustained release of the remaining drug over the next 17 hours (Allan et al 1993). The difference seen may be due to the different physico-chemical natures of the 2 drugs combined with the fact that less gluteraldehyde was utilised in the preparation of the EO9-loaded microspheres.

In the manufacture of MMC microspheres using heat denaturation methods, only 20% of the encapsulated drug was actually recoverable and this was shown to be slowly released *in vivo* in comparison to administration of free drug (Fugimoto et al 1985A). MMC is more water soluble than doxorubicin and has 3 chemically reactive groups which are unstable at acid pH. The high level of drug degradation which occurs using this method of manufacture (Mehta et al 1988) may account for the low level of drug which was recovered.

HPLC analysis of the samples which were obtained confirmed that the EO9 which was released from the microspheres was chemically intact and that it remained so until release was complete. This compares well with MMC-loaded microspheres where 90% of the released drug released was chemically intact, with the remaining 10% accounted for as the *cis*- and *trans*-hydroxy metabolites of MMC (Allan et al 1993).

EO9 is a drug whose mechanism of action is thought to involve cross-linking of DNA, single strand breaks and the generation of reactive species capable of causing cellular damage (Bailey et al 1994, Walton et al 1991). Taking this proposed mode of action into account, the best way to utilise EO9 is to maximise the peak concentration of the drug rather than aim for prolonged delivery of a low dose of the drug as this is the method most likely to result in maximum cell damage. Prolonged release of small amounts of drug is unlikely to achieve the same response because exposure of the cell to inadequate levels of the drug is likely to occur, particularly in view of the unstable nature of EO9. This is in contrast to doxorubicin, whose mode of activity involves the inhibition of the enzyme Topoisomerase II. Continuous exposure of the cell to low doses of this drug is more likely to increase its activity because it will result in prolonged enzyme inhibition.

The EO9 microspheres therefore appear to release the drug in a manner which is more appropriate for the drugs' proposed mechanism of action and are more likely to produce an antitumour effect than if the drug was released in lower doses over a prolonged time period. This would appear to be confirmed in the recent clinical trials which compared weekly schedules with higher dose, 3 weekly schedules of EO9. The 3 weekly schedule had a significantly higher level of stable disease, than the weekly schedule, although it was associated with an increase in systemic toxicity (Pavlidis et al 1996).

3.5.4 Microsphere Biodegradabililty

The length of time the microspheres remain chemoembolised could potentially affect the drug release pattern, especially if part of the drug load is covalently attached to the matrix material, because this will be released as the microspheres are broken down. This is known to occur with doxorubicin-loaded albumin microspheres. Initial studies using these microspheres suggested that they had 5 times the level of activity of free drug and that

active drug metabolism was still being recorded 1 week after their administration. Free drug given with blank microspheres did not have this effect. The use of radiolabelled doxorubicin revealed that it was the covalently bound fraction of the drug within the microspheres which was responsible for their sustained release properties. This fraction was slowly being released from the microspheres as they underwent biodegradation. The effect was shown to be dependent both on the amount of cross-linkage and the type of matrix material used (Cummings et al 1991).

It is difficult to determine from our studies whether any EO9 is actually covalently bound to the matrix material, but it is thought that from the nature of the microspheres, any such levels would be low. Regardless of this however, the chemoembolic effect of the microspheres remains important for a number of reasons:

Firstly, chemoembolism, by causing a reduction in blood flow is more likely to retain the drug at the required site of action, which should reduce the risk of systemic overflow and therefore toxicity. This has been shown using doxorubicin-loaded microspheres given into the renal artery. When compared with free drug given by the same route, the drug-loaded microspheres had a reduced peak plasma concentration and AUC and an increase in the local renal drug concentration (Kerr et al 1988). This is likely to be of benefit for EO9 as it means that the local drug concentration will be increased without increasing the systemic drug concentration, which will minimise systemic toxicity.

Secondly, chemoembolism may also induce an hypoxic environment. This has been shown using doxorubicin-loaded albumin microspheres where stimulation of anaerobic quinone reduction has been demonstrated (Willmott et al 1987A, Cummings et al 1992A & 1992B). This type of environment is likely to be ideal for bioreductive drugs such as EO9 where the presence of hypoxia could potentially increase the drugs' antitumour activity.

CHAPTER 4

The antitumour activity of EO9-loaded albumin microspheres

4 Chapter 4

4.1 INTRODUCTION

EO9 has been successfully encapsulated, chemically intact, into human albumin microspheres and these drug-loaded microspheres have now been formally characterised. The aim of this chapter is to assess the antitumour activity of the microspheres.

Several studies have been carried out using EO9 which have demonstrated significant antitumour activity against a wide variety of solid tumour cell lines (Fitzsimmons et al 1996, Phillips et al 1992, Robertson et al 1994, Smitskamp-Wilms et al 1994, Walton et al 1992A). These studies have suggested that in aerobic conditions, cell lines which are high in the activity of the 2 electron reducing enzyme DT-Diaphorase are more sensitive to EO9 than cell lines which have low levels of enzyme activity (Collard et al 1995, Fitzsimmons et al 1996, Plumb et al 1994A, Smitskamp-Wilms et al 1994, Walton et al 1992A).

The role of hypoxia in the activation of EO9 has also been studied by several groups. These studies have shown that cells with low levels of DT-Diaphorase activity demonstrate an increase in sensitivity to EO9 under hypoxic conditions. Cell lines which have high levels of enzyme activity do not demonstrate the same degree of hypoxic enhancement (Bando et al 1995, Plumb et al 1994B, 1994C, Robertson et al 1994).

However, the information obtained from the *in vitro* studies does not seem to translate directly into the *in vivo* situation. Both enzyme activity levels and antitumour activity appear to be different when the cells are grown as xenografts and treated with either intravenous or intraperitoneal EO9. These studies have suggested that the correlation

previously determined *in vitro* between antitumour activity and enzyme activity levels does not hold *in vivo* (Bibby et al 1993A, Collard et al 1995).

In view of these data, it was thought to be important to assess the drug-loaded microspheres in an *in vivo* tumour model rather than *in vitro*, because an *in vivo* method was likely to provide more relevant antitumour data. *In vivo* techniques are also a better way of assessing the activity of any potential drug delivery system.

The antitumour activity of the microspheres was therefore assessed *in vivo* in mouse models bearing four different tumour types. The microspheres were given by direct intratumoural injection rather than by the preferred intra-arterial administration because the size of the mice rendered intra-arterial administration impossible. Their antitumour effect was compared to that of the equivalent amount of free drug which was also administered intratumourally.

4.2 MATERIALS

All reagents, equipment and suppliers used are listed in Appendix 1.

4.2.1 Animal Models

4.2.1.1 Murine Tumour Models

The murine tumour model used was the inbred NMRI mouse and the subcutaneously growing murine adenocarcinomas of the colon, MAC 16 and MAC 26 (original tumours and breeding pairs kindly supplied by Professor J.A. Double and Dr M.C. Bibby, Clinical Oncology Unit, University of Bradford, Bradford, U.K.). The mice were kept under standard laboratory conditions of heating and lighting, fed a standard mouse diet (RM3(E)) and were given access to water ad lib. The MAC tumours were maintained by passaging 1-3 mg of

viable tumour via a trochar needle to animals weighing 25-30g. When tumours reached 0.02-0.1cm^3 (after 2-3 weeks), the animals were either randomised for experimental studies or sacrificed as a source of tumour material. Regular histological analyses of the tumours during their passage were carried out to confirm the consistency of their pathology.

4.2.1.2 Human xenograft tumour models

The xenograft model used was the Nu/nu mouse (originally bred at Imperial Cancer Research Fund (ICRF), London, supplied by Harlan UK Ltd or the ICRF Unit at Clare Hall, London) and the subcutaneously implanted human adenocarcinomas, HT29 and BE, established in-house from their respective cell lines. The BE cell line was kindly supplied by Dr Neil W. Gibson, Pfizer Inc, Grouton, USA via Dr Jane Plumb, CRC Department of Medical Oncology, Glasgow, UK. The Nu/nu mice were housed in Morden isolators, fed on RM3 (E) mouse diet treated by irradiation and given free access to water. The xenografts were maintained by serial subcutaneous passage of 1-3 mg of viable tumour via a trochar needle to animals weighing between 20-25g. When tumours reached 0.02-0.1cm^3 (after 4 weeks for the HT29 and 6 weeks for the BE), the animals were either randomised for experimental studies or sacrificed for collection of tumour material. Regular histological analyses of the tumours during their passage were carried out to confirm their pathology.

4.3 METHODS

4.3.1 Dose Finding Studies to Determine Tumour Sensitivity to EO9

4.3.1.1 Murine tumour model

MAC 16 bearing NMRI mice were randomised into 6 groups, each containing 7 or 8 animals, which were then treated by direct intratumoural injection with 200µl of PBS/0.5% Tween 80 containing 50, 75, 125 or 250µg of free EO9. The two control groups were treated with either 200µl of PBS/0.5% Tween 80 alone or received no treatment. Measurement of the tumours was carried out every second day for a total of 14 days. Tumour volume was determined by caliper measurement and calculated using the formula: 0.5 x length x width^2. The animals were sacrificed once the study was complete.

Antitumour activity or growth delay, was calculated as the percentage increase/decrease in tumour volume compared to day 0:

$$\frac{\text{Tumour volume (cm}^3\text{) Day X}}{\text{Tumour volume (cm}^3\text{) Day 0}} \times 100\%$$

The mean, standard deviation and standard error were then calculated for each group, and results compared using the unpaired students t-test.

4.3.1.2 Xenograft tumour model

HT29-bearing Nu/nu mice were randomised into four groups, each containing seven animals, which were then treated as above with 200µl of PBS/0.5% Tween 80 containing 125µg, 250µg or 500µg of EO9. The control group was treated with PBS/0.5% Tween 80 alone. Measurements and antitumour activity were carried out and determined as above.

4.3.2 Assessment of the Antitumour Activity of EO9-loaded Albumin Microspheres

4.3.2.1 Murine tumour model

MAC 16 and MAC 26 bearing NMRI mice were each randomised into six groups containing 6-8 animals. The groups were treated as outlined in Table 4.1 by direct intratumoural injection with 200μl of PBS/0.5% Tween 80 containing 250μg EO9 (based on data from the dose finding studies (section 4.4.1)), EO9-loaded microspheres containing the equivalent of 125μg, 250μg or 500μg EO9 (the amount of microspheres used calculated from the previously described drug loading studies (chapter 3.4.3)) blank microspheres or PBS/0.5% Tween 80 only. The microspheres were prepared as described in Table 2.3, but each batch was reconstituted in a different amount of PBS/0.5% Tween 80 so that the EO9 dose given did not affect the overall injection volume. Measurements were carried out and antitumour activity determined as described previously in 4.3.1.1. Following completion of the study, selected tumours were removed for histological assessment as outlined below (4.3.3).

For comparative purposes, the antitumour activity of EO9 was also expressed as a percentage of the mean antitumour activity of the drug treated group/mean antitumour activity of the control group (T/C) on day 14 (the time taken for approximately 3-4 tumour doublings (Double et al 1989)). The antitumour activity of 250μg of free EO9 and the equivalent dose of EO9-loaded microspheres were compared. The control groups for the free EO9 and the EO9 microspheres were PBS/0.5% Tween 80 and blank microspheres (resuspended in PBS/0.5% Tween 80) respectively. T/C estimation gives a "point" estimate with no assessment made of the confidence in that estimate and therefore its significance. In an effort to address this, the T/C values were determined using three methods which were then compared.

Control Group	*Free EO9 (μg)*	*EO9-loaded Microspheres (Equivalent free EO9) (μg)*
PBS/0.5% Tween 80		125μg
	250μg	250μg
Blank microspheres		500μg

Table 4.1 **Outline of the treatment and control groups used to determine the antitumour activity of free EO9 and EO9-loaded microspheres when given by direct intratumoural injection in the murine and xenograft models. The injection volume in each group was 200μl and the vehicle was PBS/0.5% Tween 80.**

Method 1

The first method took the mean of the treated group (T) and divided it by the mean of the control group (C) on day 14.

Method 2

In an attempt to try to obtain more information about the variability of the data, the means and standard deviations of each of the treated and control groups were calculated and then used to simulate 10000 normally distributed T values and 10000 normally distributed C values using the Minitab Release 11 programme. T/C values and their confidence intervals were calculated from these. The results obtained for each group were then compared using the unpaired students t-test.

Method 3

In the third method, because there is no correlation between the treated groups and the control groups and therefore no reason to divide any one T by any one C, the percentage tumour volumes from each of the treated group were taken and divided by each of the tumour volumes from the control group. This produced a larger sample of T/C values and a set of data from which the mean, median and standard deviation could be obtained. The data however, were not normally distributed, so the results obtained for each group were compared using the Mann-Whitney test.

4.3.2.2 Xenograft Tumour Model

4.3.2.2.1 BE xenograft

BE-bearing Nu/nu mice were randomised into six groups each containing 5 or 6 animals. Each group was then treated as outlined in Table 4.1. Measurements were carried out and antitumour activity determined as above (4.3.2.1), except that the experiment was continued until day 21 (the time taken for 3-4 tumour doublings) before the animals were sacrificed. Histological analysis was carried out as outlined in 4.3.3. below.

4.3.2.2.2 HT29 xenograft

HT29-bearing Nu/nu mice were randomised into six groups containing 6 or 7 mice and treated as shown in Table 4.1. Measurements were carried out and antitumour activity determined as outlined previously (4.3.2.1). The animals were sacrificed at 21 days. Histological analysis was carried out as outlined in 4.3.3 below.

The experiment was repeated in this tumour type with identical controls, but using lower doses of free EO9 and therefore EO9-loaded microspheres, in an attempt to try to determine whether there was any difference in antitumour activity between the free drug and the microspheres. The doses used were 30μg and 60μg in the second experiment, and 75μg and 100μg of EO9 or the microsphere equivalent in the third experiment. Antitumour activity was measured in these experiments for 30 days, following which, the animals were sacrificed.

4.3.3 Histological Preparation of Tumours Treated with EO9-loaded Microspheres

Tumours were randomly selected from animals treated with the EO9-loaded microspheres at the end of the previous experiments and following removal were fixed in 4% paraformaldehyde solution and then embedded in paraffin wax ready for sectioning. Tumour sections of approximately 3μm thickness were cut, mounted on non-coated glass slides and then stained with Haematoxylin and Eosin. Sections were examined using a Leitz Ortholux 11 microscope equipped with Leitz 10, 25, 40 and 100 objectives. Photographs were taken with a Wild Photoautomat MPS 45 camera and Fujichrome Velvia Daylight film.

4.4 RESULTS

4.4.1 Dose Finding Studies to Determine Tumour Sensitivity to EO9

4.4.1.1 Murine tumour model

This experiment contained two possible control groups: a group which received no treatment and a group which received the control vehicle PBS/0.5% Tween 80. Although some variability in growth activity was seen between the two groups, it was not statistically significant ($p<0.05$) (Figure 4.1). The PBS/0.5% Tween 80 group was chosen as the control for all future experiments because it was the vehicle which was used to resuspend the microspheres prior to their use.

The growth curves using different doses of EO9 are shown in Figure 4.2. They suggest increasing antitumour activity with increasing EO9 dosage.

When compared with the control group, using the students unpaired t-test, there was significant growth delay at all time points in the group treated with 250μg of EO9 ($p<0.005$).

The growth of the tumour in the group treated with 250μg EO9 was also significantly delayed throughout the time course of the experiment when compared with the other treatment groups of 125μg, 75μg and 50μg EO9 ($p<0.05$). No significant difference in growth was detected between the groups treated with 125μg, 75μg and 50μg EO9 and the control group during the experiment.

The dose of 250μg of EO9 was chosen for future experiments because this produced significant growth delay in comparison to the control group.

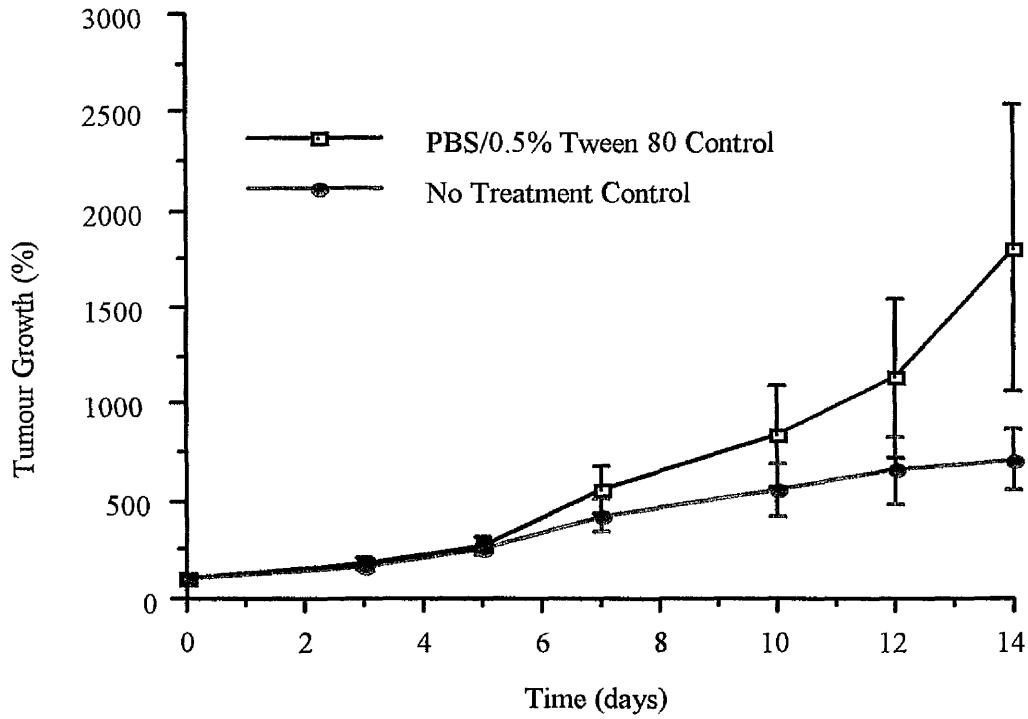


Figure 4.1 **Comparison of control groups treated with either intratumoural PBS/0.5% Tween 80 or no treatment, on the subcutaneously growing MAC 16 tumour in NMRI mice. Each time point denotes the mean ± standard error (SE) for the group.**

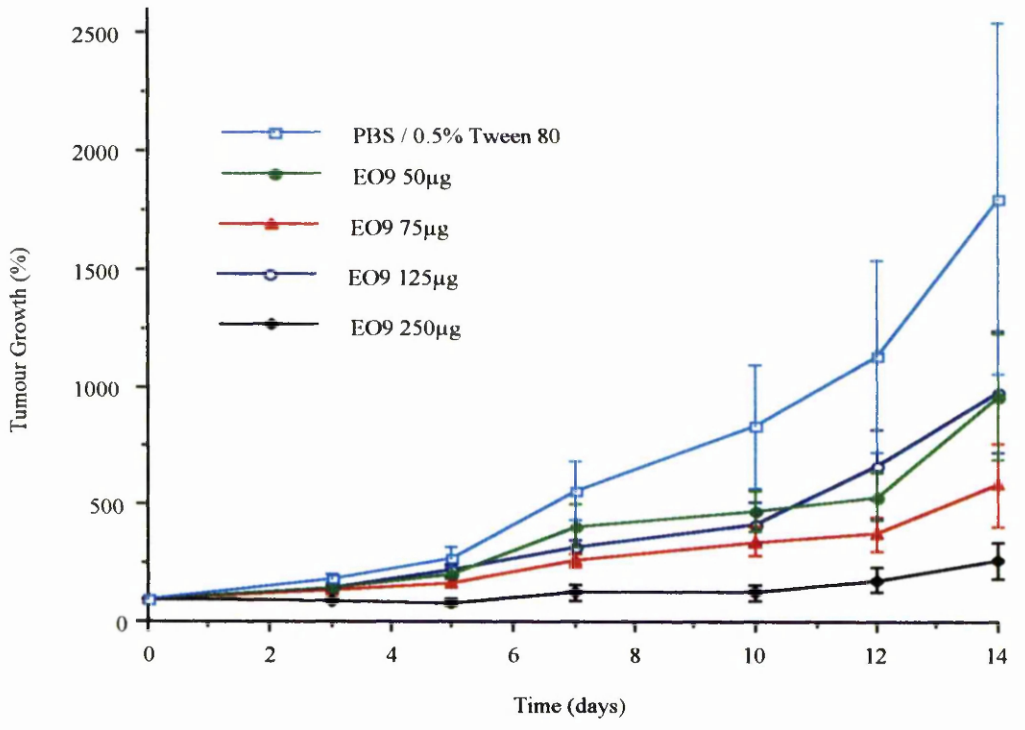


Figure 4.2 **Dose finding study to determine the antitumour activity of EO9 given by direct intratumoural injection, on the subcutaneously growing MAC 16 tumour in NMRI mice. Each time point denotes the mean ± standard error (SE) for the group.**

4.4.1.2 Xenograft tumour model

The growth delay profiles for the different doses of EO9 used in the experiment with the HT29 xenograft are shown in Figure 4.3. This shows that the three doses utilised all had significant antitumour activity in comparison to the control group. The highest dose of EO9 (500μg) produced the most significant results ($p<0.0005$ from day 7 onwards). The 125μg and 250μg doses of EO9 produced similar results which were statistically significant from day 11 onwards ($p<0.05$), when compared to the control group.

The growth delay profile in this experiment was different to that seen in the previous study. The initial increase in size of the treated tumours may be related to the extensive necrosis and formation of non-tumoural scar tissue which occurred following intratumoural injection with EO9 in this tumour type.

The dose of 250μg of free EO9 was again chosen for future experiments because it resulted in a growth delay, which was significantly different from the control group during the experiment. It also allowed for comparison to be made with the murine tumour model.

4.4.2 Assessment of the Antitumour Activity of EO9-loaded Albumin Microspheres

4.4.2.1 Murine tumour model

4.4.2.1.1 MAC 16 tumour

As for all the tumour types, no significant difference in growth delay was seen between the two control groups of PBS/0.5% Tween 80 and the blank microspheres resuspended in PBS/0.5% Tween 80 (Figure 4.4).

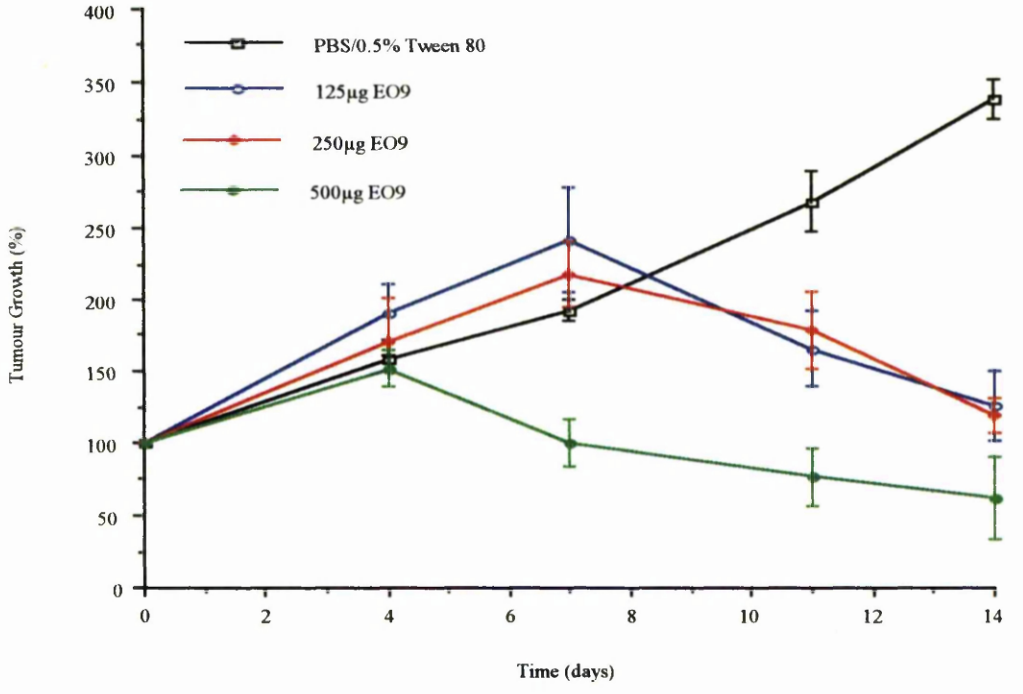


Figure 4.3 **Dose finding study to determine the antitumour activity of EO9 given by direct intratumoural injection, on the subcutaneously growing HT29 tumour in Nu/nu mice. Each time point denotes the mean ± standard error (SE) for the group.**

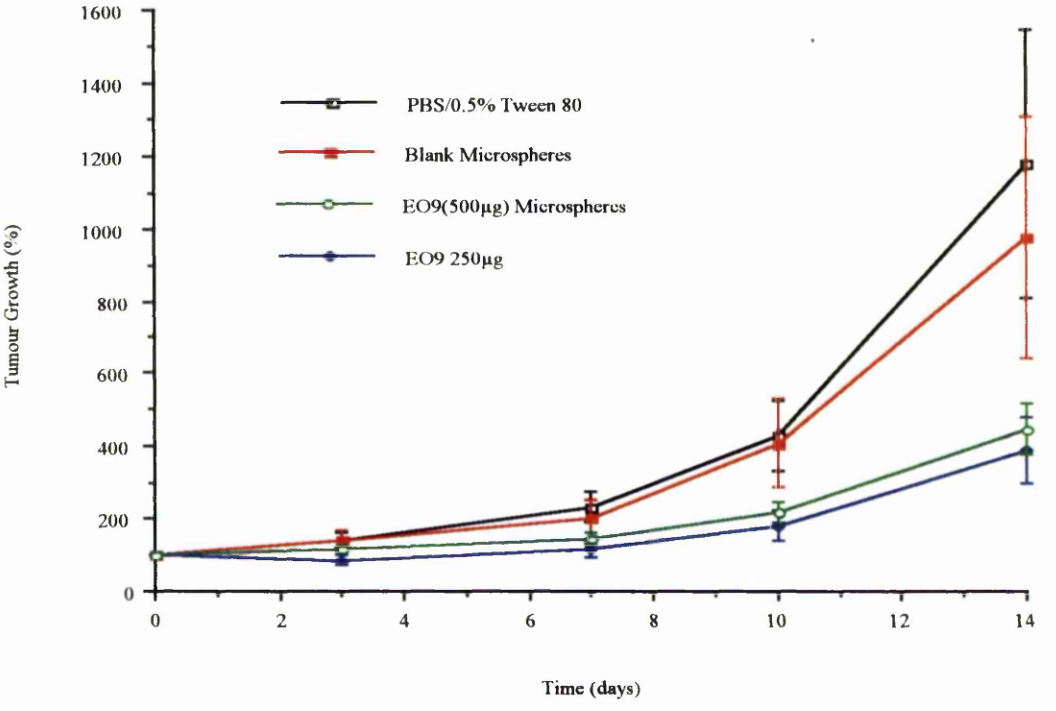


Figure 4.4 **Comparison of the antitumour activity of the control groups (PBS/0.5% Tween 80 and blank microspheres) with free EO9 (250µg) and EO9 (500µg)-loaded microspheres given by direct intratumoural injection, on the subcutaneously growing MAC 16 tumour model in NMRI mice. Each time point denotes the mean ± standard error (SE) for the group.**

In comparison to the control groups significant growth delay was detected in the free EO9 group (250μg) from day 7 onwards ($p<0.05$), which compares well with the initial dose finding study outlined in Section 4.4.1.1. The EO9 microsphere group containing the equivalent of 500μg of EO9, demonstrated significant growth delay from day 10 onwards ($p<0.05$) when compared to the control groups. No significant growth delay was seen in the group treated with the equivalent of 125μg or 250μg EO9 in microsphere form.

There was a significant difference in antitumour activity detected between 250μg of free EO9 and the EO9-loaded microspheres containing 250μg EO9 in favour of the free drug at days 3, 7 & 10 ($p<0.05$) (Figure 4.5). There was no significant difference however, at any time point between the antitumour activity of free EO9 (250μg) and the microsphere group containing the equivalent of 500μg EO9 (Figure 4.4).

These results suggest that the EO9 microspheres do have antitumour activity and that the trend is towards increased activity as the dose of EO9 in the microspheres increases (Figure 4.6). However, in this model system, a higher dose of EO9-loaded microspheres is required to produce an equivalent antitumour effect to free EO9.

4.4.2.1.2 MAC 26 tumour

The growth delay results for the MAC 26 tumour show that in comparison to the control group, free EO9 (250μg) produced antitumour activity which reached statistical significance from day 7 onwards ($p<0.05$) (Figure 4.7). The equivalent dose of EO9 (250μg) in the microsphere group failed to produce any significant antitumour effect. The higher dose of EO9-loaded microspheres (500μg) had statistically significant activity detectable only at the end time point of the study ($p<0.05$).

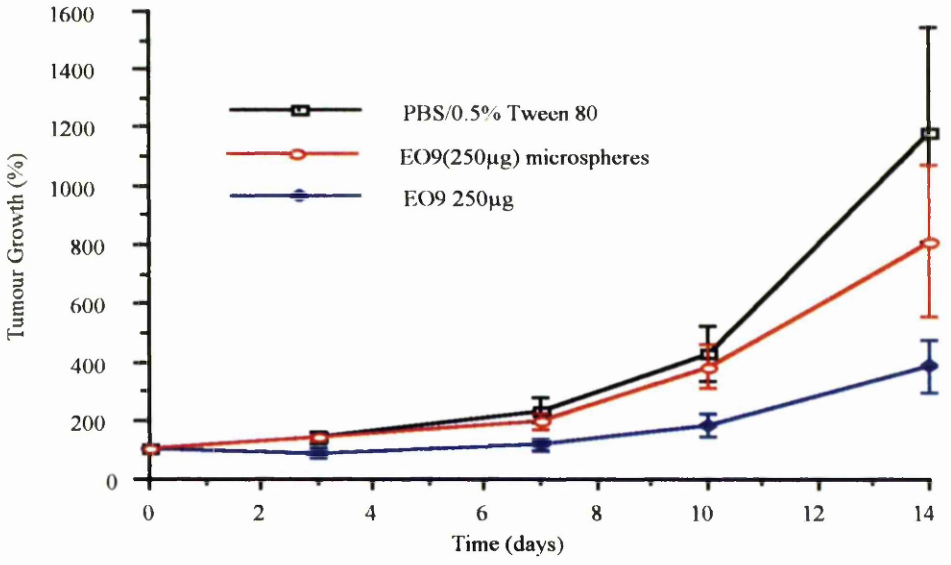


Figure 4.5 **Comparison of antitumour activity of 250µg free EO9 with the equivalent dose of EO9-loaded microspheres given by direct intratumoural injection, on the subcutaneously growing MAC 16 tumour in NMRI mice. Each time point denotes the mean ± standard error (SE) for the group.**

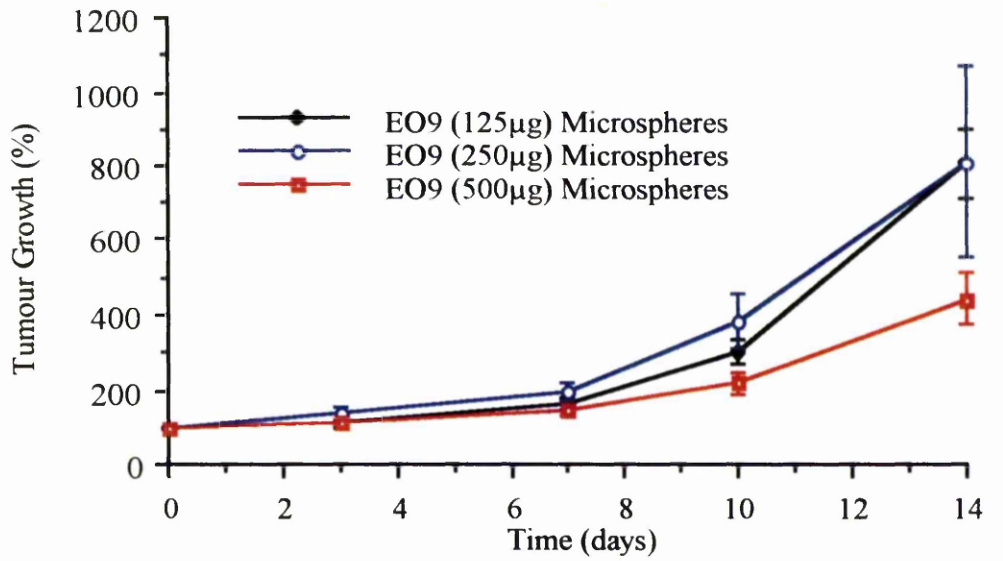


Figure 4.6 **Comparison of the antitumour activity of different doses of EO9 (125µg, 250µg, 500µg) loaded albumin microspheress given by direct intratumoural injection, on the subcutaneously growing MAC 16 tumour in NMRI mice. Each time point denotes the mean ± standard error (SE) for the group.**

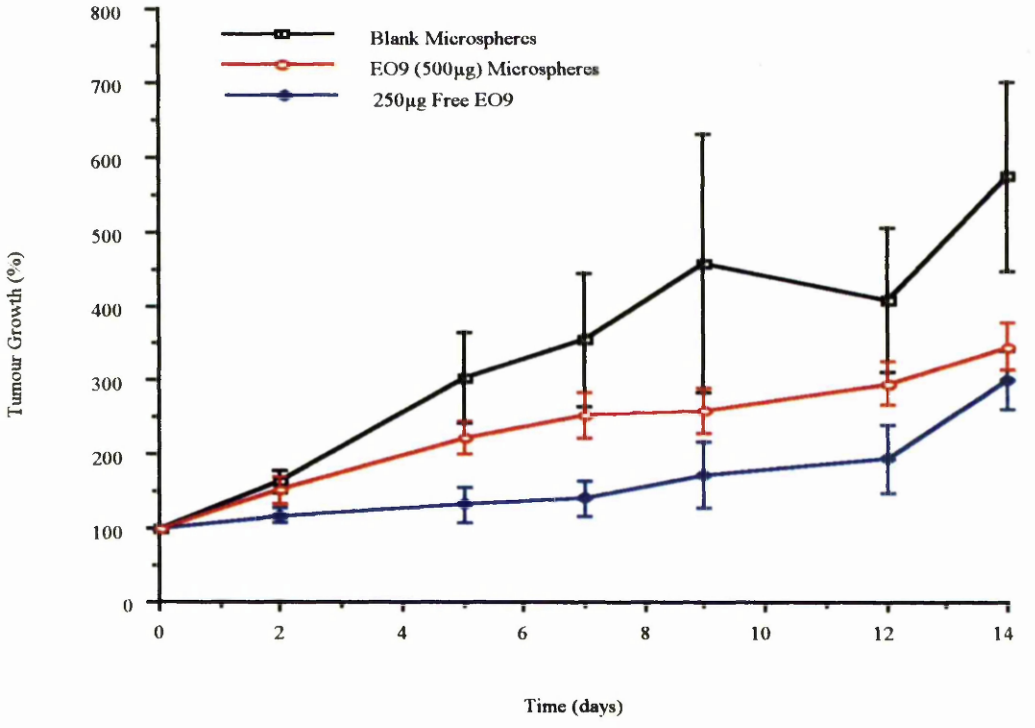


Figure 4.7 **Comparison of the antitumour activity of free EO9 (250µg) with EO9 (500µg) loaded microspheres given by direct intratumoural injection, on the subcutaneously growing MAC 26 tumour in NMRI mice. Each time point denotes the mean ± standard error (SE) for the group.**

These results suggest that the free EO9 has significant antitumour activity in relation to the MAC 26 tumour, but that this activity appears to be reduced when the drug is encapsulated into the microspheres.

4.4.2.2 Xenograft tumour model

4.4.2.2.1 BE xenograft

There was a significant growth delay associated with the administration of free EO9 compared to the control group from day 10 onwards ($p<0.05$) in the BE tumour (Figure 4.8). However, there was no significant difference in antitumour activity between the control group and either the 250μg or the 500μg EO9 microsphere group in this experiment.

These results demonstrate that free EO9 also has significant antitumour activity in the BE tumour. However, when the drug is encapsulated into the microspheres this activity appears to be reduced.

4.4.2.2.2 HT29 xenograft

The initial experiment, which compared 250μg of free EO9 with the equivalent of 250μg and 125μg of EO9-loaded microspheres, showed significant antitumour activity in all three groups in this tumour type when compared with the control groups ($p<0.005$ at days 19 and 21) (Figure 4.9). No significant difference however, could be detected between the free drug and the drug-loaded microsphere groups. Again, as in the dose finding study, there was an initial increase in tumour size in those groups treated with the EO9 due to the necrotic effect of the drug on the tumour.

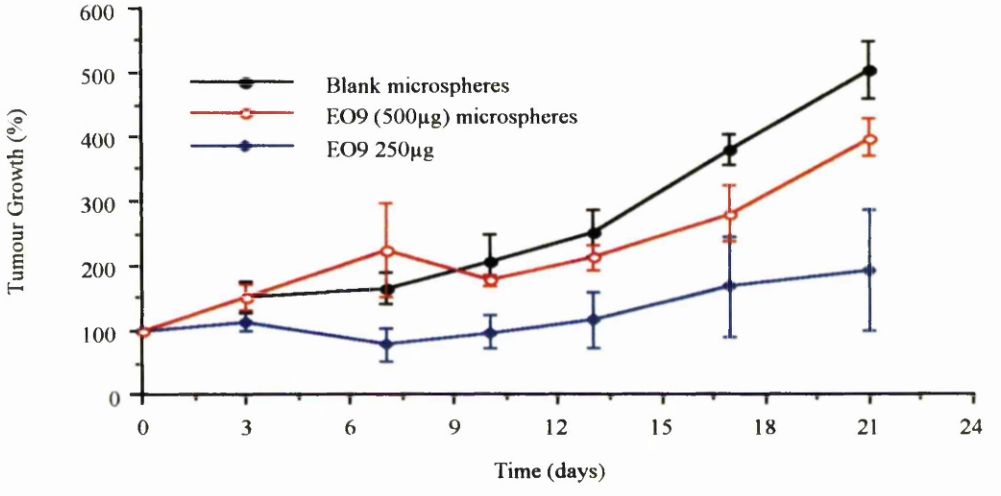


Figure 4.8 **Comparison of the antitumour activity of free EO9 (250µg) with EO9 (500µg) loaded microspheres given by direct intratumoural injection, on the subcutaneously growing BE tumour in Nu/nu mice. Each time point denotes the mean ± standard error (SE) for the group.**

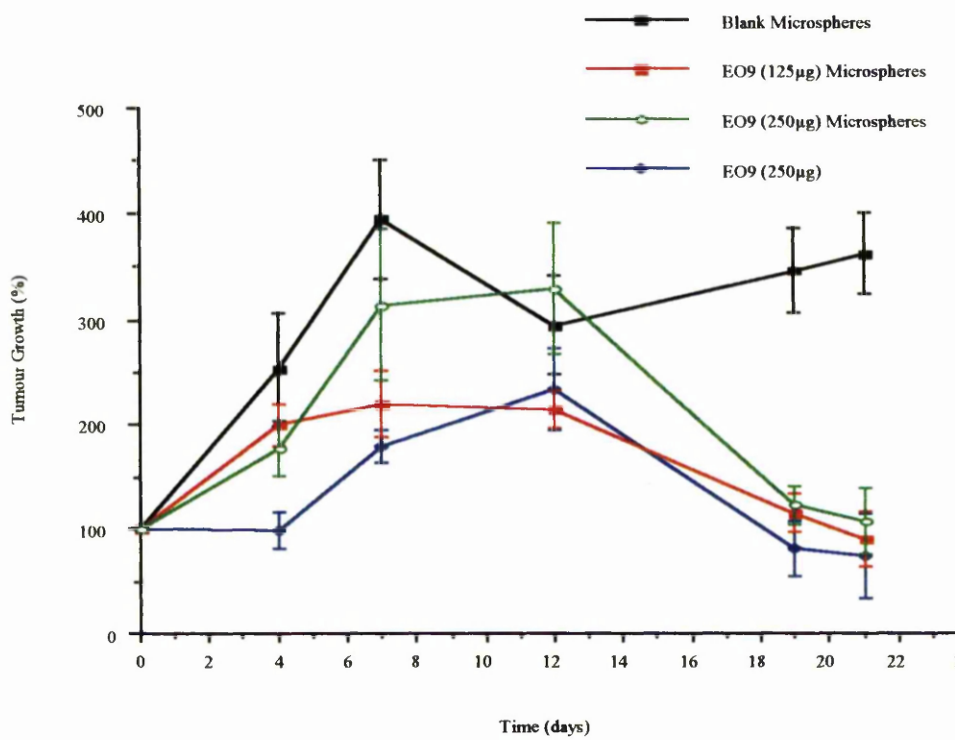


Figure 4.9 **Comparison of the antitumour activity of 250µg of free EO9 with the equivalent dose of 125µg and 250µg in EO9-loaded microspheres given by direct intratumoural injection, on the subcutaneously growing HT29 tumour in Nu/nu mice. Each time point denotes the mean ± standard error (SE) for the group.**

The experiment was therefore repeated using lower doses of both free and microsphere-loaded EO9 in an attempt to try to determine the form in which the drug was most active.

At intratumoural doses of 30μg and 60μg of free EO9 and the equivalent dose of EO9-loaded microspheres, no significant antitumour activity was detected throughout the time course of the experiment when compared to the control groups (Figure 4.10).

At higher doses (75μg and 100μg) of free EO9 and the equivalent dose of EO9-loaded microspheres a definite antitumour effect was again seen (Figure 4.11). When compared to blank microspheres, both the free drug and the EO9-loaded microspheres (75μg and 100μg) demonstrated significant antitumour activity from day 7 onwards ($p<0.05$), with the level of significance increasing towards the end of the study. There was no statistically significant difference between the free drug and its microsphere equivalent. There was also no significant difference in antitumour activity between the two doses (75μg and 100μg) for either free drug or the microsphere equivalent.

These results show that the HT29 tumour is the most sensitive tumour type to EO9. There was no significant difference between the free drug and the equivalent amount given in microspheres in terms of antitumour activity.

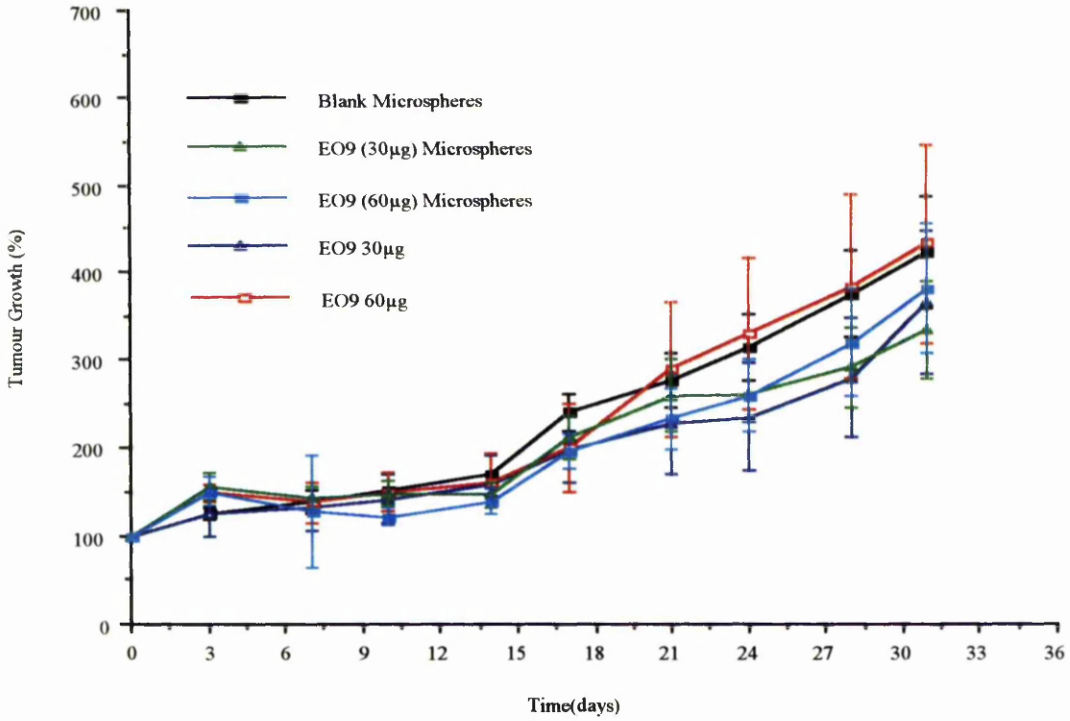


Figure 4.10 **Comparison of the antitumour activity of 30µg and 60µg of free EO9 with the equivalent dose of EO9-loaded microspheres given by direct intratumoural injection, on the subcutaneously growing HT29 tumour model in Nu/nu mice. Each time point denotes the mean ± standard error (SE) for the group.**

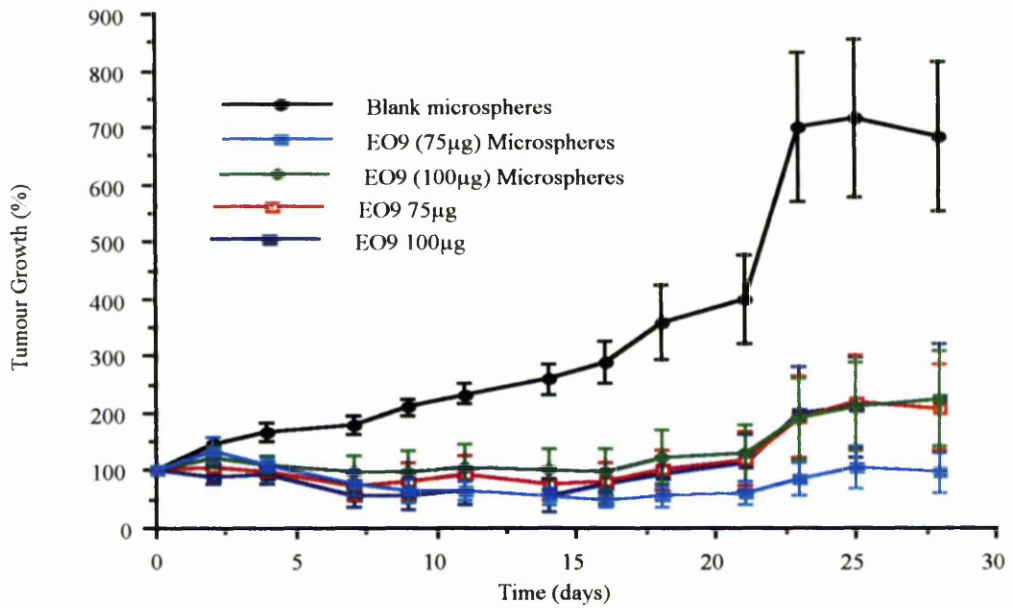


Figure 4.11 **Comparison of the antitumour activity of 75μg and 100μg of free EO9 with the equivalent dose of EO9-loaded microspheres given by direct intratumoural injection, on the subcutaneously growing HT29 tumour model in Nu/nu mice. Each time point denotes the mean ± standard error (SE) for the group.**

4.4.2.3 Assessment of antitumour activity using T/C (Tumour/Control) estimates in each of the tumour types

Method 1

The results of the T/C estimates calculated from the mean percentage tumour volumes of the treated groups (250μg free EO9 and the equivalent dose of EO9-loaded microspheres) divided by the mean percentage tumour volumes of the control groups (PBS/0.5% Tween 80 and blank microspheres) for each of the tumour types are shown in Table 4.2. These results suggested that the order of sensitivity of the tumour types differed between the free drug and the microsphere groups. In the case of the free drug, HT29 appeared to be the most sensitive (15%), followed by the BE tumour (27%) and the MAC 16 tumour (33%), with the MAC 26 tumour being the least sensitive (60%). In the microsphere groups, the HT29 tumour again appeared to be the most sensitive (29%) followed by the MAC 16 tumour (58%) and the MAC 26 tumour (74%) with the BE tumour being the least sensitive (79%). The major difference between the two groups therefore appeared to be the change in the ranking of the BE tumour. In addition, the EO9-loaded microspheres appeared to be less active than the free EO9 in all four tumour types. The main problem with this method however is that the results do not give any indication of the confidence intervals of these estimates, which made it difficult to determine whether the results held any real significance in relation to each other.

Method 2

The results obtained using a method which simulated T/C values from 10000 T and 10000 C results based on the mean and standard deviation of each of the treated and control groups are outlined in Table 4.3. The simulated T and C values were normally distributed and the results from the students t-test show that when comparing the microspheres with free drug in each of the tumour types, there was a significant difference only in the BE tumour ($t=24.09$; $p<0.0001$) in favour of the free drug.

Tumour type	*Antitumour activity (T/C) Free EO9*	*Antitumour activity (T/C) EO9 microspheres*
HT29	15%	29%
BE	27%	79%
MAC 16	33%	58%
MAC 26	60%	74%

Table 4.2 **Comparison of antitumour activity T/C values (tumour/control volumes) for free EO9 and EO9-loaded microspheres in each of the 4 tumour types, calculated by dividing the mean percentage tumour volume of the treated group with the mean percentage tumour volume of the control group.**
(For the free EO9 the control group is PBS/0.5% Tween 80. For the EO9-loaded microspheres the control group is blank microspheres resuspended in PBS/0.5% Tween 80).

Tumour Type	*Free EO9*			*EO9 Microspheres*		
	Mean	*Median*	*SEM*	*Mean*	*Median*	*SEM*
HT29	6	14	17	33.1	29.8	0.4
BE	34.4	27.4	1.9	81	78.5	0.5
MAC 16	30.9	27	8.2	53.9	40.6	17.2
MAC 26	104	58	88	-153	74	242

Table 4.3 **Comparison of antitumour activity (T/C values) of free and EO9-loaded microspheres using the simulated data based on the mean and standard deviation of each of the treated and control groups for each of the tumour types. The number of simulations in each group is 10000.**

(SEM = standard error of the mean)

There was no significant difference between each of the tumour types treated with the free drug using the unpaired students t-test. In comparing the effect of the drug-loaded microspheres, there was a significant difference in activity only between the HT29 and the BE groups in favour of the HT29 group ($t=-68.62$; $p<0.0001$). However, several of the standard deviations for the means which were used for the simulation, particularly the MAC 26 microsphere group, were large, which gave huge distributions for simulated T/C values and in the case of the MAC 26 microsphere group, a negative simulated mean value. Therefore, meaningful statistical analysis of these results was probably not possible.

Method 3

The results outlined in Table 4.4, produced a series of T/C values by dividing each of the treated values by each of the control values in each group. These T/C estimates were not normally distributed so data could not be compared using the students t-test. Therefore the Mann-Whitney test was employed. This showed a significant difference in results when comparing the free drug with the microspheres in both the BE tumour and the MAC 16 tumour, in favour of the free drug ($p<0.05$). Similarly, when comparing the effect of free EO9 between each of the four tumour types, there was significantly increased antitumour activity in the HT29 tumour compared with the MAC 26 tumour ($p<0.05$), the MAC 16 tumour compared to the MAC 26 tumour ($p<0.05$), and the BE tumour compared to the MAC 26 tumour ($p<0.05$). In the case of the EO9-loaded microspheres, there was a significant difference between the HT29 and all the other tumour types ($p<0.05$) in favour of the HT29 tumour, but not between any of the other groups. These results were similar to those obtained using the first method, in that when free EO9 was used the MAC 26 tumour was much less sensitive than the other three tumour types and when the EO9 microspheres were used, HT29 was much more sensitive than the other three tumour types.

Tumour Type	*Free EO9*		*EO9 Microspheres*	
	Median	*Number in Sample*	*Median*	*Number in Sample*
HT29	23.2	24	22.9	49
BE	31.7	20	78.1	20
MAC 16	31.4	35	109.3	35
MAC 26	63.1	15	70.4	15

Table 4.4 **Comparison of the antitumour activity (T/C) values for free EO9 and EO9-loaded microspheres in each of the tumour types obtained by dividing each of the treated values by each of the control values in each group.**

The results obtained with the simulated data were compared with the results outlined in the previous paragraph and plotted in a boxplot which used the median values with 25% percentiles on either side of the median value (Figure 4.12). This plot shows that there is a good similarity between the 'real' data and the simulated data when the median values are compared, except in the MAC 16 microsphere group.

4.4.3 Histological Preparation of Tumours Treated with EO9-loaded Microspheres

The results were similar for each of the 4 tumour types and therefore only the HT29 and BE tumours are shown (Figures 4.13 and 4.14). In comparison to the tumours treated with PBS/0.5% Tween 80, an increase in the number of inflammatory cells and stromal tissue was seen in the tumours treated with the EO9-loaded microspheres which was particularly pronounced in the HT29 tumour. The microspheres were still clearly visible 2 weeks following the intratumoural injection in both tumour types. Some disruption of the normal architecture of the tumour was also seen with loss of tumour cells in the areas of the tumour which were adjacent to the microspheres.

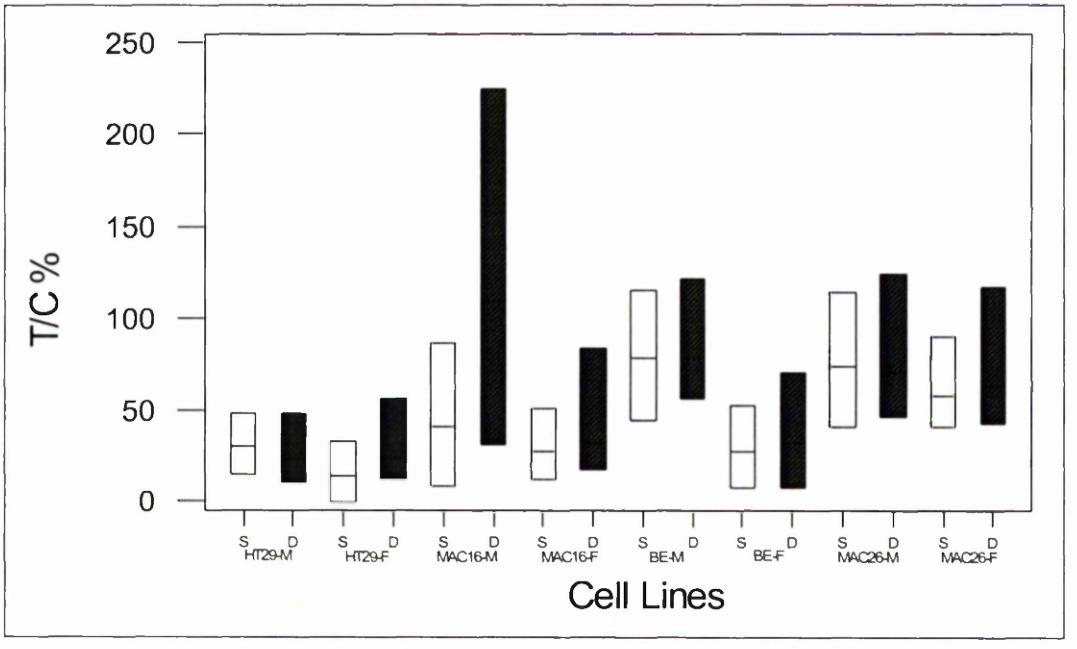
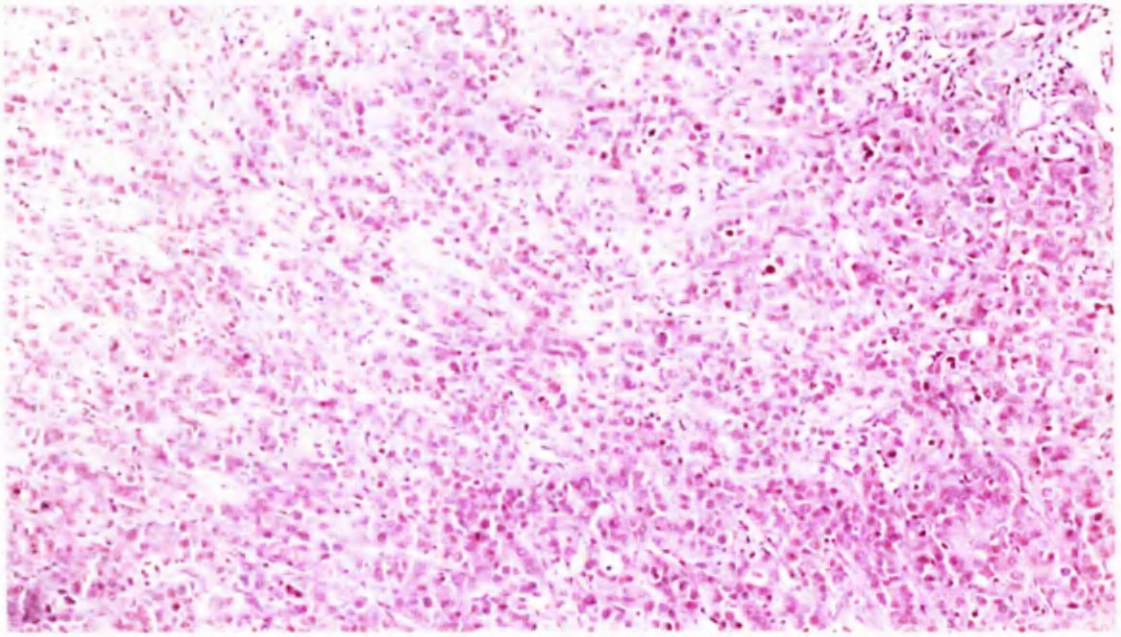


Figure 4.12 **Boxplots of simulated T/C data compared with the "real" data from Method 3 for free EO9 and EO9-loaded microspheres in each of the four tumour types.**
(M = EO9-loaded microspheres, F = free drug, S = simulated data, D = real data)

(A)



(B)

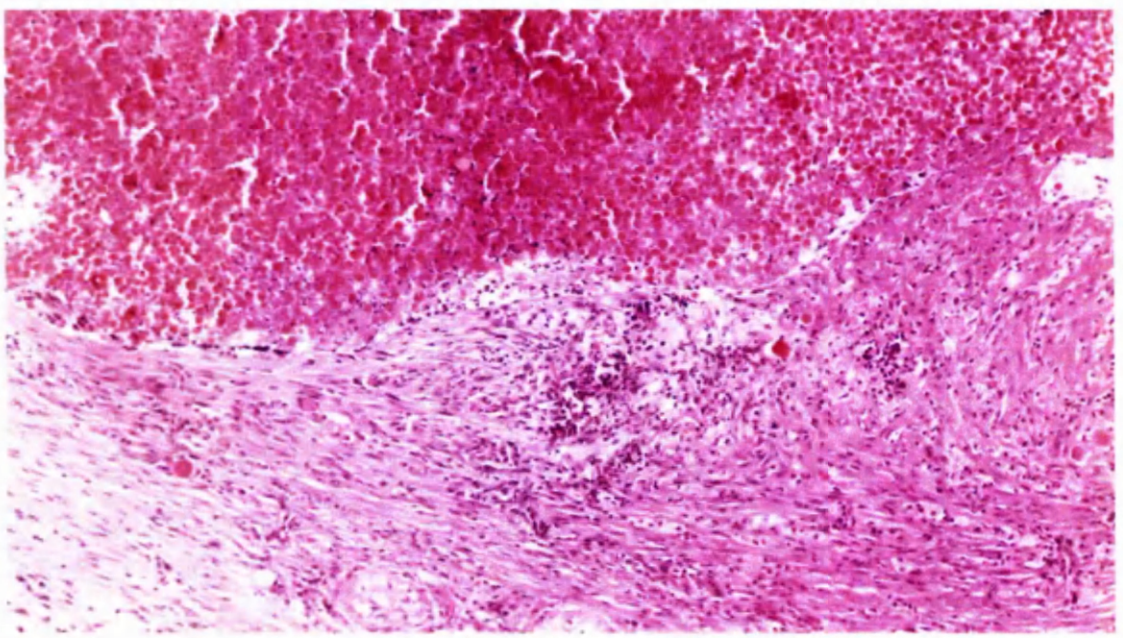
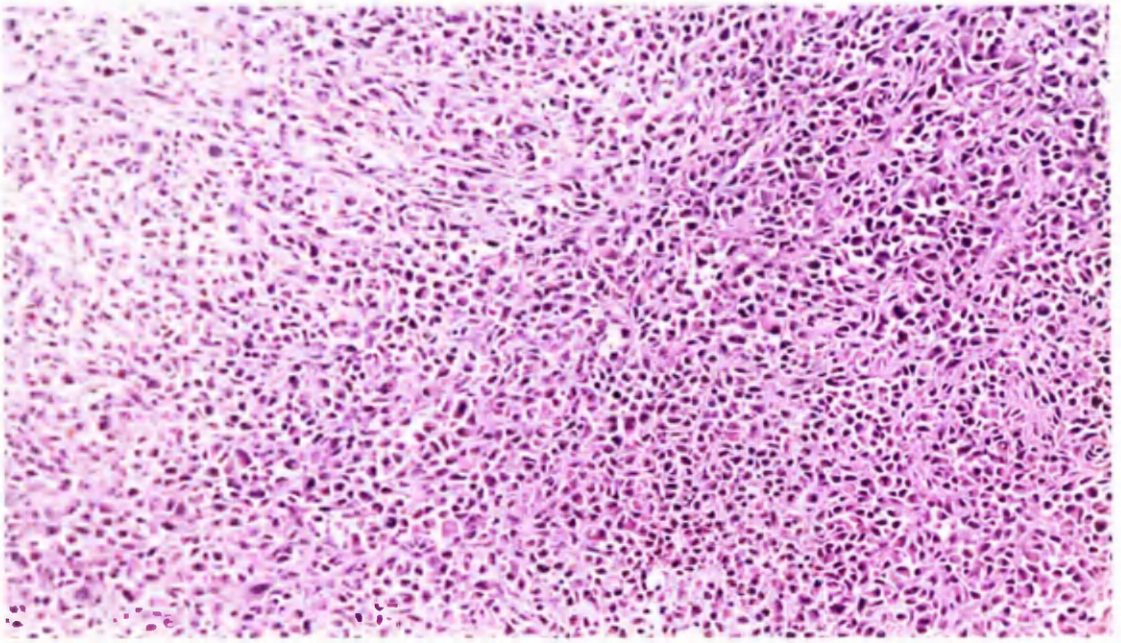


Figure 4.13 **Comparison of the histology of the HT29 tumour grown subcutaneously in the Nu/nu mouse, 14 days following intratumoural injection of 200μl of A) PBS/0.5% Tween 80 and B) 250μg EO9-loaded albumin microspheres. The microspheres are clearly visible in the upper half of Slide B.**

(A)



(B)

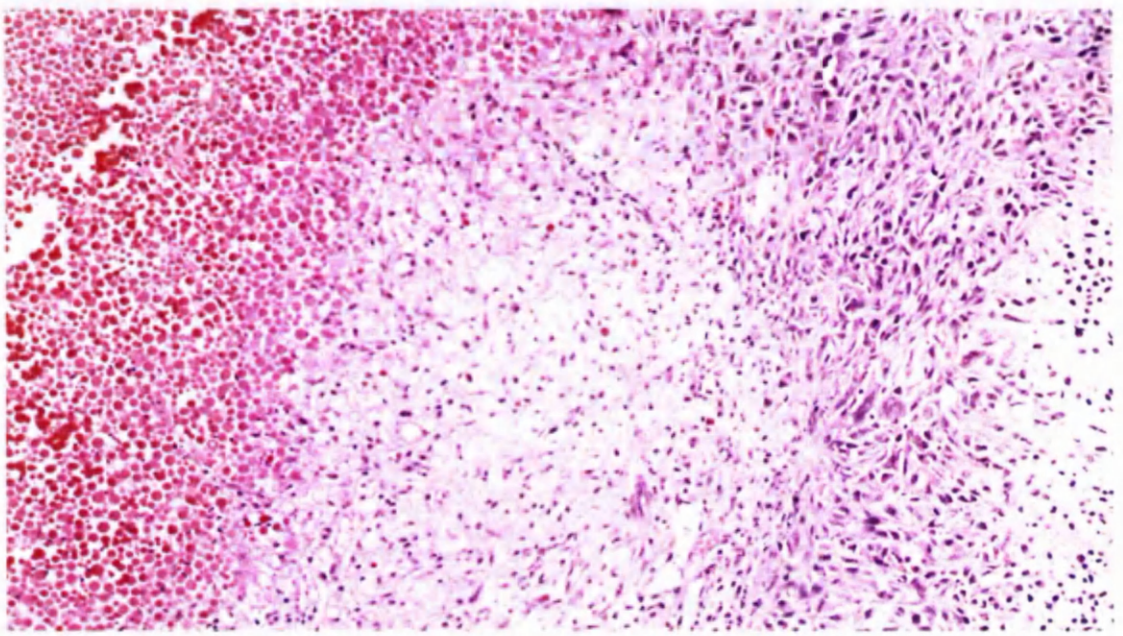


Figure 4.14 **Comparison of the histology of the BE tumour grown subcutaneously in the Nu/nu mouse, 14 days following intratumoural injection of 200μl of A) PBS/0.5% Tween 80 and B) 250μg EO9-loaded albumin microspheres. The microspheres are clearly visible on the left hand side of Slide B.**

4.5 DISCUSSION

The aims of the studies described in this chapter were to investigate the antitumour potential of the EO9-loaded microspheres and to compare the effect of these drug-loaded microspheres with the equivalent amount of free drug when given by direct intratumoural injection.

The models which have been utilised in this study are the well-established murine adenocarcinomas of colon, MAC 16 and 26 and the human colonic xenografts HT29 and BE. These were particularly useful models to study because some information was already available using EO9 in these tumour types both *in vitro* and *in vivo*, thus allowing for comparison of our results. They were also appropriate because of the potential clinical use of the microspheres as a locoregional treatment in the management of liver metastasis from colorectal cancer.

The MAC tumours have been well described by others (Cowan et al 1980, Double et al 1978, 1989, Bibby et al 1988, 1989). They are similar to human colon tumours in that they exhibit varying histological characteristics and are active mucin producers. The spectrum of chemosensitivity of these tumours has also shown good correlation with the response rates to standard therapeutic agents in colon cancer (Ball et al 1975, Double et al 1975). MAC 26 is well differentiated, well vascularised and has a relatively slow doubling time of around 4.5 days. MAC 16, on the other hand, is poorly differentiated, poorly vascularised and necrotic and its growth usually associated with marked cachexia and weight loss in the host. MAC 16, probably due to its poorer blood supply, is usually less responsive to cytotoxic therapy (particularly alkylating agents), than the MAC 26 tumour (Double et al 1989).

Less information is available with regard to the HT29 and BE xenografts, particularly the BE xenograft, which were both established in-house as subcutaneous xenografts from their respective cell lines.

The intratumoural route for delivery of drug or microspheres was the chosen method of administration for these experiments. This method has previously been successfully employed in our laboratory (Cummings et al 1991, 1992A, 1992B, 1993B, 1994, Chirrey et al 1995, Willmott et al 1987A). It was chosen because in small rodents it reproduces as closely as is technically feasible, chemoembolised particles and thus allows a study of drug disposition parameters to be made without interference from targeting variables (Cassidy et al 1993). Furthermore, previous studies which have used systemic EO9 have failed to detect EO9 or metabolites within the treated tumour (Workman et al 1992C). This may be related to the known instability and short half life of the drug, which makes it difficult to determine whether the tumour has actually been exposed to adequate doses of the drug before it is metabolised. Therefore direct intratumoural injection should ensure that the tumour is exposed to the drug and should allow for detection of both drug and metabolites in subsequent pharmacokinetic studies (See following chapter).

Dose finding studies in the MAC tumours and the xenografts were carried out prior to the formal antitumour studies so that a dose of EO9 would be utilised in these experiments which would allow for the observation of either an increase or decrease in response when the microspheres were compared with free drug. To account for any change which could be caused by the general distension and cellular destruction of the tumour as a result of the injection volume, the same volume of the vehicle, PBS/0.5 Tween 80, was chosen as the control group for each of the studies.

The HT29 tumour, as expected, was the most sensitive to both free drug and drug-loaded microspheres with significant antitumour activity being detected at lower drug concentrations than in the other tumour types. It was also the only tumour to show no significant difference in antitumour activity between free EO9 and the equivalent dose of EO9-loaded microspheres, which suggested that the tumour was equally sensitive to both methods of drug presentation. MAC 16 was also found to be sensitive to both free drug and microspheres. However, a higher dose of the microspheres was required to produce a level of antitumour activity which was comparable to that of the free EO9, which suggested that the microspheres were less active than the free drug in this tumour type. In the MAC 26 tumour, free EO9 demonstrated statistically significant antitumour activity from day 7 onwards. No significant antitumour activity was detected with the equivalent dose of microspheres, although the higher dose (500μg) of microspheres did demonstrate activity by the end of the study. This suggested that the drug-loaded microspheres were again less active than the free drug in this tumour type. Significant antitumour activity was only detected in the BE tumour when free EO9 was utilised, but not at any of the microsphere doses which were administered.

The degree of sensitivity of each of the tumour types to EO9 was also expressed as a percentage of the mean drug treated tumour volume/mean control tumour volume (T/C). To facilitate statistical comparison, three methods were used to calculate T/C. The simplest method, which divided the mean of the treated group by the mean of the control group, results of which are outlined in Table 4.2 for both free drug and drug-loaded microspheres, suggested that the degree of sensitivity to EO9 depended on the method of presentation of the EO9. In the case of the free drug, the HT29 tumour was the most sensitive (15%) with the MAC 26 tumour being the least sensitive (60%). The HT29 tumour also appeared to be the most sensitive (29%) to the EO9-loaded microspheres with the BE on this occasion appearing the least sensitive (79%). However, these results gave no indication of the degree of

confidence which could be placed in this data, especially as the spread of the results in all the groups was known to be wide. The other two statistical methods were therefore employed to try to take this into account.

The second method, which took the mean and standard deviation of the treated and control groups and simulated 10000 T and C values, allowed comparisons between the groups to be made using the unpaired students t-test. These results suggested that in each tumour type there was no statistically significant difference in antitumour activity between 250μg of the free drug and the equivalent dose of EO9-loaded microspheres, except in the BE tumour where treating with the microspheres appeared to result in a loss of antitumour activity. It also suggested that there was no real difference in antitumour activity between each of the tumour types when free drug activity was compared following intratumoural administration. When the drug-loaded microspheres were compared in each of the tumour types, the only difference in activity was between the BE and HT29 tumours in favour of increased antitumour activity in the HT29 tumour. However, the simulation of 10000 values gave huge distributions of T/C values and therefore meaningful statistical analysis was probably not achieved.

In the third method, where all measured T values were each divided by all measured C values, the data were not normally distributed and therefore had to be analysed using the Mann-Whitney test. This showed that, as for the simulated data, there was a significant difference in activity in the BE tumour between the free drug and the microspheres in favour of the free drug. It also showed that there was a difference in activity in the MAC 16 tumour again in favour of the free drug. These data also suggested that there was a significant difference in activity between the MAC 26 tumour and the other three tumour types when the free drug was administered with the MAC 26 being less sensitive to EO9. When the drug-loaded microspheres were administered, the difference between the HT29 and the 3

other tumour types was significantly in favour of the HT29. These results are similar to those obtained when the mean of the treated group was divided by the mean of the control group (Method 1), but have the advantage of statistical confidence.

EO9 has been shown previously to have activity in both MAC tumour types *in vivo* as well as *in vitro*. Although our experiments have used a different method of drug administration, our work agrees with that of others, in that activity in the usually less drug responsive MAC 16 tumour was greater than that seen in the MAC 26 tumour (Workman et al 1990B, Walton et al 1992A). This was the case for both free drug and drug-loaded microspheres.

Our results however, differ to that previously seen in HT29-bearing Nu/nu mice where little activity was seen following systemic administration of EO9 at its maximum tolerated dose of 6mg/kg given intravenously on four consecutive days. This documented lack of activity was thought to be either the result of inadequate levels of drug reaching the tumour or an alteration in the levels of metabolising enzyme levels within the tumour compared to the *in vitro* model (Collard et al 1995). Taking our results into account, which indicate that the tumour remains very sensitive to EO9 *in vivo*, this documented lack of activity is more likely to be related to the inability to target adequate doses of the drug into the tumour.

The chemosensitivity of the BE xenograft has, to the best of our knowledge, not been described previously. The fact that antitumour activity with free drug is detected in this tumour type, known to lack the 2 electron reductase DT-Diaphorase (DTD), suggests a role for the other reductase enzymes in the drug activation process.

The differences which were detected in T/C values between the free drug and the microsphere group were different to those which had been anticipated. The enzymology of EO9, as mentioned previously, is extremely complex. *In vitro* studies had suggested that

under normoxic conditions there was a strong correlation between tumour levels of DT-Diaphorase and sensitivity to EO9 (Walton et al 1992A, Robertson et al 1992, 1994, Plumb et al 1994A, 1994C, Smitskamp-Wilms et al 1994). The HT29 tumour was shown to be 15 to 30-fold more sensitive than the BE tumour and the MAC 16 tumour 28-fold more sensitive than the MAC 26 tumour (Walton et al 1992A). This has been confirmed *in vivo* using the MAC tumours where a correlation was seen between the levels of DTD and sensitivity to EO9 when the drug was given systemically (Workman et al 1990B, Walton et al 1992A). This correlation however, has not been borne out in other *in vivo* studies. The influence of other cell types which may be present within the tumour, together with the presence of cell debris, may result in an inaccurate estimation of the tumour's actual DT-Diaphorase levels (Collard et al 1995).

Previous work within our laboratory had estimated the levels of both 1 and 2 electron reductase enzymes present in each of the tumour types *in vivo* (Table 4.5) (Spanswick 1996). These results confirm that the highest level of DT-Diaphorase is present within the HT29 tumour and that the BE tumour lacks any active DT-Diaphorase. The level of DT-Diaphorase was also found to be greater in the MAC 16 tumour when compared to the MAC 26 tumour, which is in agreement with the results of others (Workman et al 1990B, Walton et al 1992A).

Based on these results and taking into account the *in vitro* studies mentioned previously, we would have expected the HT29 and the MAC 16 tumours to be the most sensitive to free EO9 with the MAC 26 and BE tumour being the least sensitive. What we found was that in both the HT29 and MAC 16 tumours free EO9 did show significant antitumour activity when compared to the MAC 26 tumour, but that in the BE tumour similar antitumour activity was seen to that in the HT29 and MAC 16 tumours.

Tumour Type	*Cytosol*			*Microsomes*			
	DT-Diaphorase			*DT-Diaphorase*		*Cytochrome P450 reductase*	*Cytochrome b5 reductase*
	NADH	*NADPH*	*Others*	*NADH*	*NADPH*		
HT29	2780 ± 210	2100 ± 370	100 ± 36	98 ± 7	51 ± 10	115 ± 10	100 ± 14
BE	ND	ND	16 ± 2	ND	ND	16 ± 2	30 ± 3
MAC 16	480 ± 20	330 ± 20	380 ± 40	5 ± 1	10 ± 3	17 ± 2	18 ± 1
MAC 26	20 ± 6	16 ± 4	50 ± 4	ND	ND	15 ± 1	17 ± 5

Table 4.5 **Quinone reductase activity in cytosolic and microsomal subcellular fractions isolated from MAC 16 and MAC 26 murine tumours and HT29 and BE human colonic xenografts. All enzyme activities are expressed as nmol cytochrome *c* reduced/min/mg protein using the extinction coefficient, e: 21.1 x 10^3 M/cm. Each value represents the mean ± SE from 3 experiments.**

ND = Not detected (reproduced with permission from Dr V.J. Spanswick)

The activity seen in the BE tumour was unexpected because of its known lack of active DT-Diaphorase and cannot be fully explained. The activity seen must be due to the other reductive enzymes present within the tumour.

There is a known difference in the reductive capacities of human and murine reductases. Murine DT-Diaphorase has been shown to be less effective than its human equivalent (Lewis et al 1994). This difference may also apply to the one electron reductases which might help to explain the antitumour activity which was seen in the BE tumour.

The role of hypoxia in EO9 activation has also been extensively investigated *in vitro* (Bando et al 1995, Plumb et al 1994A, 1994B, 1994C, Robertson et al 1994). In a panel of cell lines in hypoxic conditions, there was a greater increase in the level of sensitivity of cell lines which were low in DT-Diaphorase compared to cells which had high levels of the enzyme, with a significant inverse correlation being found between levels of DT-Diaphorase and hypoxic sensitivity to EO9 ($r=0.93$) (Plumb et al 1994B). In the case of the BE cell line, this resulted in a 1000-fold increase in sensitivity compared to HT29 which only showed a 2 to 5-fold increase in sensitivity (Plumb et al 1994C). The sensitivity of the EMT6 mouse mammary tumour cell line has been shown to increase 3-fold in the presence of hypoxia (Hendriks et al 1993) and an increase in sensitivity has also been documented in the V79 and KHT cell lines under hypoxic conditions (Adams et al 1992). This suggests that the other reductases present in the cell take on a more prominent role in low oxygen conditions (Plumb et al 1994A, 1994B, Robertson et al 1994).

Previous work, looking at doxorubicin-loaded microspheres, suggested that the microspheres appeared to induce hypoxia when given by direct intratumoural injection. This was based on the fact that stimulation of anaerobic quinone reduction of doxorubicin by cytochrome P450 reductase to its 7-deoxyglycone metabolite was increased by up to 155-fold

in tumour tissue following intratumoural injection of doxorubicin-loaded microspheres (Cummings et al 1992A). In addition, studies using magnetic resonance spectroscopy have suggested that intratumoural injection of the microspheres results in the induction of a hypoxic environment lasting approximately 20 minutes (Spanswick, personal communication). Based on this and taking into account the results of the *in vitro* work mentioned above previously, it was anticipated that the tumour types which were lower in DT-Diaphorase (MAC 26 and BE) would show some enhancement of activity when treated with the encapsulated form of EO9 compared to treatment with free EO9. The opposite effect was in fact seen to occur in that the tumour types which had least DT-Diaphorase were found to be even less sensitive in the presence of the encapsulated drug, i.e. a reduction in response compared with the free drug. These results are in agreement with previous studies carried out in our laboratory, which investigated the metabolism of EO9 in tumour homogenates of each of the four tumour types *in vitro* under aerobic and hypoxic conditions. No enhancement of activity was seen under hypoxic conditions in those tumours which were low in DT-Diaphorase (Spanswick 1996). The reason for this difference was unclear, but it may be related to the degree of hypoxia which was present. The microspheres probably induce a relative degree of hypoxia rather than absolute hypoxia or anoxia. In the case of the *in vitro* data, the hypoxic conditions described suggest that anoxia rather than hypoxia may have occurred (Plumb et al 1994A, 1994B, 1994C). Perhaps there needs to be a much lower level of oxygen present than we were able to produce for augmentation of activity to be seen.

The blood supply to each of the tumours will also be important when assessing tumour sensitivity. The MAC 26 tumour, as mentioned previously, is known to have a good blood supply and therefore the drug is likely to be cleared more quickly than in a necrotic tumour such as MAC 16. In addition, in a well-vascularised tumour, a reduction in relative oxygen concentration to levels which are significant will be more difficult to achieve. The poorly

vascularised MAC 16 tumour on the other hand, is more likely to have hypoxic regions present within the tumour. The poorer blood supply may also mean that a higher concentration of the drug is retained within the tumour, which may have significant implications in determining antitumour activity. The increase in antitumour activity seen by Bibby et al in the MAC 26 tumour following co-administration of hydralazine was postulated to be the result of hypoxia, because hydralazine had been shown to cause an 80% vascular shutdown in MAC 26 tumours. In fact, the effect may be due to more EO9 being retained within the tumour as a result of the reduced blood flow, rather than, or as well as, the presence of hypoxia (Bibby et al 1993B). This issue will be addressed in greater detail in the next chapter, when pharmacokinetic studies will be performed in each of the tumour types.

A further reason for the reduction in the antitumour activity of the microspheres may be the rate of presentation of the drug to the tumour. Although the release of EO9 from the microspheres is known to occur rapidly (see Chapter 3.4.3), the drug will be presented at a different rate to the tumour compared to the administration of free drug. EO9 is thought to act by producing single strand breaks in DNA (Walton et al 1991) as well as DNA cross-linkage (Bailey et al 1994), so perhaps it is the peak concentration of the drug rather than prolonged drug release (which may attenuate the peak), which is important for maximum DNA damage to be achieved. This contrasts with doxorubicin-loaded microspheres where, as discussed previously (Chapter 3.5.4), studies have suggested that the observed 5-fold increase in activity is the result of slow release of the doxorubicin, covalently bound to the albumin of the microspheres rather than the production of the 7-deoxyglycone metabolites which is thought to result in drug inactivation (Cummings et al 1991, 1992A). Doxorubicin is known to produce its antitumour effect by inhibition of Topoisomerase II and therefore slow release of the drug will be the more effective method of administration because it will

produce prolonged enzyme inhibition. Again, this will be considered in more detail in the next chapter.

The data presented in this chapter is in contrast to previous work with MMC-loaded albumin microspheres where the encapsulation process appeared to result in complete loss of activity (Cummings et al 1994). However, these experiments were incomplete in that only one tumour type was examined (MAC 16) and only one dose of microspheres was compared with the free drug equivalent. As noted from our results, increasing the dose of EO9-loaded microspheres resulted in significant antitumour activity in the MAC 16 tumour and it would be interesting to know whether this would also have been the case for the MMC-microspheres (Cummings et al 1994).

In conclusion, the EO9-loaded microspheres have demonstrated antitumour activity in the HT29 xenograft and the MAC 16 murine adenocarcinoma, but not in the BE xenograft or the MAC 26 murine adenocarcinoma. This contrasts with the free drug which shows significant antitumour activity in all the tumour types when given by direct intratumoural injection. These results therefore do not support the use of EO9-loaded microspheres as a method of increasing the drug's activity in this type of tumour model.

However, intratumoural injection of the EO9-loaded microspheres is unlikely to be the best method of determining the relative activity of the microspheres compared with free drug. Intratumoural injection of the free drug might be the optimal way to treat a tumour but is not practical in the majority of cases. Cytotoxic drugs are usually given systemically by the intravenous route. The result of this is that the drug may only be transiently in contact with the tumour. A further problem with EO9 is that its instability means that not only is it transiently in contact with the tumour when given intravenously, but it is also unlikely to reach the tumour in any significant concentration as a result of its rapid metabolism or

hydrolysis, a problem previous studies have identified (Workman et al 1992C). This may well contribute to its lack of activity. Therefore, the advantage of the microspheres is that they not only have the potential to deliver a high local concentration of the drug if they are administered via the tumour's arterial blood supply, but they may also help to physically retain the drug at the tumour site. A better model of studying the microspheres needs to be devised in order to obtain a more clinically relevant picture of the microsphere activity. This will be discussed further in Chapter 6, where a model will be developed which will allow the comparison of intra-arterial administration of free EO9 and EO9-loaded microspheres with free EO9 given by the intravenous route.

CHAPTER 5

Pharmacokinetic studies with EO9-loaded albumin microspheres

5 Chapter 5

5.1 INTRODUCTION

In Chapter 4, the antitumour activity following intratumoural injection of free EO9 or the equivalent dose of EO9-loaded albumin microspheres in the HT29, BE, MAC 16 and MAC 26 tumour types was presented. The results showed that the administration of free EO9 was associated with antitumour activity in all four tumours and that the HT29 xenograft was the most sensitive of the four tumour types which were studied. When the EO9-loaded microspheres were compared to free drug, only the HT29 tumour demonstrated antitumour activity at a dose of microspheres equivalent to that of free drug. The MAC 16 tumour required an EO9 dose of 500μg in microsphere form to produce an equivalent antitumour response to 250μg of free drug. No significant antitumour activity was demonstrated by any dose of microspheres in either the MAC 26 or BE tumours.

The aim of the experiments described in this chapter was to determine whether the variation seen in antitumour activity between the four different tumours and between the free drug and the microsphere treated groups in the same tumour type could be explained by pharmacokinetic differences. In particular, the metabolism of EO9 was studied to see whether any metabolites could be identified which may correlate with the differences which were observed.

Previous studies within our laboratory studying MMC-loaded microspheres in the MAC 16 tumour had suggested that although there was similar drug exposure compared to the free drug studies (30.0μg/ml x hr (microsphere group) v 46.9μg/ml x hr (free drug group)), the half life and absolute tumour concentrations in the microsphere treated group were different. Encapsulating the drug resulted in a prolonged $t_{1/2}$ of 600 minutes in the

microsphere group compared to 70 minutes in the free drug group, with generally lower MMC concentrations in the microsphere group at the early time points in the study (Allan 1994). A significant reduction in plasma MMC concentration in the microsphere-treated group was also detected (Cummings et al 1994).

In addition to the change in the pharmacokinetic profile when the microspheres were used, MMC metabolism was also altered. 2,7-diaminomitosene (2,7-DM) is thought to be the principal indicator of MMC metabolic activation in tumour tissue because it is only found after quinone reduction and levels correlate *in vitro* with cytotoxicity. It was the main metabolite found after intratumoural injection of free MMC, but was present in much lower levels following intratumoural injection of MMC-loaded microspheres, suggesting possible inhibition of its formation in the microsphere treated group. An additional four mitosane metabolites were detected in the group treated with the microspheres. The encapsulation of MMC into microspheres was associated with reduced antitumour activity, so that the level of 2,7-DM which was detected *in vivo* was thought to be an important indicator of antitumour activity (Cummings et al 1994).

An alteration in the metabolism of doxorubicin has also been demonstrated when the drug is encapsulated into microspheres. This results in the stimulation of anaerobic quinone reduction of the doxorubicin to 7-deoxyglycone metabolites by a factor of up to 155-fold when compared to the free drug (Willmott et al 1987A).

EO9, as indicated previously, is known to be very unstable with a short half-life. There are very little data available on the generation of reactive intermediates and metabolite formation *in vivo*. This is due in part to the presumed instability and short half-life of the metabolites. Previous *in vitro* work in our laboratory using EO9 has been carried out in the four tumour types mentioned above in an attempt to identify potential metabolites which

may act as indicators of tumour sensitivity (Cummings et al 1998). HPLC analysis of tumour homogenates was carried out following incubation with EO9 under aerobic or hypoxic conditions. Three main metabolites were identified in addition to EO5A that had retention times between 4.2 - 4.4 minutes, 5.4 - 6.2 minutes and 7.5 - 8 minutes. They were present in all four tumour homogenates and their formation appeared to be unaffected by the presence or absence of oxygen. No correlation was identified between any of these metabolites and tumour sensitivity *in vivo*, nor was there any correlation with these metabolites and levels of the major bioreductive enzymes, DT-Diaphorase, Cytochrome P450 reductase and Cytochrome *b*5 reductase (Cummings et al 1998). The experiments in this chapter will determine whether these metabolites are also present *in vivo* and, if so, whether the encapsulation of drug into microspheres alters metabolite formation.

Pharmacokinetic analysis has been carried out previously *in vivo* following i.v. injection of EO9. At the maximum tolerated dose in C3H/He mice (12mg/kg) the $t_{1/2}$ was 1.9 minutes with a peak plasma concentration of 1.8μg/ml. EO9 and EO5A were identified in the plasma along with several other potential metabolites. No EO9 or metabolites were detectable in the tumour or any of the tissues examined during the course of the experiment. No parent drug was detected in the urine but, 20% of the total EO9 dose was detectable in the urine as either EO5A or other hydrophilic metabolites which, as yet, have not been formally characterised. This absence of EO9 in both the tissues and urine suggested that there was rapid metabolism and clearance of the drug (Workman et al 1992C). Pharmacokinetic studies which were performed during the Phase I trial of EO9 detected EO9 and EO5A in the plasma, but minimal amounts of each in the urine (Schellens et al 1994).

No work, to the best of our knowledge, has been performed *in vivo* to look at EO9 metabolism in tumour tissue. The direct intratumoural injection of EO9 should maximise

the chance of detecting any metabolites which may be formed and will allow a direct comparison with the metabolism of EO9 incorporated into the microspheres which may, as indicated previously alter the tumour environment and thus, drug metabolism.

5.2 MATERIALS

All reagents, equipment and suppliers used are listed in Appendix 1. The High Performance Liquid Chromatography (HPLC) analyses were performed using a Waters Alliance System equipped with the following features: a 996 Photodiode Array detector, a 2690 Separations module which included a variable volume automatic injector and autosampler and a heated column compartment. System control and evaluation were performed by a Millennium software package run on a standard PC.

5.3 METHODS

5.3.1 Pharmacokinetic Studies

5.3.1.1 Comparison of free EO9 with EO9-loaded albumin microspheres administered by direct intratumoural injection

MAC 16- and 26-bearing NMRI mice and HT29- and BE-bearing Nu/nu mice were randomised into groups of three animals per time point and injected intratumourally with 200μl of PBS/0.5% Tween 80 containing either 250μg of EO9 or the equivalent dose of freshly prepared EO9-loaded microspheres. Following injection, the animals were killed using CO_2 asphyxia at the following time points; 0, 5, 10, 15, 30, 45, 60 minutes and 2, 4, 6, 18 and 24 hours following drug administration. Blood samples were immediately collected by open cardiac puncture, centrifuged (2000r.p.m for 10 minutes at 4^{0}C) and the separated plasma then placed in liquid nitrogen. The tumours were excised and immediately placed in liquid nitrogen. Both plasma and tumour samples were stored at -80^{0}C prior to analysis.

5.3.1.2 Preparation of pharmacokinetic samples using Solid Phase Extraction (SPE) for HPLC analysis

a) Plasma

The plasma was allowed to thaw at room temperature and kept on ice. The total volume was determined and 100µl of the plasma sample was then added to 800µl of 20mmol sodium phosphate buffer, pH 8.0 and 100µl of the internal MMC standard (100µg/ml). The 1cc C18-OH Bond Elut solid phase extraction columns were activated with 1ml of methanol and washed with 1ml of the above buffer. The sample was placed on the column and washed with a further 1ml of buffer. The column was allowed to vacuum dry for 20 minutes and the sample eluted in 0.5ml of methanol. This was dried down in a stream of nitrogen and then reconstituted in 300µl of a methanol: buffer (25:75 ratio) mixture. The reconstituted sample was passed through a syringe filter and a 100µl aliquot was injected onto the HPLC column.

b) Tumour

The tumour was thawed slowly on ice and weighed. Buffer was added to the whole tumour in a ratio of 2 buffer: 1 tumour and the tumour homogenised. To the volume of tumour homogenate, 100µl of the internal MMC standard (see above) was added and additional buffer to make a final volume of 2.6mls. The sample was then spun at 2500r.p.m for 10 minutes at 4^0C, following which, the supernatant was immediately removed and kept on ice. A 3cc C18-OH Bond Elut column was activated with 2.5mls of methanol and washed with 2.5mls of buffer. The sample was then placed on the column and the column and sample were washed with a further 2.5 mls of buffer. The column was allowed to vacuum dry for 20 minutes and the sample then eluted from the column in 1ml of methanol. This was dried down in a stream of nitrogen and then reconstituted in 300µl of the buffer: methanol mixture as outlined above. It was then passed through a syringe filter and a 100µl aliquot was placed in the HPLC column for analysis.

5.3.1.3 HPLC analysis of tumour and plasma pharmacokinetic samples

HPLC analysis was carried out under identical conditions to those previously described in Chapter 2.3.1. The HPLC equipment which was used was the Waters system rather than the Hewlett Packard system and is described earlier in this chapter. Extracted standards were analysed on a daily basis to ensure reproducibility of results.

5.3.1.4 Pharmacokinetic data analysis

The chromatograph obtained for each sample was carefully analysed, both to determine the amount of EO9 present in the sample (based on the height of the EO9 peak) and also to look for potential EO9 metabolites.

The total EO9 (μg) from each tumour and the plasma EO9 concentration (μg/ml) were determined for each sample using previously constructed standard curves. The mean (and standard error) and median of the total EO9 obtained following analysis of the 3 samples at each time point were then calculated and plotted against time. The results following intratumoural injection with free drug were compared with the results obtained from tumours treated with the equivalent dose of drug-loaded microspheres. Individual time points were then compared using the unpaired students t-test.

5.3.2 Timed Histological Assessment of Tumours following Direct Intratumoural Microsphere Injection

In order to try and establish how long the microspheres remained within the tumour, MAC 16- and 26-bearing NMRI mice and HT29- and BE-bearing Nu/nu mice (previously described in Chapter 4.2) were injected intratumourally with 250μg blank albumin microspheres resuspended in 200μl PBS/0.5% Tween 80. The control group were treated with 200μl PBS/0.5% Tween 80 only. There were seven animals in each group, which were sacrificed at the following time points: 0, 1, 2, 4, 7, 10 and 14 days. The final tumour volume was determined and the tumours removed and prepared for histological assessment as discussed previously in Chapter 4.3.3.

5.4 RESULTS

5.4.1 Pharmacokinetic Studies: Comparison of Free EO9 with EO9-loaded Microspheres

5.4.1.1 HT29 xenograft model

a) Tumour

After injection of free EO9 and EO9-loaded microspheres into the tumour, analysis of samples collected immediately after injection and sacrifice (t_0) showed that the mean tumour EO9 content at this time point was 12.8μg (5% of the original total of free EO9) and 28.2μg (11% of the original total of EO9 microspheres). These results were not significantly different. Following injection of free drug, EO9 was detected in the tumour for up to 24 hours, although only a small amount (< 0.2% of the total EO9 injected into the tumour) was present after the 2 hour time point. When the EO9-loaded microspheres were used, drug was also detected in the tumour up to 24 hours following injection, with less than 0.2% of the total EO9 which had been injected into the tumour being present after 6 hours (Figure 5.1).

Significant variation was present in the three replicates obtained at each time point in both groups which made statistical analysis of the data difficult e.g. in the free drug group at t_0 tumour EO9 content was 30.6μg, 7.4μg and 0.2μg respectively with a mean of 12.8 ± 15.9μg. No statistical difference between the two sets of data, apart from one isolated time point at 60 minutes ($p<0.05$) in favour of the microsphere treated group, were detected (Figure 5.1).

In an effort to eliminate the impact of this variability and exclude outliers the median values from each set of data were compared. This suggested that the rate of drug loss from the tumour was slower in the microsphere group than in the free drug group (Figure 5.2). This implied that retention of EO9 within the microsphere treated tumours may be occurring compared to the group treated with free drug.

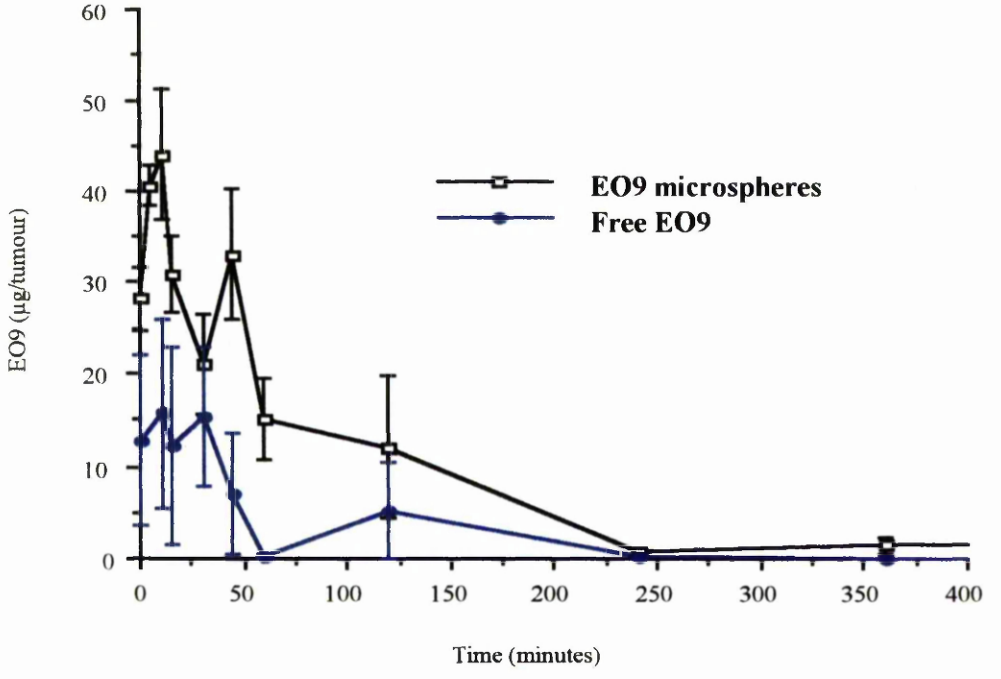


Figure 5.1 **The effect of time on the concentration of EO9 following direct intratumoural injection of 250μg free EO9 or the microsphere equivalent in the HT29 xenograft grown subcutaneously in the Nu/nu mouse. The EO9 concentration at each time point, expressed as the total drug content (μg) per tumour is the mean result ± standard error of 3 animals. No statistical difference was detected between the two sets of data except for an isolated time point at 60 minutes in favour of the microsphere group ($p<0.05$).**

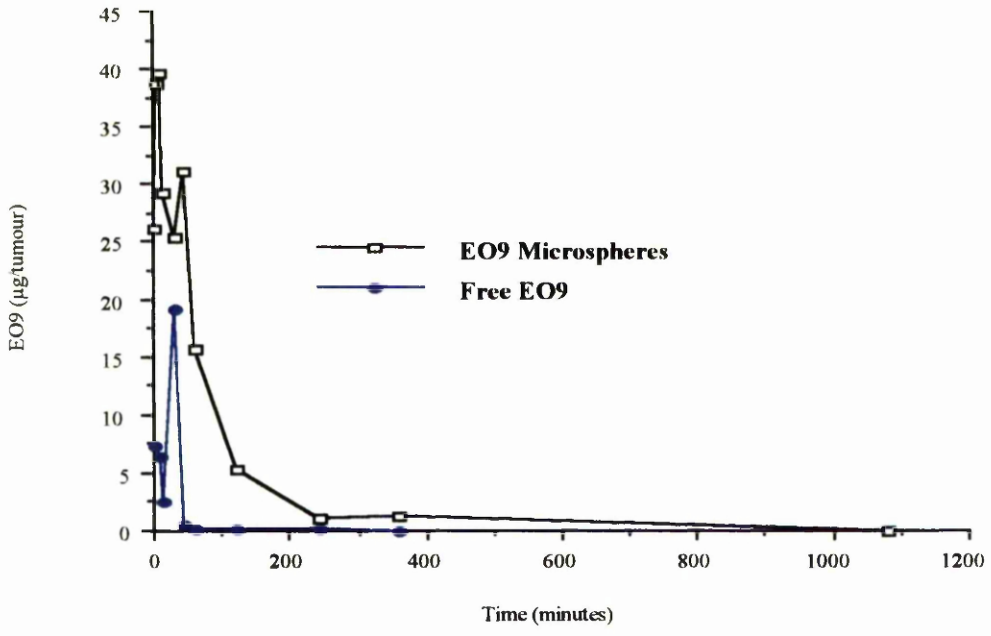


Figure 5.2 **The effect of time on the concentration of EO9 following direct intratumoural injection of 250µg free EO9 or the microsphere equivalent in the HT29 xenograft grown subcutaneously in the Nu/nu mouse. The EO9 concentration at each time point, expressed as the total drug content (µg) per tumour is the median result from 3 animals.**

However, when the experiment was repeated these results were not reproducible. When free EO9 was injected in the second experiment it was detected in the tumour throughout the 24 hour period, but when the EO9-loaded microspheres were used, EO9 was only detected in the tumour over the first 6 hours (Figure 5.3).

When the results of both these experiments were combined and the administration of EO9 and EO9 microspheres were compared using the unpaired students t-test, no statistical difference was detected between the two sets of data (Figure 5.4). This suggested that there was no difference in drug concentration over time within the HT29 tumour following the administration of free drug or drug-loaded microspheres. The variability which was seen in these experiments could be due to the method of drug administration which may result in peritumoural as well as intratumoural administration of the drug.

b) Plasma Concentration

In the first experiment, EO9 was detected in the plasma 5 minutes following intratumoural injection of free drug but was undetectable after 15 minutes. When the drug-loaded microspheres were used EO9 was again detected after 5 minutes but, remained detectable 1 hour later in all three samples and in 1 out of the three samples at 2 and 6 hours respectively. Although highly variable, this did initially suggest that retention of EO9 occured in the tumours of mice treated with microspheres, resulting in the prolonged detection of drug in the plasma. This was more apparent when the median values from each group were compared (Figure 5.5). However, these results again were not confirmed in the repeat experiment which failed to detect EO9 in the plasma of either group at any time point, thus further demonstrating the lack of reproducibility.

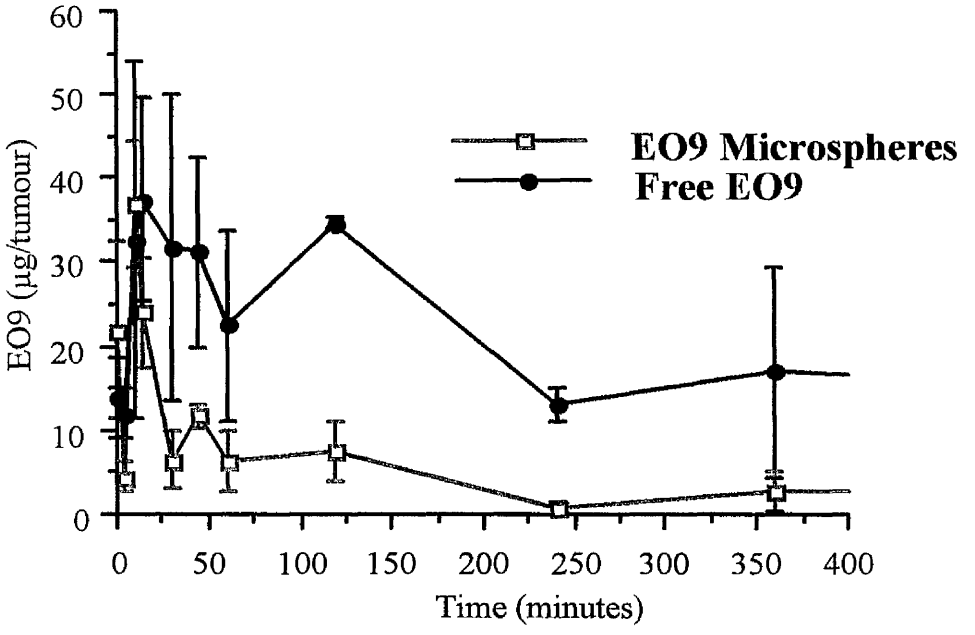


Figure 5.3 **The repeated effect of time on the concentration of EO9 following direct intratumoural injection of 250µg free EO9 or the microsphere equivalent in the HT29 xenograft grown subcutaneously in the Nu/nu mouse. The EO9 concentration at each time point, expressed as the total drug content (µg) per tumour is the mean result ± standard error of 3 animals.**

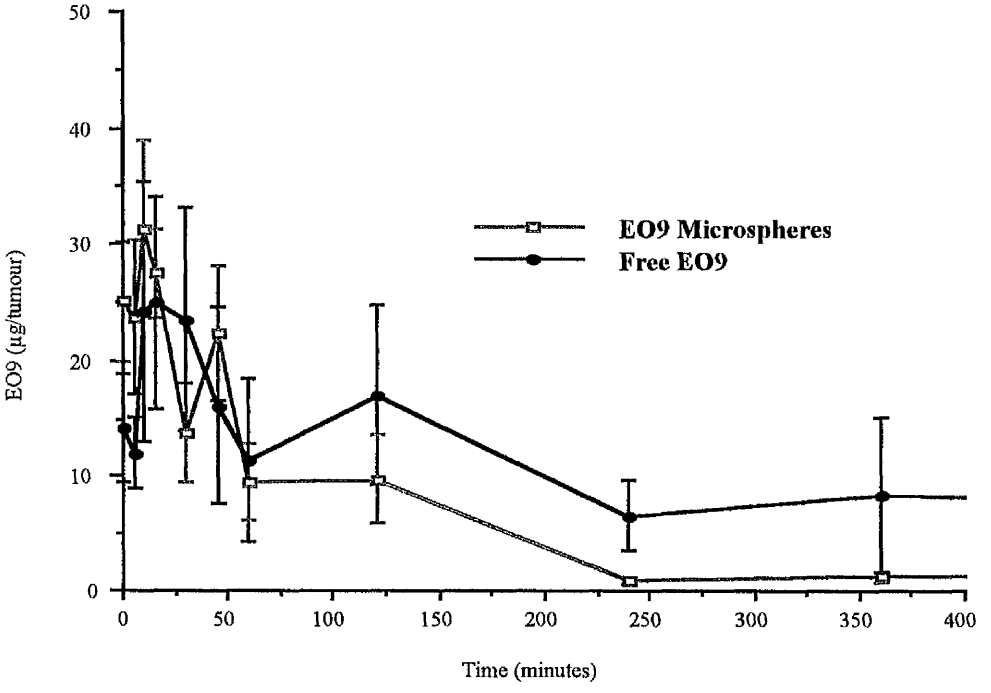


Figure 5.4 **The combined effect from two identical experiments on the concentration of EO9 following direct intratumoural injection of 250µg free EO9 or the microsphere equivalent in the HT29 xenograft grown subcutaneously in the Nu/nu mouse. The EO9 concentration at each time point, expressed as the total drug content (µg) per tumour is the mean result ± standard error for 6 animals. No statistical difference was detected between the two sets of data ($p<0.05$).**

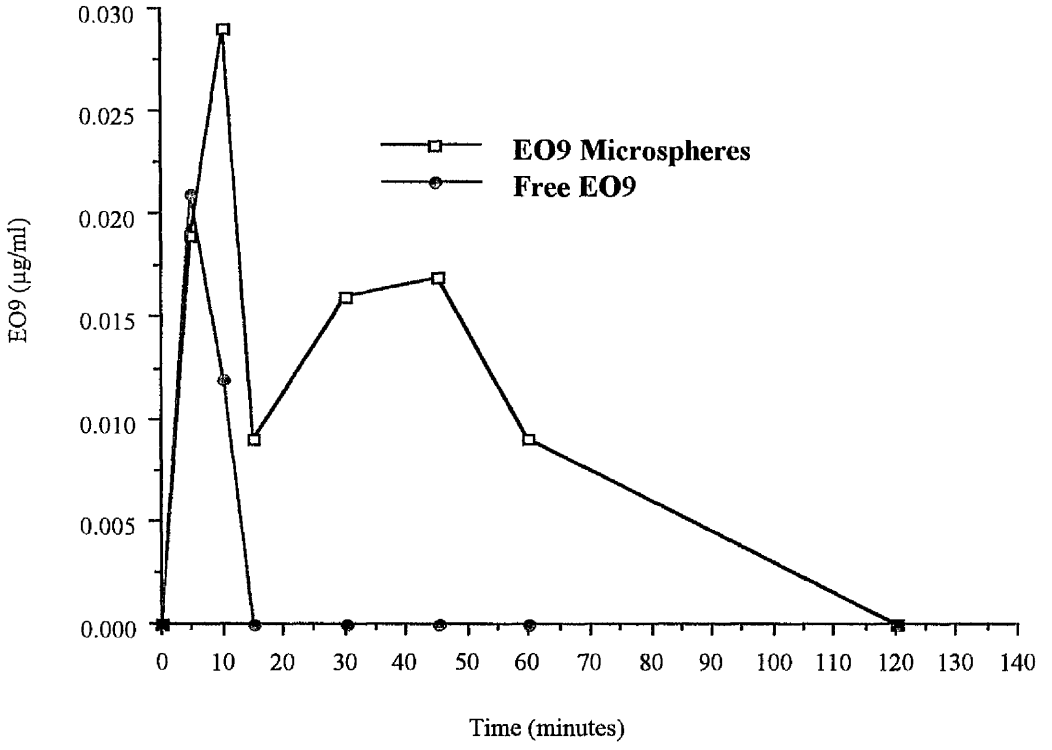


Figure 5.5 **The effect of time on the plasma concentration (µg/ml) of EO9 following direct intratumoural injection of 250µg free EO9 or the microsphere equivalent in the HT29 xenograft grown subcutaneously in the Nu/nu mouse. The EO9 plasma concentration at each time point is the median result for 3 animals.**

5.4.1.2 BE xenograft model

a) Tumour

After injection of free EO9 and EO9-loaded microspheres into the tumour, analysis of "t_0" samples demonstrated that the mean EO9 tumour content at this time point in each group was again much less than 100% (27%: free EO9 and 13.2%: EO9 microspheres). This was not statistically significantly different. EO9 was detected in both groups of tumours throughout the 24 hour time course of the experiment but tumour drug content had fallen to less than 0.2% after 2 hours in the free drug group (except for the 18 hour sample and this result appears to be an outlier suggesting mislabelling) and to less than 0.2% after 6 hours in the microsphere treated group (Figure 5.6). Variability in drug content was again seen at each time point, making analysis of the results difficult, e.g. in the microsphere treated group EO9 values of 3.5μg, 22.6μg & 73.3μg were obtained at t_0. When the median values for each group were compared the rate of drug loss from the tumour in the microsphere group appeared slower than in the free drug group (Data not shown).

However, when the experiment was repeated, the results were not reproducible. Drug was again detected in both groups throughout the 24-hour time period. In this experiment however, the amount of drug was greater than 0.2% 6 hours after injection for both the free drug and the microspheres (Figure 5.7). The results were significantly different and in favour of the free EO9 group at 15 minutes and 2 hours following intratumoural injection ($p<0.05$).

When the results of both experiments were combined and the free EO9 group compared with the EO9 microsphere group, no statistical difference was detected in drug concentration during the time course of the experiment.

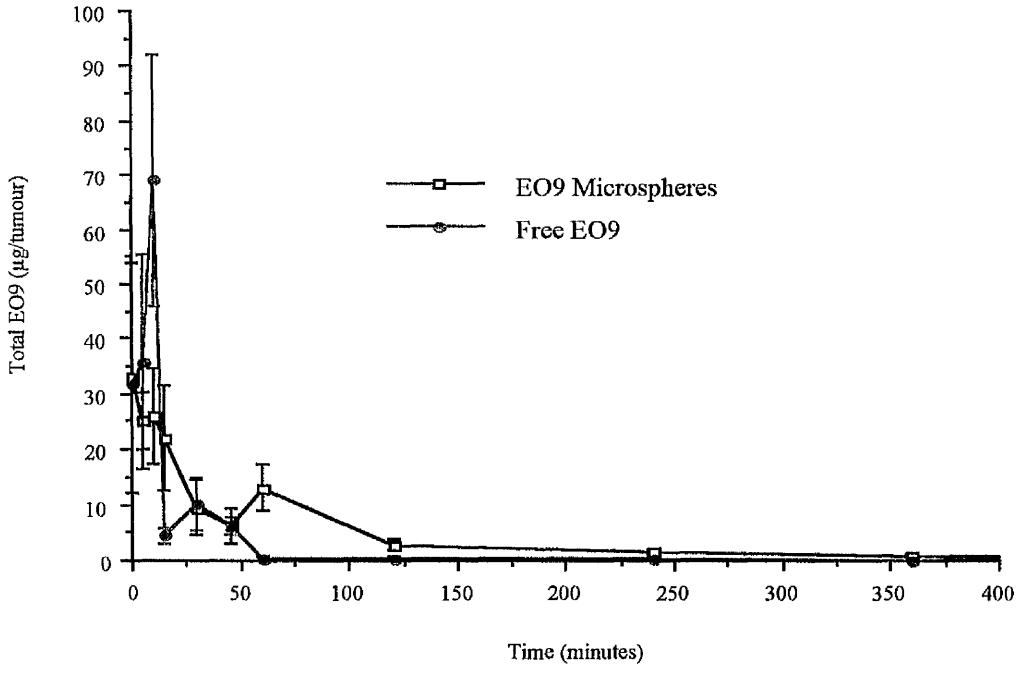


Figure 5.6 **The effect of time on the concentration of EO9 following direct intratumoural injection of 250µg free EO9 or the microsphere equivalent, in the BE xenograft grown subcutaneously in the Nu/nu mouse. The EO9 concentration at each time point, expressed as the total drug content (µg) per tumour is the mean result ± standard error for 3 animals. No statistical difference was detected between the two sets of data ($p<0.05$).**

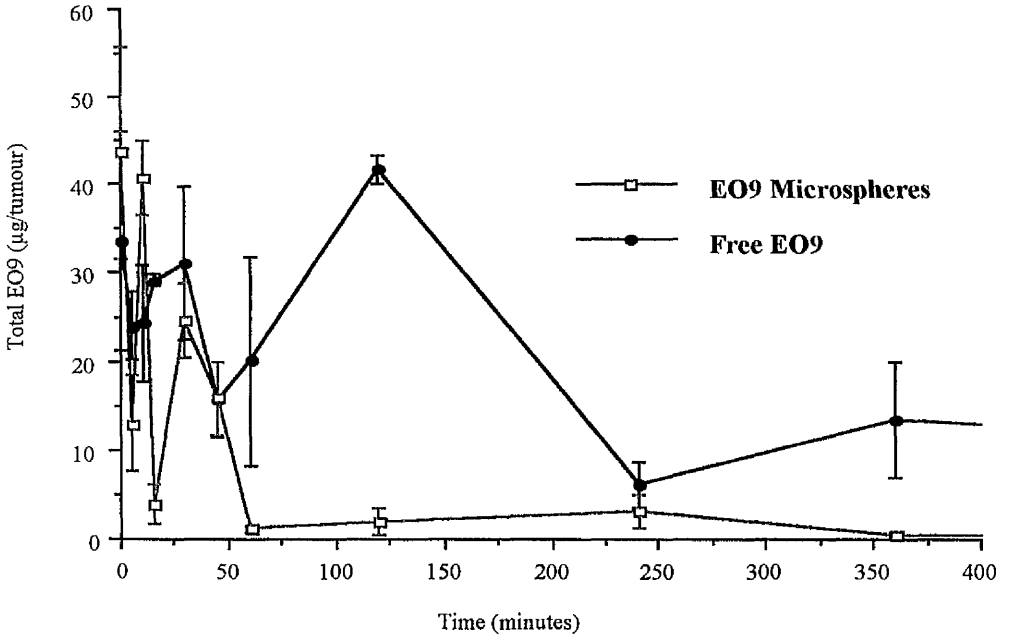


Figure 5.7 **The repeated effect of time on the concentration of EO9 following direct intratumoural injection of 250µg free EO9 or the microsphere equivalent in the BE xenograft grown subcutaneously in the Nu/nu mouse. The EO9 concentration at each time point, expressed as the total drug content (µg) per tumour, is the mean result ± standard error for 3 animals. No significant difference was detected between the two sets of data except at 15 minutes and 2 hours in favour of the free EO9 group ($p<0.05$).**

This experiment therefore failed to detect any significant difference in tumour EO9 levels between the free drug and the microsphere group in the BE tumour, a result similar to that obtained in the HT29 xenograft.

b) Plasma Concentration

In both the experiments EO9 was detected in the plasma up to 15 minutes following administration of the free drug and up to 10 minutes following administration of the microspheres (Figure 5.8). No statistical difference was detected between the two sets of results. There was no suggestion of prolonged plasma concentration of drug in the microsphere group.

5.4.1.3 MAC 16 tumours growing in NMRI mice

a) Tumour

After injection of free EO9 and EO9-loaded microspheres into the tumour, sample analysis at "t_0" demonstrated that the mean EO9 content of the tumour was 5.5% (free EO9) and 11.4% (EO9 microspheres) immediately after injection. These were not statistically significantly different. Following the intratumoural injection of free EO9, drug was detected above 0.2% of the starting dose at 6 hours but was undetectable by 24 hours. When the drug-loaded microspheres were injected EO9 was still detectable at 24 hours, although levels had fallen to less than 0.2% by 2 hours (Figure 5.9). No statistically significant difference was detected between drug levels in the tumours treated with free drug compared to those treated with the microspheres.

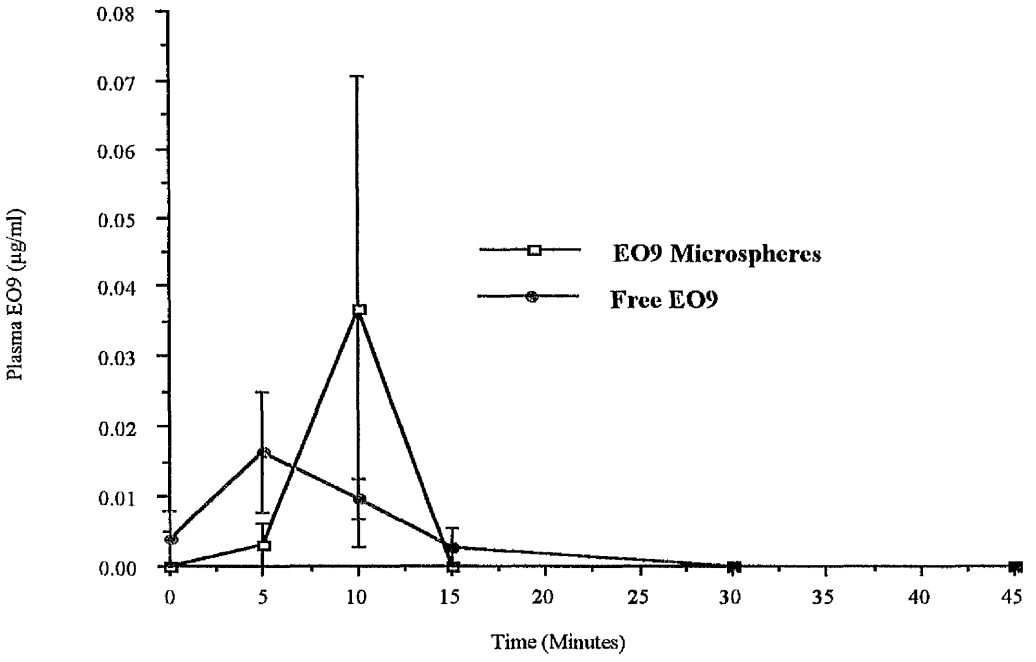


Figure 5.8 The effect of time on the plasma concentration (µg) of EO9 following direct intratumoural injection of 250µg free EO9 or the microsphere equivalent in the BE xenograft grown subcutaneously in the Nu/nu mouse. The EO9 plasma concentration at each time point is the mean result ± standard error for 3 animals. No statistical difference was detected between the two sets of data ($p<0.05$).

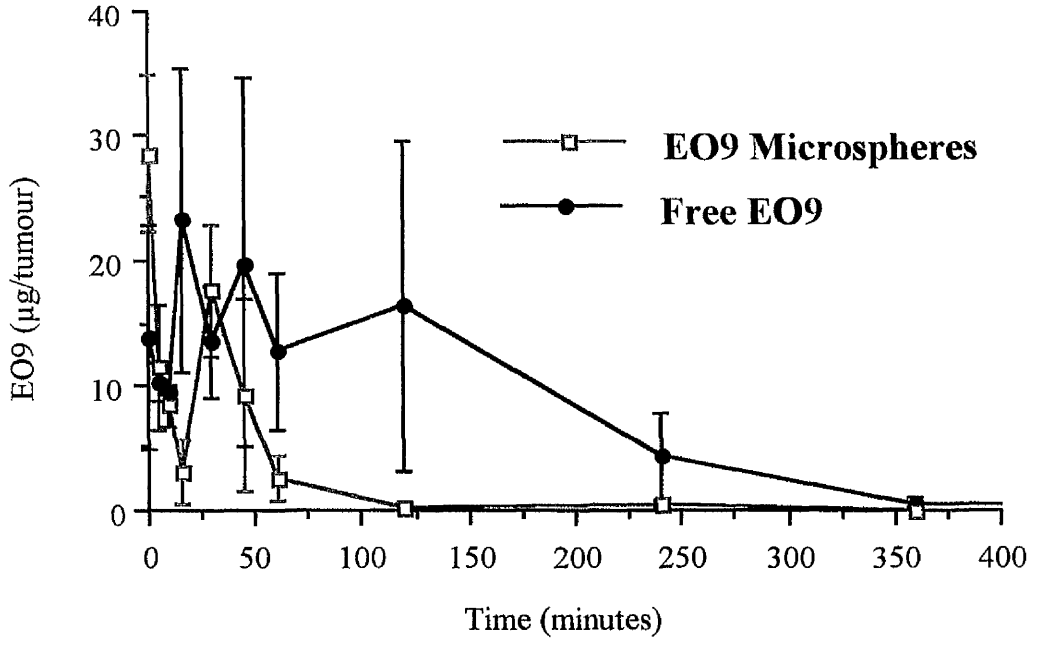


Figure 5.9 **The effect of time on the concentration of EO9 following direct intratumoural injection of 250µg or the microsphere equivalent in the MAC 16 tumour grown subcutaneously in the NMRI mouse. The EO9 concentration at each time point, expressed as the total drug content (µg) per tumour is the mean result ± standard error for 3 animals. No statistical difference was detected between the two sets of data ($p<0.05$).**

c) Plasma Concentration

When the plasma EO9 mean concentrations were compared, EO9 was detected throughout the time course of the experiment in the microsphere treated group, but only up to 2 hours in the free drug group (Figure 5.10). The results were statistically significant after 45 minutes suggesting continued release of drug from the tumour into the plasma in the microsphere-treated group.

5.4.1.4 MAC 26 tumours growing in NMRI mice

a) Tumour

After injection of free EO9 and EO9-loaded microspheres into the tumour, sample analysis at "t_0" demonstrated that the mean EO9 tumour content immediately after injection was 11.8% (free EO9) and 2.7% (EO9 microspheres) and these were not statistically different. EO9 was detected in the tumour up to 24 hours following intratumoural injection of the free drug, but only up to 2 hours following intratumoural microsphere injection (Figure 5.11). No statistically significant difference could be detected between the two groups during the first two hours of the analysis due to the variability of results within each group.

b) Plasma Concentration

When the mean EO9 plasma levels were studied, EO9 was not detected in any of the free plasma samples at any of the time points. EO9 was present in the microsphere-treated group up to 4 hours following the intratumoural injection. The result in the free drug group suggests a technical problem in the analysis of the samples which makes any comparison between the two groups difficult and requires the experiment to be repeated.

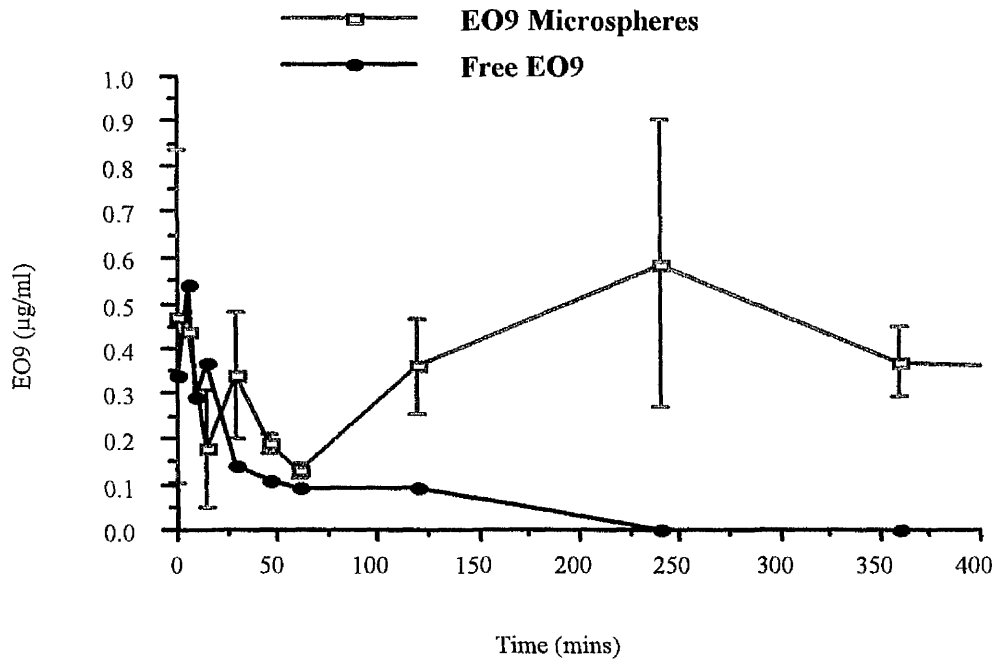


Figure 5.10 **The effect of time on the plasma concentration (µg/ml) of EO9 following direct intratumoural injection of 250µg free EO9 or the microsphere equivalent in the MAC 16 tumour grown subcutaneously in the NMRI mouse. The EO9 plasma concentration at each time point is the mean ± standard error for 3 animals. The results were statistically significant in favour of the microsphere treated group after 45 minutes ($p<0.05$).**

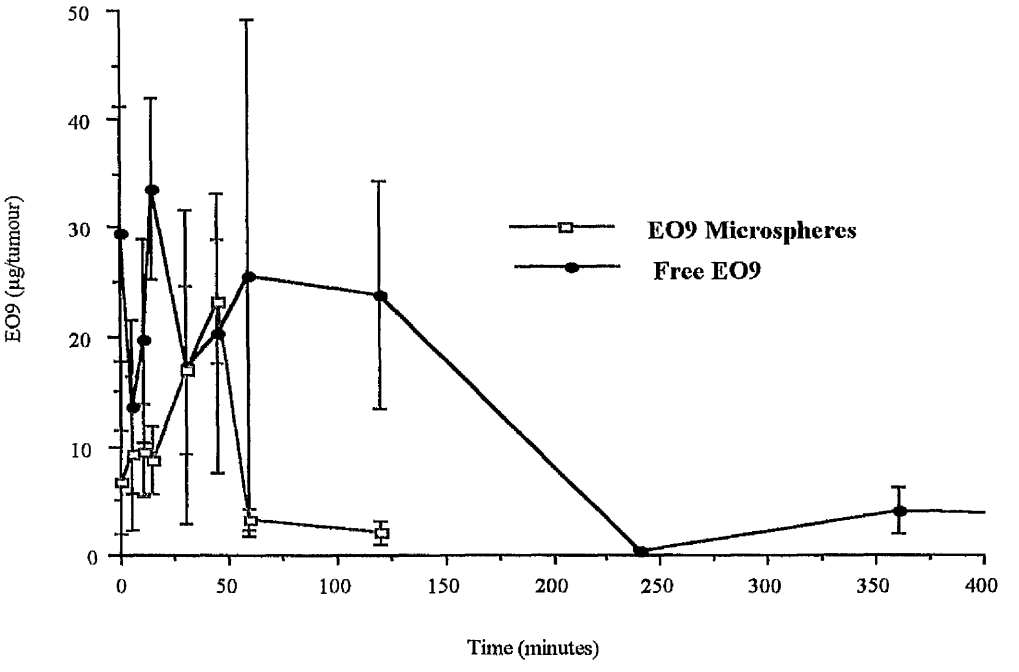


Figure 5.11 **The effect of time on the concentration of EO9 following direct intratumoural injection of 250µg free EO9 drug or the microsphere equivalent in the MAC 26 tumour grown subcutaneously in the NMRI mouse. The EO9 concentration at each time point, expressed as the total drug content (µg) per tumour, is the mean result ± standard error for 3 animals.**

5.4.2 Chromatographic Analysis Comparing Free EO9 with EO9-loaded Microspheres

Detailed chromatography was only available for the human xenograft tumours (HT29 and BE) and therefore only these data will be discussed. As standards are not available for EO5A and potential metabolites of EO9, the results can only be descriptive and comparative rather than quantitative.

5.4.2.1 HT29 xenograft

5.4.2.1.1 Chromatographic analysis of the HT29 tumour following intratumoural administration of free EO9

In comparison to the chromatography of the control tumour (Figure 5.12), after intratumoural injection of free EO9, 4 other peaks were identified in addition to EO9 and the hydrolysis product EO5A (with retention times of 24.4 and 17.5 minutes), at 5.8 minutes (Peak I_f), 6.6 minutes (Peak II_f), 11.9 minutes (Peak III_f), and 14.7 minutes (Peak IV_f) respectively (Figure 5.13 and Table 5.1).

EO9 and EO5A were both present throughout the time course of the study. The peak at 5.8 minutes (Peak I_f) was present for the first 2 hours. The peak height increased in the first 15 minutes, and began to fall after 1 hour. It had a UV spectrum similar to EO5A. The peak at 6.6 minutes (Peak II_f) was present from t_0 with peak height falling by 10 minutes and it was absent by 45 minutes. On UV spectral analysis it did not resemble the spectra of EO9 or EO5A suggesting that it was unlikely to be an EO9 related product (Figure 5.14). The peak at 11.9 minutes (Peak III_f) was a broad peak present from t_0 at low level and absent after 4 hours. It had a UV spectrum similar to EO5A. Peak IV_f was another broad peak present from t_0 at 14.7 minutes. It was not detected after 2 hours and had a UV spectrum similar to EO5A.

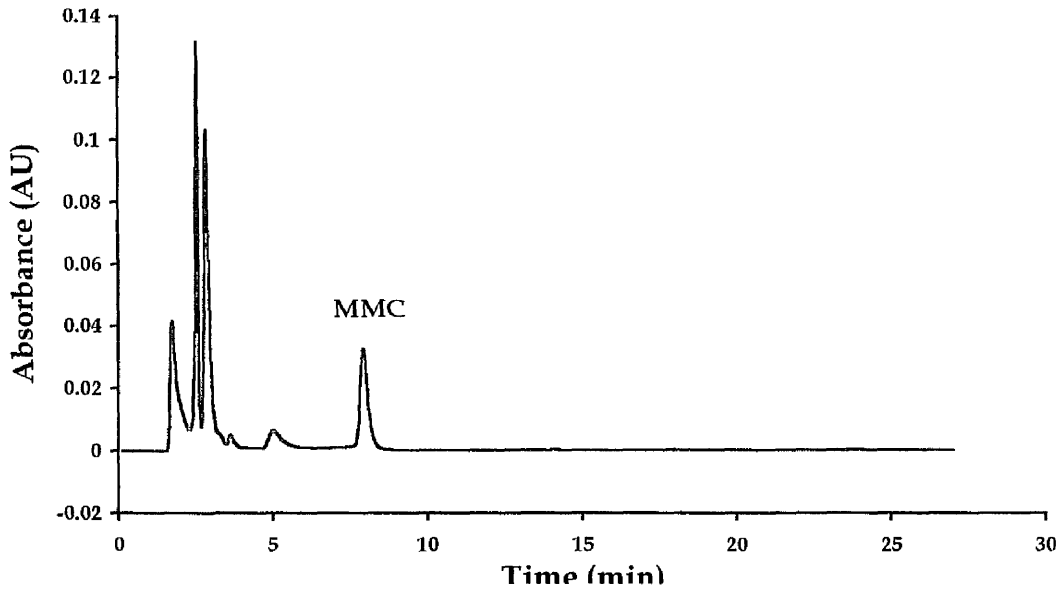


Figure 5.12 **HPLC analysis of untreated (control) HT29 tumour homogenate which shows the internal MMC standard (100μg/ml).**

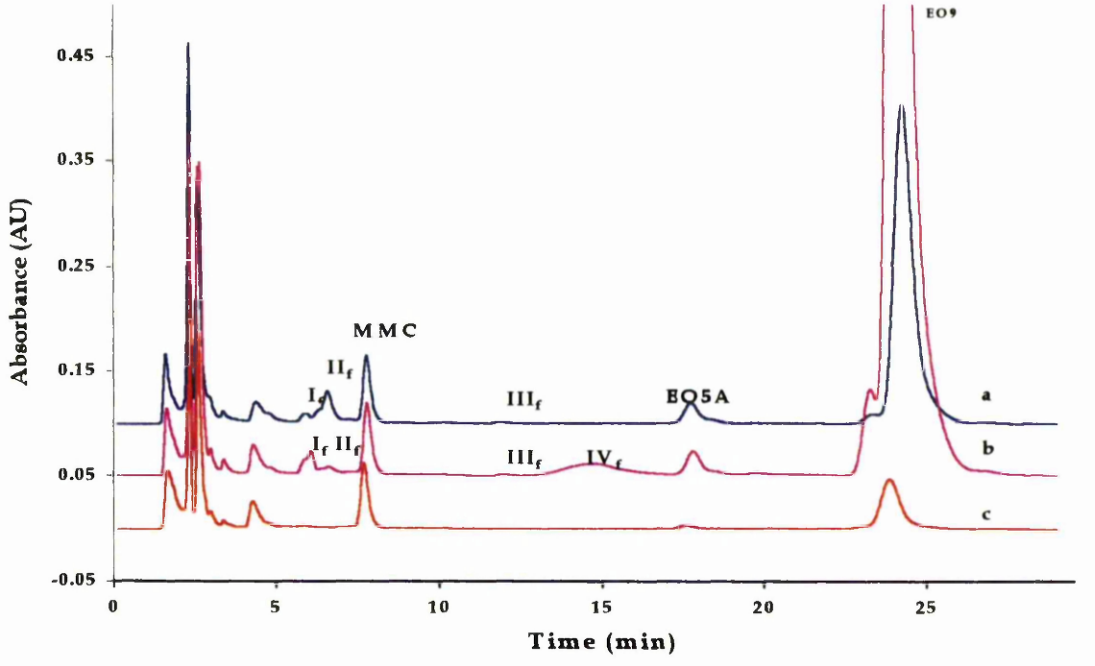


Figure 5.13 HPLC analysis of HT29 tumour homogenate at a) t = 0, b) t = 15 min, and c) t = 24 hours, following intratumoural administration of 250μg free EO9. In addition to the hydrolysis product EO5A, 4 additional peaks were identified at 5.8 min (Peak I_f), 6.6 min (Peak II_f), 11.9 min (Peak III_f), and 14.7 min (Peak IV_f).

	Free EO9				*EO9-loaded Microspheres*			
Peak	I_f	II_f	III_f	IV_f	I_m	II_m	III_m	IV_m
Retention Time (minutes)	5.8	6.6	11.9	14.7	3.4	6.2	11.7	13
Time Period (hours)	0 - 2	0 – 45 minutes	0 – 4	0 - 2	0 – 24	0 – 6	0 – 30 minutes	4 - 24
EO9/EO5A Resemblance	EO5A	No	EO5A	EO5A	No	EO5A	EO5A	EO5A
Proposed Identity	Aziridinyl Adduct	?	Aziridinyl Adduct	Aziridinyl adduct	Protein Drug Adduct	Aziridinyl Adduct	Aziridinyl adduct	Aziridinyl adduct

Table 5.1 **Summary of the peaks identified together with their proposed identity in the HT29 tumour homogenate, following *in vivo* intratumoural injection of 250μg Free EO9 or EO9-loaded microspheres.**

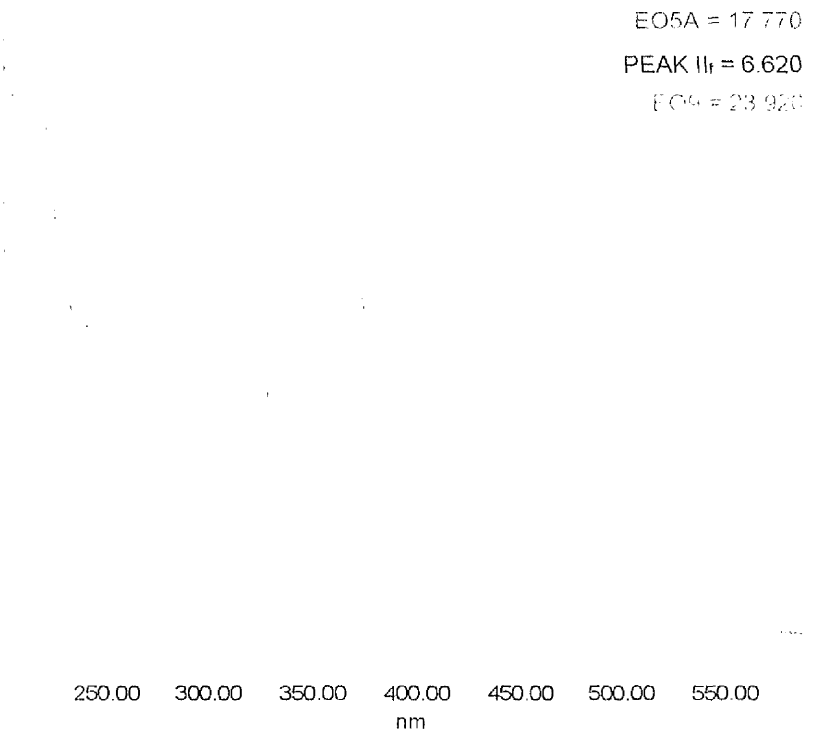


Figure 5.14 **Spectral analysis of EO9, EO5A and Peak II$_f$ in the HT29 tumour homogenate following intratumoural administration 250μg free EO9. The retention time (in minutes) for each peak is shown on the graph.**

5.4.2.1.2 Chromatographic analysis of the HT29 tumour following administration of EO9 microspheres

EO9 and the hydrolysis product, EO5A, were present throughout the time course of the study. The EO5A level remained fairly constant for the first 2 hours and then began to fall, although more slowly than the EO9, so that the proportion of EO5A relative to EO9 increased. By 18 hours, unlike the free drug group, the height of the EO5A peak was greater than the EO9 peak. In addition to the EO9 and EO5A peaks, definite peaks were also detected at 3.4 minutes (Peak I_m), 6.2 minutes (Peak II_m), 11.7 minutes (Peak III_m) and 13 minutes (Peak IV_m) when compared to the control sample (Figures 5.12 & 5.15 and Table 5.1). Although the peak at 3.4 minutes (Peak I_m) was present at a relatively constant level over the 24 hours of the study, it remained small. On UV spectral analysis, it was not similar to either EO9 or EO5A (Figure 5.16) and was not present in the analysis of the tumour treated with free drug. The peak at 6.2 minutes (Peak II_m) was also present from t_0. It remained at a constant level for the first hour and then its height began to fall relative to the MMC peak (internal standard). It was not detectable after 6 hours. The UV spectrum of this peak was similar to the spectra of EO5A (Figure 5.16). The broad peak detected at 11.7 minutes (Peak III_m) was present from t_0 but was not detected after 30 minutes. It had an identical UV spectrum to that of EO5A and was similar to Peak III_f in the free drug group. By 4 hours a further broad peak (Peak IV_m) had developed at 13 minutes which was present throughout the remaining time points. This again had a UV spectrum identical to that of EO5A.

5.4.2.1.3 Chromatographic analysis of HT29 plasma following administration of EO9 and EO9 microspheres

In both the free drug group and the microsphere group, only EO9 and EO5A were identified at low concentrations in the plasma.

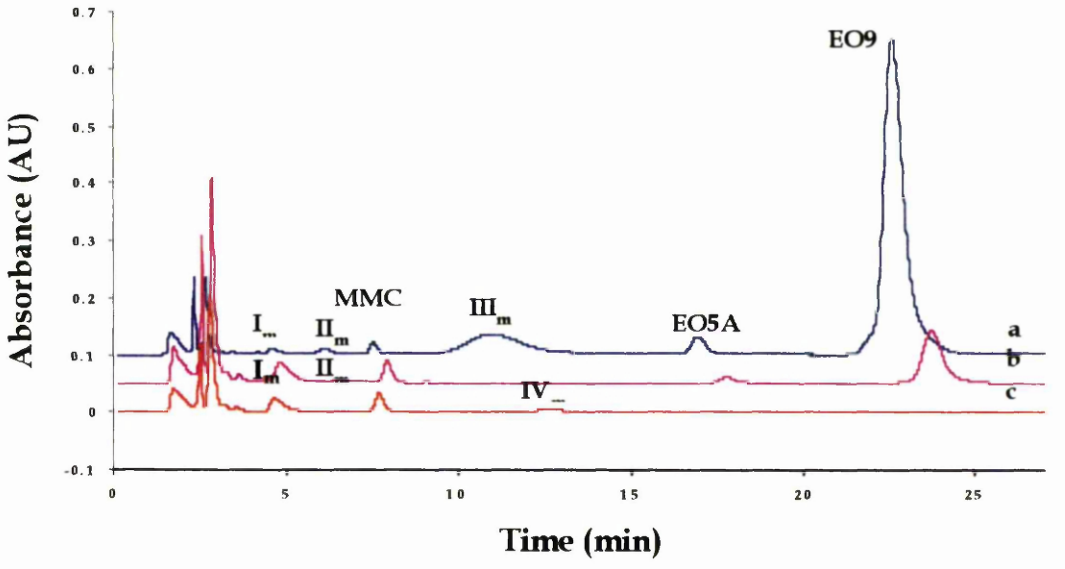


Figure 5.15 HPLC analysis of HT29 tumour homogenates at: a) t = 10 min, b) t = 2 hr, and c) t = 24 hr, following intratumoural administration of 250μg EO9 in albumin microspheres. In addition to EO9 and the hydrolysis product EO5A, 4 additional peaks were identified at 4.2 min (Peak I_m), 6.2 min (Peak II_m), 11.7 min (Peak III_m) and 13 min (Peak IV_m).

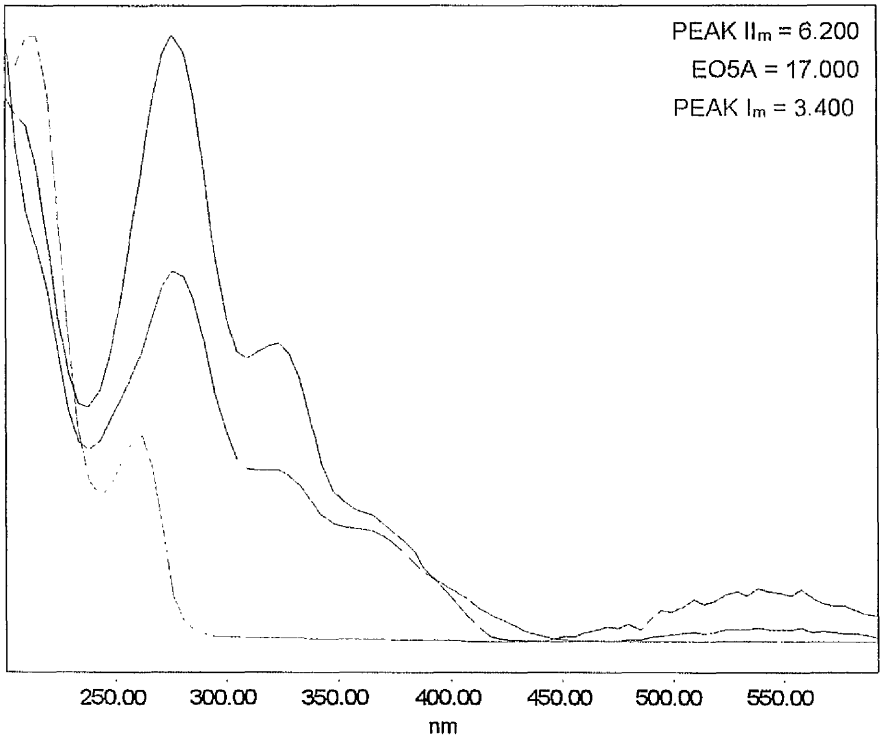


Figure 5.16 **Spectral analysis of EO5A, Peak I_m and Peak II_m in the HT29 tumour homogenate following intratumoural administration of 250μg EO9 in albumin microspheres. The retention time (in minutes) for each peak is shown on the graph.**

5.4.2.2 BE xenograft

5.4.2.2.1 Chromatographic analysis of the BE tumour following administration of free EO9

EO9 and EO5A were present throughout the 24 hour time course of the experiment (Figure 5.17). Their retention times occurred earlier than for the HT29 tumours, at 20.1 and 15.6 minutes respectively. This was likely to be related to the age of the column used for the HPLC analysis as retention times are known to shorten as the column ages. The ratio of EO9 to EO5A remained similar throughout the time course of the experiment and was similar to that seen in the HT29 tumour treated with free drug. In addition to the EO9 and EO5A, further peaks were identified at 4 minutes (Peak 1_f), 6 minutes (Peak 2_f) and 12.5 minutes (Peak 3_f) (Figure 5.17 and Table 5.2). The peak at 4 minutes (Peak 1_f) was detected from 5 minutes onwards and was present up to 1 hour. It remained a very small peak at all time points. Spectral analysis showed that it was not similar to EO9 or EO5A (Figure 5.18). The peak at 6 minutes (Peak 2_f) was present from t_0 and present for the first 6 hours of the study. Its peak height was greatest in the first 15 minutes of the study and fell thereafter. Its spectrum was similar to but not identical to that of EO9. The peak at 12.5 minutes (Peak 3_f) was present from 15 minutes but absent after 6 hours. Its spectrum was different to that of EO9 or EO5A, having no absorption present in the visible spectrum range (Figure 5.18). This peak had not been previously identified in the HT29 tumour treated with EO9.

5.4.2.2.2 Chromatographic analysis of the BE tumour following administration of EO9 microspheres

EO9 and EO5A were present throughout the analysis with retention times similar to the free EO9 study at 20 minutes and 15.7 minutes respectively.

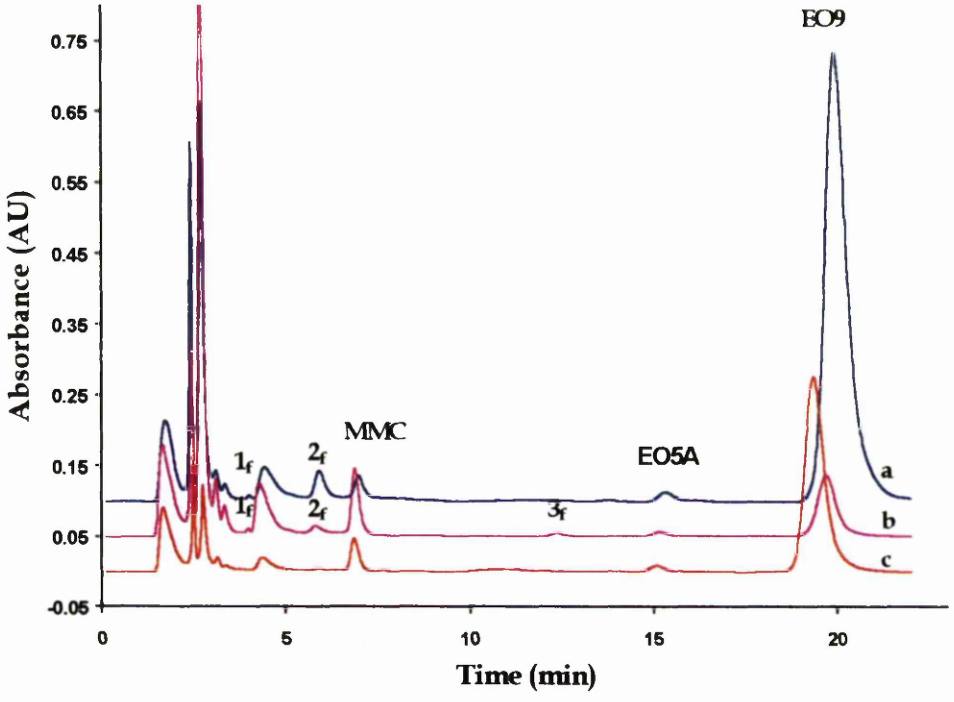


Figure 5.17 HPLC analysis of BE tumour homogenates at a) t = 5 min, b) t = 1 hr, and c) t = 18hr, following intratumoural administration of 250μg free EO9. In addition to EO9 and the hydrolysis product EO5A, 3 additional peaks were identified at 4 min (Peak 1_f), 6 min (Peak 2_f), and 12.5 min (Peak3_f).

	Free EO9			*EO9-loaded Microspheres*			
Peak	1_f	2_f	3_f	1_m	2_m	3_m	4_m
Retention Time (minutes)	4.0	6.0	12.5	4.0	6.4	12	23.5
Time Period (hours)	5 minutes – 1 hour	0 – 6 hours	15 minutes – 6 hours	0 – 6 hours	0 – 4 hours	15+30 minutes	30 minutes – 24 hours
EO9/EO5A Resemblance	No	EO9 like	No	No	EO9 like	EO5A	EO5A
Proposed Identity	Drug/Protein Adduct	Hydroquinone adduct	?	Protein Drug adduct	Hydroquinone Adduct	Aziridinyl adduct	Aziridinyl adduct

Table 5.2 **Summary of the peaks identified, together with their proposed identity, in the BE tumour homogenate following *in vivo* intratumoural injection of 250μg free EO9 or EO9-loaded microspheres.**

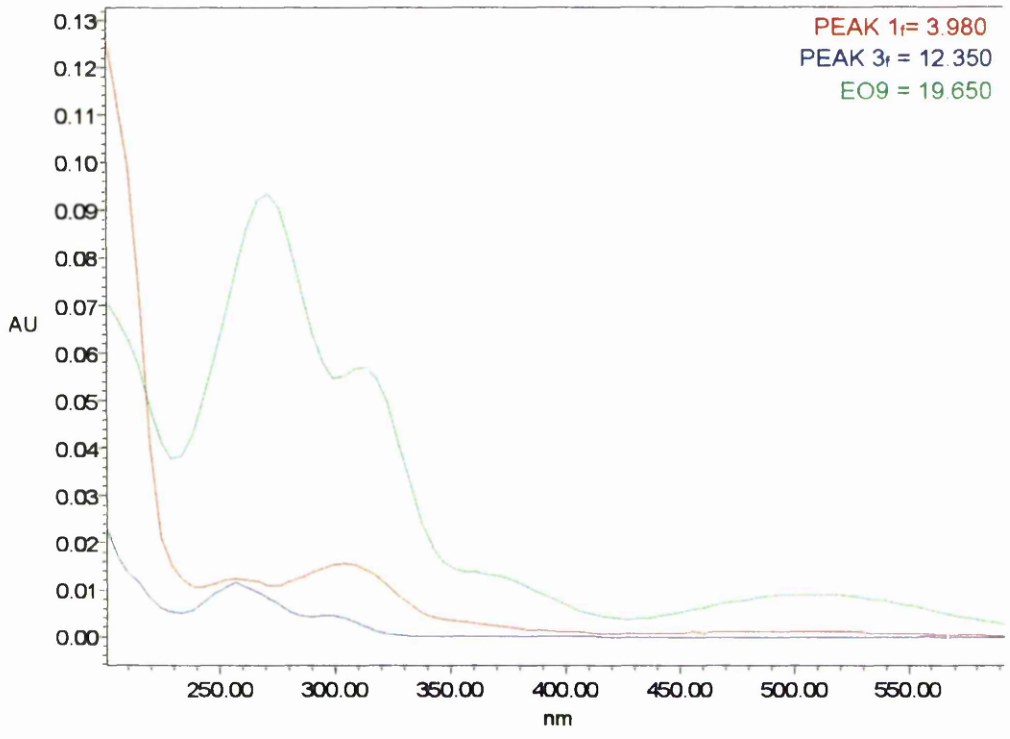


Figure 5.18 **Spectral analysis of EO9, Peak 1_f and Peak 3_f in the BE tumour homogenate following intratumoural administration of 250μg free EO9. The retention times (in minutes) for each peak are shown on the graph.**

The peak height of EO5A remained fairly constant over the first 6 hours of the study and then began to fall but more slowly than the EO9 peak, with the result that, as for the microsphere-treated HT29 tumour, the ratio of EO5A increased relative to the EO9. Further consistent peaks were identified at 4 mins (Peak 1_m), 6.4mins (Peak 2_m), 12 mins (Peak 3_m) and 23.5 mins (Peak 4_m) (Figure 5:19 and Table 5.2). The peak at 4 minutes (Peak 1_m) was detected from t_0 until 6 hours. Spectral analysis confirmed it to be identical to Peak 1_f in the free drug study (Figure 5.20). The peak at 6.4 minutes (Peak 2_m) was present from t_0 until 4 hours. Like the free drug study it was present at its maximum height for the first 15 minutes and then the peak height fell. Its spectrum was identical to the spectrum of Peak 2_f in the free drug study suggesting that it was an EO9 related product (Figure 5.20). The peak at 12 minutes (Peak 3_m) was detected at 15 and 30 minutes only. Its peak height remained very small, with a spectrum similar to that of EO5A. The broad peak at 23.5 minutes (Peak 4_m) was detected from 30 minutes and was present until the end of the study. Spectral analysis also confirmed this to be similar to EO5A.

5.4.2.2.3 Chromatographic analysis of BE plasma following administration of EO9 and EO9 microspheres

The only peaks identified in the plasma of both the free drug and the microsphere treated tumours were EO9 and EO5A, an identical result to that seen in the HT29 xenograft.

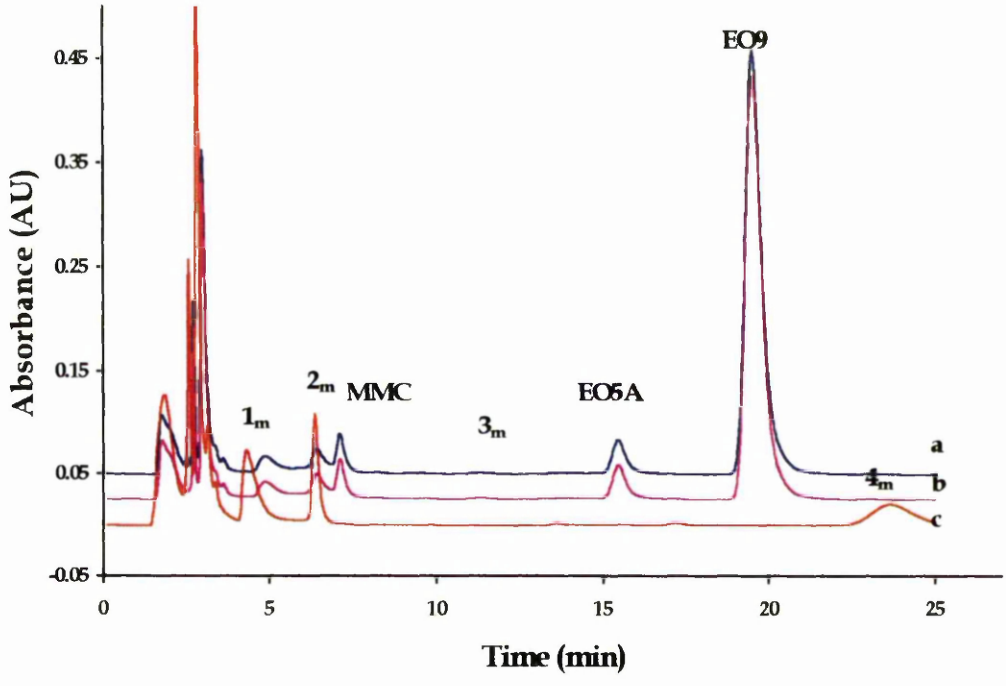


Figure 5.19 HPLC analysis of BE tumour homogenates at: a) t = 0, b) t = 30 min, and c) t = 18 hr, following intratumoural administration of 250μg EO9 in albumin microspheres. In addition to EO9 and the hydrolysis product EO5A, 4 additional peaks were identified at 4 min (Peak 1_m), 6.4 min (Peak 2_m), 12 min (Peak 3_m), and 23.5 min (Peak 4_m) (Peak1_m and 2_m on chromatograph c corresponds on spectral analysis to the control peak at 5 minutes and the MMC peak on chromatographs a and b respectively. This is due to the earlier retention times of these peaks on chromatograph c).

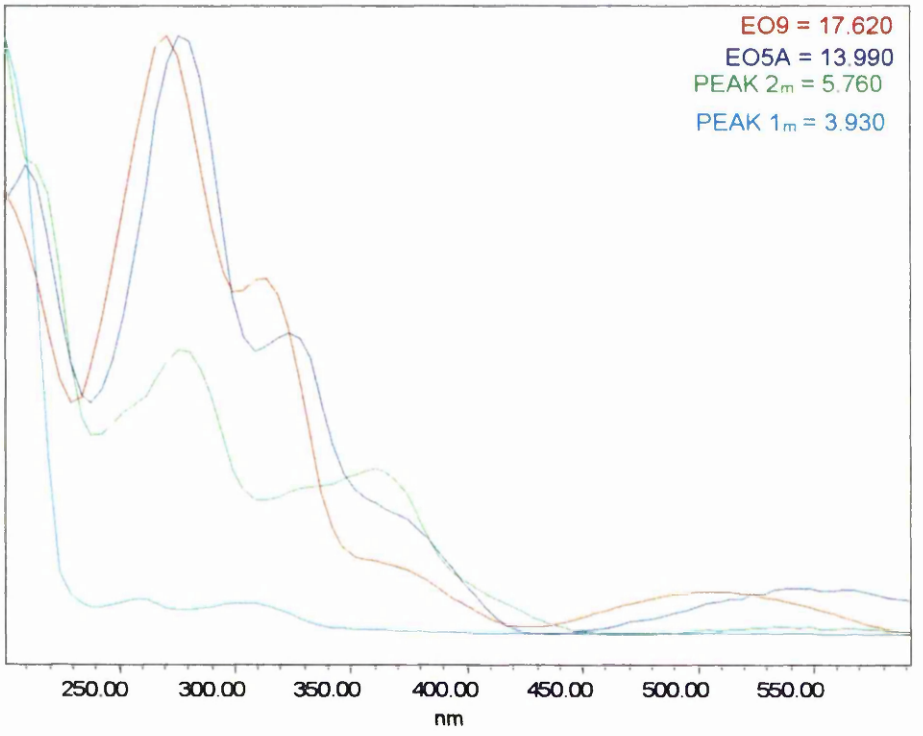


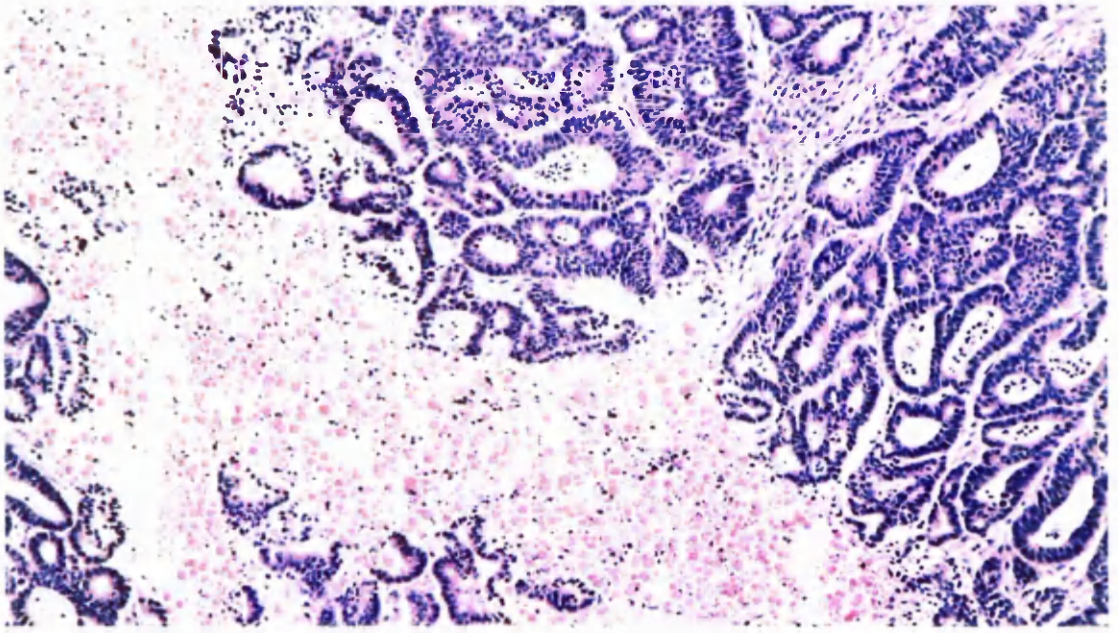
Figure 5.20 **Spectral analysis of EO9, EO5A, Peak 1_m and Peak 2_m in the BE tumour homogenate following intratumoural administration of 250μg EO9 in albumin microspheres. The retention times (in minutes) for each peak are shown on the graph.**

5.4.3 Timed Histological Assessment of Tumours following Direct Intratumoural Microsphere Injection

The results for each of the tumour types were similar and therefore only the MAC 26 tumour will be discussed. As indicated previously (Chapter 4.5), the MAC 26 tumour is a well differentiated, well vascularised tumour. This was clearly seen throughout the time course of the study in both the microsphere treated and the control group (Figure 5.21). The microspheres could be detected throughout the 14 days of the study, and do not appear to undergo rapid breakdown and digestion by the inflammatory cells present within the tumour.

Immediately following the intratumoural injection of the microspheres disruption of the architecture of the tumour is seen (Figure 5.21A). This was followed by the rapid accumulation of inflammatory cells both around and within the site of the microspheres within the tumour (Figure 5.21B). An increase in stromal tissue around the microspheres then developed which suggested that the inflammatory cell component was attempting to "wall off" the microspheres from the tumour tissue (Figure 5.21C). No changes were seen in the tumour following the intratumoural injection of PBS/0.5% Tween 80 (Figure 5.21D), which suggested that the changes which were seen relate to the microspheres rather than the intratumoural injection process itself.

(A)



(B)

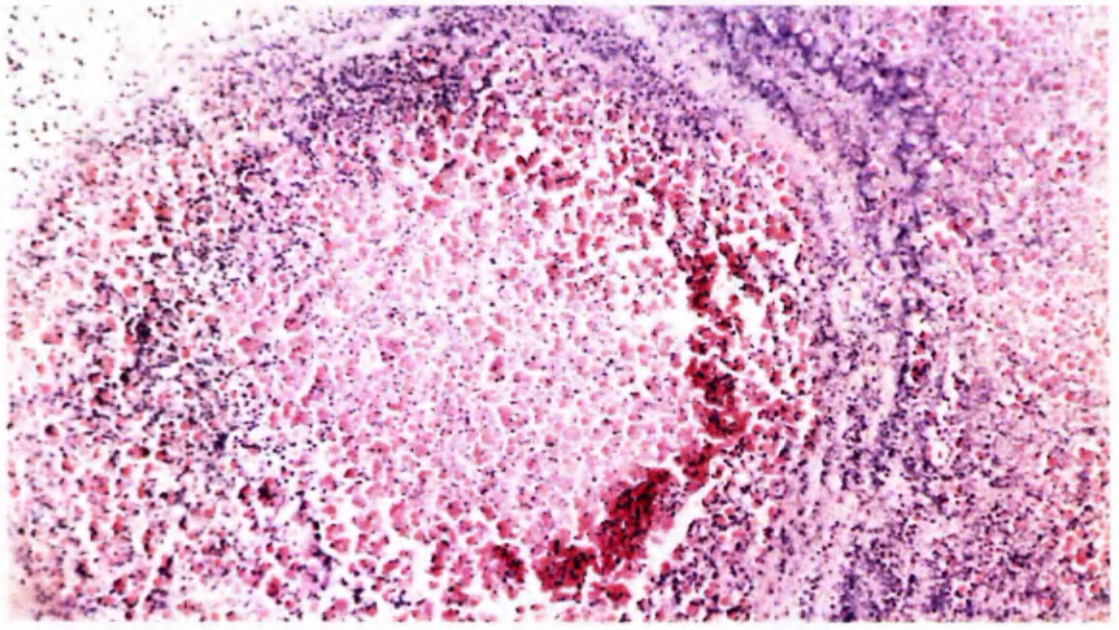
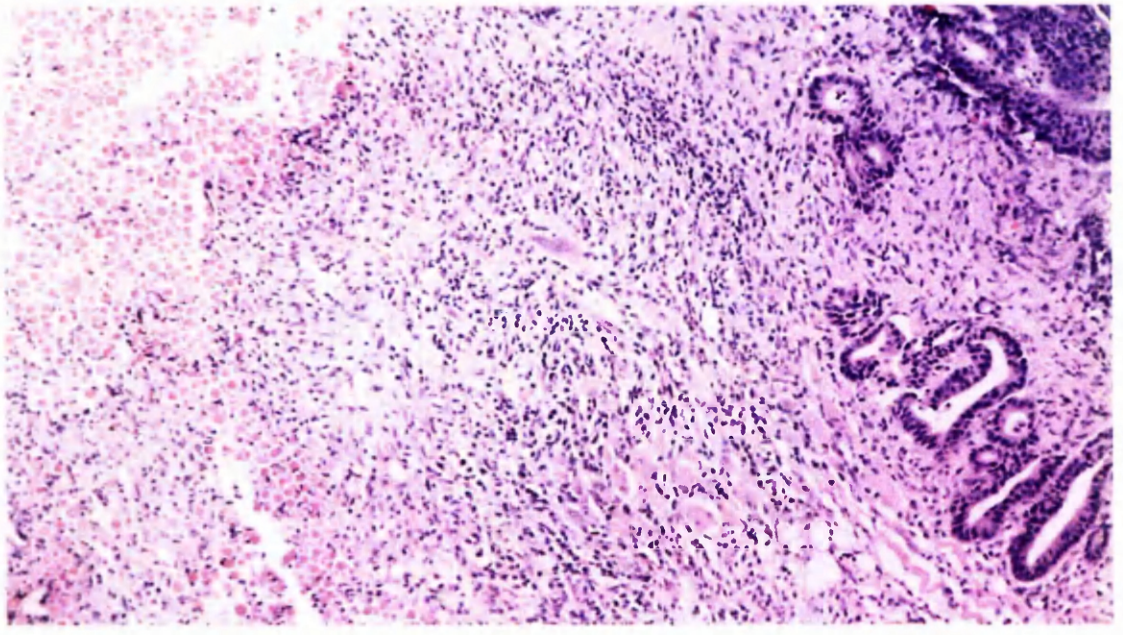


Figure 5.21 **Timed histological assessment of the MAC 26 tumour following the *in vivo* intratumoural administration of blank albumin microspheres. Comparison of tumour at A) Time 0 and B) Day 2. The microspheres are clearly visible on the left side of slide A and in the centre of slide B.**

(C)



(D)

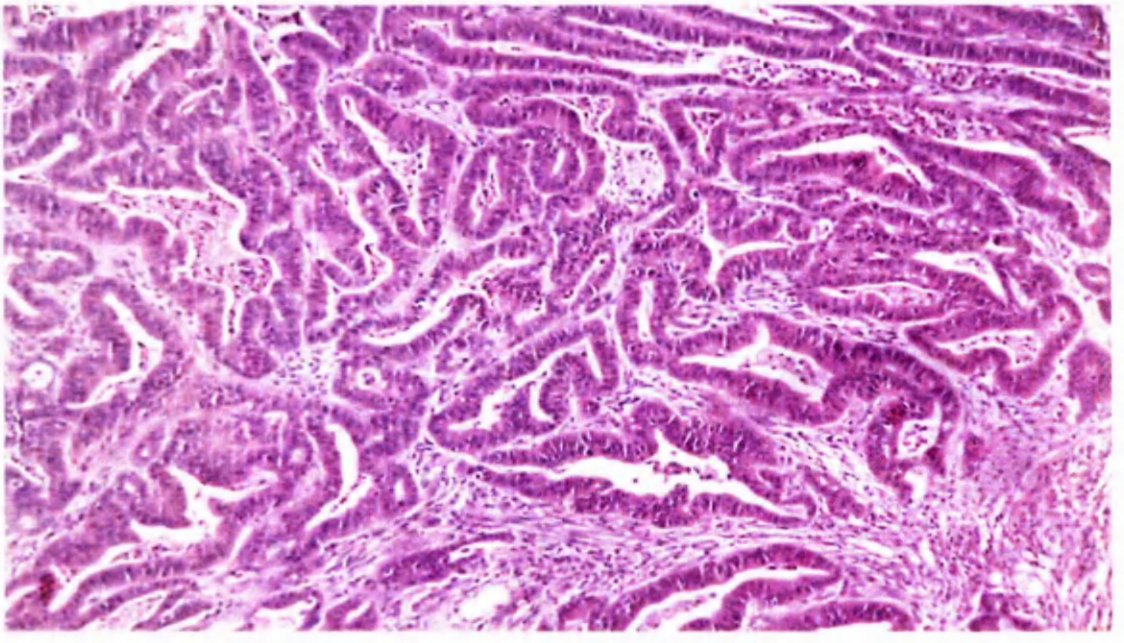


Figure 5.21 **Timed histological assessment of the MAC 26 tumour following the *in vivo* intratumoural administration of blank albumin microspheres. Comparison of tumour at C) Day 14 and, D)PBS/0.5% Tween 80 control Day 14. The microspheres are still clearly visible on the left side of slide C.**

5.5 DISCUSSION

The aim of the studies presented in this chapter was to compare the pharmacokinetics of free EO9 and EO9-loaded microspheres *in vivo* following intratumoural injection in each of the tumour types in order to determine whether encapsulating the drug into microspheres altered the pharmacokinetic profile. The presence of any potential EO9 metabolites during the study were also assessed to see if there was any relationship between metabolite formation *in vivo* and antitumour activity.

The results from the pharmacokinetic studies show that the experiments failed to detect any significant difference in drug levels within the tumour between the free drug and the microspheres. This was found in all four tumour types. These results therefore suggest that the antitumour activity which was seen was independent of the amount of EO9 which was detected within the tumour. This would be in keeping with previous *in vitro* studies which have shown that only very low levels of the drug are actually required for antitumour activity (Hendriks et al 1993, Workman et al 1992C).

There was also no evidence of significantly sustained drug release in the tumours treated with the microspheres, despite the persistence of the microspheres within the tumour (5.4.3). This agrees with the *in vitro* characterisation work in Chapter 3.4.3 where the drug was found to undergo rapid release from the microspheres. Since it is the peak concentration of drug which is thought to be important for its mode of action, as discussed in Chapter 4.5, this characteristic is perhaps less important.

The results which were obtained in these studies were much more variable than those obtained in previous antitumour studies (Cummings et al 1994, Spanswick 1996, Willmott et al 1987A). These previous studies had, however, involved relatively stable drugs such as

Adriamycin and MMC. EO9's known instability may therefore be an important factor in explaining some of the variability in the results which were obtained. In this respect, care was taken at all stages in the study to try and maintain as stable an environment as possible for the drug, both in terms of storage and in preparation for analysis.

Several problems were identified during this study which require further discussion. One of the most consistent features in each of the experiments was that although 250μg of EO9 was injected directly into each tumour, a mean of only 4.8 ± 2.9 % of the original dose of EO9 was actually detected at t_0 on tumour analysis. There was no significant difference between tumours treated with free drug or with drug-loaded microspheres.

One factor which may have contributed to this low result was the fact that t_0 was not an immediate sample. T_0 refers to the earliest time point at which plasma and tumour could be sampled. Drug was injected, the mouse killed by CO_2 asphyxia and subsequently bled by open cardiac puncture. The tumour specimen was then removed. Clearly a finite amount of time (approximately 2-3 minutes) will have passed from the intratumoural injection before sample collection is completed. This short delay may allow time for rapid metabolism of the drug to occur, such that by the time the tumour has been removed, the majority of the drug has been metabolised. This would be in agreement with previous *in vivo* studies using EO9 where rapid drug loss following systemic administration was demonstrated both in C3H/He mice ($t_{1/2}$ of 1.9 minutes) and Sprague-Dawley rats ($t_{1/2}$ of 3.0 minutes). No drug was detected in the tissues of either animal following systemic administration of EO9, presumed to be the result of rapid drug metabolism (Workman et al 1992C). Similarly, in the phase I study, rapid drug metabolism was thought to occur based on the short, though variable $t_{1/2}$ of 0.8-19 minutes (Schellens et al 1994, Smitskamp-Wilms et al 1996).

However, previous studies using intratumoural drug injections of MMC and MMC-loaded microspheres have shown equally low results (Cummings et al 1994, Spanswick 1996) which suggests that this may be a feature of the technique rather than being simply due to rapid drug metabolism or instability, although in the case of EO9, this will certainly be a contributory factor. One of the potential reasons for the low level of drug detection may be related to the intratumoural injection technique itself. It is not possible to know following the injection how much of the drug has actually been injected intratumourally and how much has been placed peritumourally. This will be of particular relevance in small and necrotic tumours. Although a defined tumour size was used in all the experiments to try and minimise this effect, it is still likely to be important. Perhaps attempting to analyse the surrounding tissues as well as the tumour following the EO9 injection may give some idea of how much drug is lost in this way.

The intra-group variation seen at each time point was often large, which made comparison of the results difficult. An alternative approach which was tried was to plot the median results from each group in order to see if any definite pattern emerged, although of course, no statistical analysis of these results could be attempted.

In the human xenograft tumours each experiment was repeated to try to determine whether the results seen in the first experiment were reproducible. Unfortunately this was not the case with the repeat experiments producing different results, which were again not statistically significant due to the intra-group variation.

The results from experiments 1 & 2 were then combined so that in essence there were six animals per time point rather than three, in order to determine whether by increasing the number of animals at each time point any pattern emerged. The presence of large error bars again meant that only large differences in drug levels would be detected, so that the

combined results still failed to demonstrate any significant difference between the free drug and the microspheres in both types of tumour.

These results are different to those seen using MMC-loaded microspheres (Cummings et al 1994). Because of the wide variability in the results obtained in these experiments, it was impossible to calculate any pharmacokinetic parameters such as half-life ($t_{1/2}$) or area under the curve (AUC). Studies with the MMC microspheres showed that although the level of drug exposure was similar for both free drug and microspheres, the pharmacokinetic profile was quite different (Cummings et al 1994). However, the characteristics of the MMC and the EO9 microspheres are different. In particular, as can be seen in Chapter 3.4.3, the rate of drug release from the EO9 microspheres occurs much more rapidly than from the MMC microspheres (Allan et al 1993). Therefore, one might anticipate that there may not be such a great difference in the pharmacokinetic profile between the free EO9 and the EO9-loaded microspheres compared to the MMC study where the microspheres did demonstrate more of a sustained release characteristic. Despite concerns about the variability and reproducibility of the studies, it was felt that given the characteristics of the EO9 microspheres, a difference in tumour pharmacokinetic profile between the free drug and the microspheres might not have been anticipated.

The results obtained from plasma analysis are also difficult to assess because, like the tumour results, they are not consistent. There is the suggestion that the microsphere treated groups may have more sustained plasma levels than the free drug groups in the HT29 and MAC16 tumours. This is in contrast to the results obtained with the MMC microspheres where lower levels of MMC were seen in the plasma of the microsphere-treated MAC 16 group (Cummings et al 1994). No relationship was seen between plasma levels and blood supply. One might have expected that those tumours with the better blood supply, such as the MAC 26 tumour, would have higher levels of drug detectable in the plasma compared to

the more necrotic MAC 16 tumour, as has been shown in previous *in vivo* studies using MMC (Spanswick 1996).

Analysis of the chromatographic data produced during the pharmacokinetic study is limited. This is because of the lack of any available data for the metabolites including EO5A, in terms of both standard curves and extraction efficiencies. Previous *in vitro* studies have also failed to detect any metabolites which may be indicative of EO9 activation (Cummings et al 1998).

In addition to EO9 and the hydrolysis product EO5A, four other peaks were identified in the HT29 tumour treated with free EO9 (I-IV_f) and four peaks in the HT29 tumour treated with EO9-loaded microspheres (I-IV_m). Similarly in the BE tumour, three additional peaks were identified in the tumour treated with free EO9 (1-3_f) and four peaks in the tumour treated with EO9-loaded microspheres (1-4_m). It was noted that in both tumour types the microsphere-treated groups had some peaks which were detected over a longer time course (24 hours) compared with the free drug treated groups (4-6 hours). Some of these peaks were common to 2 or more of the groups, though each group had at least 1 peak which was not seen in any other groups. The UV spectra of these individual peaks were often similar suggesting that, although not identical, they were related. The properties of these peaks were summarised in Tables 5.1 and 5.2.

On analysis of these peaks, Peak I_m in the HT29 microsphere treated group was similar but not identical to Peaks 1_f and 1_m (which were identical) in the BE free drug and microsphere-treated groups, with retention times of 4.2 and 4.0 minutes respectively. This peak was not identified in the free drug treated HT29 tumour. Peaks 2_m and 2_f were also identical in the BE microsphere and free drug treated groups with retention times of 6.4 and 6.0 minutes respectively. No similar peaks were identified in the HT29 treated tumours.

The remaining peaks in all the tumours except the BE free drug group peak at 12.5 minutes have spectra identical to that of EO5A. Some of these peaks were present for 24 hours in the microsphere-treated groups but were only detected for up to 6 hours in the free drug groups.

When these results were compared with the *in vitro* studies mentioned previously (Cummings et al 1998), several similarities were noted. In addition to EO5A, the peak at 4 minutes in the *in vitro* study appeared to correlate in spectral analysis, with peaks I_m, 1_f and 1_m (in the microsphere-treated HT29 tumour and the microsphere and free EO9 treated BE tumour) in our study. The peak *in vitro* at 8 minutes appeared to be similar to Peaks 2_f and 2_m (in the free EO9 and microsphere-treated BE tumour) in our study. The other peaks identified *in vivo* were not seen *in vitro*.

Based on the spectra and retention times of the peaks which were identified together with information obtained from previous *in vitro* work in tumour homogenates and using pulse radiolysis (Spanswick 1996), the possible identities of some of the peaks can be discussed. Peaks I_m, 1_f and 1_m are possibly drug-protein adducts based on the fact that protein has an absorption maxima similar to that of these peaks. The lack of visible absorption may be a consequence of the absorption spectrum of the protein masking that of the adducted EO9 metabolite. Peaks 2_m and 2_f in the BE studies may be intermediate products based on the fact that they are only present in the first part of each study. The spectrum, while similar in part to EO9, lack any visible absorption which has been demonstrated to be characteristic of certain hydroquinone intermediates (Cummings et al 1998), and suggests that they may be hydroquinone adducts. All the other peaks have spectra identical to EO5A which is characteristic of an open aziridine ring product. The variation in retention times seen for these peaks suggests variation in the water soluble nature of the aziridinyl adducts which are formed. It should also be remembered that these products are formed by the process of

hydrolysis rather than by true EO9 metabolism and that in this study they form the majority of the peaks which are seen.

It would be interesting to try to formally identify these metabolites but this is likely to be difficult and may not yield any further information on EO9's activity. High Performance Liquid Mass Spectroscopy has been used to try to identify the metabolites of EO9 following pulse radiolysis (a technique which allows controlled drug reduction to occur without the requirement for enzymes and cofactors (Salmon et al 1993), but this has been unsuccessful because the majority of EO9 ended up as products which were not detectable on analysis (Spanswick 1996).

In conclusion, these results show that no one metabolite can be identified which is related to EO9 activation, which is in agreement with previous *in vitro* studies (Cummings et al 1998) and no significant difference in tumour pharmacokinetics could be detected between those treated with the free drug and those treated with the microspheres.

CHAPTER 6

The development of a relevant tumour model to study the *in vivo* antitumour effects of EO9-loaded albumin microspheres

6 Chapter 6

6.1 INTRODUCTION

Previous studies have shown (Chapter 4.4.2) that in the HT29 tumour, the intratumoural injection of 250μg of free EO9 was equivalent in activity to the same dose of EO9 administered in microspheres. However, in the MAC 16 tumour twice the EO9 dose was required in microsphere form to give the same effect as free drug. In the MAC 26 and the BE tumours, despite antitumour activity being demonstrated with free drug, no activity was seen following the administration of EO9 microspheres. This suggested that, apart from in the HT29 tumour, encapsulation of EO9 into microspheres lead to a reduction rather than the desired enhancement of the drug's antitumour activity. The pharmacokinetic analyses, described in Chapter 5, failed to provide an explanation for the differences which were seen, both between tumour types and between the free drug and the microsphere-treated tumours.

A major concern with regard to the antitumour studies was that they compared the antitumour effect of free drug and microspheres once they were physically at the tumour site. The direct intratumoural injection of a drug such as EO9 will maximise its antitumour effect while at the same time will minimise the problems associated with its systemic administration. However, direct intratumoural injection may not be the best method of assessing the antitumour effect of a potential drug delivery system such as the microspheres.

It has been suggested that the lack of antitumour effect following systemic administration of EO9 in the Phase II studies (Dirix 1996, Pavlidis et al 1996) was due to the drug's instability and resultant short half-life. Renal toxicity prevented the dose of EO9 from being increased to overcome this problem (Schellens et al 1994, Verweij et al 1994). EO9's instability meant

that following systemic administration the amount of drug reaching the tumour would be a fraction of the administered dose. This has been confirmed by *in vivo* studies where following intravenous administration of 12mg/kg of EO9 in C3H/He mice, no drug could be detected in the tumour by HPLC analysis (Workman et al 1992C). Systemic administration of 6mg/kg of EO9 on four consecutive days in nude mice also failed to produce an antitumour effect in the HT29 tumour model (Collard et al 1995) in comparison to the studies of intratumoural administration where, the HT29 tumour was found to be sensitive to EO9 (Chapter 4.4.2.2.2). This difference in effect is clearly related to the method of drug administration.

The rationale for encapsulating EO9 into microspheres was to administer the drug locoregionally so that, unlike conventional intravenous administration, EO9 would reach the tumour in high enough concentration to have an antitumour effect whilst minimising systemic exposure to the drug and thus toxicity. The possible induction of hypoxia as a result of the administration of microspheres (as previously discussed: Chapter 1.8.3.2.6) may further enhance the drug's activity due to EO9's bioreductive activation.

Therefore, the microspheres should be assessed in a model system where they can be administered in a clinically relevant way. The rat has been used previously in studying the effects that the administration of drug and drug delivery systems given via the hepatic artery can have on tumour deposits within the liver. These studies have, amongst others, looked at the antitumour activity of MMC-loaded albumin microspheres (Fugimoto et al 1985A, 1985B, Morimoto et al 1989), Adriamycin loaded microspheres (McArdle et al 1988) and degradable starch microspheres (Teder et al 1993, 1995), as well as the relationship between tumour blood flow and microsphere administration (Anderson et al 1991B, 1992). The rat model was considered particularly useful in view of the potential for administration

via the hepatic artery mimicing the likely eventual clinical use, i.e. of intrahepatic arterial administration for liver metastases.

It was initially intended to use a rat colon tumour, WB2054M (a dimethylhydracin induced colonic rat adenocarcinoma), which was established *in vivo* in the Wistar rat (University of Edinburgh) from its cell line, which was obtained from the European collection of animal cell cultures. However, spontaneous regression of the tumour within the liver in this particular strain of Wistar rat meant the model had to be abandoned. It was therefore decided to use a rat model system which was well established elsewhere. This was the HSN sarcoma cell line (originally induced in a male Lister Hooded rat with 3-4-benzpyrene (Currie et al 1973)) grown in the Lister Hooded rat (Anderson et al 1991B, 1992). There was no information available on the sensitivity of this particular tumour to EO9 *in vitro* or *in vivo*. Similarly, there was no published data concerning the systemic effects and potential toxicity of EO9 in the Lister Hooded rat. Previous work in Sprague-Dawley rats had safely administered 3mg/kg (18mg/m^2) i.v. without any immediate toxicity. However, the animals treated at this dose were part of a pharmacokinetic study which ended at 24 hours, so there was no information available on late toxicities (Workman et al 1992C). Further studies in the Wistar rat had looked at a single intravenous dose of 0.45mg/kg and subsequently weekly doses of 0.45mg/kg for 4 doses, with no obvious long term toxicity other than local irritation at the injection site (Hendriks et al 1993).

This chapter therefore describes the preliminary studies which were carried out to assess:

i) the *in vitro* sensitivity of the HSN tumour to EO9.
ii) the *in vivo* sensitivity of the HSN tumour to EO9.
iii) the growth of the HSN tumour in the liver of Lister Hooded rats.
iv) the effect of EO9 on tumour bearing Lister Hooded rats.
v) the initial intrahepatic administration of EO9 on tumour bearing Lister Hooded rats.

6.2 MATERIALS

All reagents, equipment and suppliers used are listed in Appendix 1.

6.2.1 *In Vitro* Tumour Model

The HSN rat sarcoma cell line was kindly supplied by Dr Sue Eccles, Institute for Cancer Research, Sutton, Surrey via Dr Alison Wood, University Department of Surgery, Glasgow Royal Infirmary, Glasgow, UK. It was easily grown as a monolayer culture in Dulbeccos Modified Eagles Medium (DMEM) supplemented with 10% foetal calf serum (FCS). Penicillin-Streptomycin solution was added to the media at a final concentration of 100 IU to reduce bacterial contamination. The cells were grown and maintained in $75cm^3$ tissue culture flasks at 37^0C in a humidified atmosphere of 95% oxygen and 5% carbon dioxide (Scotlab VSL incubator). All procedures involving the cell line were carried out under sterile conditions in M.D.H Interlab Class II tissue culture hoods.

6.2.2 *In Vivo* Tumour Models

6.2.2.1 HSN rat sarcoma in Nu/nu mice

Nu/nu mice (originally bred at Imperial Cancer Research Fund (ICRF), London) were supplied by Harlan UK Ltd or the ICRF unit at Clare Hall, London. The HSN rat sarcoma, was implanted subcutaneously using $4x10^6$ HSN cells in 500µl of serum-free media into the flank of each mouse. The histology of the tumour was checked by the Department of Pathology, Western General Hospital, Edinburgh. The Nu/nu mice were housed in Morden isolators, fed on RM3(E) mouse diet treated by irradiation and given free access to water. Once established, the xenograft was maintained by serial subcutaneous passage of 1-3 mg of viable tumour via a trochar needle to animals weighing between 20 and 25g. When tumours

reached 0.1-0.5cm^3 animals were randomised for experimental studies or sacrificed for collection of tumour material.

6.2.2.2 Lister Hooded rats

Lister Hooded rats were obtained from Charles Rivers or from Harlan UK. The rats were kept under standard laboratory conditions of heating and lighting, fed a standard rat diet (CRM(E)) and were given access to water ad lib.

6.3 METHODS

6.3.1 *In Vitro* analysis of HSN sensitivity to EO9

6x10^6 HSN cells were added to 18x12.5cm^3 flasks containing 5mls of DMEM/FCS and placed in the incubator for 72 hours. The media was then changed and EO9 added in the following concentrations: 10^{-5}M, 10^{-6}M, 10^{-7}M, 10^{-8}M and 10^{-9}M with a control flask containing media only. Each concentration of EO9 and the control were set up in triplicate. After 24 hours the EO9 was removed, the flasks rinsed with sterile PBS and 5mls of fresh DMEM/FCS added to each flask. After a further 48 hours in the incubator, each flask was trypsinised with 1ml of trypsin (0.25%):versene(1mM EDTA, 0.5% phenol red) (in a ratio of 1:1). Once the cells had detached from the surface of the flask trypsinisation was neutralised by the addition of 2 mls of DMEM/FCS and the cell suspension transferred into a universal container. 200µl of this suspension was placed in a coulter counter pot containing 9.8mls normal saline to allow counting to take place.

6.3.1.1 *Analysis of cell counts*

Each sample was analysed three times with the coulter counter and the mean result obtained. The background count, from a control pot containing saline alone, was subtracted from this result to obtain the mean count for each sample. Once each sample had been counted the mean count for each concentration of EO9 was calculated. The mean cell count for each treated group (T) was then divided by the control group value (C) and expressed as a percentage in order to allow the IC_{50} (the concentration causing 50% growth inhibition compared to the control) for EO9 to be determined.

6.3.2 Dose Finding Studies to Determine HSN Tumour Sensitivity to EO9 *in vivo* in Nu/nu Mice

HSN bearing Nu/nu mice were randomised into four groups each containing seven animals, which were then treated by direct intratumoural injection with 200µl of PBS/0.5% Tween 80, either alone as a control group or containing 125µg, 250µg or 500µg of EO9. Measurement of the tumours was carried out every second day for a total of 21 days. Tumour volume was determined by caliper measurement (see Chapter 4.3.1.1) and calculated using the formula: 0.5 x length x width^2. The animals were sacrificed once the study was completed.

The antitumour activity for each group was determined using the following formula:

$$\frac{\text{Mean tumour value (cm}^3\text{) day x}}{\text{Mean tumour value (cm}^3\text{) day o}} \times 100\%$$

The results from each group were compared using the unpaired students t-test.

6.3.3 Assessment of Antitumour Activity of EO9-loaded Albumin Microspheres on the HSN Tumour *in vivo* in Nu/nu Mice

HSN bearing Nu/nu mice were randomised into five groups containing 6-10 animals per group. The groups were treated with 200μl of PBS/0.5% Tween 80 containing 125μg EO9, EO9-loaded microspheres containing the equivalent of 125μg or 250μg EO9, blank microspheres or PBS/0.5% Tween 80 only.

The microspheres were prepared as described in Chapter 2.4.3 (Table 2.3), but each batch was reconstituted in a different amount of PBS/0.5%Tween 80 so that the EO9 dose given did not affect the overall injection volume. Measurement was carried out and antitumour activity determined as described previously. The results from each group were compared using the unpaired students t-test.

The initial experiment produced unsatisfactory results (see below, Chapter 6.4.3) and was therefore repeated using smaller tumour volumes ($\leq 0.2cm^3$) at t_0.

6.3.4 Implantation of HSN Tumour Cells in the Liver of the Lister Hooded rat

The HSN cells were grown in tissue culture, as described previously, until they had reached late log phase. They were harvested immediately prior to the implantation procedure. Following trypsinisation and washing, the cell pellet was vigorously resuspended in 20mls of DMEM and the cell number counted using an haemocytometer. The cell suspension was centrifuged again at 2000r.p.m and the resulting cell pellet resuspended in serum-free media to obtain a final concentration of $5x10^6$ cells per ml.

The rat was anaesthetised by intraperitoneal injection with a combination of midazolam (2mg/ml)/ sterile water/Hypnorm (Fentanyl Citrate (0.315mg/ml) and Fluanisone (10mg/ml)) in a final ratio of 1ml:2mls:1ml at a dose of 3.3mls/kg. Once anaesthetised, a midline abdominal incision was performed and the liver identified. A suspension of $1x10^6$ HSN cells in 200µl of serum-free media was placed by subcapsular injection using a 21 gauge Microlance needle into the median and left lobes of the liver (one innoculation per lobe). The liver was seen to blanch following the injection. Prior to removal of the needle a cotton bud soaked in 70% absolute alcohol solution was placed over the exit site (to try to minimise peritoneal spread) and maintained in position for 1-2 minutes until the bleeding had stopped. This was subsequently replaced by Fibrinogen/Thrombin combination sealant. The incision was closed using standard Mersilk sutures for the muscle layer and 12x2.5mm surgical clips for the skin layer. The rats were then given butorphanol (2mg/kg) subcutaneously as pain relief and allowed to recover.

6.3.5 Assessment of HSN Tumour Growth within the Liver

Following the implantation of tumour cells within the liver of the Lister Hooded rats, as described above (6.3.4), the animals were observed daily for signs of distress relating either to the surgical procedure or the presence of tumour within the liver itself. The rats were culled at defined time points (1, 2, 3, 4 & 5 weeks depending on the experiment) following the procedure. Detailed inspection of the abdominal cavity was then carried out to look for the presence of tumour within the liver as well as for evidence of tumour spread. The liver was then removed and the weight and volume of any tumour present within each lobe documented. The mean tumour volume was determined for each time point and results compared using the unpaired student t-test.

6.3.6 The Effect of Systemic EO9 on the Tumour Bearing Lister Hooded Rat

HSN cells were prepared *in vitro* and implanted in the liver in eighteen Lister Hooded rats as described previously. The rats were divided into three groups containing six rats per group. Each group was then treated by tail vein injection with either 400µl of PBS/0.5% Tween 80 alone or containing EO9 at a dose of 3 (18mg/m^2) or 6 (36mg/m^2) mg/kg. This dose was based on available preclinical data. The animals were observed daily for signs of toxicity following the tail vein injection and a post mortem examination was performed on any animal which died and any obviously affected organs removed for histological assessment.

Due to initial problems with toxicity, a further twelve Lister Hooded rats were divided into three groups of four and treated in an identical manner with 2mg/kg (12mg/m^2), 1mg/kg (6mg/m^2) or 0.5mg/kg (3mg/m^2) EO9 in the same injection volume of 400µl PBS/0.5% Tween 80. The animals were culled 14 days following the tail vein injection.

Once a satisfactory dose of EO9 to be used had been determined (2mg/kg - this was the highest dose which could be administered with no toxic deaths), twenty-four Lister Hooded rats were divided into 2 groups and treated 7 days after tumour implantation by tail vein injection with either 400µl of PBS/0.5% Tween 80 as the control group, or EO9 (2mg/kg) in PBS/0.5% Tween 80. The animals were observed daily. Six animals from each group were culled at 3 weeks and the remaining six animals from each group at 4 weeks post tumour implantation (i.e. at 2 and 3 weeks post tail vein injection). The livers from each animal were then examined for tumour presence and size. The tumour means (based on tumour volume) were calculated at two and three weeks for both the control group and the EO9 group and plotted against time. Because each rat had two tumours present, the number of tumours present in each group was double the number of rats in each group. The tumour means were then compared using an unpaired students t-test.

6.3.7 Feasibility Study on the Administration of Blank Albumin Microspheres Via the Hepatic Artery

In order to determine the feasibility of administering the microspheres via the hepatic artery and to establish the optimal dose of microspheres for safe administration, blank human albumin microspheres were manufactured and prepared as described previously in Chapter 2.4.3 (Table 2.3).

Ten Lister Hooded rats were divided into four groups of two or three rats per group. The rats were anaesthetised as described previously (Chapter 6.3.4) and following a midline abdominal incision, the hepatic artery was identified. Each group of rats received via the hepatic artery either 5mg of blank albumin microspheres resuspended in 250μl or 500μl of PBS/0.5% Tween 80 or 10mg of blank microspheres resuspended in 250μl or 500μl of PBS/0.5% Tween 80, via a 26 gauge Microlance needle. Immediately following removal of the needle, the hepatic artery was sealed with Fibrinogen/Thrombin combination sealant. The incision was closed using continuous standard Mersilk sutures for the muscle layer and surgical clips for the skin layer. The rats were then given butorphanol (2mg/kg) subcutaneously as pain relief and allowed to recover. Following recovery the rats were observed daily for 7 days and then culled.

6.3.8 The Effect of EO9-loaded Microspheres Administered via the Hepatic Artery on HSN Tumour in the Lister Hooded Rat. Comparison with Free Drug and Blank Microspheres

HSN cells were prepared *in vitro* and implanted in the liver in sixteen Lister Hooded rats. On this occasion however, all the cells ($2x10^6$) were implanted in a single site in the left lobe of the liver in an attempt to improve the consistency of tumour growth.

Ten days following tumour implantation the rats underwent a second laparotomy. The rats who had definite tumour visible in the implanted left lobe were divided into four groups on the basis of the treatment they were to receive via the hepatic artery. The control group received 500µl of PBS/0.5% Tween 80 and the other groups received 500µl of PBS/0.5% Tween 80 containing 5mg of blank albumin microspheres, 5mg EO9-loaded albumin microspheres (containing the equivalent of 60 ± 6µg EO9) or 100µg free EO9. Following administration the hepatic artery was sealed with Fibrinogen/Thrombin glue. The midline incision was closed using continuous standard Mersilk sutures for the muscle layer and surgical clips for the skin layer. The rats were then given butorphanol (2mg/kg) subcutaneously for pain relief and allowed to recover.

Following recovery the rats were observed daily for any signs of distress. Any surviving animals were to be culled at 21 days following tumour implantation, the livers removed and the tumour size assessed.

6.4 RESULTS

6.4.1 *In Vitro* Analysis of HSN Sensitivity to EO9

The HSN cell line was shown to be sensitive to EO9 with a marked fall in cell numbers as the concentration of EO9 increased (Table 6.1 & Figure 6.1). The IC_{50} can be estimated from the graph as 4nM EO9 which is comparable with other cell lines such as HT29 (IC_{50} of 6.7nM) (Walton et al 1992A).

6.4.2 Dose Finding Studies to Determine HSN Tumour Sensitivity to EO9 *in vivo* in Nu/nu Mice

EO9 demonstrated significant antitumour activity against the HSN tumour in Nu/nu mice when administered intratumourally. There was a trend towards an increase in antitumour activity with an increase in the EO9 dose (Figure 6.2). When compared with the control group, using the unpaired students t-test, there was significant growth delay ($p<0.05$) at all doses of EO9 from day two until the end of the experiment.

Therefore, a dose of 125μg of EO9 was chosen for future experiments because it produced significant growth delay and was not associated with any overt toxicity (two toxic deaths were recorded in the 500μg EO9 group).

EO9 concentration (nM)	*Mean Cell Count (Standard Deviation)*	*Treated/Control (%)*
0	3596 (962)	100
1	3413 (1004)	95
10	638 (211)	18
100	101 (17)	2.8
1,000	8.0 (3.2)	0.22
10,000	5.3 (1.2)	0.15

Table 6.1 **The Mean (and Standard Deviation) cell counts and calculated Treated/Control (T/C) values for HSN cells treated *in vitro* for 24 hours with varying concentrations of EO9 (nM).**

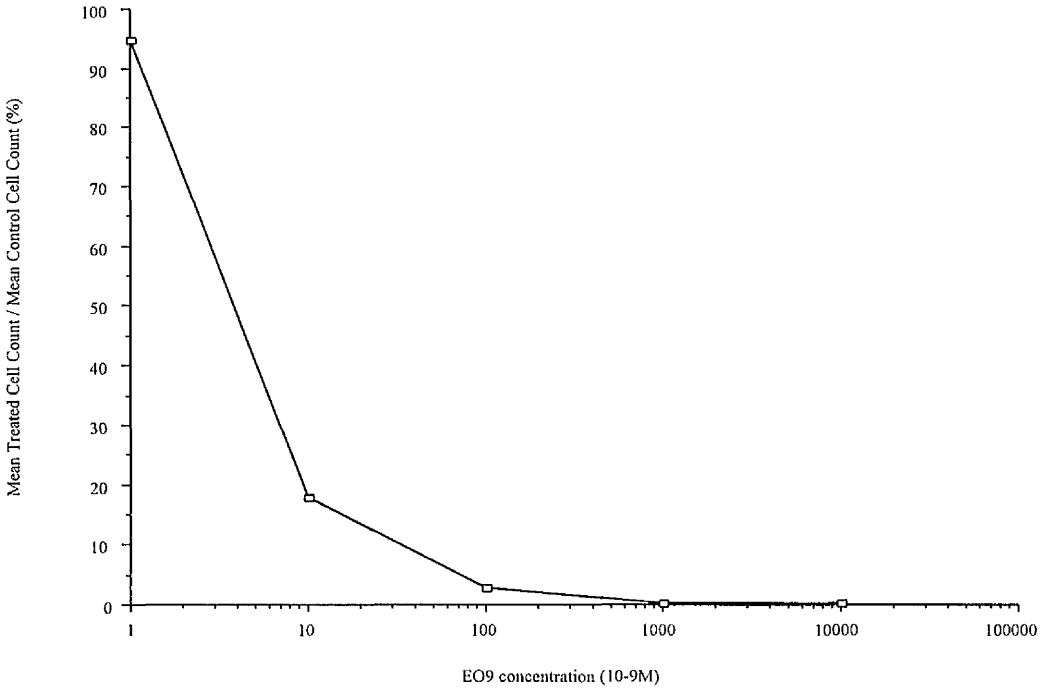


Figure 6.1 **The effect of EO9 concentration (nM) on the growth of HSN cells *in vitro*. The cell counts are expressed as a percentage of the mean treated cell count divided by the mean control cell count (T/C).**

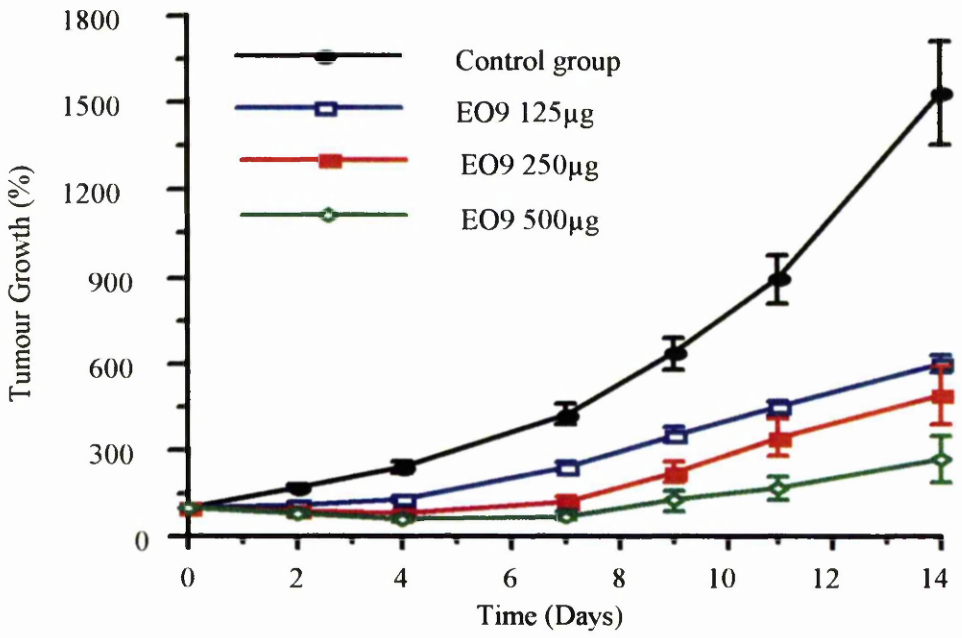


Figure 6.2 **Dose finding study to determine the antitumour activity of free EO9 given by direct intratumoural injection on the HSN rat sarcoma cell line growing subcutaneously in the Nu/nu mouse. Each time point denotes the mean ± standard error (SE) for the group. Growth delay is significant from day 2 onwards ($p<0.05$) in all groups treated with EO9 compared to the control group.**

6.4.3 Assessment of Antitumour Activity of EO9-loaded Albumin Microspheres on the HSN Tumour *in vivo* in Nu/nu Mice

The first experiment was terminated at 14 days instead of the intended 21 days due to rapid tumour growth in all groups. Indeed, only 1-2 animals in each group (of the original ten animals) survived to 14 days. There were no significant differences detected between the control group (PBS/0.5% Tween 80) and any of the treatment groups which included blank microspheres, free EO9 and EO9-loaded microspheres (Figure 6.3).

These results were not consistent with those of the EO9 dose finding study (6.4.2) where a significant antitumour effect had been detected following the administration of 125μg EO9. On further analysis of the results, it was felt that the tumours had been too advanced at the beginning of the experiment (90% tumours were > than $0.2cm^3$ at t_0) and had entered a phase of rapid growth. The increased tumour size may have also reduced the EO9 concentration within the tumour which may have resulted in reduced tumour sensitivity and hence a decreased antitumour effect.

Therefore, this experiment was repeated with the initial tumour volume in each mouse $\leq 0.2cm^3$. This second experiment continued to 21 days in the control and blank microsphere groups, when animals had to be sacrificed due to tumour size and to 28 days in the other groups. In comparison to the control group, a significant difference in tumour growth from day 2 onwards was detected in all the EO9-treated groups (Figure 6.4). No significant difference in activity however, could be detected between the 250μg and the 125μg EO9 microsphere groups and the free EO9 group at any of the time points in the experiment. No significant difference in activity was detected between the control group treated with PBS/0.5%Tween 80 and the blank microsphere-treated group (Figure 6.4).

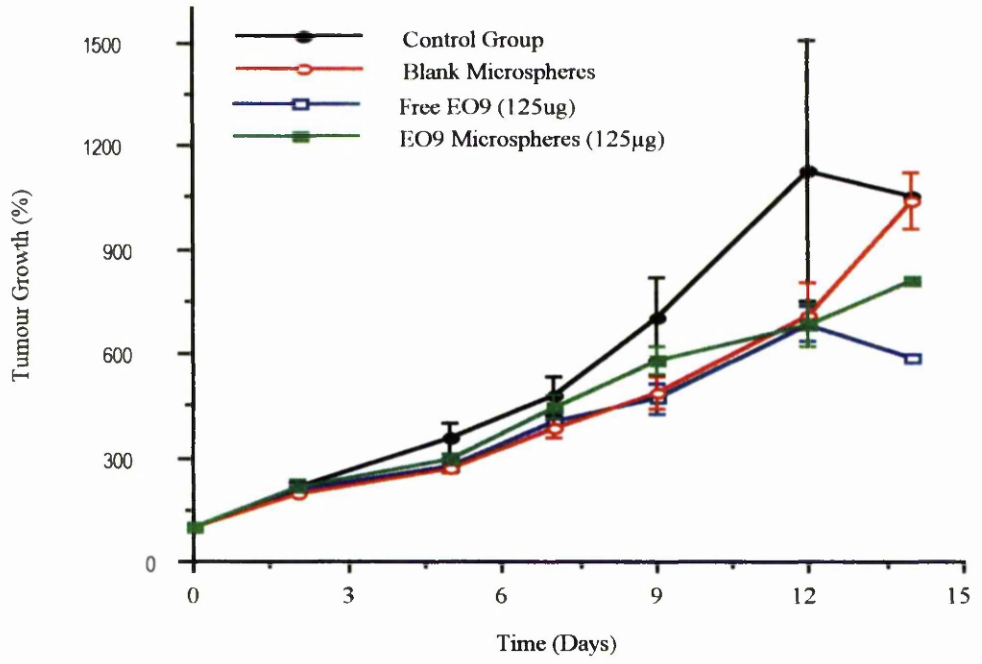


Figure 6.3 **Comparison of the antitumour activity of free EO9 with EO9-loaded albumin microspheres and blank albumin microspheres given by direct intratumoural injection on the HSN rat sarcoma cell line grown subcutaneously in Nu/nu mice. Each time point denotes the mean ± standard error for the group. No significant difference in tumour growth was detected in any of the treated groups when compared to the control group (PBS/0.5% Tween 80).**

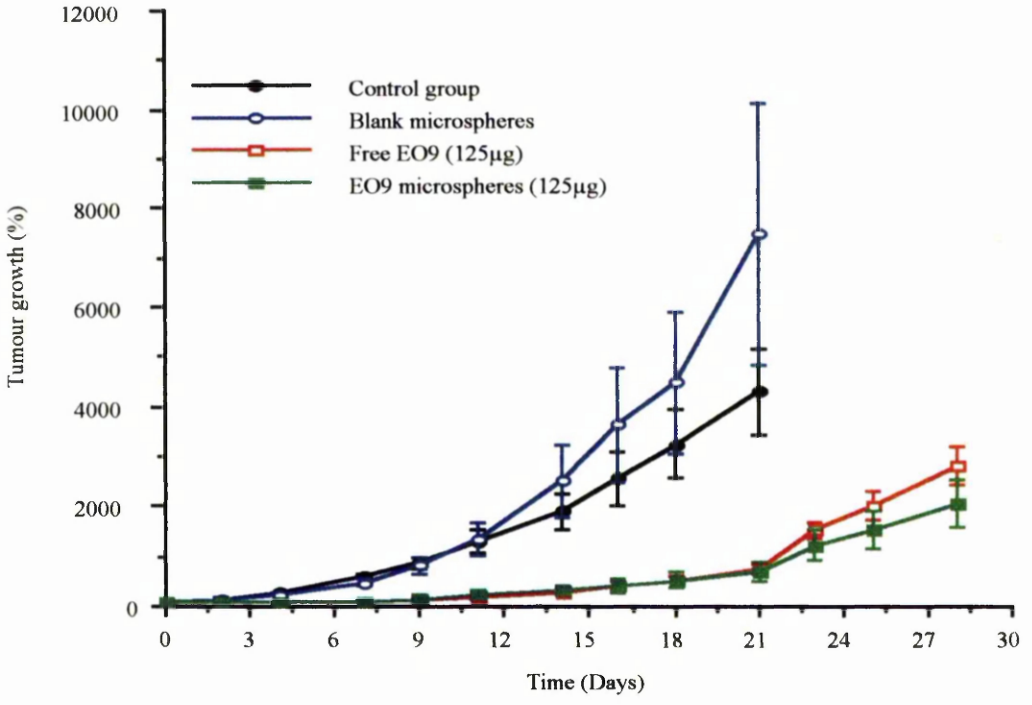


Figure 6.4 **Comparison of the antitumour activity of free EO9 with EO9-loaded albumin microspheres and blank albumin microspheres given by direct intratumoural injection on the HSN rat sarcoma cell line grown subcutaneously in Nu/nu mice. The tumour volume at t_0 was $\leq 0.2cm^3$ in each group. Each time point denotes the mean ± standard error for the group. Growth delay is significant from day 2 onwards ($p<0.05$) in all groups treated with EO9 (both free and microsphere) compared to the control groups (PBS/0.5% Tween 80 and blank microspheres).**

These results confirm the *in vitro* data and demonstrate, that the HSN tumour, when grown in the Nu/nu mouse, is sensitive to intratumoural administration of EO9. However no significant difference in antitumour activity was detected when free drug and the equivalent dose of EO9 microspheres were compared. This is similar to the result which was obtained for the HT29 xenograft (Chapter 4.4.2.2.2). The sensitivity of the HSN tumour to EO9 means that it is a suitable cell line to use in developing a more clinically relevant tumour model to study the antitumour effects of the EO9-loaded microspheres.

6.4.4 Implantation of HSN Tumour Cells in the Liver of the Lister Hooded rat

Initial experiments were performed to establish a surgical technique for implantation of the HSN tumour cells within the liver, including optimising the recovery of the rats from the anaesthetic.

Studies using Lister Hooded rats obtained from the supplier Charles River were unsuccessful for two reasons. The first was that the animals were very "anaesthetic sensitive". Using a standard anaesthetic regimen for rats, the initial experiments had a perioperative mortality rate of 60-80%. This was anaesthetic rather than surgery-related because some of the animals which died following the anaesthetic had not undergone any form of surgery. The second problem was that there appeared to be spontaneous tumour regression occurring 3-4 weeks following tumour implantation. This was shown in two separate experiments where tumour was found to increase in size from 2 weeks to 3 weeks and then decrease in size from 3 weeks to 4 weeks. This difference in tumour size between the third and fourth weeks was statistically significant in the first experiment (Figure 6.5).

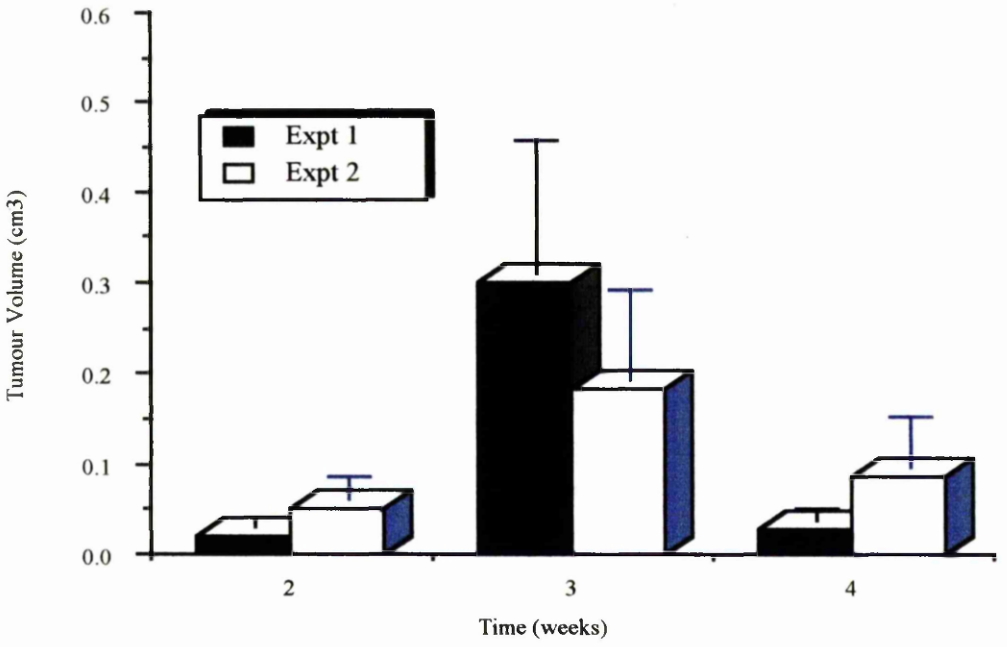


Figure 6.5 **The growth of $1x10^6$ HSN rat sarcoma cells implanted subcapsularly into the median and left lobes of the liver of the Lister Hooded rat. Each time point denotes the mean tumour volume ± standard error of 6-8 tumours. Tumour regression was seen between weeks 3 and 4, which reached statistical significance in Experiment 1 ($p<0.05$) in this particular substrain of rat.**

When the rats were obtained from a different breeder (Harlan, UK) the problem with anaesthetic sensitivity was not apparent (100% of animals survived the surgery) and spontaneous tumour regression was not seen. This difference was thought to be the result of using a different substrain of the Lister Hooded rat.

The surgical technique of subcapsular implantation of the HSN tumour cells in the liver was straightforward. Fibrinogen glue was found to be more useful in preventing leakage from the surface of the liver following needle removal than the 70% absolute alcohol solution which was initially utilised, both in terms of haemorrhage immediately post needle removal and tumour cell leakage in terms of the number of metastatic tumour sites seen at post mortem examination. There were no post-operative complications documented. The wounds healed well and the clips were removed uneventfully after 7 days.

6.4.5 Assessment of HSN Tumour Growth within the Liver

The growth of the HSN tumour within the liver was formally assessed in three separate experiments. In the initial experiment tumour size was assessed at 2 and 4 weeks post implantation with five animals present in each group (ten tumours for assessment). This confirmed that no tumour regression had occurred with the tumour at 4 weeks being significantly larger than at two weeks ($p<0.005$) (Figure 6.6). However, there was significant variation present in the size of the two tumours present within the same animal e.g. 1.10cm^3 and 0.01cm^3 in one rat at two weeks and 1.47cm^3 and 4.33cm^3 in one rat at 4 weeks.

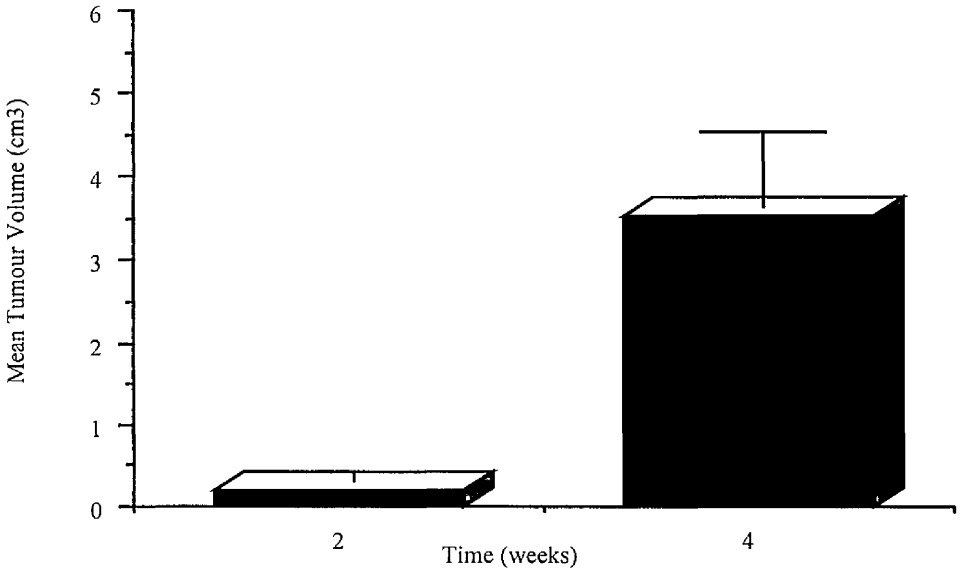


Figure 6.6 **The growth of $1x10^6$ HSN rat sarcoma cells implanted subcapsularly in the median and left lobes of the Lister Hooded rat at 2 and 4 weeks post implantation. Each value represents the mean tumour value ± standard error of 10 tumours. The mean tumour volume has increased significantly by 4 weeks compared to 2 weeks ($p<0.005$).**

In order to get an indication of the rate of tumour growth and to further compare the growth of tumour within the two lobes of the same animal, the experiment was repeated using sixteen animals with assessments at 2, 3, 4 and 5 weeks. These results confirmed the continued growth of the tumour with time, which was statistically significant by 5 weeks ($p< 0.05$) (Figure 6.7), as well as the variation in the size of the two tumours in the same rat e.g. tumour sizes of $0.13cm^3$ and $3.65cm^3$ at 3 weeks post tumour implant in one rat.

A further experiment using fifteen rats with all the cells injected into the left lobe ($2x10^6$) was then carried out just prior to the microsphere experiment. Animals were assessed at 1, 2 and 3 weeks in order to decide on the most appropriate time for antitumour treatment. These results confirmed both the variability seen between animals (calculated coefficient of variation of 108%, 114% and 42% at 1, 2 and 3 weeks post tumour implantation) as well as the continued growth of the tumour with time. Increasing the cell numbers, as might be expected, increased the size of the tumour (Figure 6.8).

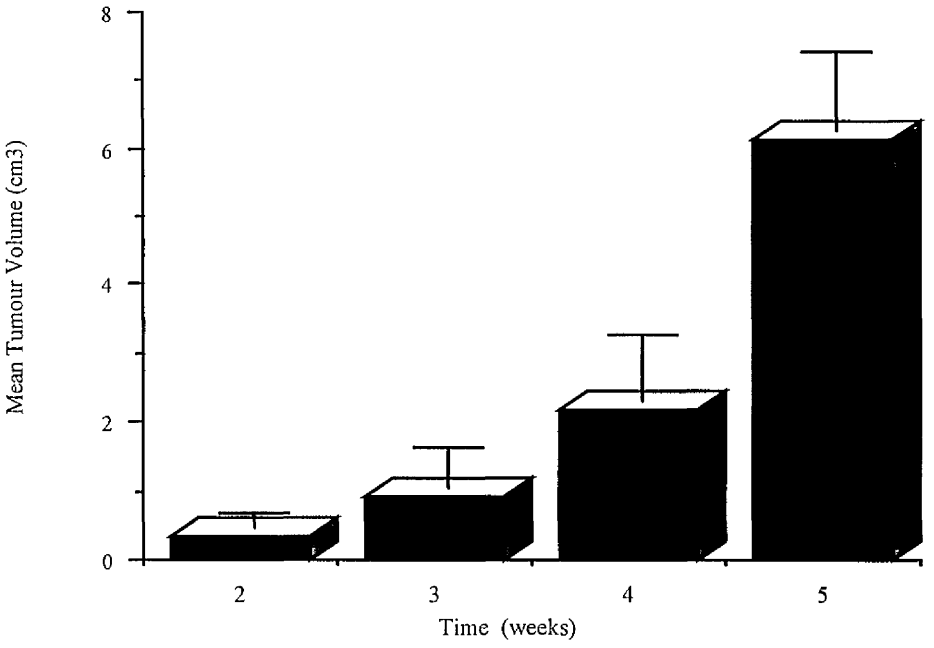


Figure 6.7 The growth of $1x10^6$ HSN rat sarcoma cells implanted subcapsularly in the median and left lobes of the Lister Hooded rat at 2, 3, 4, and 5 weeks post implantation. Each time point denotes the mean tumour volume ± standard error of 6-8 tumours. The increase in tumour volume was statistically significant by 5 weeks ($p<0.05$).

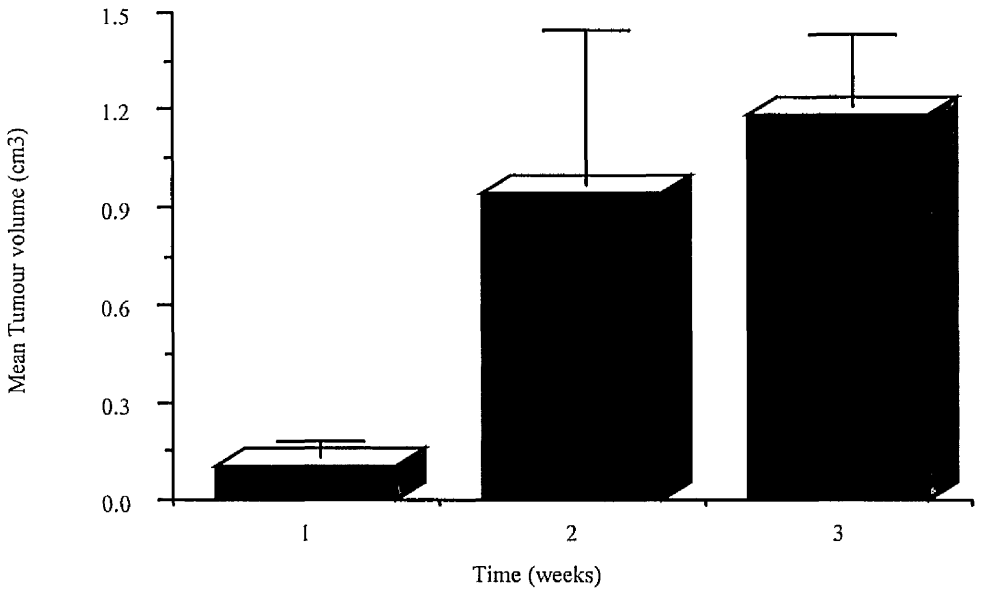


Figure 6.8 **The growth of $2x10^6$ HSN rat sarcoma cells implanted subcapsularly in the left lobe of the Lister Hooded rat at 1, 2 and 3 weeks post implantation. Each time point is the mean volume ± standard error of 5 tumours.**

6.4.6 The Effect of Systemic EO9 on Tumour Bearing Lister Hooded Rats

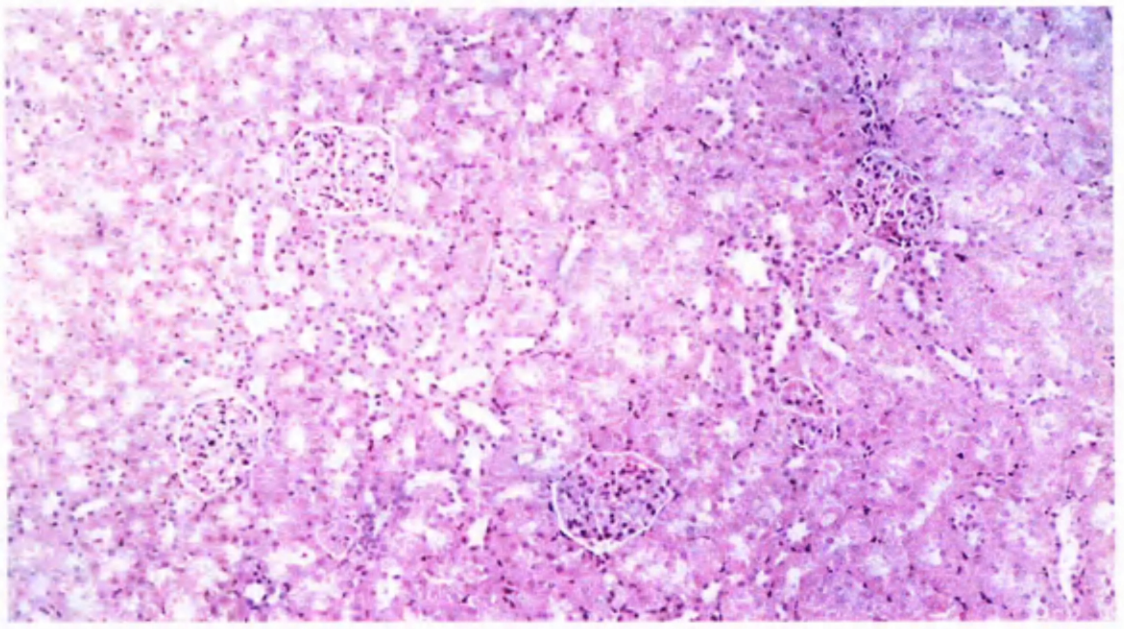
When the rats were treated with either PBS/0.5% Tween 80 alone or containing EO9 at 3 or 6mg/kg, there was no immediate toxicity obvious other than the previously documented local irritation at the injection site in the EO9-treated groups (Hendriks et al 1993). However, by 48 hours post injection all the animals in both EO9 groups were dead. The deaths were unexpected as there had been no evidence of any premorbid illness. There were no deaths in the control group which suggested that the cause of death was EO9-related. Post mortem examination was unremarkable except that the kidneys in the EO9-treated animals appeared paler than normal. Unfortunately, due to the timing of the deaths, histological analysis was not possible in the EO9-treated groups.

When the study was repeated with lower doses (2mg/kg, 1mg/kg and 0.5mg/kg) of EO9, there were no deaths in any of the groups and again no toxicity was seen other than local irritation at the injection site.

Following completion of the experiment at 2 weeks post-injection, histological analyses were performed on the kidneys of the rats treated with 2mg/kg EO9 and compared with the control group (PBS/0.5% Tween 80). No obvious differences were seen on Haematoxylin and Eosin (H+E) staining between the two groups (Figure 6.9). The dose of 2mg/kg was chosen for future experiments because this appeared to be the maximum tolerated dose.

When a larger antitumour study was performed with this dose of EO9 (2mg/kg) there was one death in the group treated with EO9 which occurred 5 days after the EO9 injection, but no other evidence of toxicity. Each group, including the control group, contained an animal which, at post mortem, had no evidence of any tumour present in either lobe of the liver.

(A)



(B)

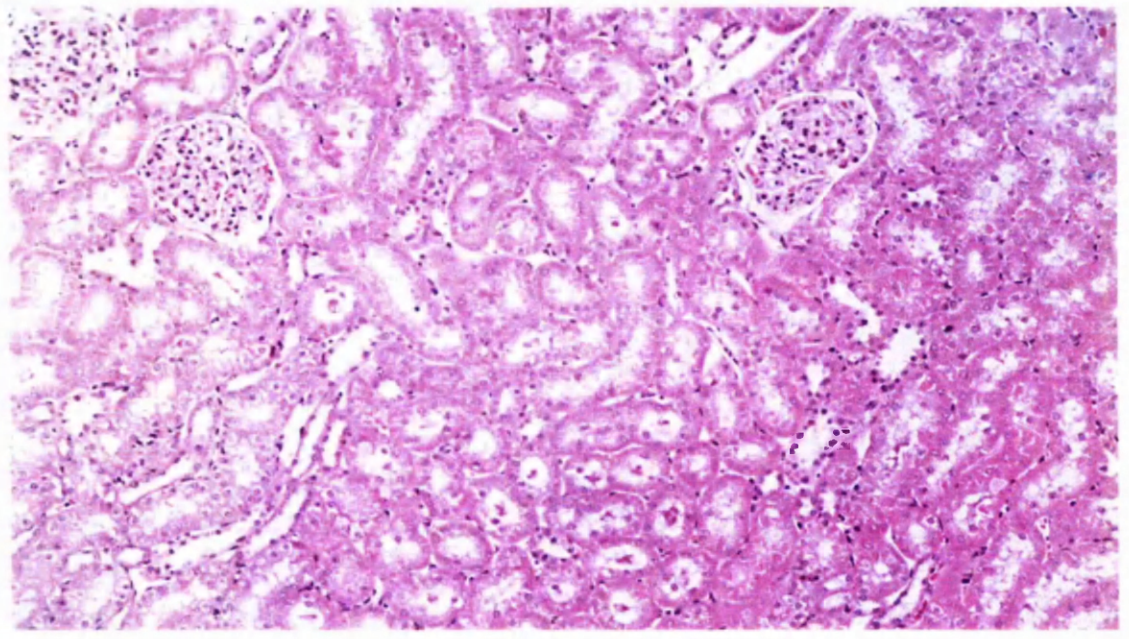


Figure 6.9 **Histology of the kidney of the Lister Hooded rat following tail vein injection of 400μl of A) PBS/0.5% Tween 80 and B) EO9 2mg/kg. No obvious differences were detected between the two groups.**

These were likely to represent animals in which the tumour cells had not "taken" at the time of implantation but they were still included in the analysis of the results. In both groups, but especially the EO9-treated group, considerable variability was seen in the size of the tumours present within both lobes of the liver, e.g. at 3 weeks post tumour implantation in the EO9-treated group, tumour volumes ranged between 0.09 and 5.89cm^3 and at 4 weeks in the same group, tumour volumes ranged between 0.03 and 20.0 cm^3. There was also some variation, although less marked, between the two treated lobes within each animal e.g. at 4 weeks in the EO9-treated group, tumour volumes of 0.75 and 2.94cm^3 were present in the left and median lobes of one rat.

The mean results from this experiment show that there was no statistically significant difference between the control group and the EO9-treated group at either 2 or 3 weeks post treatment (Figure 6.10). This suggested that the administration of systemic EO9 at this dose had no significant effect on the growth of the HSN tumour within the liver of the rats.

6.4.7 Feasibility Study on the Administration of Blank Albumin Microspheres via the Hepatic Artery

In the five Lister Hooded rats which received 10mg of microspheres in either 250 or 500µl of PBS/0.5% Tween 80, all the animals died within 24 hours of the injection, either during the procedure as a result of hepatic arterial rupture (three rats), or as a result of post operative haemorrhage (one rat), or hepatic arterial blockage by microspheres (one rat).

In five rats treated with 5mg of microspheres there were two deaths as a result of hepatic arterial rupture, but the remaining rats made a good recovery and survived the remainder of the experiment. There was no significant difference in survival between the 250µl and 500µl groups although the larger injection volume was easier for microsphere resuspension. 5mg of microspheres in 500µl PBS/Tween 80 was therefore chosen for further experiments.

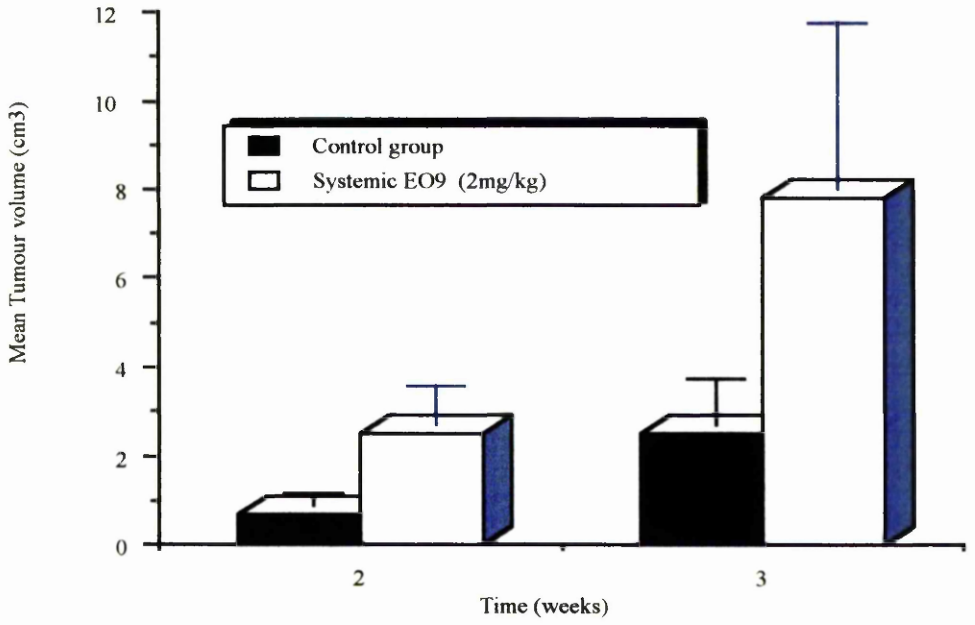


Figure 6.10 **The effect of systemic EO9 (2mg/kg) on the growth of $1x10^6$ HSN rat sarcoma cells implanted in the median and left lobes of the liver in the Lister Hooded rat at 2 and 3 weeks following administration. Each time point represents the mean tumour volume ± standard error of 10-12 tumours. No significant difference was detected between the EO9-treated group and the control group at either time point.**

6.4.8 The Effect of EO9-loaded Microspheres Administered via the Hepatic Artery on HSN tumour in the Liver of the Lister Hooded rat: Comparison with Free Drug and Blank Microspheres

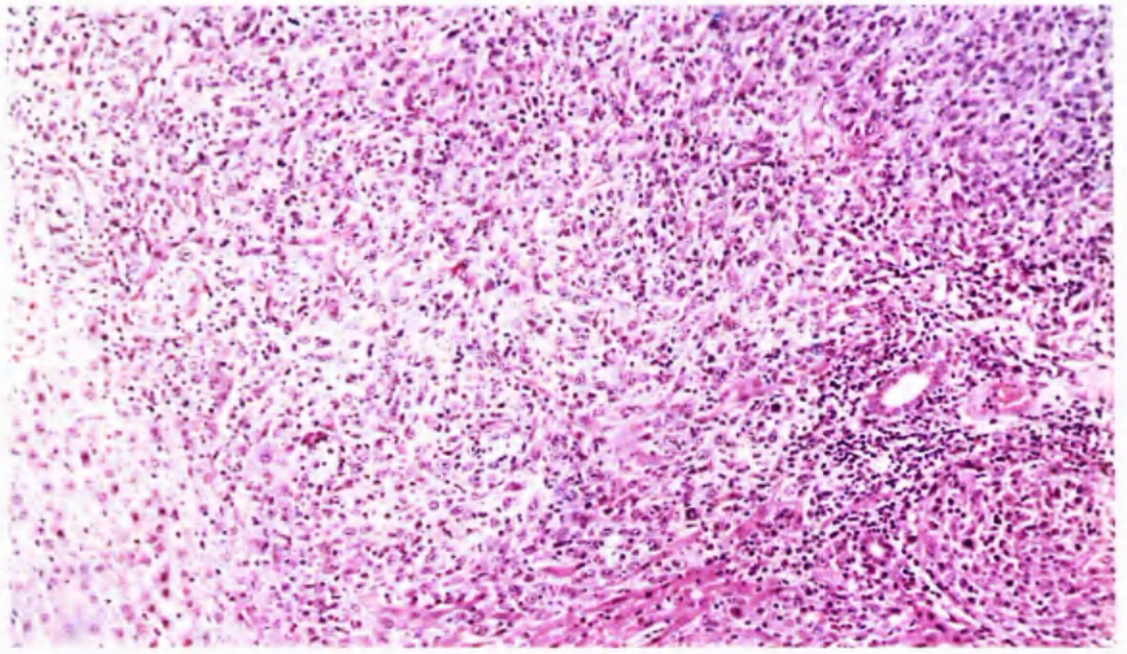
There were no problems experienced with the rats during the surgery or in the recovery phase following hepatic tumour implantation. At the second laparotomy 10 days later, five animals were found to have no evidence of tumour present within the liver and these were therefore withdrawn from the study.

Of the remaining fifteen animals which underwent the second part of the procedure involving hepatic artery injection, 10 of the animals died; two during the procedure itself (both animals in the PBS/0.5% Tween 80 control group) and eight within 24 hours (4/4 animals treated with blank microspheres; 2/3 animals treated with the EO9-loaded microspheres and 2/4 animals treated with free EO9). The 5 animals which survived the immediate post-operative period recovered well and survived the remaining time course of the experiment (21 days post implantation).

Post mortem examinations carried out on the rats which died showed that there was no evidence of hepatic artery haemorrhage in any of the rats. The reason for the deaths in the microsphere groups was thought to be related to blockage of the hepatic artery by the microspheres. Problems as a result of subjecting the rats to a second laparotomy and anaesthetic were also considered because this had not been previously attempted.

Histological analysis (using H+E staining) of the liver from the animals which survived to the end of the study, showed no obvious difference in liver architecture between the control, free EO9 and EO9 microsphere-treated groups. In the areas of tumour within the liver there was no obvious difference seen between the control group and the free EO9-treated group. However, in the EO9 microsphere-treated animal the tumour appeared to be a little less cellular with an increase in the inflammatory cell component. The significance of this observation is uncertain, as only one animal in this group actually survived to the end of the experiment. No microspheres were seen in the sections of either the liver or the tumour of the microsphere-treated animal (Figure 6.11).

(A)



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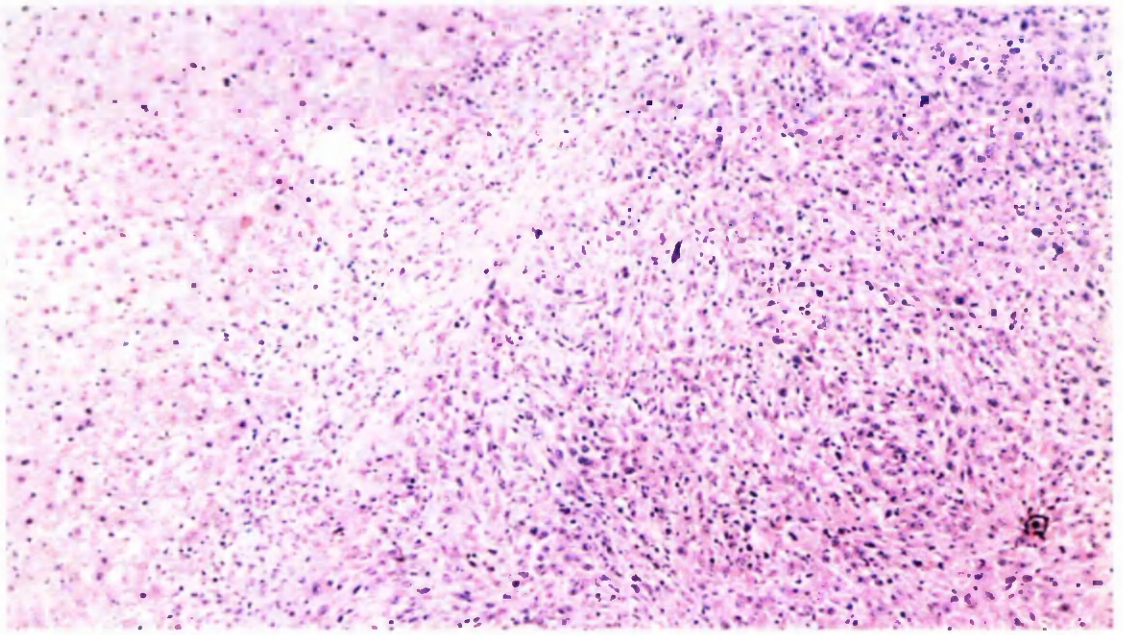

Figure 6.11 **Histology of the liver implanted with HSN tumour in the Lister Hooded rat 11 days following administration of 500μl of A) PBS/0.5% Tween 80 and B) 100μg Free EO9 via the hepatic artery.**

(C)

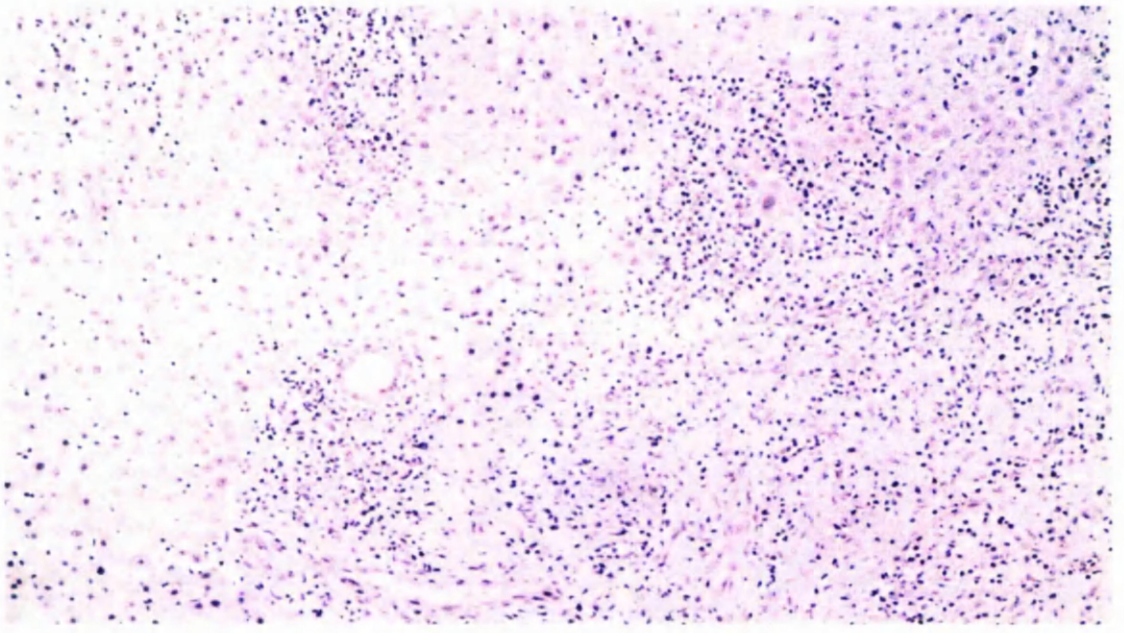

Figure 6.11 **Histology of the liver implanted with HSN tumour in the Lister Hooded rat 11 days following administration of 500μl of C) 60±6μg EO9-loaded microspheres via the hepatic artery.**

6.5 DISCUSSION

The aim of the studies presented in this chapter was to attempt to investigate the antitumour activity of the EO9-loaded microspheres in a clinically relevant tumour model system.

The sensitivity of the HSN rat sarcoma cell line to EO9 was unknown. Therefore, preliminary experiments focused on establishing the cell line *in vitro* and then, prior to commencing *in vivo* studies, establishing that it was sensitive to EO9. An IC_{50} of 4nM confirmed its sensitivity. This compared favourably with other cell lines which have been tested, particularly the HT29 tumour which has an IC_{50} of 6.7nM (Walton et al 1992A).

The EO9 sensitivity of the HSN cell line was then confirmed *in vivo* in antitumour studies in NU/nu mice. A significant antitumour response using only 125μg EO9, administered intratumourally, suggested that it was more sensitive to EO9 than other tumours previously studied such as the MAC 16, MAC 26 and BE (Chapter 4.4.2). When the antitumour effect of the microspheres was compared with free drug by direct intratumoural injection, the microspheres had an antitumour effect which was equivalent to that of the same dose of free drug (125μg), a response similar to that seen in the HT29 tumour model (Chapter 4.4.2.2.2).

Early studies with the HSN tumour in the rat were problematic, due to both anaesthetic sensitivity and more importantly, tumour regression which was confirmed in both subcutaneous and intrahepatic tumour deposits. This regression was statistically significant in the liver tumours at four weeks post implantation when compared with the tumour size at three weeks. This result obviously had important implications as such a model could not be used for antitumour studies.

After discussion with the group at The University Department of Surgery, Glasgow Royal Infirmary, who had supplied the original HSN cells and who were using this model system without any such problems, the rat supplier was changed so that the rats were obtained from the same source as the Glasgow group (Harlan, UK). As a result the problems with both anaesthetic sensitivity and tumour regression ceased to be an issue. The substrain and not just the strain of rat is therefore extremely important in establishing a tumour line *in vivo*.

No information was available concerning the toxicology of EO9 in the Lister Hooded rat. Previous *in vivo* work in rats in general was also limited but 3mg/kg EO9 in Sprague-Dawley rats (Workman et al 1992C) and weekly doses of 0.45mg/kg in the Wistar rat (Hendriks et al 1993) had both been administered without any adverse effects being documented. An initial experiment showed that 3 and 6mg/kg (18 and 36mg/m^2) was universally fatal in both groups treated with EO9 48 hours following drug administration (no deaths in the control group). Histological analysis of the kidneys of the EO9-treated rats unfortunately was not possible due to the timing of their deaths. Subsequent assessment of the kidneys of rats treated with 2mg/kg EO9 revealed no obvious abnormality on H+E staining. However, as the documented renal effect of EO9 is minimal change glomerulonephritis (Schellens et al 1994, Verweij et al 1994), electron microscopy would be required to detect this. Renal toxicity has not been identified previously in animal models (Hendriks et al 1993). Subsequent experiments found that although 2mg/kg EO9 represented the highest dose which could be safely administered, it did not appear to produce any significant antitumour activity.

During the establishment of a reliable tumour model, studies were carried out to document the growth of the tumour within the liver in order to determine the most appropriate time to initiate antitumour treatment . These studies produced variable tumour growth at each time

point although the overall growth effect was statistically significant with time. Significant variation in the two tumour sites within the same animal was also seen. This variation meant that any antitumour effect obtained with treatment would need to be large in order to be detectable at a particular time point.

There are several potential reasons for the observed variation. The main reason was thought to be ongoing problems with the technical aspects of the surgical procedure. One important factor which also needed to be considered was the length of time the cells remained viable following *in vitro* harvesting. The animals which were treated later in the morning may have received cells which were less viable and therefore did not grow as well. An *in vitro* analysis using trypan blue was performed in order to assess this further. This showed that >90% of the HSN cells were still viable 6 hours following harvesting and this was therefore unlikely to be the reason for the variation which was seen.

Another reason for continued variability in tumour growth may have been that insufficient cells were being injected on each occasion into each lobe to produce a consistent growth pattern. The experiment was repeated injecting double the number of cells into the same single lobe in each animal. However, this did not improve the consistency of tumour growth. More work is needed on the subcapsular injection of the tumour cells to achieve as consistent a growth rate as possible for future antitumour studies before the technique can be used for comparative purposes.

The other main surgical technique which needed considerable practice was the procedure used for the hepatic arterial injections. The final technique which was used differed slightly to the previously described technique (Anderson et al, 1991B) in that the needle was inserted directly into the hepatic artery rather than cannulating the gastroduodenal artery and then as the needle was removed, the injection site was immediately sealed with

Fibrinogen/Thrombin glue. This appeared to successfully prevent any problems with post operative arterial haemorrhage.

The dose of microspheres it was thought possible to inject into the hepatic artery was based on previous intra-arterial studies (Goldberg et al 1992, Anderson et al 1991B). Two different doses (5mg and 10mg) in two injection volumes (250µl and 500µl) were chosen. The microspheres were carefully resuspended prior to use in order to minimise the potential risk of blocking the hepatic artery which would result in the death of the animal. The lower dose of microspheres in the larger injection volume was found to be the easiest to administer and this was therefore chosen as the microsphere dose in the preliminary antitumour study. This compared the antitumour effect of EO9-loaded microspheres with an equivalent dose of free EO9 and blank albumin microspheres.

Unfortunately there were significant problems during this study. 5/6 of the animals treated with microspheres (blank and EO9-loaded) died during the procedure. This was thought to be the result of blockage of the hepatic artery due to either poor microsphere resuspension or the possible effects of the Fibrinogen/Thrombin glue. There were also two deaths in the control group and free EO9 groups which may have been the result of subjecting the animals to a second anaesthetic and laparotomy. Of the five animals which survived the procedure, it is not possible to draw any significant conclusion from the results which were obtained. The experiment therefore needs to be repeated once the problems which have occurred in this experiment have been addressed.

In summary, the HSN cell line has been shown to be sensitive to EO9 *in vitro* and *in vivo*. The cell line has been successfully implanted into the liver of Lister Hooded rats although further work is required in order to improve both the consistency of tumour growth within the liver and the safety of the intra-arterial injection following tumour implantation. However, such a model is likely to be an appropriate method to determine whether encapsulating EO9 into microspheres improves drug delivery to the tumour and hence antitumour activity following intra-arterial administration.

Chapter 7

Summary

The aim of the studies described in this thesis was to encapsulate the indoloquinone EO9 into human albumin microspheres and to then characterise formally the microspheres which were produced. Following characterisation the antitumour activity of the microspheres was assessed. Pharmacokinetic analysis was performed to determine whether variations in antitumour activity could be explained by pharmacokinetic differences and to look for the presence of any EO9 metabolites which might correlate with activity. Finally, a more clinically relevant model was developed to allow better assessment of the antitumour activity of the microspheres.

The bioreductive quinone, EO9, has been successfully encapsulated into albumin microspheres using chemical cross-linking of albumin by gluteraldehyde during emulsification at room temperature. This method was chosen because it avoids the use of heat and strong chemicals for cross-linkage, particularly important with an unstable drug such as EO9. The final method was a modification of the original method used, which was based on the encapsulation of MMC (Allan et al 1993). Reducing the concentration of gluteraldehyde in the cross-linkage step allowed the microspheres to be digested in the presence of trypsin. This was important because it allowed HPLC analysis of the microspheres to be performed, which potentially allowed both the chemical integrity and the total amount of EO9 incorporated into the microspheres to be determined. It also increased the probability that the microspheres would be biodegradable following *in vivo* administration. Removal of the isopropanol wash step resulted in less EO9 being lost from the microspheres during their preparation, but required the microspheres to be freeze-dried and resuspended using ultrasonication prior to use. The freeze-drying process had the additional benefit of being a useful way of preparing the microspheres for storage.

The characteristics of the EO9 microspheres were determined because of the important role they play in determining both the activity and the disposition of the encapsulated drug

(Chen et al 1994). The characteristics which we considered to be important were microsphere size and biodegradability together with the retention of the drug's chemical integrity following the encapsulation process. The degree of incorporation of EO9 into the microspheres together with the rate of drug release from the microspheres was also assessed.

Microspheres of different sizes were produced by varying the speed of the mixer used in the emulsification process. The microspheres which were produced at the slower speed of 1600r.p.m. produced particles which had a mean median size of 19.9μm. Approximately 70% of the particles were greater than 12μm in diameter, the size required to become trapped within the first capillary bed they encounter. This made them the most suitable of all the microspheres produced for locoregional administration. The incorporation of EO9 into the microspheres did not significantly alter microsphere size, though this may have been related to the amount of drug which was incorporated. It would be interesting to determine whether the final size of the particles would be altered if the amount of EO9 incorporated into the microspheres were increased.

The amount of drug which can be encapsulated is important because of the physical limitations with regard to the maximum amount of microspheres (300mg) which can be successfully administered (Goldberg et al 1988). Only a low rate of encapsulation (1.24 ± 0.2mg/100mg microspheres) was achieved, but this was comparable to results from the other studies which used a similar method (Allan et al 1993, Chen et al 1988). Unlike the methods of microsphere production which used heat denaturation (Fugimoto et al 1985A), this represents the amount of intact EO9 which is actually released from the microspheres. It may be an under representation of EO9 incorporation because it does not take into account any drug which may be covalently complexed to the albumin within the microspheres which would be released as the particles are digested *in vivo*. The release characteristics of

the EO9-loaded microspheres however, would suggest that the amount of EO9 incorporated in this way is low.

This degree of drug-loading means that if the maximum amount of microspheres (300mg) were administered via the hepatic artery, the dose of EO9 which would be given is 3-4.5mg. This is considerably less than the average systemic dose of 22-40mg (depending on the schedule) of EO9 which has previously been administered. Therefore further studies should attempt to increase drug loading by increasing the amount of EO9 used in the microsphere preparation. If this were successful, then re-characterisation of these new microspheres in terms of size and release characteristics would be required. The drug load however may be of lesser importance if altered metabolism of EO9 occurs following encapsulation into microspheres. Previous studies have suggested (Willmott et al 1987A, Cummings et al 1992A) that the microspheres induce a hypoxic environment and this may result in augmentation of EO9's activity.

Results from the *in vitro* rate release studies showed that the EO9 was rapidly released from the microspheres and had fully eluted from the column within six hours, a result similar to that obtained for the free drug, which suggests that the microspheres act as a carrier for the EO9 rather than as a means of sustained drug release. This is in keeping with EO9's mode of action (cross-linkage of DNA, single-stand breaks and the generation of reactive oxygen species (Bailey et al 1994, Walton et al 1991)) where it is the peak concentration of EO9 rather than prolonged delivery of a low dose of drug which is more likely to produce maximum cell damage. This would be in agreement with the Phase II trial data where the highest rate of stable disease was demonstrated in the group treated with the 3-weekly EO9 schedule ($22mg/m^2$) compared with the weekly schedule ($12mg/m^2$) (Pavlidis et al 1996).

Although formal characterisation of the microspheres has been performed, further studies would be required in terms of both quality control and aseptic preparation before the microspheres would be ready for use in clinical trials with patients.

The characteristics of the EO9-loaded microspheres suggested they had the potential for antitumour activity. They were compared with the equivalent dose of free EO9 (250μg), given by the same route (intratumoural injection) and were initially assessed *in vivo* in four tumour types; the murine adenocarcinomas of colon MAC 26 and MAC 16, and the human colonic adenocarcinoma xenografts HT29 and BE. Previous *in vitro* studies had shown that in aerobic conditions, cell lines which were high in the activity of the 2 electron reducing enzyme DT-Diaphorase were more sensitive to the antitumour effect of EO9 than cell lines which had low enzyme activity (Collard et al 1995, Fitzsimmons et al 1996, Plumb et al 1994A, Smitskamp-Wilms et al 1994, Walton et al 1992A). However, in the presence of hypoxia, the opposite effect was seen with cells with low DT-Diaphorase activity showing a greater increase in sensitivity compared to cells which had higher levels of enzyme activity (Bando et al 1995, Plumb et al 1994B, 1994C, Robertson et al 1994). The MAC 16 and HT29 tumours are known to have high levels of DT-Diaphorase enzyme activity when compared with the MAC 26 tumour. The BE tumour has no active DT-Diaphorase. It was felt that these four tumour types would allow the assessment of EO9's antitumour activity in relation to DT-Diaphorase levels and if, as previous studies have suggested, microspheres do induce hypoxia, the effect this might have on antitumour activity.

The *in vivo* antitumour studies showed that in the HT29 tumour the EO9-loaded microspheres demonstrated equivalent activity to that of the free drug. In the MAC 16 tumour the microspheres were found to be less efficient than the free drug and in the BE and MAC 26 tumour types no antitumour activity was seen in the microsphere-treated groups. These results suggested that in the tumour types with low DT-Diaphorase activity (MAC 26

and BE), there was no augmentation of effect in the presence of hypoxia (if the microspheres induce hypoxia), which is in contrast to previous *in vitro* studies (Bando et al 1995, Plumb et al 1994B, 1994C, Robertson et al 1994). However, several factors other than the levels of bioreductive enzymes may have influenced the results. The degree of hypoxia present within each of the tumour types could have significantly influenced the outcome, particularly as the four tumour types vary widely in their differentiation and vascularisation patterns. It would have been useful to have determined whether it was possible to induce hypoxia in each of the tumour types using the microspheres before performing antitumour studies. In this respect, preliminary experiments using Magnetic Resonance Spectroscopy in one tumour type were performed and suggested that the intratumoural injection of microspheres, results in the induction of a hypoxic environment which lasts approximately 20 minutes (Spanswick, personal communication). Tumour vascularisation as well as influencing hypoxia, will also affect the concentration of drug present within the tumour. Therefore, it may have been more useful to have studied a panel of xenografts with similar degrees of differentiation and vascularisation patterns, but differing levels of bioreductive enzymes in order to assess the relationship between the levels of bioreductive enzymes and the antitumour activity of free EO9 and EO9-loaded microspheres.

Pharmacokinetic studies were set up to try and determine whether the variation in antitumour activity between the free drug and microsphere-treated groups could be explained by pharmacokinetic differences. These studies failed to demonstrate any significant difference in the concentration of EO9 between the free drug and the microsphere-treated groups in both tumour and plasma analysis. The results were however extremely variable. Furthermore, the amount of EO9 detected at T_0, the initial time point of the analysis, was less than 5% of the original dose of EO9 given by direct intratumoural injection, which made it difficult to know just how meaningful the results were. The results suggested that antitumour activity was independent of the EO9 concentration within the

tumour and that there was minimal evidence of sustained drug release in the microsphere-treated groups, which was in agreement with the previous *in vitro* characterisation studies.

The variability in the PK data shows that initial pharmacokinetic studies should have been performed on one of the tumour types in which differences between free EO9 and EO9-loaded microspheres had been detected in the antitumour studies, e.g. BE or MAC 26. Analysis of these results would have identified the wide variation which was seen in the results at an early stage, and would have allowed the study to have been redesigned prior to analysis of the other tumour types. Changes which would have been made to the study design, are a reduction in the number of later time points, as these were of limited use together, with an increase in the number of animals used at each remaining time point.

As the relative amount of drug detected at "T_0" was small, more studies should be performed to determine the reason for this. Removal of both the tumour and peritumoural tissues for drug assessment would have given some indication as to whether a significant amount of EO9 had been placed peritumourally or whether the low concentration was in fact due to the rapid drug metabolism in the 2-3 minutes required to obtain the first sample.

The development of any metabolites which might correlate with antitumour activity was also assessed. Chromatography analysis failed to detect any one metabolite which could be related to EO9 activation, which agrees with the previous *in vitro* studies (Cummings et al 1998). There were no extraction efficiencies and standard curves available for EO5A or EO9 metabolites, so the results were qualitative and descriptive rather than quantitative. A more useful method of determining EO9's activity might have been to use a technique which measures the direct consequences of EO9 activation such as DNA adduct formation. This is based on the fact that EO9 is metabolised into short-lived and therefore largely undetectable reactive intermediates rather than long-lived stable metabolites.

The use of direct intratumoural injections compared the effect of free EO9 with the equivalent dose of EO9 in microspheres once they were physically at the tumour site. This method maximises the likely antitumour effect, particularly of the free drug, but minimises any problems associated with systemic drug administration and may therefore not be the best method of assessing a drug delivery system. Direct intratumoural injection will also fail to assess the chemoembolic vascular effects of the microspheres on the tumour. For this reason, the HSN tumour model was investigated where the HSN tumour was established in the liver of the Lister Hooded rat. The administration of free EO9 and EO9-loaded microspheres could then be compared following administration via the hepatic artery. In retrospect, because of the limited value of the mouse antitumour studies, it may have been better to have concentrated on this model system earlier in the project.

The HSN tumour was shown to be very sensitive to EO9 both *in vitro* and *in vivo* (intratumoural injection in Nu/nu mice), but there were significant problems with the *in vivo* rat model. Further work is required to improve the consistency of tumour growth within the liver. Increasing further the number of cells used for subcapsular implantation might help reduce the variation which was seen. The efficiency and safety of the hepatic arterial injection also needs to be improved. The method which we used (direct intra-arterial injection) was different from the published technique which carried out intra-arterial injection via cannulation of the gastroduodenal artery (Anderson et al 1991B). It would be useful to compare our method with the original method which, although technically more difficult, eliminates the need to seal the hepatic artery with Fibrinogen/Thrombin glue which was probably responsible for the problems we encountered with blockage of the hepatic artery.

Alternative models of tumour growth could be considered if the rat model failed to produce consistent tumour growth within the liver. One such model is the previously described VX-

2 tumour grown on the hind leg of the albino rabbit and treated under general anaesthesia by injection of microspheres/free drug/placebo via the femoral artery (Fugimoto et al 1985A). This model would have the additional advantage of allowing regular assessment of tumour growth following treatment.

Once the preclinical tumour model was reliably established, antitumour studies would compare the systemic (venous) administration of free EO9 with the intra-arterial administration of free EO9, EO9-loaded microspheres, blank microspheres and PBS/0.5% Tween 80. Different doses of EO9-loaded microspheres would be compared for antitumour activity and toxicity. Pharmacokinetic studies to include examination of tumour tissue would also be performed.

If the preclinical model suggested that EO9-loaded microspheres had better antitumour activity than either intra-arterial or systemic free EO9 and toxicity was acceptable, then clinical assessment of the EO9 microspheres would be considered. Albumin microspheres have previously been safely administered to patients via the hepatic artery (Goldberg et al 1991C). During dose finding studies the administration of a defined amount of EO9-loaded microspheres via the hepatic artery to patients about to undergo formal hepatic resection of tumour would be considered. Subsequent examination of the resected tumour would give some indication of the microsphere distribution within the tumour tissue in comparison to the normal liver. Analysis of the EO9 concentration within the tumour tissue would also be performed together with the measurement of the tumour's bioreductive enzymes. These studies would help to assess the validity of this method prior to embarking on prospective randomised trials utilising this approach.

Chapter 8

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Appendices

APPENDIX A - REAGENT SUPPLIERS

Reagent	*Supplier*
Absolute Alcohol	Hayman Ltd
Butorphanol	Willow Francis Veterinary
Carbon dioxide (solid)	BOC
Cotton seed oil	Sigma Chemical Co Ltd
CRE (E) Rat diet	SDS
Disodium Hydrogen Orthophosphate	BDH Merck Ltd
DPX Mounting Media	Fisons Chemicals Ltd
Dulbecco's Modified Eagle's Medium	Gibco Life Technologies Ltd
EO9	A gift from Kyowa Hakko Kogyo Co (under the auspices of Dr H Furukawa and Mr J Kelly) supplied by Dr H Hendriks, NDDO
Fibrinogen/Thrombin combination sealant	Scottish National Blood Transfusion Service
Foetal calf serum	Gibco Life Technologies Ltd
Gluteraldehyde: Grade I (25% Aqueous)	Sigma Chemical Co Ltd
Harris's Haematoxylin	Sigma Chemical Co Ltd
Helium	BOC
Human serum albumin (Fraction V)	Sigma Chemical Co Ltd
Hypnorm (fentanyl citrate/ fluanisone)	Janssen Animal Health
Hypnovel (midazolam)	Roche Products Ltd
Lithium Carbonate	BDH Merck Ltd
Methanol	Rathburn Chemicals Ltd
Mitomycin C	Kyowa Hakko Kogyo Co
Nitrogen (Gas/Liquid)	BOC
Penicillin-Streptomycin	Gibco Life Technologies Ltd
Petroleum spirit/ether (ANALAR) (Boiling range 120-160^{0}C)	BDH Merck Ltd
Phosphate Buffered Saline	In house
Polyoxyethylene sorbitan monolaurate (Tween 20)	Sigma Chemical Co Ltd
Polyoxyethylene sorbitan monoleate (Tween 80)	Sigma Chemical Co Ltd
Propan-2-ol (Isopropanol)	Rathburn Chemicals Ltd
RM3(E) Mouse diet	SDS
Sodium Dihydrogen Orthophosphate	BDH Merck Ltd
Sodium dodecyl sulphate	BDH Merck Ltd
Sorbitan monoleate (Span 80)	Sigma Chemical Co Ltd
Trypsin (Type III)	Sigma Chemical Co Ltd
Trypsin/Versene	Gibco Life Technologies Ltd
Xylene	Fisons Chemicals Ltd
Y Eosin (alcoholic)	Sigma Chemical Co Ltd

APPENDIX B - APPARATUS SUPPLIERS

Apparatus	*Supplier*
Beckman GPR centrifuge	Beckman Instruments Ltd
Bond Elut C18-OH (3cc & 1cc columns)	Varian Ltd
Decon FS100 Ultrasonic waterbath	Decon Ultrasonics Ltd
Edwards Modulyo Freeze-dryer	Edwards High Vacuum International
Endecott stainless steel sieve (63μm pore diameter)	Endecotts Ltd
Eppindorf Microcentrifuge	Eppendorf Ltd
Falcon (tissue culture) Flasks	Becton Dickinson
Hewlett Packard Liquid Chromatograph (Model 1090)	Hewlett Packard Ltd
Leitz Ortholux 11 microscope	E. Leitz (Instruments) Ltd
LiChrosorb RP-18 HPLC Cartridge	BDH Merck Ltd
LiChrospher 100 RP-18 precolumn	BDH Merck Ltd
LiChrocart Manu-fix Holder	BDH Merck Ltd
LKB Broma 7000 Ultrorac Fraction Collector	LKB Broma Ltd
LKB Broma 2120 Varioperspex Peristaltic Pump	LKB Broma Ltd
Malvern 2600 Particle Sizer	Malvern UK
M.D.H Interlab tissue culture hood (Class II)	M.D.H Ltd
Mersilk sutures	Ethicon
Michel clips (12 x 2.5mm)	International Market Supply
Microlance needles	Becton Dickinson
Milli-U10 water purification system	Millepore (U.K.) Ltd
Minitab Release 11 programme	Minitab Inc
Morden Isolators	Morden Scientific Ltd
Scotlab VSL incubator	Scotlab Ltd
Silverson Mixer (Model SL2T) (with emulser screen)	Silverson Machines Ltd
Syringe Filters (0.45μm)	Millepore (U.K.) Ltd
Vac Elut SPS 24	Phenomenex
Vacuum pump filters (0.45 and 0.6μm)	Millepore (U.K.) Ltd
Waters Alliance System	Waters Ltd
ZM Coulter Counter	Coulter Electronics

APPENDIX C - SUPPLIER ADDRESSES

Supplier	*Address*
Beckman Instuments Ltd.	Progress Road, High Wickham, Bucks, UK.
Becton Dickinson	Cowley, UK
BDH Merck Ltd.	Merck House, Poole, Dorset, UK
BOC	Dear Park Road, London, UK.
Charles River	Margate, UK
Coulter Electronics	Luton, Bedfordshire, UK
Decon Ultrasonics Ltd	Conway Street, Hove, Sussex, UK
Edwards High Vaccum International	Manor Royal, Crawley, West Sussex, UK
Endecotts Ltd	Lombard Road, London, UK
E. Leitz (Instruments) Ltd	Luton, UK.
Eppendorf Ltd	Merck House, Poole, Dorset, UK
Ethicon	Edinburgh, UK
Fisons Chemicals Ltd	Bishop Meadow Road, Loughborough, UK
Gibco Life Technologies Ltd	Renfrew Road, Paisley, Scotland.
Harlan UK Ltd.	Shaw's Farm, Blackthorn, Bicester, UK.
Hayman Ltd	Eastways Park, Witham, Essex, UK
Hewlett Packard Ltd	Wokingham, Berkshire, UK.
International Market Supply	Congleton, UK
Janssen Animal Health	High Wycombe, UK
Kyowa Hakko Kogyo Co	Martindale Pharmaceutics, Romford, UK
LKB Broma Ltd	Uppsala, Sweden.
M.D.H Ltd	Walwarth Road, Andover, UK
Millipore (UK) Ltd	Blackmoor Lane, Watford, UK
Minitab Inc	3081 Enterprise Drive, State College, PA 16801, USA
Morden Scientific Ltd	Penicuik, Scotland
NDDO	Free University, Amsterdam, Netherlands.
Phenomenex	Queens Avenue, Macclesfield, Cheshire, UK
Rathburn Chemicals Ltd	Walkerburn, Scotland.
Roche Products Ltd	Welwyn Garden City, UK
SDS	Witham, Essex, UK.
Scotlab Ltd	Kirkshaws Road, Coatbridge, Scotland
Scottish National Blood Transfusion Service	Protein Fractionation Centre, Edinburgh, UK
Sigma Chemical Co. Ltd	Fancy Road, Poole Dorset, UK.
Silverson Machines Ltd	Waterside, Chesham, Bucks, UK
Varian Ltd.	24201 Frampton Avenue, Harbour City, CA 90710, USA
Waters Ltd	The Boulevard, Blackmoor Lane, Watford, UK
Willow Francis Veterinary	Crawley, UK