THE CLINICAL EVALUATION OF THE PUTATIVE CANCER CHEMOPREVENTIVE AGENT SILYBININ IN COLORECTAL CANCER AND RESECTABLE HEPATIC METASTASES

Thesis submitted for the degree of
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The clinical evaluation of the putative cancer chemopreventive agent silybinin in colorectal cancer and resectable hepatic metastases

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Silybinin and its crude form silymarin exhibit cancer chemopreventive efficacy in rodents including models of colorectal carcinogenesis. Silymarin is used clinically as a hepato-protectant against alcohol- and drug-related damage. Silybinin is a strong antioxidant and modulates the insulin-like growth factor (IGF) system in mice *in vivo* by increasing circulating levels of IGF binding protein-3 (IGFBP-3). In this thesis, the hypothesis that oral consumption of silybinin affords pharmacologically active levels in blood, liver and colorectum was tested. Twelve patients with colorectal carcinoma and twelve with colorectal liver metastases received silybinin phosphatidylcholine (silipide) at varying doses for 7 days. Blood, normal and malignant colorectal or liver tissue were obtained before and after silybinin ingestion. A HPLC-UV method was developed and validated prior to quantifying levels of silybinin in plasma and tissue samples. Plasma metabolites were identified by liquid chromatography-mass spectrometry. Levels of IGFBP-3 and IGF-1 in serum and of the oxidative DNA damage pyrimidopurinone adduct of deoxyguanosine (M_1dG) in leucocytes were determined as potential pharmacodynamic markers of silybinin efficacy. Repeated administration of silipide was safe and well tolerated. Silybinin levels recovered from plasma between 1-4 h *post* final silipide dose were 0.3-4.0 μM. Silybinin monoglucuronide, silybinin di-glucuronide, silybinin mono-sulphate and silybinin glucuronide sulphate were identified as metabolites in the plasma. Silybinin concentrations in liver and colorectal tissues obtained by resection 3-6 h *post* last silipide dose were 0.3-2.5 and 20-141 nmolesg^{-1}, respectively. Intervention with silipide did not affect blood levels of IGF-1, IGFBP-3 or M_1dG. In conclusion, silipide ingestion at safe doses can achieve detectable levels of agent in plasma and liver, and silybinin concentrations reached in the colorectal tract are of the level which has been shown to elicit pharmacological effects in cells *in vitro*. The results support the further development of silybinin as a potential human colorectal cancer chemopreventive agent.

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Hoh CS, Boocock DJ, Marczylo TH, Brown VA, Cai H, Steward WP, Berry DP, Gescher AJ. Quantitation of silybinin, a putative cancer chemopreventive agent derived from milk thistle (*Silybum marianum*), in human plasma by high-performance liquid chromatography and identification of possible metabolites. *J Agric Food Chem.* 2007 Apr 4;55(7):2532-5.

Abstracts

Hoh CS, Berry DP, Garcea G, Hemingway D, Miller AS, West K, Dennison AR, Singh R, Boocock DJ, Marczylo TH, Cai H, Steward WP, Gescher AJ. A clinical pilot study of oral silybinin, a putative colorectal cancer preventive agent, in patients with primary colorectal cancer and colorectal liver metastases. *Proc Amer Assoc Cancer Res.* 2006; 47: 3191.

Hoh CS, Garcea G, Gescher AJ, Hemingway D, Miller AS, West K, Dennison AR, Steward WP, Berry DP. The emerging role of milk thistle as a colorectal cancer chemopreventive agent. *Colorectal Disease.* 2006; 8(2): 116.

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*HPB.*2006; 8(2): 114.

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Presentations

Oral Presentations

1.	May 2005	Midlands Hepatobiliary and Pancreatic Meeting, Leicester. "The role of silybinin in colorectal cancer chemoprevention".
2.	November 2005	The 5th Institute of Cancer Studies Meeting, Leicester. "Pharmacological pilot study of silybinin in patients with resectable colorectal cancer and colorectal liver metastases".

Poster Presentations

1.	October 2005	National Cancer Research Institute Conference, Birmingham. "Pharmacological pilot study of silybinin in patients with resectable colorectal cancer".
2.	April 2006	American Association of Cancer Research Conference. Washington DC – Clinical Scholar in Training Awardee. "A clinical pilot study of oral silybinin, a putative colorectal cancer preventive agent, in patients with primary colorectal cancer and colorectal liver metastases".
5.	July 2006	Association of Coloproctology Great Britain and Ireland. "The emerging role of milk thistle as a colorectal cancer chemopreventive agent."
6.	September 2006	7th World Congress of the International Hepato-Pancreato Biliary Association. "A study of the cancer chemopreventive agent silybinin in patients with resectable colorectal liver metastases."
		"A pilot study of oral silybinin, a putative colorectal cancer preventive agent, in patients with primary colorectal cancer and colorectal liver metastases."

Chapter 1. INTRODUCTION

1.1 Colorectal carcinoma

Colorectal cancer (CRC) is the second commonest cause of cancer mortality in Western societies after lung cancer. Globally, it affects around 9.4% of the world's population and causes 492,000 deaths per year with similar numbers in both genders (Flora *et al.,* 1998). Although recent years have seen marginal improvements in mortality for white US citizens, the prognosis remains poor with 25% of patients having advanced metastatic disease at the time of presentation (Flora *et al.,* 1998). Furthermore, the 5 year survival rate for a patient with lymph node involvement (Duke's C) is approximately 39% following surgery (Giovannucci, 1995). Of these patients, 80% will have macroscopic clearance of the disease but as many as 50% will suffer recurrence (Fuchs *et al.,* 2002). Distant spread of CRC is common to the liver, followed by lung and bone usually through the bloodstream or lymphatic system.

The epidemiology and molecular biology underlying CRC have been studied more thoroughly than for any other malignancy. There is a wide geographical difference in the incidence of large bowel cancer between continents: higher in North America, Western and Northern Europe, Australia and New Zealand than in Africa and Asia (Flora *et al.,* 1998). This implies that dietary and environmental factors may play a role in the development of CRC. Diets low in vegetables and folate and high in fat, red meat and alcohol appear to increase the relative risk of developing the disease (Giovannucci *et al.,* 2003; Fuchs *et al.,* 2002). Lack of exercise, cigarette smoking and progressive age are also contributing risk factors. CRC is an important public health problem worldwide.

The majority of patients with CRC (around 80%) have sporadic disease, while in the remaining 20% the disease is inherited through genetics defects (Bruce *et al.*, 2000). The development of CRC, as described by Fearon and Vogelstein (Fearon & Vogelstein, 1990), is a multi-step process characterised by a number of successive genetic changes in an orderly fashion over an extended period of time, followed by histopathological changes in the colorectal epithelium as a phenotypic expression of the genetic abnormalities.

It has been well-established and widely accepted that the adenoma – carcinoma sequence represents the process by which most, if not all, CRCs arise. Although pre–existing polyps and adenomas are common in the elderly, it is rare for individual adenomas to progress to carcinoma despite the high rate of cell division in adenomas (Bruce *et al.*, 2000). Dietary carcinogens or other environmental factors could also act as exogenous risk factors. According to the genetic model of CRC proposed by Fearon and Vogelstein, mutational activation of proto-oncogenes (K-ras) coupled with inactivation of tumour-suppressor genes, [examples include 3p21 (β catenin gene), 5q21 (APC gene), 9p (p16 and p15 gene), 13q (Rb gene) 17p (p53 gene), 17q (BRCA1 gene), 18q (SMAD 2&4, and DCC gene) and 16q (E cadherin gene)] in a background of evolving chromosomal instability produces dysplastic cells in the colorectal epithelium (Fearon & Vogelstein, 1990). Multiple mutations are required for the development of malignant tumours, whereas fewer changes are necessary for premalignant lesions to occur. Macroscopically, this growth of abnormal cells produces a visible mucosal lesion, termed the adenomatous polyp. As more cells accumulate, the polyp grows in size until the epithelial cells in the adenoma acquire the ability to break through their basement membrane and invade locally through the

bowel wall and metastasize to lymph nodes or distant organs (Figure 1.1 and Figure 1.2).

Figure 1.1 The proposed adenoma to carcinoma sequence in colorectal cancer. Adenomatous polyposis coli (APC) gene mutation and hypermethylation occur early, followed by K-ras mutations. Deleted in colon cancer (DCC) and p53 gene mutations occur later in the sequence; the exact order may vary.

Modified from Hardy *et al.* (Hardy *et al*., 2000)

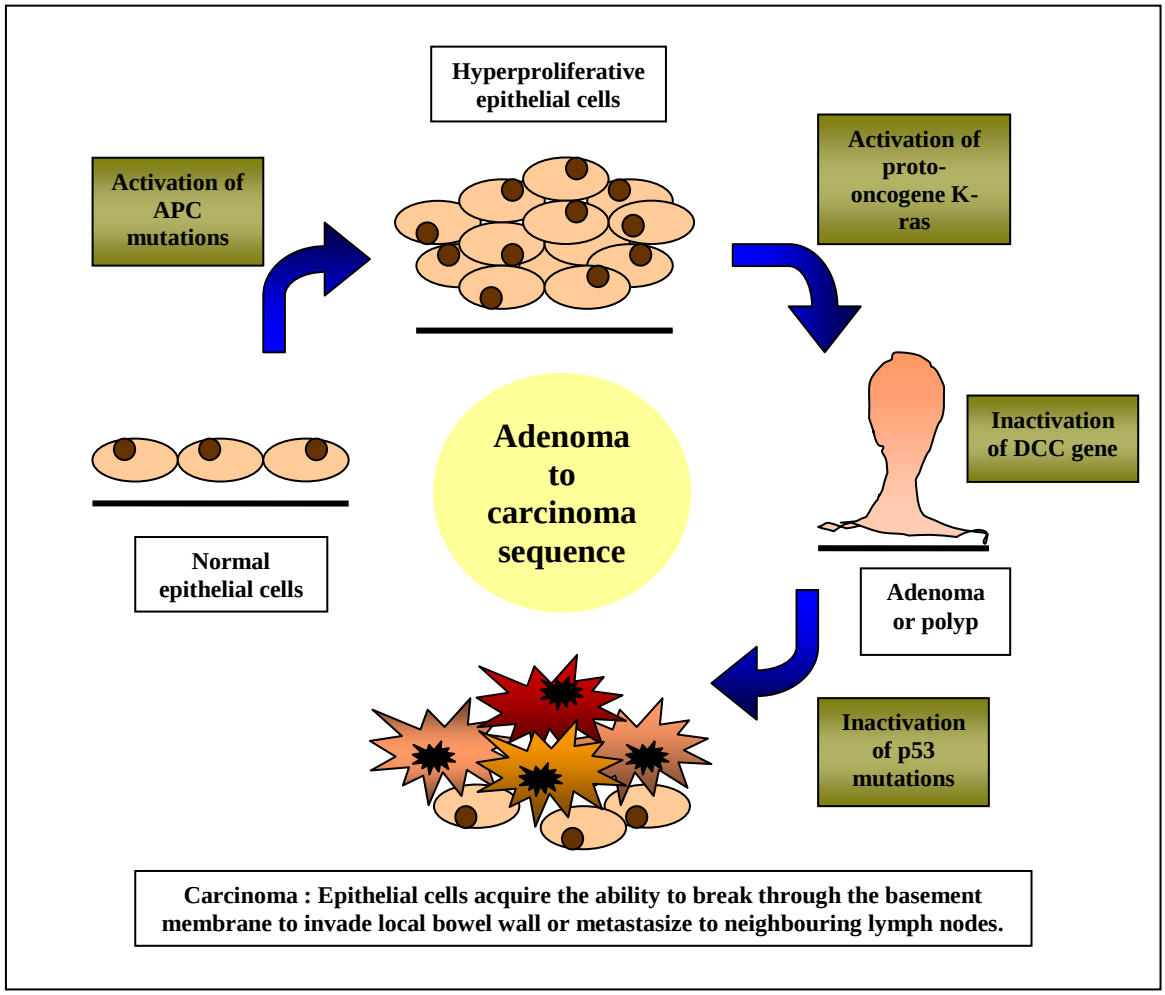
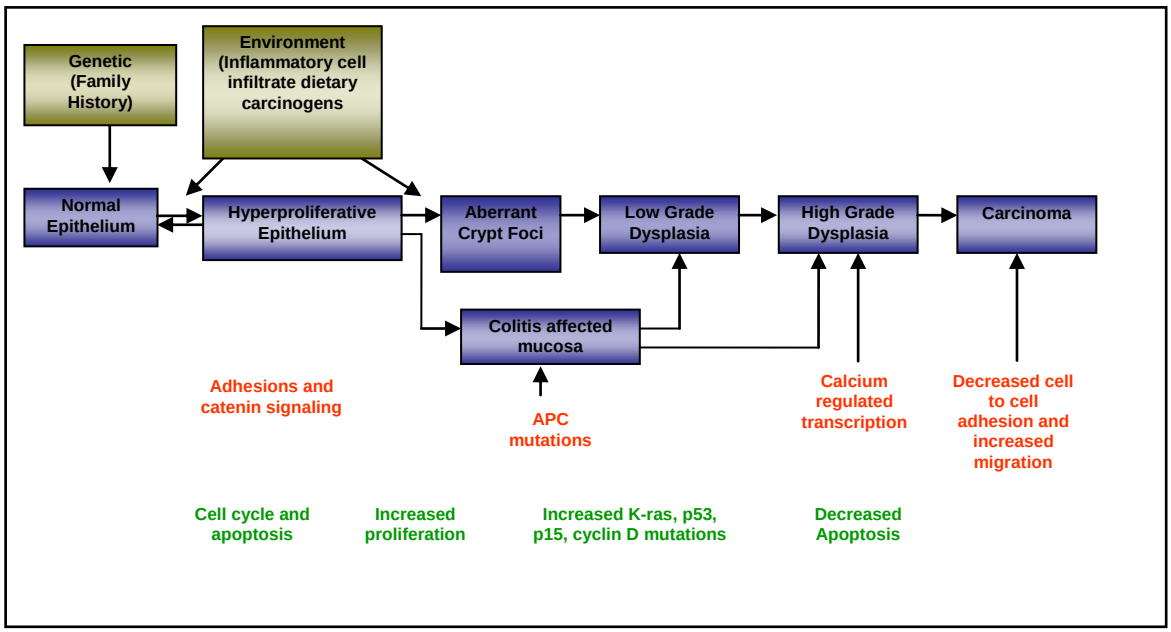


Figure 1.2 The key molecular events in colorectal premalignancy.
Modified from Hardy *et al*. (Hardy *et al*., 2000)



Despite considerable efforts in improving diagnosis and treatment modalities over the years, CRC remains a significant cause of mortality in the western world. Surgery is the mainstay of treatment once the condition is diagnosed, involving resection of the tumour and associated tissues. Adjuvant treatment including chemotherapy and radiotherapy may be used in aggressive disease. Since carcinogenesis is a diverse and multi-step process, which involves many complex pathways over several years, it is conceivable that inhibition or reversal at any point of these pathways can prevent the development of cancer before the time of its inception. This opens up several avenues for cancer prevention.

1.2 Cancer prevention

Given the substantial morbidity and mortality associated with colorectal malignancies and limited scope for treatment, cancer prevention in its many forms emerges as a very attractive approach. With the rapidly expanding fields of molecular biology and genetic studies, it may be possible to prevent CRC at different levels. Primary prevention of CRC involves the knowledge and manipulation of risk factors that may be important to the initiation of CRC carcinogenesis. An example is that of a diet rich in vegetables (Block *et al.*, 1992) and fibres (Kritchevsky, 1997) to reduce the relative risk of CRC development. Secondary prevention involves early identification and intervention of the CRC carcinogenesis pathway during its symptom-free, pre-malignant or pre-cancerous stages of development. An example of secondary prevention of CRC could be represented by the NHS Bowel Cancer Screening Programme which was introduced in the United Kingdom in July 2006, whereby anyone aged between 60 – 69 would be invited to undergo a faecal occult blood test every two years. This test examines individual patients' stool and detects hidden traces of blood in the faeces (Hardcastle *et al.*, 1996). Patients with a 'positive' result will be urgently referred to a specialist centre for colonoscopy. The presence of polyps or CRC is a common cause of blood in the faeces and bleeding from the rectum. It is well established that removal of these polyps, a precursor of CRC, reduces the incidence of CRC. Although colonoscopy and polypectomy, i.e. removal of the polyp from the colon through a flexible telescope inserted per rectum, are associated with a 76 – 90% reduction in CRC incidence, as shown in the US National Polyp Study (Bruce *et al.*, 2000), it carries significant risks. Colonoscopy is an invasive procedure and can be uncomfortable for the patient. It can cause respiratory depression and allergic reaction in patients from the potent 'cocktail' of anxiolytics

and analgesics injected intravenously for sedation. In colonoscopy, there is also a 1 in 1000 coloinc perforation risk with its associated mortality rate. In patients with predisposed risk of developing CRC, such as patients with ulcerative colitis or Familial Adenomatous Polyposis (FAP) Coli, this invasive procedure may need to be carried out repeatedly, hence multiplying its risks.

1.2.1 Concept of cancer chemoprevention

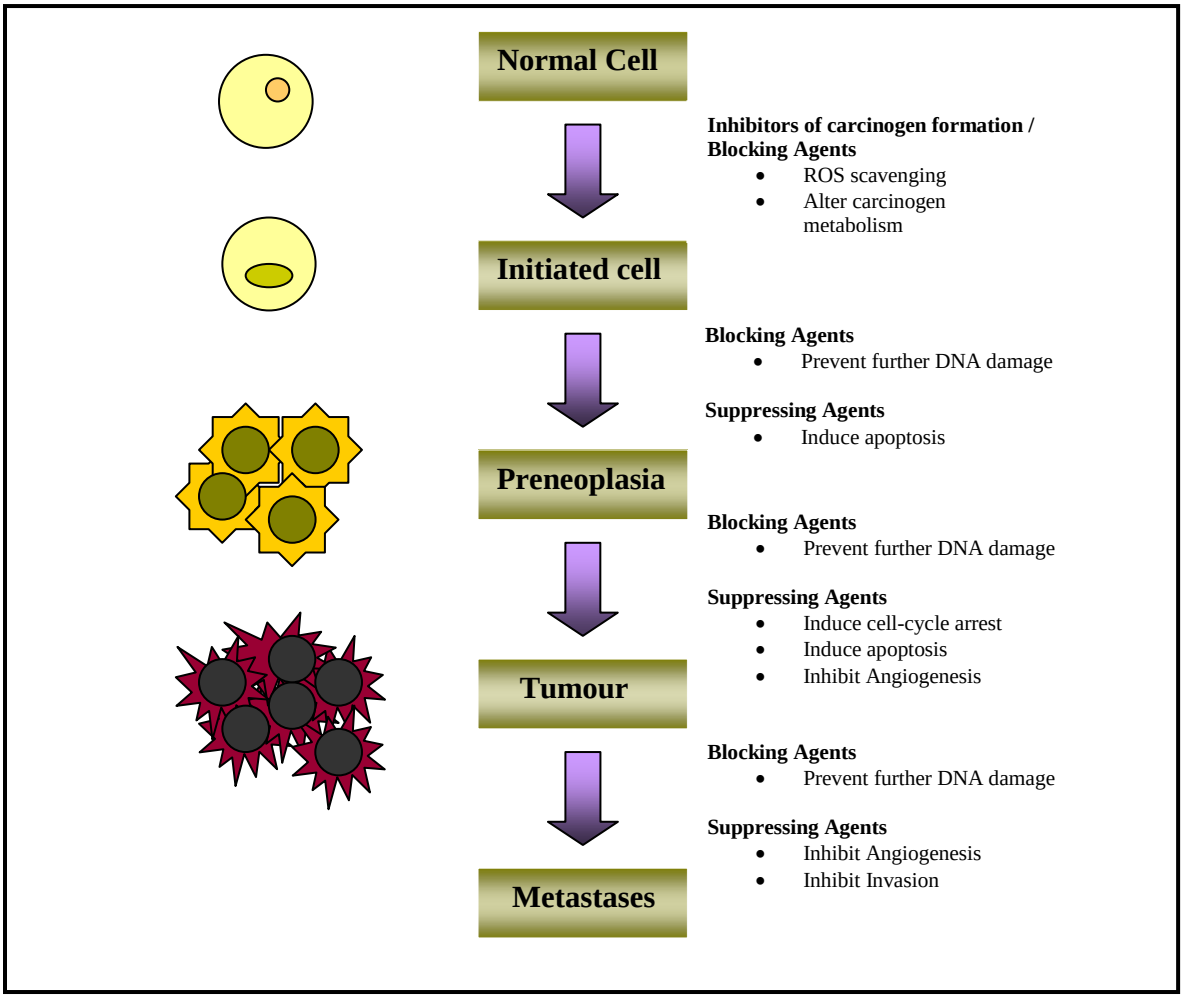
Cancer chemoprevention is an approach of utilizing drugs or natural products to interrupt the multi-step carcinogenic pathway (Wattenberg, 1985). Chemopreventive agents can be classified into 'inhibitors of carcinogen formation', 'blocking agents' or 'suppressing agents'. The agents that inhibit the formation of carcinogens tend to intervene before the initiation of carcinogenesis. 'Blocking agents' can prevent, or greatly reduce initiation of carcinogenesis, while "suppressing agents" affect later stages along the carcinogenesis pathway by reducing cell proliferation, shown in Figure 1.3.

Drugs such as tamoxifen and finasteride have been shown to prevent cancer, or postpone its onset; mammary tumours in the case of the former (Veronesi, 1995) and prostate cancer for the latter (Mellon, 2005). An interesting subgroup of cancer chemopreventive compounds is the diet-derived agents that are expected to be safe with little or no toxicity on long-term consumption in a healthy population. Examples include folate, curcumin, green and black tea polyphenols, genistein, indole-3-carbinol, resveratrol, vitamin D, vitamin E, selenium and calcium (Gescher *et al.*, 2001). Dietary products have the benefit of expected safety and, unlike synthetic pharmaceuticals, are not perceived as 'medicine', leading to their acceptable wide-

spread and long-term use in the normal risk population. Also, cancer chemoprevention requires individuals to take a compound for prolonged periods, possibly for life which makes it essential that the agent is of low cost, high efficacy, low toxicity and displays little interaction with other medications.

Figure 1.3 Potential for intervention in carcinogenesis. Chemopreventive agents can exert blocking or suppressing effects on the different stages in the pathway. Blocking mechanism prevents damage to DNA, whereas suppression slows down or inhibit the growth of transformed cells or new blood vessels.

Modified from Manson *et al*. (Manson *et al.*, 2000). *(ROS = reactive oxygen species)*



1.2.2 Cancer chemopreventive agents

One of the more promising and better tested groups of colorectal cancer chemopreventive agents is the family of non-steroidal anti-inflammatory drugs (NSAIDs). In multiple population-based case-controlled studies, aspirin has repeatedly been found to cause a 50 % risk reduction in CRC occurrence (Logan *et al.*, 1993; Giovannucci *et al.*, 1994; Kune *et al.*, 2007; Thun *et al.*, 1991). While not all studies have demonstrated an association between NSAIDs intake and CRC risk reduction (Thun *et al.*, 1991), the findings do support a relationship between the use of low dose aspirin and the decreased risk of CRC. Other NSAIDs agent that have been investigated include sulindac (Labayle *et al.*, 1991; Nugent, 1995; Giardiello *et al.*, 2002), piroxicam (Calaluce *et al.*, 2000; Rao *et al.*, 1991) and the selective COX-2 inhibitors; celecoxib (Steinbach *et al.*, 2000) and rofecoxib (Hallak *et al.*, 2003). Table 1.1 lists some of the pharmaceutical and dietary chemopreventive agents used in clinical trials.

Also, many dietary compounds have shown promise in chemopreventive efficacy in preclinical and clinical studies. There is good evidence to suggest an increased intake of calcium and Vitamin D reduces the risk of developing colorectal adenomatous polyps (Neugut *et al.*, 1996; Newmark & Lipkin, 1992) and large bowel cancer (Bostick *et al.*, 1997; Kampman *et al.*, 2000). Diets high in folate or fibre (Freudenheim *et al.*, 1991; Simopoulos, 2004), selenium (Reddy *et al.*, 1997a) or perillyl alcohol from citrus fruits (Reddy *et al.*, 1997b) have also demonstrated chemopreventive activity.

Curcumin, a component of tumeric, deserves particular attention. Previous studies from the same laboratory where the current research project was conducted showed that curcumin displayed substantial chemopreventive activity in an animal model of colorectal carcinogenesis (Perkins *et al.*, 2002) and has strong anti-proliferative effects on human colon cancer cells (Chen *et al.*, 2006; Collett & Campbell, 2005). It is one of the few dietary agents that has also shown pharmacodynamic efficacy in humans. (Sharma *et al.*, 2004; Garcea *et al.*, 2005)

Table 1.1 Agents undergoing preclinical and clinical trials for chemoprevention activity.

Modified from Kelloff et al. (Kelloff et al., 1999)

Chemopreventive agents	Target organ	E.g. of the mechanisms of action
Pharmaceuticals		
➢ NSAID • Aspirin • Sulindac • Piroxicam • Ibuprofen • Indomethacin	Colon Oesophagus	• Inhibit formation/ activation of carcinogen by inhibiting cyclooxygenase (COX). • Inhibit polyamine metabolism by inhibiting ornithine decarboxylase (ODC) induction. • Restore immune response by enhancing Langerhan's cells. • Induce apoptosis by enhancing arachidonic acid.
➢ Selective COX 2 Inhibitor • Celecoxib • Rofecoxib	Colon	• Similar to NSAIDS. • Inhibit angiogenesis by inhibiting tyrosine kinase
➢ DMFO (Dimethylfluoro-ornithine)	Colon Oesophagus Breast Prostate Bladder	• Inhibit polyamine metabolism by inhibiting ornithine decarboxylase (ODC)
➢ Antioestrogens / antiandrogens • Tamoxifen • Finasteride	Breast Prostate	• Modulate hormone / growth factor activity • Induce apoptosis by inducing TGF β.
➢ Oltipraz	Liver	• Deactivate carcinogens by enhancing Glutathione-S-Transferase (GST) enzyme.
➢ Ursodeoxycholic acid	Colon	• Inhibit carcinogen uptake by binding to bile, a known tumour promoter.

Table 1.1 (continued)**.** Agents undergoing preclinical and clinical trials for chemoprevention activity. Modified from Kelloff *et al.* (Kelloff *et al.*, 1999)

Chemopreventive Agents	**Target Organ**	**Mechanism of Action**
Dietary Compounds		
➢ Calcium	Colon	• Inhibit carcinogen uptake by binding to bile, a known tumour promoter.
➢ Folic Acid	Colon Cervix	• DNA repair by enhancing CpG island methylation.
➢ Retinoids : Vitamin A - E - C - D - β-carotene	Lung Cervix Head and neck Breast Prostate Colon	• Modulate growth factor activity by inhibiting IGF-1. • Inhibit ODC induction. • Induce terminal differentiation and apoptosis by inducing TGF-β. • Restore immune response by enhancing Langherans cells. • Inhibit angiogenesis by inhibiting thrombomodulin.
➢ Selenium	Oesophagus	• Enhancement of T-lymphocytes
➢ Curcumin (from tumeric)	Colon Breast Oral cavity	• COX inhibition • Reduction of DNA adducts • Anti-angiogenesis • Induction of Phase II detoxifying enzymes
➢ Ellagic acid	Oesophagus	• Phase I Enzyme Inhibition by inhibiting cytochrome P450.
➢ Indole-3 Carbinol	Breast	• Induce cytochrome P450 system in Phase I Metabolism.
➢ Epigallocatechin gallate (from tea and chocolate)	Colon Head and Neck Skin	• Inhibit cytochrome P450 and prevent carcinogen – DNA binding. • Inhibit COX and enhance T-lymphocytes in restoring immune response.
➢ Genistein (from cereals and pulses)	Breast Prostate Colon	• Inhibit cytochrome P450 and inhibit activation of carcinogen. • Modulate hormone activity by antagonizing oestrogen receptors. • Inhibit angiogenesis by inhibiting tyrosine kinase.

1.2.3 Biomarkers of carcinogenesis

To confirm efficacy for any chemopreventive agent, one has to administer the compound to a cohort of individuals and evaluate the difference in the incidence of the target tumour between the trial and control groups. Such trials are lengthy, expensive and a large number of subjects are required. To circumvent this, small-scale phase I pilot studies with a short time course could be carried out to assess the feasibility and appropriateness of conducting extended drug trials. The major obstacle in the design of chemoprevention pilot studies is the lack of a suitable endpoint that correlates with delay or prevention of carcinogenesis. This requires identifying and validating suitable biomarkers to act as 'surrogate' end points of the disease process.

Biomarkers of carcinogenesis are quantifiable molecules involved in physiological and pathological transformation steps which occur between exposure to exogenous or endogenous carcinogens and the subsequent development of cancer (Kelloff *et al.*, 2001). These biomarkers provide a means of diagnosing very early changes of cancer development before actual tumours or precancerous lesions are present. In contrast to histological markers, such as polyp formation or the presence of high grade dysplasia, molecular biomarkers can often be assayed in surrogate tissue such as blood or faeces, which obviates the need for tissue biopsies. Changes in the levels of these molecular biomarkers are often detectable before any malignant changes are visible, with the biomarker assays being usually quantitative, thus reducing inter-observer variation. In addition, molecular biomarkers should exhibit properties intimately linked to the particular role they play in the development of the specific disease they are intended to signify. For example, markers of the *APC* tumour suppressor gene relate

particularly to colorectal carcinogenesis. The expression of a biomarker which is to be used in surveillance or early diagnosis of cancer should correlate with early stage of the disease. Its levels should be differentially expressed in normal, premalignant and malignant tissue with this correlation being reliable and reproducible. Biomarkers should be detected sufficiently early in the carcinogenesis cascade to allow a favorable impact on mortality from early intervention (Groopman *et al.*, 1995; Einspahr *et al.*, 1997). For the purpose of chemoprevention intervention studies, levels of biomarkers must be modifiable by the particular agent tested (Einspahr *et al.*, 1997) which should correlate with a reduction in tumour incidence.

Biomarkers could be classified into three different groups according to their properties and clinical application; (i) risk biomarkers (ii) diagnostic biomarkers and (iii) intervention biomarkers (Garcea *et al.*, 2003b). Risk biomarkers are used to identify individuals who are at a high risk of developing that particular disease within the general population, e.g. the *APC gene* defect in predicting colorectal cancer. Risk biomarkers with low specificity and high sensitivity could be used for cost-effective population screening. Groups within the population with an above-average risk identified from screening can then undergo more invasive investigations. In contrast to risk biomarkers which do not predict early disease in any given individual, diagnostic biomarkers help identify individuals with early cancer before any clinical evidence of cancer. Such an example includes serum and stool testing for the proto-oncogene *K-RAS* in confirming the presence of CRC. Finally intervention biomarkers are molecular markers used as indicators of efficacy in a particular intervention or treatment. Examples of molecular biomarkers used in CRC are outlined in Table 1.2.

Table 1.2 Molecular biomarkers used in colorectal cancer.

Modified from Garcea *et al*. (Garcea *et al*., 2003b).

Class of Biomarkers	**Examples of important biomarkers**	**Application**
Risk biomarkers	• Glutathione S-Transferase • Gene defect, e.g. *APC* gene defect • DNA adducts (with reactive oxygen species)	Identify individuals at risk of developing CRC.
Diagnostic biomarkers		
➢ Serum testing for genetic defects	• *APC* gene localised to chromosome *5q21* (Bodmer WF, Nature, 1987) • Oncogene *K-RAS* (Kopreski MS, JNCI,2000) • Functional analysis of MMR mutation	Identification of carcinogenesis and followed by appropriate treatment
➢ Mass spectrometry and CDNA microarray	• Calcium-binding protein calgranulin (Stulik J, Clinica Chemical Acta, 1997)	
➢ Stool testing for genetic defects	• *K-RAS* proto-oncogene (Lev Z, J Cell Biochem, 2000) • *TP53* • *BAT-26*	
Intervention biomarkers		
➢ DNA alterations	• Oxidative DNA adducts ▪ Pyrimidopurinone adducts of deoxyguanosine (M_1dG) ▪ 8-oxo-deoxyguanosine	To monitor the efficacy of intervention (e.g. chemopreventive agents)
➢ Differences in polyamine metabolism	• Ornithine decarboxylase	
➢ Cytokine levels	• Prostaglandins (PGE2) • Regulatory enzymes such as cyclo-oxygenase-2 isoenzyme (COX-2)	
➢ Markers of cycle event	• β-catenin	

1.2.4 Development of chemoprevention agents

Chemopreventive agents need to be administered for prolonged periods of time in order to be efficacious. Hence, they need to undergo rigorous testing for toxicity before they can be subjected to extensive clinical trials. To date, there are more than 40 promising agents and agent combinations that are under evaluation for major cancer targets (Kelloff *et al.*, 2000). However, more than 80% of the several thousand agents reported to have chemopreventive activity do not get marketing approval, with many failing late in their development (sometime at phase III trials) due to unexpected safety issues, lack of efficacy or other confounding factors (Kelloff *et al.*, 1999). There is a clear need for faster, more cost-effective strategies to evaluate novel agents in suitable populations.

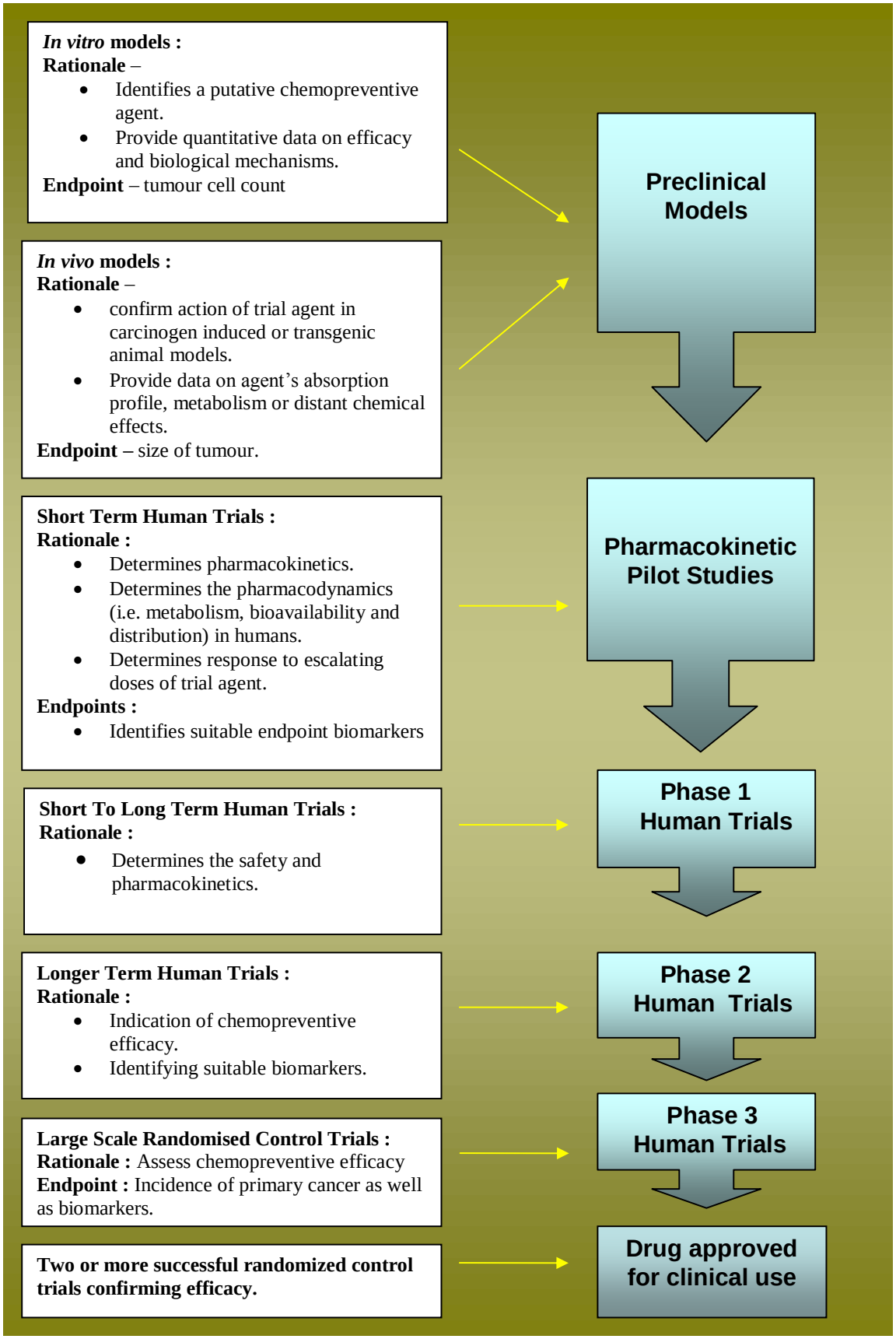
Preclinical investigations of agents for chemopreventive activity usually precede clinical trials and follow a structured design. *In vitro* mechanistic assays (Steele & Kelloff, 2005) and cell-based transformation assays are carried out to provide sufficient quantitative data on efficacy, toxicity and biological mechanisms. However, the major limitation of *in vitro* assessments is that they give no information on the agent's absorption profile, metabolism or distant chemical effects.

Animal studies allow these crucial data to be validated. *In vivo* animal models can be broadly classified into animal models of chemical carcinogenesis or genetically modified rodent models of cancer development. In CRC studies, carcinogens such as azoxymethane and dimethylhydrazine are commonly used to promote the development of colonic adenomas (Corpet & Pierre, 2003). Such an example is provided by Kohno (Kohno *et al.*, 2002) and Gershbein (Gershbein, 1994). Exposure to these chemicals causes development of intestinal adenocarcinoma on which the

chemopreventive agents can be tested. Alternatively, the genetic *Apc*$^{Min+}$ (Multiple Interstinal Neoplasia) mouse model which carries an inherited germline mutation in the *APC gene* promoting multiple small interstinal adenomas is often used to imitate the human condition familial adenomatous polyposis (FAP). In liver tumour studies, a potent carcinogen such as aflatoxin (Roebuck *et al.*, 1991) or diethylnitrosamine (Chuang *et al.*, 2000) is given to mice or rats in high doses to induce hepatocellular cancer which mimics the condition in man. Results from studies in such rodents help prioritize putative chemopreventive agents for further evaluation in short or long term human clinical trials. Although the genetic and carcinogen induced animal models of cancer offer opportunities to study chemoprevention across the different stages of the carcinogenic pathway, they cannot predict an agent's chemopreventive effect in humans. This is primarily because most humans develop tumours from sporadic *APC* mutations as opposed to germline defects and the effect of environmental carcinogens on cancer development is probably significant. Secondly, the *Apc*$^{Min+}$ mouse develops small bowel adenomas causing GI haemorrhage which eventually kills the mouse unlike FAP in humans where tumours occur in the colon and rectum and progress to malignancy and metastases. In addition, adenocarcinomas are seldom observed in this mouse model, and no typical aberrant crypt foci (ACF) arises above the intestinal mucosa. Consequently, the ACF to carcinoma progression is not established in this mouse model. Finally, *K-ras* mutations and *p53* inactivation that is frequently observed in human colon cancers are not detected in the *Apc*$^{Min+}$ model. As yet, no satisfactory animal model has been found to reproduce the liver metastases from primary CRC. Hence, pilot studies are necessary for the characterization of an agent in terms of efficacy, safety, pharmacokinetic and pharmacodynamic properties in humans. The knowledge gained in such pilot studies can help optimize the design of large-scale trials.

Figure 1.4 Stages of clinical trials of chemopreventive agents. Boxes on the right display the progression of clinical trials. Inserts on the left outlines their objectives.

Modified from Garcea *et al.* (Garcea *et al.*, 2003a)



1.3 Silybinin

Silybinin is the major constituent of silymarin, which has been widely used for decades as a herbal remedy. Silymarin is derived from the milk thistle plant (*Silybum Marianum). Silybum Marianum* is a member of the *asteraceae* family which also encompasses other herbal/weed-like plants including daisies, thistles and even artichokes. Although the plant's natural habitat originated from Europe, it is now widely grown and commercially processed across Europe and the eastern and southern parts of the United States of America. According to legend, its name 'milk thistle' originally derived from its characteristic spiked leaves with white veins which were believed to 'carry the milk of the Virgin Mary'. The mature plant has large, bright purple flowers and abundance of stout pines making it easily recognizable. Silybinin, the biologically active component, makes up about 60% of the lipophilic silymarin which is extracted from the seeds, fruit and leaves of the plant.

Figure 1.5 ***Silybum Marianum*** (Milk thistle).
Picture taken from phytochemical.info.com.

1.3.1 Silybinin : properties, chemistry and formulation

Silymarin is a mixture of flavonolignans, namely silybinin, silydianin, silycristin, and taxifolin at ratio of 3:1:1:traces (Wagner *et al.,* 1968). Recently, Mackinnon *et al.* (MacKinnon *et al.*, 2007) have isolated a new flavonolignan silyamandin from the tincture preparations of the milk thistle fruit. The word 'flavanoid' derives from the Latin word 'flavus' meaning yellow. 'Lignans' are a large group of natural products characterised by the coupling of two C_6C_3 units, for example propylbenzene. Silybinin which gives a bright yellow solution, is the most biologically active component and known for its medicinal use worldwide as a hepatoprotectant (Flora *et al.*, 1998) as well as a chemopreventive agent (Zi & Agarwal, 1999; Singh *et al.*, 1994).

The structure of silybinin was first established in 1975 by Pelter and Hansel, (Pelter & Hansel, 1975) and later confirmed by Merlini (Merlini *et al.*, 1979). What was termed 'silybinin' was isolated as the diastereoisomeric mixture of silybinin and isosilybinin (57% : 43%) which are not mirror images of each other. Later, Lee and Liu *et al*.(Lee & Liu, 2003) displayed through X-ray, that rotational movements at C-7' and C-8' positions transforms silybinin and isosilybinin to further two distinct molecules which share the same chemical formulae and functional groups but differ in biological activities. These were named silybinin A and silybinin B, isosilybinin A and isosilybinin B (Kim *et al.*, 2003) (Figure 1.6).

Commercially the names silybinin, silibinin, silybin or silibin are used interchangeably. Silybinin is the most active hepatoprotective agent in the silymarin mixture thought to be attributable to its 1,4-dioxane ring system (Ahmed *et al.*, 2003). The disadvantages of the lipophilic silybinin at the clinical level is the poor absorption of the compound after oral administration (Skottova *et al.*, 2001) which is mainly

attributed to two factors. Firstly, its large and bulky multiple ring structure makes it impossible for the molecule to be absorbed through simple diffusion. Secondly, it has poor miscibility with oils and other lipids, severely limiting its ability to pass across the lipid-rich outer membranes of the enterocytes of the small intestine. Therefore a formulation (Silipide; IdB 1016 Indena s.p.a., Milan, Italy) combining silybinin with phosphatidyl choline at a 1:1 molar ratio has been developed to increase silybinin absorption (Morazzoni *et al.*, 1992). Silipide is well tolerated in both animal models and healthy human volunteers (Morazzoni *et al.*, 1992; Barzaghi *et al.*, 1990). Other forms of silybinin-phosphatidylcholine complex utilized in clinical trials include Legalon, Thisilyn and Siliphos.

Figure 1.6 Chemical structure of silybinin A, silybinin B, isosilybinin A and isosilibinin B.

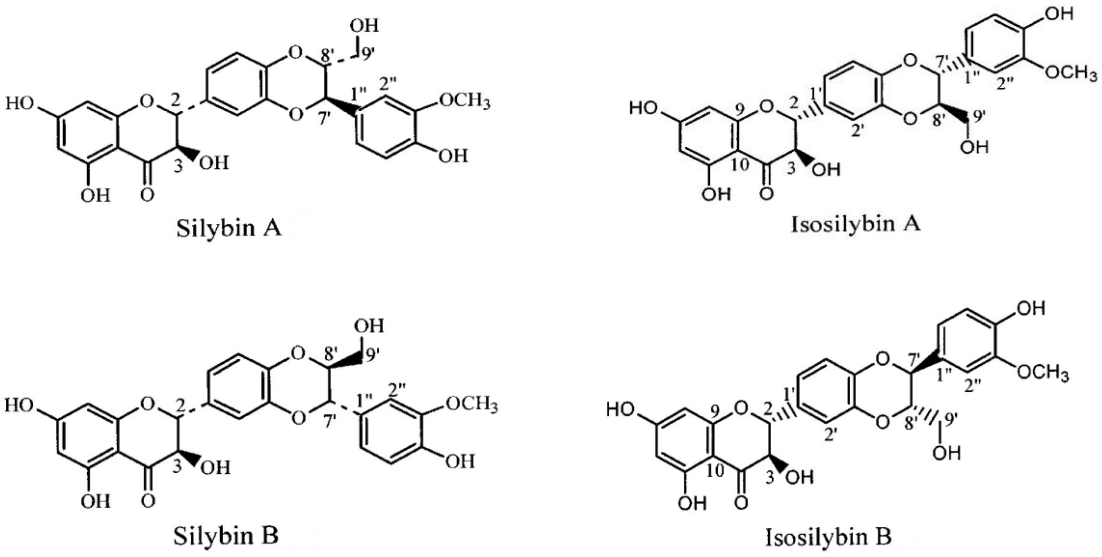
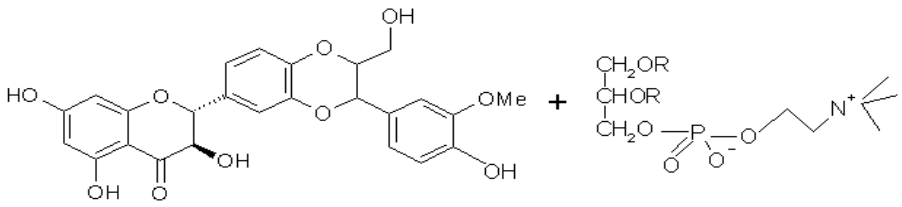


Figure 1.7 Chemical structure of silipide (IdB 1016 Indena s.p.a., Milan, Italy)
(See section 3.2 for silipide formulation and supply)



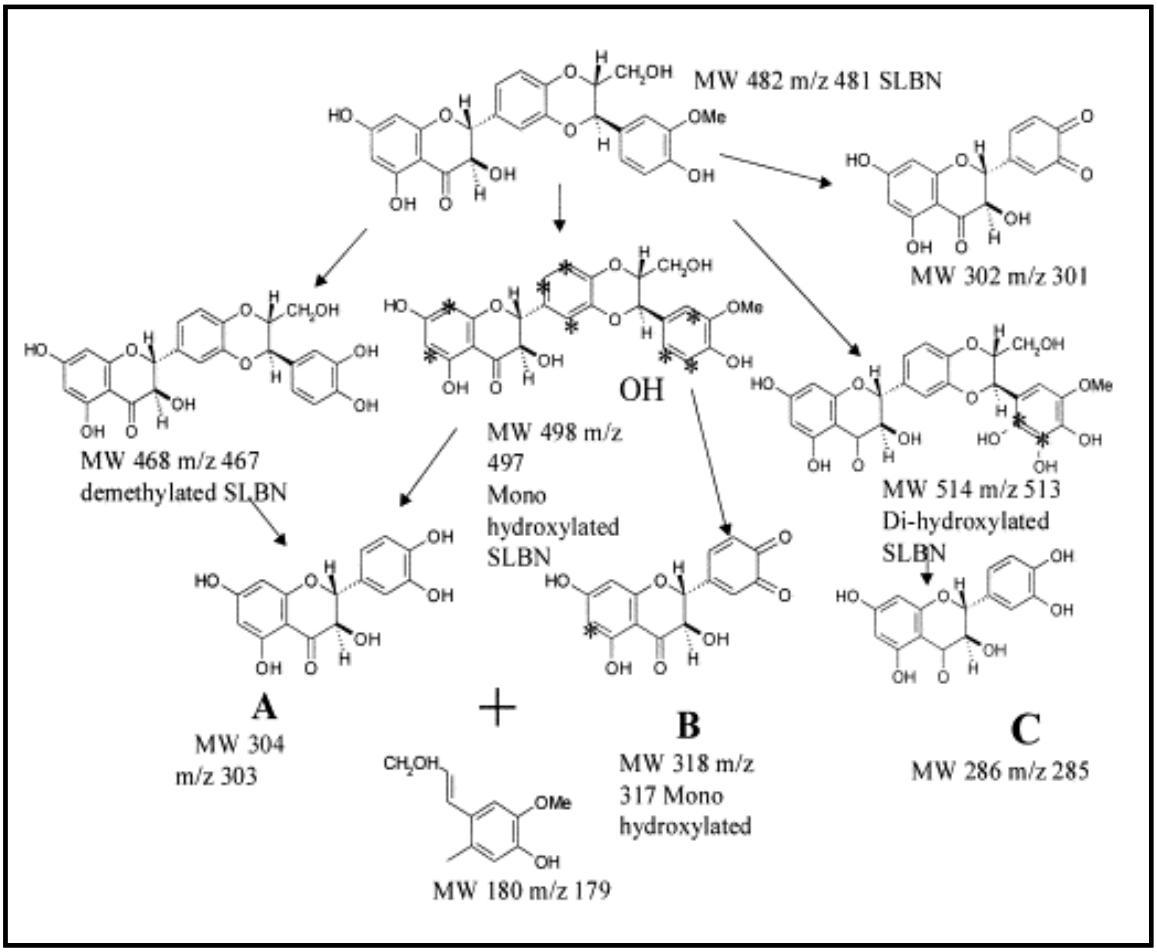
(silybinin) (phosphatidylcholine)

1.3.2 Silybinin : metabolism and pharmacokinetics

Despite the wide use of milk thistle extract as a herbal remedy, the metabolism of silybinin is still not well studied. Silybinin has been shown to undergo phase I and extensive phase II metabolism in human and rat liver preparations *in vitro* (Gunaratna & Zhang, 2003). Gunaratna suggested that demethylation of the silybinin parent compound gives rise to one major demethylated silybinin, three minor monohydroxy silybinin and one minor dihydroxylated silybinin (Gunaratna & Zhang, 2003; Jancova *et al.*, 2007) (Figure 1.8**)**. In a separate study with bovine liver microsomes *in vitro*, it was suggested that silybinin B was glucuronidated at a more efficient rate than that of its diastereoisomer (Han *et al.*, 2004; Kren *et al.*, 2000) and the glucuronidation of silybinin B was preferentially at position C-20, while that of silybinin A occurred equally at both the C-7 and C-20 positions. This suggests that the metabolism of silybinin is steroeselective. In a human pilot study *in vivo,* Gatti *et. al.* (Gatti & Perucca, 1994) showed that a large amount of orally consumed silybinin were converted to its sulphate and glucuronide metabolites before excretion from the circulation. Next to nothing is known about pharmacological properties of metabolic conjugates of silybinin, and it is conceivable that at least some of them share pharmacological activity with the parent molecule.

Figure 1.8 Demethylation of silybinin giving several metabolites as confirmed by LC-MS. The possible hydroxylated sites are denoted by an asterisk.

Modified from Gunaratna and Zhang. (Gunaratna & Zhang, *2003*)



The superior bioavailability of silipide (oral silybinin) over non-complexed silybinin has been documented in pharmacokinetic studies conducted in rats (Morazzoni *et al.*, 1992; Yanyu *et al.*, 2006) and humans (Barzaghi *et al.*, 1990). In rodents, silybinin in plasma remained virtually undetectable after oral ingestion of a single large dose of uncomplexed silybinin, while in contrast, when the same amount of silybinin was given as silipide, silybinin plasma levels were measurable within minutes (Morazzoni *et al.*, 1993). After administration, 94% of the plasma silybinin was present in conjugated form (Morazzoni *et al.*, 1992). Similar studies in humans volunteers (360 mg single dose silipide) have also shown 4.6 times greater oral bioavailability of silipide as compared to silymarin (Barzaghi *et al.*, 1990), the crude form of silybinin. Peak plasma levels were seen at 2 h post ingestion (ranged from 24 – 201 ng/mL) and the elimination $T_{1/2}$ was 6 h (Barzaghi *et al.*, 1990; Lorenz *et al.*, 1982). A follow up multiple-dose study with healthy volunteers (120 mg silipide twice daily for 8 days) confirmed that there was no decline in absorption efficiency after multiple dosing (Barzaghi *et al.*, 1990).

In animals, silybinin rapidly reaches the liver, spleen, heart and kidney before the majority of the compound (13% of the administered dose over 24 hours) is extracted through the bile and undergoes enterohepatic circulation (Morazzoni *et al.*, 1993; Lorenz & Mennicke, 1981). Urinary recovery of silybinin levels was low (Barzaghi *et al.*, 1990). In humans, the assumption that silybinin accesses the liver, a target organ for pharmacological action, is based on the high levels of the compound (up to 11% of the dose administered) recovered from the bile of 9 cholecystectomy patients with T-tube drainage following single oral doses of silipide, equivalent to 120 mg of silybinin (Schandalik *et al.*, 1992). To date, the level of silybinin in humans has only been

measured in plasma, urine and bile (Barzaghi *et al.*, 1990). Little is known about the pharmacokinetic and pharmacodynamic properties of silybinin in humans.

1.4 The pharmacology of silybinin

1.4.1 Silybinin as a hepato-protectant

Silybinin has been used as a medical remedy since ancient Greek civilization, where it was used to treat the bites of poisonous snakes (Grieve, 1931) and to treat jaundice (Foster, 1990). Gerarde in 1596 stated that milk thistle was the "best remedy against melancholy" or "black bile" (Grieve, 1931). British herbalist Culpepper discovered that silybinin was a remedy for symptoms of liver or splenic obstructive diseases, and the widespread practice of silybinin infusions was used to treat jaundice secondary to gallstone biliary obstruction (Grieve, 1931). More recently, silybinin has also been used for the treatment of hepatic disorders such as acute viral hepatitis (Magliulo *et al.*, 1978), alcoholic liver disease (Ferenci *et al.*, 1989; Pares *et al.*, 1998) and death cap mushroom poisoning (Hruby *et al.*, 1983).

Homeopathic practitioners currently advocate preparations of silybinin as a potent treatment in jaundice, gallstones, toxin-induced liver damage as well as for peritonitis, haemorrhage, bronchitis and dyspeptic symptoms. The properties of silybinin as a hepatoprotectant have been investigated in laboratories using cell lines and animal models. Collectively, *in vitro* and animal studies provide some explanations for the protective effects of silybinin in liver disease. Although the underlying mechanisms of action have not been characterized, data in the literature indicate that silybinin acts in four different ways : (i) as an antioxidant, scavenger of free radicals (Mira *et al.*, 1994) and regulator of the intracellular content of glutathione (Pietrangelo *et al.*,

1995); (ii) as a cell membrane stabiliser and permeability regulator that prevents hepatotoxic agents from entering the hepatocytes (Bosisio *et al.*, 1992; Carini *et al.*, 1992); (iii) as a promoter of ribosomal RNA synthesis, stimulating liver regeneration (Magliulo *et al.*, 1979); and (iv) as inhibitor of the transformation of stellate hepatocytes into myofibroblasts, the process responsible for the deposition of collagen fibres leading to cirrhosis (Boigk *et al.*, 1997; Schuppan & Hahn, 2001).

Most clinical trials of milk thistle have been conducted in patients with either hepatitis (Flisiak & Prokopowicz, 1997) or cirrhosis (Pares *et al.*, 1998; Ferenci *et al.*, 1989; Lucena *et al.*, 2002). Other studies have investigated silybinin in patients with hyperlipidaemia, diabetes (Velussi *et al.*, 1997) and *Amanita Phalloides* mushroom poisoning (Hruby *et al.*, 1983). Eight randomised trials have been reported in patients with hepatitis or cirrhosis, while only one randomised trial reported the use of silybinin as a prophylaxis to iatrogenic hepatic toxicity. Endpoints for these trials have generally included serum levels of bilirubin and/or the liver enzymes, SGOT / AST, and SGPT / ALT, as indicators of liver inflammation, damage or diseases, while the lowering of their serum levels is a sign of clinical improvement. In patients with Hepatitis A and B, Magliulo et al. (Magliulo *et al.*, 1978) found that silybinin at 140 mg/day for 3-4 weeks resulted in lower levels of AST / ALT, and bilirubin by day 5, compared with the placebo group. In another randomised placebo-controlled study of patients with viral hepatitis B, silybinin at 210 mg/day had no effect on either the disease course or liver enzyme levels (Flisiak & Prokopowicz, 1997).

In patients with chronic liver disease, a randomised, placebo-controlled study found normalisation of serum AST, ALT and bilirubin levels after 1 month of treatment with silybinin (140 mg three times daily), in comparison to treatment with placebo. In another large observational study involving 2,637 patients with chronic liver disease, 8 week treatment with 560 mg/day of silybinin resulted in reduction of serum

AST/ALT, and gamma-glutamyltranspeptidase (γ-GT) and a decrease in the frequency of palpable hepatomegaly. Both Hruby (Hruby *et al.*, 1983) and Enjalbert (Enjalbert *et al.,* 2002) described silybinin as the only effective antidote in patients with liver damage from death cap mushroom (*Amanita Phalloides).* The beneficial effect of silybinin on liver histology suggests it has a role in the prevention of hepatitis and/or cirrhosis. Despite its global use as a remedy for liver diseases, no study has yet measured the bioavailability of silybinin in the liver although its hepatic presence has been implicated (Schandalik *et al.*, 1992). Table 1.3 lists some previous clinical trials using silybinin as a treatment for liver disease.

Many chemotherapeutic agents cause hepatotoxicity. Invernizzi and colleagues (Invernizzi *et al.*, 1993) have shown that silymarin maintain normal liver transaminase levels in leukaemia patients, who previously experienced methotrexate and 6-mercaptopurine hepatotoxicity. In children on chemotherapy for acute lymphoblastic leukaemia (ALL) which lead to hepatotoxicity, milk thistle supplementation has been associated with a decrease in liver transaminase levels and a greater than 50% reduction in total bilirubin. Other studies have demonstrated that silymarin may play a role in adjuvant cancer therapy. Silymarin have shown to increase daunomycin accumulation, potentiated doxorubicin toxicity and inhibited efflux of these drugs from cancer cells, hence improving the chemotherapeutic drug efficacy. In a non-randomised study, patients with brain metastases receiving stereotactic radiotherapy with omega 3 fatty acids and silymarin, have been shown to have longer survival times and a decreased number of radionecroses (Ramasamy & Agarwal, 2008). Further clinical studies are necessary to examine silybinin activity in multifactorial mechanisms of action, well-designed clinical trials and clarification of adverse effects.

Table 1.3 Clinical studies investigating silybinin / silymarin in the treatment or prevention of liver disease.

Reference Citation	Type of Study	Type of Disease	Number of Patient Enrolled;Treated; Control	Findings
(Salmi & Sarna, 1982)	Double blind placebo controlled randomised clinical trial	Acute and Subacute liver disease	106 ; 47 ; 50	Decreased LFTs; improved histology
(Ferenci *et al.*, 1989)	Double blind placebo controlled randomised clinical trial	Cirrhosis	170 ; 87 ; 83	Increased survival
(Flisiak & Prokopowicz, 1997)	Controlled Randomised trial	Viral Hepatitis B	52 ; 20-silybinin ; 20-misoprostol ; 12	No significance
(Pares *et al.*, 1998)	Double blind placebo-controlled randomised clinical trial	Alcohol induced cirrhosis	200 ; 58 ; 67	No significance
(Lucena *et al.*, 2002)	Double blind placebo- controlled randomised clinical trial	Alcohol induced cirrhosis	60 ; 24 ; 25	Significant increases in erythrocyte glutathione and decreased platelet MDAvalues; no significant differences in liver function tests
(Angulo *et al.*, 2000)	Non randomised pilot study	Primary biliary cirrhosis	27 ; 27 ; 0	No significant findings
(Velussi *et al.*, 1997)	Controlled randomised trial	Diabetic patients with cirrhosis	60 ; 30 ; 30	Decrease in lipid peroxidation and insulin resistance

1.4.2 Silybinin as a cancer chemopreventive agent

Recently, silybinin has received much attention regarding its anti-proliferative and anti-carcinogenic effects with respect to several malignancies including prostate, breast, ovarian, skin, bladder, lung and colorectal neoplasia as outlined in several *in vitro* and *in vivo* studies (Singh & Agarwal, 2004b; Tyagi *et al.*, 2004b; Gallo *et al.*, 2003; Singh *et al.*, 2002b; Tyagi *et al.*, 2004a; Singh *et al.*, 2004; Kohno *et al.*, 2002). For example, silybinin has been shown to inhibit mitogenic and cell survival signaling in LNCaP (Zhu *et al.*, 2001), DU 145 (Dhanalakshmi *et al.*, 2002; Tyagi *et al.*, 2002) and PC3 (Deep *et al.*, 2006) human prostate carcinoma cell lines. In certain animal models, silybinin strongly prevents and inhibits the growth of advanced human prostate tumour (Singh *et al.*, 2002a; Singh *et al.*, 2003) and was found to increase plasma insulin-like growth factor-binding protein-3 levels (Singh *et al.*, 2002a). Since the completion of the work reported in this thesis, several clinical trials involving silybinin phytosome have been carried out. The trials include a phase I clinical trial and pharmacokinetic study of silybinin-phytosome in prostate cancer patients (Flaig *et al.*, 2007), in which thirteen patients with advanced prostate cancer received a silybinin-phytosome (namely Siliphos) at dose levels between 2.5 – 20 g in three divided doses for 4 weeks. A quarter of all patients who underwent the trial experienced asymptomatic liver toxicity, but otherwise silybinin was well tolerated (Flaig *et al.*, 2007). These results complement the findings reported in Chapter 6. Currently, a phase II clinical trial to assess the effect of silybinin administration on prostate cancer progression using surrogate biomarkers as endpoints is in progress and results are awaited (Ramasamy & Agarwal, 2008). In parallel, silymarin (along with soy, lycopene and anti-oxidants) was used in a phase III clinical trial to delay prostate

specific antigen progression after prostatectomy and radiotherapy in prostate cancer patients (Schroder *et al.*, 2005)

With respect to colorectal cancer, both silymarin and silybinin have shown a strong cytotoxic effect against colon cancer cell lines (Yang *et al.*, 2003). Recent evidence in rodent preclinical studies have also suggested that silybinin may be useful in chemoprevention of malignancies of the intestinal tract (Verschoyle *et al.*, 2008; Kohno *et al.*, 2002). Dietary silymarin delayed the development of intestinal adenocarcinomas in rats induced by dimethylhydrazine (Gershbein, 1994) or azoxymethane (Kohno *et al.*, 2002). In the former, silymarin (0.1% of diet) was given by oral gavage to 4 week old male rodents in conjunction with dimethylhadrazine for 32 weeks. There was a significant decrease in both proximal and distal colonic adenocarcinoma frequencies in the treatment group. In the latter experiment, dietary silymarin was administered for 4 weeks to male F344 rats with azoxymethane (AOM)-induced colonic aberrant crypt foci (ACF), the putative precursor lesions for colonic adenocarcinoma (Kohno *et al.*, 2002). It also suppressed ACF and colonic adenocarcinoma, but this suppression was dose-independent and did not involve induction of apoptosis (Volate *et al.*, 2005). In our laboratory, silybinin, but not silipide, showed a marginal adenoma number-reducing effect in the Apc^{Min+} mice, the genetic model of intestinal malignancy (Verschoyle *et al.*, 2008). The transgenic Apc^{Min} mouse (Section 1.2.4) has a mutated *Apc* gene, similar to that in patients with familial adenomatous polyposis (FAP) and in many sporadic cancers, in that it mimics the rapid development of adenomatous polyps. Apc^{Min+} mice were randomized to being fed either a standard diet or with oral silybinin or silipide (0.2 % w/w in terms of silybinin, approximately 300 mg/kg per day) for 21 days from weaning until death. Murine blood was obtained and their haematocrit measured. In addition their

intestinal tract was removed and small bowel examined for multiplicity, location and size of adenomas. Intervention with dietary silybinin, but not silipide, reduced adenoma number in the small intestine by 18% and raised the haematocrit by 21%, albeit failed to reach a significant level (Verschoyle *et al.*, 2008). These results cautiously indicate the chemopreventive ability of dietary silybinin against colorectal cancer and provide a scientific basis for progression to clinical trials of the chemoprevention of human colon cancer.

A number of chemopreventive mechanisms of silybinin have been proposed. Silybinin is a potent anti-oxidant involved in the inhibition of overproduction of intracellular superoxide anion (Ramasamy & Agarwal, 2008) and thus interfere with oxidative DNA adducts (Zhao *et al.*, 2000; Comoglio *et al.*, 1990). Recently, silybinin was found to modulate the IGF system (Singh *et al.*, 2002a), which has been implicated as an oncogenic regulator of several malignancies. Silybinin has demonstrated an ability to interfere with angiogenesis in various cancers (Yang *et al.*, 2003; Jiang *et al.*, 2000). In addition, silybinin has anti-inflammatory effects through inhibition of the transcription factor nuclear factor-κB (Deep & Agarwal, 2007; Ramasamy & Agarwal, 2008), which regulates and coordinates the expression of various genes involved in inflammation, cell survival, differentiation and growth. Silybinin also inhibits the nuclear cell cycle at G0/G1 or G2/M phase by inhibiting cyclin-dependant kinases (CDK) and upregulation of cyclin-dependant kinase inhibitors (CDKi) causing attenuation of cancer cell growth and perturbation of cell cycle progression in prostate (Singh & Agarwal, 2004a), ovarian (Scambia *et al.*, 1996), bladder (Tyagi *et al.*, 2007), breast (Zi *et al.*, 1998a), lung (Sharma *et al.*, 2003) and skin (Gu *et al.*, 2005) cancer cells. Several phytochemicals including

silybinin exert anti-cancer effects by inducing cellular apoptosis in cancer cells (Agarwal *et al.*, 2003).

The potent anti-oxidation properties of silybinin in intefering with oxidative DNA adducts and its ability to modulate the IGF system will be discussed in detail in the subsequent sections - these were used as pharmacodynamic biomarkers of the efficacy of silybinin in the clinical trial performed in this thesis. The other mechanisms of silybinin are mentioned and summarised briefly.

1.4.2.1 Silybinin: an anti-oxidant

A generally accepted hypothesis is that reactive oxygen (ROS) and nitrogen species (RNS) play an important role in carcinogenesis (Schreck *et al.*, 1992). These species include superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), hydroxyl radical (HO•), nitrogen oxide (NO•) and their metabolites are all products of normal metabolic pathways of the human organs. When the formation of ROS and RNS is not counter balanced by their removal in normal organ function, the ROS can induce DNA damage. ROS are unstable molecules and therefore readily react with DNA in the cell nucleus resulting in DNA strand breaks, base modification and DNA-protein cross-links. Antioxidants are a group of substances which, when present at low concentrations, significantly inhibit this oxidative process, by being oxidized themselves, hence reducing the ROS and RNS production within the cell. Antioxidants decrease oxidative stress-induced carcinogenesis by direct scavenging of ROS (Tanaka *et al.*, 1997) and by inhibiting cell proliferation secondary to the inhibition of protein phosphorylation (Schreck *et al.*, 1992).

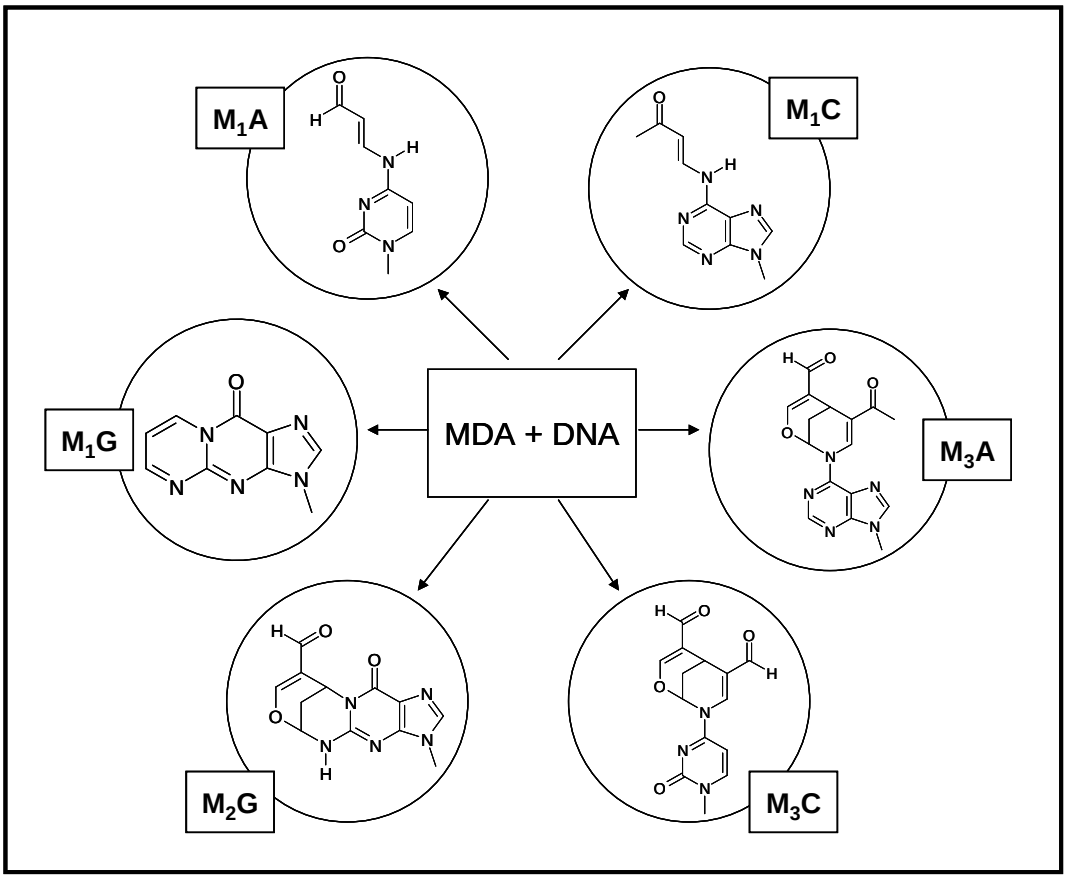
Malondialdehyde (MDA) is a mutagenic carbonyl compound generated in tissues during lipid peroxidation and during prostaglandin biosynthesis (Marnett, 1999a).

MDA binds to form multiple adducts (Figure 1.9). The most common of these directly oxidized base products is pyrimido[1,2-*a*]-purin-10(3*H*)-one, M_1dG (Marnett, 1999a). Both MDA and M_1dG are significantly raised in colorectal cancer tissue (Hendrickse *et al.*, 1994; Schmid *et al.*, 2000). DNA adducts have been found to be raised above normal in colorectal adenomas and also in hepatoma cell lines (Kawanishi *et al.*, 2000; Esterbauer *et al.*, 1990). Oxygen radicals can directly attack DNA bases, alternatively they can oxidise lipids to generate reactive intermediates that react with DNA to form adducts (Hussain *et al.*, 2000). When a cell attempts to replicate this damaged DNA, mutation may occur.

Silymarin and silybinin have been implicated as powerful anti-oxidants as a consequence of their polyphenolic structure (Zhao *et al.*, 2000; Comoglio *et al.*, 1990), and they support redox homeostasis in several *in vitro* and *in vivo* models. Kiruthiga *et al.* (Kiruthiga *et al.*, 2007) have shown that administration of silymarin increases the activites of anti-oxidant enzymes like superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase together with a decrease in the levels of MDA, a marker of lipid peroxidation. Silymarin application extensively lessens GSH depletion and ROS production a well as lipid peroxidation in UVA irradiation-induced damage in human keratinocytes (Svobodova *et al.*, 2007). Lahiri-Chatterjee and Zhao (Lahiri-Chatterjee *et al.*, 1999; Zhao *et al.*, 2000) showed that through its anti-oxidation activity, silybinin applied topically to SENCAR mouse skin resulted in a highly significant protection against skin cancer. Other studies have shown that silymarin inhibits MDA formation in epidermal microsomes in a dose-dependant manner, and also inhibits TPA- and benzoyl peroxide-caused lipid peroxidation in mouse skin epidermis (Agarwal *et al.*, 2006). These studies suggest that silybinin exerts its chemopreventive effect by

reducing oxidative stress, and therefore M_1dG levels may be a suitable marker for silybinin's anti-oxidant and also chemopreventive efficacy.

Figure 1.9 Adduct formation by the carbonyl compound MDA.
Modified from Marnett *et al*. (Marnett, 1999a)



1.4.2.2 Silybinin : a modulator of the IGF system

The IGF system comprises two ligands; IGF-1 and IGF-2, which are potent mitogens produced mainly by hepatocytes in response to growth hormone stimulation (Arany *et al.*, 1994; Olivecrona *et al.*, 1999). Both ligands bind to specific tyrosine kinase receptors and lead to phosphorylation of downstream proteins including protein kinase B (Akt/PKB), phosphatidylinositol 3-kinase (PI3K), MAPK kinase, β-catenin and other molecules implicated in cellular proliferation and survival (Hassan & Macaulay, 2002). However, more than 90% of the circulating IGFs are efficiently sequestered by insulin-like growth factor binding protein (IGFBP), of which there are 6 types. Less than 1% circulate in the free form (Grimberg & Cohen, 2000).

The IGF system has been implicated as an oncogenic regulator in a number of malignancies (Grimberg, 2000; Yu & Rohan, 2000) including those of the colorectum (Hassan & Macaulay, 2002), lung (Yu *et al.*, 1999), prostate (Wolk *et al.*, 1998; Signorello *et al.*, 1999) and pancreas (Lopez & Hanahan, 2002) by affecting proliferation, differentiation, migration and apoptosis of cancer cells. IGF-2, and less importantly IGF-1 polypeptides, have been suggested as regulators of murine tumour growth, both in early adenoma and in the progression to carcinoma in colorectal carcinogenesis (Freier *et al.*, 1999). Inhibition of the mitogenic activity of IGFs can be achieved by the sequestration of the IGF-1 and IGF-2 polypeptides by a high affinity major IGFBP-3 in plasma, thus preventing IGFs from interaction with IGF receptors (Angelloz-Nicoud & Binoux, 1995). In addition, IGFBP-3 also has IGF-independent pro-apoptosis effects as shown in advanced human prostate cancer cells, *in vivo* (Singh *et al.*, 2002a).

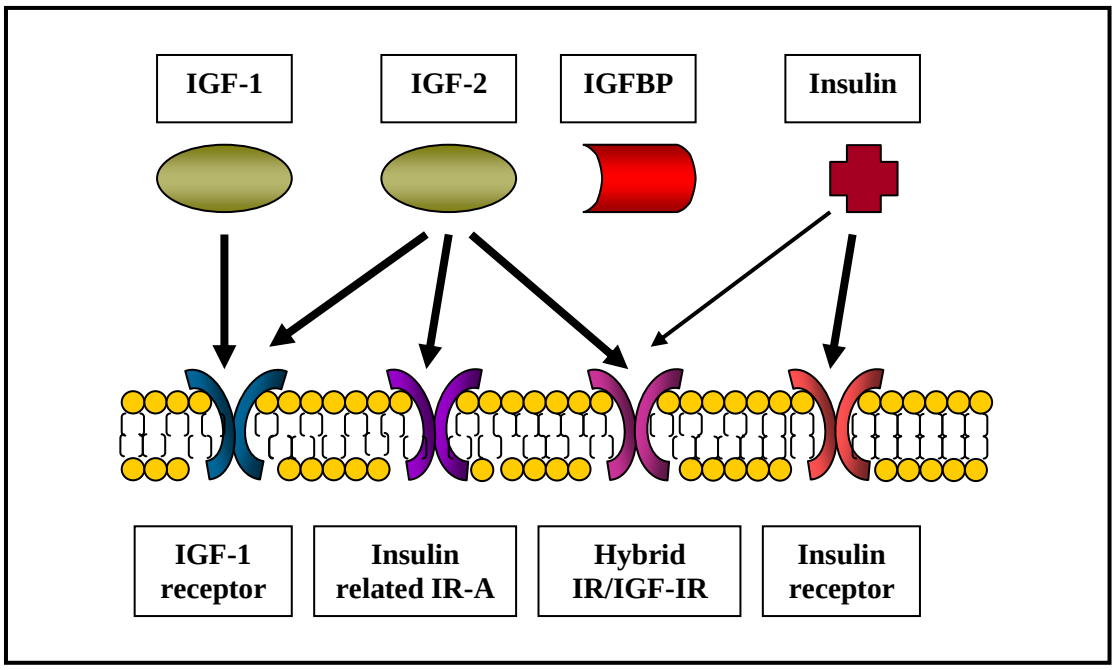
There are several laboratory and epidemiological studies suggesting that the IGF system plays a significant role in colon cancer growth and metastases (Donovan & Kummar, 2008). Wu and colleagues found that the incidence of colonic adenocarcinoma growth and the frequency or numbers of hepatic metasases were significantly reduced in liver specific IGF-1 deficient mice compared to control mice (Wu *et al.*, 2002), suggesting a direct proportional relationship between malignancy and circulating IGF-1 levels. Hakam *et al.* (Hakam *et al.*, 1999) evaluated the role of IGF pathway in human blood and normal and malignant colonic tissues and found a strong IGF-1R positive correlation with higher-grade and higher-stage tumours ($p < 0.01$). A meta-analysis of five epidemiological studies in healthy individuals comparing the highest versus the lowest categories of IGF-1 levels, there was a positive association between elevated levels of circulating IGF-1 and CRC risk, with an odds ratio of 1.58 and a 95% confidence interval of 1.11 – 2.27 (Donovan & Kummar, 2008). In a prospective case controlled study in healthy adults an increased relative risk of developing colorectal cancer was seen in subjects with pre-morbid IGF-1 levels in the highest quartile many years prior to diagnosis (Ma *et al.*, 1999). In this study about 15,000 men were followed for 14 years in the Physicians' Health Study, and men with high IGF-1 levels had a relative risk of developing colorectal cancer of 2.5:1. The effect was reciprocal with respect to the level of IGFBP-3, the main IGF binding protein, as high levels of IGFBP-3 were associated with a decreased relative risk of 0.28. More recently in a large randomised trial, plasma levels of IGF-1, IGF-2 and IGFBP-3 were measured in 527 patients undergoing first line chemotherapy for metastatic colorectal cancer, and results showed a higher baseline plasma IGFBP-3 levels were associated with a significantly greater chemotherapy response rate ($P = 0.03$) and a significantly longer time to tumor progression ($P = 0.03$). Whilst the scientific basis of the overall role of the IGF system

in neoplasia has still to emerge fully, the current hypothesis is that high IGF-1 levels, an endogenous antagonist to IGFBP-3, predisposes to colorectal cancer and may increase the risk in a certain subgroup of individuals. Figure 1.10 shows the two ligands of the extracellular component of the IGF system, IGF-1 and IGF-2, IGFBP and insulin polypeptides competing for the various receptor site on the cell membrane. IGF-1 and IGF-2 predominantly activate IGF-IR, a tyrosine kinase receptor with similar structure to insulin receptor. Both IGFs have a high affinity for the family of binding proteins, mostly IGFBP-3 its major serum carrier protein and their preferential receptor binding sites.

IGF-IR is a tyrosine kinase receptor and mediates the growth-promoting actions of both IGF-1 and IGF-2 (Ullrich *et al.*, 1986). IGF ligands bind to its specific receptors and in turn triggers a cascade of protein phosphorylation on other tyrosine or serine-threonine residues, and activate intracellular signal transduction pathways. Figure 1.11 illustrates the major tyrosine kinase signal transduction pathways.

Figure 1.10 Extracellular components of the insulin-like growth factor system showing IGF-I and IGF-II, IGF binding proteins and insulin polypeptides competing for the various receptor site on the cell membrane.

Modified from Grimberg and Cohen. (Grimberg & Cohen, 2000)

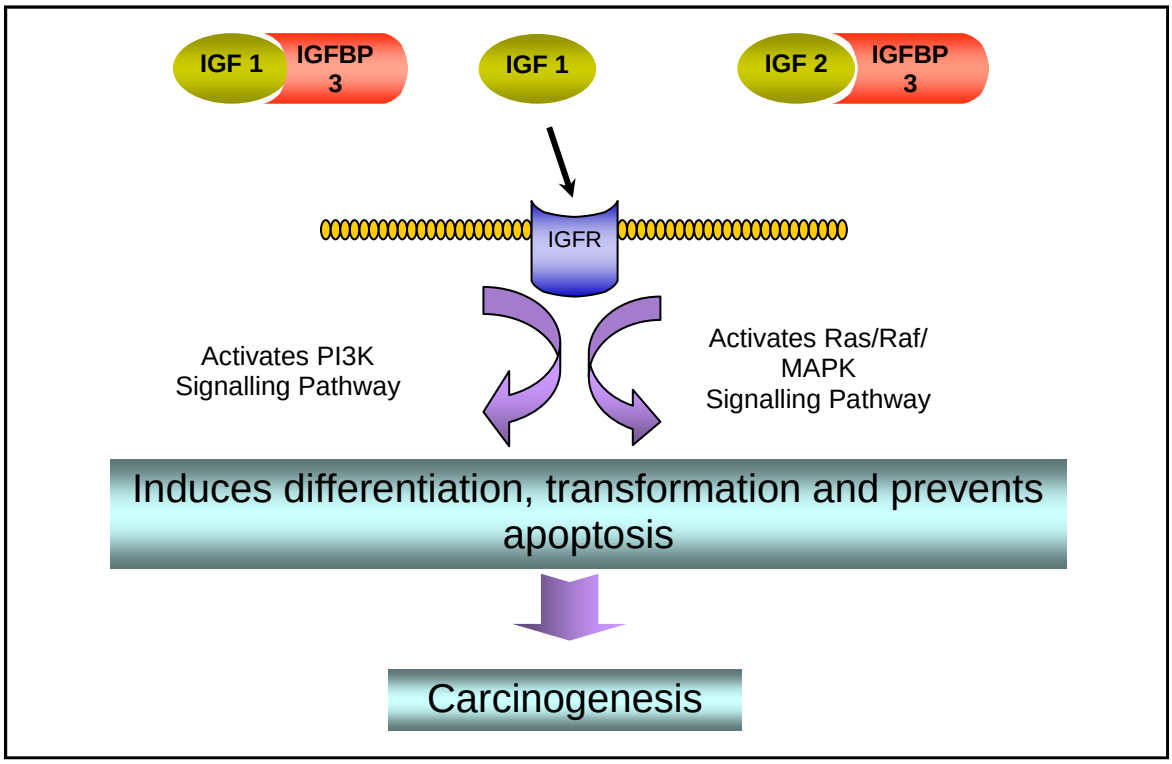


More than 90 % of IGF-1 and IGF-2 ligands are bound to IGFBP-3 and transported within the intravascular system. The remaining 10 % of the unbound ligands may interact and hence activate the tyrosine kinase activity of IGF-IR which triggers downstream activation via ras/raf/MAPK and PI3K/Akt pathways as shown in Figure 1.11. This in turn affects several growth-promoting functions, stimulating mitogenesis in different cell types (Esposito *et al.*, 1997; Hongo *et al.*, 1996) and protecting the cell from apoptosis (Chen *et al.*, 1998)

Silybinin is one of very few agents which have been shown to decrease IGF-1 and increase IGFBP-3 levels in prostate cancer cells leading to growth inhibition, *in vitro* (Zi *et al.*, 2000; Singh *et al.*, 2003) and in prostate tumour-bearing rodents *in vivo* (Singh *et al.*, 2003). Its inhibitory effect on IGF-signaling was established by using IGFBP-3 antisense, which partially reversed the effect of silybinin on insulin receptor

substrate-1 (IRS-1) tyrosine phosphorylation and cell proliferation in human PCA PC-3 cells (Zi *et al.*, 2000). A further study on transgenic adeno-carcinoma of mouse prostate (TRAMP) mouse model, observed that silybinin feeding decreases IGF-1 and increases IGFBP-3 in plasma (Raina *et al.*, 2008). Overall, based on these findings, it could be concluded that silybinin modulates IGF-receptor signaling, in part, via up-regulation of IGFBP-3.

Figure 1.11 Free IGF ligands bind and activate the IGF-1 receptor (IGF-1R) causing downstream phosphorylation and triggers the carcinogenesis pathway.
Modified from Grimberg and Cohen. (Grimberg & Cohen, 2000)



1.4.2.3 Silybinin : anti-angiogenesis

Angiogenesis is the growth of new blood vessels from the pre-existing vasculature by budding and sprouting of endothelial cells. Many studies have shown that solid tumours stimulate angiogenesis. Formation of new vessels is obligatory for tumour growth beyond 2mm in diameter because simple diffusion of oxygen and nutrients from neighboring capillaries can no longer support the rapid proliferation of malignant cells within the solid tumours (Garcea *et al.*, 2004; Folkman, 1971), hence an alternative blood supply is needed. Not only is angiogenesis an early and critical step in adenoma-carcinoma tumourgenesis of colorectal cancer (Bossi *et al.*, 1995), it is also an independent prognostic factor for the disease (Tien *et al.*, 2001; Choi *et al.*, 1998; Sternfeld *et al.*, 1999). Increased vascularity not only allows expansion in tumour size, it also encourages haemotogenous embolisation of the malignant cells resulting in metastatic spread. There are many steps which are crucial for angiogenesis and blood capillary formation including vasodilatation, endothelial permeability, periendothelial support, endothelial cell proliferation, migration, survival, organization and remodeling into three-dimensional network of tubular structure (Carmeliet & Jain, 2000).

In normal tissues, angiogenesis is dependent on the balance between stimulatory and inhibitory factors. Most cancers produce pro-angiogenic factors in excess to stimulate the characteristic formation of a highly disorganized, thin walled microvasculature. Of these pro-angiogenic factors, vascular endothelial growth factor (VEGF) is the key mediator in tumour angiogenesis, although others include platelet-derived growth factors (PDGF), platelet-derived endothelial cell growth factor (PDECGF), angiopoietins, fibroblasts growth factors (FGF), hypoxia-inducible factor (HIF),

interleukin 8 (IL-8), intergrins and thrombospondin (Garcea *et al.*, 2004). These in turn stimulate endothelial cell proliferation and migration, increased vascular permeability, tubulogenesis and microvessel formation. The VEGF family has 6 subunits classified as VEGF-A, -B, -C, -D, -E and placenta growth factor (PIGF) (Tischer *et al.*, 1991). These subunits bind to one of three receptor that have been identified; VEGFR-1, VEGFR-2 and VEGFR-3. (Ferrer *et al.*, 1999) and each receptor may function to mediate distinct intracellular functions: for example, VEGFR-1 enhances assembly of endothelial cells into tubular structure and VEGFR-2 induces proliferation and migration of endothelial cells. (Ellis *et al.*, 2001). In colorectal adenocarcinoma, VEGF-A expression is upregulated, (Takahashi, 2004; Wong *et al.*, 1999) associated with increasing likelihood of metastatic spread of primary colorectal tumours (Takahashi, 2004) and is inversely associated with long-term patient survival (Kang *et al.*, 1997; Ishigami *et al.*, 1998).

The anti-angiogenic potential of silymarin has been demonstrated in various cancers. Agarwal and colleagues have demonstrated that silymarin inhibits the growth and survival of human unbilical vein endothelial cells (HUVECS) by inhibiting capillary tube formation, and induction of cell cycle arrest and apoptosis together with a reduction in invasion and migration. The molecular events associated with these effects include an upregulation of Kip1/p27, Cip1/p21 and p53; mitochondrial apoptosis and caspase activation; down regulation of survivin and inhibition of Akt and NF-κB signaling; and matrix metalloproteinase (MMP)-2 secretion (Agarwal *et al.*, 2006; Singh & Agarwal, 2006; Jiang *et al.*, 2000) (Figure 1.13). Other *in vitro* study on colon cancer LoVo cell lines suggested silybinin reduces proliferation, migration and tubular formation of malignant endothelial cells through inhibition of VEGFR-1 and VEGFR-2 (Yang *et al.*, 2003). This is thought to be inter-related to

another *in vivo* study on athymic nude mice with human prostate carcinoma xenograft which found clear evidence that a reduction of VEGF secreted by the cytoplasm of tumour cells matched a significant reduction in microvessel density in the silybinin-fed group (Singh *et al.*, 2003). Gallo et. al. (Gallo *et al.*, 2003) have shown that administration of silybinin-phosphatidylcholine complex, IdB 1016, down regulates VEGFR-3 and upregulates angiopoietin-2 in female nude mice bearing human ovarian cancer xenografts. Other *in vivo* studies have shown that silybinin inhibits microvessel density and inhibit VEGF secretion in prostate and lung tumours (Agarwal *et al.*, 2006; Singh *et al.*, 2006). This implies that the inhibition of VEGFR-1 and VEGFR-2 is secondary to an upstream reduction in VEGF production of the malignant cell. Bearing in mind the factors that control VEGF production is still unknown, this discovery opens up a large avenue for further investigation into the anti-angiogenic property of silybinin as a chemopreventive agent.

Figure 1.12 shows each member of the VEGF family and the different angiongenesis pathway they may mediate. Interestingly, the effect of silybinin in VEGF inhibition may closely be related to its other abilities to inhibit NF-κB (Saliou *et al.*, 1998), impairment of receptor tyrosine kinase (Ahmad *et al.*, 1998; Zi *et al.*, 1998b) and inhibition of cyclin-dependant kinase (Zi *et al.*, 1998b; Manna *et al.*, 1999) which will be discussed separately in the following section. A proposed mechanism of anti-angiogenic effects of silybinin on human endothelial cells are outlined in Figure 1.13.

Figure 1.12 VEGF family comprising of 6 molecules VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and PIGF. VEGF-A exists as at least five different isoforms, VEGF-189 and VEGF-205 are cell bound proteins whilst VEGF-165 is the most common found in tumours. Three receptors for VEGF-A have been characterized VEGFR-1, VEGFR-2 and VEGFR-3, each of which may mediate different events in the angiogenesis pathway.

Modified from Garcea *et al*. (Garcea *et al.*, 2004)

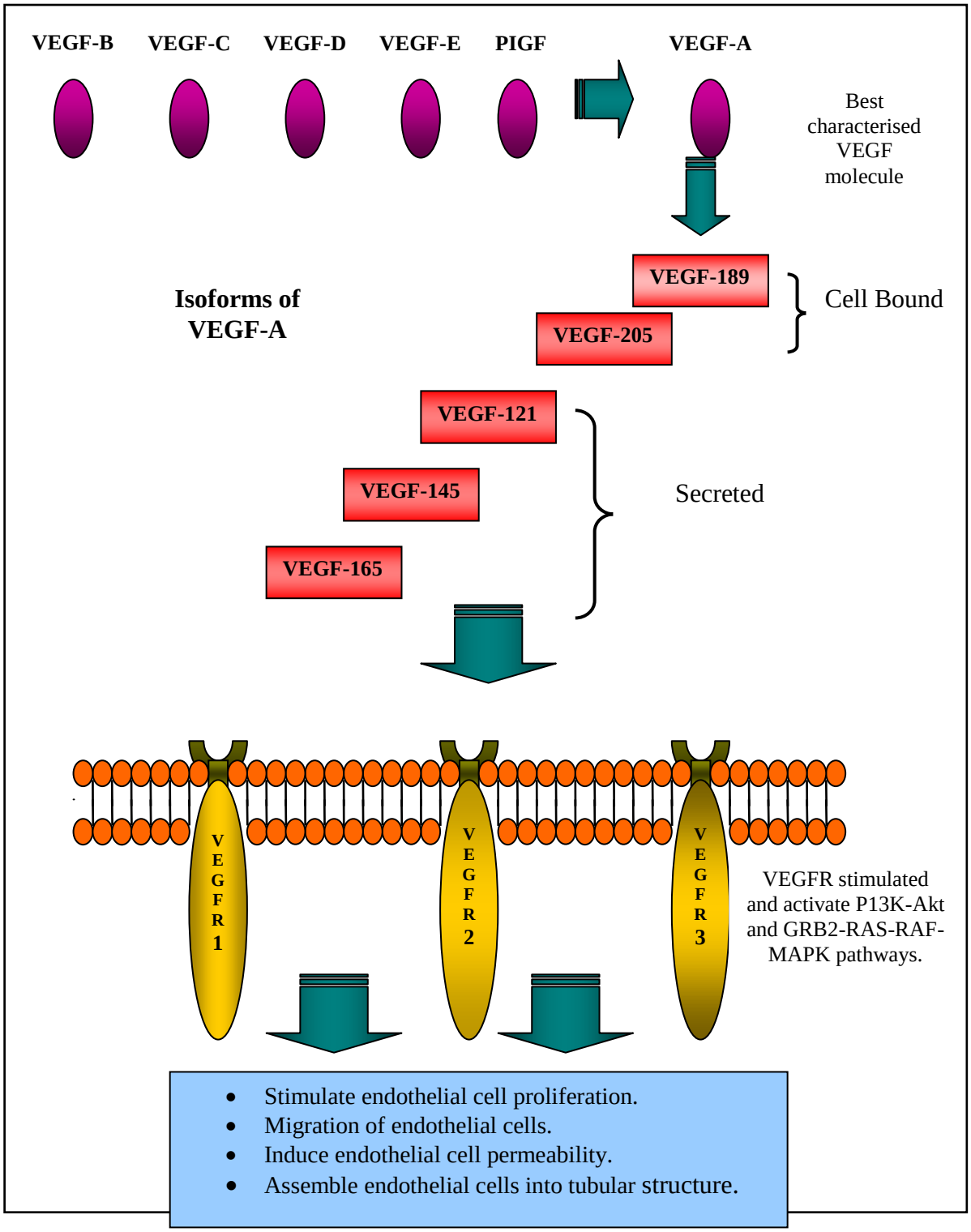
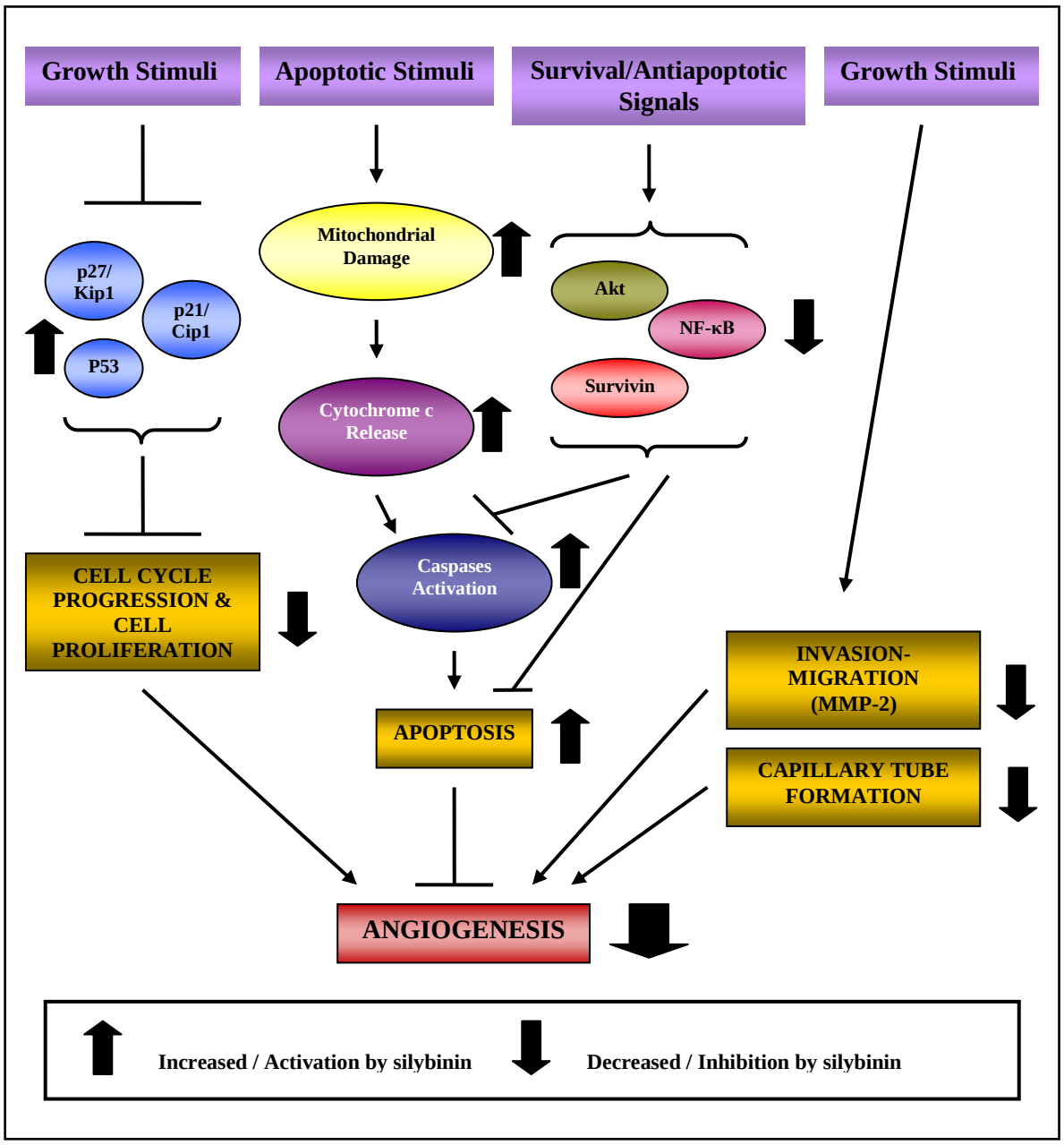


Figure 1.13 Proposed anti-angiogenic mechanisms of silybinin in human endothelial cells. Silybinin showed pleiotropic anti-angiogenic effects including inhibition of cell cycle progression and cell proliferation, induction of apoptosis and suppression of invasion, migration and capillary tube organization.

Modified from Agarwal *et al*. (Agarwal *et al.*, 2006)



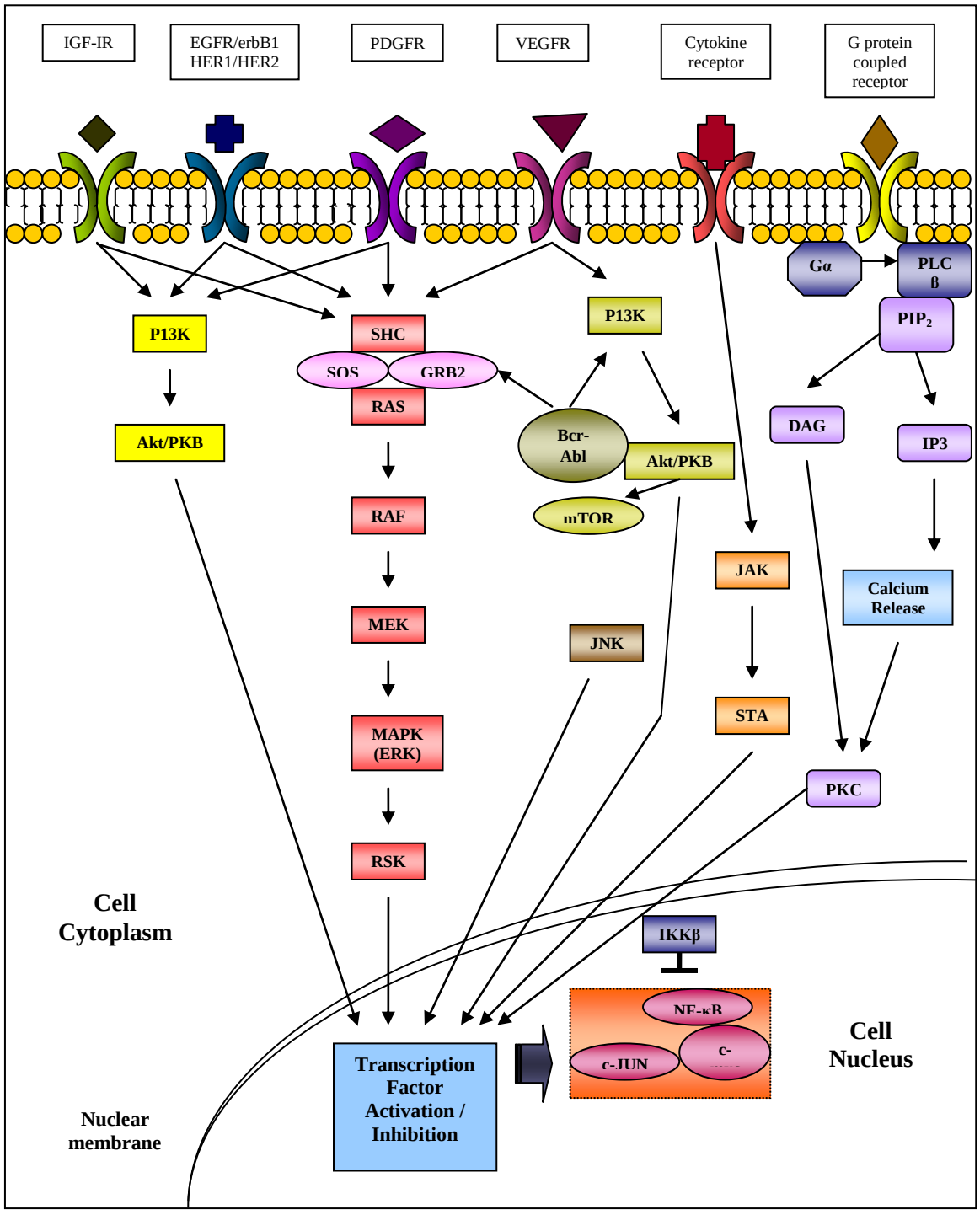
1.4.2.4 Silybinin and its anti-inflammatory effect

The anti-inflammatory effects of silybinin are related to inhibition of the transcription factor nuclear factor-κB (NF-κB), which regulates and coordinates the expression of various genes involved in inflammation, cell survival, differentiation and growth. In particular, NF-κB contributes to the production of interleukin (IL)-1 and -6, tumor necrosis factor (TNF)-α, lymphotoxin, granulocyte macrophage colony-stimulating factor (GM-CSF) and interferon (IFN)-γ. In most of the resting cells, NF-κB is sequestered in the cytoplasm by binding to the inhibitory-κB (IκB)-proteins which block the nuclear localisation sequences of NF-κB (Deep & Agarwal, 2007). Studies have demonstrated that silybinin is a potent inhibitor of NF-κB activation in response to TNF-α. This effect was mediated through the inhibition of phosphorylation and degradation of IκB (Deep & Agarwal, 2007) (Figure 1.14). It also decreases the p65 subunit nuclear translocation and NF-κB dependant reporter gene transcription. Silymarin also blocked NF-κB activation induced by phorbol ester, lipopolysaccharide, ocadaic acid and ceramide, whereas H_2O_2-induced NF-κB activation was not significantly affected. Manna *et al.* (Manna *et al.*, 1999) studied the effect of silymarin on NF-κB activation induced by various inflammatory agents. Silymarin also inhibited TNF-α induced activation of MAPK and JNK, TNF-induced cytotoxicity and caspase activation. Silymarin in combination with tetrandine attenuates NF-κB activated pathways and the induction of metallothionein gene transcription in the liver of dimetylnitrosamine (DMN) administered rats (Hsu *et al.*, 2007). In human mesangial cells, silymarin showed dose-dependent inhibition of TNF-α and IL-1β-induced NF-κB activation, and TNF-α-induced intracellular calcium and monocyte chemotactic protein-1 (MCP-1) expression (Chang *et al.*, 2006). Silymarin has a protective effect against endotoxin-induced sepsis and also has

the inhibitory effect on the production of IL-1β and prostaglandin (PG)-E2 (Kang *et al.*, 2004). Silymarin dose-dependently inhibits both cytokine-induced nitric oxide (NO) production and IFN-γ-induced NO production and β-cell dysfunction in human pancreatic islets (Matsuda *et al.*, 2005).

Figure 1.14 Major tyrosine kinase signal transduction pathways**.**

Modified from Deep and Agarwal. (Deep & Agarwal, 2007a)



1.4.2.4 Silybinin : an inhibitor of the nuclear cell cycle at G0/G1 and G2/M phase

In normal cells the cell cycle is a tightly regulated and orderly process by which one cell becomes two. Most normal animal cells lie in a quiescent state (G0 Phase) at any specific time. When stimulated by external growth factor proteins such as epidermal growth factor (EGF) and insulin-like growth factor (IGF-1), the cells are activated to proliferate and hence move into the cell cycle. The cell cycle is divided into four phases, G1, S, G2 and M (Norbury & Nurse, 1991). At G1 phase, growth factors bind to receptors on the external surface of the mammalian cell and this message is conducted across the cell membrane through signal transduction molecules which in turn activate a phosphorylation cascade of signals involving cyclins and cyclin-dependent kinases (CDK) within the cell. The active cyclin-CDK complexes then phosphorylate the retinoblastoma (Rb)-E2F complexes allowing a release of the E2F family of transcription factors which induce expression of a number of genes required for the DNA synthesis in the S phase transition (Weinberg *et al.*, 1995). Further investigation revealed yet other proteins known as inhibitors of cyclin-dependent kinases (CDKI) such as p21 / Cip1, p27 / Kip1 and p53 act to block activities by binding to cyclin-CDK complexes to terminate cell proliferation and hence block tumourigenesis (Sherr, 1996). Mitosis (M phase) depends upon completion of S phase and the G2 phase is preparatory for it.

The basis of cancer's deranged growth control is primarily located in G1 phase, shortly before initiation of DNA synthesis (Pardee, 1989). Carcinogenesis may be perceived as a consequence of cell cycle deregulation and the progressive loss of genomic integrity. Cell cycle regulators are frequently mutated in human neoplasia and result in an upregulation of the cyclin-CDK cascade as well as inactivation of the

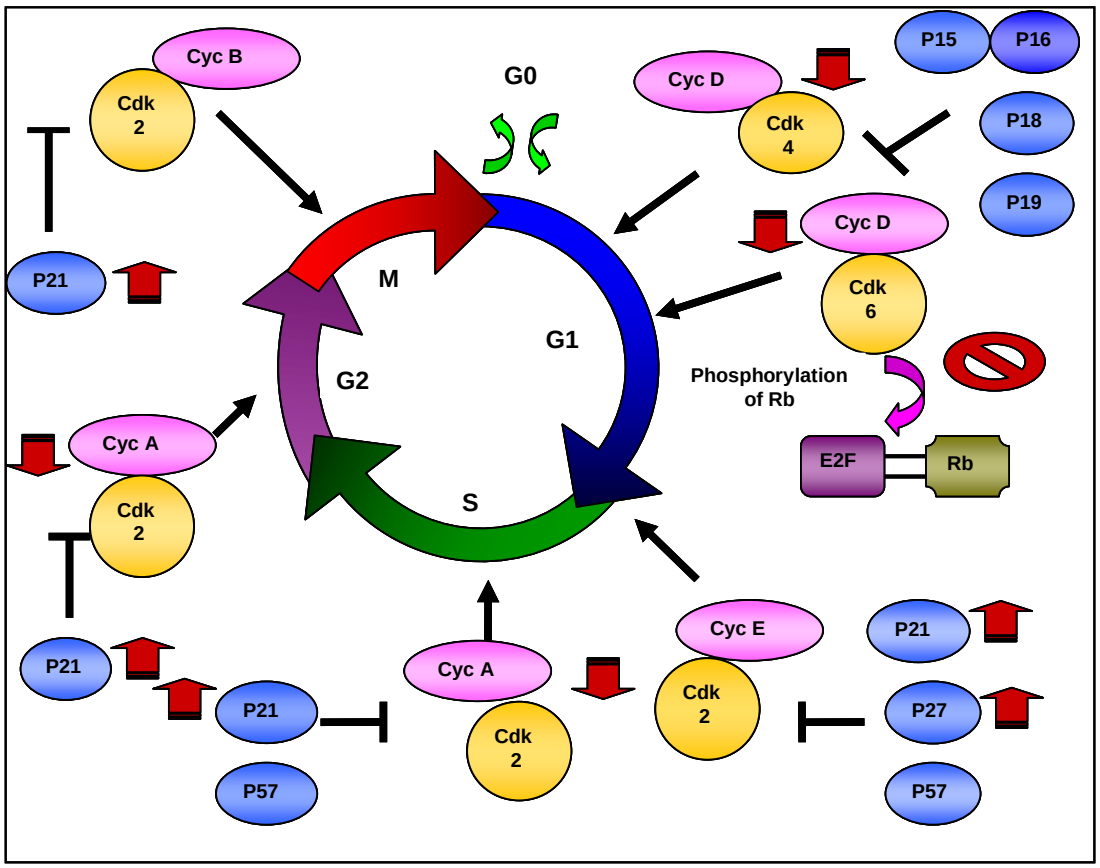
CDK inhibitors (the commonest being the p53 tumour suppressor gene) resulting in uncontrolled cellular proliferation.

Agarwal found that during the cell cycle, silybinin at 50μg/ml caused a G0/G1 arrest in HT-29 colorectal cancer cells by inducing Kip1/p27 which is a key regulator in the G0/G1 phase (Agarwal *et al.*, 2003). This caused an upregulation of the CDKI, which further inhibits the cyclin-CDK complex from further phosphorylation. The overall effect of this mechanism halts the cell cycle progression. At a higher dose of silybinin (100μg/ml), Cip-1/p21 which is a universal inhibitor of cell cycle progression, is upregulated, inhibiting the cyclin-CDK complex at both G0/G1 and G2/M phase. There is simultaneous decrease in CDK 2, CDK 4, cyclin E and cyclin D1 levels accordingly, as shown below (Dhanalakshmi *et al.*, 2004). Tyagi (Tyagi *et al.*, 2002) used silybinin as an anti-proliferative agent on androgen dependent human prostate carcinoma LNCaP cells and reported silybinin inhibits the retinoblastoma protein (Rb) at the serine arm which prevents Rb phosphorylation with the cyclin-CDK complex and hence an increase in the total Rb pool. In other studies, silybinin treatment showed dose-dependent growth inhibition together with G1/G2/M arrests in bladder (Tyagi *et al.*, 2006), breast (Zi *et al.*, 1998a), ovarian (Scambia *et al.*, 1996), lung (Sharma *et al.*, 2003) and skin (Deep & Agarwal, 2007) cancers

In summary, silybinin induces CDKI to block the cyclin-CDK complex, thus down-regulating further phosphorylation. Silybinin also inhibits Rb protein from being phosphorylated. Both these events result in termination of cell cycle progression. This concept is illustrated as a diagram in Figure 1.15.

Figure 1.15 The mechanism of action of silybinin as an anti-carcinogenic agent within the cell cycle.

Modified from Pardee *et al.* (Pardee, 1989)



Silybinin inhibiting the retinoblastoma protein at the serine site and preventing phosphorylation.

Silybinin inducing the production of Kip-1/p27 and Cip-1/p21

Silybinin down-regulating cdk 2, cdk 4, cyclin E and cyclin D1 levels.

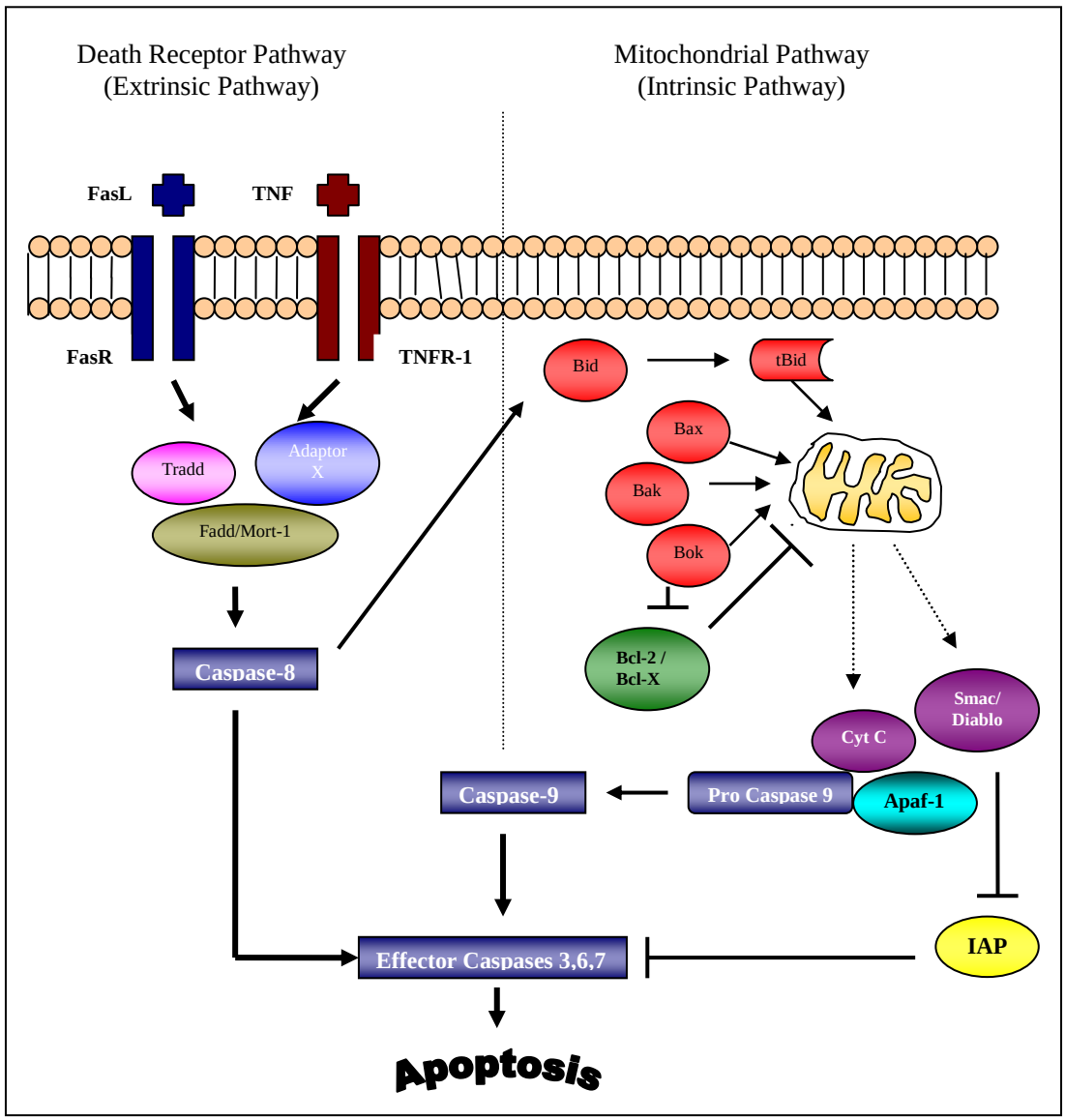
1.4.2.5 Silybinin: an initiator of apoptosis

In normal cells, programmed cell death is an essential part of physiology to control cellular proliferation which prevents carcinogenesis (Vaux & Korsmeyer, 1999). As a treatment strategy, if apoptosis can be induced in the tumour cell, it is the most desirable form of tumour cell death as it brings about their death, without damage to normal cells. Apoptosis is characterised by cytoskeletal disruption, cell shrinkage, membrane blebbing, nuclear condensation and internucleosomal DNA fragmentation (Wyllie *et al.*, 1999). It differs from the alternative cellular destruction through necrosis although both can overlap in some elements.

Classically there are two principal pathways to apoptosis, the death receptor pathway (*Extrinsic Pathway*) and the mitochondrial pathway (*Intrinsic Pathway*) (Matsuyama *et al.*, 2000). Both pathways depend on the degradation of cellular proteins by a cysteinyl-aspartate specific proteinase (caspase) cascade. The caspase cascade consists of initiator caspases, such as caspase-8, -9 and 10. These can activate effector caspases, such as caspase-3, -6 and 7, that act on a wide range of substrates to ensure destruction of the cell.

The death receptor pathway is triggered by coupling of ligand binding to cell surface receptors initiating activation of caspases. This includes the interaction between the Fas ligand (FasL) and the Fas receptor and the tumour necrosis factor (TNF) and its receptors, eg. TNF-receptor 1. Binding of the ligand to its receptor results in a conformational change so that the receptor interacts with an adaptor molecule, which then binds initiator caspases, as shown in Figure 1.16.

Figure 1.16 Induction of apoptosis by the extrinsic and intrinsic pathway. Modified from Matsuyama *et al.* (Matsuyama *et al.*, 2000)

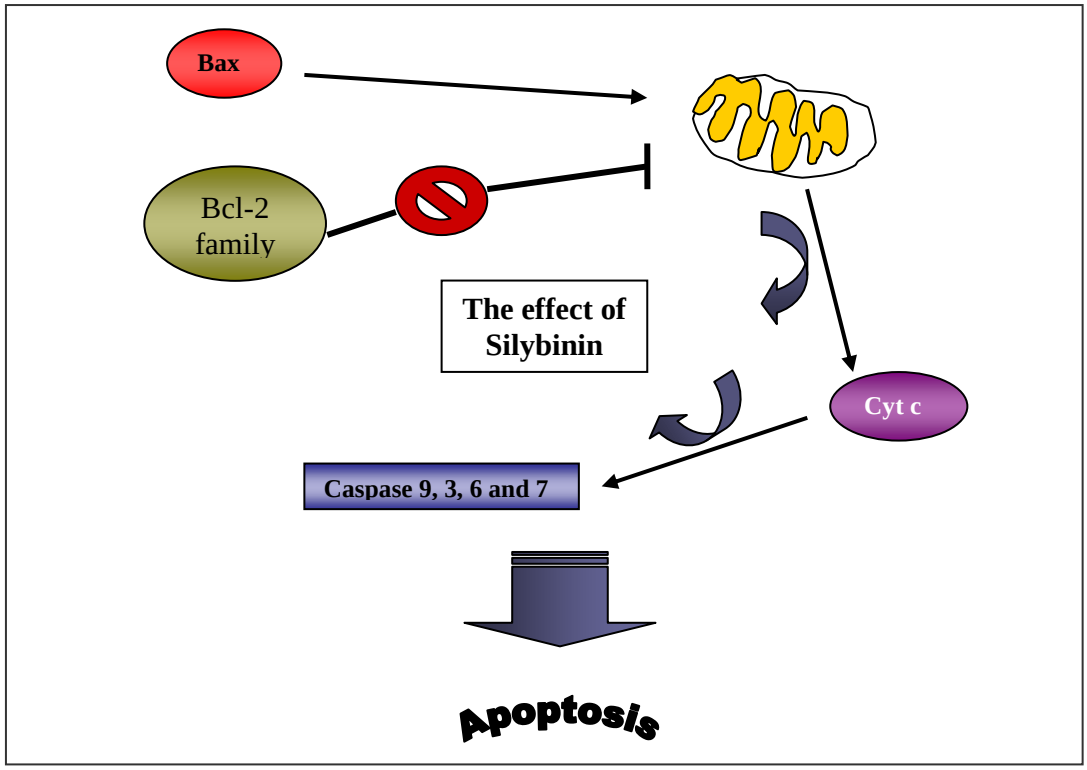


The mitochondrial pathway is characterised by the release of mitochondrial proteins including cytochrome c (cyt c) to the cytosol. Cyt c release is associated with the formation of an intracellular apoptosome complex that recruits pro-caspase 9 via adaptor protein apoptotic protease activating factor-1 (Apaf-1). The apoptosome complex activates pro-caspase 9 to caspase 9, which in turn activates effector caspases such as caspase 3. The release of cyt c is modulated by members of the B cell Lymphoma 2 (Bcl-2) family of proteins, whose members are either proapoptotic (Bax, Bak, Bok and Bid) or antiapoptotic (Bcl-2, Bcl-X_L, Bcl-W, Mcl-1, Bcl-B and Bfl-1) function. In addition to cyt c release, several other molecules are released from the mitochondria such as smac/diablo, which reverse Inhibitors of Apoptosis Proteins (IAPs). The p53 tumour suppressor protein can induce apoptosis by up-regulating the expression of the proapoptotic Bax.

In both human colorectal carcinoma cells (HT-29) (Agarwal *et al.*, 2003) and human bladder transitional carcinoma cells (RT4) (Tyagi *et al.*, 2004a), silybinin has been reported to initiate apoptosis by inducing caspase 9 followed by caspase 3 pathway (Tyagi *et al.*, 2004a; Tyagi *et al.*, 2003) and by down-regulating the inhibition of apoptosis proteins (IAPs) (Tyagi *et al.*, 2003). More recently, silybinin was found not only to be a potent inhibitor of cell proliferation but also to induce a decrease in the Bcl-2 levels without altering the Bax level (Yoo *et al.*, 2004) while promoting cytochrome release and enhancing caspase pathways. The increase in the Bax/Bcl-2 ratio triggers the apoptotic signalling in immortalized human umbilical vein endothelial cells. The same study also suggested a silybinin dose-dependent increase in the release of cyt c and upregulation of caspases 3, 6, 7 and 9 enzymes thus favouring apoptosis (Figure 1.17).

Figure 1.17 The effect of silybinin on apoptosis by the intrinsic pathway. Silybinin decreases Bcl-2 levels.

Modified from Yoo *et al*. (Yoo *et al*., 2004)



Hence, taking together all of these results intimate both the propitiousness and the feasibility of developing silybinin as a colorectal cancer chemopreventive agent. The aims of this thesis is further outlined in Chapter 2.

Chapter 2. AIMS AND OBJECTIVES

Silybinin has been shown to be a promising putative cancer chemopreventive agent in preclinical *in vitro* cells and *in vivo* rodent models of carcinogenesis (Section 1.4). The overall aim of the work described in this thesis is to gain clinical pharmalogical information which might help optimise the design of the further clinical exploration of silybinin as a cancer chemopreventive agent.

The specific objectives were as follows :

1. To assess the safety of silipide given to humans at doses of 360 to 1440 mg per day for seven days. Single doses of silybinin at 360 mg were used in healthy volunteers without showing signs or symptoms of toxicity (Barzaghi *et al.*, 1990).

2. To test the hypothesis that silybinin when administered as silipide (Section 1.3.1) furnishes levels of agent in the gastrointestinal tract which are consistent with chemopreventive efficacy. To that end, a safe, simple and robust reversed-phase UV-HPLC method for the analysis of silybinin in human colonic mucosa and plasma was developed (Section 5).

3. To define the levels of silybinin recovered from liver tissues when silipide is administered at doses that have exhibited hepatoprotective properties (Section 1.5)

4. To investigate if silybinin can be recovered from the blood in humans who have ingested silipide for 7 consecutive days.

5. To explore a potential relationship between doses of silipide and levels of silybinin recovered from the gastrointestinal tract and/or liver and/or blood.

6. To find out which silybinin metabolites are found in the human organism.

7. To test the hypothesis that consumption of silybinin in the form of silipide for 7 days modulates circulating levels of IGF-1 and IGFBP-3.

8. To find out if consumption of silybinin in the form of silipide for 7 days reduces levels of oxidative DNA adducts M_1dG in the blood or gastrointestinal tissue.

Chapter 3. MATERIALS AND METHODS

3.1 Software and hardware

All data was collated using Microsoft Excel (XP). Graphs were produced using Microsoft Excel and Prism 3.0. Figures were created using Paint shop Pro 7.0 and transferred to Microsoft Powerpoint (XP). Statistical analysis was performed with SPSS 12.0 for Windows. Other software used in the interpretation and analysis of data were Star Chromatography Work Station, version 5.5; ISIS Draw 2.5; GeneSnap version 5.0 and GeneTool version 3.0 from SynGene analytical software.

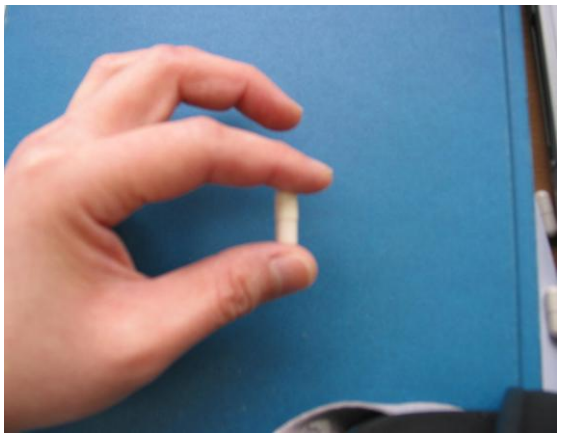
3.2 Silipide formulation and supply

Silybinin was formulated in capsules as 'silipide' (IdB 1016), a phytosome product marketed as a hepatoprotectant (Indena SpA, Milan, Italy). Each silipide capsule contained 120 mg silybinin (present as an approximate racemic mixture of the two silybinin stereoisomers) and soy phosphatidylcholine at a molar ratio of 1:1.

Contents of each silipide capsule by weight:	**Silybinin**	**120 mg**
	Phosphatidylcholine	**180 mg**

The capsules were supplied by Dr. Paolo Morazzonni and generously donated by Indena SpA. The capsules were provided in sterile blister packs and stored below 10°C in the Leicester Royal Infirmary Pharmacy Department.

Figure 3.1. The size and shape of Silipide™ capsules (IdB 1016).



3.3 Clinical method

3.3.1 Human study design

This study was designed as a phase I dose escalation trial administering silybinin to patients with resectable primary colorectal carcinoma and secondary hepatic metastases. The recruited patients were randomly divided into three dose groups, who received 360 mg, 720 mg or 1440 mg silybinin per day for the 7 days prior to their operation. The last dose of the treatment was given on the morning of the patients' resection surgery. There was no dose escalation or dose reduction within each group once the initial dose had been prescribed. Table 3.1 lists the dose levels of silipide for each patient group.

Table 3.1 Dose levels of silipide and silybinin for each patient group.

Number of silipide capsules taken daily	Total silybinin consumed per day (mg)
3	360
6	720
12	1440

The daily dose was achieved by three separate doses taken in the morning, at lunchtime and at dinner time. The maximum daily dose of silipide employed in this study was 12 capsules (corresponding to 1440 mg of pure silybinin) daily which was selected because it represented the highest dose which had previously been administered in healthy volunteers (Barzaghi *et al.*, 1990) and without any adverse effects. The proposed doses of silybinin in this study were chosen based on several

preclinical studies that have shown efficacy in inhibiting bladder (Vinh *et al.*, 2002), skin (Singh *et al.*, 2002b), prostate (Singh *et al.*, 2002a) and colon (Kohno *et al.*, 2002) carcinogenesis. (See section 1.3.2). One study in particular demonstrated clearly that mice that received silybinin at 0.05% and 0.1% *per diem*, equating to approximately 75 and 150 mg/kg body weight per day respectively, showed a raised circulating plasma IGFBP-3 levels (Singh *et al.*, 2002a). These doses extrapolated to humans on the basis of body surface area [420 mg/square meter in mice, according to Freireich (Freireich *et al.*, 1966)], would amount to ~450 or ~900 mg silybinin per person per day, assuming a body surface area of 1.8 square meters accompanying a body weight of 70 kg. Whilst such extrapolation has to be interpreted with caution, the doses proposed for this pilot study (between 360 and 1440 mg per day in humans) cover the doses shown to be efficacious in mice.

All patients gave written informed consent for the use of their blood and tissues in this study. Pre-treatment peripheral blood samples were collected in heparinized tubes and tissue biopsies (from the colorectum or liver) in biopsy cassettes, before commencing their silipide treatments. For the purpose of minimising patient risk, all tissue biopsies were co-ordinated with routine investigations conducted for staging and diagnosis of their disease. Post-treatment peripheral blood and tissue samples were taken after their 7 day treatment and before their colectomy or hepatectomy. Portal blood was taken directly from the portal vein after mobilising the liver and the portal vein is fully exposed. Both portal blood and liver tissues were taken before the Pringle manoeuvre was applied to minimise oxidative damage to the liver tissues, which may interfere with the analysis (Garcea *et al.*, 2006). All concomitant medications were carefully recorded including induction medication and inhalational agents used during general anaesthesia. Haematological profiles, urinary levels of urea and electrolytes and

hepatic function for each patient were recorded before and after silipide treatment. Other demographic data such as patient age, gender, body mass index, Duke's staging and concurrent radio- and/or chemotherapy were also noted.

A 24 h contact number was provided to all patients on the trial in the event that they experienced any problems whilst on the trial drug. An exemption licence for the trial drug was obtained from the Medicine Control Agency (Ref: MF8000/13095) (Appendix A). Approval from the Leicestershire Research Ethical Committee (LREC) was obtained prior to commencing the trial; Ethics approval numbers were UHL 9174 and UHL 9176. (Appendix B)

3.3.2 Patient recruitment criteria

The patients were initially identified at the Colorectal and Hepatobiliary multi-disciplinary team meetings (MDT) held weekly at the Leicester Royal Infirmary and the Leicester General Hospital. Only patients who satisfied the following inclusion criteria were approached for recruited.

Inclusion Criteria :

- Patients must be over 18 years of age.
- Patients must be of sound mind to give written informed consent.
- Patients must have a strong clinical suspicion of having a primary colorectal adenocarcinoma or secondary liver metastases.
- All patients must undergo a colonoscopy or a laparoscopy for colorectal or liver tissue biopsy to confirm and stage the disease.
- All patients must have disease amenable to surgical resection.

Exclusion Criteria :

- Patients unfit for general anaesthesia.

- Patients with significant impairment of gastrointestinal function or absorption, or with active peptic ulcer disease.
- Patients unwilling or unable to comply with the protocol.
- Patients with abnormal liver function tests.

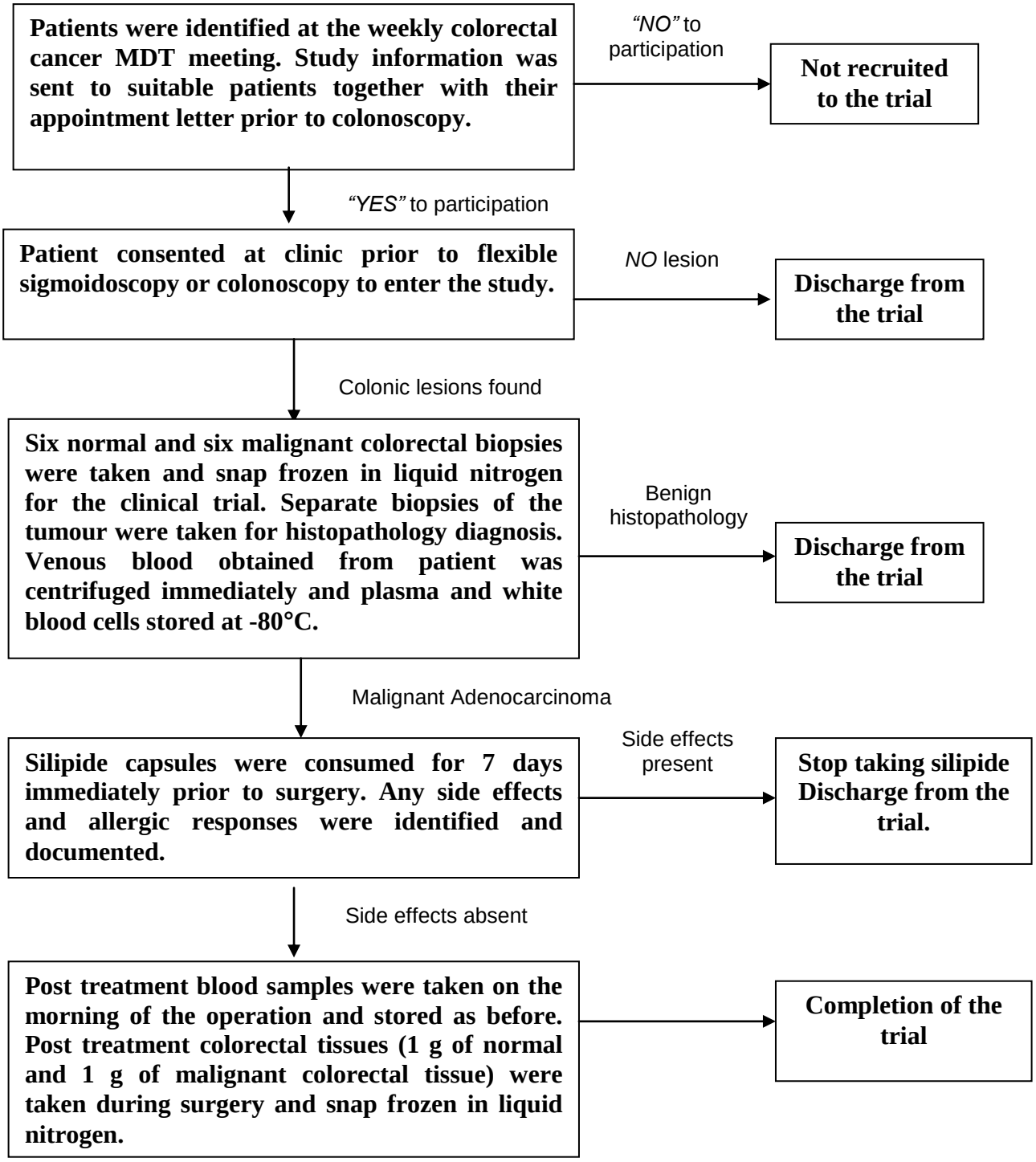
3.3.2.1 Patient recruitment for the colorectal arm of the clinical trial

Colorectal patients due to undergo colonoscopy could be classified into one of the following categories. These patients were approached and consented for this study.

- Patients presenting with rectal bleeding, weight loss and other symptoms highly suggestive of colorectal malignancy.
- Patients due to undergo a colonoscopy for histological diagnosis following a suspicious malignant lesion seen at a previous barium enema or computed tomography (CT) scan.
- Patients requiring a repeat colonoscopy and biopsy of a lesion to verify the diagnosis of cancer.
- Patients with low-lying rectal tumours, who are due to undergo routine examination under anaesthesia (EUA), to obtain detailed information on position and fixation of the tumour mass. This procedure is important in the planning of the operation and assessing whether a sphincter-preserving operation can be performed.

Blood samples and six biopsies of healthy colonic mucosa tissue (4 – 20 mg in total) and six biopsies of the malignant tumour tissue (3 - 30 mg in total) were taken during the above investigation from the patients who consented to the study. Healthy mucosa of the colon was defined as inner lining of the colon at least 5cm proximal or distal to the cancer edge.

Figure 3.2 Summary of colorectal patient recruitment



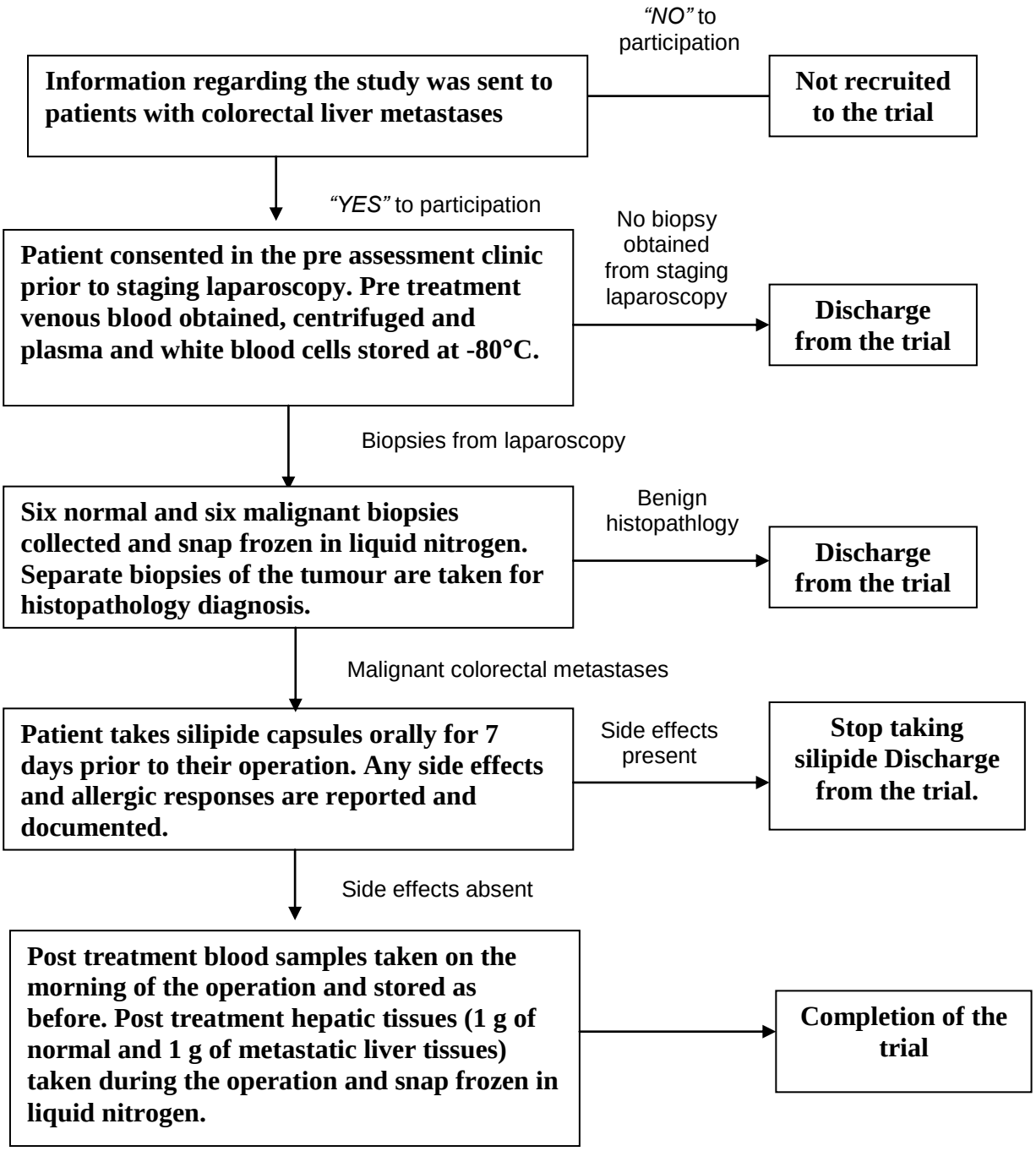
3.3.2.2 Patient recruitment for the hepatic arm of the clinical trial

The hepatic patients were identified at the weekly hepato-biliary and pancreatic multi-disciplinary team meetings (MDT). All hepatic patients suitable for liver resection surgery undergo a staging laparoscopy prior to their operation to ascertain whether a patient has any peritoneal disease beyond the resolution of computed tomography (CT). A venous blood sample was obtained from consented patients who met the inclusion criteria. During the staging laparoscopy, six normal (20 – 60 mg normal liver tissue) and six malignant liver tissue biopsies (30 – 60 mg hepatic tumour tissue) were taken and snap frozen in liquid nitrogen.

Patients received a 7 day course of oral silybinin and had further blood and tissue samples taken during their main resection surgery. In addition, portal blood and bile were obtained during the resection surgery for analysis of silybinin and metabolites.

A further six patients (three colorectal and three hepatic) were recruited as controls and tissue collection proceeded as described above. Their tissue was used in the preparation of standard curves needed to measure the concentration of silybinin in normal and malignant tissue, peripheral and portal blood (described below).

Figure 3.3 Summary of hepatic patient recruitment



3.3.3 Patients' blood and tissue collection

Peripheral blood samples (30 mL) were collected in lithium heparin tubes (S-monovette), purchased from Sarstedt (Loughborough, UK). Colorectal tissue biopsies were collected using colonoscopic biopsy forceps (by Olympus^{TM} Hamburg, Germany) and flash frozen in liquid nitrogen. It was stored at -80°C in biopsy cassettes (obtained from Histopathology Department, UHL NHS Trust) prior to analysis.

Figure 3.4 A diagram of the biopsy forceps used to collect colorectal tissue biopsies (Photograph courtesy of Olympus, UK)

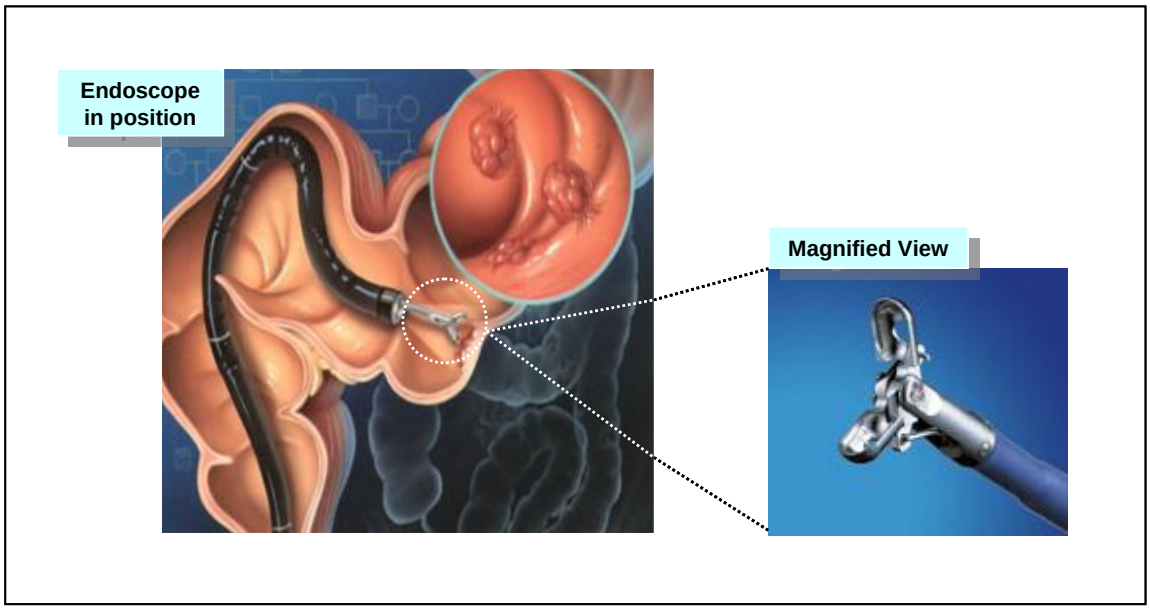


Table 3.2 Summary of the blood and tissue collection from the pre- and post-treament colorectal patients

	Pre-treatment specimens taken	**Post-treatment samples taken at operation**
Patients with confirmed colorectal cancer	• 30 ml of peripheral blood samples obtained (percutaneous venous puncture) • x6 biopsy forceps specimens obtained at routine colonoscopy of normal colorectal mucosa • x6 biopsy forceps specimens of tumour	• 30 ml of peripheral blood samples (percutaneous venous puncture) • 1 g of normal colorectal mucosa (from resected specimen) • 1 g of malignant colorectal tissue

Liver tissue samples were collected using the "Trucut^{TM}" biopsy needle obtained from Swan-Morton (Sheffield, UK), flash frozen in liquid nitrogen and stored at -80°C. Portal blood was collected by direct needle venupuncture of the portal vein during laparotomy.

Figure 3.5 The diagram below shows a Trucut™ biopsy needle (A), magnified views of the sharp biopsy needle tip and the tissue collecting column (B) and method of hepatic tumour biopsy (C).

(Picture courtesy of Swan-Morton, UK).

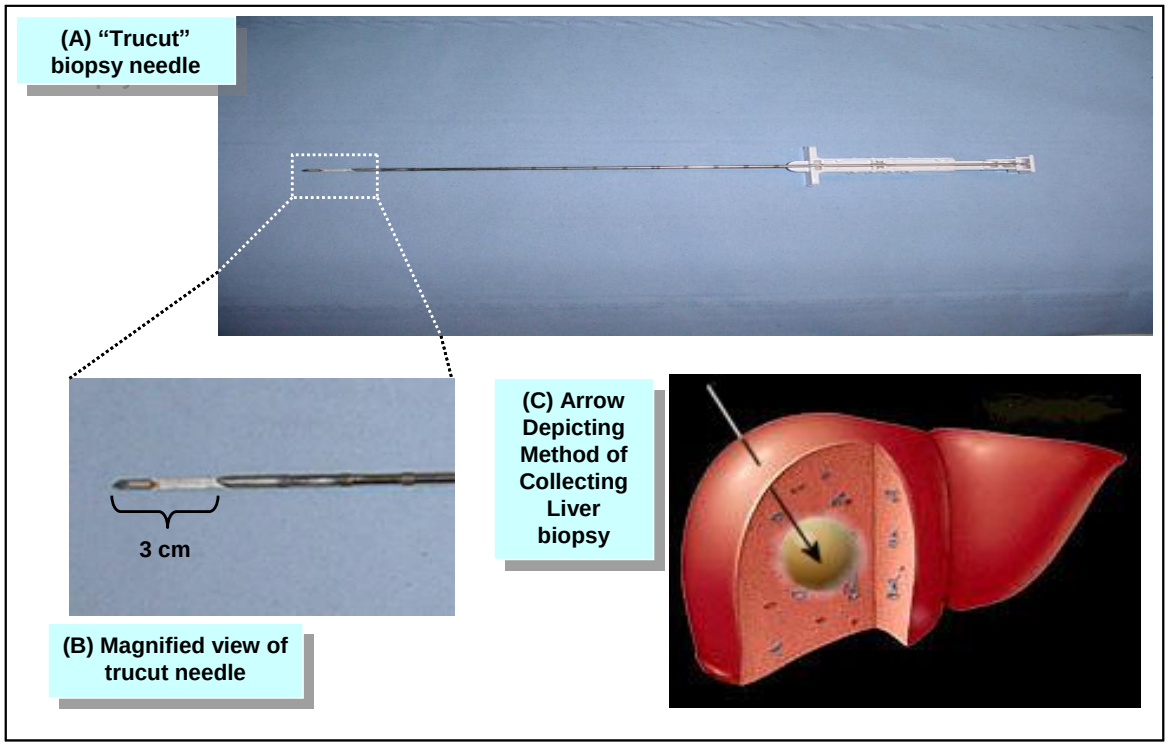


Table 3.3 Summary of the blood and tissue collection from the pre- and post-treatment hepatic patients

	Pre-treatment specimens	**Post treatment samples taken at operation**
Patients with confirmed resectable hepatic metasteses	• 30 ml of peripheral blood samples (percutaneous venous puncture) • x6 "trucut" biopsy specimens of both normal and malignant liver tissue (at routine laparoscopy)	• 30 ml of peripheral blood samples (percutaneous venous puncture) • 10 ml of bile samples (needle puncture of gall bladder or cystic duct) • 30 ml of portal blood samples (needle puncture of portal vein) • 1 g of both normal and malignant liver tissue (from resected liver specimen)

3.3.4 Timing of tissue collection

The collection of patients' blood and tissue samples were co-ordinated around the time of normal clinical care of the recruited patients. Pre-treatment blood and tissues were collected either at colonoscopy or diagnostic laparoscopy in colorectal or hepatic patients, respectively. This was approximately 2 - 3 weeks in advance of the patient commencing the 7 day course of silybinin. Collection of post-treatment blood and tissues varied slightly according to the operation performed and at the convenience of the surgeon. All patients were fasted for 4 h prior to their operation. Hence, their last dose of silybinin was taken at this time. Peripheral blood samples were taken just before the induction of anaesthesia, at 1 - 8 h after the last silipide dose. Harvesting

the tissue samples usually occurred between 2 - 10 h after the last silybinin dose in colectomy patients, and 4 – 10 h in hepatectomy patients. For the hepatic patients, portal blood was taken directly from the portal vein at 3 – 10 h after the last silipide dose. Table 3.4 outlines the timing of collection of patient samples.

Table 3.4 Timing of patients' samples collection

	Type of Samples	**Timing after Last Silipide Dose**	
		Range (h)	**Mean ± S.E.M**
Colorectal Patients	**Peripheral Blood**	**1 – 8**	**2.67 ± 0.64**
	Colonic Tissues	**2 – 10**	**6.25 ± 0.76**
Hepatic Patients	**Peripheral Blood**	**1 – 3**	**2.42 ± 0.19**
	Portal Blood	**3 – 10**	**5.75 ± 0.63**
	Hepatic Tissues	**4 – 10**	**6.42 ± 0.68**

3.3.5 Tissue weights used for the pharmacokinetic analysis

In general, greater amounts of tissue from both colorectal and hepatic patients were used for silybinin extraction and analysis than those required for DNA extraction and subsequent M_1dG and IGF analysis (Table 3.5 and 3.6). Altogether, less tumour tissue was obtained and available for analysis i.e. 792 mg of colonic and 1100 mg of hepatic tissues, compared to 1800 mg and 3800 mg of normal colonic and hepatic tissues. Strict guidelines were imposed by the Department of Pathology on the amount and position of tumour tissue harvested. It is important that the tissue sampling technique did not interfere with pathological grading nor the resection margins of the tumour. Pre-treatment colonic and hepatic tissue samples were obtained in the form of biopsies either at colonoscopy or staging laparoscopy and the total amount of tissue collected were small. Hence, in order to provide sufficient tissue for biomarker assays, there were no pre-treatment tissue samples available for pharmacokinetic analysis.

Post-treatment tissues taken at resection surgery were relatively plentiful in comparison to pre-treatment tissue sampling. Larger amounts of hepatic tissues were needed for silybinin extraction, i.e. 3800 mg in normal and 1100 mg in tumour tissues, in comparison to 1800 mg of normal and 792 mg of tumour colonic tissue obtained from colorectal patients. This was because silybinin levels detected in the hepatic tissues were very low in comparison with the levels observed in the colorectal tissues. Therefore, more tissues were needed for pharmacokinetic analysis in order to obtain a detectable level of silybinin. Inevitably, much of the silybinin in the liver would be metabolised and appear in the form of conjugated silybinin. Thus, another

reason for finding a low level of free silybinin in the liver. These findings will be further discussed in Chapter 6.

Table 3.3.4 Weights of normal and malignant colorectal tissue used for analysis.

Laboratory Assay	Colonic Tissue Type	Weight Range (mg)	Mean Weight (mg)
Silybinin Extraction	Normal Tissue	1200 - 2140	1800
	Tumour Tissue	545 - 936	792
M_1dG Assay (immunoslotblot)	Pre-treatment Normal Tissue	4 - 61	26
	Pre-treatment Tumour Tissue	10 - 251	67
	Post-treatment Normal Tissue	105 - 315	246
	Post-treatment Tumour Tissue	101 - 312	208

Table 3.3.5 Weights of normal and malignant hepatic tissue used for analysis.

Laboratory Assay	Colonic Tissue Type	Weight Range (mg)	Mean Weight (mg)
Silybinin Extraction	Normal Tissue	2500 - 4200	3800
	Tumour Tissue	900 - 1300	1100
M_1dG Assay (immunoslotblot)	Pre-treatment Normal Tissue	5 - 46	23
	Pre-treatment Tumour Tissue	3 - 70	29
	Post-treatment Normal Tissue	31 - 83	48
	Post-treatment Tumour Tissue	8 - 86	50

3.3.6 Normal and malignant; colorectal and hepatic tissues used for DNA extraction followed by M_1dG analysis

Collection of pre-treatment liver tissue using the Trucut™ needle method was more labour intensive with a higher risk of bleeding than the biopsy forceps method used for colorectal tissue. In addition, the trucut needle only allows a small cylindrical column of tissue (measuring 0.5 x 0.1 mm) to be obtained, therefore, less pre-treatment liver tissue was available when compared to tissue collection from colorectal patients. Furthermore, liver tumours were often positioned deep within the liver parenchyma making access to good quality biopsies difficult, resulting in less tissue for analysis. This explains the smaller weights of liver tissue samples used in the DNA extraction, 23 mg for normal and 29 mg for tumour, when compared to the tissue weights from colorectal patients, 26 mg and 67 mg for normal and tumour respectively. Conversely, smaller amounts of liver tissue, per mg weight, were found to yield higher amounts of DNA concentration than in colorectal tissues. This may reflect the higher mitotic activity of hepatocytes in comparison to colonic mucosal cells (Gutman & Fidler, 1995). Similarly, it was observed that both colonic and hepatic tumour tissues had a higher concentration of DNA per mg tissue than normal healthy tissue of the same organ. This could be explained by the fact that neoplastic tissues display accelerated mitotic activity within the cell, resulting in increased DNA concentration (Baribault *et al.*, 1985).

3.3.6.1 Amount of extracted DNA for M_1dG analysis

The yield of DNA obtained from blood using the Qiagen extraction kit were consistent between 0.1 – 2.6 µg/mL (Table 3.7). However, in colorectal and hepatic tissues, DNA yield appeared much higher in tumour when compared to normal tissue. The higher DNA production in tumour tissue could be explained by the greater nuclear density and mitosis in tumour tissue (Table 3.8). Table 3.8 also show that the larger the tissue volume used for DNA extraction, the higher the DNA concentration produced i.e. the abundant post treatment tissue gave higher concentration of DNA (µg/mL) than the smaller pre-treatment biopsy samples. All DNA used for analysis were of analytical purity between 1.6 – 1.8.

Table 3.7 The range and mean quantity of DNA yield from pre- and post-treatment leucocytes

Plasma source	Pre or Post Treatment Leucocytes	Range of DNA concentration (µg/ml)	Mean DNA concentration (µg/ml)
Colorectal Patients	Pre - Peripheral Leucocytes	0.1 – 2.2	1.2
	Post - Peripheral Leucocytes	0.3 – 1.9	1.3
Hepatic Patients	Pre - Peripheral Leucocytes	0.2 – 2.6	1.0
	Post - Peripheral Leucocytes	0.2 – 1.4	0.7
	Post – Portal Leucocytes	0.1 – 1.6	0.8

Table 3.8 The range and mean quantity of DNA yield from pre-and post-treatment normal and malignant colorectal and hepatic tissue from patients.

Tissue source	Tissue Type	Range of DNA concentration (µg/ml)	Mean DNA concentration (µg/ml)
Colorectal Patients	Pre – Treatment Normal Tissue	0.0 – 0.9	0.1
	Pre – Treatment Tumour Tissue	0.0 – 5.5	1.0
	Post – Treatment Normal Tissue	0.0 – 0.8	1.4
	Post – Treatment Tumour Tissue	0.0 – 2.4	1.6
Hepatic Patients	Pre – Treatment Normal Tissue	0.5 – 2.5	1.7
	Pre – Treatment Tumour Tissue	0.3 – 1.9	1.0
	Post – Treatment Normal Tissue	1.6 – 2.3	2.0
	Post – Treatment Tumour Tissue	1.4 – 3.0	2.3

3.4 Laboratory materials and methods

3.4.1 Rodent study design (Chapter 5 for results)

3.4.1.1 Mice study design - pharmacokinetic analysis of silybinin

Sixteen mice (eight male and eight female) of the strain C57BL6J, of similar age, size and weight were randomly assigned to two groups. The first group consisted of 4 male and 4 female mice were fed 0.2% silybinin with their diet for 7 days. The remaining group (4 male and 4 female) were fed 0.2% silipide (silybinin-phospholipid complex) per diem for 7 days. At the end of the 7 days, all sixteen animals were anaesthetized with halothane and terminally exsanguinated by cardiac puncture. Liver, small bowel mucosa and prostate (in males) were removed at post mortem. The organs were homogenised and stored in -20°C prior to analysis. All samples were analyzed for the presence of silybinin. Results were shown in Section 5.2.

3.4.1.2 Rat study design - identification of silybinin and its metabolites (Section 5.3.2)

Eight male rats at 10 weeks of age, of similar size and weight (i.e. weight range between 382 – 484 g, mean = 420 g) were randomly assigned to one of three groups. Two rats were assigned to the first group (*x*) which acted as the control animals (n = 2). The other two groups of rats received either a single oral dose of silybinin at 132 mg/kg (*y*) equivalent to ~1440 mg per day in humans (i.e. the human volunteers in the pilot trial who received the highest dose of silipide administration) or a single oral dose of silybinin at 1200 mg/kg (*z*). These doses extrapolated to humans on the basis of body surface area (Freireich *et al.,* 1966) (Section 3.3.1). There were 3 rats (n = 3) in each of group *y* and *z*. All animals were fasted overnight prior to the treatment but

had free access to water. Treatment was a single oral dose of either silybinin dissolved in a maximum 5000 mg/kg glycerol formal, in groups *y* and *z*, or the vehicle alone in the case of controls (*x*). After 20 minutes the animals were anaesthetized with halothane and terminally exsanguinated. Blood was drained directly from the heart and the liver removed at post mortem. The liver was homogenised and stored in -20°C prior to analysis. All samples were analyzed for the presence of silybinin and its conjugated metabolites. Results were shown in Section 5.3.2.

3.4.2 Pharmacokinetic analysis - measurement of plasma and tissue levels of silybinin

3.4.2.1 (A) Materials used in the quantitation of silybinin levels

Silybinin (for the authentic standards) (CAS 22888-70-6), apigenin (for the internal standard) (CAS 520-36-5) and β-glucuronidase enzyme from *Helix Promatia* (Type H-2) were purchased from Sigma-Aldrich (Poole, UK). Purity of silybinin was > 98% and consisted of a racemic mixture of two diastereoisomers. Human plasma was obtained from the National Blood Transfusion Centre (Sheffield, UK). Ammonium acetate, glacial acetic acid and HPLC grade methanol were obtained from Fisher Chemicals (Loughborough, UK). AnalR grade dimethyl sulfoxide (DMSO) was purchased from Sigma (Poole, UK).

The HPLC system consisted of a Varian ProStar 230 Pump, ProStar 410 autosampler and a 310 UV/Vis detector set at 290nm. (Varian Analytical Instruments, Oxford, UK). Chromatographic separation was accomplished using an Atlantis column as the stationary phase (4.6 × 150mm, 3-µm C_{18} column; Waters, Elstree, UK) in

combination with an Atlantis 4.6 × 20mm, 5-µm C_{18} guard column, at 40 °C. The collected data were analyzed using Star LC Workstation software version 5.5.

3.4.2.1 (B) Method of sample preparation for HPLC analyses

3.4.2.1.1 Silybinin extraction from human plasma

- Plasma samples were thawed to room temperature and vortexed thoroughly before use.
- 995 µL of plasma was transferred into a labelled, clean 10 ml falcon tube. Samples were run in triplicates.
- 5 µL of 100 $\mu g.mL^{-1}$ solution of the internal standard (IS), apigenin, was added to each sample.
- The samples were vortexed at high-speed setting for at least 20 s prior to adding 3 mL of ice-cold methanol to precipitate proteins. Efficient protein precipitation was achieved by keeping samples at -20°C for 30 min.
- Samples were centrifuged (6000 g for 20 min), the supernatant removed and transferred to fresh plastic tubes and dried under a constant stream of nitrogen at 20°C.
- Residues were reconstituted in 100 µL of mobile phase B (Section *4.2.1.5*) followed by a final 13,000 rpm centrifugation at 4°C and supernatant transferred to suitable HPLC vial inserts prior to HPLC analysis.

3.4.2.1.2 Silybinin extraction from human tissue

- Tissues were thawed at room temperature.
- Each tissue sample was placed on a filter paper and rinsed with PBS to remove unwanted faecal contaminant.

- Samples were air dried for 15 min, weighed and homogenized in an equal part of ice-cold potassium chloride (0.15 M) (weight of tissue in grams to volume of buffer in mL).
- Homogenization was carried out using the high speed X-1020 Ultra Turax (RS, Corby).
- Due to the varying silybinin concentrations achieved in tissues, 200 µL of colonic tissue homogenate and 500 µL of hepatic tissue homogenate were used in the silybinin extraction procedure.
- 10 µL of 10 $\mu g.mL^{-1}$ solution of IS, apigenin, was added to each sample and vortexed for at least 20 s for equilibration.
- The extraction procedure then proceeded as for plasma except 600 µL (for colonic tissue) and 1500 µL (for hepatic tissue) ice-cold methanol were employed for protein precipitation. (i.e. one part matrix to three part ice-cold methanol).

3.4.2.1.3 Preparation of stock solution :-

- Stock silybinin was dissolved in pure methanol at a concentration of 100 $\mu g.mL^{-1}$. Further dilutions were made using 70% aqueous methanol containing 5% aqueous acetic acid.

3.4.2.1.4 Preparation of the standard curve:

- Pooled control human plasma was thawed at room temperature and vortexed prior to use. Control human colonic and hepatic tissues were thawed, weighed and homogenized as mentioned above in section *3.4.2.1.2***.**
- 1ml of plasma, 200 µL colon homogenate or 500 µL liver homogenate were taken for each point of the standard curve.

- Varying concentrations of silybinin stock solutions were prepared as shown above, and then added to the plasma or tissue homogenate to make up the final concentrations as detailed in Table 3.9.
- The range of standard curves varied to ensure that analysis of silybinin levels in respective plasma or tissues fell within the range of the standard curve.
- 5 µL of apigenin [100 µg.mL^{-1}] was added to each sample as an internal standard. Sample was vortexed for at least 20 s.

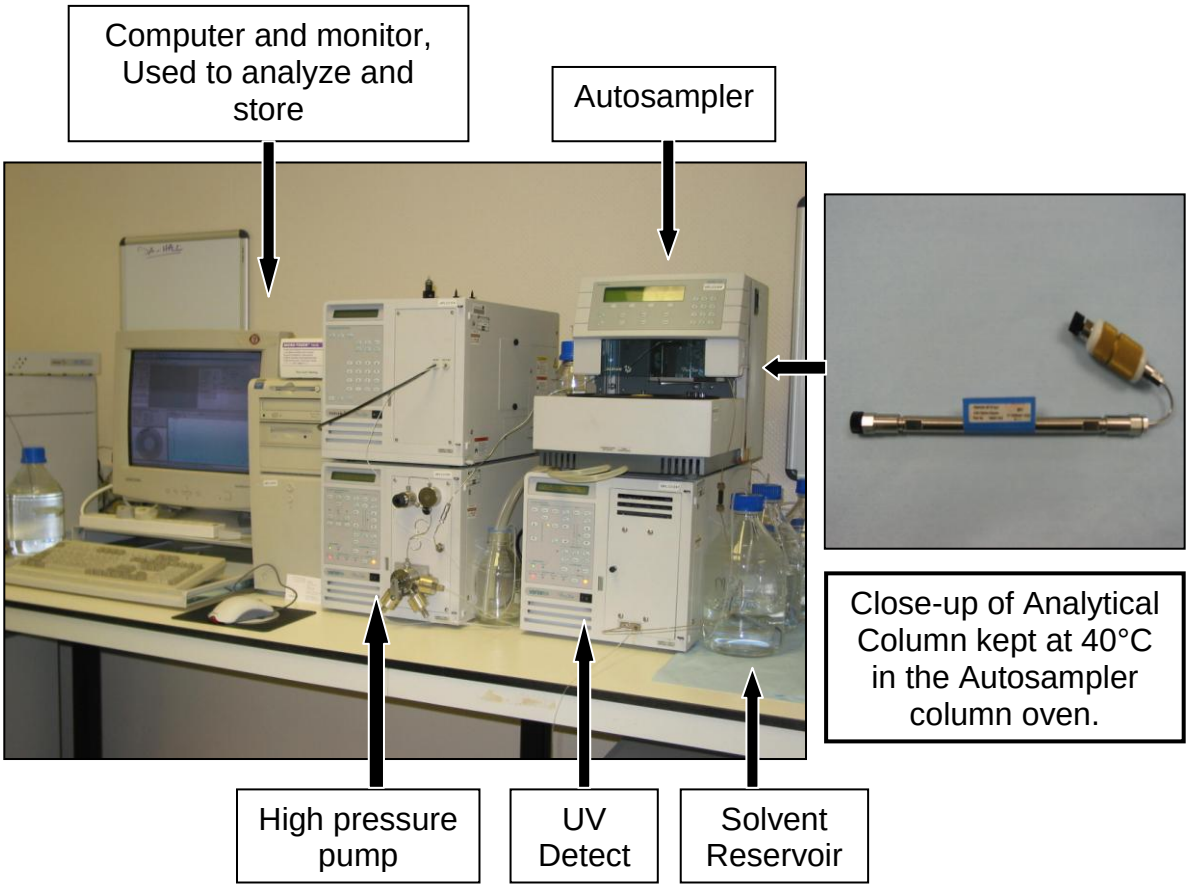
Table 3.9 Standard curve generation for analysis of silybinin in biomatrices**.**

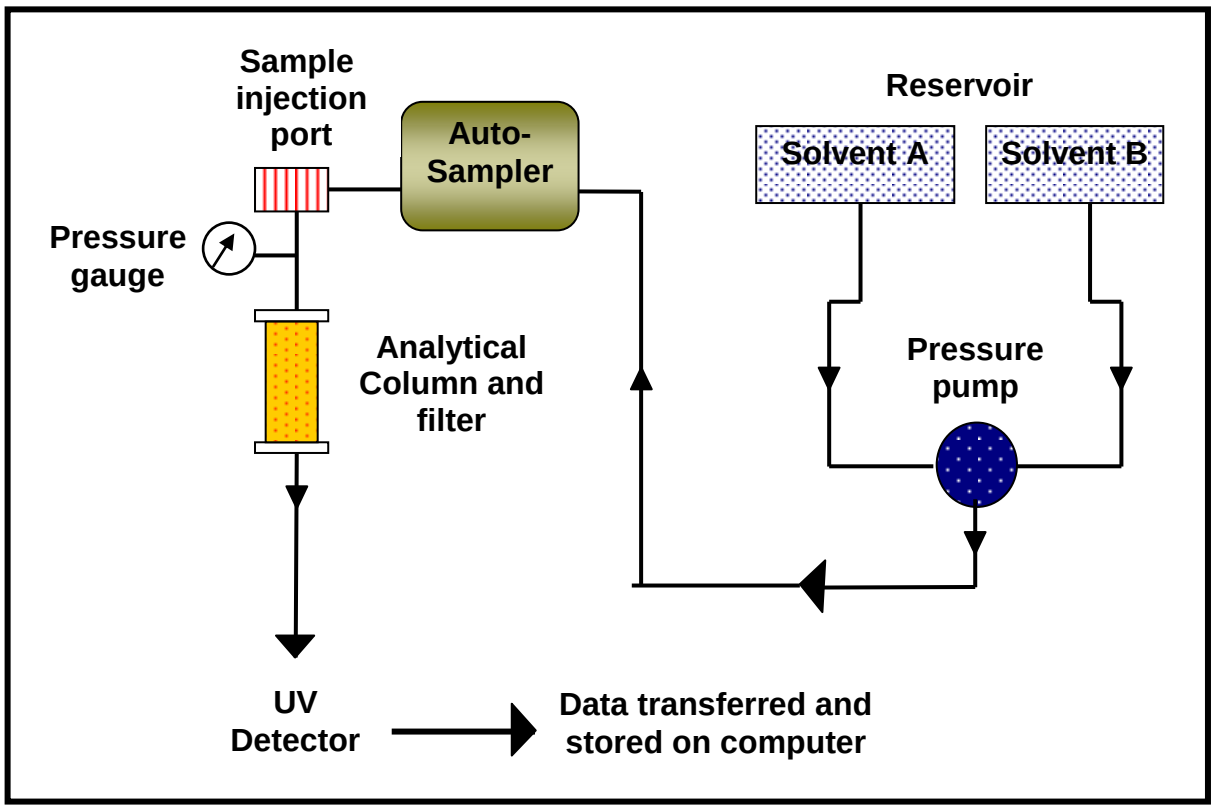
Silybinin stock solution (µg/mL)	**Amount of silybinin stock added (µL) [amount of biomatrix (µL)]**			***Final silybinin concentration (µg/mL) [Apigenin final concentration (µg/mL)]**
	Plasma	Colon Homogenate	Liver Homogenate	
0	100 [900]	20 [180]	50 [450]	0 [0]
0	100 [900]	20 [180]	50 [450]	0 [0.5]
0.5	100 [900]	20 [180]	50 [450]	0.05 [0.5]
1.0	100 [900]	20 [180]	50 [450]	0.1 [0.5]
2.5	100 [900]	20 [180]	50 [450]	0.25 [0.5]
5.0	100 [900]	20 [180]	50 [450]	0.5 [0.5]
10.0	100 [900]	20 [180]	50 [450]	1.0 [0.5]
50.0	100 [900]	20 [180]	50 [450]	5.0 [0.5]

* Silybinin concentration represents the sum of the two diastereoisomers.

- Samples were centrifuged (6000 g for 20 min), the supernatant removed and transferred to fresh plastic tubes and dried under a constant stream of nitrogen.
- Residues were reconstituted in 100 µL of mobile phase B (Section *3.4.2.1.3*) followed by a final 13,000 rpm centrifugation at 4°C and supernatant transferred to vials prior to HPLC analysis.

Figure 3.6 Schematic of HPLC-UV system and the Prostar 230 HPLC model used in the analyses of silybinin





3.4.2.1.5 HPLC conditions

Chromatographic separation was accomplished using a Waters Atlantis column (4.6 × 150 mm, 3-µm C_{18}; Waters, Elstree, UK) in combination with a Waters Atlantis (4.6 × 20 mm, 5-µm C_{18}) guard column, at 40°C. The Atlantis stationary phase was chosen for this analysis due to its ability to run under highly aqueous conditions and its ability to separate the diastereoisomers of silybinin.

Mobile phase A consisted of glacial acetic acid and distilled water at a ratio of 1:19 (pH unaltered ~2.4). Mobile phase B was glacial acetic acid and methanol in a 1:19 ratio. Flow rate was 1.0 $mL.min^{-1}$ and the total run time was 35 min. Each injection volume was 50 µL.

Table 3.10 HPLC binary gradient employed for the separation of silybinin diastereoisomers

Gradient (min)	Mobile Phase A(%)	Mobile Phase B (%)
0	95	5
2	95	5
5	70	30
20	50	50
25	40	60
30	5	95
32	5	95

Quantitation of silybinin was accomplished by reference to a calibration curve of silybinin peak area ratio (silybinin d1 or d2 to internal standard) plotted against silybinin concentration. Apigenin was used as the internal standard in all experiments. The UV detection of silybinin and its metabolites were achieved at 290 nm.

3.4.2.2 Synthesis of conjugated silybinin metabolites *in vitro*

3.4.2.2.1 Preparation of human hepatic cytosol and microsomes:

- Samples (10 g) of stored human livers purchased from UK Human Tissue Bank (Ethical approval was sought and obtained: LREC reference No. 6868) were washed with ice-cold 0.15 M potassium chloride (KCl), and placed in buffered sucrose (90 mL; 0.25 M sucrose, 10 mM TRIS, 1mM EDTA with pH adjusted to 7.4 with 1 M HCl).
- The liver was homogenised with 10 slow passes at 1100 rpm using a Potter homogeniser.
- The homogenate was centrifuged at 9000 g for 20 min at 4°C. The lipid layer was removed prior to decanting the supernatant, which represents the S9 fraction, to polycarbonate tubes and centrifuged at 100 000 g for 60 min.
- The supernatant was removed and retained as the cytosol fraction.
- The pellet was re-suspended in 5 mL of 1.15% KCl using a Teflon homogeniser and centrifuged at 100 000 g for 60 min, after which the supernatant was decanted and the microsomal pellet again re-suspended in glycerol and chloride-free 0.25 M phosphate buffer, pH 7.4 (30/70, v/v).
- Subcellular fractions were stored at -80°C for no more than 12 weeks before use.

3.4.2.2.2 Synthesis of silybinin glucuronide:

Synthesis of silybinin glucuronide was developed from previously published work by Boocock *et al*. (Boocock *et al.*, 2000) describing the sulphonation of tamoxifen. The concentration of microsomal protein was determined using the Lowry method (LOWRY *et al.*, 1951) employing bovine serum albumin as the standard.

- Stock solutions of 25 mM of silybinin (SB) in DMSO, 1 M of magnesium chloride ($MgCl_2$) in HPLC-grade water, 10 mM of uridine diphosphoglucuronic acid (UDPGA) (12.924 mg UDPGA in 2 mL O.5 M TRIS-HCl) and HPLC-grade water were aliquoted into separate eppendorfs as tabled.

Table 3.11 Incubation mixtures used in the synthesis of silybinin (SB) glucuronides.

Incubation mixture	**Liver M (µL)**	**$MgCl_2$ (1M)(µL)**	**UDPGA (10mM)(µL)**	**Silybinin (25mM)(µL)**	**Water (µL)**
Liver MC without SB	50	5	50	0	395
Liver MC with SB	50	5	50	6	389
SB without MC	0	5	50	6	439
Microsome (MC) incubation mixture sample	**Gut M (µL)**	**$MgCl_2$ (1M)(µL)**	**UDPGA (10mM)(µL)**	**Silybinin (25mM)(µL)**	**Water (µL)**
Gut MC without SB	50	5	50	0	395
Gut MC with SB	50	5	50	6	389
SB without MC	0	5	50	6	439

- The reaction was initiated by the addition of UDPGA and incubated for 60 min at 37°C.
- The reaction was terminated by adding 1.5 mL of ice-cold methanol and the solution centrifuged at 13 000 rpm for 15 min to precipitate the protein.
- The supernatant was collected and presence of metabolite investigated by HPLC analysis as described above.

- To ensure that any observed peaks were consistant with silybinin glucuronides; negative control samples were also run each omitting one of the reagents needed for glucuronidation such as substrate (silybinin), UDPGA and microsomal aliquots were incubated with β glucuronidase to see if the putative silybinin glucuronide peak disappeared. Further aliquots were analysed using mass spectrometry.

3.4.2.2.3 Synthesis of silybinin sulphate:

The sulphonation of silybinin was developed from methods described for other compounds (Boocock *et al.*, 2000)

- A 5 mM solution of silybinin was incubated with 1.25% Bovine serum albumin (BSA), 66 mM Tris-HCl buffer, HPLC-grade water and human liver cytosol for 30 min at 37°C.
- The solutions were aliquoted into separate eppendorfs as outlined in the table below.

Table 3.12 Incubation mixtures employed in the sysnthesis of silybinin (SB) sulphonates.

Hepatic incubation mixture	**Cytosol (µL)**	**PAPS (50µM)(µL)**	**1.25% BSA (µL)**	**Tris-HCl (60mM)(µL)**	**Silybinin (5mM)(µL)**	**Water (µL)**
Cytosol no SB	18.5	6	5	23	0	49.5
Cytosol with SB	18.5	6	5	23	2	47.5
SB without Cytosol	0	6	5	23	2	66.0
Intestinal incubation mixture	**Cytosol (µL)**	**PAPS (50µM)(µL)**	**1.25% BSA (µL)**	**Tris-HCl (60mM)(µL)**	**Silybinin (5mM)(µL)**	**Water (µL)**
Cytosol no SB	9.5	6	5	23	0	58.5
Cytosol with SB	9.5	6	5	23	2	56.5
SB without Cytosol	0	6	5	23	2	66.0

- The reaction was initiated by the addition of PAPS (lithium 3′-phosphoadenosine 5′-phosphosulphate) and incubated for 30 min at 37°C.
- The reaction was terminated by the addition of 300 μL of ice-cold methanol.
- The precipitation protein was centrifuged and the supernatant removed and an aliquot injected onto the HPLC column. To ensure that additional peaks observed were derived from silybinin sulphonate; negative control samples were run each omitting one of the reagents needed for sulphonation e.g. substrate (silybinin), PAPS, cytosol or Tris-HCl. and the decrease or disappearance of sulphonate peak after further incubation with sulphatase. Mass spectrometry was also utilised, as described in the earlier section.

3.4.2.2.4 Purification of silybinin metabolites:

- The glucuronide and sulphonate metabolites of silybinin were prepared as described above and then semi-purified by collection of eluant from the HPLC at the retention time consistent with the appearance of metabolite peaks. The eluant was pooled from ten separate injections run under the same conditions.
- Collected eluant fractions were dried under a stream of nitrogen at 20°C and then reconstituted with 100 μL of mobile phase B (Section *3.4.2.1.3*) and a proportion re-injected to confirm the presence of the metabolites. An aliquot was analysed by mass spectrometry to confirm metabolite identity.
- The samples were then used in co-elution studies.

3.4.2.3 Deconjugation of silybinin metabolites

- Human and rat biomatrices were kept at –20°C. The plasma were thawed and the liver tissue weighed and homogenized in an equal part of 0.15 M KCl solution prior to analyses.
- 200 μl of the biomatrices was aliquoted into clean eppendorfs in duplicate.
- 5 μl of β- glucuronidase from *Helix Promatia* (type-H2, Sigma-Aldrich, containing sulfatase) was added to one eppendorf from each sample and the other acted as control.
- 300 μl of 0.1 M ammonioum acetate buffer was added to each eppendorf (pH 6.0) and vortexed.
- The samples were incubated in a shaking water bath for 1 h set at 37°C.
- The reaction was terminated by the addition of 600 μL of ice-cold methanol.
- The protein precipitation was vortexed for 20 s and left at –20°C for 30 min.
- The samples were then centrifuged and the supernatant removed and an aliquot injected onto the HPLC column.
- To ensure that additional peaks observed were derived from silybinin glucuronides and sulphonates; negative control samples from rats and patients that had no intervention were run simultaneously. Mass spectrometry was also utilised, as described in the earlier section.

3.4.2.4 Co-elution studies

Co-elution studies utilising pure silybinin and semi-purified silybinin metabolites were performed with gut mucosa, liver, portal blood and peripheral blood samples obtained from patients post-silybinin treatment. Serial dilutions of reference compound were undertaken until the peak area of the diluted sample was similar in size to the peak area of the suspected silybinin and silybinin metabolite peaks in the patient samples. The patient sample was then spiked with the reference compound and the resultant chromatogram compared with a control sample spiked with a comparable volume of acidified methanol alone. The increase in the area of a particular peak after the addition of reference compounds identifies it as the compound of interest.

3.4.3 Mass spectrometry in the identification of silybinin and silybinin metabolites

Analyses were performed using an API 2000 mass spectrometer (Applied Biosystems MDS Sciex, Warringon, UK) equipped with an Agilent 1100 series sample delivery system employing an Atlantis 2.1 × 150 mm, 3-µm C_{18} column (Waters, Elstree, UK) in combination with a Atlantis 2.1 × 20 mm, 5-µm C_{18} guard column.

Confirmation of trace levels of silybinin and its metabolites in plasma and tissues was achieved by collecting fractions from the HPLC and subjecting them to mass spectrometry. Mass spectrometry was carried out by Dr David Boocock and Dr. Raj Singh, Department of Cancer Studies and Molecular Medicine, University of Leicester, UK. Analyses were performed using an API 2000 LC mass spectrometer (Applied Biosystems MS Sciex, Warrington, UK) equipped with an Agilent 1100 series sample delivery system. Silybinin and metabolites were analysed using electrospray ionization in negative ion mode . Conditions were as follows: declustering potential –26 V, focusing potential –350V, electrode potential –12V, cell entrance potential –16V, cell exit potential –20V, temperature 500°C. Identification of silybinin-derived species was by selected ion monitoring for silybinin *(m/z* 481), silybinin mono-glucuronide (*m/z* 657), silybinin di-glucuronide (*m/z* 833), silybinin sulphonate (*m/z* 561) and silybinin mono-glucuronide mono-sulphonate (*m/z* 737).

3.4.4 Genomic DNA extraction from blood and tissues for the analysis of M_1dG DNA adducts using the Qiagen method

3.4.4.1 DNA extraction from tissues and whole blood

The packed blood cells and tissue samples were thawed to room temperature prior to use. DNA extraction for all the blood samples and part of the tissue samples was performed using the Qiagen genomic column and buffer kit. Different quantities of buffer solutions, enzymes and column sizes were used depending on the volume of blood or tissues used (see below). Smaller quantities of tissues (as in pre-treatment biopsies, 3-60 mg) used smaller columns and less buffers as compared to larger quantities of tissues (100-300 mg) which require larger columns and larger buffer volumes.

3.4.4.1.1 Preparation of tissue samples for DNA extraction:

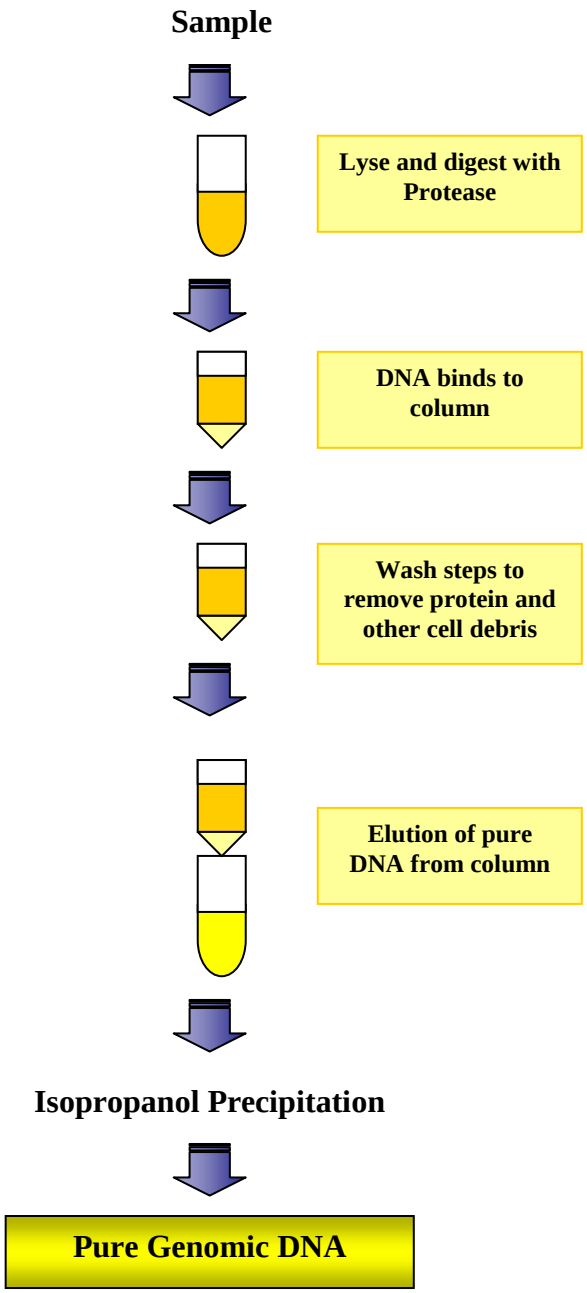
For samples less than 20 mg in weight, the 20/G Mini Qiagen column was used and volume of buffers and enzymes are shown below. For samples weighing 20 – 100 mg, the 100/G Midi Qiagen Column was used and the volume of buffers and enzymes are shown below in brackets.

- 4 µL, (19 µL) of RNAse A stock solution (100 $mg.ml^{-1}$) and 2 ml, (9.5 ml) aliquot of Buffer G2 were added to the colon or hepatic tissue and thoroughly homogenised, using a Dounce homogenizer.
- The homogenate was transferred to a 10 ml, (50 ml), screw-cap tube and 0.1 ml, (0.5 ml) of the Qiagen Proteinase K stock solution was added. The homogenate was mixed thoroughly and vortexed for 20 s.
- The homogenate was incubated for 2 h at 50°C in a water bath.

- The sample was then centrifuged at 5000 x g for 10 min at 4°C to remove any large proteins which may later 'clog' the column prior to DNA extraction.

The samples were now ready for proceeding with the Genomic - tip protocol (described below).

Figure 3.7 DNA extraction using the Qiagen method.
(Figure taken from QIAGEN Genomic DNA Handbook.)



3.4.4.1.2 Preparation of whole blood samples for DNA extraction:

- 1 volume (5 ml) of ice-cold Buffer C1 and 3 volumes of ice-cold distilled water (15 ml) were added to 3 ml of whole blood.
- The suspension was mixed by inverting the tube several times, until the suspension becomes translucent.
- The suspension was incubated on ice for 10 min.
- The suspension of lysed blood cells was centrifuged at 4°C for 15 min at 13000 x g. The supernatant was then carefully discarded.
- 1 ml of ice-cold Buffer C1 and 3 ml of cold distilled water were added to the pellet. Vortex to re-suspend the pelleted nuclei. The suspension was again centrifuged at 4°C for 15 min at 1300 x g, (see above) discarding the supernatant.
- 5 ml of Buffer G2 was added and the nuclei pellet suspended by vortexing for 10 – 30 s at maximum speed.
- 95 μL of Qiagen Proteinase K solution was added to each sample and incubated at 50°C for 30 - 60 min.
- The samples were now ready for proceeding with the Genomic - tip protocol (described below).

3.4.4.1.3 Genomic tip protocol

- The 20/G Genomic tip (100/G Genomic tip) was equilibrated with 2 ml, (4 ml) of Buffer QBT and allowed to empty by gravity flow.
- The samples prepared either from the tissue homogenate or from whole blood were vortexed for 10 s at maximum speed and applied to the equilibrated genomic tip.
- The genomic tip was then washed with 3 x 1 ml, (2 x 7.5 ml) of Buffer QC.
- The DNA was then eluted from the column with 2 x 1 ml, (1 x 5 ml) of Buffer QF which was pre-warmed to 50°C to increase the DNA yield. The eluants were collected in clean 10 ml, falcon tubes.
- The DNA was then precipitated by adding 1.4 ml, (3.5 ml) of room temperature isopropanol.
- The sample was then immediately centrifuged at >5000 x g for at least 15 min at 4°C and the supernatant carefully removed, leaving the DNA pellet at the bottom of the 10 ml falcon tube.
- The centrifuged DNA pellet was washed in 1 ml, (2 ml) of cold 70% ethanol. This was repeated and the pellet was then air dried for 5 min and re-suspended in 100 µL of HPLC grade water and stored at -80°C until required.

3.4.4.1.4 Buffer solutions

Buffer G2 (General Lysis Buffer)	800 mM guanidine hydrochloride; 30 mM ethylenediamine tetra-acetic acid (EDTA), pH 8.0; 5% Tween-20; 0.5% Triton X-100.
Buffer QBT (Equilibration Buffer)	750 mM sodium chloride; 50 mM 3-(n-morpholino) propanesulfonic acid (MOPS), pH 7.0; 15% isopropanol; 0.15% Triton X-100.
Buffer QC (Wash Buffer)	1.0 M sodium chloride; 50 mM MOPS, pH 7.0; 15% isopropanol.
Buffer QF (Elution Buffer)	1.25 % sodium chloride; 50 mM Tris-CL, pH 8.5; 15% isopropanol.

3.4.5 Ultra-violet spectrophotometry for quantification of DNA

Quartz cuvettes were purchased from Amersham Biosciences (New York, USA). Samples were analysed using GeneQuant^{TM} *pro* RNA / DNA U-V spectrophotometer (Amersham Biosciences, NY, USA). Following extraction of the DNA and re-suspension in 100 µL of HPLC grade water, 1 µL aliquot of the solution was taken and diluted by a factor of 100 in HPLC grade water. The samples were placed in a quartz cuvette and analysed with the UV spectrophotometer. The double strand DNA concentration was calculated using the following equation:

dsDNA concentration (µg/µL) = A[260]* × 0.05 × Dilution Factor

The purity of the dsDNA was calculated using the formula: $\frac{A[260]^*}{A[280]^\dagger}$

* A[260] is the absorbance value of the DNA at a wavelength of 260 nm

† A[280] is the absorbance value of the DNA at a wavelength of 280 nm

The concentration and its purity were then used to calculate the appropriate volume of DNA solution required to give 3.5 µg of DNA for immunoslotblot analysis. Only DNA with a purity value of between 1.6 – 1.8 was considered acceptable for further analysis.

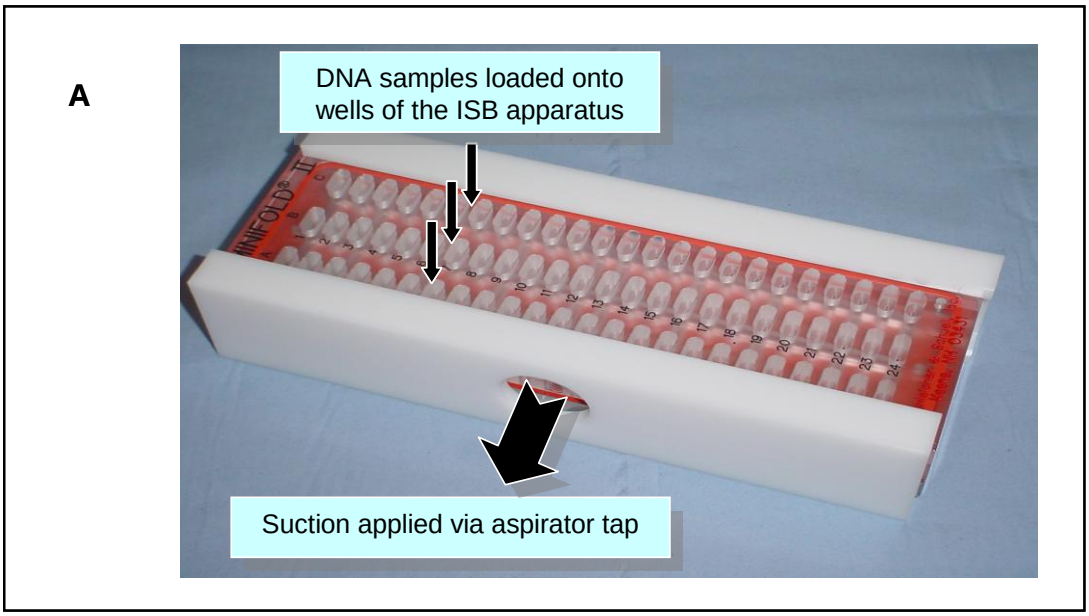
3.4.6 Quantification of the oxidative DNA adduct malondialdehyde - deoxyguanosine (M_1dG)

3.4.6.1 The immunoslot blot apparatus

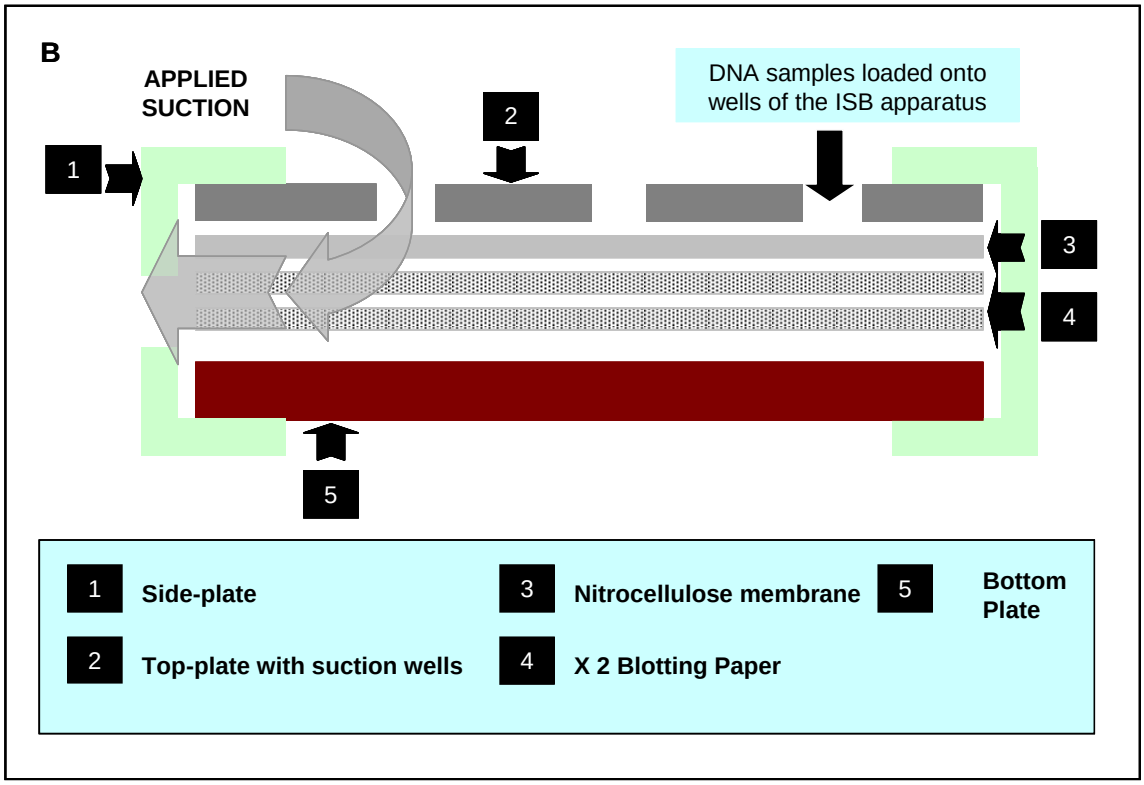
Genomic DNA extraction was performed using reagents and columns obtained from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan) and Qiagen Ltd. (West Sussex, UK). Propidium iodide, ammonium acetate crystals and polyoxyethylene-sorbitan monolaurate (Tween-20) were purchased from Sigma-Aldrich (Poole, UK). Dipotassium hydrogen-orthophosphate and HPLC grade water were obtained from Fisher Chemicals (Loughborough, UK). Supersignal west dura extended duration chemiluminescence substrate was purchased from Pierce Biotechnology (Rockford, USA). Primary antibody was generously supplied by Dr. Lawrence Marnett, Department of Biochemistry; Vanderbilt University, USA. Secondary antibody- goat anti mouse immunoglobulin horse radish peroxidase was bought from DakoCytomation (Glostrup, Denmark). Phosphate Buffered Saline tablets (PBS) were purchased from Sigma-Aldrich (Poole UK). The immunoslot blot apparatus, cellulose nitrate filter and the gel-blotting papers were supplied by Schleider and Schuell, Anderman and Company Ltd. (Surrey, UK). And finally the chemiluminescence analysis was performed using 'Genegnome' and 'Genegenius' Bioimaging systems, Synergie (Cambridge, UK). Figure 3.8 shows a photograph of the actual apparatus and a schematic diagram of its cross sectional views of the immunoslot blot (ISB) apparatus used in the determination of M_1dG.

Figure 3.8 Figure showing the immunoslot blot (ISB) apparatus (A) and a schematic diagram showing the cross-section of the ISB apparatus (B). Also shown is the loading of DNA onto the wells while the suction was applied.

(Figure was taken from Schleider and Schuell, Anderman and Company Ltd.)



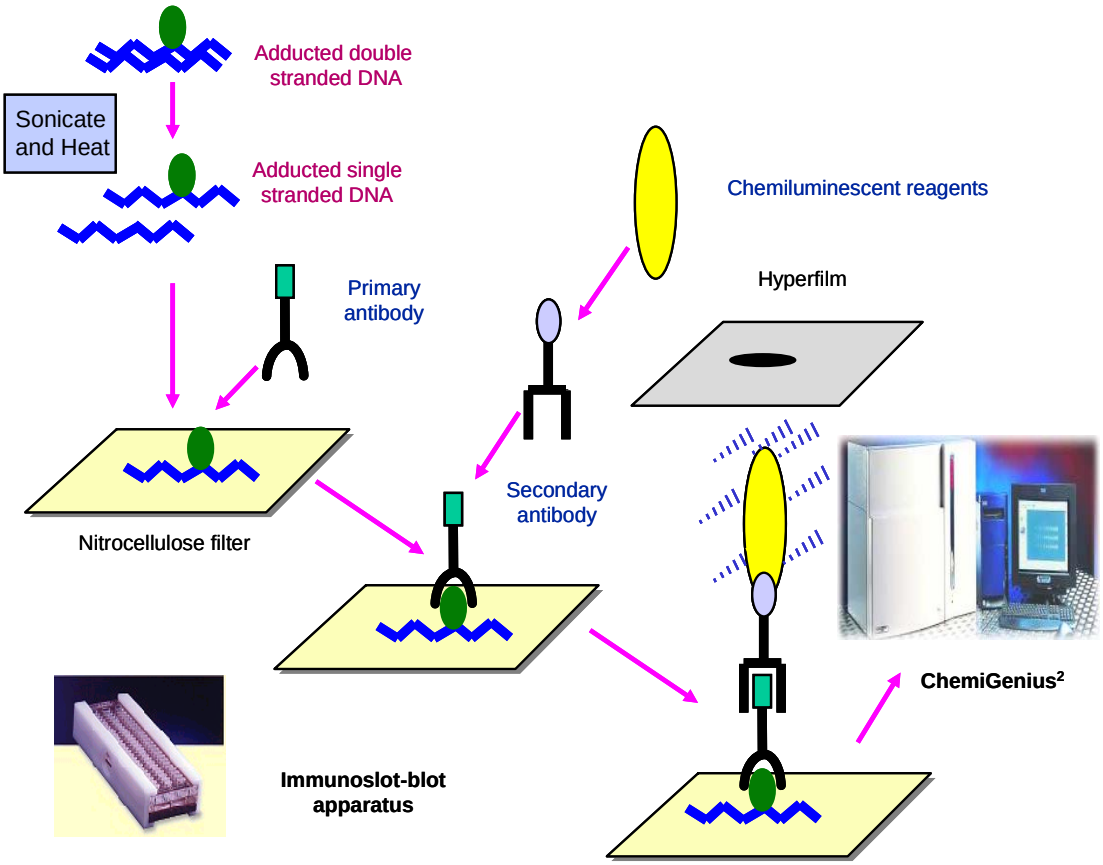
(i)



3.4.6.2 The quantification of M_1dG using the immunoslot blot method

Levels of M_1dG were measured in both the normal and malignant tissue, in pre- and post treatment tissues. The assay used involved immunoslot blot methods described by Leuratti (Leuratti *et al.*, 1998). The samples were analysed in triplicate and equal loading of DNA was determined by propidium iodide staining following adduct measurements. The intensity of adduct staining was measured using a Genegnome™ fluorescence imager and the actual adduct concentrations calculated as adducts per 10^7 nucleotides from a standard curve using authentic standards generated by treating calf thymus DNA with malondialdehyde (Leuratti *et al.*, 1999). Authentic, quantified M_1dG standards were kindly supplied by Dr Raj Singh, University of Leicester. Figure 3.9 summarises the immunoslot blot procedure.

Figure 3.9 Schematic representation of the immunoslot-blot procedure.
(Figure was modified from Syngene.com.)



3.4.6.3 Preparation of M_1dG standard curve:

- MDA-treated calf thymus DNA with a known M_1dG composition (10 fmol M_1dG/µg DNA) was diluted with untreated calf thymus DNA to give a series of standards (Table 3.13) in a total volume of 50 µl. Vortex and centrifuge at 14,000 rpm for 1 min.

Table 3.13 Standard curve generation for the analyses of M_1dG adducts

Control calf thymus DNA at 100µg/ml (µl)	MDA treated calf thymus DNA at 100 µg/mL (µl)	M1dG (fmol/µg)
50	0	0
48	2	0.4
45	5	1.0
40	10	2.0
35	15	3.0
25	25	5.0
20	30	6.0
10	40	8.0
0	50	10.0

- Pipette 35 µl (3.5 µg) of each standard into an eppendorf and then add 65 µl of KP buffer (10 mM *di*-potassium hydrogen orthophosphate (K_2HPO_4), pH 7.0). Vortex mix and centrifuge at 14,000 rpm for 1 min.
- Each standard was then sonicated$^{\otimes}$ for 20 min, this allows for the breaking up of the DNA into approximately 100 base pair long strands.

- Then add 150 μl of PBS (one tablet of Oxoid Phosphate buffered saline (Dulbecco 'A') per 100 ml of HPLC grade water) and vortex.
- Heat sample to 100°C for 5 min and then cool on ice for at least 10 min. Heating gives rise to single stranded DNA.
- Vortex and centrifuge at 14,000 rpm for 2 min and then add 250 μl of 2 M ammonium acetate, vortex and centrifuge again at 14,000 rpm for 1 min.
- Pipette 143 μl for 1 μg of each standard onto the nitrocellulose filter.

3.4.6.4 Preparation of patient samples:

- Pipette volume of sample containing 3.5 μg of DNA and make the volume up to 100 μl with KP buffer. Vortex and centrifuge at 14,000r pm for 1 min.
- Samples were then sonicated for 20 min.
- Add 150 μl of PBS and vortex mix.
- Heat to 100°C for 5 min and then cool on ice for at least 10 min.
- Vortex and centrifuge at 14,000 rpm for 2 min and then add 250 μl of 2 M ammonium acetate, vortex and centrifuge again at 14,000 rpm for 1 min.
- Pipette 143 μl for 1 μg of DNA onto the nitrocellulose filter.

Loading the immunoslot blot:

- Two gel-blotting papers (GB 002, Schleicher and Schuell, Anderman and Company Ltd., Kingston-Upon-Thames, Surrey, UK) were bathed in 1 M ammonium acetate before being put onto the immunoslot blot apparatus (Schleicher and Schuell).

- The cellulose nitrate filter (0.1 µm, BA79, Schleicher and Schuell) was bathed in distilled water (HPLC grade,) followed by 1 M ammonium acetate before being placed on top of the gel-blotting papers. The immunoslot blot apparatus was assembled together, ensuring that there were no air bubbles between the filter, and blotting paper and connected to an aspirator.
- A volume that contained 1 µg of DNA for each sample was pipetted into each well of the immunoslot blot apparatus in triplicate while the aspirator was running.
- The wells were allowed to run dry and then 200 µl of 1 M ammonium acetate was added to each well and again the wells were allowed to run dry.
- Lines were drawn using a ball point pen on the edges of the filter to mark the exterior wells and also in the left upper corner to allow for later orientation during processing.
- The filter was carefully placed between filter paper and heated at 80°C for 1.5 h in an oven.
- The filter was placed in a plastic tray and bathed in 100 ml of PBS-T (PBS-T = PBS plus 0.1% Tween) plus 5% non-fat milk powder (5. 0g, Marvel, Nestle Ltd.) and allowed to rock gently at room temperature (20ºC) for 1 h. The filter was then washed twice for 5min with PBS-T (50 ml) and sealed in a plastic bag.
- The primary M_1dG antibody was diluted 1:8000 (5 µl in 40 ml, 6.25 ng/ml) with PBS-T plus 0.5% milk powder (0.2 g) and was added to the filter and incubated in a sealed plastic bag for 2 h at room temperature then 4°C overnight with gentle rocking.
- The filter was washed with PBS-T (50 ml) by gentle rocking in a tray for 1 min followed by a further two 5 min washes with fresh PBS-T (50 mL).

- The filter was incubated in a plastic bag with the secondary antibody (goat anti mouse immunoglobulin horse radish peroxidase conjugated, Dako A/S, Denmark). The antibody was diluted 1:4000 (8 μl in 32 ml) with PBS-T plus 0.5% milk powder (0.16 g). The filter was allowed to rock at 20°C for 2 h.
- The filter was washed with PBS-T (50 ml) by gentle rocking in a tray for 15 min followed by a further two 5 min washes. Care was taken not to let the filter dry out after the final wash.
- Just before being used, the supersignal ultra chemiluminescent substrate (Pierce, Illinois, USA) working solution was prepared by mixing equal volumes (4 ml) of the ultra luminol/enhancer solution with the ultra stable peroxide solution.
- The filter was bathed in the chemiluminescence reagents for 5 min, gently blotted dry on a paper towel, covered in cling film and exposed to the ECL-hyperfilm (Amersham International plc.).
- The amount of chemiluminescence for standards and samples was determined using a Genegnome^{TM} fluorescence imager. Quantification of M_1dG levels in sample DNA was calculated by reference to the standard curve.

3.4.7 Enzyme-linked Immunosorbent Assay (ELISA) analysis of IGF-1 and IGFBP-3 from human serum

The enzyme-linked immunosorbent assay kits 10-5600 ACTIVE and 10-6600 ACTIVE, for the analysis of human IGF-1 and IGFBP-3, respectively, were obtained from Diagnostic Systems Laboratories UK (Oxon, UK). Manufacturers guidelines were strictly followed in the analysis of the samples. The laboratory method is outlined below.

IGF-1 and IGFBP-3 concentrations in patients' plasma were determined using enzyme-linked immunosorbent assay (ELISA) kits 10-5600 ACTIVE and 10-6600 ACTIVE, respectively (Diagnostic Systems Laboratories UK, Oxon, UK). The IGF-1 kit procedure contained a "one-step" acid-ethanol extraction step to separate IGFs from their binding proteins in plasma. In the IGFBP-3 kit, an additional incubation step with anti-IGFBP-3 polyclonal antibody was carried out prior to the former procedure. The assays were validated and performed according to the instructions of the manufacturer.

3.4.7.1 Extraction procedure for patient sample:

- For each sample, a 1.5 mL polypropylene eppendorf and two polypropylene 12 x 75 mm culture tubes, were prepared and clearly labelled.
- The patients' plasma was stored at -80°C and thawed and mixed thoroughly by gentle swirling prior to use.
- 50 µL of each sample was pipetted and placed to the bottom of an individual eppendorf, to which 450 µLof extraction solution, containing ethanol hydrochloride, was added.

- Eppendorfs were capped, and samples vortexed and incubated at room temperature (~20°C) for 30 min.
- During the extraction, 100 μL of the neutralizing buffer was added to each sample before centrifugation at 10 000 rpm for 3 min at room temperature.
- Immediately after centrifugation, 100 μL of each clear sample supernatant was transferred into the culture tubes and vortexed thoroughly.
- Then, 200 μL of the sample diluent (0 ng/mL standard) and another 50 μL of neutralized buffer were added to each sample and vortexed (This accounts for a 1:5 dilution, resulting in a final dilution of 1:100).
- Do not dilute standards or controls.

3.4.7.2 IGF-1 ELISA procedure for analysis of human plasma:

- Each 96 well plate coated with anti-IGF-1 antibody was labelled clearly prior to use.
- 20 μL of each standard, control or patient sample was pipetted into the appropriate wells in duplicate.
- The IGF-1 Antibody-Enzyme Conjugate (Ab-Enz-C) solution, which contains anti-IGF-1 antibody conjugated to the enzyme horseradish peroxidase in a protein based buffer, was prepared by a 1:50 dilution with a BSA buffer (provided by the kit).
- 100 μL of the Ab-Enz-C solution was added to each well incubated for 2 h at room temperature (~20°C) with constant fast speed shaking at 500 – 700 rpm on an orbital microplate shaker.
- After 2 h, each well was washed five times with a buffered saline solution with a nonionic detergent (Wash Concentrate diluted 1:25 with deionised water) using an

automatic microplate washer and blotted dry by inverting the plate on absorbent material.

- Then, 100 μL of the tetramethylbenzidine (TMB) chromogen solution was added to each well and the entire plate was wrapped in foil to prevent exposure to direct sunlight. This was incubated for a further 10mins, shaking at 500 – 700rpm on an orbital microplate shaker.
- The reaction was terminated by adding 100 μL of 0.2 M sulfuric acid to each well.
- The absorbance of the solution in each well was read within 30min, using a microplate reader set to 450 nm.

3.4.7.3 ELISA procedure for analysis of IGFBP-3 in human plasma:

- Serum samples from patients were prepared as outlined above.
- The 96 well plate coated with goat anti-IGFBP-3 polyclonal antibody was labelled.
- 25 μL of each standard, control or patient sample was pipetted into the appropriate wells in duplicate.
- 50 μL of the assay buffer were added to each well and the wells incubated for 2 h at room temperature (~20°C) with constant fast speed shaking at 500 – 700 rpm on an orbital microplate shaker.
- Following this, each well was washed five times with a buffered saline solution with a nonionic detergent (60 mL wash concentrate diluted to 1500 mL deionised water) and blotted dry by inverting the plate on absorbent material.
- The IGFBP-3 Antibody-Enzyme Conjugate (Ab-Enz-C) solution, which contains goat anti-IGFBP-3 polyclonal antibody conjugated to the enzyme horseradish

peroxidase in a protein based buffer, was prepared by a 1:50 dilution with a protein-based (BSA) buffer (provided by the kit).

- 100 μL of the Ab-Enz-C solution was added to each well using a semi-automatic dispenser and the wells incubated for 2 h at room temperature (~20°C) with constant fast speed shaking at 500 – 700 rpm on an orbital microplate shaker.
- After 2 h, each well was washed five times with wash solution and blotted dry.
- 100 μL of the tetramethylbenzidine (TMB) Chromogen solution was added to each well and the entire plate was wrapped in foil to prevent exposure to direct sunlight. This was incubated a further 10 min, shaking at 500 – 700 rpm on an orbital microplate shaker.
- The reaction was terminated by adding 100 μL of 0.2 M sulfuric acid to each well.
- The absorbance of the solution in each well was read within 30 min, using a microplate reader set to 450 nm.

Chapter 4. METHOD DEVELOPMENT

4.1 Development of an analytical method for quantification of silybinin

4.1.1 Introduction

The clinical assessment of silybinin requires the establishment of robust analytical methodology for its identification and quantitation in biological matrices. Previously, Martinelli *et al.* (Martinelli *et al.*, 1991) described a HPLC method for silybinin using normal–phase solid extraction columns for purification with tert-butylmethylether as eluent and *n*-hexane/ethanol as mobile phase. This method did not separate the diastereoisomers of silybinin, and the fact that *n*-hexane is toxic (Burgaz *et al.*, 2002; Spencer *et al.*, 2002) mitigates against its use in the laboratory. Mascher *et al.* (Mascher *et al.*, 1993) published a semi-validated HPLC method that, for the first time, separated the two diastereoisomers of silybinin, but this method employed a mobile phase composed of 0.02 M perchloric acid, which is a powerful oxidizing agent and as such raises safety concerns for large-scale use in the laboratory. Therefore, the primary purpose of this part of the study was to develop a safe, simple and robust reversed-phase HPLC method that was validated for detection of silybinin in human plasma and tissues which produces clearly separated peaks for both silybinin diasteroeisomers. The method should ideally provide a low limit of detection for the quantification of silybinin and clearly separate the parent compound and its metabolites.

4.1.2 Plasma extraction method development

Previously published studies have used solvents such as acetonitrile or ethyl acetate to extract silybinin from rat plasma (Yanyu *et al.*, 2006). Silybinin is a large molecule which contains 25 carbon atoms. ($C_{25}H_{22}0_{10}$). It has a molecular weight of 482.44 and a melting point of 158°C for anhydrous silybinin (167°C for monohydrate). Silybinin is essentially insoluble in water but dissolves in organic solvents such as acetone, ethyl acetate, methanol, ethanol and chloroform.

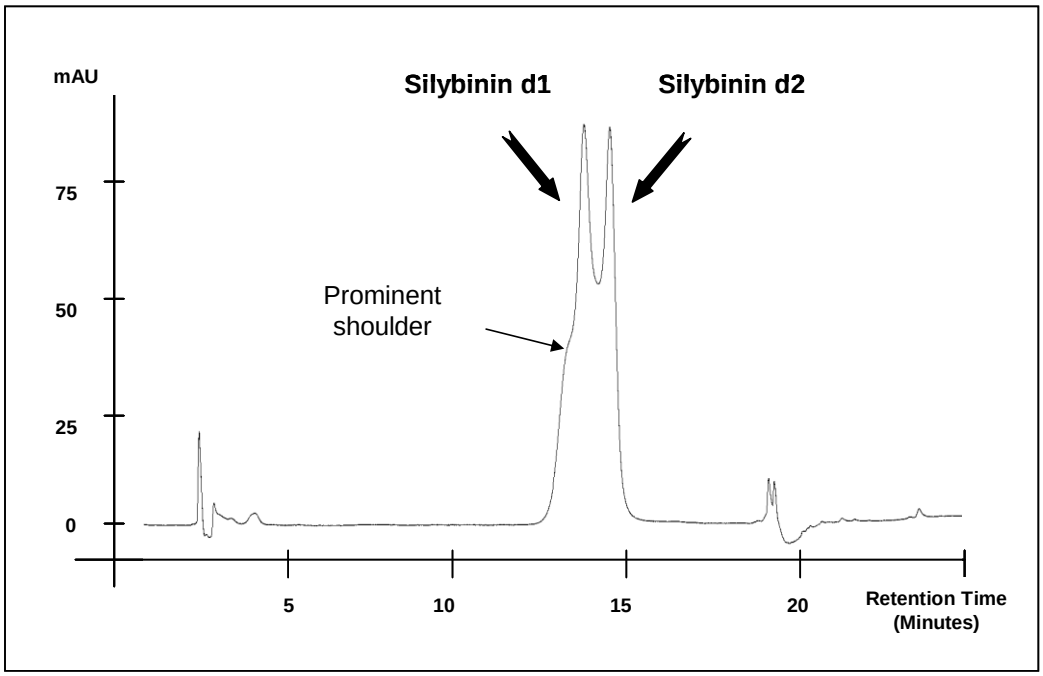
In this study, initial work focused on the development of the most efficient method for extracting silybinin from human plasma. Experiments were conducted using three types of extraction namely, solid phase extraction, liquid-liquid extraction and protein precipitation with methanol.

4.1.2.1 Solid phase extraction

For solid phase extraction, Oasis HLB columns (purchased from Waters, Elstree, UK) were used to separate silybinin from human plasma. The column has a water-wettable polymer to trap large hydrophobic molecules such as silybinin and its metabolites on active pore-sites whilst allowing small hydrophilic molecules such as water, methanol and plasma to wash through. The columns were placed on a Waters vacuum extraction chamber. The columns were primed with 1 mL methanol and 1 mL water to wet the polymer, then 1 mL human plasma samples were loaded and allowed to filter through. Each column was then washed with 1 mL water and eluted with 1 mL methanol to eliminate remaining impurities.

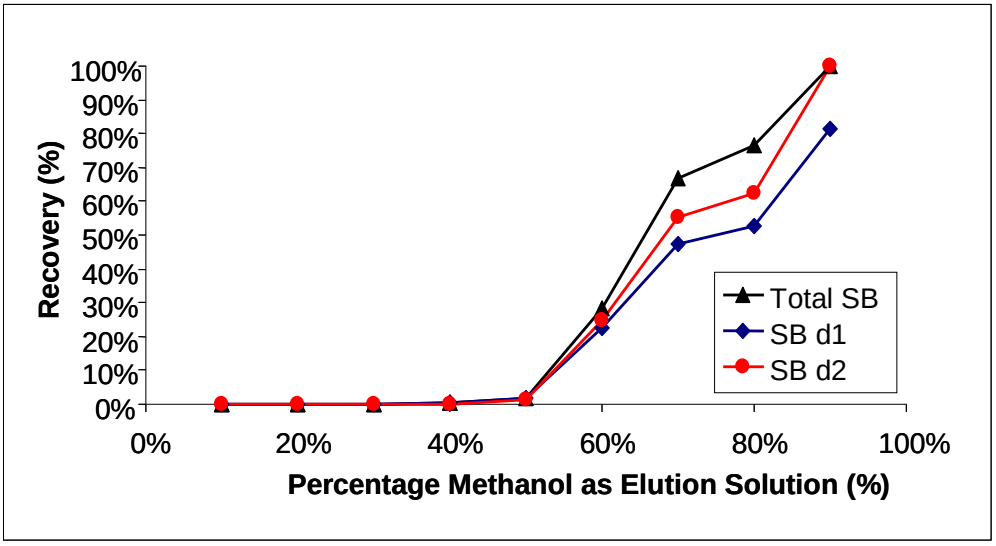
Figure 4.1 shows a typical HPLC-UV chromatogram of silybinin spiked plasma following solid phase extraction.

Figure 4.1 HPLC-UV chromatogram of eluant after solid phase extraction of a plasma sample spiked with silybinin at UV wavelength of 290 nm. The two prominent peaks which most likely represent the two silybinin diastereoisomers (d1 and d2) are poorly separated. A prominent shoulder seen before the silybinin peaks may be due to the fact that the HPLC column is old.



This method was relatively straightforward to perform and the results were reproducible. However, protein and lipid contamination within the plasma matrix caused constant blockage of the columns. Attempts were made to overcome this problem with blockage of the columns by acidifying the plasma matrix prior to loading on the columns, but this failed to resolve. Further experiments were conducted using different percentages of methanol solutions to identify the optimal eluant condition which facilitated maximal silybinin recovery. The results are shown in Figure 4.2.

Figure 4.2 The effect of different percentages of aqueous methanol as eluant for silybinin in solid phase extraction with Water Oasis HLB columns showing that the recovery of silybinin increased with content of methanol.



The extraction efficiency (EE) of a particular experiment is expressed as the percentage of silybinin recovered over the amount of silybinin within the plasma matrix at the beginning. In solid phase extraction, the EE (%) was inconsistent and varied between each run, measuring between 50 – 95%. Further work was done by increasing the percentage of methanol with either an acid or alkaline for column elution to try and improve the recovery. The process was carried out as previously described and either 2% ammonium hydroxide solution or 2% acetic acid or 5% acetic acid were added into an increasing percentage of methanol (1 mL solution altogether) were used to elute the columns for the silybinin spiked human plasma. It was found that columns acidified with 2% or 5% acetic acid did not become blocked and showed increased EE (%). This increase was not directly related to the amount of acid added. These results are illustrated in Figure 4.3 and Figure 4.4. All of the columns that had ammonium acetate were blocked and the extraction process could not be completed.

Figure 4.3 The effect of acidified plasma on the recovery of silybinin by solid phase extraction of silybinin spiked human plasma. Results show a marginal increase in the percentage recovery after acidifying the plasma samples with 2% acetic acid.

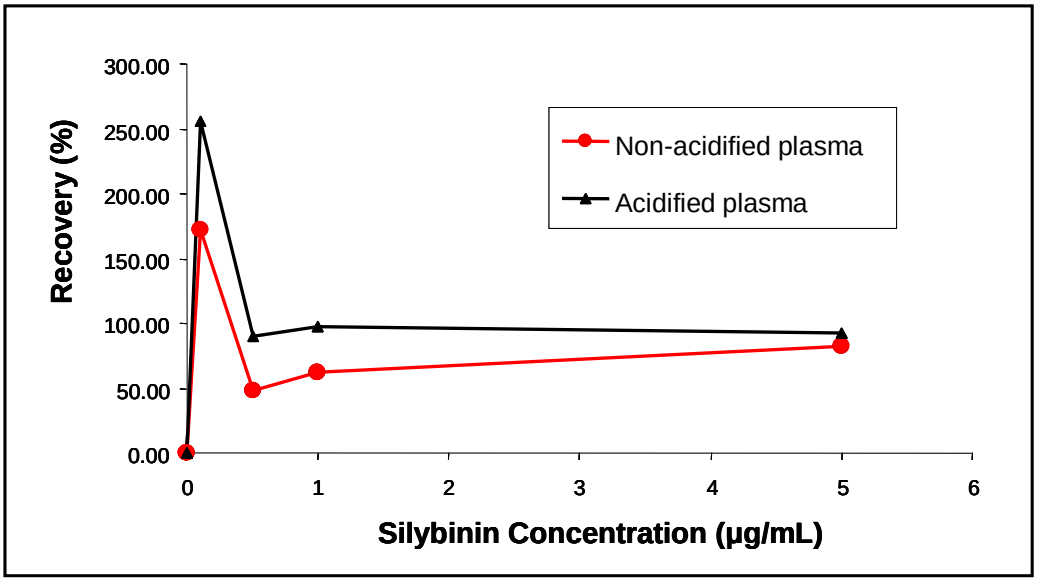


Figure 4.4 A comparison of different concentrations of acetic acid in the elution of silybinin in solid phase plasma extraction. The graph shows that there is marginal difference between 2% and 5% acetic acid influencing the recovery of silybinin from the analysed plasma samples.

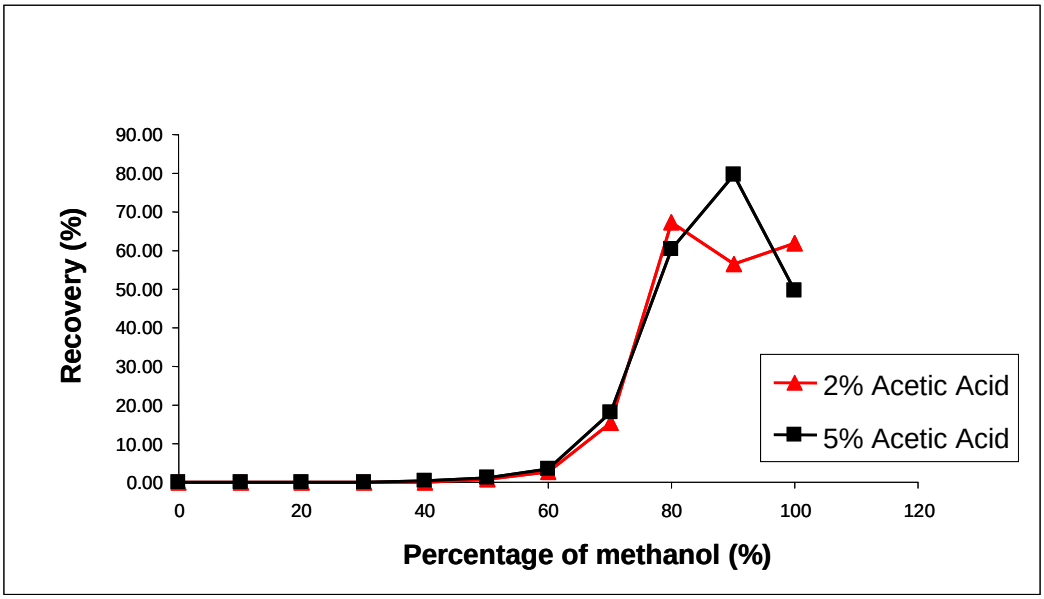


Figure 4.4 suggests that silybinin yields decent peaks in eluants of 40% and above. However, the shouldering (seen in Figure 4.1) was more pronounced in 70% methanol and above. There was little difference between using 2% or 5% acetic acid as eluant. Nevertheless, 5% acetic acid concentrations gave a slightly higher recovery of silybinin. Therefore, 5% acetic acid in 70% methanol was used in further experiments.

4.1.2.2 Liquid – liquid extraction

Liquid-liquid extraction was carried out by adding an immiscible organic solvent to the silybinin spiked plasma sample. Plasma matrix was added to 5 mL of organic solvent and vortex at room temperature for 5 min. Centrifugation at 5000 g was carried out for 5 min at 4°C to further separate the two immiscible layers. The organic layer was removed and the extraction process was repeated three times. The extracts from all three runs were pooled and evaporated to dryness under nitrogen. The residue was then dissolved in 100 µL 5% acetic acid with 70% methanol (described in Section 5.2.1. and Figure 20) prior to HPLC-UV analysis. Organic solvents investigated were ethyl acetate, methyl tert-butyl ether (MTBE) and chloroform. Ethyl acetate gave the highest EE, followed by MTBE, and chloroform, for a standard concentration of silybinin spiked human plasma. A typical HPLC-UV chromatogram of silybinin from the liquid–liquid extraction method is shown in Figure 4.6 (A).

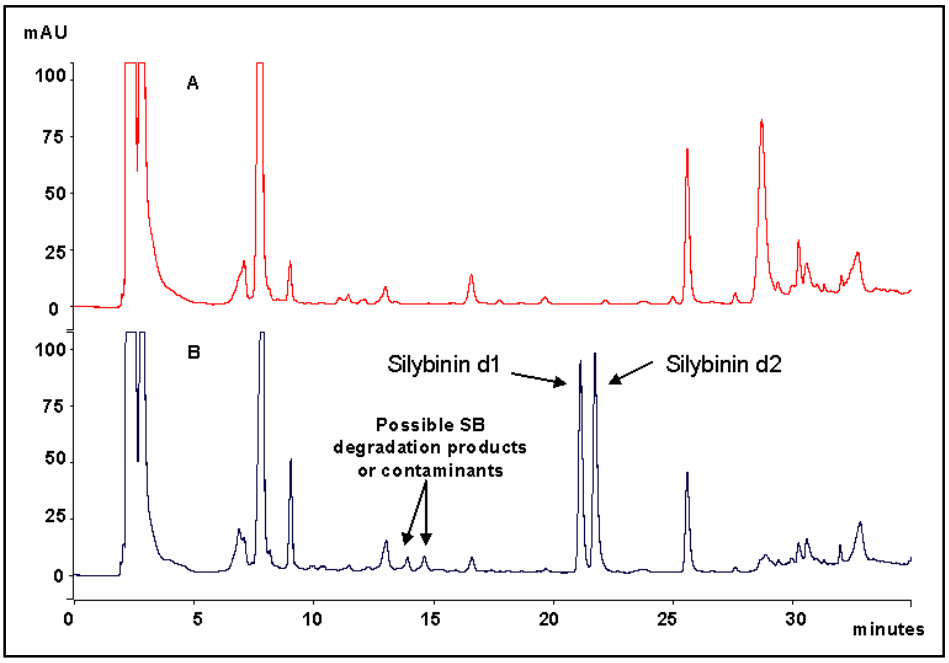
In comparison with solid phase extraction, this method was less time consuming and gave a superior extraction efficiency (EE) of 70 – 85%. It was cheap, simple to carry out and efficient. To optimise this extraction method, a comparison between using 3

or 5 mL ethyl acetate was performed. This showed that using 3 mL ethyl acetate gave a higher recovery than 5mL. An experiment where the extraction process was repeated from three to five times resulted in the same EE. In contrast to the solid phase extraction method, acidifying the plasma with phosphoric acid prior to liquid-liquid extraction showed a decline in recovery (Results not shown).

4.1.2.3 Protein precipitation

The extraction method ultimately chosen in this study used methanol precipitation of the proteins within the plasma samples. This method, which was described in Section 4.2.1, gave an extraction efficiency of between 53 – 58%. Figure 4.5 shows a typical chromatogram of silybinin spiked plasma following methanol precipitation extraction.

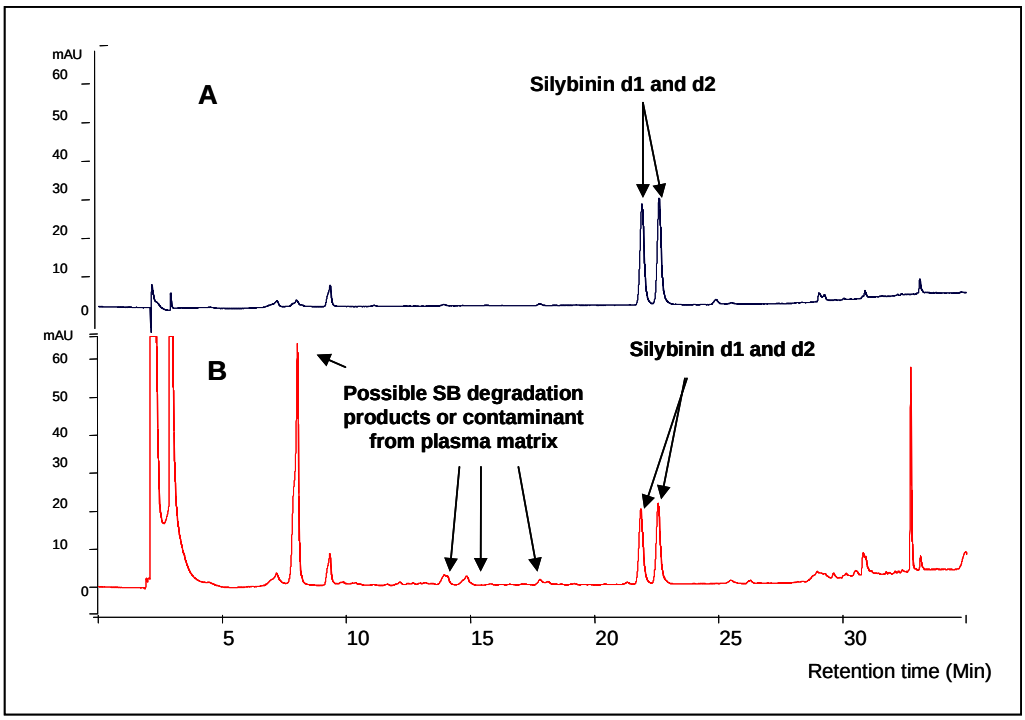
Figure 4.5 A typical HPLC-UV chromatogram of extracts from methanol precipitation extraction of unspiked human plasma (A) and human plasma spiked with silybinin at a concentration of 5 µg/mL (B). The two prominent peaks represents the silybinin diasteroeisomers. Other peaks highlighted in silybinin spiked plasma chromatogram (B) which are not present in the extracted unspiked plasma (A), may represent either silybinin degradation products or contaminants from the plasma matrix.



Experiments performed comparing methanol and ethanol in the protein precipitation extraction of silybinin from a known plasma silybinin concentration showed that methanol gave a better EE (Results not shown).

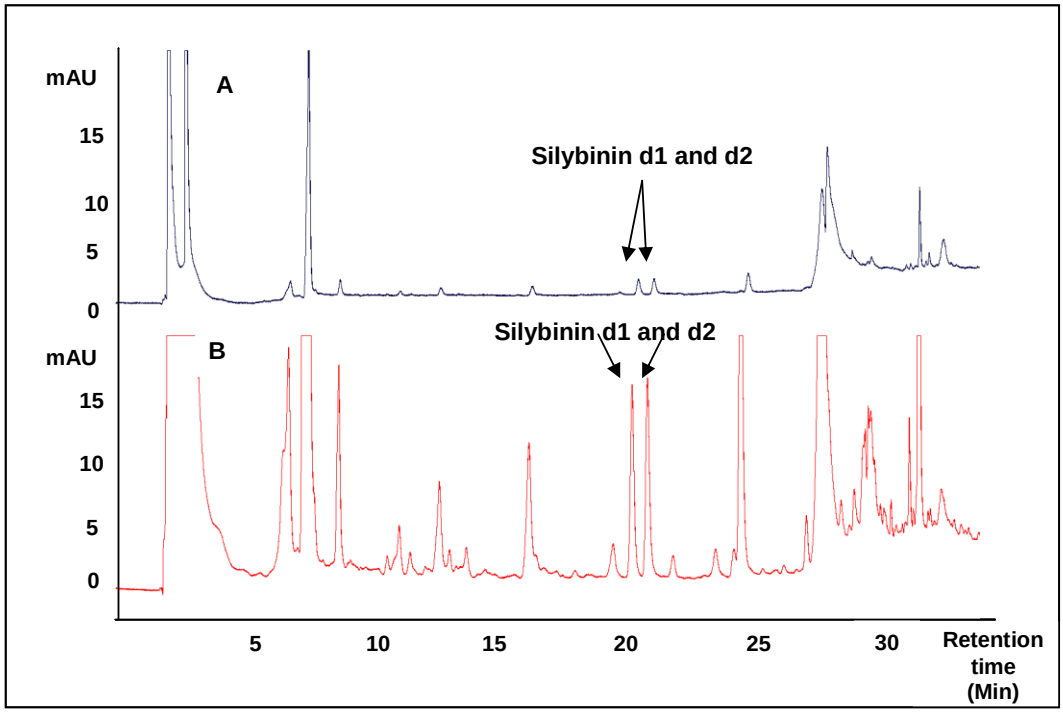
Additional work was carried out comparing the reliability, efficiency and recovery of silybinin extraction by methanol protein precipitation and ethyl acetate plasma extraction methods. Both methods were comparable in terms of reliability and reproducibility. Even though the recovery of silybinin from the methanol precipitation method was lower compared to the ethyl acetate extraction, it gave better chromatograms with resolved and measurable peaks. Results were reproducible and repeat experiments gave a consistent recovery. HPLC-UV chromatograms showing silybinin from spiked plasma following the two extraction methods are shown, for comparison, in Figure 4.6. Further silybinin extractions of patient plasma showed that following methanol protein precipitation, it was possible to extract silybinin metabolites together with its parent compound from each sample and both seemed to give separate peaks in a single HPLC chromatogram.

Figure 4.6 HPLC-UV chromatograms for ethyl acetate extraction of human plasma spiked with silybinin at 5 µg/mL (A) and methanol precipitation extraction method of human plasma with silybinin at 5 µg/mL (B). The chromatograms show that ethyl acetate extraction method gave a higher extraction efficiency (with highest peak areas) but methanol precipitation isolates several other peaks which may represent either silybinin degradation products or contaminants from the plasma matrix.



Methanol precipitation was used as the plasma extraction method in this study. For further sample optimisation following the extraction of plasma spiked with silybinin standards, the supernatants were dried under a constant nitrogen stream and then reconstituted with 100 µL of 70% aqueous methanol containing 5% acetic acid to concentrate the samples. An experiment was conducted to compare direct injection of a silybinin standard with injection of a dried and then reconstituted standard of the same concentration (Results shown in Figure 4.7).

Figure 4.7 Representative HPLC-UV chromatograms from direct analysis of the extracted supernatant without drying (A) and supernatant dried and reconstituted with 100 µL of 70% aqueous methanol containing 5% acetic acid (B). Chromatogram (B) shows increased silybinin peaks making these peaks easily quantifiable.



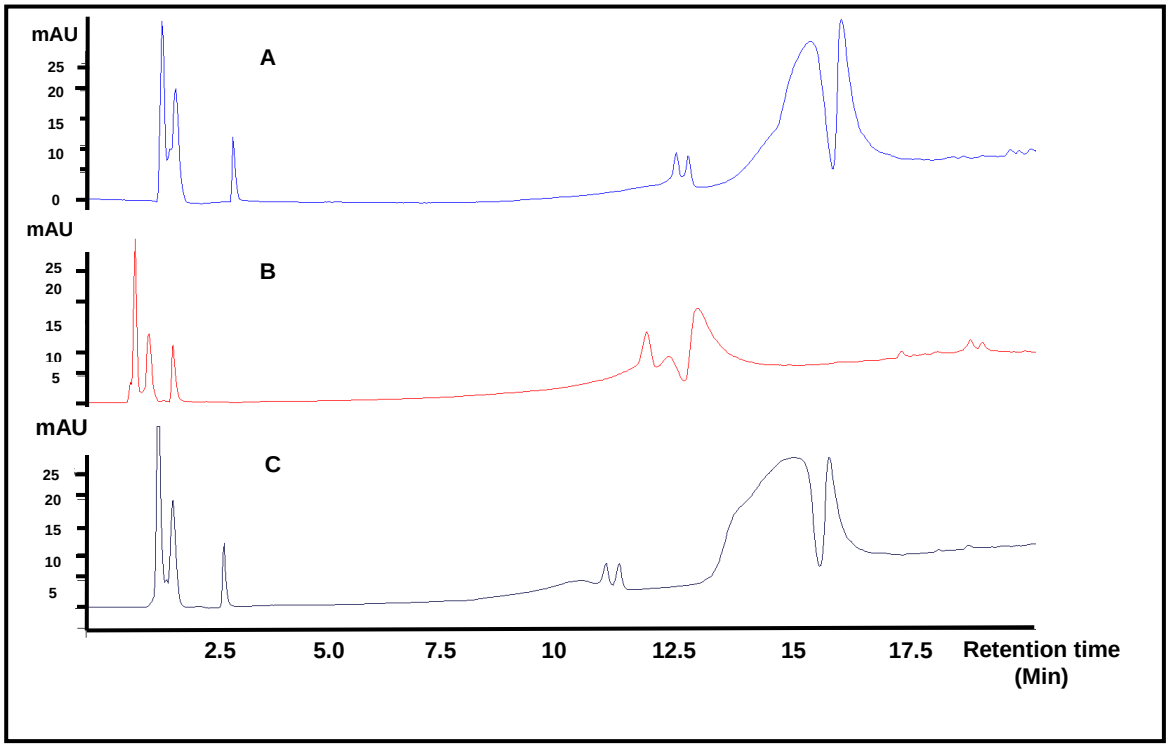
4.1.3 HPLC method development for the quantification of silybinin

It was important to achieve a clear separation of the parent compound silybinin and its metabolites. This method was based on a previous report by Indena Spa (Morazzoni *et al.*, 1993).

4.1.3.1 Selection of hplc column

The following columns were evaluated: Waters Atlantis 150 x 4.6 mm (3 μm) dC_{18} column, Waters Xterra phenol 150 x 4.6 mm (3μ), Waters Symmetry Shield 150 x 3.9 mm (5 μm), Genesis C18 150 x 4.6 mm (4 μm) and Zorbax SB C18 150 x 4.6 mm (3.5 μm). Figure 4.8 shows the chromatograms obtained using three different columns. All the hplc columns were kept at 40°C with the same mobile phase and gradient. The gradient used here was outlined in Section 3.4.2.1.5 and Table 3.5. Water Atlantis w the most suitable column giving clear separation of both diastereoisomeric peaks.

Figure 4.8 HPLC-UV chromatograms of Water Atlantis d18 column (A), Water Symmetry Shields d18 column (B) and Genesis C18 column (C).

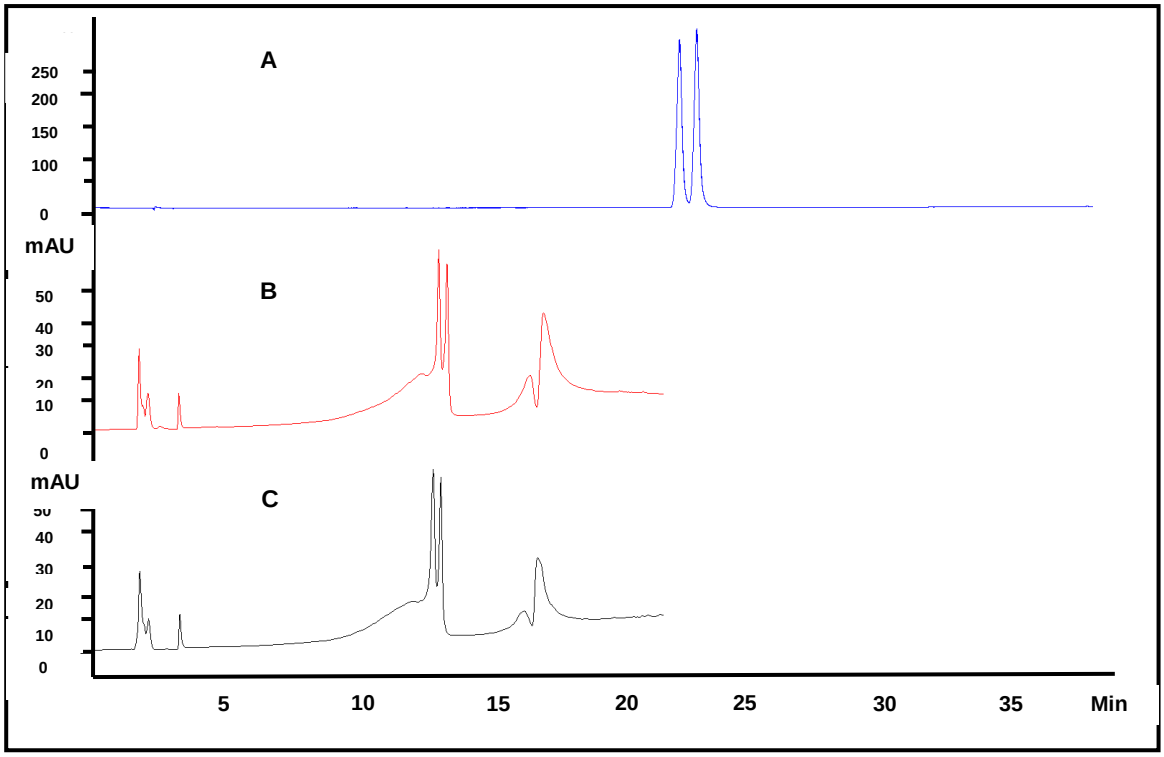


4.1.3.2 Selection of mobile phases and gradient

The objective for the method development was to obtain a gradient which would separate the two diastereoisomeric peaks of silybinin. Previous work in the laboratory had been on the quantitation of trans-resveratrol in human plasma and urine. The gradient for the separation of resveratrol used ammonium acetate and methanol containing 2% propan-2-ol (Boocock *et al.*, 2007). Therefore, initial experiments were concentrated on using a binary solvent system consisting 5 mM ammonium acetate in mobile phase A (pH 4-5 by addition of acetic acid), and methanol for mobile phase B. The chromatograms obtained were sub-optimal giving an elevated baseline and a persistent shoulder before the silybinin peaks (Figure 4.9-B). The addition of 2% propan-2-ol to both mobile phases made marginal improvement to the chromatogram,

but was not ideal for separation. However, a better separation was achieved by using a mobile phase with a lower pH. Hence, aqueous acetic acid (pH 2-3) was used in replacement of ammonium acetate. A linear gradient that was described previously by Indena Spa, was used (Morazzoni *et al.*, 1993) and minor modifications were made until optimum separation was achieved.

Figure 4.9 HPLC-UV chromatogram obtained with different mobile phases. Aqueous acetic acid - pH 2.7 (A), 5 mM ammonioum acetate with acetic acid – pH 5.0 (B) and 5 mM ammonioum acetate with phosphoric acid – pH 4.7 and the addition of 2% propan-2-ol (C).

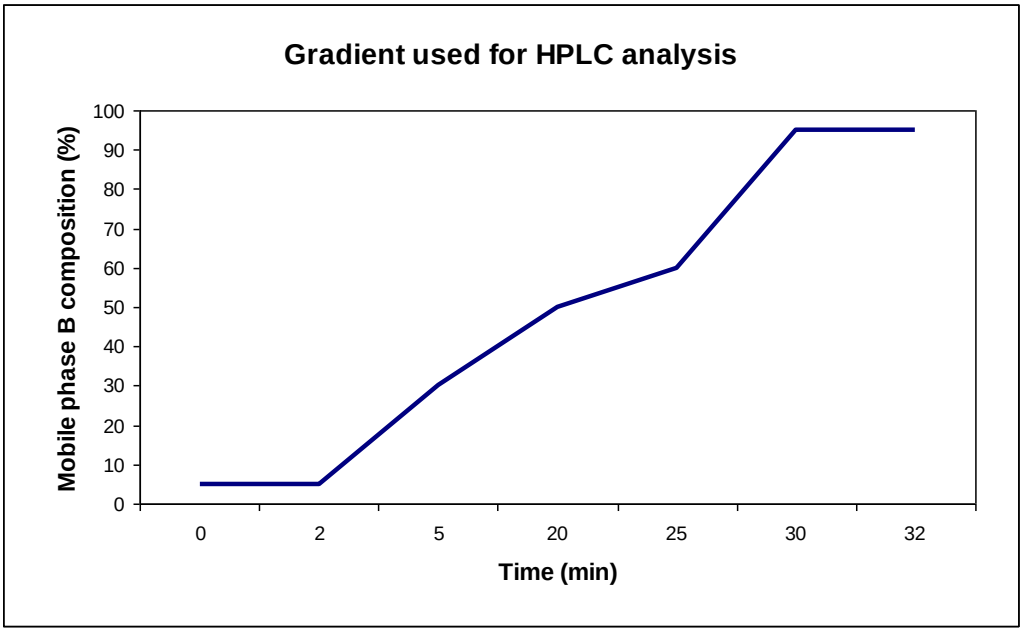


HPLC runs were carried out using mobile phases comprising a range of acetic acid concentrations from 1 – 10% acetic acid in water and methanol. The optimal separation was obtained when using 5% acetic acid in water (mobile phase A) and 5% acetic acid in methanol (mobile phase B). The HPLC gradient used is presented in Table 4.1 and Figure 4.10.

Table 4.1 The optimum gradient used for the HPLC analysis:

Gradient time (min)	Mobile phase A (%)	Mobile phase B (%)
0	**95**	**5**
2	**95**	**5**
5	**70**	**30**
20	**50**	**50**
25	**40**	**60**
30	**5**	**95**
32	**5**	**95**

Figure 4.10 A graphic representation of the gradient used for HPLC analysis.

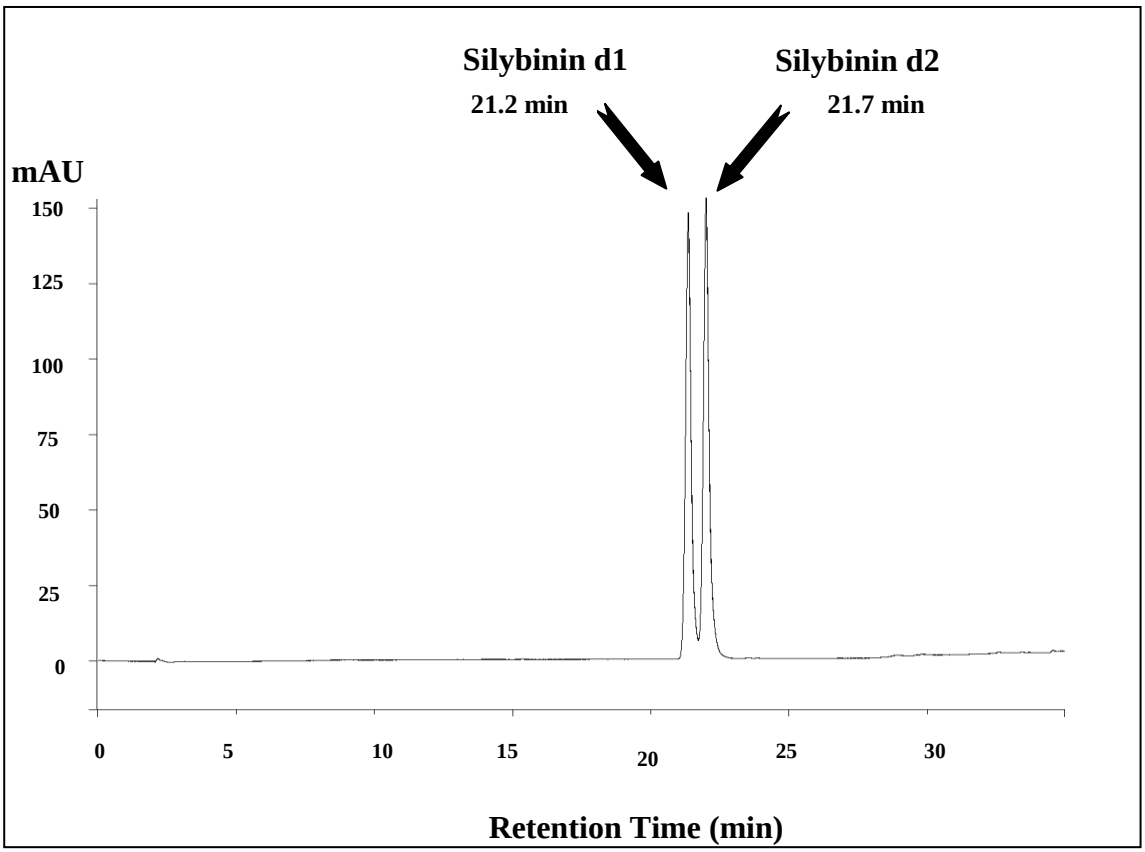


4.1.3.3 Selection of hplc column temperature

The HPLC column temperature setting was changed to optimize separation. Temperature settings at 30°C and 40°C were evaluated. The higher temperature, i.e. 40°C, reduced the shouldering before the silybinin peaks and the frequency of interfering peaks, which may represent impurities. However, at column temperature above 40°C, the silybinin in patient plasma samples may breakdown, giving an inaccurate silybinin level. Therefore 40°C was used.

In conclusion, a precise and well separated double peaked chromatogram of silybinin was obtained. This allowed accurate quantification of silybinin in human plasma samples. Figure 4.11 shows a typical HPLC chromatogram of silybinin using the method described above.

Figure 4.11 HPLC-UV chromatogram analysis at 290 nm of a standard solution of silybinin dissolved in methanol displaying the two peaks representative of the two diastereoisomers (d1 and d2) of silybinin. The gradient for hplc analysis used is outlined in Table 5.1. Mobile phase A comprised 5% aqueous acetic acid and mobile phase B was 5% acetic acid in methanol.



The two silybinin diastereoisomers mentioned throughout this manuscript are labeled as silybinin isomers d1 and d2; as the method we employed did not allow specific confirmation of which isomer was silybinin A and which was silybinin B.

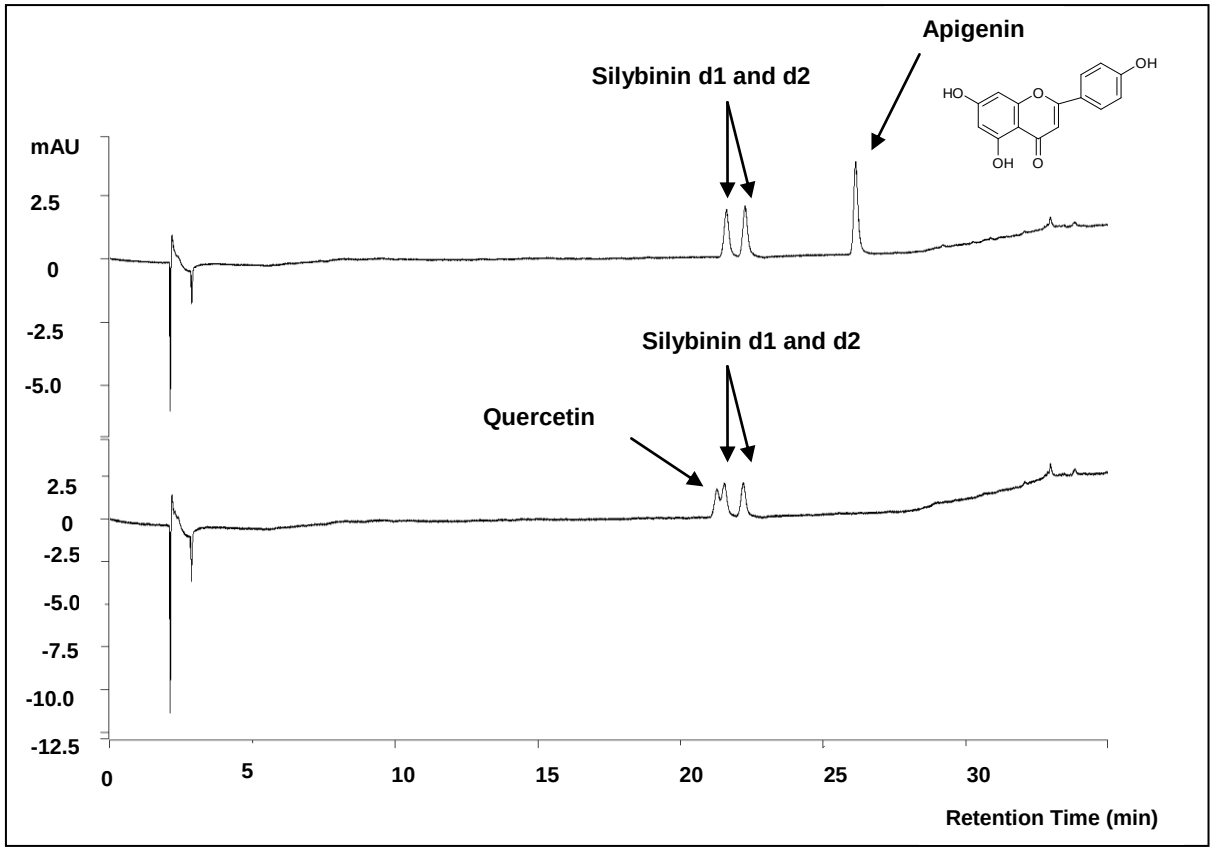
4.1.4 Method optimization for analysis of silybinin in patient plasma

4.1.4.1 Internal standard

An internal standard (IS) of a known quantity, was added in equal amounts to each patient plasma sample and standard plasma sample. The presence of an IS allows accurate quantification of silybinin in patient plasma which contain an unknown level of silybinin. The method in which this is carried out was by firstly running a standard curve of plasma spiked with silybinin and added IS. The peak area for silybinin and the IS were obtained from the chromatogram and a ratio of silybinin to the IS was calculated and refered to as the peak area ratio (PAR). The IS added to each plasma sample was of equal amount, and accounts for any variability in sample processing. A graph of PAR over silybinin concentration was plotted and a linear curve obtained. When the patient plasma samples were analyzed, the PAR was calculated and substituted into the linear equation and using linear regression analysis, an accurate quantification of the silybinin concentration in the patients' plasma was obtained.

Quercetin and apigenin were investigated as potential internal standards (IS) (purchased from Sigma-Aldrich and made into 100 μg/mL solutions using DMSO). An aliquot (5 μL) of the IS solution were added to 995 μL of spiked plasma. HPLC-UV analysis at 290 nm showed quercetin to co-elute with the silybinin d1 peak, making it unsuitable for this analysis. Apigenin was clearly separated from both silybinin peaks and eluted as a well resolved peak, making it easily quantifiable. (Figure 4.12)

Figure 4.12 HPLC-UV chromatogram analysis showing apigenin and quercetin as internal standards for HPLC of silybinin. Apigenin was well separated from the silybinin diastereoisomers while quercetin co-eluted with the silybinin d1 isomer.

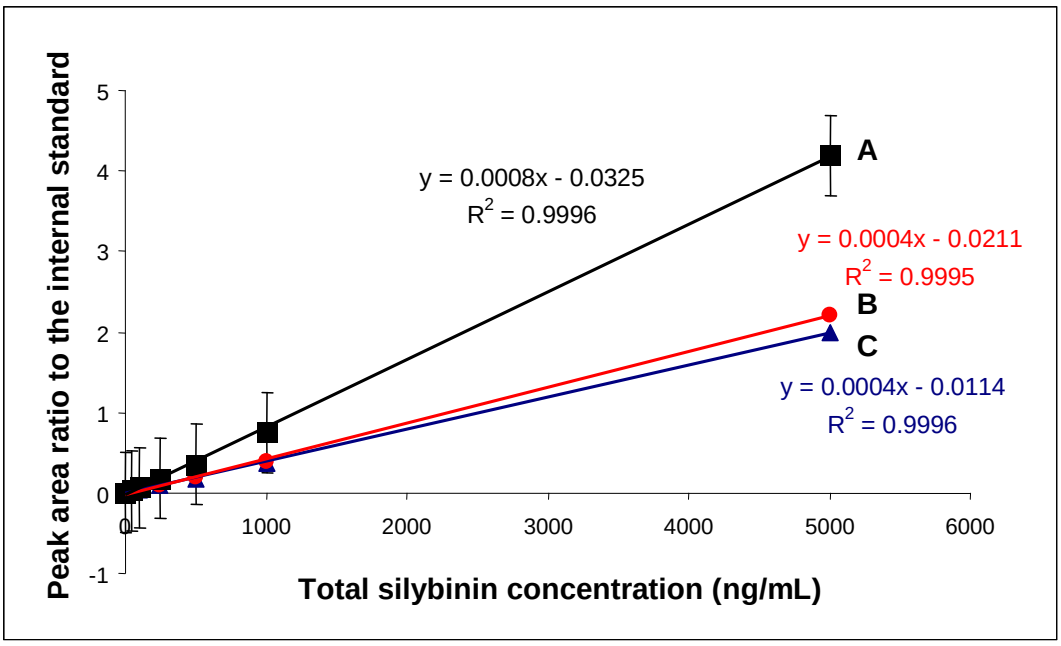


Apigenin was chosen as the internal standard for this study because it gave a well resolved separate peak eluting after both silybinin diastereoisomers and their metabolites, when analysed with the HPLC method outlined in section 3.4.2.1.4.

4.1.5 HPLC method validation

The method described in this thesis resolves the two diastereoisomers of silybinin into two baseline-separated chromatographic peaks at retention times 21.2 and 21.7 min, which were arbitrarily assigned as silybinin d1 and silybinin d2, respectively (Figure 4.11). Plasma spiked with silybinin yielded calibration curves that were linear over the observed range (0-5 μg/mL). Figure 4.13 shows a typical calibration curve of silybinin at concentrations 0, 0.05, 0.1, 0.25, 0.5, 1 and 5 μg/mL, obtained during bioanalytical validation.

Figure 4.13 Typical standard curve derived from silybinin standards used in the quantification of silybinin levels in human plasma. The BLUE line signifies silybinin d1 (C), the RED line shows silybinin d2 (B) and the BLACK line represents the sum of silybinin d1 and d2 (A).



* A = B + C

To comply with the U.S. Department of Health and Human Service: Food and Drug Administration (FDA) guidelines, six calibration curves of standard silybinin spiked plasma were performed to obtain both intra-day and inter-day mean correlation coefficient (r^2). For intra-day validation, the silybinin-spiked plasma, at concentrations 0, 0.05, 0.1, 0.25, 0.5, 1 and 5 μg/mL, were analyzed in six consecutive HPLC runs within the same day. The same concentrations were prepared, stored and analyzed at room temperature over six different days for inter-day validation.

4.1.6 Linearity, recovery, accuracy and precision

Linearity was assessed for each diastereoisomer by linear regression analysis. Recovery was assessed by comparison of silybinin isomers extracted from spiked plasma samples with non-extracted standard solutions in methanol of the same silybinin concentration (six consecutive analyses). The accuracy of the analytical method was the calculated silybinin concentration obtain after extraction compared to the actual silybinin concentration within the plasma. Precision was defined as the deviation of silybinin concentrations of multiple analysis of the same silybinin concentration from plasma samples. Recovery of silybinin from plasma samples, precision and accuracy for silybinin (d1 and d2) were determined at four concentrations (25, 50, 250 and 2500 ng/mL).

In intra-day analysis, silybinin d1 was characterized by the calibration equation $y = (0.0005 \pm 0.0001)\, x - (0.0166 \pm 0.0196)$, silybinin d2 by $y = (0.0005 \pm 0.0001)\, x - (0.0271 \pm 0.0232)$ (mean ± SD of 6 separate runs) (Table 4.2). The mean correlation

coefficient (r^2) for the intra- and inter-day analyses was consistently above 0.995 over six sets of consecutive analysis. Recovery of silybinin d1 and d2 from pooled plasma samples was 53 - 58%. The accuracy was consistently within 15% for silybinin d1 at all concentrations and silybinin d2 for concentrations 50 to 2500 ng/mL. The precision determined at each concentration level as reflected by the coefficient of variation (CV) or relative standard deviation (RSD) did not exceed 15 % (Table 4.2).

Table 4.2 Summary of recovery, precision and accuracy (n = 6) for extraction and measurement of silybinin diastereoisomers from human plasma.

Silibinin diastereoisomer	**Concentration (ng/mL)**	**Recovery (%)**	**Recovered concentration (Mean ± SD)**	**Accuracy (% ± SD)**	**Coefficient of variation RSD (%)**
d1	25	58 ± 8	29.04 ± 4.08	93.6 ± 6.2	14.04
d2	25	55 ± 7	27.44 ± 3.29	75.3 ± 6.3	11.98
d1	50	56 ± 4	55.61 ± 3.83	97.4 ± 6.9	6.89
d2	50	53 ± 7	54.41 ± 2.15	85.8 ± 4.1	4.02
d1	250	54 ± 6	270.6 ± 32.2	106.7 ± 12.1	11.92
d2	250	55 ± 6	275.6 ± 28.7	106.3 ± 11.4	11.40
d1	2500	55 ± 6	2598 ± 272	100.0 ± 10.5	10.48
d2	2500	55 ± 5	2732 ± 255	113.5 ± 10.6	9.32

4.1.7 Limit of detection and limit of quantification

The limits of detection (LOD) and quantification (LOQ) were estimated as the concentrations of silybinin diastereoisomers that generate peaks with signal to noise ratios of three and seven, respectively. Low concentrations of silybinin at 0, 10, 25, 40 and 50 ng/mL were extracted from spiked plasma and run in triplicate on the HPLC. The LOD of silybinin diastereoisomers were 2 ng/mL for silybinin d1 and 1 ng/mL for silybinin d2. Limits of quantification (LOQ) for silybinin d1 and d2 in human plasma were found to be 5 and 3 ng/mL, respectively. The co-efficient of variation (CV), expressed as [standard deviation / mean] x 100) for LOQ is within the acceptable limits of the FDA method validation guidelines i.e. within 15 %.

Table 4.3 Summary of the recovery and coefficient of variation (n = 3) for the lower limit of quantification (LOQ) of silybinin extracted from human plasma.

Total Silybinin Concentration (ng/mL)	Mean Recovery (%)	Standard Deviation Recovery (%)	Coefficient of variation RSD (%)
0	0.0	0.0	0.0
10	84.22	5.05	5.99
25	47.71	3.98	8.35
40	46.13	5.40	11.70
50	38.84	1.98	5.11

4.1.8 Silybinin stability

The stability of silybinin was assessed to ensure that the silybinin concentration in the human plasma samples would not decrease prior to its analysis. Hence, silybinin diastereoisomers at concentrations of 0, 25, 50 and 2500 ng/mL and internal standard extracted from spiked human plasma were analyzed at time 0, following sample storage at room temperature, -20 °C and -80 °C for 24 h (Table 4.4) and after three freeze-thaw cycles at room temperature, -20°C and -80°C, respectively (Table 4.5). Silybinin d1 and d2 were stable for at least 1 month at room temperature in the dark when kept in methanol (Table 4.4). Both silybinin d1 and d2 were stable when kept at -20 °C and -80 °C after three freeze/thaw cycles (Table 4.5).

Table 4.4 The stability of silybinin at room temperature after 1 month.

		Silybinin diastereoisomer concentration (ng/mL)					
		25		**50**		**2500**	
Diasterioisomer	**Storage**	**Peak Area [a]**	**RSD[b] (%)**	**Peak Area[a]**	**RSD[b] (%)**	**Peak Area[a]**	**RSD[b] (%)**
d1	20°C	0.027	16.290	0.048	12.737	2.551	5.761
d2		0.019	18.423	0.041	10.847	2.746	5.604

[a] *Mean ratio of peak area of silybinin diastereoisomer compared with that of apigenin.*
[b] *Relative standard deviation determined by dividing standard deviation by mean of peak area ratio and expressed in percentage.*

Table 4.5 The stability of silybinin at room temperature, -20°C and -80°C after three freeze/thaw cycles.

		Silybinin diastereoisomer concentration (ng/mL)					
		25		**50**		**2500**	
Diastereoisomer	**Storage**	**Peak Area [a]**	**RSD[b] (%)**	**Peak Area[a]**	**RSD[b] (%)**	**Peak Area[a]**	**RSD[b] (%)**
d1	Immediate (t = 0)	0.056	5.669	0.125	16.224	7.206	1.988
d2		0.075	2.365	0.140	8.592	8.013	1.947
d1	-20°C	0.035	5.612	0.118	6.067	6.960	0.582
d2		0.060	11.191	0.114	8.613	7.637	0.367
d1	-80°C	0.062	2.212	0.111	4.693	6.990	3.380
d2		0.069	7.270	0.116	7.791	7.863	2.391

[a] *Mean ratio of peak area of silybinin diastereoisomer compared with that of apigenin.*
[b] *Relative standard deviation determined by dividing standard deviation by mean of peak area ratio and expressed in percentage.*

4.1.9 Contamination peaks in control plasma

Human plasma contains plasma proteins, clotting factors and lipids. Most large plasma protein and clotting molecules would be extracted during the methanol precipitation process. However, the smaller protein molecules and other lipid contaminants that remained within the plasma matrix after extraction could often be detected by HPLC as contaminating peaks. Furthermore, plasma may also contain compounds from the diet and other sources. These factors explain the appearance of non-silybinin related peaks in control plasma (Figure 4.14). If these contaminating peaks co-elute with silybinin diastereoisomers, they could potentially interfere with the quantitation of silybinin in patient plasma samples.

Figure 4.14 Overlay HPLC-UV chromatograms of control plasma without silybinin or apigenin in BLACK and plasma spiked with silybinin at 100 ng/mL and apigenin in RED.

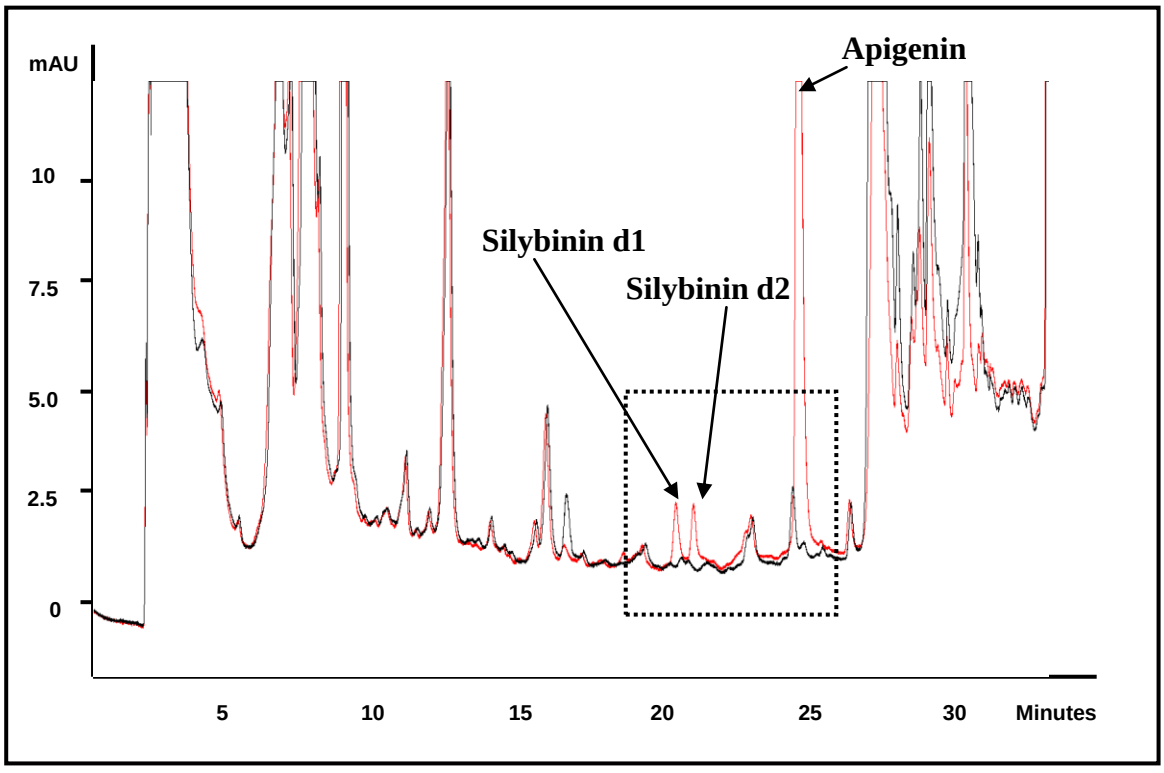
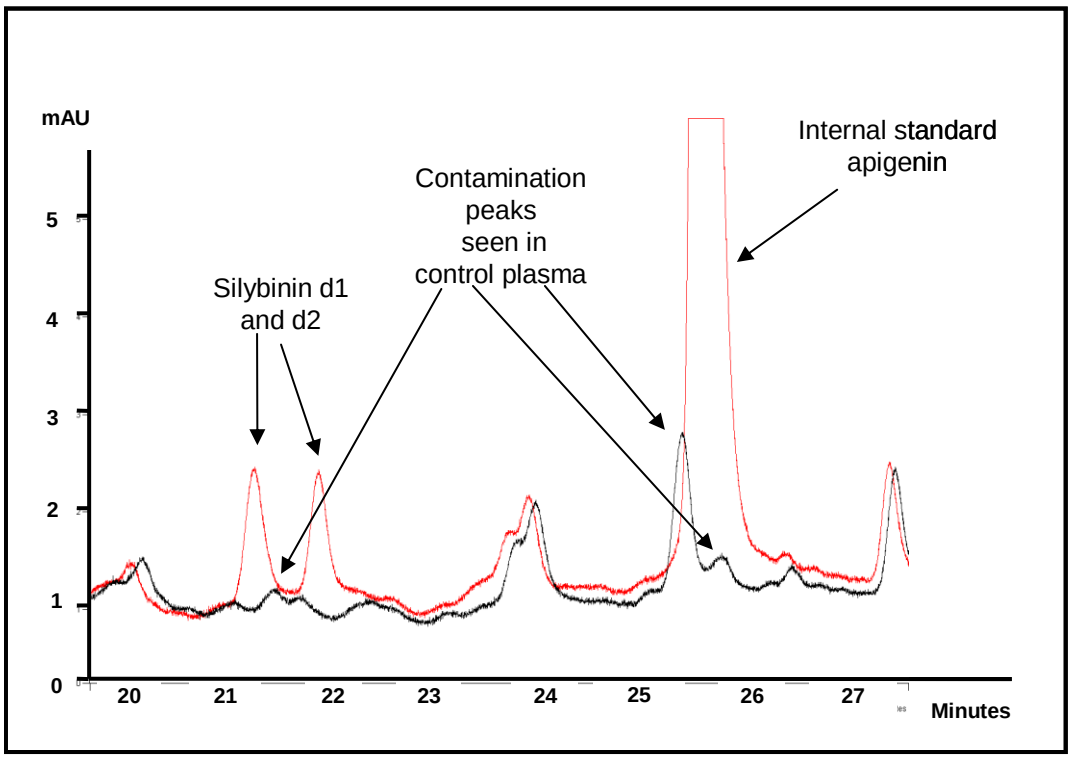


Figure 4.15 Magnification of the highlighted segment of the HPLC-UV chromatograms shown in Figure 4.14. Two small contamination peak retained between 21 and 22 minutes coincides with the separation of the silybinin diastereoisomers. A further two peaks at retention time, 25.5. and 25.9 minutes interferes with apigenin separation. These ultimately affect the quantitation of silybinin in patient plasma samples especially if very low levels of silybinin are being assessed.



To counteract the interference caused by the contamination peaks on the chromatogram, all patient samples and standards were run in triplicates. The mean of the three results were taken for further analysis. Although, this lessened the artifacts caused, it by no means completely solves the problem. However, in reality, these endogenous peaks may only interfere with plasma silybinin levels below 10 ng/mL. All patient samples analyzed were found to contain silybinin levels well above this.

4.2 Discussion

Although several methods have been developed for the analysis of silibinin in human plasma, to the best of our knowledge no simple, fully validated method suitable for the clinical evaluation of silybinin has yet been published. Here we describe a simple method utilizing plasma protein precipitation and robust, precise and reproducible reverse-phase HPLC to separate silybinin diastereoisomers with LOD and LOQ comparable with, or better than, previously published HPLC-UV detection methods (Martinelli *et al.*, 1991; Mascher *et al.*, 1993; Ding *et al.*, 2001). Ricklng *et al*. (Rickling *et al.*, 1995) published a LOD for silybinin diastereoisomers of 0.25 ng/mL, which are superior to those quoted here. However this level of sensitivity was achieved by using electrochemical detection and column switching, whereas the present method only uses simple UV/vis detection at 290 nm, without the need for column switching. Other techniques have required the use of mobile phases such as dioxane and perchlorate for separation (Martinelli *et al.*, 1991; Mascher *et al.*, 1993) that are now deemed hazardous and thus unsuitable for use in the laboratory. A published method looking at the glucuronidation of silybinin using bovine liver microsomes (Gunaratna & Zhang, 2003) was not optimized for human plasma nor was it optimized for the various phase 1 and 2 metabolites likely to be present in humans taking silybinin.

The method described here was validated according to FDA guidelines, rendering it suitable for use in the clinical assessment of silybinin. It was then used to analyse silybinin and its metabolites in patients who had received oral silybinin at dose level 360 mg, 720 mg and 1440 mg, three times daily for 7 days as described in the following clinical pilot study (Chapter 7).

Chapter 5. LEVELS OF SILYBININ AND IDENTIFICATION OF METABOLITES IN RODENT *IN VIVO* AND HEPATIC FRACTIONS *IN VITRO*

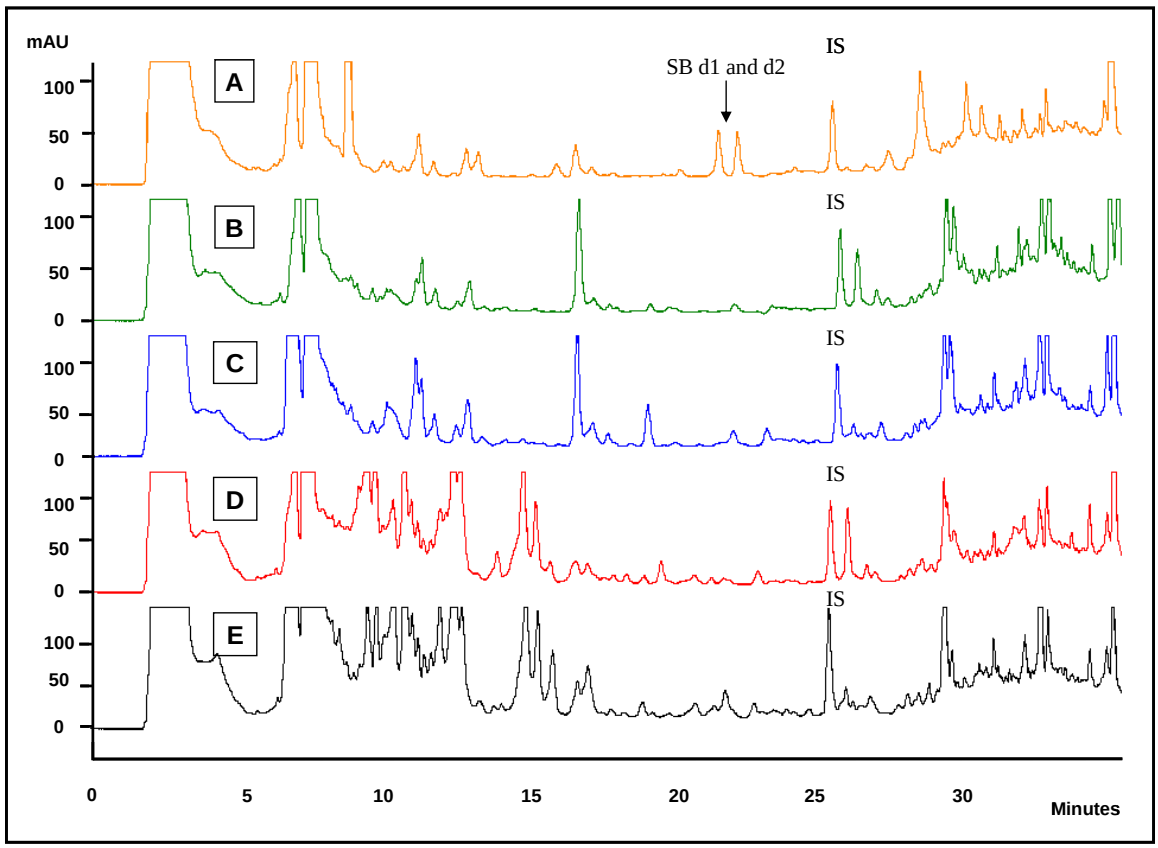
5.1 Introduction

Although silybinin is known to undergo extensive metabolism *in vivo*, the metabolism of silybinin in rodents and humans is still not well understood (Section 1.3.2). In order to better understand silybinin metabolism, several preclinical experiments were conducted in rodents to evaluate silybinin metabolism. In the first experiment, sixteen C57BL/6J mice (8 male and 8 female) were fed either silybinin or silipide (0.2% w/w of silybinin equivalents *per diem*, approximately 300 mg/kg per day) for 7 days from 21 days of life until killing. Plasma and tissue samples from the liver, small bowel mucosa and prostate were obtained and analyzed for free and conjugated silybinin, to give information on the distribution of silybinin and the proportion of agent in metabolite form. In the second set of experiments, silybinin metabolites were synthesized in rodent hepatic and gastrointestinal mucosa fractions *in vitro*, to investigate the chromatographic properties of silybinin metabolites. In the final set of rodent experiments, HPLC analysis was performed on plasma and hepatic fractions from Wistar rats that had received a single oral dose of silybinin at 132 mg/kg or 1200 mg/kg, mimicking the medium and high dose levels used in the clinical trial. Individual silybinin metabolite peaks were then collected, pooled and subjected to mass spectrometric analysis for identification.

5.2 Levels of silybinin and its metabolites in mice

The study design for the C57BL/6J mice experiment is outlined in section 3.4.1.1. Initial analysis using 0.5 mL of mouse plasma afforded the detection of no silybinin peaks in the HPLC-UV chromatogram. Silybinin levels in individual mouse plasma were suspected to be too low for detection. Therefore, murine plasma was pooled from 4 mice per group (male and female), that received either silybinin or silipide intervention. The results are shown in Figure 5.1.

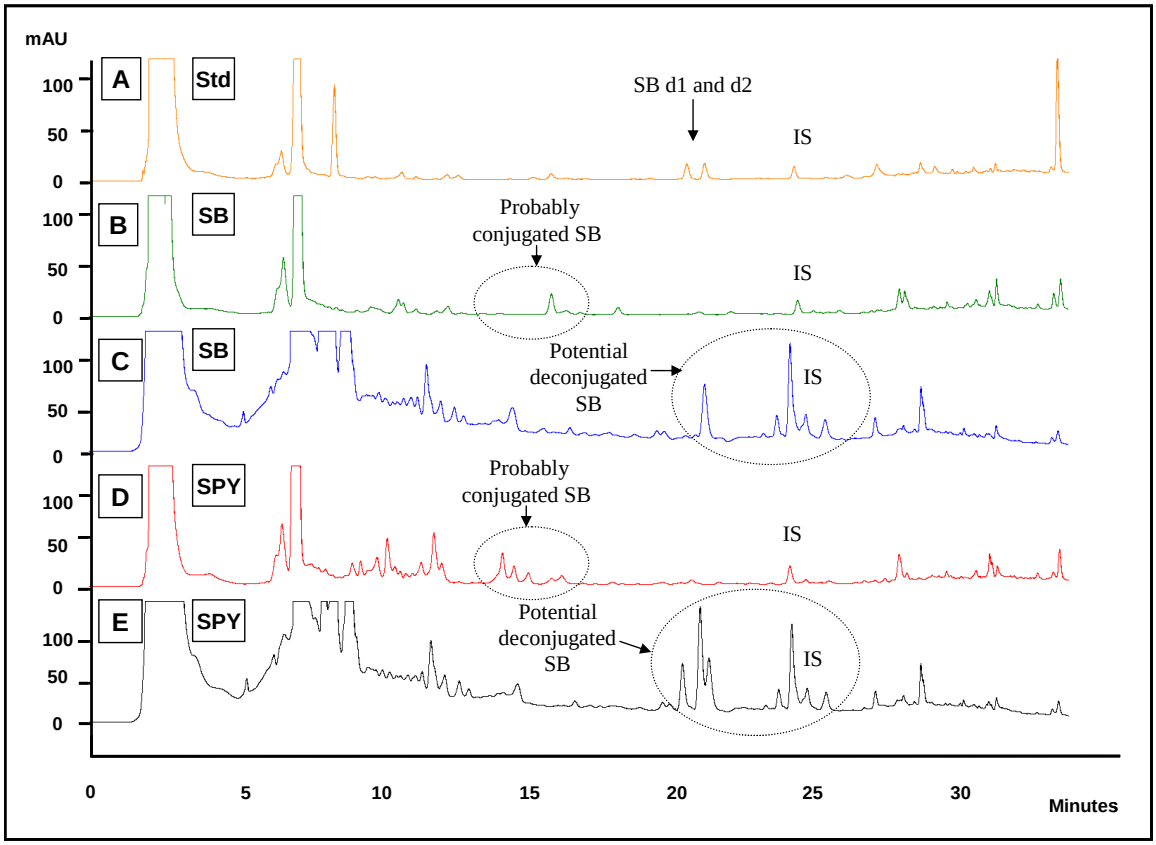
Figure 5.1 HPLC-UV chromatograms of plasma extracts from control mice spiked with silybinin (250 ng/ml) (A), plasma extracts of male (B,D) and female(C,E) mice that had received silybinin (B, C) or silipide (D, E).



SB = silybinin. IS = internal standard.

The HPLC-UV chromatogram for male murine plasma was similar to the female plasma. Two peaks of silybinin d1 and d2 were evident in the HPLC-UV chromatogram of spiked silybinin standard (Figure 5.1-A) but not in murine plasma. This suggests that levels of free silybinin in plasma were too low for detection. The total (unconjugated and conjugated) silybinin levels in the murine plasma were then evaluated after incubating murine plasma with β-glucuronidase and sulfatase deconjugation enzyme. Details of the method are outlined in section 3.4.2.3. Conjugated silybinin metabolites were thus hydrolysed to their unconjugated forms and quantified. This deconjugation step enables the detection and proportion of conjugated silybinin species in plasma. Figures 5.2 show the HPLC-UV chromatograms of the total (free and conjugated) silybinin in the plasma of mice that had consumed silybinin or silipide, respectively.

Figure 5.2 HPLC-UV chromatograms of plasma extracts from control mice spiked with silybinin (500 ng/ml) (A), or of plasma from mice which received either silybinin (B, C) or silipide (D, E); before (B, D) or after (C, E) incubation with *H.promatia.* The dashed circles highlight peaks representing probable conjugated SB and SB metabolites in B and D (before deconjugation) and promising deconjugated SB in C and E (after deconjugation).



Std = standard spiked plasma, SB = silybinin, SPY = silipide and IS = internal standard.

The emergence of the two silybinin peaks on the HPLC-UV chromatograms of mice plasma extracts after deconjugation (i.e. Figures 5.2 C and E), confirmed the presence of silybinin metabolites.

Table 5.1 Silybinin levels in pooled plasma of male and female C57BL/6J mice that received 0.2% per diem silybinin (SB) or silipide (SPY) for 7 days prior to exsanguination.

		SB d1 (µM)	SB d2 (µM)	SB d1 + d2 (µM)
Plasma from male mice that received SB (n = 4)	**Before deconjugation**	0.015*	0.103*	0.119*
	After deconjugation	0.524*	0.274*	0.798*
Plasma from female mice that received SB (n = 4)	**Before deconjugation**	0.048*	0.204*	0.251*
	After deconjugation	0.211*	0.227*	0.438*
Plasma from male mice given SPY (n = 4)	**Before deconjugation**	0.043*	0.081*	0.124*
	After deconjugation	7.742*	14.114*	21.856*
Plasma from female mice given SPY (n = 4)	**Before deconjugation**	0.096*	0.262*	0.358*
	After deconjugation	27.329*	58.357*	85.686*

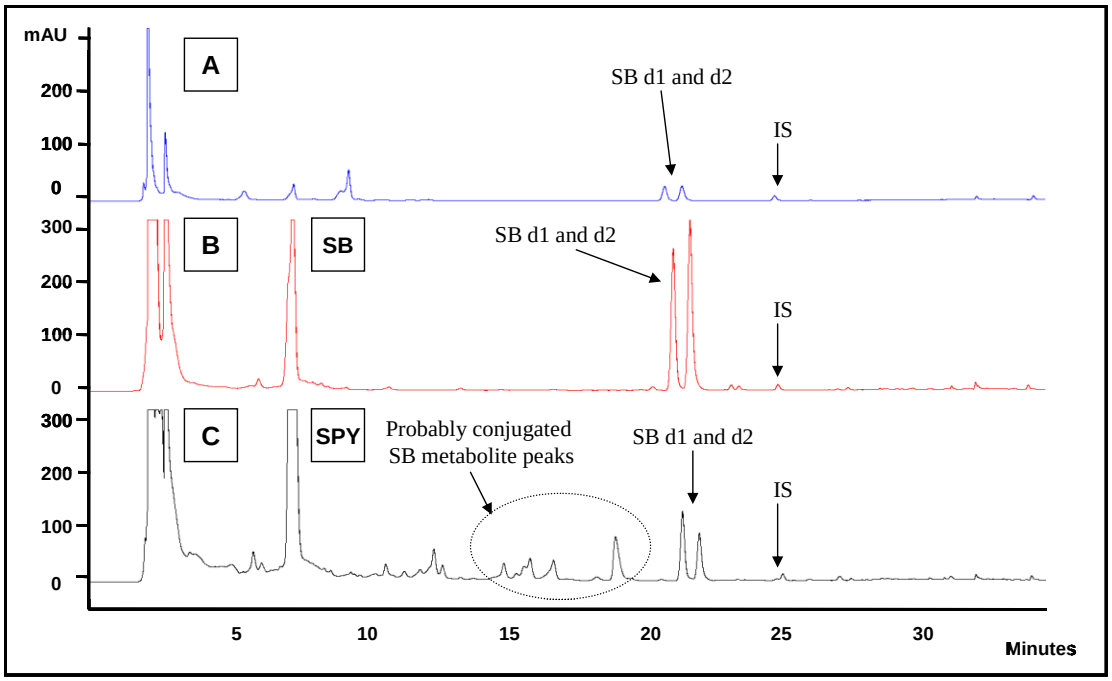
***The levels of silybinin were recorded as single figures since they were obtained from a single hplc analysis of pooled murine plasma*

The levels of silybinin (d1 + d2) found in male and female murine plasma were similar ,i.e., 0.12 : 0.12 (µM) in males and 0.25 : 0.36 (µM) in females after ingestion of silybinin and silipide. There was an apparent increase in the level of total silybinin, after deconjugation (i.e. conjugated and free silybinin), when compared to silybinin levels before deconjugation (i.e. free silybinin only). This suggests that a large proportion of silybinin absorbed from the gastrointerstinal tract in mice circulates as conjugated silybinin. The murine plasma silybinin levels were higher in the mice that

had consumed silipide than those that consumed silybinin, reflecting the superior bioavailability of silipide to pure silybinin (Table 5.1).

Further analysis were carried out on tissue homogenate from small bowel mucosa, liver and prostate. The results are shown in Figure 5.3 and Figure 5.4. The method used for the analysis of rodent small bowel mucosa was identical to that used for the analysis of large bowel mucosa in human (Section 3.3.1.2.2).

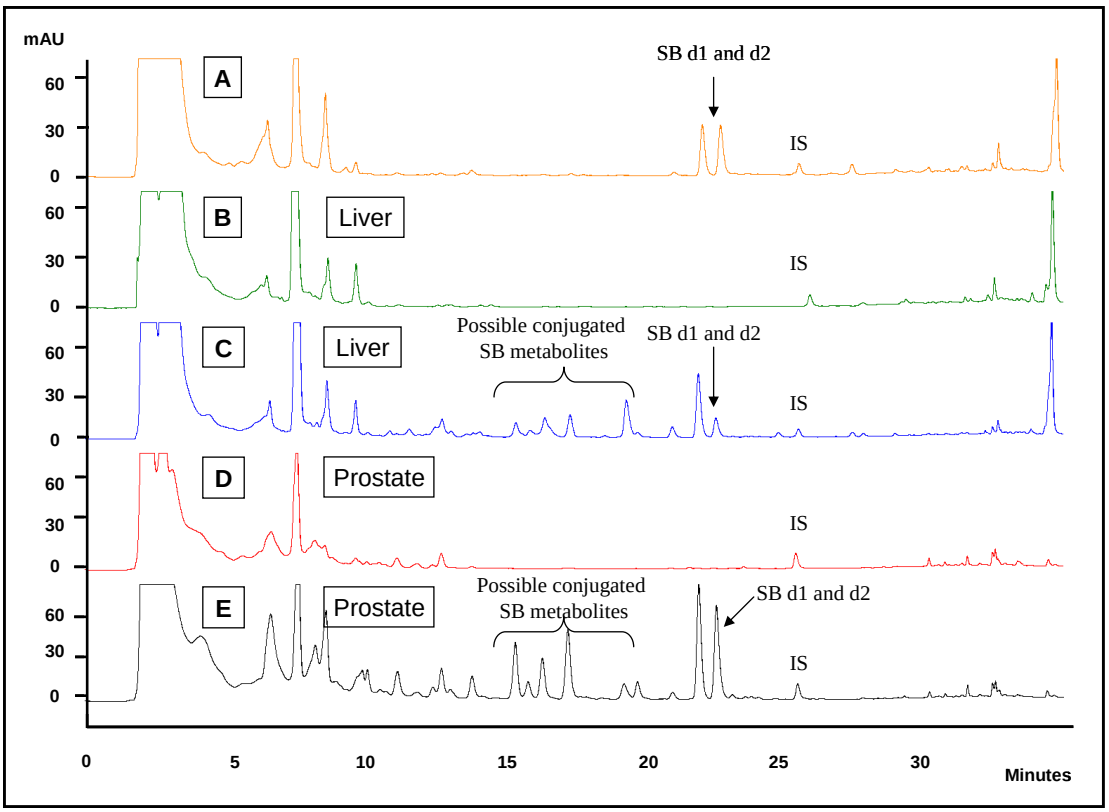
Figure 5.3 HPLC-UV chromatograms of extracts of small bowel mucosa from control mice spiked with silybinin (5 µg/ml) (A), or of extracts of small bowel mucosa obtained from a mouse fed with silybinin (B) and mouse fed with silipide (C). The dashed circle shows several silybinin metabolite peaks visible in the chromatogram of small bowel mucosa extracts from mouse fed with silipide.



Std = Standard spiked plasma, SB = Silybinin, SPY = silipide and IS = internal standard.

In contrast to the plasma analysis, levels of free silybinin in the gastrointestinal tract of mice fed with silybinin were higher than those in mice on silipide (Figure 5.3 and Table 5.2). Several peaks representing possible conjugated silybinin metabolites were observed in the gut of both these group of mice, and more peaks were seen in those fed with silipide (Figure 5.3). Figure 5.4 shows the analysis of murine liver and prostate tissues.

Figure 5.4 HPLC-UV chromatograms of extracts of liver homogenate from control mice spiked with silybinin (2500 ng/ml) (A), or of extracts of liver homogenate from mouse that had consumed silybinin (B) or silipide (C), or of extracts of prostate homogenate from mouse that had consumed silybinin (D) or silipide (E).



IS = internal standard.

Those mice that had consumed silipide afforded larger silybinin and metabolite peaks, both in the liver and prostate extracts, than in mice that had taken pure silybinin (Figure 5.4**)**.

HPLC analysis of the small bowel mucosa and liver was performed on all mice. Levels of free silybinin were calculated and data shown in Table 5.2. Extracts of prostate tissue from the eight male mice were pooled (2 groups of 4 mice) due to low amount of analyte from each mouse. Results are shown in Table 5.2.

Table 5.2 Levels of silybinin in small bowel mucosa, liver and prostate (males only) of pooled male and female C57BL/6J mice that were given 7 days of 0.2% per diem silybinin or silipide.

		SB d1 nmol/g	**SB d2 nmol/g**	**SB d1 + d2 nmol/g**
Mice on 0.2% silybinin per diem for 7 days (n = 8)	**Small bowel mucosa**	882.06 ± 148.68	985.42 ± 165.79	1867.48 ± 314.45
	Liver Homogenate	0.62 ± 0.15	0.39 ± 0.04	1.01 ± 0.18
	Prostate (male only) (n = 4)	0.85*	0.89*	1.74*
Mice on 0.2% silipide per diem for 7 days (n = 8)	**Small bowel mucosa**	370.91 ± 99.23	285.99 ± 90.69	656.90 ± 189.88
	Liver Homogenate	28.33 ± 4.60	14.71 ± 3.00	43.04 ± 7.16
	Prostate (male only) (n = 4)	86.61*	75.43*	162.04*

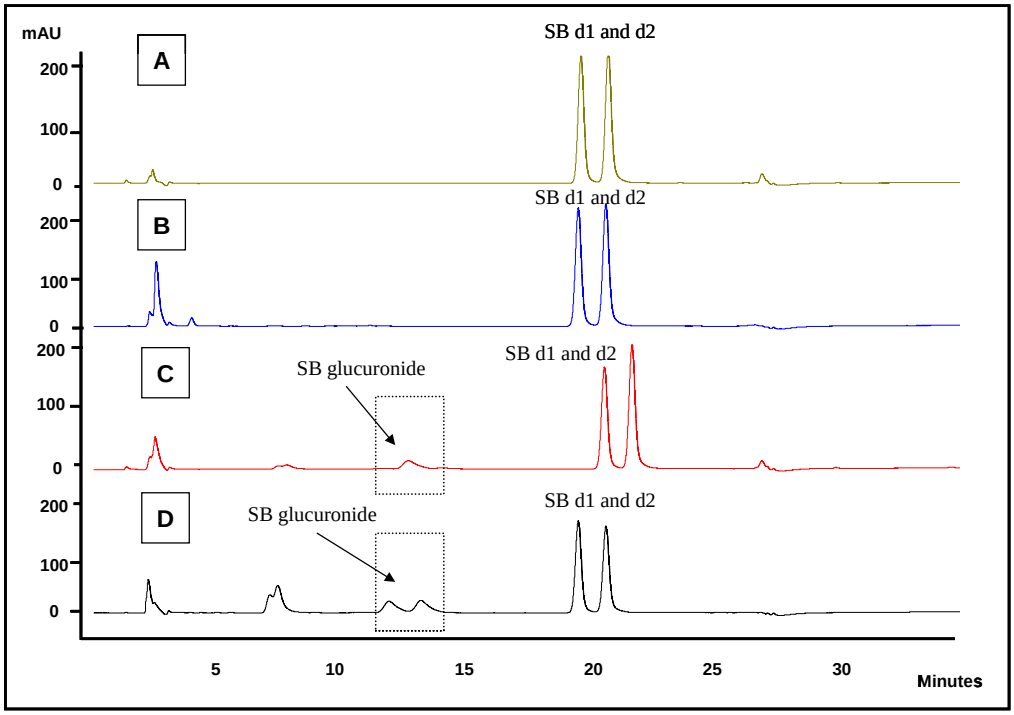
** Single sample analysis of pooled prostate extract.*
All other results shown are given as mean ± standard error of mean.

5.3 Levels of silybinin and its metabolites in rodent hepatic fractions *in vitro* and *in vivo*

5.3.1 Chromatographic characteristics of silybinin and its metabolites

The rationale for performing the *in vitro* metabolism experiments was to gain insight of the chromatographic characteristics of silybinin glucuronide. Silybinin glucuronide was synthesized using microsomes from liver and gastrointestinal tract of either rat or mouse (Section 3.4.2.2). Silybinin was incubated with microsomes in the presence of suitable cofactors. The results are shown in Figure 5.5. All samples afforded double peaks of silybinin d1 and d2. The incubation of silybinin with rat liver microsomes showed a small peak at a retention time of 13 min, probably silybinin glucuronide (Figure 5.5-C). When rat gut microsomes were used instead of rat liver microsomes, the silybinin glucuronide peak was not observed (Figure 5.5.-B). When mouse liver microsomes were analysed, there were two metabolite peaks had emerged at retention times 13 and 14 minutes. It is possible that mice liver microsomes generate two silybinin glucuronide species while rat liver microsomes only one. The other possibility is that these double peaks are the diastereoisomers of silybinin glucuronide. If the double peaks seen in Figure 5.5-D are the latter, there should have been two silybinin glucuronide peaks in rat liver microsomal incubations. Since this was not the case, it is possible that rat liver microsomes metabolise silybinin in a stereo-specific manner.

Figure 5.5 HPLC-UV chromatograms of extracts of silybinin incubated with rat microsomes without UDPGA (Control) (A), or of extracts of silybinin incubated with rat gut (B), rat liver (C) and mouse liver (D) microsomes with cofactor UDPGA. The interupted squares and arrows highlights the one and two silybinin glucuronide peaks seen in the incubates of rat liver and mice liver extracts respectively (as seen in HPLC chromatogram C and D).



5.3.2 Silybinin metabolites in rats *in vivo*

In order to indentify the different silybinin metabolites, experiments were carried out in rats *in vivo*. HPLC-UV analysis was carried out on plasma from Wistar rats that were given a single oral gavage of silybinin at 132 mg/kg or 1200 mg/kg. These doses are similar to the medium and high doses used in the clinical trial (Section 3.3). For study design see Section 3.4.1.2 and 3.4.2.3.

The HPLC-UV chromatograms obtained from the extracts of rat plasma show peaks in both the control (Figure 5.6-A) and treated groups (Figure 5.6-B and C). In the treated rat plasma, two prominent peaks at retention times 21, 22 min co-eluted with silybinin d1 and d2. Levels of free silybinin, as determined from their peak area, were higher in the higher dose group, thus suggesting levels of free silybinin in the circulating plasma was dependant on dose. Furthermore, additional 'double' peaks possibly representing conjugated silybinin were observed.

Figure 5.6 HPLC-UV chromatograms of extracts of plasma from control rat (A), or of plasma from Wistars treated with 132 mg/kg (B) or 1200 mg/kg of silybinin (C) (Magnified views shown in Figure 5.7). Arrows indicate the emergence of two silybinin peaks (SB d1 and d2) seen in the plasma of rats treated with silybinin.

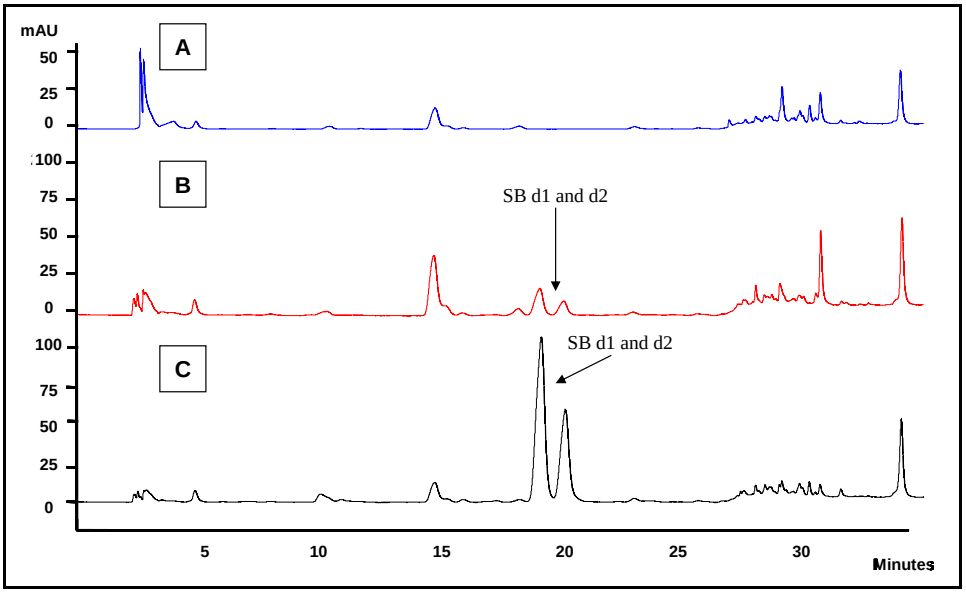
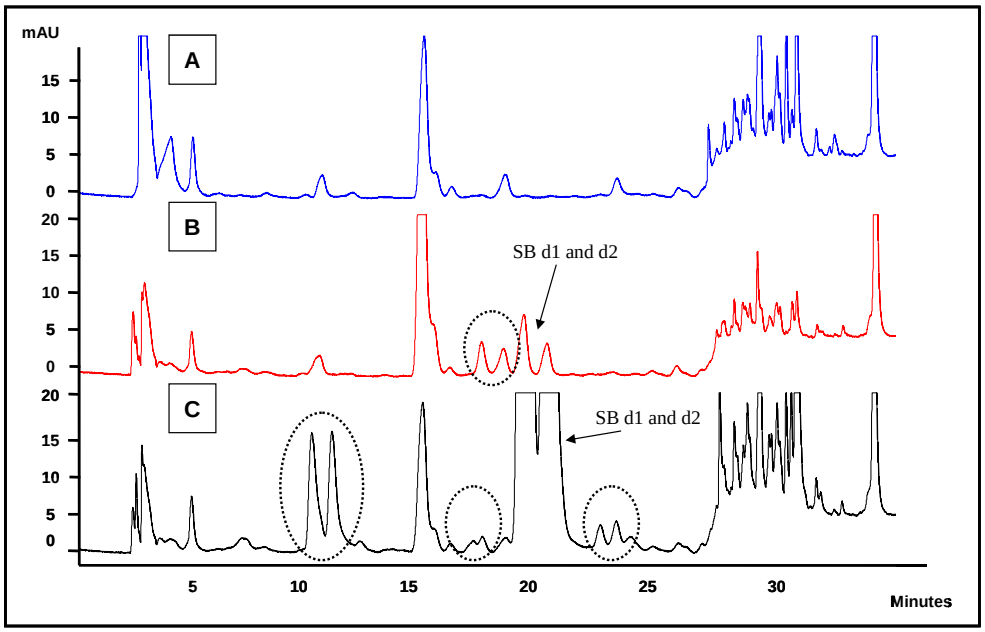


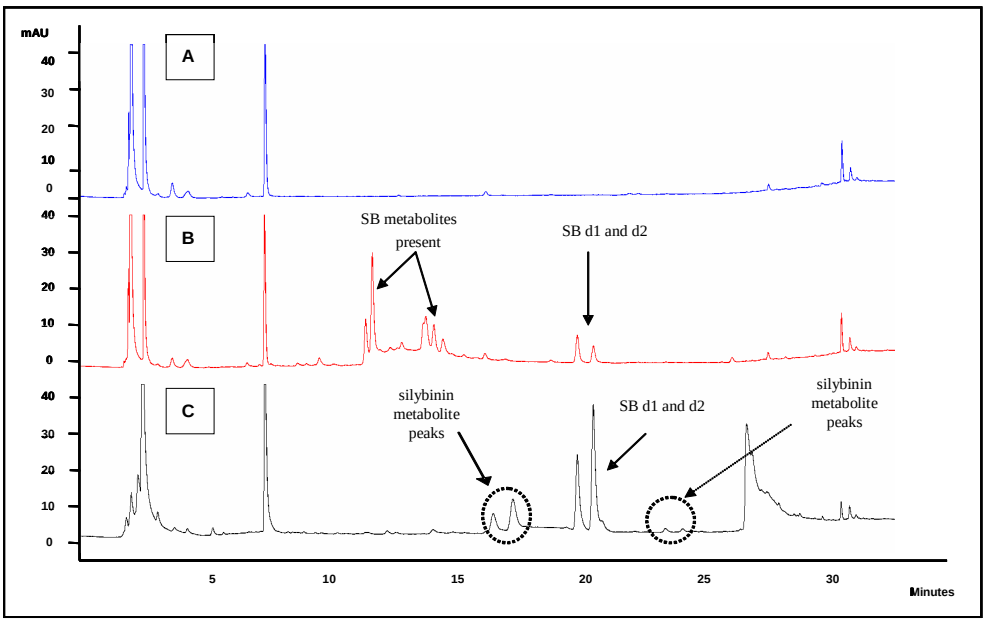
Figure 5.7 Above chromatogram at higher attenuation. The interrupted circles show the possible silybinin metabolites in the plasma of rats treated with silybinin.



In order to confirm that these peaks were indeed silybinin conjugates and to allow an appropriate estimation of the proportion of silybinin in conjugated form, plasma from rats which had received 1200 mg/kg of silybinin were incubated with sulphate- and glucuronide deconjugating enzymes from *Helix promatia* (Section 3.4.2.3 for method of analysis). A control sample without β– glucuronidase enzyme was included for comparison. There was a gradual decline in the levels of silybinin over the 1 h incubation, (results not shown) which suggests that the silybinin is unstable and that levels found in tissues and plasma may be an underestimation of original levels.

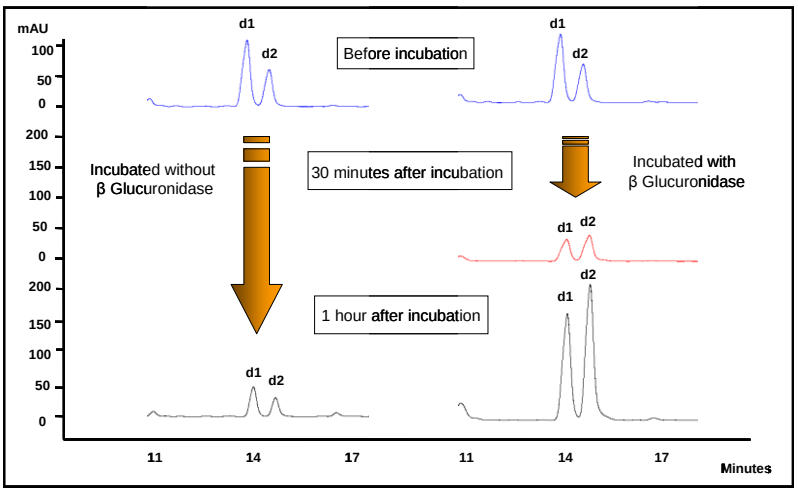
β-Glucuronidase enzyme was added to rat plasma and extracts were analysed at 30 and 60 min after incubation at 37°C. Two peaks of silybinin d1 and d2 were evident at all three time points. At 30 min after incubation, silybinin d1 and d2 peaks had both increased over time. At 1 h, both silybinin d1 and d2 had increased significantly. Figure 5.8 show that metabolite peaks disappear and silybinin d1 and d2 peaks increase. In addition, there are peaks that appear, probably representing primary silybinin metabolites (methylated species) consistent with differential polarity on the HPLC chromatogram.

Figure 5.8 HPLC-UV chromatograms of extracts of plasma from control rat without silybinin (A), or of plasma from rat that received 1200 mg/kg of silybinin (B) or of plasma from rat that received 1200 mg/kg of silybinin and incubated for 1 h with β-glucuronidase enzyme (C). The circles show the appearance of new peaks probably representing primary silybinin metabolites.



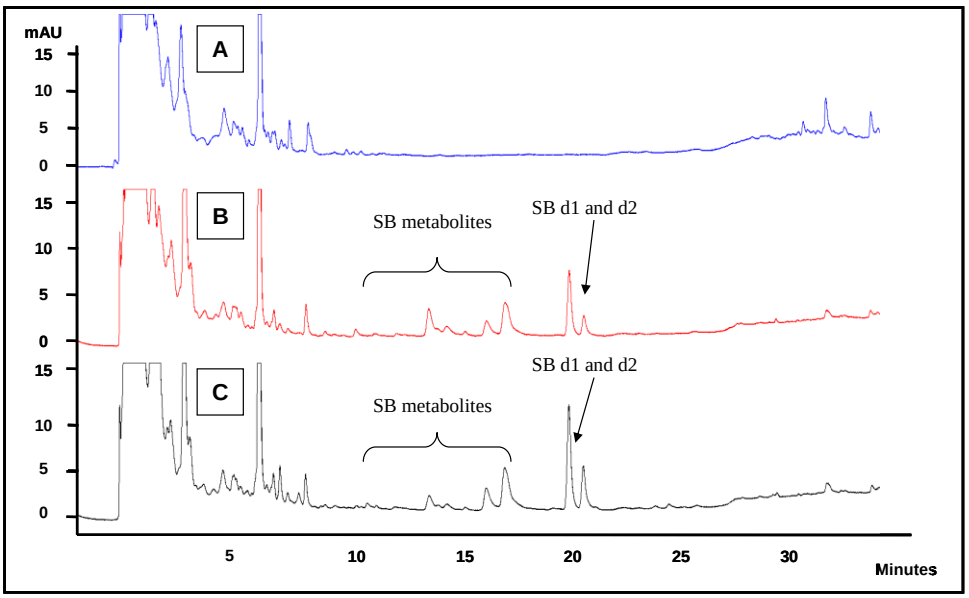
It was also observed that this increase in the silybinin d1 and d2 peaks was not at a uniform rate. After 1 h of incubation with β glucuronidase enzyme, the analysis of the rat plasma showed a greater peak area increase in silybinin d2 compared to silybinin d1, thus resulting in a reverse of the d1:d2 ratio. This increase is illustrated in Figure 5.9.

Figure 5.9 Magnified portions of silybinin d1 and d2 peaks as displayed on the plasma chromatogram of rats given 1200 mg/kg of silybinin before and after incubation with β-glucuronidase. The magnified chromatogram portions were taken between 11 – 17 min.



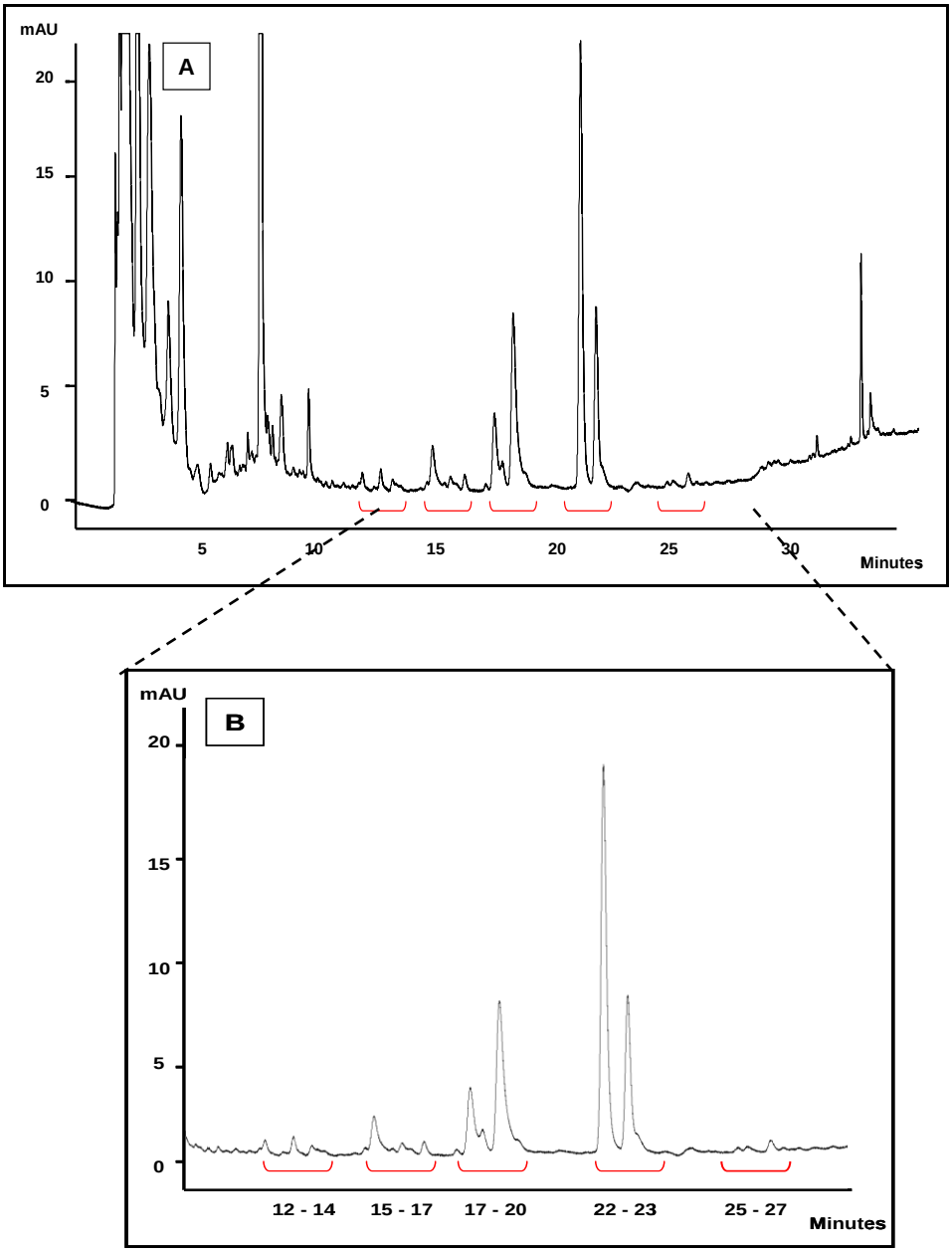
Similar to rat plasma, when rat liver homogenate was incubated with β-glucuronidase enzyme, there was an increase in silybinin d1 and d2 peak area. In this case, the silybinin metabolite peaks were still present, albeit smaller, indicating abundant levels of conjugated silybinin (Figure 5.10).

Figure 5.10 HPLC-UV chromatograms of extracts of rat liver homogenate from control rat (A), or of liver homogenate from rat that received 1200 mg/kg of silybinin (B) or of liver homogenate from rat that received 1200 mg/kg of silybinin and was incubated for 1 h with β-glucuronidase enzyme (C).



In order to characterize these silybinin metabolites, fraction collection of the individual metabolite peaks was carried out during HPLC separation (Section 3.4.2.2.4 for fractionation method). Rat liver homogenate rather than rat plasma was used for metabolite peak fractionation because of its higher content of metabolites. The fractions collected corresponded to the chromatogram peaks at 12 – 14, 15 – 17, 17 – 20, 22 – 23 and 25 – 27 min. Figure 5.11 shows silybinin metabolite fractions obtained according to their retention times. The fractionated samples of silybinin metabolites were subjected to tandem mass spectrometry analysis.

Figure 5.11 HPLC-UV chromatogram of extracts of rat liver homogenate showing silybinin d1 and d2 peaks and its putative metabolite peaks (A). A magnification of the above from 10 – 30 min is shown in (B). The RED brackets indicate the individual fraction collections and their retention times.

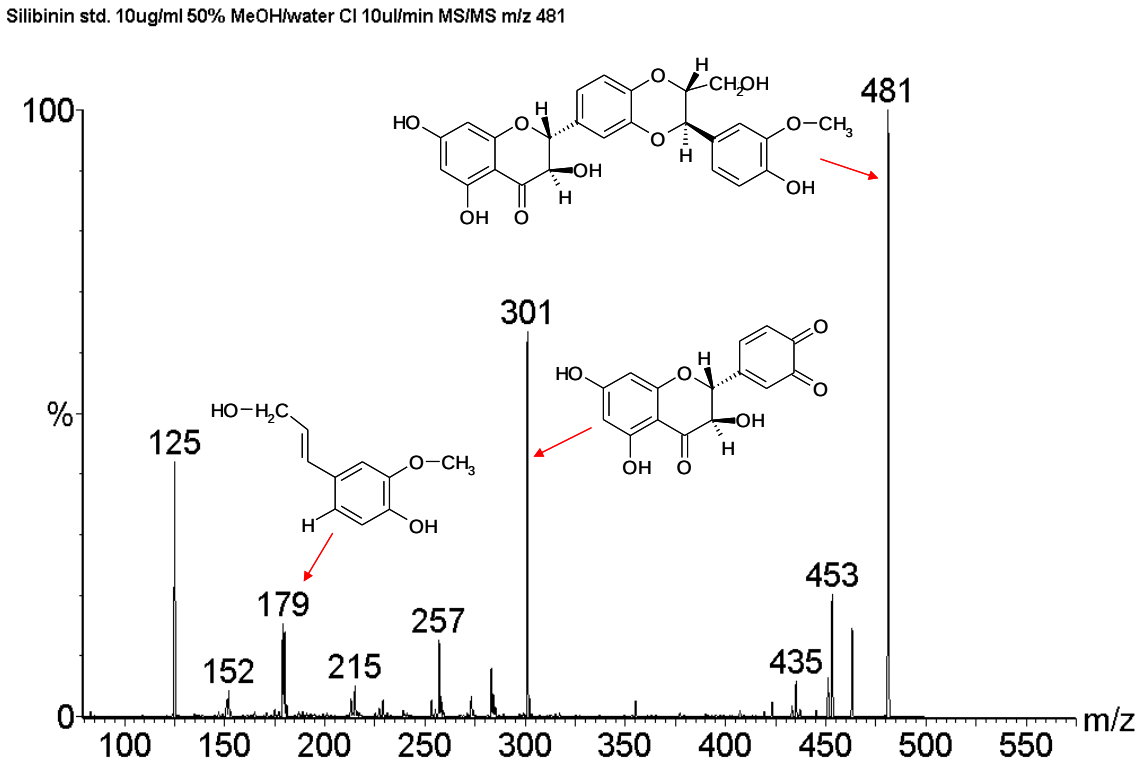


5.3.3 Identification of metabolites from rodent biomatrices by mass spectrometry

The silybinin metabolites obtained from sections 5.3.1 and 5.3.2 were characterized using tandem mass spectrometry using negative electrospray ionisation. Details of the mass spectrometric analysis have been described in section 3.4.3.

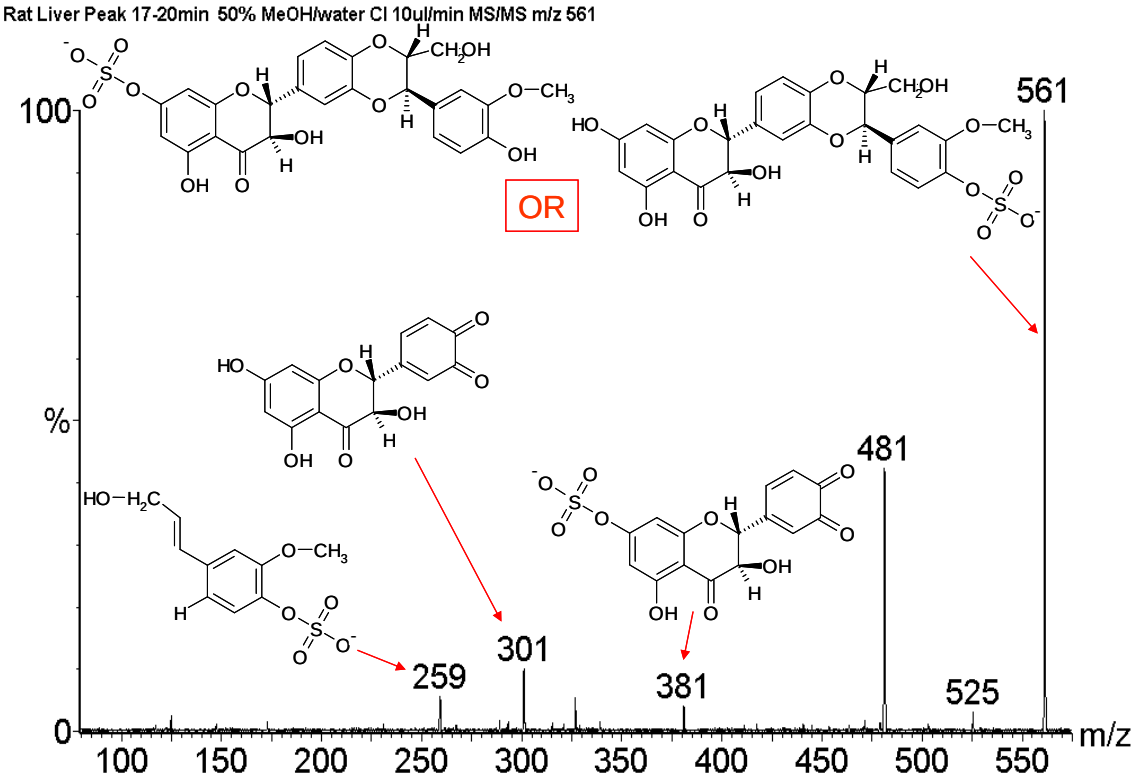
A standard solution of 10 µg/mL silybinin was used to tune the instrument and a typical product ion spectrum observed is shown in Figure 5.12 for the molecular ion at *m/z* 481 $[M-H]^-$ for silybinin. HPLC fractions collected from rat liver homogenate were then analysed for the presence of metabolites.

Figure 5.12 Product ion spectrum of a standard silybinin solution showing the molecular ion at *m/z* 481 $[M-H]^-$ and fragments at *m/z* 179 and 301.



Following mass spectrometric analysis of the HPLC fractions only the sulphate conjugate metabolite could be identified. The product ion spectrum for the HPLC fraction collected between 17 – 20 min shown in Figure 5.13 afforded fragments at *m/z* 259, 301 and 381 from the molecular ion of silybinin monosulphate at *m/z* 561 $[M-H]^-$.

Figure 5.13 Product ion spectrum for the HPLC fraction collected between 17 – 20 min for rat liver homogenate showing the molecular ion at *m/z* 561 $[M-H]^-$ for silybinin monosulphate and fragments at *m/z* 259, 301 and 381.



5.4 Discussion

In the first animal experiment utilising C57BL/6J mice, it was concluded that there were no gender differences in the absorption and metabolism of silybinin or silipide in mice. The absorption of silybinin from the gastrointestinal tract and the bioavailability of silybinin in murine plasma was superior after oral silipide administration when compared with silybinin. This finding is consistent with previous pharmacokinetic studies conducted in rats by Morazzoni (Morazzoni *et al.*, 1992) and Yanyu (Yanyu *et al.*, 2006). It is also likely that a large proportion (up to 94%) of total silybinin circulates in its metabolite form, as we have found in mice and others have found in rats (Morazzoni *et al.*, 1992). This notion is supported by the large increase in the silybinin d1 and d2 peaks sizes and the disappearance of the conjugated silybinin peaks on incubation with β-glucuronidase deconjugating enzymes.

Silipide, when given orally, was better absorbed than non-complexed silybinin and resulted in measurable levels of silybinin in murine plasma and tissues. In comparison to equivalent doses of non-complexed silybinin, there was less silybinin in the bowel mucosa of the mice that consumed silipide. One possible explanation could be that in silipide, a larger amount of silybinin was absorbed and transported across the gastrointestinal tract to yield conjugates, therefore leaving behind a reduced amount of free silybinin in the small bowel mucosa as compared to non-complexed silybinin. Hence, the silybinin d1 and d2 peaks were smaller but silybinin metabolite peaks more abundant, after oral silipide (Figure 5.3).

In the C57BL6J mice, it was shown that not only was silybinin detectable in the plasma, liver and prostate extracts of mice that were fed silipide, these levels were higher than levels of silybinin which have shown reduction colorectal and prostate tumour cell growth *in vitro* (Kohno *et al.*, 2002; Singh *et al.*, 2002a).

HPLC-UV analysis of murine liver and prostate showed multiple peaks which may relate to silybinin conjugated metabolites. It could be anticipated that *in vivo*, most of the silybinin is converted to glucuronides and to a smaller proportion, sulphates as suggested by Kren, Han and Wen (Han *et al.*, 2004; Wen *et al.*, 2008; Kren *et al.*, 2000)

The biosynthesis of silybinin glucuronide from rat liver microsomes afforded one metabolite peak at 13 min, whilst silybinin glucuronidation with mouse liver microsomes produced two peaks at 13 and 14 min. Relating this finding to Han's suggestion of stereoselectivity in silybinin metabolism (Han *et al.*, 2004), it is possible that rat liver preferentially metabolises silybinin B causing a reduction in silybinin d1 peak, furnishing one silybinin glucuronide peak at position C-20. On the other hand, mouse liver microsomes might preferentially metabolise silybinin A, and according to Han, this would produce equal amount of two silybinin glucuronides, metabolised at C-7 and C-20 positions (Han *et al.*, 2004).

In addition, it was found that the glucuronidation of silybinin occured at a higher rate in liver microsomes than in gastrointestinal microsomes in rat (Figure 5.5). This could either reflect a higher level of glucuronidation enzyme (UDP-glucuronyltrasferase) or its co-factor (UDP-glucuronic acid) that is present in liver microsomes in comparison to gut microsomes. This finding would be consistent with the liver being the main drug detoxifying organ both in rodents and humans alike (Gibson & Skett, 1986).

In the experiment employing rats, free and conjugated silybinin were detectable in the rat plasma after a single oral dose of silybinin (Figure 5.6). The peak abundance of the conjugated and free silybinin were proportional to silybinin dose. Silybinin is conjugated to generate several metabolites, hence multiple peaks were observed on the HPLC-UV chromatogram at retention times 12 - 14, 17 – 20 and 22 – 23 min. It is conceivable that these peaks may represent silybinin metabolites as they preserved the "double peak appearance" of the parent isomer mixture on the HPLC-UV chromatogram.

Following deconjugation with β-glucuronidase, a majority of the silybinin conjugates were reverted back to silybinin, thus the disappearance of the silybinin metabolite peaks coupled with an increase in the authentic silybinin peaks. A small proportion of silybinin conjugates, were converted to primary silybinin metabolites, represented by the double peaks at retention times 16 – 17 and 24 – 25 min (Figure 5.8). It is possible that these peaks are demethylated and/or hydroxylated silybinin species, as suggested by Gunaratna *et al.* and Jancova *et al.* (Jancova *et al.*, 2007; Gunaratna & Zhang, 2003). However, the identity of these peaks have yet to be established.

When silybinin doses similar to those employed in the clinical trial were given to rats, free and conjugated silybinin could be isolated from the liver. The liver is the major drug detoxifying organ and most exogenous drugs within the plasma circulation are metabolised for excretion in the liver. It was therefore expected that the levels of both conjugated and unconjugated silybinin are higher in the liver than plasma. Wu *et. al.* reported similar findings in their analysis of silybinin in rat plasma and bile for hepatobiliary excretion (Wu *et al.*, 2007).

Finally, mass spectrometric analysis of silybinin conjugates confirmed the presence of silybinin mono-sulphate which was the only silybinin metabolite identifiable with the method employed. Other metabolites are likely to be present within the biomatrices, as has been shown by other studies (Gunaratna & Zhang, 2003). However, their levels may have been much lower compared to the detectable mono-sulphate, and hence, could not be detected with this method. Furthermore, there was limited supply of rat plasma for this analysis, and despite sample pooling, the levels of other conjugates could not be establised.

Chapter 6. PATIENT DEMOGRAPHICS, THE SAFETY OF SILIPIDE AND THE PHARMACOKINETICS OF SILYBININ IN PATIENTS

6.1 Introduction

Patients were recruited to test the safety of silipide and its pharmacokinetic and pharmacodynamic effects in humans. One of the primary objectives of this clinical trial was to re-examine the safety of oral silipide. The first part of this chapter describes the demographics of the recruited patients. The safety of the drug and adverse effects were recorded and are presented. An important factor in the measurement of silybinin levels is the timing of patient sample collection. It has been documented and is shown.

The second part of this chapter describes the laboratory experiments carried out to investigate if silybinin can be recovered from blood in human patients who have ingested silipide and the potential relationship between dose of silipide and levels of silybinin in the gastrointestinal tract and/or liver and/or blood. The levels of silybinin recovered from liver tissue, after pharmacological doses of silipide, were defined. In addition, results are presented from experiments aimed at finding out which silybinin metabolites are found in the human circulation.

6.2 Patient cohorts

A total of twelve patients with only primary colorectal cancer and a further twelve patients with primary colorectal cancer and liver metastases were recruited into the study. All patients received silybinin formulated as silipide capsules at 360, 720 or 1440 mg daily for a week prior to surgery. Of the twelve patients recruited into the colorectal arm of the study, eleven were male and one female. In the hepatic cohort, five patients were male and seven female. The mean age of the colorectal patients was 66 years, with a range of 55 - 78 years and their mean weight was 73 kg. The mean body mass index (BMI) was 27. The mean age of the hepatic patients was marginally younger at 62 years of age, but with similar weight. The mean weight was 76 kg and BMI was 26. Details of the patients' physical parameters are outlined in Table 6.1.

For the colorectal patients recruited, all were found to have a normal range of routine parameters before intervention but a reduced mean haemoglobin of 10 g/dL. The laboratory reference range was 11.0 -16.0 g/dL.

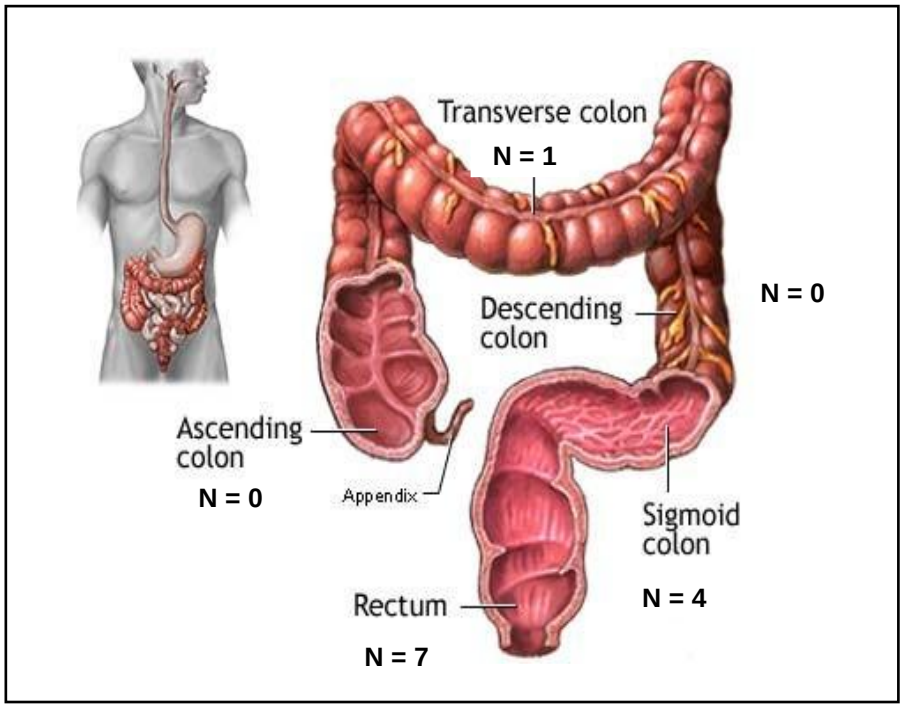
Table 6.1 Mean physical parameters and blood results for the preoperative colorectal and hepatic patients.

			Colorectal Patients	**Hepatic Patients**
Physical	Gender	Male	**11**	**5**
		Female	**1**	**7**
	Age (years)	Range	**55 - 78**	**49 -78**
		Mean	**66**	**62**
	Weights (Kg)	Range	**50 - 120**	**55 - 101**
		Mean	**73**	**76**
	Body Mass Index (Kg/m^2)	Range	**21 - 40**	**18 - 37**
		Mean	**27**	**26**
Full Blood Count	Haemoglobin (11 – 18 g/dL)*	Range	**8 – 12**	**11 - 16**
		Mean	**10**	**13**
	White cell count ($4 – 11 \times 10^9/L$) *	Range	**6 - 12**	**2 - 12**
		Mean	**7**	**7**
	Platelets ($150 – 400 \times 10^9/L$) *	Range	**170 - 380**	**160 - 260**
		Mean	**306**	**234**
Urea and Electrolytes	Sodium (133 – 145 mmol/L) *	Range	**135 - 140**	**134 - 142**
		Mean	**137**	**138**
	Potassium (3.5 – 5.3 mmol/L) *	Range	**3 – 5**	**3 - 5**
		Mean	**4**	**4**
	Urea (2.5 – 6.6 mmol/L) *	Range	**2 - 7**	**2 - 9**
		Mean	**5**	**6**
	Creatinine (70 – 120 µmol/L) *	Range	**69 - 110**	**61 - 169**
		Mean	**87**	**82**
Liver Function Tests	Bilirubin (< 21 µmol/L) *	Range	**6 - 14**	**5 - 38**
		Mean	**10**	**12**
	Alkaline Phosphatase (< 100 iU/L) *	Range	**44 - 92**	**51 – 208**
		Mean	**63**	**120**
	Alanine Transferase (< 40 iU/L) *	Range	**7 - 49**	**16 - 143**
		Mean	**24**	**36**
	Albumin (34 – 48 g/L) *	Range	**37 - 52**	**32 - 46**
		Mean	**44**	**41**

* Normal laboratory reference ranges.
RED values indicate abnormal results.

The commonest site for the tumours in the colorectal patient group was at the rectum, seen in 7 patients, followed by sigmoid colon in 4 patients. The least common location was at the transverse colon, found in one patient (Figure 6.1). Eleven patients had one tumour at their primary resection colonic segment. One patient had another synchronous large tubular villous polyp found immediately proximal to the malignant colon, and both of these were resected as a continuous colonic segment. Five patients had Dukes C tumours, another five had Dukes B tumours and two were found to have Dukes A tumours.

Figure 6.1 A diagram of the colorectum and the distribution of the carcinoma found in the twelve colorectal patients recruited into the study. (Picture taken from Macmillan.org.uk)



The Dukes grading of colorectal tumours has been used to classify the depth of tumour invasion into the bowel and whether distant metastases are present. This classification is frequently used clinically to assess the prognosis for the individual patient. The Dukes classification of colonic tumours is shown in Figure 6.2.

Figure 6.2 The TNM and the Dukes classification for colorectal cancer staging. Modified from Sanofi-Aventis.com.

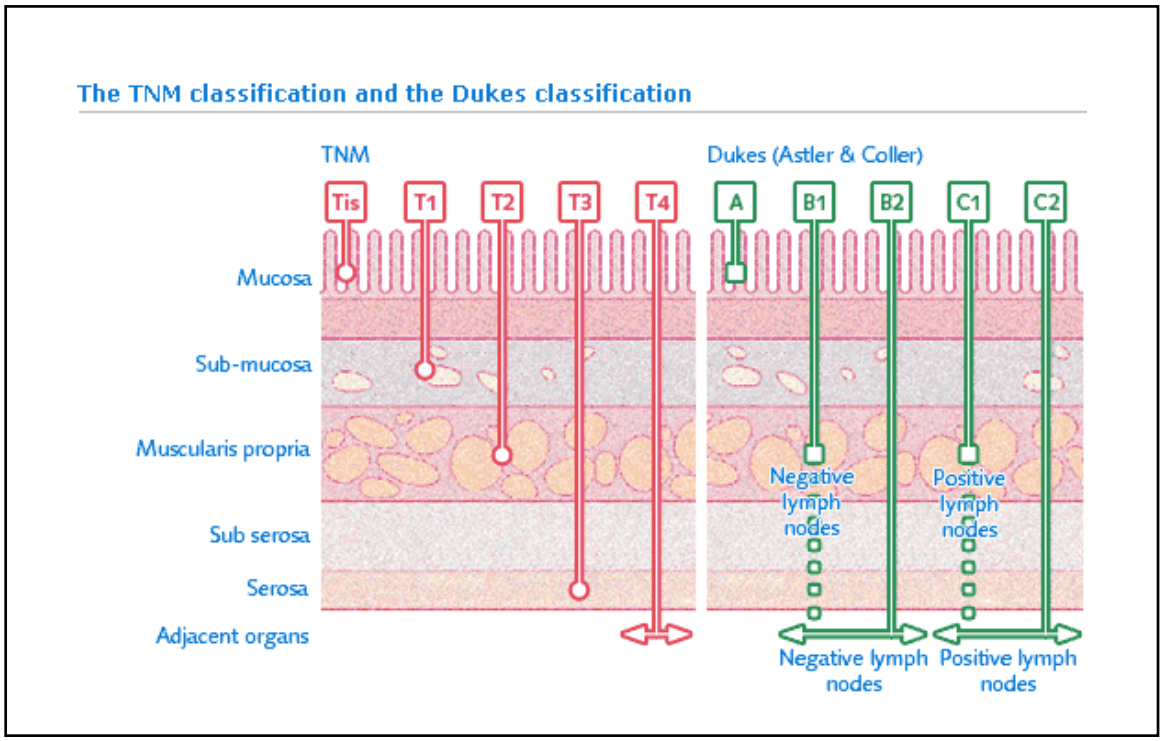


Table 6.2 The Duke's or TNM classification in colorectal cancer with 5 year survival rate.

Modified Dukes (Astler + Coller)	TNM Stage	Primary Tumour	Lymph Node Invasion	Distant Metastases	Prognosis – 5yr survival (%)
A	**Stage 0**	**T_{is}**	**N_0**	**M_0**	**100**
A1	**Stage 1**	**T_1**	**N_0**	**M_0**	**> 90**
B1		**T_2**	**N_0**	**M_0**	**85**
B2	**Stage 2**	**T_3**	**N_0**	**M_0**	**70 – 80**
B2		**T_4**	**N_0**	**M_0**	**35 – 80**
	Stage 3				
C1 / C2	**A**	**Any T**	**N_1**	**M_0**	**35 – 65**
C1 / C2	**B**	**Any T**	**$N_2 – N_3$**	**M_0**	**35 – 65**
D*	**Stage 4**	**Any T**	**Any N**	**M_1**	**< 5**

** Dukes D or Stage 4 involves tumour metastases to distant organs i.e. liver, lung or other intra-abdominal organs.*

In the hepatic patients, the mean alkaline phosphatase was elevated at 120 iU/L (normal range 60 – 110 iU/L). Other blood parameters were within normal limits. The mean blood results of all the patients and its reference ranges are shown in Table 6.1.

The primary cancer site in the hepatic patient cohort was the rectum (n = 7), or the sigmoid (n = 3), descending (n = 1) or transverse (n = 1) colon. All primary tumours had been previously resected. Ten patients had undergone and completed a course of chemotherapy before their liver resections. A list of all the concurrent medications taken by the colorectal and hepatic patients during their 7 day course of silipide capsules is shown in Table 6.3.

Table 6.3 Concurrent medications taken by the colorectal and hepatic patients over the 7 days of silipide ingestion.

Drug classifications	**Colorectal Patients**	**Hepatic Patients**
Non steroidal anti-inflammatory drugs	3	0
Proton Pump Inhibitors	2	3
Anti-hypertensives	3	4
Anti-anginals	1	1
Anti-depressants	4	2
Anti-histamine	0	1
Bronchodilators	1	1
Hormonal Preparations (e.g. Thyroxine, HRT)	0	3
Vitamins and digestive enzymes	3	1

6.3 Safety of silipide

None of the twenty four patients who consumed silipide reported any adverse effects throughout the intervention, including those who consumed the highest dose, 1440 mg per day. All patients completed their assigned treatment prior to having their blood and tissue samples taken.

6.4 Silybinin levels in blood and tissues of patients

Patient sample extracts of plasma or normal and malignant, colorectal or hepatic tissues were subjected to quantitative HPLC-UV analysis. Full details of sample preparation, HPLC method and analysis have been documented in Section 3.4.2.1. The double peak of silybinin was detected in the peripheral plasma of all patients who had consumed the course of silybinin taken at three divided portions a day for 7 consecutive days. The peak area ratio (PAR) of silybinin to apigenin (internal standard) was measured, and levels of silybinin in the plasma and tissues were calculated (Section 4.1.4). Figure 6.3 shows a chromatogram of pre- and post-treatment peripheral and portal plasma from a patient who received the highest dose of silipide. Levels of silybinin in the colorectal mucosa and liver tissues of the same patient are shown in Figure 6.4.

Figure 6.3 HPLC-UV chromatograms of extracts of pooled plasma spiked with silybinin (500 ng/ml) (A), peripheral plasma obtained from a patient prior to the first dose of silipide (B), peripheral plasma obtained 3 h after last dosage (C) and portal plasma taken 9 h after the last of seven daily doses of silipide (D) (1440 mg daily in three divided portions).

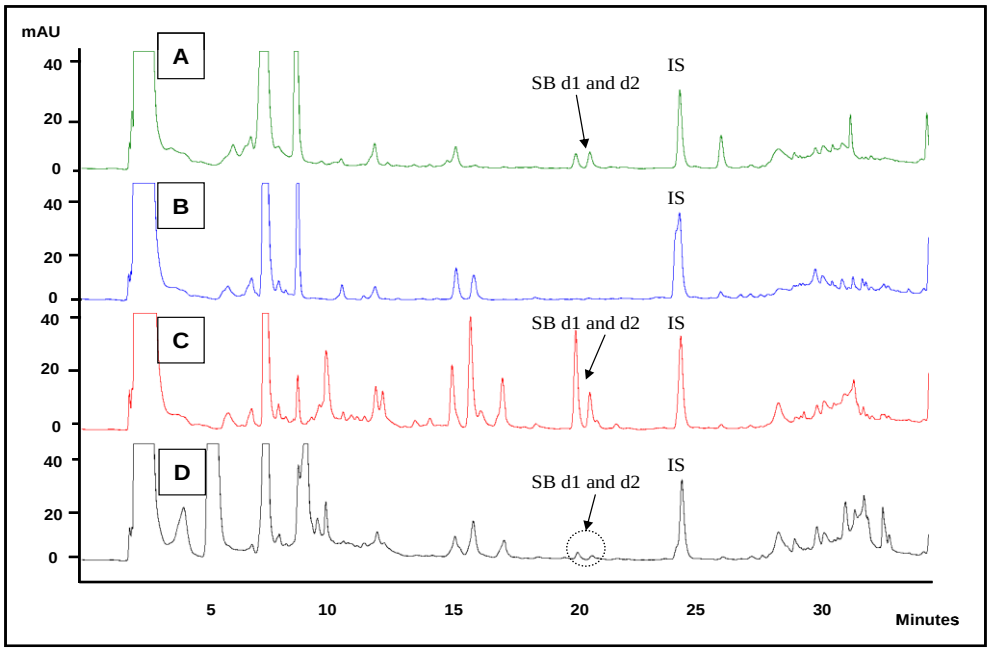
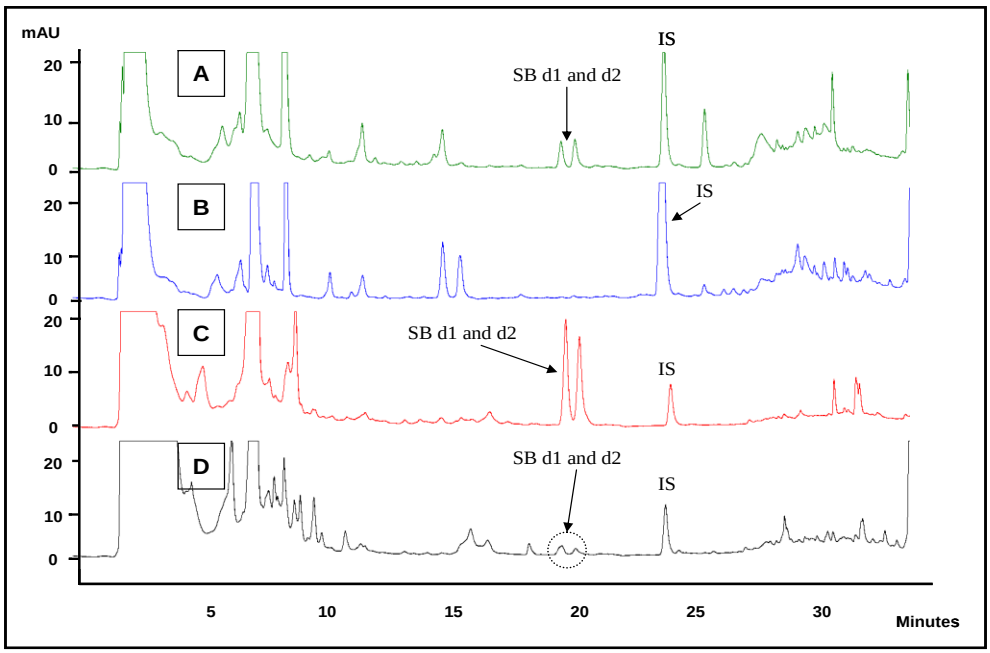


Figure 6.4 HPLC-UV chromatograms of extracts of pooled plasma spiked with silybinin (1 µg/ml) (A), control liver homogenate obtained from the Human Tissue Bank (B), colonic mucosa obtained from a patient 8 h after the last dose (C) and liver tissue taken from the same patient, 10 h after the last of seven daily doses of silipide (1440 mg daily in three divided portions) (D). Colonic and liver tissue obtained for this chromatogram was from the same patient as shown in Figure 6.3.



6.4.1 Silybinin levels in peripheral and portal plasma

The silybinin levels detected in the plasma of patients who had consumed silipide were found to be dose related (Table 6.4). Patients who had received the highest dose of silybinin of 1440 mg per day had the highest plasma silybinin levels, as measured by the size of the silybinin (d1 + d2) peaks. Peripheral plasma levels were between 0.3 and 4 µM. Silybinin levels in portal plasma were similar to those in peripheral plasma.

Table 6.4 Levels of silybinin found in the plasma of the colorectal and hepatic patients who had received their corresponding doses of silybinin daily for 7 days as silipide capsules.

	Dose of silipide (mg/day)	**Peripheral blood levels of silybinin (µM)**	**Portal blood levels of silybinin (µM)**
Colorectal patients	360	0.3 ± 0.3*	
	720	0.7 ± 0.6*	
	1440	3.0 ± 2.3*	
Hepatic patients	360	0.4 ± 0.2*	0.5 ± 0.3*
	720	1.4 ± 1.0*	0.5 ± 0.4*
	1440	4.0 ± 5.3*	1.6 ± 0.3*

* *Values are the mean ± SD of four patients.*

6.4.2 Silybinin levels in the colorectal and hepatic tissues

Silybinin levels detected in normal and malignant colorectal tissue showed considerable variation between patients (Table 6.5). Values are between 20 and 141 nmol/g and not related to silipide dose. Concentrations of silybinin in hepatic tissue were similar to those in plasma and ranged between 0.3 and 2.5 nmol/g.

Table 6.5 Levels of silybinin found in the colorectal or hepatic tissues of the recruited colorectal or hepatic patients, respectively. All patients had received their corresponding doses of silybinin daily for 7 days as silipide capsules.

	Dose of silipide (mg/day)	**Normal colorectal tissue levels (nmol/g)**	**Malignant colorectal tissue levels (nmol/g)**
Colorectal patients	360	27.8 ± 30.8*	37.0 ± 50.6*
	720	120.9 ± 180.7*	19.8 ± 26.4*
	1440	140.7 ± 168.9*	67.8 ± 98.0*
	Dose of silipide (mg/day)	**Normal hepatic tissue levels (nmol/g)**	**Malignant hepatic tissue levels (nmol/g)**
Hepatic patients	360	1.0 ± 0.9*	0.6 ± 0.5*
	720	1.2 ± 1.1*	0.3 ± 0.1*
	1440	2.5 ± 2.4*	0.6 ± 0.7*

**Values are the mean ± SD of four patients.*

6.5 Silybinin metabolites in human liver microsomes *in vitro* and in blood and tissues of patients *in vivo*

Studies in human volunteers suggest that silybinin undergoes conjugation to mainly glucuronide but also to sulphate metabolites (Wen *et al.*, 2008; D'Andrea *et al.*, 2005; Gatti & Perucca, 1994; Gunaratna & Zhang, 2003). This had not been shown in humans *in vivo* at the time when this clinical trial was performed. Furthermore, there has been little information on the pharmacological activity of the metabolic conjugates of silybinin (Section 1.3.2). *In vitro* human liver microsomes and cytosol were used to synthesise silybinin metabolites (Section 3.4.2.2) which could be detected in the clinical samples. Subsequently, peripheral plasma samples from the patients who had received silybinin were analyzed and incubated with β-glucuronidase enzyme to allow quantification of free and conjugated silybinin. Mass spectrometric analysis was carried out to identify silybinin metabolites found in human blood following oral administration of silipide.

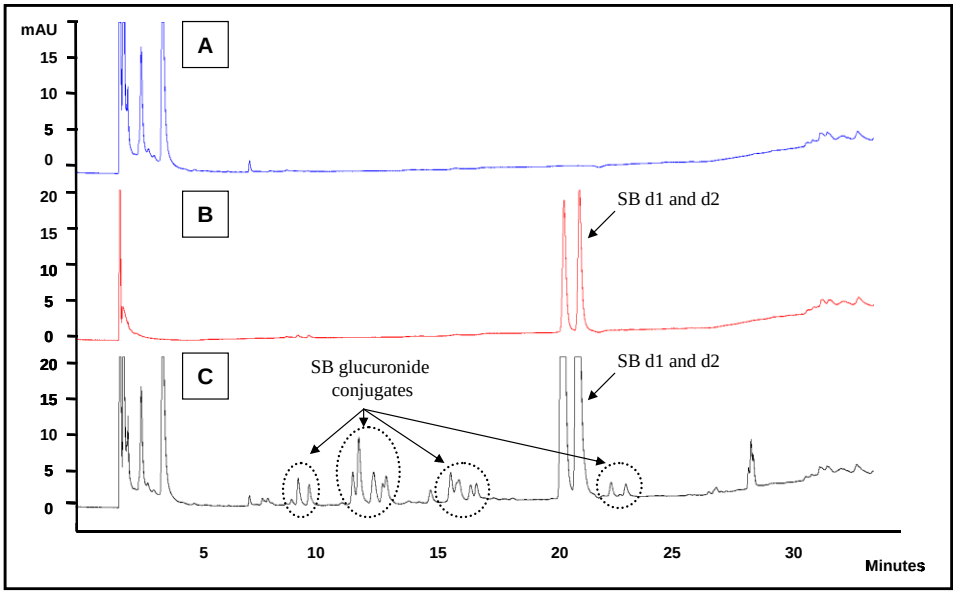
6.5.1 Analysis of silybinin metabolites in human liver and gut subfractions *in vitro*

Human liver and gut microsomes and cytosol were used to generate silybinin glucuronide and sulphate conjugates. Characterisation of their HPLC properties would help identify the metabolites in human plasma.. Details of the experimental method are described in section 3.4.2.2.2. Figure 6.5(I) shows the emergence of several peaks on the chromatogram, with retention times lower and higher than those for parent silybinin. These peaks may represent putative silybinin glucuronide conjugates (Figure 6.5).

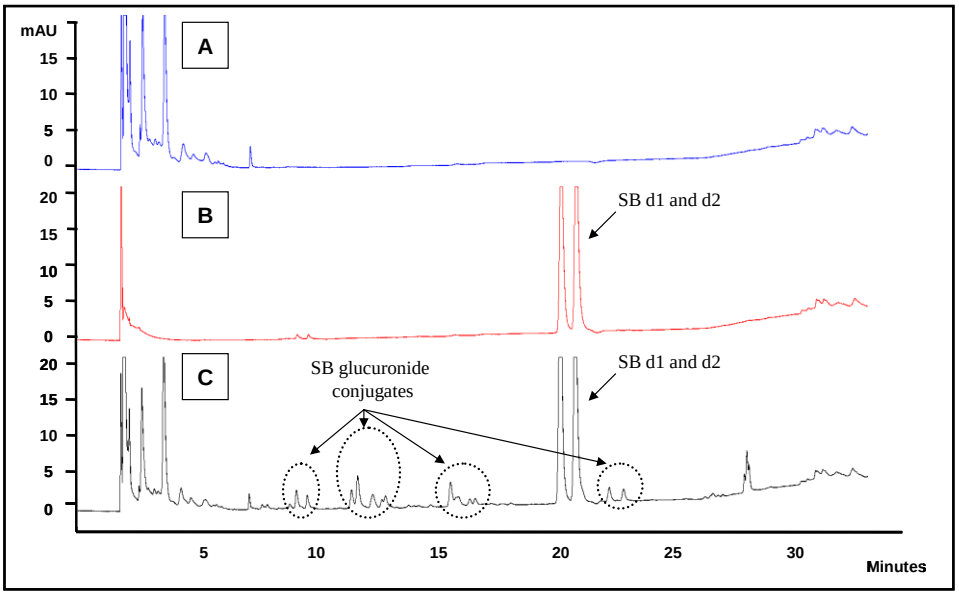
Human gut microsomes purchased from the human tissue bank were used and experiments performed under the same conditions as the liver microsomes. The results obtained were similar to the chromatogram of human liver samples and are shown in Figure 6.6(II).

Figure 6.5 HPLC-UV chromatograms of extracts of human liver (I) and gut (II) microsomes incubated with magnesium chloride ($MgCl_2$) and UDPGA but without silybinin, as control (A), silybinin and $MgCl_2$ and UDPGA but without human microsomes (B) and finally silybinin with human microsomes in the presence of $MgCl_2$ and UDPGA. All samples were incubated for 60 min at 37°C. Dashed circles highlight several silybinin glucuronide peaks present in sample (C).

(I) Human liver microsomes



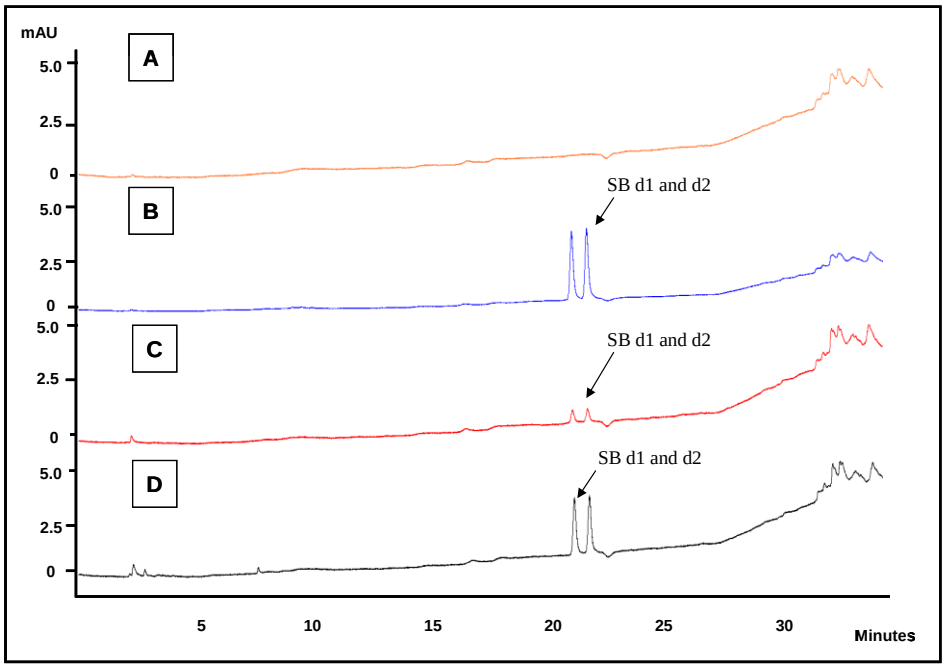
(II) Human gut microsomes



The retention times of the silybinin glucuronide peaks in both types of microsomes were identical. This implies that the same species of glucuronide conjugates were formed.

The synthesis of human silybinin sulphonate metabolites was performed utilizing human liver and gut cytosol obtained from centrifugation of human liver and gut homogenate (Section 3.4.2.2.3). Human cytosol was incubated with lithium 3′-phosphoadenosine 5′-phosphosulphate (PAPS) as a co-factor for 30 min at 37°C. HPLC-UV chromatograms of the analysis of the matrix extracts obtained are shown in Figure 6.6.

Figure 6.6 HPLC-UV chromatograms of extracts of human liver cytosol incubated in the presence of PAPS but without silybinin, as control (A), or with PAPS and silybinin but without human liver cytosol (B), or with PAPS, silybinin and with human liver cytosol (C) or with PAPS, silybinin and with human gut cytosol (D).

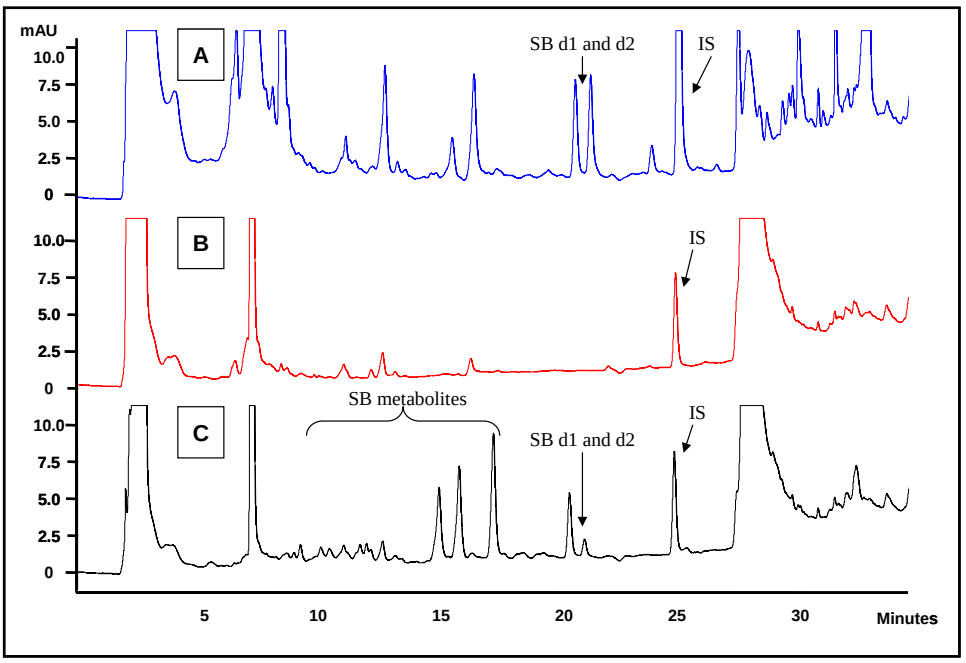


The HPLC-UV chromatogram of the silybinin glucuronide metabolites identified several metabolite peaks, however the HPLC-UV chromatograms of extracts of liver or gut cytosol incubated with silybinin only afforded parent silybinin (Figure 6.6).

6.5.2 Analysis of silybinin metabolites in blood and hepatic tissues in patients who received silipide

Patients with either confirmed colorectal cancer or colorectal liver metastases received silipide daily for 7 days prior to colorectal or hepatic surgery. Silybinin exists as two trans-diastereoisomers furnishing two peaks on HPLC analysis with retention times 21 and 22 min. All three doses afforded measurable peaks in the plasma at the retention times of authentic silybinin (Figure 6.7**)**

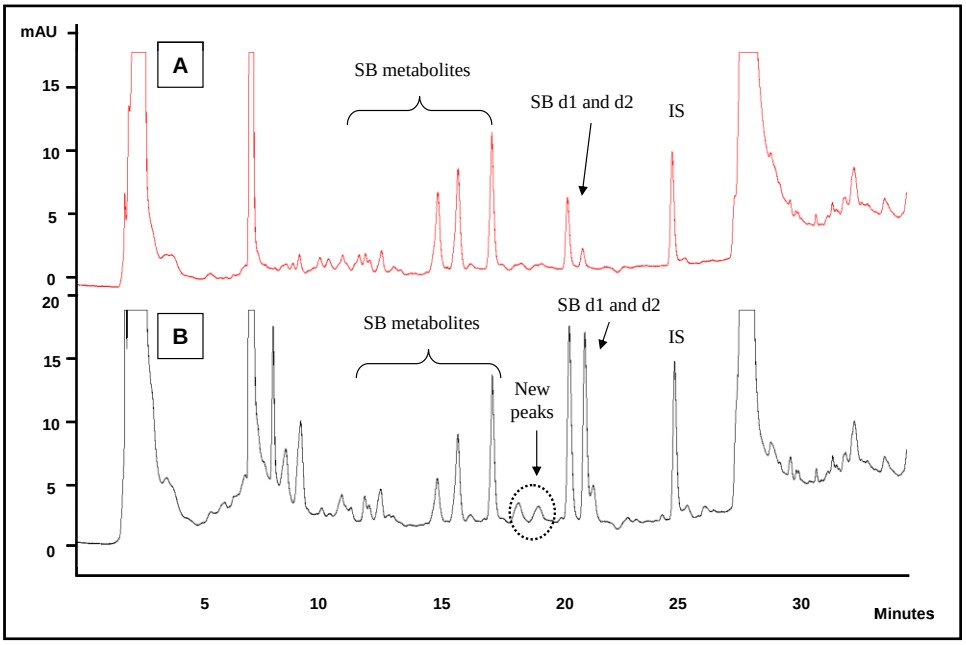
Figure 6.7 HPLC-UV chromatograms of extracts of pooled plasma spiked with silybinin (400 ng/ml) (A), plasma obtained from a patient prior to the first dose of silipide (B) and 3 h after the last of seven daily doses of silipide (1440 mg silybinin, administered in three divided portions) (C).



For the post-treatment human plasma HPLC chromatogram, the presence of silybinin d1 and d2 peaks were confirmed by co-chromatography with silybinin standards (Figure 6.7). The presence of several peaks between retention times 10 and 18 min, which were not present in the pre-treatment samples, most likely represents conjugated silybinin metabolites. These peaks may be similar to the peaks observed for the HPLC chromatograms of the analysis of Wistar rat plasma (Figure 5.10) and to the human silybinin conjugated metabolites generated in microsomes (Figure 6.5-I and II**)**.

Further incubation (at 37°C for 1 h) of the post-treatment peripheral plasma with deconjugation enzymes furnished a marked increase in the height of the parent silybinin peaks, consistent with the presence of silybinin sulfate and glucuronide conjugates (Figure 6.8). There was also a reduction in size of several peaks separated between 15 – 17 min and the emergence of a double peak between retention times' 18 – 19 min which were not previously observed (Figure 6.8). As expected, incubation with glucuronidase deconjugating enzymes made no difference to the pre-treatment plasma and therefore results are not shown.

Figure 6.8 HPLC-UV chromatograms of extracts of a patient's post treatment peripheral plasma (A) and the same patient's post treatment plasma incubated with β glucuronidase enzyme for 1 h at 37°C (B)**.** Dashed circles highlights the emergence of two new peaks after incubation with β glucuronidase enzyme. These peaks represent probable primary silybinin metabolites.



I.S. = internal standard.

6.5.3 Identification of silybinin metabolites by mass spectrometry

The HPLC peaks that were observed for the analysis of silybinin glucuronides from human liver and gut subfractions *in vitro* (Section 6.5.1 and Figure 6.5) and the *in vivo* silybinin conjugates from patients' post-treatment blood and liver tissues (Section 6.5.2 and Figure 6.7) were subjected to fraction collection followed by characterization using liquid chromatography – mass spectrometry (LC-MS) in full scan mode (*m/z* 100 – 900) as previously described (Section 3.4.3). Mass spectrometry was performed by Dr. David Boocock (Data not shown). Figure 6.9-B shows a HPLC-UV chromatograms of plasma extracts obtained from a patient after consuming oral silipide (1440 mg) daily for 7 days. The HPLC-UV chromatogram indicates the isolated silybinin metabolites labelled as A – G, which were identified by LC-MS.

The two peaks identified at 20.6 and 21.1 min on both the HPLC-UV chromatograms were unambiguously confirmed as silybinin by mass spectrometry, as they afforded molecular ion with *m/z* at 481 $[M-H]^-$. They were also consistent with the LC-MS chromatogram of authentic silybinin standards (Figure 6.10**)**. Plasma sample extracts were also analysed by mass spectrometry for metabolic species derived from silybinin. Silybinin mono-glucuronide, silybinin mono-sulphate and silybinin di-glucuronide could be detected (Table 6.6). HPLC-MS single ion monitoring (SIM) yielded multiple peaks, which could be assigned to silybinin mono-glucuronide, silybinin di-glucuronide, silybinin mono-sulphate and silybinin glucuronide sulphate (Figure 6.11). There was also some evidence for the presence of silybinin tri-glucuronide (*m/z* 1008) and O-desmethyl silybinin glucuronide (*m/z* 643), but the levels of detection were too low to provide a significant mass spectrometric

chromatogram (results not shown). Because of the occurrence of multiple diastereoisomers of silybinin derivatives and given the absence of authentic reference materials, it was not possible to assign specific positional isomers to the multiple peaks observed by LC-MS SIM.

Table 6.6 LC-MS SIM analysis results of ions related to silybinin in plasma from humans who had received silybinin (1440 mg) daily for 7 days as silipide capsules.

m/z $[M-H^+]^-$	Inference	Retention Time (min)
481	Silybinin	20.8; 21.4
561	Silybinin sulphate	17.0; 17.8; 18.8
657	Silybinin glucuronide	12.6; 15.6; 16.4; 17.8
737	Silybinin glucuronide sulphate	11.1; 13.1
833	Silybinin di-glucuronide	9.2; 10.8

Figure 6.9 HPLC-UV chromatograms of plasma extracts obtained from a patient prior to the first dose of silipide (A) and 3 h after the last of seven daily doses of silipide (1440 mg silybinin, administered in three divided portions) (B). Peaks were putatively identified by LC-MS : (A) mono-glucuronide-mono-sulphate [*m/z* 737] (B) di-glucuronide [*m/z* 833] (C, D, F) mono-glucuronides [*m/z* 657] (E, G) mono-sulphates [*m/z* 641].

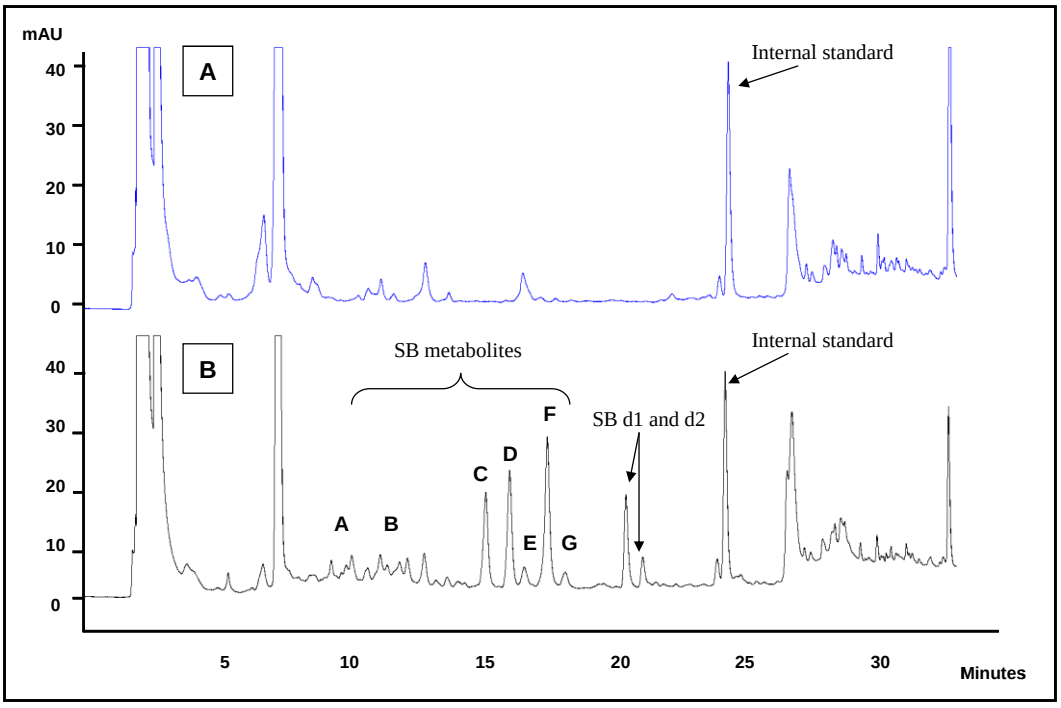


Figure 6.10 LC-MS SIM chromatograms of a standard solution of silybinin (A) and an extract of a patient's post treatment plasma, 3 h after the last of seven daily doses of silipide (1440 g silybinin) (B).

(For details of dosing and analysis see Materials and Methods.)

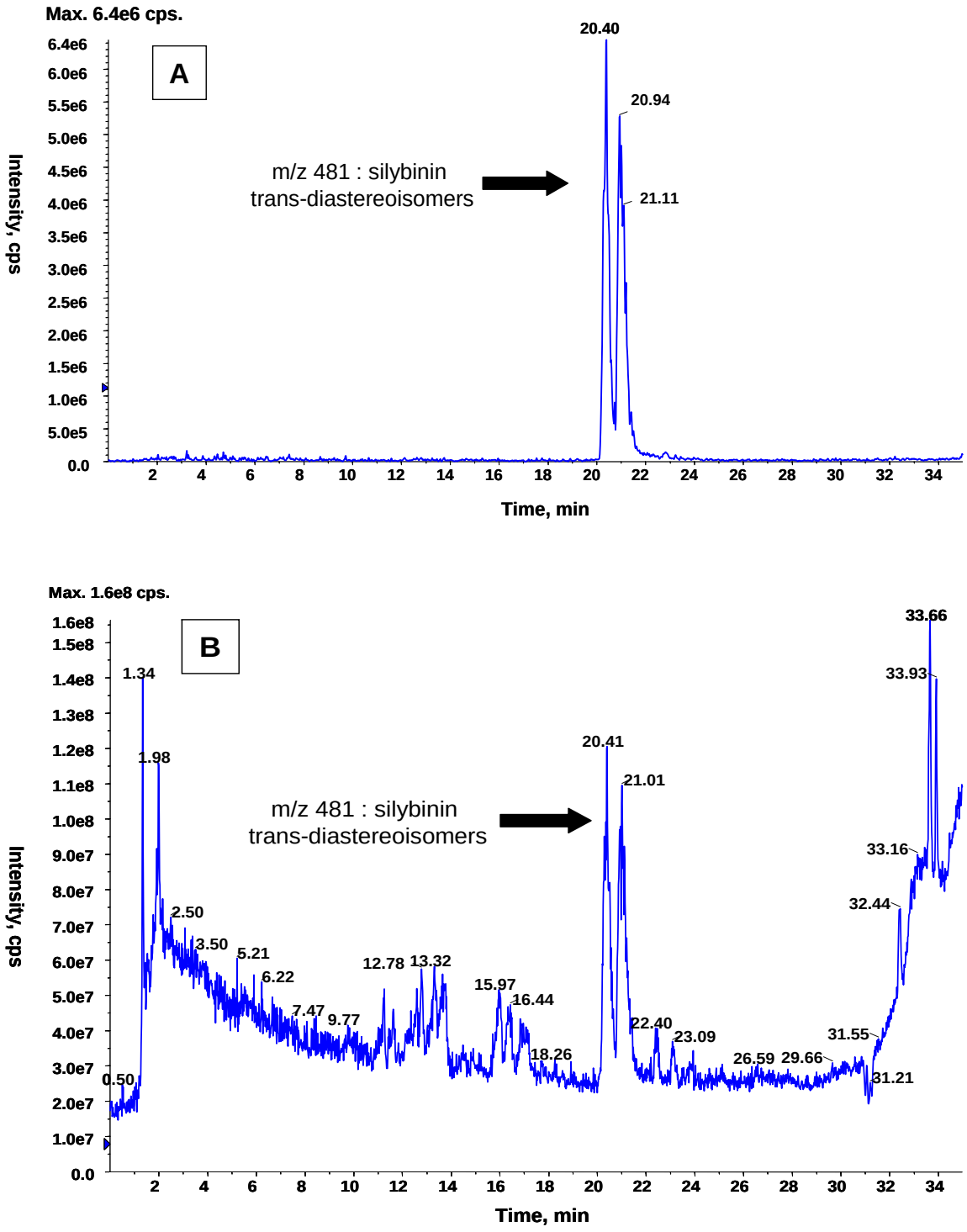
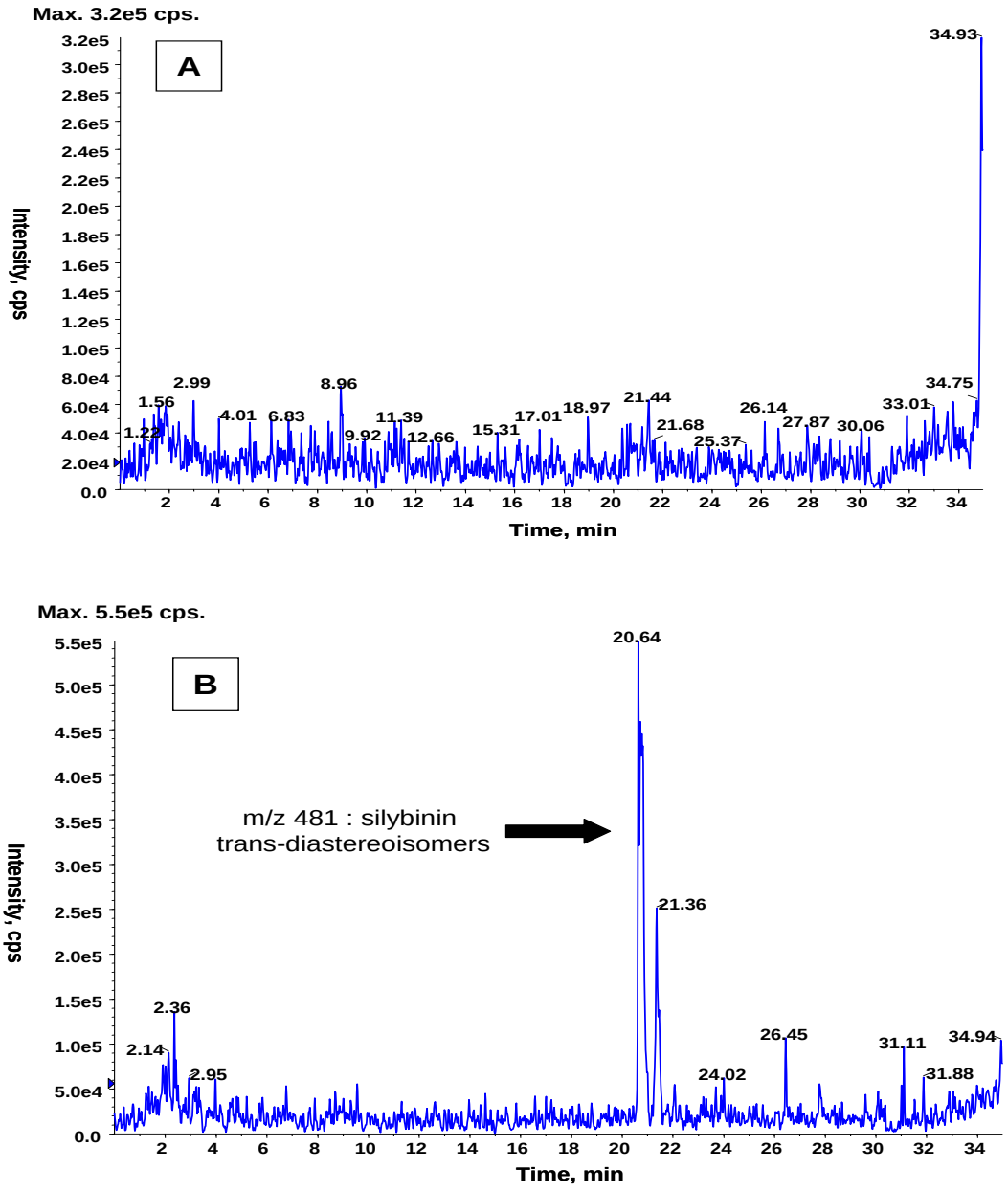
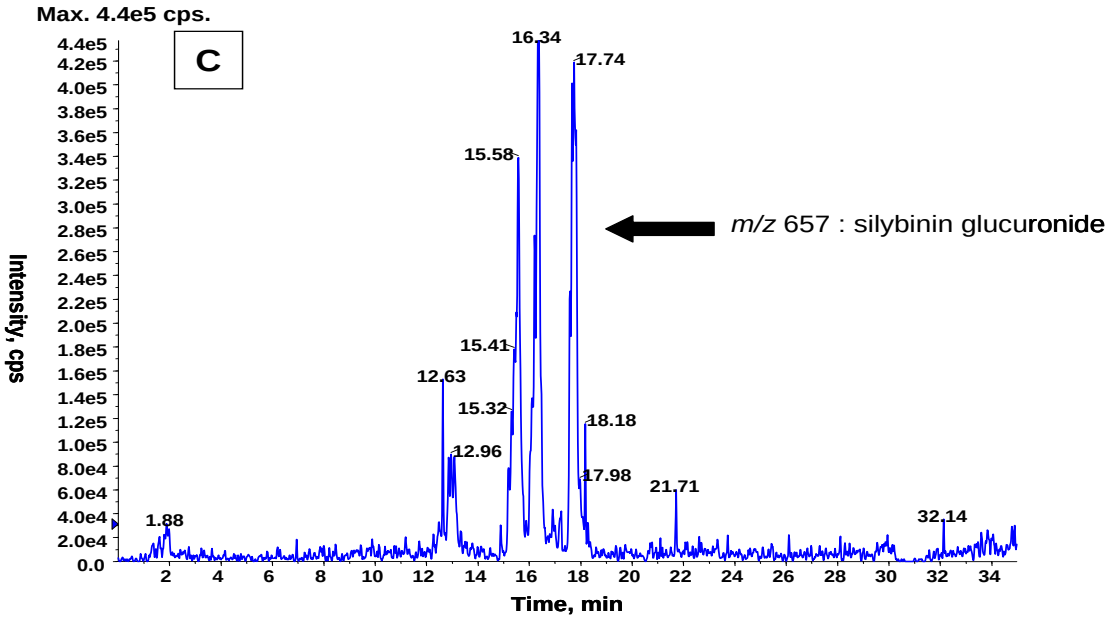
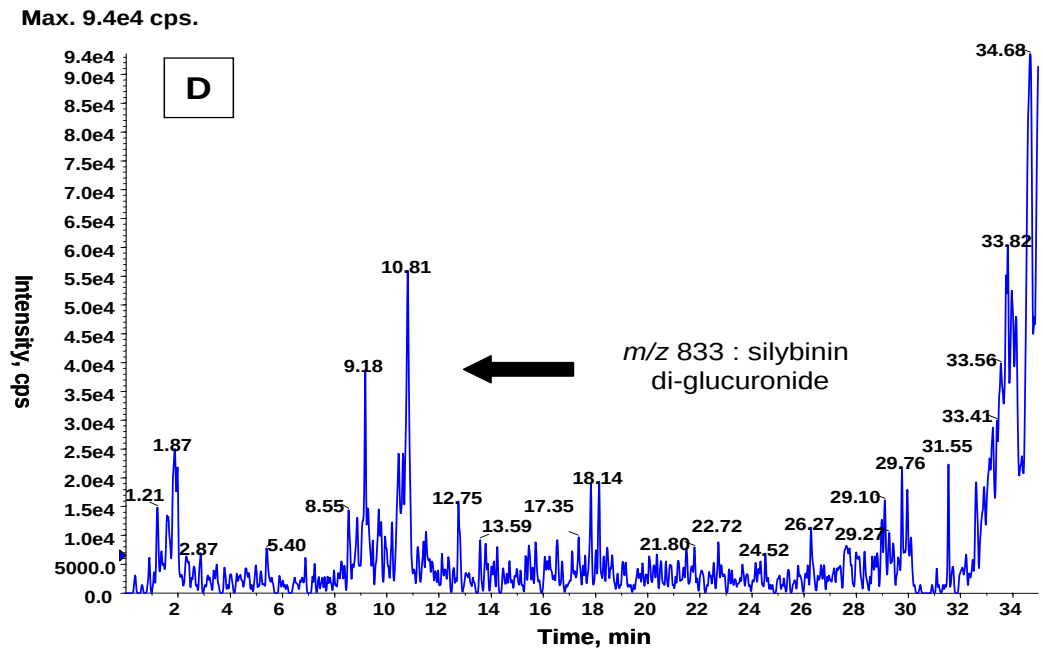


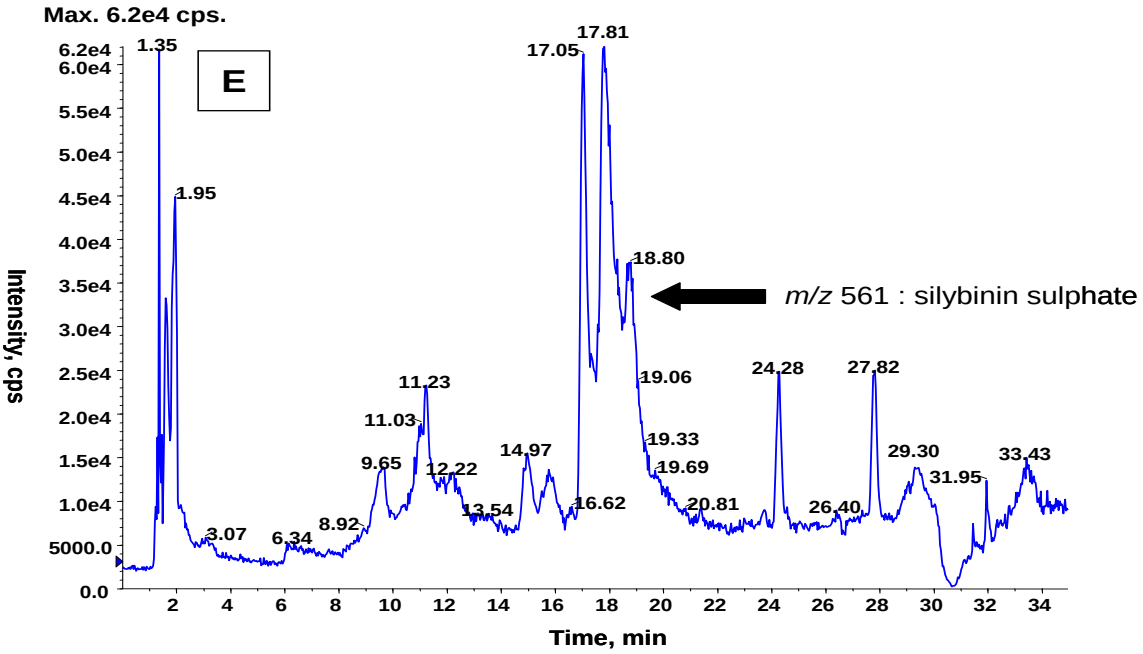
Figure 6.11 HPLC-MS-SIM chromatograms of an extract of a patient's plasma obtained before silybinin treatment (A) and 3 h after the last of seven daily doses of silipide (1440 g silybinin). In the post treatment plasma samples the following $[M-H]^-$ ions were monitored: 481 (m/z of silybinin, B), 657 (m/z of silybinin glucuronide, C), 833 (m/z of silybinin di-glucuronide, D), 561 (m/z of silybinin sulfate, E) and 737 (m/z of silybinin glucuronide sulfate, F).

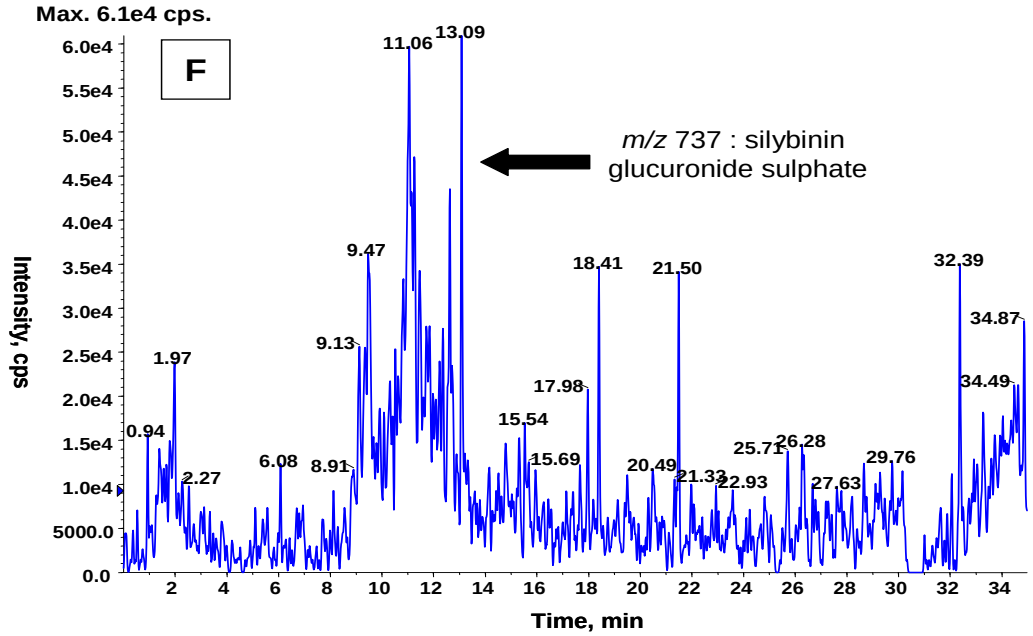
(For details of dosing and analysis see Materials and Methods.)




Max. 4.4e5 cps.
C
Intensity, cps
Time, min
m/z 657 : silybinin glucuronide
16.34
17.74
15.58
15.41
12.63
15.32
18.18
12.96
17.98
21.71
1.88
32.14


Max. 9.4e4 cps.
D
Intensity, cps
Time, min
m/z 833 : silybinin
di-glucuronide
34.68
33.82
10.81
33.56
9.18
33.41
1.87
31.55
29.76
18.14
1.21
29.10
8.55
12.75
17.35
13.59
22.72
26.27
29.27
2.87
5.40
21.80
24.52


Max. 6.2e4 cps.
E
Intensity, cps
Time, min
m/z 561 : silybinin sulphate
1.35
1.95
3.07
6.34
8.92
9.65
11.03
11.23
12.22
13.54
14.97
16.62
17.05
17.81
18.80
19.06
19.33
19.69
20.81
24.28
26.40
27.82
29.30
31.95
33.43


Max. 6.1e4 cps.
F
Intensity, cps
Time, min
m/z 737 : silybinin glucuronide sulphate
0.94
1.97
2.27
6.08
8.91
9.13
9.47
11.06
13.09
15.54
15.69
17.98
18.41
20.49
21.32
21.50
22.93
25.71
26.28
27.63
29.76
32.39
34.49
34.87

6.6 Discussion

One of the primary aims of this clinical trial was to ascertain the safety of oral silipide in humans. The outcome of both the human and rodent study outlined here and in Chapter 5 support the notion that repeated administration of silybinin at daily doses of up to 1.44 g for a week is safe. This conclusion is consistent with the result of the original evaluation of silipide in human volunteers (Barzaghi *et al.*, 1990) and a similar inference was made in the preliminary report of a phase 1 study of silybinin in hormone-refractory prostate cancer patients, in which up to 20 g silybinin was administered orally daily for a month (Flaig *et al.*, 2007).

The daily silybinin dose of 1440 mg tested here has been the highest dosage of silipide administered to humans to date. None of the twenty four patients, who consumed silipide for 7 days showed any signs or symptoms of silybinin toxicity. This is important for the planning of future clinical trials of silipide. The patients that were given silipide, had primary and secondary malignant disease, who would usually be more susceptible to drug adverse effects. However in this case, none were detected.

There was a similar number of male and female patients in both arms of the patient cohorts. The mean age of the hepatic patients at 62 years of age was marginally younger than the colorectal patients. This is likely because of the strict criteria that needed to be met in the selection of patients for liver resections. Patients are usually fitter and younger, with less underlying medical illness. Most patients over the age of seventy would not be suitable for this physically challenging procedure, and it would not be possible to recruit these patients into the trial.

In the colorectal patients group, all preoperative blood analysis except reduced mean haemoglobin of 10 g/dL was within the normal laboratory range. This finding could be explained by occult bleeding from the primary tumour, which is a common symptom of presentation in the diagnosis of colorectal cancer. In the hepatic patients group all blood parameters were normal except alkaline phosphatase levels which were elevated. Alkaline phosphatase (ALP) has previously been used as a tumour marker for monitoring liver metastases (Li *et al.*, 1998). Serum alkaline phosphatase has been suggested to be an independent prognostic marker for survival (Schindl *et al.*, 2005). ALP is a hydrolase enzyme that is found in all tissues but particularly concentrated in the liver and bile ducts. ALP lines the biliary ducts in the liver and can be elevated in clinical conditions such as cholestasis, cholecystitis, cholangitis, cirrhosis, hepatitis and liver metastases. In liver metastases, an increase in ALP is caused by external compression of the bile ducts by the tumour mass, stimulating an increased ALP secretion by the ductal cells (Watine & Friedberg, 2004).

In both the colorectal and hepatic patients that had been recruited into the clinical trial, the commonest site of the tumour occurrence within the gastrointestinal tract was in the rectum. The second commonest tumour occurrence site was found to be the sigmoid colon.

On average, peripheral blood was obtained about 3 h and portal blood about 6 h, after the last silipide dosage. Peripheral blood was obtained from venepuncture at the antecubital fossa at induction of anaesthesia. Generally, collection was easy and did not require much time. In contrast, portal blood was obtained through venepuncture of the portal vein, and much time was allowed for liver dissection and exposure of the appropriate vessels. Similarly, there was a delay in the harvesting of the colorectal

and hepatic tissues that were collected about 6 h after the last silipide dose, attributed to the time required for surgical dissection.

There were many similarities in the metabolism of silybinin in rodents and in humans as previously suggested by D'Andrea (D'Andrea *et al.*, 2005). In both species, up to 80% of silybinin is metabolised to sulphates and glucuronides (Section 5.3 and 6.5) (Wen *et al.*, 2008; Barzaghi *et al.*, 1990; Weyhenmeyer *et al.*, 1992; Morazzoni *et al.*, 1992; Schandalik *et al.*, 1992; Rickling *et al.*, 1995). In rodents, our experiment showed that silybinin mono-sulphate was the major metabolite (Section 5.3.3.3). We also demonstrated that three other minor metabolites were obtained, although unfortunately these were uncharacterizable due to low concentration. In contrast, silybinin metabolism in humans produced at least nine different metabolites, some of which could be measured and characterized by LC-MS (See Table 6.6). These silybinin conjugates included mono-glucuronide, di-glucuronide, mono-sulphate and mono-glucuronide mono-sulphate. Since silybinin has multiple phenolic and alcoholic hydroxyl sites for conjugation, there could be many isomers. Therefore, accurate characterization of each conjugate is impossible without the use of NMR or isotope-labelling and mass spectrometry.

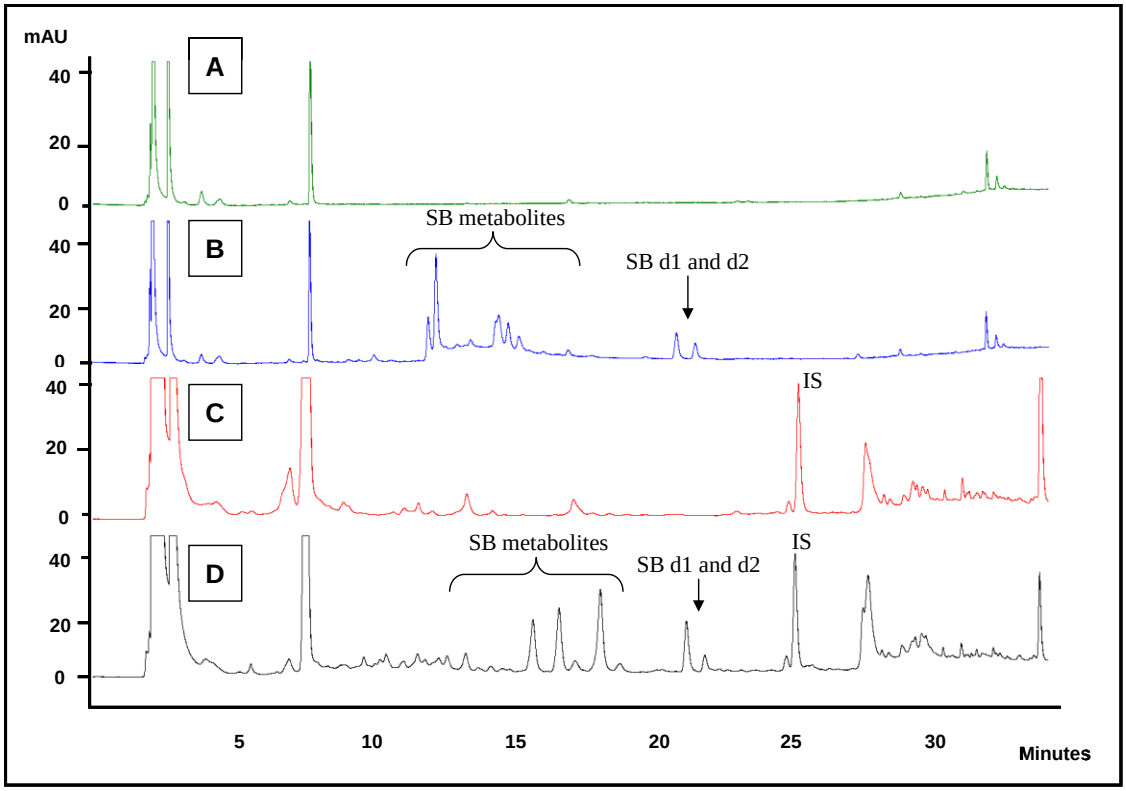
Free and conjugated silybinin could be detected in both rodent and human plasma following the oral consumption of silipide. The plasma concentration of silybinin appeared to be dose-related, and was highest in the 1440 mg per day treatment group. For the first time, we have detected conjugated and free silybinin beyond the systemic circulation, in the liver and gastrointestinal tract, in rats and humans, and prostate tissue (in rats) *in vivo*, following oral silipide.

By comparison, the levels of free silybinin detected in the human plasma (0.3 – 4.0 μM) were higher than those seen in rat plasma (0.12 – 0.36 μM). In contrast, silybinin concentrations reached in the colorectal tract and liver tissues in rodents, 650 – 850 nmol/g in colorectal and 43 – 50 nmol/g in hepatic tissue, were higher than those seen in humans, 20 – 141 nmol/g in the colon and 0.3 – 2.5 nmol/g in the liver tissue. An explanation for the different silybinin levels we see in the rodents and humans, plasma and tissues, could in part be attributed to the different mode of dietary administration of silybinin in rodents and humans. The rodents were fed 0.2% w/w silybinin which is approximately 300 mg/kg per day, this amount equates to ~ 1.8 g per human per day (Section 3.3.1). This dose is similar to high doses employed in the clinical trial. However, it is pertinent to note that the rodents were fed continously throughout the day, whilst in humans, silipide were given in three divided doses for 7 days. The variation in the mode of silybinin administration, distinguishes the circulating levels of silybinin in plasma and tissues, at each time point. Silybinin concentrations reached in the colorectal tract of both humans and rodents are of a level which has been shown to elicit pharmacological effects in cells *in vitro* (Gershbein, 1994; Kohno *et al.*, 2002; Singh *et al.*, 2002a). Levels of silybinin in the liver, were also higher than the silybinin levels found to show hepatoprotective activity (Pradhan & Girish, 2006).

Figure 6.12 shows the HPLC-UV chromatograms of plasma extracts from a rodent and a patient, pre- and post-consumption of oral silybinin. Similarly in both, silybinin d2 isomer was prefentially metabolised, furnishing a lower silybinin d2 peak in the post silybinin chromatogram, as shown below. The silybinin conjugates formed in rodents separated as a cluster of peaks between retention time 12 – 17 min in the chromatogram (Figure 6.12-B). In human silybinin metabolism, conjugates were formed and seen as evenly distributed peaks with retention times 12 – 19 min (Figure

6.12-D). Most noticeably, there were three prominent metabolite peaks seen in the human but not in rat plasma. In general, the pattern of silybinin metabolites were different in the 2 species.

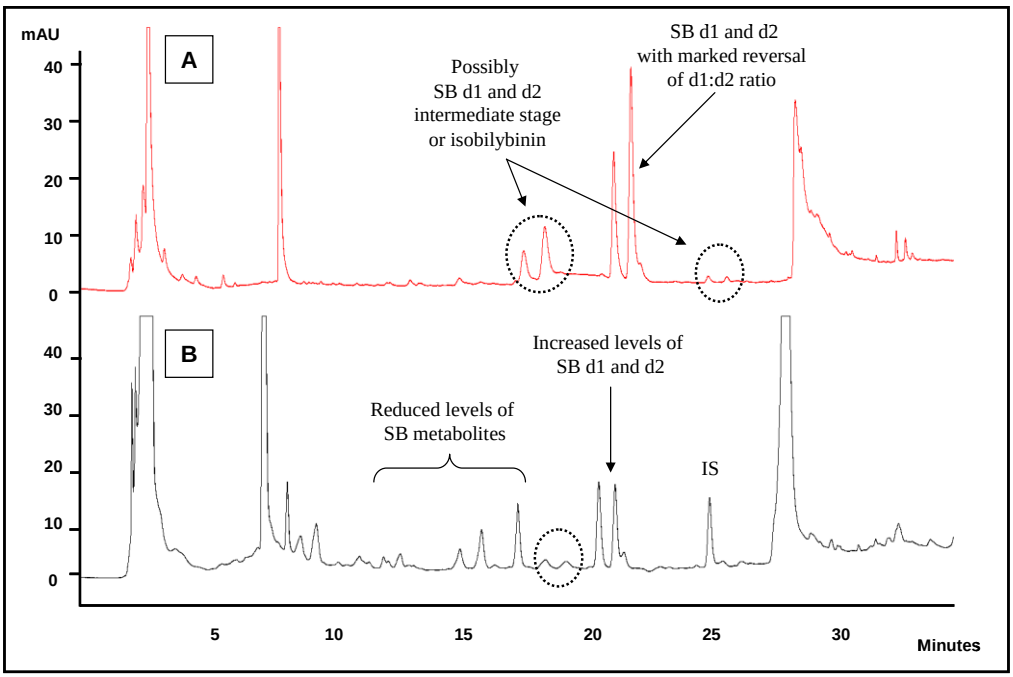
Figure 6.12 HPLC-UV chromatograms of extracts of plasma from rat before (A) or after a single oral gavage of silybinin at 1200 mg/kg (B) or plasma from patient before (C) or after silipide, 1440 mg daily for 7 days (D). Brackets and arrows show silybinin metabolites and parent silybinin.



After deconjugation (Figure 6.13), silybinin d2 levels were higher than its d1 counterpart in rodent plasma but in human plasma, both the silybinin d1 and d2 were uniformly increased. Similar observation were made by Kren (Kren *et al.*, 2000) and Weyhenmeyer (Weyhenmeyer *et al.*, 1992). It is possible that in rats, silybinin d2 is preferentially metabolised, but in humans, both d1 and d2 isomers are metabolised in equal amounts, elucidating to the stereoselective metabolism of silybinin (Han *et al.*, 2004). In the post-deconjugation rat plasma (Figure 6.13-A), there were two separate 'double peak' structures seen at retention times 16 – 17 min and 24 – 25 min (circled

in dashed lines), whilst only one was seen in the human plasma at retention time 18 – 19 min (Figure 6.13-B). These additional 'double' peaks may represent different isomers of hydrolysed silybinin, formed by hydrolysis of secondary silybinin metabolites back to primary metabolites. However, it was interesting to observe that the three dominant metabolite peaks seen in the post silybinin human plasma (Figure 6.13-B), did not decrease dramatically in size or disappear as in the rat plasma.

Figure 6.13 HPLC-UV chromatograms of extracts of plasma from rat (A) or human (B). Both (A) and (B) were incubated with β glucuronidase enzyme for 1 h at 37°C.



IS = internal standard

We describe here for the first time the identification of silybinin plasma metabolites and measurement of silybinin tissue levels in humans who ingested silybinin. Consistent with results using liver preparations incubated with silybinin *in vitro* (Gunaratna & Zhang, 2003), the results outlined here suggest that silybinin undergoes multiple conjugation reactions in humans. The presence of metabolic conjugates of silybinin in the human biomatrix has thus far been shown only indirectly (Gatti & Perucca, 1994) in that raised levels of the parent molecule after enzymatic hydrolysis

was taken to indicate the presence of conjugates. In contrast, here the conjugate species silybinin mono-glucuronide, silybinin di-glucuronide, silybinin mono-sulphate and silybinin glucuronide sulphate were unambiguously identified.

The silybinin molecule possesses five hydroxy moieties (Figure 1.6), three of which phenolic in nature, but the analysis described here does not allow the exact position of conjugation on the silybinin molecule to be elucidated. Undoubtedly, metabolic species in addition to those identified here were present in human plasma. On the assumption that the antioxidant activity of silybinin is a function of its polyphenolic structure, the presence of silybinin mono-sulphate and mono-glucuronide, which bear (at least) two intact phenol moieties, suggest that appreciable amounts of circulating silybinin-derived species may share, at least to some extent, the antioxidant potency of the parent molecule.

The plasma levels of silybinin described here need to be compared with those reported previously in healthy volunteers who received oral silipide on a repeated dose schedule (Barzaghi *et al.*, 1990). In that study silipide at 720 mg (equivalent to 240 mg silybinin) per day given for 7 consecutive days furnished a mean peak plasma level of 0.38 μmol/L (0.18 μg/mL) reached 0.9 h after administration of the last dose, and the terminal plasma half-life of the last dose was 3.4 h. This published data allows a tentative prediction of the levels achieved at the time points at which peripheral blood samples were taken for silybinin analysis in the pilot study described here (between 1 and 4 h after the last silipide dose). Blood was collected at times which coincide approximately between mean peak levels (i.e. about 1 h after last silipide dose) and the terminal plasma half-life (i.e. about 4 h after last silipide dose). The

mean plasma levels for the 360 mg daily dose observed here, which were 0.3 to 0.4 μmol/L (0.14 – 0.19 μg/mL), are broadly consistent with the earlier volunteer study.

Levels of silybinin in human liver have previously not been described, even though silybinin-containing remedies have long been marketed as liver protectants. In our study, the colorectal mucosa levels of silybinin were highly variable and not related to silipide dose, which may, at least to some extent, be the corollary of the difference between patients in time period (3 – 6 h), which elapsed between the consumption of the last dose and surgery. The recommended oral dose of, for example, Legalon (Madaus, Germany), which contains 70 mg silymarin per tablet, is two tablets taken three times a day. So the daily dose of silymarin recommended for liver protection is 420 mg. Silymarin contains silydianin and silychristin as well as silybinin. On the assumption that silymarin contains ~ 80 % silybinin, this silymarin dose would translate into approximately ~ 340 mg silybinin, which is similar to the low dose of silybinin administered in the pilot study described here. Based on this gross calculation, one may tentatively infer that silybinin concentrations in liver tissue of an order of magnitude similar to those measured here after the 360 mg dose (i.e. 0.3 – 0.5 μg/g or 0.6 – 1 μmol/L in concentration terms) can afford protection of the human liver against toxic insult. In contrast to the relatively low systemic and hepatic levels of silybinin, levels achieved in colorectal tissue, between 9.6 and 68 μg/g or 20 to 141 μmol/L in concentration terms, are highly likely to elicit pharmacological effects in the light of the concentrations reported to cause responses in cells in culture. For example, in cultured DU-145 prostate cancer cells 15 and 30 μmol/L (7.2 – 14.5 μg/mL) silybinin were sufficient to significantly compromise cell proliferation and increase IGFBP-3 in the cellular supernatant (Zi *et al.*, 2000).

Chapter 7. THE PHARMACODYNAMICS OF SILYBININ IN PATIENTS

7.1 Introduction

Several mechanisms have been proposed to explain how silybinin may interfere with carcinogenesis (Section 1.4.2). Elevated oxidative stress has been implicated as an important carcinogenic stimulus, and endogenous or exogenous oxidative events, such as those associated with lipid peroxidation, generate malondialdehyde (MDA), a mutagen (Marnett, 1999b). MDA can react with DNA to produce the pyrimidopurinone adduct of deoxyguanosine, M_1dG (Marnett, 1999a). Therefore, M_1dG levels may be a suitable marker of chemopreventive efficacy of strong antioxidants, such as silybinin (Section 1.4.2.1). Silymarin and silybinin are also powerful antioxidants as a consequence of their polyphenolic structure (Comoglio *et al.*, 1990; Zhao *et al.*, 2000).

Silybinin has been suggested to modulate the insulin-like growth factor (IGF) system. IGFs are mediators of cell survival in that they can inhibit apoptosis and influence differentiation of many normal and malignant cell types (Butt *et al.*, 1999; Lopez & Hanahan, 2002). The IGF system is regulated by IGF-binding proteins (IGFBPs), especially IGFBP-3, which binds IGFs in the extra-cellular milieu with high affinity and specificity, thus reducing the bioavailibility of IGFs (Section 1.4.2.2). Epidemiological studies have linked increased serum concentrations of IGF-1, decreased concentrations of IGFBP-3, or both with an increased risk of cancer at several sites, including the colorectum (Hassan & Macaulay, 2002; Samani *et al.*, 2004). Silybinin increased levels of IGFBP-3 in prostate cells *in vitro* (Zi *et al.*, 2000) and in tumor-bearing rodents *in vivo* (Singh *et al.*, 2002a).

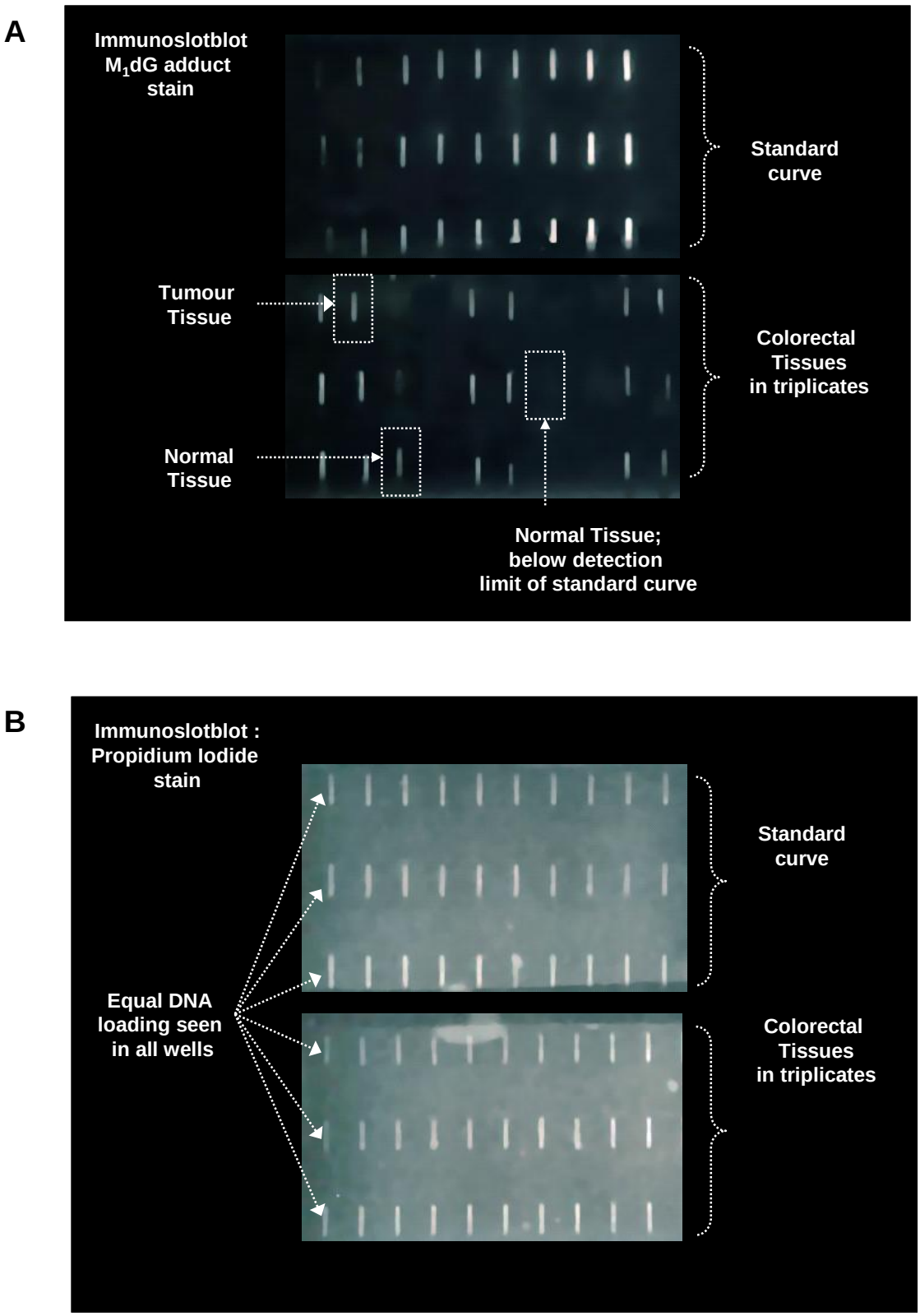
Levels of M_1dG and IGFBP-3 / IGF-1 were studied as potential markers of silybinin efficacy in the peripheral blood and tissues from 24 patients obtained before the first dose of silybinin and between 1 and 4 h after the last dose (Section 3.4.6 and 3.4.7 for details of materials and methods.)

7.2 Analysis of M_1dG adducts

7.2.1 Immunoslotblot method in colorectal tissue

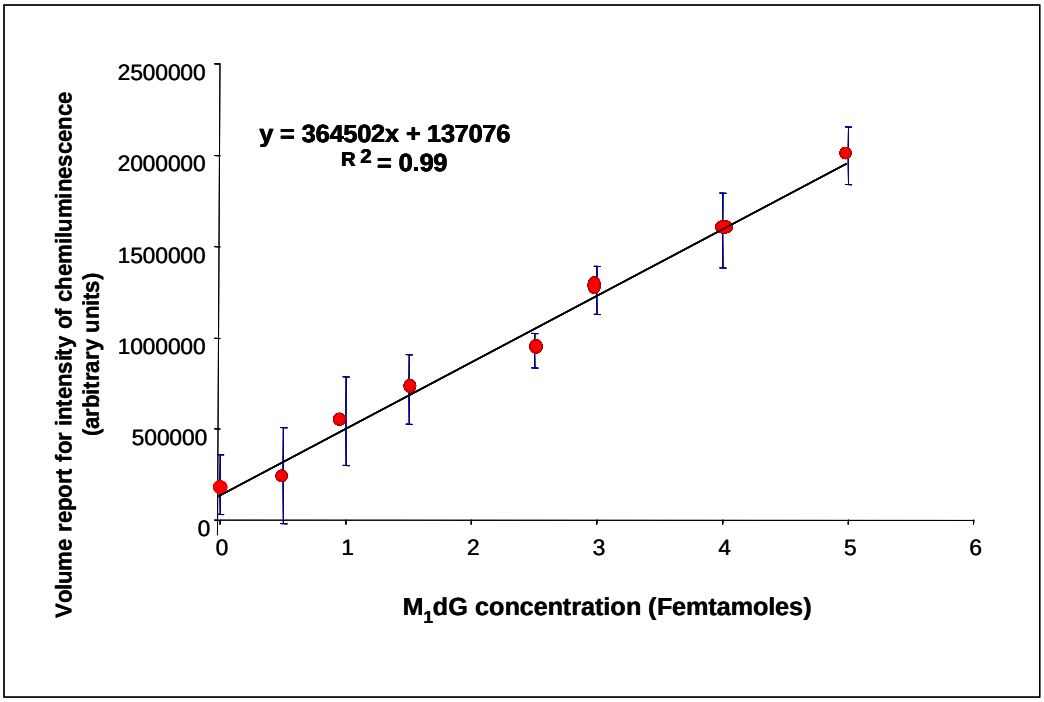
Varying concentration of the primary antibody was used with different exposure times used by the Genegnome^{TM} analyser until an optimum balance was achieved between detection of M_1dG levels in the tissue samples without saturation of the standard curve. However, very low concentration levels of M_1dG were present in the colorectal tissues. The calculations for M_1dG were on the lower limit of the standard curve derived from MDA-treated calf thymus DNA. Although the M_1dG levels in colorectal tumour tissue were consistently detectable, normal colorectal tissue frequently fell below the standard curve. In such instances, the quantity of M_1dG present were recorded as zero (Figure 7.1). Propidium iodide staining for DNA was undertaken to ensure equal loading in all wells (Figure 7.1).

Figure 7.1 Immunoslotblot image for M_1dG levels in colorectal tissue DNA demonstrating the consistent detection of M_1dG in tumour tissue and the very low levels in normal mucosa (A). Propidium iodide staining confirming equal DNA loading (B).



Immunoslot blot standard curves derived for colorectal tissue DNA displayed R^2 values of 0.97 to 0.99 (Figure 7.2).

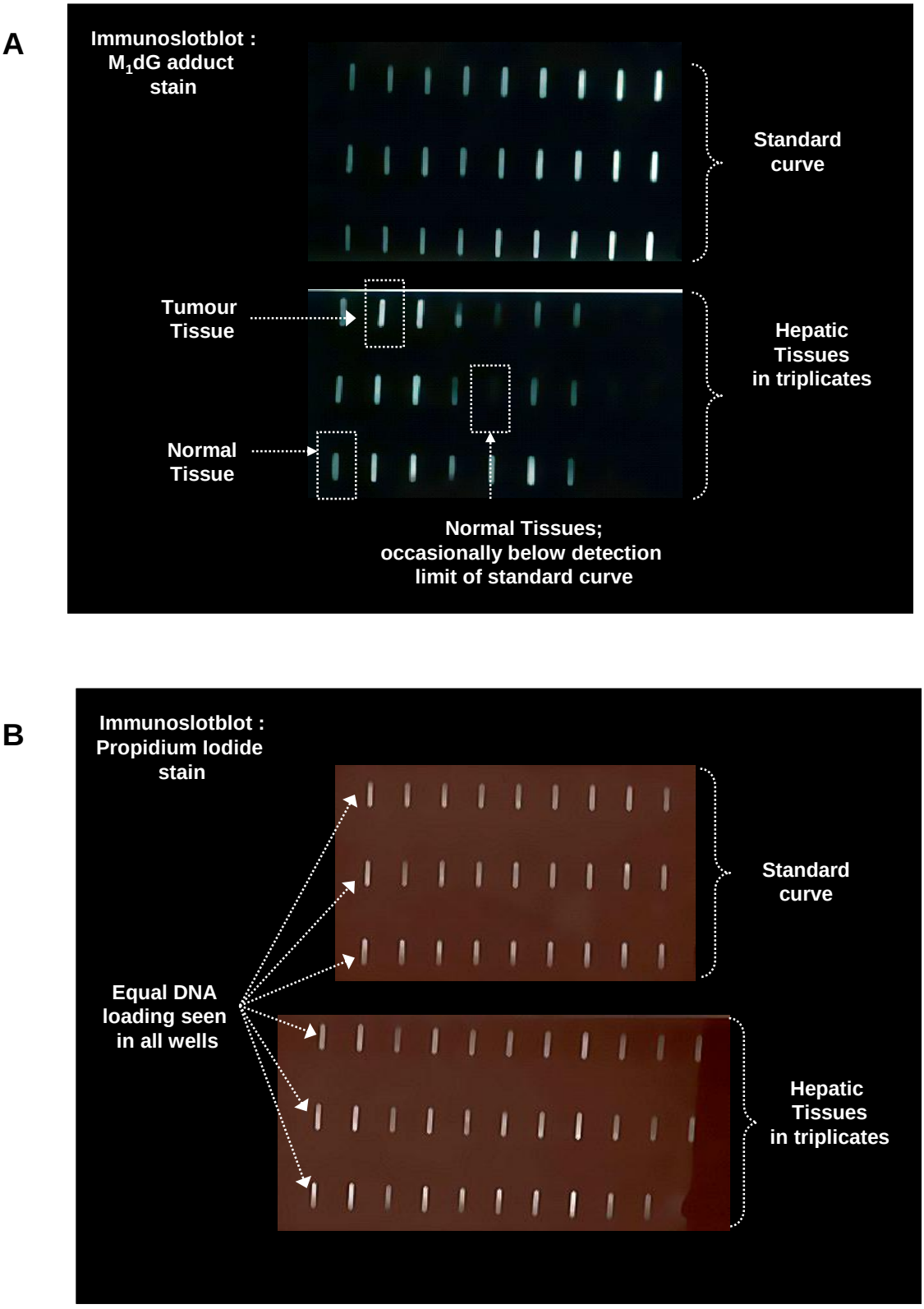
Figure 7.2 Typical calibration line for the standard curve showing regression line and R^2 values used for the analysis of colorectal tissue DNA.



7.2.2 Immunoslotblot method in hepatic tissue

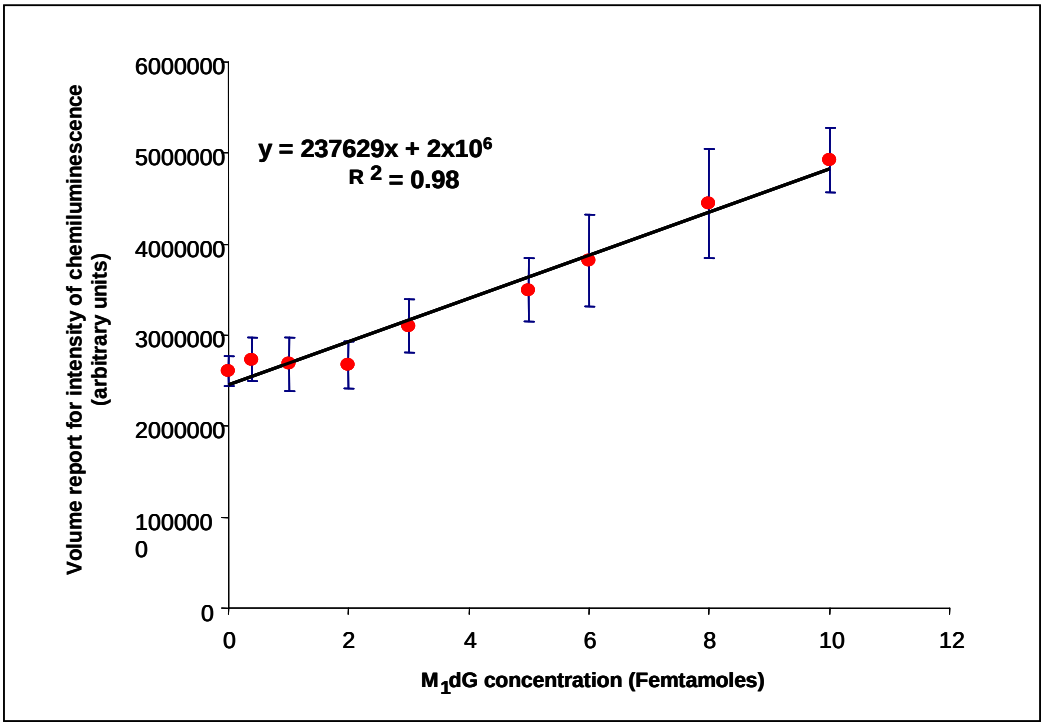
The binding of primary antibody to M_1dG was markedly higher in hepatic tissues than for colorectal tissues DNA. This was probably related to higher concentrations of M_1dG in liver tissues DNA due to the greater levels of oxidative stress being present in this metabolically active tissue. Compared to colorectal tissues, normal and tumour liver tissues only occasionally fell below the detection limit of the standard curve. Figure 7.3 shows an example of M_1dG adduct and propidium iodide staining for standards and hepatic tissue DNA determined using the Genegnome^{TM} fluorescence imager.

Figure 7.3 Immunoslotblot image for M_1dG levels in hepatic tissue DNA demonstrating the consistent detection of M_1dG in normal and tumour tissues (A). Propidium iodide staining confirming equal DNA loading (B).



Concentrations of M_1dG levels in hepatic tissue DNA was calculated from the regression lines derived from the standard curves, R^2 values of 0.97 to 0.98 were obtainable. A typical standard curve is shown below (Figure 7.4).

Figure 7.4 Typical calibration line for the standard curve showing regression line and R^2 value used for the analysis of hepatic tissue DNA**.**



7.3 The effect of silybinin on M_1dG adducts in leucocytes and tissue in patients

7.3.1 M_1dG adduct levels in peripheral blood

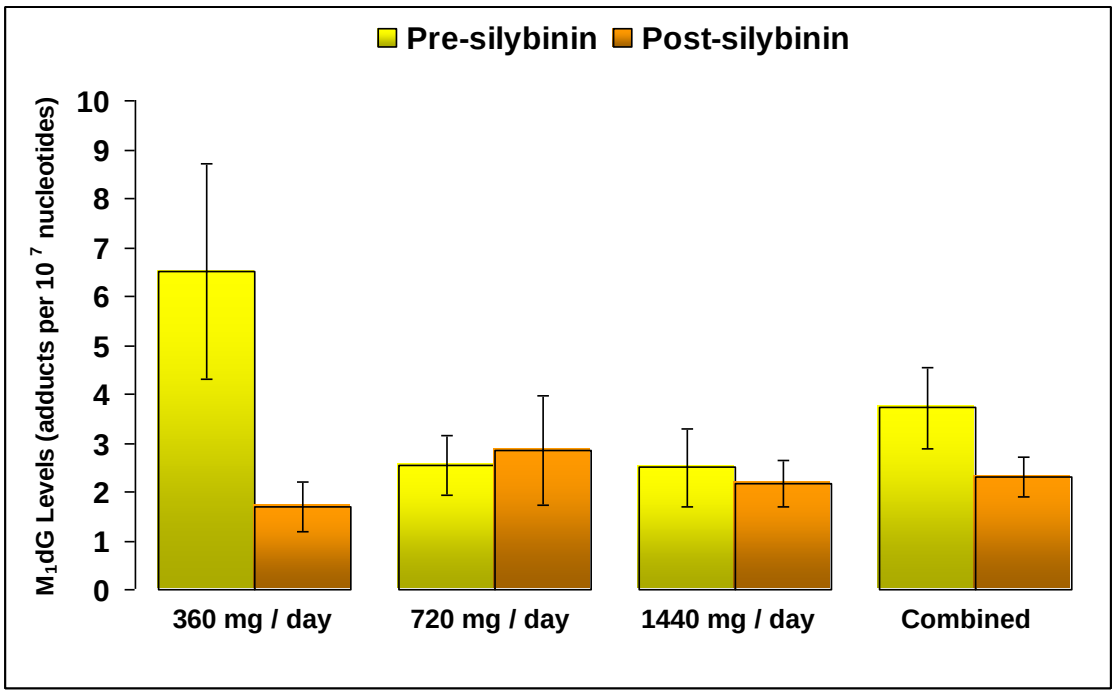
M_1dG levels in leucocytes from peripheral blood isolated before and after the intervention from twenty patients were 3.7 ± 0.8 and 2.3 ± 0.4 adducts per 10^7 nucleotides, respectively. In the remaining four individuals leucocytic pre-intervention M_1dG levels were below the limit of detection of the assay. The values are of the same order of magnitude as those reported previously for human blood (Leuratti *et al.*, 1998). Statistical comparison between pre- and post- intervention values of the individual dose groups or the combined doses failed to reveal significant differences, but in the low dose group (360 mg/day) and in the combined dose group, there was a trend towards a reduction in leucocytic M_1dG (p = 0.096) (Table 7.1**)**.

Table 7.1 Leucocytic M_1dG levels of pre and post-silybinin

Dose of Silybinin	M_1dG Levels in leucocytes (adducts per 10^7 nucleotides)		
	Pre- treatment	Post- treatment	*P* value
360 mg/day (n = 6 patients)	6.5 ± 2.2*	1.7 ± 0.5*	0.073 †
720 mg/day (n = 6 patients)	2.5 ± 0.6 *	2.8 ± 1.1 *	0.751 †
1440 mg/day (n = 8 patients)	2.5 ± 0.8 *	2.2 ± 0.5 *	0.604 †
Combined Doses (n = 20 patients)	3.7 ± 0.8 *	2.3 ± 0.4 *	0.096 †

* *Mean ± Standard Error of Mean*
† *P value was obtained using Wilcoxon (non-parametric) paired T test*

Figure 7.5 Comparison of M_1dG levels in pre- and post-treatment leucocytes of the individual and combined doses patients.



7.3.2 M_1dG adduct levels in colorectal tissues

We also compared the M_1dG levels in normal and malignant colorectal tissue obtained by biopsy and after surgery. The values measured in tissue samples were extremely variable between patients, with a substantial number of them close to or below the limit of detection, confounding meaningful interpretation. One factor which could cause such a situation would be if the quality and purity of the DNA was poor. In order to exclude this cause, re-extraction of the DNA of all the colorectal tissue samples were performed. The DNA obtained were then analysed using HPLC-UV detection following enzymatic hydrolysis to deoxynucleosites by Dr Raj Singh. Results showed definite amount of DNA were present. Doubling the amount of DNA to 2µg loading into each well did not improve the M_1dG adduct detection but caused increased background signals. All colorectal tissue samples were pooled and analyzed

in triplicates according to individual dose groups, before and after intervention. Table 7.2 and Figure 7.6, 7.7 summarises these findings.

Table 7.2 M_1dG levels in normal and malignant colorectal tissue before and after administration of silybinin.

Dose of Silybinin	M_1dG levels in normal colorectal tissue (adducts per 10^7 nucleotides)		M_1dG levels in malignant colorectal tissue (adducts per 10^7 nucleotides)	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
360 mg/day (pooled from 4 patients)	15.06	8.21	7.88	3.71
720 mg/day (pooled from 4 patients)	6.90	2.72	2.38	2.74
1440 mg/day (pooled from 4 patients)	2.88	2.41	1.69	2.73

Figure 7.6 Comparison of M_1dG levels in normal colorectal tissue before and after administration of silybinin (4 patient samples were pooled for analysis in each dose group)

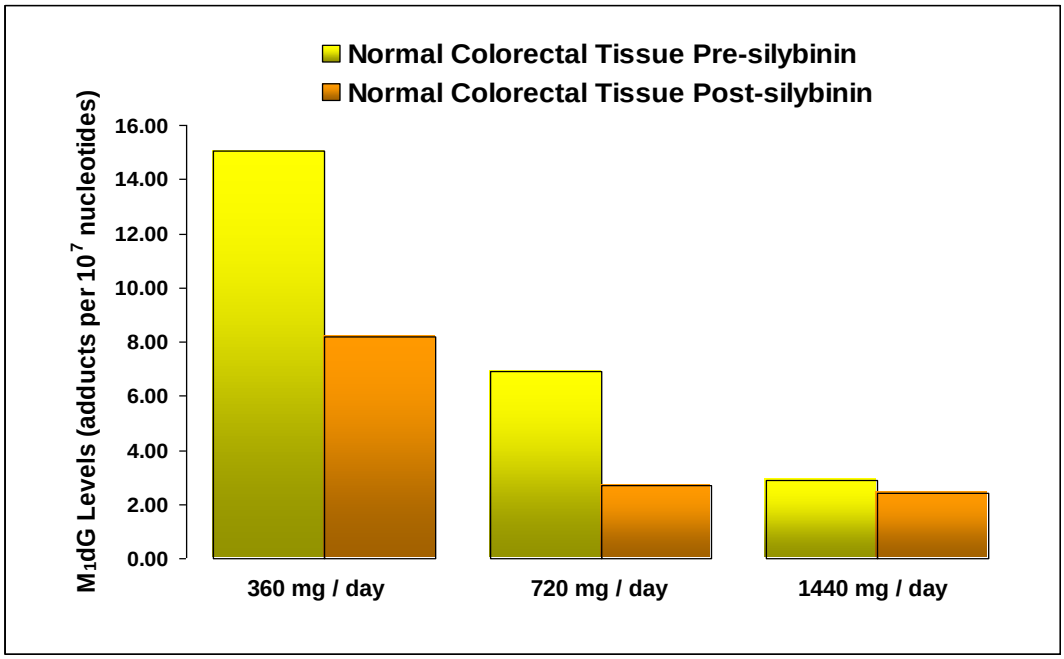
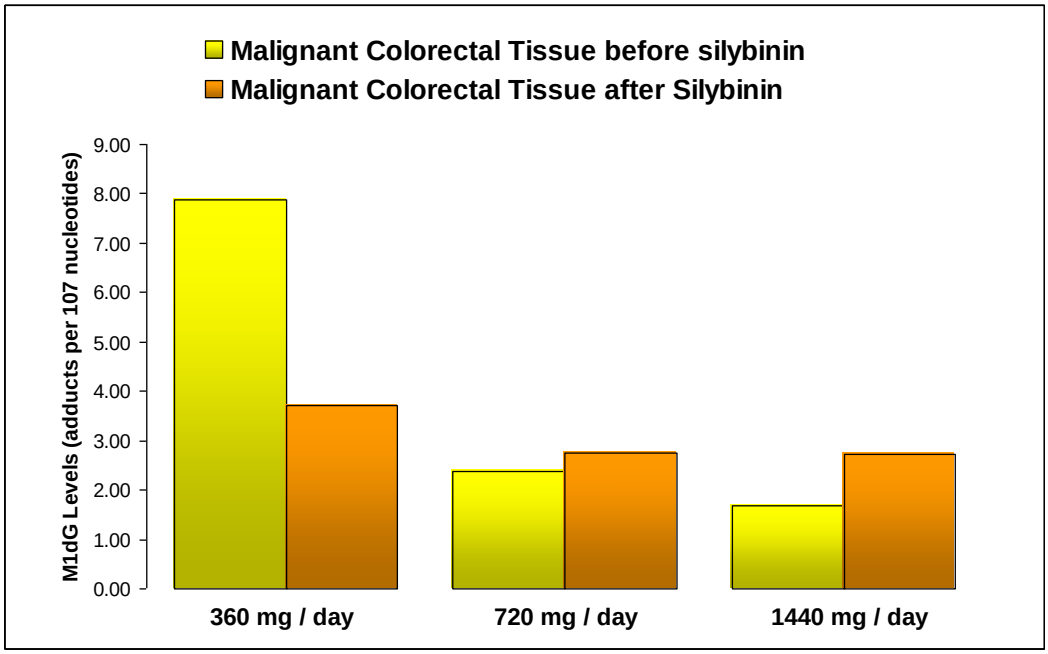


Figure 7.7 Comparison of M_1dG levels in malignant colorectal tissue before and after administration of silybinin (4 patient samples were pooled for analysis in each dose group)



7.3.3 M_1dG adduct levels in hepatic tissues

The M_1dG levels in normal and malignant hepatic tissues were measured and the values were variable and mostly below the limit of detection making it impossible to evaluate changes caused by the silybinin treatment. Hence, M_1dG analysis of the hepatic tissues were not performed.

7.4 The effect of silybinin on circulating IGF-1 and IGFBP-3 levels

Levels of IGFBP-3 and IGF-1 were studied as a potential marker of silybinin efficacy in the peripheral blood from 24 patients obtained before and after seven days treatment with silybinin. Table 7.3 and 7.4 display the levels of IGFBP-3 and IGF-1 in patients' serum before and after treatment with silybinin. Figure 7.8 and 7.9 show that there were no significant differences in concentration of IGFBP-3 or IGF-1 between pre-treatment and post-treatment serum at any of the dose levels. Neither did comparison of the molar ratio of IGF-1 to IGFBP-3 reveal any difference between pre-intervention and post-intervention serum (data not shown).

Table 7.3 Levels of IGFBP-3 in patients before and after 7-days of silybinin.

Dose of Silybinin	IGFBP-3 Levels in patients serum (x 1000 ng/mL)		
	Pre- treatment	Post- treatment	*P* value
360 mg/day (n = 8 patients)	5.8 ± 0.5 *	6.1 ± 0.2 *	0.539 †
720 mg/day (n = 8 patients)	6.2 ± 0.2 *	5.6 ± 0.3 *	0.160 †
1440 mg/day (n = 8 patients)	5.6 ± 0.2 *	5.2 ± 0.3 *	0.419 †

* *Mean ± Standard Error of Mean*
† *P value was obtained using Wilcoxon (non-parametric) paired T test*

Table 7.4 Levels of IGF-1 in patients before and after 7-days of silybinin.

Dose of Silybinin	IGF-1 Levels in patients serum (x 100 ng/mL)		
	Pre- treatment	Post- treatment	*P* value
360 mg/day (n = 8 patients)	3.01 ± 0.04 *	2.92 ± 0.04 *	0.792 †
720 mg/day (n = 8 patients)	3.04 ± 0.03 *	2.95 ± 0.03 *	0.578 †
1440 mg/day (n = 8 patients)	3.01 ± 0.03 *	2.58 ± 0.04 *	0.069 †

* *Mean ± Standard Error of Mean*
† *P value was obtained using Wilcoxon (non-parametric) paired T test*

Figure 7.8 Levels of IGFBP-3 in patients' plasma taken before and after 7 days treatment with oral silybinin.

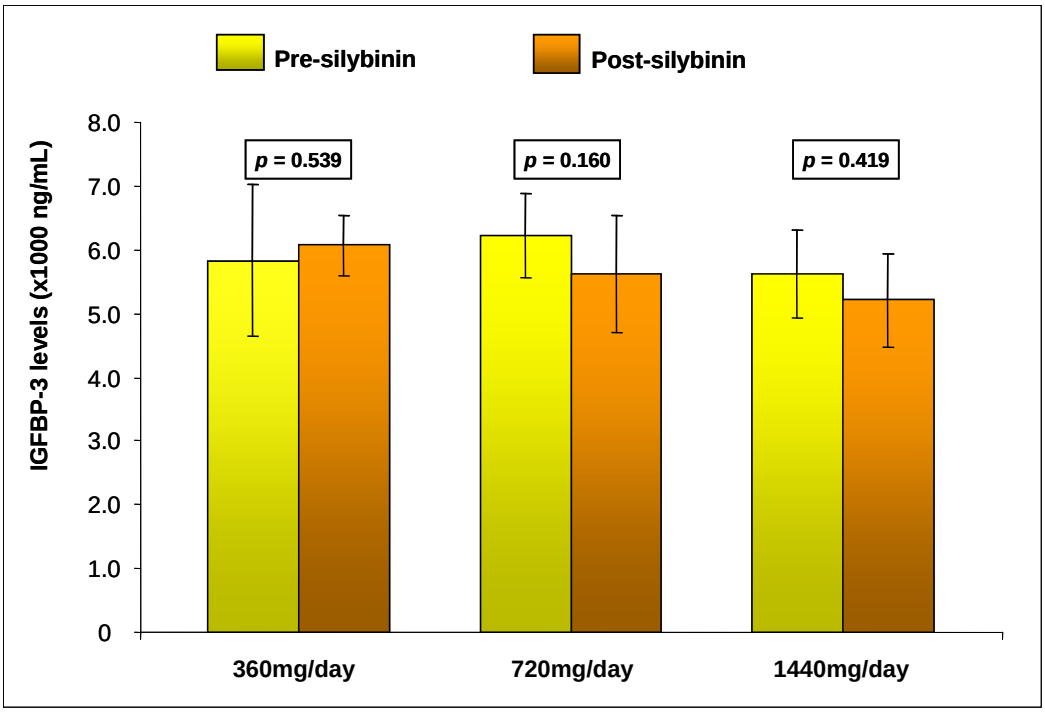
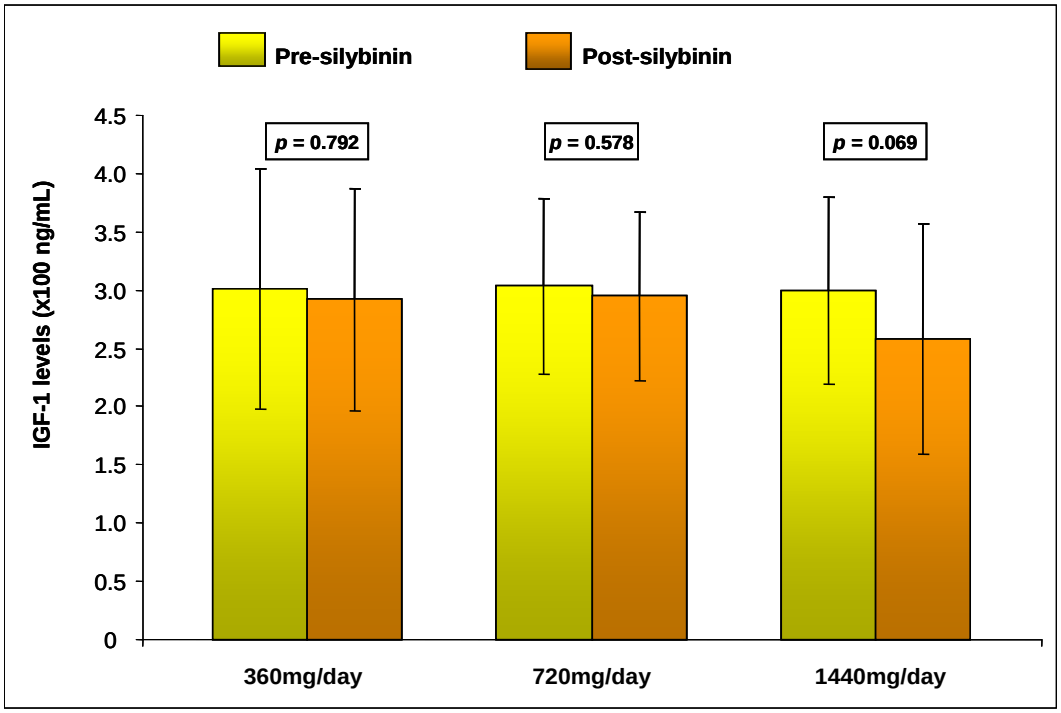


Figure 7.9 Levels of IGF-1 in patients' plasma taken before and after 7 days treatment with oral silybinin.



Statistical analysis of the difference between pre-intervention and post-intervention values of IGF-1 in serum from patients who consumed 1,440 mg silybinin (Figure 7.9) afforded $P \sim 0.07$, tentatively hinting at the possibility that, given a larger cohort of individuals and/or a longer period of intervention , this dose may decrease IGF-1 levels. When IGFBP-3/IGF-1 levels were compared between patients with different disease stage, there was no obvious difference between Duke's stage on the one side and IGFBP-3/IGF-1 levels or susceptibility towards modulation of biomarker levels by silybinin on the other (data not shown).

7.5 Discussion

In this chapter we have considered the levels of M_1dG and IGFBP-3 / IGF-1 as potential markers of silybinin efficacy in the peripheral blood and tissues of the 24 patients who received 7 days treatment with oral silybinin. Statistical comparison of the M_1dG levels in leucocytes between pre- and post-intervention values of the individual dose groups or the combined doses failed to reveal a significant difference in the intervention with silybinin. However in the low dose group (360 mg/day) and in the combined dose group, there was a trend towards a reduction in leucocytic M_1dG. This may hint of the possibility that M_1dG levels may be a suitable pharmacological marker for silybinin efficacy. Although the consumption of silybinin for a week did not significantly alter the levels of M_1dG in leucocytes, these preliminary findings provide a hint that the reduction in the leucocytic M_1dG levels might have been more substantial if the intervention period with silybinin was extended. There was little change in the antioxidant activity of silybinin recorded at the different dose levels.

As for the M_1dG levels in colorectal and hepatic tissues, the values were extremely variable and low, often falling below 2.5 adducts per 10^8 bases, the limit of detection for the immunoslotblot assay (Leuratti *et al.,* 1998). When the colorectal tissues were pooled and analyzed, there was an indication of a reduction in M_1dG levels in the post-intervention colorectal tissues. Even so, we failed to show a higher M_1dG level in malignant colorectal tissues as compared with normal colorectal tissues, as suggested by Leuratti *et al.* (Leuratti *et al.,* 2002). M_1dG levels in the hepatic tissues had been shown to be elevated after hypoxic reperfusion injury from the Pringle manoeuvre during hepatectomy (Garcea *et al.*, 2006). Consequently, measurement of M_1dG levels from post treatment liver tissues would not be representative of the antioxidant efficacy of silybinin in human liver.

The results presented here suggest that the concentration of silybinin achieved after consumption of seven daily doses of up to 1.44 g per day of silybinin is insufficient to affect circulating levels of IGF-1 or IGFBP-3. However at the highest dose group (1.44 g per day), a decrease in the post-intervention serum IGF-1 tentatively hints that given a larger cohort of individuals and/or a longer period of intervention, there may be a more significant reduction in the serum IGF-1 levels. We know from previous reports that an increase in IGF-1 protein appear to correspond to the increase invasiveness and metastatic potential of several types of cancers, in particular colorectal cancers (Freier *et al.*, 1999). In athymic mice bearing the DU-145 prostate tumor, administration of silybinin at 0.05% and 0.1% in the diet (equivalent to ~75 and ~150 mg/kg body weight, respectively, *per diem*) for the animals' lifetime caused 4-6 fold elevation of IGFBP-3 levels over controls, for the two doses respectively (Zi *et al.*, 2000). Similar findings were reported by a separate study in mice where oral administration of silybinin was found to suppress the growth of local and metastatic

tumours in xenografts model of renal cell carcinoma by increasing the plasma levels of IGFBP-3 (Cheung *et al.*, 2007).

In terms of dose extrapolation on the basis of surface area from mice to humans these doses are comparable to those used here (Section 3.3.1). Steady state plasma levels of silybinin, which accompanied the IGFBP-3 elevation activity in mice, were between 7 and 21 μM (3.4-10.1μg/mL), thus approximately two- to seven-fold above the levels measured in our human pilot study for the 1.44 g (highest) dose of silybinin. It is conceivable that plasma silybinin levels of 0.3 – 4.0 μM measured in patients from this study, was insufficient to show a statistical change of IGFBP-3 levels.

Chapter 8. FINAL DISCUSSION

In this thesis, a simple, robust, precise and reproducible analytical method for the quantification of silybinin in human plasma and tissues was developed. This method utilizes reverse-phase HPLC-UV and plasma protein precipitation techniques to separate the silybinin diastereoisomers from human plasma and tissues, giving a limit of detection and a limit of quantification that is comparable with, or better than, previously published HPLC-UV detection methods. Then, using this newly developed extraction method, levels of silybinin were obtained in mice *in vivo* and preliminary patterns of silybinin metabolites were identified. In addition, a clinical pilot study was carried out, whereby twenty four patients with resectable primary colorectal carcinoma and secondary hepatic metastases were given silipide (oral silybinin), divided in three dose groups; 360 mg, 720 mg or 1440 mg silybinin per day, for seven days. These patients' blood, colorectal and hepatic tissues were examined for silybinin levels, the presence of silybinin metabolites and levels of M_1dG and IGFBP-3 / IGF-1 as potential markers of silybinin efficacy were examined.

The outcome of the pilot study described here supports the notion that the repeated administration of silybinin at daily doses up to 1.44 g for a week is safe. This conclusion is consistent with the result of the original evaluation of silipide in human volunteers (Barzaghi *et al.*, 1990), and a similar inference was made in a phase I study of silybinin in hormone–refractory prostate cancer patients, in which up to 20 g silybinin, were administered orally daily for a month (Flaig *et al.*, 2007). We describe here for the first time the identification of silybinin plasma metabolites and measurement of silybinin tissue levels in humans who ingested silybinin. Consistent with results obtained previously using liver preparations incubated with silybinin *in vitro* (Marnett LJ *et al.* 2000), the results outlined here suggest that silybinin undergoes multiple conjugation reactions in human subjects.

The presence of metabolic conjugates of silybinin in the human biomatrix has hitherto been shown only indirectly (Gatti G. *et al.* 1994), in that raised levels of the parent molecule after enzymatic hydrolysis was taken to indicate the presence of conjugates. In contrast, here, the conjugate species silybinin mono-glucuronide, silybinin di-glucuronide, silybinin mono-sulfate, and silybinin glucuronide sulfate were unambiguously identified. The silybinin molecule possesses five hydroxy moieties (Figure 1.6), three of which are phenolic in nature, but the analysis described here does not allow the exact position of conjugation on the silybinin molecule to be elucidated. On the assumption that the antioxidant activity of silybinin is a function of its polyphenolic structure, the presence of silybinin mono-sulphate and mono-glucuronide, which bear (at least) two intact phenol moieties, suggests that appreciable amounts of circulating silybinin-derived species may share, at least to some extent, the antioxidant potency of the parent molecule.

The plasma levels of silybinin described here need to be compared with those reported previously in healthy volunteers who received oral silipide on a repeated dose schedule (Barzaghi *et al.*, 1990). In that study silipide at 720 mg (equivalent to 240 mg silybinin) given daily for 7 consecutive days furnished a mean peak plasma level of 0.38 μM (0.18 μg/mL) reached 0.9 h after administration of the last dose, and the terminal plasma half-life of the last dose was 3.4 h. These published data allow a tentative prediction of the levels achieved at the time points at which peripheral blood samples were taken for silybinin analysis in the pilot study described here (between 1 and 4 h after the last silipide dose). Blood was collected at times which coincide approximately between mean peak levels (i.e. about 1 h post dosage) and the terminal plasma half-life (i.e. about 4 h post dosage) of silybinin. The mean plasma levels for the 360 mg daily dose observed here, which were 0.3 - 0.4 μM (0.14 - 0.19 μg/mL),

are broadly consistent with the earlier volunteer study. However, in the recently completed phase I clinical trial of silybinin-phytosome (Siliphos ®) in patients with advanced prostate cancer, Flaig *et. al*. found free plasma silybinin peak levels to be as high as 100 μM.

This high level of free plasma silybinin levels obtained was mainly attributed to the high silybinin phytosome dose ingested, up to 20 g. Nevertheless, all these trials have identified that silybinin is safe when consumed orally, up to 13 g daily (Flaig *et al.*, 2007), the active drug is better absorbed when formulated with phosphatidylcholine, has a rapid peak, prominent interpatient variability and a short half-life when taken at standard doses.

The results presented here suggest that the concentration of silybinin achieved after consumption of seven daily doses of up to 1.44 g per day is insufficient to affect circulating levels of IGF-1, IGFBP-3 and M_1dG. It is of course conceivable that these putative efficacy biomarkers would be amenable to modulation by these doses of silybinin when given over longer periods of intervention. One week might have been too short to achieve a long-lasting effect on the IGF axis. In athymic mice bearing the DU-145 prostate tumor, administration of silybinin at 0.05 and 0.1% in the diet (equivalent to ~ 75 and ~ 150 mg/kg body weight daily, respectively) for the lifetime of animals caused 4- to 6-fold elevation of IGFBP-3 levels over controls, for the two doses, respectively (Singh *et al.* 2002a). In terms of dose extrapolation based on surface area from mice to humans, these doses are comparable with those used here (Section 3.3.1). Steady state plasma levels of silybinin, which accompanied the IGFBP-3-lowering activity in mice, were between 7 and 21 μM (3.4 and 10.1 μg/mL), thus approximately two- to seven-fold above the levels measured in our human pilot

study for the 1.44 g (highest) dose of silybinin. In a separate study in xenograft mice models of renal cell carcinoma, oral silybinin was found to suppress the growth of local and metastatic tumours accompanied by a 61% increase in the plasma levels of IGFBP-3 (Cheung *et al.*, 2007). Intervention with 9-*cis*-retinoic acid in former smokers was recently reported to cause a significant decrease in serum IGF-1 and an increase in IGFBP-3 (Lee *et al.*, 2005). It is pertinent to note that the period of intervention in that study was 3 months, substantially longer than the period of intervention with silybinin described here. Recently, preliminary evidence for the chemotherapeutic activity of silipide has been published. Repeated daily administration of silipide by oral gavage (at 450 mg/kg silipide, equivalent to 180 mg/kg silybinin) caused inhibition of ovarian tumor growth in nude mice, and levels of silybinin in the plasma and tumor *post* termination of the experiment were 15 μM (7.2 μg/mL) and 0.38 nmol/g (0.2 μg/g) tissue, respectively (Gallo *et al.*, 2003). Furthermore silipide at a dose of 1,800 mg/kg (equivalent to 720 mg/kg silybinin) given concomitantly with chemotherapy enhanced the antitumor activity of cis-platinum in a nude mouse model bearing the human A2780 ovarian cancer (Giacomelli *et al.*, 2002).

Levels of silybinin in human colorectal and liver tissues have not been described previously, although silybinin-containing remedies have long been marketed as liver protectants. In our study, the colorectal mucosal levels of silybinin were highly variable and not related to silipide dose, which may, at least to some extent, be the corollary of the difference between patients in time period (3 – 6 hours), which elapsed between the consumption of the last dose and surgery. The recommended oral dose of, for example, Legalon (Madaus, Germany), which contains 70 mg silymarin per tablet, is two tablets, taken three times a day. So the daily dose of silymarin

recommended for liver protection is 420 mg. Silymarin contains silydianin and silychristin as well as silybinin. On the assumption that silymarin contains ~ 80 % silybinin, this silymarin dose would translate into approximately 340 mg silybinin, which is similar to the low dose of silybinin administered in the pilot study described here. Based on this gross calculation, one may tentatively infer that silybinin concentrations in liver tissues that were measured here after the 360 mg dose (i.e. 0.3 - 0.5 μg/g or 0.6 - 1 μM in concentration terms) can afford protection of the human liver against toxic insult. In contrast to the relatively low systemic and hepatic levels of silybinin, levels achieved in colorectal tissue, between 9.6 - 68.0 μg/g or 20 and 141 μM in concentration terms, are highly likely to elicit pharmacological effects in the light of the concentrations reported to cause responses in cells in culture. For example, in cultured DU-145 prostate cancer cells, 15 and 30 μM (7.2 - 14.5 μg/mL) silybinin were sufficient to significantly compromise cell proliferation and increase IGFBP-3 in the cellular supernatant (Zi *et al.*, 2000).

In summary, these results validate the pharmacological safety of silybinin, which is needed for effective chemopreventive as well as chemotherapeutic agents. In addition, repeated administration of silipide achieved levels of silybinin in the colorectal tract similar to those known to exert pharmacological activity. Several silybinin sulphates and glucuronides have been identified in human blood, some of them retaining the intact phenol structure, a pharmacophoric feature, which may mediate, at least in part, pharmacological activity. Intervention for periods of a week seemed to be insufficient for orally consumed silybinin to affect the IGF-1/IGFBP-3 system in humans, and circulating silybinin-derived species were not abundant enough to reduce blood levels of M_1dG significantly. Nevertheless in the light of the colorectal cancer chemopreventive activity of silybinin in rodents (Gershbein, 1994; Volate *et al.*, 2005;

Kohno *et al.*, 2002) the high silybinin levels achieved in the human colorectal mucosa after consumption of safe silybinin doses support its further exploration as a human colorectal cancer chemopreventive agent. Silybinin-phosphotidylcholine doses higher than 1.44 g given for a longer sustained period, up to 4 weeks, may modulate biomarkers such as IGF-1, IGFBP-3 or M_1dG and show more convincing evidence of the chemopreventive effect of silybinin in colorectal cancer. Additional clinical research is warranted to evaluate further the chemopreventive as well as chemotherapeutic effects of silybinin against various human cancers.

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APPENDICES

MHRA
Safeguarding public health

Medicines and Healthcare products
Regulatory Agency
Market Towers
1 Nine Elms Lane, London SW8 5NQ

Telephone 020 7273 0328
Facsimile 020 7273 0443

Mr David P. Berry
Department of Hepatobiliary Surgery
The Leicester General Hospital
Gwendolen Road
Leicester
Leicestershire LE5 4PW

Our Ref: MF8000/13095

22 October 2003

Dear Mr Berry

THE MEDICINES (EXEMPTION FROM LICENCES) (SPECIAL CASES AND MISCELLANEOUS PROVISIONS) ORDER 1972
PRODUCT: Silipide

I am writing in connection with your notification under the Medicines (Exemption from Licences) (Special Cases and Miscellaneous Provisions) Order 1972 which relates to a proposed trial using **Silipide** supplied by **Indena s.p.a., Italy.**

This exemption is effective from 22 October 2003; the above-named supplier may lawfully supply the product for the purpose outlined in your notification: the Licensing Authority have decided not to issue a direction under article 4(2)(v) of the Order. There is no need for a marketing authorisation or for a clinical trial certificate for the purpose of the trial.

Please note that all serious unexpected adverse reactions occurring during the trial should be notified to the Licensing Authority.

In coming to its decision the Licensing Authority has not evaluated the safety, quality and efficacy of the product, and this notice should not be taken to imply approval of the product in terms of safety, quality or efficacy.

We shall be pleased to see a copy of any report which is produced as a result of this trial.

Yours sincerely

Mrs. S Syed.

Mrs S. Syed
CLINICAL TRIALS UNIT
ddxappro

Copy to Carmen, Andy Gedder, Wen Steward.
Please file

24 OCT 2003

MHRA
Safeguarding public health

Medicines and Healthcare products
Regulatory Agency

Market Towers
1 Nine Elms Lane, London SW8 5NQ

Telephone 020 7084 2327
Facsimile 020 7084 2443
Room 12-241

Mr David P. Berry
Department of Hepatobiliary Surgery
The Leicester General Hospital
Gwendolen Road
Leicester LE5 4PW

Our Ref:MF 8000/13095

8 January 2004

Dear Mr Berry

THE MEDICINES (EXEMPTION FROM LICENCES) (SPECIAL CASES AND MISCELLANEOUS PROVISIONS) ORDER 1972
PRODUCT: Silipide

I am writing in connection with your correspondence dated 7 January 2004 under the Medicines (Exemption from Licences) (Special Cases and Miscellaneous Provisions) Order 1972 which relates to a proposed trial using the above product.

Your current DDX will be rolled over as a CTA on 1 May 2004 without further application.

Yours sincerely

Ms D. Heffernan
CLINICAL TRIALS UNIT
ddxappro

Copy to Carmen | Wim | Andy S

Appendix C
Revised February 1992 Form MLA 163

DECLARATION BY SUPPLIER IN CONNECTION WITH A NOTIFICATION UNDER THE PROVISIONS OF THE MEDICINES (EXEMPTION FROM LICENCES) (SPECIAL CASES AND MISCELLANEOUS PROVISIONS) ORDER 1972 (SI 1200)

Name of Product: Silipide

Product Licence, Clinical Trial Certificate or Clinical Trial Certificate Exemption Number (If applicable) PL/CT/CTX .....UNIVERSITA' CATTOLICA DEL SACRO CUORE ROMA- ITALIA - ETHIC COMMITEE APPROVAL (COPY ENCLOSED)

Name of Practitioner: David P. Berry

Hospital/Practice Title: Consultant Hepatobiliary Surgeon

Address: Hepatobiliary Surgery
The Leicester General Hospital,
Gwendolen Rd, Leicester, Leicestershire LE5 4PW, UK....

In connection with the supply of the above named product to the above named practitioner for use in a clinical trial, I certify that this product is sold or supplied exclusively for this clinical trial, or if sold or supplied otherwise is sold or supplied in accordance with a Product Licence, Clinical Trial Certificate, or Clinical Trial Certificate Exemption, or in circumstances which enable sale or supply to be carried out otherwise than in accordance with such licence, certificate or certificate exemption.

Name: Dr. Paolo Morazzoni
INDENA S. p. A.
Scientific Director
(Dott. Paolo Morazzoni)

Ing. Dario Bonacorsi
INDENA S.p.A.
Chief Executive Officer

Signature: ..... **Date:** 24th September 2003

on behalf of:

Name of Supplier: Indena s.p.a

Address: Viale Ortles, 12
20139 Milano
Italy

Note: When completed this form should be sent to:

Medicines Control Agency
Clinical Trial Exemption Section
12th Floor
Market Towers
1 Nine Elms Lane
London SW8 5NQ

NHS

UHL 9174, Ethics ref: 7180

Leicestershire Local Research Ethics Committees
Lakeside House
4 Smith Way
Grove Park
Enderby
Leicester
LE19 1SS
Tel: 0116 295 7591
Fax: 0116 295 7582

Ethics Administration
Direct dial: 0116 295 7591/2

22 January 2004

7180 Please quote this number on all correspondence

Ms Carmen Hoh
Clinical Research Fellow
Cancer Biomarkers and Prevention Group
Department of Oncology
Robert Kilpatrick Clinical Sciences Building
LRI

Dear Ms Hoh

Re: Phase 1 trial of silibin and colorectal disease, ethics ref: 7180

I am receipt of your letter received on 16 January 2004 as detailed below:

Letter from C Hoh (undated)
DDX from Mrs S Syed, MHRA, dated 22 October 2003

Thank you for sending us the above. On behalf of Leicestershire Local Research Ethics Committee One, I have noted the above for information. Please be advised that ethical approval can now take full effect.

Your application has been given a unique reference number. Please use it on all correspondence with the LREC.

Yours sincerely

Dr PG Rabey
Chairman
Leicestershire Local Research Ethics Committee One

(N.B. All communications related to Leicestershire Research Ethics Committee must be sent to the LREC Office at Leicestershire, Northamptonshire and Rutland Health Authority. If, however, your original application was submitted through a Trust Research & Development Office, then any response or further correspondence must be submitted in the same way).

An advisory committee to Leicestershire, Northamptonshire and Rutland Strategic Health Authority

NHS

UHL 9176, Ethics ref: 7181

Leicestershire Local Research Ethics Committees

Lakeside House
4 Smith Way
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Fax: 0116 295 7582

Ethics Administration
Direct dial: 0116 295 7591/2

RESEARCH & DEVELOPMENT
26 JAN 2004
LIHL NHS TRUST

22 January 2004

7181 Please quote this number on all correspondence

Ms Carmen Hoh
Clinical Research Fellow
Cancer Biomarkers and Prevention Group
Department of Oncology
Robert Kilpatrick Clinical Sciences Building
LRI

Dear Ms Hoh

Re: Phase I trial of silibin and hepatic metastases, ethics ref: 7181

I am receipt of your letter received on 16 January 2004 as detailed below:

Letter from C Hoh (undated)
DDX from Mrs S Syed, MHRA, dated 22 October 2003

Thank you for sending us the above. On behalf of Leicestershire Local Research Ethics Committee One, I have noted the above for information. Please be advised that ethical approval can now take full effect.

Your application has been given a unique reference number. Please use it on all correspondence with the LREC.

Yours sincerely

P Rabey

Dr PG Rabey
Chairman
Leicestershire Local Research Ethics Committee One

(N.B. All communications related to Leicestershire Research Ethics Committee must be sent to the LREC Office at Leicestershire, Northamptonshire and Rutland Health Authority. If, however, your original application was submitted through a Trust Research & Development Office, then any response or further correspondence must be submitted in the same way).

Pilot Study of Oral Silibinin, a Putative Chemopreventive Agent, in Colorectal Cancer Patients: Silibinin Levels in Plasma, Colorectum, and Liver and Their Pharmacodynamic Consequences

Carmen Hoh,[1] David Boocock,[1] Tim Marczylo,[1] Rajinder Singh,[1] David P. Berry,[2] Ashley R. Dennison,[2] David Hemingway,[3] Andrew Miller,[3] Kevin West,[4] Stephanie Euden,[1] Giuseppe Garcea,[2] Peter B. Farmer,[1] William P. Steward,[1] and Andreas J. Gescher[1]

Abstract Silibinin, a flavonolignan from milk thistle, has intestinal cancer chemopreventive efficacy in rodents. It is a strong antioxidant and modulates the insulin-like growth factor (IGF) system by increasing circulating levels of IGF-binding protein 3 (IGFBP-3) and decreasing levels of IGF-I. Here, the hypothesis was tested that administration of oral silibinin generates agent levels in human blood and colorectal and hepatic tissues consistent with pharmacologic activity. Patients with confirmed colorectal adenocarcinoma received silibinin formulated with phosphatidylcholine (silipide) at dosages of 360, 720, or 1,440 mg silibinin daily for 7 days. Blood and biopsy samples of normal and malignant colorectum or liver were obtained before dosing, and blood and colorectal or hepatic tissues were collected at resection surgery after the final silipide dose. Levels of silibinin were quantified by high-pressure liquid chromatography-UV, and plasma metabolites were identified by liquid chromatography-mass spectrometry. Blood levels of IGFBP-3, IGF-I, and the oxidative DNA damage pyrimidopurinone adduct of deoxyguanosine (M_1dG) were determined. Repeated administration of silipide was safe and achieved levels of silibinin of 0.3 to 4 μmol/L in the plasma, 0.3 to 2.5 nmol/g tissue in the liver, and 20 to 141 nmol/g tissue in colorectal tissue. Silibinin monoglucuronide, silibinin diglucuronide, silibinin monosulfate, and silibinin glucuronide sulfate were identified in the plasma. Intervention with silipide did not affect circulating levels of IGFBP-3, IGF-I, or M_1dG. The high silibinin levels achieved in the human colorectal mucosa after consumption of safe silibinin doses support its further exploration as a potential human colorectal cancer chemopreventive agent.

Silibinin, a flavonolignan (for structure, see Fig. 1), is a major constituent of the seeds of milk thistle (*Silybum marianum* L.). Extracts of milk thistle are widely consumed as a dietary supplement especially in the United States. Silymarin, a standardized milk thistle extract, of which silibinin is the main component, has been evaluated clinically in the treatment of hepatitis and liver damage inflicted by alcohol and long-term treatment with psychotropic drugs (1–4). Recent evidence in rodents suggests that silymarin and silibinin may be useful in the chemoprevention of malignancies at a variety of sites, including the intestinal tract (5–10). Dietary silymarin delayed the development of intestinal adenocarcinomas in rats induced by dimethylhydrazine (8) or azoxymethane (9). It also suppressed aberrant crypt foci in rats, which had been exposed to azoxymethane, but this suppression was dose independent and did not involve induction of apoptosis (10). In our laboratory, silibinin interfered moderately with small intestinal carcinogenesis in the Apc^{Min} mouse model.[5] Silibinin has been formulated with phosphatidylcholine (silipide, Indena SpA, Milan, Italy) to improve its systemic availability, and clinical evaluation of this formulation at single or repeated doses reflects both its safety (summarized in ref. 11) and its improved bioavailability with respect to silymarin (12). Several mechanisms have been proposed to explain how silibinin may interfere with carcinogenesis. Among these mechanisms are impairment of receptor tyrosine kinase and erbB1 signaling and up-regulation of cyclin-dependent kinase inhibitors causing attenuation of cancer cell growth and perturbation of cell cycle progression (13, 14), induction of cancer cell differentiation (15), and antiangiogenesis (16). Silibinin has also been suggested to modulate the insulin-like growth factor (IGF) system. IGFs are mediators of cell survival in that they can inhibit apoptosis

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Requests for reprints: Andreas Gescher, Department of Cancer Studies, Robert Kilpatrick Clinical Sciences Building, Leicester Royal Infirmary, University of Leicester, Leicester LE2 7LX, United Kingdom. Phone: 44-116-223-1856; Fax: 44-116-223-1855; E-mail: ag15@le.ac.uk.

doi:10.1158/1078-0432.CCR-05-2724

[5] Verschoyle, Ho, Steward, Gescher, unpublished data.

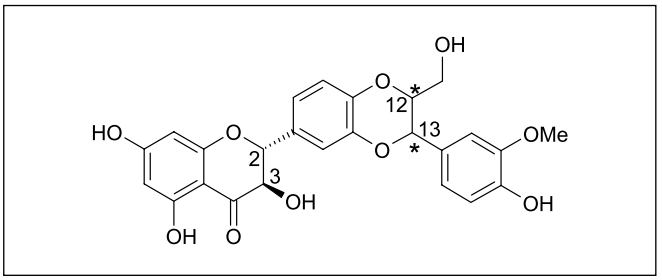

Fig. 1. Structure of naturally occurring silibinin, which is a diastereomeric mixture (1:1) of the two forms 2R, 3R, 12S, and 13S and 2R, 3R, 12R, and 13R. Asterisks, optically active centers.

and influence differentiation of many normal and malignant cell types (17–19). The IGF system is regulated by IGF-binding proteins (IGFBP), especially IGFBP-3, which bind IGFs in the extracellular milieu with high affinity and specificity, thus reducing the bioavailibility of IGFs. Epidemiologic studies have linked increased serum concentrations of IGF-I, decreased concentrations of IGFBP-3, or both with an increased risk of cancer at several sites, including the colorectum (20, 21). Silibinin increased levels of IGFBP-3 in prostate cells *in vitro* (22) and in tumor-bearing rodents *in vivo* (23). Silymarin and silibinin are also powerful antioxidants as a consequence of their polyphenolic structure (11, 24). Elevated oxidative stress has been implicated as an important carcinogenic stimulus, and endogenous or exogenous oxidative events, such as those associated with lipid peroxidation, generate malondialdehyde, a mutagen (25). Malondialdehyde can react with DNA to furnish the pyrimidopurinone adduct of deoxyguanosine M_1dG (26). M_1dG levels may be a suitable marker of chemopreventive efficacy of strong antioxidants, such as silibinin.

Silibinin undergoes extensive metabolism, especially phase II conjugation reactions, both in human liver preparations *in vitro* (27) and in volunteers *in vivo* after consumption of silibinin (28). There is no information available on the pharmacologic properties of metabolic conjugates of silibinin, and it is conceivable that at least some of them share pharmacologic activity with the parent molecule. Taken together, all of these results intimate both the propitiousness and the feasibility of developing silibinin as a colorectal cancer chemopreventive agent. To obtain pharmacokinetic and pharmacodynamic information to aid the design of future colorectal cancer intervention studies with silibinin, we carried out a pilot "presurgery model" study in colorectal cancer patients. Patients consumed silibinin daily for a week before colectomy, and blood and colorectal or hepatic tissue was obtained by biopsy and resection before and after the administration. Blood and tissues were analyzed to explore whether metabolites of silibinin can be identified in human blood and whether silibinin levels achievable in blood, colorectal, or hepatic tissue are comparable with pharmacologically active levels reported in cells *in vitro*. Furthermore we wished to find out if consumption of silibinin affects circulating levels of IGFBP-3, IGF-I, and the oxidative DNA damage adduct M_1dG. Overall, the study was designed to provide data, which helps optimize the design of long-term intervention studies of silibinin as a colorectal cancer chemopreventive agent.

Materials and Methods

Patients and tissues. Twelve patients (1 female and 11 male, ages 55-78 years, mean age 65 ± 7) with confirmed colorectal carcinoma of stages Dukes A (2 patients), B (5), or C (5), who were to undergo colorectal resection, and 12 patients (7 female and 5 male, ages 49-78 years, mean age 62 ± 9, all Dukes D) with hepatic metastatic disease originating from primary colorectal carcinoma, who were to undergo hepatic surgery, were recruited into the trial following approval by the University Hospitals of Leicester (Leicester, United Kingdom) ethics committee. All patients gave written informed consent. Hematologic profiles, plasma levels of urea and electrolytes, and hepatic function were within the reference range defined by the laboratories of the University Hospitals of Leicester. One patient who underwent colectomy had preoperative radiotherapy and none preoperative chemotherapy. All, except two patients who underwent hepatic surgery, had received 5-fluorouracil with folinic acid, oxaliplatin, and/or irinotecan before recruitment. Drug histories included antihypertensives, diuretics, antidepressants, and analgesics. Colorectal tumors in patients who underwent colorectal resection were located in the ascending/transverse colon (one patient), sigmoid colon (four patients), or rectum (seven patients). Tissue biopsy specimens taken at diagnosis weighed 3 to 60 mg (tumor biopsy) and 4 to 60 mg (normal tissue biopsy). Colectomy or hepatic resection was done 3 to 6 hours after the last dose of silipide. The weight of the surgical colorectal/liver tissue samples for chemical analysis was 1.1 to 1.5 g. Samples of peripheral blood were collected in heparinized tubes before intervention and 1 to 4 hours after the last silipide dose. Portal blood was taken at the point of hepatic surgery. Blood samples were centrifuged to generate leukocytes (for M_1dG measurement), serum (for IGF-I/IGFBP-3 measurement), and plasma [for high-pressure liquid chromatography (HPLC) analysis]. Blood components were kept on ice until storage at −80°C. Tissue samples were flash frozen in liquid nitrogen. Biomatrices were kept at −80°C for up to 6 months until analysis. Preliminary HPLC analysis had established that silibinin is stable under these conditions in tissues and plasma.

Silibinin formulation and dose. Silibinin was formulated in capsules as silipide (IdB 1016), a phytosome product marketed by Indena SpA for use as a hepatoprotectant (see www.indena.it/pdf/prodottiweb.pdf). Each silipide capsule contained 120 mg silibinin (validated by HPLC-UV analysis, see below) and soy phosphatidylcholine at a molar ratio of 1:1, which in terms of percentage weight constitutes ~40% silibinin and 60% phosphatidylcholine. Patients received silipide at dosages of either 360, 720, or 1,440 mg silibinin daily for 7 days before surgery; each daily dose was divided in three equal portions taken in the morning, at noon, and in the evening. There were eight individuals per dose level (four patients who underwent colectomy and four who had liver resection). The first and second portions of the first dose were taken at noon and in the evening, respectively, of day 1; the last dose portion was ingested in the morning of day 8 before surgery so that, in total, the seven daily doses were distributed >8 days. The doses used in this pilot study were based approximately on the dose that raised plasma IGFBP-3 levels in mice (23). In that study, mice received silibinin at 0.05% or 0.1% in the diet, which equates to ~75 and 150 mg/kg daily, respectively. These doses extrapolated to humans based on body surface area (420 mg/m^2 in mice; ref. 29) would amount to ~450 or ~900 mg silibinin/person daily, assuming a body surface area of 1.8 m^2 accompanying a body weight of 70 kg. Although such extrapolation has to be interpreted with caution, the doses proposed for the pilot study here cover the dose shown to be efficacious in mice.

Analysis of silibinin and silibinin conjugates. Biomatrices were thawed, weighed, and homogenized in an equal part of KCl solution (0.15 mol/L). Samples of plasma or tissue homogenate were mixed with 3 parts ice-cold methanol. The mixture was centrifuged, and the supernatant was decanted and dried under nitrogen, reconstituted in aqueous methanol (70%, containing 5% acetic acid), and analyzed for silibinin by HPLC with UV detection using a gradient system with a

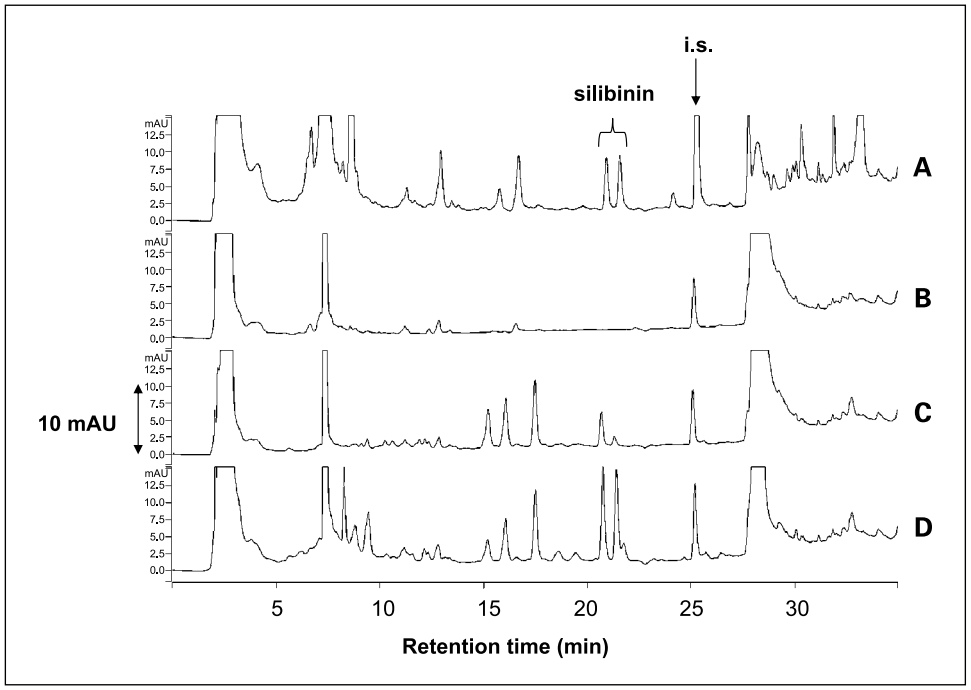

Fig. 2. HPLC-UV chromatograms of extracts of pooled plasma added to silibinin (400 ng/mL) (*A*), plasma obtained from a patient before the first dose of silipide (*B*) or 3 hours after the last of seven daily doses of silipide (1,440 mg silibinin, administered in three divided portions; (*C*) and plasma sample *C* incubated for 1 hour with sulfate- and glucuronide-deconjugating enzymes from *H. pomatia* (*D*). i.s., internal standard. For details of dosing, sample preparation, and HPLC analysis, see Materials and Methods.

two-component mobile phase. The details, characterization, and validation of the method are described in a separate article.[6] The limit of quantitation for the two silibinin diastereoisomers was 5 and 3 ng/mL. Some plasma samples were incubated [1 hour, 37°C, 0.1 mol/L ammonium acetate buffer (pH 6.0)] with β-glucuronidase from *Helix pomatia* (type H-2, containing sulfatase; Sigma-Aldrich, Poole, United Kingdom) before extraction.

Silibinin and some of its metabolites were characterized by LC-mass spectrometry (MS) using a turbospray source in negative electrospray ionization mode. Analyses were done using an API 2000 LC-MS (Applied Biosystems/MDS Sciex, Warringon, United Kingdom) equipped with an Agilent (South Queensferry, United Kingdom) 1100 series sample delivery system. Separation of silibinin and metabolites was achieved using the method mentioned above. MS conditions were as follows: declustering potential, −26 V; focusing potential, −350 V; electrode potential, −12 V; cell entrance potential, −16 V; cell exit potential, −20 V; and temperature, 500°C. Identification of silibinin-derived species was by selected ion monitoring.

Pharmacodynamic measurements. IGF-I and IGFBP-3 concentrations in patients' serum were determined using ELISA kits 10-5600 Active and 10-6600 Active, respectively, from Diagnostic Systems Laboratories (Oxon, United Kingdom). The IGF-I kit procedure contained an acid-ethanol extraction step to separate IGFs from their binding sites. The assays were validated and done according to the instructions of the manufacturer. The molar ratio of IGF-I to IGFBP-3 was calculated as [0.13 × IGF-I concentration (ng/mL)] / [0.036 × IGFBP-3 concentration (ng/mL)].

DNA was extracted from leukocytes or tissues using the Qiagen (Crawley, United Kingdom) kit, and M_1dG adduct levels were measured in triplicate by immunoslot blot using a murine monoclonal anti-M_1dG antibody provided by Dr. Lawrence Marnett (Vanderbilt University, Nashville, TN) as described previously (30). Goat and anti-mouse horseradish peroxidase–conjugated secondary antibodies were purchased from Dako Cytomation (Ely, United Kingdom). Discrepancies in the amount of DNA per slot were corrected for by staining the nitrocellulose filter with propidium iodide and doing UV light densitometry. The detection limit for M_1dG was ~1 adduct/10^7 nucleotides.

Results

Identification of silibinin and metabolites in blood. Patients with confirmed colorectal cancer received silibinin formulated as silipide at 360, 720, or 1,440 mg daily for a week before colorectal or hepatic surgery (for removal of liver metastases). This intervention was not associated with any adverse effect of silibinin. Silibinin exists as two *trans*-diastereoisomers (Fig. 1), furnishing two peaks on HPLC analysis with retention times near 21 minutes. All three doses afforded measurable peaks in the plasma at the retention times of authentic silibinin. Figure 2 shows the chromatogram of peripheral plasma from a patient who received the highest dose. These peaks were unambiguously identified as silibinin by MS, as they afforded *m/z* 481 $[M\text{-}H^+]^-$ consistent with the mass spectrum of authentic silibinin. Plasma sample extracts were also scanned by MS for metabolic species derived from silibinin. Silibinin monoglucuronide, silibinin monosulfate, and silibinin diglucuronide

Table 1. HPLC-MS analysis of species related to silibinin in plasma from humans who had received silibinin (1,440 mg) daily for 7 days as silipide capsules

m/z $[M\text{-}H^+]^-$	Inference	Retention time (min)
481	Silibinin	20.8; 21.4
657	Silibinin glucuronide	12.6; 15.6; 16.4; 17.8
833	Silibinin diglucuronide	9.2; 10.8
561	Silibinin sulfate	17; 17.8; 18.8

[6] C.S.L. Hoh, et al. Quantitation of silibinin, a putative cancer chemopreventive agent, in human plasma by high performance liquid chromatography, submitted for publication.

could be detected (Table 1). Single ion monitoring yielded multiple peaks, which could be assigned to silibinin monoglucuronide, silibinin diglucuronide, silibinin monosulfate, and silibinin glucuronide sulfate (Fig. 3). There was also some evidence for the presence of silibinin triglucuronide (*m/z* 1,008) and *O*-desmethyl silibinin glucuronide (*m/z* 643; data not shown). Because of the occurrence of multiple diastereoisomers of silibinin derivatives and given the absence of authentic reference materials, it is impossible to assign specific positional isomers to the multiple peaks seen on single ion monitoring. Figure 2 illustrates that incubation with conjugate-hydrolyzing enzymes furnished a marked increase in the height of the parent silibinin peaks, consistent with the abundant presence of silibinin sulfate and/or glucuronide conjugates.

Silibinin levels in blood and tissues. Extracts of samples of plasma or of normal or malignant colorectal or hepatic tissue were subjected to quantitative HPLC-UV analysis. Peripheral plasma levels were between 0.3 and 4 μmol/L, and they were related to silipide dose (Table 2). Silibinin levels in portal plasma were similar to those in peripheral plasma. Levels of silibinin in normal and malignant colorectal tissue showed considerable variation. They were between 20 and 141 nmol/g and not strictly related to silipide dose. Concentrations of silibinin in hepatic tissue were similar to those in plasma (Table 2).

Effect of silibinin on circulating levels of IGFBP-3, IGF-I, and M_1dG. Levels of IGFBP-3, IGF-I, and M_1dG were studied as potential markers of silibinin efficacy in the peripheral blood from 24 patients obtained before the first dose of silibinin and between 1 and 4 hours after the last (i.e., seventh) dose. Figure 4 shows that there was no significant difference in concentration of IGFBP-3 or IGF-I between pretreatment and posttreatment serum at any of the dose levels. Neither did comparison of the molar ratio of IGF-I to IGFBP-3 reveal any difference between pre-intervention and post-intervention serum (data not shown). Statistical analysis of the difference between pre-intervention and post-intervention values for IGF-I in serum from patients on 1,440 mg silibinin (Fig. 4B) afforded $P = 0.07$, tentatively hinting at the possibility that, given a larger cohort of individuals and/or a longer period of intervention, this dose may decrease IGF-I levels. When IGFBP-3/IGF-I levels were compared between patients with different disease stage, there was no obvious difference between Dukes stage on the one side and IGFBP-3/IGF-I levels or susceptibility toward modulation of biomarker levels by silibinin on the other (data not shown).

M_1dG levels in leukocytes from peripheral blood isolated before and after the intervention from 20 patients were 3.7 ± 3.7 and 2.3 ± 1.8 adducts per 10^7 nucleotides, respectively. In the remaining four individuals, leukocytic pre-intervention M_1dG levels were below the limit of detection. The values are of the same order of magnitude as those reported previously for human blood (31). Statistical comparison between pre-intervention and post-intervention values of the individual dose groups or the combined doses failed to reveal significant differences, suggesting that consumption of silibinin for a week did not markedly alter leukocytic M_1dG. We also compared M_1dG levels in normal and malignant tissues obtained by biopsy and after surgery. The values measured in tissue samples were extremely variable between patients, with a substantial number of them close to or at the limit of detection, confounding meaningful interpretation.

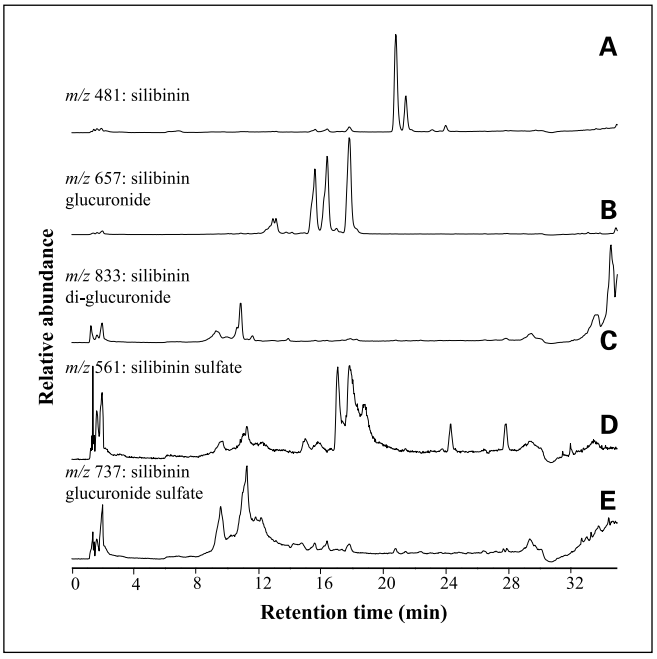

Fig. 3. HPLC-MS selected ion monitoring chromatograms of an extract of a patient's plasma obtained 3 hours after the last of seven daily doses of silipide (1,440 mg silibinin, administered in three divided portions). The following $[M\text{-}H^+]^-$ ions were monitored: 481 (*m/z* silibinin; *A*), 657 (*m/z* silibinin glucuronide; *B*), 833 (*m/z* silibinin diglucuronide; *C*), 561 (*m/z* silibinin sulfate; *D*), and 737 (*m/z* silibinin glucuronide sulfate; *E*). For details of dosing and analysis, see Materials and Methods.

Discussion

The outcome of the pilot study described here supports the notion that the repeated administration of silibinin at daily doses up to 1.44 g for a week is safe. This conclusion is consistent with the result of the original evaluation of silipide in human volunteers (12), and a similar inference was made in a preliminary report of a current phase I study of silibinin in hormone-refractory prostate cancer patients, in which up to 20 g silibinin was administered orally daily for a month (32). We describe here for the first time the identification of silibinin plasma metabolites and measurement of silibinin tissue levels in humans who ingested silibinin. Consistent with results obtained previously using liver preparations incubated with silibinin *in vitro* (27), the results outlined here suggest that silibinin undergoes multiple conjugation reactions in humans. The presence of metabolic conjugates of silibinin in the human biomatrix has hitherto been shown only indirectly (28), in that raised levels of the parent molecule after enzymatic hydrolysis was taken to indicate the presence of conjugates. In contrast, here, the conjugate species silibinin monoglucuronide, silibinin diglucuronide, silibinin monosulfate, and silibinin glucuronide sulfate were unambiguously identified. The silibinin molecule possesses five hydroxy moieties (Fig. 1), three of which are phenolic in nature, but the analysis described here does not allow the exact position of conjugation on the silibinin molecule to be elucidated. On the assumption that the antioxidant activity of silibinin is a function of its polyphenolic structure, the presence of silibinin monosulfate and monoglucuronide, which bear (at least) two intact phenol moieties,

Table 2. Silibinin levels in blood and colorectal or hepatic tissues of colorectal cancer patients who had received silibinin daily for 7 days as silipide capsules

Dose (mg/d)	Peripheral blood levels (μmol/L)	Colorectal tissue levels (nmol/g)	
		Normal	Malignant
360	0.3 ± 0.3*	28 ± 31	37 ± 51
720	0.7 ± 0.6	121 ± 181	20 ± 26
1,440	3 ± 2.3	141 ± 169	68 ± 98

Dose (mg/d)	Blood levels (μmol/L)		Hepatic tissue levels (nmol/g)	
	Peripheral	Portal	Normal	Malignant
360	0.4 ± 0.2	0.5 ± 0.3	1 ± 0.9	0.6 ± 0.5
720	1.4 ± 1	0.5 ± 0.4	1.2 ± 1.1	0.3 ± 0.1
1,440	4 ± 5.3	1.6 ± 0.3	2.5 ± 2.4	0.6 ± 0.7

*Mean ± SD of four patients.

suggests that appreciable amounts of circulating silibinin-derived species may share, at least to some extent, the antioxidant potency of the parent molecule.

The plasma levels of silibinin described here need to be compared with those reported previously in healthy volunteers who received oral silipide on a repeated dose schedule (12). In that study, silipide at 720 mg (equivalent to 240 mg silibinin) given daily for 7 consecutive days furnished a mean peak plasma level of 0.38 μmol/L (0.18 μg/mL) reached 0.9 hour after administration of the last dose, and the terminal plasma half-life of the last dose was 3.4 hours. This published data allows a tentative prediction of the levels achieved at the time points at which peripheral blood samples were taken for silibinin analysis in the pilot study described here (between 1 and 4 hours after the last silipide dose). Blood was collected at times that coincide approximately with peak levels on the one side and ~0.9 half-life beyond peak levels on the other. The mean plasma levels for the 360-mg daily doses observed here, which were 0.3 to 0.4 μmol/L (0.14-0.19 μg/mL), are broadly consistent with the earlier volunteer study. The results presented here suggest that the concentration of silibinin achieved after consumption of seven daily doses of up to 1.44 g daily is insufficient to affect circulating levels of IGF-I, IGFBP-3, and M_1dG. It is of course conceivable that these putative efficacy biomarkers would be amenable to modulation by these doses of silibinin when given over longer periods of intervention. One week might have been too short to achieve a long-lasting effect on the IGF axis. In athymic mice bearing the DU-145 prostate tumor, administration of silibinin at 0.05% and 0.1% in the diet (equivalent to ~75 and ~150 mg/kg body weight daily, respectively) for the lifetime of animals caused 4- to 6-fold elevation of IGFBP-3 levels over controls, for the two doses, respectively (23). In terms of dose extrapolation based on surface area from mice to humans, these doses are comparable with those used here (see Materials and Methods). Steady-state plasma levels of silibinin, which

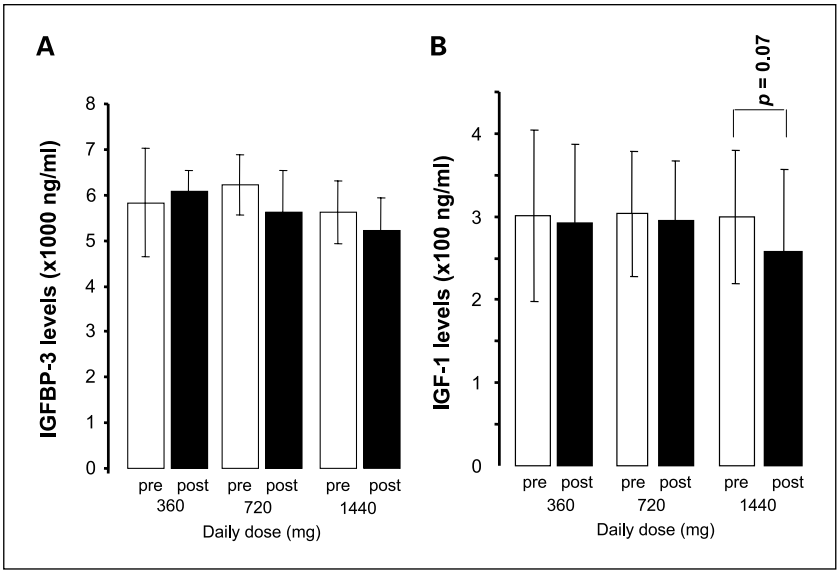

Fig. 4. Concentrations of IGFBP-3 (*A*) and IGF-I (*B*) in serum from patients obtained before dosing with silipide (*pre; white columns*) or 1 to 4 hours after (*post; black columns*) the last of eight daily doses of silipide (360, 720, or 1,440 mg silibinin, administered in three divided portions). Analysis was by ELISA using commercially available kits. Columns, mean of eight patients for the 720-mg and 1,440-mg dose levels and five patients for the 360-mg dose; bars, SD. *P* derived by statistical comparison using paired Student's *t* test. For details of IGFBP-3 and IGF-I analyses, see Materials and Methods.

accompanied the IGFBP-3-lowering activity in mice, were between 7 and 21 μmol/L (3.4 and 10.1 μg/mL), thus, ~2- to 7-fold above the plasma levels measured in our human pilot study for the 1.44 g (highest) dose of silibinin. Intervention with 9-*cis*-retinoic acid in former smokers was recently reported to cause a significant decrease in serum IGF-I and an increase in IGFBP-3 (33). It is pertinent to note that the period of intervention in that study was 3 months, substantially longer than the period of intervention with silibinin described here. Recently, preliminary evidence for the chemotherapeutic activity of silipide has been published. Repeated daily administration of silipide by oral gavage (at 450 mg/kg silipide, equivalent to 180 mg/kg silibinin) caused inhibition of ovarian tumor growth in nude mice, and levels of silibinin in the plasma and tumor after termination of the experiment were 15 μmol/L (7.2 μg/mL) and 0.38 nmol/g (0.2 μg/g) tissue, respectively (34). Furthermore, silipide at a dose of 1,800 mg/kg (equivalent to 720 mg/kg silibinin) given concomitantly with chemotherapy enhanced the antitumor activity of *cis*-platinum in a nude mouse model bearing the human A2780 ovarian cancer (35).

Levels of silibinin in human colorectal and liver tissue have not been described previously, although silibinin-containing remedies have long been marketed as liver protectants. In our study, the colorectal mucosal levels of silibinin were highly variable and not related to silipide dose, which may, at least to some extent, be the corollary of the difference between patients in time period (3-6 hours), which elapsed between the consumption of the last dose and surgery. The recommended oral dose of, for example, Legalon (Madaus, Germany), which contains 70 mg silymarin/tablet, is two tablets, taken thrice daily. So the daily dose of silymarin recommended for liver protection is 420 mg. Silymarin contains silydianin and silychristin as well as silibinin. On the assumption that silymarin contains ~80% silibinin, this silymarin dose would translate into ~340 mg silibinin, which is similar to the low dose of silibinin administered in the pilot study described here. Based on this gross calculation, one may tentatively infer that silibinin concentrations in liver tissue of an order of magnitude similar to those measured here after the 360-mg dose (i.e., 0.3-0.5 μg/g or 0.6-1 μmol/L in concentration terms) can afford protection of the human liver against toxic insult. In contrast to the relatively low systemic and hepatic levels of silibinin, levels achieved in colorectal tissue, between 9.6 and 68 μg/g or 20 to 141 μmol/L in concentration terms, are highly likely to elicit pharmacologic effects in the light of the concentrations reported to cause responses in cells in culture. For example, in cultured DU-145 prostate cancer cells, 15 and 30 μmol/L (7.2-14.5 μg/mL) silibinin were sufficient to significantly compromise cell proliferation and increase IGFBP-3 in the cellular supernatant (22).

In summary, repeated administration of silipide achieved levels of silibinin in the colorectal tract similar to those known to exert pharmacologic activity. Several silibinin sulfates and glucuronides have been identified in human blood, some of them retaining the intact phenol structure, a pharmacophoric feature, which may mediate, at least in part, pharmacologic activity. Intervention for periods of a week seemed to be insufficient for orally consumed silibinin to affect the IGF-I/IGFBP-3 system in humans, and circulating silibinin-derived species were not abundant enough to reduce blood levels of M_1dG significantly. Nevertheless, in the light of the colorectal cancer chemopreventive activity of silibinin in rodents (8–10), the high silibinin levels achieved in the human colorectal mucosa after consumption of safe silibinin doses support its further exploration as a human colorectal cancer chemopreventive agent.

Acknowledgments

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Quantitation of Silibinin, a Putative Cancer Chemopreventive Agent Derived from Milk Thistle (*Silybum marianum*), in Human Plasma by High-Performance Liquid Chromatography and Identification of Possible Metabolites

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Silibinin has recently received attention as a potential cancer chemopreventive agent because of its antiproliferative and anticarcinogenic effects. A simple and specific reversed-phase high-performance liquid chromatography method was developed and validated for the quantitation of silibinin in human plasma. Sample preparation involved simple protein precipitation, and separation was achieved on a Waters Atlantis C_{18} column with flow rate of 1.0 mL/min at 40 °C and UV detection at 290 nm. Silibinin was detected as two peaks corresponding to trans-diastereoisomers. The peak area was linear over the investigated concentration range (0−5000 ng/mL). The limits of detection were 2 and 1 ng/mL for the two diastereoisomers (d1 and d2), with a recovery of 53−58%. This method was utilized to detect silibinin in plasma of colorectal patients after 7 days of treatment with silipide (silibinin formulated with phosphatidyl choline).

KEYWORDS: Silibinin; silipide; silybin; IDB 1016; HPLC; human plasma; milk thistle; *Silybum marianum*

INTRODUCTION

Silymarin, a mixture of flavonolignans extracted from the seeds, fruits, and leaves of milk thistle (*Silybum marianum*), has been used traditionally for the treatment of hepatic disorders such as acute viral hepatitis (*1*), alcoholic liver disease (*2*, *3*), and death cap mushroom poisoning (*4*). Silymarin consists of silibinin (34%), isosilibinin (26%), silydianin (20%), silycristin (20%), and traces of taxifolin (*5*). Silibinin exists as two trans-diastereoisomers (*6*) (**Figure 1**). The names silybinin, silibinin, silibin, or silybin have been used interchangeably; here, the term "silibinin" will be used throughout.

In recent years, silibinin has received much attention regarding its antiproliferative and anticarcinogenic effects in a variety of neoplasias as reflected in experiments with cancer cell lines (*7*, *8*) and in rodents (*9*, *10*). Silibinin has previously been shown to have low oral bioavailability (*11*). Therefore, a formulation (Silipide) combining silibinin with phosphatidyl choline at a molar ratio has been developed to increase its absorption (*12*). Silipide is well-tolerated in both animal models and healthy human volunteers (*12*, *13*). Clinical development of silibinin requires the development of a robust analytical technique for its identification and quantitation in biological matrices. Previously, Martinelli et al. (*14*) described a high-performance liquid chromatography (HPLC) method for silibinin using normal-phase solid extraction columns, *tert*-butylmethylether as the eluent, and *n*-hexane/ethanol as the mobile phase. This method did not separate silibinin diastereoisomers, and *n*-hexane is known to be toxic (*15*, *16*). In 1993, Mascher et al. (*17*)

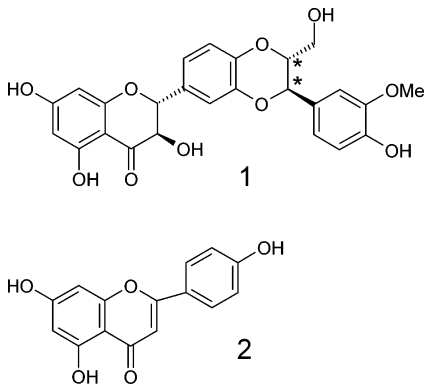

Figure 1. Molecular structure of the *trans*-diastereoisomers of silibinin (**1**) showing the chiral centers (*) and apigenin (internal standard) (**2**).

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published a semivalidated HPLC method that, for the first time, separated the two diastereoisomers of silibinin, but this method employed a mobile phase composed of 0.02 M perchloric acid, which is a powerful oxidizing agent and as such raises safety concerns for large-scale use in the laboratory. The purpose of this study was to develop a safe, simple, and robust reversed-phase HPLC method validated for detection in human plasma and suitable for use in the further clinical development of silibinin.

MATERIALS AND METHODS

Compounds and Biomatrices. Silibinin (CAS 22888-70-6) and apigenin (CAS 520-36-5) were purchased from Sigma-Aldrich (Poole, United Kingdom). Silibinin was >98% pure and consisted of a racemic mixture of two diastereoisomers. Silipide (IdB 1016: a 1:1 molar ratio of silibinin and phosphatidylcholine, i.e., 40% of silibinin and 60% of phosphatidylcholine by weight) was supplied by Indena s.p.a (Milan, Italy). Human plasma was obtained from the National Blood Transfusion Centre (Sheffield, United Kingdom). Plasma samples were also obtained from colorectal cancer patients receiving silipide capsules (containing 480 mg of silibinin) three times a day for 7 days prior to surgery. The Leicestershire Local Research Ethics Committee authorized the investigation of silibinin bioavailability in colorectal cancer patients. Patients gave informed consent prior to the commencement of the study.

Extraction of Silibinin from Human Plasma. Pooled human plasma was stored at −80 °C and thawed at room temperature prior to use. Silibinin stock solutions were prepared in methanol. Dilutions were made using 70% aqueous methanol containing 5% acetic acid. Human plasma samples spiked with silibinin (0−5 μg/mL) were analyzed in triplicate and incorporated an internal standard (apigenin) added at a final concentration of 500 ng/mL. The samples were vortexed at high-speed setting for at least 20 s prior to adding 3 mL of ice-cold methanol to precipitate the proteins. Efficient protein precipitation was achieved by keeping samples at −20 °C for 30 min. Samples were centrifuged (6000*g* for 20 min), and the supernatant was removed and transferred to fresh plastic tubes and dried under a constant stream of nitrogen. Residues were reconstituted in 100 μL of mobile phase B followed by a final 13000*g* centrifugation at 4 °C, and the supernatant was transferred to vials prior to HPLC analysis (50 μL injection volume).

Blood from colorectal cancer patients was collected into heparinized tubes before treatment and 2 h after the final dose. Plasma was isolated immediately by centrifugation and stored at −80 °C until analysis. Samples were thawed to room temperature, and 5 μL (100 μg/mL) of apigenin (internal standard) was added to patient plasma (995 μL). Silibinin was extracted as described above.

The extraction method for plasma was validated for various parameters. Linearity was assessed for each diastereoisomer by r^2 regression using Microsoft Excel 2002 software. Recovery was assessed by comparison of silibinin isomers extracted from spiked plasma ($n = 6$) with nonextracted standard solutions in methanol ($n = 6$). Precision and accuracy for silibinin diastereoisomers (d1 and d2) were determined at four concentrations (2500, 250, 50, and 25 ng/mL). The limits of detection (LOD) and quantification (LOQ) were estimated as the concentrations of silibinin diastereoisomers that generate a signal-to-noise ratio of three and seven, respectively.

HPLC Analysis of Silibininin in Human Plasma. Chromatographic separation was accomplished using 150 mm × 4.6 mm i.d., 3 μm, C_{18} Atlantis column (Waters, Elstree, United Kingdom) in combination with a 20 mm × 4.6 mm i.d., 5 μm guard column at 40 °C. The Atlantis stationary phase was chosen for this analysis due to its ability to run at high aqueous concentrations and to separate the diastereoisomers of silibinin. The HPLC system consisted of a Varian ProStar 230 Pump, ProStar 410 autosampler, and a 310 UV/vis detector at 290 nm (Varian Analytical Instruments, Oxford, United Kingdom). The collected data were analyzed using Star LC Workstation software version 5.5. Mobile phase A consisted of glacial acetic acid and distilled water at a ratio of 1:19 (pH unaltered ∼2.4). Mobile phase B contained glacial acetic acid and methanol (1:19 v/v). The flow rate was 1.0 mL/min, the total run time was 35 min, and the gradient employed was as follows (mobile phase B): 5% at 0 min, 30% at 5 min, 50% at 20 min, 60% at 25 min, and 95% at 30 min and held at 95% for 5 min. Quantitation of silibinin was accomplished by reference to a calibration curve of silibinin peak area ratio (silibinin d1 or d2 to internal standard apigenin) plotted against silibinin concentration.

Identification of Silibinin and Metabolites by Liquid Chromatography/Mass Spectrometry (LC/MS). LC/MS analysis was performed using an API2000 mass spectrometer (Applied Biosystems, Warrington, United Kingdom) with sample delivery via an 1100 series HPLC instrument (Agilent Technologies UK Ltd., South Queensferry, United Kingdom). The HPLC separation used was essentially as described above with the following modifications: 150 mm × 2.1 mm i.d., 3 μm Atlantis column with 20 mm × 2.1 mm i.d. guard column and the flow rate reduced to 300 μL/min to allow direct injection in the mass spectrometer without the need for eluant splitting. Mass spectrometric analyses were performed in negative mode under the following conditions: declustering potential, −121 V; focusing potential, −300 V; entrance potential, −10 V; collision energy exit potential, −20 V; ion spray voltage, −4500 V; and temperature, 500 °C. Silibinin was identified by a Q1 multiple ion scan looking at *m*/*z* 481 ($M - H^+$).

RESULTS AND DISCUSSION

Validation of Silibinin Extraction. The method described here resolves the two diastereoisomers of silibinin into two baseline-separated chromatographic peaks at retention times 21.2 and 21.7 min, which were arbitrarily assigned as silibinin d1 and silibinin d2, respectively (**Figure 2**). Plasma spiked with silibinin yielded calibration curves that were linear over the observed range (0−5 μg/mL). Silibinin d1 was characterized by the calibration equation [$y = (0.0005 \pm 0.0001)x - (0.0166 \pm 0.0196)$, silibinin d2 by $y = (0.0005 \pm 0.0001)x - (0.0271 \pm 0.0232)$] (mean ± SD of $n = 6$). The mean correlation coefficient (r^2) for the interday analysis was consistently above 0.995 ($n = 6$).

Recovery of silibinin d1 and d2 from pooled plasma samples and precision and accuracy was estimated at four concentrations (2500, 250, 50, and 25 ng/mL). Recovery was 53−58% (**Table 1**). The precision determined at each concentration level as reflected by the coefficient of variation (CV) did not exceed 15%. Accuracy was within 15% for silibinin d1 at all concentrations and silibinin d2 for concentrations 50−2500 ng/mL (**Table 1**).

The LODs of silibinin d1 and d2 were determined by extraction from spiked plasma. The LOD for silibinin d1 was 3 ng/mL, and for silibinin d2, the LOD was 2 ng/mL. LOQs for silibinin d1 and d2 in human plasma were found to be 7 and 5 ng/mL, respectively. The CV for LOQ is within the acceptable limits of the FDA method validation guidelines (*18*).

Silibinins d1 and d2 were stable for at least 1 month at 4 °C in the dark when kept in methanol. The stability of silibinin and apigenin in human plasma was evaluated following sample storage at −20 and −80 °C for 24 h. Both silibinins d1 and d2 were stable when kept at −20 and −80 °C after three freeze−thaw cycles (**Table 2**).

Determination of Silibinin in Human Plasma from Patients Receiving Oral Silipide. Silibinin was successfully detected at quantifiable levels in the plasma of colorectal cancer patients receiving oral silipide. **Figure 2** shows typical HPLC chromatograms of plasma obtained from a colorectal cancer patient before and after consumption of silipide capsules, at 1440 mg silibinin per day in three divided doses for 7 days. Peaks at 21.1 and 21.7 min were identified as silibinin diastereoisomers by cochromatography with authentic standard. These peaks were absent from plasma samples taken from the patient before silipide ingestion (**Figure 2D**). Several peaks appeared in

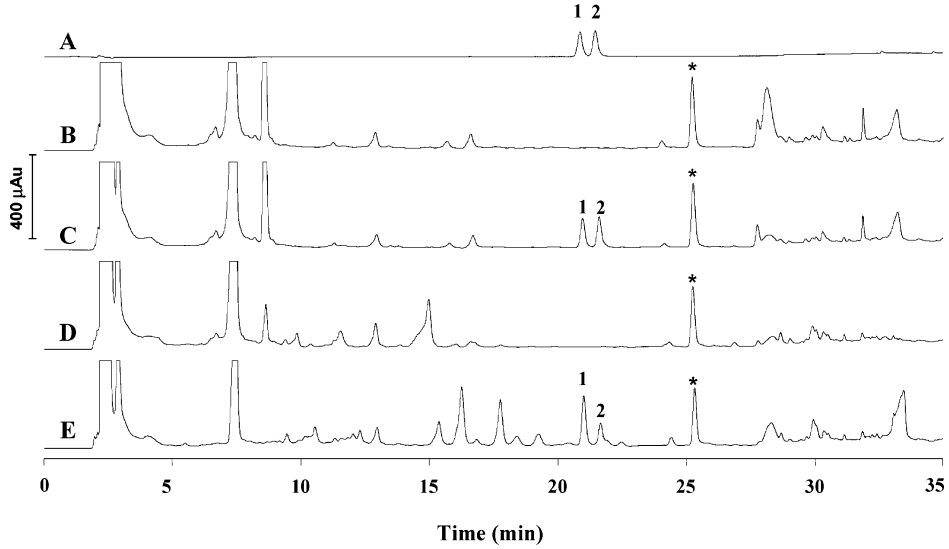

Figure 2. Separation and identification of silibinin diastereoisomers in human plasma. Silibinin d1 and d2 (peaks 1 and 2, respectively) in methanol (**A**) spiked in human plasma (**C**) and in plasma obtained from a colorectal cancer patient after a 7 day treatment of silipide capsules (containing 480 mg of silibinin three times daily) (**E**). Blank pooled human plasma (**B**) and plasma from patient prior to initiating silipide treatment (**D**) demonstrate the absence of any coeluting peaks (* indicates the internal standard, apigenin).

Table 1. Summary of Recovery, Precision, and Accuracy ($n = 6$) for Extraction and Measurement of Silibinin Diastereoisomers from Human Plasma

silibinin diastereoisomer	concn (ng/mL)	recovery (%)	recovered concn (mean ± SD)	accuracy[a] (% ± SD)	coefficient of variation RSD (%)
d1	25	58 ± 8	29.04 ± 4.08	93.6 ± 6.2	14.04
d2	25	55 ± 7	27.44 ± 3.29	75.3 ± 6.3	11.98
d1	50	56 ± 4	55.61 ± 3.83	97.4 ± 6.9	6.89
d2	50	53 ± 7	54.41 ± 2.15	85.8 ± 4.1	4.02
d1	250	54 ± 6	270.6 ± 32.2	106.7 ± 12.1	11.92
d2	250	55 ± 6	275.6 ± 28.7	106.3 ± 11.4	11.40
d1	2500	55 ± 6	2598 ± 272	100.0 ± 10.5	10.48
d2	2500	55 ± 5	2732 ± 255	113.5 ± 10.6	9.32

[a] $n = 6$.

Table 2. Stability of Silibinin Diastereoisomers in Spiked Plasma after Three Freeze–Thaw Cycles

		silibinin diastereoisomer concentration (ng/mL)					
		25		50		2500	
diasterioisomer	storage	peak area ratio[a]	RSD (%)	peak area ratio[a]	RSD (%)	peak area ratio[a]	RSD (%)
d1	immediate ($t = 0$)	0.056	5.669	0.125	16.224	7.206	1.988
d2		0.075	2.365	0.140	8.592	8.013	1.947
d1	−20 °C	0.035	5.612	0.118	6.067	6.960	0.582
d2		0.060	11.191	0.114	8.613	7.637	0.367
d1	−80 °C	0.062	2.212	0.111	4.693	6.990	3.380
d2		0.069	7.270	0.116	7.791	7.863	2.391

[a] Peak area ratio of silibinin diastereoisomer to the internal standard apigenin.

postdose plasma samples that may be attributable to silibinin metabolites (e.g., at retention times 15.3, 16.2, 17.7, 18.3, and 19.2 min).

Identification of Silibinin and Possible Metabolites in Human Plasma from Patients Receiving Oral Silipide. Plasma obtained from patients was investigated by LC/MS in Q1 scan mode (*m*/*z* 100−900) as described previously (*19*). Peaks in the mass chromatogram at 20.8 and 21.5 min (*m*/*z* 481) were identified as the two diastereoisomers of silibinin, corroborated by coelution with authentic standard, and these are the same peaks as seen by HPLC. Various *m*/*z* values were scanned for in multiple ion scan mode corresponding to probable phase I and phase II metabolites. Of these, only the following showed peaks of significant intensity: *m*/*z* 657 (monoglucuronide) 13.1, 15.6, 16.4, and 17.8 min; *m*/*z* 833 (diglucuronide) 10.8 min; *m*/*z* 561 (monosulfate) 17.0, 17.8, and 18.8 min; and *m*/*z* 737 (glucuronide sulfate) 9.1 and 11.0 min. There was also some evidence for the presence of *O*-desmethyl silibinin glucuronide (*m*/*z* 643) and silibinin triglucuronide (*m*/*z* 1008).

Although several methods have been developed for the analysis of silibinin in human plasma, to the best of our knowledge, no simple, fully validated method suitable for clinical evaluation of silibinin and its metabolites has yet been published. Here, we describe a simple method utilizing plasma protein precipitation and robust, precise, and reproducible reverse-phase HPLC to separate silibinin diastereoisomers with LOD and LOQ comparable with, or better than, previously published HPLC-UV detection methods (*14*, *17*, *20*). Rickling et al. (*21*) published a LOD for silibinin diastereoisomers of 0.25 ng/mL, which are superior to those quoted here; however, this level of sensitivity was achieved by using electrochemical detection and column switching, whereas our method has the advantage of employing simple UV/vis detection at 290 nm and without the need for column switching. Other techniques have required the use of less common constituents of mobile phases such as dioxane and perchlorate for separation (*14*, *17*). A published method looking at the glucuronidation of silibinin

using bovine liver microsomes (*22*) was not optimized for human plasma nor was it optimized for the various phase I and II metabolites likely to be present in humans taking silibinin.

The method described in this paper was used to separate silibinin and its metabolites in a patient who had received silibinin at a dose of 480 mg, three times daily oral dose for 7 days in a clinical pilot study (**Figure 2**). The results of the clinical study (*19*) suggests that silibinin is orally bioavailable and present in the systemic circulation at clearly quantifiable concentrations. The peaks eluting before silibinin in **Figure 2** suggest the presence of silibinin conjugates. Consistent with this observation, silibinin conjugates have been putatively identified (*19*) using this method, the validation for which is described here. This method seems highly suitable for the development of silibinin as a cancer chemopreventive agent in humans.

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