School of Pharmacy

Development of Novel Formulation Strategies to Improve Pharmacokinetic Profile of Resveratrol

Basavaraj

This thesis is presented for the Degree of
Doctor of Philosophy
of
Curtin University

January 2011

DECLARATION

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature:

Date:

ACKNOWLEDGEMENTS

First of all, I wish to express my deepest gratitude to my supervisor, **Dr Yan Chen** for her guidance, encouragement and patience throughout the journey. I am thankful to my co-supervisor Dr Heather A.E. Benson and the associate supervisor Dr David H. Brown for their guidance, support and encouragement. My association with my supervisors was a memorable experience.

I am thankful to Prof. Jeffery Hughes, Prof. Michael Garlepp and Prof. V B Sunderland for their support. I also acknowledge the cooperation of all academic, technical and administrative staff of School of Pharmacy.

I wish to thank Mr. Michael Boddy, for his support and help in the lab work. I wish to thank Jeorge Martinez for assistance in some of the lab work. I sincerely acknowledge cooperation of Charmaine and Kathy in ordering chemicals.

I wish to acknowledge help of Associate Professor Kevin Batty in help me to calculate pharmacokinetic parameters for my studies and I thank him for his effort and assistance. I also thank Christopher Cruickshank, Chemcentre for his assistance in running some of my samples in LC-MS/MS.

I thank Dr Beng Chua and Mark Habart for training me in animal handling and also for continuous assistance during animal experiments.

I am very lucky to have very close friends to help me to cope with the stress. I am thankful to Dhanushka, Yusuf, Chirag, Gayathri, Sarika, Muder and Fatima for all their support. I would like to acknowledge the support of my colleague Louise Whittal for helping me in recording NMR for some of my samples.

I am especially thankful to my wife Jyoti for her patience, inspiration and financial support. I express my sincere gratitude to my father, mother, sister, and brother in laws, father in law and mother in law for their encouragement and financial support.

Basavaraj

TABLE OF CONTENTS

7 APPENDIX

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ADME	Absorption distrubution metabolsim elimination
ATP	Adenosine triphosphate
AFM	Atomic force microscopy
AHR	Aryl hydrocarbon receptor
AUC	Area under the curve
BFC	7-benzyloxy-4-(trifluoromethyl)-coumarin
COX	cyclooxygenase
CYP	Cytochrome p450
cGMP	Guanosine 3’ 5’–monophosphate (cGMP)
DCM	Dichloromethane
DCC	1,3-dicyclohexylcarbodiimide
DMAP	4-dimethylaminopyridine
DMBA	7, 12-dimethylbenzanthracene
DOX	Doxorubicin
DMF	Dimethyl formamiede
DMSO	Dimethyl sulfoxide
DPPH	1,1-dipehnyl-2-picrylhydrazyl
DIC	N, N’-Diisopropylcarbodiimide
d_6	Deuterium
EDC	1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride
EPR	Enhanced permeation and retention
EDTA	Ethylene diamino tetra acetic acid
FLD	Fluorescence detector
FESEM	Field emission scanning electron microscopy
gm	gram
HCL	Hydrochloric acid
HPMA	N-(2-Hydroxypropyl) methylacrylamide
HPLC	High performance liquid chromatography
H2O2	Hydrogen peroxide
IV	intravenous
K_2CO_3	Potassium carbonate
kDa	Kilodalton

kV	Kilo-Volt
LDL	Low density lipoprotein receptor
LOD	Limit of Detection
LOQ	Limit of Quantification
MRM	Multiple reaction monitoring
mL	Millilitre
MillQ	Millipore filtered
µg	Microgram
MRT	Mean residence time
MS	Mass spectrometry
mM	Millimoles
µM	Micromoles
MeO	Methoxy
NMBA	N-nitrosomethylbenzylamine
NADP	nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance spectroscopy
NaOH	Sodium Hydroxide
PDA	Photo diode array
PEG	Polyethylene Glycol
PCL	Polycaprolactone
PLA	Polylactic acid
PTX	Paclitaxol
ROS	Reactive oxygen species
RPM	Rotations per minute
RSD	Relative Standard Deviation
SAR	Structure activity relationship
SD	Standard Deviation
TNF	Tumour necrosis factor
TPA	12-O-tetradecanoylphorbol-13-acetate
TCDD	2, 3, 7, 8,-tetrachlorodibenzo-p-dioxin
UDPGA	Uridine diphosphoglucuronic acid
UGT	UDP-glucuronosyltransferases
UVD	Ultra violet detector

Abstract

Resveratrol (RSV) is a natural polyphenol with diverse biochemical, physiological and pharmacological effects. Chemically resveratrol is a stilbene molecule with three hydroxyl group in the structure. These hydroxyl groups are prone to metabolism. Hence resveratrol is known to undergo rapid metabolism in rats as well as in human. Because of rapid metabolism there is a lack of correlation between *in vitro* and in vivo biological activities of resveratrol. Resveratrol often fails to produce the desired pharmacological activities in vivo. In order to produce or improve biological activities of resveratrol in vivo, the pharmacokinetic profile of resveratrol has to be improved. These findings led to design of this PhD project to address poor pharmacokinetic profile of resveratrol.

In this study novel formulation strategies were designed to improve pharmacokinetic profile of resveratrol. Polymeric conjugation approach was explored in this regard. The hydrolysable ester and non hydrolysable ether based polymeric conjugates of resveratrol were prepared. The resveratrol polymer esters such as MeO-PEGN-Succ-RSV 2 kDa, 20 kDa, MeO-PEGO-Succ-RSV 2 kDa, MeO-PEG-PLAO-Succ-RSV 2 kDa and 6.5 kDa were prepared and characterized by NMR, HPLC and LC-MS. In all the cases the polymer conjugates were mixture of species with 4'-Polymer-RSV as major component followed by 3-polymer-RSV and minor amount of disubstituted species. The resveratrol polymer esters were subjected to various *in vitro* tests such as stability in buffers, rat plasma and rat liver microsomes. The MeO-PEGN-Succ-RSV 2 kDa and 20 kDa were stable in pH 4.5 but hydrolyzed slowly to free resveratrol at pH 7.4. MeO-PEGO-Succ-RSV was stable at pH 4.5 and pH 7.4, this stability was attributed to self assembly of polymer conjugate into micelles. Similar trend was observed for MeO-PEG-PLAO-Succ-RSV conjugates. All resveratrol PEG esters were found to hydrolyze rapidly in the rat plasma and were completely hydrolyzed to resveratrol within 5 minutes. However MeO-PEG-PLAO-Succ-RSV conjugates were stable in rat plasma and stability was attributed to aggregation of conjugates into micelles. The plasma stability of micelle forming conjugates was found to be dependent on hydrophobicity of polymer. However this was true to a certain extent. Finally multiple parameters such as hydrophobicity, PEG corona (PEG chain length and surface density) and aggregation behavior

(micelle/nanoparticles) were found to influence plasma stability of polymer conjugates. The MeO-PEG-PLAO-Succ-RSV 2 kDa conjugate with a balanced of PEG/PLA content and ability to form compact structure was found to be stable in plasma with a half life of 3 hr.

In parallel, the resveratrol-PEG ether conjugates, with a non cleavable ether bond were prepared employing potassium carbonate mediated etherfication using MeO-PEG-Br as starting material. Resveratrol-PEG ethers were evaluated for stability in buffer, rat plasma and rat liver microsomes. Resveratrol-PEG ether 2 kDa was stable in buffers, plasma and microsomes.

Selected polymer conjugates were evaluated for preliminary pharmacokinetic profile in Wistar rats. MeO-PEG-PLAO-RSV 2 kDa although showed significantly better pharmacokinetic profile than resveratrol, was still cleared by mononuclear phagocyte systems. Resveratrol-PEG ethers 2 and 5 kDa displayed significantly better pharmacokinetic profiles than that of resveratrol and MeO-PEG-PLAO-Succ-RSV 2 kDa with improved peak plasma concentration, area under curve (AUC) and longer plasma residence time.

Solvent mediated fluorescence enhancement and quenching effects were explored to develop a highly sensitive HPLC method for analysis of MeO-PEGN-Succ-RSV 2 kDa. The effect of solvent composition on fluorescence intensity of MeO-PEGN-Succ-RSV 2 kDa conjugate was evaluated by varying concentration of methanol in solvent mixture. The fluorescence intensity of MeO-PEGN-Succ-RSV 2 kDa was found to be dependent on the methanol concentration and was maximum at 100% methanol. The HPLC assay method with a linear gradient was developed which allowed the maximum detection of the MeO-PEGN-Succ-RSV 2 kDa conjugate with methanol concentration at 95% in the mobile phase, while separation and analysis of resveratrol and its metabolites can still be achieved. The LOQ (Limit of Quantification) for resveratrol-PEG was 300 ng/mL (equivalent to 30 ng/mL resveratrol), nearly ten times more sensitive than HPLC with UV detection (3 µg/mL). The peaks detected for resveratrol-PEG, resveratrol and its metabolites in HPLC were identified qualitatively by LC/MS and LC-MS/MS (metabolites) and found to correlate with their respective molecular masses. The results of this study

demonstrate that the developed HPLC assay method can accurately and selectively quantify MeO-PEGN-Succ-RSV 2 kDa conjugates and resveratrol. This method was used for analysis of all polymer conjugates during *in vitro* and *in vivo* analysis.

A simple LC-MS method was developed to monitor PEGylation of resveratrol. The developed LC-MS method can separate and quantify unmodified MeO-PEG-OH 2 kDa, carboxylic acid terminated PEG, resveratrol and PEG-resveratrol conjugates. This methodology was able to monitor and determine the extent of conversion of MeO-PEG-OH to MeO-PEG succinylester acid (MeO-PEGO-SuccOH), which was found to be 100%. The developed method was also utilised to determine the extent of conjugation of resveratrol to carboxylic acid terminated PEG. The conversion of carboxylic acid terminated PEG to PEG-resveratrol conjugate was found to be 100% and 73%, respectively, for MeO-PEG succinylamide resveratrol (MeO-PEGN-Succ-RSV) and MeO-PEG succinylester resveratrol (MeO-PEGO-Succ-RSV). The 100% conjugation of MeO-PEGN-Succ-RSV is consistent with the result obtained from an NMR study. The average molecular weights determined by LC-MS for MeO-PEG-OH, MeO-PEGO-SuccOH and MeO-PEGO-Succ-RSV were found to be 2108, 2321 and 2423 respectively. These data correlate well with the theoretical values. This methodology proved to be simple and effective in determining the extent of fuctionalization of PEG and its conjugation to resveratrol. Overall our LC-MS method coupled with NMR permitted extensive characterization of PEGylated resveratrol and the reaction precursors. However due to mass limitations of LC-MS instruments, this method could not be applied to all polymer conjugates of resveratrol.

Overall in this project, effective formulation strategies were designed and successfully applied to improve pharmacokinetic profile of resveratrol.

CHAPTER 1

LITERATURE REVIEW AND GENERAL INTRODUCTION

1.1 INTRODUCTION

There has been increasing public awareness created these days about health benefits of consuming fruits and vegetables. There have been many reports about beneficial effects of consuming fruits and vegetables [1]. Chemoprevention, cardioprotection and anti aging are the few of health benefits of fruits and vegetables consumption. In Australia, the famous slogan "Go for 2&5" (2 serves of fruits and 5 serves of vegetables a day) has been effectively used to convey the message of health benefits of fruits and vegetables. Presence of the variety of essential vitamins, minerals and antioxidants in fruits and vegetables are said to be responsible for these health benefits[1]. Resveratrol is one of the major antioxidants found in many plant sources [1].

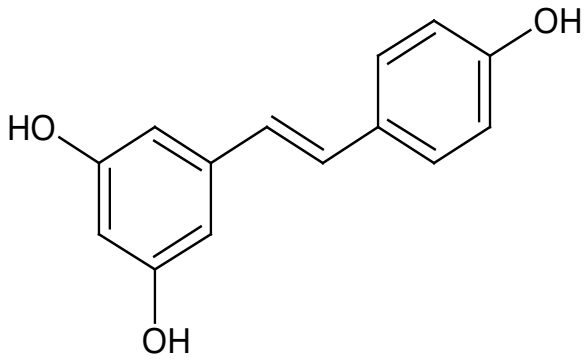


Figure 1.1: Chemical structure of *trans*-resveratrol

Resveratrol, as a natural polyphenolic compound (Figure 1.1 shows the structure of its major natural form- *trans* isomer), its therapeutic utility dates back to the ancient history. The Hindus ancient medicinal book "Ayurveda" described grape juice as an effective cardiotonic and it was referred to as a gift of God and was presumably used for cleansing body and soul. In 1940, resveratrol was first time identified as principle medicinal component of grapes [1].

1.2 SOURCES OF RESVERATROL

Resveratrol is found in many plant sources. It is believed to be produced in response to external stress such as water deprivation, exposure to UV irradiation and especially fungal infection. Thus the production of resveratrol in plants can be considered a defense mechanism. This property classifies it as a phytoalexin, the

compounds produced by plants in response to damage[1]. The major natural source of resveratrol is found in the roots of the plant Polygonum cupsidatum cultivated mainly in China and Japan. The skin of whole grapes contains around 50-100 mg of resveratrol (per grape), which is believed to be responsible for the cardio protective activity of red wine [1]. Resveratrol is found in a wide variety of other fruits such as mulberry, bilberry, lingonberry, sparkleberry, deerberry, partridgeberry, cranberry, blueberry, and jackfruit. Resveratrol is also found in peanuts, and a wide variety of flowers and leaves including: gnetum; white hellebore; corn lily; butterfly orchid trees; eucalyptus; spruce; poaceae; scots pine and rheum [1].

1.3 PHYSICOCHEMICAL PROPERTIES OF RESVERATROL

Resveratrol is an off white powder with a melting point of 253^0C. It is sparingly soluble in water, but soluble in organic solvents like DMSO, ethanol, diethyl formamide etc. Resveratrol is a hydrophobic molecule with a logP value of 3.13 [2]. Resveratrol was found to be stable in solid state for 4 years in accelerated conditions (40^0C/75%RH) and in liquid state (standard resveratrol added to grape extract) was stable for 2 years under ambient conditions [3]. When protected from light, *trans*-resveratrol was stable for at least 42 h (CV 1.0%) and for at least 28 days with a CV $\leq$ 4.7% in buffers pH 1-7. However, resveratrol is found to be unstable at higher pH conditions. The initial half-life for *trans*-resveratrol in pH 10.0 buffer was 1.6 h. The stability studies were conducted in hydro alcoholic solution with 50% alcohol (Ethanol) in the buffer solution. This was done because of the poor solubility of resveratrol in aqueous solutions.[3] Yang and colleagues [4] have studied stability of resveratrol in various buffer solutions. Their results indicated that resveratrol was unstable in base modified eagles medium (BME) containing $NaHCO_3$ and Milli Q water with $NaHCO_3$. There was no significant decrease in resveratrol content when incubated with NaH_2PO_4, other inorganic salts, amino acids, and vitamins of BME in water. It was evident from the study that presence of bicarbonate ions contributed to in-stability of resveratrol by generating free radical H_2O_2 which catalyzed oxidative decomposition of resveratrol. Trans reveratrol is stable and naturally occurring in plants, where as cis resveratrol is unstable and commercially not available [5]. The trans and cis resveratrol were found to have different spectral properties, UV max for trans resveratrol is 304 nm and for cis resveratrol 286 nm.

Both forms can be distinguished and analyzed by the UV spectropphotometer. [5] The monitoring of each form can done by HPLC with PDA (Photodiode array) detector. Trans resveratrol was found to undergo photoisomerization after exposing to sunlight for 8 h.[5]

1.4 BIOLOGICAL ACTIVITIES OF RESVERATROL

Resveratrol was reported to have diverse biochemical and physiological effects including antioxidant, anticancer, cardio protective, antiplatelet, chemopreventive, and, anti-inflammatory activities [1, 6]. Das et al [1], have summarised the various biological activities of resveratrol including chemoprevention, anti-inflammatory, cardio protection and outlined the possible mechanism for each activity. The cardioprotection and cardiac preconditioning have been extensively deliberated. In another report chemopreventive, functions as phytoestrogen, anti-inflammatory, and antiprogression activities were reviewed and potential possible mechanisms were proposed[6]. It is not the intention of this thesis to produce a comprehensive review of all biological activities of resveratrol, here the focus was given to those studied or reported extensively in the literature.

1.4.1 Anti-oxidant activity

Resveratrol is capable of scavenging intracellular reactive oxygen species due to its unique molecular structure. Although, resveratrol has shown to exhibit antioxidant activity *in vitro*, it was reported to be a weak antioxidant, when compared to ascorbic acid, in terms of its ability to scavenge hydroxyl radicals *in vitro* [1]. Depending on the biological system, resveratrol can also scavenge superoxide anion O_2^{-}. In contrast to this, resveratrol was reported to show a strong antioxidant activity *in vivo*. This strong antioxidant activity *in vivo* could be because of its ability to increase the synthesis of nitric oxide (NO) inside the body, which in turn acts as the *in vivo* antioxidant, scavenging superoxide radicals [7, 8]. In the ischemic reperfused heart, brain, or kidney, resveratrol induces NO synthesis and lowers oxidative stress. Nitric oxide having an unpaired electron possesses a strong affinity for free radicals and hence is a stronger antioxidant. These findings indicate, that resveratrol by virtue of structure, acts directly as an antioxidant, and by inducing various other enzymes. Based on these observations, it is anticipated that resveratrol would produce much stronger antioxidant effects *in vivo*.

1.4.2 Cardio-protective activity

The extracts of grapes and wines found to relax pre-contracted smooth muscles of intact rat aortic rings. [9] It was concluded that the observed muscle relation was due to the function of resveratrol. Cardio protective activity of resveratrol comes from its ability relax blood vessels. The increase in NO synthesis and the amount of guanosine 3',5'–monophosphate (cGMP) was responsible for vasorelxation. Resveratrol was believed to bring about these changes. [8]The direct evidence of NO activity was observed when cultured pulmonary endothelial cells showed increased NO synthesis when treated with resveratrol [10]. These finding clearly suggest that resveratrol protects heart against heart attack by vasorelaxation and scavenging free radicals which are harmful to heart.

1.4.3 Anti inflammatory activities of resveratrol

The anti-inflammatory activities of resveratrol have been attributed to various mechanisms in different reports. In ischemic heart experiments, treatment with resveratrol has significantly improved post ischemic ventricular function, and reduced myocardial infarct size when compared to the non treated control group. This effect was shown to be mediated by increased NO synthesis[11]. The anti-inflammatory activity of resveratrol is also attributed to the suppression of aberrant expression of tissue factor and cytokines in vascular cells. [12] In contrast to these observations, other reports, concluded that resveratrol exhibits its anti-inflammatory activity by the inhibition of cylooxygenase enzymes COX-1 and COX-2, and also by decreasing NO production [13,14]. The NF-kB, mediates inflammation through its ability to induce proinflammatory genes [15]. Resveratrol is found to suppress the activation of NF-kB in MCF-7 breast cell lines [16].

1.4.4 Resveratrol as a phytoestrogen

The phyotoestrogenic activity of resveratrol was evidenced by its capacity to bind and activate α and β-estrogen receptors [17]. The estrogenic activity of resveratrol remains ambiguous because of contradicting reports [18]. Gehm et al. showed resveratrol (3-10 μM) as super agonist when combined with estradiol (E_2) in MCF-7 cells [17]. However, Lu et al. reported that resveratrol (5 μM) exerts antagonism in presence of E_2 and mild agonism in its absence [18].

1.4.5 Resveratrol and lipoproteins, atherosclerosis

Resveratrol was found to decrease total cholesterol and total triglycerides in mice. There are various cellular mechanisms that would lead to increased low density lipoprotein (LDL)-cholestrol leading to atherosclerosis; a disruption in apolipoprotein E or low density lipoprotein receptor (LDLR) genes, and an over expression of human apolipoprotein B (apoB), contributes to an increase in the level of very low density lipoprotein (VLDL)-cholestrol. Resveratrol was reported to reduce the apoB content and secretion. Resveratrol was also found to decrease intracellular content and the rate of secretion of cholesteryl ester enzymes from hepatoblastoma cells [19-22]. In addition, resveratrol was found to reduce lipid peroxidation via copper chelation and scavenging free radicals [18]. This activity coupled with vasorelaxation may have contributed to anti atherosclerosis in humans. French people are known to consume high levels of cheese, butter, eggs, rich creamy sauces and other fatty foods, however they are considered healthy, with a low incidence of coronary heart diseases and certain types of cancer. A French diet contains 15% more saturated fat than an American diet. However, the rate of heart disease in France is 60% lower than that of the American population. Similarly France has a lower incidences of certain types of cancer. This may have been attributed to the high consumption of red wine by the French, a phenomenon referred to as the French Paradox [23].

1.4.6 Anti-platelet aggregation property

An aggregation of platelets contributes to atherosclerosis and chronic heart disease. Resveratrol showed a marked reduction in platelet aggregation in human platelet rich plasma, after induction by thrombin and adenosine-5'-diphosphate (ADP) treatment [24-26]. The thrombin down regulates the endothelial ectonucleotidase activity, resulting in high levels of ADP and ATP, which activate the platelets, leading to platelet aggregation. Resveratrol, inhibits thrombin induced secretion of adenosin triphosphate (ATP) and ADP, decreases neutrophil function and restores CD39/ATPDase [27-28].

1.4.7 Chemopreventive activity

Biotransformation is the part of our biological defence mechanisms by which body fight against any foreign chemical compounds entering the body. Phase-I biotransformation induces oxidation, reduction and hydroxylation of chemical compounds. Phase-II biotransformation brings about chemical conjugation such as sulfation, glucorinidation etc. However, phase-I biotransformation of certain chemicals, such as poly cyclic aromatic hydrocarbons and poly chlorinated biphenyls, may transform them into potential carcinogens [29-31]. Cytochrome p450 1A1 (CYP 1A1) in particular converts these chemicals into hydroxylated compounds, which are carcinogenic/mutagenic metabolites. In addition, poly cyclic aromatic hydrocarbons play a role in induction of CYP 1A1, CYP 1A2 and CYP 1B1. After phase-I transformation, these hydroxylated carcinogens are anticipated to undergo chemical conjugation to inactive compounds amenable for excretion. However, certain enzymes such as O-acetyl transferases and sulfotransferases, collectively called phase-II carcinogen activators, can trigger carcinogen formation, for example the induction of tumours by aromatic amines [32]. Resveratrol was found to inhibit CYP 1A1 [33] and also O-acetyl transferases and sulfotransferases, [34-36] thereby collectively preventing the formation of carcinogens. In addition, resveratrol decreases the activity of breast cancer resistance proteins responsible for transferring carcinogen into cells [37-38].

In another report [14], the chemopreventive activity of resveratrol was attributed to the collective action of various other biological effects, this is because resveratrol acts as an antioxidant and an antimutagen, induces phase-II drug-metabolizing enzymes (Anti initiation effect), mediates anti-inflammatory activity, inhibits cyclooxygenase and hydroperoxidase functions (Antipromotion activity) and induces human promyelocytic leukemia cell differentiation (Anti-progression activity) [38]. All these effects stop initiation, promotion and progression stages of cancer development, [14] and suggest that resveratrol has the capacity to be a strong chemopreventive agent.

1.5 ANTICANCER/CHEMOPREVENTIVE EFFECTS OF RESVERATROL ON VARIOUS CANCER CELL LINES

1.5.1 Prostate cancer

Prostate cancer is the third most frequently diagnosed cancer in USA and other western countries [22]. Studies conducted in cell culture have showed antiproliferative effects of resveratrol on prostate carcinoma cells [39]. Hsieh and Hu [40] investigated the

effects of resveratrol on growth, induction of apoptosis, and modulation of prostate specific gene expression using DU-145, PC-3 and JCA-1 human carcinoma cells [40]. The study suggested that, resveratrol reduced cell growth by affecting mitogenesis as well as inducing cell apoptosis, leading to anticancer activity.

1.5.2 Breast cancer

Breast cancer is the most common cancer affecting woman in the world. Resveratrol showed antiproliferative effects against various breast cancer cells irrespective of their estrogen receptor status *in vitro* [41, 14]. Resveratrol inhibited development of preneoplastic lesions in carcinogen treated mouse mammary organ cultures [43]. Resveratrol was also found to inhibit 4T1 breast cancer cells in time and dose dependent manner [41]. The inhibition of breast cancer cell growth could be attributed to resveratrol's ability to inhibit the induction of CYP 1A1, down modulation of p53, induction of apoptosis and various other cellular interventions [42].

1.5.3 Skin cancer

Skin cancer is one of the major cancers affecting Australian population and constitutes nearly 80% total diagnosed cases of cancer. Two in three Australians will be diagonised with skin cancer by the time they are 70 years old [43]. Resveratrol demonstrated chemopreventive effect in chemically induced skin carcinogenesis in mouse model [34]. In a study conducted by Jang and Pessuto [14], application of phorbol 12-myristate-13-acetate (TPA) to mouse skin to establish an animal model with increased oxidative stress as indicated by generation of H_2O_2 , enhanced levels of methyl peroxidise activity, oxidized glutathione reductase activity and decreases in glutathione levels and super oxide dismutase activity. The TPA treatment showed increase in expression of COX-1, COX-2 and TNF-α. Pre-treatment with resveratrol negated several of these activities. The H_2O_2, methyl peroxidise activity, oxidized glutathione reductase activity, glutathione levels and super oxide dismutase activity were restored to the level of control group levels which received no TPA treatment.

1.5.4 Liver cancer

The resveratrol was found to inhibit growth of hepatoma cell line H22 in a time and dose dependent manner via induction of apoptosis [44]. The resveratrol has also been shown to decrease the growth of tumour cells (25%) in the rats inoculated with Yoshida AH-130 ascites hepatoma cells [45]. Ciolino and Yeh have studied the effect

of resveratrol on aryl hydrocarbon receptor (AHR) and CYP 1A1 in human hepatoma cells [46]. They found that resveratrol inhibited the increase in CYP1A1 mRNA caused by AHR ligand 2, 3, 7, 8,-tetrachlorodibenzo-p-dioxin (TCDD) in a concentration dependent fashion, resulting in chemo preventive activity.

1.5.5 Colorectal and intestinal cancer

Various studies have reported anti-proliferative and chemopreventive activity of resveratrol against colorectal cancer [47, 48]. Schneider et.al [49] studied the effect of resveratrol on polyamine metabolism of CaCo-2 human colon cancer cells. The treatment with 25 µM of resveratrol showed 75% inhibition in growth of cancer cells. This was associated with significant decrease in the ornithine decarboxylase activity, a key enzyme in polyamine biosynthesis, which is normally high in cancer cells. This indicates polyamines might be one of the targets which resveratrol acted upon to produce observed activity. Wolter et.al [50] investigated the effect of resveratrol on human colonic adrenocarcinoma Caco-2 cells, and found that, resveratrol inhibited growth and proliferation of these cells. They attributed such activity to down regulation of cyclin D1 and cyclin dependent kinase. Several other studies have linked the observed anticancer effects of resveratrol to its activation of p53, a tumour suppressor [51, 52, 53, 54.]

1.5.6 Blood cancer

Various studies have reported the anti proliferative and chemo preventive effects of resveratrol against leukaemia cells [55-57]. It was reported that resveratrol, reduced the viability and DNA synthesis in cultured leukaemia (HL-60) cells (58 pap3). The study showed that antiproliferative and chemo preventive effects were due to induction of apoptosis. There was a decrease in expression of anti-apoptotic Bcl-2. Other study correlated observed effects to involvement of capsages, CD95- CD95L pathways in apoptosis .

From above mentioned studies it is evident that resveratrol is involved in many different biological process. The mechanisms of actions for various biological activities of resveratrol are summarized in Figure 1.2 and these mechanisms of actions were all established via *in vitro* cell studies [2].

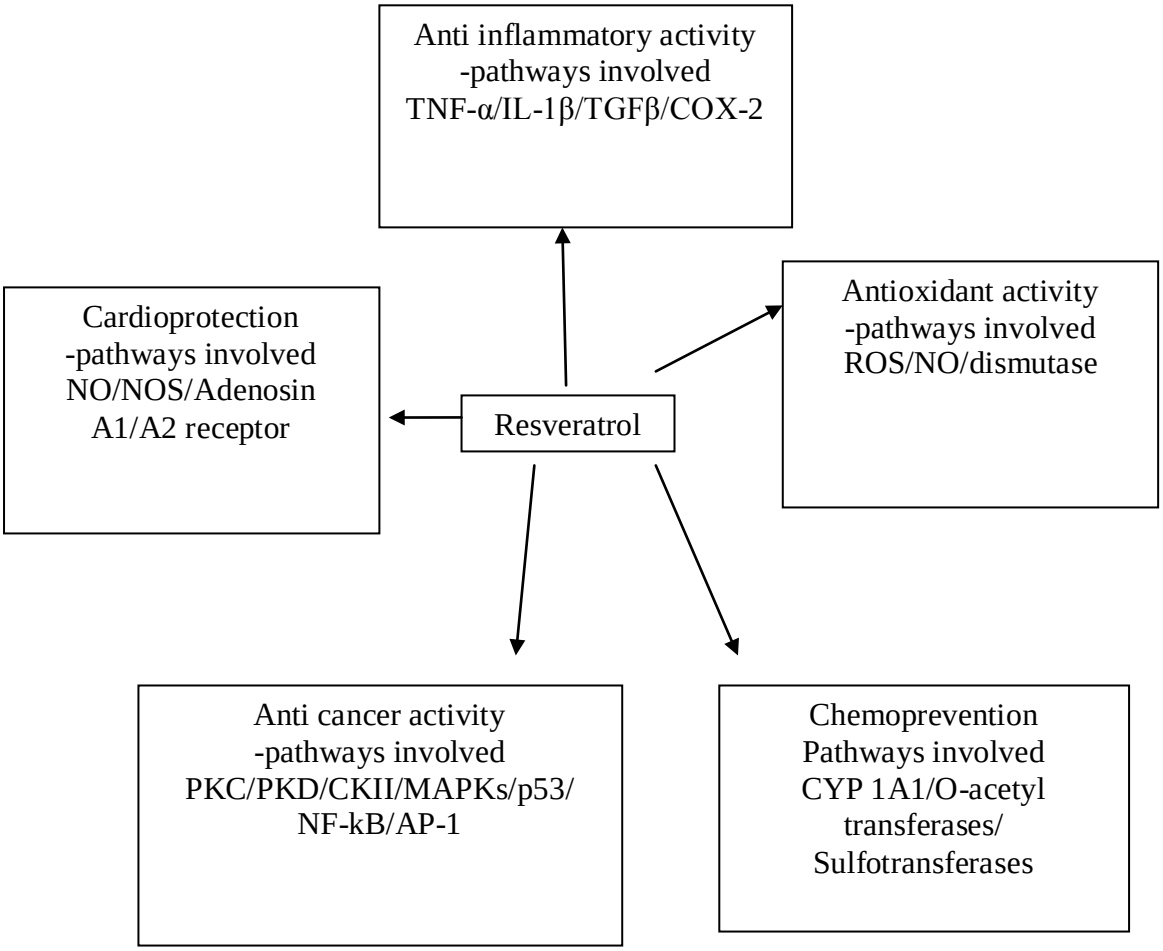


Figure 1.2: Interventions of resveratrol in various biological pathways responsible for its biological activity

1.6. IN VIVO PHARMACOLOGICAL EFFECTS OF RESVERATROL

Data obtained from these *in vitro* studies suggest that resveratrol could be a promising therapeutic agent for many diseases/disorders. Interestingly, the *in vivo* studies on animal models, sometimes revealed different stories.

Elamai et al. have carried out experiments to investigate the effect of resveratrol on osteoarthritis, using rabbits with surgically induced osteoarthritis by anterior cruciate ligament transection (ACLT), to create degenerative changes in the articular cartilage in the knee [58]. Five weeks after this procedure, one group of rabbits received 10µmol/kg of resveratrol in DMSO injected directly into the knee; another group just received the DMSO as a control once a day for two weeks. The rabbits were killed after 7 days of the last injection, and a histological examination of knee cartilage was performed. Results indicated a significant decrease in cartilage destruction with a score of 1.7 vs 2.8 for control. A loss of matrix proteoglycan content in the cartilage was also much lower in the the treatment group. Authors have attributed these effects to the inhibition of prostaglandin synthesis, mediated by COX-2 gene expression and COX-2 enzyme activity by resveratrol. The inhibition of NF-kB activation by resveratrol was also proposed as being the responsible factor for the activity.

Jang et.al [14] investigated the chemopreventive activity of resveratrol using a skin cancer mouse model. Tumeourogenesis was evaluated in two stages in mouse skin using 7, 12-dimethylbenzanthracene (DMBA) as an initiator, and 12-O-tetradecanoylphorbol-13-acetate (TPA) as a promoter. An application of resveratrol along with TPA, twice a week to the skin at a dose of 1,5,10 and 25 µmol showed a reduction of tumour per mouse by 68, 81, 76, and 98%,, suggesting resveratrol could be an effective chemopreventive agent for skin cancer. Researchers speculated that the chemopreventive activity of resveratrol is a result of reduced oxidative stress, induced by the combined antioxidant effects of resveratrol and antioxidant enzymes induced by it. Similar results were reported by Soleas et.al on resveratrol's chemopreventive activity with a 2 stage CD-1 mouse skin model produced by DMBA and TPA.

The chemopreventive effects of resveratrol on azoxymethane induced colon carcinogenesis have been investigated by Tessitore et.al [59]. In the study, rats were given orally a solution of resveratrol (200 µg/kg body weight) in drinking water (with 0.3mL of alcohol /liter) for 100 days. The resveratrol dosing began 10 days before a carcinogen injection and ceased 3 months after. Carcinogen, azoxy methane was injected twice subcutaneously one week apart, at the dose of 15 mg/kg. The growth of aberrant crypt foci (ACF) was measured at the end of the study. The results showed less numbers of ACF/colon (25.7) for the resveratrol treated group as against 39 for the control group.

Li et.al [60] studied the suppression of N-nitrosomethylbenzylamine (NMBA) induced tumerogenesis in F344 rats by resveratrol. Resveratrol was dosed at 2 mg/kg orally for 16 weeks, two groups were dosed at 1 mg/kg and 2 mg/kg for 16 weeks intraperitonially; another group received resveratrol 1mg/kg intraperitonially for 20 weeks. At the same time all the groups received 1mg/kg of NMBA subcutaneously. The resveratrol group revealed a reduction in the development of oesophageal tumours to 78, 62, 54, and 48% respectively at these doses, as against the control group.

Gao et.al [57], have compared *in vitro* and *iv vivo* antileukemic effects of resveratrol in C3H mice. Treatment of 32Dp210 leukaemia cells with resveratrol at concentration of 25-50 µmol/L showed significant and irreversible inhibition of growth in-vitro. The *in vivo* antileukemic studies with resveratrol dosed orally at 8 and 40mg/kg daily, five times per week did not show any effects on improving survival of mice injected with leukaemia cells. Allthe mice died. Only at the 80mg/kg dose, a very weak antileukemic effect was seen with 2 mice surviving out of 14 mice treated. However when mice injected with leukaemia cells pre-treated with resveratrol *in vitro* before injection, did not produce leukaemia and 92% mice survived, this effect could be due to strong chemopreventive effects of resveratrol *in vitro*. Resveratrol pretreatment could have affected proliferation ability of leukaemia cells and hence no development of leukaemia. This clearly shows that resveratrol is active *in vitro* but failed to show its effectiveness *in vivo*, possibly its pharmacokinetics (absorption, distribution, metabolism and excretion) may have affected its activity *in vivo*. In a similar study conducted by Ziegler et.al [61], the researchers reported a lack of *in vivo* effects on tumerogenesis in $Apc^{min/+}$mice following the treatment of resveratrol. The resveratrol when given orally in feed to mice at the dose of 0-

90mg/kg did not show any reduction in number of tumours per mice against control. Moreover, analysis of mice plasma by HPLC showed only resveratrol-glucoronide but not the native resveratrol present. Researchers concluded that resveratrol did not produce chemopreventive effects because of its rapid metabolism to resveratrol-glucuronide.

Wenzel et.al [62] evaluated the bioactivity and metabolism of resveratrol when given orally with food to wistar rats. The resveratrol at the dose of 50 and 300 mg/kg per day was fed to the rats for 8 weeks. The chemopreventive parameter such as phase I and phase II metabolizing enzymes, total antioxidant activity, and vitamine E status were evaluated. The plasma, urine, kidney and liver samples were analyzed for quantification of resveratrol and its metabolites. Various metabolites of resveratrol were synthesized to correlate with metabolites detected in various samples. The study revealed that resveratrol had no effect on the biochemical parameters responsible for chemopreventive action, except for antioxidant activity which was just up by 19%. The lack of a significant effect on chemopreventive parameters, was attributed to rapid metabolism of resveratrol in the body to various sulfated and glucuronide metabolites.

The review of the above studies indicates that resveratrol is more active when applied directly to the site of action such as the knee joints and skin; this showed remarkable activity when compared to those studies, where resveratrol was given orally or by other routes where the drug has to undergo absorption and distribution before it reaches target tissue. Hence there is an obvious need to understand the correlation between phamacodynamics and pharmacokinetics of resveratrol. The absorption, distribution, metabolism and excretion profile of resveratrol needs to be reviewed.

1.7 PHARMACOKINETIC PROFILE OF RESVERATROL IN RODENTS AND HUMAN

`There are numerous studies reported in the literature, concerning *in vitro* metabolism and pharmacokinetic study of resveratrol on various animal species such as rats and mice. The pharmacokinetics of resveratrol in human beings has also been reported.

Yu et al. [63] investigated the metabolism of resveratrol in humans, rats and mice. The resveratrol was incubated with human liver microsomes, rat and human hepatocytes

in vitro. In the same study, resveratrol solution in neobee oil (with 6.5 % v/v of alcohol in oil) was dosed intraperitoneally to rats at 20mg/kg body weight. Similarly the solution of resveratrol in corn oil (with 5.4% v/v of alcohol in oil) was dosed respectively to mice at 20mg/kg, and 60mg/kg intraperitoneally and orally. The urine from rat and serum from mice were processed and along with samples collected from *in vitro* incubations, were analyzed by HPLC coupled with LC-MS. The results showed that, resveratrol did not undergo any phase-I metabolism like oxidation, reduction or hydroxylation; however, resveratrol underwent extensive metabolism when incubated with rat and human hepatocytes. Resveratrol-3-O-glucuronide, Resveratrol-4'O-glucuronide were major metabolites detected with human hepatocytes. The resveratrol-3-O-sulfate was a major metabolite, with traces of resveratrol-3-O-glucuronide with rat hepatocytes. Only resveratrol-3-O-glucuronide was detected in rat urine samples tested 2 hours post dose. The mice serum samples did not show any native resveratrol but abundant resveratrol-3-O-glucuronide and resveratrol-3-O-sulfate metabolites were detected in serum samples, from initial time point of 5 minutes to 1 hour post dose.

In another study oral and an intravenous pharmacokinetic profile of resveratrol was investigated in Sprague-Dawley rats [64]. The rats were dosed at 15 mg/kg , 50 mg/kg using oral and intravenous routes respectively. The plasma profile after IV dosing, was characterized by low and rapidly decreasing levels of resveratrol with high amounts of resveratrol glucuronide. The plasma half life ($t_{½}$) of resveratrol after intravenous dosing was found to be 0.13 hrs. The plasma profile after oral dosing was slightly different, with lower levels of resveratrol detected than those samples employing IV dosing. The plasma concentration of resveratrol was found to be depleting at a slower rate. In comparison, the $t_{1/2}$ after oral administration was found to be 0.29 hrs. Although, the plasma levels were seen reaching base line in around two hours, there were slight increases in levels of resveratrol concentration after 4 hours; this was attributed to the enterohepatic recirculation of resveratrol, and was confirmed by analysing bile samples taken from the rats.

Absorption and metabolism of resveratrol in the jejunum of an isolated rat's small intestine has been reported [65]. The resveratrol solution (200 µM) when perfused through the isolated intestinal segment, showed only trace amounts of resveratrol crossing the membrane unmodified and 95% of detected compound on serosal side

of jejunum was resveratrol glucuronide. The presence of resveratrol glucuronide was confirmed by HPLC-PDA and nano ES-MS/MS. The authors concluded, that resveratrol is most likely to be in the form of glucuronide conjugate after passing through the intestine and reaching blood circulation. They pointed out that, the lack of native resveratrol entering blood circulation would impact on the in vivo biological effects of resveratrol.

Wenzel et.al [62] studied the bioactivity and metabolism of resveratrol in Wistar rats after oral administration. The rats were dosed with resveratrol in their feed at two dose levels of 50mg and 300mg/kg body weight for 8 weeks. The resveratrol and metabolites concentrations were analyzed in plasma, urine, feces, liver and kidney by HPLC. The metabolite standards were also synthesized and analysed by HPLC. The biological activity such as phase-I and Phase-II biotransformation activities, were determined by the analysis of NADPH-cytochrome-c reductase (CCR) and glutathione-S-transferase in the liver and kidneys respectively. The antioxidant activity of plasma samples were determined by inhibitory effects on linoleic acid peroxidation. The study revealed that neither resveratrol nor metabolites, could be detected in plasma, liver and kidneys at 50mg/kg dose, and was only detected in pooled urine and feces collected during 3rd and 8th week. The absence of resveratrol and its metabolites in plasma, liver and kidneys, could be because samples were collected after 24 hours of fasting (no resveratrol dosed) at the end of the 8th week. At 300 mg/kg dose, half of the animals showed only metabolites in plasma, liver and kidneys, but not free resveratrol. Based on the dose and biological samples analysed, 6 different metabolites of resveratrol such as 3,4'5-trisulfate, 3,4'-disulfate, 3,5-disulfate, 3-sulfate, 4'-sulfate, and 3-glucuronide were detected. The antioxidant and chemopreventive activity of the resveratrol fed rats did not differ from the control group. The authors concluded, that a lack of *in vivo* activity was probably due to the formation of conjugate metabolites, and similar findings were reported for resveratrol from other rat, mice and human pharmacokinetic studies.

In addition to animal studies, there are reports on pharmacokinetic profile of resveratrol in healthy human volunteers [66,67]. Walle et al examined absorption, bioavailability and metabolism of ^{14}C-resveratrol after IV and oral administration in 6 healthy volunters [66]. The 25 mg of ^{14}C resveratrol in alcoholic simple syrup

solution with 50 mg of ascorbic acid was dosed orally. The 0.2 mg of ^{14}C-resveratrol in alcohol/ saline mixture was dosed intravenously as infusion for 10 minutes. The plasma samples collected immediately after IV dosing showed unchanged resvertrol at concentrations of 3.7 to 16.4 ng/mL. There was a significant metabolite peak with an estimated concentration of 9 to 13.5 ng/mL, which was investigated and found to be sulphated metabolite of resveratrol. However, only trace amounts of unchanged resveratrol (<5 ng/ml) could be detected in plasma after oral dosing as against total radioactive compound with peak plasma levels of resveratrol and metabolites of 491 ± 90 ng/ml. The oral bioavailability of resveratrol calculated based on total radioactivity was 70%. However there was not much unchanged resveratrol in blood. Hence authors termed the pharmacokinetics of resveratrol as "high absorption but very low bioavailability. In the study five metabolites were identified by LC-MS, M1,M2, two monoglucorindes, M3 dihydromonoglucorinide, M4 monosulfate and M5 dihydromonosulfate. Detection of dihydro glucorinide and sulphate metabolites indicates possible phase I metabolism resulting in hydrogenation of double bond. The group reported the detection of sulfate conjugate M4/M5 in plasma samples within 2 hrs post iv or oral dose and suggested that the sulfation of resveratrol may be the major limiting factor to its bioavailability. Authors also speculated that, although the systemic bioavailability of resveratrol is very low, rapid uptake and accumulation of resveratrol in epithelial cells along the aerodigestive tract, together with the formation, in the colon, of dihydroresveratrol metabolites, which were shown potency slightly less than resveratrol in cell line, may contribute to the chemopreventive action of resveratrol against colon cancer [67]. They pointed out that, because of the rapid metabolism, the chemopreventive action of resveratrol at other target sites such as breast and prostate, would need to reply on the activation or activity of resveratrol metabolites such as resveratrol sulfate and glucuronide.

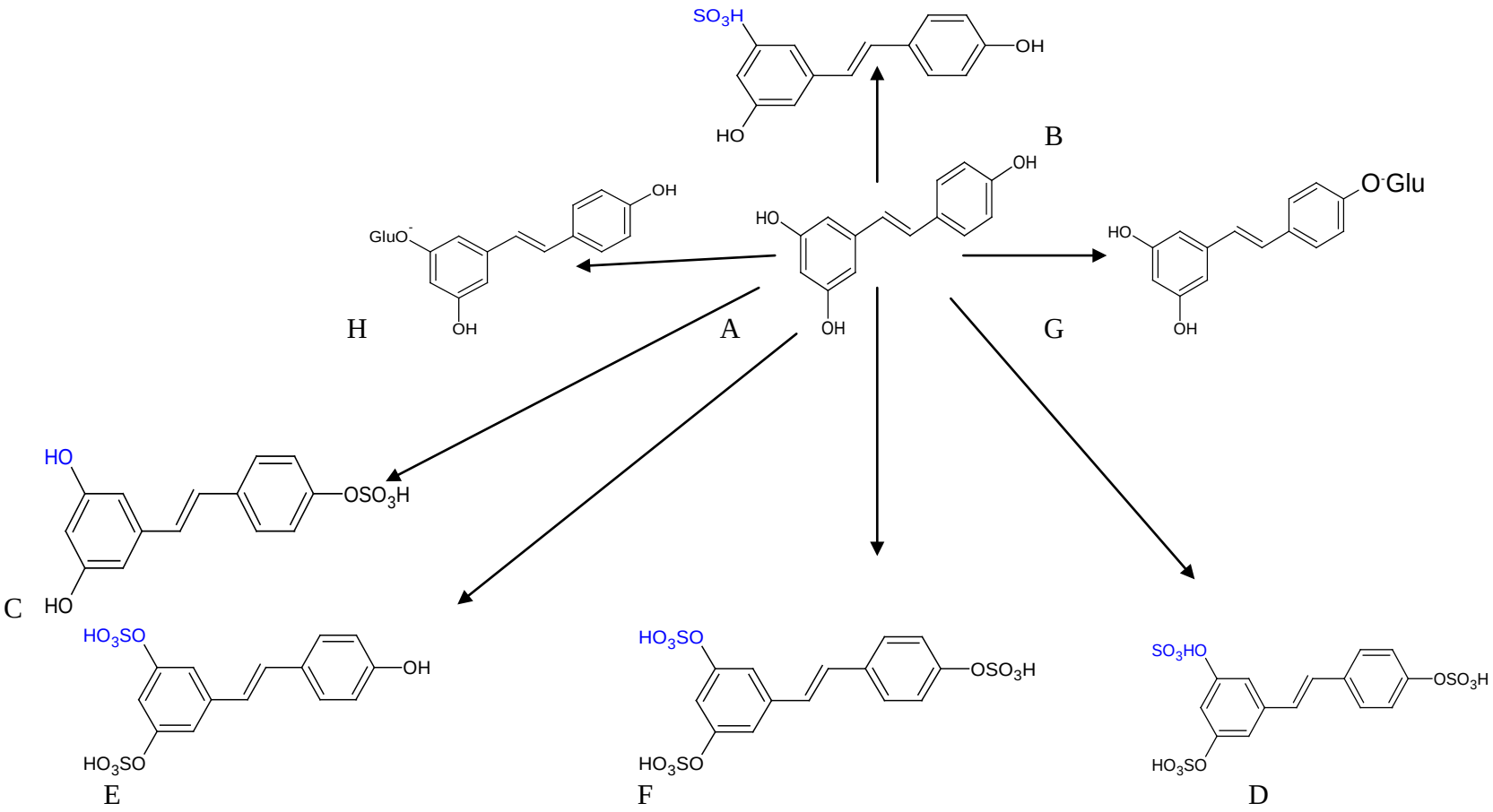


Figure 1.3: Structure of *trans*-resveratrol'smetabolic products A:Resveratrol, B:Resveratrol-3-O-sulfate, C:4'-O-sulfate, D:3,4'-O-disufate, E:3, 5-O-disulfate, F:3,4,5'-O-trisulfate, G:4'-O-glucuronide, H:3-O-glucuronide.

Boocock et.al have [67] also studied the pharmacokinetics of resveratrol after oral administration at various doses to healthy human volunteers. The doses selected for the study were 0.5, 1, 2.5 and 5 g. Consumption of resveratrol did not cause serious adverse events. Resveratrol and six metabolites were detected in plasma and urine samples. Peak plasma concentration of resveratrol at the highest dose was 539 ±384 ng/mL (2.4 µmol/L), which was observed 1.5 h post dosing. Peak levels of two monoglucuronides and resveratrol-3-sulfate were 3- to 8-fold higher than resveratrol. The area under the concentration curve (AUC) values for resveratrol-3-sulfate and resveratrol monoglucuronides were up to 23 times greater than those of resveratrol. Resveratrol and its metabolites were excreted rapidly in the urine. Authors have concluded that the levels of resveratrol observed with highest dose could be insufficient to elicit chemopreventive activity as 5 µmol/L is required for the chemopreventive activity *in vitro* conditions. However this could only be true if resveratrol metabolites lack chemopreventive activities. Researchers suggested investigation of chemopreventive activity of metabolites such as resveratrol-glucuronide and resveratrol-sulfate.

To study safety, tolerability and pharmacokinetic profile of resveratrol, a study with various doses of resveratrol administered six time a days until 13th dose was conducted in healthy volunteers [68]. The 25, 50, 100 and 150 mg of resveratrol were given orally as capsule formulation and all the doses were well tolerated without any major clinically significant adverse effects. The resveratrol produced very low plasma levels. Following the 13th dose of trans-resveratrol 25, 50,100 and 150 mg, mean peak plasma concentrations were at 3.89, 7.39, 23.1 and 63.8 ng/mL and mean area under the plasma concentration–time curves (AUC_{0-t}) were 3.1, 11.2, 33.0 and 78.9 ng.h/mL respectively. There was no significant difference in AUC between 1st and 13th dosing. The C_{max} also did not vary between repeated dosing. Only in higher dose 150 mg, the C_{max} was doubled after 13th dose. Trough (C_{min}) concentrations were≤1 ng/mL following 25 and 50 mg, 3 ng/mL following 100 mg and <10 ng/mL following 150 mg. It was concluded that the results of this study indicate metabolism of resveratrol is very fast. Repeated dosing of resveratrol at a high frequency can not compensate for its fast metabolism.

The various studies which were conducted to understand *in vitro* metabolism and pharmacokinetic profile of resverarol indicated that resveratrol undergoes rapid metabolim. Pharmacokinetic profile is similar across different animal species and human. The levels of resveratrol in plasma were below the anticipated concentrations required for biological activity. In most of the studies, the metabolites concentrations were higher than native resveratrol even at the initial sampling points. The researchers from different groups have concluded that resveratrol would not produce substantial biological effects *in vivo* because of its rapid metabolism. However some researchers speculated that there can be significant *in vivo* biological effects if metabolites are biologically active. In order to validate these speculations, Miksits et.al [69] have conducted *in vitro* experiments to evaluate anti tumour activity resveratrol sulfated metabolites and compared with activity of resveratrol. Three major sulfated metabolites of resveratrol, Resveratrol-3-O-sulfate, resveratrol-4'-O-sulfate and resveratrol-3,4'-O-disulfate metabolites were synthesized. The anti-tumour activity was evaluated against three breast cancer cell lines (two hormone-dependent: MCF-7 and ZR-75-1; one hormone-independent: MDA-MB-231) and one immortalized breast epithelial cell line (MCF-10A). It was found that, in comparison to resveratrol, all three sulfated metabolites were less potent against MCF-7, MDA-MB-231 and ZR-75-1 cells (*trans*-resveratrol 3-*O*-sulfate < *trans*-resveratrol ′4 -*O*-sulfate < *trans*-resveratrol 3-*O*-4′-*O*-disulfate) indicating that any conjugation of the phenolic groups with sulfuric acid strongly affecting the cytotoxicity. Interestingly, all sulfated metabolites displayed about 10-fold decrease in anti-tumour activity, but showed nearly equal cytotoxicity towards nonmalignant MCF-10A breast cells (IC_{50} 202-228 µM). Despite these findings, authors have concluded that observed low activity of metabolites may not actually reflect their *in vivo* performance, because ubiquitously existing human sulfatases could convert the metabolites back to resveratrol in humans [69].

In summary, the metabolism and pharmacokinetic studies revealed that resveratrol undergoes rapid metabolism to produce resveratrol glucuronides and sulfate conjugates. In rodents, mice showed little unchanged resveratrol after IV dosing and only metabolites after oral[63]. Similarly, in rats, there was very little unchanged resveratrol but metabolites after oral dosing and although resveratrol level was slightly higher after IV dosing but still very low. The half life for IV and oral were

0.13 and 0.29 hrs respectively [63]. In human, after oral dosing very low levels of resveratrol was seen in plasma [66, 67]. The repeat dosing did not improve the resveratrol levels in plasma. In all the pharmacokinetic studies , it was concluded that resveratrol levels were not high enough to elicit pharmacological activities and warranted the study on activity of metabolites [66-68]. Few studies were conducted on the activity of metabolites, it was observed that metabolites were less active than resveratrol [69] . Although it was suggested that under *in-vivo* conditions the sulfate metabolites may convert back to resveratrol but that was not seen in any pharmacokinetic studies yet. The sulfation process seems to be faster than desulfation resulting in more metabolites. Hence resveratrol belongs to a class of compounds where poor bioavailability is a cause for attrition in drug development process as shown in Figure 3. It is clear from the above review that administering resveratrol in a conventional way would not result in desired pharmacological effects. Hence there is need to develop formulation strategies to deliver the resveratrol in the native form and sustain its level *in vivo* for an adequate period of time to maximize its biological activity.

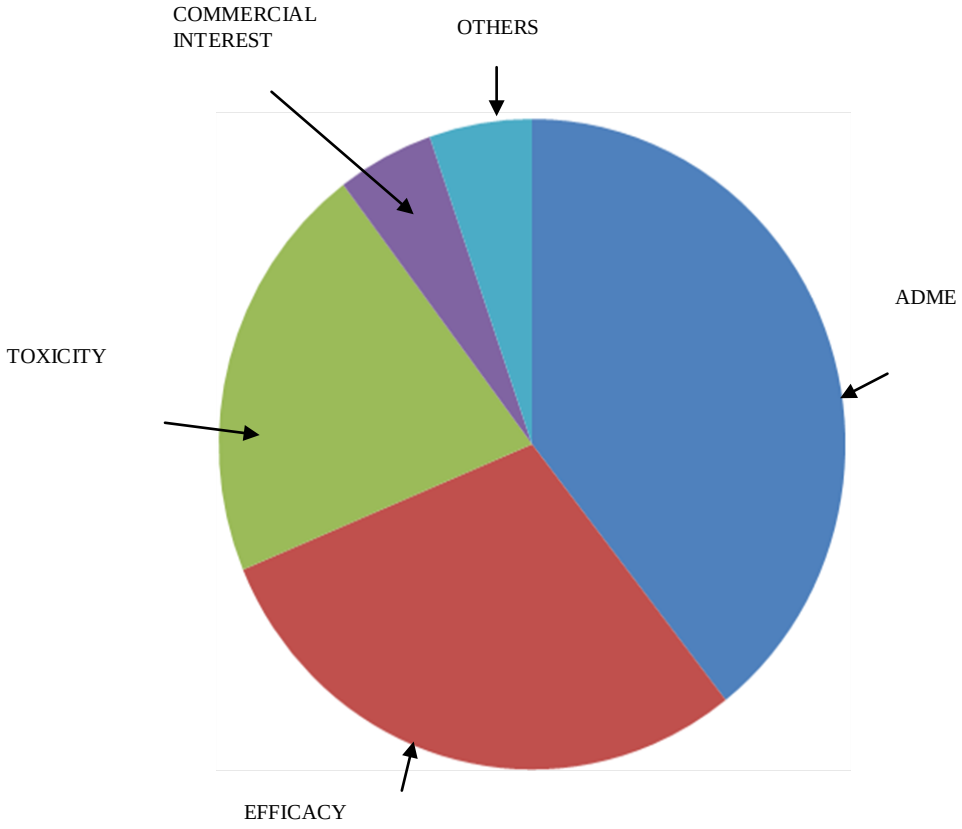


Figure 1.4: Attrition of new drugs during drug development stages for various reasons [70]. ADME stands for absorption, distribution, metabolism, excretion.

1.8 STRUCTURE ACTIVITY RELATIONSHIP (SAR)

The pharmacokinetic and metabolism studies of resveratrol revealed that resveratrol is very short lived in the body, with a half life of 0.13 hours. The reason for the rapid clearance of resveratrol from the body is due to its rapid metabolism to sulfate and glucuronide metabolites. These findings suggest a need for changes in the structure of resveratrol, in order to improve its pharmacokinetic profile. However, before making attempts to modify structural properties of resveratrol, the structure-activity relationship of resveratrol needs to be carefully reviewed.

Ovesna et.al [71] reported their work on the structure activity relationship of resveratrol. They found the chemopreventive activity of resveratrol is dependent on the number and position of the hydroxyl group, stereochemistry, double bond and intramolecular hydrogen bonding. They reported that the stilbene structure with intact 4' hydroxyl group, double bond and bearing orthodiphenoxyl or paradiphenoxyl functionalities possesses a greater antichemopreventive activity.Another group reported the structure activity relationship of resveratrol, as a COX-2 inhibitor. [72] The study revealed that, the introduction of hydroxyl groups, in addition to existing ones, has resulted in an increased COX-2 inhibitory effect of resveratrol. The 3, 3';4, 4, 5, 5' hexa- hydroxyl- trans-stilbene was a potent COX-2 inhibitor, with a IC50 value of 0.00104 µM, against 0.034 µM for celecoxib. The compound was more selective for COX-2 than COX-1, with a selectivity index of 719 compared to 546 for celecoxib. Replacement of the OH group with the methoxyl group at 3,4' and 5 positions, resulted in decreased activity, activity was lesser than resveratrol itself. However 3,3',4,4',5,5' hexamethoxyl-trans-stilbene slightly bettered the COX-2 inhibitory effect. The IC50 value for this hexa methoxy derivative was 0.35 µM. as against 0.99 µM for resveratrol. One factor that has become evident from this study is that for better COX-2 activity, existing hydroxyl functional groups are essential. Replacing only hydroxyl groups, without changing the rest of the structure leads to decreased activity. However there is no information on this activity if one or two hydroxyl groups are selectively replaced with methoxyl groups.

In another study, the same group reported [73] that hydroxylated resveratrol had exhibited better antioxidant and cytotoxic effects compared to unmodified resveatrol. The 3, 3', 4', 5 tetrahydroxystilbene was found to have 6600 fold higher antiradical activity than resveratrol. The cytotoxicity studies with HL-60 cell lines, showed that stilbenes with the orthohydroxy group exhibited 3 fold increase in antistatic activity.

Lee et.al [74] have studied the free radical scavenging activity of resveratrol derivatives. They found that, having bromo, fluromethyl and iodo groups, while preserving existing hydroxyl groups, increases the free radical scavenging activity of resveratrol. Replacing all hydroxyl groups with methoxyl groups, results in a complete loss of activity. However, substituting only one hydroxyl group with glycoside moiety, results in just doubling IC-50 value, and not a complete loss of activity.

1.9 USE OF RESVERATROL DERIVATIVES AND DRUG DELIVERY SYSTEMS TO IMPROVE PHARMACOKINETIC PROFILE AND BIOLOGICAL ACTIVITY. .

To overcome rapid metabolism of reseratrol many efforts have been made. Pujic and his coworkers [75] proposed the use of various conjugates of resveratrol for delivery of resveratrol into skin. They prepared the various ester and conjugates of resveratrol and studied their hydrolysis *in-vitro* in presence of stratum corneum and cholesterol esterase enzymes. The resveratrol was initially reacted with acetic anhydride in pyridine to get triacetate ester followed by selective enzymatic deacetylation to yield 3,5-resveratrol diacetate. The diacetate resveratrol was conjugated to lipoic acid and vitamin E succinate to yield 3,5-diacetyl-resveratrol-4'-O-lipoate and 3,5-diacetyl-resveratrol-4'-O-succinate-vitamin E respectively. The lipoate and vitamin E conjugates of resveratrol were tested for stability at pH 5.5 and 7.4 at 37^0 C. Both conjugates were stable in pH 5.5 and were less stable underwent slow hydrolysis to yield mono and diacetate esters. The lipoate conjugate hydrolysed to resveratrol and lipoic acid with stratum corneum enzymes over period of 3 days by a slow release of resveratrol thus enabling antioxidant activity. The resveratrol-vitamin E conjugate did not undergo hydrolysis in presence of stratrum corneum enzymes, instead hydrolysed in presence of esterase, cholesterol esterase. Although this paper describes the scope for sustained delivery of resveratrol across the skin but did not

provide information of stability of conjugates while passing through actual skin. Further studies such as actual permeation these compounds across the skin and their stability in skin are needed. After passing through skin, these conjugates will be taken up into systemic circulation and stability of these compounds in presence of plasma estereases need to be studied. Moreover resveratrol itself is lipophilic, solubility after conjugating with lipophilic compounds was not given. Further increase in lipophilicty would make i.v delivery difficult. Hence further investigation such as solubility, stability in plasma and pharmacokinetic profile after i.v., oral and dermal administration is required in order to know whether these conjugates are better than resveratrol and are really viable options as a delivery system.

Lucia Biasutto et.al [76] have synthesized mitochondria targeted resveratrol derivatives and evaluated their *in vitro* anti cancer activity. The 3,5' diacetylresveratrol-4'-O-(4-triphenylphosphoniumbutyl) iodide was synthesized. The compound showed good activity against the murine colon cancer cell line, fast growing mouse embryonic fibroblast (MEF) but not against slow growing MEF. The activity was better than native resveratrol. Although authors claimed that product had transient stability against metabolic conjugation but no elaborate studies on metabolism was reported. The detailed pharmacokinetic and pharmacodynamic studies are essential to assess the metabolic stability and activities *in vivo*. Further biological evaluation of these compounds in animal models is required. In addition safety and toxicity of compounds are also essential. As there was new group added onto resveratrol as ether, no hydrolysis can be expected in the body to release parent compound. If proven safe and active than resveratrol, these compounds would be candidates for clinical studies.

Same group in another study [77] have prepared and evaluated pharmacokinetic properties of 3,4',5-tri(a-D-glucose-3-O-succinyl)-resveratrol. They found that ester bond in the compound did undergo rapid hydrolysis in the blood, all was converted back to resveratrol within an hour. After oral administration this glucose ester of resveratrol, only delayed absorption with C_{max} achieved after 4 hours compared to 60 minutes for resveratrol. However there was no change in the amount of native resveratrol reaching systemic circulation. AUC of resveratrol was found to be 53,4 µg*h/mL and for 3,4',5-tri(a-D-glucose-3-O-succinyl)-resveratrol it was found to be

51,8 ± 37,2 µg*h/mL. Both resveratrol and glucose ester did undergo metabolism to the same extent.

Biasutto and co workers [78] have also evaluated various carboxy esters of resveratrol and evaluated their stability in blood and permeability across isolated rat jejunum. The resveratrol triacetate and resveratrol-tri-mPEG hydrolysed completely in blood within an hour. But resveratrol-trimethane sulfonate was stable. The resveratrol and triacetate form appeared in basolateral side as phase-II metabolites. Resveratrol-triPEG although underwent hydrolysis but metabolism of resveratrol was prevented by adjacent PEG molecules. The resveratrol triamethane sulfonate neither underwent hydrolysis nor metabolism during the permeation studies. There was no information on biological activity of these molecules. Though resveratrol trimethane sulfonate was stable, but again it is a new molecule altogether, further preclinical biological evaluation is required for advancing this molecule into clinical trials.

Li et.al [79] have reported synthesis, *in vitro* mineralization and *in vivo* work focusing on bone regeneration activity of resveratrol-polycaprolactone conjugates. There was no report on the conjugates other biological activities, their stability in plasma and metabolism. Since these conjugates were used as implants in this study, solubility of conjugates was not an issue. However for IV dosing this conjugate had to be formulated in nanoparticles. Which may lead to their clearance by reticulo endothelial system. The drug release and other activity studies need to be performed. On other hand soluble conjugates may have advantages over this compound as they can be formulated as intravenous solution.

Jiang and his colleagues reported [80] design and synthesis of resveratrol with aliphatic acid ligands to assess their impact on biological activity of resveratrol. Research also studies the solubility of various resveratrol-4'-O-aliphatic acids and their binding to human serum albumin. The author reported that reseveratrol aliphatic acid derivatives had stronger affinity towards human serum albumin. There are no details about the activity and metabolic stability of acid ligands. Although 4' hydroxyl group was modified into aliphatic ligand, there two more hydroxyl groups at position 3 and 5 of resveratrol which can undergo metabolic conjugation to glucuronide and sulfate metabolites. The detailed studies to ascertain the activity and stability of resveratrol ligands *in vivo* are needed. Since the derivatives were made through non

cleavable ether linkage with aliphatic substitution, they constitute relatively new compounds which would not undergo hydrolysis. Further animal studiesare required.

Feng et.al [81] prepared resveratrol biotin conjugate for studying protein binding pattern of resveratrol in order to understand molecular mechanism of bioactivity. The conjugate was synthesized via ester linkage between resveratrol and biotin. Biotinylated resveratrol was synthesized in the presence of 1,3-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine as catalyst. The biotinylated resveratrol and un-modified resveratrol were tested for anticancer activity. The growth inhibitory activity was tested in MCF-7 and Hep G2 cell lines. At 300 µM, the resveratrol-biotin showed growth inhibition of 30% against 40% for resveratrol and similar pattern of inhibition was observed for both cell lines. The authors have concluded that biotinylation did not affect biological activity of resveratrol. There was a small decrease in solubility of resveratrol reported after conjugation to biotin. The biotinylated resveratrol was used as substrate instead of native resveratrol in order to facilitate easy assay with enzyme linked immnosorbent assay (Elisa). The information on plasma stability and metabolism of resveratrol is not mentioned in the paper. Detailed structure elucidation of conjugate was not performed, although MS spectrum indicates mono biotinylated resveratrol but exact position of substitution was not derived. The positive outcome of the study was that mono substitution of resveratrol with bulky biotin does not impair its biological activity; however fate of conjugated molecule *in vivo* needs to be investigated.

In addition to resveratrol derivatives and conjugates, drug delivery systems have also been investigated for resveratrol. Guo et.al have investigated the antitumor effects and functional mechanism of resveratrol-bovine serum albumin nanoparticles on human primary ovarian carcinoma cells in nude mice [82]. The tumour was developed in the mice by injecting carcinoma cells subcutaneously. The tumour bearing mice were given intraperitoneally resveratrol nanoparticles, normal saline and albumin. The *in vivo* antitumor efficacy was evaluated by measurement of tumor volume, whereas morphological alterations were observed by transmission electron microscope (atomic force microscopy); TUNEL assays and immunoblotting for apoptotic and cell proliferation proteins were carried out to elucidate the possible mechanism. The administration of resveratrol nanoparticles significantly retarded the

growth of carcinomas in nude mice from the third week onwards, and the inhibition rate was markedly higher than in mice treated with resveratrol (52.43% vs. 46.34%, $p < 0.05$), without causing weight loss ($p > 0.05$). The nanoparticle formulation approach proved to be effective in enhancing biological activity of resveratrol in animal model, however formulation needs to be worked out to release all entrapped resveratrol in a reasonable period of time to maximize its activity.

Shao et.al have studied the cytotoxic effects of resveratrol loaded mPEG-PCL nanoparticles in comparison to free resveratrol in glioma cells [83]. The results revealed better uptake of resveratrol loaded naoparticles by glioma cells than free resveratrol. The resveratrol loaded nanoparticles showed significantly higher cell death compared to resveratrol. Furthermore free resveratrol was less cytotoxic than resveratrol loaded nanoparticles. The authors attributed such difference in this activity between free resveratrol and its nanoparticles to improved uptake of resveratrol into cells when it is in nanoparticles. Increased intracellular reactive oxygen species (ROS) levels was thought to be the reason for the anticancer activity. In contrast to the ability of resveratrol to scavenge ROS [1], in malignant cells it induced the ROS there by exerting cyctotoxic activity [83]. Although this formulation technology has improved the *in vitro* anticancer activity of resveratrol, authors did point out need of confirmation from in-vivo studies. It is envisaged that problems such as limited drug loading, incomplete drug release, stability of system and fast clearance by RES inside the body are likely going to be the barriers.

1.10 EFFECT OF POLYMER CONJUGATION ON METABOLISM OF DRUGS

There are many reports on possible effects of polymer conjugation on metabolism and pharmacokinetic profile of peptide and non peptide drugs. Many factors are involved in the removal of substances from the circulation. Proteins and peptides are rapidly eliminated by proteolytic degradation, renal filtration, and/or immunogenic and antigenic reactions. For small molecules such as resveratrol, its clearance from blood circulation involves biotransformation to various metabolites followed by excretion. Monfardini et.al [84] in their review, summarized various research outcomes of polymer conjugation on circulation time of peptides and proteins. Based on their survey, it can beconcluded that elimination of peptides/proteins by

proteolytic degradation, immunogenic and antigenic responses were significantly reduced and circulation times were prolonged by conjugation with polymers. They also concluded that polymer conjugation of non protein drugs leads to the long-lasting activity due to the increased half-life in the circulation, which is a result of protection against metabolism and/or reduced kidney ultrafiltration because of the increased size of the drug-polymer conjugate. They also stated that polymer modification could also impart decreased toxicity due to polymer shielding of "toxic" moieties, and the possibility of targeting to specific organs and tissues by introduction of a active targeting ligand along the polymer backbone.

Polymer conjugation approach has been successfully applied for improving safety, efficacy and pharmacokinetic profile of small molecule drug such as doxorubicin (DOX), norfloxacine (NOR), campothecine (CAMP) and paclitaxol (PTX).
Numerous reports have been published on polymer conjugation of doxorubicin. Dororubicin-dextran polymer conjugates were extensively investigated. Doxorubicin was coupled to oxidized dextran 70 chain via Schiff's base formation. Conjugate was found to have higher plasma level, persisting for long time, thus provided very effective passive targeting [85.] However phase I clinical studies of the conjugate showed that conjugate was slightly more toxic than DOX itself. WHO grade IV hepatotoxicity, grade III cardiotoxicity, grade III thrombocytopenia were some of the major clinical signs noticed. However polymer conjugate had prolonged circulation time with dose dependent elimination half life of $t_{1/2\beta}$ of 2.69 to 11.58 hours and $t_{1/2\gamma}$ of 41.44 to 136.58 hours (doses 12.5mg/m^2 to. 40mg/m^2). Based on these studies, dose of 12.5mg/m^2 was recommended for phase II clinical studies.

Doxorubicin conjugates with polyethylene glycol (PEG) were also investigated in preclinical studies. Various DOX-PEG conjugates were prepared with different tetrapeptide spacers and PEGs of varying molecular weight [86]. The purpose of research work was to achieve longer circulation and greater tumour targeting. Among the various spacers tested Glycine-phenyl alanin-leucine-glycine(GFLG), Glycine-leuci-glycine (GLG), Glycine-Glycine-arginine-arginine (GGRR), and Arginine-Glycine-Leuci-Glycine (RGLG) were found to provide porolonged release in presence of lysosomal enzymes *in vitro* (16% release in 5 hours). Thus providing slow and targeted release of DOX in cytoplasm of the cells by action of lysosomes.

The circulation time of DOX-Peptide-PEG in the body was dependent on the Mw of PEG, the longer Mw PEGs provided prolonged circulation time, however there was no exact coorelation between Mw of polymer and circulation time. This was because some of DOX-peptide-PEG with smaller PEG chain formed micelles, which eventually had longer circulation times. The conjugates were found to be 10 fold less cytotoxic than DOX (IC 50 > 2µg/mL vs 0.24µg/mL for DOX). DOX-peptide-PEG conjugates had improved tumour taragetting with releatively less DOX levels in the heart. Antitumor activity of DOX-Peptide-PEG in the sc B16F10 and ip L1210 models in black male mice shown that conjugates had superior activity compared to DOX (T/C value 146 against 120 for DOX).

Doxorubicin was successfully conjugagted to D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) [87]. The TPGS was initially derivitized to acid functional succcinyl end group, later connected to DOX via ester link. DOX-TPGS exihibited better *in vitro* activity than DOX itself against MCF-7 cell lines, with IC50 of 0.03 µM against 0.117 µM for DOX. This was attributed to improved uptake of conjugates by cells. The pharmacokinetic studies of conjugate revealed 4.5 fold increase in half life and 24 fold increase in area under the curve (AUC). Drug levels in heart, gastric and intestine were reduced, indicating passive targeting and decreased side effects.

Paclitaxol has been conjugated to soluble polymers such as polyethylene glycol [88], micelle forming diblock polymer polyethylene glycol-polylactide (PEG-PLA) [89] and triblock polymer PLA-PEG-PLA [90]. The paclitaxol-PEG conjugated made via ester link was found to undergo rapid hydrolysis in plasma with $t_{1/2}$ of 30 minutes. The PTX-PEG ester conjugates were considered ideal for prodrugs for parenteral formulation to overcome solubility issues. However PTX-PEG prodrugs were found unsuitable for the use as long circulating drug delivery systems. The paclitaxol was conjugated to PLA-PEG with an ester bond, the conjugate was found to be active against H7402 liver cancer cell line [89]. Authors concluded that paclitaxol was released in the cells and polymer conjugation did not affect the activity of paclitaxol. However there are no data on pharmacokinetic and *in vivo* activity was reported. Similarly PTX-PLA-PEG-PLA was also prepared, and micelle properties and *in*

vitro activity were evaluated. Again there were no *in vivo* studies reported on this conjugate [90].

Although polymer conjugates were reported to slow down metabolism of drugs/peptides and enhance the circulation time, the mechanism by which the polymer stabilize the drugs against metabolism needs to be reviewed.

Veronese et.al [91] conducted review on the possible mechanism for reduced metabolism and increased circulation time of drugs after conjugation to polymers. They hypothesized that shielding effect of polymer provides protection against chemical and enzymatic degradation and masks the antigenic sites of drugs which can trigger immunogenic response from the body. In the case of PEG, high molecular mobility, conformational flexibility and water binding ability of polymer chain prevent, mainly by thermodynamic effects, approach of degrading enzymes towards drugs. The large size of macromolecular conjugates reduces the renal excretion of drugs. This happens when threshold volume of molecule reaches the volume of serum albumin. Due to large molecular size, it makes possible for tumour targeting of drugs via enhanced permeation and retention (EPR) phenomenon.

In addition to shielding effects, some PEG derivatives such as Vitamin E-succinate-PEG (TPGS) has shown to have inhibitory effects on drug metaboliszing enzymes such as cytochrome P450 3A (CYP3A) thereby exerting chemical inhibition of drug metabolism [92].

Inhibitory effects of various formulation vehicles on Cytochorme P450 using human liver microsomes, monkey intestinal microsomes with 7-benzyloxy-4-(trifluoromethyl)-coumarin (BFC) as substrate has been reported [93]. The PEG 400 was found to inhibit metabolism of BFC. It was suggested that inhibition of CYP 3A was responsible for reduction in the metabolism of BFC. Gonzalez et.al [94] have investigated effect of surfactants such as solutol HS, Cremophore EL and Tween-80 on metabolism of midazolam with liver microsomes and hepatocytes. There was up to 51% reduction in the midazolam clearance observed with all surfactants. The surfactants did not show cytotoxic effects on cells. The direct enzyme inhibition and molecular interactions between surfactant and microsomes were attributed to reduction in metabolism. In case of Tween-80 and Cremophore EL, reduction in metabolism of midazolam with hepatocytes was only significant above the critical

micelle concentrations. This indicates release of midazolam from micelles or shielding effects micelles would also contribute to reduction metabolism. Hence it was suggested that physical entrapment of drugs by micelles can also be an fficient tool for stabilizing drugs against metabolism.

Biasutto et.al also have reported the inhibitory effect of PEG on intestinal metabolism of resveratrol. The resveratrol-PEG conjugates although hydrolysed while passing through intestinal membrane but adjacent PEG molecules prevented the metabolism of resveratrol [78].

Overall the polymer conjugation can be considered as an effective option for improving pharmacokinetic profile of resveratrol. By using appropriate polymers, the prodrugs /conjugates may also form polymeric micelles which could also provide opportunity to improve the stability and biological activity of resveratrol. The resveratrol can either be entrapped into the micelles or it can be conjugated to polymers which can self assemble into micelles. These formulations can be used for prolonged release of resveratrol in the body. It is believed that micelle structures can also provide additional stability of conjugates against hydrolysis by plasma esterases. The entrapment of resvertrol in mPEG-PCL micelles has shown to improve *in vitro* anticancer activity and such an effect was attributed to improved uptake of micelles by cancer cells [83]. In light of these observations exploration of micelle formulations either by physical entrapment or by chemical conjugation of the resveratrol to polymers appears to be a feasible approach to improve pharmacokinetic profile of resveratrol. Despite of these, there is very limited research conducted with resveratrol polymer conjugates (Table 1.1), further development and evaluation of resveratrol polymer conjugates will bring the benefit to not only to the therapeutic application of resveratrol but also to the research field addressing the issues of how to overcome the rapid metabolism of some therapeutic molecules.

Table 1.1: A summary of resveratrol polymer conjugates found in the literature

Polymer used	Extent of resveratrol substitituiton	Mw of polymer	Formulation	Studies conducted/Outcomes	Reference
PEG	Not mentioned	1900 Da	solution	Blood stability, intestinal stability and metabolism. The conjugate hydrolysed completely in an hour in the blood. Permeation studies showed that polymer hydrolyzed to resveratrol but adjacent polymer stabilized against metabolism.	78
Poly-caprolactone	Not given	80000 Da	Polymer porousscaffold	Bone remineralization in rats. Resveratrol-PCL increases bone remineralization.	79
PEG	Not given	2000-20000 Da	Not mentioned	RGD binding and other biological studies. Improved biological activity. No details are mentioned in the patent.	95
PLA/Polyacrylic acid	Not given	2000-20000 Da	Not mentioned	Same patent, not much of information given	95
PLA-Polylysine/ Hyaluronic acid	Not given	2000-20000 Da	Not mentioned	Same patent, not much of information given	95

1.11. OBJECTIVES OF THIS STUDY

This PhD research project aimed to develop resveratrol-polymer conjugates which could provide the prolonged circulation of resveratrol in the body and protect the resveratrol against metabolism. Various polymers with diverse chemical structure were explored for this purpose. Below are detailed *in vitro* and *in vivo* studies which were performed to achieve the overall objective and to test the effectiveness of polymeric conjugation strategies for formulation of resveratrol delivery systems.

1. Synthesized resveratrol-PEG conjugates via ester linkage and studied the effect of polymer molecular weight and linker on plasma stability.
2. Synthesized resveratrol-PLA-PEG conjugates via ester linkage.
3. Synthesized resveratrol-PEG conjugates with non hydrolysable ether linkage.
4. Characterized the polymer conjugates by NMR, IR and LC-MS for structural elucidation.
5. Studied the physicochemical properties of conjugates in terms of particles size and critical micellar concentrations.
6. Developed and validated suitable HPLC method to separate and quantify the resveratrol, its polymer conjugates and analysis of plasma samples of selected polymer conjugates and resveratrol.
7. Evaluated stability of conjugates in various buffers and rat plasma.
8. Developed LC-MS/MS method closely matching LC conditions of HPLC to identify the metabolites.
9. Conducted preliminary metabolism studies of resveratrol and its polymer conjugates in rat liver microsomes in presence of uridine diphsopho glucuronic acid (UDPGA).
10. Conducted preliminary single dose pharmacokinetic studies of resveratrol and its different polymer conjugates via i.v. injection.

1.12 SIGNIFICANCE OF PROPOSED WORK

Although resveratrol has shown diverse bioactivities *in vitro,* its *in vivo* activities have not been proven unequivocally. The reason for lack of *in vivo* effectiveness was due to its rapid metabolism in the body. It was proposed that the formulation approaches which could protect resveratrol against its metabolism *in vivo* could eventually enhance its pharmacological activities. This research work tested the strategy of using polymeric conjugates to protect resveratrol against metabolism *in vivo,* thereby prolong the its biological half life and improve pharmacokinetic profile.. The success of the project would improve possibilities of therapeutic success of resveratrol, thus provides new therapeutic opportunities for resveratrol. Furthermore this study wouldalso provide an opportunity to gain knowledge in optimization of the design of resveratrol-polymer conjugates.

1.13 THESIS OVERVIEW

The research work of the project has been compiled in four chapters, each consisting of an introduction, experimental, results and discussion section. These chapters are followed by a general discussion and conclusions chapter. The four main chapters of thesis explain four varied parts of my research work as explained below,

- **Synthesis, characterization, biological and preliminary pharmacokinetic evaluation of resveratrol-polymer ester conjugates**. This chapter describes synthesis and characterization of resveratrol-polymer conjugates formed via ester bonds. It explains how polymer conjugates of resveratrol were assessed *in vitro* for buffer stability, plasma stability, *in vitro* antioxidant function and metabolism in presence of rat liver microsomes. This chapter also reports the preliminary intravenous pharmacokinetic studies conducted for selected polymer conjugates in Wistar rats. Due to the time constrain, only single dose i.v. bioavailabilities of resveratrol and selected polymer conjugates were studied.

- **Synthesis, characterization, biological and preliminary pharmacokinetic evaluation of resveratrol-PEG ethers:** This chapter focuses on synthesis and characterization of resveratrol-PEG ethers. This chapter elaborates how

reaction conditions were optimized to achieve maximum PEG conjugation to resveratrol. It also describes *in vitro* and *in vivo* biological studies conducted to evaluate the effectiveness of polymer ether conjugates in improving pharmacokinetic profile of resveratrol.

- **Development of HPLC method for simultaneous analysis of resveratrol, resvertrol-polymer conjugates and detection of resveratrol metabolites:** This chapter describes the development of a sensitive HPLC method for detection and quantification of resveratrol, its polymer conjugates and detection of metabolites. This chapter also explains how method was optimized to get maximum sensitivity with fluroscence detection. Various validation parameters for resveratrol and some of selected conjugates were reported and discussed.

- **Developments of a LC-MS methodology to separate, detect, characterize and quantify resveratrol-PEG conjugates and the conjugation reaction precursors and intermediates:** This chapater emphasizes on how an LC-MS method was developed and applied for characterization of resveratrol-PEG conjugates. Italso explains how method was utilized to monitor PEGylation process and determine extent of PEG functionalization and conjugation to resveratrol.

  All investigations in this project were carried out using a commercially available isomeric form of resveratrol, ie **trans-resveratrol**,which is a stable form of naturally occurring polyphenol. .

The research objectives/activities associated with this PhD project are summarised in the flowchart shown inFigure 1.4.

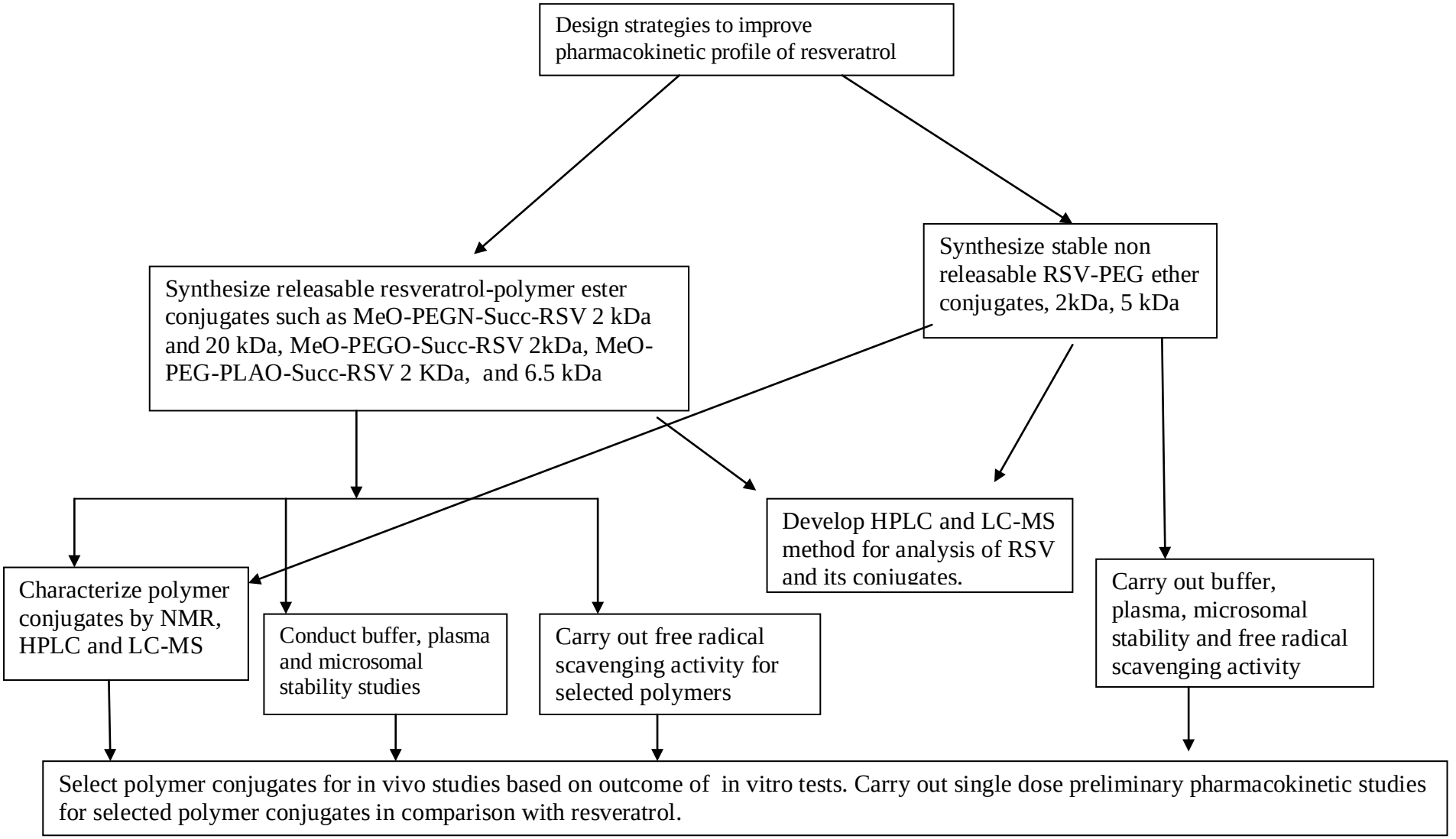


Figure 1.5: Summary of objectives of this PhD project.

1.14 REFERENCES:

1. Das DK, Maulik N. Resveratrol in cardioprotection: a therapeutic promise of alternative medicine. Mol Interv. 2006 Feb;6(1):36-47.
2. Budavari S. Resveratrol. Merck Index. 1996; 12th edition,:1402.
3. Prokop J, Abrman P, Seligson AL, Sovak M. Resveratrol and its glycon piceid are stable polyphenols. J Med Food. 2006 Spring;9(1):11-4.
4. Yang NC, Lee CH, song TY, Evaluation of resveratrol oxidation in vitro and crucial role of bicarbonate ions.Biosci.Biotechnol.Biochem, 2010, 74(1),63-68
5. Camont L, Cottart CH, Rhayem Y, Nivet-Antoine V, Djelidi R, Collin F, Beaudeux J L, Rousselot DB. Simple spectrophotometric assessment of the trans-/cis-resveratrol ratio in aqueous solutions. Analytica Chimica Acta:2009 534,121–128.
6. Le Corre L, Chalabi N, Delort L, Bignon YJ, Bernard-Gallon DJ. Resveratrol and breast cancer chemoprevention: molecular mechanisms. Mol Nutr Food Res. 2005 May;49(5):462-71.
7. Hattori R, Otani H, Maulik N, Das DK. Pharmacological preconditioning with resveratrol: role of nitric oxide. Am J Physiol Heart Circ Physiol. 2002 Jun;282(6):H1988-95.
8. Cadenas S, Barja G. Resveratrol, melatonin, vitamin E, and PBN protect against renal oxidative DNA damage induced by the kidney carcinogen KBrO3. Free Radic Biol Med. 1999 Jun;26(11-12):1531-7.
9. Fitzpatrick DF, Hirschfield SL, Coffey RG. Endothelium-dependent vasorelaxing activity of wine and other grape products. Am J Physiol. 1993 Aug;265(2 Pt 2):H774-8.
10. Klinge CM RK, Watts MB, Beck V, Eder R, Jungbauer A. Estrogenic activity in white and red whine extracts. JAgriFoodChem. 2003;51, 1850-7.
11. Das S, Das DK. Anti-inflammatory responses of resveratrol. Inflamm Allergy Drug Targets. 2007 Sep;6(3):168-73.
12. Pendurthi UR WJ, Rao LV. Resveratol, a polyphenolic compound found in wine, inhibits tissue factor expression in vascular cells:A possible mechanism for the cardiovascular benefits associated with moderate consumption of wine. ArteriosclerThromVascuBiol. 1999;19:419-26.

13. Kampa M, Hatzoglou A, Notas G, Damianaki A, Bakogeorgou E, Gemetzi C, et al. Wine antioxidant polyphenols inhibit the proliferation of human prostate cancer cell lines. Nutr Cancer. 2000;37(2):223-33.
14. Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW, et al. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science. 1997 Jan 10;275(5297):218-20.
15. Baldwin AS. THE NF-κB AND IκB PROTEINS: New Discoveries and Insights. Annual Review of Immunology. 1996;14(1):649-81.
16. Kundu JK, Surh YJ. Molecular basis of chemoprevention by resveratrol: NF-kappaB and AP-1 as potential targets. Mutat Res. 2004 Nov 2;555(1-2):65-80.
17. Gehm BD, McAndrews JM, Chien PY, Jameson JL.. Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. Proceedings of the National Academy of Sciences of the United States of America. 1997;94(25):14138.
18. Lu, R. and Serrero, G. Resveratrol, a natural product derived from grape, exhibits antiestrogenic activity and inhibits the growth of human breast cancer cells. Journal of Cellular Physiology, 1999, 179: 297–304
19. Delmas D, Jannin B, Latruffe N. Resveratrol: preventing properties against vascular alterations and ageing. Mol Nutr Food Res. 2005 May;49(5):377-95.
20. Fukao H, Ijiri Y, Miura M, Hashimoto M, Yamashita T, Fukunaga C, et al. Effect of trans-resveratrol on the thrombogenicity and atherogenicity in apolipoprotein E-deficient and low-density lipoprotein receptor-deficient mice. Blood Coagul Fibrinolysis. 2004 Sep;15(6):441-6.
21. Pal S, Ho N, Santos C, Dubois P, Mamo J, Croft K, et al. Red wine polyphenolics increase LDL receptor expression and activity and suppress the secretion of ApoB100 from human HepG2 cells. J Nutr. 2003 Mar;133(3):700-6.
22. DM, Hahn SE, Parkes JG. Beyond alcohol: beverage consumption and cardiovascular mortality. Clin Chim Acta. 1995 Jun 15;237(1-2):155-87.
23. Aziz MH, Kumar R, Ahmad N. Cancer chemoprevention by resveratrol: in vitro and in vivo studies and the underlying mechanisms (review). Int J Oncol. 2003 Jul;23(1):17-28.

24. Pace-Asciak, C. R.; Rounova, O.; Hahn, S. E.; Diamandis, E. P.; Goldberg, D. M., Wines and grape juices as modulators of platelet aggregation in healthy human subjects. Clinica chimica acta 1996, 246 (1-2), 163-182.
25. Olas B, Wachowicz B, Stochmal A, Oleszek W. Anti-platelet effects of different phenolic compounds from Yucca schidigera Roezl. bark. Platelets. 2002 May;13(3):167-73.
26. Bertelli AA, Giovannini L, Giannessi D, Migliori M, Bernini W, Fregoni M, et al. Antiplatelet activity of synthetic and natural resveratrol in red wine. Int J Tissue React. 1995;17(1):1-3.
27. Wang Z, Huang Y, Zou J, Cao K, Xu Y, Wu JM. Effects of red wine and wine polyphenol resveratrol on platelet aggregation in vivo and in vitro. Int J Mol Med. 2002 Jan;9(1):77-9.
28. Kaneider NC, Mosheimer B, Reinisch N, Patsch JR, Wiedermann CJ. Inhibition of thrombin-induced signaling by resveratrol and quercetin: effects on adenosine nucleotide metabolism in endothelial cells and platelet-neutrophil interactions. Thrombosis research. 2004;114(3):185-94.
29. Davis CD, Schut HAJ, Snyderwine EG. Enzymatic phase II activation of the N-hydroxylamines of IQ, MelQx and PhIP by various organs of monkeys and rats. Carcinogenesis. 1993 October 1, 1993;14(10):2091-6.
30. Falck F, Ricci A , Wolff MS, Godbold, J, Deckers P. biphenyl residues in human breast lipids and their relation to breast cancer. Archives of environmental health. 1992;47(2):143.
31. Denison MS, Whitlock JP. Xenobiotic-inducible transcription of cytochrome P450 genes. Journal of biological chemistry. 1995;270(31):18175.
32. Garner RC MC, Clayson DB editor. Carcinogenic aromatic amines and related compounds. 2nd Edition ed. Washington DC: American Chemical Socitey 1984.
33. Lee J-E, Safe S. Involvement of a post-transcriptional mechanism in the inhibition of CYP1A1 expression by resveratrol in breast cancer cells. Biochemical pharmacology. . 2001;62(8):1113-24.
34. Davis CD, Schut HAJ, Snyderwine EG. Enzymatic phase II activation of the N-hydroxylamines of IQ, MelQx and PhIP by various organs of monkeys and rats. Carcinogenesis. 1993 October 1, 1993;14(10):2091-6.

35. Turesky RJ, Lang NP, Butler MA, Teitel CH, Kadlubar FF. Metabolic activation of carcinogenic heterocyclic aromatic amines by human liver and colon. Carcinogenesis. 1991 October 1, 1991;12(10):1839-45.
36. Dubuisson JG, Dyess DL, Gaubatz JW. Resveratrol modulates human mammary epithelial cell O-acetyltransferase, sulfotransferase, and kinase activation of the heterocyclic amine carcinogen N-hydroxy-PhIP. Cancer letters.2002;182(1):27-32.
37. Doyle LA, Ross DD. Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2). Oncogene. 2003;22(47):7340.
38. Maliepaard M, Scheffer GL, Faneyte IF, van Gastelen MA, Pijnenborg AC, Schinkel AH, van De Vijver MJ, Scheper RJ, Schellens JH. Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues. Cancer research. 2001;61(8):3458.
39. Surh Y-J, Hurh Y-J, Kang J-Y, Lee E, Kong G, Lee SJ. Resveratrol, an antioxidant present in red wine, induces apoptosis in human promyelocytic leukemia (HL-60) cells. Cancer letters. 1999;140(1-2):1-10.
40. Hsieh TC, Wu JM. Differential effects on growth, cell cycle arrest, and induction of apoptosis by resveratrol in human prostate cancer cell lines. Experimental cell research. 1999;249(1):109.
41. Bove K, Lincoln DW, Tsan M-F. Effect of Resveratrol on Growth of 4T1 Breast Cancer Cells in Vitro and in Vivo. Biochemical and biophysical research communications; 2002;291(4):1001-5.
42. Hsieh TC, Burfeind P, Laud K, Backer JM, Traganos F, Darzynkiewicz Z, et al. Cell cycle effects and control of gene expression by resveratrol in human breast carcinoma cell lines with different metastatic potentials. International journal of oncology. 1999;15(2):245-52.
43. Australian Institute of Health and Welfare and Australasian Association of Cancer Registries, Cancer in Australia, an overview 2008, Canberra, 2008.
44. Sun ZJ, Pan CE, Liu HS, Wang GJ Anti-hepatoma activity of resveratrol in vitro. WORLD JOURNAL OF GASTROENTEROLOGY. 2002;8(1):79.
45. Carbó N, Costelli P, Baccino FM, López-Soriano FJ, Argilés JM. Resveratrol, a Natural Product Present in Wine, Decreases Tumour Growth in a Rat Tumour Model. Biochemical and biophysical research communications. 1999;254(3):739-43.

46. Ciolino HP, Yeh GC. The effects of resveratrol on CYP1A1 expression and aryl hydrocarbon receptor function in vitro. Adv Exp Med Biol. 2001;492:183-93.
47. Delmas, D, Jannin B, Latruffe N. Resveratrol, a chemopreventive agent, disrupts the cell cycle control of human SW480 colorectal tumor cells. International Journal of Molecular Medicine. 2002;10(2):193.
48. Wolter F, Clausnitzer A, Akoglu B, Stein J. Piceatannol, a natural analog of resveratrol, inhibits progression through the S phase of the cell cycle in colorectal cancer cell lines. The Journal of nutrition. 2002;132(2):298.
49. Schneider Y, Vincent F, Duranton B, Badolo L, Gossé F, Bergmann C, et al. Anti-proliferative effect of resveratrol, a natural component of grapes and wine, on human colonic cancer cells. Cancer Letters. 2000;158(1):85-91.
50. Wolter F, Akoglu B, Clausnitzer A, Stein J. Downregulation of the Cyclin D1/Cdk4 Complex Occurs during Resveratrol-Induced Cell Cycle Arrest in Colon Cancer Cell Lines. The Journal of Nutrition. 2001 August 1, 2001;131(8):2197-203.
51. Mahyar-Roemer M, Roemer K.p21 Waf1/Cip1 can protect human colon carcinoma cells against p53-dependent and p53-independent apoptosis induced by natural chemopreventive and therapeutic agents. Oncogene. 2001;20(26):3387.
52. Mahyar-Roemer M, Katsen A, Mestres P, Roemer K. Resveratrol induces colon tumor cell apoptosis independently of p53 and precede by epithelial differentiation, mitochondrial proliferation and membrane potential collapse. International journal of cancer. 2001;94(5):615.
53. Soleas GJ, Goldberg DM, Grass L, Levesque M, Diamandis EP. Do wine polyphenols modulate p53 gene expression in human cancer cell lines? Clinical Biochemistry.2001;34(5):415-20.
54. She Q.B, Huang C, Zhang, Y, Dong Z. (2002), Involvement of c-jun NH_2-terminal kinases in resveratrol-induced activation of p53 and apoptosis. Molecular Carcinogenesis, 33: 244–250.
55. Gao X, Xu YX, Divine G, Janakiraman N, Chapman RA, Gautam SC. Disparate In Vitro and In Vivo Antileukemic Effects of Resveratrol, a Natural Polyphenolic Compound Found in Grapes. The Journal of Nutrition. 2002;132(7):2076-81.

56. Gautam SC, Xu Yx, Dumaguin M, Janakiraman N, Champman RA, Resveratrol selectively inhibits leukemia cells: a prospective agent for ex vivo bone marrow purging. Bone marrow transplantation. 2000;25(6):639.
57. Pervaiz S. Resveratrol-from the Bottle to the Bedside? Leukemia & lymphoma. 2001;40(5):491.
58. Elmali N, Esenkaya I, Harma A, Ertem K, Turkoz Y, Mizrak B. Effect of resveratrol in experimental osteoarthritis in rabbits. Inflammation Research. 2005;54(4):158-62.
59. Tessitore L, Davit A, Sarotto I, Caderni G. Resveratrol depresses the growth of colorectal aberrant crypt foci by affecting bax and p21CIP expression. Carcinogenesis. 2000;21(8):1619-22.
60. Li ZG, Hong T, Shimada Y, Komoto I, Kawabe A, Ding Y, et al. Suppression of N-nitrosomethylbenzylamine (NMBA)-induced esophageal tumorigenesis in F344 rats by resveratrol. Carcinogenesis. 2002;23(9):1531-6.
61. Ziegler CC, Rainwater L, Whelan J, McEntee MF. Dietary resveratrol does not affect intestinal tumorigenesis in Apc(Min/+) mice. J Nutr. 2004 Jan;134(1):5-10.
62. Wenzel E, Somoza V. Metabolism and bioavailability of trans-resveratrol. Mol Nutr Food Res. 2005 May;49(5):472-81.
63. Yu C, Shin YG, Chow A, Li Y, Kosmeder JW, Lee YS, et al. Human, Rat, and Mouse Metabolism of Resveratrol. Pharmaceutical Research. 2002;19(12):1907-14.
64. Marier JF, Vachon P, Gritsas A, Zhang J, Moreau JP, Ducharme MP.Metabolism and disposition of resveratrol in rats: extent of absorption, glucuronidation, and enterohepatic recirculation evidenced by a linked-rat model. The journal of pharmacology and experimental therapeutics. 2002;302(1):369.
65. Kuhnle G, Spencer JPE, Chowrimootoo G, Schroeter H, Debnam ES, Srai SKS, et al. Resveratrol Is Absorbed in the Small Intestine as Resveratrol Glucuronide. Biochemical and Biophysical Research Communications. 2000;272(1):212-7.
66. Walle T, Hsieh F, DeLegge MH, Oatis JE, Jr., Walle UK. High absorption but very low bioavailability of oral resveratrol in humans. Drug Metab Dispos. 2004 Dec;32(12):1377-82.

67. Boocock DJ, Faust GES, Patel KR, Schinas AM, Brown VA, Ducharme MP, et al. Phase I Dose Escalation Pharmacokinetic Study in Healthy Volunteers of Resveratrol, a Potential Cancer Chemopreventive Agent. Cancer Epidemiology Biomarkers & Prevention. 2007;16(6):1246-52.
68. Almeida L, Vaz-da-Silva M, Falcao A, Soares E, Costa R, Loureiro AI, et al. Pharmacokinetic and safety profile of trans-resveratrol in a rising multiple-dose study in healthy volunteers. Mol Nutr Food Res. 2009 May;53 Suppl 1(1):S7-15.
69. Miksits M, Wlcek K, Svoboda M, Kunert O, Haslinger E, Thalhammer T, et al. Antitumor Activity of Resveratrol and its Sulfated Metabolites against Human Breast Cancer Cells. Planta Med. 2009; 75:1227,30.
70. Prentis RA. Lisa Y, Walker S. Pharmaceutical innovation by the seven UK-owned pharmaceutical companies (1964-1985). British journal of clinical pharmacology. 1988;25(3):387.
71. Ovesna Z, Horvathova-Kozics K.Structure—Activity Relationship of trans-Resveratrol and Its Analogues.Chem Inform. 2008;39(28).
72. Murias M. Resveratrol analogues as selective cyclooxygenase-2 inhibitors: synthesis and structure-activity relationship. Bioorganic & medicinal chemistry. 2004;12(21):5571.
73. Murias M, Jäger W, Handler N, Erker T, Horvath Z, Szekeres T, et al. Antioxidant, prooxidant and cytotoxic activity of hydroxylated resveratrol analogues: structure-activity relationship. Biochemical Pharmacology. 2005;69(6):903-12.
74. Lee HJ, Seo JW, Lee BH, Chung K-H, Chi DY. Syntheses and radical scavenging activities of resveratrol derivatives. Bioorganic & Medicinal Chemistry Letters 2004;14(2):463-6.
75. Gelo-Pujic M, Desmurs JR, Delaire S, Adao A,Tawil, D Synthesis of new antioxidant conjugates and their in vitro hydrolysis with Stratum corneum enzymes. International journal of cosmetic science. 2008;30(3):195.
76. Biasutto L, Mattarei A, Marotta E, Bradaschia A, Sassi N, Garbisa S, et al. Development of mitochondria-targeted derivatives of resveratrol. Bioorganic & Medicinal Chemistry Letters. 2008;18(20):5594-7.

77. Biasutto L, Marotta E, Bradaschia A, Fallica M, Mattarei A, Garbisa S. Synthesis and bioavailability of 3, 4', 5-tri ([alpha]-d-glucose-3-O-succinyl) resveratrol. Bioorganic and medicinal chemistry letters. 2009;19(23):6721.
78. Biasutto L, Marotta E, Mattarei A, Beltramello S, Caliceti P, Salmaso S. Absorption and metabolism of resveratrol carboxyesters and methanesulfonate by explanted rat intestinal segments. Cellular physiology and biochemistry. 2009;24(5-6):557-66.
79. Li Y, Dnmark S, Edlund U, Finne-Wistrand A, He X, Norgrd M, et al. Resveratrol-conjugated poly-ε-caprolactone facilitates in vitro mineralization and in vivo bone regeneration. Acta Biomaterialia. 2011;7(2):751-8.
80. Jiang YL. Design, synthesis and spectroscopic studies of resveratrol aliphatic acid ligands of human serum albumin. Bioorganic & medicinal chemistry. 2008;16(12):6406.
81. Feng L, Jin J, Zhang L-F, Yan T, Tao W-Y. Analysis of the resveratrol-binding protein using phage-displayed random peptide library. Shengwu Huaxue Yu Shengwu Wuli Xuebao. 2006;38(5):342.
82. Guo L, Peng Y, Yao J, Sui L, Gu A, Wang J. Anticancer Activity and Molecular Mechanism of Resveratrolâ“Bovine Serum Albumin Nanoparticles on Subcutaneously Implanted Human Primary Ovarian Carcinoma Cells in Nude Mice. Cancer biotherapy & radiopharmaceuticals. 2010;25(4):471.
83. Shao J, Li X, Lu X, Jiang C, Hu Y, Li Q. Enhanced growth inhibition effect of Resveratrol incorporated into biodegradable nanoparticles against glioma cells is mediated by the induction of intracellular reactive oxygen species levels. Colloids and surfaces B, Biointerfaces. 2009;72(1):40.
84. Monfardini C, Veronese FM. Stabilization of substances in circulation. Bioconjugate chemistry. 1998;9(4):418.
85. Danhauser-Riedl S, Hausmann E, Schick H-D, Bender R, Dietzfelbinger H, Rastetter J,. Phase I clinical and pharmacokinetic trial of dextran conjugated doxorubicin (AD-70, DOX-OXD). Investigational new drugs. 1993;11(2-3):187.

86. Veronese FM. PEG Doxorubicin Conjugates: Influence of Polymer Structure on Drug Release, in Vitro Cytotoxicity, Biodistribution, and Antitumor Activity. Bioconjugate chemistry. 2005;16(4):775.
87. Cao N, Feng S. Doxorubicin conjugated to d-[alpha]-tocopheryl polyethylene glycol 1000 succinate (TPGS): Conjugation chemistry, characterization, in vitro and in vivo evaluation. Biomaterials. 2008;29(28):3856.
88. Greenwald RB, Gilbert CW, Pendri A, Conover CD, Xia J, Martinez A. Drug Delivery Systems: Water Soluble Taxol 2-Poly (ethylene glycol) Ester ProdrugsDesign and in Vivo Effectiveness. Journal of medicinal chemistry. 1996;39(2):424.
89. Zhang X, Li Y, Chen X, Wang X, Xu X, Liang Q. Synthesis and characterization of the paclitaxel/MPEG-PLA block copolymer conjugate. Biomaterials. 2005;26(14):2121.
90. Xie, Z., Lu, T., Chen, X., Lu, C., Zheng, Y. and Jing, X. (2007), Triblock poly(lactic acid)-b-poly(ethylene glycol)-b-poly(lactic acid)/paclitaxel conjugates: Synthesis, micellization, and cytotoxicity.Journal of applied polymer science. 2007;105(4):2271.
91. Veronese FM, Morpurgo M.. Bioconjugation in pharmaceutical chemistry. Il farmaco. 1999;54(8):497.
92. Ciolino HP, Yeh GC. The effects of resveratrol on CYP1A1 expression and aryl hydrocarbon receptor function in vitro. Adv Exp Med Biol. 2001;492:183-93.
93. Mountfield RJ, Senepin S, Schleimer M, Walter I, Bittner B. Potential inhibitory effects of formulation ingredients on intestinal cytochrome P450. International journal of pharmaceutics. 2000;211(1-2):89.
94. González RC, Huwyler J, Boess F, Walter I, Bittner B. In vitro investigation on the impact of the surface-active excipients Cremophor EL, Tween 80 and Solutol HS 15 on the metabolism of midazolam. Biopharmaceutics & drug disposition. 2004;25(1):37.
95. Bridges AM, Mousa AS, Davis PJ. Polyphenol conjugates as RGD binding compounds and method of use, US patent No US20100209382A1

CHAPTER 2

SYNTHESIS, CHARACTERIZATION, BIOLOGICAL AND PRELIMINARY PHARMACOKINETIC EVALUATION OF RESVERATROL-POLYMER ESTER CONJUGATES.

2.1 INTRODUCTION

Due to natural abundance and diverse biological activities, resveratrol has attracted much of research.[1-4] Resveratrol being polyphenolic in nature has tendency to undergo phase II metabolism to form metabolites via glucuronidation and sulfation. [5-9] Despite showing good biological activity under *in vitro* conditions, resveratrol failed to produce the desired pharmacological activity in animal models. [10-12] However, the presence of hydroxyl groups in resveratrol provides an opportunity for chemical modification to produce ester or ether prodrug derivatives. This could be explored to design polymeric prodrugs/conjugates for masking molecules against metabolism. Although the structure of resveratrol is favourable for modification to form polymeric conjugates, the structure activity relationship of resveratrol should be taken into consideration in developing polymer conjugates. Studies of resveratrol revealed that the hydroxyl group in resveratrol is essential for the biological activity. The structural modifications with bromo, iodo and fluromethyl groups on aromatic rings have shown better activity than resveratrol; however replacement of hydroxyl groups with any other functional moieties has resulted in reduced activity. [13, 14] This indicates that hydroxyl groups in structure contribute to its bioactivity. In order to sustain resveratrol activity in the body, simple but reversible chemical modification of resveratrol, which can mask the molecule against metabolism, as well as being able to release native resveratrol in the body would be desirable. In this regard, developing polymeric conjugates would be an effective formulation strategy for overcoming the rapid metabolism and prolonging resveratrol residence time *in vivo*. As reviewed in chapter one, the polymer conjugation strategy has been successfully applied to small molecules such as paclitaxol, campothecin, docetaxol, doxorubicin, norfloxacin. The polymer conjugation proved effective in improving solubility, circulation time, enhanced cellular uptake and tumour targeting. [15]

2.1.1 Review of synthetic procedures for the synthesis of drug-polymer ester conjugates.

The release of drugs from the polymer conjugates depends on the nature of polymer, the type of linker and the drug. [16, 17] The hydrolysis of polymer conjugates (esters) in plasma is a rate limiting process. Not all chemical modification or polymer conjugation will enhance the activity of drug molecules. For instance, PEGylation of paclitaxol using carbamate bonding reduced the drug activity. [18] The reduced activity

was attributed to two reasons, the large bulky PEG substitution blocked the activity at the target cell and the possibility that paclitaxol failed to reach the target site. [18] The half life of polymeric conjugates can depend on the nature of the drug and the position of the functional group to which the polymer is attached; for example, paclitaxol when PEGylated via ester linkage at 2' hydroxyl group had a very short plasma half life (hydrolysis) of 0.4h. In contrast to this, the paclitaxol-7-PEG with the same linker had $t_{1/2}$ of >400 h. [19]

The prolonged release of drugs from a polymer conjugate can be achieved by varying the type of linker. The plasma hydrolysis of campothecin-PEG ester was strongly dependent on the structure of the linker. The PEG esters with glycine linker hydrolysed more slowly in rat plasma than the plain PEG ester. [20] The plasma stability further varied as the electronegativity of the α amido group changed. The highest plasma stability of ester was observed with the most electronegative substitution on the α amido group of the linker [20]. The alkylations of nitrogen also lead to a significant increase in plasma stability. Although the glycine PEG ester was more stable than carboxy PEG ester in plasma, it was less stable compared to the other N-substituted glycine linker, attributed to anchimeric participation of a basic NH group in a-lactam intermediate formation in the campothecin-glycine-PEG molecule. [20] Tripartate PEG conjugates have been extensively investigated for drug delivery. Essentially, the system consists of two prodrug moieties; one actually cleaves by enzymatic hydrolysis and another by chemical decomposition. Tripartate PEG drugs were not only soluble, but also had extended plasma half life. This technology has been successfully applied to anticancer drugs containing amine group. Benzyl elimination systems were explored for this purpose, the amine containing anticancer drug daunorubicin was coupled to the benzyl linker via the carbamate bond, and the benzyl linker was conjugated to the PEG with different bonding chemistry such as ester, carbonate, and carbamate. The PEG was removed in plasma by hydrolysis which was followed by a classical 1, 4- or 1, 6- benzyl elimination reaction to produce the free amine drug. [21-23] The stability of polymer conjugate can be varied by changing the bonding chemistry between the PEG and the benzyl linker, and also by increasing the stearic hindrance on the benzyl group. [17] However, detachment of PEG from the benzyl group was found to be a rate limiting step in the overall stability of conjugate in plasma. The benzyl conjugation and elimination technology was also successfully applied for antifungal agent

amphotericin B. [24, 25] The permanently linked PEG-urethane-amphotercin B, showed similar activity to that of native amphotericin B; however, the PEG prodrugs with benzyl elimination technology with a plasma stability of 1-3 h, were found to be safe, with approximately six times less toxicity than the native drug. The maleamide spacer was reported to be a versatile linker for the polymer conjugation of anthracyclines with simple conjugation chemistry and prolonged circulation times of anthracyclines. [26]

Apart from the effect of the linker on plasma stability of polymer conjugate, the physical and chemical properties of the polymer linked to the drug also play a significant role in the stability of the polymer conjugate. N-(2-Hydroxypropyl) methylacrylamide (HPMA) copolymer has been used to conjugate drugs. Several factors, such as the inter and intra-molecular association of side chains, have been shown to affect the enzyme catalysed release of drugs from the conjugate. [27] The polymers that have the ability to form micelles have advantages over other polymers in their application in drug delivery. Possible aggregation of polymer chains in micelles could have impeded hydrolysis. The micelles can provide additional benefits such as the long circulation, internalization into the tumour, and the better plasma stability of the drug-polymer conjugate. [27] In case of water soluble polymers such as PEG, the polymer with Mw < 30 kDa would be excreted via the kidney, because threshold of kidney filtration is not reached. [15-17] However, the micelle forming polymer can overcome this limitation as the size of micelles prevents kidney filtration. The paclitaxol conjugates PLA-PEG diblock and triblock PLA-PEG-PLA polymers have been developed for this pupose and unfortunately not much information on plasma stability of conjugates has been reported. [28-29]

There are several approaches reported to produce polymer conjugates which are stable in plasma but undergo hydrolysis at specific sites. The controlled and site specific release of drugs can only be achieved by proper selection of spacers. [30] The pH controlled drug release was successfully developed by using acid sensitive but serum peptide resistant spacers. When macromolecular polymeric drugs are taken into cells by endocytosis, the conjugate is anticipated to be expose to the acidic environment of lysosome. Moreover, the pH in and around the tumour cells was reported to be a little lower than the healthy tissues. [30] This can be exploited to

design polymer conjugates with acid sensitive linkers to release the drug at the site of action. [30]

The acid labile spacers were first developed by Shen and Ryser.[31] They synthesized the daunorubicin-linked aminoethyl polyacrylamide beads and poly(d-lysine) using a *N*-*cis*-aconityl spacer. The pH-controlled hydrolysis of the *cis*-aconityl was demonstrated by measuring the half lives at pH 5 ranging from 1 to 4 h, depending on the conjugate and the type of buffer used. The *cis*-aconityl spacer was readily hydrolysed at pH 4 but negligible at pH 6. [31] Such results were also reported for adriamycine [32]. Similarly, the example involving streptomycin coupled to dextran and to poly-(*N*-(2-hydroxyethyl)-l-glutamine) via a carboxylic hydrazon linkage were reported [33]. The conjugate showed pH dependent release of streptomycine at lysosomal pH (4.5). After internalization of conjugates into cells by endocytosis, the drug-polymer conjugate was not only exposed to acidic pH but also to lysosomes and any spacer which can be selectively cleaved by the lysosomes and would provide a excellent opportunity for controlled and targeted release of polymer conjugates in the body [34-39]. The lysosomes are often over expressed in tumours [34-39]. Several lysosomal proteases such as cathepsins B, D and metalloproteinases promote tumour growth and formation of metastases [34-39]. This provides a rationale for polymer conjugate based anticancer drug delivery. If the spacer is oligopeptide, a substrate of lysosome enzymes, cyctotoxic drugs can be delivered to the tumour and subsequently released. The rate of drug release would be dependent on the amino acid sequence of oligopeptide. A series of hydroxyl poly methacrylic acid (HPMA)-Doxorubicin conjugates have been prepared and evaluated for lysosomal sensitive drug delivery. *In vitro* release study of doxorubicin in media with lysosomal enzymes suggested that drug release can be tailored by length and composition of amino acids [40].

However, the possibility of using various spacers would be limited for resveratrol as hydroxyl groups are the only sites for conjugation. Any efforts to conjugate polymer to resveratrol should be based on the chemistry of the hydroxyl group. The peptide spacers used in the polymer conjugation of doxorubicin provide resistance to hydrolysis of the amide bond between the drug and peptide in the conjugate by the carboxy peptidases of plasma, but they can be hydrolysed by lysosomes. Similar things cannot be anticipated for resveratrol, as there are no amine groups in the

structure; peptide spacer even if used in the polymer conjugation, would contain an ester link with resveratrol. The role of the peptide spacer in stabilizing ester bond against plasma esterases has not been documented. However, for resveratrol exploring different polymers with varying physiochemical properties and using a suitable ester linker would be good option for resveratrol, in order to achieve reasonable stability of polymer conjugate in the plasma.

As mentioned early, synthesis of polymer conjugates of resveratrol can be achieved by exploiting hydroxyl groups of resveratrol. The phodophyllotoxin, having the hydroxyl group in the structure, has been coupled to PEG with various bonding chemistry such as ester using PEG with acid end group and Diisopropylcarbodiimide, 4-Dimethylaminopyridine (DIPC-DMAP), carbonate using PEG paranitrophenol carbonate (and DMAP) and as carbamate using PEG isocynate (and DMAP) [43]. These approaches have been demonstrated in polymer conjugation of other drugs. For instance, paclitaxol has been conjugated to PEG acid, PEG-PLA acid and PLA-PEG-PLA di-acid. In all of these couplings, the carbodiimide coupling reactions were employed [19, 28, 29]. The carbodiimide coupling agents are also referred as zero length cross linkers as they are the smallest linking agents [30]. These coupling agents mediate the formation of bond between two molecules without any additional spacer atom. The carbodiimides activate the COOH group by forming an O-acylisourea active intermediate which upon reaction with amine or hydroxyl groups forms amide or ester bonds and the by-product a urea derivative is formed, which in many cases is insoluble in organic solvents and can be separated by filtration. Since the carbodiimide mediated esterification needs hydroxyl and a COOH group, either the drug or the polymer must bear a COOH group. In the case of resveratrol, only hydroxyl functionality is present in the structure, which can be converted into acid ester (Example using succinic anhydride) or directly coupled to acid fuctinalized polymer. There are a few PEG acids available commercially or the polymers can be easily derivitized to have an acid end group. PEG chain was converted to a PEG acid for paclitaxol conjugation by reacting with PEG-OH with ethylbromoacetate in the presence of tert-butoxide to form ethyl ester. The ethyl ester was treated with NaOH and the HCl to produce PEG-acid. [19] In another approach, the MeO-PEG-PLA-OH was converted to carboxyl terminated polymer by treating with succinic anhydride in the presence of DMAP. [19] In

addition, PEG can also be succinylated by treating with succinic anhydride in presence of pyridine in 1,4-dioxane [44] . The simple procedure for succinylating PEG was reported by Kuang et.al, where PEG was refluxed with excess succinic anhydride in toluene [45].

The polymer conjugation approaches have been reported for resveratrol; however, most of approaches offered limited or no protection against metabolism due to the rapid hydrolysis of polymer conjugates in plasma. Biasutto et al. prepared and evaluated various esters, such as PEG caboxyester, methanesulfonate and acetate ester of resveratrol. [46] The PEG conjugation to resveratrol was achieved by reacting PEG acid (PEG-glycine) with resveratrol in dimethyl formamide (DMF), in the presence of DMAP and N,N'-dicyclohexylcarbodiimide (DCC). The PEG esters of resveratrol underwent rapid hydrolysis in blood (hydrolyzed completely in an hour), so was acetate ester; whereas the methane sulfonate ester was stable in blood. Intestinal permeation studies revealed that for resveratrol-PEG ester, although hydrolyzed while passing through the intestine, adjacent PEG molecules prevented metabolism of resveratrol. In contrast, acetate ester was also hydrolyzed while passing through the membrane, but did not offer any protection against metabolism. Methanesulfonate ester was found to be stable and permeated as is, across the intestine membrane. In a patent covering polymer conjugation of various polyphenols [47], resveratrol was conjugated with different polymers such as PEG, PLA, polylysine-PLA, hyaluronic acid etc. But no details on the hydrolysis of polymer conjugates in plasma, metabolism and the pharmacokinetic profile of conjugates were given in the patent. Resveratrol was also coupled to biotin using DCC and DMAP in DMF [48]. The conjugate was used in biological assays to deduce the mechanism of actions responsible for various activities of resveratrol. The plasma stability, metabolism and pharmacokinetic profile of conjugate have not been reported.

2.1.2 Objectives of the study

The objectives of the work described in this chapter were to design and synthesize ester based polymer conjugates of resveratrol, and to characterise and evaluate them in comparison with resveratrol. Various acid linkers and polymers with different Mw and physicochemical properties were synthesized and characterized to gain the

knowledge on how to obtain stable but slowly hydrolysable polymer conjugates. The polymer conjugates were studied by NMR, HPLC and LC-MS for structural characterization and were investigated for stability in buffers and plasma. The micelle forming polymer conjugates were evaluated for critical micellar concentration (CMC), micelle size and morphology by microscopy. The polymers conjugates with reasonable stability in rat plasma were then selected and evaluated for single dose preliminary pharmacokinetic studies *in vivo* in comparison to resveratrol.

2.2. MATERIALS AND METHODS

2.2.1 Materials

Trans-Resveratrol was purchased from DND Pharma-Tech Co., Inc, Shanghai, China, with 99% purity. Polyethylene glycol carboxylic acid monomethyl ether (MeO-PEGN-SuccOH) of molecular weight Mw 2 kDa and 20 kDa were obtained from IRISH biotech GMBH, Germany. Monomethoxy polyethylene glycol (MeO-PEG-OH, Mw 2 kDa), monomethyl polyethylene glycol-co-polylactide (MeO-PEG-PLA-OH, Mw 2 kDa) and succinic anhydride were procured from Sigma Aldrich, Australia. MeO-PEG-PLA-OH, Mw, 3.7 kDa, 6.5 KDa was purchased from Akina Inc, Indiana, USA. N, N'-Diisopropylcarbodiimide (DIC) and 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDC.HCl) were obtained from GL Biochem, Shanghai, China. Rat liver microsomes (20mg/mL protein) were obtained from Invitrogen, Australia. UDP-glucuronosyltransferases (UGT A) and UGT B solutions were purchased from BD Bioscience, Australia.Dimethyl yellow was obtained from Sigma, Australia. Wistar rat plasma was obtained from Animal resource centre, Perth. 0.1M acetate buffer pH 4.5 and 0.1M phosphate buffer pH 7.4 were prepared in house. All remaining chemicals such as pyridine, toluene, and diethyl ether were of analytical grade and used without any further purification.

2.2.2 Synthesis of MeO-PEGO-SuccOH

MeO-PEG-OH (Mw 2 kDa, 5 g , *ca* 2.5 mmol)) was dissolved in toluene (50 mL) and succinic anhydride (1.2g, 12 mmol) was added to the mixture. The reaction mixture was refluxed at 90^0C for 4 h in a round bottom flask fitted with a condenser. At the end of the reaction, the solution was concentrated to 10 mL in vacuo at 110^0C. The solution was cooled to room temperature and diethyl ether (150 mL) was added

to precipitate the MeO-PEGO-SuccOH. The precipitate was collected by filtration, air driedand was characterized by NMR and LC-MS.. The development and details of LC-MS method described in the chapter 5.

2.2.3 Synthesis of MeO-PEG-PLAO-SuccOH

MeO-PEG-PLA-OH, (Mw 6.5 kDa, 1 g, ca 0.15 mmol) was dissolved in toluene (10 mL) and an excess of succinic anhydride (0.2 g, 2 mmol) was added to mixture. The reaction mixture was refluxed at 90^0C for 4 hours in a round bottom flask fitted with a condenser. At the end of the reaction, the mixture was cooled to room temperature and diethyl ether (100 mL) was added to precipitate the MeO-PEG-PLAO-SuccOH. The precipiate was isolated by filtration and was characterized by NMR. A similar process was used to prepare MeO-PEG-PLAO-SuccOH Mw 2 kDa.

2.2.4 Synthesis of Resveratrol-PEG conjugates

DIC (0.1 g, 0.79 mmol) was added to a solution of MeO-PEGN-SuccOH (Mw 2 kDa, 1 g, *ca*. 0.5 mmol) and excess resveratrol (0.25 g, 1.09 mmol) in pyridine (2 mL) and the resulting solution was stirred in darkness, at room temperature for 24 h. The solution was filtered (0.44 μm filter) and the filtrate was diluted with diethyl ether (150 mL). The resulting precipitate was then washed with a solution of ethyl acetate/diethyl ether (20 mL/80 mL), a solution of ethyl acetate/diethyl ether (50 mL/50 mL), and then dried *in vacuo* to afford an off white material (300 mg, 30% yield). A similar procedure was followed for preparation of resveratrol conjugates with MeO-PEGN-SuccOH 20 kDa and MeO-PEGO-SuccOH 2 kDa.

2.2.5 Synthesis of Resveratrol-PLA-PEG conjugates

MeO-PEG-PLAO-SuccOH, (Mw 6.5 kDa, 1g, ca 0.15 mmol) was dissolved in pyridine (5.0 mL). The excess of resveratrol (0.15g, 0.65 mmol) and EDC.HCl (0.15 g , 0.78 mmol) were added to the mixture. The reaction mixture was stirred at room temperature for 48 hours under dark conditions. At the end of the reaction, the mixture was added to diethyl ether (150mL) and the product was isolated by filtration. The product was dissolved in dichloromethane (50 mL) and was extracted with 0.1M HCl (50 mL) twice to remove any unreacted EDC.HCl and its urea by-product. Finally DCM layer was evaporated in vaccum to get off white powder

(500mg, 50% yield). The MeO-PEG-PLAO-Succ-Resveratrol, Mw 2 kDa was also prepared with the same procedure.

2.2.6 Characterization of resveratrol-polymer conjugates

2.2.6.1 Analysis of polymer conjugates and their precursors by ^{1}H NMR

Dried powder samples of reaction products obtained from section 2.2.2 to 2.2.5 were analysed by ^{1}H NMR to elucidate the chemical structures of compounds/mixtures. All ^{1}H NMR analyses were performed on a Bruker AVN400 spectrometer (400.1 MHz for ^{1}H, Bruker, USA, country) with spectra referenced to solvent signals.

2.2.6.2 Analysis of Polymer conjugates by LC-MS

Qualitative and quantitive LC-MS methodology was developed to characterize resveratrol-PEG conjugates and intermediates. Details about methods and results of analysis are described in Chapter 5.

2.2.6.3. Micelles preparation and characterization

A solution of 10-40 mg of MeO-PEG-PLAO-Succ-RSV conjugates in acetonitrile (2 mL) was prepared and the solution was added to water (4 mL). The acetonitrile was removed by bubbling inert nitrogen gas at room temperature in the dark. The final volume of micellar/nanoparticle dispersion was adjusted to the required volume with water. The micelles of MeO-PEGO-Succ-RSV were formed spontaneously after dispersing in water.

2.2.6.3.1 Measurement of micelles size

Particle size and size distribution of the resveratrol polymer conjugate micelles were measured by a Zetasizer (3000HS, Malvern Instruments, UK), using a quartz cell with the automatic mode at 25^{0}C and a detection angle of 90^{0}. Mean diameters (Z-average) were obtained after 10 repeated measurements.

2.2.6.3.2 Determination of critical micellar concentration values (CMC) for resveratrol polymer conjugates.

The critical micellar concentrations for some micelle forming resveratrol polymer conjugates were determined by a UV spectrophotometery employing dimethyl yellow as a probe. The extent of dye solubilisation and absorption maximum (λmax)

shifts were monitored as a function of the concentration of polymer conjugates. A concentrated solution of dimethyl yellow (10mg/mL) was prepared in acetone. Aliquots of 25 µl were dispensed into different vials and dried in a fume hood at ambient temperature to remove the solvent. Stock solutions of MeO-PEG-PLAO-Succ-RSV (20 mg/mL) conjugates were prepared in acetone (for 2 kDa) and acetonitrile (for 6.5 kDa). The initial concentrated micelle solutions were prepared by diluting 125 µL of polymer conjugate stock solution to 100 mL with millQ water. The solution was spurged with nitrogen gas for an hour to remove solvent, and finally made up to 100 mL with water. This solution was further diluted serially with water to get solutions with various concentrations of polymer conjugates (1-50 mg/L). The MeO-PEGO-Succ-RSV solutions were prepared by dissolving 40 mg of conjugate in 10 mL of MillQ water, further serial dilution to get solutions of concentrations 0.005 to 0.4% (50 to 4000 mg/L). These polymer conjugate solutions were added separately to vials containing dimethyl yellow and sonicated for 10 minutes. The solutions were stored in the dark for 3 hours to equilibrate. These solutions were scanned for λ max using a UV-Visible spectrophotometer between 300 and 510 nm. The change in wavelength of maximum absorption and absorbance at fixed wavelengths 441 and 423 were noted as a function of polymer conjugate concentration. The CMC values were calculated from a plot of the log of concentration (log C) of polymer conjugates versus λ max (or absorbance at 441 nm).

2.2.6.3.3 Micelle morphology by atomic force microscopy (AFM) and field emission scanning electron microscopy (FESEM)

Atomic Force Microscopy was used to visualise the samples. All measurements were taken in Tapping Mode operation with a Digital Instruments Dimension 3000 SPM system (Veeco, Santa Barbara, USA). Standard silicon Tapping Mode probes type NCH (NanoWorld, Switzerland) with resonant frequency 330 kHz, spring constant 42 N/m and tip radius < 8 nm.The samples were prepared by drop casting the micelle solution onto a freshly cleaved mica substrate. The sample was placed in a desiccator attached to a vacuum pump to remove excess solvent

Morphological evaluation of micelles was also performed using FESEM, Zeiss Neon 40 ESB,(Oberkocken, Germany). For FESEM, one drop of micelle solution was

mounted on a carbon coated stub. Excess sample was removed carefully. A Polaron E5100 sputter-coater was used to sputter coat the samples with chromium. Samples were then examined under the FESEM with an accelerating voltage of 1 kV.

2.2.6.4 Stability of resveratrol-polymer conjugates in physiological relevant buffers

The stability of resveratrol polymer conjugate was evaluated at different pH buffers 4.5 (acetate buffer 0.1M) and 7.4 (phosphate buffer 0.1M) to mimic lysozomal and blood pH. The resveratrol polymer conjugates of different Mw and linkers were incubated in the two buffers at a concentration of 10 mg/mL at 37^0C, the samples were withdrawn at 0, 1, 2, 4, 6 and 24 hours and were diluted with water/acetonitrile (50:50) and were analysed by HPLC. A similar procedure was used to study the stability of MeO-PEG-PLAO-Succ-RSV in buffers, micelles were prepared as per section 2.2.6.2 and diluted 1:5 with buffer solutions and incubated at 37^0C. All samples were protected against light during the experiments,

2.2.6.5 Stability of resveratrol-polymer conjugates in rat plasma

The stability of various resveratrol polymer conjugates in rat plasma was tested in order to assess the stability of the ester linkages towards plasma esterases. The MeO-PEGN-Succ-RSV, 2 kDa, 20 kDa and MeO-PEGO-Succ-RSV 2 kDa at the 10 mg/mL were incubated in plasma at 37^0C. The micelles of MEO-PEG-PLAO-Succ-RSV 2kDa and 6.5 kDa were prepared and, finally the micelles were mixed with plasma and incubated at 37^0C. Aliquots of plasma (0.1 mL) were withdrawn at regular intervals and mixed with acetonitrile (0.9 mL). The mixtures were centrifuged at 10000 rpm for 5 minutes. The supernatant (0.25 mL) was diluted with water (0.25 mL) and analysed by HPLC. All samples were protected against light during the experiments,

2.2.6.6 *In vitro* preliminary metabolism studies of resveratrol and its polymer conjugates in rat liver microsomes.

To evaluate the effect of polymer conjugation on metabolism of resveratrol, metabolism studies of resveratrol and its polymer conjugates were conducted in rat liver microsomes following the protocol provided by BD bioscience (Appendix xx). Since resveratrol reportedly to undergoes only phase II metabolism, such as glucuronidation and sulfation[6], the metabolism studies were conducted in the

presence of uridine diphosphoglucuronic acid (UDPA). The stock solutions of resveratrol (0.13 mM) and polymer conjugates (0.13 mM resveratrol eqvivalents) were prepared in 10% DMSO in water. The rat liver microsomes, UGT (UDP-glucuronosyltransferases) solution A (contains UDPA) and UGT solution B were thawed at 37^0C. The 200 µl of UGT solution B, 80 µl of UGT solution A and 660 µl of water were mixed, and 10 µl of the test substance stock solution (resveratrol or conjugates) was added to the mixture. The mixture was kept at 37^0C. The reaction was initiated by adding 50 µl of microsomes (1mg protein). Samples from reaction mixtures were withdrawn at 0, 10, 20, 30, 40 and 60 minutes and mixed with equal volumes of cold acetonitrile. The mixtures were centrifuged at 10000 RPM for 10 minutes and supernatans were collected and analyzed by HPLC. Similary, control incubations of test substances were performed in reaction mixtures without microsomes. All samples were protected against light during the experiments,

2.2.6.7 *In vitro* antioxidant assay of resveratrol and its polymer conjugates

The free radical scavenging effects of resveratrol and its polymer conjugates were evaluated using 1,1-dipehnyl-2-picrylhydrazyl (DPPH) inhibition assay. Initially series of solutions of resveratrol and resveratrol-PEG ester conjugates were prepared in methanol. The solution of DPPH (2×10^{-4}M) was prepared in methanol. The DPPH solution of 1mL was mixed with 1 mL of resveratrol solution. Final concentration of DPPH was maintained at 1×10^{-4}M. The procedure was repeated for other resveratrol solutions of various concentrations and also for resveratrol-PEG ester solutions. Finally the concentration of resveratrol in testing solutions was ranged from 21-877 µMol/L and 210 to 8770 µMol/L for polymer conjugates. After 30 minutes of incubation at room temperature, absorbances of these solutions were read at 518 nm using a Hewelett Packard 8452A, UV-VIS spectrophotometer (Waldbrann, Germany). Remaining DPPH concentration in solution was calculated by comparing with the control, a standard solution of DPPH without any resveratrol or conjugate. The IC 50 values were obtained from plot of concentration of RSV vs % DPPH remaining.

2.2.6.8 Preliminary pharmacokinetic study of resveratrol and its polymer conjugates in Wistar rats with iv injection

The pharmacokinetic studies were conducted in male Wistar rats (170-210g) and rats were housed in the animal facility for at least 3 days prior to experiments. All dosing and bleeding procedures were conducted under anaesthesia. The volatile anaesthetic agent isoflurane at 5% for the induction and 2% for the maintenance was used along with 1.5% oxygen. A resveratrol solution (6 mg/mL) was prepared in 20% hydroxyl-propyl-β-cyclodextrin. The solution was dosed to Wistar rats at 10 mg/kg body weight via the tail vein. Similarly the MeO-PEG-PLA-Succ-RSV 2 kDa and 6.5 kDa micelles were prepared as per section 2.5.2 (10 mg/mL) and dosed to rats at a dose of 2mg/kg of resveratrol equivalent. The blood was collected at 0.083, 0.5, 1, 2, 4 and 8 hours into EDTA coated tubes. The blood samples were immediately kept on ice. The blood samples were then immediately placed into a centrifuge maintained at 4^0C. The plasma was separated by centrifuging at 12000 RPM. The plasma was then mixed with an equal volume of acetonitrile and centrifuged again for 12 minutes. The supernatant was collected and analysed by HPLC. All samples were protected against light during the experiments, Due to the time constraints, only plasma concentration profiles of resveratrol and resveratrol equivalent (polymer conjugates) were determined. Metabolites were identified using LC-MS/MS (chapter 4). Non compartmental analysis of plasma vs time profile was performed using Kinetica™ Version 4.4 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Area under the curve total (AUC total) , Mean residence time (MRT) and other parameters were obtained from software. (Table 7.1, Appedex). All animal experiments were approved by the Animal Ethics Committee of Curtin University.

2.3. RESULTS AND DISCUSSION

2.3.1 Synthesis of succinic acid terminated polymers for resveratrol conjugation

The addition of an acid end group in the polymer by esterification with succinic anhydride is essential for conjugation with the polymer which did not bear any acid group in their chain. The esterification of polymers in this study was achieved by a ring opening reaction mediated by heat in toluene. This was performed using modified procedure adopted from Kuang et.al (Figure 2.1). [45] The reaction worked well for both MeO-PEG-OH and MeO-PEG-PLA-OH irrespective of their polymer chain length and composition with approximate 80% yield. The esterification of polymer was confirmed by the presence of ethylene protons of succinic acid end group at 2.3 and 2.4 ppm in NMR analysis and 7.1 in Appendix), which are characteristic triplet peaks of ethylene protons. The formation of succinic acid terminated MeO-PEG-OH was also supported by qualitative and quantitative LC-MS analysis. The new peak at 2.8 minutes for succinylated PEG was observed in LC-MS chromatogram in contrast to unmodified MeO-PEG-OH at 4.5 minutes. The quantitative analysis revealed that the conversion of MeO-PEG-OH to MeO-PEGO-SuccOH was 100% as there was no peak observed at 4.5 minutes, the retention of native MeO-PEG-OH, in the final MeO-PEGO-SuccOH solution. Figure 2.1 shows the schemes for the synthesis of MeO-PEGO-SuccOH and MeO-PEG-PLAO-SuccOH.

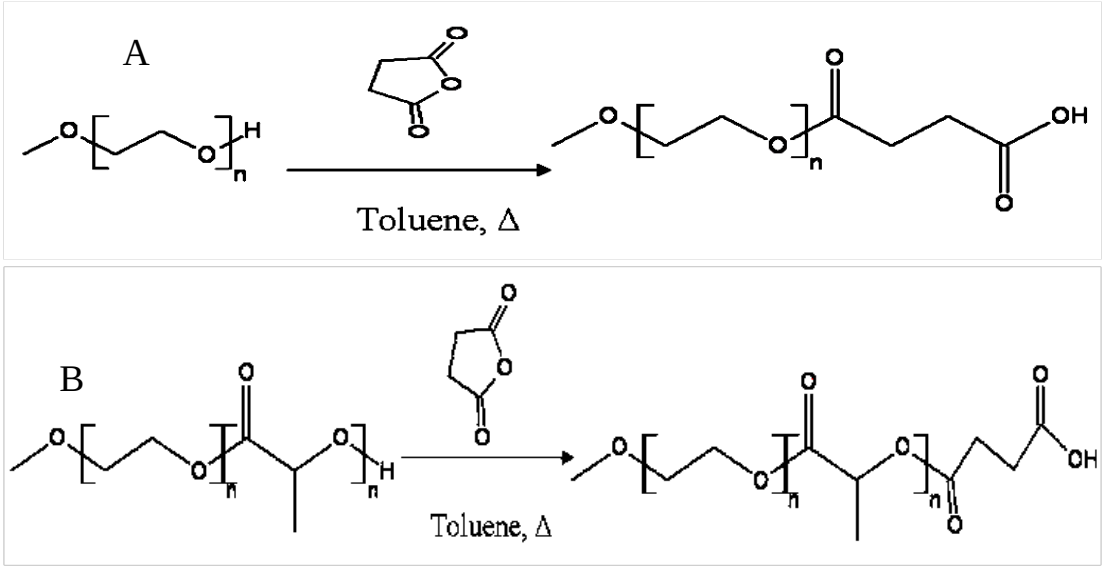


Figure 2.1: A: Reaction scheme for the synthesis of MeO-PEGO-SuccOH; B: Reaction scheme for the synthesis of MeO-PEG-PLAO-SuccOH

2.3.2 Synthesis of resveratrol-PEG ester conjugates

The conjugation of various molecular weight PEGs to resveratrol was achieved by carbodiimide coupling, using modification of a procedure reported by Greenwald et al for the PEGylation of 6-hydroxyquinoline [49] as shown in Figure 2.2. Detailed NMR analysis of MeO-PEGN-Succ-RSV 2 kDa was performed to understand the chemistry of the conjugation. The mixture (MeO-PEGN-Succ-RSV 2 kDa) included two mono-PEGylated resveratol conjugates 4'-MeO-PEGN-Succ-RSV (*ca.* 50%) and 3-MeO-PEGN-Succ-RSV (*ca.* 35%) as the major products, and is presumably disubstituted derivative 3,4'-di(MeO-PEGN-Succ)-RSV as a minor product. The identification of the mixture, and the substitution pattern, was based on the ^{1}H NMR spectra for the resveratrol-PEG conjugate mixture (see Figure 2.3,Figures 2.4 and 2.5). The ^{1}H NMR spectra revealed that the conjugate mixture contained no free resveratrol, as indicated by the absence of a signal at *ca*. 6.1 ppm in Figure 2.4(a). This signal, observed in Figure 2.4(b), is due to H-4 of free resveratrol (see Figure 2.5 for assigned spectra). As a control, a solution of resveratrol and MeO-PEG-CO_2H in d_6-DMSO was analysed by ^{1}H NMR spectroscopy. The chemical shifts of the signals for resveratrol in the mixture were identical to that shown in Figure 2.4(b) for resveratrol in d_6-DMSO. The ^{1}H NMR spectra for the resveratrol-PEG conjugate

mixture also indicated that no free MeO-PEGN-SuccOH remained in the material. Signals corresponding to the succinyl moiety in MeO-PEGN-SuccOH were absent in the spectra for the resveratrol-PEG conjugate mixture (see Figure 2.4). The assignment of signals corresponding to 3-MeO-PEGN-Succ-RSV and 4'-MeO-PEGN-Succ-RSV in Figure 2.5 was based on a comparison with the spectra for resveratrol and 2D NMR spectra (COSY). Additional minor signals remain unassigned in Figure 2.5. These signals presumably are due a third product, 3,4'-di(MeO-PEGN-Succ)-RSV which was formed as a minor component. To confirm this assumption a sample of the resveratrol-PEG conjugate mixture in d_6-DMSO was treated with water and then heated at 90°C for *ca*. 1 week, to hydrolyse the phenolic ester linkages. The ^{1}H NMR spectrum for the sample, shown in Figure 2.3(c) (and Figure 2.4), revealed the majority of the material had hydrolysed to afford free resveratrol. The remaining signals shown in Figure 2.4(c) can be attributed to residual 3-MeO-PEGN-Succ-RSV and 4'-MeO-PEGN-Succ-RSV. Interestingly, the hydrolysis of the resveratrol-PEG conjugate did not afford MeO-PEGN-SuccOH as the other hydrolysis product, though this may have formed as an intermediate. The ^{1}H NMR spectra for the solution after the hydrolysis reaction are consistent with the formation of a succinimide-terminated PEG chain (Figure 2.6). Figure 2.4(d) displays a singlet at *ca*. 2.5 ppm consistent with the succinimidyl ring methylene protons, and the absence of the succinyl amide acid protons observed for MeO-PEGN-SuccOH (*c.f*. Figures 2.4(a) and 2.4(d)). The formation of a succinimide ring is consistent with literature observations for the hydrolysis of succinyl ester amide moity. [50-52] Integration of the signals for resveratrol and the succinimide-terminated PEG in Figure 2.4(d) indicated that there was more succinimide-terminated PEG than free resveratrol, which supports the assumption that the minor component of the resveratrol-PEG conjugate was the disubstituted product 3,4'-di(MeO-PEGN-Succ)-RSV. The NMR spectra (full drawn) of MeO-PEGN-Succ-RSV, MeO-PEGN-SuccOH and MeO-PEGN-Succ-RSV after hydrolysis are shown Figures 2.7, 2.8 and 2.9. The ^{1}H NMR spectra for other PEG ester conjugates of resveratrol showed that the conjugation pattern was the similar to that of MeO-PEGN-Succ-RSV 2 kDa. However the extent of PEG conjugation to resveratrol varied between PEGs of different Mw and acid functional groups. The conjugation was almost *ca* 100% for MeO-PEGN-SuccOH 2 kDa, ca 70% and 50% respectively for MeO-PEGO-SuccOH, 2 kDa and MeO-PEGN-SuccOH 20 kDa, This was based on the amount of

free PEG acid/acyl urea peaks in NMR. However MeO-PEGO-Succ-RSV 2 kDa and MeO-PEGN-Succ-RSV 20 kDa contained significant amount of an N-acyl urea as a by-product. In the reaction of a carboxylic acid with a carbodiimide the reactive O-acylisourea is formed, which would normally react to form the desired product. However, O-acylisourea, can decompose to the non-reactive N-acylurea (Figure 2.6 B) [53, 54]. The presence of N-acyl urea indicates the reaction is slow and incomplete with MeO-PEGO-SuccOH and MeO-PEGN-SuccOH, 20 kDa. These results clearly indicate that, in given reaction conditions, the extent of PEG conjugation to resveratrol depends on the structure of the acid end group and the PEG chain length. Different reaction conditions, such as prolonged reaction time, the increased amounts of base pyridine and the DIC did not improve extent of conjugation. The reactions involving MeO-PEGO-SuccOH were also carried out with a different base 4-Dimethylamino pyridine (DMAP); however, there was no significant improvement in the extent of PEG conjugation. The presence of unreacted PEG in the form of N-acylurea is not uncommon.Similar results were reported for PEGylation of paclitaxol, significant amount of N-acylurea was observed in the reactions involving PEG acetic acid and paclitaxol [19]. Authors reported that product was used as is, without any purification since the PEG-N-Acyl urea was found to be biologically inert.

The formation of MeO-PEGN-Succ-RSV was also confirmed by LC-MS and HPLC. The new peak believed to be of MeO-PEGN-Succ-RSV 2 kDa was observed at 21.5 minutes in HPLC and 6.5 minutes in LC-MS. The new peak in HPLC showed a UV spectrum similar to that of resveratrol. The peak at 6.5 minutes in LC-MS showed a mass spectrum with a range of intense peaks between a mass range of 600-1000, which are reported to be fragmented peaks of PEG. Simultaneous monitoring of the same peak in PDA showed a UV spectrum similar to resveratrol. This clearly confirms the formation of a new structure, resveratrol-PEG conjugate. Results of HPLC and LC-MS are dealt with in detail in chapters 3 and 4.

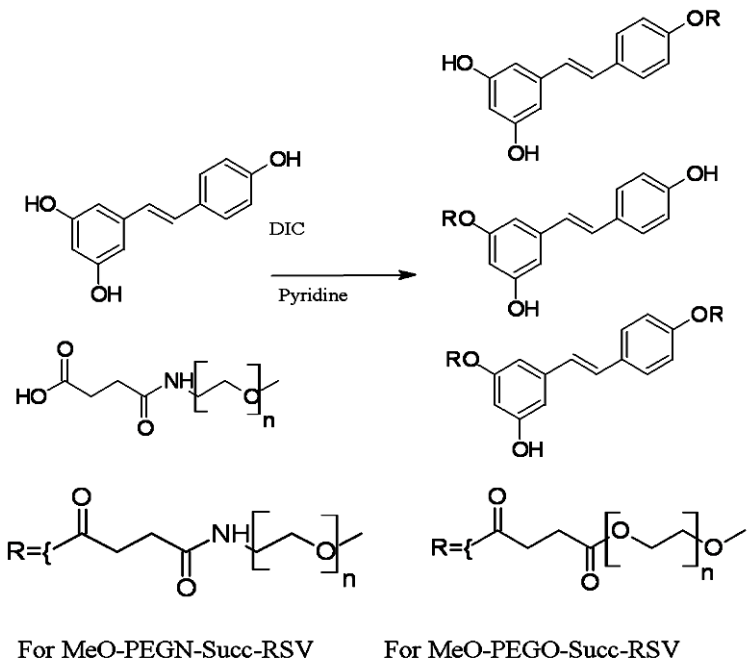


Figure 2.2: Synthesis of PEG-resveratrol ester conjugates

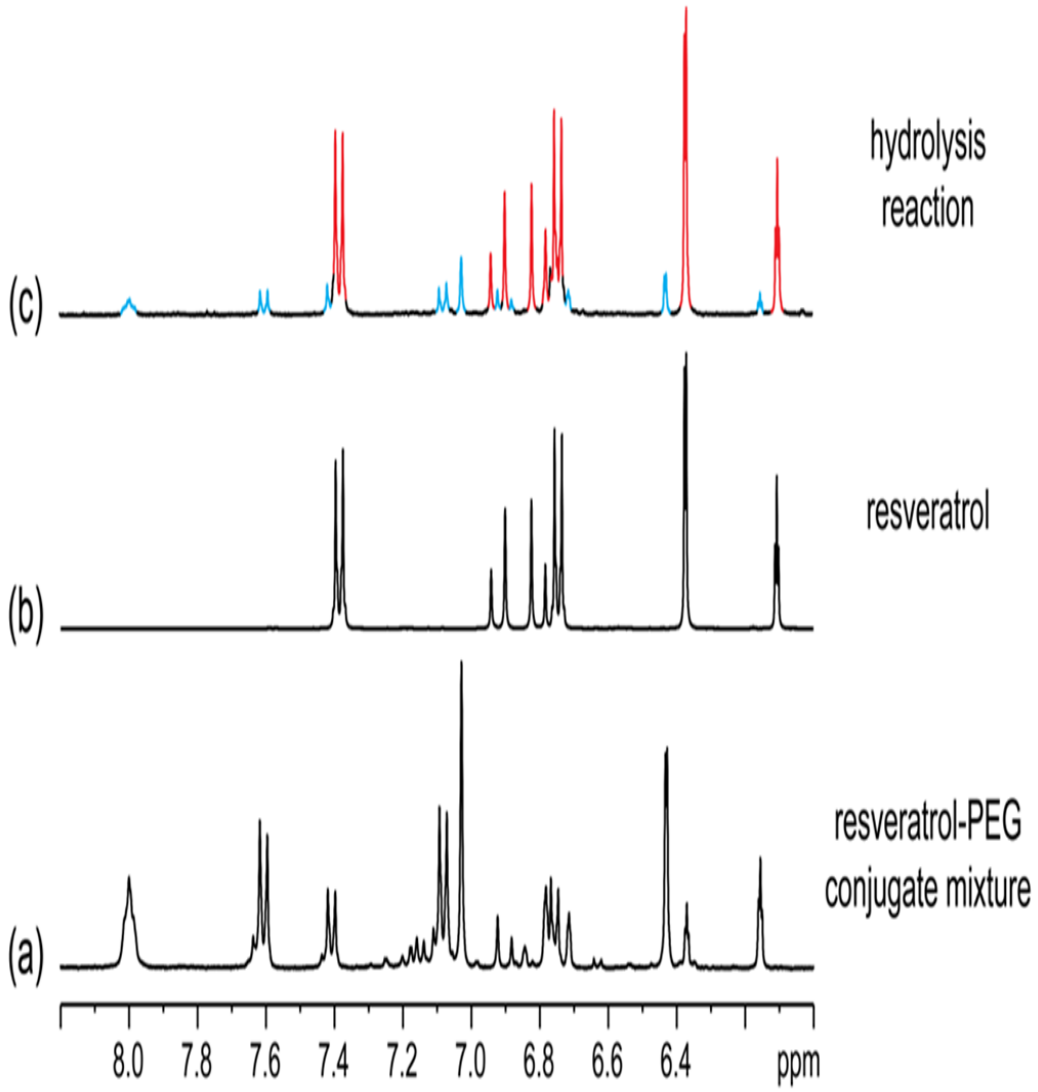


Figure 2.3: The down field region of ^{1}H NMR spectra (400 MHz) for: (a) a solution of the MeO-PEGN-Succ-RSV 2 kDa mixture in d_6-DMSO; (b) a solution of resveratrol in d_6-DMSO; and (c) the solution from (a) after treatment with water and heat (90 °C) for *ca.* 1 week. In (c) the signals for free resveratrol are highlighted in red and those for the remaining resveratrol-PEG conjugates are highlighted in blue.

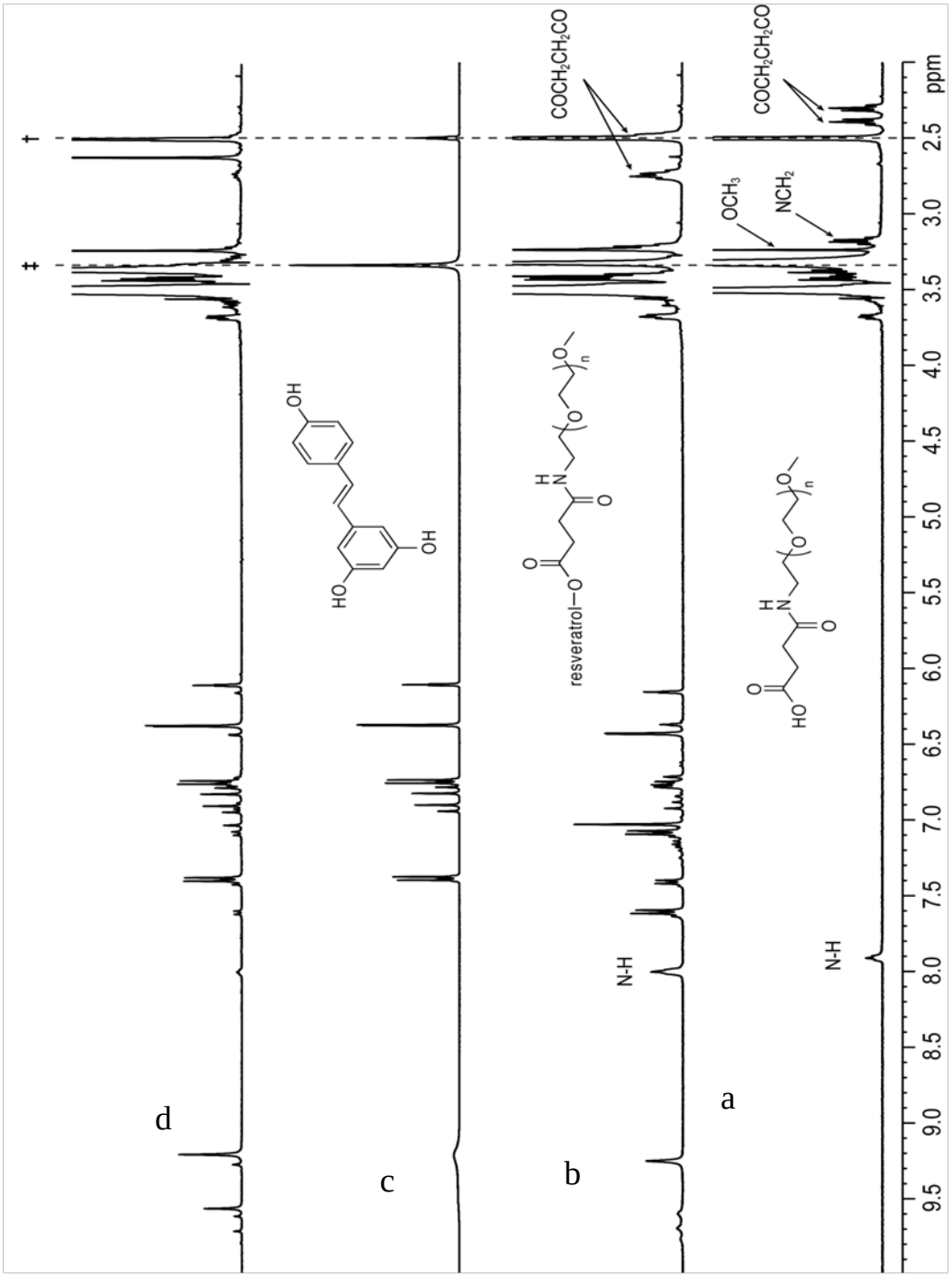


Figure 2.4. ^{1}H NMR spectra (400 MHz) for: (a) MeO-PEGN-SuccOH 2 kDa; (b) MeO-PEGN-Succ-RSV 2 kDa mixture; (c) resveratrol; and (d) the solution from (b) after treatment with water and heat (90 °C) for *ca.* 1 week). All solutions are in d_6-DMSO. Key: † = d_5-DMSO, ‡ = H_2O. To identify the low intensity down-field signals the spectra in (a), (b) and (d) have been expanded vertically.

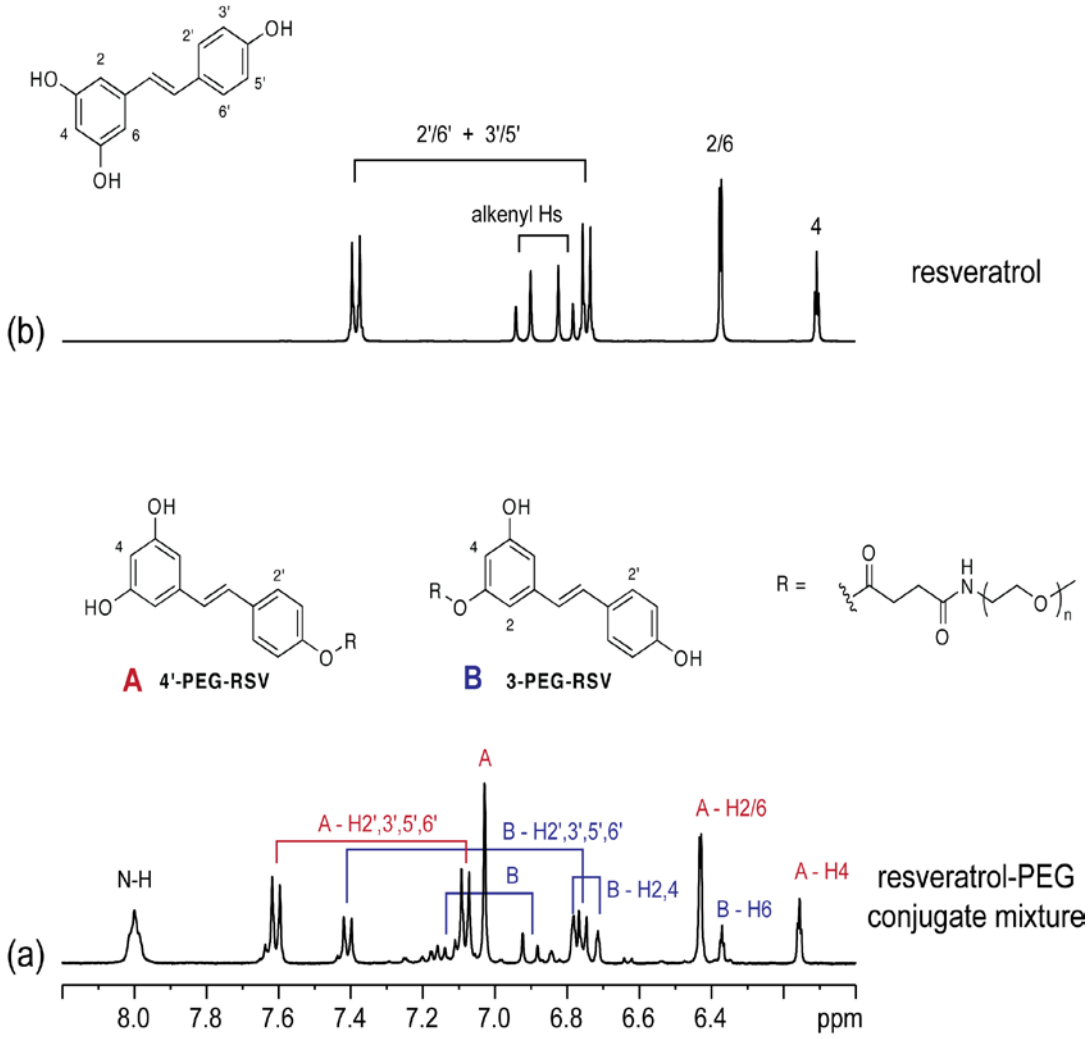


Figure 2.5: The down field region of the ^{1}H NMR spectra (400 MHz) for: (a) a solution of the resveratrol-PEG conjugate mixture in d_6-DMSO showing the assigned signals for the two major products 4'-MeO-PEGN-Succ-RSV 2 kDa and 3-MeO-PEGN-Succ-RSV 2 kDa; and (b) a solution of resveratrol in d_6-DMSO.

2

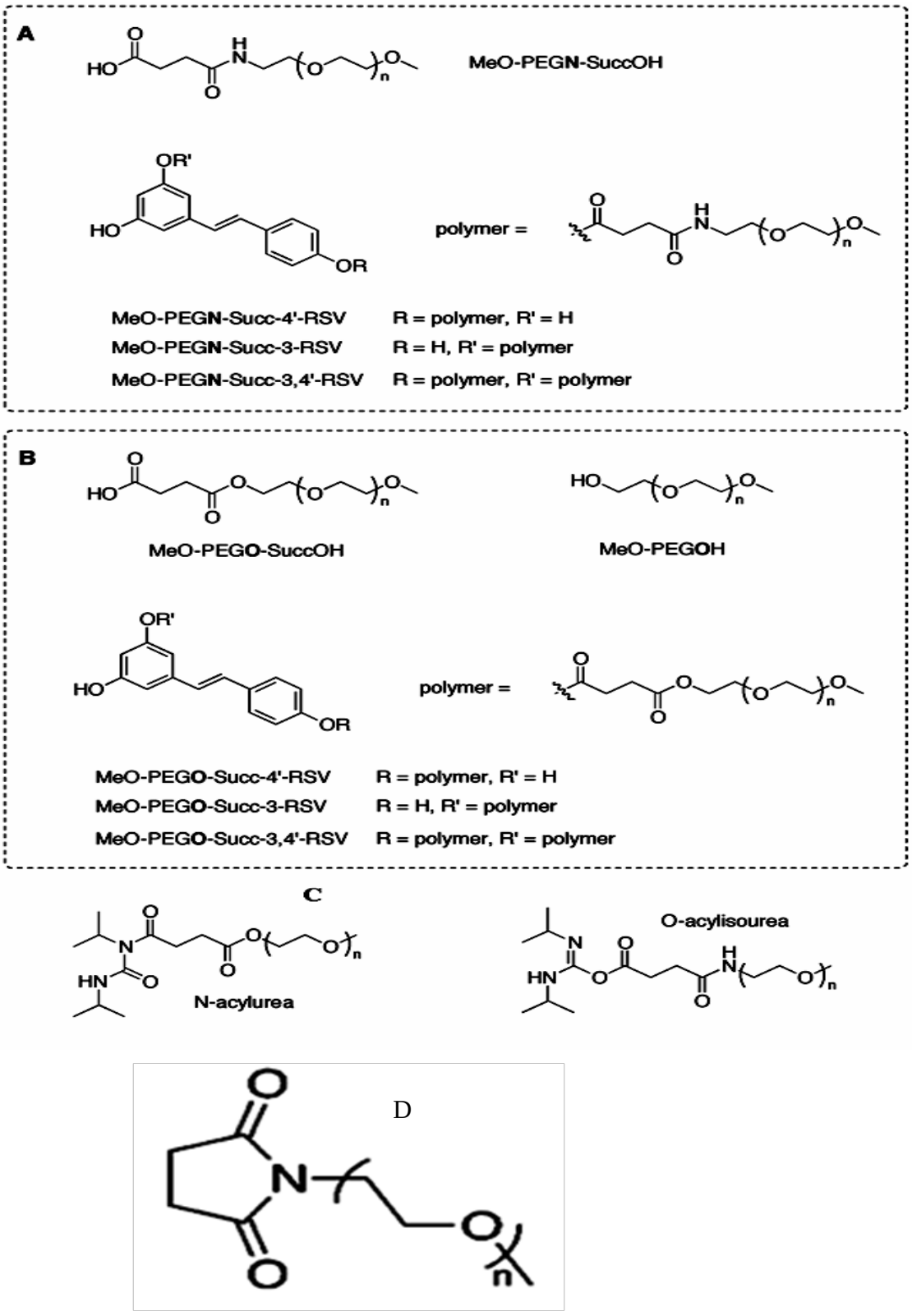


Figure 2.6: A &B Structures of Resveratrol-PEG conjugates and intermediates. C Structures of O and N-acylurea. D: The succinimide-terminated PEG chain that formed from the hydrolysis of the resveratrol-PEG conjugates

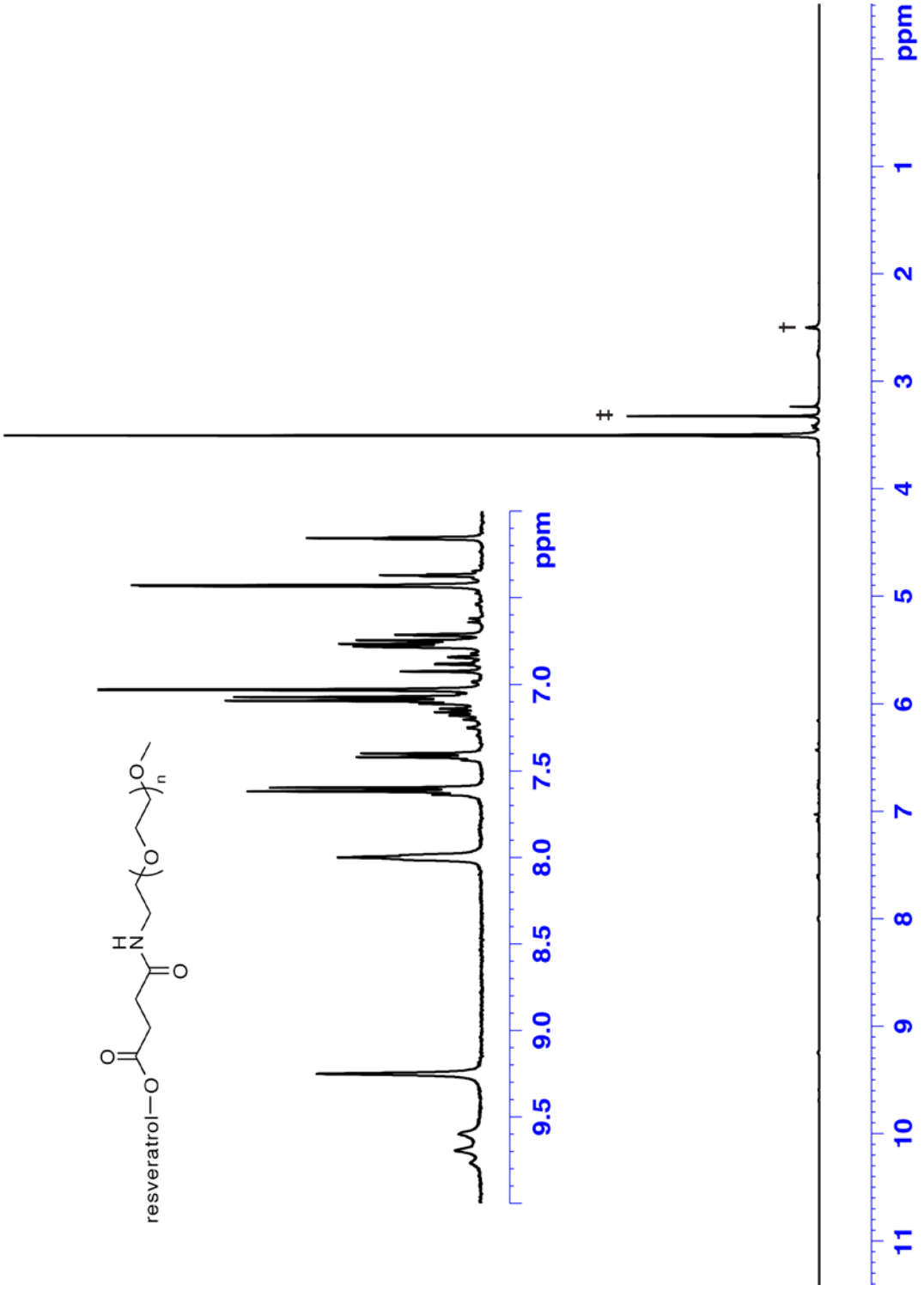


Figure 2.7. ^{1}H NMR spectra for the MeO-PEGN-Succ-RSV 2 kDa conjugate mixture in d_6-DMSO, recorded at 400 MHz. Key: † = d_5-DMSO, ‡ = H_2O.

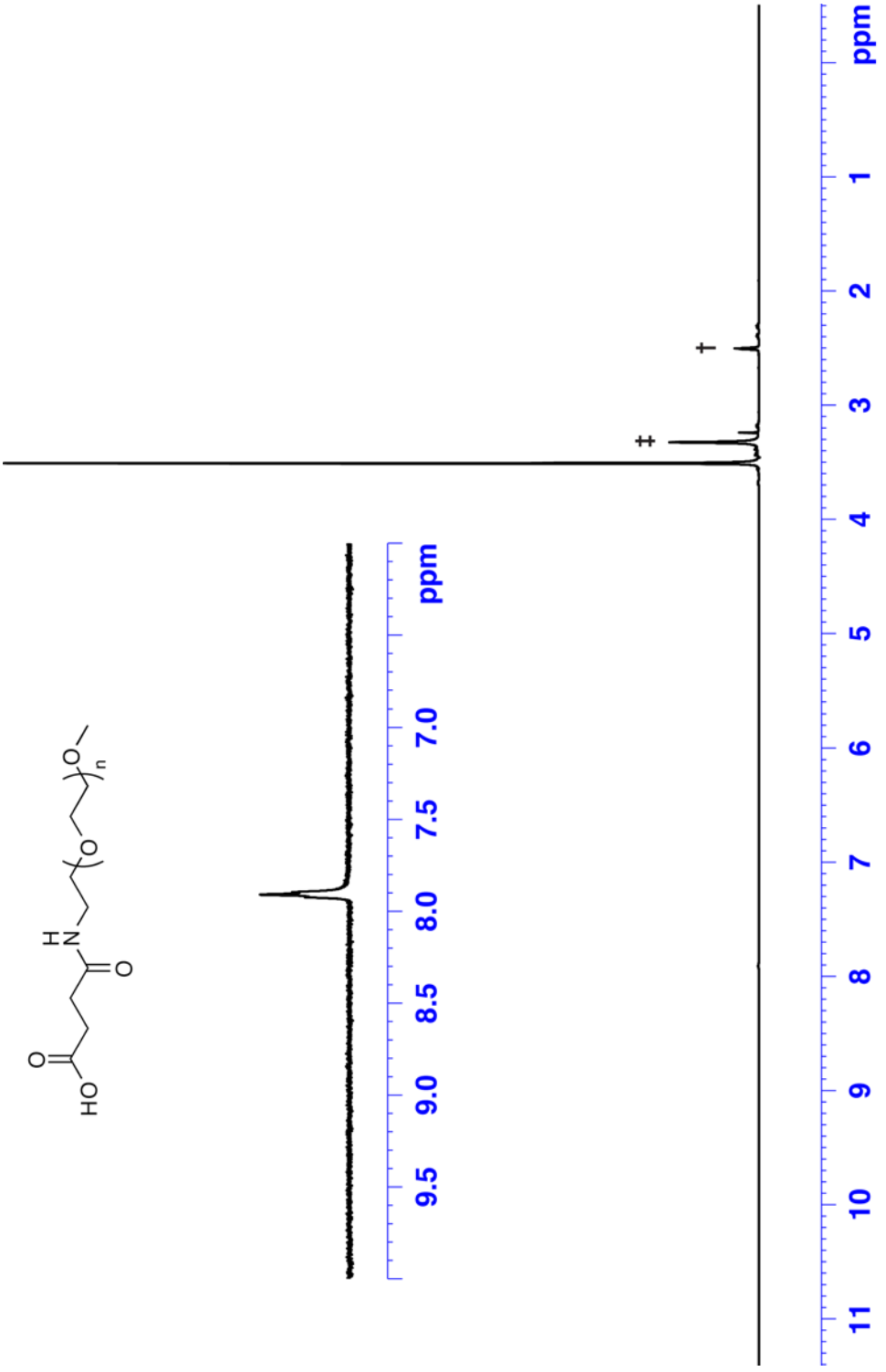


Figure 2.8. ^{1}H NMR spectra for MeO-PEGN-SuccOH 2 kDa in d_6-DMSO, recorded at 400 MHz. Key: † = d_5-DMSO, ‡ = H_2O.

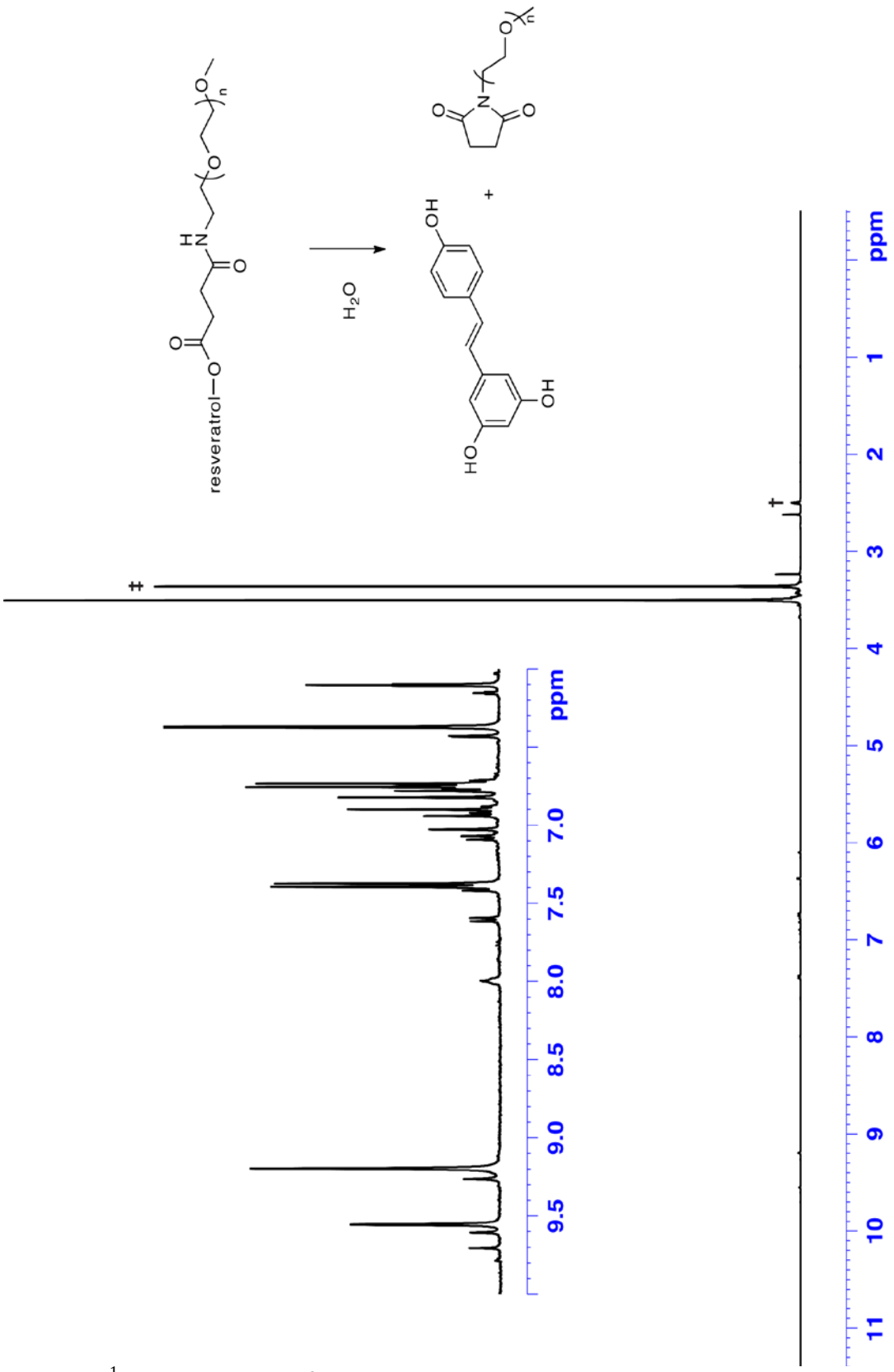


Figure 2.9 ^{1}H NMR spectra of the MeO-PEGN-Succ-RSV 2 kDa conjugate mixture after treatment with water and heat (90 °C) for *ca*. 1 week, in d_6-DMSO, recorded at 400 MHz. Key: † = d_5-DMSO, ‡ = H_2O.

2.3.3 Synthesis of resveratrol-PLA-PEG conjugates

The synthesis of MeO-PEG-PLAO-Succ-RSV was achieved by, again, using the carbodiimide coupling method modified from that reported by Zhang for the conjugation of paclitaxel to PLA-PEG [28]. The successful conjugation chemistry was optimized by using EDC.HCl as the coupling agent and pyridine as the base (Figure 2.10). The obtained product was purified by precipitation with diethyl ether and extraction with 0.1 M HCl. The NMR analysis of MeO-PEG-PLAO-Succ-RSV, 2 kDa (Figure 7.2 of Appendix) indicated that the product is a mixture of conjugates **4'-** MeO-PEG-PLAO-Succ-RSV (ca. 50%) and **3-** MeO-PEG-PLAO-Succ-RSV (ca. 35%) as the major products, and disubstituted derivative **3,4'-di(**MeO-PEG-PLAO-Succ**)-RSV** as a minor product (Figure 2.10). The HPLC analysis of MeO-PEG-PLAO-SUCC-RSV showed a new peak appearing at 21.5 minutes, similar to the one observed for MeO-PEGN-Succ-RSV, 2 kDa. The photo diode array (PDA) spectrum of the peak revealed the presence of a UV spectrum similar to resveratrol. Again it unequivocally proved conjugate formation and corroborated NMR observations. The NMR observations for the substitution pattern remained the same for MeO-PEG-PLAO-Succ-RSV 6.5 kDa but there was a considerable amount of un-conjugated resveratrol. The exact pattern of the conjugation could not be determined because of the interference from the un-conjugated resveratrol. Since the HPLC was able to separate and quantify free resveratrol and the polymer conjugate, all the *in vitro* studies were unaffected. Moreover MeO-PEG-PLAO-Succ-RSV, 6.5 kDa did not show significant stability in rat plasma and was not the suitable candidate for pharmacokinetic studies, hence further purification of product was not performed.

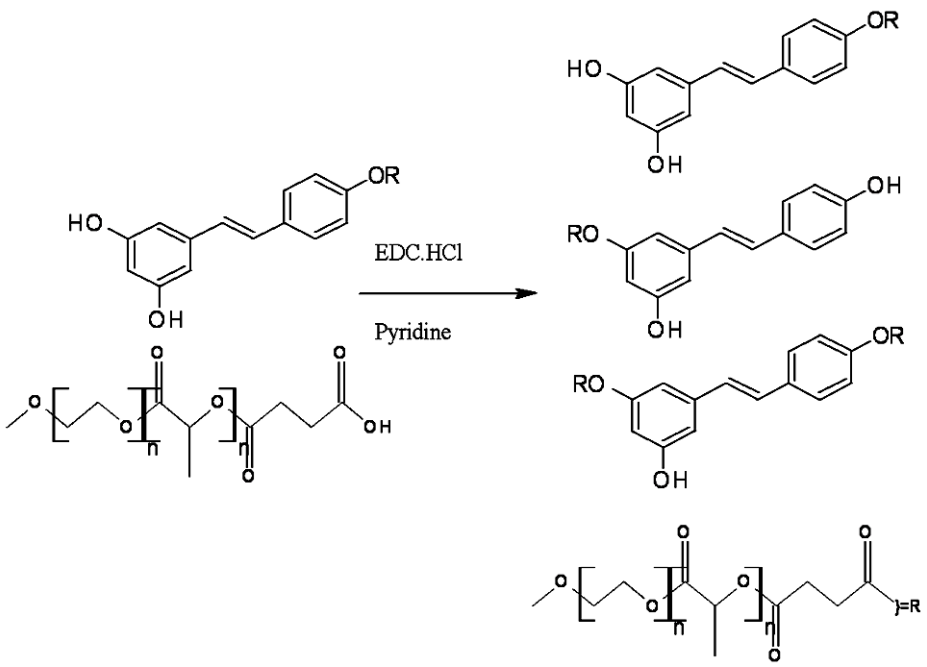


Figure 2.10: Reaction scheme for synthesis of MeO-PEG-PLAO-Succ-RSV conjugates.

2.3.4 Characterization of micelles of resveratrol-polymer conjugates

The PLA-PEG is a copolymer of hydrophilic polyethylene glycol and hydrophobic polylactic acid. Since this polymer is insoluble in water, they were converted into polymeric micelles/nanoparticles for all *in vitro/in vivo* studies. Interestingly the MeO-PEGO-Succ-RSV was found to self aggregate into micelles in water. This was observed during *in vitro* stability studies in buffers. The solutions at higher concentration turned slightly cloudy after some time, initially it was speculated that, this was because of the hydrolysis of conjugate to free resveratrol, which is insoluble in water. However, HPLC analysis did not indicate significant hydrolysis. The MeO-PEGO-Succ-RSV, made with a PEG of molecular weight 2000 Daltons, only had resveratrol as a hydrophobic moiety, but the succinic acid linker would also impart some hydrophobicity to the structure. Succinic anhydride, which has a closed ring structure, is more hydrophobic and water insoluble and only becomes soluble in water after hydrolysis. It is hypothesized that combined structure of resveratrol and succinic acid linker would provide sufficient hydrophobicity to counter the

solubilising effects of PEG with the shorter molecular weight of 2000 Da, and hence the micelle structure was formed in the solution at high concentrations. In stark contrast to this, MeO-PEGN-Succ-RSV 2 kDa was soluble and formed a clear solution with no cloudiness, even at a higher concentration (10 mg/mL). This improved solubility and lack of aggregation could be due to the presence of nitrogen in linker, which imparts additional polarity to molecules, promotes hydrogen bonding with water, and prevents hydrophobic intermolecular interaction. Veronese et al. have reported similar findings with doxorubicin (DOX)-PEG conjugates with the tetra peptide linker GFLG. They found that the tendency of polymer conjugate to aggregate into micelles was dependent on the molecular architecture and Mw of polymer. [40] The DOX-GFLG-PEG with PEG5000 aggregated more readily than with PEG10000.

The critical micelle concentration for MeO-PEG-PLA-Succ-RSV, 2 kDa, 6.5 kDa, and MeO-PEGO-Succ-RSV 2 kDa were found to be 4 mg/L, 8 mg/L and 500 mg/L respectively (Figure 2.11-2.13 and Table 2.1). This clearly indicates that CMC is strongly dependent on the hydrophobic content in the chain and the Mw of polymer. The CMC values reduced as the hydrophobic chain length increased in the polymer. However, this observation was true to a certain extent of hydrophobic chain length. There are other molecular mechanics that drive the assembly of molecules into micelles, and are discussed in detail in the **section 2.3.6, plasma stability of polymer conjugates.** The CMC values were determined by using a UV probe as opposed to familiar fluorescent probes such as pyrene. This was because of the interference of resveratrol in the fluorescent measurements involving pyrene. The resveratrol has a very broad excitation (290-350 nm) and emission (380-420 nm) ranges. Although excitation and emission maxima were 320 and 400 nm respectively, the broad peak overlapped with those of the fluorescent probe, such as pyrene. The dimethyl yellow employed in this study has poor aqueous solubility (13mg/L), this property of molecule facilitated monitoring parameter, absorption as a function of surfactant concentration in addition to change in wavelength [55]. The CMC values obtained by both dye solubilisation and wavelength shift have correlated well. This indicates the efficiency of dimethyl yellow as an alternative UV probe in CMC determination.

The micelles size and polydispersibility index of resveratrol polymer conjugates are given Table 2.1. There was no exact correlation between micelle size and polymer architecture, such as chain length and hydrophobic content of polymers. In general, polymer conjugates with lesser hydrophobic content have formed micelles with smaller diameter. The micelles diameter was found to increase as the polymer concentration was increased. This could be due to the formation of large aggregates at higher polymer concentration.

Morphology studies with FESEM were not completely successful and only images of MeO-PEG-PLAO-Succ-RSV, 2 kDa and MeO-PEGO-Succ-RSV 2 kDa micelles could be captured. The images of the other micelles of resveratrol conjugates with PLA-PEG could not be detected in FESEM. Generation of heat during imaging, from electron beam, may have destroyed the polymer matrix. The images of MeO-PEG-PLAO-Succ-resveratrol 2 kDa micelles showed that micelles were rather solid nanoparticles once dried (Appendix, Figure 7.16). The hollow micelle-like structures could not be seen. The micelles would have been fused to form a solid structure during drying at room temperature or polymer itself would have aggregated into nanoparticles. The formation of nanoparticles could be the result of the inherent physicochemical properties of the polymer. Since the polymer has a short chain length with more than 60% PLA, at higher concentrations, the close and compact of assembly of the polymer chains leading to formation of solid nanoparticles cannot be ruled out. This close and compact structure with reasonably good PEG corona on the surface may also contribute to the improved plasma stability of the drug-polymer ester bond in the plasma (section 2.3.6). The FESEM image of MeO-PEGO-Succ-Resveratrol showed a melted wax-like material (Appendix, Figure 7.16). This could be due to a rupture of the micellar structure and a melting of the polymer due to the heat generated by the electron beam. The slides of MeO-PEG-PLAO-Succ-RSV 6.5 kDa did not show any particulate matter, that could be again due to melting. Because of these limitations of FESEM analysis, morphology studies were later performed with AFM. The images of micelles of all polymer conjugates of resveratrol were recorded. This was possible because AFM allows to capturing of images without generation of heat. AFM images of MeO-PEG-PLAO-Succ-RSV 2 kDa and 6.6 kDa showed a solid nanocrystalline structure. The images of MeO-PEGO-Succ-RSV showed a few distinct micelles with more of the collapsed PEG mass. All FESEM

images are presented in Figure 7.16 (Appendix) and AFM images in the Figures 7.17-7.19 (Appendix).

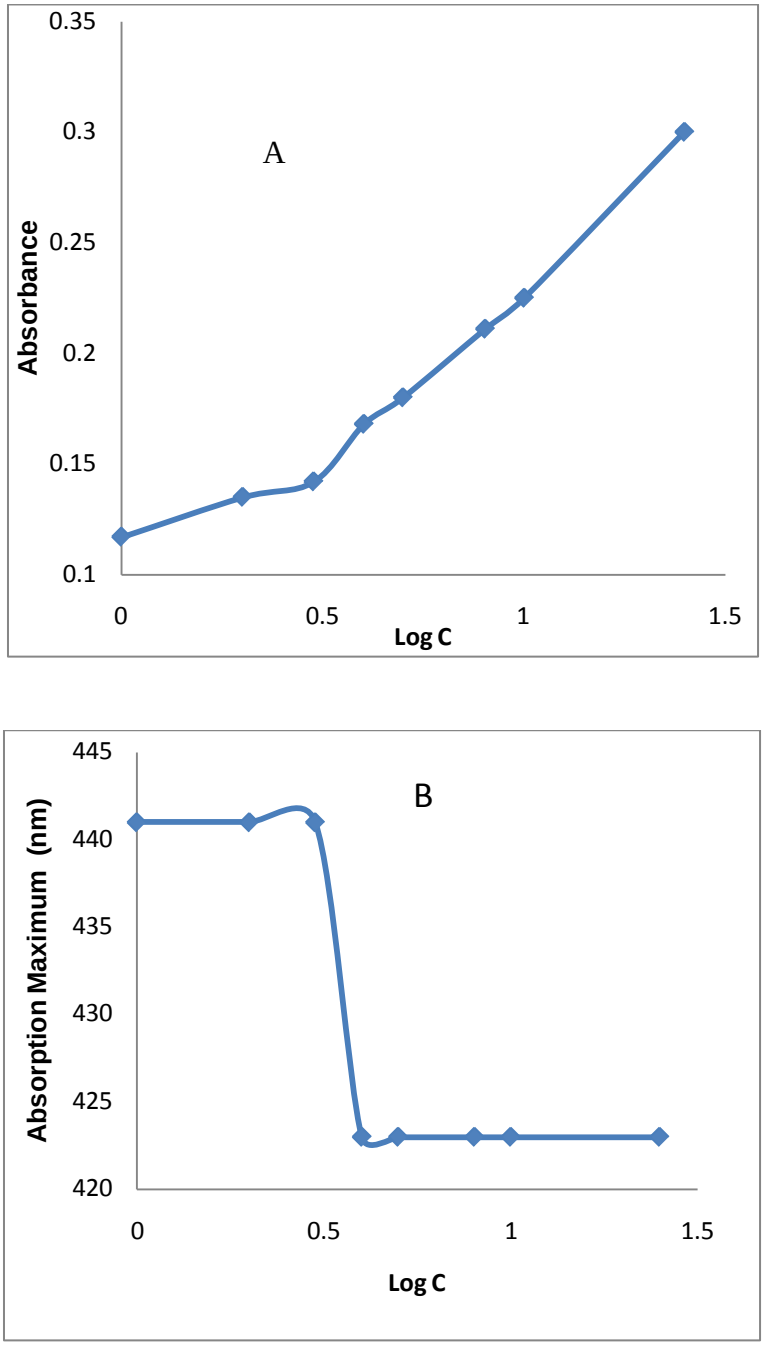


Figure 2.11: Determination of CMC for MeO-PEG-PLAO-Succ-RSV 2kDa A: plot of log (Concentration mg/L) vs Absorbance for dimethyl yellow at 441. B: plot of log (Concentration mg/L) vs Absorption maximum (nm) for dimethyl yellow.

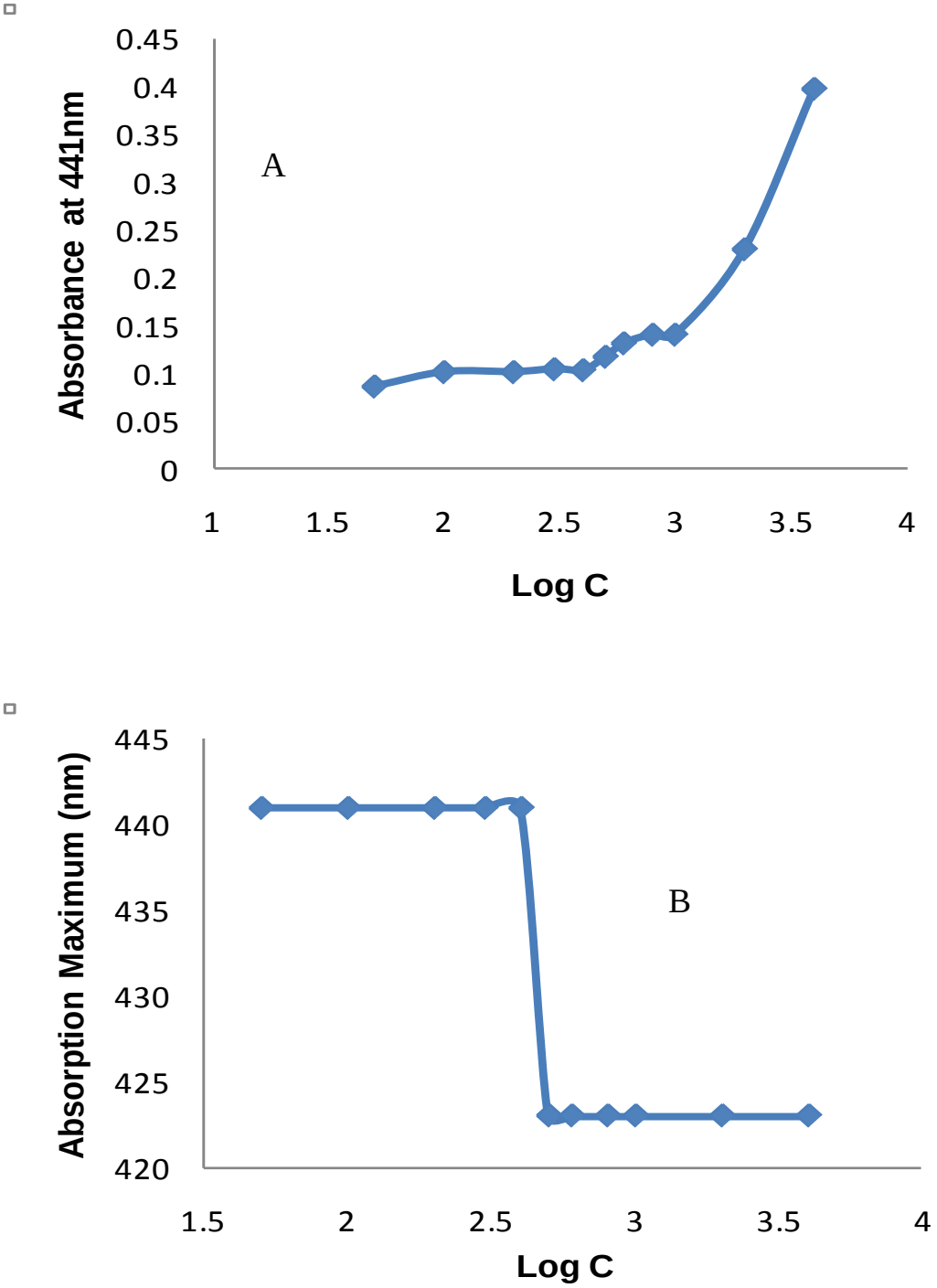


Figure 2.12: Determination of CMC value for MeO-PEGO-Succ-RSV A: plot of log (Concentration mg/L) vs Absorbance for dimethyl yellow at 441. B: plot of log (Concentration mg/L) vs Absorption maximum (nm) for dimethyl yellow.

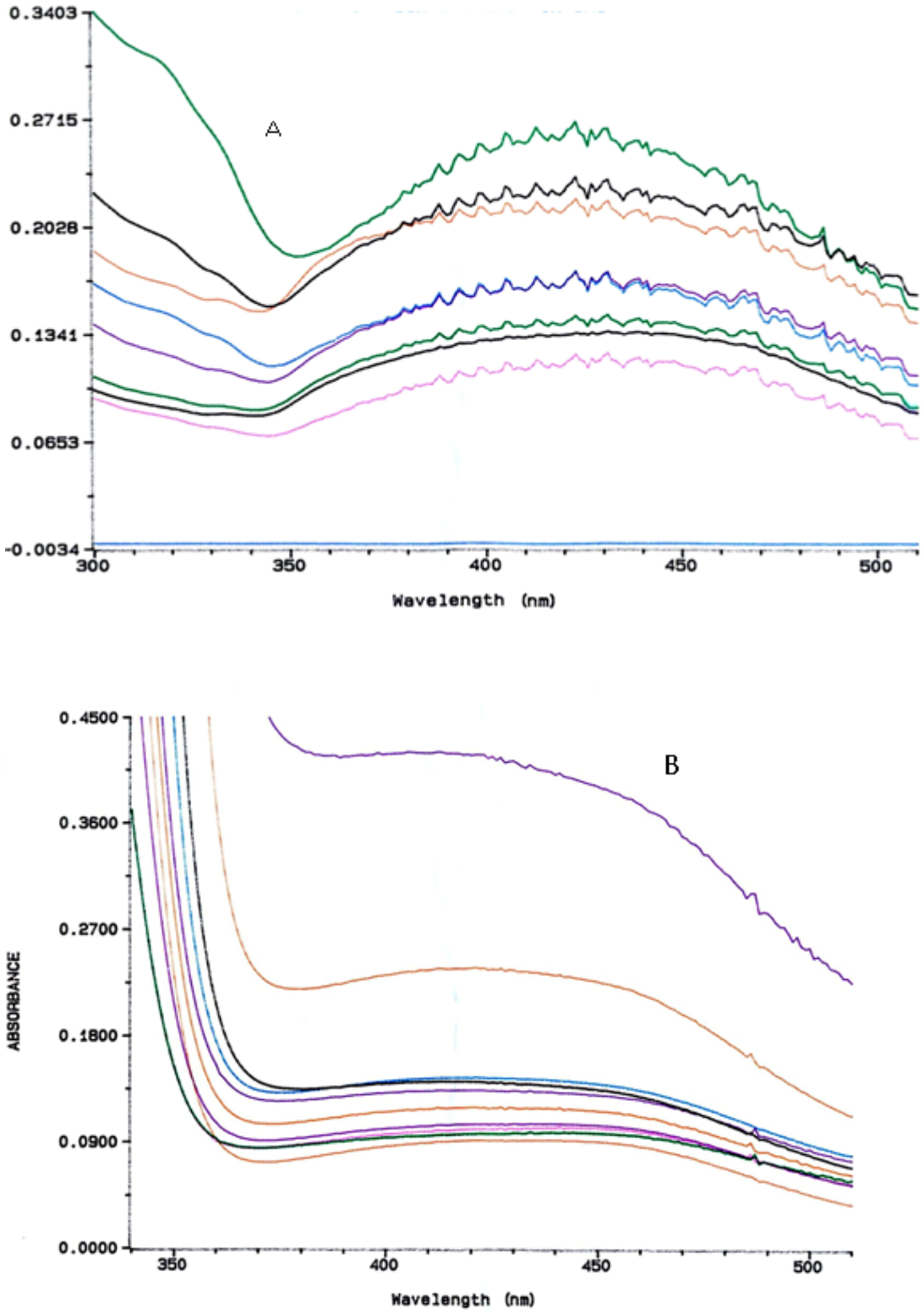


Figure 2.13: Plot of absorbance vs wavelength for dimethyl yellow in various concentration of A: MeO-PEG-PLAO-Succ-RSV, 2 kDa (1-25 mg/L). B:MeO-PEGO-Succ-RSV, 2 kDa (50-4000 mgL)

Table:2.1 Micelles size and polydispersibility of resveratrol-polymer conjugates.

Concentration (%)	Micelle Size nm(SD)	Poly dispersibility Index (SD)	Micelle Size nm(SD)	Poly dispersibility Index(SD)
	Meo-PEG- PLAO- Succ RSV 2kDa		**Meo-PEG-PLAO-Succ RSV 6.6K**	
0.05	170(0.63)	0.21(0.051)	147(3)	0.25(0.079)
0.1	249(10.44)	0.59(0.03)	147(6.11)	0.342(0.070)
0.2	255(3.0)	0.489(0.079)	165(14.42)	0.75(0.15)
	MeO-PEGO-Succ-RSV 2 kDa			
0.1	116(1.52)	0.121(0.024)		
0.25	136(1.15)	0.076(0.05)		
0.5	181(2.51)	0.19(0.03)		

2.3.5 Stability of polymer conjugates in buffers at different pH

The stability of polymer conjugates was studied in buffers of pH 4.5 and 7.4 at 37^0C, physiologically relevant to the pH of the lysosomes and the blood. The concentration of polymer conjugates in buffers was monitored by HPLC. All the polymer conjugates of resveratrol were found to be stable in pH 4.5, with nearly 90% of polymer conjugates remaining intact after 24 hours (Figure 2.14). The MeO-PEGN-Succ-RSV, 2 K and 20 K were found to be unstable at pH 7.4, with only about 9% of polymer conjugates remaining intact after 24 hours, which indicates that the ester link is unstable in slightly alkaline conditions. Similar results were reported for PEG-scutellarin conjugates and PEG-Taxol conjugates [19, 56]. MeO-PEGO-Succ-RSV was found to be relatively stable in pH 7.4 with nearly 65% of polymer conjugate found intact after 24 hours. This could be attributed to the self assembly of polymer conjugates to micelles. The micelles could form a barrier for ion catalysing hydrolysis (H^+/OH^-). The fact was further confirmed from the results of MeO-PEG-

PLAO-Succ-RSV conjugates of different Mw, which were found to be stable in pH 4.5 and 7.4 with >80% polymer conjugates found intact after 24 hours.

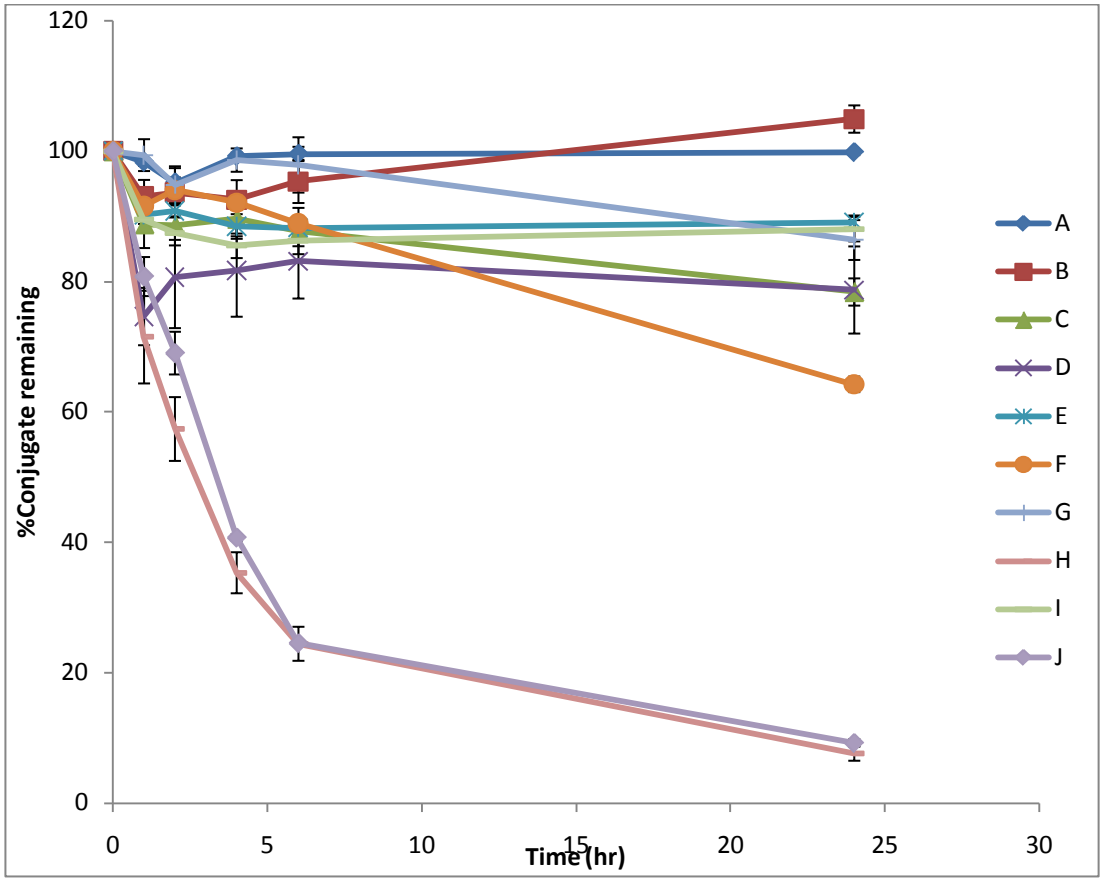


Figure 2.14: Stability of resveratrol polymer conjugates in buffers, A: MeO-PEG-PLAO-Succ-RSV, 2 kDa pH 4.5 ; B: MeO-PEG-PLAO-Succ-RSV, 2 kDa pH 7.4; C: MeO-PEG-PLAO-Succ-RSV, 6.5 kDa pH 4.5 ; D:MeO-PEG-PLAO-Succ-RSV, 6.5 kDa pH 7.4 ;E: MeO-PEGO-Succ-RSV, 2 kDa pH 4.5; F: MeO-PEGO-Succ-RSV, 2 kDa pH 7.4; G: MeO-PEGN-Succ-RSV, 2 kDa pH 4.5; H: MeO-PEGN-Succ-RSV, 2 kDa pH 7.4; I: MeO-PEGN-Succ-RSV, 20 kDa pH 4.5 ; J: MeO-PEGN-Succ-RSV, 20 kDa pH 7.4 All the data are presented as mean ± SD (n = 3)

2.3.6 Stability of resveratrol-polymer conjugates in rat plasma

The effectiveness of drug-polymer conjugates with an ester link intended for the sustained release of drugs would depend on the stability of ester bonds in plasma, as plasma esterase would catalyse the hydrolysis. All PEG conjugates of resveratrol, irrespective of the linker and polymer chain length, hydrolysed rapidly to free resveratrol (Figure 2.15) when exposed to plasma. The hydrolysis was so rapid that

almost no intact polymer conjugate was detected in HPLC at the initial time point of 0 hr. The rate of hydrolysis was more rapid than that observed for campothecin-20-PEG and taxol-2'-PEG with plasma $T_{1/2}$ of 2 and 0.4 hours respectively [19, 20]. The reason for this rapid hydrolysis could be because of the phenolic OH being more acidic than the aliphatic OH group, and electron delocalization and resonance stabilization of phenoxide ions would contribute to ester bond cleavage. However, phenols would not be ionized at a physiological pH of 7.4, since the pKa of phenols is >10. Hence electron delocalization contributes significantly to the instability of the ester link catalysed by plasma esterases. Although there are a few reports suggesting the application of more electronegative acid linkers, such as glycine for campothecine and peptides in the case of doxorubicin for greater plasma stability, for resveratrol, pegylating via a glycine linker did not provide any improvement in stability [46]. The PEG-peptide attached to doxorubicin through an amide linker has provided better stability against carboxy peptidases in plasma. Introducing a peptide linker between PEG and resveratrol demands an ester link between resveratrol and peptide, for there is no amine group in resveratrol. So the role of peptide in slowing down ester hydrolysis would be minimal. Hence the possibility of micelle forming polymeric conjugates to slow the ester hydrolysis was explored in this study. Accordingly the MeO-PEG-PLAO-Succ-RSV of different Mw and with a PLA/PEG ratio, were prepared and evaluated for plasma stability. The plasma $t_{1/2}$, Mw of conjugate, Mw of PLA/PEG segments are tabulated in Table 2.2 and the stability profile is shown in Figure 2.15. The results indicate that MeO-PEG-PLAO-Succ-RSV, 2 kDa was found to be more stable than other MeO-PEG-PLAO-Succ-RSV, 6.5 kDa conjugate. The other micelle forming conjugate MeO-PEGO-Succ-RSV 2 kDa was no different from MeO-PEGN-Succ-RSV, 2 and 20 kDa in terms of plasma stability (Figure 2.15). It had hydrolysed rapidly with no intact conjugate being detected at the initial time point. The trend indicates that the plasma stability depends on hydrophobic chain length in polymer conjugates. Stability increased as hydrophobicity increased. However the contrasting trend between MeO-PEG-PLAO-Succ-RSV, 6.5 kDa and 2 kDa, where the plasma stability decreased as the PLA chain length increased, indicates there are other parameters affecting the plasma stability. The plasma stability of the polymeric drug conjugate is a complex phenomenon, Pan et al. reported in their work on plasma a stability of various HPMA polymer-PGE_1 conjugates [27]. The authors attributed the difference in plasma

hydrolysis of conjugates of various species of poly HPMA to physicochemical properties such as conformation, inter and intra molecular forces, and the hydrophobicity. The authors have also reported that adsorption of plasma proteins, such as albumin onto polymer conjugates may favour enzyme substrate formation and activate enzyme-substrate, thereby leading to rapid hydrolysis. In another report, the higher amount of the hydrophobic drug bound to the polymer increased the plasma stability [57]. Association between heptapeptide side chains of HPMA copolymer have been shown to decrease the enzymatically catalysed release of doxorubicin from conjugate [58]. Riley et al. [59] studied the effect of PEG corona (PEG chain length and suface density) on the colloidal stability of PEG-PLA nanoparticles. With a fixed PEG chain length of 5 KDa, the colloidal stability was higher when the PLA chain varied between 3-5 kDa. A further increase in the PLA chain length resulted in reduced colloidal stability and the authors attributed this to the presence of naked PLA patches on nanopartilces. In another study, Gref et.al [60] reported the influence of PEG corona on plasma protein adsorption and the phagocytic clearance of PLA-PEG nanoparticles. They concluded the protein adsorption and phagocytic uptake was high when the PLA content in the nanoparticles is high, and they attributed this to the unprotected PLA surface. Jay Prakash Jain and Neeraj Kumar [61] reported the effect of PEG content on the formation of the polymersomes (micelles) of PEG-PLA structure. They concluded that the formation of self assembled polymersomes was dependent on the PEG chain length and assembly was favoured when the PEG content is high. They reported trisubstituted PLA (with 3 PEG chains and one PLA chain using a citrate linker) has the tendency to form a vesicle even at 10% PEG content because of the complete coverage of PLA by a branched PEG structure around the PLA chain. Considering these observations and reports, for a better plasma stability of the conjugate, it is concluded that there has to be optimum hydrophobicity, micelle forming ability and colloidal stability, with adequate surface protection by the PEG. In this regard, MeO-PEG-PLAO-Succ-RSV 2 kDa would form compact, colloidally stable micelles, with a strong PEG corona, thereby providing maximum stability against ester hydrolysis. MeO-PEG-PLAO-Succ-RSV 6.5 kDa, with a longer PLA chain length would lead to the formation of colloidally unstable micelles, with significantly higher naked PLA surfaces providing little protection against ester hydrolysis. In addition, solid nanoparticle-like structures were observed in AFM for air dried samples of MeO-

PEG-PLAO-Succ-RSV 2 kDa micelles. Formation of this solid nanoparticle-like structure would impart more resistance to hydrolysis.

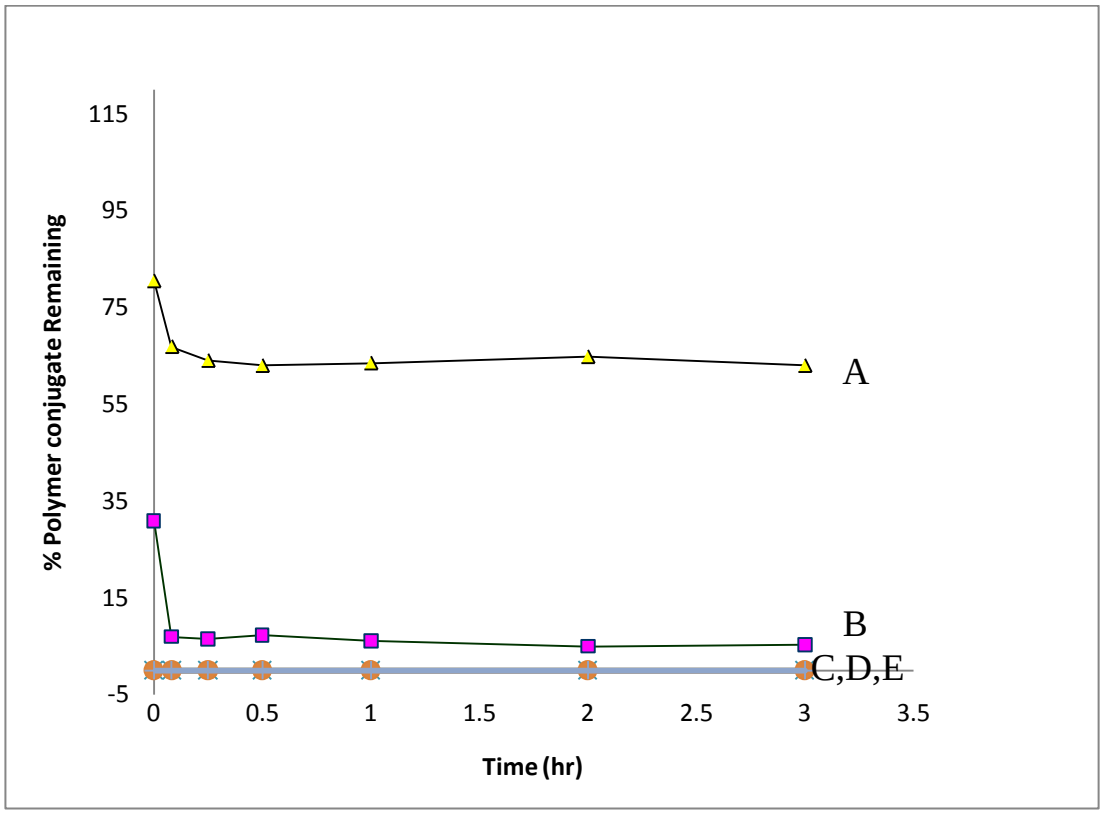


Figure 2.15: Plasma hydrolysis profile of resveratrol polymer conjugagtes A: MeO-PEG-PLAO-Succ-RSV, 2 kDa B: MeO-PEG-PLAO-Succ-RSV, 6.5 kDa C,D,E: merged profiles represents MeO-PEGN -Succ-RSV, 2 kDa, MeO-PEGN-Succ-RSV, 20 kDa and MeO-PEGO-Succ-RSV, 2 kDa respectively. All the data are presented as mean (n = 2).

Table 2.2: Various RSV-PLA-PEG conjugates with CMC values and Plasma stability

RSV-polymer conjugates	**PLA Mw**	**PEG Mw**	**Plasma $t_{1/2}$**	**CMC mg/L**	**CMC Moles/L**
MeO-PEG-PLAO-Succ-RSV 2 kDa	1000	750	3 hours	4	0.002
MeO-PEG-PLAO-Succ-RSV 6.5 kDa	4600	2000	5 minutes	8	0.0012
MeO-PEGO-Succ-RSV 2 kDa	Not applicable	2000	<5 minutes	500	0.25

2.3.7 *In vitro* metabolism studies of resveratrol and its polymer conjugates in rat liver microsomes

Since resveratrol undergoes phase II metabolism, such as glucuronidation and sulfation and minimum phase I metabolism, such as oxidation, reduction, and hydroxylation. The microsomal incubations were supplemented with a UGT reaction mixture A (contains uridine diphospho glucuronic acid, UDPA) and mixture B (buffer system), in order to bring about glucuronidation of resveratrol. Results of microsomal studies are shown in Figure 2.16 and 2.17. This study showed that resveratrol readily underwent metabolism to produce two monoglucuronide metabolites (Figure 2.18). The similar metabolites were also observed in the plasma samples of rats from the pharmacokinetic studies and were characterized by LC-MS (chapter 4). The metabolism of resveratrol was rapid and almost all of the resveratrol was metabolized within 10 minutes. The incubations of MeO-PEG-PLAO-Succ-RSV, 2 kDa with microsomes over 60 minutes did not result in any detectable level of metabolites in HPLC and presumably there was no metabolism occurring. However there was a fluctuation in the concentration of MeO-PEG-PLAO-Succ-RSV, 2 kDa in the microsomal incubations over the same period of time, errors in the analysis could not be excluded as there was only single incubation due to a shortage of microsomes and time constrain in getting supply. The control incubations without microsomes did not show any appreciable decrease In contrast, the incubation samples of MeO-PEGN-Succ-RSV 2 kDa, showed formation of moderate amount of glucuronide metabolites. However the control incubations of MeO-PEGN-Succ-RSV showed some degree of conjugate hydrolysis to free

resveratrol and which was subsequently metabolized. Hence metabolites were observed in microsomal incubations. These observations clearly demonstrated that, to provide protection against the metabolism of resveratrol, the polymer conjugates need to be intact and with micelle forming ability; the physical mixture of polymer conjugates with resveratrol would not be helpful in reducing metabolism. These observations are in contrast to those observed with metabolism studies of resveratrol-PEG conjugates carried out with intestinal membrane[46]. In that study, despite the hydrolysis of resveratrol polymer conjugates, resveratrol did not undergo metabolism and was attributed to the inhibitory effects of adjacent polymer molecules. This could be because of the weak enzymatic activity in intestinal membrane compared to isolated and concentrated liver microsomal preparation.

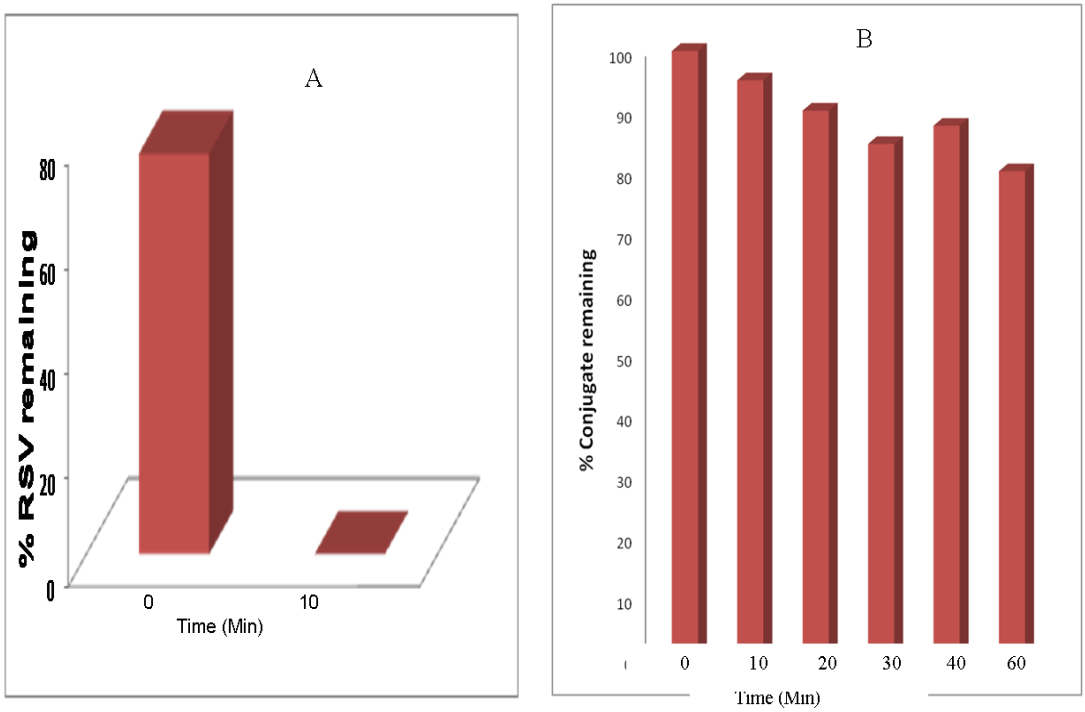


Figure 2.16 : The degradation profile of A: Resveratrol and B: MeO-PEG-PLAO-Succ-RSV 2 kDa in microsomal incubations with UDPA.

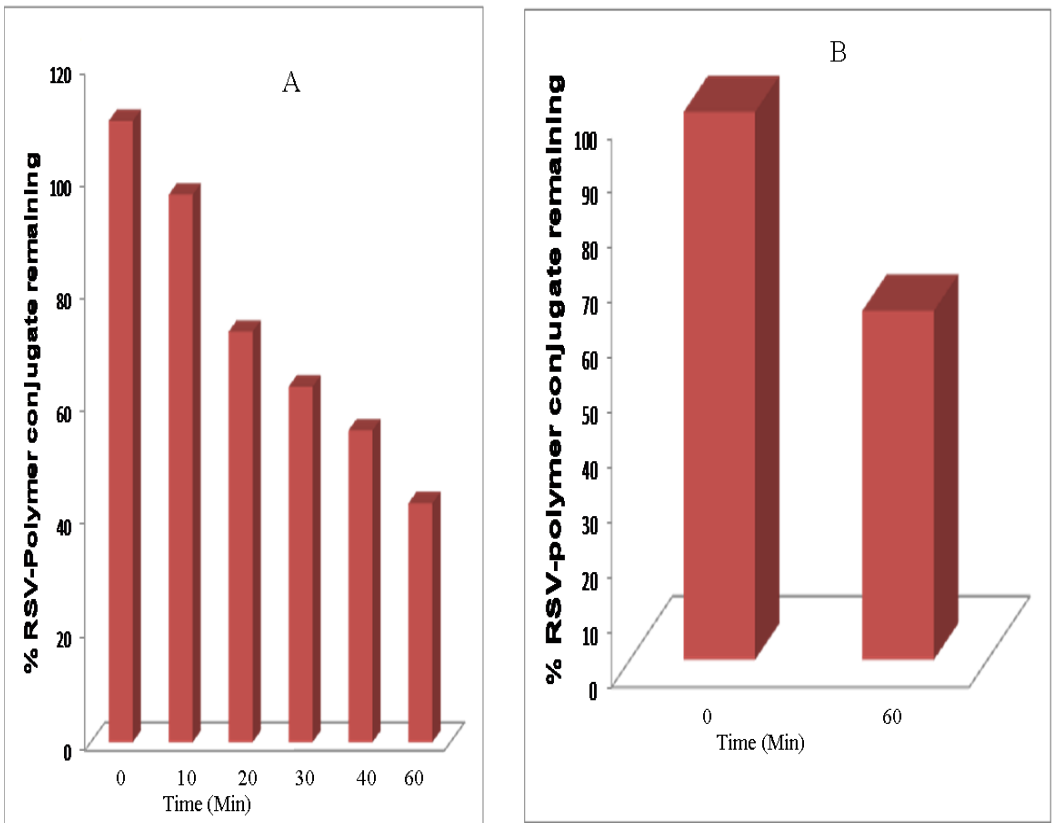
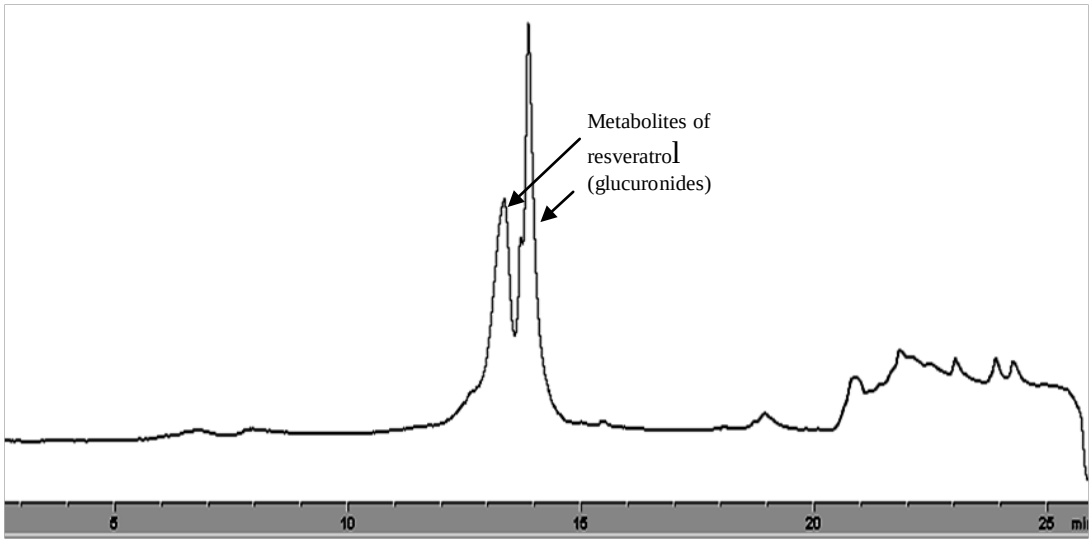


Figure 2.17 Degradation profile of A:MeO-PEGN-Succ-RSV 2 kDa in micosomal incubation with UDPA. B: :MeO-PEGN-Succ-RSV 2 kDa in control incubations in reaction mixture without microsomes.



Time

Figure 2.18: Chromatogram of resveratrol in microsomal incubations at 10 minutes.

2.3.8 *In vitro* antioxidant assay

In vitro antioxidant activities of resveratrol and its polymer conjugates were assessed based on their ability to scavenge the free radical DPPH. The results of antioxidant assay revealed that the free radical scavenging ability of polymer conjugates (MeO-PEGN-Succ-RSV 2kDa and MeO-PEG-PLAO-Succ-RSV 2 kDa) was decreased (IC 50 values 219 µM vs 60 µM for RSV) as shown in Figure 2.19. This decrease in the activity may not really affect *in vivo* activity profile of the polymer conjugates, as these ester conjugates would hydrolyze in the body to produce native resveratrol. The antioxidant activity is deliberated in details in the chapter 3, section 3.3.3.

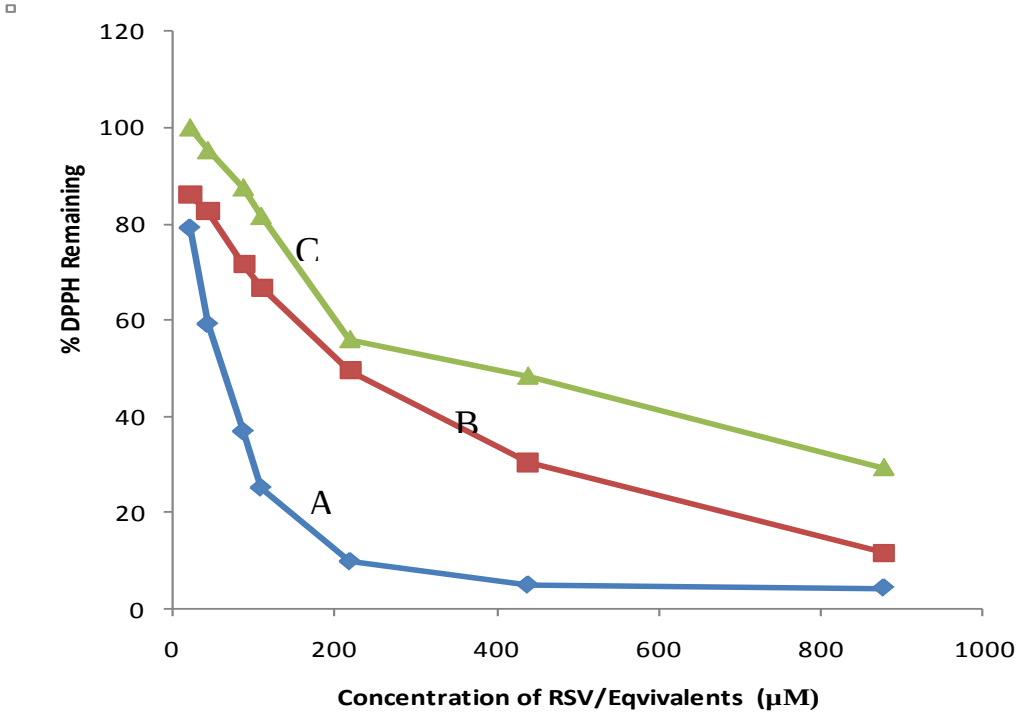


Figure 2.19 DPPH inhibition profile of A: Resveratrol; B: MeO-PEGN-Succ-RSV, 2kDa; C: MeO-PEG-PLAO-Succ-RSV, 2kDa All the data are presented as mean ± SD (n=3)

2.3.9 Single dose preliminary pharmacokinetic study

The plasma concentration profile of resveratrol, MeO-PEG-PLAO-Succ-RSV 2 kDa is shown in Figure 2.20 and the HPLC chromatogram of RSV plasma sample with metabolites in Figure 2.21. After intravenous dosing of resveratrol at 10 mg/Kg, the

plasma concentration profile showed rapid elimination of resveratrol from the plasma. The plasma levels dropped to almost no detectable levels within an hour. There were four major metabolites detected in HPLC and characterized by LC-MS/MS. The four metabolites could be assigned to 3- and 4'- monoglucuronides, 3-, 4'- mono sulphates. The area under the curve (AUC total) for resveratrol was found to be 0.63 µg*h/L with T ½ of 2.3 h and mean residence time (MRT) of 1.7 h. The $T_{1/2}$ was overestimated; because software calculations were based on plasma concentration at few terminal time points, where the concentrations are low and were approaching base line. When MEO-PEG-PLAO-Succ-RSV 2 KDa was dosed to rats at 2 mg/kg equivalent to resveratrol. There was a comparatively higher plasma concentration observed at the initial time point of 5 minutes, however there was a rapid decline in the plasma concentration thereafter. This can be attributed to clearance by mononuclear phagocyte systems (MPS) [62]. There was no appreciable amount of conjugate in plasma after 2 hours, as the level was approaching the detection limits. The AUC total, $t_{1/2}$ and MRT (5.54 µg*h/L, 2.3 h and 2.5 h) were significantly higher compared to resveratrol. Interestingly, some of the free drug released after hydrolysis of polymer conjugates was not completely metabolized as was in the case of free resveratrol. The intensity of the resveratrol peak with polymer conjugates was greater than that of metabolites. This was in stark contrast to free resveratrol profile, wherein, metabolite concentration was more from the initial time point of 5 minutes. This clearly demonstrates that the majority of resveratrol produced from hydrolysis was still in micelles, which prevented the metabolism of resveratrol.

The plasma profile of MeO-PEG-PLAO-Succ-RSV 6.6 kDa was very different from that of MeO-PEG-PLAO-Succ-RSV 2 kDa. There was no appreciable amount of polymer conjugate detected in the plasma, even at initial time point of 5 minutes. The rapid decline or non-availability of polymer conjugate could be due to the combined effect of hydrolysis and MPS clearance. Unlike the MeO-PEG-PLAO-Succ-RSV 2 kDa , MeO-PEG-PLAO-Succ-RSV 6.6 kDa displayed higher level of metabolites, possibly due to a naked PLA surface bearing resveratrol, which in turn released resveratrol upon hydrolysis, and the latter was subsequently metabolized. These observations correlate to results obtained from the *in vitro* plasma stability study .

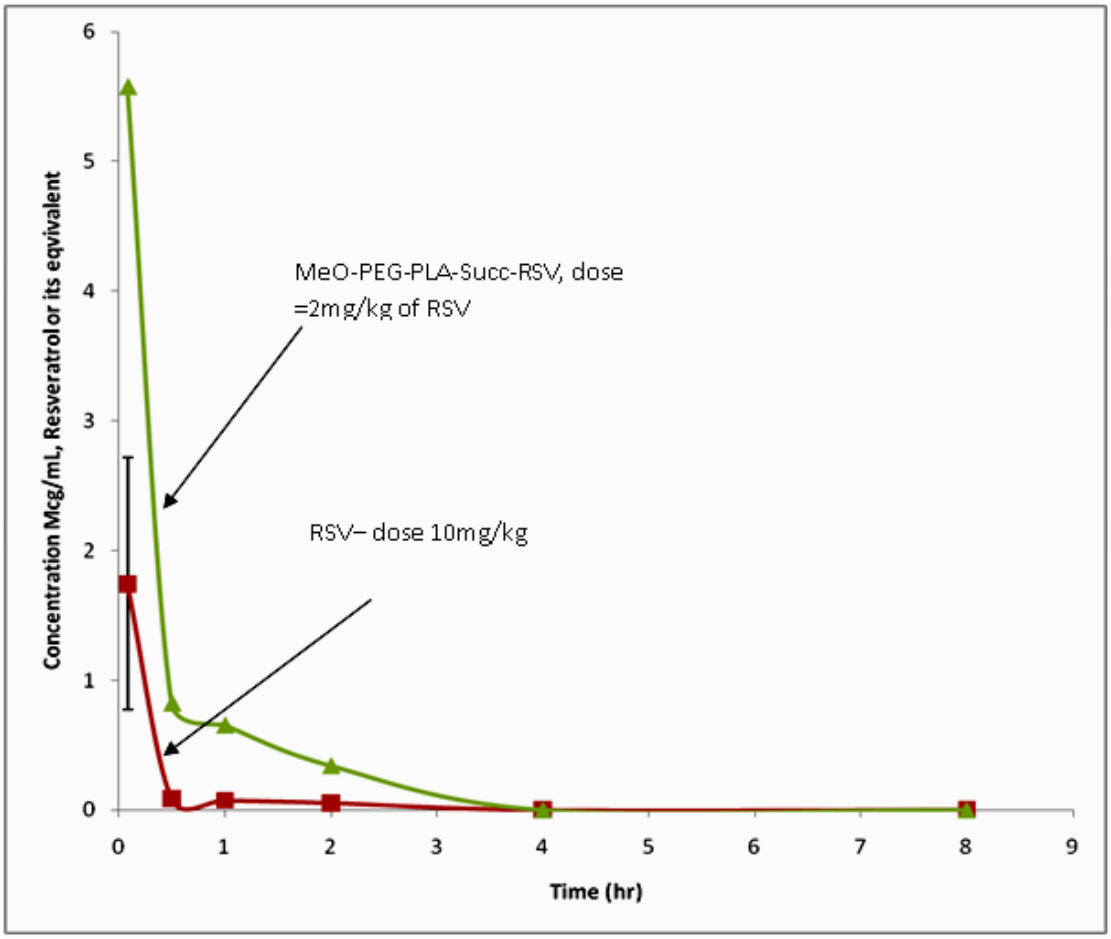


Figure 2.20: Single dose IV plasma concentration–time profile of resveratrol (RSV) and MeO-PEG-PLAO-Succ-RSV2 kDa conjugate. All the data are presented as mean ± SD (n = 6 for conjugate and n=4 for RSV).

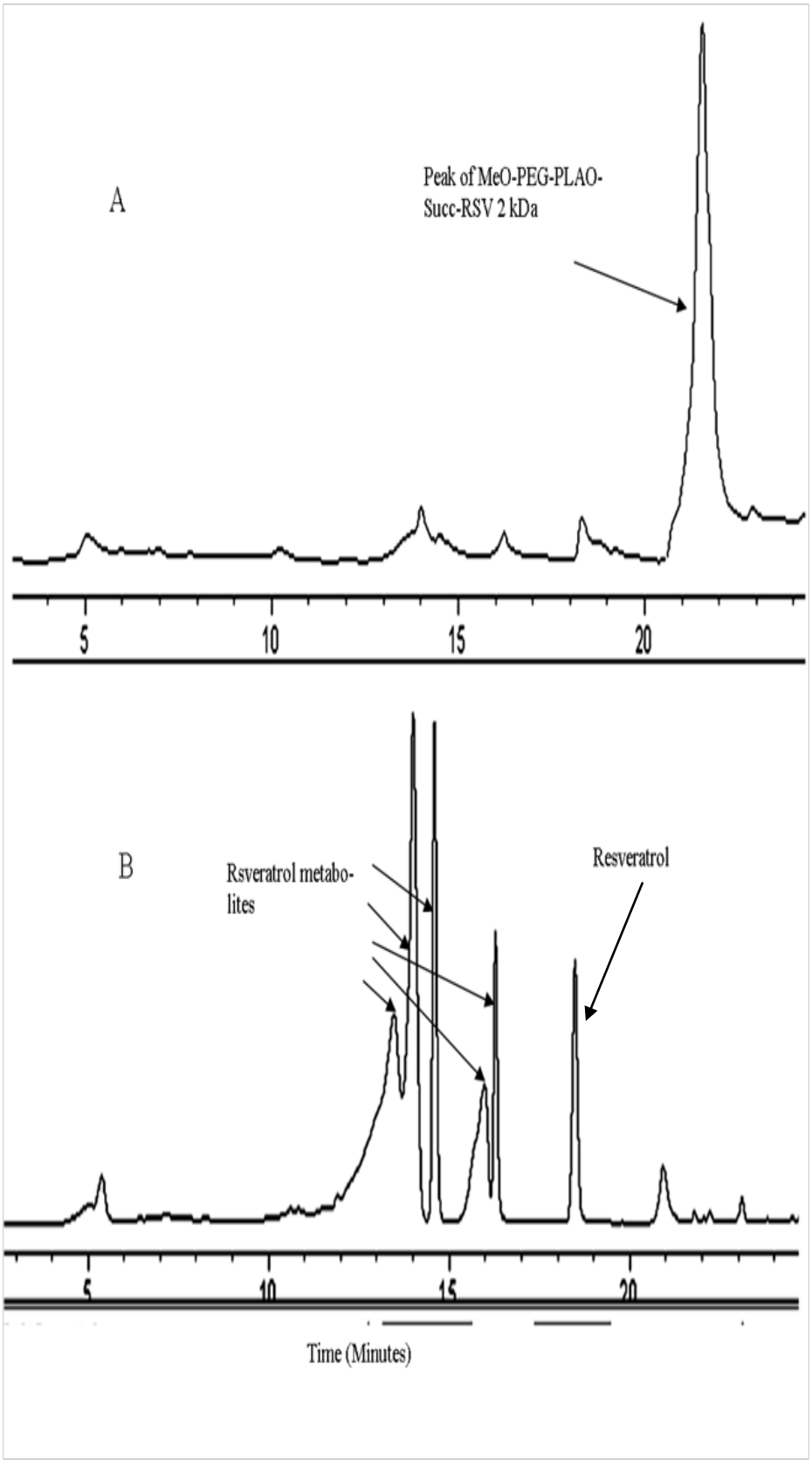


Figure 2.21: Chromatogram of plasma samples of rats at 5 minutes A: MeO-PEG-PLAO-Succ-RSV, 2 kDa. B: Resveratrol

2.4 References

1. Ludovic LC, Chalabi.N, Delort. L, YvesJB, Bernard-Gallon.D.J. Resveratrol and breast cancer chemoprevention: Molecular mechanisms. Mol Nutr Food Res. 2005 49:462-471.
2. Das DK, Maulik N. Resveratrol in cardioprotection: a therapeutic promise of

   alternative medicine. Mol Interv. 2006; 6(1):36-47.
3. Delmas D, Jannin B, Latruffe N. Resveratrol: preventing properties against vascular alterations and ageing. Mol Nutr Food Res. 2005; 49(5):377-95.
4. Le Corre L, Chalabi N, Delort L, Bignon YJ, Bernard-Gallon DJ. Resveratrol and breast cancer chemoprevention: molecular mechanisms. Mol Nutr Food Res. 2005; 49(5):462-71.
5. Goldberg DM, Yan J, Soleas GJ. Absorption of three wine-related polyphenols in three different matrices by healthy subjects. Clin Biochem. 2003; 36(1):79-87.
6. Yu C, Shin YG, Chow A, Li Y, Kosmeder JW, Lee YS, et al. Human, rat, and mouse metabolism of resveratrol. Pharm Res. 2002; 19(12):1907-14.
7. Wenzel E, Somoza V. Metabolism and bioavailability of trans-resveratrol. Mol Nutr Food Res. 2005; 49(5):472-81.
8. Marier JF, Vachon P, Gritsas A, Zhang J, Moreau JP, Ducharme MP.Metabolism and disposition of resveratrol in rats: extent of absorption, glucuronidation, and enterohepatic recirculation evidenced by a linked-rat model. J Pharmacol Exp Ther. 2002; 302(1):369-73.
9. Walle T, Hsieh F, DeLegge MH, Oatis JE, Jr., Walle UK. High absorption but very low bioavailability of oral resveratrol in humans. Drug Metab Dispos. 2004; 32(12):1377-82.
10. Luciana T, Annalisa D, Ivana S, Caderni. G. Resveratrol depresses the growth of colorectal aberrant crypt foci by affecting bax and p21CIP expression Carcinogenesis. 2000; 21(8):1619-1622.
11. Wenzel E, Soldo T, Erbersdobler H, Somoza V. Bioactivity and metabolism of trans-resveratrol orally administered to Wistar rats. Mol Nutr Food Res. 2005; 49(5):482-94.

12. Gao X, Xu YX, Divine G, Janakiraman N, Chapman RA, Gautam SC. Disparate in vitro and *in vivo* antileukemic effects of resveratrol, a natural polyphenolic compound found in grapes. J Nutr. 2002; 132(7):2076-81

13. Ovesn Z, K. Ht-K. Structure-activity relationship of trans-resveratrol and its analogues. Neoplasma. 2005; 52(6):6.
14. Lee HJ, Seo JW, Lee BH, Chung KH, Chi DY. Syntheses and radical scavenging activities of resveratrol derivatives. Bioorg Med Chem Lett. 2004; 14(2):463-6.
15. Monofaedini C, Veronese FM. Stabilization of substances in circulation. Biconjuate Chem.1998,9, 418-450
16. Veronese FM, Morpurgo M. Bioconjugation in pharmaceutical chemistry. II Farmaco. 1999; 54(8):497-516.
17. Greenwald RB, Choe YH, Mcguire J, Conover CD. Effective drug delivery by PEGylated drug conjugates. Adv.Drug.Del.Rev, 2003,55, 217-250.
18. Greenwald RB, Pendri A, Bolikal D. Highly water soluble taxol 7-polyethylene glycol carbamates and carbonates. J.Org.Chem. 1995,60,331-336.
19. Greenwald RB, Gilbert CW, Pendri A, Conover CD, Xia J, Martinez A. Drug delivery systems: water soluble taxol 2'-poly(ethylene glycol) ester prodrugs-design and in vivo effectiveness. J Med Chem. 1996; 39(2):424-31.
20. Greenwald RB, Pendri A, Conover CD, Lee C, Choe YH, Gilbert C, Martinez A, Xia J, Wu D, Hsue M. Camptothecin-20-PEG Ester Transport Forms: the E€ect of Spacer Groups on Antitumor Activity. Bioorganic & Medicinal Chemistry 6 (1998) 551-562.
21. P.L. Carl, P.K. Chakravarty, J.A. Katzenellenbogen, novel connector linkage applicable in prodrug design, Med. Chem. 24 (1981) 479–480.
22. W.A. Denny, W.R. Wilson, The design of selectively-acti-vated anti-cancer prodrugs for use in antibody-directed and gene-directed enzyme-prodrug therapies, J. Pharm. Pharmacol. 50 (1998) 387–394.
23. M.Wakselman, 1,4- and 1,6-eliminations from hydroxy and amino-substituted benzyl systems: Chemical and biochemical applications Nouvean.J. de Chemie 7 (1983) 439–447.

24. M. Sedlak, V. Buchta, L. Kubicova, P. Simunek, M. Holcapek, P. Kasparova, Synthesis and characterization of new amphotericin B-methoxypoly(ethylene glycol) conjugate. Bioorg. Med. Chem. Lett. 11 (2001) 2833–2835.

25. H. Zhao, C.D. Conover, P. Reddy, A. Martinez, C.B. Longley, R.B. Greenwald, The Utility of Polyethylene Glycol Conjugation to Create Prodrugs of Amphotericin B, delivery systems employing 1,6-elimination: National Biotechnology Conference, June 24–26 (2002). San Diego, CA.

26. Sann CL. Maleimide spacers as versatile linkers in the synthesis of bioconjugates of anthracyclines. Nat. Prod. Rep., 2006, 23, 357–367.

27. Pan H, Kopeckova P, Liu J, Wang D, Millre SC, Kopecek J. Stability in plasmas of various species of HPMA copolymer-PGE_1 conjugates. Pharm.Research 2007.12,2270-2280.

28. Zhang X, Li Y , Chen X, Wang X, Xu X, Liang Q,Hu J, Jing X. Synthesis and characterization of the paclitaxel/MPEG-PLA block copolymer conjugate. Biomaterials.2005;26:2121
29. Xie Z, Lu T, Chen X, Lu C, Zheng Y, Jing X. Triblock poly(lactic acid)-b-Poly(ethylene glycol)-b-Poly(lactic acid)/Paclitaxol Conjugates:Synthesis, Micellization, and Cytotoxicity. J.App.Polymer Sci, DOI 10.1002.
30. K. Hoste, K. De Winne, E. Schacht. Polymeric prodrugs. Int.J.Pharmaceutics, , 2004, 277, 119–131.
31. Shen, W.C., Ryser, H.J.P. Cis-aconityl spacer between daunomycin and macromolecular carriers: a model of pH-sensitive linkage releasing drug from a lysosomotropic conjugate. Biochem. Biophys. Res. Commun. 1981. 102, 1048–1054.
32. Kaneko, T., Willner, D., Monkovic, I., Knipe, J.O., Braslawsky, G.R., Greenfield, R.S., Dolatrai, M.V., New hydrazone derivatives of adriamycin and their immunoconjugates. A correlation between acid stability and cytotoxicity. Bioconjugate Chem. 1991. 2, 133–141.

33. Coessens, V., Schacht, E., Domurado, D., Synthesis of polyglutamine and dextran conjugates of streptomycin with an acid-sensitive drug–carrier linkage. J. Control. Release, 1996. 38,141–150.
34. Vassalli, J.D., Pepper, M.S., Tumor biology—membrane proteases in focus. Nature 1994. 370, 14–15.
35. Rochefort, H., Capony, F., Garcia, M., Cathepsin D a protease involved in breast-cancer metastasis. Cancer Metast.Rev. 1990. 9, 321–331.
36. Osmak, M., Babic, D., Abramic, M., Vrhovec, I., Milicic, D.,Skrk, J.Cathepsin D content in malignant tumours of corpus uteri. Eur. J. Cancer. 1997. 33, 699–700.
37. Keppler, D., Fondanèche, M.C., Dalet-Fumeron, V., Pagano, M., Burtin, P., Immunohistochemical and biochemical study of a cathepsin B-like proteinase in human colonic cancers.Cancer Res. 1988.48, 6855–6862.
38. Stetler-Stevenson, W.G., Type IV collagenases in tumor invasion and metastasis. Cancer Metast. Rev. 1990.9, 289–303.
39. Ginestra, A., Monea, S., Seghezzi, G., Dolo, V., Nagase, H., Mignatti, P., Vittorelli, M.L., Urokinase plasminogenactivator and gelatinases are associated with membrane vesicles shed by human HT1080 fibrosarcoma cells. J. Biol. Chem. 1997.272, 17216–17222.
40. Veronese FM, Schiavon O, Pasut G, Mendichi R, Andersson L, Tsirk A, Ford J, Wu G, Kneller S, Davies J, Duncan R. PEG-Doxorubicin Conjugates: Influence of Polymer Structure on Drug Release, in Vitro Cytotoxicity, Biodistribution, and Antitumor Activity Bioconjugate Chem. 2005, 16, 775-784
41. Kare NV, The conjugation of proteins with poly(ethylene glycol) and other polymers.Altering properties of proteins to enhance their therapeutic potential, Adv.Drug.Deliv.Rev. 1993,16, 91-114.
42. Cuatrecases P, Anfinsen CB, Affinity chromatography, Method Enzymol 1971, 22, 345-378.
43. Greenwald RB, Conover CD, Pendri A, Choe YH, Martinez A, Wu D, Guan S, Yao Z, Shum KL, Drug Delivery of anticancer agents:Water soluble 4-poly(ethylene glycol) of derivatives of lignin, podphyllotoxin, J.Control.Rel. 1999,61, 281-294.

44. Laurienzo P , Malinconico M , Motta A , Vicinanza A Synthesis and characterization of a novel alginate–poly (ethylene glycol) graft copolymer. Carbohydrate Polymers 2005, 62 274–282.
45. J. Kuang, J. Yuan, M. Zhou, W. Yuan, X.Sui, Z.Li. Materials Letters, (2008), 62, 4078-4080.
46. Biasutto L, Marotta E, Mattarei A, Beltramello S, Caliceti P, Salmaso S, Bernkop-Schnurch A, Garbisa S, Zoratti M, Paradisi C. Absorption and Metabolism of Resveratrol Carboxyesters and Methanesulfonate by Explanted Rat Intestinal Segments Cell. Physiol. Biochem.24 (2009) 557-66.
47. Feng L, Feng. Analysis of the resveratrol-binding protein using phage-displayed random peptide library. Shengwu Huaxue Yu Shengwu Wuli Xuebao. 2006;38(5):342.
48. Bridges AM, Mousa AS, Davis PJ. Polyphenol conjugates as RGD binding compounds and method of use, US patent No US20100209382A1.
49. Greenwald, R. B.; Choe, Y. H.; Wu, D., Selective phenolic acylation of 10-hydroxycamptothecin using poly (ethylene glycol) carboxylic acid. Bioorg Med Chem Lett 2003, 13 (3), 577-80.
50. Alla A, Rodríguez-Galán A, Muñoz-Guerra S. Polymer 2000; 41: 6995.
51. Anderson, D.; Conradi, R.A.; Knuth, K.E.J.Pharm.Sci 1985;74,365
52. Villuendas, I.; Molina, I., Regaño,C., Bueno, M.; Ilarduya, A.M.D.; Galbis, J.A. Macromolecules 1999; 32: 8033
53. Stadler A, Kappe CO. The Effect of Microwave Irradiation on Carbodiimide-Mediated Esterifications on Solid-Support. Tetrahedron. 2001; 57:3915.
54. Bates H, Jones J, Witty MJ.Direct observation of an alkoxycabonylamino acid O-acylusourea. 1980; 773.
55. Voronov A, Vasylyevb S, Kohuta A, Peukertb W, Surface activity of new invertible amphiphilic polyesters based on poly(ethylene glycol) and aliphatic dicarboxylic acids. J Colloid Interface Science 323 (2008) 379–385.

56. ZHOU QS, JIANG XH, YU JR, LI KJ. Synthesis and Characterization of PEG-scutellarin Conjugates, a Potential PEG Ester Prodrug for the Oral Delivery of Scutellarin. Chinese Chem Letters 2006,17, 85-88
57. Pan HZ, Kopeckova P, Wang D, Yang JY, Miller S, Kopecek J.Water soluble HPMA copolymer-postaglandin E-1 containing a cathepsin sensitive spacer. J.Drug.Targetting 2006. 14 425-435.
58. Ding H, Kopeckova P, Kopecek J. Self association of properties of HPMA containing an amphipatic heptapeptide. J.Drug.Targetting. 2007. 15,465-474.
59. Riley T, Govender T, Stolnik S, Xiong CD, Garnett MC, Illum L,Davis SS. Colloidal stability and drug incorporation aspects of micellar-like PLA–PEG nanoparticles. Colloids and Surfaces B: Biointerfaces 1999. 16 147–159
60. Gref R, Luck M, Quellec P, Marchand M, Dellacherie E,Harnisch S, Blunk T, Muller RH. Stealth corona-core nanoparticles surface modified by polyethylene glycol (PEG): influences of the corona (PEGchain length and surface density) and of the core composition on phagocytic uptake and plasma protein adsorption. Colloids and Surfaces B: Biointerfaces 18 (2000) 301–313

61. Jain JP, Kumar N. Self Assembly of Amphiphilic (PEG)3-PLA Copolymer as Polymersomes: Preparation, Characterization, and Their Evaluation As Drug Carrier. Biomacromolecules 2010, 11, 1027–1035
62. Li Y , RongQi X, Maitani Y, Nagai T. PEG–PLA diblock copolymer micelle-likenanoparticles as all-trans-retinoic acid carrier: in vitro and in vivo characterizations. Nanotechnology, 2009,20, 055106

CHAPTER 3

SYNTHESIS, CHARACTERIZATION, BIOLOGICAL AND PRELIMINARY PHARMACOKINETIC EVALUATION OF RESVERATROL-PEG ETHERS

3.1 INTRODUCTION

Resveratrol has shown remarkable activities *in vitro* [1, 2], which have not been able to be translated into the activities *in vivo*. The very low availability of native resveratrol in the circulation was attributed to this effect [3, 4]. The metabolic conjugation was responsible for rapid clearance of resveratrol from systemic circulation, and the metabolites are reported to be either less active or inactive [5, 6, 7]. Poor pharmacokinetic properties would impede clinical efficacy [8]. Polymeric conjugates (prodrugs) have been reported for providing a masking effect against the metabolism of conjugated drugs [9]. The polymeric conjugate strategy involves covalent attachment of a polymer to drugs mainly via amide or ester bonding. After administration into the body, these polymeric systems release the drug by action of plasma esterases/carboxy peptidases in the blood [9, 10]. The rate of release depends on the type of spacer used for the ester/amide link, and the physicochemical properties of the polymer and the drug. Peptide spacers have reported to produce stable and long circulating polymer conjugates [10] However to introduce a peptide spacer between the drug and polymer, there has to be specific functional groups, for example, $NH_2$and SH and would not always result in enhanced circulation times of polymeric drugs [10, 11]. Peptide spacers may provide protection against carboxy peptidases of the plasma, but cannot be anticipated for esterases if the spacer is connected to the drug via an ester link. The polymeric ester conjugates have been reported to undergo rapid hydrolysis in the plasma. Similarly, resveratrol PEG conjugates have been reported to hydrolyse very rapidly in the plasma [13]. The efforts described in the chapater 2 to design ester based PEGylated resveratrol with reasonable stability were partially successful and desired goal have not be achieved. So far, only micelles forming PEG-PLA block polymer conjugates have shown a certain degree of resistance against plasma esterases but improvement in the pharmacokinetic profile was not up to expectation. In this regard, permanent PEGylation of resveratrol would be the best option to protect resveratrol against metabolic conjugation. Although permanent PEGylation via linker stronger than

esters, has been reported to slightly reduce the biological activity of some of the small molecule nonpeptide drugs, the increased plasma concentration and long circulation time may compensate for this effect and even provide overall advantage.

3.1.1 Review of procedure for synthesis of permanently PEGylated drug conjugates

Various approaches have been tested reported for permanent PEGylation of small molecules. The paclitaxol was conjugated by a carbamate bond [14]. Drugs with thiol groups have been modified into stable PEG conjugates by a carbon-sulphur bond or a thiocabamate link [12]. The possible chemistry for permanent PEGylation of resveratrol is either through a carbamate or an ether bond. However , PEGylation via a carbamate link needs modification of one of the hydroxyl groups of resveratrol. On the other hand, linking PEG via an ether bond appears to be less complicated, given commercial availability of PEGs with an end group bearing halides. Careful review of the literature of structural activity relationship studies of resveratrol leads to the conclusion that converting one hydroxyl group into ether or any other small chain substituent would not completely destroy the bioactivity of resveratrol; in fact some derivatives showed better anticancer activity than native resveratrol [15-19].

It has been reported that small chain alkyl substitution on the 4'-OH of resveratrol could be accomplished by use of an alkyl bromide in the presence of K_2CO_3 [15, 16]. The K_2CO_3 was reported to abstract proton selectively at 4' OH group, thus enabling the formation of monosubstituted resveratrol ether derivatives, but yields of reactions were oftern low (10-20%) after purification . Such reactions can be explored to synthesize resveratrol-PEG conjugates with ether links. A similar approach for PEGylation of butylated hydroxyl toluene has been reported, where in the methyl group of BHT has been modified into a benzyl halide and reacted with HO-PEG-OH in the presence of NaH/DMF [21].

3.1.2 Objectives of the study

The objectives of the study described in this chapter were to: design and optimize synthetic schemes for the preparation PEGylated resveratrol via an ether link; characterize resveratrol-PEG ethers by NMR for structural confirmation; evaluate the selected PEG ether conjugates for stability in physiologically relevant buffers

and plasma; assess the effect of PEGylation on the bioactivity of resveratrol through *in vitro* antioxidant assay; and, finally to evaluate the pharmacokinetic profile of conjugates in Wistar rats following intravenous administration.

3.2. MATERIALS AND METHODS

3.2.1 Materials

Trans-Resveratrol was purchased from DND Pharma-Tech Co., Inc, Shanghai.P.R.China, with 99% purity. Monomethoxy polyethylene glycol bromide (MeO-PEG-CH_2-CH_2-Br) of different molecular weights were obtained from, IRISH biotech GMBH, Germany(>99% purity). N, N-Dimethyl formamide (DMF, 99.8%) was purchased from Sigma Aldrich, Australia. Potassium carbonate (K_2CO_3) was obtained from BDH chemicals, Poole, England. Other chemicals, such as Diethyl ether, and Dicloromethane (DCM), were of labarotary grade and were used without any further purification.

3.2.2 Methodologies for synthesis of resveratrol-PEG ethers

3.2.2.1 Potassium carbonate mediated etherification in acetone

MeO-PEG-CH_2-CH_2-Br, (2 kDa, 1 g ca 0.5 mmol) and resveratrol (0.25 g, 1.09 mmol) were dissolved in acetone (20 mL). K_2CO_3 (0.8g, 5.79 mmol) was added to the solution and the mixture was heated at 60^0 C for 24 hours in the dark. At the end of the reaction, mixture was analysed by HPLC.

3.2.2.2 Potassium carbonate mediated etherification in DMF

The reaction conditions were optimized to achieve maximum PEG conjugation by varying the amount of K_2CO_3 and solvent DMF. The reactions that were attempted are described as below.

A) MeO-PEG-CH_2-CH_2-Br, (Mw 2 kDa, 0.2 g, ca 0.2 mmol) and resveratrol (0.05g, 0.219 mmol) were dissolved in DMF (2 mL). K_2CO_3 (0.04 g, 0.289 mmol) was added to solution and mixture was stirred at room temperature for 24 hours in dark. At the end of the reaction, mixture was analysed by HPLC.

B) MeO-PEG-CH_2-CH_2-Br, (Mw 2 kDa, 1 g, 0.5 mmol) and resveratrol (0.15g, 0.657 Mmol) were dissolved in DMF (5 mL). K_2CO_3 (0.15g, 1.08 Mmol) was added to solution and mixture was stirred at room temperature for 24 hours in dark. At the

end of the reaction, the mixture was added to DCM (50 mL), solution was filtered to remove potassium bromide produced in the reaction and also unreacted K_2CO_3. Filtrate was extracted with 0.1M HCl (50 mL) solution to remove traces of KBr and K_2CO_3. The extraction process was repeated 2 more times and DCM layer was evaporated in vacuo to yield off white to slightly brown product (500 mg, 50%).

C) MeO-PEG-CH_2-CH_2-Br, (Mw 2 kDa, 1g, 0.5 mmol) and resveratrol (0.15g, 0.657 mmol) were dissolved in of DMF (5 mL). K_2CO_3 (0.25 g, 1.81 mmol) was added to solution and mixture was stirred at room temperature for 24 hours in dark. At the end of the reaction, the mixture was added to DCM (50 mL), solution was filtered to remove potassium bromide produced in the reaction and also unreacted K_2CO_3. Filtrate was extracted with of 0.1M HCl solution (50 mL) to remove traces of KBr and K_2CO_3. The extraction process was repeated 2 more times and DCM layer was evaporated in vacuo to yield off white to slightly brown product (500 mg, 50%). The resveratrol-PEG ether with MeO-PEG-Br Mw 5 kDa was also prepared with the same procedure.

3.2.3 ^{1}H NMR analysis of polymer conjugates

^{1}H NMR analysis was performed on a Bruker AVN400 spectrometer (400.1 MHz for ^{1}H, Bruker, Australia) with spectra referenced to solvent signals. The HPLC analysis was performed to qualitatively characterize the extent of conjugation.

3.2.4 HPLC monitoring of conjugate synthesis

HPLC method described in chapter 4, was used to qualitatively analyse or monitoring the completion of the reaction. The same method was also used for quantitative analysis of conjugate samples in buffer and plasma stability studies, and also for the analysis of plasma samples in preliminary pharmacokinetic studies. Briefly, the method employed a gradient elution with solvent A, buffer pH-7.0 with triethyl amine-formic acid (0.05 and 0.2% v/v in water) and solvent B, methanol. A 30 minute linear gradient was programmed as follows: 0 min 5% B, 4 min 20% B, 7 min 20% B, 16 min 55% B, 18 min 55% B, 18.5 min 95% B, 23 min 95% B,and 24 min 5% B. The flow rate was maintained at 1mL/min. The injection volume was fixed at 50 µL. An analysis was performed using an Agilent HPLC-1100 series (Agilent Technologies, , USA). The fluorescence detection with excitation at 320 nm and emission at 400 nm was used to monitor the analyte.

3.2.5 Stability of resveratrol-PEG ether conjugates in physiological relevant buffers

The stability of the resveratrol polymer conjugate was evaluated at different pH buffers, 4.5 (0.1 M acetate buffer) and 7.4 (0.1M phosphate buffer), to mimic the lysozomal and the blood pH. The resveratrol -PEG ether conjugate was incubated in two buffers at concentration of 10 mg/mL at 37^0C; the samples were withdrawn at 0, 1, 2, 4, 6 and 24 hours and were diluted with water/acetonitrile (50:50) and analysed by HPLC.

3.2.6 Stability of RSV-polymer conjugates in Rat plasma

The resveratrol -PEG ether, 10 mg/mL was incubated in Wistar rat plasma at 37^0C. The aliquots of plasma (0.1 mL) were withdrawn at regular intervals and mixed with acetointirle (0.9 mL). The mixture was centrifuged at 10000 RPM for 5 minutes. The 0.1 mL of supernatant was diluted to 10 mL with an acetonitrile/water mixture and analysed by HPLC.

3.2.7 *In vitro* antioxidant activity of resveratrol and its polymer conjugates

Antioxidant activity of resveratrol and PEG ether conjugates was assessed based on their ability to scavenge free radical DPPH. Details of procedure are mentioned in chapter 2, section 2.2.6.7.

3.2.8 *In vitro* metabolism studies of resveratrol and its polymer conjugates

The metabolism of resveratrol and its polymer conjugates was carried out with rat liver microsomes in presence of UDPA. Details of study given chapter 2, section 2.2.6.6. In addition, metabolism studies were also conducted with microsomes in presence nicotinamide adenine dinucleotide phosphate **(**NDPH) regenerating system to check the possibility of polymer detachment from resveratrol by demethylation.

3.2.9 Preliminary pharmacokinetic study of resveratrol- PEG ether polymer conjugates in Wistar rats following intravenous administration

Prelminary pharmacokinetic studies were conducted as per the procedure described in chapter 2. Briefly, 26 mg/mL of resveratrol-PEG solutions were prepared in water

and a dose of 2 mg/Kg body weight given to rats intravenously. The blood was collected at 0.083, 0.5, 1, 2, 4 and 8 hours into EDTA coated tubes. The blood samples were immediately placed on ice. The blood samples were then immediately transferred into a centrifuge maintained at 4^0C. The plasma was separated by centrifuging at 12000 rpm for 12 minutes. The plasma was then mixed with an equal volume of acetonitrile and centrifuged again for 12 minutes. The supernatant was collected and analysed by HPLC. The standard calibration curve for each resveratrol-PEG tested for pharmacokinetic studies was constructed in plasma, by spiking stock solution of conjugate (in acetonitrile) into plasma. Subsequents dilutions were performed with plasma to get series of solutions. The plasma samples were then mixed with equal volme of acetonitrile to precipitate proteins. Supernatant collected after centrifugation were analyzed by HPLC. This standard calibration curve was utilized for calculating the resveratrol-PEG content in the plasma samples from rats dosed with resveratrol -PEG ether. The resveratrol plasma concentration-time profiling was performed as per descriptions in chapter 2. All animal experiments were approved by the Animal Ethics Committee of Curtin University.

3.3. RESULTS AND DISCUSSION

3.3.1 Synthesis of Resveratrol-PEG ether conjugates

The synthesis of resveratrol -PEG ether conjugates were achieved by the modification of the procedure used for the synthesis of small chain alkyl ethers of resveratrol using alkyl halides in the presence of mild base K_2CO_3 with different solvents [15, 16]. Initially synthesis of resveratrol-PEG ether was attempted using PEG-Br by adopting a procedure reported by Jiang for the synthesis of resveratrol aliphatic ligand, in which reactions were carried out in acetone at 60^0C with K_2CO_3 [16]. The reaction did not yield any desired product as indicated by the HPLC analysis. This could be due to the slow reaction rate, and similar results with low yield of 14% were reported for etherification of resveratrol with small chain alkyl halide. As

expected, the reactions would progress much slower with long chian alkyl halides. After adopting and modifying the procedure reported for synthesis of 4'-(4-O-chlorobutyl)resveratrol[15] , a favourable conjugation was achieved with improved yield.. Our experiment results demonstrated that the amount alkali K_2CO_3 in the reaction mixture played an important role in etherification. The extent of conjugation was increased as the concentration of alkali K_2CO_3 was increased in the reaction mixture. This was based on a comparison of the normalised HPLC area of the new peak corresponding to resveratrol -PEG ether appearing at 21.5 minutes. The area of peak was low when 0.2 mM of K_2CO_3 was used, and it gradually increased, and reached the highest when K_2CO_3 was at 1.81 mM and DMF was used as the solvent.. In addition to concentration of alkali K_2CO_3, solvent type was identified as critical. For instance, when very high concentration of K_2CO_3 (5.79 mM) was employed in the reaction with acetone as the solvent, there was no significant conjugation. This could be due to the solvent effect as K_2CO_3 is more soluble in DMF. It can be concluded that DMF provides a favourable environment for the abstraction of protons from resveratrol by K_2CO_3. The reaction schemes are shown in Figure 3.1 and HPLC data in Tabl-3.1.

The NMR analysis of the final product showed that the product is a mixture of different PEGylated resveratrol species, i.e resveratrol PEGylated at different positions. The **4'-PEG-RSV** was a major product (ca 60%) with **3-PEG-RSV**(ca 30%) and a minor **3,4'-di(PEG)-RSV** (ca 10-15%). Although the extent of di-substituted resveratrol was slightly more than that observed for resveratrol-PEG esters, 4'-PEG-RSV is still a major product. The substitution pattern observed here was slightly different from that reported for 4'-(4-O-chlorobutyl) resveratrol and 4'-aliphatic lingands [10, 12], where substitution at 4' of the position of resveratrol was reported. However the procedures included column separation and minor products could have been separated. The modest substitution at 3 positions could be due to an excess of K_2CO_3 in the reaction mixture. Although K_2CO_3 was a mild base, it was reported to abstract protons at the 4' position of resveratrol. Its high concentration in the mixture could have lead to an abstraction of the proton from 3 positions of resveratrol.

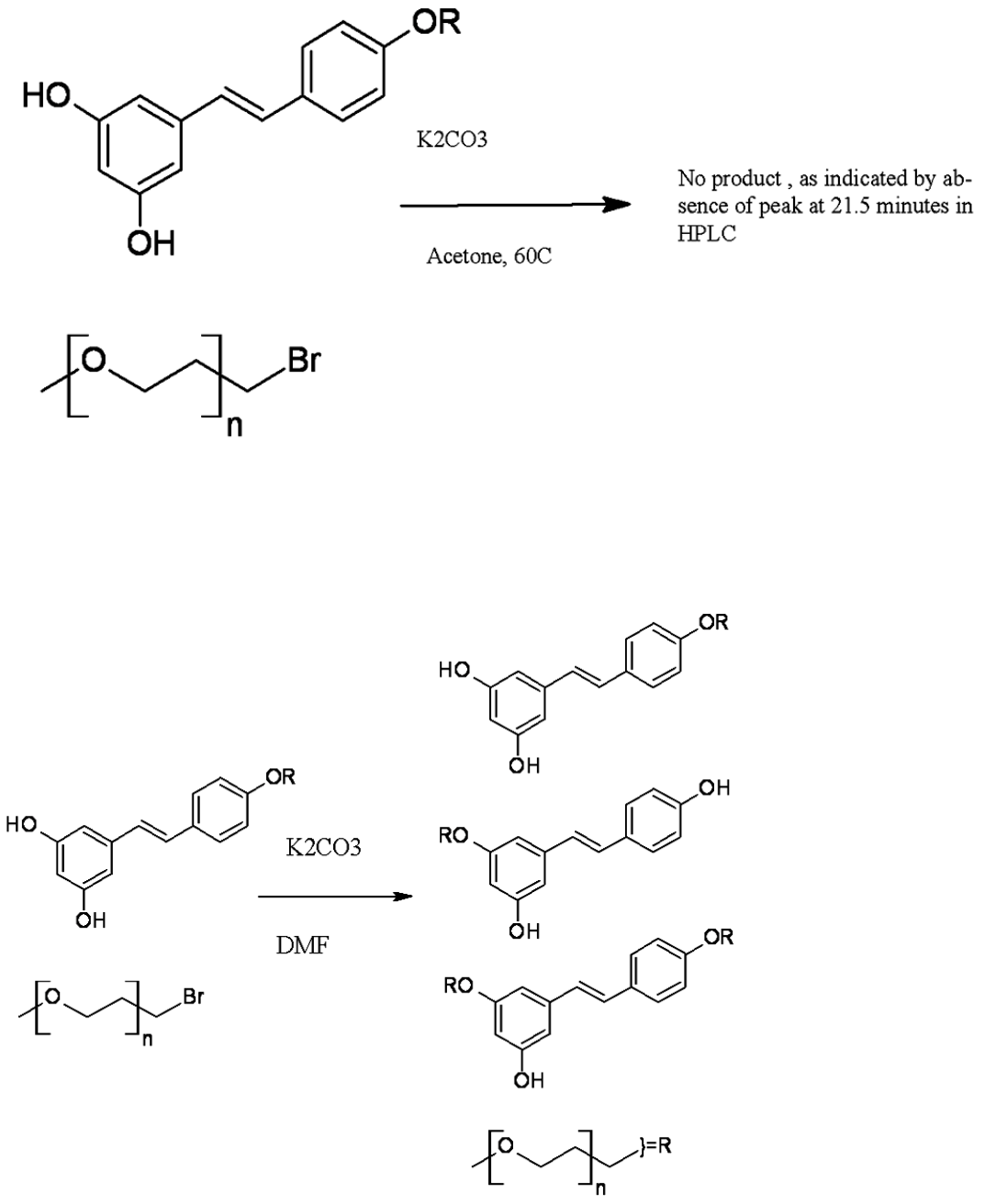


Figure 3.1: Synthesis of resveratrol-PEG ethers

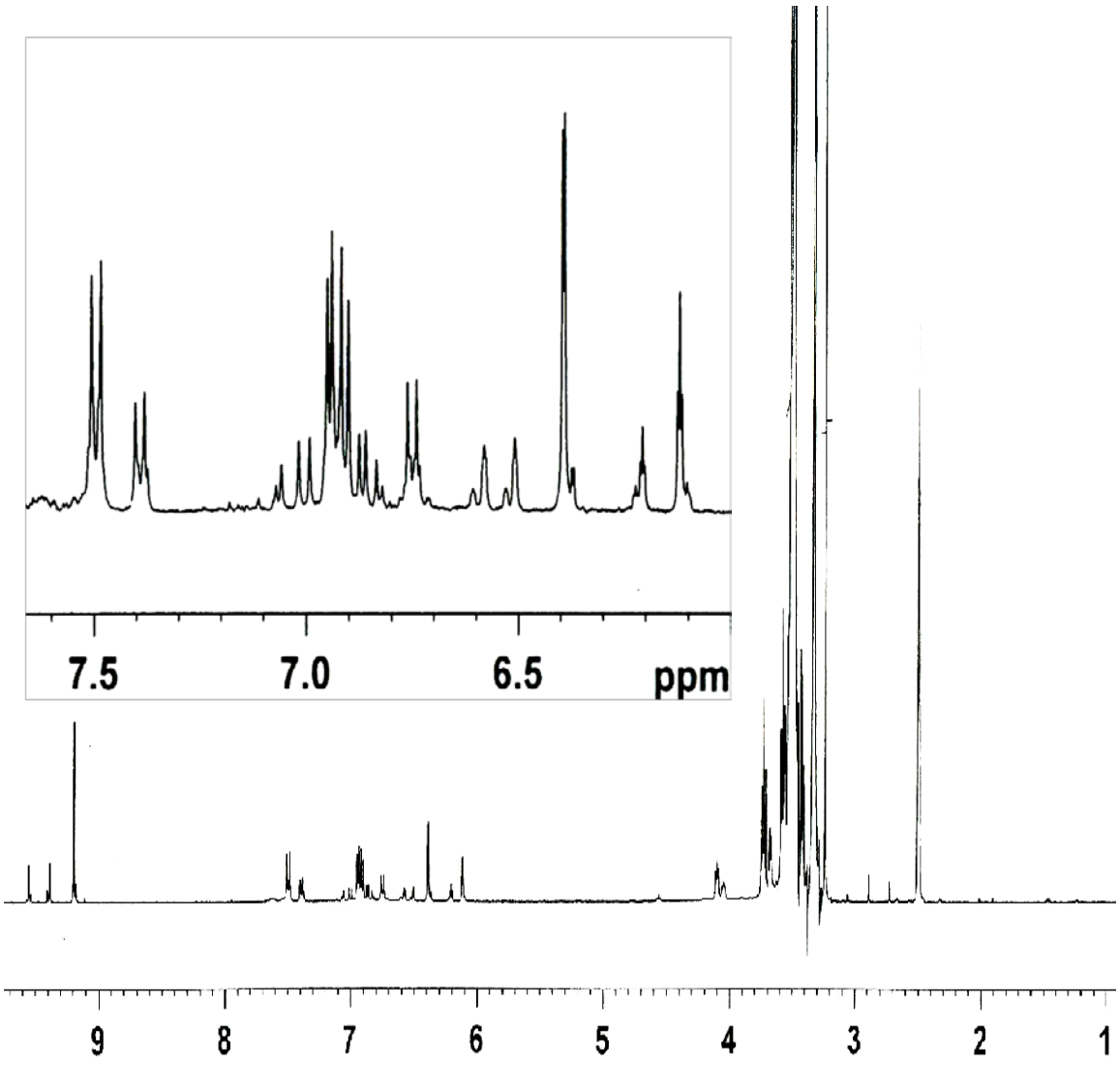


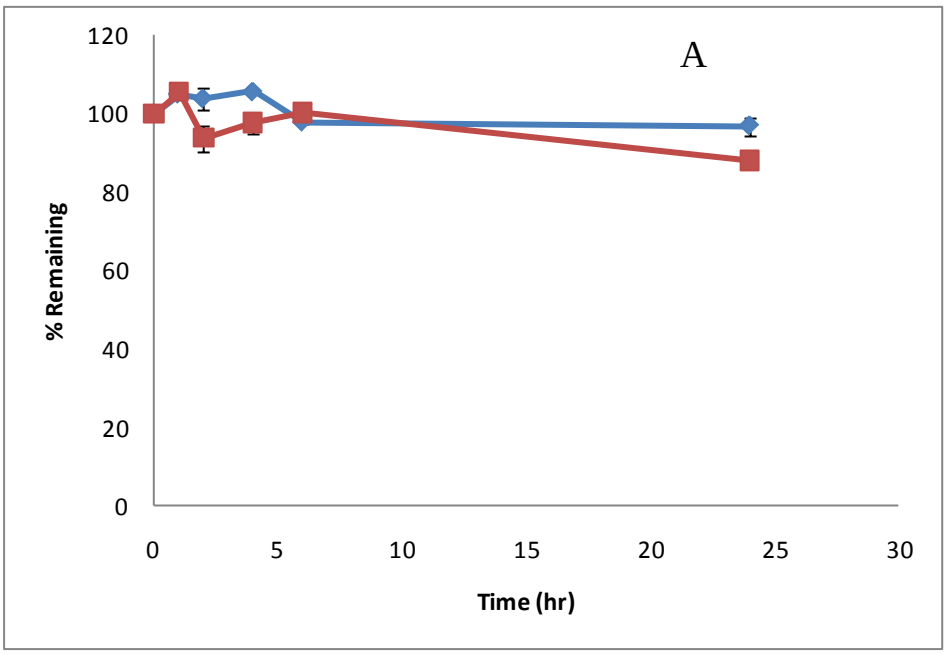
Figure-3.2 NMR spectrum of RSV-PEG 2K, insert expanded version of aromatic region.

Table 3.1: Monitoring of reaction completion in synthesis of PEG-RSV ether conjugates based on the area of conjugate peak in HPLC

Reaction conditions (Amount of K_2CO_3 and solvent used)	**Area of peak at 21.5 minutes (100 µg/mL of conjugate product in acetonitrile)**
5.79 mM- Acetone	Peak not detected
0.289 mM- DMF	188
1.08 mM- DMF	829
1.81 mM- DMF	1735

3.3.2 Stability of resveatrol-PEG ether conjugates in buffers and plasma

Stability profiles of resveratrol-PEG conjugate in buffers and plasma are shown in Figure 3.3. The results indicate that resveratrol-PEG ether conjugate was stable in both buffers pH 4.5 and 7.4 and plasma. There was no hydrolysis detected in the plasma. These results were expected, as the ether bond is very stable and does not undergo hydrolysis either in the buffer or plasma. The conjugates were freely soluble (>10mg/mL) in the water and the buffers tested.


A
120
100
80
60
40
20
0
% Remaining
0
5
10
15
20
25
30
Time (hr)

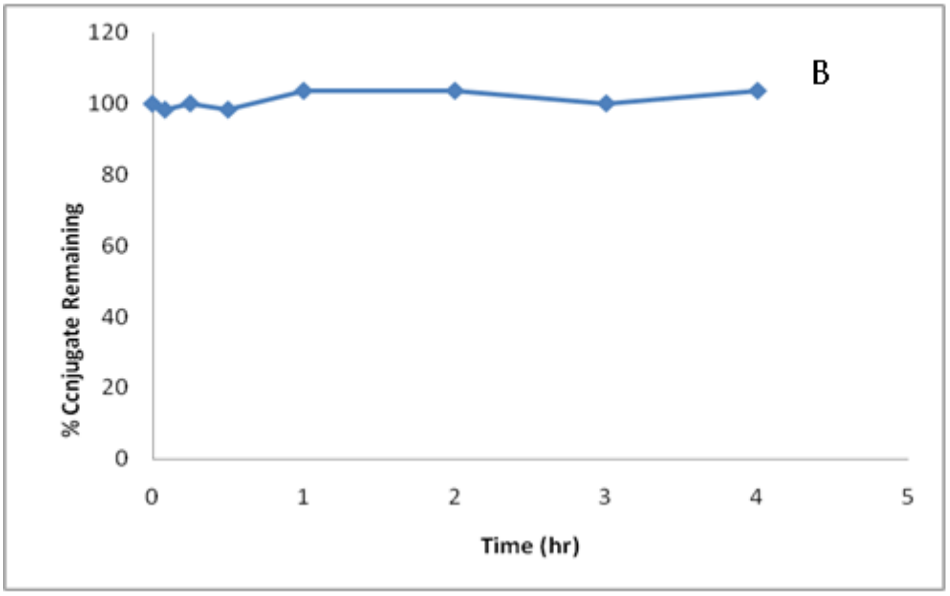

B
120
100
80
60
40
20
0
% Ccnjugate Remaining
0
1
2
3
4
5
Time (hr)

Figure 3.3: Stability of resveratrol-PEG ether 2K in A: in buffers.Data presented as mean±SD (n=3) ; red curve is at pH 4.5; blue curve at pH 7.4 . B: in rat plasma. Data presented as mean (n=2).

3.3.3 *In vitro* antioxidant assay of resveratrol-PEG ether conjugates

The *in vitro* antioxidant profile is shown in Figure3.4. The IC-50 value of resveratrol was found to be 65 µM, which is in close agreement with the value reported by Fauconneau et al[17]. The IC 50 value for resveratrol-PEG was 2190 µM (equal to 219 µM of resveratrol). The profile also shows that PEGylated resveratrol is less active. There was an almost 3.5 fold increase in IC 50 values compared to resveratrol. The decrease in antioxidant activity indicates that all the hydroxyl groups of resveratrol are involved in reaction with free radicals. Hence, blocking one or more hydroxyl groups would lead to a decrease in activity. However, the activity was not completely abolished as reported for 3, 4', 5-triemethoxy stilbene wherein all the OH groups of resveratrol were modified to methoxyl groups[18]. The activity indicated by this assay method actually was based on direct neutralization of free radicals and may not reflect actual *in vivo* activity, as resveratrol exerts *in vivo* antioxidant activity by many other mechanisms. The literature reported that resveratrol could mediate the release or production of nitric oxide, which has stronger affinity for O_2^{-}.[1] Resveratrol is believed to induce enzymes dismutase and NAD(P)H:quinine oxidoreductase, both of which scavenge the reactive oxygen species. [1, 2] Modifying of one hydroxyl group of resveratrol would not lead to a drastic decrease in these activities. Although cyclooxygenase (COX-2) inhibition of 3,3', 4,4', 5,5'-hexahydroxystilbene was much higher than resveratrol [15], the introduction of additional OH groups made the structure more susceptible to metabolic conjugation [19]. In the same report, methoxylated analogues of resveratrol did not lose COX-2 inhibitory activity, in fact, it was slightly better than the resveratrol. These observations suggest that selectively modifying one of the OH groups of resveratrol may not jeopardize the pharmacological effectiveness *in vivo*. However, blocking more than one OH of resveratrol would lead to the complete loss of certain activities, such as antioxidants[18]. To produce a successful clinical candidate, there has to be a balance of all biological properties such as activity, toxicity and metabolism. The

molecule which is best, in terms of biological activity would not necessary make it to clinical studies or the market due to other issues such as metabolism [21]. Similarly, having an unmodified resveratrol with higher activity but prone to metabolism would not be useful for clinical studies. Modified resveratrol, such as PEGylated resveratrol as discussed here with the better plasma profile would be a better choice for clinical studies despite the moderate decrease in the antioxidant activity.

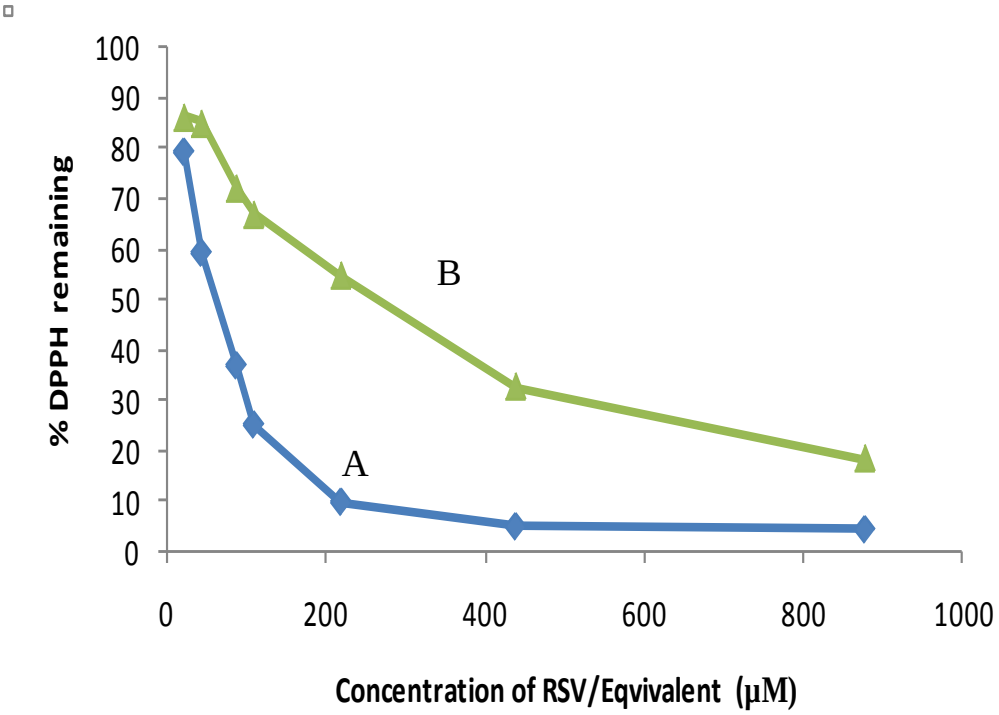


Figure 3.4: DPPH inhibition profile for antioxidant assay of A: Resveratrol B: Resveratrol-PEG ether 2 kDa. The data presented as mean±SD (n=3)

3.3.4 *In vitro* metabolism studies of resveratrol and its polymer conjugates

Resveratrol underwent rapid metabolism with microsomes in presence of UDPA. Resveratrol PEG ethers did not undergo metabolism with microsomes in presence of either UGT enzymes or NADPH regenerating system.(Data in appendix Figure-7.13)

3.3.5 Single dose preliminary pharmacokinetic study of resveratrol-PEG ether conjgates

The plasma concentration-time profile of resveratrol-PEG ether and resveratrol are presented in Figure 3.5 and the corresponding representative HPLC chromatograms in Figure 3.6. The profile indicates that resveratrol undergoes rapid elimination, and mainly by metabolism. The initial plasma concentration of resveratrol at 5 minutes was very low (1.74±0.67 µg/mL) with high intense peaks of metabolites. The plasma concentration of resveratrol declined rapidly and fell almost below the detection limits within two hours. On the contrary, the resveratrol-PEG ether 2 kDa displayed a better profile with a higher initial plasma concentration (7.24 µg/mL) and depleting more slowly than the resveratrol. The AUC total for resveratrol--PEG ether 2 kDa was 15.660 µg*h/L compared to 0.63 µg*h/L for resveratrol. The MRT for resveratrol-PEG ether 2 kDa was also higher 6.2h (1.7 h for resveratrol). Although the resveratrol-PEG displayed a significantly better profile than resveratrol, the trend still showed a rapid decline followed by stagnation after 1hour. The rapid clearance of resveratrol--PEG could be attributed to increased solubility after the PEG conjugation, which facilitates kidney filtration. The steady levels after 1 hour were initially thought to be because of **3, 4'-di(PEG)** resveratrol, which has a larger Mw (4200 KDa). The resveratrol--PEG ether 5 kDa was prepared and the pharmacokinetic profile was evaluated. Surprisingly there was no difference between the elimination patterns of resveratrol-PEG 2 and 5 kDa. Only plasma concentration at 5 minutes post dose was higher with the PEG ether 5 kDa. This suggests that an increase in Mw from 2 kDa to 5 kDa has a marginal effect on kidney filtration and a rise of Mw was not sufficient to reach the threshold limit of filtration (Serum albumin volume) [17]. Pharmacokinetic studies with radio labelled PEGs of various Mw reported by Reza Mehvar [22] clearly showed that a significant effect of Mw on clearance of PEG can be observed only after Mw of 20 kDa. The low but steady plasma levels of RSV-PEG after 1 hour could be due to the intrinsic distribution and elimination properties of PEG. It was possible that the special conformational arrangements of **3, 4'-di (PEG)** may give a more hydrodynamic volume, in turn lead to slow kidney filtration [9]. Fee et al. have studied the size comparison between proteins PEGylated with linear and branched chain PEGs [23]. They concluded that branched PEG would have a higher viscosity and larger radius than proteins PEGylated with linear PEGs of same molecular weight. Based on the same reasoning, disubstituted resveratrol with a PEG of 2 kDa would appear like branched PEG and with a butterfly like shape. This would eventually give rise to

more hydrodynamic volume than monosubstituted resveratrol- with PEG of 5 kDa. However these findings need further confirmation from studies using suitable analytical techniques.

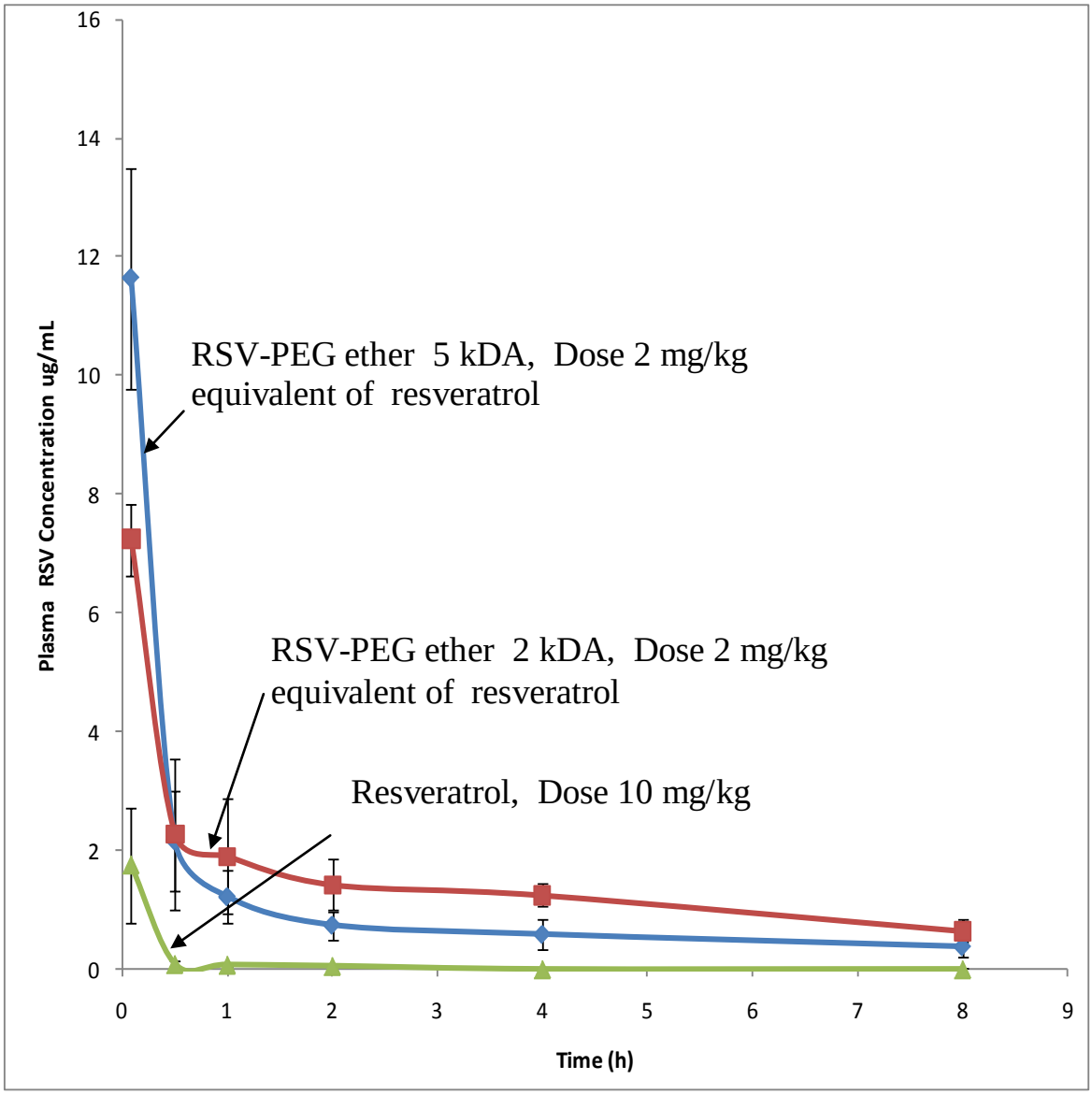


Figure 3.5: Pharmacokinetic profile of resveratrol and Resveratrol-PEG ether.Data presented as mean±SD (n=6 RSV-PEG 5 kDa, 5 for RSV-PEG 2 kDa and 4 for Resveratrol)

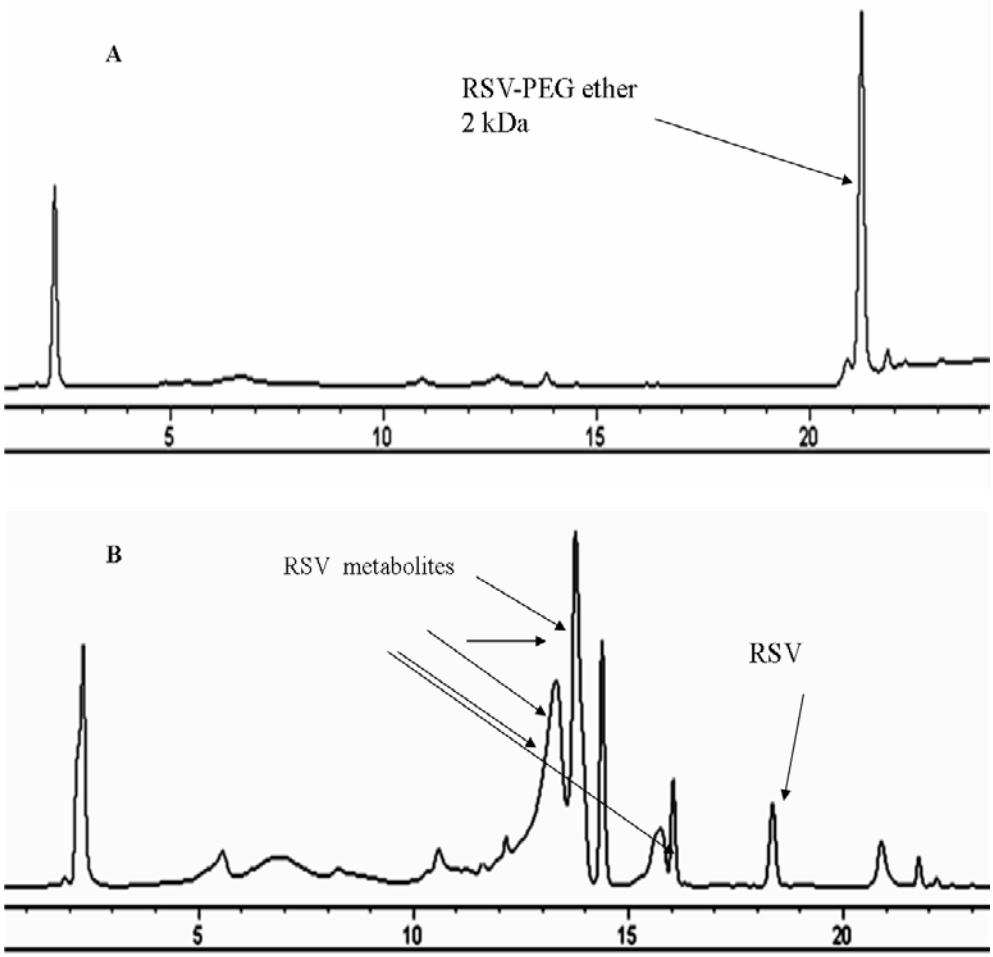

A
RSV-PEG ether
2 kDa
5
10
15
20
B
RSV metabolites
RSV
5
10
15
20

Figure 3.6: HPLC chromatogram of A: resveratrol-PEG (RSV-PEG) ether sample from rat at 5 minutes post dose (2mg/kg resveratrol- equivalent), B: resveratrol-(RSV) plasma sample from a rat at 5 minutes post dose(10mg/kg).

3.4 REFERENCES

1. Das DK, Maulik N. Resveratrol in cardioprotection: a therapeutic promise of
   alternative medicine. Mol Interv. 2006; 6(1):36-47.
2. Le Corre L, Chalabi N, Delort L, Bignon YJ, Bernard-Gallon DJ. Resveratrol and breast cancer chemoprevention: molecular mechanisms. Mol Nutr Food Res. 2005; 49(5):462-71.
3. Wenzel E, Soldo T, Erbersdobler H, Somoza V. Bioactivity and metabolism of trans-resveratrol orally administered to Wistar rats. Mol Nutr Food Res. 2005; 49(5):482-94.
4. Gao X, Xu YX, Divine G, Janakiraman N, Chapman RA, Gautam SC. Disparate in vitro and in vivo antileukemic effects of resveratrol, a natural polyphenolic compound found in grapes. J Nutr. 2002; 132(7):2076-81
5. Marier JF, Vachon P, Gritsas A, Zhang J, Moreau JP, Ducharme MP.Metabolism and disposition of resveratrol in rats: extent of absorption, glucuronidation, and enterohepatic recirculation evidenced by a linked-rat model. J Pharmacol Exp Ther. 2002; 302(1):369-73.
6. Walle T, Hsieh F, DeLegge MH, Oatis JE, Jr., Walle UK. High absorption but very low bioavailability of oral resveratrol in humans. Drug Metab Dispos. 2004; 32(12):1377-82.
7. Miksits.M, Wlcek K, Svoboda M, Kunert O, Haslinger E, Thalhammer T, Szekeres T, Jäger W. Antitumor Activity of Resveratrol and its Sulfated Metabolites against Human Breast Cancer Cells. Planta Med 2009; 75(11): 1227-1230.

8. Prentis RA, Lis Y, Walker SR. Pharmaceutical innovation by the seven UK-owned pharmaceutical companies (1964-1985) Br. J. clin. Pharmac. (1988), 25, 387-396.
9. Veronese FM, Monfardini C. Stabilization of substances in circulation. Bioconjugate Chem, 1998,9,418-450.
10. K. Hoste, K. De Winne, E. Schacht. Polymeric prodrugs. Int.J.Pharmaceutics, 277 (2004) 119–131.
11. Greenwald RB, Choe YH, Mcguire J, Conover CD. Effective drug delivery by PEGylated drug conjugates. Adv.Drug.Del.Rev, 2003,55, 217-250.
12. Veronese FM, Morpurgo M. Bioconjugation in pharmaceutical chemistry. II Farmaco, 1999,54, 497-516.
13. Biasutto L, Marotta E, Mattarei A, Beltramello S, Caliceti P, Salmaso S, Bernkop-Schnurch A, Garbisa S, Zoratti M, Paradisi C. Absorption and Metabolism of Resveratrol Carboxyesters and Methanesulfonate by Explanted Rat Intestinal Segments *Cell. Physiol. Biochem.24 (*2009) 557-66.
14. Greenwald RB, Pendri A, Bolikal D. Highly water soluble taxol 7-polyethylene glycol carbamates and carbonates. J.Org.Chem. 1995,60,331-336.
15. Biasutto L, Mattarel A, Marotta E, Bradaschia A, Sassi N, Gabisa S, Zorattit M, Paradisi C.Development of mitochondria-targetted derivatives of resveratrol. Biorg.Med.Chem.Letters, 2008, 18, 5594-5597.
16. Jiang YL. Design, synthesis and spectroscopic studies of resveratrol aliphatic acid ligands of human serum albumin. 2008,16 640-6414.
17. Ffauconneau B, Waffo-teguop P, Huguet F, Barrier L, Decendit A, Mjzrillont JM. Comparative study of radical scavenger and antioxidant Properties of phenolic compounds from vztzs vznzfera Cell cultures using in *vitro* tests. Life.Sci.1997, 61, 2103-2110.
18. Lee HJ, Seo JW, Lee BH, Chung KH, Chi DY. Synthesis and radical scavenging activities of resveratrol derivatives. Biorg.Med.Chem.Letters,2004,14,463-466.
19. Murias M, Handler N, Erker T, Pleban K, Ecker G, Saiko P, Szekeres T, Jager W. Resveratrol analogues as selective cyclooxygenase-2

inhibitors:synthesis and structure-activity relationship. Biorg.Med.Chem.2004,12,5571-5578.

20. Bebbington D, Dawson CE, Gaur S, Spencer J. Prodrug and Covalent Linker Strategies for the Solubilization of Dual-Action Antioxidants/Iron Chelators.
Bioorg.Med.Chem.Letters 2002,12, 3297-3300.
21. Beresford AP, Selick HE, Tarbit MH. The emerging importance of predictive ADME simulation in drug discovery. DDT 2002,2,109-116.
22. Mehvar R. Modulation of the pharmacokinetics and pharmacodynamics of proteins by polyethylene glycol conjugation.J.Pharm.Pharmaceut Sci 2000. 3, 125-136.
23. Fee CJ. Size comparison between proteins PEGylated with branched and Linear poly(ethylene glycol) molecules. Biotechnol Bioeng 2007 ; 98 : 725 -31

CHAPTER 4

DEVELOPMENT OF HPLC METHOD FOR SIMULTANEOUS ANALYSIS OF RESVERATROL, RESVERTROL-POLYMER CONJUGATES AND DETECTION OF RESVERATROL METABOLITES

4.1. INTRODUCTION

One of the most effective approaches to reduce or even prevent drug metabolism and improve therapeutic efficacy is to use a polymeric prodrug or conjugate strategy. This involves protecting the drug's reactive sites with cleavable groups to mask the native drug. This approach has been utilised previously to reduce the rate of resveratrol metabolism [1]. Using the same principle, various resveratrol polymeric ester conjugates have also been synthesized in this project for the same purpose (chapter 2). In order to evaluate these systems and to conduct pharmacokinetic profiling of these products, a simple but highly sensitive HPLC method is essential for detection and quantification of native resveratrol, polymeric conjugates as well as metabolites. There are a few reports on resveratrol quantitification by HPLC using UV, fluorescence and electrochemical detection [2-5]. The sensitive LC-MS methods reported for resveratrol [6] bear some limitations for analysis of resveratrol-polymer conjugates, for instance, there are mass detection limits for polymer conjugates with higher molecular weights and lack of specificity. Since there would be narrow molecular weight differences between drug-polymer conjugates and polymers, the specific detection and quantification would not possible unless there is adequate separation between them. Moreover the polymers are composed of a mixture of polymer with various molecular weights, narrow difference would not be noticed. Hence the simple and sensitive HPLC method is essential of resveratrol-polymer conjugates. So far, there is no reported method that author is aware of which can detect both resveratrol and its polymeric conjugate simultaneously in a sensitive manner. Developing a single HPLC method which can be applied for separation and quantification of number of resveratrol-polymer conjugates would be advantage.

4.1.1 Objectives of study

Objectives of the study described in this chapter were to develop a highly sensitive HPLC method for separation and quantification of resveratrol and its polymer conjugates and also to detect the metabolites. In the current investigation, a systematic approach was used to develop a highly sensitive HPLC assay with fluorescence detection to simultaneously analyse resveratrol and its polymer conjugates, and also to allow the detection of metabolites. Efforts were also made to increase the fluorescence intensity of compounds of interest and to study the effect of solvent modulation on the sensitivity of the HPLC method. All the analytes such a resveratrol, resveratrol-polymer conjugates and metabolites, which were detected in HPLC, were also qualitatively analyzed by LC-MS or LC-MS/MS. The LC-MS method was developed as such that compounds of interest were separated and eluted with retention times matching closely with the HPLC assay using the same column. This HPLC method was initially developed and validated for MeO-PEGN-Succ-RSV 2 kDa conjugate, later it was applied for quantification of other resveratrol-polymer conjugates. Hence, this chapter focuses on the methodology and mechanistic studies that lead to development a highly sensitive HPLC method for MeO-PEGN-Succ-RSV 2 kDa, as well as the extensive validation of the method. The same method was applied for analysis of other polymer conjugates and some of these data have been presented in previous chapters (chapter 2 &3) and some in this chapter.

4.2.EXPERIMENTAL

4.2.1 Materials and Reagents

Trans-Resveratrol was purchased from Shanghai DND Pharm-Technology Co., Inc., Shanghai, China, with 99% purity. Resveratrol-polymer conjugates: MeO-PEGN-Succ-RSV, 2 kDa, 20 kDA, MeO-PEGO-Succ-RSV, 2 kDA, MeO-PEG-PLAO-Succ-RSV, 2 kDa and 6.5 kDa, Resveratrol-PEG ether 2 kDa and 5 kDa were synthesized and characterised in house (Chapter 2 section 2.2.4 and 2.2.5 and Chapter 3, section 3.2.2.2). Acetonitrile, triethylamine and formic acid were analytical grade and purchased from Sigma (Sydney, Australia). They are used

without any further purification. The HPLC column, ODS C-18 150 x 4.6 mm, 5 µm was purchased from Altech (Sydney, Australia)

4.2.2 Fluorescence and UV Scanning.

Excitation and emission wavelengths for resveratrol were determined using a Varian Carry Eclipse spectroflurometer (Varian Australia, Australia). A resveratrol standard solution of 1 µg/mL was prepared in methanol and scanned for excitation and emission. UV absorption maxima were determined using a Hewlett Packard 8452A, UV-VIS spectrophotometer (Hewlett Packard, Germany). Based on these results, excitation and emission were set at 320 nm and 400 nm respectively for HPLC and independent fluorescence analyses. Solutions of 1 µg/mL of resveratrol and its equivalent of MeO-PEGN-Succ-RSV were prepared separately in buffer pH 7.4, and buffer-methanol mixtures. The fluorescence intensity of these solutions was recorded at 400 nm with an excitation wavelength of 320 nm. The results obtained from these experiments were then used to develop solvent gradient parameters for HPLC.

4.2.3 Reverse-phase HPLC Chromatography

The HPLC analyses were performed using an Agilent Technologies chromatographic system (Santa Clara, CA, USA) HPLC-1100 series with dual detection (G1321A Fluorescence and G1315B UV-DAD), G1311A pump, G1316A column oven, G1329A autosampler and Chemstation B.04.02 software. An Altech Apollo C18 (150 x 4.6 mm, 5 µm) column was employed.

The HPLC gradient system consisted of solvent A, buffer pH 7.0 with triethyl amine-formic acid (0.05 and 0.2% v/v in water) and solvent B, methanol. The 30 minute linear gradient was programmed as follows: 0 min 5% B, 4 min 20% B, 7 min 20% B, 16 min 55% B, 18 min 55% B, 18.5 min 95% B, 23 min 95% B, 24 min 5% B. The flow rate was maintained at 1mL/min and the injection volume was 50 µL.

4.2.3.1 Linearity

A series of standard solutions of resveratrol in methanol/solvent A mixture (50:50) were prepared with concentrations ranging from 5 ng/mL to 1000 ng/mL. Similarly MeO-PEGN-Succ-RSV standard solutions were prepared using acetonitrile/water mixture (50:50). Standard solutions were analysed by the HPLC in triplicate.

4.2.3.2 System and method precision

Standard solutions of resveratrol and MeO-PEGN-Succ-RSV at one concentration were injected six times and standard deviation and %RSD were calculated. Similarly the method precision was determined by injecting six different standard solutions of same concentration.

4.2.3.3 Accuracy

Standard solutions of resveratrol and MeO-PEGN-Succ-RSV at three different concentrations (2, 4, and 6 µg/mL eqvivalent to 200, 400 and 600 ng/mL of RSV) were analyzed in triplicate. The accuracy was calculated by comparing the concentration determined against that of prepared.

4.2.3.4 Intra-day and inter-day repeatability

Standard solutions of resveratrol and MeO-PEGN-Succ-RSV 2 kDa at three different concentrations (2, 4, and 6 µg/mL eqvivalent to 200, 400 and 600 ng/mL of RSV) were injected two times a day. Standard solutions prepared separately on day 2 were also analysed. The concentrations were determined from calibration curve and standard deviation and %RSD were calculated.

4.2.3.5 Stability at room temperature

Resveratrol solutions at three different concentrations were stored in HPLC vials at room temperature under dark. Samples were analyzed after 3 days.

4.2.3.6 Stability at refrigeration

Resveratrol and MeO-PEGN-Succ-RSV standard solutions at three different concentrations were stored at 2-8ºC and analyzed by HPLC.

4.2.3.7 Recovery of resveratrol and MeO-PEGN-Succ-RSV from a mixture

The solutions containing a mixture of resveratrol and MeO-PEGN-Succ-RSV at three different concentrations were prepared in water/acetonitrile mixtue (50:50). They were analysed by HPLC and their recovery was calculated based on concentration obtained from analysis against the prepared concentration.

4.2.3.8 Recovery of resveratrol from plasma

The recovery of resveratrol from rat plasma was determined by spiking blank plasma with the resveratrol standard to obtain the final concentration of 50, 100, 500 and 1000 ng/mL in duplicates. Plasma samples were then mixed with an equal volume of acetonitrile to precipitate proteins, and the supernatants obtained after centrifugation were analysed by HPLC. Recoveries were calculated by comparing the measured concentration to the spiked concentration of resveratrol.

4.2.4 LC-MS Analysis

To confirm the detection and quantification of the MeO-PEGN-Succ-RSV conjugates performed using HPLC-fluorescence/UV detection, an LC-MS method was developed to analyze qualitatively the MeO-PEGN-Succ-RSV conjugates. LC-MS analysis was performed using a Shimadzu LC-MS system LC-MS 2020 (Kyoto, Japan). The System consisted of a binary pump 20AD, auto injector SIL 20 AC HT, column oven CTO 20A, PDA detector SPD M 20A, mass analyser MS2020 and communication unit CBM 20A. Data were acquired, processed and analysed using chromatographic software LABSOLUTIONS®.

The LC gradient system consisted of solvent A, water and solvent B, methanol. The 30 minute linear gradient was programmed as follows: 0 min 5% B, 4 min 20% B, 7 min 50% B, 12 min 50% B 16 min 55% B, 18 min 95% B, 23 min 95% B, 24 min 5% B. The flow rate was maintained at 0.4 mL/min. The same column used for HPLC analysis was used in LC-MS. Samples were analysed with injection volume of 40 µL. The optimised ESI conditions employed included a DL temperature at 300^0C, interface temperature at 350^0C. The heat block temperature was maintained at 220^0C. The drying gas, nitrogen flow, was maintained at 10L/minute. Other mass parameters were set as per auto-tuning results. The scan mode was used to record the abundance of ions corresponding to resveratrol in negative mode and PEG in positive mode. The scan was performed with a mass range of 200-2000 Daltons. Similar LC parameters were used to detect metabolites using LC-MS/MS system (Applied Biosystems, USA) with multiple reaction monitoring (MRM) at 307/227 and 403/227 as reported in the literature [6].

4.2.5 Detection of resveratrol metabolites in rat plasma by HPLC.

An aqueous solution of 2 mg/mL resveratrol in 10% w/v of hydroxyl propyl-β-cyclodextrin was prepared and sterilized by filtration. An aliquot of 0.3 mL of this solution was injected intravenously into Wistar rats *via* the tail vein. Blood was collected from the tail vein at 10 min, post dose, by vein puncture. The plasma was separated by centrifugation at 10,000 RPM using a mini spin, Effendorf centrifuge (USA). The 100 µL of plasma was mixed with 200 µL of acetonitrile to precipitate proteins. The supernatant was collected by centrifugation. The obtained supernatant was mixed 1:1 with water and analysed by HPLC as described in section 4.2.3. All animal experiments were approved by the Animal Ethics Committee of Curtin University.

4.3. RESULTS AND DISCUSSION

4.3.1 Fluroscence and UV analysis

Analysis of a solution of resveratrol in methanol using fluorescence spectroscopy indicated that the maximum excitation and emission wavelengths were 320 nm and 400 nm respectively. UV absorbance spectra for methanol solutions of both resveratrol and the MeO-PEGN-Succ-RSV conjugate mixture indicated absorption maxima at 307 nm for both solutions. The UV and fluorescence spectrum is shown in Figure 4.1.

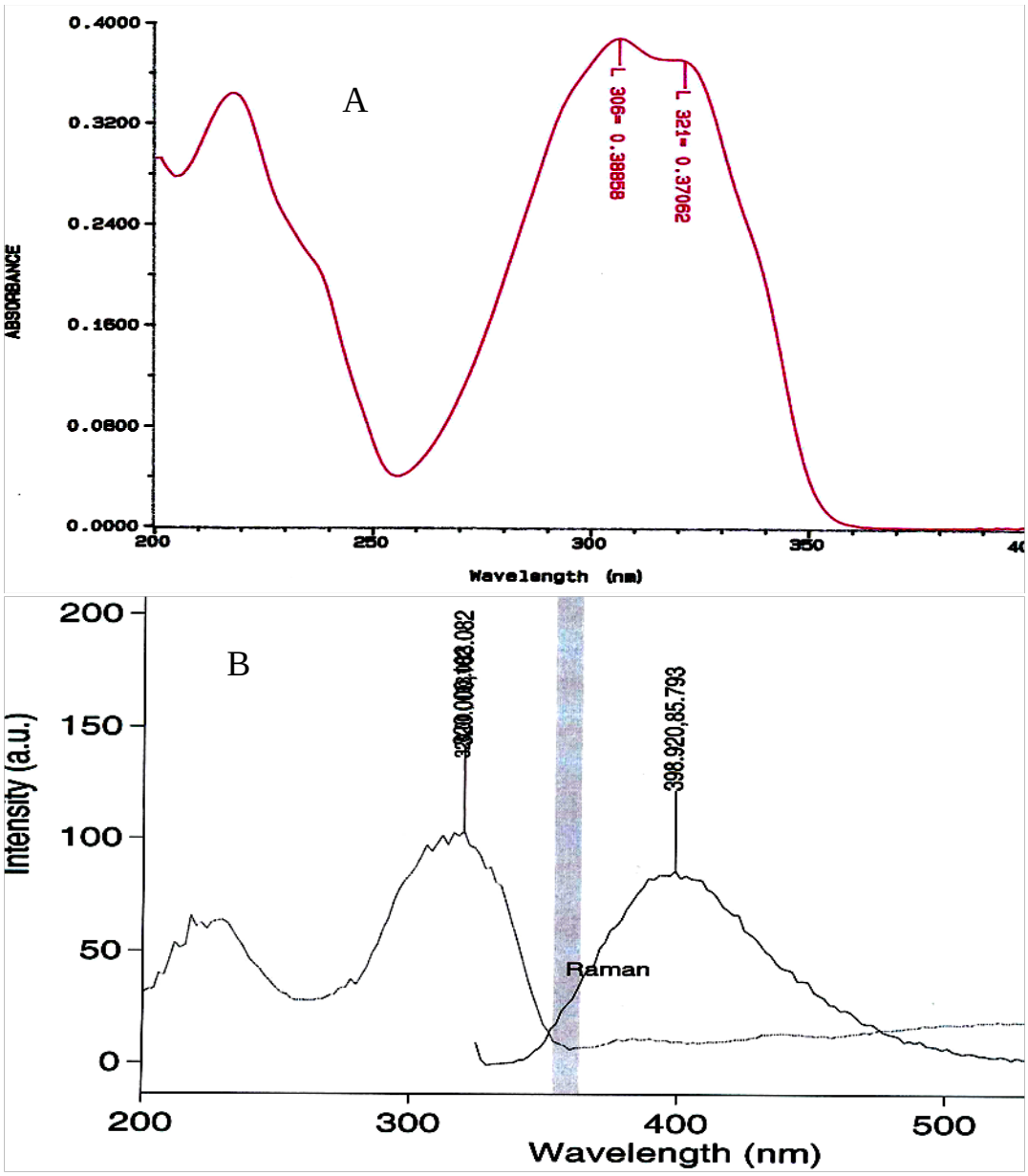


Figure 4.1: A: UV spectrum of resveratrol in methanol. B: fluorescence spectrum of resveratrol in methanol.

In order to evaluate the effect of solvent on the fluorescence intensity, a series of fluorescence intensity measurements were recorded for resveratrol and MeO-PEGN-Succ-RSV with the solutions prepared from mixture of methanol and an aqueous buffer. The results indicated an increasing fluorescence intensity of the MeO-PEGN-Succ-RSV conjugates as the methanol concentration increased (Figure 4.2). The highest fluorescence intensity was achieved when the methanol concentration was 100%. The fluorescence intensity of the MeO-PEGN-Succ-RSV conjugates in each

of the solvent mixtures was always higher than that of the equivalent amount of resveratrol. The fluorescence intensity of resveratrol itself did not show significant change as the solvent polarity changed, this was in stark contrast to the MeO-PEGN-Succ-RSV conjugate which displayed a drastic increase in fluorescence as the methanol concentration increased above 60% (Figure 4.2). To validate these findings, further HPLC analyses were performed with isocratic elution comprising of 75% and 80% methanol with all other parameters unchanged. The analysis was performed for MeO-PEGN-Succ-RSV solution 300ng/mL (equivalent to 30ng/mL of resveratrol). Results clearly indicated fluorescence intensity increased with methanol concentration in the mobile phase (Figure 4.3). The increase in intensity was almost double for every 5% increase in methanol concentration. These results are in agreement with the fluorescence measurements performed with varying concentration of methanol. Although isocratic runs looks provide better LOQ due to more flat baseline, at this high concentration of methanol, separation cannot be achieved between RSV, metabolites and polymer conjugate. These findings lead us to design the HPLC mobile phase in such a way that the analytes of interest eluted at high methanol concentrations, where the fluorescence intensity would be at a maximum.

The increase in fluorescence intensity of resveratrol after the coupling to PEG could be attributed to increased hydrophilicity and dispersing effect of PEG, which in turn may prevent intermolecular hydrophobic interactions between resveratrol molecules. Our findings are in close agreement with those reported for phthalocyanines by Jian et al [7]. The group reported that fluorescence of phthalocyanines was rarely observed in aqueous media due to aggregation and hydrophobic interactions. Upon conjugation with PEG the fluorescence intensity was greatly increased, presumably due to reduced hydrophobic interactions and an increased 'monomeric' structure which was attributed to the dispersant properties of PEG. The increase in fluorescence intensity as a function of methanol concentration, for the MeO-PEGN-Succ-RSV conjugates can be attributed to the change in the environment of the resveratrol. In the low methanol solutions the PEG would interact strongly with water and the resveratrol units would presumably aggregate to avoid contact with the aqueous phase. This process would lead to significant intermolecular interactions, leading to fluorescence quenching [8]. At high methanol concentrations presumably the resveratrol interacts more strongly with the solvent and less aggregation occurs.

At this point the PEG may prevent intermolecular interactions between resveratrol molecules leading to an enhanced fluorescence. Similar findings were reported for butadiene in water-dioxane mixture [9].

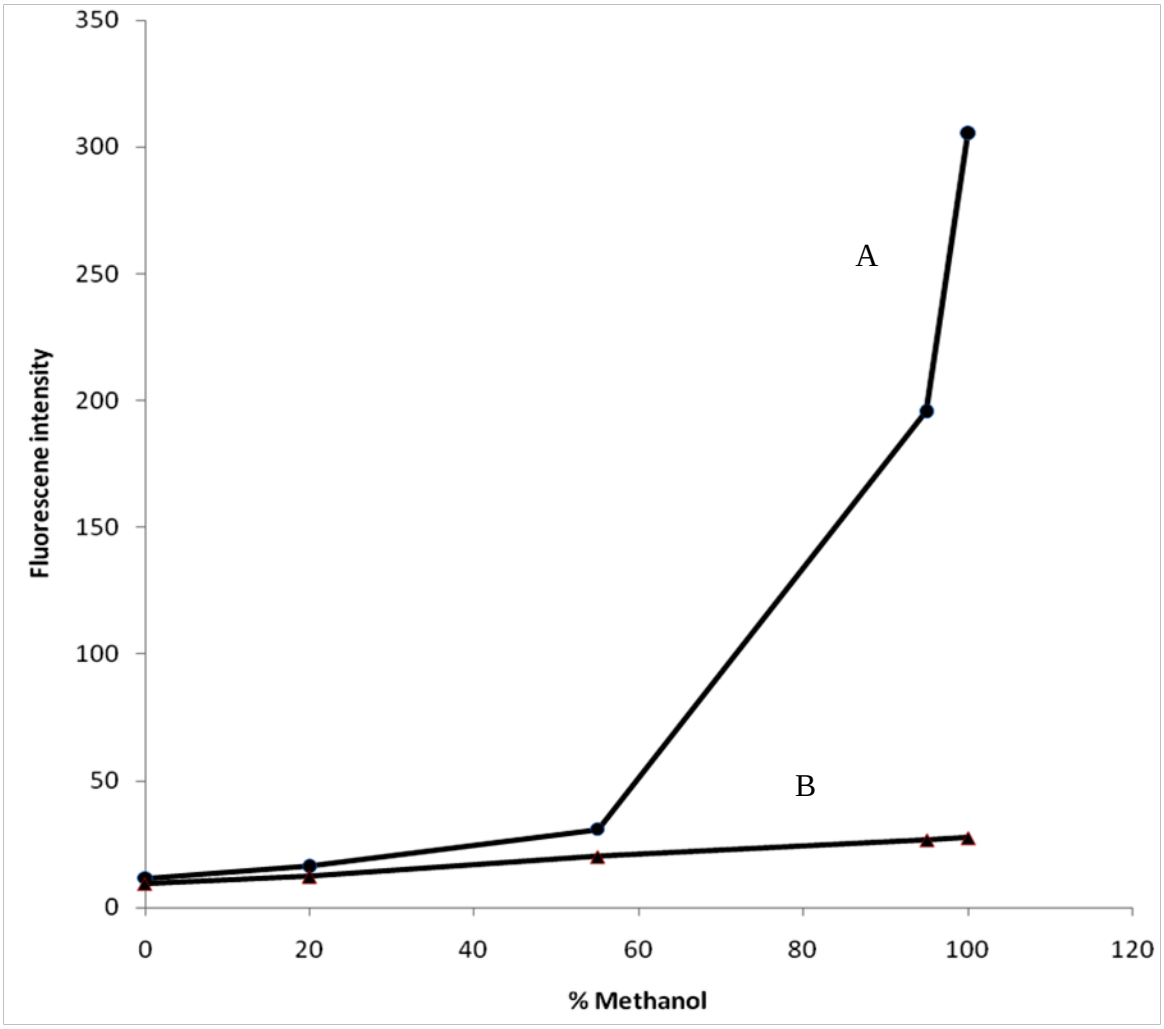


Figure 4.2: Effect of solvent composition on fluorescence of A-MeO-PEGN-Succ-RSV and B-resveratrol, analyses were performed at excitation wavelength of 320 and emission wavelength of 420 with resveratrol concentration of 1 µg/mL and MeO-PEGN-Succ-RSV concentration equivalent to 1µg/mL of resveratrol.

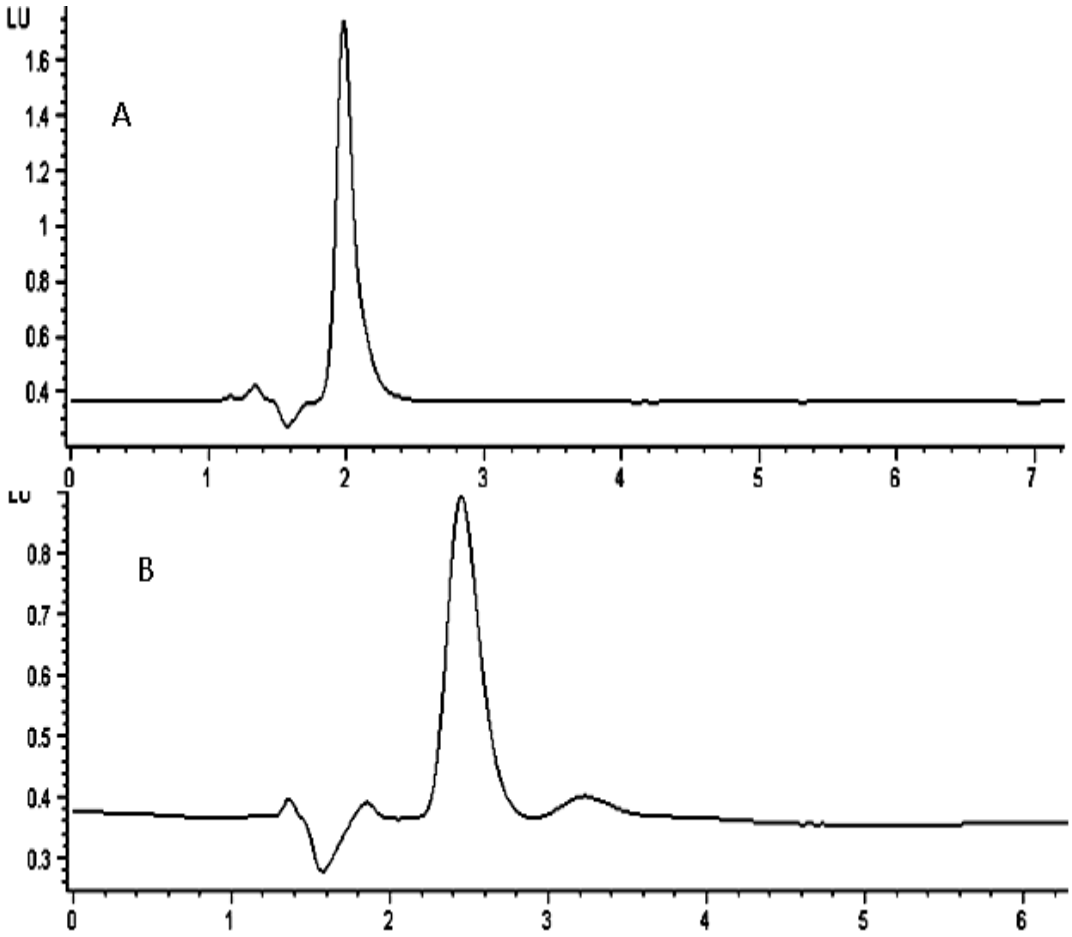


Figure 4.3: Effect of methanol concentration on MeO-PEGN-Succ-RSV (RSV-PEG) florescence peak intensity. A: Isocratic chromatogram with 80% methanol; B: isocratic chromatogram with 75% methanol.

4.3.2 Chromatographic analysis

The increased fluorescence intensity associated with the MeO-PEGN-Succ-RSV conjugates permitted development of a sensitive HPLC assay method for analysis of resveratrol and the PEG conjugates because the UV detection can be replaced by more sensitive fluorescence detection, particularly for analysis of PEG conjugates of resveratrol. Representative chromatograms for resveratrol and MeO-PEGN-Succ-RSV conjugate with both UV and fluorescence detection are shown in Figure 4.4. In the absence of fluorescence detection, quantification of MeO-PEGN-Succ-RSV conjugates at low concentration would have proved more difficult, demanding either alternative techniques or hydrolysis of PEG esters back to resveratrol in order to

quantify it accurately. Such an approach is more complex and time consuming. In our HPLC assay method, the solvent gradient is such that MeO-PEGN-Succ-RSV conjugates elute when the methanol concentration in the mobile phase is 95% and at that particular concentration, the fluorescence intensity of the MeO-PEGN-Succ-RSV conjugates is close to a maximum. This ultimately results in the high sensitivity of this method. Such a sensitive method is very useful for physiochemical, metabolic and pharmacokinetic profiling of these compounds, where dealing with quantification of resveratrol, its PEG conjugates at low concentration is often anticipated. Moreover, this HPLC quantification is based on the fluorescence properties of resveratrol in resveratrol-PEG conjugate, the method is more specific and there would not be any interference from un-cojugated polymer even if co-eluting. This overcomes limitations of LC-MS method which is based on mass number of analytes. The narrow difference between molecular weights of PEG and RSV-PEG would complicate specific detection and quantification individual species if there is no adequate separation between two. In addition. some LC-MS instruments have mass detection limit up to 2000, the polymer conjugates with Mw >2000, which do not undergo fragmentation could not be analyzed with such LC-MS instruments.

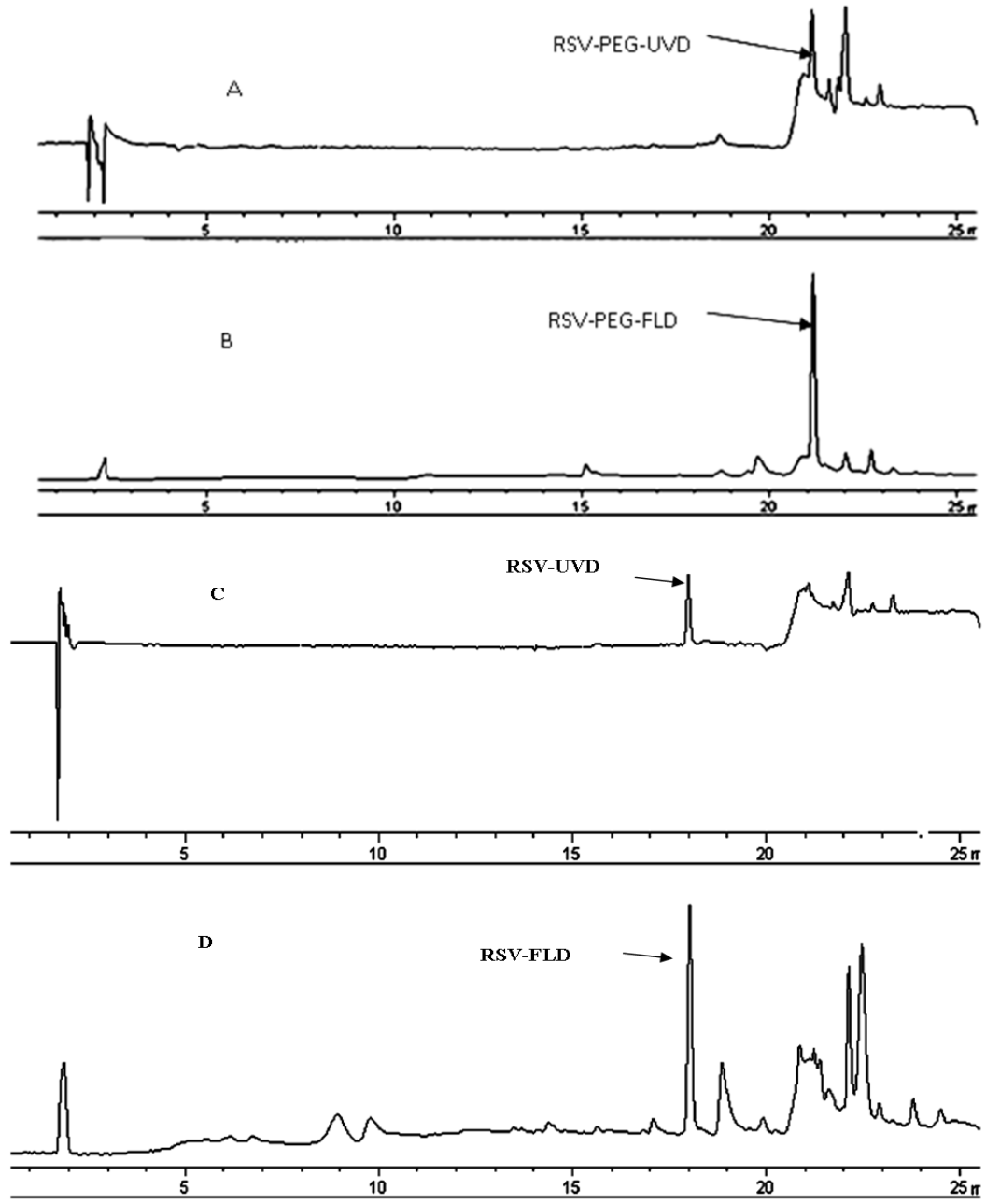


Figure 4.4: HPLC chromatograms of resveratrol and MeO-PEGN-Succ-RSV. A: MeO-PEGN-Succ-RSV (RSV-PEG ~30ng/mL of resveratrol) chromatogram with UV detection (UVD): B: MeO-PEGN-Succ-RSV (~30ng/mL of resveratrol) chromatogram with Fluorescence detection (FLD); C: resveratrol 30ng/mL chromatogram with UV detection: D: resveratrol 30ng/mL chromatogram with Fluorescence detection.

4.3.3 Linearity, accuracy, recovery and limit of quantification

Linearity curves were obtained for resveratrol and the MeO-PEGN-Succ-RSV conjugates by plotting mean area of peaks against concentration. Table 4.1 lists the linear regression coefficient, linear range, intercept, slope, limit of detection (LOD), limit of quantification (LOQ) and Table 2 show the recovery results obtained from HPLC analysis of resveratrol and the MeO-PEGN-Succ-RSV conjugate mixture. LOD and LOQ were calculated using three and ten times the standard deviation of baseline noise and the calibration curve obtained using peak height against concentration. LOQ was found to be 5 ng/mL for resveratrol and 300 ng/mL for its PEG conjugate (~30 ng/mL of resveratrol). The slight increase in LOQ value for MeO-PEGN-Succ-RSV is due to the baseline shift and subsequent noise at the retention time due to the change of gradient. In the absence of this, the LOQ may be improved. However, any move to reduce the methanol concentration to achieve a flatter base line at the time of MeO-PEGN-Succ-RSV elution would risk the sensitivity as was observed in fluorescence and HPLC analysis with lower methanol concentration. On the other hand, increasing the methanol concentration earlier than this may lead to poor separation between resveratrol, metabolites and PEG conjugate. The full validation results for resveratrol and MeO-PEGN-Succ-RSV analyses are given in Table 4.3 and 4.4. The HPLC data for other polymer conjugates such as MeO-PEGN-Succ-RSV 20 kDa, MeO-PEGO-Succ-RSV 2 kDa. MeO-PEG-PLAO-Succ-RSV, 2 kDa and 6.6 kDa are presented in Table 4.5-4.6. Only limited data such as linearity, LOD and LOQ were obtained for other polymer conjugates in this project whereas a full set of method validation data was obtained for MeO-PEGN-Succ-RSV, 2 kDa conjugate to demonstrate the feasibility and application of the assay method.The representative chromatograms of polymer conjugates are shown in Figure 4.5-4.8.

Plasma recovery data were all within acceptable limits for bioanalytical method validation [10]. The average recovery of resveratrol in plasma was 92-102% (SD 0.1-5.3%), suggesting high reproducibility and extraction efficiency of the method. The recovery of MeO-PEGN-Succ-RSV in plasma was not done because it broke down very quickly in the plasma.. For the same reason, plasma recoveries for MeO-PEGN-

Succ-RSV 20 kDa and other ester conjugates were not performed because they hydrolyzed very quickly back to resveratrol in the plasma. The MeO-PEG-CH_2CH_2-O-RSV ether conjugates were stable in plasma and recovery data was presented in chapter 3. For all samples, a simple protein precipitation method using acetonitrile was used to extract analytes from plasma because of the sensitivity of the analytes to light and heat.

Table 4.1: HPLC calibration parameters for resveratrol and MeO-PEGN-Succ-RSV 2 kDa.

Sample	**Slope**	**Intercept**	**R^2**	**Linear Range[1] (ng/mL)**	**LOQ (ng/mL)**	**LOD (ng/mL)**
Resveratrol	0.0751	0.3699	1.0000	5-1000	5	2.5
MeO-PEGN-Succ-RSV, 2 kDa	0.044	1.1534	0.9999	300-1000*	300 (3µg/mL for UV)	100

*The value is equivalent to 30-1000 ng/mL of resveratrol

[1] Concentration range for the caliberation curve.

Table 4.2: Recovery of HPLC analysis of resveratrol (RSV) and MeO-PEGN-Succ-RSV 2 kDa conjugates (RSV-PEG) in a mixture.

Concentration (µg/mL)		**Recovery (%)**	
(RSV)	**(RSV-PEG)***	**RSV-PEG**	**RSV**
0.1	1	107.0(±3.2)	100.0(±5.2)
0.5	5	108.5(±2.4)	103.0(±1.9)
1	10	109.0(±0.7)	107.0(±3.6)

*10 µg/mL RSV-PEG is equivalent to 1 µg/mL RSV

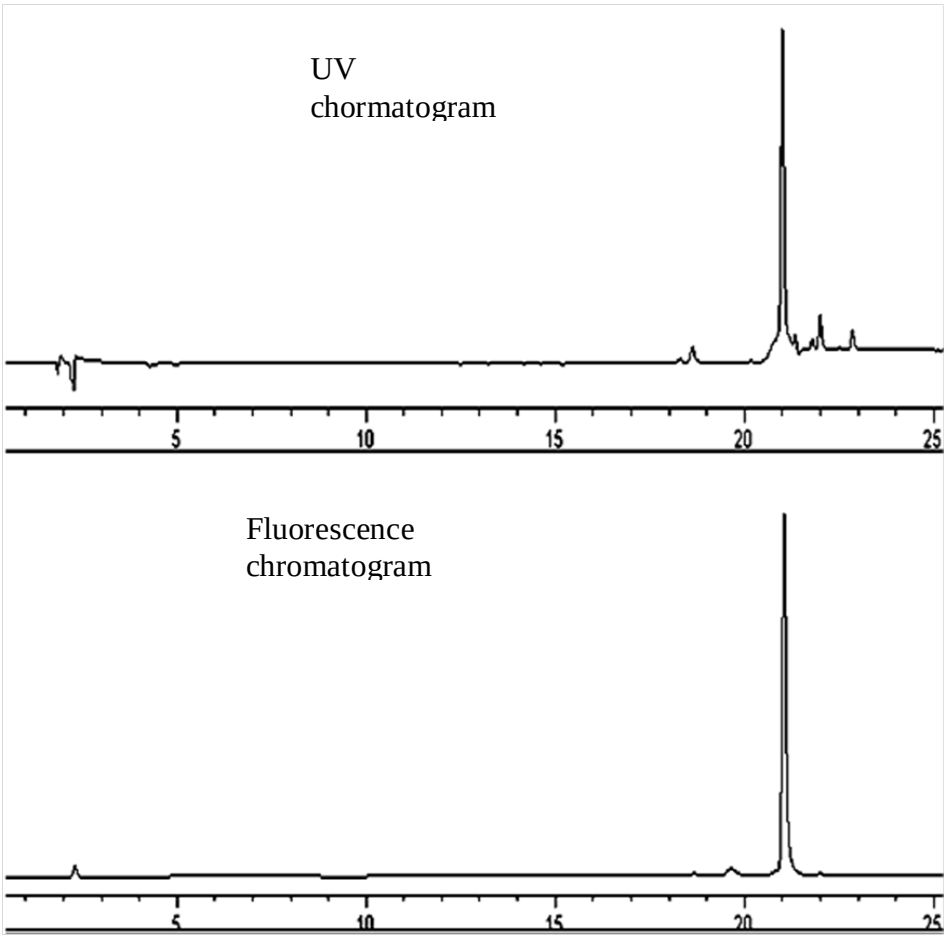


Figure 4.5: HPLC chromatogram of MeO-PEGN-Succ-RSV 20K, 100 µg/mL

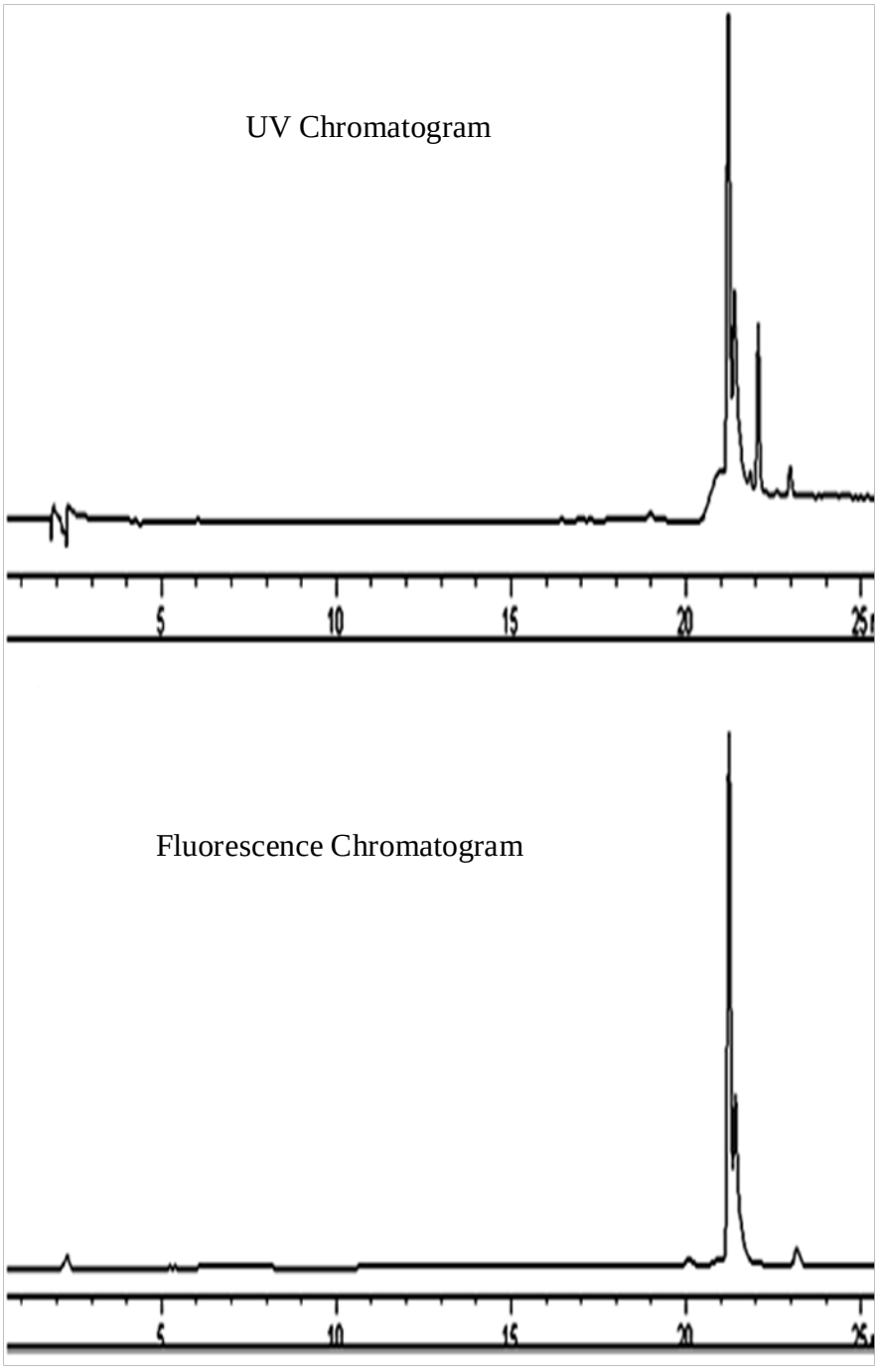


Figure 4.6: MeO-PEGO-Succ-RSV 2 kDa, 10 µg/mL

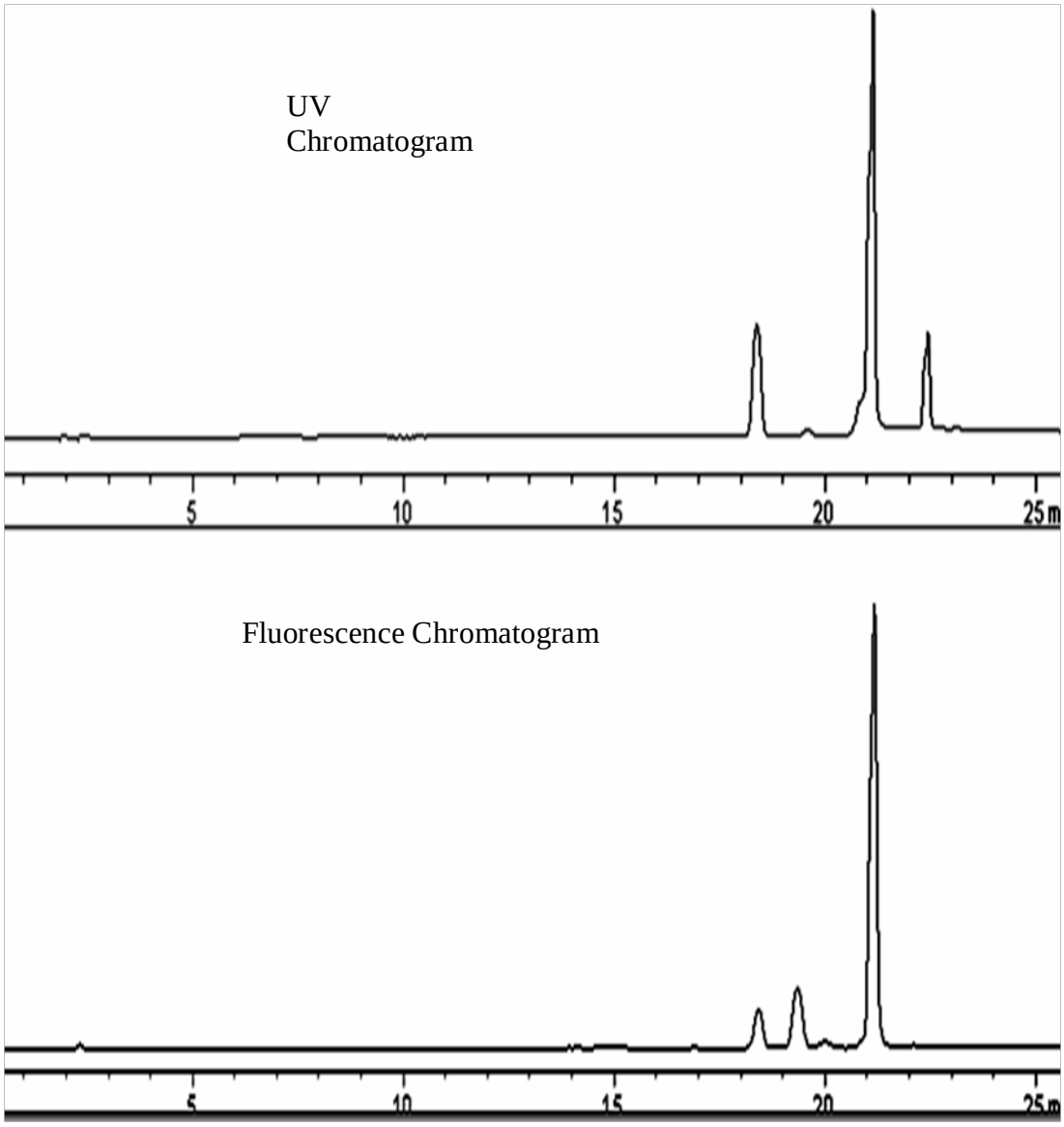


Figure 4.7: MeO-PEG-O-CH2-CH2-RSV ether 5 kDa, 50 µg/mL

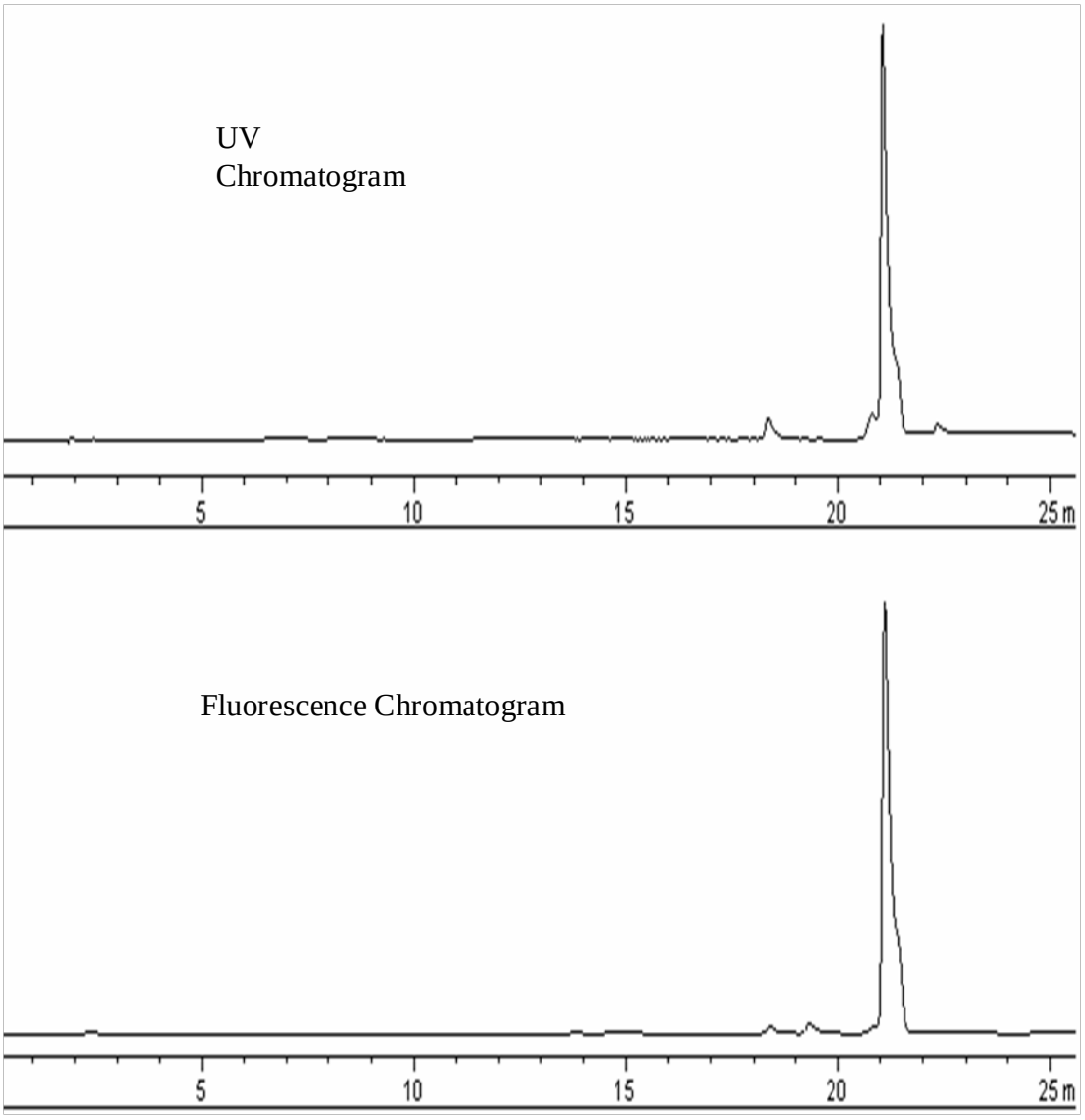


Figure 4.8: HPLC chromatogram of MeO-PEG-O-CH2-CH2-RSV 2 kDa, 25 µg/mL

Table 4.3: Validation parameters for resveratrol

Study	**Concentration (ng/mL)**	**Results ng/mL (SD)**	**%RSD**
System precision	1000	1000.4 (2.02)	0.20
Method precision	1000	1005.0(13.00)	1.30
Interday	50	50.2(0.58)	1.16
	100	101.4(0.54)	0.53
	1000	1000.4(2.02)	0.20
Intraday	50	49.5(0.61)	1.24
	100	101.0(0.64)	0.63
	1000	1005.5(13)	1.30
Accuracy (%)	50	99.0(1.23)	1.24
	100	101.0(0.64)	0.63
	1000	100.5(1.31)	1.30
Stability at room temperature, 3 days	50	48.2(1.25)	1.25
	100	102.0(1.13)	1.10
	1000	1032.1(11.38)	1.10
Stability at 2-8 ^{0}C, 3 days	50	50.3(0.13)	0.26
	100	103.3(0.53)	0.51
	1000	1019.9(0.65)	0.06

Table 4.4: Validation data for MeO-PEGN-Succ-RSV, 2 kDa

Study	**Concentration (µg/mL)**	**Results µg/mL (SD)**	**%RSD**
System precision	6	6.0(0.00)	0.11
Method precision	6	6.0(0.12)	2.14
Interday	2	1.9(0.00)	0.18
	4	4.0(0.05)	1.41
	6	6.0(0.00)	0.11
Intraday	2	1.9(0.01)	1.02
	4	3.9(0.03)	1.00
	6	6.0(0.12)	2.14
Accuracy (%)	2	95.9(0.18)	0.18
	4	101.5(1.48)	1.46
	6	100.1(0.11)	0.11
Stability at 2-8 ^{0}C, 24 hrs	2	1.9(0.03)	2.07
	4	3.8(0.03)	1.00
	6	5.8(0.02)	0.50

Note: 10µg of MeO-PEGN-Succ-RSV, 2 kDa is theoretically equal to 1 µg of resveratrol.

Table 4.5: Validation data for MeO-PEGN-Succ-RSV, 20 kDa

Study	**Concentration (µg/mL)**	**Results µg/mL (SD)**	**%RSD**
System precision	80	80.7(0.23)	0.29
Method precision	80	76.3(0.63)	0.83
Interday	40	38.9(0.89)	2.29
	60	61.2(1.27)	2.07
	80	80.7(0.23)	0.29
Intraday	40	38.4(0.60)	1.56
	60	57.1(0.23)	0.41
	80	76.3(0.63)	0.83
Accuracy (%)	40	97.4(2.24)	2.29
	60	102.1(2.11)	2.07
	80	100.9(0.29)	0.29
Stability at 2-8 ^{0}C, 24 hrs	40	37.3(0.86)	2.30
	60	57.3(0.37)	0.64
	80	74.6(1.9)	2.66

Note: 100µg of MeO-PEGN-Succ-RSV 20 kDa is theoretically equal to 1 µg of resveratrol.

Table 4.6: Linearity, LOQ and LOD data for various resveratrol-polymer conjugates

Sample	**Slope**	**Intercept**	**R^2**	**Linear Range* (µg/mL)**	**LOQ (µg/mL)**	**LOD (µg/mL)**
MeO-PEGN-Succ-RSV, 20 K[a]	0.0021	0.79	0.9994	5-100	8.0	2.3
MeO-PEGO-Succ-RSV, 2 K[b]	0.0300	1.94	0.9985	0.5-10	0.9	0.3
MeO-PEG-PLAO-Succ-RSV[c], 6.6K	2.2101	3.87	0.9953	20-100	19.5	5.3
MeO-PEG-PLAO-Succ-RSV, 2K[d]	26.7400	43.95	0.9979	5-100	3.2	2.2
RSV-O-PEG Ether, 2K[e]	6.4803	2.46	0.9996	5-200	5.0	0.7

*Concentration range used for the caliberation curve.

a- 100 µg/mL of conjugate is eqvivalent to 1 µg/mL of RSV

b- 10 µg/mL conjugate is eqvivalent to 1 µg/mL of RSV

c- 33 µg/mL conjugate is eqvivalent to 1 µg/mL of RSV

d- 10 µg/mL conjugate is eqvivalent to 1 µg/mL of RSV

e- 10 µg/mL conjugate is eqvivalent to 1 µg/mL of RSV

4.3.4 Analysis of MeO-PEGN-Succ-RSV and resveratrol by LC-MS

The elution of resveratrol and MeO-PEGN-Succ-RSV conjugate observed in LC-MS analysis was very similar to that achieved in HPLC, with resveratrol eluting at 17.8 minutes and MeO-PEGN-Succ-RSV at 22 minutes. LC-MS chromatogram of resveratrol showed a peak at the retention time of 17.5 minutes with m/z 227 (Mw of

resveratrol is 228) as shown in Figure 4.9B. Similarly there is a peak at retention time of 22 minutes with corresponding mass spectrum showing a series of intense mass peaks in the range of m/z 550-900 (Figure 4.9A). These mass spectrum peaks are believed to be characteristic fragmented peaks of PEG [11]. The HPLC peak with retention time of 22 minutes, suspected to be MeO-PEGN-Succ-RSV, did not show resveratrol ion in mass spectrum, but its PDA spectrum did in fact reveal a spectrum almost matching that of resveratrol, indicating the formation of MeO-PEGN-Succ-RSV structure (Figure 7.14-7.15 in Appendix). These data suggest that the new peak observed at 22 minutes is of MeO-PEGN-Succ-RSV. The findings of this experiment together with HPLC results obtained suggest that the developed HPLC and LC-MS methodology can separate, identify and accurately quantify resveratrol and MeO-PEGN-Succ-RSV.

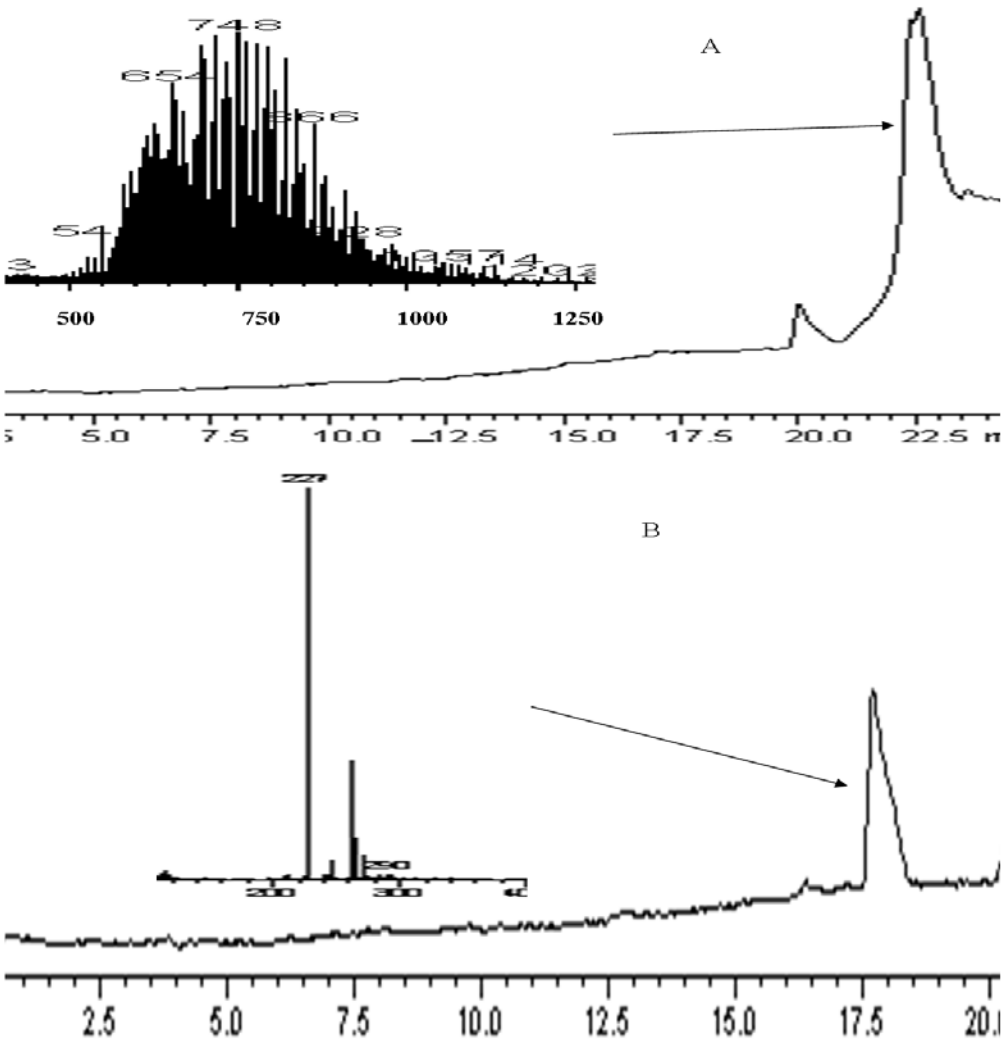


Figure 4.9: LC-MS chromatogram of A: MeO-PEGN-Succ-RSV 2000 in scan mode (insert: Mass spectrum of MeO-PEGN-Succ-RSV which corresponds to the peak at retention time of 22.5 minutes) and B-:resveratrol (insert: mass spectrum of resveratrol from the peak at 17.8 minutes)

4.3.5 Detection of resveratrol metabolites by HPLC

The present HPLC method with fluorescence detection was found to be very sensitive for metabolite detection (Figure 4.10). Peak intensities of metabolites were higher using fluorescence detection than UV detection. The five additional peaks observed in the HPLC chromatograms and are believed to be of metabolites with UV spectra matching with that of resveratrol. The LC-MS/MS monitoring of metabolites with multiple ion monitoring (MRM) at 307/227 and 403/227 revealed the presence of two monosulfates peak 1 and 4 and two monoglucuronides 2 and 3 as shown in Figure 4.10. These observations are in close agreement with those reported in literature [6]. There was a fifth peak observed in HPLC and LC-MS/MS with MRM 307/227, which could be due to di or tri sulphate conjugate of resveratrol and could have appeared in MS chromatogram after loss of one or more of the sulphate moieties. All these findings need further confirmatory studies like isolation or synthesis of resveratrol metabolites and subsequent NMR analysis to confirm exact structures. However, changing the solvent gradient in the method to maximize the intensity of MeO-PEGN-Succ-RSV did increase the likelihood of detection of of metabolites while still offered a good separation of metabolites from resveratrol and it polymer conjugates.

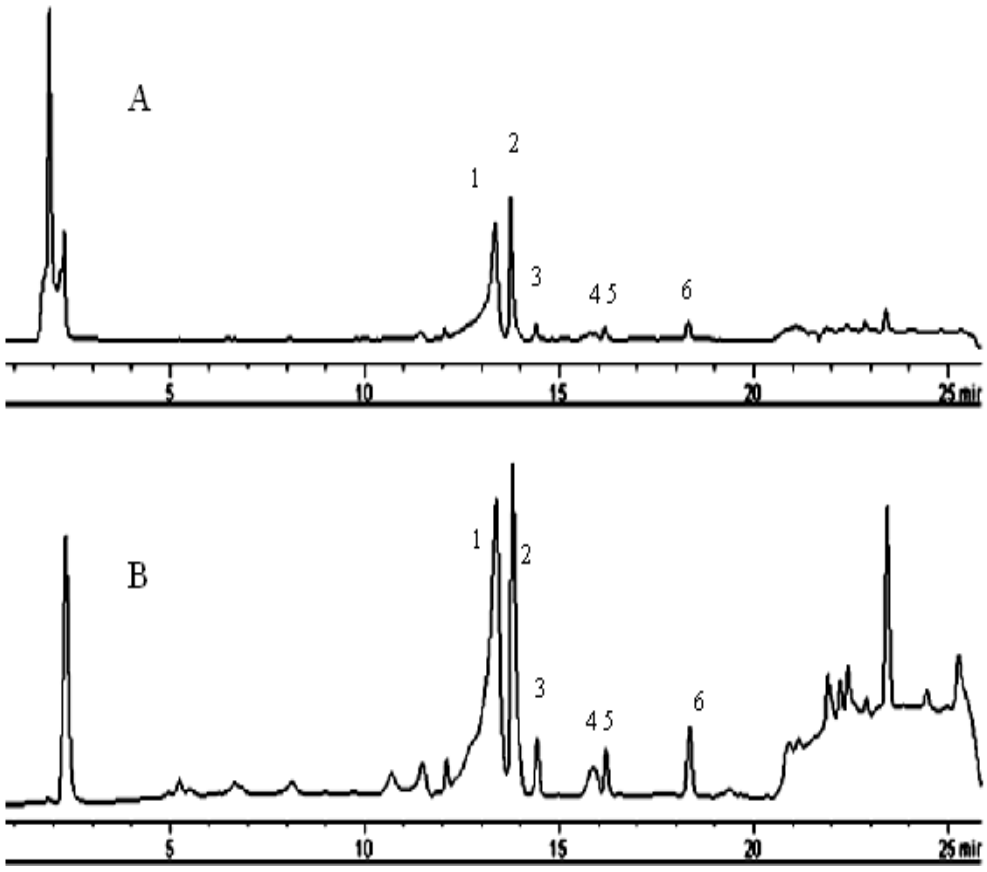


Figure 4.10 HPLC chromatograms of resveratrol and resveratrol metabolites in rat plasma. A: Chromatogram with UV detection. B: Chromatogram with fluorescence detection. 1, 2,3,4,5 are metabolites. 6 is resveratrol.

4.4 RFERENCES

1. Biasutto L, Marotta E, Mattarei A, Beltramello S, Caliceti P, Salmaso S, et al. Absorption and metabolism of resveratrol carboxyesters and methanesulfonate by explanted rat intestinal segments. Cell Physiol Biochem. 2009;24(5-6):557-66.
2. Z.Pineeiro, M.Palma,C.G. Barroso, Determination of trans-resveratrol in grapes by pressurised liquid extraction and fast high-performance liquid chromatography. J .Chromatogr A.2006; 1110 (1-2) 61-65.

3. Lopez R, Dugo P, Mondello L. Determination of trans-resveratrol in wine by micro-HPLC with fluorescence detection. J Sep Sci. 2007;30(5):669-72.
4. R.Pezet, M. Pont, P. Cuenat, Method to determine resveratrol and pterostilbene in grape berries and wines using high-performance liquid chromatography and highly sensitive fluorimetric detection. J .Chromatogr A 1994; 663(2), 191-97.
5. Boocock DJ, Patel KR, Faust GE, Normolle DP, Marczylo TH, Crowell JA, et al. Quantitation of trans-resveratrol and detection of its metabolites in human plasma and urine by high performance liquid chromatography. J Chromatogr B Analyt Technol Biomed Life Sci. 2007;848(2):182-7.
6. Urpi-Sarda M, Jauregui O, Lamuela-Raventos RM, Jaeger W, Miksits M, Covas MI, et al. Uptake of diet resveratrol into the human low-density lipoprotein. Identification and quantification of resveratrol metabolites by liquid chromatography coupled with tandem mass spectrometry. Anal Chem. 2005 May 15;77(10):3149-55.
7. D.J.Huang,S. Wang,C.P Lo,P.W. Fong,H.W. Ko,P.K.D Ng. Halogenated silicon(IV) phthalocyanines with axial poly(ethylene glycol) chains. Synthesis, spectroscopic properties, complexation with bovine serum albumin and in vitro photodynamic activities. New .J. Chem.2004; 28, 348-354.
8. Chen, C.L. Chiang, Y.C. Lin, L.H. Chan, C.H. Huang, C.T.Chen. Ortho-Substituent Effect on Fluorescence and Electroluminescence of Arylamino-Substituted Coumarin and Stilbene. Org. Lett.2003;5, 1261-1264.
9. Fayed TA, Etaiw S, Saleh NJ, Micellar effects on the molecular aggregation and fluorescence properties of benzazole-derived push–pull butadienes J.Lumin. 2006;121, 431-44
10. Juan ME, Maijo M, Planas JM. Quantification of trans-resveratrol and its metabolites in rat plasma and tissues by HPLC. J Pharm Biomed Anal. 2010 Jan 20;51(2):391-8.
11. Huang L, Gough PC, Defelippis MR. Characterization of poly(ethylene glycol) and PEGylated products by LC/MS with postcolumn addition of amines. Anal Chem. 2009;81: 567

CHAPTER 5

DEVELOPMENTS OF A LC-MS METHODOLOGY TO SEPARATE, DETECT, CHARACTERIZE AND QUANTIFY RESVERATROL-PEG CONJUGATES AND THE CONJUGATION REACTION PRECURSORS AND INTERMEDIATES

5.1 INTRODUCTION

Monitoring the conjugation process, level of reaction intermediates and characterizing the polymeric conjugates is a vital part of the process that requires suitable analytical techniques that permit monitoring each step of the process in order to develop effective synthetic methodologies. Analytical techniques such as HPLC, IR, NMR and mass spectroscopy are currently used for this purpose with limited success [1,8]. All these techniques have some limitations for instance, HPLC, although capable of separating different compounds, cannot directly identify the polymeric compound unless there is a standard or UV spectrum of the compound available; thus the structural attributes and degree of substitution cannot be obtained from this technique. ^{H}NMR can give useful information for structural identification and quantification of simple structures and mixtures, however, in certain cases, overlapping signals can make obtaining quantitative information difficult. The determination of end group modification of a polymer, using NMR, can also be a difficult prospect. Hence the quantification becomes complex and may need other complimentary techniques for quantification. The development of an effective analytical methodology in addition to NMR, which can overcome some of these limitations, will be invaluable for understanding and controlling conjugate synthesis, and for characterisation of the synthesised polymeric conjugates or conjugates.

The qualitative structure elucidation, molecular weight determination and quality checks by LC-MS have reported for PEGylated proteins [3]. The PEG is known to undergo fragmentation in mass detector to small Mw daughter ions. However post column addition of amines such dimethyl and triethyl amines prevented fragmentation thereby allowing characterization of PeGylated proteins for their structure and molecular weight (Mw). The molecular weight determination by LC-MS was accurate with <0.01% variation [3]. The modifications of Pegylated proteins by oxidation and maleamide ring opening were also detected by LC-MS. Furthermore, the LC-MS was utilized to determine chemical composition of block copolymers such as polyethylene glycol-polycaprolactone (PEG-PCL) and polyethylene glycol-polycaprolactone-linoleic acid (PEG-PCL-:LA) [4] . The various ionization techniques such as atmospheric pressure chemical ionization and electrospray ionization were utilized for mass analysis. Combination of both

techniques was helpful in understanding the chemical composition of block copolymers. Such a method was proved to be useful in reaction monitoring. This was confirmed by a separate study, in which LC-MS was successfully used for monitoring the conversion of PEG to mPEG [5].

5.1.1 objectives of the study

Objectives of the current study were to develop a suitable LC-MS method to characterize various stages of PEGylation process of resveratrol, which included. to detect, characterize and quantify PEGylated resveratrol and reaction intermediates. It is essential that the various stages in pegylation such as modification of MeO-PEG-OH to MeO-PEGO-SuccOH, conjugation of MeO-PEG-SuccOH to resveratrol were monitored qualitatively and quantitatively in order to monitor the progress of the reaction and optimise the synthesis. In this study, The extent of PEG conversion to succinyl ester and extent PEG acid conjugation to resveratrol were monitored and determined by LC-MS. Finally LC-MS was also used to estimate the Mw of various PEG derivatives and conjugates.

5.2. MATERIALS AND METHODS

5.2.1 Materials

Trans-Resveratrol was purchased from DND Pharma-Tech Co. Inc, Shanghai, China, with 99% purity. MeO-PEG-OH was purchased from Sigma-Aldrich, Australia. MeO-PEGO-SuccOH 2 kDa, MeO-PEGO-Succ-RSV 2 kDa and MeO-PEGN-Succ-RSV, 2 kDa were prepared as per description in section 2.2.3, 2.2.4 of chapter 2.

5.2.2 LC-MS analysis of resveratrol, resveratrol PEG conjugates and reaction intermediates

The LC-MS analysis was carried out using a Shimadzu (Tokyo, Japan) LC-MS single quadrupole mass spectrometer (LC-MS, 2020) equipped with a binary pump 20AD, thermostated auto sampler SIL 20ACHT, thermostated column compartment CTO 20A, PDA detector SPD M 20A and mass analyser MS2020 with both electro spray ionization (ESI) and atomic pressure chemical ionization (APCI) systems. Data were acquired, processed and analysed by LABSOLUTIONS® chromatographic software.

LC-MS analysis was performed using a linear gradient system consisting of solvent A: water and solvent B: acetonitrile. The 10 min linear gradient was programmed as follows, 0 min 40%B, 1 min 40%B, 4 min 95%B, 8 min 40%B. Flow rate was maintained at 0.4 mL/minute. Elution was performed using a Zorbax SBC-18, 150 x 4.6 mm, 3.5 µm column maintained at 30ºC and the injection volume was 5 µL. The optimised ESI conditions employed included a DL temperature at 300^0C, interface temperature at 350^0C and heat block temperature was maintained at 220^0C. The drying gas, nitrogen flow was maintained at 10L/minute. The abundance of ions corresponding to resveratrol; free MeO-PEG-OH, MeO-PEG acids and PEG-resveratrol conjugates were recorded using simultaneous negative and positive ion detection mode. The scanning mass range was fixed at 50-2000 mass units. The selected ion monitoring system (SIM) including a series of ions with m/z of 700, 720, 730, 900, 1000 corresponding to PEG and 227 corresponding to resveratrol was used for quantitative analysis.

5.2.3 LC-MS qualitative analysis of resveratrol and PEG derivatives

Solutions of 10 µg/mL resveratrol, MeO-PEG-OH, MeO-PEG**O**-SuccOH, MeO-PEG**N**-SuccOH, and MeO-PEG-Resveratrol conjugates, prepared in chapter-2, were injected separately into LC-MS and analysed in scan mode to assess the selectivity of the LC-MS method and to determine the molecular masses of corresponding peaks.

5.2.4 Determination of extent of MeO-PEG-OH conversion to MeO-PEGO-SuccOH

The extent of functionalization of PEG (eg. MeO-PEG-OH) to acid (MeO-PEG**O**-SuccOH) was determined by analysis of the level of MeO-PEG-OH in the synthesised MeO-PEG**O**-SuccOH. Solutions of MeO-PEG**O**-SuccOH and a standard solution of MeO-PEG-OH, 1000 ng/mL prepared in acetonitrile/water mixture (50:50), was analysed in SIM mode by the LC-MS system in chromatographic conditions similar to those used for the MeO-PEG**N**-SuccOH and MeO-PEG**O**-SuccOH analysis as described above. The extent of functionalization of PEG was calculated following the determination of the concentration of MeO-PEG-OH in MeO-PEG**O**-SuccOH solution.

5.2.5 Determination of extent of PEG conjugation to resveratrol by LC-MS

A series of standard solutions of PEG acids (MeO-PEG**N**-SuccOH and MeO-PEG**O**-SuccOH) in the concentration range of 100 to 1000 ng/mL was prepared in acetonitrile/water mixture (50:50) and injected onto the LC-MS system. The analysis was performed in SIM mode to quantify concentrations of PEG acids. A standard calibration curve was then constructed by plotting area under the curve of PEG acid peak against concentration. A solution of resveratrol-PEG conjugate (1000 ng/mL) was also analysed in a similar fashion. The extent of conjugation of resveratrol to PEG was determined based on the concentration of free carboxylic acid terminated PEG detected in the conjugate mixture.

5.2.6 Determination of extent of PEG conjugation to resveratrol by UV-vis spectrophotometery

The resveratrol standard solutions with a concentration range of 1-10 µg/mL were prepared in acetonitrile. The absorbance of solutions was measured at 307 nm using a UV-VIS spectrophotometer (Waldbrann, Germany). Similarly 50 and 100 µg/mL solutions of resveratrol-PEG conjugates (equivalent to 5 and 10 µg/mL of resveratrol) were prepared and the absorbance recorded at 307 nm. The total resveratrol content in polymer was determined from the calibration curve of resveratrol. The average extent of PEG conjugation to resveratrol (%) was calculated based on the resveratrol content obtained experimentally against the theoretical value, assuming product is monosubstituted resveratrol.

5.2.7 Molecular weight determination of PEG derivatives and MeO-PEG-resveratrol conjugates

Molecular weight determination of PEG derivatives and resveratrol conjugates was performed by LC-MS analysis with an Applied Biosystems 4000 QTRAP LC-MS/MS system (Toronto, Canada). The analysis was performed by directly injecting analyte solutions via a T- junction with mobile phase consisting of 0.5% v/v triethyl amine in water. The analyte entered the mass detector without passing through any column. Ionization was induced with APCI ionization mode and positive ions were monitored with a Q1 MS scan. Heated nebulizer with gas flow of 20 L/min and with current of 3.0 µA was maintained, while source temperature was set at 600 ℃ and declustering potential 80 V. 1 mg/mLanalyte solutions of MeO-PEG-resveratrol,

MeO-PEG-OH, MeO-PEG**O**-SuccOH, and MeO-PEG**N**-SuccOH were infused separately at 20 µL/min with the mobile phase pumped at 0.5 mL/min. The average molecular weight of the polymeric compound was calculated using the following equation [3]

$$Mw = \sum \frac{M \times I}{I}$$

Where M is mass number of individual peak with intensity I.

5.3 RESULTS AND DISCUSSION

5.3.1 Structure of PEG-resveratrol conjugates and intermediates

Synthesis of resveratrol PEG conjugates was discussed in Chapter 2, section 2.3.2. The structures of polymers polymer conjugates and intermediates which were separated and analysed by LC-MS are shown in Figure 5.1.

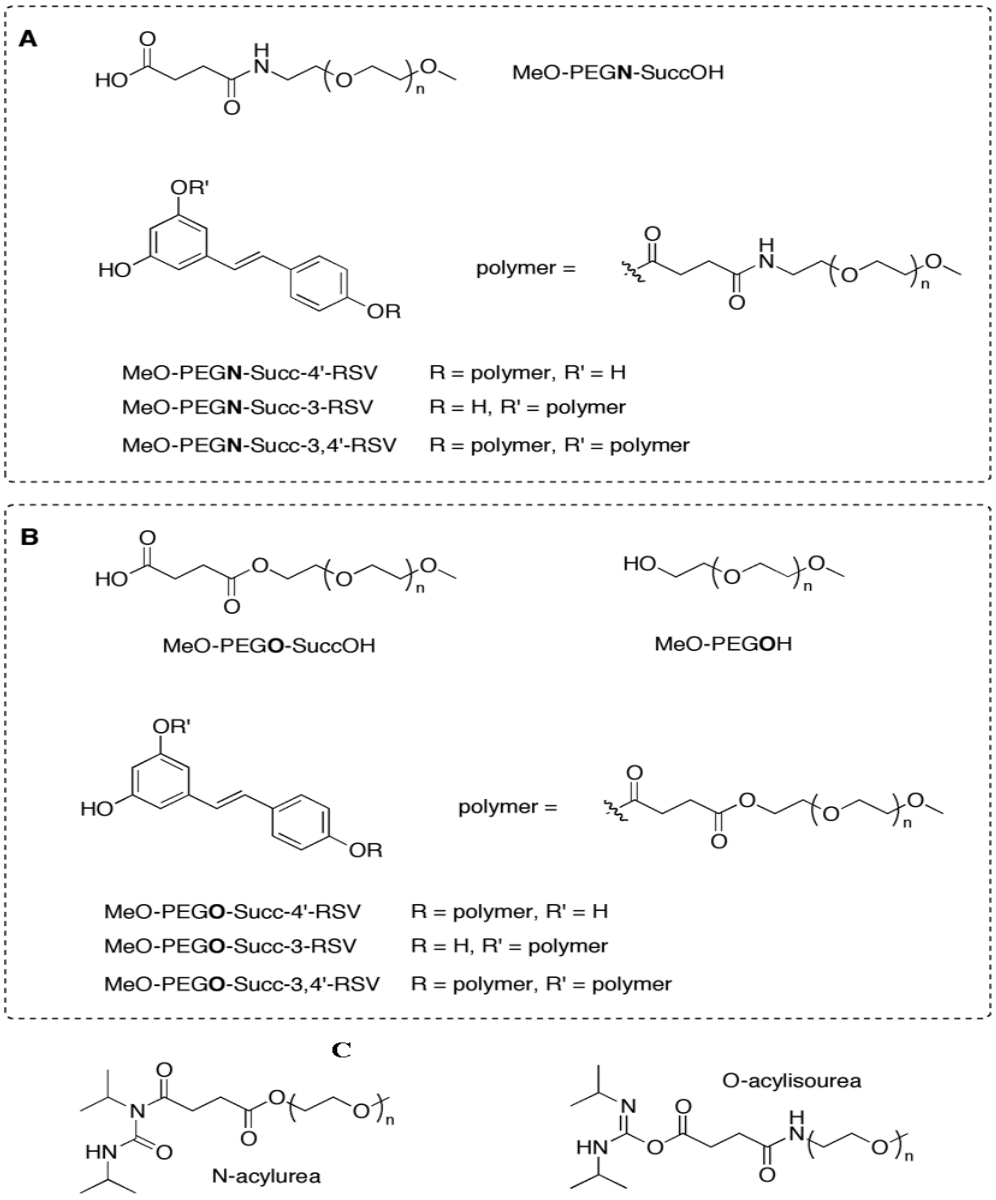


Figure 5.1: (A & B) Structures of Resveratrol-PEG conjugates and intermediates. (C) Structures of O-acylisoureaand N-acylurea.

5.3.2 LC-MS detection of MeO-PEG-resveratrol conjugates and MeO-PEG derivatives

The optimum chromatographic conditions for achieving the satisfactory separation of MeO-PEG acids, MeO-PEG-resveratrol conjugates, and MeO-PEG-OH with short retention times and good peak shape were obtained after studying the effects of column type and column temperature. Under these optimum conditions, resveratrol and its PEG conjugates were eluted at 5.4 and 6.6 min respectively (Figure 5.2);

whereas MeO-PEG acids and unmodified MeO-PEG-OH eluted at 2.9 and 4.6 min respectively as shown in Figure 5.3. Irrespective of the type of acidic linker in the structure, both MeO-PEG- resveratrol conjugates eluted at 6.6 min and the same was observed with the PEG acids, MeO-PEGN-SuccOH and MeO-PEGO-SuccOH, which were all eluted at 2.9 min. A representative chromatogram of a mixture of resveratrol-PEG, PEG acids and MeO-PEG-OH is shown in Figure 5.4. The mass spectra of peaks corresponding to MeO-PEG-OH, MeO-PEG acids and MeO-PEG-resveratrol showed a series of intense peaks within the 650-1000 (m/z) range in positive ion scan mode, which are characteristic fragmented peaks observed for PEG as shown in the Figure 5.4. The fragmentation of high molecular weight PEGs to low mass number in the mass detector at neutral or acidic pH conditions is a common observation and similar results were reported by Huang et al. for the PEG with Mw of 40 KDa [3].

LC-MS chromatograms of resveratrol and MeO-PEG-resveratrol with inserts of mass and UV spectra are shown in Figure 5.2. The mass spectrum of resveratrol showed a single intense peak of m/z 227 in negative mode, which correlates to the molecular weight of resveratrol. Little distinction could be made between the mass spectrum of MeO-PEG**N**-SuccOH, MeO-PEG**O**-SuccOH and MeO-PEG-resveratrol due to fragmentation, and there was no ion observed for resveratrol in either the positive or negative ion scan for MeO-PEG-resveratrol. However a simultaneously recorded UV spectrum of the MeO-PEG-resveratrol did in fact show a spectrum similar to that of resveratrol. Such a spectrum was not observed with either MeO-PEG**N**-SuccOH or MeO-PEG**O**-SuccOH samples. This unequivocally confirms that the peak eluted at 6.6 min belongs to MeO-PEG-resveratrol, demonstrating that the developed methodology can detect the formation of MeO-PEG-resveratrol structure in the conjugation process.

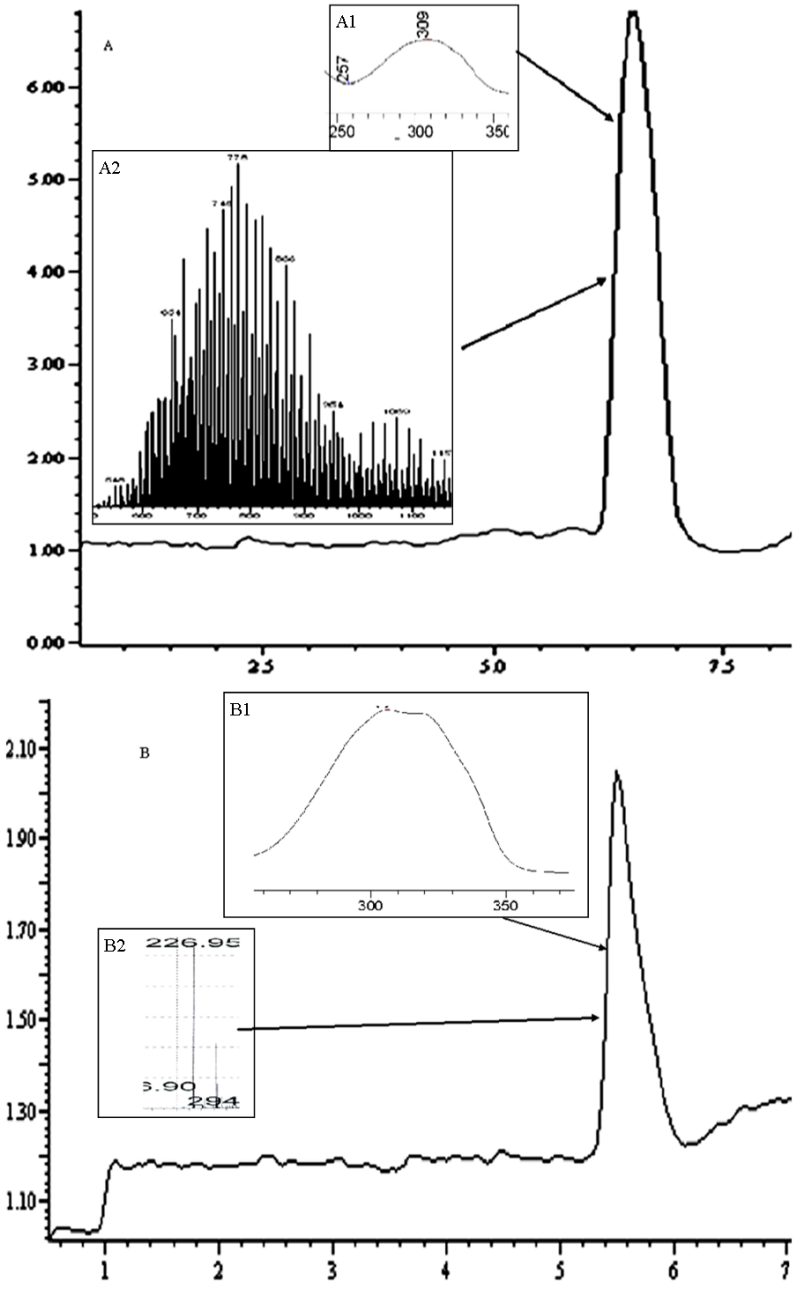


Figure 5.2. LC-MS chromatograms (Scan mode) of A: MeO-PEGN-Succ-RSV; B: RSV. Inserts are UV (A1, B1) and Mass spectra (A2, B2) of corresponding peaks.

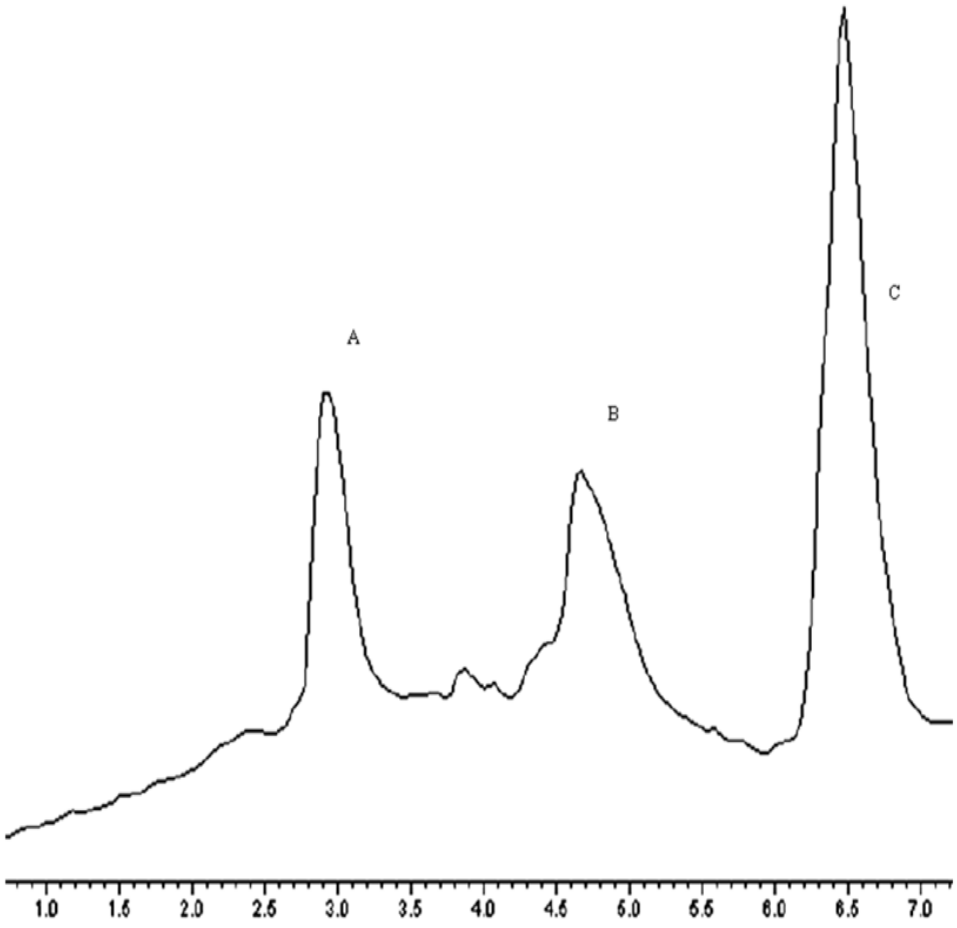


Figure 5.3. LC-MS chromatogram of the mixture of resveratrol derivatives (SIM mode) A: MeO-PEGN-SuccOH; B: MeO-PEG-OH; C: MeO-PEGN-Succ-RSV

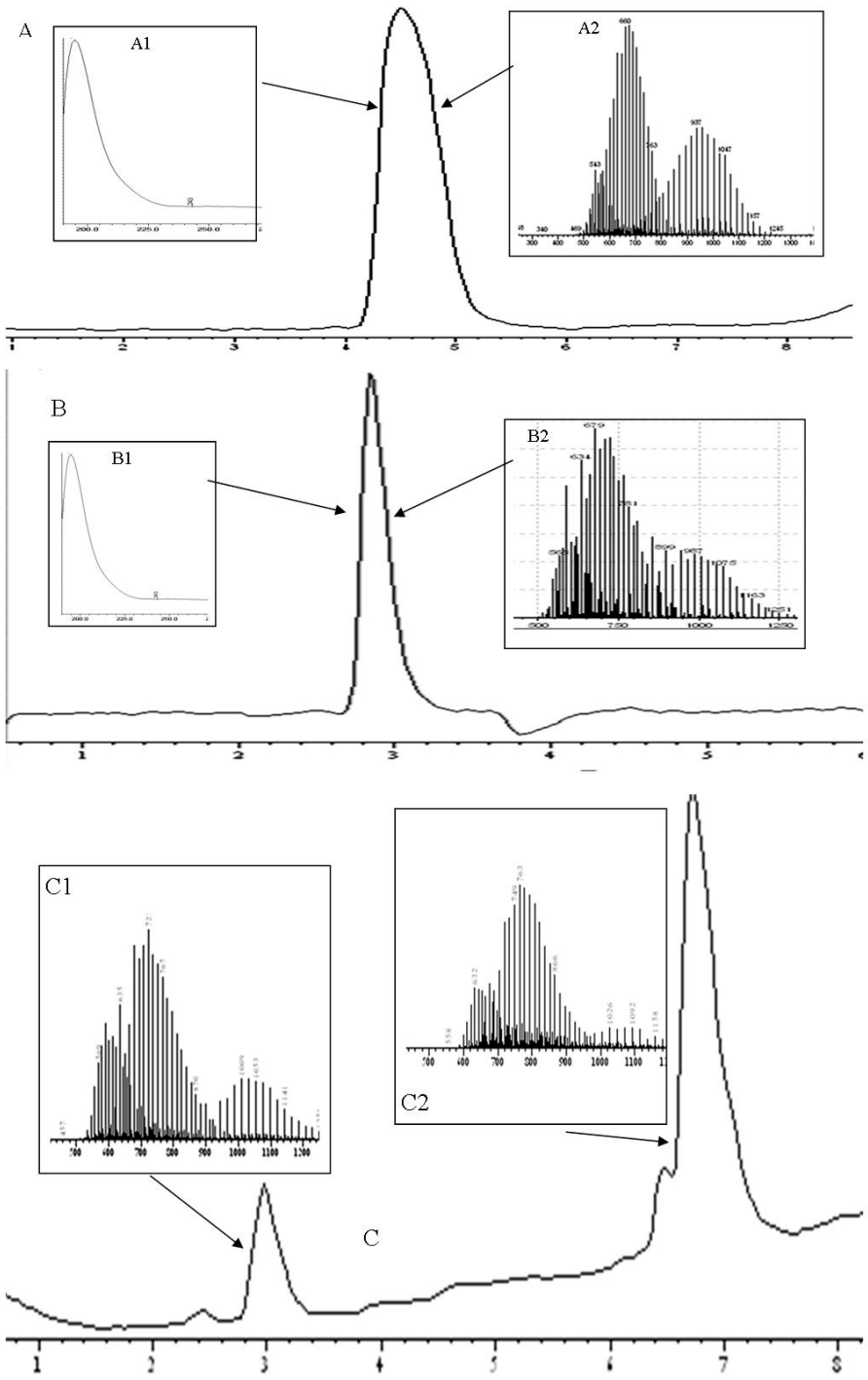


Figure 5.4. LC-MS chromatogram of A: MeO-PEG-OH; B: MeO-PEGO-SuccOH C: MeO-PEGO-Succ-resveratrol with small quantity of free MeO-PEGO-SuccOH at 2.9 minutes. Inserts are UV (A1, B1) and Mass spectra (A2, B2, C1,C2) of corresponding peaks

5.3.3 Determination of extent of functionalization of MeO-PEG-OH to MeO-PEGO-SuccOH

In the present study, the LC-MS analysis was found to be an effective tool for determining the extent of MeO-PEG-OH conversion to the functionalized acid derivative MeO-PEG**O**-SuccOH. The complete separation of MeO-PEG-OH and MeO-PEG**O**-SuccOH permitted the accurate quantification of free MeO-PEG-OH (Figure 5.3 & 5.4). The separation achieved in the present study was better than that reported for MeO-PEG-OH and HO-PEG-OH, which could be due to polarity differences [5]. In the present study, there was no free MeO-PEG-OH detected in MeO-PEGO-SuccOH solutions prepared at various concentrations, indicating complete conversion of MeO-PEG-OH to the acid. Various studies have reported the use of NMR for analysing the level of conversion or functionalization of MeO-PEG-OH to acid derivatives by calculating ratio of area of specific ethylene protons to that of the MeO end group of polymers [8]. Employing the same technique, conversion of MeO-PEG-OH to MeO-PEGO-SuccOH was calculated from NMR and it was found to be complete, supporting our LC-MS determination.

5.3.4 Determination of extent of PEG conjugation to resveratrol

LC-MS analysis in SIM mode with multiple ions being monitored was found to be a very sensitive methodology for detection of MeO-PEG acids despite the noise created by multiple ion monitoring. The method is very sensitive, such that the lowest concentration,100 ng/mL, with 5µl injection volume, produced a signal that was multiple times higher than the noise and well above the signal to noise ratio of 10 for quantification. The free MeO-PEG**N**-SuccOH was not detected in the conjugation product (i.e MeO-PEG**N**-Succ- resveratrol solution) therefore the degree of substitution was considered to be 100%. However in the case of resveratrol coupled to MeO-PEG**O**-SuccOH, a significant amount of free MeO-PEG**O**-SuccOH was detected in the conjugate solution (27%). Accordingly the degree of substitution was found to be 73%. In the absence of such a method, the degree of substitution could only be determined by hydrolysing the conjugate back to the parent drug resveratrol, the actual free resveratrol generated has to be quantified and compared against theoretical value [6] However, the accurate determination of resveratrol after hydrolysis could be difficult as resveratrol is highly light, carbonate and alkali sensitive [7]. The use of NMR has been suggested for determination of polymer

conjugation to drugs [8]. The ratio of the area for specific signals for protons of the drug molecule to that of signals for protons from the MeO end group of the polymer can be calculated and compared against the theoretical value. However in certain drug molecules where there are multiple sites for polymer conjugation, the resulting product could be a mixture of multiple conjugated species. In such a complex situation calculating total conjugated drug to polymer ratio would become difficult. The similar findings were observed for resveratrol where two major monosubstituted species are formed. Given these findings, the present LC-MS method is proven to be simple and reliable for determining the extent of resveratrol conjugation to PEG.

The NMR analysis showed that non-conjugated PEG in the product was a mixture of an N-acylurea by-product and free MeO-PEG**O**-SuccOH. There was no separate peak observed for the N-acylurea derivative in the LC-MS, indicating it presumably eluted at the same time as that of MeO-PEG**O**-SuccOH. Hence, the LC-MS quantification performed is a total of two species. Moreover under neutral pH mobile phase conditions PEG derivatives undergo fragmentation in the mass detector to produce smaller daughter ions and only major ions, which are common in all PEG derviates, were selected in selected ion monitoring (SIM) method for quantification. As such the presence of different species of non-conjugated PEG, whether it is in the form of free MeO-PEG**O**-SuccOH or the N-acylurea does not affect actual quantification of total non-conjugated PEG.

Table 5.1. Comparison of various methods for determining the extent of PEG conjugation to resveratrol (RSV)

	Extent of PEG conjugation to RSV %		
Test substance	UV-Spectroscopy [1]	NMR[2]	LC-MS[2]
MeO-PEGN-Succ-resveratrol	74 %	100%	100%
MeO-PEGO-Succ-Resveratrol	40%	Not determined[3]	73%

[1]Based on resveratrol content in the final product.

[2]Based on free PEG acid in the final product.

[3] Value could not be obtained from NMR because of complex nature of sample due overlapping signals from mixture of species.

5.3.5 Determination of extent of PEG conjugation to resveratrol by UV spectroscopy

The extent of PEG conjugation to resveratrol determined by UV spectrophotometer measurements are substantially different from those obtained by LC-MS and NMR experiments (Table 5.1). It should point out that both LC-MS and NMR analyses the free PEG in the samples, whereas the UV spectroscopy method determines the resveratrol content. The UV spectroscopy analysis showed that the extent of conjugation of MeO-PEGN-Succ-resveratrol was 74%, compared to 100% by LC-MS and NMR. This result was obtained based on assumption that the conjugate contained a monosubstituted resveratrol. As a some fraction of disubstitued resveratrol conjugate was in fact present in the mixture (5-10%, by NMR), thus it is expected that the conjugation of PEG to resveratrol detected by UV analysis should be less than 100%. This underestimation of PEG conjugation by UV analysis may be caused by a change in UV extinction coefficient of resveratrol after coupling to PEG.

5.3.6 Molecular weight determination of MeO-PEG, MeO-PEG acids and MeO-PEG- resveratrol conjugates

The average molecular weights of MeO-PEG-OH, MeO-PEG acids and MeO-PEG-resveratrol conjugates were determined by the mass spectral analysis of PEG derivatives with 0.5% v/v triethylamine as mobile phase, which prevented fragmentation of PEG to ions with lower m/z values (Figure 5.5) and thus the observed mass spectrum consisted of ions of mass number closely correlating to actual PEG molecular weight. This observation is in close agreement with Huang et.al [3]. There was clearly a gradual increase in molecular weights observed for MeO-PEG-OH, MeO-PEGO-SuccOH and MeO-PEGO-Succ-resveratrol as shown in Figure 5. Similar observations were found with MeO-PEGN-SuccOH and MeO-PEG-COO-resveratrol conjugates (data not shown). The average molecular weight of MeO-PEG, MeO-PEGO-SuccOH and MeO-PEGO-Succ-resveratrol determined by the developed method was 2108, 2321 and 2423 respectively (Table 2). These correlate with the theoretical value for MeO-PEG-OH and MeO-PEGO-SuccOH and MeO-PEGO-Succ-resveratrol The differences in average molecular weights of MeO-PEGO-SuccOH and MeO-PEGO-Succ-resveratrol (determined versus theoretical) are slightly more than expected (10.5%), possibly due to the presence of free PEG acid in the sample and the possibility of overestimation of PEG acids (Table 2). The differences between the determined and the theoretical values with consideration of the level of PEG substitution suggest that this method provides reasonably accurate molecular weight estimation. The average molecular weight calculated by this approach matches closely with those obtained by spectral deconvulation and summation using the ABI software system [3]. However in the absence of such software, manual calculation performed as reported in this study was found to be satisfactory and matched well to the reported values generated by other analytical techniques such as gel permeation chromatography (values from raw material suppliers). The average molecular weight calculations of MeO-PEG-resveratrol conjugates were based on the assumption that the majority of substitution is mono substitution either at 4' or 3 hydroxyl groups of resveratrol. The disubstituted resveratrol species could not be detected due to limitations of the Mass Analyser (<2800 Da). However comparison of the intensity of major ions in PEG acids and their respective conjugates revealed that intensities were similar, which indicates that disubstituted species are very minor, if they do exist.

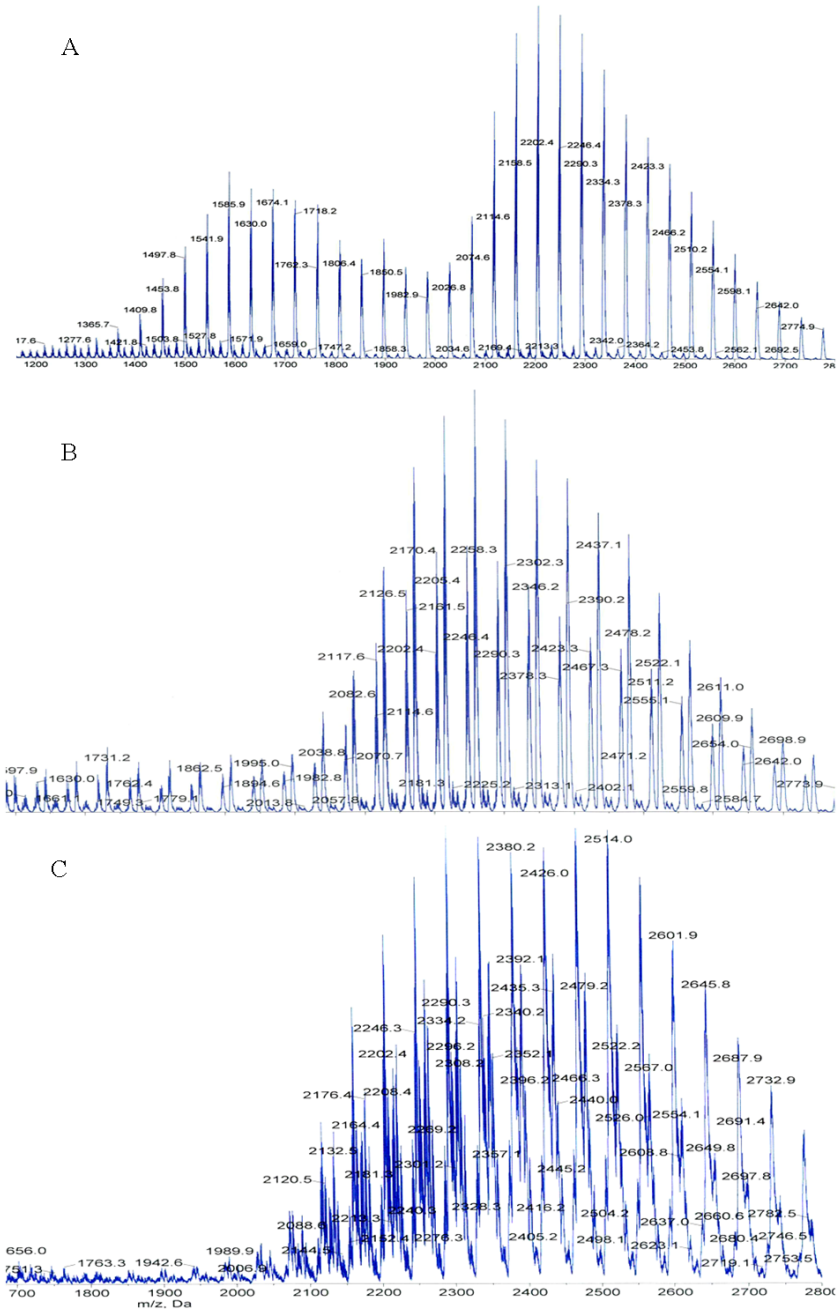


Figure 5.5. Mass spectra of PEG derivatives with 0.5% v/v triethylamine as mobile phase solvent A: MeO-PEG-OH; B: MeO-PEGO-SuccOH; C: MeO-PEGO-Succ-resveratrol

Table 5.2. Comparision of average molecular weights calculated based on chemical structure and that determined by mass spectra, for resveratrol, MeO-PEG-OH, MeO-PEGO-SuccOH and MeO-PEGO-Succ-RSV

Test substance	**Theoretical Average Molecular Weight (Da)**	**Average Molecular weights Determined by Mass Spectra (Da)**	**Difference in Average Molecular Weight (%)**
Resveratrol	228	227.9	-
MeO-PEG-OH	2000[1]	2108	5.4
MeO-PEGO-SuccOH	2100	2321	10.5
MeO-PEGO-Succ-RSV (100% PEG substitution)	2328	2423	4.1
MeO-PEGO-Succ-RSV (73% PEG substitution)[2]	2266	2423	9.2

[1] Average molecular weight provided by manufacturer.

[2] 73% PEG substitution is weight based.

\

5.4 REFERENCES

1. Greenwald RB, Choe YH, Mcguire J, Conover CD. Effective drug delivery by PEGylated drug conjugates. Adv.Drug.Del.Rev, 2003,55, 217-250.
2. Cao N, Feng S.S. Doxorubicin conjugated to D-alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS): conjugation chemistry, characterization, in vitro and in vivo evaluation. Biomaterials. 2008; 29: 3856.
3. Huang L, Gough PC, Defelippis MR. Characterization of poly(ethylene glycol) and PEGylated products by LC/MS with postcolumn addition of amines. Anal Chem. 2009;81: 567.
4. Van-Leuwen SM, Tan B, Grijpma DW, Feijen J, Karst U. Rapid.Commun.Mass Spectrom 2007,21, 2629-2637.
5. Barman BN, Champion DH, Sjoberg. SL. Identification and quantification of polyethylene glycol types in polyethylene glycol methyl ether and polyethylene glycol vinyl ether.J Chromatogr A. 2009;1216: 6816.
6. Veronese FM, Schiavon O, PasutG, Mendichi R, Andersson L, Tsirk A, Ford J,Wu G, Kneller S, Davies J, Duncan R. PEG-doxorubicin conjugates: influence of polymer structure on drug release, in vitro cytotoxicity, biodistribution, and antitumor activity Bioconjug Chem. 2005;16:775.
7. Yang N.C, Lee CH, Song TY. Evaluation of resveratrol oxidation in vitro and the crucial role of bicarbonate ions. Biosci Biotechnol Biochem. 2010;74: 63.
8. Zhang X, Li Y , Chen X, Wang X, Xu X, Liang Q,Hu J, Jing X. Synthesis and characterization of the paclitaxel/MPEG-PLA block copolymer conjugate. Biomaterials. 2005;26:2121

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

6.1 GENERAL DISCUSSION

Despite numerous data available on the beneficial properties of resveratrol, many studies confirmed that resveratrol is rapidly absorbed and highly metabolized by conversion to sulfo- and glucuro-conjugates with biological half life of 0.13 hours in rats and similar trend was observed in human [1,2]. Although it has been suggested that conjugated metabolites may contribute to resveratrol activity, but their potency is weaker than that of resveratrol [3]. Hence it is logical to take the approach to investigate the effect of extending the resveratrol residence time in the body with the aim to produce the desired pharmacological effects which were shown *in vitro* but not *in vivo* due to the high metabolic rate of resveratrol [4] The objective of this PhD project was to develop novel formulation strategies to address poor pharmacokinetic profile of resveratrol. Review of literatures in this area suggests that polymer conjugation is one of the most feasible approaches to mask the molecule against metabolism and extend the circulation time of drugs [5]. Accordingly a strategy was designed to develop polymer conjugates of resveratrol using permanent and cleavable linker chemistry. The various polymer conjugates investigated in this project and results of those studies carried out are tabulated in Table 6.1 for easy reference.

6.1.1 Synthesis of resveratrol polymer ester conjugates

Generally ester conjugates are developed to address problems associated with solubility or stability of drugs. Upon reaching systemic circulation, such ester conjugates are anticipated to hydrolyze rapidly to produce parent drug molecules. The polymeric ester conjugates, in addition to have potential in improving solubility, can provide protection against potential metabolism. Based on polymer architect and linker chemistry, these polymer conjugates can provide extended circulation time and gradually release drug over an extended period of time. With appropriate ligand molecule and polymer , it can also provide targeting potential to tissues such as tumour.

In this study, simple ester based polymer conjugates were developed for resveratrol against metabolism. Soluble polymers such as PEG and micelle forming diblock polymer such as PLA-PEG were selected and investigated for this purpose. Initially

resveratrol conjugates with PEG were prepared by employing simple carbodiimide coupling chemistry..The carbodiimide coupling involves creation of a covalent link between the hydroxyl (OH) group and carboxyl (COOH) group of polymer. It is usually brought about by employing a base and a carbodiimide such as DIC, EDC. The carbodiimide coupling agent, activates COOH group of polymer by forming an reactive intermediate O-acylisourea which in turn facilitates the formation of ester bond while producing urea as a by-product. The carbodiimide coupling agents are also referred to as a zero length spacer since they don't introduce any additional atoms during the conjugation process.

Initial reactions for conjugation of PEG to resveratrol were carried out using DIC-DMAP in THF/dichloromethane solvent system. However, in order to achieve the ease of separation of product from reactants, reaction conditions were optimized to include pyridine as base and carbodiimide DIC as the coupling agent. A simple precipitation process using diethyl ether was adopted for purification of resveratrol-PEG conjugates. The urea by-product N,N'-diisopropylurea, excess of pyridine, DIC, were found to be either soluble or miscible with diethyl ether, whereas the conjugates were not, making purification process easier. The resveratrol was not freely soluble in ether, so multiple washing of product with ether/ethyl acetate mixture was required to purify the product in order to remove trace amount of resveratrol. This was done to prevent interference of resveratrol in NMR spectral interpretations. The purification by dialysis using benzyolated (MWCO 2000 Da) and regenerated cellulose (MWCO 1000 Da) tubing's was not successful because resveratrol did not diffuse, possibly bound to membrane.

The resveratrol-PEG conjugation was performed with different linker group and PEG of different Mw. The extent of PEG conjugation to resveratrol varied. The 100% conjugation was achieved for MeO-PEGN-SuccOH 2 kDa; 70% for MeO-PEGO-SuccOH, 2 kDa and 50% for MeO-PEGN-SuccOH 20 kDa. These results clearly indicated that the rate of reaction was dependent on Mw of polymer and chemistry of carboxyl end group in polymers. Similarly resveratrol was conjugated to MeO-PEG-PLAO-SuccOH using the same conjugation chemistry and achieved success as indicated by HPLC analysis . With respect to linker chemistry, DIC failed to produce consistent results in conjugation. Hence EDC was used instead of DIC,

for conjugation of resveratrol to MeO-PEG-PLAO-SuccOH. The MeO-PEG-PLAO-Succ-RSV conjugates were purified by extraction using DCM/0.1M HCl. All resveratrol polymer ester conjugates were characterized by NMR, HPLC and selected conjugates by LC-MS. All the techniques indicated formation of conjugates. The yield was ranged between 60-65% for all conjugates before washing/purification and 30-40% after purification. The NMR indicated that the polymer conjugates were mixture of species with 4'-PEG-RSV (or 4'-PEG-PLA-RSV) as a major product (50%), followed by 3-PEG-RSV (40%) and a minor 3,4'-di(PEG)-RSV.

6.1.2 Synthesis of Resveratrol-PEG ether conjugates

The permanent non cleavable PEGylation of resveratrol was achieved via ether bond. The resveratrol-PEG ethers were prepared to have stable conjugates as an alternative strategy as esters are too prone to hydrolysis in plasma by plasma esterases. The permanent PEGylation was achieved by simple etherfication using MeO-PEG-Br and potassium carbonate. The reaction was reported for preparation of resveratrol small chain ethers[7, 8]. However, the reaction conditions as reported did not yield desired results with PEG conjugation and hence considerable efforts were made to optimize reaction conditions to achieve maximum conjugation of PEG with resveratrol. During these experiments it was found that the amount of potassium carbonate was very critical in reaction and it determined the extent of PEGylation. The higher the concentration of potassium carbonate, the greater the PEGylation. The final product can easily purified by a simple extraction procedure using DCM/HCl. The NMR again indicated that product is mixture of species, this time 4'-PEG-RSV slightly more (60%).

6.1.3 *In vitro* evaluation of resveratrol polymer conjugates

Resveratrol polymer conjugates were tested for solubility, stability in buffers, plasma and in rat liver microsomes. Resveratrol-PEG conjugates, irrespective of polymer chain length and linker chemistry were all soluble in water, pH 4.5 and 7.4. The resveratrol conjugates with PLA-PEG were insoluble in water and hence they were dissolved in acetonitrile and then dispersed in water to form solid micelles or nanoparticles in aqueous medium after solvent vaporization. Such conjugate dispersion was used for all *in vitro* and *in vivo* studies. The CMC values of micelle forming polymer conjugates were determined by dye solubilization method using

water insoluble dimethyl yellow. The CMC value was found to be 4 Mg/L for MeO-PEG-PLAO-Succ-RSV, 2 kDa, 8 Mg/L for MeO-PEG-PLAO-Succ-RSV, 6.5 kDa and 500 Mg/L for MeO-PEGO-Succ-RSV 2 kDa. The average micelle diameter for MeO-PEG-PLAO-Succ-RSV, 2 kDa micelles was found to be 170-250 nm (0.05 to 0.2%), 147 to 165 nm (0.05% to 0.2%) for MeO-PEG-PLAO-Succ-RSV, 6.5 kDa and for MeO-PEGO-Succ-RSV 116 to 181 nm (0.1 to 0.5%). These findings suggest that, at lower concentration polymer conjugates aggregate as micelles and at higher concentration would form nanoparticles.

In terms of chemical stability, all of resveratrol polymer conjugates were stable in buffer pH 4.5. But in buffer pH 7.4, MeO-PEGN-Succ-RSV 2kDa and 20 kDa were found to be unstable, whereas MeO-PEGO-Succ-RSV 2 kDa was stable. It was speculated that the self aggregation and possible formation of micelles by MeO-PEGO-Succ-RSV 2kDa was the reason for the results.. A similar trend was observed for MeO-PEG-PLAO-Succ-RSV 2 kDa which was also stable in both pH 4.5 and pH 7.4. Plasma stability study indicated that resveratrol-PEG esters underwent instant hydrolysis in rat plasma, no intact polymer conjugates were detected immediately after its exposure to the plasma (near 0 minute). It is possible that the conjugates could be hydrolyzed during the lag time of sampling and dilution with acetonitrile. However the micelle forming conjugate, MeO-PEG-PLAO-Succ-RSV 2 kDa, was stable in rat plasma with $t_{1/2}$ of 3 hours. In contrast, MeO-PEG-PLAO-Succ-RSV 6.5 kDa was much less stable, with $t_{1/2}$ of only 5 minutes. From these results, it is evident that the micelle forming resveratrol ester conjugates were relatively more stable in rat plasma and the length of hydrophobic chain can have an impact on the plasma stability of the conjugate. This is not unexpected as it had been reported that an optimum hydrophobic core with relatively strong PEG corona on micelle surface offered greater plasma stability to conjugate [9, 10].

AFM and FESEM images revealed that the conjugate of MeO-PEG-PLAO-Succ-RSV 2 kDa formed solid nanoparticle like structure which resisted structural deformation during the process of drying. Whereas micelles of MeO-PEGO-Succ-RSV 2 kD, and MeO-PEG-PLAO-Succ-RSV 6.5 kDa polymer conjugates appeared to be fused during drying process. A consistent phenomena was observed with AFM images, with conjugates of MeO-PEG-PLAO-Succ-RSV, 2 kDa showing individual

particles and conjugates of MeO-PEG-PLAO-Succ-RSV, 6.5 kDa and MeO-PEGO-Succ-RSV, 2 kDa displaying fused patches. The formation of compact nanoparticle like structure in the case of MeO-PEG-PLAO-Succ-RSV 2 kDa would also explain for observed stability of conjugate in plasma.

Resveratrol-PEG ethers with non-cleavable ether link, as anticipated, did not hydrolyze in buffers and plasma. Further investigations are necessary to design PEG-resveratrol ester conjugates which can release the drug slowly in the plasma. Various linkers such as acid liable spacers *cis*-aconityl/hydrazone linker need to be evaluated as an alternative to produce better plasma stability of resveratrol-PEG ester conjugates [11, 12]. Although peptide spacers (eg. amide bonds) between drug and PEG were found to stabilize conjugate in the plasma, feasibility of inclusion such linkers for resveratrol, a molecule with hydroxyl groups only, with an appropriate polymer is yet to be assessed [13].

In vitro preliminary metabolism studies of resveratrol and its polymer conjugates were performed in rat liver microsomes. Rat liver microsomes were supplemented with uridine diphosphoglucuronic acid (UDPA) in order to bring about phase II metabolism, glucuronidation. The results revealed that polymer conjugates were stable in microsomal incubations containing UDPA, with more than 80% conjugates remaining after 60 minutes. In contrast, resveratrol on its own metabolized rapidly with almost none resveratrol left and only metabolites were detected after 10 minutes. The MeO-PEGN-Succ-RSV, 2 kDa was found to hydrolyze in microsomal reaction mixture and free resveratrol generated was metabolized. From these studies, it is evident that polymer conjugates must be intact in order to prevent the metabolism of resveratrol. The physical mixture of resveratrol and polymer would not help in slowing down metabolism or improving pharmacokinetic profile of resveratrol.

In vitro antioxidant assay of resveratrol and its polymer conjugates was performed using DPPH inhibition assay. The free radical scavenging ability of resveratrol-polymer conjugates, MeO-PEGN-Succ-RSV, 2 kDa, MeO-PEG-PLAO-Succ-RSV 2 kDa and RSV-PEG ether 2 kDa decreased slightly against resveratrol. The IC 50 value of resveratrol was found to be 65 µM, whereas for polymer conjugates it was

about 219 µM (resveratrol equivalent) , nearly 4 fold decrease in activity. Although the direct free radical scavenging activity of resveratrol was decreased after polymer conjugation, resveratrol can also act as *in vivo* antioxidant by inducing enzymes such as dismutase. Such activities of conjugates need to be evaluated. How the improved plasma levels and prolonged circulation of polymer conjugates would affect *in vivo* antioxidant activity of resveratrol is yet to be investigated.

6.1.4 *Single* dose preliminary pharmacokinetic studies of resveratrol and its polymer conjugates in a rat model

Single dose preliminary pharmacokinetic studies of resveratrol and selected polymer conjugates (those showed stability in rat plasma) were performed in Wistar rats to evaluate effectiveness of polymer conjugation in improving plasma concentration profile of resveratrol. As expected, resveratrol exhibited rapidly declining plasma profile, reaching almost non-detectable level within 2 hours, due to rapid metabolism of resveratrol. Peak areas of metabolites were always higher than resveratrol since the initial sampling time at 5 minutes post injection. The pharmacokinetic profile of MeO-PEG-PLAO-Succ-RSV showed different profile from resveratrol and other PEG ether conjugates. The MeO-PEG-PLAO-Succ-RSV 2kDa showed highest plasma concentration (three times of resveratrol concentration) at 5 minutes and rapidly declined thereafter. Interestingly, the sharp decline in conjugate levels in plasma may not be actually due to hydrolysis and subsequent metabolism but possibly due to its uptake by mononuclear phagocyte systems (MPS) [14]. The fact that a relatively high level of resveratrol compared to metabolites was detected in polymer conjugates also supports this reasoning. In addition, the larger size of micelles/nanoparticles produced by the presence of hydrophobic of polymer made the conjugate susceptible for MPS uptake [14]. On the other hand, resveratrol PEG ether 2 kDa produced another distinct profile which was different from resveratrol and MeO-PEG-PLAO-Succ-RSV, 2 kDa. The profile was characterized with higher plasma concentration than that of native resveratrol and PEG-PLA conjugate. Although there was sharp decline in plasma concentration after 5 minutes, the levels were still much higher and almost stagnated after 30 minutes until 4 hours. Despite the dose of conjugate (5kDa, ether conjugate) was equivalent to 1/5 of resveratrol, its plasma concentration was still more than 10 folds higher than that of resveratrol. The resveratrol-PEG ether conjugates were stable in plasma and did not hydrolyze to

native resveratrolas there was neither free resveratrol nor metabolites detected in plasma in any of time point. Since PEG ether conjugate was soluble, it was expected to be extensively excreted via the kidney. As the 2 kDa molecule was not big enough to reach threshold volume to overcome kidney filtration [15], it was thought that the stagnant levels after 1 hour could possibly due to the presence of disubstituted resveratrol (3, 4' di (PEG)-resveratrol) with Mw of 4 kDa. The pharmacokinetic profile of resveratrol-PEG ether 5 kDa indeed showed marginally slower elimination after 5 minutes, suggesting size of conjugate only has limited impact on the elimination of conjugates. The rapid elimination, in first 30 minutes, of RSV-PEG ether 5 kDa could be due to increased polarity. The slower terminal elimination of RSV-PEG ether may be attributed to the intrinsic distribution and elimination properties of molecules or conformational arrangement of di (PEG)-RSV which gives more volume to molecule leading to slower elimination. However all these findings need further confirmation with suitable analytical techniques. The AUC tot, MRT for RSV-PEG ether 2 kDa were found to be 15.66 µ*h/mL and 6.7 h respectively (11.51 µ*h/mL, 6.2 h respectively for RSV-PEG ether 5 kDa). To achieve further improvement in pharmacokinetic profile of resveratrol-PEG ethers, synthesis of conjugates with polymer of Mw > 20 kDa or polymer conjugate capable of forming stable micelles or nanoparticles is recommended. This strategy may overcome rapid conjugateexcretion through kidneys and potentially prolong the plasma residence with relatively higher plasma concentrations. However, the ratio of PEG to hydrophobic polymer is important to minimise the uptake of conjugates by MPS when high molecule weight polymers are to be used.

6.1.5 Development of HPLC and LC-MS for monitoring and analysing the resveratrol-polymer conjugates and detection of reaction intermediates and resveratrol metabolites.

A reliable, reproducible, sensitive HPLC method with fluorescence detection was developed. The method allowed detection and quantification of resveratrol and MeO-PEGN-Succ-RSV conjugates at 5 and 30 ng/mL (resveratrol equivalent) respectively. This study revealed that the PEG conjugation enhanced the fluorescence properties of resveratrol, and this enhancement could be modulated by methanol concentration. These findings were subsequently applied to the

development of a simple but sensitive HPLC method for simultaneously analysis of resveratrol and it polymer conjugates in plasma samples.

An LC-MS methodology was developed for the separation, characterization and quantification of resveratrol-PEG conjugates and reaction intermediates. The purpose LC-MS method development was to monitor and optimize reactions conditions for small Mw PEG and adopt same reaction conditions for other polymer conjugates. The LC-MS method was able provide to information on extent of MeO-PEG-OH conversion to MeO-PEGO-SuccOH, extent of PEG conjugation to resveratrol and Mw of polymer conjugates. Overall LC-MS method was helpful in understanding conjugation process. However due to Mw limitations of mass detector of LC-MS instruments, all polymer conjugates could not be analyzed by present LC-MS method and analysis using LC-MS with greater mass detection limits can be considered for future work.

6.2 Conclusion

In this study various polymer conjugates of resveratrol were prepared and evaluated for their impact on plasma concentration profile of resveratrol. The polymer conjugates were investigated for their surface properties and tested for stability in buffers, rat plasma and microsomes, the preliminary pharmacokinetic profile of selected polymers were evaluated in Wistar rats.

Resveratrol polymer esters were successfully prepared using carbodiimide coupling reactions. The NMR analysis revealed that conjugates were mixture of mono and disubstitued resveratrol with 4'-PEG-RSV as major component (ca 50%) , followed by 3'-PEG-RSV (35%). And minor amount of 3, 4'-di (PEG)-RSV. The substitution pattern was almost same for all polymer conjugates. LC-MS analysis of some of resveratrol polymer conjugates and polymers showed that reaction adopted for synthesis did not cause any degradation of polymer.

The resveratrol PEG esters were unstable in plasma and hydrolyzed very rapidly. Micelle forming PEG-PLA conjugates were stable in plasma, rate of hydrolysis was found to be dependent on PLA/PEG content in polymer, colloidal stability of micelles/nanoparticles. Based on AFM and SEM images, MeO-PEG-PLAO-Succ-

RSV 2 kDa was formed nanoparticle like structure at higher concentration and hence was stable in plasma. Resveratrol-PEG ethers were stable in both buffers and plasma. Free radical scavenging activity was reduced 4 folds after conjugation of polymers however improved plasma levels may compensate for reduced activity and improve overall pharmacological activity *in vivo*.

.Finding that the resveratrol-PEG conjugates exhibits enhanced fluorescence properties compared to that of resveratrol, and this enhancement could be modulated by methanol concentration has lead to the development of a simple, yet reproducible and sensitive HPLC method with fluorescence detection for simultaneous detection and quantification of resveratrol and resveratrol polymer conjugates, as well as detection of resveratrol metabolites *in vitro* and *in vivo*.

The LC-MS methodology developed has enabled the separation, characterization and quantification of resveratrol-PEG conjugates and reaction intermediates, which provided us with a better understanding of conjugation process. This made the optimisation of conjugation process easier.Pharmacokinetic studies revealed that MeO-PEG-PLAO-Succ-RSV did not offer the same level of advantage as resveratrol-PEG ether, in terms of extending the circulation time as it was rapidly cleared from circulation. On the other hand resveratrol-PEG ether 2 kDa displayed significantly better profile than resveratrol and other polymer conjugates. Plasma concentration and duration were improved. The improved pharmacokinetic profile with resveratrol-PEG ethers demonstrated that they are good candidates for further biological studies such as efficacy and toxicity studies in animal models. Findings of this study revealed that polymer conjugation is suitable approach to improve pharmacokinetic profile of resveratrol. The selection of polymer and linker chemistry was found to be more stable than those reported by Biasutto et.all [16].. Overall, this present study provided insight into conjugation chemistry for the stable polymer conjugates and reported the development of novel formulation strategy, stable polymer conjugates, which improved pharmacokinetic profile (plasma concentration–time profile) of resveratrol.

Table 6.1 Summary of resveratrol-polymer conjugates synthesized and evaluated in this Ph.D project.

Polymer conjugate	**Tests performed**					
	NMR/LC-MS/HPLC	**Buffer stability**	**Plasma stability**	**Microsomal stability**	**Pharmacokinetic (PK) studies**	**Conclusions**
MeO-PEGN-Succ-RSV, 2 kDa	Results indicate conjugate formation with 4'-PEG-RSV as major product	Stable in pH 4.5 and hydrolyse slowly in pH 7.4	Rapid hydrolysis	Stable and slowly hydrolyse in reaction buffers. Free RSV produced; underwentrapid metabolism	Not done	Rapid hydrolysis and metabolism; Not suitable for PK studies
MeO-PEGN-Succ-RSV, 20 kDa	Results indicate conjugate formation with 4'-PEG-RSV as major product	Stable in pH 4.5 and hydrolyse slowly in pH 7.4	Rapid hydrolysis	Not done	Not done	Rapid hydrolysis ;Not suitable for PK studies
MeO-PEGO-Succ-RSV, 2 kDa	Results indicate conjugate formation with 4'-PEG-RSV as major product	Stable in pH 4.5 and pH 7.4	Rapid hydrolysis	Not done	Not done	Rapid hydrolysis; Not suitable for PK studies
MeO-PEG-PLAO-Succ-RSV, 2 kDa	Results indicate conjugate formation	Stable in pH 4.5 and pH	Stable with $t_{1/2}$ of 3 hours	Stable	Slightly better than RSV but	Needs further improvement in

	with 4'-PEG-PLA-RSV as major product	7.4			underwent rapid clearance by RES macrophages	PK profile
MeO-PEG-PLAO-Succ-RSV, 6.5 kDa	Results indicate conjugate formation with 4'-PEG-PLA-RSV as major product	Stable in pH 4.5 and pH 7.4	Not stable in plasma $t_{1/2}$ < 5 minutes.	Not done	Slightly better than RSV but underwent rapid clearance by RES macrophages	Needs further improvement in PK profile biological studies
RSV-PEG ether 2 kDa	Results indicate conjugate formation with 4'-PEG-RSV as major product	Stable	Stable	stable	Better than RSV and other conjugates tested	suitable for further biological studies
RSV-PEG ether 5kDa	Results indicate conjugate formation with 4'-PEG-RSV as major product	Stable	Stable	stable	Better than RSV and PEG-PLA-RSV	suitable for further biological studies

6.3 SCOPE FOR FUTURE WORK

Although significant amount of work was done in this project, still there is a wide scope of research can be undertaken to further understand the properties of conjugates and their correlation with improving pharmacokinetic profile and biological activities of resveratrol. The synthetic procedure adopted in this study has some limitations such as extent of conjugation was not 100% with certain polymers, multiple conjugated species were formed in the reaction and moderate yield of final product, The resveratrol-polymer ester conjugates did not show desired stability in rat plasma. Some of the polymer conjugates such as MeO-PEG-PLAO-Succ-RSV were insoluble in water and needed to be converted into micelles. Based on these limitations, future work should focus on following issues:

- The PEG conjugation to resveratrol via hydrolysable ester link was attempted in this study but the plasma stability was limiting, therefore, reasonably stable PEG esters could not be obtained. In this regards screening various spacer moieties such as cis aconityl, hydrazone, carbamate and peptides could be beneficial and should be considered in the future studies. If sustained hydrolysis of PEG ester is controlled, it will be very useful for pharmacokinetic and biological evaluation.
- The resveratrol conjugates with PLA-PEG needs to be further optimized for PLA/PEG content, Mw and micelle diameter. The micelles with right amount of PLA/PEG and smaller diameter would probably improve plasma residence time of conjugate.
- In the case of resveratrol-PEG ethers, although large improvement in pharmacokinetic profile was achieved, in order to further extend the circulation time and improve plasma level, PEG ethers of higher Mw PEGs need to be synthesized and evaluated. The resveratrol-PEG ethers tested in this study, can be considered for further *in vivo* pharmacological studies such as xenograft mice model to evaluate the translation of improved pharmacokinetic into *in vivo* biological effects.
- Resveratrol polymer conjugates with targeting ligands can be considered for tumour targeting and improved anticancer activity.
- More extensive *in vitro* metabolism studies should be performed to understand the mechanism by which polymers modulate metabolism of resveratrol. The intact polymer conjugate in the microsomal incubation needs to be hydrolysed, after reaction is stopped, to see whether resveratrol bound to polymer is metabolite or

native resveratrol. A suitable process for hydrolysis of polymer conjugate should be developed without causing degradation of resveratrol.

- Although LC-MS analysis was performed for some of conjugates for Mw determination, an LC-MS with greater Mass detection limit should be considered for other polymers in order to obtain Mw of all polymer conjugates.

6.4 REFERENCES

1. Yu C, Shin YG, Chow A, Li Y, Kosmeder JW, Lee YS, et al. Human, rat, and mouse metabolism of resveratrol. Pharm Res. 2002; 19(12):1907-14.
2. Walle T, Hsieh F, DeLegge MH, Oatis JE, Jr., Walle UK. High absorption but very low bioavailability of oral resveratrol in humans. Drug Metab Dispos. 2004; 32(12):1377-82.
3. Miksits M. Antitumor activity of resveratrol and its sulfated metabolites against human breast cancer cells. Planta medica. 2009;75(11):1227.
4. Gao X, Xu YX, Divine G, Janakiraman N, Chapman RA, Gautam SC. Disparate in vitro and in vivo antileukemic effects of resveratrol, a natural polyphenolic compound found in grapes. J Nutr. 2002; 132(7):2076-81
5. Monofaedini C, Veronese FM. Stabilization of substances in circulation. Biconjuate Chem.1998,9, 418-450
6. Veronese FM, Morpurgo M. Bioconjugation in pharmaceutical chemistry. II Farmaco. 1999; 54(8):497-516.
7. Biasutto L, Mattarel A, Marotta E, Bradaschia A, Sassi N, Gabisa S, Zorattit M, Paradisi C.Development of mitochondria-targetted derivatives of resveratrol. Biorg.Med.Chem.Letters, 2008, 18, 5594-5597.
8. Jiang YL. Design, synthesis and spectroscopic studies of resveratrol aliphatic acid ligands of human serum albumin. 2008,16 640-6414.
9. Riley T, Govender T, Stolnik S, Xiong CD, Garnett MC, Illum L,Davis SS. Colloidal stability and drug incorporation aspects of micellar-like PLA–PEG nanoparticles. Colloids and Surfaces B: Biointerfaces 1999. 16 147–159
10. Gref R, Luck M, Quellec P, Marchand M, Dellacherie E,Harnisch S, Blunk T, Muller RH. Stealth corona-core nanoparticles surface modified by polyethylene glycol (PEG): influences of the corona (PEGchain length and surface density) and of the core composition on phagocytic uptake and plasma protein adsorption. Colloids and Surfaces B: Biointerfaces 18 (2000) 301–313

11. Shen, W.C., Ryser, H.J.P. *Cis*-aconityl spacer between daunomycin and macromolecular carriers: a model of pH-sensitive linkage releasing drug from a lysosomotropic conjugate. Biochem. Biophys. Res. Commun. 1981. 102, 1048–1054.
12. Kaneko, T., Willner, D., Monkovic, I., Knipe, J.O., Braslawsky, G.R., Greenfield, R.S., Dolatrai, M.V., New hydrazone derivatives of adriamycin and their immunoconjugates. A correlation between acid stability and cytotoxicity. Bioconjugate Chem. 1991. 2, 133–141.
13. Veronese FM, Schiavon O, Pasut G, Mendichi R, Andersson L, Tsirk A, Ford J, Wu G, Kneller S, Davies J, Duncan R. PEG-Doxorubicin Conjugates: Influence of Polymer Structure on Drug Release, in Vitro Cytotoxicity, Biodistribution, and Antitumor Activity Bioconjugate Chem. 2005, 16, 775-784
14. Li Y , RongQi X, Maitani Y, Nagai T. PEG–PLA diblock copolymer micelle-likenanoparticles as all-trans-retinoic acid carrier: *in vitro* and *in vivo* characterizations. Nanotechnology, 2009,20, 055106
15. Fee CJ. Size comparison between proteins PEGylated with branched and Linear poly(ethylene glycol) molecules. Biotechnol Bioeng 2007 ; 98 : 725 -31.
16. Biasutto L, Marotta E, Mattarei A, Beltramello S, Caliceti P, Salmaso S, et al. Absorption and metabolism of resveratrol carboxyesters and methanesulfonate by explanted rat intestinal segments. Cell Physiol Biochem. 2009;24(5-6):557-66.

CHAPTER 7

APPENDIX

7.1 Additional NMR spectrums of the resveratrol polymer conjugates discussed in chapter 2 and 3.

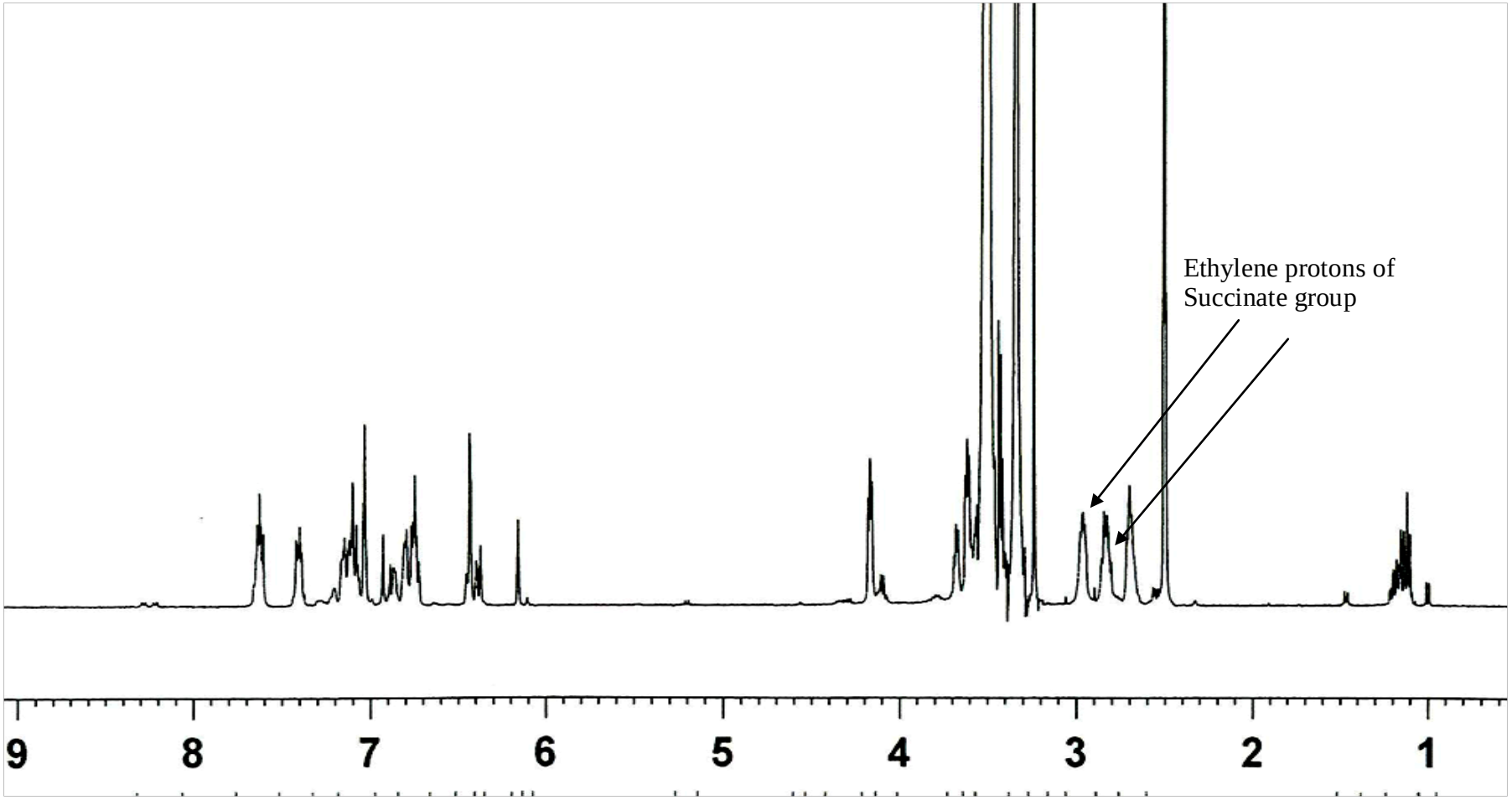


Figure 7.1: ^{1}H NMR spectra of the MeO-PEGO-Succ-RSV 2 kDa conjugate mixture in d_6-DMSO, recorded at 400 MHz.

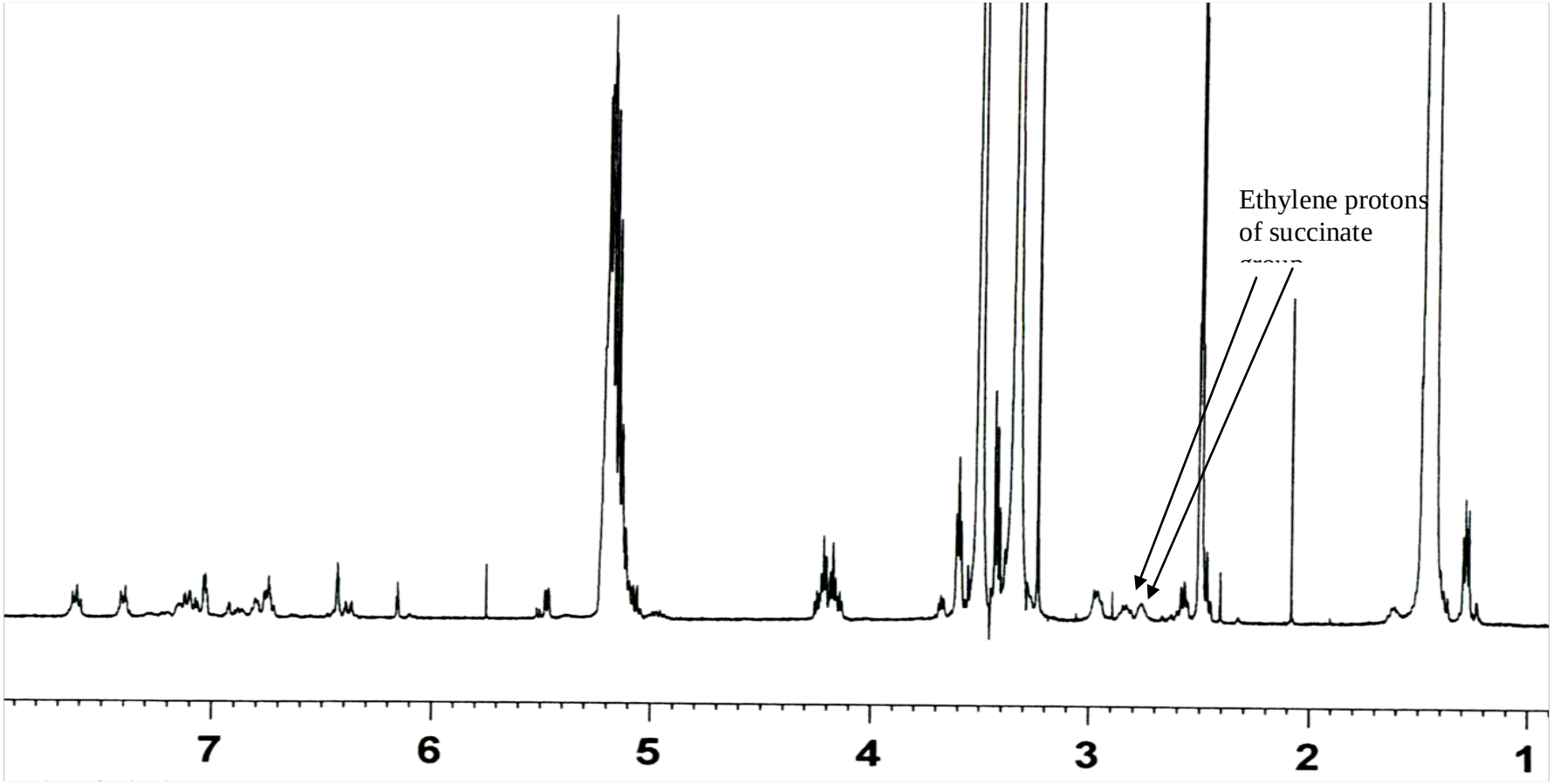


Figure 7.2: ^{1}H NMR spectra of the MeO-PEG-PLAO-Succ-RSV 2 kDa conjugate mixture in d_6-DMSO, recorded at 400 MHz.

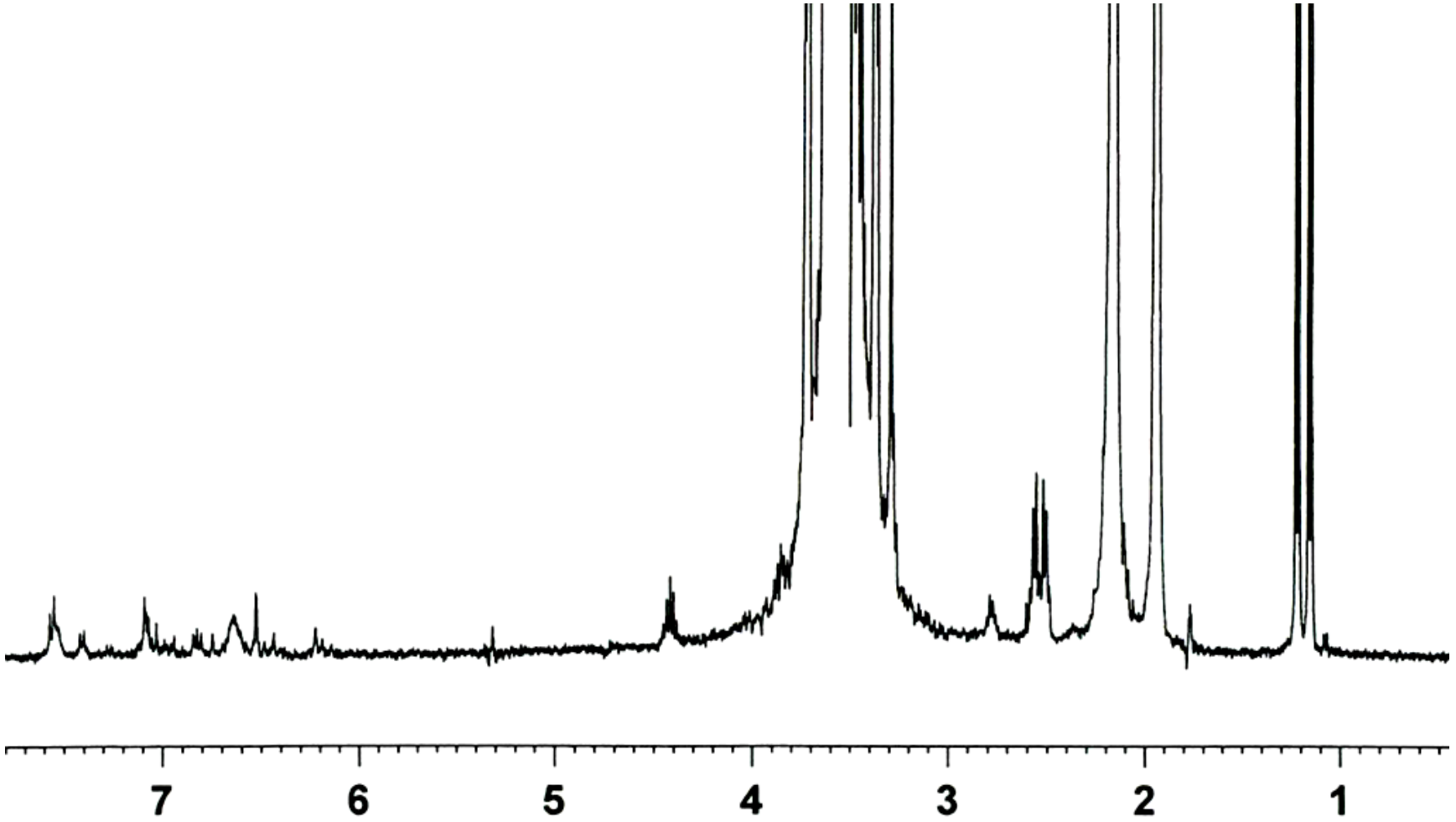


Figure 7.3: ^{1}H NMR spectra of the MeO-PEGN-Succ-RSV 20 kDa conjugate mixture in *CD_3CN*, recorded at 400 MHz.

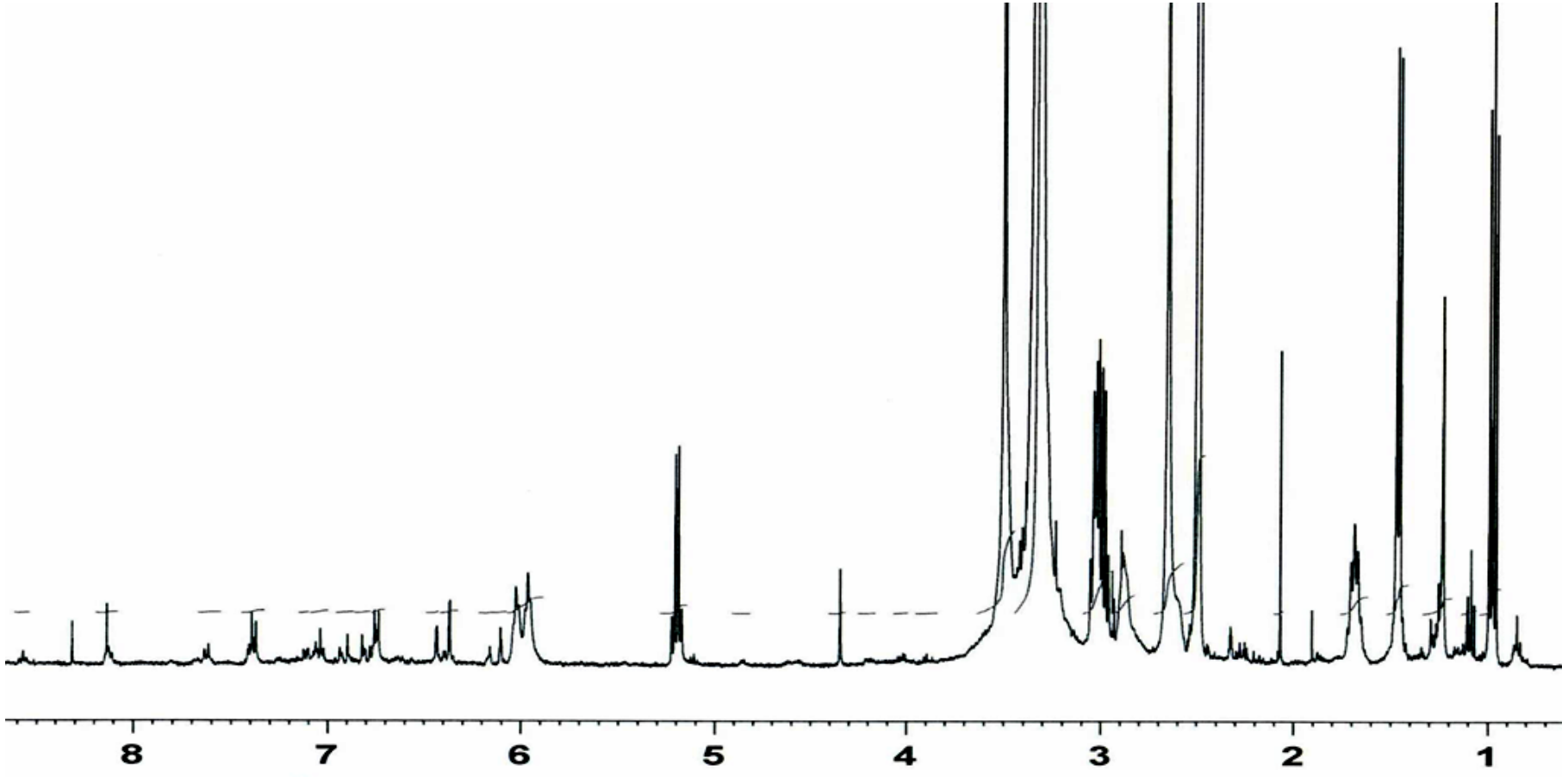


Figure 7.4: ^{1}H NMR spectra of the MeO-PEG-PLAO-Succ-RSV 6.5 kDa conjugate mixture in d_6-DMSO, recorded at 400 MHz.

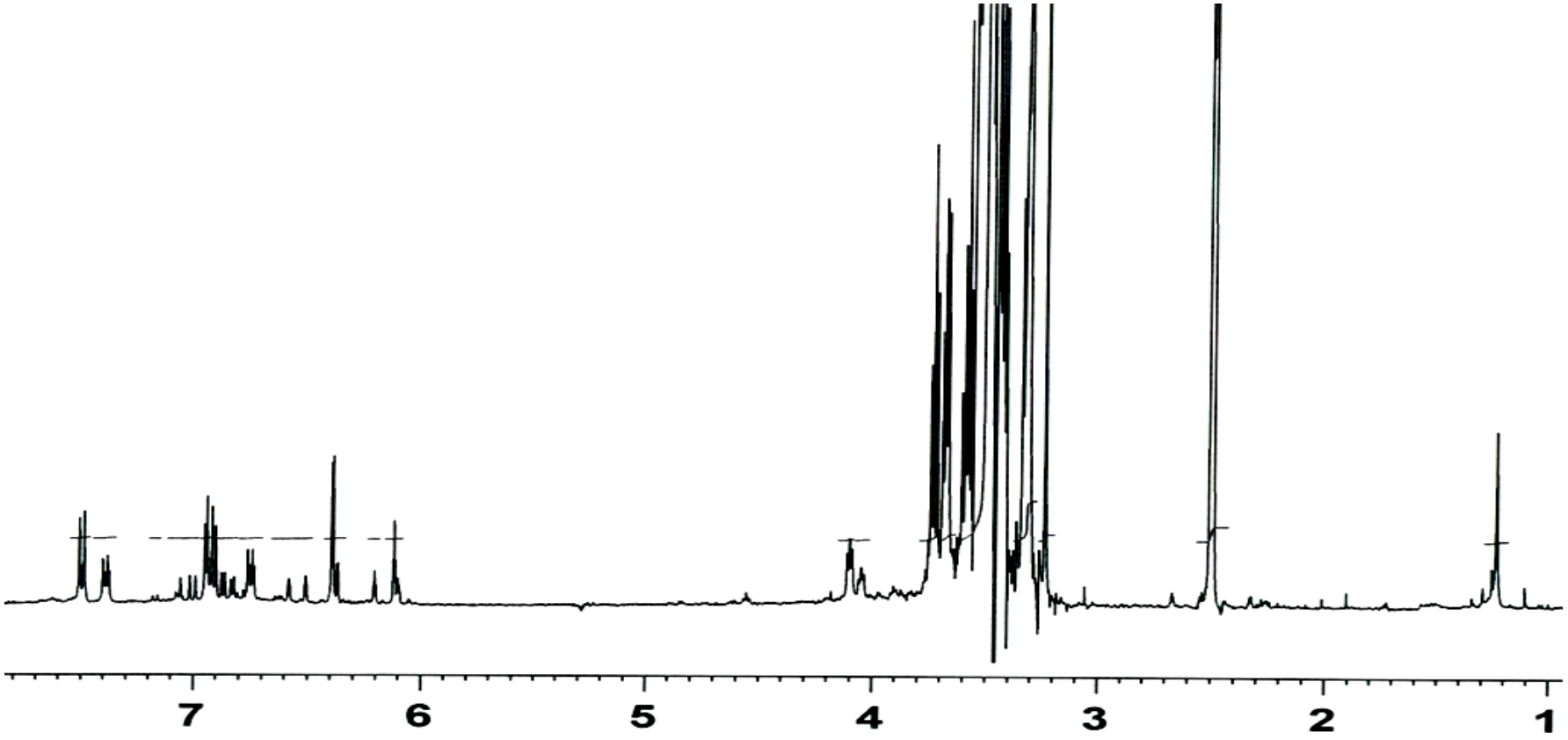


Figure 7.5: ^{1}H NMR spectra of the resveratrol-PEG ether kDa conjugate mixture in d_6-DMSO, recorded at 400 MHz.

7.2 Identification of resveratrol metabolites in rat plasma by LC-MS/MS

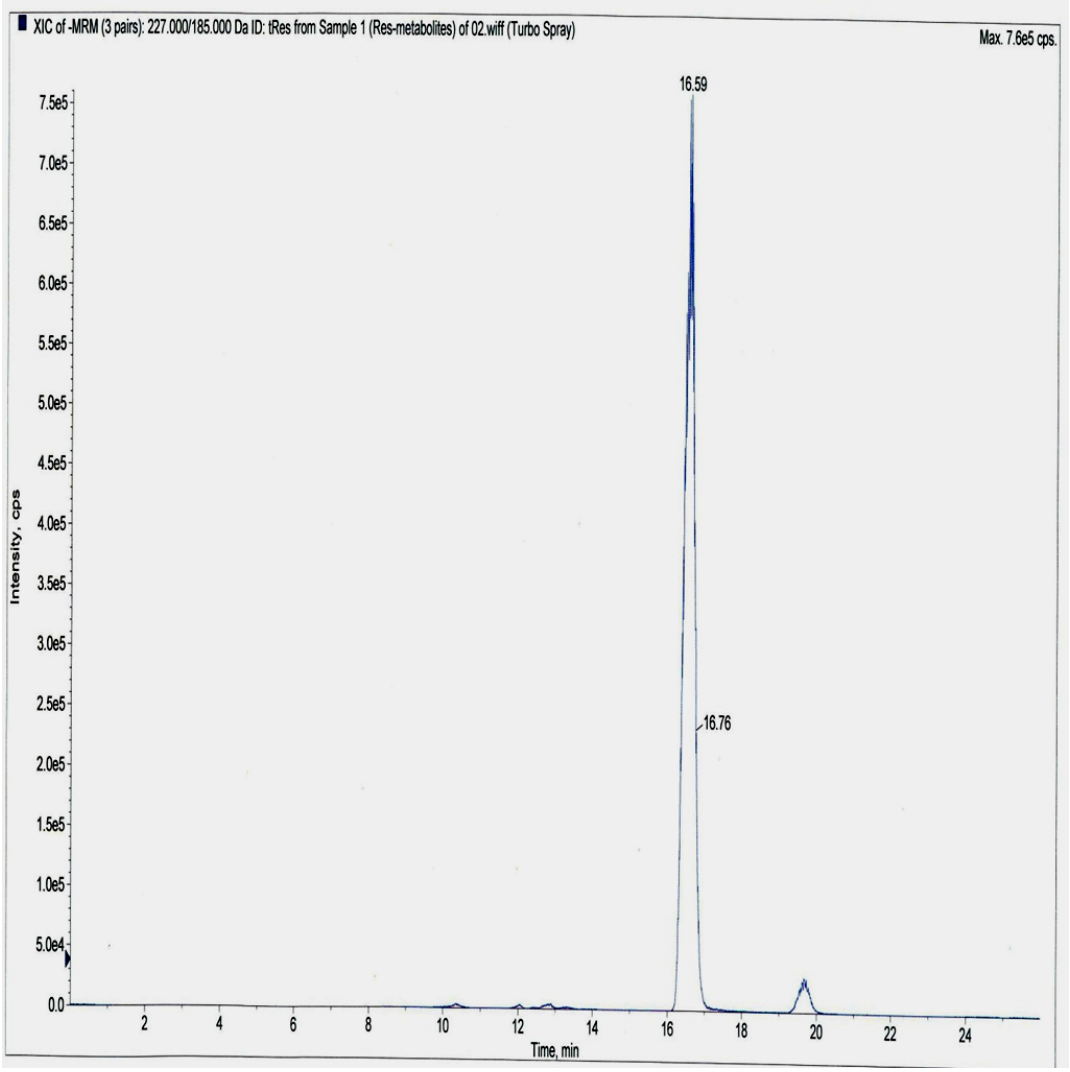


Figure 7.6: LC-MS/MS chromatogram (MRM) of resveratrol, analysis carried out as per section 4.2.5 of chapter 4.

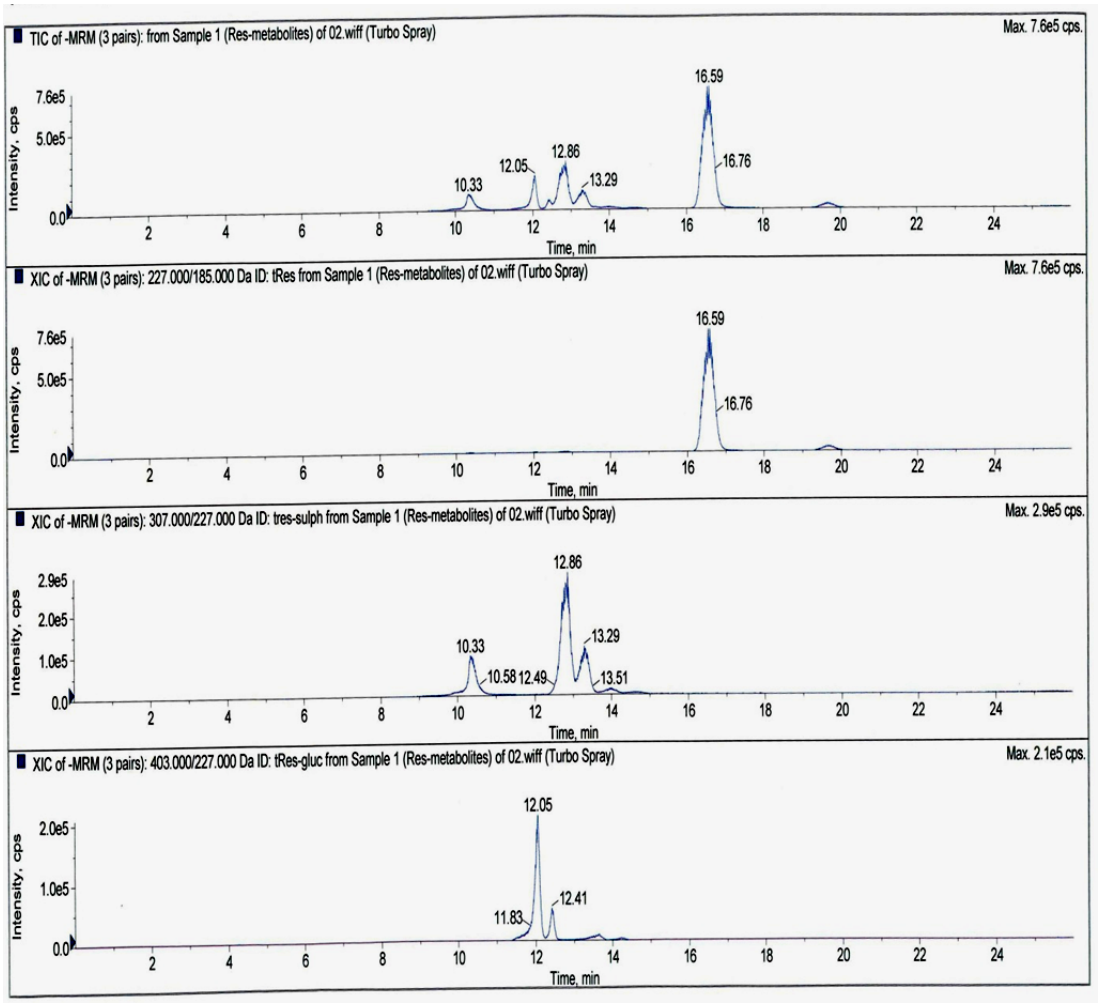


Figure 7.7: LC-MS/MS (MRM) chromatogram of rat plasma sample (obtained as per section 4.2.6) analyzed as per section 4.2.5.

7.3 Data for molecular weight determination of MeO-PEGN-SuccOH and MeO-PEGN-Succ-RSV.

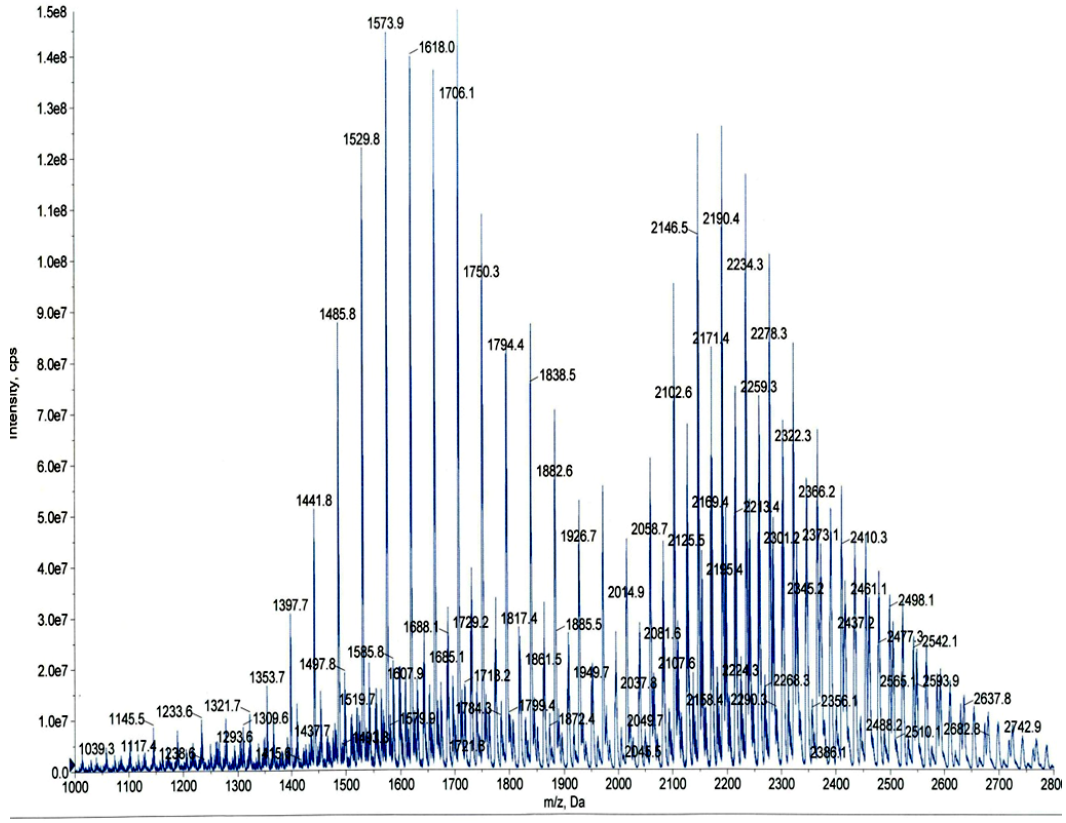


Figure 7.8: Mass spectrum of MeO-PEGN-SuccOH 2 kDa recorded using 0.5% v/v triethyl amine as solvent in LC-MS.

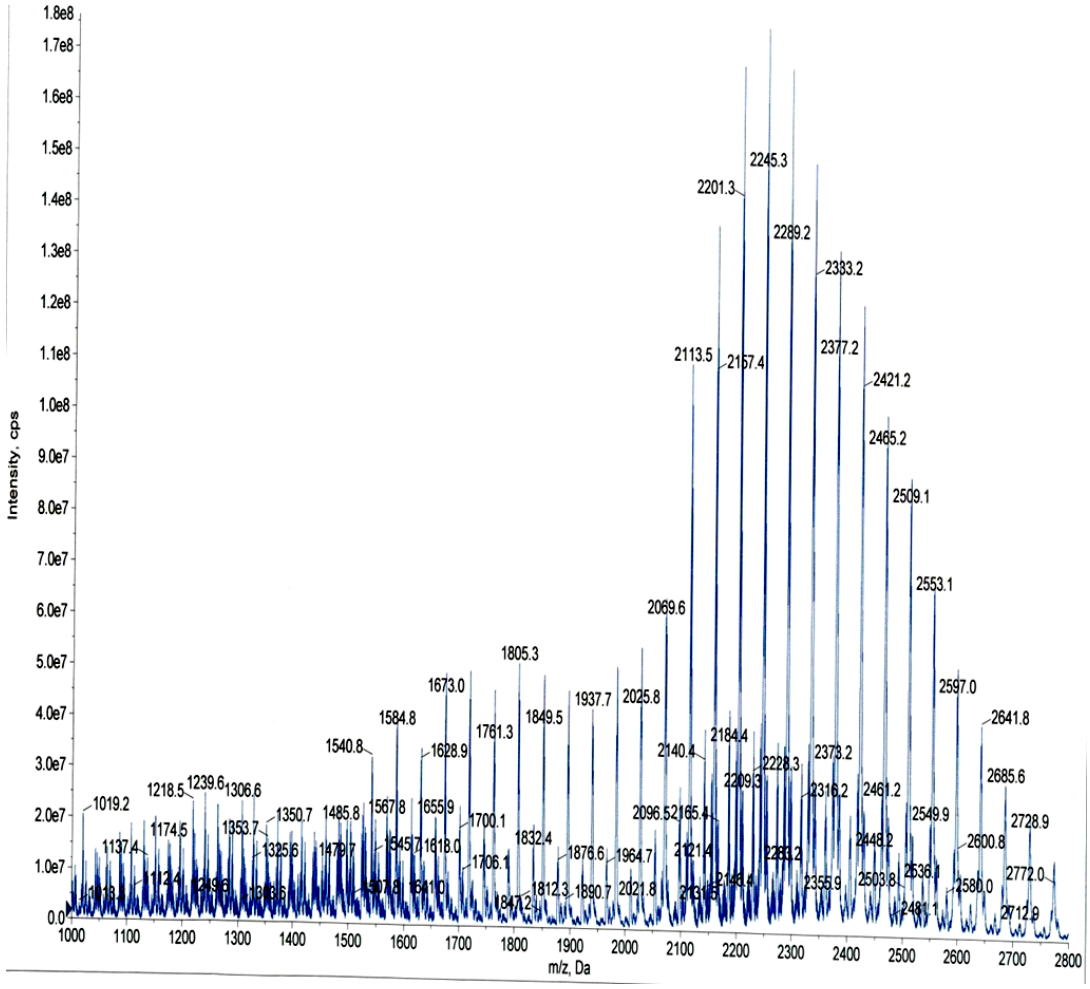


Figure 7.9: Mass spectrum of MeO-PEGN-SuccOH 2 kDa recorded using 0.5% v/v triethyl amine as solvent in LC-MS.

7.4 : CMC values determination by surface tension method

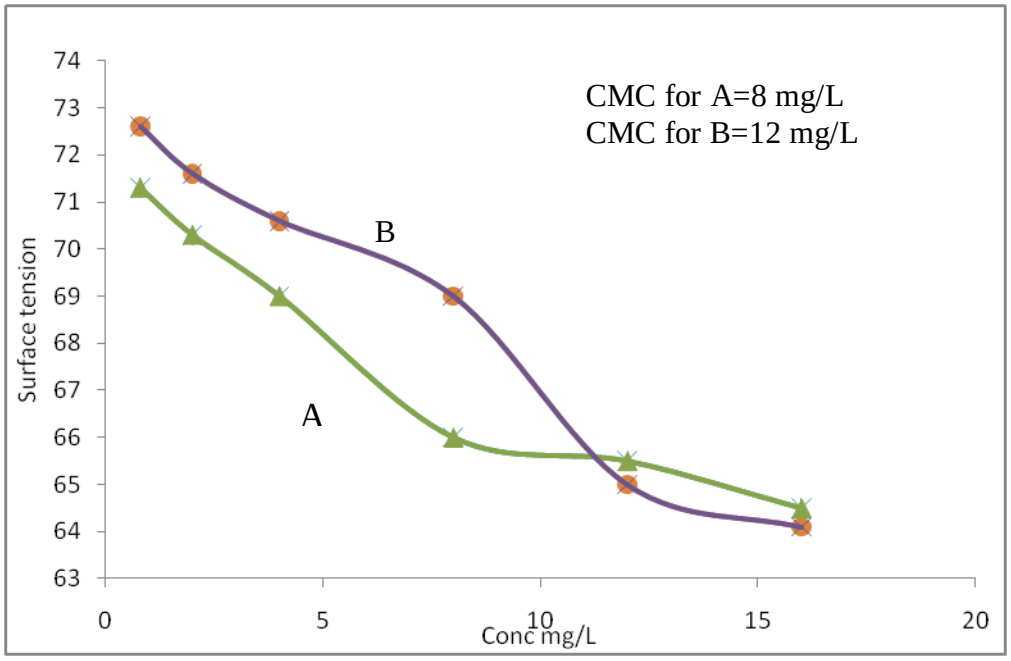


Figure 7.10: Plot of concentration vs suface tension for the determination of CMC value A:MeO-PEG-PLAO-Succ-RSV 2 kDa. B: MeO-PEG-PLAO-Succ-RSV 6.5 kDa

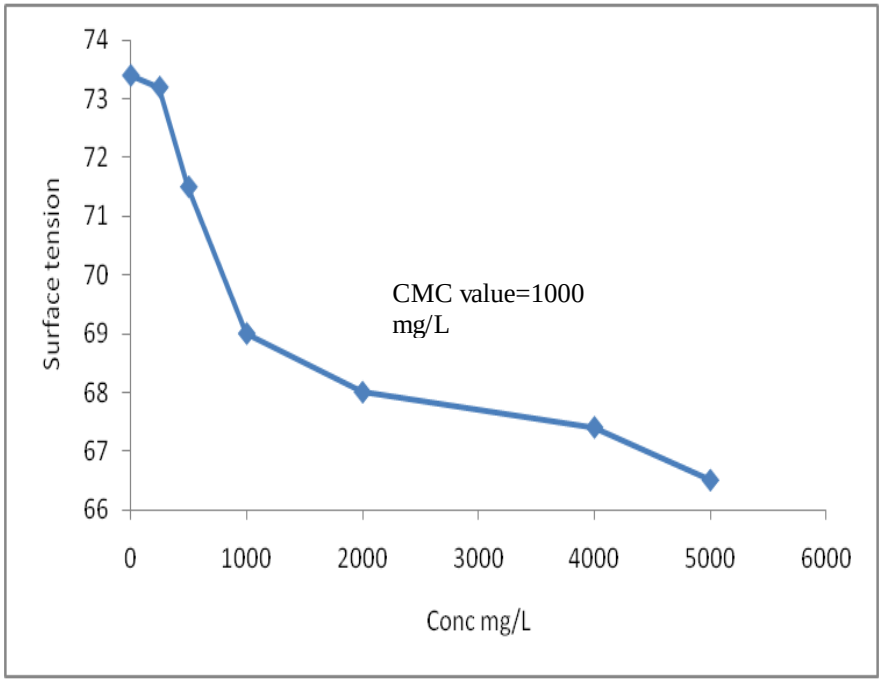


Figure 7.11: Plot of concentration vs suface tension for the determination of CMC for A:MeO-PEG-Succ-RSV 2 kDa.

7.5 Scheme for synthesis of resveratrol-chitosan conjugate

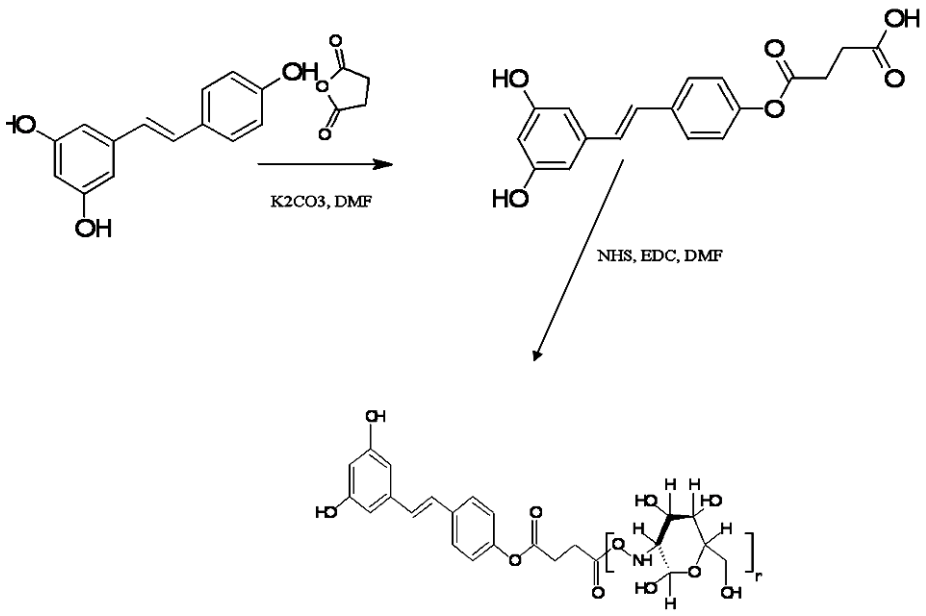


Figure 7.12: Reaction scheme for synthesis of rsveratrol-chitosan conjugates

7.6 BD bioscience protocol for *in vitro* metabolism studies with microsomes

Introduction

Mammalian liver is the principal organ for drug and other foreign compound (xenobiotic) metabolism. Microsomes are prepared by differential centrifugation from a crude tissue (liver) homogenate. Tissue is homogenized in buffer and subjected to a 9000 x g centrifugation. The Supernatant from this centrifugation (often referred to as "S9") is recovered and then centrifuged at 100,000 x g. The pellet from this high speed centrifugation is referred to as "microsomes" and the supernatant is referred to as "cytosol1." The microsomes from this first 100,000 x g centrifugation are resuspended in buffer and centrifuged again at 100,000 x g2. The pellet from this final centrifugation is resuspended in 250 mM sucrose and the protein concentration adjusted to 20 mg/mL. Microsomes are principally derived from the membranes of the endoplasmic reticulum. However, membranes from other cellular organelles are typically present in these relatively crude tissue fractions. Microsomes provide an enriched source of membrane bound drug metabolizing enzymes. Principal among these are the cytochrome P450 (CYP) superfamily of oxidative hemoprotein enzymes and uridine glucuronosyl transferase (UGT) enzymes but many other enzymes are present.

Each lot of material is characterized for the level of activity for a series of CYP, UGT and other enzymes. Use of these products to study metabolism requires fortification with the appropriate cofactors for the enzyme class to be studied. In the case of CYPs, fortification with NADPH is recommended. In the case of UGTs, fortification with uridine diphosphate glucuronic acid (UDPGA) is recommended. UGTs demonstrate the property of "latency" in microsomes. Latency is believed to be due to restricted access of the substrate and/or cofactors to the UGT active site3. Pore forming agents such as alamethicin are used to mitigate the latency of UGT enzymes4.

This *Guidelines for Use* document provides information intended to aid in experimental design. It contains three parts: (1) a discussion of the major components of the assay and the potential influence on assay results, (2) a suggested general assay procedures and (3) further considerations for specific applications.

(1) Components of the Assay

Enzyme: Consult the product insert (batch data sheet) for important product information including CYP content (expressed as pmole enzyme per mg of product protein), protein content (typically 20 mg/mL using the method of Lowry), level of various enzyme activities. All mammalian liver microsomes should be stored at -80oC and thawed rapidly in a 37oC water bath and then stored on wet ice prior to use. BD recommends that if all the material is not to be used at once, that aliquots be prepared to minimize freeze thaw cycles and any potential variability associated with freeze thaw.

Protein concentration is an experimental variable and will vary depending on the application and the susceptibility of the substrate to metabolism. In the absence of any other information, an enzyme concentration of 0.5 mg/mL is a good starting point.

Buffer: CYPs and UGTs are active in a range of buffers of different concentrations. The product insert contains the buffer composition for the QC assay. This is a buffer which BD has found to function well with the specific cytochrome P450 product. Generally, 50 mM to 100 mM Tris HCl (pH 7.5) or potassium phosphate (pH 7.4) work well and these can be prepared by diluting BD Biosciences Cat. Nos. 451202 (0.5M Tris Buffer) or 451201 (0.5 M phosphate buffer). The activity of CYPs and UGTs can vary with buffer and its ionic strength. UGTs have generally been found to be more active in Tris buffers relative to phosphate buffers. A recommended UGT buffer, containing alamethicin is available from BD Biosciences (Cat. No. 451320, 5x concentrate). When evaluating new buffers it may be advisable to test new compositions relative to the buffer used for the QC assay.

NADPH: NADPH cofactor (or some other source of reducing equivalents) must be supplied for CYP activity. We recommend the use of an NADPH generating system which can be prepared from a 1:20

1 The material in BD Biosciences cytosol products are subjected to a second 100,000 x g centrifugation and the supernatant from this second centrifugation is isolated, assayed and packaged. The purpose of this second centrifugation is to reduce any potential contamination with microsomal materials.

2 The purpose of this second 100,000 x g centrifugation of the microsomes is to reduce any potential contamination with cytosolic materials.

3 The active site of UGTs is generally present in the luminal side of the endoplasmic reticulum which becomes the interior of the microsomes upon homogenization. In contrast, CYPs are present on the opposite side of the ER and have freer access to substrate and cofactors.

4 Fisher, M.B. et al., Drug Metab. and Disp. 28 (2000) 560-566. http://dmd.aspetjournals.org/content/28/5/560.abstract TF000017 Rev 1.0 Mammalian Liver Microsomes Guidelines for Use

dilution of BD Biosciences Cat. No. 451220 (Solution A) and a 1:100 dilution of BD Biosciences Cat. No. 451200 (Solution B). An NADPH solution can also be added directly. Typically a concentration around 1 mM is used to mitigate depletion of this cofactor during longer incubations.

UDPGA: UPDGA cofactor must be supplied for UGT activity. We recommend use of an UDPGA cofactor system which can be prepared from a 1:12.5 dilution of BD Biosciences Cat. No. 451300 (Solution A, 25 mM UDPGA) to result in a final UDPGA concentration of 2 mM. UDPGA concentration can be an experimental variable. Different UGT enzymes have differing affinities for UDPGA.

Vessel: A variety of vessels and materials can be used with polypropylene and glass being most commonly used (we recommend polypropylene). Polystyrene plates are commonly used for some assay applications. You should check for compatibility with any organic solvents (e.g. stop solutions).

Solvent: CYPs and UGTs are well known to be inhibited by a variety of organic solvents and the impact of this inhibition will vary depending on the application. For CYP reactions DMSO solvent concentration should not exceed 0.2%. Acetonitrile and methanol are less inhibitory towards CYPs and can have final concntrations of 2% and 1%, respectively. DMSO and methanol are least inhibitory towards UGT isoforms and can be used at final concentration of up to 2% with little impact on activity. If acetonitrile is to be used, a solvent concentration of 1% or less is recommended for UGT reactions.

Substrate Concentration: The tested substrate concentration will vary depending on the application. If metabolic stability is being measured, it is customary to use a low substrate concentration (e.g. 1 uM) based on an assumption that this is well below the apparent Km and the observed rate

approximates the Intrinsic Clearance5. If inhibition of the cytochrome P450 is being measured, it is customary to use a substrate concentration which is near the apparent Km value as this allows easier estimation of the apparent Ki from an IC50. If metabolite formation is being measured for reaction phenotyping or kinetic parameters are being determined, a range of substrate concentrations (above and below the apparent Km value) may be needed.

Assay Linearity: The degree of linearity will vary among substrates and should be determined experimentally for new substrates.

Order of Addition of Assay Components: The combination of substrate, enzyme and cofactors (NADPH and UDPGA) will cause metabolism to begin. We recommend that to initiate metabolism by pre-warming the substrate, buffer and cofactors to 37oC and then adding cold liver microsomes in a small volume of buffer. An alternative approach is to pre-warm the enzyme, substrate and buffer and initiate metabolism by the addition of cofactors. You may wish to compare these two approaches to determine which works best for your specific assay.

Agitation: After an initial mixing (e.g. by pipetting, inverting a sealed tube or vortexing) no further agitation is typically needed.

Stop Solutions: An example stop solution is provided in the batch data sheet QC assay. The stop solution serves two purposes: to inactivate the enzymes and to precipitate the protein so it does not interfere with metabolite analysis. A 0.5x to 2x volume of acetonitrile is commonly used as a stop solution. Acidification of the stop solution with acetic acid (or some other acid) may be needed to control the ionization state of the substrate and metabolite (e.g. for chromatography or mass spectrometry). Protein is typically removed by centrifugation (e.g. 10,000 x g for 3 minutes in microcentrifuge tubes or 4000 x g for 20 minutes in multiwell plates).

Metabolite Analyses: A basic method for metabolite analysis by HPLC separation, fluorometric or spectrophotometric detection is provided in the batch data sheet. The analytical method should be adapted based on the metabolite(s) to be detected.

5 Intrinsic Clearance is the ability of the liver to remove a drug absent other, confounding factors. In *in vitro* assays it is defined as the Vmax divided by the Km.

(2) Suggested General Assay Procedures

Cytochrome P450 and other NADPH Dependent Enzymes

I. Thaw liver microsomes (20 mg protein per mL), NADPH Regenerating System Solutions A & B and keep on wet ice.

II. Prepare 5 mM Substrate in DMSO. Store appropriately based on substrate stability.

III. Also needed, 0.5 M potassium phosphate pH 7.4 (BD Biosciences Cat. No. 451201), acetonitrile, 1.7 mL microcentrifuge tubes, pipettors and 37oC water bath.

IV. Combine the following

i. 713 uL purified water

ii. 200 uL 0.5 M Potassium phosphate pH 7.4 (BD Biosciences Cat. No. 451201, 100 mM final concentration)

iii. 50 uL NADPH Regenerating System Solution A (BD Biosciences Cat. No. 451220)

iv. 10 uL NADPH Regenerating System Solution B (BD Biosciences Cat. No. 451200)

v. 2 uL Substrate in DMSO (10 uM final concentration)

V. Warm to 37oC for 5 minutes in a water bath.

TF000017 Rev 1.0 Mammalian Liver Microsomes Guidelines for Use

VI. Initiate by the addition of 25 uL (0.5 mg) liver microsomes. Mix by inverting the capped tube twice. Return to the 37oC water bath.

VII. After 0, 5, 10, 20, 30, 40, 50 and 60 minutes, withdraw 100 uL from the incubation and add to 100 uL acetonitrile. Mix and place on wet ice.

VIII. Centrifuge 10,000 x g (or higher) for 3 minutes.

IX. Withdraw the supernatant from the protein pellet.

X. Analyze according to your analytical method.

UGT Enzymes

I. Thaw liver microsomes (20 mg protein per mL), UGT Reaction Mix Solutions A & B and keep on wet ice.

II. Prepare 5 mM Substrate in DMSO. Store appropriately based on substrate stability.

III. Also needed acetonitrile, 1.7 mL microcentrifuge tubes, pipettors and 37oC water bath.

IV. Combine the following

i. 693 uL purified water

ii. 200 uL UGT Reaction Mix Solution B (BD Biosciences Cat. No. 451320)

iii. 80 uL UGT Reaction Mix Solution A (BD Biosciences Cat. No. 451300)

iv. 2 uL Substrate in DMSO (10 uM final concentration)

V. Warm to 37oC for 5 minutes in a water bath.

VI. Initiate by the addition of 25 uL (0.5 mg) liver microsomes. Mix by inverting the capped tube twice. Return to the 37oC water bath.

VII. After 0, 5, 10, 20, 30, 40, 50 and 60 minutes, withdrawn 100 uL from the incubation and add to 100 uL acetonitrile. Mix and place on wet ice.

VIII. Centrifuge 10,000 x g (or higher) for 3 minutes.

IX. Withdraw the supernatant from the protein pellet.

X. Analyze according to your analytical method.

7.6.1 Data for Micosomal metabolism studies of RSV-PEG ethers

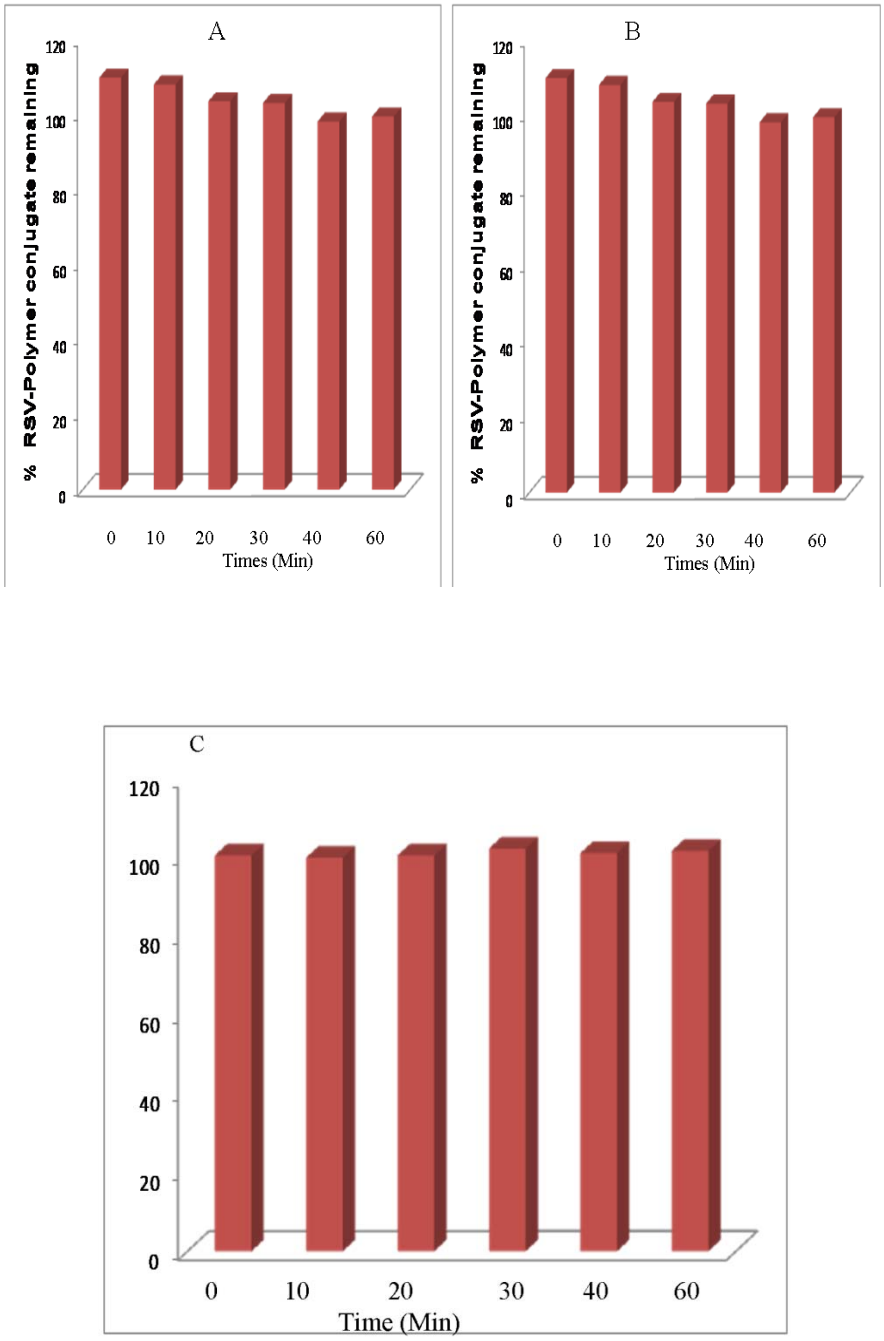


Figure 7.13 Degradation profile various resverarol PEG ethers in microsomal incubations A: RSV-PEG ether 2 kDa with UDPA B: RSV-PEG ether 2 kDa with NDPH regenerating system C: RSV-PEG ether 5 kDa with UDPA

7.7 Pharmacokinetic studies of resveratrol and conjugates in Wistar rats

Table 7.1: Pharmacokinetic data for resveratrol and its polymer conjugates in Wistar Rats (Intravenous dosing)

							Average		
RSV-PEG ether 5 kDa; 2 mg/kg									
Weight (g)	190	210	214	195	202	210		AUClast	8.24671
Dose (mg)	0.38	0.42	0.428	0.39	0.404	0.42	**0.407**	AUCtot	11.5159
Time (hr)	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6		AUMClast	17.3996
0.083	9.678296	10.77793	9.989858	12.22577	12.51901	14.58997	**11.63014**	AUMCtot	72.3483
0.5	1.247804	3.648662	1.559366	1.760965	2.274125	2.457397	**2.158053**	thalf	6.10532
1	0.82628	1.925909	0.936243	0.789626	1.33944	1.46773	**1.214205**	MRT	6.28248
2	0.478064	0.773864	0.643008	0.533045	0.82628	1.156169	**0.735072**	Clearance	0.035343
4	0.459737	0.643008	0.445075	0.239811	0.734644	0.95457	**0.579474**	Vz	0.3113
8	0.5697	0.276465	0.276465	0.166502	0.368101	0.5697	**0.371155**	MRTlast	2.10989
MeO-PEG-PLAO-Succ-RSV, 2kDa; 2 mg/kg								AUClast	3.26876
Weight (g)	200	205	196		210			AUCtot	5.54573
Dose (mg)	0.4	0.41	0.392		0.42		**0.4055**	AUMClast	1.92808
Time	R1	R2	R3		R6			AUMCtot	14.0707
0.083	18.06253	5.624233	2.497831		3.799252		**7.495961**	thalf	2.31013
0.5	2.610022	0.390726	0.8		0.418549		**1.054824**	MRT	2.53722
1	1.607779	0.443979	1.256245		0.620494		**0.982124**	Clearance	0.073119
2	1.271204		0.367315		0.41107		**0.683196**	Vz	0.243694
								MRTlast	0.589852
RSV-PEG ether 2 kDa; 2 mg/kg									
Weight (g)	170	183	210	172	180			AUClast	11.3284
Dose (mg)	0.34	0.366	0.42	0.344	0.36		**0.366**	AUCtot	15.667
Time	R1	R2	R3	R4	R5			AUMClast	32.5119
0.083	6.627187	8.029139	7.727453	6.840142	6.982112		**7.241207**	AUMCtot	96.7324
0.5	1.480781	4.302431	1.480781	1.374303	2.758509		**2.279361**	thalf	4.71485
1	1.143602	2.492316	1.232334	1.285572	3.326389		**1.896043**	MRT	6.17426
2	1.232334	1.551766	0.895155	1.356557	2.04866		**1.416894**	Clearance	0.023361
4	1.232334	1.374303	0.930648	1.25008	1.409796		**1.239432**	Vz	0.158905
8	0.486992	0.486992	0.628962	0.628962	0.957267		**0.637835**	MRTlast	2.86994
Resveratrol; 10 mg/kg								AUClast	0.460658
Weight (g)	214	210	215	208				AUCtot	0.638259
Dose (mg)	2.14	2.1	2.15	2.08			**2.1175**	AUMClast	0.17655
Time	R1	R2	R3	R4				AUMCtot	1.10923
0.083	1.090125	2.641339	0.736049	2.503137			**1.742662**	thalf	2.2538
0.5	0.096885	0.134059	0.039344	0.075673			**0.08649**	MRT	1.7379
1	0.071915	0.118353	0.025069	0.083842			**0.074795**	Clearance	3.31762
2	0.06434	0.041503	0.075223	0.037416			**0.054621**	Vz	10.7874
								MRTlast	0.383256

Note: All plasma concentrations and parameters are in µg/mL

7.8 HPLC peak purity for resveratrol and its polymer conjugate

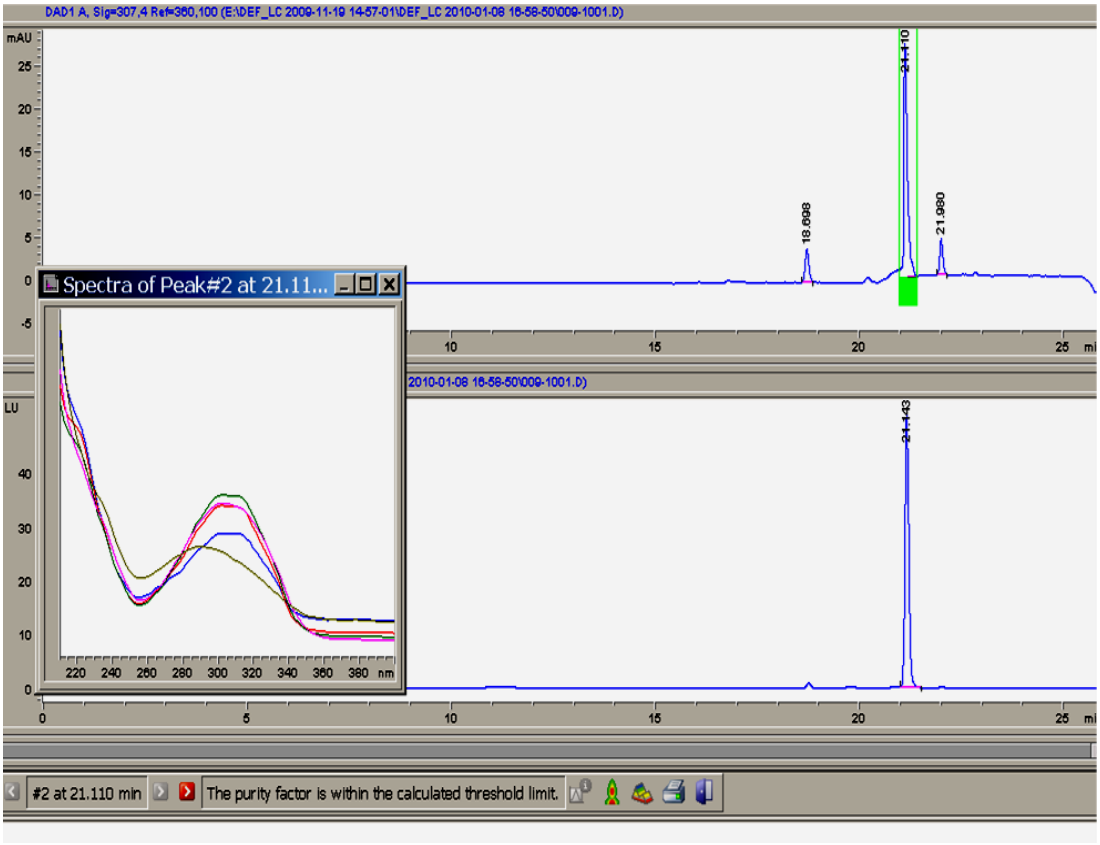


Figure 7.14: HPLC chromatogram with peak purity and spectrum for MeO-PEGN-Succ-RSV 2 kDa.

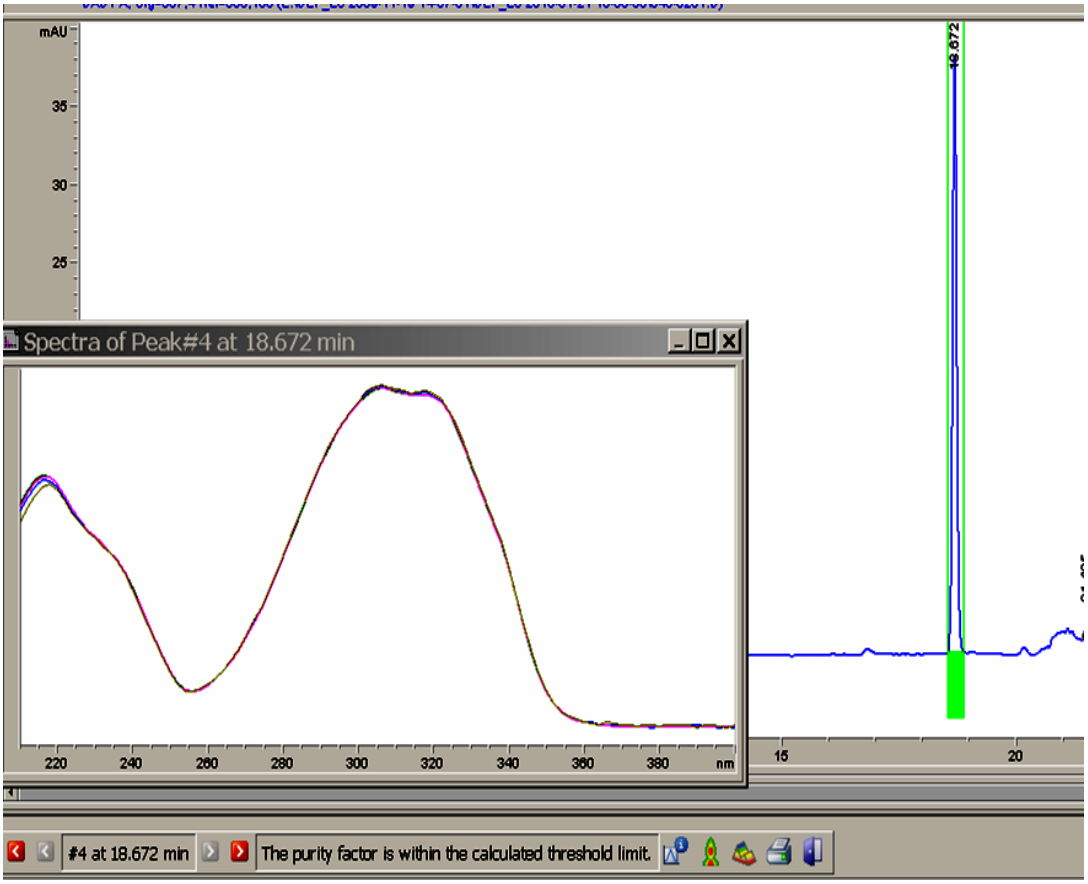


Figure 7.15: HPLC chromatogram with peak purity and spectrum for resveratrol

7.9: Plasma recovery data for resveratrol and its polymer conjugates

Table 7.2 :Plasma recovery of resveratrol

Concentration ng/mL	Average Recovery	SD
50	103.9	2.19
100	103.98	2.88
500	90.62	1.35
1000	99.8	1.49

Table 7.3: Plasma recovery of RSV-PEG ether, 2 kDa

Concentration spiked (µg/mL)	% Recovery against Spiked concentration	SD
5	116.33	2.47
10	105.48	1.77
25	96.80	0.023
50	96.08	0.21

Note: 10 µg of RSV-PEG ether is equal to 1 µg of RSV.

7.10: Source of polymers used with catalogue numbers

Table 7.4 List some important chemicals used and their codes, suppliers

Chemical Name	Purity	Code	Company
mPEG COOH 2 kDa	>99%	PEG1158	Irish biotech, GMBH, Germany.
mPEG COOH 20 kDa	>99%	PEG1159	Irish biotech, GMBH, Germany.
mPEG Br 2 kDa	>99%	PEG1132	Irish biotech, GMBH, Germany.
mPEG Br 5 kDa	>99%	PEG1134	Irish biotech, GMBH, Germany.
mPEG-PLA 2 kDa	>99%	659657	Sigma, Australia.
mPEG-PLA 5 kDa	>99%	AK04	Akina Inc, USA.
Rat liver microsomes (Sprague-Dawley) microsomes	Not applicable	RTMC-PL	Invitrogen, Australia.

Table 7.5: Molar extinction coefficient of Resveratrol and its polymer conjugates

Conjugate name	Molar Extiction Coefficient
Resveratrol	298.7
MeO-PEGN-Succ-RSV 2kDa	217.1*
MeO-PEGO-Succ-RSV 2kDa	127.0*
RSV-PEG ether 2 kDa	150.7*
MeO-PEGN-Succ-RSV 20 kDa	86.2*

*molar absorption coefficients are interms of resveratrol content.

Molar extinction coeffiecient was calculated using formula
A=abc (a is molar extinction coefficient, b is path length, i.e constant 1 cm, c is concentration in moles/L)

7.11 FESEM and AFM images of RSV-polymer micelles

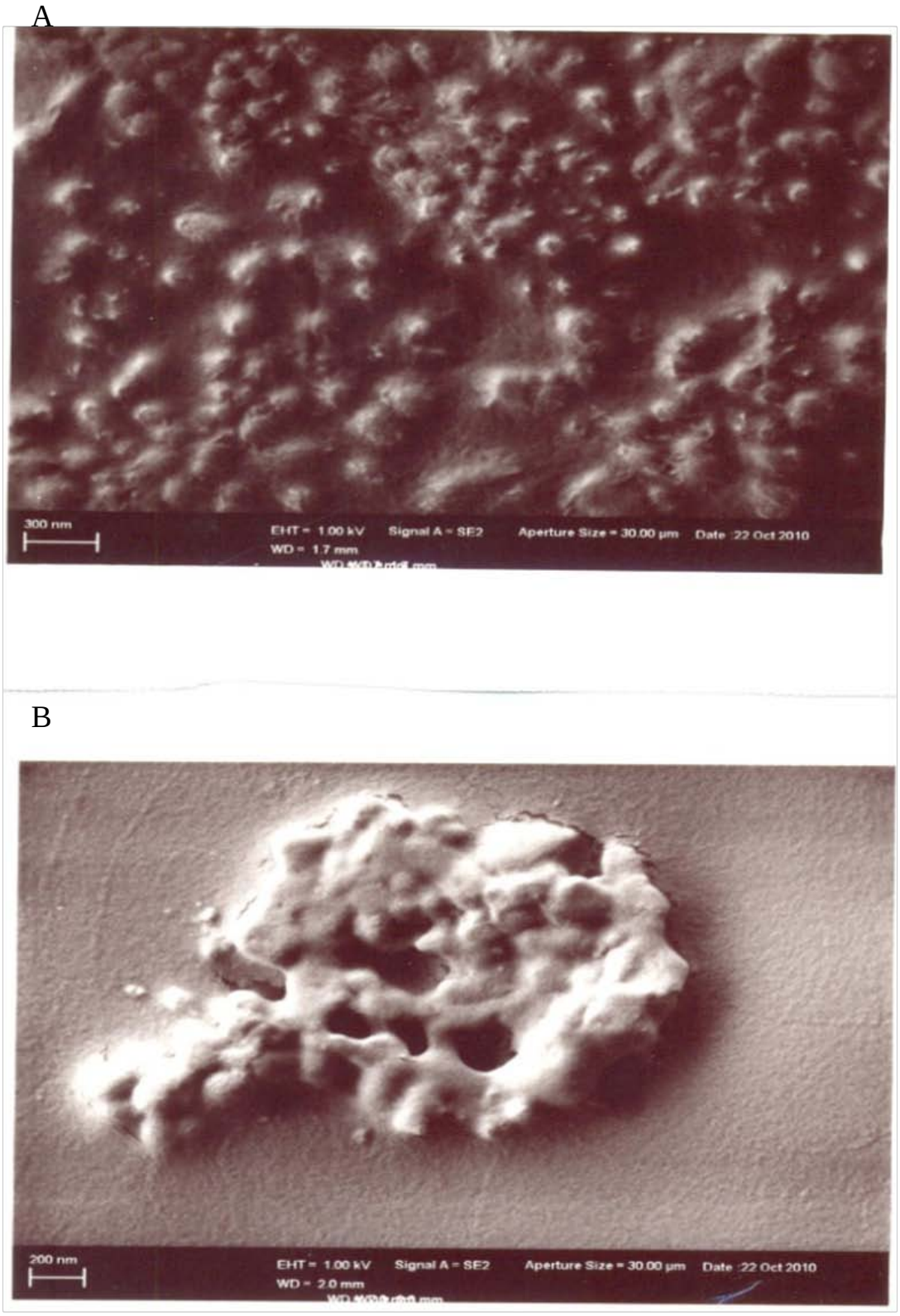


Figure 7.16: FE-SEM images of A: MeO-PEG-PLAO-Succ-RSV 2 kDa micelle solution air dried and B: MeO-PEGO -Succ-RSV 2 kDa micelle solution air dried.

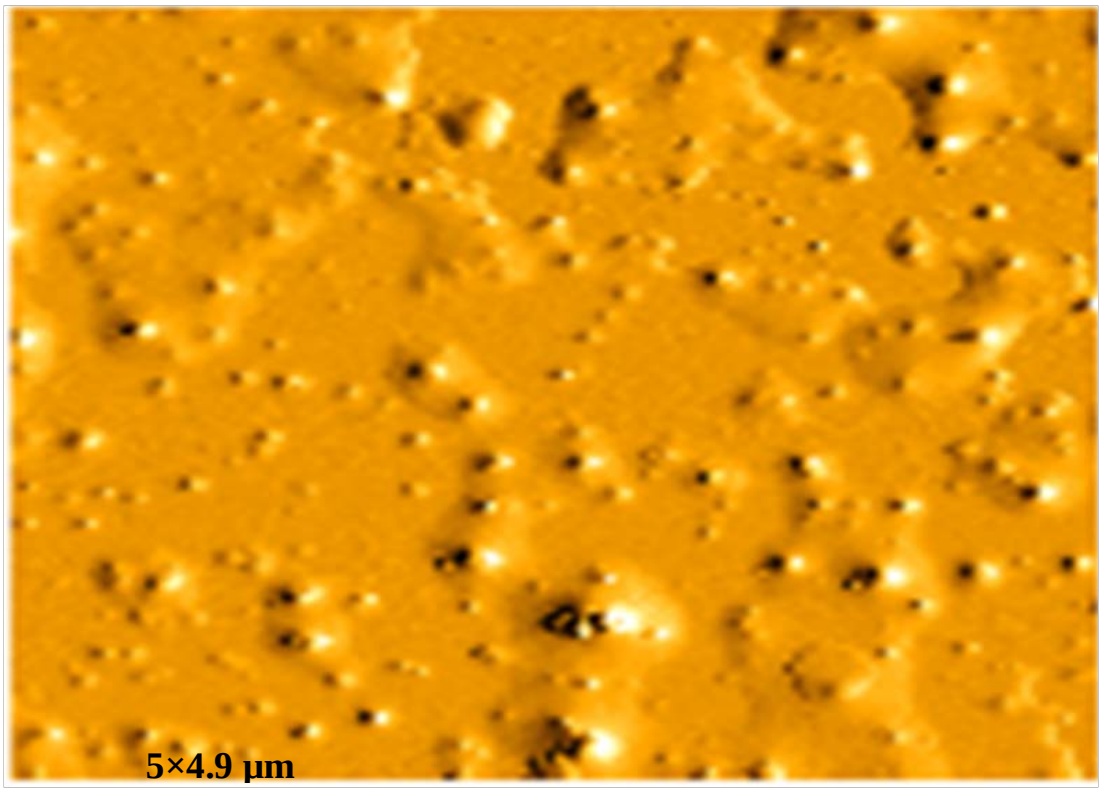


Figure 7.17: AFM images of MeO-PEG-PLAO-Succ-RSV 2 kDa micelles dried under vaccum on mica surface.

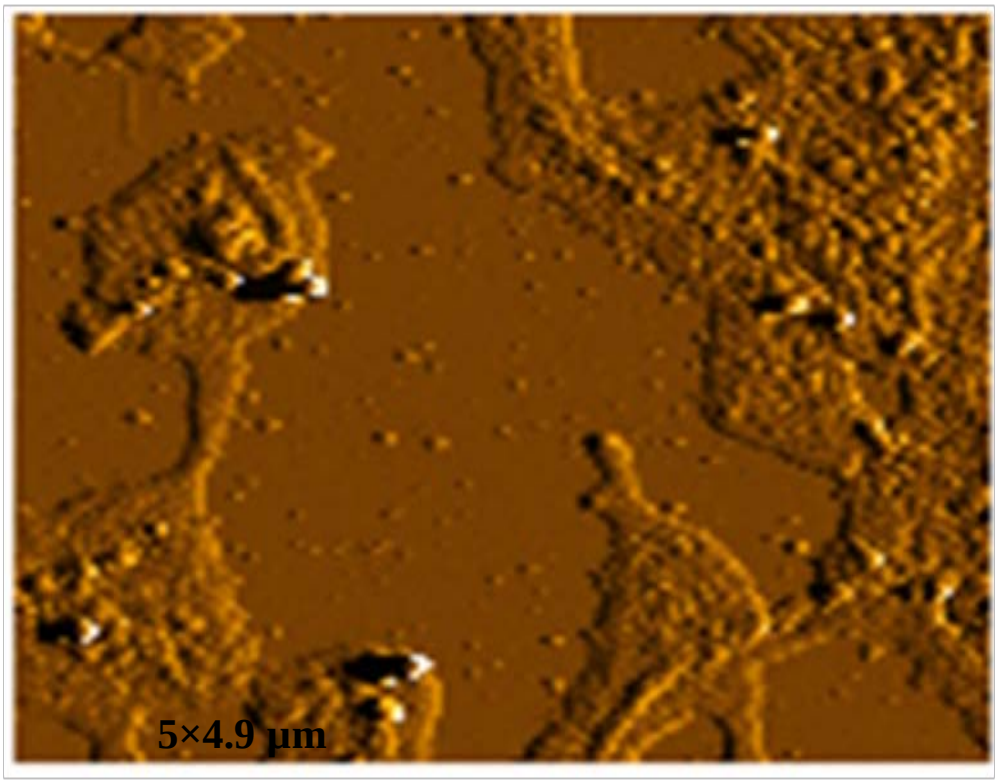


Figure 7.18: AFM images of MeO-PEG-PLAO-Succ-RSV 6.6 kDa micelles dried under vaccum on mica surface.

.

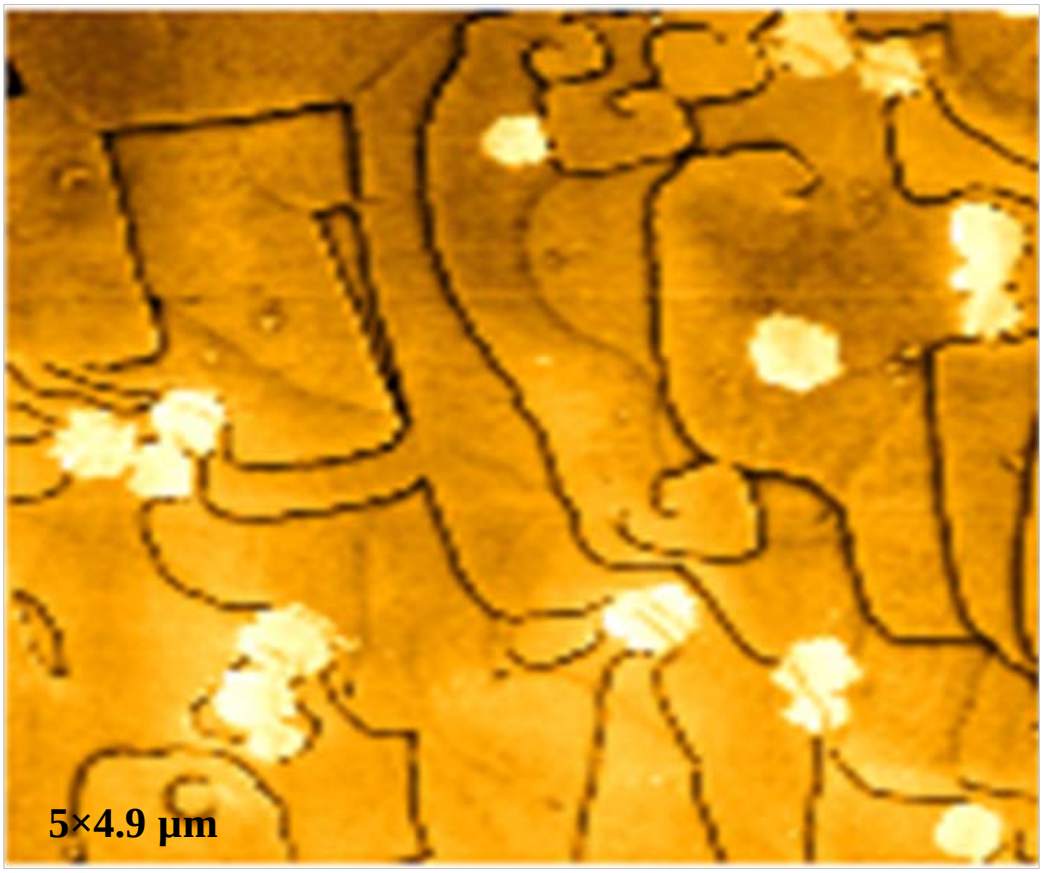


Figure 7.19: AFM images of MeO-PEGO-Succ-RSV 2 kDa micelles dried under vaccum on mica surface.

7.12 List of Publications and posters from PhD student

1. Chen.Y, **Basavaraj.S**, Chan.PHH, Heather.HAE. Development of chitosan nanoparticles for delivery of dalargin. **Biopolymer (Peptide science),** Volume 90 / Number 5, 663-670.

Status: Published

2. **S. Basavaraj**, Heather A.E. Benson, Christopher Cruickshank, David H. Brown, Yan Chen "Development of a LC-MS methodology to separate, detect, characterize and quantify resveratrol-PEG prodrugs and the conjugation reaction precursors and intermediates" Rapid Commun. Mass Spectrom. 2011, 25, 1543–1551**.**

Status: Published

3. **S.Basavaraj** et al. "Novel formulation strategies to improve solubility and pharmacokinetic profile of resveratrol" **Poster accepted for presentation at Product Development Forum-Formulation of poorly soluble drugs, organized by CRS,** to be held on 26-29, Jan 2011 at Miami, Florida, USA.

Status: Presented poster in the conference.

4. **S. Basavaraj**, Heather A.E. Benson, David H. Brown, Yan Chen "Application of solvent influenced fluorescent quenching and enhancement to develop a highly sensitive HPLC methodology for analysis of resveratrol-PEG conjugates" Drafted**.**

5. **Another minimum of two manuscripts yet to be drafted from PhD thesis.**