Preparation, characterisation and in vivo evaluation of DHEA-protein conjugates

by

Sasitorn Kittivoravitkul, B. Pharm, MSc.

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Centre for Drug Delivery Research
The School of Pharmacy
University of London

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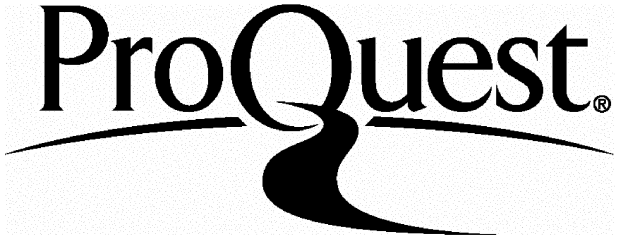

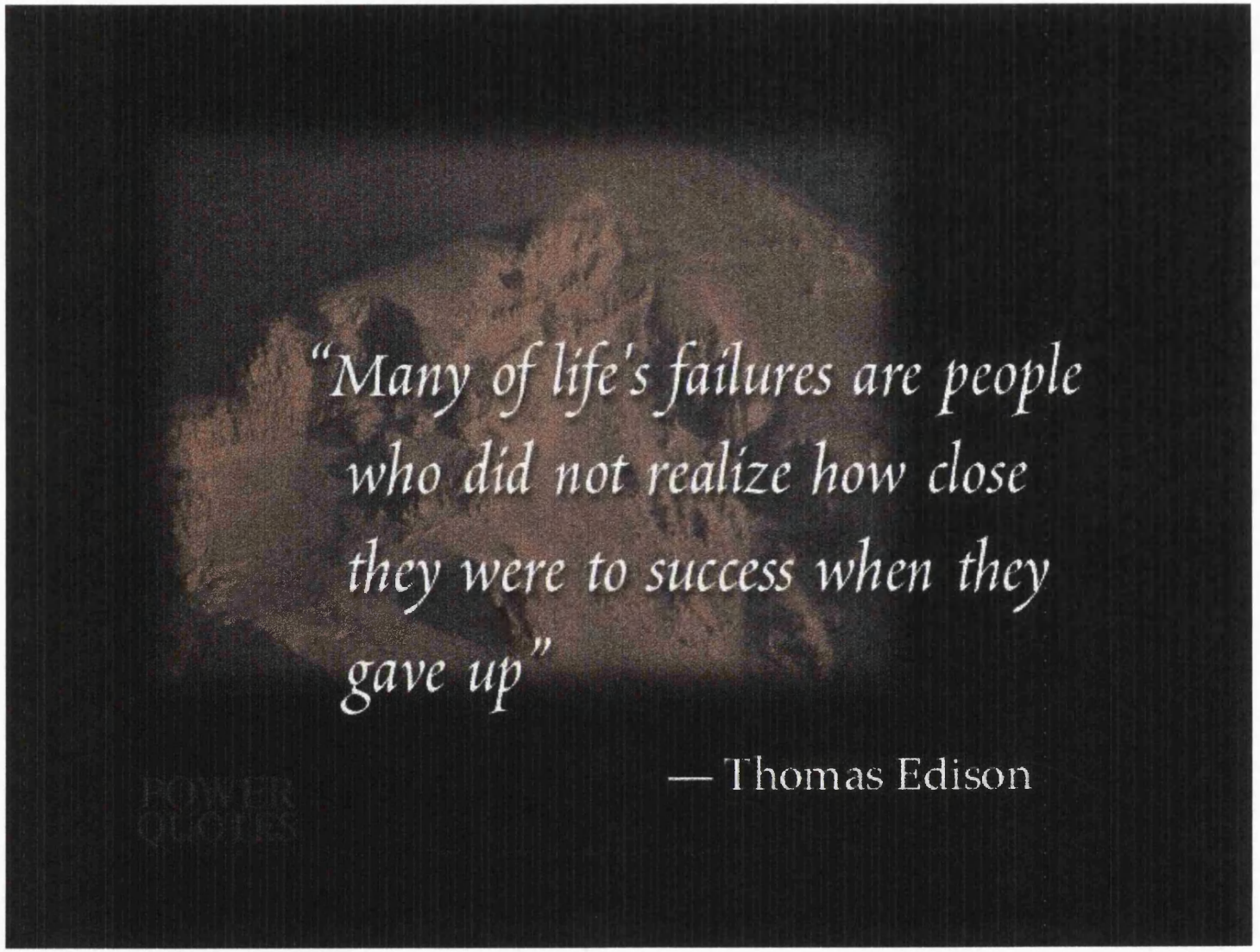
"Many of life's failures are people who did not realize how close they were to success when they gave up"
— Thomas Edison

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Abstracts

Dehydroepiandrosterone (DHEA) is the major secretory steroid product of the adrenal gland, and it has been found that its endogenous level declines with ageing. Exogenous administration has been suggested for cardiovascular diseases, cancer, rheumatoid arthritis, weight loss, diabetes etc. DHEA used here as a model lipophilic drug is highly insoluble in water making its administration parenterally difficult to achieve. In attempt to improve the water solubility of DHEA, it was covalently linked to the ε-amino groups of the lysine residues of natural macromolecular carriers such as immunoglobulin G (IgG) and bovine serum albumin (BSA) through a succinyl spacer which provides an opportunity to formulate soluble DHEA without toxic solubilisers.

The 3'-hydroxy group of DHEA was esterified with succinic anhydride. The evidence for 3'-succinyl-DHEA was obtained by HPLC, UV-visible spectroscopy and mass spectrometry. After the reaction of 3'-succinyl-DHEA with N-hydroxy-3-sulfo-succinimide in the presence of carbodiimide, the carboxylate function on the succinyl spacer produced an active ester which is highly reactive to the lysyl amino groups of the proteins. Methods for the characterisation of the IgG-succinyl-DHEA and BSA-succinyl-DHEA included gel filtration chromatography, UV-visible spectroscopy , the TNBS method, radiolabelling and SDS-PAGE electrophoresis. It was found that the degree of derivatisation (number of drug molecules per molecule of protein) was related to the concentration of 3'-sulfoNHS-succinyl-DHEA in DMF and the ratio of the protein: 3'-sulfoNHS-succinyl-DHEA.

Double radiolabelling was used to follow the two moieties of the conjugates (DHEA and protein) simultaneously for *in vitro* and *in vivo* studies. The conjugates were stable in mouse plasma after incubation for 24h. After intravenous administration, the clearance of the conjugates was evaluated and compared to that of the free drug. The clearance of the conjugates was found to be much slower than that of the free drug in a two compartment model. The pharmacokinetic parameters showed that the clearance of the conjugates was not dependent on the degree of derivatisation. Judging from radioactivity measurements, the tissue distribution study of the conjugates revealed that the conjugates altered the disposition of DHEA and prolonged the retention of DHEA in the tissues as compared with the free drug. The results suggested that IgG and BSA could be used as drug carriers to improve the solubility of insoluble drugs, prolong their circulation in the blood and alter their biodistribution in the tissue.

Table of Contents

List of Figures

List of Tables

Chapter I

Introduction

The clinical use of therapeutic agents is limited by their association to some degree with toxic side effects. In addition, the therapeutic response of drugs is normally diminished by their inability to gain access to the diseased site or the site of action of the drug at an appropriate concentration. In 1906, the great biologist, Paul Ehrlich, described the phrase "the magic bullet" which might selectively direct drugs to their specific site of action. It is clear that achievement of site-specific delivery of drug molecules would be a clear advantage as shown in Table 1.1. It has been argued that for site specific drug delivery to occur, therapeutic systems have to be designed such that they have the functions of site access, and retention, since the issue of timing of release may be critical to successful drug use.

Table 1.1: Rationale for site specific drug delivery

Exclusive delivery to specific compartments (and/or diseases)
Access to previously inaccessible sites (e.g. intracellular infections)
Protection of the body from unwanted drug deposition which could lead to untoward reactions and metabolism, etc
Controlled rate and modality of drug delivery to pharmacological receptor
Reduction in the amount of active principle employed

(Tomlinson, 1986)

Recent advances in molecular biology and in pharmacology have allowed the development of new classes of highly effective therapeutic agents. While drugs are now available to combat a wide range of genetic, malignant, and infectious diseases, the therapeutic efficacy of these agents is often diminished by their inability to gain access to the diseased site at an appropriate dosage. This has followed from an understanding of both anatomical and (patho) physiological opportunities and constraints, as well as from a proper consideration of the nature of the drug's interaction with the disease, target cell responses, dose-response relationship, the intravascular to extravascular localisation required, potential side effects and clinical use of developed therapeutic systems. Table 1.2 gives the properties that appear to be needed for site-specific carriers (Tomlinson, 1987).

Table 1.2: Idealised characteristics of a site specific drug delivery system

Biological factors
Vascular carriage to site of action
Placement at site (via active and passive means)
Epi-and/or endothelial passage
Restricted drug distribution to target site
Drug and host protected from one another
Release controlled by biological processes
Release related to the responsiveness of the target
Drug-related factors
Controlled modality and frequency of release
No premature drug release during transit
Adequate levels of drug carried
Carrier-related factors
Biologically compatible
Biodegradable/excreted
No carrier modulation of the disease
Convenient and cost-effective to prepare and to formulate
System chemically and physically stable in its dosage form

(Tomlinson, 1987)

1.1 Drug Carrier Systems

Carrier systems play a major role in the present attempts to achieve site specific delivery of therapeutic agents. Carriers modulate the pharmacokinetics of the drug by changing its elimination and distribution profile. Many carrier systems have been proposed over the years. The different carrier systems can be roughly divided into three categories: macromolecule, cellular and particulate systems as shown in Table 1.3. Even systems in the same category can strongly differ in their behaviour *in vivo* (Crommelin and Storm, 1998).

Table 1.3: The options of drug carrier systems

Macromolecule	Cellular	Particulate
antibodies	erythrocytes	liposomes
hormones	leukocytes	nanoparticles
lectins	hepatocytes	microspheres
DNA	fibroblasts	emulsions
Carbohydrates		LDL
Polymers		

(Adapted from Gregoriadis, 1978)

The initial study on developing site-specific macromolecular carriers was the radioimmunolocalisation of tumours using antibodies by Ghose and Cerini (1969). This report was followed closely by that of Ghose and Nigam (1972) on the direct linking of chlorambucil to an antibody. In 1971, Rogers and Kornfeld demonstrated the use of asialofetuin to direct chemically bound proteins to the liver. One year later, Trouet et al.(1972) proposed that specific intracellular drug action could occur through endocytosis

of a drug carrier conjugate. Szekerke et al.(1972) reported the use of glutamyl-containing synthetic polypeptides as carriers for cytotoxic drugs and Rowland et al. (1975) proposed polymers interventing between drug and antibody. For particulate carriers, early proposals on the use of carriers are attributable to Sessa and Weissmann (1969); to Gregoriadis et al. (1971) on liposomes , and to Kramer (1976) on monolithic microspheres. Gregoriadis and Ryman (1972) proposed the use of liposomes as carriers of enzymes in the treatment of lysosomal storage disease. Friend and Pandburn (1987) have detailed an excellent study on the use of various type of carriers. Other articles and various reviews detailing the attributes of various particulate and soluble conjugate systems have also appeared (e.g., Gregoriadis (1977), Tomlinson (1983), Poste and Kirsh (1983), Poznansky and Juliano (1984), Friend and Pandburn (1987), Singh et al. (1996), Duncan et al. (1996), Langer (1998), Brocchini and Duncan (1999). An overview of carrier-drug conjugates is given in Table 1.4.

Table 1.4: Overview of drug carrier systems

Carrier	Agents delivered	References
Antibodies	Metrotrexate	Mathe et al., 1958
Antibodies	Chlorambucil	Ghose et al., 1972, 1975, 1978; Rubens and Dulbecco, 1974; Rowland et al., 1975
Antibodies	Adriamycin	Hurwitz et al., 1975
Antibodies	Daunomycin	Arnon and Sela, 1982; Hurwitz, 1982; Hurwitz et al., 1975, 1976, 1978,1979
Antibodies	Diphtheria toxin	Moolten et al., 1972, 1982; Chang et al., 1977[a,b]; Gilliland et al., 1978, 1980 [a,b]
Antibodies	Glucose oxidase	Knowles et al., 1973
Antibodies	α-Glucosidase	Poznansky and Bhardwaj, 1981
Antibodies	Ricin A	Vitetta et al., 1982; Krolick et al., 1982 [a,b] ; Raso and Griffin, 1981; Raso, 1982; Raso et al., 1982
Antibodies	Pseudomonas exotoxin	Fitzgerald et al., 1983
Antibodies	Doxorubicin	Trail et al., 1997
Albumin	5-fluorodeoxyuridine	Balboni et al., 1976
Albumin	Antifungal agents	Fiume et al., 1979
Albumin	Uricase	Paillot et al., 1974
Albumin	Methotrexate	Magnenat et al., 1969; Harding, 1971; Chu and Whiteley, 1977; Yoon et al., 1991
Albumin	α-Glucosidase	Poznansky and Bhardwaj, 1981
Albumin	Asparaginase	Poznansky et al., 1982; Nerker and Gangadharan, 1989
Albumin	Paclitaxel	Dosio et al., 1997
Albumin	Mitomycin C	Tanaka et al., 1995; Kaneo et al., 1990
Albumin	Doxorubicin	Ohkawa et al., 1993
Albumin	Folate	Shinoda et al., 1998
Dextran	α-Amylase	Marshall et al., 1977
Dextran	Asparaginase	Benbough et al., 1979; Wileman et al., 1983, 1986
Dextran	Catalase	Marshall et al., 1977
Dextran	Superoxide dismutase	Miyata et al., 1988
Dextran	Uricase	Fujita et al., 1990
Liposomes	Amphotericin B	Taylor et al., 1982; Lopez-Berenstein et al., 1983
Liposomes	Cancer drugs, antimicrobial agents	Kirby and Gregoriadis, 1999; Perez-Soler and Priebe, 1990; Perez-Soler et al., 1993
Liposomes	Vaccines	Gregoriadis, 1990
Liposomes	Gene therapy	Kirby and Gregoriadis, 1999
Liposomes	Doxorubicin	Forssen and Tokes, 1983; Rahman et al., 1985; Rahman et al., 1990; Gregoriadis, 1995

1.2 Macromolecule-Drug Conjugates

Macromolecule drug carrier systems have been developed in order to alter the tissue localisation of drugs; therefore, the effects of drugs at the desired site of action are enhanced while preventing any interaction with non-target sites. Drugs and hormones conjugated to natural and synthetic macromolecular carriers are receiving widespread attention and have numerous advantages (Gregoriadis, 1979; Juliano and Layton, 1980; Gregoriadis et al, 1982; Venter, 1982; Takakura and Hashida, 1996). The possible advantages for the use of such macromolecular conjugates can be divided into the following categories (Table 1.5).

Table 1.5: Rationale for the use of macromolecules as carriers

Pharmaceutical

- Stabilisation of the drug in its active form.
- Increased solubility, which facilitates the acceptability of the drug.
- Improving retention of drugs in vesicle carriers and prolonged release of the drug at some desired site.
- In vitro loading of drugs into cellular drug carriers.

Pharmacokinetic

- Increased circulation half-life by restricting the metabolic and immune attack and renal excretion of the drug.
- Confinement of the drug to a chosen compartment.
- Targeted distribution to the specific site, increasing the activity to the desired site and minimising the exposure to the other tissues.

Pharmacodynamic

- Cell-specific interaction and overcoming of drug transport barriers, which enables the achievements of the ultimate specificity.
- Selective activation at the target site.

Others

As a tool for drug mechanism studies such as " target site" and drug resistance studies.

(Sezaki and Hashida, 1985)

In contrast to these advantages, there are objections and problems in their potential clinical usage, the most obvious one being the occurrence of adverse immunologic reactions. Prolonged and unwanted retention in the body is another serious problem.

1.3 Barrier to Selective Drug Delivery

The goal of any controlled drug delivery system is to assure the transit of drug molecules from the circulation or other site of administration to a specific locus (or receptors) on or within a particular population of cells within a particular organ or tissue. There are a multitude of physiological barriers standing between a drug-carrier complex in the circulation (or other body compartments) and the ultimate target of the drug within a cell. A detailed understanding of the physiological barriers will allow the development of carrier systems that are modified to deliver a drug to the target tissue or organ.

1.3.1 The Endothelial Barrier

The vascular system provides the mechanism by which oxygen, nutrients and hormones are supplied to the tissues. The exchange between blood and tissues occurs in capillaries and postcapillary venules of the microvasculature. The endothelium is the main component of the wall of capillaries and postcapillary venules and represents an active and selective barrier for fluid, solutes, macromolecules and white blood cells between the blood and the tissue. Extravasation of molecules is actively and selectively

controlled by the endothelium and varies in different parts of the body (Hinsbergh, 1992). In humans, the total surface area of the capillaries bed system has an estimated area of 60 m^2 (Simionescu et al., 1973) and the capillary endothelium is a single thin (0.2-0.5 μm) layer of endothelial cells (Hammersen and Hammersen, 1985). Fig. 1.1 shows the difference in the endothelial lining of the blood vessels at different sites of the body under physiological conditions. This endothelial barrier may interfere with the efficiency of a drug , on the other hand, the differences in the various endotheria of the body may be used to address a drug to a specific target organ.

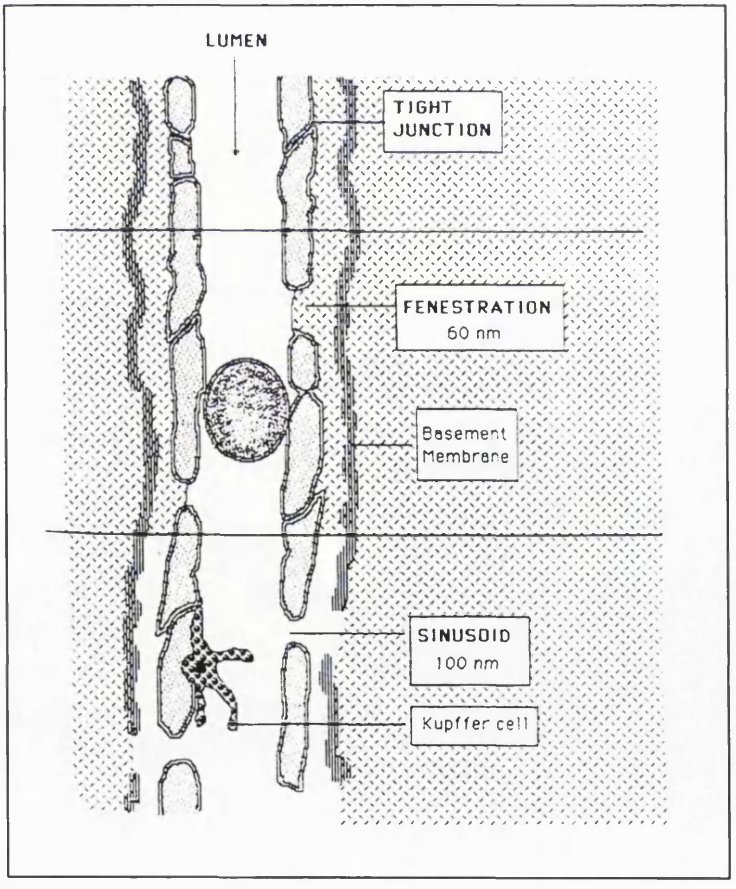

Fig. 1.1 *The major types of endothelium (Davis and Illum, 1986)*

In the design of drug delivery systems, understanding of how the drug-carrier complex might leave the vascular space seems essential. The following describes the potential pathways for the transfer of macromolecules across capillary endothelium (Fig. 1.2 and Table 1.6).

Table 1.6: Potential pathways for the transfer of various nutrients, fluids and macromolecules across the endothelium (see also Fig. 1.2)

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1) Direct diffusion through the two plasma membranes and the cytoplasm. Oxygen can be exchanged in this way.
2) Lateral diffusion of small lipid molecules through the lipid bilayers of the intercellular junctions.
3) Exchange of fluid and solutes through the narrow slits of intercellular junctions. The permeability is regulated at the tight junctions and probably by a fibre matrix that fills the junctional areas.
4) Bulk exchange of solutes and macromolecules via vesicles.
5) Exchange of solutes and macromolecules via transendothelial channels, which are formed by several fused vesicles.
6) Receptor-mediated transcytosis (active transport and facilitated diffusion) of macromolecules and essential nutrients via specific carrier proteins.
7) Exchange of fluid, solutes and possibly macromolecules via fenestrae with closed diaphragms.
8) Exchange of fluid, solutes and macromolecules via open diaphragms. The basal membrane underneath these openings can prevent the escape of many macromolecules by sieving and electrostatic repulsion.
9) Bulk exchange of solutes and large macromolecules via gaps in sinusoidal endothelial cells of liver and via intercellular gaps in postcapillary venules upon inflammatory activation.

(Hinsbergh, 1992)

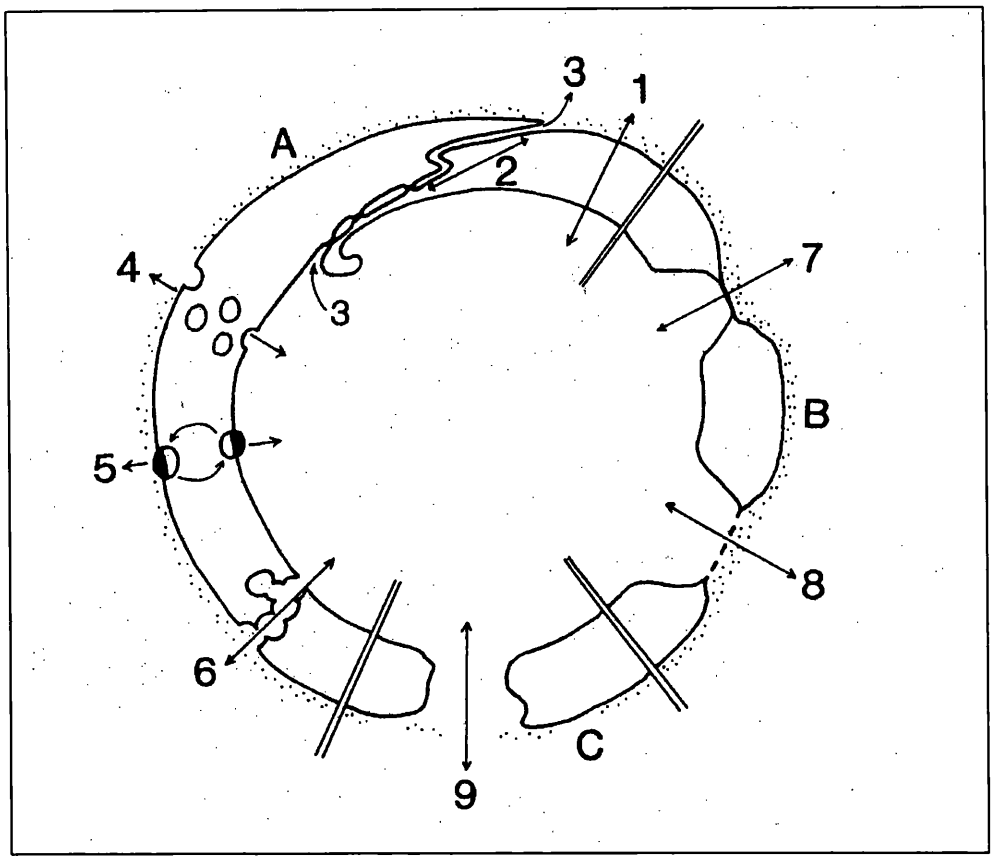


Fig. 1.2 Potential pathways for the transfer of various nutrients, fluids and macromolecules across the endothelium (see also table 1.6)

1.3.1.1 Continuous capillaries

The most common capillary barrier is the continuous endothelium where the cells are close to each other with tight junctions between adjacent endothelial cells. The cells are extremely thin at their peripheral (to less than 0.3 μm) and only thickened in the region of nucleus. their surface is covered with a thin glycolyx which is negatively charged. The subendothelial basement membrane of the continuous endothelium is also continuous. A continuous endothelium is found in most tissues such as the capillaries of all muscles and connective tissues.

1.3.1.2 Fenestrations

Fenestrations are circular transendothelial openings in the endothelial cell membrane that may be either open or covered with a diaphragm. Open fenestra are typical of the renal glomerular capillaries where the openings are 600-800 °A in diameter or large enough to permit free permeation of blood-borne macromolecules. The glomerular capillary is unusual, however, in that the basement membrane is thick enough to pose a barrier to the permeation of plasma proteins and macromolecules (Deen et al., 1983; Levick and Smaje, 1987). Other visceral capillary endothelia may exhibit closed or diaphragmed fenestra which, like capillary endothelia with open fenestra, are highly permeable to water, ions, and small molecules (Levick and Smaje, 1987). In fenestrated endothelia, the channel may be diaphragmed on both sides (Clementi and Palade, 1969). The capillary endothelium of a given tissue (e.g., intestine) may also exhibit mixtures of both closed and open fenestra (Simionescu and Simionescu, 1983). The diaphragm of a closed fenestration is 40-60 °A thick. Although the chemical nature of the diaphragm is not entirely clear, the luminal surface of this structure is known to be coated with heparan sulphate proteoglycans, giving the diaphragm an electronegative charge (Simionescu et al., 1984). Due to the existence of a diaphragm, closed fenestrated capillary endothelia share similar permeability characteristics with respect to macromolecules. Endothelial cell fenestrations with diaphragms are considered permeable to macromolecules under certain conditions. Cationic macromolecules, for instance, may be adsorbed on the surface of a fenestral diaphragm and subsequently cross the diaphragm. Polycationic substances may also neutralise the diaphragm's net negative charge, altering the overall permeability characteristics of the closed fenestra (Clementi and Palade, 1969; Granger et al., 1986).

1.3.1.3 Sinusoidal endothelium

Sinusoidal endothelium is found in the liver and the spleen, where the basal membrane is either absent or present as a fragmented interrupted structure, the fenestrae between endothelial cells are large and reach dimentions in the 100-200 nm size range . Relatively large molecules and particles, such as low density lipoproteins, can exchange easily between the blood and the space of disse underneath the endothelium and can then be taken up by the liver parenchyma. Only very large particles such as chylomicrons are excluded and have to be partly catabolised in peripheral tissues before their remnants gain access to the hepatocytes. However, liver sinusoidal endothelial cells are very rich in receptors, coated pits and vesicles, which are involved in the removal of specific and modified molecules from the circulation (Davis and illum, 1986; Crommelin and Storm, 1998; Hinsbergh, 1992).

1.3.1.4 Intercellular Junctions

The permeability of capillary endothelial intercellular junctions varies with tissue location (Renkin, 1988). Interjunctional diffusion or filtration of macromolecules will be limited by the effective molecular size of the particular substance. From physiological studies, it was found that the passage of small water-soluble molecules proceeds via intercellular junctions in continuous endothelia (except at the blood brain barrier and similar very tight endothelia where glucose and amino acids are transported via specific receptors).Generally, fenestrated and continuous endothelium of peripheral capillaries have intercellular junctions permeable to substances with apparent effective diameters of approximately 40-90 °A (Renkin, 1979,1988; Palade et al.,1979). For reference, serum

albumin (molecular weight ≈ 69000), with an effective diameter of about 72 °A, does not readily diffuse across most capillary endothelia. This is a critical feature of endothelia since albumin is a circulating protein important physiologically for maintaining the osmotic balance between blood and tissues (Audus and Borchardt, 1991). Interjunctional filtration or diffusion of macromolecules through enthothelia may be influenced by conditions aside from simply the size limitations imposed by the width of the junctional opening. For example, lateral diffusion of plasma membrane-associated lipophilic macromolecules (e.g., chylomicrons) through intercellular junctions has been suggested (Scow et al., 1976). Under certain pathophysiological conditions, vasoactive substances may stimulate intercellular junction alterations causing leakiness to macromolecules and water and subsequently vasogenic edema (Svensjo and Grega, 1986). Small lipophilic molecules can exchange by lateral diffusion via the intercellular junctions (Blanchette-Mackie and Scow, 1981), but may also be partly carried by albumin molecules across certain endothelial barriers (Galis et al., 1988).

1.3.1.5 Endocytosis and Phagocytosis

Endocytosis, a process used vesicular carriers by cells to internalised a variety of macromolecules, supramolecular complexes, and organism, is the main cellular activity involved in the internalisation of extracellular particulate material (phagocytosis) or uptake of small solutes or small droplets of extracellular fluid (pinocytosis) (Schwartz, 1995). Phagocytosis is used mainly by specialised cells such as macrophages and neutrophils for the engulfment of large particles, cells and microorganisms (Silverstein et al., 1977).

Generally, free and targeted macromolecule-bound drugs enter cells by different mechanism. Free drugs enter the cell interior via transmembrane transport (diffusion) or adsorptive pinocytosis (nonspecific endocytosis), while cellular uptake of macromolecule-bound drugs is mostly restricted to receptor-mediated endocytosis. It was found that the drug will reach the cytoplasm of the cell when transported by diffusion, whereas pinocytic process are lysosomotropic (DeDuve et al., 1974). However, the rate of uptake by adsorptive pinocytosis is considerably slower if compared with the uptake by receptor-mediated processes (Shepherd, 1989). Normally, The reason for covalent drug conjugation to a macromolecular targeted carrier is the limitation of the cellular uptake of the drug to the mechanisms of receptor-mediated endocytosis (Duncan, 1987).

Receptor-mediated endocytosis is a process where by extracellular macromolecules and particles gain entry to the intracellular environment. The cells use receptor-mediated endocytosis for nutrition, defense, transport and processing. Generally, ligands first bind to specific cell surface receptors that carry an internalisation signal recognised by adaptors on the cytosolic side of the plasma membrane. The receptors are then clustered into the coated pits and enter very efficiently the cells together with their ligand. The cytoplasmic coat of coated pits is composed of a family of proteins with the major species having a molecular weight of 180 kDa, termed 'clathrin' (Pearse, 1976; Pearse and Crowther, 1987). The coated vesicles comprise 1-2% of the plasma membrane surface area of most cells and appear as a cagelike structure (Heuser and Evans, 1980). Clathrin-mediated endocytosis from the plasma membrane was one of the first vesicular coat proteins to be identified by electron microscopy (Robinson, 1994). Following the internalisation, the receptor and the ligand may both be recycled to the cell surface, they may both be directed to the lysosomes where degradations occurs or the receptor may be recycled to the cell surface and the ligand directed to the lysosomes (Rihova, 1998). Some

receptor molecules recycle in a few minutes (low density lipoprotein, transferrin), other recycle less efficiently (insulin receptor, Fc receptor) (Brown and Anderson, 1983; Mellman and plutner, 1984).

Intact human red blood cells have very limited endocytosis. Only a very small portion of the youngest cord red blood cells demonstrate receptor-mediated endocytosis after binding lectin, concavalin A or anti-A IgG antibody (Matovcik et al., 1985). The new approach to the treatment of cancer or to immunomodulation is the restriction of the action of the effective compound only to the treated tissue. This can be accomplished by using targeted instead of the free drug. Targeting should deliver the drug to the sites of its action. A variety of targeting ligands, such as asialoglycoproteins, transferrin, hormones, growth factors, lectins, folic acid, carbohydrates, antibodies (monoclonal or polyclonal) or glycoproteins, are used as targeting moieties.

1.3.2 The Blood Brain Barrier

The tightness of the endothelial barrier surrounding the central nervous system, sometimes called the blood brain barrier (BBB), is an extreme example of the heterogeneity of vascular beds. Unlike endothelia of many peripheral tissues, the endothelia of the cerebrovasculature have tight intercellular junctions, no fenestra, and an attenuated pinocytotic activity (Reese and Karnovsky, 1967; Brightman and Reese, 1969). The BBB is a specialised population of endothelia with mechanisms elaborated for tightly controlling the movement of substances between the blood and the brain. As a result of these features, the BBB plays an essential role in maintaining a consistent extracellular environment for the central nervous system (Cornford, 1985; Pardridge,

1987). A few of the basic features distinguishing brain capillary from peripheral endothelium are illustrated in Fig. 1.3. The apparent strategy of the BBB is to force all substances to undergo transcellular transfer between the blood and the brain. This strategy allows the BBB to regulate both the types and the amounts of substances that may influence the central nervous system. While several classic transport systems are available to facilitate transendothelial passage of nutrients such as hexoses, amino acids, amines, and nucleosides (Oldendorf, 1971; Goldstein and Betz, 1986; Hawkins, 1986), only a few transport mechanisms for macromolecules have been recognised (Banks and Kastin, 1988). The current interest in biotechnology products with potential applications in the therapy of central nervous system maladies has created a particular need for identification of mechanisms and schemes to deliver macromolecules across the BBB. This particular barrier complicates the delivery of even small water soluble drug molecules to brain tissue.

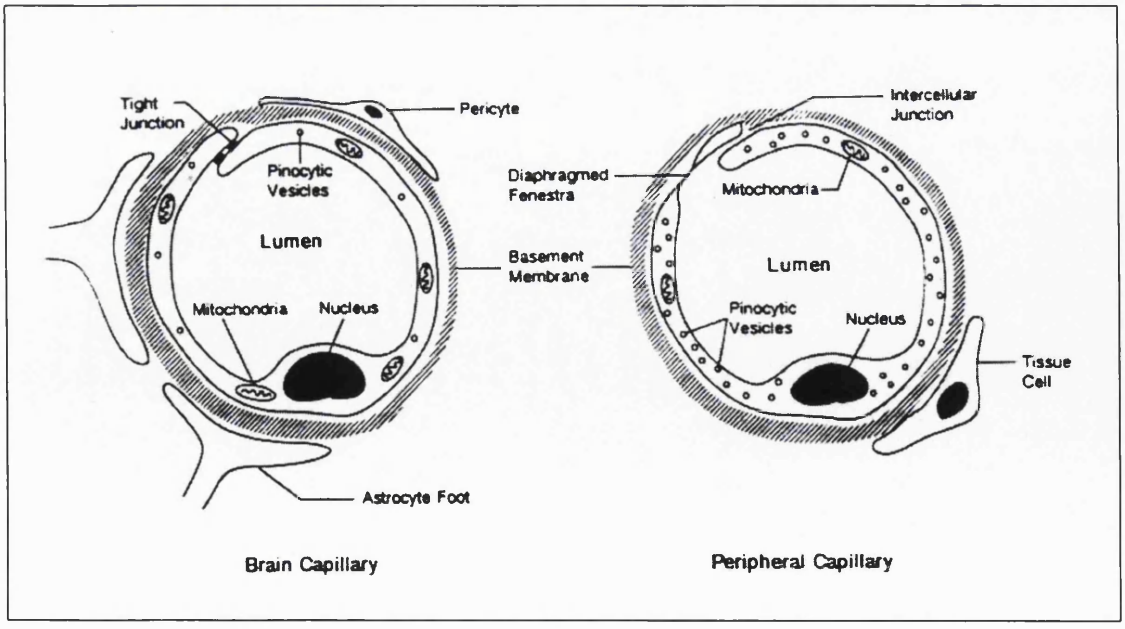


Fig. 1.3 *Major differences between the endothelium of the blood-brain barrier and the peripheral vasculature. (Audus and Borchardt, 1991)*

The BBB has tight intercellular junctions, excluding molecules with effective sizes greater than the Lanthanum ion (Stokes radius 10 °A) (Bouldin and Krigman, 1975; Cserr and Bundgaard, 1984). Solutes and macromolecules have to pass via specific receptors. On the other hand, the special metabolic activities of brain microvascular endothelial cells convert or degrade many molecules during their passage through the cell. At the BBB, astrocytes have been shown to influence tight junction permeability of the endothelia (Raub et al., 1989).

1.3.3 The Basal Lamina Barrier

The capillary endothelium is subtended by a layer of dense fibrillar material which is termed the basal lamina or the basement membrane (Martnez-Herandez, 1981). A major component of the basal lamina is type IV collagen organised in microfibrillar arrays. In addition, the basal lamina contains laminin, a high molecular weight protein that promotes adhesion of cells, as well as several different proteoglycans especially those of the heparin sulphate type.

For the purpose of devising and understanding drug delivery systems, we must be concerned with the ability of the basal lamina to act as an ultrafilter for macromolecules and macromolecular structures (Martnez-Herandez, 1981). Charge and size characteristics are important in determining the rates of diffusion through the basal lamina. Part of the charge discrimination of the basement membrane may be due to the proteoglycan component. It is important to keep in mind that macromolecule components of the basement membrane have specific binding capacities for macromolecules. For example, fibronectin has binding sites for collagen, DNA, fibrin, actin and other macromolecule

(Ruoslahti et al., 1982). Thus, the ability of macromolecular drug-carrier complexes to transit the basal lamina of capillary endothelial will depend not only on the size and charge of the carrier complex, but also on specific macromolecular binding characteristics.

1.3.4 The Reticuloendothelial Barrier

In order for a drug-carrier complex to successfully reach target cells within a certain tissue, it must not only be able to exit from the circulation, passing the endothelial and basal lamina barriers, but it must also be able to escape the grasp of the endothelial system, the body's disposal mechanism for foreign particles and macromolecules. Numerous investigators in the past two decades have shown that the majority of particulate colloids and drug carriers injected intravenously is retained by organs of the reticuloendothelial system (RES) comprising the liver, the spleen, and the bone marrow (Moghimi et al., 1994). It is well recognised that the elements of the RES is regulated by the presence and balance of serum components promotes phagocytosis (opsonins) and suppresses the process (dysopsonin) (Absolom, 1986). Opsonins promote phagocytosis primarily by affecting the critical first step of attachment, in which opsonins form a bridge between the phagocytic substrate and phagocyte. Opsonins are not generally regarded as being those factors which influence subsequent events in the process such as internalisation and/or digestion of phagocytic substrates. In many cases, cooperation of a number of opsonic molecules may influence particle recognition by phagocytes (Patel, 1992). Classical examples of opsonins include various subclasses of immunoglobulins (e.g. IgG1 and IgG3 in humans), certain components of the complement system (e.g. C3b,

iC3b, C1q), fibronectin, lipopolysaccharide-binding protein, apolipoprotein E and pentraxins (e.g. C-reactive protein and serum amyloid P component). Recently, both thrombospondin and von Wille brand factor can also act as opsonins and trigger phagocytosis of sulfatide-rich and -coated particles (Serra et al., 1992). Some opsonic entities such as tuftsin binds to the phagocytic cell rather than to the particle and enhances phagocytic ingestion two to three folds. Dysopsonins, on the other hand, are known to inhibit phagocytic ingestion (Absolom, 1986). Generally, dysopsonins act either by altering the surface properties of the phagocyte or particle which explain the mechanistic role in RES avoidance, by altering the metabolic activity of the phogocyte (Moghimi and Patel, 1998). The best known dysopsonins are Immunoglobulin A (IgA) and secretory IgA (sIgA) (Patel, 1992).

After the particle opsonised by serum components, the receptor-mediated events then destine the system to the intrinsic cellular pathway mediated by the cells of the reticulo-endothelial system (RES), mainly macrophages. Immunoglobulin (mainly IgG) is an important component of the opsonisation process, which activate the classical complement pathway to present particulates or colloidal carriers to phagocytes. It is recognised that the phagocytosis of particulates is dependent on their interaction with serum components, their size and surface properties. The absorbability of opsonins to surface of the particles play an important role in the phagocytic uptake of particulate delivery systems. The opsono-phagocytosis is the relatively higher protein adsorbability on hydrophobic surfaces as compared to hydrophilic surfaces (Vyas and Sihorkar, 2000). It should be understood that not all particles in the circulation are rapidly cleared by macrophages, for example, healthy erythrocytes circulate for 110 days in humans (Poznasky and Juliano, 1984). Various surface manipulations of particles will lead to qualitative and quantitative alterations in opsonisation and macrophage uptake. In recent

years, it has been speculated coupling of polyethyleneglycol (PEG) to the liposome surface can drastically prolong the blood circulation of liposomes (Senior et al. 1991).

1.3.5 The Targeting Problem

One of the most fundamental barriers to selective drug delivery involves the so called "targeting" problem. Much of the intellectual excitement in the drug delivery area revolves around the concept of directing therapeutic agents to a particular cell population where desirable effects can be achieved without exposing other cell populations where toxic effects may occur. The most obvious example of this would be a toxic agent that can discriminate between neoplastic and non-neoplastic cells.

First this approach presumes the existence of something to aim at, that is, some molecular characteristic that differs between target and nontarget cells. Clearly the first task is establishing that a discrete and accessible molecular difference does exist between target and nontarget cells. The second task is to develop a reagent that will show a high degree of selectivity in bringing a toxic drug into target cells. Drug-carrier conjugates showing excellent discrimination between target and nontarget cells.

1.4 Technologies for controlled drug delivery

1.4.1 Liposomal drug delivery systems

Water-insoluble polar lipids in an excess of water give rise to highly ordered assemblages, which eventually arrange themselves into a system of concentric, closed, bilayered membranes called liposomes. One of the popular experimental approaches to controlled drug delivery has been the use of liposomes, or phospholipid vesicles. Liposomes are formed spontaneously when amphipathic lipids are dispersed in excess water. The lipid molecules arrange themselves by exposing their polar head groups towards the water phase, while the apolar hydrocarbon moieties stick together in the bilayer thus forming closed concentric bimolecular lipid leaflets separated by aqueous compartments (Roerdink et al., 1987). Their design allows water soluble drugs to be encapsulated in the aqueous core of the liposome and hydrophobic/lipophilic drugs to be incorporated within the phospholipid bilayer. Since 1972, when Gregoriadis and Ryman proposed the use of liposomes as carriers of enzymes in the treatment of lysosomal storage diseases, the application of liposomes has been extended to a variety of drugs such as antineoplastic agents, antimicrobial compounds, immunomodulators, etc. (Gregoriadis et al., 1982). There is now a wide variety of methods available for the synthesis, characterisation, drug incorporation and targeting of liposomes (Weiner et al., 1989) and liposomal technology has matured to the extent that liposomal products are now commercially available. The purpose of using liposomal carriers was to increase the uptake of drugs by specific cells or tissues, thereby enhancing the potency and/or reducing the toxicity of the encapsulated agents. Among the various routes of administration of liposomes the intravenous injection is the most widely applied. The half-lives of liposomes in the blood stream can range from a few minutes to many hours

depending on the size and lipid composition of the vesicles. In the blood stream after IV injection, liposomes may be susceptible to destabilising effects of serum proteins, resulting in leakage of encapsulated water-soluble compounds. The high density lipoproteins (HDL) were found to be responsible for penetration into the liposomal bilayers, a process which was accompanied by the net loss of phosphatidylcholine from the liposomes to the HDL (Krupp et al., 1976; Scherphof et al., 1978). Low density lipoproteins may also increase liposomal permeability, taking lipid components from the liposomal membrane, especially when large doses of liposomes are used (Shahrokh and Nichols, 1982; Comiskey and Heath, 1990). However, the incorporation of cholesterol, causing increased packing of phospholipids in the lipid bilayer, reduces the transfer of phospholipids to the HDL (Damen et al., 1981; Hunt, 1982). Large-size vesicles like MLV or LUV are rapidly cleared from the blood and taken up by cells of the reticuloendothelial system (RES), especially macrophages in liver (Kupffer cells) and spleen (Roerdink et al., 1981, 1984 a,b). In addition to liver and spleen macrophages, circulating monocytes have been reported to take up significant amounts of liposomes. In the lung, the liposome-loaded monocytes can subsequently migrate to the alveoli to become alveolar macrophages (Poste et al., 1982). The first approach to the development of stabilised liposomes, called "stealth liposomes" or "ninjasomes", used SUV composed of saturated phospholipids such as DSPC and cholesterol (Gregoriadis and Davis, 1979; Senior and Gregoriadis, 1982; Senior, 1987; Senior et al., 1985; Chow et al., 1989). To reduce the RES uptake, the first strategy is to prepare liposomes mimicking the erythrocyte membrane to be recognised by the RES as "self" instead of "foreign" structures; since the sialic acid portion of erythrocyte glycoproteins is thought to be important for immune recognition. Liposomes are modified by gangliosides and sialic acid derivatives such as monosialoganglioside (GM1) (Goto et al., 1987; Allen and

Chonn, 1987; Gabizon and Papahadjopoulos, 1988; Allen et al., 1989). The second strategy consists of increasing the hydrophilicity of the liposomal surface. Polyethyleneglycol (PEG) was successfully introduced into liposome structures as PEG-phosphatidylethanolamine (PE) (Klibanov et al., 1990; Blume and Cevc, 1990; Allen et al., 1991; Senior et al., 1991) and although PEG is still the best known material for this aim, many other polymers have been investigated as liposome stabilisers (Monfardini and Veronese, 1998).

1.4.2 Microparticles and Nanoparticles as drug carriers

The use of particles as drug carriers has been developed as a method for improving drug bioavailability and for reaching the therapeutic aims of controlled drug release and drug targeting. Particles are classified as microparticles (1-50 μm diameter) or nanoparticles (10-1000 nm diameter) according to their size. Microparticles may circulate in the blood and pass through the heart, but they cannot pass through capillaries because of their size. On the basis of this property, biodegradable microspheres prepared from starch (Dakhil et al., 1982), albumin (Gupta, 1990), polylactic acid (Flandroy et al., 1990), and microcapsules of ethycellulose (Kato et al., 1981; Nemoto et al, 1981) have been studied for chemoembolisation; where, drugs localise in the nearby tissue surrounding the capillaries that have accumulated microparticles by upstream intra-arterial injection. Microspheres are also used as depot systems, especially by subcutaneous injection and also by the intravenous or intraperitoneal routes. In this case, the advantage of their size allows easy administration by injection, while the entrapped drug is slowly and continuously released into the circulation from a system that protects it

from degradation. The rate of drug release may be designed by properly choosing the polymer, i.e., pore size, degradation, and swelling properties. The best example of this application is the release from polylactide microcapsules of peptides used to treat prostate cancer, a somatostatin analogue RC-160 (Pinsky et al., 1994) and a LH-RH antagonist SB-75 (Yano et al., 1992). A novel lipid microsphere (LM) emulsified by lecithin with saturated fatty acids was investigated as a drug carrier (Inoue et al., 1997).

Nanoparticles are capable of not only releasing the active molecule at the target site but also carrying it there. Their size makes them suitable for systemic use, as they can pass through capillaries. The reticuloendothelial system (RES) is responsible for eliminating nanoparticle drug carriers from circulation with the following biodistribution: 80-90% in liver, 5-8% in spleen, and 1-2% in bone marrow. Many drugs have been loaded into nanoparticles such as doxorubicin, ampicillin, insulin etc. Many attempts have been made to prolong the circulation time of drugs entrapped in particulate systems after intravenous injection. Particles with polar and charged surfaces have an increased circulation time and reduced uptake by the RES organs (Wilkins and Myers, 1966). The uptake of nanoparticles by RES may be prevented by coating particles with a hydrophilic surface based on PEG-type materials, like block polymers of ethylene oxide/propylene oxide (POE/POP) or poloxamine-908, which are either adsorbed or covalently linked to the particles (Illum et al., 1987; Moghimi et al., 1991, 1993a,b; Porter et al., 1992).

1.4.3 Albumin as drug carriers

This protein is very easily available from blood banks, and is widely used to modify proteins of therapeutic interest. Albumin possesses a total of 59 lysine ε-amine

groups (with only 30-35 of these typically available for derivatisation), 1 free cysteine sulfhydryl (with 17 disulfides buried within its three-dimentional structure), 19 tyrosine phenolate residues, and 17 histidine imidazoles. The presence of numerous carboxylate groups gives BSA its net negative charge (pI 5.1). Its polyfunctional ability allows a variety of derivatives to be prepared, but it is also responsible for intra- and intermolecular cross linkages, with the final formation of heterogeneous products. Albumin is an especially attractive choice as a carrier for both drugs and enzymes by virtue of the following properties: ready availability, ease of purification, chemical and physical stability, excellent storage properties, lack of toxicity, favourable rate of degradation, lack of antigenicity and immunogenicity of homologous albumins, ease of conjugation with a wide range of drugs and enzymes, ability to form complexes which are slowly or rapidly cleared form the circulation, depending on the degree of heat or chemically induced denaturation. Isliker et al.(1964) first suggested that albumin , which had been shown to be taken up intact into tumour cells by pinocytosis, might be used as a carrier.

Albumin is one of the natural polymers most frequently used for protein modification. There are at least five available reactive groups on albumin for modification: epsilon amino groups of lysine residues, alpha amino groups found on many different proteins, phenolic hydroxyl groups of tyrosine residues, alpha amino groups on proteins, sulfhydryl groups of cysteine residues, imidazole groups of histidine residues. Drug modification by albumin is performed in aqueous buffer in the presence of cross-linking agents such as glutaraldehyde, carbodiimide, and sodium periodate. A number of groups have reported the successful use of albumin as a drug carrier. Magnenat et al. (1969) conjugated methotrexate and a methylhydrazine derivative to albumin by a diazo linkage and the conjugates were found to be at least twice as active as equivalent

molar quantities of free methotrexate. Barbanti-Brodano and Fiume (1973) have cross-linked the peptide amanitin (AMA) to albumin using carbodiimide. Amanitin is toxic to eukaryotic cells because it binds to and inhibits RNA polymerase II. The AMA-BSA complex appears selectively toxic to highly phagocytic cells. In *in vitro* experiments, the AMA-BSA complex is more toxic to macrophages than to lymphocytes or fibroblasts. Balboni et al. (1976). Tsong and Koide (1974) found that estrogen-BSA conjugates prolonged stability of the steroid *in vivo* followed by hydrolysis to release free estrogen, which can subsequently be detected in the cytosol. Uricase modified by albumin has an enhanced plasma life (Remy and Ponansky, 1978), and recombinant human granulocyte colony stimulating factor (rhG-CSF) conjugated to both rat and human serum albumin through a spacer of heterobifunctional maleimidocarboxyl PEG (1:1 albumin - rhG-CSF conjugate) increase its serum stability (Paige et al., 1995). Modification of earthworm fibrinolytic enzyme with fragmented HSA (Mw ≈10000-30000 Da) causes loss of its antigenicity, increased stability toward protease inhibitors in rat plasma, and retention of its potent proteolytic activity (Nakajima et al., 1996).

1.4.4 Antibodies as drug carriers

At the beginning of this century, Ehrlich (1906) anticipated the use of antibodies as effective targeting agents to introduce drugs to specific tissues. The first drug-antibody conjugate was reported in 1958, when Mathe et al., linked the drug methotrexate (MTX) by diazotisation to antibodies raised against L1210 leukemia cells and used the conjugate in the successful treatment of L1210-bearing DBA/2 mice. Although the initial results with drug-antibody conjugates appeared promising, almost no work was carried out in

this field for fourteen years and when it began again, it involved entirely different drugs and antibodies.

Physically, antibodies are complex proteins, consisting of multiple polypeptide chains that contain a variety of reactive chemical groups, such as amino, carboxyl, hydroxyl, and sulhydryl. The best characterised and best understood immunoglobulin is Immunoglobulin G (IgG); other classes of immunoglobulin are IgA, IgM, IgD, and IgE.

Immunoglobulin G, the most basic immunoglobulin molecule, is composed of two light and two heavy chains, held together by noncovalent interactions as well as a number of disulfide bonds. This arrangement renders the molecule a characteristic Y configuration (Fig. 1.4). The light chains are disulfide-bonded to the heavy chains in the C_L and C_H^1 regions, respectively. The heavy chains are in turn disulfide-bonded to each other in the hinge region. The heavy chains of each immunoglobulin are identical. Depending on the class of immunoglobulin, the molecular weight of these subunit ranges from about 50,000 to around 75,000 Daltons. Similarly, the two light chains of an antibody are identical and have a molecular weight of about 25,000 Daltons. For IgG molecules, the overall molecular weight representing all four subunits is in the range 150,000 to 160,000 Daltons (Hermanson, 1996). Fragments Fab and Fc, shown in Fig. 1.5, are obtained after cleavage by the proteolytic enzyme, papain. The arms, called the Fab fragments, contain the variable regions that create the unique binding sites for the antigen. The base of the molecule, the Fc fragment, retains the antigenicity and is responsible for the recognition of the molecule by other components of the immune system.

Antibody molecules possess a number of functional groups suitable for modification or conjugation purpose. The earliest coupling method used for drug-antibody conjugation was diazotisation (Mathe, 1958), a technique which favours

coupling to tyrosine residues of proteins. Lysine residues are generally present in immunoglobulins and the epsilon amino side chain is commonly the preferred site for drug conjugation. Carboxylate groups, plentiful in antibodies, may also be coupled to another molecule. In order to achieve delivery of a drug to its target site by means of an antibody, the coupling procedure should fulfil certain agreed criteria which include the need for both the drug and the antibody to retain their respective activities and for the conjugate or complex to remain stable in transit to the target site (Rowland, 1983).

In 1963, Israels and Linford showed chlorambucil to be bound avidly to serum proteins and the rate of hydrolysis of the drug to be reduced in the presence of serum proteins particular serum albumin. Blakeslee and Kennedy (1974) claimed that chlorambucil could be linked non-covalently to rabbit IgG, provided that suitable reaction conditions pertained and that as many as 64.5 moles drug per mole IgG could be incorporated. In 1975, Ghose and colleagues developed chlorambucil noncovalently linked to antibodies used in the treatment of a melanoma patient. The most obvious problem regarding the use of non-covalent conjugates concerns their stability in the body fluids. Robinson et al.(1973) linked methotrexate to anti-L1210 antibodies and used the conjugate to attack L1210 leukemia cells growing in mice. The conjugate was shown to be more effective therapeutically than unconjugated antibody and drug given together. Burstein and Knapp (1977) coupled methotrexate to an antibody by a carbodiimide and a mixed anhydride reaction.

Several advantages may be seen when drugs are delivered as antibody conjugates. The conjugate may specifically reach the target cells without significant distribution to normal tissues. The binding of drugs with an antibody might impart enhanced stability of drug molecules against enzymatic degradation and prevent rapid excretion, thereby increasing the drug half-life. In addition, the antibody molecules are fairly rigid and can

withstand conditions employed in labelling. The molecules are little affected by the chemical manipulations required for conjugate synthesis (Ross et al., 1980). Selective delivery to the target site may reduce the doses of drugs necessary for treatment. Thus, the advantages are clear; less side effects, small doses and selective targeting. Most research workers in the field of antibody drug targeting have focused their attention on the delivery of anticancer drugs (Rowland et al., 1975; Rowland, 1977). A major aim of work in anticancer drug delivery with antibodies should therefore be to improve the therapeutic index (Larson et al., 1983; Brown et al., 1981; Hellstrom and Hellstrom, 1985).

In designing drug-antibody conjugates, consideration must be given to the studies on the structure-activity relationship of the drugs as well as to the methods of conjugation. An ideal method of conjugation should provide the maximum number of incorporation without altering much of the antibody activity with an acceptable protein recovery. In general, substitution ratios of greater than 10:1 with respect to IgG produce a marked loss of antibody reactivity; and in many cases, substitution of as few as four drug residues per IgG molecule renders unacceptable antibody damage (Baldwin, 1985). The method should not also cause any aggregation and/or precipitation during the reaction. Hydrophobic drugs, upon conjugation with the antibodies through carboxylic or amino groups of the antibody molecule, may give rise to a conjugate with reduced aqueous solubility leading to aggregation and precipitation. However, with chlorambucil even a substitution ratio of 30:1 resulted in substantial protein recovery and good antibody activity (Smyth et al., 1986; 1987). Care should be taken during conjugation to ensure the process does not yield a homopolymer. The conjugation method should be simple and straightforward, and must produce a covalent linkage between the drug and the antibody.

A major difficulty in the use of antibodies for drug delivery has been their heterogeneity with respect to their size, charge, antigen specificity and affinity. Heterogeneity is also displayed if such antibodies are administered in vivo, either alone or with agents coupled to them (Zurawski et al., 1978; Steinitz et al., 1977). The specificity of the antibodies is of paramount importance for successful antibody-mediated drug delivery. Polyclonal antibodies show high affinity and avidity, and the molecules are capable of withstanding fairly rigorous chemical procedures. Monoclonal antibodies, on the other hand, tend to display weaker affinities and avidities, and their immunological specificity may be profoundly influenced by the local chemical environment (Mossman et al., 1980).

Early work on drug targeting with polyclonal antibodies suggested very little effect of conjugation methodology on the binding affinity, which may be due partly to the heterogeneity of such antibodies. Rowland et al.(1988) conjugated vindesine to three different monoclonal antibodies by the same procedure. The conjugates produced the following antibody activities relative to the unconjugated antibody: 3:1, 84%; 7:1, 76% and 9:1, 70%.

Embleton and Garnett (1985) have also reported that a particular antibody is inactivated to a different extent when different drug molecules are coupled by an equally mild reaction, e.g., 6 moles of vindesine or 8 moles of 14-bromodaunomycin can be coupled to 1 mole of monoclonal antibody with hardly any loss of activity. Substitution with 3 moles of MTX however produced 70% loss of activity. the degree of retention of binding activity decreases with increasing substitution for any one drug molecule. Therefore, it is necessary to choose an antibody that withstands chemical manipulations and that allows adequate substitution without appreciable damage to the antibody binding property.

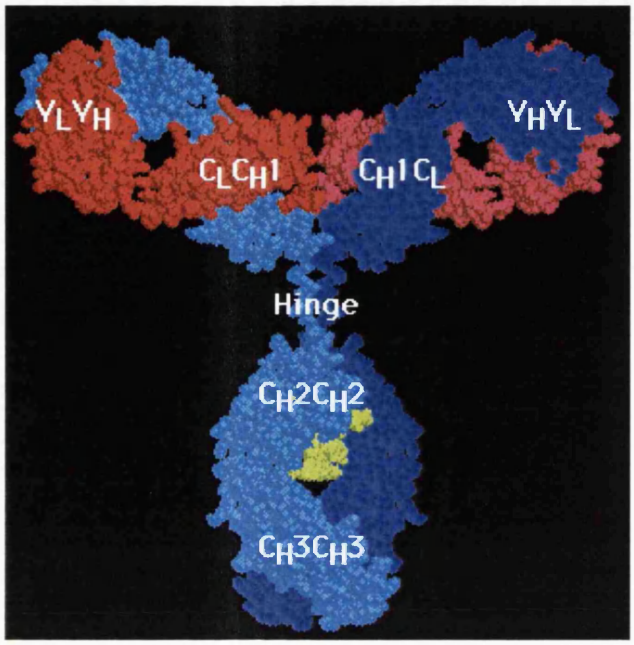


Fig. 1.4 *The structure of the IgG molecule*

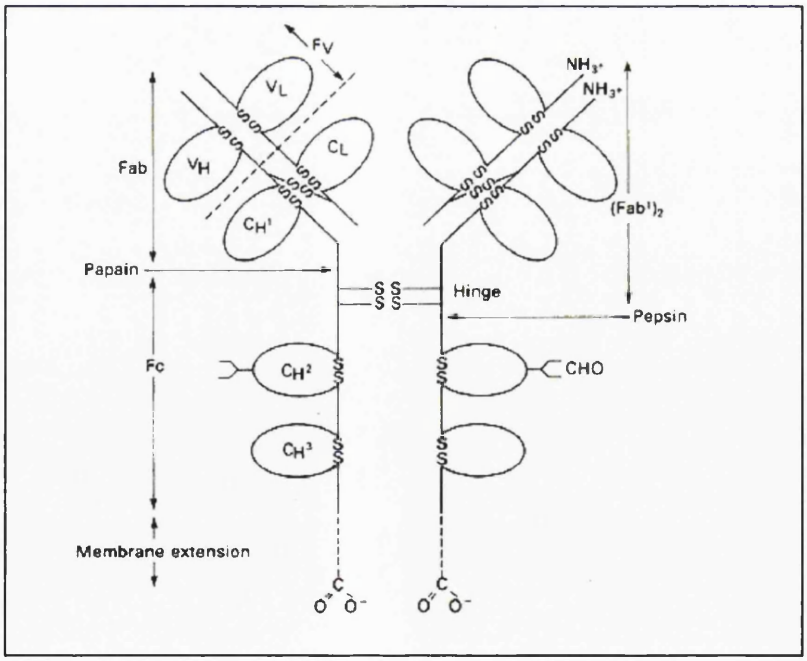


Fig. 1.5 *Schematic representation of the four-chain structure of IgG showing Fab and Fc, inter and intra-chain disulphide bridges, papain and pepsin fragment.*

1.5 Drug conjugation to protein : Coupling Reactions

The design and development of a therapeutically active macromolecule-drug conjugate requires considerable and precise multidisciplinary knowledge. Some earlier studies with drug molecules such as daunomycin or methotrexate bound to antibodies or DNA molecules in a non-covalent manner have been reported (Trouet et al., 1980), but the specific benefits of the conjugation have been questioned. For successful targeted drug delivery, it is essential that the macromolecule-drug is stable during transport to the target cell. It might be that the drug does not possess its therapeutic action while still conjugated to the carrier and the cleavage of the bond might be a necessary step for drug action. Under such circumstances the bond must be sensitive to endogenous breakdown if the drug is to be released following delivery.

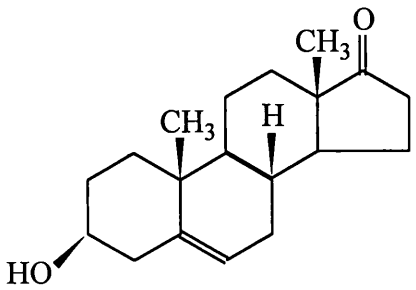
Prior to consideration of the cross-linking step, a detailed understanding of the available reactive groups on the carrier and drug or therapeutic agent is essential. In choosing a cross-linking procedure, while there is a certain amount of empiricism involved, a knowledge of what side groups are available and are remote from the regions of both drug and protein activity is beneficial. The object of any conjugation procedure is to effect the attachment without altering the desired properties of the drug (or enzyme) and carrier particle. The conjugation procedure must be gentle enough not to destroy either drug or carrier activity. Consideration must be given to the cross-linking conditions to assure that protein and drug maintain the desired properties. Conditions of the cross-linking such as temperature, pH, time, and protection of sensitive sites must be considered. For example, in the use of antibodies as carriers of drugs it is necessary that, following conjugation, the antibody retains its specificity towards its binding site while the drug retains its required activity (Poznansky and Juliano, 1984). The following

represents some examples of coupling reactions that might serve to link drug molecules to carriers.

Table 1.7: Coupling reactions for drugs conjugation to antibodies and albumins

Carriers	Active agents	Method of preparation	Reference
Antibodies	Methotrexate	Mixed-anhydride	Burnstein and Knapp (1977)
Antibodies	Heptens	Mixed-anhydride	Erlanger et al. (1967, 1973)
Antibodies	Horseradish peroxidase	Carbodiimide	Clyne et al. (1973)
Antibodies	Lecithin	Carbodiimide	Mizushima and Igarashi (1991)
Antibodies	Ricin	*m*-maleimidobenzoyl *N*-hydroxysuccinimide ester	Youle and Neville (1980)
Antibodies	Vinblastine	*N*-hydroxysuccinimide ester	Bumol et al. (1988)
Antibodies	Alkaline phosphatase/ etoposide phosphate	*N*-hydroxysuccinimide ester/ 4-(methylenemaleimido) cyclohexylcarboxylic acid.	Senter et al. (1988)
Anitbodies	Doxorubicin	Acid-labile hydrazone bond	Trail et al. (1997)
Anitbodies	Doxorubicin	Branched hydrazone linker	King et al. (1999)
Antibodies	Daunomycin	Carbodiimide	Hudecz et al. (1990)
HSA	Methotrexate	Carbodiimide	Chu and Howell (1981); Chu and Whiteley (1977,1979,1980); Hartung et al. (1997)
HSA	Methotrexate	Carbodiimide/ *N*-hydroxy-succinimide	Bures et al. (1988, 1990); Bostik et al. (1988)
HSA	Chlorambucil	Maleimide spacer	Kratz et al. (1998)
HSA	Paclitaxel	*N*-hydroxy-3-sulfosuccinimide ester	Dosio et al. (1997)
HSA	Mitomycin C	Carbodiimide (glutaric anhydride spacer)	Tanaka et al. (1991)
RSA	Methotrexate	Carbodiimide	Stehle et al. (1997 a,b)
BSA	Polyethylene glycol	Cyanuric chloride	Abuchowski et al. (1977)
BSA	Glucosiduronic acid	*N*-hydroxy-succinimide/ dicyclohexylcarbodiimide	Mattox and Nelson (1979)
BSA	*p*-amino benzoic acid	Diazotization	Sashidhar et al. (1994)

1.6 Dehydroepiandrosterone (DHEA)



$C_{19}H_{28}O_2$

Mw 288.43

Dimorphous, needles, mp. 140-141 °C

Soluble in benzene, alcohol, ether

Sparingly soluble in chloroform, petroleum ether

***Fig. 1.6** Structure of Dehydroepiandrosterone (DHEA)*

Dehydroepiandrosterone (DHEA) unconjugated and its sulphated metabolised (DHEA-S), are the major secretory products of the adrenal gland (Ebeling and Koivisto, 1994). Scientists have known about these hormones since 1934. These hormones have been widely publicised in both the lay press (Jaroff, 1995; Perlman, 1995) and in the scientific literature (Morale et al.,1994; Lopez, 1984; Rudman et al., 1990) for their supposed anti-age effects. Investigators have even postulated that DHEA and/or DHEA-S concentrations can be viewed as possible markers of physiologic ageing (Lopez, 1984; Rudman et al., 1990). Interest in these hormones is considerable, as indicated by a recent burst of scientific publications. A Medline search of articles published from 1963 through 1997 identified 1,477 publications in English that had DHEA and/or DHEA-S as a focus, with 31% (459) of those published during the 5 years from 1993 to 1997.

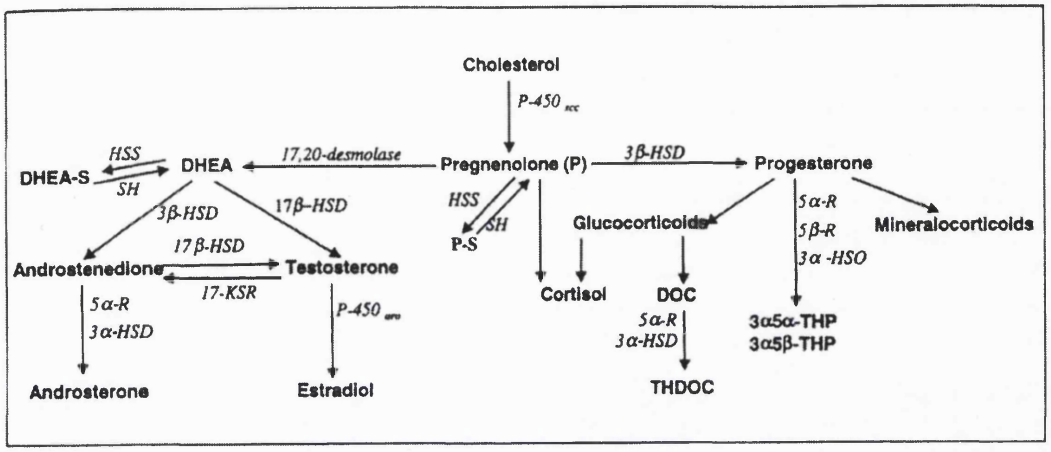


Fig. 1.7 *Biosynthesis and metabolic pathway of DHEA and DHEA-S (Kroboth et al., 1999)*

DHEA is a 19 carbon steroid also known as 5-androsten-3β-ol-17-one (Fig. 1.14). Fig. 1.15 is a schematic that describes the synthesis and major metabolic pathways of DHEA (Mw = 288.43 Da), DHEA-S (Mw = 371 Da), and other related steroids (Majewska, 1992; Mellon, 1994; Robel and Baulieu, 1994; Regelson et al., 1994; Lancaster, 1994). As indicated in Figure 1.15, pregnenolone is the precursor to DHEA as well as other androgens, mineralocorticoids, and glucocorticoids. Pregnenolone is derived from cholesterol after side chain cleavage by cytochrome P450scc, now referred to as CYP11A1. Cytochrome P45017α (CYP17), a 17α-hydroxylase with 17,20-desmolase activity, catalyses the synthesis of DHEA from pregnenolone. Hydrosteroid sulphatases convert DHEA to DHEA-S, which is the most abundant circulating steroid hormone in humans. The specific cytosolic enzyme responsible has been commonly referred to as dehydroepiandrosterone sulfotranferase (DHEAST) because of its high affinity for catalysing this reaction. The adrenal cortex is the primary source of circulating concentrations of DHEA and DHEA-S, with DHEA-S being the most abundant product

of the adrenals (Flier and Foster, 1998). In healthy women, the synthesis of DHEA and DHEA-S occurs exclusively in the adrenal cortex; in men, it is estimated that 5% of DHEA-S and 10% to 25% of the circulating DHEA are secreted by the testes (Verneyken, 1980). Once in circulation, DHEA-S can be metabolised back to DHEA by sulfohydrolases in peripheral and adrenal tissues (Kishimoto and Hoshi, 1972). Bird et al.(1984) reported that 64% and 74% of the daily produced DHEA-S is converted to DHEA in women and men, respectively, but only about 13% of DHEA is metabolised back to DHEA-S (Poortman et al., 1980). DHEA and DHEA-S serve as the precursors to approximately 50% of androgens in adult men, 75% of active estrogens in premenopausal women, and almost 100% of active estrogens after menopause (Labrie et al., 1997). DHEA itself has a 3- to 10- fold predominance of androgenic over estrogenic activity (Labrie et al., 1996). The circulating concentrations of DHEA-S are approximately 250 and 500 times higher than those of DHEA in women and men, respectively (Labrie et al., 1997). DHEA has a shorter half-life of 1 to 3 hours, while the half-life of DHEA-S is 10 to 20 hours (Rosenfeld, 1975). DHEA is weakly bound to albumin, whereas DHEA-S is relatively strongly bound to albumin. While DHEAS is bound to albumin and forms a circulating reservoir, DHEA probably is more active at the tissue level. DHEA secretion was greater in young than in older adults.

Plasma concentrations of DHEA are age and gender dependent. DHEA concentrations decline from the first month until 5 years of age, then rise rapidly from age 9 in boys (Sulcova et al., 1997) until concentrations reach their peak between the ages of 20 and 30 (Sulcova et al., 1997; Carlstorm et al., 1988). In girls, the rise begins at age 7, and DHEA concentrations reach maxima at approximately 20 and again at 40 years of age (Sulcova et al., 1997).

Acute physiologic trauma such as burns (Lephart et al, 1987) and serious illness (Parker et al., 1985) are associated with decreases in DHEA and DHEA-S concentrations. Lephart et al (1987) evaluated the time course of DHEA-S decline in men after burn trauma and showed that by day 10, DHEA-S concentrations were significantly lower than in healthy men. Testosterone and androstenedione concentrations also declined.

DHEA and DHEA-S are classified among the group of steroids known as neurosteroids, so named because they can be synthesised de novo in the central nervous system (CNS) (Majewska et al., 1990). Concentrations of DHEA and DHEA-S are considerably higher in the brain than in other organs (Corpechot et al., 1981). Studies of CNS effects of DHEA and DHEA-S include correlation of hormone concentrations with mood, alterations in diurnal variation in patients with specific psychiatric diagnoses, and studies of the effects of DHEA administration on depression and cognition (Kroboth et al., 1999). In studies of the effect of DHEA on food choices, Porter and Svec (1995) reported that DHEA decreases fat intake of both lean and obese Zucker rats by modulating hypothalamic neurotransmitters. Nestler et al.(1988) reported the effects of DHEA on body fat mass, serum lipid levels, and tissue sensitivity to insulin in 5 young healthy men.

Data from animal studies suggest that DHEA enhances the immune function to protect mice from viral, bacteria, and parasitic infections (Padgett et al., 1995). This has led to the evaluation of the role of DHEA and DHEA-S in modulating immune function, with application to ageing as well as to a variety of disease states. As a vaccine adjuvant of DHEA, Araneo et al (1993) demonstrated an enhanced immune response to the hepatitis B antigen following immunisation in aged mice. Relative to concentrations in control subjects, DHEA concentrations are significantly lower in patients with an advanced stage of HIV infection (Christeff et al., 1996). Epidemiological studies have

suggested that DHEA is linked to the progression of HIV infection to AIDS (Centurelli and Abate, 1997). DHEA and DHEA-S have also been postulated as useful for the treatment of systemic lupus erythematosis (SLE) and rheumatoid arthitis (Kroboth et al., 1999). Several studies have been conducted to assess the relationship of cardiovascular disease to DHEA and DHEA-S (Alexandersen et al., 1997). Barrett-Connor and Khaw (1987) reported that in 289 women (60 to 79 years old), cardiovascular disease was highest in those with the highest concentrations of DHEA-S. The information described above indicates that ageing, gender, and various diseases influence circulating concentrations of DHEA and DHEAS. DHEA is now marketed as a drug, which has been found to have effects on anti-age, immune response, weight loss, diabetes, mood improvement, cardiovascular disease, cancer etc. However, its physiological importance is still unclear.

1.7 Outline of work

The object of this study is to enhance the water solubility of insoluble drugs, which are difficult to administer parenterally, by attaching them to a hydrophilic macromolecule. This provides the possibility of circumventing the problems associated with poor drug solubility and the toxicity of the formulations usually chosen to solubilise these hydrophobic compounds. An additional aspect is to prolong drug circulation in the blood and biodistribution in tissues after intravenous injection. Drug side effects and toxicity are generally associated with drug action at non-target sites and this limits the amount of drug that can be administered, thereby reducing its potential effectiveness. For this reason, coupling a therapeutic agent to a macromolecule drug carrier can temporarily inactivate the drug which is converted to an active drug only at the required site of action. This can also increase the plasma half-life by reducing the renal clearance thus potentially resulting in a dramatic effect on the drug distribution in the body, ultimately providing a greater therapeutic effect.

DHEA (MW 288.43 Da), a commercially available drug, was used as a model lipophilic drug in this study. In an attempt to improve the water solubility of DHEA and to prolong its circulation in the plasma, the drug was covalently coupled to natural macromolecule carriers such as immunoglobulin G (IgG) and bovine serum albumin (BSA). BSA and IgG contains an abundance of functional groups available for conjugation with drug molecules. Lysine residues are generally present in IgG and BSA, and the epsilon amino side chain is commonly the preferred site for drug conjugation. IgG is the major immunoglobulin accounting for 70-75 percent of the total immunoglobulin pool, and has a molecular weight of 150k Da with approximately 86 ε-amino groups of lysine. BSA, containing approximately 59 ε-amino groups of lysine, has a molecular

weight of 66k Da and is very easily available from blood banks. These two proteins are attractive for conjugating with DHEA as they are water soluble, have adequate functional groups for coupling and are very well distributed in the organism.

In this study, DHEA is covalently linked to the ε-amino groups of the lysine residues of IgG or BSA through a succinyl spacer which provides an opportunity to formulate soluble DHEA without toxic solubilisers. These conjugates may serve as a means to increase the biological half-life of insoluble drugs in plasma, target drugs to specific tissues in order to increase drug concentration at the target site, and may also reduce side effects.

Methodology (Chapter II) and the results of this study are described in the following chapters. The coupling method and characterisation of IgG and BSA conjugates are discussed in Chapter III. The in vivo behaviour of these two conjugates is evaluated in Chapters IV and V.

Chapter II

Materials and Methods

2.1 Materials

Deionised water was obtained from an Elgastat Option 4 water purification unit (Elga Ltd, UK). In brief, the water is pre-treated in a reverse osmosis cartridge and then further purified via an ion/organic removal cartridge, UV chamber and a 0.2 μm filter. The resistivity of the purified water was above 5 MΩ cm at 25 °C and its pH was neutral.

Ultra high quality (UHQ) water was obtained in an Elgastat UHQ-PS unit (Ultra High Quality Polishing System; Elga Ltd, UK) fed with water pre-purified by deionisation as mentioned above and then further purified by a combination of organic adsorption, deionisation, microfiltration and photo-oxidation. The final water had a resistivity above 18 MΩ cm at 25 °C.

A Wallac Compuspec UV/Visible spectrophotometer connected to a PC, (Wallac UK Ltd., UK) was used in all spectrophotometric determinations.

The materials used in the experiments are given in Table 2.1. All reagents were of analytical grade. All materials were used as obtained from suppliers without further purification.

Table 2.1 Materials and sources

Material	Source
Acetronitrile	Rathburn Chemicals Ltd, UK
Bovine serum albumin (BSA)	Sigma, Dorset, UK
Bromocresol green	Sigma, Dorset, UK
Chloroform	BDH, UK
Chloramine-T	Sigma, Dorset, UK
Cellulose membranes	Medicell International Ltd, UK
Coomasie brilliant blue (R-250)	Sigma, Dorset, UK
Dehydroepiandrosterone (DHEA)	Sigma, Dorset, UK
1,2,6,7 (^{3}H) DHEA	Dupont (UK), New England Nuclear Products (Stevenage, Herts, UK)
Dichloromethane (DCM)	Applied Biosystems, Cheshire, UK
N,N-dimethylformamide (DMF)	Sigma, Dorset, UK
4-dimethylaminopyridine	Sigma, Dorset, UK
1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDAC)	Sigma, Dorset, UK
Heparin	Sigma, Dorset, UK
Hydrochloric acid (HCl)	BDH, UK
N-hydroxy-3-sulfo-succinimide	Sigma, Dorset, UK
Bovine immunoglobulin G (IgG)	Sigma, Dorset, UK
Kieselgel PF_{254} plates	Sigma, Dorset, UK
$KHSO_4$	Sigma, Dorset, UK
The Lane Marker Reducing Sample Buffer	Pierce, Rockford, IL
The Low Range Prestained SDS-PAGE standards	BioRad, UK
Liquid scintillation cocktail OptiPhase 'HiSafe' 3	Fison Chemicals, UK
L-glutamic acid	Sigma, Dorset, UK
L-lysine	Sigma, Dorset, UK
Methanol	BDH, UK
$MgSO_4$	Sigma, Dorset, UK
Microcapillary pipette	Supracaps
$Na^{125}I$	Amersham International plc, Bucks, UK
$NaHCO_3$	Sigma, Dorset, UK
PD-10 gel column	Pharmacia, UK
4-15% Polyacrylamide Precast Gels	BioRad, UK
Potassium iodide	Sigma, Dorset, UK
Pyridine (dry)	Sigma, Dorset, UK
Sephadex G-50	Pharmacia, UK
Sodium azide	Sigma, Dorset, UK
Sodium borate	Sigma, Dorset, UK
Sodium dodecyl sulphate (SDS)	Sigma, Dorset, UK
Sodium hydroxide	BDH, UK
Sodium metabisulfate	Sigma, Dorset, UK
Succinic anhydride	Sigma, Dorset, UK
Trichloroacetic acid (TCA)	Sigma, Dorset, UK
Trifluoroacetic acid (TFA)	KMZ Chemicals Ltd, Surrey, UK
Trinitrobenzenesulfonic acid (TNBS)	Sigma, Dorset, UK
Whatman filter paper	Whatman Scientific Ltd, UK

2.2 Methods

The methodology used throughout the preparation and characterisation of IgG-succinyl-DHEA and BSA-succinyl-DHEA conjugates is described below. For more details on the composition of buffers and reagent solutions see Appendix 1.

2.2.1 Gel filtration chromatography

Originally introduced by Porath and Flodin (1959), the dextran-based materials of Pharmacia chemicals, Sephadex gel, quickly acquired a reputation as a gel filtration medium for the rapid separation of macromolecules. In gel filtration chromatography, molecules in solution are separated, as they pass through a column packed with a gel, according to their size. While small molecules enter the gel beads and are retarded in their passage down the column, larger molecules that cannot diffuse into the gel move together with the eluent in the forefront. Molecules thus leave the column in order of decreasing size due to the sieving effect of the gel and for this reason this technique is also known as molecular sieve, size exclusion and gel permeation chromatography. One parameter which is critical in this technique is the necessity to be certain that no significant quantity of material is adsorbed irretrievably to the column. Pharmacia's handbook, Gel Filtration: Principles and Methods (1991) gives an excellent theoretical and practical overview of this technique.

Procedure:

Sephadex G-50 gels were supplied as dry powder. Thus, Sephadex gels were suspended in D.I. water and allowed to swell for 3 h at 20°C. The columns used were pipettes or burettes, with a glass wool plug at the bottom. The column was filled with the gel suspension carefully added, while the outlet was simultaneous opened. During the process of settling, the gel was continuously poured into the column until about 2 cm from the top. The column was then washed and equilibrated with at least 3 volumes of 20mM borate buffer (pH 9.5). Samples (0.5-1.0ml) were carefully applied on the top of the gel bed to avoid disturbance. The sample was let into the gel and washed by the addition of only a small volume (1.0-2.0 ml) of borate buffer. Once all the sample had entered the gel, borate buffer contained in a reservoir was added onto the gel under vacuum. Typically, 1ml fractions were collected with a fraction collector (RediFrac, Pharmacia LKB Biotechnology, UK) equipped with a drop counting device. The number of drops equivalent to 1 ml was pre-determined. Columns were washed as described above, prior to the addition of a different sample. If not in use for more than 2 days, columns were equilibrated with D.I. water containing 0.02% (w/v) sodium azide to prevent microbial growth.

2.2.2 Chloramine-T method for the radioiodination of proteins

Radiolabelling techniques have been used extensively in biochemical studies for the measurement of very low concentrations or small amounts of substances. The major advantage of the use of radioactively-labelled tracers is the ease, rapidity, reliability and

precision of their quantitation. The preferred method for labelling soluble proteins is the addition of ^{125}I because the isotope is cheap and easily detected. The radioiodination occurs by electrophilic addition of ^{125}I to tyrosine residues and to a lesser extent to histidine and trytophan .

Chloramine-T (CT), the sodium salt of *N*-chloro-*p*-toluenesulfonamide, has been used as an oxidant for radioiodination since the early 1960s (Hunter and Greenwood, 1962; Greenwood *et al.*, 1963; Hunter, 1970). Chloramine-T is a water soluble reagent and it has strong oxidising properties that readily lead to the formation of the required electrophilic halogen species that results in iodine incorporation into target molecules (Figure 2.1). The reactions of CT are well documented, being suitable for both macromolecule protein iodination and small molecule modification (Wilbur, 1992; Amersham's handbook, 1993).

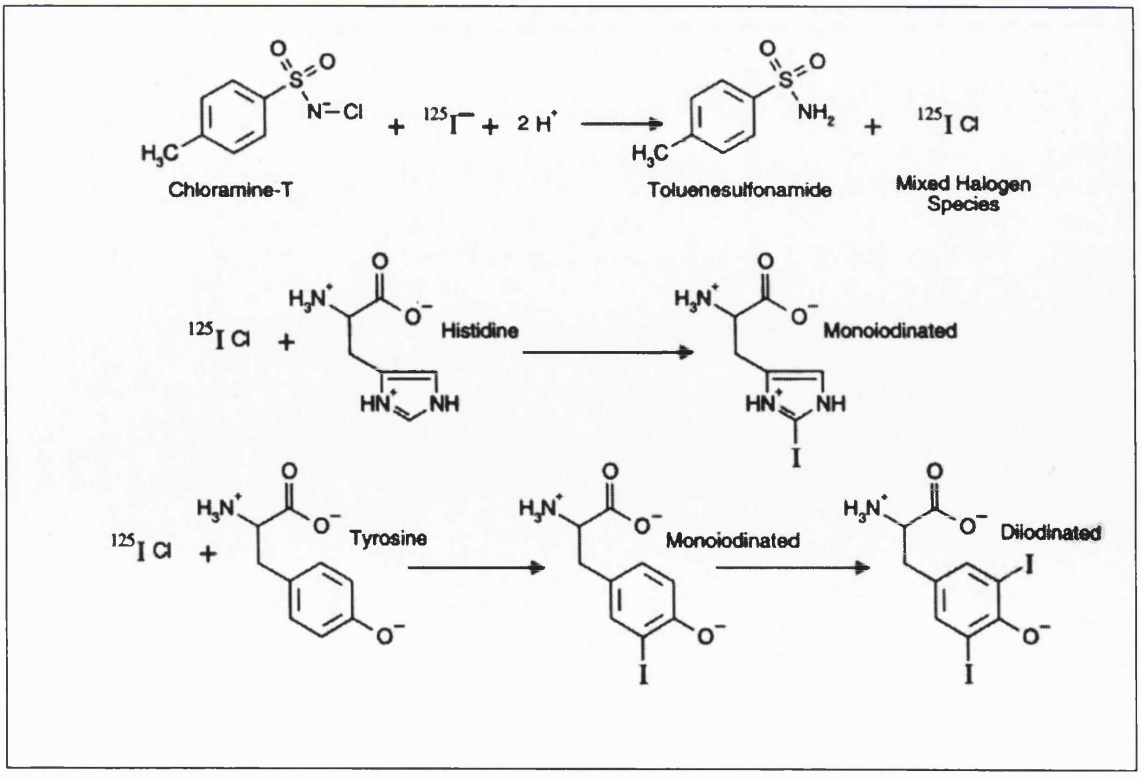


Fig. 2.1 *Chloramine-T and its sequence of reactions in aqueous system.*

The reaction of CT with iodide ion in solution results in oxidation with subsequent formation of a reactive, mixed halogen species (^{125}ICl). The ^{125}ICl then rapidly reacts with any sites within target molecules that can undergo electrophilic substitute reactions. Within proteins, any tyrosine and histidine side-chain groups can be modified with iodine within 30s to 30 min. Sonoda and Schlamowitz (1970) have studied more possibilities of optimising the conditions of iodination to obtain higher incorporation of radioactive iodine into proteins and to avoid damage of the proteins. The optimisation that has been suggested is a pH over the range 6-8, 2°C and around 10 min.

Procedure:

IgG or BSA (100 µl, 0.5 mg/ml), dissolved in phosphate buffer (0.05M, pH 7.5) was added to Na^{125}I solution (2 µl, 200 µCi) in 10 µl phosphate buffer (0.25M, pH.7.5). Chloramine-T (10 µl, 5mg/ml) in phosphate buffer (0.05M, pH 7.5) was added into the mixture. After 10 min at 2°C, the reaction was stopped by the addition of sodium metabisulfite (20 µl, 1.2 mg/ml) in phosphate buffer (0.05M, pH 7.5). Potassium iodide (368 µl, 1 mg/ml) in phosphate buffer (0.05M, pH 7.5) was added to adjust the final volume. The iodinated protein was purified by gel filtration using PD-10, allowing the column to equilibrate with phosphate buffer (0.05M, pH 7.5) before applying the mixture directly to the top of the gel bed. The fractions corresponding to the iodinated protein were pooled and dialysed against 0.05M phosphate buffer pH 7.5 (3x1000ml) in order to remove unbound ^{125}I. The extent of iodinated protein was assessed by trichloroacetic acid (TCA) precipitation. The radiolabelled protein (5µl) was mixed with BSA (1 drop, 10% w/v) in D.I water. Then 1.25 ml TCA (20% w/v) in D.I.water was added into the mixture and the mixture was incubated at 4°C for 1 h. After centrifugation at 3000 rpm for 10min,

the radioactivity content of the pellets and the supernatant was determined. The radiolabelled protein giving a percentage of less than 90 was again subjected to dialysis until an over 90% value was achieved.

2.2.3 Dialysis

Dialysis offers one of the cheapest and simplest techniques available for the separation of proteins from low-molecular-mass impurities. The principle underlying dialysis is very simple. The protein solution to be purified is contained within a semi-permeable membrane (generally manufactured from cellulose or cellulose acetate), whose pores are small enough to retain the protein but large enough to allow free diffusion of small ions and molecules. The sample of impure protein solution, securely sealed within the membrane, is placed in a surrounding volume of aqueous solution. The small species present will diffuse through the membrane until an equilibrium situation is reached, in other words, until the concentration of all the small species is identical both inside and outside the membrane. A large surface area will increase the opportunity for diffusion of solutes but the large area of tubing also provides the opportunity for loss of protein due to adsorption. Clearly, if the volume of clean solution outside the membrane is very large compared to that of the sample, the concentration of impurities within the membrane can be reduced to a very small fraction of its original concentration. Repeating the process with fresh solution further reduces the remaining impurities, so that an extremely low level can be reached within a relatively few changes.

Dialysing a 10 ml sample against 10 litres of buffer reduces the level of impurities to 0.1% of the original value; with only two further changes of buffer, the residue will be

one part per billion of the starting concentration, in the absence of any effects such as binding to the protein. It is usually best to dialyse against 0.5 litres several times; e.g. impurities in 10 ml can be reduced to less than 0.1% after 2-3 changes.

Procedure:

The cellulose membranes used for dialysis may be soaked in water or buffer before use. Samples were placed within the semi-permeable cellulose tubing with a 12000 to 14000 MW cut-off . Samples, after being sealed with plastic clips, were placed in buffer and usually dialysed overnight at 4 °C.

2.2.4 The Quantification of ε-amino groups with trinitrobenzenesulfonic acid

The TNBS reagent has been used for the determination of free amino groups in proteins (Habeeb, 1966; Kakade and Liener, 1969; Cayot and Tainturier, 1997). TNBS reacts equally well with both α-amino and ε-amino groups of amino acids (Fields, 1972). In this study, L-glutamic acid (MW 147.13) was chosen because of its proximity to the molecular weight of L-lysine (146.19). L-lysine contains two reactive primary amino groups (α- and ε-amino groups) which react equally well with the TNBS reagent. It was observed that the TNP derivative of L-lysine had twice the absorbance of the TNP derivative of L-glutamic acid, when they were matched mole to mole.

The difference in absorbance of TNP-L-lysine and TNP-L-glutamic acid gives the true estimate of the ε-amino groups present in L-lysine. The number of ε-amino groups present in proteins and drug-protein conjugates was directly determined from a standard

curve generated from the difference in the absorbance at 340 and 420 nm. for TNP-L-lysine and TNP-L-glutamic acid. The difference in absorption accurately accounts for the free ε-amino groups. The standard curve was based on linear regression analysis using the equation $y = A + Bx$ (Sashidhar et al., 1994)

Procedure:

1 ml of amino acid, L-lysine (MW 146.19) and L-glutamic acid (MW 147.13) or protein conjugate (50-200 μg) solution, were added to 1 ml of 4% $NaHCO_3$ (pH.8.5) and 1 ml of 0.01% or 0.1% freshly made trinitrobenzenesulfonic acid (TNBS). The reaction was carried out at 42 ± 2 °C for 2 h and was followed by the addition of 1 ml 10% sodium dodecyl sulphate (SDS) and 0.5 ml of 1 N HCl. The absorbance of the solution was monitored at 340 and 420 nm. A Wallac CompuSpec UV/Visible spectrophotometer connected to a PC, (Wallac UK Ltd., UK) was used in the spectrophotometric determinations.

The number of ε-amino groups present in the conjugates was directly determined from a standard curve generated from the difference in the absorbance at 340 or 420 nm of TNP-l-lysine and TNP-l-glutamic acid. The difference in absorption accurately accounts for the free ε-amino groups. The standard curve was based on linear regression analysis using the equation $y = A + Bx$. Furthermore, the percentage of conjugation was calculated using the following equation:

$$\% \text{ conjugation} = \frac{(\text{conc.of } \varepsilon\text{-amino group in carrier protein} - \text{conc.of } \varepsilon\text{-amino group in conjugates})}{\text{conc.of } \varepsilon\text{-amino group in carrier protein}} \times 100$$

2.2.5 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is the most common form of electrophoresis of proteins, used to assess purity and determine apparent MW. When denatured by heating in the presence of excess sodium dodecylsulphate (SDS) and a reducing agent (usually β-mercaptoethanol or dithiothreitol (DTT)), most proteins bind to the negatively charged SDS in a constant weight ratio so that they acquire identical charge densities and migrate in polyacrylamide gels of adequate porosity according to their size (Shapiro et al., 1967; Hames, 1990; Westermeier, 1993).

Sodium dodecyl sulphate (SDS) is a powerful anionic detergent, due to its ability to bind avidly to proteins in a manner virtually independent of all parameters except molecular size. SDS was originally included in electrophoresis formulations to dissociate and solubilise protein complexes (Maizel, 1966; Shapiro et al., 1966). The addition of an excess of SDS to protein solutions also prevented the aggregation of the proteins.

Coomassie Brilliant Blue R is a sensitive protein stain which can be used to locate protein bands after electrophoresis in polyacrylamide gel. This protein stain varies among proteins but 0.5 μg/band is usually detectable (Johnstone and Thorpe, 1982).

Procedure:

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (1970). An equal volume of the Lane Marker Reducing Sample Buffer (100 μl) containing SDS and DTT was added to each sample (100 μl, 2 mg/ml of proteins) and the mixture heated for 3 min at 95°C. The low range prestained SDS-PAGE standards containing phosphorylase B (103 kDa), bovine

serum albumin (77 kDa), ovalbumin (48 kDa), carbonic anhydrase (34.2 kDa), soybean trypsin inhibitor (28.4 kDa) and lysozyme (20.5 kDa) were used as markers. Electrophoresis of the samples was performed on precast gels from the Ready Gel Cell (BioRad, UK) using a power supply (model 1000/500) (BioRad, UK) with a linear 4-15% polyacrylamide gradient. Reservoir buffer as described in the appendix was poured into the electrode reserviour. 20 µl of molecular weight marker and sample mixture were applied in each well using gel loading tips. Gels were run at a constant voltage of 200 volts. Electrophoresis was stopped when the tracking dye had migrated up to 0.7 cm from the bottom of the gel. Gels were stained with Coomassie Brilliant Blue (R-250) solution (see appendix) overnight at room temperature. Gels were rinsed with water and placed in a destaining solution (see appendix) after discarding the stain solution. The destaining solution was changed frequently until a clear background was obtained.

2.2.6 Mass Spectrometry

Mass Spectrometry (MS) has been practised as an analytical technique since the early part of the century. MS has capabilities that enable it to be used to solve structural problems not easily handled by other techniques. It is able to provide accurate molecular weight information on picomole amounts of substances. Its principle is relatively simple; charged species are generated from an atom or molecule of interest, and separated on the basis of their mass-to-charge (m/z) ratio. This separation can be achieved with magnetic sector, time-of-flight or quadrupole mass analyses of which the latter is the most commonly used.

Procedure:

Mass spectra were run on a VG analytical ZAB-SE instrument, using the fast-atom bombardment (FAB) technique. 20 kV Cs^+ ion bombardment was used, with 2 μl of an appropriate matrix consisting of a solution of either 3-nitrobenzyl alcohol in methanol, or thioglycerol with NaI in methanol. The MALDI-TOFMS analysis were performed on a Fisons Instruments (Manchester, UK) TofSpec using 337 nm radiation from a nitrogen laser. The mass spectrometer was operated in linear mode, at 28 kV accelerating voltage, giving an ion flight path of 0.9 m. Some samples were analysed by Electrospray ionisation mass spectrometry (ESI-MS). ESI-MS was performed on a Finnigan Navigator mass spectrometer (Manchester, UK) equipped with a Waters alliance 2690. The sample was pumped into a 75 μm i.d. stainless steel capillary which was held at a voltage of 3-5 kV.

2.2.7 Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) is a mode of liquid chromatography in which the sample is applied as a small spot to the origin of a thin sorbent layer supported on a glass, plastic, or metal plate. The mobile phase moves through the stationary phase by capillary action, sometimes assisted by gravity or pressure. TLC separations take place on the open layer with each component having the same total migration time but different migration distances. TLC has been regarded traditionally as a simple, rapid, and inexpensive method for the separation, tentative identification, and visual

semiquantification of a wide variety of substances (Fried and Sherma, 1999). Positions of separated zones on TLC are described by the R_f value of each substance, where

$$R_f = \frac{\text{Distance compound has moved from origin}}{\text{Distance of solvent front from origin}}$$

Procedure:

Reaction progress was monitored by TLC on Kieselgel PF_{254} using chloroform-methanol (9.75:0.25 v/v) as the solvent system. The separating compounds were observed as dark spots on a fluorescent background when viewed under ultraviolet light. The coloured spots were also detected on TLC by spraying bromocresol green solution as an acid-base indicator.

2.2.8 High Performance Liquid Chromatography (HPLC)

Since 1969, the major development in liquid chromatography has been the introduction of high-performance apparatus, which utilise columns of very homogeneous small bead particles. Such particles create a high resistance to liquid flow, so the equipment is designed to generate and operate at high pressures (Jeffery et al., 1991). HPLC consists of a solvent delivery system which includes a pump, a sample injection system, the column, the detector, strip chart recorder, data handling device and microprocessor control. The choice of a suitable mobile phase is vital in HPLC. Optimum

separating conditions can often be achieved by using a mixture of two solvents, and gradient elution is frequently used where sample components vary widely in polarity.

Procedure:

Preparative HPLC was carried out using an Applied Biosystems 400 Solvent Delivery System, Mixer and Injector, with solvent gradients effected by two micro-processor-controlled Gilson 302 single piston pumps. HPLC grade solvents, 0.1% TFA/H_2O (A) (KMZ Chemicals Ltd, Surrey, UK) and 80% acetronitrile/0.1% TFA/H_2O (B), were filtered under vacuum through a 25 μm filter prior to use. Compounds were detected with an Applied Biosystems Programmable Absorbance Detector at 214 nm, and chromatographs were recorded with an LKB 2210 single channel chart recorder. Analytical separations were carried out using a Beckman Ultrasphere Octyl Column (5 μm, 4.6 mm x 250 mm) with guard column (5 μm, 4.6 mm x 45 mm) and a solvent gradient that increased from 0-100 % solvent B (80% acetronitrile (Rathburn Chemicals Ltd, UK)) over a period of 30 min, with a constant flow rate of 0.7 ml/min (Hillery et al., 1996).

2.2.9 Preparation of IgG-succinyl-DHEA and BSA-succinyl-DHEA

2.2.9.1 Synthesis of 3'-succinyl-DHEA

DHEA (500 mg, 1.7 mol) mixed with tracer 1,2,6,7 (^{3}H) DHEA (0.5 ml, 0.5 mCi) was added to succinic anhydride (50 mg, 0.5 mol) in the presence of 4-dimethylaminopyridine (50 mg, 0.4 mol). Then 5 ml of dry pyridine was added and the

solution was stirred for 24 h at 20°C. The completion of the reaction was monitored by TLC. The residue, after the evaporation of the pyridine, was dissolved in 20 ml dichloromethane. The solution was washed with 1M $KHSO_4$ (3x10ml), dried with $MgSO_4$ and filtered. After filtration, the 3'-succinyl-DHEA in dichloromethane was evaporated to dryness. The resultant compound was analysed by TLC, HPLC and MS.

2.2.9.2 The conjugation of succinyl-DHEA to IgG and BSA

The 3'-succinyl-DHEA (50 mg, 0.129 mmol), dissolved in 5 ml of dimethylformamide, was reacted with N-hydroxy-3-sulfo-succinimide (13.98 mg, 0.065 mmol) and 1-(3-dimethylaminopropyl) 3-ethylcarbodiimide hydrochloride (12.35 mg, 0.065 mmol). After 6 h, the reaction produced a sulfo-NHS active ester (3'-sulfo-NHS-succinyl-DHEA) which is highly reactive with amines on proteins.

IgG (2 mg) dissolved in 1 ml borate buffered solution (20mM, pH. 9.5) was reacted with excess of 3'-sulfo-NHS-succinyl-DHEA (1:10 to 1:300 molar ratio of IgG to drug) for 2 h at 20°C. The water soluble conjugates were isolated from excess reagents by gel filtration on Sephadex G-50 eluted with borate buffered solution (20mM, pH. 9.5). The fractions of IgG-succinyl-DHEA conjugates were pooled and lyophilised and kept at -20°C. The lyophilised IgG-succinyl-DHEA conjugates were dissolved with 2 ml PBS buffer (pH.8).

BSA (2 mg) dissolved in 1 ml borate buffered solution (20mM, pH. 9.5) was reacted with excess of 3'-sulfo-NHS-succinyl-DHEA (1:10 to 1:300 molar ratio of BSA to drug) for 2 h at 20°C. The water soluble conjugates were isolated from excess reagents by gel filtration on Sephadex G-50 eluted with borate buffered solution (20mM, pH. 9.5).

The fractions of BSA-succinyl-DHEA conjugates were pooled and lyophilised and kept at -20°C. The lyophilised BSA-succinyl-DHEA conjugates were dissolved with 2 ml PBS buffer (pH.8).

2.2.10 Stability of the conjugates

(^{125}I)IgG-s-(^{3}H)DHEA (2 mg IgG and 0.33 mg DHEA) and (^{125}I)BSA-s-(^{3}H)DHEA (2 mg BSA and 0.12 mg DHEA) in 0.2 ml 0.15M phosphate buffered saline pH 8 (PBS) were mixed with fresh mouse plasma at 37 °C and incubated at the same temperature. After 1, 3, 6, 10 and 24 h, samples (0.2 ml) were collected and stored at -20°C prior to isolating the conjugates and free drug by gel filtration (Sephadex G-50). Each of the fractions of the conjugates and free drug were monitored for ^{125}I and ^{3}H in a gamma counter and a liquid scintillation counter.

2.2.11 Pharmacokinetics study after a single intravenous injection

Animals: Male T/O outbred mice (20-25 g body weight) were purchased from Harlan-OLAC, UK, and acclimatised for at least one week prior to any experiment. A conventional diet and water were available *ad libitum*.

(^{125}I)IgG (2 mg), (^{125}I)BSA (2 mg), (^{125}I)IgG-s-(^{3}H)DHEA (2 mg IgG and 0.17-0.33 mg DHEA) and (^{125}I)BSA-s-(^{3}H)DHEA (2 mg BSA and 0.09-0.12 mg DHEA) in 0.2 ml 0.15M PBS (pH 8) were injected intravenously into the tail veins of the mice (n=4).

The tail was warmed before the injection by the use of a desk lamp with a 100 W bulb supplies sufficient heat to keep the vein dilated. In another experiment, animals (n=4) were injected with 0.3 mg of (^{3}H)DHEA in 0.05 ml ethanol-PBS (1:1 v/v). At time intervals after injection, 50 µl blood samples were collected from the lateral tail vein up to 24h into heparinised micropipettes, diluted into 450 µl of PBS, and centrifuged at 3000 rpm for 10 min. Supernatants (diluted plasma) were then assayed for ^{125}I and ^{3}H in a gamma counter and a liquid scintillation counter respectively.

2.2.12 Tissue distribution after a single intravenous injection

For tissue distribution studies, (^{125}I)IgG-s-(^{3}H)DHEA (2 mg IgG and 0.33 mg DHEA) and (^{125}I)BSA-s-(^{3}H)DHEA (2 mg BSA and 0.12 mg DHEA) in 0.2 ml 0.15M PBS (pH 8) were injected intravenously into the tail veins of male outbred mice (n=4). Also, mice (n=4) were injected with 0.3 mg of (^{3}H)DHEA in 0.05 ml ethanol-PBS (1:1 v/v). After intravenous injection, the mice were kept in metabolic cages in order to collect urine and faeces at time intervals. Animals were sacrified at 5 min, 2h, 6h, 12h and 24h and tissues (liver, kidney, spleen, thyroid, brain, lung, muscle, intestine and intestine washings) were dissected, homogenised in 1M NaOH and then assayed for ^{125}I and ^{3}H.

Chapter III

Preparation and Characterisation of IgG-s-DHEA and BSA-s-DHEA

3.1 Introduction

This chapter describes the coupling method used to prepare IgG-s-DHEA and BSA-s-DHEA conjugates in which the drug is covalently attached through a succinyl (s) spacer. The conjugates were characterised by gel filtration chromatography, the TNBS method, radiolabelling and SDS-gel electrophoresis.

In designing the conjugates, the functional groups available for conjugation on both the carrier and the drug to be used must first be identified. Then, a cross-linking agent under appropriate conditions of reaction and solvent systems must be chosen. The conjugation method should be simple and straightforward. An ideal method of conjugation should provide the maximum number of incorporated drug molecules without altering much of the antibody activity and acceptable protein recovery. The method should also not cause any aggregation and/or precipitation during the reaction. The conjugation of hydrophobic drugs through the carboxylic or amino groups of protein molecules may reduce aqueous solubility leading to aggregation and precipitation. Care should be taken during conjugation to ensure that the process does not produce protein-protein cross-linking (dimer or oligomer formation) or intraprotein cross-linking. The

introduction of a spacer molecule between the drug and protein, to increase the drug load and to provide a favourable steric orientation, may be selected.

It has been apparent that drug molecules linked directly to antibodies are often poorly released from their conjugates by the action of lysosomal enzymes. Such slow release rates might be due to a simple steric effect, since success has been achieved by introducing a spacer molecule between the two components. Thus, the advantage of a spacer is twofold: it reduces the steric effect and it also helps to increase the drug load per immunoglobulin molecule. The spacers are designed in such a way that the conjugates remain stable during their transit through the blood, but release free drug in the lysosomal milieu. Most often, amide linkages between the components in the ternary conjugates are chosen because of the presence of higher amidase activity in lysosomes and the absence of similar activity in the serum.

Immunoglobulin G and albumin molecules possess a number of functional groups suitable for modification especially the ε-amino groups of lysine and the carboxylate groups which are plentiful in most proteins. The coupling procedure in this study involves mainly the ε-amino groups of lysine residues in proteins. DHEA has a hydroxyl group and a keto group which can be used for chemical modification. A hydroxyl group of DHEA was used for conjugation in this study. The coupling involves a two-step derivatisation. Firstly, the esterification of a hydroxyl group on DHEA with succinic anhydride to form hemisuccinates is a simple mechanism used to generate a carboxyl group that can be used for further reaction with proteins (Mitra and Ghose, 1990). Secondly, the formation of amide bonds between carboxyl groups and amino groups present on succinyl spacer and protein respectively in the presence of carbodiimide (Dosio et al., 1997).

1-ethyl-3-(3-dimethylaminpropyl)carbodiimide hydrochloride (EDC) is the most popular carbodiimide used in conjugating biological substances. Its water solubility allows direct addition to a reaction without prior organic solvent dissolution. However, the reagent is labile in the presence of water and should be stored desiccated at -20°C. The bottle must be warmed to room temperature before opening to prevent condensation that will cause decomposition of the reagent over time.

N-hydroxysulfosuccinimide (sulfo-NHS) esters are hydrophilic active groups that react rapidly with amines on target molecules (Staros, 1982). Unlike nonsulfonated NHS esters that are relatively water insoluble and must be first dissolved in organic solvent before being added to aqueous solution, sulfo-NHS esters are relatively water soluble, long-lived and hydrolyse more slowly in water.

The advantage of adding sulfo-NHS to EDC reactions is to increase the stability of the active intermediate, which ultimately reacts with the attacking amine. EDC reacts with a carboxylic group to form an active ester (O-acylisourea) leaving group. Unfortunately, this reactive complex is subject to rapid hydrolysis in aqueous solutions, having a rate constant measured in seconds (Hoare and Koshland, 1967). If the target amine does not find the active carboxylate before it hydrolyses, the desired coupling cannot occur. This is especially a problem when the target molecule is in low concentration compared to water.

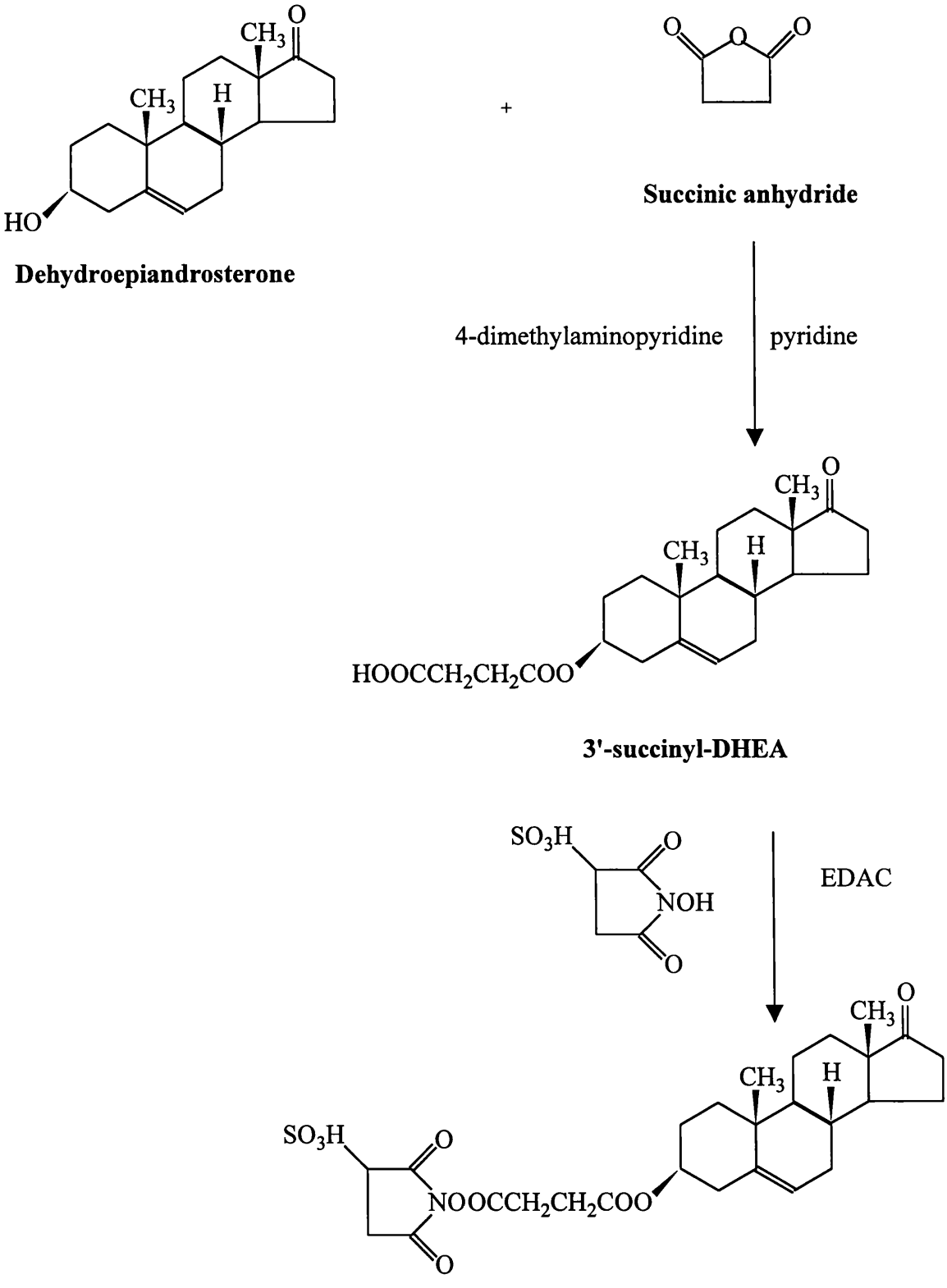


Fig. 3.1 Synthesis of 3'-(3-sulfo-N-hydroxysuccimidyl)-succinyl-DHEA

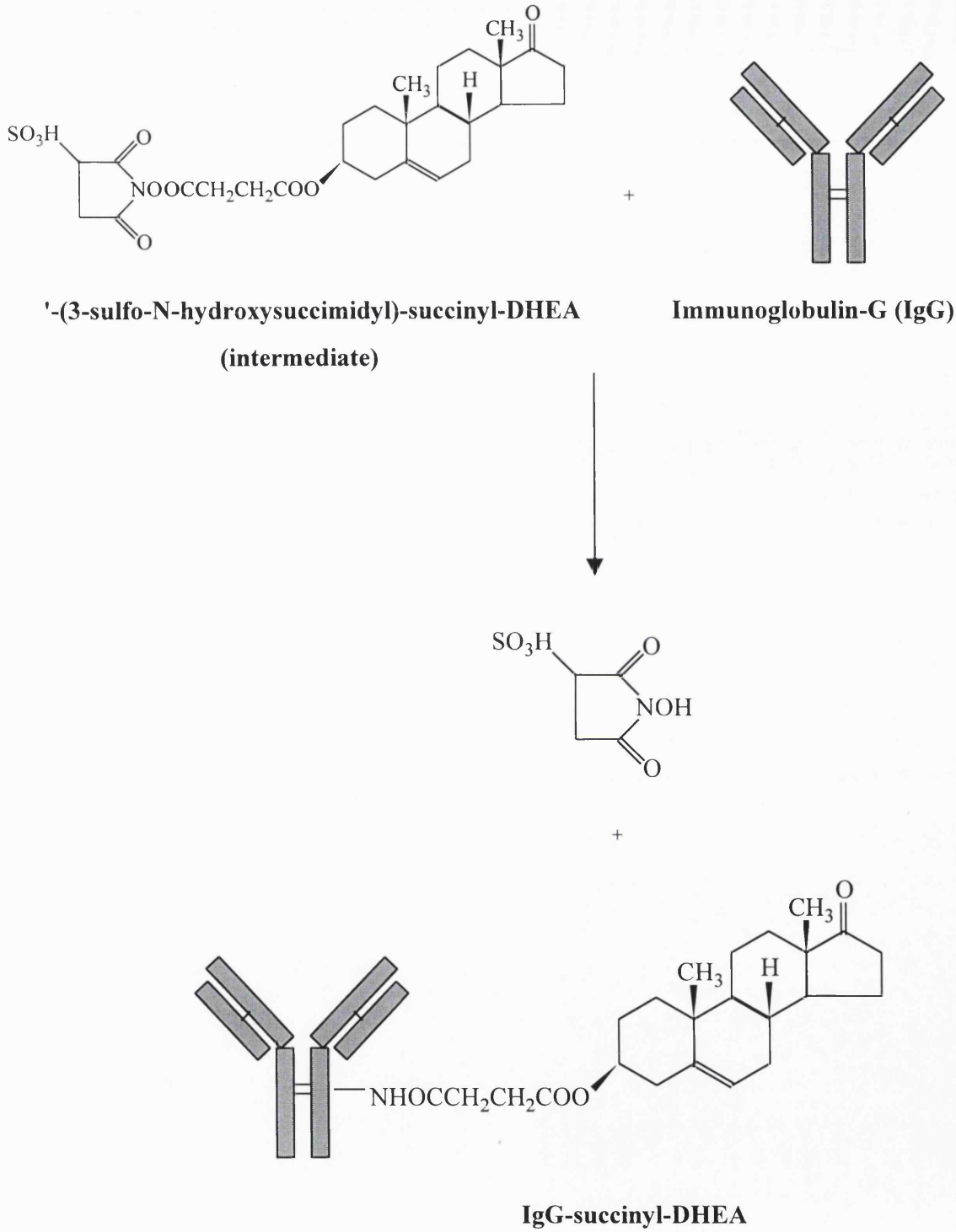


Fig. 3.2 *Conjugation of dehydroepiandrosterone to IgG/BSA through a succinyl spacer*

The DHEA molecule is first linked by its 3'-hydroxyl to succinic anhydride in dry pyridine and 4-dimethylaminopyridine. This reaction yields hemisuccinate, which introduces a carboxyl group at the terminal of a succinyl spacer for conjugation to proteins. The 3'-succinyl-DHEA after purification, is incubated with N-hydroxy-3-sulfo-succinimide, in the presence of carbodiimide reagent to produce a sulfo-NHS active ester (Fig. 3.1). This ester is highly reactive towards nucleophilic groups (ε-amino groups of lysine). The active ester solution is then used directly to derivatise the protein and to give a stable amide linkage (Fig. 3.2). This two-step process allows the active species control over the conjugation without protein-protein cross-linking or intraprotein cross-linking.

3.2 Results and Discussion

3.2.1 Preparation of IgG-s-DHEA and BSA-s-DHEA

3.2.1.1 Synthesis of 3'-succinyl-DHEA

A hydroxyl group and a keto group available on the DHEA molecule can be used for chemical modification. The 3'-hydroxyl group of DHEA allows for the introduction of a succinyl spacer to give an ester linkage and carboxylate functions for further derivatisation purposes. This short chain can serve as a spacer to enhance steric accommodations and the terminal carboxylate group can facilitate subsequent coupling with amines. Succinic acid is a four carbon molecule with carboxylic acid groups on both ends. The anhydride has a five atom cyclic structure that is highly reactive toward nucleophiles. Reaction of the anhydride with available -OH groups opens the anhydride ring, forming an ester bond with hydroxyl and create carboxylates for further conjugation. This anhydride reaction is similar to that of acyl halides. Anhydrides are somewhat less

reactive than acyl halides but are more difficult to hydrolyse than acyl halides. Acids and bases are often used as catalysts. In this study, pyridine and 4-dimethyaminopyridine are used as a catalyst in the reaction mixture. The 3'-succinyl-DHEA was prepared according to the synthetic scheme shown in Fig. 3.1. The reaction progress was monitored by thin-layer chromatography (TLC) using chloroform-methanol (97.5:2.5 v/v) as the solvent system (R_f= 0.26). It was found that the yield of 3'-succinyl-DHEA after purification was 80.33%. It was observed that the presence of a succinyl spacer in the drug conjugate resulted in the increased lipophilicity of DHEA. The purified sample was characterised by HPLC, UV-visible spectroscopy and by mass spectrometry.

Fig. 3.3 and 3.4 illustrates the mass spectra obtained from DHEA and 3'-succinyl-DHEA samples. The mass spectra in Fig. 3.3 shows a sodium adduct ion of DHEA at 311 Da $[M + Na]^+$, while sodium adduct ion of 3'-succinyl-DHEA were at 411 Da $[M+Na]^+$ and 433 Da $[M+2Na]^+$ respectively (fig. 3.4). The molecular weight of 388 Da for 3'-succinyl-DHEA represents a difference of +100 Da to the mass of DHEA (288 Da). This proved the coupling of a succinyl spacer to DHEA. The mass spectra obtained for 3'-succinyl-DHEA also showed an ion of 3'-succinyl-(^{3}H)DHEA at a molecular weight of 400 $[M^+]$.

Analysis of the 3'-succinyl-DHEA and DHEA by HPLC showed that the succinyl spacer was bound to DHEA due to the differences of the retention time. The retention time of DHEA and 3'-succinyl-DHEA was 14.38 min and 15.63 min respectively (Fig. 3.5a,b) using a solvent gradient as described in chapter II. The HPLC profiles also showed a single peak for 3'-succinyl-DHEA, indicating the absence of free DHEA after purification (Fig. 3.5b).

The 3'-succinyl-DHEA and DHEA analysis can also be performed by UV-Visible spectrophotometry. The position of maximum absorbance is dependent on the distribution of electrons in the molecule. Absorbance characteristics can therefore respond to conformational changes in structure. The UV-Visible spectrum of DHEA (Fig. 3.6) and 3'-succinyl-DHEA (Fig. 3.7) showed maximum absorptions for DHEA and 3'-succinyl-DHEA at 295 nm and 270 nm respectively. This confirmed that a UV absorbance maximum in the DHEA spectrum had shifted towards a shorter wavelength when a succinyl spacer was linked to the DHEA.

Nuclear magnetic resonance spectrometry (NMR) can also be used to confirm that the succinyl spacer was attached to DHEA due to the different environments of hydrogen atoms in DHEA and 3'-succinyl-DHEA molecules. For NMR preparation, $CDCl_3$ was used to dissolve DHEA, 3'-succinyl-DHEA and pyridine. The NMR spectrum of DHEA (Fig. 3.8) shows δ 0.89 (3H, s, CH_3), δ 1.04 (3H, s, CH_3), δ 2.44-1.03 (19H, m, CH_2 and CH), δ 3.53 (1H, m, CHOH), δ 5.38 (1H,brs, 3'H), while the spectrum of 3'-succinyl-DHEA (Fig. 3.9) are δ 0.88 (3H, s, CH_3), δ 1.04 (3H, s, CH_3), δ 2.17 (4H, s, -CO-CH_2-CH_2-CO-), δ 3.53 (1H, m, CHOH), δ 3.72 (1H, q, -CHO-CO-). It was found that the peak at δ 3.53 (DHEA) has been shifted to δ 3.72 (3'-succinyl-DHEA) and also the spectrum of 3'-succinyl-DHEA shows a higher peak at δ 2.17 as compared to the spectrum of DHEA. This shows that the succinyl spacer was linked to -OH group of DHEA at 3' position. However, a small peak of DHEA (δ 3.53) was also found in the spectrum of 3'-succinyl-DHEA (Fig. 3.9), indicating the presence of free DHEA after purification. Due to the presence of free DHEA, it is not possible for the -OH group of DHEA to react with ε-amino groups of proteins. Thus, the presence of DHEA will not cause any problems for further reaction with proteins.

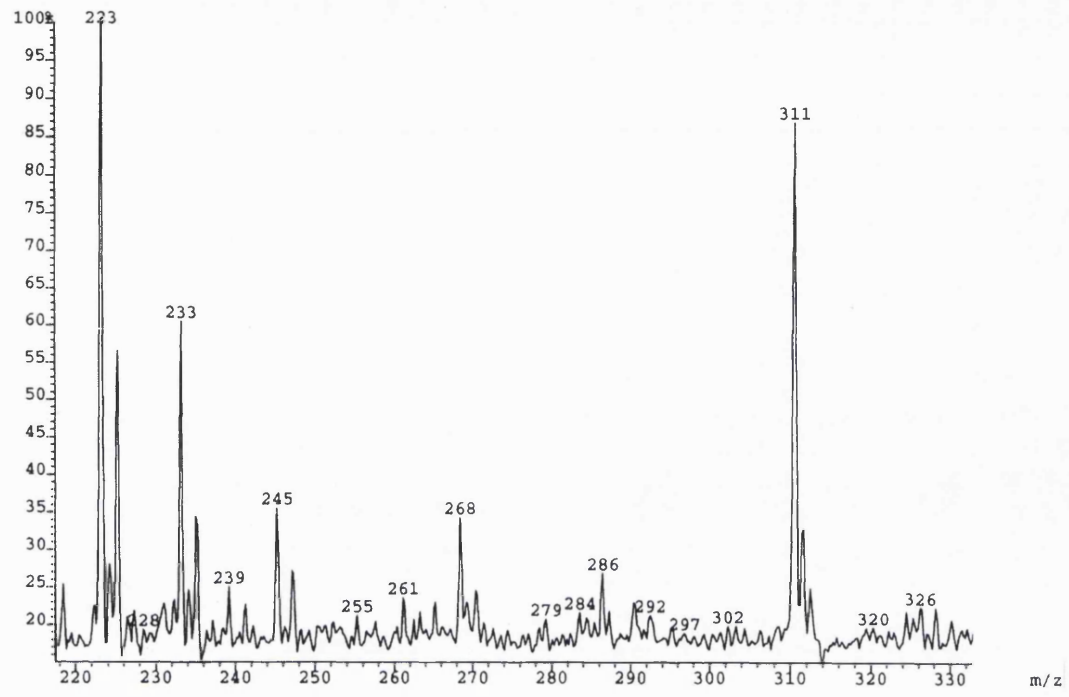


Fig. 3.3 *Mass spectra of Dehydroepiandrosterone (DHEA)*

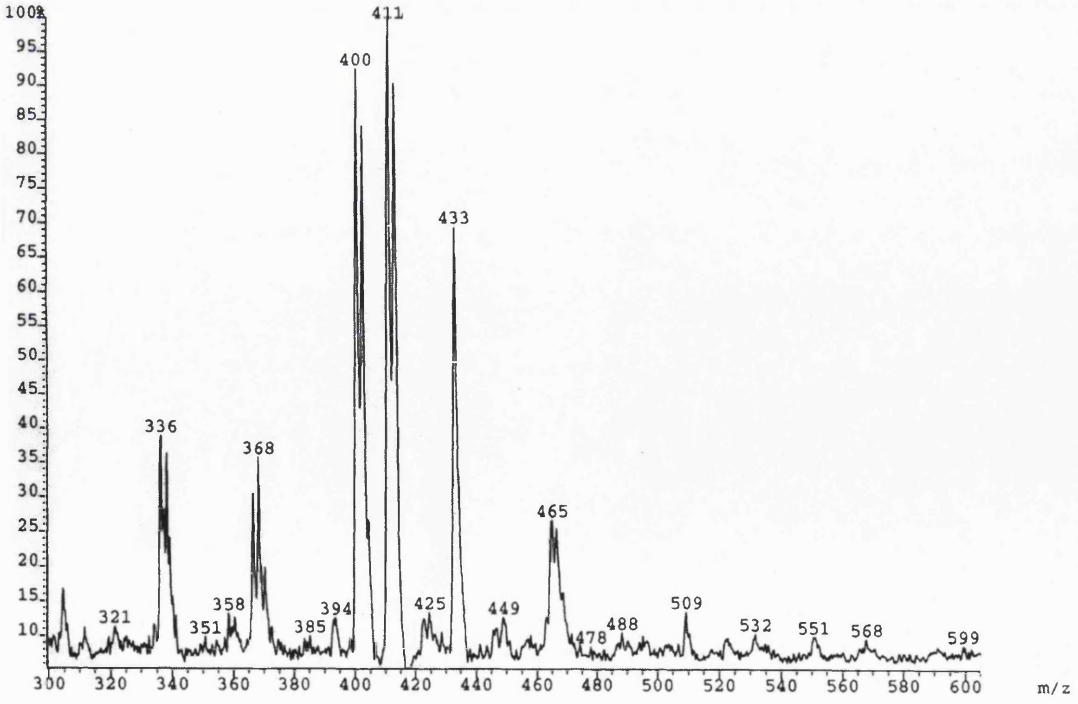
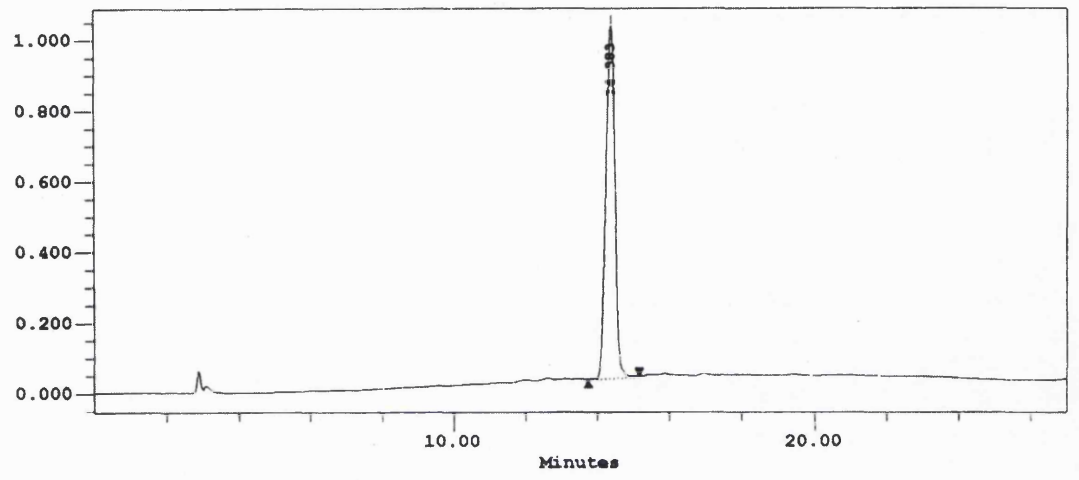


Fig. 3.4 *Mass spectra of Succinyl-dehydroepiandrosterone (s-DHEA)*

(a)



(b)

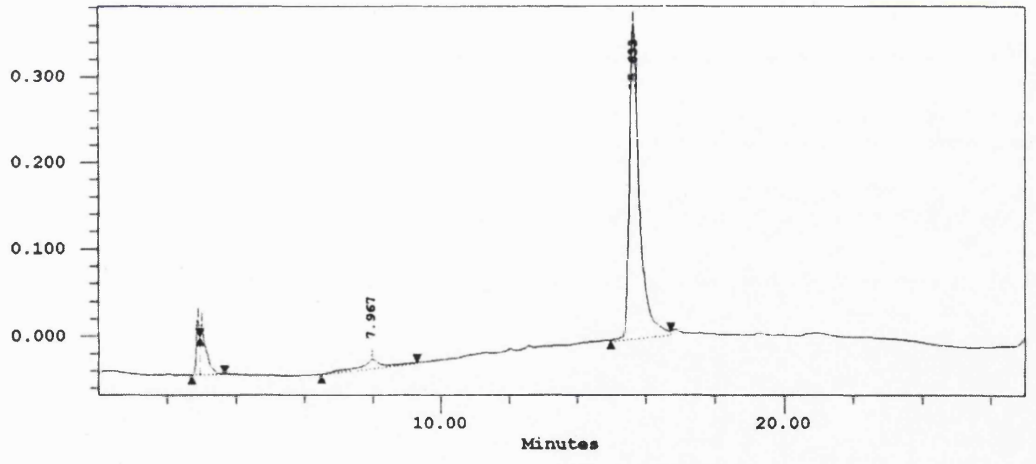


Fig. 3.5 *HPLC profiles of (a) DHEA (b) s-DHEA after purifcation. Analysis was performed using a Beckman Ultrasphere Octyl column.*

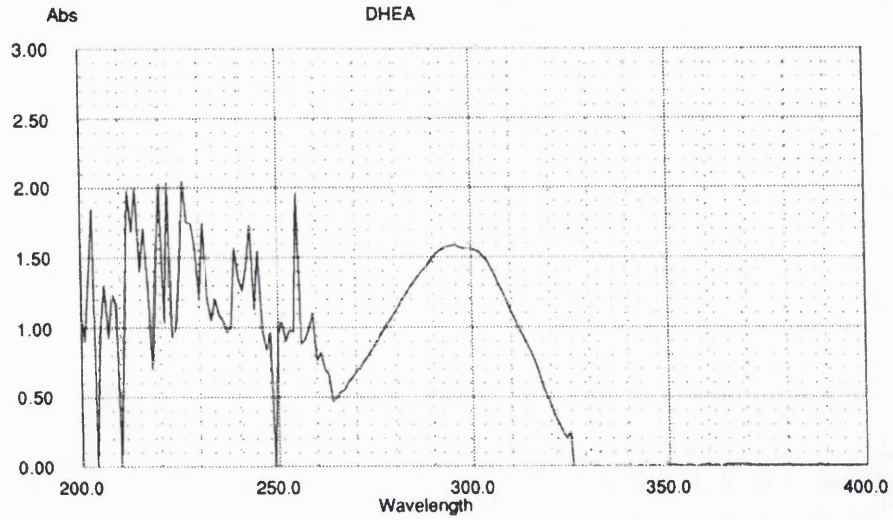


Fig. 3.6 *UV-Visible spectrum of Dehydroepiandrosterone (DHEA)*

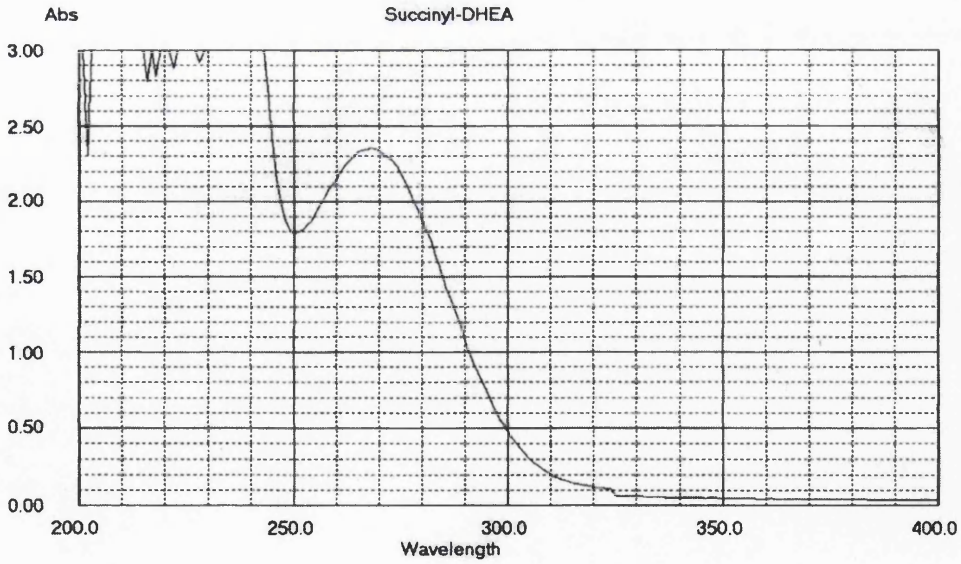


Fig. 3.7 *UV-Visible spectrum of 3'-Succinyl-dehydroepiandrosterone*

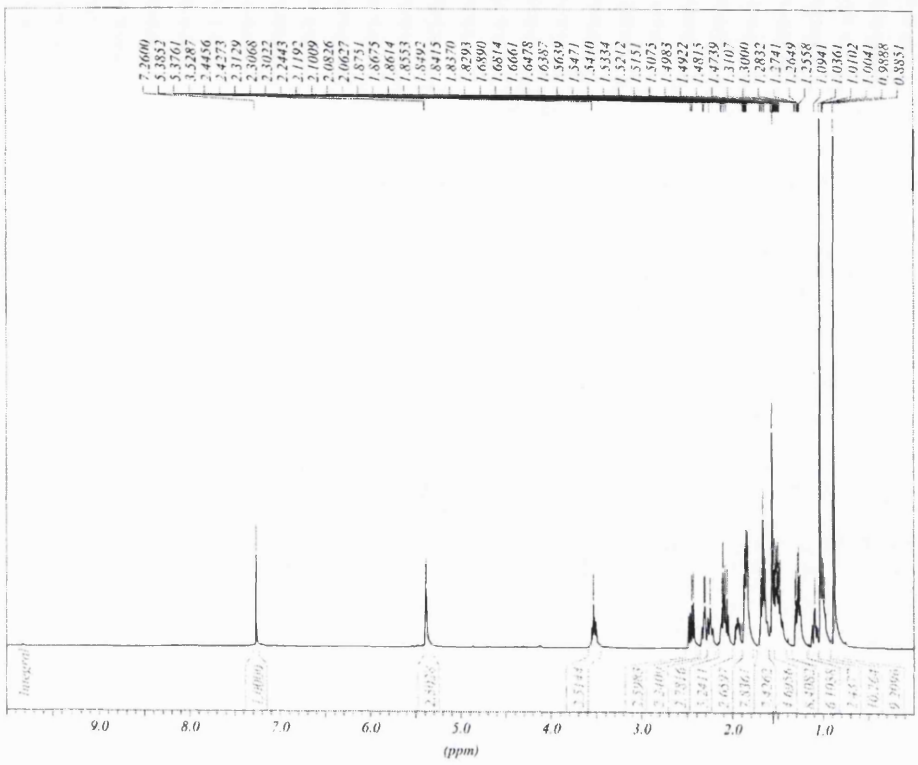


Fig. 3.8 *NMR spectrum of Dehydroepiandrosterone (DHEA)*

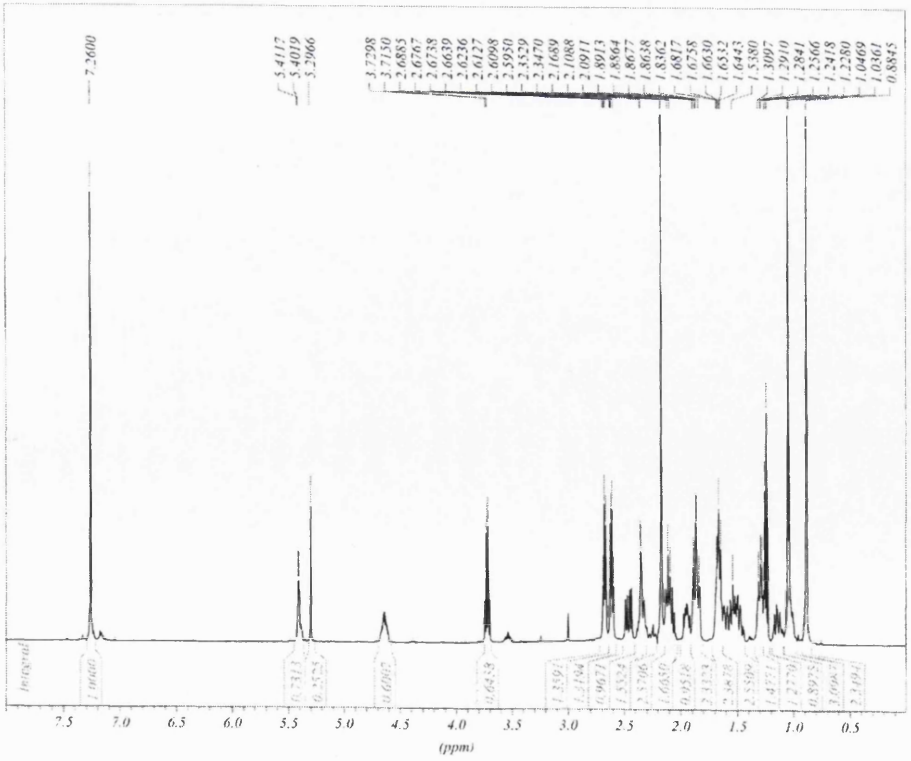


Fig. 3.9 *NMR spectrum of Succinyl-dehydroepiandrosterone (s-DHEA)*

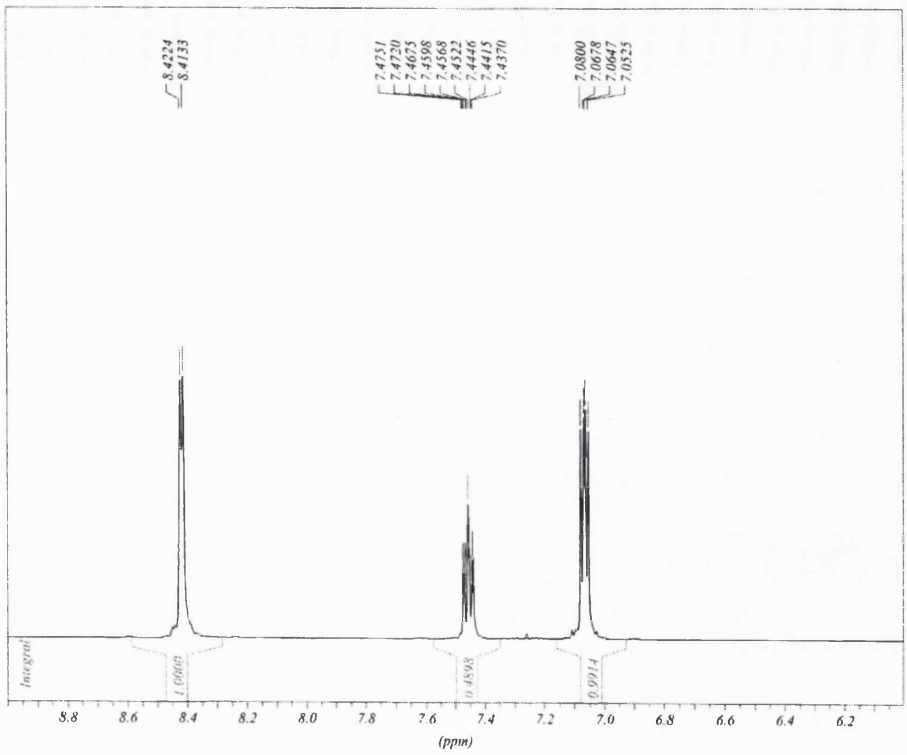


Fig. 3.10 *NMR spectrum of Pyridine*

Fig. 3.10 illustrates the NMR spectrum of pyridine at δ 7.06 (1H, dd, meta-H), δ 7.45 (1H, m, para-H), δ 8.42 (1H, d, ortho-H). This confirmed that there was no pyridine contamination in 3'-succinyl-DHEA after purification due to the absence of pyridine peaks in 3'-succinyl-DHEA spectrum.

In order to detect the degree of modification of the protein or monitor the *in vivo* behaviour of the conjugates, the 3'-succinyl-DHEA was prepared containing (^{3}H)DHEA as tracer. The (^{3}H)DHEA was tritium-labelled at positions 1, 2, 6, and 7.

1,2,6,7 (^{3}H)DHEA (0.5 ml, 0.5 mCi) was mixed with 500 mg of unlabelled DHEA, then the synthesis of 3'-succinyl-DHEA was carried out as described in Chapter II. It was found that the radiolabelled DHEA was also linked with succinic anhydride by a hydroxyl group available at position 3. The evidence for 3'-succinyl-(^{3}H)DHEA was determined by a liquid scintillation counter after purification.

3.2.1.2 Conjugation of 3'-succinyl-DHEA to IgG/BSA

The esterification of the 3'-hydroxyl group on DHEA with succinic anhydride generated a carboxyl group that was used for further reaction with the ε-amino groups of lysine residues of proteins. In this study, 3'-succinyl-DHEA molecules were incubated in the presence of EDC/sulfo-NHS to form the isolated active ester and then added to the IgG/BSA molecule for conjugation (Fig. 3.2). This two step process allows only drugs to form amide bond on proteins, preventing polymerisation of proteins during the conjugation.

Forming a sulfo-NHS ester intermediate from the reaction of the hydroxyl group on sulfo-NHS with the EDC active ester complex extends the half-life of the activated carboxylate to hours. EDC/sulfo-NHS coupled reactions are highly efficient and usually increase the yield of conjugation dramatically over that obtainable solely with EDC. The sulfo groups, increase the hydrophilicity, rendering the NHS active ester more reactive and a higher degree of derivatisation results. In conjugation reactions, the use of EDC/sulfo-NHS often causes severe precipitation of the conjugate. It appears that scaling back the amount of EDC/sulfo-NHS added to the reaction solves this precipitation problem (Hermanson, 1996). It was observed that precipitation was obtained when using EDC/sulfo-NHS to 3'-succinyl-DHEA in a 1:1 ratio, suggesting that scaling back the ratio of EDC/sulfo-NHS to 3'-succinyl-DHEA to a 0.5:1 ratio solved this problem.

As mentioned above, the purified 3'-succinyl-DHEA was dissolved in dimethylformamide (DMF) in the presence of EDC and sulfo-NHS. This reaction produced an active ester named 3'-sulfoNHS-succinyl-DHEA. This reaction was performed in the mixture of DMF and buffered solution in order to dissolve both drugs and proteins. Dimethylformamide was used as a cosolvent because of the following

desirable properties: (a) it is inert to many of the reactive reagents used in preparing conjugates, (b) it is miscible with water in all proportions, and (c) it is compatible with most aqueous protein solutions even at up to 30% v/v ratios. The 3'-sulfo-NHS-succinyl-DHEA solution was used directly to derivatise the proteins. This ester is highly reactive toward nucleophilic groups such as the ε-amino groups of lysine.

This reaction was similar to that reported for paclitaxel-albumin conjugates. The paclitaxel active ester was reacted with human serum albumin (HSA) in a phosphate buffered solution (0.1M, pH 7.3). Dosio et al. (1997) reported that the modification of lysyl groups with a hydrophobic drug involved a drastic decrease in protein solubility. The determination of the degree of derivatisation was estimated using a labelled succinyl spacer and then evaluating the ratio between radioactivity associated with linked paclitaxel and the protein concentration obtained by a modified Lowry method. It was reported that the increase in the degree of derivatisation, from 10 to 30 drug molecules, significantly reduced conjugate solubility and increased the formation of hydrophobic aggregates.

In this study, the 3'-succinyl-DHEA active ester in DMF was linked to IgG or BSA, dissolved in buffered solutions at different pH values (pH 4, 7.4, 9.5), by the same coupling procedure. It was apparent that the precipitation of the conjugates occurred at pH 4 and 7.4, while the conjugates were solubilised in bufferred solutions at pH 9.5. It was observed that the pH value of the buffered solutions greatly affected the precipitation of the conjugates. The observed water solubility of the conjugates is of particular importance because the ultimate goal is to administer intravenously.

In the modification of amines, only the unprotonated form is reactive, and therefore it is necessary to maintain a pH at which a significant number of amines are unprotonated. An average pKa above 9 for lysines indicates that the higher the pH, the

better conjugation is achieved. Therefore, a pH around 9.5 should be used for protein conjugation.

Considering the degree of modification, the mixture of 3'-s-DHEA active ester in dimethylformamide as solvent was added in different ratios to the same concentration of IgG or BSA. It has been found that the degree of derivatisation of the protein is proportional to the excess used after purification. The methods to isolate the conjugates and quantify their degree of derivatisation will be described further.

3.2.2 Characterisation of IgG-s-DHEA and BSA-s-DHEA

3.2.2.1 Ultraviolet-visible spectrophotometry for the conjugates determination

UV-visible spectroscopy is a convenient, non-destructive technique which has found widespread application for protein quantification. Typically, spectrophotometric quantification of proteins is carried out at 280 nm, where the absorbance is due to residues such as tryptophan (maxima at 288 nm, 279 nm) and tyrosine (maxima at 275 nm, 294 nm) (Beaven and Holiday, 1952). The strongest absorbances which are attributed to the peptide bonds (Goldfarb et al., 1951) normally occur in the lower wavelength (215-230 nm) region, however, many buffers and other molecules also absorb at these lower wavelengths. Measurements can therefore be made at 280 nm with high precision, thus avoiding many non-protein interference issues. Unfortunately, measurement at 280 nm is really only sensitive enough to determine protein concentrations down to around 100 μg/ml. However, the concentration of IgG/BSA (2 mg/ml) used in this experiment was sufficient for measurement by UV-Visible spectrophotometry.

As shown in Fig. 3.11 and 3.12, the strongest absorbances of IgG and BSA were those occurring in the 200-230 nm region, which were attributed to the peptide bonds. As stated, this region is affected by the interference of non-protein species such as the buffered solutions, therefore, the second strongest peak at 280 nm was chosen for protein determination.

In an attempt to quantify the incorporation of DHEA or 3'-succinyl-DHEA into IgG/BSA, the ideal situation would be for 3'-succinyl-DHEA and IgG/BSA each to possess at least one absorbance maximum which is not overlapped significantly by the spectrum of the other. Unfortunately, IgG and BSA had an absorbance maximum at 280 nm, where also the 3'-succinyl-DHEA had an absorbance at the same wavelength. It was therefore impossible to determine the degree of derivatisation by this method. However, this method could still be used with gel filtration chromatography as a rapid and rough method to follow the elution profiles of both the conjugates and free drug.

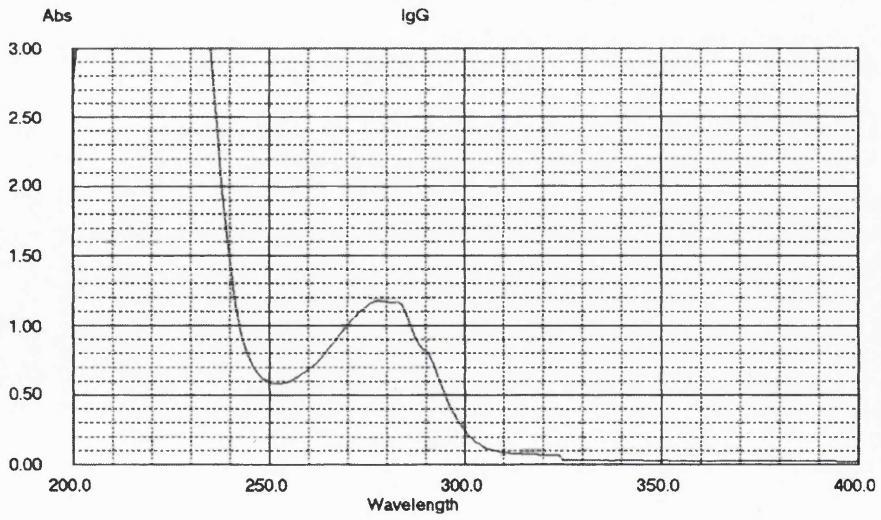


Fig. 3.11 *UV-Visible spectrum of immunoglobulin G (IgG)*

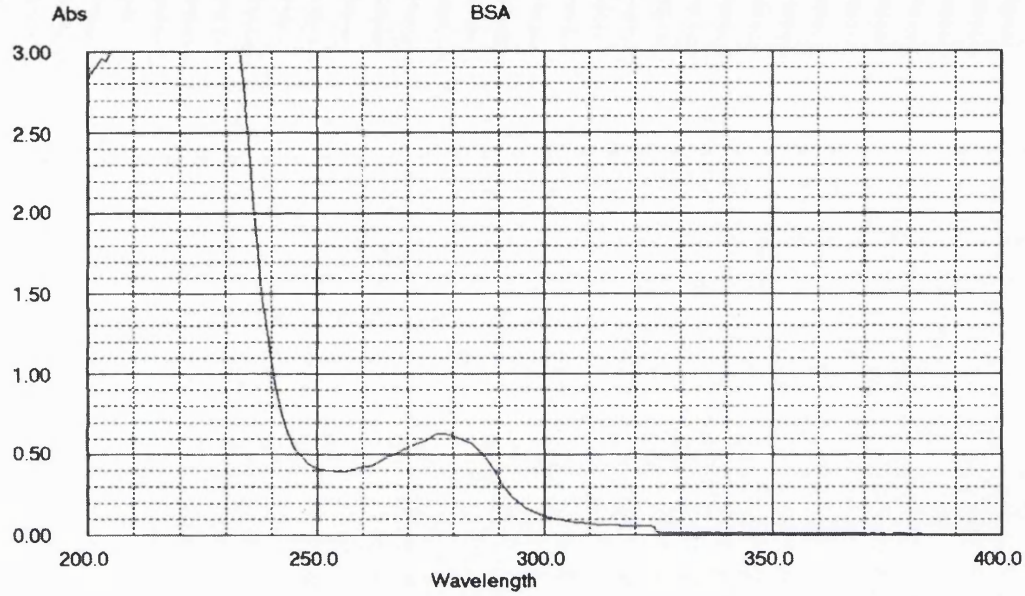


Fig. 3.12 *UV-Visible spectrum of bovine serum albumin (BSA)*

3.2.2.2 Characterisation of IgG/BSA-s-DHEA by gel filtration chromatography

Separation of the conjugate constructs from unreacted drug, the conjugates, s-DHEA and small molecular weight reagents was attempted by GFC. Ideally, GFC is based both on size and shape by which physical interaction between sample and the separating media occurs. This non-destructive method allows for the isolation of the constructs without irreversible denaturation of the protein. In theory, if both or at least one of the molecules were in the fractionation range of the separation media, due to differences in their MW, they would be separated by GFC. The IgG, BSA and 3'-succinyl-DHEA were passed separately through the column in order to test the separation ability before passing the reaction mixture.

As discussed previously, both IgG and BSA had absorbance maxima at 280 nm and despite interference from the absorbance of 3'-succinyl-DHEA at the same wavelength, for convenience 280 nm was chosen to follow the elution profiles of both the conjugates and unreacted drug.

When IgG and BSA were chromatographed separately, elution profiles showed their presence were found in fraction numbers 8-15 and 7-12 respectively (Fig. 3.13, 3.14). 3'-Succinyl-DHEA was also passed through the column and was found in fraction number 15-30 (Fig. 3.15). This showed that the difference in their MW was sufficient for resolution to occur. As described in Chapter II, molecules in solution are separated, as they pass through a column packed with a gel, according to their size. While small molecules enter the gel beads and are retarded in their passage down the column, larger molecules that cannot diffuse into the gel move together with the eluent in the solvent front. The MW of IgG, BSA and 3-succinyl-DHEA are 150 kDa, 66 kDa and 388 Da respectively. Therefore, IgG/BSA leave the column first followed by the smaller molecules (3'-succinyl-DHEA) in the order of their size. IgG and 3'-succinyl-DHEA active ester in molar ratios of 1:100 were passed through the column. Fig. 3.15 shows the gel filtration profiles of the separation of IgG-succinyl-DHEA and 3'-succinyl-DHEA in the reaction mixture. The first peak represents the elution point for IgG or the conjugates, while the absorbance due to excess unreacted drug was contained in the second peak. IgG or IgG-succinyl-DHEA were found in fractions 8-14 and free 3'-succinyl-DHEA was found in fractions 15-27.

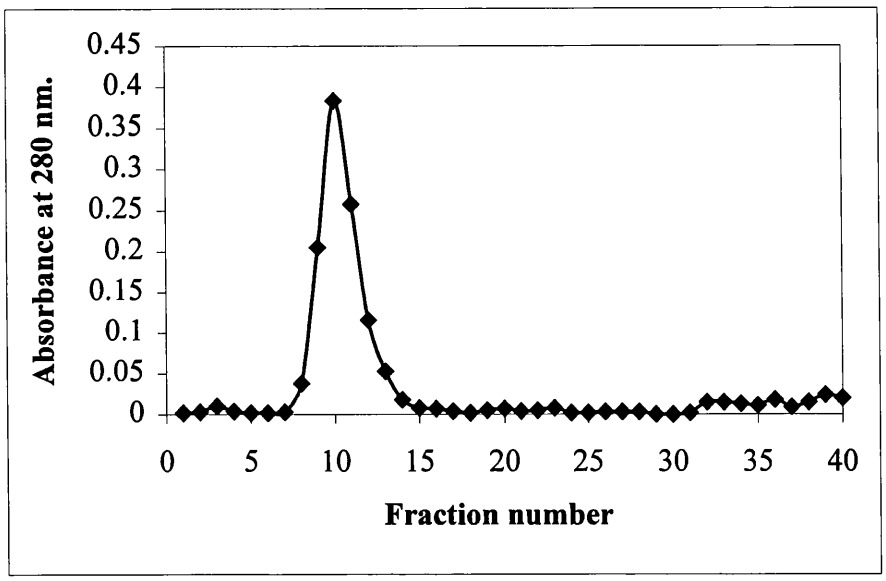


Fig. 3.13 Molecular sieve chromatography of immunoglobulin G. Sample was chromatographed on Sephadex G-50 (column: 25.0 x 1.1 cm; sample volume,500 µl; eluent, 20 mM borate buffer; flow rate, 4 ml/min).

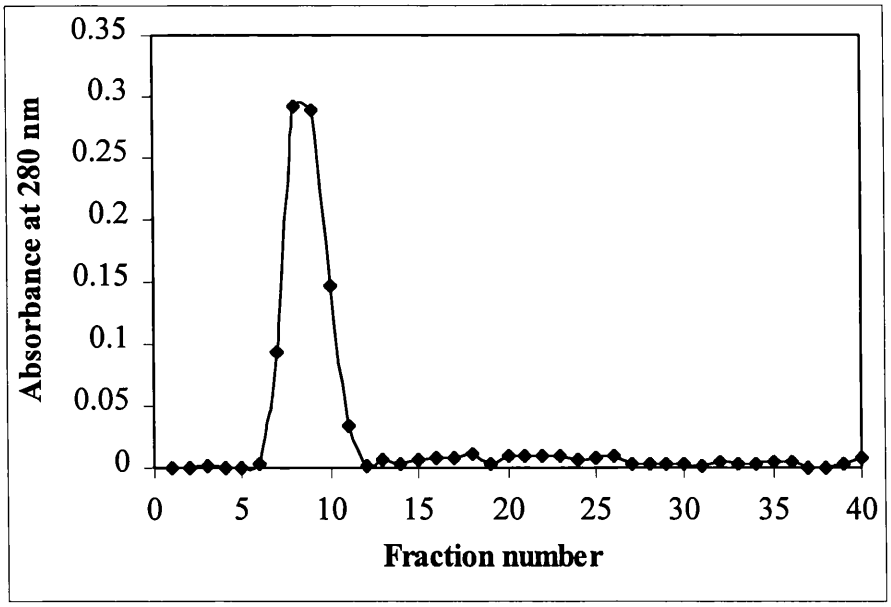


Fig. 3.14 Molecular sieve chromatography of bovine serum albumin. Sample was chromatographed on Sephadex G-50 (column: 25.0 x 1.1 cm; sample volume,500 µl; eluent, 20 mM borate buffer; flow rate, 4 ml/min).

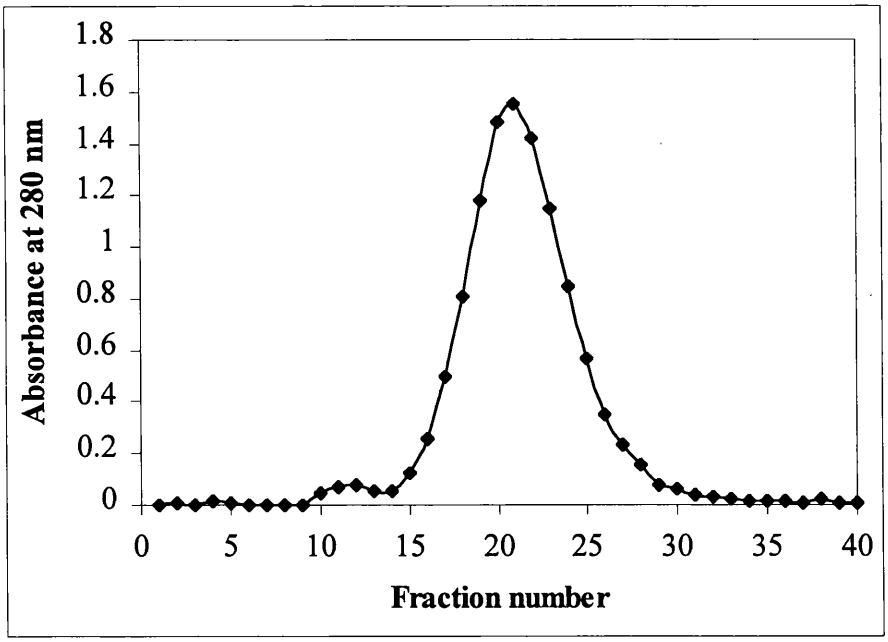


Fig. 3.15 *Molecular sieve chromatography of 3'-succinyl-DHEA. Sample was chromatographed on Sephadex G-50 (column: 25.0 x 1.1 cm; sample volume, 500 µl; eluent, 20 mM borate buffer; flow rate, 4 ml/min).*

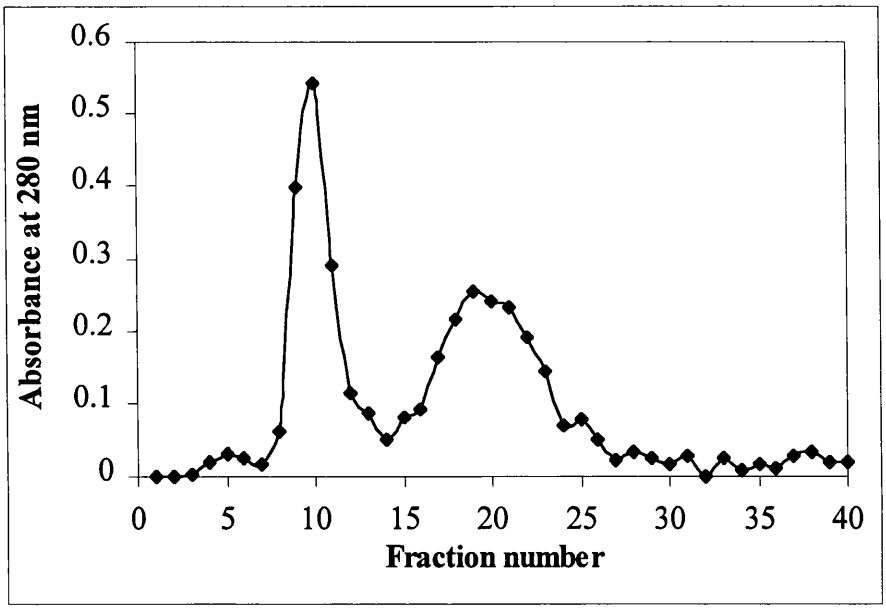


Fig. 3.16 *Molecular sieve chromatography of 1:100 IgG-s-DHEA conjugate. Sample was chromatographed on Sephadex G-50 (column: 25.0 x 1.1 cm; sample volume, 500 µl; eluent, 20 mM borate buffer; flow rate, 4 ml/min).*

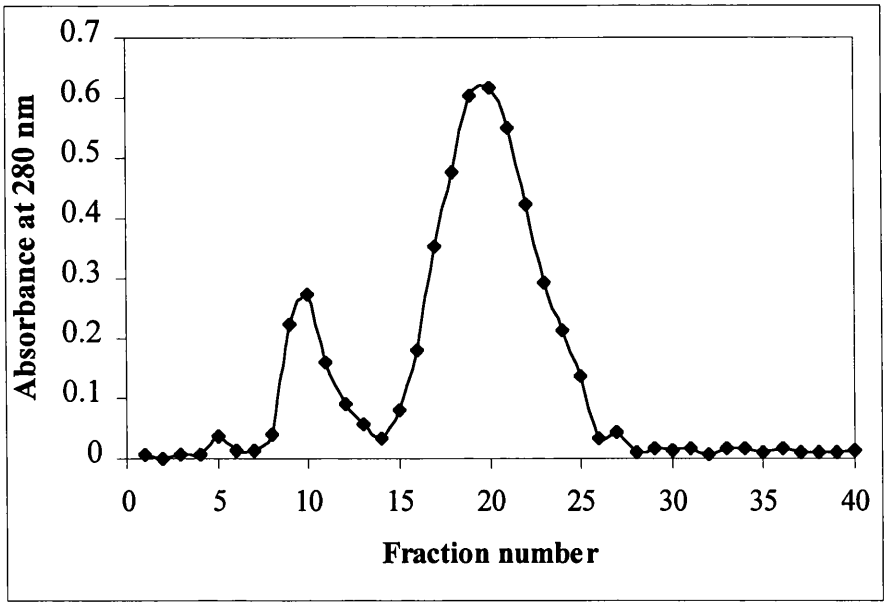


Fig. 3.17 *Molecular sieve chromatography of 1:100 BSA-s-DHEA conjugate. Sample was chromatographed on Sephadex G-50 (column: 25.0 x 1.1 cm; sample volume, 500 µl; eluent, 20 mM borate buffer; flow rate, 4 ml/min).*

Fig. 3.17 also shows the elution profiles of a gel filtration separation after 3'-succinyl-DHEA was conjugated to BSA. The first peak (fractions 8-14) was BSA or BSA-succinyl-DHEA conjugates, while the second peak (fractions 15-26) was excess unreacted 3'-succinyl-DHEA. These results demonstrated the ability of gel filtration to fractionate these molecules according to their molecular weight and molecular size.

As shown in Figs. 3.16 and 3.17, the absorbance of the first peak was higher or identical to that of unbound IgG or unbound BSA. However, it is not possible to identify the extent to which the first peak was the conjugates or unbound proteins by UV-Vis spectrophotometry measurement. Other methods are required to identify and determine the degree of derivatisation of the conjugates after the purification step.

3.2.2.3 Determination of free amino groups in conjugates by TNBS method

Several methods have been developed for the determination of protein conjugate ratios, based on the measurement of radioactivity, molar extinction coefficients and amino acid analysis (Erlanger, 1980; Makela and Seppala, 1986; Shuler et al., 1992). Earlier, the hapten to carrier protein ratio was determined on the basis of the differential UV absorption spectra of hapten and carrier protein. However, the accuracy of the UV absorption method is limited whenever there is an overlap of the absorption of carrier protein over that of the hapten. Mattox et al. (1979) reported that the ratio of steroid to BSA was higher by 13% when quantitated by UV absorbance alone, as compared to measuring the steroid by UV alone and the protein by the Lowry method.

TNBS was originally developed for the determination of the free amino groups of amino acids, peptides and proteins (Satake, et al, 1960, Habeeb, 1966, Fields, 1972). Due to the specificity of TNBS for the amino groups of proteins, this reagent has been successfully used for the quantitation of protein in microgram quantities and the method may be used for the quantitation of carrier proteins where it is more sensitive than the Lowry and Bradford methods (Hazra et al., 1984).The absorption spectra of TNP-L-lysine and TNP-L-glutamic acid are shown in Fig. 3.18. The amino acid, L-glutamic acid (MW 147.13) was chosen because of its proximity to the molecular weight of L-lysine (146.19) and the presence of α-amino groups. L-lysine contains two reactive primary amino groups (α-amino and ε-amino groups) which react equally well with the TNBS reagent. TNBS reacts equally with both α-amino and ε-amino groups of amino acids (Fields, 1972).

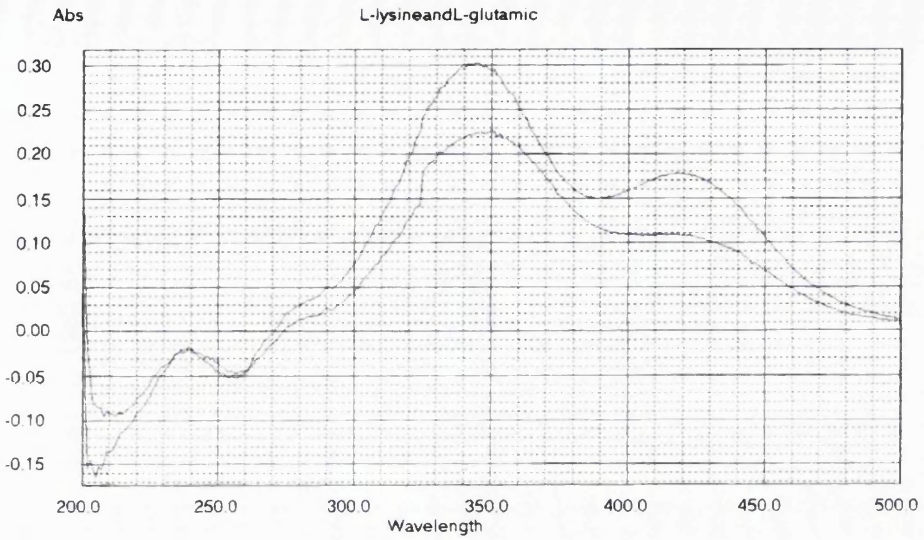


Fig. 3.18 *Absorption spectra of trinitrophenyl derivatives of L-lysine and L-glutamic acid.*

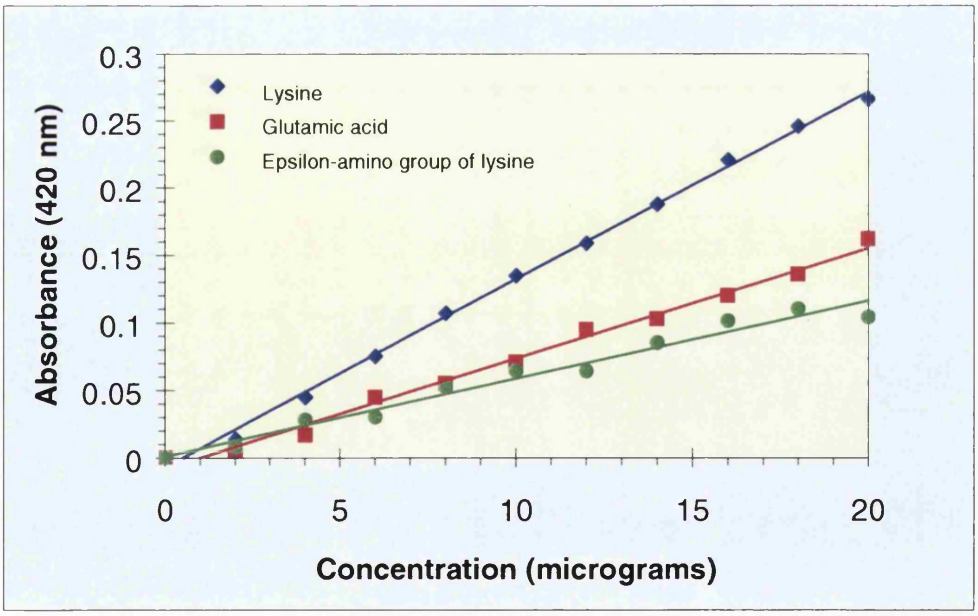


Fig. 3.19 *Regression lines of amino groups in lysine, glutamic acid and ε-amino groups of lysine by TNBS method. Lysine ($y = 0.0139x - 0.0069$, $r^2 = 0.9978$); glutamic acid ($y = 0.0082x - 0.008$, $r^2 = 0.9914$); ε-amino group of lysine ($y = 0.0058x + 0.001$, $r^2 = 0.9729$)*

The standard curve was based on linear regression analysis using the equation y = A + Bx. The difference in absorbance of TNP-L-lysine and TNP-L-glutamic acid gives the true estimate of the ε-amino groups present in L-lysine. The ε-amino group of lysine content on the protein carriers was analysed by this standard curve.

In this study, the TNBS reagent was used at a concentration of 0.1% which was found to exhibit a linear relationship with a reference amino acid concentration of up to 20 μg/ml of ε-amino group of lysine. The number of ε-amino groups present in the proteins was directly determined from a standard curve generated from the difference in the absorbance at 420 nm for TNP-L-lysine and TNP-L-glutamic acid. The absorbance at 420 nm was chosen instead of 340 nm because the picric acid, produced by the side reaction between TNBS and hydroxide ions, exhibited a maximum at 352 nm. IgG and BSA alone were analysed for their lysine content. It was found that this method did not give the correct number of lysine residues being present in these proteins by comparison with previous literature. The reason could be that it is incorrect to use the difference in absorbance of TNP-L-lysine and TNP-L-glutamic acid as a standard curve. Sashidhar et al. (1994) mentioned that TNP-L-lysine had twice the absorbance (335 nm) of the TNP-L-glutamic acid by matching mole to mole of amino acid reference standards. In this assay, it was observed that the TNP derivative of L-lysine did not exhibit twice the absorbance of the TNP derivative of L-glutamic acid, when they were matched mole to mole. Furthermore, the complete accessibility of amino groups must be considered even under optimal experimental conditions. In globular proteins, many lysyl residues are possibly locked in the core of the ternary structure. This region could be therefore inaccessible to the TNBS. Unfortunately, an attempt in using a denaturing agent like SDS or urea does not solve the problem.

Table 3.1: Degree of modification from the conjugation of IgG with s-DHEA as determined by the TNBS method. Results are mean ± S.D. of three different preparations.

IgG/s-DHEA molar ratio in the reaction mixture	Degree of modification (% amino groups reacted)	IgG/s-DHEA molar ratio in the conjugate
1:10	8.56 ± 0.58	1:7.4
1:50	18.66 ± 2.32	1:16.0
1:100	21.14 ± 4.82	1:18.2
1:200	44.35 ± 3.03	1:38.1
1:300	48.14 ± 1.03	1:41.4

Table 3.2: Degree of modification from the conjugation of BSA with s-DHEA as determined by the TNBS method. Results are mean ± s.d. of 3 different preparations.

BSA/s-DHEA molar ratio in the reaction mixture	Degree of modification (% amino groups reacted)	BSA/s-DHEA molar ratio in the conjugate
1:10	24.17 ± 2.96	1:14.3
1:50	40.34 ± 1.29	1:23.8
1:100	53.75 ± 8.29	1:31.7
1:200	59.43 ± 10.66	1:35.1
1:300	51.42 ± 11.58	1:30.3

Methods based on the extinction coefficient of the TNP derivative of the protein have been used in the determination of carrier protein-hapten ratios before and after the coupling reaction. The procedure is very simple to perform: the protein is allowed to react with TNBS in a buffered medium, giving *N*-trinitrophenyl-protein derivatives (TNP-protein). The difference in the absorbance of TNBS bound to free amino groups of carrier protein alone and the conjugates is assumed to give the number of amino groups occupied by small molecules. IgG and BSA were reacted with TNBS as already described for L-lysine and L-glutamic acid. The 3'-succinyl-DHEA was coupled to IgG and BSA (at varying molar ratios) as mentioned for the IgG/BSA-s-DHEA conjugates. After the purification step, the conjugates were reacted with TNBS and the absorbance compared at 420 nm with that of TNP-IgG and TNP-BSA. The percent conjugation of 3'-succinyl-DHEA to IgG or BSA was calculated by the difference in absorbance between the conjugates and the proteins divided by the absorbance of proteins before coupling and multiplied by 100.

Tables 3.1 and 3.2 show the percentage of occupied amino groups in the IgG-s-DHEA conjugates ranging from 8 to 48, and from 24 to 59 for BSA-s-DHEA. As reported in the literature, the number of lysine residues present in IgG and BSA were 86 are 59 respectively (Hirayama et al., 1990; Meares, 1993). The drug to protein mole ratio for IgG-s-DHEA and BSA-s-DHEA was calculated from the percentage of modification to the total number of lysine residues contained in IgG and BSA ,which was assumed to be 100%. It is apparent that the highest ratios for IgG-s-DHEA and BSA-s-DHEA were 41.4moles of s-DHEA/mole IgG and 30.3 moles of s-DHEA/ mole BSA respectively. It was found that this method could be used as a rapid and approximate method for evaluating the modification of proteins at its lysyl residues. It was possible that once an amino group has been coupled with 3'-succinyl-DHEA or TNBS, the neighbouring lysyl

residues become inaccessible to TNBS because of the steric hindrance of the fixed trinitrophenyl group or 3'-succinyl-DHEA. For greater accuracy, a comparison between different methods is necessary. A more convenient and direct method for the determination of the conjugate ratios is the radiolabelling technique.

3.2.2.4 Radiolabelling for the determination of drug conjugation to proteins.

Radioactive isotopes with suitable decay rates and energies provide a powerful approach to conjugate characterisation. In order to measure very low concentrations of compounds with precision, a radioactively labelled tracer is required. There are a large number of radioisotopes available from commercial sources, but the number of these which are useful for radiolabelling is fairly small. Radioisotopes which emit β- or γ-emitters are generally favoured. The ideal radiolabel should be incorporated with no extraneous atoms being introduced. This can only be achieved by replacing one or more atoms in the molecule with a radioactive isotope of the same element such as ^{3}H or ^{14}C. For small molecules, it may be possible to buy material already incorporating a radiolabel or it may be necessary to incorporate a radioactive element into a synthetic procedure. For proteins, it is more difficult to incorporate a radiolabel directly into the molecule. It is much more common to radiolabel proteins by the introduction of an extraneous group, typically iodine (^{125}I or ^{131}I).

In all cases of iodine labelling, iodine is not a part of the structure of the natural molecule. This can be contrasted with the situation in ^{14}C or ^{3}H labelling where the radioactive nuclide usually replaces a naturally occurring non-radioactive isotope of the same element which is present in the native molecule. There are likely to be some

differences in behaviour between radioiodinated and unlabelled materials and these differences are likely to be more marked than those between, for example, tritiated and unlabelled steroids. The transmission of γ-radiation through most materials is sufficiently large that it can be measured directly from the sample. Thin sample vessels of glass or plastic will cause no significant loss of signal. The transmission of β-particles is relatively poor, it is more usual to transfer the energy to a suitable fluorescent molecule, then detect the light produced. The greatest advantage of radioiodination is a minimum requirement for sample preparation. This can be particularly important when large numbers of samples are involved or tissues are to be tested. Beta-emitting radionuclides require liquid scintillation counting systems which are expensive in term of scintillation fluids and often require somewhat time-consuming and tedious sample preparation procedures.

Once one of the components of a conjugation has been obtained in radiolabelled form, incorporation data can be obtained very simply after the coupling reaction. Generally, the specific activity of a radiolabelled material is so high that only a small fraction needs to be included together with the unlabelled 'cold' material. Provided that the radiolabelled material has identical chemical reactivity to its cold analogue, it will be incorporated in a random fashion in the conjugate which will accurately reflect the overall incorporation of this component.

In this study, the degree of derivatisation was determined from the ratio between radiolabelled drugs and proteins. Tritium radiolabelled drug was chosen because of its long half life, its relatively low cost and its commercial availability. 1,2,6,7 (^{3}H) DHEA, purchased from Dupont (UK), was mixed together with DHEA (cold drug) for the esterification with succinic anhydride as described in Chapter II. The 3'-succinyl-(^{3}H)DHEA was then linked to IgG or BSA using the general procedure for unlabelled DHEA.

As mentioned before, purification by gel filtration is an effective way to obtain the conjugates in high yield and is often used to stop reactions. Harsh elution conditions can also be avoided. Therefore, the water soluble conjugates were isolated from excess reagents in reaction mixtures by gel filtration on Sephadex G-50 eluted with 20 mM borate buffer. Evidence for the presence of the conjugates and unreacted drugs was obtained by measuring tritium (^{3}H-DHEA). Fig. 3.20 and 3.21 show that fractions from the first peak contained the desired conjugates while fractions from the second peak presumably contained unbound 3'-succinyl-DHEA. The pattern obtained from gel filtration showed that the molecular weight of the conjugates were increased by increasing the molar ratio of 3'-sulfo-NHS-succinyl-(^{3}H)DHEA) reacted with IgG or BSA.

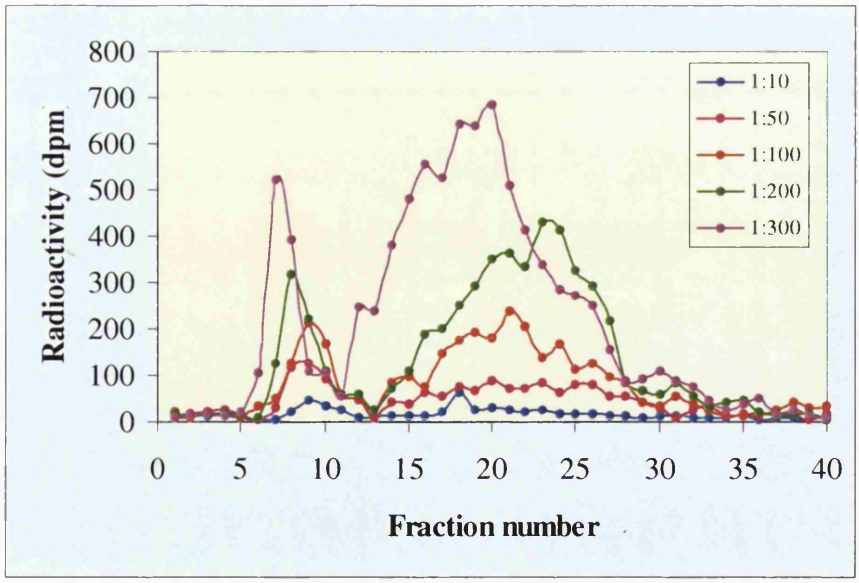


Fig. 3.20 *Molecular sieve chromatography of IgG-s-(^{3}H)DHEA (1:10 to 1:300 molar ratio of IgG to 3'-sulfo-NHS-succinyl-(^{3}H)DHEA). Sample was chromatographed on Sephadex G-50 (column: 25.0 x 1.1 cm; sample volume, 500 µl; eluent, 20 mM borate buffer; flow rate, 4 ml/min).*

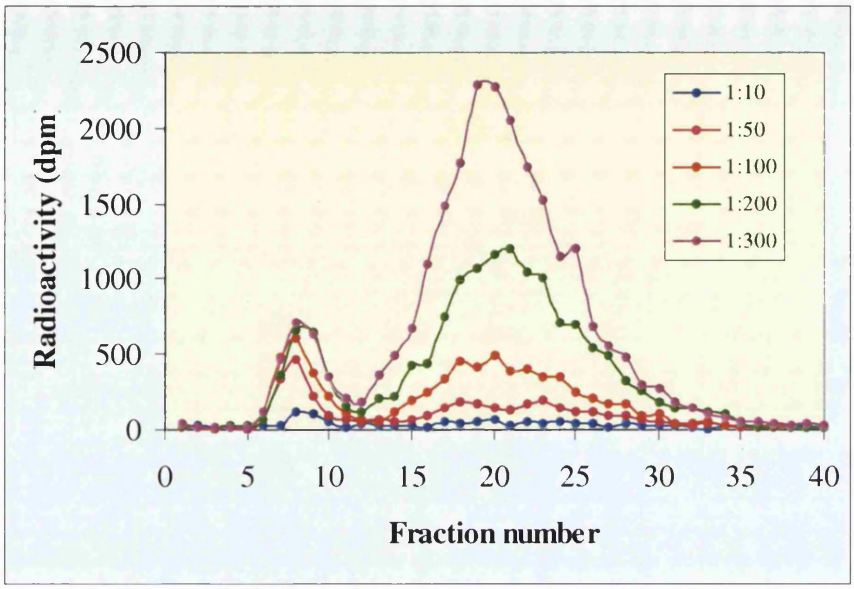


Fig. 3.21 *Molecular sieve chromatography of BSA-s-(^{3}H)DHEA (1:10 to 1:300 molar ratio of BSA to 3'-sulfo-NHS-succinyl-(^{3}H)DHEA). Sample was chromatographed on Sephadex G-50 (column: 25.0 x 1.1 cm; sample volume, 500 µl; eluent, 20 mM borate buffer; flow rate, 4 ml/min).*

After the purification step, the first peak fractions were pooled and the (^{3}H) radioactivity measured to determine the extent of drug binding to IgG or BSA via the succinyl spacer.

It is apparent that the extent of DHEA conjugation is dependent on the molar ratio of 3'-sulfo-NHS-succinyl-(^{3}H)DHEA to IgG or BSA used in the coupling reaction. The conjugation yields were expressed in terms of IgG:s-DHEA or BSA:s-DHEA molar ratios as shown in Tables 3.3 and 3.4.

Table 3.3: Degree of modification from the conjugation of IgG and 3'-sulfo-NHS-succinyl-(^{3}H)DHEA expressed in terms of IgG:s-DHEA as determined by ^{3}H radioactivity. Values denote means ± S.D. (3 different preparations).

Molar ratio of IgG: 3'-sulfo-NHS-succinyl-DHEA in the reaction mixture	Amount of 3'-succinyl -DHEA conjugated to IgG (mg/mg) (mean± SD)	Molar ratio of IgG-3'-succinyl-DHEA in the conjugate
IgG 1:10	0.025±0.003	1:4.8
IgG 1:50	0.082±0.009	1:15.8
IgG 1:100	0.131±0.008	1:25.4
IgG 1:200	0.209±0.067	1:40.3
IgG 1:300	0.254±0.044	1:49.1

Table 3.4: Degree of modification from the conjugation of BSA and 3'-sulfo-NHS-succinyl-(^{3}H)DHEA expressed in terms of BSA:s-DHEA as determined by ^{3}H radioactivity. Values denote means ± S.D. (3 different preparations).

Molar ratio of BSA: 3'-sulfo-NHS-succinyl-DHEA in the reaction mixture	Amount of 3'-succinyl -DHEA conjugated to BSA (mg) (mean± SD)	Molar ratio of BSA-3'-succinyl-DHEA in the conjugate
BSA1:10	0.065±0.007	1:5.5
BSA1:50	0.266±0.007	1:22.6
BSA1:100	0.424±0.046	1:36.0
BSA1:200	0.478±0.023	1:40.6
BSA1:300	0.532±0.024	1:45.2

By increasing the molar ratio of 3'-sulfo-NHS-succinyl-(^{3}H)DHEA to IgG (up to 300), the degree of modification was also increased to obtain a maximum value of 49.1 molecules of 3'-succinyl-DHEA to one molecule of IgG. Under the same procedure, the highest ratio of BSA:s-DHEA was 45.2 moles of s-DHEA/mole BSA, which was achieved by the use of a 300 fold excess of 3'-sulfo-NHS-succinyl-(^{3}H)DHEA in the reaction mixture. Since IgG and BSA molecules contain 86 and 59 lysine residues, these ratios correspond to IgG-s-DHEA and BSA-s-DHEA with an average 57.1% and 76.6% of the available lysine groups respectively occupied by the drug.

As mentioned before, it is difficult to incorporate a radiolabel into the molecule of proteins, therefore, ^{125}I was used to radiolabel IgG and BSA. In this study, IgG and BSA, which were conjugated to the tritiated drug, were labelled with ^{125}I in order to trace the behaviour of the conjugates in vitro and in vivo. This double labelling provides the ability to follow two substances simultaneously. It was found that the protein recovery in the IgG-s-DHEA and BSA-s-DHEA conjugates after the purification was 95.19 ± 1.02% and 92.33 ± 1.11% respectively.

3.2.2.4.1 Radiolabelling of IgG and BSA

IgG and BSA were radiolabelled by the chloramine-T method as described in Chapter 2. After the reaction, gel filtration (Sephadex G-25, PD 10 column) was used to separate iodinated protein from unreacted iodide (Fig. 3.22 and 3.23). The iodinated IgG and BSA were detected in the first peak related to the molecular weight of proteins because iodinated proteins cannot enter the pores of the beads and so are eluted from the column in the void volume. On the other hand, free iodine, which is found in the second

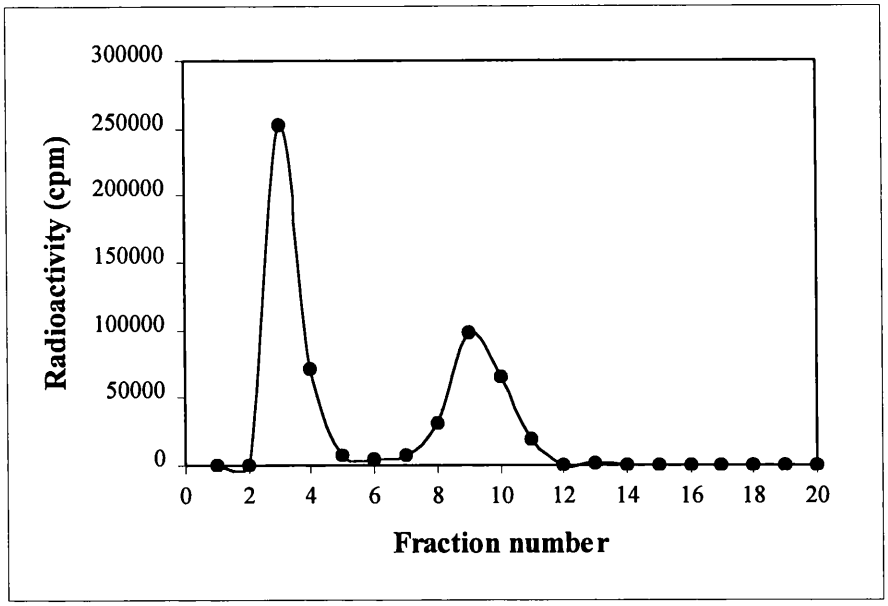


Fig.3.22 Removal of free ^{125}I from labelled immunoglobulin G

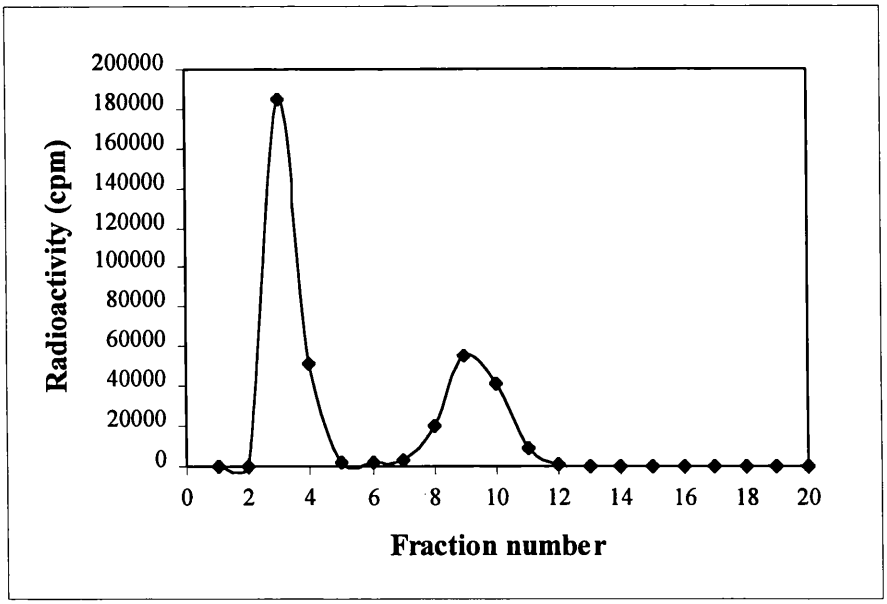


Fig.3.23 Removal of free ^{125}I from labelled bovine serum albumin

peak, enters the pores and so has to pass through the total volume of the column before being eluted. It was necessary to ensure that the proteins did not contain free iodine (^{125}I). The fractions corresponding to the iodinated protein were pooled and the unbound iodine removed by the dialysis technique. The percentage of radiolabelled protein and free iodine was determined by TCA precipitation. The purity of iodinated protein should be over 90%. Table 3.5 showed that the purity of iodinated IgG and BSA were 98.64% and 98.84% respectively.

Table 3.5: Purity of iodinated proteins. Results are expressed as a percentage of iodine recovered with the protein (mean ± s.d., n=3).

Proteins	Purity
Immunoglubulin G	98.64 ± 0.89
Bovine serum albumin	98.84 ± 0.22

3.2.2.5 Electrophoretic characterisation of the IgG/BSA-s-DHEA conjugates by sodium dodecyl sulphate polyacylamide electrophoresis (SDS-PAGE)

All electrophoretic techniques are based on the separation of solutes under the influence of an electric field, and their resolving properties are therefore linked to the charge of the solutes under the operational conditions. Gels based on carbohydrates such as cellulose and agarose have been employed for electrophoresis. Agarose, for example, has very wide pores which are amenable to very large analytes. However, polyacrylamide has found the widest popularity, to the extent that polyacrylamide gel electrophoresis (PAGE), is often referred to as a discrete technology (Raymond and Weintraub, 1959; Chrambach and Rodbard, 1971). These gels are formed by the copolymerisation of acrylamide monomers with a cross-linking agent such as N,N'-methylene bisacrylamide. Various concentrations of monomer and cross-linker can be employed to produce gels of different properties. These gels can be formed under a single set of polymerisation conditions to yield a uniform gel, often characterised by the percentage of acrylamide which they contain: for example a 3% gel will allow the free passage of solutes with a molecular mass greater than 100 kDa, while a 15% gel has a cut-off at 15 kDa (Dunn, 1989).

There are sometimes certain advantages to the use of gradient gels, which are prepared by modifying the polymerisation conditions as the gel is constructed. These gels allow powerful resolution of solutes over a mass range wider than would be possible from a uniform gel. In this study, the molecular weight of native IgG/BSA and IgG/BSA-s-DHEA conjugates were estimated on 4-15% polyacrylamide gradient ready gels. This gradient gel allowed a wide range of molecular weights, from a low molecular weight of 6.5 kDa to a high molecular weight of 200 kDa, to be visualised on the same gel. The low

range prestained SDS-PAGE standards contain phosphorylase B (103 kDa), bovine serum albumin (77 kDa), ovalbumin (48 kDa), carbonic anhydrase (34.2 kDa), soybean trypsin inhibitor (28.4 kDa) and lysozyme (20.5 kDa).

The Lane Marker Reducing Sample Buffer containing 5% SDS, 50% glycerol and 100mM DTT was mixed with proteins or proteins conjugates as described in chapter II. These Lane Marker Samples Buffers contained a bright pink hydrophobic tracking dye for SDS-PAGE as an alternative to the typical bromophenol blue dye. Normally, the free dye migrates faster than any proteins and was used to monitor the progress of the electrophoresis.. Dithiothreitol (DTT) was included in the reducing sample buffers in order to cleave disulfide bonds, to ensure the protein is fully unfolded into a rod -like structure. The incorporation of the reducing agents has been reported to reduce aggregation-related problems such as streaking and entrapment at the origin (Fairbanks et al., 1971). Mercaptoethanol, which is the commonly used disulfide cleaving reagent in reducing sample buffers, was replaced with DTT since mercaptoethanol had a strong odour and tended to auto-oxidise. Glycerol was required in reducing sample buffers to make the density of the sample higher than the density of buffer. This causes the sample "sink" to the bottom of the well preventing any cross-contamination (Blackshear, 1984). The addition of SDS to sample buffers was required for unravelling intramolecular associations such as hydrophobic interactions or hydrogen bonds. Furthermore, the SDS bound to the proteins generates a constant anionic charge-to-mass ratio for all the unravelled protein chain so that SDS-PAGE separates proteins according to size only, not charge differences.

To detect the fractionated bands on a gel, the commonest approach is to employ a staining agent with a broad affinity for proteins. Most dyes used tend to be attracted by positively charged groups such as lysine, arginines and histidines on the protein;

consequently proteins with higher proportions of these groups, generally more basic proteins, tend to stain more strongly. The commonest protein stain for gel electrophoresis is probably Coomasie Brilliant Blue R-250. A Coomasie Blue staining solution for gel electrophoresis contains 0.1% (w/v) CBB R-250 in a fixative, methanol-water-acetic acid (45:45:10) solvent. The staining procedure may take a few hours depending on the thickness of the gel and its composition. Destaining, which reduces the background colour, is carried out using the same solvent without the dye.

IgG and IgG conjugates were analysed by SDS-PAGE electrophoresis under non-reducing conditions. It was observed that IgG and IgG conjugates were precipitated at the origin of the gels. This could be explained by the fact that proteins were too large to penetrate the gel pores due to the size of proteins or the aggregation. Therefore, a reducing agent (DTT) was incorporated with the IgG preventing the precipitation at the application point. Under reducing conditions, IgG molecules, which consist of disulphide-linked subunits, was splitted into their two heavy and two light chains. Fig. 3.24 shows the presence of two bands, with the estimated molecular weight of 29 kDa and 50 kDa corresponding to the heavy and light chains respectively. It was also found that the increase in the degree of derivatisation, from 4.8 to 49.1 drug molecules, slightly decreased the migration of the conjugates in both heavy and light chains. This suggested that drug molecules were linked to both the heavy and light chains of IgG. In lane F and G, the 1:40.3 and 1:49.1 IgG-s-DHEA conjugates stained less intensely than in lanes B-E, presumably due to the lesser availability of basic groups for interaction with Coomasie blue R-250.

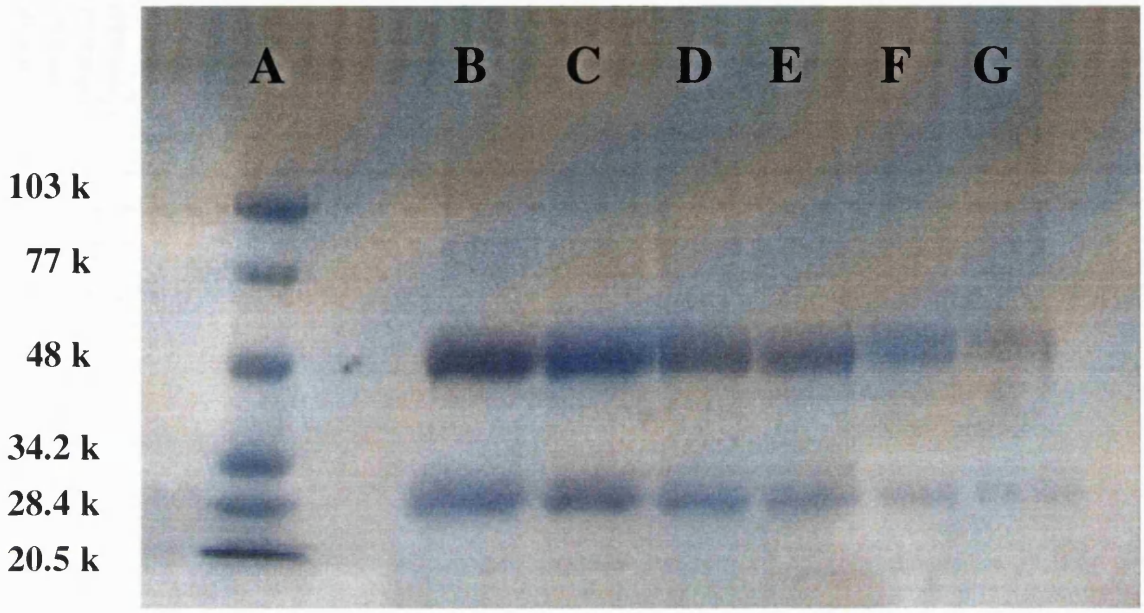


Fig. 3.24 *SDS-PAGE of IgG and IgG-s-DHEA conjugates under reducing conditions. (4-15% polyacrylamide gradient gels (BioRad), stained with Coomasie brilliant blue R-250) A) prestained low molecular weight marker; B) IgG; C) 1:4.8; D) 1:15.8; E) 1:25.4; F) 1:40.3; G) 1:49.1 (Molar ratio of IgG : 3'-succinyl-DHEA)*

Fig. 3.25 *SDS-PAGE of BSA and BSA-s-DHEA conjugates under reducing conditions. (4-15% polyacrylamide gradient gels (BioRad), stained with Coomasie brilliant blue R-250) A) prestained low molecular weight marker; B) BSA; C) 1:5.5; D) 1:22.6; E) 1:36; F) 1:40.6; G) 1:45.2 (Molar ratio of BSA : 3'-succinyl-DHEA)*

Furthermore, gel electrophoresis of BSA exhibited a single band with an estimated molecular weight of 60 kDa (Fig. 3.25, lane A) in reducing conditions. Fig. 3.25 showed that the molecular weight of BSA-s-DHEA conjugates, containing 5.5-45.2 mol of 3'-succinyl-DHEA per 1 mol of BSA, become larger than BSA with increasing succinylated drug to protein molar ratio (slower migration of the conjugate bands as compared to the BSA band). As mentioned earlier, the colour intensity of the bands, especially lane F and G (Fig. 3.25) was decreased because of the increased binding of the succinylated drug to the ε-amino groups of the lysine on BSA. As shown in Fig. 3.24 and 3.25, this method confirmed that the 3'-succinyl-DHEA was covalently linked to IgG and BSA.

3.2.3 Optimisation of the method

A further series of observations were made concerning the extent of DHEA conjugation. As mentioned in 3.2.2.4, it was found that the degree of modification was increased by increasing the molar ratio of 3'-sulfo-NHS-succinyl-(^{3}H)DHEA to IgG or BSA. By varying the reaction times, it may well also be possible to increase the conjugation yield . It was observed that the maximum amount of IgG-s-DHEA formed after 2 h (Table 3.6), while the maximum amount of BSA-s-DHEA was achieved after 15 min (Table 3.7). However, a significantly greater degree of derivatisation was not shown after increasing the reaction time. Therefore, the reaction time for conjugation was held at 2h. Longer reaction times are acceptable since the degree of modification is generally limited by the ratio of the reagent to protein, rather than the reaction time.

Table 3.6: The effect of reaction time on the degree of derivatisation of IgG-s-DHEA. Results are mean ± S.D. of 3 separate experiments.

Reaction time	Degree of derivatisation (molar ratio of drug conjugation to IgG)
15 min	36.39 ± 0.03
1 h	36.51 ± 0.02
2 h	39.13 ± 0.11
4 h	36.63 ± 0.03
6 h	36.58 ± 0.01

Table 3.7: The effect of reaction time on the degree of derivatisation of BSA-s-DHEA. Results are mean ± S.D. of 3 separate experiments.

Reaction time	Degree of derivatisation (molar ratio of drug conjugation to BSA)
15 min	41.54 ± 0.80
1 h	40.55 ± 0.69
2 h	37.28 ± 0.46
4 h	37.32 ± 0.70
6 h	37.18 ± 0.19

Table 3.8 : Optimisation of degree of modification from the conjugation of IgG and 3'-sulfo-NHS-succinyl-(^{3}H)DHEA. The conjugates were expressed in terms of IgG:s-DHEA as determined by ^{3}H radioactivity. The concentration of 3'-succinyl-DHEA in DMF was 20 mg/ml instead of 10 mg/ml. Values denote means ± S.D. (3 different preparations).

Molar ratio of IgG: 3'-sulfo-NHS-succinyl-DHEA in the reaction mixture	Amount of 3'-succinyl -DHEA conjugated to IgG (mg/mg) (mean± SD)	Molar ratio of IgG-3'-succinyl-DHEA in the conjugate
IgG 1:100	0.842±0.036	1:34.6
IgG 1:200	1.58±0.004	1:63.9
IgG 1:300	1.6±0.059	1:64.4

Table 3.9: Optimisation of degree of modification from the conjugation of BSA and 3'-sulfo-NHS-succinyl-(^{3}H)DHEA. The conjugates were expressed in terms of BSA:s-DHEA as determined by ^{3}H radioactivity. The concentration of 3'-succinyl-DHEA in DMF was 20 mg/ml instead of 10 mg/ml. Values denote means ± S.D. (3 different preparations).

Molar ratio of BSA: 3'-sulfo-NHS-succinyl-DHEA in the reaction mixture	Amount of 3'-succinyl -DHEA conjugated to BSA (mg/mg) (mean± SD)	Molar ratio of BSA-3'-succinyl-DHEA in the conjugate
BSA 1:100	0.485 ± 0.026	1:41.2
BSA 1:200	0.582 ± 0.034	1:49.5
BSA 1:300	0.549 ± 0.009	1:46.6

In an attempt to maximise the degree of modification, the maximum yield of IgG-s-DHEA and BSA-s-DHEA conjugates were observed when two fold concentrations of 3'-sulfo-NHS-succinyl-(^{3}H)DHEA in DMF were employed. At 10 mg/ml of 3'-succinyl-DHEA in DMF, the highest yields of IgG-s-DHEA and BSA-s-DHEA were 49.1 moles of s-DHEA/mole IgG and 45.2 moles of s-DHEA/mole BSA respectively (Table 3.3 and 3.4). Increasing the concentration to 20 mg/ml of 3'-succinyl-DHEA in DMF, Table 3.8 and 3.9 showed that the maximum conjugation yields of IgG-s-DHEA and BSA-s-DHEA were 64.4 and 49.5 molecules of 3'-succinyl-DHEA to one molecule of IgG on BSA respectively.

Chapter IV

Pharmacokinetics of IgG-s-DHEA and BSA-s-DHEA

4.1 Introduction

The purpose of the work described in this chapter was to study the behaviour of the water-soluble IgG-s-DHEA and BSA-s-DHEA in animals as compared to that of the corresponding native IgG, BSA and DHEA, with a view to determine whether the DHEA modified by the attachment to IgG and BSA is likely to have properties that make this drug therapeutically more potent.

The objective of drug targeting is to deliver a drug to its site of action and, at the same time, minimise any toxic effects (Tomlinson, 1987). In pharmacokinetics terms, site-specific drug delivery aims to maximise the ratio of the drug concentration at its site of action to that at sites of potential toxicity. Generally, the lack of selectivity of most conventional drugs is closely related to their pharmacokinetic properties. The *in vivo* fate of a drug given by a particular administration route is determined by both the physicochemical properties of the drug and anatomical and physiological characteristics of the body.

Retention of drugs in circulation is one of the main goals in drug delivery. Although various factors, such as proteolytic degradation, reticuloendothelial uptake, and

receptor-mediated clearance are involved in the rapid elimination, one major problem inherent in small molecule drugs is their susceptibility to glomerular filtration. Macromolecules with a molecular weight of less than 50,000 (approximately 6 nm in diameter) are susceptible to glomerular filtration and are excreted into the urine. Therefore, conjugation with a larger macromolecular carrier, that escape sieving through the glomerulus, is a simple and effective way to reduce their glomerular filtration and subsequently prolong their blood circulation. Enhanced retention of a small molecule drug in the circulation may be useful to increase the chance of the drug to reach the target site.

4.1.1 IgG, BSA, DHEA and conjugates pharmacokinetics after a single injection

Pharmacokinetics involves the study of the rates of absorption, distribution, metabolism and elimination after a drug is given to a subject. Parenteral drug delivery, especially intravenous injection, can gain easy access to the systemic circulation with complete drug absorption and therefore the site of drug action being reached rapidly. In humans, the amount of drug in the body can never be directly measured. However, blood or plasma drug levels are practical and convenient measurements for determining drug in the body.

Several methods can be employed for graphically displaying plasma concentration-time data. One common method is to plot concentration against time. Another method uses the same plot, but the concentration scale is logarithmic. The shape of the log C versus t curve following intravenous dosing gives information on the mathematical model that applies to a particular pharmacokinetics profile. On the semilogarithmic plot, the plasma concentration is observed to fall rapidly after

administration. Thereafter, the decline is slower and appears to continue linearly. The early phase is commonly called the distribution phase and the latter, the elimination phase.

A. Distribution Phase

Distribution is the process of reversible transfer of a drug to and from the site of measurement, usually the blood or plasma. The distribution phase is so called because distribution primarily determines the early rapid decline in plasma concentration. The rapidity of this process implies that the drug must have already left the plasma and been distributed into other tissues., including the liver and kidney. Some of the drug entering these elimination organs is also cleared from the body, however, the administered dose lost during the distribution phase is small.

B. Elimination Phase

During the distribution phase, changes in the concentration of the drug in plasma reflect primarily the movement of the drug within, rather than loss from, the body. However, with time, a distribution equilibrium is established with more and more tissues, and eventually, changes in the plasma concentration reflect a proportional change in the concentration of drug in all other tissues and, hence, in the amount of drug in the body. During this proportionality phase, the body acts kinetically as a single container or compartment. The decline of the plasma concentration reflects elimination of the drug

from the body and this phase is often called the elimination phase. However, the distinction between the distribution phase and the elimination phase is sometimes not clear-cut. Usually, elimination is depicted as occurring directly from the central compartment but the elimination can sometimes occur in tissues of the peripheral compartment. The elimination phase is characterised by two parameters, the elimination half-life ($t_{1/2}\beta$) and the apparent volume of distribution (V_d).

If one thinks of the body as a single compartment, pharmacokinetic calculations are relatively simple. When thinking about drug distribution, elimination and pharmacologic effect, it is more appropriate to conceptualise the body as two or more than two compartments. After intravenous dosing of a drug, the drug mixes rapidly in the plasma (central compartment) and then starts to distribute into the tissues (tissue compartment). The first compartment can be thought of as a rapidly-equilibrating volume, usually made up of blood and those organs or tissues that have high blood flow. This first compartment has a volume referred to as Vi or initial volume. The second compartment equilibrates with the drug over a somewhat longer time period. This volume is referred to as Vt or tissue volume. The sum of Vi and Vt is the apparent volume of distribution (Vd).

4.1.1.1 The apparent volume of distribution (Vd)

The apparent volume of distribution for a drug is the size of a compartment necessary to account for the total amount of drug in the body if it were present throughout

the body at the same concentration as found in the plasma. The following is the equation for the volume of distribution:

$$Vd = A/C$$

where Vd is the apparent volume of distribution, A is the total amount of drug in the body, and C is the plasma concentration of drug. The amount of drug in the body is known immediately after an intravenous bolus since it is the dose administered. This calculation requires that a distribution equilibrium be achieved between the drug in tissues and that in plasma.

Apparent volumes of distribution which are larger than the plasma compartment (>3L) indicate that the drug is also present in tissues or fluids outside that compartment. The apparent volume of distribution is a function of the lipid versus water solubilities, of the plasma and tissue protein binding properties of the drug. Factors which tend to keep the drug in the plasma or increase the plasma concentration of the drug (such as low lipid solubility, increased plasma protein binding, or decreased tissue binding), reduce the apparent volume of distribution.

4.1.1.2 The half-life ($t_{1/2}$)

The half-life (t1/2) of a drug is the amount of time required for the total amount of drug in the body or the plasma drug concentration to decrease by one-half. Half life is more conveniently determined from a linear semilog plot which follows first-order pharmacokinetics. In a two compartment model, the half life for the distribution phase is

referred to as the alpha half-life($t_{1/2\alpha}$), and the half-life for drug elimination from the blood circulation is referred to as the beta half-life ($t_{1/2\beta}$).

$$t_{1/2} = 0.693 / k$$

where k is the rate constant.

4.1.1.3 Clearance (Cl)

Clearance can be thought of as the intrinsic ability of the body or its organs of elimination (usually kidneys and the liver) to remove drug from the blood or plasma. Clearance is expressed as volume per unit of time. It is important to emphasise that clearance is not an indicator of how much drug is being removed; its only represents the theoretical volume of blood or plasma which is completely cleared of drug in a given period of time.

$$Cl = Vd * k$$

or

$$Cl = (0.693*Vd) / t_{1/2}$$

4.1.1.4 Area under the curve (AUC)

The area under the concentration-time curve is related to the total amount of drug in the systemic circulation following the administration of a single dose of that drug.

Several methods exist for measuring the area under the plasma concentration-time curve. However, a simple estimation of area can be made by using the trapezoidal rule. The total blood volume of mice was assumed as 2 ml, i.e. ~ 7% of the body weight (Senior et al., 1991).

4.1.1.5 The mean residence time (MRT)

The mean residence time is the average time the amount of drugs introduced reside in the body. After an intravenous bolus dose, the MRT is a measure of the average time a substance spends in the body. When a drug is given by an extravascular route, the drug spends additional time at the site of administration (e.g. gastrointestinal tract, muscle or subcutaneous tissues). Therefore, the observed MRT is the sum of the mean times at these sites and in the body.

In this study, pharmacokinetics parameters were calculated on the basis of an open two-compartment model (single i.v. dosing), using MW/Pharm (version 3.15E) which is a Dutch Pharmacokinetics software program.

4.2 Results and Discussion

Prior to any in vivo experiments it was deemed appropriate to label both the protein (IgG or BSA) and the drug molecules. The method of choice for the double isotopic labelling, was radioiodination of the proteins (Greenwood et al., 1963) and the tritium radiolabel of DHEA as described in Chapter III.

4.2.1 Stability of the conjugates

The IgG-s-(^{3}H)DHEA employed (1:64 molar ratios of IgG:s-DHEA) and BSA-s-(^{3}H)DHEA employed (1:50 molar ratios of BSA:s-DHEA) conjugates, equivalent to 2 mg of IgG/BSA, were incubated at 37°C in the presence of mouse plasma to determine the stability of the conjugates. After 1, 3, 6, 10 and 24 h, samples (0.2 ml) were collected, applied on Sephadex G-50 and eluted with PBS, pH.7.4. To check the extent of (^{3}H)DHEA remaining in the conjugates, gel filtration chromatography was used to isolate the conjugates and the free drug. Each fractions was collected and monitored for ^{125}I in a gamma counter and ^{3}H in a liquid scintillation counter. As shown in Figs. 4.1 and 4.2, free (^{3}H)DHEA, released from the conjugates, could not be detected after the isolation of the conjugates and the free drug. This is presumably because of the small amount of free drug released from the conjugates. Therefore, the stability of conjugates was assessed by measuring the quantity of drug bound to IgG or BSA. However, it is possible that the free drug can bind to the plasma proteins after incubation in mouse plasma. Thus, a control experiment between the free drug and mouse plasma may be able to find out the possibilities of the free drug bound to the plasma proteins.

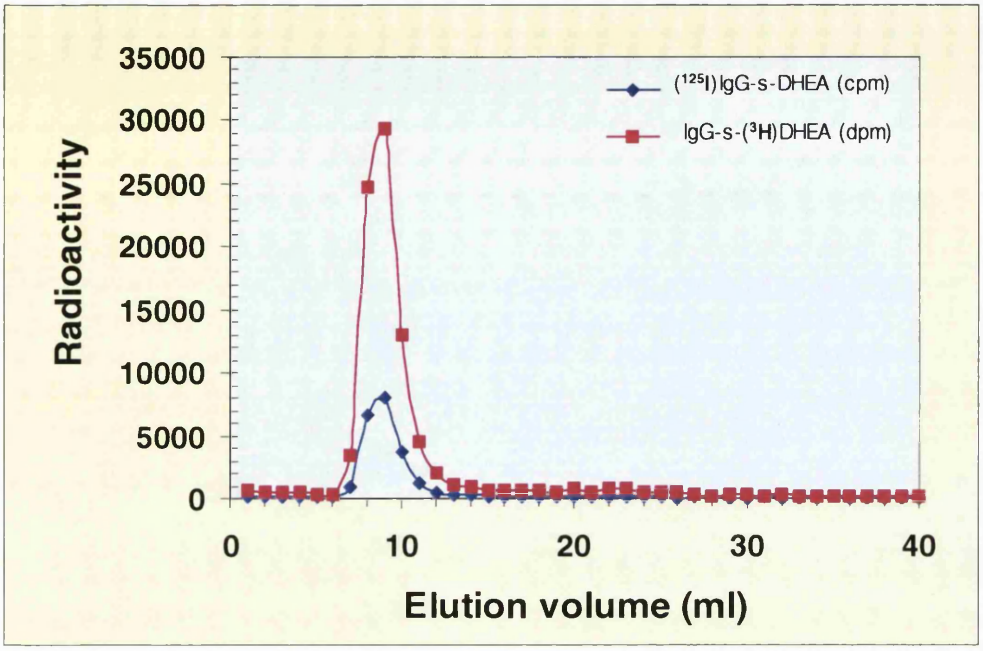


Fig. 4.1 *Molecular sieve chromatography on Sephadex G-50 of (^{125}I)IgG-s-(^{3}H)DHEA after incubation in mouse plasma at 37 °C. The column was equilibrated with PBS, pH 7.4.*

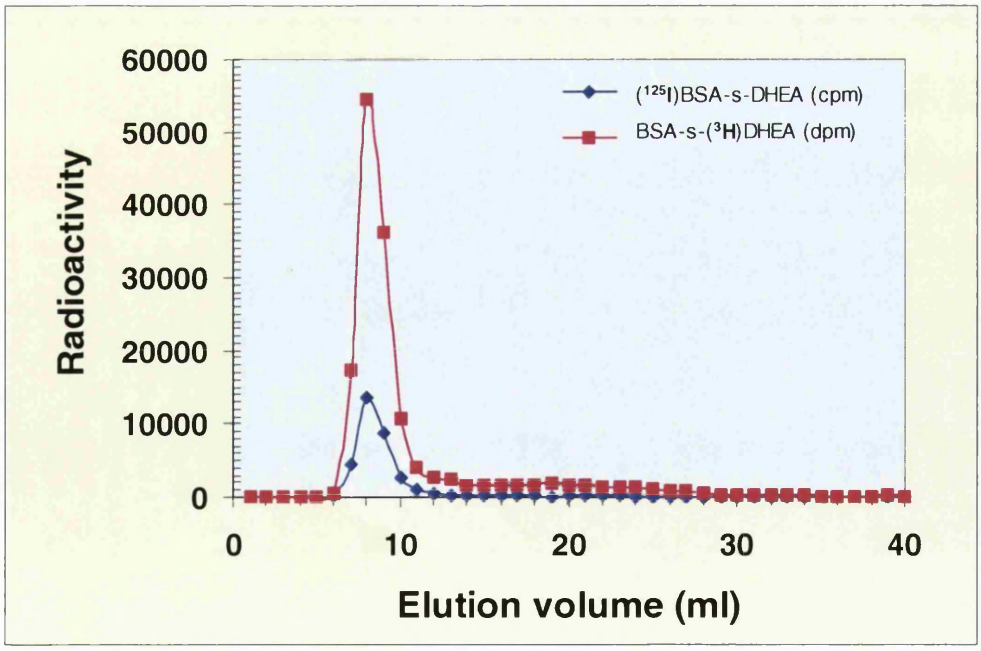


Fig.4.2 *Molecular sieve chromatography on Sephadex G-50 of (^{125}I)BSA-s-(^{3}H)DHEA after incubation in mouse plasma at 37 °C. The column was equilibrated with PBS, pH 7.4.*

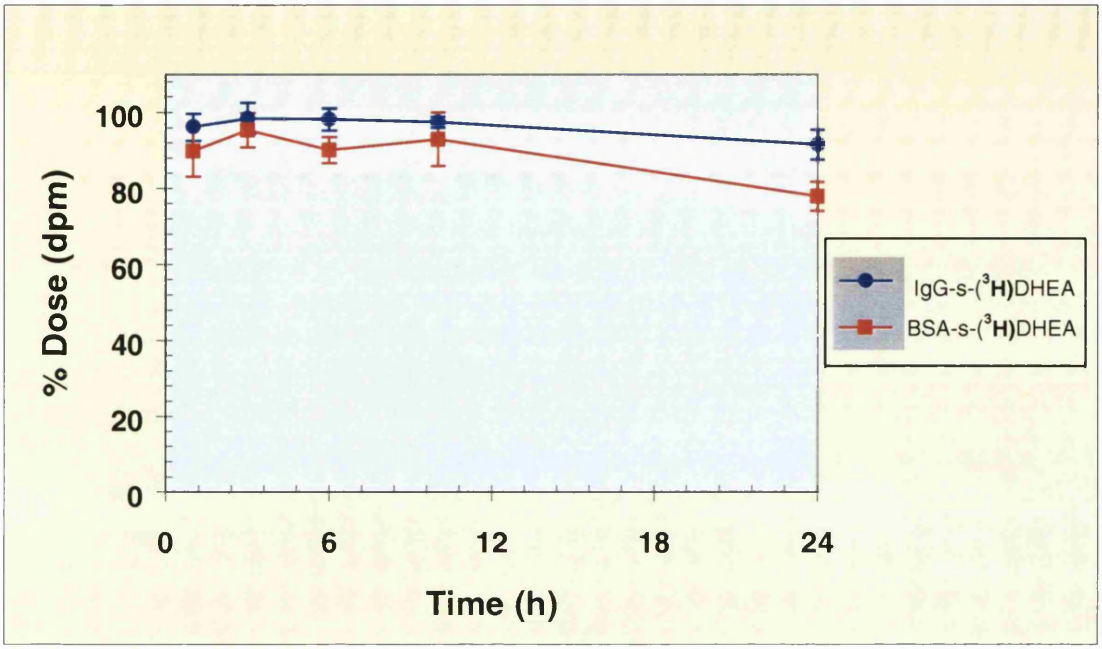


Fig.4.3 *(^{3}H)DHEA remaining with IgG-s-(^{3}H)DHEA and BSA-s-(^{3}H)DHEA after incubation of the conjugates in the presence of plasma at 37℃. Data are means ± s.d. of 3 different preparations.*

In addition, ion exchange chromatography may be introduced in this experiment in order to differentiate between the drug bound to IgG/BSA or plasma proteins.

Fig. 4.3 shows the percentages of (^{3}H)DHEA remaining with IgG-s-DHEA and BSA-s-DHEA conjugates after incubation with mouse plasma. After 6h of incubation, 98.15 ± 2.86% and 90.08 ± 3.46 % of (^{3}H)DHEA were bound to (^{125}I)IgG and (^{125}I)BSA respectively. After 24 h, at least 91.49±3.92 % and 77.86±3.85 of (^{3}H)DHEA were still linked to (^{125}I)IgG and (^{125}I)BSA respectively. This results indicate that the IgG-s-DHEA conjugate was more stable in plasma than the BSA-s-DHEA conjugate. The data presented in Fig. 4.3 demonstrate that the release of (^{3}H)DHEA from the conjugates was very modest, so the drug appeared to be only covalently bound. It showed that the release was not due to being "physically adsorbed".

4.2.2 Clearance of IgG-s-DHEA and BSA-s-DHEA conjugates from the circulation

Pharmacokinetics involves the study of the rates of absorption, distribution, metabolism and elimination of drugs after administration (Shargel and Yu, 1985). Here, the pharmacokinetics of IgG-s-DHEA and BSA-s-DHEA were compared to that of the native IgG and BSA in intravenously injected mice. To characterise the pharmacokinetic behaviour of the conjugates after intravenous injection, several parameters were estimated experimentally: a) area under the curve (AUC), b) clearance (Cl), c) the apparent volume of distribution (Vd), d) plasma half-life for distribution phase ($t_{1/2\alpha}$), e) plasma half-life for elimination phase ($t_{1/2\beta}$) and f) mean residence time (MRT).

Groups of four mice were injected into the tail vein with 2 mg of (^{125}I)IgG, (^{125}I)IgG (process), (^{125}I)BSA, (^{125}I)BSA (process), (^{125}I)IgG-s-(^{3}H)DHEA (2 mg IgG and 0.17-0.33 mg DHEA) and (^{125}I)BSA-s-(^{3}H)DHEA (2 mg BSA and 0.09-0.12 mg DHEA) in 0.2 ml 0.15M PBS (pH 8). In another experiment, animals (n=4) were injected with 0.3 mg of (^{3}H)DHEA in 0.05 ml ethanol-PBS (1:1 v/v). IgG (process) and BSA (process) are IgG or BSA used in the process. These IgG or BSA come from the chemical reaction without drug.

The shape of the semi-logarithmic plot of percentage dose *vs* time indicated that the behaviour of the conjugates and free drug after intravenous injection was a two compartment open model with elimination occurring from the central compartment. The first part of the curve is the α phase during which the drug is being distributed out of the plasma into tissues or extravascularly. The second, straight line part, is the β phase where the drug is being eliminated from the body.

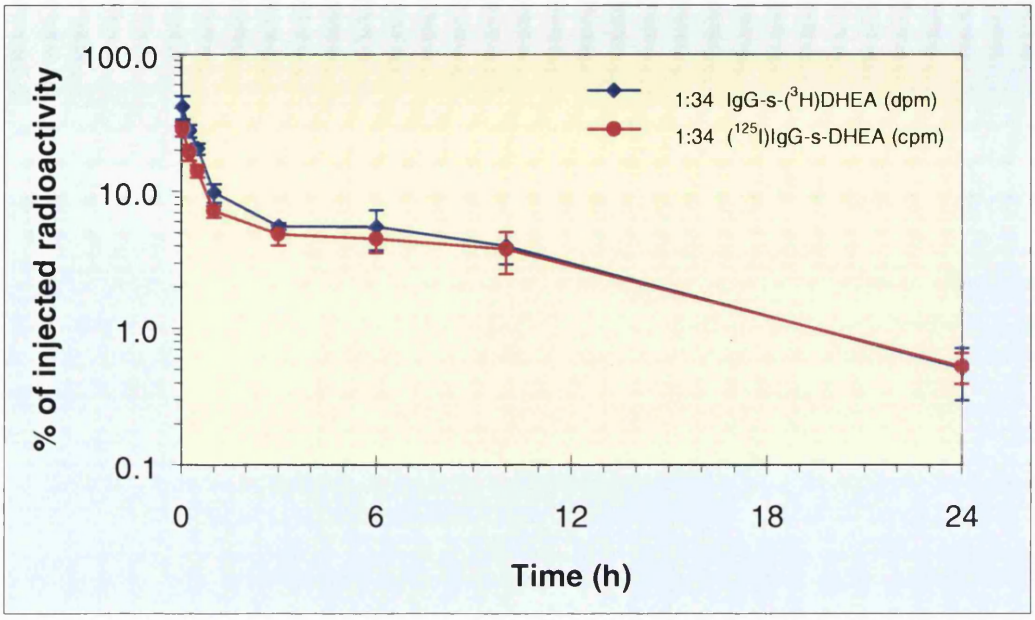


Fig. 4.4 *Clearance of radiolabelled 1:34 (^{125}I)IgG-succinyl-(^{3}H)DHEA conjugate from the blood of intravenously injected mice. Results are means ± s.d. (n=4).*

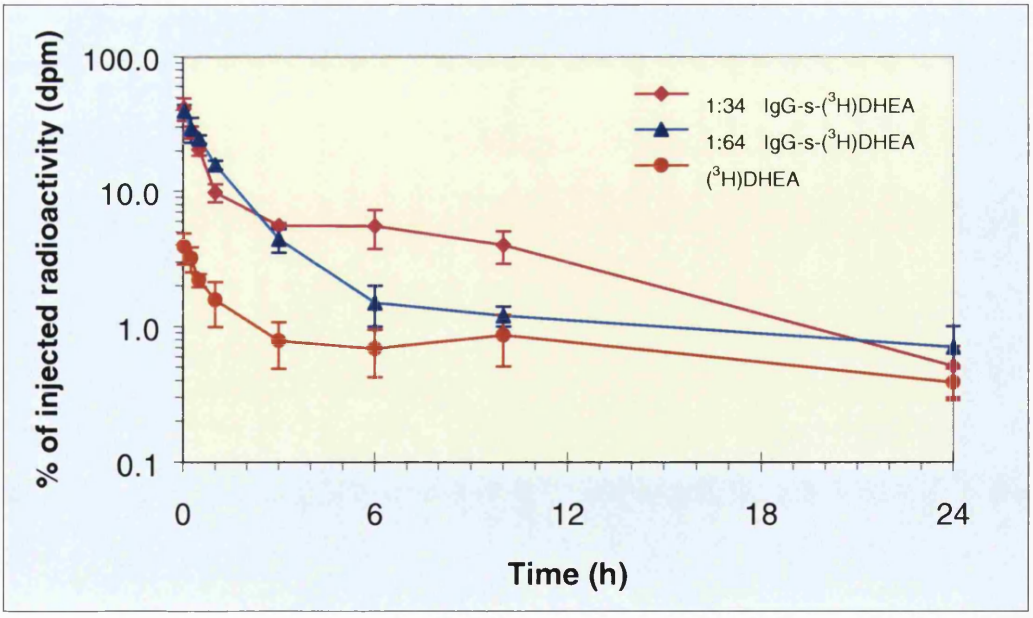


Fig. 4.5 *Clearance of ^{3}H radiolabelled dehydroepinadrosterone (DHEA), 1:34 IgG-succinyl-DHEA conjugate and 1:64 IgG-succinyl-DHEA conjugate from the blood of intravenously injected mice. Results are means ± s.d. (n=4).*

As shown in Fig. 4.4, (^{125}I)IgG and (^{3}H)DHEA in 1:34 IgG-succinyl-DHEA conjugate were gradually removed from the circulation at the same percentage of injected dose, revealing that (^{125}I)IgG was still linked to (^{3}H)DHEA with a resistance to plasma enzymes in the circulation up to 24 h.

Results from Fig. 4.5 showed that 1:34 IgG-s-DHEA and 1:64 IgG-s-DHEA were removed from the circulation at slower rates than the free drug. Thus, the percentage of (^{3}H)DHEA given as free was 3.9 ± 1.0 after 2 min and 1.6 ± 0.6 after 1h. On the other hand, the percentage of (^{3}H)DHEA from 1:34 IgG-s-DHEA was 41.1 ± 7.9 after 2 min and gradually decreased to 9.79 ± 1.5 within 1 h. With the 1:64 IgG-s-DHEA, the percentage of (^{3}H)DHEA was 39.4 ± 4.4 at 2 min and decreased to 15.7 ± 1.2 after 1 h.

Comparative pharmacokinetics parameters are shown in Table 4.1. The area under the plasma concentration curve (AUC) of (^{3}H)DHEA , 1:34 IgG-s-(^{3}H)DHEA and 1:64 IgG-s-(^{3}H)DHEA were estimated to be 0.03, 0.16 and 0.24 mg*h/ml respectively. The AUC of 1:34 and 1:64 IgG-s-(^{3}H)DHEA were 5.3-fold and 8-fold higher than that with free DHEA. Clearance of the conjugates from mouse plasma was 8.6-10.8 fold slower than that of the free drug. The apparent volume of distribution of free DHEA was about 5.7-8.3 times larger than for IgG-s-(^{3}H)DHEA conjugates. The distribution half-life of the conjugates was 1.9-3.6 times longer than the free drug and the elimination half-life of the conjugates was 1.3-1.5 fold longer than (^{3}H)DHEA. This indicated that the pharmacokinetic behaviour of (^{3}H)DHEA in blood circulation was improved by coupling DHEA to IgG. For comparison of the pharmacokinetic behaviour between 1:34 and 1:64 IgG-s-(^{3}H)DHEA conjugates, the clearance of the conjugates was found to be independent of the increased drug load.

By measuring the (^{125}I)IgG radioactivity of the conjugates (Fig. 4.6), the areas under the plasma curve of 1:41 (^{125}I)IgG-s-DHEA and 1:64 (^{125}I)IgG-s-DHEA was estimated to be 1.56 ± 0.19 and 1.57 ± 0.49 h*mg/ml respectively. This demonstrated that the clearance of (^{125}I)IgG-s-DHEA conjugates did not seem to depend on the degree of derivatisation although pharmacokinetic parameters for both of the conjugates were different for the native IgG and the process IgG (Fig. 4.7, Table 4.1). As shown in Fig. 4.7 and Table 4.1, the area under the plasma curve, elimination half-life and mean residence time of the native IgG was 3 fold higher or longer than for the process IgG while the apparent volume of distribution of the native IgG showed no difference to the process IgG. As mentioned earlier, the process IgG comes from the chemical reaction without drug. It is apparent that the pharmacokinetic behaviour of the IgG molecules was affected by the chemical manipulations required for conjugate synthesis.

Table 4.1 Mean pharmacokinetic parameters of dehydroepiandrosterone (DHEA), native immunoglobulin G (IgG), the process immunoglobulin G (IgG(process)),1:34(^{125}I)IgG-s-(^{3}H)DHEA and 1:64(^{125}I)IgG-s-(^{3}H)DHEA after intravenous administration in mice Results are means ± s.d. (n=4).

	AUC (h*mg/ml)	Cl (ml/h)	Vd (ml)	$t_{1/2}\alpha$ (h)	$t_{1/2}\beta$ (h)	MRT (h)
(^{3}H) DHEA	0.03± 0.00	11.73± 1.18	83.03±13.27	0.15± 0.04	4.89± 0.37	6.68± 0.43
1:34 IgG-s-(^{3}H) DHEA	0.16± 0.03	1.09± 0.23	9.96± 1.54	0.29± 0.05	6.55± 1.66	8.06± 2.16
1:64 IgG-s-(^{3}H) DHEA	0.24± 0.01	1.36± 0.08	14.63± 0.68	0.54± 0.05	7.44± 0.17	7.52± 0.37
1:34 (^{125}I)IgG-s-DHEA	1.56± 0.19	1.22± 0.15	13.31± 2.14	0.27± 0.02	7.57± 0.72	9.72± 0.71
1: 64 (^{125}I)IgG-s-DHEA	1.57± 0.49	1.31± 0.37	8.36± 1.53	0.40±0.07	4.61± 1.22	5.09± 1.45
(^{125}I)IgG	10.32±1.14	0.20± 0.02	4.75± 0.84	0.70± 0.18	16.82± 2.14	23.40± 2.80
(^{125}I) IgG (process)	3.06± 0.22	0.62± 0.05	4.84± 0.98	0.27± 0.15	5.45± 1.37	7.23± 1.58

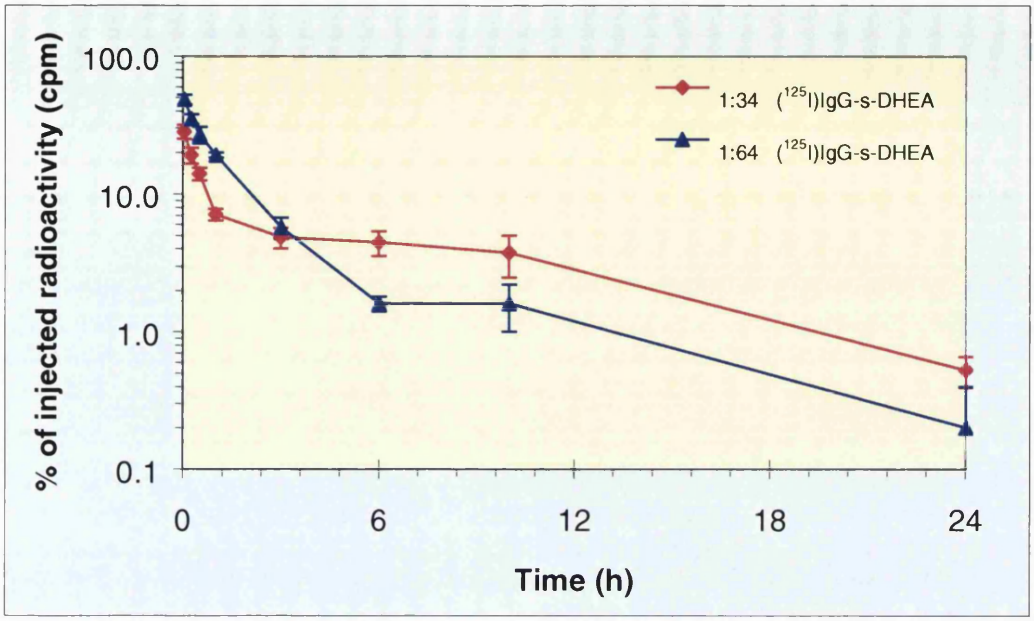


Fig. 4.6 *Clearance of ^{125}I radiolabelled 1:34 IgG-succinyl-DHEA conjugate and 1:64 IgG-succinyl-DHEA conjugate from the blood of intravenously injected mice. Results are means ± s.d. (n=4).*

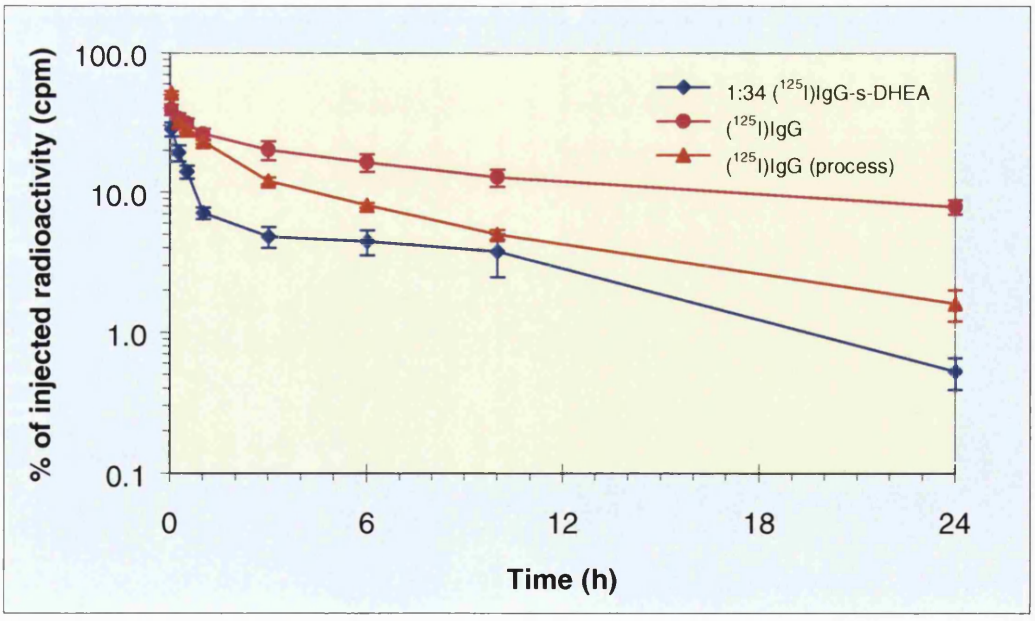


Fig. 4.7 *Clearance of ^{125}I radiolabelled native immunoglobulin G (IgG), the process immunoglobulin G (IgG (process)) and 1:34 IgG-succinyl-DHEA conjugate from the blood of intravenously injected mice. Results are means ± s.d. (n=4).*

As observed with BSA conjugates, Fig. 4.8 showed that (^{125}I)BSA was still bound to (^{3}H)DHEA in the blood circulation. By following (^{3}H)DHEA as a tracer, the percentage of 1:41 BSA-s-(^{3}H)DHEA was found to be 56.25 ± 2.84 after 2 min and slowly decreased to 27.33 ± 2.41 within 1 h while the percentage of 1:50 BSA-s-(^{3}H)DHEA was 45.35 ± 3.38 for 2 min and decreased to 22.35 ± 3.03 after 1 h. Fig. 4.9 shows that both of the conjugates were eliminated from the circulation at slower rates than the free drug.

Pharmacokinetic parameters are presented in Table 4.2. The area under the curve of 1:41 and 1:50 BSA-s-(^{3}H)DHEA were calculated to be 0.76 ± 0.08 and 0.80 ± 0.05 h*mg/ml respectively. It was found that the AUC of 1:41 and 1:50 BSA-s-(^{3}H)DHEA were 25 fold and 27 fold larger than free DHEA while (^{3}H)DHEA was cleared 18.3 and 17 times faster than 1:41 and 1:50 BSA-s-(^{3}H)DHEA respectively. This showed that conjugation of (^{3}H)DHEA to BSA improved the pharmacokinetic behaviour of the free drug in plasma circulation. However, it was also observed that there was no difference in the pharmacokinetic behaviour with an increased degree of modification.

Based on (^{125}I)BSA radioactivity measurements, the calculated area under the curve for 1:41 (^{125}I)BSA-s-DHEA, 1:50 (^{125}I)BSA-s-DHEA, the native BSA and the process BSA were 2.38 ± 0.27, 3.38 ± 0.24, 7.24 ± 0.77 and 3.68 ± 0.63 h*mg/ml respectively (Table 4.2). It was noted that the plasma pharmacokinetics of 1:41 (^{125}I)BSA-s-DHEA, 1:50 (^{125}I)BSA-s-DHEA and the process BSA showed insignificant differences with modification or the degree of derivatisation. However, the difference between the pharmacokinetic parameters of the native BSA and the process BSA indicated the influence of the chemical reactions in conjugate synthesis.

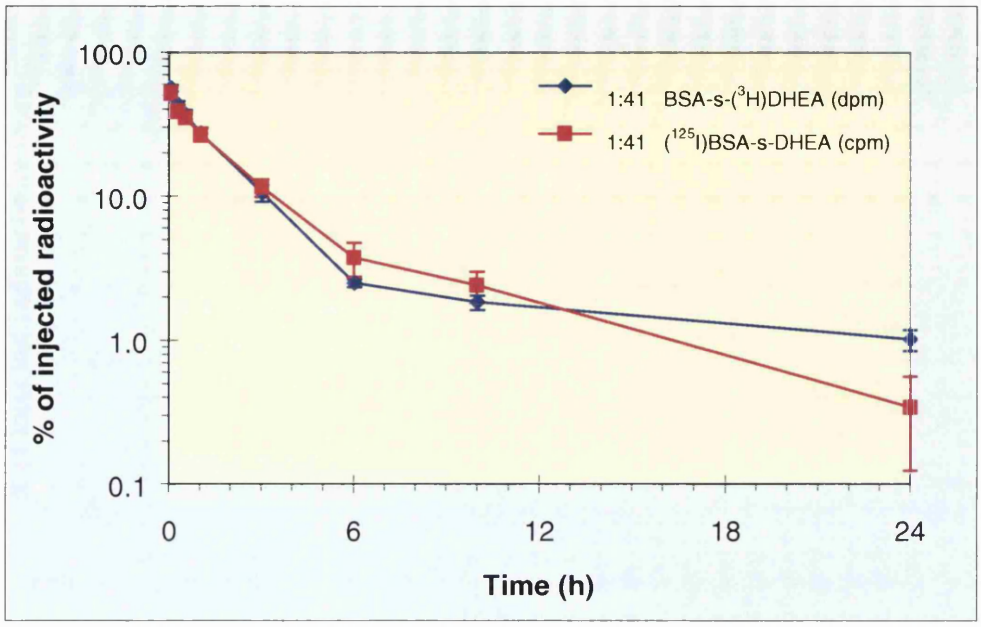


Fig. 4.8 *Clearance of radiolabelled 1:41 (^{125}I) BSA-succinyl-(^{3}H)DHEA conjugate from the blood of intravenously injected mice. Results are means ± s.d. (n=4).*

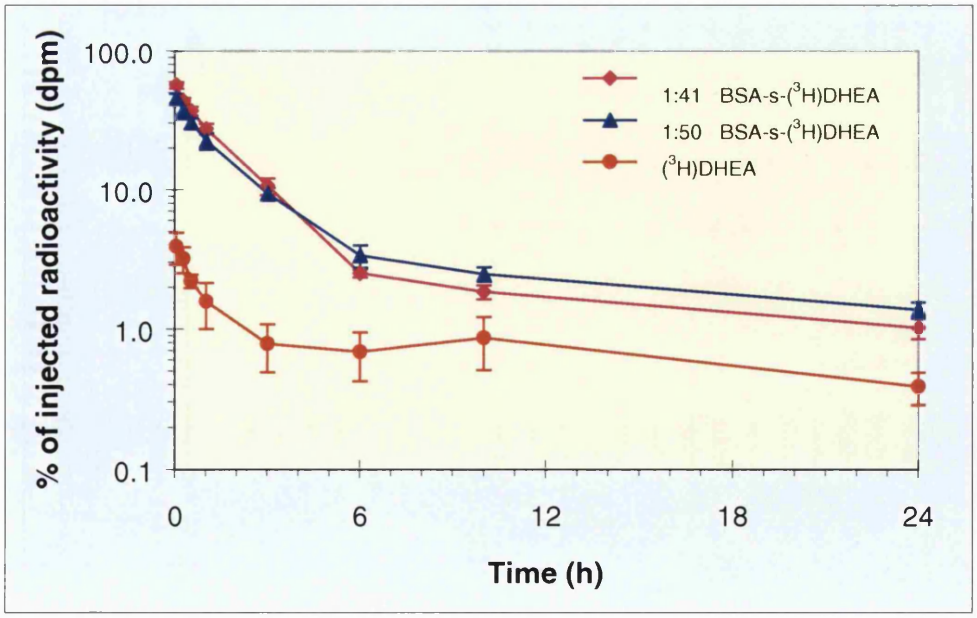


Fig. 4.9 *Clearance of ^{3}H radiolabelled dehydroepinadrosterone (DHEA), 1:41 BSA-succinyl-DHEA conjugate and 1:50 BSA-succinyl-DHEA conjugate from the blood of intravenously injected mice. Results are means ± s.d. (n=4).*

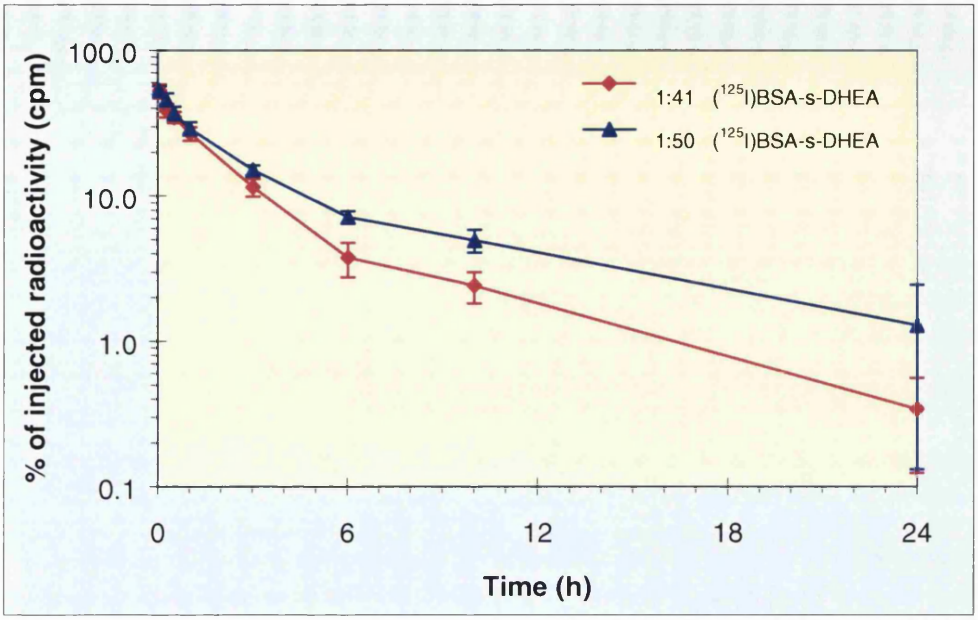


Fig. 4.10 *Clearance of ^{125}I radiolabelled 1:41 BSA-succinyl-DHEA conjugate and 1:50 BSA-succinyl-DHEA conjugate from the blood of intravenously injected mice. Results are means ± s.d. (n=4).*

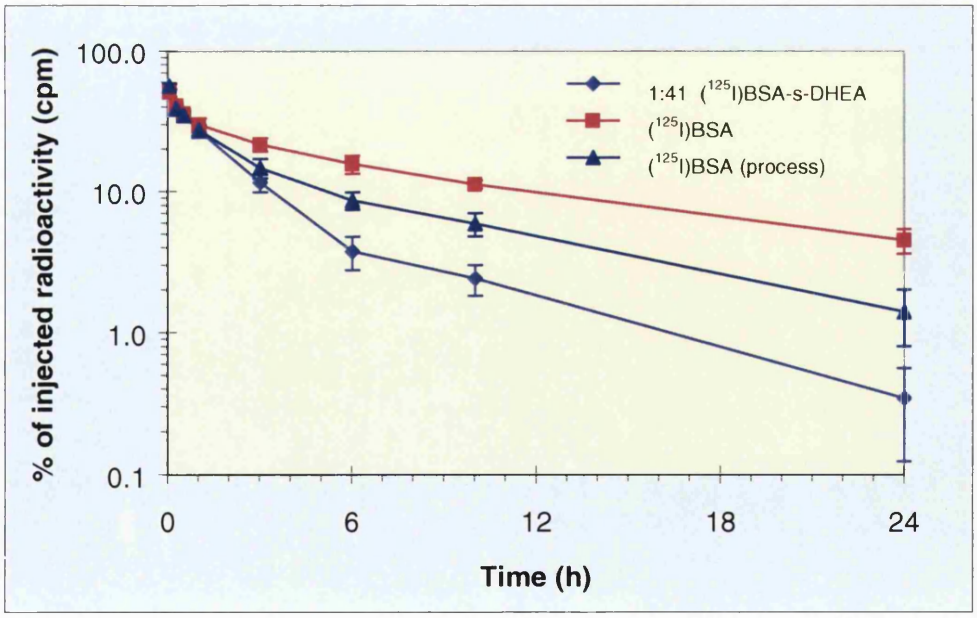


Fig. 4.11 *Clearance of ^{125}I radiolabelled the native bovine serum albumin (BSA), the process bovine serum albumin (BSA (process)) and 1:41 BSA-succinyl-DHEA conjugate from the blood of intravenously injected mice. Results are means ± s.d. (n=4).*

Table 4.2 Mean pharmacokinetic parameters of dehydroepiandrosterone (DHEA), the native bovine serum albumin (BSA), the process bovine serum albumin (BSA(process)), 1:41 (^{125}I)BSA-s-(^{3}H)DHEA and 1:50 (^{125}I)BSA-s-(^{3}H)DHEA after intravenous administration in mice. Results are mean ± s.d. (n=4).

	AUC (h*mg/ml)	Cl (ml/h)	Vd (ml)	$t_{1/2}\alpha$ (h)	$t_{1/2}\beta$ (h)	MRT (h)
(^{3}H) DHEA	0.03± 0.00	11.73± 1.18	83.03±13.27	0.15± 0.04	4.89± 0.37	6.68± 0.43
1:41 BSA-s-(^{3}H) DHEA	0.76±0.08	0.64±0.06	23.65±2.29	1.07±0.05	25.63±3.26	19.20±2.93
1:50 BSA-s-(^{3}H) DHEA	0.80±0.05	0.69±0.05	16.84±3.00	1.05±0.11	17.03±3.76	15.02±2.63
1:41 (^{125}I)BSA-s-DHEA	2.38±0.27	0.85±0.10	6.44±1.08	1.01±0.29	5.39±1.44	4.58±1.16
1:50 (^{125}I)BSA-s-DHEA	3.38±0.24	0.59±0.04	4.30±0.81	0.77±0.16	5.04±1.05	5.90±1.27
(^{125}I)BSA	7.24±0.77	0.28±0.03	3.98±0.44	0.61±0.45	9.94±0.97	13.46±1.53
(^{125}I) BSA (process)	3.68±0.63	0.53±0.10	4.93±0.44	0.60±0.16	6.61±1.20	8.16±1.45

Generally, human serum albumin and human immunoglobulin G exhibit an average half-life 0f 19 and 25 days respectively. However, it was observed that the native IgG and BSA were eliminated from the circulation so quickly. It is possible that the conformation of the native IgG/BSA has been changed due to the influence of the radioiodination by chloramine-T method. Thus, the other methods for the radioiodination such as bolton-hunter, iodogen or iodo-beads may minimise the damage of the proteins.

The pharmacokinetic values presented in Table. 4.1 and 4.2 showed that the AUC for 1:41 BSA-s-(^{3}H)DHEA (equivalent to 0.49 mg DHEA) was 4.75 fold higher than 1:34 IgG-s-(^{3}H)DHEA (equivalent to 0.84 mg DHEA). This demonstrated that the content of 1:41 BSA-s-(^{3}H)DHEA circulated in the blood for a longer period than 1:34 IgG-s-(^{3}H)DHEA, although the amount of DHEA in BSA conjugates was 0.35 mg less than the

IgG conjugates. It was also found that the AUC of 1:50 BSA-s-(^{3}H)DHEA (0.58 mg DHEA) was 3.3 times higher than 1:64 IgG-s-(^{3}H)DHEA (1.6 mg DHEA) (Table. 4.1) despite the fact that the IgG conjugates contained approximately 1.02 mg more than the BSA conjugates. This observation demonstrated that the pharmacokinetics of IgG-s-DHEA conjugates was not as good as the BSA-s-DHEA conjugates. Therefore, the molecular mass, shape and charge of the conjugates should be taken into consideration. This study has shown that IgG and BSA show promise as soluble drug carriers after intravenous administration.

Chapter V

Biodistribution of IgG-s-DHEA and BSA-s-DHEA conjugates

5.1 Introduction

For effective treatment, it is necessary to deliver therapeutic agents selectively to their target sites, since most drugs are associated with both beneficial effects and unfavourable actions. Most conventional drugs diffuse freely throughout the body and show relatively even tissue distribution due to their low molecular weight. Among the various strategies for site-specific drug delivery, macromolecular carriers can be a formidable tool because of their diversity in physicochemical and biological properties. The rationale for the macromolecular carrier approach in site-specific drug delivery lies in the altered disposition of a carrier-conjugated drug in the body which is largely dictated by the properties of the carrier and accordingly differs from that of the free drug administered by the same route (Takakura and Hashida, 1996).

As already discussed in Chapter IV, the pharmacokinetic behaviour of dehydroepiandrosterone (DHEA) in the blood circulation was improved by coupling DHEA to immunoglobulin G (IgG) or bovine serum albumin (BSA). The aim in this chapter is to determine the potential of IgG and BSA as site specific drug carriers for an insoluble drug (DHEA) in the body. In this study, the tissue distribution of the water-soluble IgG-s-DHEA and BSA-s-DHEA conjugates was investigated and compared with that of free DHEA after intravenous injection into mice.

5.1.1 The processes of ADME

Following administration, a drug is absorbed and distributed by blood flow to all sites within the body including the drug-eliminating organs. The plasma concentration of a drug is one of the major factors in determining the response of the subject to that drug. The concentration of a drug in the plasma circulation depends on the route of administration. A compound which is given intravenously reaches a high blood concentration immediately, and then tails off as the compound is eliminated. With oral administration, the uptake of a drug depends on its absorption from the gastrointestinal tract. The processes of absorption, distribution, metabolism and excretion (ADME) of a drug are shown in Fig. 5.1.

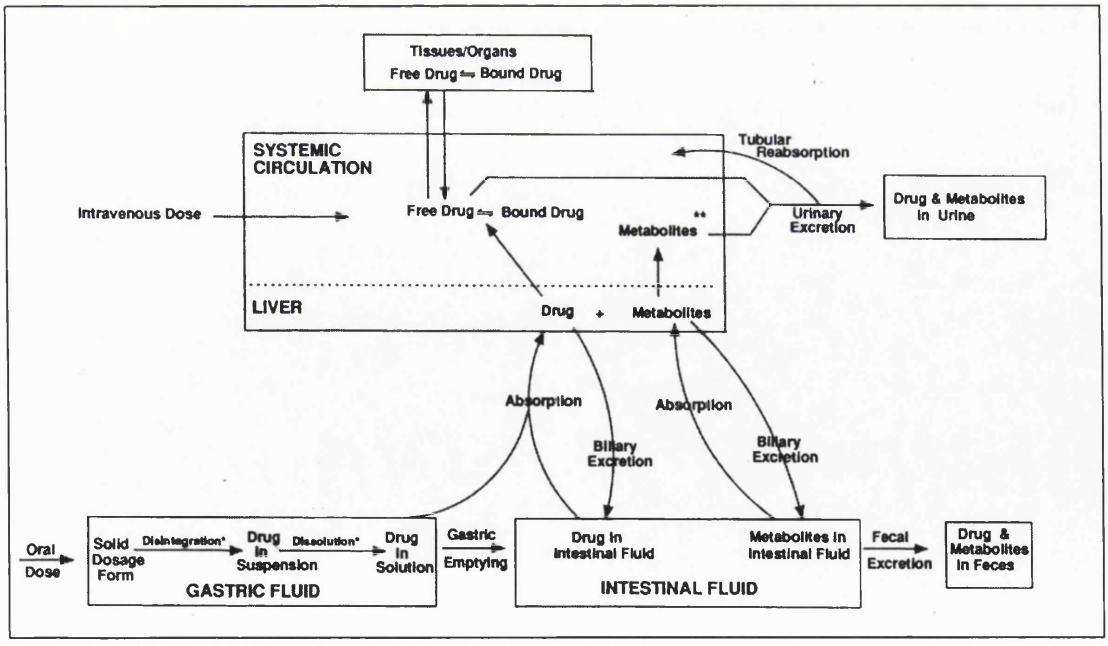


Fig. 5.1 *The processes of absorption, distribution, metabolism and excretion in the body (Tse and Jaffe, 1991)*

Animal models are often employed in the development of new dosage forms or formulations. ADME studies can describe the disposition characteristics of the drug and can serve as the basis for the selection of a suitable dosage form. ADME data obtained are often useful in the interpretation of drug effects.

5.1.1.1 Absorption

Absorption is the process by which a test compound and its metabolites are transferred from the site of administration to the systemic circulation. The rate of absorption, by the oral route for instance, can be markedly influenced by how rapidly the compound dissolves in the gastrointestinal fluids. The absorption process is often the rate-limiting step and can subsequently influence the onset, duration, and intensity of the pharmacological activity of the compound. Obviously, the absorption process does not need to be taken into consideration when drugs are administered intravenously as all of the dose enters the circulation directly. Absorption is not restricted only to oral administration. It also occurs after intramuscular, subcutaneous, and other extravascular routes.

5.1.1.2 Distribution

As depicted in Fig. 5.1, the molecules of the administered compound mix with body fluids after entering the blood stream and are then distributed to the various organs of the body. Distribution is influenced by how well each organ is perfused with blood,

organ size, binding of the drug to other entities within the blood and tissues, and the ability of the drug to cross tissue and cell membranes. Like other equilibrium processes, tissue distribution is usually reversible between the inside and outside of the tissue cells. The rate of tissue uptake is generally dependent on the blood flow to that area. Thus, equilibrium occurs rather rapidly in the highly perfused organs such as the liver and the kidney, relatively more slowly in moderately perfused sites such as muscle tissue and skin, and much more slowly in fat tissue.

Molecules, that are not bound to plasma proteins in the blood and do not have molecular weights of more than 500-600 Da, can penetrate the capillary walls and reach the interstitial spaces. For lipid soluble compounds, nonprotein-bound molecules pass through the membranes by a passive diffusion process. For central nervous system (CNS) drug delivery, the brain capillaries are surrounded by a cellular sheath that makes them substantially less permeable to water-soluble materials than capillaries found in other areas of the body. Thus, the ability of compounds to penetrate the blood brain barrier is highly dependent on their lipid solubility and polarity.

5.1.1.3 Metabolism

Metabolism is the process by which the administered compound is structurally changed in the body by either enzymatic or nonenzymatic reactions. Drug metabolism is normally divided into two phases (Phase I and Phase II) which are associated with the chemical reactions. Phase I metabolism includes oxidation, reduction, hydrolysis and hydration reactions while phase II, often termed conjugation reactions, involves coupling between the compound or its metabolite and endogenous substrates, especially glucuronic

or sulphuric acids. Although compounds are predominantly metabolised in the liver by microsomal enzymes located in the endoplasmic reticulum., other sites may also play a part in the metabolism of some drugs such as enzymes in the blood, the kidney, the gastro-intestinal tract (GIT) and the lung. Since metabolites are generally more polar than the original compound, their volumes of distribution are reduced and their ability to be eliminated via the kidneys is greatly increased.

5.1.1.4 Excretion

Compounds are eliminated from the body as unchanged molecules or as metabolites. In most excretory organs except lungs, water soluble substances are excreted more efficiently than lipid soluble substances. Excretion can take place through numerous pathways such as the bile, faeces, milk, saliva, sweat, tears and lungs , however, the most significant organ for elimination is the kidneys. Renal excretion involves three processes: passive glomerular filtration, active tubular filtration and passive tubular reabsorption. In the proximal and distal tubules, passive reabsorption of compounds from the glomerular filtrate back into the blood is influenced by the intrinsic lipid solubility of the compound, its ionisation constant and the pH of the urine. Thus, compounds with high lipid solubility do not appear in the urine in large proportions because most of the molecules are filtered at the glomerulus and return to the blood by diffusing across the lipid-like boundary of the tubular cells. On the other hand, compounds of low lipid solubility are excreted in the urine because they are poorly reabsorbed in the tubule.

Hepatic elimination can also play an important role in the excretion process. Compounds that are metabolised in the liver are often excreted in the bile into the

intestinal tract. The metabolites or unchanged drug can then be reabsorbed by passive diffusion into the blood (enterohepatic circulation) or eventually excreted in the faeces. Excretion by other routes (sweat, saliva, milk, tears) is quantitatively insignificant in relation to the urine and faeces. However, mammary excretion can be of potential importance for highly potent compounds that could induce toxic manifestations in the nursing of the newborn whose hepatic and renal detoxification capacities are usually limited.

In this study of tissue biodistribution of injected IgG-s-DHEA and BSA-s-DHEA, the Student's t-test was used for unpaired comparisons. All statistical calculations were performed using GraphPad InStat (GraphPad Software, version 2.0, 1995).

5.2 Results and Discussion

In Chapter IV, the clearance of radioactive free DHEA was compared with that of IgG-succinyl-DHEA and BSA-succinyl-DHEA after intravenous injection into mice. For tissue distribution studies, (^{3}H)DHEA, (^{125}I)IgG-succinyl-(^{3}H)DHEA and (^{125}I)BSA-succinyl-(^{3}H)DHEA (as mentioned in Chapter II) were injected intravenously into the tail veins of male outbred mice. The mice were kept in metabolic cages for the collection of urine and faeces at time intervals. At 5 min, 2h, 6h, 12h and 24h, the mice were killed and their tissues (liver, kidneys, spleen, thyroid, brain, lung, muscle, intestine and intestine washings) were dissected and analysed (in terms of radioactivity) for the amount of compound present in each organ. It is emphasised here that radioactivity measured in tissues and excretions may represent intact drug and/or its radiolabelled metabolites. No attempt was made to identify DHEA metabolites as this falls beyond the scope of this study.

Table 5.1: Distribution of (^{3}H)DHEA after intravenous administration into mice. Results are expressed as percentage of injected radioactivity (means ± SE; n = 4).

Organs or excretions	Time after injection				
	5 min	2 hours	6 hours	12 hours	24 hours
Liver	9.73±1.22	4.36±0.55	1.23±0.38	2.79±0.60	0.68±0.14
Kidneys	1.59±0.18	0.95±0.30	0.40±0.06	0.61±0.13	0.19±0.07
Spleen	0.55±0.12	0.41±0.10	0.25±0.06	1.15±0.33	0.47±0.04
Thyroid	0.04±0.01	0.08±0.02	0.03±0.01	0.07±0.00	0.04±0.01
Brain	1.17±0.07	0.19±0.03	0.20±0.03	0.22±0.06	0.20±0.02
Lung	0.62±0.11	0.81±0.11	0.28±0.04	1.04±0.20	0.68±0.24
Muscle (a)	1.10±0.14	0.57±0.09	0.43±0.10	0.75±0.28	0.64±0.20
Small intestine	3.54±0.18	3.32±0.74	0.87±0.14	0.41±0.09	0.25±0.06
Large intestine	0.80±0.10	1.10±0.34	1.27±0.14	0.34±0.07	0.15±0.05
Intestine washings	1.11±0.17	20.18±1.86	8.03±1.52	2.69±0.61	1.29±0.25
Urine			10.15±1.65	17.31±1.68	24.64±3.26
Faeces			20.47±1.45	54.92±6.67	62.68±6.19
Blood	1.65±0.30	0.56±0.19	0.43±0.05	0.35±0.10	0.19±0.05
Total recovered (b)	20.80±1.82	31.95±2.87	43.61±2.44	81.89±7.64	85.73±10.52

(a): Values are expressed as per gram muscle tissue. (b): Values are excluded muscle tissue.

The tissue distribution of (^{3}H)DHEA presented in Table 5.1 shows that the majority of the free drug radioactivity was found in liver, intestine washings, urine and faeces. Uptake of radioactivity by the liver was nearly 10% of the dose after 5 min but it gradually decreased to nearly 1% after 24h. As mentioned in Chapter IV, rapid clearance from the plasma was observed after intravenous injection of radiolabelled free drug into mice. Only about 2% of the free drug radioactivity was found in total blood after 5 min (Table 5.1). The percentage of total recovery of the administered radioactivity dose was found to be 21% after 5 min and this increased to 86% after 24h (recovery included radioactivity excreted in the urine and faeces).

Due to its low molecular weight and lipid solubility, DHEA diffused freely throughout the body showing a high volume of distribution (Chapter IV). This

demonstrated that the free DHEA molecules, which are in the form of a lipophilic uncharged compound, are able to pass through cell membranes and the body fat by passive diffusion. Approximately 20% of the free drug radioactivity after 2h was found in the intestine washings (Table 5.1). After 24h, 25% of the injected free drug radioactivity was found in urine and 63% in the faeces. This suggests that the main route of elimination for DHEA radioactivity was the biliary tract. Bile is produced in the liver, passes into the gall bladder and is then secreted into the intestine. It is possible that DHEA was distributed in the liver by entering the hepatocytes by lipophilic diffusion or transported there by plasma proteins. The free drug was then metabolised in the liver, the metabolites dissolved in the bile and then secreted into the intestine after storage in the gall bladder. The liver may have also secreted intact drug into the bile and then into the intestine from where the drug may be either excreted in the faeces or reabsorbed back into the plasma. Generally, a lipophilic drug such as DHEA can be reabsorbed in the renal tubules due to its small molecular weight or low polarity; this would explain the relatively limited amount of drug found in urine. However, the drug may have also been converted to a more polar metabolite in the liver before excretion in the urine as sulphate or glucuronide forms.

Previous studies (Belanger et al., 1990, Belanger, 1993) showed that, in addition to the unconjugated and sulphate ester forms of DHEA, conjugated fatty acid ester (-FA) derivatives of DHEA are also present in the circulation. Jone and James (1985) reported that 10-20% of labelled DHEA can be converted to non-polar derivatives in the human plasma by esterification of the hydroxy group of DHEA to a fatty acid moiety. It was suggested that the plasma may contain an esterifying enzyme, called lecithin:cholesterol acyltransferase (LCAT), which is found in the circulation bound to high density lipoproteins (HDL) and is responsible for cholesterol ester formation in plasma (Lavallee

et al., 1996). Generally, HDL is known to play a role in the removal of the cellular cholesterol excess. Cholesterol is esterified by LCAT after incorporation into HDL and the resulting cholesteryl esters become incorporated into the core of the particle. HDL transfer cholesterol to the liver from where it can be eliminated through the biliary tract. Therefore, this may explain the elimination pathway of DHEA due to its similar structure with cholesterol.

Table 5.2: Distribution of 1:64 (^{125}I)IgG-succinyl-(^{3}H)DHEA conjugate after intravenous administration into mice. Results are expressed as percentage of injected radioactivity (means ± SE; n = 4).

a) IgG-s-(^{3}H)DHEA

Organs or excretions	Time after injection				
	5 min	2 hours	6 hours	12 hours	24 hours
Liver	20.96±1.24	27.79±2.49	12.33±1.62	5.15±0.24	3.98±0.89
Kidneys	2.86±0.09	1.90±0.38	0.88±0.20	0.61±0.06	0.32±0.06
Spleen	1.19±0.10	1.64±0.08	0.63±0.11	0.34±0.02	0.39±0.13
Thyroid	0.05±0.01	0.67±0.08	2.08±0.33	2.99±0.33	2.53±0.28
Brain	0.38±0.03	0.11±0.02	0.08±0.01	0.10±0.03	0.18±0.01
Lung	2.18±0.35	1.44±0.06	0.63±0.09	0.39±0.02	0.20±0.05
Muscle (a)	1.54±0.20	1.00±0.07	0.76±0.16	0.25±0.02	0.10±0.01
Small intestine	1.89±0.28	2.63±0.53	1.03±0.20	0.68±0.10	0.36±0.04
Large intestine	0.39±0.03	0.81±0.07	0.86±0.07	0.54±0.05	0.10±0.03
Intestine washings	0.88±0.31	22.57±1.42	27.18±1.92	11.66±2.72	1.97±0.47
Urine			27.18±2.55	19.19±4.12	40.39±7.52
Faeces			12.65±4.84	52.64±7.97	43.29±3.23
Blood	48.27±1.84	9.56±1.09	2.41±0.07	1.14±0.18	0.54±0.22
Total recovered (b)	79.05±2.31	69.12±2.57	77.18±2.71	95.45±8.54	94.25±4.46

(a): Values are expressed as per gram muscle tissue. (b): Values are excluded muscle tissue.

b) (^{125}I)IgG-s-DHEA

Organs or excretions	Time after injection				
	5 min	2 hours	6 hours	12 hours	24 hours
Liver	14.63±0.14	14.46±0.35	6.31±1.00	2.00±0.10	0.92±0.07
Kidneys	2.08±0.12	1.64±0.07	0.61±0.05	0.31±0.05	0.25±0.09
Spleen	0.82±0.06	0.90±0.06	0.41±0.02	0.15±0.02	0.29±0.20
Thyroid	0.06±0.00	0.99±0.33	4.90±0.43	6.07±0.58	5.13±0.56
Brain	0.30±0.03	0.20±0.10	0.06±0.01	0.03±0.01	0.07±0.02
Lung	1.71±0.02	0.92±0.25	0.45±0.01	0.22±0.01	0.12±0.03
Muscle (a)	1.55±0.21	0.97±0.16	0.71±0.13	0.39±0.04	0.04±0.06
Small intestine	0.77±0.08	1.67±0.09	0.75±0.02	0.45±0.13	0.17±0.01
Large intestine	0.28±0.02	0.40±0.03	0.21±0.01	0.09±0.01	0.06±0.01
Intestine washings	0.04±0.02	1.51±0.22	1.92±0.16	1.16±0.20	0.19±0.04
Urine			15.01±2.85	23.69±4.29	40.78±8.39
Faeces			1.25±0.34	11.79±4.72	9.69±4.42
Blood	52.79±3.67	10.57±0.85	2.72±0.35	0.76±0.18	0.15±0.04
Total recovered (b)	73.48±3.55	33.26±1.16	34.60±2.87	46.74±2.82	57.81±3.94

(a): Values are expressed as per gram muscle tissue. (b): Values are excluded muscle tissue.

Table 5.3: Distribution of 1:50 (^{125}I)BSA-succinyl-(^{3}H)DHEA conjugate after intravenous administration into mice. Results are expressed as percentage of injected radioactivity (means ± SE; n = 4)

a) BSA-s-(^{3}H)DHEA

Organs or excretions	Time after injection				
	5 min	2 hours	6 hours	12 hours	24 hours
Liver	24.78±1.77	43.55±1.60	17.38±2.19	6.18±1.20	1.34±0.21
Kidneys	5.15±0.80	7.36±0.93	1.90±0.48	1.10±0.23	0.87±0.12
Spleen	1.87±0.03	5.52±0.88	2.63±0.62	0.85±0.20	0.27±0.03
Thyroid	0.04±0.01	0.33±0.05	0.79±0.19	0.64±0.07	1.07±0.23
Brain	0.91±0.20	0.36±0.03	0.43±0.16	0.27±0.01	0.21±0.03
Lung	3.91±0.47	5.42±1.14	1.99±0.28	0.79±0.13	0.20±0.04
Muscle (a)	1.87±0.19	1.11±0.15	0.92±0.08	0.57±0.10	0.29±0.08
Small intestine	2.31±0.23	2.74±0.79	1.61±0.09	0.61±0.07	0.29±0.06
Large intestine	0.58±0.01	0.96±0.21	1.00±0.09	0.71±0.17	0.19±0.03
Intestine washings	2.20±0.26	19.87±1.84	16.43±1.85	8.62±0.67	2.60±0.68
Urine			16.80±3.74	19.01±1.61	21.94±5.54
Faeces			15.10±5.82	36.63±1.61	35.27±3.30
Blood	55.59±2.51	16.18±0.72	4.32±1.24	1.00±0.20	0.87±0.15
Total recovered (b)	97.33±5.09	102.30±0.82	80.39±6.63	76.41±1.46	65.12±7.54

(a): Values are expressed as per gram muscle tissue. (b): Values are excluded muscle tissue.

b) (^{125}I)BSA-s-DHEA

Organs or excretions	Time after injection				
	5 min	2 hours	6 hours	12 hours	24 hours
Liver	8.16±0.20	8.57±0.60	6.45±0.11	1.79±0.09	0.38±0.03
Kidneys	3.43±0.21	3.20±0.14	0.61±0.04	0.16±0.01	0.12±0.02
Spleen	0.61±0.03	0.91±0.05	0.39±0.03	0.10±0.02	0.03±0.01
Thyroid	0.03±0.01	0.89±0.05	2.60±0.66	2.63±0.31	4.50±0.61
Brain	0.30±0.05	0.17±0.02	0.06±0.01	0.01±0.00	0.04±0.03
Lung	1.36±0.08	1.27±0.09	0.40±0.03	0.14±0.01	0.08±0.01
Muscle (a)	1.00±0.04	1.23±0.06	0.38±0.09	0.17±0.02	0.07±0.01
Small intestine	0.91±0.02	1.82±0.17	0.73±0.08	0.33±0.04	0.09±0.01
Large intestine	0.25±0.02	0.38±0.06	0.17±0.02	0.05±0.01	0.04±0.01
Intestine washings	0.14±0.03	0.63±0.05	0.90±0.17	0.49±0.11	0.22±0.04
Urine			19.38±3.69	29.52±1.42	31.68±7.77
Faeces			0.27±0.11	1.69±0.27	1.96±0.46
Blood	72.86±2.09	24.79±0.64	7.70±2.01	0.78±0.09	0.35±0.13
Total recovered (b)	88.04±2.09	42.62±1.05	40.18±2.48	39.79±0.61	39.51±7.47

(a): Values are expressed as per gram muscle tissue. (b): Values are excluded muscle tissue.

Observations of the fate of (^{125}I)IgG-succinyl-(^{3}H)DHEA and (^{125}I)BSA-succinyl-(^{3}H)DHEA (Table 5.2 and Table 5.3) revealed that the majority of ^{125}I and ^{3}H radioactivity was found in blood, liver, intestine washings, urine and faeces. A comparison of the relevant radioactivity for the DHEA, IgG-s-DHEA and BSA-s-DHEA in each of the organs is made in the following Figures.

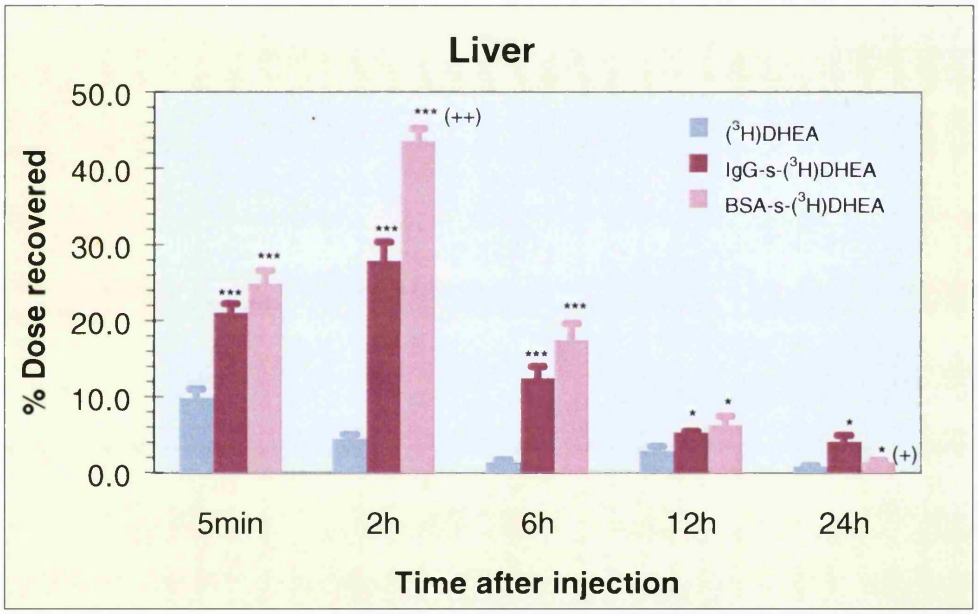


Fig. 5.2 *The percentage uptake of (^{3}H)DHEA, IgG-s-(^{3}H)DHEA and BSA-s-(^{3}H)DHEA in liver after intravenous administration into mice. Results are means ± SE (n=4). (* p<0.05, *** p<0.001 as compared to the (^{3}H)DHEA, + p<0.05, ++ p<0.01 as compared between IgG-s-(^{3}H)DHEA and BSA-s-(^{3}H)DHEA)*

Table 5.2a shows that 21% of the IgG-s-(^{3}H)DHEA radioactivity was found in the liver after 5 min. After 2h, the uptake in liver was elevated to 28% and then declined to 4% after 24h. On the other hand, although the uptake of BSA-s-(^{3}H)DHEA radioactivity by the liver at 5 min was similar to that of the IgG construct (25%), it increased to 44% after 2h and then declined to approximately 1% at 24h. Fig. 5.2 indicates that the IgG and

BSA conjugates of DHEA significantly increased the hepatic uptake of the tritiated drug at 2h: by 6.4 fold for the IgG conjugate and by 10 fold for the BSA conjugate as compared with the free drug ($p < 0.001$).

Dunn et al. (1981) reported that the endogenous DHEA circulates in the blood in non-protein bound form or bound to albumin and sex-hormone-binding globulin. According to Leszczynski and Schafer (1989), nearly 80% of circulating DHEA is bound to albumin. However, the binding of albumin to DHEA is relatively weak and has little effect on its metabolism (Longcope, 1995). In this study, DHEA was linked to the two proteins covalently by conjugation. As shown in Fig.5.2, the maximum uptake of free DHEA radioactivity in the liver was observed after 5 min whereas that of IgG-s-(^{3}H)DHEA and BSA-s-(^{3}H)DHEA was reached after 2h. This could be explained either as the result of a greater but slower transport of the drug by the protein (IgG or BSA) to the tissue and/or a slower catabolism of the drug in the form of a conjugate, leading to a more delayed elimination of the ^{3}H-labelled metabolites.

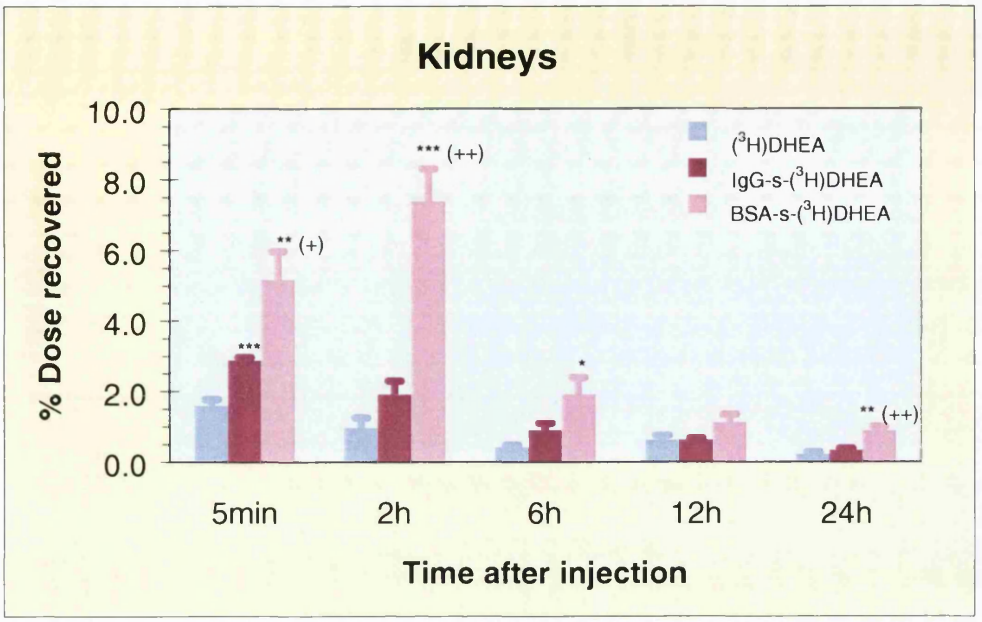


Fig. 5.3 *The percentage uptake of (^{3}H)DHEA, IgG-s-(^{3}H)DHEA and BSA-s-(^{3}H)DHEA in kidneys after intravenous administration into mice. Results are means ± SE (n=4). (* p<0.05,** p<0.01, *** p<0.001 as compared to the (^{3}H)DHEA, + p<0.05,++ p<0.01 as compared between IgG-s-(^{3}H)DHEA and BSA-s-(^{3}H)DHEA)*

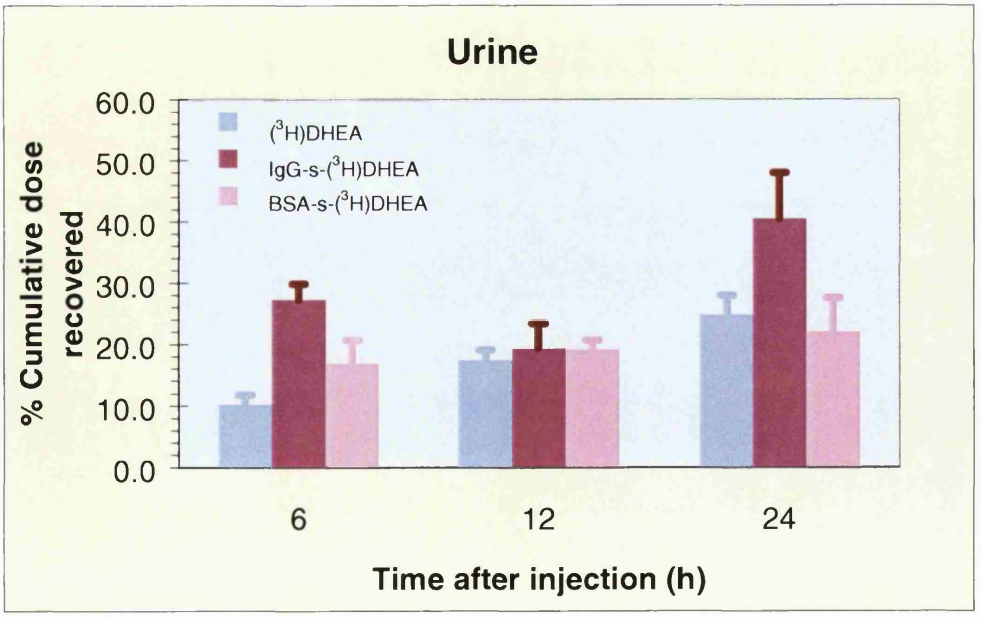


Fig. 5.4 *The percentage of (^{3}H)DHEA, IgG-s-(^{3}H)DHEA and BSA-s-(^{3}H)DHEA in urine after intravenous administration into mice. Results are means ± SE (n=4).*

Fig. 5.3 shows that a higher percentage of ^{3}H radioactivity from the BSA-s-DHEA conjugate was found in the kidneys at 2h (as compared to both the tritiated IgG-s-DHEA and tritiated free drug). However, Fig. 5.4 also shows that 10-40% of the administered dose of ^{3}H radioactivity from the DHEA, IgG-s-DHEA and BSA-s-DHEA was recovered in the urine with no statistically significant differences ($p > 0.05$). In view of the unknown contribution of intact drug or its metabolites (in term of ^{3}H radioactivity) to the values shown, the significance of these findings cannot be explained.

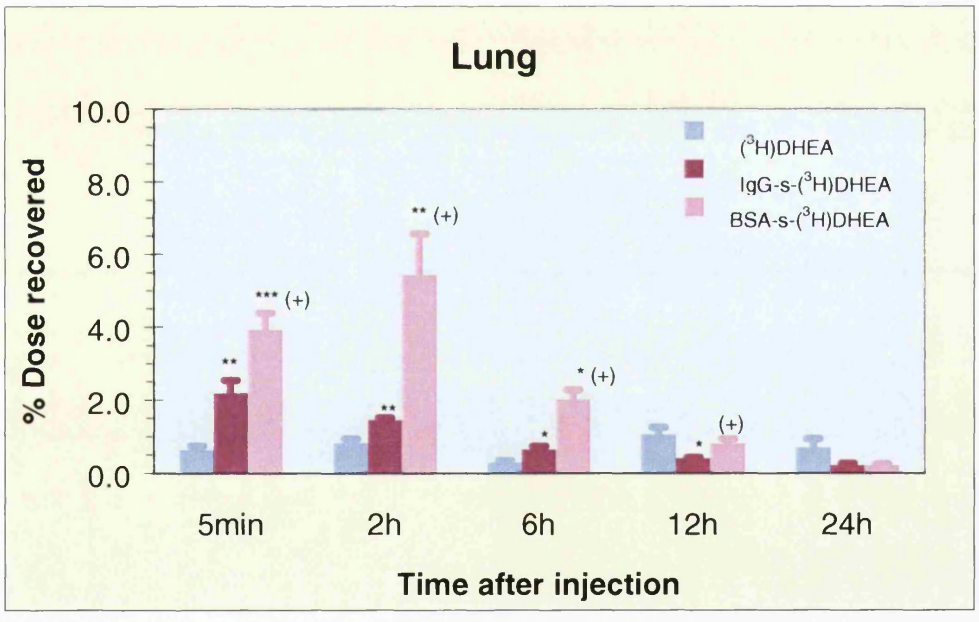


Fig. 5.5 *The percentage uptake of (^{3}H)DHEA, IgG-s-(^{3}H)DHEA and BSA-s-(^{3}H)DHEA in lung after intravenous administration into mice. Results are means ± SE (n=4). (* p<0.05,** p<0.01, *** p<0.001 as compared to the (^{3}H)DHEA, + p<0.05 as compared between IgG-s-(^{3}H)DHEA and BSA-s-(^{3}H)DHEA)*

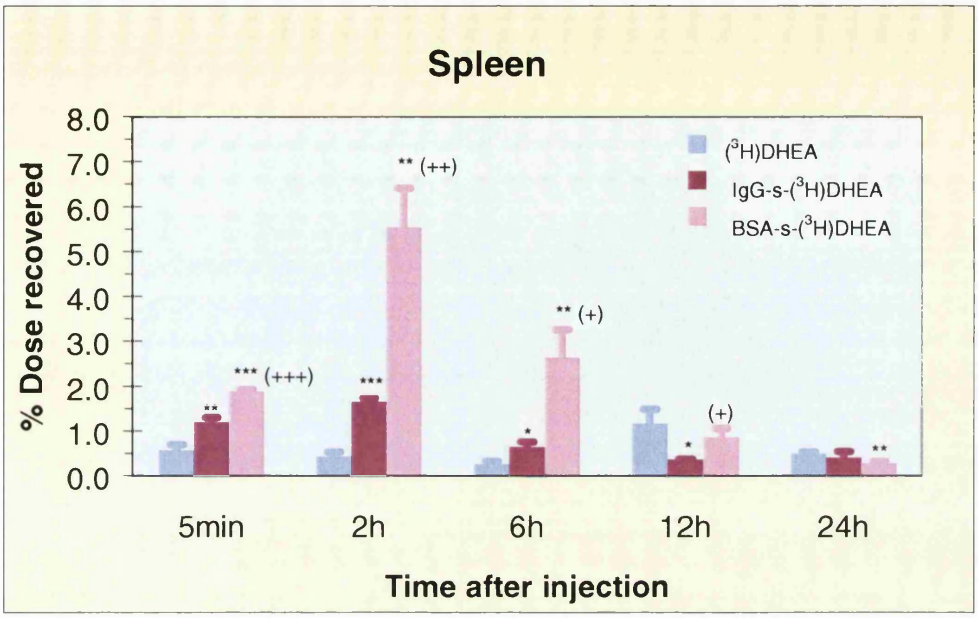


Fig. 5.6 *The percentage uptake of (^{3}H)DHEA, IgG-s-(^{3}H)DHEA and BSA-s-(^{3}H)DHEA in spleen after intravenous administration into mice. Results are means ± SE (n=4). (* p<0.05,** p<0.01, *** p<0.001 as compared to the (^{3}H)DHEA, + p<0.05,++ p<0.01, +++ p<0.001 as compared between IgG-s-(^{3}H)DHEA and BSA-s-(^{3}H)DHEA)*

As depicted in Figs. 5.5 and 5.6, a higher percentage of the tritiated DHEA from the BSA-s-DHEA was found in the lung and spleen respectively compared to both the free DHEA and IgG-s-DHEA (including the liver, Fig.5.2). These results suggest that uptake of the BSA conjugate by these tissues (probably the reticuloendothelial system component) is greater than that of the IgG conjugate.

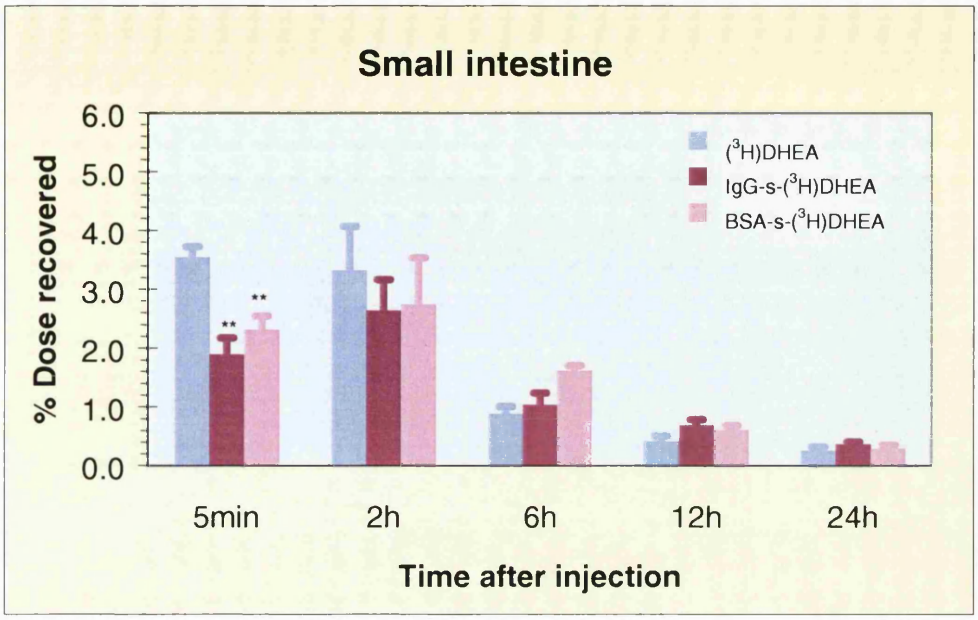


*Fig. 5.7 The percentage uptake of (^{3}H)DHEA, IgG-s-(^{3}H)DHEA and BSA-s-(^{3}H)DHEA in small intestine after intravenous administration into mice. Results are means ± SE (n=4). (** p<0.01 as compared to the (^{3}H)DHEA)*

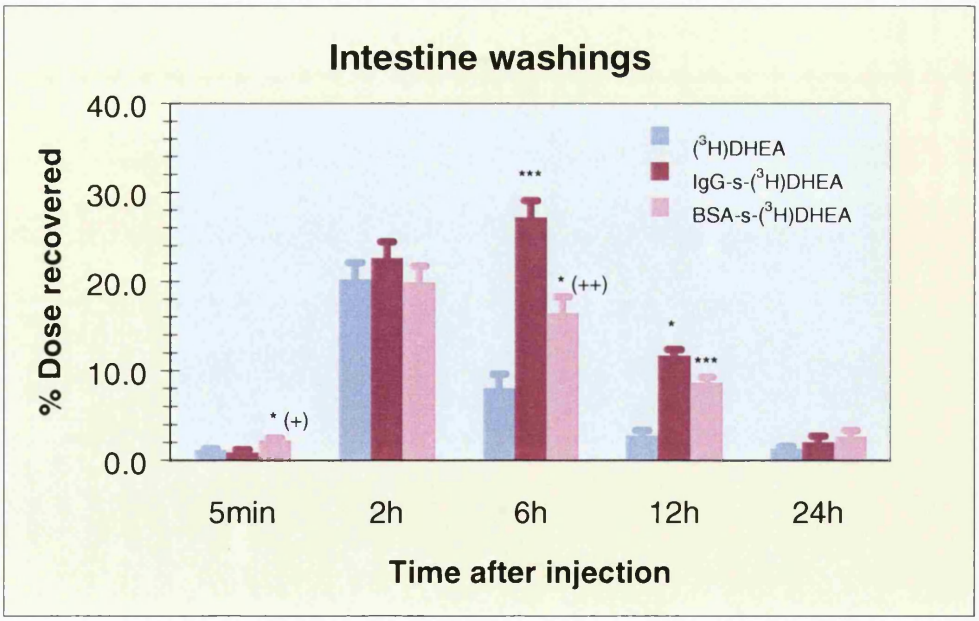


Fig. 5.8 The percentage of (^{3}H)DHEA, IgG-s-(^{3}H)DHEA and BSA-s-(^{3}H)DHEA in intestine washings after intravenous administration into mice. Results are means ± SE (n=4). (p<0.05, *** p<0.001 as compared to the (^{3}H)DHEA, + p<0.05, ++ p<0.01 as compared between IgG-s-(^{3}H)DHEA and BSA-s-(^{3}H)DHEA)*

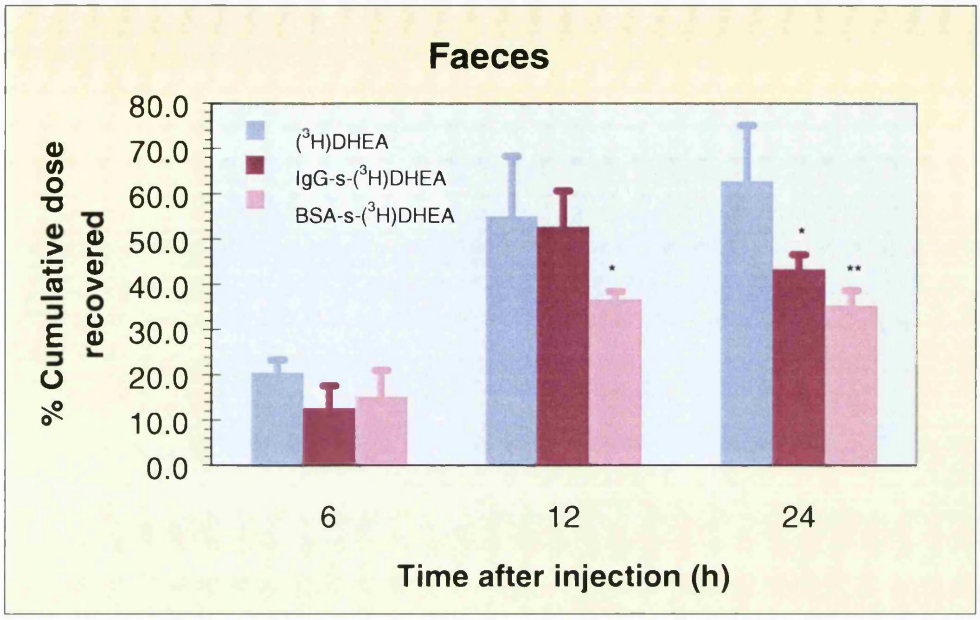


Fig. 5.9 *The percentage of (^{3}H)DHEA, IgG-s-(^{3}H)DHEA and BSA-s-(^{3}H)DHEA in faeces after intravenous administration into mice. Results are means ± SE (n=4). (* p<0.05,** p<0.01 as compared to the (^{3}H)DHEA)*

Fig. 5.7 shows no significant difference between (^{3}H)DHEA, IgG-s-(^{3}H)DHEA and BSA-s-(^{3}H)DHEA uptake in the small intestine after 2h and up to 24h while the uptake of the tritiated DHEA from both of the protein conjugates was significant compared to that of the tritiated free drug after 5 min.

The elimination pathway of free (^{3}H)DHEA and conjugated drug (IgG-s-(^{3}H)DHEA and BSA-s-(^{3}H)DHEA) (in terms of ^{3}H radioactivity) was examined by collecting intestine washings, urine and faeces. As shown in Fig. 5.8, about 20% of the free drug radioactivity was found in intestinal washings after 2 h, declining to 8% at 6h. However, conjugated drug levels, although similar to those of free drug at 2h, remained high (27% and 16%) at 6 hours and declined less rapidly than that the free drug level (Fig. 5.8). Such differences between the levels of free drug radioactivity and those of the conjugated drug probably reflect differences in their metabolic rates in the tissues to

which the free and conjugated drug is distributed after intravenous injection. The same applies to the values observed in the faeces (Fig. 5.9) although in this case values for free drug radioactivity appear to be higher than those of the conjugated drug (12 and 24 hours), possibly effecting to some extent the level in the intestinal washings. Generally, these results indicate that much of the intact drug or its metabolic products are removed through the biliary tract.

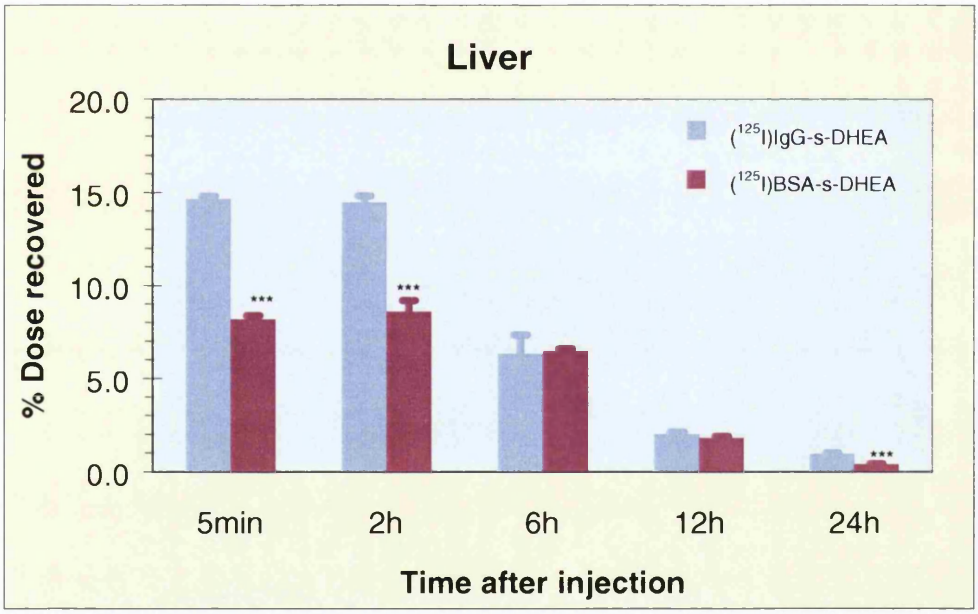


Fig. 5.10 *The percentage uptake of (^{125}I)IgG-s-DHEA and (^{125}I)BSA-s-DHEA in liver after intravenous administration into mice. Results are means ± SE (n=4). (*** = p<0.001 as compared between (^{125}I)IgG-s-DHEA and (^{125}I)BSA-s-DHEA)*

Typically, tissue distribution studies involving proteins have employed ^{125}I labelled molecules as tracers. By measuring the radioiodinated protein moieties of the conjugates (Table 5.2b and 5.3b), the uptake of IgG in the liver was about 15% at 5 min

and 2h and gradually declined to nearly 1% at 24h. However, the uptake of (^{125}I)BSA by the liver was lower i.e. 8% at 5 min and 2h and declined to nil at 24h.

As shown in Figs. 5.2 and 5.10, the ^{3}H and ^{125}I radioactivities from the conjugates, representing the drug and proteins respectively, were found in the liver. This suggests that (^{125}I)IgG-s-(^{3}H)DHEA and (^{125}I)BSA-s-(^{3}H)DHEA were distributed from the blood circulation into the liver after intravenous administration, presumably as intact conjugates. However, it was also observed that lower percentages of ^{125}I radioactivity (corresponding to the protein moieties) were recovered in the liver compared to percentages of ^{3}H radioactivity of the protein-bound DHEA. This could be attributed to a more rapid catabolism of the proteins (with ^{125}I released in the circulation) than that of the drug. Fig. 5.10 also shows a higher level in liver for the IgG than the BSA which can be the result of either a more rapid uptake of the IgG conjugate, or a greater catabolic rate of the BSA conjugate.

It was noted (Figs. 5.11 and 5.12) that ^{125}I radioactivity was recovered in the kidneys and urine, even though the molecular weight of the proteins exceeds the renal threshold. As shown in Tables 5.2b and 5.3b, 41% and 10% of the (^{125}I)IgG was found in the urine and faeces respectively after 24h. The corresponding values for (^{125}I)BSA were 32% and 2%. This suggests that the major elimination pathway for (^{125}I)IgG and (^{125}I)BSA radioactivities was through urine. It is likely that the proteins were catabolised in the liver with the iodinated metabolites or even free iodine eliminated via the kidneys and then excreted in the urine. However, some of the ^{125}I radioactivity was also recovered in the thyroid (Fig. 5.13) which uses it to make thyroid hormone as expected free iodide (Ganong, 1995)

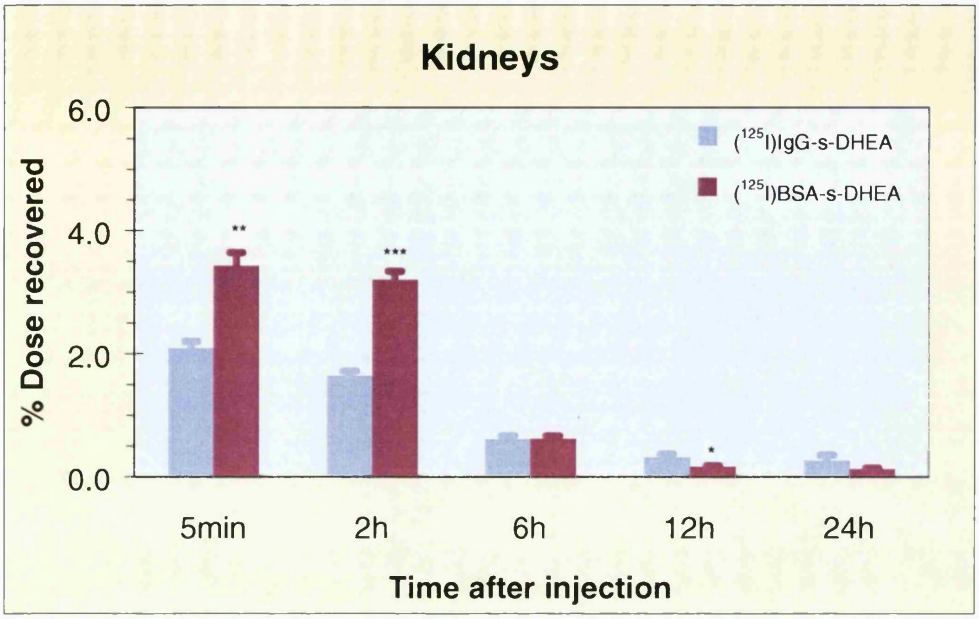


Fig. 5.11 *The percentage uptake of (^{125}I)IgG-s-DHEA and (^{125}I)BSA-s-DHEA in kidneys after intravenous administration into mice. Results are means ± SE (n=4). (* = p<0.05, ** = p<0.01, *** = p<0.001 as compared between (^{125}I)IgG-s-DHEA and (^{125}I)BSA-s-DHEA)*

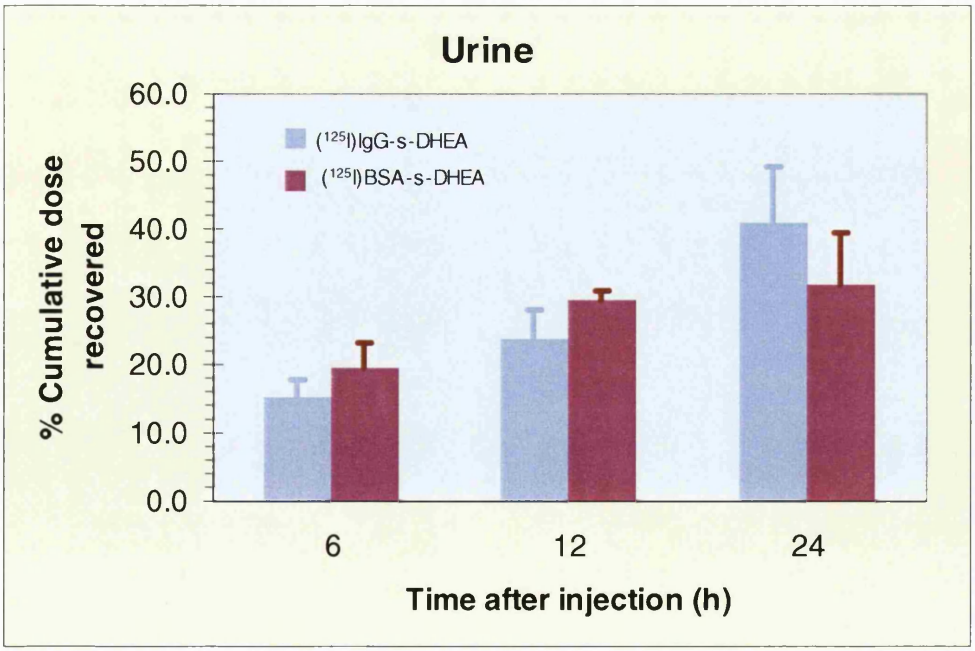


Fig. 5.12 *The percentage of (^{125}I)IgG-s-DHEA and (^{125}I)BSA-s-DHEA in urine after intravenous administration into mice. Results are means ± SE (n=4).*

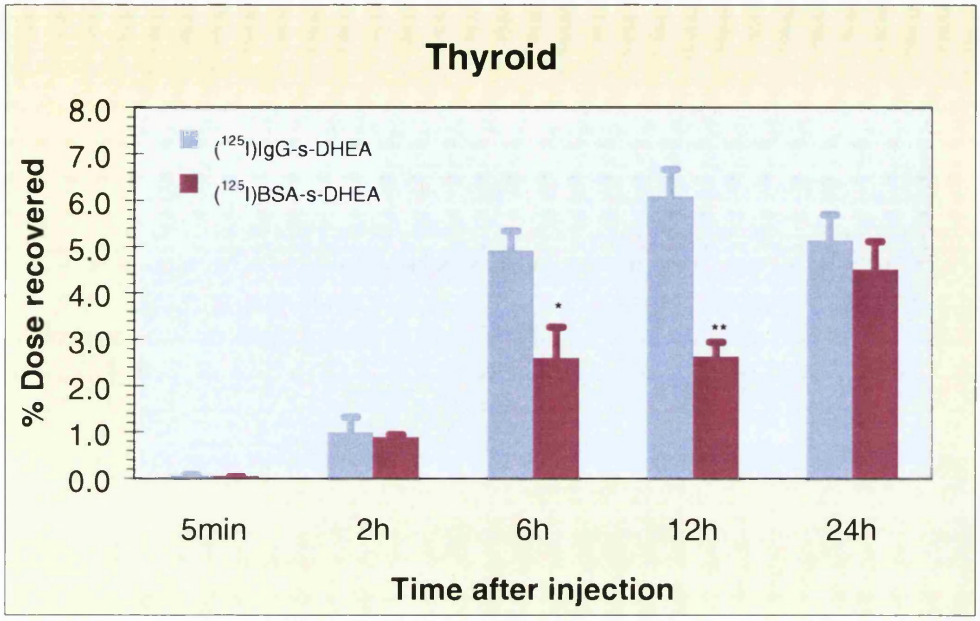


Fig. 5.13 *The percentage uptake of (^{125}I)IgG-s-DHEA and (^{125}I)BSA-s-DHEA in thyroid after intravenous administration into mice. Results are means ± SE (n=4). (* = p<0.05, ** = p<0.01 as compared between (^{125}I)IgG-s-DHEA and (^{125}I)BSA-s-DHEA)*

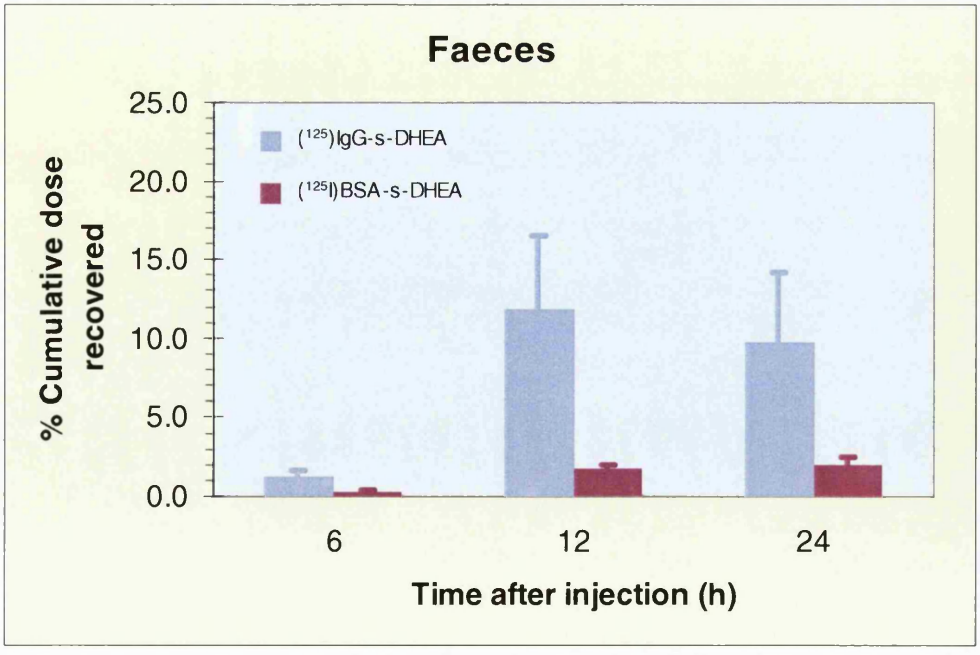


Fig. 5.14 *The percentage of (^{125}I)IgG-s-DHEA and (^{125}I)BSA-s-DHEA in faeces after intravenous administration into mice. Results are means ± SE (n=4).*

As shown in Fig. 5.14, less than about 11% of ^{125}I radioactivity from the IgG and BSA conjugates was found in the faeces. This confirms that the main elimination pathway of ^{125}I radioactivity corresponding to the proteins was via the kidneys into the urine, possibly in the form of the free iodide (^{125}I) (Table 5.2b and 5.3b).

Chapter VI

Conclusions and perspectives

The work described in this thesis represents an attempt to use immunoglobulin G (IgG) and bovine serum albumin (BSA) in the delivery of a model lipophillic drug, dehydroepiandrosterone (DHEA). The use of IgG and BSA improves the solubility of the DHEA for intravenous administration and potentially its therapeutic efficacy. Thus, these conjugates appear to circumvent the problems associated with poor drug solubility and the possible toxicity of formulations usually chosen to solubilise water insoluble compounds.

IgG and BSA were chosen for conjugation with DHEA as they are biocompatible and water soluble, have adequate functional groups for coupling and are very well distributed in the organism. The method of conjugation of IgG/BSA with DHEA involved a two-step modification. Evidence for the formation of 3'-succinyl-DHEA following the purification step was obtained by HPLC, UV-visible spectroscopy, NMR and mass spectrometry. It was found that the characterisation of 3'-succinyl-DHEA through HPLC and mass spectrometry did not show the presence of free DHEA where as NMR spectra shows a trace of DHEA. It was presumed that 3'-succinyl-DHEA was reasonably pure enough to carry out further experiments. However, the presence of DHEA in purified compound did not cause any problems for further reaction with proteins due to unreacted -OH group of DHEA with proteins. Protein-protein or intra-protein cross-linking was also not observed in this two-step coupling method as shown in SDS-PAGE. Optimisation of the coupling method so as to obtain the highest degree of modification (i.e. highest number of DHEA molecules on the

protein) without the problem of precipitation was achieved. It was found that the pH value of the buffered solutions affected both the solubility of the conjugates and the degree of modification; to that a pH end, around 9.5 was found optimal and thus used for the coupling reaction in this study. Moreover, the degree of drug coupling was directly related to the amount of 3'-sulfoNHS-succinyl-DHEA used in the coupling reaction. It was found that the extent of 3'-succinyl-DHEA coupling to IgG or BSA is amenable to manipulation by increasing the concentration of 3'-sulfoNHS-succinyl-DHEA in DMF and the amount of 3'-sulfoNHS-succinyl-DHEA in the coupling media.

The conjugates were purified by gel filtration chromatography and the elution profiles of both conjugates and the unreacted drug were monitored at 280 nm by UV-visible spectroscopy. The TNBS method was used to determine the degree of modification due to the overlapping of the absorption spectra of the DHEA and the proteins. It was shown that this method could provide a rapid, approximate evaluation of protein modification at the lysyl residues. However, a problem with the TNBS method is the steric hindrance of the 3'-succinyl-DHEA which limited the accessibility of the TNBS to its target sites on the protein. Thus, radiolabelling, a more convenient, direct and much more accurate method, was introduced for the determination of drug conjugation to proteins using tritiated DHEA. It was found that covalent coupling of DHEA with the ε-amino groups of lysine residues on IgG and BSA was achieved through a succinyl spacer. This provides an opportunity to formulate soluble DHEA with 64.4 and and 49.5 molecules of 3'-succinyl-DHEA to one molecule of IgG and BSA respectively. In this study, double labelling (^{125}I for protein and ^{3}H for the drug) was used to follow the behaviour of the conjugates in vitro and in vivo. Furthermore, SDS-PAGE electrophoresis confirmed that the molecular size of the conjugates was affected by the degree of modification compared to that of the native proteins.

In evaluating the stability of the conjugates in biological fluids, it was shown that the percentage of DHEA released from the conjugates was low in the presence of plasma, even after 24h. Moreover, the results indicated that the IgG-s-DHEA conjugate was more stable in the plasma than the BSA-s-DHEA conjugate.

The need to maintain biologically active agents in the circulation is one of the main goals in therapeutic and diagnostic applications. Thus, retention of drugs in the circulation is a key requirement in drug design and delivery. Even the most active compound in vitro is of no use if it does not reside in the blood long enough to reach its target, while managing to avoid to some extent premature metabolism, immunological reactions and interception by non-target tissues.

There are at least two approaches to increase the circulatory half life of drugs. The older strategy causes an increase in the residence time of the drug simply by modifying its hydrophilicity and generally protecting it from metabolism. The newer approach (conjugation to a biopolymer such as proteins), may increase the stability of the drug by protecting it from metabolic enzymes and prevents its renal ultrafiltration because of the high molecular mass of the construct. It was recognised that conjugation of DHEA to IgG or BSA was likely to improve the hydrophilicity of the drug, at the same time increasing its molecular size. In Chapter IV, it was found that the removal of DHEA as a conjugate from the blood circulation was slower than for the free drug after intravenous administration into mice, presumably reflecting the rate of clearance of the protein moiety of the construct.

It has been reported that a DHEA dosage form suitable for long term therapy will still have to be developed, since the only available route of administration is oral, which suffers from an important first pass effect and low and variable bioavailability (Casson et al., 1998). Thus, an alternative route of administration, for instance by injecting soluble DHEA intravenously may overcome the first pass effect problem. Another alternative to improve

the pharmacokinetics of the conjugates would be pre-injection of the native IgG and BSA. This pre-injection may saturate the receptors and prolong IgG-s-DHEA and BSA-s-DHEA conjugates in the circulation.

The tissue distribution study (Chapter V) showed that IgG-s-DHEA and BSA-s-DHEA altered the disposition of the DHEA moiety and prolonged its retention in a number of tissues (e.g. liver, kidneys, spleen, thyroid etc.) as compared to the free drug. Generally, it is believed that the prolonged blood circulation of drugs can result in accumulation at accessing target sites (passive targeting). As discussed in Chapter IV, IgG and BSA conjugates significantly increased the pharmacokinetics of DHEA , therefore, it is possible that the conjugates may be advantageous in accumulation more drugs to a target site as compared to the injection of the same dose of free drug. In Chapter V, the hepatic uptake of the tritiated DHEA as compared with the free drug. Therefore, IgG-s-drug or BSA-s-drug constructs may serve as a means to target to liver, to increase drug concentration and to prolong drug retention in the liver. In this study, the native IgG and BSA have not been used as control experiments for tissue distribution because both of IgG and BSA are naturally existed in the circulation. It was thought that externally injected proteins may behave similar as native proteins.

DHEA is available over the counter drug in the USA. As discussed earlier, DHEA has effects on various diseases such as anti-age, immune response, cancer, weight loss, cardiovascular disease, diabetes, mood improvement etc., and it is still puzzling how DHEA achieve these effects. Thus, coupling DHEA to IgG/BSA may possibly restrict unfavourable effects.

Recent advances in recombinant DNA technology and knowledge of antibody gene structure have been applied to the engineering of rodent antibodies to make them less immunogenic. A humanised antibody is constructed by transferring the murine

complimentarity determining regions (CDRs) on to the human framework region (Chari, 1998). Since CDRs form the antigen combining site, a humanised or CDR-grafted antibody preserves the murine antigen specificity, but because most of the antibody structure is human, it is likely to be less immunogenic than the parent mouse antibody. It has been reported that CDR-grafted antibodies did not induce a primary immune response in 46 patients as compared to humanised antibodies, even after several courses of treatment. Therefore, the newer conjugates would be with CDR-grafted antibodies that are expected to be non-immunogenic allowing for use in repeated cycles of therapy. Moreover, it has been reported that cationic proteins, such as cationised albumin and cationised immunoglobulin G can be transported across the brain capillary endothelium (Bickel et al., 1993). This approach may therefore be useful for delivering DHEA, for instance, to the brain across the blood-brain barrier and prolonging the retention of DHEA in the brain.

Polyethylene glycol (PEG), a non-biodegradable synthetic polymer, has been used extensively to modify protein drugs as well as the surface of liposomes (Fuertges and Abuchowski, 1990; Katre, 1990; Klibanov et al., 1990; Senior et al., 1991). PEG modification of protein drugs enables them to avoid rapid clearance from the systemic circulation by a simple size effect in glomerular filtration and a "stealth" effect against recognition by the reticuloendothelial system. Recently, polysialic acids, the highly hydrophilic and biodegradable *N*-acetylneuraminic acid polymers, have been proposed as an alternative to PEG (Gregoriadis et al., 1993; Fernandes and Gregoriadis, 1997). This approach is very attractive for improving the retention of therapeutic proteins, peptides and small drugs in the body and reducing their uptake by the RES.

In an alternative approach, Laneri et al. (1999) have synthesised ionised dehydroepiandrosterone (DHEA) prodrugs with greater water solubility and for iontophoretic transdermal application. The prodrugs are mainly used for transdermal

iontophoretic delivery, but an important object is to have DHEA in the receptor compartment. This concept also requires the prodrugs to generate the parent drug during its passage through the skin by local esterase. In this study, it was shown that, in addition to DHEA, the prodrugs was also found in the receptor compartment. This was attributed to a faster permeation rate than hydrolysis rate by esterase in the skin. Our conjugates may therefore be introduced by this route of administration to prevent the permeation of the prodrugs, prolong the hydrolysis rate by enzymes in the skin and enhance only the parent drug transport through the skin into the circulation. In yet another approach, McCormack and Gregoriadis (1996) suggested the use of DHEA-cyclodextrin inclusion complexes entrapped in liposomes for the delivery of insoluble drugs. IgG and BSA could be alternative carriers in controlling the action of a wide range of therapeutic agents by entrapping drug conjugates into liposomes or by using cyclodextrin to modify the surface of the conjugates.

Over the past 30 years, various carrier systems such as liposomes, monoclonal antibodies, soluble polymers, colloidal and non-colloidal particles have been developed. Drug delivery and targeting has become the Holy Grail for pharmaceutical scientists. Many of the problems with the new delivery systems will have to be addressed if only to generate a full understanding of the problem. However, the improvement of a drug's performance by refining these strategies clearly has important repercussions in the pharmaceutical industry.

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Appendix

All solutions were made with deionised water except in the case of the electrophoresis buffers were UHQ water was used.

Reservoir buffer

Tris base	15 g/l
glysine	72 g/l
SDS	5 g/l

Make up to 1000 ml with water. The reservoir buffer was diluted 1:5 and pH adjusted to 8.3 prior to use.

Lane Marker Reducing Sample Buffers

Tris-HCl pH. 6.8	0.3 M
SDS	5%
Glycerol	50%
Dithiothreitol (DTT)	100 mM

Coomassie blue stain solution

Coomassie brilliant blue (R-250)	0.025	% w/v
Methanol	45	% v/v
Acetic acid	10	% v/v

The Coomassie blue was dissolved in methanol before making up to volume with acetic acid and water.

Destain solution

Acetic acid	10 % v/v
Methanol	45 % v/v

Make up to volume with water.

0.15 M Phosphate buffered saline, pH 8.0 (PBS)

NaCl	8.00 g
$Na_2HPO_4*2H_2O$	0.025 g
$NaH_2PO4*2H_2O$	0.05 g
KCl	0.20 g

Make up to 1000 ml with water and adjust pH to 8.0

Properties of isotopes used for radiolabelling

Nuclide	Half-life	Type of emission	Principal energies (MeV)
Tritium	12.43 years	β	0.0186
Carbon-14	5730 years	β	0.156
Sulfur-35	87.4 days	β	0.167
Iodine-125	60.0 days	γ and x-ray	0.035 and 0.027-0.032

List of publications and presentations

Abstracts

Kittivoravitkul, S., Flinn, N.S., Toth, I., Gregoriadis, G. (1998) Preparation and characterisation of a IgG-dehydroepiandrosterone conjugate. *J. Pharm. Pharmacol.*, **50**, 243

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List of Abbreviations

AIDS	Acquired immune deficiency syndrome
AUC	Area under the curve
BBB	Blood brain barrier
BSA	Bovine serum albumin
CBB	Coomasie Brilliant Blue
Cl	Clearance
CNS	Central nervous system
cpm	Counts per minute
CT	Chloramine-T
Da	Daltons
DCM	Dichloromethane
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone sulphate
DHEAST	Dehydroepiandrosterone sulfotransferase
D.I.	Deionised
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
dpm	Disintegrations per minute
DSPC	Distearoyl phosphatidyl choline
DTT	Dithiothreitol
EDC	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
ESI	Electrospray ionisation
FAB	Fast atom bombardment
GFC	Gel filtration chromatography
GIT	The gastro-intestinal tract
HCl	Hydrochloric acid
HDL	High density lipoproteins
HIV	Human immunedeficiency virus
HPLC	High performance liquid chromatography
HAS	Human serum albumin
IgG	Immunoglobulin G

i.v.	Intravenous
LDL	Low density lipoproteins
LUV	Large unilamellar vesicle
MALDI	Matrix-assisted laser desorption ionisation
MLV	Multi-lamellar vesicle
MPS	Mononuclear phagocyte system
MRT	The mean residence time
MS	Mass spectrometry
MTX	Methotrexate
Mw	Molecular weight
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PE	Phosphatidylethanolamine
PEG	Polyethylene glycol
pI	Isoelectric point
RES	Reticuloendothelial system
s.d.	Standard deviation
S-DHEA	3'-succinyl-dehydroepiandrosterone
SDS	Sodium dodecyl sulphate
SLE	Systemic lupus erythematosis
Sulfo-NHS	N-hydroxysulfosuccinimide
SUV	Small unilamellar vesicle
$t_{1/2}$	The half-life
$t_{1/2\alpha}$	Plasma half-life for distribution phase
$t_{1/2\beta}$	Plasma half-life for elimination phase
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
TNBS	Trinitrobenzenesulfonic acid
TOF	Time of flight
UV	Ultraviolet
Vd	The apparent volume of distribution